Indirect impacts on a phytophagous insect of birch:

The role of fungal phytopathogens & leaf-mining insects

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A B S T R A C T

Communities are woven from complex inter-species interactions, comprising both direct interactions (*e.g.* interference competition or predation) and indirect interactions, mediated through a third species. This study focussed on two plant-mediated indirect interactions affecting the birch aphid *Euceraphis betulae* Koch (Homoptera: Drepanosiphinae) via its host plant *Betula pendula* Roth (Betulaceae). The indirect impacts of a plant pathogen *Marssonina betulae* (Lib.) Magn. (Deuteromycotina: Melanconiales) and the leaf-mining moth *Eriocrania* spp. Zeller (Lepidoptera: Eriocraniidae) were investigated.

A strong positive correlation existed between natural populations of *Euceraphis betulae* and leaves infected with *M. betulae*. *Euceraphis betulae* preferred to feed on fungal-inoculated leaves, and showed enhanced performance when so doing. When feeding on inoculated leaves, aphid biometric features increased by more than 10%, and developmentally mature embryos were both more numerous and larger. Free amino acid concentrations in inoculated leaves were more than double those of fungi-free leaves, akin to changes in plant chemistry occurring during leaf senescence. It is suggested that this mechanism underpins the positive effects on aphid performance. In contrast, *E. betulae* mortality rose by more than 25% when reared with the leaf-miner, *Eriocrania*. Like fungal-infected leaves, mined leaves contained elevated phenolic concentrations, but these had no discernible impact on the aphid. Leaf-miner damage to the leaf midrib was pinpointed as the cause of higher aphid mortality, probably because it disrupted phloem hydraulics.

These interactions illustrate the potential importance of plant-mediated indirect effects in phytophagous insect communities. Different indirect effects can have both beneficial and deleterious consequences for the same focal species and may arise through induced chemical or physical changes to the plant. Moreover, the fungal impacts demonstrate the potential magnitude of indirect interactions on insect performance and abundance, whilst the leaf-miner effects illustrate how indirect interactions can arise through unexpected mechanisms that are potentially more commonplace than hitherto recognised.

C O N T E N T S

C H A P T E R T W O Methodology 35

LIST OF FIGURES, TABLES & PLATES

CHAPTER FOUR

CHAPTER FIVE

CHAPTER SIX

CHAPTER SEVEN

P R E F A C E

Two of the chapters in this thesis (5 & 6) have been presented in a format appropriate for peer-review journals. Some repetition was unavoidable where recurrent methodologies were used therefore.

Co-authors for these papers were: Dr S. E. Hartley (Chapter 5 & 6), Dr A.E. Douglas (Chapters 5 & 6), Dr P.J. Mayhew (Chapter 6) and Dr. S. Woodward (Chapter 5).

Citations, references and text in this thesis are set out in accordance with the stipulations laid down by the British Ecological Society for publication in their journals.

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[1] Now in sunnier climes at the University of Sussex

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DECLARATION

I alone compiled this thesis and declare that none of the material has been submitted in any previous application for a degree. Other than the assistance described in the acknowledgments, the work presented was carried out by myself. The co-authors in Chapters 5 & 6 (written for peer-review journal) have made contributions to these chapters befitting the extent of their authorship.

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SCOTT JOHNSON
NOVEMBER 2001

To my Parents

CHAPTER ONE

Introduction

1.1. INTRODUCTION

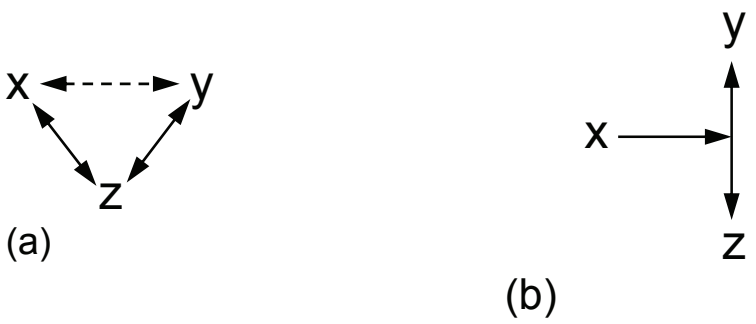
One of the most fundamental and enduring questions in community ecology is how the complex structure of natural communities is maintained, in essence the question of community stability. This debate has preoccupied ecologists for almost half a century, with the earliest contributors (MacArthur 1955; Elton 1958) suggesting that the complexity of interactions between species in ecological systems underpins community stability. May (1972, 1973), however, suggested that complexity actually led to community *instability* - a view that prevailed for a number of years. Recently the debate has come full circle, and there is now growing recognition that complexity of species interactions does indeed contribute to the stability of a community (McCann, Hastings & Huxel 1998; Polis 1998). Central to this, is that in addition to the many overt direct pair-wise interactions between species (*e.g.* predation or parasitism), many indirect interactions, involving the mediation by a third species, operate within any given community (Strauss 1991). Whilst the existence of indirect interactions in ecological communities has never been denied, attempts to address their importance in relation to the more widely understood direct interactions, is a relatively recent development in community ecology (Wootton 1994a, 2001; Menge 1995; Fox & Olsen 2000).

There are several ways of classifying indirect interactions based on the mechanisms underpinning their effects (*e.g.* Miller & Kerfoot 1987; Strauss 1991; Wootton 1994a; Menge 1995). The usefulness of the definitions depends on system being considered, but they essentially fall into two types of qualitative indirect interaction. The first type, termed 'interaction chain' by Wootton (1994a) (Figure 1.1a) involves one species, *x*, altering the abundance, morphology, chemistry or physiology of an intermediary species, *y*, which then in some way affects a third, *z*. The second type, termed 'interaction modification' by Wootton (1994a) (Figure 1.1b) occurs when the presence of one species, *x*, affects the interaction between two other species (*y* and *z*) without actually directly acting on either, usually through behavioural mechanisms.

It is generally recognised that the more complex the community, the greater the role of indirect interactions in shaping that community (Menge 1995). Phytophagous insects account for approximately 25% of all extant species on the planet (Masters & Brown

1997) and their communities can often be complex, so indirect interactions have the

Figure 1.1. Schematic representation of the two types of indirect interaction between two species involving a third. (a) The indirect interaction between species and *x* and *y* via mediation of *z*, and (b) the modification of the direct interaction between species *y* and species *z* by the presence of species *x*. Direct interactions are represented by solid lines and indirect interactions by dashed lines (after Wooton 1994a).



potential to play a major role in how insect-herbivore communities are structured.

The debate about whether phytophagous insect communities are driven predominantly by top-down effects, such as predators and parasites (Hairston, Smith & Slobodkin 1960; Lawton & Strong 1981) or bottom-up effects such as resource limitation (Hairston *et al.* 1960; McNeil & Southwood 1978; Lawton & Strong 1981; Price 1990) has shifted to determining the *relative* importance and patterns of interplay of these forces (Hunter & Price 1992; Walker & Jones 2001). It is perhaps more useful to take a pluralistic approach when considering indirect effects however, since they can operate via top-down and bottom-up mechanisms simultaneously (Figure 1.2) (Karban 1997).

As Walker & Jones (2001) observe, studies of indirect interactions within insect-herbivore communities (including this thesis) tend to emphasise top-down (*e.g.* Bonsall & Hassell 1997) or bottom-up (*e.g.* Fisher, Hartley & Young 2000) forces to the exlusion of the other (Figure 1.3). To a certain extent, this is because simultaneous studies of bottom up and top down indirect effects in complex communities are likely to be difficult to accomplish. Studies that examine the scope and processes of indirect

Figure 1.2. Schematic representation of an indirect interaction between two phytophagous insects (a & b) via modification of a host plant with the result that herbivore b indirectly interacts with the predator of herbivore a, which in turn indirectly interacts with the plant. Direct interactions are represented by solid lines and indirect interactions by dashed lines.

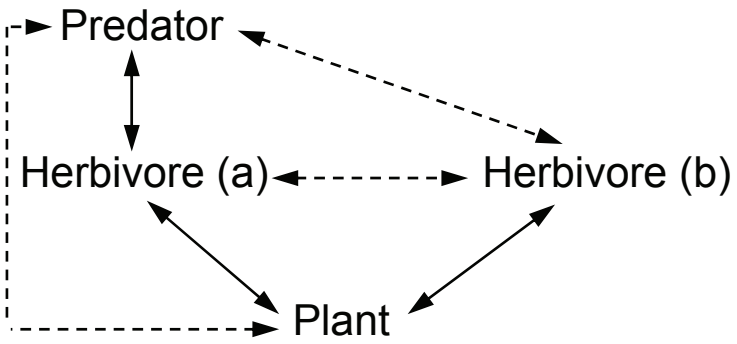
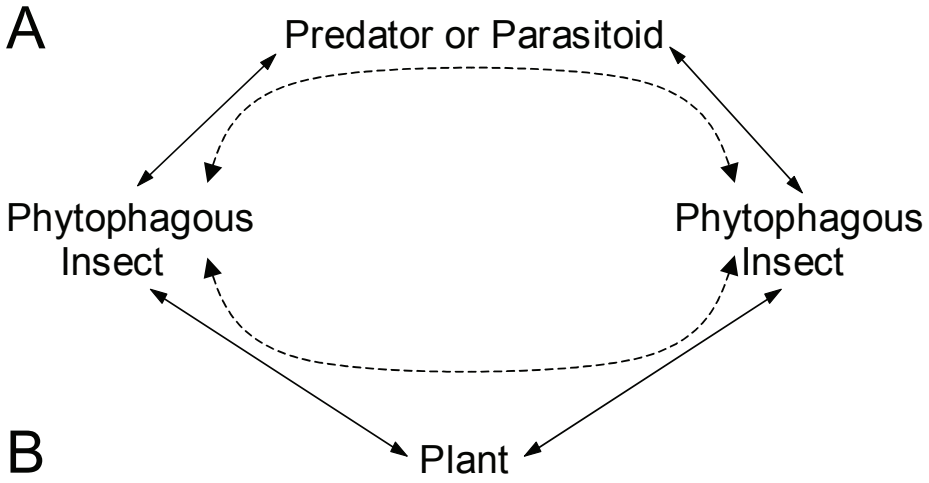


Figure 1.3. Schematic representation of reported indirect interactions between phytophagous insects which tend to emphasise either (A) top-down (*e.g.* apparent competition between two insects) or entirely (B) bottom-up (*e.g.* competitive effects via plant modification) effects, in contrast to the more realistic pluralistic nature of indirect interactions (Figure 1.2). Direct interactions are represented by solid lines and indirect interactions by dashed lines.

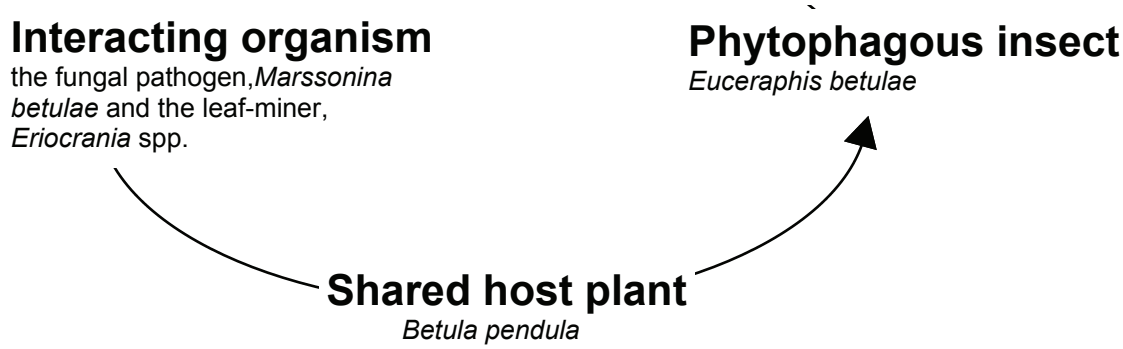


interactions operating in relatively simple systems therefore, are invaluable before more ambitious approaches can be adopted.

Insects and fungi are thought to be the two most numerous groups of extant organisms on the planet (Hawksworth 1991) many of which live solely on terrestrial plants, so the scope for indirect interactions between these groups is considerable. As well as being intuitively interesting to the community ecologist, understanding the impacts of plant-microbes and phytophagous insects on plant-herbivore interactions could have important implications for applied problems. It is known that indirect interactions can offset or exacerbate the direct effects of environmental manipulation, making predictions of ecological outcomes difficult (Wootton 1994a). Species introductions and removal of pest species can have widespread and counter-productive effects on the rest of the system if indirect interactions are common in that particular system. For example, in a phytophagous insect community of cotton plants, where the initial treatment with insecticides in 1950 led to the eradication of the two pests (the boll weevil and Alabama leafworm) but also a massive decline in natural enemies and parasitoids of three other potential pest species (cotton bollworm, cotton aphid and the pink bollworm). Without such natural enemies the populations of these phytophagous insects displaced the original two species, and became even more prevalent pests of cotton. In the case of fungi-plant-insect interactions, a better understanding of how phytophagous insects and fungal plant pathogens interact would provide a clearer insight into some plant diseases, for instance in the evolution of vector-pathogen relationships (Webber & Gibbs 1989). Furthermore, the interest in the use of plant dwelling fungi as a means of conferring herbivore resistance on agronomically important plants must surely be reliant on a better understanding of the processes involved (Prestidge & Ball 1997; Kluth, Kruess & Tscharntke 2001).

The aim of this research was to investigate the indirect effects of plant-fungi and leaf-mining insects on phloem feeding insects, via chemical and physical modification of their shared host plant (Figure 1.4). The focal species of these indirect interactions was the birch aphid *Euceraphis betulae* Koch (Homoptera: Drepanosiphidae), and the interacting species were a fungal pathogen *Marssonina betulae* (Lib.) Magn (Deuteromycotina: Melanconiales) and a leaf-mining insect *Eriocrania* spp. Zeller

Figure 1.4. Schematic representation of the indirect effects of a fungal pathogen, *Marssonina betulae* and the leaf-miner *Eriocrania* spp. on the birch aphid, *Euceraphis betulae* via changes to their shared host plant, *Betula pendula*.



(Lepidoptera: Eriocraniidae), all of which are primary consumers of *Betula pendula* Roth (Betulaceae) leaves.

This system has a number of advantages that make it a particularly suitable model to study the impact of indirect interactions on phytophagous insects. These are:

i. Two of the interacting organisms (the leaf-miner and the fungus) are temporally separated. The leaf-miner feeds on birch at the beginning of the growing season whereas the fungal pathogen occurs in the second half of the season. This allows pair-wise, indirect interactions with *E. betulae* to be considered independently of the other. Similarly, the leaf-miner, fungal pathogen and aphid are spatially separated. The leaf-miner is an internalised feeder of leaf tissue between the upper and lower leaf lamellae, the fungal pathogen is largely restricted to the leaf lamina and the aphid feeds from the basal midrib and petiole. The potential for direct interactions between these organisms, therefore, is minimal.

ii. Aphids are useful experimental organisms when identifying indirect interactions because their rapid growth and phenotypic plasticity allows performance parameters to be assessed easily. In addition, *E. betulae* has only one parthenogenetic adult morph (winged), simplifying experimental design.

iii. As the extensive literature on the birch-herbivore system illustrates (*e.g.* Haukioja & Neuvonen 1985; Hartley & Lawton 1987; Haukioja *et al.* 1990; Fisher, Hartley & Young 2000) this system is relatively well understood and amenable to the investigation of indirect interactions; an invaluable approach for identifying causal relationships in complex interactions. Species interactions on *B. pendula* leaves at the field site were relatively simple, being dominated by *E. betulae* and *M. betulae* which can be very abundant on birch leaves. *Eriocrania* spp. leaf-miners were less common, but sufficiently abundant during Spring to allow experimentation.

1.2. PLANT-MEDIATED INDIRECT INTERACTIONS

Plant-mediated indirect effects on phytophagous insects are important because some phytophagous insects escape the effects of predators, but none escape the effects of their host plant (Hunter & Price 1992). To understand how these indirect interactions might arise through the plant, it is necessary to discuss what features of plants are important to the insect-herbivores in terms of their nutritional requirements, how these might be modified by interacting organisms, and ultimately how these changes might affect phytophagous insects. The host plant changes brought about by herbivory and fungal infection are as numerous and complex as the indirect effects on phytophagous insects that they may cause. The introduction that follows therefore, only discusses the salient features of herbivore and fungal interactions with plants, which are discussed in more detail by Karban & Baldwin (1997) and Barbosa, Krischik & Jones (1991), respectively.

1.2.1. What makes plants poor food for insect herbivores?

The suitability and acceptability of a host plant to a phytophagous insect depends to a large extent on chemical composition of that plant (Pimm 1991; Hartley & Jones 1997). Plants represent poor food for phytophagous insects because they have very low nutritional quality (McNeil & Southwood 1978) and they often possess substances or attributes that prevent herbivores from obtaining what little nutrition is available.

1.2.1.1. Nutritional quality

Nitrogen compounds are an essential part of insect herbivore nutrition, and more often than not are the limiting factor in their diets (Mattson 1980; Waring & Cobb 1992). This is largely due to the low total nitrogen content of plant tissues, and even lower concentrations of useable forms of nitrogen (*e.g.* soluble or extractable protein). The nitrogen content of birch leaves for instance, is typically around 2 % of the dry weight (Allen 1989), although this is highly variable with leaf age and position on the tree (Lehtilä *et al.* 2000).

1.2.1.2. Allelochemicals

Plants possess a spectacular array of secondary compounds (so-called because they do not appear to have a primary metabolic function), the compositional characteristics of which can be specific to that plant species (Haslam 1988). They are widely recognised as having defensive functions against plant consumers ranging from bacteria to insect and mammalian herbivores (Rosenthal & Berenbaum 1992), although they have additionally been implicated in many other processes (Hartley & Jones 1997). The effects of secondary compounds on insect herbivores are now well studied, and have been seen to have many deleterious impacts including deterrence (Bernays 1990), reductions in growth and fecundity (Berenbaum & Feeny 1981; Klocke, Vanwagenen & Balandrin 1986) and digestive inhibition (Rosenthal & Berenbaum 1992). Secondary compounds have generally been classified as either 'digestibility reducers or toxins', based on their mode of action or as 'constitutive or induced defences', based on their residency in plants. Constitutive defences are present in plant tissues permanently whereas induced defences are produced *de novo* in response to wounding (Karban & Baldwin 1997; Speight, Hunter & Watt 1999).

1.2.1.3. Physical characteristics

In addition to this array of secondary compounds, plants often possess physical attributes that make it difficult for phytophagous insects to obtain sufficient nutrition.

Many plant surfaces possess trichomes that may act purely as a physical barrier to the insect (structural) or else may actively secrete sticky or noxious substances (glandular) to hinder insect herbivores (Speight *et al.* 1999). The physical properties of the plant itself might also serve to prevent insects feeding efficiently, for instance by possessing waxy cuticles and tough fibrous tissues (Vicari & Bazely 1993).

1.2.2. Modification of plant quality by phytophagous insects and fungal pathogens

Nutritional quality and the ease with which these nutrients might be obtained (due to the presence of secondary compounds and defences) are the key mechanisms by which plant-mediated effects on phytophagous insects manifest themselves. Modifications of one or both of these plant characteristics could render plants more or less suitable to phytophagous insects and potentially affect their performance. These modifications may occur in response to a range of intrinsic factors (*i.e.* phenological or diurnal variation), or extrinsic factors (*i.e.* environmentally driven modification) (Hartley & Jones 1997). Important environmental modifications include climatic effects such as drought and elevated atmospheric CO_2 (Major 1990; Bezemer & Jones 1998), but also the effects of organisms that exploit plants, including phytophagous insects (Karban & Baldwin 1997) and plant-associated fungi (Saikkonen *et al.* 1998; Dixon 2001).

1.2.2.1. Insect modification of plant suitability

Insects are ubiquitous consumers of plants, whose effects can drastically reduce plant fitness and even cause mortality. Reduced leaf area, turnover, and abundance in addition to diminished plant stature and seed production are amongst the deleterious effects of insect herbivory on plant performance (Louda, Keeler & Holt 1990). In addition to exploitative damage to the plant as a resource, insect herbivory is known to induce a number of more subtle physiological and chemical responses in the plant (Masters & Brown 1997).

The nutritional quality of foliage may change in response to insect herbivory. This may occur because of compensatory increases in nutrient production and their subsequent movement around the plant to counteract the loss of foliage (Danell & Hussdanell 1985; Haukioja *et al.* 1990). Conversely, insect herbivory may lead to the formation of an additional sink for plant nitrogen and carbon, for instance phloem feeding and leaf-

mining insects were seen to create a sink which reduced levels of foliar nitrogen throughout the rest of the plant (Gange & Brown 1989; Masters & Brown 1992).

Plants are extensively attacked by insects, but the maintenance of a permanent suite of secondary compounds to deter herbivores may be costly, especially when the risk of herbivory is minimal. Because of this, many plants show induced responses to herbivory, whereby allelochemicals are produced only when plant tissues are damaged (Karban *et al.* 1999). Over a hundred plant species have been shown to respond to insect damage with changes in levels of secondary compounds (Karban & Baldwin 1997) over different spatial and temporal scales. Induction of elevated secondary compounds can remain localised in the leaf where the damage is occurring or can become systemic, whereby a response occurs in other parts of the plant (Hartley & Firn 1989; Karban *et al.* 1999). The time-scale of induction and persistence of secondary compounds is also highly variable, ranging from a few hours to many years (Hartley & Lawton 1991b; Neuvonen & Haukioja 1991).

Phytophagous insects can influence the morphology of their host plant, for instance by causing an increase in the density of prickles, spines and trichomes (Bazely, Myers & Dasilva 1991) or by modifying plant phenology (Williams & Whitham 1986). Modifications to the vasculature and structural characteristics of the leaves (Fritz, Sacchi & Price 1986; Mattson 1986; Hunter & Willmer 1989) are also common features of insect herbivory.

1.2.2.2. Fungal modification of plant quality

Fungal plant pathogens, like phytophagous insects, can bring about the general decline and sometimes death of their host plants, although their effects more often involve less lethal physiological and morphological modifications of their hosts (Hammond & Hardy 1988; Barbosa *et al.* 1991; Hatcher 1995 and references therein). Changes to the nutritional quality of the host plant can come about directly by mycologically synthesised compounds, or else plant-initiated changes to plant chemistry in response to fungal infection. In leaves infected by a fungal pathogen, there is generally a decline in photosynthesis and the export of photoassimilates can be either reduced or elevated (Hatcher 1995) with dramatic effects on carbon and nitrogen compound concentrations. The interplay of catabolic and anabolic processes in *both* the fungus

and plant also contribute to the chemical composition of the plant. For example, increases in carbohydrates (Ramsell & Paul 1990) and nitrogenous compound concentrations (Reddy & Rao 1976; Ramsell & Paul 1990) have been reported, as have decreases in carbohydrates (Hatcher & Ayres 1998) and nitrogenous compound concentrations (Lam 1985).

Whilst there is now little doubt that insect herbivores can induce and influence concentrations of allelochemicals in their host plant (Karban & Myers 1989), it is indisputable that fungi do so (Dixon 2001). Induced plant responses to fungi, most notably overt fungal pathogens, are rather better understood than induced plant responses to insect herbivores, although in some cases the responses can be similar (Hartley & Lawton 1991a; Karban *et al.* 1999). An example of a common defensive response is in tomato leaves where herbivory and fungal infection induced the production of the same proteinase inhibitors, known to interfere with both insect and microbial metabolism (Farmer & Ryan 1992). Induction of a defensive response by plants to fungal attack typically involves a hypersensitive response, in which cells surrounding the site of infection are killed, thereby isolating the pathogen. This is closely associated with the production of allelochemicals belonging to three major classes; phytoalexins, proteinase inhibitors and lipoxygenase, any of which may be damaging to the fungal pathogen or phytophagous insects feeding on infected leaves (Dixon, Harrison & Lamb 1994). One dissimilarity between herbivore and fungal induced responses in plants is that the fungus can also contribute to the allelochemical profile of the plant by synthesising its own allelochemicals, which may be detrimental to the plant or insects feeding on the plant (*e.g.* Wilkinson *et al.* 2000).

One further type of host plant modification associated with fungal pathogen infection is morphological change, usually as one of the many symptoms characteristic of a particular disease. The most obvious being stunted and reduced growth, although developmental abnormalities such as irregular, twisted or rolled leaf margins are also common (Barbosa 1991). Many leaves also change colour when infected, most noticeably when a fungal pathogen causes leaf chlorosis due to reduced photosynthesis and chlorophyll catabolism.

1.2.3. Plant-mediated effects of insect

herbivores and plant fungi on phytophagous insects

Having broadly defined what types of host plant modification might mediate the effects of indirect interactions, it is clear that the specific types of modification to a plant can be complex, and so too are the responses of phytophagous insects to such modification.

1.2.3.1. Insect-plant-insect interactions

Interspecific interactions between phytophagous insects have been reviewed by Denno, McClure & Ott (1995) who found extensive evidence of interspecific interactions between insect herbivores, many of which were indirect interactions mediated via the plant. Plant-mediated indirect interactions are based on host-plant modification, so their effects can manifest themselves between spatially and temporally distinct phytophagous insects. The classification of plant-mediated interactions between phytophagous insects devised by Masters & Brown (1997) is outlined in Table 1.1 with examples from the literature where appropriate. In short, spatially separated insect herbivores share their host plant at the same time and may share the same feeding guild but they exploit different niches; temporally separated insect herbivores use the same niche but feed at different times. Spatio-temporally separated insect herbivores feed at different times and may or may not share guilds and niches (Table 1.1).

There is a growing catalogue of examples of phytophagous insects interacting via induced changes to plant nutritional quality. In addition to well-known phenomena such as nutritional deterioration induced by large numbers of aphids feeding on the same plant (Dixon 1998), there are many other examples of phytophagous insects causing nutritional changes that impact on other phytophagous insects that can occur elsewhere on the plant or even at different times. Masters & Brown (1992) illustrated how a leaf-miner (*Chromatomyia syngenesiae*) brought about a reduction in plant nitrogen with negative impacts on a spatially separated root feeding beetle (*Phyllopertha horticola*). Denno *et al.* (2000) and Petersen & Sandstrom (2001) recently reported temporally discrete competitive effects of between two phloem feeders, also via nutritional modification of their shared host plant.

Several authors have also evoked induced secondary compounds as the mechanism behind the interaction between insect-herbivores, most notably for early versus late

Table 1.1. Classification of possible plant-mediated interactions between insect herbivores devised by Masters & Brown (1997), with relevant examples drawn from the literature where possible. Chapter 6 of in this thesis reports the interactions of spatially separated insects of different feeding guilds.

INDIRECT INTERACTION	**Species separation**	**Example**	**Effects**	**Reference**
SPATIAL	Niche	Phloem feeders on different parts of the leaf	+/+	Kidd *et al*. 1985
	Guild	Leaf-miner & phloem feeding insect sharing a leaf	0 / -	Chapter 6
	Niche & Guild	Leaf-miner and root-feeding beetles	0 / -	Masters & Brown 1992
TEMPORAL	Feeding time	Two leafhoppers feeding at different times	0 / -	Denno *et al*. (2000)
SPATIO-TEMPORAL	Niche	Early season leaf feeder and late season flower feeder		
	Guild	Early season leaf chewer and late season leaf-miner	0 / -	West (1985)
	Niche & Guild	Early season phloem feeder and late season leaf chewer		

season feeding insects on oak (*Quercus robuur*). West (1985), for instance, demonstrated that caterpillars feeding on leaves in spring had negative effects on a late season leaf-miner and feeding by the winter moth (*Operophtera brumata*) in spring was seen to have negative effects on an aphid (*Tuberculoides annulatus*) later in the season (Silva-Bohorquez (1987) cited in Masters & Brown (1997)). Indirect interactions via induced defence have also been suggested for a number of insect-herbivores of birch (Haukioja & Niemela 1979; Fowler & Lawton 1985; Hanhimaki 1989), but the effect has seldom proved to be clear-cut (*e.g.* Hartley & Lawton 1987; Fisher, Hartley & Young 2000). There is even some doubt about the role of induced allelochemicals in mediating insect herbivore interactions (Masters & Brown 1997), with several authors suggesting that changes in nutritional quality are more potent mediators of such interactions in most circumstances (Denno *et al.* 2000).

Indirect interactions between phytophagous insects that are mediated through morphological changes to their shared host plant are less well represented in the literature compared to nutritional or allecochemical mechanisms. These interactions arise because one insect herbivore modifies plant parts in a way that makes it less easy for another to utilise that plant (Denno *et al.* 1995). Examples of such morphological manipulation are nearly always temporally separated and occur because one phytophagous insect induces changes in the developmental architecture of a plant, for example bud feeding by a cecidomyid (*Arcivena kielmeyerae*) brings about developmental changes that result in modified stamens that cannot be fed upon by the weevil (*Anthonomus biplagiatus*) (Mattson 1986). Intraspecific interactions via morphological modification of a host plant are relatively common in nature, with many inducing such changes in order to ameliorate the host plant for themselves, their immediate progeny or subsequent generations (Price & Louw 1996). Whether these physical changes impact on different species of contemporaneous insect herbivores is as yet a little explored avenue of research, which forms the basis for investigating the indirect impacts of *Eriocrania* spp. on *Euceraphis betulae* in Chapter 6.

1.2.3.2. Fungi-plant-insect interactions

Plant-associated fungi have been seen to have indirect effects on phytophagous insects through their modification of the plant so as to make it more or less nutritious to phytophagous insects (Barbosa 1991). Depressed nitrogen content in fungal-infected leaves, for instance, have been associated with depressed performance of phytophagous insects (Hatcher *et al.* 1995) whilst high concentrations have been associated with enhanced performance (Gange 1996). Leaves infected with fungi quite often have higher carbohydrate concentrations because of assimilate movement or aberrant starch metabolism, and this too has been associated with positive impacts on insect performance or abundance (Carruthers, Bergstrom & Haynes 1986). As these examples illustrate, indirect interactions between fungi and insects via changes to plant nutritional quality are quite common, but few empirical studies have managed to define the mechanistic basis of the interaction. The interaction between the *M. betulae* and *E. betulae* reported in Chapter 5 is the first time that the mechanism underpinning the indirect impact of a tree pathogen and a free-living tree-aphid has been demonstrated using manipulation experiments.

There are many more examples of plant-associated fungi affecting phytophagous insects via altered allelochemical profiles, either directly by producing mycologically synthesised allelochemicals (Breen 1994) or by inducing the plant to do so (Krause & Raffa 1992). Where phytophagous insects are affected by mycologically rather than plant produced allelochemicals it is arguable that these are not indirect interactions at all, although where the interaction is based on the insect feeding on plant rather than fungal tissues, it may be justifiable to classify the interaction as indirect (Strauss 1991). However interpreted, a number of studies have shown indirect interactions between fungi and phytophagous insects via altered alleochemical profile of their shared host plant. These impacts can arise through fungal-produced compounds (Miller, Strongman & Whitney 1985; Clay 1988) those produced by the plant in response to their infection (Karban, Adamchak & Schnathorst 1987; Krause & Raffa 1992) or possibly both (Lappalainen, Helander & Palokangas 1995).

There are somewhat fewer examples in the literature of fungal pathogens indirectly interacting with phytophagous insects through morphological manipulation of their

shared host plant, though some insects with low water requirements, such as grasshoppers, found infected leaves that had begun to wilt more palatable than asymptomatic leaves (Lewis 1979). The actual chlorosis of leaves is also known to be important cue for certain aphids who associate yellow colouration with immature or senescent leaves which they find more nutritious than mature foliage (Dixon 1998).

1.2.4. Summary

The indirect effects of fungal pathogens and insect herbivores on other insect herbivores via their shared host plant are most likely to occur when the plant's nutritional quality or the plant's possession of attributes or substances that make nutrition less available, are altered. Both insect herbivores and fungal pathogens can cause these modifications, either through direct manipulation or else inducing the plant to undergo these chemical changes. Fungal pathogens can additionally alter the nutritional and allelochemical profile of the host plant by directly producing compounds *in situ*.

1.3. STUDY OBJECTIVES

In essence, the aim of this study was to answer two core questions concerning birch mediated indirect impacts of two types of organisms, the fungal pathogen (*M. betulae*) and leaf-mining insect (*Eriocrania* spp.), on the birch aphid (*E. betulae*).

1. What is the impact of fungal infection of birch leaves on aphid abundance and performance, and what is the mechanistic basis for the effect?
2. What is the impact of leaf-mining on aphid performance, and what is the nature of the mechanism underpinning this effect?

1.4. THESIS OUTLINE

This study examines two plant mediated indirect interactions: (a) between a fungal pathogen and an insect herbivore, and (b) between two insect herbivores. The mechanistic basis and evidence for their existence in the literature is reviewed in this Chapter.

Chapter 2, 'Methodology', introduces the study site and system, highlighting the salient features of the host plant, *Betula pendula* (the silver birch) and the leaf-miner which do not have separate chapters dedicated to them. The experimental and investigative procedures used in this study are outlined, together with the associated technical information and the rationale behind their use.

Chapter 3, 'Distribution and abundance of *Euceraphis betulae* on *Betula pendula*', reviews information in the literature about the birch aphid *E. betulae*, the focal species of these indirect interactions, and describes its abundance and underlying spatial distribution on *B. pendula*. The patterns are discussed in relation to climatic factors and host plant phenology.

Chapter 4, 'Naturally occurring birch leaf-spot fungi and its impact on aphids', focuses on research that involved naturally occurring leaf-spot pathogen, *Marssonina betulae*. The general pathology of the disease is discussed, and its occurrence on *B. pendula* is described. Results of experiments rearing *E. betulae* on naturally infected leaves are presented.

Chapter 5, 'Microbial impacts on plant-herbivore interactions: the indirect effects of a birch pathogen on a birch aphid' examines the indirect interaction between *M. betulae* and *E. betulae*. The spatial co-occurrence between the two species is reported and manipulation experiments in which asymptomatic leaves were artificially inoculated with *M. betulae* are described. Microbial modification of foliage quality for phytophagous insects is considered in the wider context of insect community manipulation and its impacts on life-history strategies.

Chapter 6, 'Insects as leaf engineers - can leaf-miners alter leaf structure for birch aphids?' explores the impact of the leaf-miner, *Eriocrania* spp. on *Euceraphis betulae* using manipulation and simulated leaf-miner damage experiments together with observations of aphid abundance on differently mined leaves. The mechanism underpinning the interaction is addressed.

CHAPTER TWO

Methodology

*The study system was situated in North East Scotland, at a site dominated by juvenile (*i.e. *non-flowering)* Betula pendula *trees. The study system consisted of four organisms from three Kingdoms, whose identification was complicated by: similarity with sister species (*i.e. *birch and the aphid); indistinguishable life-stages (*i.e. *the leaf-miner) or incomplete descriptions (*i.e. *the fungal pathogen). Descriptions and references, and where possible, methodologies are put forward in this chapter for their identification in the field. Recurring empirical procedures are described, as is the survey protocol that forms the basis of Chapters 3 & 4. Methods for plant chemical analysis and the use of Meteorological Office weather data are described, together with the statistical procedures used for the analysis of the large survey data sets obtained during this research.*

2.1. SITE DESCRIPTION AND STUDY SYSTEM

2.1.1. Birch

Three species of *Betula* are native to the United Kingdom, the two tree species *B. pendula* Roth (Betulaceae) and *B. pubescens* Ehrh. and the shrub *B. nana*. L. In addition to these species, hybrids between *B. pendula* and *B. pubescens* are also common (Atkinson 1992). In Scotland, approximately half of all broad-leaved trees are birch (Parr 1981) and pure birch stands are common. Birch is considered a pioneer species in primary and anthropogenic secondary successions, largely because it does not have specific soil type requirements (*e.g.* pH tolerant) and is able to grow in nutrient-poor soils. Indeed, birch is renowned for its ability to improve soils (Gardiner 1968), for instance by deacidifying soils to the extent that other species of plant colonise the same area and frequently out-compete birch. A complete account of *B. pendula* and *B. pubescens* is given by Atkinson (1992) and references therein.

The study site used in this research, Dalhaikie Flat, was located in North East Scotland and consisted of an almost continuous birch thicket of *B. pendula* with a few *B. pubescens* (Figure 2.1) details of which are given in Chapters 5 and 6. In Aberdeenshire, *B. pendula* tends to be dominant below 300 m above sea level, whereas *B. pubescens* is predominant above this altitude (Forbes & Kenworthy 1973).

The two native tree species, *B. pendula* and *B. pubescens* are superficially alike, but can be reliably distinguished from one another in the field using differences in leaf morphology described by Atkinson & Codling (1986) (Plate 2.1). To distinguish between the two species, three measurements were taken from each of five leaves in the lower canopy.

i. LTF – the line between the tips of the third and fourth lateral vessels. Subtract the number of teeth extending beyond this line from the number of teeth between the two vessels (in Plate 2.2 this is 2 - 0).
ii. DTF - distance to first leaf tooth from the petiole in mm.
iii. LTW - the shortest distance across the tip of the leaf one quarter of the dis tance between the apex and leaf base.

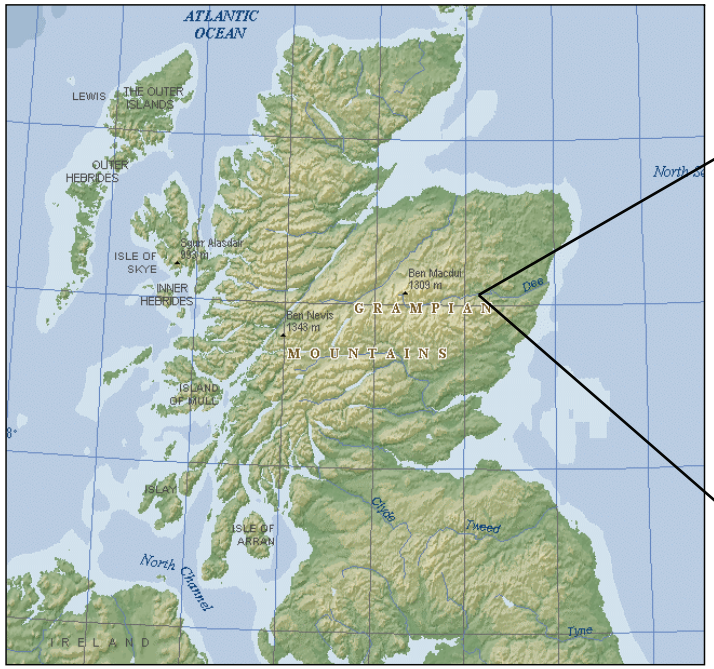


Figure 2.1. (a) Location of area within Scotland (b) Specific location of site (from OS NO69/70). Each square is equal to 1000m × 1000m

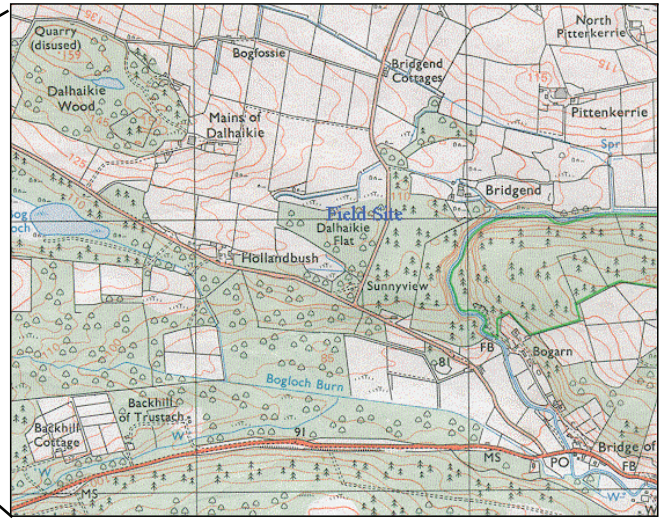


Figure 2.1. (c) 180° Panoramic view of Dalhaikie Flat, Aberdeenshire

If the solution to equation 2.1. is greater than zero, then the tree is *B. pendula* whereas negative solutions are indicative of *B. pubescens*. This technique was 93% accurate in the field when tested against chromosome number (Atkinson & Codling 1986).

$$(12 \times LTF) + (2 \times DTF) - (2 \times LTW) - 23 \qquad \textbf{Equ. 2.1}$$

In practice, *B. pendula* was relatively easily distinguished from *B. pubescens* without this procedure, but it was used at the beginning of this research to demonstrate that 41 out of

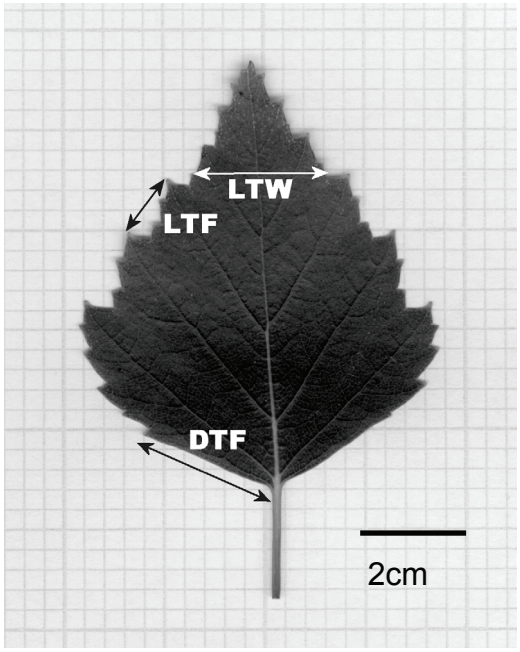


Plate 2.1. The three leaf biometric measurements described by Atkinson & Codling (1986) for distinguishing between *B. pendula* and *B. pubescens*.

50 birch trees at Dalhaikie Flat were *B. pendula* and also helped to classify 'border-line' individuals when they arose.

All trees used in this research were juvenile (*i.e.* not flowering), an important consideration for birch which grows particularly vigorously at this stage. Even so, latitudinal factors and the strong competitive effects operating in such dense birch thickets probably ensured that trees seldom reached their potential growth rate of 3.4cm week^{-1} (Kelly & Mecklenburg 1980). Ninety-five percent of survey trees for instance, only achieved between 9 and 23cm of vertical growth within the 1999 growing season (18-22 weeks).

2.1.2. The aphid

The aphid *Euceraphis betulae* Koch (Homoptera: Drepanosiphidae) was the focal species of the indirect interactions investigated in this research, and as is discussed in Chapter 3 was the dominant insect-herbivore of *B. pendula* at Dalhaikie Flat. Until their distinction on cytological and morphological grounds by Blackman (1976, 1977) only one species, *Euceraphis punctipennis*, was believed to feed on both *B. pendula* and *B. pubescens*. Box 2.1 and Table 2.1 show the main morphological differences between the two species, based largely on the original descriptions of Blackman (1977). With their distinction came the realisation that *E. betulae* is found predominantly on *B. pendula* and *E. punctipennis* predominates on *B. pubescens* (Blackman 1977). This conclusion was tested empirically by Mahdi & Whittaker (1993) at their site in Northern England, where 97% of *Euceraphis* aphids feeding on *B. pendula* were identified as *E. betulae*. Of those collected from

Hind tarsus is proportionately or actually longer in *Euceraphis betulae* compared to *E. punctipennis*, hence the ratio of ;

$$\frac{\text{Length of basal antennal segment VI}}{\text{Length of hind tarsal length II}}$$

is an absolute partition between the two species;

	Eb	*Ep*
Fundatrix	< 1.23	> 1.25
Virginopara	< 1.32	> 1.34
Sexupara	< 1.32	> 1.32
Ovipara	< 1.12	> 1.15
Males	< 1.36	> 1.35 (*sic*)

Box 2.1. Distinction between *E. betulae* and *E. punctipennis* can be made by biometric differences common for all morphs, and definitively with the ratio of antennal and tarsal lengths (after Blackman 1977).

Table 2.1. Distinction between *E. betulae* and *E. punctipennis* can be made by small, but consistent differences in pigmentation (after Blackman 1977).

	Differences in Pigmentation	
Life Stage	***Euceraphis betulae***	***Euceraphis punctipennis***
Fundratrices	Dark sclerotic transverse bars on the dorsal abdomen – particularly on tergites III, IV, V and VI.	Either without pigmentation on dorsal abodomen or else just on tergites III and IV. Never have complete tranverse bars.
First generation virginoparae larvae	Pale yellow-green.	Pale green.
First generation virginoparae adults	Pigmentation on antennal segments III and IV.	No pigmentation on antennal segments III and IV.
	Fore tibia is distally pigmented and rough.	Only the extremity of fore tibia is pigmented and rough.
Second generation virgioparae adults	*Euceraphis betulae* is much paler than earlier generations so differences in pigmentation are unreliable.	
Sexuparae	Dorsal abdomen often has pigmentation on tergites IV and V and complete transverse bars on the tergite sometimes seen.	Dorsal abdomen often has pigmentation on tergites IV and V but complete transverse bars on the tergite never seen.
Oviparae and Males	Intra-specific vartiation in pigmentation makes differentiation unreliable.	

B. pendula (30 individuals) at Dalhaikie Flat, all were identified as *E. betulae*. *E. betulae* is described further in Chapter 3.

2.1.3. The fungal pathogen

Leaf-spot pathogens of birch are poorly understood and only a few species have a described sexual phase to their life-cycle, although there is little doubt that most possess a sexual morph (Edmonds, Agee & Gara 2000). The asexual phases that dwell on living

tissues are better understood, although it is indicative of the state of knowledge about birch fungal pathogens, that some of the best descriptions are a century old (*e.g.* Allescher 1901). Using existing descriptions (Allecher 1901; Peace 1962; Bennell & Millar 1984; Sinclair, Lyon & Johnson 1987; Strouts & Winter 1994; Kurkela 1995) and recent research into birch leaf-spot pathogens (Paavolainen, Hantula & Kurkela 2000; Paavolainen *et al.* 2001) the main fungal pathogen associated with necrotic leaf spots at Dalhaikie Flat was identified as *Marssonina betulae* (Lib.) Magn, (Deuteromycotina: Melanconiales), the pathology of which is described in Chapter 4.

2.1.4. The Leaf-miner

The leaf-mining larval stage of the Lepidopteran genus *Eriocrania* Zeller (Eriocraniidae) was also found at the site, albeit infrequently during 1999, 2000 and 2001. The exclusively birch feeding *Eriocrania* genus consists of six individual species, five of which are indistinguishable as larvae and whose life history is essentially identical (Heath 1976) allowing them to be treated as a single taxon in this and other research (Koricheva & Haukioja 1994; Fisher, Hartley & Young 1999, 2000). The *Eriocrania* life-cycle is univoltine (one generation per year) and closely parallels the phenology of their host plant (Figure 2.2). Adults fly in spring and oviposit under the epidermis of newly-emerged buds (Koricheva & Haukioja 1994). The larvae emerge from the eggs 2-3 weeks later and begin to feed internally on leaves, forming a blotch mine as they progress (Plate 2.2). Once leaf-miners are ready to emerge they tear open the leaf lamina and drop to the soil to overwinter, and being univoltine, only emerge as adults in the following spring. Adult *Eriocrania* spp. were scarce at Dalhaikie Flat and so sweep-net capturing was limited to a few individuals which were identified as *Eriocrania semipurpella* Stephens. The interaction between *Eriocrania* spp. leaf-miners and *Euceraphis betulae* is examined in Chapter 6.

2.2. EMPIRICAL PROCEDURES

2.2.1. Confinement Experiments

All manipulation experiments relied upon aphids being confined on one or more birch leaves and being isolated from other insects. This was achieved using clipcages, modified Blackman boxes and polyester Organza™ bags, depending upon the number of leaves required.

Figure 2.2. The life-cycle of the *Eriocrania* spp. leaf-miner. Whilst the months shown are typical of the leaf-miner life-cycle at Dalhaikie Flat, the actual timing can be relatively variable, for instance the 2001 generation was approximately one month later than the months shown.

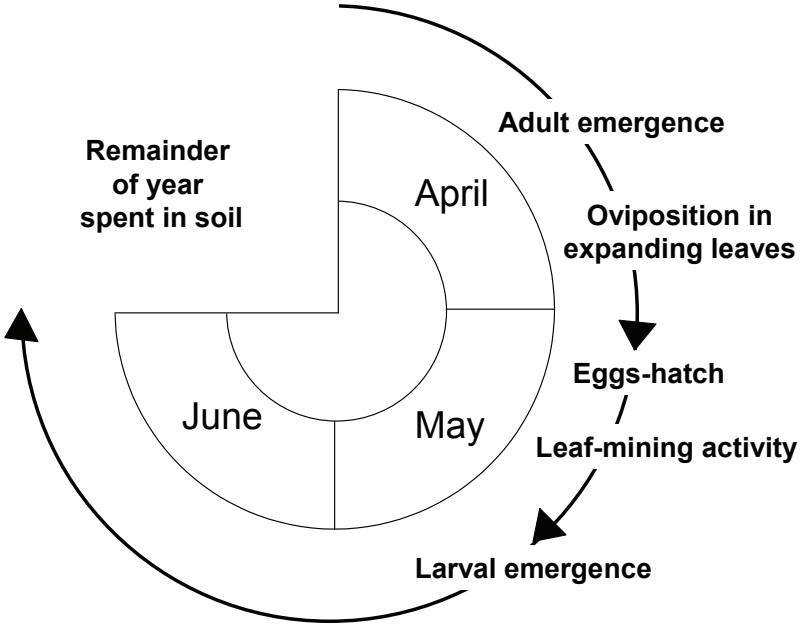


Plate 2.2. Blotch mine of the *Eriocrania* spp. leaf-miner on *B. pubescens*. Note larva in the central region and convoluted frass deposits. (Photograph courtesy of S.E. Hartley)

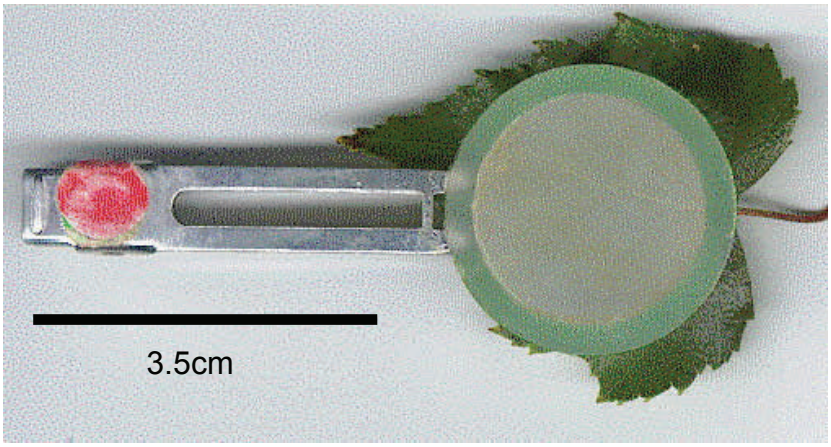


Plate 2.3. Mesh covered clip-cages were used for confining aphids on single leaves.

Mesh covered clip-cages (Plate 2.3), with an internal chamber area of 4.5cm^2 were used where aphids were to be confined on single leaves. It is acknowledged that although they are widely used in such experiments, clip-cages may inadvertently impose experimental artefacts, for example changes in plant physiology, on the system (Crafts-Brandner 1999). Where several leaves were required, for instance in choice tests, modified Blackman boxes (Adams & Douglas 1997) (Plate 2.4) were used. Each box (75mm x 44mm x 22mm) was constructed from transparent Perspex™ but modified so that it bore an aperture (∅ 5mm) at the top and the internal partition was removed. A mesh covered window on the retractable door allowed air movement and putty was inserted into the gap between the stem and box aperture. All experiments involving Blackman boxes were conducted over a relatively short period of time (<36 hours).

Plate 2.4. Modified Blackman boxes were used when aphids were caged on several leaves (inoculated leaf choice test pictured).

Leaves inside clip-cages and Blackman boxes showed no physical signs of deterioration such

as desiccation, chlorosis or wilting (except when inoculated) and stringent controls were used in all experiments so any effects would be seen in all treatments.

Experiments involving more than ten leaves were conducted using polyester Organza™ bags (Plate 2.5) (∅ 160mm x 700mm) which were secured on branches using foam strips and wire. As with clip-cages, there may be incidental effects of enclosing branches in

Plate 2.5. Polyester Organza™ bags were used where more than ten leaves were required. The bag is secured around the branch with foam and wire.

such bags, though similar procedures have been shown to have negligible effects on internal temperature (Way & Banks 1968) and plant chemistry (West 1985).

2.2.2. Simulating leaf-miner damage and pathogen inoculation

In addition to experiments with naturally occurring *Eriocrania* spp. leaf-miners and the *M. betulae* pathogen, experiments were also conducted using inoculated foliage (Plate 2.6) and simulated leaf-miner damage. These techniques are discussed in detail in Chapters 5 and 6 respectively.

2.2.3. Assessing aphid performance

Assessing how well aphids perform in experimental situations was a critical consideration throughout this research and has received a good deal of attention elsewhere (van Emden

Plate 2.6. Leaves were inoculated with the spore suspension using a modeller's airbrush to apply an even dose.

1972). Measures of performance used here represent those that were: reliably recognisable, quantifiable and showed low variability within the species.

The fresh mass of both individual and populations of aphids was used as indices of performance. Individual adult aphids were recovered in the clip-cages they had occupied throughout the experiment. They were subsequently manipulated using ultra-fine artists' paintbrushes into pre-weighed 0.2 ml Eppendorf tubes so that they could be weighed on a micro-balance (Cahn C-31, Cahn Instruments ™, California) to an accuracy of ± 1 µ g. The total biomass of aphid populations was measured by swiftly transferring all aphids from polyester Organza ™ bags into pre-weighed Petri-dishes under cool conditions (2.5 ° C ± 1.0 ° C) and weighing each dish on a balance (Precisa 125A ™, Zurich, Switzerland) to an accuracy of ± 0.1mg.

Fresh and preserved aphids were dissected to quantify external biometric features and characterise embryo content. Aphids were preserved by placing them in successively higher concentrations (50, 65 and finally 80%) of ethanol (>99% pure, Fisher™), before being transferred into 0.2 ml Eppendorf tubes and stored at 2 ° C ± 1.0 ° C. Dissection of

fresh and preserved aphids was carried out using pins in a drop of ice-cold 50mM Tris-HCl pH 7.5 (>99% pure, Fisher). With the aid of a dissecting microscope (Leica MZ3) at × 160 magnification the length of both hind tibiae was measured and number of embryos scored. Where present, the length of the most basal embryo bearing pigmented eyespots was also determined. All measurements were made using a calibrated eyepiece micrometer.

The performance of aphids was also assessed *in situ* during field trials. For individual aphids, development (using morphology and exuviae counts) and survival were monitored. In population experiments, the mortality, fecundity and adult/larval ratio of the aphid population was scored at the end of the experiment.

2.3. SURVEY PROCEDURES

As well as being essential information in this study, survey data about the occurrence of aphid *E. betulae* on *B. pendula* is rare, and at present there are no published data on the occurrence of *M. betulae* on *B. pendula*. Surveys carried out at Dalhaikie Flat aimed to redress this imbalance, by systematically recording the occurrence of aphid and pathogen on *B. pendula* in a non-destructive manner during the *Betula* growing seasons of 1999 and 2000. Whilst in practice surveys of the aphid and pathogen were carried out simultaneously on the same trees, their occurrences on *B. pendula* are reported separately in Chapters 3 and 4 respectively, and the influence of *M. betulae* on the distribution of *E. betulae* is considered in Chapter 5.

Thirty *B. pendula* trees of approximately the same height (1.5-2.2m) were selected and labelled for study during April 1999, whilst bud burst was underway. Fifteen branches from the upper canopy (top half) and 10 from the lower (bottom half) were chosen as sampling units. Labels were placed below the fifth leaf node from the apex of each branch and leaf nodes numbered consecutively from this point to the apex. The unpredictable growth of branches, for instance from leaf nodes within the initial sampling unit, was countered by continually redefining the sampling unit as the first three or more leaf nodes from the original label (Figure 2.3). Leaves were classified as basal, intermediate or apical according to their position of the branch (basal being closest to the label and apex being furthest away). Leaves were considered to be at the same node if their axes were less than

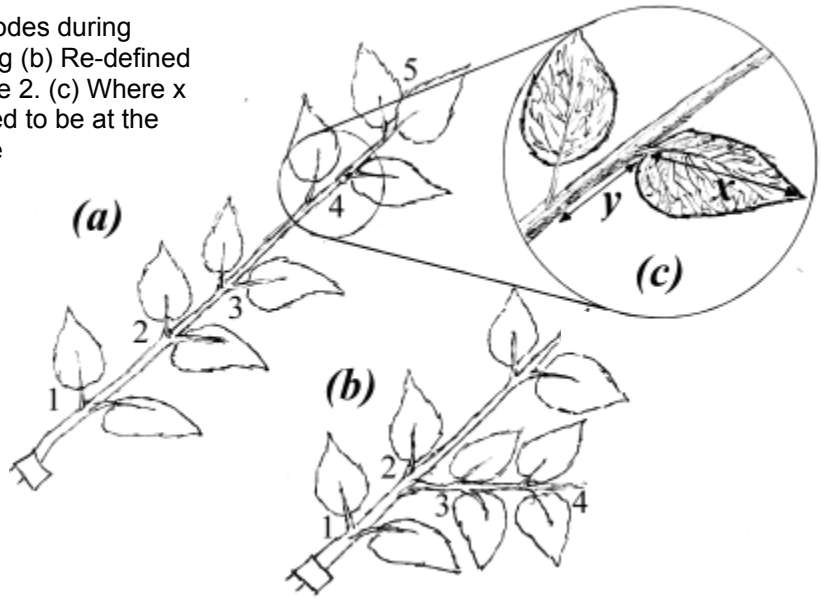


Figure 2.3. Numbering of nodes during surveys. (a) Original labelling (b) Re-defined labelling after growth at node 2. (c) Where x > y the leaves are considered to be at the same node. Where x < y the leaves are separate nodes. In the original labelling (a) Basal node is 1; intermediate nodes are 2,3 & 4; apical node is 5. After growth (b), basal is 1; intermediates are 2 & 3 and apex is 4.

perpendicular to each other and the distance that separated them was less than the length of the leaf (Figure 2.3).

The number of adult and larval aphids occurring on the stem or adaxial and abaxial leaf surfaces was recorded non-destructively for each node, as was the occurrence of other insects found within the sampling unit (see Chapter 3). When fungal lesions became apparent on leaves (late May – June) they too were recorded (see Chapter 4). Surveys completed in 2000 were carried out using 12 of the original trees that displayed the highest and lowest levels of fungal infection during 1999 (six trees of each).

2.4. PLANT CHEMICAL ANALYSIS

Leaves for chemical analysis were excised from trees using sharp scissors and immediately transferred to a darkened coolbox where they were stored on ice to prevent further changes in plant chemistry; a precaution suggested by Waterman & Mole (1994).

2.4.1. Analysis of plant nitrogen concentrations

The main carbon and nitrogen sources used by aphids are sugars (notably sucrose), and amino acids (Dixon 1998) which are obtained from the phloem sap on which they feed. The amino acids are considered to be particularly important, as dietary amino-nitrogen is often the limiting factor in aphid nutrition (Dixon 1998). Total nitrogen content of leaves

is often used as a surrogate, albeit a poor one, for the amino-nitrogen concentration of phloem sap, since the sap itself can be difficult to assay (Douglas 1993).

In this research, the free amino acid concentration of foliage was investigated using reverse-phase high performance liquid chromatography. One leaf from each tree was used for soluble amino acid analysis by macerating 30 mg leaf in 0.5ml 80% HPLC grade methanol on ice. After centrifugation at 1200 rpm, the supernatant was stored at –18 ° C until assayed using a similar procedure to that described by Inaba *et al.* (1994). Supernatants were loaded on a Dowex 50 cation exchange column followed by 7 x 300 µ l of deionised water. Amino acids were eluted with 2 x 300 µ l and 4 x 600 µ l of 5M NH_4OH. The eluant was evaporated to dryness for 24 h, and the residue dissolved in 0.5ml 2M HCl and analysed by reverse-phase high performance liquid chromatography (HPLC) with pre-column derivitisation using *o*-phthaldialdehyde according to Jones, Paabo & Stein (1981). Amino acids were separated on a Xorbax ™ Eclipse XDB-C8 column at 20 ° C using a Hewlett-Packard HP 1100 delivery system and fluorescence detector. Amino acids were quantified by comparison of sample peak areas to three level calibration plots of a reference amino acid mixture, AA-S-18 (Sigma, UK), supplemented with glutamine, asparagine and tryptophan. All protein amino acids except proline and cysteine were detected to an accuracy of 0.5 pmol per sample.

Eriocrania is a leaf-mining, chewing insect that feeds on leaf tissue between the upper and lower lamellae, and is probably like most phytophagous insects in that availability of nitrogen is limiting in its diet (Mattson 1980; Waring & Cobb 1992). Unlike *E. betulae* which is a free-living phloem feeder, *Eriocrania* larvae cannot move between feeding sites and must consume the tissues that surround it to ensure growth. The total amount of nitrogen in leaves is a more relevant measure of leaf quality for *Eriocrania* therefore, and whilst it may not represent the absolute nutritional value of a leaf to *Eriocrania* (*e.g.* not all nitrogen might be in a useable form), it provided a nutritional index in *Eriocrania* performance experiments. It was also used to examine any broad differences in foliar nitrogen content of differently mined leaves.

Total carbon and nitrogen content of birch leaves were analysed using a C, H, N analyser. This analytical technique measures the amount of carbon and nitrogen in materials by the

combustion of samples in pure oxygen at high temperatures. The combustion products are separated and quantified by passing them through a thermal conductivity detector, which uses the different electrical conductivity properties of gases to quantify the amount of carbon and nitrogen in samples.

Leaves were freeze-dried for 36 h before being milled through a 1 mm mesh and stored in anhydrous conditions at room temperature prior to nitrogen analysis (NA2100 Brewanalyser, CE Instruments). Leaf samples (*ca.* 5mg) were loaded into tin capsules (Elemental Microanalysis, UK) and combusted in the presence of a chromium oxide catalyst (Chromosorb W, Elemental Microanalysis, UK). Cobalt oxide catalysts were used to remove S, Cl, Br, I, P and F from the gaseous product. Gases were then separated in a Poropack 25 separation column before being quantified in a thermal conductivity chamber using a calibration curve produced from a urea standard (100 mg l^{-1} HPLC grade water) (Thermoquest, UK).

2.4.2. Analysis of phenolic compound concentrations

Phenolic compounds are defined as compounds that possess one or more hydroxyl groups bonded to an aromatic carbon ring, the simplest being phenol. They are ubiquitous in plants having functions ranging from structural support to signaling (Waterman & Mole 1994 and references therein). Phenolic compounds have traditionally been regarded as important in herbivore-plant interactions, having several important functions including roles in the defensive response by the plants to herbivore attack. Phenolic compounds in birch are thought to play a major role in plant resistance to herbivore or pathogen attack, essentially for three reasons: (a) birch foliage contains very high concentrations of phenolics (often above 10% of dry mass), (b) one group of birch phenolic compounds, the tannins, cause protein precipitation, which is generally deleterious to insect digestion and pathogen colonisation (c) after leaf damage, foliar phenolic compound concentrations increase and remain at high levels for some time after defoliation (Hartley & Lawton 1987; Ossipov *et al.* 2001).

The measurement of total phenolic compound concentrations in leaves is common in many ecological studies, and although it provides no compositional information about

particular compounds, it gives a good indication about likely relative palatability and digestibility of foliage to herbivores. The most common methods to quantify total phenolic compound concentrations of leaves are the Folin-based procedures (Waterman & Mole 1994). These colorimetric procedures rely upon a reduction-oxidation reaction in which the phenolate ion of phenolic compounds becomes oxidized whilst the phosphomolybdic complex in the reagent (*e.g.* Folin-Denis or Folin-Ciocalteau reagent) is reduced in the presence of an alkali (sodium carbonate) turning it into a blue-coloured solution, the intensity of which can be measured using a spectrophotometer and compared against a standard.

Birch leaves were freeze-dried for approximately 36h prior to analysis for phenolic content, milled through a 1 mm mesh, before analysis for total phenolic compound content using the Folin-Ciocalteau method (Waterman & Mole 1994; Kerslake, Woodin & Hartley 1998). 10mg of each sample was extracted in 10ml 50% methanol at 80 ° C for 30 minutes. The samples were centrifuged and the supernatant added to 2.9 ml distilled water together with 0.25ml Folin-Ciocalteau reagent and 1ml saturated Na_2CO_3. The absorbance of these samples was measured at 760nm using a UV-visible spectrophotometer (Camspec ™, UK) and phenolic content derived from a standard curve produced from a range of tannic acid (Fisher, UK) concentrations (9.78 - 78.38 pM).

2.5. CLIMATE DATA

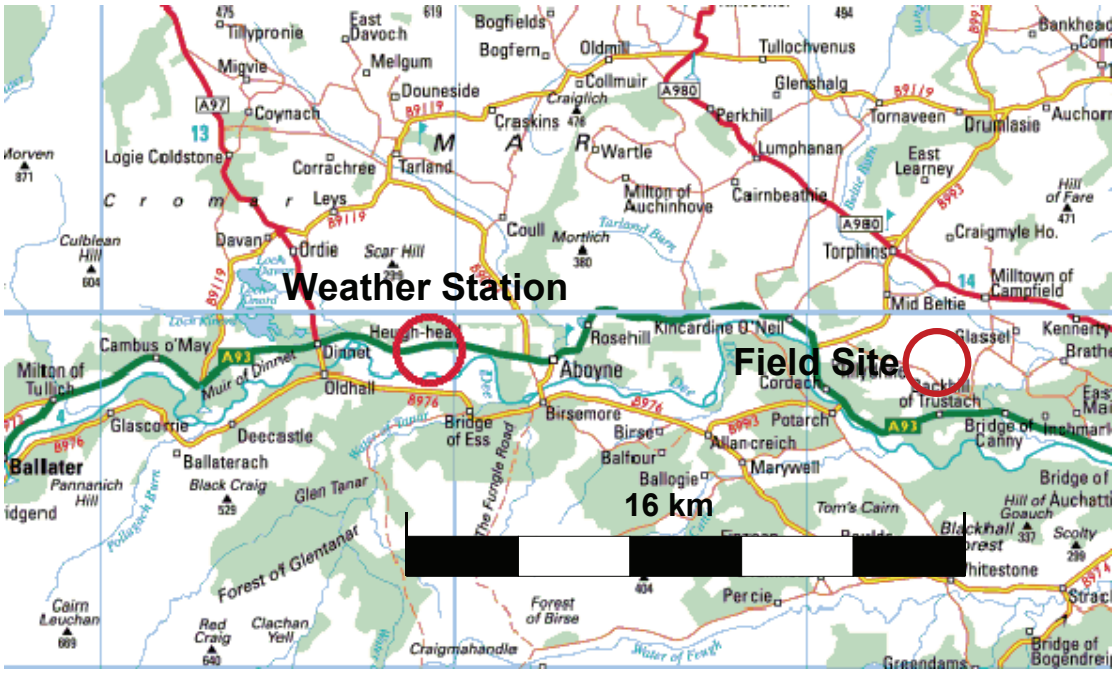
2.5.1. Meteorological Office Weather Station

Whilst some measurements of temperature were personally undertaken at Dalhaikie Flat, the climatic data recorded at the Aboyne weather station in 1999 and 2000 are used in Chapters 3 and 4. This synoptic automatic weather station (Met Office 2001) made hourly climatic measurements and was located 16km to the East of Dalhaikie Flat in Aboyne, Aberdeenshire (57 ° 76'N, 2 ° 836'W) (Ordnance Survey Ref. NO493 987) (Figure 2.4).

2.5.2. Climatic data

Air temperature was measured to an accuracy of ± 0.1 ° C using electrical resistance thermometers in which the quadratic relationship between the electrical resistance of platinum and temperature is used to measure the current air temperature. Wind speed was

Figure 2.4. Location of weather station in relation to the field site at Dalhaikie Flat.



measured in 0.515 ms^{-1} units (1 knot) using a Munro cup anemometer for 10 minutes every hour and averaged to an accuracy of ± 0.515 ms^{-1}. Precipitation was measured using a tipping bucket raingauge to an accuracy of ± 0.2 mm (Met Office 2001).

2.6. GENERALISED LINEAR MIXED MODELS

Generalised linear and generalised linear mixed models are a powerful and increasingly employed type of statistical analysis. Whilst their practice and theory is not part of this thesis, they were used recurrently in the analysis of survey data, so some basic information is appropriate.

A generalised linear mixed model is an extension of a fixed-effect linear model which basically permits the violation of two existing assumptions about a given data set; that the errors are normally distributed and that the response variable is equated to a linear combination of fixed and random effects.

$$y = x\beta \qquad \text{Equ. 2.2}$$

A fixed effect linear model with normal error distribution might be represented thus:

where the response, y, is related to the vector of

$$E(y) = x\beta \qquad \text{Equ. 2.3}$$

covariates, x, and coefficients β. The predicted values of the observations E(y) could also be written as:

So $x\beta$ becomes a linear model of $E(y)$. The basic premise of generalized linear models is that the parameters of a linear model, $x\beta$, can be estimated using maximum likelihood procedures (described below) to fit $x\beta$ to a *function* of $E(y)$ – not $E(y)$ itself.

Generalised linear models have three components which are central to their implementation: (a) the error structure (b) the linear predictor and (c) the link function.

2.6.1. Error structure

Ecological data quite often do not have normally distributed errors meaning that the response variable must either be transformed or else analysed using (frequently less powerful) non-parametric analysis. Generalised linear models however permit the error distribution to be specified. Count data (*e.g.* aphids per leaf) might be assumed to have a Poisson distribution, whereas survivorship (*e.g.* aphid is alive or dead) would have a binomial distribution.

2.6.2. The Linear Predictor

The linear predictor works by relating the mean of each observed response value, y, to a function of the predicted value E(y). The predicted value is derived by transforming (see the link function below) the value produced by the linear predictor, η. The linear predictor is the sum of the effects of the explanatory variables. The number of terms in the linear predictor is equal to the number of parameters to be estimated, so for a simple one-way ANOVA with three treatments, η is the sum of three terms; mean of the first treatment and the differences of the other two treatment means compared with the first.

2.6.3. The link function

The link function (g) links the mean value (μ) of the response variable (y) to its corresponding linear predictor (η) (Nelder & Wedderburn 1972):

$$\eta = g(\mu) \qquad \textbf{Equ. 2.4}$$

Table 2.2. The link functions used in the generalised linear mixed models in this research – determined in effect by the choice of error structure.

Type of Data	Link function	Formula	Error Structure
Aphid counts (*e.g.* aphids per leaf)	Log	$\eta = \log \mu$	Poisson
Aphid survivorship and occurrence of fungi on leaves (*e.g.* live or dead and infected or asymptomatic)	Logit	$\eta = \log\left(\frac{\mu}{n-\mu}\right)$	Binomial

The maximum likelihood procedure, as already mentioned, is a means of assessing which values of the model parameters best fit the data. The best fitting model, is the most minimal but adequate model to describe the data with the smallest amount of residual deviance. For analogy, the maximum likelihood estimate for linear regression would be an estimate of the intercept and slope of a relationship using least squares procedure. Generalised linear models use more general procedures to estimate the value of the model's parameters but the concept is essentially the same (see McCullagh & Nelder 1989). The degree of fidelity between fitted values produced by a generalised linear model and the actual data is revealed by the coefficient of dispersion value in the output.

Generalised linear *mixed* models differ from generalised linear models by allowing random effects to appear in the linear predictor. This is appropriate in situations where 'treatments' or factors are applied to a hierarchical organisation of an experimental system. In generalised linear mixed models, the parameters of a model are not estimated using full maximum likelihood procedures, but are estimated using quasi-likelihood procedures

(Littell *et al.* 1996). These procedures allow random effects to be fitted into the model in a hierarchical manner. For instance in the analysis of survey data here, survey tree number was fitted as a random term, and the branches examined belonging to that particular tree (themselves a random effect) nested within this term.

2.6.4. The 'GLIMMIX' macro

The GLIMMIX macro is a program devised for the SAS system (SAS Institute 1999) that provides a number of useful extensions to the conventional generalised linear models. A common problem with ecological data is that the data do not conform exactly to a described distribution, for instance count data often do not fit a Poisson distribution exactly because the variance is normally greater than the mean – termed 'overdispersion'. In addition to specifying the error distribution, generalised linear mixed models carried out using the GLIMMIX macro take into account this overdispersion by adjusting the variance using an overdispersion parameter (McCullagh & Nelder 1989).

2.6.5. Example of aphid survey data

The spatial occurrence of *E. betulae* on *B. pendula* was believed to be dependent on a number of factors such as position of leaves in the canopy and presence of fungal infection etc, therefore the mean number of aphids was modelled as being dependent on canopy position, position on branch, leaf fungal-infection and branch number within tree number. Aphid count data are most closely related to Poisson distribution, so the linear dependency of the model was via a log - link function to the mean number of aphids. The explanatory variables used in the model are shown in Table 2.3.

Table 2.3. An example of the explanatory variables used in the generalised linear mixed model analysis of the occurrence of *E. betulae* on *B. pendula*.

Explanatory variable	Variable type	Description
Canopy position	Fixed categorical	Upper or lower branches
Branch position	Fixed categorical	Basal, intermediate or apex leaves
Fungal-infection§	Fixed categorical	Infected or asymptomatic[1] High or low fungal infection[2]
Branch replicate	Random categorical	10 replicates of upper and 15 replicates of lower branches
Tree replicate	Random categorical	30 replicates (branch replicate nested within tree replicate)

§ Two indices of fungal infection were used in this research: [1] presence or absence of lesions in Chapter 4 and [2] low or high fungal infection in Chapters 3 & 5.

CHAPTER THREE

Distribution and abundance of *Euceraphis betulae* on *Betula pendula*

The aphid Euceraphis betulae *is a common and abundant herbivore of* Betula pendula*, apparently occurring over the full geographical range of its host plant. It was the dominant insect herbivore of birch at Dalhaikie Flat during 1999 and 2000, although its population was considerably smaller in 2000 than the previous year, probably due to a complex interplay of factors including later egg-hatch and the small number of egg-laying oviparae in the previous autumn. The high proportion of larvae in the population in the 1999 season suggested that the aphids were reproducing throughout the season. The spatial dynamics of the aphid population within a tree paralleled host plant phenology, generally being more uniformly distributed throughout the canopy at bud-burst and leaf-fall, but switching to the actively growing upper branches and apical leaves in between.*

3.1. INTRODUCTION

3.1.1. Rationale

The unifying theme of this thesis is how the aphid *Euceraphis betulae* Koch (Homoptera: Drepanosiphidae) is indirectly affected by other organisms that share a common host plant, *Betula pendula* Roth. To understand the processes that underpin these interactions, it was first necessary to explore the occurrence of *E. betulae* on *B. pendula* in natural situations. The abundance and spatio-temporal distribution of the aphid helped to assess the importance of any indirect interactions in the context of birch-feeding insects in general, and exploring the occurrence of other insects on *B. pendula* gave some indication of the relative abundance of the aphid in relation to sessile and easily observable insect herbivores at this site. Furthermore, the evaluation of any association between aphids and birch leaves infected with a fungal pathogen in natural situations (discussed in Chapter 5) relied upon a wider understanding of the underlying spatial distribution of *E. betulae* on asymptomatic birch leaves (*i.e.* those that lacked visible evidence of fungal infection).

3.1.2. Aphids on birch

Apart from oak (*Quercus*) and willow (*Salix*), birch (*Betula*) trees possess the largest number of insect species of all deciduous and coniferous trees in Britain (Southwood 1961, Southwood *et al.* 1982). Between 5-10% of these are aphids, accounting for 20 species belonging to 10 genera (Blackman & Eastop 1994).

Euceraphis betulae is one of the more abundant and common aphids of birch, considered to occur in the United Kingdom wherever *B. pendula* is found (Stroyan 1977) and is normally recorded in large numbers by suction traps used by the Rothamsted Insect Survey (IACR 2001). Despite this fact, *E. betulae* has received relatively little attention compared with other boreal aphids (*e.g.* the sycamore aphid, *Drepanosiphum platanoidis*), and existing research on this species has largely concentrated on Scandinavian (*e.g.* Heie 1972) and North American populations (*e.g.* Hajek & Dahlsten 1988).

Part of the reason for the apparent disinterest in *E. betulae* might lie in the fact that birch is commercially unimportant in most countries and, unlike many plants, is thought to tolerate aphid infestations reasonably well. The tolerance of *Betula* to aphid infestation

may be overestimated however; Nieminen *et al.* (2000) for instance, recently suggested that *Euceraphis* infestations caused early senescence and developmental abnormalities in many young birch trees in Finland.

3.1.3. Phylogeny and morphology

Euceraphis betulae belongs to the family Drepanosiphinae, tribe Phyllaphidini and occurs throughout Europe on native *Betula pendula* and on trees introduced to North America, Australia and New Zealand (Blackman & Eastop 1994). The name *Euceraphis betulae* is additionally applied to a species complex of morphologically-similar aphids occurring on a number of birch species throughout the world. They are distinguishable by their karyotypes alone, and have been reported on numerous birch species including *Betula occidentalis* and *Betula papyrifera* in North America and *Betula ermanii* and *Betula platyphylla* in East Asia (Blackman & Eastop 1994).

As discussed in Chapter 2, *E. betulae* was considered to be the same species as *E. punctipennis* until their distinction on cytological and morphological grounds by Blackman (1976, 1977). Despite these small differences, their life-cycles and ecologies are essentially synonymous and reference is made to research on *E. punctipennis* (Wratten 1974; Parry 1985) in this thesis.

Euceraphis betulae is a comparatively large aphid, with a body length of 3.5 – 4.2mm. The life-cycle (Section 3.1.4.) consists of several morphs, and as with the rest of the genus, all but the oviparae are winged aphids that secrete a blueish-white wax forming irregular tufts on the legs and antennae. The immature *E. betulae* fundatrix is dark yellow – brown (cf. the yellow-green appearance of *E. punctipennis*) which at adulthood (like the successive generations of virginoparae) becomes pale green with dark patches on the thorax and head. The virginoparae (Plate 3.1 & Figure 3.1) possess long antennae which may equal their body length, a truncate siphunculi which can range from pale to a black appearance (cf. *E. punctipennis*), and a pale or brownish knobbed, constricted cauda (Figure 3.1). Successive generations of virginoparae show less pronounced dark pigmentation than the first. The pigmentation of successive generations of virginoparae shows considerable intra-specific variability making generalisations difficult. The sexuparae are very similar to virginoparae and could not be distinguished by Blackman (1977) or at Dalhaikie Flat.

Plate 3.1 *E. betulae* virginopara on *B. pendula* leaf (probably first generation). Photograph courtesy of G.W. Hopkins.

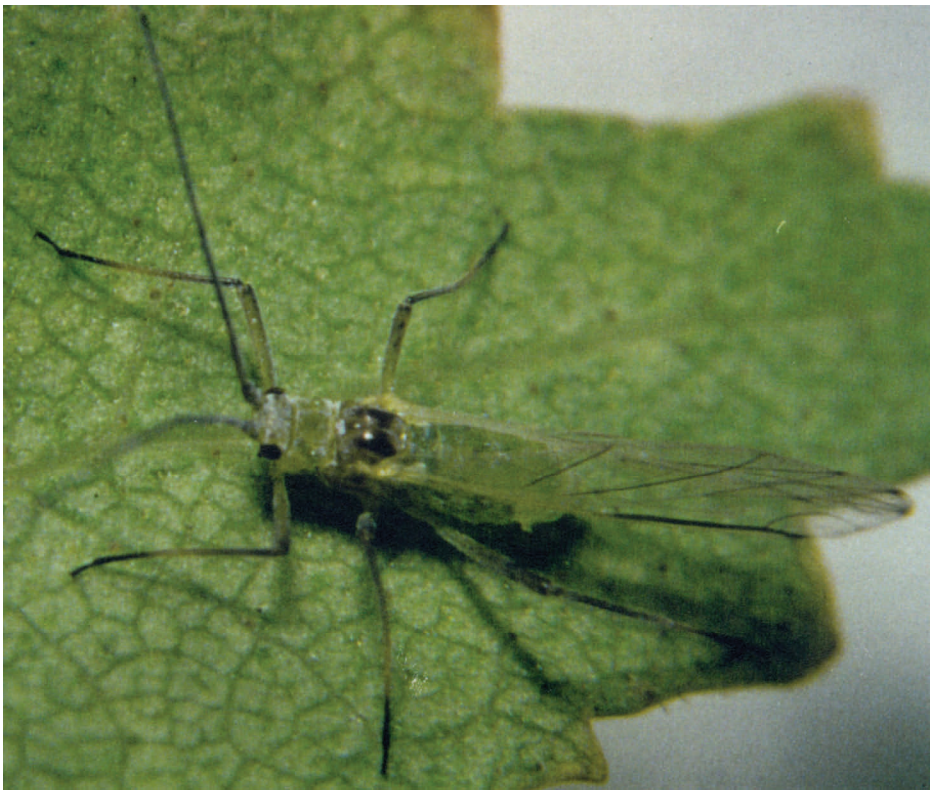
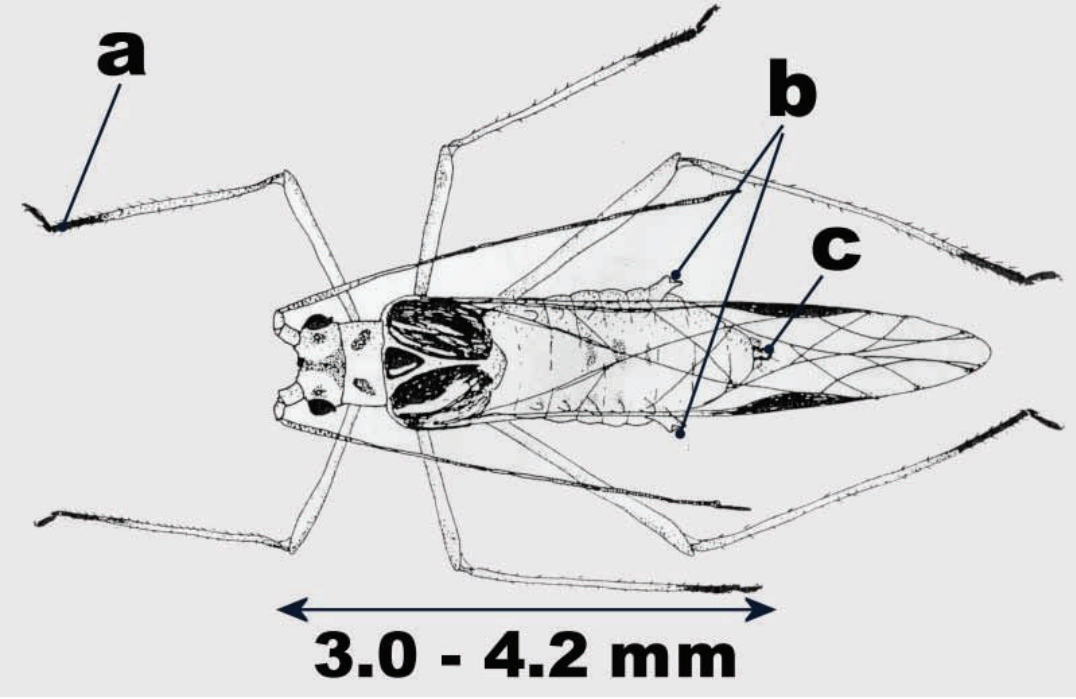


Figure 3.1. Diagram of first generation *E. betulae* virgionopara (after Heie 1982) as above. Note the distal pigmentation of fore tibia (a) which distinguishes *E. betulae* from *E. punctipennis* in which it is only present at the extremities. Note also, the truncate siphunculi (b) and knobbed, constricted cauda (c) which distinguish larval virgiopara from larval ovipara.



Males could not be distinguished reliably in the field either, but are generally smaller and more slender than virginoparae and normally possess darker antennae. Oviparae were distinguishable in the field by their yellowish appearance and their prolonged and conical abdomen that function as an ovipositor. In contrast to immature virginoparae, oviparae do not secrete wax, their siphunculi have a developed flange and the cauda is unrestricted (Blackman 1977; Stroyan 1977; Heie 1982; Blackman & Eastop 1994).

3.1.4. Life Cycle

The life-cycle of *E. betulae* is holocyclic and monoecious, meaning that it contains sexual and asexual phases that both live entirely on *B. pendula* (Figure 3.2). *E. punctipennis* in contrast is found on *B. pubescens* (Blackman 1977; Heie 1982; Mahdi & Whittaker 1993; Blackman & Eastop 1994) which has not been planted widely outside its natural range, thereby limiting the geographical range of *E. punctipennis*.

Over-wintered eggs hatch in late April or May in Scotland; in late March or early April in Southern England (Wratten 1974) and Denmark (Heie 1972) and late May in Russia (Kula 1993). Egg-hatch normally coincides with budburst and the fundatrices often feed on swelling buds and young leaves. Fundatrices usually reach adulthood a few weeks later and larviposit without a reproductive delay, giving rise to the first generation of virginoparae (the fundatrigenia) which reproduce parthenogenetically. This initial phase of the life-cycle occurs during a period when leaves and phloem sap of birch possess high levels of soluble nitrogen (Haukioja *et al.* 1978) and as a result aphid reproduction is high (Wratten 1974; Hajek & Dahlsten 1988). As the season progresses the levels of soluble nitrogen in leaves and phloem sap become lower and *Euceraphis* often persist as non-reproducing adults for a number of weeks during this period (Wratten 1974; Hajek & Dahlsten 1986, 1988). The sycamore aphid, *Drepanosiphum platanoidis*, also undergoes a similar mid-summer reproductive diapause (Dixon 1975) for the same reason, a strategy in which maternal growth is effectively decoupled from embryonic growth and adults possess only developmentally immature embryos (Douglas 2000). The cessation of growth and maintenance of only developmentally immature embryos, allows diapausing adults to incur relatively low nutritional costs whilst retaining the capacity to respond rapidly and develop embryos when conditions become more favourable.

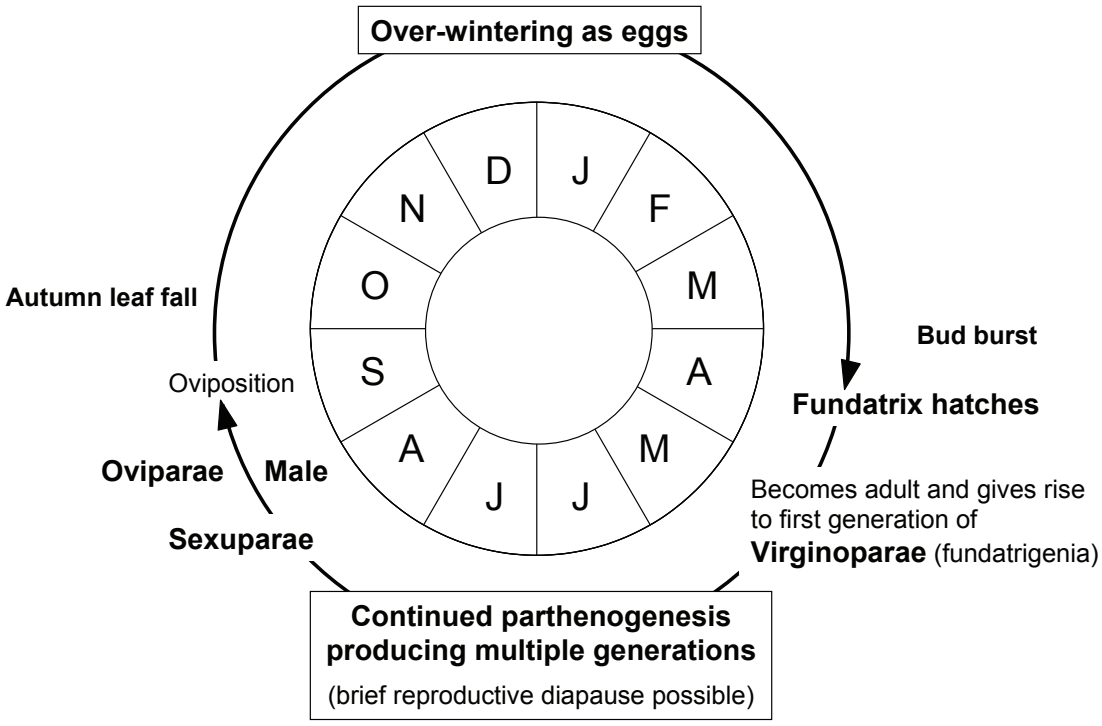


Figure 3.2. Life-cycle of *E. betulae* which occurs entirely on *B. pendula*.

Reproduction normally resumes shortly before the onset of autumn when soluble nitrogen levels in birch leaves and phloem sap increase due to translocation of soluble nitrogen which occurs in many deciduous trees during leaf senescence (Chapin & Kedrowski 1983). Because birch continually flushes new foliage throughout the season and some leaves become prematurely senescent it is thought that *Euceraphis* is not deprived of developing or senescing foliage for long periods during summer (Wratten 1974; Hajek & Dahlsten 1988). The nutritional condition of sycamore leaves, in contrast, is likely to be more uniform as these leaves are phenologically synchronous (Dixon & MaKay 1970), perhaps depriving *D. platanoidis* of nutritious foliage to a greater extent in the middle of summer .

Sexuparae are observed from September in Southern England (Blackman 1977) and Denmark (Heie 1972) although they could not be distinguished reliably in the field in this research. Oviparae are produced by the sexuparae in September in Scotland and October

in Russia (Kula 1993). Oviparae oviposit eggs in the axil buds and stems of birch which then over-winter until the following spring.

3.1.5. Ecology

Aphids feed almost exclusively on phloem vessels located in many plant parts ranging from roots to fruiting bodies (Raven 1983). With the exception of *Monaphis antennata*, all birch aphids feed predominantly on the abaxial surfaces of leaves (Blackman & Eastop 1994) and sometimes on the adjoining petiole (Hajek & Dahlsten 1986). *E. betulae* has a particularly long stylet (approximately 0.4 - 0.5mm long at adulthood) compared with other birch aphids, allowing it to penetrate the largest leaf veins where the phloem is more deeply embedded, but the highest flux of phloem sap occurs (Hajek & Dahlsten 1986). *Euceraphis* shows a marked preference for developing and senescing leaves (Wratten 1974; Fowler & Lawton 1984; Hajek & Dahlsten 1986, 1988), sometimes even feeding on swelling buds before leaves have begun to emerge (Heie 1982). *E. betulae* normally feeds on the major leaf veins on the abaxial leaf surfaces and the adjoining petiole, with gravity apparently being an important cue in locating feeding sites on a particular leaf (Hopkins & Dixon 2000). *Euceraphis* is extremely mobile and can move around the tree canopy, allowing it to settle on the more nutritious developing and senescing leaves (Wratten 1974; Hajek & Dahlsten 1986, 1988).

As might be expected from a highly mobile aphid, *E. betulae* does not live in colonies in the strictest sense (Heie 1982), but several authors have noted patterns of aggregation during certain periods of the birch growing season (Wratten 1974; Hajek & Dahlsten 1988). As already stated, birch is a highly heterogeneous resource and it might be supposed that *E. betulae* displays patterns of aggregation that correspond with changing plant phenology. For instance, *E. betulae* might aggregate on the most nutritious foliage (that which is either developing and senescing) which is not abundant during mid-summer, but become randomly or uniformly distributed when developing and senescing leaves are widespread during Spring and Autumn respectively.

Coexistence between *E. punctipennis* and *E. betulae* with other birch aphids has been reported in the past (Table 3.1). There is a general consensus that morphological features (stylet length and body mass) and consequent differential feeding site mediate niche

differentiation within the guild, reducing the scope of inter-specific interactions between birch aphids except when population densities are very high. When high densities do arise birch aphids may potentially affect one another through host plant deterioration.

Table 3.1. The co-existence of birch aphids on shared birch leaves.

Euceraphis Species	Coexisting Aphid Species	Reference
E. betulae	*Callipterinella calliptera* *Betulaphis brevipilosa*	(Hajek & Dahlsten 1986)
E. punctipennis	*Betulaphis quadrituberculata* *Callipterinella minutissma*	(Dixon 1998)

Euceraphis betulae populations introduced to North California are known to have a number of natural enemies including aphid-specific and polyphagous predators and several parasitoids (Hajek & Dahlsten 1988). The most abundant predator of *E. betulae* in North California was the coccinellid, *Adalia bipuncta*, for which *E. betulae* is particularly suitable and nutritious prey (Kalushkov 1998). Despite this, Hajek & Dahlsten (1988) conclude that the presence of considerable numbers of *A. bipuncta* did not have a significant impact on the development of large populations of *E. betulae*. This may in part be due to the ability of *E. betulae* to avoid predation by *A. bipuncta*. For example in 'arena experiments' the apparently acute visual perception of approaching predators and the long legs of *E. betulae* allowed it to escape predation from *A. bipuncta* more than any other birch aphid investigated (Hajek & Dahlsten 1987).

Euceraphis betulae is not attended by ants (Stroyan 1977; Heie 1982), and several authors have reported the predation of *E. betulae* (Hajek & Dahlsten, 1988; Mahdi & Whittaker 1993) and *E. punctipennis* (Karhu 1998) by the wood ants *Iridomyrmex humilis*, *Formica rufa* and *Formica aquilonia*. The significance of ant predation in shaping *E. betulae* populations however, was uncertain.

Of the parasitoids of *E. betulae*, Hajek & Dahlsten (1988) suggest that the hymenopteran *Praon flavinode* is amongst the most common in North California, and yet they observed mummies infrequently and those that were, had either been partially eaten by predators or were desiccated.

3.1.6. Aims of surveys

Field surveys were undertaken to investigate the distribution and dynamics of *E. betulae* on *B. pendula* at Dalhaikie Flat during 1999 and 2000. The specific objectives of these surveys were to:

a. investigate the spatial and temporal variation in *E. betulae* abundance in relation to host plant phenology, temperature, wind velocity and between-year effects,
b. explore the underlying spatial distribution and feeding site preferences of *E. betulae* on a natural stand of *B. pendula*, and how they may change within the season,
c. record the occurrence of insect herbivores and natural enemies on leaves.

3.2. MATERIALS AND METHODS

3.2.1. Surveys of aphid occurrence on *Betula pendula*

The survey protocol outlined in Section 2.3 of Chapter 2 was used to investigate the occurrence of *E. betulae* and other insects on *B. pendula.*

Surveys began on 28th April in 1999 and 2000 and were repeated at approximately 17 and 11 day intervals, respectively. Each survey was completed within 48 hours.

Whilst not reported in this Chapter, these surveys were modified to simultaneously record the occurrence of fungal pathogens on leaves (see Chapter 4) from 12th June 1999 and 29th May 2000. In addition to those factors considered here (canopy and leaf position), leaf infection by *M. betulae* proved to be an important factor in determining aphid spatial distribution (discussed in Chapter 5). Fungal infection was fitted into the statistical analysis model (described below), but only the effects of canopy and leaf position are reported in this Chapter. Surveys completed in 2000 were carried out using 12 of the original survey trees that displayed the highest and lowest levels of fungal infection (See Chapters 4 and 5) during 1999 (six trees of each).

3.2.2. Statistical analysis

Analysis was carried out in SAS version 8.2 (SAS Institute 1999). Survey data were analysed using a GLIMMIX (SAS Institute 1999) generalised linear mixed model with a Poisson error structure and log link function. Branch number was fitted within tree number and both treated as a random terms in the model. Canopy position (upper and lower branches) and branch position (basal, intermediate and apical leaves) were fitted as sources of variation within the model. Degrees of freedom for all generalised linear mixed models were calculated using the Satterthwaite formulas (Littell *et al.* 1996). The first survey in 2000 (28th April) was excluded from the analysis because of the scarcity of observations.

3.3 RESULTS

Examination of 1999 aphid occurrence data using only those 12 trees used in 2000 (*i.e.* those displaying the heaviest and lowest infection levels in 1999) showed no substantial differences in aphid occurrence when the difference in fungal infection (see Chapters 4 and 5) was accounted for. Therefore, data presented for 1999 is derived using all 30 trees.

3.3.1. Temporal distribution of *Euceraphis betulae*

The total number of aphids scored on surveyed birch leaves over the growing season of 1999 (Figure 3.3a) and 2000 (Figure 3.3b) shows that the population in 1999 was considerably larger than that observed in 2000. Apart from the obvious difference in magnitude, the two populations had different temporal patterns, with the population peaking shortly after budburst in 1999, whereas the population only peaked by mid-summer in 2000. The aphid population in 1999 underwent a rapid decline by the end of August, whereas the population in 2000 declined more steadily. Both populations remained buoyant throughout mid-summer.

Population composition, in terms of adult and larval aphids for 1999 and 2000 is shown in Figure 3.4a and Figure 3.4b respectively. Fundatrices hatch in larger numbers in 1999 compared with 2000, most of which have become adult by the end of May in both years when the number of adults exceeds the number of larvae for the first time. Parthenogenetic reproduction at the level of the population was underway by mid-June, when the number of larvae increased again (the first increase being during egg-hatch), particularly rapidly in 1999 when larval numbers quickly exceeded adult numbers, compared with the more gradual increase in larval numbers during 2000. The composition of the two populations during the middle of the growing season was completely opposite; in 1999, the population was dominated by larvae, which outnumbered adults by almost 2:1; whereas in 2000, adult aphids were recorded more frequently than larvae (with the exception of the 8th July). Both populations were in decline by the middle of August although the more rapid decline in adult numbers in 1999 reduced the population to similar levels as 2000 (~ 0. 25 aphids per leaf node examined).

The mean air temperature at the nearby Aboyne weather station during the 1999 and 2000 seasons is subsequently discussed in Chapter 4 (Figure 4.3a). For exploratory purposes, degree days accumulated were compared with *E. betulae* abundance at Dalhaikie Flat (Figure 3.5). Whilst this type of comparison can only be a guide to the importance of temperature on the *E. betulae* population in the field, there appeared to be no obvious relationship between number of degree days accumulated and aphid abundance. Degree days were calculated on the basis that the lower development threshold for *E. betulae* is 1.52°C (Hopkins 1996). The mean wind velocity during 1999 and 2000 (Figure 3.6.), again show no substantial differences between the two years. The mean temperature and wind velocity recorded during survey days in 1999 and 2000 is reported in Figures 3.7 (a) and (b) respectively.

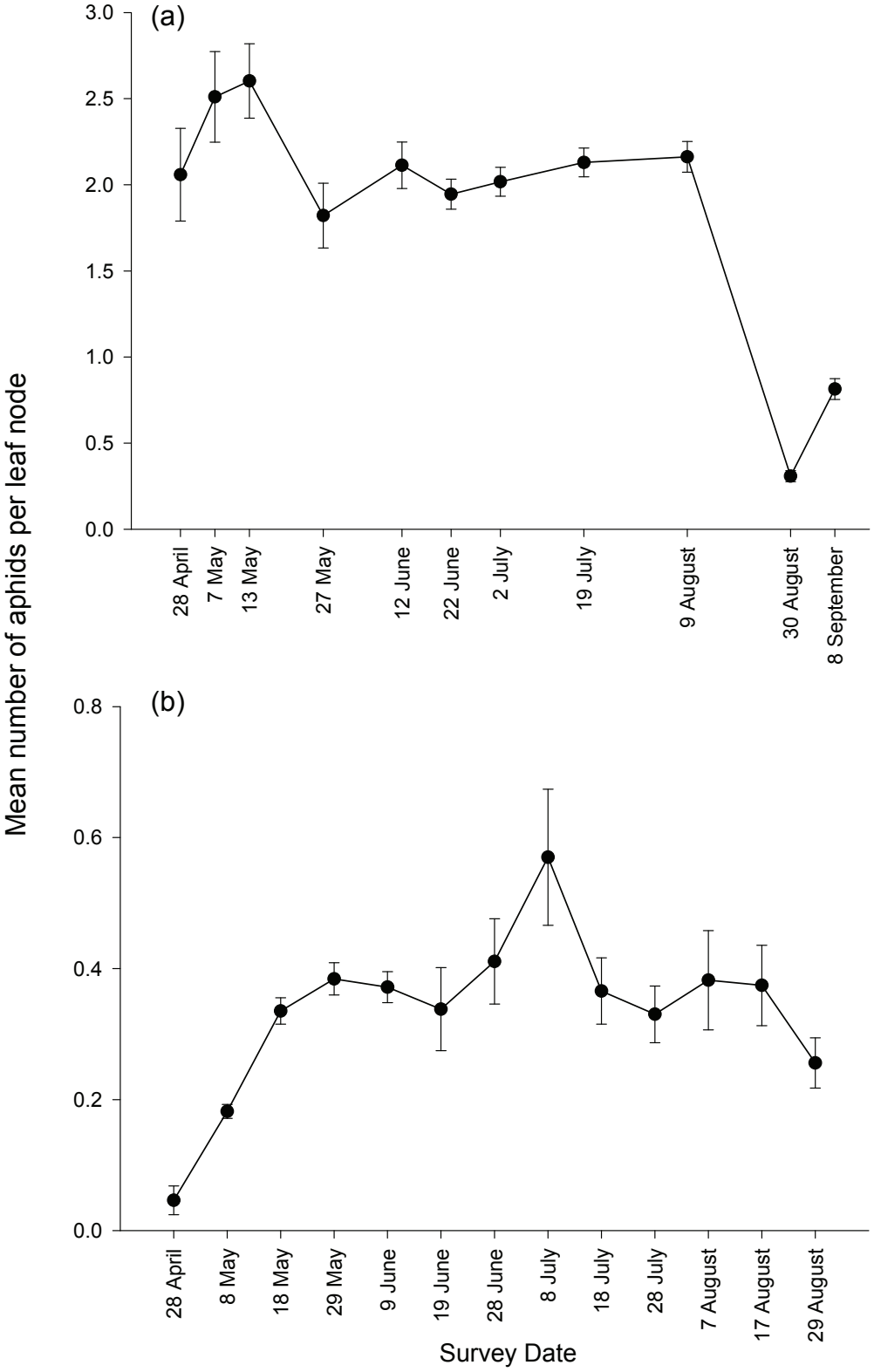


Figure 3.3. Seasonal abundance of *E. betulae* (mean number per leaf node examined ± standard error) on *B. pendula* during (a) 1999 and (b) 2000. Note different scales on the two x and y axes.

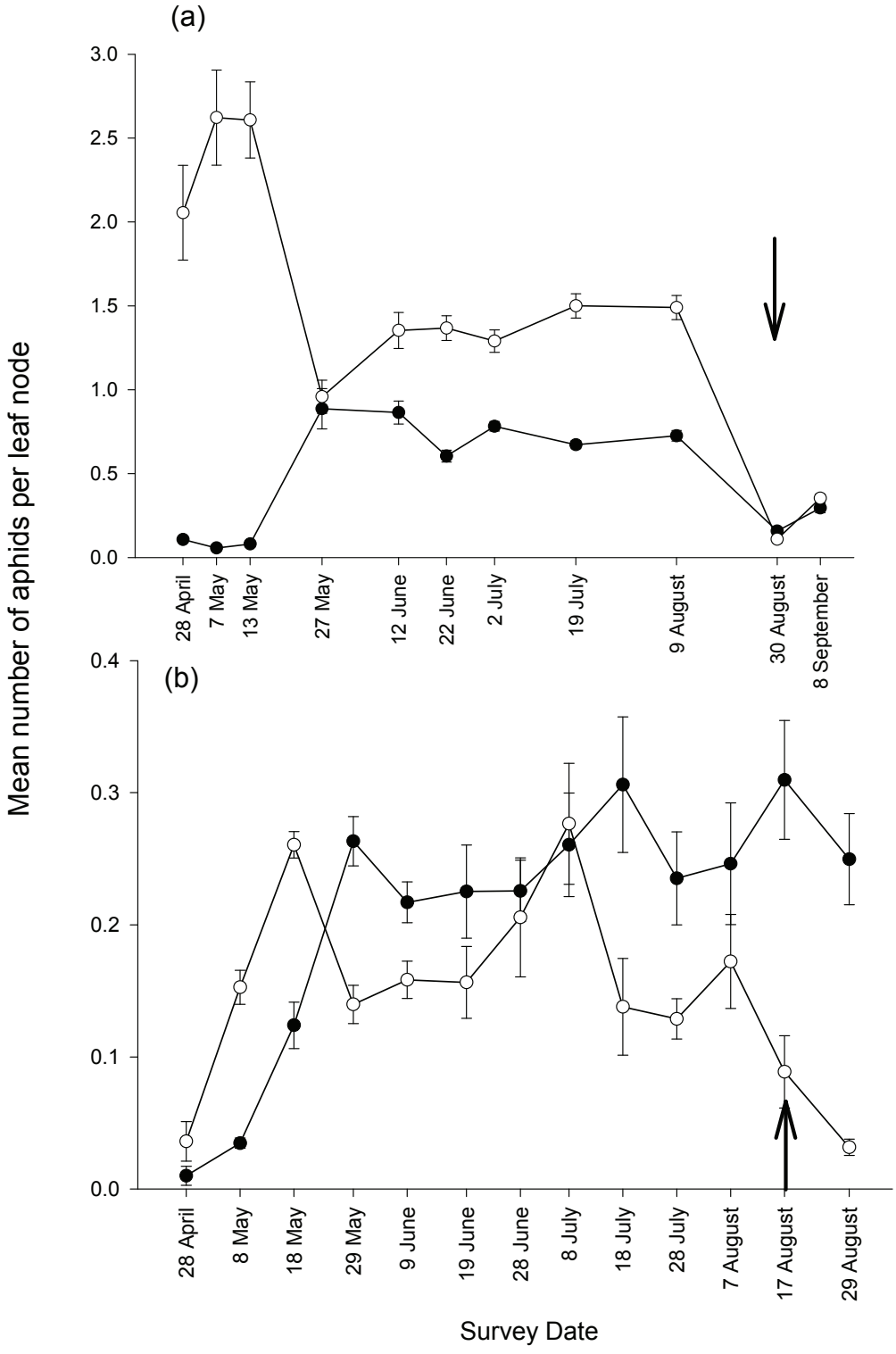


Figure 3.4. Seasonal abundance of *E. betulae* (mean number per leaf node examined ± standard error) larvae (○) and adults (●) on *B. pendula* during (a) 1999 and (b) 2000. Note the different scales on the two x and y axes. The first field observation of oviparae (abundance not shown for clarity) is depicted by an arrow.

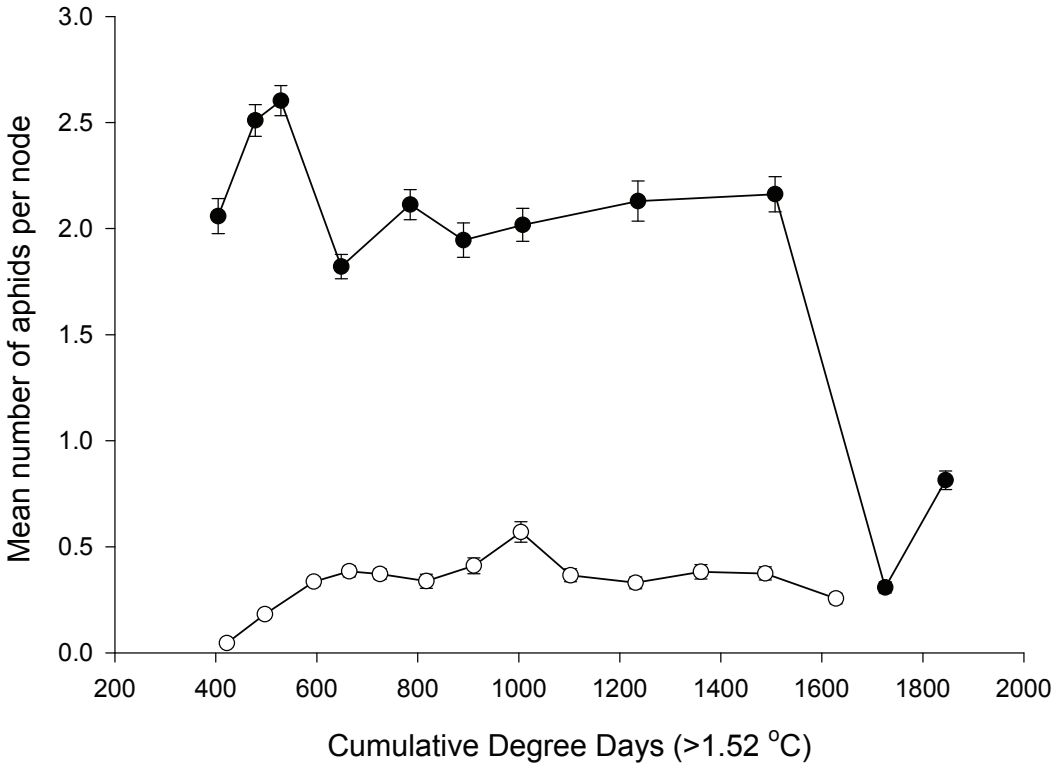


Figure 3.5. Mean abundance of *E. betulae* (± standard error) with cumulative degree days above the known lower development threshold of *E. betulae* (1.52°C) during 1999 (●) and 2000 (○).

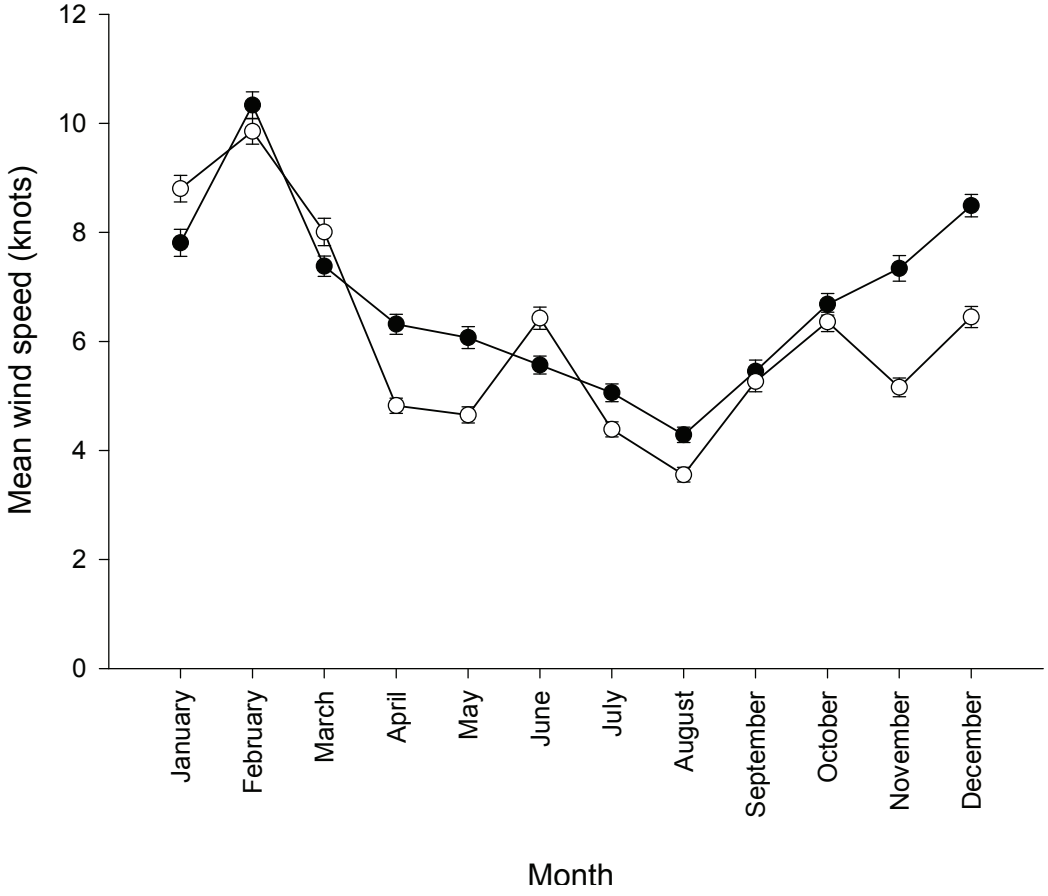


Figure 3.6. Mean wind speed (knots ± standard error) recorded at the nearby Aboyne weather station during 1999 (●) and 2000 (○). Standard error calculated using hourly measurements for each month.

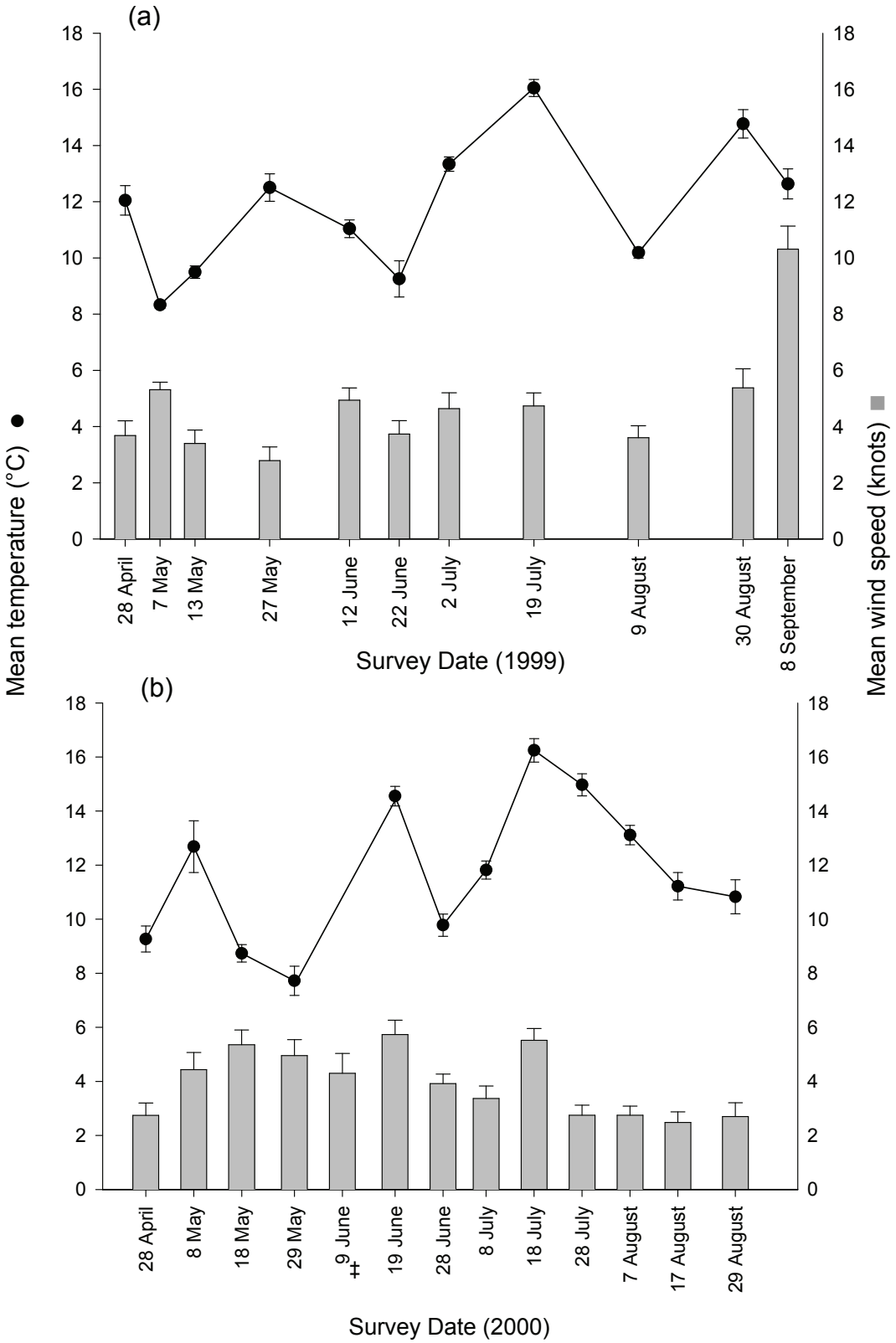


Figure 3.7. Mean temperature (●) and wind speed (■) (± SE) on survey days during 1999 and 2000. ‡ No temperature data were available. Standard error calculated as in Figure 3.6.

3.3.2. Spatio-temporal distribution of *Euceraphis betulae*

Euceraphis betulae was recorded more frequently on upper branches than lower branches in the tree canopy of *B. pendula* during the growing season in 1999 and 2000 (Figure 3.8, 3.9 respectively and Table 3.2). The disparity between abundances on the two types of branch was greatest at the beginning of the 1999 growing season, when comparatively few aphids were recorded on lower branches (Figure 3.8). During the beginning of the growing season in 2000, more aphids were also observed on upper branches, but the difference between the two branch types was not as pronounced although still statistically significant (Figure 3.9). The difference between the two branch types was also smaller at the end of both growing seasons when the aphid population was in decline. Despite the very small difference between branch type at the end of the 1999 growing season, aphids remained significantly more abundant (albeit in much lower numbers than earlier in the season) on the upper rather than lower branches.

Patterns of aphid distribution within individual branches (Table 3.3) were less consistent than distribution within the canopy during both 1999 (Figure 3.10 & Table 3.4) and 2000 (Figure 3.11 & Table 3.5). Note that the least square means estimates calculated in the generalised linear mixed model (Tables 3.4 & 3.5) do not necessarily equate to the exponent of the numerical mean values shown in Figures 3.10 and 3.11. In general, aphids appeared to be found more frequently on basal and intermediate leaves than apical leaves at the very beginning of the growing season, but this trend was reversed by late June in 1999 and late May in 2000, when aphids became significantly more abundant on apical leaves. Aphids were found consistently more frequently on apical leaves thereafter until late August in both years when there was very little difference in aphid abundance along the branch.

Examination of the interaction between canopy position × branch position showed that the general trend of initial aphid abundance on basal and intermediate leaves followed by movement to apical leaves was the same for both upper and lower branches, although the trend was generally more pronounced on upper branches.

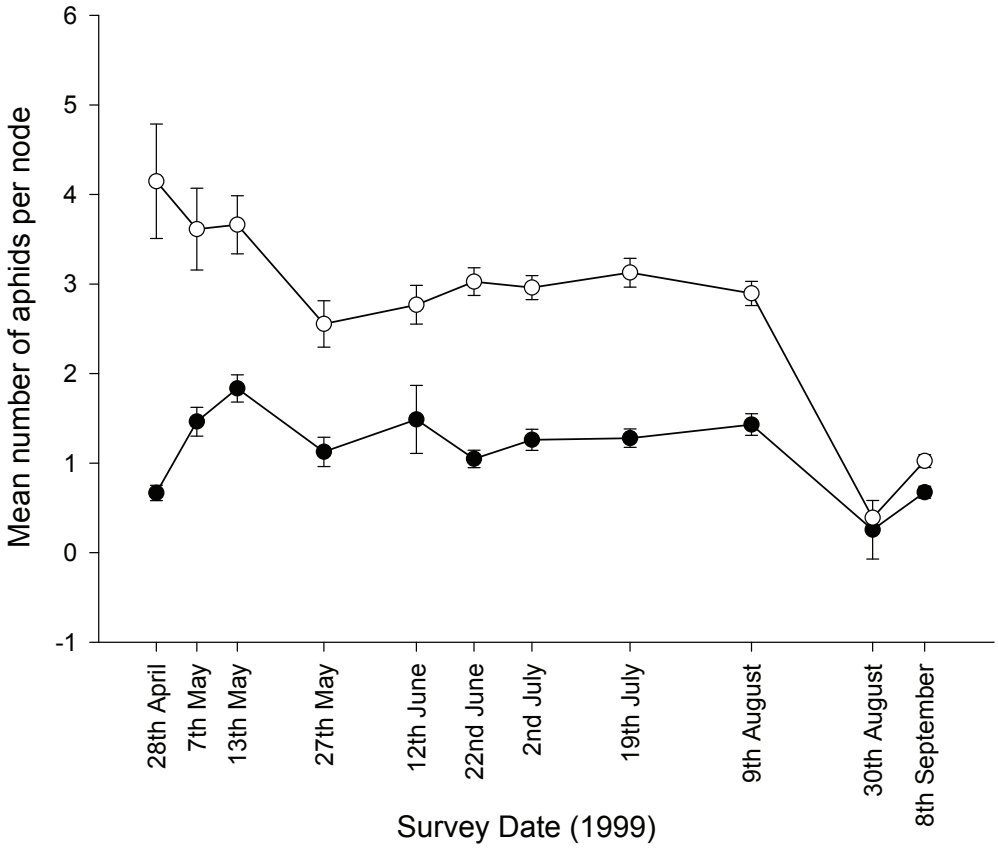


Figure 3.8. Seasonal distribution of *E. betulae* (mean number per leaf node examined ± standard error) on upper (○) and lower branches (●) within the canopy of *B. pendula* trees during 1999.

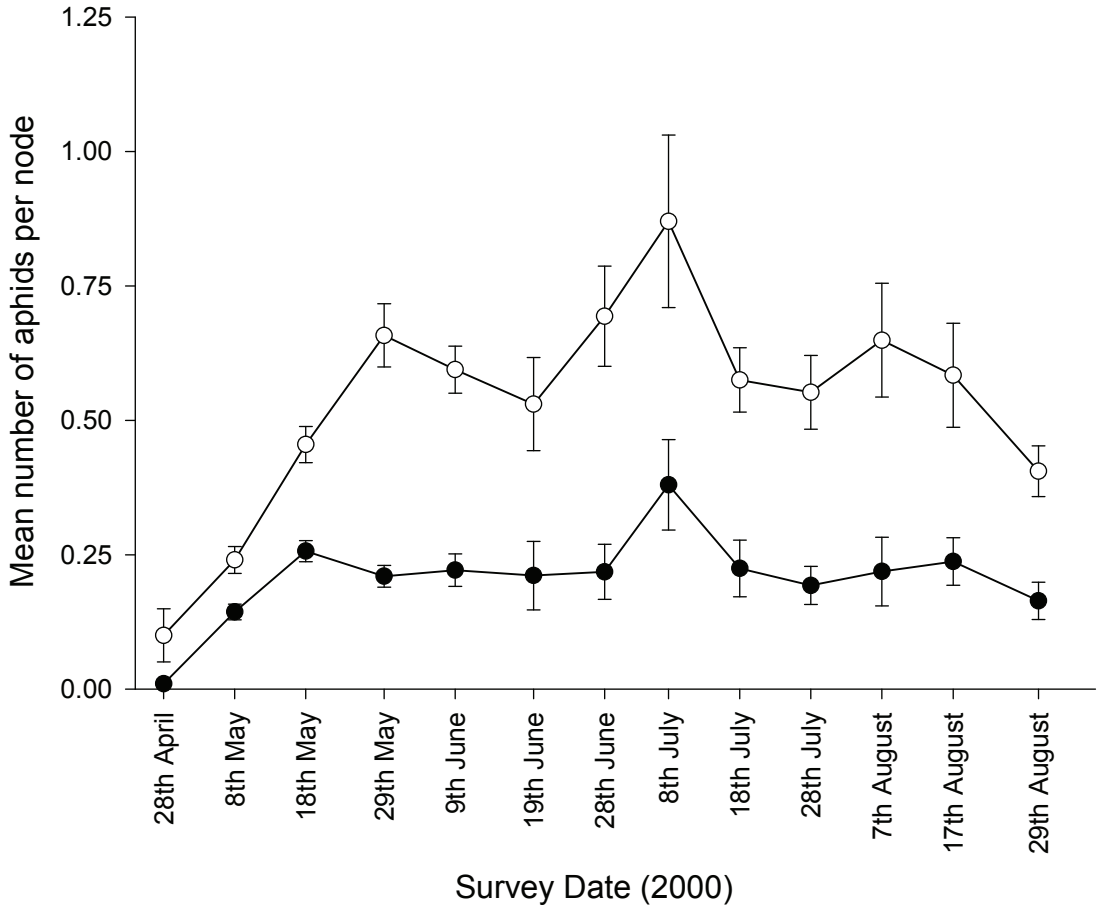


Figure 3.9. Seasonal distribution of *E. betulae* (mean number per leaf node examined ± standard error) on upper (○) and lower branches (●) within the canopy of *B. pendula* trees during 2000.

Table 3.2. Summary of generalised linear mixed model results examining position of branch in the canopy (upper or lower) as a source of variation in the distribution of *E. betulae* on *B. pendula* trees. Dates shown are for the more frequent surveys in 2000 with 1999 dates in parentheses. § 2nd July 1999

Survey Date	(1999)			2000		
	df	*F*	*P*	df	*F*	*P*
(28) April	1, 678	296	<0.0001		-	
8 (7) May	1, 606	104	<0.0001	1, 308	9	0.0029
18 (13) May	1, 557	91	<0.0001	1, 323	45	<0.0001
29 (27) May	1, 531	91	<0.0001	1, 346	67	<0.0001
9 (12) June	1, 743	111	<0.0001	1, 344	60	<0.0001
19 (22) June	1, 865	258	<0.0001	1, 299	36	<0.0001
28 (2§) June	1, 585	182	<0.0001	1, 309	52	<0.0001
8 July		-		1, 276	36	<0.0001
18 (19) July	1, 646	190	<0.0001	1, 302	60	<0.0001
28 July		-		1, 265	58	<0.0001
7 (9) August	1, 667	167	<0.0001	1, 253	67	<0.0001
17 August		-		1, 227	26	<0.0001
29 (30) August	1, 592	30	<0.001	1, 206	24	<0.0001
(8) September	1, 577	11	0.0011		-	

Table 3.3. Summary of generalised linear mixed model results examining position of leaf on the branch (basal, intermediate or apical leaves) as a source of variation in the distribution of *E. betulae* on *B. pendula* trees. Dates shown are for the more frequent surveys in 2000 with 1999 dates in parentheses. § 2nd July 1999.

Survey Date	(1999)			2000		
	df	*F*	*P*	df	*F*	*P*
28 (2) July	2, 3127	145	<0.0001		-	
8 (7) May	2, 2206	198	<0.0001	2, 1263	23	<0.0001
18 (13) May	2, 1957	14	<0.0001	2, 1415	2	0.1151
29 (27) May	2, 1870	389	<0.0001	2, 1143	17	<0.0001
9 (12) June	2, 2124	27	<0.0001	2, 1141	6	<0.0001
19 (22) June	2, 1974	217	<0.0001	2, 1035	5	0.0044
28 (2§) June	2, 1947	106	<0.0001	2, 1041	42	<0.0001
8 July		-		2, 975	44	<0.0001
18 (19) July	2, 1623	270	<0.0001	2, 1035	30	<0.0001
28 July		-		2, 949	68	<0.0001
7 (9) August	2, 1589	131	<0.0001	2, 953	76	<0.0001
17 August		-		2, 908	93	<0.0001
29 (30) August	2, 1806	9	0.0001	2, 671	1	0.3916
(8) September	2, 1030	16	<0.0001		-	

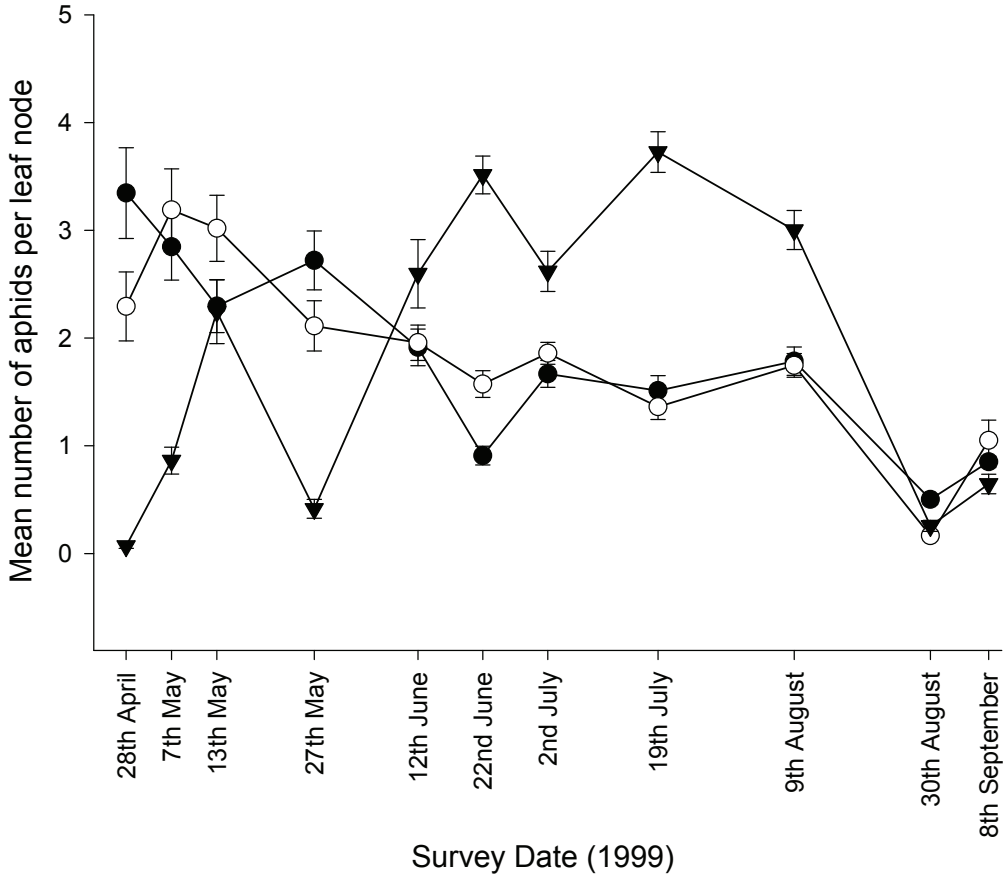


Figure 3.10. Seasonal distribution of *E. betulae* (mean number per leaf node examined ± standard error) on basal (●) intermediate (○) and apical (▼) leaves of *B. pendula* branches during 1999.

Table 3.4. Summary of generalised linear mixed model least squares mean estimates (log scale) for the spatial distribution of *E. betulae* on *B. pendula* leaves during 1999 comparing position on branch (basal, intermediate and apical leaves) (see Figure 3.10).

Comparisons between leaf positions	Least squares means estimates (Log scale)			Maximum Standard Error
Survey Date (1999)	Basal	Intermediate	Apex	
28th April	0.61	-0.05	-3.83	0.44
7th May	0.68	0.64	-0.52	0.13
13th May	0.64	0.84	0.61	0.12
27th May	0.52	0.05	-1.38	0.13
12th June	0.53	0.46	0.88	0.07
22nd June	-0.37	0.14	1.16	0.09
2nd July	0.75	1.00	1.67	0.08
19th July	0.58	0.64	1.96	0.87
9th August	0.55	0.79	1.64	0.07
30th August	-0.92	-1.31	-0.99	0.15
8th September	-0.54	-0.42	-0.92	0.10

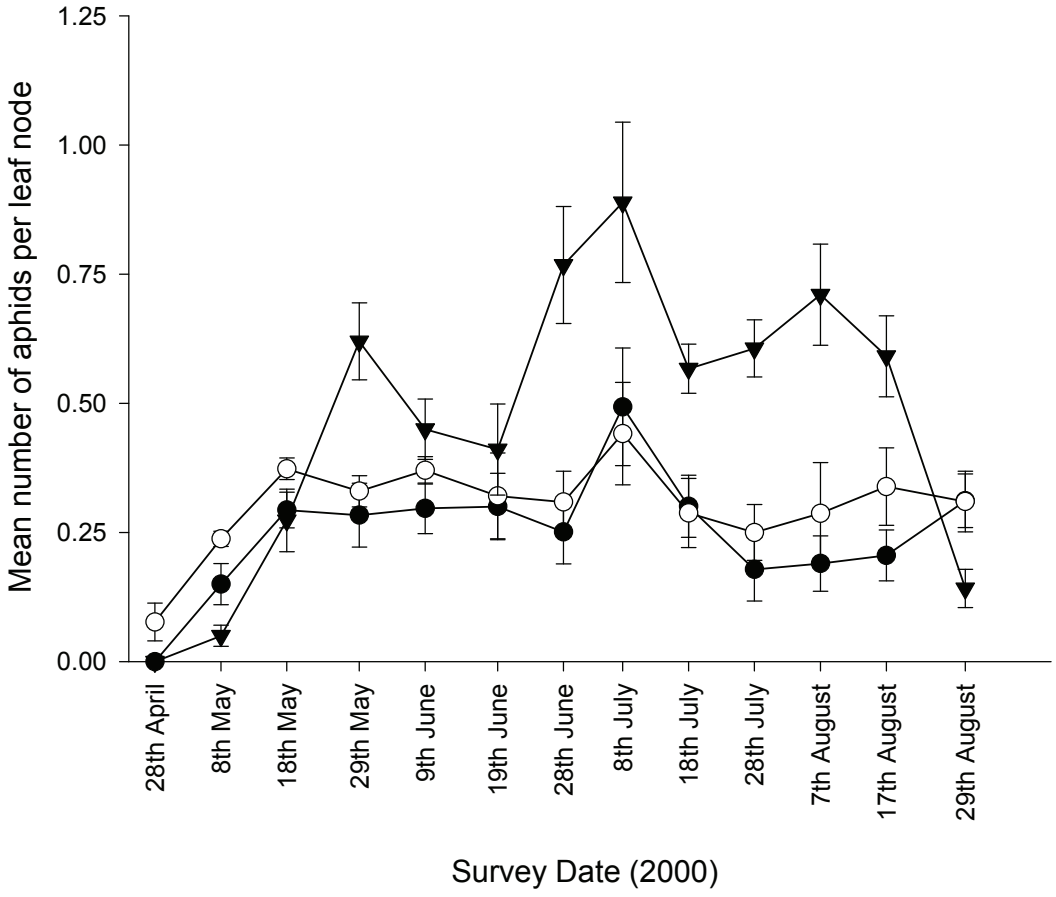


Figure 3.11. Seasonal distribution of *E. betulae* (mean number per leaf node examined ± standard error) on basal (●) intermediate (○) and apical (▼) leaves of *B. pendula* branches during 2000.

Table 3.5. Summary of generalised linear mixed model least squares mean estimates (log scale) for the spatial distribution of *E. betulae* on *B. pendula* leaves during 2000 comparing position on branch (basal, intermediate and apical leaves) (see Figure 3.11).

Comparisons between leaf positions	Least squares means estimates (Log scale)			Maximum Standard Error
Survey Date (2000)	Basal	Intermediate	Apex	
8th May	-2.16	-1.67	-3.25	0.25
18th May	-1.22	-0.98	-1.29	0.16
29th May	-1.38	-1.19	-0.62	0.14
9th June	-1.38	-1.16	-0.85	0.13
19th June	-1.78	-1.66	-1.27	0.15
28th June	-1.74	-1.57	-0.50	0.16
8th July	-1.29	-1.47	-0.35	0.13
18th July	-1.59	-1.77	-0.81	0.13
28th July	-2.35	-2.10	-0.94	0.15
7th August	-2.56	-2.01	-0.87	0.16
17th August	-2.46	-2.53	-0.92	0.14
29th August	-2.16	-2.20	-2.38	0.15

3.3.3. Occurrence of other insects

Euceraphis betulae dominated the insect fauna on *B. pendula* at Dalhaikie Flat and very few other insects were observed on survey trees during 1999 and 2000. Those that were recorded during 1999 are shown in Table 3.6. The same species of insects were observed in 2000 but occurred less frequently, the only exception being the two leaf-miners which were more abundant.

The *Eriocrania* spp., leaf-miner (whose interaction with *E. betulae* is discussed in Chapter 6) was the most numerous of the other insects, but was still recorded infrequently overall. Another leaf-miner, the case bearing moth *Coleophora serratella* (Plate 3.2) was also observed mining leaves in late Spring. *Operoptera brumata*, the polyphagous winter moth larvae, was observed feeding on birch leaves early in Spring. The predatory two spot ladybird (both adults and larvae), *Adalia bipuncta*, was also recorded on leaves but never witnessed eating *E. betulae*. Other insects (not identified to species) included leaf-hoppers (Cicadellidae) and frog-hoppers (Cercopidae), although the frog-hoppers had almost certainly moved onto birch trees as they tend to feed on grasses (Chinery 1993).

Plate 3.2. The case-bearing leaf-miner *Coleophora serratella* L. (Lepidoptera: Coleophoridae) was one of the more numerous insects recorded on *B. pendula* but was still much less abundant than *E. betulae*.

Table 3.6. The occurrence of insects other than *E. betulae* on *B. pendula* at Dalhaikie Flat during survey observations. The absolute number recorded during each survey is shown together with the number of leaf-nodes examined in that survey.

		INSECTS						
		Leaf-miners		Leaf-chewers	Sap-feeders		Potential natural enemies	
DATE (1999)	Number of leaf nodes examined	*Eriocrania* spp.	*Coleophora serratella*	*Operophtera brumata*	Cecopidids (Froghopper)	Cicadellids (Leafhopper)	*Adalia bipunctata*	Formicidae Ants
28 April	3750	0	0	2	15	17	0	0
7 May	2854	0	0	1	4	18	0	0
13 May	2592	0	0	8	5	7	0	0
27 May	2611	14	0	0	5	3	0	0
12 June	2568	39	18	2	5	3	0	2
22 June	2260	38	18	5	0	0	7	11
2 July	2229	5	0	5	0	0	7	5
19 July	1888	1	1	0	0	9	1	7
9 August	1843	0	0	0	0	10	3	3
30 August	2007	0	0	0	0	0	0	1
8 September	1438	0	0	0	0	0	0	0

3.4 DISCUSSION

3.4.1. Factors affecting tree aphid populations

A complex interplay of factors are known to influence the abundance and distribution of aphid populations, often including the nutritional quality of the host plant, temperature, wind conditions and the abundance and occurrence of natural enemies (Dixon 1998).

Seasonal changes in host plant quality are thought to be very important in determining the temporal and spatial abundance of tree-dwelling aphids (Kindlmann & Dixon 1996). Growth and reproduction of individual aphids is generally thought to be limited by the amino-nitrogen concentration of foliar phloem sap on which they feed (Dixon 1998), and the amino-nitrogen concentration of the phloem sap is likely to vary with plant phenology. As birch continuously flushes new foliage throughout the season, it is potentially a spatially and temporally heterogeneous resource to aphids.

Temperature, both through its effects on host plant phenology, and directly upon aphids, is also known to play an important role in influencing the magnitude and dynamics of tree-dwelling aphid populations (Kindlmann & Dixon 1996). Warmer temperatures have been associated with advanced aphid phenology and improved individual performance in the past (Harrington *et al.* 1995). Like many tree aphids, *E. betulae* performs best at moderate temperatures. *E. betulae* grows fastest at around 20°C (Hopkins 1996), above which growth declines for both individuals in culture (Hopkins 1996) and naturally occurring populations on trees (Hajek & Dahlsten 1988). In addition, tree-dwelling aphid populations are often affected by windiness (Dixon 1978) because aphids become dislodged from leaves and most do not manage to rejoin the population (Dixon & MacKay 1970).

3.4.2. Temporal dynamics of the population

The *E. betulae* population at Dalhaikie Flat was larger in 1999 than 2000, and the occurrence of large numbers of larvae early in the season during 1999 might have underpinned the subsequent success of the population throughout the rest of the season. Synchronisation between the hatching of aphid eggs and bud-burst of deciduous trees in spring is vital if the aphid is to feed on the highly nutritious developing foliage, and those aphids that hatch too early or too late are likely to suffer heavy mortality (Dixon 1976).

E. betulae frequently hatches well before bud-burst as its possession of a long stylet permits it to feed on unfurled leaves (Hopkins 1996). If egg-hatch occurs prematurely (before buds begin to swell) the aphid would be deprived of food altogether and be subject to the disadvantages associated with earlier egg-hatch (*i.e.* cooler temperatures, adverse weather), whilst late egg-hatch would deny it the advantage of feeding on the especially nutritious unfurled leaves in the presence of fewer inter-specific competitors. *E. betulae* was observed feeding on swelling birch buds well before bud-burst during 1999 and as a result its population was already very abundant by the first survey at bud-burst, whereas egg-hatch occurred after bud-burst (and the first survey) in 2000 so the population was still increasing steadily whilst leaves were maturing. Bud-burst actually occurred about three weeks earlier in 1999 than 2000, so aphid egg-hatch was particularly late in 2000. In the absence of data about the lower development threshold of *E. betulae* eggs it is difficult to determine whether temperature was involved in the earlier egg-hatch in 1999, but the later egg-hatch in 2000 was completely out of step with *B. pendula* bud-burst.

The effect of temperature on *E. betulae* after egg-hatch could not be properly assessed with the data available, but the mean temperature was generally a little higher during the 1999 season compared with the 2000 (see Chapter 4, Figure. 4.3). There was some evidence of individual aphids having responded to the different ambient temperatures in field trials conducted over both years. In identical control experiments, aphids reared in clip-cages in 2000 (Chapter 5) took longer to develop to adulthood and possessed longer hind tibiae (~20% longer) when they did so compared with aphids reared in 1999 (Chapter 4). Larger appendages and delayed development are among the characteristics of the sycamore aphid *Drepanosiphum platanoidis* when it is affected by cooler temperatures (Dixon 1975). It is unlikely that windy conditions were responsible for the smaller population size in 2000, since wind conditions in 2000 were not substantially different from 1999. The last survey in 1999 (8th September) was conducted on windy days, which may have contributed to the small number of aphids recorded, however the preceding survey (30th August) also recorded lower numbers, so there does appear to be a genuine steep decline towards the end of the season.

Between-year effects offer an additional, but not necessarily alternative explanation for the

much lower occurrence of *E. betulae* at the beginning of 2000 compared with 1999. Dixon (1978) reported that spring populations of *D. platanoidis* on sycamore were very much lower when the previous autumn egg-laying stages were themselves less abundant. In the present study, the 1999 population of *E. betulae* underwent a dramatic decline at the end of the growing season and the oviparae were recorded slightly later than in 2000. The fact that the population was small and the oviparae were present for less time before leaf-fall could have contributed to fewer eggs been laid, thereby limiting the number of *E. betulae* that hatched in the following spring. Such between-year correlations in aphid populations involve multiple factors and are notoriously more complicated than they might first seem (Dixon 1998). Nonetheless, Heie (1972) also reported smaller autumn populations of birch aphids being followed by smaller spring populations in the following year. The over-wintering mortality of *E. betulae* eggs may have been greater during the 1999-2000 winter compared with the 1998-1999 winter, although not driven by temperature as winter temperatures never fell below levels which are lethal to *Euceraphis* eggs (Parry 1985).

A lack of developing birch foliage at the beginning of the 2000 growing season is unlikely to be responsible for the initial low numbers of *E. betulae* as the quantity and size of birch leaves at bud-burst (and hence the flux of resources allocated to them via the phloem) is known to correlate with climatic conditions experienced the previous year (1999) (Haukioja *et al.* 1985; Lehtillä *et al.* 2000) which were generally favourable.

Another feature of the *E. betulae* population studied at Dalhiakie Flat was the presence of large numbers of larvae in the middle of the growing season during 1999. This is in contrast to other studies of *Euceraphis* populations in California (Hajek & Dahlsten 1988) and Southern England (Wratten 1974) where low numbers of larvae were recorded in the middle of the season, albeit for a relatively short period. This was attributed to seasonal reproductive diapause in response to the lower levels of soluble nitrogen in mature foliage, as is the case for the sycamore aphid *D. platanoidis* (Dixon 1975). The high proportion of larval *E. betulae* in 1999 is an indication that a depression in reproductive activity is not occurring at the scale of the population. If *E. betulae* is comparable with *D. platanoidis*, and the other similarities in aphid phenology suggest that it is, then reproductive diapause is likely to be a genotypically hard-wired response to changing day-length (Dixon 1975).

Tree-dwelling aphids that hatch early (*e.g. D. platanoidis* and *E. betulae*) tend to undergo this reproductive diapause, whereas late hatching tree aphids (*e.g. D. acerinum*) reproduce throughout the season. Aphids that undergo reproductive diapause are thought to cope poorly with hot temperatures and those generations that experience the hottest temperatures in mid-summer frequently show reduced melanic pigmentation, which exacerbates heat stress by absorbing solar radiation (Dixon 1998). The *E. betulae* population investigated here, hatched early in the season and second generation virginoparae showed reduced pigmentation (see Chapter 2), but the cooler temperatures at this latitude possibly place less of an environmental stress on the aphid so the reproductive diapause may be absent. Dissections of aphids reported in Chapters 4 and 5 suggest that this was not the case, since a significant proportion of the population were in a state of reproductive diapause. If the reproductive diapause was sufficiently asynchronous across the population however, this may have kept larval numbers high because there were always some individuals in the population who were reproducing.

Because reproductive diapause is a life-history strategy in response to the poor nutritional quality of foliage in the middle of the season, the absence of a reproductive depression in 1999 might point to an amelioration of this situation to which *E. betulae* is able to respond. The effects of poor quality food in mid-summer may be lessened by the continual flushing of new (and more nutritious) foliage by birch throughout the season. In addition to this potential factor, Chapters 4 and 5 consider the effects of a fungal leaf pathogen that occurred widely at Dalhaikie Flat as a possible cause of improved plant nutritional quality at this time.

3.4.3. Spatio-temporal dynamics of the population

The high degree of mobility shown by *E. betulae* is thought to enable it to move extensively around the tree canopy, allowing it to only settle at feeding sites which are highly nutritious (Hajek & Dahlsten 1986, 1988). The most nutritious leaves for aphids to feed upon are those which have high concentrations of amino-nitrogen concentration in the phloem, typically leaves with high total nitrogen concentrations that are either developing (Haukioja *et al.* 1978) or senescing (Chapin & Kedrowski 1983). The widespread occurrence of developing leaves at the beginning of the growing season and senescent leaves at the end are thought to be explain the corresponding enhancement of

aphid reproduction at the level of the population (Dixon 1998). Between bud-burst and senescence however, the tree is probably a much more heterogeneous resource. The distribution of resources via the phloem from photosynthesising leaves has been shown to vary in birch depending on the relative sink strengths of particular sections of the canopy (Lehtilä *et al.* 2000). Upper branches and specifically apical buds and leaves represent the strongest sinks and so it is to these sections of the plant that the greatest concentrations of soluble nitrogen are translocated for growth.

Euceraphis betulae is probably more abundant on upper branches rather than lower branches in the tree canopy because the upper branches of *B. pendula* have greater exposure to light and therefore grow more quickly. Furthermore, the growth of upper branches depends on the translocation of nutrients to developing tissues, and it is during the movement of soluble nitrogen in the phloem that aphids feed most successfully (Raven 1983).

At the beginning of the growing season, bud-burst is occurring on all branches irrespective of their position in the canopy so there is probably little difference in the nutritional quality of phloem sap being translocated to developing leaves on upper and lower branches. Likewise, at the end of the growing season, leaves are senescing equally on upper and lower branches, so again the difference in the nutritional quality of phloem sap (this time being transported away from leaves) is unlikely to be different between the two types of branches. The greatest difference between upper and lower branches would be expected to be in the middle of the growing season, when upper branches are growing more actively than lower branches. Patterns of *E. betulae* abundance in 2000 generally coincide with changing host-plant phenology, in that they are much more abundant on upper branches in the middle of the growing season, but the difference between upper and lower branches is less striking at the beginning and end of the growing season. The main deviation from this pattern in 1999 is that aphids are much more abundant on upper branches at the beginning of the season as well as in the middle of it. Part of the reason for this may be that bud-burst occurred about three or four weeks earlier in 1999 compared with 2000, and significant growth of upper branches may have already commenced by the time of the first survey, helped perhaps by the generally favourable climatic conditions at this time.

Where the eggs are oviposited by the oviparae during the previous winter probably has some bearing on early spatial distribution of young larvae in the following spring, which are generally less mobile than adults. For instance, the *E. betulae* population was spread more evenly between upper and lower branches at the end of the 1999 growing season, and so too were the newly hatched fundatricies at the beginning of the 2000 season. If very young larvae are less able to move to nutritious foliage, their abundance might initially be determined by the oviposition of the previous generation. The location of older larvae and subsequent generations is more likely to reflect location of resources.

Hajek & Dahlsten (1988) found that *E. betulae* was slightly more abundant on leaves of the outer canopy compared with the inner canopy, but not significantly so and nor was there any significant difference between leaves of the lower and middle canopy. It is difficult to relate these categories to those used here (upper and lower branches) but the fact that the upper canopy was not investigated suggests that they used taller (and older) birch trees which generally show less vigorous vertical growth than the juvenile trees used here.

As discussed in Section 3.4.2., *E. betulae* fits the profile of a tree-dwelling aphid which copes poorly with high temperatures in the middle of summer and yet the predominance of *E. betulae* on upper branches during mid-summer in 1999 and 2000 is atypical of such an aphid. *D. platanoidis* for instance, moves to the more shaded lower branches in the canopy during the hottest parts of the season (Dixon & MaKay 1970), whereas the more heat-tolerant *D. acerinum* shows no such downward movement (Dixon 1972). None of the surveys were conducted on particularly cool days (Figure 3.7) and the trend is very consistent for both 1999 and 2000. It is reasonable to assume that *E. betulae* did not migrate downwards because they were not adversely affected by temperature on upper branches, perhaps because of the generally cooler temperatures at this latitude, indeed the mean temperature rarely approached levels which were harmful to *E. betulae* in culture (> 22.5°C (Hopkins 1996).

Ovipositional and plant phenological explanations might account for the distribution of aphids within particular branches as well as within the tree canopy as a whole. At the beginning of the growing season in both 1999 and 2000, aphids are found infrequently on apical leaves at the tips of branches. *Euceraphis* eggs are oviposited in cracks and crevices

along the branch (Parry 1985; Hajek & Dahlsten 1988) so it might be supposed that basal and intermediate leaves would initially be closer to oviposition sites rather than leaves at the very tips of branches. Whilst bud-burst was underway, leaves were probably nutritionally similar to one another, but once the initial flush of leaves matured, buds and leaves which were actively growing (*i.e.* the apical buds and leaves) probably became relatively more nutritious than non-growing leaves further down the branch. Aphids may switch from basal and intermediary leaves to track this change in host plant quality. It has been suggested that *D. platanoidis* also aggregates on apical leaves of sycamore during the middle of the season, apparently to take advantage of this nutritionally favourable 'micro-climate' (Dixon & MacKay 1970). *E. punctipennis* was also found to be more abundant on apical leaves of *B. pubescens* during the middle of summer, but to be found equally throughout the branch at the beginning and end of the growing season (Wratten 1974).

3.4.4. Insect fauna on *Betula pendula*

The design of the survey was very much aimed at investigating the occurrence of *E. betulae* on *B. pendula* rather than the insect fauna in general, so the absence of large numbers of other insects in these surveys must be treated with caution. Surveys for instance were conducted diurnally, so nocturnal insects would not be recorded. However, the low frequency of sessile insects (*e.g. Eriocrania* spp. and *Coleophora serratella*) probably is an accurate reflection of their true abundance. The low abundance of other insects in comparison to the abundance of *E. betulae* on *B. pendula* leaves does suggest that *E. betulae* is the dominant insect herbivore of birch at this site.

Over 50,000 aphid occurrences were recorded in 1999 and 2000, and yet no aphid mummies were ever observed - perhaps suggesting that aphid parasitoids are either absent or present in very small numbers. Dixon (1998) suggests that the longer development times of some hymenopterous primary parasitoids relative to their aphid hosts, and their tendency to oviposit in small numbers within a patch of aphids to avoid attracting hyperparasites (which would decrease fitness), reduces both their occurrence and impacts on aphid populations.

One of the main predators of *E. betulae*, and tree-dwelling aphids in general, is the two-spotted ladybird, *Adalia bipuncta*, which was recorded infrequently during 1999 and 2000.

Whilst treating observational data on a mobile predator with caution, it nevertheless might reflect a low occurrence on birch leaves at Dalhaikie Flat. There is good experimental evidence that suggests there is a selective advantage to *A. bipuncta* laying a small number of eggs over a brief period of time during aphid egg-hatch. The lengthy development time of *A. bipuncta* could mean that aphids have migrated away from the egg-patch by the time they mature, leading to heavy mortality, and asynchronous egg-hatch which would result from egg-laying over a longer period time could lead to cannibalism of younger eggs by emergent *A. bipuncta* larvae (Dixon 1998). In addition to this, particularly large densities of *Euceraphis* aphids are needed for *A. bipuncta* to start ovipositing in that patch, more so than for any other tree aphid examined (*Eucallipterus tiliae*, *D. platanoidis* and *Myzocallis boerneri*), perhaps because *Euceraphis* frequently escapes from predators (Dixon 2000).

3.4.5 Conclusions

The underlying spatial dynamics of *E. betulae* on its host plant probably reflect the changing suitability of *B. pendula* as a resource to the aphid. This spatial heterogeneity is driven by plant phenology for the most part, but other organisms could influence the suitability of leaves for *E. betulae*. Having described the underlying distribution of *E. betulae* in the absence of other interacting species in this Chapter, the distribution of the fungal pathogen *Marssonina betulae* on *B. pendula* is discussed in the next Chapter, before a consideration of how the pathogens spatial distribution might affect that of the aphids in Chapter 5.

CHAPTER FOUR

Naturally occurring birch leaf-spot fungi and its impact on aphids

Leaf spot is a common and normally non-lethal disease caused by a number of fungal pathogens occurring on birch foliage. Two such pathogens, Marssonina betulae *and to a much lesser extent* Discula betulina *were found to occur on* Betula pendula *in the field. Infection was more prevalent in 1999 than 2000, and was predominantly found on leaves of the lower branches initially, but upper branches succumbed to infection later in the season. In addition, leaves closest to the trunk of the tree showed greater levels of infection and more acute symptoms than those at the branch tip. Infected leaves that became chlorotic were generally distributed equally between upper and lower branches. There was no relationship between increase in tree height and infection load, although some trees consistently bore greater levels of infection than others. Higher concentrations of phenolic compounds were found in heavily infected leaves. When reared on heavily infected foliage* Euceraphis betulae *became heavier, possessed longer hind tibiae and showed enhanced embryo development. Survivorship and development time remained the same when reared on infected and asymptomatic leaves.*

4.1. INTRODUCTION

4.1.1. Rationale

An important element in the study of indirect effects of *Marssonina betulae* (Lib.) Magn (Deuteromycotina: Melanconiales) on *Euceraphis betulae* Koch (Homoptera: Drepanosiphidae) was to examine both the pathology of the disease on *Betula pendula* Roth in the field and possible effects of infected foliage on aphid preference and performance. The extent and incidence of *M. betulae* infection on trees helped to assess the ecological importance of any indirect interaction in shaping the dynamics of *E. betulae* populations, and empirical measurement of aphid behaviour and performance in relation to naturally occurring infection provided a foundation for experiments using fungal inoculation (see Chapter 5) and also helped gauge the relevance of these experiments to the natural situation.

4.1.2. Fungal pathogens of birch

Whilst some detailed studies of mycorrhizal fungi occurring on birch have been undertaken in the past (Mason 1982), the paucity of research into fungal pathogens is remarkable given that they may be implicated in the general decline of birch trees after 30 years of age (Bennell & Millar 1984). This is perhaps in part due to the lack of commercial interest in birch and also due to the more obvious susceptibility of birch to climatic rather than biotic stress, for instance during the summer droughts of 1975 and 1976 in Britain (Phillips & Burdekin 1992).

Nonetheless, Bennell and Millar (1984) report the existence of 18 different fungal pathogens on birch foliage, 13 of which have been reported in the United Kingdom. The most common, and certainly that which has received most attention, is that causing birch rust disease, *Melamsporidium betulinum*, but birch leaf spot disease is also relatively common (Bennell & Millar 1984; Phillips & Burdekin 1992). Several fungi have been reported as causal agents of leaf spot disease on *Betula pendula* and *Betula pubescens* (Table 4.1). The classification of these different agents is often confused in the literature (Kurkela 1995; Paavolainen, Hantula & Kurkela 2000) and some authors still use the name *Gloeosporium* even though its validity is questionable on taxonomic and nomenclatural grounds (von Arx 1970). Classification of the birch leaf spot fungi is frequently ambiguous, for example Kurkela (1995) cites Bennell & Millar's (1984) description of *Gloeosporium betulae* to

describe *Asteroma microspermum* and subsequently lists *Gloeosporium betulae* as a distinct fungus. Bennell & Millar (1984) themselves possibly mistranslate their German reference (Paetzholdt & Schneider 1965) when suggesting that *Myxosporium devastans* is a synonym of *Discula betulina,* as the latter authors stipulate that this disease is only found on birch twigs and buds and the pathology they describe is somewhat dissimilar to *Discula betulina*.

Table 4.1. Fungi described as causal agents of leaf spot disease on birch. Synonyms used by authors are shown in parentheses with their preferred name shown without them. [1]found on dead or moribund leaves alone, [2]never recorded in Europe, [3]lesions are blotch like so possibly shouldn't be considered as causing leaf spot disease, [4]lesions are ulcer-like, [5]possibly the imperfect state of *Venturia ditricha,* [6]possibly the same fungi as *Gloeosporium betulae* (Kurkela 1995). Fungi within box are those present at Dalhaikie Flat and the names used in this thesis are in bold. See Bennell & Millar (1984) for full authorities.

Bennell & Millar (1984)	**Sinclair** *et al.* **(1987)**	**Phillips & Burdekin (1992)**	**Paavolainen** *et al.* **(2000)**
Ceuthospora betulae[1] *(Gloeosporium betulae)*	-	*Gloeosporium betulae (Ceuthospora betulae)*	*Gloeosporium betulae*
Discula betulina *(Myxosporium devastans) (Gloeosporium betulinum)*	***Discula betulina*** *(Gloeosporium betulinum) (Gloeosporium betulicola)*	***(Discula betulina)*** *Gloeosporium betulinum (Myxosporium devastans)*	*Gloeosporium betulinum*
Marssonina betulae	***Marssonina betulae***	***Marssonina betulae***	***Marssonina betulae***
Septoria betulae[2]	-	-	*Septoria betulae*[2]
Septoria betulina[2]	Three further unnamed (*sic*) fungi	-	*Septoria betulina*[2]
Glomerella cingulata[2]	-	-	*Glomerella cingulata*[2]
Calycellina leucella[3] *(Helotium leucellum)*	-	-	-
Phyllosticta betulina[3,5] *(Mycosphaerella punctiformis)*	-	*Phyllosticta betulina*[3,5]	-
Fusicladium betulae[4] *(Venturia ditricha*[1]*)*	-	*Fusicladium betulae (Venturia ditricha*[1]*)*	-
-	-	*Melanconium betulinum (Melanconis stilbostoma)*	-
-	-	*Asteroma microspernum*[6] *(Gloeosporium betulae-albae)*	-
-	-	-	*Pyrenopezia betulicola*

The causal fungal pathogens of leaf spot at Dalhaikie Flat were identified as *M. betulae* and to a much lesser extent, *D. betulina* on the basis of fungal and lesion morphology together with known pathology. As with many fungal pathogens, the entire life-cycle of either pathogen has yet to be described definitively (Sinclair *et al.* 1987) but the important features of their pathology on living tissues have been reported (Grove 1935; Peace 1962; Sinclair *et al.* 1987). In addition, the lifecycles of '*Gloeosporium* or *Discula* type' leaf spot pathogens of birch have been described recently (Kurkela 1995; Paavolainen *et al.* 2000, 2001) and probably do not differ significantly between species, indeed the life-cycles of Ascomycete foliage pathogens as a whole are remarkably similar (Edmonds, Agee & Gara 2000). The generalised life-cycle of a leaf pathogen based on Strouts & Winter (1994) and (Edmonds *et al.* 2000) is shown in Figure 4.1. In brief, primary ascospore infection of recently emerged leaves probably occurs during May in Scotland. Lesions first appear on leaves around June and acervuli, the asexual fruiting body in which conidiospores are produced, become visible by July. Secondary infection by conidiospores begins shortly afterwards and probably continue until leaf fall in September or October. It is most likely that ascospores are produced in the apothecia on fallen leaves until ascospore dispersal during Spring of the following year. Without explicit information about the complete life-cycle of *M. betulae*, aspects of the sexual perfect state can only be inferred from descriptions of related birch leaf spot pathogens (Kurkela 1995, Paavolainen *et al.* 2000, 2001). Only the asexual stage is considered here, as only this stage shares living birch foliage with the aphid *Euceraphis betulae*.

Discula betulina was found infrequently at Dalhaikie Flat, but the taxonomic and symptomatic similarity between the two makes comparisons pertinent at this stage. From late May or June, *M. betulae* produces brown-black lesions on birch leaves. Lesion margins have been variously described as radiate, stellate (Sinclair *et al.* 1987) or dentate (Phillips & Burdekin 1992). Lesions were always small (<10mm) on infected leaves at Dalhaikie Flat but became numerous on leaves after initial infection. *D. betulina* lesions are larger and have indefinite margins by comparison (Sinclair *et.al.* 1987), but like *Marssonina betulae* they are frequently associated with interveinal leaf chlorosis which is normally followed by leaf abscission (Worf 1986; Sinclair *et al.* 1987; Strouts & Winter 1994). Subcuticular acervuli form on abaxial leaf surfaces for *D. betulina* and the adaxial leaf surfaces for *M. betulae* (Sinclair *et al.* 1987 and Kurkela 1995). Like the leaf spots of *Pyrenopeiza betulicola*

(Paavolainen *et al* 2000), 'green-islands' developed around lesions on heavily chlorotic leaves (Plate 4.1.), perhaps suggesting that fungal cytokinins are retarding plant-initiated leaf senescence in these leaves (Thomas & Stoddart 1980).

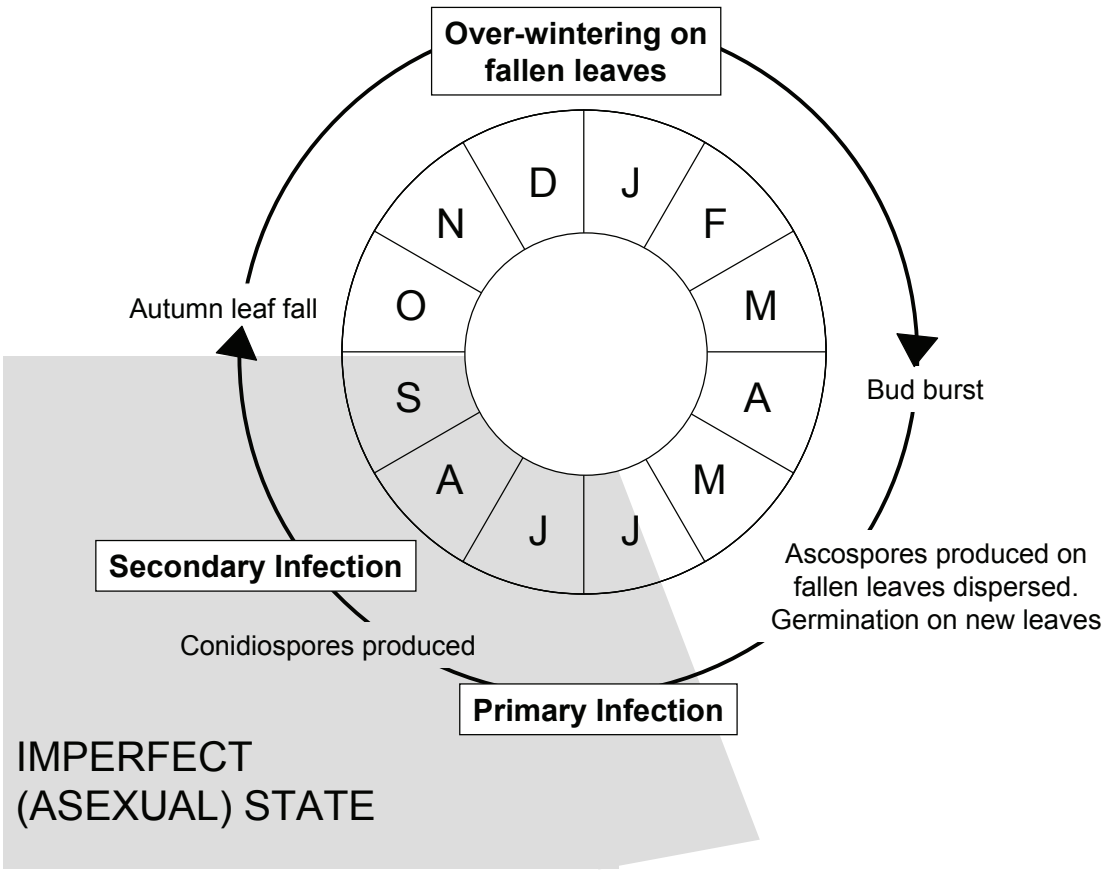


Figure 4.1. Generalised life-cycle of a fungal leaf pathogen on birch. The perfect state of *M.betulae* has yet to be described definitively. Shaded area represents the occurrence of *M. betulae* on living birch leaves in its asexual state.

Birch leaf-spot disease is not considered to damage trees permanently (Phillips & Burdekin 1992) and seldom warrants chemical treatment except on aesthetic grounds (Worf 1986). Despite this, localised and chronic defoliation of susceptible trees sometimes occurs relatively early in the season (Plate 4.2) and there is also anecdotal evidence for stunting or even death of nursery plantings (Worf 1986).

The aim of this investigation was to establish temporal and spatial patterns of *M. betulae* occurrence on *B. pendula* at Dalhaikie Flat and to explore the possibility of indirect effects of infection on the aphid *E. betulae*, specifically any preference for infected leaves and effects on aphid performance that might arise from so doing.

Plate 4.1. 'Green islands' or green zones surrounding fungal lesions on chlorotic leaves.

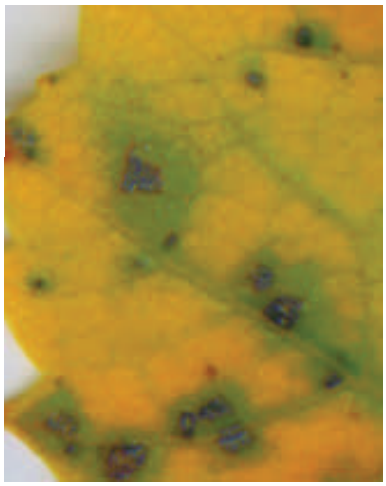


Plate 4.2. Chronic defoliation by leaf-spot pathogens on particularly susceptible trees can occur early in the season. Cf. diseased tree on the right with neighbouring healthy tree during July 2000

4.2. MATERIALS AND METHODS

4.2.1. Survey of birch leaf spot at Dalhaikie Flat

The survey protocol outlined in Section 2.3. of Chapter 2 was used to investigate the occurrence of *M. betulae* on birch leaves.

On the 10th June 1999, 50 leaves representing the spectrum of disease severity were removed from neighbouring trees and digitally scanned against a high-resolution graph paper background (Hewlett Packard DeskScan II ™, USA). Lesion area, both as an absolute value and as a proportion of the leaf surface, were measured electronically (Scion Image Beta 4.02 ™, Scion Corp., Maryland, USA).

In each survey, the area of each leaf covered with *M. betulae* lesions was estimated using the scanned images as a reference and any visible yellowing of leaf tissue (chlorosis) noted. Surveys that included fungal observations commenced on 12th June 1999 and 29th May 2000 and were repeated at approximately 17 and 11 day intervals, respectively. Surveys completed in 2000 were carried out using 12 of the original trees that displayed the highest and lowest levels of infection during 1999 (six trees of each). Tree height was measured on the initial and final surveys and also once in the middle of the growing seasons. All surveys were typically completed within 48 hours of initiation and were carried out on trees that were infected with *M. betulae* and not *D. betulina.*

4.2.2. Aphid choice tests

Forty aphid choice tests were conducted during July 2000 on 20 additional trees, which were similar in terms of height, aspect and infection level, as those used for surveys and the performance experiment (described below). On each tree, two branches having both asymptomatic and similarly infected leaves on the terminal 6cm, were selected and enclosed within a modified Blackman Box (described in Section 2.2.1) (Adams & Douglas 1997). Infected leaves showed slight chlorosis (~ 10% of leaf area) in all cases. Apart from one asymptomatic and one infected leaf, all leaves were excised (Plate 2.4 in Chapter 2) and either a young larva or adult aphid placed at the bottom of the box, so that each tree possessed an example of both tests. The location of individual aphids (*i.e.* infected leaf, asymptomatic leaf or neither) was then recorded at 120 minute intervals for 12 hours, and finally 24 and 36 hours later.

4.2.3. Aphid performance on infected leaves

Twenty birch trees that neighboured survey trees and were approximately the same height (1.5-2.2 m), and that shared a common aspect and degree of pathogen infection to one another were selected on the 1st July 1999. From each tree, three leaves of similar size and position were chosen to represent gradations of *M. betulae* infection; a leaf without infection, a moderately infected leaf (15-25% of leaf area bearing lesions) and a heavily infected leaf (25-35% of leaf area bearing lesions and displaying chlorosis).

Foliage containing aphids was collected from neighbouring trees. Recently deposited (young) larvae without wingbuds were isolated from the foliage and randomly divided into groups of three. Each group was weighed collectively using a microbalance (Cahn C-31, Cahn Instruments ™, California, USA) to an accuracy of ± 1 μ g and placed inside the same mesh-covered clipcages described in Section 2.2.1. Clip-cages were randomly allocated and secured to the three leaves on the 20 trees. Inspection of the clip-cages was carried out daily until the aphids became adult, at which time they were removed. Development time, number of exuviae and survivorship were recorded for each clip-cage. Aphids were again weighed in groups on the microbalance before being dissected with pins in a drop of ice-cold 50mM Tris-HCl pH 7.5 and viewed through a dissecting microscope (Leica MZ3, Leica Micosystems (UK), Milton Keynes, UK) at × 160 magnification using a calibrated eyepiece micrometer to measure the lengths of both hind tibiae. The number of developmentally mature embryos (bearing pigmented eyespots) was recorded. Where present, the length of the basal developmentally mature embryo was also determined. Relative growth rate was calculated using the equation according to Leather & Dixon (1984);

$$RGR = \frac{(\ln f - \ln i)}{T} \qquad \textbf{Equ. 4.1.}$$

where f is the final mass of the aphid upon reaching adulthood, i is initial mass of larval aphid at the beginning of the experiment and T is the time that elapsed between these events.

4.2.4. Phenolic concentration of leaves infected with *Marssonina betulina.*

Samples of leaves representing the corresponding levels of infection as used in the performance experiment were collected from surrounding trees, in addition to samples from each experimental tree. Samples were frozen (-18 ° C) for subsequent phenolic compound analysis as described in Section 2.4.2. Care was taken to remove and use only the basal portions of the leaves (which were generally the least symptomatic) in order to minimise contamination of subsequent phenolic analysis with fungi-produced phenolics.

4.2.5. Statistical analysis

Analysis was carried out in Minitab version 13.2 and SAS version 8.2 (SAS Institute, 1999). Occurrence of *M. betulae* was analysed using a GLIMMIX (SAS Institute 1999) generalised linear mixed model with a binomial error structure and logistic link function. Position of branch in the canopy (upper and lower branches) and leaf position on branch were sources of variation for 1999 and 2000 data. Tree type (heavily or moderately infected) was additionally fitted to the model for 2000 data, both as an individual source of variation and as an interaction with canopy and leaf position. Occurrence of fungi-induced chlorotic leaves was similarly analysed. The first survey of 2000 was omitted from the analysis because of the scarcity of observations. Branch number was fitted within tree number and both treated as a random terms in the model. All pair-wise comparions were made within the GLIMMIX macro by examining values of least squares means and maximum standard error. Degrees of freedom were calculated using the Satterthwaite formulas (Littell *et al.* 1996). Spearman's rank correlation test was used to test for any correlation between growth in tree height and infection load.

Analysis of the performance of aphids reared on infected and infection-free leaves was evaluated with several tests. Where data were normally distributed (larval and adult aphid mass, mean aphid tibia length and basal embryo length), analysis was carried out using general linear models. Phenolic concentration of infected leaves was similarly analysed. Aphid relative growth rates were not normally distributed and so evaluated using a Kruskal-Wallis test. Binary logistic regression was used to examine differences in aphid survivorship and development. A chi-square test was used to evaluate differences in the aphids' possession of developmentally mature embryos with pigmented eyespots. Where

4.3. RESULTS

4.3.1. Occurrence of *Marssonina betulae* at Dalhaikie Flat

Levels of fungal infection and leaves showing chlorosis over the growing seasons of 1999 and 2000 are shown in Figure 4.2. For comparison with 2000 data, the 1999 data are derived using only those twelve trees that were subsequently surveyed in 2000 (*i.e.* the heaviest and least infected trees in 1999). The pathogen became apparent at the field site at approximately the same time (within 15 days) in 1999 and 2000, but infection seemed to spread more rapidly in 1999 with 32% of surveyed leaves becoming infected by 12th June compared with just 14% of surveyed leaves on 9th June 2000. Levels of infection during 2000 subsequently increased at an approximately constant rate until they had reached similar levels to those seen in 1999 by mid July and possibly by the beginning of July from interpolation of Figure 4.2. Levels of infection remained relatively stable for the remainder of July in both years, but began to increase again after the beginning of August. The increase in surveyed leaves becoming infected was more rapid in 1999 with more than half of leaves examined showing symptoms by the end of August compared with 38% in 2000.

Leaves appeared to become chlorotic as a result of fungal infection slightly earlier during 2000 compared with the previous year, although levels of leaf chlorosis remained similar thereafter (Figure 4.2). Small decreases in levels of infection and fungi induced leaf chlorosis were probably the result of leaf abscission as there was never any alleviation of symptoms once leaves became infected (pers obs.).

The mean air temperature and total monthly rainfall at the nearby Aboyne weather station (see Section 2.5) for 1999 and 2000 is shown in Figure 4.3. The air temperature was slightly higher at the beginning of the year in 2000 compared with 1999, but the temperature in 1999 increased more rapidly as the season progressed and remained generally higher than 2000 for the rest of the growing season with the exception of August when it dropped by a mean of 2°C. The proportion of measurements that fall within 8-12°C[1] during April, May and June is consistently higher during 1999 compared with 2000. Rainfall is generally greater during 2000 compared with 1999, and especially so during April, October and November.

[1] the optimum temperature of *Melamsporidium betulinum* being 10 °C

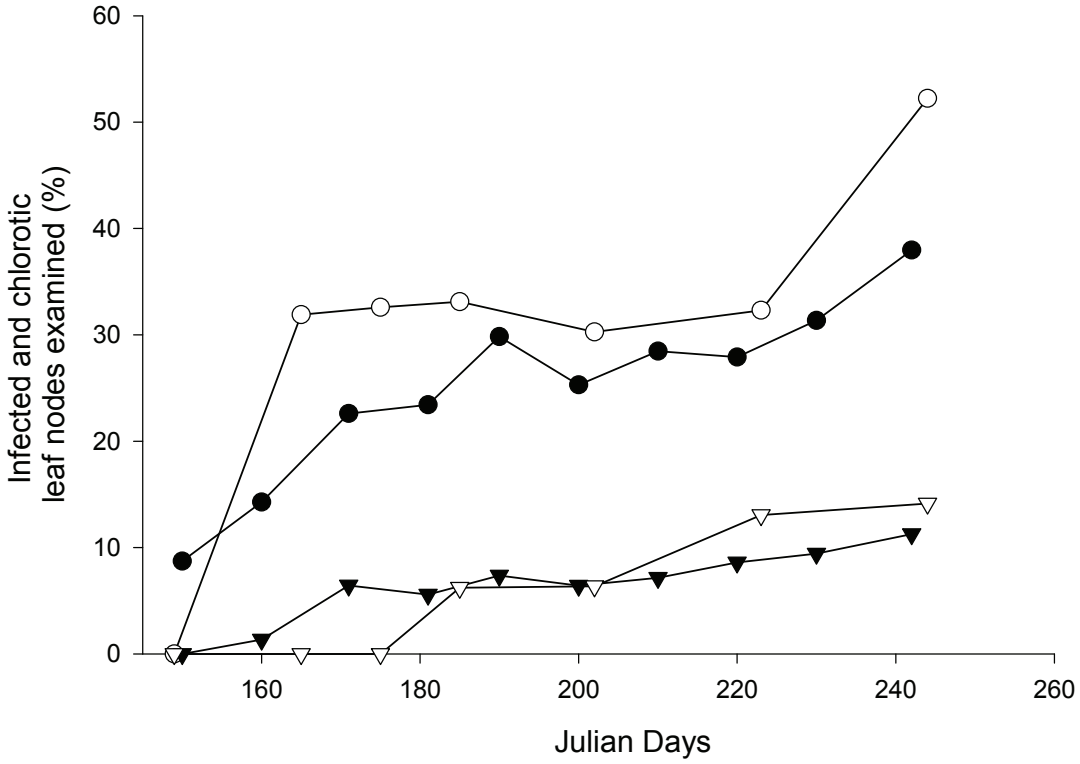


Figure 4.2. Percentage of leaf nodes with foliage displaying infection or leaf chlorosis during the growing seasons of 1999 (open symbols) and 2000 (closed symbols). Percentage infected with *M. betulae* in 1999 (○) and 2000 (●) and percentage becoming chlorotic in 1999 (▽) and 2000 (▼). 1999 data is derived using only from those trees that were subsequently surveyed in 2000.

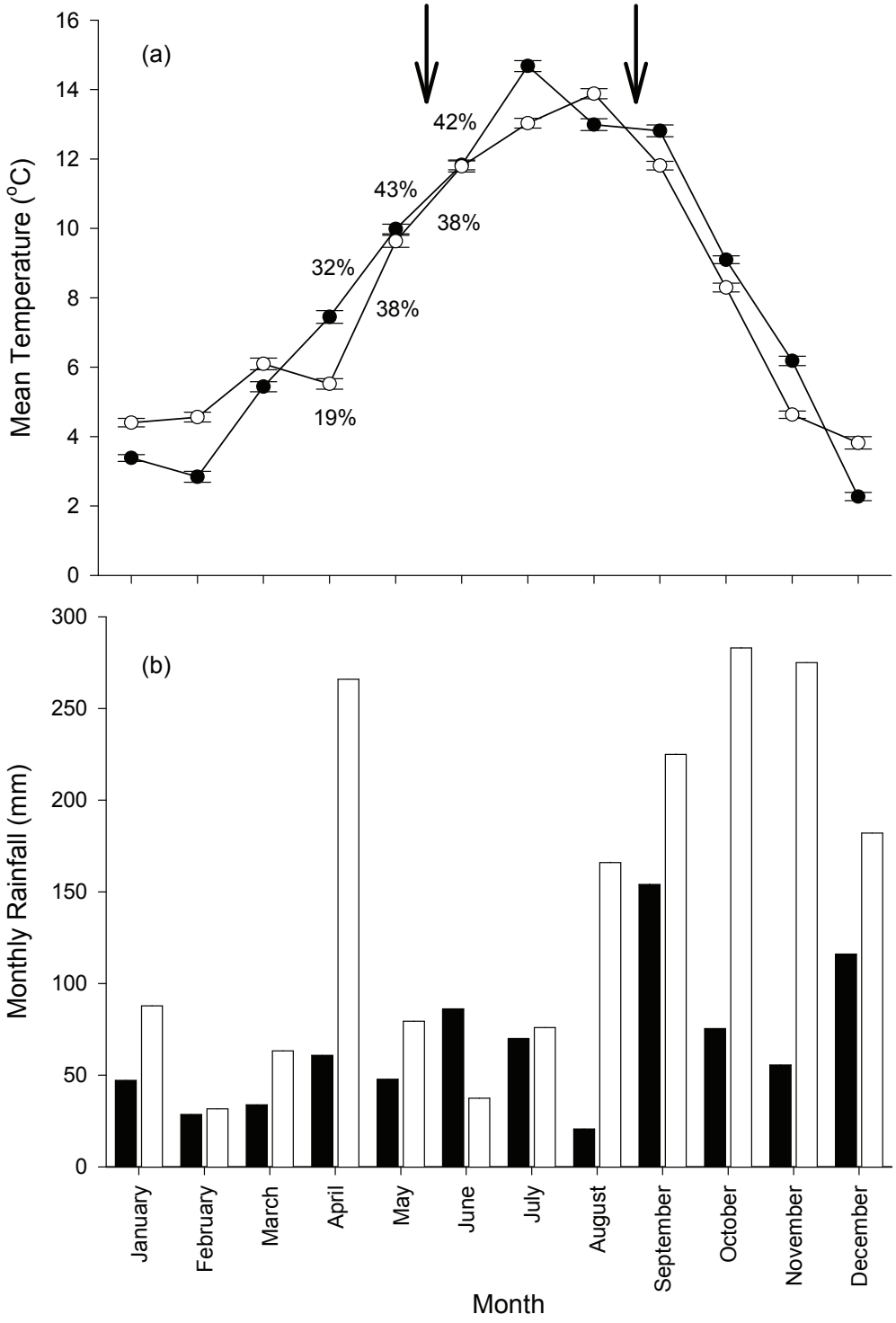


Figure 4.3. (a) Mean air temperature (°C ± SE) and (b) total monthly rainfall (mm) at Aboyne weather station (~ 16km from Dalhaikie Flat) during 1999 (●) and 2000 (○). The first arrow indicates the widespread occurrence of *M. betulae* lesions and the second indicates the onset of leaf fall. Proportion of hourly measurements between 8-12 °C (the optimum spore germination temperature of *M. betulinum*) in 1999 and 2000 are shown above and below the plots respectively.

significant, differences between the three leaf types were analysed with *post hoc* Tukey's tests.

The spatial distribution of *M. betulae* on *Betula pendula* is described in Figures 4.4-4.10, Tables 4.3-4.7 and the accompanying text. To aid interpretation, these figures and tables are outlined in Table 4.2 overleaf.

Marssonina betulae was found significantly more often on leaves of lower branches of the tree canopy for the first three consecutive surveys during 1999, the fourth narrowly missing the 95 % confidence interval (Figure 4.4). From the 9th August 1999, upper branches became equally as infected and remained so for the remainder of the growing season. The pattern was more complex for 2000 (Figures 4.5a & 4.5b and Table 4.3), when trees initially showed greater infection on leaves of lower branches (on the 9th and 28th June 2000) but the upper branches became equally and then more infected by 17th August 2000. Infection is clearly much less abundant in 2000 on those trees designated as least infected during 1999 (Figure 4.5b) and the pattern of infection initially being on lower branches then switching to upper branches later in the season is the same for both heavily and moderately infected trees (Table 4.3).

Leaves became infected by *M. betulae* to varying degrees depending on their position along the branch. In 1999, leaves which were closest to the trunk of the tree (*i.e.* basal and intermediate) showed greater incidence of fungal infection than those leaves which at the branch apex (*i.e.* apical leaves) in all surveys (Figure 4.6 and Table 4.4). Infection was consistently less frequent on apex leaves, whereas basal and intermediate leaves were similarly infected (Table 4.4). Likewise, basal and intermediate leaves examined on heavily and moderately infected trees during 2000 (Figures 4.7a & 4.7b respectively and Tables 4.5 & 4.6) showed greater incidence of fungal infection compared with leaves at the branch apex. Basal and intermediate leaves were again similarly infected throughout 2000 (Table 4.6). This pattern was consistent for both heavily and moderately infected trees apart from 18th and 28th July 2000 when apex leaves of heavily infected trees became more

Table 4.2. Overview of graphs and tables subsequently discussed in relation to the spatial distribution of *M. betulae* and chlorotic leaves on *B. pendula* at Dalhaikie Flat.

Description		Figure	Corresponding Statistics	Page
1. Spatial occurrence of infection				
Position of branch in tree canopy (e.g. upper or lower)	1999	4.4	Fig. 4.4 legend	15
	2000	4.5	Table 4.3	16-17
Position on leaf on the branch (e.g. basal, intermediate or apical)	1999	4.6	Fig 4.6 legend & Table 4.4	18-19
	2000	4.7	Table 4.5 & Table 4.6	21-22
2. Symptom severity on leaves				
Frequency of leaves with degrees of symptom severity over the season	1999	4.8	-	24
	2000	4.9	-	25
3. Spatial occurrence of chlorotic leaves				
Position of branch in tree canopy (e.g. upper or lower)	2000	4.10	Table 4.7	26-27

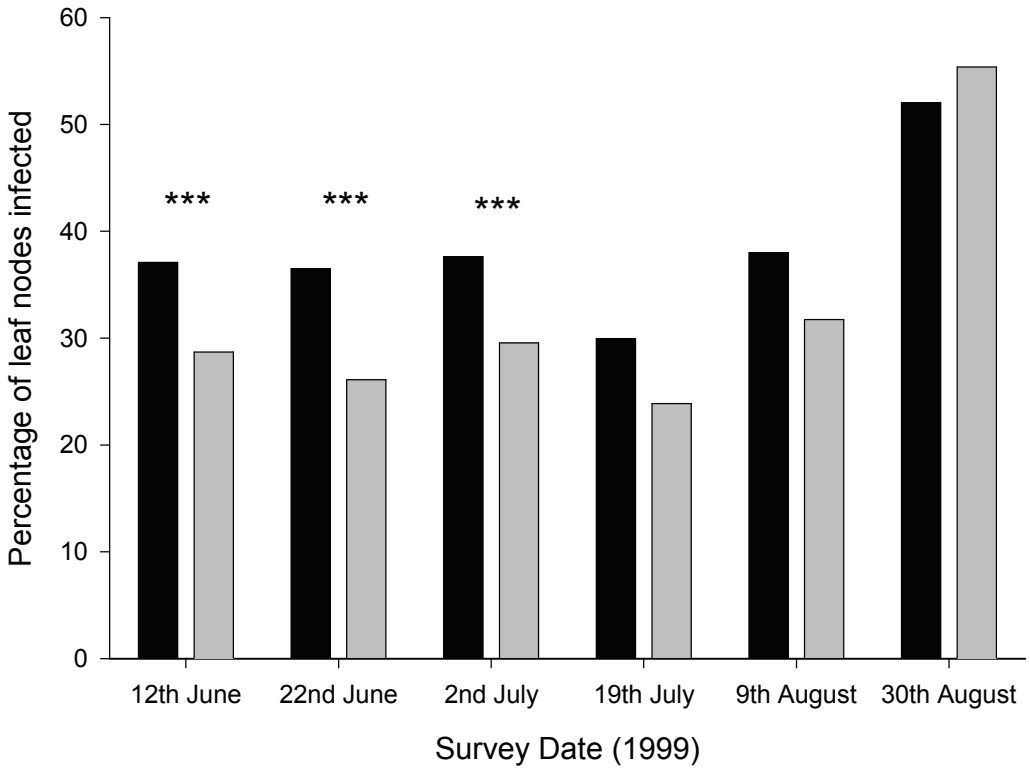


Figure 4.4. The percentage of leaf nodes that possess foliage infected with *M. betulae* on upper (■) and lower (▒) branches within the tree canopy during the growing season of 1999. 12th June, $F_{1,603} = 26.72$, $P < 0.0001$; 22nd June, $F_{1,551} = 19.29$, $P < 0.0001$; 2nd July, $F_{1,560} = 13.91$, $P = 0.0002$; 19th July, $F_{1,507} = 3.63$, $P = 0.0571$; 9th August, $F_{1,430} = 2.51$, $P = 0.1141$; 30th August, $F_{1,576} = 1.17$, $P = 0.2790$. Statistical significance of the effect of branch position in the canopy on levels of infection is shown with asterisks *** $P < 0.001$.

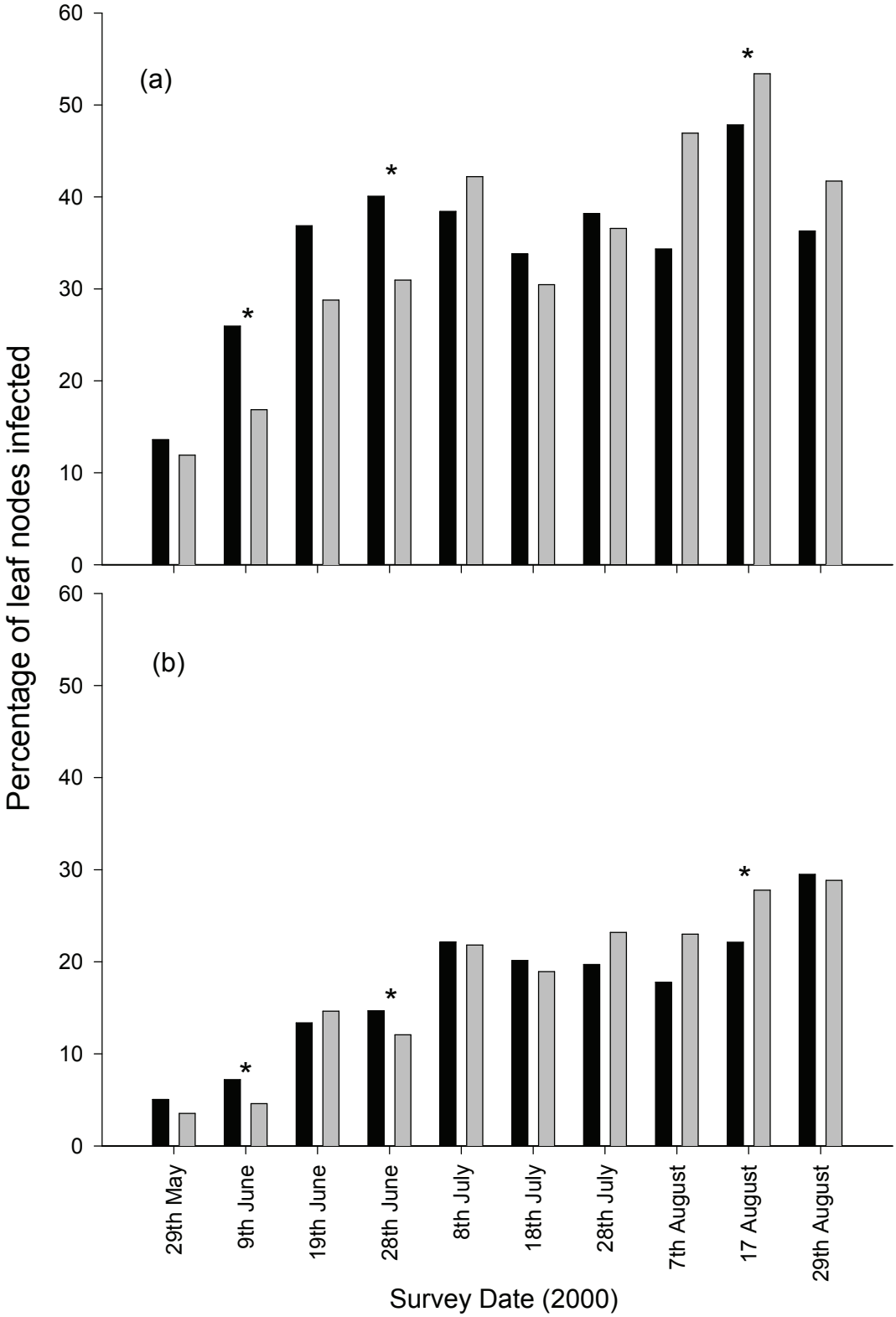


Figure 4.5. The percentage of leaf nodes that possess foliage infected with *M. betulae* on upper (■) and lower (▒) branches within the tree canopies of (a) heavily infected and (b) moderately infected trees during the growing season of 2000. Statistical significance of the effect of branch position in the canopy on levels of infection is shown with asterisks * $P < 0.05$ (Table 4.3).

Table 4.3. Results of generalised linear mixed model analysis for the spatial occurrence of *M. betulae* on *B. pendula* during 2000 in relation to the effect of canopy position (upper and lower branches) (see Figure 4.5) and the interaction with tree type (heavily and moderately infected trees) as sources of variation in distribution of fungi infected leaves. * $P < 0.05$.

Survey Date (2000)	Position in Canopy			Tree type × Position in Canopy		
	df	*F*	*P*	df	*F*	*P*
9th June	1, 298	5.34	0.0216*	1, 298	0.01	0.9640
19th June	1, 255	0.84	0.3590	1, 255	1.66	0.1987
28th June	1, 282	4.20	0.0413*	1, 282	0.71	0.3999
8th July	1, 237	0.11	0.7425	1, 237	0.07	0.7905
18th July	1, 245	0.55	0.4590	1, 245	0.26	0.6126
28th July	1, 255	0.15	0.7035	1, 255	0.45	0.5024
7th August	1, 273	2.97	0.0860	1, 273	0.17	0.6761
17th August	1, 258	6.64	0.0105*	1, 258	0.51	0.4770
29th August	1, 226	0.35	0.5545	1, 226	0.43	0.5119

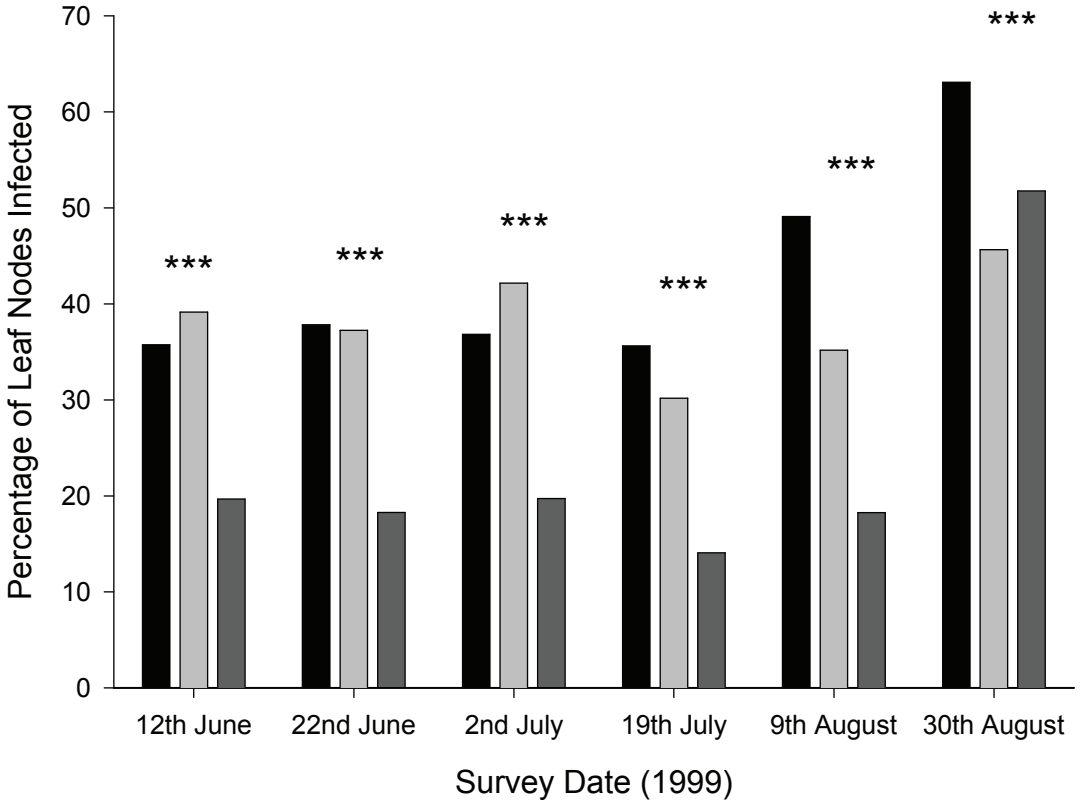


Figure 4.6. The percentage of leaf nodes that possess foliage infected with *M. betulae* on basal (■), intermediate (■) and apical (■) *B. pendula* leaves during the growing season of 1999. Basal leaves were those closest to the trunk and apical leaves those furthest away. 12th June, $F_{2,1924} = 85.92$, $P < 0.0001$; 22nd June, $F_{2,1615} = 76.06$, $P < 0.0001$; 2nd July, $F_{2,1620} = 72.56$, $P < 0.0001$; 19th July, $F_{2,1312} = 104.86$, $P < 0.0001$; 9th August, $F_{2,1133} = 192.20$, $P < 0.0001$; 30th August, $F_{2,1372} = 38.51$, $P < 0.0001$. Statistical significance of leaf position on the branch is shown with asterisks *** $P < 0.001$.

Table 4.4. Summary of generalised linear mixed model least squares mean estimates (logistic scale) for the spatial occurrence of *M. betulae* on *B. pendula* leaves during 1999 comparing position on branch (basal, intermediate and apex leaves) (see Figure 4.6) as sources of variation in fungal distribution.

Comparisons between leaf positions	Least squares means (Logistic scale)			Maximum Standard Error
Survey Date (1999)	Basal	Intermediate	Apex	
12th June	-0.87	-0.60	-2.06	0.18
22nd June	-0.69	-0.72	-2.02	0.14
2nd July	-0.71	-0.44	-1.81	0.13
19th July	-0.82	-1.35	-2.82	0.17
9th August	0.10	-0.98	-2.70	0.22
10th August	0.70	-0.18	0.03	0.14

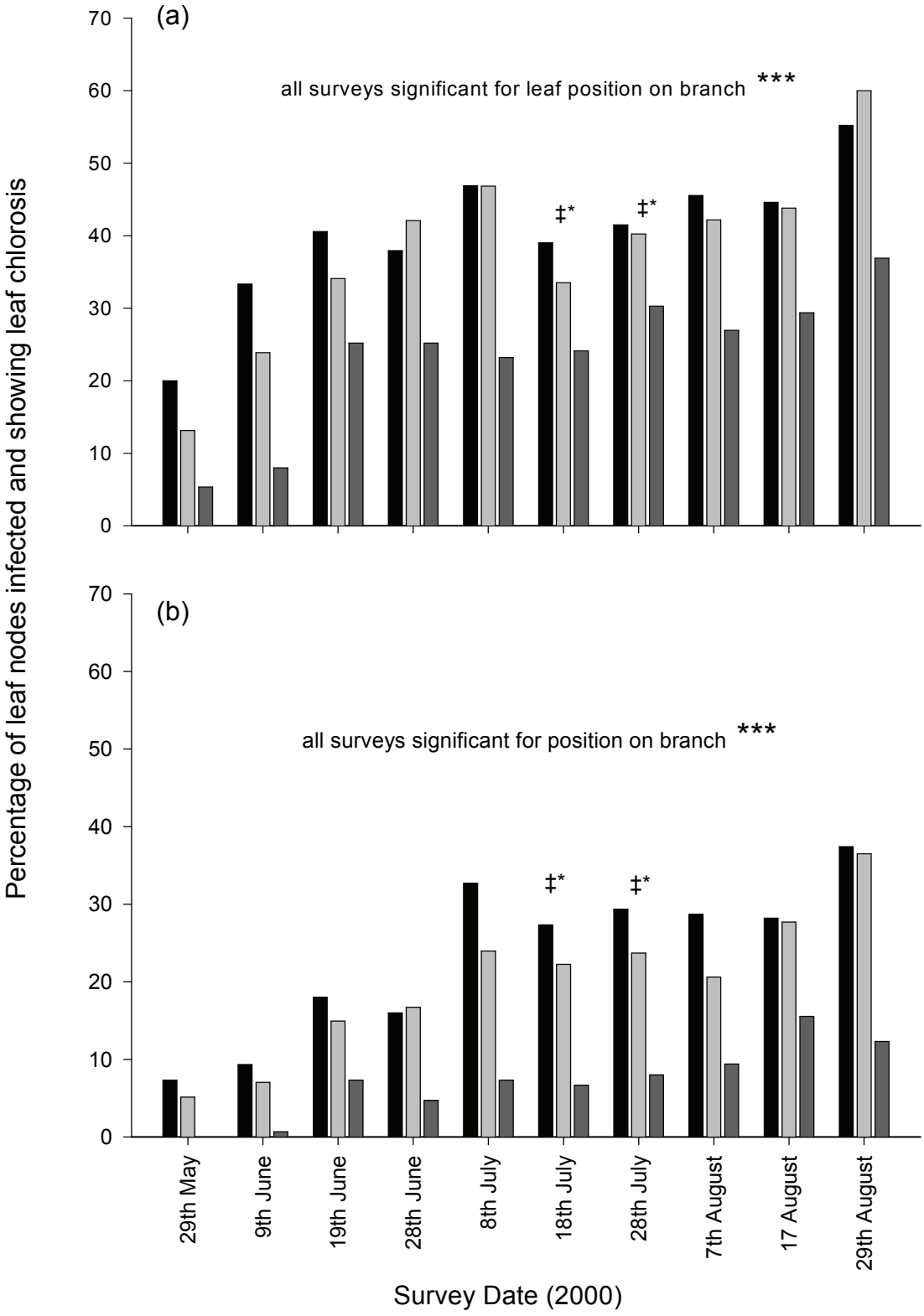


Figure 4.7. The percentage of leaf nodes that possessed foliage infected with *M. betulae* on basal (■), intermediate (□) and apex (■) leaves on branches of (a) heavily and (b) moderately infected trees during the growing season of 2000. Basal leaves were those closest to the trunk and apex leaves those furthest away. ‡Only the statistical significance of the 'leaf position × tree type' interaction is shown as leaf position on branch is significant for every survey * $P < 0.05$.

Table 4.5. Results of generalised linear mixed model analysis for the spatial occurrence of *M. betulae* during 2000 showing the effect of position on branch (basal, intermediate and apical leaves) (see Figure 4.7) and its interaction with tree type (heavily and moderately infected trees) as sources of variation in location of infected leaves.

Survey Date (2000)	Position on Branch			Tree type × Position on Branch		
	df	*F*	*P*	df	*F*	*P*
9th June	2, 1112	28.36	<0.0001	2, 1112	2.51	0.0816
19th June	2, 870	18.77	<0.0001	2, 870	1.15	0.3167
28th June	2, 862	18.18	<0.0001	2, 862	1.46	0.2327
8th July	2, 805	37.17	<0.0001	2, 805	2.66	0.0706
18th July	2, 805	24.71	<0.0001	2, 805	3.72	0.0245
28th July	2, 748	20.05	<0.0001	2, 748	4.61	0.0103
7th August	2, 742	17.78	<0.0001	2, 742	1.11	0.3285
17th August	2, 687	13.42	<0.0001	2, 687	0.02	0.9796
29th August	2, 512	23.12	<0.0001	2, 512	1.50	0.2246

Table 4.6. Summary of generalised linear mixed model least squares mean estimates (logistic scale) for the spatial occurrence of *M. betulae* on *B. pendula* leaves during 2000, comparing position on branch (basal, intermediate and apical leaves) (see Figure 4.7) as sources of variation in fungal distribution.

Comparisons between leaf positions	Least squares means (logistic scale)			Maximum standard error
Survey Date (2000)	Basal	Intermediate	Apex	
9th June	-2.34	-2.79	-5.16	0.52
19th June	-1.27	-1.62	-2.47	0.27
28th June	-1.23	-1.01	-2.29	0.24
8th July	-0.48	-0.72	-2.18	0.22
18th July	-0.86	-1.08	-2.26	0.23
28th July	-0.71	-0.83	-1.88	0.18
7th August	-0.54	-0.81	-1.68	0.16
17th August	-0.59	-0.58	-1.34	0.15
29th August	-0.15	-0.08	-1.36	0.17

The frequency of leaf nodes possessing leaves with increasing symptom severity over the growing seasons of 1999 and 2000 was also compared by defining symptom severity as the proportion of leaf displaying fungal lesions, classified as 1-10 %, 11-20 %, 21-30 % and > 30 % lesion cover. (Figures 4.8 and 4.9 respectively). The large majority of infected leaves showed mild symptoms (1-10 % leaf area symptomatic) in both years, but examples of increasing symptom severity became more frequent as the growing season progressed. The disease appears to be developing more slowly at the level of leaf in 2000 as no leaves display severe infection (>30 % of leaf area symptomatic) until three weeks after the pathogen becomes apparent on survey trees. In contrast, some leaves (albeit very few), showed severe symptoms during the first survey of 1999. Surveys from both years suggest a general upward trend of symptom severity as leaves displaying more acute symptoms (*i.e.* larger and/or more numerous lesions) become more frequent as the growing season progresses.

Fungal-induced leaf chlorosis occurred throughout the tree canopy without any discernible trends, except that it was more frequent on heavily infected trees than on moderately infected trees during 2000 (Figure 4.10). Chlorotic leaves were found equally dispersed between upper and lower branches however (Table 4.7).

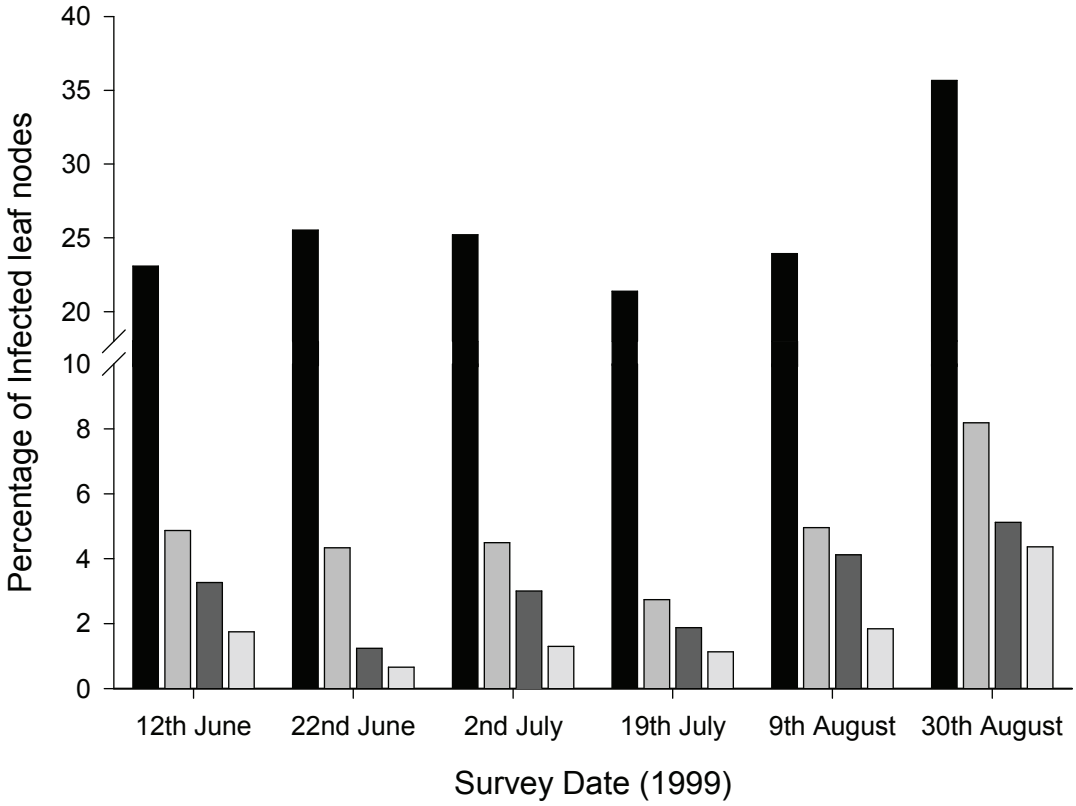


Figure 4.8. Percentage of infected leaf nodes possessing *B. pendula* leaves with varying degrees of infection by *M. betulae*; 1-10% of leaf area infected (■), 11-20% infected (■), 21-30% infected (■) and >30% infected (■) during the growing season of 1999.

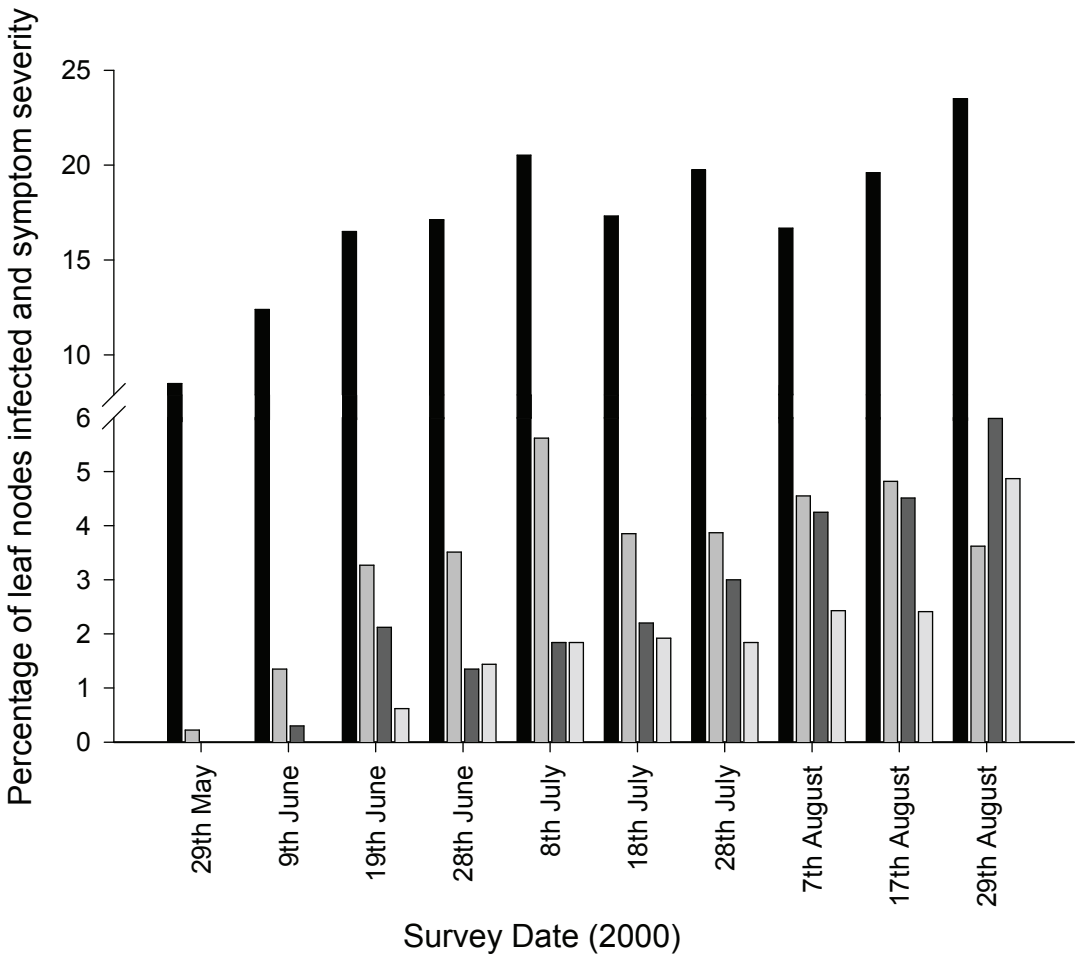


Figure 4.9. Percentage of infected leaf nodes possessing *B. pendula* leaves with varying degrees of infection by *M. betulae*; 1-10% of leaf area infected (■), 11-20% infected (■), 21-30% infected (■) and >30% infected (■) during the growing season of 2000.

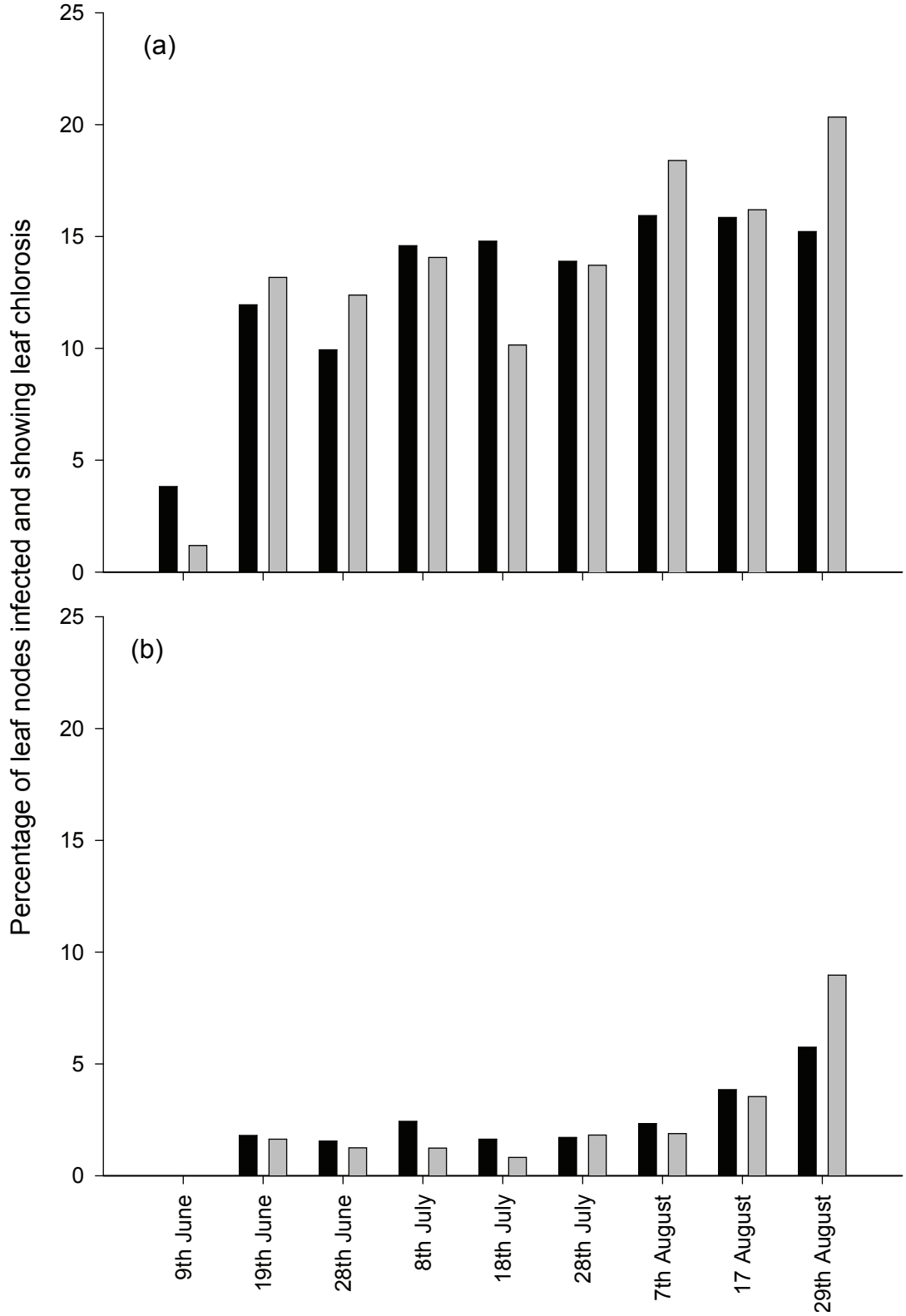


Figure 4.10. The percentage of leaf nodes that possess *B. pendula* leaves infected with *M. betulae* and show leaf chlorosis on upper (■) and lower (■) branches within the tree canopies of (a) heavily infected and (b) moderately infected trees during the growing season of 2000.

Table 4.7. Results of generalised linear mixed model analysis for the spatial occurrence of *M. betulae* induced leaf-chlorosis on *B. pendula* during 2000 in relation to the effect of canopy position (upper and lower branches) (see Figure 4.10) and the interaction with tree type (heavily and moderately infected trees) as sources of variation in distribution of chlorotic leaves.

Survey Date (2000)	Position in Canopy			Tree type × Position in Canopy		
	df	*F*	*P*	df	*F*	*P*
19th June	1, 334	0.05	0.824	1, 334	0.01	0.923
28th June	1, 306	0.04	0.834	1, 306	0.23	0.631
8th July	1, 394	0.38	0.540	1, 394	0.47	0.495
18th July	1, 533	1.07	0.302	1, 533	0.00	0.978
28th July	1, 448	0.23	0.635	1, 448	0.01	0.922
7th August	1, 431	0.00	0.989	1, 431	0.26	0.610
17th August	1, 379	0.09	0.768	1, 379	0.07	0.788
29th August	1, 286	2.71	0.101	1, 286	0.04	0.847

infected than apex leaves of moderately infected trees relative to neighbouring basal and intermediate leaves.

No relationship could be found between the growth of survey trees (in terms of increases in height) and their individual levels of infection measured as either mean number of leaf nodes with infected foliage (Figure 4.11a) or as the mean number of leaf-nodes with chlorotic leaves. Several trees showed no increase in height between the beginning and end of the growing season, but these trees reflected the full spectrum of infection seen on survey trees.

Some trees consistently bore the highest levels of fungal infection and there was a strong positive correlation between fungal infection on trees in 1999 and 2000, $r_s = 0.755$ $P = 0.005$ (Figure 4.12).

4.3.2. Aphid choice tests

In choice tests conducted in Blackman boxes that were placed on a branch with an infected and asymptomatic leaf, larval and adult aphids showed similar patterns of preference for the infected leaf, so the data were pooled. After just four hours significantly more aphids were found on the infected leaf rather than the asymptomatic leaf. The number of aphids on infected leaves gradually increased as time progressed (Figure 4.13).

4.3.3. Aphid performance on infected leaves

The performance of aphids upon reaching adulthood when reared on leaves with different degrees of infection is seen in Table 4.8. Survivorship and development time to adulthood was not significantly different for aphids reared on each leaf type. There was also no significant difference between the larval masses of aphids randomly allocated to the different leaves, but those aphids on heavily infected leaves did become significantly heavier than those reared on asymptomatic or moderately infected leaves, which remained statistically indistinguishable from one another. The development time and relative growth rate was not significantly different. Mean hind tibiae length showed a similar pattern to mean final mass in so far as those aphids reared on heavily infected leaves developed

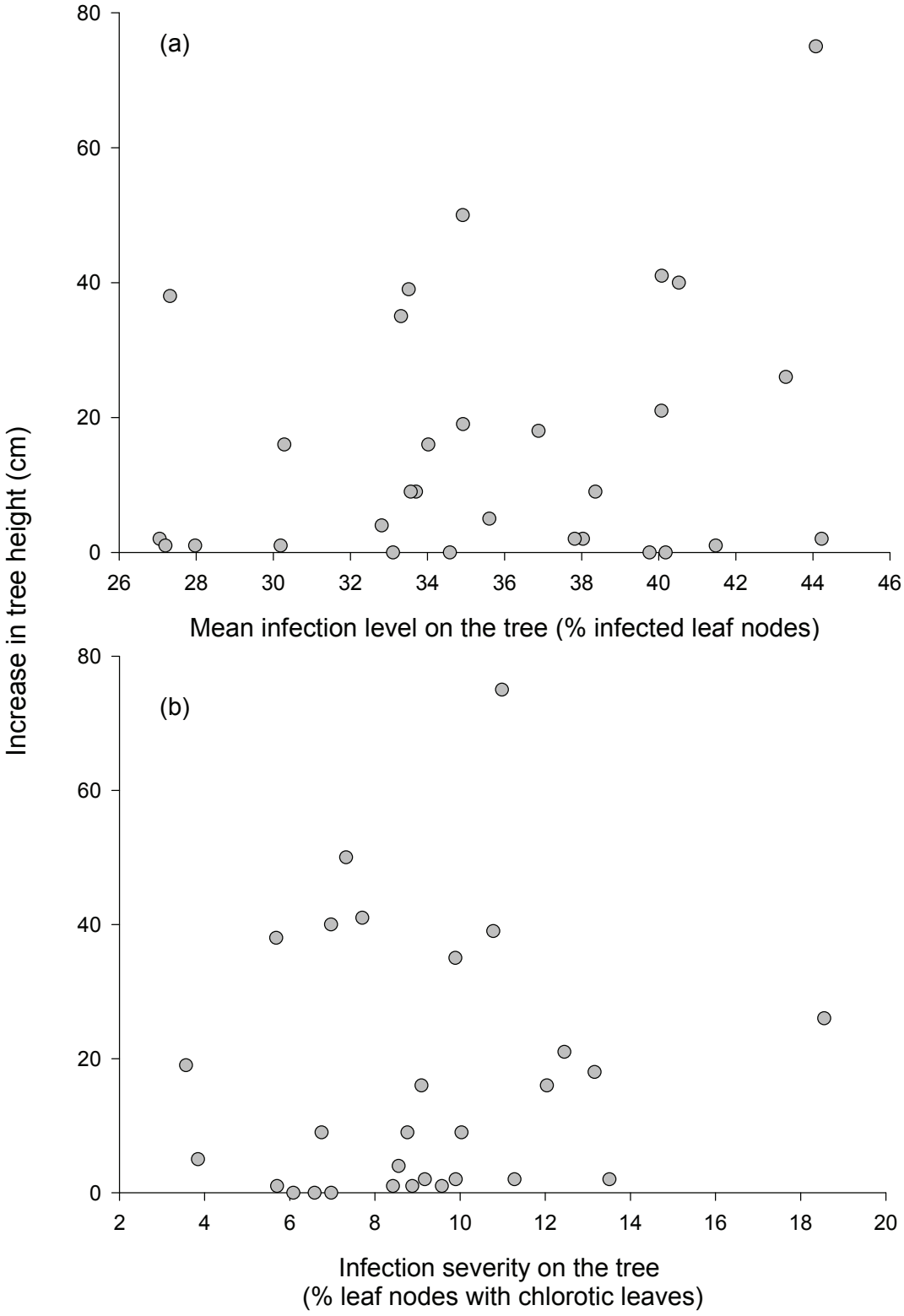


Figure 4.11. Increase in tree height and infection load over the growing season during 1999. (a) Infection level was defined as the mean percentage of leaf nodes examined that possessed infected leaves and (b) infection severity as proportion of leaf nodes with fungal-stimulated chlorotic leaves. Spearman's correlation was carried out to compare infection load and increase in tree height: r_s = 0.178 *P* = 0.348.

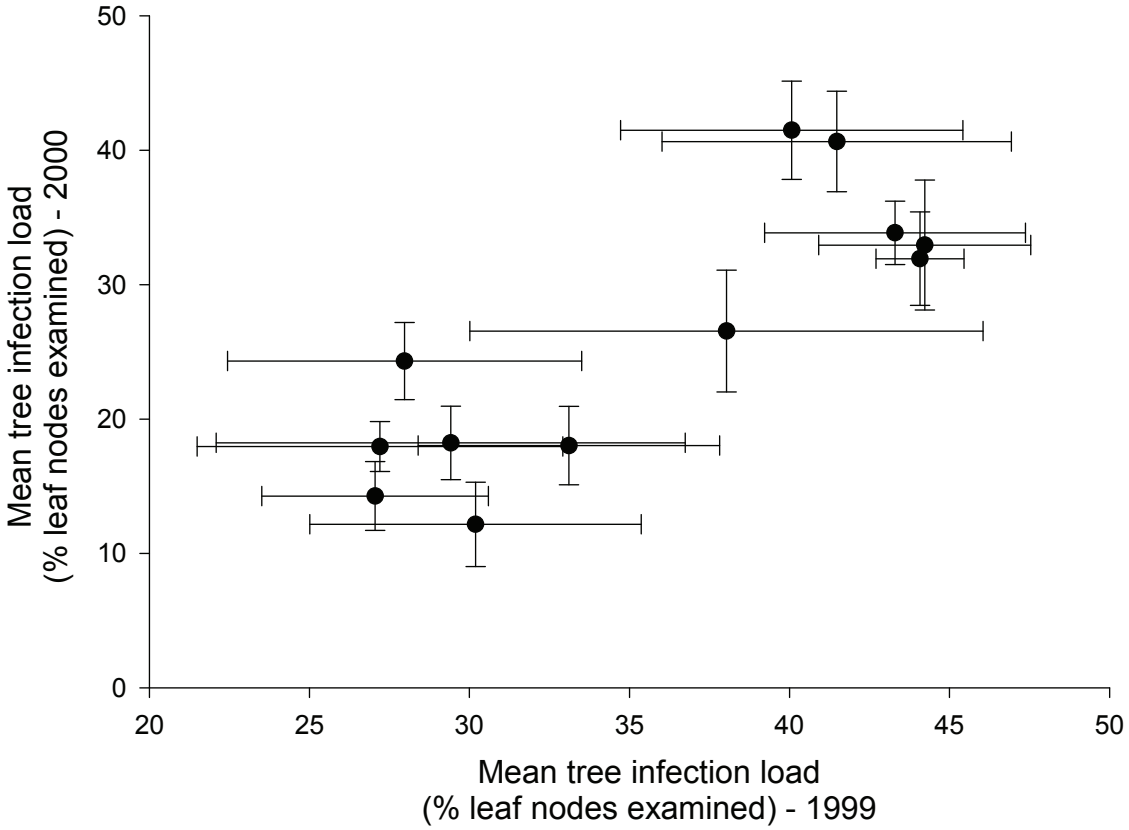


Figure 4.12. Mean infection loads per survey tree in 1999 and 2000, defined as mean number of leaf nodes examined bearing fungi-infected leaves ± SE.

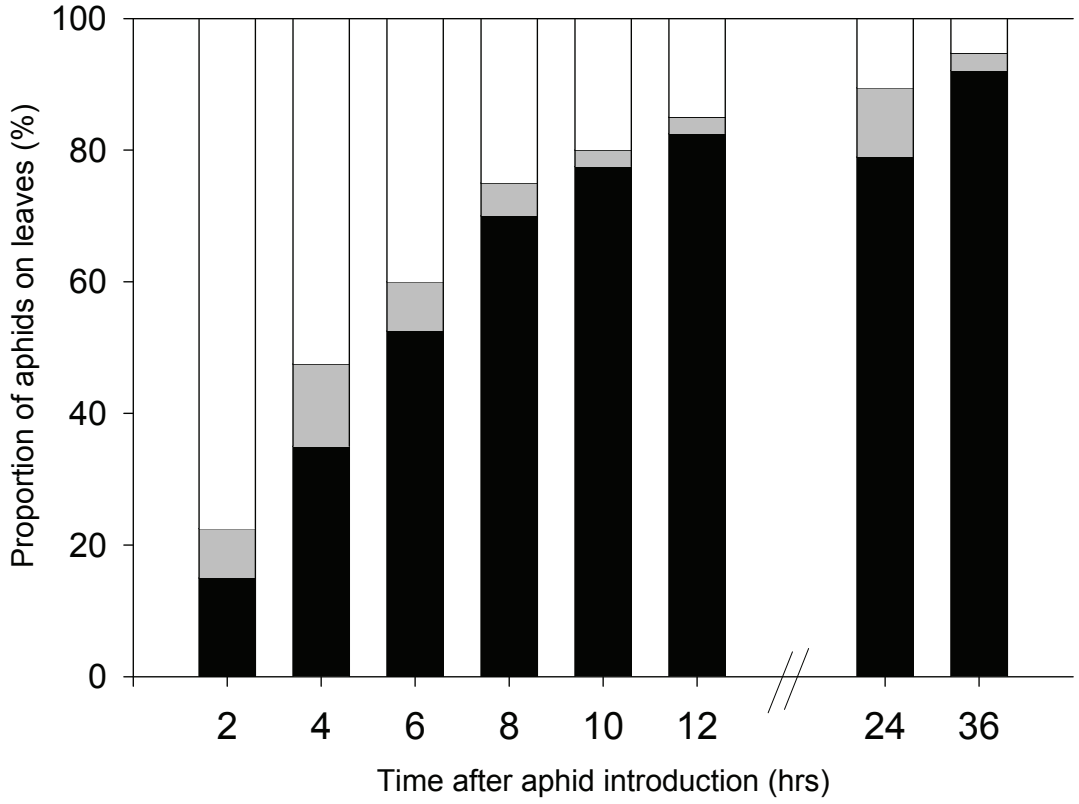


Figure 4.13. Aphid choice tests showing the proportion of aphids (adult and larval data pooled) located on fungi-infected (■) and asymptomatic (▒) leaves or elsewhere (□) in the Blackman Box. Aphids were significantly more abundant on inoculated rather than asymptomatic leaves from 4 hrs onwards. $\chi_1^2 = 4.26$, $P < 0.05$.

Table 4.8. Performance of aphids caged on leaves with varying degrees of fungal infection. Approximately 15-25% of leaf surface area bore fungal lesions in moderately infected leaves, whereas heavily infected leaves possessed 25-35% cover and showed signs of chlorosis. Survivorship to adulthood $G_1 = 0.248$, $P = 0.0619$; Development time to adulthood DF = 1, G = 1.40, $P = 0.237$; Initial mass $F_{2,57} = 1.39$, $P = 0.257$; Final mass $F_{2,37} = 16.41$, $P < 0.001$; Relative growth rate $H_2 = 4.47$, $P = 0.107$; Hind tibiae length $F_{2,64} = 17.62$, $P < 0.0001$. Significant differences between leaf types are indicated with lower case superscripts (as determined by a Tukey's *post-hoc* test).

Leaf Type	Aphid Performance					
	Survivorship (%)	Development time (days)	Mass (mg)		Relative growth rate (mg^{-1} mg^{-1} day^{-1})	Mean Hind Tibia Length (mm)
			Initial (larval)	Final (adult)		
Asymptomatic	56.9 (n = 16)	13.63 ± 0.125	0.388 ± 0.027	0.999^{a} ± 0.043	0.068 ± 0.005	2.17^{a} ± 0.056
Moderately infected	54.8 (n = 15)	13.27 ± 0.118	0.336 ± 0.030	0.886^{a} ± 0.033	0.079 ± 0.009	2.02^{a} ± 0.041
Heavily infected	63.0 (n = 9)	13.44 ± 0.176	0.397 ± 0.049	1.270^{b} ± 0.062	0.097 ± 0.014	2.47^{b} ± 0.061

statistically longer hind tibia than those reared on asymptomatic or moderately infected leaves, the latter two being statistically indistinguishable from each other.

Dissection of aphids revealed that all but one of the aphids reared on heavily infected leaves possessed at least one developmentally mature embryo with pigmented eyespots, whereas only about half of aphids on asymptomatic and moderately infected leaves did so. In aphids raised on the heavily infected leaves, the basal embryo was significantly longer than those possessed by aphids on moderately infected leaves, but not more so than those on the asymptomatic leaf. Basal embryo length was not significantly different in aphids on asymptomatic and moderately infected leaves (Table 4.9).

4.3.4. Phenolic analysis of infected leaves

The concentration of phenolic compounds in heavily infected leaves was significantly higher than concentrations in moderately and asymptomatic leaves, which were not significantly different from each other (Figure 4.14). There was no significant difference

Table 4.9. Embryo characteristics of aphids caged on leaves with varying degrees of fungal infection as defined in Table 4.8 legend. Adults possessing developed embryos with pigmented eyespots $\chi_2^2 = 11.99$, $P < 0.01$; Length of basal embryo $F_{2,74} = 3.87$, $P = 0.03$. Lowercase superscripts indicate significant differences between leaf types.

	Embryo Characteristics	
Leaf Type	Number of adults possessing pigmented embryos / adults examined	Basal embryo length (mm)
Asymptomatic	14 / 26	$0.331^{ab} \pm 0.037$
Moderately Infected	10 / 24	$0.246^{b} \pm 0.026$
Heavily Infected	16 / 17	$0.398^{a} \pm 0.0522$

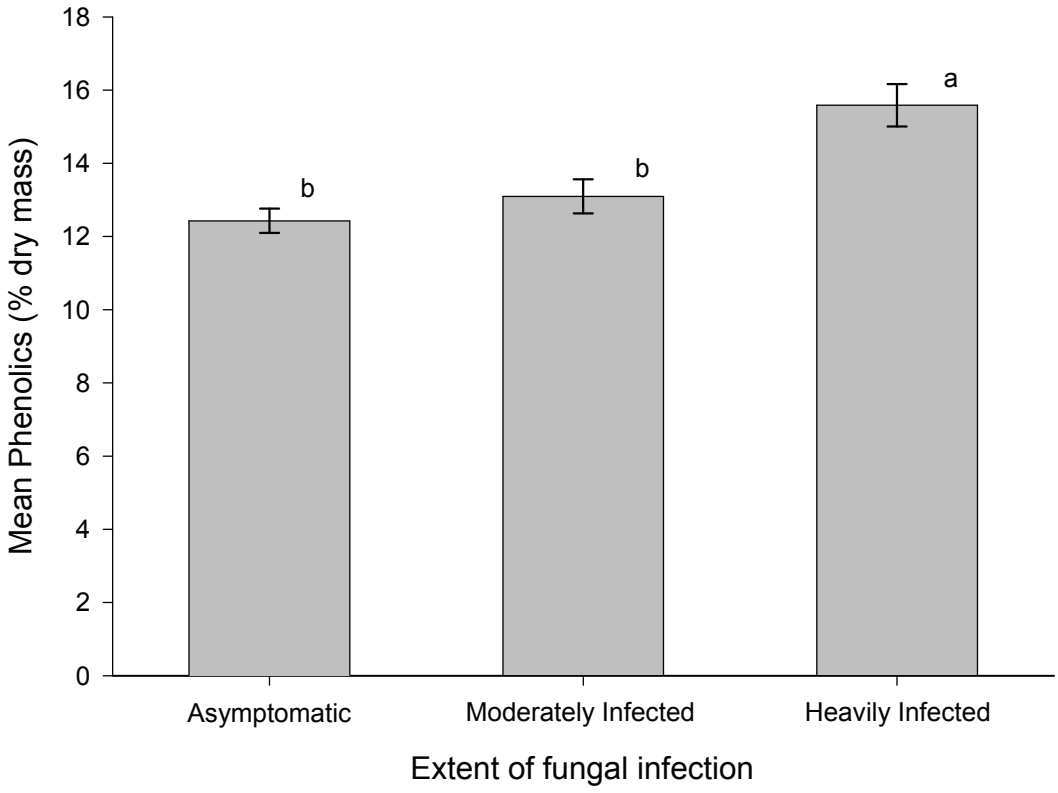


Figure 4.14. Mean phenolic concentration (± SE) of asymptomatic leaves and leaves showing moderate and heavy fungal infection (as defined in Table 4.8 legend). $F_{2,87}$ = 12.56, *P* <0.001. Lowercase superscripts indicate significant differences between leaf types.

between the phenolic levels of leaves of the experimental trees, on which aphids were reared, and those of neighbouring trees so all data within treatments was pooled.

4.4. DISCUSSION

4.4.1. Occurrence of *Marssonina betulae* at Dalhaikie Flat

Warm and wet weather early in spring are thought to promote primary infection of leaf-spot pathogens (Strouts & Winter 1994). Temperature and rainfall are considered to be very important factors for the germination and penetration of the rust pathogen, *Melamsporidium betulinum*, on birch (Doodley 1984; Helander *et al.* 1998) and this may in part explain why the *Marssonina betulae* infection appeared to develop more rapidly in 1999 than 2000, although with only two years of data such explanations are exploratory rather than definitive.

During 1999, the mean temperature was slightly higher leading up to and during spore dispersal and primary infection (April-June) than in 2000. Dooley (1984) found that *M. betulinum* spores germinated on birch leaves most successfully at 10°C. The number of hourly measurements that are between 8 - 12°C is consistently higher in 1999 than 2000 during April - June (Figure 4.3). In addition, although 2000 is generally a wetter year, rainfall during June 1999 when lesions first became widespread is more than double that which fell in June 2000. Even these short periods of favourable conditions may be enough to lead to successful initial infection if they occur at the critical time (Hamelin *et al.* 1992; Helander *et al.* 1998). For instance, Elamo *et al.* (2000) suggested that small increases in temperature (1-2°C) and rainfall (43-51mm) at crucial points in the infection cycle were enough to account for the large between-year differences in infection level of *M. betulinum* on birch trees.

Marssonina betulae was more abundant for the remainder of the growing season in 1999 compared with 2000. The extent of secondary infection would depend, amongst other things, on the amount of warm and wet weather during the rest of the growing season (Strouts & Winter 1994). The mean temperature generally remained slightly higher during 1999 than in 2000 but 1999 tended to be drier so it may be that the successful and rapid infection achieved early in 1999 provided a foothold for subsequent infection throughout the remainder of the growing season. Symptom severity on individual leaves developed more rapidly during the initial surveys of 1999 than 2000 so this early advantage may have

laid the foundation for further infection. The heavy rainfall in August 2000 may also explain the escalation in infection that occurred during the same period.
At the beginning of the season there was a trend (albeit less clear-cut in 2000) toward lower branches being more infected than upper branches. Towards the end of the season when the infection levels were higher, upper branches became equally infected (and even more so in 2000). There have been some anecdotal reports of the occurrence of birch leaf spot disease occurring primarily on lower branches, sometimes leaving only crowns of healthy leaves (Worf 1986). Disease progression from the base of the tree has been observed for *Discula betulina* (Redfern *et al.* 1981; Bennell & Millar 1984; Phillips & Burdekin 1992), but again these were not quantitative observations.

The continual flushing of birch foliage (Atkinson 1992) on upper branches would mean that a smaller proportion of leaves were mature on these branches relative to those on lower branches. Phenology of leaves could be important in determining their susceptibility to pathogen infection as leaves at the apex of branches (which by definition are youngest) always possessed much lower levels of infection than neighbouring leaves closer to the trunk of the tree. A similar trend, whereby phenologically advanced birch foliage possessed greater levels of *M. betulinum* infection than foliage in a less advanced state was reported by Elamo *et al.* (2000).

The high incidence of *M. betulae* on mature foliage early in the season may be as a result of a time-lag in which the younger foliage becomes infected but symptoms do not manifest themselves until later. This may explain why in the latter surveys when the age difference between leaves on the upper and lower branches is proportionately at its lowest, there is no statistical difference in levels of infection.

The specific morphological and chemical characteristics of leaves vary depending on their position on the tree, and such characteristics could be important in determining the success of *M. betulae* spores in either germinating or penetrating the leaf epidermis or else post-penetration development. For example, *M. betulinum* obtained from *B. pendula* leaves

could not successfully infect *B. pubescens* leaves, perhaps because of subtle differences in the leaf architecture (Poteri & Ryynänen 1998).

Survey trees were randomly selected in 1999 without prior knowledge of infection history, but those that bore high levels of infection in 1999 again displayed high infection levels in 2000 (Figure 4.12). It is difficult to make generalisations about differential phenotypic resistance of individual trees to pathogen attack based on two years results, but personal observations made in 2001 do at least suggest that those survey trees that were most susceptible in 1999 and 2000 continue to be so in June 2001. Birch trees can show consistent genotypic variation in their resistance to fungal pathogen attack, for instance in their resistance to rust pathogen, *M. betulinum* (Helander *et al.* 1998), so it is conceivable that some individual birch trees are intrinsically more likely to succumb to *M. betulae*. This type of predisposed genetic susceptibility might well have serious implications for the genotypic diversity of natural birch populations during periods of chronic infection, and as is discussed in Chapter 7, may make certain birch genotypes both vulnerable to pathogen infection and aphid infestation.

Fungal pathogen induced leaf chlorosis was equally abundant on upper and lower portions of the trees, unlike phenologically induced leaf senescence in which 'pockets' of prematurely senescent leaves occur on the lower and inner branches of sycamore (Dixon 1978). The sycamore aphid, *Drepanosiphum platanoidis*, often fed on prematurely senescent leaves during mid-summer when nitrogen content of leaves and sap was at its lowest. Wratten (1974) and Hajek & Dahlsten (1988) report a similar preference by *Euceraphis* on birch, suggesting that the flushing of foliage and asynchrony of senescence help to maintain the population during mid-summer when food quality is poor.

There was no relationship between the growth of tree and its disease load, perhaps because of the use of tree height as an assessment of plant growth was too crude, but possibly because *M. betulae* normally has no serious impact on plant health and other factors are more important. *M. betulinum* has been shown to retard growth in tree height (Lilja 1973) so tree height might be a reasonable index of plant performance. There is a general consensus however, that leaf-spot disease rarely causes long term damage to plants except under exceptional circumstances (Bennell & Millar 1984; Sinclair *et al.* 1987;

Phillips & Burdekin 1992; Strouts & Winter 1994) so it may be that growth of these trees is restricted by other environmental factors and strong intra-specific competition rather than disease.

4.4.2. Aphid choice tests

Aphids were recorded more frequently on birch leaves infected with *M. betulae* rather than infection-free birch leaves. The sycamore aphid *D. platanoidis* became significantly more abundant on sycamore leaves which had become infected with the fungal *Rytisma* tarspots in natural situations and performed significantly better when reared on such leaves (Gange 1996).

4.4.3. Aphid performance on infected leaves

Aphid performance is significantly higher in terms of final mass and hind tibiae length at adulthood when reared on heavily infected leaves compared with asymptomatic and moderately infected leaves, but survivorship and development time remained unaffected by fungal infection, as did the mean relative growth rate, a result which initially appears to be contradictory. Because initial mass was the same, but final mass was significantly different during the same period of development, it is intuitive to assume that relative growth rate must be higher for those aphids that became heaviest. However, when the mean *E. betulae* larval mass is compared with adult mass there is no correlation between the two which suggests that growth rate is not uniform across the population, this could then confound statistical analysis. The highly significant difference in aphid mass on reaching adulthood from statistically identical initial masses, however, does suggest that the enhancement to aphid performance when feeding on heavily infected foliage is genuine.

The embryo characteristics of aphids reared on differently infected leaves suggests that individual aphid growth is paralleled by the growth of their progeny, with almost all aphids on heavily infected leaves possessing developed embryos compared to approximately half of those on asymptomatic or moderately infected leaves. The length of basal embryos was also variable between aphids reared on leaves with varying degrees of infection, with those aphids on heavily infected leaves having the longest. There was

only a statistically significant difference between those aphids from heavily and moderately infected leaves however.

Many aphids, including *E. betulae*, persist as non-reproducing adults for several weeks during summer when the nitrogen content of the leaves and phloem sap of birch is low (Wratten 1974; Hajek & Dahlsten 1988). The sycamore aphid, *D. platanoidis*, also undergoes a similar mid-summer reproductive diapause (Dixon 1975) for the same reason. One of the characteristics of the reproductive diapause in *D. platanoidis* was the absence of developmentally mature embryos in adults (Douglas 2000) as maternal growth is effectively decoupled from embryonic growth in diapausing *D. platanoidis*. By maintaining only developmentally immature embryos, diapausing adults incur relatively low nutritional costs whilst retaining the capacity to respond rapidly and develop embryos when conditions become more favourable.

In Chapter 3, the high incidence of *E. betulae* larvae throughout mid-summer appeared to suggest that this reproductive diapause was absent, perhaps because of the cooler temperatures experienced at this latitude. These results suggest otherwise, since a large number of *E. betulae* lacked developed embryos - a good indication that the experiment was conducted during a period of reproductive diapause, albeit a rather heterogeneous one. Moreover, with the exception of one individual, *E. betulae* reared on heavily infected leaves always possessed developmentally mature embryos, suggesting that the possible enhanced nutritional quality of infected foliage allowed maternal and embryonic growth to be re-coupled in those individuals that otherwise would have maintained only developmentally immature embryos. As was discussed in Chapter 3, *E. betulae* did not migrate to the lower shaded branches in the middle of summer, which is a formulaic trait of aestivating aphids that cope poorly with high temperatures. This strongly implies that the temperatures at Dalhaikie Flat were not too high for *E. betulae*, which may have removed the other obstacle to continued reproduction throughout summer. In other words, the cooler temperatures at this latitude and the improved nutritional quality of infected foliage may have removed the necessity for the aphid to undergo a reproductive diapause in mid-summer, which after all is an adaptation to high temperatures and low foliar nutritional quality that occur during mid-summer.

That aphids did not perform significantly better on moderately infected leaves compared with asymptomatic leaves suggests that the nature of fungal infection is important, either quantitatively (*i.e.* how much leaf tissue was infected) or qualitatively (*i.e.* presence of chlorosis reflecting changes in leaf chemistry) or a combination of both. Aphids often respond to increased availability of soluble nitrogen in senescing leaves (Dixon 1978), so it might be hypothesised that the leaf senescence that occurs in response to fungal infection is the more likely cause rather than the specific density of infection. Changes in leaf chemistry (specifically free amino acid and phenolic compound content) associated with fungal-stimulated leaf senescence are discussed in Chapter 5.

4.4.4. Phenolic concentration of infected foliage

Heavily infected foliage had higher levels of phenolic compounds compared with asymptomatic and moderately infected leaves. Phenolic compounds are an important group of defence compounds in birch (Haukioja *et al.* 1990; Ossipov *et al.* 2001) and their induction in foliage by both insect herbivores and fungal pathogens has been documented in the past (Karban 1999). Fungal pathogens are often responsible for the inducing secondary compounds (Moran 1998), and the results presented here suggest that *M. betulae* may induce such a response. Whether the elevated concentrations of phenolic compounds in infected leaves are a direct defensive response to infection is unclear, but any serious impact on the fungal pathogen in this case seems to be limited, judging by the widespread occurrence of this pathogen at Dalhaikie Flat.

Elevated levels of phenolics in heavily infected foliage also seemed to have little detrimental impact on *E. betulae* since they performed significantly better on the heavily infected leaves. The possible reasons for this apparent insusceptibility, and the potential effects on phytophagous of different feeding guilds is discussed in Chapter 5.

4.4.5. Conclusions

Marssonina betulae became widespread at Dalhaikie Flat with more than half of the surveyed birch leaves displaying symptoms on some heavily infected trees. Without detailed results about plant performance in response to infection, it is difficult to assess

the importance of this disease to *B. pendula.* However, the extensive occurrence of *M. betulae* on *B. pendula* provides the potential for it to influence the population dynamics of the co-occurring aphid, *E. betulae,* particularly given the enhanced embryo development seen by aphids reared on heavily infected leaves. Aphids preferred to feed on infected leaves and performed better as a result. However, the effects of *M. betulae* on plant chemical composition and indirectly on *E. betulae* cannot be disentangled from factors correlated with infection in these experiments. Only with artificial inoculation of foliage, to produce infection without the other variables associated with natural infection, can the indirect effects of the fungus on the aphid be determined with certainty; this approach is attempted in the next Chapter. The results presented in this Chapter do however underpin those discussed next.

CHAPTER FIVE

Microbial impacts on plant-herbivore interactions: the indirect effects of a birch pathogen on a birch aphid

1. *The processes underpinning the indirect effects of a fungal plant pathogen,* Marssonina betulae *(Lib.) Magn. (Deuteromycotina: Melanconiales) on an aphid,* Euceraphis betulae *Koch (Homoptera: Drepanosiphinae), via their shared host plant* Betula pendula *(silver birch) Roth (Betulaceae), were investigated in field trials.*
2. *There was a strong positive correlation between leaves with high fungal infection and the aphid in field surveys. In choice tests, aphids settled on leaves inoculated with the fungus rather than asymptomatic leaves, a tendency that resulted in improved aphid performance. Aphid population growth rate on branches inoculated with the fungal pathogen was positively correlated with increasing fungal infection. Individual aphids reared on inoculated leaves were significantly heavier, possessed longer tibiae and displayed enhanced embryo development compared with those reared on asymptomatic leaves.*
3. *Inoculated leaves contained higher concentrations of free amino acids, perhaps reflecting a plant-initiated response to fungal attack in which free amino acids from the degradation of mesophyll cells are translocated out of infected leaves via the phloem. These changes in plant chemistry are similar to those occurring during leaf senescence, and are proposed as the mechanistic basis for the positive interaction. The elevated phenolic content of fungal infected leaves had no discernible impact on aphid performance.*
4. *All aphids reared on inoculated leaves possessed developmentally mature embryos, whereas over a quarter of those reared on asymptomatic leaves were in a state of reproductive diapause; a hard-wired event associated with low nutritional availability in the middle of summer. It is proposed that aphids reared on inoculated leaves may be able to respond to the improved nutritional conditions in a phenotypically plastic manner.*

5.1. INTRODUCTION

It is widely recognised that insect-plant interactions can be influenced by those interactions plants have with other organisms (Crawley 1983; Southwood 1985). Although most recent research to date has concerned plant-mediated attraction of natural enemies of phytophagous insects (*e.g.* Dicke 1994; Pare, Alborn & Tumlinson 1998; Kessler & Baldwin 2001), there is good evidence that micro-organisms associated with plants may also influence plant suitability to phytophagous insects (Hammon & Faeth 1992; Hatcher 1995; Saikkonen *et al.* 1998; Omacini *et al.* 2001). In particular, colonisation of plants by fungi, including mycorrhizal fungi, endophytic fungi and overt pathogens may modify the acceptability and/or the suitability of plants to insects.

There is some evidence that the processes underlying the impact of plant-associated fungi on phytophagous insects are predominantly indirect, mediated through the effects of the fungi on the plant. The interplay of metabolic processes in both the fungus and the plant following fungal infection can cause major changes in the chemical composition of the plant (Barbosa, Krischik & Jones 1991; Hatcher 1995). Elevated and depressed concentrations of carbohydrates and nitrogenous compounds can occur, as well as elevated levels of secondary compounds, either as part of a defensive response by the plant (Dixon 2001), or as a result of compounds synthesised by the fungus (Saikkonen *et al.* 1998). The magnitude of these effects and their impact on phytophagous insects is anticipated to vary with environmental factors and with the taxonomic and ecological characteristics of the fungus, plant and insect. The relationship between the plant and fungus also varies; a single fungal species, for instance, can have a spectrum of interactions ranging from being purely endophytic, where it lives asymptomatically with the plant, to being highly pathogenic (Hammon & Faeth 1992; Saikkonen *et al.* 1998).

There is a growing catalogue of examples in which endophytic fungi confer herbivore resistance to plants, most notably in agronomically important grass systems (*e.g.* Breen 1994; Wilkinson *et al.* 2000; McLeod *et al.* 2001), but the processes involved in plant-mediated effects of pathogenic fungi on phytophagous insects are generally less well understood. The purpose of this study was to determine the processes underlying the indirect effects of a fungal plant pathogen on a phytophagous insect. Our experimental system involved a fungal pathogen and an insect that exploit leaves of the deciduous tree,

Betula pendula Roth (Betulaceae) (silver birch). As the extensive literature on *Betula*-herbivore interactions illustrates (*e.g.* Haukioja & Neuvonen 1985; Hartley & Lawton 1987; Haukioja *et al.* 1990; Fisher, Hartley & Young 2000), this system is amenable to experimental manipulation in the field, an invaluable approach for identifying causal relationships in complex interactions. At the study site in NE Scotland, the species interactions on *B. pendula* leaves were relatively simple, being dominated by the fungal pathogen *Marssonina betulae* (Lib.) Magn (Deuteromycotina: Melanconiales) and the aphid *Euceraphis betulae* Koch (Homoptera: Drepanosiphinae). The scope for direct interactions between the two taxa is minimal since they are spatially separated; the fungal pathogen occurs on the leaf lamina, whereas the aphid feeds from the basal midrib and petiole (Hajek & Dahlsten 1986).

The aphid, *E. betulae*, lives and feeds entirely on birch, reproducing asexually from spring until autumn, whereupon a sexual generation produces eggs that over-winter until the following spring. *M. betulae* is a fungal pathogen that causes necrotic leaf spots on birch leaves. Symptoms occur on mature birch leaves, frequently causing leaf chlorosis (*i.e.* yellowing associated with reduced photosynthesis and chlorophyll content) and premature abscission (Sinclair, Lyon & Johnson 1987).

This multidisciplinary investigation took a mechanistic approach using mycological, entomological and plant biochemical techniques to explore the indirect interaction between the fungus and the aphid. A comprehensive survey of *E. betulae* on *B. pendula*, in which several thousand aphid occurrences were recorded, was undertaken to characterise patterns of aphid abundance on birch and ascertain whether infection by *M. betulae* had any bearing on this distribution. To allow the effects of fungal infection to be clearly separated from the correlated effects of natural infection on aphid performance observed in the field, the fungal pathogen was isolated, cultured and inoculated onto asymptomatic leaves. Because insect preference and performance parameters are sometimes poorly correlated (*e.g.* Cronin & Abrahamson 2001), *E. betulae* preference for fungal-inoculated leaves was assessed in parallel with performance parameters. In terms of dietary nutrition, aphid growth and reproduction are generally thought to be limited by the amino-nitrogen content of plant phloem sap (Dixon *et al.* 1993; Douglas 1993; Dixon 1998). The major

group of allelochemicals in birch, whose concentrations are known to increase in response to leaf damage are phenolic compounds (Hartley & Firn 1989; Ossipov *et al.* 2001). Because of this, the free-amino acid and phenolic compound concentrations in fungal-inoculated leaves were measured to evaluate potential mechanisms for the interaction.

The specific objectives of this study were to; (a) establish the spatial distribution of *E. betulae* on its host plant *B. pendula* in natural situations, and determine whether leaf-infection by *M. betulae* had any impact on this distribution; (b) experimentally test whether *E. betulae* preferred to feed on leaves inoculated with the fungus and what effect feeding on such leaves had on individual aphid performance and population increase; (c) quantify the important nutritional and allelochemical changes in leaves that accompany inoculation with *M. betulae*.

5.2. MATERIALS & METHODS

5.2.1. Site Description

The study site, Dalhaikie Flat, Aberdeenshire, UK (57°075' N, 2°582' W; OS 3645 8992), comprised *ca.* 3 ha of almost continuous birch thicket dominated by *Betula pendula* (with small numbers of *B. pubescens*) that had regenerated after mature trees were felled in 1989. The abundance of aphids and fungi on leaves of *B. pendula* was surveyed July-August 1999, and manipulative experiments were conducted in summer 2000. The dominant aphid species was identified as *Euceraphis betulae* Koch (Homoptera: Drepanosiphinae) on morphological criteria (Blackman 1977; Blackman & Eastop 1994) and the principal fungal pathogen was identified as *Marssonina betulae* (Lib.) Magn (Deuteromycotina: Melanconiales) (Peace 1962; Sinclair *et al.* 1987), although the taxonomy of foliar pathogens of birch is confused, see Kurkela (1995) and Paavolainen, Hantula & Kurkela (2000). In addition to adult aphids, first and second instar larvae (referred to as 'young larvae') were collected from foliage at the site for manipulation experiments.

5.2.2. The distribution of aphids and fungi on *Betula pendula*

Aphids and fungi were surveyed on 2 and 19 July and 9 and 30 August 1999, on leaves of 30 trees of *B. pendula* of uniform height (1.5-2.2m), selected for study during April 1999 whilst bud-burst was underway. For each tree, 15 branches from the upper canopy and 10 branches from the lower canopy were selected as sampling units. Leaf nodes within each of the 25 branches were numbered consecutively from the fifth leaf node in from the branch apex, and classified as 'basal' (closest to the tree trunk), 'intermediate' and 'apical'. Where branch growth occurred from leaf nodes within the initial sampling unit, the sampling unit was redefined as the first three or more leaf nodes from the 'basal' leaf node as originally defined in April.

Each leaf within every sampling unit was scored for (a) number of aphids and (b) fungal infection. Disease severity was classified as being either 'low' or 'high' fungal infection. Leaves classified as 'low infection' ranged from being asymptomatic (no visible fungal lesions) to 20 % of leaf area being covered with lesions; leaves with 'high infection' had lesions on > 20% of leaf area and were invariably chlorotic. Consistent assessment of leaf area covered by fungal lesions was assisted by reference to a chart of scanned images with the full spectrum of disease severity, in which lesion cover had been digitally measured

using image analysis software (Scion Image Beta 4.02™, Scion Corp., Maryland, USA). Multiple (usually two) leaves at a single leaf node could be collectively classified as either 'low' or 'high' infection since they always displayed similar levels of infection.

5.2.3. Isolation and inoculation of the fungal pathogen

The fungal pathogen was isolated from leaves of *B. pendula* during 1999. Infected leaves were surface sterilised by wiping with cotton wool soaked in 70% ethanol and the leaf tissue surrounding and including lesions was excised with a sterile scalpel. The plant material was placed on malt extract agar [2% malt extract; 1% bacteriological agar (Oxoid, w/v)] supplemented with antibiotics [50mg l^{-1} penicillin-G (Sigma, UK); 100mg l^{-1} streptomycin sulphate (Glaxo, UK) and 50 mg l^{-1} chloramphenicol (BDH, UK)]. Petri dishes were incubated at 20°C ± 2°C and inspected daily for fungal growth, whereupon sub-cultures were taken and incubated as above. Leaves of *B. pendula* were inoculated with the cultured fungus, following the procedures of Redlin (1995) and Paavolainen *et al.* (2000). Conidiospores were harvested from 14-day-old agar cultures and suspended in 0.025% surfactant (Tween-20™) in distilled water (v/v) at a density of 10^6 spores ml^{-1}. The suspension was applied evenly to leaves using a modeller's airbrush. Control (asymptomatic) leaves were sprayed with distilled water and the surfactant alone.

5.2.4. Aphid choice tests

In August 2000, 30 trees without visible fungal infection were selected for study. One leaf in the terminal 6-7 cm of each of two branches on each tree was inoculated with the fungal spore solution whilst the other leaves of the branch section were sprayed with distilled water and surfactant alone (see above). Branch sections were placed inside polythene bags to promote fungal infection. Two weeks later, 20 trees had two suitable branch sections with a single infected leaf (lesions on ~ 20% of leaf area). All but one of the asymptomatic leaves and the inoculated leaf were excised from both branch sections on each tree, which were inserted into Perspex™ cages (75mm × 44mm × 12mm), following Adams & Douglas (1997). Each of the two boxes had either a single adult or young larval *E. betulae* placed inside the cage away from either leaf, so that each tree had an example of both tests. The location of the aphid in the cage was scored as being on the infected leaf, asymptomatic leaf or elsewhere in the cage at 120 min intervals for 12 h, and finally 24 h later.

5.2.5. Aphid performance

The first experiment investigated aphid population performance when reared on fungal-inoculated leaves. In July 2000, all insects were cleared from two asymptomatic branches with *ca.* 100 leaves on each of 30 trees. The trees were assigned randomly to two groups: 'treatment' and 'control'. One of the branches from the 'treatment' group of trees was sprayed with distilled water and surfactant solution (control branch 1) whilst the other branch (treatment branch) was inoculated with the fungal spore solution (as above). In case 'control branch 1' inadvertently became too infected because of the inoculation of the neighbouring treatment branch, a second control branch from the separate 'control' group of trees was sprayed with distilled water and surfactant as before (control branch 2). Each branch was inserted into a polyester Organza™ bag, fixed securely at both ends and enclosed in a polythene bag for one week to promote fungal infection. Two weeks after inoculation, 10 adult and 10 young larval aphids were introduced to each polyester Organza™ bag. After three weeks, all 'bagged' branches were excised and the aphids were counted. The number of infected leaves in each bag was scored and expressed as a percentage of the total.

The second experiment addressed the performance of individual aphids reared on asymptomatic leaves and leaves inoculated with the fungal pathogen. Fifteen trees of uniform height (1.7-2.0 m) were selected in July 2000 and replicate leaves either inoculated with the fungal pathogen (treatment leaves) or sprayed with distilled water and surfactant alone (control leaves). All leaves were enclosed in individual polyester bags for one week to promote fungal infection. Two weeks after inoculation, 63% of the treatment leaves showed visible fungal infection and chlorosis; the unsuccessfully inoculated leaves and any control leaves that had become inadvertently infected were discarded. A young larval *E. betulae* was clip-caged to half of the treatment leaves and half of the control leaves (one per leaf) and monitored daily. The remaining treatment and control leaves were removed for chemical analysis (see below). Upon reaching adulthood (14-15 days later) one aphid was randomly selected from a treatment and control leaf from each of the 15 trees, weighed (Cahn Instruments™, California USA) to an accuracy of ± 1 μg and dissected with pins in a drop of ice-cold 50 mM Tris-HCl pH 7.5 at × 160 magnification. Both hind tibiae lengths were determined using a calibrated eyepiece micrometer, as was the length

of the basal embryo in the gonads. The total number of developmentally mature embryos (*i.e.* with pigmented eyespots) was also scored.

5.2.6. Chemical analysis of leaves

Leaves prepared for the second performance experiment (see above) were used. Two weeks after inoculation, one inoculated leaf and one asymptomatic leaf on each tree (all aphid-free) were used for phenolic compound and free-amino acid analysis. For the phenolic assay, leaves were frozen individually at –18°C, freeze dried for 36 h and analysed using the Folin-Ciocalteau method outlined by Waterman & Mole (1994) and Kerslake, Woodin & Hartley (1998). For the amino acid analysis, leaves were macerated individually in 0.5ml ice-cold 80% methanol (HPLC grade) centrifuged at 1200 rpm, and the supernatant stored at –18°C until analysed. Each sample was loaded on a Dowex 50 cation exchange column followed by 7 × 300 ml of deionised water. Amino acids were eluted with 2 × 300ml and 4 × 600ml 5 M NH_4OH. The eluant was evaporated to dryness for 24 h, and the residue was dissolved in 0.5ml 2M HCl and analysed by reverse-phase high performance liquid chromatography (HPLC) with pre-column derivitisation using *o*-phthaldialdehyde (Jones, Paabo & Stein 1981). Amino acids were separated on a Zorbax™ Eclipse XDB-C8 column at 20°C using a Hewlett-Packard HP 1100 delivery system and fluorescence detector. Amino acids were quantified by comparison of sample peak areas to three level calibration plots of a reference amino acid mixture, AA-S-18 (Sigma, UK), supplemented with glutamine, asparagine, γ-amino butyric acid and tryptophan. All protein amino acids except proline and cysteine were detected to an accuracy of 0.5 pmol per sample.

5.2.7. Statistical analysis

Analysis was carried out in SAS version 8.2 (SAS Institute 1999). Survey and aphid population data were analysed using a generalised linear mixed model GLIMMIX (SAS Institute 1999) with a Poisson error structure and log link function. For survey data, branch and tree replicate were treated as a random terms in the model. Canopy position (upper and lower branches), leaf position (basal, intermediate and apical leaves) and fungal infection level (low or high) were fitted as explanatory variables within the model. In population experiments, tree replicate was a random term in the model and comparisons between branch types in terms of aphid abundance and fungal infection were made using

differences of least squares means tests calculated within the GLIMMIX macro (SAS Institute 1999). Degrees of freedom for fixed effects in generalised linear mixed models were adjusted using Satterthwaite formulas (Littell *et al.* 1996). The relationship between aphid number and the proportion of infected leaves in the population experiment were additionally analysed using Spearman's rank order correlation. For individual aphid performance, response variables that were normally distributed were analysed using general linear models PROC GLM (SAS Institute 1999). Number of developmentally mature embryos in aphid gonads were transformed [$\log_{10}(n+1)$] and analysed in the same manner; differences in possession of developmentally mature embryos were evaluated using a generalised linear model with binomial error structure and logistic link function PROC GENMOD (SAS Institute 1999). Choice test data were analysed using either Chi-square tests or Fisher's exact test for independence (Sokal & Rohlf 1995). Natural logarithms of amino acid and phenolic compound concentrations were compared using a paired *t*-test.

5.3. RESULTS

5.3.1. Spatial distribution of aphid and fungus on *Betula pendula*

In both study years, 1999 and 2000, the aphid *E. betulae* was first observed on *B. pendula* in April. Thereafter, multiple parthenogenetic generations were scored until late August/ September, when sexual morphs were produced; overwintering eggs were laid late September/early October. Fungal pathogenic lesions were observed on leaves of *B. pendula* as brown necrotic lesions of diam. 1-2 mm in June. The lesions became larger and more numerous as the season progressed, and leaf chlorosis (*i.e.* yellowing) was evident from July. Disease incidence was widespread in 1999. By the end of August 1999, 53 % of the 2,007 leaf nodes examined bore leaves with some degree of infection, 10 % being chlorotic.

Aphid abundance and fungal infection were simultaneously monitored in July and August 1999, during which 12,967 aphid occurrences were scored (Figure 5.1). Aphid abundance was significantly elevated on leaves with high fungal infection, relative to low fungal infection. Aphids were significantly more abundant on upper rather than lower branches in the canopy (Figure 5.1a) and on leaves at the branch apex than those closer to the trunk of the tree (Figure 5.1b) for leaves with either low or high levels of fungal infection. The spatial difference in aphid abundance within the canopy and on branches (*i.e.* being more abundant on upper branches and apical leaves) was significantly less pronounced when leaves bore high fungal infection compared with those with low infection, except for 30 August when infection had no interactive effect on aphid spatial distribution (Table 5.1).

5.3.2. Aphid choice tests

The position of aphids when caged with a single fungal-infected and asymptomatic leaf in field-based choice tests is shown in Figure 5.2. Of the 20 larval and 20 adult aphids placed inside the cages, 19 adults and all 20 larvae were feeding on leaves at the end of the 24 h trial, 17 larval and adult aphids (89% and 85% respectively) being on fungus-infected leaves. Since leaf selection did not differ between adults and larvae (Fisher's exact, P = 0.81), data were pooled, indicating a significantly higher incidence of aphids on fungal-infected leaves (χ_1^2 = 21.56, P <0.001). The data were examined to determine how preferential colonisation and arrest contributed to this pattern. All 40 aphids colonised a leaf during the experiment; 17 (43 %) colonised the fungal-infected leaf first and 23 (57%)

selected the asymptomatic leaf ($\chi_1^2 = 0.9$, $P > 0.05$). Of the 17 aphids colonising the fungal-infected leaf, all but three individuals remained on the leaf for the remainder of the trial (one settling on the asymptomatic leaf and two moving away from both leaves). In contrast, 20 of the original 23 aphids colonising the control leaf moved to the inoculated leaf ($\chi_1^2 = 19.13$, $P < 0.0001$).

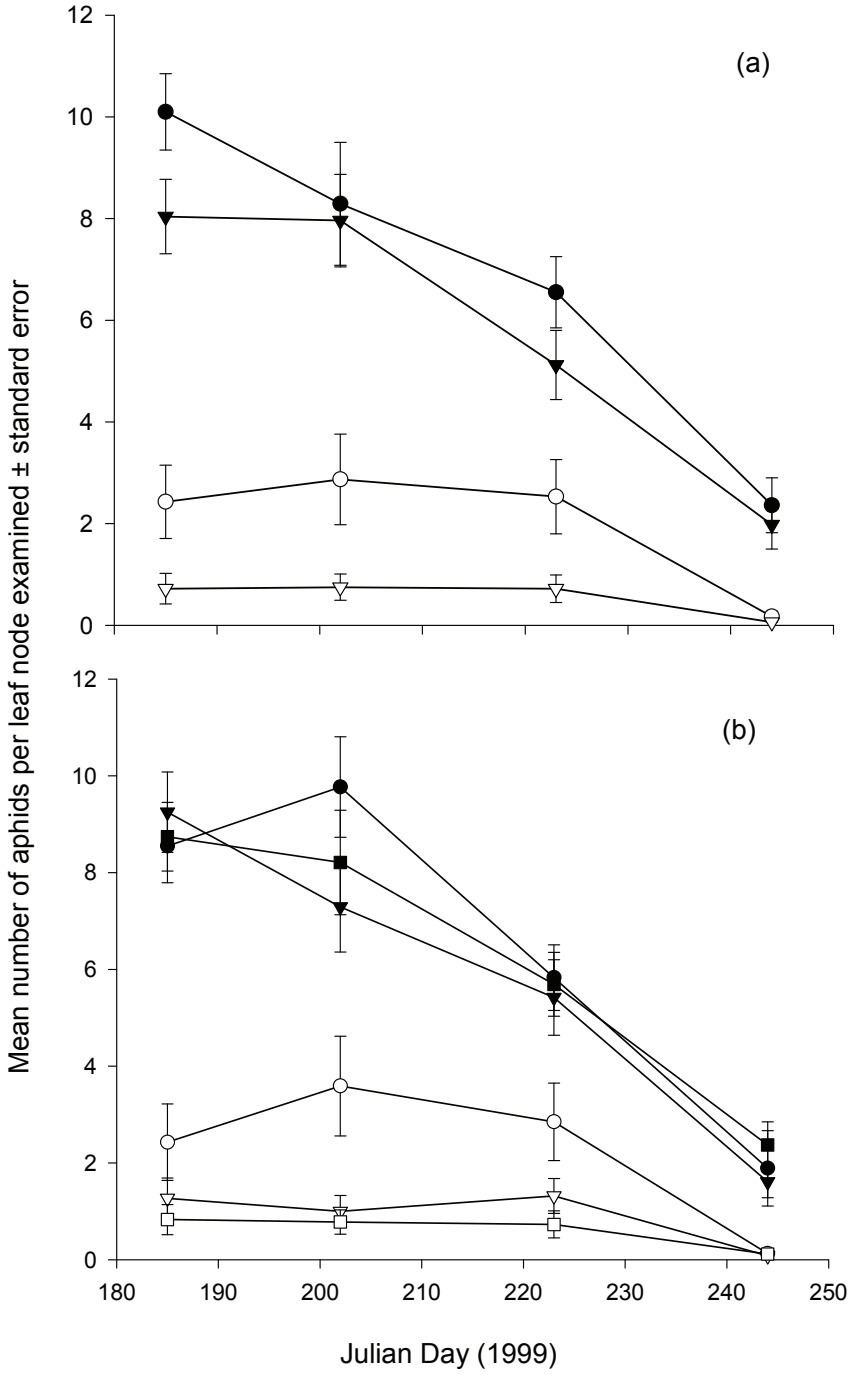


Figure 5.1. (a) Seasonal spatial density of *E. betulae* on upper (●-○) and lower branches (▼-▽) in the canopy of *B. pendula* and (b) seasonal spatial density of *E. betulae* on basal (■-□), intermediate (▼-▽) and apical leaves (●-○) within branches. Open symbols represent 'low fungal infection' and closed symbols represent 'high fungal infection' with *M. betulae*.

Table 5.1. Summary of generalised linear mixed model results describing the spatial distribution of *E. betulae* on *B. pendula* in 1999 shown in Figure 5.1'Canopy position' (upper or lower and branches), 'position on branch' (basal, intermediate or apical) 'fungal infection' and interactions between fungal infection and the two spatial factors were fitted as explanatory variables.

Survey Date (1999)	2 July			19 July			9 August			30 August		
Source of variation	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*
Canopy position	1, 517	82	<0.0001	1, 624	54	<0.0001	1, 617	83	<0.0001	1, 506	27	<0.0001
Position on branch	2, 1880	16	<0.0001	2, 1365	32	<0.0001	2, 1681	28	<0.0001	2, 1832	5	<0.0061
Fungal infection	1, 1421	652	<0.0001	1, 1198	431	<0.0001	1, 1381	380	<0.0001	1, 1554	701	<0.0001
Fungal infection × canopy position	1, 1524	38	<0.0001	1, 1391	60	<0.0001	1, 1556	51	<0.0001	1, 1264	3	0.1002
Fungal infection × position on branch	2, 1824	11	<0.0001	2, 1300	15	<0.0001	2, 1683	18	<0.0001	2, 1857	1	0.4701

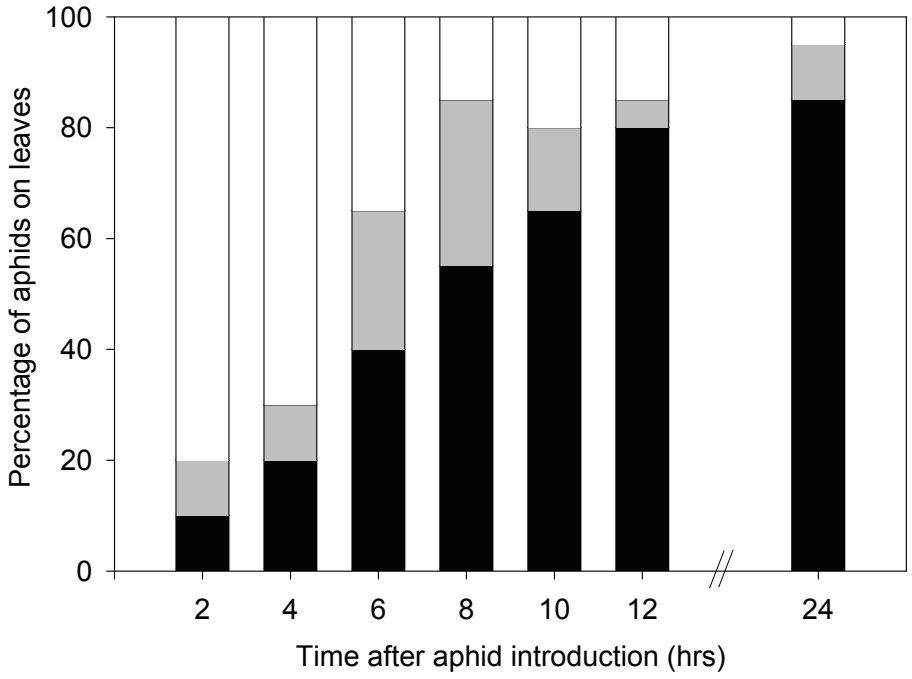


Figure 5.2. Aphid choice tests showing the proportion of aphids (adult and larval data pooled) located on inoculated (■) and asymptomatic (▒) leaves or elsewhere (□) in the Perspex™ cage. Aphids were significantly more abundant on inoculated rather than asymptomatic leaves from eight hours onwards ($\chi_1^2 = 5.14$, $P < 0.05$).

5.4.3. Aphid performance

The first experiment addressed the impact of fungal infection of leaves on the population increase of *E. betulae*. All the branches of *B. pendula* bagged after spraying with fungal spore solution (treatment branches) or distilled water and surfactant (control branches) bore at least 5 leaves with visible fungal infection, but treatment branches were significantly more infected than control branches, either from the same tree (control branch 1) (t_{40} = -5.27, P <0.001) or from the separate control trees (control branch 2) (t_{40} = -7.75, P <0.001). Aphid population increase was significantly higher on those branches that had been inoculated with the fungal pathogen than those which had been sprayed with distilled water and surfactant alone ($F_{2,40}$ = 8.34, P <0.001). Pair-wise comparisons showed that aphid populations recovered from bagged branches were significantly larger on treatment branches compared with control branches on the 'control trees' (control 2) (t_{40} = -3.96 P <0.001), and only narrowly missed significance at the 95% confidence interval when compared with control branches from the 'treatment trees' (control 1) (t_{40} = -1.98, P = 0.055). Pooling the data for all branches (Figure 5.3) revealed a strong positive correlation between the proportion of leaves infected inside each polyester Organza™ bag and the final number of aphids recorded after 21 days (r_s = 0.817, P <0.001).

The second performance experiment explored aphid performance during larval development to adulthood (14-15 days after caging). Aphids reared on fungal inoculated leaves were significantly heavier and possessed longer hind tibiae (mean per aphid) at adulthood than those reared on asymptomatic leaves (Table 5.2). All of the aphids on fungal infected leaves possessed completely developed embryos upon reaching adulthood, whereas a significant proportion of those reared on asymptomatic leaves possessed only developmentally immature embryos. Furthermore, developmentally mature embryos were both more numerous and individually larger in aphids reared on inoculated leaves compared to those aphids reared on asymptomatic leaves that possessed them.

5.4.3. Leaf chemistry

The concentration of phenolic compounds was significantly elevated in fungal inoculated leaves than in asymptomatic leaves, and the concentration of free amino acids in inoculated leaves was more than double that of asymptomatic leaves (Table 5.3).

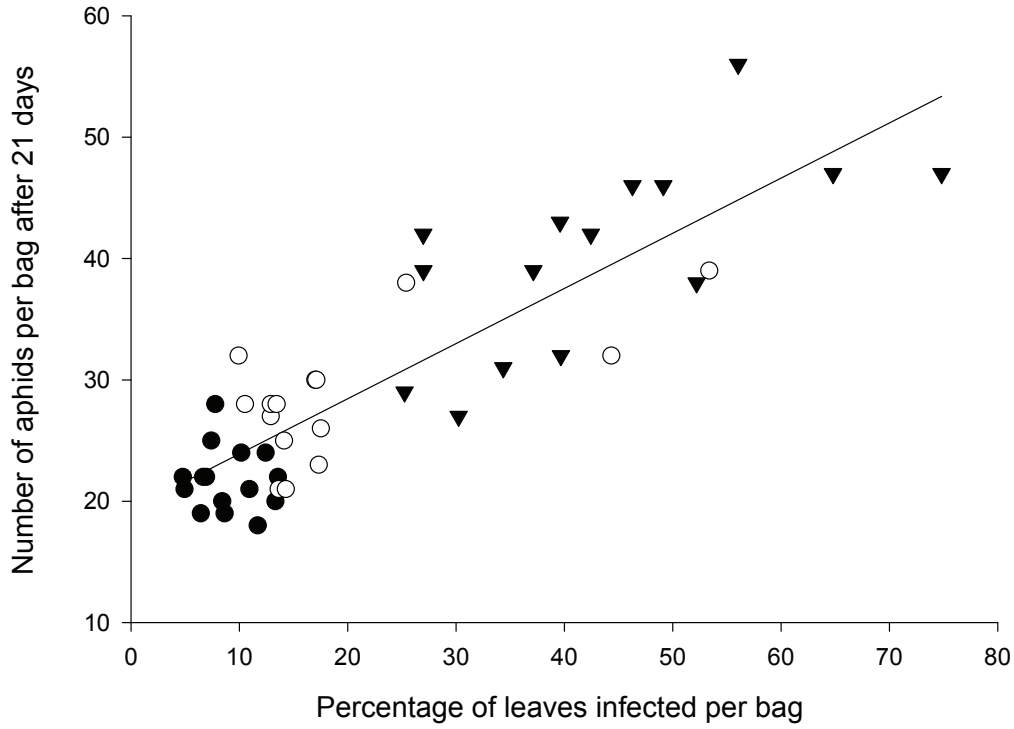


Figure 5.3. Number of aphids recorded after 21 days when reared on branches with differing degrees of fungal infection inside polyester Organza™ bags - (●) inoculated branches on 'treatment trees', (○) non-inoculated branches on 'treatment trees' and (▼) non-inoculated branches on separate 'control trees'.

Table 5.2. Size and reproductive characteristics of *E. betulae* reared to adulthood on leaves inoculated with the fungus *M. betulae* (infected leaves) and leaves without fungal infection (asymptomatic). 15 replicates unless otherwise stated.

	Aphid performance				
Leaf Type	Adult mass (mg) (mean ± SE)	Adult hind tibia length (mm) (mean ± SE)	Individuals possessing developed embryos (15 examined)	Number of developed embryos per aphid (mean ± SE)	Basal developed embryo length (mm) (mean ± SE)
Asymptomatic	1.79 ±0.06	2.46 ± 0.07	11	4.1 ± 0.74	0.75 ± 0.03 [1]
Infected	2.04 ± 0.07	2.78 ± 0.08	15	7.1 ± 0.44	0.91 ± 0.04
	$F_{1,28} = 7.06$	$F_{1,28} = 9.13$	$P = 0.013$	$F_{1,28} = 11.68$	$F_{1,24} = 8.22$

[1] 11 replicates used since developed embryos absent in 4 aphids

Table 5.3. Concentration of phenolic compounds and free amino acids in leaves of *B. pendula* leaves. Data displayed as mean ± SE, 15 replicates.

Leaf type[1]	Phenolic concentration (mg tannic acid equivalents g^{-1} dry weight)	Free amino acid concentration (nmol g^{-1} fresh weight)
Asymptomatic	145 ± 7.6	114 ± 18
Infected	186 ± 7.4	272 ± 62
	$t_{14} = 3.66$ $P = 0.003$	$t_{14} = 2.33$ $P = 0.035$

[1] Definition as Table 5.2.

5.4. DISCUSSION

The aim of this study was to establish whether *M. betulae* had an indirect impact on *E. betulae* performance and identify the processes underpinning this interaction. *E. betulae* were much more abundant on leaves infected with *M. betulae* in the field, and they both preferred to feed on leaves inoculated with *M. betulae* and displayed enhanced performance when so doing. The mechanism for these effects was most probably due to altered leaf chemistry caused by *M. betulae* infection.

The underlying spatial distribution of *E. betulae* on *B. pendula* is characterised by aphids being significantly more abundant on upper, rather than lower branches in the canopy and on the apical rather than basal leaves within branches. This perhaps reflects the differential allocation of resources in *Betula* during the growing season, with developing foliage in the upper canopy and at the branch apices representing the strongest sinks for nutrients via the phloem (Lehtilä *et al.* 2000). Given that aphid growth and development is thought to be dependent on the availability of amino-nitrogen in the phloem sap (Dixon 1998), aphids might be expected to feed at these potentially more nutritious sites. The most striking observation was that aphids were more abundant on leaves with high fungal infection compared with low infection, irrespective of their position in the canopy or on individual branches.

The results from the inoculation experiments suggest the association between aphids and the pathogen is the result of aphids becoming abundant on infected leaves, rather than the fungal pathogen infecting leaves that are infested with aphids, for instance by aphid-mediated fungal transmission. Some fungal pathogens do occasionally proliferate in response to honeydew produced by aphids (*e.g.* Stadler, Solinger & Michalzik 2001) and some infect leaves via insect wounds (*e.g.* Raffa & Smalley 1995), but *M. betulae* was never associated with honeydew deposits and the highly specialised feeding niche of *E. betulae* on the basal midrib and petiole would result in lesions being localised around these areas if fungal germ tubes entered through stylet pathways. The results of the choice tests in which aphids were overwhelming observed on the inoculated rather than asymptomatic leaves also help to refute this possibility. The higher incidence of *E. betulae* on infected leaves arose because aphids arrested on fungal leaves and moved onto them from asymptomatic leaves, rather than colonising the infected leaf first. It was surprising that

E. betulae should not preferentially colonise the chlorotic infected leaves first, as many aphids are known to use visual cues such as yellow colouration to locate host plants, since the more nutritious young and senescent leaves tend to be more yellow than mature leaves (Dixon 1998).

5.4.1. Processes underpinning the interaction

Selection is expected to favour individual phytophagous insects that select and feed on foliage that leads to the greatest performance, a decision that may sometimes be influenced by fungal infection (Wilson & Faeth 2001). The enhancement of *E. betulae* performance by *M. betulae* seemed to be evident at the level of the population, as isolated aphid populations became larger with increasing fungal infection. It is unlikely that those branches that were inoculated in the field became infected solely as a result of this inoculation and not also from ambient pathogen activity, but disease levels were significantly higher than on branches sprayed with water and surfactant alone. The proportion of leaves infected was correlated with aphid population increase, suggesting that in addition to being more abundant on infected leaves in natural situations, *E. betulae* were probably reproducing at a higher rate when feeding on them too. Enhanced aphid performance in response to fungal inoculation was borne out by the experiments using individual *E. betulae*. Those aphids reared on leaves inoculated with *M. betulae* performed better than those reared on asymptomatic leaves, both in terms of their own growth (*i.e.* they were heavier and had longer tibiae) and that of their future progeny (*i.e.* developmentally mature embryos were always present, and were both more numerous and bigger).

The causal mechanism of enhanced aphid performance when reared on inoculated leaves was likely to be the result of an improvement in leaf nutritional quality. The compositional and quantitative changes in plant chemistry caused by pathogen infection are probably numerous and complex, but nitrogen availability is probably most critical to phytophagous insects (Mattson 1980; Waring & Cobb 1992) including aphids, for whom it is commonly the limiting nutrient (Dixon *et al.* 1993; Douglas 1993; Dixon 1998). Many pathogens, including *M. betulae* (Phillips & Burdekin 1992), stimulate a plant-initiated response to fungal attack that is akin to the changes in plant chemistry that occur during leaf senescence. Free amino acids from the degradation of mesophyll cells are translocated out

of infected leaves via the phloem as part of this response (Thomas & Stoddart 1980). Inoculated birch leaves possessed higher concentrations of free amino acids compared to asymptomatic leaves, and whilst only correlated with enhanced aphid performance, this result is a strong indication that these fungal-stimulated changes in plant chemistry are the cause of the positive effects.

Like many aphids, *E. betulae* prefer to feed on senescent leaves in natural situations (Fowler & Lawton 1984; Hajek & Dahlsten 1988) because the flux of amino acids is thought to be higher in the phloem sap of senescent leaves (Chapin & Kedrowski 1983). Furthermore, *E. betulae* has been seen to perform better on birch trees treated with simulated acid rain; the mechanism suggested being a stress-related improvement in nutritional quality (Neuvonen & Lindgren 1987). Increased translocation of amino acids in the phloem as a result of fungal infection has been proposed as a possible mechanism for increased aphid performance in the past (Barbosa 1991; Zebitz & Kehlenbeck 1991; Kluth, Kruess & Tscharntke 2001), but to our knowledge this hypothesis has never been tested before using manipulative experiments.

5.4.2. Microbial impacts on phytophagous insect community structure

Leaves inoculated with *M. betulae* possessed higher concentrations of phenolic compounds than asymptomatic leaves. Phenolic compounds are regarded as one of the major groups of damage-induced allelochemicals in birch (Haukioja & Niemela 1979; Ossipov *et al.* 2001) and many anti-microbial toxins produced in response to pathogen attack are phenolic compounds (Bailey & Mansfield 1982). Despite using lesion-free parts of the leaf for phenolic analysis, it is impossible to say whether the elevated phenolic content of inoculated leaves was due to a plant-initiated defensive response to fungal infection or as a consequence of direct phenolic synthesis by the fungus itself. However produced, the elevated phenolic compound levels in inoculated leaves were not correlated with depressed *E. betulae* performance, despite their having deleterious effects on aphid performance when administered in artificial diets (Todd, Getahun & Cress 1971). Part of the reason for this apparent lack of response might be that phenolic compounds are known to occur in low concentrations in phloem sap (Raven 1983; Karban & Myers 1989). Furthermore, aphid performance has previously been seen to be unaffected by elevated phenolic compounds in birch leaves (Martin, Cappuccino & Ducharme 1994),

and the effects of phenolic compounds on phloem-feeding insects are generally believed to be weak (Hartley & Jones 1997).

Whilst the elevated phenolic compound levels in leaves inoculated with *M. betulae* did not adversely affect *E. betulae*, their effects might render birch leaves unsuitable to phytophagous insects of different feeding guilds. Many studies have demonstrated that phytophagous insects are negatively affected by elevated phenolic levels in birch (Bergelson, Fowler & Hartley 1986; Fowler & Macgarvin 1986; Hartley & Lawton 1987; Valladares & Hartley 1994). By rendering foliage more suitable for some phytophagous insects and less suitable for others, plant-associated fungi could play a major role in shaping the composition of insect herbivore communities.

Aphids could be major beneficiaries of such microbial manipulation of resources. Empirical data shows that aphid performance can be depressed (Pesel & Poehling 1988; Zebitz 1988), or enhanced (Zebitz & Kehlenbeck 1991; Gange 1996; Moran 1998) when feeding on plants infected with fungal pathogens, but improvements to aphid performance might generally be more common than for other phytophagous insects of different feeding guilds (Barbosa 1991; Hatcher 1995; Kluth *et al.* 2001). Aphids are known to be able to respond rapidly to changes in resource quality and, whilst far from universal, some host-specific aphids show reduced susceptibility to the effects of allelochemicals. Many aphids encounter lower concentrations of allelochemicals in phloem sap (Martin *et al.* 1994), some can detoxify or even sequester allelochemicals (Dixon 1998) and phloem feeding often circumvents the effects of many digestion-inhibiting compounds (Hartley & Jones 1997).

5.4.3. Microbial impacts on insect life-history strategies

The magnitude of plant-mediated fungal effects on phytophagous insects will be dependent on the taxonomic and ecological characteristics of the organisms involved, but if the fungal effects are as significant as the system discussed here, they could potentially be influential on insect life-history traits and ultimately insect population dynamics. That over a quarter of *E. betulae* reared on asymptomatic leaves did not possess any developmentally mature embryos is compatible with some of the *E. betulae* population in the field being in a state of reproductive diapause. Reproductive diapause is a common

life-history strategy for many heat-intolerant tree aphids, including this species (Hajek & Dahlsten 1988), during summer when the nutritional quality of foliage is depressed (Dixon 1975; Dixon 1998). Developmentally mature embryos are also absent in the sycamore aphid, *Drepanosiphum platanoidis* (Schr.) during the mid-summer reproductive diapause (Douglas 2000). Whilst maintaining only developmentally immature embryos, *D. platanoidis* incurs low nutritional costs and retains the ability to recommence embryo development when conditions become more favourable. *E. betulae* reared on fungal inoculated leaves always possessed developmentally mature embryos, which were more numerous and individually larger than those of aphids reared on asymptomatic leaves, four of whom did not possess them at all. This pattern was also seen in an identical experiment (data not shown) conducted in the previous season (July 1999) using naturally infected leaves; only 24 out of 50 aphids reared on asymptomatic leaves possessed developmentally mature embryos compared with 16 out of 17 aphids reared on fungal infected leaves ($\chi_1^2 = 9.62$, $P = 0.002$).

The widespread occurrence of *M. betulae* from July onwards may improve the nutritional quality of foliage sufficiently to maintain the population at high levels when otherwise it may be static or in decline due to reproductive diapause in summer. A true reproductive diapause in aphids is a hard-wired response to certain environmental cues (Tauber, Tauber & Masaki 1986), so the enhanced embryo development seen in *E. betulae* reared on infected leaves could be a phenotypically plastic response to improved nutritional quality (Stadler 1995; Nager, Keller & van Noordwijk 2000). Given that the causal mechanism believed to be responsible for enhancing *E. betulae* performance, namely the translocation of nutrients out of infected leaves, is a relatively simple and common response by plants to microbial infection (Vanderplank 1982), the modification of insect life-history traits by plant fungal pathogens may be more prominent in phytophagous insect communities than hitherto recognised.

In conclusion, this study uses a mechanistic approach to demonstrate the significant impact that plant-associated fungi can have on phytophagous insects via plant-mediated mechanisms, and to identify one of the processes involved. It also points to the, previously unreported, impacts of plant-associated fungi on insect life-history strategies

such as reproductive diapause, and the wider impacts on insect-herbivore communities through manipulation of the host-plant so as to make it more or less suitable to different herbivores.

CHAPTER SIX

Insects as leaf engineers – can leaf-miners alter leaf structure for birch aphids?

1. *Many insect herbivores affect one another through changes to their shared host plant, most usually through alterations in host plant chemical composition, but it is also possible that herbivores could structurally modify their shared host plant. The indirect impacts of a leaf-mining insect,* Eriocrania *spp. Zeller (Lepidoptera: Eriocraniidae) on a phloem-feeding insect,* Euceraphis betulae *Koch (Homoptera: Drepanosiphinae) were investigated.*
2. Euceraphis betulae *mortality was higher when caged on leaves with* Eriocrania *leaf-miners. Mortality was not affected by the area of leaf mined or elevated phenolic compound concentrations in mined leaves, but leaf-miner induced damage to the midrib was strongly correlated with poor aphid survival.* E. betulae *was less abundant on mined leaves with midrib damage than on mined leaves with just lamina damage, or mine-free leaves.*
3. *Experiments simulating leaf-miner damage to the midrib confirmed that only this type of damage was associated with higher* E. betulae *mortality, whereas lamina damage had no effect on mortality.*
4. Eriocrania *larvae mining leaves with manually damaged midribs weighed more than those in which the midrib was intact. There was also a trend towards higher nitrogen concentrations in leaves in which* Eriocrania *had damaged the midrib.*
5. *33% of the* Eriocrania *larvae in a field population damaged the midrib, so the interaction with aphids may be common. There could also be a selective advantage to leaf-miners that damage the midrib if severance renders the leaf unsuitable to a potential competitor and improves leaf nutritional quality. Midrib damage, however, may cause higher* Eriocrania *mortality, because of more rapid leaf abscission due to reduced structural integrity.*

6.1. INTRODUCTION

It is widely acknowledged that inter-species interactions comprise of both direct pair-wise interactions (*e.g.* exploitative competition and predation) and indirect interactions, mediated by a third species (Wootton 1994; Polis 1998; Fox & Olsen 2000). The importance of plant-mediated indirect interactions in shaping insect-herbivore communities in particular, is becoming widely recognised (Masters & Brown 1997) and illustrated by a growing catalogue of empirical studies (*e.g.* Denno *et al.* 2000; Fisher, Hartley & Young 2000; Petersen & Sandstrom 2001). Herbivore-induced changes to plant chemical composition frequently involve reductions in the availability of nitrogen compounds (Denno *et al.* 2000) and increases in secondary compound concentrations (Haukioja *et al.* 1990). Both of these changes have been associated with detrimental effects on other phytophagous insects and hence may underpin competitive indirect interactions between herbivores (Denno *et al.* 1995). A third type of plant modification by insect herbivores, which is overlooked in comparison, but could be equally important, is structural modification of plant tissues in a way that affects other phytophagous insects. This possibility has generally been disregarded (Masters & Brown 1997) or reported as an idiosyncratic phenomenon (*e.g.* Mattson 1986), but the growing realisation that many organisms may act as 'ecosystem engineers' by physically manipulating resource availability for other organisms (Jones, Lawton & Shachak 1994, 1997), has recently stimulated interest in how physically-mediated interactions between insect-herbivores could arise (Leather 2000; Fukui 2001).

Physical modification of a plant by an insect herbivore might be an incidental and inconsequential effect of feeding, or it may ameliorate resource quality for itself or its offspring (Tuomi *et al.* 1994). Herbivore-induced disruption of the vasculature, for instance, is a common physical modification of plant tissues (Price & Louw 1996). Benefits derived from modifying vasculature include evasion of plant defence compounds (Carroll & Hoffman 1980; Dussourd & Eisner 1987; Dussourd & Denno 1994), predisposing the plant to subsequent attack (West 1947; Coutts & Dolezal 1966) and causing nutrient accumulation in disconnected tissues (Forcella 1982; White 1984). Gall-forming insects that improve leaf nutritional quality by physically modifying vasculature are good examples of such 'resource regulation' (Price & Louw 1996; Hartley 1998; Wool *et al.* 1999). Gall-formers cause short-term structural modification of plant vessels, as well

as longer-term changes in plant growth and architecture that could potentially affect other insect species feeding on shared leaves. Phloem-feeding insects, whether gall-forming or free-living, might be particularly susceptible to vasculature manipulation by other insects, as they are reliant on high flux rates of phloem sap (Raven 1983; Hartley & Jones 1997). Whilst most attention to date has focussed on the impacts of gall-forming insects on leaf vasculature, leaf-mining insects may also cause similar effects by virtue of their similar endophagous lifestyle (Connor & Taverner 1997).

The purpose of this study was to identify the processes by which two insect-herbivores of different feeding guilds might interact via changes to their shared host plant, focusing on the physical changes to leaves caused by insects when feeding. The system examined how a free-living aphid, *Euceraphis betulae* Koch (Homoptera: Drepanosiphinae) is affected by co-occurring leaf-mining moths of the genus *Eriocrania* Zeller (Lepidoptera: Eriocraniidae). The genus consists of six species, five of which are indistinguishable during larval stages, but whose identical life-histories have led to them being treated as a single taxon in this, and other research (*e.g.* Koricheva & Haukioja 1994; Fisher, Hartley & Young 1999, 2000). The aphid and leaf-miner feed on leaves of the deciduous tree *Betula pendula* Roth (Betulaceae) (silver birch). The two species are spatially separated and do not compete directly for the same resource; *Eriocrania* feeds internally between the upper and lower lamellae, whereas *E. betulae* feeds on phloem sap from the basal midrib and petiole (Hajek & Dahlsten 1986). *Eriocrania* have the potential to affect *E. betulae* indirectly however, if their internalised feeding disrupts the vascular system.

The specific aims of this investigation were to: (1) establish whether there was a competitive interaction between the *Eriocrania* leaf-miner and the aphid *E. betulae* on shared *B. pendula* leaves; (2) determine the mechanistic basis for any competition observed, and its implications for both the aphid and leaf-miner. To achieve these aims we measured aphid performance when reared on leaves that were being mined by *Eriocrania*, and quantified both the amount of leaf damaged by mining activity and its effect on foliar phenolic compound concentrations – the main group of secondary compounds in birch (Haukioja, Niemela & Siren 1985; Hartley & Lawton 1991; Ossipov *et al.* 2001). Whether leaf-mining effects on aphid performance were the result of damage to the primary vasculature was tested by comparing the effects of simulated leaf-miner damage to the leaf

midrib and lamina on aphid survivorship. We also conducted surveys to (a) ascertain the frequency at which field populations of *Eriocrania* damaged the midrib and (b) compare aphid occurrence on *Eriocrania* mined leaves with and without midrib damage. Lastly, we examined whether midrib damage had beneficial effects on *Eriocrania* performance, for example by causing nitrogen compounds to accumulate in leaves.

6.2. MATERIALS AND METHODS

6.2.1. Site description & study system

The study site, Dalhaikie Flat, Aberdeenshire, UK (57°075' N, 2°582' W; OS 3645 8992), comprised *ca.* 3 ha of almost continuous birch thicket dominated by *Betula pendula* (with small numbers of *B. pubescens*) that had regenerated after mature trees were felled in 1989. All field investigations were conducted between May and July of 1999, 2000 and 2001. The aphid, *E. betulae*, lives and feeds entirely on birch, reproducing asexually from spring until autumn, whereupon a sexually reproducing generation produces eggs that over-winter until the following spring. Third instar aphid larvae (referred to as 'young larvae') and fourth instar larvae ('old larvae') were collected from foliage at the site for manipulation experiments. The adult *Eriocrania* moth oviposits under the epidermis of newly-emerged birch buds during spring. The leaf-mining larvae emerge from the eggs 2-3 weeks later and feed internally, forming a blotch mine as they progress. Once larval development is complete, *Eriocrania* exits the leaf-mine and overwinters in the soil until the following spring whereupon it oviposits as an adult (Heath 1976).

6.2.2. Aphid performance on *Eriocrania* mined leaves

In May 1999, 20 saplings with at least 5 mined leaves were labelled. On each tree, two of the mined leaves and two adjacent mine-free leaves were clip-caged. Pairs of either 'young' or 'old' aphid larvae were randomly assigned to each cage, so that each tree possessed: (a) a mined leaf with two young larvae; (b) a mined leaf with two old larvae; (c) a mine-free leaf with two young larvae; and (d) a mine-free leaf with two old larvae. After seven days, aphid larvae were recovered with the leaf and surviving aphids scored.

Mined leaves were digitally scanned to measure mined and total leaf surface area using image analysis software (Scion Image Beta 4.02™, Scion Corp, USA). Leaf-mining damage to the midrib was recorded. All leaves (mine and mine-free) were frozen (-18°C) within 3 h of recovery from the field, freeze-dried for 36 h, and assayed for total phenolic compound content using the Folin-Ciocalteau method outlined by Waterman & Mole (1994) and Kerslake, Woodin & Hartley (1998).

6.2.3. Simulated leaf-miner damage experiment

To test whether leaf-miner damage to the midrib affected aphid survivorship, leaf-miner damage was simulated on birch leaves. In May 2000, six mine-free leaves from similar regions of the canopy of 20 birch saplings (1.7 – 2.0m height) were selected. On each tree, two leaves had approximately 30mm^2 of the abaxial lamina cauterised using an electronics soldering iron, two leaves had 30mm^2 of the abaxial midrib region cauterised, and the remaining two leaves were undamaged. After 24 h, all leaves were clip-caged, and pairs of either young or old aphid larvae were randomly assigned to the leaves in a factorial manner (as above). After 7 days, aphids and leaves were recovered from the field. Leaves were stored and analysed for phenolic compound content (as above), and aphid survivorship and larval instar recorded for each leaf. Live aphids were weighed (Cahn Instruments™, California, USA) to an accuracy of ± 1μg.

6.2.4. Surveys of leaf-mining damage and aphid abundance on mined leaves

The closest 300 trees to a random coordinate at the site were surveyed for *Eriocrania* mines (4 min per tree) between 6 - 8 June 2000. Patterns of leaf-miner damage were classified according to which zones of the leaf had been mined (Figure 6.1). Leaf-mines were re-examined three weeks later and leaf-mining patterns were re-classified using the same protocol.

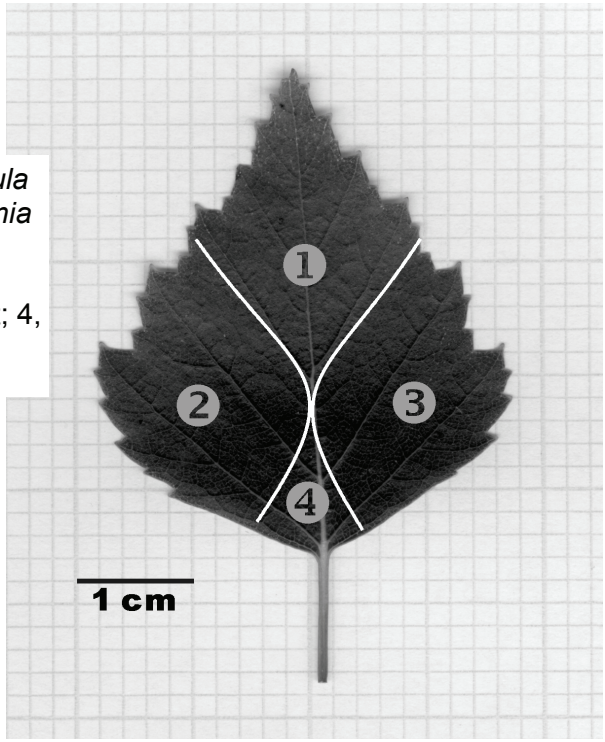


Figure 6.1. Zones on *B. pendula* leaves used to assess *Eriocrania* blotch mine development and orientation on the leaf. Leaf zones: 1, apical; 2, left; 3, right; 4, basal midrib.

Aphid abundance on mined leaves, with and without midrib damage, was measured on 63 of the original 300 trees that possessed such leaves in similar regions of the canopy. From each tree, a mined leaf with midrib damage and one without, together with a neighbouring mine-free leaf were monitored. The number of aphids on each was scored on alternate days between 11 – 25 June 2000. If the leaf-miner died, or if a leaf-miner on a mined leaf classified as 'midrib intact' subsequently damaged the midrib, an alternate tree was adopted for the survey.

6.2.5. Leaf-miner performance on midrib damaged leaves

This experiment addressed how damage to the midrib might affect leaf-miner performance. The shortest distance between the mine and the midrib was measured on 100 leaves with recently initiated *Eriocrania* mines in May 2001. After seven days, 15 trees bore at least 4 mined leaves with midrib damage and at least 8 mined leaves with just lamina damage ('midrib intact'). On each of the 15 trees, one of 'midrib intact' mined leaves had the midrib manually severed using a mounted dissection pin, whilst the other two leaves were left unmodified. All three leaves were clip-caged and monitored until leaf-miner emergence from the mine, whereupon the leaf and the emergent larva were recovered from the field. The larva was weighed (Cahn Instruments™, California, USA) to an accuracy of ± 1μg, and the leaf digitally scanned and measured as above. Leaf area was converted to leaf biomass using a regression equation (biomass (mg) = 17.05 × surface area (cm^2) – 3.9129) derived from leaf mass and surface area measurements from 100 leaves collected from the field ($r = 0.966$).

6.2.6. Mined and mine-free leaf chemistry.

The 15 trees used in the leaf-miner performance experiment (see above) were also used for leaf chemical analysis. Mined leaves labelled in May 2001 that were not used in the performance experiment were classified as either 'midrib damaged' or 'midrib intact'. Half of the 'midrib intact' leaves had the midrib manually severed, 48 h prior to collection from the field for C/N analysis. At the end of the leaf-miner performance experiment (mid June 2001), 5 mine-free leaves that neighboured the caged leaf-miner leaves were removed from each of the 15 trees for C/N analysis. All leaves were oven dried, milled to a fine powder, and *ca.* 5 mg of leaf assayed for total carbon and nitrogen concentrations using a C H N combustion analyser (Carlo Erba Instruments, Model E1110, Milan, Italy).

6.2.7. Statistical analysis

Statistical analysis was carried out in SAS version 8.12 (SAS Institute 1999). Aphid survivorship on mined and mine-free leaves was analysed using a generalised linear model with binomial distribution and logistic link function (PROC GENMOD) (SAS Institute 1999). Tree number, larval age ('young' or 'old'), and leaf-miner presence were analysed together and the least significant ($P > 0.10$) explanatory variable removed sequentially until all were significant. This procedure was also used to identify which aspect of leaf-mining affected aphid survivorship. Aphid survivorship and development on artificially damaged leaves were also analysed this way. Leaf type comparisons were made in which *P* values were calculated based on the asymptotic χ^2 distribution of the χ^2 statistic (SAS Institute 1999). Aphid mass conformed to normality and was compared using a general linear model (PROC GLM) (SAS Institute 1999), although low survivorship on midrib damaged leaves meant this category was excluded from the analysis. Aphid abundance on mined leaves, with and without midrib damage, and mine-free leaves was analysed using a generalised linear mixed model (GLIMMIX) (SAS Institute 1999) with Poisson distribution and log link function. Tree replicate was fitted as a random term on the model, and between leaf type comparisons made using differences in least squares means tests calculated within the macro. Degrees of freedom for fixed effects were adjusted using the Satterthwaite formulas (Littell *et al.* 1996). In the leaf-miner performance experiment, those response variables conforming to normality were analysed with a general linear model (PROC GLM) (SAS Institute 1999). Leaf-miner mass at emergence was analysed in the same way with amount of leaf eaten and nitrogen content of adjacent leaves fitted as covariates. Comparisons made using Tukey's *post hoc* tests. Arcsine square root transformations of leaf C/N data were analysed using a general linear model (PROC GLM).

6.3. RESULTS

In all three study years, 1999 – 2001, the aphid *E. betulae* was first observed on *B. pendula* in April. Multiple parthenogenetic generations were observed thereafter until last August / September, when sexual morphs produced overwintering eggs from late September / early October. *Eriocrania* adults were observed ovipositing into newly emerged *B. pendula* leaves in late April / early May and blotch mines began to develop 2-3 weeks later. Leaves were typically mined for 2-3 weeks until larval emergence.

6.3.1. Aphid survivorship on *Eriocrania* mined leaves

Euceraphis betulae survivorship was significantly depressed when caged in the presence of an *Eriocrania* leaf-miner for both 'young' and 'old' aphid larvae (Figure 6.2 & Table 6.1a). Young larvae survivorship was lower than for their older counterparts when caged on leaves, but there was no interaction between the effects of the leaf-miner and aphid age on survivorship (Table 6.1a). Examining aphid survivorship on mined leaves alone (Table 6.1b) showed that the amount of leaf mined (both the absolute area and as a proportion of the whole leaf) and phenolic compound content had no significant effect on *E. betulae* survivorship. Leaf-mining damage to the midrib, in contrast, was strongly correlated with low aphid survivorship, affecting both young and old aphid larvae equally (Table 6.1b).

6.3.2. Simulated leaf-miner damage experiment

Euceraphis betulae survivorship was significantly lower and its development retarded when reared on leaves in which the midrib had been damaged compared to those in which the lamina had been damaged or the leaf remained intact (Table 6.2). There was no significant difference in aphid survivorship and development on lamina damaged and undamaged leaves. There was also no significant difference in aphid mass upon reaching adulthood when reared on lamina damaged and undamaged leaves (1.33 and 1.35 mg, respectively) or the final larval instar 'old larvae' (1.26 and 1.24 mg, respectively) ($F_{1,83} = 0.07$, $P = 0.791$). As with the caging experiment with leaf-miners, young aphid larvae survived and developed less well than their older equivalents in clip-cages, but both age groups were equally affected by the type of damage imposed on the leaf (Table 6.2). Phenolic compound concentrations were significantly higher in cauterised leaves, whether the

damage was imposed on the midrib (154 mg g^{-1} dry weight) or the lamina (152 mg g^{-1} dry weight) compared to undamaged leaves (134 mg g^{-1} dry weight) ($F_{2,57}$ = 3.94, P =0.025). Phenolic concentrations in cauterised leaves were statistically indistinguishable (t = -0.158 P = 0.986).

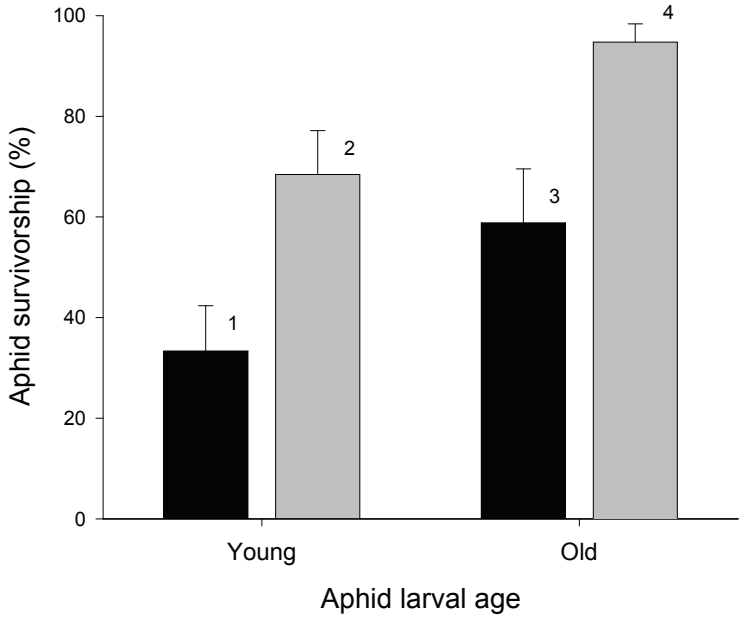


Figure 6.2. *E. betulae* survivorship (mean % ± SE) when caged in the presence (■) and absence (▒) of an *Eriocrania* leaf-miner. Replicates [1] 18, [2] 19, [3] 17, [4] 19. Young aphid larvae are instar III and old larvae are IV (final) instar.

Table 6.1. (a) Summary of generalised linear model results for *E. betulae* survivorship when caged on *Eriocrania* mined and mine-free leaves (b) Summary of generalised linear model results for *E. betulae* survivorship on mined leaves alone, exploring effects of midrib damage, amount of leaf mined and foliar phenolics concentration on aphid survivorship. Significant terms in bold. *F* and *P* values for non-significant terms were derived from individually adding these terms to the model containing all significant terms.

(a)

Sources of Variation	df	*F*	*P*
Larval age[1]	1,70	8.40	<0.01
Leaf-miner presence	1,70	14.76	<0.001
Non-significant terms			
Tree Number	19,51	0.71	0.796
Larvae age[1] × Leaf-miner presence	1,69	0.91	0.342

(b)

Sources of Variation	df	*F*	*P*
Larval age[1]	1,32	3.85	0.058
Midrib damage	1,32	12.97	0.001
Non-significant terms			
Area mined (cm^2)	1,31	1.56	0.222
Area mined (% of leaf)	1,31	1.67	0.206
Phenolic concentration	1,31	0.08	0.781
Larval age[1] × Midrib damage	1,31	<0.01	0.949

[1] Larval age defined as Figure 6.2.

Table 6.2. *E. betulae* performance when reared on *B. pendula* leaves with simulated leaf-mining damage to the midrib and lamina and undamaged leaves. Total number of aphids shown (40 replicates except where indicated §). Larval age definition as Figure 6.2. Lowercase superscripts indicate significant differences between leaf types. F and *P* values for non-significant terms calculated as Table 6.1.

	Aphid survivorship		Aphids developing to next instar§	
Leaf Damage Type	Young	Old	Young	Old
Leaf midrib cauterised	7^{a}	11^{c}	0	2^{f}
Leaf lamina cauterised	35^{b}	38^{d}	11^{e}	32^{g}
Leaf undamaged	32^{b}	39^{d}	10^{e}	33^{g}
Significant terms				
Damage type	$F_{2,116} = 65.04$	$P < 0.001$	$F_{2,91} = 10.53$	$P < 0.0001$
Larvae age	$F_{1,116} = 7.90$	$P = 0.005$	$F_{1,91} = 39.82$	$P < 0.0001$
Non-significant terms				
Tree number	$F_{19,97} = 0.97$	$P = 0.507$	$F_{19,72} = 1.30$	$P = 0.129$
Larvae age ´ Damage type	$F_{2,114} = 1.34$	$P = 0.266$	$F_{2,89} = 0.11$	$P = 0.851$
Phenolic concentration	$F_{1,115} = 0.66$	$P = 0.41$	$F_{1,90} = 0.45$	$P = 0.507$

§ surviving aphids only (replicates as per survivorship column).

6.3.3. Surveys of leaf-mining damage and aphid abundance on mined leaves

There was no obvious pattern to how leaf-miners mined *B. pendula* leaves. Of the 221 mined leaves examined on 27-29 June 2000; 91 (41 %) were orientated to the left of the leaf, 77 (35 %) to the right and 53 (24 %) had no overall inclination to the left or right. Seventy three (33 %) of leaf-miners damaged the midrib, although they seldom completely severed it (pers obs.), whereas 148 (67 %) left the midrib intact.

Aphids were significantly less abundant on mined leaves with midrib damage than either mined leaves with the midrib intact or mine-free leaves, for which aphid abundance was statistically indistinguishable (Figure 6.3).

6.3.4. Leaf-miner performance on midrib damaged leaves

Mine initiation always occurred at similar distances from the midrib, regardless of whether the midrib was subsequently damaged (Table 6.3), and all juvenile mines touched the leaf perimeter at the beginning of mining activity (pers obs.). There was no difference in the mass of mined leaves with and without midrib damage (Table 6.3), nor with the mine-free leaves collected to derive a regression equation relating leaf biomass and surface area ($F_{2,143} = 0.13$, $P = 0.72$). The time taken until *Eriocrania* emerged from mines did not differ significantly between the three types of mine, and nor did the amount of leaf eaten whilst mining the leaf (Table 6.3). *Eriocrania* larval mass at emergence from leaf-mines was, however, positively correlated with the quantity of leaf eaten ($F_{1,40} = 82.52$, $P < 0.001$), (Figure 6.4), but there was no significant relationship with nitrogen concentrations of adjacent leaves ($F_{1,40} = 1.38$, $P = 0.247$).

Eriocrania larval mass was significantly higher when the midrib was damaged compared to when it was intact ($F_{2,40} = 4.22$, $P = 0.022$). *Post hoc* analysis indicated that the difference was only significant between leaf-miners with manually severed midribs and leaf-miners which mined the lamina ($t = -2.83$, $P = 0.0193$), and not between leaf-miners from leaves with naturally damaged midribs ($t = -0.81$, $P = 0.70$). There was no significant difference between the masses of leaf-miners emerging from leaves with naturally damaged midribs and leaves with intact midribs ($t = -2.01$, $P = 0.122$).

6.3.5. Leaf chemistry in mined and mine-free leaves

The total carbon and nitrogen concentrations, and the C/N ratio in mined and mine-free leaves were not significantly different (Figure 6.5). However, there was a trend ($F_{3,98}$ = 2.41, P = 0.072) towards higher nitrogen concentrations in leaves in which *Eriocrania* had damaged the midrib.

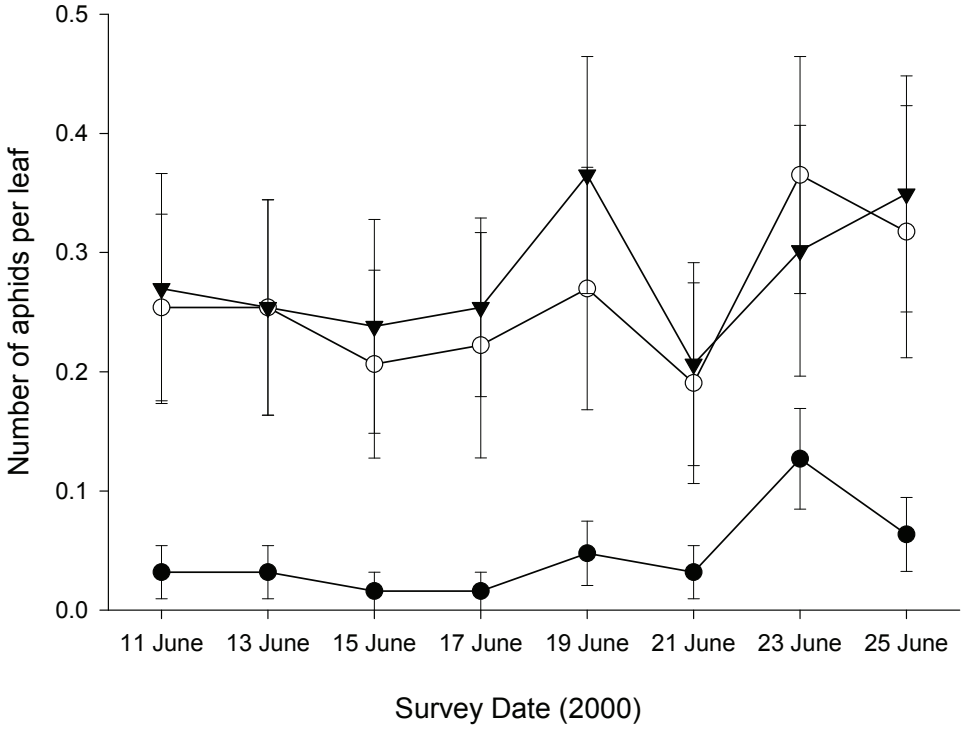


Figure 6.3. Mean number of *E. betulae* aphids recorded per leaf examined (± SE) on alternate days for two weeks during *Eriocrania* mine development. Leaf types were: mined leaves possessing midrib damage (●), mined leaves with the midrib intact (O) and mine-free leaves (▼). Survey date $F_{7,1427} = 1.73$ $P = 0.099$; Leaf type $F_{2,1427} = 22.00$ $P < 0.0001$; Survey date × leaf type $F_{14,1427} = 0.41$ $P = 0.972$. Least square mean tests between leaf types: (O - ●) t = -6.29 P <0.001; (▼ - ●) t = -6.57 P <0.0001 and (O-▼) t = - 0.55 P = 0.580.

Table 6.3. Characteristics of leaves and leaf-mining activity in which *Eriocrania* larvae mined leaves and damaged the midrib, mined the lamina alone (midrib intact) and mined the lamina alone, but the midrib was manually damaged. Data displayed as mean ± SE, 15 replicates.

	Leaf characteristics		Leaf-mining characteristics	
Mined Leaf Type	Initial distance of mine from midrib (mm)	Initial leaf mass (mg)	Time from caging to larval emergence (days)	Leaf eaten (mg)
Midrib damaged by larvae	7.7 ± 0.30	81.9 ± 6.15	9.93 ± 0.35	69.20 ± 4.73
Lamina damaged by larvae (midrib intact)	8.5 ± 0.31	85.4 ± 6.92	10.13 ± 0.32	65.69 ± 5.57
Lamina damage by larvae (midrib manually damaged)	7.7 ± 0.21	88.2 ± 6.10	10.27 ± 0.42	67.17 ± 4.29
	$F_{2,42} = 2.59$ $P = 0.087$	$F_{2,42} = 0.25$ $P = 0.782$	$F_{2,42} = 0.21$ $P = 0.81$	$F_{2,42} = 0.13$ $P = 0.88$

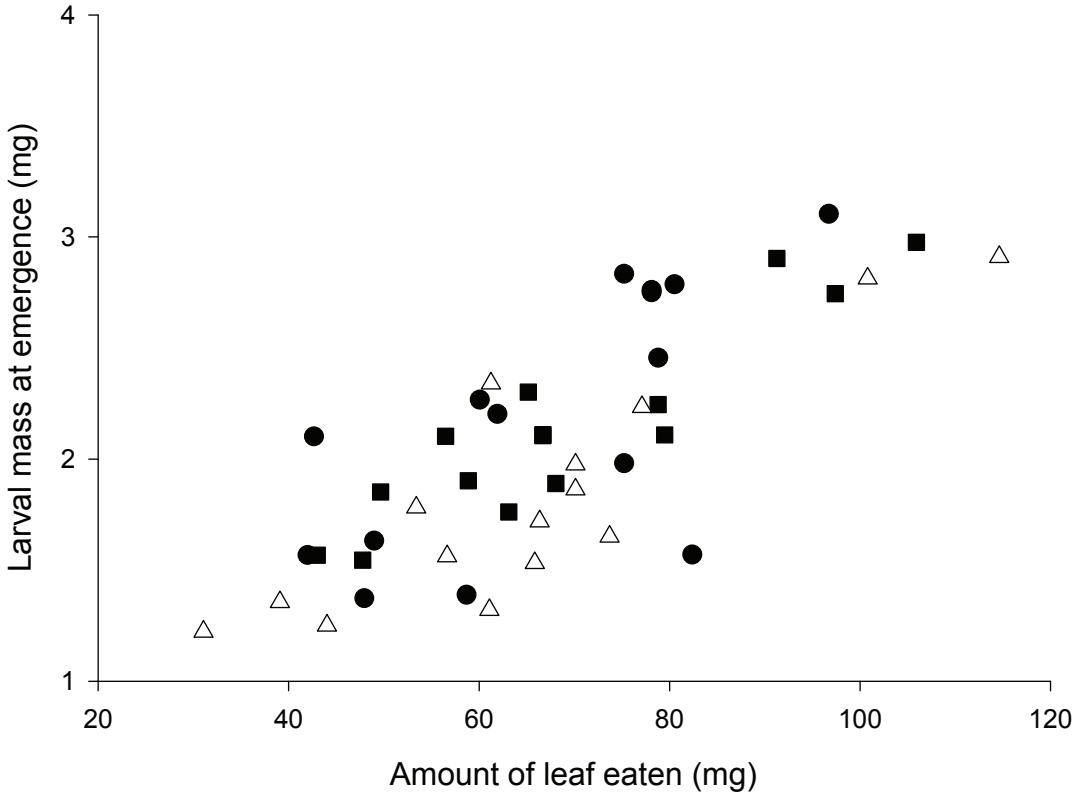


Figure 6.4. Relationship between amount of *B. pendula* leaf eaten by *Eriocrania* larvae and larval mass at emergence from leaves in which the midrib was damaged by the larvae (■), the lamina alone was damaged by the larvae (△) and the lamina was damaged by the larva and the midrib was manually damaged (●).

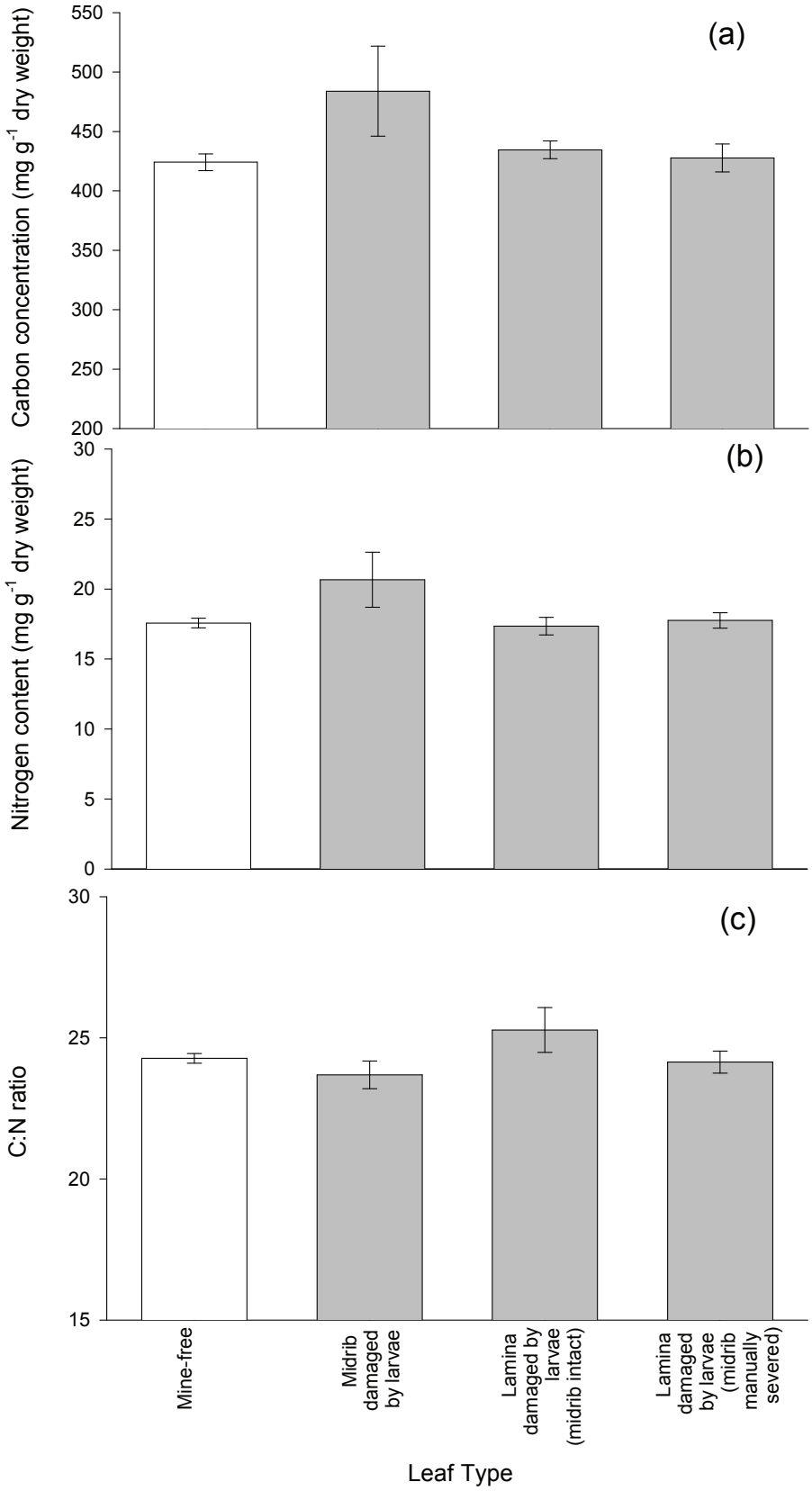


Figure 6.5. Leaf chemistry of mine-free *B. pendula* leaves (□) and leaves bearing *Eriocrania* leaf-miners (■) with the midrib intact and with (natural and manual) midrib damage (± SE). (a) total carbon concentration ($F_{3,98}$ = 1.74 *P* = 0.164); (b) total nitrogen concentration ($F_{3,98}$ = 2.41 *P* = 0.072) and (c) C:N ratio ($F_{3,98}$ = 1.82 *P* = 0.148).

6.4. DISCUSSION

The aim of this study was to investigate the plant mediated indirect effects of a leaf-mining moth, *Eriocrania*, on a phloem feeding aphid, *Euceraphis betulae*, on birch. This study focused on the physical changes that occur during leaf-mining activity. *E. betulae* was negatively affected by *Eriocrania* when the leaf-miner damaged leaf vasculature, and *E. betulae* was less abundant on mined leaves with this type of damage in the field. Structural damage to the leaf midrib was not a prerequisite of *Eriocrania* leaf-mining, but leaf-miner performance was positively affected when the midrib was manually damaged.

6.4.1. Mechanistic basis for the interaction

The negative effect of one insect herbivore (*Eriocrania*) on the survivorship of another of a different feeding guild (*E. betulae*) is usually considered in terms of nutritional or allelochemical changes induced by one that subsequently affects the other (Masters & Brown 1997). *Eriocrania* was associated with higher concentrations of foliar phenolic compounds, the main group of anti-herbivore allelochemicals in birch (Hartley & Lawton 1991; Ossipov *et al.* 2001), but this together with the actual amount of leaf mined, was unrelated to *E. betulae* survivorship. Birch aphid performance, including that of *E. betulae* (unpublished data), has been shown to be unaffected when reared on birch leaves with elevated phenolic compound concentrations (Martin, Cappuccino & Ducharme 1994), perhaps because of their low concentrations in phloem sap (Raven 1983; Karban & Myers 1989).

The actual region of the leaf mined rather than leaf-miner presence *per se* was strongly correlated with *E. betulae* survivorship. Specifically, when *Eriocrania* mines impinged on the leaf midrib, aphid survivorship was significantly lower than when leaf-mining was restricted to the lamina. Similarly, when *E. betulae* was reared on leaves with simulated leaf-miner damage, aphid performance was significantly lower on leaves with midrib damage compared to either lamina damaged or undamaged leaves. Aphid survivorship, development and mass was statistically indistinguishable when reared on lamina damaged and undamaged leaves, despite lamina damaged leaves having higher phenolic compound concentrations. This provides further support for secondary compounds not being responsible for the depressed *E. betulae* performance. Artificial damage is known to produce slightly different effects to natural herbivory (Baldwin 1990), but these

manipulations allowed potentially confounding effects to be uncoupled from damage type and permitted standardisation of leaf damage.

Euceraphis betulae were significantly less abundant on mined leaves with midrib damage compared to mined leaves with an intact midrib or mine-free leaves. That *E. betulae* were equally abundant on leaves with damaged and undamaged lamina reinforces the suggestion that it is a particular consequence of leaf-mining, namely damage to the primary vasculature, that makes a leaf unsuitable as a resource to the aphid, rather than leaf-miner presence as such.

6.4.2. Implications of leaf midrib damage for the aphid

It is unlikely that *Eriocrania* caused changes in the nutritional chemistry of phloem sap only in leaves with midrib damage and not in lamina damaged leaves. Whilst not an ideal indication of phloem sap quality, the slightly higher nitrogen concentrations of mined leaves with midrib damage also makes this possibility look doubtful. A more likely explanation for the negative effects on the aphid, is that damage to the midrib disrupts phloem hydraulics on which phloem feeders are dependent (Dixon 1998). A comparable situation arose when an aphid gall (*Forda formicaria*) was situated on the midrib of a Pistachio leaf, thereby diverting nutrients away from a second aphid gall (*Geioica* sp.) (Inbar *et al.* 1995).

Euceraphis betulae might be particularly susceptible to interference with phloem hydraulics because of its specific feeding sites on the larger primary veins, namely the basal midrib and petiole. The phloem is more deeply embedded in primary veins than elsewhere on the leaf but since *E. betulae* possesses a particularly long stylet (0.4 - 0.5 mm at adulthood) it can tap into them successfully (Hajek & Dahlsten 1986). Whilst the flow of phloem sap is greater in the larger veins, the concentration and quality tends to be lower than in neighbouring smaller vessels (Dixon & Logan 1973), perhaps suggesting that *E. betulae* is adapted to feeding on lower quality sap but at higher flux rates. Any interference with the phloem hydraulics might then be especially detrimental to *E. betulae* compared to an aphid which is adapted to feed on vessels with a lower flow of phloem sap. Indeed, Prestidge & McNeil (1982) have suggested that the availability of phloem sap has selected for two discrepant life-history styles in phloem feeding insects. Those phloem-feeders which have

highly specific demands for phloem sap (*e.g.* high flux rates) are highly mobile so as to meet those demands, whereas phloem-feeders that are more tolerant of fluctuating phloem sap availability tend to be less mobile. Under such circumstances, vasculature damage by leaf-mining insects might disproportionately affect large and mobile aphids such as *E. betulae*, whereas sessile aphids might be less adversely affected.

6.4.3. Implications of leaf midrib damage for the leaf-miner

Most leaf-mining insects avoid mining major veins (Kimmerer & Potter 1987; Scheirs, Vandevyvere & DeBruyn 1997; Scheirs, De Bruyn & Verhagen 2001 and references therein) because they are nutritionally unfavourable (Scheirs *et al.* 2001) and frequently the toughest tissues in the leaf (Choong 1996). Such avoidance results in distinctively shaped mines between the veins (Stiling, Simberloff & Anderson 1987; Scheirs *et al.* 1997). *Eriocrania,* in contrast, apparently did not avoid large leaf veins, since there was no obvious pattern to how the leaf was mined and the midrib was actually mined in a third of the leaf-mines examined. As the majority of *Eriocrania* left the midrib intact, mining the midrib cannot be considered to be an essential part of their feeding, unlike the holly leaf-miner, *Phytomyza ilicis*, which must initially mine the midrib to enter the laminal parenchyma (Valladares & Lawton 1991). Whether *Eriocrania* proceeds to mine the midrib was not determined by proximity of the young mine to the midrib, signifying that leaf-miners did not mine the midrib because it was close by. Leaf-miners that mine the midrib may be compelled to do so because the available lamina is becoming limited, but those leaf-miners selected for this field trial had (by necessity) mined into the midrib whilst the mine was still young, so this cannot fully explain midrib damage either.

There was a strong positive correlation between larval mass of leaf-miners at emergence from their mines and the amount of leaf they had consumed. Perhaps more interesting, is that leaf-miners that fed on leaves where the midrib was artificially damaged performed disproportionately well compared with leaf-miners from leaves with intact midribs, when amount of leaf eaten and nutritional quality of adjacent leaves were taken into account. Leaf-miner larvae which developed quickly initially may have been able to mine tough midrib tissues by virtue of their more developed mouthparts (Scheirs *et al.* 1997), whereas those that developed more slowly were not. This does not account for the significant differences in final mass however, as leaf-miners from the manipulated leaf (where the

lamina was mined by *Eriocrania* and the midrib was manually damaged) were the heaviest at emergence, despite coming from the group of leaf-miners which were destined to mine the lamina alone. The identical mine occupancy time for all types of mined leaf also helps disqualify developmental differences in emergence weight (*i.e.* that miners may have weighed more because they ate for longer).

Most phytophagous insects are nitrogen limited (Waring & Cobb 1992) and damaging the midrib might increase the nutritional value of the leaf for *Eriocrania*. In addition to restricting the supply of water to leaves via the xylem, damaging the midrib could also curb the export of nutrients via the phloem. Accelerated protein degradation as a result of water stress, could cause nitrogen to accumulate in leaves (White 1984). This phenomenon is known to be made use of by a range of phytophagous insects including the sawfly, *Eriocampa ovata* (MacKay & Wellington 1977); the lepidopterans *Danaus plexippus* (Brewer 1977; Rothschild 1977) and *Manduca sexta* (Heinrich 1971) and the coleopteran *Oncideres cingulata* (Forcella 1982). Phloem-feeders like *E. betulae* would not be able to take advantage of this nutritional improvement, since they are reliant on the flux of amino acids rather than the total amount *per se* (Dixon 1998). There was a trend towards higher nitrogen concentrations in mined leaves with miner-induced midrib damage, which may have reflected an accumulation of nitrogenous compounds in leaves with damaged midribs. This may ultimately benefit the leaf-miner, but as Choong (1996) demonstrated, the severance of the tough midrib is energetically demanding and nutritionally unfavourable.

It is also possible that damaging the midrib is an incidental effect of leaf-mining by *Eriocrania*, and may even be disadvantagous for the leaf-miner in some cases. Preszler & Price (1993) for instance, showed that rapid leaf-miner development on young willow leaves caused early leaf abscission by the plant resulting in high leaf-miner mortality. Whether birch leaves are abscissed in direct response to *Eriocrania* mining is unknown but damaging the midrib may reduce the structural integrity of the leaf, since the midrib contains high levels of structural compounds such as lignin (Choong 1996), making such weakly attached leaves prone to dislodgement. Alternatively, damage to the midrib may actually impede the plant chemical processes that cause leaf abscission in response to

environmental stress (Taylor & Whitelaw 2001), thereby prolonging leaf attachment to the parent plant.

6.4.4. Insect competition through physical modification of leaves

Regardless of whether damaging the midrib is advantageous to *Eriocrania*, these findings demonstrate that if the midrib is damaged, the leaf is rendered unsuitable for *E. betulae* to feed upon. Mining part of the midrib does not deprive *E. betulae* of its feeding site in the strictest sense, but it could disrupt phloem turgor on which aphids are at least partially dependent (Auclair 1965; Raven 1983). In contrast to *E. betulae*, all *Eriocrania* larvae were alive at the end of the caging experiments, suggesting that this is a largely asymmetric interaction and *E. betulae* has little discernible impact on *Eriocrania*. This supports the findings of Fisher *et al.* (2000), who also found that birch aphids had no impact on *Eriocrania* performance in field trials. This competitive interaction, like many direct (Lawton & Hassell 1981) and indirect (Bonsall & Hassell 1997) interactions between insects, is very probably asymmetric. The asymmetric indirect effect of *Eriocrania* on *E. betulae* is likely to be fundamentally more stable than a symmetric interaction between the two insects, since there is no mechanism for a positive feedback on *Eriocrania* that would tend towards the exclusion of *E. betulae* (Lawton & Hassell 1981).

This is believed to be the first time that the indirect effects of a leaf-miner on a phloem feeder via structural changes to the vasculature of a shared host plant have been reported. We suggest that anatomical modification of a shared resource could be more widespread than previously reported. If so, the extent of inter-specific competition between insect-herbivores may have been underestimated, particularly since most studies of competitive interactions between insect herbivores focus on changes in plant chemical composition rather than plant structure.

CHAPTER SEVEN

Discussion

7.1 INTRODUCTORY REMARKS

The focal species of this study, *Euceraphis betulae*, was both a beneficiary and casualty of indirect interactions with organisms that simultaneously exploit *Betula pendula* leaves through chemical and physical mechanisms. The wider implications of these indirect impacts on *E. betulae* are considered in this chapter, focusing on the possible changes to aphid population dynamics, how the effects of climate change might modify these interactions and how this might affect birch. The ecological importance of plant-mediated insect competition is also discussed, before considering how plant responses to both fungi and insects may affect each other.

7.2. APHID PHENOLOGY – PROCESSES AND STRATEGIES

The processes and regulatory mechanisms that drive tree-dwelling aphid population dynamics have recently received renewed interest (*e.g.* Sequeira & Dixon 1997; Jarosik & Dixon 1999). Whilst many studies make use of long-term data sets (often more than 15 years), these are insensitive to the short-term processes operating within a single year which can only be identified with more frequent non-destructive observations like those presented in Chapter 3. The survey of *E. betulae* population abundance and distribution on *B. pendula* reported in this thesis, is believed to be the first for this species in the United Kingdom.

7.2.1. Resource tracking

Seasonality is an important feature of aphid population dynamics since their short generation time and probable hard-wired anticipation of seasonal trends allows them to track changes in habitat quality very closely (Kindlmann & Dixon 1996; Sequeira & Dixon 1997; Dixon 1998). The seasonal changes in the free-amino acid content of phloem sap discussed in Chapter 3 almost certainly contribute to these seasonal trends in aphid abundance (Dixon *et al.* 1993; Sequeira & Dixon 1996; Dixon 1998).

E. betulae is a common herbivore of *B. pendula*, whose dynamics were probably linked to changes in the nutritional quality of its host plant. *E. betulae* abundance tracked host plant phenology with remarkable fidelity, generally occurring more evenly throughout the tree canopy at the beginning and end of the growing season and moving to the most actively growing (and probably most nutritious) parts of the tree (upper branches and apical

leaves) in between. Such an intimate association with host plant phenology is probably crucial to *E. betulae* and one reason suggested for the smaller *E. betulae* population in 2000 compared with 1999 was that egg-hatch was not synchronised with bud-burst in 2000.

It would be inappropriate to predict between-year trends in *E. betulae* population dynamics on the basis of two years of results, but some within-year and between-year trends in 1999 and 2000 are at least compatible with the so-called 'see-saw effect' identified by Dixon (1975, 1978), for which there is growing evidence for several tree-dwelling aphids (*e.g.* Liao & Harris 1985; Sequeira & Dixon 1997; Day & Kidd 1998). The effect describes a negative correlation between abundance of first generation (fundatrices) in spring and the abundance of sexuals in autumn, and a positive correlation between the latter and the abundance of fundatrices in the following spring. As a result of the 'see-saw effect', tree-dwelling aphid populations superficially follow a two-year cycle, being abundant one year and scarce the next. There are many biological and statistical complications associated with this general effect (Kindlmann & Dixon 1996), but it seems to occur in most tree-dwelling species of aphid whenever it is looked for (Dixon 1998). The current view is that the effect could be driven by density-dependent aphid infestation, operating either through deterioration in host plant quality or directly because of aphid crowding, or possibly both. Whether natural enemies play a role in this phenomenon, and to what extent it is a functional rather than a numerical response to aphid populations remains unclear.

It would be interesting to explore how the effects of *M. betulae* in the middle of the season would interact with the anticipated seasonal trends in resource quality. Since the effect of *M. betulae* on host plant leaves is physiologically similar to autumn leaf senescence (the translocation of higher fluxes of amino acids via the phloem), widespread occurrence of *M. betulae* might function as an 'early autumn', thereby shortening the seasonal decline in host quality during mid-summer. The interplay of plant phenology and fungal-impacts on *E. betulae* populations may be difficult to predict, but there is some evidence that one important aphid strategy that occurs in response to seasonal changes in plant quality is being modified by the effects of *M. betulae* – reproductive diapause.

7.2.2. Reproductive diapause

Many tree-dwelling aphids that hatch from eggs relatively early in the season (e.g. *E. betulae* and *D. platanoidis*) are poorly adapted to warmer temperatures and the accompanying lower nutritional quality of phloem sap experienced during mid-summer and undergo a period of reproductive diapause (Dixon 1975, 1998; Douglas 2000). Reproductive diapause in aphids is characterised by the uncoupling of maternal and embryonic growth, absence of developmentally mature embryos, and possibly the suppression of symbiotic bacterial function (Douglas 2000). Reproductive diapause is widely viewed as a life-history strategy to cope with warmer temperatures and poor nutrition, since the maintenance of only developmentally immature embryos incurs relatively low nutritional costs whilst enabling the aphid to respond rapidly and develop embryos when conditions become more favourable (Dixon 1998; Douglas 2000). Diapause is a genetically hard-wired event (in contrast to facultative 'quiescence') in response to environmental cues, possibly including low plant nutrients, high temperature and crowding (Chambers 1982; Dixon *et al.* 1993), mediated through the effects of juvenile hormone in the aphid (Denlinger 1985). Behavioural changes are often correlated with these physiological changes, for instance many aphids such as *D. platanoidis* move to lower branches in the canopy which are generally more shaded during summer (Dixon & McKay 1970).

In this study, the high number of larval *E. betulae* throughout the season and the lack of migration to the lower canopy suggest that the conditions in the mid-summer were not unduly adverse, and reproductive diapause may be less prominent than in other *Euceraphis* populations (*e.g.* Wratten 1974; Hajek & Dahlsten 1988), possibly because of the generally cooler temperatures experienced at this latitude. Reproductive diapause was not absent from the population altogether however, since a significant proportion of those aphids reared on asymptomatic leaves (*i.e.* those without fungal lesions) in mid-summer possessed only developmentally immature embryos (Chapters 4 & 5) – a characteristic of aphids in a state of reproductive diapause (Douglas 2000). With the exception of one individual in 1999, none of the aphids reared on fungal-infected leaves were in a state of reproductive diapause. This suggests that *M. betulae* may be ameliorating leaf quality in a way that permits individuals to develop embryos, when otherwise they may possess only developmentally immature embryos - a phenotypically plastic response to favourable

conditions (Nager, Keller & van Noordwijk 2000). This may take the form of either not committing to diapause at all, or else recovering from the condition more rapidly than would otherwise occur.

These findings raise the possibility of microbial influences on the evolution of insect life history strategies. The cooler temperatures experienced by *E. betulae* at these latitudes may allow the first obstacle of high summer temperatures to be overcome, whilst the effects of *M. betulae* on plant chemistry may permit the second, the low nutritional quality of phloem sap, to be surmounted. There is fragmentary evidence that the reproductive diapause seen in *Euceraphis* populations may be less rigid than for *D. platanoidis*, because unlike sycamore, birch flushes new foliage throughout the season and so may not be as nutritionally unfavourable to aphids during mid-summer (Wratten 1974; Hajek & Dahlsten 1988). Given the more benign summer temperatures at this latitude and greater plasticity in reproductive diapause in this genus, microbial impacts on the evolution of *Euceraphis* life history strategies look distinctly feasible. Whether such fundamental changes could occur under less favourable (hotter) summer temperatures or in other aphid species is unknown, but a greater understanding of how microbial effects can deactivate (or possibly activate in some cases) reproductive diapause in aphids is a key issue, since their prodigious reproductive capacity is the reason they are pests in the first place. A better understanding of the physiological and hormonal processes that govern reproductive diapause in *E. betulae* would provide a clearer insight into how these genetically hard-wired events may be overridden by the effects of *M. betulae*, and under what circumstances *E. betulae* is able to display such remarkable phenotypic plasticity.

7.3. IMPACTS OF CLIMATE CHANGE

Chapter 3 suggested that temperature alone was not responsible for between year differences in the size of *E. betulae* populations, but gradual climate change may have more subtle effects on *E. betulae* either directly via changes to insect physiology or indirectly through its host plant. Potentially just as important are the effects of climate change on the indirect interaction between *M. betulae* and *E. betulae*, since climate change is widely anticipated to affect plant-pathogen dynamics (Manning & Vontiedemann 1995).

7.3.1. Direct impacts on *E. betulae*

Studying the impacts of climate on insects is not a recent development (*e.g.* Uvarov 1931), but research effort into the impacts of climate change on phytophagous insects has burgeoned in the past decade (Bale, Jones & Gibbons 1996; Coley 1998). Aphids in particular, have the potential to show rapid and dramatic responses to climate change because of their short generation times and prodigious reproductive rates. Temperature is generally regarded as the main abiotic factor controlling aphid development, fecundity and movement (Harrington, Bale & Tatchell 1995). Current predictions for North Scotland are a 0.9 – 2.6 °C rise in average temperature by 2080, and an increase in precipitation characterised by frequent heavy showers, but fewer 'rain days' (Hulme & Jenkins 1998; Houghton *et al.* 2001).

As birch is only found in temperate regions, *E. betulae* normally lives at temperatures below its developmental optimum of 20°C (Hopkins 1996), therefore warmer temperatures associated with climate change will almost certainly increase developmental and reproductive rates. In this study, a total of 1845 and 1628 day-degrees above a lower developmental threshold of 1.52 °C (Hopkins 1996) were accumulated between January and aphid oviposition (September) in 1999 and 2000 respectively. Based on the requirement of 296 degree days per generation (Hopkins 1996), this would produce approximately six *E. betulae* generations per season in both years. On the basis of this, an increase of 2 °C would result in seven to eight *E. betulae* generations in each season. This increase in developmental and reproductive rate may be offset however by the constraints of a reproductive diapause which may become more prominent under warmer conditions, as is the case for *Euceraphis* populations at more southerly latitudes (*e.g.* Wratten 1974; Hajek & Dahlsten 1988). Behavioural changes may occur with warmer temperatures too. Unlike the observations reported in this study (Chapter 3) *E. betulae* may migrate from the upper to the lower canopy during mid-summer, as is common for many tree-dwelling aphids that are intolerant of high temperatures (Dixon 1998). Movement away from the actively growing upper branches in summer may place less of a nutritional drain on birch and allow better growth which is generally anticipated to increase under warmer climatic regimes and higher CO_2 concentrations (Wayne, Reekie & Bazzaz 1998).

Warmer temperatures are unlikely to result in much higher mortality of *E. betulae* at this latitude, since they seldom exceed levels known to have deleterious effects on *E. betulae* (Hopkins 1996). An increase of 2 °C would result in 58 instead of 14 hours of adverse heat (> 25 °C) in 1999 and 10 instead of one hour in 2000. *Euceraphis* eggs are extremely cold-hardy (Parry 1985), so it is unlikely that increases in winter temperatures will have discernible improvement on overwintering mortality which is largely thought to be due to developmental abnormalities such as incomplete egg fusion (Hajek 1984).

7.3.2. Indirect impacts on *E. betulae*

The effects of climate change on *E. betulae* may manifest themselves indirectly via climatic effects on *M. betulae*. Warmer and wetter weather are generally regarded as being more favourable to fungal pathogen infection of plants (Strouts & Winter 1994), so predicted climate change would suggest that *M. betulae* will become a more prevalent disease of *B. pendula*. On the basis of the findings reported in this study (Chapters 4 & 5), widespread infection by *M. betulae* could cause large increases in *E. betulae* populations. On the scale investigated in this study, the positive effects of *M. betulae* on *E. betulae* were clear-cut, but the situation may become more complicated when the effects are considered on larger spatial and temporal scales. *M. betulae* causes an improvement in nutritional quality of phloem sap because the plant mobilises amino-nitrogen out of infected leaves, but *M. betulae* neither increases the amount or composition of foliar amino-nitrogen potentially available to the *E. betulae*, it just makes it available earlier in the year. If *M. betulae* were to become even more prevalent, its effects might be functionally similar to an 'early autumn' as mentioned above. *E. betulae* numbers would still be relatively high after a spring peak so more aphids would be able to take advantage of the mobilisation of amino-nitrogen than during autumn. *E. betulae* populations could become very large during summer because of this, but widespread fungal induced leaf senescence (and abscission) in summer would result in the reduced autumn leaf senescence, so conditions may become less favourable at this time - perhaps resulting in population crashes. The 'see-saw' effect illustrates how smaller aphid populations in autumn are correlated with smaller spring populations so whether the larger assemblages of *E. betulae* would exist to take advantage of the effects of *M. betulae* infection in summer is uncertain. It is intriguing therefore to consider how the effects of widespread *M. betulae* infection would interplay with the 'see-saw' effect as both strengthened and weakened effects can be envisaged.

These effects cannot be considered in isolation either, since climate change is likely to affect birch phenology, possibly lengthening its growing season and increasing photosynthetic and growth rates. Increased growth could generally be favourable to *E. betulae* since they feed on actively growing tissues, but plant vigour might also make birch more resistant to *M. betulae* infection. Conversely, birch is particularly susceptible to drought which may arise due to the greater number of 'dry days', despite the higher total amount of precipitation. Drought-stressed birch may be more susceptible to *E. betulae*, for instance *E. betulae* performance was seen to increase through a stress-related improvement to nutritional quality in birch (Neuvonen & Lindgren 1987). Equally if growth were to be limited by drought, *E. betulae* would be deprived of actively growing feeding sites on which it was particularly abundant. Drought infected trees are widely believed to be more susceptible to disease (Bennell & Millar 1984), so it could reasonably be assumed that *M. betulae* would become more prevalent, which would generally promote *E. betulae* performance.

With so many compounding effects, it is very difficult to make predictions about how the indirect interaction between *M. betulae* and *E. betulae* via their shared host plant could be modified by climate change, and even more difficult to predict what the net effects on *E. betulae* populations in Scotland would be. Nevertheless, some of the more likely effects of climate change on *E. betulae* are shown Table 7.1. In summary, *E. betulae* might be generally expected to benefit from climate change because;

(a) it benefits from both plant vigour (actively growing leaves and buds) and plant stress (*M. betulae* infection),
(b) it generally occurs at latitudes with characteristic temperatures currently below its developmental optimum temperature of 20 °C,
(c) phloem feeders may be less adversely affected by the higher C:N ratios in plant tissues anticipated to occur with higher CO_2 concentrations (Bezemer & Jones 1998; Hughes & Bazzaz 2001).

Table 7.1. Some of the more likely direct and indirect effects of climate change on *E. betulae*, and the associated implications for *B. pendula* and *M. betulae*. Positive outcomes are envisaged to be favourable on performance and abundance and negative outcomes to be detrimental. Where no effect is anticipated, the outcome is denoted zero.

EFFECT TYPE ON *E. BETULAE*	IMPACT ON ORGANISM		
DIRECT EFFECTS	Aphid	Birch	Fungi
1. Increased aphid reproduction and developmental rates.	+	-	0
2. More prominent aphid reproductive diapause and vertical migration in summer.	-	+	0
INDIRECT EFFECTS			
1. Increased growth of birch	+	+	+/-
2. Increased infection by *M. betulae*	+	-	+
3. Adverse effects of drought on birch	+/-	-	+/-

7.4. IMPLICATIONS FOR BIRCH

That two common pest species of birch such as *E. betulae* and *M. betulae* should have received relatively little attention in the past partly reflects their non-lethal effects and the low commercial value of birch in most countries. Recently however, attention has focused on birch woodland as an important habitat in terms of wildlife biodiversity, especially in upland areas of Britain (Patterson 1993; Humphrey, Holl & Broome 1998) and there is even some interest in the commercial exploitation of birch (Lorrain-Smith & Worrell 1991; Seaman 1994). Successful management of birch woodlands, will depend amongst other things, on a better understanding of the pest species which exploit birch and may reduce plant health (Humphrey *et al.* 1998).

Heavy infestations of aphids are known to reduce tree growth by placing a nutrient drain on the tree (Day *et al.* 1998; Dixon 1998). Despite this, many of the insects that exploit

birch, such as *E. betulae,* have been generally regarded as having little impact on plant health (Hajek 1984), but this study demonstrates that some organisms that exploit birch may act in tandem and potentially be more harmful than hitherto recognised. That *M. betulae* lesions are often surrounded by 'green islands' which arise through fungal inhibition of leaf senescence, suggests that translocation of nutrients out of fungal infected leaves is largely a plant rather than a fungal initiated response. *E. betulae* exploits this movement of nutrients in the phloem, and consequently the plant retrieves less from infected leaves than it otherwise would. This might not immediately affect plant health, but could have a cumulative effect over several seasons. Because *E. betulae* reproduces more when feeding on infected leaves, widespread *M. betulae* infection could lead to large assemblages of *E. betulae* on birch. In addition to fungal infected leaves, *E. betulae* also feeds on actively growing stems and leaves where it takes advantage of the higher fluxes of phloem sap being transported to developing tissues. If large assemblages of *E. betulae* removed nutrients destined for growth, this could seriously hinder birches otherwise rapid growth rate; the main advantage for the use of birch in silviculture.

Chapter 4 demonstrated that some individual birch trees bore consistently higher infections of *M. betulae* than others at Dalhaikie Flat in 1999 and 2000. Individual plants often vary in their phenotypic responses to pathogens because of genotypic differences, the virulence of the pathogen and biotic or abiotic influences or interactions of any these factors (Helander *et al.* 1998). Birch genotypes, for instance, were seen to vary in their susceptibility to infection by birch rust, *Melamsporidium betulinum* (Helander *et al.* 1998), so it is conceivable that some individual trees may be intrinsically more susceptible to *M. betulae* infection than others. There is a strong positive association between *E. betulae* and leaves infected with *M. betulae* at Dalhaikie Flat (Chapter 5), so some individual trees may be predisposed to *E. betulae* infestation because of their susceptibility to *M. betulae*. Lappalainen, Helander & Palokangas (1995) suggested that some individual birch trees were less likely to be defoliated by the autumnal moth, *Epirrita autumnata*, because the trees were more susceptible to infection by *M. betulinum*, which reduced leaf quality and so caused reduced insect performance. Whilst the negative interaction between *M. betulinum* and *E. autumnata* made it less likely that birch genotypes would be exploited by both *M. betulinum* or *E. autumnata*, the positive impact of *M. betulae* infection on *E. betulae* may

cause some genotypes to be vulnerable to both pathogen infection and aphid infestation. There is good evidence that those trees that were heavily infected with the fungal pathogen, *M. betulae*, in 2000 were also the most heavily infested with *E. betulae* (Figure 7.1) ($r_s = 0.85$, $P < 0.0001$), so there is a distinct possibility that some birch genotypes may be susceptible to both fungal attack and insect herbivory because of this indirect interaction. This could potentially have implications for the genotypic diversity of natural birch populations, and hence could have wider impacts on floral and faunal biodiversity that is influenced by that genetic diversity (Humphrey *et al.* 1998). Section 7.6 of this Chapter discusses why some plants that are attacked by pathogens may also have their anti-herbivore defences compromised.

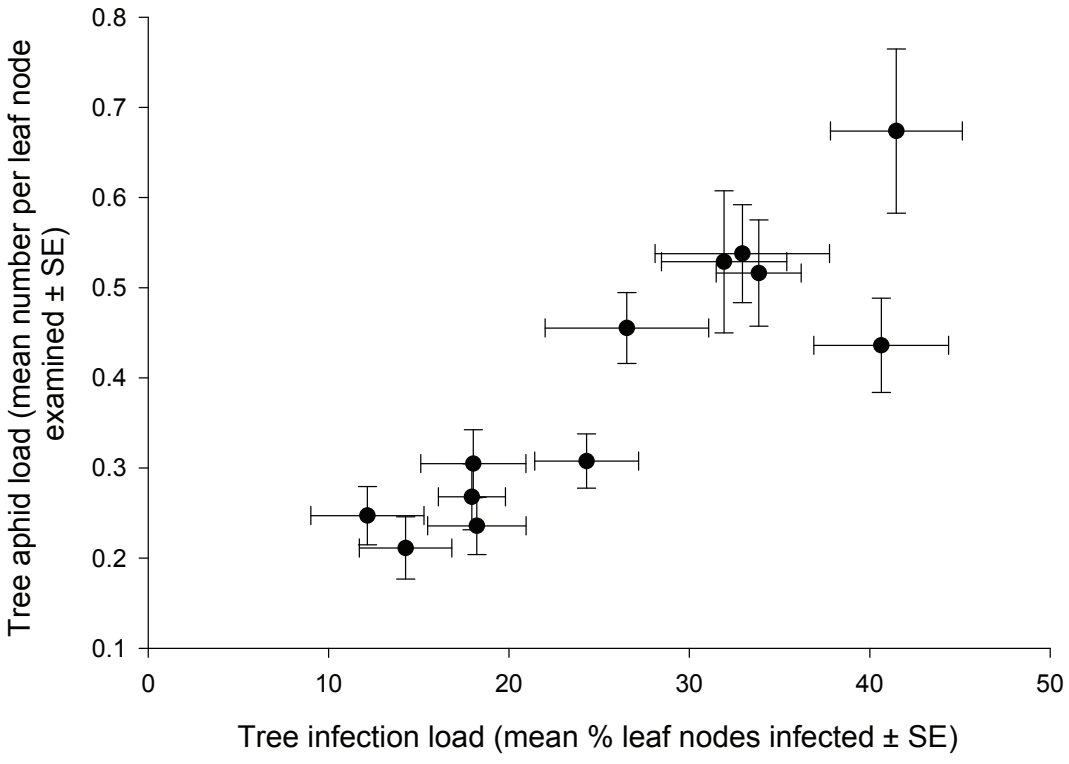


Figure 7.1. Fungal infection and aphid infestation on trees during 2000.

Treatment of fungal disease in trees is dependent on the extent of the damage caused by the pathogen and the whether the damage warrants costly and time-consuming applications of fungicides or altered nursery practice (Strouts & Winter 1994). Such assessment often depends on the 'economic injury level' (EIL) or 'control action threshold' (CAT) that defines the level beyond which treatment would result in dividends above and beyond what the treatment costs, in other words, the degree of disease severity at which treatment becomes viable (Begon, Harper & Townsend 1996). Because birch leaf-spot diseases have traditionally been viewed as having very little impact on plant health (Strouts & Winter 1994), the EIL and CAT would be high and infection would have to be chronic before use of fungicides were warranted. The findings presented in this thesis demonstrate that impact of *M. betulae* infection on birch go beyond the effects that it is directly responsible for because it indirectly promotes the performance of a second pest of birch, *E. betulae*. This could lead to a severe overestimate in the degree of *M. betulae* infection that can be tolerated by birch before serious impacts on growth occur, since the interactive effects of *E. betulae* would not be accounted for. Therefore, any meaningful and coordinated attempt to control birch disease should consider the additional impacts of phytophagous insects, since they may either exacerbate (*e.g.* Chapter 4 & 5) or ameliorate (*e.g.* Lappalainen, Helander & Palokangas 1995) the net effects on plant health.

7.5. PLANT-MEDIATED INSECT COMPETITION

Many textbooks refer to indirect interactions as 'unexpected interactions' (*e.g.* Begon *et al.* 1996) which betrays a fundamental, but often forgotten aspect of these interactions; that they regularly go unnoticed because they arise through unanticipated mechanisms. The indirect competitive effect of *Eriocrania* on *E. betulae* reported in Chapter 6 demonstrates this amply. The higher phenolic compound concentrations of mined leaves might have initially appeared to be the mechanism behind the competitive effect had not higher phenolic concentrations in fungal infected (naturally or artificially inoculated – Chapters 4 and 5) and artificially damaged leaves (Chapter 6) been seen to have no discernible effect on *E. betulae* performance. Only when different regions of the leaf were damaged artificially was midrib severance, and the likely impediment to phloem hydraulics, revealed as the more probable mechanism underpinning the interaction.

Whether damage to the midrib by *Eriocrania* was a trait that arose to ameliorate the host

leaf for itself, or else make a resource unusable to a potential competitor may be difficult to know for certain. The findings reported in Chapter 6 do not refute the possibility that damage to the midrib is purely incidental and that *Eriocrania* does not secure any tangible benefits by damaging it, but this is inconsequential in the context of plant-mediated interactions between phytophagous insects. The fact that this modification occurs in a significant number of *Eriocrania* mined leaves demonstrates that this competitive interaction can occur between two insect-herbivores of different feeding guilds, regardless of the origin of the mechanism. This highlights two features of plant-mediated indirect interactions between insects that could have potentially compounded their identification in the past; (1) the interaction may not be present throughout the entire species (*i.e.* only 33 % of *Eriocrania* interacted), and (2) the mechanism underpinning the interaction may be masked or correlated with incidental plant changes which have no effect (*i.e.* higher concentrations of phenolic compounds in mined leaves).

Rather than a influential regulator of aphid populations, this interaction is presented as a mechanistic example of a plant-mediated insect interaction that is based purely on structural modification to plant tissues. It is quite possible however, that higher densities of *Eriocrania* could limit the number of suitable leaves for large assemblages of *E. betulae* that occur during mining activity in spring. Not all leaves in the canopy are suitable for *E. betulae* to feed on, so this smaller subset of leaves could be further reduced by *Eriocrania* during a time when *E. betulae* growth, development and feeding rates are at their maximum.

7.6. PLANT RESPONSES TO FUNGI AND INSECT HERBIVORES: DEFENCE AGAINST MULTIPLE ENEMIES

Marssonina betulae and *Eriocrania* were associated with compositional changes to leaf chemistry. Most attention in this study focussed on the nutritional and physical changes they respectively brought about in birch leaves, since these affected *E. betulae* most, but both *M. betulae* and *Eriocrania* were also associated with higher concentrations of phenolic compounds which are known to be implicated in plant defence against both phytopathogens and insect herbivores (Schultz, Hunter & Appel 1992). An emerging area of research concerns how the plant defence pathways induced by both phytophagous insects and plant pathogens can interact with one another, namely the issues of 'cross-talk'

and signal conflicts/synergies (Bostock 1999; Bostock *et al.* 2001). The possibility of interactions between signalling pathways is especially interesting, since this illustrates how defence pathways induced by insect herbivores can sometimes suppress defensive pathways that respond to pathogen damage and *vice versa* (Felton *et al.* 1999; Thaler *et al.* 1999; Hunter 2000). Whilst this is just one aspect of signalling pathway interactions, several authors have demonstrated that the two signalling pathways that tend to be triggered by either insects or pathogens, the jasmonic acid and salicylic acid pathways respectively, can be inhibitory towards one another (Bostock 1999; Felton *et al.* 1999; Thaler *et al.* 1999), though see Stout *et al.* (1999). If this suppression occurs between defensive pathways it may cause some plants that are attacked by phytophagous insects to become more vulnerable to pathogen attack and equally that diseased plants may become susceptible to insect herbivory. That some birch trees were more susceptible (and by implication some were more resistant) to fungal infection and aphid infestation in this study (see section 7.4) is at least compatible with signal suppression occurring between defensive responses to fungal attack and the defensive response to aphids, effectively making it easier for aphids to feed on fungal infected leaves.

In contrast, Bostock (1999) reported that induction of plant defences by certain aphids was closer to the (pathogen inducing) salicylic acid pathway than jasmonic acid pathways more usually associated with insect herbivory. In effect, the pathogen and aphid might induce a defensive response in plants that could operate against either organism. If the higher concentrations of phenolic compounds observed in fungal inoculated leaves were indeed a defensive response against fungal attack, their effects on aphids were clearly outweighed by the increases in phloem nutritional quality. Furthermore, there was no evidence of solitary *E. betulae* eliciting a phenolic response in birch, since fungus-free leaves with aphids caged upon them contained phenolic compound concentrations that were not significantly different from surrounding leaves that were aphid and fungus free.

A simplistic model of discernible and directly opposable herbivore and pathogen pathways is probably unrealistic. Modification of plant chemistry, especially nutritional quality, may ameliorate, exacerbate or conceal the effects of defensive pathway inhibition by one primary consumer on another. Nevertheless, the system used in this study could be a good example for identifying defensive signal interactions for three reasons (but see

Section 7.7.2):

(a) there was an increase in phenolic compound concentrations in fungal inoculated leaves - traditionally a good indication of a defensive response in birch;
(b) aphids fed on fungal infected leaves with impunity;
(c) aphid performance was demonstrably higher when feeding on infected leaves.

7.7. FUTURE WORK AND CONCLUDING REMARKS

There is growing interest in how indirect interactions between species contribute to the structure of complex ecological communities (Fox & Olsen 2000; Wootton 2001), together with a new appreciation of their relevance to applied issues. Understanding the mechanisms and processes that underpin indirect interactions is likely, therefore, to preoccupy ecologists for many years to come. The growing catalogue of empirical studies alluded to throughout this thesis, together with modelling and integrative approaches of (Wootton 1994b,c 2001) and others, have demonstrated our understanding of indirect interactions is increasing, but there are still many unanswered questions. With better understanding of the processes governing indirect interactions, it may become clearer to what extent community interactions can be broken down into their component parts and whether it is possible to predict the consequences of anthropogenic impacts on the environment.

The indirect interactions studied in this research have raised several promising questions, which would be interesting to explore further. The first pair of questions relate to the responses shown by *E. betulae* to indirect impacts, whilst the second pair concern the fungi – plant – aphid interaction specifically.

7.7.1. Can an insect species be useful as a model for testing the effects of environmental change on insect-plant interactions?

Studying these indirect interactions was undoubtedly aided by the choice of *E. betulae* as the focal species of these interactions, the specific advantages of which are outlined in Section 1.1. of Chapter 1. Predicting how environmental change may affect phytophagous insects is reliant on a good understanding of the direct and the less easily detected indirect impacts (*e.g.* plant-mediated effects) that it could cause. *E. betulae* would appear to be a

strong candidate for unravelling such interactions, since it appears to respond rapidly and in a measurable way to changes in its host plant, *Betula pendula*. Indeed, *E. betulae* has already been used to test the indirect effects of pollutants on insect assemblages; responding positively to both aboveground (simulated acid rain) and belowground (nitrate and ammonium deposition) pollutant impacts (Neuvonen & Lindgren 1987; Port *et al.* 1995). Furthermore, *Betula*-herbivore systems are comparatively well understood, which could help to identify the mechanistic basis for any effects observed.

7.7.2 Why do some aphids appear to be insusceptible to elevated levels of phenolic compounds in leaves?

Unlike many other phytophagous insects of birch (*e.g.* Bergelson, Fowler & Hartley 1986; Fowler & Macgarvin 1986; Hartley & Lawton 1987; Valladares & Hartley 1994), *E. betulae* performance was not depressed when it was reared on leaves with elevated phenolic compound concentrations, regardless of whether they arose from fungal inoculation, leaf-mining activity or artificial damage. There are several possible reasons for this trend, including the potentially lower concentrations of phenolic compounds in the phloem sap and the fact that phloem feeding circumvents the effects of many digestion-inhibiting phenolic compounds. All of the examples mentioned above, for example, are leaf-chewers, so phloem-feeders may generally be less adversely affected (*e.g.* Martin, Cappuccino & Ducharme 1994).

Another possibility mooted in Section 7.6, is that attack by a pathogen may result in a plant response that inhibits subsequent defensive response to insect herbivory, via interactions between signalling pathways. The main problem with this idea is that *E. betulae* appeared to insusceptible to the elevated phenolic compound concentrations associated with *both* pathogen and herbivore damaged leaves. Improved identification of the specific phenolic compounds associated with damaged birch leaves (*e.g.* Ossipova *et al.* 2001; Ossipov *et al.* 2001) and a better understanding of the individual signalling pathways involved (*e.g.* Paul, Hatcher & Taylor 2000) would help to explain why *E. betulae* appeared to be less adversely affected. A more integrative approach using ecological and molecular techniques, advocated by Hunter (2000) and Paul *et al.* (2000), might also give some

suggestion as to whether overt insusceptibility was common amongst phloem feeding insects.

7.7.3. Could microbial impacts render previously unsuitable plants more suitable to insect herbivores and expand plant range?

As well as possibly making some plants unsuitable to certain insect herbivores via elevated concentrations of phenolic compounds, *M. betulae* brought about changes to the nutritional quality of the plant that could potentially render leaves more suitable for insect herbivores that might not normally be able to feed on *B. pendula*. An example of microbial modification of plant suitability is illustrated by the leafhopper *Dalbulus maidis* which feeds on maize and teosnite but cannot usually feed on asters, except when they are infected with yellows mycoplasma-like organism (MLO) (Purcell & Nault 1991).

As was mentioned in Chapters 2 and 3, *Euceraphis betulae* is closely related to a sister species, *Euceraphis punctipennis* - the two only being distinguished within the last 30 years (Blackman 1976, 1977). One of the differences observed was that *E. betulae* appeared to feed entirely on *B. pendula* and *E. punctipennis* on *B. pubescens* (Blackman 1977; Mahdi & Whittaker 1993). Whilst the physiological and behavioural mechanisms for this distinction have not been rigorously tested, some authors suggest that the two aphid species are incapable of feeding on each other's host plant species (Hopkins 1996). Given the relatedness of these two aphid species, it is reasonable to assume that any physiological and behavioural barriers that may restrict each aphid to a species of birch are weaker than for two distantly related phytophagous insects. As *M. betulae* infects both *B. pendula* and *B. pubescens*, it is intriguing whether the tangible effects of *M. betulae* on the nutritional quality of birch leaves could be sufficiently influential to make both birch species equally suitable to both aphid species. *E. punctipennis* appears to be limited by the geographical range of *B. pubescens*, which unlike *B. pendula*, has not been planted beyond its natural range (Blackman 1977), so any broadening of this aphids host specificity could allow it to significantly extend its geographical range. Microbial influences on plant chemistry could effectively cause new plant phenotypes to arise more rapidly than through evolutionary changes in plant populations (Clay 1996).

7.7.4. Could aphids become vectors as well as beneficiaries of plant diseases?

Because the fitness of *E. betulae* increases when it feeds on birch leaves infected with *M. betulae*, there may be a selective advantage for a closer relationship between the species (Wootton 1994a), for example if *E. betulae* were to become a vector of the disease. There are many examples of mutualistic relationships evolving between insects and plant-fungi (*e.g.* Schultz 1999), including pathogen dissemination by insects (Webber & Gibbs 1989), so it is intriguing why no such relationship has evolved between *E. betulae* and *M. betulae*. There may be a physiological barrier that prevents fungal spores being vectored on *E. betulae*, but such hurdles have been frequently overcome in other species via subtle physiological adaptation over evolutionary time (Barbosa, Krischik & Jones 1991). Another reason might be that the advantage to a virulent fungal pathogen like *M. betulae* in a vector-pathogen relationship is doubtful, since horizontal transmission is achieved by the huge volume of spores released into the atmosphere, and whilst vectoring by *E. betulae* might reduce this reproductive investment, the benefits might not be significant enough to abandon the virulent and successful transmission achieved by inanimate means.

7.7.5. Concluding remarks

The findings presented in this thesis illustrate two mechanistic indirect interactions that could also effect many other phytophagous insects. It also demonstrates two important components of indirect interactions; (a) the potential magnitude of their effects and (b) their capacity to arise though unexpected mechanisms. The positive impact of the fungal pathogen, *M. betulae*, on the birch aphid, *E. betulae*, demonstrates the potential importance of indirect effects on insect performance and abundance. As well as increasing population growth, there was evidence for indirect fungal effects on genetically hardwired traits in insects (reproductive diapause) and also modification of the aphid's spatial distribution on its host plant. The negative indirect impacts of the leaf-miner *Eriocrania* on *Euceraphis betulae* are no less dramatic in the sense that they cause high aphid mortality when the leaf-miner damages the midrib, but more importantly this interaction demonstrates that indirect interactions often arise through unanticipated mechanisms that are probably more commonplace than hitherto recognised.

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