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Physiological, ecological and environmental factors that predispose trees, stands and landscapes to infestation by tree-killing *Dendroctonus* beetles

by

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To Frauke Godlinski: Thank you for convincing me to become a scientist.

Abstract

In the last century the frequency and severity of outbreaks of tree-killing *Dendroctonus* beetles (Coleoptera: Curculionidae) have increased. Small-scale drivers within trees likely drive outbreak dynamics across landscapes. At a small scale, variation in carbohydrate availability within the stems of lodgepole pines (*Pinus contorta* var. *latifolia*) impacts the fungal symbionts of the mountain pine beetle (*D. ponderosae* Hopkins). I found that, during the growing season, carbohydrates were less available in the lower stems of pines than in their upper stems. After inoculation with a fungal symbiont of the mountain pine beetle however, trees mobilized carbohydrates to lesion fronts regardless of inoculation height along the stem. Interestingly, lesions that formed in response to fungal inoculation were larger in the lower portion of the stem than in the upper stem, likely due to due to lower initial concentrations of carbohydrates available to fund responses to fungal attack.

I evaluated the consequences of common silvicultural treatments in stands attacked by bark beetles and found that small-scale interactions remained important in these systems. Fertilization reduced carbohydrate reserves in the

roots of lodgepole pine trees by promoting tree growth. As trees use carbohydrate reserves to fund defensive responses, fertilized trees may therefore exhibit weakened defenses against bark beetle attack. In a separate experiment I found that fertilization increased beetle survival in bolts that overwintered in the Crowsnest Pass —an effect that was mediated by their fungal symbionts.

In a landscape-scale analysis of a 30-year dataset, I found no evidence that defoliation by a lepidopteran (*Choristoneura biennis* Freeman) facilitates local spruce beetle (*D. rufipennis* Kirby) outbreaks in British Columbia. Thus, small-scale characteristics of bark beetle biology undoubtedly impact their populations whereas I was unable to confirm the importance of landscape-scale ecological interactions.

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I hope to continue interacting with as many of you as possible as I continue my research.

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Chapter 1

Thesis Introduction

Despite research on bark-beetle management that has accrued since the early nineteenth century, devastating bark beetle outbreaks continue to impact large regions of forest in North America and Europe. Outbreak species of *Dendroctonus* bark beetle (Coleoptera: Curculionidae) in North American forests include the mountain pine beetle (*D. ponderosae* Hopkins), the spruce beetle (*D. rufipennis* Kirby), and the southern pine beetle (*D. frontalis* Zimmerman). Two recent outbreaks of mountain pine beetle, one in the early nineteen eighties and the current outbreak which began in the nineties, have destroyed large forested areas in British Columbia and Alberta (Taylor and Carroll, 2004). The current mountain pine beetle outbreak, which has spread from its epicentre in British Columbia into Alberta, is the largest on record (Kurz et al., 2008). Spruce beetles have undergone large outbreaks in the Kenai peninsula in Alaska, and Kluane National Park in the Yukon as well as in the mountainous Western regions of British Columbia (Berg et al., 2006). Large outbreaks of the southern pine beetle in the southeastern United States have occurred in the seventies, eighties and nineties (Price et al., 1998). Although we know that more frequent and severe bark-beetle epidemics can be caused in

part by shortened life cycles caused by climate warming (Bentz et al., 2010), large areas of susceptible forests due to fire suppression (Taylor and Carroll, 2004), and homogeneity in forested landscapes (Raffa et al., 2008), we remain unable to manage bark beetle populations to prevent large outbreaks.

Dendroctonus females initiate the attacks on host conifers by boring through the outer bark and entering the phloem of living trees. As they excavate, they inoculate Ophiostomatoid fungal symbionts, and emit aggregation pheromones to attract males to the entrance holes (Byers et al., 1988). Males also emit aggregation pheromones until enough beetles have arrived and the tree succumbs (Raffa and Berryman, 1983). It is this aggregation mechanism and their ability to exploit synergistic reactions between host defense chemicals and with their own pheromones which permits *Dendroctonus* beetles to so efficiently kill their host trees (Raffa and Berryman, 1983). Beetles mate and female beetles construct maternal galleries where they lay eggs. Hatched larvae feed on phloem that is infected by fungal symbionts as they excavate larval galleries perpendicular to the maternal gallery (Raffa et al., 2008). By consuming phloem colonized by their fungal symbionts, bark beetles obtain important nutritional benefits that may enable faster development and higher survival of brood (Barras, 1973; Ayres et al., 2000; Cook et al., 2010). Understanding interactions between bark-beetles and their host conifers requires the integration of microscopic and macroscopic factors influencing the system. Microscopic ecological interactions include the emission of semiochemicals by trees and beetles as well as bark-beetle-fungal symbioses. These factors relate to macroscopic determinants such as environmental and climatic conditions, which together impact both trees and bark-beetle populations and result in large-scale patterns in

bark-beetle epidemiology across forested landscapes (Raffa et al., 2008).

One impediment that hinders management of bark beetle populations is our ignorance of cross-scale drivers of outbreak dynamics (Raffa et al., 2008) whereby changes at a small scale can accumulate in a non-linear way leading to disproportionate effects at a larger scale. For example, Rykiel et al. (1988) reported that lightning strikes of individual loblolly pine trees (*Pinus taeda*) may decrease their resistance to southern pine beetles. Trees struck by lightning are an ephemeral resource providing suitable habitat immediately after the lightning strike and enabling higher reproductive rates in southern pine beetle populations. Therefore, they are able to successfully colonize nearby healthy trees due to their increased ability to attack in aggregate. This leads to pockets of infestation on the landscape which, under epidemic conditions, eventually coalesce into continuous expanses of beetle-killed trees (Rykiel et al., 1988). Thus, the fecundity of bark beetles in weakened trees may enable them to colonize landscapes full of vigorously growing trees in ways that are difficult to predict if the system is only considered at a large scale.

We know that such cross-scale drivers of population irruptions exist but they are difficult to study due to the inherent challenges involved in synthesizing results at different scales. However, by choosing to ignore variation across spatial scales, we homogenize our understanding and lose information. Moreover, we risk not detecting cross-scale drivers of insect epidemics, thereby precluding synthesis that would otherwise prove useful for minimizing future outbreaks. In this thesis, I describe very small-scale variation in carbohydrate availability and defense within trees but I also investigate the ramifications of ecological interactions and management at a stand and landscape level. I

examine the impact of fertilization and thinning on tree-level vigour before focusing again on small scale impacts of fertilization on mountain pine beetle development and survival within individual beetle galleries. Finally, I consider the macroecology of positive interactions between a defoliator and a bark beetle and how interactions might impact bark beetle epidemiology on a landscape level.

1.1 Spatial variation in defense along tree stems

Long branch-free stems predominate in mature pine forests as trees compete for light by growing vertically, with crowns only at the top of their stems. If vulnerability to bark beetles varies along these stems, evaluating vigor at a whole-tree level may obscure biologically important details. For example, spatial variation in defense investment along tree stems may lead to chinks in their armour, enabling bark beetles to attack their weakest points. Carbohydrates fund many processes in trees including maintenance, growth, and the production of defense structures and chemicals (Chapin et al., 1990). Therefore, measuring carbohydrate availability along tree stems may reveal weaknesses and disclose the health of tissues in different regions along the stem. Growth, maintenance and defense sinks nearer to the crown likely withdraw from the downward flowing carbohydrate stream before sinks lower along the stem. Therefore, based on a conceptual model proposed by Landhäusser and Lieffers (2012), we hypothesized that the shape and structure of tree stems in mature forests leads to diminishing carbohydrate availability in the stem with distance from the crown. Monoterpenes are a class of carbon-based defense compounds that are important both for preformed constitutive defenses and

induced responses as they are a major component of tree resin. Moreover, monoterpenes are produced in response to invasion by the fungal symbionts of bark beetles (Raffa et al., 2005). Because monoterpene synthesis occurs in plastids (Chappell, 2002), where carbohydrates are stored, it likely depends on a ready supply of sugars. Therefore, the hypothesized gradients in carbohydrate availability may result in similar gradients in monoterpene production along the boles of conifers.

Carbohydrate mobilization to sinks depends on carbohydrate concentrations in source tissues (Minchin and Lacointe, 2005). Therefore, reduced carbohydrate availability in the lower stem may make it more susceptible to colonization by bark beetles and fungal pathogens than the upper stem. Indeed on a per area basis, bark beetle attacks are more numerous in the lower bole than in the upper bole (Cole and Amman, 1983). Once bark beetles have overcome the induced defenses of a host tree, parent beetles and their brood still have to contend with chemicals remaining in the phloem (Erbilgin et al., 2006). Therefore, gradients in chemical defenses along the stems of trees may help to explain why mountain pine beetle larvae are more abundant in the lower stem than in the upper stem (Cole and Amman, 1983). Two objectives motivated my study of spatial variation in carbohydrate availability and defense in the crownless section of tree stems. I wished to test whether variability in defense and resistance to colonization by fungal symbionts of the mountain pine beetle exist in lodgepole pine stems. My second objective was to relate variation in defense and vulnerability to the availability of carbohydrates along stems.

1.2 The effect of fertilization and thinning on carbohydrate reserves

To manage the diseases and pathogens that outbreak we need to decrease the scale at which we perceive the outbreak from the population level to the individual level. Thus we may treat individual stands or even individual trees in order to minimize the devastation caused by the mountain pine beetle across landscapes (Carroll et al., 2006). Facing the mountain pine beetle outbreaks in Western Canada and the United States in the seventies and eighties, forest managers considered thinning, and sometimes fertilization as methods to manage stand resistance to mountain pine beetles (Waring and Pitman, 1985; McGregor et al., 1987; Amman et al., 1988). Drastic thinning from below, can reduce the number of attacks in treated stands relative to in untreated stands if remaining trees are spaced four to five metres apart (Whitehead and Russo, 2005). Under these conditions tree resistance appears to be less important in protecting trees than stand habitability for mountain pine beetles (Whitehead and Russo, 2005). Increased solar radiation on the lower boles of trees may interfere with normal landing rates (Bartos and Amman, 1989) and increased wind flow through the stand may reduce beetle host finding abilities by dispersing pheromones (Geizler and Gara, 1978; Bartos and Amman, 1989).

In addition to increasing stand resistance to colonization, silvicultural treatments may increase the vigour of individual trees enabling a more rigorous defense against attack by bark beetles. Thus by fertilizing and thinning a stand, the resistance of many individual trees in that stand may be increased beyond the threshold necessary for the successful repulsion of a mountain pine beetle attack (Waring and Pitman, 1985). The authors argue that this hap-

pens on the basis of the carbon allocation hierarchy hypothesis (Waring and Schlesinger, 1985) which states that radial growth is a lower priority sink than carbohydrate storage and therefore rapid growth indicates that the tree has a rich supply of carbohydrate reserves available for defense. More recent work has suggested that some plants exhibit a trade-off between growth and defense when resources, including nutrients and light, are abundant (Herms and Mattson, 1992; Viiri et al., 1999). However, trees do not always exhibit a negative relationship between growth and defense (Wainhouse et al., 1998)

Obviously I am critical of the carbon allocation hierarchy hypothesis in light of my statement above that stem sinks, including sinks created by radial growth, likely consume carbohydrates *en route* to storage sites lower in the tree. If this counter-hypothesis is true, fertilization may in fact decrease the ability of individual trees to fend off mountain pine beetle attacks by encouraging growth and thereby depleting carbohydrate reserves. My objective in this study was to determine whether increased growth rates in the crown and stem of fertilized and thinned trees corresponds to increased root carbohydrate storage. I also evaluated several commonly used tree measurements, including the length of the crownless bole (height-to-live-crown), as predictors of carbohydrate reserves in the roots of fertilized and thinned trees as well as in untreated trees.

1.3 Mountain pine beetle symbioses and productivity in fertilized trees

Although increasing stand vigour by fertilization and thinning may minimize mountain pine beetle success in certain situations (Berryman, 1982; War-

ing and Pitman, 1985), ignoring temporal variation in outbreak dynamics may lead to management solutions that are disastrous when used out of context. The behaviour of bark beetle populations has been divided into four stages (Safranyik and Carroll, 2006): In their endemic stage beetle populations are relatively small and inhabit weakened trees. In their incipient-endemic stage, populations expand due to favourable conditions or local immigration until they reach their epidemic stage in which their impacts are evident on a landscape scale. During the subsequent post-epidemic stage, populations decline, usually due to adverse weather and host depletion. Factors that predispose trees to attack when bark beetle populations are in their endemic stage include drought and insect-induced stress, whereas in their epidemic stage bark beetles prefer large vigorous host trees (Safranyik and Carroll, 2006). This vigour paradox (Berryman, 1982) means that silvicultural strategies, such as fertilization and thinning, that maximize tree vigor and resistance in the endemic stage likely promote the growth of mountain pine beetle populations in the epidemic stage—especially if mountain pine beetles survive better in high nutrient phloem. Proponents of thinning to increase stand-level resistance to mountain pine beetle attack acknowledge that when population densities of beetles are high, no treatment can save stands (Whitehead and Russo, 2005) and therefore, these strategies are most useful if used to prevent populations from reaching epidemic levels. Fertilization of stands near Kenneth Creek, British Columbia, did not enable them to successfully avoid insect attack (Sanborn et al., 2011). In fact, fertilized stands were destroyed by mountain pine beetles which did not preferentially attack untreated trees nearby (Kathy Lewis, personal communication).

When their populations are large enough, bark beetles can successfully colonize even vigorous trees, and they likely benefit from consuming more nutritious phloem when they do (Berryman, 1982). Subcortical bark beetles feed on nutrient-poor food in comparison to folivorous insects and therefore, bark beetles may be sensitive to small increases in the nutritional quality of their food (Ayres et al., 2000). Symbiotic relationships wherein fungi vectored by bark beetles enhance the nutrition of the phloem for beetle brood (Barras, 1973; Ayres et al., 2000; Klepzig et al., 2001; Bleiker and Six, 2007; Cook et al., 2010) provide a mechanism whereby small-scale drivers may have macroscopic effects on bark beetle populations (Six and Klepzig, 2004).

If mountain pine beetles successfully colonize trees in stands that have been fertilized to increase stand vigor, improved phloem nutrition in fertilized trees may enable further population irruptions. Although this makes intuitive sense, it remains unconfirmed as no study has demonstrated that mountain pine beetles survive or reproduce better in phloem with high concentrations of nitrogen. Thus my primary objective was to determine whether mountain pine beetle survival would increase if they were reared in higher nutrient phloem. Because the ecology and population dynamics of mountain pine beetles is linked to how well their fungal symbionts perform (Six and Klepzig, 2004), I also wished to determine whether the fungal associates of mountain pine beetle colonize more area and concentrate more nitrogen in high nutrient phloem than low nutrient phloem. A secondary objective was to determine whether improved phloem nutrition might boost the fat reserves in mountain pine beetle larvae and improve their overwinter survival in the more northerly regions of their range.

1.4 Insect interactions on a landscape scale

At a tree-level, defoliation by lepidopterans likely depletes energy reserves available for defense rendering trees vulnerable to attack by bark beetles (Wright et al., 1984; Bowers et al., 1996; Raffa et al., 1998). This may enable bark beetles to overcome thresholds that normally restrict their ability to colonize host trees thereby helping them to reach epidemic population levels (Raffa et al., 2008). In this case we should see evidence of tree level interactions between defoliators and bark beetles at a landscape scale.

A defoliator, *Choristoneura biennis* Freeman (Lepidoptera: Tortricidae) and a tree-killing bark beetle, *Dendroctonus rufipennis* Kirby are two outbreak insect species in British Columbia (BC) with overlapping ranges. Over seven million m^3 of lumber from 1974 to 1999 were lost in BC due to mortality caused by *D. rufipennis* (Holsten et al., 1999). Although defoliation by *C. biennis* generally does not kill trees unless they are defoliated several years in a row, this insect causes top-kill, and loss of timber volume in large-scale periodic outbreaks (Zhang and Alfaro, 2002). Both insects share Engelmann spruce (*Picea engelmanni* Parry) and interior spruce (*Picea engelmanni* $\times$ *Picea glauca* Moench Voss) host trees.

Maps of historical outbreaks of *C. biennis* and *D. rufipennis*, which have been archived by the British Columbia Ministry of Forests, Lands and Natural Resource Operations and the Canadian Forest Service, may contain information on interactions between these insects. However, due to the variability in the dynamics of each insect through time and space, it is difficult to determine whether outbreaks of *C. biennis* may predispose stands to outbreaks of *D. rufipennis* by visual inspection alone. My objective in studying these two

insects was to use spatiotemporal logistic regressions to determine whether defoliation by *C. biennis* promotes establishment by *D. rufipennis* in their overlapping ranges. However, pre-outbreak predictors of population dynamics are often no longer predictive during outbreaks as density dependent effects take over as the dominant drivers of outbreak dynamics (Raffa et al., 2008). Therefore, another objective of this research was to determine whether interactions with *C. biennis* may be more important determinants of *D. rufipennis* infestations when local outbreaks of *D. rufipennis* are building than when they are declining.

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Chapter 2

Variation in carbon availability, defense chemistry and susceptibility to fungal invasion along the stems of mature trees

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A version of this chapter is in press: Goodsman, D.W., Lusebrink, I., Landhäusser, S.M., Erbilgin, N., Lieffers, V.J. In Press. Variation in carbon availability, defense chemistry and susceptibility to fungal invasion along the stems of mature trees. *New Phytologist.*

2.1 Introduction

Bark beetles that kill healthy mature trees when their populations are sufficiently large include the mountain pine beetle, *Dendroctonus ponderosae* Hopkins, the southern pine beetle, *Dendroctonus frontalis* Zimmerman, and the European spruce bark beetle, *Ips typographus* Linnaeus (Franceschi et al., 2005). These beetles kill mature trees by attacking their stems, yet with the

exception of one study on *Pinus ponderosa*, which reported resin exudation at two heights (Kolb et al., 2006), researchers have not investigated whether defenses vary along tree stems.

Carbohydrate reserves fund many processes which act as carbon sinks in trees including growth and the production of defense chemicals. Thus, carbohydrate reserves, which comprise primarily sugars and starch (Chapin et al., 1990), are converted to soluble sugars and transported to carbon sinks throughout the tree. Before arriving in the roots, soluble sugars produced in the crown must descend the stem which is itself a carbon sink, especially during the period of wood growth (Vose and Ryan, 2002). Radial ring growth and respiration sinks extract soluble sugars from the phloem as they are transported down from the crown (Sevanto et al., 2003). This is likely the reason why the reserves in the roots of mature aspen trees that were defoliated by forest tent caterpillar (*Malacasoma disstria* Hübner) recovered slower than the reserves in the woody parts of the crown (Landhäusser and Lieffers, 2012). Moreover, sinks in tree stems likely also explain negative correlations between the length of lodgepole pine stems and the quantity of carbohydrate reserves in their roots (Goodsman et al., 2010). If a tree is attacked mid-stem, the resources used to fund the local defensive response originate in the crown and therefore must have been previously transported down the stem.

Although carbohydrate transport in stems is recognized as an important factor impacting defense (Christiansen et al., 1987), the mechanics of long distance carbohydrate transport are still in dispute. There is disagreement in the literature about whether the pressure-induced flow mechanisms originally proposed by Münch (1930) can adequately describe carbohydrate transport

in long stems (Thompson, 2006; Jensen et al., 2012). Thompson (2006) contends that the dual function of the sieve element-companion cell complexes, which includes both long distance transport and lateral movement of carbohydrates to surrounding tissues, is not accounted for in the original Münch hypothesis. Apoplastic unloading is an example of lateral movement which makes phloem transport conduits appear to leak (Thompson, 2006). Leaky phloem transport in apple seedlings provides a mechanism that may explain the existence of carbohydrate reserves in the stem phloem of trees, as well as how they can be remobilized (McQueen et al., 2005). While the literature on long distance phloem transport is unsettled, we know still less about how the concepts outlined above relate to carbon availability and defense in the stems of large trees.

Conifers use carbohydrate reserves located within their stems to fund defense reactions there (Guérard et al., 2007), but it is unknown how carbohydrate reserves are allocated to attack zones at different locations along the length of the stem. Although sink/source relationships are typically used to explain the impacts of defoliation on defense within tree crowns (Honkanen et al., 1999), they are also relevant along tree stems because they explain variation in defense based on rules that govern how carbon sinks access carbon resources (Honkanen and Haukioja, 1998). The most explicit rule of this framework is that strong growth sinks lead to high sugar influx in growing tissues resulting in strong defensive responses due to high carbohydrate availability. Thus sink tissues in the crown that are growing rapidly are highly inducible because of high sugar influx rates and varying sink strength and sink priority lead to spatial and temporal variation in suitability for herbivores within plants

(Honkanen and Haukioja, 1998). One limitation of the sink/source framework as described by Honkanen and Haukioja (1998), however, is that it is based on competing modular sinks. To my knowledge it has not been extended to include non-modular plant organs such as tree stems. I contend that the sink/source framework is equally useful in this context provided the concept of discrete competing modules is replaced by a vertical continuum of competing sinks along the length of the stem. As sinks in the stem likely reduce downstream carbohydrate availability, we hypothesized that long crownless stems would create vertical hierarchies in chemical defenses dependent on distance from the crown.

The current North American mountain pine beetle outbreak motivated our study of vulnerability along the stems of mature pine trees. The mountain pine beetle is one of the most destructive pests in North American forests due to its ability to use aggregation pheromones to mass-attack and kill healthy pines (Safranyik and Carroll, 2006). After landing on a suitable host, beetles bore through the outer bark and inoculate the phloem and xylem with symbiotic fungi, including *Grosmannia clavigera* (Rob.-Jeffr. & R.W. Davidson) Zipfel, Z.W. de Beer & M.J. Wingf., *Leptographium longiclavatum* Lee S., J. J. Kim, & C. Breuil, and *Ophiostoma montiu*m (Rumbold) von Arx (Six, 2003). Although constitutive defenses in the phloem are the first obstacles encountered by invading bark beetles, pine trees also respond to attack by synthesizing defense chemicals, pitching out beetles with resin, and by forming resin-filled lesions to quarantine and kill the beetles and their fungi (Franceschi et al., 2005). Shorter lesions are believed to indicate more efficient defenses (Bonello and Blodgett, 2003; Bonello et al., 2006; Krokene et al., 2008). Monoterpene

concentrations in the phloem are a relevant measure of defense in this context as they are a major component of tree resin, and pine trees produce them when responding to invasion by bark beetles and their fungal symbionts (Raffa et al., 2005). The objectives of our study were to test whether gradients in defense and vulnerability to fungal attack exist in lodgepole pine stems, and to relate these to local carbohydrate availability and distance from carbon sources. We quantified defense by measuring monoterpene concentrations in the phloem and vulnerability by measuring lesion lengths formed by trees after they were inoculated with *G. clavigera*.

2.2 Materials and Methods

Individual trees were selected in three pure fire-origin lodgepole pine stands that were 55 years old, had similar stand density and productivity as measured by site index (height over age), and with similar understory composition. Stands were located south of Hinton, Alberta, Canada: Stand 1 was located at N 53.233, W 117.360; stand 2 at N 53.224, W 117.348; stand 3 at N 53.227 W 117.355 (WGS 84 Map datum). In each of the three stands, 12 dominant and 12 intermediate lodgepole pine trees were selected. Dominant trees were 15 m tall on average, and had crowns which were partially above those of neighbouring trees. The crowns of dominant trees received full sunlight throughout most of the day. Intermediate trees had crowns with tops below the general canopy, but they still received direct light from above. The mean diameter at breast height (1.3 m) of dominant trees was 16.85 cm while the mean diameter at breast height of intermediate trees was 9.19 cm. We included both dominant and intermediate trees in our study because mountain pine beetles prefer

larger diameter dominant trees when their populations are high (Safranyik and Carroll, 2006). Experimental trees were separated by a minimum of 30 m (approximately two tree lengths) to minimize non-independence among trees. They had symmetrical crowns, and stems free of visible signs of damage or disease. The lodgepole pine trees in these stands were well suited to this experiment as we were able to access the full length of their crownless stems using a 13 m extension ladder stabilized with guy lines.

In mid-July 2010, we randomly assigned three dominant and three intermediate trees at each site to one of four treatments such that each site had a total of 12 dominant and 12 intermediate trees assigned to treatments. The four treatments were (1) inoculation near the base of the live crown; (2) inoculation at a height of 1.3 m; (3) inoculation halfway between the base of the live crown and 1.3 m, and (4) no inoculation. We chose to inoculate at the base of the live crown because the direction of carbohydrate mobilization below the crown would presumably be downward towards sinks in the stem and roots whereas carbohydrates can be moved upwards within the crown. We also inoculated at 1.3 m above the ground because this is a standard sampling location in trees, and the location where most fungal-inoculation studies insert inoculums. Having an inoculation point between the base of the crown and 1.3 m permitted us to detect gradients along the stem. Throughout the current study we refer to the inoculation near the base of the live crown as the top inoculation; the inoculation between the base of the crown and 1.3 m as the middle inoculation and the inoculation at 1.3 m as the bottom inoculation (Fig. 2.1a). Trees that were not inoculated served as a baseline for carbohydrate levels at each height to be compared to the fungus-inoculated trees at

the end of the study. The *G. clavigera* that was inoculated was isolated from living mountain pine beetle larvae as described in Goodsman et al. (2012) and grown on malt extract agar (1.5 % agar and 1.5 % malt extract by volume) at room temperature (21 ° C).

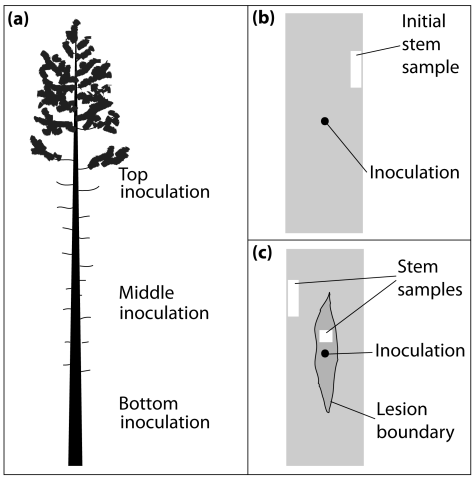

Figure 2.1: Sampling and inoculation methods showing a) the location of inoculations along the crownless stem of a lodgepole pine tree, b) the location of the initial stem sample with respect to the inoculation point and c) the location of the late-August stem samples with respect to the lesion that formed in response to fungal inoculation. At each inoculation height (Top, middle and bottom) trees were inoculated twice such that inoculations were on opposite sides of the stem.

To inoculate with *G. clavigera* we bored a hole through the phloem and into the first layer of the xylem using a sterilized cork borer (0.9 cm in diameter). We then inserted a circular fungus-agar plug and held it in place with a small piece of sterilized wooden dowel. Trees were inoculated on opposite sides of the stem at the same height (two inoculations per tree). At the time of inoculation, we also collected two samples of phloem (3 cm long $\times$ 1 cm wide) from opposite sides of the stem, 4 cm to the right and 4 cm above each inoculation point using sterilized chisels (Fig. 2.1b). Samples were immediately frozen on dry ice

in the field and remained frozen at -20°C until carbohydrate and monoterpene analysis. Inoculation treatments across all three sites were completed within a week.

In late-August, approximately six weeks after inoculation, we collected a 1 cm × 1cm sample of phloem from inside each lesion above the point of inoculation and a 3 cm × 1 cm sample from 4 cm to the left of the lesion boundary (Fig. 2.1c). Lesions were measured from their highest to lowest extents based on discoloration of the xylem just under the vascular cambium (Fig. 2.2). Thus, for each inoculated tree we collected two samples from outside the lesions and two samples from inside the lesions. At this time we also sampled the untreated trees (not inoculated) at the three respective heights to be comparable to samples taken from inoculated trees. Untreated trees were not sampled when the study was initiated because we assumed that the samples we took in mid-July from trees that we subsequently inoculated would be representative of carbohydrate and monoterpene levels in trees at that time. However, by inoculating, we changed the way trees allocated carbohydrates relative to in unmolested trees, so we needed untreated trees to serve as a baseline in late-August. All samples were placed on dry ice in the field and frozen at -20°C until carbohydrate and monoterpene analysis. Mid-July and late-August sample periods corresponded to the time when mountain pine beetles attack and colonize new hosts as well as the time of rapid radial growth in the stem.

In the laboratory, we removed the outer bark and xylem from our frozen stem samples using a box cutter and split each sample in half to obtain living phloem. Half was immediately returned to the freezer and remained frozen

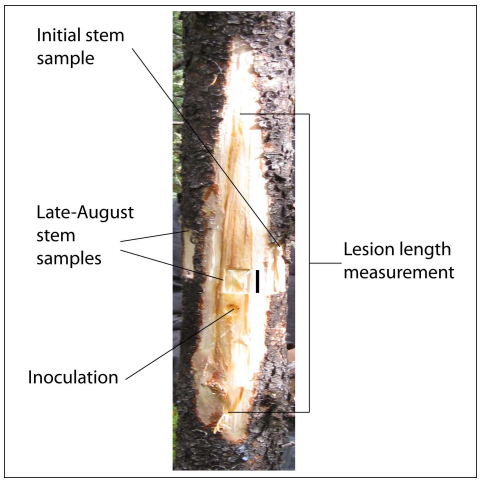

Figure 2.2: A lesion that formed on a lodgepole pine tree responding to inoculation with *G. clavigera*. To determine lesion length, lesions were measured from the highest point of discoloration on the sapwood down to the lowest point. The location of the initial stem sample collected at the time of inoculation and the location of stem samples collected 6 weeks later when lesion lengths are shown. Bar, 1 cm.

at -20°C until monoterpene analysis. The other half was oven dried at 100°C for one hour to stop enzymatic conversion of starch to sugar, and then dried for another three days at 70°C. After drying, samples were ground using a Wiley Mini Mill fitted with a 40 mesh (0.4 mm) screen (Wiley, Thomas Scientific, Swedesboro, NJ, USA). After grinding, the two samples from each tree were pooled and used to determine total starch and water-soluble sugar concentrations in the phloem according to the protocol described by Chow and Landhäusser (2004). Briefly, water soluble sugars were extracted from 50 mg of ground tissue in 80% hot ethanol and reacted with phenol-sulphuric acid before colorimetric measurement using a spectrophotometer (Pharmacia LKB Ultrospec III, Sparta, NJ, USA) at a wavelength of 490 nm. We enzymatically digested starches remaining in the residual pellet and combined the resul-

tant glucose hydrolyzate with peroxidase-glucose oxidase/o-dianisidine (color reagent) before measuring glucose hydrolyzate (starch) concentrations at a wavelength of 525 nm.

For the monoterpene analyses, the remaining half of each frozen phloem sample was ground in liquid nitrogen, and 100 mg of the tissue was transferred to a 1.5 mL micro-centrifuge tube. Samples were extracted with 0.5 mL dichloromethane and 0.01% (v/v) tridecane as a surrogate standard. After adding the solvent, the samples were vortexed for 30 s, sonicated for 10 min, centrifuged at 13200 rpm for 15 min, and placed in a freezer overnight to freeze the pellet. The following morning, we transferred the extract to GC vials and repeated the extraction on the pellet before discarding it. One μl of extract was injected from the GC vials into an Agilent 7890A/5062C Gas Chromatograph/Mass Spectrometer (Agilent Technologies, Santa Clara, California, USA) equipped with an HP Innowax (Agilent Technologies) column (I.D. 0.25 mm, length 30m). The helium carrier gas flow was set at 1.0 ml/min and the following temperature program was applied: the temperature was set to 50°C for two min, increased to 60°C by 1°C per min and then elevated to 250°C by 20°C per min. We used the following standards for quantification: borneol, pulegone, α-terpinene, γ-terpinene, α-terpineol (Sigma-Aldrich, St. Louis, Missouri, USA), camphor, 3-carene, α-humulene, terpinolene, α-thujone and β-thujone, (-)-α-pinene, (-)-β-pinene, (S)-(-)-limonene, sabinene hydrate, myrcene, (-)-camphene, p-cymene (Fluka, Sigma-Aldrich, Buchs, Switzerland), bornyl acetate, cis-ocimene, α-phellandrene (SAFC Supply Solutions, St.Louis, Missouri, USA), β-phellandrene (Glidco Inc., Jacksonville, Florida, USA). Agilent software (MSD ChemStation) allowed us to

determine the concentrations of each chemical by integrating the area under each peak measured by the GC.

We used the R program to analyze and graph our data (R Development Core Team, 2011) as well as the nlme package (Pinheiro et al., 2011) for mixed models in R. Our statistical models were either ANOVA or regression models. ANOVA models had two main effects: inoculation height along the stem (three levels) and dominance class (two levels). There were never statistical interactions between the two main effects and, based on AIC values, statistical models with separate random intercepts (mixed effects models) for each site were never better than models in which all sites were pooled together. For our untreated trees, which were sampled at all three heights, having a random intercept for each tree improved the models. All of our data were normally distributed or were transformed to resemble normally distributed data. When present, heterogeneity of variance at different inoculation heights was accommodated in the models using weighting functions (Zuur et al., 2009).

For F-tests, F-values followed by numerator and then denominator degrees of freedom were reported along with associated p-values. Sample sizes used to calculate estimates were not perfectly balanced due to missing data. Our sample size for inoculated trees included 7 intermediate trees inoculated at the top position; 9 intermediate trees inoculated at the middle position; 7 intermediate trees inoculated at the bottom position; 9 dominant trees inoculated at the top position; 8 dominant trees inoculated at the middle position and 9 dominant trees inoculated at the bottom position (N = 49). Our sample size for untreated trees was balanced. We therefore had 9 dominant and 9 intermediate trees that were sampled at three heights along their stems that

corresponded to the top, middle and bottom inoculation positions (N = 54). Rather than perform a myriad of post-hoc tests, we used confidence intervals to visually compare means. Details on this approach and its relationship to fixed alpha testing are given in Cumming and Finch (2005). We used the boot package (Canty and Ribley, 2012) for bootstrapping confidence intervals in R. To bootstrap confidence intervals we re-sampled our data with replacement such that we had a bootstrapped sample size equivalent to our true sample size. We re-sampled the data this way 2000 times and fitted our ANOVA models to each of the 2000 re-sampled datasets. From the re-fitted models we acquired 2000 estimates for each coefficient (mean) corresponding to the treatments. We calculated the 95% CI using these estimated coefficients. A similar procedure was used to estimate the 95% CI for untreated trees.

2.3 Results

2.3.1 Constitutive carbohydrate reserves & monoterpenes

While there was a decreasing trend in soluble sugar concentrations with distance from the live crown in mid-July (Fig. 2.3a), starch concentrations in the phloem did not differ at different heights along the stem (Fig. 2.3b). Dominant trees had higher overall starch concentrations in their phloem than intermediate trees (Fig. 2.3b, $F_{1,43} = 35.6$, $p < 0.001$) but there was only weak evidence that they had higher overall soluble sugar concentrations in mid-July (Fig. 2.3a,$F_{1,43} = 3.24$, $p = 0.079$).

In mid-July constitutive monoterpene concentrations in the stem phloem were higher near the top inoculation than near the lower inoculations in both

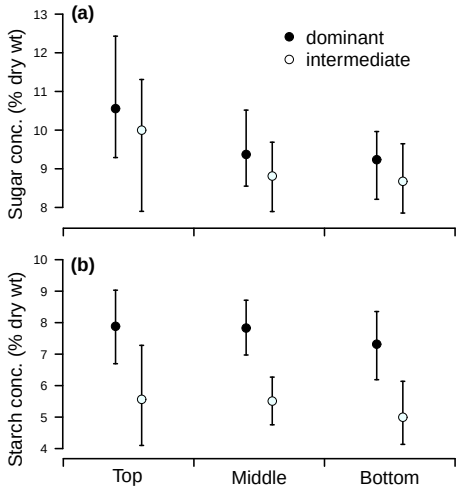

Figure 2.3: Mid-July concentrations of a) soluble sugar, and b) starch in lodgepole pine phloem sampled at the base of the live crown (Top), halfway between the top and bottom (Middle), and 1.3 m above the ground (Bottom) in dominant and intermediate trees. Points represent means and whiskers indicate width of bootstrapped 95% CI.

dominant and intermediate trees (Fig. 2.4a, $F_{2,43}$= 10.9, $p < 0.001$). Moreover, dominant trees, which had higher overall carbohydrate concentrations, also had higher overall concentrations of constitutive monoterpenes than intermediate trees (Fig. 2.4a, $F_{1,43} = 12.4$, $p = 0.001$). This pattern of higher monoterpene concentrations in dominant than in intermediate trees was consistent for the three most abundant monoterpenes produced by our study trees (β-phellandrene, β-pinene, and 3-carene). Furthermore, at the time of inoculation, concentrations of each of these monoterpenes were consistently higher near the top inoculation than near the lower inoculations in both dominant and intermediate trees (Fig. 2.4b,d).

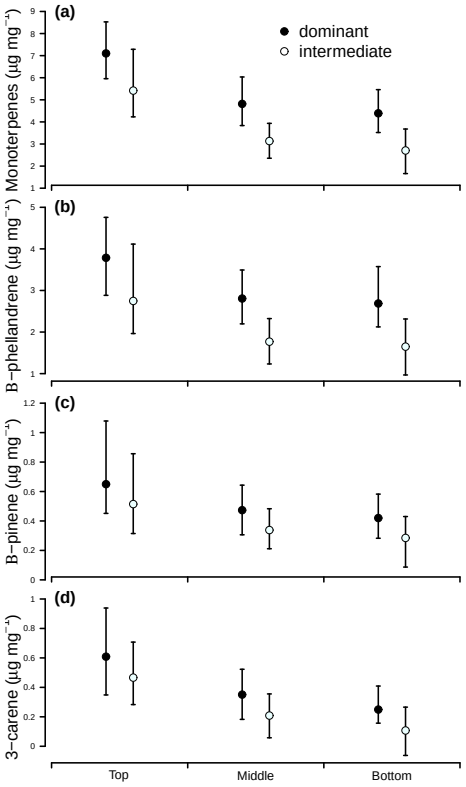

Figure 2.4: Mid-July concentrations of constitutive (preformed) monoterpenes in lodgepole pine phloem at the time of inoculation in mid-July: a) total monoterpenes, b) β-phellandrene, c) β-pinene, and d) 3-Carene. Samples were taken below the base of the live crown (Top), halfway between the top and bottom (Middle), and 1.3 m above the ground (Bottom) in dominant and intermediate trees. Points represent means and whiskers indicate width of bootstrapped 95% CI.

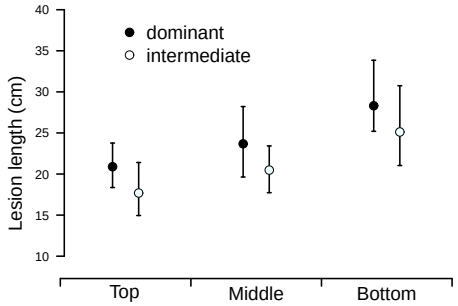

Figure 2.5: Lesion lengths formed on lodgepole pine stems just under the vascular cambium at inoculation points near the base of the live crown (Top), halfway between the top and bottom (Middle), and at 1.3 m (Bottom) in dominant and intermediate trees. Points represent means and whiskers indicate width of bootstrapped 95% CI.

2.3.2 Lesion length and chemistry

Lesion length depended on height of inoculation along the stem ($F_{2,45} = 4.97$, $p = 0.011$). In both dominant and intermediate trees, lesions were longer near the bottom inoculation and shorter near the top inoculation (Fig. 2.5). In addition, near the top and middle inoculations there was no relationship between lesion length and local soluble sugar concentration (Fig. 2.6a,b). However, near the bottom inoculation, local lesion length exhibited a negative relationship with the quantity of sugars nearby prior to inoculation that was curvilinear and consistent within both dominant and intermediate trees (Fig. 2.6c).

Concentrations of induced monoterpenes inside lesions were 10 times higher than constitutive monoterpene levels and they did not vary based on inoculation height (Fig. 2.7). Thus, within a tree dominance class, large and small lesions had similar internal monoterpene concentrations. However dominant trees did have higher overall monoterpene concentrations inside their lesions

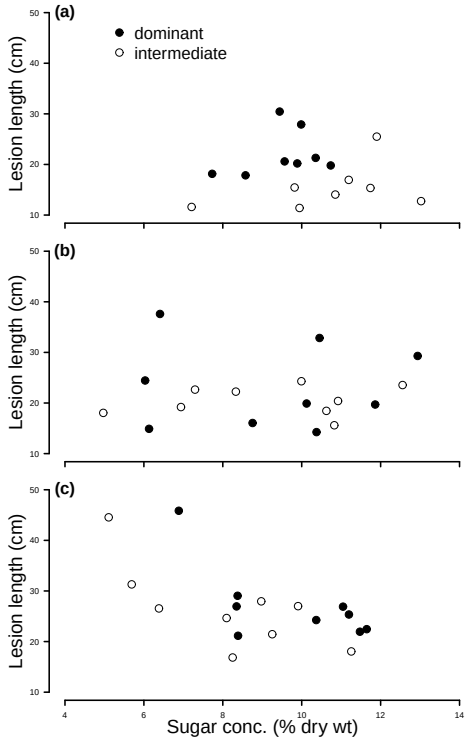

Figure 2.6: Lesion lengths formed on lodgepole pine stems in relation to the concentration of soluble sugars in a phloem sample taken from the inoculation point at the time of inoculation in mid-July. Points represent measurements from single dominant or intermediate trees a) at the base of the live crown (Top), b) halfway between the top and bottom inoculations (Middle) and c) at 1.3 m above the ground (Bottom).

than intermediate trees (Fig. 2.7, $F_{1,43} = 14.7$ $p < 0.001$).

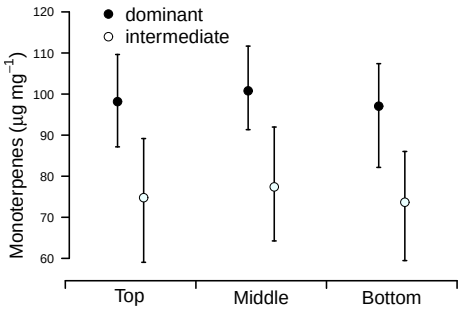

Figure 2.7: The total concentration of monoterpenes accumulated inside lesions in the phloem. Samples were collected at inoculation points near the base of the live crown (Top), halfway between the top and bottom (Middle), and 1.3 m above the ground (Bottom) in dominant and intermediate trees. Points represent means and whiskers indicate width of bootstrapped 95% CI.

2.3.3 Carbon sinks created by fungal infection

In infected trees, soluble sugar concentrations in the phloem outside the lesions did not differ depending on inoculation height, whereas they were still lower near the middle and bottom than near the top in uninfected trees (Fig. 2.8a square symbols, $F_{2,34}$= 11.1, $p < 0.001$). Note that in uninfected trees, concentrations of soluble sugars and their downward trend were similar to those in our inoculated trees prior to inoculation (Fig. 2.3a) but the 95% CI were much narrower due to within-tree replication. Like soluble sugar concentrations, starch concentrations in the phloem of untreated trees decreased with distance from the crown (Fig. 2.8b). Moreover, soluble sugar and starch concentrations in defense zones at all inoculation heights were consistently higher than in samples taken from corresponding heights in uninfected trees (Fig. 2.8). Near the bottom inoculation, dominant and intermediate trees,

allocated more soluble sugars (concentration after - concentration before) to defense zones when lesions were longer (Fig. 2.9).

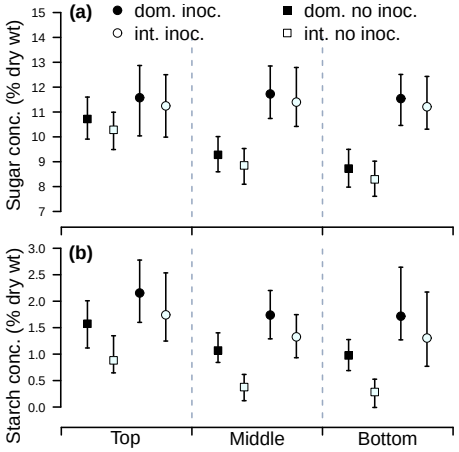

Figure 2.8: Late-August concentrations of a) soluble sugar, and b) starch in the phloem of lodgepole pine trees that were inoculated with *G. clavigera* and in trees that were uninfected. Phloem was sampled at the base of the live crown (Top), halfway between the top and bottom (Middle), and 1.3 m above the ground (Bottom) in dominant and intermediate trees. Points represent means and whiskers indicate width of bootstrapped 95% CI.

2.4 Discussion

Our results demonstrate the utility of the sink/source framework for explaining patterns in defense in non-modular tree stems which are outside its traditional domain. Consistent with the predictions of the sink/source framework as outlined by Honkanen and Haukioja (1998), we observed variation in susceptibility to fungal infection along the stems of lodgepole pines that depended on carbon import from sources to sinks. Prior to inoculation with *G. clavigera*, carbohydrate concentrations in the phloem and constitutive monoterpene defenses decreased with distance from the crown. At the time

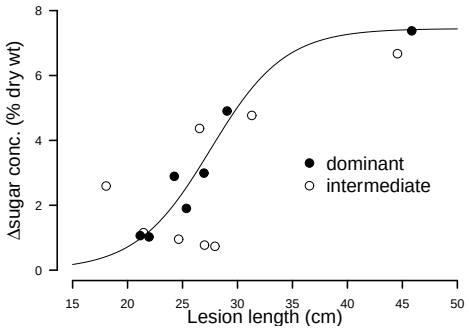

Figure 2.9: Changes in soluble sugar concentrations as a function of lesion length near inoculation points at the bottom of lodgepole pine stems (1.3 m above the ground) six weeks after inoculation with *G. clavigera*. The fitted line is a logistic curve fitted only to the data for dominant trees.

of inoculation, higher sugar influx rates near the crown likely led to superior defensive responses relative to lower in the stem as lesions were smaller near the crown. After inoculation, sink priority in the stem changed and trees imported more carbohydrates to the phloem near large lesions than near small lesions even though larger lesions were generally further from the crown. We discuss these findings in detail below.

2.4.1 Constitutive carbohydrates & monoterpenes

Carbohydrate concentrations in the phloem of dominant and intermediate trees decreased with distance from the crown in both the July and August samples. As peak cambial growth occurs in the summer, gradients in carbohydrate concentrations in stems were likely the result of active growth sinks along their length. Sinks closer to carbon sources diminish the quantity of carbohydrates available to more distal sinks, provided their sink strengths are similar (Minchin and Lacointe, 2005). Therefore, in accordance with a conceptual

model proposed by Landhäusser and Lieffers (2012), we observed diminishing carbohydrate availability in the crownless stem with distance from the crown.

We did not measure reserves in the stem xylem even though it is widely regarded as the principal storage organ in mature trees (Hoch et al., 2003; Sala et al., 2012) due to its size. However, we contend that concentrations of starch and soluble sugars in the phloem are better indicators of carbohydrate availability. This is partly due to the phloem's role as an avenue for carbohydrate transport, but also because the carbohydrate content of the xylem may be disconnected from carbon availability if carbohydrates have become sequestered there (Millard et al., 2007). In aspen (*Populus tremuloides* Michx.) phloem carbohydrate concentrations, and in particular starch concentrations, are consistently higher throughout the year than in the xylem (Landhäusser and Lieffers, 2003). Carbohydrate concentrations in the phloem of ponderosa pines (*Pinus ponderosa* (Douglas) are 5 to 6 times higher than those in the xylem (Pruyn et al., 2005). Because carbohydrates are transported along concentration gradients, sinks in the xylem are likely supplied by resources in the phloem. However, because carbon import into local sinks in the xylem is likely constrained by carbohydrate availability in the nearby phloem, we believe that the concentrations of starch and soluble sugars in the outer rings of the xylem would exhibit similar patterns to those we found in the phloem.

Constitutive monoterpene concentrations were highest where carbohydrate availability was highest—near the tops of the crownless stems. We anticipated that monoterpene production would depend on local carbohydrate availability because the mevalonate-independent pathway for monoterpene synthesis occurs in plastids where carbohydrate reserves are stored (Chappell, 2002).

Honkanen et al. (1999) found that growth rates and concentrations of monoterpenes in Scots pine needles were positively related, as they had predicted based on sink/source relationships. The sink/source framework also provides a viable explanation for high monoterpene concentrations in the stem phloem near active growth regions: high carbohydrate availability coupled with strong growth sinks near the tops of stems likely resulted in high rates of soluble sugar import and consequently, high availability of carbohydrate reserves for monoterpene synthesis.

2.4.2 Lesion lengths & sugar concentrations

Lesions on the stem were shorter near the crown where local carbohydrate concentrations were high, and longer near its base, where local carbohydrate concentrations were lower. One explanation for this finding is that the rate of soluble sugar import into sinks created by fungal infection likely dictates the efficacy of defensive responses (Lieutier et al., 1993). Sugar concentrations in tissues near inoculation points prior to fungal infection likely corresponded to rates of soluble sugar import in those regions and consequently, they also impacted the speed of response to fungal invasion. Thus, we propose that longer lesions on the lower stem resulted from delayed sugar import into defense zones due to lower initial concentrations of soluble sugars in nearby reserves.

Near the crown, where concentrations of sugars in the phloem, and presumably, soluble sugar influx rates from the crown were high, there was no relationship between local sugar concentration prior to infection and lesion size. Near the base of the stem however, where influx from the crown was likely diminished, lesion length was negatively related to sugar concentrations

in the phloem prior to inoculation. The finding that trees with more local reserves displayed more effective responses near their bases suggests defensive responses depended on the mobilization of nearby reserves to the region of attack. This assertion is corroborated in a tracer study on Scots pine saplings inoculated with a blue-stain fungus, which showed that saplings used reserves located in the stem to fund defense reactions (Guérard et al., 2007). When carbohydrate reserves in the phloem were lower, moving sugars over a greater distance likely delayed defensive responses allowing longer lesions to develop.

2.4.3 Carbon sinks created by infection

Because the allocation of soluble sugars to defense zones increased with lesion size, the decreasing trend in soluble sugar concentrations with distance from the crown was no longer evident after inoculation. Moreover, sugar accumulation near lesions likely reflected the cost of battle rather than preparedness for war. The lesions of dominant trees contained higher concentrations of monoterpenes than those of intermediate trees, and dominant trees also had higher concentrations of soluble sugars and starch near defense zones. Moreover, carbohydrate concentrations in the phloem near longer lesions increased more than in the phloem near shorter lesions even though longer lesions were further from the crown. Thus, when locally stressed, the size of sinks created by lesions superseded the influence of sink proximity to the crown as a determinant of carbohydrate import. High quantities of carbohydrate reserves have also been recorded near latex tapping locations at the base of rubber trees (Silpi et al., 2007; Chantuma et al., 2009). Based on the sink/source framework, we hypothesize that after normal rates of sugar influx along the

stem have been perturbed by attack, local sugar accumulation corresponds to the sink created by tree responses to fungal infection.

2.4.4 Ecological Implications

Terpenes and phenolics are carbon-based defense chemicals (Keeling and Bohlmann, 2006), and thus, the ability to mobilize carbohydrates to defense zones is likely critical for their synthesis in response to attack. For this reason carbohydrate mobilization along tree stems is likely a highly adaptive response since long branchless stems are vulnerable to attack. Our study showed that the sinks created by tree responses to *G. clavigera* inoculations eventually lead to carbohydrate mobilization to the lesion front. However, trees initially had low levels of carbohydrates and constitutive defenses in their lower stem relative to their upper stem. The delay associated with importing the necessary carbohydrates likely impeded defensive responses in the lower stem, and resulted in heavier damage to the xylem and phloem. Thus, we propose that the lower stem is in a poor position to quickly mobilize the large quantities of carbohydrates necessary for the production of the defensive compounds used to contain attacks. This may contribute to higher mountain pine beetle colonization of the lower stem as observed by (Cole and Amman, 1983). Although mountain pine beetles preferentially attack healthy dominant trees when their populations are large (Safranyik and Carroll, 2006), we have confirmed that dominant trees within a stand are better defended than intermediates. When bark beetles mass-attack however, the mobilization of large quantities of carbon to reaction zones in dominant trees likely results in rapid depletion of carbohydrate reserves. Thus, during mass-attacks by bark beetles the defen-

sive responses elicited by bark beetle fungi may exhaust carbohydrate reserves and hasten tree death (Lieutier et al., 2009).

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Chapter 3

Fertilization of lodgepole pine trees increased diameter growth but reduced root carbohydrate concentrations

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3.1 Introduction

In the context of tree resistance to insect herbivory, tree vigor relates to the carbohydrate reserves available to produce defensive chemicals and defensive structures (Dunn et al., 1987; Dunn and Lorio, 1992; Lombardero et al., 2000; Dunn et al., 1990). However, in many cases, tree vigor is estimated indirectly

by measuring growth rates rather than by quantifying energetic resources directly. Vigor index (Waring et al., 1980) and growth efficiency (Waring and Pitman, 1985) are constructed by expressing stem growth relative to sapwood area in the case of vigor index or relative to total leaf area for growth efficiency. Both vigor index and growth efficiency measure the relative allocation of carbon to stem wood, but Waring and Pitman (1985) suggested vigor index and growth efficiency are also indicative of the amount of carbohydrates stored by the tree and they proposed a carbon allocation hierarchy hypothesis to support this argument. In it they argued that high concentrations of soluble carbohydrates have been measured in the sapwood of stressed lodegepole pine trees despite very low radial growth rates (Webb, 1981) and therefore stem growth must be a lower priority than storage (Waring and Pitman, 1985; Waring, R H and Schlesinger, W H, 1985). Thus rapid radial expansion is interpreted as evidence that all carbohydrate reserves have been well supplied and supplemental carbon is allocated to ring growth. On this basis, vigor index and growth efficiency have been widely used to predict and explain tree susceptibility to insects and pathogens (Coops et al., 2009; Haavik et al., 2010; Lieutier et al., 1993; Waring and Pitman, 1985; Waring et al., 1992).

A contrasting viewpoint is that growth, along with other carbon sinks related to tree size negatively impact carbohydrate reserves within trees. Rapid stem growth due to fertilization decreased carbon reserves in the roots and stem of loblolly pine (Ludovici et al., 2002; Warren et al., 1999). Also, the relatively large quantity of carbon needed for sapwood respiration in large trees may penalize their carbon reserves (Ryan, 1989, 1990). Furthermore, the large cambial surface area in the bole of large trees is likely proportional

to the energetic cost of diameter growth since plant surface area is correlated to growth respiration (Landsberg, 1986). An added factor for large trees is that they have a lower proportion of photosynthetic structures relative to respiring tissue. Smaller live crown ratios (LCR) in large lodgepole pine trees (Fish et al., 2006) reflect a decline in leaf area relative to structural tissues. Variables corresponding to tree size, such as diameter at breast height (DBH), LCR, cambial surface area and sapwood area are likely correlated with carbon assimilation rates as well as increases in carbon demands. These may therefore be useful predictors of carbohydrate reserves and vigor in trees.

Silviculture treatments that boost tree growth are attractive to forest managers because they have the potential to increase annual productivity and might also reduce the susceptibility of trees to insects and diseases. Thinning reportedly decreased lodgepole pine susceptibility to mountain pine beetle and other bark beetles (Safranyik et al., 1999). Influential research by Waring and Pitman (1985) suggested that the combination of fertilization and thinning reduced lodgepole pine mortality due to mountain pine beetle in the Western United States and that this lower mortality corresponded to increased growth efficiency.

The current study tests the hypothesis that increased growth rates due to fertilization and thinning correspond to increased root carbohydrate storage. The objectives were to manipulate lodgepole pine growth rates by fertilizing and thinning, and to describe how this impacts carbohydrate reserves in the roots. Root tissue was used in this study as an indicator of stored energy reserves since in conifers, roots contain higher starch concentrations than the bark, needle and branches throughout the growing season (Ludovici et al.,

2002). We also compared DBH, sapwood area, height-to-live-crown, growth rates in the stem, LCR, cambial surface area, and vigor index as predictors of the root carbohydrate concentrations of fertilized and unfertilized lodgepole pine trees.

3.2 Materials and Methods

3.2.1 Experimental Design

The experiment was conducted in the Upper and Lower Foothills natural subregions on the eastern slopes of the Rocky Mountains in Alberta, Canada. This area is characterized by sandy Brunisolic and Luvisolic soils, a mean annual temperature of 3°C, and a mean annual precipitation of 501 mm (Beckingham et al., 1996). Ten experimental sites were established in pure lodgepole pine stands ranging in elevation from 1041 m to 1473 m above sea level. Trees had stem diameters at breast height (DBH at 1.3 m) from 8 cm to 25 cm and ages from 13 to 52 years. Trees smaller than the smallest diameter class usually attacked by montain pine beetles were included to elucidate mechanisms that might explain beetle preference for larger diameter host trees. Although the majority of the sites were of harvest origin, the two oldest sites were of fire origin.

At each site, four 35 m × 35 m (1225 m^2) plots were established in level areas of pure lodgepole pine (95-100% pine stem composition). The tree density ranged from 2800 pine stems ha^{-1} in the oldest sites and 7700 pine stems ha^{-1} in the youngest sites. Plots were separated by a buffer of at least 20 m. In the winter of 2005 and spring of 2006 two plots per site were thinned from

below to a density of 2500 coniferous stems ha^{-1}. All non-coniferous trees and shrubs were cut from the thinned plots. To complete a 2 $\times$ 2 factorial design, two plots per site were fertilized in May 2006 with 300 kg ha^{-1} nitrogen from urea and mono-ammonium phosphate, 100 kg ha^{-1} of phosphorus from mono-ammonium phosphate, 100 kgha^{-1} of potassium from muriate of potash and potassium magnesium sulphate, and 32.5 kg ha^{-1} of magnesium from potassium magnesium sulphate, 75 kg ha^{-1} of sulphur from mono-ammonium phosphate and potassium magnesium sulphate, and 3 kg ha^{-1} of boron from granular borate. Thus, each site had one thinned plot; one fertilized plot; one thinned and fertilized plot; and one untreated control plot. This protocol was replicated at all sites except the two fire-origin sites where only untreated and fertilized plots were established, resulting in 36 treatment plots on 10 sites (8 sites with 4 plots + 2 sites with 2 plots). Natural self-thinning in the two fire-origin sites made thinning treatments similar to those applied in the younger sites impractical as the density of pine stems in these plots was only 1500-1700 pine stems ha^{-1}. Four dominant pine trees within each treatment plot were randomly selected in the spring of 2008. All sample trees had full crowns, were free from stem defects and had no sign of abnormal needle loss or infection.

3.2.2 Measurements

Foliar nitrogen was analysed one and three years after fertilization; needles were collected in late fall from opposite branches in the upper third part of the live crown of 3 dominant trees per plot using a pruning pole or a shotgun on the taller trees. Branch samples were kept frozen at -20 °C until chemical analysis. A subsample of 100 needle fascicles of the youngest age class from

each tree were dried at 70°C, and ground in a Wiley Mill (Thomas Scientific, NJ, USA) fitted with a 40 mesh screen (0.42mm). Samples were analyzed for total foliar nitrogen using the Kjeldahl digestion method (Bremner and Mulvaney, 1982). Nitrogen concentrations were measured colorimetrically at 660 nm.

In June 2008, DBH was measured with a diameter tape, and height and height-to-live-crown were measured using a clinometer (Vertex IV, Haglöf, Sweden) for all sample trees. At the same time, root samples (1 ± 0.2 cm diameter) were collected for carbohydrate analysis by following a root out from the bole and sampling between 1 m and 2 m from the bole of each sample tree. The rationale for this protocol was to minimize variability in root carbohydrate reserves due to root location and root size. Root samples were bagged and placed on ice in the field and frozen at -20 °C until they were processed for carbohydrate analysis.

Root samples were thawed, oven dried at 100 °C for 1 hour to stop enzymatic conversion of starch to sugars, then dried to constant weight at 70 °C. Dried samples were ground using a Wiley Mill fitted with a 40 mesh screen (Thomas Scientific, USA). Total starch and sugar concentrations were determined colorimetrically (Pharmacia LKB Ultrospec III, Sparta, NJ, USA) following the protocol described by Chow and Landhäusser (2004). Briefly, 50 mg of ground tissue was extracted in 80% hot ethanol. Sugars were reacted with phenol-sulphuric acid before colorimetric measurement at 490 nm. Starch remaining in the residual pellet left after sugar extraction was digested enzymatically and the resultant glucose hydrolyzate was reacted with peroxidase-glucose oxidase/o-dianisidine (color reagent). Glucose hydrolyzate (starch)

concentrations were quantified on a spectrophotometer at 525 nm.

In early August 2008, shoot growth for each tree was measured on two actively growing branches collected from opposite sides of the upper third of the crown to control the effects of location in the crown and sun exposure on shoot growth. Branch samples were collected using a pruning pole or a shotgun in taller trees. Twig growth increment for 2007 was assessed using bud scars and the measurements of the two branches were averaged. Only the growth in 2007 was measured because the most recent (2008) shoots had not finished growing at the time of collection.

In late August 2008, two increment core samples were taken on opposite sides of the bole at 1.3 m. Increment cores were glued to grooved boards, dried and sanded with progressively finer sand paper before staining with an aqueous solution of Bromocresol Green (Fisher Scientific, NJ, USA) to distinguish sapwood. Cores were then scanned (HP, ScanJet 4c/t) and the width of the current year's growth, sapwood width, and total core length from the pith to the vascular cambium were determined using pixel-based measurement tools in SigmaScan Pro (Jandel Scientific, CA, USA). Sapwood area and basal area increment were calculated from sapwood width and the width of the current year's ring.

Ring volume increment in the current year (2008) was estimated by subtracting diameter growth, determined from the two increment cores taken at 1.3 m, from the current DBH to estimate DBH in the last year assuming there was no change in bark thickness between the two years. Using reported diameter-height relationships for the Foothills region of Alberta (Huang, 1994), height for the last year was estimated from DBH in the last year. Taper equa-

tions for the Alberta Foothills region (Yang et al., 2009) were used to calculate stem volumes based on DBH and height from the current and previous year. Stem volume in the previous year was subtracted from stem volume in the current year to estimate volume increment.

In addition to basic tree characteristics such as size and growth, complex variables (Table 3.1) derived by combining basic tree measurements were also used as predictors of root starch concentrations. Complex variables included ring volume increment, live crown ratio, which is the ratio of crown length to tree height, vigor index, and an estimate of the cambial sink in the stem based on surface area. Vigor index was calculated by dividing basal area increment in the latest year (2008 in this study) by total sapwood area which gave a rough estimate of productivity per unit of leaf area (Waring et al., 1980). Instead of estimating total cambial surface area (Landsberg, 1986), we estimated stem cambial surface area below the live crown (CSA) because the crownless stem is strictly a carbon sink. Therefore, the CSA variable approximates cambial surface area between the stump and the base of the live crown using the formula for the area of a conical frustum (Harris and Stöcker, 1998): $CSA = \pi[r(r+s) + R(R+s)]$, where R is the radius of the bole at stump height, r is the radius of the bole at the base of the live crown and s is the length of surface area being calculated: $s = \sqrt{(R-r)^2 + HLC^2}$, where HLC is height to live crown. Bole radius at the base of the live crown was calculated using geometric relationships between bole radius at the base of the live crown and bole radius at stump height.

3.2.3 Statistical analysis

The four trees sampled per plot were considered subsamples as the treatments were applied to the plot; in all analyses the experimental unit was the plot. Plots in this study were clustered by site therefore mixed models were used to isolate random effects due to variability between sites from treatment effects. The effects of fertilizer and thinning treatments on basal area increment (BAI in mm^2), vigor index (mm^2 mm^{-2}), shoot growth (mm), and root sugar concentrations (% dry weight), were evaluated using analysis of variance (ANOVA) models The effects of fertilizer and thinning treatments on root starch concentrations was analyzed using an analysis of covariance (ANCOVA) model with DBH as the covariate (COV). Model formulae for mixed model ANOVAs and ANCOVAs were of the following form:

$$y = \alpha_i + COV + T + F + T * F + \epsilon_{i,j} \tag{3.1}$$

This is a random intercept ANOVA (COV excluded), or ANCOVA (COV included), where y is the mean treatment effect, α_i is the intercept which varies by site (i=sites 1 through 10), T is the thinning effect, F is the fertilizer effect, T*F is the treatment interaction term and $\epsilon_{i,j}$ is the error term which contains the random site effect (i subscript) and the residual error (j subscript). The covariate was only required when analyzing thinning and fertilizer effects on root starch concentrations. DBH was used as the covariate as it had a strong linear effect on root starch concentrations in all treatments.

The statistics in the current study are presented as treatment means and main effects means including 95% confidence intervals rather than as p-values. For post hoc tests of treatment effects on BAI, vigor index, lateral branch

growth, starch concentration and sugar concentration, if the 95% confidence intervals of the main effects means do not overlap zero, they are significantly different from zero. Significant p-values are less informative than 95% confidence intervals because they do not distinguish between large treatment effects and small sample variances which have differing biological relevance and interpretation (Nakagawa and Cuthill, 2007). The 95% CI for post hoc tests of treatment effects were not adjusted for family-wise type I error because the comparisons were always orthogonal.

Mean root starch concentrations in each plot were regressed against predictors. The single variable predictors were, DBH, sapwood area, HLC, ring width, and basal area increment. Complex predictors were ring volume increment, CSA, LCR and vigor index (Table 3.1). Both linear and non-linear regressions were used to explore the relationships. The predictive power of different regressions was compared using residual mean square error (RMSE), AICc statistics and Akaike weights (model probabilities denoted ω_i). AICc is a form of the commonly used AIC statistic which is corrected for small sample bias (Anderson, 2008). Akaike weights describe model probabilities, relative to other models in the model set, given the data (Anderson, 2008). The purpose of these regressions was not to create models to predict root starch concentrations outside of the sample collected in this experiment as this would require regression equations with multiple predictor variables. Instead, the purpose was to determine which tree characteristics are the best predictors of root starch concentration and to suggest possible mechanisms for why these predictive relationships exist. For comparing the predictive power of the complex variables, a truncated dataset which excluded the youngest

plots was used. The youngest plots contained trees with live branches below 1.3 m where sapwood area is usually measured precluding accurate estimation of leaf area from sapwood area using principles of the pipe model theory (Grier and Waring, 1974). Although sapwood area is not converted to leaf area in the current study, the youngest plots were eliminated for the sake of fair comparisons between vigor index and the other complex variables. The fit statistics of these regressions cannot be compared to the fit statistics of the individual tree variables which correspond to the complete dataset.

Table 3.1: Tree characteristics used as predictors of root starch concentrations. For complex predictors that are calculated using multiple individual variables, a list of components is provided.

Variable	Acronym	Components
Diameter at breast height	DBH	-
Sapwood area	-	-
Height-to-live crown	HLC	-
Ring width	-	-
Basal area increment	BAI	-
Ring volume increment	-	DBH, height
Cambial surface area	CSA	Stump diameter, height, HLC
Live crown ratio	LCR	Height, HLC
Vigor index	-	BAI, sapwood area

All statistical analyses were done in the R program (R Development Core Team, 2009) using the lme4 package for mixed models and the gplots package for mean and confidence interval graphs.

3.3 Results

3.3.1 The impact of fertilization & thinning on vigor, growth & carbohydrates

Mean (± 95% CI) foliar nitrogen concentration in the year following fertilization was 1.48 ± 0.04 % of dry weight in fertilized plots which was significantly higher than in unfertilized plots (1.12 ± 0.02 %). After three growing seasons, foliar nitrogen concentration (1.17 ± 0.06 % of dry weight) in fertilized plots was still significantly higher than in unfertilized plots (1.07 ± 0.03 %).

To assess the effect of fertilization and thinning on BAI, vigor index, lateral shoot growth, root starch concentrations and root sugar concentrations, main effect means and 95% confidence intervals were compared (Table 3.2). Trees in fertilized plots had larger BAI than trees in unfertilized plots. The average untransformed BAI in fertilized plots was 1080 mm^2 but only 836 mm^2 in unfertilized plots. Although thinning appeared to increase BAI, it was not significantly larger than in unthinned plots due to high variability in the sample. Both fertilization and thinning increased vigor index, but only fertilization increased vigor index significantly. Fertilization significantly increased lateral shoot growth (measured as the average 2007 internode length from branch samples) in the upper crown whereas thinning had no effect. Mean root starch concentrations were significantly lower in fertilized plots than in unfertilized plots. Neither fertilization nor thinning significantly altered root soluble sugar concentrations. Interactions between fertilization and thinning treatments were not significant for any of the response variables tested above

and thus were not reported.

Table 3.2: Raw treatment means and main effect means with 95% confidence intervals (Diff ± 95%CI) for vigor-related variables measured in fertilized plots versus unfertilized plots, and thinned plots versus unthinned plots. The variables shown are the natural log of basal area increment, vigor index, lateral branch growth (mm), root starch concentration (% dry weight) and root sugar concentration (% dry weight). All analyses are without covariates except for root starch which had DBH as a covariate. In bold are the treatment variable combinations with significant treatment effects.

Variable	Fert	Unfert	Diff ± 95%CI	Thin	Unthin	Diff ± 95%CI
Ln of BAI	**6.96**	**6.69**	**0.27 ± 0.19**	6.93	6.74	0.19 ± 0.20
Vigor index	**0.115**	**0.098**	**0.017 ± 0.012**	0.117	0.098	0.006 ± 0.014
Lat. branch	**195**	**156**	**38.7 ± 23.3**	146	199	2.96 ± 29.8
Root starch	**5.89**	**9.42**	**3.41 ± 0.95**	8.16	7.25	0.21 ± 1.74
Root sugar	5.69	5.73	0.04 ± 0.41	5.92	5.55	0.30 ± 0.41

3.3.2 Relationships between tree characteristics & root starch concentrations

To compare the relative performance of various tree characteristics as predictors of root starch concentrations, univariate regressions were fitted to the root starch concentration data with either DBH, sapwood area, HLC, ring width, BAI, ring volume increment, CSA, LCR, or vigor index as the predictor variable. In all of the regression analyses of basic and complex variables as predictors of root starch concentrations, fertilized plots clearly separated from unfertilized plots whereas thinned plots did not separate from unthinned plots because thinning had little effect on root starch concentrations (Table 3.2). Root starch concentrations decreased as tree size increased irrespective

of whether DBH, sapwood area, or HLC was used as a predictor in both fertilized and unfertilized plots (Fig. 3.1A-C). The fit statistics suggest that the HLC predictor variable explained the root starch concentration data better than any of other single-variable predictors with a model probability (ω_i) 20 times higher than the next best predictor (Table 3.3). The next best predictors of root starch reserves were DBH and sapwood area, followed by ring width and volume increment. Although ring width and volume increment had consistently poorer fits, they still had R^2 values between 0.3 and 0.5 (Table 3.3). Mean root starch concentrations of trees growing in unfertilized plots and fertilized plots were positively related to 2008 ring width (Fig. 3.1D); BAI and root starch concentrations were uncorrelated in both treatments (Fig. 3.1E); and volume increment and root starch concentrations were negatively correlated (Fig. 3.1F).

Table 3.3: Fit statistics for tree characteristics as predictors of root starch concentrations (y) in unfertilized and fertilized trees: residual mean square error (RMSE), second order AIC (AICc), and Akaike weights (w). Predictors (x) include ring width, basal area increment (BAI), ring volume incement, diameter at breast height (DBH), sapwood area, and height-to-live crown (HLC). The best model (with the lowest AICc and the smallest RMSE) in this set is bolded. NL denotes relationships or which no R^2 value is provided due to the non-linear fit; (-) indicates an insignificant correlation.

	Unfertilized				Fertilized			
	RMSE	AICc	ω_i	R^2	RMSE	AICc	ω_i	R^2
DBH	2.04	-18.7	0.07	NL	1.68	-25.6	0.04	NL
Sapwood area	2.25	-15.2	0.01	NL	1.76	-23.9	0.02	NL
HLC	**1.78**	**-23.7**	**0.90**	**NL**	**1.41**	**-32.0**	**0.94**	**NL**
Ring width	2.33	-13.8	0.01	0.42	2.11	-17.4	0.00	0.50
BAI	-	-	-	-	-	-	-	-
Increment V	2.28	-14.6	0.01	0.45	2.45	-12.0	0.00	0.33

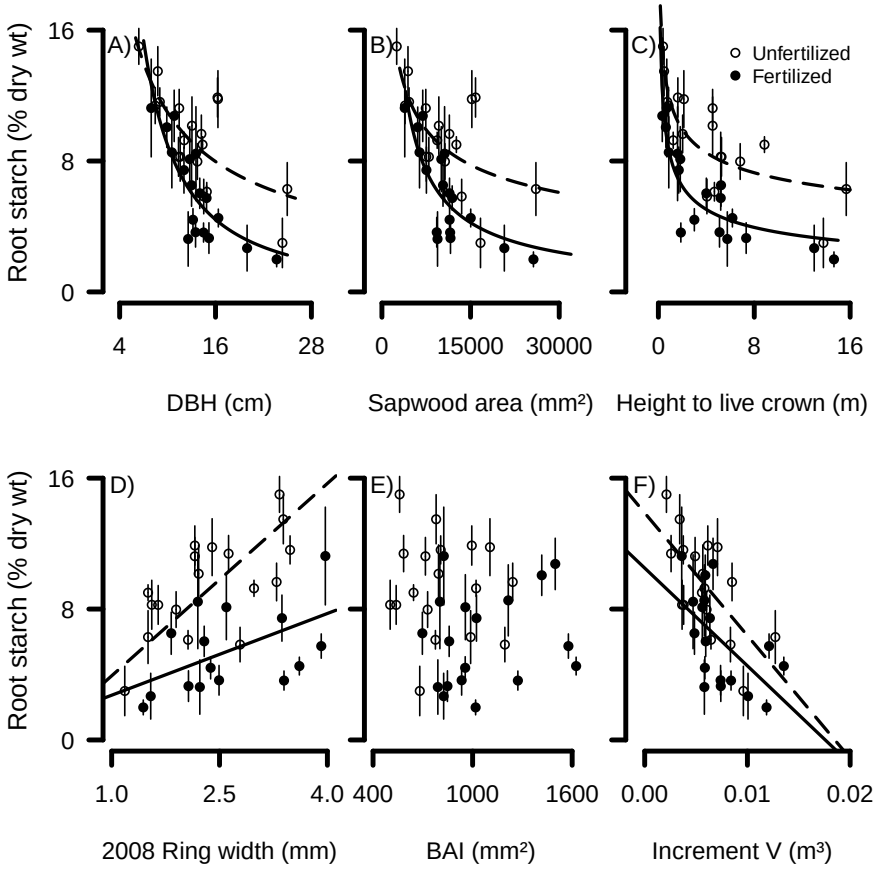

Figure 3.1: Tree characteristics as predictors of root starch concentrations with separate linear relationships shown for unfertilized (open/dashed) and fertilized (solid) trees: (A) diameter at breast height, (B) sapwood area, (C) height-to-live crown, (D) ring width, (E) basal area increment, and (F) ring volume increment. All ring measurements shown were for the latest year (2008). Data are mean root starch concentrations in each plot (and standard errors).

Of the three complex-variable predictors of root starch concentration, CSA was the most predictive, followed by LCR, and vigor index (Table 3.4). The regression of starch against CSA had a model probability (ω_i) more than 30 times that of the vigor index regression (Table 3.4). Fertilization changed some of the estimates of the coefficients for the regression analyses relating complex variables to root starch concentrations; the "a" coefficient for the CSA regression was significantly smaller in the fertilized plots than the "a" coefficient for the analogous regression in the unfertilized plots (Fig. 3.2A). Root starch concentration increased with increasing LCR (Fig. 3.2B) and vigor index (Fig. 3.2C) and this relationship was consistent in both fertilized and unfertilized plots. In addition, the intercept of the LCR regression in the fertilized sample tended to be lower than in the unfertilized sample although the difference was not significant at an alpha level of 0.05 (Fig. 3.2B).

Table 3.4: Fit statistics for each of the complex predictors of root starch concentrations. Predictors include cambial surface area below the live crown (CSA), live crown ratio (LCR) and vigor index. The best model (with the lowest AICc and the smallest RMSE) in this set is bolded. NL denotes relationships for which no R^2 value is provided due to the non-linear fit. AICc values in this table are not comparable to those in Table 3 as these models were fit to a smaller dataset (see Section 2).

	Unfertilized				Fertilized			
	RMSE	AICc	ω_i	R^2	RMSE	AICc	ω_i	R^2
Cambial surface area	**2.03**	**-8.69**	**0.74**	**NL**	**1.24**	**-22.6**	**0.77**	**NL**
Live crown ratio	2.20	-6.42	0.24	0.37	1.35	-20.1	0.22	0.50
Vigor index	2.59	-1.90	0.02	0.13	1.79	-12.3	0.01	0.14

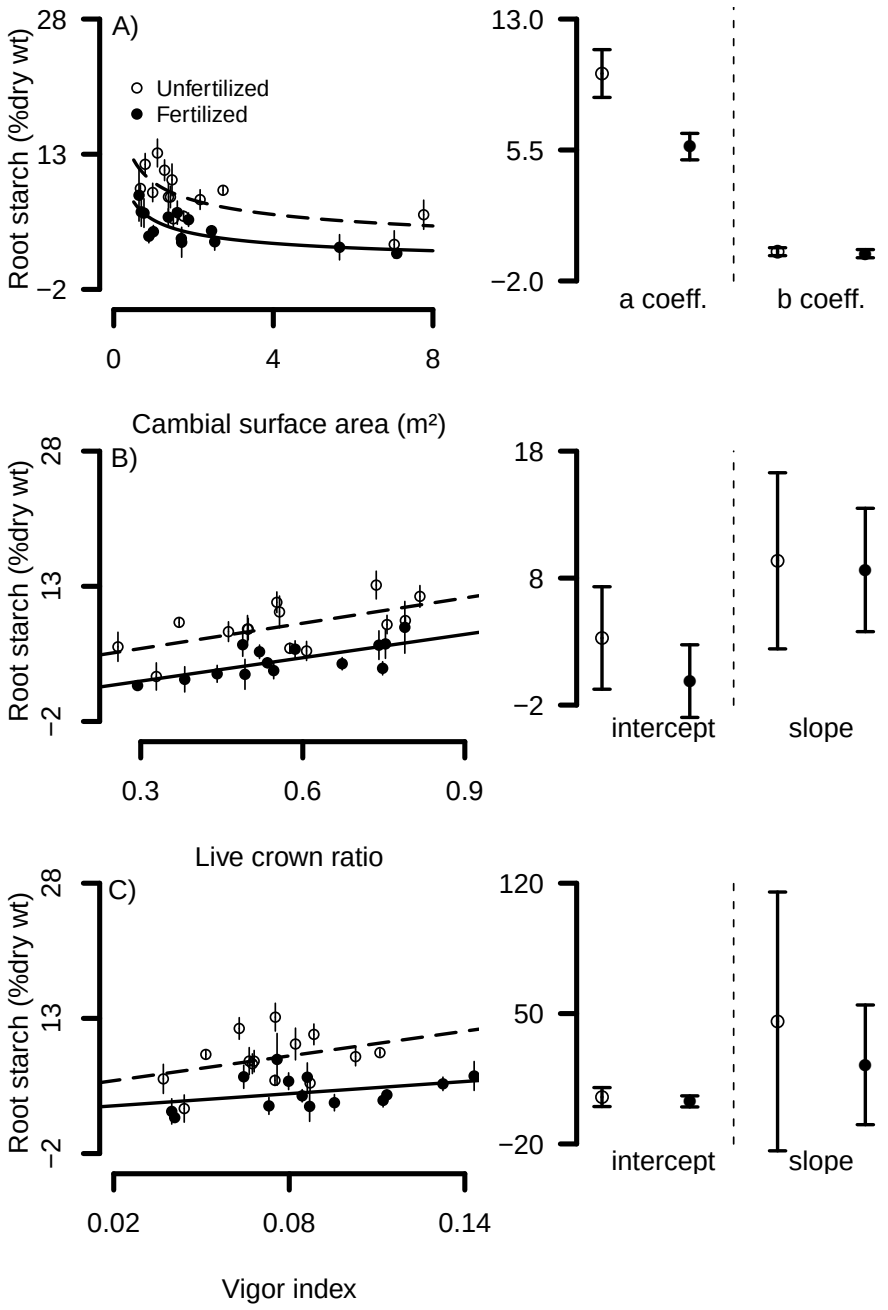

Figure 3.2: Root starch concentrations in relation to complex variables: (A) cambial surface area below the live crown (CSA), (B) live crown ratio (LCR) and (C) vigour index. Data are mean root starch concentrations in each plot (and standard errors). The regression coefficent estimates and 95% confidence estimates are shown to the right of plots for the CSA, LCR, and vigor index regressions.

3.4 Discussion

Contrary to our hypothesis, our results showed that fertilization increased growth of stem-wood but decreased root starch concentrations. Although thinning appeared to increase stem-wood growth, the effect was not strong, and thinning had no detectable effect on root starch concentrations. This is in contrast to the carbon allocation hierarchy hypothesis, which suggests that root carbon reserves should increase as diameter growth and vigor index increase (Waring and Pitman, 1985; Waring, R H and Schlesinger, W H, 1985). Others have also found that increased site nutrition negatively impacts carbon allocation to below ground tissues in Monterey pine (Beets and Whitehead, 1996) and loblolly pine (Ludovici et al., 2002). Therefore it is questionable whether stem growth is a good indicator of carbohydrate reserves when comparing trees with differing access to resources.

Unlike the fertilization treatment, thinning had no discernible impact on root starch concentration. The discrepancy between root starch concentrations in fertilized versus thinned plots might be explained by an additional carbon sink created by fertilization. Diameter growth in fertilized plots and in thinned plots was similar but only fertilization increased shoot growth in the crown. Therefore, fertilized trees likely allocated more carbon to the crown at the expense of carbohydrates in the roots. This is consistent with findings that increased foliar nitrogen content is linked to increased branch production in conifers (Amponsah et al., 2004; Mead et al., 1984; Snowdon and Benson, 1992).

Root starch concentrations consistently declined with increasing sapwood area, DBH, HLC, and CSA. Since the concentration of root starch in pines

mirrors the overall starch concentration throughout the tree (Ludovici et al., 2002), the small trees in our study likely had more carbohydrate reserves relative to their size than the larger trees. Therefore declining root starch concentration with increasing tree size is consistent with the finding that tree vigor declines with increasing tree height (Reid et al., 2004; Robichaud and Methven, 1991; Ryan, 1989). However, we should note that there were differences in both age and size of trees among our sites and we measured root starch concentration rather than root starch content. Hence we have difficulty isolating the effect of age and size on root carbohydrate reserves.

Of the predictors of root starch concentration that we compared, HLC and CSA were the most predictive. As a predictor of root starch concentration, HLC's predictivity may arise from an inverse relationship between carbohydrate translocation distance in the phloem and carbohydrate concentration in carbon pools (Minchin and Lacointe, 2005). Furthermore, strong negative relationships between root starch concentration and CSA suggest that the vascular cambium in the bole might function as an important carbon sink during the growing season thereby limiting the quantity of carbohydrates that reaches the root system. Others have noted the importance of growth in the stem as a sink for carbon due to the correlation between stem respiration and diameter growth (Maier, 2001). We suspect that growth, and carbohydrate translocation in the stem draw upon energy reserves in the stem before they reach the roots. Such a mechanism is consistent with the current understanding of carbon allocation priority: Carbon is first allocated to shoots and leaves, then to the vascular cambium, then to roots growth and finally to storage (Dickson, 1989; Minchin and Lacointe, 2005).

Fertilization compounded the negative effect of tree size on root carbohydrate reserves. This phenomenon is supported by reductions in the estimated regression coefficients relating tree characteristics and root starch concentrations in fertilized plots. The "a" coefficient of the CSA regression represents the rate at which root starch concentrations decrease with increasing cambial surface area. Lower "a" coefficients indicate that as CSA increases, root starch concentrations approach zero at a faster rate. The estimate for the "a" parameter was significantly lower in fertilized plots (Fig. 3.2D) than in the unfertilized plots. Thus the penalty on root starch concentration due to stem size was increased by fertilization and the starch reserves in the roots of large trees, which were already low, decreased still more when they were fertilized. Models of carbon partitioning in forests predict similar decreases of carbon allocation to the root system with increasing site nutrition (Landsberg and Waring, 1997).

In both the unfertilized and fertilized plots, the concentration of carbohydrate reserves in the root system increased with ring width. However, root starch concentrations decreased as ring volume increment increased. This apparent contradiction can be reconciled by considering how ring width and volume increment relate to tree size. Ring width was negatively correlated with DBH (Fig. 3.3) while ring volume was positively correlated with DBH. In this study, larger trees had smaller ring widths and lower concentrations of starches in their roots and smaller trees had larger ring widths and higher root starch concentrations in their roots. Therefore, when ring size is used as a predictor of root carbohydrate reserves across tree size classes, its predictive power comes from its correlation with tree size. To properly test ring size

(width, area or volume) as a predictor of carbohydrate reserves, root starch concentrations need to be regressed against ring size within each size class to control the confounding effect of size. Due to small numbers of trees within the plots and sites in this study, this was not possible. Nevertheless, the fact that trees in fertilized plots had larger rings but lower concentrations of root starch, indicates that diameter growth is not always a reliable predictor of carbohydrate reserves.

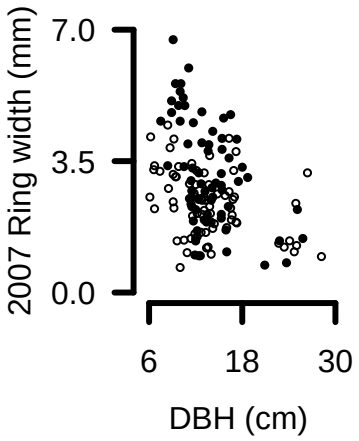

Figure 3.3: Ring width and diameter at breast height for individual trees from fertilized (solid) and unfertilized (open) plots.

Our results indicate that assuming a positive relationship between carbohydrate reserves and growth rates in the stem is tenuous when trees are fertilized. Past research supports this assertion: Fertilization reduced soluble carbohydrates in the stem despite rapid stem growth in Norway spruce (Kyto et al., 1996) and loblolly pine (Ludovici et al., 2002; Warren et al., 1999). Although Waring and Pitman (1985) proposed that reduced susceptibility of lodgepole pine after fertilization and thinning was the result of higher carbohydrate reserves, we believe increased carbohydrate storage after these

treatments was unlikely. The reduction in susceptibility observed by Waring and Pitman (1985) may be due to positive correlations between some tree defenses and radial growth rates: Resin duct densities, and resin exudation were positively correlated with growth rates of the xylem and phloem in ponderosa pine (McDowell et al., 2007).

When vigor index is used across a range of tree sizes and sites with varying resource availability, we found it to be poor predictor of carbohydrate reserves. In addition, expressing tree vigor as a ratio has two potential pitfalls. Firstly, ratio variables obscure the interpretability of their individual components by blending them together. The second problem arises when vigor index is calculated from increment cores wherein the measurement error associated with the calculation of the numerator (BAI) is likely correlated with the error in the denominator (sapwood area). In such cases estimates of error variance are unreliable (Jackson and Somers, 1991; Kronmal, 1993; Prairie and Bird, 1989).

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Chapter 4

The impact of phloem nutrients on overwintering mountain pine beetles and their fungal symbionts

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4.1 Introduction

The mountain pine beetle (*Dendroctonus ponderosae* Hopkins) outbreak in western Canada has killed 16.3 million ha of lodgepole pine forest in British Columbia over a ten-year period and has expanded northward and eastward beyond the previous limits of its range. Possible drivers of this expansion include climate change, winds favouring long distance dispersal (Jackson et al., 2008),

and an abundance of suitable host trees leading to large populations (Safranyik and Carroll, 2006). The growth of fungal symbionts may also influence mountain pine beetle population dynamics and epidemiology by increasing beetle fitness and winter survival (Safranyik et al., 2010).

When they attack pine trees, female mountain pine beetles bore through the outer-bark and inoculate the phloem and xylem of their host tree with fungal symbionts (Cole and Amman 1983, Safranyik and Carroll 2006) including *Grosmannia clavigera*, *Leptographium longiclavatum* and *Ophiostoma montium* (Rice et al., 2008). These fungi colonize the xylem and phloem alongside the maternal galleries where larvae later hatch and develop. Although mountain pine beetle brood can be reared without their symbiotic fungi (Whitney and Spanier, 1982), blue-stain fungi positively impact the survival of mountain pine beetle brood and *G. clavigera* is more beneficial to larval survival than *O. montium* (Six and Paine, 1998). The fungal symbionts of bark beetles likely benefit bark beetles by exhausting tree defences during mass-attack (Lieutier et al., 2009), by expediting the development of immature beetles (Barras, 1973), and by increasing phloem nutrients (Ayres et al., 2000; Klepzig et al., 2001; Bleiker and Six, 2007). Recently, Cook et al. (2010) found that the fungal associates of mountain pine beetle concentrate nitrogen in fungal-infected tissues and that beetles maturing in fertilized bolts are higher in nitrogen than those in unfertilized trees. Although the amplification of phloem nutrients by symbiotic fungi is often evoked to explain how bark beetles benefit from such symbioses, whether higher nutrient levels in the phloem do in fact lead to improved survival in immature bark beetles is unknown. Furthermore, no research has addressed how symbiotic fungi may impact other types of phloem

nutrition such as carbohydrate and lipid levels, and how this may impact bark beetles.

Mountain pine beetles overwinter as larvae. The later larval instars of mountain pine beetle can tolerate temperatures near -40 °C for several days (Yuill, 1941). However, in order to survive harsh winter temperatures, developing mountain pine beetles must reach their later larval instars prior to the coldest winter temperatures which generally occur between December and February. The egg and early instar larval life stages which occur before this period as well as the pupa and adult life-stages which occur after it, are all more susceptible to freezing (Safranyik and Carroll, 2006). Therefore, unseasonal temperatures early or late in the development cycle of the mountain pine beetle can lead to high mortality (Safranyik and Linton, 1991). The common fungal associates of the mountain pine beetle also have different temperature and moisture niches in which they perform optimally: *G. clavigera* and *L. longiclavatum*, are well adapted to the cool and moist climates typical of fall and spring in the boreal forest, while *O. montium* performs better in dryer and warmer conditions (Six and Paine, 1998; Rice et al., 2008).

Our primary objective was to determine whether the quality (as measured by lipid levels) and quantity of surviving mountain pine beetle progeny increase when they are reared in higher nutrient phloem. We also wished to determine whether the fungal associates of mountain pine beetle colonize more area and concentrate more nitrogen in high nutrient phloem than low nutrient phloem as this may influence the response of the beetles to fertilization. Because we expected mountain pine beetles and their fungal symbionts to respond differently to fertilization at different temperatures, we addressed these questions at

two locations: a northern location with cooler mid-winter temperatures, and a southern location with warmer mid-winter temperatures.

4.2 Materials and Methods

We selected 30 dominant or co-dominant lodgepole pine trees from a 120 year-old fire-origin stand 144 km West of Edmonton Alberta, Canada. We chose trees that were 25-35 cm in diameter at breast height (1.35 m), free of visible signs of damage or disease. Experimental trees were separated by at least 30 m and had a maximum of two large-tree competitors within an 8 m radius of the focal tree. We randomly selected 15 of the 30 trees for fertilizer treatment and fertilized a radius of 8 m around the bole (201.1 m^2 total area per tree) with a blend fertilizer in May 2009. The fertilizer prescription included 300 kg ha^{-1} nitrogen from urea and mono-ammonium phosphate; 100 kg ha^{-1} of phosphorus from mono-ammonium phosphate, 100 kg ha^{-1} of potassium from muriate of potash and potassium magnesium sulphate; and 32.5 kg ha^{-1} of magnesium from potassium magnesium sulphate; 75 kg ha^{-1} of sulphur from mono-ammonium phosphate and potassium magnesium sulphate, and 3 kg ha^{-1} of boron from granular borate.

In Late August 2009, we collected a phloem sample (3 $\times$ 3 cm) from the stem at a height of 1.5 m for measurement of phloem nitrogen levels. We felled the trees and cut four 50 cm long bolts from the bole between 1.2 m and 5 m from stump height (0.3 m). For the sake of brevity, we refer to bolts cut from fertilized trees as fertilized bolts and to bolts cut from unfertilized trees as unfertilized bolts. We randomly assigned each of the four bolts per tree to one of the following treatments: (1) fungus-inoculated southern location; (2)

fungus-inoculated northern location; (3) beetle-infested southern location; (4) beetle-infested northern location. We designed the experiment in two parts. In the first part we investigated fertilizer × location effects on mountain pine beetle development, survival and fat reserves. In the second part we investigated fertilizer × location effects on fungal growth and fungal concentration of nutrients in the phloem. We transported all of the bolts to the laboratory to be sealed top and bottom with paraffin wax and infested with mountain pine beetle or inoculated with their fungal symbionts.

Bolts were infested with mountain pine beetles caught in pheromone traps in the Saddle Hills North of Grande Prairie (North Central Alberta). For each of four insertion points, we placed a female beetle in an empty gel pill capsule that we taped against a 0.5 cm hole drilled through the bark at the lower end of the bolt. Once the female had commenced excavating a gallery and the female abdomen was no longer visible, we placed a male in the same capsule and taped it once again to the opening in the bark. If the female rejected the male, after 24 hours we replaced that male with another. We introduced four pairs of beetles in each of our 60 beetle-infested bolts at equally spaced intervals on the bole circumference. We introduced beetles to bolts in random order with respect to fertilizer and location treatment so that the fertilized bolts were not infested before or after unfertilized bolts and likewise for the bolts going to the northern and southern overwinter sites. We covered all beetle-infested bolts with aluminum screen to prevent accidental escape or predation. We stored the beetle-infested bolts in a covered but unheated shed at a research station South of Edmonton, Alberta for two weeks prior to transporting them to the northern and southern overwinter locations. Thus the mountain pine beetles

inside the experimental bolts were subjected to seasonal temperatures similar to what they would experience outside while they were stored.

For fungal inoculations, we isolated mutualistic blue-stain fungi from larvae and infected wood adjacent to galleries in infested logs from Grande Prairie Alberta, Canada. We incubated tissue samples or living larvae on malt extract agar (MEA) amended with 0.02% oxytetracycline dihydrate (Sigma-Aldrich) at room temperature in inverted sterile petri dishes sealed with parafilm. Once fungi had colonized the entire plate we isolated *G. clavigera* by re-plating samples taken from regions of the mixed culture that had morphological traits consistent with *G. clavigera*. We incubated the resultant fungal cultures at 4 °C and then repeated the previous step. After several iterations, we inspected the fungus under a light microscope and compared samples to pure cultures obtained from the Northern Forestry Centre in Edmonton, Alberta Canada to ensure that we had pure cultures. We isolated *L. longiclavatum* using the same method. For *O. montium* we used similar methods except we incubated at 30 °C rather than at 4 °C.

We inoculated two bolts per tree with our pure fungal cultures. Each fungus-inoculated bolt had four equally spaced inoculation positions on the circumference 25 cm above the base of the bolt. Three of these positions were occupied by the three fungal species that we isolated as described above, leaving a space without inoculation to serve as the phloem control. To inoculate, we bored a hole through the phloem and into the first layer of xylem using a sterilized cork borer (0.9 cm in diameter) as described by Colgan and Erbilgin (2011). We re-sterilized the cork borer by dipping it in ethanol and combusting the remaining alcohol, and then used it to cut a circular plug from the fungal

culture on MEA, and inserted the plug in the inoculation point. We held each inoculum in place with a sterilized wooden dowl and a strip of weather-proof duct tape. We inoculated in random order such that the fertilized bolts were not inoculated before or after unfertilized bolts and likewise for the bolts going to the northern and southern overwintering sites.

In mid-September 2009 we transported the 30 beetle-infested and 30 fungus-inoculated bolts to the Saddle Hills in North central Alberta, Canada (Lat. 55° 59' 0.02", Long. 119° 25' 0.53"). The elevation at the northern site was approximately 655 m. We hung the bolts from the boles of large pine trees so that they would remain above the snowline throughout the winter and thus be subjected to typical winter temperatures. We attached two temperature loggers (Hobo, Bourne, Massachusets, USA) to the boles of trees at a height similar to the heights of the suspended bolts (2 m). We followed the same procedures for the 30 beetle-infested and 30 fungus inoculated bolts in the southern site in Crowsnest Pass Alberta, Canada (Lat. 49° 38' 7.09", Long. 114° 27' 7.81") where the elevation was approximately 1310 m.

At the end of May 2010, we returned to the two overwinter locations to collect the beetle and fungal inoculated bolts. For fungal inoculated bolts, we used a sterilized chisel to sample a 9 cm^2 section (3 × 3 cm) of phloem from the area directly above the point of inoculation for each fungal species. We also collected a phloem sample from the uninfected region of the bolt and placed all phloem samples on dry ice. In the laboratory we sub-sampled a small portion of fungal infected phloem to confirm the identity of our fungi by re-culturing it on 2% MEA. We recovered the original fungal species from 10 of the 15 subsamples we re-cultured for *Grosmannia clavigera*; from 12 of the

15 subsamples we re-cultured for *Leptographium longiclavatum*; and from 10 of the 15 subsamples we re-cultured for *Ophiostoma montium*. In our subsequent analyses, we excluded samples if we did not successfully re-culture the fungi that we originally inoculated. We dried the remaining sample at 70°C in preparation for total nitrogen and total non-structural carbohydrate analysis. To record fungal growth we removed the bark from the fungus-inoculated bolts and traced onto plastic film the visible extent of fungal spread as seen at the vascular cambium (Fig. 4.1). We later quantified the area of phloem colonized by each fungus using a scanner and image analysis software (SigmaScan, San Jose, California, USA).

Unlike the fungus-inoculated bolts which we processed in the field, we brought the beetle-infested bolts back to the laboratory between May 27 and May 30, 2010 where they were stored at room temperature until dissection on June 1 and 2. Therefore bolts remained indoors for up to five days at room temperature before we dissected them. Fertilized and unfertilized bolts did not differ in the amount of time that they were stored but bolts that overwintered at our northern site arrived two days prior to the bolts that overwintered in the South. On June 1 and 2, we carefully removed the bark from the beetle-infested bolts. From the four locations per bolt where a pair of beetles was introduced, we excluded galleries in which it was difficult to make out pupal chambers due to the activity of saprophytic fungi and bacteria. We randomly selected a gallery from those that remained and traced the maternal gallery, larval galleries, and pupal chambers onto plastic film. Of the 240 locations where we introduced beetles, 223 had full length maternal galleries with larval galleries and pupal chambers. We collected all living mountain pine beetle

Figure 4.1: An example of the extent of fungal spread at the end of May for fungal symbionts of the mountain pine beetle. The fungi visible in the centre and on the left were inoculated into the waxed bolt the previous fall (September 2009).

stages from all of the galleries in each bolt and placed them in the freezer for follow-up analysis.

4.2.1 Chemical analysis

Prior to our measurement of larval lipids, we separated dark-coloured larvae that were still alive from white larvae. Dark coloured larvae resembled white larvae in that they were still moving, and still plump. However, outlines suggested the presence of dark material within the larval bodies (Fig. 4.2). We assumed that this dark-coloration indicated the presence of fungal infected phloem in the alimentary canal of the larvae but this has not been confirmed.

Figure 4.2: A living larva exhibiting the dark tinged characteristic in the middle and anterior regions mentioned in the text.

To quantify total lipids in larvae, we used the method of Hagen and Atkins (1975). We transferred beetle larvae from the -20 °C freezer to a 70 °C oven. Throughout the drying process, we reweighed one larval carcass until its weight no longer changed. We dried the larval corpses for one additional day (a total of three days) before recording the weight of each individual corpse. We then

extracted fats for eight hours using petroleum ether in a soxhlet apparatus and re-dried the carcasses under a fume hood before weighing them again. We estimated the total lipids (in g) by taking the difference of the pre and post-extraction weights.

We oven dried the frozen fungal infected phloem samples at 100 °C for 1 hr to stop enzymatic conversion of starch to sugars, and then dried them to constant weight at 70 °C. After drying, we ground the dried samples using an electric mill fitted with a 40 mesh screen (Wiley, Thomas Scientific, USA). We determined total starch and sugar concentrations colorimetrically (Chow and Landhäusser, 2004). Briefly, we extracted sugars from 50 mg of ground tissue in 80% hot ethanol. We then reacted the sugars with phenol-sulphuric acid before colorimetric measurement at a wavelength of 490 nm. We enzymatically digested starches remaining in the residual pellet and reacted the resultant glucose hydrolyzate with peroxidase-glucose oxidase/o-dianisidine (color reagent). Using a spectrophotometer (Pharmacia LKB Ultrospec III, Sparta, NJ, USA), we measured glucose hydrolyzate (starch) concentrations at a wavelength of 525 nm.

We measured fats in a subset of our phloem samples infected with *G. clavigera* that we dried and ground as described above. We employed a modified Bligh & Dyer method combined with a Folch wash (Nelson and Dickson, 1981) to extract the fats followed by thin layer chromatography (TLC) to separate the triglycerides, free fatty acids and diglycerides from other lipids. We eluted the triglycerides, free fatty acids and diglycerides from the TLC strip and then re-absorbed them into pre-weighed filter paper for gravimetric measurement.

For total nitrogen analysis, we quantified the TN concentrations in fungal

infected phloem as well as in uninfected phloem that we had dried and ground as described above. We ran the samples on an elemental analyzer (Costech Elemental Analyzer 4010 CHNS, Pioltello, Milano, Italy) using the Dumas combustion method.

4.2.2 Statistical analysis

In general, a split-plot statistical analysis is appropriate for our experimental design in which we divided fertilized and unfertilized trees into two sections and subjected these to one of two winter temperature treatments. However, the statistical models we used to analyze different components of our experiment varied slightly from one analysis to the next and so we provide details for each below.

To test for a fertilizer effect on phloem percent nitrogen, we used a Welch's t-test on log transformed percent nitrogen data.

For counts of larval galleries and pupal chambers, we used a generalized linear mixed model (glmm) based on the Poisson distribution to estimate main effects and interactions between main effects. The glmm we used preserved the split-plot structure of our experiment:

$$y_{i,j,k} \sim Poisson(\Theta_{i,j,k}),$$
$$\log \Theta_{i,j,k} = \mu + \tau_i + \omega_k + \tau * \omega_{i,k} + \beta_j,$$

The y term represents the observed number of galleries or pupal chambers. The Θ parameter represents the expected number of galleries or chambers. The μ term is roughly the mean population effect, the τ term represents the

treatment effect (i = 1 or 2 for fertilized or control), the ω term represents the effect of overwinter location (i = 1 or 2 for the northern site or the southern site), and the $\tau * \omega$ term represents the interaction between the fertilizer treatment and the overwinter location treatment. The β term represents the random effect of tree j where the mean random effect is centred on the population mean (μ):

$$\beta_j \sim (0, {\sigma_r}^2).$$

Glmm statistical models are currently in development and provide only rough estimates for p-values (Zuur et al., 2009). As standard ANOVA tables are unavailable for these types of models, we tested the importance of the main effects (Fertilizer treatment, overwinter location treatment, and interaction) by building nested models that included or excluded each main effect and using likelihood ratio tests to compare them—an approved approach for inference for glm models (Venables and Ripley, 2009). In our statistics for likelihood ratio tests, we report 1 degree of freedom. This does not refer to a small sample size for constructing the models but rather to the nested nature of the models. To calculate confidence intervals for each treatment mean, we separated our raw data according to treatment, we then sampled from the data 2000 times under the assumption that they followed a Negative Binomial distribution (overdispersed Poisson) to build bootstrap samples from which we calculated means and approximate 95% CI.

To compare the total number of living life stages in our southern bolts, we used glm models assuming a Negative Binomial distribution:

$$y_{i,j} \sim NB(\Theta_{i,j}, k),$$
$$Var(y_{i,j}) = \Theta_{i,j} + \frac{{\Theta_{i,j}}^2}{k},$$
$$\log \Theta_{i,j} = \mu + \tau_i.$$

Here the y term represents the count data for total number of living life stages that we collected or the number of pupae and teneral adults collected. The Θ parameter roughly represents the expected number of counts of living life stages. The k parameter represents the lack of overdispersion. As $k \to \infty$, the Negative Binomial distribution converges to the Poisson distribution (Zuur et al., 2009). The μ and τ parameters have the same interpretations as in the models we describe above. We used a likelihood ratio test to evaluate the support in the data for a fertilizer effect.

For larval lipid content we natural log transformed the lipid data (% dry wt). We then fit a split-plot ANOVA model as follows:

$$y_{i,j,m} \sim N(\Theta_{i,j,m}, {\sigma_e}^2),$$
$$\Theta_{i,j,m} = \mu + \tau_i + c_m + \tau * C_{i,m} + \beta_j,$$
$$\beta_j \sim N(0, {\sigma_r}^2).$$

The y term represents the natural log transformed lipid data on a bolt level (where m is the index for dark or light larvae). The Θ parameter represents the expected value of the log transformed lipid data. The τ parameter represents the fertilizer treatment as before. However, the c parameter is new and represents the effect of larval color on lipid levels, while the $\tau * c$ term represents

the interaction between larval color and fertilizer treatment. In addition, the variance term for the likelihood and the variance for the random effect are distinguished by their subscripts (e and r respectively). We used F-statistics and the associated p-values from standard ANOVA tables to evaluate the evidence against main effects. We built 95% CI using the standard errors returned by the statistical model in R.

To quantify the effect of fertilization and overwinter location on phloem area colonized and the concentration of nitrogen in phloem tissue we applied normal ANOVA models to natural logarithm transformed data:

$$
\begin{aligned}
y_{i,j,k} &\sim N(\Theta_{i,j,k}, {\sigma_e}^2), \\
\Theta_{i,j,k} &= \mu + \tau_i + \omega_k + \tau * \omega_{i,k} + \beta_j, \\
\beta_j &\sim N(0, {\sigma_r}^2).
\end{aligned}
$$

The y term represents the natural log transformed data. The remaining parameters are interpreted as described above. We used F-statistics and the associated p-values from standard ANOVA tables to evaluate the evidence against main effects. We built 95% CI using the standard errors returned by the statistical model in R.

We did not require a test to determine that the concentration of total non-structural carbohydrates in the phloem of our experimental bolts prior to beetle-infestation was different in the fall and the following spring as the data did not overlap at all. We used a Welch two sample t-test to evaluate the null hypothesis that there was no difference in nitrogen concentration in phloem that was inoculated or not inoculated with the three fungal symbionts of mountain pine beetle used in this study. We performed separate tests for

each of the three fungal species and for phloem from fertilized trees and unfertilized trees. We also used a Welch two sample t-test to evaluate the null hypothesis that there was no difference in the major lipid levels in phloem inoculated with *Grosmannia clavigera* and phloem that was not inoculated.

Because each of the treatments in our analyses contained only two levels, post hoc-tests were unnecessary except when interactions were evident. In such cases, we visually compared the overlap of 95% CI rather than perform post-hoc tests. Confidence intervals can be used by readers to visually approximate two sample comparison tests at whatever alpha level they choose (Cumming and Finch, 2005; Payton et al., 2000). If confidence intervals of two means overlapped by less than half of the length of the confidence intervals, we considered the evidence to be strong that the means are not the same. This interpretation is roughly equivalent to performing a conservative two sample t-test on normally distributed data with an alpha level less than 0.05 (Cumming and Finch, 2005). We did all of our statistical analyses using the R program (R Development Core Team, 2011) and the lme4 (Bates et al., 2011) and nlme (Pinheiro et al., 2011) packages for mixed models. We also used the MASS (Venables and Ripley, 2009) package in R for analyses involving negative binomially distributed data.

4.3 Results

The fertilizer treatment resulted in higher mean total nitrogen concentrations in the phloem of fertilized trees than in unfertilized trees ($p = 0.004524$, df = 27.095). Mid-winter minimum temperatures (November 2009 to March 2010) were consistently 4-6 °C cooler at the northern site than at the south-

ern site (Table 4.1). However, minimum temperatures in October were 10°C cooler in the South and equivalent in the North and South in April and May (Table 4.1). In addition, 93 degree-days above 5°C were accumulated at the northern location by the end of May whereas only 31 degree-days above 5°C were accumulated at the southern location by this time.

Table 4.1: Monthly minimum temperatures and cumulative degree days per month in which temperatures exceeded 5 °C at the northern and southern overwinter sites

Month	Min. N	Min. S	DD.N	DD.S
Oct.	-8.9	-18.8	4.88	3.06
Nov.	-14.1	-7.3	4.88	4.56
Dec.	-35.1	-27.1	4.88	4.56
Jan.	-31.3	-25.3	4.88	4.56
Feb.	-17.4	-13.4	4.88	4.56
Mar.	-13.8	-8.5	10.76	4.56
Apr.	-8.1	-8.9	42.16	15.34
May	-4.8	-5.2	93.10	31.02

4.3.1 Fertilization and location effects on mountain pine beetles

There was little support in the larval gallery data for a model with a fertilizer treatment by location treatment interaction ($p = 0.5532$, df = 1) nor was there support for a fertilizer effect ($p = 0.8692$, df = 1) or a location effect ($p = 0.7118$, df =1). Therefore there was little evidence that the fertilizer treatment impacted the number of larval galleries in our experimental bolts. However there was strong evidence of an interaction between the fertilizer treatment and the location treatment in the number of pupal chambers in the

experimental bolts (emphp = 0.001322, df = 1). There were higher counts of pupal chambers in fertilized than unfertilized bolts at the northern location and the 95% CI did not overlap (Fig. 4.3a).

Few mountain pine beetles survived until early June at the northern site in any life-stage. Individuals that survived were equally divided amongst the larval, pupal and teneral adult life-stages (Fig. 4.3b). There was some evidence, however, that fertilized bolts produced more living individuals than unfertilized bolts at the southern location ($p = 0.08233$, df = 1). In addition, at the southern location, there was strong evidence that more individuals reached the pupal and teneral adult stages by early June in fertilized bolts than in unfertilized bolts ($p = 0.005562$, df = 1).

In the South, where we collected more living larvae, white larvae from fertilized bolts had higher lipid levels than white larvae from unfertilized bolts ($p = 0.0046$, df =154). Dark-coloured larvae had high lipid levels that were equivalent to those we observed in white larvae reared in fertilized bolts regardless of whether they were reared in fertilized or unfertilized bolts (Fig. 4.4). However, when the dark and light coloured larvae were pooled, there was much less evidence for an effect of fertilization on larval lipids ($p = 0.15$, df = 17).

4.3.2 Fertilization and location effects on mountain pine beetle fungi

Neither fertilization nor overwinter location had an obvious effect on the area of phloem colonized by *G. clavigera*, *L. longiclavatum*, or *O. montium* (Fig. 4.5). However, there was strong evidence that the total nitrogen concen-

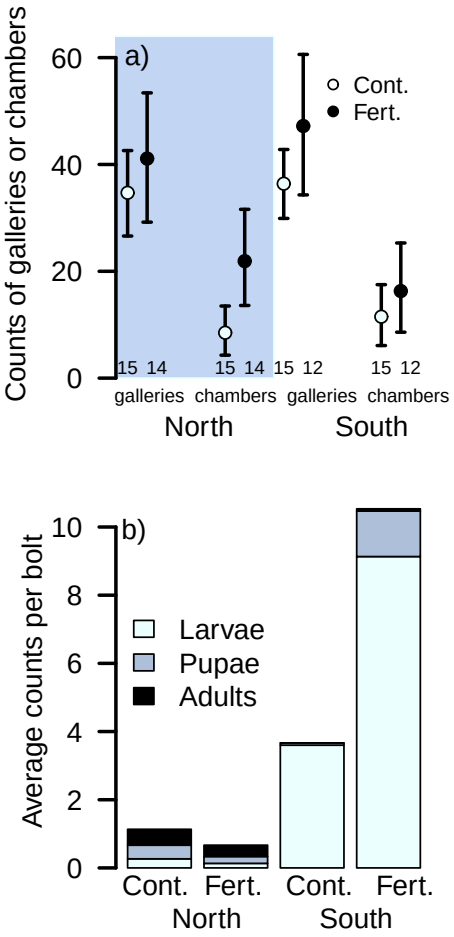

Figure 4.3: The mean counts of a) larval galleries and pupal chambers (and 95% CI) in unfertilized (Control) and fertilized bolts that overwintered at the northern and southern sites. The mean number of b) living beetle larvae, pupae and teneral adults (and 95% CI) collected per bolt in bolts cut from fertilized and unfertilized trees stored at the northern and southern sites. The sample sizes for each mean and 95% CI are shown in the figure below the points.

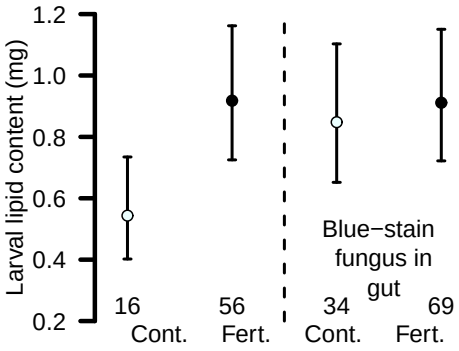

Figure 4.4: Mean lipid content (and 95% CI) in larvae in unfertilized and fertilized bolts that overwintered at the southern site. Larvae were divided into two groups according to whether they had dark stain, possibly from having consumed blue-stain fungi, in their digestive tracts. The sample size (n) for each mean and 95% CI are shown in the figure below the points. Note that the n values listed are for larvae within bolts in the statistical analyses presented.

tration in the phloem of fertilized bolts that we inoculated with *G. clavigera* or *L. longiclavatum* was much higher than the concentration in the phloem of fertilized bolts that were not inoculated ($p = 0.003168$, df = 6.133; and $p = 0.01812$, df = 4.371 respectively). There was little evidence in our data to support a finding of a similar increase due to inoculation in our unfertilized bolts (Fig. 4.6).

Total non-structural carbohydrates dropped from approximately 6% to about 1% in all phloem samples over the course of the winter whether they were inoculated or not (Fig. 4.7a). There was little evidence that lipid levels (sum of triglyceride, fatty acid and diglyceride concentrations) in phloem inoculated with *G. clavigera* were different from those in phloem that was not inoculated ($p = 0.2876$, df = 4) (Fig. 4.7b).

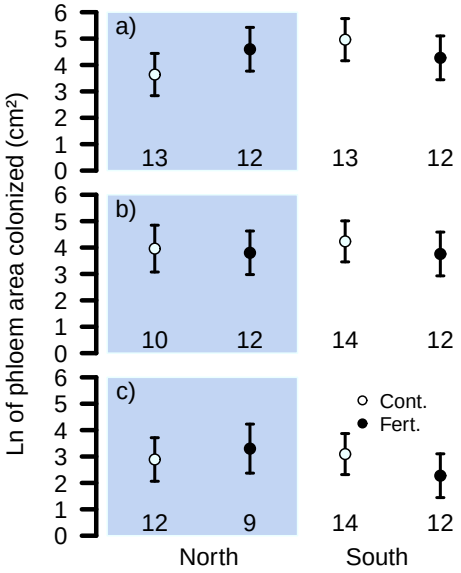

Figure 4.5: The mean area of phloem (and 95% CI) colonized by a) *Grosmannia clavigera* (GC), b) *Leptographium longiclavatum* (LL) and c) *Ophiostoma montium* (OM), in bolts cut from unfertilized (Cont.) and fertilized (Fert.) lodgepole pine. Bolts were stored overwinter either at the northern or southern site. The data were natural logarithm transformed. The sample sizes for each mean and 95% CI are shown in the figure below the points.

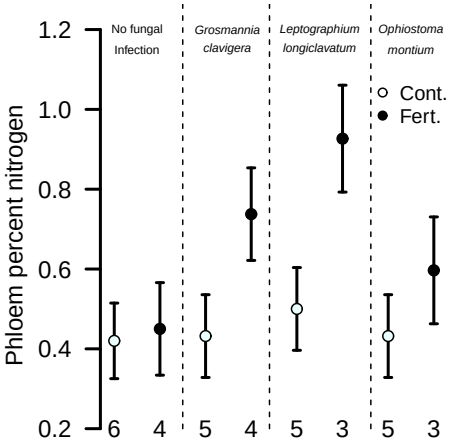

Figure 4.6: The mean total nitrogen concentration of phloem (and 95% CI) from bolts cut from unfertilized (Cont.) or fertilized trees 9 months after they had been left uninfected, or inoculated with *G. clavigera*, *L. longiclavatum*, or *O. montium*. Due to similar responses in the northern and southern locations, results for the location treatment are pooled. The sample sizes for each mean and 95% CI are shown in the figure below the points.

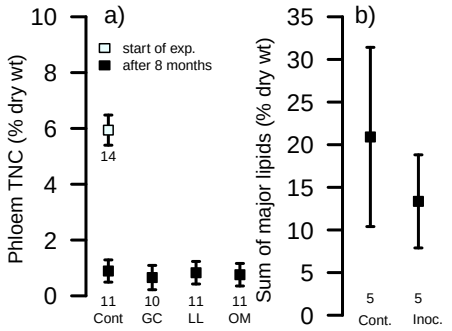

Figure 4.7: Means (and 95% CI) for a) non-structural carbohydrate concentrations in lodgepole pine phloem at the beginning of the experiment and after an 8 month overwinter period. Phloem was either uninfected (Cont.), or inoculated with *G. clavigera* (GC), *L. longiclavatum* (LL), or *O. montium* (OM). Means (and 95% CI) for b) concentration of major lipids (triglycerides, fatty acids and diglycerides) in uninfected phloem (Control) and phloem inoculated with *G. clavigera*. Due to similarity, results for unfertilized and fertilized bolts and the northern and southern locations are not shown separately. The sample sizes for each mean and 95% CI are shown in the figure below the points.

4.4 Discussion

Fertilization impacted beetles differently at the northern and southern overwinter locations. At the southern location, the enhanced pupation rate in fertilized bolts corresponded to an increased number of surviving pupae and teneral adults while at the northern location increased pupation rates in fertilized bolts were evident in increased numbers of pupal chambers. However by June, progeny had died in high numbers after constructing pupal chambers in both fertilized and unfertilized bolts that overwintered at the northern site. Although consistently cooler temperatures from November to March may have resulted in higher cumulative mortality at the northern location than at the southern location (Table 4.1), we are uncertain of the cause of high mortality of individuals that constructed pupal chambers in fertilized bolts. At the southern location, there were more pupae and teneral adults in fertilized bolts than in unfertilized bolts indicating that fertilization may increase development rates. Thus, accelerated development rates in high nutrient phloem may have resulted in higher mortality rates at the northern site as mountain pine beetle progeny developed more quickly into pupae and teneral adults which are more susceptible to cold than are larvae (Bentz and Mullins, 1999). In a modeling study Jonsson et al. (2007) reported that accelerated development rates render *Ips typographus* more susceptible to mortality due to cold temperatures. The current temperature-based model for mortality in mountain pine beetles incorporates only the impact of minimum winter temperatures on survival (Regniere and Bentz, 2007). Our results suggest that the realism of mountain pine beetle mortality models may be enhanced by incorporating spring mortality.

In the current study, we distinguished white and dark-coloured larvae and found that white larvae reared in fertilized bolts had higher fat reserves than those reared in unfertilized bolts. However, dark larvae had elevated lipid levels similar to those of white larvae reared in fertilized bolts, regardless of whether they came from fertilized or unfertilized bolts. We suspect that dark tinged larvae may have appeared so because they had large quantities of phloem and blue-stain fungi in their digestive tract. However, finding dark coloured larvae was not an objective of this study and we therefore have no means of determining the cause of the dark color we observed. Other researchers have not reported similar dark tinged larvae even in larvae that had consumed blue stain fungi. Furthermore, when dark and white coloured larvae are pooled, there is much less evidence that fertilization increased larval lipids. Thus conclusions from our larval lipid data are tentative and this area of enquiry requires further research.

The growth of the fungal symbionts of mountain pine beetle did not appear to be limited by nutrients as they did not colonize more phloem in fertilized bolts. However, *G. clavigera* and *L. longiclavatum* consistently colonized larger areas of phloem than *O. montium*. We suspect this occurred because *G. clavigera* and *L. longiclavatum* had higher growth rates than *O. montium* at temperatures between 5 and 15°C whereas *O. montium* grew faster between 25 and 30°C as suggested by Rice et al. (2008). In the current study, temperatures in the fall when mountain pine beetle fungi would be growing (Bleiker and Six, 2009*a*), never reached 25°C and were usually below 15°C. Furthermore, the conditions inside waxed bolts are likely moister than inside naturally infested trees. Both *G. clavigera* and *L. longiclavatum* respond positively to

moist growing environments whereas *O. montium* grows better in drier conditions (Bleiker and Six, 2009*b*). Therefore growing conditions likely favoured *G. clavigera* and *L. longiclavatum*. The trend we anticipated, that colder temperatures would limit the growth of mountain pine beetle fungi at the northern site, was not evident in part because our overwinter location treatment did not correspond as closely as we anticipated to cooler winter conditions in the North and warmer winter conditions in the South. There were equal numbers of degree-days above 5°C in the northern and southern locations in the fall and more degree days above 5°C in the spring at the northern site. As the fungal symbionts of mountain pine beetle are likely most active in the fall, these patterns in degree-days at the northern and southern locations may explain why we did not see the reduced fungal growth in the North that we expected.

All fungal symbionts of mountain pine beetle increased the nitrogen concentration near the point of inoculation in fertilized bolts and this was particularly apparent for *L. longiclavatum* and *G. clavigera*. Our results appear to corroborate the *in vitro* findings of Cook et al. (2010) that *O. montium* is a less efficient nutrient concentrator than *G. clavigera*. We did not observe a concentrating impact of symbiotic blue-stain fungi on nutrient levels in unfertilized phloem but our sample size was small. The fungal associates of bark beetles likely concentrate nutrients in their hyphae and conidia which inhabit areas near the feeding chambers of bark beetles (Ayres et al., 2000). In our study, the fungal symbionts of mountain pine beetles were less capable of concentrating nitrogen in unfertilized than in fertilized trees. Thus we propose that the hyphae of these symbionts absorb available forms of nitrogen such as nitrates, ammonium or amino acids, much like the roots of plants, but they

are less able to access nitrogen that is bound in structural tissues.

Due to cellular respiration, as well as the action of fungi and other microbes, the nutritional quality of phloem likely changes throughout the development period of mountain pine beetles. In our experiment, the TNC concentration dropped from around 6% to around 1% between mid-September 2009 and the end of May 2010. This occurred whether or not we inoculated the phloem with blue-stain fungus and regardless of the species of blue-stain fungus. Respiration in plant cells continues after they are removed from the plant (Bett-Garber et al., 2011). Because TNC levels also dropped in uninfected phloem, we suspect that the drop in TNC occurred not due to fungal consumption of TNC, but because phloem cells remained alive and respired carbon during the fall and spring.

Phloem fats may be another important source of nutrition for mountain pine beetles. We found large quantities of lipids (13-20%) in the phloem of our experimental trees. Although infection of the phloem with *G. clavigera* appeared to cause a slight decrease in the major phloem lipids, this difference may have resulted from the conversion of lipids into ergosterol or other fat sources accessible to mountain pine beetles (Bentz and Six, 2006). Over the course of the winter, TNC concentrations dropped but phloem lipids remained high in phloem that was uninfected and infected with blue-stain fungus. Due to their continuous availability, phloem lipids in pine trees may be a crucial source of nutrition for bark beetles.

In the present study, we fertilized lodgepole pine trees and provided evidence that bark beetles benefit from inhabiting fertilized trees: larvae in fertilized bolts were more likely to build pupal chambers at our northern site and

we collected more living pupae and teneral adults from fertilized bolts than unfertilized bolts at our southern site. Furthermore, our inoculation of the phloem of fertilized trees with *G. clavigera* and *L. longiclavatum* resulted in a two-fold increase in total nitrogen concentrations relative to phloem that received no fungal inoculum. It is known that the fungal symbionts of bark beetles transmit the nutritional benefits of inhabiting nutrient rich phloem to bark beetles when they are consumed by beetle larvae. In this study we found evidence that this effect is especially pronounced when the concentration of nutrients in the phloem has been increased by fertilization.

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Chapter 5

Do large outbreaks of a lepidopteran defoliator facilitate a stem colonizing bark beetle at a landscape-scale?

5.1 Introduction

Interspecific competition and facilitation are ubiquitous and important factors shaping insect communities (Kaplan and Denno, 2007). In facilitation, one species benefits from an association without harming its benefactor (Bruno et al., 2003). Although such interactions between insects typically happen on relatively small scales, small changes may have large-scale consequences if they enable insects to overcome thresholds that normally limit their population growth (Raffa et al., 2008). Thus, if facilitation at a smaller scale impacts the population dynamics of herbivorous insects, effects may accumulate until they become evident across landscapes when outbreaks occur. However, factors that predict population processes in pre-outbreak conditions are often no longer predictive during outbreaks because positive feedbacks and density-dependent effects take over as the dominant drivers of outbreak dynamics (Raffa et al., 2008). Therefore, if facilitation enables insect populations to

irrupt, its role as a driver of population dynamics may be diminished once outbreaks have peaked. Although there are some studies of predator-prey interactions at larger scales (Thies et al., 2003; Bianchi et al., 2010), landscape studies of interactions between insects remain sparse (Cronin and Reeve, 2005). No prior study has investigated facilitative interactions between insect species at a landscape level.

Our knowledge about plant-mediated interactions between phytophagous insect species originates from studies that determined how changes in plants instigated by one insect species affect the survival, behaviour or reproductive success of a second attacking species (Wright et al., 1984; Bowers et al., 1996; Raffa et al., 1998; Annila et al., 1999). The initial attack may render a tree more resistant by priming its defenses (Annila et al., 1999; Erbilgin et al., 2006). Alternatively, the tree may become more susceptible if the earlier attack weakens its defenses or increases the palatability of its tissues (Raffa et al., 1998). Defoliation of conifers by lepidopterans often has a beneficial impact on bark beetle species that subsequently attack the boles of defoliated trees (Wright et al., 1984; Bowers et al., 1996; Raffa et al., 1998). Thus, tree-mediated interactions between herbivorous insects may be competitive when an attack by one species results in increased tree resistance to other species, or they may be facilitative when an attack by one species increases tree susceptibility. Regardless of the outcome, tree-mediated interactions provide mechanisms whereby indirect relationships between phytophagous insects may impact their reproduction, survival and possibly, their epidemiology.

In British Columbia (BC), Canada, the ranges of the two-year cycle spruce budworm (*Choristoneura biennis* [Freeman]) and the spruce beetle (*Dendroc-*

tonus rufipennis [Kirby]) overlap in time and space, and both insects cause widespread damage (Fig. 5.1). Outbreaks of *D. rufipennis* in BC resulted in the loss of over seven million m^3 of lumber from 1974 to 1999 (Holsten et al., 1999). Meanwhile in large-scale outbreaks that have occurred periodically at approximately 32 year intervals, *C. biennis* causes top-kill, tree mortality, and loss of timber volume (Zhang and Alfaro, 2002). Subalpine fir (*Abies lasiocarpa* [Hook.] Nut.), Engelmann spruce (*Picea engelmanni* [Parry]), and interior spruce (Engelmann × white spruce hybrid) trees are defoliated by *C. biennis* (Zhang and Alfaro, 2002). All North American species of spruce are susceptible to *D. rufipennis* attack (Holsten et al., 1999). Thus in BC, *D. rufipennis* and *C. biennis* share two host species: Engelmann and interior spruce. The objectives of this study were to determine whether defoliation by *C. biennis* facilitates *D. rufipennis* outbreaks at a landscape-scale and to determine whether this effect varies in importance depending on the stage of spruce beetle outbreaks.

5.2 Materials and Methods

5.2.1 Study Area

My study area included all regions of British Columbia, Canada, where outbreaks of either *C. biennis* or *D. rufipennis* occurred between the years 1978 and 2010—a diagonal strip running from the southeast to the northwest corner of the province (Fig. 5.1). Most of the outbreak activity of both insects occurred in the sub-boreal spruce (SBS) and the Engelmann spruce-subalpine fir (ESSF) biogeoclimatic zones. The climate of the SBS zone is

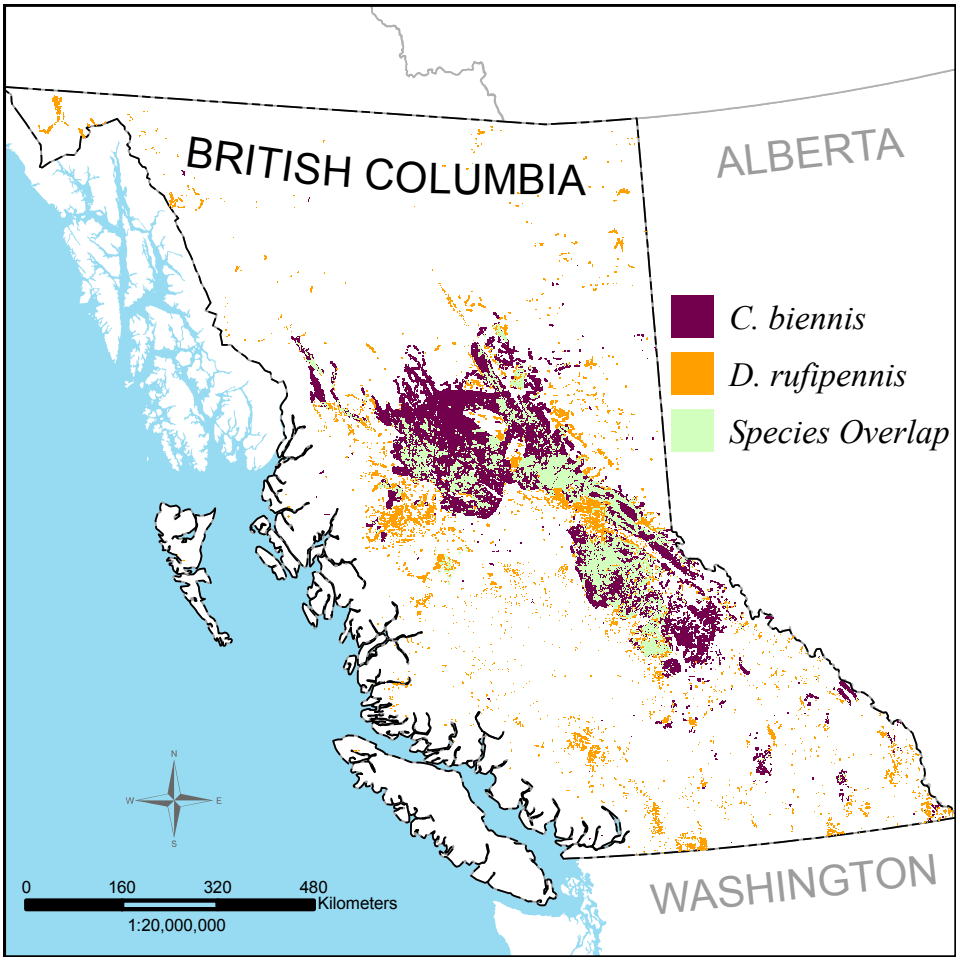

Figure 5.1: The ranges of the two-year cycle spruce budworm (*Choristoneura biennis*) and the spruce beetle (*Dendroctonus rufipennis*) in British Columbia, Canada, and their overlap.

continental, and it therefore exhibits seasonal extremes resulting in severe winters and warm, moist summers (Meidinger et al., 1991). The sub-boreal landscape is characterized by upland coniferous forests wherein interior spruce and subalpine fir trees predominate as climax species (Meidinger et al., 1991). The climate of the ESSF zone is more extreme than that of the SBS zone in that growing seasons are cooler and shorter while winters are colder (Coupé et al., 1991). At lower elevations Engelmann spruce usually dominates the canopy of mature stands, but at high elevations, and in some wetter areas, subalpine fir may out-compete Engelmann spruce (Coupé et al., 1991).

5.2.2 Study Insects

Choristoneura biennis moths emerge as adults from mid-July to early August, mate, and oviposit about 150 eggs on the undersides of needles. Eggs hatch within two weeks and larvae emerge without feeding to overwinter in hibernacula in the bark of host trees in their second instar. In late-May to early June of the following year, larvae begin to mine needles for the first time. They spend their second winter as fourth instar larvae and emerge the following spring to continue feeding. Damage in this second year of feeding is more extensive than in the first (Nealis and Turnquist, 2003). Individuals pupate and then eclose as adults in mid-July to complete their two year life cycle.

The life-cycle of *D. rufipennis* occurs over one to three years, but throughout most of its southern range in North America, it is completed in two years or less (Holsten et al., 1999). The life cycle information that follows is summarized from Holsten et al. (1999). Adult female beetles typically emerge in early summer and colonize new host trees by boring through the outer bark

to excavate maternal galleries. Host tree mortality, mediated by mass-attack, is required at this stage for successful production of beetle broods. Male and female beetles mate and females deposit eggs along the gallery walls. Eggs hatch in August, and newly hatched larvae bore outwards from the maternal gallery, initially feeding as a group, before excavating individual feeding tunnels. Immature spruce beetles typically spend their first winter as larvae—some of which will then pupate in the early summer. Prior to their second winter, pupae have typically eclosed as juvenile adults. Some juvenile adults overwinter in pupal chambers, but the majority emerge and re-enter the lower section of boles in the fall to minimize mortality due to cold and woodpeckers. Individuals emerge and colonize new host trees early in the following summer. Although functional tree death occurs in the year of successful attack, it is not visible until the following year when foliage begins to fade.

5.2.3 Data

The data on *C. biennis* and *D. rufipennis* infestations in BC were collected by aerial sketch-mappers who mapped infected areas from an aircraft as part of the forest insect monitoring programs of the Canadian Forest Service and the BC Ministry of Forests (Protocol described in the Forest Health Aerial Overview Survey Standards for British Columbia). Damage caused by *D. rufipennis* infestation was detected based on distinctive foliage discoloration which is visible in the second summer after attack (Holsten et al., 1999). Therefore, infestation by *D. rufipennis* was observed one year after the initial attack occurred, whereas defoliation by *C. biennis* was recorded in the year of occurrence. Areas affected by bark beetles and defoliators were

typically mapped in the same flights which were scheduled between mid-June and early-September.

In addition to data on the area of infestations, the maps contained nominal information on infestation severity: local attacks were categorized as trace when less than 1% of trees in the area were affected, light when 1 – 10% were affected, moderate when 11 – 30% were affected, severe when 31 – 50% were affected and very severe when more than 50% of trees were affected. As the trace and very severe categories were only added in 2004, we reverted these to light and severe respectively. The Canadian Forest Service digitized the data in the maps using the Albers Equal Area projection (Datum: NAD83). Thus, infested areas on the maps were represented by polygons in the resulting ArcGIS (ESRI, Redlands, CA) shape files. Aerial survey data of this type are bound to contain some inaccuracies including detection bias, and errors due to the lack of ground truthing (Aukema et al., 2008). However, these deficiencies are less important when searching for patterns at a landscape scale (Aukema et al., 2006, 2008).

Using ArcGIS software, I converted polygon data to grid data by overlaying a lattice of 1 $\times$ 1 km squares. Grid squares were assigned an integer value depending on the severity of attack (1-light, 2-moderate, 3-severe) in the polygon underneath. When a grid square was intersected by polygons of differing severities in a single year, it was defined as belonging to the more severe class (rounding up). Using the same grid, we also converted data for each insect to binary form (0-not infested, 1-infested) so that we could use logistic regressions to predict insect infestations in grid squares. To avoid the effects of edges near provincial boundaries and bodies of water on the distribution

of the study organisms, I excluded grid cells in a 100 km buffer around the outside edge of our study region. Provincial boundaries do not limit insects, but they do limit the availability of data. The data only included areas inside the province of BC.

Based on the biology and ecology of *D. rufipennis*, I chose variables that would be useful for predicting its presence or absence on the landscape (Table 5.1). Some of these variables were dynamic as we had data recording how they changed over the study period, while others were static. Most dynamic variables comprised climate data that were spatially interpolated over the study area using ANUSPLIN software (Hancock and Hutchinson, 2006) which interpolates by fitting thin plate smoothing splines to climate data (McKenney et al., 2011).

Table 5.1: Climate and environmental variables used to predict locations of spruce beetle (*Dendroctonus rufipennis*) infestation in British Columbia, Canada. Data were available on a yearly basis for climate variables but not for environmental variables.

Variable type	Variable	Description
Climate	dd	Degree days ($> 5°C$)
	SpPrecip	Mean spring precip. (Mar, Apr, May)
	MinT	Min winter temp
Environmental	EngSpCov	Percent Engelmann spruce cover
	WtSpCov	Percent white spruce cover
	SubFirCov	Percent subalpine fir cover
	elev	Digital Elevation Model (DEM) elevation estimates

When using climate-related variables to predict the dynamics of insect infestation, I was careful to account for the timing of infection. For example, when using cumulative degree days over 5°C (dd in Table 1) to predict damage

caused by *D. rufipennis*, the time-span prior to the initiation of attack was considered (two years before infestation became evident). Spring precipitation levels likely influence the stand-level risk of infestation by *D. rufipennis* in the same year (Zhang et al., 1999). Therefore I used a version of this variable that was lagged by one year to predict visible signs of damage that appeared one year after the initiation of attack. Low winter temperatures lead to increased winter mortality for *D. rufipennis* individuals (Frye et al., 1974). Therefore I used minimum winter temperature in the year of attack initiation as a predictor of subsequent *D. rufipennis* infestations.

Static variables included the percent cover of Engelmann spruce, white spruce and subalpine fir in our study area. Tree distribution maps containing the average percent ground cover of each species were provided by Hamann and Wang (2006). I overlaid the same 1×1 km grid on the tree distribution maps and computed the percent cover of each species in each grid square. When several polygons with differing percent cover attributes overlapped a grid square, the value assigned to it was calculated by averaging all of the percent cover estimates for each overlapping polygon.

5.2.4 Models

Outbreaks of *C. biennis* and *D. rufipennis* do not behave uniformly across the landscape and outbreaks are, by definition, not persistent through time. Determining whether these two species interacted, therefore, required a spatiotemporal approach. I used logistic regression models that incorporated Markov processes to account for temporal dependencies and autocovariates to account for spatial dependencies. A probabilistic process is defined as Marko-

vian when current states depend on a limited number of past states (Allen, 2011). In first order Markov processes, the current state depends on the state in the previous time step; in second order Markov processes the current state depends on the states in two previous time steps. Due to its life cycle, which is completed in one to three years, the dynamics of *D. rufipennis* resemble a Markov process because the presence of the insect on the landscape depends on where it was up to three years previously. Thus my models of *D. rufipennis* dynamics incorporated third order temporal dependencies.

I accounted for spatial autocorrelation in *D rufipennis* infestations in my logistic regression models using a spatial covariate defined as follows:

$$W_t = X_t \cdot \Omega$$

Where W_t is the covariate value assigned to the grid square centered at coordinates (x, y) in year t. The covariate was calculated by computing the dot product of two matrices which equates to multiplying them element by element and then summing the result over all columns and rows. The X_t matrix is an $M \times M$ sub-matrix sampled from the grid (data) at year t and centered on a focal grid cell located at (x, y). The Ω matrix is an $M \times M$ weighting matrix where M corresponds to the number of grid cells contained in the neighbourhood of the focal cell (including the focal cell) in the vertical and horizontal directions. Based on the spatial extent of autocorrelation in our data and on computational limitations, I chose M = 201.

Although written in compact form, the above spatial covariate function differs only from those used by other researchers employing spatial logistic regressions (Augustin et al., 1996) in that I did not scale it by dividing by

the sum of all elements of the weighting matrix. I calculated the value of the spatial covariate for every grid square in our lattice for every year in the study interval except for cells that were located in a 100 km buffer zone around the outer edge of the study region. Due to the computation required to calculate a spatial covariate within a neighbourhood this large ($201 \times 201 = 40401$ cells), we programmed our weighting function in C++ and implemented it in R (Development Core Team, R, 2012) using the inline (Skylar et al., 2010) and Rcpp packages (Eddelbuettel and Romain, 2012).

To specify the weighting matrix (Ω) for the spatial covariate, I estimated spatial autocorrelation in *D. rufipennis* presence on the landscape using the Sncf function in the ncf (Bjornstad, 2012) package for R. The data were extensive so I evaluated spatial autocorrelation in a random sample of 2000 locations from our original dataset. The Sncf function fits a smoothing spline that relates autocorrelation to Euclidian distance between cells (Fig. 5.2(a)). The Euclidian distances between the central focal cell of a 201×201 grid and all of the other cells in the grid were computed (Fig. 5.2(b)). The fitted values of the smoothing function were then used to calculate the degree of autocorrelation between a focal cell at the center of the 201×201 grid, and all other cells within that grid (Fig. 5.2(c)). This information was then converted to a matrix (Ω) and used to weight each cell in our dataset based on *D. rufipennis* infestations in the nearby cells.

The logistic models of insect dynamics were fitted using functions in the biglm library for R (Lumley, 2011). Note that the response variable (insect presence or absence) in the current year was not used in constructing the spatial covariates and therefore this logistic regression is not the classic autol-

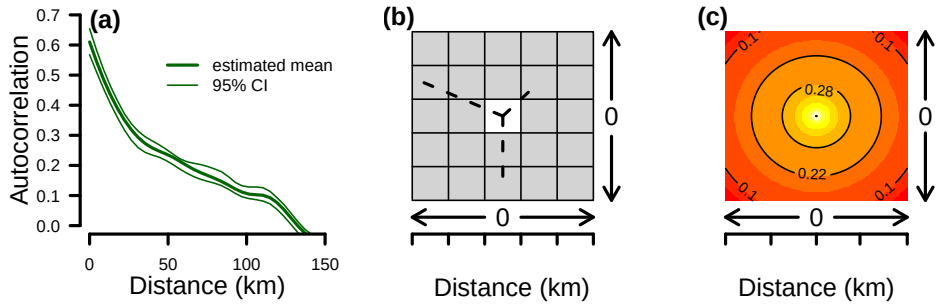

Figure 5.2: Computing an autocovariate to describe spatial autocorrelation in lattice data on spruce beetle (*Dendroctonus rufipennis*) infestations in British Columbia, Canada involved (a) fitting a smoothing spline to estimate spatial autocorrelation across the entire dataset, (b) computing the Euclidian distances between a focal cell and all other cells within a 201 × 201 km grid, and (c) using the smoothing spline to estimate the amount of autocorrelation between all of the grid cells in a 201 × 201 km grid and the central focal cell based on Euclidian distance.

ogistic regression as described by Besag (1972).

5.2.5 Model selection

I built one candidate model for *D. rufipennis*, that incorporated the relevant predictor variables, lagged states to accommodate temporal dependencies and spatial covariates (also lagged) to account for spatial autocorrelation. To determine whether *D. rufipennis* outbreaks are impacted by previous defoliation by *C. biennis*, I added the severity of *C. biennis* defoliation as a variable in the regression predicting *D. rufipennis* presence or absence on the landscape. Thus, the candidate model incorporated the following dependencies:

$$Beetle_t \sim W_{t-1} + W_{t-2} + W_{t-3} + Beetle_{t-1} + Beetle_{t-2} + Beetle_{t-3} + dd_{t-2} + SpPrecip_{t-1} + MinT_{t-1} + BudWSev_{t-1} + BudWSev_{t-2} + elev + EngSpCov + WtSpCov + SubFirCov.$$

In all cases a t subscript indicates no lag while a *t-1* subscript indicates a one year lag and so on. The Beetle term indicates whether a particular grid cell was infected by the *D. rufipennis* at a given time, and W corresponds to the spatial covariate for *D. rufipennis*. The *BudWSev* terms refers to the severity of budworm infection in the cell at time t or *t-1*. All of the remaining variables in the regressions are defined in Table 1. My approach was to build the model above, and then to compare it to a model that excluded interactions between the insect species (*BudWSev* terms). For each year in my time-series, parameters were estimated and then fitted models were used to predict the subsequent year that had not yet been used for model fitting. Thus my model fitting and selection procedure was as follows:

1. Fit model for year t including all of the relevant lagged predictor variables (Some are lagged by up to three years).

2. Predict the outcome of year *t+1* based on the parameters estimated above.

3. Calculate AUC values based on comparison of predictions to data for the year *t+1*.

4. Proceed to fit the model again for year *t+1* and run through the loop again.

The full candidate model and the reduced model without the *BudWSev* terms were then compared on a yearly basis where higher AUC scores indicate better model performance (Rosset, 2004). Comparing models on a yearly basis in this way permitted me to evaluate the relative importance of defoliation by *C. biennis* as a predictor of *D. rufipennis* dynamics during different stages of spruce beetle outbreaks. Moreover this method does not assume that parameters remain stationary over the entire time series. It is identical to the prequential model evaluation method (Dawid, 1984) except that I have not generated any test statistics as these rely on assumptions of spatial independence of observations which are violated in my models.

Whereas model selection approaches do not provide insight into the relative importance of predictor variables, the Random Forest (tm) algorithm of Breiman and Cutler can rank variables based on their predictive performance. Therefore, I used the randomForest (Liaw and Wiener, 2012) package in R to evaluate the relative importance of the predictor variables over the whole time series based upon the decrease in Gini impurity (Breiman, 1996). Gini impurity is a measure of how likely a model without a particular variable is to mistakenly categorize an experimental unit that has been randomly chosen from the dataset (See Breiman, 1996 for computational details).

5.3 Results

Models of the dynamics of *D. rufipennis* that included variables relating to the severity of previous infestation by *C. biennis* were not more predictive

than those without them and exhibited similar predictive power to models that excluded these variables over the whole time series (Fig. 5.3).

Based on Gini impurity computed by the Random Forest (tm) algorithm, spatial covariates were consistently the most important predictors of spruce beetle infestation over the whole time series (Table 5.2). However, spring precipitation, minimum temperature and degree days also ranked high in importance while budworm severity variables were less important (Table 5.2).

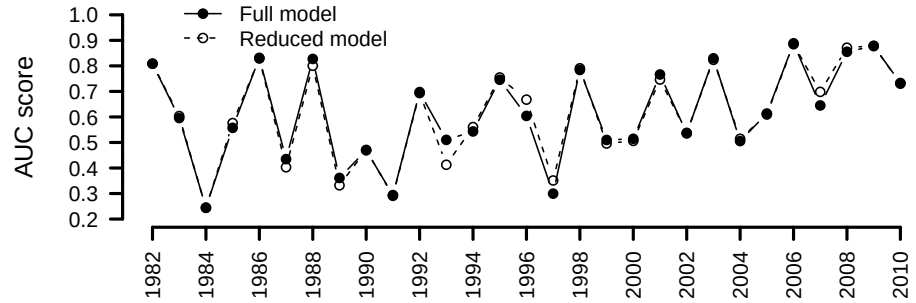

Figure 5.3: Time series showing a) the predictive performance of a full model predicting spruce beetle (*Dendroctonus rufipennis*) infestations in British Columbia, Canada which includes predictors based on previous defoliation by the two year cycle spruce budworm (*Choristoneura biennis*) compared to a reduced model that excludes them.

5.4 Discussion

My data and models suggest that *D. rufipennis* and *C. biennis* do not interact on a landscape-scale. Therefore, we will discuss other biotic and abiotic factors in the epidemiology of *D. rufipennis* that were important predictors of infestation.

Spatial autocovariates were the most important variables predicting spruce beetle occurrence. This suggests that infestations were strongly spatially de-

Table 5.2: Rankings of the importance of predictors of spruce beetle (*Dendroctonus rufipennis*) presence on the landscape in British Columbia (BC) based on a Random Forest (tm) model fitted to the entire time series (1981 - 2010). Importance rankings are based on out-of-bag error estimates generated by the Random Forest (tm) algorithm.

Variable	Abbreviation	Mean decrease in Gini impurity
Spatial covariate (lag 1)	W_{t-1}	2393
Spatial covariate (lag 3)	W_{t-3}	1897
Spatial covariate (lag 2)	W_{t-2}	1774
Spring precipitation (lag 1)	$SpPrecip_{t-1}$	1196
Minimum temperature (lag 1)	$MinT_{t-1}$	866
Degree days (lag2)	dd_{t-2}	571
Elevation	elev	530
Engelmann spruce cover	EngSpCov	283
White spruce cover	WtSpCov	173
Budworm severity (lag 1)	$BudWSev_{t-1}$	122
Subalpine fir cover	SubFirCov	96
Budworm severity (lag 2)	$BudWSev_{t-2}$	74
Beetle (lag 1)	$Beetle_{t-1}$	64
Beetle (lag 2)	$Beetle_{t-2}$	47
Beetle (lag 3)	$Beetle_{t-3}$	22

pendent through time. The spatial covariance function that I estimated for *D. rufipennis* dynamics in BC was similar to the one reported in Økland et al. (2005). Possible causes of spatial dependence include spruce beetle dispersal, the clumped distribution of host trees and spatially correlated weather phenomena (Økland et al., 2005). Beetles in the genus *Dendroctonus* have at least two types of dispersal: in long distance dispersal they use updrafts and wind currents to disperse up to 110 km d^{-1}, whereas short distance dispersal involves self-propelled flight under the forest canopy (Jackson et al., 2008). I predict that long distance dispersal may decrease rather than increase spatial interdependence near source populations due to the stochastic nature of weather phenomena that drive it. By itself, short distance dispersal also cannot explain the observed spatial autocorrelation over 130 km because *Dendroctonus* beetles typically fly only a few hundred m per day (Safranyik et al., 1992; Turchin and Thoeny, 1993). Instead, spatially correlated wind events, a well known driver of *D. rufipennis* population dynamics (Holsten et al., 1999), may generate spatially aggregated infestations by blowing down trees that then become spruce beetle nurseries. This mechanism has been proposed as a driver of spatial covariance in the Eurasian spruce bark beetle (*Ips typographus*) in Scandinavia where large windfall events promote population growth resulting in landscape-scale outbreaks (Økland et al., 2005).

Although windfall data were not available, several other climate-related variables were consistently important predictors of *D. rufipennis* infestation. Spring precipitation, cumulative degree days above 5 °C, and minimum winter temperatures were positively related to infestation. My results confirm the positive relationship between the incidence of *D. rufipennis* outbreaks and

spring precipitation levels that was initially reported in a dendrochronological study (Zhang et al., 1999). While no study has provided a mechanistic explanation for this positive relationship, I hypothesize that spring precipitation falling as snow at high elevations may insulate adults overwintering in the lower sections of tree trunks against woodpecker predation and low spring temperatures.

Besides being inherently interesting, interactions between herbivorous insects may have important consequences for large-scale insect epidemiology as facilitative interactions may lead to greater damage to forests than predicted based on climate variables alone. However, my results suggest that if they exist for *C. biennis* and *D. rufipennis*, facilitative interactions are typically not strong enough to manifest on a landscape scale and they therefore need not inform management policy.

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Chapter 6

Thesis Discussion

Interactions between conifers and bark beetles occur at various levels but ultimately depend on small-scale chemical and ecological processes, including the ability of trees to mobilize carbohydrates to fund defensive reactions to attack. Once beetles kill their host tree, variation in phloem nutrition that is amplified by their fungal associates may impact beetle dynamics in stands and landscapes by modulating how quickly they develop and how many survive to emerge the following year (Goodsman et al., 2012*a*). The stands that bark beetles infest after emerging in large numbers may depend on tree-mediated interactions with other phytophagous insects. My thesis highlights the importance of these ecological interactions at different spatial scales as determinants of bark beetle biology and epidemiology.

6.1 Interactions between trees and the fungal symbionts of bark beetles

Until very recently, I was convinced that large dominant trees in any given stand are, in fact, less defended than subordinate trees since mountain pine

beetles preferentially attack dominant trees during outbreaks (Cole et al., 1976). Although my reasons for believing that dominants were less defended than subordinates seem spurious now, they were based on knowledge that productivity tends to decline with tree-size (Mencuccini et al., 2005). As productivity likely depends on adequate carbohydrate availability, I assumed that larger trees would have lower quantities of carbohydrate reserves with which to construct defense chemicals. In Chapter Two, I illustrated that dominant trees are not less defended than subordinate trees and I have thus been forced to realign my opinion with those of the majority of scientists that study bark beetles (Raffa and Berryman, 1982; Safranyik and Carroll, 2006).

Although dominant trees are not weaker than subordinate trees in terms of carbohydrate reserves or defense chemistry, their strength in these respects becomes a weakness when bark beetle populations are sufficiently high to mass-attack them. The results of my second chapter illustrate how the mountain pine beetle's symbioses with blue-stain fungi are ideal for killing large and well-defended trees: Dominant trees within a stand respond heavily to fungal invasion by mobilizing carbohydrates to the lesion front and by synthesizing monoterpenes that accumulate inside lesion boundaries. Due to these energetically expensive responses, large trees are more easily forced to exhaust their carbohydrate reserves during mass-attacks (Lieutier et al., 2009). They defend themselves to death.

The study in my second chapter was also the first to report decreasing gradients in carbohydrate availability along the stems of mature trees with distance from carbon sources (Goodsman et al., 2012*b*). Based on this finding, I extended ideas on how carbon sinks and sources impact tree defenses so

that they could be applied to tree stems. The sink/source framework (Honkanen and Haukioja, 1998) had previously only been applied to explain patterns of defense within tree crowns. By extending it I was able to explain why carbohydrate reserves are relatively low in the lower stem, and therefore why the lower sections of pine stems are particularly vulnerable to mountain pine beetles (Cole and Amman, 1983). I believe that this extension of the sink/source framework will elucidate mechanisms underlying the interactions between trees and a wide variety of insects and fungi that attack their boles.

6.2 Tree-mediated interactions between humans and bark beetles

The majority of prior research on silvicultural management of bark beetle populations—termed indirect control—contended that tree productivity and resistance to bark beetles could be maximized at the same time (Berryman, 1982; Raffa and Berryman, 1982; Waring and Pitman, 1985). In fact all of the studies referenced above suggested using the width of tree rings as a proxy for carbohydrate reserves and defensive vigor. In my second chapter I too have argued that carbon influx and concentrations of defense chemicals are higher near the tops of crowns where tree rings are wide. Thus, I do not deny that radial growth in tree stems is necessarily linked to carbohydrate availability there. However, in my third chapter I argue that fertilization, which increases radial growth in the stem, changes carbohydrate allocation priority such that growth is temporarily favoured over storage. The relationship between the width of tree rings and the amount of carbohydrates available for storage, maintenance and defense is therefore changed after fertilization (Goodsman

et al., 2010).

The idea, discussed in my third chapter that growth in the stem consumes carbohydrates before they reach the roots seems to contradict my earlier claim that strong sinks in tree stems correspond to high rates of carbon influx. However, the findings reported in my second and third chapters can be reconciled if one realizes that although radial growth in the stem consumes carbohydrates, this does not negate the possibility that ring growth may reflect or even drive local carbohydrate availability. If the supply of carbohydrates is greater than or equal to demand, sinks may control the influx of soluble sugars into their own tissues (Minchin and Lacointe, 2005), whereas carbon supply and allocation priority may dictate the activity of sinks when this is not so.

Not only does fertilization reduce carbohydrate reserves that would likely be used to defend against bark beetle attack, but it also increases the amount of nitrogen available to bark beetles once trees have been overwhelmed. The subcortical environment inhabited by bark beetles is notoriously low in nutrients (Ayres et al., 2000), and even when trees are fertilized, the nutritional content of phloem is poor compared to that of foliage. In Chapter 4 I illustrate how the nutritional benefits enjoyed by mountain pine beetles inhabiting nutrient rich trees are likely mediated by their fungal symbionts which concentrate nitrogen in the phloem consumed by beetles. As the concentration of nitrogen in the phloem that is available to bark beetles is amplified by their fungal symbionts, and because increased nitrogen positively impacts bark beetle overwinter survival (Goodsman et al., 2012*a*), small changes in phloem nutrition may have disproportionate effects on beetle populations.

6.3 Landscape-scale interactions between insect species

I was unable to detect landscape-scale interactions between *C. biennis* and *D. rufipennis*. However several other variables proved to be important predictors of *D. rufipennis* dynamics on the landscape: Degree days above 5°C, minimum temperatures and spring precipitation were consistently good predictors of the probability of spruce beetle infestation when used in conjunction with autocovariates.

Where bark beetles will go on a landscape once they have emerged *en masse* is difficult to predict, partly because dispersal is impacted by environmental factors, such as weather patterns, wind speed and wind direction (Jackson et al., 2008), which are irregular. Defoliation variables were not sufficient to predict *D. rufipennis* presence on the landscape. Fortunately, bark beetles dynamics are spatially synchronous through time, making it possible to predict where they will go based on where they have been (Økland et al., 2005; Aukema et al., 2006). Thus, autocovariates computed based on this principle were indispensable components of my models.

6.4 Management implications

Tree-mediated interactions are not only limited to arthropods. Management of forests may change the resources that individual trees have available to respond to bark beetle attack (Goodsman et al., 2010) or their suitability for overwintering bark beetles (Goodsman et al., 2012*a*). Thus management of bark beetle damage, whether it is preventative or reactive, cannot afford to ignore aspects of bark beetle biology that enable small and seemingly insignif-

icant changes to amplify bark beetle fecundity or survival (Raffa et al., 2008). It is therefore critical that managers incorporate ecological understanding into management decisions.

Managers should not only consider possible ramifications of management in terms of bark beetle biology, but they should also consider what might happen if management fails: While thinning from below has been shown to decrease bark beetle colonization of host trees when their populations are low (Whitehead and Russo, 2005), if their populations are large, they will kill all of the overstory trees leaving no understory trees to be released. Thus a natural feature of the ecology of bark beetle outbreaks, which has been recorded in numerous dendrochronological studies throughout North America (Zhang et al., 1999; Berg et al., 2006), is precluded.

6.5 Future research

I can foresee four areas of research that would clarify aspects of bark beetle biology that I only grazed in my doctoral work. The study described in my second chapter is the first to report gradients in carbohydrate availability along tree stems and to propose an explanation based on tree physiology (Goodsman et al., 2012*b*). However, these gradients have not been confirmed throughout the year and they are likely a function of rapid mid-summer growth in the stem. Therefore, there is an opportunity for future research to clarify mechanisms that lead to variation in carbohydrate availability within stems by collecting similar data at intervals throughout the year. Moreover, the functional relationship between radial growth at different heights along tree stems and local carbohydrate availability has yet to be described and substantiated.

In my study of bark beetle survival and development in fertilized bolts, I measured larval lipids as well as lipids in the phloem they inhabited. I found that lipids in the phloem of pine trees were quite high (up to 20%) and they remained high when other aspects of phloem quality declined. Therefore lipids in the phloem may be a crucial source of nutrition for bark beetles, especially as they prepare to emerge as juvenile adults. Larval lipids tended to be higher in bolts from fertilized trees but the results were not conclusive unless beetle larvae were separated into dark and light-colored larvae. Dark-colored larvae were still living when I collected them and they had much higher fat content than light-colored larvae. The cause of the dark-coloration was a source of contention with the referees, when we submitted the research for publication. One referee disagreed with our claim that the dark coloration was caused by the consumption of fungus-infected phloem. Having observed it on numerous occasions, I believe that dark-coloration is actually a common trait amongst mountain pine beetle larvae. The cause of this dark coloration needs further investigation as it may have important ramifications for bark beetle overwinter survival and population dynamics.

In my fifth chapter I failed to find evidence on a landscape scale that defoliation by *C. biennis* renders trees and stands more susceptible to spruce beetles. However, I was able to corroborate findings reported by Zhang et al. (1999) that spring precipitation is positively related to the probability a stand will be infested with *D. rufipennis*. I hypothesized that this may be due to the insulating effect of snow falling at high elevations and the concurrent reduction in predation by woodpeckers in the lower stems of trees underneat the snow. This mechanism requires experimental testing or field assessment.

The causes of spatial covariance in bark beetle dynamics that I observed in spruce beetles in my fifth chapter remain unknown (Økland et al., 2005). If we knew the causes of spatial synchrony in bark beetle dynamics, adding the relevant variables to our models should eliminate most of the spatial autocorrelation of model residuals and make autocovariates unnecessary. Since I was unable to accomplish this in my own models of spruce beetle dynamics, I contend that spatial patterns in degree days, minimum temperature, precipitation and tree cover do not adequately explain the aggregated behaviour of spruce beetle infestations on their own. Nor do short or long distance dispersal on their own since short distance dispersal occurs over distances less than one km and long distance dispersal would likely result in irregular infestation patterns and outbreaks across the landscape. Thus the causes of spatial synchrony in insect populations remain mysterious and beguiling.

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