The Impact of Phloem Nutrients on Overwintering Mountain Pine Beetles and Their Fungal Symbionts

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ABSTRACT In the low nutrient environment of conifer bark, subcortical beetles often carry symbiotic fungi that concentrate nutrients in host tissues. Although bark beetles are known to benefit from these symbioses, whether this is because they survive better in nutrient-rich phloem is unknown. After manipulating phloem nutrition by fertilizing lodgepole pine trees (*Pinus contorta* Douglas var. latifolia), we found bolts from fertilized trees to contain more living individuals, and especially more pupae and teneral adults than bolts from unfertilized trees at our southern site. At our northern site, we found that a larger proportion of mountain pine beetle (*Dendroctonus ponderosae* Hopkins) larvae built pupal chambers in bolts from fertilized trees than in bolts from unfertilized trees. The symbiotic fungi of the mountain pine beetle also responded to fertilization. Two mutualistic fungi of bark beetles, *Grosmannia clavigera* (Rob.-Jeffr. & R. W. Davidson) Zipfel, Z. W. de Beer, & M. J. Wingf. and *Leptographium longiclavatum* Lee, S., J. J. Kim, & C. Breuil, doubled the nitrogen concentrations near the point of infection in the phloem of fertilized trees. These fungi were less capable of concentrating nitrogen in unfertilized trees. Thus, the fungal symbionts of mountain pine beetle enhance phloem nutrition and likely mediate the beneficial effects of fertilization on the survival and development of mountain pine beetle larvae.

KEY WORDS blue-stain, *Dendroctonus*, nitrogen, pine, symbiosis

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 10-year period and has expanded northward and eastward beyond the previous limits of its range (http://cfs.nrcan.gc.ca/pages/49?lang=en_CA, 2011). Possible drivers of this expansion include climate change, winds favoring 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 (Safranyik et al. 2010).

When they attack conifers, female mountain pine beetles bore through the outer-bark and inoculate fungal symbionts (Cole and Amman 1983, Safranyik and Carroll 2006) including *Grosmannia clavigera*, *Leptographium longiclavatum*, and *Ophiostoma montium* (Rumbold) von Arx (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), Six and Paine (1998) reported that blue-stain fungi positively impacted the survival of mountain pine beetle brood and that *G. clavigera* was more beneficial to larval survival than *O. montium*. The fungal symbionts of bark beetles likely benefit bark beetles by exhausting tree defenses 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 concentrated nitrogen in their tissues and that beetles maturing in fertilized bolts were higher in nitrogen than those in unfertilized trees. Although the amplification of phloem nutrients by symbiotic fungi is often invoked 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. In their later instars that occur between December and February, some larvae are able to tolerate temperatures near −40°C for several days (Yuill 1941). The egg and early instar larval life stages that occur before this period as well as the pupa and adult life-stages that

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occur after it, are all more susceptible to freezing (Safranyik and Carroll 2006). Therefore, unseasonable 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, 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 midwinter temperatures, and a southern location with warmer midwinter temperatures.

Materials and Methods

We selected 30 dominant or codominant lodgepole pine trees from a 120 yr-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 at least 30 m away from other experimental trees 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 nitrogen from urea and mono-ammonium phosphate; 100 kg/ha of phosphorus from mono-ammonium phosphate; 100 kg/ha of potassium from muriate of potash and potassium magnesium sulfate; 32.5 kg/ha of magnesium from potassium magnesium sulfate; 75 kg/ha of sulfur from mono-ammonium phosphate and potassium magnesium sulfate; and 3 kg/ha of boron from granular borate.

In late August 2009, we collected a phloem sample (3×3 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 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; and 4) beetle-infested northern location. We designed the experiment in two parts. In the first part we investigated fertilizer X location effects on mountain pine beetle development, survival, and fat reserves. In the second part, we investigated fertilizer X 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, Canada). 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 building 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 h 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, Canada, for 2 wk before 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. We incubated tissue samples or living larvae on malt extract agar (MEA) amended with 0.02% oxytetracycline dihydrate (Sigma-Aldrich, St. Louis, MO) at room temperature in inverted sterile petri dishes sealed with parafilm. Once fungi had colonized the entire plate we isolated *G. clavigera* by replating 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 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 circum-

ference 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 resterilized the cork borer, 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 dowel 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 (Lat. 55° 59′0.02″, Long. 119° 25′0.53″). 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, MA) 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″).

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 subsampled a small portion of fungal infected phloem to confirm the identity of our fungi by reculturing it on 2% MEA. We recovered the original fungal species from 10 of the 15 subsamples we recultured for *G. clavigera;* from 12 of the 15 subsamples we recultured for *L. longiclavatum;* and from 10 of the 15 subsamples we recultured for *O. montium.* In our subsequent analyses, we excluded samples if we did not successfully reculture the fungi that we originally inoculated. We dried the remaining sample at 70°C in preparation for total nitrogen and total nonstructural 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. 1). We later quantified the area of phloem colonized by each fungus using a scanner and image analysis software (SigmaScan, San Jose, CA).

Unlike the fungus-inoculated bolts that we processed in the field, we brought the beetle-infested bolts back to the laboratory between 27 and 30 May 2010 where they were stored at room temperature until dissection on 1 and 2 June. Therefore, bolts remained indoors for up to 5 d at room temperature before we dissected them. Fertilized and unfertilized

Fig. 1. An example of the extent of fungal spread in June for fungal symbionts of the mountain pine beetle. The fungi visible in the center and on the left were inoculated into the waxed bolt the previous fall (September 2009). (Online figure in color.)

bolts did not differ in the amount of time that they were stored but bolts that overwintered at our northern site arrived 2 d before the bolts that overwintered in the south. On 1 and 2 June, 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 because of 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 stages from all of the galleries in each bolt and placed them in the freezer for follow-up analysis.

Chemical Analysis. Before our measurement of larval lipids, we separated dark-colored larvae that were still alive from white larvae. Dark colored 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. 2).

To quantify total lipids in larvae, we used the method of Hagen and Atkins (1975). We transferred

Fig. 2. A living larva exhibiting the dark tinged characteristic in the middle and anterior regions mentioned in the text. (Online figure in color.)

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 3 d) before recording the weight of each individual corpse. We then extracted fats for 8 h using petroleum ether in a soxhlet apparatus and redried the carcasses under a fume hood before weighing them again. We estimated the total lipids (in grams) 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 h 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). We determined total starch and sugar concentrations colorimetrically (Chow and Landhausser 2004). Briefly, we extracted sugars from 50 mg of ground tissue in 80% hot ethanol. We then reacted the sugars with phenol-sulfuric 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), 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 used a modified Bligh & Dyer method combined with a Folch wash (Nelson and Dickson 1981) to extract the fats followed by thin-layer chromatography to separate the triglycerides, free fatty acids, and diglycerides from other lipids. We eluted the triglycerides, free fatty acids, and diglycerides from the thin-layer chromatography strip and then reabsorbed them into preweighed 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.

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:

$$y_{ijm} \sim Poisson(\theta_{ijk}).$$

$$\log(\theta_{ijk}) = \mu + \tau_i + \omega_k + \tau\omega_{ik} + \beta_j.$$

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 centered on the population mean (μ):

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

The glmm statistical models are currently in development and provide only rough estimates for *P* values (Zuur et al. 2009, p. 339). As standard analysis of variance (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 glmms (Venables and Ripley 2002). In our statistics for likelihood ratio tests, we report one 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 CIs for each treatment mean, we separated our raw data according to treatment, we then sampled from the data 2,000 times under the assumption that they followed a Negative Binomial distribution (overdis-

persed 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 glmms assuming a Negative Binomial distribution:

$$y_{ij} \sim NB(\theta_{ij}, k).$$

$$Var(y_{ij}) = \theta_{ij} + \frac{\theta_{ij}^2}{k}.$$

$$\log(\theta_{ij}) = \mu + \tau_i.$$

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 $k \rightarrow \infty$ 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 weight). We then fit a split-plot ANOVA model as follows:

$$y_{ijm} \sim \mathbf{N}(\theta_{ijm}, \sigma_r^2).$$

$$\theta_{ijm} = \mu + \tau_2 + c_m + \tau c_m + \beta_j.$$

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

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 τc 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 SEs returned by the statistical model in R (R Development Core Team 2011).

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:

$$y_{ijk} \sim \mathbf{N}(\theta_{ijk}, \sigma_r^2).$$

$$\theta_{ijk} = \mu + \tau_2 + \omega_k + \tau\omega_{ik} + \beta_j.$$

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

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 SEs returned by the statistical model in R (R Development Core Team 2011).

Table 1. Monthly min. temperatures and cumulative DDs per month in which temperatures exceeded 5°C at the northern and southern overwinter sites

Month	Min. north	Min. south	DD north	DD south
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
April	−8.1	−8.9	42.16	15.34
May	−4.8	−5.2	93.10	31.02

We did not require a test to determine that the concentration of total nonstructural carbohydrates in the phloem of our experimental bolts before 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 that from fertilized trees and for phloem from 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 *G. clavigera* and phloem that was not inoculated.

Because each 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. CIs can be used by readers to visually approximate two sample comparison tests at whatever alpha level they choose (Payton et al. 2000, Cumming and Finch 2005). If CIs of two means overlapped by less than half of the length of the CIs, 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 <0.05 (Cummings 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 2002) package in R for analyses involving negative binomially distributed data.

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). Midwinter minimum temperatures (November 2009 to March 2010) were consistently cooler (4–6°C) at the northern site than at the southern site (Table 1). However, minimum temperatures in October were 10°C cooler in the south and equivalent in

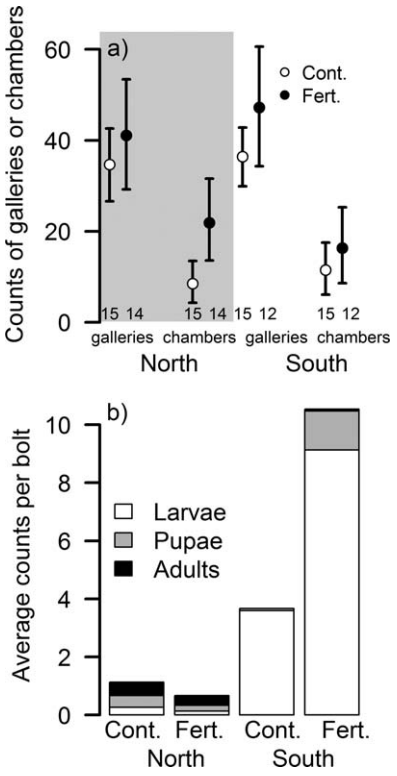

Fig. 3. The mean counts of (a) larval galleries and pupal chambers (and 95% CI) in unfertilized (Control) and fertilized bolts that overwintered at the northern and southern sites. The mean number of (b) living beetle larvae, pupae, and teneral adults (and 95% CI) collected per bolt in bolts cut from 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.

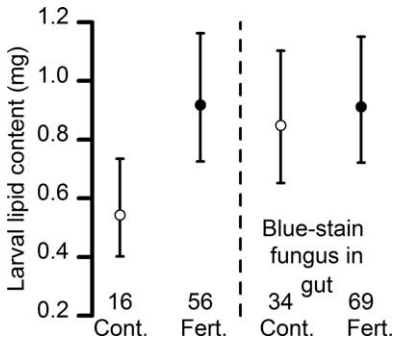

Fig. 4. Mean lipid content (and 95% CI) in larvae in unfertilized and fertilized bolts that overwintered at the southern site. Larvae were divided into two groups according to whether they had dark stain from blue-stain fungi in their digestive tracts. The *n* for each mean and 95% CI are shown in the figure below the points. Note that the *n* values listed are for larvae within bolts in the statistical analyses presented.

the north and south in April and May (Table 1). In addition, 93 DD above 5°C were accumulated at the northern location by the end of May whereas only 31 DD above 5°C were accumulated at the southern location by this time.

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 ($P = 0.001322$; 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. 3a).

Few mountain pine beetles survived until early June at the northern site in any life-stage. Individuals that survived were equally divided among the larval, pupal, and teneral adult life-stages (Fig. 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-colored 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). However, when the dark and light colored larvae were pooled, there was much less evidence for an effect of fertilization on larval lipids ($P = 0.15$; df = 17).

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. 5). However, there was strong evidence that the total nitrogen concentration 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 because of inoculation in our unfertilized bolts (Fig. 6).

Total nonstructural carbohydrates dropped from ≈6 to ≈1% in all phloem samples over the course of the winter whether they were inoculated or not (Fig. 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. 7b).

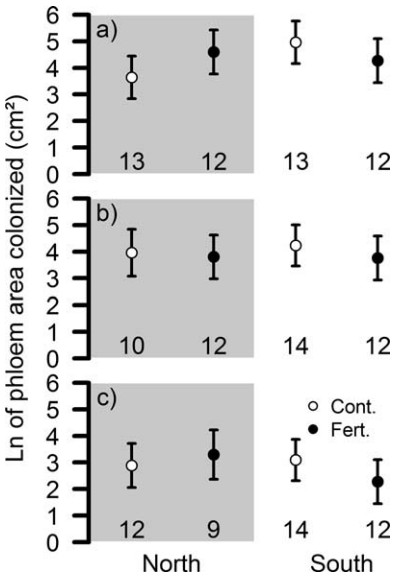

Fig. 5. The mean area of phloem (and 95% CI) colonized by (a) *G. clavigera* (GC), (b) *L. longiclavatum* (LL), and (c) *O. montium* (OM), in bolts cut from unfertilized (Cont.) and fertilized (Fert.) lodgepole pine. Bolts were stored overwinter either at the northern or southern site. The data were natural logarithm transformed. The sample sizes for each mean and 95% CI are shown in the figure below the points.

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.

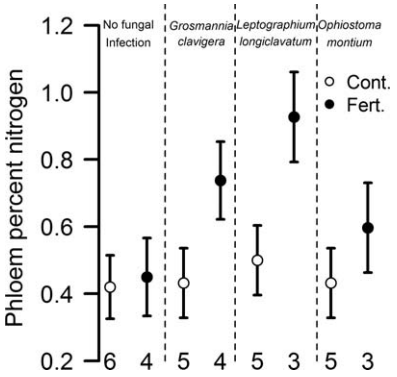

Fig. 6. The mean total nitrogen concentration of phloem (and 95% CI) from bolts cut from unfertilized (Cont.) or fertilized trees 9 mo after they had been left uninfected, or inoculated with *G. clavigera*, *L. longiclavatum*, or *O. montium*. Because of similar responses in the northern and southern 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.

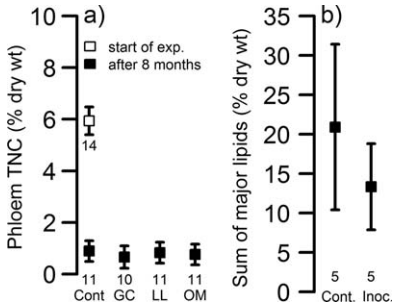

Fig. 7. Means (and 95% CI) for (a) nonstructural carbohydrate concentrations in lodgepole pine phloem at the beginning of the experiment and after an 8 mo overwinter period. Phloem was either uninfected (Cont.), or inoculated with *G. clavigera* (GC), *L. longiclavatum* (LL), or *O. montium* (OM). Means (and 95% CI) for (b) concentration of major lipids (triglycerides, fatty acids, and diglycerides) in uninfected phloem (Control) and phloem inoculated with *G. clavigera*. Because of 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.

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 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 that 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* (DeGreer, 1775) more susceptible to mortality because of 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-colored 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

colored 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 colored 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 2009a), 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 2009b). Therefore, growing conditions likely favored *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 DD above 5°C in the northern and southern locations in the fall and more DDs 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 DDs 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 that 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.

Because of 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 total non-structural carbon (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 because of 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. Schoonmaker et al. (unpublished) found similar quantities of lipids in lodgepole pine phloem. 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. Because of their continuous availability, phloem lipids in pine trees may be a crucial source of nutrition for bark beetles.

In the current 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 twofold 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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