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Evidence of a direct chemical plant defense role for maltol against spruce budworm

Martin Williams, Eldon Eveleigh, Glen Forbes, Rosanna Lamb, Lucas Roscoe & Peter Silk*

Canadian Forest Service – Atlantic Forestry Centre, Natural Resources Canada, PO Box 4000, Fredericton, NB, E3B 5P7, Canada

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Abstract

This study examines the direct chemical defensive role of maltol, a previously identified secondary metabolite found in balsam fir, *Abies balsamea* (L.) Mill. (Pinaceae), that was detected during herbivory of spruce budworm, *Choristoneura fumiferana* (Clemens) (Lepidoptera: Tortricidae). Although used extensively in many industries, in addition to being found in multiple plant species, its functional role in plants remains unknown. The objectives of this study were to quantify the amount of free maltol and its potential conjugated form, maltol glucoside, in various foliage age classes and to evaluate whether constitutive foliage levels of maltol have an impact on spruce budworm fitness in maltol supplementation assays. Gas chromatography–mass spectrometry (GC-MS) analysis of balsam fir foliage showed that maltol is produced in all foliage age classes tested; however, concentrations were significantly higher in older foliage. Liquid chromatography–mass spectrometry–mass spectrometry (LC-MS-MS) analysis showed that maltol also exists in balsam fir in its glucosylated form, a unique discovery in conifers. Similar to maltol, maltol glucoside is also present in current and 1-year-old balsam fir foliage and in significantly higher concentration in older foliage. We investigated the impact of maltol-treated diet on spruce budworm fitness. Maltol additions that reflected constitutive foliage concentrations caused a significant reduction in larval development rate and pupal mass, whereas higher concentrations were required to cause significant mortality. These results suggest that maltol may be an important component of a direct defense strategy in balsam fir against spruce budworm herbivory.

Introduction

One of the most destructive insect defoliators in Canada is the spruce budworm (SBW), *Choristoneura fumiferana* (Clemens) (Lepidoptera: Tortricidae). Population outbreaks of this defoliating pest of spruce [*Picea glauca* (Moench) Voss, *Picea mariana* (Mill.) BSP, and *Picea rubens* Sarg.] and balsam fir [*Abies balsamea* (L.) Miller] (all Pinaceae) usually happen every 30–40 years (Royama, 1984) and impact forest productivity through tree mortality and diminished growth and yield due to defoliation (MacLean, 2016). Use of insecticides such as *Bacillus thuringiensis* Berliner var. *kurstaki* (*Btk*) and MIMIC® (insect growth regulator, tebufenozide) have been the most prevalent options used to minimize tree mortality or yield loss (Cadogan et al., 1998; Carisey et al., 2004). Although effective against SBW, economic and ecological impacts on non-target organisms such as natural enemies, as well as potential resistance development in target insects (Ferré & van Rie, 2002; Janmaat & Myers, 2003) from repeated long-term use are important concerns. Consequently, the development of cost-effective and ecologically safe management techniques for SBW is important.

A potentially effective approach may involve exploiting host plant-derived secondary metabolites such as terpenoids, alkaloids, flavonoids, and phenolics, as well as defensive proteins (Howe & Jander, 2008) that are produced either constitutively, induced following herbivory, or both. In conifers, some of these compounds are well known, and they can impact herbivore fitness, including that of SBW. For example, monoterpenes are naturally produced secondary metabolites in conifers, and some

*Correspondence: E-mail: peter.silk@canada.ca

have an impact on survival and development rates of SBW when added to artificial diets of female sixth instars (L6) (Kumbasli & Bauce, 2013). In white spruce, Delvas et al. (2011) demonstrated the presence of the phenolic acetophenones (piceol and pungenol) as well as their glucosylated precursors, picein and pungenin. Addition of the acetophenones to artificial SBW diet significantly impacted the fitness of SBW by decreasing survival, retarding development, and decreasing pupal mass (Delvas et al., 2011). Further analyses of these glycosylated and non-glycosylated acetophenones among other conifer species confirmed that many species did not have all four compounds, and some species, including balsam fir, lacked all four (Parent et al., 2018). This suggests that acetophenones are likely not involved in defending balsam fir against herbivory. Many other secondary metabolites are similarly produced in conifers, with recent research demonstrating that various volatile organic compounds were produced upon herbivory by SBW second instars (L2) on spruce and fir host species (LeClair et al., 2015). Although some of the volatiles, including several monoterpenes, were common among the tree species tested, one compound, maltol, was only emitted from balsam fir, with the relative quantities of this compound increasing as herbivory progressed (LeClair et al., 2015).

Maltol is a naturally produced secondary metabolite found in several plant species, including conifers (LeBlanc & Akers, 1989; Tiefel & Berger, 1993). Maltol is extensively used in multiple industries, such as food, beverage, pharmaceutical, health, and personal care; however, information related to its function in plants is scarce. Research has been largely restricted to work completed by Blenis et al. (1988), who observed possible antifungal properties of maltol against gall rust infection in lodgepole pine (*Pinus contorta* Dougl. ex. Loud). Maltol is a pyrone (3-hydroxy-2-methyl-4*H*-pyran-4-one) biosynthesized through the polyketide sugar unit biosynthesis pathway, which itself is part of the metabolism of terpenoids and polyketides (Kanehisa et al., 2017). In this pathway, maltol is listed as an 'end product' (Kanehisa et al., 2017). Given its emission as a volatile during SBW herbivory assays (LeClair et al., 2015) and its specificity to balsam fir relative to white, red, and black spruces, maltol was investigated for its potential role in direct chemical plant defense. Maltol can also be glucosylated to form maltol glucoside (maltol β-D-glucopyranoside), and this metabolite has been found in other plants, including the manuka tree (*Leptospermum scoparium* JR Forst & G Forst; Adams et al., 2015) and the katsura tree (*Cercidiphyllum japonicum* Sieb. et Zucc; Tiefel & Berger, 1993). The presence of maltol glucoside has never been reported for any conifer species. Glycosylation is common in plants and can result in increased solubility, translocation, and compartmentalization of the aglycones and prevention of phytotoxicity (Neilson et al., 2013; Pentzold et al., 2014). Quantifying both bound and free forms of maltol is important to fully quantify the concentration attainable in foliage, which may in turn be used as a direct defense against herbivory in balsam fir.

To evaluate the role of maltol in direct chemical plant defense against SBW, we attempted to answer four questions: (1) Is maltol endogenously produced in balsam fir foliage, and if so, at what concentration? (2) Does foliage age affect maltol concentration? (3) Is maltol glucosylated in balsam fir? And (4) does maltol supplementation have an impact on SBW fitness?

Materials and methods

Analysis of foliage for maltol and maltol glucoside

Four-year-old overwintering balsam fir seedlings were transferred from outdoors to a greenhouse with climatic control to break dormancy. The greenhouse conditions were as follows: L16(20 °C):D8(16 °C) photo-thermoperiod and L60%:D40% r.h. Before moving the trees to the greenhouse, they were treated with dormant oil to protect against potential overwintering pests. Trees were watered when needed (2–3× a week) with 20-8-20 (N-P-K) grower fertilizer (Plant-Prod Forestry Special, Master Plant-Prod, Brampton, ON, Canada) at a 100-p.p.m. N concentration. Current-year foliage (C) and 1-year-old foliage (C-1) from 4-year-old balsam fir seedlings were collected after 1 month of growth under greenhouse conditions, overlapping with the feeding period (June) of SBW in the field (i.e., when new buds had fully flushed and were in the elongation process). In total 10 individual trees were sampled with foliage (C and C-1) collected from the same branch for each individual tree.

Foliage was frozen in liquid nitrogen, ground to a fine powder using a mortar and pestle, and stored at −80 °C until extraction. Samples were extracted and analyzed by Research and Productivity Counsel (RPC, Fredericton, NB, Canada). For extraction, 1 ml of methanol (Sigma-Aldrich, St. Louis, MO, USA) was added to 200 mg of ground foliage and placed in an ultrasonic bath for 30 min. Extract was then microcentrifuged for 5 min at 423 ***g***, and supernatant was filtered through a 0.22-µm nylon syringe filter before analysis. Maltol was analyzed on an Agilent 5973 MSD gas chromatograph-mass spectrometer (GC-MS) connected to an Agilent 6890N GC (Agilent Technologies, Santa Clara, CA, USA). GC-MS conditions were as follows – column: DB-5 MS 30 m × 0.32 mm, 1 µm film (Agilent); injection: 2 µl, splitless; oven temperature program: 50 °C, hold 5 min, 25 °C per min to 300 °C, hold 13 min. The liquid chromatography-mass

spectrometry (LC-MS) analysis was conducted on a Waters TQ-S micro with an Acquity H-Class UPLC (Waters, Mississauga, ON, Canada). LC conditions were as follows – column: Kinetex 2.6 μ PFP, 50 × 4.6 mm (Phenomenex); mobile phase: A, 0.1% formic acid in water; B, acetonitrile mobile phase gradient; 0.5 min 10% A, 2.5 min 50% A, hold 0.5 min, 3 min 90% A, hold 1 min, 5 min 10% A; flow rate: 0.4 ml per min; injection volume: 5 μl. MS-MS conditions: ionization mode, electrospray positive (ES+); cone voltage: 10 V; source temperature: 125 °C; desolvation temperature: 450 °C; desolvation gas flow: 550 l h^{-1}. Transitions monitored: quantitation, 289.1>126. Both compounds were quantified using a standard curve with a maltol standard (Sigma-Aldrich) and maltol glucoside standard (Ensol Biosciences, Daejeon, South Korea). The lower reporting limit was 25 mg kg^{-1} for maltol and 0.05 mg kg^{-1} for maltol glucoside.

Maltol supplementation assays

To determine whether maltol affects the survival, development time, pupal mass, sex ratio, and mating success of SBW, L2 obtained from the Insect Production Services (IPS) of the Canadian Forest Service (Sault Ste. Marie, ON, Canada) were reared on treatment diets of six maltol concentrations: standard McMorran artificial diet (control) (McMorran, 1965) and standard McMorran diet supplemented with maltol (99% pure; Sigma Aldrich) added at 0.5, 1, 2, 3, or 4% of the diet's volume. The experimental setup was similar to that described by MacKinnon et al. (2016). Briefly, McMorran diet, obtained from IPS in solid state, was placed in a glass beaker and heated in a 700-W microwave oven for 5 min, stirred, and heated for another 3 min until liquefied. For each treatment, the volume of diet was measured and placed in an industrial blender, and maltol was added and thoroughly blended. In all treatments, diet was poured into trays to a depth of ca. 1 cm and refrigerated over night. Glass vials (17 mm diameter, 60 mm high; 2 dram) were plugged with diet by cutting the diet using the open end of the vials and then pushing the plugs to the bottom of the vials with a plastic plunger. Strips of cheesecloth containing diapausing L2 larvae were placed in a glass bowl and misted with water twice daily until the L2 emerged from diapause. The L2 were randomly selected from the colony, with one larva placed into each of (n =) 100 vials per treatment diet using a small paint brush. The order in which vials received a larva was randomized among the six treatment diets. The vials were sealed with foam plugs and stored upside down in trays with the diet upward. Mortality and the development time to reach L6, pupal stage, and adult eclosion were monitored daily. The instar at death was determined by measuring head capsules (McGugan, 1954). Pupae were weighed, sexed, and placed in clean glass vials to eclose. The experiment was conducted in a laboratory at a constant 22 °C, ca. 40% r.h., and L16:D8 photoperiod.

Adults from the six treatments were used to determine the effects of maltol on mating success by pairing males and females that emerged within each treatment. Up to five 2- to 3-day-old males and five 1- to 2-day-old females per replicate were placed in mesh cages (60 × 60 × 90 cm) for each treatment. The number of replicates for each treatment was determined by the number of adults of each sex that eclosed. The pairs were left to mate for 72 h in a room at 22 °C, ca. 40% r.h., and L16:D8 photoperiod, with an oscillating fan set on low for air movement. The mating status of the females was determined by removing the bursa copulatrix and examining for the presence or absence of a spermatophore under a dissecting microscope.

Statistical analysis

Maltol and maltol glucoside concentrations in C and C-1 foliage were analyzed with a two-tailed paired t-test. Larval and pupal mortality (%), sex ratio (% females), and mating success (% females mated) were analyzed using the GENMOD procedure (dist = binomial, link = logit; SAS Institute, 1999), followed by comparisons of each treatment with the control by Wald tests. The effect of maltol treatments on (1) male and female development times (days) from L2 to L6, from L6 to pupae, and from pupae to adults, and (2) male and female pupal mass, were analyzed using one-way ANOVA followed by Dunnett's test (Minitab v.16; Minitab, State College, PA, USA). The level of significance for all tests was set to $\alpha = 0.05$.

Results

To measure the foliage concentrations of maltol, two foliage age classes (C and C-1) from the same branch of the same tree were collected from a total of 10 balsam fir trees. The range found in the current-year foliage was 0.20–0.54% (mean ± SD = 0.36 ± 0.12%), whereas maltol concentrations in 1-year-old foliage ranged from 0.87 to 1.80% (1.12 ± 0.28%). Although maltol was found in high quantities in both foliage age class samples, concentration was higher in the 1-year-old foliage samples vs. the new foliage (Figure 1A).

To evaluate whether maltol is glucosylated in balsam fir, the same foliage samples were analyzed by LC-MS-MS against a pure maltol glucoside standard. Maltol glucoside was found in both foliage age classes (current-year foliage: 0.000027 ± 0.000022%; 1-year-old foliage:

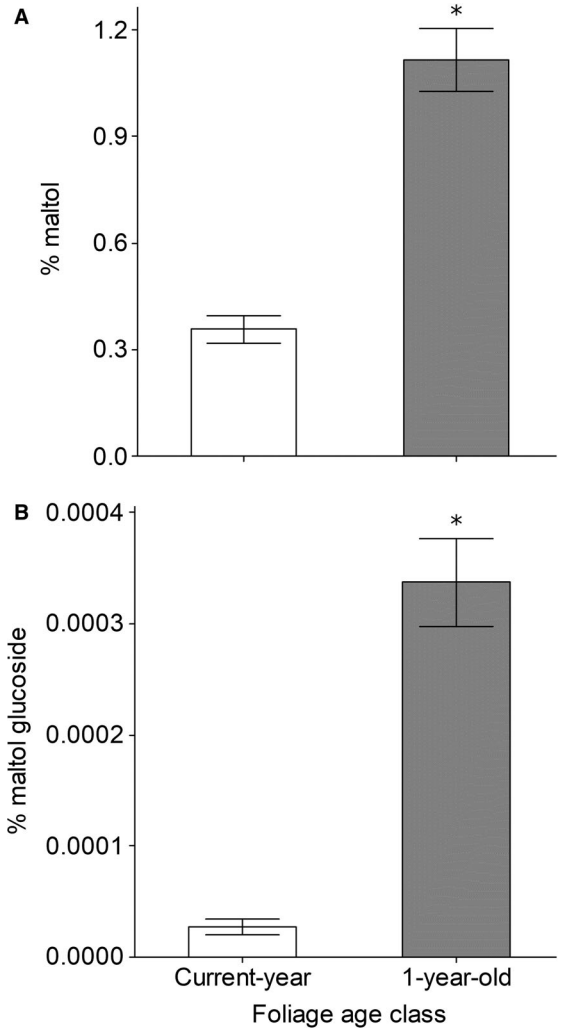

Figure 1 Mean (± SD) concentrations (% wet weight) of (A) maltol and (B) maltol glucoside in current-year and 1-year-old balsam fir foliage. * indicates a significant difference between foliage age class (two-tailed paired t-test: P<0.001; n = 10 per foliage age class).

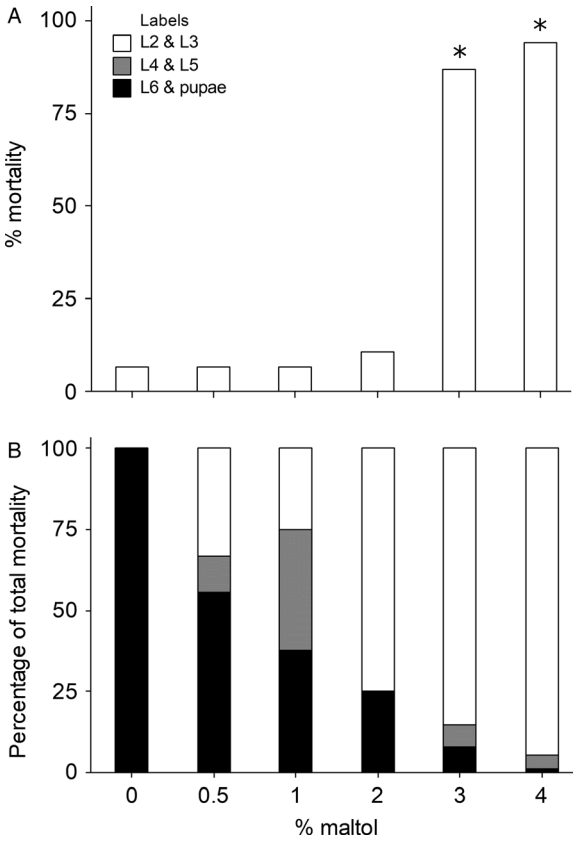

Figure 2 (A) Effects of maltol on spruce budworm (SBW) mortality from L2 to adult eclosion, and (B) percentage of SBW that died as L2 and L3, L4 and L5, and L6 and pupae, when fed maltol incorporated into artificial diet at concentrations of 0 (control), 0.5, 1, 2, 3, and 4% (wet weight) of the diet's volume. * indicates a significant difference from the control (Dunnett's test: P<0.05; n = 100 per treatment).

0.00034 ± 0.00013%; Figure 1B) but at much lower concentrations compared with maltol (>13 200 × lower in current-year foliage, >3 300 × lower in older foliage). However, the concentrations of maltol glucoside were higher in 1-year-old foliage than in younger foliage (Figure 1B). Ratio differences were also higher with maltol glucoside (12.5-fold change) than that measured for maltol (3.1-fold change) when comparing 1-year-old with current-year foliage.

Artificial diet supplemented with maltol affected SBW larval and pupal mortality (χ^2 = 437.89, d.f. = 5, P<0.0001), with significant mortality occurring at 3% maltol (87% mortality; χ^2 = 83.21) and at 4% maltol (94% mortality; χ^2 = 86.11, both d.f. = 1, P<0.0001), compared with the control (7% mortality; Figure 2A). Of the subset of SBW that died, most of the mortality that occurred at maltol concentrations >1% occurred in the early instars (L2 and L3), whereas most of the mortality in the control and 0.5% concentration occurred in the late instar (L6) and the pupal stage (Figure 2B). Development times from L2 to L6 were affected by maltol concentrations for both males ($F_{4,206}$ = 22.38) and females ($F_{4,162}$ = 17.12, both P<0.001), with the development being longer than the controls at concentrations >0.5% for male larvae and at concentrations >1% for female larvae (Figure 3A). Similarly, development times from L6 to pupae were affected by maltol concentrations for both males ($F_{4,206}$ = 31.00) and females ($F_{4,162}$ = 8.06, both P<0.001). Compared with the controls, the development times of male larvae from L6 to pupae were longer only at the two highest

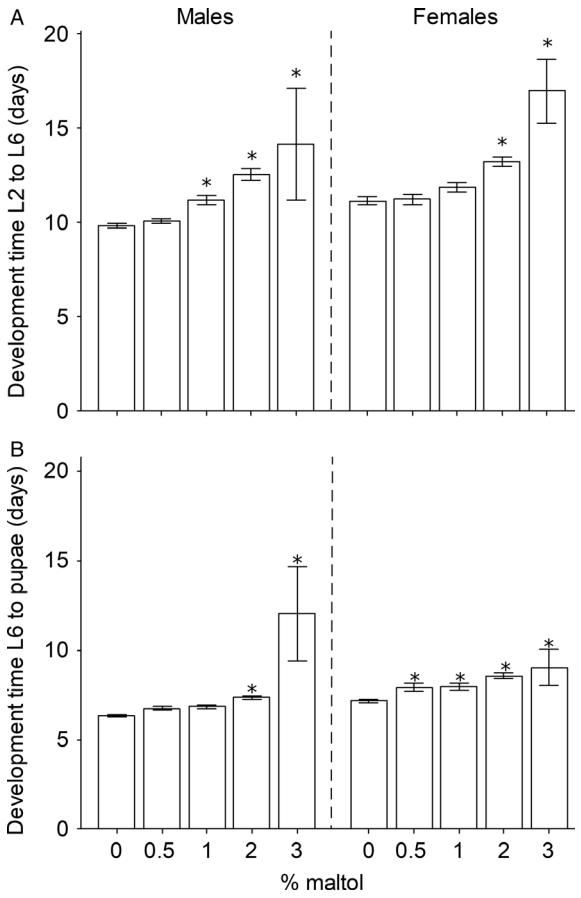

Figure 3 Effects of maltol incorporated into artificial diet at concentrations of 0 (control), 0.5, 1, 2, and 3% (wet weight) of the diet's volume on mean (± SE) development time (days) of male and female spruce budworm from (A) L2 to L6, and from (B) L6 to pupae. * indicates a significant difference from the respective control (Dunnett's test: $P<0.05$).

concentrations (2 and 3%), whereas the development times of female larvae were longer at all concentrations (Figure 3B). The development times of both male and female pupae were not significantly affected by any of the maltol concentrations tested (males: $F_{4,195} = 1.5$, $P = 0.20$; females: $F_{4,158} = 0.88$, $P = 0.48$; data not shown). Pupal mass (Figure 4) was affected by maltol concentrations for both males ($F_{4,206} = 3.89$, $P<0.005$) and females ($F_{4,162} = 23.33$, $P<0.001$), with the pupal mass being lower at all concentrations for males and females, compared with controls. Neither the sex ratio (% females) nor mating success (% females mated) were affected by maltol concentrations (sex ratio: $\chi^2 = 2.39$, d.f. = 4, $P = 0.66$; mating success: $\chi^2 = 3.08$, d.f. = 3, $P = 0.38$; data not shown). Too few adults survived at the 3 and 4% concentrations to test effects on mating success.

Discussion

Maltol is produced and glucosylated in balsam fir foliage

In this study, we found that maltol was present in all samples tested but at higher concentrations in 1-year-old foliage than in current foliage. These higher concentrations in 1-year-old foliage could be due to either an increased level of maltol production or decreased moisture content in older foliage compared with new foliage. As buds flush and elongate during spring, moisture content in current-year foliage can be 2–3× higher than that found in 1-year-old foliage (Little, 1970). This is phenologically matched to the feeding period of SBW larvae (Miller, 1963). Because our objective was to evaluate actual amounts of maltol in the foliage that SBW would be exposed to during herbivory, fresh weight values were assessed instead of dry weights. Using fresh weights minimizes the effect on the weight variable due to the loss of volatile secondary

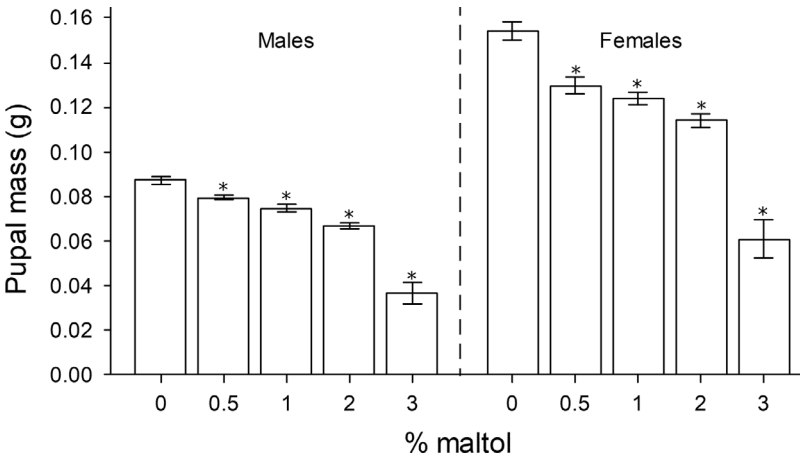

Figure 4 Effects of maltol incorporated into artificial diet at concentrations of 0 (control), 0.5, 1, 2, and 3% (wet weight) of the diet's volume on mean (± SE) mass (g) of male and female spruce budworm pupae. * indicates a significant difference from the respective control (Dunnett's test: $P<0.05$).

metabolites such as maltol related to drying foliage and allows for the measurement of actual in-plant concentrations of toxic metabolites present during SBW feeding. Spruce budworm will occasionally mine into needles of older foliage when it is the only foliage available in the spring before host budbreak (Miller, 1963). However, this feeding reduces pupal size and fecundity (Blais, 1952) and could be an important mortality factor for emerging L2 feeding on older foliage with higher concentrations of secondary metabolites. Whether SBW has adapted its behavior to select foliage with lower levels of secondary metabolites remains unknown, but its preference for new foliage may increase its fitness as lower levels of maltol are found in current-year foliage. Constitutive production of some conifer foliar secondary metabolites, including monoterpenes and acetophenones, varies from year to year and throughout a season, and is influenced by factors such as tree age, foliage age, and environmental conditions (Bauce et al., 1994; Kumbasli et al., 2011; Despland et al., 2016; Mageroy et al., 2017; Parent et al., 2017). The phenological mismatch between foliage development in the spring and early-instar emergence could potentially expose larvae to higher concentrations of toxic secondary metabolites and impact early-instar survival rates.

Another factor that changes herbivore exposure to toxic metabolites is the potential for the metabolite to exist as a bound form, such as being glycosylated. Evaluating both bound and free concentrations of secondary metabolites is important to consider if exposure levels are to be assessed. It is possible that the herbivory process itself could increase exposure to higher concentrations of toxic metabolites after release from its bound form (Neilson et al., 2013; Pentzold et al., 2014). Although glucosylated forms of maltol have been found in some plant species (Tiefel & Berger, 1993; Adams et al., 2015), to our knowledge, this is the first time maltol glucoside has been found in a conifer species. We assessed free and bound levels of maltol at a time that correlates to SBW feeding in the spring to select appropriate foliage concentrations of maltol to be used for supplementing the diet. At this time, seasonal fluctuations of these compounds in balsam fir remain unknown. However, at the time used for measurement, the proportion of maltol glucoside to maltol found in balsam fir foliage is so small that conversion to maltol would not significantly increase exposure of SBW to maltol during feeding. Further evaluation of seasonal fluctuations of these compounds might help determine whether maltol is produced from the cleavage of a maltol glucoside precursor similar to what is found in the phenolic acetophenones in spruce (Delvas et al., 2011), or if maltol glucosylation has another function. Such a temporal shift in concentrations has been observed in *C. japonicum*, where maltol is present during spring and summer, decreases at the end of summer, and increases for a short period of time in the fall (Tiefel & Berger, 1993). In *C. japonicum*, maltol glucoside was only found in the fall, although no mechanistic explanation for this was given, but it could involve one or more features of glycosylation to avoid losses during leaf senescence. Evaluating the inducibility of both of these compounds after herbivory (same season or in subsequent seasons) would also be valuable to see whether some populations or at least individuals can adapt and defend against herbivory.

Maltol impacts SBW fitness

Given that maltol is emitted from balsam fir during SBW herbivory (Leclair et al., 2015), and its potential role as a defensive secondary metabolite, we investigated the impact on SBW fitness by supplementing artificial diet with maltol. Using a range of maltol concentrations either attainable or higher than what is found in current-year or 1-year-old foliage, significant mortality in SBW was observed but only at diet concentrations that were higher than foliar concentrations of maltol. Although a gradual decrease in survivorship was expected as maltol concentration increased, our results showed that a significant increase in mortality occurred when diet concentrations exceeded 2%. Mortality was not significantly affected at lower concentrations, but occurred at earlier instars at those concentrations. Earlier instar mortality is consistent with other fitness parameters that showed a significant decrease in development rate and pupal mass for both sexes at those lower concentrations (<2%) that are attainable in foliage. Longer development time could indirectly lead to increased mortality due to longer periods of exposure to natural enemies (Parry et al., 1998), as well as decreased adult fecundity due to lower pupal size (Blais, 1952). Sublethal effects that influence feeding behavior could also contribute to lower egg production (Haynes, 1988). To investigate whether maltol has a stronger effect on SBW fitness at early instars, each instar would have to be evaluated separately at those concentrations. Presently, the mode of action by maltol on SBW remains unknown, but could involve its potent metal-chelating activity (Yasumoto et al., 2004). Metal chelation to maltol has shown that it can act as a pro-oxidant (Murakami et al., 2006). This generates reactive oxygen species, which, upon further modification, can produce the powerful cytotoxic hydroxyl radical ($OH^{\cdot}$) that can contribute to antimicrobial activity and induce lethal oxidative damage to lipids, DNA, and proteins (Imlay, 2003).

In a monoterpene study done on balsam fir, mortality rates were significantly higher for α-pinene and Δ^3-carene at concentrations similar to that in foliage, whereas

mortality was higher in individuals when bornyl acetate and camphene were supplemented at concentrations exceeding those found in foliage (Kumbasli & Bauce, 2013). However, they used female L6 in their study. When compared with the phenolic acetophenones (Delvas et al., 2011), the synergistic effect of piceol and pungenol on larval mortality was higher than that of maltol at their natural foliage concentrations. At concentrations higher than that found in foliage, larval mortality increased significantly for both maltol and the phenolic compounds, although the instar at which mortality occurred was not evaluated in the acetophenone study. Future studies that determine this would be useful. Although most (ca. 90%) food consumption happens at the sixth instar in SBW (Miller, 1977), feeding on high concentrations of defensive secondary metabolites at earlier instars can have a bigger impact on larval survival due to the higher relative growth rate, consumption rate, metabolic rates, a higher assimilation efficiency, and lower net growth efficiency of these early instars (Scriber & Slansky, 1981; Johnson & Zalucki, 2007). These factors, along with their potential increased vulnerability to plant defense compounds (Zalucki et al., 2002), may contribute to current-year foliage being the preferred food source for early-instar SBW.

As maltol is known to be glucosylated in some plant species, we investigated and confirmed the presence of a glucosylated form of maltol in balsam fir, which has not been previously found in a conifer species. At the time measured in this study, concentrations of maltol glucoside are very low compared with maltol, and release of this conjugated form to maltol upon herbivory would not significantly change the exposure level of SBW to maltol. However, similar to the monoterpenes and acetophenones, temporal changes in the production of both metabolites during feeding could alter SBW exposure to maltol and impact survival or development. Further evaluation in mature forest stands is needed to assess if maltol can occur naturally in balsam fir foliage, either due to individual variation or by induction after herbivory, to be used as a defense against SBW. Exploiting natural variants producing higher levels of maltol in balsam fir foliage could enable the development of a tree improvement program similar to what is currently being done for many forest trees (Sniezko et al., 2012). Significant research effort will be needed to fully investigate this prospect, but it could be useful for a variety of stakeholders, including Christmas tree growers. At this point, further characterization of maltol is worth investigating, such as its mechanism of action in SBW and whether it is involved as a kairomone of natural enemies, given its volatility. Using trees that constitutively produce or that can be induced to produce high levels of defensive secondary metabolites phenologically matched to early-instar herbivore feeding might be beneficial for the development of tree improvement strategies to breed resistance against insects and diseases to limit severe defoliation and mortality of forest trees (Sniezko & Koch, 2017).

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