Spruce Budworm (Lepidoptera: Tortricidae) Oral Secretions II: Chemistry

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ABSTRACT As sessile organisms, plants have evolved different methods to defend against attacks and have adapted their defense measures to discriminate between mechanical damage and herbivory by insects. One of the ways that plant defenses are triggered is via elicitors from insect oral secretions (OS). In this study, we investigated the ability of second-instar (L2) spruce budworm [SBW; *Choristoneura fumiferana* (Clemens)] to alter the volatile organic compounds (VOCs) of four conifer species [*Abies balsamea* (L.) Mill., *Picea mariana* (Miller) B.S.P., *Picea glauca* (Moench) Voss, *Picea rubens* (Sargent)] and found that the emission profiles from all host trees were drastically changed after herbivory. We then investigated whether some of the main elicitors (fatty acid conjugates [FACs], β-glucosidase, and glucose oxidase) studied were present in SBW OS. FACs (glutamine and glutamic acid) based on linolenic, linoleic, oleic, and stearic acids were all observed in varying relative quantities. Hydroxylated FACs, such as volicitin, were not observed. Enzyme activity for β-glucosidase was also measured and found present in SBW OS, whereas glucose oxidase activity was not found in the SBW labial glands. These results demonstrate that SBW L2 larvae have the ability to induce VOC emissions upon herbivory and that SBW OS contain potential elicitors to induce these defensive responses. These data will be useful to further evaluate whether these elicitors can separately induce the production of specific VOCs and to investigate whether and how these emissions benefit the plant.

KEY WORDS β-glucosidase, elicitor, fatty acid conjugate, glucose oxidase, volatile organic compound

The spruce budworm (SBW) is among the most significant conifer defoliators in Canada, and population outbreaks are known to occur every 30–40 yr (Royama 1984, Hix et al. 1987). Much effort has been devoted over the past few decades to understanding this native pest's ecology to monitor and mitigate its impacts on forest productivity (Royama 1984). Among the management options used in dealing with SBW, the most prevalent is the use of chemical insecticides. Because of environmental regulations, the only formulations available for use today to protect foliage against SBW are the bacterium *Bacillus thuringiensis kurstaki* and the insect growth regulator tebufenozide (MIMIC) (Cadogan et al. 1998, Carisey et al. 2004). In addition, the female-produced sex pheromone (Silk and Kuenen 1988, Silk and Eveleigh 2016) is registered for use in Canada as a mating disruptant (Hercon Disrupt Micro-Flake Spruce Budworm Mating Disruptant).

It is well known that when herbivores feed on their host plants, the host responds with various direct or indirect defensive measures. Direct defense is made possible by the production of secondary metabolites (e.g., alkaloids, terpenoids, and phenolics) or can be physical in nature, making use of various external structures to create barriers or obstacles to avoid herbivory (e.g., trichomes, spines, and thorns). Indirect defense relies on the ability of the host plant to attract natural enemies of herbivores for protection against herbivory (Turlings et al. 1990, Kessler and Baldwin 2001). The benefit of both of these defensive strategies is reduced herbivory. Because of the high metabolic cost of inducing this chemical defense system, plants have developed a sophisticated means of discriminating between wounding and herbivory and they do this by identifying insect elicitors found in insect larval oral secretions (OS) (Howe and Jander 2008). It has been demonstrated that SBW produce varying amounts of OS under various disturbance scenarios and conditions (Rhainds et al. 2011, Eveleigh et al. submitted).

Four main types of elicitors are known: fatty acid conjugates (FACs) (Alborn et al. 1997), β-glucosidases (Mattiacci et al. 1995), inceptins (Schmelz et al. 2006), and caeliferins (Alborn et al. 2007). FACs, the class of elicitors most reported to date, have been found in herbivore OS of many families of Lepidoptera (Turlings et al. 1990, Pare et al. 1998, Kessler and Baldwin 2001, Mori and Yoshinaga 2011). Volicitin (Fig. 1), the first FAC reported (Alborn et al. 1997), is a (*S*)-17-hydroxy derivative of linolenic acid, conjugated to glutamine. The glutamic acid-based FACs as well as 17-hydroxylinoleic and linoleic acid-based FAC derivatives of

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volicitin are often found in herbivore OS. Although present in many families of Lepidoptera, few functions have been confirmed for FACs, but they could act as biosurfactants in the insect gut to micellize dietary fats during digestion (Spiteller et al. 2000, Halitschke et al. 2001). This biosurfactant property could also be useful as a defensive measure against predators (e.g., ants: caterpillars disgorge OS when under attack, and this results in increased grooming time by ants to get rid of the OS, which could give the caterpillars more time to escape [Rostas and Blassmann 2009, Eveleigh et al. submitted]). Another role for FACs could be in nitrogen assimilation as a means of storage via conversion of glutamic acid to glutamine to its FAC (Yoshinaga et al. 2008, Mori and Yoshinaga 2011). β-glucosidases are part of a two-component chemical defense system in many plants that involves the activation of inactive compounds (alkaloid, benzoxazinoid, cyanogenic, and iridoid glucosides) as well as glucosinolates and salicinoids (Halkier and Gershenzon 2006, Morant et al. 2008, Dobler et al. 2011, Pentzold et al. 2014). These compounds are usually compartmentalized separately from the β-glucosidases, and upon herbivory, the mixing of both components results in the release of toxic aglucones. In insects, β-glucosidases are involved mainly in the digestion of cellulose obtained from the ingested plant material but have been found to elicit an indirect defensive response in, e.g., the cabbage white butterfly *Pieris rapae* (Lepidoptera, Pieridae) (Mattiacci et al. 1995). β-glucosidase was found in *P. rapae* regurgitant and elicited the emission of volatiles that enhanced the attraction of parasitoids of *P. rapae*; the insect β-glucosidase-induced volatile emission was more efficient at attracting the parasitoids than that of the cabbage β-glucosidase obtained from a leaf extract.

Herbivorous insects have also developed strategies to avoid triggering plant defensive processes using chemical compounds from their OS. Among the many enzymes found in insect OS, glucose oxidase (GOx) has been proposed to be involved in many functions, including the suppression of direct and indirect plant defenses (Musser et al. 2002, 2005, Zong and Wang 2004, Bede et al. 2006, Delphia et al. 2006, Weech et al. 2008, Diezel et al. 2009). GOx belongs to the family of GMC-oxidoreductases (Iida et al. 2007) and catalyzes the reaction between D-glucose and oxygen to produce D-gluconic acid and hydrogen peroxide.

The molecular mechanisms in defense suppression involved are unknown, but GOx might impact the production of salicylic acid through the increased production of hydrogen peroxide, which in turn would attenuate jasmonate levels through the octadecanoid pathway (Diezel et al. 2009). GOx activity has been found in insect species, such as the honey bee *Apis mellifera* (Iida et al. 2007, Li et al. 2008), the aphid *Myzus persicae* (Harmel et al. 2008), and many Lepidoptera species, including two species of Tortricids [*Ancylis plantana* Clemens, *Cydia pomonella* (L.)] (Eichenseer et al. 2010).

In the first article in this series, we focused on various aspects of the biology and function of SBW OS depicted in a conceptual model of potential functions of OS (Fig. 1, Eveleigh et al. submitted) and reported that SBW OS appears to be multifunctional, serving as both an intra- and interspecific epideictic pheromone and as an antipredator defensive component. In this second article, we investigate other aspects of SBW OS shown in the conceptual model, in particular, the ability of SBW larvae to induce or augment volatile organic compound (VOC) emissions in host plants. We also investigate the composition and relative quantities of the major types of

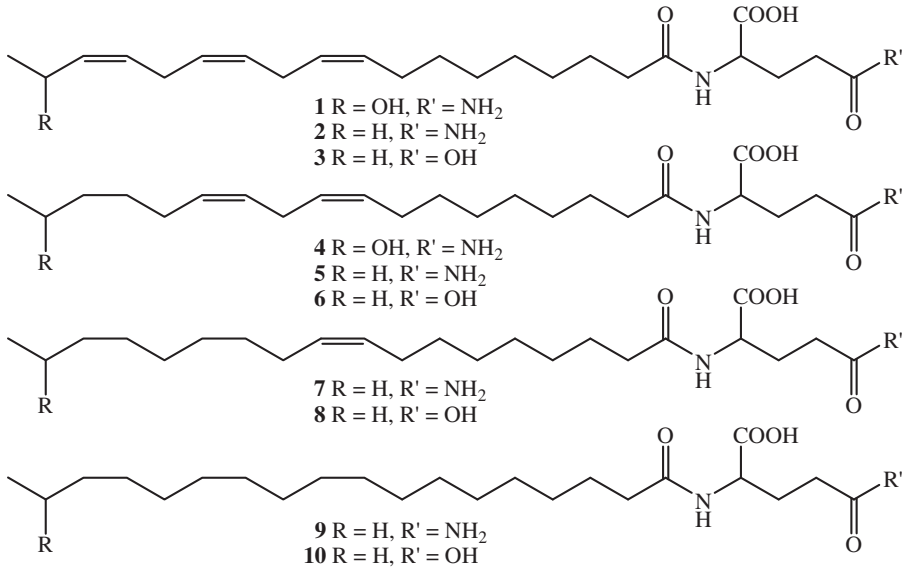

Fig. 1. Chemical structures of volicitin (1) and 17-hydroxy linoleyl-glutamine (4) as well as the glutamine (gln) and glutamic acid (glu) FACs examined in this study: linolenyl-gln (2), linolenyl-glu (3), linoleyl-gln (5), linoleyl-glu (6), oleyl-gln (7), oleyl-glu (8), stearyl-gln (9) and stearyl-glu (10).

elicitors (FACs, β-glucosidases, and GOx) in SBW OS that could be involved in the induction of these VOC emissions, and we evaluate SBW frass as a potential secondary source of VOCs. The information from this work will bring us closer to understanding the chemical interactions between SBW, VOCs (from host tree and SBW), and SBW parasitoids and predators.

Materials and Methods

SBW Rearing. Second-instar SBW larvae (L2) were obtained from Insect Production Services (IPS), Canadian Forest Service, Sault Ste. Marie, Ontario, Canada, and reared on artificial diet (AD, supplied by IPS), wild foliage [red spruce, *Picea rubens* Sarg., white spruce, *Picea glauca* (Moench) Voss, or black spruce, *Picea mariana* (Mill.) B.S.P., or balsam fir, *Abies balsamea* (L.) Mill.,], or greenhouse seedlings (spruces or balsam fir) at 23°C and a photoperiod of 16:8 (L:D) h.

VOCs From Seedlings With vs. Without Budworm Feeding. Volatiles were collected from branches of potted conifer seedlings that were being fed upon by SBW larvae and compared with volatiles collected from undamaged, control seedlings. A single branch per seedling was sampled from 3-yr-old seedlings of balsam fir and white, red, and black spruces. The trees were brought indoors from nursery stock before bud burst. One tree species was tested at a time, with three replicates (seedlings) of each of two treatments: 1) control and 2) 10 L2 SBW enclosed within a sleeve cage on a 12–15-cm-long branch. Baseline volatile profiles were established by sampling daily a few days before bud burst and a number of days following burst before adding SBW, which was timed on a Friday (day 0 in Fig. 2), giving them time to come out of dormancy and settle into a feeding routine. This was confirmed by observed frass pellets the following Monday. VOC sampling continued daily until the end of the week. Branches on control seedlings were also fitted with the same kind of sleeve as a further means of control. VOC sampling was achieved using a solid-phase microextraction (SPME) technique with a polydimethylsiloxane-coated fiber (Aldrich, Oakville, Ontario, Canada). The volatiles were channeled via a 4-cm diameter Pyrex tube, 15 cm in length with a tapered end, 1.5 cm in diameter, and 2 cm in length (custom glassware order; ChemGlass Inc., Vineland, NJ) that was slipped over the branch to be sampled and held in place on an upward angle (30–45°) using clamps. The SPME assembly was clamped to expose the fiber at the narrow part of the tapered section and allowed to collect volatiles for 1 h between 07:00 and 08:00. The glass sleeve was removed to prevent condensation from plant transpiration. The SPME fiber and glass tube that were assigned to a branch were reused on that same branch for the duration of the experiment. The fibers were then placed in labeled screw-cap test tubes and kept at −20°C until analysis onto an Agilent 6890 gas chromatograph (GC) coupled to a 5973 series mass spectrometer (MS) with a Zebron ZB-5 Inferno column (Phenomenex Inc., Torrance, CA). The injection port was kept at 250°C and had a 4 mm i.d. liner (no glass wool). The temperature program was as follows: 70°C held for 2 min, ramp to 245°C at 15°C/min, and held for 3 min. Once desorbed (4 min), the SPME was retracted and stored in its screw-cap test tube and kept at −20°C.

Frass Analysis. We observed feeding and frass production in each one of the seedlings on which L2s were sleeve caged. To verify what VOCs frass could be contributing to the mix during sampling, freshly produced frass from fourth- to fifth-instar larvae being reared on foliage was collected off drop sheets, which would ensure a maximal VOC content. Twenty pellets were placed in a 1.8-ml vial with a septum cap. A polydimethylsiloxane SPME fiber was introduced through the septum and left to adsorb volatiles for 15 min at room temperature. The fibers were desorbed and analyzed as described above to obtain a qualitative profile of VOCs ($n = 1$).

OS Collection and Processing. In addition to harvesting OS from well-fed larvae as described below, we also harvested OS from food-deprived larvae. This is because during outbreaks and especially when heavy defoliation of host trees occurs, it can be expected that SBW larvae experience periods of food deprivation. It has also been documented that OS volume from food-deprived larvae increases compared with regularly fed larvae (Rhainds et al. 2011). To study whether this fasting and volume change have an impact on FAC and fatty acid (FA) production or content, fifth- or sixth-instar SBW larvae were deprived of their food source for a 24-h period before harvesting.

Fifth- or sixth-instar larvae were placed in a Petri dish and gently tapped on the head until they regurgitated a globule of OS, which was siphoned into a 5-μl glass pipette via capillary action. Once the tube was almost full (4.8–5.0 μl), which often required pooling OS from a few SBW specimens, it was sealed at both ends with warmed paraffin wax and stored in a freezer at -17°C or used immediately for analysis in the case of GOx and β-glucosidase activity assays (see sections "GOx Activity Assay and Protein Determination" and "β-Glucosidace Activity Assay"). Prior to FA and FAC analysis, the contents of the tube were transferred to a 0.2-ml microcentrifuge tube containing 100 μl methanol, vortexed for a few seconds, and centrifuged for 10 min at 12,000 revolutions per minute. The supernatant was collected and concentrated to dryness using a CentriVap concentrator (Labconco Inc., Kansas City, MO), where the rotor chamber was heated to 45°C. The remaining extract was reconstituted in 60 μl 1:1 water:acetonitrile and filtered using a 0.45-μm syringe filter into a vial and kept at −20°C until analysis (each processed sample represented approximately 5 μl OS).

SBW Glands for Ultra-performance Liquid Chromatography–Mass Spectrometry analysis. Mandibular and labial glands were dissected from a number of sixth-instar larvae, preserved in pairs in 0.1 ml of methanol, and kept at −20°C. Whole-head samples were also retained. Each sample was homogenized using a tissue grinder with a plastic sterile pestle for 1.5 ml microcentrifuge tubes, filtered into a new tube, and the previous tube and filtrate rinsed three times with 0.1 ml portions of methanol. The filtrate was rotovapped to dryness and reconstituted as with the OS

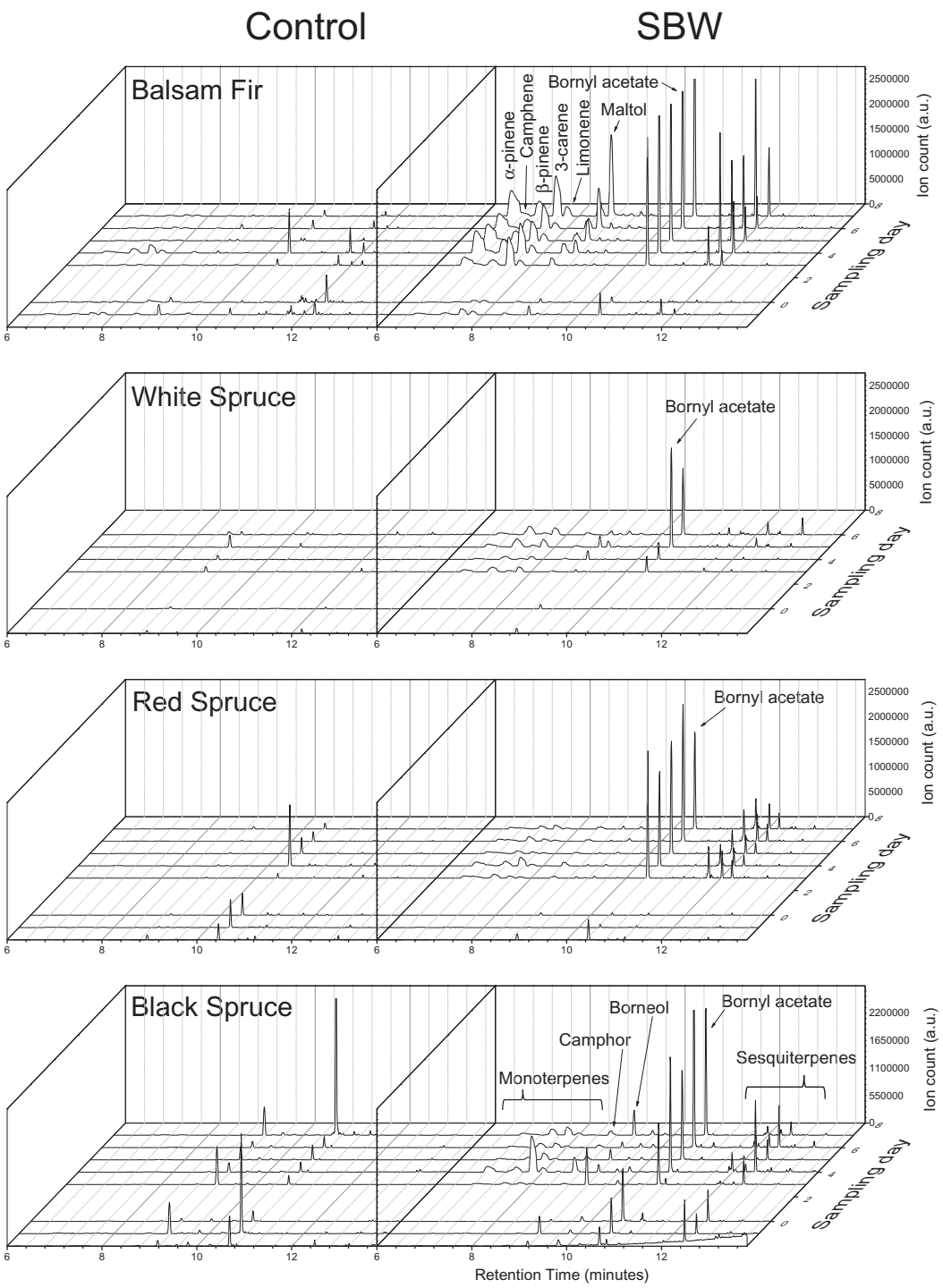

Fig. 2. GC-MS chromatograms (mean, $n = 3$) of daily sampling of VOCs emitted from seedlings with SBW feeding and controls (no SBW) on four conifer species (10 L2 SBW were added to the sleeved shoot on day 0, see Materials and Methods).

samples above. Labial gland preparations for enzymatic assays are discussed later.

FAC Standards. A series of eight FACs were synthesized from linolenic, linoleic, oleic, and stearic acids, conjugated to either glutamine or glutamic acid according to Spiteller et al. (2004). FA and amino acid standards were obtained from Chem-Impex International Inc. (Wood Dale, IL).

Ultra-performance Liquid Chromatography Electrospray Ionization Time-of-Flight Mass Spectrometry (UPLC-ESI-TOF-MS). FACs were analyzed by ultraperformance liquid chromatography (Waters nano-acquity UPLC, Mississauga, Ontario, Canada) coupled to a time of flight mass spectrometer (Waters Xevo QTof, Mississauga, Ontario, Canada), using an electrospray ionizing source operating in negative-ion mode. The capillary voltage was set at 1.8 kV, sampling cone voltage at 39 V, extraction cone voltage at 2.9 V, source temperature at 100°C, desolvation temperature at 450°C, cone gas flow at 76 liters/h, desolvation gas flow at 625 liters/h, and purge gas flow at 300 liters/h. The UPLC column used was a Waters Acquity HSS T3, 1.8 µm, 1.0 mm by 100 mm with a constant flow rate of 40 µl/min. Solvent A was water with 0.1% formic acid, and solvent B was acetonitrile with 0.1% formic acid. The solvent gradient of A:B was as follows: 60:40 B for 3 min, increased to 5:95 over the next 23 min, held for 5 min, and returned to the original 60:40 ratio over the last 5 min. The sample injection volume was 5 µl. The highly intense [M-H] negative ion and retention time of each FAC were compared against standards to confirm the analyte identity (Yoshinaga et al. 2010).

GOx Activity Assay and Protein Determination. As GOx activity in Lepidoptera is mostly found in labial glands (Eichenseer et al. 2010), we analyzed GOx activity from labial glands of sixth-instar SBW (wild and reared on AD) and late-instar *Trichoplusia ni* (Hübner), which acted as our positive control for GOx activity. Early instar *T. ni* were obtained from IPS and reared on SBW AD and cabbage in the laboratory under the same conditions as SBW (see SBW Rearing). Proper negative (phosphate-buffered saline [PBS] 1X) and positive controls (12.5 µg/ml [17,300 U/g] stock solution of fungal GOx from *Aspergillus niger* [G6125, Sigma, Oakville, Ontario, Canada]) were used for the assays. Before labial gland dissection, actively feeding caterpillars were anesthetized by placing them in a freezer at −17°C for 10 min, and 5–10 pairs of labial glands (each pair being a sample) were removed using forceps and inserted into a 1.5-ml microcentrifuge tube containing 500 µl PBS (1X) and 1 µM phenylmethylsulfonylfluoride (PMSF) as protease inhibitor. The glands were either processed immediately or frozen overnight at −20°C before GOx activity measurements. Labial glands were homogenized in PBS buffer using a sterile blue plastic pestle, and thoroughly homogenized glands were centrifuged at 14,000 RPM for 10 min at 4°C. Supernatant was filtered through a 0.22 µm low protein binding filter before GOx activity assay. We measured GOx activities as described (Eichenseer et al. 1999). The assay measures the rate of dianisidine oxidation by hydrogen peroxide, which is a product of the GOx reaction. Twenty microliters of homogenate were added to 180 µl of master mix (1.8% D-glucose, 0.054 mg/ml o-dianisidine, and 0.02 mg/ml [156 U/mg] in PBS 1X buffer at pH 7.0). Oxidized dianisidine was measured every min for 20 min at 460 nm using a Spectramax M2e multimode microplate reader with Softmax Pro software (Molecular Devices LLC., Sunnyvale, CA) using patented PathCheck PathLength Measurement Technology. Plate temperature was set at 37°C for the duration of the assay. Protein determinations were done using the Bradford assay using the 96-well plate assay protocol (Bradford 1976). Three GOx and protein measurements were made for each sample, and specific activities were calculated.

β-Glucosidase Activity Assay. To identify whether β-glucosidase activity could be a potential insect elicitor of conifer VOCs, β-glucosidase assays were done on labial glands from SBW that were either wild collected or laboratory reared on balsam fir and on OS from SBW reared on AD, using the activity assay kit (MAK129, Sigma, Oakville Ontario, Canada). AD was also assayed to verify whether β-glucosidase activity was present in the diet. OS were collected, and labial gland samples were prepared as mentioned above for the GOx activity assays. AD samples were prepared by grinding approximately 100 mg of AD with a plastic pestle in three times the amount of PBS buffer pH 7.0. The mix was kept at ambient temperature for 30 min before the solution was centrifuged for 10 min at $20{,}000 \times g$ in a microcentrifuge. Supernatant was transferred to a new tube and recentrifuged another 5 min at $20{,}000 \times g$ before being retransferred to a new tube for analysis. To check if the activity of the β-glucosidase could be lost by thermal denaturation, we boiled SBW OS for 10 min and remeasured the activity. A stock solution (0.4 U/ml) of β-glucosidase (from almonds, cat#.49290 [Sigma, Oakville, Ontario, Canada]) was used as positive control for the assay. The positive control was also boiled for 10 min to denature the enzyme. A no β-NPG (*p*-nitrophenyl-b-D-glucopyranoside) (assay substrate) negative control was also added to the assay to verify whether change over time was not due to the oxidation of the OS. Twenty microliters of each sample were used for the assay. Two samples of five pairs of labial glands were analyzed, and three samples of OS (boiled and unboiled) and AD slurry were analyzed. β-glucosidase activity was measured at 405 nm before and after 20 min using a Spectramax M2e multimode microplate reader with Softmax Pro software (Molecular Devices LLC.). Plate temperature was set at 37°C for the duration of the assay.

Statistical Analyses. To assess the effects of food source (AD, balsam fir, white spruce, red spruce, black spruce) and feeding history (fed or food deprived) on the quantities of each FAC and each FA in SBW OS, we used a general linear model: $x = \text{food source} + \text{feeding history} + \text{food source*feeding history} + \text{error}$. Both food source and feeding history were considered as fixed effects. We also used general linear model to determine whether quantities of each FAC and each FA were influenced by food source and purported storage sites of OS (tissue type: labial and mandibular glands and whole head): $x = \text{food source} + \text{tissue type} + \text{food source} * \text{tissue type} + \text{error}$. Both food source and tissue type were considered as fixed effects. If the raw data for each FAC and FA did not meet the assumptions of normality using the Anderson–Darling test, the data were transformed using either Box-Cox transformation or Johnson transformation. All analyses were performed using Minitab 16. Differences are considered significant at $\alpha = 0.05$.

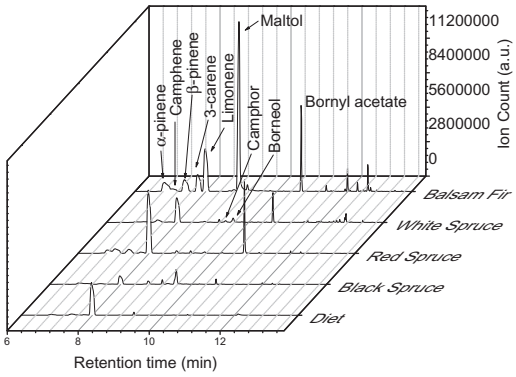

Fig. 3. GC-MS chromatograms of VOCs emitted from frass samples of SBW fed on various dietary sources (balsam fir, white spruce, red spruce, and black spruce).

Results

VOCs From Seedlings With vs. Without Budworm Feeding. VOC sampling of all trees prior to SBW feeding showed traces of compounds, including low levels of bornyl acetate (Fig. 2). However, 3 d after introducing L2 SBW (day 3 and thereafter in Fig. 2), the VOC profile changed for all tree species (Fig. 2); the monoterpenes (α-pinene, β-pinene, 3-carene, limonene, and α-terpinolene) (Silk et al. 2010), bornyl acetate, and several sesquiterpenes were now measurable above baseline sampling abundance levels. Maltol was unique to balsam fir, whereas spruces produced more borneol and camphor. Sesquiterpenes were also more prevalent in seedlings that had been fed on by SBW compared with profiles recorded prior to their addition. Balsam fir and spruces have two major sesquiterpenes in common, tentatively assigned (NIST 2008 MS library search) as β-caryophyllene and humulene (α-caryophyllene), respectively, whereas spruces have additional compounds: longifolene, cadinene, α-farnesene, and elemene for white spruce, germacrene-D or α-cubebene (top two hits for a single unknown) for red spruce, and longifolene, copaene or farnesene or muurolene (top three library hits for a single unknown) for black spruce. White spruce does not appear to respond as strongly as red and black spruces (the chemical identity of the sesquiterpenes was not confirmed by authentic standards).

VOC Analysis in Frass. Frass VOCs were analyzed from each diet-dependent source (Fig. 3). Frass from diet-fed larvae contained traces of volatiles, including limonene and methyl paraben (an antifungal agent added to AD). In addition to the usual mono- and sesquiterpenes, frass from balsam fir-fed larvae also contained maltol and bornyl acetate as dominant VOCs. Frass from spruce-fed larvae had small amounts of monoterpenes, including limonene, similar amounts of bornyl acetate to that in balsam fir frass, low amounts of borneol and camphor, and only traces of sesquiterpenes.

Assessment of FACs and FAs in SBW OS. OSs collected from SBW fed on AD contained all eight FACs, and their relative abundance diminished following a 24-h fast, except for linolenic-glutamine (Fig. 4). Conversely, the OS collected from larvae that were feeding on spruces contained much lower quantities of FACs than OS from diet-fed larvae, and FAC abundance increased following the 24-h fast (Fig. 4). OSs from larvae that fed on balsam fir had the lowest quantities of FACs of all food sources, but most were present in measurable amounts. FAC abundance was significantly affected by the larval food source and, with the exception of oleic-gln and stearic-gln, by feeding history (Table 1). The interaction between food source and feeding history was significant for five FACs and, in most cases, appeared to be due to the negative effect of fasting on FAC levels in OS from larvae that were fed on AD and a positive effect of fasting on FAC levels in OS from larvae that fed on the conifers, especially spruces (Table 1).

FAs were present in all samples regardless of food source or feeding history, with unsaturated FAs being present in greater abundance than stearic acid (Fig. 5). Of the four FAs detected in OS, linolenic acid and stearic acid were not significantly affected by larval food, and only linoleic acid was significantly affected by feeding history. Both linolenic and linoleic acid were significantly affected by the interaction between larval food source and feeding history (Table 1). Fasting reduced levels of linolenic and linoleic acid in larvae that had fed upon AD, red spruce, or black spruce but increased their levels in larvae that fed on balsam fir and white spruce (Fig. 5).

With the exception of stearic-glu, which was found only in head samples of red spruce-fed larvae, all eight FACs were observed in all samples from excised whole-head preparations, and mandibular and labial gland extracts (Fig. 6). All FACs were significantly affected by food source (Table 2). Only quantities of linolenic-glu differed significantly among tissue extracts, and none of the interactions between food source and tissue extracts were significant (Table 2).

FAs were ubiquitously present in all samples (Fig. 7). The quantity of linolenic acid differed significantly among food sources and tissues, with most in the whole-head extracts of larvae that fed on balsam fir and white spruce and least in the mandibular glands of larvae that fed on any food source (Table 2; Fig. 7). The amount of linolenic acid, linoleic acid, and oleic acid differed significantly among tissues, with greatest amounts in the whole-head extracts, and none of the interactions between food source and tissue extracts were significant (Table 2, Fig. 7).

Assessment of GOx Activity in SBW OS. No GOx activity was detected in SBW labial glands, whether larvae were reared on AD or collected from the field (Fig. 8). To confirm the validity of our assay, labial glands from a positive-control species [*T. ni* (Hübner)], measured in the Eichenseer et al. (2010) survey and shown to have GOx activity, were tested. GOx activity was found in this species, both in the cabbage-fed (40 ± 1 nmol/min/mg) and AD-fed (209 ± 2 nmol/min/mg) samples. These activities from *T. ni* confirm the efficiency of the assay and were found to be a little higher for AD-fed insects than what was measured in the survey (Eichenseer et al. 2010). To check for possible GOx activity other than in the labial glands, we tested L2 SBW larvae, fifth- and sixth-instar SBW whole bodies

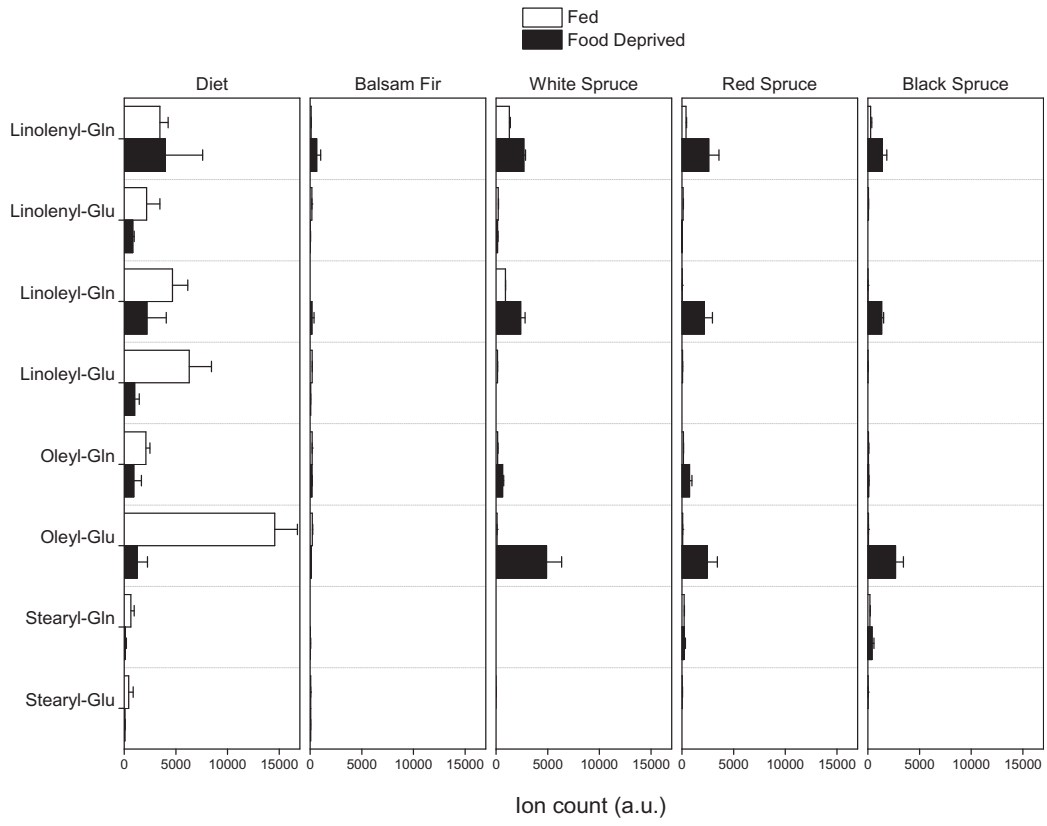

Fig. 4. Mean (±SE; $n = 3$) amounts of FACs extracted from SBW OSs collected during normal feeding and after a 24-h fasting period from each of five different food sources (balsam fir, white spruce, red spruce, and black spruce). Gln, glutamine; Glu, glutamic acid.

Table 1. Results from general linear models (GLMs) assessing the effects of food source and feeding history on quantities of FACs and FAs in SBW OSs

	Food source[a]		Feeding history[b]		Interaction[c]	
	$F_{4,20}$	P	$F_{1,20}$	P	$F_{4,20}$	P
FAC						
Linolenic-gln	6.72	0.001	8.59	0.008	2.93	0.047
Linolenic-glu	38.89	<0.001	34.42	<0.001	1.92	0.146
Linoleic-gln	18.19	<0.001	15.14	0.001	5.86	0.003
Linoleic-glu	37.31	<0.001	66.67	<0.001	1.58	0.219
Oleic-gln	16.67	<0.001	3.39	0.08	6.71	0.001
Oleic-glu	15.63	<0.001	16.29	0.001	27.37	<0.001
Stearic-gln	7.89	0.001	0.30	0.591	0.87	0.497
Stearic-glu	5.08	0.005	4.67	0.043	4.02	0.015
FA						
Linolenic acid	1.23	0.329	2.70	0.116	7.41	0.001
Linoleic acid	7.01	0.001	10.63	0.004	8.48	<0.001
Oleic acid	2.98	0.044	0.01	0.934	1.62	0.208
Stearic acid	2.07	0.123	0.02	0.890	0.41	0.798

[a] Food sources = AD, balsam fir, white spruce, red spruce, and black spruce.
[b] Feeding history = fed or food deprived for 24 h prior to "milking."
[c] Food source * feeding history interaction.

(males and females), SBW heads, and frass, and no GOx activity was found (data not shown).

Assessment of β-Glucosidase in SBW Labial Glands and OS. β-glucosidase activity was detected in the OS of SBW reared on AD (82 ± 3 U/liter) but not in labial glands of SBW reared on white spruce under greenhouse conditions or in feral SBW (Fig. 9). To determine whether the β-glucosidase we measured may have been derived from the diet, we measured the activity in the AD itself and found very low β-glucosidase activity. This confirms the β-glucosidase activity that we observed in the OS is specific to SBW. As thermal denaturation of β-glucosidase inactivates the enzyme, we boiled the SBW OS samples as well as samples of β-glucosidase from almonds (pure) for 10 min and found an almost 90% decrease in β-glucosidase activity in the SBW OS and near 100% decrease in β-glucosidase activity in the β-glucosidase from almonds, compared with untreated samples. The minimal activity remaining in the OS samples after denaturation was not due to actual β-glucosidase activity but to a slight change in absorbance during the assay at 405 nm, which we confirmed with the no substrate negative control and which could be due to the degradation of the sample during the assay.

Discussion

VOC Profile From Herbivory Activity. Once L2 SBW come out of dormancy and begin feeding on host foliage, there is an immediate response from the host

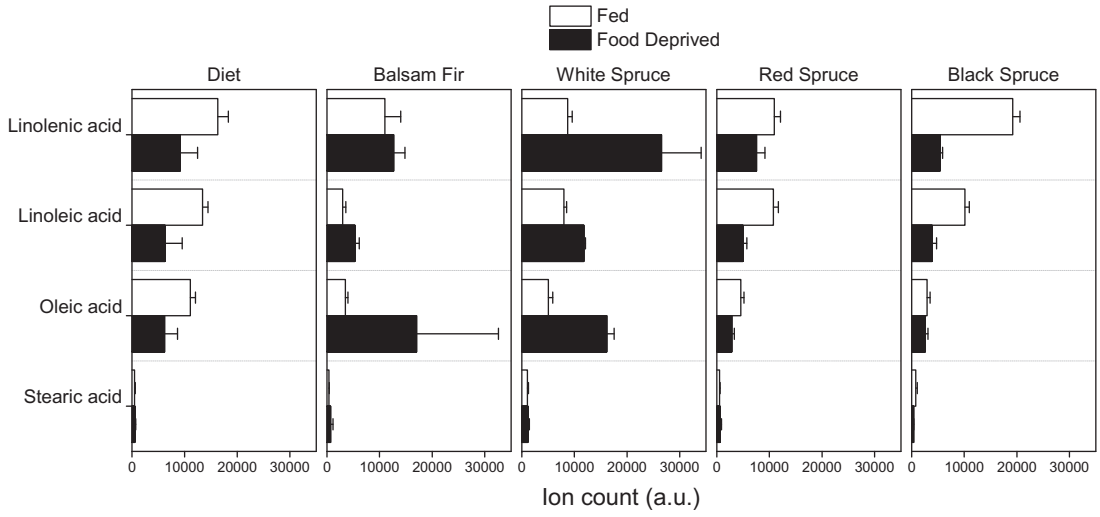

Fig. 5. Mean (±SE; $n = 3$) amounts of FAs extracted from SBW OSs collected during feeding and after a 24-h fasting period from each of five different food sources (balsam fir, white spruce, red spruce, and black spruce).

(Fig. 2). These chromatograms of the collected volatiles (Fig. 2) show that monoterpenes elute first, followed by some hydroxylated compounds, then bornyl acetate, and lastly, a series of sesquiterpenes. In contrast, hosts not experiencing herbivory by SBW emitted low levels of bornyl acetate and trace monoterpenes only. Unique to balsam fir was the increased production of maltol (odor similar to that of cotton candy), whereas the spruces emitted larger quantities of camphor and borneol. There appears to be unique sesquiterpenes to each spruce species, such as longifolene, cadinene, cubebene, copaene, elemene, muurolene, and farnesene. However, without authentic standards, the identification remains tentative. These unique compounds might possibly be used as kairomones by SBW parasitoids (see Eveleigh et al. 2007 for SBW and its natural enemies complex). GC-MS analyses show that frass (Fig. 3) still retained many of the VOCs found from branch feeding. It is known that SBW actively maintains a clean feeding tunnel (Poirier and Borden 1995), possibly to minimize their odor signature and limit parasitism risk.

FACs and FAs in SBW. As the budworm starts to feed on its balsam fir and spruce hosts, several defense signals appear to be induced, leading to different defense responses. Among them are the features of compound recognition in the insect OS, including FACs. Common constituents of OS insect-derived FACs identified so far are either L-glutamine or L-glutamic acid conjugated via an amide bond to linolenic acid, 17-hydroxy linolenic acid, or linoleic acid compounds, with the linolenic acid derivatives being the most potent (Tumlinson and Engelberth 2008). We have now found significant amounts of glutamine and glutamic acid FACs of the stearic (C18:0), oleic (C18:1), linoleic (C18:2), and linolenic (C18:3) series in SBW (Fig. 4), except that levels of saturated stearic-gln and stearic-glu appear to be relatively low compared with the other unsaturated FACs. In FAC biosynthesis, there is a clear preference for unsaturated FAs compared with saturated ones (Aboshi et al. 2007). In this case, the levels of available stearic acid are indeed lower, which could be a limiting factor in the biosynthesis of saturated-based FACs. Interestingly, we did not find hydroxylated FACs such as volicitin in SBW OS. Another tortricid, the light brown apple moth [*Epiphyas postvittana* (Walker)], has been reported to produce glutamine and glutamic acid-based FACs (Yoshinaga et al. 2010), and similar to SBW, hydroxylated FACs, such as volicitin, appear to be absent in this species as well. When comparing the FACs found in fed and food-deprived SBW OS (Fig. 4), we found that there appeared to be visible differences in the biosynthesis of FACs between fed SBW depending on the nutrition source. This may be attributed to AD containing higher amounts of FAs or easier access to these from the AD, which includes processed raw materials such as casein, wheat germ, and linseed oil (McMorran 1965, Grisdale and Wilson 1988). However, AD does not seem to contain any more or less FAs than the other nutrition sources tested (Fig. 5). The level of FACs produced between fed and food deprived in plant-based diets are all lower when fed. This is surprising because FAs are known to be obtained from the plant, so one would expect higher quantities of FACs being produced due to their increased availability. One explanation is that food-deprived SBW are increasing their production of FACs, which is highly unlikely as they are not eating, and FA levels between fed and food deprived are not significantly different except for linoleic acid. Another possibility is that production of FACs is lower when SBW is fed plant-based diets due to potential inhibitors in the plant-based diets. Alternatively, the limiting factor could involve the amino acid component (glutamine and glutamine acid) required for the production of FACs, either by their de novo production by SBW, from their bio-availability in the various diets tested or both. In the majority of cases,

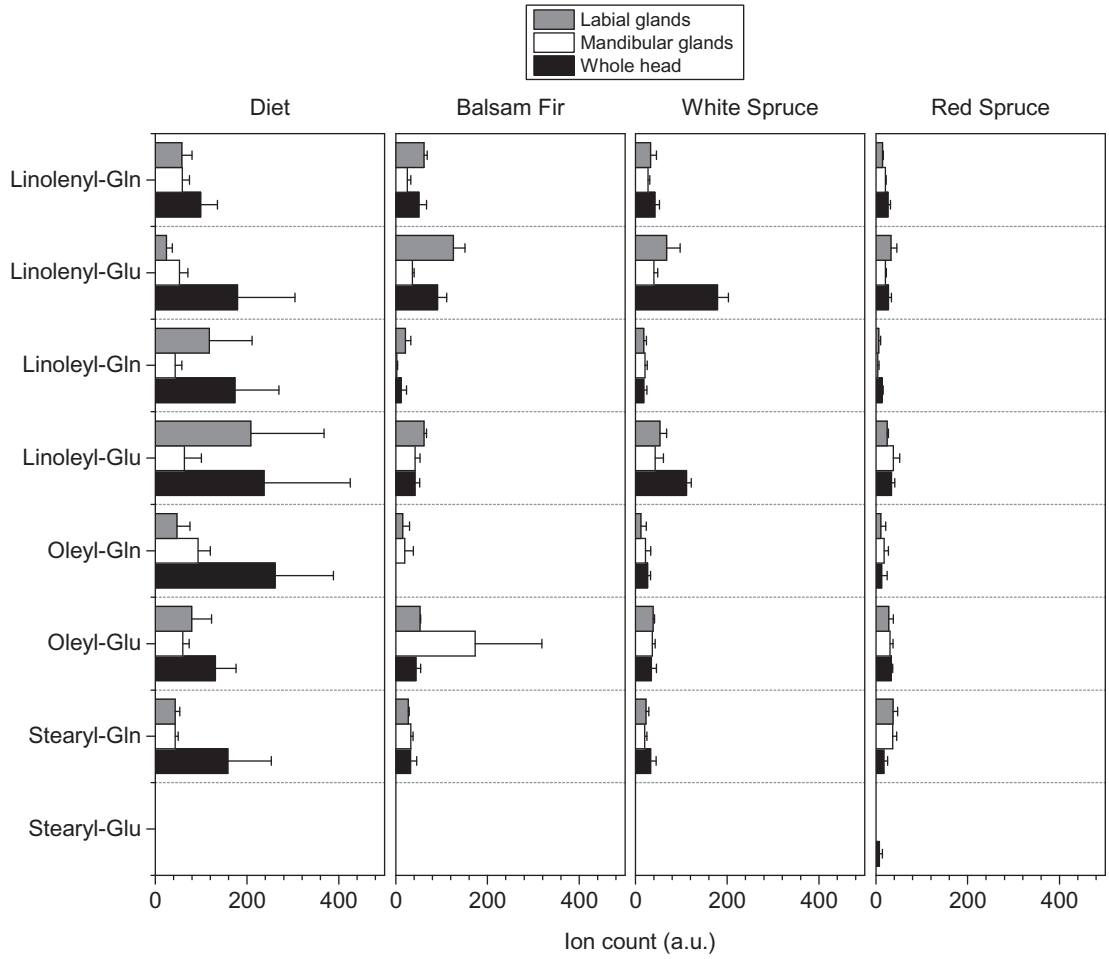

Fig. 6. Mean (±SE; $n = 3$) amounts of FACs extracted from select dissected tissues of SBW sixth-instar larvae that had been reared on various food sources (balsam fir, white spruce, red spruce, and black spruce). Gln, glutamine; glu, glutamic acid.

Table 2. Results from general linear models (GLMs) assessing the effects of food source and tissue extracts (labial and mandibular glands and whole head) on quantities of FACs and FAs in SBW

	Food Source[a]		Tissue[b]		Interaction[c]	
	$F_{3,24}$	P	$F_{2,24}$	P	$F_{6,24}$	P
FAC						
Linolenic-gln	8.30	0.001	2.30	0.122	1.11	0.384
Linolenic-glu	7.79	0.001	5.91	0.008	2.05	0.098
Linoleic-gln	9.92	<0.001	1.18	0.325	0.74	0.625
Linoleic-glu	3.59	0.028	1.95	0.165	1.06	0.413
Oleic-gln	4.27	0.015	0.94	0.406	0.70	0.653
Oleic-glu	3.66	0.027	0.08	0.926	0.34	0.910
Stearic-gln	4.46	0.013	0.05	0.953	1.23	0.326
FA						
Linolenic acid	12.07	<0.001	33.65	<0.001	1.13	0.375
Linoleic acid	2.71	0.068	5.26	0.013	0.29	0.934
Oleic acid	2.94	0.054	3.66	0.041	0.55	0.764
Stearic acid	0.23	0.871	0.43	0.654	0.18	0.979

Note: results for Stearyl-glu were not analyzed because it was found only in the tissues of SBW that fed on red spruce.

[a] Food source = AD, balsam fir, white spruce, red spruce.

[b] Tissue = labial glands, mandibular glands, whole head.

[c] Food source * tissue interaction.

FAC and FA levels in SBW OS were present in much higher quantities than were found in the samples of different insect parts (labial, mandibular glands, and whole head) (Figs. 4 and 5 vs. Figs. 6 and 7). This is understandable as the biosynthetic activity that conjugates the FAs to their amino acid component to produce FACs is known to happen in the insect gut (Paré et al. 1998).

GOx Activity Is Absent From SBW OS. Multiple studies have shown that GOx is an enzyme with multifunctional roles, such as detoxification, digestion, and suppression of plant defense (Eichenseer et al. 1999, Musser et al. 2002, 2005, 2006, Diezel et al. 2009, Afshar et al. 2010). Although GOx has been previously surveyed in other organisms, the most comprehensive study was done on lepidopteran species (Eichenseer et al. 2010), and it was found that most species contained a certain level of GOx activity in their labial glands. Out of the 85 Lepidoptera species (from 23 families) assessed in Eichenseer et al. (2010), two species were members of the Tortricidae, and both

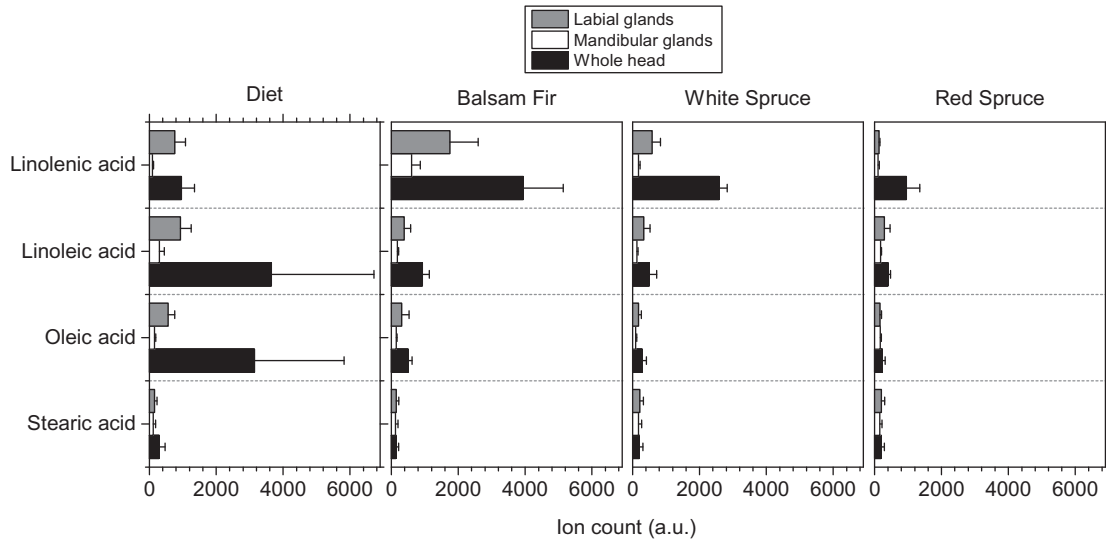

Fig. 7. Mean (±SE; $n = 3$) amounts of FAs extracted from select dissected tissues of SBW sixth-instar larvae that had been reared on various food sources (balsam fir, white spruce, red spruce, and black spruce).

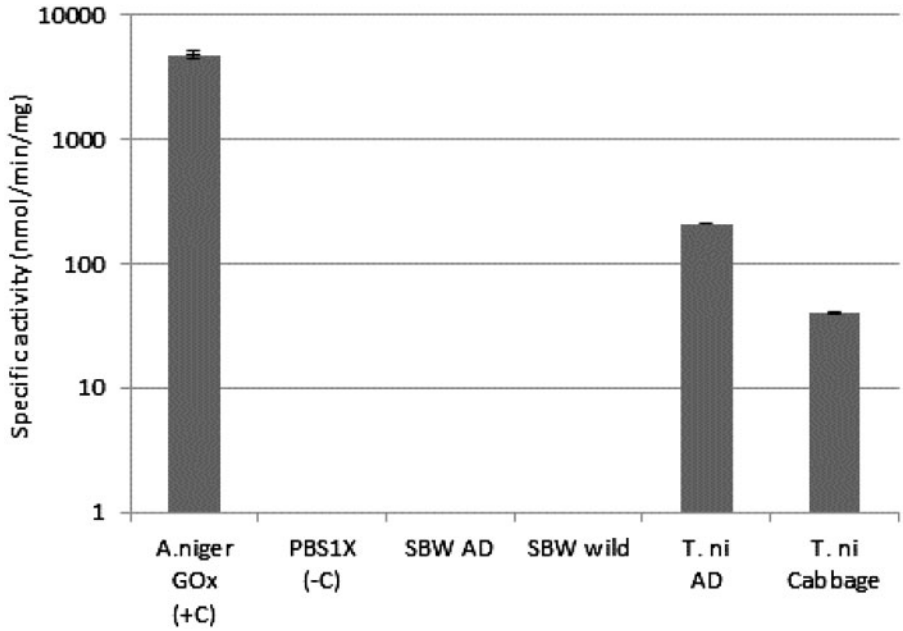

Fig. 8. Mean (±SD; $n = 3$) Glucose oxidase specific activity in labial glands spruce budworm and *Trichoplusia ni (T. ni)* under different diet regimens. AD = artificial diet, wild = feral spruce budworm (diet unknown), PBS = phosphate buffered saline, SBW = spruce budworm.

contained some level of GOx activity, whereas only five species were found to have no detectable GOx activity. In this article, we evaluated the presence of GOx activity in the labial glands of another member of the Tortricid family, *Choristoneura fumiferana,* and found no activity in the labial glands of this species. This result was surprising because both Tortricids previously evaluated had GOx activity but not improbable since GOx activity in species from the same family varied widely and included species with no detectable activity (e.g., Papilionidae and Noctuinae [Eichenseer et al. 2010]).

β-Glucosidase Activity Is Present in SBW OS. Both β-glucosidase and FACs have been tested and proven to be elicitors of indirect plant defense through parasitoid attraction (Boland et al. 1992, Mattiacci et al. 1995, Turlings et al. 2000). β-glucosidase activity was measured in *Pieris brassicae* (L.) OS, and the blend of volatiles emitted by artificially damaged

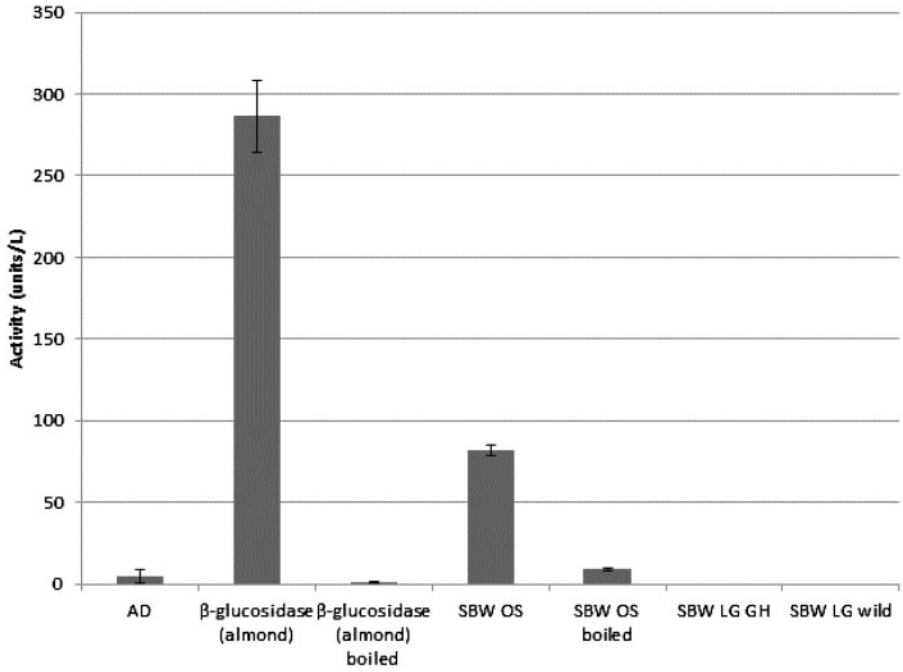

Fig. 9. Mean (±SD; $n =$) β-glucosidase activity in spruce budworm. AD = artificial diet, OS = oral secretions, LG = labial glands, GH = balsam fir fed spruce budworm in greenhouse conditions, wild = feral spruce budworm (diet unknown), SBW = spruce budworm.

cabbage leaves inoculated with OS or β-glucosidase (from almonds) was found to be highly attractive to the parasitic wasp (*Cotesia glomerata* (L.)), a natural enemy of *P. brassicae* (Mattiaci et al. 1995). Similar results were found by treating artificially wounded lima bean leaves with β-glucosidase (Boland et al. 1992). The natural enemy complex of SBW is large (Eveleigh et al. 2007), and attraction of natural enemies by elicitating VOCs through β-glucosidase has yet to be investigated for SBW. Although β-glucosidase activity is present in SBW OS, the origin of the enzyme is not clear: it could be produced by SBW or other organisms (e.g., fungi and bacteria) residing in the gut.

Production of β-glucosidase by microorganisms other than the SBW would indicate that these microorganisms are involved in the digestion of cellulose, but at the same time, could be responsible for the elicitation of plant VOCs. Another drawback for the insect of having endogenous β-glucosidase activity is that it could potentially hydrolyze plant glucosides to toxic aglycons through the plant's two-component system (Pentzold et al. 2014) and retard development and increase mortality (Delvas et al. 2011). For instance, a recent study (Mageroy et al. 2015) found that host resistance against SBW in white spruce was linked to β-glucosidase expression and was due to the presence of higher levels of the acetophenone aglycons, piceol, and pungenol, produced from their respective glucosides, picein, and pungenin. This finding is surprising because β-glucosidase activity tends to be rendered inactive in alkaline guts (Pentzold et al. 2014) to protect the insect from the plant's two-component system. One possibility is that the alkalinity of the gut inactivates the plant β-glucosidase (Pentzold et al. 2014) but not the insect β-glucosidase (either from the insect itself or gut microflora) or that a constitutive de novo production of the insect β-glucosidase is sufficient to enable the digestion of plant material. Either way, the presence of β-glucosidase in SBW OS and its ability to act as an elicitor of plant defense remain to be tested.

In summary, we have shown that feeding L2 SBW can trigger unique volatile production profiles from various hosts. SBW frass contained some of the same volatiles emitted by host trees, including maltol in the case of balsam fir. After confirming that SBW herbivory induced VOC emissions, a variety of major elicitors were investigated from different insect parts and secretions (OS). For example, SBW produced the FACs of glutamine and glutamic acid, which is similar to other herbivorous larvae. When the appropriate building blocks are available, it seems that SBW can produce a wide range of FACs, derived from both saturated and unsaturated FAs. It is also possible that other amino acids are conjugated to the FAs, but these were not examined. Enzyme activities for two other elicitors (β-glucosidase and GOx) were assessed, and the first was found to be present in SBW OS, whereas the second was absent from labial glands. We are currently investigating the impact of these identified elicitors (individual FACs, β-glucosidase, etc.) and their ability to induce specific plant VOCs that were identified during feeding. Separating the volatiles emitted due to mechanical damage from ones that might be induced by mechanical damage and insect elicitors from OS

would be highly beneficial in understanding tree host–herbivore–natural enemy complex for SBW and help in the development of mitigation strategies for this destructive insect.

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