Spruce Budworm (Lepidoptera: Tortricidae) Oral Secretions I: Biology and Function

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ABSTRACT The potential roles of the oral secretions (OS) of spruce budworm (SBW; *Choristoneura fumiferana* Clemens) larvae and factors that may affect the volume of OS disgorged were investigated in the laboratory. Experiments revealed that diet-fed SBW larvae readily disgorge OS when induced ("milked"), with minimal overall cost to their development and eventual pupal weight. Exposure of conspecific larvae to OS throughout larval development negatively affected survival and male pupal weight; however, male development time was faster when exposed to OS. Female pupal weight and development time were not affected. Preliminary experiments suggested that OS had a repellent effect on a co-occurring herbivore, the false hemlock looper, *Nepytia canosaria* (Walker). OS produced by larvae that fed on three host tree species and on artificial diet significantly increased the grooming time of ants (*Camponotus* sp.), indicating that SBW OS have an anti-predator function. The volume of OS is significantly greater in L6 than in L4 or L5, with the volume produced by L6 depending on weight and age as well as feeding history at time of milking. These findings indicate that SBW OS function as both an intra- and interspecific epideictic pheromone and as an anti-predator defensive mechanism, while incurring minimal metabolic costs.

KEY WORDS *Choristoneura fumiferana*, oral secretion, epideictic pheromone, anti-predator defense, natural enemies

In the past few decades, research has demonstrated that larval oral secretions (OS; mixture of saliva and regurgitant) of many herbivorous insects have a wide range of functions (Musser et al. 2006). In addition to involvement in the digestion of nutrients and/or the detoxification of food (Miles 1999), studies indicate that OS can play a role as a defense against predators and parasitoids (Eisner et al. 1974, Gentry and Dyer 2002, Grant 2006, Rostas and Blassmann 2009), as epideictic (spacing) pheromones that deter feeding by conspecific larvae (Corbet 1971; Poirier and Borden 1996, 2000), and as a mediator (elicitor) of both direct and indirect inducible plant defenses against herbivores (Felton 2008). OS may also have antimicrobial properties (Liu et al. 2004, Musser et al. 2005). However, the range of potential functions of the OS of any given herbivorous insect, particularly those species that feed on coniferous trees, has received scant attention.

Here, we investigate aspects of the OS of the spruce budworm (SBW), *Choristoneura fumiferana* (Clem.) (Lepidoptera: Tortricidae), as depicted in a conceptual model constructed mainly from published information about the OS of various herbivorous larvae in agroecosystems (Fig. 1). The SBW, a major forest insect pest in eastern North America, feeds on members of the Pinaceae, mainly balsam fir (*Abies balsamea* (L.) Mill.), white spruce (*Picea glauca* (Moench) Voss), red spruce (*Picea rubens* Sarg.), and black spruce (*Picea mariana* (Mill.) B.S.P.) (Eveleigh et al. 2007). During outbreaks, severe defoliation caused by very high larval densities can lead to reduced growth and even mortality of host trees (Royama 1992) and, consequently, can reduce the food supply for larval growth. SBW larvae are particularly vulnerable to attack by a large, diverse array of natural enemies (predators and parasitic insects) and share their host plants with several herbivore species that serve as alternate or alternative hosts for a variety of SBW parasitoids and predators (Eveleigh et al. 2007). Hence, it is of considerable interest to investigate: 1) the potential roles played by SBW OS in shaping ecological interactions with other organisms in its food web and with its host plants, and 2) the potential of OS constituents as control agents (kairomones, epideictic pheromones, elicitors).

As with many herbivorous insects, SBW larvae regurgitate or "spit" quite readily when disturbed (Rhainds et al. 2011). This predilection for spitting when disturbed may serve as an effective defensive mechanism that is often used by vulnerable, soft-bodied larvae to deter attacks by ants (Peterson et al. 1987, Rostas and Blassmann 2009), spiders (Smedley and Ehrhardt 1993, Theodoratus and Bowers 1999), and parasitic wasps (Gentry and Dyer 2002), and/or it may have repellent effects on coinhabiting herbivorous species (Fig. 1). SBW OS may also act as an epideictic

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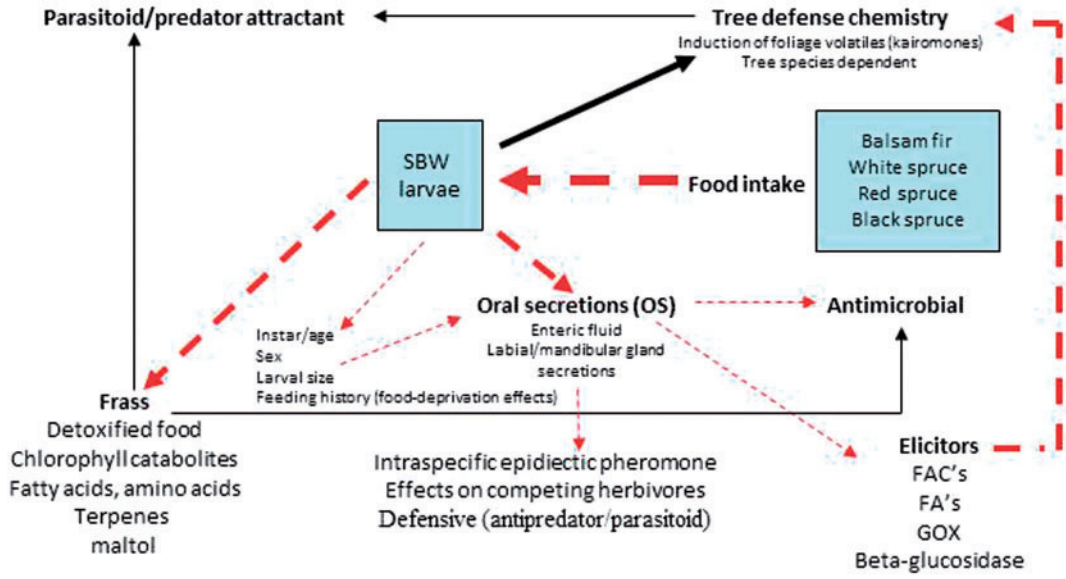

Fig. 1. Conceptual model of the potential functions of spruce budworm OS and factors that may affect its production based on published studies of various herbivorous insects. FAC = fatty acid–amino acid conjugates; FA = fatty acids, GOX = glucose oxidase.

pheromone that deters feeding among conspecific larvae (Poirier and Borden 1995, 1996, 2000; Fig. 1).

Many studies have shown that the OS of some herbivore species contain chemical components that can elicit plant defenses to herbivory (Felton 2008, Howe and Jander 2008; Fig. 1), whereas in other species, the OS can suppress plant defenses (Chung et al. 2013). These insect-induced defenses may be direct, involving the release of compounds that have repellent, antinutritive or toxic effects on the herbivores (Howe and Jander 2008), or they may be indirect, involving the release of plant volatiles that attract herbivore natural enemies or enhance their host-finding ability by exposing herbivore feeding sites (Dicke and Sabelis 1988, Hilker et al. 2002, Turlings and Wäckers 2004, Mumm and Hilker 2006, Ozawa et al. 2008). Certain elicitors in herbivore OS have been identified, for example, β-glucosidase, fatty acid–amino acid conjugates (FACs), inceptin, and caeliferins (Yoshinaga et al. 2014), but neither the identification of potential elicitors in SBW OS nor the potential involvement of SBW OS in eliciting tree defenses has been investigated.

Besides its role as an important elicitor of plant responses to herbivory (Musser et al. 2006), the saliva component of OS, secreted from labial and mandibular glands (Peiffer and Felton 2009), is also purported to have antimicrobial properties (Liu et al. 2004, Musser et al. 2005). For example, the salivary glands of *Bombyx mori* (L.) (Bombycidae) contain a prophenoloxidase-activating enzyme involved in antimicrobial activity (Satoh et al. 1999). Lysozyme, an antimicrobial, has been reported in the saliva of several insects (Liu et al. 2004). In addition, glucose oxidase, another potent antimicrobial, has been found in the labial glands of many insect herbivores (Eichenseer et al. 2010). However, as noted by Liu et al. (2004), few studies have investigated the antimicrobial role of saliva in caterpillars, including SBW.

The volume of OS that a larva can produce in a given bout, and throughout its larval period when repeatedly attacked, will determine, in part, the effectiveness of spitting as a defensive strategy (Grant 2006). However, repeated use of OS as a defensive tactic against attackers and/or coinhabiting herbivores may adversely affect growth and development of immature stages through losses of essential nutrients by disgorgement (Bowers 2003). Some larval regurgitators may compensate for this loss by reimbibing OS (Grant 2006). The factors that may influence the amount of OS produced by SBW during the larval period, as well as the important interplay between the potential defensive/repellent benefits of repeated OS disgorgement and the costs of lost nutrition (Bowers 2003; Fig. 1), remain unexplored.

In this study on SBW OS, we investigate those aspects of the conceptual model represented by the dashed lines in Figure 1, and we report our findings in two separate articles. The experiments presented here focus on aspects of the biology and function of SBW OS (Fig. 1). Our primary objectives are to: 1) determine the effects of repeated disgorgement of OS on SBW life-history performance (i.e., cost of repeated regurgitation); 2) determine if constant exposure to OS influences SBW survival, larval developmental time, and pupal weight; 3) determine if OS repel competing, co-occurring herbivores; 4) determine if SBW OS are used as a mechanism of defense against predators; and 5) examine certain factors that may affect the volume of SBW OS produced. The source and chemical composition of SBW OS, as well as the volatile blends

emitted by host trees in response to SBW feeding and those emitted by SBW frass (Fig. 1), are reported in a companion article (LeClair et al. 2015).

Materials and Methods

Insects. Overwintering, second-instar SBW larvae (L2) were obtained from the Canadian Forest Service's Insect Production Services (IPS) in Sault Ste. Marie, Ontario, Canada. Some larvae were maintained in the laboratory at 22°C and 16-h photoperiod in translucent, 22-mL plastic cups, and fed ad libitum with artificial budworm diet (McMorran 1965), available from IPS. Other larvae were reared on 3- to 4-yr-old potted seedlings of balsam fir, white spruce, red spruce, and black spruce in a greenhouse environment (25–30°C, natural light) or laboratory (22°C, 16-h photoperiod), or on branches cut from the midcrown of mature trees in the University of New Brunswick woodlot (45° 55′ 18.34° N; 66° 39′ 46.18° W). Cut branches were placed in large plastic containers in the laboratory (22°C, 16-h photoperiod), and replaced by freshly cut branches as needed.

OS Collection. Quantities of OS were obtained from SBW larvae by tapping individuals on the head using a 5-μL micropipette as described by Poirier and Borden (1995) and Rhainds et al. (2011). The volume of OS was quantified by multiplying the length (mm) of the column of OS in the micropipette by factor 0.185 μL/mm (Rhainds et al. 2011). The ends of the tubes were sealed with warm paraffin wax and stored at −17°C for later use in experiments described below.

Effects of Inducing OS (Milking) on SBW Life-History Performance. To determine the effects (costs) of repeated inductions of OS throughout most of larval development on SBW survival, development, and growth (weight), we placed 300 newly emerged second-instar (L2) larvae individually into numbered 50-mm plastic dishes containing a 25-mm diameter disk of artificial diet. Using a random number generator, we divided the 300 larvae into three equal groups: 100 control, 100 disturbed but not milked (disturbed group), and 100 disturbed + milked (milked group). The disturbed group was used to determine if any effects of milking could be due partly to being disturbed prior to milking, a situation that is likely to occur in nature when a larva is lightly touched by another larva or natural enemy and backs away without necessarily spitting. Larval development was monitored daily by counting molted head capsules. When the majority had reached the fourth-instar (L4), larvae in both treatment groups were gently encouraged to move into a clean, empty dish using a fine-hair artist's brush. Larvae in the milked group were gently tapped on the head 10 times with a 5-μL micropipette to collect and measure OS (see above) and then returned to their original dish to resume feeding. Larvae in the disturbed group were returned to their original dish without being milked. If the disturbed larvae spit during transfers between dishes, the OS was collected and measured. Larvae in the two treatment groups were manipulated twice during the fourth instar, once during the fifth instar, and three times during the sixth instar for a total of six times from L4 to the prepupal stage. Manipulations of larvae were not performed prior to L4 because there is a high risk of damaging small L2 and L3 and it is not possible to collect and measure the minute amounts of OS they produce. Control larvae were not touched during the whole experiment; only their development was recorded. Pupal weight (to nearest 0.001 gm) was recorded for all groups using a Metler DeltaRange balance.

The effects of milking and disturbance on survival were tested using chi-square goodness-of-fit tests. Differences in pupal weights and larval development times of each sex caused by the experimental treatments were tested using one-way ANOVA.

Effects of Constant Exposure of OS on SBW. The effects of constant exposure to SBW OS throughout SBW larval and pupal development on survivorship, time to pupation, and pupal weight for each sex were examined. Two hundred vials containing artificial diet and one L2 were set up; 100 vials were treated with 5 μL of OS, obtained from artificial diet-fed SBW, every 6 d throughout the larval period from L2 to the prepupal stage, and 100 vials were treated every 6 d with 5 μL of distilled water (control). On each treatment day, the OS and water were applied to the surface of the diet using 5-μL micropipettes. Because large quantities of OS were needed in this experiment, OS stored at −17°C for different lengths of time had to be used. This enabled us to also examine the effects of OS age on intraspecific competition with respect to each of the factors mentioned above.

We conducted a chi-square test to determine the effects of exposure to OS on survival from L2 to the adult stage. The effects of exposure to OS on the pupal weights and development times of each sex were tested using general linear models (GLM). The relationships between the age of OS and pupal weights, and the age of OS and development times, of each sex were analyzed using general regression analyses (GRA).

Repellent Effect of OS on Competing Herbivores. A complex of insect herbivores feed on the same host trees as SBW larvae, many serving as alternate/alternative hosts for SBW parasitoids and as competitors for resources (Eveleigh et al. 2007). We tested the hypothesis that SBW OS have a repellent effect on competing herbivores using late-instar larvae of the false hemlock looper (*Nepytia canosaria* (Walker)) (Geometridae) collected from balsam fir near Sussex, New Brunswick. Two disks (7 mm in diameter by 5 mm in depth) of artificial diet were placed, with centers of disks 22 mm apart, in 50 mm diameter, clear-plastic petri dishes. One disk was ringed with 5 μL of OS from balsam fir-fed larvae and the other disk by 5 μL of distilled water (control). Care was taken not to touch the disks with the OS or water. The looper larvae were food-deprived for 24 h prior to use in the experiment. Each disk was recorded as not fed on, or fed on as either the first or second choice. Owing to low populations of loopers, only a total of 12 larvae were found for use in the experiment ($n = 12$). All larvae were observed at 3 and 4 d after the experiment was

set up. We tested the hypothesis that OS have a repellent effect on the loopers using a chi-square goodness-of-fit test.

OS as a Defense Against Natural Enemies. SBW larvae are attacked by many generalist invertebrate predators, such as ants and spiders. We used ants (*Camponotus* sp.) as model predators in this study. The ants were excavated from a colony in a natural field near Fredericton, NB, transported to the laboratory, and reared in a container of soil from the colony. A single ant was placed in a clean 90-mm-diameter plastic petri dish and kept in a freezer at −17°C for 1.5 min to immobilize it. A 2.5-μL droplet of OS from SBW larvae that fed on one of the three host tree species or on artificial diet (food source) was applied to the head/thorax with a 5-μL micropipette. OS from larvae deprived of food for 24 h after feeding on each host tree species or on artificial diet (feeding history) were also tested. All OS were obtained as described above. Ants receiving 2.5 μL of distilled water or no applications of test liquids served as controls. Ten new ants were used per treatment. The time spent grooming by the ants during a 10-min observation period was recorded for each ant/treatment.

The effect of all 10 treatments on the grooming time of ants was analyzed using a one-way ANOVA. To determine the effects of food source and feeding history on grooming time, the water and nothing controls were dropped, and the data analyzed using GLM. Both food source and feeding history were considered as fixed effects.

Factors Affecting Volume of OS Produced During Sixth Instar. The effects of weight, age, and food deprivation at time of milking on the volume of OS produced by each sex of L6 milked on different days after molting to L6 were examined. Two hundred L2 were reared individually on artificial diet until they reached L6. Each age group of L6 (i.e., 1-, 2-, 3-, 4-, and 5-d-old L6) was then divided into two groups: one group was food deprived for 24 h, while the other group was allowed to continue feeding over the same 24-h period. Individual larvae in both groups were then weighed and milked by tapping the head 50 times and collecting the OS. After milking, larvae in both groups were provided with artificial diet and left undisturbed until pupation. For example, one group of 1-d-old L6 was food-deprived for 24 h, whereas the other 1-d-old L6 group was allowed to feed over the 24-h period. Individuals in both groups were then weighed and milked as 2-d-old larvae and provided with food until pupation. The same procedure was followed for 2-, 3-, 4-, and 5-day-old L6. All individuals were sexed as pupae.

The effects of age of L6 (days within the instar) on the volume of OS produced by each sex were analyzed using one-way ANOVA, followed by Fisher's LSD. The relationships between pupal weight and the volume of OS produced by each sex were analyzed using GRA. The effects on the volume of OS produced by L6 by weight of L6 on day milked, and by feeding history (fed or food deprived) for each sex, were analyzed using GRA.

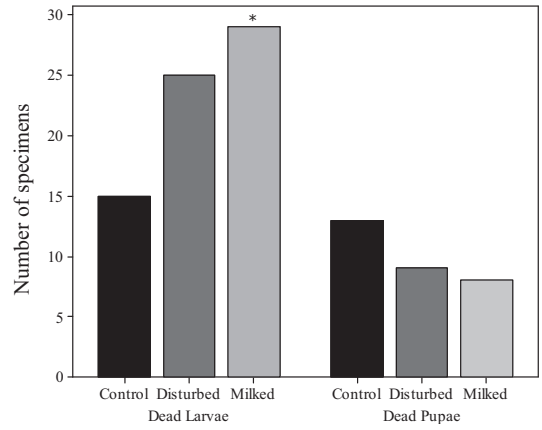

Fig. 2. Number of spruce budworm larvae and pupae that died from being milked, being disturbed without being milked, and being left alone (control) throughout the experiment. (See Materials and Methods for details). Asterisk represents significant difference between treatment and control ($n = 100$ for each group).

Defensive Behavior and Gut Morphology. We used the experimental protocol developed by Grant (2006) to examine the relationship between defensive behavior and gut morphology. Briefly, defensive behavior was assessed by pinching L6 larvae slightly behind mid-body, using blunt, feather-weight forceps to simulate the bite of a predator. The number of pinches required to produce OS was recorded. All larvae were food-deprived for 6 h prior to the experiment to eliminate any effects of previous meal on defensive responses. Gut morphology was measured by pinning freeze-killed L6 larvae, ventral side up, to a dissecting dish and cutting the fully stretched larvae from the head to the anus. The foregut (crop), mid-gut, and total gut length were measured to the nearest 0.1 mm with an ocular micrometer.

Data Analyses. All data analyses were conducted using Minitab 16. The level of statistical significance was set at $\alpha = 0.05$ for all analyses.

Results

Effects of Inducing OS (milking) on SBW Life-History Performance. Although more larvae died in both treatment groups than in the control (Fig. 2), only milked larvae incurred significantly greater mortality than control larvae ($\chi^2 = 4.45$, $P < 0.035$). Once the pupal stage was reached, mortality was similar in all treatments ($\chi^2 = 1.40$, $P = 0.497$; Fig. 2). The pupal weight of neither females (ANOVA: $F_{2,108} = 0.10$, $P = 0.902$) nor males (ANOVA: $F_{2,115} = 1.63$, $P = 0.20$) was adversely affected by milking or disturbance. However, larval development was significantly affected by the experimental treatments (ANOVA: $F_{2,289} = 4.34$, $P = 0.014$), with both disturbed and milked larvae developing significantly faster than control larvae (Fisher LSD, $P < 0.05$), although they did not differ in age at the start of the experiment (ANOVA: $F_{2,297} = 0.81$, $P = 0.445$).

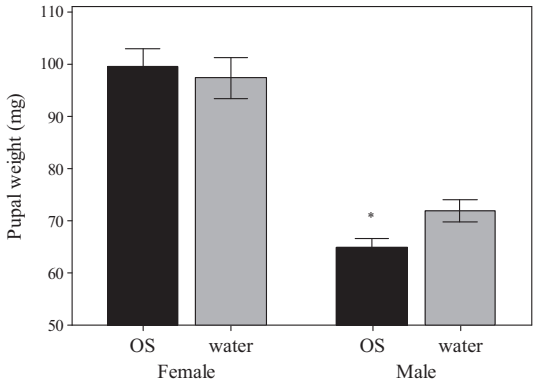

Fig. 3. Mean (± SE) weight of female and male spruce budworm pupae when larvae were exposed continuously from L2 to adult eclosion to either spruce budworm OS or water. Asterisk represents significant difference between treatment (OS) and control (water) in males (n = 100 for each group at start of experiment).

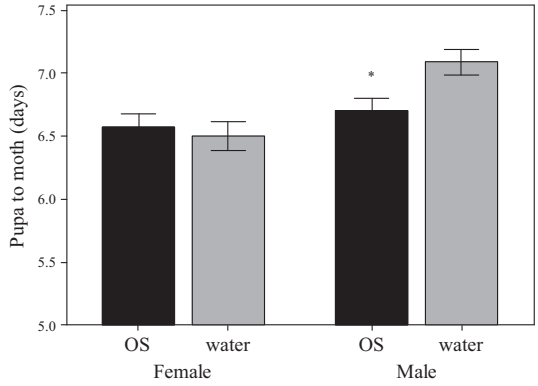

Fig. 4. Mean (± SE) development time (days) of spruce budworm from pupa to adult eclosion when all larvae instars (L2 to L6, inclusively) were exposed continuously to either spruce budworm OS or water. Asterisk represents significant difference between treatment (OS) and control (water) in males (n = 100 for each group at start of experiment).

Effects of Constant Exposure of OS on SBW. Exposure of immature SBW to OS throughout their development negatively impacted survival from L2 to the adult stage ($\chi^2 = 7.86$, $P = 0.02$), particularly the larval stage; nearly twice as many died in the OS treatment (38) than in the water treatment (20). Compared with controls, male pupae weighed significantly less when exposed to OS (GLM: $F_{1,82} = 5.52$, $P = 0.021$), but female pupal weight was not affected (GLM: $F_{1,57} = 0.19$, $P = 0.667$; Fig. 3). Development time from pupa to moth was significantly shorter when exposed to OS for males (GLM: $F_{1,55} = 7.13$, $P = 0.01$), but not for females (GLM: $F_{1,39} = 0.20$, $P = 0.656$; Fig. 4). However, neither the development time from L2 to pupa nor from L2 to moth was affected by exposure to OS in either sex (all $P > 0.23$).

There was a significant positive relationship between the age of OS (i.e., time OS were in stored at −17°C) and female pupal weight (GRA: $F_{1,27} = 9.261$, $P = 0.005$), and a significant negative relationship between age of OS and developmental time of females from L2 to moth (GRA: $F_{1,19} = 5.106$, $P = 0.036$). The pupal weight and developmental time of males from L2 to moth were not affected by age of OS (GRA: $F_{1,31} = 2.217$, $P = 0.147$ and $F_{1,22} = 0.0346$, $P = 0.854$, respectively).

Repellent Effect of OS on Competing Herbivores. The number of false hemlock looper larvae feeding on the OS-ringed disks and on the water-ringed disks as first or second choices did not differ significantly on the first observation day ($\chi^2 = 4.5$, $P = 0.105$), owing to the low numbers of looper available for testing and the number of specimens that did not feed (Fig. 5). However, when those loopers that did not feed were excluded from the analysis, there was evidence of a repellent effect of OS ($\chi^2 = 4.5$, $P = 0.034$). Only 1 of the 12 larvae fed exclusively on the OS-ringed disks (8.3%), and two of the three larvae that fed on the OS-ringed disks did so only after completely consuming the water-ringed disks. Four of the 12 water-ringed disks were completely consumed versus only one of the OS-ringed disks. On the second observation day (4 d after larvae were exposed to the diet disks), eight of the water-ringed disks were consumed, and feeding on the OS-treated disks had increased (8 of 12 were fed on), suggesting any repellent effect of OS diminished when larvae had no other feeding choice.

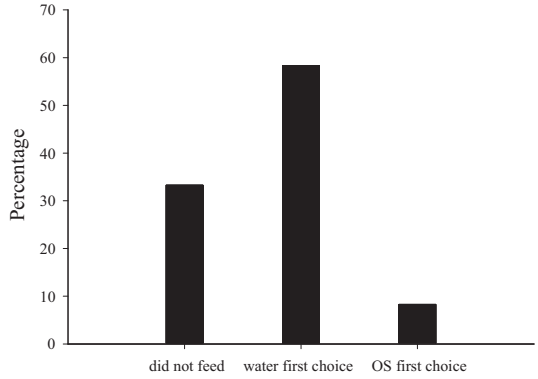

Fig. 5. Percentage of late-instar larvae of the false hemlock looper (*Nepytia canosaria*) that fed on neither the spruce budworm OS-ringed diet disk nor the water-ringed diet disk, fed first on a water-ringed diet disk, or fed first on an OS-ringed diet disk, 3 d after given a choice between both diet disk treatments (n = 12).

OS as a Defense Against Natural Enemies. The grooming time (sec) of ants was significantly affected by the application of OS (ANOVA; $F_{9,91} = 9.73$, $P < 0.0001$; Fig. 6). Regardless of the source of OS (food source and/or feeding history of larvae that produced the OS), the grooming time of ants to which OS were applied was significantly longer than for ants in both of the controls. However, neither the food source (GLM: $F_{3,72} = 1.07$, $P = 0.368$) nor feeding history ($F_{1,72} = 0.01$, $P = 0.909$) affected the grooming time, and the

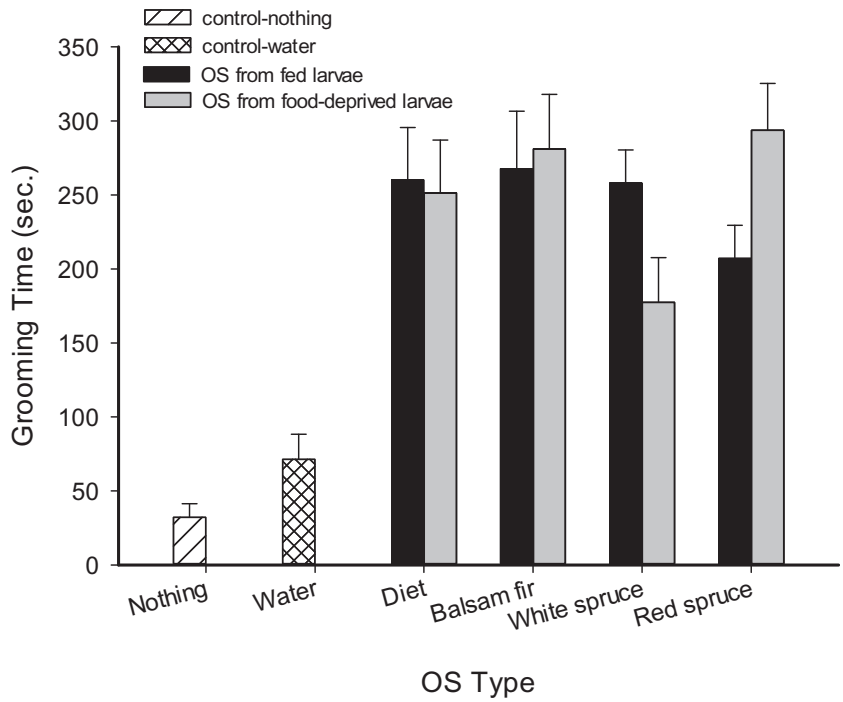

Fig. 6. Effect of spruce budworm OS on the grooming time of ants (*Camponotus* sp.). Ants in the treatment groups were treated with OS from spruce budworm larvae that were either fed ad libitum on artificial diet, balsam fir, white spruce, and red spruce or food-deprived on each of these food sources. Ants in the control groups were treated with either water or nothing. Bars represent mean ± SE grooming time (sec) ($n = 10$ per treatment).

interaction between food source and feeding history was not significant ($F_{3,72} = 2.28$, $P = 0.087$).

Factors Affecting Volume of OS Produced During L6. The volume of OS increased throughout larval development from L4 to L6, with L6 producing about 6–11 times more volume of OS than L5 (Fig. 7). There was a significant difference in the amount of OS produced by male L6 as they aged (ANOVA: $F_{2,86} = 6.47$, $P = 0.002$), with Treatment 6 being significantly lower than Treatments 4 and 5 (Fisher LSD, $P < 0.05$). However, the amount of OS produced by female L6 did not differ with age (ANOVA: $F_{2,108} = 0.69$, $P = 0.504$; Fig. 7). The volume of OS increased significantly with pupal weight in females (GRA: $F_{1,35} = 8.12$, $P < 0.007$), but not males (GRA: $F_{1,35} = 1.36$, $P = 0.252$; Fig. 8). Also, as revealed in the experiment on repeated inductions of OS, simple disturbance of larvae without being milked can also influence the volume of OS produced, with larger females and males showing a tendency to produce larger volumes of OS than smaller individuals; females produced 0.04 ± 0.01 µL of OS (mean ± SE) below median weight and 0.74 ± 0.29 µL above median weight ($n = 35$), and males produced 0.05 ± 0.03 µL of OS below and 0.50 ± 0.16 µL above median weight ($n = 38$).

There was a significant positive relationship between the volume of OS produced by L6 and L6 weight on day "milked" in females ($F_{1,76} = 9.555$, $P = 0.003$), but not in males ($F_{1,69} = 3.334$, $P = 0.072$) when age and feeding history (i.e., fed vs food-deprived) are ignored (Fig. 9). These results would be expected when a random sample of L6 of unknown history is collected in the field, weighed, and milked. However, there was a significant positive relationship between volume of OS and weight in both females ($F_{1,31} = 5.375$, $P = 0.03$) and males ($F_{1,37} = 13.134$, $P = 0.0009$) that had been fed, but not in either females ($F_{1,42} = 2.733$,$P = 0.106$) or males ($F_{1,30} = 1.056$, $P = 0.312$) that had been food-deprived.

The volumes of OS produced by fed and food-deprived female and male L6s at different ages are shown in Figure 10. In females, the volume of OS produced tended to decline with increasing age regardless of feeding history, whereas in males, this decline in OS production was evident only in food-deprived larvae.

Defensive Behavior and Gut Morphology. We found that L6 larvae regurgitated after 2–6 pinches (mean ± SE = 3.5 ± 0.42; $n = 8$), mostly in a nondirected manner, and did not reimbibe OS. The mid-gut proportion of the total gut was 0.66 ± 0.04 and the foregut (crop) proportion was 0.15 ± 0.02 ($n = 8$).

Discussion

Our observations and those of Poirier and Borden (1995) indicate that SBW larvae display aggressive behavior when encountering conspecific larvae and/or when handled, and often respond by disgorging OS. Larvae of the western SBW, *Choristoneura freemani* Razowski (Tortricidae), exhibit similar behavior (Poirier

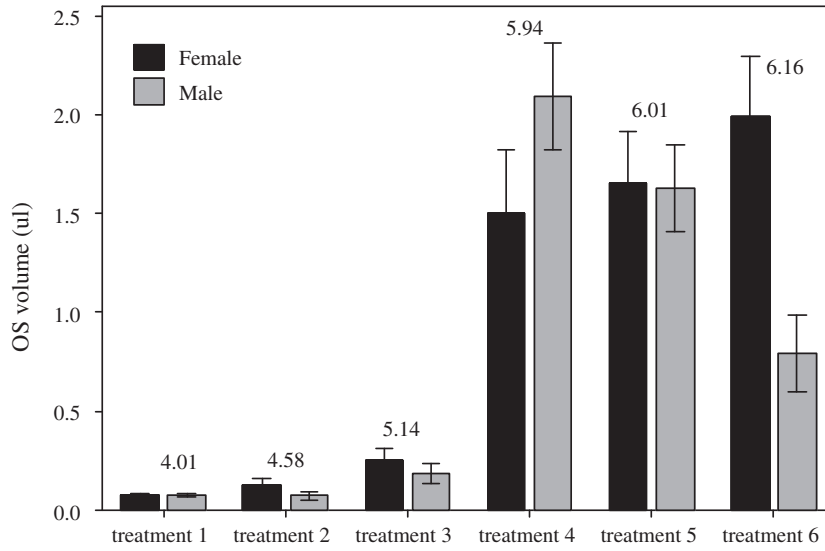

Fig. 7. Mean (± SE) volume of OS (μL) produced by late-instar spruce budworm larvae. Larvae were milked twice in the fourth instar (Treatment 1, 2), once in the fifth instar (Treatment 3), and three times on three consecutive days in the sixth instar (Treatments 4–6). Numbers above bars represent average larval instar at time of milking ($n = 200$ at start of experiment).

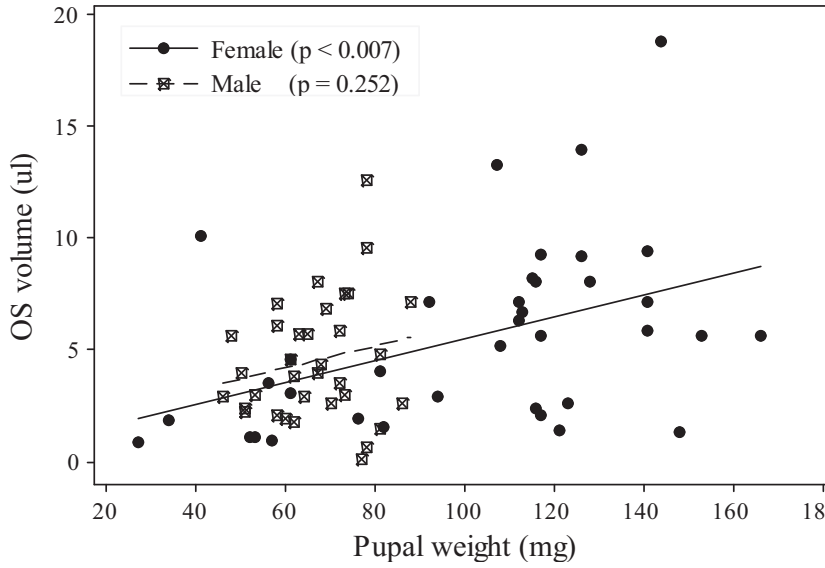

Fig. 8. Relationships between pupal weight (mg) and volume (μL) of OS produced by male and female spruce budworm larvae ($R^2 = 0.19$ and 0.04 for females and males, respectively) ($n = 200$ at start of experiment).

and Borden 1995). However, the nutritional cost of repeated OS disgorgement over a period of time, particularly at outbreak populations when the frequency of encounters with conspecific larvae and natural enemies is most likely to be high, was not addressed.

We simulated repeated encounters of SBW larvae by conspecifics and natural enemies in the lab. Our results show that both disturbance and repeated inductions of OS [disturbance + a total of 60 head taps throughout the later larval instars (L4–L6)] have limited overall effects on SBW survival, pupal weight, and development. In fact, milked larvae that survived repeated inductions of OS developed faster than control larvae. Thus, the cost of regurgitation, in terms of loss of nutrients for growth and development through repeated OS production, appears to be minimal in SBW. This is unlike the Catalpa sphinx, *Ceratomia catalpa* (Boisduval) (Sphingidae), in which disturbed, regurgitating larvae grew more slowly than those that were not disturbed (Bowers 2003).

If costs are minimal, then what are the potential benefits of regurgitation? In some species of Lepidoptera,

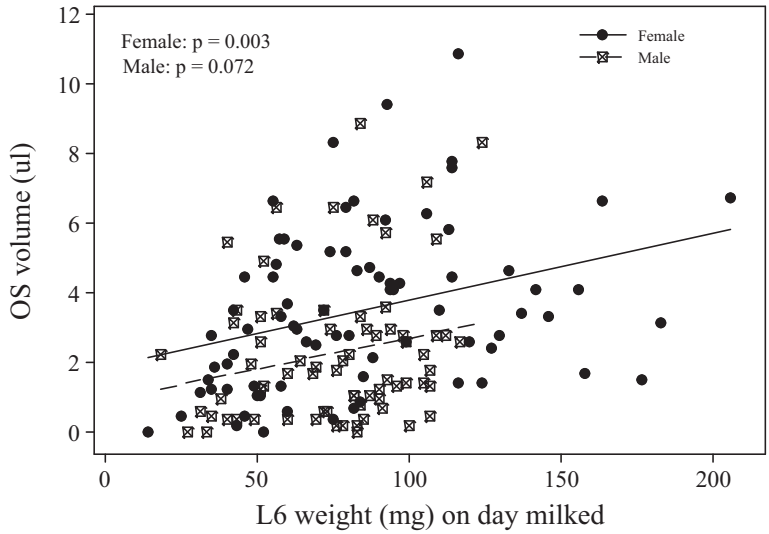

Fig. 9. Relationship between the weight (mg) of sixth-instar spruce budworm larvae on day milked and OS volume (μL) on day milked for both female and male spruce budworm ($R^2 = 0.11$ and 0.05 for females and males, respectively; $n = 200$ at start of experiment).

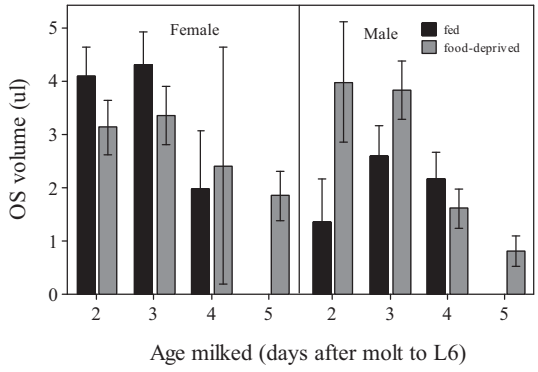

Fig. 10. Mean (± SE) volume of OS (μL) produced by female and male sixth-instar spruce budworm larvae milked 2, 3, 4, or 5 d after molting to L6. Larvae had been either fed ad libitum or food-deprived for 24 h prior to milking. (See Materials and Methods for further details) ($n = 200$ at start of experiment).

exposure of larvae to the OS of conspecifics can mediate competition for food and space through effects on life-history characteristics. For example, last-instar larvae of the flour moth, *Ephestia kuehniella* Zeller (Pyralidae), pupated sooner when exposed to an "appropriate" amount of mandibular gland secretions than larvae exposed to higher or lower amounts of the secretion (Corbet 1971). Furthermore, the amount of secretions laid down by the larvae was related to larval density (Corbet 1971). We found that continuous exposure of SBW larvae to SBW OS throughout larval development from L2 to pupation significantly reduced their survival from L2 to adults compared with controls, with males being more severely affected than females. The age of the OS (time in storage before use) was also an important factor; when exposed to fresher OS, females, but not males, tended to weigh less and their development was prolonged compared with those exposed to older OS. This deterioration of SBW OS over time was also noted by Poirier and Borden (2001). These negative effects on the life-history performance of conspecific larvae exposed to fresh OS support the suggestion that SBW OS may function as an epideictic pheromone through feeding repellency, thereby increasing individual spacing on overcrowded resources at outbreak densities, at least in the short term (Poirier and Borden 1996, 2000). We did not address the possibility that exposure of adult female SBW to OS could affect fecundity and oviposition behavior; *E. kuehniella* females laid fewer eggs when exposed to high concentrations of secretions than controls, but laid more eggs than controls when exposed to intermediate concentrations (Corbet 1973).

Interspecific interactions between SBW and other herbivores on host trees are common (Eveleigh et al. 2007). Our study indicates that, in addition to acting as an intraspecific epideictic pheromone, the disgorgement of OS by SBW larvae when disturbed/attacked may also function interspecifically to repel other competing herbivores feeding on the same host plants. We found evidence that feeding by false hemlock looper larvae was suppressed by the presence of SBW OS near the food source. Poirier and Borden (1996) also found that the OS of both SBW and *Choristoneura freemani* act as a feeding deterrent both intra- and interspecifically. Thus, SBW OS may function as an effective strategy to deter competing defoliators, many of which also serve as alternate/alternative hosts for SBW parasitoids (Eveleigh et al. 2007), from feeding on the same resources.

Finally, our study suggests that regurgitation of OS by SBW has a defensive function against natural

enemies. The application of a droplet of SBW OS to the head/thorax of ants (*Camponotus* sp.) caused the ants to engage in extensive grooming more than three times longer than ants that received a water droplet. Furthermore, in contrast to water droplets, we observed that OS droplets caused many of the ants to drag their bodies on the substrate in attempts to remove the OS from the ventral body surface. The time spent by the ants in these activities was certainly sufficient for a SBW larva to escape an ant attack. However, we do not know the effectiveness of such a defensive strategy because we did not expose SBW larvae to the ants.

Although the degree of effectiveness of OS against enemies is largely unexplored; it appears that the OS of many species are possibly toxic because of the repellent or deleterious effects of OS on potential attackers (Gentry and Dyer 2002, Oliveira and Freitas 2004). This may be the case for SBW OS functioning as an intra- and interspecific epideictic pheromone (see above), causing both conspecific and nonconspecific larvae to be repelled. However, we found that the ant grooming responses to SBW OS were independent of the food source on which the SBW larvae fed and, with the exception of red spruce, the OS from food-deprived larvae did not differ from larvae fed ad libitum. These results suggest that if toxic secondary plant compounds sequestered by host plants (allelochemicals) are present in OS from plant-fed larvae, they are either not responsible for the response by ants or the ants are not affected by these compounds. This is because the OS from artificial diet-fed larvae produced the same response in ants as the OS from plant-fed larvae, and all ants appeared to behave normally after grooming. Unlike water, the OS from all food sources did not roll off the ant's cuticle, suggesting that SBW OS are amphiphilic. Thus, as with the OS of *Spodoptera exigua* (Hubner) (Noctuidae) (Rostas and Blassmann 2009), the defensive properties of SBW OS may be owing more to surfactant properties than any toxic properties they may contain. It has been postulated that FACs in the insect gut may act as surfactants, in addition to being elicitors of plant volatiles (Tumlinson and Engelberth 2008; see LeClair et al. 2015).

The volume of OS that a caterpillar is able to produce will also determine, in part, the effectiveness of OS as a repellant and/or defensive response (Grant 2006). We found that L6, the largest and final larval instar, produced significantly larger amounts of OS than L4 and L5. Larger female larvae tended to produce more OS than smaller females, whereas in males, there was no such relationship. Also, both larger females and males tended to produce more OS when disturbed than smaller individuals. These data suggest that larger individuals of the most vulnerable and conspicuous instar—L6—are probably more capable of repelling conspecifics and enemies than smaller individuals by being able to produce more OS in a given bout. Thus, there is a competitive advantage to being large and well-fed, although food-deprived larvae, particularly males, are still able to disgorge the same, or greater, volumes of OS when attacked/disturbed (Fig. 10; Rhainds et al. 2011). Our results also show that the amount of OS produced by L6 depends on their age when attacked/disturbed, with older L6 producing less OS per bout than younger L6. This is probably owing to gradual emptying of the gut in preparation for molting to the pupal stage (Poirier and Borden 1995).

The use of OS as a defensive strategy varies considerably among herbivore species. Grant (2006) classified regurgitators into three types based on regurgitation behavior and gut morphology: 1) primary regurgitators: species whose initial defensive response is to regurgitate readily (after 1–3 pinches) and accurately at the attacker, reimbibing regurgitant when possible to minimize nutrient losses, and mean proportion of mid-gut to total gut = 0.50 ± 0.03 (mean ± SE) and mean crop proportion = 0.34 ± 0.02; 2) secondary regurgitators: species that regurgitate less readily (3–6 pinches) in a nondirected manner, regurgitating most often after attempts to bite and/or flee fail to deter attack, not reimbibing regurgitant after attack, and mean proportion of mid-gut to total gut = 0.65 ± 0.02 and mean crop proportion = 0.21 ± 0.02; and 3) nonregurgitators: species that reluctantly regurgitate (8–10 pinches), not reimbibing regurgitant after attack, and mean proportion of mid-gut to total gut = 0.73 ± 0.03 and mean crop proportion = 0.09 ± 0.01. Using this classification, our results indicate that SBW behave more like a secondary than a primary regurgitator. Interestingly, these results are similar to those reported for *Choristoneura rosaceana* (Harris) (Tortricidae), which was also classified as a secondary regurgitator (Grant 2006).

A noteworthy finding is sex-specific differences in the volume of OS disgorged by male and female larvae, and in the effects of constant exposure to conspecific OS during larval development. Larger female larvae disgorged larger volumes of OS than smaller females and developed into larger pupae despite disgorging large volumes of OS during repeated L4–L6 milking. This was not the case for males; larger male larvae did not disgorge larger volumes of OS than smaller males (Fig. 8). Thus, it appears that female larvae adjust the amount of OS disgorged according to body size with minimal nutrient loss for subsequent body growth, whereas males do not. Also, in contrast to males, neither the pupal weight nor the development time of females from pupae to moth was affected by exposure to OS. The reason(s) for these sex-specific differences is unclear but, as shown in studies on other lepidopteran species, probably involves differences in the ways that males and females 1) regulate accumulation and post-ingestive utilization of macronutrients for body growth and reproductive success (Telang et al. 2001, Lee 2010), and 2) deal with exposure to potentially toxic material (assuming that SBW OS contain undigested toxic compounds). Studies on several insects have shown sexual dimorphism in susceptibility to toxic material such as insecticides (De Lame et al. 2001, Wei and Fadamiro 2013).

In summary, it is well-known that the OS of herbivorous insects have multiple roles in ecological interactions (Vadassery et al. 2012). However, this study is the

first to examine a number of potential roles of OS in a single herbivore species. It appears that SBW OS are multifunctional, serving as both an intra- and interspecific epideictic pheromone and as an anti-predator defensive mechanism. Despite such multiple demands on OS production, SBW larvae disgorge OS readily with minimal metabolic costs on life-history performance. The identity of the biologically active components of SBW OS and their potential use as natural biological control agents remain to be explored. In a companion article (LeClair et al. 2015), we investigate the chemical composition of SBW OS and the potential role of SBW to enhance the production of herbivore-induced plant volatiles.

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