Qualitative analyses of larval oral exudate from eastern and western spruce budworms (Lepidoptera: Tortricidae)

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ABSTRACT

A two-choice feeding bioassay was used to investigate the effects of dilution, centrifugation, storage and autoclaving on the repellency of the oral exudate of eastern and western spruce budworms, *Choristoneura fumiferana* (Clem.) and *C. occidentalis* Free., to their respective conspecifics. The exudate from insects reared on either artificial diet or foliage was active at a volume equal to the amount emitted by one larva when disturbed with a pipet in the laboratory, but repellency was lost at lower doses. Centrifugation did not partition the exudate into active and inactive fractions. Exudate from both diet- and foliage-reared insects was active for at least 48 h at room temperature. However, after being frozen for one month, exudate from diet-reared insects was still active, while that from foliage-reared insects was not.

Key words: *Choristoneura fumiferana*, *Choristoneura occidentalis*, spruce budworm, oral exudate, regurgitant, epideictic pheromone

INTRODUCTION

Many insects release oral secretions when they are disturbed or handled (Davies and McCauley 1970, Corbet 1971, Eisner *et al.* 1974). While these secretions may serve to protect the emitters from predators and parasitoids (Eisner *et al.* 1974), they may also be used as epideictic pheromones (Prokopy 1981), to repel competing individuals from potentially overcrowded resources (Corbet 1971). Larvae of the spruce budworm and the western spruce budworm, *Choristoneura fumiferana* (Clem.) and *C. occidentalis* Free., respectively, have been shown to produce an oral exudate that repels conspecifics (Poirier and Borden 1995). In two-choice feeding bioassays, larvae of both species avoided feeding stations that had been treated with conspecific exudate (Poirier and Borden 1996). Larvae of both species responded to con- and heterospecific oral exudate in the same manner, as did laboratory-reared and wild-caught larvae. Larvae reared on artificial diet responded to exudate from both diet- and foliage-reared larvae, whereas foliage-reared larvae responded only to exudate from other foliage-reared larvae (Poirier and Borden 2000).

When the bioactive components are known, it may prove possible to exploit the activity of the oral exudate in the management of spruce budworms. Isolation and identification of the active components (Brand *et al.* 1979) can be facilitated by an understanding of the properties of the exudate. We investigated the threshold concentration for repellency, whether or not the active components are carried in the exudate in suspension or solution,

the thermostability of the repellent, its persistence under bioassay conditions, and the duration the exudate may be stored without losing its bioactivity.

METHODS

Insects. Laboratory-reared insects of both species were obtained as eggs or diapausing second instars from the Canadian Forest Service, Sault Ste. Marie, Ontario, from colonies maintained under laboratory conditions for many years at high population densities. Wild *C. occidentalis* larvae were collected in June 1994 near Kamloops, BC by L.M.P.

All insects were reared in the laboratory at approximately 24°C, 60% RH, and a photoperiodic regime of 16L:8D. Diet-fed larvae of both species were reared on an agar-based artificial spruce budworm diet (Bio-Serv Inc., Frenchtown, New Jersey) in 30 ml disposable vials. Two larvae were kept in each vial. Foliage-fed *C. occidentalis* larvae were reared on potted 3-year-old Douglas-fir, *Pseudotsuga menziesii* (Mirb.) Franco.

Bioassay. Each experiment (Exp.) employed a two-choice, diet-station bioassay, modified from Poirier and Borden (1996) to preclude any interaction between organic solvents and the diet stations, if such solvents were to be used during chemical procedures. Circular glass cover slips (18 mm diam.) were attached to the ceiling of the petri dish bioassay chambers using chloroform, so that diet stations on the cover slips had their centers 3 cm apart. Exudate or water treatments (2 µl) were applied to the glass in a ring around the outer edge of one cover slip; third- to fifth- instar larvae produce an average of 2 µl of oral exudate when disturbed with a pipet in the laboratory (Poirier and Borden 1996). The other cover slip was left untreated. A drop of molten artificial diet was then applied to the center of each cover slip within, but not contacting, the exudate treatment ring. Separate dishes, with one station treated with 2 µl of distilled water, served as controls. A conspecific third- to fifth-instar larva, randomized with respect to age, was then placed in the center of the bottom of each petri dish chamber, and left undisturbed for 24 h under the above rearing conditions. These test larvae were taken from the same food source as the larvae that provided the exudate. At the end of 24 h, the stations were checked for signs of feeding or establishment, such as feeding cavities or silk feeding tunnels. Larvae that did not establish on a feeding station were included in the sample, unless they had molted or pupated over the 24 h test period. Earlier studies by Poirier and Borden (1996, 2000) showed that non-feeding larvae provide important information about the degree of repellency of various treatments. Larvae were thus categorized as not feeding, feeding on the untreated station, or feeding on the treated station.

For each experiment, the numbers of larvae in the three categories were compared between experimental and control dishes using Fisher's Exact Test for a 2 X 3 contingency table, α =0.05 (Steel and Torrie 1980, Mehta and Patel 1983; Schlotzhauer and Littell 1987). Possible variation due to larval age was not taken into account.

Threshold for Bioactivity. Exp. 1 used third- to fifth-instar diet-reared *C. fumiferana*. Exp. 2 used third- to fifth-instar wild-collected *C. occidentalis*, reared on foliage. Exudate was collected by drawing it into a 5 µl micropipet after touching a larva with the pipet. The exudate from several larvae was pooled in a glass vial kept on ice. The exudate was then diluted 10-, 100- and 1000-fold by serial dilution in distilled water. One station in each bioassay dish was treated with 2 µl of distilled water (control dishes), 2 µl of undiluted exudate or 2 µl of one of the three exudate dilutions. Twenty dishes were prepared for each of the five treatments. An uninduced test larva, *i.e.* a larva that had not been induced to produce exudate, was placed in each dish, and left undisturbed for 24 h.

Solubility. Exp. 3 used third- to fifth-instar diet-reared *C. occidentalis* larvae, from the laboratory colony. Exp. 4 used third- to fifth-instar wild-collected *C. occidentalis*, reared

on foliage. For both experiments, exudate was collected from larvae using a 5 µl micropipet, and pooled in three Eppendorf tubes kept on ice. Two tubes were centrifuged at 13,000 rpm for 5 min in a Micro-Centaur benchtop Eppendorf centrifuge. The supernatant was drawn off one tube with an Eppendorf Pipetman automatic pipet and retained, and distilled water was added to the pellet fraction to bring it back to the pre-centrifugation volume. In the second tube, the pellet was resuspended in the supernatant with an automatic pipet. The third tube was left untreated. One station in each bioassay dish was treated with 2 µl of either distilled water (control dishes), whole exudate, supernatant, pellet suspension, or centrifuged and then reconstituted exudate. Forty dishes (replicates) were prepared for each of the five treatments. An uninduced test larva was placed in each dish, and left undisturbed for 24 h.

Storage Duration. Exp. 5 used third- to fifth-instar diet-reared *C. fumiferana*. Exp. 6 used third- to fifth-instar wild-collected *C. occidentalis* larvae, reared on foliage. On three consecutive days, exudate was collected from larvae using a 5 µl micropipet, and pooled in a glass vial kept on ice until the exudate was applied to the feeding stations. One feeding station in each dish was treated with 2 µl of either distilled water or exudate. Test larvae were introduced to the exudate-treated dishes. Exudate was added to the exudate-treated dishes 0, 24 or 48 h prior to the introduction of test larvae. The water-treated control dishes were tested immediately (0 h delay). The treatments were staggered so that larvae were added to all dishes on the same day. The dishes for 24 and 48 h delayed testing were held under the above rearing conditions until larvae were added. Twenty dishes (replicates) were prepared for each of the four treatments in each experiment. An uninduced test larva was placed in each dish, and left undisturbed for 24 h.

In Exp. 7, third- to fifth-instar, diet-reared *C. fumiferana* larvae were used. Exudate was collected from larvae using a 5 µl micropipet, and pooled in glass vials kept on ice. One vial was used immediately. The other vials were stored at approximately -4°C for one week or one month. One feeding station in each dish was treated with 2 µl of either distilled water (control dishes), fresh exudate, week-old exudate, or month-old exudate. Twenty dishes were prepared for each of the four treatments. An uninduced test larva was placed in each dish, and left undisturbed for 24 h.

Thermal Stability. All insects used in Exp. 8 were third- to fifth-instar diet-reared *C. occidentalis* larvae from the laboratory colony. Oral exudate was collected from larvae using a 5 µl micropipet, and pooled. Half the exudate was autoclaved for 15 min at 15 kPa and 122°C. One feeding station in each dish was treated with 2 µl of distilled water (control dishes), 2 µl of fresh exudate, or 2 µl of autoclaved exudate. Twenty dishes (replicates) were prepared for each of the three treatments. An uninduced test larva was placed in each dish, and left undisturbed for 24 h.

RESULTS

The results of all eight experiments are given in Table 1.

Threshold for Bioactivity. When the exudate was diluted by any of the three dilution rates, the numbers of larvae in the three response categories were not significantly different between experimental and control dishes in either experiment (Exp. 1, 2). Only larvae in dishes treated with undiluted exudate were significantly deterred from feeding on the treated station, with most larvae establishing on the untreated feeding station. Results were similar for *C. fumiferana* reared on artificial diet (Exp. 1) and *C. occidentalis* reared on foliage (Exp. 2).

Solubility. In Exp. 3, diet-fed larvae were significantly deterred from feeding only on diet stations treated with whole or reconstituted exudate. There was no difference between

Table 1

Feeding responses by *Choristoneura fumiferana* or *C. occidentalis* larvae to treated or untreated diet stations in eight experiments testing the potency, solubility, longevity and stability of larval oral exudate.

Experiment and Treatments	n	Number of larvae			*P* value (Fisher's Exact Test)
		Not feeding	Feeding on untreated station	Feeding on treated station	
Experiment 1,*C. fumiferana*, threshold for bioactivity, test larvae diet-reared					
Control: distilled water *vs.* untreated	19	2	9	8	
Diet-reared exudate *vs.* untreated	17	3	11	3	0.043
Diet-reared exudate, 0.1X *vs.* untreated	20	2	11	7	0.897
Diet-reared exudate, 0.01X *vs.* untreated	18	0	8	10	0.513
Diet-reared exudate, 0.001X *vs.* untreated	18	1	10	7	1.00
Experiment 2, *C. occidentalis*, threshold for bioactivity, test larvae foliage-reared					
Control: distilled water *vs* untreated	20	2	8	10	
Diet-reared exudate *vs.* untreated	20	1	16	3	0.032
Diet-reared exudate, 0.1X *vs.* untreated	19	2	8	9	1.00
Diet-reared exudate, 0.01X *vs.* untreated	20	1	10	9	0.805
Experiment 3, *C. occidentalis*, solubility, test larvae diet-reared					
Control: distilled water *vs.* untreated	19	0	9	10	
Diet-reared exudate *vs.* untreated	33	2	24	7	0.043
Diet-reared exudate supernatant *vs.* untreated	34	5	14	15	0.280
Diet-reared exudate pellet *vs.* untreated	35	4	20	11	0.183
Reconstituted diet-reared exudate *vs.* untreated	33	2	24	7	0.043

Experiment and Treatments	n	Number of larvae			*P* value (Fisher's Exact Test)
		Not feeding	Feeding on untreated station	Feeding on treated station	
Experiment 4, *C. occidentalis*, solubility, test larvae foliage-reared					
Control: distilled water *vs.* untreated	24	1	12	11	
Foliage-reared exudate *vs.* untreated	36	0	30	6	0.011
Foliage-reared exudate supernatant *vs.* untreated	40	0	32	8	0.017
Foliage-reared exudate pellet *vs.* untreated	33	0	27	6	0.026
Reconstituted foliage-reared exudate *vs.* untreated	23	1	19	3	0.030
Experiment 5, *C. fumiferana*, storage duration, test larvae diet-reared					
Control: distilled water *vs.* untreated	19	1	10	8	
Diet-reared exudate, 0 h old *vs.* untreated	18	1	12	5	0.019
Diet-reared exudate, 24 h old *vs.* untreated	20	1	15	4	0.036
Diet-reared exudate, 48 h old *vs.* untreated	19	1	15	3	0.019
Experiment 6, *C. occidentalis*, storage duration, test larvae foliage-reared					
Control: distilled water *vs.* untreated	17	0	8	9	
Foliage-reared exudate, 0 h old *vs.* untreated	18	3	13	2	0.010
Foliage-reared exudate, 24 h old *vs.* untreated	19	1	13	5	0.033
Foliage-reared exudate, 48 h old *vs.* untreated	17	3	6	8	0.284

Experiment and Treatments	n	Number of larvae			*P* value (Fisher's Exact Test)
		Not feeding	Feeding on untreated station	Feeding on treated station	
Experiment 7, *C. fumiferana*, storage duration, test larvae diet-reared					
Control: distilled water *vs.* untreated	19	2	9	8	
Fresh diet-reared exudate *vs.* untreated	19	1	15	3	0.011
1 week old diet-reared exudate *vs.* untreated	17	3	11	3	0.043
1 month old diet-reared exudate *vs.* untreated	18	2	9	7	1.00
Experiment 8, *C. occidentalis*, thermal stability, test larvae: diet-reared					
Control: distilled water *vs.* untreated	20	1	10	9	
Fresh diet-reared exudate *vs.* untreated	19	5	12	2	0.035
Autoclaved diet-reared exudate *vs.* untreated	20	3	10	7	0.604

control dishes and dishes treated with supernatant or the pellet resuspension. However, in Exp. 4, the numbers of foliage-fed larvae in the three response categories were significantly different from the control dishes in all treatments.

Storage Duration. In Exp. 5, more diet-reared larvae fed on the untreated than exudate-treated station for all delay times, demonstrating persistent bioactivity of exudate from diet-reared larvae for at least 48 h under laboratory conditions. However, in Exp. 6, the bioactivity of exudate from foliage-reared larvae persisted for >24 h, but <48 h. When the exudate from diet-reared larvae was frozen at -4°C, bioactivity persisted for at least a week, but less than one month (Exp. 7).

Thermal Stability. The numbers of larvae in the three response categories were significantly different from the control dishes in the fresh exudate treatment only (Exp. 8), indicating that autoclaving for 15 min destroyed all deterrent activity.

DISCUSSION

Poirier and Borden (1995) showed that each larva of *C. fumiferana* and *C. occidentalis* produced about 2 µl of oral exudate per induction. The results of Exp. 1 and 2 show clearly that application of one larval equivalent of exudate is necessary to elicit a response from test larvae in the laboratory bioassay. Because this biologically realistic dose is necessary for bioassays, it is probable that large amounts of starting material will be needed for chemical fractionation, bioassay and analysis (Brand *et al.* 1979), if the active constituents in the exudate are to be identified.

The results from Exp. 3 and 4 were not conclusive. Centrifugation did not destroy the bioactivity of exudate from either diet- or foliage-reared larvae, since reconstituted exudate was as deterrent as whole exudate. Exudate from diet-reared insects may have had some of the repellent components carried as a suspension, but the particulate matter alone was not repellent. Exudate from foliage-reared insects appeared to have the repellent components equally distributed between the pellet and the supernatant, with both fractions being as repellent as whole exudate. It is possible that the centrifugation used was not sufficient to separate the materials in the exudate, and that better results might have been achieved by using higher centrifugation speeds or a longer duration. However, since the exudate apparently consists of a partially digested substrate (Poirier 1995), it is also possible that a portion of the repellent component is still associated with the particulate matter suspended in the exudate, while the remainder is free in solution. Diet-reared larvae may produce lower concentrations of repellent than foliage-reared insects or may lack an independently repellent component that is present in the regurgitate of foliage-reared larvae (Poirier and Borden 2000). In either case, for diet-reared larvae, dividing the exudate between two fractions could reduce the concentration in one or both fractions below the response threshold.

Exp. 5, 6 and 7 indicate that exudate from both diet- and foliage-reared larvae deteriorates over time, although the breakdown appears to be faster in the latter case. While this deterioration may be mediated by microbial activity, it seems likely that the repellent components will also be subject to the action of larval enzymes contained in the regurgitant. The retention of residual foliar enzymes in the regurgitant could account for the shorter longevity of repellency in the regurgitant of foliage-reared larvae than in that of diet-reared larvae. It is possible to store the exudate at -4°C for at least a week, but storage at a lower temperature may increase longevity by halting further enzymatic activity.

Autoclaving appears to destroy the bioactivity of the exudate from diet-reared larvae (Exp. 8). Because a number of materials may be significantly altered by high temperatures, this result does little to indicate the type of compound involved in the repellency. However,

the bioactive material may prove to be unstable in certain laboratory procedures, *e.g.* gas chromatography, that involve heat.

These experiments provide considerable insight into the nature of the bioactive constituent(s) in the larval oral exudate of these two *Choristoneura* spp. It persists for up to 48 h at room temperature, and survives storage when frozen at -4°C for at least one week. However, it is thermally unstable under pressure at 122°C. When centrifuged, the bioactive component(s) from foliage-reared larvae does not partition completely, suggesting that some fraction remains associated with the particulate matter in the exudate, while some is in solution. Lastly, below a biologically realistic concentration of one larval equivalent, bioactivity is lost. Because diet can influence bioactivity (Poirier and Borden 2000), and because the components of the artificial diet are known, modification of this diet could potentially provide further insight into the nature of the bioactive component(s).

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