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Unsaturated Cuticular Hydrocarbons Enhance Responses to Sex Pheromone in Spruce Budworm, *Choristoneura fumiferana*

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Abstract The primary sex pheromone components of the female spruce budworm, *Choristoneura fumiferana* (Clem.) (Lepidoptera: Tortricidae), are (*E*)- and (*Z*)-11-tetradecenal, produced in 95:5 ratio. However, male flight responses to calling females in a wind tunnel were faster and maintained longer than responses to any synthetic aldehyde blend. Analyses of cuticular extracts from spruce budworm adults revealed series of *n*-alkanes and *n*-monoalkenes with predominantly odd numbers of carbon atoms from C_{23}- C_{29} in both sexes. (*Z,Z,Z*)-3,6,9-tricosatriene and (*Z,Z,Z*)-3,6,9-pentacosatriene were identified only in cuticular extracts from females. Pheromonally naïve males showed wing fanning and circling responses to forewing scales from females but not to scales from males. Males also exhibited the same strong responses to scales excised from pharate females, indicating that the pheromone components are produced by females prior to emergence. (*Z*)-11-hexadecenal and (*Z*)-5-tricosene enhanced male responses to the primary sex pheromone aldehydes in wind tunnel bioassays, including higher proportions of in-flight and copulatory responses by males and increased time on the source. Addition of (*Z,Z,Z*)-3,6,9-tricosatriene to the 95/5 blend of (*E*)- and (*Z*)-11-tetradecenal released close-range copulatory responses including abdomen curling on treated septa. We propose that the sex pheromone blend of *C. fumiferana* is composed of the 95/5 blend of (*E*)- and (*Z*)-11-tetradecenal as primary components, with (*Z*)-11-hexadecenal, (*Z*)-5-tricosene and (*Z,Z,Z*)-3,6,9-tricosatriene fulfilling secondary roles in orientation and close-range courtship.

Keywords Unsaturated cuticular hydrocarbons; secondary pheromone components; (*E*)-11-tetradecenal · (*Z*)-11-tetradecenal; (*Z*)-11-hexadecenal · (*Z*)-5-tricosene · (*Z,Z,Z*)-3,6,9-tricosatriene

Introduction

The primary sex pheromone components of *Choristoneura fumiferana* (Clemens) (Lepidoptera: Tortricidae) were identified many years ago as (*E*)- and (*Z*)-11-tetradecenal (E11–14:Ald and Z11–14:Ald) in a 95/5 ratio (Sanders and Weatherston 1976; Silk et al. 1980), and traps baited with this blend trapped males as effectively as traps baited with virgin females. The saturated aldehyde, tetradecanal (14:Ald), was identified in effluvia by Silk et al. (1980), but addition of this compound to the blend of unsaturated aldehydes at 2% of E11–14:Ald did not increase trap catches in the field and caused only marginally more males to initiate flight and reach the source in wind-tunnel bioassays (Alford and Silk 1983; Silk and Kuenen 1986).

There is indirect evidence that (*Z*)-11-hexadecenal (Z11–16:Ald) may also be a minor component in the pheromone blend of *C. fumiferana* as the potential biosynthetic precursor, (*Z*)-11-hexadecenyl acetate (Silk and Kuenen 1988) was detected in trace amounts in the sex pheromone gland, although Z11–16:Ald was not detectable in effluvia (Dunkelblum et al. 1985; Wolf and Roelofs 1987). More males flew upwind to

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septum loaded with 3 μg of 95/5 E/Z11–14:Ald with 3% Z11–16:Ald added than in response to the primary components alone, but the response was still not equivalent to that elicited by virgin females (P.Silk, unpublished results; Silk and Kuenen 1988). The mating behavior of *C. fumiferana* has been partially described from wind-tunnel observations of upwind flight and copulatory activity of males to calling females (Sanders and Lucuik 1992), with the conclusion that several components were likely missing from the pheromone blend.

The use of cuticular hydrocarbons as chemical signals in insects in general is well established (Howard and Blomquist 2005), and in the Lepidoptera, in particular, semiochemical functions have long been known. Recently, blends of Type I and Type II compounds (sensu Millar 2000) have been discovered as sex pheromones in pyralid (Hall et al. 2017; Millar et al. 2005; Strong et al. 2008; Wang et al. 2010) and crambid species (Cabrera et al. 2001; Gibb et al. 2007; Xiao et al. 2011, 2012), indicating that such combinations may be more widespread than originally thought (Millar et al. 2005).

It was shown that wing scales from *C. fumiferana* of either sex applied to filter paper release copulatory attempts from pheromone-stimulated, conspecific males (Grant 1987), but pulverized scales and scale extracts did not, leading to the conclusion that the cues eliciting copulation were likely not chemical but were associated with the mechanical properties of the scales only. Our objective was to test the hypothesis that additional pheromone components derived from body scales of females and males are part of the sexual communication system in *C. fumiferana*. We identified a homologous series of C_{23}-C_{29} mono-alkenes in male and female body wax and two trienes in females only and present evidence that some of these alkenes function as additional components of the sex pheromone communication system in *C. fumiferana*.

Methods and Materials

Laboratory-Reared Insects Overwintering, second-instar larvae (L2) of *C. fumiferana* were obtained from Insect Production Services (IPS), Canadian Forest Service, Sault Ste. Marie, Ontario, Canada, and reared from L2 to L6 in the laboratory at 22 °C and 16:8 hr L:D photoperiod. Larvae were fed ad libitum with artificial diet (McMorran 1965) available from IPS in translucent, 22-ml plastic cups. The pupae were separated by sex and placed in cages for adult emergence in separate rooms under the same conditions with lights off at 0800 hr. Males and females were 2–4 d old when tested.

Feral Insects Branches of balsam fir (*Abies balsamea* [L.] Mill.) containing late-instar budworm larvae and/or pupae were collected from various locations in Quebec, Canada. Larvae were kept in large plastic containers and fed ad libitum freshly cut balsam fir branches in the laboratory at 22 °C and 16:8 hr L:D photoperiod. Pupae and adults were maintained as described above for laboratory-stock insects.

Preparation and Fractionation of Extracts Whole body extracts were prepared from 2- to 4-d-old virgin females and males during early scoto- and photophase. A whole insect was soaked in hexane for ca. 10 min with gentle stirring, filtered through a cotton filter in a pipette to remove scales, pooled, and stored at −20 °C until use.

Hexane extracts of both sexes were passed through a 1 g SPE silica gel cartridge (Phenomenex, Torrance, CA) and eluted with hexane to remove fatty acids. Extracts of both females and males were then further fractionated on a $AgNO_3$-impregnated SPE cartridge column (10%, 200 mesh, SigmaAldrich, Oakville, ON, Canada). The column was successively eluted with 2 ml each of hexane, 10%, 20% and 50% diethyl ether in hexane. Fractions were concentrated under argon and stored as above.

Analyses of Extracts Extracts and synthetic compounds were analyzed by gas chromatography/mass spectrometry (GC/MS) on an Agilent 7890 GC and a 5975 mass selective detector (Agilent, Santa Clara, CA 95051) in electron impact (EI; 70 eV) mode. The column was either a ZB-5-HT capillary (30 m × 0.25 mm × 0.25 μm film thickness) or a ZB-FFAP capillary (30 m × 0.25 mm × 0.25 μm film thickness) (Phenomenex, Torrance, CA 90501–1430) in splitless mode with helium as carrier gas. The injection port was at 220 °C and the oven temperature was programmed from 70 °C, held for 3 min, and then increased at 15 °C/min to 220 °C and held for 30 min.

Chemicals *n*-Alkanes and aldehydes (C_{20} – C_{29}) used as analytical standards were obtained from SigmaAldrich or Alltech (Woodridge, IL, USA). All other compounds were obtained from SigmaAldrich, Alfa-Aesar (Tewksbury, MA, USA) or were synthesized as described below. The pheromone blend of *C. fumiferana* (95/5 E/Z11–14:Ald) and Z11–16:Ald were obtained from Bedoukian Research (Danbury, CT).

Monounsaturated hydrocarbons were synthesized in high yield by *Z*-selective Wittig reaction of an *n*-alkylltriphenylphosphonium bromide and the appropriate *n*-aldehyde in THF at −78 °C using sodium bis(trimethylsilyl)amide (NaHMDS) (1.0 M solution in THF) as base (Ginzel et al. 2003; Xiao et al. 2011). Thus, (*Z*)-5-heneicosene (Z5–21:H), (Z)-5-tricosene (Z5–23:H), and (Z)-5-pentacosene (Z5–25:H) were synthesized by coupling *n*-pentyltriphenylphosphonium bromide to hexadecanal, octadecanal and eicosanal, respectively. 1-tricosene (1–23:H) was synthesized in similar fashion by Wittig reaction of methyltriphenylphosphonium bromide and docosanal. The

long-chain aldehydes were freshly prepared from commercially available 1-alkanols by oxidation with pyridinium chlorochromate in dichloromethane, followed by column chromatographic purification. (*Z*)-9-tricosene (Z9–23:H) was obtained from SigmaAldrich and was used without further purification.

(*Z,Z,Z*)-3,6,9-tricosatriene (ZZZ3,6,9–23:H) and (*Z,Z,Z*)-3,6,9-pentacosatriene (ZZZ3,6,9–25:H) were synthesized by reduction of methyl linolenate to the corresponding alcohol, iodination, and chain extension with pentyl- or heptylmagnesium bromide respectively in the presence of a catalytic amount of Li_2CuCl_4 (Underhill et al. 1983). All synthetic compounds were >98% pure as determined by GC/MS analysis.

Identification of Chemicals Hydrocarbons were identified by comparing mass spectra and retention times with those of standards with reference to the parent ion (M^+) and molecular formulae. Double bond positions in the mono-alkenes were determined by epoxidation with 3–chloroperbenzoic acid in methylene chloride followed by GC/MS analysis, revealing characteristic fragments formed by cleavage either side of the epoxide function (Ginzel et al. 2003). To confirm double bond configuration, ca. 10 mg of synthetic (*Z*)-monoene was isomerized with 5.0 μl thiophenol in a sealed vial at 110 °C for 1 hr and, after work-up, was epoxidized as described above. Monounsaturated hydrocarbons from males and females (~ 200 insect equivalents; 10% ether/hexane fraction) were treated similarly and compared to standards by GC/MS. The (*E*)-isomers eluted before the (*Z*)-isomers on the ZB-5-HT column (Ginzel et al. 2003).

Moth Scale Bioassays To determine if pheromonally naïve males respond to pheromone components present on forewing scales, scales were taken from excised forewings of 2- to 3-d-old virgin female or male moths. Using forceps, a wing was held over a 25 mm filter paper disk and scales were gently scraped onto the disk using a scalpel. All tools were washed with ethanol between each disk preparation. Each disk was placed in the center of a filter paper substrate at the center of a 90 × 17 mm glass Petri dish. New disks and filter paper substrates, and clean Petri dishes were used for each replicate. Adult male *C. fumiferana* moths (2- to 3-d old) were held in a dark room in glass vials with one adult per vial for 30 min prior to the start of each experiment. Adults were released by allowing them to crawl out of the vials into the dishes. A replicate consisted of one adult per dish. Each adult was observed for 20 min at 19–23 °C, 41–65% RH, under red light. The incidence and duration of wing fanning and circling round the source were recorded.

Forewing scales from females prior to eclosion were also tested. Pupae were inspected against a background light source and when judged to be <12 hr from eclosion, the pupa was carefully removed from the case and the unfolded forewings excised. Scales were gently scraped onto a filter paper disk as described in the assays above.

Wind-Tunnel Bioassays The wind-tunnel (90 × 90 × 240 cm) was fabricated as described by Kuenen and Rowe (2006). The floor consisted of factory-painted white medium-density fiberboard which was patterned using green vinyl adhesive disks of varying diameter, arranged in a random pattern. The sides and top were covered with 6 mm-thick Lexan® panels. At the upwind end of the tunnel, the ducting was reduced to 63 × 63 cm and a filter box added for a single 5 cm thick charcoal filter (Filterfast.com, product # FFOKGM). A tube (64 cm diameter, 92 cm long) housed the 61-cm fan blade and a Dayton 0.25HP 90VDC variable speed motor (Dayton, OH; #4Z248). An array of eight incandescent light fixtures was fixed above the tunnel and controlled via a Lutron (Coopersburg, PA) dimmer. Tunnel air velocity and temperature were recorded using a heated wire anemometer/thermometer (VWR #21800–024), luminosity with a lux meter (VWR #21800–014), and relative humidity with an analog household meter (Accu-Temp #99047).

All wind-tunnel tests were conducted at 20–25 °C and 40–60% RH under a red incandescent light at 2 lx. Test extracts and solutions were loaded either onto pre-extracted red rubber septa (Wheaton, NJ, USA) or strips of filter paper (1 cm × 2 cm; Whatman #1, Buckinghamshire, UK), and the source placed 35.6 cm from the floor and 35.6 cm from the upwind end of the tunnel. A septum loaded with 3 μg of 95/5 E/Z11–14:Ald gave emission rates of ~2–4 ng/h, shown previously to be very similar to that released by a calling female (Silk and Kuenen 1986). After a brief acclimatization period (20–30 min), males (2– to 4-d old) were released at the downwind end of the tunnel from small mesh cages (3.2 cm × 3.2 cm) placed 168 cm from the septum. Male moths were allowed to leave the cage after setting a stimulus source at the upwind end of the tunnel. Various behaviors and their duration (where applicable) were recorded.

The following treatments on septa were tested in the tunnel in two separate tests. For Component Test 1: (1) 3 μg 95/5 E/Z11–14:Ald; (2) 3 μg 95/5 E/Z11–14:Ald + 0.2 μg Z11–16:Ald; (3) 3 μg 95/5 E/Z11–14:Ald + 0.2 μg Z11–16:Ald + 0.2 μg Z5–23:H; (4) 3 μg 95/5 E/Z11–14:Ald + 0.2 μg Z11–16:Ald + 0.2 μg 1–23:H; (5) 3 μg 95/5 E/Z11–14:Ald + 0.2 μg Z5–23:H; (6) 0.2 μg Z5–23:H. For Component Test 2: (1) 3 μg 95/5 E/Z11–14:Ald; (2) 3 μg 95/5 E/Z11–14:Ald + 0.2 μg Z11–16:Ald; (3) 3 μg 95/5 E/Z11–14:Ald + 0.2 μg Z11–16:Ald + 0.2 μg (Z)-7-tricosene (Z7–23:H); (4) 3 μg 95/5 E/Z11–14:Ald + 0.2 μg Z11–16:Ald + 0.2 μg Z9–23:H; (5) 3 μg 95/5 E/Z11–14:Ald + 0.2 μg Z11–16:Ald + 0.2 μg Z7–23:H + 0.2 μg Z9–23:H.

Bioassays of Copulatory Behavior The potential importance of ZZZ3,6,9–23:H and ZZZ3,6,9–25:H in the mating sequence of *C. fumiferana* was determined using a simple observational bioassay in which the behaviors of individual males caged with a septum treated with one or more chemical blends were monitored. Arenas consisted of a clear plastic Petri dish base (9.0 cm diameter) holding a single filter paper disk (Whatman Cat. No. 1002 090, No. 2,) and a 7.5 cm tall cone-shaped plastic screen cage. A rubber septum was fixed to the center of the filter paper base using a small piece of clear plastic tape. Using a 10 μl syringe, one of seven chemical blends was applied to the septum: (1) 1 μg 95/5 E/Z11–14:Ald; (2) 1 μg 95/5 E/Z11–14:Ald + 60 ng ZZZ3,6,9–23:H; (3) 1 μg 95/5 E/Z11–14:Ald + 60 ng ZZZ3,6,9–25:H; (4) 1 μg 95/5 E/Z11–14:Ald + 60 ng ZZZ3,6,9–23:H + 60 ng ZZZ3,6,9–25:H; (5) 60 ng ZZZ3,6,9–23:H; (6) 60 ng ZZZ3,6,9–25:H; and (7) 60 ng ZZZ3,6,9–23:H + 60 ng ZZZ3,6,9–25:H.

For each bioassay, an individual male (approx. 3 d old) was introduced into the arena and was observed for 3 min. Wing fanning, curling of abdomen onto or toward the septum, and contact with the septum were observed. Time (sec) each male spent both wing fanning and while in contact with the septum was recorded, along with the total incidences of abdomen curls. Bioassays were conducted under conditions identical to those of the wind-tunnel bioassays. Males were used once and a fresh septum was used every 1.5 hr.

Statistical Analyses Differences in the proportion of moths responding to treatments in all behavioral bioassays were assessed using a generalized linear model (proc GENMOD, dist = binomial, link = logit; SAS Institute 1999), followed by CONTRAST statements (Wald tests) to compare all possible pairs of treatments for a given behavior where appropriate. The time spent in various activities in response to treatments in all bioassays was analyzed using one-way ANOVA followed by a post hoc Tukey's test where appropriate. The level of statistical significance was set at $\alpha = 0.05$ for all analyses.

Results

Identification of Hydrocarbons Initially, we used laboratory-reared insects for extraction and chemical analyses of alkanes and alkenes. However, many hydrocarbons, particularly the triterpene squalene, originating from the linseed oil and other ingredients in the artificial diet (McMorran 1965) confounded the analyses. Therefore, only insects reared on balsam fir foliage collected from the field were used for chemical analyses.

The hexane fractions from fractionation on $AgNO_3$-impregnated silica gel of whole-body washes of both laboratory-reared males and females appeared almost identical, containing *n*-alkanes with predominantly odd-numbers of carbon atoms from C_{23} to C_{29}, and smaller amounts of *n*-alkanes with even-numbers of carbon atoms.

The 10% ether/hexane fraction from the $AgNO_3$-impregnated silica gel column showed groups of Z-monoalkenes with odd numbers of carbon atoms from C_{23} to C_{29} (Fig. 1a). These had double bonds at 11, 9, 7, 5, 3, and 1, with the 7- and 9-isomers predominating in each group (Fig. 1a). Similar amounts (~3–16 ng/moth) and proportions were found in both male and female body washes. Epoxidation and GC/MS analysis (Millar 2000; Silk et al. 2011) confirmed the

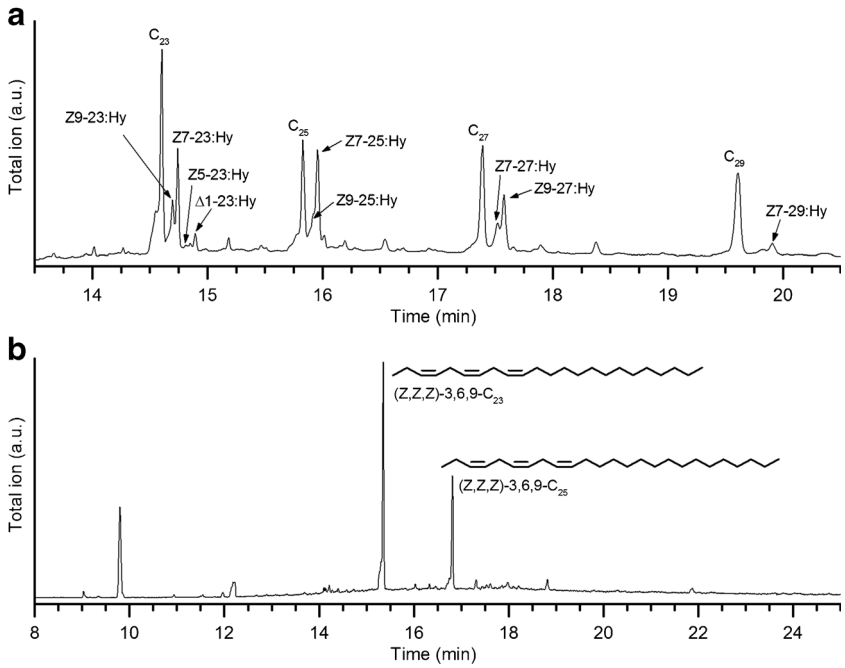

Fig. 1 GC/MS analyses (ZB-FFAP column) of fractions from fractionation on $AgNO_3$-impregnated silica gel of hexane body washes of adult female *Choristoneura fumiferana*: **(a)** full-scan analysis of 10% ether/hexane fraction; **(b)** selected ion scan at *m/z* 108 of 50% ether/hexane fraction

double-bond positions. The configuration was confirmed to be the (*Z*)-isomer in each homologous group (Fig. 1a) by separation of the (*E*)- and (*Z*)-isomers on the FFAP column after thiophenol isomerization (Ginzel et al. 2003).

The 50% ether/hexane fraction from the $AgNO_3$-impregnated silica gel column showed two major peaks at 15.36 min and 16.82 min on the FFAP column (Fig. 1b) only present in body washes of females. The full-scan EI mass spectra of these compounds were similar, with molecular ions at *m/z* 318 ($C_{23}H_{44}^+$) and 346 ($C_{25}H_{48}^+$) respectively. Both spectra contained ions at *m/z* 79 and *m/z* 108, assigned as $[(CH = CH)_3H^+]$ and $[CH_3CH_2(CH = CH)_3H^+]$ (Millar 2000). Diagnostic ions at *m/z* 262 and *m/z* 290 correspond to $[M^+ - C_4H_8]$. The structures were assigned as ZZZ3,6,9–23:H and ZZZ3,6,9–25:H respectively, and comparison of retention times on both columns and EI mass spectra with authentic synthetic compounds confirmed these assignments. Shorter retention times of both these compounds on the nonpolar ZB-5-HT capillary column compared with those of corresponding saturated alkanes confirmed that none of the double bonds in either molecule was conjugated (Millar 2000).

Bioassays of Moth Scales Adult male *C. fumiferana* moths responded to the presence of female, but not male, scales (Table 1). A similar percentage of males fanned in the presence of female scales regardless of whether the scales came from eclosed or uneclosed females, but they spent significantly more time fanning at scales from eclosed than uneclosed females (Table 1). Also, a similar percentage of males circled the scales from eclosed and uneclosed females, but spent significantly more time circling scales from eclosed females (Table 1). In the absence of scales, males showed little activity (Table 1). Females did not respond behaviorally to the presence of either male or female scales, moving for only 12.5% of the total time in each assay (data not shown).

Wind-Tunnel Bioassays Wind-tunnel bioassays focused on the hypothesis that Z11–16:Ald was a pheromone component as determined through fatty acid analyses (Dunkelblum et al. 1985; Silk and Kuenen 1988), predictions from pheromone biosynthesis (Wolf and Roelofs 1987), and literature precedents for the possible alkene but not alkane components (Gibb et al. 2007; Xiao et al. 2011, 2012). The nonpolar silica gel fraction, which contained the odd-numbered saturated straight-chain alkanes, showed no activity in short-range bioassays on septa with or without 3 μg 95/5 E/Z11–14:Ald and so were not tested further (data not shown).

Behavioral responses of male *C. fumiferana* to the first series of blends of synthetic compounds are shown in Tables 2 and 3. The percentage of males that performed wing fanning in the cage did not differ significantly among treatments. However, a significantly higher percentage took flight and were stimulated to fly in the plume in blend 3 (95/5 E/Z11–14:Ald + Z11–16:Ald + Z5–23:H) versus blend 1 (95/5 E/Z11–14:Ald alone). The percentage of males both finding and fanning at the septum was significantly higher with blend 3 versus all other treatments except blend 5 (95/5 E/Z11–14:Ald + Z5–23:H). Blend 3 also elicited a significantly higher percentage of males attempting copulation with the septum and remaining in constant contact with the septum than in any other treatment (Table 2). No males ($N = 20$) responded to blend 6 (Z5–23:H alone), and this blend was excluded from the analyses.

With this first series of blends, the mean time spent in the cage prior to flight and the mean time fanning in the cage differed significantly among treatments, with the most time spent in these behavioral responses in response to blend 1 (Table 3). There was no significant difference in the mean time flying in the plume among treatments, although males spent more time flying in the plume in blend 3 than in other treatments. Upon reaching the septum, males in treatment 3 spent significantly more time fanning at the septum than in other treatments (Table 3). These results indicated that blend 3 containing both Z11–16:Ald and Z5–23:H in addition to the primary pheromone components had the greatest effect, eliciting the full behavioral repertoire of males, unlike blend 1 containing only the primary component blend, which did not differ from the other blends for most responses. The other

Table 1 Behavioral responses of adult male *Choristoneura fumiferana* to conspecific forewing scales

		Wing fanning		Circling	
Treatment	*N*	%	Mean time ± SE (sec)	%	Mean time ± SE (sec)
Female scales	30	53	184.0 ± 40.3 a	47	166.0 ± 40.9 a
Male scales	30	0	0.0 ± 0.0 b	0	0.0 ± 0.0 b
Females scales pre-eclosion	30	57	78.1 ± 22.5 b	33	2.0 ± 0.6 b
No scales (control)	20	15	5.2 ± 4.3 b	0	0 b
		$P < 0.001$[a]	$P < 0.001$[b]	$P < 0.001$[a]	$P < 0.001$[b]

[a] χ^2 test

[b] ANOVA; means in a column that do not share a letter are significantly different (Tukey's test; $P < 0.05$)

Table 2 Behavioral responses of adult male *Choristoneura fumiferana* to pheromone blends in first series of wind-tunnel bioassays (% showing response; values within a column that do not share a letter are significantly different by Wald test, $P < 0.05$)

		Response (%)					
Blend[a,b]	*N*	Fan in cage	Fly in plume	Contact septum	Fan at septum	Attempt copulation	Stay on septum[c]
1	72	63	33 c	28 c	28 c	25 b	20 bc
2	77	62	43 bc	35 bc	35 bc	32 b	22 b
3	76	51	59 a	58 a	58 a	58 a	91 a
4	68	47	51 ab	28 bc	28 bc	19 b	11 bc
5	78	60	49 abc	44 ab	44 ab	23 b	3 c
P (χ^2)		0.2110	0.0239	< 0.001	< 0.001	< 0.001	< 0.001

[a] Blends (1) 3 μg 95/5 E/Z11–14:Ald; (2) 3 μg 95/5 E/Z11–14:Ald + 0.2 μg Z11–16:Ald; (3) 3 μg 95/5 E/Z11–14:Ald + 0.2 μg Z11–16:Ald + 0.2 μg Z5–23:H; (4) 3 μg 95/5 E/Z11–14:Ald + 0.2 μg Z11–16:Ald + 0.2 μg 1–23:H; (5) 3 μg 95/5 E/Z11–14:Ald + 0.2 μg Z5–23:H

[b] Blend (6) (0.2 μg Z5–23:H) did not stimulate any of the above behaviors in males, and responses to this blend were not included in statistical analyses

[c] % based on those males that found the septum

monounsaturated compound tested, 1–23:H, did not show any effect (Table 3, treatment 4).

The behavioral responses of males to blends containing Z7–23:H and Z9–23:H were evaluated in a second series of tests and results are shown in Tables 4 and 5. With the exception of the proportion of males fanning at the cage, the addition of Z7–23:H or Z9–23:H to the primary components did not significantly affect any of the behavioral responses of the males (Table 4). No males attempted copulation with the septum in response to any of these pheromone blends. Likewise, the time spent by males in the various behavioral activities was not affected by the addition of Z7–23:H or Z9–23:H to the primary components, with the exception of time spent fanning in the cage, which was significantly reduced in response to blend 5 compared with blend 1 (Table 5).

Addition of the trienes, ZZZ3,6,9–23:H or ZZZ3,6,9–25:H, to the primary pheromone components applied to filter paper did not affect the behavioral responses in the wind tunnel compared with the primary components alone (data not shown).

Bioassays of Copulatory Behavior Proportions of males wing fanning, abdomen curling, and contacting the septum were highly dependent on the chemical blend present on the septum (Table 6). In trials where ZZZ3,6,9–23:H and/or ZZZ3,6,9–25:H were present without 95/5 E/Z11–14:Ald, the occurrence of any mating behavior was extremely low (<1% for all three behaviors across treatments), and these results are not presented in Table 6. Although proportions of males wing fanning and contacting the septum were not significantly influenced by chemical blend, treatment blend had a significant effect on the proportions of males exhibiting abdomen curls (Table 6). Pairwise comparisons indicated that the occurrence of abdomen curls was significantly greater in

Table 3 Mean durations of behavioral responses to pheromone blends by adult male *Choristoneura fumiferana* in first series of wind-tunnel bioassays

		Mean time spent ± SE (sec)[c]			
Blend[a,b]	*N*	In cage	Fan in cage	Fly in plume	Fan at septum
1	72	157.3 ± 28.6 a	18.0 ± 4.2 a	12.6 ± 3.0	9.4 ± 2.4 a
2	77	138.4 ± 25.7 ab	12.3 ± 2.8 ab	14.9 ± 2.6	11.4 ± 3.0 a
3	76	88.5 ± 21.9 ab	5.4 ± 1.3 bc	20.2 ± 2.9	34.2 ± 5.2 b
4	68	60.6 ± 17.0 b	5.4 ± 1.9 bc	14.0 ± 2.5	9.2 ± 2.6 a
5	78	100.4 ± 21.3 ab	2.7 ± 0.5 c	15.6 ± 2.8	11.6 ± 3.1 a
ANOVA		$P = 0.033$	$P < 0.001$	$P = 0.362$	$P < 0.001$

[a] Blends (1) 3 μg 95/5 E/Z11–14:Ald; (2) 3 μg 95/5 E/Z11–14:Ald + 0.2 μg Z11–16:Ald; (3) 3 μg 95/5 E/Z11–14:Ald + 0.2 μg Z11–16:Ald + 0.2 μg Z5–23:H; (4) 3 μg 95/5 E/Z11–14:Ald + 0.2 μg Z11–16:Ald + 0.2 μg 1–23:H; (5) 3 μg 95/5 E/Z11–14:Ald + 0.2 μg Z5–23:H

[b] Blend (6) (0.2 μg Z5–23:H) did not stimulate any of the above behaviors in males, and times relating to this blend were not included in statistical analyses

[c] Column means that do not share a letter are significantly different (Tukey's test; $P < 0.05$)

Table 4 Behavioral responses of adult male *Choristoneura fumiferana* to pheromone blends in second series of wind-tunnel tests (% showing response; values within a column that do not share a letter are significantly different by Wald test, $P < 0.05$)

		Mean response (%)						
Blend[a]	*N*	Fan in cage	Fly in plume	Fan at septum	Hover at septum	Hover after septum	Stay on septum	Display hair pencils[b]
1	34	64.7 a	32.4	32.4	5.9	5.9	2.9	32.4
2	30	53.3 ab	33.3	33.3	6.7	16.7	6.7	33.3
3	38	36.8 bc	26.3	18.4	5.3	7.9	2.6	18.4
4	34	44.1 abc	23.5	20.6	8.8	5.9	0.0	17.7
5	30	26.7 c	20.0	16.7	3.3	6.7	3.3	16.7
P (χ^2)		0.022	0.722	0.308	0.920	0.578	0.535	0.290

[a] Blends: (1) 3 μg 95/5 E/Z11–14:Ald; (2) 3 μg 95/5 E/Z11–14:Ald + 0.2 μg Z11–16:Ald; (3) 3 μg 95/5 E/Z11–14:Ald + 0.2 μg Z11–16:Ald + 0.2 μg Z7–23:H; (4) 3 μg 95/5 E/Z11–14:Ald + 0.2 μg Z11–16:Ald + 0.2 μg Z9–23:H; (5) 3 μg 95/5 E/Z11–14:Ald + 0.2 μg Z11–16:Ald + 0.2 μg Z7–23:H + 0.2 μg Z9–23:H

[b] No males attempted copulation with the septum in response to any of the pheromone blends;

groups where ZZZ3,6,9–23:H was present (blends 2 and 4) than when absent. Mean number of abdomen curls and mean time spent wing fanning were not significantly affected by chemical blend. However, chemical blend did significantly affect the mean time that males spent in contact with the septum, with mean contact time with blend 2 being significantly greater than that for males exposed to blend 4 (Table 6).

Discussion

Based on the above results, we propose that the sex pheromone blend of *C. fumiferana* includes 95/5 E/Z11–14:Ald as primary components, with Z11–16:Ald, Z5–23:H, and ZZZ3,6,9–23:H fulfilling secondary roles in orientation and close-range courtship. This is the first report of the occurrence of blends of Type I and Type II pheromone components (Millar 2000) in a tortricid.

It is presumed that Z11–16:Ald is produced in the pheromone gland and Z5–23:H in the cuticular waxes. Although they are both behaviorally inactive alone, they significantly enhanced male responses when presented with the primary sex pheromone aldehyde blend in wind-tunnel tests. Results were very similar to responses of males to "calling" females, as previously observed in earlier studies (Alford et al. 1983; Silk and Kuenen 1986, 1988). These new compounds clearly enhance close-range behaviors, as observed in the proportions of males exhibiting in-flight behaviors and copulatory responses and in the increased time spent on the source. The sex pheromone of *C. fumiferana* is, therefore, better represented by a mixture of 95/5 E/Z11–14:Ald with both Z11–16:Ald and Z5–23:H enhancing in-flight responses. Addition of either of the triene compounds detected in cuticular extracts from female *C. fumiferana* did not enhance upwind flight parameters, but addition of ZZZ3,6,9–23:H to the primary components released copulatory behavior by males on contact as evidenced by significant abdomen curling. These new

Table 5 Mean durations of behavioral responses to pheromone blends by adult male *Choristoneura fumiferana* in second series of wind-tunnel bioassays

		Mean time spent ± SE (sec)[b]					
Blend[a]	*N*	In cage	Fan in cage	Fly in plume	Fan at septum	Hover before septum	Hover after septum
1	34	61.3 ± 24.6	2.00 ± 0.51 a	16.76 ± 5.13	8.74 ± 2.60	0.29 ± 0.22	0.24 ± 0.16
2	30	55.4 ± 27.2	1.17 ± 0.26 ab	18.70 ± 5.88	11.97 ± 3.96	0.57 ± 0.41	0.87 ± 0.38
3	38	61.4 ± 26.2	0.97 ± 0.31 ab	16.13 ± 4.72	3.47 ± 1.37	0.32 ± 0.22	0.58 ± 0.35
4	34	33.1 ± 17.5	1.18 ± 0.33 ab	9.24 ± 3.44	3.18 ± 1.22	0.59 ± 0.35	0.59 ± 0.41
5	30	13.7 ± 3.2	0.40 ± 0.13 b	6.17 ± 2.81	6.00 ± 2.88	0.10 ± 0.10	0.40 ± 0.28
ANOVA		$P = 0.488$	$P = 0.032$	$P = 0.261$	$P = 0.070$	$P = 0.733$	$P = 0.747$

[a] Blends: (1) 3 μg 95/5 E/Z11–14:Ald; (2) 3 μg 95/5 E/Z11–14:Ald + 0.2 μg Z11–16:Ald; (3) 3 μg 95/5 E/Z11–14:Ald + 0.2 μg Z11–16:Ald + 0.2 μg Z7–23:H; (4) 3 μg 95/5 E/Z11–14:Ald + 0.2 μg Z11–16:Ald + 0.2 μg Z9–23:H; (5) 3 μg 95/5 E/Z11–14:Ald + 0.2 μg Z11–16:Ald + 0.2 μg Z7–23:H + 0.2 μg Z9–23:H

[b] Column means followed by the same letter are not significantly different (Tukey's test, $P < 0.05$)

Table 6 Courtship behavioral responses of adult male *Choristoneura fumiferana* to pheromone blends in arena bioassay

		Wing fanning		Curling abdomen		Contacting septum	
Blend[a,b]	*N*	%	Time ± SE (sec)	%[c]	Number ± SE (sec)	%	Time ± SE (sec)[d]
1	48	83.0	45.4 ± 8.00	31.3 b	3.44 ± 0.78	79.2	55.3 ± 9.47 ab
2	49	83.6	49.1 ± 5.86	51.0 a	5.56 ± 0.69	73.5	86.5 ± 10.6 b
3	52	65.4	27.4 ± 5.96	21.2 b	3.82 ± 0.89	53.8	67.6 ± 11.6 ab
4	43	83.7	48.5 ± 5.97	46.5 a	4.31 ± 0.61	67.4	44.9 ± 7.58 a
P (χ^2)		0.150		0.006		0.068	
P (ANOVA)			0.082		0.286		0.010

[a] Blends: (1) 1 μg 95/5 E/Z11–14:Ald; (2) 1 μg 95/5 E/Z11–14:Ald + 60 ng ZZZ3,6,9–23:H; (3) 95/5 E/Z11–14:Ald + 60 ng ZZZ3,6,9–25:H; (4) 1 μg 95/5 E/Z11–14:Ald + 60 ng ZZZ3,6,9–23:H + 60 ng ZZZ3,6,9–25:H; (5) 60 ng ZZZ3,6,9–23:H; (6) 60 ng ZZZ3,6,9–25:H; (7) 60 ng ZZZ3,6,9–23:H + 60 ng ZZZ3,6,9–25:H

[b] In experiments with blends where 95/5 E/Z11–14:Ald was not present (Blends 5–7), the occurrence of any mating behavior was extremely low (<1% of males observed, $N = 41$ to 52), and these were not included in the statistical analyses

[c] Values within a column that do not share a letter are significantly different (Wald test)

[d] Column means that do not share a letter are significantly different (Tukey's test)

compounds appear to be required together, although the addition of other isomers and homologs such as Z5–25:H, Z7–23:H, Z9–23:H, Z7–25:H or Z9–25:H appears not to affect behavioral responses.

Grant (1987) found that *C. fumiferana* males stimulated by the primary pheromone component blend do not attempt copulation unless an appropriate release stimulus (visual, tactile or chemo-tactile) is presented. Males attempted copulation with filter paper impregnated with pheromone but only when the filter paper was pinned to a rubber septum, and Alford et al. (1983) reported similar results. Grant (1987) also showed that conspecific wing scales from either sex applied to filter paper did release copulatory attempts from pheromone-stimulated males, but pulverized scales and scale extracts did not, concluding that the cues eliciting copulation were not chemical but were associated with the mechanical properties of the scales only. In *C. fumiferana*, similar amounts of monoalkenes were found in body waxes of both sexes. As males do not produce the aldehyde pheromone components 95/5 E/Z11–14:Ald, alkene presence in males would not be expected to elicit responses from other males (Xiao et al. 2012). This offers an explanation also for the results of Grant (1987) where males, prestimulated with primary component aldehydes, gave responses to both male and female scales. In our studies, primary component-naïve males, i.e., not exposed to 95/5 E/Z11–14:Ald, did not exhibit this behavior. Scales taken from pre-eclosed females also elicited highly significant responses in males. This implies that the primary component aldehydes are being produced by females prior to emergence. With the protandrous nature of this insect (Bergh et al. 1988), it would, therefore, be expected that females would be mated very quickly upon emergence and close to the site of eclosion.

Contact or copulation-inducing pheromones are typically present on the body surface of signaling insects. Although they only appear effective at short range, they often add to the attractiveness of long-range sex or aggregation pheromones. Close-range pheromones have been noted in several orders of the Insecta, including Diptera, Hymenoptera (Blomquist and Bagnères 2010), Coleoptera (Ginzel et al. 2003), Isoptera (Clément 1982), and Lepidoptera (Grant 1987).

The Canadian registration in 2007 of Hercon Disrupt SBW Micro-flakes®, a pheromone-based product for control of *C. fumiferana*, paved the way for large-scale trials to test pest management theories and concepts related to an early intervention strategy and population suppression (Kettela and Silk 2005; Kettela et al. 2006; Palaniswamy et al. 1982; Rhainds et al. 2012; Sanders and Silk 1982; Silk and Kuenen 1984). This new spruce budworm sex pheromone blend may be much more efficacious for disruption than the two-component blend and perhaps efficacious at higher insect densities. It also may be useful in development of an "attracticide" for budworm mitigation (Evenden and McLaughlin 2004).

Unanswered questions that may be of significance in *C. fumiferana* population ecology are what triggers moth dispersal and whether the sex pheromone is involved in the dispersal process. Sanders (1984) showed that female moths, who can perceive their own pheromone (Palaniswamy and Seabrook 1978), are more active in pheromone-permeated air, which suggests that pheromones may be involved, and that dispersal might increase with increasing concentrations of pheromones in the local air space.

Finally, the finding that Z11–16:Ald and Z5–23:H are synergistic components to the primary aldehydic pheromone components that modify in-flight behavior and that the triene ZZZ3,6,9–23:H releases copulatory attempts in *C. fumiferana* may be of fundamental importance to the

understanding of the pheromone systems throughout the *Choristoneura* genus. This may be particularly important for those species producing aldehydic pheromone components (Silk and Eveleigh 2016; Silk and Kuenen 1988) and might help in completing the sex pheromone blends of all species and in chemotaxonomic studies. Pheromone similarities and differences may, in fact, be important factors in species isolation and in defining phylogenetic relationships in this complex group of species (Harvey 1996; Lumley and Sperling 2011a, b).

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