FACTORS AFFECTING SPRUCE BUDWORM (*CHORISTONEURA FUMIFERANA*) (CLEM.) MATING AND MATING DISRUPTION WITH PHEROMONE IN THE LABORATORY

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Abstract

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Factors influencing spruce budworm (*Choristoneura fumiferana*) mating and mating suppression in an enclosed environment in the laboratory were investigated to develop a quantitative assay suited to statistical analysis. Mating in the absence of the two major components of spruce budworm sex pheromone (control) was not affected by changes in moth population density nor by increasing the experimental duration from 20 to 44 h. The proportions mated increased with an increase in the male:female ratio to 1.5:1 and when the experimental duration was prolonged to 68 h. Using a population density, sex ratio combination of 15:10 (male:female) the proportions of mated females decreased with increasing source concentrations of the two major spruce budworm sex pheromone components (95:5 *E*/*Z*-11–14-tetradecenal). This effect was diminished with increases in the population density and with extended test duration. Mating in the presence of pheromone remained lower than controls over all durations tested.

Résumé

On a fait une enquête sur les facteurs qui influencent l'accouplement et l'élimination de l'accouplement de la toudeuse de l'épinette (*Choristoneura fumiferana*) dans un milieu clos en laboratoire pour développer un essai qualitatif approprié aux analyses statistiques. L'accouplement en absence des deux principaux composants du pheromone sexuel (contrôle) de la toudeuse de l'épinette n'a pas été affecté par les changements dans la densité de la population du papillon de nuit, ni par l'augmentation de la durée de l'expérience de 20 en 44 h. La proportion des accouplés augmente avec une augmentation dans le ratio mâle:femelle à 1.5:1 et quand la durée de l'expérience a été prolongée à 68 h. Utilisant une combinaison densité de population, ratio sexuel de 15:10 (mâle:femelle) les proportions de femelles accouplées diminuent avec une augmentation des concentrations de source de deux principaux composants du phéromone sexuel de la toudeuse de l'épinette (95:5 *E*/*Z*-11–14-tétradécenal). Cet effet était diminué avec les augmentations dans la densité de population et avec une prolongation de la durées du test. L'accouplement en présence du pheromone restait inférieur aux contrôles par rapport à toutes les durée des tests.

Introduction

In the laboratory, the mating of many species of Lepidoptera has been distrupted by exposing them to artificially high concentrations of the appropriate sex pheromone or sex pheromone components (Shorey *et al.* 1967; Fluri *et al.* 1974; Sower *et al.* 1975; Charmillot *et al.* 1976; McLaughlin and Hagstrum 1976; Vick *et al.* 1978; Carles *et al.* 1979; Hagstrum and Davis 1982; Hirai and Mitchell 1982). Mating disruption in confined populations has been shown to be moth density dependent (Sower *et al.* 1975; McLaughlin and Hagstrum 1976; Vick *et al.* 1978) and pheromone concentration dependent (Shorey *et al.* 1967; Sower *et al.* 1975: Charmillot *et al.* 1976; Vick *et al.* 1978; Carles *et al.* 1979; Hagstrum and Davis 1982; Hirai and Mitchell 1982).

In small-cage field experiments using high-density populations, Schmidt and Seabrook (1979) demonstrated that spruce budworm (SBW) (*Choristoneura fumiferana*) mating can be distrupted by dissemination of its major pheromone components. Subsequently

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Palaniswamy *et al.* (1982) showed that mating suppression in small cages was dependent on pheromone concentration and population density. Schmidt *et al.* (1980), using a population of 10 pairs of SBW moths confined in a 500-mL flask, achieved a reduction in mating with a source concentration of 1 mg of the two major pheromone components although under these conditions control mating was low.

In this study we demonstrated a simple and quantitative laboratory SBW mating system suitable for a variety of purposes (e.g. Bergh and Seabrook 1986; and see Discussion).

We investigated factors affecting the mating of confined populations of SBW moths in the laboratory and we defined optimal population density and sex ratio combinations that elicit consistent mating success under these conditions. Changes in mating success resulting from the permeation of the mating chambers with a range of source concentrations of the two major components of the SBW sex pheromone were examined. The application of this technique as an assay for the screening of semiochemicals and their analogues also was demonstrated.

Materials and Methods

Insects. Second-instar SBW larvae from the Forest Pest Management Institute, Sault Ste. Marie, were reared on synthetic diet (McMorran 1965) as modified by Grisdale (1973), with the exception of the inclusion of linseed oil. Rearing conditions were 17L:7D photoperiod (range = 5.1–8.4 lx according to shelf position), 25 ± 1°C, ca. 60% RH. Pupae were sexed and placed in separate rearing rooms with independent ventilation systems. Females 0–24 h old and males 48–72 h old were used to maximize mating success (Outram 1971; Sanders 1971).

Mating Chambers. Corning Pyrex™ 2500-mL crystallizing bowls (190 mm diameter × 100 mm height; #3140) were used as mating chambers. The vertical walls of these allow females to support males during copulation, overcoming the problems associated with previous attempts to mate moths in 500-mL Erlenmeyer flasks (Ponder, pers. obs.). Glass tops were affixed to the bowls with parafilm strips. A 2.25-cm hole in each top, plugged with a tinfoil-wrapped stopper, permitted the introduction of moths into the mating chambers. The bottoms were lined with Fisher Scientific 9-795-J filter paper. Domtar (#51-170, Montreal) notepaper was found to be equally suitable.

A glass fiber filter paper (Reeves angel) 1 cm^2 was pinned to plasticine affixed to the underside of the glass top. Depending upon the experiment, 0 to 320 μg 95:5 *E*/*Z*-11–14:Ald (hereafter referred to as "pheromone") or pheromone analogue (see below) in 60 μL of hexane solvent (ASC spectrograde, Caledon) or hexane alone (control) was applied to the filter paper. After solvent evaporation (3 min) the bowls were sealed. Up to 1 h was allowed for pheromone diffusion prior to male moth introduction. Female moths were added 1 h after male introduction. No attempt was made to quantify the aerial concentration of test compounds in the bowls.

Experiments were prepared in the laboratory ca. 6 h prior to scotophase and transferred to an incubator (Percival 1–35 Series with vertical lights) in a 17L:7D cycle (ca. 23 lx) at 21 ± 1°C. Unless otherwise indicated, tests were run between 1400 hours (Day 1) to 1000 hours (Day 2). Females were scored as mated or unmated by the presence or absence of a spermatophore in the *bursa copulatrix*.

"Mating suppression" (MS) is used to index the phenomenon of mating reduction under the conditions described. Abbott's formula (Abbott 1925), as modified by Sower *et al.* (1975), was used to measure mating suppression:

$$MS = \frac{(\%\text{ mated in control}) - (\%\text{ mated in treatment})}{\%\text{ mated in control}} \times 100.$$

Chemicals. The SBW pheromone components *E*- and *Z*-11-tetradecenal (*E*/*Z*-11–14-Ald) (for review see Silk and Kuenen 1986) were prepared as described by Lonergan (1986).

The analogue, 12-tetradecenal, was prepared as follows: the 11-bromo-1-undecanol (Aldrich) was protected as the pyranyl ether with dihydropyran in the presence of *p*-toluenesulfonic acid catalyst and subsequently alkylated with propyne in Na/NH_3 at $-78°C$. Without isolation the product was reduced with excess sodium to the alkene. This reduction step provided the alkene in a convenient (95:5) *E*/*Z* ratio. The tetrahydropyranyl protecting group was removed in aqueous acidic methanol and the resulting 12-tetradecenol was oxidized to the corresponding aldehyde with pyridinium chlorochromate (pcc) in methylene chloride.

The analogue, 11,13-tetradecadienal, was prepared utilizing 11-bromo-1-undecanol as well. The bromo alcohol was protected as the pyranyl ether and the bromine function transformed to the corresponding aldehyde by treatment with pyridine N-oxide. Wittig reaction of the aldehyde with the triphenyl phosphine salt of bromo propene in THF/*n*-butyl Lithium at 0°C followed by removal of the tetrahydropyranyl protecting group produced the 11,13-tetradecadienol. Oxidation with pcc gave the desired dienaldehyde with a 1:1 *E*/*Z* ratio. This analogue was used in all tests as the 1:1 *E*/*Z* mixture.

The final products and appropriate intermediates were purified either by "flash" column or preparative thin-layer chromatography. Final products were found to be greater than 98% pure utilizing a Varian 3700 gas chromatography/capillary system.

Statistical Analysis. Portions of the results are presented in purely descriptive form. Where indicated, hypothesis testing relied upon analysis of variance procedures (Sokal and Rohlf 1981) and distribution-free methods (Daniel 1978; Zar 1984). Specific procedures include (model I) 1- and 2-way ANOVA, the binomial test for two or multiple proportions, corrected chi-square analyses (when df = 1), linear regression of transformed data, and the Tukey test for multiple comparisons.

Experiments. I. Sex ratio and population density. The sex ratios (male:female) tested were as follows: 1:1, 1.2:1, and 1.5:1. Moth densities ranged from 12 to 45 individuals per mating chamber (Table 1).

II. Pheromone source concentrations and population density. Unless specified, the sex ratio and moth density combination of 15 males : 10 females was used in the following tests.

SBW mating success was examined over a broad range of pheromone source concentrations (0–320 μg, 30 replicates) at 20 h duration. Using an intermediate pheromone source concentration (60 μg) we then compared the mating success at four moth densities after 20 h (10 replicates).

III. Experimental duration. The duration experiment resolves two independent questions. First, what is an acceptable experimental duration for the other tests described herein? Second, what is the effect of extended duration on mating in the pheromone-treated chambers relative to the 20-h treatment? As described above, 60 μg of pheromone was introduced into the mating chambers; mating success was recorded after three time durations (0–20 h, 0–44 h, and 0–68 h) and compared with hexane controls.

IV. Sources of sample variability. We characterized the sample variability found in control and treatment trials. To do this we examined 53 replicates of three treatments (air, 60 μL hexane controls, and 60 μg/60 μL pheromone:hexane treatment), hereafter called AIR, HEX, and PHER, respectively. Each replicate contained 15 males and 10 females. We tested the hypotheses (*a*) that the AIR and HEX controls are equivalent in the mean proportion of mated females, $\hat{p}$, and (*b*) that the variability about $\hat{p}$ for both treatments comes from the same binomial frequency distribution. We then compared the HEX and PHER treatments in a likewise manner.

By examining sample variability and characterizing it, we confirmed that any residues remaining from the evaporated hexane solvent did not influence mating.

V. Analogues. Two pheromone analogues that elicit positive electroantennogram (EAG) responses [Ponder, unpubl. (Methods: Ross *et al.* (1979)] and reduce pheromone trap-catch [Lonergan and Ross, unpubl. (Methods: Ross *et al.* 1982)] were tested in the mating chambers to determine their effect on mating success. The analogue source concentration used was 60 μg dissolved in 60 μL hexane.

Results and Discussion

In the control mating chambers, we observed all of the post-landing, precopulatory behaviors previously described for male SBW orienting to females in a wind tunnel (Sanders 1979). The females exhibited calling postures and were readily mated. If permitted, females oviposited in the chamber during the second or third scotophase.

I. Sex Ratio and Population Density. Sex ratio had a greater effect on the proportion of mated females per trial than did moth density (Table 1). As the ratio of males to females was increased the proportion of mated females increased correspondingly. Optimal mating was achieved with a 1.5:1 (male:female) ratio. Among the population densities examined within this ratio, the 9:6 (male:female) combination resulted in the highest mating. However, by using the 15:10 (male:female) combination, we are better able to measure random and induced variability in mating success. Rearing restrictions made impractical the use of higher ratios.

II. Pheromone Source Concentrations and Population Density. The mean number of mated females per treatment declined over the range of pheromone source concentrations tested (Fig. 1) (1-way ANOVA, $p<0.001$), but mating could not be entirely eliminated with extreme dosages. The lowest PHER source concentration (1 μg) significantly reduced mating relative to the controls ($\alpha = 0.05$, Tukey test). The means could not be made linear after a log/probit transformation. The proportion of mated females per container was regressed on the PHER source concentration using the following transformations: $Y' = \mathrm{SQT}(Y + 0.5)$; $X' = \mathrm{LOG}(X + 1)$. The significant regression ($t = -17.01$, df = 358, $p<0.001$) is best described as $Y' = 2.78 - 0.49X'$, with $r^2 = 0.447$.

Dose-dependent mating suppression under confined conditions in the laboratory has been demonstrated for *Plodia interpunctella* (Sower *et al.* 1975). *Laspeyresia* (= *Cydia*) *pomonella* (Charmillot *et al.* 1976), *Sitotroga cerealella* (Vick *et al.* 1978), *Lobesia botrana* (Carles *et al.* 1979), *Ephestia cautella* (Hagstrum and Davis 1982), and *Spodoptera frugiperda* (Hirai and Mitchell 1982). The extent of semiochemical-mediated mating suppression in the laboratory appears variable among species (Rothschild 1981).

Among all moth densities tested, pheromone exposure resulted in lower mean proportions of mated females than did their respective controls (Table 2). There was added variance among pheromone treatments ($p<0.001$) with significant (pheromone × density) interaction ($p<0.002$), but no noticeable added variance due strictly to moth densities ($p>0.10$) (2-way ANOVA). None of the arcsine-transformed control mating proportions differed significantly from the others ($\alpha = 0.05$, Tukey test for multiple comparisons, Table 2). The mating success within pheromone treatments was greater at higher moth densities ($\alpha = 0.05$, Tukey test).

Other moth species also appear to exhibit pheromone × density interaction in laboratory studies (Sower *et al.* 1975; McLaughlin and Hagstrum 1976; Vick *et al.* 1978). In the SBW, this interaction has been documented in small-cage field studies (Palaniswamy *et al.* 1982).

Three density-dependent factors may explain changes in SBW mating success in pheromone-permeated environments: pheromone degradation, pheromone adsorption, or random encounters. The possibility that pheromone may be degraded or adsorbed at rates proportional to the moth density could account for the nearly three-fold differences in the proportion of mated females in the lowest and highest moth densities (Table 2). Pheromone

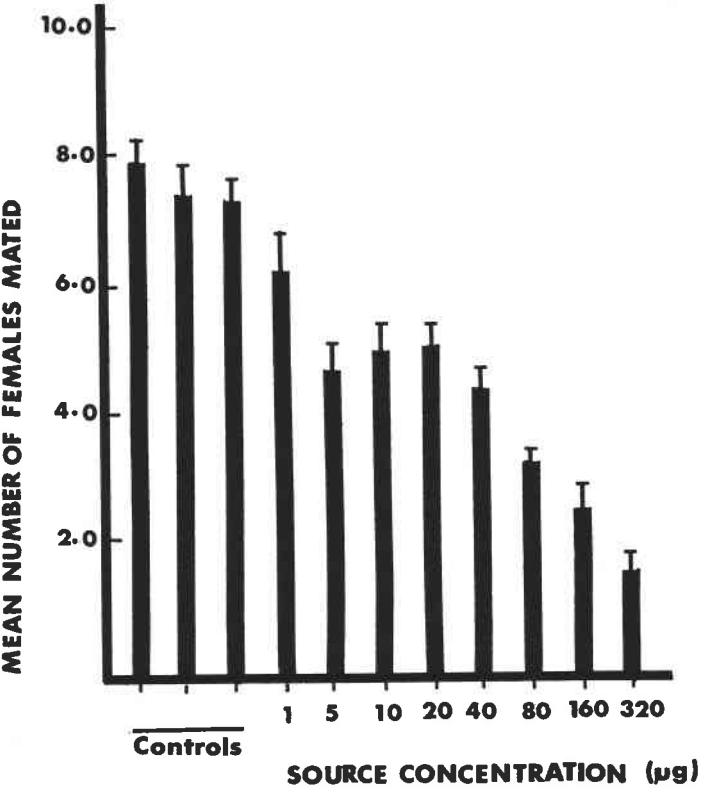

FIG. 1. Relationship between the mean proportion of mated spruce budworm females per replicate (N = 30) and 20 h exposure to different synthetic pheromone source concentrations. Sex ratio – density combination = 15 males : 10 females. Vertical bars are standard errors of the mean.

adsorption to SBW bodies is known to occur. Ramaswamy and Cardé (1984) showed a 75% adsorption of pheromone onto five SBW females after 3 h exposure to a 100-µg source concentration in a 250-mL static air system. As well, adsorption of significant amounts of pheromone onto the glass cannot be ruled out (Baker *et al.* 1980). Lonergan

Table 1. Mating success of spruce budworm females at different sex ratio – density combinations (10 replicates per combination)

Sex ratio ♂:♀	Total adults ♂:♀	Mean no. of mated females/trial (±SD)	Females tested	Percentage mated females
1:1	6:6	1.9(0.99)	60	31.6
1:1	10:10	4.1(1.73)	100	41.0
1:1	14:14	7.0(1.15)	140	50.0
1:1	18:18	8.5(2.80)	180	47.2
1.2:1	12:10	5.2(1.75)	100	52.0
1.2:1	17:14	7.7(2.00)	140	55.0
1.2:1	22:18	10.6(2.50)	180	58.9
1.5:1	9:6	4.7(0.67)	60	78.3
1.5:1	15:10	7.3(1.70)	100	73.0
1.5:1	21:14	9.1(0.74)	140	65.0
1.5:1	27:18	13.4(2.55)	180	74.4

Table 2. Mating success of female spruce budworm moths at different densities with and without exposure to a source concentration of 60 μL 95:5 *E/Z*-11–14:Ald (10 replicates)

Experiment	Total adults ♂:♀	Mean mated females/trial (± SD)	Females tested	Proportion females mated*	MS†
Control‡	9:6	4.7(0.67)	60	0.78a	
Pheromone	9:6	0.8(0.79)	60	0.13c	83%
Control	15:10	7.3(1.34)	100	0.73a	
Pheromone	15:10	2.6(1.84)	100	0.26bc	64%
Control	21:14	9.1(0.74)	140	0.65a	
Pheromone	21:14	5.3(2.11)	140	0.38b	42%
Control	27:18	13.4(2.55)	180	0.74a	
Pheromone	27:18	6.8(2.20)	180	0.38b	49%

*Proportions with the same letter are not significantly different, $\alpha = 0.05$, Tukey multiple comparisons test.
†MS = percentage mating suppression relative to control experiment.
‡All control experiments used blank pheromone dispensers to which 60 μL hexane was applied and evaporated for 3 min prior to insertion into mating chambers.

(1986) and Morse and Meighen (1984) demonstrated that pheromone is degraded by SBW body parts.

Random encounter may account for all or part of the density-related mating increases found in Table 2. Sower *et al.* (1975) reported that male *Plodia interpunctella* within 2 cm of females often mated while in the presence of high pheromone titers (see also Smith *et al.* 1978). However, Fluri *et al.* (1974) reported that in *Laspeyresia pomonella* there was no evidence that olfactory cues could be over-ridden by visual, tactile, or other stimuli; *T. ni* may respond similarly (Shorey *et al.* 1967). In the SBW, probably all three factors (i.e. random encounter, pheromone degradation, and adsorption) are affected by changes in moth density. But density-dependent random encounter does not explain the equality in mated proportions found in the control chambers. If random encounter is increased with increases in density, then the control mating at higher densities should have been greater than observed. This is currently under study.

III. Pheromone and Duration. There was significant added variance between pheromone and control treatments and among duration treatments, but no significant ($p = 0.08$) (treatment × duration) interaction ($\alpha = 0.05$, 2-way ANOVA). Between the 0–20 and 0–44 h control tests, there was no difference in the proportion of females mated (Table 3). Beyond 44 h, mating increased. Mating in the pheromone-treated chambers increased with increasing duration but mating at the 68-h duration still did not surpass the mating observed in the 20-h control experiment ($\alpha = 0.05$, Tukey test for multiple comparisons, Table 3). Roehrich and Carles (1977) observed similar mating delays for the tortricid *Lobesia botrana* when moths were exposed to pheromone.

The increased mating in the 68-h control chambers may be explained by the random encounter hypothesis, whereas the increased mating in the treatment chambers over the duration of the experiment may result from random encounter, pheromone adsorption, and/or pheromone degradation.

IV. Characterization of Sample Variability. It was our original purpose only to characterize the sample variability within and between AIR and HEX controls. However, the frequency distribution of mated females for both AIR and HEX controls was found to approximate the binomial distribution. This permits use of our mating assay as a screening technique employing the binomial test to detect mating suppression with minimum replication. We demonstrate the screening technique in the Analogue section.

In the AIR (control) experiment we observed 381/530 female moths that were mated (53 replicates). The proportion ($\hat{p}$) of mated moths to be expected in any one replicate of

Table 3. Mean mating proportions (± SD) of female spruce budworm in control (HEX) and pheromone-treated (60 μg) mating chambers; 10 replicates of three durations using the 15:10 male:female ratio

	20 h	44 h	68 h
Control	7.3(1.3)b*	7.4(1.8)b	8.1(1.0)a
Pheromone	2.6(1.8)e	4.9(2.0)d	6.2(1.7)c

*Proportions with the same letter are not significantly different, Tukey multiple comparison test, $\alpha = 0.05$.

10 female moths is $\hat{p} = 0.719$ and the expected proportion of unmated females is $1 - \hat{p} = \hat{q} = 0.281$. The observed range of mated females per replicate was highly variable (median = 7, range = 3–10, Table 4). We tested the hypothesis that the proportion of mated and unmated females per replicate follows a binomial distribution. The analysis, which pooled the expected frequencies for $r <= 4$ (r = number of mated ♀♀ per replicate), is consistent with this hypothesis ($\chi^2 = 3.4$; df = 5; $p > 0.50$; binomial goodness of fit test).

We then compared the AIR controls with the HEX controls (Table 4) to justify the use of HEX controls elsewhere. We tested the null hypothesis: the mating status of females (r) in the HEX experiment is from a binomial distribution with $\hat{p} = 0.730$ ($\chi^2 = 6.5$; $p > 0.50$; binomial goodness of fit test). If any vapors from the 3-min-evaporated, hexane-treated blank squares did volatilize in the mating chambers, then such vapors did not significantly influence the overall mean proportion of mated females, or the frequency distribution of r, the number of mated females per replicate.

The frequency distribution of r in the PHER treatments (Table 4) was tested against a binomial distribution with $\hat{p} = 0.289$. The analysis failed to support the null hypothesis ($\chi^2 = 16.8$, df = 5, $p < 0.005$). We have not characterized it as was done with the AIR and HEX control data. What seems important here is that the PHER exposure (*a*) reduced the mean proportion of mated females (e.g. Fig. 1) and (*b*) affected the frequency distribution function of r, the number of mated females per replicate (Table 4). Why the frequency distribution of the number of mated females per replicate in the PHER treatment fails to follow the binomial is unknown.

V. Analogues. Of the two analogues tested in the mating assay, 11,13-tetradecadienal reduced mating by 38% whereas the addition of 12-tetradecenal resulted in no mating reduction, relative to controls. Both analogues, at 0.2 mg source concentration, elicited

Table 4. Frequency distribution of the number of mated female spruce budworm per replicate, r, exposed to three treatments: AIR, hexane (HEX), and pheromone (PHER)

r		AIR	HEX	PHER
0		0	0	3
1		0	0	13
2		0	1	10
3		1	0	6*
4		1	3	12
5		2	5	4
6		13	15	3
7		13*	15*	1
8		13	5	0
9		9	8	1
10		1	1	0
	Σ =	53	53	53
	$\bar{x}$ =	7.2	6.7	2.9

*Median.

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EAG responses of 4.86 mV (SD ± 1.58) and 3.73 mV (SD ± 0.6) for 11,13-tetradecadienal and 12-tetradecenal, respectively (Ponder, unpubl.). The corresponding pheromone control responses were 4.08 mV (SD ± 0.6) and 4.32 mV (SD ± 0.74) (0.1 mg source concentration). Both analogues reduced trap-catch when added to pheromone lures. Pheromone trap-catch was reduced from a daily mean of 74.7 males (SD ± 18.8) to 14.0 by the addition of 11,13-tetradecadienal; and mean daily catch was reduced from 83.7 (SD ± 4.7) with pheromone to 13.7 (SD ± 13.4) when 12-tetradecenal was added (Lonergan and Ross, unpubl.).

Roelofs and Comeau (1971), working with *Argyrotaenia velutinana*, demonstrated that when pheromone analogue trap-catch reduction occurs, then positive EAG responses can be expected. However, positive results using these two criteria do not necessarily imply that the compound in question will suppress mating (Bartell 1982). The contrasting results of the 11,13-tetradecadienal and the 12-tetradecenal presented here exemplify this phenomenon. We are currently investigating the possibility that mating success, used in conjunction with other laboratory assays and trapping studies, may be useful in the screening of semiochemical blends and their analogues prior to mating suppression trials in the field.

Conclusions

The proportion of mated females in 20-h AIR or HEX controls was not significantly affected by population density but increased significantly with larger male:female ratios. Over all population densities examined, optimal mating was achieved with a 1.5:1 (male:female) sex ratio. Using the 15:10 (male:female) population density, sex ratio combination, the proportion mated was unaffected by extending the experimental duration to 44 h but increased significantly when the duration was prolonged to 68 h.

The presence of *E*/*Z*-11–14:Ald reduced the proportions of mated females. This relationship is dose and population density dependent where the proportion mated decreases with increasing dose and increases with increasing moth density at one dose. The effect of pheromone on the mated proportion diminished as the experimental duration was prolonged but even at the longest duration, treatment mating remained significantly lower than control.

Two pheromone analogues with known EAG and trapping activity were tested. Mating was suppressed by 11,13-tetradecadienal but not by 12-tetradecenal.

The mating assay is reliable, rapid, and inexpensive and may be effectively employed to screen potential mating suppressants. Close-range precopulatory behaviors may be easily observed and measured.

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