Sex Pheromone Components of Satin Moth, *Leucoma salicis* (L.)

(Lepidoptera: Lymantriidae)

By

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B.Sc. Simon Fraser University, 1994

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE

REQUIREMENTS FOR THE DEGREE OF MASTER OF PEST MANAGEMENT

In the Department

Of

Biological Sciences

August 2000

Abstract

Analyses of pheromone gland extract of female satin moths, *Leucoma salicis*, by coupled gas chromatographic-electroantennographic detection (GC-EAD) employing fused silica columns coated with DB-210, DB-23 or DB-5 revealed six antennally active compounds. (3*Z*,9*Z*)-*cis*-6,7-Epoxy-heneicosadiene (**1**) and (3*Z*,6*Z*)-*cis*-9,10-epoxy-heneicosadiene (**2**) were identified based on retention index calculations and comparison with known synthetic standards. Evidence in support of (3*Z*)-*cis*-6,7-*cis*-9,10-diepoxy-heneicosene (**4**), termed leucomalure, as the major pheromone component includes: 1) greatest abundance and EAD-activity; 2) identical retention and mass spectrometric characteristics of synthetic and female-produced **4**; 3) comparable EAD-activity of synthetic and female-produced **4** when tested at equivalent quantities; and 4) captures of male SM in traps baited with synthetic **4**. GC-EAD analysis of synthetic stereoisomeric **4** revealed that stereoisomers separated in the form of two diastereomers (each containing two stereoisomers), and that female SM produced at least one stereoisomer of each diastereomer (**4a** and **4b**). Of all four stereoselectively synthesized stereoisomers, ***SRSR*-4** elicited the strongest antennal response, and by itself attracted male SM, suggesting that it is the major SM pheromone component. Addition of ***SRRS*-4** to ***SRSR*-4** at a wide ratio, as found in pheromone gland extracts, significantly enhanced attractiveness of ***SRSR*-4**, further suggesting that ***SRRS*-4** is a second pheromone component. EAD-active monoepoxides **1** and **2** were not behaviourally active with male SM, but may be pheromone components in other *Leucoma* spp. For pheromone-based monitoring of SM, a suboptimal but economical mixture of stereoisomers of **4** (in a 1:1:1:1 ratio) is

recommended as a trap lure to obtain a correlation of captured males with the actual population density in the sampling area. For development of pheromone-based detection surveys of SM, which rely on optimal attraction of male moths, further testing of ***SRSR*-4**, ***SRSR*-4** plus ***SRRS*-4** and stereoisomeric **4** is required to determine the most attractive bait.

Dedication

To Suzanne, Edith, Gordon, Ron, and Wies

and my son David Keith

Acknowledgements

I am indebted to Dr. Gerhard Gries my senior supervisor for allowing me to work on this exciting project, for his enthusiastic approach in teaching me about the field of chemical ecology, and for his guidance. I sincerely thank Regine Gries for her teaching and technical assistance. Thanks are due to my committee member, Dr. John Borden, for discussions, advice and editing; Priyantha Wimalaratne, for syntheses of test chemicals; Michael Shannon for the excellent volunteer fieldwork, Chris Saunders for expressing an interest and initiating this project; Akbar Syed and Bruce Leighton for assistance in insect rearing; and Edith and Gordon Holden for support and kindly allowing the use of their vehicle for fieldwork. I deeply thank my fellow MPM students, Marnie Duthie, Troy Kimoto, Christian Krupke, David Onyabe, and Sherah VanLaerhoven, for the discussions, advice and good friendship. Finally, I am most grateful to my wife, Suzanne, for everything. The research was supported, in part, by a graduate fellowship from the Department of Biological Sciences to D.G.H. and by an NSERC grant to G.G.

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1. Introduction

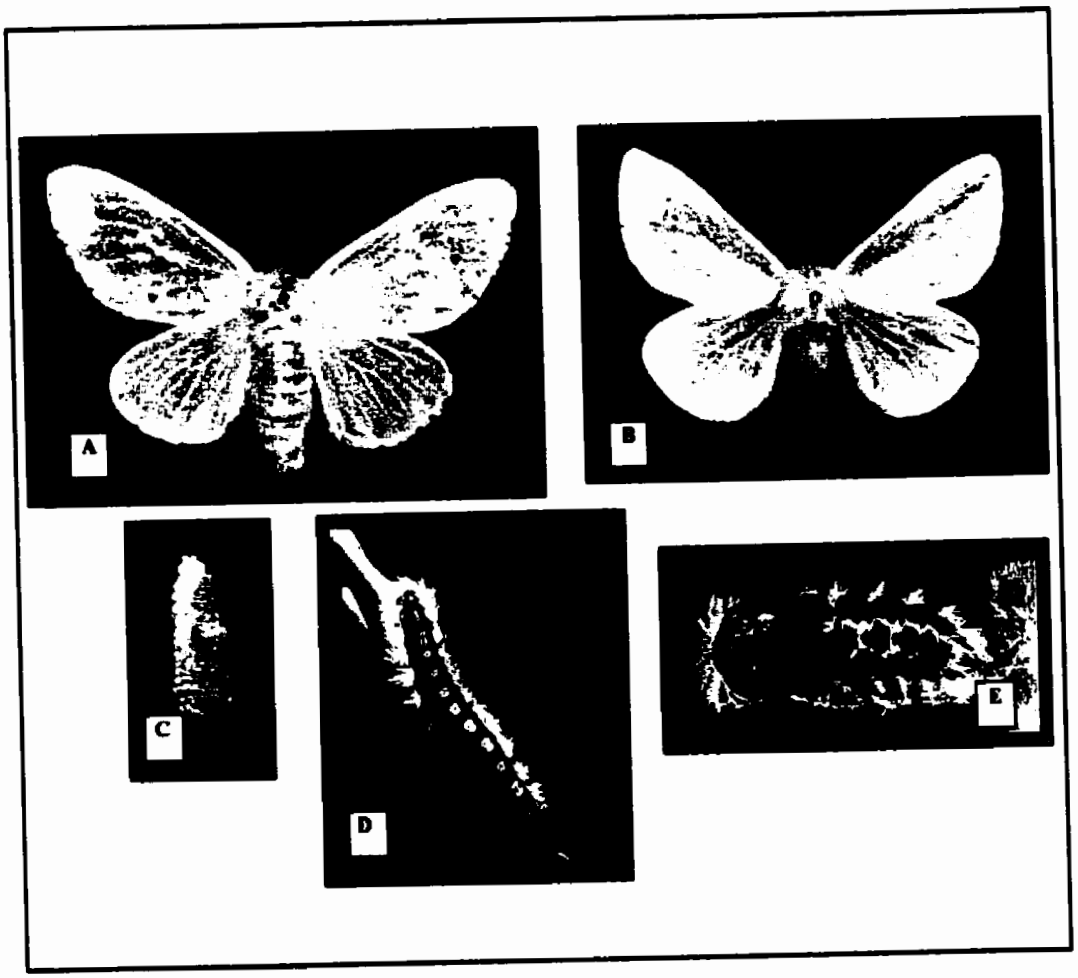
1.1. Morphology of the Satin Moth (SM)

Male and female satin moths, *Leucoma salicis* (L.), (SM) are black-bodied but appear satin white due to a dense covering of white scales (Fig. 1). The SM resembles the fall webworm, *Hyphantria cunea* (Drury), except that the SM has a black banding pattern on its legs (produced from alternation of black and white scales) and is usually larger in size. Males and females are 14-21 mm and 18-26 mm long, respectively. Wing span ranges between 35-60 mm, with males being generally smaller. Unlike females, males have plumose antennae.

The flat egg mass (Fig. 1) may be found on a variety of host and non host surfaces, and consists of several hundred ovoid light green eggs covered by a whitish frothy secretion. The length of SM larvae ranges from 3 mm in the first instar to 48 mm in the seventh instar. The last instar (Fig. 1) has a black head capsule and a medium grey-brown body with a row of conspicuous cream-coloured dots dorsally, running from the first thoracic segments to the tip of the abdomen. There is also a subdorsal row of small cream-coloured spots along the length of the body. The lateral verrucae are reddish-brown and bear uniform tufts of brown to light-yellow hair. There are paired eversible glands dorsally on the first and second abdominal segment; as in other lymantriid larvae, two eversible glands occur dorsally on the sixth and seventh abdominal segments.

Pupae (Fig. 1) are shiny black with whitish-yellow markings laterally between abdominal segments. There is a sparse covering of light yellow hair arising from tufts on the lateral and dorsal regions. Pupae are ca. 25 mm long. In general, male pupae are

Fig. 1 Satin moth, *L. salicis*, collected near Merritt, British Columbia: A) adult female; B) adult male; C) egg mass; D) seventh instar; E) pupa.


A
B
C
D
E

smaller than female pupae. Pupae reside in a loosely spun web of silk usually wrapped up in leaves or crevices of a tree.

1.2. Distribution and Host Plants

The SM is native to parts of temperate Europe and Asia, and is introduced to North America (Fig. 2). The distribution of SM in British Columbia (BC) likely ranges (Humphreys 1984) from Victoria to Campbell River on Vancouver Island, from Vancouver to Powell River on the mainland coast, and from to Hope to Moyie (south of Cranbrook) and north to Prince George (Fig. 2). Recent reports of SM in Edmonton Alberta indicate that SM continues to spread eastward (Langor 1995; Brandt 1995).

SM larvae defoliate deciduous trees and shrubs (Table 1). Poplar, *Populus* spp., and willow, *Salix* spp., are preferred hosts, whereas oak, *Quercus* spp., crabapple, *Malus* spp., and saskatoon, *Amelanchier* spp., are fed upon occasionally (Humphreys 1984). In China, larvae may also feed on filbert, *Corylus* spp., and maple, *Acer* spp. (Sun 1988). In BC, I have observed larvae feeding on trembling aspen, *Populus tremuloides* Michaux, black cottonwood, *P. trichocarpa* Torrey & A. Gray, and willow, *Salix* spp.

1.3. Life History

In North America and throughout most of Europe, the SM is univoltine. It may be bivoltine in the most southern extent of its European range (Grijpma 1988). In BC, SM larvae develop through seven instars (Lejeune and Silver 1961). Neonate larvae emerge

Fig. 2 Global (top) and local maps depicting the distribution (shaded) of satin moth, *L. salicis*. The field site was located 40 km southwest of Merritt, British Columbia. Note: Drawings are schematic and do not imply continuous distributions of *L. salicis* in western and eastern North America or Eurasia.

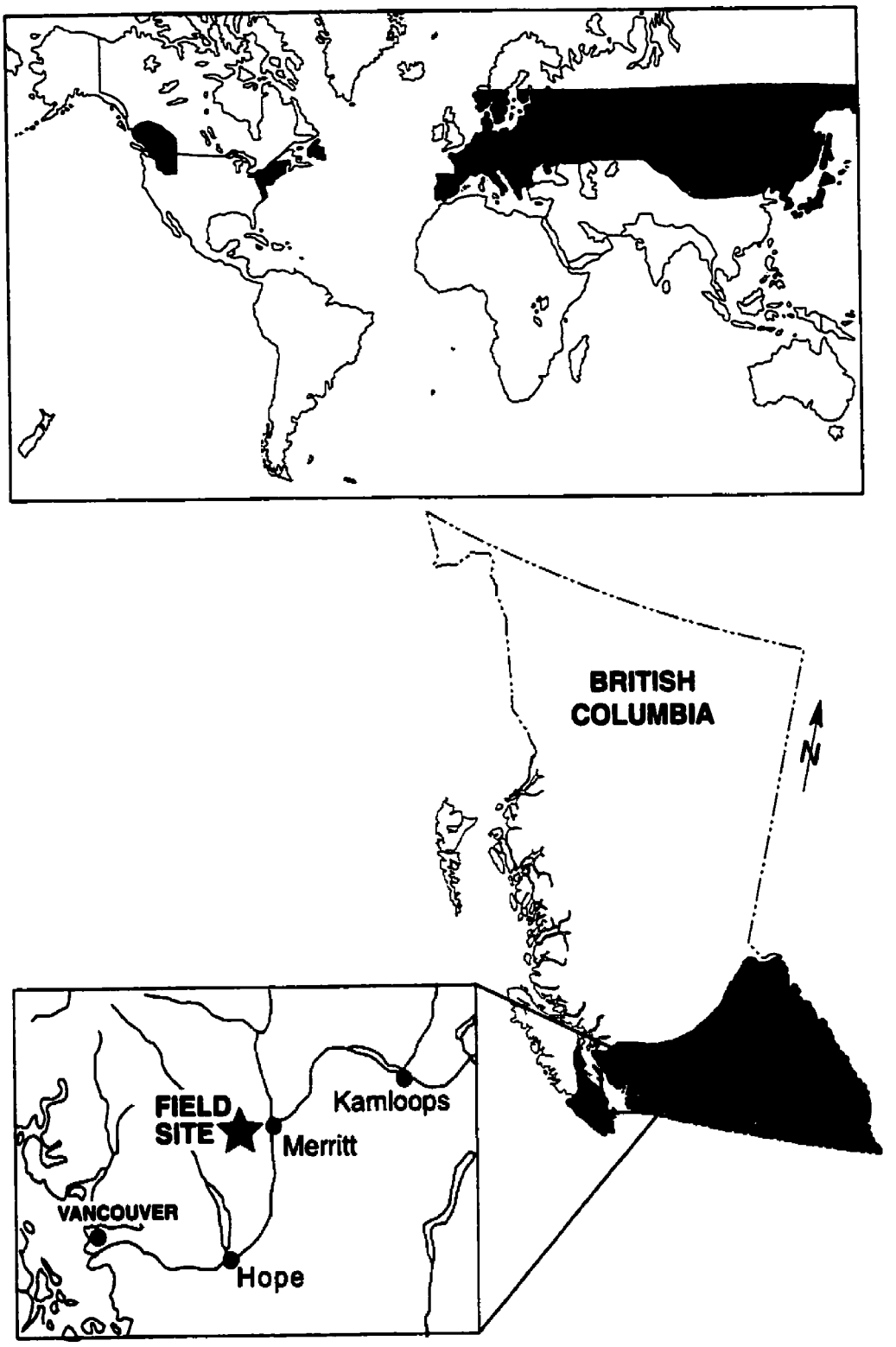

BRITISH
COLUMBIA
N
FIELD
SITE
Kamloops
Merritt
VANCOUVER
Hope

Table 1. List of host plants consumed by the satin moth, *L. salicis*.

Common Name	Scientific Name	Reference
Trembling aspen	*Populus tremuloides* Michaux	Wagner and Leonard (1979)
Largetooth aspen	*P. grandidentata* Michaux	
European white poplar	*P. alba* L.	Humphreys (1984; 1996)
Lombardy poplar	*P. nigra* L. cv. Italica Muench	
Trembling aspen	*P. tremuloides* Michaux	
Willow spp.	*Salix* spp.	
Oak spp.	*Quercus* spp.	
Crabapple spp.	*Malus* spp.	
Saskatoon spp.	*Amelanchier* spp.	
Lombardy poplar	*P. nigra* L. cv. Italica Muench	Burgess and Crossman (1929)
Eastern cottonwood	*P. deltoides* Bartr. ex Marsh	
Balm-of-Gilead poplar	*P. candicans* Ait.	
European white poplar	*P. alba* L.	
Largetooth aspen	*P. grandidentata* Michaux	
Trembling aspen	*P. tremuloides* Michaux	
Golden willow	*Salix vitelluna* L.	
Scrub oak	*Quercus ilicifolia* Wang	
Black oak	*Q. velutina* Lamarck	
Lombardy poplar	*P. nigra* L. var. *italica* Muench	Grijpma (1988)
Eastern cottonwood	*P. deltoides* Bartr. ex Marsh	
White poplar	*P. alba* L.	
Grey poplar	*P. canescens*	
European aspen	*P. tremula* L.	
Trembling aspen	*P. tremuloides* Michaux	
Popular	*Populus* spp.	Sun (1988)
Willow	*Salix* spp.	
Filbert	*Corylus* spp.	
Maple	*Acer* spp.	
Black cottonwood	*P. trichocarpa* Torrey & A. Gray	Magasi and Van Sickle (1984)
Balsam poplar	*P. balsamifera* L.	

in mid to late August, locate foliage and start to feed. They skeletonize leaves revealing the veins on the upper or lower surface. After ca. 7 days of skeletonization feeding, larvae seek cracks or crevices in the bark and spin a temporary web in which to moult. Second instars continue to feed until photoperiod, decreased temperature, and/or deteriorating leaf quality signal the onset of fall. Larvae overwinter in small indentations of the bark or under detached bark of dead trees, where they spin silken hibernacula and moult into third instars. In April, they resume feeding on leaves for 5-10 days and then moult. Fourth to seventh instars feed on the entire leaf except the midrib. During this stage, defoliation of trees becomes apparent (Fig. 3). When larval development is complete in late June, larvae void their guts, find a suitable pupation site, and spin a silken cocoon. Eight to 12 days later adult moths eclose. Protandrous males may await eclosion of a nearby female or take flight in search for females that emit pheromone (Priesner 1975). Males follow a pheromone plume upwind, alight near the female, and wing fan and probe with their abdomen before copulation ensues. Mated females deposit several hundred eggs before they take flight seeking further oviposition sites. Neonate larvae emerge after 12–17 days later.

1.4. Pest Status and Management

The SM is a minor pest of poplar and willow trees worldwide (Burgess and Crossman 1927; Grijpma 1988). Introduced from Europe around 1920, it is established in eastern and western North America and continues to spread into the interior of the continent (Reeks and Smith 1956; Langor 1995). Defoliated trees usually suffer reduced

Fig. 3 Stand of trembling aspen, *Populus tremuloides* Michaux, near Merritt, British Columbia (12 July, 1996) defoliated by larvae of the satin moth, *L. salicis*.

growth rates and some branch mortality in the current year (Burgess and Crossman 1927). Heavy defoliation in successive years causes mortality of trees (Burgess and Crossman 1927). Under favourable conditions, SM is capable of reaching outbreak levels (Humphreys 1996). In vast stands of trembling aspen in Alberta, the establishment of SM could threaten the integrity of the forest industry (Brandt 1995). In urban environments, larvae (and moths) become nuisance pests, like the gypsy moth, *Lymantria dispar* (L.), inflicting damage on shade, wind break and ornamental trees (Humphreys 1996). Because both male and female adults can fly (female *L. dispar* can not fly), egg masses can be carried on surfaces of goods and vehicles, and larvae can disperse by "ballooning" on silken threads, the SM has the potential to become established quickly in new areas (Reeks and Smith 1956; Condrashoff 1957).

Since the arrival of the exotic SM in North America, the United States Bureau of Entomology imported the braconid wasps *Apanteles solitarius* (Ratzeburg) [now recognized as *Cotesia melanoscelus* (Ratzeburg)] and *Meteorus versicolor* (Wesmael) into New England as biological control agents for the SM (Clausen 1956). Both braconids were also introduced into BC and Washington State (Lejeune and Silver 1961). In BC, *C. melanoscelus* and *M. versicolor* are promising biological control agents, but their effectiveness can be strongly reduced by hyperparasitoids (Lejeune and Silver 1961). The tachinid fly, *Compsilura concinnata* (Meigen), an introduced parasitoid of *L. dispar*, may also be effective against SM (Lejeune and Silver 1961). Native parasitoids have not proven very effective in reducing SM populations (Reeks and Smith 1956). The bacterium, *Bacillus thuringiensis* Berliner (Bt), and the fungus, *Beauveria* sp., have

helped alleviate the impact of SM outbreaks (Humphreys 1996). A cytoplasmic and nuclear polyhedrosis virus are associated with SM populations, but their virulence has yet to be determined (Fry 1987). I witnessed American robins, *Turdus migratorius* L., chipmunks, *Eutamias* spp., and dragonflies, *Aeshna* spp., feeding on adult SMs.

Chemical insecticides, such as lead arsenate and DDT, were once used for control of SM larvae (Reeks and Smith 1956), but are now replaced by the environmentally acceptable Bt, which is applied in early spring when larvae consume new foliage. Manual removal and destruction of egg masses, hibernacula and larvae may only be effective when very few trees are infested. Wrapping burlap or corrugated cardboard around the tree's base provides overwintering sites, which can be destroyed.

Pheromone-baited traps may be used to determine the presence, distribution and density of SM populations. Captures of male SM in female-baited traps may be followed by immediate eradication measures to prevent SM establishment in uninfested areas. Increasing trap captures from year to year may signal an incipient outbreak, allowing the forest manager to consider and plan control measures. While there is evidence that female SM emit a sex pheromone to attract males (Priesner 1975; Wagner and Leonard 1979), the pheromone is yet to be identified. Several outbreaks of SM in British Columbia (BC) and Alberta in 1995-1999 provided the opportunity to study the pheromone biology of this lymantriid moth.

1.5. Objectives

My objectives were:

1. to identify candidate pheromone components of female SM, and
2. to field test these components.

2. Materials and Methods

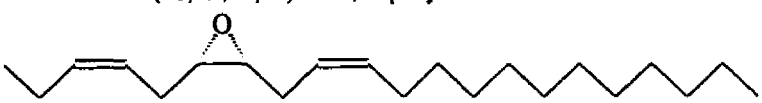
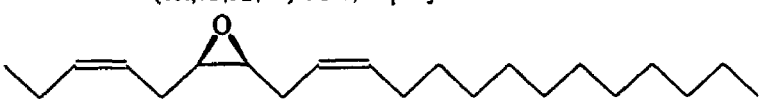
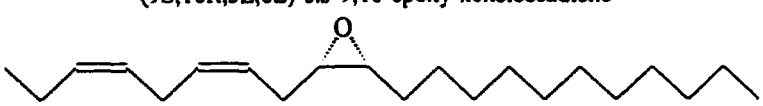
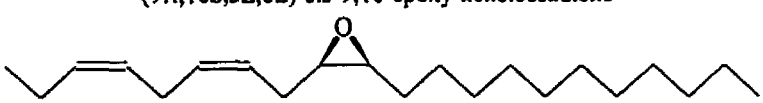
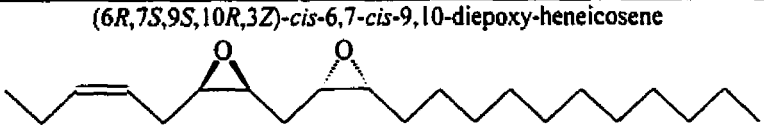
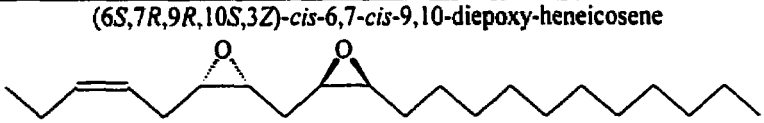
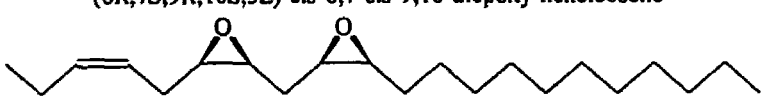
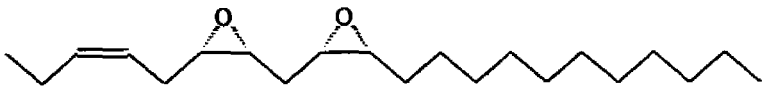
2.1. Experimental Insects and Pheromone Analyses

SM pupae were collected from balsam poplar in Edmonton, Alberta. They were reared to adults in the laboratory at ~70% RH, ~26°C and a photoregime of 16:8 (light:dark). Male and female pupae were distinguished by size and kept in separate Petri dishes to avoid mating of eclosed moths. Voucher specimens of *L. salicis* have been deposited in the entomological collection of Simon Fraser University.

During peak calling activity 3-4 h into the scotophase (Wagner and Leonard 1979), pheromone glands of 1-2 day old virgin females were removed and extracted for 5-20 min in HPLC-grade hexane. Extracts of female SM were analyzed by coupled gas chromatographic-electroantennographic detection (GC-EAD) (Arn *et al.* 1975), employing fused silica columns (30 m X 0.25 or 0.32 mm ID) coated with DB-5, DB-210 or DB-23 (J&W Scientific, Folsom, California). For retention index calculations of EAD-active compounds (Van Den Dool and Kratz 1963), synthetic straight chain C_{14}–C_{23} hydrocarbon standards were chromatographed under identical conditions.

Candidate pheromone components were provided by the National Research Council, Saskatoon, Saskatchewan or synthesized by P.D.C. Wimalaratne (Simon Fraser University) (Table 2). All chemicals were > 90 % chemically and ≥ 90 % geometrically pure.

Table 2 List of candidate pheromone components identified in pheromone gland extracts of female satin moth, *L. salicis*. Compounds are numbered according to their order of elution in GC-EAD analyses (DB-5 column).

	Compound	Chemical Name and Molecular Structure	Source
	***SR*-1**	(6*S*,7*R*,3*Z*,9*Z*)-*cis*-6,7-epoxy-heneicosadiene 	National Research Council
	***RS*-1**	(6*R*,7*S*,3*Z*,9*Z*)-*cis*-6,7-epoxy-heneicosadiene 	National Research Council
	***SR*-2**	(9*S*,10*R*,3*Z*,6*Z*)-*cis*-9,10-epoxy-heneicosadiene 	National Research Council
	***RS*-2**	(9*R*,10*S*,3*Z*,6*Z*)-*cis*-9,10-epoxy-heneicosadiene 	National Research Council
	3	Unknown	NA
4a	***RSSR*-4**	(6*R*,7*S*,9*S*,10*R*,3*Z*)-*cis*-6,7-*cis*-9,10-diepoxy-heneicosene 	Synthesized by P.D.C. Wimalaratne
	***SRRS*-4**	(6*S*,7*R*,9*R*,10*S*,3*Z*)-*cis*-6,7-*cis*-9,10-diepoxy-heneicosene 	Synthesis by P.D.C. Wimalaratne
4b	***RSRS*-4**	(6*R*,7*S*,9*R*,10*S*,3*Z*)-*cis*-6,7-*cis*-9,10-diepoxy-heneicosene 	Synthesis by P.D.C. Wimalaratne
	***SRSR*-4**	(6*S*,7*R*,9*S*,10*R*,3*Z*)-*cis*-6,7-*cis*-9,10-diepoxy-heneicosene 	Synthesized by P.D.C. Wimalaratne
	5	Unknown	NA

2.2. Field Testing of Candidate Pheromone Components

Sixteen field experiments (Exp.) were conducted from 1996-1999 in stands of *P. tremuloides*, near Merritt, BC (Fig. 2), with treatments, dates, and number of replicates as in Table 3. For each experiment, sticky delta traps (Gray *et al.* 1984) were suspended from trees circa 1.5 m above ground in randomized complete blocks with inter-trap spacing of 15-25 m. Traps were baited with rubber septa (The West Company, Phoenixville, PA) impregnated with candidate pheromone components.

For Exps. 1- 8 (1996), synthetic diastereomers **4a** and **4b** (Table 2) were separated by high performance liquid chromatography [(HPLC): Waters LC 625 equipped with a Waters 486 variable wavelength UV-visible detector set at 220 nm, and a Nova-pak TMC_{18} (3.9 x 30 mm) column with 1ml/min of acetonitrile flow]. For Exps. 9 – 16 (1998-1999), stereospecifically synthesized stereoisomers were used as trap baits.

Exp. 1 tested **4a** (20 μg) and **4b** (20 μg) singly and in combination. Exp. 2 tested **4b** (20 μg) singly and in combination with **4a** at 0.2, 2, and 20 μg. Exps. 3-7 tested whether the attractiveness of the diepoxide **4b** could be enhanced by the addition of the monoepoxides **1a, 1b, 2a** and/or **2b.** Specifically, Exp. 3 tested **4b** (20 μg) singly and in combination with ***RS*-1** (0.5 μg) plus ***RS*-2** (0.5 μg), ***SR*-1** (0.5 μg) plus ***SR*-2** (0.5 μg), or all 4 enantiomers combined. Exp. 4 tested **4b** (20 μg) singly, in binary combination with **4a** (20 μg), in ternary combination with ***RS*-1** (0.1 μg) plus ***RS*-2** (0.1 μg), and in quartenary combination with **4a** (20 μg), ***RS*-1** (0.1 μg) and ***RS*-2** (0.1 μg). Exp. 5 tested **4b** (20 μg) singly, and in binary and ternary combinations with ***RS*-1** (0.5 μg) and ***RS*-2** (0.5 μg). Exp. 6 tested **4b** (20 μg) singly, and in binary and ternary combination with

Table 3 List of field experiments conducted near Merritt, British Columbia, to determine the sex pheromone of female satin moth, *L. salicis*.

Exp. No. [a]	Treatments [b]	Time
1	**4a** (20 µg) **4b** (20 µg) **4a** (20 µg), **4b** (20 µg) **unbaited**	18-22 July 1996
2	**4b** (20 µg) **4b** (20 µg), **4a** (0.2 µg) **4b** (20 µg), **4a** (2 µg) **4b** (20 µg), **4a** (20 µg)	28-30 July 1996
3	**4b** (20 µg) **4b** (20 µg), ***SR*-1** (0.5 µg), ***SR*-2** (0.5 µg) **4b** (20 µg), ***RS*-1** (0.5 µg), ***RS*-2** (0.5 µg) **4b** (20 µg), ***SR*-1** (0.5 µg), ***SR*-2** (0.5 µg), ***RS*-1** (0.5 µg), ***RS*-2** (0.5 µg)	22-24 July 1996
4	**4b** (20 µg) **4b** (20 µg), ***RS*-1** (0.1 µg), ***RS*-2** (0.1 µg) **4b** (20 µg), **4a** (20 µg) **4b** (20 µg), **4a** (20 µg), ***RS*-1** (0.1 µg), ***RS*-2** (0.1 µg)	11-14 July 1996
5	**4b** (20 µg) **4b** (20 µg), ***RS*-1** (0.5 µg) **4b** (20 µg), ***RS*-2** (0.5 µg) **4b** (20 µg), ***RS*-1** (0.5 µg), ***RS*-2** (0.5 µg)	29-31 July 1996
6	**4b** (20 µg) **4b** (20 µg), ***SR*-1** (0.1 µg) **4b** (20 µg), ***SR*-2** (0.1 µg) **4b** (20 µg), ***SR*-1** (0.1 µg), ***SR*-2** (0.1 µg)	31 July - 2-Aug. 1996
7	**4b** (20 µg) **4b** (20 µg), ***SR*-1** (0.5 µg) **4b** (20 µg), ***SR*-2** (0.5 µg) **4b** (20 µg), ***SR*-1** (0.5 µg), ***SR*-2** (0.5 µg) unbaited	19-23 July 1997
8	**4b** (20 µg) 2 virgin females	26-28 July 1996

Table 3 continued.

9	***SRSR*-4** (20 µg) ***RSRS*-4** (20 µg) ***SRRS*-4** (20 µg) ***RSSR*-4** (20 µg) ***SRSR*-4** (20 µg), ***RSRS*-4** (20 µg), ***SRRS*-4** (20 µg), ***RSSR*-4** (20 µg) **unbaited**	15-17 July 1998
10	***SRSR*-4** (20 µg) ***SRSR*-4** (20 µg), ***RSRS*-4** (0.2 µg) ***SRSR*-4** (20 µg), ***RSRS*-4** (2 µg) ***SRSR*-4** (20 µg), ***RSRS*-4** (20 µg)	17-22 July 1998
11	***SRSR*-4** (20 µg) ***SRSR*-4** (20 µg), ***RSSR*-4** (0.2 µg) ***SRSR*-4** (20 µg), ***RSSR*-4** (2 µg) ***SRSR*-4** (20 µg), ***RSSR*-4** (20 µg)	17-22 July 1998
12	***SRSR*-4** (20 µg) ***SRSR*-4** (20 µg), ***SRRS*-4** (0.2 µg) ***SRSR*-4** (20 µg), ***SRRS*-4** (2 µg) ***SRSR*-4** (20 µg), ***SRRS*-4** (20 µg)	17-22 July 1998
13	***SRSR*-4** (20 µg) ***SRSR*-4** (20 µg), ***SR*-1** (0.5 µg) ***SRSR*-4** (20 µg), ***SR*-2** (0.5 µg) ***SRSR*-4** (20 µg), ***SR*-1** (0.5 µg), ***SR*-2** (0.5 µg)	22-25 July 1998
14	***SRSR*-4** (20 µg) ***SRSR*-4** (20 µg), ***RSRS*-4** (2 µg) ***SRSR*-4** (20 µg), ***RSSR*-4** (2 µg) ***SRSR*-4** (20 µg), ***SRRS*-4** (2 µg) ***SRSR*-4** (20 µg), ***RSSR*-4** (2 µg), ***SRRS*-4** (2 µg) ***SRSR*-4** (20 µg), ***RSRS*-4** (2 µg), ***RSSR*-4** (2 µg) ***SRSR*-4** (20 µg), ***RSRS*-4** (2 µg), ***SRRS*-4** (2 µg) ***SRSR*-4** (20 µg), ***RSSR*-4** (2 µg), ***SRRS*-4** (2 µg), ***RSRS*-4** (2 µg)	27 July - 2-Aug. 1999
15	***SRSR*-4** (20 µg) ***SRSR*-4** (20 µg), ***RSSR*-4** (2 µg) ***SRSR*-4** (20 µg), ***SRRS*-4** (2 µg)	30 July - 1-Aug. 1999
16	***SRSR*-4** (20 µg) ***SRSR*-4** (20 µg), ***RSSR*-4** (2 µg) ***SRSR*-4** (20 µg), ***RSSR*-4** (20 µg), ***SRRS*-4** (20 µg), ***RSRS*-4** (20 µg) **unbaited**	31 July - 1 Aug. 1999

[a] All experiments employed 10 replicates except for experiment 4 with 8 replicates.
[b] Compound abbreviations as in table 2.

***SR*-1** (0.1 μg) and ***SR*-2** (0.1 μg). Exp. 7 was identical to Exp. 6 except that quantities of ***SR*-1** and ***SR*-2** were increased to 0.5 μg each. Exp. 8 tested 2 virgin females *versus* the most attractive synthetic bait (**4b**, 20 μg), as determined in 1996. Virgin females were contained in a plastic cylinder (4 x 7 cm) with the top and bottom covered with 1mm fiberglass mesh. Exps. 9-12 and 14-16 investigated attractiveness of the stereoisomers of **4** singly and in combinations. Exp. 9 tested ***SRSR*-4** (20 μg), ***RSRS*-4** (20 μg), ***RSSR*-4** (20 μg), and ***SRRS*-4** (20 μg) singly and in quartenary combination. Exp. 10 tested ***SRSR*-4** (20 μg) singly and in combination with ***RSRS*-4** at 0.2, 2, and 20 μg. Exp. 11 tested ***SRSR*-4** (20 μg) singly and in combination with ***RSSR*-4** at 0.2, 2, and 20 μg. Exp. 12 tested ***SRSR*-4** (20 μg) singly and in combination with ***SRRS*-4** (20 μg) at 0.2, 2, and 20 μg. Exp. 13 tested ***SRSR*-4** (20 μg) singly, and in binary and ternary combination with ***SR*-1** (0.5 μg) and ***SR*-2** (0.5 μg). Exp. 14 tested ***SRSR*-4** (20 μg) singly and in all binary, ternary and quaternary combinations with ***RSRS*-4** (2 μg), ***RSSR*-4** (2 μg), and ***SRRS*-4** (2 μg). Exp. 15 tested ***SRSR*-4** (20 μg) singly and in binary combinations with ***SRRS*-4** (2 μg) and ***RSSR*-4** (2 μg). Exp. 16 tested ***SRSR*-4** (20 μg) singly, in binary combination with ***RSSR*-4** (2 μg), and in quartenary combination with ***RSRS*-4** (20 μg), ***SRRS*-4** (20 μg), and ***RSSR*-4** (20 μg). Numbers of captured male SM were recorded at the end of each experiment.

Statistical analyses were conducted using the SAS statistical package (SAS Institute Inc., Cary, North Carolina). Despite transformation, data from field experiments were not normally distributed and were therefore subjected to nonparametric analysis of

variance by ranks (Friedman's test), followed by nonparametric comparison of means (Student-Newman-Keuls test) (Zar 1984; SAS 1988). In all cases α=0.05.

3. Results

3.1. Pheromone Analyses

Analyses of female SM pheromone extract by GC-EAD revealed six antennally-active compounds (Fig. 4). Retention indices (Van Den Dool and Kratz 1963) and EAD-activity of compounds **1** and **2** on GC columns coated with DB-210, DB-23 or DB-5 were identical to those of synthetic (3*Z*,9*Z*)-*cis*-6,7-epoxy-heneicosadiene (**1**) and (3*Z*,6*Z*)-*cis*-9,10-epoxy-heneicosadiene (**2**), respectively. FID-detectable and most EAD-active **4b** was hypothesized to be (3*Z*)-*cis*-6,7-*cis*-9,10-di-epoxy-heneicosene because: a) inter-column differences of retention indices (DB-23 to DB-5: 875; DB-5 to DB-210: 575; and DB-23 to DB-210: 300) were ca. twice as great as those of monoepoxides **1** or **2**; b) GC-mass spectra (Hewlett Packard 5985B) in electron impact (Fig. 5) and chemical ionization (isobutane) modes were consistent with a monounsaturated C21 di-epoxide; c) epoxy positions in **1** and **2** were *cis*-6,7 (**1**) and *cis*-9,10 (**2**); and d) epoxidation of synthetic (3*Z*,6*Z*,9*Z*)-heneicosatriene resulted in mono- and di-epoxides, one of which had retention and EAD-characteristics consistent with SM-produced **4b**. GC-EAD analyses of stereoselectively synthesized stereoisomers of **4** revealed that ***SRSR***-4 elicited the strongest anntenal response (Fig. 6).

3.2. Field Experiments

Traps baited with **4b** captured significantly more male SM than those baited with **4a** or **4a** + **4b** (Fig. 7, Exp. 1). Attractiveness of **4b** could not be enhanced by addition of

Fig. 4 Flame ionization detector (FID) and electroantennographic detector (EAD) responses to one female equivalent of *L. salicis* pheromone gland extract. Chromatography: Hewlett Packard 5890 equipped with a fused silica column (30 m x 0.25 mm ID) coated with DB-5; splitless injection, temperature of injection port 220°C; temperature program: 100°C (1 min), 15°C/min to 280°C. EAD-active compounds 3 and 5 are yet to be identified.

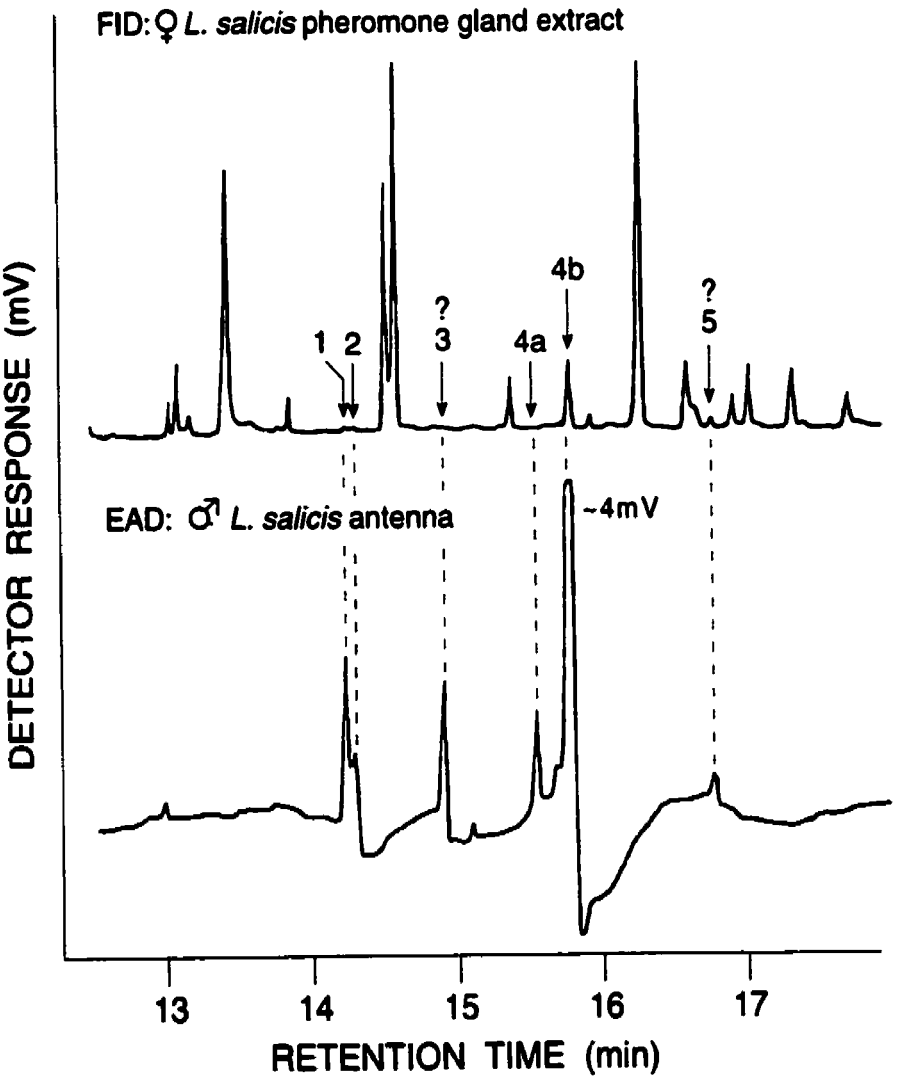

FID: ♀ L. salicis pheromone gland extract
1
2
?
3
4a
4b
?
5
EAD: ♂ L. salicis antenna
~4mV
DETECTOR RESPONSE (mV)
13
14
15
16
17
RETENTION TIME (min)

Fig. 5 Electron impact GC-mass spectrum (Hewlett Packard 5985B, GC-MS) of compound **4b** in Figure 4 that elicited the strongest antennal response.

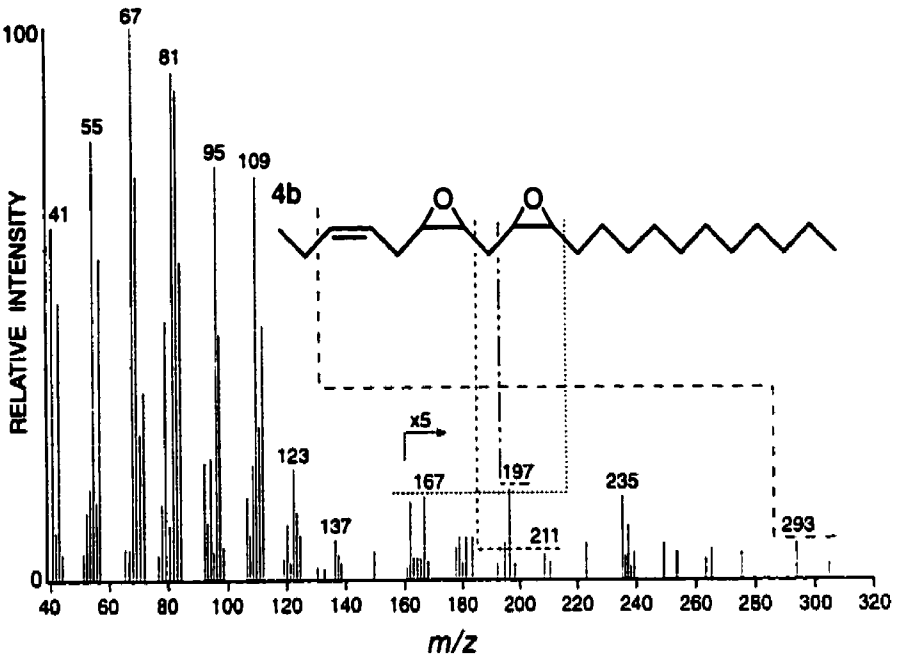

100
0
RELATIVE INTENSITY
41
55
67
81
95
109
123
137
167
197
211
235
293
x5
4b
O
O
40
60
80
100
120
140
160
180
200
220
240
260
280
300
320
m/z

Fig. 6 EAD responses from male *L. salicis* antenna to 50 ng of synthetic standards of ***RSSR*-4**, ***SRRS*-4**, ***RSRS*-4** and ***SRSR*-4**. Enantiomeric (3Z,9Z)-*cis*-6,7-epoxy-heneicosadiene (50 ng) served as internal standard (IS). Chromatography: Hewlett Packard 5890 equipped with a fused silica column (30 m x 0.32 mm ID) coated with DB-5; split injection, temperature of injection port 220°C, temperature program: 250°C isothermal. Note: FID traces not depicted; quantities of compounds take split injection into account.

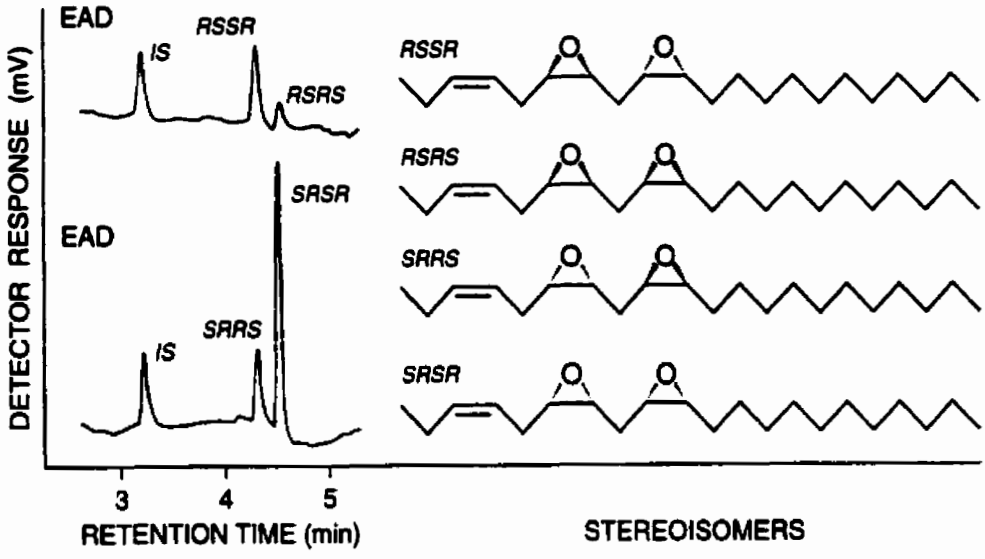

EAD
IS
RSSR
RSRS
EAD
IS
SRRS
SRSR
DETECTOR RESPONSE (mV)
3
4
5
RETENTION TIME (min)
RSSR
RSRS
SRRS
SRSR
STEREOISOMERS

Fig. 7 Comparison of numbers of male *L. salicis* captured in Exps. 1 and 2 in sticky Delta traps baited with different mixtures of candidate pheromone components. For each experiment, bars with the same letter superscript are not significantly different, non parametric ANOVA followed by the Student Newman Keuls test, $p < 0.05$.

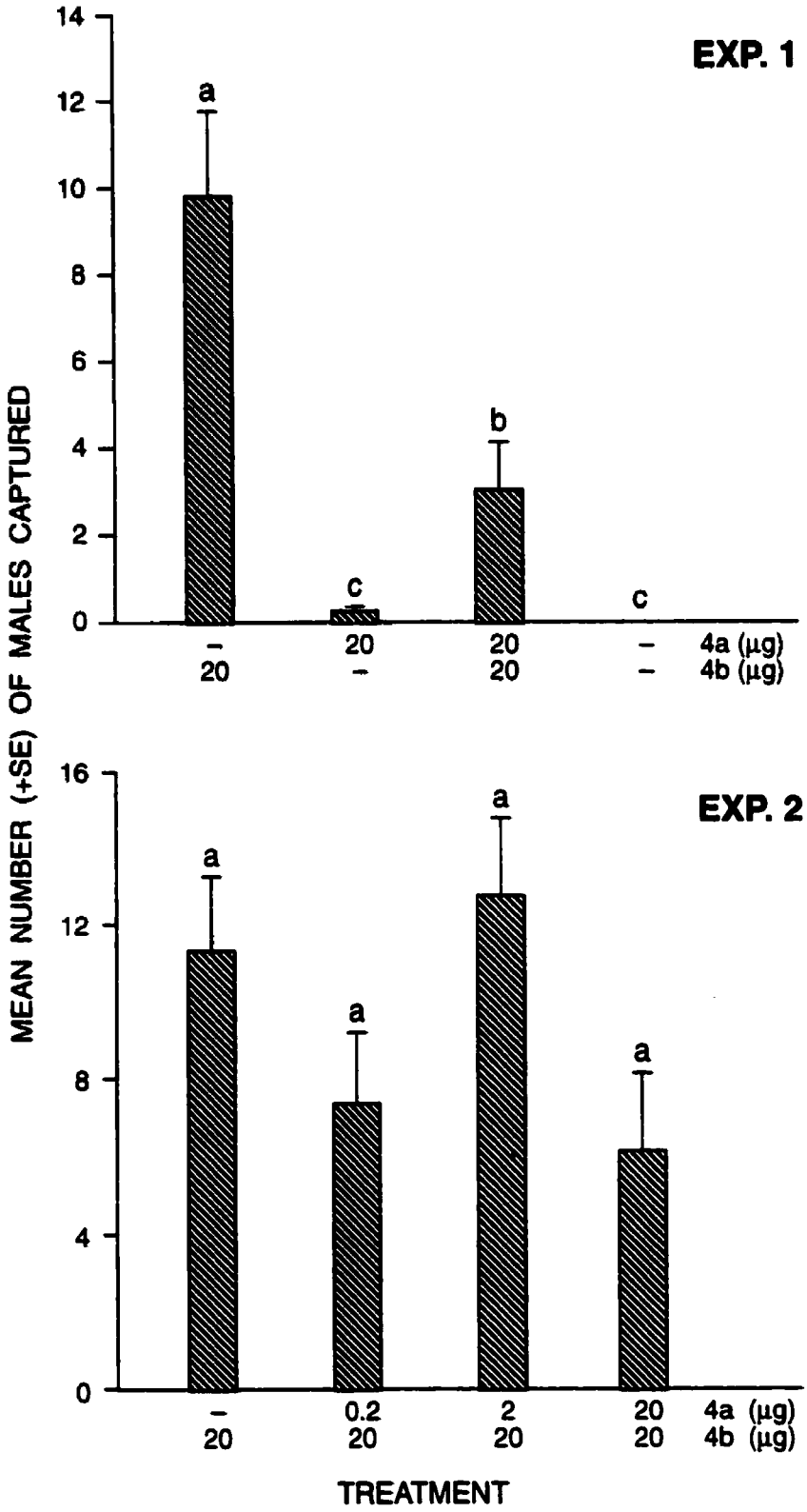
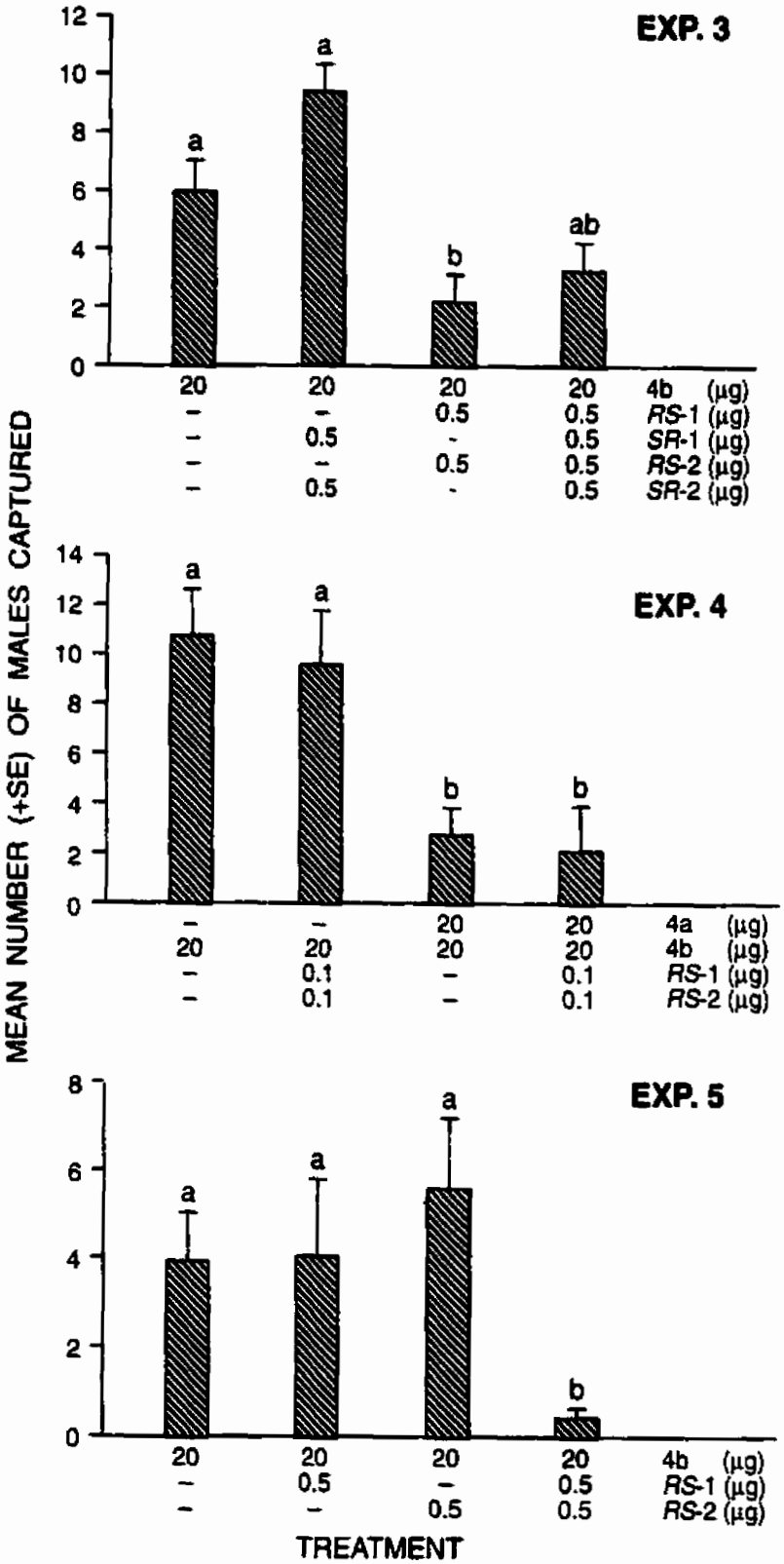
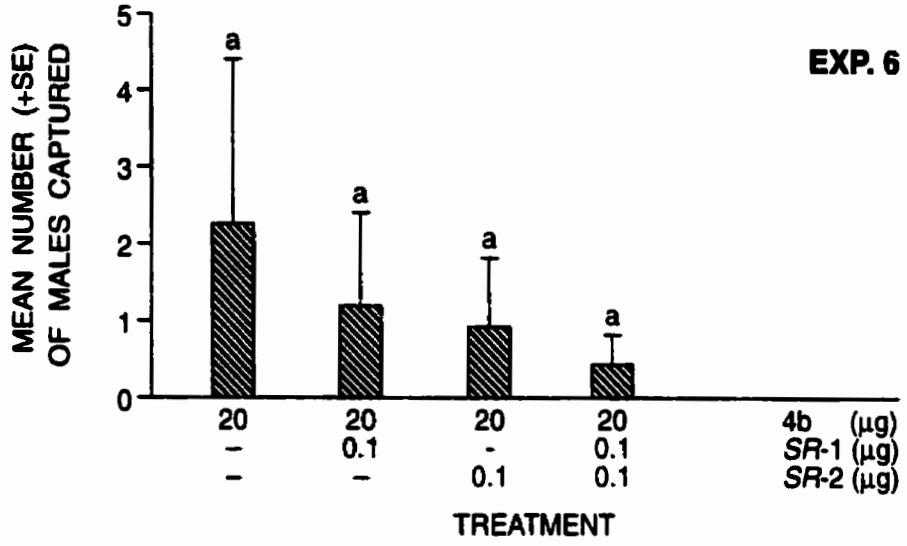

EXP. 1
14
12
10
8
6
4
2
0
a
c
b
c
–
20
20
–
20
–
20
–
4a (µg)
4b (µg)
EXP. 2
16
12
8
4
0
a
a
a
a
–
20
0.2
20
2
20
20
20
4a (µg)
4b (µg)
MEAN NUMBER (+SE) OF MALES CAPTURED
TREATMENT

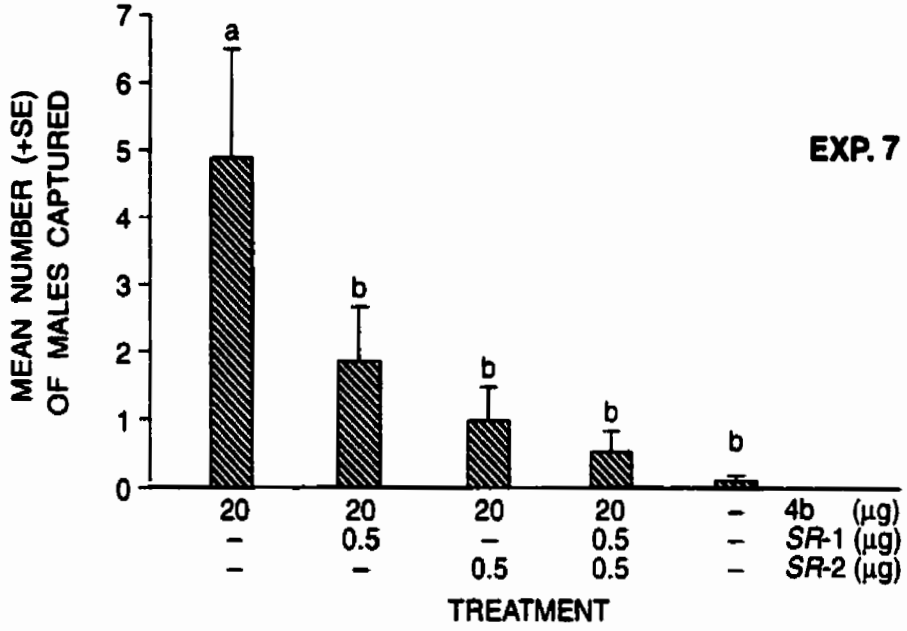
Fig. 8 Comparison of numbers of male *L. salicis* captured in Exps. 3-5 in sticky Delta traps baited with different mixtures of candidate pheromone components. For each experiment, bars with the same letter superscript are not significantly different, non parametric ANOVA followed by the Student Newman Keuls test, $p < 0.05$.


EXP. 3
EXP. 4
EXP. 5
MEAN NUMBER (+SE) OF MALES CAPTURED
TREATMENT
4b (µg)
RS-1 (µg)
SR-1 (µg)
RS-2 (µg)
SR-2 (µg)
4a (µg)

4a at various ratios (Fig. 7, Exp. 2). Addition of ***RS*-1** and ***RS*-2** (Exps. 3, 5) or **4a** (Exp. 4) to **4b** reduced trap captures (Fig. 8). Similarly, addition of ***SR*-1** and ***SR*-2** to **4b** also reduced trap captures, although the apparent reduction in trap captures in Exp. 6 (using a lower dose) was not significant (Fig. 9, Exps. 6, 7). Traps baited with **4b** or with virgin females captured male moths between 18:00 hr and 2:00 hr (Fig. 9, Exp. 8). The stereoisomer ***SRSR*-4** singly was significantly more attractive than any of the other three stereoisomers and as attractive as all four stereoisomers combined (Fig. 10, Exp. 9). Addition of ***RSRS*-4** (Exp. 10), ***RSSR*-4** (Exp. 11) and ***SRRS*-4** (Exp. 12) to ***SRSR*-4** had no effect on trap captures in Exp. 10, slightly reduced trap captures in Exp. 11, and significantly increased or decreased, respectively, trap captures in a ratio-dependent manner in Exp. 12 (Fig. 11). Stereoisomer ***SRSR*-4** singly and in admixture with ***SR*-1**, ***SR*-2** or both were equally attractive (Fig. 12, Exp. 13). There were also no differences in captures of male moths when traps were baited with ***SRSR*-4** singly and in all possible binary, ternary and quaternary combinations with ***RSRS*-4**, ***RSSR*-4** and ***SRRS*-4** (Fig. 13, Exp. 14), but trap captures were low and highly variable, making interpretation of results difficult. Addition of ***RSSR*-4** to ***SRSR*-4** increased trap captures both in Exp. 15 and 16, although the increase was statistically significant only in Exp. 16 (Fig. 14). Combinations of ***SRSR*-4** with ***RSSR*-4** or with all 3 other stereoisomers were equally attractive and significantly more attractive than ***SRSR*-4** alone (Fig. 14, Exp. 16).

Fig. 9 Comparison of numbers of male *L. salicis* captured in Exps. 6-8 in sticky Delta traps baited with different mixtures of candidate pheromone components and with two virgin females in Exp. 8. For each experiment, bars with the same letter superscript are not significantly different, non parametric ANOVA followed by the Student Newman Keuls test, $p < 0.05$.


EXP. 6
MEAN NUMBER (+SE) OF MALES CAPTURED
5
4
3
2
1
0
a
a
a
a
20 20 20 20 4b (µg)
– 0.1 - 0.1 SR-1 (µg)
– – 0.1 0.1 SR-2 (µg)
TREATMENT


EXP. 7
MEAN NUMBER (+SE) OF MALES CAPTURED
7
6
5
4
3
2
1
0
a
b
b
b
b
20 20 20 20 – 4b (µg)
– 0.5 – 0.5 – SR-1 (µg)
– – 0.5 0.5 – SR-2 (µg)
TREATMENT

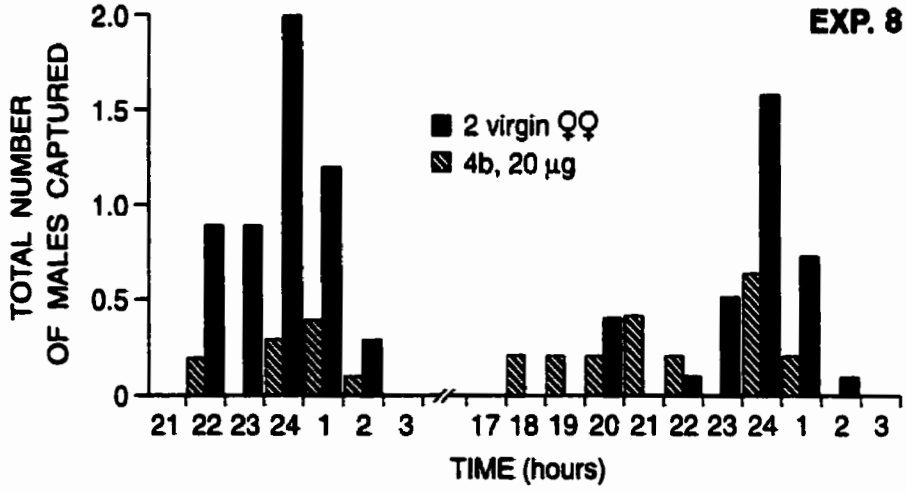

EXP. 8
TOTAL NUMBER OF MALES CAPTURED
2.0
1.5
1.0
0.5
0
2 virgin ♀♀
4b, 20 µg
21 22 23 24 1 2 3 17 18 19 20 21 22 23 24 1 2 3
TIME (hours)

Fig. 10 Comparison of numbers of male *L. salicis* captured in Exp. 9 in sticky Delta traps baited with different mixtures of candidate pheromone components. For each experiment, bars with the same letter superscript are not significantly different, non parametric ANOVA followed by the Student Newman Keuls test, $p < 0.05$.

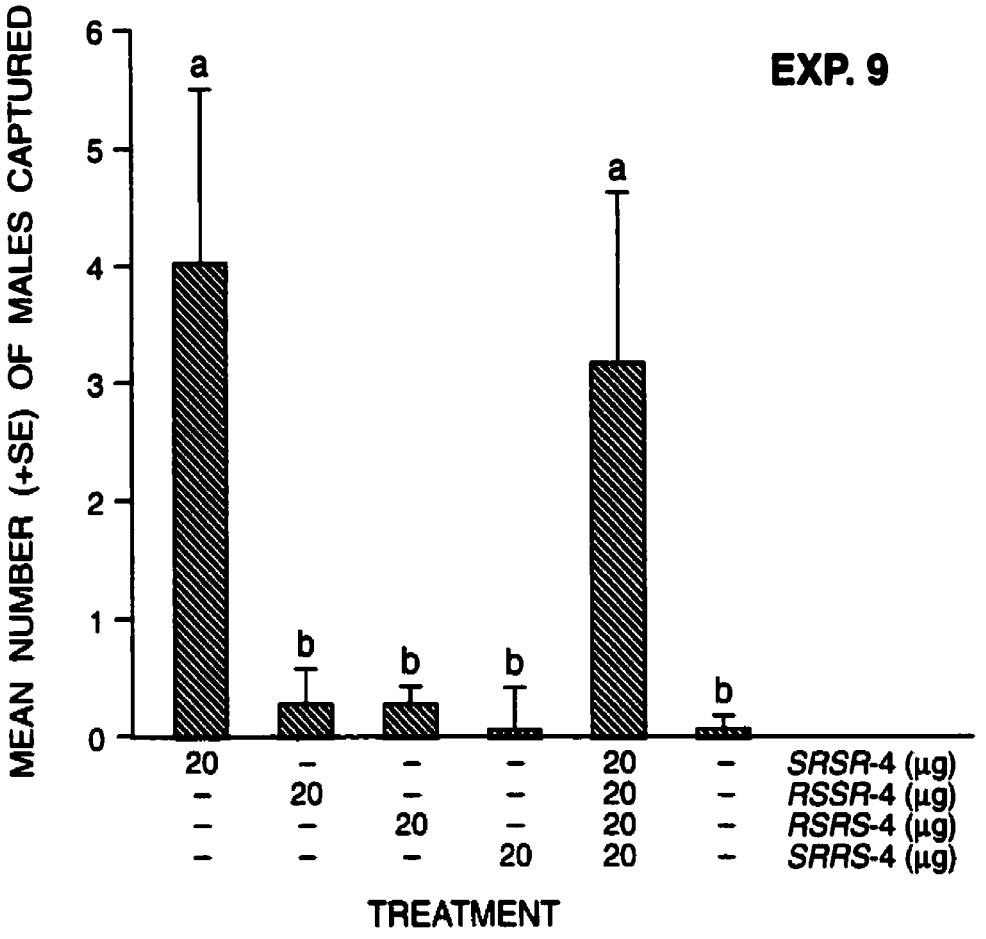

EXP. 9
MEAN NUMBER (+SE) OF MALES CAPTURED
6
5
4
3
2
1
0
a
b
b
b
a
b
20 – – – 20 – SRSR-4 (µg)
– 20 – – 20 – RSSR-4 (µg)
– – 20 – 20 – RSRS-4 (µg)
– – – 20 20 – SRRS-4 (µg)
TREATMENT

Fig. 11 Comparison of numbers of male *L. salicis* captured in Exps. 10-12 in sticky Delta traps baited with different mixtures of candidate pheromone components. For each experiment, bars with the same letter superscript are not significantly different, non parametric ANOVA followed by the Student Newman Keuls test, $p < 0.05$.

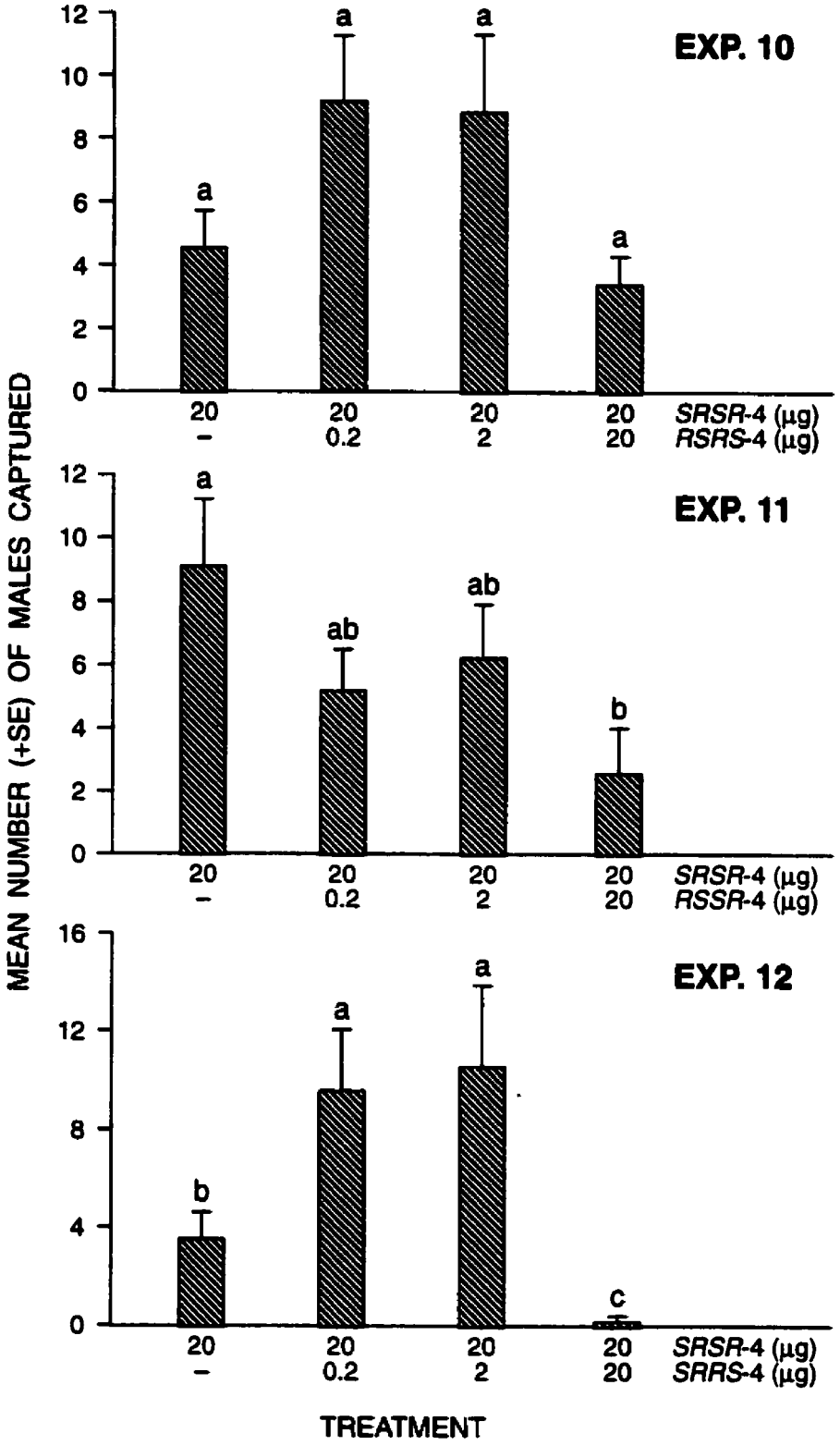

EXP. 10
EXP. 11
EXP. 12
MEAN NUMBER (+SE) OF MALES CAPTURED
TREATMENT
SRSR-4 (µg)
RSRS-4 (µg)
RSSR-4 (µg)
SRRS-4 (µg)
20 20 20 20
– 0.2 2 20

Fig. 12 Comparison of numbers of male *L. salicis* captured in Exp. 13 in sticky Delta traps baited with different mixtures of candidate pheromone components. For each experiment, bars with the same letter superscript are not significantly different, non parametric ANOVA followed by the Student Newman Keuls test, $p < 0.05$.

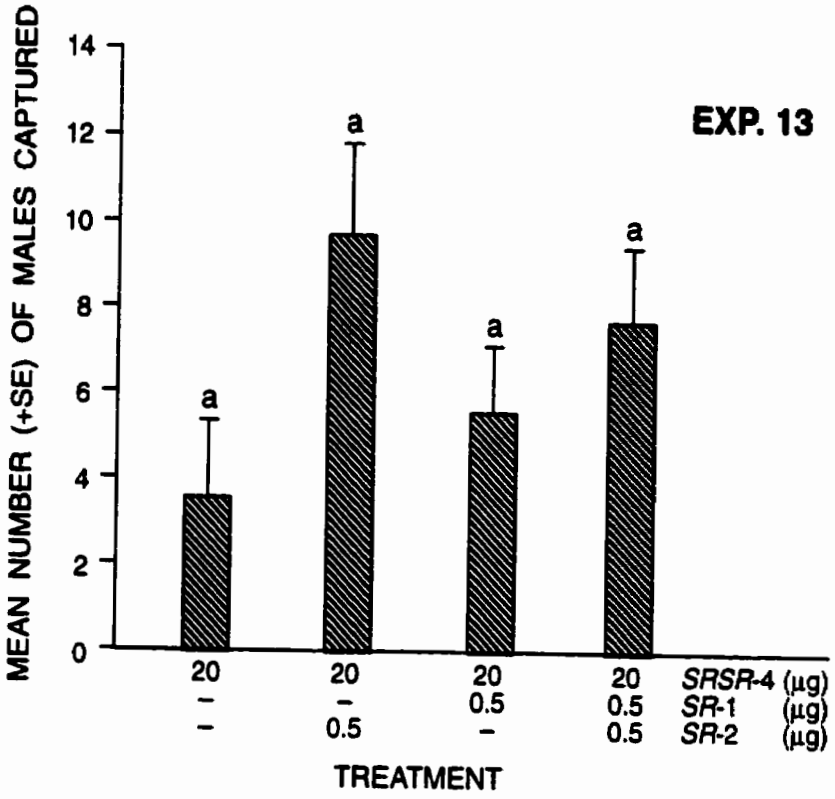

MEAN NUMBER (+SE) OF MALES CAPTURED
14
12
10
8
6
4
2
0
a
a
a
a
EXP. 13
20 20 20 20 SRSR-4 (µg)
– – 0.5 0.5 SR-1 (µg)
– 0.5 – 0.5 SR-2 (µg)
TREATMENT

Fig. 13 Comparison of numbers of male *L. salicis* captured in Exp. 14 in sticky Delta traps baited with different mixtures of candidate pheromone components. For each experiment, bars with the same letter superscript are not significantly different, non parametric ANOVA followed by the Student Newman Keuls test, $p < 0.05$.

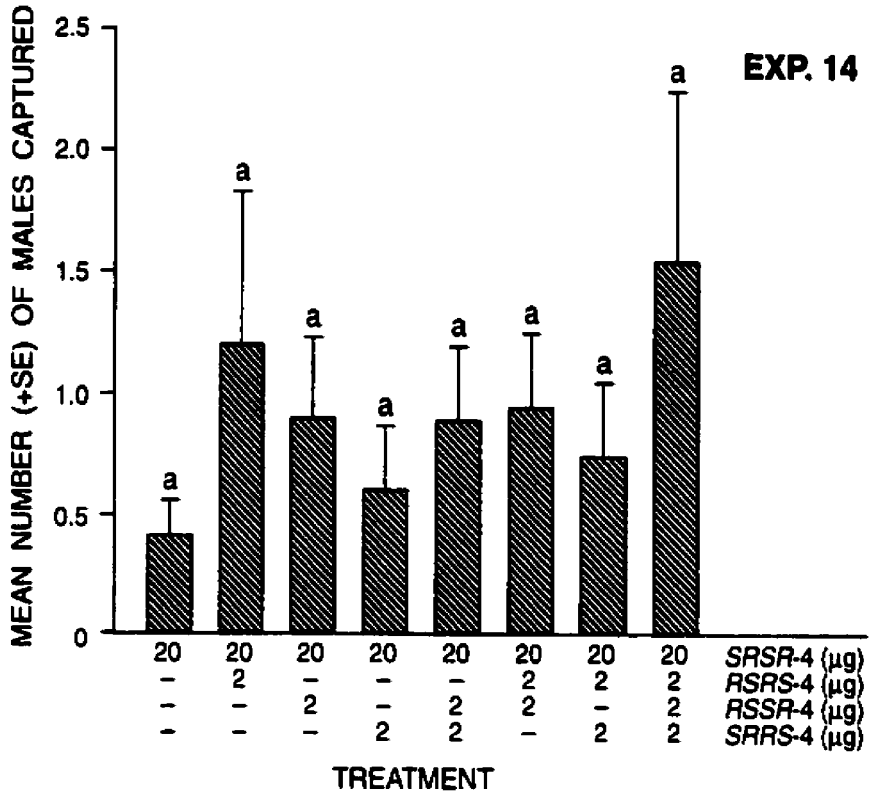
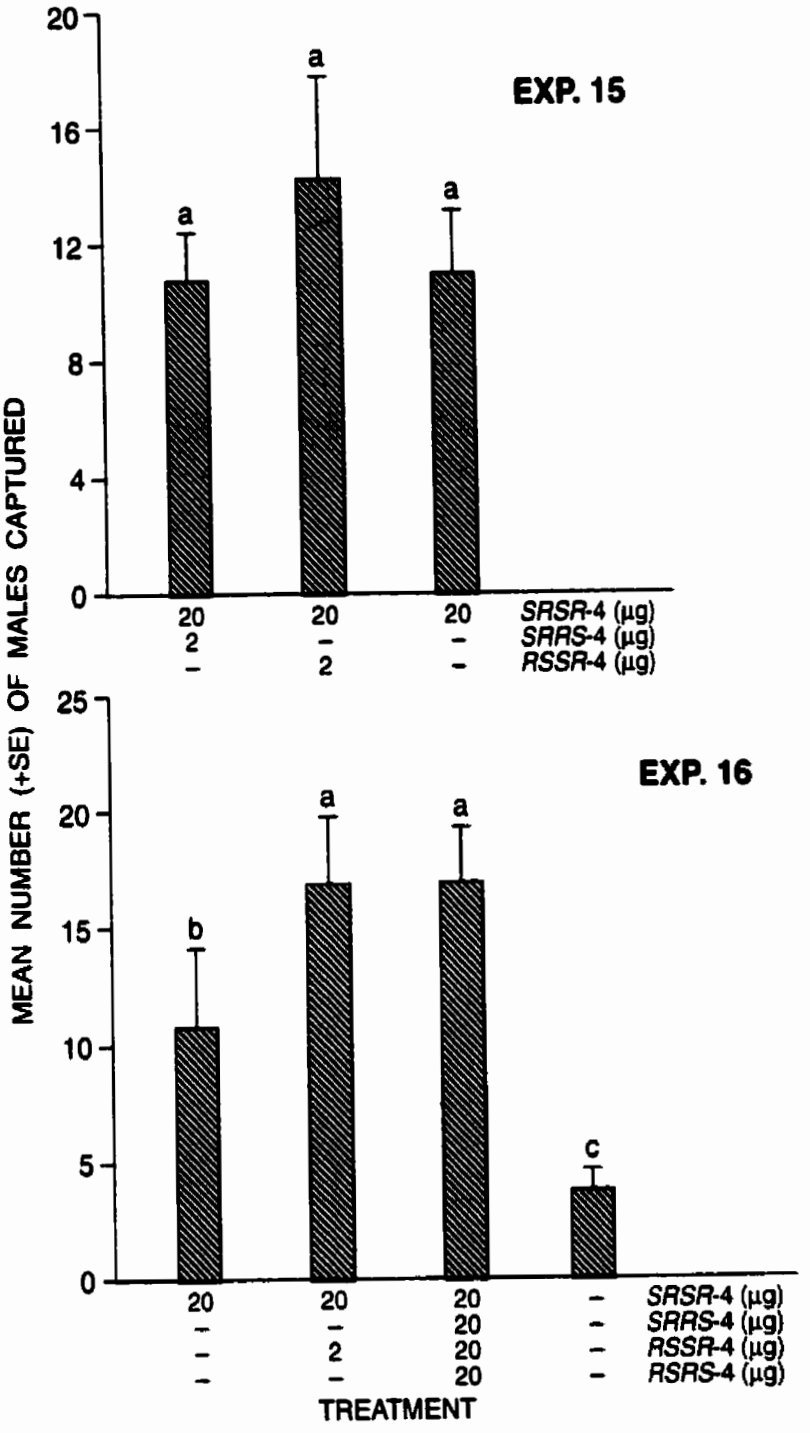

EXP. 14
MEAN NUMBER (+SE) OF MALES CAPTURED
2.5
2.0
1.5
1.0
0.5
0
a
a
a
a
a
a
a
a
20 20 20 20 20 20 20 20 SRSR-4 (µg)
– 2 – – – 2 2 2 RSRS-4 (µg)
– – 2 – 2 2 – 2 RSSR-4 (µg)
– – – 2 2 – 2 2 SRRS-4 (µg)
TREATMENT

Fig. 14 Comparison of numbers of male *L. salicis* captured in Exps. 15 and 16 in sticky Delta traps baited with different mixtures of candidate pheromone components. For each experiment, bars with the same letter superscript are not significantly different, non parametric ANOVA followed by the Student Newman Keuls test, $p < 0.05$.


EXP. 15
MEAN NUMBER (+SE) OF MALES CAPTURED
20
16
12
8
4
0
a
a
a
20 20 20 SRSR-4 (µg)
2 – – SRRS-4 (µg)
– 2 – RSSR-4 (µg)
EXP. 16
25
20
15
10
5
0
b
a
a
c
20 20 20 – SRSR-4 (µg)
– – 20 – SRRS-4 (µg)
– 2 20 – RSSR-4 (µg)
– – 20 – RSRS-4 (µg)
TREATMENT

4. Discussion

Laboratory analyses of pheromone gland extracts of female SM and field tests of synthetic candidate pheromone components revealed that (3*Z*)-*cis*-6,7-*cis*-9,10-diepoxy-heneicosene (**4**), termed leucomalure, is the major pheromone component of female SM. Evidence in support of this conclusion includes: 1) greatest abundance and EAD-activity of compound **4** in GC-EAD analyses (Fig. 4); 2) identical retention and mass spectrometric characteristics of synthetic and female SM-produced **4**; 3) comparable EAD-activity of synthetic and female SM-produced **4** when tested at equivalent quantities; and 4) captures of male SM in traps baited with synthetic **4** (Figs. 7-14).

Gas chromatographic analysis of synthetic stereoisomeric **4** revealed that stereoisomers separated in the form of two diastereomers (each containing two stereoisomers), and that female SM produced at least one stereoisomer of each diastereomer (**4a** and **4b** in Fig. 4). Of all four stereoselectively synthesized stereoisomers, ***SRSR*-4** elicited the strongest antennal response (Fig. 6), and by itself attracted male SM in Exp. 9 (Fig. 10), suggesting that it is the major SM pheromone component. Addition of ***SRRS*-4** to ***SRSR*-4** at a wide ratio, as found in pheromone gland extracts (Fig. 4), significantly enhanced attractiveness of ***SRSR*-4** (Fig. 11, Exp. 12), further suggesting that ***SRRS*-4** is a second pheromone component of female SM.

Synergism between pheromonal optical isomers has rarely been reported in the Lepidoptera. A 1:1 enantiomer ratio of (*Z*6,*Z*9)-*cis*-3,4-epoxy-heptadecadiene attracted 3 times more males of the geometrid moth, *Epelis truncataria* (Walker) than did either enantiomer alone (Millar *et al.* 1990). Similarly, a 1:1 and more strongly a 1:4 ratio of

(6*R*,7*S*,*Z*3,*Z*9)- and (6*S*,7*R*,*Z*3,*Z*9)-*cis*-6,7-epoxy-nonadecadiene attracted males of the noctuid moth, *Bleptina caradrinalis* Guenée (Millar *et al.* 1991). Finally, attraction of male pink gypsy moth, *Lymantria mathura* Moore, in Japan required the same 1:4 ratio of (9*R*,10*S*,*Z*3,*Z*6)-*cis*-9,10-epoxy-nonadecadiene and (9*S*,10*R*,*Z*3,*Z*6)-*cis*-9,10-epoxy-nonadecadiene as produced by conspecific females (Gries *et al.* 1999a). The absolute configuration and 1:4 ratio of *L. mathura*-produced pheromone enantiomers could be determined through GC-MS and GC-EAD analyses of pheromone extract and authentic standards on a custom-made column coated with a 1:1 mixture of heptakis-(2,6-di-*O*-methyl-3-*O*-pentyl)-*β*-cyclodextrin and OV-1701 (König *et al.* 1992; Pietruska *et al.* 1992). An equivalent chiral GC or HPLC column that separates the four stereoisomers of **4** needs to be developed to conclusively prove that ***SRSR*-4** and ***SRRS*-4** constitute the absolute configuration of leucomalure stereoisomers produced by female SM.

Although present in pheromone gland extracts and EAD-active, the monoepoxides (*Z*3,*Z*9)-*cis*-6,7-epoxy-heneicosadiene (**1**) and (*Z*3,*Z*6)-*cis*-9,10-epoxy-heneicosadiene (**2**) are not pheromone components of female SM. When tested together with ***SRSR*-4**, SR-enantiomers of **1** and **2** combined were behaviourally benign (Fig. 12, Exp. 13), whereas RS-enantiomers even reduced trap captures (Fig. 8, Exps. 3, 5). Both ***RS*-1** and ***RS*-2** may be pheromone components in other *Leucoma* spp., but may have lost pheromonal activity in *L. salicis*. 2-Methyl-(*Z*)7-octadecene (2me-*Z*7-18Hy), for example, is a common EAD-active compound in pheromone glands of female *Lymantria* spp., including *L. serva*, *L. monacha* (nun moth), *L. fumida*, and *L. dispar*. While 2-me-*Z*7-18Hy is the major pheromone component of *L. serva* [G. + R. Gries (Simon Fraser University) and P.

Schaefer (Beneficial Insects Introduction Research Lab, Newark, DE, USA), pers. comm.], and a synergistic pheromone component of *L. monacha* (Grant *et al.* 1996; Gries *et al.* 1996) and *L. fumida* (Schaefer *et al.* 1999), it inhibits response of male *L. dispar* to the major pheromone component (+)-disparlure [(7*R*,8*S*)-*cis*-7,8-epoxy-2-methyloctadecane] (Cardé *et al.* 1973). Analogously, EAD-active compounds **1** and **2** (Fig. 4) in *L. salicis* may be pheromone components of a *Leucoma* congener that inhibits response of male *L. salicis* to ***SRSR*-4**. Alternatively, monoepoxides **1** and **2** may represent biosynthetic precursors to ***SRSR*-4** and/or ***SRRS*-4**.

With two sympatric *Leucoma* congeners (*L. salicis* and *L. candida*) in Japan, and possibly 6 congeners in Hong Kong [Roger Kendrick (Dept. of Ecology & Biodiversity, University of Hong Kong, Hong Kong), pers. comm.], stereospecificity in the production of and/or response to leucomalure may have evolved to contribute to species-specific sexual communication. Assuming that monoepoxides **1** and **2** and diepoxide **4** are pheromone components in the genus *Leucoma* (see above), chirality of their mono- and diepoxides offers a wide variety of possible pheromone blends. There are four stereoisomers of **4** (leucomalure) and two enantiomers each of compounds **1** and **2**. Discounting ratio and inhibitory effects of optical isomers (Fig. 8, Exp. 5), and assuming that one stereoisomer of leucomalure invariably represents the major pheromone component, there are 54 possible pheromone blends with either one, two or three components. If speciation in the Lepidoptera were linked to the evolution of species-specific pheromone blends (Bell and Cardé 1984), species that produce chiral pheromone components may give rise to new species more readily than those that produce nonchiral

pheromone components. If this theory were true, it may explain the species diversity particularly of geometrid and lymantriid moths which typically produce chiral pheromone components.

In keeping with previous conclusions (Roelofs and Comeau 1969; Roelofs and Brown 1982), taxonomic classification of insects should be based on diverse criteria, such as morphometrics, molecular comparisons, ecological analyses and pheromone biology. The identification of epoxy pheromones in *Lymantria* (Bierl *et al.* 1970; Gries *et al.* 1996, 1999b) ketones in *Orgyia* (Smith *et al.* 1975; Gries *et al.* 1997a; 1999c; unpublished data; Wei 1999), esters in *Euproctis* (Tan *et al.* 1984; Leonhardt *et al.* 1991; Gries *et al.* unpublished data) and a diepoxide in *Leucoma* (Gries *et al.* 1997b) is consistent with the taxonomic placement of lymantriid moths into their respective genera. Whether diepoxy pheromones are characteristic of *Leucoma* spp. will be established as pheromone blends of congeners are analyzed.

For future development of pheromone-based monitoring and/or detection of SM populations, it was essential to determine an economically viable lure. Use of stereoisomeric **4**, instead of stereospecific ***SRSR*-4**, as trap bait would help alleviate expenses associated with stereospecific syntheses. However, tests of stereoisomeric mixtures generated inconsistent results that are not easily explained. For example, diastereomers **4a** plus **4b** were significantly less attractive than **4b** in Exp. 1 (Fig. 7), but were as attractive as **4b** in Exp. 2 (Fig. 7). Similarly, ***SRRS*-4** significantly reduced attractiveness of the major pheromone component ***SRSR*-4** when tested in binary (1:1) combination, but did not inhibit trap captures when tested in quaternary (1:1:1:1)

combination with ***RSSR*-4**, ***RSRS*-4** and ***SRSR*-4** (Fig. 10, Exp. 9). These conflicting data could have resulted from: 1) genetically related subsets of male SM, exhibiting diverging preference for different pheromone blends of optical isomers, as demonstrated in the leaf-mining moth *Eriocrania semipurpurella* (Löfstedt *et al.* 1998); 2) males of two *Leucoma* spp. expressing contrasting pheromonal preference; and/or 3) release dynamics of dispensers being affected by the overall amount of solvent they received. While genetic relatedness of captured moths was not investigated, analyses of genitalia from 32 male moths removed from light traps and traps with different pheromone baits, disclosed no significant differences between specimens, suggesting that males of only one species (SM) responded to pheromone-baited traps. A large amount of solvent furthers homogenous impregnation of dispensers with test chemicals, thus conceivably improving release dynamics of candidate pheromone components. The overall amount of solvent pipetted onto dispensers may have differed between Exps. 1 and 2, thereby contributing to inconsistent results.

Even if a mixture of stereoisomers (at a 1:1:1:1 ratio) of **4** were a suboptimal trap lure, it consistently attracted male moths (Fig. 7, Exp. 1, 2; Fig. 8, Exp. 4; Fig. 14, Exp. 16). For pheromone-based monitoring of SM, suboptimal trap lures may even be desirable. They may prevent immigration of males from adjacent forest stands, thereby improving the likelihood of obtaining a realistic correlation of captured males with the actual population density in the sampling area (Sweeney *et al.* 1990; Faccioli *et al.* 1993). For development of pheromone-based detection surveys of SM, which rely on optimal

attraction of male moths, further testing of ***SRSR*-4**, ***SRSR*-4** plus ***SRRS*-4** and stereoisomeric **4** is required to determine the most attractive bait.

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