INTERSPECIFIC RESPONSES OF DIPRIONID SAWFLIES TO NATURAL AND OPTICALLY ACTIVE AYNTHETIC PHEROMONES

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ABSTRACT

INTERSPECIFIC RESPONSES OF DIPRIONID SAWFLIES TO NATURAL AND OPTICALLY ACTIVE SYNTHETIC PHEROMONES

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In this study, the responses of diprionid sawflies to mixtures of optical isomers of 3,7-dimethylpentadecan-2-yl acetate or proprionate were studied both in the field and by electroantennogram (EAG). Various aspects of behavioral response of male sawflies to sex pheromones were studied in the field and greenhouse. The nature of chirality of the natural pheromones from the females of different species was determined by gas liquid chromatography (GLC).

The field studies show that (1) all the fall-flying species respond mainly to the acetate while the spring/early summer-flying species respond either to acetate (designated as "A") or proprionate (designated as "P"); (2) the (2S,3S,7S)-3,7-dimethylpentadecan-2-yl acetate (2S,3S,7S-A) or proprionate (2S,3S,7S-P) isomer was active against all Neodiprion species, but 2S,3R,7R-P was the active isomer for Diprion similis and Gilpinia frutetorum (Fabricius); (3) the effectiveness of the 2S,3S,7S isomer was significantly improved by addition of varying concentrations of 2S,3R,7R or 2S,3R,7S isomer. Only N. nanulus nanulus and N. lecontei

appeared not to respond synergistically to mixtures of isomers.

Through EAG analysis, synergistic interaction was found among the optical isomers only when submicrogram amounts of the compounds were used to stimulate the male antennae. The synergists appeared to increase the maximum response value in N. sertifer, N. virginianus, and N. pinetum.

By using capillary GLC analysis on samples purified on TLC and preparative GLC it was found that the erythro (i.e., 2S,3S or 2R,3R) isomers can be separated from the threo (i.e., 2S,3R or 2R,3S) isomers, with the threo isomers having longer retention time.

Analysis of the natural pheromone showed that only 2S,3S,7S isomers were detectable in N. lecontei, N. nanulus nanulus, and N. sertifer. However, in D. similis and N. pinetum two isomers 2S,3S,7S and 2S,3R,7R were detectable. The same set of isomers is believed to be present in N. pratti banksianae. Two peaks corresponding to 2S,3S,7S and 2S,3R,7S are indicated in N. virginianus. Natural pheromones of two other species, N. rugifrons and N. nigroscutum appeared to have these isomers. There were variations in the amount of pheromone extracted from different species. Behavioral response to pheromone source is similar in D. similis, N. virginianus and N. sertifer. The optimum height at which males respond to pheromone on the host plant is about 3.5 m.

To my children:

Bolarinwa Tiwalola, Olubunmi Ikeolape and
Olufunmilola Eniola

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CHAPTER ONE

Field Evaluation of Chiral Isomers of the Sex Pheromone of the European Pine Sawfly, *Neodiprion sertifer*

INTRODUCTION

A female-produced sex pheromone component of the red-headed pine sawfly *Neodiprion lecontei* (Fitch), has been isolated and identified as the acetate of 3,7-dimethylpentadecan-2-01 (Jewett et al. 1976). They observed, by using nuclear resonance spectroscopy, that the pheromone has an erythro configuration of the adjacent optically active carbons (i.e., carbons 2 and 3). Electroantennographic studies have shown that males of many species of diprionid sawflies are responsive to either the acetate or proprionate of diprionol isolated from *Neodiprion sertifer* (Geoffroy) or *N. lecontei* females. However, the responses of *Diprion similis* (Hartig) and *Gilpinia frutetorum* (Fabricius) males appeared different from those belonging to the *Neodiprion* genus (Jewett et al. 1976). Matsumura et al. (1979) found that the chiral arrangement of the pheromone for *N. lecontei* were 2S,3S. The chirality of all the carbons of the sex attractant of *Neodiprion pinetum* (Norton) was also established as the acetate of 2S,3S,7S-diprionol. This was the only active erythro isomer for that species (Kraemer et al. 1979). In

the present study, we have extended our work to N. sertifer, a common species in the Great Lakes Region.

MATERIALS AND METHODS

Field Tests

All chemicals were dissolved in 1 ml. of n-hexane and stored in sealed glass ampules. In the field, the ampule was broken and its contents were poured into a 2.5 cm. cotton wick held with a pair of forceps. The wick was then attached to the inside roof of a Pherocon II trap (Zoecon Corp., Palo Alto, California). To avoid contamination, the forceps were washed with alcohol after each use. All the field trapping of N. sertifer took place in Rose Lake Wildlife Research Station, Sec. 33, Bath and at Arboretum, Lansing, Ingham County, Michigan. Larval colonies were collected at these locations on scotch pine, Pinus sylvestris L. Rose Lake, where the majority of the studies were carried out, covered an area of about 8 square kilometers. Stands of scotch pine were mostly wind breaks separated by grasses, hardwoods and patches of other pines, notably, red pine Pinus resinosa Ait and white pine Pinus strobus L.

Synthetic Pheromone

The acetate of (2S,3S,7S)-3,7-dimethylpentadecan-2-01 (2S,3S,7S-A) was obtained from Dr. Mori (Mori et al. 1978). From the same chemist was obtained 2R,3R,7R-A from which we synthesized 2R/S,3R,7R-A by the method described by Kikukawa et al. (1982a). The 2S,3R,7R/S-A was obtained from Dr. Tai

(Tai et al. 1978). NMR analyses of these compounds showed that each erythro isomer was free from contaminations by the other. On the other hand, 2S,3R,7R/S-A contained approximately 5% of 3S contamination. The 2S,3R,7R-A; 2S,3R,7S-A and one isomer synthesized through a Grignard reaction designated 2S,3R,7R-AG were obtained from Dr. Tai's laboratory (Kikukawa et al. 1982b).

Partial Purifications of Natural Pheromone from KOH-Treated Body

Two hundred N. sertifer virgin females were immersed in ether overnight. The solvent was decanted and the insects were washed two more times with the same solvent. The solvent was evaporated by a rotary evaporator under reduced pressure. The residue was hydrolyzed by the addition of 1 g KOH in 50 ml. methanol. This mixture was refluxed for 5 hrs. The methanol was removed by rotary evaporation. The organic material was separated in a separatory funnel (hexane-water). The hexane fraction was dried over Na_2SO_4 to give the crude alcohol fraction. The hexane was removed by rotary evaporation and the residue was esterified by adding 2 ml. pyridine and 1 g. of acetic anhydride. The reaction was left overnight, in the dark and at room temperature. The crude ester fraction thus obtained was purified by TLC (activated silica gel $HF_{254+366}$ with hexane-ether (4:1) mobile phase). The most field-active fractions (R_f = 0.65 - 0.39) was further purified by a second TLC system using n-hexane first and benzene second as the mobile phase.

Purification of 2S,3S,7S-A by
Gas Liquid Chromatography (GLC)

The original 2S,3S,7S-A from Mori et al. (1978) was subjected to GLC with a carbowax 20 M (4m) column on chromosob AW. In this system, erythro isomers come slightly ahead of threo isomers. To achieve a partial purification, the front, center, and tail parts of the peak due to 2S,3S,7S-A were separately collected (Figure 1-1). These fractions were separately biossayed on the field.

RESULTS

Kikukawa et al. (1983) tested many isomers of diprionol and established that (1) males of N. sertifer respond best to 2S,3S,7S-A (2) TLC-charcoal purification of 2S,3S,7S-A improves field effectiveness (3) such purifications alone could not bring the level of field effectiveness of the synthetic pheromone to that of natural pheromone and (4) female N. sertifer has about 10 ng of pheromone shortly after adult emergence.

The original 2S,3S,7S-A preparation (Mori et al. 1978) when separated into fractions through GLC (Figure 1-1) and field tested, the separated fractions were relatively more effective than the original 2S,3S,7S-A (Table 1-1). The central portion was most active, as 0.5 ug of this preparation attracted more males than 10 ug of the original 2S,3S,7S-A. The front and tail portions of the same concentration as the central were less active, but those preparations attracted similar numbers of males as did 10 ng of the

Figure 1-1. GLC fractionation of 2S,3S,7S-A peak into I front, II center, and III back fractions on carbowax 20M column.

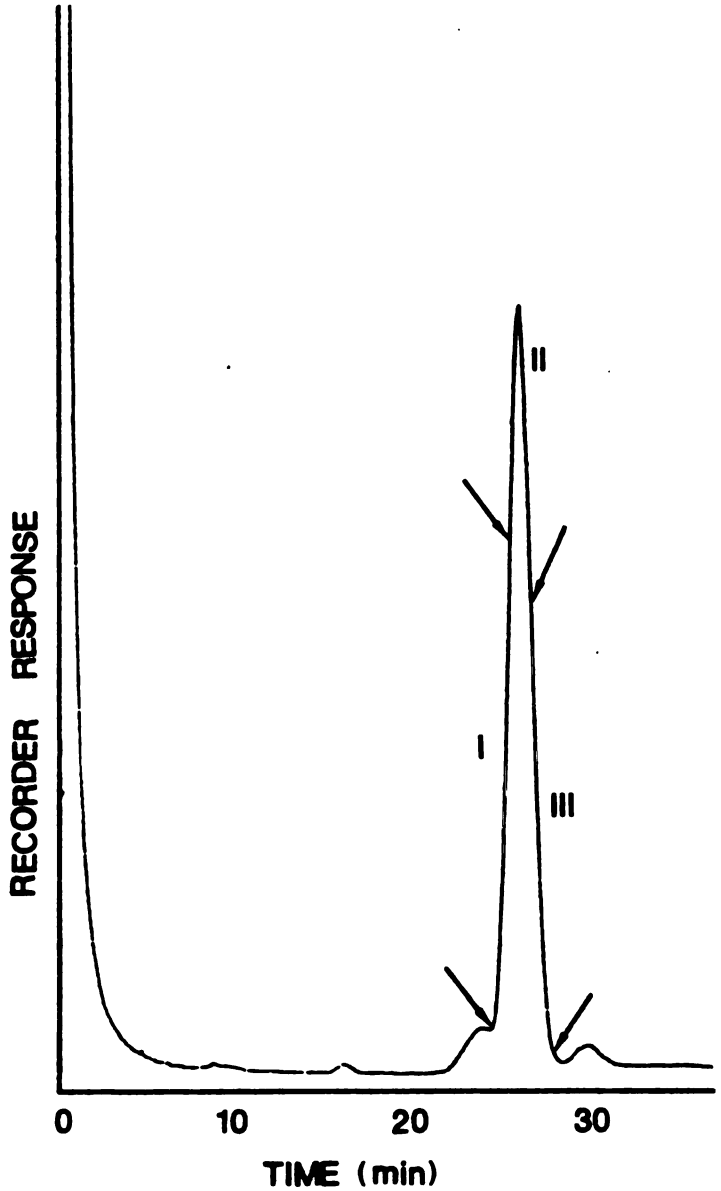
RECORDER RESPONSE
I
II
III
0
10
20
30
TIME (min)

Table 1-1. Comparison of N. sertifer trap catch between original 2S,3S,7S-A preparations and that purified by GLC, September 19 to October 26, 1981 at Rose Lake, Lansing, Michigan.

Preparations	Amount (ug/trap)	Mean/Trap[c] ± S.E.
2S,3S,7S-A original[a]	10.0	5.0 ± 0.8
2S,3S,7S-A GLC[b] peak front	0.5	3.7 ± 0.8
2S,3S,7S-A GLC[b] peak center	0.5	7.7 ± 1.4
2S,3S,7S-A GLC[b] peak back	0.5	3.7 ± 1.0

[a] 2S,3S,7S-A contaminated by impurities.

[b] The front, center and back portions of the 2S,3S,7S-A peak were collected separately as shown in Figure 1.

[c] Means of 3 replicates. Analysis of variance insignificant.

original 2S,3S,7S-A. Despite improved performance of this GLC purified 2S,3S,7S-A, it is clear from this table that purification alone cannot drastically increase the effectiveness of 2S,3S,7S-A over the original 2S,3S,7S-A.

In a series of experiments summarized in Table 1-2, I have explored a possibility that the presence of a trace amount of a threo isomer in the purified 2S,3S,7S-A preparations could increase the effectiveness of the trap catch. The experiment was conducted in Fall 1981, when a new preparation 2S,3R,7R-A became available. It was shown by three independent tests that the presence of 0.01-0.003 ug of 2S,3R,7R-A in 5 ug of 2S,3S,7S-A was advantageous in terms of effective catching of N. sertifer males. In the next test the synergistic effectiveness of several threo preparations containing 2S,3R,7R-A component was compared at a fixed ratio of 5:0.003 ug. Among them 2S,3R,7R-A and 2R/S,3R,7R-A were most synergistic followed by 2S,3R,7R/S-A and 2R/S,3R,7S-A. In 1982, four threo isomers were mixed with 2S,3S,7S-A and field tested. The results (Table 1-3) showed that 2S,3S,7S-A/2S,3R,7R/S-A, 2S,3S,7S-A/2S,3R,7S-A and 2S,3S,7S-A/2S,3R,7R-AG mixtures at blend ratio 5:0.003 were more effective than 2S,3S,7S-A alone. At this ratio, the importance of chirality at 7-carbon of the synergists were tested (Table 1-4). The result indicates that the chirality of the 7th carbon is not important.

In view of the high effectiveness of these mixtures, an attempt was made to compare the potency of naturally

Table 1-2. Stimulatory effect of trace amount of 2S,3S,7R-A on effectiveness of trap catch by 2S,3S,7S-A. The test was conducted at Rose Lake, Michigan, September 19 to November 9, 1981.

2S,3S,7S-A & 2S,3R,7R-A (ug/trap)	Series A[a] 9/19 - 11/9 (x ± SE)	Series B[a] 9/27 - 11/9 (x ± SE)	Series C[a] 10/9 - 11/9 (x ± SE)
5/0	c	5.0 ± 1.2	2.7 ± 2.2
5/0.005	c	4.7 ± 0.9	2.3 ± 0.9
5/0.001	c	4.0 ± 1.0	5.0 ± 0.6
5/0.003	87.7 ± 39.1[b]	13.7 ± 1.5[b]	7.0 ± 3.2[b]
5/0.01	43.7 ± 16.8[b]	24.3 ± 13.9[b]	12.7 ± 4.8[b]
5/0.03	7.7 ± 4.7	4.7 ± 2.7	3.0 ± 1.0
5/0.1	22.3 ± 4.3	14.3 ± 5.0[b]	2.7 ± 0.9
5/0.3	1.7 ± 0.3	1.3 ± 0.3	0.0 ± 0.0
5/1	0.7 ± 0.7	0.7 ± 0.7	0.7 ± 0.7

[a] The result of catches by three sets of traps for each concentration.

[b] All means significantly different from others by Duncan's multiple range test at 5% level.

[c] These traps were inadvertently left out of Series A. During the same period and at the same locations, a trap baited with 20 ug of 2S,3S,7S-A alone caught 37 males.

Table 1-3. Stimulatory effect of different threo isomers on 2S,3S,7S-A in attracting male N. sertifer on the field. Test conducted at Rose Lake, Michigan, September 25 to October 19, 1982.

Amount (ug/trap)	2S,3S,7S-A/[a] 2S,3R,7R/S-A	2S,3S,7S-A/[a] 2S,3R,7S-A	2S,3S,7S-A/[a] 2S,3R,7R-AG	2S,3S,7S/[a] 2R/S,3R,7R-A
5/0	3	3	10	6
5/0.003	14	7	13	2
5/0.03	5	2	2	2
5/0.3	0	0	0	1
5/1	0	0	0	0

[a] The total by three sets of traps for each concentration.

Table 1-4. Importance of configurations at C-7 of threo isomers on effectiveness of trap catch by 2S,3S,7S-A. The test was conducted at Rose Lake 10/5 - 11/5/81 and 9/22 - 10/19/82.

Pheromone	Series A[a] 10/5 - 11/5/81 (x ± SE)[b]	Series B[a] 9/22 - 10/19/82 (x ± SE)[b]
2S,3S,7S-A / 2S,3R,7R/S-A	4.0 ± 1.2	4.6 ± 1.2
2S,3S,7S-A / 2 S/R,3R,7R-A	5.6 ± 1.3	0.7 ± 0.6
2S,3S,7S-A / 2S,3R,7S-A	2.6 ± 0.9	2.3 ± 0.8
2S,3S,7S-A / 2S,3R,7R-A	4.3 ± 1.1	4.3 ± 1.3

[a] The result of catches by three sets of traps for each treatement.

[b] Analysis of variance insignificant and no significant difference among the means by Duncan's multiple range test.

occurring diprionol which was purified as earlier described. As a result of a comparison test (Table 1-5), it was established that the synergized preparation containing 2S,3S,7S-A and a trace amount of 2S,3R,7R-A at a ratio of 5:0.003 was roughly as active as the naturally occurring pheromone preparation.

DISCUSSION

This work demonstrates that a trace addition of threo acetate containing 2S,3R,7R-A configurations certainly increased the field effectiveness of 2S,3S,7S-A. A question must be raised as to the optical purity of these synthetic epimers themselves. At this stage there is no convenient method to measure the optical purity of each active carbon in the final synthetic product per se. NMR spectroscopic analysis, however, gives information as to the diasteroemetric arrangement on 2-carbon and 3-carbon (Jewett et al. 1976). As judged by this approach, none of the erythro isomers provided by Dr. Mori's group is contaminated with threo isomers. The limit of detection is approximately 1%. On the other hand, the threo compounds 2S,3R,7R/S-A, synthesized via Wittig reaction (Tai et al. 1978) contained approximately 5% of epimerization product at carbon 3. The 2S,3R,7R-A, 2S,3R,7R-AG and 2S,3R,7S-A were synthesized through Grignard reaction and are known to contain no optical impurity (Kikukawa et al. 1982b). Meanwhile, it appears from our data that the addition of 3R to 3S of 2S,3S,7S-A is

Table 1-5. Comparison of field effectiveness of natural and synthetic pheromone of N. sertifer; Rose Lake, Michigan, October 9 - November 9, 1981.

Pheromone	Amount (ng/trap)	Replicates A	B	C	Total	Mean/Trap
Synthetic Mixture						
2S,3S,7S-A/ 2S,3R,7R-A	30.0	0	0	4	4	1.3
	10.0	0	7	5	12	4.0[a]
	3.0	2	3	10	15	5.0[a]
	1.0	0	3	5	8	2.7
Natural Pheromone (acetate)	3.0 (= 0.3 FE)	4	6	15	25	8.3[a]
	1.0 (= 0.1 FE)	1	0	8	9	3.0[a]
	0.3 (= 0.03 FE)	0	0	0	0	0.0

[a] Means significantly different by Duncan's multiple range test at 5% level from other values.

the crucial point in the case of field attraction of N. sertifer males, as mixing of a small amount (0.06%) of 2S,3R,7R/S-A, 2R/S,3R,7R-A, 2S,3R,7R-A and 2S,3R,7S-A gave an equally stimulatory effect.

In conclusion, the current work established that 2S,3S,7S-A is the chiral configuration most preferred by males of N. sertifer. The same configuration is reported for males of N. pinetum (Kraemer et al. 1979) and N. lecontei (Kraemer et al. 1981). The most intriguing aspect of our finding is that small amounts of 2S,3R,7R-A stimulates the field effectiveness of the 2S,3S,7S-A preparation. The significance of this is rather early to predict, but this signals that sex pheromones of diprionid sawflies might involve mixtures of optical isomers.

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CHAPTER TWO

Response of Male Red Pine Sawfly, *Neodiprion nanulus nanulus* (Hymenoptera: Diprionidae) to Natural and Mixtures of Optically Active Synthetic Sex Pheromone 3,7-dimethylpentadecan-2-yl acetate

INTRODUCTION

The red pine sawfly, *Neodiprion nanulus nanulus* Schedl is an important native defoliator of the red pine (*Pinus resinosa* Ait), jack pine (*P. banksiana* Lamb.) and lodgepole pine (*P. contorta* Dougl.). The range of the latter two host species overlap, making *N. nanulus nanulus* a cosmopolitan species throughout North America. Red pine is the predominant conifer being planted in the Great Lakes region. According to one reported case, one complete defoliation of red pine caused a 21% reduction in height growth. Two successive defoliations reduced as much as 64% (Kapler and Benjamin, 1960). Yet such defoliations are uncommon in Michigan where this study was done.

Monitoring tools for this pest at the present consist of scouting for the larval stage and estimating the injury level. Jewett et al. (1976) isolated and identified diprionid sawfly sex pheromone as 3,7-dimethylpentadecan-2-ol (diprionol), the acetate or proprionate ester of which was active in attracting males on the field. Kraemer et al. (1983) reported that the optical isomer (2S,3S,7S)-3,7-

dimethylpentadecan-2-yl acetate (2S,3S,7S-A) and proprionate (2S,3S,7S-P) as well as (2S,3S,7R)-3,7-dimethylpentadecan-2-yl acetate (2S,3S,7R-A) were highly attractive, but 2S,3S,7S-A was significantly superior to others at lower concentrations. The 2S,3S,7S-A thus appeared to be the major pheromone in N. nanulus nanulus as in N. pinetum (Kraemer et al. 1979), N. lecontei (Matsumura et al. 1979; Kraemer et al. 1981). However, in another sympatric species N. sertifer where 2S,3S,7S-A is the major pheromone, combination with small amount of (2S,3R,7R)-3,7-dimethylpentadecan-2-yl acetate (2S,3R,7R-A) stimulates the field effectiveness of the 2S,3S,7S-A preparation (Kikukawa, et al. 1983). Here we report results of field studies conducted to examine if such interaction of optical isomers extends to N. nanulus nanulus.

MATERIALS AND METHODS

Field Tests

The red pine sawfly trapping was carried out in Vogel Center, Sec. 32, Aetna Loop, Misaukee Co., Michigan. A few trappings were also done at two other locations-Sec. 7, Manistee River, Garfield Township, Kalkaska County, and at Sec. 36, Higgins Lake, Old I-27, Beaver Township in Roscommon Co., Michigan. The main experimental area, Vogel Center, is about 1.2 Kilometer square. The red pine stand is surrounded by farm lands such that within 13 Kilometers radius only scattered plots of red pine could be found.

Ground cover is mostly grasses. Outbreak conditions in 1981 left many pine trees completely defoliated. In all the test areas, the characteristic injury of larvae consuming just three-fourths of the needles leaving stubs 1.9-0.6 cm. long (Wilson, 1970) were clearly evident. On several occasions in late spring and early summer 1981, 1982, and 1983, larval colonies of N. n. nanulus were collected from these sites.

Pherocon II^{R} traps were used in all tests. Traps were hung at a height of 2.0 to 2.5 m. and spaced at no closer than 15 m. within and between trap rows. Observations were made weekly or at least twice per month during which the traps are rotated randomly within and between rows. Pheromones were prepared in 1 ml. hexane solvent and sealed in glass ampules before taken to the field. At the test site, the contents of the ampules are dispensed onto a 4.0-5.0 cm. dental cotton roll which is positioned at the center on one side inside the Pherocon trap. The randomized block experimental design was used in all tests with at least three replicates of each treatment. Data were analyzed by analysis of variance, and the means compared by Duncan's multiple range test at 5% level.

Laboratory Rearing of Larvae and Pupae

The female insect from which pheromone was extracted were reared from larvae and pupae collected from the test sites. Larvae were reared outdoors under shade near the greenhouse. The larvae were fed fresh host twigs dipped in

water to maintain turgidity. At pupation, the last larval instar made cocoons under the heap of fecal droppings inside the cage. Such cocoons, as well as field collected cocoons, were separated by sex according to size, the larger cocoons being females. Individual cocoons were then placed in plastic capsules and left outside in cages under shade till adult emergence. Individual virgin females were placed in 3x5 cm. vials with strips of teflon simulating pine needles for a few days before being frozen till use.

Synthetic Pheromones

Two groups of chemists from Japan synthesized and supplied us with the optical isomers used in this study. Mori's group synthesized the 2S,3S,7S-A and the corresponding alcohol (Mori et al. 1978). These materials contained some unknown percent of optical impurities, but is believed to be less than 1%, the approximate limit of detection by PMR assay. Tai's group synthesized the 2S,3R,7R/S-A with a known 5% erythro contamination (Tai et al. 1978). The same group later synthesized 2S,3S,7S-A (unpublished) and 2S,3R,7R-A and 2S,3R,7R-AG (Kikukawa et al. 1982). In this instance the 2S,3S,7S-A and 2S,3R,7R-A contained less than 1% optical impurities but 2S,3R,7R-AG synthesized through Grignard was greater than 99.9% optically pure.

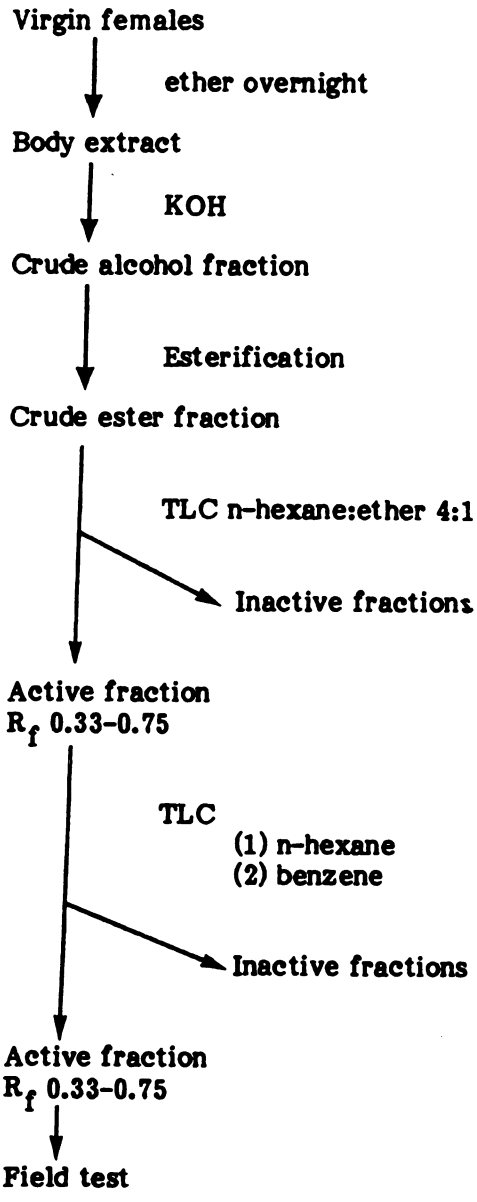
Extraction of Natural Pheromone for Field Tests

Virgin females were immersed in ethyl ether overnight. After decanting the solvent, they were rinsed with ether two

times and combined. The combined solvent was evaporated in a rotary evaporator and the residue was hydrolyzed by the addition of 5-10 ml. of 2% KOH in methanol. This mixture was refluxed for 5 hrs. The methanol was then removed by rotary evaporation. The residue was picked up in hexane and washed with water. The hexane fraction was dried over Na_2SO_4 to give the crude alcohol fraction. The hexane was removed by rotary evaporation and the residue was esterified into acetate by adding 2 ml. pyridine and 1 gm. of acetic anhydride and refluxing for 5 hrs. The reaction mixture was then dissolved in n-hexane and extracted with water. The hexane phase was dried over $MgSO_4$ and evaporated.

The residue was spotted on preparative TLC [silica gel 60G $HF_{254+366}$ (MC/B Manufacturing Chemists Co., Cincinnati, Ohio); 2 mm. thick] which was first developed to 1 cm. of the top edge in an acetone tank to remove impurities. The plates were developed until 15 cm. from the origin with a mixture of hexane and ether (4:1). The ester zone corresponding to R_f 0.45-0.56 was collected and eluted with ether. After the ether was removed, the residue was spotted on another TLC plate and developed first with n-hexane and then with benzene. Thereafter, the zone corresponding to R_f 0.75-0.85 was removed and eluted with ether. The residue remaining after evaporation of ether was then adjusted to female equivalents as necessary for field testing. The scheme for this extraction procedure is depicted in Figure 2-1.

Figure 2-1. A scheme for the preparation of diprionol for field tests.


Virgin females
ether overnight
Body extract
KOH
Crude alcohol fraction
Esterification
Crude ester fraction
TLC n-hexane:ether 4:1
Inactive fractions
Active fraction
R_f 0.33-0.75
TLC
(1) n-hexane
(2) benzene
Inactive fractions
Active fraction
R_f 0.33-0.75
Field test

RESULTS

The data summarized in Table 2-1 indicate that the 2S,3S,7S-A isomer alone was more active than any other combinations of 2S,3R,7R/S-A or 2S,3R,7R-A. At lower concentrations, 2S,3R,7R/S-A and 2S,3R,7R-A appeared to increase the effectiveness of 2S,3S,7S-A isomer, however, the increase was not statistically significant (Table 2-2). One possibility is that the effectiveness of the synergist may not express at high populations as this particular tests caught as many males. Lower concentrations of 2S,3S,7S-A was used in 1983 so that fewer males might be attracted in order to bring on clear differences if any, among the treatments. The result (Table 2-3) clearly shows that the traps baited with 2S,3S,7S-A alone attracted more males than mixtures. This again confirmed the results of Tables 2-1 and 2-2 that the 2S,3S,7S-A alone is the major pheromone of N. nanulus nanulus. Also, these data agree with the work of Kraemer et al. (1983). However, since these workers found 2S,3S,7R/S-A as active as 2S,3S,7S-A, these two isomers were compared in a side by side set up on the field. With a total catch of 640 males by 2S,3S,7S-A baited traps to 253 males in 2S,3S,7R/S-A baited traps, we proved the superiority of 2S,3S,7S-A isomer over 2S,3S,7R/S-A and the importance of S on 7-carbon on the major pheromone.

Based on apparent injury level at three locations in Roscommon County, Michigan, and the level of larvae population sampled on twenty 78 cm. branches, Manistee (with low

Table 2-1. Field response of male N. nanulus nanulus to the increasing concentrations of two isomers, 2S,3R,7R/S-A and 2S,3R,7R-A on 2S,3S,7S-A isomer. Test was conducted in Roscommon County between September 4 and October 16, 1981.

Amount (ug/trap)	2S,3S,7S-A + 2S,3R,7R/S-A (x ± S.E.)*	2S,3S,7S-A + 2S,3R,7R-A (x ± S.E.)**
10/0	582.5 ± 9.1	98.0 ± 5.6[a]
8/2	92.5 ± 3.7	35.3 ± 3.0[a]
6/4	98.5 ± 4.5	35.7 ± 3.3[a]
4/6	107.5 ± 3.8	9.3 ± 1.6[b]
2/8	60.5 ± 3.5	18.7 ± 2.6[b]
0/10	69.5 ± 2.3	18.6 ± 2.5[b]

* Mean of 2 replicates.

** Mean of 3 replicates. Means followed by same letter not significantly different at 5% level by Duncan's multiple range test.

Table 2-2. Field response of N. nanulus nanulus to the increasing concentration of two isomers 2S,3R,7R/S-A and 2S,3R,7R-A on 2S,3S,7S-A isomer. The test was conducted at two locations in Michigan from September 4 - October 16, 1981.

Amount (ug/trap)	2S,3S,7S-A + 2S,3R,7R/S-A (x* ± S.E.)**	2S,3S,7S-A + 2S,3R,7R-A (x* ± S.E.)***
10/0	413.7 ± 10.2^{abc}	47.0 ± 4.5^{a}
10/0.1	383.3 ± 8.8^{abc}	81.7 ± 5.3^{a}
10/0.3	440.3 ± 9.5^{ab}	87.0 ± 5.9^{a}
10/1	524.3 ± 11.9^{a}	50.7 ± 3.8^{a}
10/3	64.5 ± 4.5^{c}	18.3 ± 2.2^{a}
10/10	261.3 ± 10.1^{abc}	20.0 ± 2.2^{a}
10/30		19.7 ± 2.4^{a}

* Mean of 3 replicates; means followed by same letter not significantly different at 5% by Duncan's multiple range test.

** Tested at Vogel Center, Misaukee County, Michigan.

*** Tested at Higgins Lake, Roscommon County, Michigan.

Table 2-3. Field response of N. nanulus nanulus to 2S,3S,7S-A and mixtures of 2S,3S,7S-A with serial dilutions of 2S,3R,7R-A isomer. Test conducted at Vogel Center, Michigan, September 10 to October 7, 1983.

2S,3S,7S-A / 2S,3R,7R-A (ug/trap)	Mean Catch/Trap (± S.E.)[a]
5/0	41.0 ± 2.9
5/0.001	16.3 ± 0.9
5/0.003	27.3 ± 1.9
5/0.01	16.3 ± 1.3
5/0.03	30.0 ± 2.1
5/1	18.3 ± 1.4
5/5	26.0 ± 2.0
5/30	17.7 ± 1.7
5/100	33.0 ± 3.0

[a] Means of 3 replicates. Analysis of variance not significant at 5% level.

population), 0.3 ± 0.7 SD/branch, Roscommon (with medium populations), 14.5 ± 10.9 SD/branch, and Vogel Center (with high populations), 28.05 ± 8.7 SD/branch, were chosen to compare field effectiveness of the synthetic mixture and natural pheromone. At these three locations, trap catches increased with increasing concentration of the synthetic compound (Table 2-4). That agrees with the suggestion that an attractant with a correct optical configuration shows a dose-related response (Carde et al. 1977). Traps baited with the natural pheromone, however, could not catch any adults in Manistee and Roscommon, apparently due to lower population. The effectiveness of the synthetic and natural pheromone may be computed. If one assumes that one female N. nanulus nanulus contains about 10 ng. pheromone as female of N. sertifer (Kikukawa et al. 1983), any valid comparison between the synthetic and natural pheromone can not be made with the data in Table 2-4 because the synthetic pheromone concentration tested was not low enough. At lower concentration, the effectiveness of the synthetic was comparable to the natural pheromone (Table 2-5). One noticeable feature of the natural pheromone is that it was not dose related. Catches were generally lower at higher concentrations. There is a possibility that the extracts may contain certain impurities which at higher concentration was inhibitory. In 1982, all the natural preparations were purified one step further after acetylation by passing through one extra TLC system-preparative TLC $HF_{254+366}$ and developed

Table 2-4. Comparison of field effectiveness of the synthetic and natural pheromone of *N. nanulus nanulus*. Test conducted at three locations in Michigan between September 5 and October 16, 1981.

Pheromone	Amount (ug/Trap)	Locations			Total	Mean/ Trap
		Roscommon	Manistee	Vogel		
Synthetic mixture						
2S,3S,7S-A + 2S,3R,7R/S-A (5:1)	3.00	162	22	664	848	282.7
	1.00	123	16	429	568	198.3
	0.30	29	5	124	158	52.7
	0.01	12	1	55	68	22.7
Natural Pheromone (acetate)						
	1.00 FE	0	0	6	6	2.0
	0.30 FE	0	0	3	3	0.7
	0.10 FE	0	0	4	4	1.3
	0.03 FE	0	0	18	18	6.0

Table 2-5. Comparison of field effectiveness of the synthetic and national pheromone of N. nanulus nanulus. Test conducted at Vogel Center, Michigan, September 16 to October 16, 1981. Three replicates randomized three times.

Pheromone	Amount (ug/Trap)	Mean Catch/trap* (± S.E.)
Synthetic		
2S,3S,7S-A + 2S,3R,7R/S-A (5:1)	3.000	70.7 ± 2.9[a]
	1.000	33.0 ± 0.8[b]
	0.300	19.3 ± 1.7[bc]
	0.100	5.7 ± 0.8[cd]
	0.030	3.0 ± 0.8[d]
	0.010	7.0 ± 1.4[cd]
	0.003	3.0 ± 1.0[d]
	0.001	5.0 ± 1.2[d]
Control	0	0.0 ± 0.0[d]
Natural pheromone (acetate)	3.000 FE	3.0 ± 1.0[d]
	1.000 FE	1.7 ± 0.6[d]
	0.300 FE	15.7 ± 2.2[cd]
	0.100 FE	19.3 ± 2.2[bc]
	0.030 FE	11.3 ± 2.0[cd]
	0.003 FE	1.7 ± 0.9[d]

* Means followed by same letter not significantly different at 5% level.

with 20% ether in hexane as the mobile phase (Kikukawa et al. 1983). The synthetic 2S,3S,7S-A (Mori et al. 1978) was also purified through a charcoal-celite column. Pure form of 2S,3S,7S-A prepared by different routes of synthesis by another group of chemists (Kikukawa et al. 1982) was also purified. The 2S,3S,7S-A from the two sources established threshold at 0.003 ug. (Table 2-6). Also, both the synthetic and natural pheromones were favorably comparable.

To determine indirectly the form in which natural pheromone is stored in the females in nature, the ester and alcohol fractions of N. nanulus nanulus females were first purified by TLC, the latter fraction was acetylated and then tested for relative activity on the field. The data indicate that the alcohol fractions was more active by a factor of 4.5 (Table 2-7). To test whether unesterified diprionol itself might have any effect on effectiveness of synthetic pheromone, varying amounts of 2S,3S,7S-alcohol was added to a fixed amount of 2S,3S,7S-A isomer. It is clear from these data that alcohol is inhibitory and the inhibition was dose related (Table 2-8). The responses of males of N. nanulus nanulus and N. sertifer to different combinations of 2S,3S,7S-A and 2S,3R,7R-AG were different from each other (Table 2-9). As observed earlier (Table 2-2), the 2S,3R,7R-A isomer was not synergistic or inhibitory to the field effectiveness of 2S,3S,7S-A isomer against N. nanulus nanulus. Males of N. sertifer, however, showed a preference for

Table 2-6. Threshold of the synthetic pheromone 2S,3S,7S-A isomer from two sources and comparison with natural pheromone on the field against N. nanulus nanulus at Vogel Center, Michigan, September 18 to October 17, 1982.

Pheromone	Amount/Trap (ng)	Mean* Trap Catch in Three Replicates ± S.E. 2S,3S,7S-A**	2S,3S,7S-A***
Synthetic	10.000	131.0 ± 2.4[a]	298.7 ± 2.7[a]
	5.000	93.0 ± 2.1[b]	195.0 ± 4.1[b]
	1.000	39.0 ± 1.9[c]	119.7 ± 3.5[c]
	0.300	37.7 ± 3.2[c]	26.0 ± 2.2[d]
	0.100	5.3 ± 1.2[d]	6.0 ± 0.8[d]
	0.030	0.7 ± 0.4[d]	2.3 ± 0.8[d]
	0.010	1.0 ± 0.6[d]	0.3 ± 0.4[d]
	0.003	0.7 ± 0.6[d]	1.0 ± 0.6[d]
	0.001	0.0 ± 0.0[d]	0.0 ± 0.0[d]
Control	0	0.0 ± 0.0[d]	0.0 ± 0.0[d]
Natural pheromone (acetate)	10.000 FE	1.7 ± 1.0[d]	0.3 ± 0.4[d]
	3.000 FE	1.0 ± 0.6[d]	0.0 ± 0.0[d]
	1.000 FE	1.7 ± 0.6[d]	2.3 ± 0.7[d]
	0.300 FE	1.0 ± 0.8[d]	0.3 ± 0.4[d]
	0.100 FE	0.0 ± 0.0[d]	0.0 ± 0.0[d]

* Means followed by same letter not significantly different at 5% level by Duncan's Multiple Range Test.

** From Mori et al. 1978.

*** From Kikukawa et al. 1982 (unpublished).

Table 2-7. Field effectiveness of the TLC fractions of alcohol and ester from the female *N. nanulus nanulus*. Test conducted at Vogel Center from September 16 through October 16, 1981.

TLC fractions tested[a]	Female equivalents	Replicates A	B	C	Total trap catch	Mean Catch/ trap ± S.E.
Ester	1	2	1	1	4	1.3 ± 0.6
Alcohol[b]	1	4	7	7	18	6.0 ± 1.0

[a] R_f values for each fraction in this TLC system was alcohol fraction 0.07-0.30 [corresponding to band between (cinnamyl alcohol and cinnamyl acetate), ester fraction 0.39-0.65 (corresponding to 10 (E)-dodecen-1-yl acetate)].

[b] Acetylated before tested on the field.

Table 2-8. Effect of 2S,3S,7S-diprionol on the field effectiveness of 2S,3S,7S-A against *N. nanulus nanulus*. Test conducted at Vogel Center, Michigan, September 18 to October 17, 1982.

2S,3S,7S-A / 2S,3S,7S-OH (ug)	Mean catch/trap ± S.E.*
5/ 0.000	107.7 ± 3.7[a]
5/ 0.001	73.0 ± 3.5[a]
5/ 0.003	46.0 ± 3.6[b]
5/ 0.010	55.3 ± 2.4[a]
5/ 0.030	83.3 ± 2.1[a]
5/ 0.100	66.3 ± 1.6[a]
5/ 0.300	40.7 ± 2.3[b]
5/ 1.000	46.0 ± 3.9[b]
5/ 5.000	12.3 ± 1.8[b]
5/10.000	7.7 ± 1.3[b]

* Mean catch of 3 replicates. Means followed by same letter may not be significantly different at 5% level by Duncan's Multiple Range Test.

Table 2-9. Response of males of *N. nanulus nanulus* and *N. sertifer* to different combinations of optical isomers of 3,7-dimethylpentadecan-2-yl acetate. Test conducted in a red pine stand at Vogel Center, Michigan, October 16-31, 1982.

2S,3S,7S-A / 2S,3R,7R-AG (ug/trap)	*N. nanulus nanulus*[a]	*N. sertifer*[a]
5/0.000	2	2
5/0.001	0	22
5/0.003	4	9
5/0.010	3	10
5/0.030	4	3
5/0.100	1	0
5/0.300	6	0
5/1.000	4	0
5/5.000	2	0

[a] Total catch of 3 replicates.

the combination of 2S,3S,7S-A and 2S,3R,7R-A between ratio 5:0.001 and 5:0.01.

DISCUSSION

One intriguing aspect of males of N. nanulus nanulus response to synthetic pheromone is that while 2S,3S,7S-A undoubtedly is the pheromone of this species, addition of 2S,3R,7R/S-A, 2S,3R,7R-A, and 2S,3R,7S-A was not inhibitory or synergistic to the effectiveness of 2S,3S,7S-A. This fact was evident in Table 2-2 for 2S,3R,7R-A and 2S,3R,7R/S-A and when varying concentrations of 2S,3R,7S-A were combined with 2S,3S,7S-A in 1982, no treatment was superior. The importance of the pheromone as a monitoring tool for this insect is indicated by the fact that the synthetic pheromone, either as 2S,3S,7S-A alone or as mixtures, was as effective as the natural pheromone extracted from females (Table 2-5).

The red pine, no doubt, is not a preferred host for N. sertifer. However, when the traps baited with synthetic pheromone were placed in a red pine stand in mid October 1982, both N. nanulus nanulus and N. sertifer were caught. The males of N. sertifer were recognized by the black abdominal tip ventrally. They are also larger and longer than males of N. nanulus nanulus. There was a distinct range for optimum catch of N. sertifer around isomer ratio 5:0.001, 5:0.003 and 5:0.01 of 2S,3S,7S-A to 2S,3R,7R-A, while N. nanulus nanulus at the same time showed no preference for

any isomer combination. Catch for N. nanulus nanulus was low in this test (Table 2-9) due to nearness to end of adult flight season which leaves us with the data of Table 2-3 for comparison in this instance. The 2S,3S,7S-A alone was superior to combinations of isomers in attracting N. nanulus nanulus (Table 2-3) while addition of submicrogram concentration of 2S,3R,7R-A significantly improved the effectiveness of 2S,3S,7S-A against N. sertifer (Table 2-9). This result agrees with our earlier findings on N. sertifer (Kikukawa et al. 1983) and supports the theory that the 2S,3R isomer may be used as a chemical discriminator against alien species while the 2S,3S,7S remain the major pheromone of Neodiprion species (Olaifa et al. 1984). Also in 1979 at two N. sertifer infested locations near Lansing, Michigan, it was found that traps baited with natural pheromone of N. nanulus nanulus caught no male sawflies while those baited with natural pheromone of N. sertifer caught 38 male N. sertifer. In the same experiment, addition of natural pheromone of N. nanulus nanulus to that of N. sertifer decreased the catch of male N. sertifer (kikukawa et al. 1983). It is thus clear when natural pheromones were used as above and when synthetic pheromones were used as in this study that critical species specific blend of optical isomers is the major mechanism for reproductive isolation in diprionid sawflies. So far, it is in N. nanulus nanulus that the threo isomers do not appear to play any major role in the pheromone system. From the catch of two species in the same

trap, even though response of N. sertifer appeared more specific to a particular isomer combination, it can be speculated that where chemical discrimination alone fails to separate species reproductively, other isolation factors--such as copulatory behavior--may come to play.

Two incipient races have been reported in N. nanulus nanulus, one race feeding on jack pine and the other on red pine, but no morphological differences have been detected in the adult populations (Knerer and Atwood, 1973). To investigate whether these two races respond to synthetic pheromones, two active isomers 2S,3S,7S-A and 2S,3S,7R/S-A were used in traps set out at the same time at two locations, Vogel Center where N. nanulus nanulus eggs, larvae, and pupae were collected from red pine and along highway M-55 near Houghton Lake, Michigan, were N. nanulus nanulus larvae were collected from jack pine. At Houghton Lake, the 2S,3S,7S-A traps with a total catch of 154 males were more active than the 2S,3S,7R/S-A traps. At Vogel Center, similar results were obtained as described earlier. The results indicate that both incipient races of N. nanulus nanulus respond to the same sex-pheromone.

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CHAPTER THREE

Response of Male Jack Pine Sawfly *Neodiprion pratti banksianae* (Hymenoptera: Diprionidae) to Mixtures of Optical Isomers of the Sex Pheromone, 3,7-dimethylpentadecan-2-yl acetate

INTRODUCTION

The major sex pheromone of various diprionid pine sawfly are either acetate or propionate esters of 3,7-dimethylpentadecan-2-ol (diprionol) (Jewett et al. 1976). In field testing the synthetic pheromone of the redheaded pine sawfly *Neodiprion lecontei* (Fitch), it was recognized that the racemic synthetic counterpart was not as effective as the purified natural pheromone. The difference in the optical configurations between these two preparations was suspected (Matsumura et al. 1979). With three asymmetric carbon centers in the structure of diprionol, eight optical isomers are possible.

Various optical isomers were synthesized (Tai et al. 1978; Mori et al. 1978; and Kikukawa et al. 1983) and supplied to us for use in various field studies (Matsumura et al. 1979; Kraemer et al. 1979, 1981, 1983; Kikukawa et al. 1983). These studies established that (a) most Neodiprion species respond to 2S,3S,7S-3,7-dimethylpendadecan-2-yl acetate or propionate (abbrev. 2S,3S,7S-A or P) depending on the species, and (b) two Old World species *Diprion similis*

and Gilpinia frutetorum most favorably responded to propionate and acetate respectively of 2S,3R,7R-diprionol (Kikukawa et al. 1982a). Another major improvement in our knowledge of pine sawfly pheromone was the study by Kikukawa et al. (1983), which established that the presence of a small level of 2S,3R,7R-A in 2S,3S,7S-A drastically increased the latter's effectiveness in the field. In this study we have examined such interplay of optical isomers in the field against Neodiprion pratti banksianae Rohwer another important defoliator of jack pine in North America.

MATERIALS AND METHODS

All the field tests were conducted in a jack pine stand at Hartwick Pines, Michigan. This area is located in the middle of 40 hectares of State Forest. Jack pine comprises approximately 90% of the forest composition. The test area, 1.6 kilometer square, consists of pure jack pines, including a virgin forest.

Neodiprion pratti is a complex with several subspecies. In the test area only N. pratti banksianae and N. pratti paradoxicus Ross, have been recorded. However, despite several attempts, we have found only N. pratti banksianae in this location as judged by the larvae collected and reared to the adult stage in our laboratory. The females from such laboratory reared larvae were extracted for their pheromone (Kikukawa et al. 1982). Trap preparation and field distribution have been described (Matsumura et al. 1979; Kikukawa

et al. 1983). The number of males caught in each trap was recorded. They were removed from the traps, washed with n-hexane to remove the sticky material, and stored in 70% alcohol. Each trapped male was examined microscopically and compared to laboratory reared specimens.

The synthetic pheromones used were from Japan. They came either as pure, racemic isomers, or mixtures. We refer to carbon 2,3 and 7 of diprionol as either R or S, or R/S when racemic. All the isomers were esters. Four chiral isomers 2R,3R,7R; 2R,3R,7S; 2S,3S,7R; and 2S,3S,7S were synthesized by Mori et al. (1978). Tai et al. (1978) synthesized 2S,3S,7R/S and 2S,3R,7R/S used for this study. The same group (Kikukawa et al. 1983) later synthesized and supplied 2S,3S,7S; 2S,3R,7R and 2S,3R,7S isomers. These isomers probably contained varying levels of optical impurities. In one instance 2S,3S,7R/S there was a known 5% contamination with 2S,3R,7R/S isomer on the basis of PMR (Matsumura et al. 1979). Other isomers contained less than 1% of erythro in threo or threo in erythro contamination as this was the approximate limit of PRM assay.

RESULTS

In a series of field tests involving twelve propionate and acetate isomers, the males of *N. pratti banksianae* were attracted to 2S,3S,7S-A as with *N. pinetum* and *N. lecontei* (Kraemer et al. 1979, 1981 and Matsumura 1979) and *N. sertifer* (Kikukawa et al. 1983). That 2S,3S,7S-A was more active

than 2S,3S,7R-A established the importance of S at the 7-carbon for the major pheromone for this species. No optical isomer containing 2R or 3R was active. In the second series of experiments, traps baited with 2S,3S,7R/S-A showed an outstanding catch of males (Table 3-1). Several replicates comparing 2S,3S,7S-A with 2S,3S,7R/S-A were later tried. As 2S,3S,7R/S is made of two isomers-2S,3S,7S and 2S,3S,7R in the ratio 1:1 (determined by PMR), we first considered that the mixture of these isomers might be important. Several combinations of 2S,3S,7S-A and 2S,3S,7R-A were tried. This was done by holding 2S,3S,7S-A constant and by varying 2S,3S,7R-A. However, none of the combinations was active. Even at the ratio 1:1 the mixture was not effective. Another possibility is that the 2S,3S,7R/S-A preparation contained either 2R or 3R impurities. Several possible combinations of synthetic pheromones were tried by increasing the proportion of R at 2 or 3 carbon positions (Table 3-2). The results indicated that none of the mixtures of 2S,3S and 2R,3R were significantly attractive. However, several combinations where 2S,3S was mixed with isomers containing 3R were highly attractive. The most males were caught when 2S,3R,7R/S-A was added to 2S,3S,7S-A. An attempt was made to determine which of the 2S,3R isomers and at what concentrations would provide maximum activity. Although flight season was almost over when the traps were set out, the data (Table 3-3) indicated that 2S,3R,7R/S-A acted as a synergist to 2S,3S,7S-A. The 2S,3R,7R/S-A isomer used

Table 3-1. Field response of males N. pratti banksianae to traps baited with synthetic pheromones. The test was conducted from September 13-October 9, 1980 at Hartwick Pines, Michigan.

Optical Isomer	Amount (ug)	Replicates (Catch/Trap) A	B	Total
2S,3S,7S-A	5	25	19	44
2S,3S,7S-P	5	17	33	50
2S,3R,7R/S-A	10	0	1	1
2S,3R,7R/S-P	10	2	0	2
2S,3S,7R/S-A	10	226	252	478
2S,3S,7R/S-P	10	0	9	9
2S,3R,7R/S-A + 2S,3S,7S-A	10/5	228	294	522
2S,3R,7R/S-P + 2S,3S,7S-A	10/5	41	24	65
2S,3R,7R/S-A + 2S,3S,7S-P	10/5	1	2	3
2S,3R,7R/S-A + 2S,3S,7S-P	10/5	6	1	7

Table 3-2. Field response of N. pratti banksianae males to traps baited with synthetic pheromones or admixing of synthetic pheromones. The test was conducted from September 13-October 9, 1980 at Hartwick Pines, Michigan.

Optical Isomer	Amount (ug)	Mean Catch/Trap ± S.E.*
2S,3S,7S-A	5	2.0 ± 0.6[b]
2R/S,3R,7R-A	10	0.0 ± 0.0[b]
2R/S,3R,7S-A	10	0.3 ± 0.4[b]
2R,3R,7R-A + 2S,3S,7S-A	5/5	1.3 ± 0.4[b]
2R,3R,7S-A + 2S,3S,7S-A	5/5	1.0 ± 0.8[b]
2R/S,3R,7R-A + 2S,3S,7S-A	10/5	38.3 ± 3.1[a]
2R/S,3R,7S-A + 2S,3S,7S-A	10/5	8.7 ± 1.2[b]
2S,3R,7R/S-A + 2S,3S,7S-A	10/5	31.7 ± 1.7[a]
Control (no pheromone)	0	0.0 ± 0.0[b]

* Means of 3 replicates. Means followed by same letter not significantly different at 5% level by Duncan's multiple range test.

Table 3-3. Synergistic effect of the 2S,3R,7R/S-A on 2S,3S,7S-A isomer on attraction of male N. pratti banksianae in the field. Test was conducted September 26-October 16, 1981 at Hartwick Pines, Michigan.

Optical Isomer	Amount (ug)	Mean Catch/Trap ± S.E.*
2S,3S,7S-A	5	0.0 ± 0.0[b]
2S,3R,7R/S-A	5	0.0 ± 0.0[b]
2S,3R,7R-A	5	0.0 ± 0.0[b]
2S,3S,7S-A + 2S,3R,7R/S-A	5/1	4.0 ± 0.0[a]
2S,3S,7S-A + 2S,3R,7R/S-A	1/5	0.0 ± 0.0[b]
2S,3S,7S-A + 2S,3R,7R-A	5/1	0.0 ± 0.0[b]
2S,3S,7S-A + 2S,3R,7R-A	1/5	0.3 ± 0.4[b]

* Means of 3 replicates. Means followed by same letter not significantly different at 5% level by Duncan's multiple range test.

in this particular experiment, however, still contained about 5% of 3S.

In 1982 a new 2S,3R,7R-A preparation was synthesized through a Grignand preparation reaction and was designated as 2S,3R,7R-AG (Kikukawa et al. 1982b). This preparation contained +99.9% optical purity at carbon 3. By varying concentrations of 2S,3R,7R-AG; 2S,3R,7R-A; 2S,3R,7S-A and 2S,3R,7R/S-A against 2S,3S,7S-A (Table 3-4 and Figure 3-1), we showed that inhibition and stimulation were caused by the same combinations.

Using the synthetic 2S,3S,7R/S-A and the 5:1 optimum combination of 2S,3S,7S-A and 2S,3R,7R-AG, the response threshold concentration was determined. The threshold concentrations of 2S,3S,7R/S-A was high (100 ng.) whereas the 5:1 combinations of 2S,3S,7S-A and 2S,3R,7R-AG gave a threshold of 1.0 ng. (Table 3-5). Expressed as female equivalent, this threshold of 1.0 ng. corresponds to 0.1 female equivalent assuming N. pratti banksianae contains a 10 ng. pheromone per female as does N. sertifer (Kikukawa et al. 1983).

DISCUSSION

Several Neodiprion species not only feed on the same host plant, but their males fly at the same time and respond to the same ester of 2S,3S,7S isomer as their major pheromone component. These sawfly species apparently maintain reproductive isolation and there is evidence that the males

Table 3-4. Effect of the 2S,3R,7R-AG; 2S,3R,7R-A; 2S,3R,7S-A and 2S,3R,7R/S-A isomers on the 2S,3S,7S-A isomer in stimulation of male N. pratti banksianae attraction to baited traps in the field. Testing was done August 30-November 14, 1982 at Hartwick Pines, Michigan.

Amount (ug/trap)	Mean trap catch			
	2S,3S,7S-A[a] + 2S,3R,7R-AG	2S,3S,7S-A[b] + 2S,3R,7R-A[d]	2S,3S,7S-A[b] + 2S,3R,7S-A[d]	2S,3S,7S-A[b] + 2S,3R,7R/S-A
5/0	14.7	0	0	0
5/0.001	12.3	0	0	0
5/0.003	6.0	1.3	0	0.7
5/0.01	10.7	1.0	0	0.3
5/0.03	17.7	4.3	0	1.3
5/0.1	30.0[c]	6.7	0.3	3.3
5/0.3	43.0[c]	19.3[c]	1.3[c]	7.0
5/1	80.0[c]	3.3	5.3[c]	20.0[c]
5/5	11.7	13.7[c]	17.3[c]	5.7
5/10	24.7	1.3	11.7[c]	2.0

[a] Tested 9/10-10/16 and 2S,3S,7S-A from Kikukawa et al. 1982b.

[b] Tested 8/30-11/14 and 2S,3S,7S-A from Mori et al. 1978.

[c] All means significantly different from others in the same column by Duncan's multiple range test. $P \leq 0.05$.

[d] Newly synthesized by Kikukawa et al. unpublished.

Figure 3-1. Stimulation and inhibition of the field response of N. pratti banksianae males to the major sex pheromone isomer 2S,3S,7S-A by the 2S,3R,7R-A isomer.

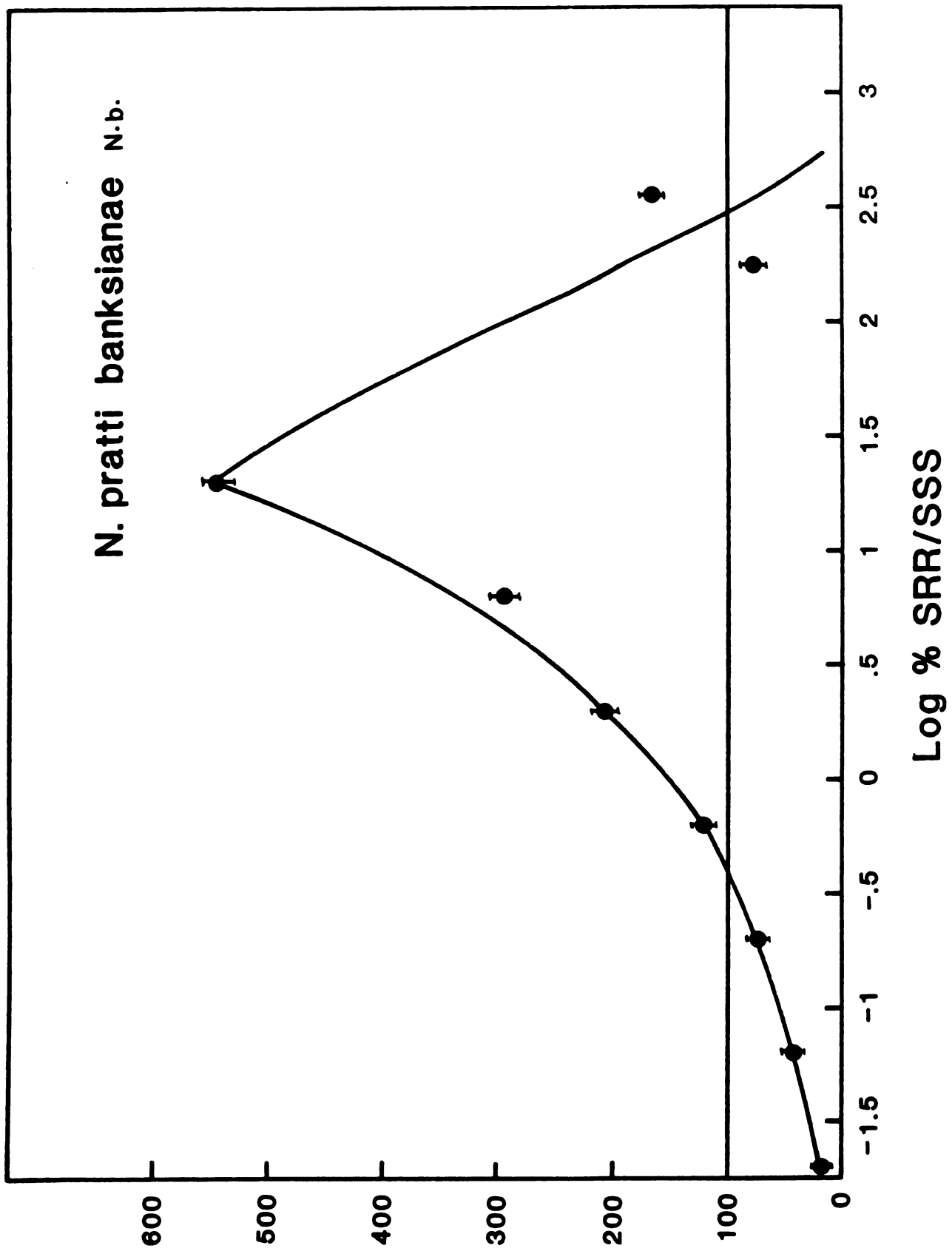
N. pratti banksianae N.b.
Male Catch as % of SSS Isomer
Log % SRR/SSS
0
100
200
300
400
500
600
-1.5
-1
-.5
0
.5
1
1.5
2
2.5
3

Table 3-5. Threshold of the synthetic pheromone of N. pratti banksianae. Test was conducted from September 27-October 16, 1982 at Hartwick Pines, Michigan.

Pheromone	Amount (ng/trap)	Replicates A	B	C	Total	Mean/* Trap
Synthetic mixture 2S,3S,7S-A / 2S,3R,7R-AG (5:1)	10,000	43	27	14	84	28.0[a]
	3,000	28	38	17	83	27.7[a]
	1,000	15	11	10	36	12.0[b]
	300	2	2	4	8	2.7[c]
	100	2	1	3	6	2.0[c]
	30	3	0	5	8	2.7[c]
	10	9	1	2	12	4.0[4]
	3	3	0	0	3	1.0[c]
	1	1	0	0	1	0.3[c]
Natural Pheromone (acetate) N. pratti pratti	10 (= 1 FE)	5	5	2	12	4.0[b]
	3 (= 0.3 FE)	0	2	0	2	0.7[c]
	1 (= 0.1 FE)	1	0	0	1	0.3[c]
	0.3 (= 0.03 FE)	0	0	0	0	0.0
	0.1 (= 0.01 FE)	0	0	0	0	0.0

* Means followed by same letter not significantly different by Duncan's multiple range test at 5% level.

respond best to the purified natural pheromone from females of their own species. Kikukawa and Matsumura (unpublished data) found that highly purified pheromone from N. sertifer females caught N. sertifer males in a red pine area in Roscommon, Michigan, where synthetic 2S,3S,7S-A preparations catch only N. nanulus nanulus. Conversely, a purified nanulus pheromone caught only nanulus males in a scotch pine stand where N. sertifer males were normally caught. Assuming minute amounts of impurities in these natural pheromone preparations, the only possible mechanisms for reproductive isolation would be either the differences in mixtures of optical isomers or a second component. The former assumption is based upon the observation that despite repeated attempts (Kikukawa et al. 1982a, 1983; Jewett et al. 1976) no one has yet found a second pheromone component which is either active alone, or synergistically active in combination with the major pheromone in at least two well-studied species (i.e., N. sertifer and D. similis). As demonstrated herein for N. pratti banksianae and N. sertifer (Kikukawa et al. 1983) earlier, the action of 2S,3R,7R/S-A, 2S,3R,7S-A and 2S,3R,7R-A on 2S,3S,7S-A is highly synergistic at optimum concentrations. At higher concentrations and ratios, however, these synergists become highly inhibitory. The 2S,3R,7R-A was the best synergist in this study, however, because 2S,3R,7R/S-A and 2S,3R,7S-A were also synergistic, the specificity of chirality at 7-carbon for the synergists could not be established by the current study. On the other

hand, to our knowledge no such mechanism utilizing mixtures of optical isomers of pheromones for species differentiation is yet demonstrated in any other species of insects. The closest examples may be the noctuid moth pheromones (Steck et al. 1980a, 1980b; Struble 1981; Kaae et al. 1973) where trace components synergize the major pheromone components. All of these examples involve geometric isomers. The 2S,3R,7R isomer has the same chiral configuration as the alcohol moiety of the most active synthetic pheromone Diprion similis and Gilpinia frutetorum. Thus the females of the diprionid sawflies are apparently capable of producing these two specific chiral forms. It is possible that the routes of biosynthesis of diprionol are such that these two forms are readily synthesized. Because of the variations in ratios among females in synthesizing these two components, males responding to specific mixture ratios may have been selected through evolution. In the case of diprionid sawflies, the females play a key role in selecting the host plants by choosing the oviposition sites. Males, on the other hand, are able to fly for long distances and may be found in areas with non-host plants. The esters of diprionol are used as long distance sex-attractants. The long distance species recognition plays a very important role in reproductive isolation of these species. After all, some Neodiprion species are capable of interbreeding when they are placed in confined quarters and their emergence synchronized. Such interbreeding may rarely take place in

nature where males must rely upon the effectiveness of the sex pheromone.

In conclusion, N. pratti banksianae, as in the case with N. sertifer (Kikukawa et al. 1983) apparently utilizes a combination of two isomers, 2S,3S,7S-A and 2S,3R,7R-A. The mixture activity is maximized at about 20% of the former and 0.06% of the latter. The females of these species, therefore, may be using this second isomer 2S,3R,7R-A as a chemical discriminator which selectively inhibits the approach of alien species. Whether this second isomer exists as a trace component in other Neodiprion species is a question which appears worthy of investigation.

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CHAPTER FOUR

Field Response of Male Redheaded Sawfly *Neodiprion lecontei* (Hymenoptera: Diprionidae) to Mixtures of Optical Isomers of the Sex Pheromone 3,7-dimethylpentadecan-2-yl acetate

INTRODUCTION

The existence of a sex attractant in *N. lecontei*, *N. sertifer* (Geoffroy) and *Diprion similis* (Hartig) provided the material from which diprionid sex pheromone was first identified as the acetate or propionate ester of 3,7-dimethylpentadecan-2-ol (diprionol) (Jewett et al. 1976). The same workers found that *N. lecontei* responded more to the acetate when tested by electroantennogram. However, when the compound was synthesized and tested against *N. lecontei* and 3 other species, it was not as active as the naturally occurring pheromone (Matsumura et al. 1978). This discrepancy was interpreted to mean that the naturally occurring pheromone has a rigid optical requirement, and that some of the optical isomers in the synthetic racemic mixture were actually inhibitory to the attractiveness of the pheromone. To prove this point, three optically pure pheromones with respect to 2-carbon and 3-carbon were synthesized and tested against *N. lecontei*. Only (2S,3S,7R/S)-3,7-dimethylpentadecan-2-yl acetate (2S,3S,7R/S-A) was active and response was roughly comparable to the natural pheromone

(Matsumura et al. 1979). It was then concluded that the chirality of sex pheromone of N. lecontei was the acetate of (2S,3S)-diprionol; the chirality at the 7-carbon remained unknown. Availability of chirally defined synthetics made it possible to identify its chemical identity to be 2S,3S,7S-A as the sex pheromone of N. lecontei (Kraemer et al. 1981). Study by the same group in Michigan on N. sertifer (Kikukawa et al. 1983) and N. pratti banksianae Rohwer (Olaifa et al. 1984) showed that these species respond mainly to 2S,3S,7S-A but such response is significantly synergized by addition of 2S,3R,7R-A or 2S,3R,7S-A at a species defined optimum concentration. In the earlier study, Kraemer et al. (1981) combined a racemic isomer 2S,3R,7R/S-A at 3 concentrations or 2R,3S,7R/S-A at one concentration with the major isomer 2S,3S,7S-A. The former inhibited the effectiveness of 2S,3S,7S-A against N. lecontei while the latter stimulated it. Since some types of isomers have been shown to elicit inhibitory response--in species which naturally respond to one enantiomeric form (Vite et al. 1976), a question has been raised in this study whether any of the optical isomers present in the preparations tested by Kraemer et al. (1981) has a property to influence the effectiveness of the major pheromone in this species.

MATERIALS AND METHODS

Field Tests

Stands of slash pine, Pinus elliotti Engelm var. elliotti, at Hague, Austin Cary Forest and Honey Plant West near Gainesville, Florida were the study area for the first generation of the redheaded pine sawfly. For the third generation, field tests were carried out on the edges of sand pine Pinus clausa (Chapm ex Engelm) Vasey ex Sarg. at Prosper Headquarters, 2° N of Highway 77 and 2° near Panama City and at Holt in Florida. Because of the location involved, all field experiments for this species were conducted by Dr. R. C. Wilkinson in Gainesville, Florida. The samples were prepared and the experimental designs were determined by us.

For the first generation trappings, five-foot lengths of PVC pipe were drilled and fitted with an eye-bolt and nut at each location. Using tie wire, Pherocon II sticky traps were attached to the eye-bolt. A rubber stopper was then placed into the eye-bolt to prevent the tie wire from coming loose. A hair clip was then stuck through the side of a trap to hold a dental wick in the center of the trap. PVC pipes were placed in the ground ca. 46 cm. deep, so that sticky traps were ca. 1 m. above ground. One and two year old pine stands were used. The pheromone was poured onto a cotton dental wick starting with the least concentration and working up and then placed in the hair clip in the trap. Different sets of forceps were used for the three isomers

used to avoid cross-contamination. Traps for the third generation population were placed on sand pines at a height of ca. 2 m. Experimental design used for the first and third generations was randomized complete block design. Two mixtures 2S,3S,7S-A/2S,3R,7S-A and 2S,3S,7S-A/2S,3R,7R-A were used for the first generation, only the latter was used for the third generation. Each block consisted of one series of dilutions of one mixture above and at one location. Where 2 mixtures were used in the first generation, the blocks were separated by at least 30 m. Rerandomization within and between blocks at each location was done weekly for 3 weeks. We did not expect variability among locations to affect difference among treatments for a particular pheromone mixture since each treatment appeared at every location. Catches were recorded weekly for 3 weeks. Data were analyzed by analysis of variance and the means compared, where possible, by Duncan's new multiple range test at 5% level.

Synthetic Pheromone

The 3 isomers used in this study were synthesized from the same laboratory in Japan. Synthesis of 2S,3R,7R-A and 2S,3R,7S-A have been described (Kikukawa et al. 1982). The same group synthesized 2S,3S,7S-A (unpublished). The 3 isomers, however, were known to be +99.9% optical purity at chiral centers and free of any contaminants by GLC checking. Each isomer was prepared at the desired concentrations in 1 ml. n-hexane and sealed in glass ampules until use.

RESULTS

From the results shown in Table 4-1 and 4-2, it is clear that two isomers 2S,3R,7R-A and 2S,3R,7S-A behaved somewhat similarly in their interaction with the major pheromone 2S,3S,7S-A by lowering field effectiveness of the latter against the first generation male N. lecontei. At one location, Hague, the effect of 2S,3R,7R-A on 2S,3S,7S-A was not very clear but at two other locations, Austin Cary and Honey Plant West, the inhibitory effect of 2S,3R,7R-A on 2S,3S,7S-A became clearer (Table 4-1). At these locations the 2S,3R,7S-A isomer was clearly inhibitory to 2S,3S,7S-A especially at concentrations of 2S,3R,7S-A greater than 1 ug (Table 4-2). The third generation male populations of N. lecontei at three locations showed a clear dose related inhibition of 2S,3S,7S-A isomer by the 2S,3R,7R-A isomer (Table 4-3). This result also show that the 2S,3R,7R-A isomer by itself was not active and that addition of 0.006-0.02 ug to 10 ug of 2S,3S,7S-A did not significantly reduce field effectiveness of 2S,3S,7S-A isomer.

DISCUSSION

From the response of male N. lecontei to 2S,3S,7S-A/2S,3R,7R-A mixture (Table 4-1), 2S,3R,7R-A/2S,3R,7S-A mixture (Table 4-2) and 2S,3S,7S-A/2S,3R,7R-A mixture (Table 4-3), it can be concluded that 2S,3R,7S-A isomer reduces the effectiveness of 2S,3S,7S-A at high concentrations while 2S,3R,7R-A showed a less pronounced inhibitory tendency.

Table 4-1. Catches of first generation male N. lecontei by traps baited with 2S,3S,7S-A/2S,3R,7R-A mixture at 3 locations in Florida. Test conducted on slash pine stand mid-May through June 1, 1983.

Mixture of 2S,3S,7S-A/ 2S,3R,7R-A isomer (ug/trap)	Hague	Austin Cary	Honey Plant West	Total catch
5/0	35	10	26	71
5/0.001	38	8	12	58
5/0.003	36	5	6	47
5/0.01	24	0	11	35
5/0.03	31	9	11	51
5/0.1	32	3	10	45
5/0.3	29	5	10	44
5/1	32	2	19	55
5/5	48	8	12	68
5/10	27	3	17	47

Table 4-2. Catches of first generation male N. lecontei by traps baited with 2S,3S,7S-A/2S,3R,7S-A mixture at 3 locations in Florida. Test condutced on slash pine stand mid-May through June 1, 1983.

Mixture of 2S,3S,7S-A/ 2S,3R,7R-A isomer (ug/trap)	Hague	Austin Cary	Honey Plant West	Total catch
5/0	36	6	1	43[a]
5/0.001	32	9	6	47[a]
5/0.003	30	6	11	51[a]
5/0.01	27	13	10	50[a]
5/0.03	30	3	7	40[a]
5/0.1	21	9	6	36[a]
5/0.3	20	11	4	35[a]
5/1	8	4	1	13
5/5	1	0	1	2
5/10	0	2	0	2
Control (No pheromone)	0	0	0	0

[a] Significant different by comparison of the means.

Table 4-3. Catches of third generation male N. lecontei by traps baited with 2S,3S,7S-A/2S,3R,7R-A mixture at 3 locations in Florida. Test conducted on sand pine stand mid-November through mid-December 1983.

2S,3S,7S-A/ 2S,3R,7R-A (ug/trap)	Prosper Headquarters	Panama City	Holt	x Catch/ trap*
10/0	32	19	41	30.7[a]
10/0.006	22	25	18	21.7[a]
10/0.02	24	27	17	22.7[a]
10/0.2	20	8	5	11.0[b]
10/2	6	9	26	13.6[b]
10/5	0	0	2	0.7[c]
10/10	2	1	3	2.0[b]
10/30	0	2	0	0.7[c]
0/10	0	0	0	0.0[c]

* Means followed by same letter not significantly different at 5% by Duncan's new multiple range test.

Such less pronounced inhibitory tendency of 2S,3R,7R-A got for the first generation may be attributed to higher male population which diluted treatment effects especially at one test site, Hague. For a better interpretation of this field data, the original NMR of the natural pheromone from female N. lecontei (Jewett et al. 1976) was consulted and no threo signal was evident (at the detection limit of 1%). N. lecontei, like another sympatric species N. nanulus nanulus does not utilize mixtures of optical isomers in its pheromone. However, N. lecontei was found to respond well to 2S,3S,7R/S-A with a known 5% 3R contamination (Matsumura et al. 1979). It is thus clear that while 2S,3S,7S-A is the main pheromone, the males are not so discriminatory therefore not fit for fine species differentiation.

One logical question at this point is how are these two species N. lecontei and N. nanulus nanulus which utilize 2S,3S,7S-A isomer solely as their pheromone able to maintain reproductive isolation? This is done by spatial separation in the south where N. nanulus nanulus is not found and where N. lecontei maintains a three generation a year population. In the north, however, temporal separation provides effective reproduction isolation between these two species because N. nanulus nanulus is fall-flying and N. lecontei maintains only one early summer population around the Great Lakes Region and Canada (Matsumura et al. 1978). Further studies will be required to completely elucidate the nature of sex pheromone of other southern diprionid sawfly species,

especially N. pratti pratti (Dyar), N. excitans and N. merkeli which co-fly with N. lecontei (Wilkinson, 1980).

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CHAPTER FIVE

Field Response of Male *Neodiprion virginianus* (Hymenoptera: Diprionidae) to Mixtures of Optical Isomers of the Sex Pheromone 3,7-dimethylpentadecan-2-ylpropionate

INTRODUCTION

From the point of view of taxonomic identity and pheromonal response of males, *Neodiprion virginianus* Rohwer is a complex species indeed. At first there was inclination towards referring to this species as *N. nigroscutum* Middleton but males of *N. nigroscutum* are known to have red lower abdomens and occur at very low populations (Wilson 1970, Becker and Benjamin, 1967), while the present species are trapped in the thousands. Attempts to locate the larval colonies in this area failed. As judged by the trapping data, this species must overwinter in cocoons and there must be two adult generations per season. On June 13, 1983, eggs were found on twigs of jack pine at the test site. There were 7 eggs per row per needle and there were clusters of eggs on one twig. Each egg is separated from the other by a distance equal to its own length, and these eggs were on old needles. The eggs hatched into light green larvae with black head capsules. Body color changed gradually from light green to whitish yellow by June 19, 1983. This color was maintained till June 22, 1983, when dark spots began to appear on the body. A full grown larval instar has two

dorsal greenish stripes and supraspiracular line-spot complement, and an epiproctal spot against a yellow-green background. The headcapsule is black. The black head capsule at first suggested N. pinetum (Norton), N. nanulus nanulus Schedl, N. Swainei Middleton, N. pratti banksianae Rohwer or N. rugifrons Middleton. However, the solid dorsal stripes would eliminate N. pinetum. The egg laying patterns of these species are: N. pinetum 3-4 eggs (Baker 1972), N. swainei 1-2 eggs in pairs on two needles arising from a common base, N. maurus Rohwer 3-6 eggs, and N. pratti banksianae 3, 4 or at most 5 eggs per row (Atwood and Peck, 1943). Therefore, these species could be eliminated. N. dubiosus Schedl could be another alternative, but this species is known to strictly have one generation per year in this area--unlike this species. Also, N. dubiosus overwinters in egg stage (Maxwell, 1958) while this present species overwinters in cocoons. The adults from these eggs did not emerge in late July or early August as expected, probably because of combined effects of artificial photo period and temperature in the laboratory rearing conditions. These two factors are known to be critical for a second generation of N. rugifrons (King and Benjamin, 1965). Three male cocoons were artificially opened in September 1983. Fully developed alive males with black lower abdomens were thus obtained. These males were found to have identical morphological characteristics with the field trapped males when compared under a dissecting microscope. Female samples

were similarly obtained. Their morphological characteristics were similar to those described for N. rugifrons. While no record of the description of male N. rugifrons exists, it is known that the female abdomen is mainly black (Atwood and Peck, 1943; Middleton, 1933; Becker et al. 1966). Baker (1972) indicates that the N. virginianus complex is made up of three species N. virginianus, N. rugifrons and N. dubiosus--though he pointed out that the question whether N. rugifrons is a true species or whether it is a member of the group known as the virginianus complex is not settled. Beck et al. (1966), through biotaxonomic investigations, identified two sawflies, N. rugifrons and N. dubiosus, as members of the N. virginianus complex. Ross (1955) coined the name N. virginianus complex and Atwood (1961) proposed N. rugifrons as a synonym to cover the rather variable species whose host is almost exclusively jackpine, whose larvae have heads ranging from orange to black, and which pass the winter in the cocoon. Also, Maxwell (1958) synonymized N. virginianus with N. rugifrons. N. rugifrons male specimens, collected as larvae and reared at University of Wisconsin by Dr. Kraemer and Dr. Coppel, have brown abdomens. Also, they report that the larvae have the orange head capsule. The color of the head capsule of the larvae in the present investigation was black. N. rugifrons has two generations to one of N. dubiosus. The adult populations of the species currently investigated is two per season.

On the basis of the observations that N. rugifrons has the closest characteristics to the present species being investigated, and on the indirect evidence that this species responds to the propionate ester of diprionol, as well as female extract of N. dubiosus as will be pointed out later, it may be concluded that this species is a member of N. virginianus complex (which Atwood referred to as N. rugifrons). The male responses of N. rugifrons towards mixtures of synthetic chiral isomers are somewhat different from those found for this species (Kraemer, personal communication). It appears best to call this species as N. virginianus Rohwer under this circumstance.

Earlier we reported that the isomer (2S,3S,7S)-3,7-dimethylpentadecan-2-yl acetate (2S,3S,7S-A) is the major sex pheromone of N. sertifer (Geoffroy) (Kikukawa et al. 1983) as well as for many Neodiprion species (Matsumura et al. 1979; Kraemer et al. 1979, 1981, and 1983), while the 2S,3R,7R-A and (2S,3R,7R)-3,7-dimethylpentadecan-2-yl propionate (2S,3R,7R-P) are likely to be the sex pheromones of Gilpinia frutetorum (Fabricius) and Diprion similis (Hartig), as judged by attractiveness of synthetic 2S,3R,7R esters (Kikukawa et al. 1982) respectively. We also reported that the threo isomers, 2S,3R,7R-A; 2S,3R,7S-A and 2S,3R,7R/S-A among other in mixture with 2S,3S,7S-A confer significant synergistic effect on the latter against N. sertifer (Kikukawa et al. 1983) and N. pratti banksianae (Olaifa et al. 1984). The optimum blend of the mixture has

a differing concentration of the threo-isomer for each species. This paper reports on the response of N. virginianus males to the synthetic optical isomers of diprionid sawfly pheromone tested singly and in various mixtures on the field.

MATERIALS AND METHODS

Field Tests

Trapping for this species occurred at Mio, Hartwick Pines, and Kalkaska, Michigan. These locations are open areas of jack pine stands covering approximately 20 square kilometers each. The ground cover is mainly grasses and reindeer moss on sandy soils. Test chemicals were poured onto dental cotton wicks 4-5 cm. long placed at the center on the inside of Pherocon II traps (Zoecon Corp.). Traps were positioned in jack pine trees at a height of 2.5 m. and separated by 15-20 m. All tests utilized a randomized block design. Data were subjected to analysis of variance and where necessary the means were graded by Duncan's new multiple range test at a 5% level.

Natural Pheromone

Virgin females of N. pinetum, N. rugifrons, and N. dubiosus were derived from field collected larvae reared to adults at University of Wisconsin. The pheromone extractions, purifications, and esterification follows that of N. sertifer (Kikukawa et al. 1983).

Synthetic Pheromone

The acetate and propionate forms of 2R,3R,7R; 2R,3R,7S; 2S,3S,7R and 2S,3S,7S were obtained from Mori et al. (1978), and 2R,3S,7R/S; 2S,3R,7R/S; 2S,3S,7R/S, and mixtures of these isomers from Tai et al. (1978). Kikukawa et al. (1982a) supplied us with 2S,3R,7S and 2S,3R,7R isomers. The modifications of pure isomers from Mori et al. (1978) into 2R/S,3S,7S; 2R/S,3R,7R and 2R/S,3R,7S was done in our laboratory by the method described by Kikukawa et al. (1982b). The purity of these chemicals was ascertained by GLC (columns 7.2 m. x 3 mm. packed with 3% S.E. 30, and 6 m. x 3 mm. packed with 10% carbowax 20 m.). A flame ionization detector was used. Materials from Kikukawa et al. (1982a) appeared pure while those from the other two sources contained varying levels of impurities and were purified as described below.

Purifications of Synthetic Isomers by Charcoal-Celite Column

Thirty-six gm. of charcoal (Norit S., J. T. Baker Chemical Co., New Jersey) preheated for 2 hrs. at 180°C, was mixed thoroughly with 9 gm. celite (Fisher Scientific Co.) and washed with 20 ml. acetone in a funnel with Whatman No. 2 filter paper. The charcoal-celite obtained from above was stirred into a 200 ml. beaker with 140 ml. 10% ether in hexane using a glass rod. The slurry obtained was packed in a 50 cm. x 2 cm. i.d. glass column. A 20 ml. solvent was first used to wash the slurry down the walls of the column

followed by 80 ml. in 2 equal volumes to stabilize the column. A maximum of 7 mg. sample was introduced to the column and was eluted with 10% ether in n-hexane at 0.2 ml./min. flow rate. Recovery was about 60% with samples less than 500 ug. it was less. Sixty-three 10 ml. fractions were collected using a microfractionator (model FC -80K, Gilson Medical Electronics Inc., Middleton, Wisconsin). A small aliquot from each fraction was injected into a GLC as described above. The active fractions 33-42, 43-60, and 61-63 were referred to as front, center, and back fractions respectively. The center fractions of 2S,3S,7S and 2S,3R,7R/S-A isomer was used throughout unless otherwise indicated. The scheme for this purification is described in Figure 5-1.

RESULTS

The males of N. virginianus respond to the propionate ester of diprionol (Table 5-1). This is the first species encountered among Neodiprion species which responded solely to the propionate and not acetate in the field. However, the major isomer still is the 2S,3S,7S as reported for N. pinetum Norton (Kraemer et al. 1979), N. lecontei (Fitch) (Matsumura et al. 1979; Kraemer et al. 1981), N. sertifer (Kikukawa et al. 1983), N. taedae linearis and N. nanulus nanulus (Kraemer et al. 1983) and N. pratti banksianae (Olaifa et al. 1984). This data also showed distribution of N. virginianus covering the entire north central region of

Figure 5-1. Scheme for the purification of isomers of diprionol by charcoal-celite column.

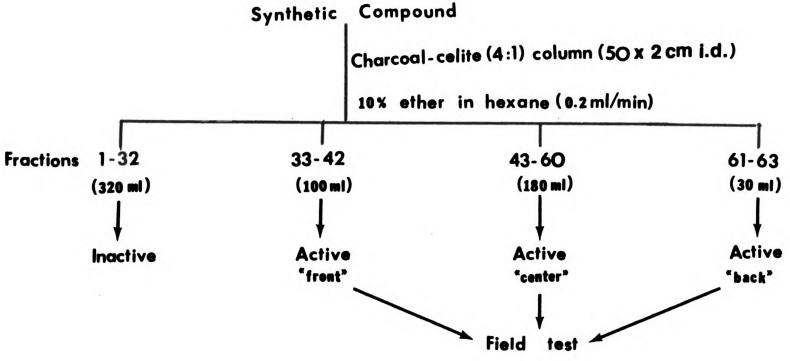
Synthetic Compound
Charcoal-celite (4:1) column (50 x 2 cm i.d.)
10% ether in hexane (0.2 ml/min)
Fractions 1-32
(320 ml)
33-42
(100 ml)
43-60
(180 ml)
61-63
(30 ml)
Inactive
Active
"front"
Active
"center"
Active
"back"
Field test

Table 5-1. Field response of N. virginianus to optical isomers of 3,7-dimethylpentadecan-2-yl propionate or acetate. Test conducted at 5 locations in Roscommon, Crawford and Kalkaska counties, Michigan, May 15-July 10, 1981. Two replicates at 4 locations and 3 replicates at one location, all randomized twice.

Isomer	Amount (ug/trap)	Total catch per treatment
2R,3R,7R-P	20	0
2R,3R,7S-P	20	0
2S,3S,7R-P	20	0
2S,3S,7S-P	20	659
2S,3R,7R-P	20	0
2S,3R,7R/S-P	20	0
2S,3S,7S-P / 2S,3R,7R-P	20/20	44
2S,3S,7S-A	20	0
2S,3S,7R/S-A	20	0
2S,3S,7R-A	20	0
2S,3S,7S-A / 2R/S,3R,7S-A	20/20	0

the lower peninsula, Michigan. There was an indication also, that the 2S,3R,7R-P isomer at the concentration tested might be inhibitory to the 2S,3S,7S-P isomer.

Actions of different propionate isomers on the major isomer were then tested (Table 5-2). Four pure or racemic isomers (mixtures not included) were found to be active in combination with the 2S,3S,7S-P isomer. The data also showed traps baited with 2R/S,3S,7S-P catching significantly more males than the major isomer. However, when 2R,3S,7S-P was added to the major isomer at ratio 1:1, there was a slight-but-insignificant reduction in catch. It must be noted that apart from 2R/S,3S,7S-P isomer, apparently because of similarities to major isomer, three other isomers which improved effectiveness of the major isomer were not active by themselves. Three other isomers 2S,3R,7R-P; 2S,3R,7S-P and 2R,3R,7S-P and those that were earlier found to improve effectiveness of the major isomer were compared for their synergistic activity (Table 5-3). Of these 7 candidate synergists, 3 of them, 2S,3R,7S-P; 2S,3R,7R/S-P and 2R/S,2R,7S-P isomers, were superior in increasing field effectiveness of the major isomer. A side by side comparison of these 3 best synergists at four concentrations showed that 2S,3R,7S-P isomer was the superior synergist at a blend ratio 1:2 of 2S,3S,7S-P and 2S,3R,7S-P (Table 5-4). These data also showed a dose (synergists) related response (trap catch) especially with 2S,3R,7S-P and 2R/S,34,7S-P isomers.

Table 5-2. Field response of N. virginianus to mixtures of optical isomers of 3,7-dimethylpentadecan-2-yl propionate. Test conducted at Mio, Michigan, July 21-August 4, 1981. Three replicates r andomized three times.

Optical Isomers	Amount (ug/trap)	$\bar{x}$ male/trap
2S,3S,7S-P	20	8.7
2R/S,3S,7S-P	20	30.7[a]
2R/S, 3S,7R-P	20	0.0
2R/S,3R,7S-P	20	0.0
2S,3R,7R/S-P	20	0.0
2R,3S,7R/S-P	20	0.0
2S,3R,7R/S / 2R,3S,7R/S-P	20	0.0
2S,3R,7R/S / 2R,3R,7R/S-P	20	0.0
2R,3S,7R/S / 2S,3S,7R/S-P	20	0.0
2S,3S,7S-P / 2S,3R,7R/S-P	20	45.0[a]
2S,3S,7S-P / 2R,3S,7R/S-P	20/20	1.3
2S,3S,7S-P / 2S,3R,7R/S / 2R,3S,7R/S-P	20/20	14.0[a]
2S,3S,7S-P / 2S,3R,7R/S / 2R,3R,7R/S-P	20/20	8.0
2S,3S,7S-P / 2R,3S,7R/S / 2S,3S,7R/S-P	20/20	3.0
2S,3S,7S-P / 2R/S,3R,7S-P	20/20	30.7[a]
2S,3S,7S-P / 2R/S,3S,7R-P	20/20	0.0
2S,3S,7S-P / 2R/S,3S,7S-P	20/20	20.3[a]
2S,3S,7S-P / 2R,3R,7R-P	20/20	13.3[a]
2S,3S,7S-P / 2R,7R,7S-P	20/20	2.7
2S,3S,7S-P / 2S,3S,7R-P	20/20	0.0
Control	0	0.0

[a] Mean significantly different from others at 5% level.

Table 5-3. Field response of N. virginianus to mixtures of candidate optical synergist-isomers. Test conducted at Sharon, Michigan, May 14-June 20, 1982. Three replicates randomized three times.

Optical Isomers	Amount (ug/trap)	$\bar{x}$ catch/trap ± S.E.
2S,3S,7S-P	10	1.0 ± 0.6
2R/S,3S,7S-P	10	1.3 ± 0.7
2S,3S,7S-P / 2S,3R,7R-P	10/10	0.3 ± 0.4
2S,3S,7S-P / 2S,3R,7S-P	10/10	58.7 ± 3.1[a]
2S,3S,7S-P / 2S,3R,7R/S-P	10/10	65.3 ± 4.3[a]
2S,3S,7S-P / 2R/S,3R,7S-P	10/10	33.3 ± 2.9[a]
2S,3S,7S-P / 2R/S,3S,7S-P	10/10	4.7 ± 1.0
2S,3S,7S-P / 2R,3R,7R-P	10/10	5.3 ± 1.2
2S,3S,7S-P / 2R,3R,7S-P	10/10	1.7 ± 0.4

[a] Significantly different from other means at 5% level.

Table 5-4. Field response of N. virginianus to mixtures of three best candidate synergist-isomers. Test conducted at Sharon, Michigan, May 15-June 20, 1982. Three replicates randomized three times.

Amount (ug/trap)	2S,3S,7S-P / 2S,3R,7R/S-P	2S,3S,7S-P / 2R/S,3R,7S-P	2S,3S,7S-P / 2S,3R,7S-P
5/ 0.0	0.3	5.7	0.0
5/ 0.1	2.0	5.3	0.7
5/ 1.0	10.3	1.3	3.3
5/ 5.0	30.7	5.0	33.3
5/10.0	18.3	24.0	71.0[a]

[a] Significantly different from other means at 5% level.

The optimum blend for 2S,3S,7S-P and 2S,3R,7R/S-P appeared to be at 1:1 ratio, even though not significant.

In 1983, the 2S,3R,7S-P and 2S,3R,7R-P isomers were further investigated regarding their synergistic influence on the major isomer. The data derived from two locations show that traps baited with a 1:2 blend of 2S,3S,7S-P and 2S,3R,7S-P significantly caught more males (Tables 5-5 and 5-6). Also, 2S,3R,7R-P isomer exhibited a subtle broad synergism to 2S,3S,7S-P with the highest catch at ratio 10:0.6. This type of synergism was not dose specific as 2S,3S,7S-P/2S,3R,7S-P mixture.

The next approach was analytical; purifying the synergist isomer 2S,3R,7R/S-P through a charcoal-celite column and collecting fractions as described in Figure 5-1. Fraction nos. 34-42 designated as 2S,3R,7R/S-P front was generally more active than the 2S,3R,7R/S-P center (fraction nos. 43-60) and 2S,3R,7R/S-P back (fraction nos. 61-63) (Table 5-7). Also the 5:5 blend ratio for 2S,3S,7S-P/2S,3R,7R/S-P front significantly attracted more males than any other fractions or blends. This result not only confirmed the results of Table 5-2 and 5-3 concerning 2S,3R,7R/S-P, it also indicated the presence of synthetic contaminant(s) which eluted with the 2S,3R,7R/S-P center and 2S,3R,7R/S-P back. Further evidence for this inhibitor is presented in Table 5-8 where the blend with 2S,3R,7R/S-P front attracted significantly more males than other blends. The ratio for an optimum catch was again found to be 1:1.

Table 5-5. Field response of N. virginianus to varying concentrations of 2S,3R,7S-P and 2S,3R,7R-P mixed with major pheromone isomer. Test conducted at Hartwick Pines, Michigan, June 10-July 14, 1983. Three replicates randomized three times.

Amount (ug)	2S,3S,7S-P/2S,3R,7S-P*	2S,3S,7S-P/2S,3R,7R-P*
10/0	5.7^{b}	1.0^{b}
0/10	1.3^{c}	4.3^{b}
10/0.002	1.7^{c}	7.0^{a}
10/0.006	5.7^{b}	17.3^{a}
10/0.02	15.0^{b}	8.7^{a}
10/0.06	28.3^{b}	6.0^{b}
10/0.2	2.7^{c}	9.0^{a}
10/0.6	4.7^{c}	34.7^{a}
10/2	7.3^{b}	30.0^{a}
10/10	56.3^{a}	33.3^{a}
10/20	66.3^{a}	3.0^{b}
10/100	4.0^{b}	1.3^{b}

* Means followed by same letter not significantly different at 5% level by Duncan's new multiple range test.

Table 5-6. Synergistic effect of admixing 2S,3R,7S-P and 2S,3S,7S-P isomers in attracting male N. virginianus on the field. Test conducted at Sharon, Michigan, June 10-15, 1983. Three replicates randomized three times.

Optical Isomers	Amount (ug/trap)	$\bar{x}$ catch/trap* ± S.E.
2S,3S,7S-P	5	5.0 ± 1.4[b]
2S,3R,7S-P	5	2.3 ± 0.7[c]
	30	9.7 ± 1.8[b]
2S,3S,7S-P / 2S,3R,7S-P	5/5	47.7 ± 2.8[ab]
	5/10	69.7 ± 4.1[a]
	5/30	23.7 ± 2.5[b]
	5/100	12.3 ± 1.7[c]

* Means followed by same letter not significant.

Table 5-7. Field response of N. virginianus to the front, center, and back charcoal-celite column fractions of 2S,3R,7R/S-P* mixed with 2S,3S,7S-P. Test conducted at Sharon, Michigan, June 30-July 23, 1982. Three replicates randomized three times.

Amount/trap mixed with 5 ug 2S,3S,7S-P	2S,3R,7R/S-P (front)	2S,3R,7R/S-P (center)	2S,3R,7R/S-P (back)
0.0	1.0	0.3	0.3
0.1	2.0	0.3	2.0
1.0	11.0	2.3	2.3
5.0	147.3[a]	5.3	2.3
10.0	56.0	5.0	9.7

* Fractions as described in Figure 1.

[a] Mean significantly different from others at 5% level.

Table 5-8. Field response of N. virginianus to the crude and the front, center, and back fractions of 2S,3R,7R/S-P isomer mixed with the major pheromone 2S,3S,7S-P isomer.* Test conducted at Sharon, Michigan, August 2-31, 1982. Three replicates randomized three times.

Preparations	Amount (ng/trap)	Mean catch/trap ± S.E.
Control (no pheromone)	0	0.0 ± 0.0
2S,3S,7S-P / 2S,3R,7R/S-P_{crude}	10/10	9.3 ± 1.9
2S,3S,7S-P / 2S,3R,7R/S-P_{front}	10/10	43.3 ± 2.5[a]
2S,3S,7S-P / 2S,3R,7R/S-P_{center}	10/10	3.7 ± 0.8
2S,3S,7S-P / 2S,3R,7R/S-P_{back}	10/10	5.7 ± 0.7

* Fractions as described in Figure 1.

[a] Significantly different from other means by Duncan's multiple range test at 5%.

The data in Table 5-9 indicate that N. virginianus males were attracted by natural pheromone of N. dubiosus another member of the N. virginianus complex. The data also show that propionate ester moiety is preferred. Using the optimum blend of the synthetic pheromone 2S,3S,7S-P / 2S,3R,7R/S-P mixture, the male catch showed a dose response relationship with threshold limit being 10 ng. (Table 5-10).

DISCUSSION

The sex pheromone of N. virginianus is a mixture of 2S,3S,7S-P and 2S,3R,7S-P isomers. This represents the most complex pheromone system in the Neodiprion species because of divergence from the acetate moiety. This two-component pheromone also illustrates the natural significance of synergistic response to pheromone optical isomers of providing means of reproductive isolations between sympatric species. Three isomers 2S,3S,7S; 2S,3R,7S and 2S,3R,7R appear to be common components in the pheromone system of Neodiprion species with one species utilizing a combination of 2S,3S,7S and any one of the other two isomers. In N. sertifer and N. pratti banksianae 2S,3R,7R appeared to be the preferred second component (Kikukawa et al. 1983 and Olaifa et al. 1984). Analysis by capillary GLC indicates that the natural pheromone is comprised of 2S,3S,7S and 2S,3R,7S isomers in N. virginianus. The details of GLC analysis is published elsewhere. The abbott sawfly N. abbotti (unpublished) and N. virginianus appear to utilize 2S,3R,7S

Table 5-9. Species specificity and ester moiety preference of *N. virginianus*. Test conducted at Sharon, Michigan, May 14-August 13, 1982. Three replicates randomized three times.

Natural pheromone	Female equivalents	Total catch
Series 1 (May 14-June 20, 1982)		
N. rugifrons (acetate)	1	0
N. pinetum (acetate)	1	0
N. dubiosus (proprionate)	1	4
N. dubiosus (acetate)	1	0
Series 2 (June 29-August 13, 1982)		
N. dubiosus	2	6
	1	2
	0.3	0
Blank	0	0

Table 5-10. Threshold limit of the synthetic pheromone of N. virginianus. Test conducted at Sharon, Michigan, June 29-August 13, 1982. Three replicates randomized three times.

Amount ng/trap (2S,3S,7S-P / 2S,3R,7R/S-P 1:1)	Mean catch/trap ± S.E.*
20,000	111.3 ± 3.8[a]
10,000	92.7 ± 1.9[a]
2,000	47.7 ± 3.4[b]
1,000	22.3 ± 2.2[b]
200	14.0 ± 1.6[c]
100	9.7 ± 1.6[c]
20	8.0 ± 1.6[c]
10	0.3 ± 0.4[c]
2	0.0 ± 0.0[c]
1	0.0 ± 0.0[c]
0.2	0.0 ± 0.0[c]
0.1	0.0 ± 0.0[c]
0	0.0 ± 0.0[c]

* Means followed by the same letter not significantly different at 5% level by Duncan's new multiple range test.

isomer. However, attractant blends that are highly species specific appear to play a major role of species isolations despite common usage of common components. Such method of species isolation has been described among the sympatric tortricine moths (Carde et al. 1977) which utilize geometric isomers. Sympatric Gnathotrichus species also utilize critical ratios of R and S sulcatol for species isolation (Borden et al. 1980) but that involved mixtures of one enantiomer and its antipode. In the Neodiprion species, the major pheromone component is the 2S,3S,7S isomer, its antipode 2R,3R,7R has never been active or synergistic. The utilization of critical ratios of 2S,3S,7S with a threo isomer 2S,3R,7S or 2S,3R,7R or 2S,3R,7R/A for species isolation as evidenced in N. sertifer (Kikukawa et al. 1983), N. pratti banksianae (Olaifa, et al. 1984) and N. virginianus in this study is novel to science. Further studies will be needed to completely elucidate the mechanism of reproductive isolation among species complexes.

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CHAPTER SIX

Neodiprion pinetum: Synergistic Response to Mixtures of Optical Isomers of the Sex Pheromone 3,7-dimethylpentadecan-2-ylacetate

INTRODUCTION

The sex pheromone of diprionid pine sawflies was described as the acetate or propionate 3,7-dimethylpentadecan-2-01 (diprionol) (Jewett et al. 1976). However, when tested on the field against three diprionid sawfly species, the synthetic compound was not as active as the natural pheromone from the field. When *Neodiprion lecontei* (Fitch) showed significant response to (2S,3S,7R/S)-3,7-dimethylpentadecan-2-ylacetate (2S,3S,7R/S-A) on the field (Matsumura et al. 1979), it then became clear that only certain optical isomers of diprionol was active. This observation was also confirmed in *N. pinetum* (Kraemer et al. 1979) which showed response to 2S,3S,7S-A, but not to 2R,7R,7R-A, and 2R,3R,7S-A. The 2S,3S,7R-A isomer also showed some activity. This study was the first to show that a *Neodiprion* species responded to 2S,3S,7S-isomer, although this has been speculated when 2S,3S,7R/S-A baited traps attracted male *N. lecontei* on the field (Matsumura et al. 1979). Further investigations by the same group revealed that *N. Lecontei*, *N. nanulus nanulus* Schedl, *N. sertifer*

(Geoffroy) and N. taedae linearis Ross respond mainly to 2S,3S,7S isomer (Kraemer et al. 1981 and 1983), while two Old World species Diprion similis (Hartig) and Gilpinia frutetorum (Fabricius) prefer the 2S,3R,7R isomer (Kikukawa et al. 1982a). Another field studies in 1980 at Higgins Lake Michigan by Kikukawa and Matsumura (unpublished) established that N. pinetum respond mainly to 2S,3S,7S-A and that males respond better to the acetate than the propionate isomers. However, no definite conclusion was made by their data as well as those of Kraemer et al. (1979) as to the effect of mixing isomers. This study reports interaction of optical isomers in field response of N. pinetum, an important defoliator of Pinus strobus Linnaeus in North America.

MATERIALS AND METHODS

Field Tests

Field trapping of N. pinetum was carried out in the States of Michigan and Wisconsin. In Michigan, two tests areas, Sec. 25 and 30, Higgins Lake State Forest, Roscommon County and Sec. 33, McGee on Highway M66, Kalkaska County, were used. Both consisted of pure white pine stands, each about 1.6 kilometer square. The ground cover consisted of grasses and fern, Pteridium aquilium (L.) Kuhn. In Wisconsin, pure white pine stands 2 miles west of Siren were used. The larval colonies of N. pinetum were collected by Dr. Mark Kraemer of University of Wisconsin and reared to adulthood in the laboratory. These laboratory reared adults

from Wisconsin were morphologically identical to the field trapped N. pinetum from Michigan. Pherocon II traps were used throughout this study. Trap preparations have been described (Kraemer et al. 1979). Randomized complete block design was the experimental design used with the block choice based on rows of clearings at the two locations. At least one re-randomization of treatments within and between blocks was done, and the record of trap catch taken weekly. The data so collected are evaluated with analysis of variance and the differences among means graded at P = 0.05 according to Duncan's new multiple range test. Trapped males were removed from the traps, washed with n-hexane to remove the sticky material and stored in 70% alcohol. Each trapped male was examined microscopically and compared to laboratory reared specimens.

Synthetic Pheromone

Four isomers, 2S,3S,7S-A and 2S,3S,7S-P; 2S,3R,7R/S-A; 2S,3R,7R-A and 2S,3R,7S-A were used in this study. The R or S refers to chirality on 2-carbon, 3-carbon and 7-carbon of the structure of diprionol. The first two isomers were from the laboratory of Dr. Kenji Mori (Mori et al. 1978). These isomers were later synthesized by Kikukawa et al. (unpublished). The synthesis of 2S,3R,7R/S-A has been described (Tai et al. 1978). The first synthesis of threo isomers in optically pure forms was made by Kikukawa et al. (1982b) who supplied us with 2S,3R,7R-A and 2S,3R,7S-A. These compounds

are prepared in desired concentrations in 1 ml n-hexane, sealed in glass ampules until use on the field.

Purification of Synthetic Isomers by Charcoal-celite Column

Thirty-six gm of charcoal (Norit A., J. T. Baker Chemical Co., New Jersey) pre-heated for 2 hrs. at 180°, was mixed thoroughly with 9 gm celite (Fisher Scientific Co.) and washed with 20 ml acetone in a funnel with Whatman No. 2 filter paper. The charcoal-celite obtained from above was stirred into a 200 ml beaker with 140 ml 10% ether in hexane using a glass rod. The slurry obtained was packed in a 50 cm x 2 cm i.d. glass column. A 20 ml solvent was first used to wash the slurry down the walls of the column followed by 80 ml in 2 equal volumes to stabilize the column. A maximum of 7 mg sample was introduced to the column and was eluted with 10% of ether in n-hexane at 0.2 ml/min of flow rate. Recovery was about 60%, with samples less than 500 ug it was less. Sixty-three 10 ml fractions were collected using a micro fractionator (Model FC-80K, Gilson Medical Electronics, Inc., Middleton, Wisconsin).

A small aliquot from each fraction was injected into a gas liquid chromatograph (GLC) equipped with a flame ionization detector (FID). Fractions 33-63 contained the sample, but fractions 33-42 and 61-63 contained the sample and impurities while fractions 43-60 contained the pure sample. It should be noted that columns less than 35 cm in height failed to purify the samples in this manner.

RESULTS

Combination of 2S,3S,7S-A and 2S,3R,7R/S-A at ratio 1:2, a significant synergism of male catch was recorded (Table 6-1). Synergism was directly related to the concentration of the 2S,3R,7R/S-A isomer. The result of a similar experiment in Siren, Wisconsin (Table 6-2) proved the same point. The synergist 2S,3R,7R/S-A, in each case, was not active by itself. It is not clear from Table 6-2 the role of 2S,3S,7S-P which was not active alone but which appeared to improve effectiveness of 2S,3S,7S-A. In this study, the traps were re-randomized once but vandalism of some of the traps made a definite conclusion of the role of 2S,3S,7S-P impossible. As indicated in previous studies (Kraemer et al. 1979) and the unpublished data in 1980 by Kikukawa and Matsumura it is not likely that both propionate and acetate occur in one species as natural pheromones, and hence, 2S,3S,7S-P was not used in subsequent studies.

Traps baited with mixtures of 2S,3S,7S-A and 2S,3R,7R-A recorded a significant trap catch (Table 6-3). This experiment compared two pure isomers 2S,3R,7S-A and 2S,3R,7R-A with the racemic 2S,3R,7R/S-A which was synergistic in the earlier results. The synergistic effect of 2S,3R,7R-A was manifested when mixed with 2S,3S,7S-A from both Mori et al. (1978) and Kikukawa et al. (unpublished). Because of the superior performance of 2S,3S,7S-A from Kikukawa (Table 6-3), we utilized this preparation in subsequent experiments. In Kalkaska, the males of N. pinetum were also attracted to

Table 6-1. Synergistic effect of the 2S,3R,7R/S-A isomer on the effectiveness of 2S,3S,7S-A isomer in attracting wild male N. pinetum at Higgins Lake State Forest Michigan, May 15-July 20, 1981 on white pine.

Preparation	Amount (ug/trap)	Mean male catch/trap* ± S.E.
2S,3S,7S-A	20	7.0 ± 1.4^{cde}
2S,3R,7R/S-A	20	0.0 ± 0.0^{e}
2S,3S,7S-A / 2S,3R,7R/S-A	20/5	7.7 ± 1.2^{cd}
2S,3S,7S-A / 2S,3R,7R/S-A	20/10	8.7 ± 1.7^{c}
2S,3S,7S-A / 2S,3R,7R/S-A	20/20	18.7 ± 1.1^{b}
2S,3S,7S-A / 2S,3R,7R/S-A	20/40	30.3 ± 1.4^{a}
Control	0	0.0 ± 0.0^{e}

* Means followed by same letter not significantly different at 5% level. Means of 3 replicates.

Table 6-2. Field response of male N. pinetum to traps baited with isomers of acetate or propionate diprionol. Test conducted at Siren, Wisconsin, May 15-June 30, 1981.

Preparations	Amount (ug/trap)	Total catch in 3 replicates
2S,3S,7S-A	12-5	4
2S,3S,7S-A	25	6
2S,3S,7S-P	12-5	0
2S,3R,7R/S-A	25	0
2S,3S,7S-A / 2S,3R,7R/S-A	12-5/25	44*
2S,3S,7S-A / 2S,3S,7S-P	12-5/12-5	40**

* Total in one trap; 2 other traps vandalized.

** Total of two traps, 1 other trap vandalized.

Table 6-3. Comparison of three threo isomers 2S,3R,7R-A, 2S,3R,7S-A and 2S,3R,7R/S-A in synergizing the effectiveness of 2S,3S,7S-A isomer against N. pinetum males. Test conducted at Higgins Lake, Michigan, May 7-June 10, 1982.

Preparation of isomers	Amount (ug/trap)	Mean catch/ trap ± S.E.
2S,3S,7S-A[a]	5	0.0 ± 0.0
2S,3S,7S-A[b]	5	1.3 ± 0.6
2S,3R,7R-A	5	0.0 ± 0.0
2S,3R,7S-A	5	0.0 ± 0.0
2S,3R,7R/S-A	5	0.0 ± 0.0
2S,3S,7S-A[a] / 2S,3R,7R-A	5/10	5.0 ± 1.0[c]
2S,3S,7S-A[b] / 2S,3R,7R-A	5/10	6.3 ± 1.1[c]
2S,3S,7S-A[b] / 2S,3R,7S-A	5/10	1.0 ± 0.6
2S,3S,7S-A[b] / 2S,3R,7R/S-A	5/10	0.3 ± 0.4
Control	0	0.0 ± 0.0

[a] 2S,3S,7S-A form Mori et al. 1978.

[b] 2S,3S,7S-A from Kikukawa et al. (unpublished).

[c] Means significantly different from others at 5% level by Duncan's multiple range test.

a mixture of 2S,3S,7S-A and 2S,3R,7R-A at 1:1 and 1:2 ratios but the latter ratio was superior (Table 6-4). The 2S,3R,7S-A and 2R/S,3R,7S-A were not active as synergist in this experiment. By holding the concentration of 2S,3S,7S-A constant at 5 ug and varying the concentration of the synergist isomer 2S,3R,7R-A from 0.001 to 20 ug, it was established that maximum effectiveness of the mixture was achieved at a concentration of 10 ug of the synergist. This implies that a 1:2 ratio gave the most active blend. When the ratio increased to 1:4, a significant reduction in catch was recorded (Table 6-5). Using the 2S,3R,7R/S-A as the synergist of the 2S,3S,7S-A isomer in Siren, Wisconsin, we again demonstrated significant effectiveness of the mixture at a blend ratio of 1:3 of 2S,3S,7S-A to 2S,3R,7R/S-A (Table 6-6).

DISCUSSION

The 2S,3S,7S-A isomer again proved to be the major pheromone of N. pinetum. This time not only because it is more effective than other isomers tested singly, but because other isomers 2S,3R,7R/S-A and 2S,3R,7R-A acted as synergists to improve its effectiveness in a dose-related manner. This study also showed that the mixture of the major and synergist isomers at the right combination was better than the major isomer alone.

The chiral differences between the pheromone of Neodiprion species and the Old World species must play a major

Table 6-4. Comparison of three threo isomers 2S,3R,7R-A 2S,3R,7S-A and 2R/S,3R,7S-A in synergizing the effectiveness of 2S,3S,7S-A isomer against N. pinetum males. Test conducted in Kalkaska, Michigan, May 5-June 10, 1982.

Preparation	Amount (ug/trap)	Mean Catch/Trap ± S.E.*
2S,3S,7S-A	5	0.0 ± 0.0[b]
2S,3S,7S-A / 2S,3R,7S-A	5/5	0.0 ± 0.0[b]
2S,3S,7S-A / 3S,3R,7S-A	5/10	0.0 ± 0.0[b]
2S,3S,7S-A / 2S,3R,7R-A	5/5	2.7 ± 1.1[a]
2S,3S,7S-A / 2S,3R,7R-A	5/10	4.7 ± 1.4[a]
2S,3S,7S-A / 2R/S,3R,7S-A	5/5	0.0 ± 0.0[b]
2S,3S,7S-A / 2R/S,3R,7S-A	5/10	0.0 ± 0.0[b]
Control	0	0.0 ± 0.0[b]

* Mean of 3 replicates. Means followed by same letter not significantly different at 5% by Duncan's Multiple Range Test.

Table 6-5. Determination of the optimum blend of the mixture of 2S,3S,7S-A and 2S,3R,7R-A isomers in attracting N. pinetum males at Kalkaska, Michigan, June 29-July 7, 1982.

Isomer preparations	Amount (ug/trap)	Mean male catch/trap* ± S.E.
2S,3S,7S-A	5	0.0 ± 0.0[c]
2S,3S,7S-A / 2S,3R,7R-A	5/0.001	0.0 ± 0.0[c]
	5/0.01	0.0 ± 0.0[c]
	5/0.1	0.0 ± 0.0[c]
	5/1	0.0 ± 0.0[c]
	5/5	10.3 ± 0.9[b]
	5/10	21.0 ± 1.6[a]
	5/20	18.3 ± 1.6[ab]

* Means followed by the same letter not significantly different at 5% level. Means of 3 replicates.

Table 6-6. Determination of the optimum blend of the mixture of 2S,3S,7S-A and 2S,3R,7R/S-A isomers in attracting N. pinetum males at Siren, Wisconsin, May 15-June 30, 1982.

Isomer preparations	Amount (ug/trap)	Mean male catch/trap ± S.E.
2S,3S,7S-A*	10	0.0 ± 0.0
2S,3S,7S-A**	10	0.3 ± 0.4
2S,3S,7S-A* / 2S,3R,7R/S-A	10/0.001	1.0 ± 0.6
	10/0.01	1.0 ± 0.6
	10/0.1	2.3 ± 0.9
	10/1	1.7 ± 0.7
	10/3	0.7 ± 0.4
	10/10	3.0 ± 0.8
	10/30	15.0 ± 1.7[C]

* 2S,3S,7S-A from Mori et al. (1978).

** 2S,3S,7S-A from Kikukawa et al. (unpublished).

[C] Mean significantly different from others by Duncan's multiple range test at 5% level.

role in species recognition. Thus, the N. pinetum and D. similis which are the two species particularly feeding on white pine, must be separated by this mechansim since N. pinetum do not respond to 2S,3R,7R isomer alone as demonstrated in Table 6-1 and 6-3. One other important mechanism of species recognition between these two species is the preference by D. similis for the propionate (Jewett et al. 1976) and acetate by N. pinetum (Kraemer et al. 1979). Such chiral differences have not been observed among Neodiprion species and as such cannot explain their mechanism of species recognition. Using purified mixture of optical isomers on the field, Kikukawa et al. (1983) demonstrated that a small addition of the 2S,3R,7R-A isomer significantly improved the effectiveness of 2S,3S,7S-A isomer against N. sertifer. That study was the first to demonstrate interaction of optical isomers in field response of diprionid sawflies to their sex pheromone. A similar interaction of optical isomers was also observed in field response of male N. pratti banksianae Rohwer to synthetic pheromones (Olaifa et al. 1984). One important difference in these two species is that while the former species utilizes a 5:0.003 combination, the latter utilizes a 5:1 combination of 2S,3S,7S-A:2S,3R,7R-A. A demonstration of 5:10 combination of the same set of optical isomers in N. pinetum in this study, is a clear indication that interaction of optical isomers is a mechanism of species recognition among Neodiprion species.

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CHAPTER SEVEN

Attractancy of Optically Active Isomers of Pheromone 3,7-dimethylpentadecan-2-yl propionate for Male *Diprion similis* (Hymenoptera: Diprionidae) on the Field

INTRODUCTION

The major sex pheromone of *Diprion similis* (Hartig) was stereochemically established by field tests as the propionate of (2S,3R,7R)-3,7-dimethylpentadecan-2-ol (2S,3R,7R-P) (Kikukawa et al. 1982a). That conclusion, however, was based on field response of males to racemic compounds 2S,3R,7R/S-P; 2S,3R,7R/S / 2R,3S,7R/S-P and 2R/S,3R,7R-P isomers. Of these racemates, the 2R/S,3R,7R-P was the most active but not significantly above the others. In the same study, it was found that the natural pheromone was far superior, the difference being in the neighborhood of 2500 fold. The discrepancy between the natural and synthetic was attributed, among others, to the racemic nature of the synthetics.

There is evidence for enantiomeric specificity in the pheromones of many scolytid Coleptera (Hedden et al. 1976; Wood et al. 1976; Borden et al. 1976 & 1980). It is also hypothesized that racemates elicit response-inhibitory effects on the receptor system of insects which naturally respond to one enantiomeric form only (Vite et al. 1976).

Experience with other diprionid sawflies showed that species respond mainly to one enantiomeric form (2S,3S,7S)-3,7-dimethylpentadecan-2-yl acetate (2S,3S,7S-A) but such response may be synergized by another one enantiomeric form 2S,3R,7R-A or 2S,3R,7S-A depending on species (Kikukawa et al. 1983; Olaifa et al. 1984). This study reports on the field response of D. similis to the racemates and one enantiomeric forms as well as mixtures of one enantiomeric forms of sex pheromone of diprionid sawflies.

MATERIALS AND METHODS

Field Trapping

Most of the field trappings took place on white pine stands at Sec. 33, McGee on M-66 highway, Kalkaska County, Michigan. All the white pine stands at this location are shrubby and fully exposed to sun. Undergrowths are mainly grasses and sweet fern, Comptonia peregrina (L.) Coult. Also used on a few occasions were Sec. 25 and 30, Higgins Lake State Forest in Roscommon County and at Sec. 33, Rose Lake Wildlife Experimental Stations at Bath Township in Ingham County, Michigan. At all these locations, eggs, larvae, cocoons as well as adult D. similis were collected. Methods of bioassay have been described (Kikukawa et al. 1982). On a few occasions, Siren, Wisconsin was used as a trapping site.

Natural Pheromone

The adult females reared from field collected larvae and pupae were extracted of their pheromone by the method described by Kikukawa et al. (1982). Purification was by TLC $HF_{254 + 366}$ developed 15 cm first in hexane:ether 4:1. After the pheromone fractions (R_f 0.21-0.33) has been removed and esterified to propionate, it is purified further by a second TLC $HF_{254 + 366}$ developed first in n-hexane and then benzene. The zone corresponding to R_f 0.75-0.85 contained the purified propionate of natural pheromone of D. similis. The diprionol band comes right between the band of cinnamyl alcohol and cinnamyl acetate, while the propionate diprionol comes about 5.0 mm above cinnamyl acetate. These two standards were chosen to take advantage of UV light detection.

Synthetic Pheromones

The propionate esters of 2S,3S,7S; 2R,3R,7R; 2R,3R,7S and 2S,3S,7R-diprionol were synthesized by Mori et al. (1978). Synthesis of the racemic compound 2S,3R,7R/S-P and 2S,3R,7R/S / 2R,3S,7R/S-P was by Tai et al. (1978). By the method described by Kikukawa et al. (1982a), the 2R/S,3R,7R-P and 2R/S,3S,7S-P were modified from one enantiomeric forms from Mori et al. (1978). Kikukawa et al. (1982b) synthesized for the first time one enantiomeric forms of diastereomers 2S,3R,7S-P and 2S,3R,7R-P.

Purification of Racemic Isomer 2S,3R,R/S-P by Charcoal-celite Column

Thirty-six gm of charcoal (Norit A., J. T. Baker Chemical Co., New Jersey) preheated for 2 hrs at 180°C, was mixed thoroughly with 9 gm celite (Fisher Scientific Co.) and washed with 20 ml acetone in a funnel with Whatman No. 2 filter paper. The charcoal-celite obtained from above was stirred into a 200 ml beaker with 140 ml 10% ether in hexane using a glass rod. The slurry obtained was packed in a 50 cm x 2 cm i.d. glass column. A 20 ml solvent was first used to wash the slurry down the walls of the column followed by 80 ml in 2 equal volumes to stabilize the column. A maximum of 7 mg sample was introduced to the column and was eluted with 10% ether in n-hexane at 0.2 ml/min flow rate. Recovery was about 60%, with samples less that 500 ug it was less. Sixty-three 10 ml fractions were collected using a microfractionator (Model FC-80K, Gilson Medical Electronics, Inc., Middleton, Wisconsin). A small aliquot form each fraction was injected into a FID GLC (stainless steel columns 7.2 m x 3 mm packed with 3% S.E. 30 and 6 m x 3 mm 10% carbowax 20 M) for purity. The active fractions 43-60 were free of any impurity by GLC detection and was used as pure compound for the field test.

Preparative GLC Fraction Collection

A Varian 1700 preparative gas chromatograph fitted with stainless steel column 6 m x 3 mm packed with 10% carbowax 20 M on 80-100 mesh chrom Q were used for fractions

collection of seven isomers. The fractions consisted of the front and back of GLC peaks of 2S,3R,7R-P; 2S,3R,7S-P; 2S,3S,7S-P; 2S,3S,7R-P; 2R,3R,7R-P; 2R,3R,7S-P and 2R/S,3S,7S-P as depicted in Figure 7-1. A 9:1 post column splitter was used which directed 90% of the injected material for collection and 10% to the detector. A 15 cm pyrex tubing bent into a 'U' at the middle was used for collection. Hexane moistened glass wool was used to plug the distal end of the tubing and the bent portion of the tubing contained a small amount of hexane solvent to monitor the flow of carrier gas. Using the solvent also increased trapping efficiency of the collecting tube. The unplugged proximal end of the tubing was inserted into the metal outlet from the detector oven at the appropriate retention time being monitored by a Linear chart recorder. The 'U' tubing was placed in ice during collection and the collected material in the tube was washed with hexane. Trapping efficiency was about 80%. The front and back fractions were bioassayed separately on the field.

RESULTS

When compared with two racemic isomers that were found active earlier (Kikukawa et al. 1982a), the 2S,3R,7R-P attracted more males. However, there was no significant difference among the treatments (Table 7-1). The discrepancy between the activity of the synthetic and natural pheromone of D. similis was partly attributed to either some impurity

Figure 7-1. GLC pattern of the 2S,3R,7R-P on 5% carbowax 20M column. The fractionation pattern into front and back is shown by arrows.

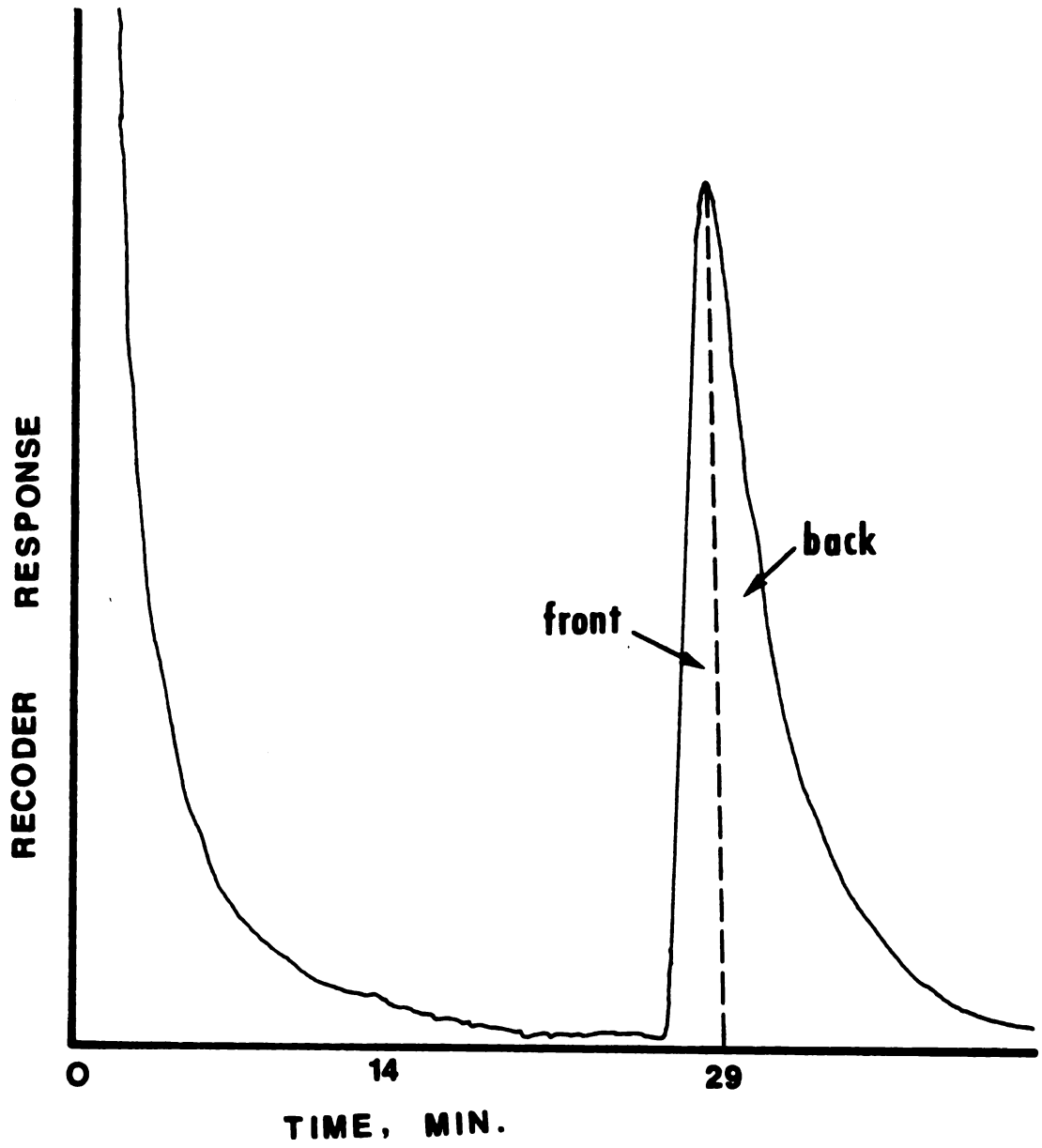
RECODER RESPONSE
front
back
0
14
29
TIME, MIN.

Table 7-1. Field response of D. similis to three enantiomers of 3,7-dimethylpentadecan-2-yl propionate at five locations in Michigan and Siren, Wisconsin. Test conducted June 6-July 20, 1981. Three replicates at each location.

Isomers (20 ug/trap)	Cattle Lake Michigan	Kalkaska Michigan	Mio Michigan	Lansing Michigan	Siren Wisconsin	Total catch
2S,3R,7R-P	2	13	11	0	0	26
2S,3R,7R/S-P	0	14	0	1	0	15
2S,3R,7R/S / 2R,3S,7R/S-P	0	3	5	1	4	13

in the synthetic compound or to a synergist in the natural preparations which they could not find or to the racemic nature of the synthetics used (kikukawa et al. 1982a). First, an attempt was made to purify 2S,3R,7R/S-P. In the results shown in Table 7-2, one can see a surprising finding that the crude 2S,3R,7R/S-P performed better than the pure 2S,3R,7R/S-P (purified through a charcoal-celite column; fraction nos. 43-60). In a 2-day field study using another racemic isomer 2S,3R,7R/S / 2R,3S,7R/S-P, purified in the same way as 2S,3R,7R/S-P, it was found that crude preparations caught 2 males, traps with pure preparations did not catch males. The impurity of 2S,3R,7R/S / 2R,3S,7R/S-P which represented the impurities contained in fraction nos. 33-42 was further concentrated and collected on GLC as described earlier, and added to 2S,3R,7R/S-P and 2S,3R,7R-P isomers. In this trial, the 2S,3R,7R/S-P with a catch of 14 males performed better than 2S,3R,7R-P plus impurity with 2 males and 1 male in the 2S,3R,7R/S-P plus impurity. However, the activity of impurity appeared to increase with dose. It appears to be that there is a synthetic synergist of which retention volume in the celite-charcoal system used in this study comes around fractions 33-42. Four isomers were mixed with 2S,3R,7R-P but no apparent synergistic interactions were present (Table 7-3). Because of low catch in this trial, it can only be speculated that increased concentrations of 2S,3S,7S-P and 2R,3R,7R-P inhibit the activity of 2S,3R,7R-P. A similar conclusion may be made

Table 7-2. Field response of D. similis to crude and purified 2S,3R,7R/S-P isomer. Test conducted at Higgins Lake and Kalkaska, Michigan, May 15-July 20, 1981.

2S,3R,7R/S-P preparation (40 ug/trap)	Series A[a]	Series B[b]	Total Catch
Crude	7	24	31
Pure[c]	1	4	5

[a] Total of 3 replicates at Higgins Lake, May 15-July 20, 1981.

[b] Total of 4 replicates at Kalkaska, July 2-August 4, 1981.

[c] Purified by charcoal-celite column, fraction No. 43-60.

Table 7-3. Response of D. similis to a mixture of 2S,3R,7R-P with four other isomers of diprionid pine sawfly sex pheromone. Conducted May 29-July 23, 1982 in Kalkaska, Michigan.

Amount (ug/trap)	2S,3R,7R-P/ 2S,3S,7S-P[a]	2S,3R,7R-P/ 2S,3R,7S-P[a]	2S,3R,7R-P 2S,3S,7R-P[a]	2S,3R,7R-P/ 2R,3R,7R-P[a]
20/0	6	3	5	5
0/40	0	0	0	0
20/1	0	2	0	1
20/5	1	8	3	2
20/10	3	2	0	0
20/20	1	4	0	0
20/40	7	2	0	0

[a] Total of catches from two replicates.

for 2S,3S,7R-P but a mixture of 2S,3R,7R-P and 2S,3R,7S-P at a ratio of 20:5 attracted more males than the 2S,3R,7R-P alone. The 2R/S,3R,7R-P was indeed more active than 2S,3R,7R-P (Table 7-4) and a clear additive effect was evident when these two isomers were tested as mixtures. Attempt was made to compare the activity of 2R/S,3R,7R-P and one to one mixture of 2R,3R,7R-P and 2S,3R,7R-P. The result showed that the latter mixture was not active (Table 7-5). The most plausible explanation is that 2R/S,3R,7R-P does not contain inhibitory synthetic impurities, while 2R,3R,7R-P and/or 2S,3R,7R-P contain some. It might be possible that this species is very sensitive to inhibitory actions of minor impurities and that the former preparation contains less impurities than the latter. Compared to natural pheromone, assuming a 10 ng pheromone complement per female as in N. sertifer (Kikukawa et al. 1983), the natural pheromone was about 200 fold more active than 2R/S,3R,7R-P (Table 7-6). The synthetic isomer set a threshold at 20 ng is this trial. In the same experiment, a 20:5 mixture of 2S,3R,7R-P:2S,3R,7S-P did not attract any males.

Up to this point, it is not clear why 2S,3R,7R-P was less active towards D. similis than 2R/S,3R,7R-P if the most active chiral arrangement of diprionol for this species is supposed to be 2S,3R,7R. The problem of impurity did not first appear to be the case since 2S,3R,7R-P appeared clean by GLC checking. No other isomer appeared to exercise any appreciable synergistic effect. Two possibilities to

Table 7-4. Response of D. similis to a mixture of 2R/S,3R,7R-P and 3S,3R,7R-P isomer. Test conducted June 30-August 31, 1982 at Kalkaska, Michigan.

Preparations	Amount (ug/trap)	Replicates A	B	C	Total catch
2S,3R,7R-P	5	1	1	0	2
2R/S,3R,7R-P	5	8	6	0	14
2R/S,3R,7R-P / 2S,3R,7R-P	5/0.01	1	0	0	1
	5/0.1	3	1	2	6
	5/1	1	4	2	7
	5/5	7	11	0	18

Table 7-5. Response of D. similis to synthetic 2R/S,3R,7R-P and a mixture of 2S,3R,7R-P and 2R,3R,7R-P. Test conducted at Kalkaska, July 23-August 13, 1982.

Preparations	Amount (ug/trap)	Replicates A	B	C	Total catch
2S,3R,7R-P	20	10	21	21	52
2R,3R,7R-P / 2S,3R,7R-P	10/10	0	0	0	0

Table 7-6. Field response of D. similis to varying concentrations of 2R/S,3R,7R-P isomer and natural pheromone. Test conducted in Kalkaska, Michigan, June 9-July 23, 1983.

Preparations	Amount	x Catch/trap*
2R/S,3R,7R-P	20.00 ug	21.7
	5.00 ug	4.7
	0.02 ug	0.1
Natural Pheromone		
D. similis female equivalent (F.E.)	2.00 FE	12.1
	0.20 FE	2.3
	0.02 FE	0.1

* Means of 3 replicates for synthetic, 7 for natural.

explain the above problem were considered. First, the inhibitory substance is one of the enantiomer of 2S,3R,7R-P. Second, it is possible that a synthetic inhibitor which is not an enantiomer, but has the same retention time with the 2S,3R,7R-P on the chromatographic systems (GLC, TLC and column chromatograph) is present. Testing the first possibility was not an easy one because there is no convenient method to measure the optical purity of each active carbon in the final synthetic product. Even the NMR spectroscopic analysis which gives information on the diastereometric arrangement on 2-carbon and 3-carbon (Jewett et al. 1976) is reliable up to about 99%. However, it is known that the erythro and threo isomers could be separated by a carbowax 20 M column even though the order of retention was not indicated (Mori et al. 1978). However, an attempt was made to collect the front and back portion of the peak of 2S,3R,7R-P as depicted in Figure 7-1. The same approach could also test the second possibility that 2S,3R,7R-P peak contain other inhibitors. When that was done and field tested (Table 7-7), the results clearly indicated that the front part was much more active than the back part. We expected all erythro isomers to come at the front part, therefore this observation excludes the possibility that any of erythro enantiomers are the inhibitors. The observation, therefore, favors the second possibility that contaminants which align with the back part of the peak inhibit the effectiveness of 2S,3R,7R-P. Indeed for the first time, we

Table 7-7. Response of *D. similis* to synthetic 2R/S,3R,7R-P and a mixture of 2S,3R,7R-P and 2R,3R,7R-P. Test conducted at Kalkaska, July 23-August 13, 1982.

Preparations	Amount (ug/trap)	Replicates A	B	C	Total* catch
2S,3R,7R-P GLC front	40	6	14	6	26
	20	0	0	2	2
	10	2	4	6	12
2S,3R,7R-P GLC back	40	0	0	2	2
	20	0	1	3	4
	10	1	1	2	4
2S,3R,7R-P GLC back/ 2S,3S,7R-P	40/1	2	1	0	3
	40/0.1	2	4	14	20
2S,3R,7R-P GLC front/ 2S,3R,7R-P GLC back	10/10	1	0	3	4
2R/S,3R,7R-P	40	0	1	1	2
D. similis (propionate)	0.2 FE	17	0	0	17

* Analysis of variance not significant.

could show that a 2S,3R,7R-P preparation (GLC front) was more effective than 2R/S, 3R,7R-P. Further combinations of 2S,3R,7R-P GLC front with 2S,3S,7R-P and 2S,3S,7S-P showed that 2S,3S,7R-P was a better synergist and the most effective combination occurred at ratio 40:0.003 (Table 7-8). The mixture again was far more superior than R/S,3R,7R-P and the natural pheromone in this trial. A more thorough comparison with the natural pheromone showed that there appeared for the first time to be identical effectiveness of the synthetic mixture and natural pheromone (Table 7-9). Synthetic mixture as low as 1 ng attracted males while control traps did not. One other isomer, 2S,3R,7S-P, showed synergism to 2S,3R,7R-P GLC front preparation at ratio 40:0.003 (Table 7-10). Large variance between replicates when 2R,3R,7R-P; 2R,3R,7S-P and 2R/S,3S,7S-P were used diluted their consideration as good synergists even though at some concentrations they attracted a fair number of males. It should be noted that the 2S,3R,7R-P GLC back preparation as well as back fraction of all the candidate synergist isomers when mixed with 2S,3R,7R-P GLC front was not active whatsoever. It is thus clear that there is a synthetic inhibitor whose retention time aligns with the back fraction.

In 1984, we pursued further the aspect of synergistic interaction of some of the isomers with 2S,3R,7R-P as observed earlier and found that 2R/S,3S,7R-P; 2S,3S,7R-P and 2S,3R,7S-P isomers are indeed synergistic to 2S,3R,7R-P in

Table 7-8. Field response of D. similis to mixtures of isomers 2S,3R,7R-P front/2S,3S,7R-P and 2S,3R,7R-P front/2S,3S,7S-P at varying ratios. Test conducted in Kalkaska, Michigan, June 15-28, 1983.

Amount (ug/trap)	2S,3R,7R-P front/ 2S,3S,7R-P[a]	2S,3R,7R-P front/ 2S,3S,7S-P[a]
40/0	10	10
40/0.001	6	6
40/0.003	23	5
40/0.01	8	6
40/0.03	13	2
40/0.1	4	5
40/0.3	3	0
40/1	0	1
40/0	0	0

[a] Trap catch of 3 replicates. Forty ug of 2R/S,3R,7R-P and 1 female equivalent of D. similis natural pheromone set on the field at the same time and replication did not catch males.

Table 7-9. Threshold limit and comparison of the synthetic with the natural pheromone of *D. similis*. Test conducted in Kalkaska, Michigan, June 21-July 30, 1983.

Preparations	Amount (ug/trap)	Replicates A	B	C	Total catch
Synthetic mixture					
2S,3R,7R-P/2S,3S,7R-P 40:0.003	40	0	2	2	4
	10	0	0	0	0
	3	0	2	3	5
	1	0	0	1	1
	0.3	0	0	1	1
	0.1	0	0	0	0
	0.03	1	1	0	2
	0.01	0	0	1	1
	0.003	0	0	0	0
	0.001	1	0	0	1
Control (no pheromone)	0	0	0	0	0
D. similis (propionate)	3 FE	0	0	0	0
	1 FE	0	0	2	2
	0.3 FE	1	0	0	3

Table 7-10. Response of D. similis to mixtures of 2S,3R,7R-P with six isomers at varying concentrations. Test conducted in Kalkaska, Michigan, June 15-28, 1983.

Preparations	Amount (ug/trap)	Replicates A	Replicates B	Total catch
2S,3R,7R-P / 2R,3R,7R-P	40/0.003	0	5	5
	40/0.001	3	1	4
	40/0.003	1	2	3
	40/0.01	0	48	48
	40/0.03	2	0	2
	40/0.1	0	17	17
2S,3R,7R-P / 2R,3R,7S-P	40/0	2	3	5
	40/0.001	1	0	1
	40/0.003	3	2	5
	40/0.01	30	0	30
	40/0.03	0	5	5
	40/0.1	0	41	41
2S,3R,7R-P / 2S,3S,3R-P	40/0	1	4	5
	40/0.001	2	2	4
	40/0.003	8	8	16
	40/0.01	2	3	5
	40/0.03	0	10	10
	40/0.1	2	1	3
2S,3R,7R-P / 2S,3S,7S-P	40/0	1	8	9
	40/0.001	4	1	5
	40/0.003	0	4	4
	40/0.01	1	0	1
	40/0.03	1	1	2
	40/0.1	0	3	3
2S,3R,7R-P / 2R/S,3S,7S-P	40/0	0	0	0
	40/0.001	2	8	10
	40/0.003	40	2	42
	40/0.01	4	0	4
	40/0.03	0	2	2
	40/0.1	0	0	0
2S,3R,7R-P / 2S,3R,7S-P	40/0	3	2	5
	40/0.001	1	0	1
	40/0.003	41	21	62
	40/0.01	3	1	4
	40/0.03	5	11	16
	40/0.1	0	8	8
2R/S,3R,7R-P	40	1	0	1

attracting male D. similis on the field (Table 7-11). The 2S,3S,7R-P isomer was the best synergist of the three at blend ratio of around 80:0.06 and 80:0.006 of 2S,3R,7R-P to 2S,3S,7R-P. The poorer performance of 2R/S,3S,7R-P as a synergist indicated that interaction on 2-carbon is not important. That 2S,3S,7R-P and 2S,3R,7S-P isomers were synergistic suggested that the synergist interaction occurred mainly on 3-carbon and to some extent on 7-carbon. Synergistic effect of 2S,3S,7R-P was again proved and optimum blend ratio of 80:0.006 of 2S,3R,7R-P to 2S,3S,7R-P confirmed (Table 7-12). At the same location and time, 2S,3S,7S-P isomer at a higher concentration than earlier tested and at a much higher concentration than 2S,3S,7R-P isomer, gave a superior synergism to 2S,3R,7R-P isomer (Table 7-13). The optimum blend ratio is 80:16 of 2S,3R,7R-P to 2S,3S,7S-P.

DISCUSSION

One common observation about D. similis in the field is that the males are more discriminating than the Neodiprion species in response to sex pheromone regarding optical and non-optical impurities, correctness of the chirality of the diastereomers and choice of ester moiety. For example, while species like Neodiprion sertifer (Geoffroy) (Kikukawa et al. 1983) and N. nanulus nanulus Schedl (Kraemer et al. 1983) two normally acetate species, would respond

Table 7-11. Synergistic interactions of three isomers on 2S,3R,7R-P isomer in attracting male D. similis. Test conducted at Kalkaska, Michigan, June 1-16, 1984.

Isomer	Amount (ug)	Mean Catch/Trap ± S.E.*
2S,3R,7R-P	80	8.3 ± 0.7[b]
2S,3R,7R-P / 2R/S,3S,7R-P	80/0.02	14.3 ± 1.2[a]
	80/2	10.0 ± 0.8[b]
2S,3R,7R-P / 2S,3R,7S-P	80/0.006	14.7 ± 0.9[a]
	80/0.06	21.0 ± 1.7[a]
2S,3R,7R-P / 2S,3R,7S-P	80/0.006	8.3 ± 0.7[b]
	80/0.06	13.3 ± 1.4[a]
2S,3R,7S-P	80	1.3 ± 0.4[b]
	312	9.7 ± 0.7[b]

* Means of 3 replicates. Means followed by same letter not significantly different at 5% level by Duncan's Multiple Range Test.

Table 7-12. Synergistic interaction of 2S,3S,7R-P isomer with 2S,3R,7R-P isomer in attracting male D. similis on the field. Test conducted at Kalkaska, Michigan, June 7-16, 1984. Three replicates randomized twice.

Isomer	Amount (ug)	Mean Catch/Trap* ± S.E.
2S,3R,7R-P / 2S,3S,7R-P	80/0	8.3 ± 6.7[b]
	80/0.006	35.0 ± 2.1[a]
	80/0.06	26.0 ± 2.3[a]
	80/0.6	27.7 ± 3.0[a]
	80/6	14.3 ± 1.5[b]
	80/25	18.0 ± 1.4[a]
	80/80	0.3 ± 0.4[b]
	0/0.006	0 ± 0[b]
	0/25	0 ± 0[b]
	0/80	0 ± 0[b]
Control	0	0 ± 0[b]

* Means followed by same letters not significantly different at 5% level by Duncan's Multiple Range Test.

Table 7-13. Synergistic interaction of 2S,3S,7S-P isomer with 2S,3R,7R-P isomer in attracting male D. similis on the field. Test conducted at Kalkaska, Michigan, June 7-16, 1984. Three replicates randomized twice.

Isomer	Amount (ug)	Mean Catch/Trap* ± S.E.
2S,3R,7R-P / 2S,3S,7S-P	80/0	8.3 ± 0.7[c]
	80/4	45.3 ± 1.7[a]
	80/16	51.0 ± 1.9[a]
	80/20	17.0 ± 1.7[b]
	80/40	20.7 ± 1.3[b]
	80/80	1.7 ± 0.8[c]
	0/16	0 ± 0[c]
	0/80	0 ± 0[c]
Control	0	0 ± 0[c]

* Means followed by same letters not significantly different at 5% level by Duncan's Multiple Range Test.

considerably to nonpurified pheromone and propionate ester of (2S,3S,7S)-3,7-dimethylpentadecan-2-ol (2S,3S,7S-P), males of D. similis strictly prefer propionate and any impurity in the pheromone will result in little or no response as demonstrated in this study. The practical implication of this strict chemical and optical requirements may be higher cost of production of synthetic pheromone.

There are two intriguing aspects of the results presented. First, that the charcoal-celite purified pheromone was less active than the crude and that the front portion of pheromone peak was active and the back portion of the same peak was not. The fractions 34-42 of the charcoal-celite column must be seen as equivalent to the GLC front portion of pheromone peak because they elicited the same positive behavioral response on the field. This study proved conclusively the presence of a synthetic inhibitor which aligns with the back part of the pheromone peak as the removal of the back part for all the isomers increased activity of the front part.

Second, and most intriguing result is that 2S,3S,7R-P and 2S,3S,7S-P interact synergistically with 2S,3R,7R-P in attracting male D. similis on the field. This constitutes a novel information as this was the first time an isomer that is R on the 3-carbon and 7-carbon will be synergized by one that is S on 2-carbon and 3-carbon, among diprionid sawflies. It is a direct opposite of the situation found among pheromone system of Neodiprion species where 2S,3S,7S isomer

is the major and 2S,3R,7R or 2S,3R,7S is the synergist (Kikukawa et al. 1983; Olaifa et al. 1984). However, one similarity is evident, in all cases synergism occurs at a specific optimum combination, response is dose related up to that optimum and above the optimum response-inhibition sets in. In D. similis optimum blend appeared at concentrations where 2S,3S,7R-P constituted about 0.06-0.2% of the mixture and when 2S,3S,7S-P constituted about 4.8-16% of the mixture.

The evolutionary significance of this type of pheromone system in D. similis may be reproductive isolation from the Neodiprion species and N. pinetum (Norton) in particular. These two species share the white pine Pinus strobus Linnaeus as the preferred host plant. Apart from being widely divergent with respect to choice of ester moiety, acetate for N. pinetum and propionate for D. similis, the males of the former do not respond to 2S,3R,7R isomer alone even though this isomer constituted about 66% of its optimum pheromone mixture (unpublished data). It may be concluded from our experience with the pheromone systems in these two species that the sex pheromones constitute effective reproductive isolation as no male N. pinetum was ever caught in traps baited with pheromone of D. similis be it synthetic or natural at the concentrations tested.

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CHAPTER EIGHT

Gas Liquid Chromatograph (GLC) Separation of the Optically Active Synthetic and Natural Sex Peromone of Diprionid Pine Sawfly Species

INTRODUCTION

Shortly after the first identification of an insect pheromone (Karlson and Butenandt, 1959), Coppel et al. (1960) presented evidence for a potent sex attractant in the in the introduced pine sawfly, Diprion similis (Hartig) based on field studies. Casida et al. (1963), despite extensive chromatographic purification of either extracts of 200 adult virgin female of D. similis, could not elucidate the chemical nature of the sawfly sex pheromone. A fifteen-year effort at chemical elucidation by the group of scientists in University of Wisconsin culminated in the report by Jewett et al. (1976) of esters of 3,7-dimethylpentadecan-2-ol (diprionol) as the major components of the sex pheromone from females of three species belonging to two genera of pine sawflies--D. similis, Neodiprion sertifer (Geoffroy) and Neodiprion lecontei (Fitch). The acetate and propionate of diprionol are utilized by many sawfly species as the major sex attractant. When bioassayed on the field, the acetate of synthetic racemic diprionol was active only at the level of 750 ug and 200 ug/trap for N. lecontei and N.

swainei respectively in the field. Further studies by the same group later found that male sawfly responds only to optical isomers and that the major pheromones in many Neodiprion species consists of 3,7-dimethylpentadecan-2-yl acetate (2S,3S,7S-A) or propionate (2S,3S,7S-P) (Matsumura et al. 1979; Kraemer et al. 1979, 1981 and 1983). Males of D. similis and Gilpinia frutetorum Fabricius, on the other hand, respond mainly to 2S,3R,7R-P and 2S,3R,7R-A respectively (Kikukawa et al. 1982). It was later found that while 2S,3S,7S-A remained the major isomer in N. sertifer and Neodiprion pratti banksianae Rohwer, addition of optimum concentration of threo isomer (2S,3R isomers) was synergistic to the field effectiveness of the former isomer (kikukawa et al. 1983, Olaifa et al. 1984). This information provided behavioral evidence that sex pheromone of diprionid sawfly is multi-isomer in composition. There is a morphological evidence too in the olfactory receptors in males of N. sertifer being innervated by 8-12 neurons as against few in the moth Bombyx mori (Hallberg and Lofquist, 1981). It is believed that such multineuron innervating the dominant receptor is an indication of a well-developed multicomponent pheromone. Mori et al. (1978) indicated that the erythro (i.e., isomers with either 2S,3S or 2R,3R) can be separated from the threo (i.e., isomers with 2S,3R or 2R,3S) isomers by GLC using carbowax 20 M column. However, these authors did not specify whether the erythro or threo comes first. Capillary GLC analysis using carbowax 20 M column

showed that the natural pheromone from body extracts of female N. sertifer was identical with the synthetic acetate of diprionol in its GLC behavior (Kikukawa et al. 1983). In that study, only one pheromone peak was found and one female was estimated to produce 10 ng pheromone. This study reports on the separation of the erythro-threo synthetic optical isomers as well as separation of optical isomers in the natural pheromone of diprionid sawflies.

MATERIALS AND METHODS

Virgin females were extracted with ether as described for D. similis (Kikukawa et al. 1982). The combined ether extract was evaporated in a rotary evaporator. The oily residue was picked up in ether and spotted on preparative TLC Silica gel 60 G HF $_{254+366}$ (Mc/B Manufacturing Chemists Co., Cincinnati, Ohio). The plate was developed in hexane ether (4:1) until 15 cm from the origin. Zone corresponding to alcohol (R_f 0.06 - 0.1) was collected and eluted with ether. Ether was removed and the alcohol fraction was acetylated by adding 2 ml pyridine and 1 gm of acetic anhydride to the residue in the reaction tube. The mixture as refluxed for 5 hrs. The reaction product was dissolved in hexane and washed with water four times. The organic phase was dried over $MgSO_4$ and evaporated in a rotary evaporator. The residue was spotted on a second TLC and developed with hexane:ether (4:1). The zone corresponding to ester fraction (R_f 0.45 - 0.56) was collected, eluted with ether,

spotted on another TLC plate and developed first in n-hexane and then in benzene. The ester zone corresponding to R_f 0.75 - 0.85 was collected and eluted with ether. This semi pure ester fraction was concentrated in n-hexane for further purification by the preparative GLC fraction collection.

Preparative GLC Fraction Collection

A Varian 1700 preparative gas chromatograph fitted with stainless steel column, 7.2 m and 3 mm i.d., packed with 10% carbowax 20 M on 80-100 mesh chrom Q was used for fraction collection of acetate of diprionol. A 9:1 post column splitter was used which directed 90% of the injected material for collection and 10% to the detector. A length of pyrex tubing bent into a 'U' at the middle was used for collection. Hexane moistened glass wool was used to plug the distal end of the tubing. Also, the bent portion of the tubing contained a small amount of hexane solvent to monitor the flow of carrier gas. The unplugged proximal end of the tubing was inserted into the heated metal outlet from the detector oven. The retention time of natural pheromone was determined first by injecting synthetic standard, and then by coinjecting the standard with natural pheromone at two column temperatures 200° and 220°. The appropriate retention was monitored by a linear chart recorder. The 'U' tubing was placed in ice during collection. After collection first at 200°, the collected pheromone in the tube was washed with hexane. This solution was concentrated and injected again into the GLC for a second collection at 220°.

The collected pheromone in the tube is again washed with hexane, and concentrated for capillary GLC analysis.

Capillary GLC Analysis

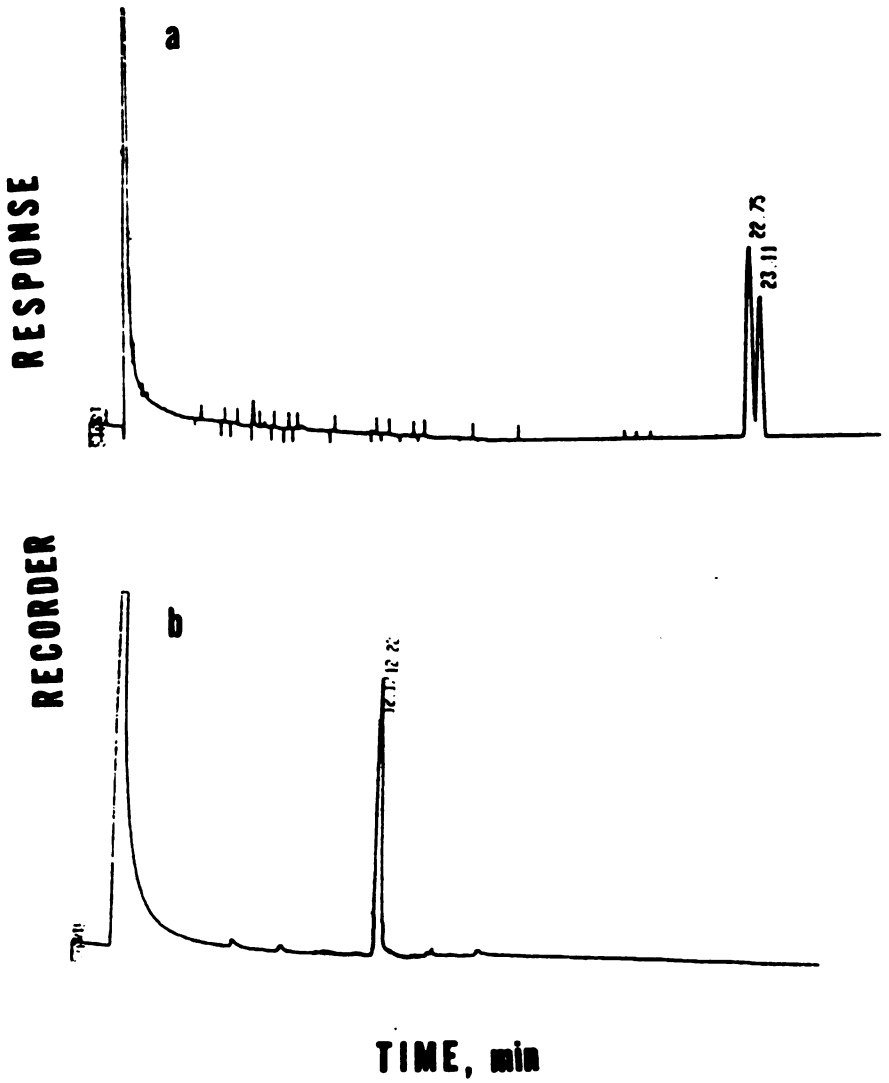
A Varian 3700 capillary GLC equipped with two FID was used. Synthetic standards containing 1 mg/ml of 2S,3S,7S-A, 2S,3R,7S-A and mixtures at ratios 1:2, 1:1, 3:2 and 2:1 of 2S,3S,7S-A / 2S,3R,7R-A and 2S,3S,7S-A / 2S,3R,7S-A were used. Natural extracts derived from purification above was used. Concentration of natural pheromone was in the order of 5000 female equivalents/ml. Two capillary columns, both fused silica, carbowax 20 M, 30 m x 0.25 mm i.d., and DB5, 40 m x 0.25 mm i.d. were used. These systems were found to be capable of resolving threo diastereomers from erythro counterparts. Runs were made isothermally at 180°C and temperature programmed from 130-190° at 4°/min, with initial hold at zero minute, and final hold of 18 min. such that total analysis time for one injection was 32 minutes.

RESULTS

By using two capillary systems DB5 (40 m x 0.25 mm i.d.) and carbowax 20 M (30 m x 0.25 mm i.d.) 2S,3S,7S-A and 2S,3R-7R-A were separated. At 180° (isothermal), retention time of 2S,3S,7S-A was 12.17 minutes and 22.75 minutes on carbowax 20 M and DB5 columns respectively. The retention time for 2S,3R,7R-A in the same system was 12.22 minutes and 23.11 minutes respectively (Figure 8-1). Also separated on DB5 from 2S,3S,7S-A was 2S,3R,7S-A with a retention time of

Figure 8-1. Capillary GLC separation of 2S,3S,7S-A / 2S,3R,7R-A mixture.

(a) Ratio 3:2, 80 ng, using DB5 column
(b) Ratio 2:3, 80 ng, using carbowax 20 M column


a
RESPONSE
22.75
23.11
b
RECORDER
TIME, min

20.69 and 20.91 minutes respectively. On the average, 0.38 minutes separated 2S,3S,7S-A and 2S,3R,7R-A, while 0.20 minutes separated 2S,3S,7S-A and 2S,3R,7S-A on DB5 column at 180°C. While the acetate can be separated, none of these systems could separate the alcohol preparations of 2S,3S,7S and 2S,3R,7R. However, injected singly, 2S,3S,7S-OH has a retention of 26.42 minutes while 2S,3R,7R-OH has a retention time of 27.07 minutes on DB5 temperature programmed 130-190°C at 4°/minute. Also, when temperature programmed, the synthetic 2S,3S,7S-A has a retention time on the average of 30.20 minutes while 2S,3R,7R-A was retained for 30.53 minutes. They were thus separated by 0.33 minutes. Under the same condition, 2S,3R,7S-A had a retention time of 30.40 minutes, thus being separated from 2S,3S,7S-A by 0.20 minutes and from 2S,3R,7R-A by 0.13 minutes. This result therefore shows for the first time that 2S,3S,7S-A, 2S,3R,7S-A and 2S,3R,7R-A can be separated and recognized by using retention time. Also, separations of 2S,3S,7S-A and 2S,3R,7R-A as well as 2S,3S,7S-A and 2S,3R,7S-A were by the same retention times on DB5 column either when run at isothermal 180° (Figure 8-2) or temperature-programmed 130-190°C at 4°/minute (Figure 8-3). However, the mixture of the 2S,3R,7R-A / 2S,3R,7S-A mixture could not be separated in our system. Separation occurs only when either 2S,3R,7R-A or 2S,3R-7S-A is in mixture with 2S,3S,7S-A.

We were able to isolate diprionol from nine species of diprionid sawflies (Figure 8-4). In the N. lecontei, N.

Figure 8-2. Capillary GLC separation on DB5 column at isothermal 180°C.

(a) 2S,3S,7S-A / 2S,3R,7S-A mixture, 100 ng
(b) 2S,3S,7S-A / 2S,3R,7R-A mixture, 100 ng

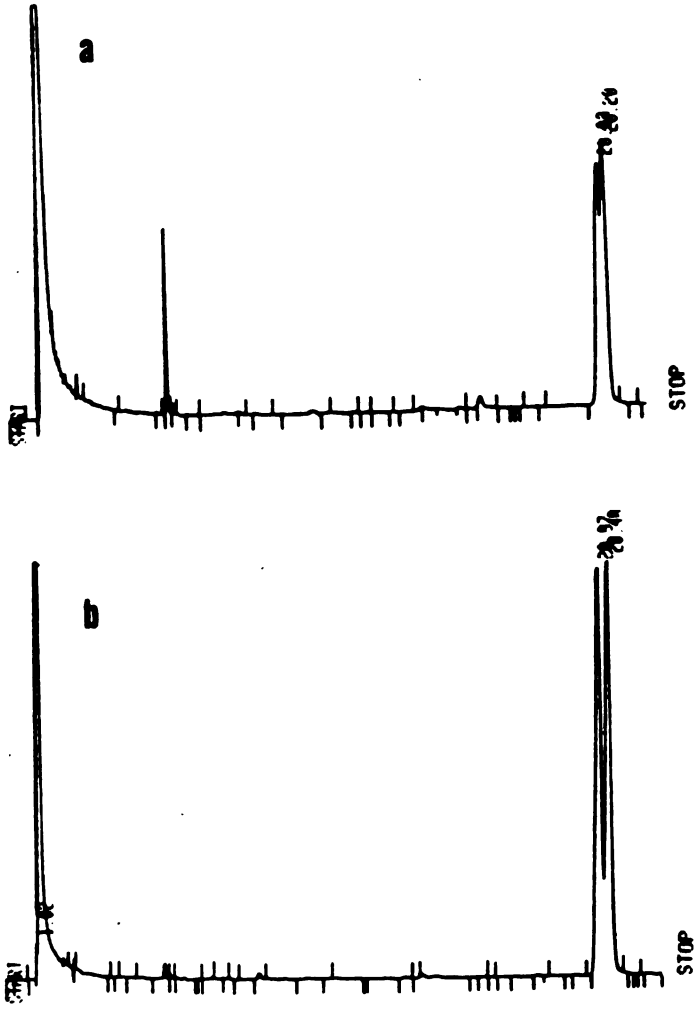
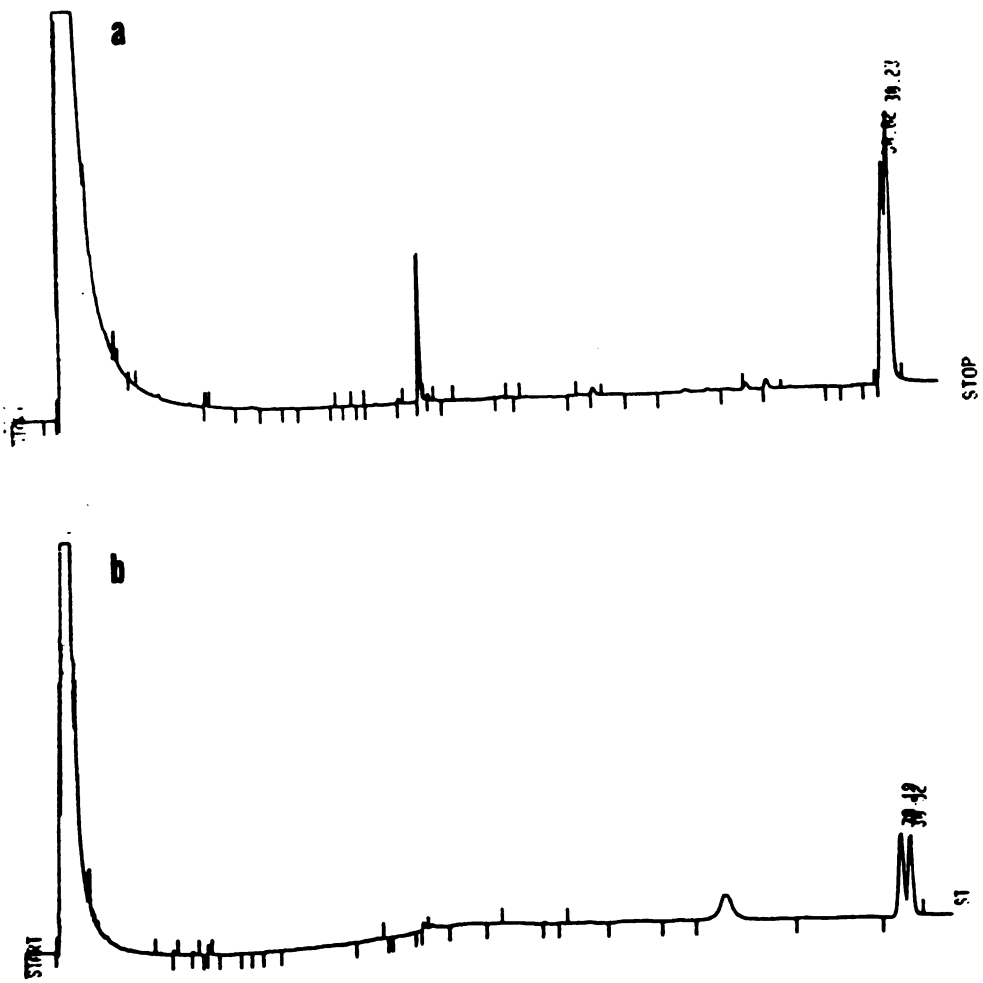

a
STOP
b
STOP

Figure 8-3. Capillary GLC separation on DB5 column temperature programmed 130-190°C at 4°/min.

(a) 2S,3S,7S-A / 2S,3R,7S-A mixture 100 ng
(b) 2S,3S,7S-A / 2S,3R,7R-A mixture 50 ng

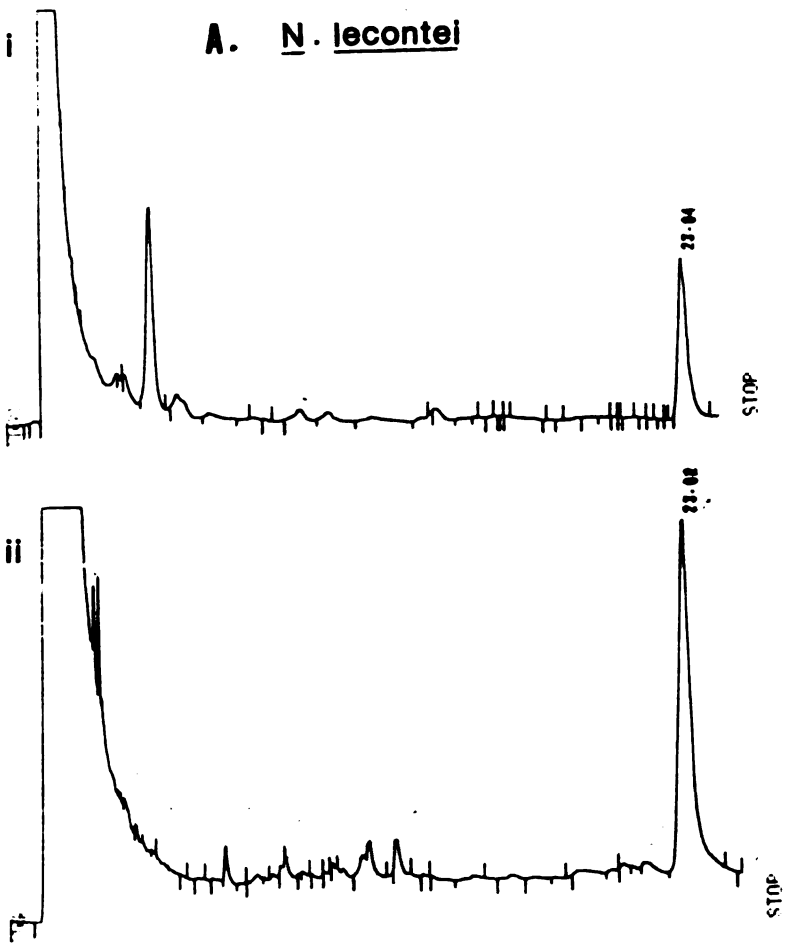

a
STOP
b

nanulus nanulus Schedl and *N. sertifer* only 2S,3S,7S isomer was detectable. On spiking the samples with 2S,3S,7S-A, only one peak was detected for each of the species (Figure 8-4 a, b and c). In the *N. pratti banksianae*, *N. rugifrons* Middleton and *N. nigroscutum* Middleton, fairly fat peaks corresponding to 2S,3R,7S-A were observed. When spiked with 2S,3S,7S-A, two peaks were observed for each of the species (Figure 8-4 d, e and f). In the case of *D. similis*, two peaks were observed. The major of the two peaks corresponds to 2S,3R,7R-A while the smaller peak corresponds to 2S,3S,7S-A (Figure 8-4 g, h and i). The measurement of the peak interval gave the indication that the natural pheromone could be 2S,3S,7S-A and 2S,3R,7R-A. However, on spiking the sample with 2S,3S,7S-A, two peaks were also detected with the smaller peak in the natural pheromone corresponding to 2S,3S,7S-A. The measurement of the peak interval this time gave the indication that the major peak in the natural pheromone could be 2S,3R,7S-form. The natural pheromone of *N. virginianus* indicated two peaks corresponding to 2S,3S,7S and 2S,3R,7S forms in ratio 1:1 (Figure 8-4 h). On spiking with 2S,3S,7S-A, one off-scale peak was observed which was separated out by computer printout of retention times into two peaks with peak interval corresponding to that between 2S,3S,7S-A and 2S,3R,7S-A. Also, the natural pheromone of *N. pinetum* indicated two peaks (Figure 8-4 i). On spiking the sample with 2S,3S,7S-A, one fat off-scale peak was observed which was separated out by the computer into peaks

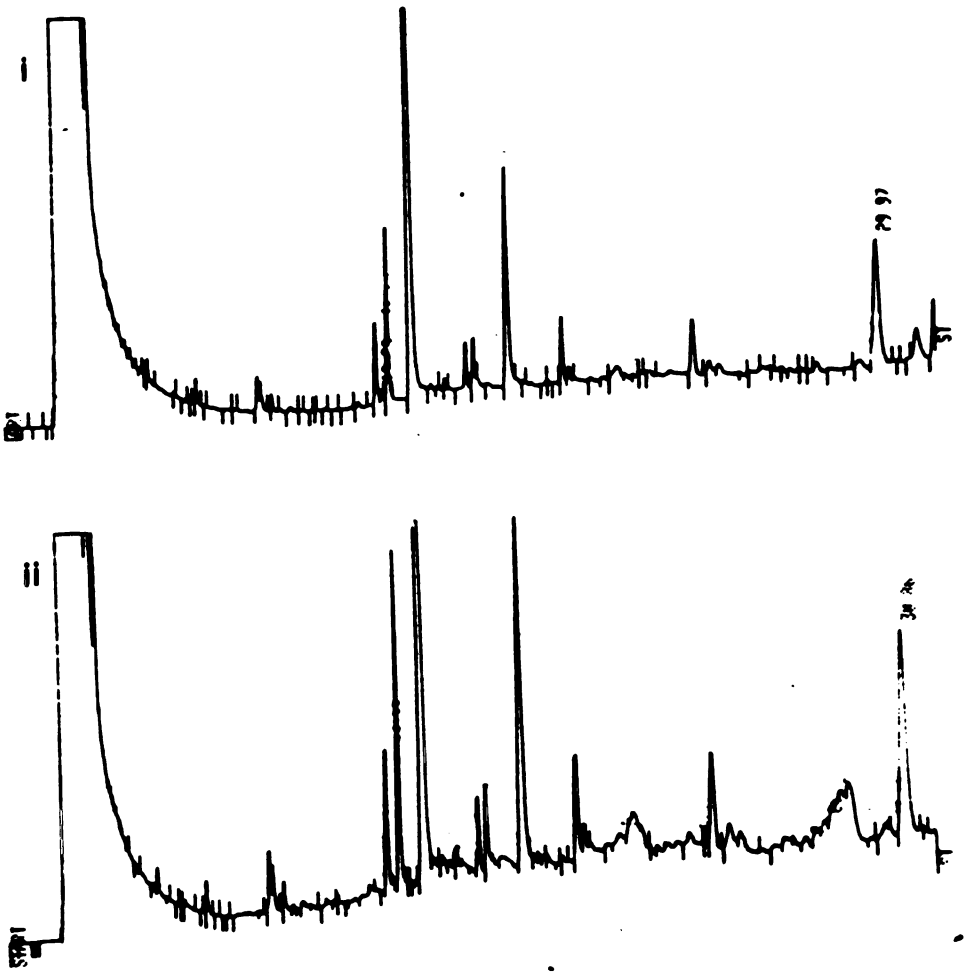
Figure 8-4. (i) Capillary GLC recordings of acetylated natural pheromone.

A. Neodiprion lecontei 20 Female equivalent (FE)
B. N. nanulus nanulus 60 FE
C. N. sertifer 6 FE
D. N. pratti banksianae 10 FE
E. N. rugifrons 16 FE
F. N. nigroscutum 4 FE
G. Diprion similis 10 FE
H. N. virginianus 8 FE
I. N. pinetum 10 FE

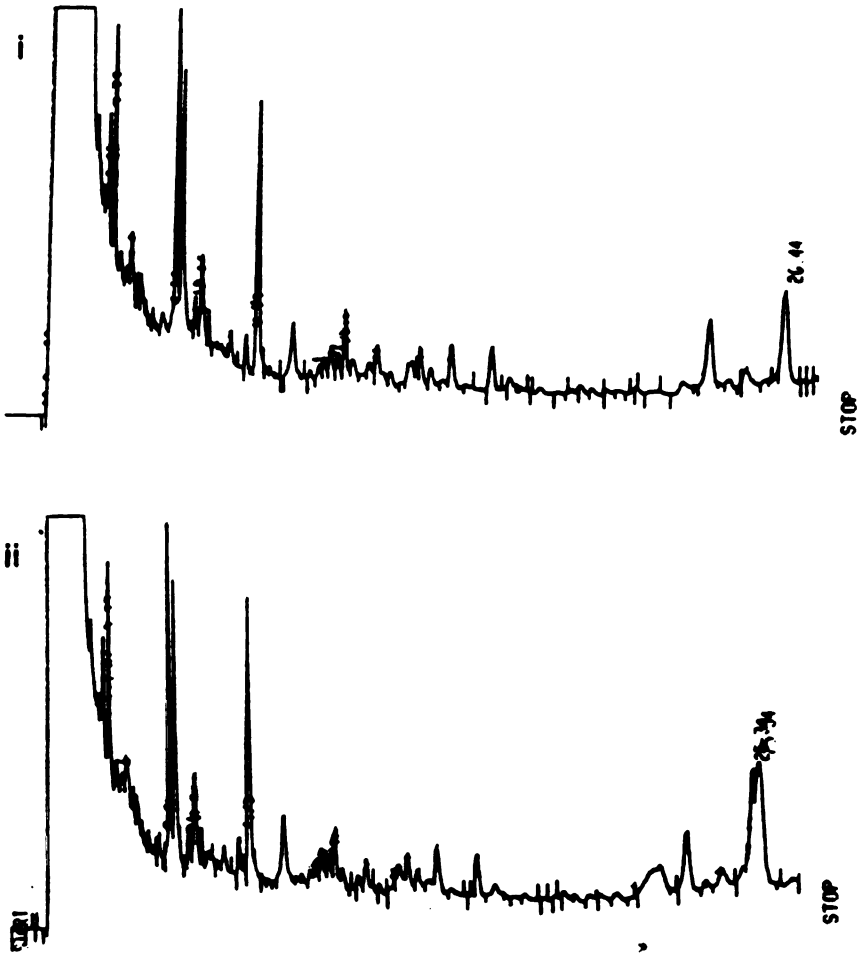
(ii) Capillary GLC recordings of acetylated natural pheromone of species A-I spiked with 2S,3S,7S-A.

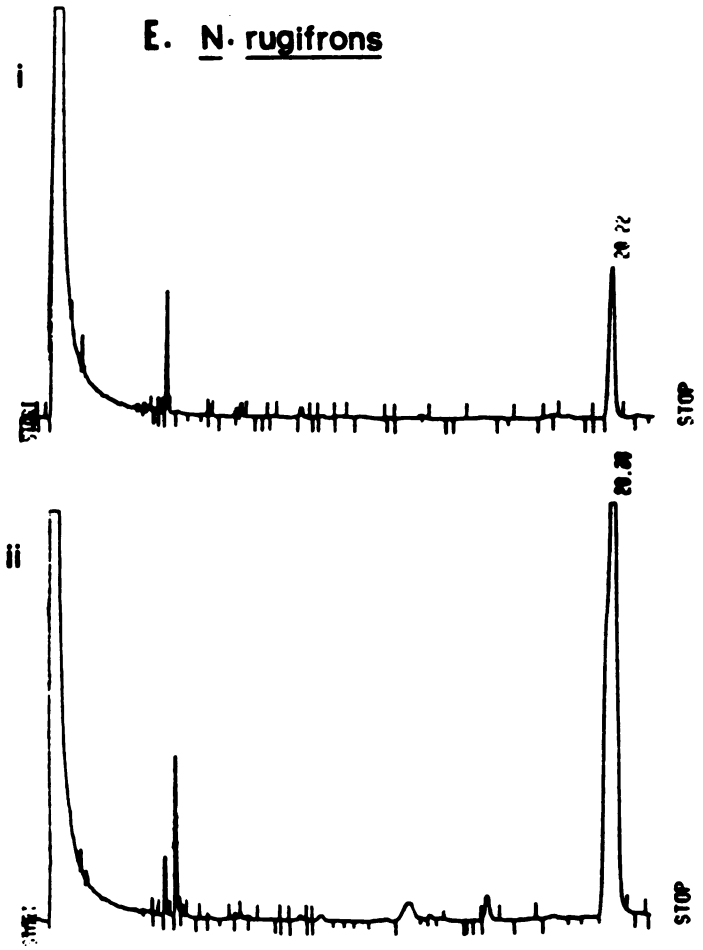

A. N. lecontei
i
ii
STOP
STOP

C. N. sertifer

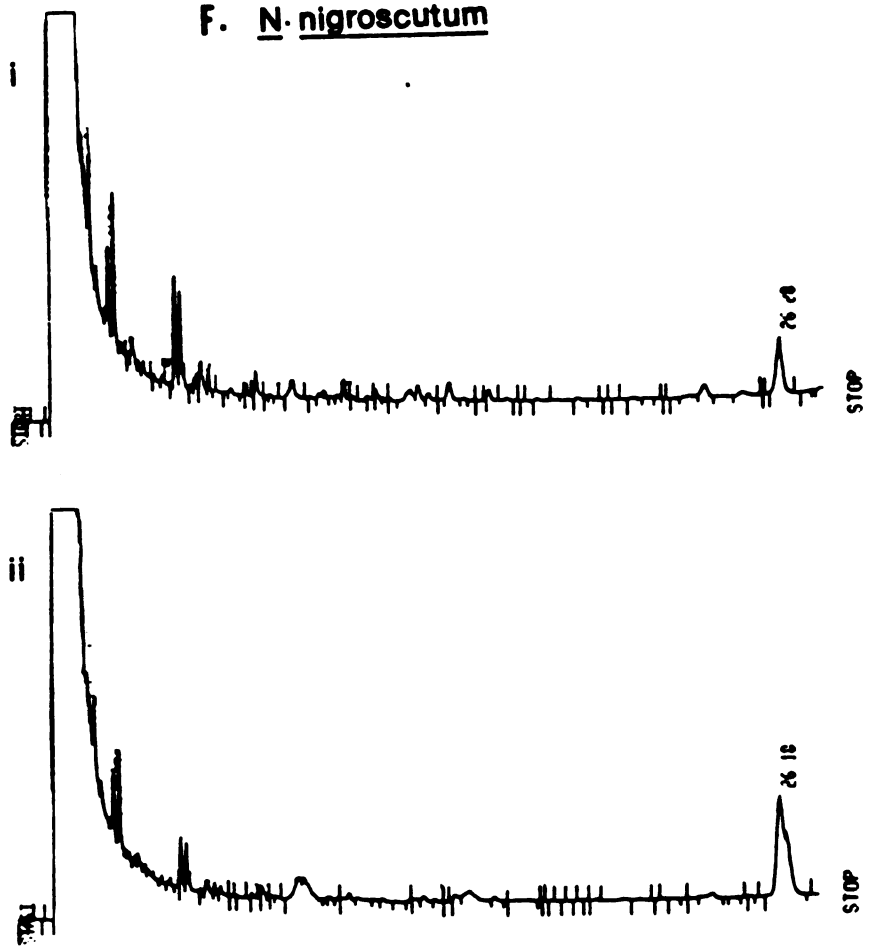


D. N. pratti banksianae

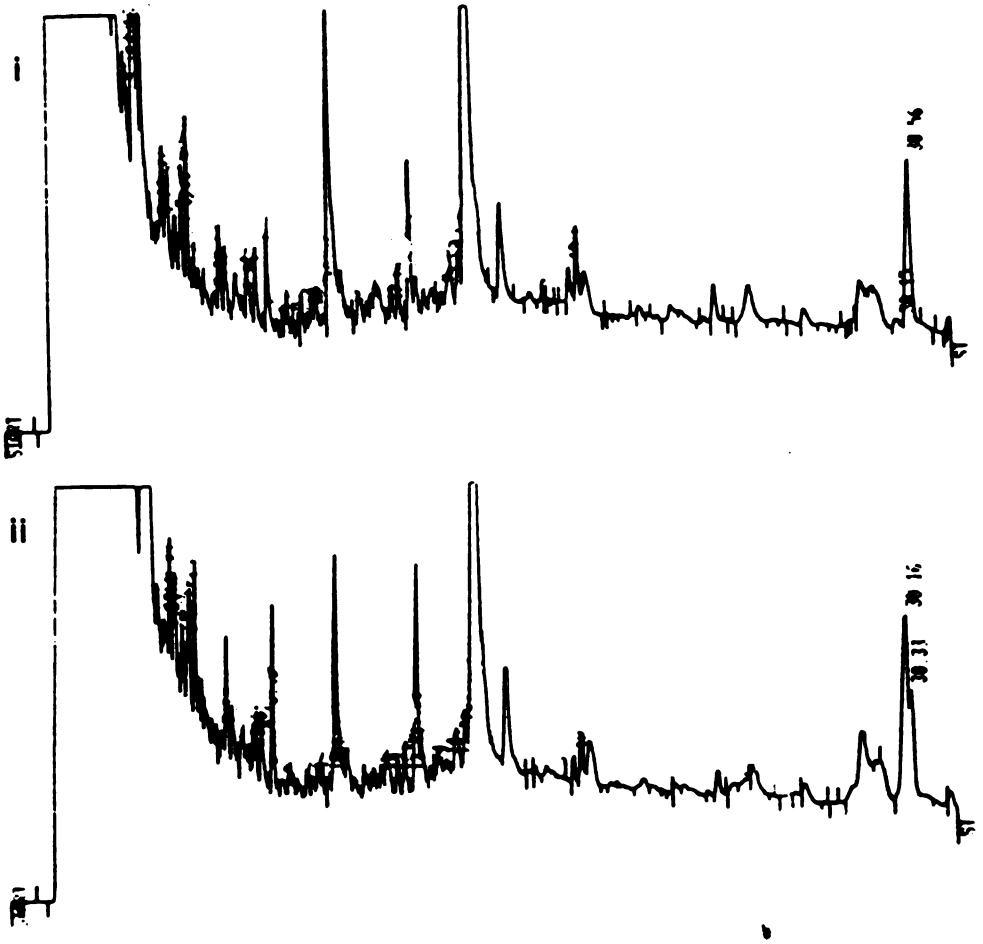



E. N. rugifrons
i
STOP
ii
STOP

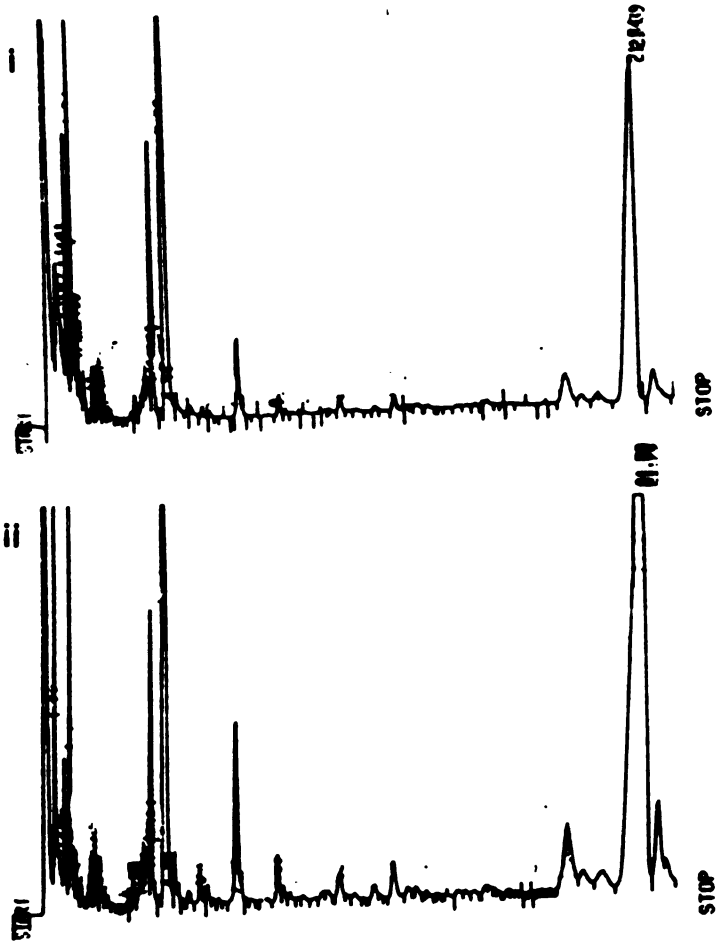
F. N. nigroscutum



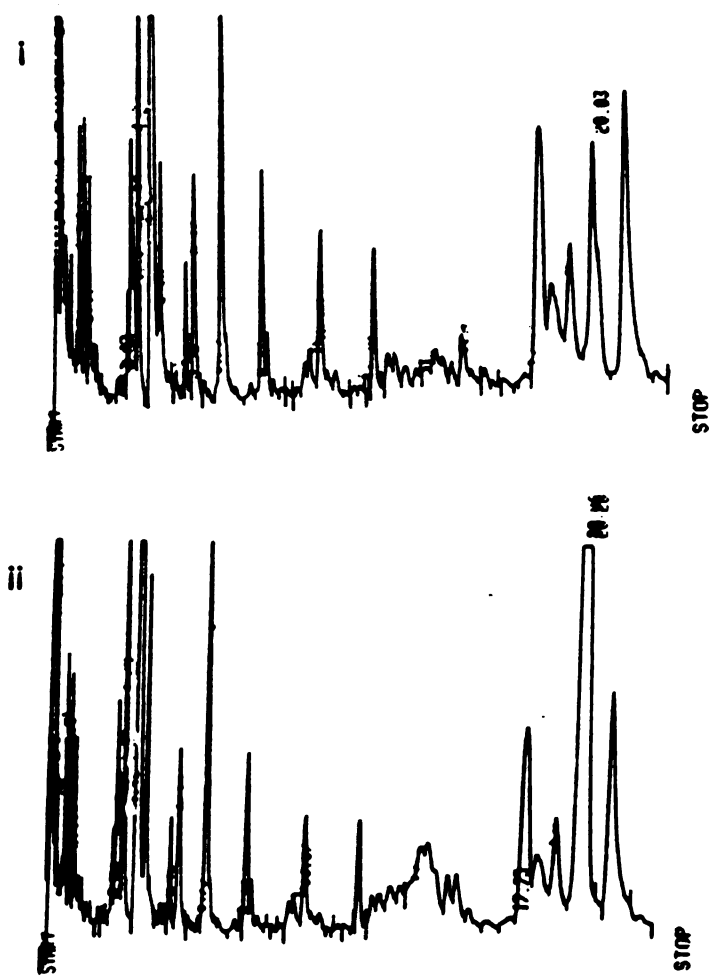
6. D. similis



H. N. virginianus



I. N. pinetum



corresponding to retention times 20.06 and 20.28 minutes respectively. The peak interval of 0.22 minutes thus indicates that 2S,3S,7S and 2S,3R,7S forms may be present.

There were variations in the amount of pheromone extracted from the different species. About 10 ng pheromone per female was extracted from N. sertifer, N. pinetum, D. similis and N. nigroscutum. The female N. lecontei and N. rugifrons had 5 ng each, while N. pratti banksianae had 6 ng. The least amount (2 ng/female) was recorded for N. nanulus nanulus while N. virginianus had the highest (13 ng/female). We could not find any pheromone peak when the initial TLC ester fraction R_f 0.45 - 0.56 was used instead of the alcohol fraction in the case of N. pinetum.

DISCUSSION

Certain assumptions are made in choosing the standard samples, 2S,3S,7S-A; 2S,3R,7R-A and 2S,3R,7S-A isomers, used in this study. The 2S,3S,7S is expected to match with 2R,3R,7R and 2R,3R,7S isomers in retention times and as such the latter ones are not tested. Also, it is expected that 2S,3R,7S should match with 2R,3S,7R, therefore the latter is not tested. By the same reasoning, 2S,3R,7R is expected to match with 2R,3S,7S and 2S,3S,7R and as such the latter ones are not tested. Therefore, an assumption has been made that the natural pheromone isomers should be 2S. This assumption was found to be true on the field. The 2R/S,3R,7R-P isomer markedly attracted more male D. similis than the 2R/S,3R,7S-

P isomer (Kikukawa et al. 1982). However, when 2R/S,3R,7R-P was compared to 2S,3R,7R-P on the field, the latter isomer significantly attracted more male D. similis than the former. Both 2R/S,3R,7S-P and 3S,3R,7S-P were not so active on the field but by electroantennogram (EAG) studies, the 2S,3R,7S-P showed a greater response than the 2R/S,3R,7S-P isomer in D. similis. The details of the field studies and EAG on D. similis is published elsewhere. This evidence thus shows that the assumption that the natural pheromone is 2S is right and that the major pheromone in D. similis is 2S,3R,7R.

Among the Neodiprion species examined in this study, field studies (details elsewhere) showed that N. virginianus, N. nigroscutum, and N. rugifrons utilize mixtures of 2S,3S,7S and 2S,3R,7S isomers in their pheromone, while N. pinetum and N. pratti banksianae combine 2S,3S,7S and 2S,3R,7R. The latter species also utilizes 2S,3R,7S or 2S,3R,7R/S (Olaifa et al. 1984) but N. pinetum strictly responds to 2S,3S,7S in combination with an appreciable amount of 2S,3R,7R isomer. Two species, N. lecontei and N. nanulus nanulus, only utilize 2S,3S,7S while N. sertifer combines a submicrogram concentration of 2S,3R,7R with 2S,3S,7S (Kikukawa et al. 1983). It is therefore not surprising that only 2S,3S,7S isomer was detected in this study in N. lecontei, N. nanulus nanulus and N. sertifer. What appears as discrepancy in other species between field studies and GLC data can be explained on the basis of GLC

behavior of the isomers and the insect extracts. GLC data of the standard samples show the order of elution as first 2S,3S,7S; second, 2S,3R,7S; and third, 2S,3R,7R based on retention times. When a large quantity of solvents or the insect extracts are injected, they promote earlier elution and low resolution. As a result, all the natural with 2S,3S,7S and 2S,3R,7S will appear as in Figure 8-4 d or f and not like Figure 8-2 i as expected. Similarly, all the naturals with 2S,3S,7S and 2S,3R,7R will appear as in Figure 8-4 (i) and not as Figure 8-2 (ii) as expected. Evidence for this explanation is also derived from D. similis which showed a clear 2S,3S,7S and 2S,3R,7R (Figure 8-4 g(i)) when natural is injected alone but a 2S,3S,7S and 2S,3R,7S (Figure 8-4 g (ii)) when a larger amount of sample is injected upon spiking with standard. Determination of 2S,3R,7R or 2S,3R,7S is based upon peak interval with 2S,3S,7S. Another common trend is that when the natural is spiked with 2S,3S,7S, 2 of the 3 species expected to contain 2S,3R,7R separated out from 2S,3S,7S whereas 2 of the 3 species expected to contain 2S,3R,7S did not separate out from 2S,3S,7S.

In conclusion, this study shows that the chirality of the natural pheromone of diprionid pine sawflies can be characterized and isomers separated by the the GLC systems used. Thus, three species, N. nanulus nanulus, N. lecontei and N. sertifer have 2S,3S,7S isomer; N. virginianus, N. rugifrons and N. nigroscutum have 2S,3S,7S and 2S,3R,7S

isomers, while N. pinetum, D. similis and probably N. pratti banksianae have 2S,3S,7S and 2S,3R,7R isomers.

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CHAPTER NINE

Electroantennogram Responses of Diprionid Pine Sawflies to the Natural and Optically Active Synthetic Pheromones and to TLC Fractions of Body Extracts

INTRODUCTION

Male pine sawflies possess bipectinate antennae that contain an array of olfactory receptors for perceiving the female produced sex pheromone. Female antennae are essentially moniliform but are also capable of sex pheromone perception. When biologically active compounds interact with the receptors, generator potentials evoked. The summation of these generator potentials may be measured by connecting the entire antenna to an amplifier (EAG). Differences in the amplitude of EAG responses is believed to reflect differences in the numbers of individual neurons in sensila responding to olfactory stimulus per unit time (Kaissling 1971).

The sex pheromone of diprionid pine sawflies was identified as the acetate or propionate ester of 3,7-dimethylpentadecan-2-ol (diprionol) (Jewett et al. 1976). This compound has 3 asymmetric carbons at 2-carbon, 3-carbon and 7-carbon. Males of many Neodiprion species of sawflies respond mainly to (2S,3S,7S)-3,7-dimethylpentadecan-2-yl acetate (2S,3S,7S-A) or propionate (2S,3S,7S-P) isomer in

the field (Kraemer et al. 1979, 1981 & 1983), whereas two old world species Diprion similis (Hartig) and Gilpinia frutetorum Fabricius respond mainly to 2S,3R,7R-P and 2S,3R,7R-A respectively (Kikukawa et al. 1982a). Kikukawa et al. (1983) and Olaifa et al. (1984) observed a synergistic effect in the response of male Neodiprion sertifer (Geoffroy) and N. pratti banksianae Rohwer to 2S,3S,7S-A when optimum concentration of 2S,3R,7R-A was mixed with the former. Earlier studies have demonstrated differential EAG responses to optical isomers but no synergistic responses of mixtures of isomers have ever been recorded. This study focuses on the EAG response to optical isomers, dose-response to major isomers and the female pheromone responses.

MATERIALS AND METHODS

The EAG technique used for this study was originally developed by Jewett et al. (1977). Some modifications were made on their methodology as follows:

Preparation of antenna for EAG

Antennae of males reared in the laboratory from field collected larvae were used for this study. Adult males 1-2 days old were used. After emergence from cocoon, the male is kept in a 5 cm x 2.5 cm vial, supplied with a moistened 1 cm dental cotton wick and covered with cheese cloth until ready for use. The test insect is held abdomen up and with a steady hand and the aid of a microdissecting extra fine

forcep (Sargent-WElch, Skokie, Illinois), one antenna is removed from its socket.

Electrodes and Recording Instrument

Two pyrex tubing diameter 0.7 cm were drawn out at the tip to a diameter of 10-20 um. Pasteur pipet could also be used but they tend to break more easily. The tubings were about 3/4 filled with Kaissling solution: NaCl (7.5 g/l); $CaCl_2$ (0.21 g/l); KCl (0.35 g/l); and $NaHCO_3$ (0.2 g/l) (Roelofs 1976). Two short pieces of chloridized silver wire were used, one as the reference electrode the other as the recording electrode. The two were soldered to the input lead of the storage oscilloscope (Model 5130 N). Each time before use, the silver electrodes were chloridized by the method of Bruce Oakley and Rollie Schaffer (1982) as follows: The silver wire electrode was burnish with a 320 grade sandpaper, then the positive terminal of a 1.5 volt battery was connected to the silver electrode to be chloridized. The negative terminal of the battery was connected to a 5 K ohm resistor which is connected to the reference electrode. Both wires were placed in a solution of 0.2 M NaCl for currents to pass for 20-30 minutes. The silver wire connected to the positive terminal turning gray marked the end of chloridizing. The terminals are stored in the dark in 0.2 M NaCl because the coating is photosensitized. Equilibrium was ensured by momentarily shorted together of the two silver wire electrodes. The base of the excised antenna is positioned to the tip of the reference electrode

and the tip of the recording electrode is set to make contact with the tip of the antenna. The antenna had an effective life span of 10-30 minutes. These operations were done under a dissecting microscope. All the components so far involved, the electrodes, oscilloscope, microscope, micromanipulators are all grounded; in addition, the tubing holding the electrodes were wrapped with aluminum foil. No Faraday cage was used.

Pheromone Delivery System:

Charcoal filtered air from a central supply flowing at 666 ml/min. was moistened by passing through distilled water in a 250 ml flask. The rubber tubing carrying the air was connected to a 15 cm glass tubing which was held and directed to the antenna by a clamp and terminated within 2 cm of the fixed preparation. This set-up was in many respects similar to that of Visser (1979). The isomers of diprionol tested were dissolved in 0.1 ml hexane which was pipetted onto 7.0 cm x 7.0 mm #1 Whatman filter paper inside a short pasteur pipette 14.5 cm long. A 2 ml pulse of air produced by pressing the plunger of a hypodermic syringe passes through a 3-way joint hose, a 1 meter long plastic tubing and the pipette containing pheromone. The tip of pipette is inserted into the hole near the tip of the tubing which carries moistened air. The 3-way joint hose was connected between the syringe and plastic tubing so that fresh air could be drawn from the outside when pulling the plunger backward. The plastic tubing in between the pasteur pipette

and the 3-way joint hose allows the operator to stand well away from the antenna when the pulse is delivered, thus avoiding mechanical and electrical noise (Jewett et al. 1977).

Response Evaluation Method:

The choice of the isomers tested for D. similis was based on the knowledge that D. similis prefer the 2S,3R,7R-P diprionol (Kikukawa et al. 1982a). In view of better performance of the natural pheromone and excellent performance of this EAG system, attempts were made in this study to test a range of isomers for synergism and to observe any EAG characteristic that may be of use in planning field experiments. The amplitude of the response as well as time for each signal were recorded. To correct for changes in the antennae response during the experimental period, the antenna was stimulated alternatively by the test isomer and hexane. Relative electroantennogram responses to test isomers was prepared in bar graphs as described for N. lecontei by Kraemer et al. (1981), and the dose-response curve was plotted where necessary.

Synthetic Pheromones

The optical isomers used for this study were the same used by Kraemer et al. (1979, 1981 and 1983) and by Kikukawa et al. (1983). The natural pheromones were prepared by the methods of Kikukawa et al. (1982a). Fractionation of the optical isomers into 'front' and 'back' fractions was done

by collecting the front and back halves of gas liquid chromatograph (GLC) peak of the isomers by cold trap. A 9:1 post column splitter, and a 7.2 m x 3 mm carbowax 20 M stainless steel column were used in Varian 1700 flame ionization detector gas chromatograph.

RESULTS

Using the technique described above, detailed shape of the waveform of the pheromone induced EAG in D. similis was produced (Figure 9-1 a-c). The waveforms (a & b) showed hyperpolarization preceding depolarization. In the waverform b, there was a decrease in hyperpolarization when 2R/S,3R,7R-P was added to 2S,3R,7R-P and in c, no hyperpolarization was produced by stimulation with 2R/S,3R,7R-P alone. Hyperpolarization nature of the waveform could not be observed before the first 15 minutes of hooking up the excised antenna and stimulation. This can be interpreted to mean a sort of decay in the antenna or that the hyperpolarization was measurable only when an inhibitor present in the sample reached a threshold concentration. Whatever caused this diphasic feature could not be understood at present. This type of waveform was observed in the EAG produced not only from 2S,3R,7R-P and 2S,3R,7R/S-P synthesized by Kikukawa et al. (1982b) and Tai et al. (1978) but also from isomers synthesized by Mori et al. (1978). The alcohol 2S,3R,7R-OH also produced hyperpolarization (Figure 9-2). The cause cannot therefore be source related. This figure

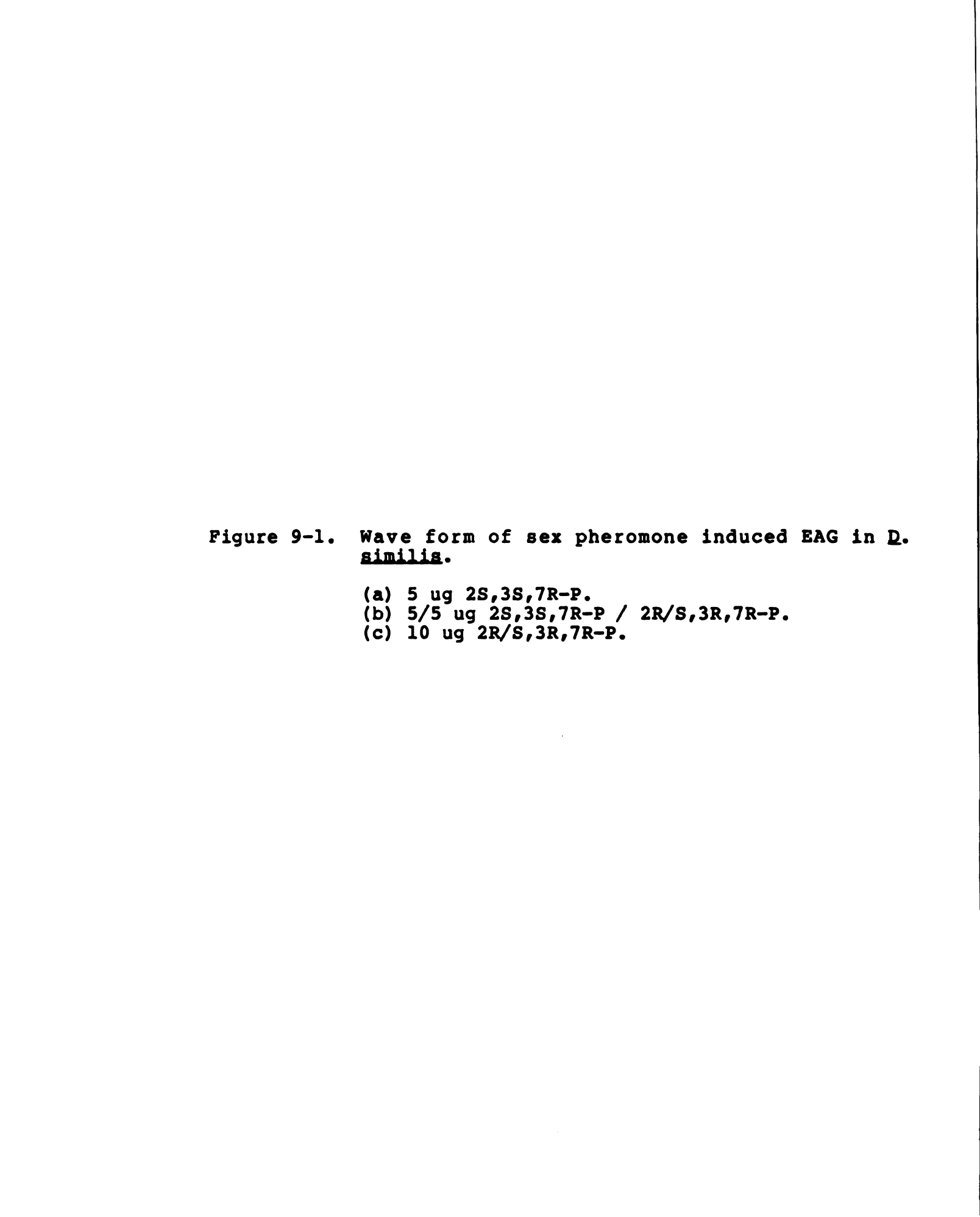
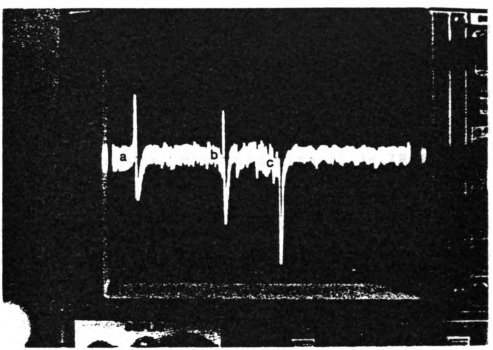


Figure 9-1. Wave form of sex pheromone induced EAG in D. similis.

(a) 5 ug 2S,3S,7R-P.
(b) 5/5 ug 2S,3S,7R-P / 2R/S,3R,7R-P.
(c) 10 ug 2R/S,3R,7R-P.

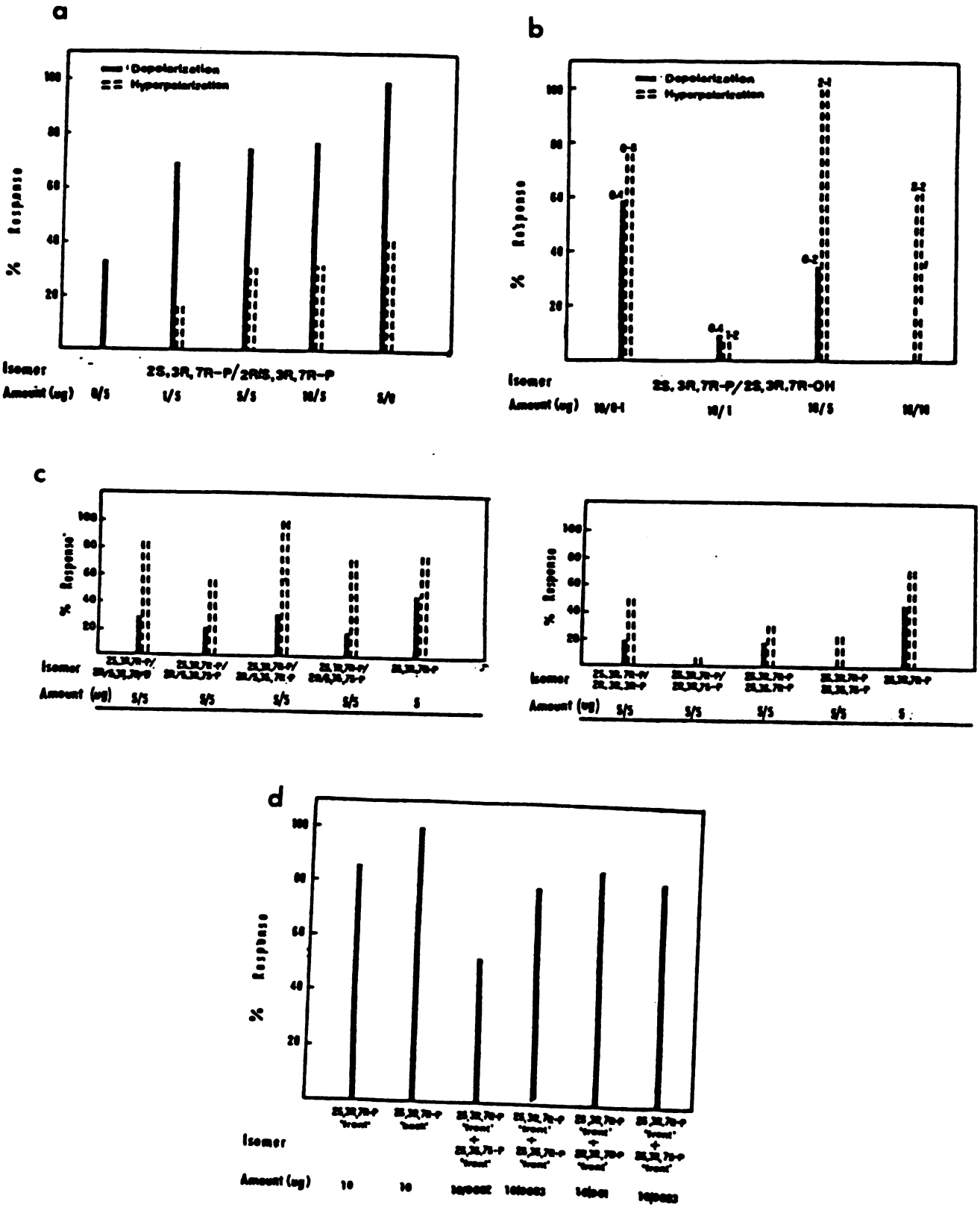

a
b
c

also shows that 2S,3R,7R-P produced amplitude about 3 times that produced by 2R/S,3R,7R-P alone. However, hyperpolarization produced by the former was lacking in the latter. Increasing amount of 2S,3R,7R-P added to 2R/S,3R,7R-P produced additive effect (Figure 9-2a). In Figure 9-2b, the alcohol tended to decrease depolarization caused by 2S,3R,7R-P. Also, recovery time of depolarization decreased and that of hyperpolarization increased with increasing addition of the alcohol 2S,3R,7R-OH. Figure 9-2c showed inhibition of 2S,3R,7R-P by various isomers. It is noteworthy that 2S,3S,7S-P and 2R,3R,7S-P totally suppressed depolarization when combined with 2S,3R,7R-P at ratio 1:1 and that 2R/S,3S,7R-P produced highest hyperpolarization. Generally all the isomers racemic at 2-carbon showed higher depolarization than the corresponding isomers not racemic at any chiral center.

In Figure 9-2 d, it is observed that the 2S,3R,7R-P back gave a relatively bigger amplitude than the 2S,3R,7R-P front, though not significantly. Also, addition of GLC front fraction of 4 isomers, which earlier proved highly inhibitory (Figure 9-2c) to 2S,3R,7R-P, appeared to produce better response. However, the responses elicited by the mixtures were not as good as that from 2S,3R,7R-P. One major observation in this figure is that the hyperpolarization response was no longer there. For the subsequent EAG studies, all the optical isomers used were fractionated by using GLC into GLC front and back and only the front

Figure 9-2. Response of male D. similis to optical isomers.

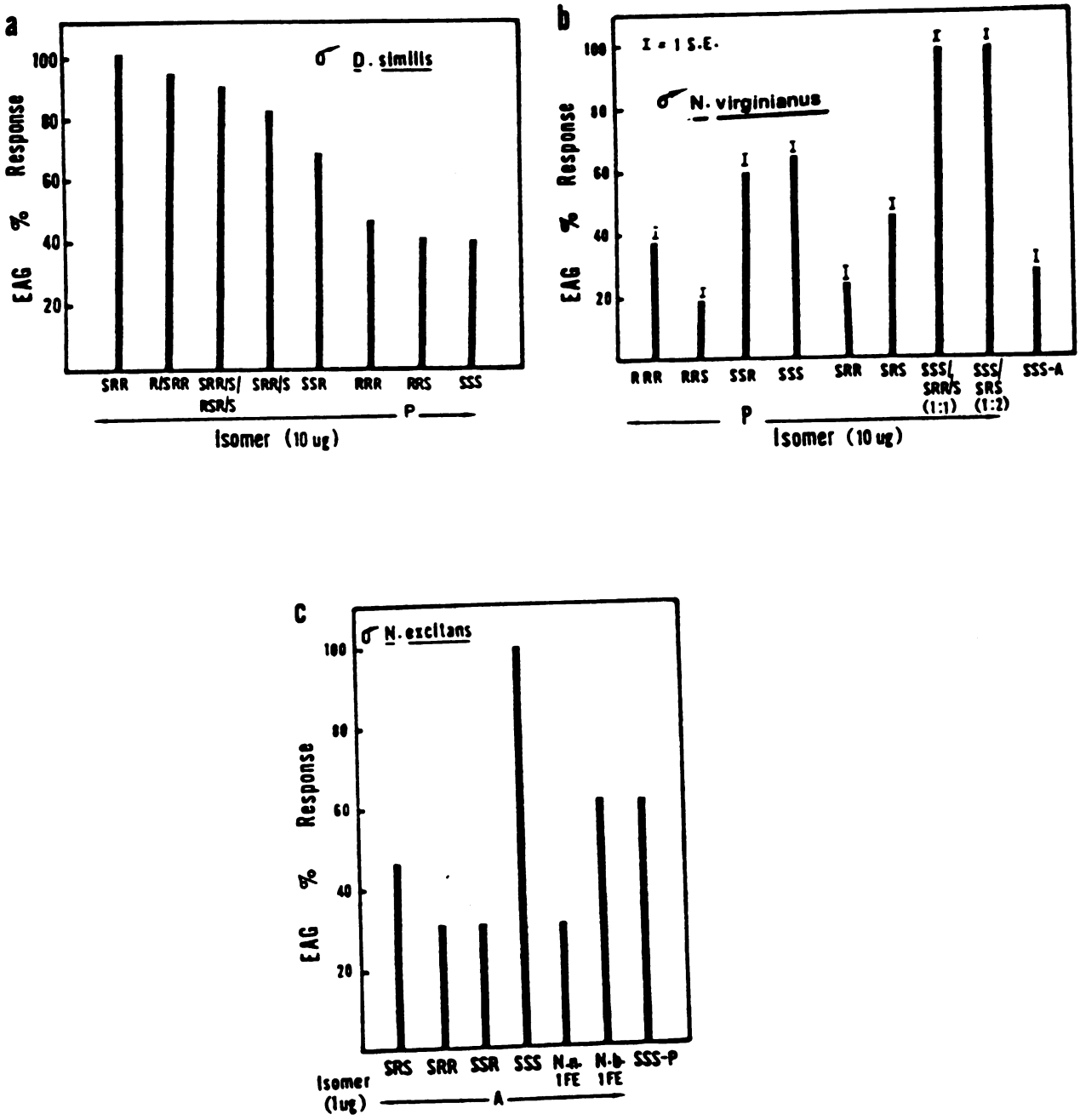
(a) additive effect of 2S,3R,7R-P / 2R/S,3R,7R-P mixture.
(b) inhibition of 2S,3R,7R-P by 2S,3R,7R diprionol.
(c) inhibition of 2S,3R,7R-P by various isomers.
(d) effect of fractionation by GLC peak of 2S,3R,7R-P.


a
Depolarization
Hyperpolarization
% Response
Isomer
2S,3R,7R-P/2RS,3R,7R-P
Amount (ug)
b
Depolarization
Hyperpolarization
% Response
Isomer
2S,3R,7R-P/2S,3R,7R-OH
Amount (ug)
c
% Response
Isomer
Amount (ug)
% Response
Isomer
Amount (ug)
d
% Response
Isomer
Amount (ug)

fraction was used. The GLC front was preferred because it performed better than the back on the field.

There were differential EAG responses to the GLC front optical isomers by male D. similis, N. virginianus and N. lecontei (Figure 9-3). Male D. similis showed maximum response to 2S,3R,7R-P, followed by 2R/S,3R,7R-P. The 2S,3S,7S-P elicited least response. Among the optical isomers tested alone, the 2S,3S,7S-P produced maximum response in male N. virginianus (Figure 9-3b). The corresponding acetate produced about half a response as that of propionate counterpart. However, the 2S,3S,7S-P / 2S,3R,7S-P and 2S,3S,7S-P / 2S,7R,7R/S-P mixtures produce response greater than any of the isomers tested alone. These two mixtures produced about equal response. This is the first time that mixture of optical isomers will produce EAG response greater than 2S,3R,7R-P alone. The latter is the optical isomer found most active on the field against D. similis and Gilpinia frutetorum (Kikukawa et al. 1982a). This improved effect of the mixture was not also realized until the optical isomers were fractionated by using GLC into front and back. The black-headed pine sawfly N. excitans also showed greatest response to the 2S,3S,7S isomer with the acetate better than propionate. This species also showed greater response to 2S,3R,7S-A than 2S,3R,7R-A, the latter being most active in D. similis. Also indicated was the importance of chirality of the 7-carbon as the 2S,3S,7R-P showed only about one-third of 2S,3S,7S-P response. These data

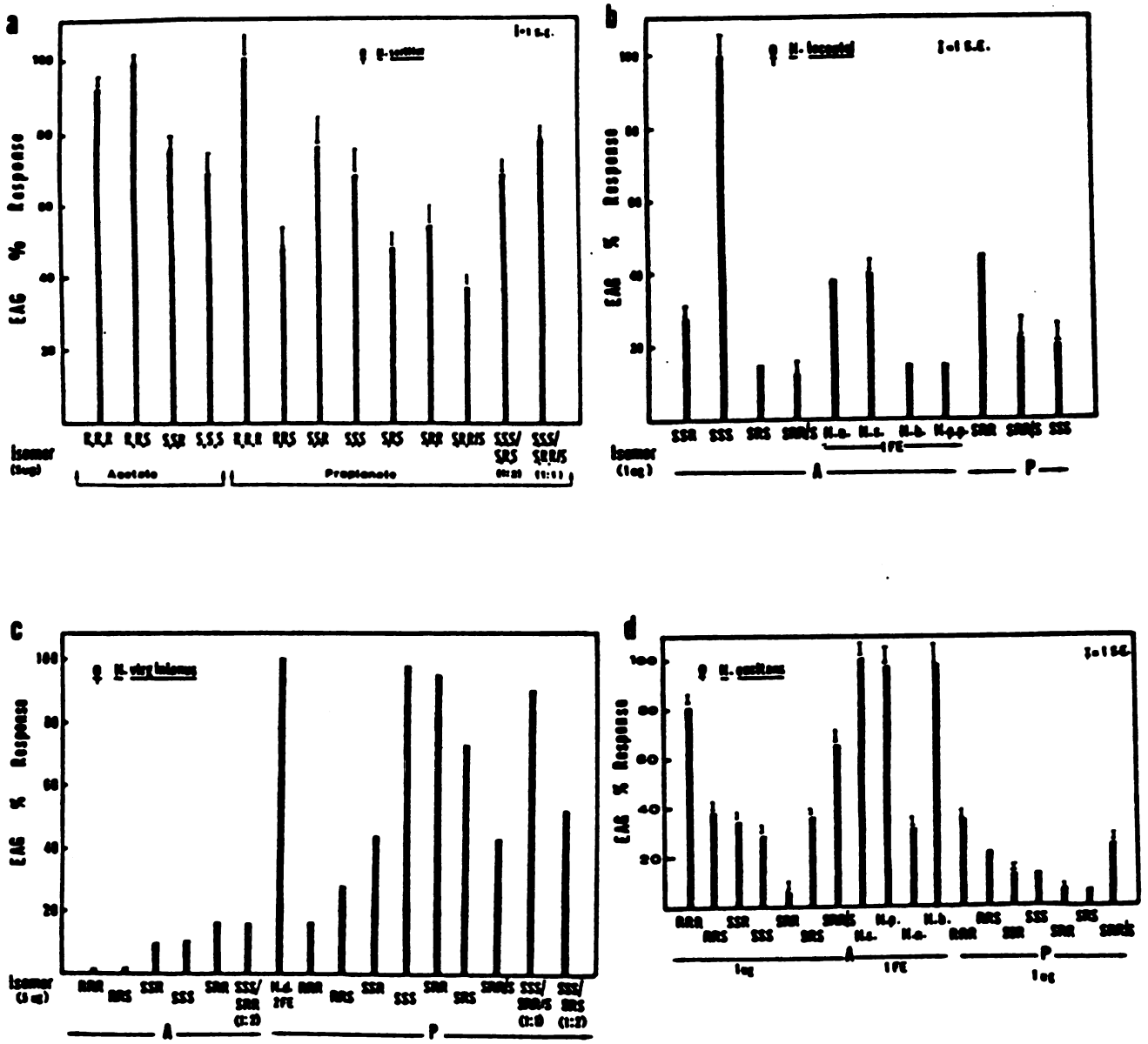
Figure 9-3. Relative EAG response of male of 3 species of diprionid sawfly to various isomers of sex pheromone.


a
EAG % Response
100
80
60
40
20
♂ D. similis
SRR
R/SRR
SRR/S/ RSR/S
SRR/S
SSR
RRR
RRS
SSS
P
Isomer (10 ug)
b
I = 1 S.E.
♂ N. virginianus
RRR
RRS
SSR
SSS
SRR
SRS
SSS/ SRR/S (1:1)
SSS/ SRS (1:2)
SSS-A
P
Isomer (10 ug)
c
♂ N. excitans
SRS
SRR
SSR
SSS
N.a. 1FE
N.b 1FE
SSS-P
Isomer (1ug)
A

show that male N. excitans showed differential responses to the natural pheromone of other species--the pheromone of N. pratti banksianae elicited about twice response as that of N. nanulus nanulus (Figure 9-3c). Partly from the fact that two mixtures of optical isomers produced equal EAG response in N. virginianus and partly from the fact that less amount of pheromone produced measurable EAG not in any way different from that produced by higher concentration, smaller concentrations of pheromone was used in subsequent studies. For the first time in diprionid sawflies, the females are shown to produce EAG to sex pheromone (Figure 9-4). Female N. sertifer (Figure 9-4a) showed response to both the acetate and propionate esters, and 2R,3R,7R-P produced greatest response. This was followed closely by 2R,3R,7S-A, 2R,3R,7R-A in that order. Only 3 optical isomers 2R,3R,7S-P, 2S,3R,7S-P and 2S,3R,7R/S-P produced response less than 50%. Response by female N. lecontei (Figure 9-4b) was more discriminating. Only the 2S,3S,7S-A elicited greatest response and the only optical isomer whose response is greater than 50%. Of the natural pheromone, that from N. sertifer and N. nanulus nanulus showed about 40% response. The females of N. virginianus showed greatest preference for the propionate pheromone (Figure 9-4c). Although synthetic isomers 2S,3S,7S-P showed greatest response but this was about 1% better than the response produced by 2 female equivalent natural pheromone of N. dubiosus. The 2S,3R,7R-P as well as 2S,3S,7S-P / 2S,3R,7R/S-P mixture produced

Figure 9-4. Response of female diprionid sawflies to sex pheromone.

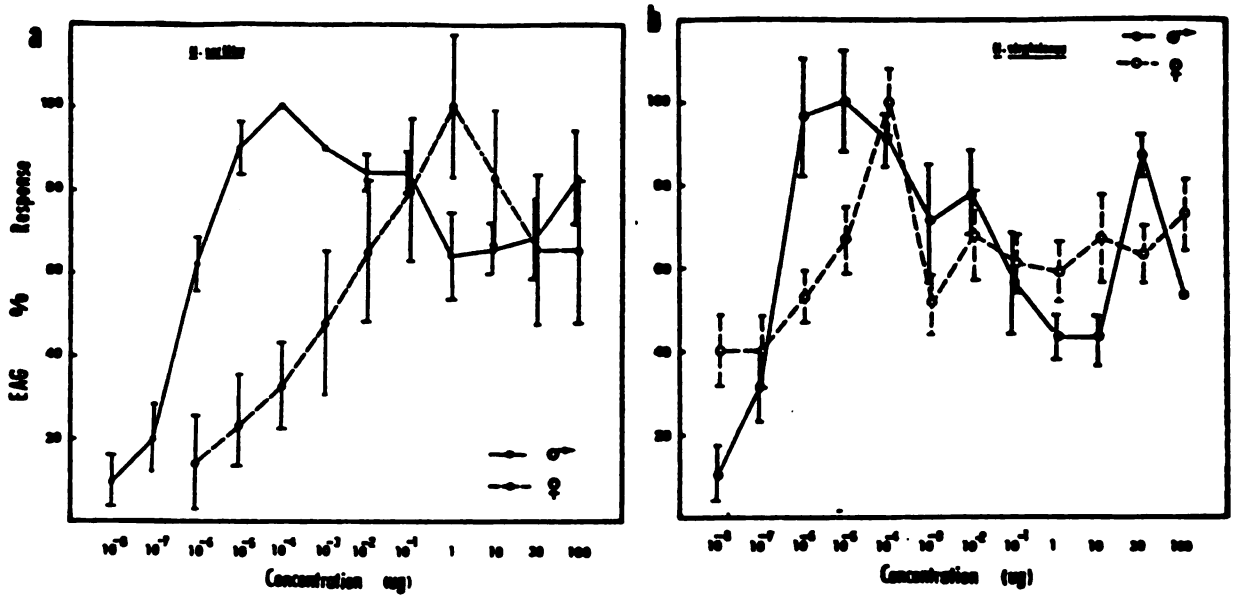
(a) N. sertifer.
(b) N. lecontei.
(c) N. virginianus.
(d) N. excitans.


a
EAG % Response
Isomer
Acetate
Propionate
b
EAG % Response
Isomer
A
P
c
EAG % Response
Isomer
A
P
d
EAG % Response
A
P

response above 80%. Response produced by female N. excitans (Figure 9-4d) was similar to that produced by female N. sertifer in two respects (1) both species respond maximally to 2R,3R,7R isomer however acetate in the former and propionate in the latter, and (2) there was relatively good response to both the acetate and propionate even though it is clearer that N. excitans prefer the acetate. However, in this species, the natural pheromones elicited greater responses than the synthetic. The pheromone of N. sertifer produced greatest response, followed closely by N. pratti banksianae and N. pinetum. The pheromone of N. nanulus nanulus produced only about 30% response.

The dose-response curves show the sensitivity of male N. lecontei, and male and female N. virginianus and N. sertifer (Figure 9-5). These curves indicate that the sensitivity for the different species is different; the EC_{50} for the males being in the neighborhood of 1 pg, 0.1 ng and 10 ng in N. virginianus, N. sertifer and N. lecontei respectively (Figure 9-5) and 1 ng for N. pinetum (Figure 9-6c). Females are less sensitive than the males, the EC_{50} being 1 ng for both N. virginianus and N. sertifer. It is also shown by using submicrogram samples of optical isomers that 2S,3R,7S-P isomer was indeed synergistic to 2S,3S,7S-P isomer (Figure 9-6a). The mixture not only produced greater response than 2S,3S,7S-P isomer alone at the same concentration, but about equal response that 2S,3S,7S-P alone elicited at 1×10^{-5} ug was also elicited by the mixtures at

Figure 9-5. Mean EAG response of 3 species of sawfly to a range of concentration of sex pheromone.


a
EAG % Response
♂
♀
Concentration (ng)
b
♂
♀
Concentration (ng)

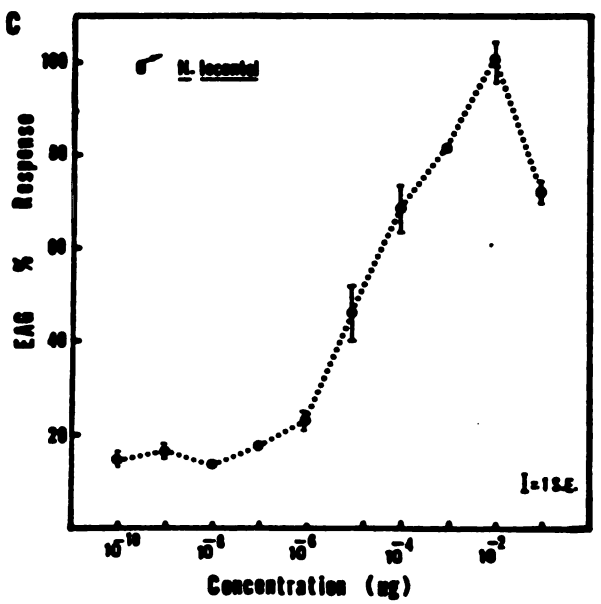
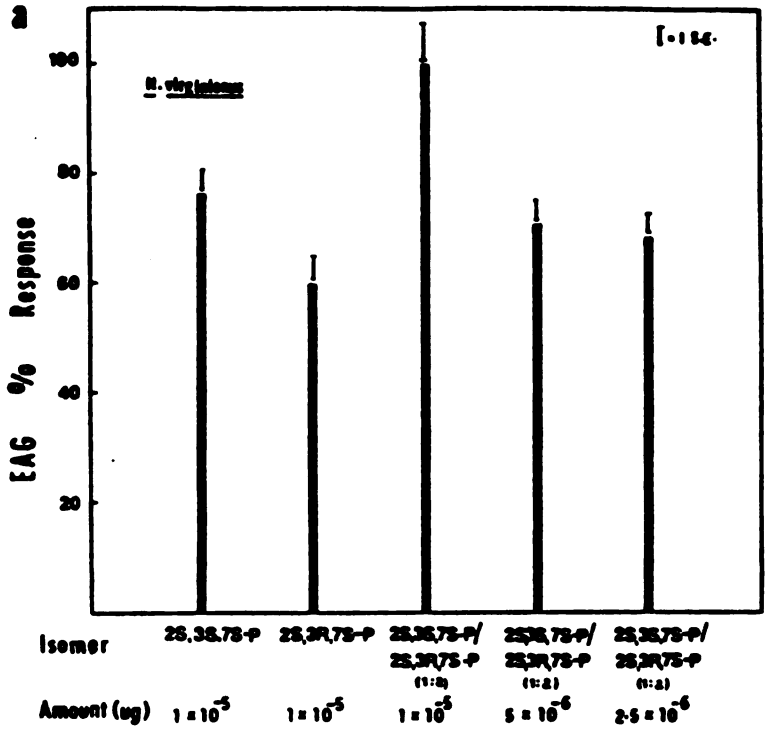
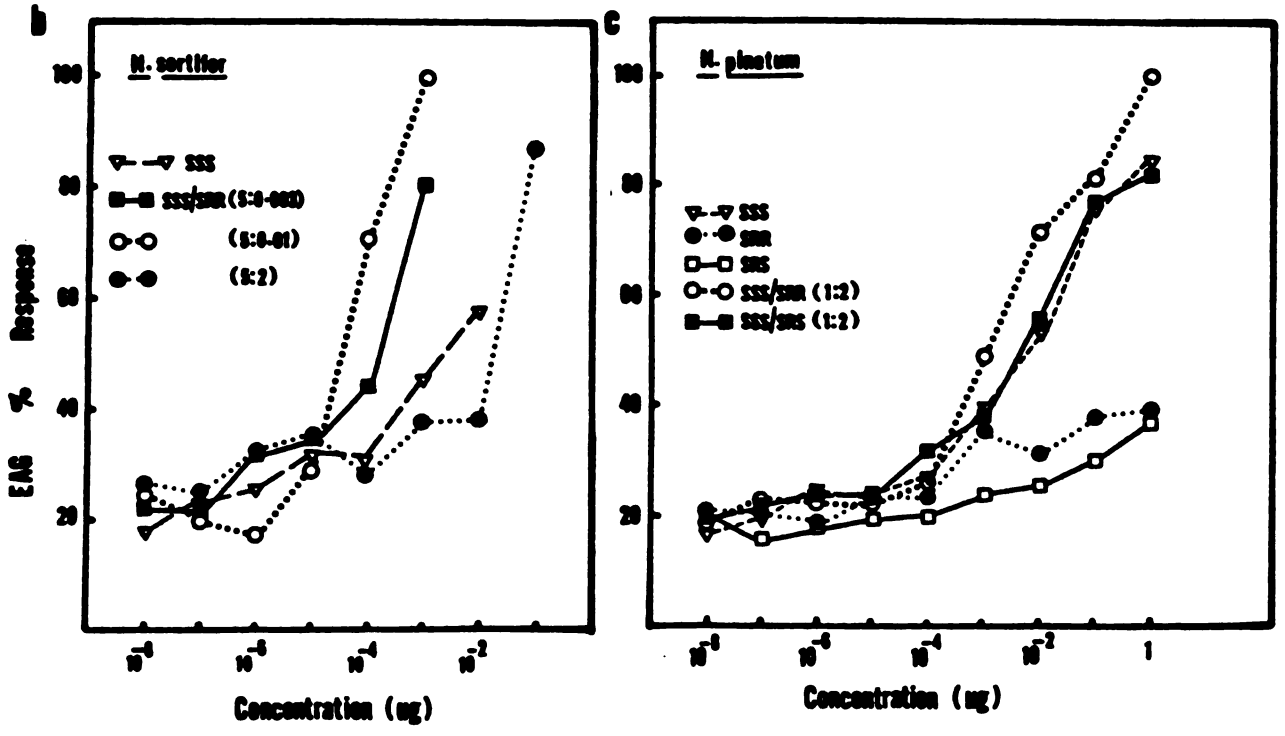

c
♂ H. locontei
EAG % Response
100
80
60
40
20
I=1S.E.
Concentration (ng)

Figure 9-6. Synergistic interactions of optical isomers in EAG of 3 species.

(a) 2S,3R,7S-P as synergist of 2S,3S,7S-P in N. virginianus.
(b) 2S,3R,7R-A as synergist of 2S,3S,7S-A in N. sertifer.
(c) 2S,3R,7R-A and 2S,3R,7S-A as synergists of 2S,3S,7S-A in N. pinetum.

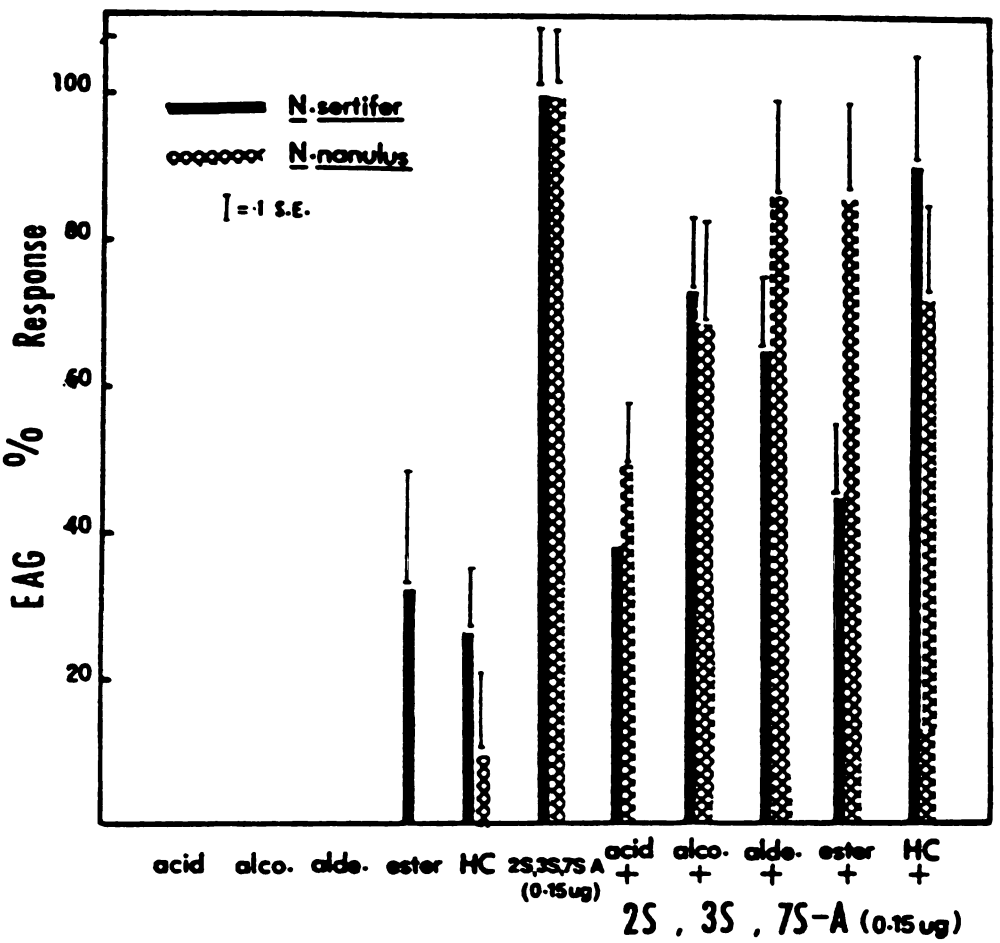

a
EAG % Response
Isomer
Amount (ug)


b
H. sertifer
SSS
SSS/SSR (5:1)
(5:1)
(5:2)
EAG % Response
Concentration (ug)
c
H. pinetum
SSS
SSR
SRS
SSS/SSR (1:2)
SSS/SRS (1:2)
Concentration (ug)

one-half and even one-quarter of this concentration. This result suggested that the mechanism by which 2S,3R,7S-P elicited synergism is probably to increase the maximum response by additive effect. In two other species N. sertifer (Figure 9-6b) and N. pinetum (Figure 9-6c) addition of optimum amount of the synergist 2S,3R,7R-A in the former species and 2S,3R,7R-A and 2S,3R,7S-A in the latter species to 2S,3S,7S-A increased the maximum response. The response of the mixture at ratio 1:0.002 and 1:0.006 in N. sertifer was greater than that of 2S,3S,7S-A alone at all the doses tested. Similar observations were made for the mixtures over 2S,3S,7S-A alone in N. pinetum at all the doses tested. The optimum combination of 2S,3S,7S-A / 2S,3R,7R-A and 2S,3S,7S-A / 2S,3R,7S-A was 1:2 as determined by field test, the result of which is published elsewhere. It should be noted that the synergists by themselves elicited low EAG response.

Using the TLC fractions of the natural body extract of female N. sertifer and N. nanulus nanulus, the antennae of male N. sertifer were stimulated with these preparations singly or in combination with major pheromone isomer of N. sertifer. The EAG response to the fractions from N. nanulus nanulus and N. sertifer was almost identical (Figure 9-7). The acid, alcohol, aldehyde, and ester fractions from N. nanulus nanulus did not elicit any response on the antennae of N. sertifer. Only the hydrocarbon fraction showed about 10% response relative to the response produced by 2S,3S,7S-A

Figure 9-7. EAG response of male N. sertifer to the TLC fractions of the body extract from female N. nanulus nanulus and N. sertifer.


EAG % Response
100
80
60
40
20
N.sertifer
N.nanulus
I = 1 S.E.
acid
alco.
alde.
ester
HC
2S,3S,7S A (0.15ug)
acid +
alco. +
alde. +
ester +
HC +
2S , 3S , 7S-A (0.15ug)

isomer. The ester and hydrocarbon fractions from female N. sertifer produced some responses. All these fractions when added to the 2S,3S,7S-A isomer proved inhibitory. The acid, aldehyde and ester fractions from N. sertifer were more inhibitory than those from N. nanulus nanulus. One observation made three out of six times the stimulations were made was the fact that the hydrocarbon fraction increased recovery time of the depolarization from 0.4 m sec to 1.0 m sec, however, this fraction did not increase the amplitude.

DISCUSSION

Two interpretations have commonly been given for the diphasic (positive and negative) impulses produced by chemical stimulation of antannae as in Figure 9-1 a & b. One is that the positive (hyperpolarization) and negative (depolarization) phases of the impulses have independent origins. The positive phase is generated on or near the cell body and spreads in both directions along the axon and back up along the dendrite towards the constriction at the base of the hair. When the impulse passes through the constricting space and enters the lumen of the hair, the opposite situation prevails and the impulse appears negative. The transition from the positive to the negative phase is very rapid, reflecting the short time that the impulse takes to pass the small distance of the constriction (Wolbarsht and Hanson, 1965). This interpretation precludes the presence of two or more specific receptor types existing on each or most of the

neurons which is the second interpretation proposed by Pfaffman (1959) and Boeckh (1967). The latter investigator also proposed that receptor cell is capable of responding either by excitation or by inhibition depending upon the qualitative nature of the stimulus. However, for this interpretation to be valid, single sensillum recordings will be necessary. The interpretation of Pfaffman (1959) and Boeckh (1967) appeared more attractive in the present investigation. It is suspected that hyperpolarization was induced by one or some of enantiomers of the diastereomers used in this study. With regards to optical purity, the critical carbon center prone to contamination is the 3-carbon, in which case with 2S,3R,7R isomer, there could be contamination of 3R with 3S; and in 2S,3S,7S isomer, there could be contamination of 3S with 3R due to epimerization in the Wittig reaction during synthesis (Matsumura et al. 1979). Fortunately, the erythro isomers (i.e., 2S,3S and 2R,3R) can be separated from the threo (i.e., 2S,3R, and 2R,3S) isomers by the GLC using DB5 and carbowax 20 M columns. The details of this separation is described elsewhere. When the GLC peak of the optical isomers were fractionated into the front and back, the ideal behind this was that the back fraction should elicit greater EAG response if indeed 2S,3R,7R-P is the pheromone of D. similis (Kikukawa et al. 1982a). The results show that 2S,3R,7R-P GLC back was indeed better than 2S,3R,7R-P GLC front but not significantly. This was a circumstantial evidence to suggest that

the receptors on the antennae of D. similis and probably the other diprionid sawflies are keyed to the same compound. Here, the enantiomers were acting as competitive blockers at the receptor cells. Also, as these results show enantiomeric interaction at the receptor cells, this is another circumstantial evidence that in these species a multicomponent pheromone is precluded.

Differential response of male sawflies to different optical isomers of sex pheromone is not new (Kraemer et al. 1979, 1981 and 1983). However, it is novel that males respond to natural sex pheromone of other species of sawfly. It is also reported here for the first time that diprionid sawfly females respond to synthetic sex pheromone and that such response in most cases is mainly to the enantiomers of major isomers of the male counterpart. The interpretation of this observation is not clear at present. However, one implication of this female enantiomeric specific response could be that perhaps males also do produce long or close range pheromone or aphrodisiac which may be optically active and chemically related to the female produced sex pheromone. This needs to be investigated in view of presence of abdominal intertergal gland in the male N. sertifer (Hallberg and Lofqvist, 1981). It is also not known whether female EAG responses could translate to behavioral responses. On a few occasions, few female N. sertifer were caught in traps baited with 2S,3S,7S-A / 2S,3R,7R-OH; 2S,3S,7S-A / 2S,3R,7S-A and 2S,3S,7S-A / 2S,3R,7R-A at various combinations. One

other observation in this study is the superior response obtained from stimulation of the female antennae by natural pheromone of different species. Also, when two or more female are confined, they always engage in fights which often result in breaking of legs. Such fights are observed in 20 ml to 4 liter rearing jars and fights occur irrespective of species. The strong response to other species pheromone by the females could therefore be interpreted to mean that perhaps a means of avoiding breeding sites of sympatric species or signal for dispersal is by chemical means. This may be worthy of future pursuit.

Using submicrogram concentration of optical isomers, synergistic response to mixtures of optical isomers is measurable. This raises the question of how did such synergism occur with mixing of optical isomers. There could be four possibilities. One, the synergist isomer may lower the response threshold but this does not appear to be the case as response curves of 2S,3S,7S-A alone and the mixtures tended to rise at about the same concentration (Figure 9-6 b & c). Two, there could be no quantitative differences on EAG but qualitative recognition of optical isomers may be the key. This possibility does not appear attractive at present. Three, the synergist isomer may act to increase the maximum response of the major isomer. This possibility seems to be part of the answer as evidenced in Figure 9-6. Since most tests were done with such high concentrations (Figure 9-2, 3 & 4), they also measure the maximum response.

Therefore, these observations agree with those in Figure 9-6, that the maximum response is important. The fourth possibility is that the synergist isomer may provide additive effect to the major isomer. Our data also support this possibility because synergists by themselves show little activity. It can therefore be concluded that the synergists increased the maximum response by additive effect with the main isomer.

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CHAPTER TEN

Behavioral Response to Optical Isomers of Sex Pheromone and Development of Non-Saturating Trap for Diprionid Sawflies

INTRODUCTION

Courtship behavior is believed to have been conditioned by selection pressure to maintain species integrity in insects (Grant et al. 1975). In the lepidopterous species where a lot of work has been done in this regard, courtship behavior is found to be species specific and chemical stimuli plus other stimuli (visual and tactile) in some cases were necessary to initiate these stereotype behavior (Shorey and Gasteon, 1970; Carde et al. 1975; Carde 1981; Grant 1981; and Teal et al. 1981). Studies on courtship behavior of pine sawflies have been virtually neglected. Researchers on sawfly have directed much attention to the biotaxonomic studies and recently to the chemical elucidation of the female sex pheromone. Chemical behavior can contribute to the yet unsolved taxonomy of sawflies and can shed more light to the role of interacting isomers of the sawfly pheromone. Coppel and Benjamin (1965) reported that males approach the females in zigzag pattern with forward progress, gradually approaching to within about 1 ft., and then it appears that a visual stimulus becomes predominant. They

also reported end-to-end mating position and a mating period of 30 min. or more. Since their report represented the state of art in courtship behavior of pine sawflies, it became necessary as part of this study to prepare a full descriptive courtship behavior of three species of pine sawfly N. sertifer, N. virginianus, and D. similis based on direct observation on the field. This study also tested visual stimuli and various fractions of the female natural extract as they affect courtship behavior.

Thomas 1983, evaluated three types of traps and two types of pheromone dispensers for the introduced pine sawfly, D. similis. The author found that larger sticky traps and cigarette filter baited traps caught more males than the Pherocon II traps and the dental roll baited traps. Another important component of traps is the trap placement on the field. For example, experience with many species of sawfly in this study showed that traps placed on the host plant in the open always caught more males than traps placed in a closed canopy. Another aspect of trap placement is the height above the ground. In a field trapping experiment against the webspinning larch sawfly Cephalcia lariciphila Wacht utilizing vertical board traps affixed at heights of 0-0.5, 1, 2 and 4m to four trees 20m apart, Borden et al. (1978) found that traps placed at 0-0.5m performed better than other heights by a factor of about seven.

The use of sex attractant baited traps has become a conventional method of monitoring population trends and

estimating population density of many lepidopterous pests and bark beetles (Ryan and Molyneux, 1981; Vite 1970; Carde, 1979 and Sanders, 1981). Such use of traps for pine sawfly is still in its infancy due to recent development of sex attractants for this group of insects. The experience we have gained over the years on the use of sticky traps Pherocon II for testing pine sawfly sex pheromone has led to the realization of inherent disadvantages of using sticky traps for sawfly species. First, sticky traps are saturated by target and non-target species and this introduced errors into estimating population density or effectiveness of attractants used. The flower of pine trees are wind pollinated therefore sticky traps set out on the field for summer flying sawflies are often heavily saturated by debris of pollens and abborted flowers, but this problem is hardly noticeable in the fall season or in white pine stands which do not flower heavily like the jack pine. Second, trapped males are often mired by the sticky material to the extent that to remove them for further investigations without losing the leg, wings or antennae becomes almost impossible. Our experience in cleaning the insects removed from sticky traps also showed that only benzene could thoroughly remove the sticky substance but that means dealing with a carcinogen hazard. The third, and most pressing problem during the present investigations was the need to conduct a chemotaxonomic study in terms of pattern of cuticular hydrocarbon in pine sawfly species. Vegetable wax constituted one of the

major ingredients of the sticky substance in Pherocon II traps (Zoecon Co., personal communication) therefore, males removed from sticky traps could not be used for such studies because of similarities of the waxes from plants and insects (Kolattukudy, 1976). There was therefore, a need for the development of nonadhesive traps which would preserve trapped males for chemotaxonomic investigations in view of chaos in sawfly taxonomy which has not yet been resolved. Such traps eventually may have to replace conventional sticky traps for field population studies on summer species of sawfly in view of aforementioned problems. The present study also describes efforts at developing a cost effective non-sticky traps with evaluation concentrating on number of male sawfly trapped, the durability of the traps and their ability to exclude non-target insects and debris.

MATERIALS AND METHODS

Courtship Behavioral Studies

Tests were conducted in the middle of a pine stand for the species concerned. Test dates were chosen such that the weather was bright with low winds. A 40 ug pheromone in 1 ml hexane was used in all the species tested to attract the male sawflies to the vicinity of the test site. The pheromone was dispensed in an open 7.5 cm glass petri dish; the petri dish was placed on top of a 1.5 m aluminum pipe pole erected 1 meter from a pine stand. Sometimes 2 or 3 such petri dishes were used in a single experiment. As soon as

up to 15 males are sighted, the petri dishes are covered and removed into an air tight polythene bag. The open source is replaced with a point source chemical stimuli either from a rubber septum decoy cut approximately to the size of a female sawfly or a pinned live female or a pheromone treated solvent-washed dry female sawfly pinned onto a mating cage. The mating cage (15 x 15 x 15 cm) with one side open was made from aluminum mesh and painted green. The paint at some areas of the top of the cage was thick to allow anchorage for the pinned decoys. The cage was put on a 18 x 18 cm ground glass plate on the top of the aluminum pole. The account of the courtship behavior on the field was recorded from the time the male was first sighted approaching the source for N. sertifer, N. virginianus, and D. similis. For completeness, the pre-copulatory and post-copulatory behaviors of N. sertifer were recorded in a set-up in the greenhouse where males were kept in a giant screen cage (2.4 m x 2.4 m x 2.4 m) made with a plastic mesh. A young potted scotch pine 2 m tall placed at the center inside the cage served as the vertical silhouette where the males rested. Also recorded in this set-up was another experiment testing the effect of the acid, aldehyde, and parafiin TLC fractions of the natural extracts form female N. sertifer. The point source pheromone was presented as in the field. To test the effect of color on the response of N. sertifer and N. nanulus nanulus, the ground glass plates on which the cages stood were painted white, gray, black, yellow, orange, red,

green, and blue colors. These colors served as a background to the cage. Different screen sizes and colors of cages were also tested against N. nanulus nanulus. The effect of color on N. nanulus nanulus and N. sertifer was further tested on the field test using sticky traps Pherocon II painted black, blue, green, gray, orange, red, white and yellow.

Effect of Trap Height

In order to test the importance of trap height in response of male sawflies to pheromone, five heights 1.5, 2.5, 3.5, 5.5, and 8.0 m were chosen. The choice of heights was based on the fact that the ground cover, mainly grasses, were of height, 1.5 m in N. sertifer trapping site in Rose Lake, Michigan; ground cover at banksianae trapping site at Hartwick Pines, Michigan was generally, 1 m. The height 2.5 m represented the height by which most trapping had been done in this study. The 8 m represented the height of > 90% of the scotch pine and jack pine stands on which this study was carried out. The heights 3.5 m, and 5.5 m were randomly picked. The sawfly species tested were N. sertifer and N. pratti banksianae because of former's abundance and its important ancestral relationship to many species (Ross 1955) and latter's notoriety for attacking the trees of all sizes (Wilson, 1970). For N. sertifer, the trap heights were simultaneously tested on both the host plant and angle steel pole erected first at 12 m away from the nearest host plant

and then 3 m away after one week at the first location. Trapping lasted two weeks with one week each for each distance of angle steel pole from the host plant. The Pherocon II traps baited with 20 ug of 2S,3S,7S-A / 2S,3R,7R-A diprionol mixture (5:0.003) was used at each height. At the end of the first week, the number of males trapped at different heights on both the angle steel pole and scotch pines was counted. There were three replicates for each treatment. After then the poles were moved to 3 m from the host plants. Trap heights were also simultaneously tested on both the host plant, the jack pine, Pinus banksianae Lamb and the angle steel pole erected 3 m from the host plant. Duration of study was 11 days and Pherocon II traps baited with 20 ug of 2S,3S,7S-A / 2S,3R,7R-A diprionol mixture (5:1) were used.

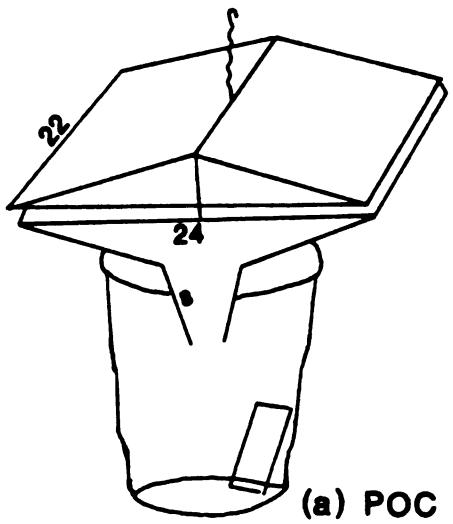
Trap Designs

There were 4 traps of our design which were compared with three other traps--Pherocon IC and Pherocon II, Zoecon Corp. Palo Alto, California, and the covered funnel trap CFT developed by Ramsawamy and Carde (1982). The Pherocon IC trap was also modified in this study by replacing the base with non-sticky top. Thus, only one trap, the Pherocon II trap was sticky. The 4 new designs are described in Figure 10-1 and Table 10-1 gives a detailed description of all the traps tested.

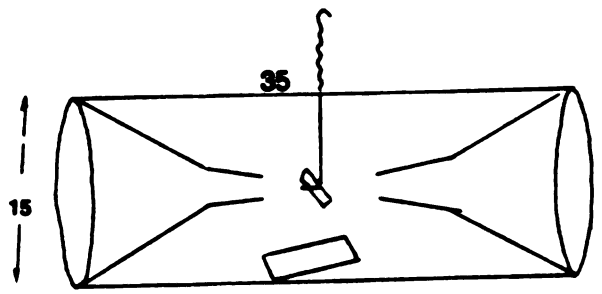
Three of the designs (DFT, IPT, ICT) were exposed in two ways and are such that will generate an elongated plume

Figure 10-1. Trap designs for sawflies (all measurements in cm.).

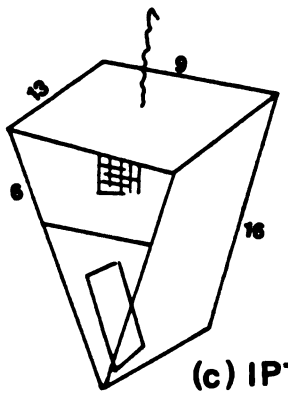
(a) Pherocon on cup trap (POC)
(b) Double funnel trap (DFT)
(c) Inverted pyramid trap (IPT)
(d) Inverted cup trap (ICT)



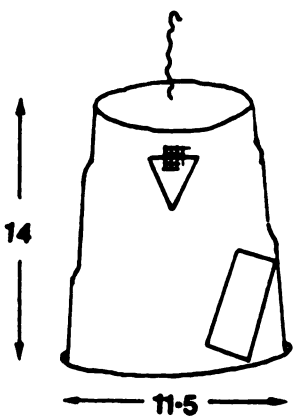
(a) POC



(b) DFT



(c) IPT



(d) ICT

Table 10-1. Descriptive data for trap designs

Model	Material	Entry space (sq. cm)	Color	Weight (g)
POC	Cover - coated cardboard Funnel - plastic Cup - plastic Polyethylene funnel Plastic body	250 cm^2	near white top and sky blue bottom	118
DFT	Polyethylene funnel Plastic body	15 cm^2	clear	226
IPT	Cardboard body stapled together with duct tape side base of entry hole	80 cm^2	white	45
ICT	3202 plastic drinking cup	18 cm^2	sky blue	52
Pheroocon IC	Coated cardboard	250 cm^2	near white	95
Pheroocon II	Coated cardboard	96 cm^2	near white	31
CFT	Polyethylene funnel with plastic dish cover	70 cm^2	near white cover, clear bottom	52

with a visual tracer (Lewis and Macaulay, 1976). Such design might be more relevant for pine sawfly whose males are great fliers (Coppel et al. 1960). The POC opens round and produced erratic plume of a visual tracer (Lewis and Macaulay, 1976). Perhaps sawfly males would take advantage of these characteristics because a more erratic odor plume is more likely to be easily detected by males presumed to be flying at random. Each of the traps was baited with 20 ug of pheromone applied as a 1 ml aliquot in hexane onto a 4 cm cotton dental wick. The wick was held with a paper clip inside a cylindrical aluminum screen (4 cm x 2.5 cm diameter), attached to the center room inside the IPT and ICT. No screens were used for POC and DFT but, in all cases, the wick was held with a clip and was aligned with the openings. Dichlorvos strip, Raid , 36 sq. cm was used as the killing agent. Two Neodiprion species N. virginianus and N. nanulus nanulus were trapped with the different designs at Sharon and Vogel Center, Michigan respectively.

RESULTS

Pheromone Source Parameters

Open sources of pheromone attracted more males N. nanulus nanulus to land than a point source (Table 10-2). However, all the sources appeared to attract the males to their vicinity. The open source (petri dish) when placed on the mating table covered with grasses attracted more males to land. In another experiment involving two species and

Table 10-2. Effect of different types of pheromone sources on response of wild N. nanulus nanulus in the field within 40 min. observation period with 2S,3S,7S-A (10 ug).

Pheromone sources	# Males[a] visiting	# males[b] landing on source
Point source (head of pin)	3	0
Watch glass (5 cm diam.)	4	1
Glass petri dish (7.5 cm diam.)	2	2
Glass petri dish (7.5 diam.) painted green	4	2
Glass petri dish (7.5 cm diam) on mating table (0.75 m^2) covered with grasses	4	5

[a] Males visiting were those that made some circular flight around the source.

[b] Males landing were those that actually landed on source.

grasses and host pine needles compared as background, the redpine needles attracted more males of N. nanulus nanulus to land than grass or plain glass background alone (Table 10-3). The males of N. sertifer however, did not exhibit any preference and were equally attracted to the 3 backgrounds. Also, in another experiment with the former species, the effects of other types of background for pheromone source (Table 10-4) were tested. The mating table covered with grass and green ground grass plates attracted more males to land. Even though transparent glass had more males visiting but could not attract equally high landing of males. The legs of the male sawfly are adapted for walking on pine needles. Their overly long and folding tarsi makes this possible but at the same time makes them a poor walker on flat surfaces. It therefore became necessary to develop a surface on which the males could land and walk easily to the pheromone source in order to gain useful knowledge on how males approach the pheromone source. Wire nettings were considered appropriate for this purpose and different types were tested against N. nanulus nanulus (Table 10-5). Crossing aluminum 0.5 cm mesh green color attracted more males to land and was as good as transparent glass in the number of visiting males. This aluminum mesh was therefore made into the mating cage which was used in all the courtship behavior studies of N. nanulus nanulus, N. sertifer and D. similis.

Background color did not appear to be important to N. sertifer when 8 different background colors were compared

Table 10-3. Effect of grass leaves and host pine needles* as background for the pheromone source** on males of N. sertifer and N. nanulus nanulus on the field. Test conducted for 60 minutes at Rose Lake and 30 minutes at Vogel Center respectively for N. sertifer and N. nanulus nanulus.

Nature of background	# Visiting only		# Visiting and landing	
	N. sertifer	N. n. nanulus	N. sertifer	N. n. nanulus
Ground glass only				
A	0	2	6	0
B	5	3	6	0
Ground glass covered with grass				
A	6	3	4	8
B	11	3	4	0
Ground glass covered with host pine needles				
A	8	7	2	13
B	4	8	7	10

* Scotch pine needles for N. sertifer; red pine needles for N. n. nanulus.

** Each source in petri dish, 10 ug 2S,3S,7S-A/2S,3R,7R-A 5:0.003 for N. sertifer; 10 ug 2S,3S,7S-A for N. n. nanulus.

Table 10-4. Effect of the nature of background object on wild N. nanulus nanulus males when the pheromone source was glass petri dish. Test conducted for 50 minutes with 10 ug 2S,3S,7S-A.

Source background	# visiting	# visiting and landing
Transparent glass	12	4
Mating table covered with grass	2	15
Metal plate	6	5
Green ground glass plate	3	7
Translucent ground glass plate	3	2

Table 10-5. Effect of mesh size of the mating cage on the response of wild N. nanulus nanulus males to 10 ug 2S,3S,7S-A. Observation was made for 60 minutes.

Mesh type	# visiting	# visiting and landing
Non-crossing steel 2 cm mesh		
A	8	8
B	-	-
Crossing aluminum 1 cm mesh		
A	1	8
B	-	-
Crossing aluminum 0.5 cm mesh gray color		
A	2	4
B	1	4
Crossing aluminum 0.5 cm mesh green color		
A	4	12
B	12	10
Crossing aluminum 0.5 cm mesh brown color		
A	4	10
B	2	4
No cage (transparent glass)		
A	5	4
B	11	6

(Table 10-6). There was no clear cut differences among the number of male visiting or landing with all the background colors tested. Each background color was made of a ground glass plate (18 x 18 cm). However, when sticky traps with different colors were compared for N. sertifer (Table 10-7), traps with the blue, green, orange, white and yellow colors significantly attracted more males. Yellow traps with no pheromone did not catch any males. The red color caught the least number of males. However, for N. nanulus nanulus, there was no significant difference among catches with different colors, but the colors with the highest catches were yellow, green, and white. The results in Table 10-8 show that pheromone source placed on mating cage at the height of 15 m attracted more males of N. sertifer when source was 1 m away from the nearest distal end of scotch pine branch. Fifteen to twenty year old stands were used. In the courtship behavior studies, the mating cage was therefore placed 1 m from the host plant. It was also found that males of N. sertifer on the average spent the longest time with the source when parafin fraction of body extract of virgin females was added to the pheromone source (Figure 10-2). The aldehyde fraction by itself showed a little activity but the number of males tested was small therefore no definite conclusion can be made in the regard.

Courtship Behavior of Male N. virginianus in the Field

The first male was sighted about 5 minutes after the petri dish containing pheromone was opened at the test site

Table 10-6. Effect of background color of pheromone source on the response of wild N. sertifer males on the field. Each treatment contained 10 ug 2S,3S,7S-A/2S,3R,7R-A (5:0.003) and observation lasted 80 minutes.

Background color	# visiting	# visiting and landing
White	4	2
Gray	5	3
Black	4	3
Yellow	4	2
Orange	5	4
Red	5	4
Green	1	2
Blue	2	3

Table 10-7. Field response of male N. sertifer and N. nanulus nanulus to different colors of sticky trap Pherocon II baited with sex pheromone. Test for N. sertifer conducted at Rose Lake (October 2-5, 1982) and for N. nanulus nanulus at Vogel Center, Michigan (September 25-October 16, 1982). Three replicates randomized 3 times.

Trap color	Mean catch/trap ± S.E.*	
	N. sertifer**	N. nanulus nanulus***
Black	24.3 ± 1.0[b]	89.0 ± 4.5[a]
Blue	32.7 ± 2.9[a]	75.3 ± 3.0[a]
Green	37.7 ± 2.6[a]	122.3 ± 3.9[a]
Red	23.3 ± 2.3[a]	104.7 ± 2.7[a]
White	53.0 ± 2.1[a]	115.0 ± 3.9[a]
Yellow	37.0 ± 2.5[a]	152.3 ± 0.0[a]
Gray	24.3 ± 1.8[b]	****
Orange	38.0 ± 1.9[b]	****
Yellow (blank)	0 ± 0[c]	0 ± 0[b]

* Means followed by same letter not significantly different.

** Traps baited with 10 ug 2S,3S,7S-A/2S,3R,7R-A 5:0.003.

*** Traps baited with 5 ug 2S,3S,7S-A.

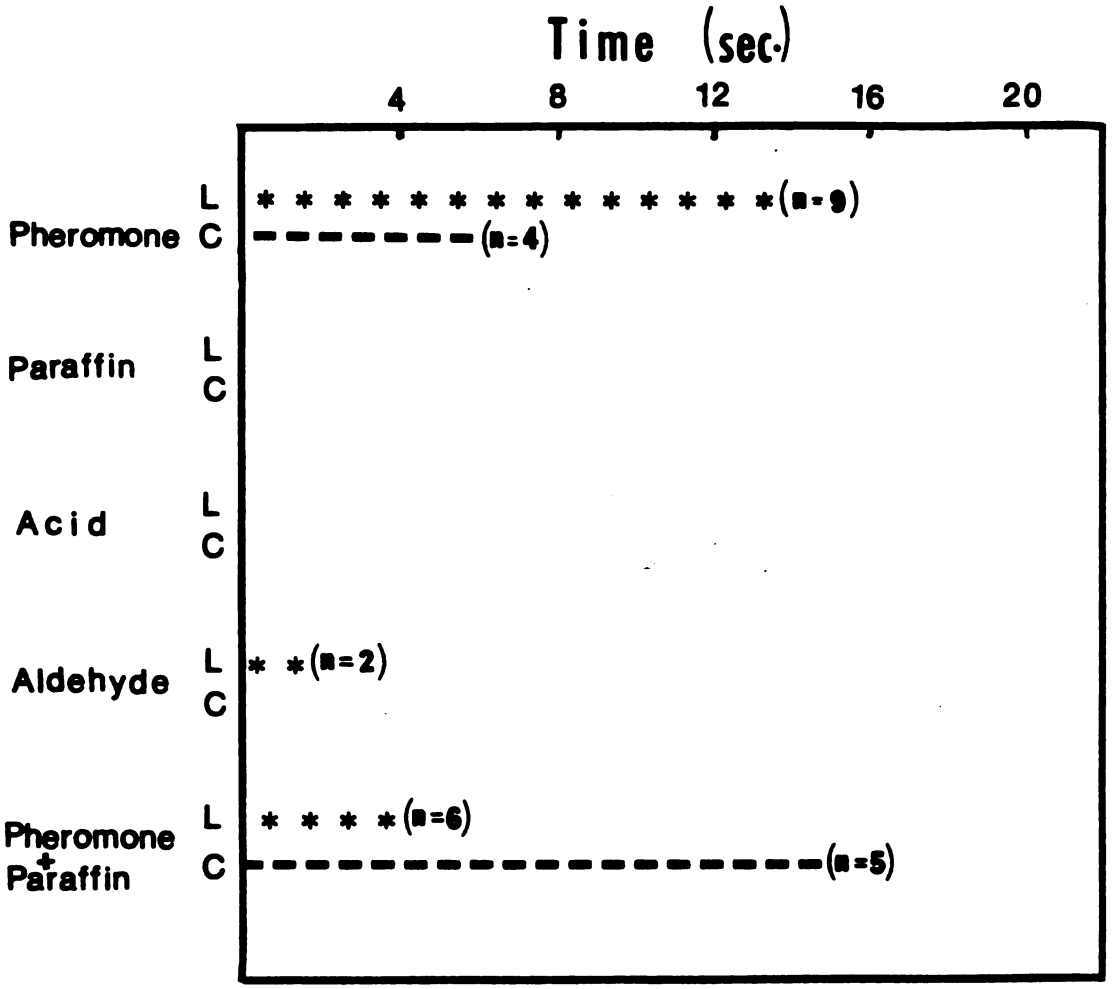
**** Inadvertently left out.

Table 10-8. Effect of distance of synthetic pheromone source used for courtship behavior of male N. sertifer on the field from the nearest scotch pine stand. Test conducted at Rose Lake, Michigan, 10/8-10/15, 1982 and at Pinckney, Michigan 10/16/82. Pheromone source was 40 ug 5:0.003 2S,3S,7S-A/2S,3R,7R-A mixture.

Pheromone placement from nearest pine tree (m)	x males observed landing on nearest scotch pine	x males that landed on mating cage within 30 min.*
0.1	6.3 ± 0.8	0.5 ± 0.4[b]
0.5	6.8 ± 0.6	1.5 ± 0.6[b]
1.0	7.3 ± 0.6	5.0 ± 0.6[a]
5.0	6.5 ± 0.5	0.5 ± 0.4[b]

* Means followed by same letter not significantly different at 5% level by Duncan's new multiple range test.

Figure 10-2. Average time (secs.) male spent within circle 2 cm. radius (L) or time male spent with dead N. sertifer female treated with chemicals by climbing up the pin holding the specimen.


Time (sec.)
4
8
12
16
20
Pheromone
L
C
* * * * * * * * * * * * * (n=9)
(n=4)
Paraffin
L
C
Acid
L
C
Aldehyde
L
C
* * (n=2)
Pheromone
+
Paraffin
L
C
* * * * (n=6)
(n=5)

in Sharon, Michigan. The male at first made a fast circular flight around the upper half of the tree nearest to the cage holding the decoy. After about one or two such turns, the male made a vertical flight down and landed on the pine branch about 1 m away from the decoy. Wing fanning started immediately after landing while the insect walked from the base to the tip of each needle. Up to 10 needles were searched. One male out of 10 even walked around the apex of the jackpine flower. Also, abdominal tip was raised intermittently for about 1 to 5 secs. The males were observed engaged in short flights to land on the cage. There were two patterns of short flights: the first of the two was zigzag and only about 60-66 cm in length, the second type was 30-36 cm and was direct, precise on the target (septum or pheromone treated solvent washed N. nanulus nanulus female) and with an apparent increase in speed. After landing, ambulation in search of the pheromone source followed. Copulation attempts with the source started within 5-10 secs ($x = 5.9 \pm 2.5$ secs S.D.; $n = 10$) upon landing on either the septum or the dry N. nanulus nanulus female. The male could find the pheromone source within 30 sec if landed on the cage. Copulation attempts with septum on the average lasted only 2.62 ± 0.83 mins. S.D. Mating with the female took a different form from the septum's in that the male attempted mating with every part of the female. The frons, cervix of neck, underside abdomen, abdominal terga, sides of abdomen and the abdominal tip. Mating with female lasted 32.3 min.

± 9.7 min. S.D. During copulation attempts, there was intermittent wing fanning, abdominal stroking, leg stroking, but no rear leg holding. Two males were observed to leak pheromone vials with their mouth parts. After copulation, the male walked down the insect pin holding the decoy. The male walked about 5 cm away from the decoy before making about 1 m vertical flight onto the canopy of pine tree. There post copulatory activities continued with cleaning of 1st the legs, then the antennae and lastly the wings. No more wing fanning this time and after the cleaning the male remained motionless for about 2 minutes before flying away. Where two males making ambulation met, they fought momentarily before each continued its searching. One of them often took off immediately after that.

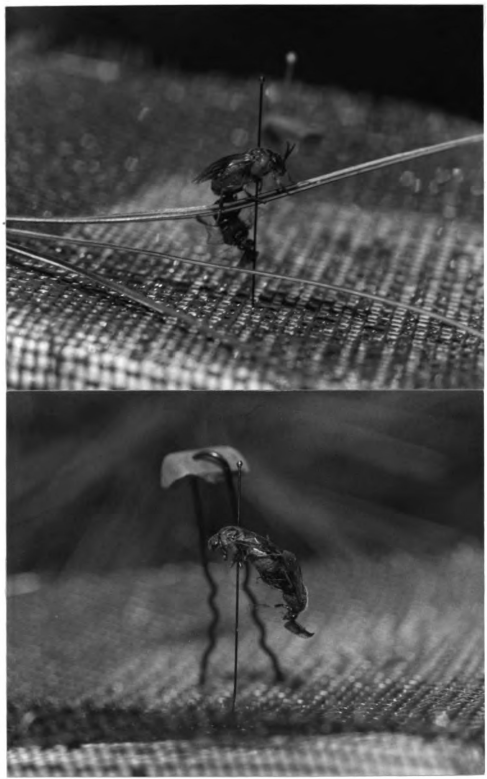
Courtship Behavior of Male
D. similis in the Field

The first male arriving is usually sighted about 5 m away from the source flying a zigzag pattern likely in a positive anemotactic flight towards the source. The male landed on pine branch about 1 m away from source. The wings were spread, and fanning and abdominal tip raised. Then the male made some 2 or 3 short flights from on twig to the other. It raced up and down the pine needles, usually walked from base to about 2/3 up each needle. Sometimes the male jumped to another needle from there or traced its path down in a sort of reversing ambulation. All along, wing fanning continued until the source is discovered. Source

was discovered after about 2 minutes when the source was a pheromone treated rubber septum. The male then climbed the pin holding the septum to the top and remained on it for about 20 secs, motionless, no mating. All the 10 males observed showed this arrestment. Two males also walked all around the septum i.e., top, side, underside of the septum. There was no copulation of the septum but a momentary copulation was observed when the pheromone was a mixture of 2S,3R,7R-P nd 2R,3R,7S-P isomers in ratio 40:0.1. The pheromone used for this study was 2S,3R,7R-P.

However, when a live female was used (female pinned with #3 minuten insect pins to the cage), or a dead, solvent washed female that is treated with pheromone, the male climbed the pin onto the female and quickly positioned himself rear to rear with the female (Figure 10-3). The positioning was completed with 3 secs. Following this was a series of tactile movements. First, the male made 3 to 4 strokes on the lateral side of the abdomen with its rear legs. This was followed by leg strokes, the two metathoracic legs of both male and female were involved in this leg stroking. The male using its metathoracic legs to stroke from femoro-tibial joint to as far down as tarsus of the female rear legs. This was supposed to acquiesce the female as the female remained motionless upon stroking. The stroking continued intermittently throughout the copulation period which lasted on the average 9 minutes 29 secs ± 4.4 secs S.D.). Occasionally during copulation when the female

Figure 10-3. Copulation in D. similis. The male is copulating a restrained live female at the top picture; copulation below is with a dead solvent washed female that is pheromone treated. Note that in both pictures, male did not copulate with the pheromone treated septum at the background. However the male climbed the septum first.



showed some movement, the male fanned wings, followed by abdominal stroking, followed by leg stroking. Where the female was motionless, no stroking occurred but the male and female rear legs were touching. Immediately after copulation, the male disengaged himself and flew away rapidly. It was observed that there was no antennal touch during copulation and the wings remained wide open and motionless during this time. There was wing fanning when copulation proved difficult. The pro and mesothoracic legs of the couple were essentially used for support.

COURTSHIP BEHAVIOR OF MALE N. SERTIFER

Precopulatory Behavior

In the greenhouse where the precopulatory behavior was studied the latency period was about 10 secs. before a quiescent male made a quick turn of 180° facing the direction of the source. Antennae movement followed and then a brief rubbing of the back with metathoracic legs. About 1 min. after the pheromone was present, the male started to move from its resting site on the scotch pine twig towards the source. Having covered the whole length of a pine needle, the male took a somewhat zigzag flight towards the source.

On the field, the male was first sighted when making a circular flight at the upper half of the vertical silhouette closest to source which was always a scotch pine about 3-4 meters in height. The circular flight was followed by a

vertical flight downwards and landing about 1 m on the pine twig away form the source. Like N. virginianus, the N. sertifer searched the pine needles around it by making a series of reversing ambulation covering the entire length of pine needles on the twig. A short flight, slow but zigzag always took the male to the cage, the center top of which was holding the pheromone source--the septum (SP), washed dry N. sertifer female treated with synthetic pheromone (W♀P) or a N. sertifer female, unwashed but dry (UW♀). Whatever the source and whether field or greenhouse, approach to the source upon landing on the cage was the same. This involved a zigzag ambulation with intermittent wing fanning every 2-3 secs and abdominal tip raising and an occasional attempt to copulate the cage. It took about 1 min to discover the source when the source was synthetic pheromone i.e., SP and W♀P, but only 30 secs when the source was natural pheromone UW♀.

Copulatory Activities

Even though male N. sertifer would climb SP and W♀P, only momentary copulatory attempts were made on these decoys. However, when the decoy was an UW♀, there was copulation which lasted an average 18.2 min. with about 4 breaks. The longest copulation time at a stretch observed was 10 minutes. The males often mated several times. However, it is not known whether multiple mating is a common behavior of live females. Certain stereotyped male behaviors were clear from this study. Upon climbing the pin

holding UW♀, the male first made a wing stroking of the female with his forelegs. Then the male quickly dropped into a rear-to-rear position making genital contact. The male stroked the abdomen with its rear legs; this was followed by leg stroking of the female rear legs by the male's and the male held the female firmly with his mesolegs. Probably another series of stereotyped behavior was generated in the mating male after about 5 min. copulation. The male then copulated the dorsum of abdomen of the female momentarily and stroking the wings of the female with his rear legs at the same time. It quickly changed to rear-to-rear position making genital contact again and mated for varying lengths of time from 1 min. to 10 min. This interchange of genital mating and the dorsal abdominal mating went on for 3 or 4 times. Another observation with other males was that instead of dorsal abdominal mating some males used the lateral side of female abdomen to interchange with normal rear-to-rear genital mating. There was no mixture of dorsal abdominal with lateral abdominal mating in a single male as observed in N. virginianus. The abdomen of the male is bent inward for male to assume a 'C' shape in carrying out this dorsal abdominal or lateral abdominal mating. Probing wings of the female with its prolegs however, was a common feature of the two styles. When another intruding male approached during copulation, the copulating male stopped mating to ward off the intruder, after which he came back to resume copulation.

Post Copulatory Activities

After copulation, the male often traced its way back the pin holding the female, then walked about 10 cm down to the side of the mating cage or sometimes to the surface of the ground glass on which the cage was placed. There was no more wing fanning, no more raised abdominal tip this time, the male halted on the ground glass, first cleaned its antennae with the aid of its forelegs for about 2 minutes. It spent another 2 minutes to clean its wings with its hind legs and lastly spent another 2 minutes to clean its antennae the second time before it walked a small distance and flew away. The courtship behavior of the male N. virginianus, D. similis and N. sertifer is summarized in Figure 10-4.

Trap Height

Traps placed at height 3.5 m on the host, the scotch pine Pinus sylvestris L. caught more males than those placed at 1.5, 2.5, 5.5 or 8.0 m (Table 10-9). Next to that was traps at 2.5 m, the commonly employed height of trap placement in diprionid sex pheromone research (Kikukawa et al. 1983). The number of males caught decreased with trap height on the host plant. These data also demonstrate that the optimum height of trapping on the host plant and artificial poles placed the open grass field near the pine stand was not the same. Traps placed at 1.5 m above the ground on the angle steel caught significantly more males than traps at higher heights. The results also show that trap catch on

Figure 10-4. Model of location of female and courtship behavior of wild male diprionid sawfly.

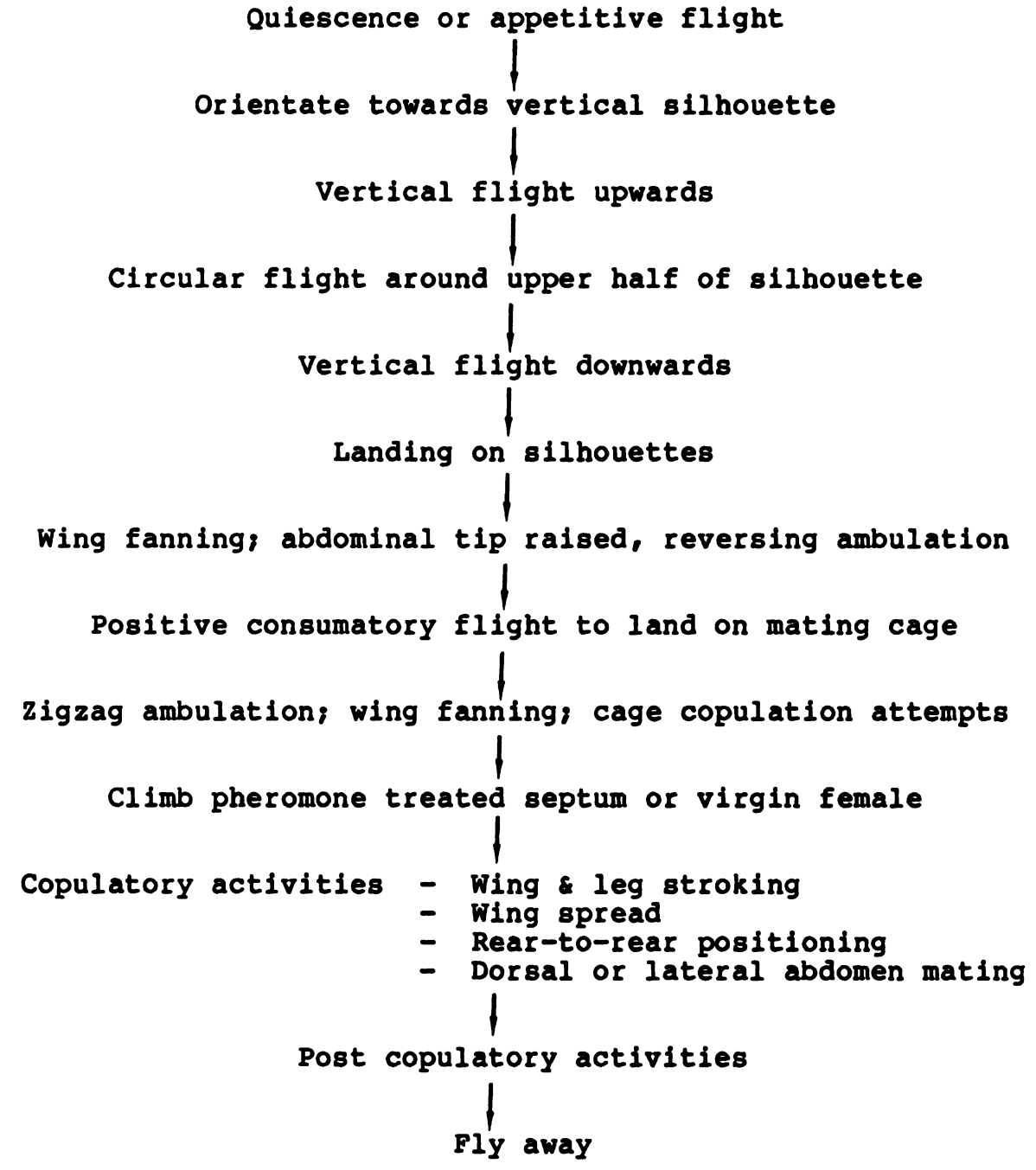

Quiescence or appetitive flight
Orientate towards vertical silhouette
Vertical flight upwards
Circular flight around upper half of silhouette
Vertical flight downwards
Landing on silhouettes
Wing fanning; abdominal tip raised, reversing ambulation
Positive consumatory flight to land on mating cage
Zigzag ambulation; wing fanning; cage copulation attempts
Climb pheromone treated septum or virgin female
Copulatory activities
- Wing & leg stroking
- Wing spread
- Rear-to-rear positioning
- Dorsal or lateral abdomen mating
Post copulatory activities
Fly away

Table 10-9. Effect of trap height on the response of male N. sertifer to synthetic pheromone. Traps placed on scotch pine stands and artificial poles erected at 12 m or 3 m from the nearest host stands. Test conducted at Rose Lake, Lansing, Michigan, September 25-October 6, 1983.

height (m)	Traps on steel pole*		Traps on* scotch pines
	12 m from host	3 m from host	
8.0	0.3 ± 0.4^{b}	0.3 ± 0.4^{b}	24.0 ± 3.2^{a}
5.5	0.7 ± 0.4^{b}	0.7 ± 0.4^{b}	21.0 ± 2.4^{a}
3.5	1.7 ± 0.6^{b}	2.3 ± 0.6^{b}	57.0 ± 2.7^{a}
2.5	1.7 ± 0.6^{b}	3.3 ± 0.7^{b}	38.7 ± 1.4^{a}
1.5	8.3 ± 1.1^{a}	9.0 ± 1.2^{a}	32.3 ± 1.4^{a}

* Means of 3 replicates. Means followed by same letter not significantly different at 5% level by Duncan's new multiple range test.

the artificial pole decreased with height above the ground. There was more catch at heights 1.5, 2.5, and 3.5 m when the artificial pole was nearer (3 m) the host plant. Table 10-10 showed that trap catch of male N. pratti banksianae at heights 1.5, 2.5, and 3.5 m was significantly superior to catches at 5.5 m or 8.0 m in 1983. There was no significant difference among the mean trap catches at 1.5, 2.5 and 3.5 m and no catch at all on traps placed on artificial pole. Also in 1981, catches at these 3 heights were superior to traps at 5.5 and 8.0 m.

Trap Designs, N. virginianus

The result of this testing (Table 10-11), showed that Pherocon II traps caught significantly more sawfly males than any of the other designs. The CFT and POC caught only 2 males while DFT caught only one. However, the funnels in two of the three replicates of DFT were lost as a result of high winds. Pherocon IC suffered the same fate from winds as in field trapping I. Pherocon II traps caught a total of 153 non-target species mainly dipterous and few lepidopterous; the POC 15, CFT 20 and Pherocon IC zero. With regards to debris, Pherocon II topped other designs with tangled twigs, pine needles and flowers. Other designs just had traces of debris. In these two series, the two traps designed to collect males without contamination with the sticky substance, POC and DFT, did not appear to perform well. To continue research in this direction, we came up with two other designs, the IPT and ICT (Figure 10-1).

Table 10-10. Effect of trap height on the response of male N. pratti banksianae to pheromone. Traps were placed on jack pine, replicated 3 times at Hartwick Pines, Michigan in 1981 and 1983.

Trap height (m)	Mean* catch/trap ± S.E.		
	Traps on jack pine		Traps on steel pole
	9/26-10/16/81	10/7-10/18/83**	10/7-10/18/83
8.0	0.7 ± 0.6	0.7 ± 0.5[b]	0 ± 0
5.5	0.3 ± 0.4	0.7 ± 0.5[b]	0 ± 0
3.5	1.0 ± 0.8	3.3 ± 0.4[a]	0 ± 0
2.5	1.3 ± 0.7	2.3 ± 0.4[a]	0 ± 0
1.5	1.3 ± 0.7	2.3 ± 0.6[a]	0 ± 0

* Means of 3 replicates.

** Means followed by same letter not significantly different at 5% by Duncan's new multiple range test.

Table 10-11. Field effectiveness of five trap designs against male N. virginianus. Test conducted at Sharon, Michigan, June 10-July 15, 1983. Three replicates randomized three times.

Design	Mean catch/trap ± S.E.*
POC	0.7 ± 0.6[b]
DFT	0.3 ± 0.4[b]
CFT	0.7 ± 0.6[b]
Pherocon II	16.0 ± 2.1[a]
Pherocon IC	0 ± 0[b]

* Means followed by same letter not significantly different at 5% level by Duncan's new multiple range test.

Trap Designs, D. similis

Four trap designs, IPT, ICT, CFT, and Pherocon II were compared. The POC, DFT, and Pherocon IC which were ineffective in earlier studies were dropped. Each trap was baited with 20 ug purified 2S,3R,7R-P. The purified preparations of this isomer was effective on the field. This was a one replicate experiment designed not only to test the efficacy of sawfly trapping of the designs but also to study the efficiency of each design and to study the behavior of the approach of sawfly into the traps. For all designs, the males landed less than 1 m from the pheromone source. With 2-3 short flights, the male landed on the pine needles closest to the entry space of the traps. From there, the male walked towards the trap, then onto the trap and finally entered the trap. In this trial, the ICT caught 2 males, Pherocon II 1, while the IPT, Pherocon IC and CFT did not catch any males. The 2 males trapped by ICT represented only about 16% of all the males that landed within the vicinity of the trap. The 1 male caught by the Pherocon II represented about 10%. The IPT and CFT trapped no males, even though 10-12 males hovered around and landed less than 1 m from these traps. The DDVP strip has a strong penetrating odor which might have negative effect to the approach of male into the trap. It was also observed that some males entered into the traps baited with DDVP strip and then escaped alive. This killing agent either needs to be more concentrated or be changed for a more effective one.

Increasing active ingredient of DDVP implied using a wider strip. Another approach was to find a means of either increasing the length of time the males have to stay inside the trap once it entered or to design the trap such that the vapor pressure concentration inside the trap is high enough to kill the male once it entered. This approach was pursued in succeeding series of field trapping that follows.

Trap Designs, N. virginianus and N. nanulus nanulus

The ICT with 9 sq cm entry space was the most effective among the 3 types of ICT tested against male N. virginianus and N. nanulus nanulus. However, the insertion of pine twigs clearly improved the catch for ICT 18 and ICT 36 but not for ICT 9. The ICT with the best performance was about 19% and 15.4% as effective as the sticky Pherocon II trap respectively for N. virginianus and N. nanulus nanulus. (Table 10-12).

DISCUSSION

Location and Recruitment of a Mate

The adult life of diprionid sawflies is specialized to propagate progeny. Adults hardly feed and do not diapause. In order to perform their reproductive function efficiently, the female has developed a potent chemical stimuli and the male in turn has developed an equally elaborate olfactory system to perceive the chemical stimulus from the female.

Table 10-12. Field effectiveness of Inverted Cup Trap (ICT) and Pherocon II traps against two species of pine sawfly. Test conducted for N. nanulus nanulus at Vogel Center, and for N. virginianus at Sharon, Michigan. Three replicates randomized three times.

Model	Mean catch/trap ± S.E.*	
	N. virginianus 6/10-7/15/83	N. nanulus nanulus 9/10-10/7/83
ICT 9	4.7 ± 10.0[b]	5.3 ± 0.7[b]
ICT 18	0.3 ± 0.4[b]	0 ± 0[b]
ICT 36	0 ± 0[b]	0 ± 0[b]
ICT 9 + pine twig	4.3 ± 0.7[b]	5.0 ± 1.2[b]
ICT 18 + pine twig	4.3 ± 0.4[b]	5.7 ± 1.7[b]
ICT 36 + pine twig	1.0 ± 0.6[b]	6.6 ± 1.5[b]
Pherocon II	25.0 ± 3.0[a]	41.0 ± 2.9[a]

* Means followed by same letter not significantly different at 5% level by Duncan's new multiple range test.

This chemical stimuli is the sex pheromone, acetate or propionate 3,7-dimethylpentadecan-2-ol (diprionol) (Jewett et al. 1976). During calling, the pheromone is released from the exoglands at the intertergal of the abdomen. While doing this the female flaps the wings to drive the pheromone into the prevailing winds. This study shows that the males, having perceived the pheromone, obey certain rules or are stereotyped to certain behavior. First, response appeared to be dose related as open source attracted more males to visit and land. The implication of this is that a female which produces more pheromone is more likely to be favored with more males. Second, as observed in the results of pheromone source parameters, the pine needles play some role in landing of males. Perhaps, volatiles from pine needles are involved in directing males to land on a suitable host plant. Color appeared not to influence response of males to pheromone probably because of the well-developed chemical communication system in these species.

In a study of sexual activity of the gypsy moth on the field, Richerson et al. (1976) reported that all males oriented to vertical silhouettes such as trees, stumps, shrubs and upright boulders whether females are present or not. Using synthetic pheromone source, we also found this to be true to a great extent for male sawflies. The first response of males when pheromone is perceived is to move out from the thicket to the open. Evidence for this comes from the fact that pheromone traps set in the thicket or bole or

branch with a lot of overstories never catch males; only traps set in the open do. In moving out of the thicket, the males fly just a few centimeters above the ground cover and orient towards a vertical silhouette. Evidence for this is seen in the significant catch of male N. sertifer at height 1.5 m on artificial poles (Table 10-9) where ground covers were approximately 1 m in height. For sawflies, only the host plant appeared to be the major vertical silhouette to which the males orient for the location of the virgin female. Evidence for this is also seen in the superior catch of male N. sertifer and N. pratti banksianae at all the heights tested on the scotch and jack pines respectively compared to catch on the angle steel poles (Tables 10-9 and 10-10). This fact is also supported by the fact that trap catch on the artificial poles did not increase drastically whether the poles were near the host plants or far from them (Table 10-9). The third step in locating the female having approached the vertical silhouettes is for the male to make a vertical flight to cover the whole height of the silhouette. This is similar to the situation in the gypsy moth where males are known to spend more time of their appetitive flight in tree oriented vertical flight (Elkinton and Carde, 1983). Circular flights, which are essentially cross wind flights to pinpoint the approximate location of female on the host canopy, presumably starting from top and proceeding downwards, are the logical searching strategy. Indeed, circular flights were observed in D. similis, N. sertifer

and N. virginianus males. Vertical flights downwards are also observed in these species. It can be speculated that males are probably able to measure the height of a particular host tree silhouette using a series of vertical and circular flights and aided by optomotor cues to know the approximate locus of female location on the host tree. This proposition may be logical in view of turbulence in forest habitat to explain why more males are trapped at a particular height (3.5 m) on the host tree. It is suspected that female sawflies too are evolutionarily guided to sit at this height to release the pheromone and the males too evolutionarily guided to locate the calling females. According to Cardé and Baker (1984), to an organism responding to sex pheromone, proximate cues and orientation mechanisms notwithstanding, such outcomes are the result of evolutionary selection. Kuenen and Baker (1982) interpreted the preferential capture of males at a particular optimum height to reflect pre-pheromone stratification of flying males in the population, or may be indicative of optimal positioning of plumes over visual patterns for the average wind velocities of an area. It is not known whether the latter explanation is applicable to diprionid sawflies but the former explanation does not seem attractive for male sawflies. The males are hardly seen flying except when pheromone is present. Appetitive flight may be physiologically expensive for these non-feeding adult males.

Courtship Behavior of Male
Diprionid Sawflies in the Field

Studying courtship behavior in the wild removes the artificial nature that is perrenial to such study in the laboratory. However, it must be mentioned that it is rather circumstantial for us to do this because both the laboratory reared and field collected male sawfly did not respond to either synthetic pheromone or virgin female in the wind tunnel. Once released, they fly straight to the side or top of the wind tunnel and stay there even when translucent screens were used to diffuse the lightings. There are striking similarities among the three species tested in their courtship behavior (Figure 10-4). However, certain close range copulatory behaviors were evident. First, ambulation locus of the searching male on the twig nearest to pheromone source appeared species specific. Both male N. sertifer and N. virginianus cover the whole length of one needle to and fro before taking on another, male D. similis only covers about 67% of any particular needle. Second, D. similis, despite repeated attempts, failed to mate septum despite the fact that septums are always the first that the males climb when presented with two choice pheromone sources--live female and septum or solvent washed pheromone treated female and septum. The Neodiprion species tested were not as discriminating as D. similis This difference can not be due to pheromone because males of all species were able to execute all acts involved in courtship as well as behavior which facilitated copulation using the most

active synthetic pheromone by each species. Also, the same pheromone that was applied to the septum was also applied to the solvent washed female (which are sometimes of different species from the male being studied, and sometimes without legs or antennae) which are mated by male D. similis (Figure 10-3b). Rather, tactile cues are likely to be involved, it is probably more developed in D. similis, intermediate in N. sertifer and least developed in N. virginianus. Thirdly, within 1 m from pheromone source, male N. virginianus appeared to jump precisely onto the source; this precision was lacking in the other two species studied. It is suspected that visual cues play a role in close range courtship behavior in diprionid sawflies, and perhaps this cue is more developed in N. virginianus in this instance. Fourth, the lateral or tergal abdominal mating of N. sertifer and the mating of every part of the female body of N. virginianus were lacking in D. similis. The dry nature of the dead females or lack of response from females on which these acts were performed cannot be the cause, because male D. similis also mated dead females. Rather, it is suspected that the act is species specific courtship behavior designed to stimulate the female.

Trap Design

Trapping experiments with ICT and Pherocon II traps show that the latter capture significantly more males than the former. The reason for this is that DDVP does not kill quickly. This was obvious from the fact that the ICT design

with the least entry space performed best. The smaller entry space not only limits escape but also keeps the vapor concentration of DDVP inside the trap higher because of limited access of winds. Also supporting this fact is that catches increased drastically when pine twigs were inserted into designs with wider entry spaces, ICT 18 and ICT 36. With twigs, males are retained inside longer for DDVP to effect a knockdown.

It is observed on the field that more males at relatively less time arrive at the ICT 9 than the Pherocon II traps. The key to better efficiency of ICT 9 is to use a killing agent that act faster than DDVP. As it is now, ICT 9 can be very useful as survey traps for new species and as monitoring tool for delimitation of known populations. ICT 9 can also be modified to collect live males when DDVP or any killing agent is not used. Such modification is done by fitting a necked cone shaped wire mesh about same size as ICT into a 3 cm diameter hole at the center of ICT lid. Males N. sertifer were trapped live with this modified ICT 9 in fall 1983 at Rose Lake, Lansing. This modified trap had an efficiency of about 6-8% of the sticky Pherocon II traps. Added to these are the advantages that ICT 9 had no debris and non-target species caught in them are minimal, 0-3 in most cases as against 9-10 in the sticky traps. The trapped males inside the ICT 9 preserved well before collection. The plastic cup used for ICT 9 is available in any food stores and it requires less than 8 minutes to make one

ICT 9 in the laboratory. Strong winds and many thunderstorms at the time of this study left the ICT intact, and the traps used for 3 trappings in spring, summer, and fall are reusable for another year. It is a design with a lot of potential in sawfly research.

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APPENDICES

APPENDIX I

Field Response of Male Abbott Sawfly, *Neodiprion abbotti* (Hymenoptera: Diprionidae) to Mixtures of Optical Isomers of the Sex Pheromone 3,7-dimethylpentadecan-2-ylacetate or propionate

INTRODUCTION

One of the potentially destructive diprionid sawflies, because of multi-generation (up to three in Virginia) occurrence in one season, is the Abbott's sawfly *Neodiprion abbotti* (Leach). It has been recorded in Ontario, Canada, Wisconsin, Virginia, North Carolina, South Carolina, Georgia, and Florida (Atwood and Peck, 1943) and is presently recorded in Michigan. The larvae have a pale spot on the front of the dark head (Atwood and Peck, 1943; Ross, 1955). The *N. abbotti* complex consists of *N. nigroscutum* Middleton, *N. compar* (Leach) and *N. abbotti*. These are regarded as the most primitive offshoot of the lecontei group (Ross, 1955). The larvae of *N. abbotti* feed on various hard pines including *Pinus carribea*, *P. echinata*, *P. heterophylla*, *P. palustris*, *P. resinosa*, and *P. taeda* (Atwood and Peck, 1943) and *P. banksiana* (Atwood, 1961).

The new record of *N. abbotti* in Michigan is made at Sec. 15, Hartwick Pines State Forest, Crawford County, from where larvae and rows of eggs were collected. Each row has 15 closely spaced eggs. The eggs, however, failed to hatch

in the laboratory. The larvae collected in that vicinity are yellow green with two broad dark green stripes dorsally and a narrower pair latero-ventrally. The black head has a faint light spot on the frons. All the six larvae spun cocoons from which adults emerged. The larvae of N. nigroscutum and N. compar, the members of the abbotti complex differ respectively by having reddish brown to black head without a frontal spot and dark brown head with additional white marking on the lower part of the face (Wilson, 1970). No other diprionid pine sawfly larval headcapsule, except Gilpinia frutetorum (Fabricius) with a frontal black triangular marking on a brown headcapsule, appears close to N. abbotti. With this apparent expansion of distribution of this currently inconspicuous species whose injury is often confused with those of other abundant species, sex pheromone baited trap could be a useful monitoring tool.

Casida et al. (1963) first reported the presence of a female produced pheromone in diprionid sawflies which is capable of attracting males from a few hundred meters away. Jewett et al. (1976) purified and identified the precursor of the pheromone as 3,7-dimethylpentadecan-2-ol (diprionol), the acetate or propionate of which is active against the males of many sawfly species. Synthesis of this compound around the time that this report was made produced a racemic compound (Jewett et al. 1976; Kocienski and Ansell, 1977). When tested on the field, these synthetic pheromones caught sparingly few males and was far less active as well as being

inhibitory to natural pheromone (Jewett et al. 1978). However, when optical isomer of this compound were tested, various field studies established that N. lecontei (Fitch) are attracted by (2S,3S,7S) 3,7-dimethylpentadecan-2-yl acetate (2S,3S,7S-A) (Matsumura et al. 1979; Kraemer et al. 1981) while males of Diprion similis (Hartig) are mainly attracted by (2S,3R,7R) 3,7-dimethylpentadecan-2-yl propionate (2S,3R,7R-P) (Kikukawa et al. 1982a). About five other Neodiprion species N. pinetum (Norton) Kraemer et al. 1979), N. sertifer (Geoffroy) (Kikukawa et al. 1983), N. pratti complex, N. taedae linearis Ross and N. nanulus nanulus Schedl are known to be mainly attracted by 2S,3S,7S-A isomer. Through an extensive field investigation (Kikukawa et al. 1983) found that addition of a small amount of 2S,3R isomer was synergistic to the field effectiveness of 2S,3S,7S-A against N. sertifer. The research reported here was undertaken to define the stereospecific response of N. abbotti and to elucidate the effect of mixtures of optical isomers in attracting males on the field.

MATERIALS AND METHODS

Field Tests

The field tests were conducted mainly in a mixed pine stand at Hartwick Pine, Michigan. The red pine, P. resinosa is the predominant species, with the white pine P. strobus and jack pine P. banksiana found scattered around the edges. A few trappings were also done in a jack pine stand on

Fletcher Road in Roscommon County, Michigan. The egg and larval collections were made on jack pine twigs on the edges of clearings in the middle of Hartwick Pine State Forest.

Pherocon II traps were used throughout this study. Trap preparations have been described (Kikukawa et al. 1983). Traps were set out on the field in a randomized complete block design, with the block choice based on past trapping experience. Throughout the trapping season, frequent re-randomization of treatments within and between blocks were done usually at the time the number of males caught in each trap was being recorded. The data so collected are evaluated with an analysis of variance and with differences among means graded at P = 0.05 according to Duncan's new multiple range test. Trapped males were removed from the traps, washed with n-hexane to remove the sticky material and stored in 70% alcohol. Each trapped male was examined microscopically and compared to laboratory reared specimens.

Synthetic Pheromones

The synthetic pheromones used came either as pure, racemic isomer or mixtures. We refer to carbon 2, 3, and 7 of diprionol as either R or S or R/S when racemic. All the isomers were esters. Four chiral isomers 2R,3R,7R; 2R,3R,7S; 2S,3S,7R; and 2S,3S,7S were synthesized by Mori et al. (1978). Tai et al. (1978) synthesized 2S,3S,7R/S and 2S,3R,7R/S and a mixture of the two isomers used in this study. Kikukawa et al. (1982b) synthesized an supplied

2S,3R,7R and 2S,3R,7S isomers. The 2R/S,3R.7R and 2R/S,3R,7S were modified from 2R,3R,7R and 2R,3R,7S by the method described by Kikukawa et al. (1982b). These isomers contained varying levels of synthetic impurities which were removed by the method described below. The isomers from Tai et al. (1978) contained a known 5% contamination of optical impurities and those from the other two chemists less than 1% of erythro-in-threo or threo-in-erythro contamination.

Purification of Synthetic Isomers by Charcoal-Celite Column

Thirty-six gm of charcoal (Norit A, J. T. Baker Chemical Co., New Jersey) pre-heated for 2 hrs. at 180°C, was mixed thoroughly with 9 gm celite (Fisher Scientific Co.) and washed with 20 ml acetone in a funnel with Whatman No. 2 filter paper. The charcoal-celite obtained from above was stirred into a 200 ml beaker with 140 ml 10% ether in hexane using a glass rod. The slurry obtained was packed in a 50 cm x 2 cm i.d. glass column. A 20 ml solvent was first used to wash the slurry down the walls of the column followed by 80 ml in 2 equal volumes to stabilize the column. A maximum of 7 mg sample was introduced to the column and was eluted with 10% of ether in n-hexane at 0.2 ml/min of flow rate. Recovery was about 60%, with samples less than 500 ug it was less. Sixty-three 10 ml fractions were collected using a micro fractionator (model FC-80K, Gilson Medical Electronics, Inc., Middleton, Wisconsin).

A small aliquot from each fraction was injected into a gas liquid chromatograph (GLC) equipped with a flame ionization detector (FID). Fractions 33-63 contained the sample, but fractions 33-42 and 61-63 contained the sample and impurities while fractions 43-60 contained the pure sample. It should be noted that columns less than 35 cm in height failed to purify the samples in this manner.

RESULTS

Of the four isomers tested in Table AI-1, traps baited with 2S,3S,7S-A caught the most males. The 2S,3R,7R/S-P and 2S,3S,7R/S-A also showed some activity, about one third and one quarter respectively that of 2S,3S,7S-A. However, the corresponding propionate 2S,3S,7S-P was not active though 2S,3R,7R/S-P showed some activity. This was our first encounter with a species responding to 2S,3S,7S-A and 2S,3R,7R/S-P but not to 2S,3S,7S-P. In the case of N. sertifer (Kikukawa et al. 1983) and N. pratti banksianae (Olaifa et al. 1984), while responding to 2S,3S,7S-A as the major isomer, these species were also attracted to a little extent by 2S,3S,7S-P and we interpreted that to mean an overriding stereospecific response to the 2S,3S,7S isomer. Also, in Wisconsin, N. pratti pratti which responded to 2S,3R,7R/S-P also showed activity towards 2S,3S,7S-P (Kraemer et al. 1983). The unexpected activity of 2S,3R,7R/S-P prompted a further investigation of other available acetate and propionate diprionol isomers against

Table AI-1. Field response of males N. abbotti to four optical isomers of acetate or propionate diprionol. Test conducted at Hartwick Pines, Michigan, May 15-July 10, 1981.

Optical isomers (25 ug/trap)	Mean catch/trap[a] ± S.E.
2S,3S,7S-A	45.3 ± 4.5
2S,3S,7R/S-A	11.0 ± 2.2
2S,3S,7S-P	0.0 ± 0.0
2S,3R,7R/S-P	16.3 ± 2.3

[a] Means of 3 replicates. Analysis of variance not significant.

this species (Table AI-2). Here, 2S,3S,7S-A again showed an outstanding activity towards N. abbotti. The 2S,3S,7R/S-A isomer also showed some activity, but 2S,3R,7R/S-P this time caught only one male. The result also demonstrates the importance of acetate isomers for this species and the fact that propionate isomers were not active against N. abbotti. Also demonstrated from this table is the importance of S configuration on 7-carbon as 2S,3S,7S-A was active, 2S,3S,7R/S-A with little activity and 2S,3S,7R-A inactive. It is thus established that 2S,3S,7S-A is the major pheromone of N. abbotti, a characteristic shared with many Neodiprion species as described earlier.

To test whether some combinations of threo isomers, i.e., 2S,3R or 2R,3s isomers, give high biological activity, five threo acetate isomers occurring as either pure or racemic forms were added to 2S,3S,7S-A (Table AI-3). Three isomers in increasing order of effectiveness in increasing the activity of the major isomer towards this species were 2R/S,3R,7R-A, 2S,3R,7R/S-A and 2S,3R,7R-A. Because of relatively large variance among the replicates for the 2S,3S,7S-A plus 2R/S,3R,7R-A treatment, the data of the two other active synergists were regarded more reliable. In another test during the following year, three threo isomers were compared and the importance of chirality on 2-carbon and 7-carbon was tested. The results show that 2S,3R,7S-A isomer was a better synergist of 2S,3S,7S-A than either 2S,3R,7R-A or 2R/S,3R,7S-A (Table AI-4). At a combination ratio of 1:2

Table AI-2. Field response of males N. abbotti to different optical isomers of acetate or propionate diprionol. Test conducted at Hartwick Pines, Michigan, June 6-July 20, 1981.

Optical isomers	Amount (ug/trap)	Replicates A	B	Total
2R,3R,7R-P	20	0	0	0
2R,3R,7S-P	20	0	0	0
2S,3S,7R-P	20	0	0	0
2S,3S,7S-P	20	0	0	0
2S,3R,7R/S-P	40	1	0	1
2S,3R,7R-P	20	0	0	0
2S,3S,7R-A	20	0	0	0
2S,3S,7S-A	20	27	26	53
2S,3S,7R/S-A	40	4	3	7
Control	0	0	0	0

Table AI-3. Field response of males N. abbotti to mixtures of optical isomers of acetate diprionol. Test conducted at Hartwick Pines, Michigan, June 5-July 11, 1981.

Preparations	Amount (ug/trap)	Mean catch/trap* ± S.E.
2S,3S,7S-A	20	3.3 ± 0.7
2S,3S,7S-A / 2S,3R,7R/S-A	10/15	6.3 ± 0.8
2S,3S,7S-A / 2S,3R,7R-A	10/15	11.7 ± 1.7
2S,3S,7S-A / 2R/S,3R,7R-A	10/15	5.3 ± 1.7
2S,3S,7S-A / 2R/S,3R,7S-A	10/15	1.6 ± 0.8
2S,3S,7S-A / (2S,3R,7R/S / 2R,3S,7R/S-A)	10/15	1.3 ± 0.6
Control	0	0.0 ± 0.0

* Mean catch of 3 replicates. Analysis of variance not significant.

Table AI-4. Field response of males N. abbotti to mixtures of 2S,3S,7S-A with 2S,3R,7R-A; 2S,3R,7S-A and 2R/S,3R,7S-A isomers. Test conducted at Fletcher Road near Sharon, Michigan, May 7-June 10, 1982.

Preparations	Amount (ug/trap)	Mean catch/trap ± S.E.
2S,3S,7S-A	5	5.3 ± 1.3
2S,3S,7S-A / 2S,3R,7R-A	5/5	1.0 ± 0.8
	5/10	1.0 ± 0.6
2S,3S,7S-A / 2S,3R,7S-A	5/5	6.7 ± 1.7
	5/10	6.3 ± 1.4
2S,3S,7S-A / 2R/S,3R,7S-A	5/5	1.0 ± 0.0
	5/10	3.0 ± 0.9

[a] Means of 3 replicates. Analysis of variance not significant.

2S,3S,7S-A:2R/S,3R,7R-A, more males were attracted than 2S,3S,7S-A:2S,3R,7R-A at the same ratio. It then appeared that S configuration at both 2-carbon and 7-carbon was preferred by this species.

In two separate tests, one at Hartwick Pines in 1981 where 2S,3R,7R/S-A was the synergist, the other at a low population area on Fletcher Road, Crawford County, in 1982 where 2S,3R,7S-A was used, the effectiveness of these isomers as synergists of 2S,3S,7S-A was confirmed (Tables AI-5 and 6). A significant synergism at a blend ratio of 1:1 was achieved when 2S,3R,7R/S-A was the synergist (Table AI-5). Also, at the same ratio, 2S,3R,7S-A isomer increased the effectiveness of the major isomer. Beyond this optimum ratio, inhibition appeared to set in.

DISCUSSION

Our data indicate that males of N. abbotti respond mainly to 2S,3S,7S-A, but increased response is obtained by employing 1:1 mixture of 2S,3S,7S-A and 2S,3R,7S-A, the latter isomer serving as a synergist. It has become clear that most Neodiprion species utilize combinations of optical isomers as their pheromone. This is the first report of a 1:1 combination. Other combinations are: a 5:0.003 for N. sertifer (Kikukawa et al. 1983) and 5:1 for N. pratti banksianae (Olaifa et al. 1984). Further studies are required for other species to identify the isomer combinations of their sex pheromone. It can only be speculated at the

Table AI-5. Synergistic effect of the 2S,3R,7R/S-A isomer on the field effectiveness of 2S,3S,7S-A against N. abbotti. Test conducted at Hartwick Pines, Michigan, May 15-August 4, 1981.

Preparations	Amount (ug/trap)	Mean catch/trap* ± S.E.**
2S,3S,7S-A	20	9.7 ± 1.1[c]
2S,3R,7R/S-A	20	0.0 ± 0.0[c]
2S,3S,7S-A / 2S,3R,7R/S-A	20/5	5.3 ± 1.1[c]
	20/10	29.7 ± 3.6[b,c]
	20/20	77.0 ± 2.7[a]
	20/40	59.0 ± 2.2[a,b]
Control	0	0.0 ± 0.0[c]

* Means of 3 replicates.

** Means followed by same letter not significantly different by Duncan's multiple range test at 5% level.

Table AI-6. Field response of male N. abbotti to a varying mixtures of 2S,3S,7S-A and 2S,3R,7S-A isomers. Test conducted at Fletcher Road, Garfield Township, Michigan, May 25-August 13, 1982.

Preparations	Amount (ug/trap)	Series A[a] I	Series A[a] II	Series B[b] I	Series B[b] II	Series B[b] III	Total catch
2S,3S,7S-A	5	0	0	0	1	0	1
2S,3S,7S-A / 2S,3R,7S-A	5/0.001	1	1	1	2	0	5
	5/0.003	0	0	0	0	0	0
	5/0.01	1	0	1	0	0	2
	5/0.03	0	1	0	0	2	3
	5/0.1	1	0	0	0	1	2
	5/0.3	1	0	0	0	0	1
	5/1	0	0	0	1	2	3
	5/3	1	1	0	0	0	2
	5/5	3	3	2	0	2	10
	5/10	2	1	1	0	2	6
	5/30	3	0	-[c]	-[c]	-[c]	3
Control	0	0	0	0	0	0	0

[a] Test conducted May 25-June 19, 1982.

[b] Test conducted June 30-August 13, 1982.

[c] Inadvertently left out.

moment that second component would be serving an important isolation mechanism for the sympatric species and for the members of complexes. It will also be worthwhile to investigate the occurrence of these isomers in the female produced sex pheromone. Unlike other conspicuous species like N. sertifer and N. nanulus nanulus, it was impossible to obtain enough females of N. abbotti from the field for extraction of pheromone. Thus, comparison of the natural and synthetic pheromone was not done in this study. The current study, however, established that the 1:1 mixture of 2S,3S,7S-A and 2S,3R,7S-A is a potent attractant of N. abbotti.

Apart from its pheromone being unique with the 1:1 blend of 2S,3S,7S-A and 2S,3R,7S-A isomers, this species appears to be the only spring flying Neodiprion species caught in non-white pine area with acetate. The Swaine sawflies--N. virginianus, N. swainei, N. rugifrons, and N. dubiosus--respond to the propionate. D. similis and N. pinetum are strictly white pine species and the former responds to the propionate while the latter is attracted with acetate. The only possible mixing up is N. pinetum. However, the male N. pinetum is generally smaller and thinner than male N. abbotti. Male abbotti is more robust than any other spring flying Neodiprion species so far encountered in Michigan. Male N. abbotti is reported to be more plump than males of known Neodiprion species in eastern Virginia (Hetrick, 1956). Freshly caught male N. pinetum is

light orange at the lower abdomen while male N. abbotti is dark orange. Thus, by size, shape and abdominal coloration, male N. pinetum and N. abbotti can be separated. Separation can also be achieved by species stereospecific blend 1:2 2S,3S,7S-A to 2S,3R,7R-A for N. pinetum. However, until the nature of the sex pheromone of other members of abbotti complex and other species that have been reported in Michigan--such as N. maurus Rohwer--is known and the males of these species caught and compared, we cannot be fully sure of the identification of the present species.

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APPENDIX II

Cuticular Hydrocarbon of Ten Species of Pine Sawfly

INTRODUCTION

Hydrocarbons are often an abundant component of cuticular lipid of insects (Jackson and Arnold, 1977). There is a great variation in hydrocarbon composition between species and along with other components of cuticular wax, these compounds are known to be highly conserved during evolution therefore, they must serve a function (Jurgen Jacob, 1978). In insects apart from the primary physiological role of forming a water impermeable barrier on the surface of a cuticle and performing a communicative role as semiochemicals (Howard, 1982), the cuticular hydrocarbon along with other wax components also protect insect from abrasive damage, serve as a barrier to the penetrating microorganisms and affect the absorption of insecticides and other chemicals from the environment (Blomquist and Jackson, 1979). Another function currently being assigned insects cuticular hydrocarbon is chemotaxonomy (Jackson and Blomquist, 1976; Lockey 1976). The basis for that is still being debated.

In considering hydrocarbon composition in chemotaxonomy work in insect, several insects have been examined and interesting qualitative differences have been found between

families of Orthoptera (Nelson and Sukkestad, 1975) and between different species within the same genus of Periplaneta (Jackson, 1972). The complex nature of the hydrocarbon composition and the interesting patterns of methyl branching in many insects suggest that such possibility might exist as previously suggested for fungi and higher plants (Jackson and Blomquist, 1976). It is also clear in many insects examined that only the branched hydrocarbons might be relevant for chemotaxonomy in insects because by using labelled precursors, Blomquist and Jackson (1979), confirmed the view that insects synthesize branched alkane fraction of the hydrocarbon. The same authors also found that in insects, the n-alkane constituents of cuticular lipids are not synthesized but are mainly derived directly from the diet. In the light of that, the n-alkane pattern of a cuticular lipid will be related more to the insect's diet than to its genotype so that a correlation between n-alkane pattern and systematic grouping is not to be expected (Lockey, 1976 and 1980). Other workers (Kolattukudy, 1976) confirmed this observation by finding large amount of n-alkanes in acridids to be common constituents of plant waxes. It is believed that hydrocarbon biosynthesis is under hormonal control (Armold and Regnier, 1975), occurs in oenocytes (Wiggleworth, 1976) and the enzyme concerned in the synthesis are determined by the genotype. It could therefore be reasoned that the genotypic differences between

the species of sawfly should be reflected in differences between their branched alkane patterns.

MATERIALS AND METHODS

The adult males reared in the laboratory from the field collected larvae were used for this study. The males of N. swainei, N. lecontei, N. pratti paradoxicus (with black lower abdomen) and N. pratti paradoxicus (with red lower abdomen), N. rugifrons and N. pinetum were secured from the laboratory of Dr. H. C. Coppel, University of Wisconsin. The other species N. nanulus nanulus; N. pratti banksianae, N. sertifer, and D. similis were reared in our laboratory. A number of extraction procedures have been described in the literature (Jackson and Armold, 1977), most of them involved some type of a short solvent wash utilizing commonly hexane, methylene chloride, diethyl ether or chloroform. The point of disagreement among various authors is the length of the washing time, some believed that short extraction periods may not remove the cuticular lipid quantitatively and others argued that long extraction periods may extract internal lipids. All the species used for this study were extracted for 10 hours in 2 ml hexane per species. For each species, 2-4 males were used. After the 10 hours the hexane was removed and was partitioned with 2 ml acetonitrile. The hexane phase was then purified of the polar components by running through florisil column chromatography, packed dry 7 cm tall inside glass pipette 0.8 cm i.d. and 1.1 cm o.d.

Florisil was activated by heating in oven at 230° for 1 hr. before use. The column was eluted with hexane and the first 10 ml fraction was collected. The solvent was removed with dry nitrogen gas to a final volume of 0.1 ml. Of this final volume, 0.05 ml was injected into a 20 ft. 1/8" i.d. stainless steel 3% S.E. 30 column, at column, injector and detector oven temperatures of 260°, 300°, and 310°, using a FID varian GC model 2400 with nitrogen carrier gas flow of 30 ml/min. H_2 30 ml/min. and air 300 ml/min. Linear recorder was used at 30 cm/hr. Attempt to clean the extracts of males collected through sticky traps from the field first by activated florisil and second by a 1:1 mixture of activated florisil and charcoal columns failed. Therefore, no males from sticky traps was used for this study. No attempt was made in this study to identify any of the cuticular hydrocarbon for any of the species. This study only compared the gas liquid chromatographic spectra of nine species of pine sawfly. This was done by first developing a spectrum for each species and then compile a sort of master spectrum comprising all the maximum peaks obtainable from all the spectra. All the peaks are renumbered according to the retention time. Identical peaks have identical numbers in all species.

RESULTS

Figure AII-1 were the chromatogram of the cuticular hydrocarbon of the species examined. One obvious

Figure AII-1. GLC chromatogram of cuticular hydrocarbon of ten diprionid sawfly species.

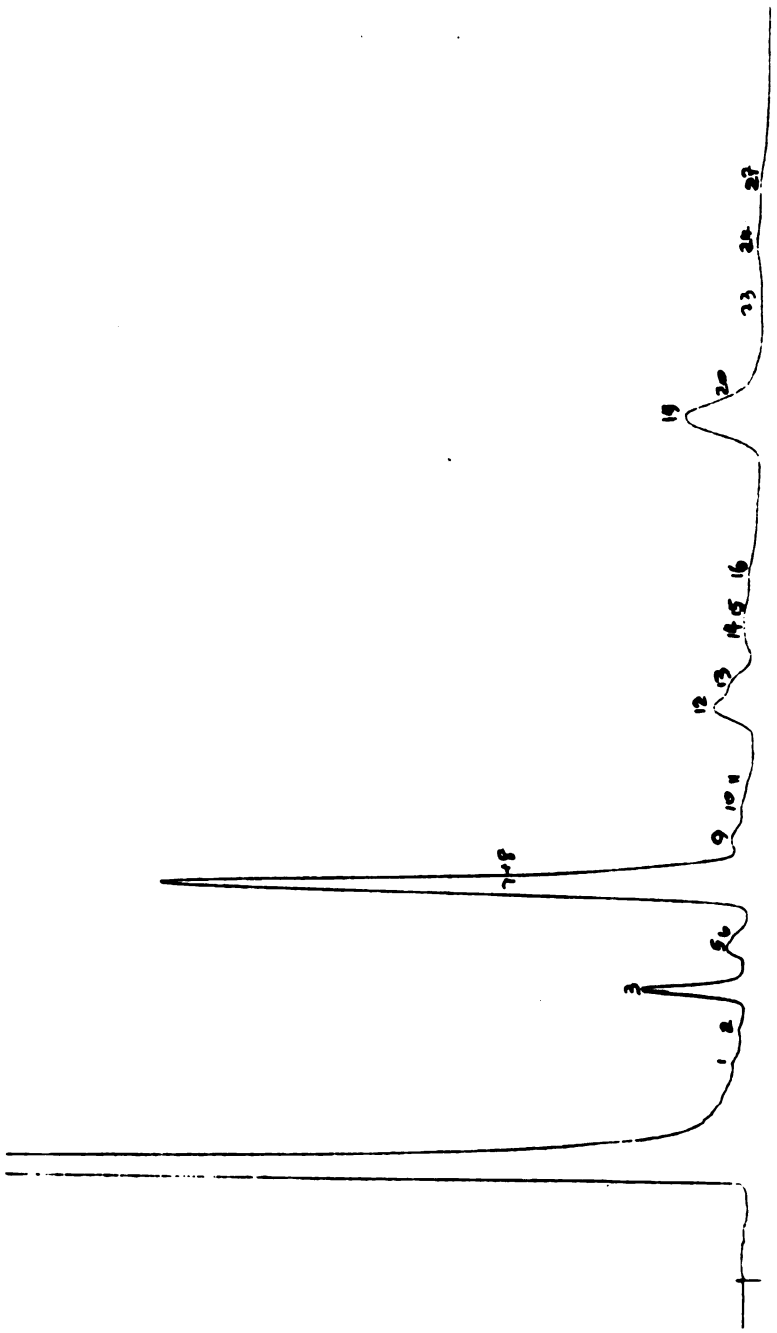

Figure AII-1a. GLC chromatogram of cuticular hydrocarbon of N. pratti paradoxicus (red lower abdomen).

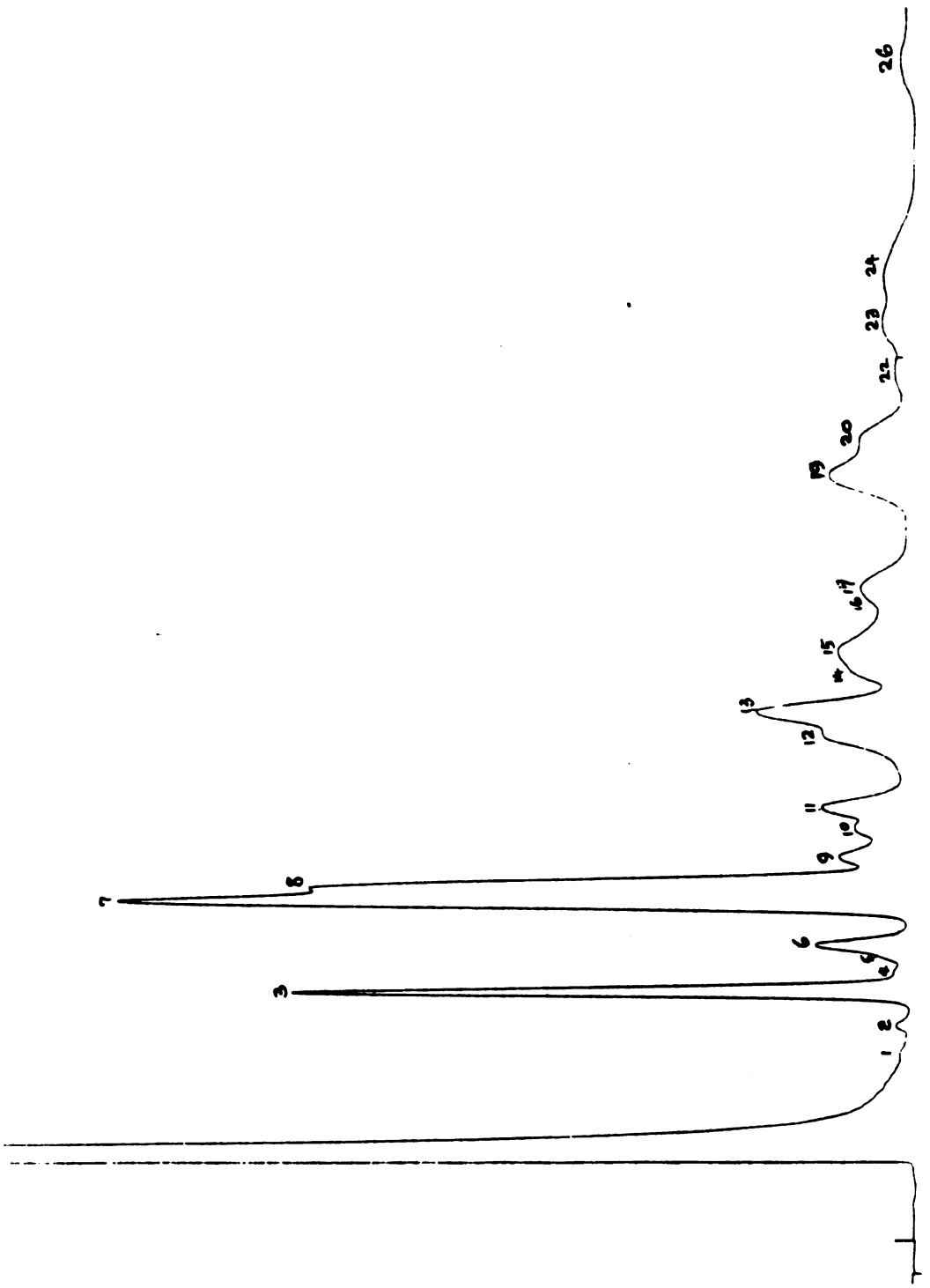

Figure AII-1b. GLC chromatogram of cuticular hydrocarbon of N. pratti paradoxi-cus (black lower abdomen).

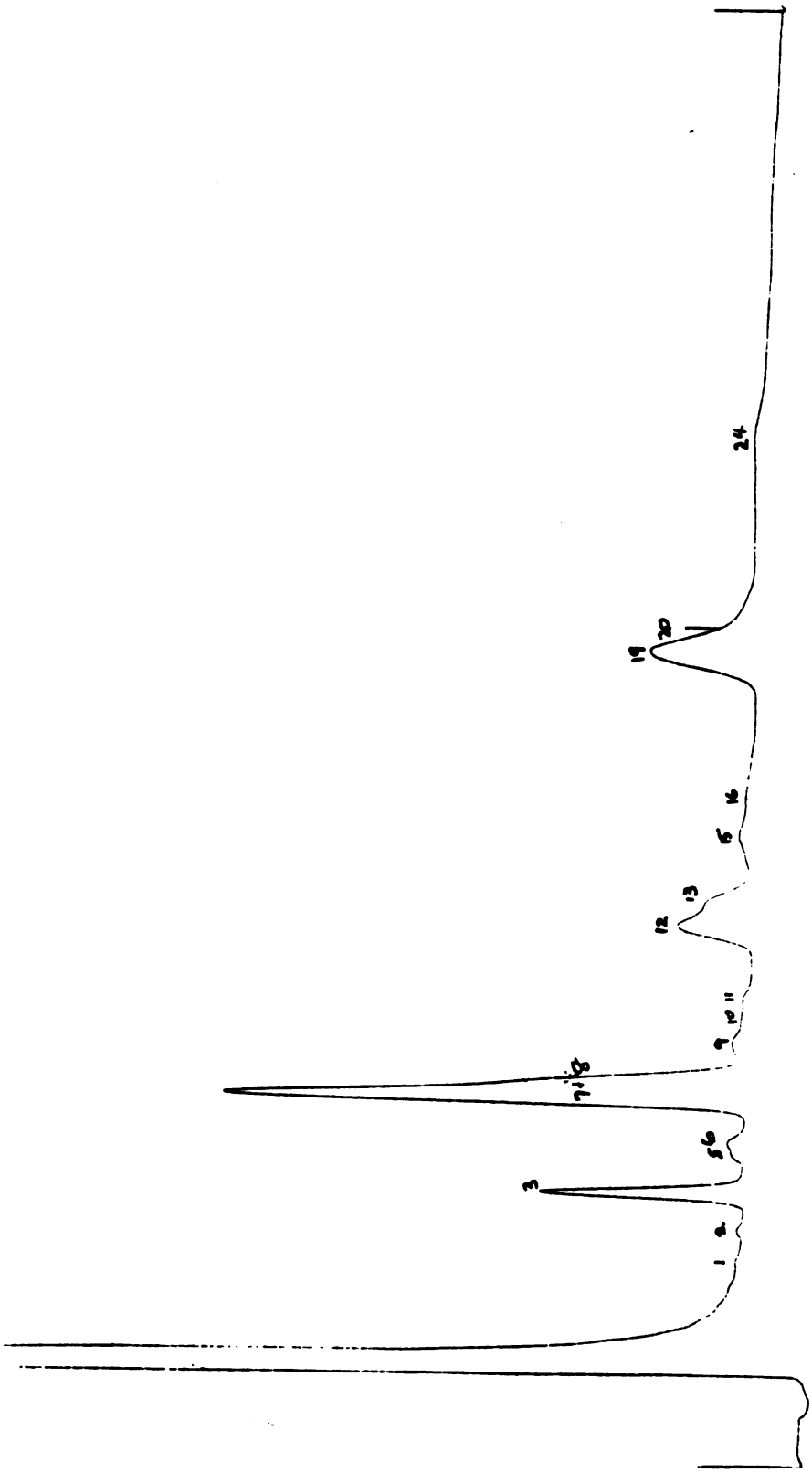

Figure AII-1c. GLC chromatogram of cuticular hydrocarbon of N. dubiosus.

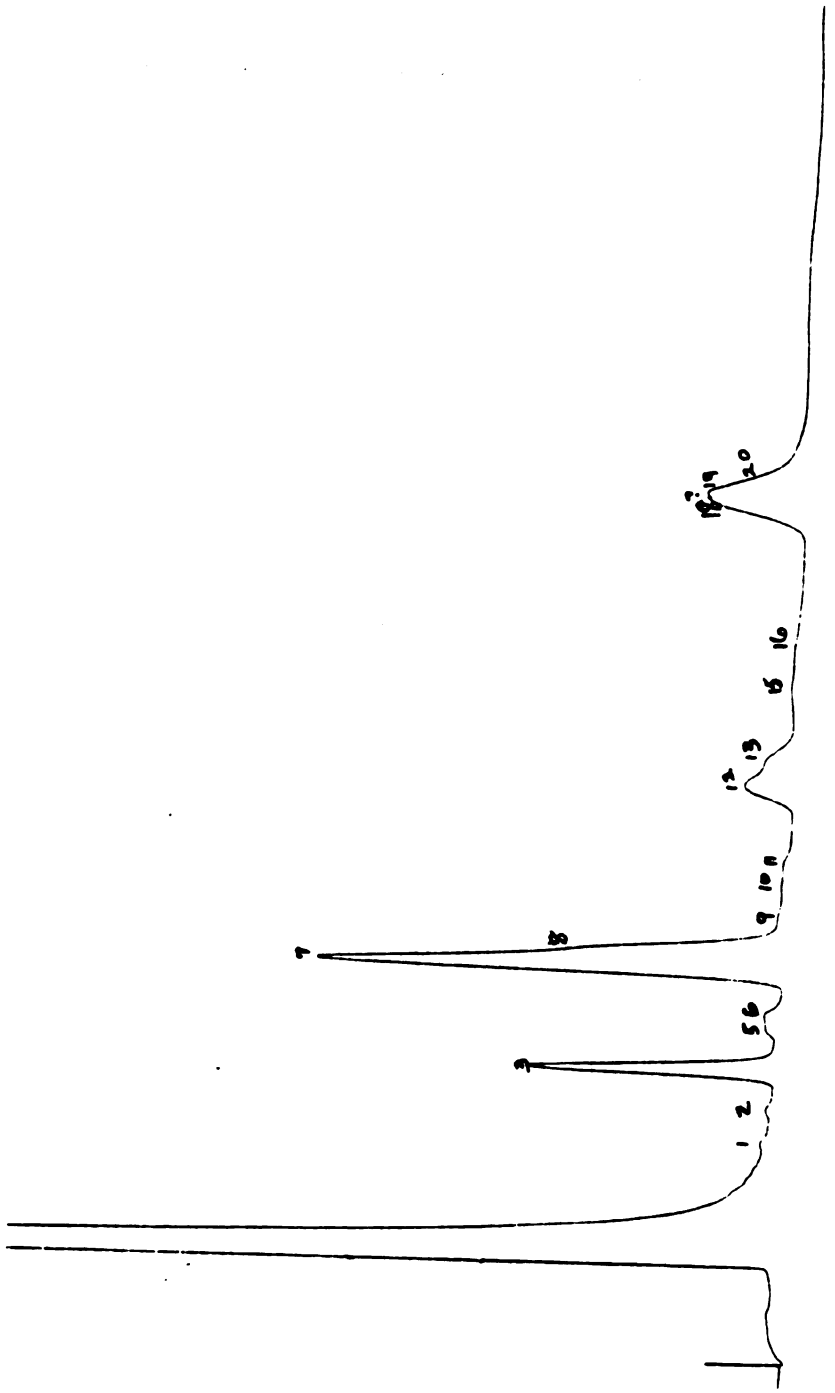

Figure AII-1d. GLC chromatogram of cuticular hydrocarbon of N. pinetum.

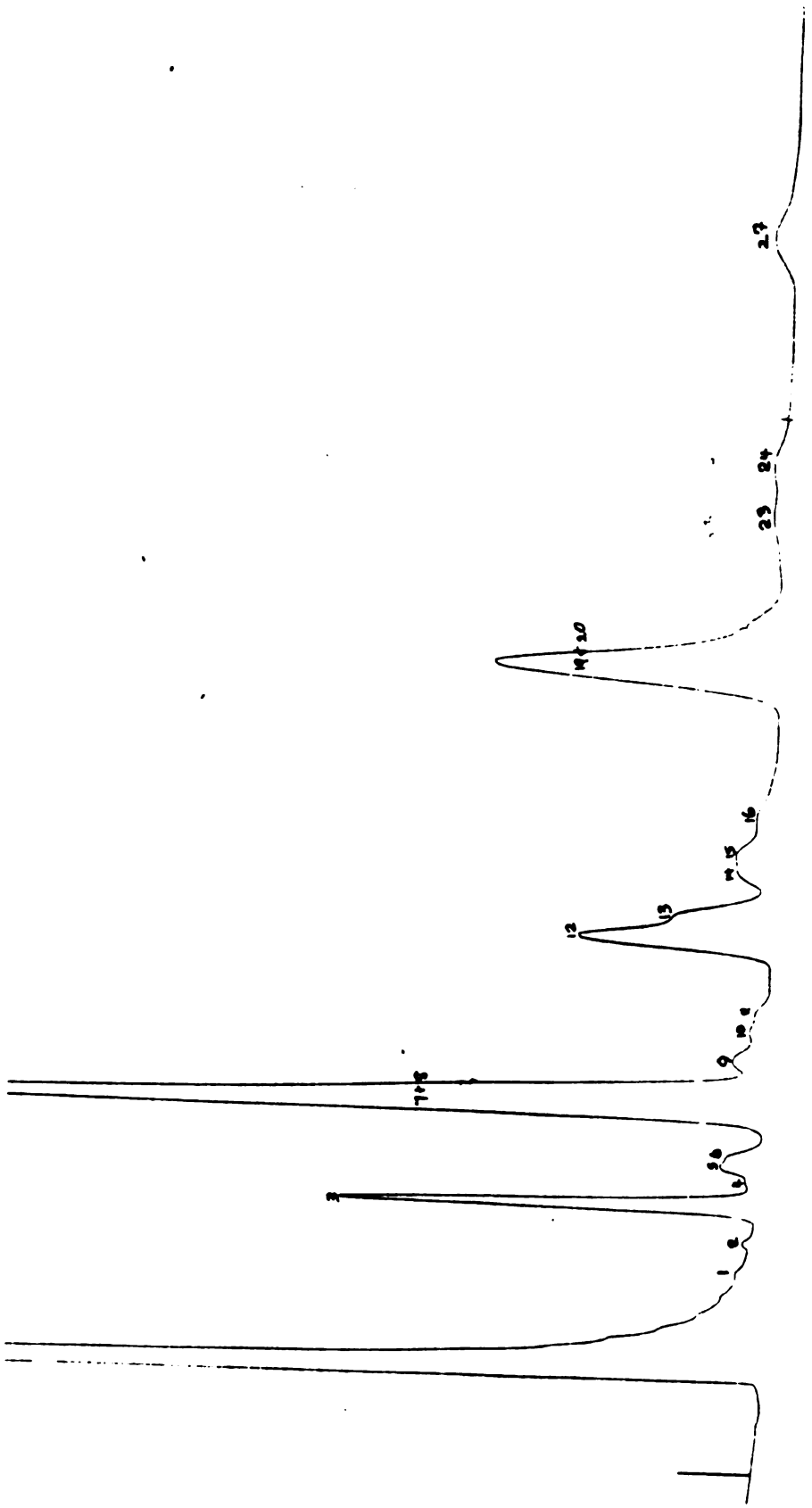

Figure AII-1e. GLC chromatogram of cuticular hydrocarbon of N. swainei.

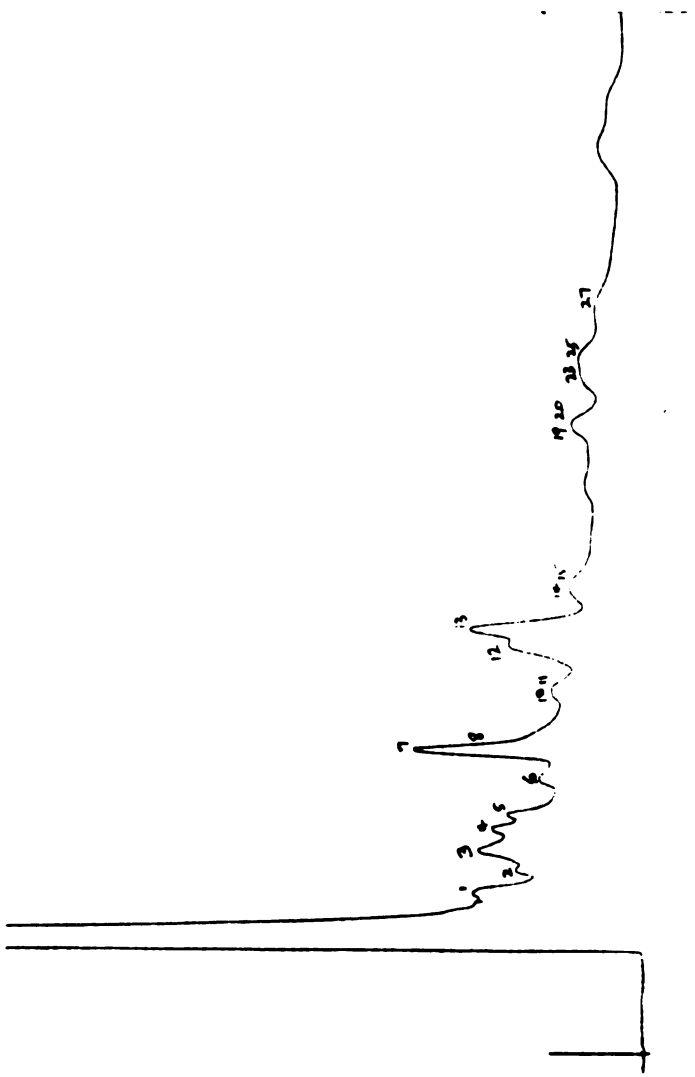

Figure AII-1f. GLC chromatogram of cuticular hydrocarbon of N. virginianus.

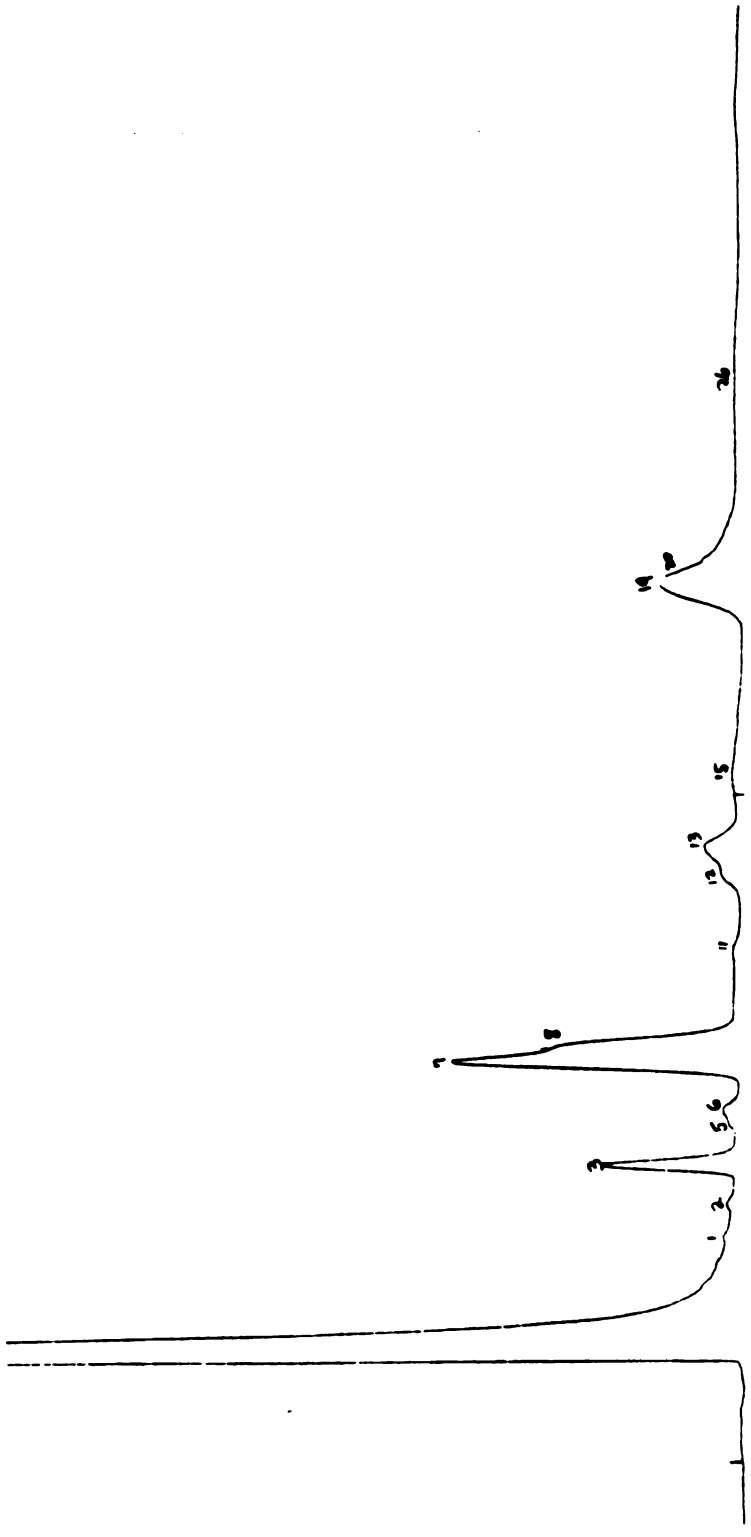

Figure AII-1g. GLC chromatogram of cuticular hydrocarbon of N. rugifrons.

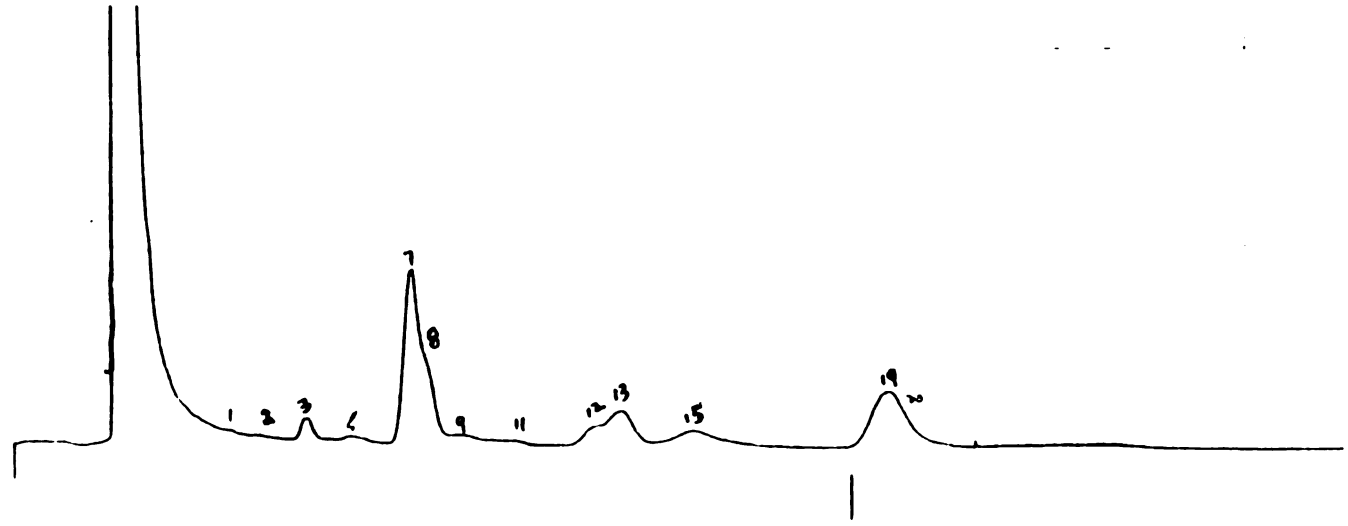

Figure AII-1h. GLC chromatogram of cuticular hydrocarbon of N. nanulus nanulus.

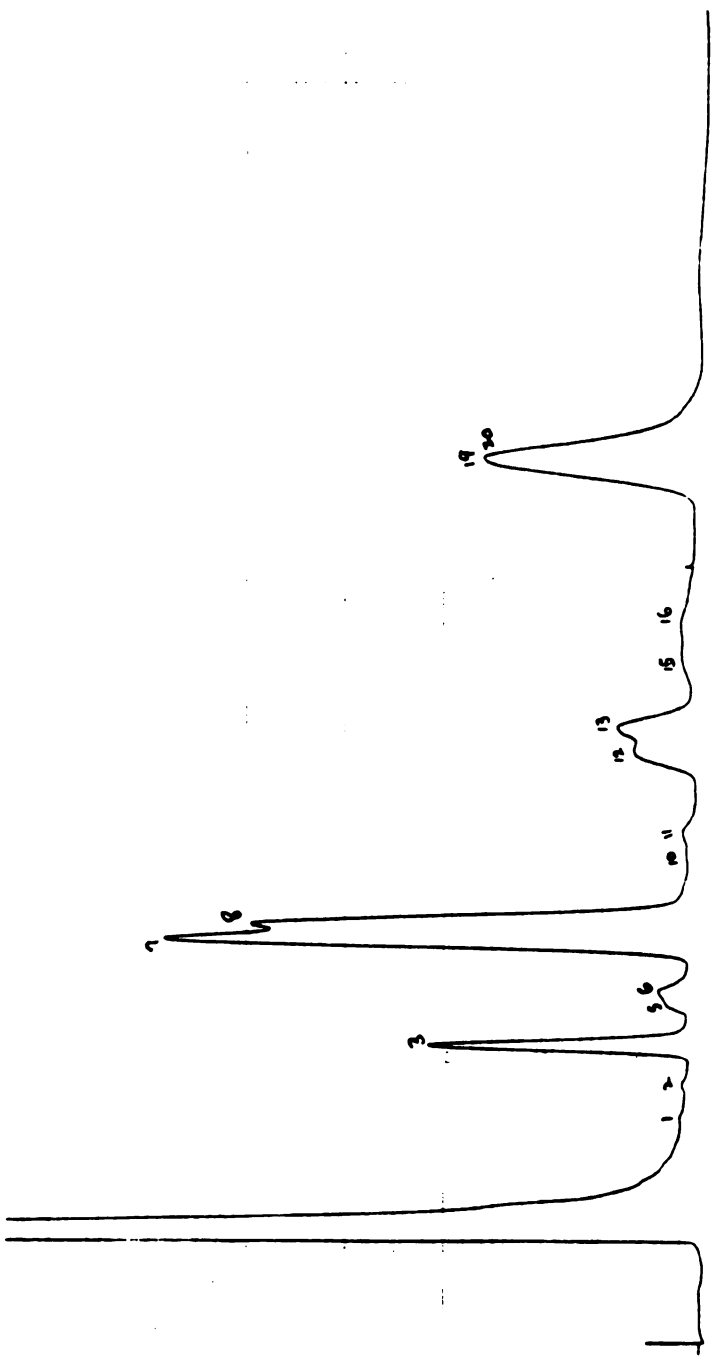

Figure AII-11. GLC chromatogram of cuticular hydrocarbon of N. lecontei.

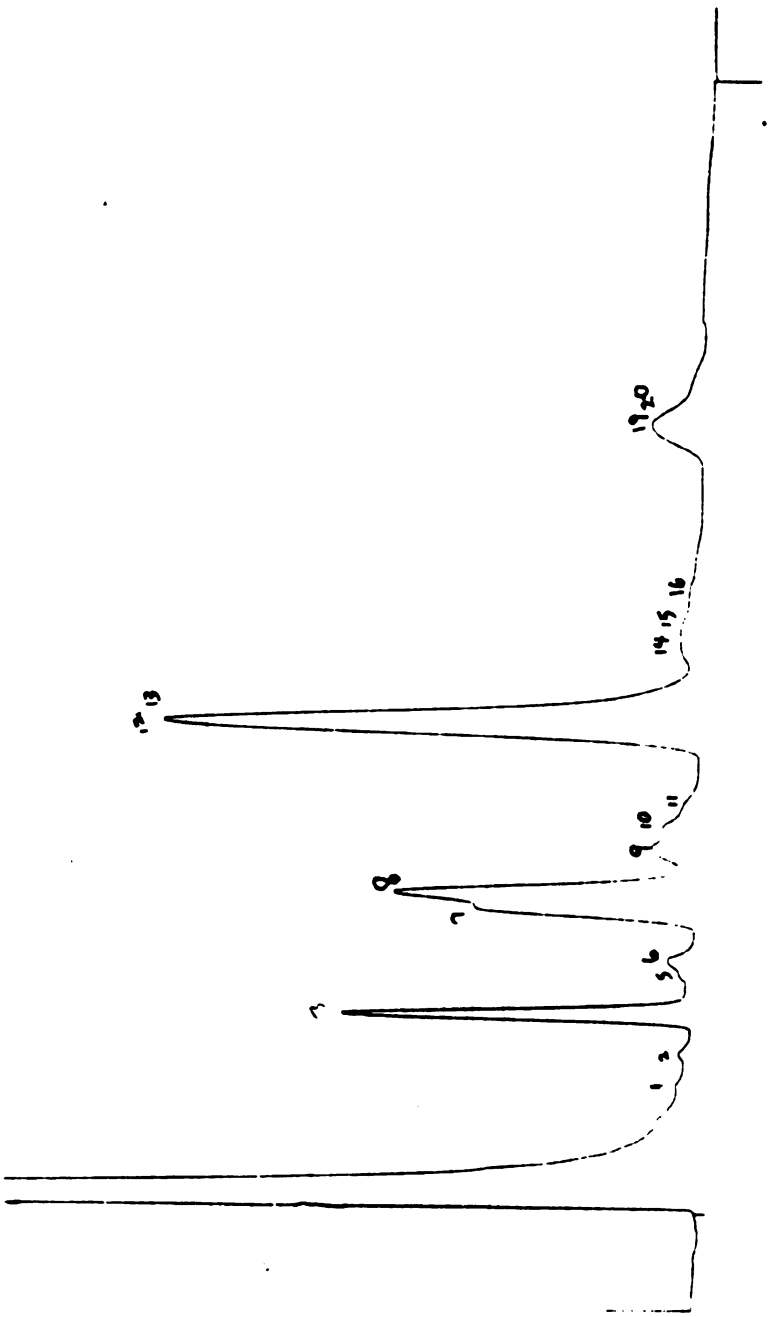

Figure AII-1j. GLC chromatogram of cuticular hydrocarbon of D. similis.

observation is the striking similarities of most of the peaks and quantitative differences among species was hardly noticeable. One quantitative difference however, separated the genus Diprion from the genus Neodiprion: in all Neodiprion species examined, peak 8 was lower than peak 7 while in Diprion similis, the reverse was true. For the D. similis three other replicates were prepared from another group of males and similar situation was observed. Another noticeable quantitative difference which grouped the nine species examined into two was the ratio of peaks 12 and 13; the ratio was in favor of peak 12 for N. p. paradoxicus (red), N. dubiosus, N. swainei, N. pinetum and D. similis but in favor of peak 13 for N. p. paradoxicus (black), N. virginianus, N. lecontei, N. rugifrons and N. nanulus nanulus. These two quantitative differences clearly presented a circumstantial evidence for separation of N. virginianus from N. dubiosus on one hand and N. rugifrons from N. dubiosus on the other. Also separated by the same reasoning was N. p. paradoxicus (red abdomen) from its black abdomen counterpart.

To bring out any qualitative difference among species, the chart (Figure AII-2) was prepared to compare the hydrocarbon peaks. From this chart it is clear that peaks numbered 1, 2, 3, 5, 6, 7, 8, 11, 12, 13, 15, 19, and 20 were common to all the species. These peaks therefore, could not be considered for chemotaxonomy for the pine sawfly species studied. The other peaks which showed qualitative

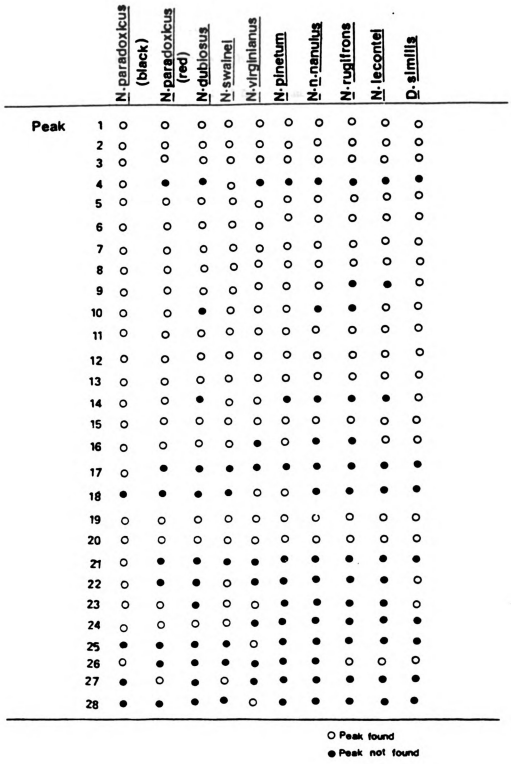

Figure AII-2. Qualitative differences among GLC peaks of the cuticular hydrocarbon of ten species of sawfly.

differences among species are tabulated in Figure AII-3. In order to show similarities among species, a method of scoring was developed where one species is taken at a time, the number of peaks that species has is scored and that number is used as a denominator for the number of peaks in another species common to the denominator's. The bigger that fraction was towards integer 1 the closer was this second species towards the first. An example of this scoring system is seen in N. p. paradoxicus (black abdomen) with 11 peaks, compare N. p. paradoxicus (red abdomen) to this with 6 common peaks; score = 6/11 = 0.545. Other species, N. dubiosus 3/11 = 0.273; N. swainei 3/11 = 0.727; N. virginianus 4/11 = 0.364; N. pinetum 3/11 = 0.273; N. n. nanulus 1/11 = 0.091; N. lecontei 3/11 = 0.273 and D. similis 6/11 = 0.545. Using N. p. paradoxicus (red) as denominator; other species compared to it as follows: N. p. paradoxicus (black), 0.857; N. dubiosus, 0.429; N. swainei, 1; N. virginianus, 0.571; N. pinetum, 0.429; N. n. nanulus, 0.143; N. rugifrons, 0; N. lecontei, 0.286; and N. similis, 0.571. In the same way, comparing others to N. dubiosus there appeared 3 groups: N. n. paradoxicus (black), N. p. paradoxicus (red), and N. swainei with 1 each; N. pinetum formed another group with 0.667 and N. n. nanulus, N. lecontei, N. virginianus, and D. similis with 0.333 each. Using N. swainei classified the nine species into 4 groups, N. p. paradoxicus (black and red) with 0.888 and 0.777 respectively as one group; N. dubiosus, N. virginianus and N. pinetum with

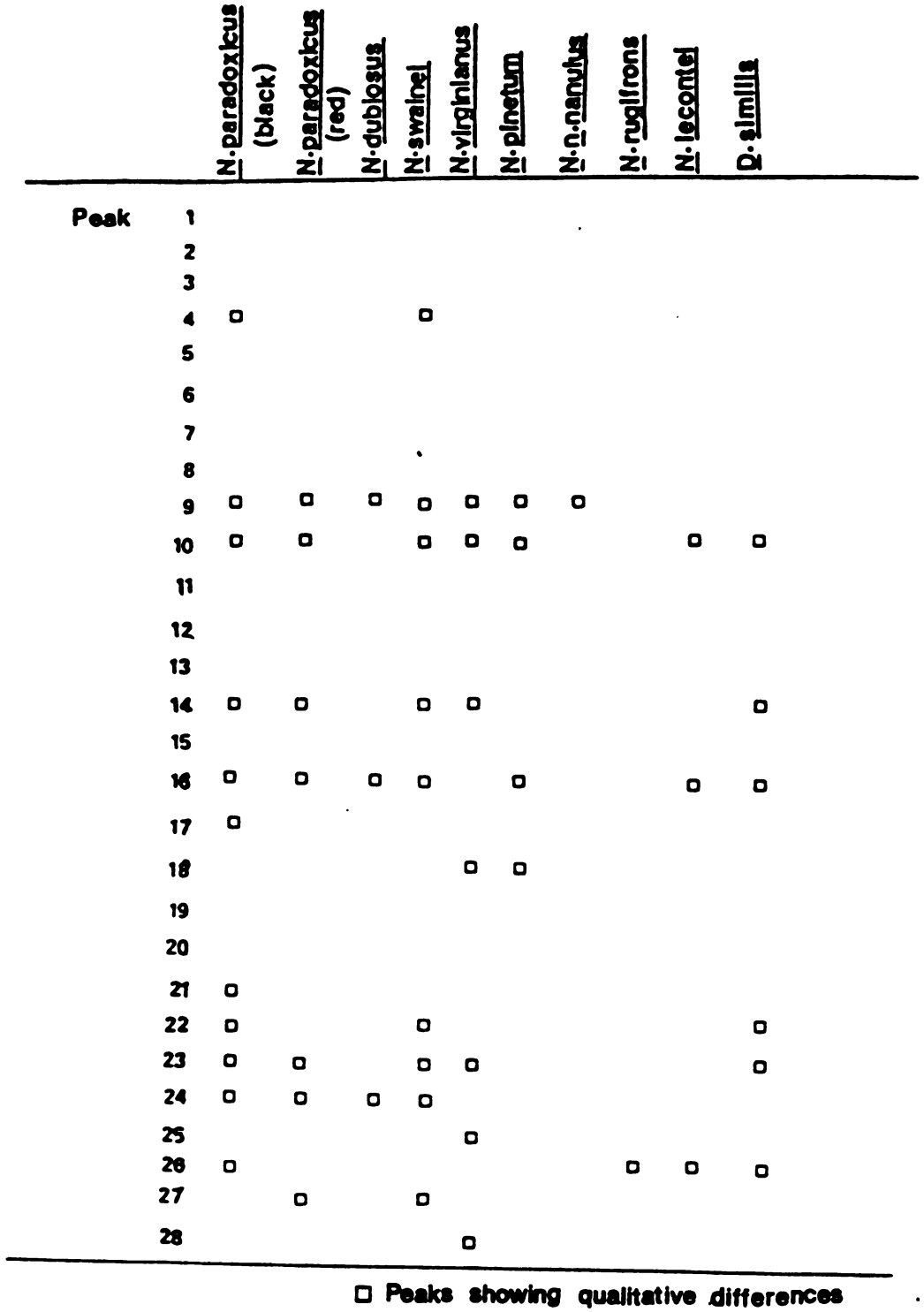

Figure AII-3. Qualitative differences among GLC peaks of the cuticular hydrocarbons of ten species of sawfly.

0.333, 0.444 and 0.333 respectively as another group; N. n. nanulus with 0.111 as one group and D. similis with 0.555 as the last group.

Other species were so compared to produce Table AII-1. The final grouping is based on the number of occurrence together in the same group of one species with other species. If the occurrence together of two species was greater or equal to 3 times, they are put in the same group. On the basis of such classification, 6 groups are formed from the nine species examined.

Group 1: comprised of N. p. paradoxicus (black); N. p. paradoxicus (red) and N. swainei.

Group 2: comprised of N. dubiosus and N. virginianus.

Group 3: comprised of N. nanulus nanulus and N. rugifrons.

Group 4: comprised of Diprion similis.

Group 5: comprised of N. pinetum.

Group 6: comprised of N. lecontei because its peaks occurred greater than 3 times with at least 3 species.

DISCUSSION

It must be pointed out that species like N. p. paradoxicus (black and red abdomen) and N. swainei where enough sample were available to be injected had obvious advantages over others with less sample. Efforts were made however, to scrutinize the chromatograms produced very well before

Table AII-1. Grouping of a species of sawfly based on degree of occurrence of identical GLC peaks of their cuticular hydrocarbon.

Species to which comparison was made	Percentage of identical GLC peaks 100-70	69-40	39-20	19-0
N. pratti paradoxicus (black lower abdomen)	N. swainei	D. similis	N. pinetum N. lecontei N. dubiosus	N. n. nanulus
N. swainei	N. p. paradoxicus (red)	D. similis	N. virginianus N. dubiosus	N. lecontei N. n. nanulus N. rugifrons
N. pratti paradoxicus (red lower abdomen)	N. swainei N. p. paradoxicus (black)	D. similis N. virginianus	N. dubiosus N. pinetum N. lecontei	N. n. nanulus N. rugifrons
N. dubiosus	N. swainei N. p. paradoxicus (black) N. p. paradoxicus (red)	N. pinetum N. rugifrons	N. virginianus N. n. nanulus N. lecontei D. similis	--
N. pinetum	N. swainei N. p. paradoxicus (red) N. p. paradoxicus (black)	N. dubiosus N. rugifrons	N. n. nanulus N. lecontei	N. rugifrons

Table AII-1. (Cont.)

Species to which comparison was made	Percentage of identical GLC peaks 100-70	69-40	39-20	19-0
N. virginianus	--	N. pinetum N. p. paradoxicus (red) N. p. paradoxicus (black) N. swainei N. similis	N. lecontei	N. dubiosus N. n. nanulus N. rugifrons
N. lecontei	N. p. paradoxicus (red) N. p. paradoxicus (black) N. swainei N. similis	N. pinetum	N. dubiosus N. virginianus N. rugifrons	N. n. nanulus
D. similis	N. p. paradoxicus (black) N. swainei	N. paradoxicus (red) N. lecontei N. virginianus	N. pinetum N. dubiosus N. rugifrons	N. n. nanulus

making judgment. If not, features of chromatograms of species like N. virginianus and D. similis with scanty samples would not have been so clearly presented. It must be emphasized that lack of identification of the peaks and absence of enough samples in most species presented a problem not only in running the test but also in interpreting the results. It is suspected that the 13 peaks which were common to all the species were mostly n-alkanes in homologous series. The n-alkanes are known to be derived directly from diet (Blomquist and Jackson, 1973) and since all the species feed on pine shoots, the n-alkanes in their cuticular hydrocarbon are likely to be similar. By the same reasoning, the peaks listed in Figure AII-3 would be mostly branched hydrocarbons. The results so far presented pointed out clearly that cuticular hydrocarbon could be considered for taxonomic use in pine sawflies. There were similarities with taxonomic groupings of this six grouping and those of Ross (1955) and Knerer and Atwood (1973).

The group 6 in this study, N. lecontei interestingly had peaks occurring about 3 times with 3 species. It was about the only species with such characteristics in this study and that suggested that N. lecontei might actually have some genetic connections with other species. This suggestion would be in line with the lecontei and sertifer group of Ross (1955). It was also of interest that N. dubiosus and N. virginianus fell together into one group. The view of earlier workers on taxonomy of N. virginianus

complex (Ross, 1955; Becker et al. 1966) supported this grouping. However, the obvious quantitative difference in GLC peaks 12 and 13 is enough to separate them into species.

Apart from these fine qualitative differences it was evident as earlier pointed out that quantitative differences also exist between genera Diprion and Neodiprion and among species of sawflies. If this classification based on cuticular hydrocarbon is considered in conjunction with larval characters, host plant oviposition pattern, life history and male courtship behavior it will go a long way at solving the taxonomy of pine sawflies. There is no doubt that cuticular hydrocarbon pattern has a role to play in chemotaxonomy of sawflies as demonstrated in this study.

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