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Preliminary Studies on Olfaction in Second Instar Eastern Spruce Budworm, *Choristoneura fumiferana*: Behaviour, Morphology, and Electrophysiology.

Ann Ascoli

A Thesis

in

The Department

of

Biological Sciences

Presented in Partial Fulfillment of the Requirements
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ABSTRACT

Preliminary Studies on Olfaction in Second Instar Eastern Spruce Budworm, Choristoneura fumiferana: Behaviour, Morphology, and Electrophysiology.

Ann Ascoli

A two choice, wind tunnel olfactometer was designed and constructed to determine whether second instar eastern spruce budworm larvae, Choristoneura fumiferana (Clem.), could detect and discriminate between host plant volatiles.

Volatiles of current year's growth of Picea glauca were preferred over P. rubens, P. mariana, or air. Abies balsamea volatiles were preferred over P. mariana, or air. Those of P. rubens and P. mariana were preferred over air.

Two-year old growth of P. glauca, A. balsamea, P. rubens, and P. mariana were all preferred over air. Current year's growth of these host evergreen species was usually preferred over former year's growth of the same species.

The olfactory sense organs were found to be on the antennae of this insect, as determined by ablation studies. The innervation of this appendage was tentatively mapped. Chemoreceptor cells appeared as larger groups of cells, located further away from the base of the sensilla to which they extend dendrites, than did mechanoreceptor cells. The pores allowing for the passage of volatiles from the air into the sensilla basiconica were revealed by light and scanning electron microscopy. Electrophysiological recordings were obtained from the antenna of the larvae, in response to host plant volatiles and to some pure terpenes.

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To Paul Albert, a very special person.

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INTRODUCTION

The eastern spruce budworm, Choristoneura fumiferana (Clem.) (Lepidoptera: Tortricidae), is a serious pest of spruce and fir forests of Eastern Canada and of the United States. Second instar larvae emerge in early May, and feed on developing buds of newly opened shoots of coniferous host trees. Balsam fir (Abies balsamea) is usually the most damaged species, followed by white spruce (Picea glauca), red spruce (P. rubens), and black spruce (P. mariana) (Blais, 1957; 1964; 1981.) This order may be related to host tree phenology: the emergence of larvae from their hibernacula coincides with the budburst of balsam fir, four days prior to that of white spruce, and about thirteen days prior to that of red and black spruces (Swaine and Craighead, 1924). Though the adult females select trees which should be suitable for larval development on which to deposit their egg masses (Stadler, 1974), the second instar larvae may disperse (Shaw & Little, 1973.)

Responses to odour by spruce budworm adults have been studied (Thorsteinson, 1960), and the antennae were identified as the site of pheromone receptors (Albert et al. 1970). However, no studies of this species have been done on larval olfaction, a component of host selection. Olfactometers have been used to investigate qualitative and quantitative aspects of insect responses to odours. Most olfactometers consist of wind tunnels in which the insect is

exposed to odour-treated air streams, while other systems (static olfactometers) rely on diffusion gradients of particular odours in still air. Both systems have been used with Coleoptera (Anderson and Fisher, 1960; Wood and Bushing, 1963; Mustaparta, 1975; Rust and Rierson, 1977; Khono et al., 1983; Mikolajczak et al., 1984; Kao et al., 1984;), Diptera (Nettles, 1980; Katsoyanos et al., 1980; Hoffmann, 1983; Ishikawa et al., 1983;), Hymenoptera (Ferriera et al., 1979; Brewer, 1983; Vet et al., 1983; Stafford et al., 1984;), Lepidoptera adults (Guerra, 1968; Katsoyanos et al., 1980; Lecomte and Thibout, 1981;), and Lepidoptera larvae (Sutherland, 1972; Khattar and Saxena, 1978; Saxena and Rembold, 1984.)

To determine the location of sensory structures, a technique known as ablation is used. Upon the removal of the olfactory organs, an insect will no longer respond to volatiles. This technique has obvious pitfalls. Ablation causes trauma in the form of blood loss, carbon dioxide poisoning, electrical shock, and physical pressure on the parts of the body which are restrained. For this reason, "sham"-operated insects, that is insects which have undergone all of the operation procedures without being subjected to the actual amputation, should be tested under the same bioassay conditions as are the insects with ablated appendages. Following these control experiments, one can safely conclude that the sensilla in question were

on the amputated part. Naturally, the smaller the ablated area, the more precise one can be about the location of the olfactory sensilla. Such studies have been performed on several insects: (Stadler, 1974; Yamada et al., 1981; Devitt, 1983.)

Once the sensilla have been approximately located, it is advisable to look at the area using microscopy to determine whether any structures which may house olfactory neurons are present. Such structures are characterized by a cuticular covering pierced by numerous pores. These sensilla often display an intricate pore tubule system which passes odourous molecules through the cuticle into the sensillum's interior. Once inside, the odourous molecules dissolve in the sensillum liquor, a liquid of unknown composition, which bathes the sensory dendrites and through which the molecules diffuse to impinge on the dendritic membrane. These dendrites are distal extensions of bipolar primary sensory cells which in turn are connected, without synapses, to the central nervous system. A more detailed description of olfactory sensillar ultrastructure can be found in a review by Steinbrecht (1969).

A number of staining methods have been developed to elucidate certain aspects of sensillar structure. The methylene blue stain (Zacharuk, 1962) is a method that selectively stains live insect neurons. As a rule, several whole preparations are studied, allowing a composite drawing of the innervation of the animal to be made.

Sections of sensory organs can be stained to emphasize the structure of the neurons within them. Mallory-Heidenhain (Casson, 1950) and Hubschman's azan (Hubschman, 1962) stains colour most tissues differentially and thus facilitate the identification of neurons.

In order to determine whether particular sensilla are olfactory in nature, whole insects may be immersed in water-soluble dyes such as crystal violet, nigrosin, or acid fuchsin. (Slifer, 1960). If the sensilla are olfactory, the stain will pass through the many small pores in their surface making the sensillar interior appear lightly stained. These can then be differentiated from contact chemoreceptors which have a single large pore or a few large pores and, as a result, stain deeply. Mechanoreceptors, bearing no pores, do not allow the passage of stain in an aqueous medium.

Olfactory pores may also be detected by placing the insect in glycerol (Slifer, 1969), a refractive medium which enhances the contrast of transparent tissues.

Scanning electron microscopy is useful for revealing the shape and number of pores on the sensilla. The preparation technique is simple for most insects: fixation, clearing, drying, and gold coating. However, the spruce budworm is a particularly "dirty" animal because it regurgitates when stressed, spins silk, and produces exudates from the pores of its basiconic sensilla. These

three features often combine to obliterate the view of the olfactory pores, or even of grosser structures on the antenna. A protease treatment has been designed specifically to remove the coating on the sensilla (Dyer et al., 1982).

Studies of the morphology of sensory structures often imply the function of these organs. It is essential however, that both behavioural and electrophysiological studies support the suggested function. If the neurons within the structures proposed to be of sensory nature do indeed respond to host volatiles or to other air-borne chemicals, one can definitively conclude that the organ is olfactory. The electroantennogram (EAG) which measures the total response to odours of the cells within the antenna (Schneider, 1955), and the single cell recording (Hodgson & Roeder, 1952), a technique which shows the action potentials emanating from individual neurons, were developed for this purpose. For both of these techniques, two electrodes are in contact with the insect's internal fluids. The "recording" electrode communicates with the haemolymph or with the sensillum liquor surrounding the sense cell, and the "reference" electrode pierces the body surface and acts as a grounding device. The potential differences caused by ion concentration fluxes from the stimulated sensory cells (receptor potentials and action potentials) are picked up by the recording electrode, amplified by a high impedance amplifier and displayed on an oscilloscope or some other

recording device.

In this study, the orientation of the second instar eastern spruce budworm to its host's odours was assessed in order to determine whether the insect could detect these odours and whether it could discriminate between volatiles (Ascoli & Albert, 1985); ablation studies were performed to determine the location of the olfactory organs; the structure and innervation of the antenna's sensilla were studied; and recordings from the antennal neurons in response to host plant volatiles and to pure terpenes were obtained, allowing confirmation of the olfactory function of some receptors within the antennae.

MATERIALS AND METHODS

Behaviour:

Experimental animals:

Post-diapause second instar larvae of Choristoneura fumiferana (Clem.) were obtained from the Forest Pest Management Institute, Great Lakes Forest Research Center, Sault-Ste-Marie, Ont.. Before being tested in the olfactometer, animals were maintained on gauze for 0-3 days at 4 °C without food or water, and were then fed for 24 hours at 28 °C on an artificial diet (McMorran, 1965). Only healthy animals were chosen for the tests. Larvae with a characteristic pale colour, that were feeding and moving normally were considered healthy. Animals were used only once in tests.

Odour source:

Foliage samples of white spruce (Picea glauca (Moench.) Voss), balsam fir (Abies balsamea (L.) Mill), red spruce (Picea rubens Sarg.), and black spruce (Picea mariana (Mill, B.S.P.)) were collected from the Maritimes Forest Research Center, N.B. on June 23, 1981, and on June 15, 1982 at a time when larvae were in their fifth and sixth instars in the field. Foliage was also collected on June 6, 1983 when larvae in the field were in their third and fourth

instars. All samples were obtained from the mid-crown level of each tree. The current year's growth and the previous year's growth were placed in separate sealed plastic bags over dry ice (for transport), then stored at -18°C (in the laboratory). Four needles were used as a sample in the bioassay chamber; these were left at room temperature for 5 minutes prior to the commencement of the assay. Needles were replaced each hour.

Bioassay:

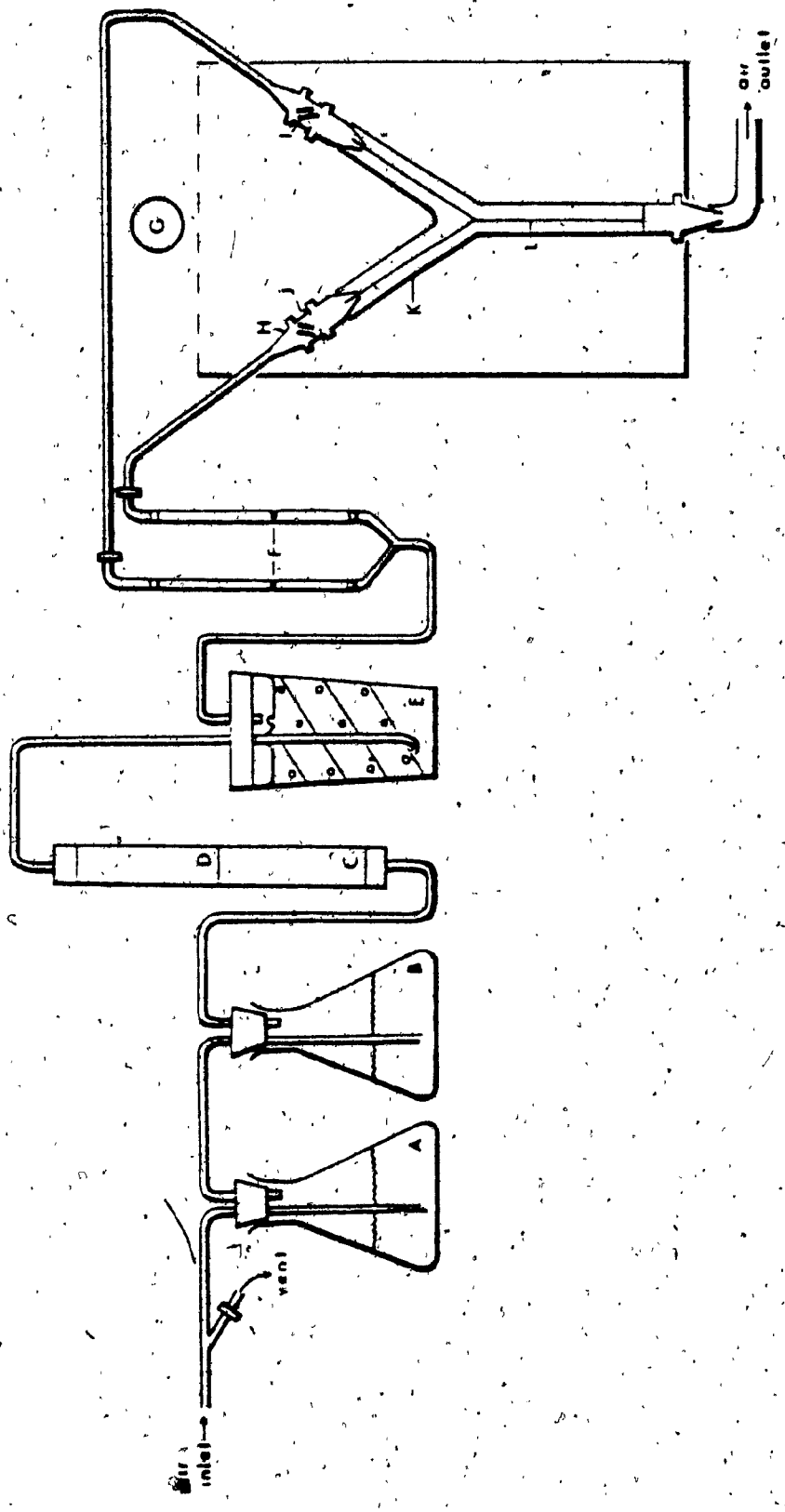
A diagram of the olfactometer used is shown in Figure 1. Air flow was controlled with a tap for coarse adjustment, and with a vent clamp for fine adjustment. The air was purified by passing through a charcoal filter (A), a drierite filter (B), a gas purifier including a second drierite filter (C), and a molecular sieve (D), followed by a gas washer (E) containing deionized glass distilled water. This provided air with a constant purity, relative humidity, and an ambient temperature between 20 and 25 °C. This air stream was divided into two equal parts by a polypropylene Y-type connector, and its flow was monitored by Roger Gilmont ® flowmeters (F). Flow rates were controlled by metal clamps on plastic tubing of 0,3 cm diameter. As a control, the position of the Y-tube was randomly (Sokal & Rohlf, 1969) switched (right or left side). The the air flow was determined by the position of the Y-tube. The Y-tube was enclosed in a black plywood box

and illuminated through a light diffusor by a fluorescent light source (G). Air descended into the two branches of the tube at a flow rate of 175 ml/min with a velocity of 614 cm/min (the diameter of the Y-tube was 0,6 cm), after passing through polypropylene joints (H) glued to the tube with epoxy resin. These joints held a layer of gauze (H) which was changed after every hour of use. The gauze supported the foliage sample (I). In this manner, the larva could not contact the foliage directly. The glass Y-tube (K) held within it a Y-shaped copper wire (L), on which the insects were allowed to crawl. Eight such tubes were constructed and each was tested for bias. (Bias was assessed by observing whether 20 larvae, when individually presented with an air/air choice, displayed preference for one branch of the tube over the other.) Tubes in which randomness of direction was observed, were used in experiments. All odourous air was carried away in a 0,7 cm Tygon tube to a fume hood in order not to expose future experimental animals to test odours.

To speed up the bioassay, this olfactometer system takes advantage of the fact that the larvae are positively phototropic and negatively geotropic. Since the Y-tube is in an upright position, and the apparatus is lit from above, the larva's progress is hastened.

Insects were transfered using a paintbrush (to avoid injury to their delicate cuticles) onto a straight wire from

Figure 1. Schematic diagram of olfactometer. A, charcoal filter, B, drierite filter, C & D, gas purifier, E, gas washer, F, flowmeters, G, light source, H, joint, I, foliage, J, gauze, K, Y-tube, L, copper wire.


Air outlet
K
E
D
C
A
Vent
Air inlet

which they crawled onto the 0,1 cm diameter wire within the olfactometer. The test began when the insect was one centimeter from the bottom of the latter wire, and ended when the insect had crawled one centimeter past the junction of the "Y". The Y-tube was then inverted, and the insect was allowed to crawl out. The time to complete the test was noted, as was the larva's behaviour during the test.

Contingency tests were applied to data from different sessions. If the Chi-square value was non-significant, data sets were pooled. Values obtained from foliage collected at different times of the year were also treated this way . The data were interpreted using a chi- square test with the null hypothesis that the air stream choice was random. Thus, odour preferences were established. For all chi-square tests, Bate's correction for small sample sizes was used (Sokal & Rohlf, 1969).

Y-tubes were cleaned after each test, and care was taken to remove all silk produced by the larva.

Ablation:

Insects were individually anaesthetized with carbon dioxide at room temperature and mounted with their dorsal side downwards on a copper ground wire, 1 mm in diameter, embedded in a block of plasticine. Three fine tungsten "clips", 0,1 mm in diameter, held the insect's head, thorax and abdomen in place without any apparent damage to these

structures. The cauterizing electrode, held by a micromanipulator, was a tungsten wire, electrolytically sharpened in a potassium nitrite solution to a diameter of 1-2 µm. Using a foot switch, a potential of 9 V was applied across the insect's antenna or mouthpart thus completely obliterating the structure. The hole created was rapidly plugged with coagulating hemolymph. The insects were allowed a recovery period of 24 hours with artificial diet (McMorran, 1965) at 28 °C. The performance of these insects was then observed in the olfactometer where their preference for new white spruce needles over air was assessed. Right or left antennae, both antennae, or both maxillary palps were removed from 230 insects. The data were interpreted in the same manner as for the bioassay described above.

Morphology:

Light microscopy:

The innervation of the antenna was studied using a modification of the methylene blue vital stain method (Zacharuk, 1962). Second instar larvae were held on a glass microscope slide with a thin ribbon of Scotch brand Magic Transparent tape (TM) placed over the abdomen. Glass micropipettes, cut with a diamond knife to a diameter of 20 µm, were used to double inject 1 µl of 2:1 2,0% methylene blue in 0,75% NaCl. To prevent excessive bleeding, injections were made through the intersegmental membranes

into the region of the dorsal aorta. Insects were kept at 4 °C for 30 min between injections and then placed overnight in a saturated aqueous solution of ammonium molybdate at 4 °C, dehydrated to 100% ethanol, cleared in xylene, and mounted in Permount (Fisher Scientific Co.). Wholemounts were observed under oil immersion with a Leitz microscope at 1000 X.

To rapidly detect any porous sensilla on the body surface of the budworm, live material and insects fixed in Bouin's for at least 24 hours, were immersed in one of the following aqueous stain solutions: (Slifer, 1960) saturated acid fuchsin, 0,5% crystal violet, or saturated nigrosin solution for 10 to 60 minutes. Stained material was then cleared in distilled water, heat dried at about 80 °C for 2 min, and mounted in Permount. The heating step caused the separation of underlying tissues from the endocuticle, but this phenomenon did not hinder the viewing of the stain which had entered the sensilla. Photomicrographs were taken immediately, as these stains, especially acid fuchsin, fade with time.

Insects fixed in 10% formalin (Fisher Scientific Co.) were perfused with glycerol overnight, then mounted in glycerine jelly. This is a technique adapted from Slifer (1968) and it ephances the contrast of the surface texture of the antennal sensilla when viewed at 400 or 1000 X.

Electron microscopy:

The following technique was used to provide good specimens for scanning electron microscopy.

Live insects were placed in Ringer's physiological saline for 15 minutes, then dehydrated in an ethanol series in Ringer's solution (30%, 50%, 70%) for 30 minutes each. This step killed the insects and prevented further regurgitation or silk production. The insects were then rehydrated back to Ringer's solution and placed in a warm 10% acetic acid solution in Ringer's solution at 40 °C for 5 minutes. This process, has been shown to remove extraneous material from pollen (Kapoor, personal communication) and appeared to do the same to the larval body surface. After three five-minute washes in Ringer's solution, the insects were placed in a warm, 1,0 % potassium hydroxide solution in Ringer's solution for 5 minutes at 40 °C. This bleaching step presumably removed the material left behind by the acetic acid. Following three more washes in Ringer's solution, the insects were transferred to a concentrated protease solution (40 µg/ml) (Dyer et al., 1982) for 20 min at 25 °C. This step removed the proteinaceous coating secreted through the pores onto the sensillar cuticle. Following three washes in Ringer's solution to terminate the enzymatic reaction, the insects were once again placed in warm acetic acid to remove undigested protein from the sensilla. Care was taken in the above steps not to allow

the insects to float. Those which did so were discarded since they may not have been impregnated with the bathing solution. In all solutions, the specimens were shaken very gently, as the cuticle of the antennal sensilla is extremely delicate. Sonication should be avoided as it always caused the breakage of the cuticle.

After three more washes in Ringer's solution, and a slow dehydration, in an ethanol series in Ringer's solution, (30%, 50%, 70%, 90%) the specimens were placed overnight in a 1:3 2,2-dimethoxypropane : 100% ethanol mixture at 4 °C, acidified with a single drop of 25% HCl. This was the fixation step and was preferable to the more traditional gluteraldehyde/osmiun tetroxide fixation due to its speed, cost , and relative safety. It is also the only known fixative to readily penetrate insect cuticle (Bjerke, 1979). Insects were then placed in three 100% acetone washes for 15 minutes. This step was optional since both acetone and ethanol, the products of the fixation reaction, are readily miscible with liquid carbon dioxide, the exchange agent in the critical point drying step. For this purpose, a Polaron Equipment Ltd., England model # E 300-II critical point drying unit was used. This step was occasionally replaced by simple air drying of specimens from propylene oxide. Both methods for desiccating the antennal sensilla yielded satisfacory results, though critical point drying maintained the complete body shape and prevented the shrinkage which

occurred in a small percentage of sensilla on air dryed specimens. Specimens were mounted on a stub with sticky tabs, coated with a 10 nm layer of gold using a sputter system E-5100 by Polaron Equip. Ltd., England, and viewed with a Hitachi S-520 scanning electron microscope. Polaroid electron micrographs of significant preparations were taken.

Electrophysiology:

Unfed larvae were secured to a glass microscope slide with three ribbons of Scotch brand Magic tape. Such individuals remained alive for over 48 hours and could later be removed and reared to maturity. Electrodes were tungsten wires, electrolytically sharpened in a potassium nitrite solution to a tip diameter of 0,5 to 1,0 µm. The ground electrode was placed anterior to the intersection of the frons and the two vertices. The recording electrode was inserted in the medial portion of the base of the antennal pedicel.

Pure humidified air was passed over the preparation at a flow rate of 250 ml/min. Stimulation was achieved by injecting 10 ml of air into the system from a syringe containing 10 needles from a host plant or 1 λ of pure monoterpene on filter paper. Stimulation lasted approximately 3,0 seconds. A light vacuum close to the preparation carried residual odourant molecules to a fume hood.

The a.c. signal produced in response to the stimuli was recorded on magnetic tape and filmed for permanent storage. Action potentials were separated according to amplitude into 8 classes. The frequency of each class was analysed.

RESULTS

Behaviour:

The most common behaviour exhibited in the olfactometer by the larvae (1) was a persistent crawl with moderate spiralling around the wire on the way up. This was followed by a rapid choice of direction at the junction of the Y. The insect crawled up the chosen branch more rapidly than it did the stem. In addition, several combinations of exceptional behaviours were displayed: (2) larvae exhibited klinotaxis* at the junction of the Y; (3) larvae exhibited klinotaxis 0,5 to 2,0 cm below the junction of the Y; (4) larvae crawled down the stem of the wire and then back up within 0,75 to 2,0 cm from the junction of the Y; (5) larvae crawled up the wire in a tight spiral; (6) larvae crawled up one branch of the Y for a few mm, then came down and went up the other side (this behaviour was interpreted as a "correction" of the first choice: analysis of these results revealed that larvae which exhibited this behaviour usually chose the same odour stream as the majority of insects tested); (7) larvae began to crawl up the wire for a short while, but then crawled back down, thus not completing the test; (8) larvae crawled all the way up on one side of the

* Klinotaxis is a reaction to a stimulus in which regular symmetrical deviations from a straight line are a necessary part of the orientation mechanism (Fraenkel & Gunn, 1961.)

wire and were thus never presented with a choice; (9) larvae crawled very slowly, with many pauses on the way up; (10) larvae exhibited klinotaxis after passing the junction of the Y; (11) larvae exhibited circus movement, where they moved in a circle for at least 15 minutes.

The percentages of the total number of larvae tested which exhibited each of these behavioural sequences is presented in Table 1.

Preferences:

Table 2 outlines the choices made by the second instar larvae in the olfactometer. Foliage collected in early June yielded the same results as foliage collected in later June. A significant preference was demonstrated for the host tree volatiles over air alone (Ln. # 5, 8, 9, 11, 12, 13, 14, 15, 16). New white spruce was preferred over new red or black spruces (Ln. # 3, 4); new balsam fir was preferred over new black spruce (Ln. # 7). New foliage was usually preferred over the old foliage from the same host (Ln. # 17, 18, 20). Table 3 is a compilation of the mean times taken for at least 40 insects to complete the test and the length of time taken for each choice to be made. There was no significant difference between the times taken to complete the test in the presence or absence of an odour source, as determined by confidence limits set using the Student's t-test and by a one-way analysis of variance.

Table 1. Behaviour exhibited by second instar larvae of Choristoneura fumiferana, expressed as a percentage of the total number of insects tested. Refer to results section for detailed description of behaviour.

Behaviour*	% of Total Insects Treated				
	intact	2 ant off	left ant off	right ant off	2 max palps off
	(n=681)	(n=53)	(n=22)	(n=29)	(n=60)
1	56	15	0	7	3
2	18	23	32	14	12
3	27	40	72	48	22
4	16	8	9	7	20
5	12	7	9	4	3
6	12	7	36	20	8
7	2	17	9	31	67
8	2	0	0	4	0
9	2	11	5	4	10
10	1	4	5	0	3
11	0	2	0	0	0

*: (1) Most common behaviour; (2) klinotaxis at the Y; (3) klinotaxis below the Y; (4) crawling down then up; (5) spiraling; (6) up one branch of the Y for a few mm, then up the other branch; (7) unfinished test; (8) crawling all the way up on one side of the wire; (9) slow crawl with pauses; (10) klinotaxis above the Y; (11) circus movement.

N.B. Some insects demonstrated more than one type of behaviour during a test.

Table 2. Results of olfactometer tests with second instar larvae of Choristoneura fumiferana.

Choice I	Choice II	Choice III	f I	f II	f III	Chi-square for I and II	ρ^2	Preference
A	B	X	53	48	6	0,26	e	none
WSn	BFn	"	21	19	2	0,12	e	none
WSn	RSn	"	28	12	2	6,42	c	I
WSn	BSn	"	28	12	7	6,42	c	I
WSn	air	"	30	10	1	10,02	a	I
BFn	RSn	"	24	16	5	1,62	e	none
BFn	BSn	"	30	10	2	10,02	a	I
BFn	air	"	32	8	4	14,42	a	I
BFn*	air	"	17	3	4	9,85	a	I
RSn	BSn	"	18	22	2	0,50	e	none
RSn	air	"	31	9	3	12,12	a	I
BSn	air	"	31	9	2	12,12	a	I
WSo	air	"	15	5	2	5,05	c	I
BFo	air	"	17	3	1	9,85	a	I
RSo	air	"	17	3	4	9,85	a	I
BSo	air	"	15	5	2	5,05	c	I
WSn	WSo	"	16	4	5	7,25	b	I
BFn*	BFo*	"	16	4	6	7,25	b	I
RSn	RSo	"	14	6	3	3,25	e	none
BSn	BSo	"	15	5	2	5,05	c	I
R	L	"	272	308	65	2,23	e	none

BF= balsam fir; BS= black spruce; RS= red spruce; WS= white spruce.
R= right; L= left.
n= current year's growth; o= two-year-old growth.
*= foliage collected June, 1982; all others from 1981 and 1983 collection.
1- larvae did not complete the test.
2- probability values: a(<0,005); b(<0,01); c(<0,025); d(≤0,05); e(>0,05).
Chi-square statistics: d.f.= 1.

Table 3. Mean times (min) taken for completion of olfactometer tests by second instar Choristoneura fumiferana in the presence of odours or in a pure air stream. Confidence limits were set using Student's t-distribution.

Choice I	Choice II	I n	I mean time (min)	I 95% C.L.	II n	II mean time (min)	II 95% C.L.
air	air	53	2,12	1,60-2,64	48	1,85	1,45-2.25
WSn	air	30	1,67	1,38-2,06	10	1,45	1,07-1,82
BFn	air	32	1,79	1,43-2,14	8	2,20	1,37-3,02
RSn	air	31	2,02	1,62-2,41	9	2,36	1,41-3,31
BSn	air	31	2,10	1,75-2,45	9	2,16	1,23-3,08

WSn = 1983 new white spruce
BFn = 1983 new balsam fir
RSn = 1983 new red spruce
BSn = 1983 new black spruce
95% C.L. = 95% confidence limits.

ANOVA statistics: F (4, 257)= 0.9; (not significant)

Table 4 summarizes the choices made by insects whose antennae or maxillary palps were ablated. Insects with both antennae ablated no longer discriminated between host volatiles and air. Those with unilateral ablations of antennae or those with their maxillary palps removed still oriented toward their host odours, with the exception of the first ablation experiment on the left antenna. Insects with unilateral ablations did not orient their heads in a consistent pattern. They were capable of finding their hosts whether their intact antenna was positioned towards or away from the odour source.

The data from the first ablation experiment on the left antenna may have been due more to faulty ablation technique than to a true lack of preference. Early ablation attempts were often done repeatedly to burn off the entire antenna, and the electrode was often inserted too deeply. This may have resulted in damage to the central nervous system and accompanying behavioural abnormalities. A repeat of this experiment indicated that the larvae could indeed discriminate.

Table 5 presents a comparison between the mean time for intact insects to choose between new white spruce and a plain air stream, and for insects with ablated sensory organs to complete the same test. Insects which had undergone an operation were slightly slower in making decisions than were intact insects, as determined by confidence limits set using the Student's t-test and by a

Table 4. Results of olfactometer tests with second instar larvae of Choristoneura fumiferana with ablated antennae and maxillary palps.

treatment	frequency of choice WS	air	X	Chi-square for WS and air	p*	preference
2 ant. ablated	22	18	13	0,50	e	none
left ant. ablated	8	12	18	0,85	e	none
right ant. ablated	15	5	9	5,05	b	WS
2 max.palps ablated	17	3	40	9,85	a	WS
left ant. ablated (repeat)	15	5	2	5,05	b	WS

WS= white spruce foliage.
X = incomplete test.
* = probability values: a (<0,005); b (<0,01); c (<0,025); d (<0,05); e (>0,05).
Chi-square statistics: d.f.= 1.

Table 5. Mean times taken for the completion of olfactometer tests by second instar Choristoneura fumiferana when presented with new white spruce or air following ablation experiments. Confidence limits were set using Student's t-distribution.

treatment	WS n	WS mean time (min)	WS 95% C.L.	air n	air mean time (min)	air 95% C.L.
intact	30	1,67	1,28-2,06	10	1,45	1,07-1,82
2 ant. ablated	22	4,80	4,19-5,41	18	3,55	2,92-4,18
left ant. ablated	8	3,46	2,90-4,02	12	2,58	2,06-3,10
right ant. ablated	15	3,63	3,07-4,18	5	2,21	0,09-4,33
2 max.palps ablated	17	4,15	3,83-4,46	3	6,37	3,70-9,04
left ant. ablated (repeat)	15	2,71	2,48-2,94	5	2,50	1,72-3,28

WS = new white spruce
95% C.L.= 95% confidence limits

ANOVA statistics: $F(4, 135) = 6,27$; $p < 0,005$.

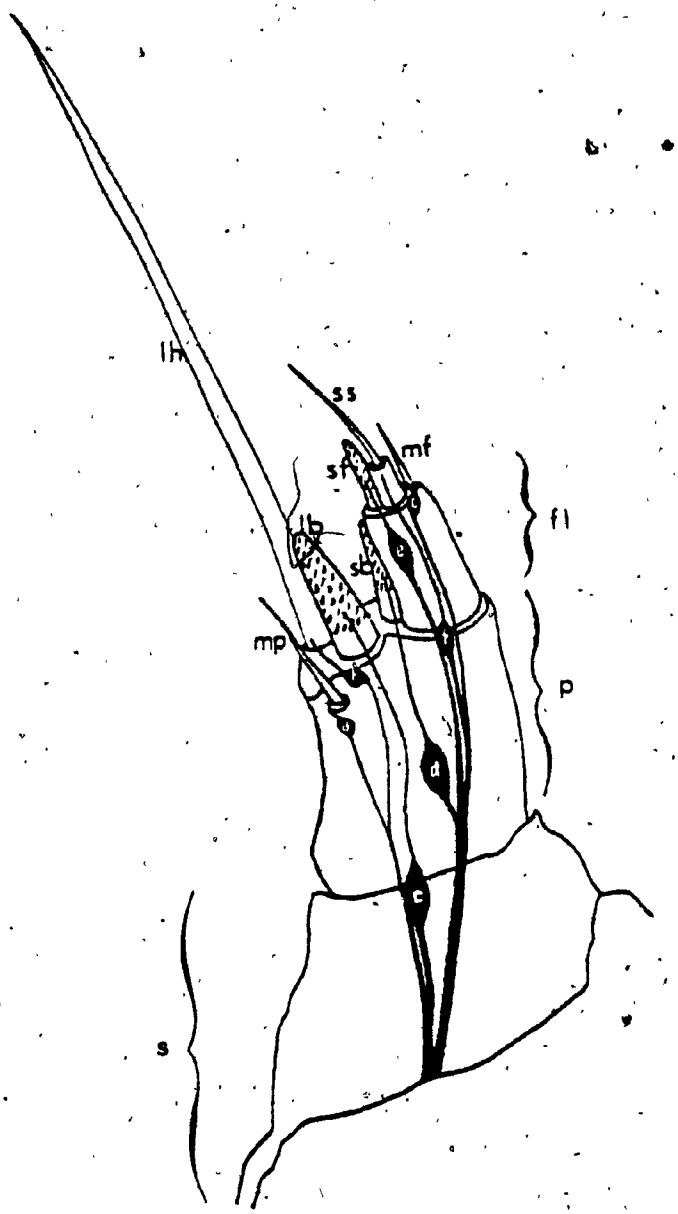
one-way analysis of variance. There was no significant difference between the times for one group of insects to choose either air or white spruce (Table 5).

Morphology of the antenna:

Direct microscopical observations:

Larval antennae in this species are located on the vertices of the head, just medial to the five ocelli. In the second instar, these retractable appendages are approximately 125 µm long. The antenna is composed of the following segments (See Figure 2.) The scape is a very flexible and mobile structure 17 µm long and 32 µm at its proximal and widest end and appears to bear no chemosensory structures. The pedicel, a segment which is 30 µm long and 18 µm wide bears a great number of sensilla. The most prominent of these is a hair, 80 µm long. Medial and dorsal to this hair is a large thin-walled sensillum basiconicum, 12 µm long. Medial to the large hair is a smaller sensillum basiconicum 7 µm in length. A few campaniform organs and a variable number of hairs are present on the pedicel. The medial portion of the pedicel supports the flagellum, which is 26 µm long and 6 µm wide and bears a sensilum styloconicum 5 µm long and 2 µm wide, which has an 11 µm long peg. Lateral to the styloconicum is an elongated sensillum basiconicum 8 µm long. This is sometimes accompanied by a second stout sensillum, the length of which

Figure 2. Proposed innervation of the antenna of a second instar larva of Choristoneura fumiferana as defined by methylene blue staining: (a) the cell innervating the small mechanoreceptive hair (mp) on the pedicel (p); (b) the cell proceeding to the large tactile hair (lh) on the pedicel; (c) the group of cells leading to the large sensillum basiconicum (lb) on the pedicel ; (d) the group of cells innervating the small sensillum basiconicum (sb) on the pedicel; (e) the group of cells proceeding to the sensillum basiconicum (sf) on the flagellum (fl); (f) the group of cells leading to the sensillum styloconicum (ss) on the flagellum; (g) the cell innervating the mechannoreceptive hair (mf) on the flagellum; (s) the scape.


lh
ss
mf
sf
lb
sb
mp
fl
p
s

is variable. A sensillum trichodeum rests medial to the styloconicum.

Apparent sizes of most of the antennal structures are subject to variations due to changes in hydrostatic pressure and muscular activity within the antenna which causes the "telescoping" of the antennal segments.

Methylene blue stain:

The antennae were always the last of the head appendages to stain. For this reason, no one preparation offered a view of the complete innervation of this structure. A composite diagram (Figure 2) was made from information gathered from 100 wholemounts, some of which are illustrated in Figure 3. The cell body clusters were clear, but the representation of the cell processes (axons and dendrites) is only tentative.

Water-soluble staining of sensilla:

Water soluble stains entered any broken structures freely and care had to be taken not to interpret these as natural openings on a sensillum. The best results were obtained with crystal violet and with acid fuchsin. These faintly stained the three sensilla basiconica on the antennae, suggesting that these structures have multiporous surfaces as opposed to the intensely staining contact chemoreceptors which have a single large apical pore. Such results support the conclusions made by studies of

Figure 3. Light micrographs of spruce budworm antennae, methylene blue staining.

3-1. A group of cells (arrowhead) innervating the large basiconic sensillum (lb) on the pedicel. The dendrites (d) are faintly stained. (X 400)

3-2. The cell body (arrowhead) of a neuron sends a process to a hair (mp) on the pedicel. The dendrites (d) in the large basiconic sensillum (lb) may also be seen. (X 1000)

3-3. The arrowhead marks the cell projecting to the hair on the flagellum. (X 400)

3-4. A group of cells (arrowhead) innervates the sensillum styloconicum (ss). (X 400)

3-5 & 3-6. The dendrites (d) proceeding to the sensillum styloconicum. (X 1000)

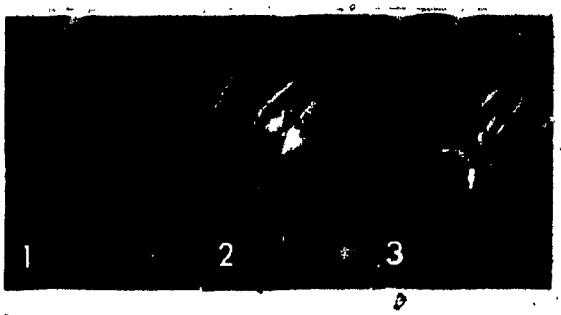
Figure 4. Light micrograph of the sensilla basiconica on the antenna of the spruce budworm, wholemounts in glycerol.

4-1. The texture of the large basiconic sensillum (lb) on the pedicel can be seen. (X 400)

4-2. The texture of the small basiconic sensillum (sb) on the pedicel is enhanced in this preparation. (X 400)

4-3. The basiconic sensillum (sf) on the flagellum also has a texture suggestive of numerous small pores. (X 400)


mp
1
2
4
5
6


1
2
3

olfactory appendages in other insects (Slifer, 1968).

Glycerol mounts:

Specimens mounted in glycerine jelly confirmed the findings made with those perfused with water-soluble stains. The sensilla basiconica appeared to have several pores. Careful focussing was needed to see the pores of the peg surface (Figure 4).

Electron microscopy:

The results obtained from protease-treated specimens indicate that the sensilla basiconica of this insect are covered with a proteinaceous or possibly lipoidal secretion. With this layer removed, a pattern of slit-shaped pores piercing the wall in the distal half of the sensilla was disclosed. Figure 5 is a plate of scanning electron micrographs illustrating the cuticle of the three basiconic sensilla, the natural appearance of the smaller basiconic sensillum on the pedicel, and a view of the entire antenna.

Electrophysiological studies:

The responses of a whole antenna to host plant volatiles and to some pure terpenes found in these hosts are presented in Figure 6. The sense cells in the antenna respond frequently to most odourous stimuli which were presented in this study. The largest action potentials have a potential difference of 0,5 mV. Pure air elicits a slight response both in amplitude and in frequency. These results

Figure 5. Scanning electron micrographs of the antenna of the second instar Choristoneura fumiferana.

5-1. Shows the entire left antenna: the scape (s) supports the pedicel (p), the next segment is the flagellum (f). Three basiconic sensilla (1-3) are found on the antenna.

5-2. The large sensillum basiconicum on the pedicel has been treated with protease to reveal its surface texture. Arrowheads point to the moulting scars.

5-3. The sensillum basiconicum on the flagellum, when treated with protease, reveals slit-like pores on the distal half of its surface.

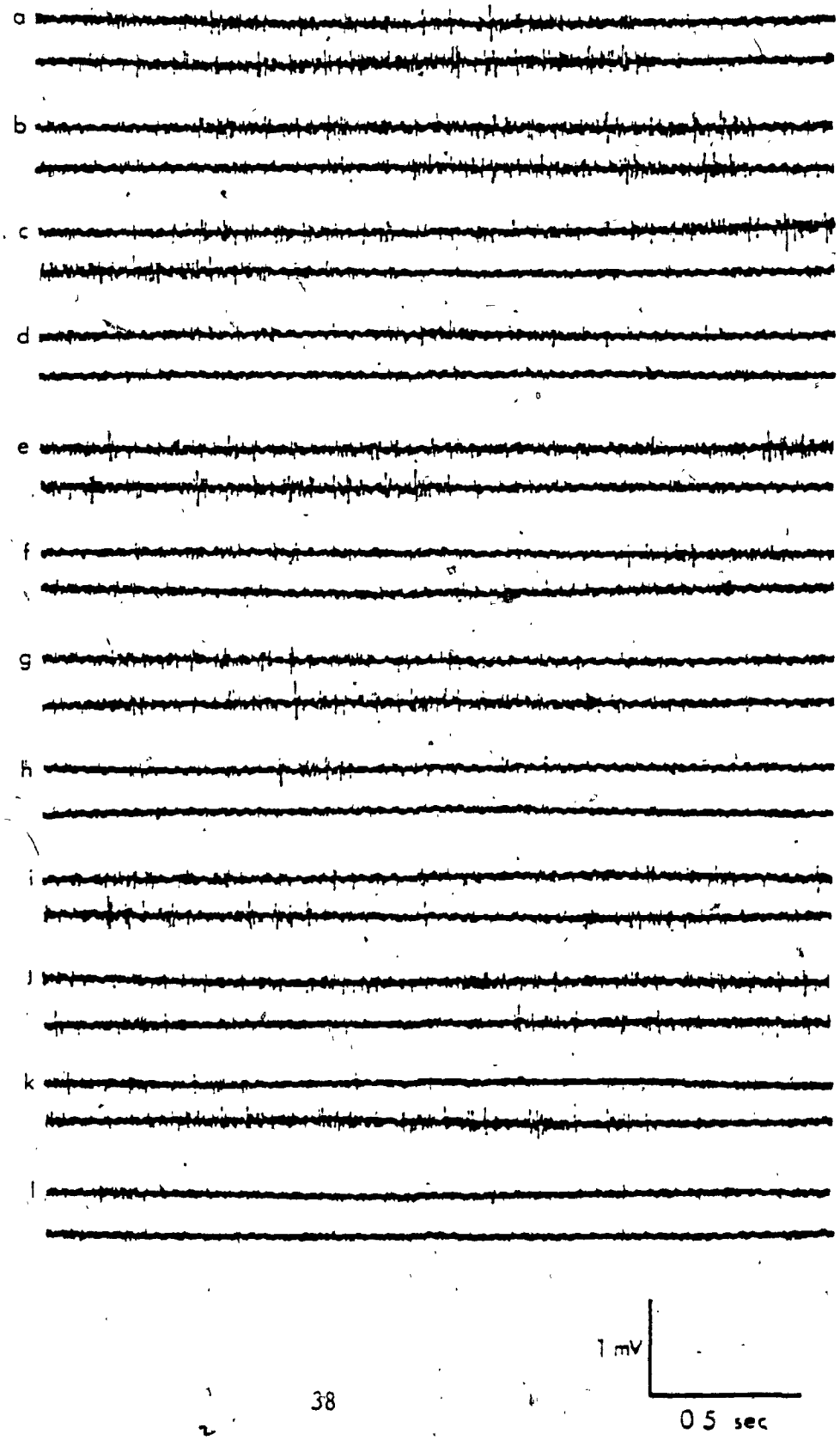
5-4. The same sensillum is infrequently accompanied by a stout sensillum (s) in early instars. Pores are indicated by arrowheads, the moulting scar by an arrow.

5-5. The short sensillum basiconicum on the scape, when treated with protease, reveals numerous pores (arrowheads) on its surface.

5-6. The same short sensillum basiconicum on the scape, without protease treatment to remove its natural coating; pores are concealed. Only a moulting scar (arrow) is apparent.

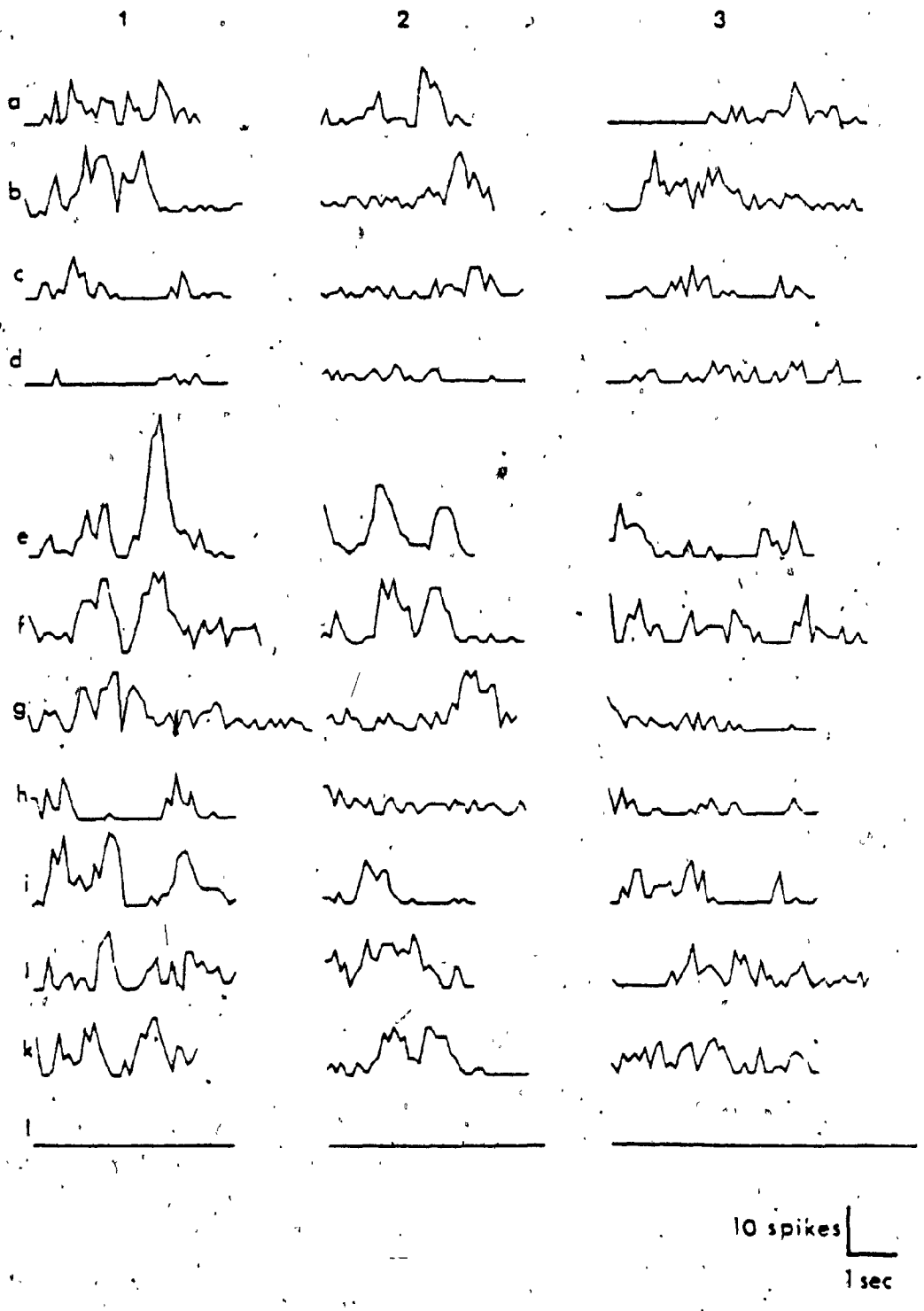

1
2
3
F
P
S
10μm
2
10μm
3
10μm
4
10μm
5
10μm
6
10μm

Figure 6. Representative single cell recordings from the antenna of Choristoneura fumiferana. a: white spruce foliage, b: balsam fir foliage, c: red spruce foliage, d: black spruce foliage, e: +- ∝pinene, f: - ∝pinene, g: + ∝ pinene, h: +- bornyl acetate, i: + limonene, j: - limonene, k: +- camphor, l: air. Onset of stimulation is at the begining of these traces.


a
b
c
d
e
f
g
h
i
j
k
l
1 mV
0 5 sec

were difficult to duplicate since we could not know which lumen of the antenna had been penetrated and thus from which cells recordings had been obtained. Neither could we tell how close the recording electrode was to a given cell; this is a factor determining the size of the action potentials recorded from that neuron. For this reason, the frequency of total action potentials, rather than those of individual action potential classes, was calculated and graphed for comparison (Figure 7). This data was normalized with respect to air. As a rule, action potentials were produced more frequently from the first insect than from the other two. In all three preparations however, response was greatest to white spruce, balsam fir, and the racemic mixture of α pinene.

Figure 7. Frequency of total antennal nerve impulses versus time (sec) for three insects when stimulated with a: white spruce foliage, b: balsam fir foliage, c: red spruce foliage, d: black spruce foliage, e: +- ∝pinene, f: - ∝pinene, g: + ∝pinene, h: +- bornyl acetate, i: - limonene, j: + limonene, k: +- camphor, l: air.


1
2
3
a
b
c
d
e
f
g
h
i
j
k
l
10 spikes
1 sec

DISCUSSION

A problem with this olfactometer system is that it requires a considerable amount of time to perform the test, to remove the insect from the Y-tube, and then to clean the tube. All of this however was required to ensure that insects were not following each other's trails, and that behaviour was not being influenced by the distribution of other previous insects within the olfactometer.

Within the olfactometer system developed for this study, the second instar eastern spruce budworm larvae were able to detect odours since they consistently moved in the direction of a given odour stream. They also chose the same hierarchy of volatiles as follows: new or old foliage > air; new foliage > old foliage; new white spruce foliage = new balsam fir foliage; new white spruce foliage > new red or black spruce foliage; new balsam fir foliage = new red spruce foliage; new balsam fir foliage > new black spruce foliage; new red spruce foliage = new black spruce foliage.

Chemical differences have been found to exist between the volatiles of spring buds and those of older foliage (Von Rudloff, 1972; Von Rudloff, 1975.) However, in this study, behavioural data obtained from using foliage collected when the insects were in their fifth and sixth instars in the field did not differ significantly from the data obtained using foliage collected when the insects were

in their third and fourth instars.

It has been postulated (Thorsteinson, 1960) that host plant volatiles serve as locomotor arrestants in lepidopterous insects. The data from the olfactometer experiments demonstrate that, in the case of the second instar eastern spruce budworm, host plant odours induce positive chemotaxis. It is worth noting in this context that there was no significant difference in the time taken to complete the test in the presence or absence of odours. It was also observed that, after the junction of the Y, the insect crawled more rapidly. This phenomenon may have been due to the resultant decrease in air flow or to the fact that the insect was exposed to an air stream from a single sample. Olfaction thus appears to help orient the insect towards its host. At higher concentrations, the host odour may act as an arrestant. The insect would then stop to feed, having reached its goal. Experiments using high volatile concentrations in time-released plastic caps could be performed in this olfactometer to test this hypothesis, though it is likely that the leaf-related tactile and gustatory stimuli are also necessary to fully inhibit locomotion.

In the field, a second instar larva finding itself on an inadequate host, can disperse by "ballooning" (Shaw & Little, 1973), that is, by suspending itself on a silk thread and being carried on the wind. Olfaction may play a

role in the orientation of the larvae during this period. It was observed, during the course of this study, that the larvae can shorten their silk thread, by ingesting it. The odourous environment may influence the insect's behaviour in lengthening and shortening the silk during ballooning. During this dispersal period, larvae of the spruce budworm are subjected to a high mortality rate (Miller, 1958). Since olfaction may play a significant role in the dispersal of the second instar larvae, an understanding of this sensory system could contribute to the control of this pest's population.

Once the ablation technique was perfected, cauterization of the whole antennae or maxillary palps did not affect the vigour of the larvae. These still actively searched for food and when fed on artificial medium, developed at a rate comparable to intact insects. Insects from early ablation trials took longer to complete the bioassay than intact insects. Later studies, such as the "left antenna repeat", showed no significant increase in the time taken by operated insects to complete the test. The series of experiments using ablated maxillary palp insects show an increase in the time taken to complete the test. This phenomenon is not surprising as the palp ablation operation required more handling.

The data from the ablation bioassay were as follows: no odour discriminaton by insects without antennae, odour discrimination with unilaterally ablated antennae, and no

effect on odour discrimination with amputation of the maxillary palps. These results indicate that the olfactory receptors responsible for a directed response to an odour source are all located on the antenna.

Although no sham-operated insects were used in ablation trials, the fact that insects with both maxillary palps removed and those with unilateral ablations performed as well as intact insects indicates that the operation itself has no effect on the ability of the larvae to discriminate between host-plant volatiles and pure air.

The behaviour exhibited by operated larvae was essentially the same as that of intact animals with the following exceptions. Insects with either one or both antennae removed showed significantly more klinotaxis on their way up the wire. This behaviour could have been predicted, since it is associated with a loss or a decrease in the concentration of the stimulus (Fraenkel & Gunn, 1961). In these bioassays however, no actual concentration changes were occurring. Insects which underwent removal of olfactory chemoreceptors merely perceived concentration decreases. Insects without antennae were constantly "searching" for some olfactory cue and insects with one antenna were subject to a greater number of perceived concentration fluxes, since they did not have their other antenna which contributes to the determination of their position in the air stream. Also, insects with no antennae

exhibited less of behaviour # 6 in which insects crawled up one branch of the Y for a few mm, and then came back to the junction to go up the other side. Insects with one antenna displayed quite a bit more of this behaviour perhaps owing to a loss of directionality. These results support the thesis that budworms "smell" with their antennae. The pausing behaviour (#9) was observed slightly more often in insects without antennae. This is in complete agreement with the proposal that host plant volatiles induce positive chemotaxis. The antennae of second instar Choristoneura fumiferana closely resemble those of the later instars (Albert & Zacharuk, personal communication), though the dimensional proportions of the segments and of the sensilla differ. The pedicel and flagellum are more elongated in the sixth instar than in the second and the three prominent sensilla basiconica (figures 5-1, 1-3) are proportionately larger in the second instar. This suggests that these structures which develop early must be functional at this stage of the insect's life cycle, a phenomenon which is confirmed by electrophysiological results included in this study. These sensilla are presumably utilized in the location of host plants, as suggested by the ablation bioassays. The stubby thin-walled sensilla, sometimes absent in early instars, must have greater physiological importance in those more mature individuals in which they are more prominent and consistently present.

The main problem with the utilization of the methylene

blue injection technique on the second instar eastern spruce budworm, was the size of the insect and the resulting difficulty in locating the dorsal aortic region. As a result, hundreds of unsuccessfully stained preparations were discarded. If the stain did not rapidly penetrate the circulatory system, it simply diffused to all body tissues. Only live nervous tissue oxidized the stain differentially. (See for eg. Zacharuk, 1962.) The time required for diffusion to transport the dye to the antennal nerves is too long for these cells to survive. For this reason, mounting the live heads on stain-filled pipettes failed to stain these neurons.

Though a major artery supplies the antenna, this area seemed to be the last to stain. The reason for this phenomenon is unknown.

Refrigeration of the specimen at 4 °C between injections appeared to prolong the life span of the stained insect. This, together with the double injection of the specimen yielded acceptable results.

The groups of cell bodies appear larger when these proceed from dendrites arising in chemoreceptive sensilla. Cell bodies which process information from what appear to be mechanoreceptive hairs (that is hairs whose articulating membranes are innervated by dendrites, and whose surfaces are not apparently pierced by pores) are smaller and are found closer to the base of these structures.

The observation of large groupings of cell bodies proceeding to chemoreceptive sensilla is consistent with work done on the sixth instar larvae by Albert & Zacharuk (personal communication). In this instar, the large sensillum basiconicum was shown in transmission electron micrographs, to house 22 dendrites, the smaller sensilla to posess 11, and the sensilla on the flagellum have 8. Three cells innervate the styloconicum; this is consistent with the results obtained in this study; the group of cells in the latter area is smaller than the groups leading to the sensilla basiconica. Due to the morphology of the styloconicum, its apparent lack of pores, and its small number of sensory cells, this organ is not presumed to have an olfactory function. The cell groups innervating the mechanoreceptive hairs are the smallest. This information coincides with the fact that only one cell innervates each of these hairs on the sixth instar.

The choice of stains was inappropriate for this phase of the work. Mallory-Heidenhain stains nervous tissue red, but it also stains other structures, such as fibrin-containing muscle, this same colour making differentiation difficult at times. Hubschman's Azan gives the same type of confusing picture. Neurons are stained pale lavender, a colour not easily differentiated from the pale blues of the cuticle, muscles and certain glands.

There exist more appropriate stains for insect nervous tissue such as the silver stains which stain the soma and

cell processes black, or the differential stains with nickel and cobalt, the resultant colours of which are dependent on the concentrations of these salts (Sakai & Yamaguchi, 1983). Though these techniques could yield information concerning the position of the nerve processes within the antenna, transmission electron microscopy (TEM) would further elucidate the anatomy of this structure. Ultra-thin sections could be used to resolve the ultrastructure of elements such as pore tubules. A TEM study would also facilitate the localization and the counting of the cells and their processes.

The appearance of the pale purple or pink colour in the sensilla basiconica, when treated with crystal violet or acid fuchsin respectively, promoted the SEM search for numerous small pores on the surface of these structures. Such an arrangement of pores is highly suggestive of an olfactory sensillum.

The sensillum styloconicum did not stain. This phenomenon suggests that this structure is not a contact chemoreceptor. Albert & Zacharuk (personal communication) have suggested that this sensillum is a thermo/hygro-receptor.

The lack of stain in the long hairs indicates an absence of pores in these structures which suggests that they are solely mechanoreceptive. They are probably involved in the reception of tactile stimuli.

The glycerol mounts show that the pores are distributed on the distal half or two thirds of the sensilla basiconica. The slit-like appearance of the pores on these sensilla as seen in the light microscope was confirmed by scanning electron microscopy.

The electrophysiological work is preliminary. It's main value is that it provides definitive evidence that some of the antennal sensory cells respond to odours. The response to pure air was always negligible. Chemoreceptors, as opposed to mechanoreceptors or thermoreceptors, are most likely to represent the majority of cells firing during stimulation. Interestingly, among the four host plant odours tested, white spruce and balsam fir, the two preferred trees from the olfactometer experiments, yielded the greatest response from the chemoreceptors. The action potentials in the response to red spruce and black spruce were fewer than those in the response to the former two hosts. It is possible therefore that these signals elicit positive chemotaxic behaviour. Of course, one must not ignore the possibility of inhibitory potentials, synergism or a very complex interaction among the sensory signals which elicit olfactory-mediated behaviour.

It should also be noted that +- α pinene, the major constituent of the four host plant volatiles (Von Rudloff, 1972, 1975), elicited the greatest response from all animals tested. Further studies should be performed to assess, both behaviourally and physiologically, if mixtures of

monoterpenes cause interactions at the sensory level. Such studies could prompt a search for repellent chemicals, or for a mixture useful in baiting traps.

Two out of three insects gave similar response patterns to each given volatile, though the response frequency was consistently greater in one insect compared to the other two. The action potentials obtained from the third insect gave a totally different frequency distribution than the other two tested, suggesting that the generalist cells in the different sensilla respond in varying ways to the same odour.

Since the cells innervating the three sensilla basiconica are not all in the same lumen (Albert & Zacharuk, personal communication), it was difficult to obtain a consistent total antennal response to a given odour. The solution to this problem would be to record from the base of each sensillum, but their small size in the second instar would make this very difficult.

CONCLUSION

The multimodal nature of this study has contributed to a greater understanding of the second instar eastern spruce budworm's olfactory system. The behavioural tests first prove that the larvae can detect and discriminate between the odours of their host plants. The ablation studies show that the sensory organs responsible for the reception of these volatiles are located on the antennae. The microscopy studies suggest that the sensilla responsible for this reception are probably the sensilla basiconica which bear numerous small pores and are innervated by several cells. This would suggest a generalist nature of the odour receptors. Finally, electrophysiological studies demonstrate that the cells within the antennae do respond to host plant volatiles and to some pure terpenes found in these hosts.

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