TWO OLFACTORY ATTRACTANTS IN ALFALFA (*MEDICAGO SATIVA*) FOR ALFALFA WEEVILS (*HYPERA POSTICA*)

by

KAREN LEE CURREY

B. A., Ohio Northern University, 1975

A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Chemistry

KANSAS STATE UNIVERSITY

Manhattan, Kansas

1977

Approved by:

Clyde E. Melton

Major Professor

TABLE OF CONTENTS

page

LIST OF FIGURES

LIST OF FIGURES - continued

LIST OF TABLES

INTRODUCTION

It is estimated that insects have a total world population of 10^{18}, that they are responsible for half of all human deaths and deformities caused by disease, and that they consume or destroy 1/3 of what man grows and stores. Yet only 0.1% of all the insects are agricultural pests and disease carriers. Pesticides that are used against them are often harmful not only to the pest, but also to the beneficial wildlife and to man as well.[2]

The alfalfa weevil attacks only alfalfa at an economically serious level and is considered one of the more serious pests of the Midwest. In Kansas, with over a million acres of alfalfa, the weevil reached a peak infestation in 1974 when at least 95% of the acreage was infested. More than 824,000 acres had to be treated with insecticides in 1974 compared to 220,000 in 1973 and 49,000 in 1972.[32]

The objective of this research was to identify compounds in alfalfa that might possibly be involved in olfactory chemical communication between the weevils and their host plant, such as feeding stimulants and egg laying (oviposition) stimulants. If such chemicals can be found, then it might be possible to breed a species of alfalfa that changes these chemical communication patterns so that the weevils can be controlled without the use of pesticides. Seven compounds have been separated and ~~three~~ identified. Two have been shown to elicit a positive olfactory response from the weevils.

Oviposition responses were studied and a stimulus found to be present, but no specific compound has as yet been identified. Evidence of an alfalfa weevil repellent in red clover has been well documented. The response of the weevils to red clover was studied, but no compounds were separated that elicited a deterring effect.

An understanding of insect--plant relationships in general, and the relationship of the alfalfa weevil to its host plant in particular, are helpful in a study of the weevil and will be discussed further.

Insect-Plant Relationships

Classification of the phytophagous, or herbivorous, insects include polyphagous, oligophagous, and monophagous feeding behavior.[9] Polyphagous refers to the fact that many plant species from different families are eaten. For oligophagous insects, only some plant species usually belonging to a few related families are acceptable as a food host. True monophagy, selection of one plant species, occurs very rarely. Many species of insects are primarily associated with only one plant species and are classified as monophagous, though erroneously, due to their common name.[7] Two examples of this are the Colorado potato beetle and the alfalfa weevil.

In finding the host plant many signals, such as visual, olfactory, tactile and gustatory, come into play. None of these various signals from a single plant though is likely to be picked up by the insect at, for example, one kilometer.[7]

Except at close range, visual signals do not contribute significantly to the unique identification of the host plant by the insect.[7] For some phytophagous insects, the plant silhouette plays an important part in the orientation phase of food finding, but it would be a very tentative identification if it is used at all.[7] The spectral composition of the light reflected from the surface of the plant is relatively narrow in range, since the leaves of most of the plants are green, or a yellow green. Some phytophagous insects can perceive certain of the color differences, though the color of the plant is probably not a critical cue for finding the host.[7]

Olfactory signals are generally credited with a major role in the food finding process. The insects do not, however, orient to the plant beyond a few meters. It may be that foraging is random until the insect is in the immediate vicinity of the host plant.[7]

Moncrieff in 1951 and Wright in 1958 stated that the acuity of odor perception in insects far exceeds that of man. Insects rely greatly on olfaction in their search for mates and food.[26] In 1960, Dethier defined compounds that elicit a response by olfaction with the following terms: "arrestant," "stimulant," "attractant," "repellent."[26]

The attractant odor is derived from a substance that is generally peculiar to that particular plant. There are some 2000 species of plants from 60 different families that synthesize hundreds of different chemicals as their "essential oils."[1] These "essential oils" are odorous substances found in the plant material (flowers, leaves, bark and wood). They possess an odor that is characteristic of that particular plant species and may repel or attract. Often the "oils" are deterrents and may even act like juvenile hormones and affect the life cycle of the insect, or the various odors may be used by the insect to locate its host plant.

These oils consist of mixtures of compounds that may be quite complex. Almost any type of organic compound may be found, though terpenes and terpenoids are the most common.[1] The basic chemical composition of the "oils" may be divided on the basis of their biosynthetic origin into two classes: 1) terpene bases formed from the acetate mevalonic pathway, and 2) aromatic compounds from the shikimic acid-phenyl propanoid pathway.[8]

If the odor of the plant serves as an attractant, and the insect explores the plant, it may either be repelled by other odors or

further attracted. One theory states that the insect finds its host by avoiding the plants with the repellent odors. If the insect is hungry it may take a test bite and both the odors from the injured plant and the chemicals ingested determine if feeding is to continue.[9]

Chemoreceptors are classified as contact or olfactory receptors. The olfactory receptors are generally restricted to the antennae although they may be present on the maxillary and labial palpi.[9] The odor of the plant is critical in the first stage of the feeding process. Insects that have their antennae and the third segment of the maxillary palpi amputated can be induced to test bite plants that would normally be rejected.[9]

There is a vast network of chemoreceptors on the insect that must receive the stimuli from the plant. The receptors are associated with the behavioral responses and are not the same for all insects. They are found on the thorax, antennae, legs, mouthparts, wings, or scattered over the general body surface.[26]

Gustation is a form of contact chemoreception and is associated with a tactile component. Without chemotactic signals, the tactile stimuli do not elicit a response from the insect.[7] Sugar is an example of a gustatory stimulus and has a marked influence on the behavior of many insects. It appears to be of importance in the regulating of feeding by phytophagous insects.[7]

Water has a chemotactic influence on gustation, as insects can be "thirsty," and will drink pure water. Water probably provides some sensory stimuli but it is not an essential feeding stimulus in the strict chemotactic sense for leaf-eating insects. Water vapor is a regulating factor on the behavior of the insect but it is not a significant factor in host finding.[7]

Although the plants differ in their physical properties, none of these factors taken alone or in combination, provides a substrate adequate to account for the diverse effects between the plants and the insects. Shape, size and color are not unique enough to allow the insect to be so discriminating. However, the chemical makeup of the plant provides an almost inexhaustible supply of a variety of substances. It is largely a chemotactic basis for food plant preference.[7]

Factors influencing ovipositioning

It may be that the ovipositioning adult performs the actual selection of the host plant. Besides optical stimuli, the female is guided by gustatory and olfactory stimuli. In many species, tactile stimuli are extremely important.[7,9] When the adult and the larvae use the same food plant, one way to check a plant's susceptibility is tc test for the acceptability of the plant as far as tasting the plant. Some insects are known to take a test bite from the plant before they oviposit.[9] Thus, either characteristic volatile factors or contact chemoreceptors of compounds typical of the host, or both, control ovipositional behavior and are in this way associated with plant selection.[9]

The oviposition response and the factors affecting it are of vital importance to the insect. Richardson, in 1925, showed that the stimuli which affected such a response in an insect are both internal (insect's state of nutrition, age, fertility, and endogenous rhythms) and external (temperature, humidity, light, air, and water flow).[26]

In 1965 Beck listed a series of complex acts that cover the process of ovipositioning. These include plant recognition, orientation to a plant or a particular part of the plant, deposition and departure. All the mechanisms involved incorporate chemoreceptive, visual, proprioceptive, and tactile stimuli.[26]

Chesnokov, in 1953, stated that some insects select a plant host specifically for the purpose of ovipositioning. The female Colorado potato beetle for instance, selects the plant.[26]

The orientation of the insect to the host for the purpose of laying eggs is shown to be directed in some cases by olfactory mediation. The response to olfactory stimuli alone, however, should not be used as a criterion for the selection of the ovipositional site. Contact chemoreception is important for some as well as a tactile stimuli. Often both tactile and volatile factors are necessary. The proportion may differ for the different species.

Environmental factors that may also influence oviposition are the age of the plant material, the smoothness and the curvature of the stem, and the stem diameter. The stem diameter has been shown to be especially important for some insects, including the alfalfa weevil. Crowding may play a part as well as the time of day and the photoperiod.[26] Succulence, toughness, pithiness, and pilosity are not stimuli but must be taken into account.[7]

Dietary factors may also affect the reproductive behavior of insects. The female of most species requires some specific source of a dietary protein before ovarian development can begin.[25]

Theories of food selection

The insect follows a general pattern of random movement, orientation, biting response, sustained feeding and dispersal. Whether to feed or not is often controlled by definite chemical factors such as attractants, stimulants or deterrents.[7,25]

One way to consider all of the aspects to the concept of stimulus is to consider the older concept of response chains. A given link in the chain influences the following link and each unit response is likely to require more than one stimulus. This applies to the interaction of two stimuli, such as color and odor, and also to the summation effects of several stimuli of the same general type, for example, chemotactic stimuli. The links in the chain thus occur in parallel, as well as sequential order. By recognizing this aspect, overemphasizing any one particular stimulus can be avoided. While the unit stimulus can be studied alone, their total significance can only be seen as a part of the whole.[7]

In 1950 Paech proposed that it was the secondary plant substances present in all the plant groups but seeming to serve no clear function for the plants themselves, that determined the susceptibility of a particular plant to a particular insect. In 1951 and 1958 Fraenkel proposed that the green leaves of the different plant species differ only slightly in their chemical characteristics. Therefore it was these "unusual" materials, or secondary substances that brought about the preferential selection of one plant over another.[26] Materials that have been

identified as secondary plant substances include glucosides, tannins, alkaloids, "essential oils," saponons and organic acids.[26] These secondary plant substances may act as deterrents or function as stimulants in ovipositioning and food finding.[7] Lipke and Fraenkel both say that these chemicals are solely responsible for guiding the phytophagous insect in general to their preferred host. Other researchers, such as Dethier say that the role remains open to question.[7] These secondary chemicals are believed to have no nutritive value to the plant. Some researchers feel that the secondary chemicals may be involved, but that the nutrients play a role too.[7]

The point that secondary plant substances or the nutritive material solely determine the plant host is out of date now. Either of the two groups, or a combination of the two, play an important role. The emphasis depends on different insect-plant relationships. This is the dual discrimination theory proposed by Kennedy and Booth.[9] In some cases the accent is on the effect of the odd substances, but in others the amounts or the ratios of the nutritive constituents may form the decisive factors.

Monophagy and oligophagy could then well be based on a fairly subtle combination of a number of common plant components, combined with the absence or presence of secondary substances. The attractiveness of the secondary substances can then be obscured by the activity of the common substituents.[9]

Final selection of the host plant then involves many factors, alone and in combination. It is unlikely though that it is a "one plant--one insect," or a "one chemical attractant--one insect" concept[25] that entirely determines the choice. However, the absence of one critical factor may affect the choice.

Insects' internal chemicals

Insects use their internal chemicals for many purposes. They use them to attract the opposite sex, mark a trail and identify friends or enemies. A chemical messenger may be used to trigger a series of physiological changes; molting, new growth, physical and sexual development.

The chemicals produced by the insect can be divided into two groups, those that influence the same species, and those that are meant for another species. The definitions of the types are as follows:[2]

Pheromones-secreted by the insect into its environment and influences the behavior of insects of the same species

Allomones-cause behavioral or developmental reactions in another species that is favorable to the transmitter

Kairomones-response is favorable to the recipient (of another species)

Chemical messengers within the insects own body are termed juvenile hormones, or JH. They regulate the life processes and are very essential for the normal growth of the insect. The timely application of the proper hormone can prevent eggs from hatching, cause premature development, and can cause sterility and death in the adult. They can also be used to alter the life cycle of the insect, as insects are unlikely to build a resistance to their own hormones.[2]

Hormones that have been made by man have been termed Insect Growth Regulators, or IGR's. They have an absence of any

undesirable effects on man, wildlife, or the environment. They are used with the intention of controlling pests. They may behave similarly to a juvenile hormone, and if the effect is to produce an abnormal morphogenesis, generally the process is irreversible.[16]

Many insects undergo a time of their life when they are dormant; this stage is referred to as diapause. This is the time they usually sexually mature and may also be the time of the year to avoid bad weather conditions. During diapause, factors such as certain hormones act on the feeding center in the central nervous system and induce complete inhibition of feeding.[9] The diapause stage may be wholly or primarily induced by a specific JH deficiency and therefore easily disrupted by the exogeneous application of an active IGR. The number of eggs then laid by the female appears to be dose dependent, quite different from the normal breaking of diapause.[16]

Weevils appear to be relatively insensitive to IGR's. They require larger doses than even beetles. Substantial doses are often needed and yield only minimal effect. For the alfalfa weevil the adult does respond positively to the exogenous application of an IGR for the breaking of diapause.[16]

Alfalfa weevil's life cycle

The alfalfa weevil, Hypera postica (Gyllenhal), family Curculionidae, was first found in the United States in 1904 near Salt Lake City, Utah. It was introduced there from southern Europe. It was not until 1951 that the weevil was discovered on the east coast, in Maryland. These are two separate strains that have

spread across the country infecting nearly every state by now. Kansas was the first state where the two strains met.[30] The Western strain entered Cheyenne and Hamilton counties in 1960. In nine years it had spread throughout 40 counties. The Eastern strain entered Cherokee county in 1967 and spread north and west. By 1972, all counties in Kansas had become infested.[30]

The weevil moves from place to place by three main ways: flying, crawling and hitch-hiking. The primary way is flying; the weevil may rise 10-15 feet in the air and by riding air currents travel long distances. The weevils may also crawl from field to field or they may hitch-hike during the harvest by riding along with the hay.

The alfalfa weevil generally produces only one generation during the growing season unless there is an especially warm, long Fall when a second partial generation may be produced. The Southern states often have a second partial generation due to their warmer climate.

The life cycle of the weevil includes four stages: egg, pupa, larva, adult.

Egg stage

The eggs are a bright lemon yellow when they are first laid and turn brown before hatching. They are oval and about 1/32 of an inch long.[11] They are laid in the green alfalfa stem, in alfalfa stubble or in hollow grasses and weeds. They are laid in clusters of 2-25 eggs on the average. There may be several hundred eggs laid in one stem. They are laid either in the spring when they will hatch in 1-2 weeks, or in the fall when they will overwinter until the following spring.

Larval stage

After hatching, the larvae emerge through the puncture and climb up the outside of the stem. If the eggs were laid in stubble, the larvae must migrate over the ground to reach a plant. If there is a heavy infestation in a certain area, the larvae may migrate from plant to plant.[10] The larvae feed for 3-4 weeks, molting three times. When fully grown they are about 3/8 of an inch long. The fourth instar stage has a green body with white stripes and a dark head. It is the larval stage that is the most voracious. Even the first instar stage has been kept in the laboratory for four days without food and has been observed to be constantly on the move, apparently in search of food.[10]

The first cutting of alfalfa is primarily damaged by the larvae stage. They feed within the plant tips and on the upper leaves and then on the lower foliage, skeletonizing the leaves.[11] The damaged fields take on a grayish to whitish cast that is somewhat similar to severe frost damage.[10] Once infested there is yearly damage by the weevil.

Pupal stage

The larva spins a cocoon when it is full grown and in a few days enters the pupal stage. The pupa is 1/4 inch in diameter and brown in color. It is found on the lower portions of the plant, in dead leaves, ground litter and on the bare earth. The pupa stage lasts 1-2 weeks.[10,30]

Adult stage

The adult weevil has a distinct short snout and is about 3/16 of an inch long. They are light brown in color with a dark stripe starting at their head and running down their back. Figure

(1) shows the adult weevil. They grow darker as they age turning a dark brown to almost black. The adult survives for 10-14 months.

The feeding by the adult is not extensive enough to damage the crops. Most of the young adults leave the field for nearby protected areas in the summer. The weevil then enters a dormant period, called diapause. The weevil matures sexually during this time and returns to mate and feed in the fall.[11]

The adult then overwinters by crawling down into the crown of the alfalfa or into a sheltered place in the field. They may leave the area for nearby shelter also. The adults return to the fields in the spring where they may again mate, and the female lays the rest of her eggs. If the weather warms during the winter, the female will often emerge for a short time to deposit more eggs that will then overwinter. The adults are usually dead before the first harvest. It appears that the female weevil needs to mate only once, and is capable of laying several hundred eggs.

A picture of the weevil's feeding pattern on alfalfa is presented in Figure (2).

The major influence on the weevil is the climate. There are important temperature ranges for the eggs to hatch and the female to oviposit. Oviposition appears to stop at 85°, though the female will oviposit as low as 45-50°. The lower limit for egg hatching is not known, but it is probably over 50°. Eggs will hatch at 75-85°. Thus if there is unseasonably warm weather in the winter, the females can emerge to oviposit, but any of the eggs that are laid will not hatch until warmer weather. Increasingly high temperatures in the late spring and early summer

FIGURE (1)

The Alfalfa Weevil

FIGURE (2)

Feeding on an Alfalfa Leaf

by an Alfalfa Weevil

inhibit oviposition since the larvae would not survive well if the eggs were laid very late in the summer.[10]

The characteristics of the adult weevils that are used to distinguish between the sexes are the shape of the caudal abdominal sternite and tergite. For the male, the last abdominal tergite extends down over the tip of the abdomen. It is easily seen from the ventral side. The last abdominal sternite terminates posteriorly in a broadly rounded projection. On the female, the last tergite is not easily seen from beneath. The last sternite extends to the end of the abdomen and is smoothly and broadly rounded without a projection. Other characteristics such as movement, size and speed are not distinctive enough to differentiate between the sexes.[22]

Literature review of bioassay methods

A reliable biological assay method is essential for the isolation and identification of an attractant. Some of the work that has been done by other researchers is presented here.

There are many literature reports of the larvae and the adults feeding or ovipositing on many different plants in the field. Larvae have been observed to develop and pupate on isolated plants of sweetclover in the field. The adult weevil can also feed and develop on plant species other than Medicago sativa (alfalfa). The alfalfa plant, however, is the only plant that is attacked at an economic destructive level.[25]

Byrne and others[25] ran tests on various plants and found that both alfalfa and sweetclover were acceptable to the adult and to the larvae. The adults were also observed to feed on

red clover, but there was no larval feeding. Field results appear to show the same results.[27] Alfalfa and sweet clover were acceptable as host plants. Stray plants of alfalfa in the red clover plots would be severely damaged but the red clover plants would not.

It was discovered that the female weevil would not feed or oviposit in hop clover alone, but did so in the presence of alfalfa.[25] When a water extract of the hop clover was sprayed on the alfalfa, ovipositioning was inhibited, but had no effect on feeding. Ether extracts and water extracts of alfalfa and of sweetclover were applied to hop clover, but no increase in oviposition was observed.

Pass and his coworkers discovered alfalfa weevil eggs in henbit stems near an alfalfa field.[21] They set up lab tests offering alfalfa and henbit together and separately. A great number of eggs were laid in the henbit, with the alfalfa present, but very few when the henbit was offered alone. The henbit is suitable for oviposition for tactile or physical reasons, but it is apparent that the presence of alfalfa is important. In the field, Pass found no eggs in the henbit farther than 18 inches from the nearest alfalfa plant. The larvae were not observed to feed on the henbit; therefore they would have to crawl to the nearest alfalfa plant to survive. Whether they were capable of doing this was not investigated.

Byrne[25] ran tests on adult feeding, oviposition, and larval survival on ten Medicago and related species. He found that if a plant is a poor larval host, it is also a poor or undesirable adult food host. The further a plant is from types related to

Medicago or Melilotus (sweetclover) the less suitable it is as a host. All the legumes being tested were in close proximity; therefore Byrne did not feel that it was an olfactory stimulus that determined the choices.

While pursuing this test[25], Byrne noticed that the number of eggs laid significantly and positively correlated to the stem diameter. Even on the preferred plants, if the diameter was too small, there were no eggs laid.

In all the tests run[25], Medicago sativa and Melilotus species were found to be suitable for all stages of the alfalfa weevil. Other species may be acceptable at one stage or another during the life cycle of the weevil, but no others were acceptable for all stages.

In another study,[26] Byrne ground fresh alfalfa and filtered it. He dipped red clover plants in the filtrate and recorded the number of eggs laid. There was a definite increase in the number of eggs laid, though it was not as high as in alfalfa alone. He repeated this with red clover juice on alfalfa. The juice from the red clover plant inhibited the level of ovipositioning greatly. It appears that the red clover inhibitor is more potent than the alfalfa stimulant since there is a much greater decrease in the level of ovipositioning than there was an increase using the alfalfa juice. It may be the absence or the reduction of deterrents in the alfalfa rather than the presence of stimuli that elicits a response from the weevil.

Various solvent extracts, hexane, diethyl ether, 70% ethanol at 27° and at 60°, and water at 27° and at 70°, plus a steam distillate were tried.[26] These extracts of alfalfa were then

applied to red clover as before. None of them appeared to isolate the material that stimulates ovipositioning.

The concentration of any extract studied is extremely important. It is possible that they had isolated the stimulant, but the concentration was incorrect.

The same procedure was tried with the red clover.[26] The hexane extract was found to reduce the level of ovipositioning by 75%, while hexane alone only reduced the level by 25%. The repellent in the red clover is apparently hexane soluble.

The removing and the counting of eggs from an alfalfa stem is often time consuming and difficult. Hower and Ferrer[15] found a technique and an ovipositioning medium which provided an efficient and fast removal of eggs from a stem. They constructed tubes out of parafilm and found that the alfalfa stems inside these tubes had more eggs in them than in the normal alfalfa stems. It is a simple matter to remove the tubes and retrieve the eggs. A hollow tube alone, though it had more punctures in it, was not suitable as an ovipositioning medium. Likewise the large tubes and the larger stems were not suitable. Cotton placed inside the tubes was not acceptable to the female weevil. Apparently the tactile stimulus was not correct.

Byrne and Steinhauer[20] examined extracts of the alfalfa and their attractiveness by olfaction. They steam distilled fresh alfalfa, extracted with diethyl ether, added water and then fractionally distilled the mixture. The fractions between 40° and 98° were collected together. This distillate was washed with diethyl ether and the ether then evaporated.

These extracts were diluted by volume and the various dilu-

tions and the concentrated extract along with the fresh steam distillate were tested. The tests were run with and without water offered beforehand. A 1/2 inch diameter section of pith was soaked with the substance to be tested and placed in the middle of a petri dish. Nylon mesh covers were placed over the top.

The response from the weevil occurred within the first few minutes. Attractants by their nature should exert their influence within the first few minutes, and this was found to be the case.

Offering the water before the test reduced the orientation to the extract. It is apparent that the water vapor itself stimulates a response from the insect. The response to the extract may be secondary if the need for water is high. However, even with the water offered beforehand, the orientation to the extracts was greater than to the water control. The 0.025% by volume dilution elicited the maximum response. The undiluted extract and the 0.0125% dilution elicited no response.

Water requirements of the insect are an important factor governing the response of the weevil to an attractant. It appears that the attractant must be in an aqueous solution to elicit a response. It may be that the water molecules carry the molecules of the attractants to the chemoreceptor sites.[19]

Many researchers have tried olfactometer tests on the weevils. Very often the results of using olfactometers, even of different designs, are very poor. This may be due to the restriction of the normal movement of the weevil or to external stimuli that are unaccounted for.[7] Byrne and Steinhauer[20] found that the position of the alfalfa was very important as the weevil has a

strong negative geotaxy response; that is, they prefer to orient upwards at all times.

Work has been done to try and locate receptors on various parts of the weevils' body.[26] Various portions were removed and the results studied. It appears that neither the front, the middle, nor the hind pair of tarsi individually appear to be receptive to any plant produced stimuli which affect the ovipositional response. The antennae were removed and were found to have no indispensable function also.

The sealing or the removing of the mouthparts had a significant effect on the response. The female may have to touch or ingest a part of the plant to be cued to lay her eggs. If the mouthparts were unsealed, the female returned to normal levels of ovipositioning.

Environmental factors affecting ovipositioning have also been examined.[26] Higher populations per test jar appear to reduce the level of ovipositioning. Although, in these same jars, if the food was changed daily, the level increased. The age of the plant material did not affect the levels. Possibly the female may deposit a substance to deter other females from laying eggs in the same general vicinity and the daily changing of food may account for the increased level as a fresh source for egg laying was available daily.

The rate of egg laying is not governed by any time regime. Test conditions of 16 light hours and 8 dark hours and the reverse had equal effects. The time of day is apparently not important, as there were as many eggs laid at 8 AM as at 8 PM. The weevils do need an alternating light and dark period, though. Weevils

kept under total dark conditions, or total light conditions, had much lower levels of egg laying.[26]

In comparing the stem and the leaf of the alfalfa plant, it appears that both are necessary for normal levels of ovipositioning to occur, however the leaf seems to be the main source of the chemical stimulant.[26]

Hsiao in 1969, found feeding stimulants for the fourth instar larval stage.[29] The compounds he identified were adenine and adenosine. These do not appear to need any other compound present to elicit a response. Hsiao therefore classified them as specific or true feeding stimulants, whereas something like glucose is a general one. Adenine was found to be present in the leaf at a concentration of 0.012%.

Another method that was used satisfactorily for the cotton boll weevil and was tried in this project for the alfalfa weevil, was to use what Neff and Vanderzant[24] termed the "hidden method". The plant part or the substance to be tested is placed in a container and parafilm is used to seal it. Water is also placed inside if the test substance is not already aqueous. Holes are then punched through the parafilm to allow any odors to escape. The punctures and the eggs are recorded.

Under field conditions, the weevil attacks only Medicago sativa, alfalfa, at economically serious levels. The reasons are many and interrelated. It is certain that the alfalfa plant is not the only plant that provides all the requirements, but it is the preferred host.[25]

Criteria such as attraction by odors or the response to a feeding stimuli when considered alone may not be as critical in

THIS BOOK CONTAINS NUMEROUS PAGES WITH DIAGRAMS THAT ARE CROOKED COMPARED TO THE REST OF THE INFORMATION ON THE PAGE.

THIS IS AS RECEIVED FROM CUSTOMER.

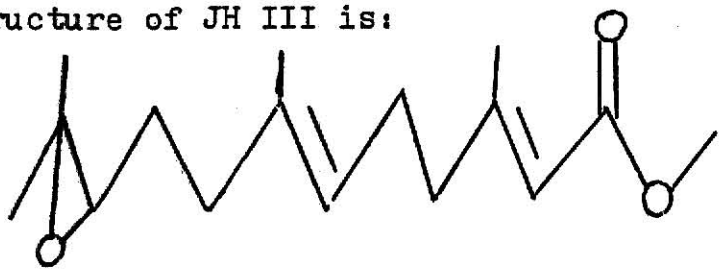
selection of a host as once thought. None of these criteria alone is the controlling influence in seeking the host plant. It may be that the "perfect" combination of the desirable factors for this insect are found only in alfalfa.[25]

Experimental, Results and Discussion

Due to the multitude of completely different bioassays run with the alfalfa weevil, it was considered more realistic to divide this project into six parts and to combine the experimental procedures with the discussion of the results for each part.

PART I Termination of Diapause Using an IGR

Part of the weevil's life cycle includes diapause during the hot summer months. At that time the weevils are incapable of mating and of being active in general. This limited weevil research to only a few months each year. In order to use weevils for tests on a year round basis, it was necessary to find a way to break diapause and activate the weevils. In the literature,[16] JH III has been reported to break the diapause stage of the alfalfa weevil. The structure of JH III is:



The application of some IGR's is known to break diapause. Since Altozar was available, this method was chosen. As far as could be determined, this particular IGR had not been used before.

The dose level and the mortality rate were unknown; therefore an experiment was devised to determine the proper dose level and the resulting mortality for each dose.

Experimental

The IGR used in this experiment was Altozar, a product of Zoecon Corp. The Altozar used was a sample that was given to Dr. Hopkins of the Entomology Department at Kansas State University in September, 1973. The chemical name for Altozar is ethyl (2E,4E)-3,7,11-trimethyl-2,4-dodecadienoate, and the formula is $C_{17}H_{30}O_2$. The structure of the compound is:

Most IGR's are unstable in sunlight[16] and with increasing temperature. Therefore the Altozar was kept in the freezer at all times when not being used.

The Altozar was dissolved in acetone and topically applied to the weevil's abdomen using a microapplicator calibrated to deliver 1.0 ul. The weevils were first anesthetised under CO_2 to facilitate handling of them when applying the IGR. The doses of

the Altozar used were 100, 50, 10, 5 and 1 ug/ul. Two control sets of weevils were used, one undergoing no treatment and the other having 1.0 ul of acetone applied.

Four replicates for each treatment with three containing ten females and ten males were setup. The fourth replicate was used as replacement weevils for any that died or were missing from the other three.

The two types of alfalfa used were Kansas Common and Kanza varieties. Each new setup was entirely one variety or the other. Ten alfalfa stems per replicate were stripped of all the leaves except the top set of leaves and inserted inside a parafilm tube. The parafilm tubes were 10 cm by 4 mm approximately. The growing tip of each plant was pinched off since early in the study it was discovered that the plants continued to grow and thus the amount of exposed plant material varied. The group of ten stems were then wrapped in a strip of cotton and placed in a 14 dram plastic vial filled with water. An example of a stem inside a parafilm tube is shown in Figure (3). One vial was then placed inside of a one gallon capacity glass jar with a screw top lid. A large hole had been cut in the top and a wire mesh screen put in place to permit air circulation. One paper towel per jar was cut and placed on the bottom of the jar to help retain excess moisture from the transpiration of the plants, and to allow the weevils a place to crawl under and hide.

The jars were randomly placed on a table in the Rearing Room of Waters Hall. Fluorescent lights were hung above them. The lights were set for an 8 hour light period, from 9 AM to 5 PM. The humidifier was set for 80% and the temperature set at 72°F.

FIGURE (3)

Alfalfa Stem Inside

a Parafilm Tube

The setup used is shown in Figure (4).

Bouquets of fresh alfalfa were added and the dead weevils replaced three times a week. The punctures in the parafilm and the number of egg masses and the number of eggs within each mass were recorded. The experiment was run from July 22, 1976, to September 10, 1976.

Results

The mortality rate turned out not to be a problem. The 100 ug dose level had the highest rate with 17 males and 10 females dying over the course of the experiment. For any short term work, this rate would not affect the results as this experiment was run longer than most bioassays would be.

The number of eggs laid per treatment yields the best results of the effectiveness of the IGR. The results are presented in Table 1.

TABLE 1

Results of IGR Experiment

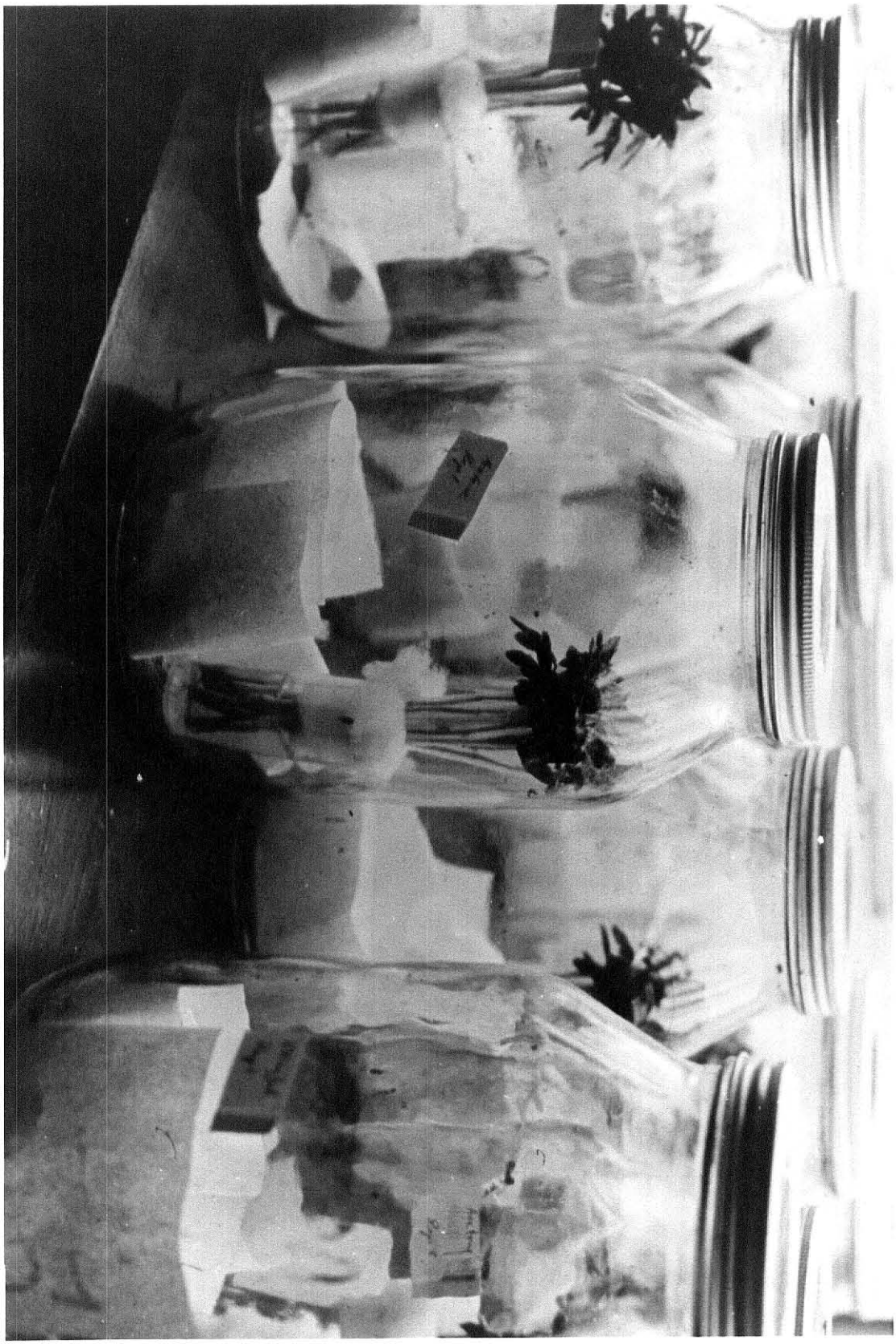
Treatment (ug/ul)	Total number of eggs
Controls	
Zero (no treatment)	506
Acetone (1 ul)	1788
Test	
1	688
5	901
10	1268
50	767
100	1702

The results of this experiment agree with the work of Staal[16] in that the number of eggs is dependent on the dose level. The discrepancy of the acetone and the 50 ug/ul dose can not be explained at the present time. The acetone should have had no

FIGURE (4)

Termination of Diapause

Experimental Setup



more effect than the no-treatment group.

On the basis of this work, it was concluded that any further work with the weevils where it would be necessary to treat them with an IGR, the 100 ug/ul dose of Altosid would be the preferred dose. If an IGR is used that is fresher, however, undoubtedly a lower level could be employed.

It appeared to take about 7-10 days for the effect of the IGR to manifest itself. Therefore any time the IGR is used, time should be allowed for the weevils to respond to treatment. The net result is that it is possible to use an IGR to bring weevils out of diapause so that year round research can be done.

PART II Bioassays on the alfalfa weevil

Several bioassays were set up based on the results obtained by Hower and Ferrer,[15] Byrne, Steinhauer, and Blickenstaff[20,25,26] and by Neff and Vanderzant.[24] Their various methods formed a starting point for our studies on the alfalfa weevil and its response. Adaptations of their work was also carried out.

Experimental

Nine bioassay tests were performed as a group. The following conditions were the same for all the tests, unless specifically noted under a test.

All stems and leaves for each vial were always from the same plant. The stems, three per vial, were wrapped in a strip of cotton and placed in a 14 dram plastic vial that was approximately 3/4 full of water. The vials were then placed in a glass, one gallon jar with a lid that had a wire mesh screen in it. Paper towels were placed on the bottom of the jars. The growing tip of all the alfalfa plants was pinched off.

The alfalfa used was field grown Kanza and Kansas Common. Any leaves that were used loosely from these plants were always from the top trifoliate groups of leaves. The red clover that was used was found along the roadside on West Anderson, west of Route 113.

Plants were changed three times a week, and the loose leaves were changed daily to avoid drying them out. Dead weevils were replaced with a weevil of the same sex.

Tests were conducted in a growth chamber with a light period of 8 hours. The temperature was 72° during the "day" and 68° at "night." The position of the jars in the chambers was always randomly chosen. White styrofoam sheets were placed between the rows of jars in the chamber to prevent interference from any visual cues from the other jars.

The weevils used were spring collected adults. The time of these tests was in the early fall, To insure a high mating level and ovipositioning level, it was decided to treat the weevils with Altozar, an IGR. The dose used was 100 ug/ul. Males and females were separated and kept in gallon jars for at least one week before being used in the tests. Fresh alfalfa was kept in the jars allowing the weevils plenty of food. Four replicates of each test was set up. Five males and five females were used in each replicate.

Test 1

The purpose of this test was to obtain a set of controls for the entire group of tests. It was necessary to know the number of eggs that the female is capable of laying at this stage of her

life cycle. The control vial for this test was alfalfa stems stripped of all the leaves except the top two trifoliate groups. The test vial contained alfalfa within a parafilm tube. Since many of the tests would be using parafilm stems it was necessary to examine the weevil's response to it compared to alfalfa alone. The test ran for 2½ weeks.

Test 2

The purpose of this test was to check for the presence of either a tactile or a chemical stimuli that the female weevil must contact before she lays her eggs. The control for this test was alfalfa stems inside parafilm tubes with two detached leaf trifoliates inserted in the top of the tube. The test vial consisted of empty parafilm tubes with leaves also inserted into the top of the tubes. This test ran for 2½ weeks.

Test 3, 3a, 3b

The purpose of this test was to determine the type of stimulus the female weevil needs to oviposit, either tactile or chemical. The control was a leafless alfalfa stem inside a parafilm tube with detached leaves in the top. The test condition was wooden applicator sticks inside the tubes. The sticks were 15.2 cm by 2 mm. Detached leaves were also inserted into the top of the tube. The test 3 was run for 2 weeks. Tests 3a and 3b were run later and carried out for 10 days.

Test 3c

The purpose of this test was to remove any possibility that the female may orient to the test condition due to the green leaves at the top. The control condition was Kansas Common alfalfa inside a parafilm tube. The test was a stick inside the

parafilm tubes. Only two stems were used per vial. The parafilm tubes were pinched shut at the top and the leaves were placed on the bottom of the jar midway between the two vials. The weevils used were randomly chosen from test 3a and 3b. The test was carried out for 1 week.

Test 4

It is often a lack of deterrents rather than the presence of a stimulus that causes the female to oviposit. This test was setup to examine the possibility of ovipositional repellents in red clover.

The control was two leafless Kanza alfalfa stems inside a parafilm tube with detached leaves at the top. The test vial was two red clover stems within a parafilm tube with their leaves removed. Alfalfa leaves were inserted in the top. The test was carried out for 10 days.

Test 4a

It was decided to repeat test 4 only using Kansas Common alfalfa instead of Kanza and also placing the leaves at the bottom of the jar. The weevils used were from test 4. This test was carried out for 1 week.

Test 5

This experiment was designed to test for the presence of a repellent in red clover. The control consisted of alfalfa that was dipped in alfalfa juice. The juice was obtained by grinding the leaves of Kanza alfalfa in a hand operated meat grinder and squeezing the ground up material through a cheesecloth to obtain the juice. The test vial consisted of alfalfa that was dipped in red clover juice. The red clover juice was obtained in the same

manner as the alfalfa juice. The replicates for this test were placed in a separate growth chamber from the previous tests, and kept in the dark for the full 24 hours every day. The test was carried out for 4 days.

Test 5a

The setup was the same as for test 5 with the exception that the plants used in the setup were rubbed with the ground up material rather than dipping them in just the juice of the plant. The plants were allowed to dry overnight before being used in the experiment. The weevils used were from test 5. The test was carried out for 6 days.

Test 5b

The conditions were the same as for test 5 and 5a but the alfalfa used was Kansas Common. It was thought that this variety might be more attractive to the weevils than the Kanza variety so it was decided to use it in the same setup. This test was carried out for 5 days.

Test 6

The purpose of this test was to compare the results between the weevils response to alfalfa and to the red clover plants. The control was two Kanza stems with the top leaves left on. The test consisted of two red clover stems with their leaves attached. The red clover plants have three large leaves at the apex and these were left on the stem. The test was carried out for 10 days.

Test 7

The purpose of this test was to look at the weevils' response to red clover when not in the presence of an alfalfa plant.

This test was designed as a no choice test with the only vial in each replicate containing two red clover stems with their leaves intact. The test was carried out for 10 days.

Test 8

Since test 7 involved a no choice test for the weevil, a control was still necessary. Therefore this test was designed to provide the control for test 7. This was also a no choice test with the only vial containing two Kanza stems with their top two leaf groups attached. This test was carried out for 10 days.

Test 9

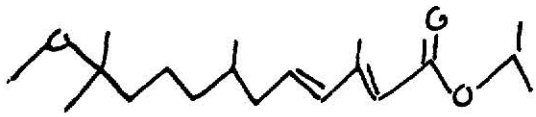
The purpose of this test was to determine if the weevils preferred one variety of alfalfa over the other. The two looked at were the two used in the previous tests, Kansas Common and Kanza. One vial contained Kanza alfalfa and the other vial Kansas Common. The weevils used were from test 8. The test was carried out for 3 days.

Hidden method test

The purpose of this test was to look for the presence of an olfactory stimulus in alfalfa that caused the female weevil to lay her eggs. This bioassay setup was similar to that described by Neff and Vanderzant.[24] Small petrie dishes, 60 by 15 mm., were used for this test. The bottoms and tops were used separately but not in the same replicate. A circle of filter paper was cut to fit the dishes. The control consisted of 0.5 ml of water added to the paper, while the test dish contained either water and an alfalfa leaf, or 0.5 ml of the steam distillate of alfalfa. The dishes were then covered and sealed with parafilm. A 3 inch

wooden stick was inserted in a parafilm tube and the top pinched shut. The tube was then pushed through the parafilm in the middle of the dish. A #3 insect pin was used to punch holes in the parafilm allowing the odors to escape. The dishes were then put in gallon jars and the jars were placed in a metal cabinet and the doors sealed with tape to prevent light leakage.

The weevils used were Fall collected and Spring collected. The weevils used were (1)untreated, (2)treated with Altozar, and (3)treated with Altosid. The Altosid is another IGR from Zoecon. The chemical name for Altosid is Isopropyl (2E,4E)-11-methoxy-3,7,11-trimethyl-2,4-dodecadienoate, and the formula is $C_{19}H_{34}O_3$. The structure of the compound is:



Olfactometer test

A Y-tube olfactometer was employed to test for any olfactory response from the weevil. The olfactometer was placed in a cardboard box that was lined with white styrofoam sheets. An air pump, run by a variac unit, provided the source of air. The air flow split to two air flow meters. Each of these then led to an Erlenmeyer flask, one the control and the other containing fresh alfalfa. Water was added to both of the flasks to avoid any moisture preference. The tubing from the flasks to the olfactometer entered the box through the two holes punched in the end of the box. The end of the olfactometer, where the two arms joined and formed a large chamber, was sealed with a rubber stopper with an air vent to allow the air currents to leave at this point.

Both this stopper, and the arms of the tube connected to the tubing from the flasks, were sealed with a wire mesh screen to allow the air to pass but not the weevils. About 1½-3 grams of fresh greenhouse alfalfa was used in the test flask.

The olfactometer was divided into sections for data purposes, though not actually marked on the olfactometer. The weevils were placed in the large end and the stopper inserted at the start of the experiment. Readings were taken every two minutes and the number of weevils in each section recorded. Three or four weevils were used for each test. The test was repeated several times varying the side that the alfalfa was placed, and the sex of the weevils. The test was run totally in the light and also with a lid on the box that was raised only long enough to take the readings. The flow rate was varied throughout all the tests, starting low and increasing every 10-12 minutes.

The day before the tests the weevils to be used were placed in petrie dishes with moist filter paper but no food. The weevils used were Fall collected weevils.

Results

For the first series of bioassays, tests 1-9, data were taken by counting punctures, egg masses and the number of eggs. Punctures were recorded only for the parafilm tubes as it is very difficult to determine punctures on a plain stem.

For comparing results, only the punctures and the total number of eggs are necessary. Both of these were totaled for the entire run of a particular test and the average calculated per replicate. Whenever a plain stem was used a 0 is recorded in the data table to indicate no puncture counts were made.

Test 1

The results of this test are presented in Table 2:

TABLE 2

Alfalfa versus Alfalfa within Parafilm Tubes

	Punctures	Av/rep	Eggs	Av/rep
Test	541	135	394	98.5
Control	0	0	1394	348.5

The female weevil greatly preferred the plain alfalfa stem over the stem inside the tube. This differs from the results by Hower and Ferrer[15], and also from preliminary tests that were conducted where the results indicated the parafilm was preferred. However, the parafilm is suitable for ovipositioning, and the results do yield the level of egg laying that the female is capable of at this time. The use of the parafilm is preferred in order to obtain puncture counts and to expedite the removal of any eggs. Use of the parafilm in other experiments, such as the termination of diapause experiment showed that it was very satisfactory as an ovipositional medium. Therefore it was decided to continue to use the parafilm for the tests.

Test 2

The results are presented in Table 3:

TABLE 3

Alfalfa versus Hollow Tubes

	Punctures	Av/rep	Eggs	Av/rep
Test	675	169	268	67
Control	947	237	1238	309.5

The results of this test are in good agreement with Hower and Ferrer;[15] the hollow tube is not preferred as an oviposition site. The number of punctures is about 2/3 that in the control, so it is apparent that the female weevil did explore the test

conditions, but the number of eggs is only 1/5 that of the control. It is possible that there is need for a tactile stimulation, from the pith inside the stem, or there may be a chemoreceptor on the female's ovipositor. Either or both of these factors could be involved, but can not be distinguished by this test.

In comparing the total number of eggs laid in this test with the results of test 1, it is obvious that the parafilm is very acceptable to the female for egg laying purposes.

Test 3, 3a, 3b

The results of test 3 were not conclusive so test 3a and 3b were also run, yielding a total of 12 replicates for this experiment. In the individual replicates, the preference varied between the test and the control. Within test 3 two replicates favored the test condition while the other two strongly favored the control condition. With 12 replicates, any discrepancies should be worked out and the overall picture should be a fairly accurate representation of the female's choice. The results of each test and the overall total are presented in Table 4.

The results of this test clearly indicate that it is a tactile stimulus that is important. Chemoreception on the ovipositor plays little if any part in the selection of the egg laying site.

Test 3c

The results of this test are presented in Table 5. The results of this test are inconclusive. The females have apparently neared the end of their egg supply. This test was setup to show if the choice of egg laying sites appeared to be by tactile reasons, or if other factors came into play, such as visual.

TABLE 4

Test for tactile stimulus

	Punctures	Av/rep	Eggs	Av/rep
#3				
Test	301	75	1375	344
Control	640	160	819	205
#3a				
Test	520	130	1248	312
Control	604	151	867	217
#3b				
Test	442	110.5	1249	312
Control	462	115.5	395	99
TOTAL				
Test	1263	105	3872	323
Control	1706	142	2081	173

TABLE 5

Removal of visual aids

	Punctures	Av/rep	Eggs	Av/rep
Test	29	7	33	8
Control	59	15	42	11

Thus the removal of the leaves at the top of the tubes removed any visual cue as far as the green alfalfa was concerned.

Test 4

The results of this test are presented in Table 6.

TABLE 6

Test for ovipositional repellents

	Punctures	Av/rep	Eggs	Av/rep
Test	200	50	99	25
Control	328	82	170	42.5

The weevils in this test and the following tests were treated with the Altozar at the same time as those in the earlier tests, but the time span from treatment to their use in the tests was much greater for these last tests. The number of eggs is down in all the tests from this point on. However tentative results can be made since even with a lower number of eggs laid, often a distinct preference will still show up.

It appears in this test that the alfalfa is preferred over the red clover stem within the parafilm tube. The red clover stems have little hairs along the stems that the alfalfa plants do not. It may be the tactile difference that the female notices and thus not lay as many eggs. Whether it is actually a chemical repellent cannot be shown by this test, especially since a fair number of eggs were laid in the test stems.

Test 4a

The same weevils were used as in test 4. These weevils had apparently ceased egg production and no results were obtained for this experiment.

Test 5, 5a, 5b

The results of these tests and the total are presented in Table 7.

TABLE 7

Test for a repellent in red clover juice

	Eggs	Av/rep
#5		
Test	126	31.5
Control	77	19
#5a		
Test	51	13
Control	116	29
#5b		
Test	29	7
Control	65	64.5
TOTAL		
Test	206	51.5
Control	258	64.5

It appears from these results that the red clover did not exert a repellent effect. This differs with the results in the literature. With low levels of egg laying though, the presence of a repellent may not be as evident as it would be at normal levels of ovipositioning.

This set of bioassays was conducted in a growth chamber that was kept in a dark period of 24 hours. It is possible that this could have affected the ovipositional response since literature reviews have stated that an alternating photoperiod is important for the weevils.[26]

Test 6

The results are presented in Table 8.

TABLE 8

Response to red clover versus alfalfa

	Eggs	Av/rep	
Test	379	95	(Red clover)
Control	435	109	(Alfalfa)

There is some preference for the alfalfa but not significantly so. It may be that the presence of the alfalfa caused the female to lay in the red clover. It appears from these results that the red clover did not exert a repellent effect.

Test 7 and Test 8

The results of these two tests are presented in Table 9.

TABLE 9

Response to "no-choice" setups

	Eggs	Av/rep	
Test 7	278	69.5	(Red clover)
Test 8	207	52	(Alfalfa)

The results of this test seem to indicate that the red clover is acceptable as a host plant. This is not in agreement with the literature findings. It is possible that other factors may be involved that are not accounted for. In looking at the individual replicates of the two tests indicates part of the problem. These results are presented in Table 10.

TABLE 10

Individual replicates of test 7 & 8

	Rep.1	Rep. 2	Rep. 3	Rep. 4
Red clover	78	67	25	108
Alfalfa	19	15	103	70

It can be seen from these results that the alfalfa had two very low replicate results. These two, number 1 and 2, had eggs on the first day only and produced no eggs after that. The female weevils are no longer producing eggs at the level necessary for study. Whether the red clover exerts a repellent effect or not would be better able to tell if the egg levels were at that level that was produced in the earlier tests, for example test 1. The female weevil may lay some eggs in red clover, but at a normal level the small number would be more conclusive of the fact that the red clover was not preferred. With the sharp drop in response on the alfalfa test, comparing the results of the two does not accurately reflect the weevil's response.

Test 9

It was thought that the response of the weevils in the other tests might also depend on the variety of alfalfa that was used. This test was setup up to determine any preference; however, the ovipositional level had dropped so low that no results were obtained. These last few tests, with the low egg levels, were run several weeks past the time of the treatment of the weevils with the Altozar. Based on the results of the weevils in the termination of diapause experiment, they should have still been laying eggs. The weevils were treated at a later stage of their

life and it is possible that they responded faster, or the effect did not last as long.

The male and female weevils were kept separated after the treatment with the Altozar until used in a test. It was discovered that some weevils had been incorrectly sexed and some males were found in the female jar. These males may have fertilized the females and the females may have laid their eggs in the "waiting" jar. Thus when the females were used in the tests, their egg production was down.

Hidden Method Test

No results were obtained for any of the setups of this experiment. The age of the weevil is very critical for any test. For these tests, the weevils were nearing the end of their life cycle and may have been unresponsive to any stimulus. The tests were run in the dark, to avoid any visual cues, but this may have affected their egg laying as other researchers have indicated that the level of ovipositioning drops with a non-alternating photoperiod.[26]

It was thought that treatment with the Altozar would stimulate the weevils but the IGR produced no response. Since IGR's deteriorate rapidly in the air and the sunlight, and since the sample had been out of the freezer several times, it was decided that the IGR may have lost its activity. Altosid was then tried since this is rated as being a more potent IGR than the Altozar. This IGR had an extremely high mortality rate at the 100 ug/ul dose level. The experiment was repeated with a dose level of

53 ug/ul. The mortality rate was still high, but not as bad. Some of the females started to produce eggs, but when placed in the setups no further eggs were laid.

The full strength of the distillate was used in these experiments. From later studies on the distillate, it was found that the concentration is extremely important. The full strength of the distillate was found to be too concentrated in the later studies, thus it could have been exerting a 'repellent' effect on the weevils since an attractant at too high a concentration will often deter rather than attract.

Hower and Ferrer[15] had studies similar to Test 1 and 2 presented here. It was decided to check further for the presence of a tactile stimulus, and the wooden applicator stick was used. The results of Test 3 were so positively in favor of this stick, that when the "hidden method" test was used, based on Neff and Vanderzant's[24] work, the stick inside the parafilm was added to their setup. It is obvious that there is a physical or tactile component that is very important in stimulating an ovipositional response. It is possible that without the proper tactile stimulus, the female will not lay at optimal rates.

The use of juices of alfalfa and red clover was suggested by Byrne.[26] It was decided to adapt this and instead of filtering, to rub the ground up material over the plant. This was done since the supply of red clover was limited and less was needed for this method.

The red clover was expected to exert a strong repellent effect on the alfalfa weevil based on literature studies.[26] This was not

shown by these tests. Even when offered red clover alone, as in Test 7, no repellent effect was observed.

Due to the low levels of egg laying, the presence of a repellent may not be obvious. It is possible that an unaccounted for variable was affecting the weevils, but none could be determined. Work is currently being done to investigate further the possible repellency of red clover.

Olfactometer test

This type of setup did not appear to work for the weevils as very little response was obtained. For the most part they remained fairly inactive during the test. No significant number of weevils explored the arm of the Y-tube that had the air flow plus the odor of alfalfa. Literature studies have agreed that the results of olfactometer tests are often very poor.

The way that this was set up, the weevils had to orient along a horizontal axis to follow the air streams. The weevils have a strong negative geotaxy response, as reported by Byrne and Steinhauer[20], and this was also observed for this test. The weevils would crawl to the top of the Y-tube in the chamber where they were put in at the start of the test and remained there for the duration of the test. Few weevils explored further.

It was decided that this was not a satisfactory method for examining the response of the weevils to olfactory stimulants. Though weevils are observed to be very active normally, this type of setup may have confined their normal movement as very little activity was observed when they were placed in the Y-tube.

PART III Preparation of alfalfa

Byrne and Steinhauer[20] had discovered a bioassay that attracted the weevils apparently by olfaction. It was decided to use their system with the exception that the solutions used were dilutions of just the steam distillate of alfalfa and not from the ether extractions of the distillate as they had done. Based on the results of the bioassays, the identification process was then carried out. For the bioassays and the anaylsis of the compounds, the alfalfa used had been collected in the Fall of 1976 and frozen. The frozen alfalfa was then steam distilled. The procedures for these processes are presented below.

Experimental

Storage of alfalfa

Large quantities of alfalfa were picked, keeping plants from the same field together. The two varieties chosen were Kansas Common, field grown on McCall's road, and Kanza. This variety is grown by the agronomy department on Browning Ave. The two varieties were kept separated. The leaves were stripped from the plants and ground in a hand operated meat grinder.

Liquid nitrogen was added to a porcelein mortar and small quantities of the ground alfalfa were added and ground into the liquid nitrogen with the pestle. Enough liquid nitrogen was used to completely cover the alfalfa while it was being frozen. The frozen alfalfa was then placed in a large glass storage bottle, either the 8 oz or the 16 oz wide mouth glass stoppered type.

When the bottle was nearly full of the frozen alfalfa, liquid nitrogen was poured in on top of the alfalfa. The lid was then placed on the bottle and as soon as the excess nitrogen gas had escaped and the pressure equalized, the lid was put securely in place and covered with foil. The bottle was then placed in an ice bath until transfer to the storage locker. The locker used was at the Manhattan Ice and Storage Co. on Yuma Ave. The temperature in the locker room was 1^{o}-10^{o}F

Steam distillate of alfalfa

A simple distillation setup was used to steam distill the alfalfa. A 500 ml 3-necked flask was used for the distilling pot, with one neck closed off with a stopper. The middle neck was the inlet for the steam, and the third led to the condenser. A measured amount of the frozen alfalfa was added to the pot, usually 50-75 grams, and 100 ml of deionized water was added. The tube carrying the steam from the steam lines in the lab, was connected to a glass rod that came through the rubber stopper used to seal the middle neck of the flask, and extended into the flask and under the alfalfa mixture. The rod was not allowed to touch the bottom of the flask. The steam thus came up through the mixture causing agitation and making stirring unnecessary. A simple takeoff head was used leading to the condensor. All of the compounds were desired so no attempt was made to collect various fractions. The collection flask was kept in an ice bath to prevent any volatile components from escaping.

The distillation was carried out for about 45 minutes each time, collecting from 30-50 ml as needed.

Results

Storage of alfalfa

Greenhouse plants often have a different proportion of chemicals than the field-grown varieties. Field-grown plants are unavailable during the winter; thus freezing of the field plants provided fresh field samples all winter.

The reason for the liquid nitrogen is to prevent any bacteria or aerobic molds from growing. By grinding the alfalfa, all parts of the leaves would be frozen simultaneously. The two varieties frozen were kept separate in order that any difference between them could be investigated.

Steam distillation of alfalfa

The longer the time used for the distillation and the less collected, yields a higher concentration of the compounds to be studied. The first 20 ml generally contained all the compounds and the concentration of them started decreasing afterwards. Thus when only small amounts were needed, it was decided to collect only a small amount of the distillate to obtain a high concentration for study.

Background runs were made at all times. The steam line was checked as well as the deionized water. Morpholine is added to the water lines to help prevent corrosion. The presence of morpholine or any other impurity was not observed. It was decided to use the steam as it came from the lines without any further purification.

Both the Kanza and the Kansas Common varieties were used. The same compounds and in the same proportions were found.

Part IV

Weevils' Response to the Steam Distillate

The following bioassay setup was based on the work by Byrne and Steinhauer[20]. Preliminary test were run to determine the effectiveness of the setup.From the results it was decided to use the steam distillate of alfalfa without the ether extractions as Byrne and Steinhauer had done.[20]

Experimental

Large plastic petrie dishes, 100mm by 15 mm, were used for this test. A large hole was punched out of the top by using a metal lid screwed onto a thick wooden stick. The lid was heated in a flame and then pushed through the top of the petrie dish. Wire mesh screens were cut and placed over the holes. At first, they were taped down, but this required re-taping frequently. A plastic glue was dissolved in $CHCl_3$ and used to seal the mesh screens to the dish. On the underside of the lid, strips of parafilm were used to seal any possible gaps to prevent the weevils from escaping. The setup is shown in Figure (5).

Filter paper was cut in $\frac{1}{2}$ inch diameter circles and one piece placed in the center of each dish. Using an eye dropper two drops of the solution to be tested were placed on the paper. All 5 replicates for one test solution were done before using another test solution. Three replicates were run together and then two more were carried out. Thus two setups were needed to do all five replicates. While one set of tests were being run, the weevils for the next setup were placed in a separate dish containing filter paper moistened with several drops of water. This allowed the weevils access to water for about 12 minutes before being used.

FIGURE (5)

Experimental Setup Used to Test the Olfactory Response of the Alfalfa Weevil to the Steam Distillate of Alfalfa

After 12 minutes, the weevils were taken off the water and put in plastic vials in groups of 10 per vial. The dishes to be used in the test were prepared with the filter paper and the solution on each paper. The weevils from one vial were placed in one of the dishes near the edge, the lid put on, and the timer started. A stopwatch was used for a timer. The other vials of weevils were added to their dishes at 10 second intervals if three replicates were being run, of after 15 seconds if only two were run. Every 30 seconds for each dish, the number of weevils on or beside the filter paper was recorded. The tests ran for 10 minutes. By spacing the addition of the weevils to the dishes, the data readings were thus spread out between the replicates and facilitated the recording of the data.

The weevils used in the tests were unsexed adults. Those used in the test 6/13/77 were collected on June 6, 1977 and kept in the refrigerator. Two days prior to the test the weevils were given fresh alfalfa and kept at room temperature. The day prior to the test, they were removed from the alfalfa and placed in a dry, pint size ice cream carton. A piece of paper toweling was folded and placed in the carton.

The weevils for the test 6/15/77 were collected on June 6, 1977 and kept in the refrigerator. Two days prior to the test, the weevils were removed and placed in a dry, pint size ice cream carton. A paper towel was folded and added to the carton.

Kansas Common alfalfa was steam distilled and the distillate used in both of the tests. Dilutions were made by volume with the distillate being the 100% solution. The following solutions were used: 100%, 10%, 1%, 0.1%, 0.05%, 0.025% and 0.0125%. Water was used as the control. Five replicates were run on the average

unless the results indicated that more were needed. The counts were averaged for the number of replicates used for each test.

Results

From preliminary tests, it was discovered that the tests did not need to be carried out past 10 minutes. This agrees with the work by Byrne and Steinhauer.[20] The response of the weevils occurs in minutes.

The counts for each replicate at each reading were summed and then divided by the number of replicates to obtain an average. Thus if more replicates were used for one dilution than another, the averages should still present an accurate picture of the response. The results were then plotted with the average number of weevils versus time. The results can be seen in Figures (6) and (7).

Only the dilutions that had a positive response in relation to water, the control, are shown on the graphs. The concentration of the solution is extremely important. Too high a concentration will elicit no response as well as too weak a concentration. Even between these two tests, the dilution that was the most active differed. For the test 6/13/77, the 0.025% and the 0.05% solutions were the best, while for the test 6/15/77, the 1.0% solution had the highest response. The dilutions were made fresh for each test and the chance of error in measuring the solutions could well account for this difference.

FIGURE (6)

TEST 6/13/77

Plot of the Average Number of
Weevils Responding Versus Time

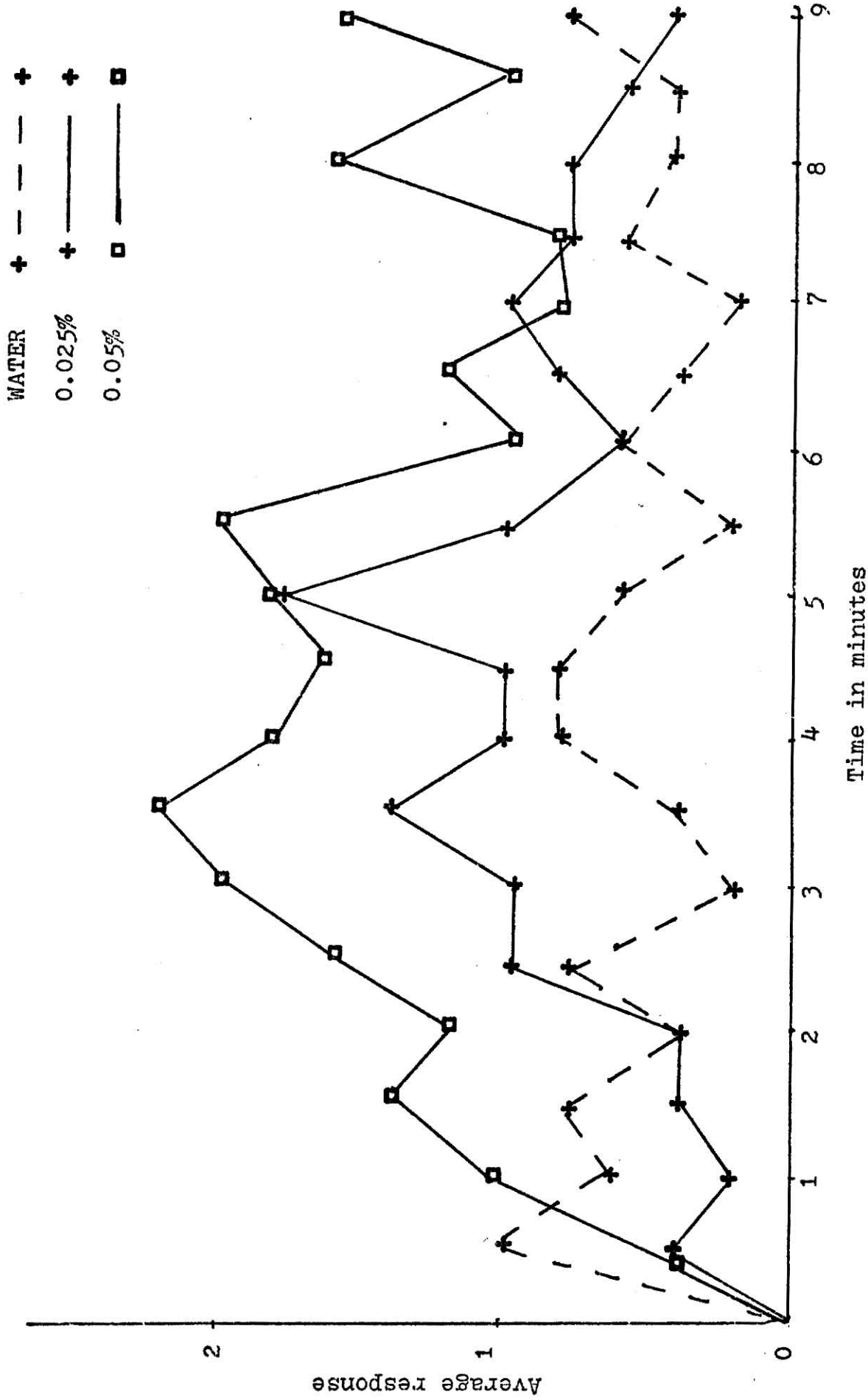
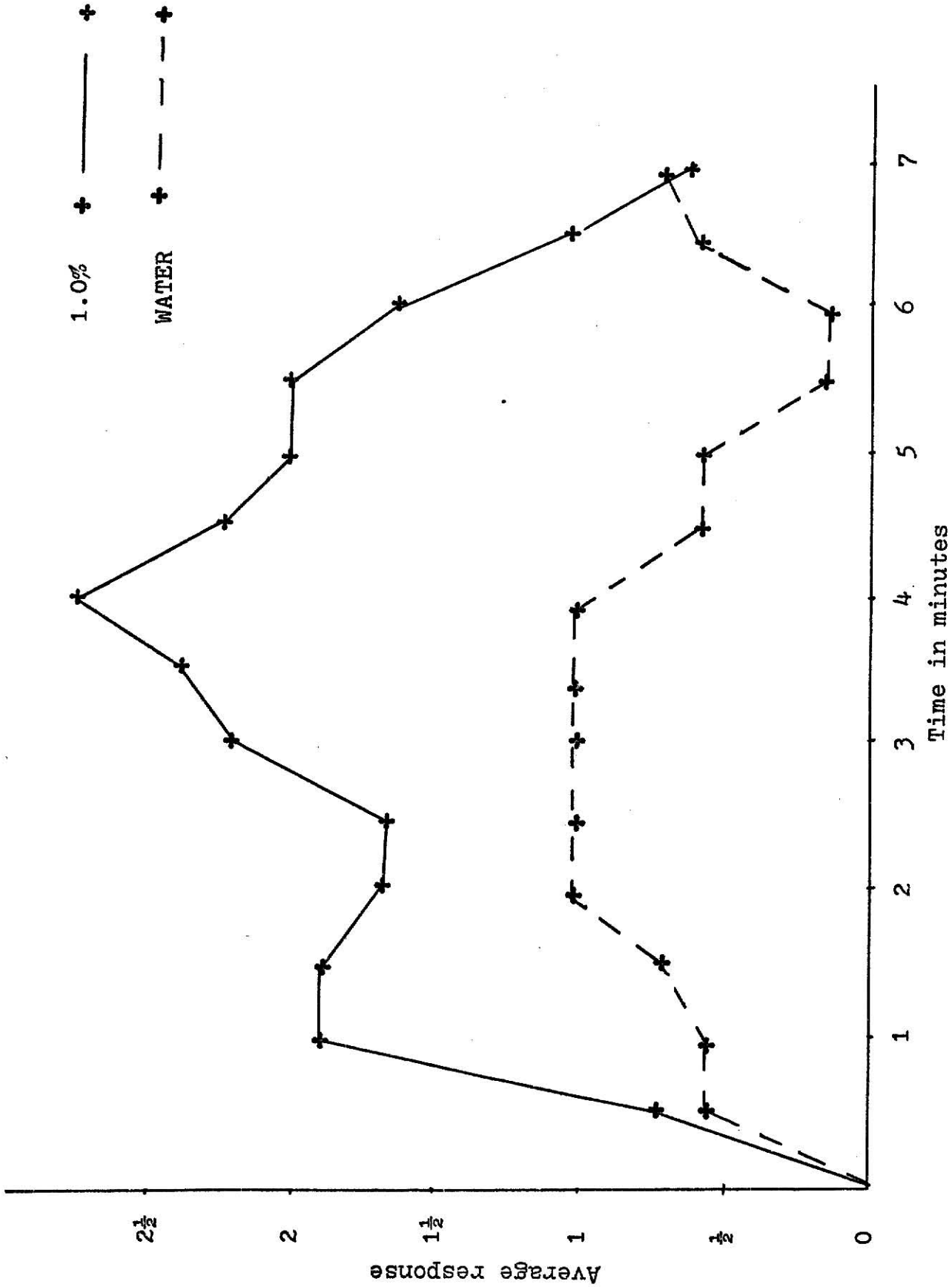

WATER
0.025%
0.05%
Average response
0
1
2
Time in minutes
1
2
3
4
5
6
7
8
9

FIGURE (7)

TEST 6/15/77

Plot of the Average Number of
Weevils Responding Versus Time


1.0%
WATER
Average response
0
½
1
1½
2
2½
1
2
3
4
5
6
7
Time in minutes

These tests indicate that there is an olfactory attractant in the alfalfa and that this attractant is present in the steam distillate of the alfalfa. It may be one compound or a combination of several compounds in the distillate that attracts the weevil. These can easily be checked once the compounds are identified, however. With the weevils unsexed it is unknown if this is a general attractant for both sexes, or specific for one. Work is currently being done to answer this question.

PART V

Identification of Compounds in the Steam Distillate

Gas chromatography/Mass spectrometry

With the solvent being water, it was necessary to find a column packing for the GC work and later the Mass spec work, that would handle water well and if possible elute it early. The large quantity of water present was too much to have go through a Mass spec system. If the water would elute early, the system could be vented while it was eluting and then be turned on afterwards to receive the sample.

The column used for this work was a mixture of Porapaks Q and R in a 4:1 ratio. Porapak was chosen since this mixture had been used in the research group previously and was known to handle water well and elute it early.

Porapaks yield sharp symmetric peaks and exhibit short retention times for water, alcohols, and glycols. They have no

adsorption of polar compounds. The position of a water peak can be adjusted by using the various porapaks or mixing them together. Several were tried for this work, and the 4:1 ratio of the Q and R turned out to be the best.

Experimental

A 6 foot 1/8 inch diameter stainless steel tubing was cleaned and packed with the Porapak mixture and then coiled to fit the GC oven. The GC used was a Bendix model 2200. Both the FID and the TC detectors were used in the work. The TC detector was used to locate the water peak but was not sensitive to the other compounds. The FID was used for the compounds in the distillate.

The sample size of the steam distillate varied from 5 to 10 ul. Both the Kansas Common and Kanza were examined. Background runs included distilled water, deionized water, and a blank distillate. This was accomplished by running the distillation process through without the alfalfa present and collecting a few ml of water.

The Mass spec work was done at Midwest Research Institute in Kansas City, Missouri. The column used in the Bendix GC was taken to their labs and used in their GC/Mass spec instrument. The Mass Spectrometer used was a Varian Mat-CH 4 model.

To avoid allowing too much water to enter the Mass Spec, after injection of the sample, the system was vented for 5 minutes. Then the system was closed and the sample allowed to enter the Mass spec. Printouts of the gas chromatogram, the mass specs of the compounds and a listing of the peaks and their intensities were obtained.

Results

A typical gas chromatogram is presented in Figure (8). Though the FID is insensitive to water as far as detecting it as a compound, it did react to the large quantity of water that was injected. This response occurs early on the chromatogram and is not shown in Figure (8). Generally it appears as a broad peak.

Using the TC detector to obtain the water retention time, and the FID for the compounds, it could be determined if the water was being separated from the rest of the sample. By holding the sample at 80° for 4 minutes most of the water was found to elute before the first compound. Then the temperature program was turned on at 10°/minute. For the Mass spec work, the water was not as well separated as appeared, and interferred with the beginning compound in the distillate. Even after venting, when the system was closed, some water was still coming through.

The first two compounds were not completely resolved and their mass specs appeared to be mixtures. Identification of three of the other major peaks was carried out. These are peaks 3, 4, and 6 on the chromatogram in Figure (8). They have been identified as ethanol, propionaldehyde and N- propanol. These have been confirmed with GC retention times. The Mass spec patterns are presented in Figures (9), (10), and (11). The analysis of the patterns is presented in Tables (11), (12) and (13).

The steam distillate was then run with a known amount of sample size. This same sample size was then combined with a mixture of the three compounds and run on the GC. The distillate is shown in Figure (12) and the "spiked" sample in Figure (13).

FIGURE (8)

Gas Chromatogram of the Steam Distillate of Alfalfa

Conditions

Sample	Kanza distillate
Sample Size	5 ul
Helium Carrier gas flow rate	21 ml/min
Initial Temp.	60°
Final Temp.	200°
Program Rate	10°/min
Detector	FID
Attentuator	20

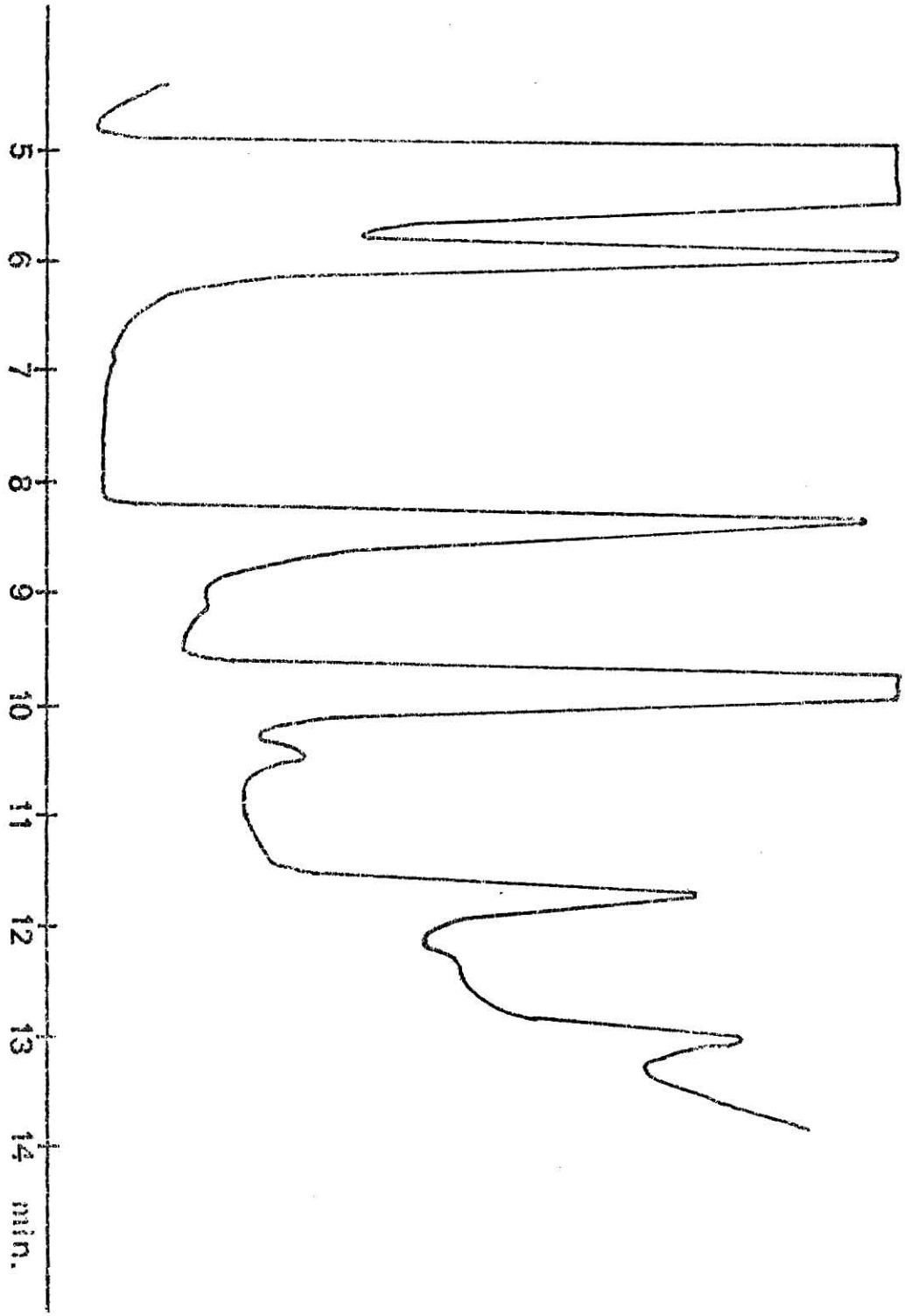

5
6
7
8
9
10
11
12
13
14
min.

FIGURE (9)

Mass Spectrum of Ethanol

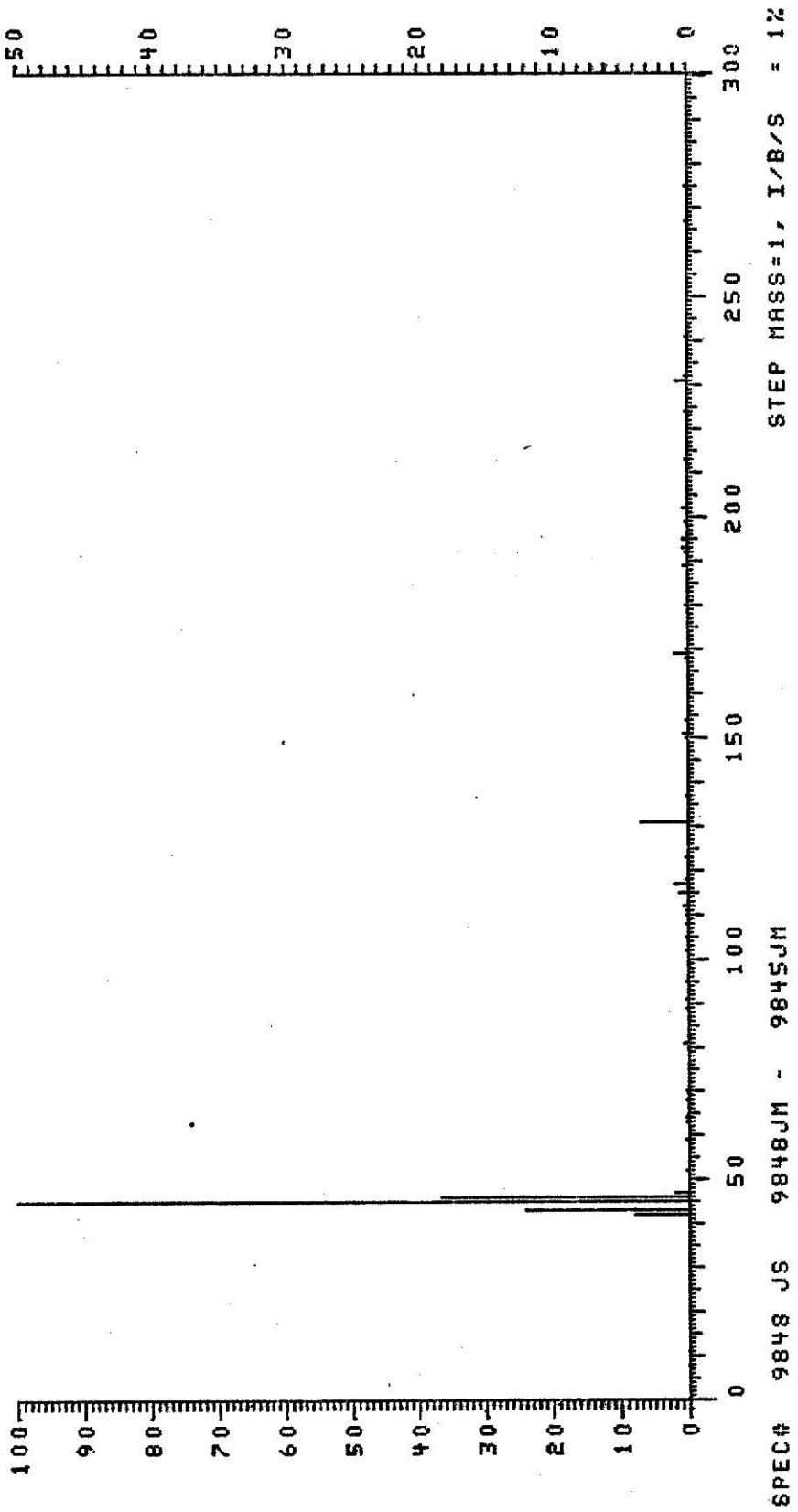

SPEC# 9848 JS 9848JM - 9845JM
STEP MASS=1, I/B/S = 12

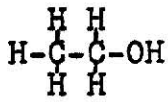
TABLE (11)

Mass Spec Analysis of Peak #3

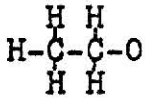
Mass	Intensity/base
45	100%
46	37%
43	24%

Proposed Structures

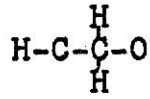
46



45



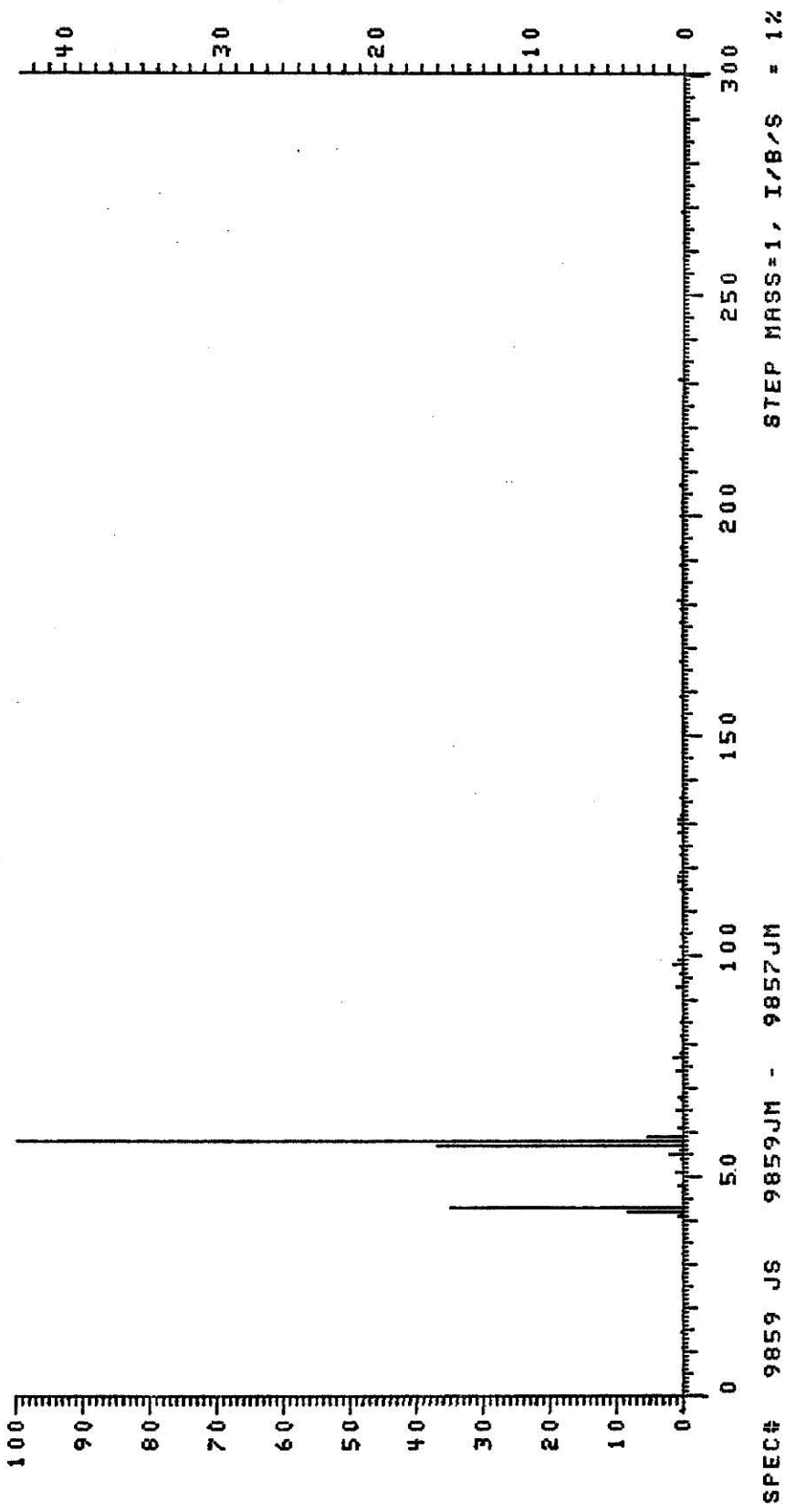
43



Chemical Name: Ethanol

FIGURE (10)

Mass Spectrum of Propionaldehyde


SPEC# 9859 JS 9859JM - 9857JM
STEP MASS=1, I/B/S = 1%

THIS BOOK CONTAINS NUMEROUS PAGES WITH MULTIPLE PENCIL AND/OR PEN MARKS THROUGHOUT THE TEXT.

THIS IS THE BEST IMAGE AVAILABLE.

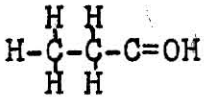
TABLE (12)

Mass Spec Analysis of Peak #4

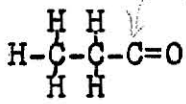
Mass	Intensity/base
58	100%
57	37%
43	35%

Proposed Structures

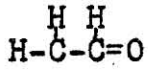
58



57



43



Chemical Name: Propionaldehyde

FIGURE (11)

Mass Spectrum of N-propanol

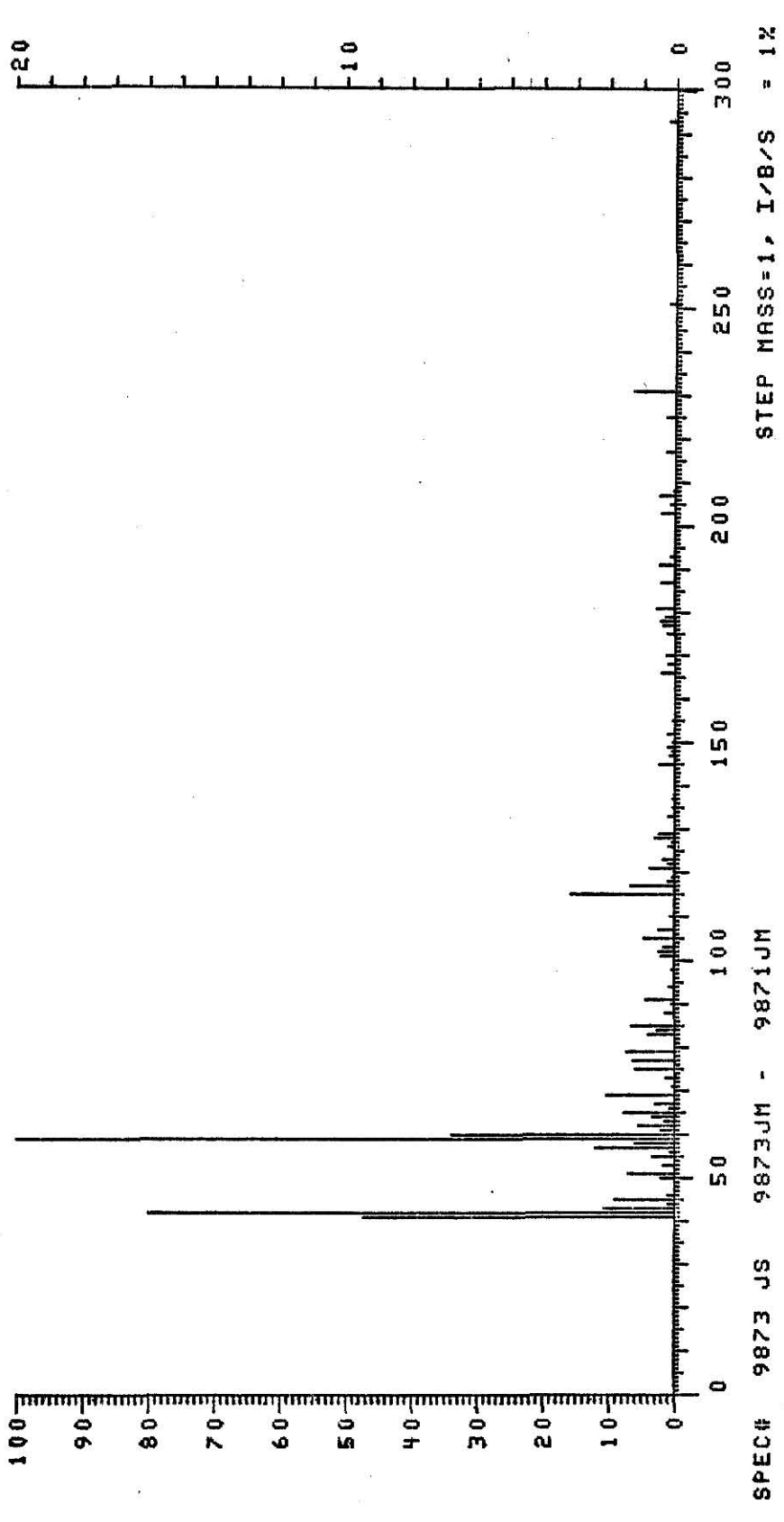

SPEC# 9873 JS 9873JM - 9871JM
STEP MASS=1, I/S = 1%

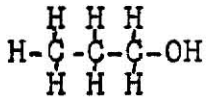
TABLE (13)

Mass Spec Analysis of Peak #6

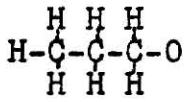
Mass	Intensity/base
59	100%
42	80%
41	48%
60	34%

Proposed Structures

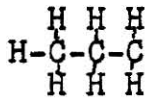
60



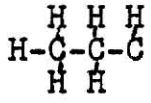
59



42



41



Chemical Name: N-Propyl Alcohol

FIGURE (12)

Gas Chromatogram of Three
Compounds of the Steam Distillate

Conditions

Sample	Kanza Distillate
Sample Size	6 ul
Helium Carrier Gas flow rate	25 ml/min
Initial Temp.	60°
Final Temp.	200°
Program Rate	10°/min
Detector	FID
Attenuator	50

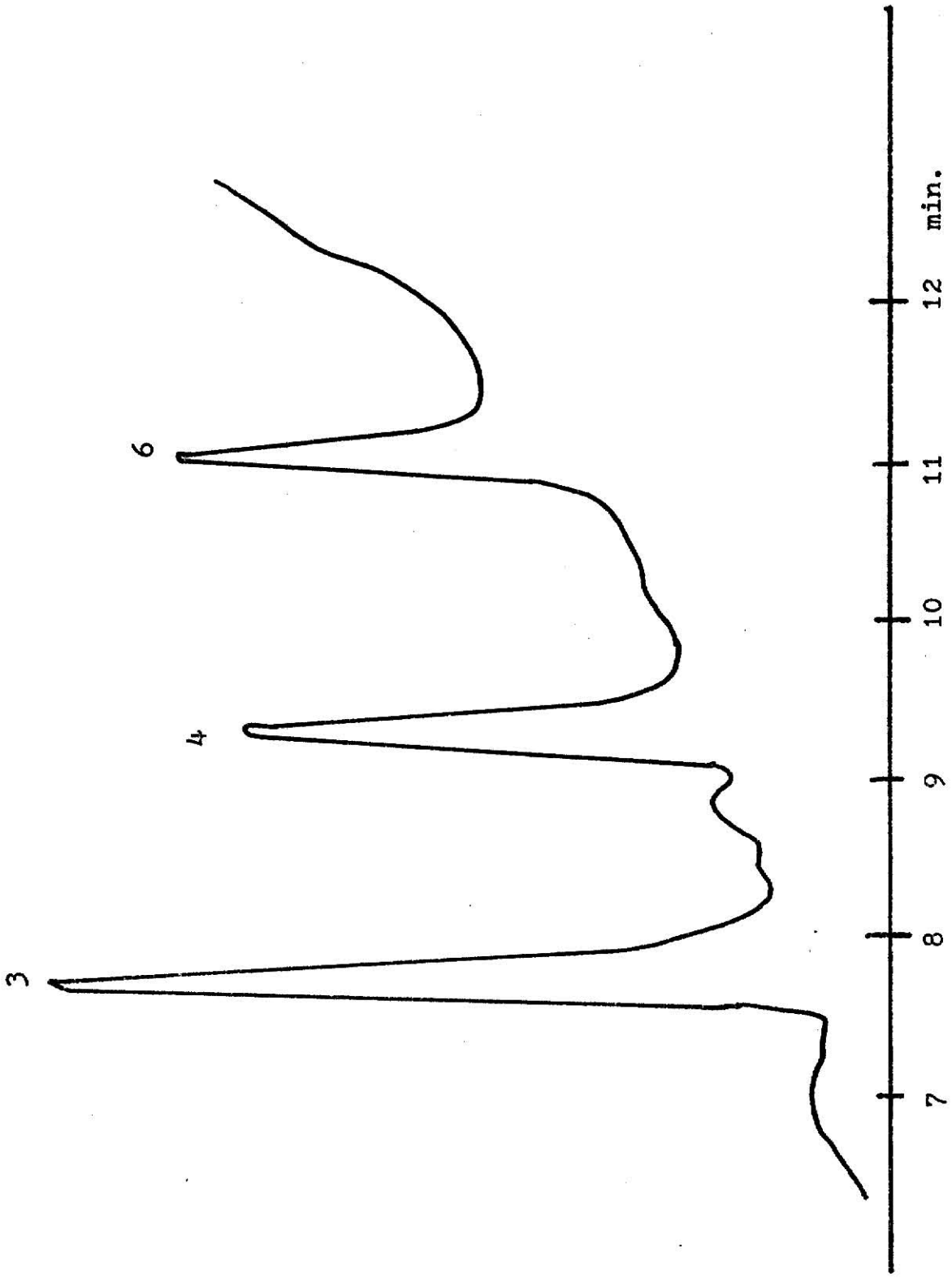
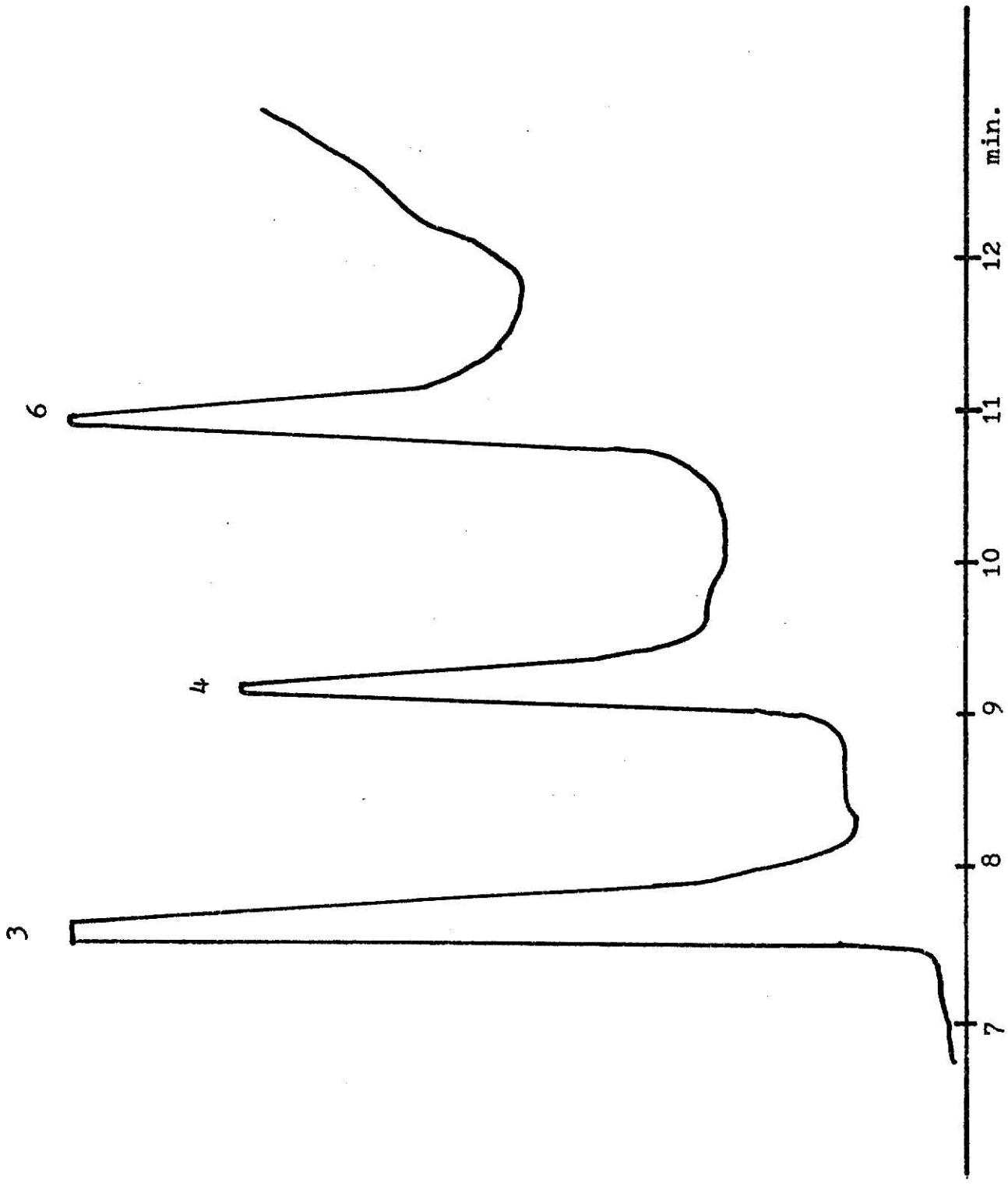

3
4
6
7
8
9
10
11
12
min.

FIGURE (13)

Gas Chromatogram of the Alfalfa
Steam Distillate Spiked with
a Mixture of the Three
Identified Compounds

Conditions

Sample	Kanza distillate plus the mixture
Sample size	6 ul distillate 2 ul mixture
Composition of Mixture in 100 ml water	16 ul of ethanol 3 ul propionaldehyde 5 ul n-propanol
Helium Carrier Gas Flow Rate	25 ml/min
Initial Temp.	60°
Final Temp.	200°
Program Rate	10°/min
Detector	FID
Attenuator	50


3
4
6
7
8
9
10
11
12
min.

An NMR was run on the propionaldehyde after trapping the compound as it came through the GC. Forty trappings were collected but the sample was still too small to show on the NMR.

PART VI

Weevils' Response to Compounds in the Steam Distillate

The ethanol and propionaldehyde were tried in bioassays that were set up similarly to the one that was used on the steam distillate. Water was again provided for the weevils for about 12 minutes before the tests. Instead of running all the replicates of one solution before changing concentration, it was decided to run one replicate of all solutions simultaneously. One replicate of the water control and one of each of five test solutions was set up.

The weevils were taken off the water supply and placed in six vials in groups of ten. The weevils in one vial were placed in the petri dish, the lid put in place, and the stopwatch started. At 5 sec intervals, the remaining 5 vials of weevils were added to the rest of the petri dishes, one vial per dish. The number of weevils on or by the filter paper was recorded every 30 sec for each dish. All solutions were made by volume dilutions of the 100% solution. The concentration for the 100% solution was calculated by matching the respective peak heights on the GC with a steam distillate sample. Thus the 100% solutions approximately matched the concentration of the same compound in the steam distillate as used for the 100% solution in the previous tests.

Test 7/6/77-Propionaldehyde

The weevils used for this test were those used in the previous tests 6/13/77 and 6/15/77. The weevils had been kept in

the refrigerator since these last tests. The weevils were removed from the refrigerator the day before the test and placed in a dry pint-size ice cream carton. They had been on alfalfa since the previous tests, so no preliminary feeding was necessary. A paper towel was folded and placed in the carton.

The 100% solution used for this test was 3.0 ul of propionaldehyde in 100 ml of water. The solutions tested were: 100%, 0.1%, 0.05%, 0.025%, 0.0125% and water.

Test 7/7/77-Ethanol

The weevils used for this test were those used in the previous test 6/15/77 and some from earlier unrecorded preliminary tests. They had been kept in the refrigerator since the last tests, and had been on alfalfa. The weevils were removed from the refrigerator and taken off the alfalfa the day before the test. They were placed in a dry pint-size ice cream carton containing a folded paper towel.

The 100% solution was 1.8 ul of 95% ethanol in 100 ml water. The various solutions tested were: 100%, 0.1%, 0.025%, 0.025% and water.

For the following three tests the preliminary treatment of the weevils was the same. Two days prior to the tests, the weevils were removed from the refrigerator and placed in dry pint-size ice cream cartons, one per each test group. Fresh alfalfa was added to the cartons. After 9 hours, the weevils were removed from the alfalfa and paper towels were added to the cartons.

Test 7/13/77-Propionaldehyde

The weevils used for this test were collected 6/2/77 and 6/9/77 and kept in the refrigerator until used. The 100% solution for this test was 3 ul of propionaldehyde in 100 ml water. The solutions tested were: 100%, 0.1%, 0.05%, 0.025%, 0.0125% and water.

Test 7/13/77-Ethanol

The weevils used in this test were collected on 6/9/77 and kept in the refrigerator until used. The 100% solution was 1.8 ul of 95% ethanol in 100 ml water. The solutions tested were: 100%, 0.1%, 0.025%, 0.0125% and water.

Test 7/13/77-Mixture

The weevils for this test were collected on 6/2/77 and kept in the refrigerator until used. The 100% solution was 1.8 ul of 95% ethanol and 3.0 ul propionaldehyde in 100 ml water. The solutions tested were: 1.0%, 0.1%, 0.05%, 0.025%, 0.0125% and water.

Results

In using tests of this nature, it is important to have accurate water control results. By running one replicate of each solution with the water control at the same time, any unusual responses from the weevils would be reflected throughout all solutions. Thus, if the weevils did not receive enough water in preparation for the test, they might have a high response to water when placed in the test dish. This would occur in all dishes however, since the weevils had been treated similarly beforehand. Any attractant effect should still show with this method. By spacing the addition

of weevils by 5 sec all later readings were thus spaced 5 sec apart and 6 setups were easily run at one time.

The tests that were conducted on 7/13/77 had lower responses than expected. It is possible that the weevils were in diapause at this time. The water response to the water offered before the tests was also down. Even with an overall lower response however, there were still response to test solutions that were greater than to the water control; therefore it was decided that these responses were still significant.

The results of the tests were treated in the following manner. The number of weevils at each time reading for all the replicates of a certain test were summed and then divided by the number of replicates to obtain an average response for that particular solution. This was then plotted versus time.

Statistical analysis was not performed on the results of the tests. It was decided that by graphing any attraction effect would be indicated. Further work that is being done with these tests will be analyzed statistically. Also the drops of solution used should be accurately measured to ensure same amount always is offered.

Test 7/6/77-Propionaldehyde

The results of this test are presented in Figure (14). The 0.025% solution had the best response. Usually the weevils' response occurs within the first 6 minutes. For this test the water control had an unusual sudden high response at the 6 minute mark. However, it was decided that there was still evidence of an attractant response based on the early part of the test.

Test 7/7/77-Ethanol

The results from this test are presented in Figure (15). The most attractive solutions were plotted, though the other solutions also indicated a response. The most attractive solutions appeared to be the 0.0125% and the 0.1%. It is obvious from the results of this test that ethanol elicits a positive olfactory response from the weevils.

Test 7/13/77-Propionaldehyde

The results of this test are presented in Figure (16) and (17). The solutions that exhibited the best response were the 0.025%, 0.05% and the 100% solution. Though the response was lower than expected, there is still an indication of preference for the solution over the water control.

Test 7/13/77-Ethanol

The results from this test are presented in Figure (18) and (19). The solutions that had the best response were the 0.05% and the 1.0% solutions.

Test 7/13/77-Mixture

The results of this test are presented in Figure (20) and (21). The solutions that showed the best response were the 0.05% and the 1.0% solutions.

Fresh solutions were prepared for each of the tests each time. With the volatility of the compounds and the chance of error in making such small dilutions, the variations in the concentrations that showed to be effective were not surprising. Concentration is very critical to the insect. Thus, variations can be expected,

especially since some of the solutions would sit longer than other ones before being used and the chance of any of the compounds escaping from solution would be greater.

It is obvious from these results that the two compounds ethanol and propionaldehyde, found in the steam distillate, are an olfactory stimulus for the weevil. The mixture of the two compounds also elicited a response which is expected if the compounds are active alone.

The compound N-propanol was identified after the tests on ethanol and propionaldehyde were conducted. With the weevils in the diapause stage it was decided to postpone any bioassays using this compound until a later date.

Since it is important to know when a weevil was used for a test, all tests were labeled with the date of the test. This gives an indication of the approximate age of the adults. It is necessary to always know when a test was done, so that the response can also be based on the time of the weevils' life cycle. When repeating a test, different results might be obtained from different times of the adults' lives; thus the date of all tests is very critical.

FIGURE (14)

Test 7/6/77-Propionaldehyde

The Alfalfa Weevil's Response
to Propionaldehyde and a
Water Control

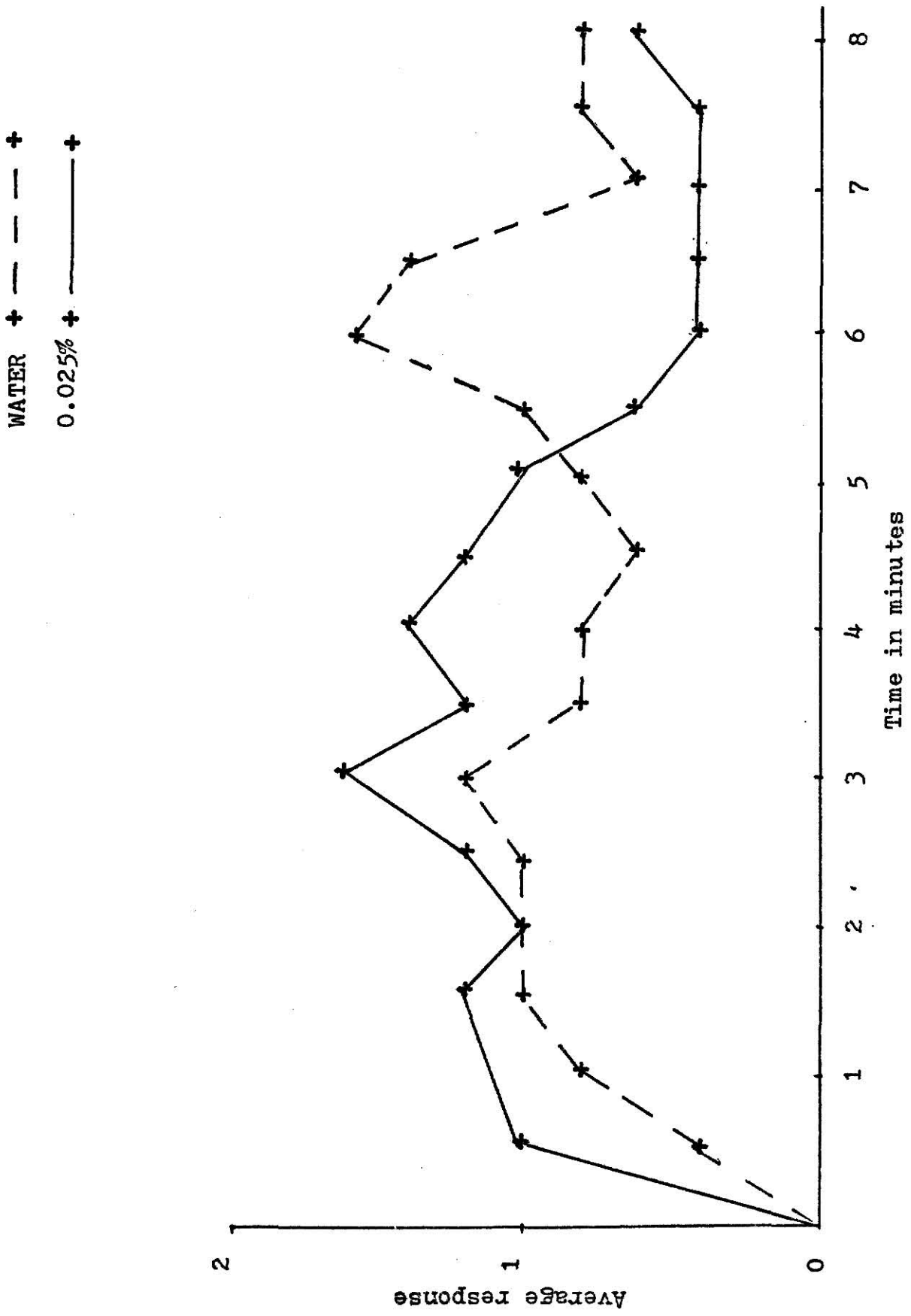

WATER
0.025%
Average response
0
1
2
Time in minutes
1
2
3
4
5
6
7
8

FIGURE (15)

Test 7/7/77-Ethanol

The Alfalfa Weevil's Response
to Ethanol and a
Water Control

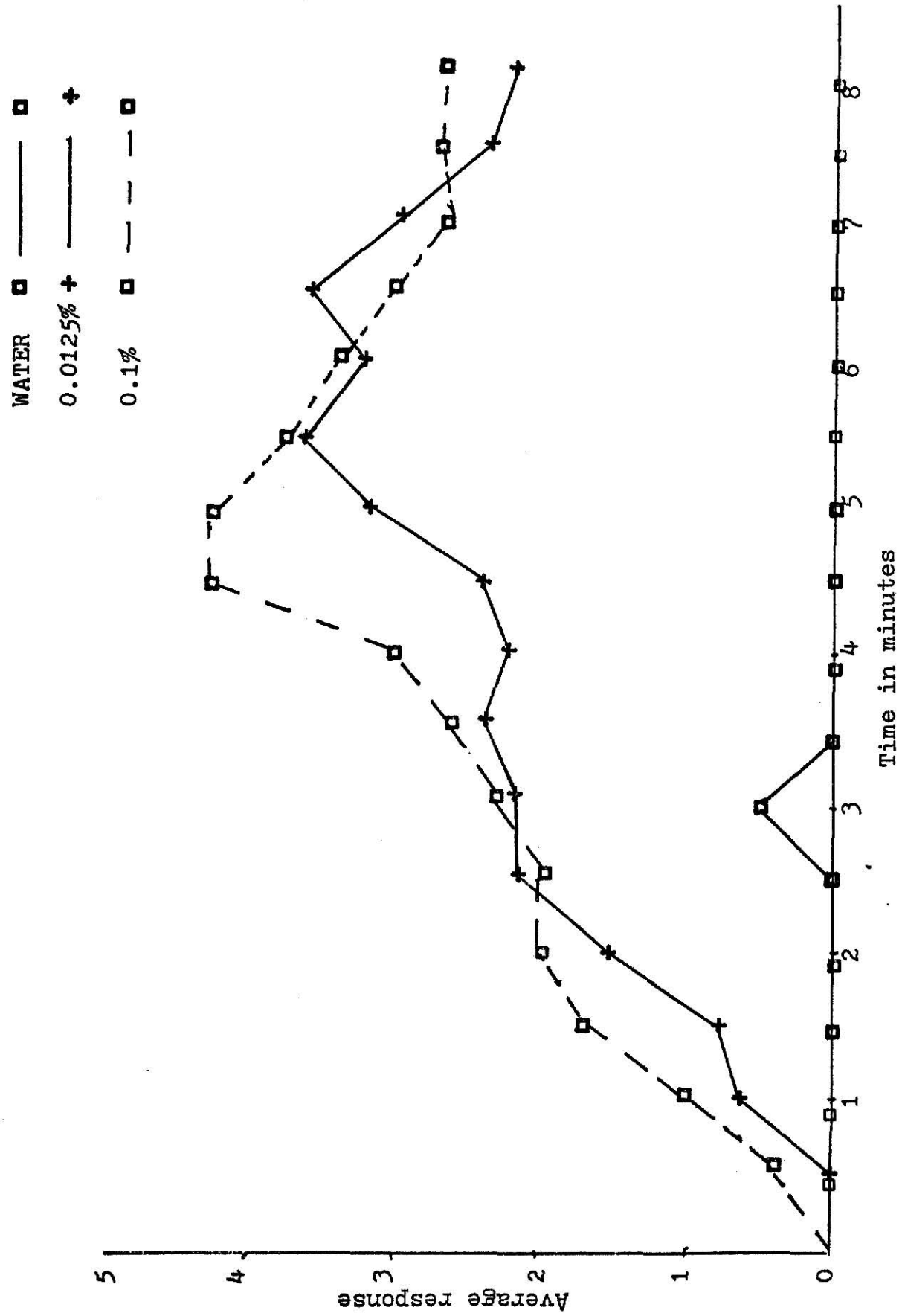

WATER
0.0125%
0.1%
Average response
Time in minutes
0
1
2
3
4
5
1
2
3
4
5
6
7
8

FIGURE (16)

Test 7/13/77-Propionaldehyde

The Alfalfa Weevil's Response
to Propionaldehyde and a
Water Control

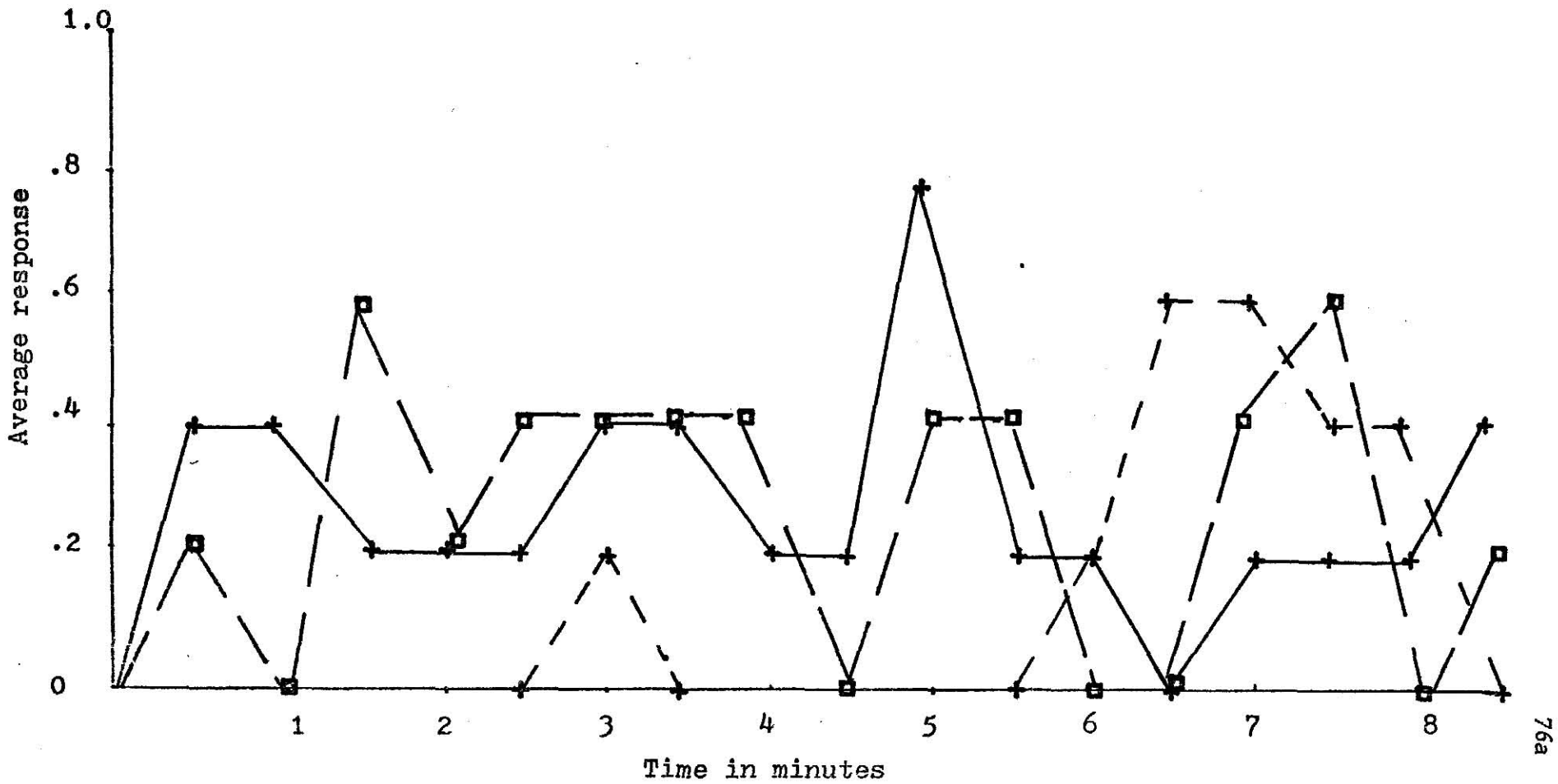

WATER
0.025%
0.05%
1.0
.8
.6
.4
.2
0
Average response
1
2
3
4
5
6
7
8
Time in minutes

FIGURE (17)

Test 7/13/77-Propionaldehyde

Part 2

The Alfalfa Weevil's Response

to Propionaldehyde and a

Water Control

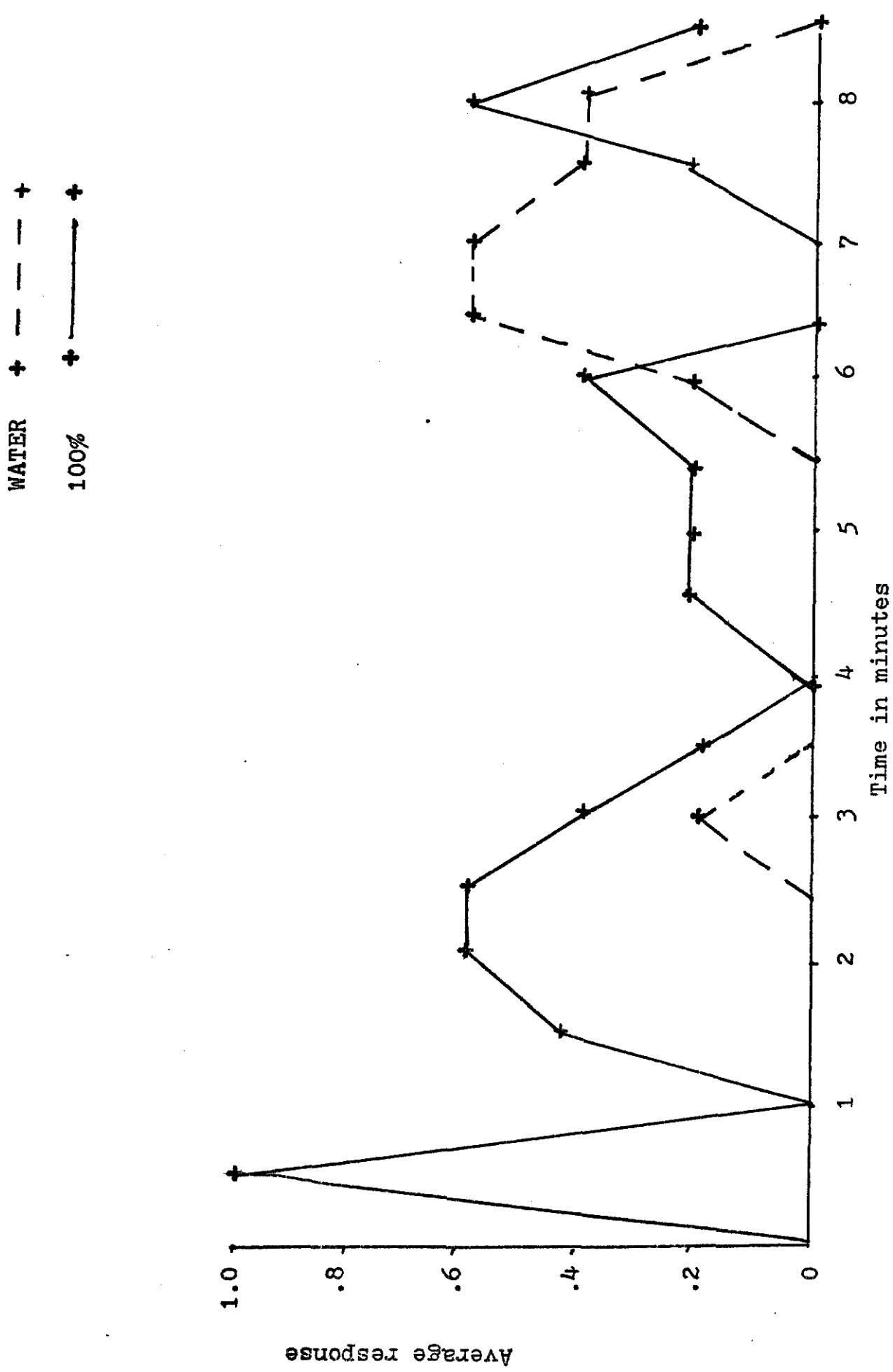

WATER
100%
Average response
1.0
.8
.6
.4
.2
0
1
2
3
4
5
6
7
8
Time in minutes

FIGURE (18)

TEST 7/13/77-Ethanol

The Alfalfa Weevil's Response
to Ethanol and a
Water Control

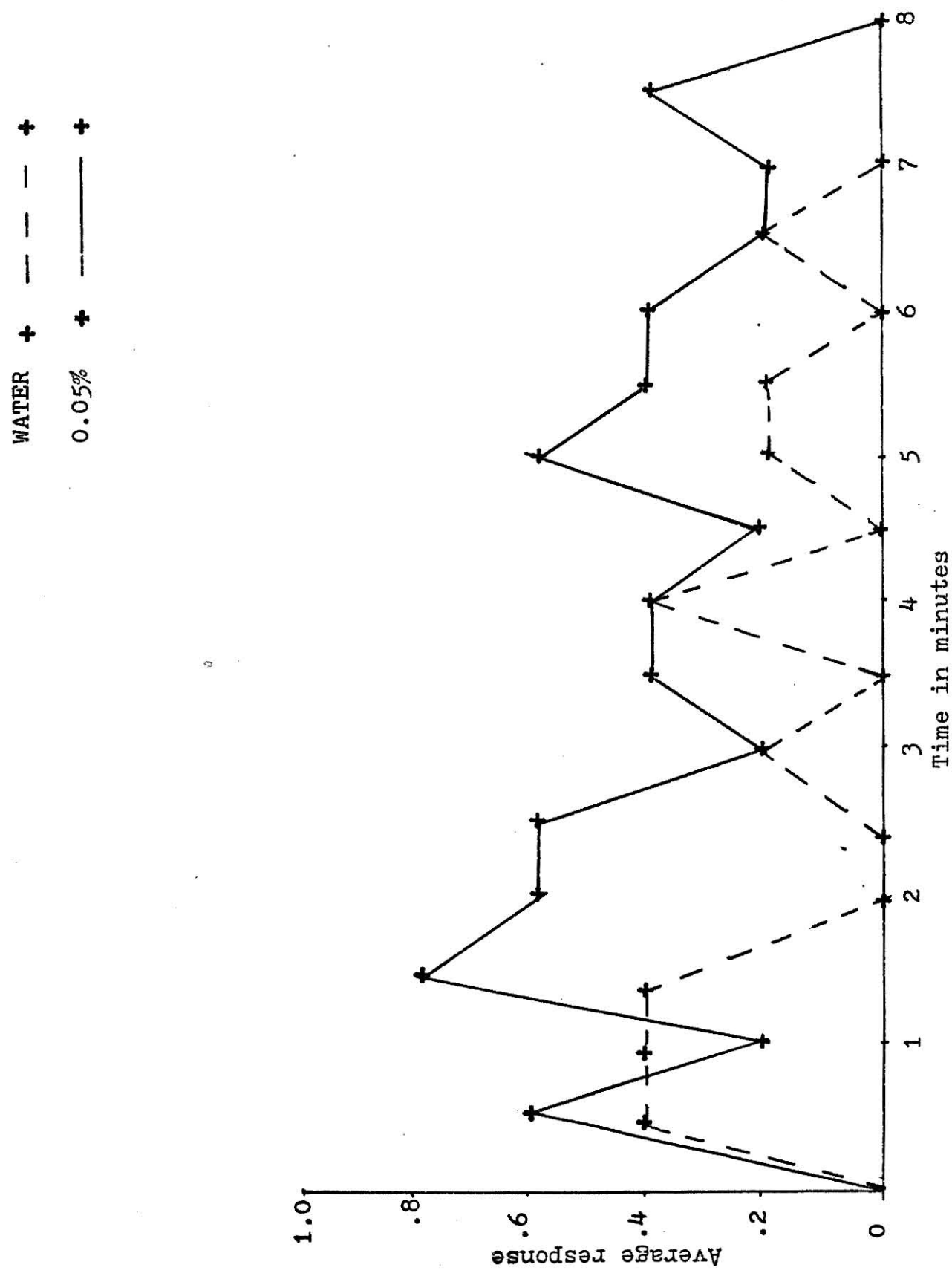

WATER
0.05%
Average response
1.0
.8
.6
.4
.2
0
1
2
3
4
5
6
7
8
Time in minutes

FIGURE (19)

TEST 7/13/77-Ethanol

Part 2

The Alfalfa Weevil's Response
to Ethanol and a
Water Control

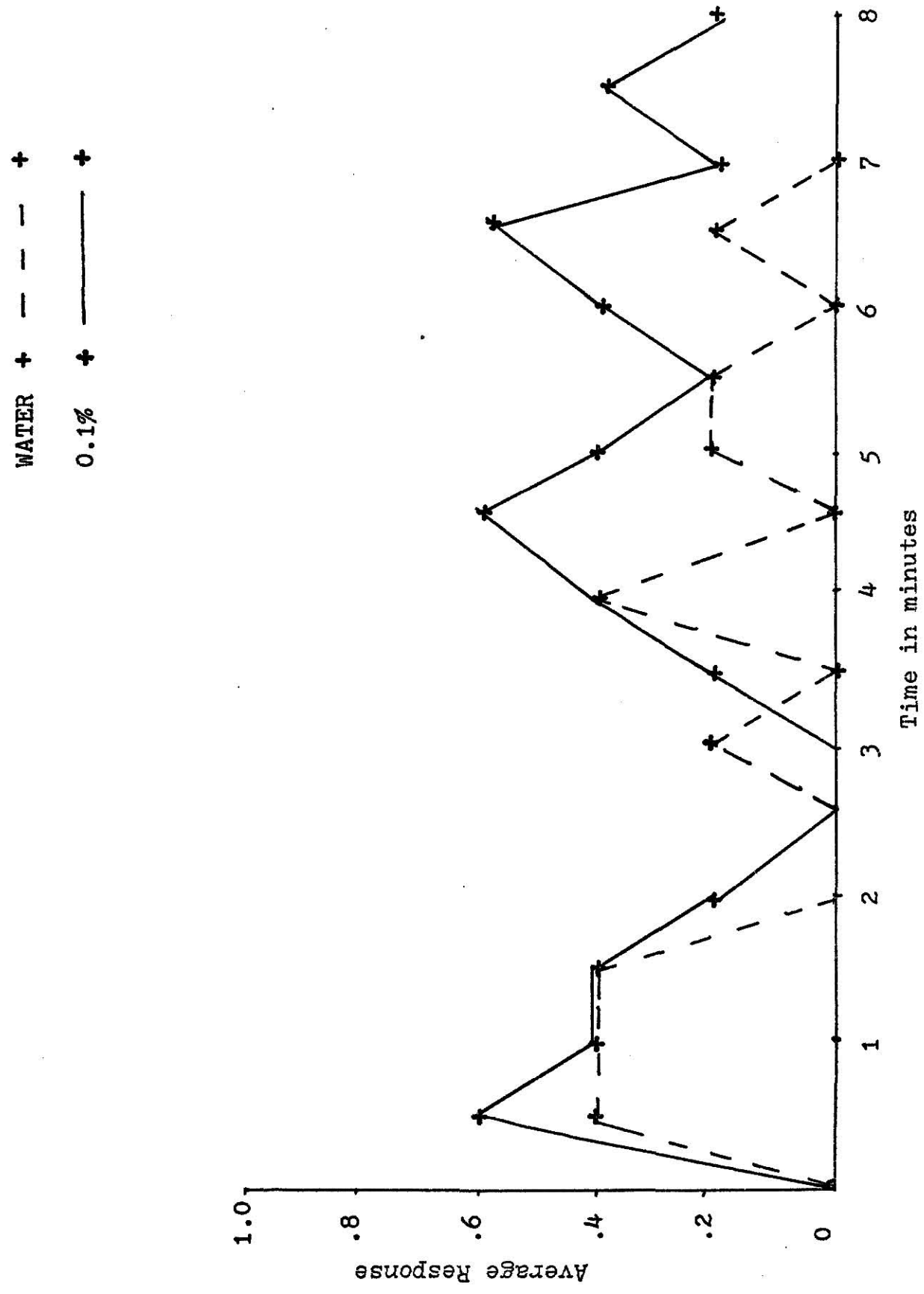

WATER
0.1%
Average Response
1.0
.8
.6
.4
.2
0
1
2
3
4
5
6
7
8
Time in minutes

FIGURE (20)

TEST 7/13/77-Mixture

Part 1

The Alfalfa Weevil's Response
to a Mixture of
Propionaldehyde and Ethanol
and a Water Control

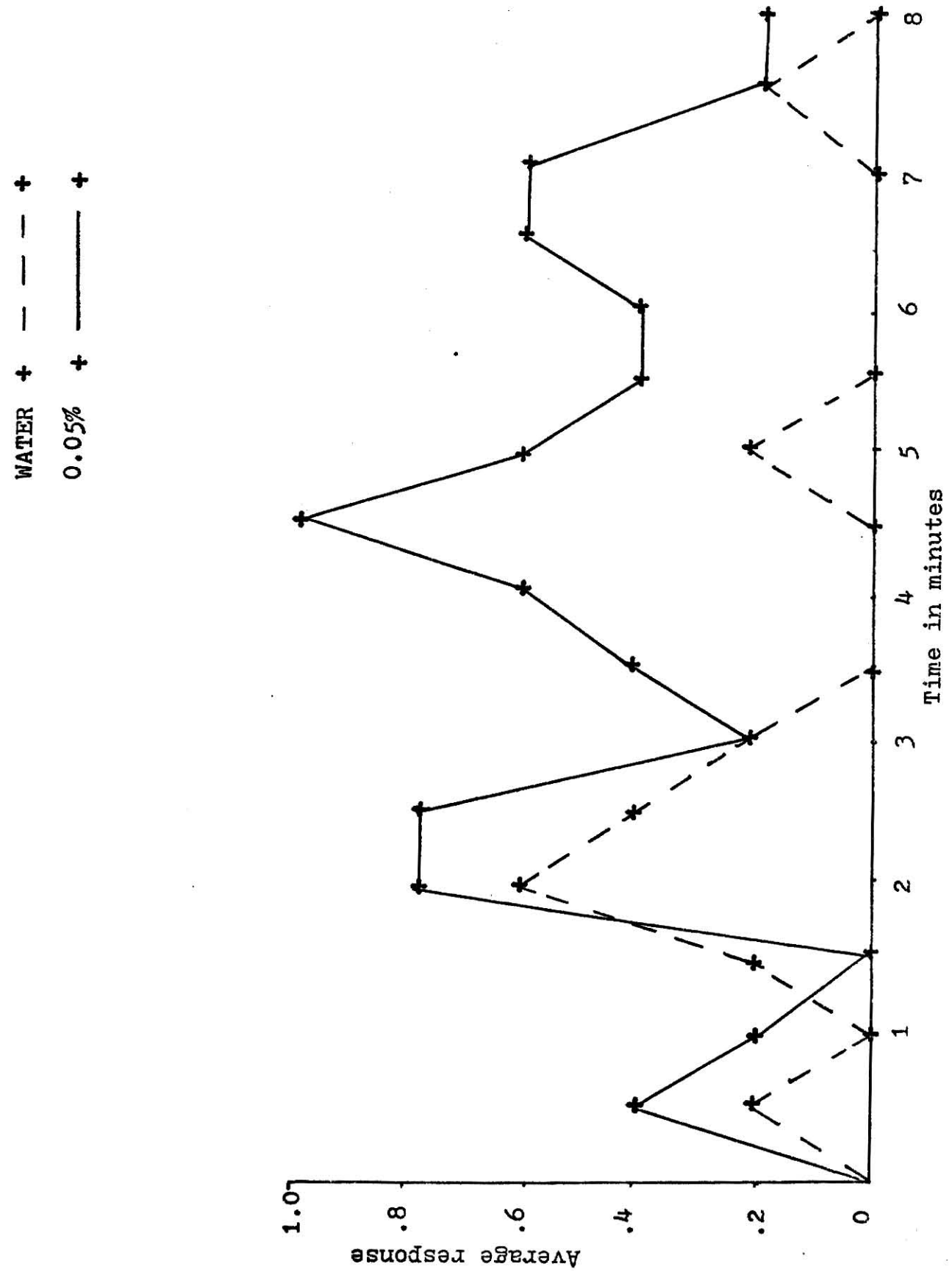
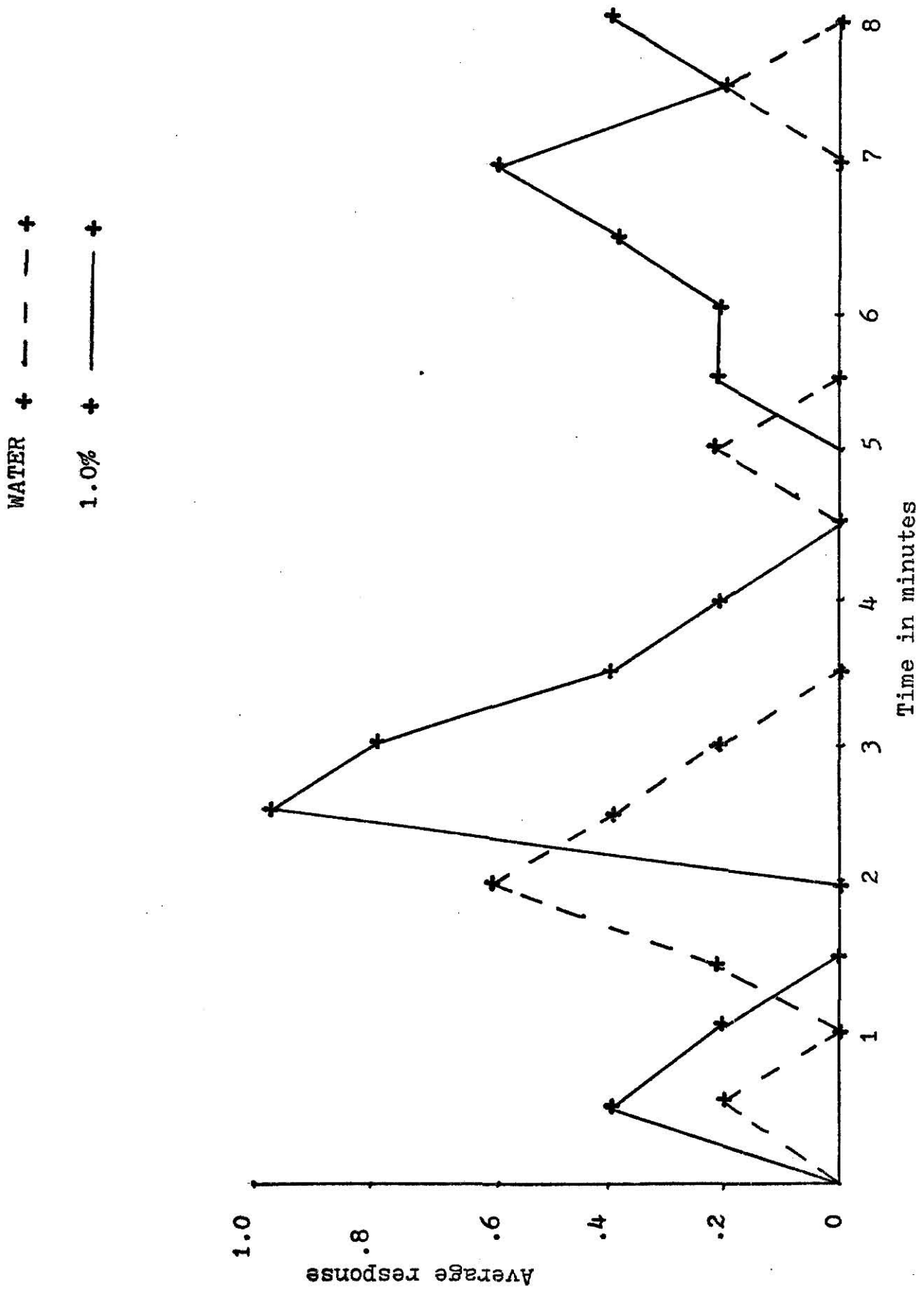

WATER + — — — +
0.05% + ——— +
Average response
1.0
.8
.6
.4
.2
0
1
2
3
4
5
6
7
8
Time in minutes

FIGURE (21)

TEST 7/13/77-Mixture

Part 2

The Alfalfa Weevil's Response
to a Mixture of
Propionaldehyde and Ethanol
and a Water Control


WATER
1.0%
Average response
1.0
.8
.6
.4
.2
0
1
2
3
4
5
6
7
8
Time in minutes

Suggestions for Future Work

From the work presented here, other areas of study are apparent. These can be divided into three classifications: (1) studies on alfalfa, (2) on red clover, and (3) other plants.

Alfalfa

Three major peaks in the steam distillate, #1, 2, and 7, were not identified and one minor peak, #5, also was not. These need to be identified. The first two peaks were not well separated and the mass spectra patterns appear to be mixtures. Another type of column packing might be useful to try and separate these further, and to resolve them better.

Terpenes and terpenoids are common "essential oils" in plants. If the unidentified peaks do not turn out to be these types of compounds, a method might be found where they could be separated from the plants and thus different varieties of the alfalfa could be examined for differences in these compounds.

The Entomology Department is growing 'resistant' varieties of alfalfa that have been noted to possess glandular hairs on the plants. The chemistry of these plants could be examined and compared to varieties that are very susceptible to the weevil.

The bioassay performed on the steam distillate and the resulting identified compounds, ethanol and propionaldehyde, were done with unsexed adult weevils. These should be repeated with the individual sexes. It may be the female that orientates to the plant and not both males and females. Any results that are obtained need to be statistically analyzed. This is more accurate than graphing the data.

The "hidden method" bioassay would be a way of testing for an ovipositional stimulant in the steam distillate. It should

be run in an alternating photoperiod however. The steam distillate is colorless so this should be no problem. The eggs laid could then be counted and the number laid on the test versus the control conditions could then be compared.

For any of the tests that are performed it is very important that controls should always be carried out at the same time as the tests are run. This is very critical to accurately judge the response of the weevils. It is also necessary to control all other factors as close as possible so no outside factors enter into the bioassay that are unaccounted for and might affect the results.

One method that was used on the Cotton Boll weevil might be applicable to the alfalfa weevil. This was performed by Neff and Vanderzant,[24] the same group that did the "hidden method" test. This other test involves examining solutions of the host plant applied to a feeding dish. The diet used for the purpose of the test is described in the article. To test a substance, two dishes of the diet are used. The diets are in an agar base and put in small dishes. A hole is removed from the middle of two dishes and in one a drop of the test solution is added and in the other a drop of the solvent for control purposes. After the solvent evaporates, the dishes are placed in the test cages with the weevils. The number of weevils on the test dishes are recorded at specific time intervals. The results are then compared.

This method could be adapted to the alfalfa weevil. Various extracts of the alfalfa could be used for the test. The steam distillate could easily be used for this with water as a control.

Red Clover

The hexane extract of the Red clover has been reported in the literature to be repellent to the alfalfa weevil. This extract could be looked at on the GC and if it proves repellent, the compounds could then be identified.

The bioassays that have been run on the red clover did not have significant results. These could be tried again using weevils that are at an optimum level of egg laying. Also bioassays should be run using the hexane extract of the red clover on the alfalfa plant.

Other Plants

Sweetclover has been identified as very acceptable to the alfalfa weevil. Comparisons with the alfalfa to check for identical compounds would be helpful. The presence of similar compounds in the two plants would be a key to the chemicals that the weevil prefers.

Bromegrass has been reported to be repellent to the weevil, though not in the literature. The possibility could be examined and the compounds then compared with red clover. If the hexane extract of the red clover proves to be repellent, a hexane extract of the bromegrass should be examined for the presence of similar compounds.

Work is currently being done in this laboratory to answer these questions.

REFERENCES

1. T.R.E. Southwood, Insect/Plant Relationship- and Evolutionary Perspective, H.F. Van Emden, ed. Symposium, 1972.
2. J.J. Vollmer and S.A. Gordon, Chemistry, 47, No. 10, 6(1974).
3. J.J. Vollmer and S.A. Gordon, Chemistry, 48, No. 4, 6(1975).
4. J.J. Vollmer and S.A. Gordon, Chemistry, 48, No. 5, 6(1975).
5. P.R. Ehrlich and P.H. Raven, Scientific American, 105(1967).
6. P.G. Seybold, Chemistry, 48, No. 8, 9(1975).
7. A.J. Thorsteinson, Ann. Rev. Entomol., 5, 193(1960).
8. "Application Highlight- Essential Oils", HPLC Applications Handbook, Dept. of Grain Science and Industry, KSU, Manhattan, Kansas.
9. L.M. Schoonhoven, "Chemosensory Bases of Host Plant Selection", 1967.
10. W.G. Evans, "The Biology and the Control of the Alfalfa Weevil in Virginia."
11. S. Coppock and N.W. Flora, "The Alfalfa Weevil and its Control", Oklahoma State Univ., Div. of Agric., Cooperative Extension Service, March 3, 1972.
12. "Field Observations on the Alfalfa Weevil in Nebraska,1969", U.S. Dept. of Agric., Ag. Res. Service, Ent. Res. Div., Grain and Forage Insects Res. Branch, Special Report X-306, Jan. 1970.
13. "Field Observations on the Alfalfa Weevil in Nebraska, 1971", U.S. Dept. of Agric., Ag. Res. Service, Ent. Res. Div., Grain and Forage Insects Res. Branch, Special Report X-333, Feb. 17, 1972.

14. J.H. Beynon, R.A. Saunders and A.E. Williams, "The Mass Spectra of Organic Molecules", Elsevier Publishing Co., Amsterdam, 1968.

15. A.A. Hower, Jr. and F.R. Ferrer, J. of Econ. Ent., 63, 761(1970).

16. G.B. Staal, Annual Rev. of Ent., 20, 417(1975).

17. G.B. Quistad, L.E. Staiger, and D.A. Schooey, J. Agr. Food Chem., 23, 299(1975).

18. C.A. Henrick, W.E. Willy and G.B. Staal, J. Agr. Food Chem., 24, 207(1976).

19. H.D. Byrne, A.L. Steinhauer and R.E. Menzer, Annals of the Entom. Soc. of Am., 59, 1013(1966).

20. H.D. Byrne and A.L. Steinhauer, Ann. Entomol. Soc. Amer., 59, 303(1966).

21. B.C. Pass, J. of Econ. Enotomol., 60, 288(1967).

22. R.L. Pienkowski, F.K. Hsieh and G.L. LeCato III, Ann. Entomol. Soc. Amer., 62, 1268(1969).

23. E.J. Armbrust, et al., J. Econ. Entomol., 59, 384(1966).

24. D.L. Neff and E.S. Vanderzant, J. Econ. Entomol., 56, 762(1963).

25. H.D. Byrne, et al., "Laboratory Studies of Factors Determining Host Plant Selection by the Alfalfa Weevil, Hypera postica (Gyl.)", Univ. of Maryland Ag. Expt. Station Bulletin A-147, June 1967.

26. H.D. Byrne, "The Oviposition Response of the Alfalfa Weevil Hypera postica (gyl.)", Univ. of Maryland Ag. Expt. Station Bulletin A-160, March 1969.

27. H.D. Byrne and C.C. Blickenstaff, J. Econ. Enomol., 61, 334(1968).

28. R.M. Silverstein and G.C. Bassler, "Spectrometric Identification of Organic Compounds", second edition, John Wiley and Sons, Inc., New York, 1967.

29. T.H. Hsiao, J. Insect Physiol., 15, 1785 (1969).

30. E.L. Esbaugh, "Alfalfa Weevil Summary", Dept. of Entomol., Kansas State Univ., Manhattan, Kansas, 1972.

31. "Atlas of Mass Spectral Data", E. Stenhagen, et al., eds., Vol. 1, Interscience Publishers, New York, 1969.

32. E.L. Esbaugh, E.L. Sorensen, and W.A. Moore, "Two Ways to Fight Alfalfa Weevils", AES Keeping Up with Research, Ag. Exp. Station, Kansas State Univ., Manhattan, Kansas, 14, Jan. 1975.

ACKNOWLEDGEMENTS

The author would like to thank the Kansas State Agricultural Experiment Station for their financial support of this project.

The author would like to thank the people at Midwest Research Institute at Kansas City, Missouri for the use of their GC-Mass Spec Data System. This was invaluable in the identification of the compounds.

Dr. T.L. Hopkins and Mr. E.L. Eshbaugh of the Entomology Dept. contributed their time and advice on the various biological setups and their cooperation is greatfully acknowledged.

The author would like to thank Kathleen Johnson of the Entomology Dept. for assistance on this project. Kathleen provided the initial instruction on the handling of the weevils and provided the source of the weevils for the tests. Her help in designing and setting up experiments in Parts 1 and 2 in this project was greatly appreciated.

The author would especially like to thank her research director, Professor Clifton E. Meloan, for his guidance and encouragement throughout this project.

Most of all, the author would personally like to thank her husband, Don, for his love and tremendous patience in waiting for her to finish the project.

VITA

The author was born in Youngstown, Ohio, on March 5, 1953. Her parents are Rev. Robert Lyle and Helen Jean Smith. Her father is a United Methodist minister currently serving a church in Ashland, Ohio. Her mother is a Licensed Practical Nurse working at the Ashland Hospital.

The author attended Marion L. Steele High School in Amherst, Ohio, and also Admiral King High School in Lorain, Ohio, from which she graduated in June, 1971.

She attended Ohio Northern University receiving her B. A. in chemistry in May, 1975.

She entered graduate school at Kansas State University in August, 1975, and completed requirements for an M.S. in chemistry in August, 1977.

She married Donald Ray Currey who is serving in the U.S. Army. He is currently stationed at Fürth, Germany. She will join her husband after completion of her degree requirements.

TWO OLFACTORY ATTRACTANTS IN ALFALFA (MEDICAGO SATIVA) FOR ALFALFA WEEVILS (HYPERA POSTICA)

by

KAREN LEE CURREY

B. A., Ohio Northern University, 1975

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Chemistry

KANSAS STATE UNIVERSITY

Manhattan, Kansas

1977

Two Olfactory Attractants in Alfalfa (*Medicago sativa*) for Alfalfa Weevils (*Hypera postica*)

by

Karen Lee Currey

Department of Chemistry
Kansas State University
Manhattan, Kansas 66506

Abstract

Alfalfa weevils infest a large proportion of the alfalfa crops, and are considered a serious economic pest of alfalfa. The identification of attractants in alfalfa might make it possible to breed a species of alfalfa that would change the chemical communication pattern between the weevil and the plant.

Bioassays were developed to determine the active constituents of the alfalfa. The steam distillate of the alfalfa leaves showed a postive olfactory response from adult weevils.

The compounds present in the steam distillate were then separated by gas chromatography and identified via their mass spectra. Three compounds have been identified. Two of these, ethanol and propionaldehyde, have been shown to elicit a positive olfactory response from adult weevils.