AN ABSTRACT OF THE THESIS OF

Gary Edward Daterman for the Ph. D. in Entomology
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Title: REPRODUCTIVE BIOLOGY OF THE EUROPEAN PINE SHOOT MOTH
RHYACIONIA BUOLIANA (SCHIFFERMÜLLER) (LEPIDOPTERA: OLETHREU-
TIDAE), WITH SPECIAL REFERENCE TO MATING BEHAVIOR, SEX
ATTRACTION, AND FECUNDITY.

Abstract approved: Redacted for privacy
(Major professor)

Dr. Paul O. Ritcher

Major objectives of the study involved development of a labora-
tory procedure to obtain mating, determining characteristics of female
sex attraction, and determination of the effects of temperature and
humidity on mating and fecundity. The second of two methods devised
to obtain mating, "the vertical airflow technique," was convenient to
employ and most efficient in producing mated females. This method
resulted in 73 percent of the females mating within 48 hours. Mating
was triggered by decreased illumination, and slow airflow directed
from females toward males greatly facilitated sexual activity.

Females varied in attractant potency, with some apparently incap-
able of attracting any males to sticky traps and others capturing
high numbers. The source of pheromone production was a gland on the
dorsum of the abdominal tip between the penultimate and terminal
segments. Males could be attracted over distances up to 100 yards
to sex attractant baits, and responded best to traps located in host
pines as compared to traps in the open or in nonhost foliage.

Mating efficiency decreased for both sexes after they were 4.5 days old. Females older than 2.5 days at the time of mating oviposited fewer eggs than females fertilized at a younger age. Average fecundity for females less than 2.5 days old when mated was 126 eggs. Males were capable of multiple matings, whereas females normally mated only once. Males copulated with an average of 2.24 females, although a small percentage never mated and others paired up to 5 and 6 times.

Matings were observed at temperatures ranging from 54 to 92° F. Within the range of 65 to 85° F. there were no significant differences in mating efficiency. Fecundity was also unaffected within these limits with an accompanying high humidity. At 92° F., fecundity decreased and there was total egg mortality despite a high humidity. Fecundity decreased proportionately to decreasing air moisture (high evaporation rates) at temperatures of 85° F. or below. These detrimental influences of high evaporation rates and temperature could limit the geographic distribution of the European pine shoot moth, since most western pine regions are noted for hot, dry summer weather.

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OLETHREUTIDAE), WITH SPECIAL REFERENCE TO MATING
BEHAVIOR, SEX ATTRACTION, AND FECUNDITY

by

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CHAPTER I

GENERAL INTRODUCTION AND LITERATURE REVIEW

The European pine shoot moth, *Rhyacionia buoliana* (Schiffer-
müller), infests young pines throughout Europe and in much of North
America. In North America damage has been limited to ornamentals
and plantation-grown trees. Many species are infested but red pine,
Pinus resinosa Ait., Scotch pine, *P. sylvestris* L., lodgepole pine,
P. contorta Dougl., and Swiss mountain pine, *P. mugo* Turra are the
most common hosts in the United States. Larvae feed in needle
fascicles, buds, and elongating shoots causing growth reduction and
distortion in tree form. Economically this is considered highly
detrimental, although the trees are seldom actually killed. Damage
is primarily restricted to regeneration growth less than 20 feet in
height, but infestations can be found on lateral branches of larger
trees.

Busck (1914) reported the first known establishment of *R.*
buoliana in the United States from Long Island, New York; infested
nursery stock imported from Europe to the East Coast was the
apparent cause. The moth was first reported in western North
America on Vancouver Island, British Columbia, in 1927 (Downes 1928).
It was not until 1959 that the shoot moth was identified in the

western United States (Furniss 1962). The insect is now established in ornamental pines and Christmas tree plantations in the Puget Sound area of western Washington.

In the western United States, a major concern has been the inadvertent movement of infested stock from the Puget Sound area into natural stands of ponderosa pine, *Pinus ponderosa* Laws. This concern is justifiable since the insect is classified as a pest of natural forests in Europe (Friend and West 1933), and ponderosa pine has been ranked with Scotch pine in its degree of shoot moth susceptibility when planted in the Lake States (Miller and Heikkenen 1954). Flora (1965) speculated that 4 to 23 million acres of ponderosa pine type could be seriously affected by European pine shoot moth. If such damage occurred, long-run impacts would include great loss of allowable timber cut and loss of up to 74,000 jobs per year (Flora 1965). The eventual results of this economic evaluation was a decision to try to eradicate this insect from the western United States. The relative isolation of the Puget Sound infestation, ringed on three sides by large bodies of water or a mountain range, seemed encouraging. Concentration of the insect in metropolitan areas did not favor pesticides for the proposed eradication task. A study on testing the feasibility of applying the sterile male technique (Knipling 1960) was finally initiated through the award of a cooperative U.S. Forest Service grant to entomologists at Washington State University. Briefly, this control technique involves the mass rearing, sterilization, and release of males of the species.

Wild females mating with these individuals produce sterile eggs and the population is thus reduced. Repetitive application over a period of years theoretically results in eradication of the species from an area.

Among aspects of biological information necessary to application of the sterile male technique is a basic understanding of reproductive biology, including mating behavior and fecundity. Clearly, laboratory mating techniques are necessary to evaluate sterilization procedures and methods to obtain maximum reproduction in the laboratory are required for mass rearing. It was primarily because of these needs that the study described in this paper was initiated.

There is abundant literature on European pine shoot moth biology, but few papers offer explicit information on the reproductive aspects. Friend and West (1933) offered the first comprehensive paper on the history and biology of R. buoliana in the United States. Miller and Neiswander (1955) went into greater detail on biology and also reviewed control practices. Miller (1967) stressed host susceptibility, types of damage, and population dynamics in the Lake States. Miller (1967) also reviewed control procedures including fumigation techniques developed by Flink and Brigham (1959); Carolin, Klein, and Thompson (1962); and Carolin and Coulter (1962). Pointing and Green (1962) reviewed the history and biology of the shoot moth in Ontario, Canada. The most useful information, pertinent to further investigation of reproduction, was contributed by Pointing (1961), Pointing and Green (1962), and Green (1962; 1965) in studies on

flight activity, dispersal, and the influence of certain physical factors. Green particularly emphasized the influence of physical factors, reporting the effects of wind, light, and temperature on emergence and mating and oviposition flights. Pointing (1961) described mating behavior in the field, studied fecundity, and found evidence of a female sex pheromone. Miller and Neiswander (1955) observed *R. buoliana* mating in an emergence cage located in a field insectary; this was the only reported case of European pine shoot moths mating in a caged situation. Unfortunately, information was lacking on efficiency of the arrangement and the specifics on physical factors and structural characteristics necessary to duplicate the results.

All of the above studies were field oriented and information was lacking for laboratory application. The need for more specific information is pointed out in the recent paper by Chawla and Harwood (1968). These workers developed an artificial wheat germ diet for laboratory rearing of the shoot moth, but their study was hampered by the scarcity of first-instar larvae. An efficient laboratory mating technique would have benefitted their study by providing abundant fertile eggs for rearing first instars.

The major objectives of my study were:

(1) To develop an efficient laboratory procedure for obtaining mated females.

(2) To determine characteristics of female sex attraction, particularly in reference to mating and male behavior.

(3) To evaluate other biotic factors that could influence reproduction such as adult age and mating frequency.

(4) To determine the effects of certain physical factors on reproduction, particularly temperature and evaporation rates.

With approval of the Oregon State University Graduate School, some preliminary results of this study have been published (Daterman 1968). The published information deals primarily with techniques to obtain mating of the shoot moth in the laboratory and characteristics of mating behavior.

CHAPTER II

LABORATORY TECHNIQUES TO OBTAIN MATED FEMALES

It was reasoned that if field conditions could be approximated with an appropriate caging technique, a reliable method for breeding this insect would result. Field studies by Pointing (1961) established that mating occurs in the evening when males take flight and receptive females move to the tips of pine needles. In the present study, artificial decrease of light intensity in the laboratory also stimulated adult activity, but no matings occurred in cages used for preliminary observations. In size, these cages varied from 1.5x6-inch plastic cylinders to larger 22x24x30-inch screened structures; all were similar in that no provision was made for directed airflow through them. Since these cages failed and because females were known to produce an attractant (Pointing 1961), the relative positioning of the sexes with respect to a directed airflow was surmised to be a key factor. If females could be kept upwind from an aggregation of males, air movement would assist transport of attractant to the males and presumably stimulate mating. Experiments with two cage arrangements designed to induce mating of R. *buoliana* are described.

Study Procedures

Cage with Horizontal Airflow

The test cage utilizing a horizontally directed flow of air was

18x18x24 inches long. The sides were constructed of transparent plastic to permit observation, and the two ends of fiber-glass screening to allow free airflow through the long axis of the cage. One screened end faced a white reflective surface which was illuminated by an incandescent lamp connected to a rheostat permitting manual regulation of light intensity. A fan, situated at the opposite screened end, forced air through the interior (Figure 1). The illuminated end of the cage was further restricted in size by covering the upper and lower quarters of the screening with black cloth. Preliminary observations had shown males to be attracted to light of low intensities. This design provided a smaller illuminated area at which males congregated and through which attractant-laden air passed out of the cage.

Prior to actual testing, moths were subjected to high light intensities (140-300 foot candles) for a period of 12 hours or more. For both pretest and test periods, relative humidity was kept above 50 percent and ambient temperature kept relatively constant ($\pm$ 1.0° C) within a range of 21-23° C. Airflow through the cage was slow, ranging from 0.3 to 0.5 ft./sec.

Moths used in this and other laboratory experiments were reared on a wheat germ diet developed by Berger (1963) and modified for the shoot moth by Chawla and Harwood (1968). An important change in Harwood and Chawla's formula was the addition of aureomycin to prevent growth of bacterial contaminants causing larval mortality (see Appendix p. 101). After emergence, moths were sexed and confined
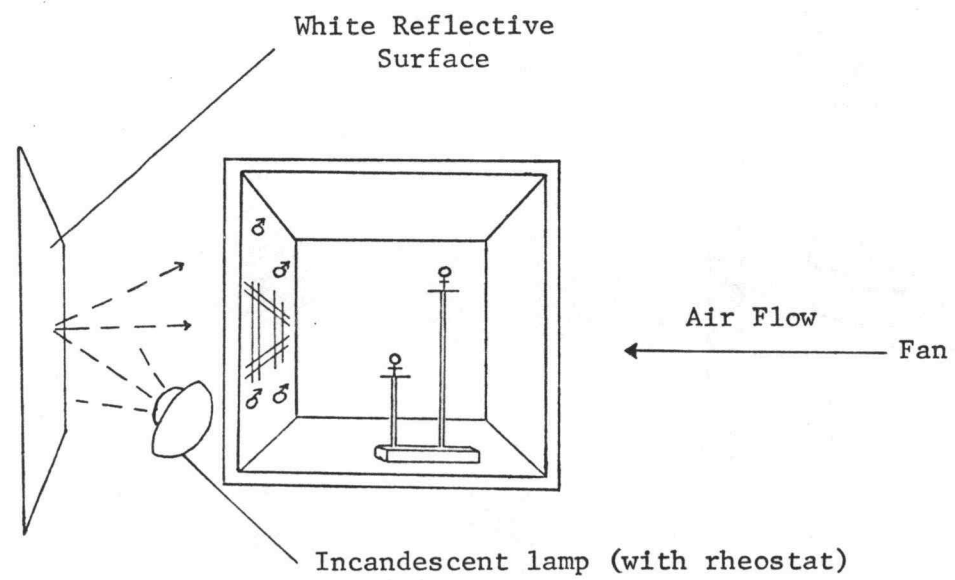
Figure 1. Cage arrangement with horizontal airflow for mating *R. buoliana*.

(two moths of the same sex per dish) in plastic 100x15 mm. petri dishes. This precaution was taken to avoid sexual contact prior to testing and to avoid damage that often resulted when large numbers were confined in the same storage container.

In each of 14 tests, 4 to 6 females were caged with 8 to 12 males. After removal of the distal half of the wings of one side, females were placed on pine needles or toothpicks anchored in modeling clay in the center of an open 100x15 mm. dish. Inside walls of the dishes were lined with Fluoroglide 200,[1] a Teflon lubricating powder. In this arrangement females could not fly and were further confined since the powder prevented their gaining traction to climb the walls of the dish.

Actual testing was begun by decreasing illumination to about 5-foot candles within 45 minutes. Each trial was run an additional 60 minutes while light intensity was decreased to less than 1-foot candle.

Cage with Vertical Airflow

A second cage arrangement was designed to correct certain drawbacks encountered in the first. The same principal of female confinement upwind of males was used. Basic differences in this design involved direction of airflow, the lighting arrangement, and shape and size of the mating chambers.

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[1] Available from Chemplast, Inc., East Newark, New Jersey.

Mating chambers, consisting of transparent plastic cylinders 6 inches high and 4.5 inches in diameter, were vertically oriented and contained within a larger (24x20x18 inches high) compartment (Figure 2). Rapid airflow (2-3 feet/second) directed through the larger compartment and across the tops of the mating chambers, caused a decreased air pressure at the screened tops, resulting in a slower airflow (.17-.5 feet/second) upward through the vertically oriented cylinders. Generally, only one or two chambers were used in a large compartment at one time. The mating chamber units were always placed directly in line with the air intake and outlet of the larger compartment. More mating chambers could be used per compartment providing air baffles were constructed to equalize airflow across the top of the larger compartment. A 14-watt Venturi "Muffin" fan[2] was used to draw air through each compartment.

Tops of the outer compartments were constructed of glass to admit light. Illumination was provided by three 15-watt fluorescent bulbs secured to a plywood base which was suspended a few inches above the glass (Figure 2). Each fluorescent tube was connected to a time switch. Gradations in illumination were accomplished by shading the fluorescent tubes with fiber-glass window screen and black cloth. The transition from light to dark was made by timing an unshaded bulb to switch off first; a lightly shaded bulb to switch off 15 minutes later, and the remaining heavily shaded bulb stayed

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[2] Available from Allied Electronics, 4000 North Aurora Ave., Seattle, Washington.
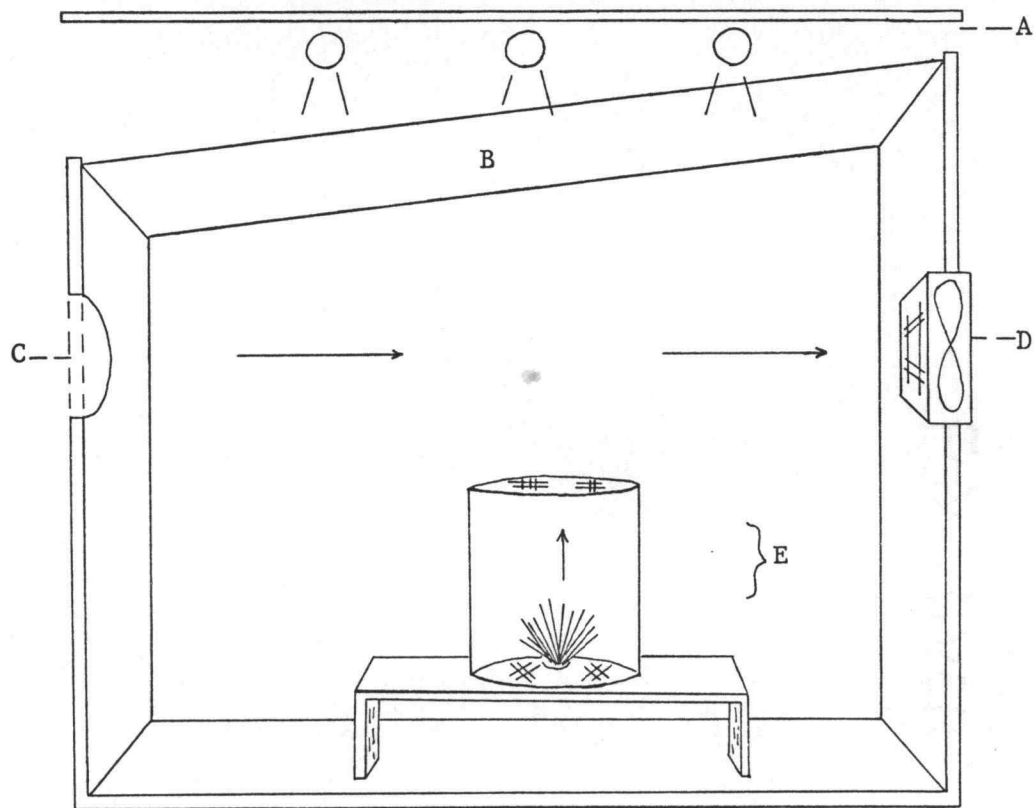
Figure 2. Vertical airflow cage arrangement for mating *R. buoliana*. (A) Fluorescent lamps and time switches. (B) Glass top. (C) Air intake. (D) Fan. (E) Mating chamber. Arrows show direction of airflow. (See text.)

on for an additional 60 minutes, providing the "twilight" condition.
The resulting gradation of light intensities varied from 230 to 70
to less than 2-foot candles when measured 1 foot below the glass top
with a Weston Illumination Meter (Model 256). Standard testing
procedure consisted of 16 hours of light, 1 hour of "twilight", and
7 hours of darkness.

Each mating chamber (Figure 3) had a porous top constructed of
cotton gauze and was fitted over a porous (16 mesh screen) floor to
permit free airflow through the cylinder. Pine needles anchored in
modeling clay were placed in the center of the screen floor. Inside
walls of the plastic cylinder were treated with "Fluoroglide 200"
lubricating powder to prevent females from climbing to the top. The
distal half of each female's wings were clipped on one side to pre-
vent flight. A standard number of three females and six males were
placed in each mating chamber. Females were dropped onto the pine
needles after removing the plastic cylinder. The cylinder was then
placed in an inverted position and the males dropped in. Most of
the males would cling to the gauze fabric of the cylinder top as it
was turned upright and fitted over the pine needles, completing the
set-up. Standard procedure was to leave the moths in the cages for
48 hours (two light/dark cycles).

Evaluating Mating Success

Success of the first caging technique was measured simply by
observing the number of mating pairs. Evaluation of the second
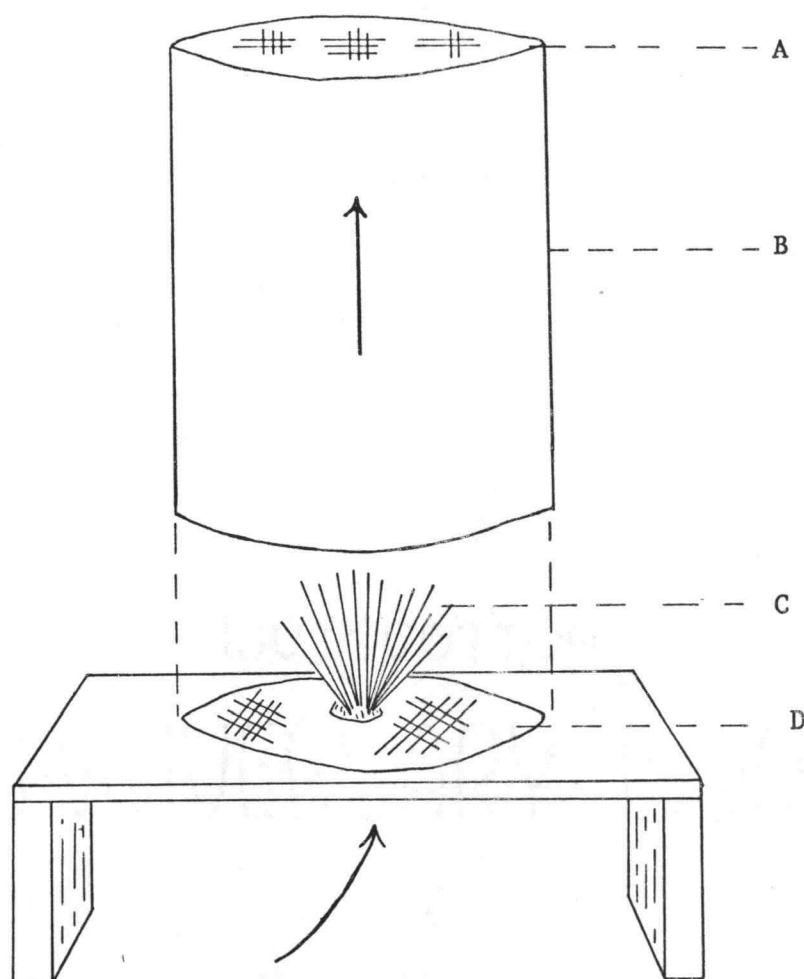
Figure 3. Exploded, enlarged view of mating chamber for
R. buoliana used in vertical airflow cage
arrangement. (A) Gauze or fine mesh screen.
(B) Transparent plastic cylinder. (C) Pine
needles. (D) Porous (screened) cage floor.
Arrows show direction of airflow.

technique (vertical airflow) was aided by the discovery of an external feature unique to mated females. During copulation the light colored scales surrounding the genital opening are removed by the clasping action of the male valvae. The resulting exposure of dark cuticle (compare Figure 4A and B) is evidence that the female has mated. Observations of hundreds of specimens showed that only females so marked produced fertile eggs. Since females were found to mate only once, the number of females so marked in a given test was equal to the number of matings.

Assessment of Design Characteristics

Following initial testing of the caging method utilizing vertical airflow, additional tests were run to evaluate the relative importance of certain characteristics involved in the technique. To evaluate the importance of the "twilight" period, an equal number of replicates were run in continual darkness, continual light, and with an abrupt light-dark transition in place of the usual twilight period. A series with the "twilight" period served as a control.

A series was also run with an interrupted airflow in the cylindrical mating chambers. This was done by sealing the cylinder tops and chamber floors with plastic sheets. A comparison was also made in the percent mating in tests lasting 24 hours (one light-dark cycle) and 48 hours (two light-dark cycles).
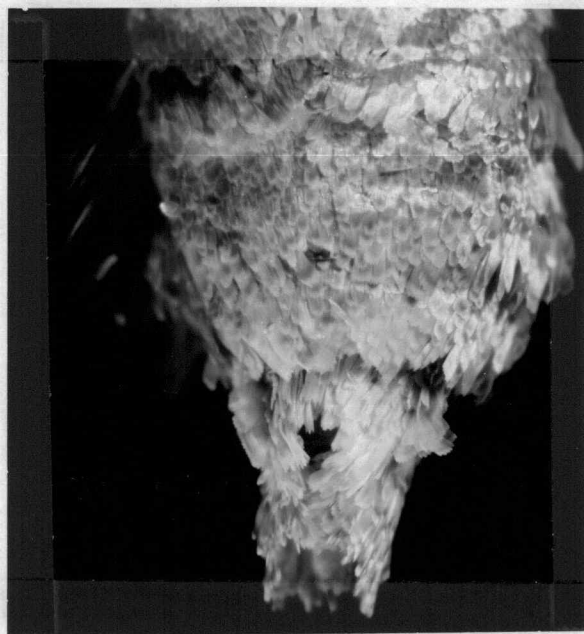
A B

Figure 4. Ventral view of abdominal tips of unmated (A) and
mated (B) females.

Results and Discussion

Cage with Horizontal Airflow

Mating behavior.--As light intensity was decreased below 10-foot
candles, males became active and moved rapidly about on the lighted
wall of the cage. Females also became more active, particularly
below the 5-foot candle level, and those not previously on needles
generally assumed a terminal position at this time. As illumination
was decreased further, some of the males became sexually stimulated,
presumably by the female scent. Stimulated males exhibited pre-
copulatory movements of rapid wing fluttering, anteriorly curved
abdomens, and opening and closing of the valvae. With a weaving
pattern of flight, the stimulated males "homed in" on the attractant
source. After landing in the vicinity of an attractive female, males
continued the precopulatory movements and moved rapidly in circular
fashion over the area and up and down pine needles in obvious attempts
to locate the female. Once the female was located, there was no
further courtship activity, and copulation occurred immediately. At
the relatively constant laboratory temperatures, copulation lasted
at least 50 minutes, and most terminated within 90 minutes.

Mating almost invariably was initiated on pine needles or a
similar object such as a toothpick. Only rarely was copulation begun
while a female was resting on a flat surface, and the structure of
pine foliage is evidently important in this regard. Since mating
was also induced on very old, dry pine needles or toothpicks, it is

doubtful that any host plant odor is required to trigger the mating response as in the case of at least one other moth (*Antherea polyphemus*, Cramer) (Riddiford and Williams 1967).

Mating success.--Although evidence of sex attraction was evident in virtually all trials, only 11 of 14 produced mated females. Only 36 percent of the available females mated, and a further drawback to the horizontal airflow technique was the inconvenience involved in manually regulating light intensity. The main value of the method was that sexual activity could be induced within a stipulated time period, enabling observations of mating behavior under artificial conditions.

Cage with Vertical Airflow

Generally, the same principles of sexual behavior were observed in this second mating cage apparatus. Mating usually occurred during the "twilight" period, although some pairings occurred in complete darkness shortly after termination of "twilight." Males apparently became sexually stimulated by female attractant transported upward by the slow, continual airflow in the plastic cylinders. Stimulated males dropped or flew to the chamber floor in the near vicinity of the females. While performing their typical precopulatory motions, the males attempted to locate the attractive females in the same manner described previously.

Mating success.--The smaller cylindrical mating chambers utilizing vertically directed airflow were more successful in producing

mated females than the other caging technique. In groups caged for a 48-hour period (two light-dark cycles), 73 percent of the females mated (Table 1). This was approximately twice as efficient as the first technique employed. Besides this increase in efficiency, the technique was also more convenient in that no manual regulation of lighting apparatus or other maintenance was necessary during the test period.

Assessment of Cage Design Characteristics

After the effort involved in devising an artificial "twilight" system, it was startling to find that such an elaboration of the lighting regime was unnecessary. No significant differences were found in percent females mated in tests having a twilight period and those having either constant darkness or only an abrupt change between the light and dark phases of the daily cycle (Tables 2 and 3). The comparison of means (Table 3) did show significantly higher mating for the control (twilight period) than either the constant light or sealed chamber treatments.

Clearly, a "twilight" period is not necessary to induce mating of the European pine shoot moth; although, a controlled 24-hour light-dark cycle seems to be favored. While the constant dark treatment did not show a significant difference from the control or the abrupt light-dark change treatment, the level of success was on the borderline of significance (Table 3). The evaluation experiment also illustrated the importance of directed airflow since the sealed chamber treatment was the least successful.

Better insight of the effects of these conditions on the mating response can be obtained from Table 4 in which the degree of reproductive activity during the first 48 hours of a given test condition is compared. It is apparent that mating was stimulated by the light-dark transition whether the illumination change was gradual (having a twilight period) or abrupt. In tests having a twilight and/or a dark phase following full illumination, over 50 percent of the females mated in the first 24 hours. In tests lacking this stimulus, there was only 13 and 23 percent mating respectively for constant light and constant dark treatments. In both the latter cases, however, there was more mating during the second half (24-48 hours) of a test. Mating activity was induced at a given time only by the light-dark transition; the reverse sequence of illumination conditions (dark-light change) did not stimulate sexual activity.

Apparently the adults have a circadian mating rhythm which is primarily stimulated by decreasing illumination. Lacking this stimulus, however, mating eventually occurs which apparently illustrates that the rhythm is not a fixed, obligatory feature in this insect. The higher mating percentages resulting when the stimulus is present are probably due to the moths being activated at roughly the same time; that is, the females are likely to be receptive and attractive when males are stimulated to fly since the drop in illumination has synchronized their activity periods.

CONCLUSIONS

Two methods were devised to obtain matings of the European pine shoot moth in the laboratory. The first of these, "the horizontal airflow technique," required continual attention by the worker, and was primarily useful for studying behavior since sexual activity could be induced at a specific time. The second method, "the vertical airflow technique," was more efficient in producing mated females and considerably more convenient in that no manual regulation of equipment or observation of the test was necessary once the moths were caged. The latter method resulted in 73 percent female mating within a 48-hour period. A flow of air directed from females to males was employed in both techniques, and was found to be a necessary factor for maximum mating activity.

A period of high illumination (230-foot candles) followed by a period of darkness or artificial twilight was necessary to induce mating at a given time. Some mating did occur in constant light, however, and also to a considerable degree in constant darkness. Apparently mating activity follows a circadian rhythm triggered by a sudden or gradual decrease in illumination. Lacking the light-dark transition, mating still occurs but no longer is synchronized in time.

TABLE 1. SUMMARY OF MATINGS OF *R. BUOLIANA* OBTAINED
IN TWO TYPES OF CAGING APPARATUS.

Type of cage	Number males	Number females	Number females mated	Percent females mated
Cage with horizontal airflow	134	66	24	36
Cage with vertical airflow	724	362	263	73

TABLE 2. INFLUENCE OF CAGE DESIGN CHARACTERISTICS ON NUMBER
OF MATINGS IN VERTICAL AIRFLOW CAGE. ANALYSIS OF
VARIANCE OF RESULTS (FREESE 1967).

Number females mated (12 males:6 females per replicate)				
Sealed chamber	Constant dark	Constant light	Abrupt light dark change	Control (twilight)
1	4	5	3	4
1	4	2	5	5
2	4	2	5	5
2	2	6	4	6
3	2	2	4	4
0	3	2	6	3
2	4	3	6	5
3	4	5	4	6
2	5	1	5	4
3	3	3	5	4
19 (31%)	35 (58%)	31 (52%)	47 (78%)	46 (77%)

Source of variation	Degrees of freedom	Sums of squares	Mean squares
Treatments	4	53.52	13.38
Error	45	58.80	1.3067

$F = 13.38/1.3067 = 10.239$, Tabular $F_{.01} = 3.77$

Conclude: Significant difference among treatments.

TABLE 3. INFLUENCE OF CAGE DESIGN CHARACTERISTICS ON NUMBER OF
MATINGS IN VERTICAL AIRFLOW CAGE. TUKEY'S TEST FOR
COMPARISONS AMONG MEANS (SNEDECOR 1956). DIFFERENCES
GREATER THAN 1.45 (D) SIGNIFICANT AT 5-PERCENT LEVEL.

Treatment	Ranked mean	Treatment differences			
		$\bar{x} - 1.9$	$\bar{x} - 3.1$	$\bar{x} - 3.5$	$\bar{x} - 4.6$
Abrupt light-dark change	4.7	2.8[1]	1.6[1]	1.2	.1
Control (twilight)	4.6	2.7[1]	1.5[1]	1.1	
Constant dark	3.5	1.6[1]	.4		
Constant light	3.1	1.2			
Sealed chamber (no airflow)	1.9				

[1] Denotes significant difference.

TABLE 4. COMPARISON OF MATING ACTIVITY DURING 48-HOUR
TEST UNDER DIFFERENT LIGHTING REGIMES.

Lighting condition	Number females tested	Percent total females mating 1st 24 hours	Percent total matings 1st 24 hours	Percent total mating 2nd 24 hours
Abrupt light-dark change	42	60	75.7	26.3
Control (twilight)	90	51	76.7	23.3
Constant light	30	13	30.8	69.2
Constant dark	30	23	41.2	58.8

CHAPTER III

CHARACTERISTICS OF FEMALE SEX ATTRACTION

An insect sex pheromone fulfills the fundamental role of bringing the sexes together for purposes of reproduction which insures the perpetuation of bisexual species. During the past five to ten years, entomological literature has so proliferated on this subject that it seems almost every insect species must possess a sex pheromone. The European pine shoot moth is no exception in this regard. Pointing (1961) and Torgerson (1964) demonstrated a female sex attractant in *R. buoliana*. Pointing (1961) observed that only unmated females were attractive, and Torgerson (1964) found that attraction ceased within one minute after a pair had begun copulation.

This study was concerned with the necessity of attraction for the occurrence of each mating, the variation in attractiveness among females, the location of pheromone production in the female, and the distance males respond to the pheromone.

Study Procedures

Necessity of the Attractant for Mating

Observation of moths in mating cages was relied on for information concerning the necessity of the pheromone for each mating.

Variations in Female Attractiveness

Tanglefoot traps were constructed and individual, unmated females used as bait. During two flight seasons these traps were placed within infested pines in the vicinity of Seattle, Washington. Records were kept on the duration of attraction and numbers of males lured to each trap. A total of 56 virgin females were separately evaluated for their relative attractiveness using this technique. For these tests the bait females were never more than 48 hours old when placed in the field.

The tanglefoot traps consisted of quart-size cylindrical ice cream cartons with one-half of each end removed to permit male entry. The interiors were lined with tanglefoot,[3/] and the live females confined in screen envelopes suspended on wire in the center of the trap. This design is basically the same as that used by Butt and Hathaway (1966).

Source of Attractant Production

Jefferson, *et al.* (1966) stated that scent organs of Lepidoptera are typically situated on the abdomen close to the genital openings. Götz (1951) reported that scent organs originate from an intersegmental fold, usually between the eighth and ninth abdominal segments. A number of other workers (Dickens 1936; George 1965; Barnes,

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3/ Available through Forestry Suppliers, Inc. 205 W. Rankin Street, Jackson, Mississippi.

Peterson, and O'Connor 1966; Jefferson, Shorey, and Rubin 1968;
Roelofs and Feng 1968; Weatherston and Percy 1968) have also pin-
pointed attractant production in the abdominal tips of female Lepi-
doptera. To verify that the *R. buoliana* attractant was produced
on the end of the abdomen, abdominal tips of virgin females were
removed and extracted in organic solvents. The remainder of the
female bodies were also extracted as a control. The extraction pro-
cedure consisted of grinding the insect tissue in the solvent with a
glass mortar and pestle. This material was then filtered and the
liquid impregnated on cotton rolls and tested in cylindrical tangle-
foot traps for bioassay.

Female abdominal tips were also fixed in Bouins solution, imbed-
ded in paraffin, and sectioned with a rotary microtome for closer
inspection. Staining of the 8-12 micron sections was with Delafield's
haematoxylin using eosin as a counterstain. The exact procedure is
listed in the Appendix (page 103). Tissues that resembled pheromone
glands of other insects were bioassayed by inserting sharp probes
into the suspect tissue of an intact female, and then introducing the
probe to the airstream of a laboratory olfactometer. This method was
used as a bioassay technique by George (1965) when working on location
of the pheromone gland in the Oriental fruit moth. Positive bioassay
required male sexual stimulation which consisted of characteristic
wing fluttering, and anterior-ventral curvature of the abdomen. Pre-
paratory to bioassay, males were held at high illumination conditions
(230-foot candles) at least 12 hours prior to decrease in light

intensity and exposure to candidate attractive material. Figure 5 is a diagram of the bioassay chamber. Except for modification to permit control of illumination, this apparatus follows the design described by Wright (1965).

Distance of Male Response

Male flights to sources of female attractant were studied by releasing males prescribed distances from traps baited with attractant. Males were liberated by placing them at the release point in open, 1/2-gallon containers or shaking them onto foliage (not necessarily the host plant) growing at the release point. Liberation was during midday or early afternoon to permit their becoming acclimated to field conditions and to avoid handling them just before or during the evening flight period. The released males usually took positions on nearby foliage until evening, although a few would fly completely out of the area when set free. Day-Glo fluorescent powder[4] was used to tag the males by anesthetizing them for a few seconds with carbon dioxide and gently brushing the distal half of the wings in the dry pigment. Attractant baits consisted of both live, virgin females and solvent extracts of unmated females. These tests were run at two study locations, both relatively open, with few native pines, and no apparent infestation. The uninfested plots were chosen to avoid the confusion of competitive attraction to released males that could be

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[4] Day-Glo Color Division, Switzer Brothers, Inc., 4732 St. Clair Avenue, Cleveland, Ohio 44103.
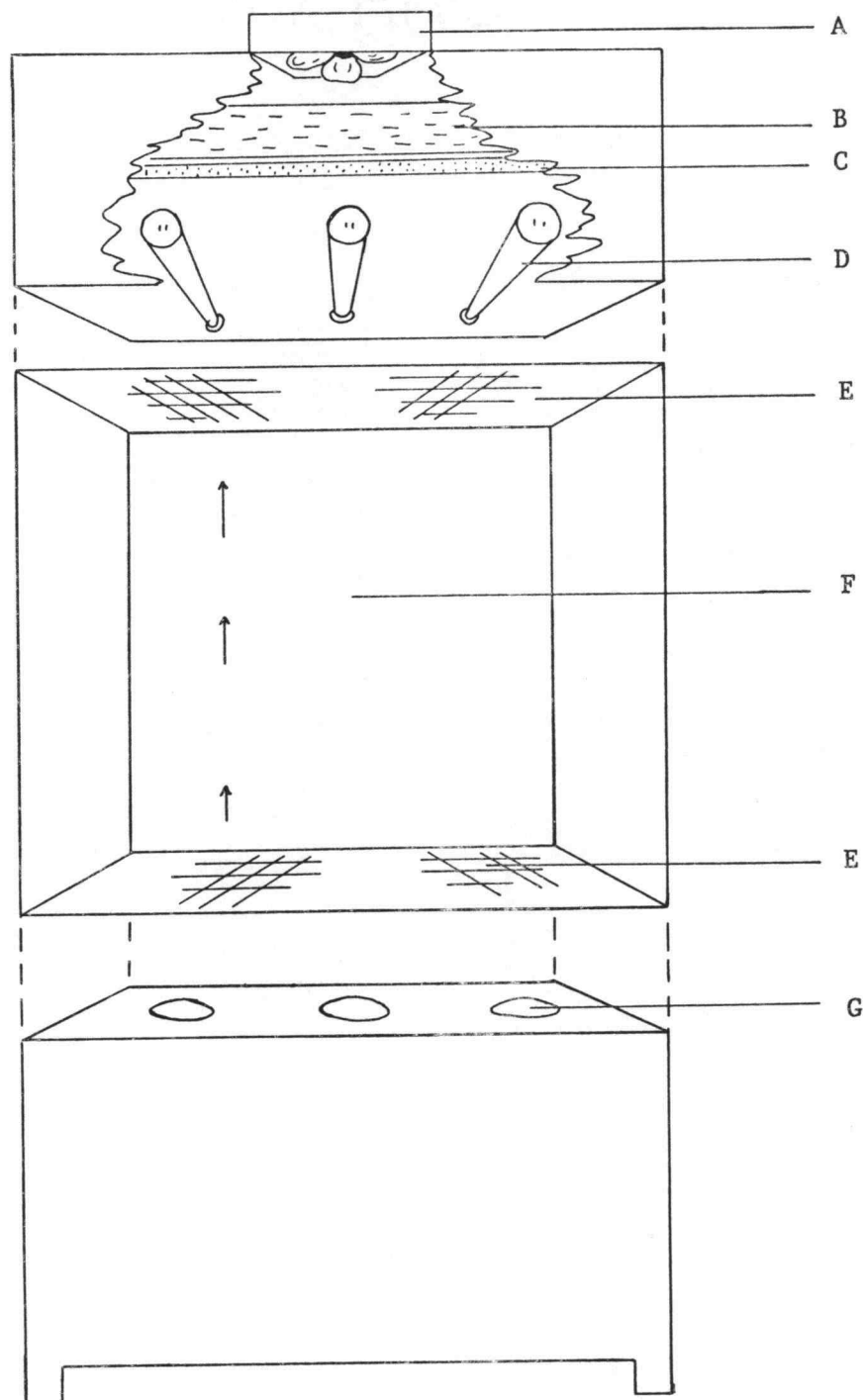
Figure 5. Exploded view of olfactometer for bioassay of attrac-
tants. Sections from top: control panel, flight
chamber, base. (A) Fan, (B) glass wool air baffle,
(C) perforated foil (D) fluorescent lamp, (E) screen-
ing, (F) transparent window, (G) air entry port.
(Arrows show direction of airflow).

caused by wild females in an infested area. The two study plots were located near Bellvue and Auburn, Washington.

A variation of the release test was run at the Auburn plot to study the influence of a host tree on male moths released downwind of the attractant. Attractant traps were put out in the absence of host foliage entirely; and as three traps with one each in a host pine, a broadleaf species, and in the absence of foliage (on a post). The latter was done by maneuvering the trap on the post until the three traps were equidistant and upwind from the release point. The traps were three to four yards apart and baited with an equal amount of attractant extract taken from a common batch.

The male release point was located 50-100 feet downwind of the traps. Most of the males in these experiments were not marked with any fluorescent pigments, since it was felt that marking could be detrimental to the moths' response capabilities and because there were no wild males in the area to confuse with the released males.

Results and Discussion

Necessity of the Attractant for Mating

The attractive female scent appeared necessary for each mating. Males became sexually stimulated only when downwind from a source of the attractant. The potency of this material was illustrated in mating tests when attractive females suddenly moved a few inches or were manually removed from their positions. Stimulated males continued to orient to the recently vacated position for several minutes after the female had moved. Males responding to such an area moved

about in circular fashion while exhibiting precopulatory motions.
This behavioral characteristic of *R. buoliana* males relegates the
importance of their visual perception of females to a very minor
role, since the female responsible for the attraction was often
positioned only a few inches away. This is in contrast with the
behavior of male gypsy moths (*Porthetria dispar* (L.)). Doane (1968)
observed that sexually stimulated males, which had responded to an
attractive decoy, would respond visually to virgin females located
6 inches downwind of them. In any case, however, visual perception
of females is of minor importance when compared to scent perception.

Usually only one or two females in a group of four to six were
attractive at a given moment. When other females in the vicinity of
the attractive one were inadvertently contacted by advancing males,
there was no attempt to copulate. This occurred when the other
females (sometimes manually positioned) were only a few inches from
the attractive one; however, stimulated males seemed unaware of their
presence and continued to respond to the immediate vicinity of the
attractive female. This was accepted as further evidence that the
attractant is prerequisite for each mating. Apparently the situation
described for the Oriental fruit moth, in which the mating of one
pair of insects seems to induce the mating of others (George 1965),
does not hold true in the case of the European pine shoot moth.

Variations in Female Attractiveness

A quantitative evaluation of female attractiveness was not

possible because of variations in numbers of males available at the different trapping locations. Gross differences in the ability of females to attract males were readily apparent, however. Numbers of males captured in a trap baited with a single unmated female ranged from 0 to 76. Some females (14 or 25 percent) captured no males even though located within infested trees. During the 2-year period, 11 of the 56 females (19.6 percent) captured 260 (72 percent) of the 351 males caught. Some of this disparity can be attributed to the placement of some traps in positions where greater numbers of males were available. This is not the full explanation, however, and much of the variation may be due to individual differences in the attractive potency of females. The female that lured 76 males to a trap, for example, was located in a very lightly infested pine approximately 100 yards from any other source of moths. Another trap, located in the same tree caught only four males.

These data demonstrate the great variation in attractiveness among females. Butt and Hathaway (1966) found similar evidence in studying the codling moth. Using field cages containing released males, it was found that 20 percent or less of the codling moth female baits attracted more than 60 percent of the trapped males. Doane (1968) reported that some gypsy moth females were incapable of attracting males. These differences in attractiveness could have an impact on reproduction since the more attractive females could be expected to complete fertilization with dispatch, whereas this process might be delayed or never consummated in the case of less favorably endowed females.

Several tests were also conducted using dead virgin or live mated females as bait. All such traps failed to attract males. Pointing (1961) also found no attraction to mated females. The possibility that mated females still contained but did not release pheromone was checked by preparing methylene chloride extracts of mated female abdomens. When tested as baits in the field, these extracts also failed to attract males. In contrast to this condition, Brady and Smithwick (1968) found that "noncalling" female *Plodia interpunctella* females were unattractive, but attractant could be extracted by solvent from either virgin or mated females.

Source of Pheromone Production

Methylene chloride was adopted as the standard solvent for extracting attractant from female tissue. The results of extraction with various solvents are shown in Table 5. Xylene was also consistent in producing active extracts; but, there was evidence that xylene extracts were repellent to males until the xylene had evaporated. Since xylene has a low evaporation rate when compared with methylene chloride it was not used.

Only the abdominal tips of unmated females yielded attractive extracts. Solutions of methylene chloride and other female body parts were unattractive.

The pheromone gland of female *R. buoliana* was found on the dorsum between the penultimate and terminal abdominal segments. These are the eighth and ninth abdominal segments which are comparatively small,

and are normally telescoped within the seventh segment. The gland is
a somewhat flattened, rounded structure composed of columnar epithe-
lial cells (Figure 6). It is very similar in appearance to the phero-
mone glands of the Oriental fruit moth, *Grapholitha molesta* (George
1965), the codling moth, *Carpocapsa pomonella* (Barnes, *et al.* 1966),
and a number of other olethreutids (Roelofs, *et al.* 1968). Extracts
of dissected gland tissues yielded a positive bioassay, but it proved
difficult to dissect only the glandular cells. George's (1965) bio-
assay procedure appeared more suitable. Unmated females were dorso-
ventrally compressed to forcibly protrude the terminal abdominal seg-
ments. The abdomen was pinned in this position leaving the gland
bearing region exposed. The gland, *in situ*, appeared maroon red
against the surrounding whitish intersegmental membrane. Fine needles
were inserted through the gland while carefully avoiding other tissue.
When introduced to the airstream of the olfactometer, the material on
the needles induced an immediate sexual response among males caged
under "twilight" conditions after first being held at high illumina-
tion for a 24-hour period.

Like the Oriental fruit moth (George 1965), but unlike the cab-
bage looper, *Trichoplusia ni* (Hübner) (Jefferson, *et al.* 1966), the
pheromone gland of *R. buoliana* is not eversible. The segments in
which it is contained, however, are often protruded during the "call-
ing" or attraction period preceding mating. During the "calling"
period the eighth and ninth segments are simply extended posteriorly.
The abdomen is never arched dorsally as in the case of some
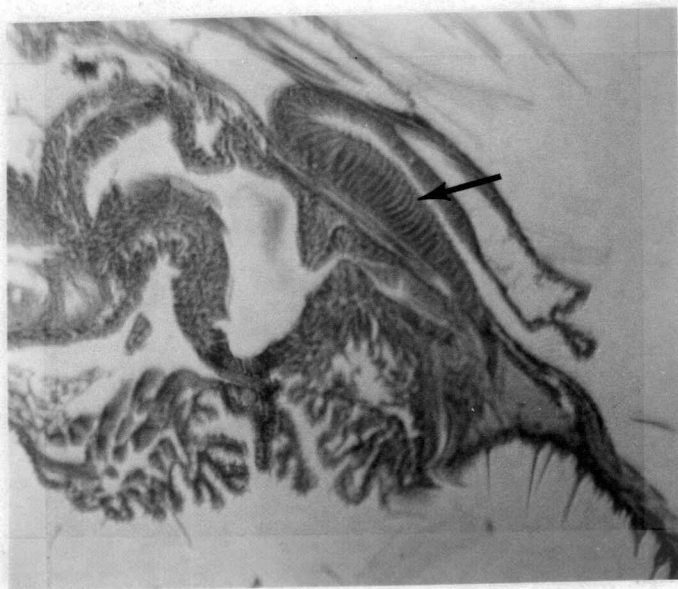
Figure 6. Lateral view of abdominal tip of R. *buoliana* female showing pheromone gland.

Lepidoptera (Brady and Smithwick 1968). Apparently, the gland-bearing segments of *R. buoliana* females are not always protruded during the receptive, attractive period for males have been observed responding to females which have not had the gland-bearing segments exposed. In order to see protrusion of these segments, the observer must first clip the distal half of the females' wings since they normally overlap the abdominal tip. Presumably, protrusion of the gland increases air exchange about the tissue, thereby aiding dissemination of the attractant. Another possibility is the protrusion of the surrounding segments exerts pressure on the tissue causing increased secretion.

The gland was slightly smaller in longitudinal dimension than in cross section. Average length was 275 microns compared to an average "width" of 325 microns (average of 8 females). Length of columnar cells, taken at the thickest portion of the gland center, ranged from 38 to 46 microns. In diameter the cells varied from 4 to 6.5 microns.

Sections of glandular tissue taken from mated females (48 hours after mating) showed no changes discernible by light microscopy. Although mated females no longer produce attractant, the gross gland structure apparently does not change.

Distance of Male Flight Response

The longest distance of male shoot moth response to the female attractant was 282 feet or approximately 100 yards (Table 6). Only one test was conducted over a longer distance (400 feet), and this resulted in no response. Adverse meteorological conditions or some

other factors, however, could have interfered in that particular test.

The distance response results could be challenged from the standpoint that captured males coincidentally flew toward the traps and actually responded to the scent only over the last few feet. It is known, however, that only minute quantities of attractant are necessary for male stimulation (Jacobson 1965) and this observation seems to support olfactory response over a distance of 100 yards. Further, previous testing with live, virgin female baits showed that traps positioned in areas of low moth population sometimes caught excessive numbers of males. A designation of "low population" refers to trees having so few damaged buds that a cursory inspection reveals little or no evidence of infestation. In contrast, a few minutes inspection of trees having heavy infestations reveals numerous damaged buds (10-100 depending on available foliage). The only explanation for the phenomenon of excessive male response was males responding from some distance away. Most conclusive in this regard was the capture of over 70 males in 2 traps located in a lone pine which showed very little signs of infestation. This tree was in the open and the nearest source of additional infested pines was just over 100 yards distant. The work of Rau (1929) also lends strong support to this view. Working with large silk moths (*Samia* spp.), Rau released marked males one-half mile from his room in which he had confined female moths. The released males sometimes took little more than an hour to return to the room through an open window. Surely, this cannot be attributed to random flight to the vicinity of the open window!

Released males were capable of locating extract traps positioned on stakes away from any kind of tree foliage. It became evident in subsequent tests, however, that foliage did provide some kind of supplementary attraction to stimulated males. Significantly higher numbers of males responded to baits located in host pines as opposed to traps in the open or in nonhost trees (Tables 7 and 8). Traps located in foliage of nonhost trees (*Alnus* or *Corylus* spp.) captured more than those located on stakes and away from any foliage (Table 8).

The stimulus responsible for the supplementary attraction is unknown. Perhaps it is associated with orientation toward the host silhouette such as Henson (1962) found for a bark beetle. Or, possibly flying males detect particular wave lengths of infra-red radiation given off by foliage as has been theorized by Callahan (1965). In any case, knowledge that males respond to other stimuli when sexually stimulated is of interest. Attractant traps designed to control or detect shoot moths would, for example, be more effective according to their positioning in relation to the host plant.

CONCLUSIONS

Production of the sex pheromone by each female was necessary for mating to occur. Based on their capacity to attract numbers of male moths, individual females varied considerably in attractant potency. When used as individual baits, a few females captured a majority of the males even when traps were located side by side. Other females failed to attract any males at all.

The source of pheromone production was a gland located on the dorsum of the abdominal tip in the intersegmental area between the penultimate and terminal segments. These segments are small, and normally telescoped within the seventh segment. The gland is oval, somewhat flattened dorso-ventrally, and is composed of columnar epithelial cells.

Males were attracted from distances up to 100 yards to sex attractant baits. Males responded best to traps located in host pines, as compared to traps in the open or in nonhost foliage.

TABLE 5.   ATTRACTION OF EXTRACTS OF UNMATED FEMALE
ABDOMINAL TIPS USING VARIOUS SOLVENTS.
(A) = ATTRACTIVE, (U) = UNATTRACTIVE, (NT) =
NO TEST.

Solvent	Replication		
	Extract 1	Extract 2	Extract 3
Methylene chloride	A	A	A
Benzene	U	U	U
Ethanol (95%)	U	U	U
Methanol	U	U	NT
Xylene	A	A	A
Acetone	U	U	NT
Toluene	U	U	NT

TABLE 6. DISTANCE RESPONSE OF RELEASED *R. BUOLIANA* MALES
TO SOURCES OF FEMALE ATTRACTANT.

Lure	Number males	Distance from traps in feet	Males captured
Virgin female	30	65	4
" "	25	150	4
" "	26	282	3
" "	30	400	0
$CHCl_2$ extract of female abdomens	80	50	6
" "	65	50	2
" "	52	50	9

TABLE 7. *RHYACIONIA BUOLIANA* MALES CAPTURED IN PHEROMONE
TRAPS LOCATED IN HOST TREES, NONHOST TREES, AND
IN THE OPEN. ANALYSIS OF VARIANCE OF RESULTS
(FREESE 1967).

Test	Males released	Treatment		
		Traps + pine	Traps + nonhost	Trap on stake
1	135[1]	9	7	6
2	"	26	13	5
3	"	20	15	9
4	"	27	20	5
		82	55	25
		$\bar{x} = 20.5$	$\bar{x} = 13.75$	$\bar{x} = 6.25$

Source of variation	Degrees of freedom	Sum of squares	Mean squares
Treatment	2	406.5	203.25
Error	9	302.5	33.61
Total	11	709.0	

F = 203.25/33.61 = 6.058, Tabular $F_{.05} = 4.26$

Conclude: Significant difference among treatments.

[1] Average of 135 males per replicate. Numbers actually
varied due to differential mortality.

TABLE 8. *R. BUOLIANA* MALES CAPTURED IN PHEROMONE TRAPS
LOCATED IN HOST PINES, NONHOST TREES, AND IN
THE OPEN. TUKEY'S TEST FOR COMPARISON AMONG MEANS
(SNEDECOR 1956). DIFFERENCES GREATER THAN 5.7156
SIGNIFICANT AT 5% LEVEL.

Treatment	Treatment differences		
	Ranked means	$\bar{x} - 6.25$	$\bar{x} - 13.75$
Attractant trap in pine	20.5	14.25[1]	6.75[1]
Attractant trap in nonhost	13.75	7.50[1]	
Attractant trap in open (on stake)	6.25		

[1] Denotes significant difference.

CHAPTER IV

OTHER BIOTIC FACTORS INFLUENCING REPRODUCTION

Mating efficiency and fecundity are very likely to be influenced by such factors as adult age and mating frequency. Males, for example, may require a maturation period before they are responsive to the female attractant. Females could vary in their receptivity to males according to age, and fecundity could also vary accordingly. These factors must be known to reach a full understanding of the insect's biology and to achieve maximum production in laboratory culture.

The objectives of this portion of the study were to analyze the influence of adult age on mating efficiency and fecundity. Mating frequency of both sexes was also considered, particularly from the standpoint of the possible influence on egg fertility.

Study Procedures

Influence of Adult Age on Reproduction

Male age.--Males were classified into six age categories before exposure to females. Aging was accomplished by holding males in 100 x 15 mm. dishes at room temperature (72° F. $\pm$ 3°) for the specified time period. No more than two males were confined in a single dish, and a water saturated cotton roll was supplied to prevent dessication.

The six male age categories ranged from 0.5-1.5 days (12-36 hours) to 8.5-10.5 days. These ages were based on the age males would

reach at the time mating occurred in the tests. A given test ran for two light-dark cycles so that matings could have occurred a full 24 hours apart within a given category. Standard lighting conditions consisted of 16 hours of light (230-foot candles), 1 hour of twilight (< 2-foot candles), and 7 hours of darkness. For a given treatment (age category), 120 males were tested with 60 females. In each of 10 replicates, 12 males and 6 females (6 males and 3 females per chamber) were tested in two of the vertical airflow mating chambers (see Chapter II). Females used in these tests were all 48 hours old or less. A standard temperature of 73° F. ($\pm$ 2°) was maintained with relative humidity 50-65 percent. Mating efficiency was based on number of copulations per replicate.

A number of males were also kept in the aging dishes until death to determine male longevity.

Female age and mating efficiency.---The aging procedure for females and experimental arrangement for testing the influence of age on mating was generally the same as used for testing males. Females were categorized into seven age groups rather than six. These age groups ranged from 0.5-1.5 days to 6.5-7.5 days. Ten replicates were run for each treatment with 6 females exposed to 12 males in each replicate (3 females and 6 males per mating chamber). All males were 1-3 days old when placed with the females.

Female age and fecundity.---The purpose of this test was to record the effect on fecundity when mating is delayed. Females were classified into four age groups and aged as described above. These age

categories were 0.5-1.5, 1.5-2.5, 2.5-4.5, and 4.5-6.5 days. These females were exposed to 1-3 day-old males until 20 fertile females in each category had been obtained. After mating, females were individually placed in 100 x 15 mm dishes to oviposit, each dish being provided with a cotton roll saturated with water. Again, temperature and relative humidity were kept at 72° F. ($\pm$ 3°) and above 50 percent. Females were held for oviposition until death; fecundity was then compared between age groups to detect any significant differences.

Records were also kept on longevity of females from the youngest age group (0.5-1.5 days) and the relative numbers of eggs laid each day after mating.

Mating Frequency

Mating frequency of males.--Single males, less than 36 hours old, were caged with three virgin females in vertical airflow mating chambers. The cages were checked daily, and any mated females removed and individually confined in 100 x 15 mm dishes for oviposition. These females were replaced in the mating chamber with freshly emerged, unmated females so that an excess of females was always available to the male. This process was continued for each male until the male died, or failed to mate on three successive evenings. Records of fecundity and fertility were kept separately for all females mated by a particular male. After oviposition was completed, each female was dissected and checked for presence and

condition of a spermatophore (vesicle containing spermatozoa).

Males for this test were collected as last-instar larvae in the field and brought into the laboratory to complete development. This seemed advisable to avoid any artifact that might arise from use of laboratory stock. A total of 50 males were painstakingly evaluated in this manner. With this procedure, it was hoped that mating frequency of males under optimal conditions could be determined. Also, if multiple matings occurred, it was expected that information could be obtained on the effects of later matings and time intervals between matings on fertility.

Female mating frequency.--Williams (1941) determined that with each pairing a Lepidoptera male deposits a spermatophore in the female bursa copulatrix. Thus, mating frequency of female moths can be determined by dissecting females and counting spermatophores. For this purpose, R. buoliana females were captured in infested areas using nylon insect nets. Only undisturbed, free-flying females were captured since Pointing (1961) found that female flight rarely occurred prior to mating.

Laboratory experiments were also conducted in mating cages using previously mated females. Direct observations were made to detect evidence of sex attraction or additional matings. Both horizontal and vertical airflow mating cages were used for this purpose.

Results and Discussion

Influence of Adult Age on Reproduction

Male age and mating efficiency.--Males in the four youngest age classes, ranging from 0.5 to 4.5 days, had the highest mating efficiency. No significant differences in mating efficiency were found among these four age categories, and they were all significantly higher than any of the older age groups tested (Tables 9 and 10). The fact that mating efficiency of the youngest age group (0.5-1.5 days) was among the highest leads to the conclusion that _R. buoliana_ males do not require a maturation period between emergence and mating.

From these data it was evident that males 4.5 days old or less should be used in laboratory breeding programs when maximum progeny production is the primary objective. This situation holds despite the fact that 4.5 days is only half the average longevity of males. Tests showed 71 males lived an average of 9.1 days; the range of longevity was 3-15 days. Males aged 4.5-8.5 days did accomplish considerable mating (Table 9), but apparently sufficient vigor is lost after 4.5 days to cause the relative decrease in efficiency.

Influence of female age on mating and fecundity.--Female age up to 4.5 days had no significant influence on mating efficiency (Tables 11 and 12). Tests with older females also produced considerable mating (over 50 percent), but significantly less than females less than 4.5 days old. These results indicated that females up to 4.5 days old should be used for laboratory breeding; however, the fecundity of aged females must also be considered. Tables 13 and 14 show significantly fewer eggs oviposited by females more than 2.5 days old at the time of fertilization. These data showed the

breeding of females 2.5 days old or less would be most productive.

The rapid loss of egg potential by aging could have importance in field populations if inclement weather or some other factor prevented mating for any length of time. The reason for the loss in fecundity is probably associated with female behavior. Females held in dishes for aging, commonly oviposited infertile eggs as early as the second evening following emergence. While the number of infertile eggs was fewer than if mating had occurred, they were substantial and sufficient to depress fecundity. This phenomenon of virgin females ovipositing infertile eggs was also observed by Pointing (1961) and Torgerson (1964). Torgerson claimed that such females no longer produced sex attractant, and Pointing reported that at least some of them were unattractive. Both of these observations imply that mating may not occur with such females. In the present study, however, laboratory tests showed that such females mated and produced fertile offspring, and that methylene chloride extracts of abdomens from these females were attractive to males.

Despite a relatively long life span, the majority of eggs were deposited during the first few evenings after mating (Figure 7). Longevity of females at 72° F. ($\pm$ 3°) was slightly greater than found for males, averaging 12.3 days for 78 individuals. The life span ranged from 5-31 days. Eggs were continually produced throughout the females' life span although at an ever decreasing rate. This resulted in a significantly higher fecundity for the longer lived females (Table 15).
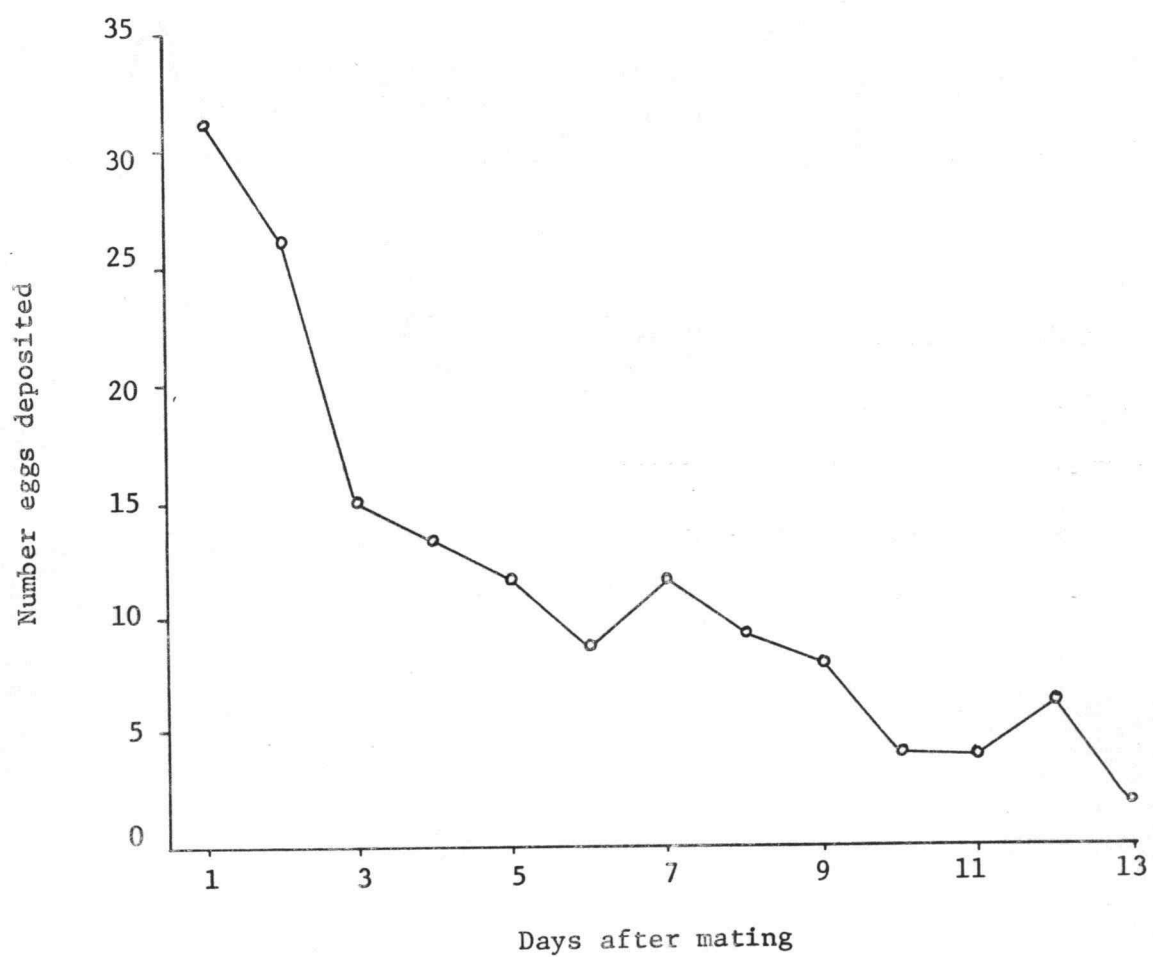
Figure 7. Average daily oviposition by surviving females of 16 member group.

The 50 females represented in the two youngest age groups of Table 13, oviposited an average of 126 eggs. This is somewhat higher than a mean of 109 eggs deposited by 36 wild females captured in the field while in copula. These figures are not greatly different from the fecundity of 116 reported by Pointing (1961), but differ considerably from the 74.5 eggs per female reported by Friend and West (1933) or the 168 eggs per female reported by Miller and Neiswander (1955). The low figure reported by Friend and West could have been caused by adverse caging conditions which may have resulted in female aging prior to fertilization. Miller and Neiswander's high figure was based on six females, which was probably too small a sample. The possibility for error in the latter case is clear when the variation in fecundity is considered. Eggs deposited by the 50 females in the first two age groups of Table 13, for example, ranged from 27 to 245.

Mating Frequency

Mating frequency of males.--European pine shoot moth males are capable of multiple matings. Sixty-six percent of the 50 males tested mated at least twice (Figure 8); only six specimens (12 percent) failed to mate at all. Three males mated more than four times with two fertilizing five females and one fertilizing six females. Previously, no higher than four matings per male had been reported (Pointing 1961). Under the optimal laboratory conditions provided, the 50 males mated an average of 2.24 times.
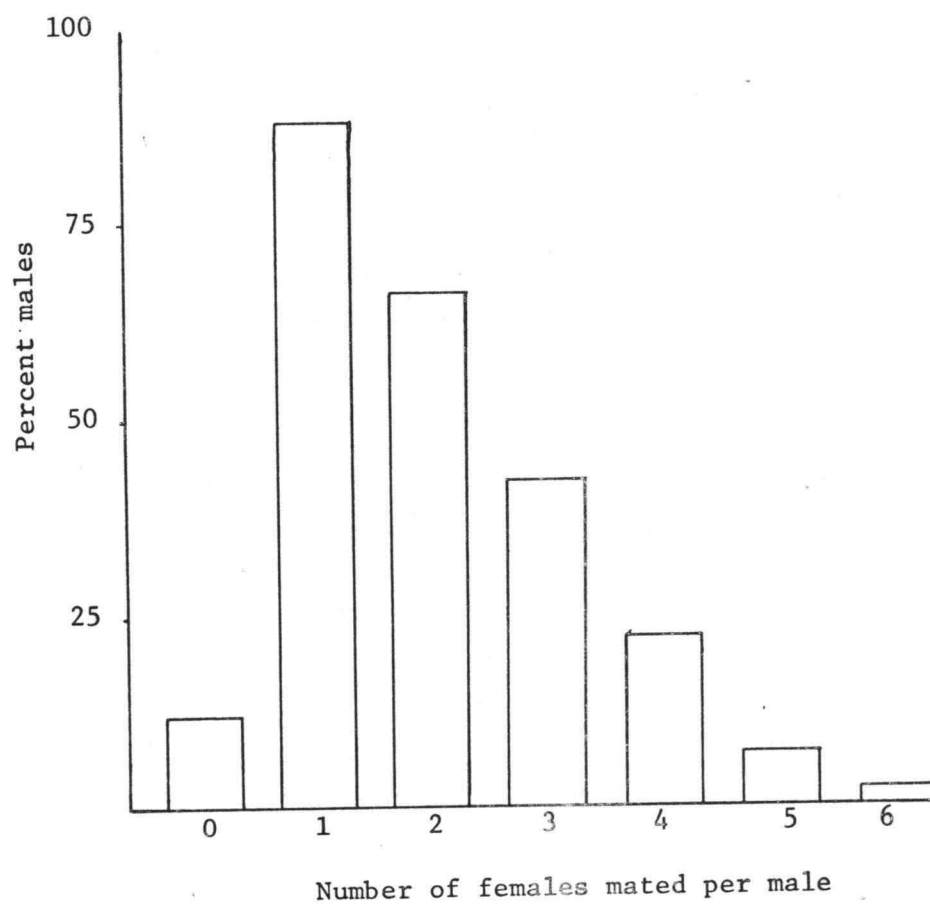
Figure 8. Mating frequency of 50 pine-reared *R. buoliana* males.

Under field conditions it is doubtful that such frequent male mating would occur. In the laboratory an excess of females was provided each male with literally no competition from other males. A female predisposed to mating under field conditions is generally approached by several males even though only one is eventually successful in pairing with her. This competition among males for available females undoubtedly lowers the mating frequency of an individual male as compared to that shown in the laboratory.

Pointing (1961) showed an apparent increase in sterility with successive male matings, but stated that sterile eggs were seldom observed in the field. Table 16 which related multiple male matings and incidence of sterile eggs appears to show little difference between mating categories. The time interval between successive matings was also a factor checked for its effect on fertility. Table 17 compares number of sterile eggs produced by females mated 24 and 48 hours after the respective male's previous pairing. No significant difference between these categories was found. Based on these data, male mating frequency and interval between male matings were not the factors responsible for the occurrence of sterility.

Evidently, there is a natural incidence of sterility in the field despite Pointing's (1961) remark to the contrary. Friend and West (1933) also found partial sterility of the egg complement of some females. In the present study, some field-captured females were observed to deposit only sterile eggs, although copulation had definitely taken place. In the laboratory study on male mating

frequency (which utilized wild males), 6 of the 112 mated females (5.4 percent), deposited only sterile eggs. The remaining 106 females produced primarily fertile eggs, but 43 of these oviposited at least some infertile eggs (8.9 per female).

Female mating frequency.--Behavioral observations and morphological evidence indicated that females mate no more than once if copulation occurs with a normal male and a spermatophore and sufficient sperm are deposited in the female's bursa. Mating trials with previously mated females caused no sexual stimulation in males. Females, once fertilized, apparently are never again sexually attractive.

Dissections of spermatophores from mated females confirmed the limitation of one mating per female. Figure 9A illustrates a female bursa containing a freshly deposited male spermatophore, and Figure 9B shows the same bursa with the spermatophore removed. The relative size of these structures would seem to preclude the possibility of a second functional mating. The corpus or bulbous head of the spermatophore collapses with the passage of the sperm and associated fluids to the spermatheca. In this condition it still fills the bursa to capacity, however, leaving little space for an additional spermatophore. In some females the corpus end of the spermatophore was found to eventually break up but this occurred toward the end of the life span when most of the female's eggs were deposited, and the likelihood of such a female mating a second time would be negligible. Further morphologic evidence was obtained by dissecting 103 females that had been in flight or ovipositing when captured in
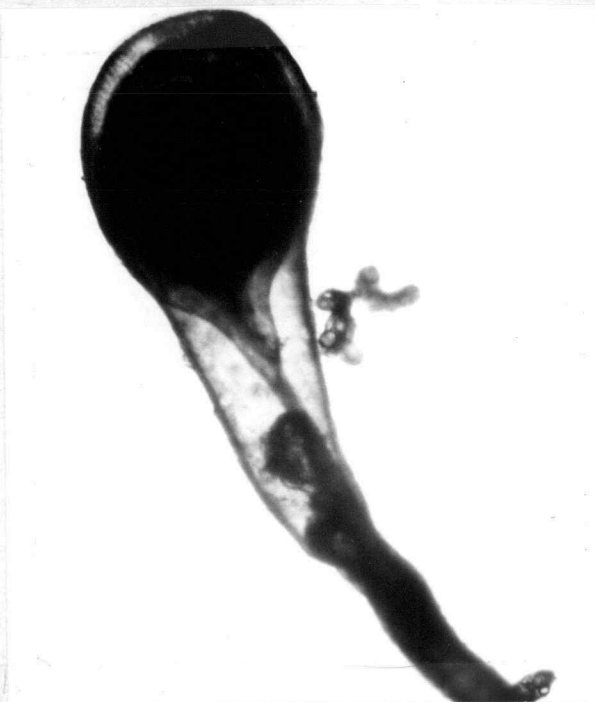
A

B

Figure 9.   A.   Female bursa copulatrix containing male spermatophore; B.   spermatophore
removed (three hours after copulation).

the field. Two of these females contained no spermatophore; the remaining 101 contained a single spermatophore.

As a result of Williams' (1941) study, many workers have depended on dissections of female moths for counts of spermatophores as a reliable estimate of successful matings by the female. George and Howard (1968), however, found this method to be unreliable in studies of the Oriental fruit moth, *Grapholitha molesta*. Successive male matings produced small spermatophores or none at all, even though sperm was transferred and fertile eggs laid by the female. Forty-three of 152 fertile Oriental fruit moth females were found to contain no spermatophore. If this situation were also true with *R. buoliana*, multiple female matings could occur with no evidence in the form of a spermatophore. In the case of *R. buoliana*, however, insemination without spermatophore deposition was rare. Dissections of the 112 mated females in the male mating frequency test showed that only three contained no spermatophore and yet laid fertile eggs. Even those paired with males mating for the fourth or fifth time contained spermatophores although the one paired with the male mating six times did not. From these data, it can be concluded that presence of a spermatophore is not necessary for fertilization in European pine shoot moth females, but for practical purposes it is a reliable indication of number of matings.

A laboratory artifact in female behavior.--Although the above information points conclusively to a limitation of one mating per female, multiple female mating was observed in the laboratory. This

phenomenon resulted from the use of defective laboratory reared males. These matings were normal, however, in the sense that copulation lasted at least 50 minutes. Dissection of females mated by these males showed no spermatophores deposited, and a total lack or subnormal quantity of sperm in the spermatheca. These females laid fewer eggs than normal, and less than 24 percent of them deposited fertile eggs. The spermatheca of a female mated with a defective male appeared translucent in saline solution. In contrast, the spermatheca from a female mated with a normal male was slightly swollen, opaque white, and yielded large quantities of sperm. Apparently, the lack of a spermatophore in the bursa, subnormal quantities of sperm, or some other male factor caused these females to remain receptive to males and mate again.

The specific cause for the defective male performance is still unknown. The situation can be corrected, however, by the exchange of food medium for freshly prepared diet three to four weeks after larval eclosion. Larval development requires an extended time period (average 9-10 weeks), and apparently a breakdown in some nutrient essential to sperm and/or spermatophore development occurs.

This phenomenon is only briefly mentioned here, because it resulted in an aberration of normal behavior. Cursory observation of this insect in the laboratory could lead to erroneous conclusions if the worker was unaware of these results of inadequate larval rearing.

Miscellaneous male behavior.--Another behavioral aspect influencing mating efficiency concerns the male "fatigue" factor. In both

field and laboratory, it was not uncommon to observe several males attracted to a single female. Even after mating was in progress, the extra males remained stimulated for several minutes and continued to approach the copulating female. Eventually the unsuccessful males became quiescent and remained in this fatigued condition even when a second female became attractive. This behavior was observed when fresh males were added to cages during a test. Usually only the fresh males responded to the second attractive female.

The quantitative effect on male response caused by the fatigue factor is illustrated in Figure 10. Caged males, conditioned 12 hours at 200-foot candles then held at simulated twilight, were exposed to attractant laden air for successive 2-minute periods following 10-minute rest intervals. Fewer males responded and for shorter periods with successive exposures. This could have some relevance in the field with low populations or elsewhere that male numbers were in low supply. Females that are naturally more attractive (see Chapter III) would attract more males than others. Only one male would mate with such a female, but due to the "fatigue" factor other males would be less responsive to female attractant and probably useless as potential mates to other females during that evening. Less attractive females would then be subject to aging plus hazards of predation for another 24 hours before having another opportunity to be fertilized.
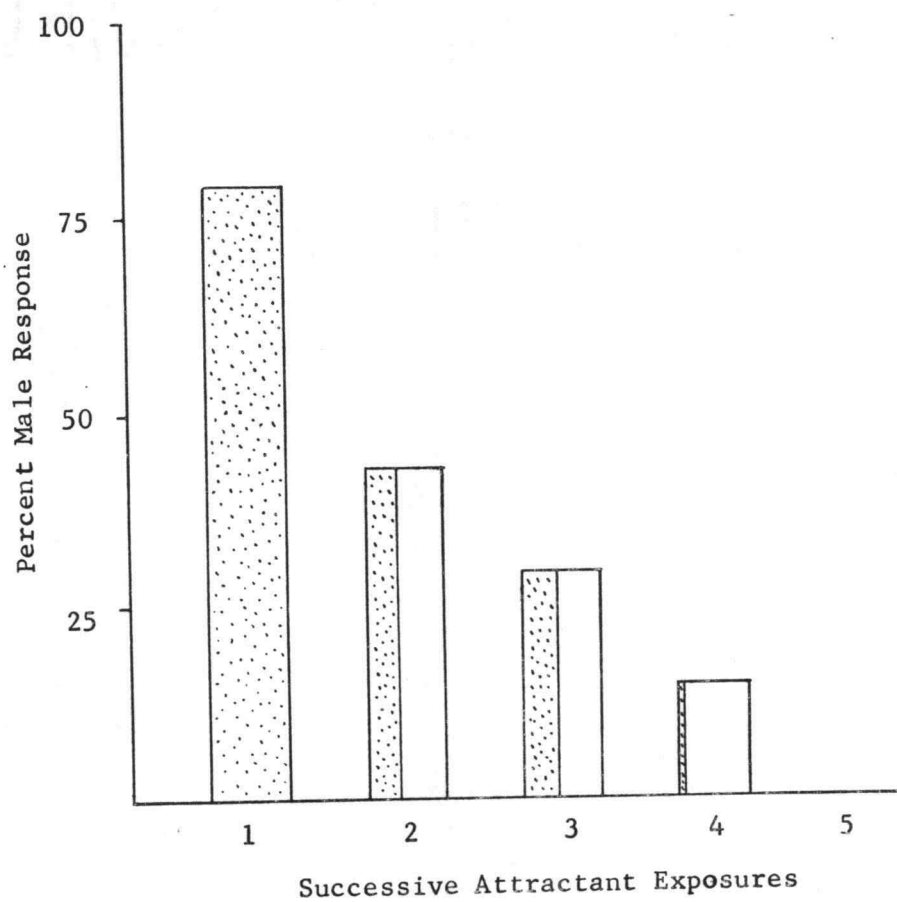
Figure 10. Quantity and duration of male response to sex attractant during successive 2-minute exposures with 10-minute rest intervals. (Shaded area on bars show relative time period that at least one male remained stimulated.)

CONCLUSIONS

Mating efficiency decreased for both sexes at age 4.5 days or older. Females older than 2.5 days at the time of mating oviposited significantly fewer eggs than younger females. For maximum progeny production males less than 4.5 days old and females less than 2.5 days old should be bred. Although females produced eggs continually throughout their life span, the majority of oviposition occurred the first few evenings after mating. Average fecundity for females less than 2.5 days old when mated was 126 eggs.

European pine shoot moth males were capable of multiple matings. Only 6 of 50 pine-reared males failed to mate, while 66 percent mated at least twice. Three males successfully fertilized five females and one performed a sixth viable mating. Average incidence of copulation was 2.24 females per male.

Females that had mated with normal males were not receptive to additional matings and no longer produced sex attractant. Some females that had paired with defective, laboratory reared males were observed to mate more than once. These defective, laboratory reared males transferred no spermatophore and little or no sperm. This implied that a spermatophore or some other male factor deposited in the female controls receptivity of females for mating. Normally, males transfer a spermatophore even after multiple matings and females mate only once.

The cause of the sterile, laboratory-reared males is as yet unknown, but has been traced to the breakdown of some dietary

constituent. A lesser degree of sterility is also present in field populations of this insect. Five percent of 112 females that had mated with pine-reared males deposited only sterile eggs, and 41 percent of the remaining 106 females oviposited at least some infertile eggs (8.9 per female).

TABLE 9. EFFECT OF MALE AGE ON MATING EFFICIENCY. ANALYSIS
OF VARIANCE OF RESULTS (FREESE 1967).

Male age in days (6 possible matings per replicate)					
0.5-1.5	1.5-2.5	2.5-4.5	4.5-6.5	6.5-8.5	8.5-10.5
3	4	5	2	2	0
6	4	4	6	2	0
6	2	1	6	5	1
5	4	5	1	2	1
5	4	4	3	3	1
2	4	6	4	3	0
6	5	5	3	3	2
3	5	5	4	4	3
3	6	6	3	5	0
2	6	4	3	2	3
41	44	45	35	31	11

Source of variation	Degrees of freedom	Sums of squares	Mean squares
Treatment	5	80.75	16.15
Error	54	104.10	1.928
Total	59	184.85	

F = 16.15/1.928 = 8.377, tabular $F_{.01}$ = 3.38

Conclude: Significant difference among age groups.

TABLE 10.   EFFECT OF MALE AGE ON MATING EFFICIENCY.
            TUKEY'S TEST FOR COMPARISON AMONG MEANS
            (SNEDECOR 1956).   DIFFERENCES GREATER
            THAN .582 (D) SIGNIFICANT AT 5 PERCENT
            LEVEL.

Treatment		Treatment differences				
Male age in days	Ranked means	$\bar{x} - 1.1$	$\bar{x} - 3.1$	$\bar{x} - 3.5$	$\bar{x} - 4.1$	$\bar{x} - 4.4$
2.5-4.5	4.5	$3.4^{1/}$	$1.4^{1/}$	$1.0^{1/}$	.4	.1
1.5-2.5	4.4	$3.3^{1/}$	$1.3^{1/}$	$.9^{1/}$	.3	
0.5-1.5	4.1	$3.0^{1/}$	$1.0^{1/}$	$.6^{1/}$		
4.5-6.5	3.5	$2.4^{1/}$	.4			
6.5-8.5	3.1	$2.0^{1/}$				
8.5-10.5	1.1					

$^{1/}$ Denotes significant difference.

TABLE 11. EFFECT OF FEMALE AGE ON MATING EFFICIENCY. ANALYSIS OF VARIANCE OF RESULTS (FREESE 1967).

Female age in days (6 possible matings per replicate)						
0.5-1.5	1.5-2.5	2.5-3.5	3.5-4.5	4.5-5.5	5.5-6.5	6.5-7.5
5	3	5	4	6	4	3
6	6	5	6	5	1	4
5	4	6	4	5	2	3
5	5	5	4	2	4	5
6	6	6	5	4	3	1
5	4	6	6	4	5	5
5	5	3	5	3	5	4
3	4	4	4	1	5	3
4	6	5	2	3	3	2
4	4	3	5	1	5	3
48	47	48	45	34	37	33

Source of variation	Degrees of freedom	Sums of squares	Mean squares
Treatment	6	27.54	4.59
Error	63	100.40	1.59
Total	69	127.94	

$F = 4.59/1.59 = 2.887$, $F_{.05} = 2.25$

Conclude: A significant difference among age classes.

TABLE 12.--EFFECT OF FEMALE AGE ON MATING EFFICIENCY. TUKEY'S TEST FOR COMPARISON AMONG MEANS (SNEDECOR 1956). DIFFERENCES GREATER THAN .525 (D) SIGNIFICANT AT 5 PERCENT LEVEL.

Treatment		Treatment differences				
Female age in days	Ranked means	$\bar{x}$ – 3.3	$\bar{x}$ – 3.4	$\bar{x}$ – 3.7	$\bar{x}$ – 4.5	$\bar{x}$ – 4.7
0.5–1.5	4.8	1.5[1]/	1.4[1]/	1.1[1]/	.3	.1
2.5–3.5	4.8	1.5[1]/	1.4[1]/	1.1[1]/	.3	.1
1.5–2.5	4.7	1.4[1]/	1.3[1]/	1.0[1]/	.2	
3.5–4.5	4.5	1.2[1]/	1.1[1]/	.8[1]/		
5.5–6.5	3.7	.4	.3			
4.5–5.5	3.4	.1				
6.5–7.5	3.3					

[1]/ Denotes significant difference.

TABLE 13.   FECUNDITY AS INFLUENCED BY FEMALE AGE AT TIME OF
FERTILIZATION.   ANALYSIS OF VARIANCE OF RESULTS
(FREESE 1967)

Female age in days when mated			
0.5-1.5	1.5-2.5	2.5-4.5	4.5-6.5

Number eggs oviposited

63	111	119	37
121	116	22	27
178	87	64	108
128	149	53	24
97	135	132	27
164	34	74	128
204	201	28	2
161	61	187	71
148	162	133	13
245	37	102	48
101	192	41	77
88	118	183	10
154	50	37	196
105	27	86	122
222	55	66	158
59	224	80	114
180	111	107	194
59	181	24	76
93	118	24	60
33	191	118	93
114	138	53	131
68	83	28	85
112	215	37	136
220	158	55	20
120	124	120	48
3,237	3,078	1,973	2,005

$\bar{x} = 129.48$ $\bar{x} = 123.12$ $\bar{x} = 78.92$ $\bar{x} = 80.20$

Source of variance	Degrees of freedom	Sums of squares	Mean squares
Treatment	3	55141.79	18380.597
Error	96	293152.72	3053.674
Total	99	348294.51	

$F = 18380.597/3053.674 = 6.019$, $F_{.01} = 3.99$

Conclude: Significant difference among age classes.

TABLE 14.   FECUNDITY AS INFLUENCED BY FEMALE AGE AT
            TIME OF FERTILIZATION. TUKEY'S TEST FOR
            COMPARISONS AMONG MEANS (SNEDECOR 1956).
            DIFFERENCES GREATER THAN 41.003 (D)
            SIGNIFICANT AT THE 5 PERCENT LEVEL.

Treatment		Treatment differences		
Female age when mated	Ranked means	$\bar{x} - 78.92$	$\bar{x} - 80.20$	$\bar{x} - 123.12$
0.5-1.5	129.48	50.56[1]	49.28[1]	6.36
1.5-2.5	123.12	44.20[1]	42.92[1]	
4.5-6.5	80.20	1.28		
2.5-4.5	78.92			

[1] Denotes significant difference.

TABLE 15.   INFLUENCE OF FEMALE LONGEVITY ON FECUNDITY.
THE "T" TEST FOR UNPAIRED PLOTS (FREESE 1967).

Longevity of mated females	
A (greater than 8 days)	B (less than 8 days)
199	131
118	145
163	178
184	151
144	105
142	98
180	220
187	17
158	110
26	32
165	83
85	24
185	192
91	120
221	33
161	98
158	1,737
124	
169	$\bar{x} = 108.56$
215	
104	
3,179	

$\bar{x} = 151.38$

$$T = \frac{\bar{x}_A - \bar{x}_B}{\bigcup s^2 \dfrac{(n_A + n_B)}{(n_A)\,(n_B)}} = 2.4103$$

$$T_{.05} = 2.031$$

Conclude: A significant difference in fecundity between groups

TABLE 16. EGG FERTILITY AS RELATED TO MULTIPLE MALE MATING.

Number of: male matings :	Frequency :	:Females at least partially fertile	:Percent fertile eggs :produced by fertile : females
1	33	31	97.0
2	26	23	95.8
3	18	18	93.7
4	9	8	97.5
5 & 6	4	4	96.8

TABLE 17.   RELATIONSHIP OF STERILE EGG PRODUCTION AND TIME INTERVAL
BETWEEN MULTIPLE MALE MATINGS.   PAIRED "T" ANALYSIS
(FREESE 1967).

Interval between: male matings	Number of sterile eggs produced by mated females													Mean
24-hr. interval	0	5	0	0	0	0	3	0	17	9	0	15	3	4.0
48-hr. interval	0	0	0	0	0	13	0	1	6	3	5	3	0	2.15
Difference	0	5	0	0	0	-13	3	-1	11	6	-5	12	3	1.85

$$T = \frac{\bar{x}_{24\ hr.} - \bar{x}_{48\ hr.}}{\sqrt{\dfrac{s^2_d}{n}}}$$

where: n = number of pairs

s^2_d = variance of difference

$$T = \frac{1.85}{\sqrt{\dfrac{47.97}{13}}} = .963 \quad T_{.05} = 2.179$$

Conclude: No significant difference.

CHAPTER V

INFLUENCES OF SOME PHYSICAL FACTORS ON REPRODUCTION

This chapter deals primarily with the effects of temperature and humidity on mating and oviposition. The influences of light have been covered previously (Chapter II, Green 1962, 1965), and this factor will be discussed only briefly in this section.

Rather than defining precise physical limits, this portion of the study was designed to determine suitable ranges of temperature and air moisture for optimum mating and oviposition in the laboratory. Green (1962) has reported the only definitive work regarding the effect of temperature on adult activity. He related temperature to the degree of evening flight activity. This was probably roughly analogous to mating and ovipositional activity since these were undoubtedly the purposes of the moth flight. Green recorded the greatest activity at temperatures of 20-24° C. (68-75° F.), during periods of favorable light intensity.

Apparently no work has been done regarding the effect of varying air moisture on reproductive biology of *R. buoliana*. This aspect could be important since it is known that varying humidities can modify the influence of temperature on a particular life process (Lowry 1967). Pilon (1966), for example, found the influence of temperature on the life span of male sawflies varied with relative humidity. More pertinent to reproductive biology, Hamilton (1950) found female locusts laid higher numbers of egg pods at relative

humidities of 65-75 percent regardless of the temperature. For these reasons, it was decided to test the effects of varying humidity on *R. buoliana* reproduction.

Study Procedures

Temperature and Humidity Control Apparatus

Temperature and humidity were controlled in two vertical airflow mating cages by connecting them in a closed airflow system to a cooling unit and an air mixing chamber. Figure 11 illustrates this overall cage arrangement. The cooling unit[5/] was a surplus portable unit used originally for controlling temperature in oxygen tents. The mixing chamber consisted of a glass-wool insulated plywood box 22x24x34 inches high. It has a small fan[6/] mounted in the top center for continual mixing of air. Two incandescent lamp plugs were also mounted on the top to provide a heat source for higher temperature experiments. Heavy gauge aluminum foil lined the chamber interior to prevent overheating of the wooden frame.

With this arrangement a temperature range of 65 to 92° F. was available in the laboratory. Within this range, a given temperature could be maintained $\pm$ 1° F. Only one thermostat was used which was mounted inside a mating chamber and connected to the cooling unit. Only one thermostat was necessary since the main problem was in

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5/ Manufactured by Continental Hospital Industries.

6/ Sprite Fan, Model SPsAs, 12 watt, Allied Electronics 4000 Aurora Ave., North, Seattle, Washington.
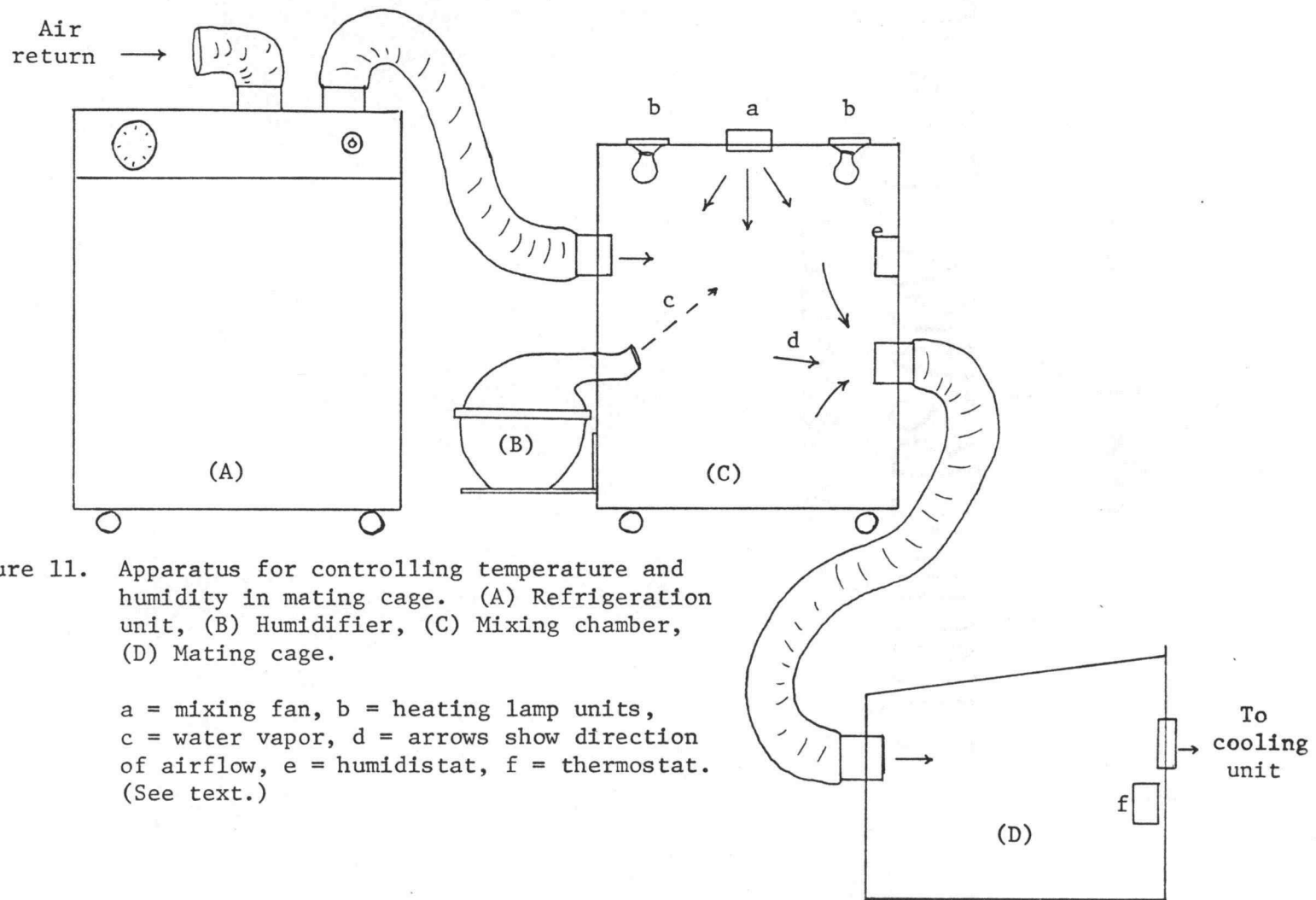
Figure 11. Apparatus for controlling temperature and humidity in mating cage. (A) Refrigeration unit, (B) Humidifier, (C) Mixing chamber, (D) Mating cage.

a = mixing fan, b = heating lamp units, c = water vapor, d = arrows show direction of airflow, e = humidistat, f = thermostat. (See text.)

keeping the temperature from climbing too high. The heat sources
(when required) were on continuously; the cooler maintained a con-
stant condition by periodically lowering the temperature to the
prescribed level. This was aided to some degree by varying the
wattage of the lamps used for heating. For instance two 150-watt
lamps were used to obtain the highest test temperature, and one 150
watt and one 40 watt were sufficient for the next level.

To provide a source of moisture, a Walton humidifier[7] was
mounted at the base of the mixing chamber. This apparatus was con-
trolled by an automatic humidistat, and increased humidity by blowing
water vapor into the mixing chamber. Air moisture could not be so
precisely controlled as temperature in the mating cages. This was
due, in part, to the "overshoot" of vapor delivered by the humidifier
before the humidistat sensor could react and shut off. The "over-
shoot" was lessened to a degree by mounting the humidistat in the
mixing chamber where the sensor was in contact with vapor immediately
after the humidifier had started.

An additional factor which added amplitude in the range of
relative humidity at a given test setting was the dehumidifying
influence of the refrigeration coils in the cooler. Whenever the
cooler was on, moisture condensed on the coils and air moisture
decreased.

These problems in humidity control are difficult to circumvent

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[7] Model WF-HP Humidifier, Walton Laboratories, Inc., 1188 Grove St.,
Irvington, New Jersey.

in any small environmental control unit. Because of the difficulty
in maintaining a precise setting, the effect of air moisture was
based on different ranges of humidities.

Temperature experiments.--Four constant temperatures were select-
ed for mating and oviposition tests. Equal numbers of adults of
optimal age (Chapter IV) were tested for mating and fecundity at 65°,
73°, 85°, and 92° F. The first temperature was slightly below the
preferendum shown by Green (1962), the second is within the prefer-
endum, and the latter two were considerably higher. For these tem-
perature evaluations relative humidities were kept uniformly high,
so that VPD (vapor pressure deficit) did not exceed 11.0 mm Hg for
any test. The decision to maintain high humidities for the temperature
evaluation was based on field conditions in areas where the shoot
moth has been most successful. The Puget Sound area and Lake States
region both represent regions of comparatively high late spring-early
summer humidities, and they also represent areas of successful shoot
moth establishment.

Humidity experiments.--Mating and oviposition were evaluated at
three ranges of relative humidity, low, medium, and high. Air tempera-
ture was held at a constant 73° F. (± 1°) for this evaluation. This
was considered an optimal temperature since it was well within the
preferendum shown by Green's (1962) isopleths. The low range of
relative humidity varied from 28 to 42 percent with an approximate
average of 34 percent and VPD (vapor pressure deficit) of 14 mm
of mercury. The medium humidity range was from 40 to 62 percent with
an average of 50 percent and VPD of 10 mm of mercury. The high range

of humidity varied from 55 to 85 percent with an average of 70 percent and VPD of 6 mm of mercury.

For further comparisons, additional tests were also run at higher temperatures with greater amplitude in relative humidity. As in the temperature evaluations, equal numbers of optimal aged females were tested for mating efficiency and fecundity under the respective physical conditions.

Results and Discussion

Temperature Effects on Mating

Fertilization took place at the extremes of 54 and 92° F. The mating occurrences at 54° were observed in the field. Mating efficiency was quantitatively lower at the 92° level, and probably at 54°. At the other temperature levels (65°, 73°, 85° F.), however, there were no significant differences in mating efficiency (Table 18). At the 85° level, it looked as though mating was started to decrease (Figure 12, Table 18), but this was not statistically significant.

It would not have been surprising had the decrease at 85° been significant, since adults are apparently influenced in their behavior at temperatures approaching 30° F. (86° F.). Green (1965) found that adults seek cooler, more sheltered positions when temperatures approach that level. Presumably, this activity could interfere with normal mating behavior.

The matings observed at 54° F. undoubtedly represent a near minimum for this activity. Moths were extremely abundant on this
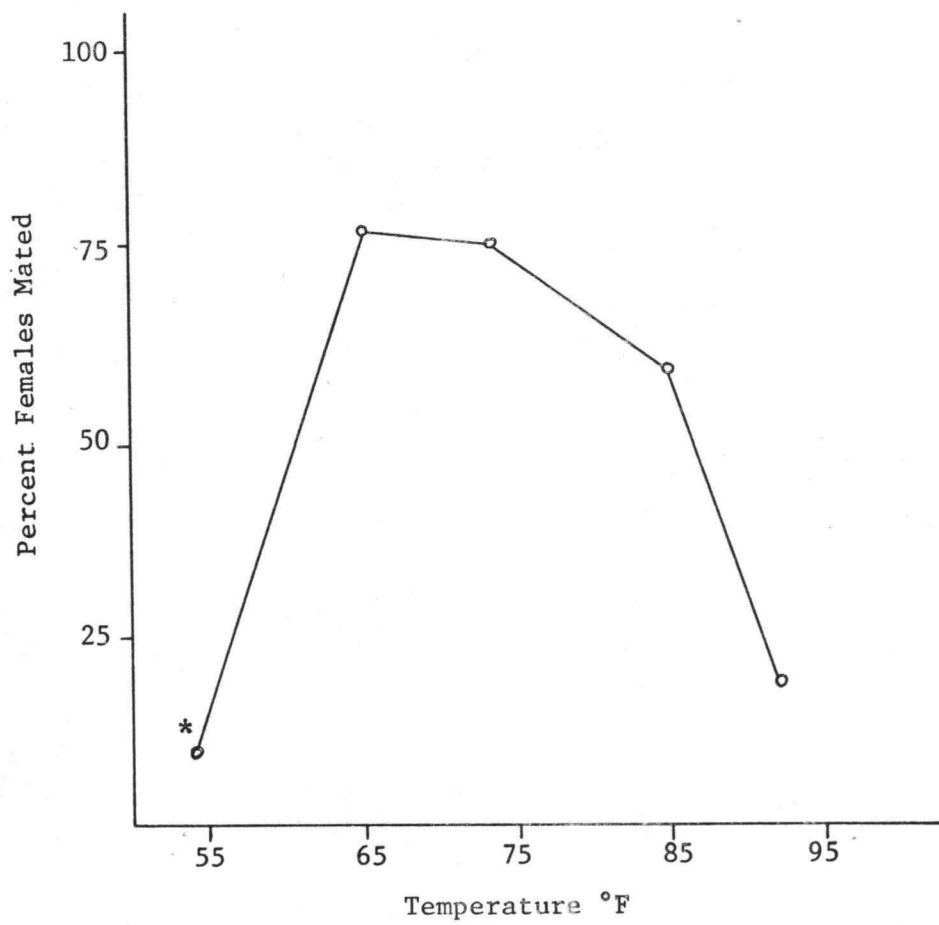
Figure 12. Effect of temperature on mating efficiency.
*Estimated from field observations at 54° F.

particular occasion, and the incidence of mating would have been much higher with higher temperature. The moths were apparently incapable of maintaining sustained flight at this temperature which agrees closely with Green's (1962) records on flight limitations. A few short flights of one foot or less were observed and apparently it was these males which were able to locate females and initiate the few matings seen.

Temperature Effects on Fecundity

As with mating efficiency, fecundity was not significantly influenced by temperature except at the extreme of 92° F. Again, with a cursory examination of the data, it looked as though fecundity was lower at the 85° F. level; however, it was not statistically significant (Table 19).

An evaluation of 92° F. was difficult because females mated at that temperature were scarce. The 11 females that were mated at 92° F. and held for oviposition at that level, laid few eggs and all of these failed to hatch. This will be discussed further under humidity effects, since air moisture was apparently involved. Pointing and Green (1962) reported that hatching success in "moist" air dropped to 40 percent at 34° C. and in "dry" air to 10 percent at 34° C. It is interesting to note that the damage was suffered by the eggs after they were laid, since females mated at 92° F. could be moved to a 73° F. chamber where they oviposited viable eggs.

Humidity Effect on Mating

At the air moisture levels tested (VPD 6-14 mm Hg) at the constant temperature of 73° F., there was no significant influence on mating efficiency (Table 20). A later test at a VPD of 22 mm Hg resulted in a mating incidence of only 45 percent, but this test involved an accompanying temperature of 85° F. This could imply a temperature-humidity interaction, but in this case the low humidity (high VPD) was probably the primary factor, since it was shown previously (Table 18) that there is not a statistically significant decrease in mating at 85° F. with a low VPD.

Humidity Effect on Fecundity

Unlike mating, number of offspring was markedly effected by variations in air moisture at a constant temperature of 73° F. Significantly fewer eggs were produced at the lower range of relative humidity (VPD of 14 mm Hg) tested at 73° F. (Tables 21 and 22, Figure 13). Since the temperature evaluation (Table 19) showed no differences in fecundity between 65-85° F. with a low VPD, this is evidence that dryness of the air is the primary factor governing fecundity.

Further statistical analysis added support to this conclusion. A scatter diagram of mean fecundity plotted over VPD suggested a possible linear relationship between these variables. Linear regression showed a high relationship (correlation coefficient $(r) = .953$) between decreasing fecundity and increasing VPD (Figure 14). This analysis might be criticized from the standpoint that all VPD values
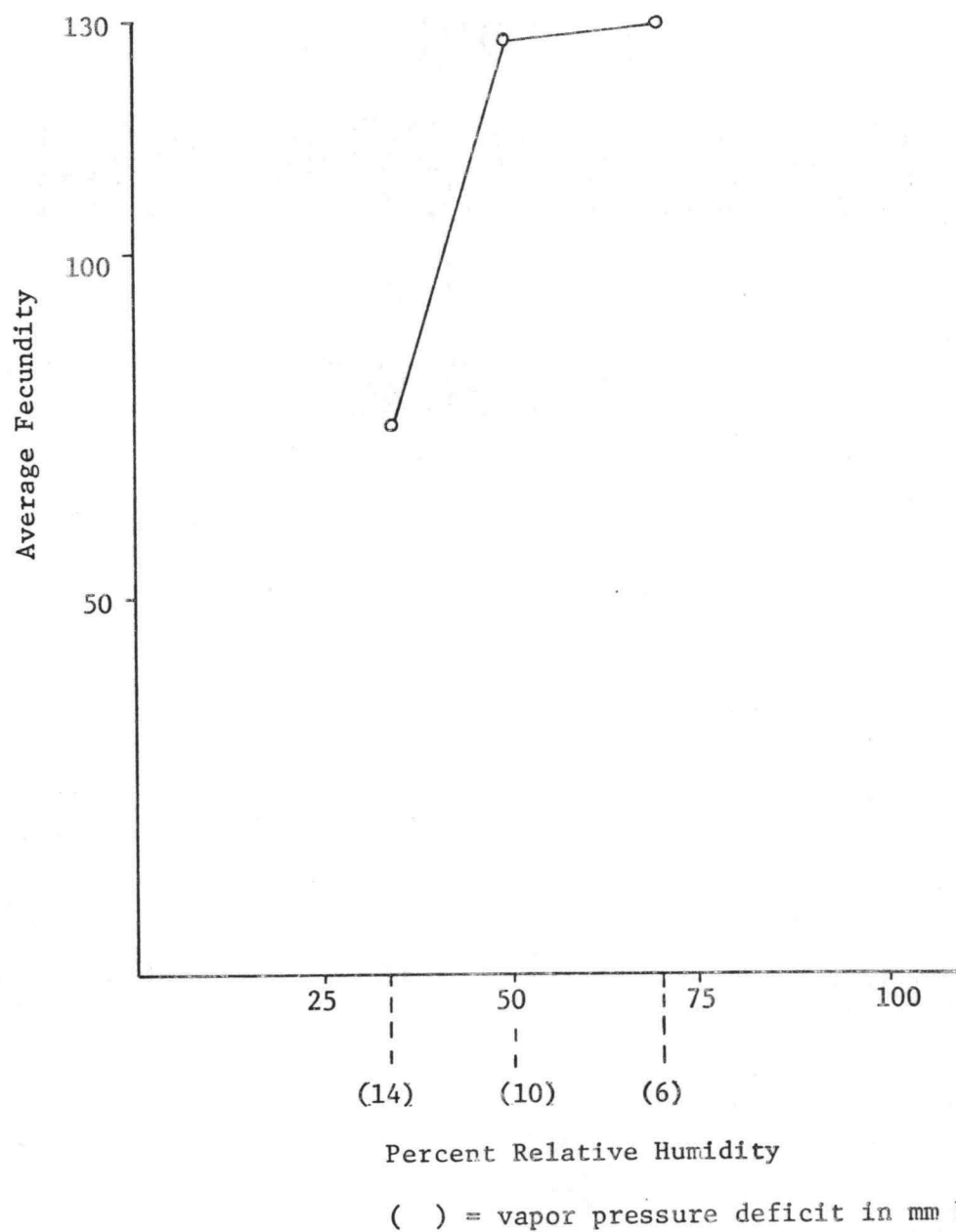
Figure 13.   Effect of air moisture on average fecundity at 73° F. (± 1°).

were not obtained at the same temperature. In answer to this criticism, however, it has already been shown (Table 19) that there is no significant effect on fecundity at temperatures of 65-85° F. so long as there is a low VPD (high humidity) condition. Therefore, temperature exerted no discernable effects within that range, and all VPD values shown in Figure 14 were obtained at temperatures within the range of 65-85° F.

It is apparent from Table 21 and Figure 14 that fecundity decreases in proportion to increasing evaporation rates at temperatures of 85° and below. Somewhere above 85° F. there is an interaction of these factors and temperature becomes more critical. This effect is shown at 92° F. where all eggs died and few were laid despite a high air moisture content (low VPD). Pointing and Green (1962) also showed high temperature mortality at 34° C. (93° F.), but in their tests air moisture was still of importance. "Dry" air resulted in 10 percent egg survival as opposed to 40 percent in "moist" air. Pointing and Green were not very explicit in describing air moisture as "dry" or "moist," nor were they specific in defining other experimental conditions, so it is difficult to compare results. The difference of no survival at 92° F. as compared to Pointing and Green's 10 and 40 percent survival at 93° F. could have resulted from population differences since their study was conducted in Ontario, Canada, with a field population.

Some Ecological Considerations

Field studies would be necessary to evaluate the importance of
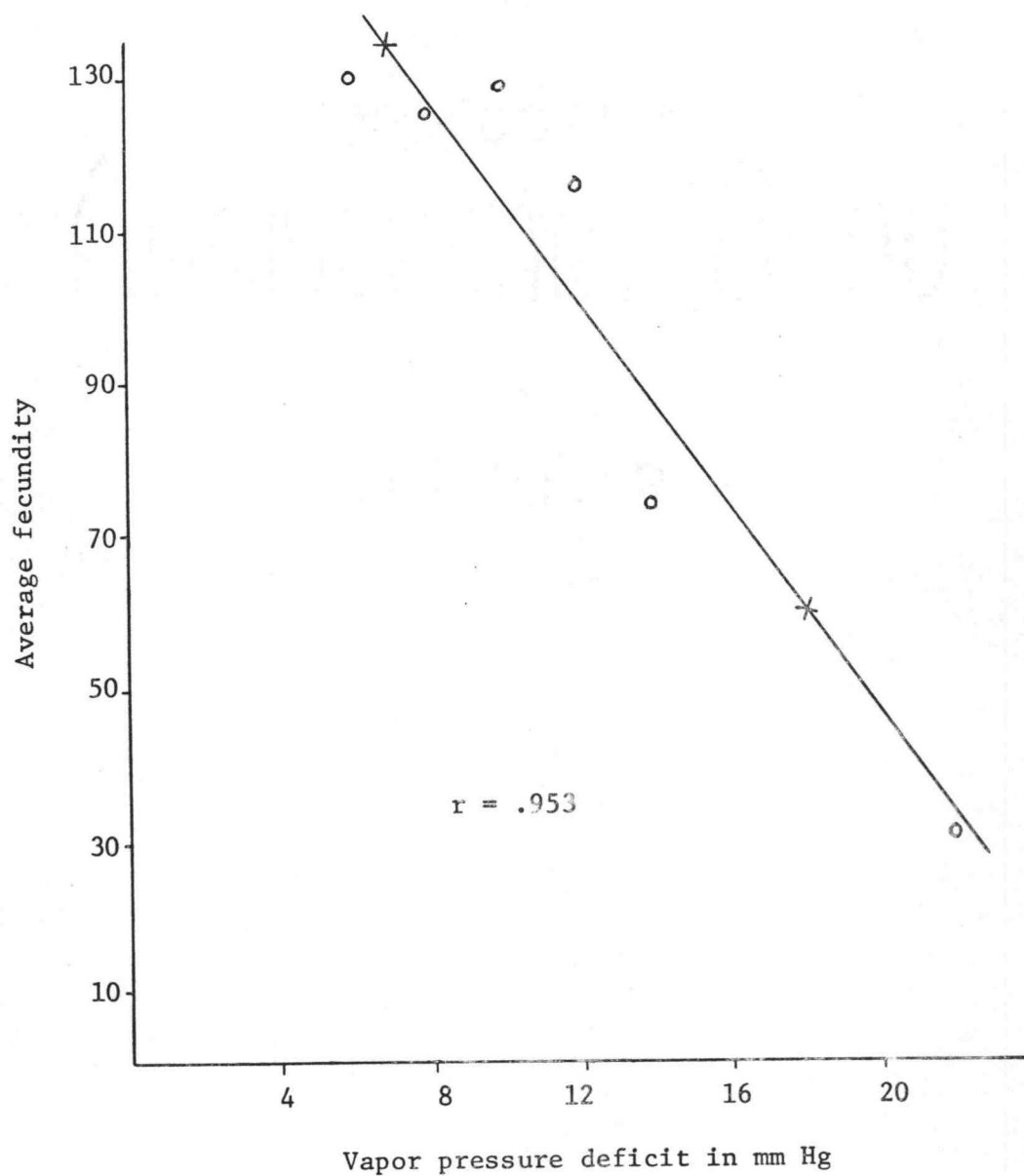
Figure 14. Linear relationship between fecundity and vapor pressure deficit (VPD). Correlation coefficient (r) is significant at one percent level.

evaporation rate, but there could be a significant impact on the population due to the detrimental effect of dry air on fecundity. As mentioned previously, the area of main concern for potential European pine shoot moth damage lies east of the summit of the Sierra Nevada and Cascade Mountain ranges in California and the Pacific Northwest. The weather in these areas is predominantely warm and dry during the summer months. Maximum daily temperatures of 90 ° F. or more and minimal relative humidities of 20-30 percent are not at all uncommon from late June through August. These conditions would result in a VPD of 25-29 mm Hg which could cause a drastic reduction of the reproductive potential of this insect (Figure 14). Further, it was shown that constant temperatures of 92° F. or higher caused egg mortality, and maximum daily temperatures often go this high and higher in the ponderosa pine region.

In favor of the insect, it must be pointed out that there are counteracting factors in the field that would probably temper the effects of the above factors. While the laboratory evaluations were conducted at constant conditions this is not the case in the field. A typical day in a pine forest, for example, might have a high of 90° F. with a night time low of 40° F. This rapid temperature drop often results in saturated air, so that temperature changes would be accompanied by a change in VPD of 20 to 0 mm Hg. These common diurnal fluctuations could decrease the detrimental effects to fecundity and egg viability caused by high temperatures and high evaporation rates. For this reason, final evaluation of these factors must be in the field.

Evolutionary aspects must also be considered here, since there is some survival at the higher evaporation rate. It is possible that over a period of generations a strain could develop that would be less sensitive to high temperature and high evaporation rates. In time, such a development could result in a more damaging shoot moth population in the ponderosa pine region.

Some Influences of Light

Temperature and humidity are clearly important factors in governing the rate or degree to which adult activity takes place. Light, however, is equally important in its function of governing when these activities occur. *R. buoliana* is a crepuscular insect, and decreasing illumination is the factor triggering flight, mating, and oviposition. Pointing (1961) reported that flight and mating took place in the evening, and this was confirmed in the present study (Chapter II). Field and laboratory observations have also disclosed that oviposition occurs primarily in the evening.

Green (1965) has also shown adult emergence to be dependent on light. He found emergence to be controlled by the dark-light transition with the daily peak of activity occurring before 0900 hr. This rhythm disappears under constant light or darkness, and can be reversed by changing the time of the dark-light transition. Similarly, in the present study, it was found that mating can be induced any hour of the day if the proper light conditions are used. These conditions involve a minimal period of 8-12 hours high light

intensity and 20-30 minutes of twilight, followed by exposure to the female sex pheromone.

Green (1965) concluded that the European pine shoot moth adult emergence rhythm is a field rhythm (Pittendrigh 1958) occurring only in the presence of light cycles with favorable temperature. Mating apparently also functions as a circadian field rhythm being closely tied to the occurrence of twilight. In contrast to this condition *Drosophila* has a persistent, endogenous emergence rhythm, the phasing of which is set by a dark-light transition that may occur in any but the prelarval stage (Pittendrigh 1960). Under natural conditions both types of insects would be synchronized in daily emergency activity. In the laboratory, however, *Drosophila* could be governed by chance dark-light conditions that existed during the immature stages, possibly causing activity patterns such as emergence to be unpredictable. In contrast, the shoot moth is governed in its activity patterns by the light conditions existing during a particular life stage.

CONCLUSIONS

Matings occurred at temperatures ranging from 54 to 92° F. Quantitatively, the degree of mating activity was much lower at these extremes, and they are probably near the minimal and maximal limits for this activity. Within the range of 65 to 85° F. there was no significant effect on mating. Fecundity was also unaffected within these limits with an accompanying high humidity (low VPD). At 92° F., however, fecundity was severely curtailed and there was total mortality

of eggs despite a high degree of air moisture.

Fecundity decreased proportionately to decreasing air moisture (high VPD) at temperatures of 85° F. or below. This influence of evaporation rate could have an impact on geographic distribution of the insect, but field studies are required for final analysis of this factor.

TABLE 18.   EFFECT OF TEMPERATURE ON MATING EFFICIENCY.   ANALYSIS
OF VARIANCE OF RESULTS (FREESE 1967).

Number females mated (6 available per replicate)

65°	73°	85°
3	4	4
4	5	4
6	4	2
4	6	2
6	4	6
6	3	3
4	5	2
5	6	5
3	4	5
5	4	2
46 = 76.7%	45 = 75%	35 = 58.3%

Source of variation	Degrees of freedom	Sums of squares	Mean square
Treatment	2	7.4	3.7
Error	27	41.4	1.53
Total	29	48.8	

$F = 3.7 \div 1.533 = 2.41$ Tabular $F_{.05} = 3.35$

Conclude: No significant difference among temperature

treatments.

TABLE 19. EFFECT OF TEMPERATURE ON FECUNDITY. ANALYSIS OF
VARIANCE OF RESULTS (FREESE 1967).

Number eggs per female		
65°	73°	85°
79	184	77
114	144	80
165	142	155
177	180	56
130	131	122
75	187	178
149	117	155
241	41	85
163	133	185
111	67	91
221	159	45
86	44	131
161	62	65
121	224	103
147	193	201
75	160	146
134	97	53
101	123	215
78	101	24
58	21	164
2,586	2,510	2,327
$\bar{x} = 129.3$	$\bar{x} = 125.5$	$\bar{x} = 116.35$

Source of variation	Degrees of freedom	Sum of squares	Mean square
Treatment	2	1,772.44	886.22
Error	57	167,937.75	2,946.28
Total	59	169,710.19	

$F = 886.22 \div 2946.28 = .3008$ Tabular $F_{.05} = 3.17$

Conclude: No significant difference among temperature treatments.

TABLE 20. EFFECT OF HUMIDITY ON MATING AT CONSTANT TEMPERATURE OF 73° F. ANALYSIS OF VARIANCE OF RESULTS (FREESE 1967).

Number of females mated

Low range R.H. VPD – 14 mm Hg	Medium range R.H. VPD = 10 mm Hg	High range R.H. VPD – 6 mm Hg
5	3	4
4	6	4
5	3	6
4	4	5
4	2	5
3	5	6
5	5	5
5	6	3
5	6	4
4	4	5
44 = 73.3%	44 = 73.3%	47 = 78.3%

Source of variation	Degrees of freedom	Sum of squares	Mean square
Treatment	2	.6	.3
Error	27	30.9	1.144
Total	29	31.5	

F = .3 ÷ 1.144 = .262 Tabular $F_{.05}$ = 3.17

Conclude: No significant difference among humidity treatments.

TABLE 21. EFFECT OF HUMIDITY ON FECUNDITY AT 73° F. ANALYSIS OF VARIANCE OF RESULTS (FREESE 1967).

Number eggs per female

Low range R.H. VPD = 14 mm Hg	Medium range R.H. VPD = 10 mm Hg	High range R.H. VPD = 6 mm Hg
40	128	215
48	122	112
113	109	158
38	21	124
69	166	169
48	135	105
82	126	104
102	151	98
41	118	55
183	163	197
83	229	115
53	49	102
60	69	125
70	160	169
23	193	17
90	224	196
81	67	122
37	121	83
91	196	192
72	20	159
1,416	2,567	2,617
$\bar{x} = 70.8$	$\bar{x} = 128.4$	$\bar{x} = 130.9$

Source of variation	Degrees of freedom	Sum of squares	Mean square
Treatment	2	46,161.7	23,080.85
Error	57	143,338.3	2,514.71
Total	59	189,500	

F = 23,080.9 ÷ 2,514.7 = 9.178 Tabular $F_{.01}$ = 5.00

Concluded: Significant difference among humidity treatments.

90

TABLE 22. EFFECT OF HUMIDITY ON FECUNDITY AT 73° F. TUKEY'S
TEST FOR COMPARISON AMONG MEANS (SNEDECOR 1956).
DIFFERENCES GREATER THAN 38.237 (D) SIGNIFICANT AT
FIVE PERCENT LEVEL.

Treatment		Treatment differences	
Humidity level	Ranked means	$\bar{x} - 70.8$	$\bar{x} - 128.4$
High range VPD = 6 mm Hg.	130.9	60.1[1]	2.5
Medium range VPD = 10 mm Hg.	128.4	57.6[1]	
Low range VPD = 14 mm Hg.	70.8		

[1] Denotes significant difference.

CHAPTER VI

SUMMARY

This study on reproductive biology of the European pine shoot moth, *Rhyacionia buoliana* (Schiff.), emphasized mating behavior, sex attraction, and fecundity. Major objectives were development of a laboratory procedure to obtain mated females, determining characteristics of sex attraction, evaluation of other biotic factors influencing reproduction, and determination of effects of temperature and humidity on mating and fecundity.

Two methods were devised to obtain matings in the laboratory. The first of these required continual observation by the worker and was primarily useful for studying mating behavior. The second method "the vertical airflow technique" was more convenient to employ and more efficient in producing mated females. The latter method resulted in 73 percent of the females mating within 48 hours. Mating activity was triggered by a decrease in illumination, and a slow airflow directed from females toward males greatly facilitated mating activity.

Production of sex attractant by each female was necessary for her to secure a mate. Individual females varied considerably in attractant potency, with some apparently incapable of attracting any males to a sticky trap and others capturing an unexpectedly high number. The source of pheromone production was a gland on the dorsum of the abdominal tip in the intersegmental area between the penultimate and terminal segments. The gland is oval, flattened dorso-

ventrally, and composed of columnar epithelial cells. Males could be attracted over distances of 100 yards to sex attractant baits. and responded best to traps located in host pines as compared to traps in the open or in nonhost foliage.

Mating efficiency decreased for both sexes after they were 4.5 days old. Females older than 2.5 days at the time of mating oviposited significantly fewer eggs than females fertilized at a younger age. Although females produced eggs continually throughout their life span, most oviposition occurred the first few evenings after mating. Average fecundity for females less than 2.5 days old when mated was 126 eggs.

Males were capable of multiple matings. Only 6 of 50 pine-reared specimens failed to mate, whereas 3 males successfully fertilized 5 females, and 1 performed a sixth viable mating. Average incidence of copulation was 2.24 females per male. Females normally mated only once, and no longer produced sex attractant after copulation. Some females that had paired with defective, laboratory-reared males, however, did mate more than once. Characteristic of these matings was lack of a spermatophore and little or no sperm being deposited in the female. This was an implication that a spermatophore or some other male factor deposited in the female during copulation controls female receptivity for mating.

The cause of the sterility in the laboratory-reared males has been traced to the breakdown of an unknown constituent in the artificial diet. To a lesser degree, sterility is also present in

field populations of this insect. Five percent of 112 females mated with pine-reared males deposited only sterile eggs, and 41 percent of the remaining 106 females oviposited at least some (8.9 per female) infertile eggs.

Matings were observed at temperatures ranging from 54 to 92° F. Quantitatively, the degree of mating was low at these temperatures, and they are probably near the limits for this activity. Within the range of 65 to 85° F. there was no significant effect on mating. Fecundity was also unaffected within these limits with an accompanying high humidity. At 92° F., however, fecundity decreased and there was total egg mortality despite a high humidity.

High evaporation rates were found to be detrimental to egg production. Fecundity decreased proportionately to decreasing air moisture (high VPD) at temperatures of 85° F. or below. This negative influence of drying power of the air could have a limiting impact on the geographic distribution of this insect, since many of the pine regions in the west are noted for hot, dry, summer weather.

At this point it might be beneficial to consider some of the practical applications of the results of the study. It is apparent that the vertical airflow mating technique should be utilized in mass-rearing programs or for fertility evaluation of males treated to induce sterility. Rearing programs could benefit through increased production by breeding only males less than 4.5 days old with females less than 2.5 days, since evaluation of the age factor showed this combination most efficient in terms of mating incidence and egg

production. Further work is necessary to correct the nutritive deficiency in the wheat germ diet which has resulted in the partial sterility of laboratory-reared males. Although it was found that periodic replacement with fresh food greatly alleviated the problem, a better solution is necessary because exchanging the food medium in culture containers is very time consuming and therefore detrimental to a large scale rearing operation.

Tests evaluating the effects of temperature and humidity on reproduction also yielded results useful for laboratory rearing of the European pine shoot moth. On the basis of these results, which generally showed dry air to be detrimental to egg production, it is suggested that VPD (vapor pressure deficit) should be maintained at less than 11 mm Hg. and temperatures between 65 and 85° F. The detrimental effects on fecundity and egg viability caused by dry air and high temperature point out a biological weakness which may ultimately rule out this insect as an economic pest in the ponderosa pine region where hot, dry summers predominate. Further work is necessary to evaluate this possibility including a rigorous study under field conditions.

Determination of mating frequency which showed females limited to one mating and males capable of several, could be beneficial in planning manipulative control procedures such as sterile male releases or male trapping programs. In sterile male release programs, for example, knowledge of mating frequency is generally required for planning the "overflood" ratio of sterile to wild males.

The attractive power of methylene chloride extracts of female

abdomens demonstrated a trapping procedure applicable for detection or possibly control of shoot moth populations. The described techniques for extracting the attractant and trapping procedure, have, in fact, been utilized to conduct limited operational surveys by the Division of Timber Management of Region Six of the U.S. Forest Service. Knowledge of the pheromone gland location plus the described bioassay technique will benefit studies aimed at chemical isolation and identification of the attractant. Since the gland location is known, only that portion of the female moth need be extracted in solvent; this results in a "cleaner" extract with less superfluous organic material, which presumably simplifies the purification of the attractant. A bioassay technique is basic to any purification study, since a means of testing the chemical fractions is necessary to evaluate their physiological activity.

If a decision is made to use the sterile male technique for control of *R. buoliana*, this study indicates that mass rearing is possible if recommended procedures for laboratory reproduction are followed. Because of the amount of labor involved with larval feeding, however, such rearing would be extremely expensive. Male release tests to pheromone traps showed that relatively few respond over distances, and this would also increase the expense since more sterilized males would be required. Detrimental effects of high temperature and evaporation rates to egg production might make application of the sterile male technique or other control procedures for European pine shoot moth unnecessary in the ponderosa pine region.

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APPENDIX

APPENDIX

Ingredients for wheat germ diet used to rear European pine
shoot moth.

Wheat germ	30.0 grams
Sucrose	25.0 "
Casein (vitamin free)	25.0 "
Soy protein	7.0 "
Alphacel	7.0 "
Wesson salts	2.5 "
NBC vitamin fortification mix (Nutritional Biochemicals Co.)	11.0 "
Choline chloride	0.6 "
Ascorbic acid	2.3 "
Cholesterol	0.5 "
Beta-sitosterol	0.5 "
Sorbic acid	0.6 "
Aureomycin (powder)	0.4 "
Distilled water	300 ml.
Formaldehyde	0.8 "
15% methyl-para-hydroxybenzoate in 95% ethanol	10.0 "
Potassium hydroxide (4M)	6.0 "
Corn oil	2.0 "
Wheat germ oil	3.0 "

These ingredients are mixed thoroughly in a blender with the 300 ml. of water. An additional 340 ml. of water is autoclaved with 16 grams agar. The autoclaved agar mixture is cooled to about 75° C. and mixed into the diet. The diet is then completed and ready to pour. This list of ingredients makes about one quart of nutritive medium. The diet was poured into five dram vials to which first-instar larvae were added, or else presented to the larvae as chunks in petri dishes. Fresh diet was added to rearing containers approximately every three or four weeks.

Steps for Preparing Slides of Female Abdominal Tips

A. Abdominal tips of fresh-killed females were stored in Bouins fixative for an indefinite period (at least 24 hours) before imbedding in paraffin.

Bouins fixative

Picric acid (saturated solution)	75 parts
Formalin	25 parts
Glacial acetic acid	5 parts

B. Most of the excess picric acid was removed by soaking in 50 percent ethanol saturated with lithium carbonate (4 changes at 30 minutes each).

C. The specimens were then dehydrolyzed in dioxan (4 changes at 30 minutes each, held in a fifth change overnight).

D. Two changes of xylol were used to rinse the dioxan. Specimens were then imbedded in Tissue-Mat[7] paraffin (melting point 56° F.)

E. A rotary microtome was used to cut sections that varied from 8-12 microns in thickness. The sections were affixed to glass slides using egg albumin as the adhesive.

F. The slides were run through the following sequence of reagents and stains.

    1. Xylol, two baths at two minutes each.

    2. Rinsed in absolute ethanol (100 percent).

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[7] Available from Van Waters and Rogers, Inc., Scientific Supplies Division, Box 9532, Portland, Oregon 97210

3. Hydrated with 95 percent ethanol (two minutes).

   Hydrated with 70 percent ethanol (two minutes).

   Hydrated with 50 percent ethanol with lithium

   carbonate (two minutes).

   Hydrated with distilled water (two minutes).

4. Delafields haematoxylin (five minutes).

5. Rinsed in tap water, stored in distilled water.

6. Destained in acid 50 percent ethanol.

7. Rinsed in tap water and blued in sodium bicarbonate.

8. Dehydrated to 95 percent ethanol (two minutes each in

   50, 70, and 90 percent ethanol).

9. Counterstained in eosin (about 30 seconds).

10. Washed in 95 percent ethanol.

11. Dehydrated in absolute (100 percent) ethanol.

12. Cleared in xylol.

13. Mounted with coverslip over Permount.