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REPRODUCTIVE BEHAVIOR OF THE PINK BOLLWORM MOTH

by

Norman Carey Leppla

A Dissertation Submitted to the Faculty of the

DEPARTMENT OF ENTOMOLOGY

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

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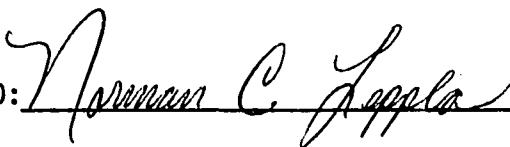
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BIOGRAPHICAL

Norman Carey Leppia was born in Wolf Point, Montana, on July 25, 1945. He received his elementary education in the Scottsdale and Mesa school systems of Maricopa County, Arizona. Secondary education was completed at Mesa High School in 1963 and he entered Arizona State University that same year. In 1967 requirements were completed for a Bachelor of Science degree in Zoology which was received in 1968. Prior to graduation, graduate studies were initiated emphasizing entomology.

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ABSTRACT

Laboratory investigations were conducted in an attempt to thoroughly analyze male and female mating behavior and other activities associated with reproduction in the pink bollworm moth (Pectinophora gossypiella (Saunders)). Information was accumulated and expanded by experimentation in the following categories, (1) circadian rhythms of behavior, (2) courtship and mating, (3) oviposition, and (4) ecological factors affecting mating. Observation procedures, remote monitoring equipment, and laboratory techniques were developed to derive detailed information about the Tucson Strain. This research was incorporated with comparable data derived from the Texas Strain, thus yielding a basic behavioral repertory for the local moth.

Circadian periodicity is regulated by photoperiod, being relatively independent of temperature, nutrition, moth age, genetic variability, gamma irradiation, and most other ecological factors within tolerance limits. Moths adapt to lights-on within 30 min and habituate to continuous light or dark within five days. During the day, moths remain inactive in secluded aggregations, whereas nocturnal activities include emergence, feeding, mating and oviposition. The mating period is initiated by the release of female sex pheromone which presumably stimulates male antennae and releases stereotyped

male courtship and, ultimately, mating. Female calling, male response, and generally gregarious behavior support the localized nature of this chemical communication.

Mating is influenced by photoperiod, moth density, sex ratio, and age, all of which were analyzed in terms of total spermatophores transferred, spermatophores transferred per female, mated/virgin females, and multiple/total matings. A bimodal pattern of receptivity occurs with age and excess females enhance mating. Extra males, above a ratio of four males to one female, increase the frequency of spermatophore transfer. Oviposition is affected by the same factors as mating, as well as substrate texture, nutrition, and related variables. Egg deposition is bimodal with age and enhanced by excess females, or males below a 4:1 male to female ratio. Mating and oviposition produce similar patterns but are mutually exclusive in time with a four to six day interim between peaks. Optimum levels may result from a compromise between the two activities in nature, but unbalanced sex ratios and advanced ages are probably never achieved.

Multiple mating and the secondary resurgence of reproductive behavior are potentially more important than previously suspected. Most experiments indicate statistically significant effects due to these parameters. In addition, stereotyped behavioral sequences are relatively independent of environmental factors including photoperiod; however, synchronization requires specific environmental cues. The pink bollworm exhibits a remarkably wide range of behavioral adaptability.

INTRODUCTION

The pink bollworm has been a potentially serious pest of cotton in the United States for over fifty years following its apparent introduction from Mexico in about 1915 (Noble, 1969). Almost every aspect of its existence has been investigated, at least superficially, during this time but much remains to be revealed. Accordingly, in most cotton producing regions, this insect is the subject of extensive research programs aimed at effecting its control. Although many outstanding contributions have resulted from these efforts, investigations concentrating on adult behavior have been infrequent. Accounts of adult ethology are usually not detailed, and an attempt has never been made to compile them into a comprehensive collection of useful information.

The primary objective of this research was to assemble and expand all available laboratory and field-related information concerning the reproductive behavior of this moth. Material derived from direct observation, active experimentation, and the literature has been formulated into a basic behavioral life history. Experimentation included studies of circadian periodicity, mating behavior, oviposition, and factors which affect these activities. Normal behavior patterns were documented and altered in an attempt to discover the causal mechanisms which effect their overt expression. Thus, the

foundation has been prepared for a much more quantitative physiological investigation.

Behavioral data may now be refined and applied to more practical problems involving management of the pink bollworm moth in the laboratory and field. In the future, additional information may be added to these original areas in attempts to solve some of the remaining perplexities. Hopefully, this contribution will provide insight and direction for these studies, and facilitate their initiation.

Circadian Rhythms and Photoperiodic Induction

Circadian periodicity of the pink bollworm has been investigated in terms of specific, isolated behavior patterns but not as an integrated series of events. The moths are active at night but little information is available concerning their nocturnal habits, and reported diurnal behavior has been limited to anecdotal observations. This situation is presumably due to the sensory limitations of investigators in relation to the small size, nocturnality, and secretive habits of the insect. Thus, much of the initial research reported here was devoted to the development of improved observational techniques and systems of detection.

Activities related to flight and orientation to light have been studied by standard black-light trapping methods (Graham, Glick, and Martin, 1964). In Brownsville, Texas, during 28 nights from June through September 3, 1961, most of the males and females captured were active between 7:30 and 11:30 PM; however, males exhibited a resurgence

of activity from 1:30 to 3:30 AM. This bimodality has also been observed in Egypt (Ballou, 1920) and Hawaii (Busck, 1917); however, in Texas (Glick, Hollingsworth, and Eitel, 1956) and Algeria (Hussain, Khan, and Ram, 1934) observations have been conflicting. In Texas, hourly collections made between 7 PM and 6 AM for seven days indicated only the second male peak, and that females were most active between 10 PM and 2 AM. In India, a second male peak occurred from 10 PM to 2 AM, and maximum female activity was exhibited between 6 and 10 PM (Hussain et al., 1934). Also, the Indian population produced a shift in this female peak from the last four to the first four nocturnal hours as the season progressed. Experiments eliminating the influence of male-female interaction and differential phototaxis were not conducted even though these factors were mentioned (Graham et al., 1964).

Subsequent investigations have concentrated on the reproductive segment of the cycle and circadian periodicity of the system. Copulation occurs between 2 and 5 AM with a peak between 3 and 4 AM (Lukefahr and Griffin, 1957). Oviposition commences shortly after sunset and continues for about seven hours, with the median egg being deposited approximately 2.5 hours after the onset of darkness (Minis, 1965). Egg hatching (Minis and Pittendrigh, 1968) and adult sensitivity to x-irradiation (Haverty and Ware, 1970) are also known to be circadian.

Photoperiodic induction, free-run in constant dark or light, and the effects of 15-minute interruptions were previously investigated using oviposition as an indicator of activity (Minis, 1965). In these

experiments continuous arrhythmic conditions reduced amplitudes and extended periods of diel egg deposition. Light pulses, applied at specific intervals, were used to entrain moths to "Arbitrary Zeitgeber Time" skeletons (AZT's).

Entrainment was normal up to an L:D 13:10 skeleton. Exceeding this broke the rhythm, which became multimodal at AZT 13:11. When subjected to a 15-hr photoperiod, moths became entrained to the shorter segment and the phase of the rhythm jumped. Light pulses beyond AZT 16 functioned as the initiators of simulated 14:10 photoperiods. Thus, the limits of entrainment, evidenced by oviposition behavior, were established for the female moth.

Oviposition continued at statistically reliable levels for at least seven days and was therefore useful in characterizing the intrinsic circadian period for females maintained at 26°C. A value of 22 hr and 40 min was established by calculating the average daily phase shift in peak oviposition during dark free-run, following L:D 14:10 entrainment. Peak behavior, rather than the conventional parameter of phase initiation, was used for the estimation which was based on the single activity of oviposition.

Cobalt 60 gamma radiation sources have been used to sterilize insects for use in sterile-release studies, and a unit which emitted from 530 to 590 roentgens per minute (av. 560 r/min.) was used to sterilize insects for fecundity tests (Ouye et al., 1964). The optimum dosage for five to seven day old pupae was determined to be 30 to 60 kr. This level compromised the lower susceptibility to

sterilization and increased resistance to malfunctions exhibited by the older pupae. In field-cage experiments, male moths sterilized with 25 to 40 kr as five to seven day old pupae have been highly competitive with normal moths in terms of mating; however, no assay has been developed to determine basic viability or vigor. Competitiveness was enhanced by reducing the dosage to 25 kr (Richmond and Graham, 1970) and currently attempts are being made to use 10 kr (Ridgway, 1971).

Pink bollworm moths from the Texas Strain have been irradiated for large scale sterile-release experiments (Mangum, Ridgway, and Brazzel, 1969). Moths presently being reared by the USDA Plant Protection Division in Phoenix, Arizona, for release in California, were used for evaluating the effects of radiation on circadian rhythms. Actograms were derived from 1, 10, and 25 kr, gamma-irradiated moths. Comparisons were also made between Tucson (Maricopa) and Brownsville Strains in terms of periodicity in locomotor activity.

Typically actogram traces were reduced to base line during the diurnal phases of 24-hr periods. Experiments were designed to disclose preferred resting sites where moths might pass this subjective day. In addition, efforts were made to describe the ensuing behavior and interpret its adaptive significance.

The secretive nature of these moths has been documented in the form of brief anecdotes and fragmentary observations scattered through the literature. Therefore, the information has been difficult to accumulate and has provided little basis for further investigation.

These earlier experiments were necessarily somewhat superficial even though they have provided much insight into the significance of specific behavior patterns.

These experiments established photoperiodic entrainment as the controlling influence over cyclic behavior of the pink bollworm. They also documented many of the fundamental activities in which the moth engages, but the segments remained essentially unrelated to the organism as a functional unit. The adaptive or ecological significance of this extremely organized system could only be evaluated by analyzing a composite program of daily behavior. My research efforts were therefore organized around a synthesis of the most pertinent studies and recent work into a more unified assemblage of information.

Courtship and Mating

Pink bollworm ethology includes many different stereotyped appetitive behavior patterns. In this study reproductive behavior was emphasized with emergence, feeding, oviposition, and other activities receiving secondary consideration. Specific autochthonous behavioral sequences involved in mating were documented in terms of primary events and formulated into a detailed ethogram. This scheme provided a system for analyzing alternative motor patterns and pursuing in depth studies of each stage.

Although many important studies have referred to adult ethology, few have considered the intricacies of mating behavior (Squire, 1937; Flaschentrager, Amin, and Jarczyk, 1957; Jacobson, 1969). Most of these initial investigations involved the accumulation of life history

data. Moths of equal age confined as pairs in an insectary were found to have multiple mating habits, with 28% mating at least twice (Lukefahr and Griffin, 1957). Sixty percent remained virgin the first night after emergence; however, this was reduced to less than 20% by the third night. First matings lasted up to two hours but succeeding ones were shorter.

Later, it was determined that each spermatophore in the bursa copulatrix of a mated female represented a separate mating (Ouye et al., 1965a). Successful transfer required at least 30 min and males can mate only once during their first 24 hr as imagoes. However, females are capable of multiple mating on their first night. Males or females, caged for their entire lives with abundant moths of the opposite sex, exhibited 95% and 97.6% mating, respectively. Potential mating frequencies averaged 4.2 times for males and 2.3 times for females, under laboratory conditions. My observations, made during moth courtship, indicated a stimulation of extra males in the vicinity just prior to clasping. While the pair was coupled, male activity subsided, but at the termination of copulation a brief resurgence occurred.

Accounts of male courtship have occasionally referred to, but have not described, a premating dance (Ouye et al., 1964; Berger et al., 1964; Jones, Jacobson, and Martin, 1966). Complete but still somewhat superficial accounts became available when this information was used to bioassay extractions from female abdomens for potential pheromones. The dance presumably included excitation, rapid wing vibrations, and

intermittent upcurving of the male abdomen while stationary or walking (Ouye and Butt, 1962). A more specific pattern of male response included the following movements: (1) antennal flickering (upward and forward position); (2) antennal preening with front tarsi; (3) erratic and rapid locomotion; and (4) fluttering (vibration of wings) and flying (Guerra, 1968).

Previous research did not document specific observations in terms of stereotyped behavioral sequences even though a precedent for formulating this type of data on economically important Lepidoptera had been established with Heliothis zea (Boddie) (Callahan, 1958; Agee, 1969). Information concerning some of the sensory mechanisms responsible for successful copulation, as well as these basic behavior patterns are necessary before further progress can be made. Therefore I have tried to provide additional information on the ethology and physiology of pink bollworm courtship and mating.

Ecological Factors Affecting Mating Frequency

The pattern of events involved in circadian periodicity and mating behavior was documented during initial phases of my research. Then, the effects of photoperiod, radiation, moth interaction and other environmental factors were studied in relation to established behavior. An attempt was made to deduce causal factors underlying observed behavioral events and, ultimately, to relate these elements to the biological success of this moth. Subsequent research was designed to evaluate, under laboratory conditions, the influence of

photoperiod, moth density, sex ratio, and age on mating frequencies. In addition, the timing and duration of this behavior was recorded.

Intensive studies on the mating habits of pink bollworm moths were begun in Brownsville, Texas, in 1954 (Lukefahr and Griffin, 1957). It was determined by direct observation that mating was dependent on light intensity below three fc for at least seven hours, that pairing occurred primarily between 2 and 5 AM, and that copulation took place for up to eight days after emergence. Seventy two percent of the females had single matings, some successfully copulated as many as five times.

After these initial findings, emphasis was placed on using light cycles to manipulate larval diapause for control (Lukefahr, Noble, and Martin, 1964; Lukefahr, Fife, and Adkisson, 1962) and for general studies on animal photoperiodism (Adkisson, 1966; Ankersmit and Adkisson, 1967). However, oviposition was used to establish basic photoperiodic responsiveness for the moth (Minis, 1965). Studies involving the cabbageworm, Pieris rapae (L.), and other Lepidoptera suggested the possibility of using photoperiodism for population control (Barker, Cohen, and Mayer, 1964; Hayes et al., 1970). Therefore, a superficial re-evaluation of some typical photoperiodic responses for the pink bollworm moth was undertaken.

Detailed but more unnatural laboratory experiments were conducted under continuous subdued light to determine the total number, duration, and temporal pattern of matings (Ouye et al., 1964). Pairs of moths were caged individually and of 94 pairs 74.5% mated.

Subsequent dissection showed that 71.0, 20.0, 7.1, and 1.4% mated 1, 2, 3, and 7 times, respectively. The mean duration of copulation was 2 hr 54 min with a range from 3 min to 26 hr 50 min. The last mating occurred at 16 days after emergence, but 41% were completed by the sixth day. Later it was determined that successful copulation required at least 30 min, with almost 100% of the moths participating and that potential frequencies ranged from 0-10 for males and 0-8 for females (Ouye et al., 1965b).

Feasibility studies, involving the use of sterile-male release for controlling pink bollworm populations, necessitated field evaluation of mating frequency (Graham, Glick, and Martin, 1964; Graham, H. M., P. A. Glick, M. T. Ouye, and D. F. Martin, 1965. Females collected with light traps were dissected for spermatophores. Presumably, each spermatophore represented a single copulation and provided a measure of mating frequency. Dissections of 2570 females, collected over a 2-year period, averaged 11.0, 72.8, 12.9, 2.5, 0.5, 0.1, and 0.04% for 0, 1, 2, 3, 4, 5, and 6 matings, respectively. The proportion of unmated individuals was lower than previously reported, and it declined progressively during the growing season until late August, when it began to increase. During the early season when population density was low, the mean number of matings was also significantly lower. The population at that time contained primarily single-mated females. It was concluded that the most effective use of a sterile-male technique against pink bollworms would be to release large numbers of sterile males in the early season to take advantage of the

small populations, high natural mortality, and low mating frequency of the native insects.

It has been estimated that field populations of the pink bollworm must be held to less than a 3-fold increase per generation, about 80% control, to effect their overall control by the sterile-moth release technique (Ouye et al., 1965b). Achievement of 81.1% control in field cages was accomplished by a single release of a 9:1 ratio of sterile to normal males. Similar tests provided 98% reduction in the normal population, with a seasonal average ratio of 7S to 1N, released on a daily basis (Ouye and Graham, 1967). However, the range varied from 301S:1N to 1S:1N, from week to week depending on the normal population.

Other investigations have concentrated on population dynamics (Graham, Fife, and Bryan, 1965; Slosser and Watson, 1972), cultural practices (Martin and Lewis, 1962), and other information directly applicable to field control programs (Graham et al., 1962). Field-cage studies and laboratory experiments have been employed to investigate the influence of sterile to normal moth ratios on population development; however, the effects of similar manipulations on non-irradiated insects have not been considered (Ouye et al., 1964, 1965a, 1969; Ouye and Graham, 1967; Richmond and Graham, 1970).

The effects of density, sex ratio, age, and other aspects of the adult stage were superficially investigated using Egyptian pink bollworms (Hussien et al., 1962). Females normally lived longer than males, although above 90% RH, the longevity of the sexes was equal.

The optimum population density, in terms of longevity, was between one and two pairs per 0.25-liter milk bottle. Incredibly, egg production under these conditions was elevated from an average of 16.8 per female to 58.4 by the addition of an additional male per pair. It was assumed that an increased opportunity for mating occurred which stimulated oviposition.

Temperature is the only factor which has been thoroughly studied for its effects on mating, oviposition, and fecundity (Graham, Glick, and Ouye, 1967; Philipp and Watson, 1971). Elaborate tests have been conducted at the population level; however, no attempt has been reported to isolate and study mating itself as a separate factor. Variability due to moth strain, laboratory environment, rearing method, experimental technique, and similar factors remain to be evaluated. Therefore, specific experiments were initiated in an attempt to fill this void of information.

Oviposition Behavior

Oviposition has been studied more intensively than any other single behavioral event exhibited by pink bollworm moths. Approaches have ranged from agricultural considerations to basic physiological research; however, much of this work probably remains in the form of unpublished reports. Accounts have often been obscured in studies on bionomics or included in poorly circulated reports. This behavior has been particularly manageable, measurable, and useful as an indicator of activity for this insect. It was, however, a poor choice for assay in many cases because of the interacting influences of so many

uncontrollable factors. The exchange between insect and environment is more elaborate than with locomotor activity, feeding, mating, or eclosion. It was therefore important to further refine available information concerning input from the insect as well as the habitat. In addition, oviposition provided the possibility for manipulation of field populations and enhancement of laboratory rearing.

Pink bollworm moths have been observed feeding and ovipositing throughout the night in cotton fields (Squire, 1937). The temporal pattern of oviposition within this period was determined by directly observing moths confined in field cages (Owen and Calhoun, 1932). Oviposition never occurred before the second night after emergence, and a peak was exhibited on the third. Nightly activity began shortly after dark, with a maximum rate from 8 to 10 PM, a decline from 10 to 12 PM, and a low from 12 to 8 AM (dusk at 8:00). Feeding, as previously reported, was continuous throughout the scotophase. The oviposition period ranged from 2 to 15 days, averaging approximately 4.5 days for all moths tested. Maximum numbers of eggs were produced on the first night, declining each night thereafter until death, which usually occurred one to four days after the end of oviposition. Averages of 105.87 and 180.72 eggs per female, with maximums of 289 and 448, were produced by long-cycle and short-cycle moths, respectively.

Inspection of field-collected cotton plants provided an estimate of site preference for oviposition by these insects (Brazzel and Martin, 1957). Previous reports indicated a preference for green

cotton bolls; however, percentages varied according to plant age, moth density, and a multitude of related factors (Fullaway, 1909; Willcocks, 1916; Busck, 1917; Loftin, McKinney, and Hanson, 1921; Garcia, 1925; Hunter, 1926; Ohlendorf, 1926; and Fenton and Owen, 1953). Many of these conflicting reports were anecdotal in nature and none of them included continuous sampling throughout a growing season. Early in the season moths preferred plant terminals, particularly the heavy pubescence on young leaves and unfolding leaves; however, many other sites including the soil were occasionally used. Later, two to three week old, green bolls became available and were preferred. The egg-laying rate increased as the season advanced, and it was concluded that this was due to the earlier lack of suitable sites rather than increased fecundity.

Field cages and bioclimatic cabinets were used to examine oviposition under carefully controlled conditions (Lukefahr and Griffin, 1957). Moths were fed sucrose solution and offered fresh cotton terminals at hourly intervals. In these experiments, peak egg deposition occurred on the third night after emergence, and more than 50% of the daily complement were laid from 8 to 10 PM, with 80% by midnight. Activity began when the light intensity dropped below 3 fc at 7:30 PM, and feeding was continuous throughout the night. Deposition was reduced at lower temperatures and higher light intensities; however, humidity within the range of 50 to 90% RH produced no effect. At light intensities above 20 fc no eggs were laid, and at 0.02 fc, under optimum conditions, oviposition was continuous.

Another investigation further refined existing information by using direct field counts of infested bolls to determine preference for boll site on the plant (Slosser, 1971). Central bolls were most infested but the cause remained unknown. It was concluded that female moths either select these bolls or only the larvae that reach them survive. Thus, the problem was reduced to a question of insect behavior in relation to the host plant. The positioning of eggs inside the involucre, under the calyx, along sutures, or other such protected places was substantiated; however, the underlying physiological processes of site selection were not investigated.

Certain environmental effects on the frequency and rate of oviposition have been studied by direct observation in the field (Fenton and Owen, 1953; Pearson, 1958). However, laboratory studies providing precisely controlled conditions produced surprising results (Hussien et al., 1962). Mean egg deposition increased from 14.35 to 33.44 eggs per female when the temperature was raised from 18 to 22°C. At 90% and 70% RH, females averaged 29.4 and 58.4 eggs per female, respectively. Furthermore, egg production decreased as moth density increased from three to five pairs per 0.25-liter container.

Temperature effects on reproduction and longevity were thoroughly investigated in order to determine the most favorable conditions for mass-rearing the insects (Graham et al., 1967). Maximum longevity of males ranged from 17 days at 32.5°C to 127 days at 18.5°C, whereas females ranged from 15 days to 92 days at these regimes. Maximum increase per generation occurred at 26.9°C even

though peak multiplication by adult females per day was at 32.5°C. Later, it was determined that the intrinsic rate of increase and net reproductive rate were greatest and the mean generation time shortest at a constant 28.3°C. In this study the oviposition rate was isolated by recording daily egg counts. The tendency for a bimodal pattern, evidenced by a later resurgence of oviposition, was evident particularly at a daily fluctuating temperature of 19.7 to 36.7°C.

The most detailed analysis of cyclic egg deposition for the pink bollworm was derived by using oviposition as an assay of photoperiodism (Minis, 1965). These experiments, as previously discussed, documented the primary influence of photoperiod on the daily timing of this activity.

Extra-floral nectaries provide the most dependable source of nutriment for these moths in the field; therefore, samples were collected and analyzed for available nutrients (Clark and Lukefahr, 1956). Sucrose, glucose, fructose, ribose, rhamnose, and raffinose were isolated from samples; however, only the first three were present in appreciable amounts. No amino acids or other suspected nutrients were found. Moths fed readily on a diet prepared in the following proportions: Sucrose 40, glucose 30, fructose 20, raffinose 5, rhamnose 5, ribose 2 and water 100. The sugar diet had no apparent effect on longevity, but an increase of approximately 100% occurred in the egg output (Lukefahr and Griffin 1956). Later experiments also determined the importance of nutrition on the rate of population increase (Adkisson, 1961).

Much of the earlier information was employed in the development of a relatively efficient mass-rearing regime for this moth (Richmond and Ignoffo, 1964). This method is now used in applied research, but it also provides a useful means for accomplishing more basic physiological and behavioral research (Minis and Pittendrigh, 1968; Bruce and Minis, 1969). Recently, the sterile-release program has provided an additional stimulus for continued research, due to the need for more efficient mass-rearing methods (Mangum et al., 1969).

Ultimately this research may explain mechanisms involved in the timing of physiologically important events and aid in interpreting their adaptive significance in relation to insect control. For the pink bollworm this aspect has recently been investigated in terms of circadian sensitivity (Haverty and Ware, 1970), and of emergence from diapause (Adkisson, Bell, and Wellso, 1963; Pittendrigh et al., 1970). It would be extremely valuable to determine specific times of vulnerability and be able to manipulate their durations.

METHODS AND MATERIALS

Moths for this research, unless otherwise stated, were obtained as late instar, nondiapausing larvae from a culture maintained at the Western Cotton Insects Research Laboratory, Tucson, Arizona (Patana, 1969). The culture was established using moths from Maricopa County with periodic additions from the Tucson area; therefore, it has been designated the Tucson (Maricopa) Strain. Larvae were reared in 1-gallon cardboard cartons held in constant darkness at 86°F for about 10 days, until they cut through the bottoms and dropped into collection jars. These collection containers contained folded facial tissues in which larvae formed pupal cells. A variety of methods were employed in manipulating pupae, depending on experimental design; however, most of these techniques were commonplace and do not warrant discussion.

Occasionally, insects were sexed as larvae by observing the dark dorsal testes, visible through the cuticle. This is relatively inaccurate, since testes are not apparent in many of the younger larvae; therefore, pupae were usually used for the determinations (Butt and Cantu, 1962). When necessary, a post-experimental check was also made by examining moths externally for claspers or ovipositor, or by dissecting for testes or bursa copulatrix. Dissection also provided an assay for mating frequency (Ouye et al., 1964).

Insects were generally housed, and experiments conducted, in the observation room under standard conditions. This environment included annual temperatures ranging from 23 to 29°C with readings in excess of 36°C occurring for 1 to 2 hr during several July and August afternoons. The yearly environment was essentially $30 \pm 7^\circ\text{C}$, $22 \pm 10\%$ RH, and L:D 14:10 photoperiod (lights on at 2000 MST); however, monthly fluctuations were typically of less than one-third this magnitude.

Observation Facilities and Equipment

A laboratory bench-top dark room or observation chamber was constructed to facilitate direct viewing and provide partially controlled rearing conditions (Figure 1). It was built almost entirely of wood with 2 x 4 framing, plywood sides, and a prefabricated, 2 foot door. The ceiling, front, and outer side were constructed as separate units, while the back and inner side were provided by the laboratory bench and associated walls. Thus, the entire structure was portable, being assembled with bolts and sealed with opaque black plastic and tape. Ventilation was provided by baffled ducts positioned near the floor and in the ceiling. These allowed room air but not light to enter the chamber. This unit was designed to eliminate many extraneous stimuli while furnishing suitable working conditions and a relatively uniform environment. Essential equipment included a bank containing two, 40-watt fluorescent tubes for subjective daylight, an electric timer to activate the lights, several filtered light sources to facilitate observation (Callahan, 1965; Agee, 1969), and adequate shelf and drawer space.

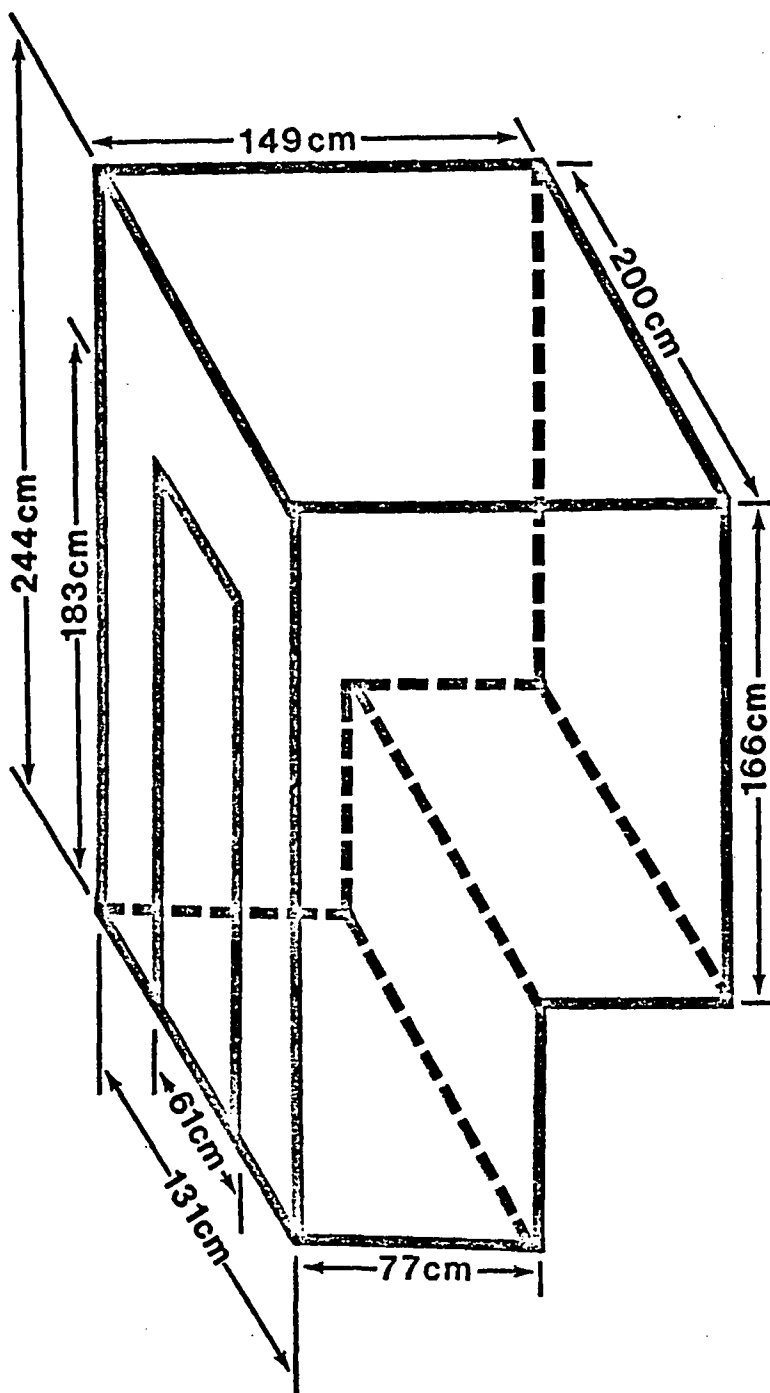


Figure 1. Laboratory bench-top observation room used for viewing and rearing pink bollworm moths under controlled conditions.

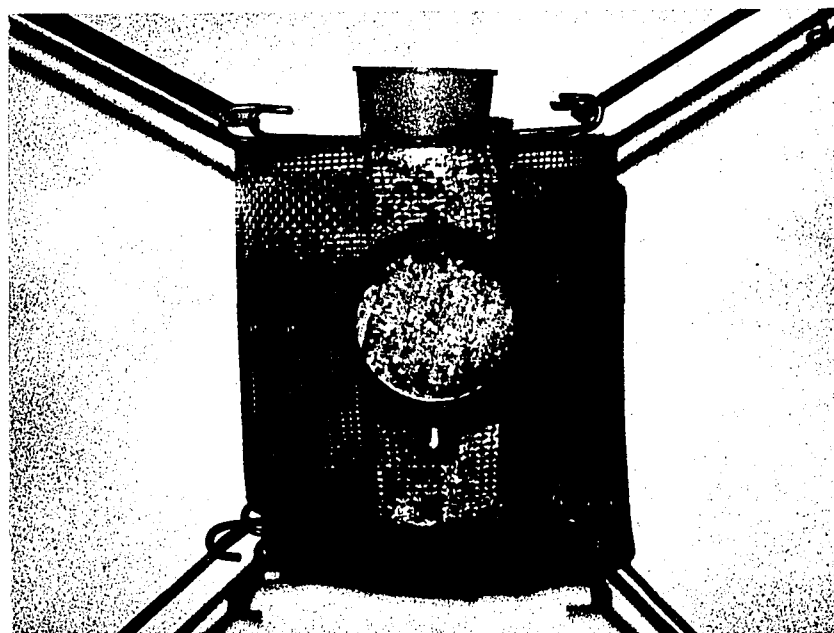
After several months of direct observation, it became apparent that experimental methods were affecting the insects. Light, applied at levels necessary for observation, suppressed the rhythmic expression of behavior and altered typical patterns. Varying the wavelengths and intensities of light failed to alleviate the problem; a remote system for automatically monitoring activity was therefore sought. The need for an instrument which would provide the desired sensitivity without altering the normal experimental environment led to the development of a new actograph. It was simple relatively inexpensive, and ideally suited for use with small flying insects (Leppla and Spangler, 1971).

Activity Detection and Recording

The activity-detecting and recording system was basically a vibration detector attached to a moth cage isolated from external vibrations (Figure 2). A Pixie silica-chip transducer (Endevco Corporation) was used in the vibration pickup device. It was isolated from extraneous vibrations by suspending the cage by rubber bands in the center of a wooden frame (Figure 3). Any movement of the cage, caused compression and expansion of the silicon element on the transducer. The resulting signal was fed through a preamplifier to a graphic level recorder modified for long-period usage.

In preparation for recording insect rhythms, moths were placed in the actograph cage which was isolated in a quiet, light-tight room. Temperature and humidity were maintained relatively constant while the photoperiod was manipulated throughout experimentation.

Figure 2. Detection system used for sensing pink bollworm moth locomotor activity. -- (a) Metal can containing the transducer with strap connecting top and bottom of cage. (b) The solder-weighted lug attached to the rigidly supported Pixie transducer within the can.



b

Figure 2. Detection system used for sensing pink bollworm moth locomotor activity.

During entrainment, light was provided by two 40-watt incandescent bulbs connected to a 24-hour timer. These were placed 1.8 m from the cage, in a position adequate for entrainment.

Prior to actograph testing for the effects of gamma irradiation, moths from the USDA Plant Protection Division in Phoenix, Arizona, were maintained under constant favorable conditions including an L:D 14:10 photoperiodic entrainment. Samples of 20 moths (10 males and 10 females) were placed in the actograph cage in succession beginning with the ten kr population (2-3 days after emergence) and ending with the 0 kr control group 12 days later. During the tests natural daylight was provided through a window.

Pheromone Extraction and Testing

In order to investigate male receptivity and learn more about the female sex pheromone, natural substances were extracted using established methods (Guerra, 1968). Ten female abdomens were macerated in 2 ml of methylene chloride (CH_2Cl_2), with 10 ml of additional solvent being added during filtration through Watman no. 30 paper, and the homogenizer was washed twice for each batch. The extracts were stored in screw-top vials placed in a refrigerator maintained at $11 \pm 4^\circ\text{C}$.

Four days after preparation, the extracted substances were tested on males during peak sexual activity on their fifth day after emergence. Tests were conducted in the observation room under standard conditions, where the moths had been housed. Males were induced to cling to the screen lids of 1-pint mason jars by placing sucrose-soaked cotton pads against the outside surfaces. Lids, with their

suspended moths, were then transferred to positions above petri dishes containing filter paper soaked with 40 drops of extract. The following treatments were applied: (1) extract in open petri dishes with filter paper held for 1.5 to 2 hr prior to testing, (2) 2.5 to 3 hr prior, (3) 3 days prior, and (4) CH_2Cl_2 controls at the same intervals. Moths were rotated and retested following 30-min recovery periods.

Another experiment involved the use of moths 1, 10, and 20 days after emergence. A randomized block design was employed to match all possible combinations of males and extracted females of these ages. Extract applied at the rate of 40 drops per filter paper, was allowed to evaporate at room temperature for 45 min prior to testing in subdued light.

Unused extracts were returned to the refrigerator and stored as before. After one month an oily material separated from the solvent and floated to the surface of each vial. When tested, this substance elicited a slight response from virgin males.

Several simple manipulations were performed in an attempt to locate male pheromone receptors. Ten male and ten female moths were housed for 20 days in 1-pint mason jars under standard conditions in the observation room. Prior to pairing, male antennae were amputated at the base of the flagellum. Dissection for transferred spermatophores, a measure of mating frequency, provided the assay and tests were replicated four times (80 moths).

Similar experiments were conducted using 40 single pairs of moths in vials with cotton plugs. Thus, contact was enforced between

paired moths, with no interference until death. Again, males were antennaless but proximity was insured, precluding the necessity for directed taxis or orientation by flight. Presumably, males were capable of mating if stimulation could have been mediated through sensory tissue not located on the antennae.

Additional information was sought by replicating the mason jar test using intact males and antennaless females. Restriction of locomotor abilities, if important, should have been evident in females as well. These aberrancies would then be reflected in altered mating frequencies. Controls were run in each case and analysis of variance was employed to determine significance.

Resting Locations

In addition to available darkness and usable space tactile stimulation, from the environment and other moths, was evaluated in the first series of tests. Arenas were constructed from 150 x 25 mm plastic petri dishes. These provided moths with a choice of (1) open dark, (2) confined dark, (3) confined light, (4) or the open dish for location during each photophase (Figure 4). Densities of approximately 25, 50, and 100 moths were utilized with data being recorded for 16, 10, and 3 consecutive days, respectively. Thus, density dependent factors were treated by offering moths unlimited choice, restricted choice, and levels of forced interference.

Photoperiod, Density, Sex Ratio, and Age Effects

Adult moths of the Tucson (Maricopa) Strain were removed daily and placed in clean 1-pint mason jars during the photophase

Figure 3. Equipment used for detecting moth movement. -- The cage is suspended by rubber bands within the wooden frame. The transducer circuitry, preamplifier and power supply are to the right of the frame.

Figure 4. Test arena allowing the selection of (1) open dark, (2) confined dark, (3) confined light, or (4) the open petri dish by pink bollworm moths during diurnal periods.

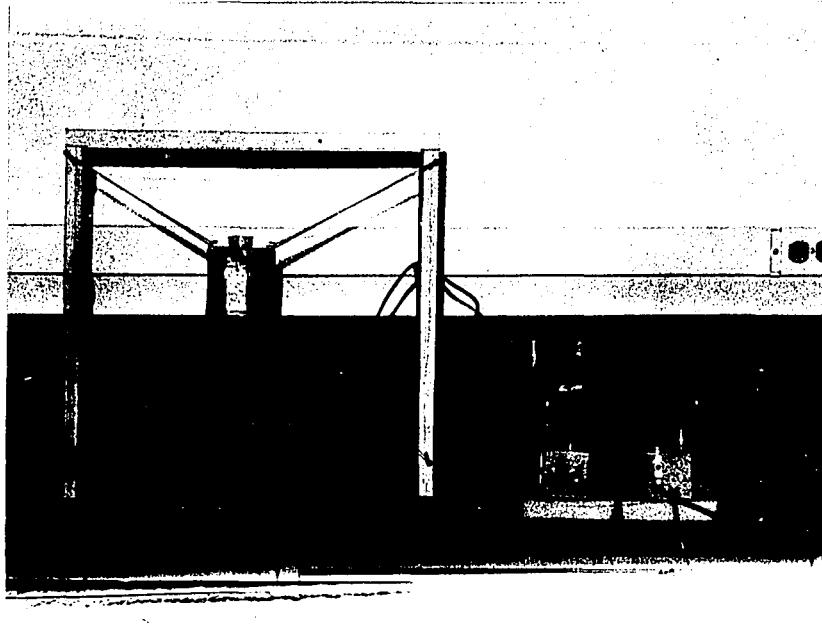


Figure 3. Equipment used for detecting moth movement.

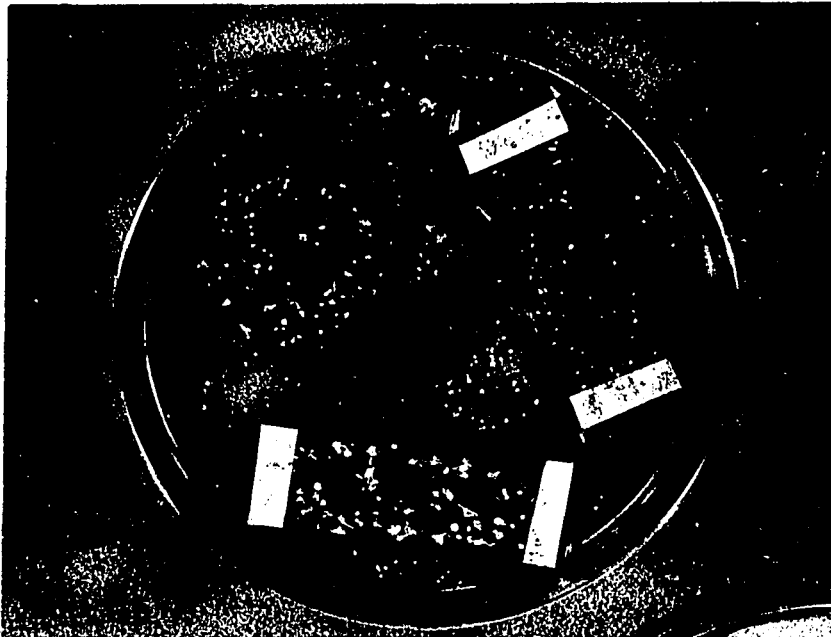


Figure 4. Test arena allowing the selection of (1) open dark, (2) confined dark, (3) confined light, or (4) the open petri dish by pink bollworm moths during diurnal periods.

when they were least active. These containers were 73 mm in diameter and 120 mm high, each providing support for the moths on the bottom and around the lid. Moths less than two days old were narcotized with CO₂ and paired in fresh jars according to the experimental design. They were then housed in the observation room under standard conditions. After 20 days the moths were killed and preserved with a solution containing 10% glacial acetic acid, 10% glycerin, and 80% absolute ethyl alcohol. Each female was dissected for spermatophores as an indication of mating success.

The first parameter to be investigated was the relationship between photoperiod and mating success. Ten male and ten female moths were used for each replicate and there were 16 replicates per treatment (320 moths). Number of spermatophores transferred and state of egg development were determined after 20-day exposures to photoperiods of L:D 14:10, 24:0, and 0:24.

The effect of moth density on mating frequency was similarly investigated. Densities of 10, 20, 30, and 40 individuals were provided by pairing 5, 10, 15, or 20 males and females per container. Treatments were replicated 12 times, utilizing 1200 moths.

The influence of sex ratio on mating frequency, a parameter of primary importance, could be investigated only after density effects were eliminated. Densities ranged from 10 to 25 individuals per 1-pint container, with sex ratios of 1:4, 1:2, 1:1, 2:1, and 4:1. These resulted from combinations of 5-20, 5-10, 5-5, 10-5, and 20-5 males to females, respectively. Approximately 900 moths were required to complete 10 replications.

Various age distributions were tested by pairing progressively older moths (2-20 days, alternating days) or those of equal age. Ten male and ten female moths from each category were maintained for 20 days prior to dissection, and five replicates required over 3000 moths.

Mating duration and timing were recorded from 30 moths sampled from a group of 500 housed in 25 x 150 mm plastic petri dishes, 50 per container. These were maintained under standard conditions and observed daily during the scotophase by using a darkroom safe light fitted with a Kodak no. 2 red safe light filter and a 15 watt bulb.

Criteria used in the analysis include total spermatophores transferred, spermatophores per female, the ratio of mated to virgin females, and the ratio of multiple to total matings. The parameter most critical to each experimental population is probably spermatophores per female; therefore, this is used for direct comparisons. However, the ratio of multiple to total matings is particularly important with respect to sterile-male release potentialities. The ratio of mated to virgin females is also important to this situation; whereas, spermatophores transferred primarily indicates the sum of interacting individuals. Further substantiation of the results was achieved by computing an analysis of variance "F" test for each relationship, and a Student-Newman-Keuls Multiple Range Test for those indicating significance.

Determination of Oviposition Rates

Oviposition studies were conducted under standard observation room conditions utilizing 15 x 100 mm plastic petri dishes modified according to each experiment. The lids were left intact or fitted with 17 mm holes covered with cotton pads. Twenty-five randomly selected pupae were added to each dish, except for sex ratio tests, and no other manipulations were performed other than daily observations to determine the number of live moths, egg deposition, hatching, and distribution. Additional modifications were made in the experimental environment depending on the information desired.

Initially, substrates other than cotton pads were offered to determine the range of acceptable sites for oviposition. Various textures were provided by using paper, plastic, glass, metal, cloth, and wax. In addition, data were recorded on moth behavior in the absence of a suitable site. Temporal patterns were noted by compiling hourly rates of oviposition on sucrose-soaked cotton pads. Daily totals yielded an overall cycle for each generation tested.

Site-selection tests required the use of specially modified plastic lids, each with one hole at the center and another near the edge. Moths were offered a choice between sucrose-soaked or dry cotton at a given hole, depending on replicate. Thus, a 2 x 2 factorial randomized block design was established to evaluate response to substrate and position.

Experimental environments, including the site selection alternatives, were as follows: (1) plastic petri dishes only, (2)

dishes with a paper towel liner, (3) paper and dry cotton at top center, (4) paper and water-soaked cotton, (5) paper and cotton soaked in 10 % sucrose solution, (6) sucrose-soaked cotton only, (7) sucrose-soaked cotton center and dry cotton side choice, and (8) 2 sucrose-soaked cotton sites. These were prepared and maintained in the observation room in a fashion identical to the other oviposition tests. The situations were designed to evaluate the influences of substrate texture, prevailing humidity, site position, available moisture, usable carbohydrate nutriment, location of media, and combination effects. Egg counts provided the only assay; however, moth mortality was recorded.

Mode of deposition and average size of egg masses were derived from 40 tests. Total number of eggs, their distributions as scattered or massed, and the number of eggs per mass were recorded for each sample. The favored orientation with respect to gravity was similarly investigated by suspending a paper substrate within the petri dishes. Sucrose-soaked cotton provided for maximum production, but eggs deposited on it were ignored.

The influence of male presence on oviposition was evaluated by isolating females or altering normal sex ratios. Females maintained in the absence of males were also dissected and examined for ovary development. Sex ratios were the same as those used in mating frequency experiments and, in this case, egg deposition was the assay.

RESULTS

Normal Rhythms of Activity

The actograph was very useful in obtaining daily activity rhythms under experimental conditions. This information was then compared with direct observations, thus aiding the formulation of natural behavior patterns. It provided a means of monitoring biological events such as hatching, emergence, and diel movement. It was also used to evaluate the influence of physical factors from the natural environment and interactions between organisms. Photoperiod, temperature, and other electromagnetic radiations were investigated in detail.

One of the problems limiting application of the actograph system was its sensitivity to external vibrations which influenced the instrument as well as the enclosed insects. The equipment recorded relative levels of activity which incorporated individual movements, but often obscured the effects of extraneous stimuli. The response was therefore indicative of the entire population rather than single insects. For this reason, all experimentation was conducted under partially controlled isolated conditions. Landing after flight and saltatory movements produced particularly useful recordings.

Composite rhythms produced by 10 male and 10 female moths contained certain stereotyped elements (Figure 5). Transients or

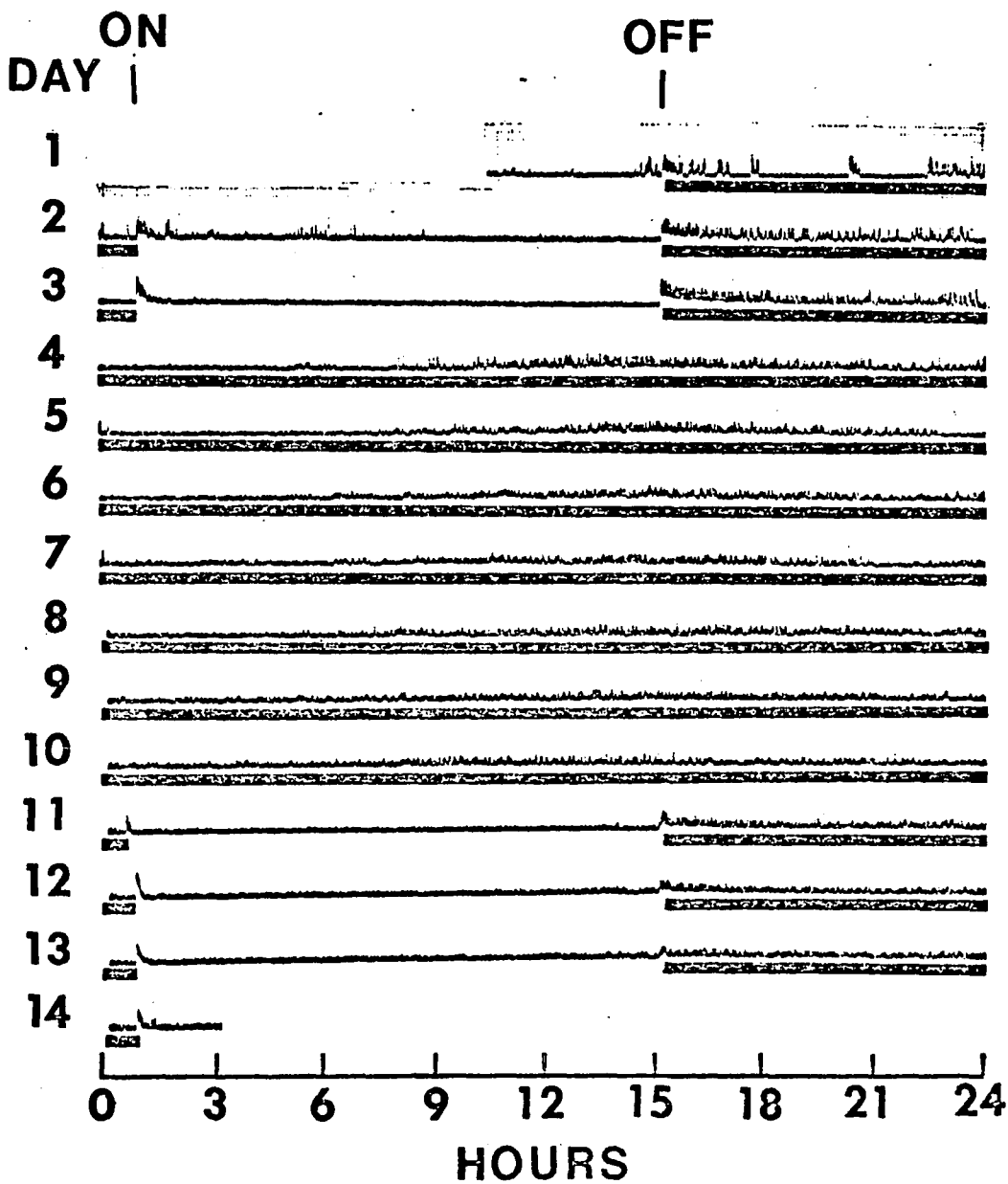


Figure 5. Circadian rhythms produced by 10 male and 10 female pink bollworm moths. -- The first 3 days show entrainment to an L:D 14:10 regime (lights on at 0600 and off at 2000 MST). Free-run in continuous dark follows, producing a phase-shift. Re-entrainment to the original scheme begins on day 11.

patterns from moths not yet entrained appeared during the first day of testing followed by characteristic behavior on the second and third days. A typical response to light was evident on the third day at 0600. Occasional movement of single or few individuals was observed during this time which could have been due to displacement by crowding or to stress produced in the confined experimental environment. At the termination of scotophase on day 3, entrainment was considered complete and the insects were allowed to free-run in constant darkness through the tenth day.

Arhythmic activity was followed by re-entrainment on day 11 and the photoperiod was identical to the original scheme. Transients did not appear and entrainment was complete by the twelfth day. The record indicated a reduced amplitude of activity, probably due to a 50% mortality of moths, which continued through the fourteenth day after which the experiment was terminated.

Entrained Patterns

When 20 female moths were tested a very similar pattern emerged (Figure 6). Activity was continuous throughout the scotophase; however, more transients appeared during entrainment. The first day of free-run, on day four, produced an extreme phase-shift but the peak remained near the entrained position. The pattern was continuous by day nine and the usual reduction in amplitude was observed. The light response during re-entrainment indicated the relatively weakened condition of the moths.

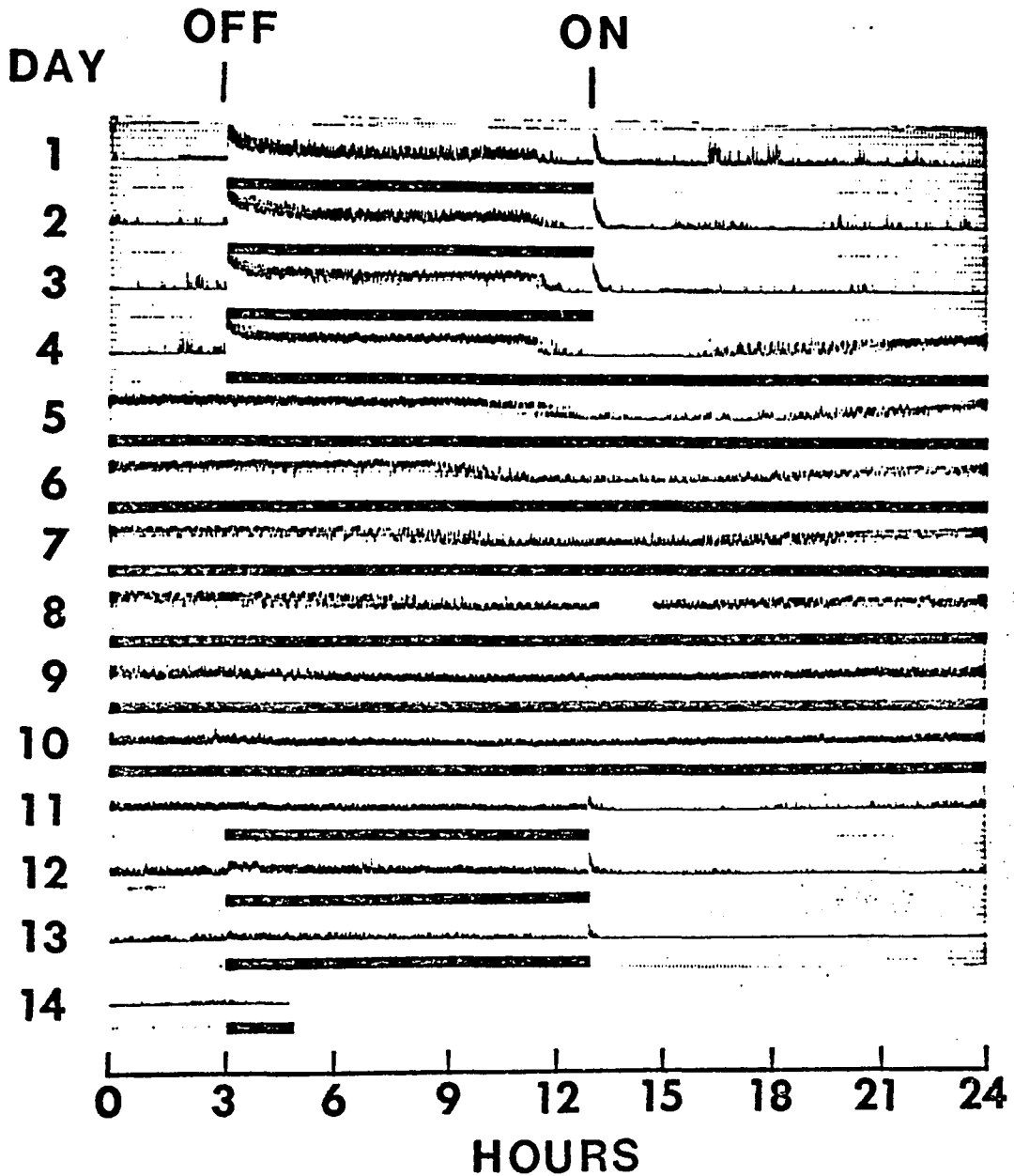


Figure 6. Circadian rhythms produced by 20 female pink bollworm moths. -- The first 4 days involve entrainment to an L:D 14:10 regime (lights on at 0600 and off at 2000 MST). Free-run in continuous dark follows on day 4 and re-entrainment to the original scheme begins on day 11.

Twenty males produced a bimodal pattern which was previously obscured by the behavior of the female (Figure 7). Male and female rhythms were similar except that males produced a second daily peak following approximately 7.5 hours after the initiation of activity. This was about 2.5 hours in duration and would have occurred at nearly 3:30 AM in the field. The remainder of the pattern was typical.

Naïve Moths

Naïve moths reared in continuous darkness were tested for possible endogenous rhythms (Figure 8). In this case, natural light impinging on the insects from a distant window was utilized. Emergence was unsynchronized, spreading over the first 3 days, without light as an environmental cue. During days 4 and 5 emergence was completed and activity became relatively continuous. Free-run progressed for the next 4 days followed by entrainment. Again, the imposed pattern was expressed as a result of suppression by light during each photophase (subjective day). This was evidenced by a reduction in the number of transients on successive days.

Periodicity in the Arizona pink bollworm is well correlated with behavior of the moth in Texas reported by Graham et al. (1964). The actograph system showed that female moths were continuously active with an early maximum during each scotophase. Males were bimodal, exhibiting the same initial peak and a second resurgence approximately six hours later. Although influences of female pheromone, other male-female interaction, age, and the prevailing ecological conditions of

Figure 7. Circadian rhythms produced by 20 male pink bollworm moths. -- Entrainment to an L:D 14:10 regime (lights on at 0600 and off at 2000 MST) resulted in the production of a bimodal pattern. The second peak occurred approximately 6 hours after the initiation of activity.

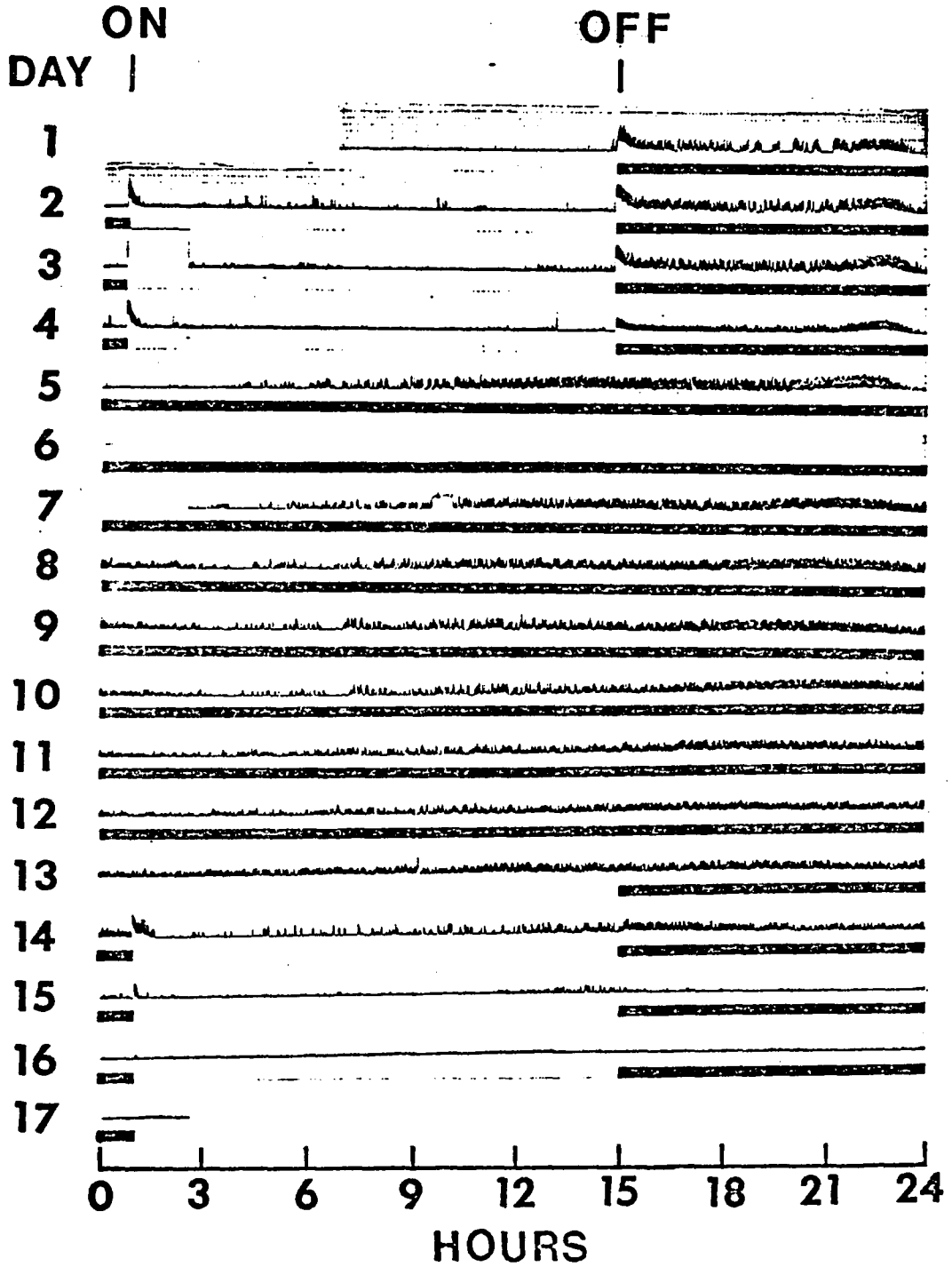


Figure 7. Circadian rhythms produced by 20 male pink bollworm moths.

the field were partially eliminated in the laboratory, the patterns persisted. Reported disparities in the periodicity of moths from different geographic localities may be due to genetic variation between populations, inconsistent environmental pressures, or combined interactions resulting in different functional niches. More probably, however, such diversity could result from the inadequacies of light trap sampling techniques.

Pink bollworm moths exhibited a biphasic response to light and other agents that suppress locomotor activity. As with carbon dioxide anesthesia, the onset of light produced an almost immediate burst of activity followed by immobility. Agitation increased, reaching peak levels within a few minutes and subsiding in less than 30 minutes. This response was constant and predictable, occurring each day throughout testing (Figure 5).

Transients which appeared early in the record were also consistent but of uncertain origin. They could have resulted from incomplete entrainment of newly emerged adults or from disturbance during transfer to the actograph cage. The former seemed more likely because of records derived from older moths.

Alteration of Normal Periodicity

Two factors of practical application, photoperiod and gamma irradiation, were investigated for their disruptive influence on normal rhythmic activity. Periodicity of the moths dissipated under conditions of constant light or darkness, whereas gamma radiation

administered at dosages of 10 and 25 kr produced no measurable effects. The importance of moth age, sex, strain, and prior exposure was also evaluated during these tests.

Free-running Patterns

In these studies, photoperiodically entrained rhythms were altered by allowing the insects to free-run in constant darkness (Figure 5). An immediate phase-shift and prolonged activity occurred on the fourth day, resulting in earlier initiation and delayed termination of movement. Simultaneously there was a distinct reduction in amplitude, indicating reduced levels of movement. The actual total amount of daily activity did not necessarily fluctuate, but merely the rhythm of its expression.

The obvious differences between entrained and free-running patterns were accompanied by more subtle changes. The rhythm became discontinuous, particularly during the early segments of each active phase. Also, near the termination of each arbitrary day, a short burst of movement occurred between 0400 and 0500 hr (days 6-9). Superficially there appeared to be little variation in the peak activity time.

The initiation of locomotor activity for each diel cycle was estimated in order to delimit population periodicity. This measurement included all activity associated with overt movement but was somewhat arbitrary due to individual variability among moths. Experiments, utilizing both sexes, conducted at $26 \pm 3^\circ\text{C}$, yielded angular shifts which approximated the previously reported 22.7 hr period. The L:D

14:10 photoperiod skeleton was not manipulated but, under this schedule, there were no discernible differences in rhythmicity between males and females.

The appearance of transients during each scotophase in these tests indicated a suppression of locomotion by light. In order to test this hypothesis, ten male and ten female moths were maintained in continuous light (Figure 9). The lights remained on following entrainment on day one. During the second day activity increased and diverged from the lights-on point of the previous day. Activity was continuous by the third day and normal amplitudes were reached by day four. Insects adapted to the light and essentially duplicated the pattern exhibited during free-run in continuous darkness.

Gamma-Irradiated and Normal Moths

In these tests the first day was disrupted due to handling, but the third of each sequence may be used for comparison (Figure 10). Superficially, the patterns from Brownsville and Tucson moths were equivalent. The increased number of transients in the Texas Strain was probably due to age (Texas 13 days and Tucson 5 days). Transients increased proportionately in 10 and 25 kr populations but both maintained distinct patterns which were in phase. The duration of diel activity increased in some gamma-irradiated moths. This would probably shorten the life span but have little effect on competitiveness. Locomotion was essentially equivalent by the fourth day after emergence in all the experimental populations.

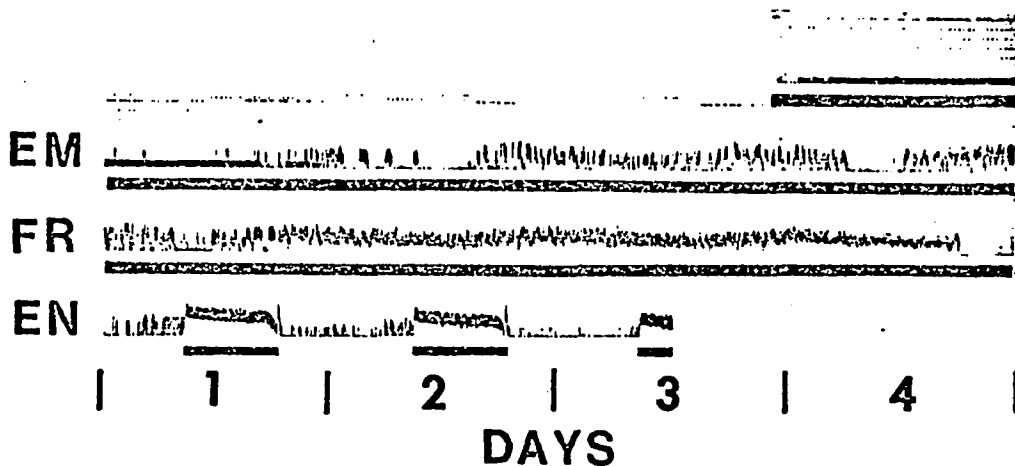


Figure 8. Circadian rhythms produced by 10 male and 10 female naive pink bollworm moths during emergence (days 1-4), free-run in constant darkness (days 4-8), and entrainment (days 9-12). -- Emergence (EM), free-run (FR), and entrainment (EN) are indicated during 12 consecutive days.

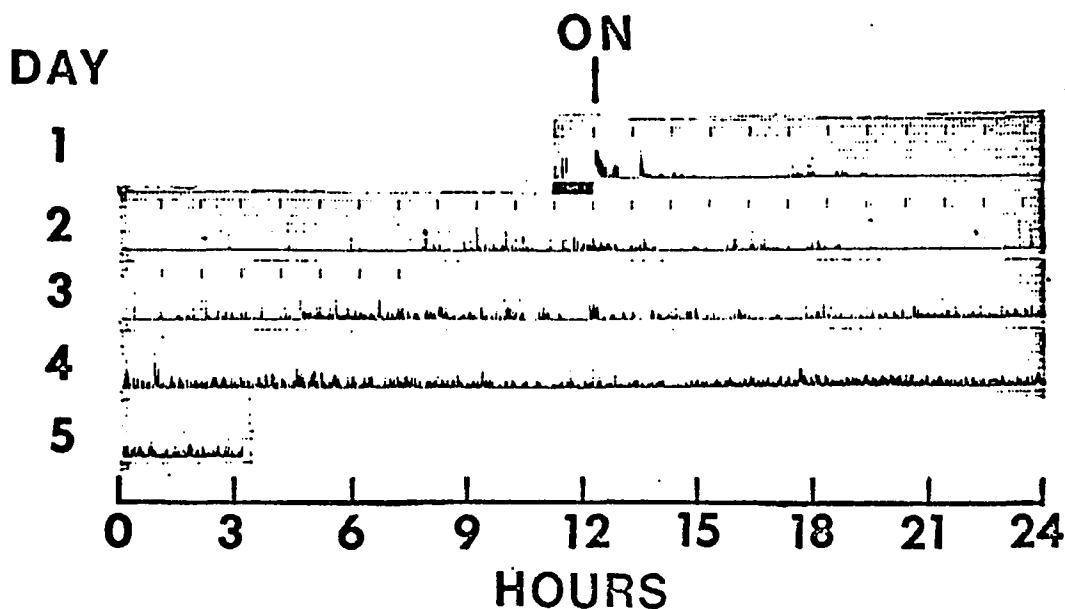


Figure 9. Diel activity patterns produced by 10 male and 10 female pink bollworm moths during free-run in constant light following entrainment to an L:D 14:10 regime (lights on at 0600 MST).

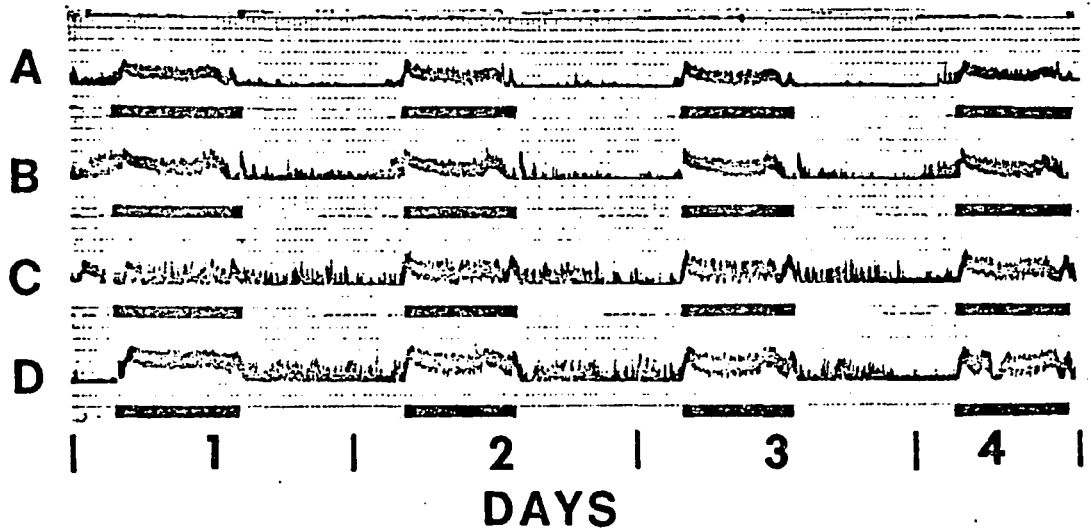


Figure 10. Rhythmicity of gamma-irradiated and normal pink bollworm moths from Tucson, Arizona (A) and Brownsville, Texas (B, C, and D). -- The actograms were each produced by 10 male and 10 female moths irradiated with doses of either 0 (A, B), 10 (C), or 25 kr (D).

Periods of Inactivity

Aggregation in Resting Sites

Experiments on resting location during the scotophase were each replicated twice and analyzed for variance by employing the Student-Newman-Keuls-Multiple Range Test (Table 1). Moths were offered a choice of (1) open dark, (2) confined dark, (3) confined light, or (4) the open dish. No statistically significant differences occurred between treatments 3 and 4; however, the mean was always higher for 3. Moths did not prefer the tactile stimulation provided by parallel glass slides, over the corners of objects within the open dish. Conversely, significantly higher numbers of moths selected the first and second treatments, with open dark being favored above all. Thus, the order of choice was 1, 2, 4, and 3, with significance at the .05 level between the dark and light, and the two dark treatments.

When 100 moths were used, confined dark was slightly favored; however, this measurement was probably erroneous due to counting difficulties. The $q \bar{S}_x$ values were slightly less than the differences between means; therefore, treatments 1 and 2 were essentially equal in effect. Tests with 50 and 25 moths probably reflected the normal selection, with 50 providing the highest counts and best test.

Table 1. Percent pink bollworm moths exhibiting microhabitat preference for (1) open dark, (2) confined dark, (3) confined light, or (4) the open dish. -- Densities of 100, 50, or 25 moths per arena were used.

Moth Density	<u>Moth Location</u>				d.f.	F- Value	\bar{Sx}
	1	2	3	4			
100	43.65	29.16	10.13	17.06	8	15.24*	8
50	35.07	51.20	4.90	8.76	36	38.03*	36
25	28.62	50.92	5.78	14.68	60	22.06*	60

*Significantly different at the 5% level

Mating Behavior

Sex Pheromone Release

The mating cycle was documented by direct observation with the use of filtered light in the dark room. It is initiated and controlled by the release of sex pheromone from the female (Figure 11). This is circadian in nature with the phase set by photoperiod. During release, females assume characteristic postures while the substance probably volatilizes from a terminal gland into the surrounding air. Pheromone stimulation is mediated through male antennae which, at this time, typically project forward and upward. Males are prompted to begin searching and orientation movements and flights increase in frequency as they presumably pursue the odor. Simultaneously, the

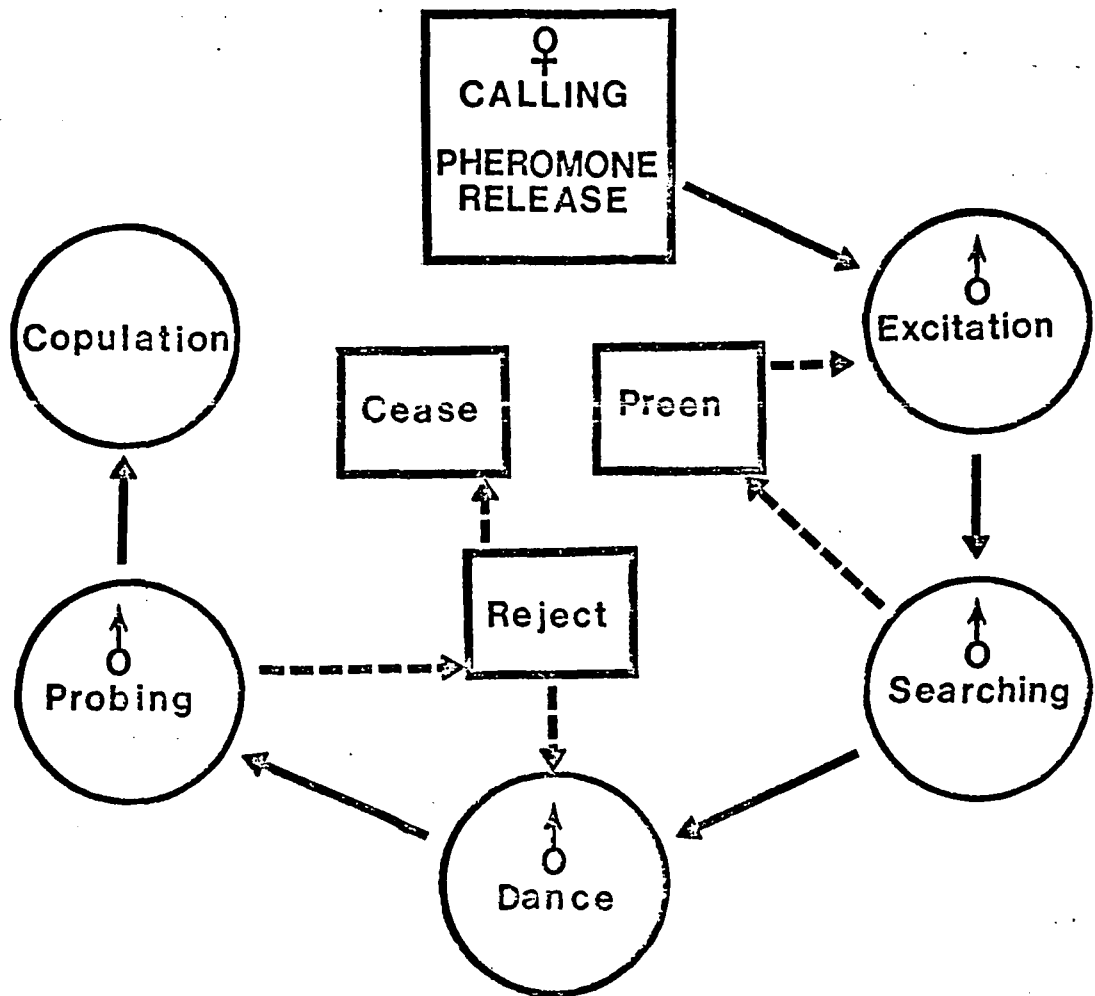


Figure 11. Ethogram depicting the mating behavior of pink bollworm moths. -- The successful sequence is indicated by the outside series of circles, and the squares within show the incomplete or unsuccessful cycle.

front antennae are preened, being drawn through a pathway formed by flexing the tarsus toward the tibia. This initial series of events is often repeated several times before success or cessation results.

Courtship

Encounters between males and females elicit the male pre-mating dance. During this display, males exhibit rapid flying, wing vibration (fluttering), and mounting of objects in the environment. Typically, males circle violently around the calling females, alternating the dance with lateral probing by the abdomen. Males extended their claspers from the side by side normal position until contact is made with the female terminalia. This behavior is also subject to repetition, with individuals being rejected and either reentering the contest or ceasing sexual activity.

Many couples paired for less than 60 sec; others engaged in bouts lasting many minutes. During these contests females employed an array of disruptive activities including rapid movement, wing lifting, tarsal pushing, flight, and hiding. Oddly, attractive females which participated in mating bouts did not necessarily pair and often hid themselves among resting individuals for the remainder of the mating period.

Copulation and Spermatophore Transfer

Following successful courtship and coupling, moths assumed the typical lepidopteran copulatory position with the individuals facing opposite directions. Genital segments were locked under

tightly compressed wings during spermatophore transfer which required at least 30 min. Legs were braced against the substrate and antennae were extended laterally and anteriorly. If not disrupted moths eventually exhibited less tense postures. Antennae were rested against relaxed wings, and legs were withdrawn to allow their bodies to contact the supporting surface. Pairs were not usually approached by other males except when they were pairing or parting (Table 2).

Female Sex Pheromone

Calling

The initial activity, calling or pheromone release, was the subject of a still more detailed investigation. Young females tended to call while suspended upside down or attached to a vertical surface. After a few days they favored an upright position on a horizontal surface, and continued this preference for life. They were always stationary while calling and, when disturbed, withdrew their telescoped terminalia but maintained their posture. The tip of the abdomen is directed downward during calling, and the behavior includes two stereotyped postures. These were termed "initial" and "overt" calling.

During initial calling the wings are extended, elevated and separated, with the antennae resting against the anterior wing margins. Legs are braced laterally, maintaining the body parallel to the substrate with the head slightly raised. The ovipositor, with its associated structures, is projected posteriorly and then curled downward and forward toward the venter (Figure 12). During this

Table 2. Summary of the successful mating sequence for the pink bollworm moth.

Pheromone Release

Specific calling postures

Stimulation of Male Antennae

Pheromone reception probably restricted to male antennae

Searching and Orientation

Flights increase in frequency

Male Premating Dance

Rapid flying near female

Wing vibration (fluttering)

Mounting of objects in the experimental environment

Side to Side Lateral Probing with Genitalia

Claspers extended

Abdomen probes from a lateral position

Pairing of individuals

Copulation and Spermatophore Transfer

Male and female face opposite directions during intro-
mission

Wings are folded down against each other

Successful transfer requires at least 30 minutes

Figure 12. Initial calling in the female pink bollworm moth depicting the pheromone gland in its ventral position.
-- (A) Lateral aspect disclosing the location of the gland in relation to the ovipositor. (B) Dorsal aspect showing the posterior protrusion of the gland.



Figure 12. Initial calling in the female pink bollworm moth depicting the pheromone gland in its ventral position.

early phase the ovipositor and pheromone gland contact the substrate and are manipulated against it with short circular motions. The ovipositor also probes anteroventrally, compressing the gland against the supporting surface.

Overt calling is an exaggeration of initial calling, with the wings elevated at about 45° , more widely separated and tensely extended. Antennae are held perpendicular to the body, away from the wings and palps. The legs further elevate the abdomen, which is held halfway between the substrate and the wings while the head was bent forward and down (Figure 13). The gland is drawn off the substrate and the ovipositor vibrated primarily dorso-ventrally, with rhythmic lateral movements producing a circular motion approximately 1 mm in diameter. In this position the gland is nearly 2 mm above, and parallel to, the substrate.

The gland is incorporated into a folding structure between the ostium bursae and the papillae anales (Wellso and Adkisson, 1962; R. N. Jefferson, L. L. Sower, and R. S. Rubin, 1971). It is everted dorsally and attains full size when the ovipositor is reflexed ventrally. Size relationships have been determined by tracing the apophysis posterioris from the terminal abdominal tergite near the ostium bursae (invisible externally) to its insertion in the basal portion of the ovipositor (Figure 13). The bulbous gland projects above the apophysis about midway along its course and is sparsely setose with some concave depressions and

Figure 13. Overt calling in the female pink bollworm moth with dorsal positioning of the pheromone gland. -- (A) Lateral aspect revealing the maximum distention of the gland. (B) Posterior aspect showing the medial location of the gland.



Figure 13. Overt calling in the female pink bollworm moth with dorsal positioning of the pheromone gland.

several small opaque spots. The gland is translucent with a shiny reflective surface, as if moist.

Extraction and Testing

Methylene chloride extracts of 10 abdomens from three-day-old females were allowed to volatilize from filter paper in open petri dishes for 1.5 to 2 hr. Forty drops of the material elicited typical mating behavior from three-day-old males. When suspended above the dishes they immediately dropped to the filter paper below and began to dance. Genitalia probed the dish, paper, and other moths while the antennae were extended and frequently preened. Response waned after three min, while after five min arousal occurred only sporadically, and after 20 min all were quiescent. Extracts held for 2.5 to 3 hr effected similar results, whereas volatilization for three days eliminated active response. Moths exhibited much less activity of relatively short duration. The methylene chloride control produced no response.

Extracts from 1, 10, and 20 day old females, administered to virgin males of these ages maintained in isolated groups elicited three levels of response. These included: (1) immediate and long lasting, (2) latent but long lasting, and (3) no activity. Males 1 to 3 days old exhibited the first response when confronted with extracts from females of all 3 ages. Behavior of the second type resulted when males 10 ± 1 days old contacted the female substances. Males 20 ± 1 days of age seldom responded and no activity was recorded for the controls (Table 3).

Table 3. Relative responsiveness of male pink bollworm moths 1, 10 and 20 days after emergence to female sex pheromone. -- The substance was crudely extracted with methylene chloride from 1, 10, and 20 day old females.

Female Age at Extraction	<u>Age of Male at Testing*</u>		
	1 day	10 days	20 days
1 day	+ + +	+ +	0
10 days	+ + +	+ +	0
20 days	+ + +	+ +	0

*
 + + + = Immediate and long lasting response
 + + = Response lags but becomes long lasting
 0 = No response

Male Antennal Receptors

Removal of male antennae and subsequent pairing under various conditions resulted in essentially no successful mating (spermatophore transfer). In mason jars, males without antennae survived but failed to inseminate a single female, whereas controls averaged nearly 20 matings per replicate. Similarly in vials, antennaless males transferred only 2 spermatophores, and it is possible that these were exchanged prior to testing as a result of an error in sexing pupae. Amputated females were not impaired, as evidenced by mating frequencies equivalent to the normal populations (Table 4).

Table 4. Mating success of pink bollworm moths following removal of the antennae. -- Insects were maintained at densities of 10 males and 10 females per 1-pint mason jar.

Replicates	<u>Number of Spermatophores Transferred After Treatment</u>		
	Male Antennae Amputated ^a	Female Antennae Amputated ^b	Male and Female Antennae Intact ^b
1	0	2	5
2	0	6	6
3	0	2	5
4	0	3	4

^aSignificantly different at the 5% level

^bNo significant difference at the 5% level; $F = 6.0$; $S\bar{x} = .730$

Factors Affecting Mating Frequency

Photoperiod

Experiments involving photoperiod produced no statistically significant differences among any of the samples at the 0.05 level, except in the ratio of multiple to total matings (Table 5). Means of .589, .409, and .232 spermatophores per female were recorded for continuous dark, continuous light, and 14:10 photoperiods, respectively. Thus, multiple mating was enhanced by conditions of continuous dark. Fecundity has also been favorably affected by the dark environment, but the parameters responsible for this have not been

Table 5. Effect of photoperiod on mating success in the pink bollworm moth indicated by the mean number of spermatophores transferred.

Analysis Criteria	Experimental Photoperiods			d.f.	F-Value	\bar{S}_x
	24:0	14:10	0:24			
Total Spermatophores Transferred	9.69	8.94	13.13	30	2.446	1.428
Spermatophores Transferred Per Female	0.97	0.89	1.13	30	0.025	0.014
Mated/Virgin Females	2.36	3.64	2.86	30	0.734	0.754
Multiple/Total Matings	0.41	0.23	0.59	30	9.150*	0.006

*Significantly different at the 5% level

established (Ridgway, 1971). Therefore, the significance of multiple mating remains obscure.

During laboratory tests on the effects of photoperiod, all other measurements indicated the same high levels of mating under continuous dark or light. This was also evident in rhythm experiments under free-running conditions, which strongly suggests a relatively unimportant role for natural cyclic photoperiodicity in the biological success of this insect. It was another example of the tremendous behavioral plasticity so characteristic of the pink bollworm. Apparently, they reach the same high levels of productivity under all continuous photoperiodic regimes even though they never encounter these situations in nature.

Influence of Density

Mating frequency was not affected by changes in population density, since no significant differences were indicated for any of the criteria tested (Table 6). However, subsequent experiments involving various densities within this range suggested that optimum limits exist, 30 moths per container being ideal. Maximum mating frequencies, as evidenced by all four measurements (total spermatophores transferred, spermatophores transferred per female, mated/virgin females, and multiple/total mating), were produced by combining 15 males with 15 females. Fortunately, more precise relationships were produced in the male to female sex ratio experiments, with significant differences occurring in total spermatophores transferred and spermatophores transferred per female.

Table 6. Effect of insect density on mating success in the pink bollworm moth indicated by the mean number of spermatophores transferred

Analysis Criteria	Pairs of Moths				d.f.	F-Values	\bar{Sx}
	5	10	15	20			
Total Spermatophores Transferred	7.42	11.42	21.17	17.25	47	11.23*	0.910
Spermatophores Transferred per female	1.48	1.14	1.41	0.86	47	2.81	0.084
Mated/Virgin Females	3.26	3.40	5.17	2.23	47	1.84	0.440
Multiple/Total Matings	0.45	0.46	0.48	0.31	47	1.25	0.036

* Significantly different at the 5% level

Sex Ratio and Spermatophore Transfer

As expected, more matings occurred with an excess of female moths, the 5 male to 20 female ratio being optimum. These conditions provided a continuous supply of receptive females throughout the reproductive lives of the male. However, when the values were adjusted by conversion to the ratio of spermatophores per female, excess males produced the maximum value. Thus, an abundance of females allowed more mating to occur and additional males increased the frequency of transfer (Table 7).

Table 7. Effect of insect sex ratio on mating success in the pink bollworm moth indicated by the mean number of spermatophores transferred.

Analysis Criteria	<u>Male : Female Sex Ratios</u>					d.f.	F- Value	S \bar{x}
	1:4	1:2	1:1	2:1	4:1			
Total Spermatophores Transferred	9.60	8.10	4.90	4.70	5.50	36	5.22*	0.952
Spermatophores Transferred per female	0.48	0.81	0.98	0.79	1.10	36	5.09*	0.011
Mated/Virgin Females	0.76	1.71	1.88	2.25	1.88	36	1.62	0.439
Multiple/Total Matings	0.17	0.23	0.37	0.27	0.20	36	2.37	0.005

* Significantly different at the 5% level

Another unexpected finding appeared during these experiments. Male to female ratios of 2:1 produced statistically significant lower

mating frequencies than 1:1 and 1:2; whereas, 4:1 was highest. An increased mating frequency in proportion to the number of added males was expected, as occurred with increased oviposition rates in other experiments (Hussien et al., 1962). Perhaps excess males interfered in some way, but their disruptive influence was more than compensated for by the addition of more males. Alternative explanations are difficult to formulate.

Effect of Age

Experiments on the influence of moth age on mating efficiency produced rather startling results (Tables 8, 9, and 10). Fresh young males 1-3 days old paired with females at 2-20 days after emergence, the reciprocal of this with fresh females, and tests with even-aged moths paired at 2-20 days of age all produced similar results. Each group exhibited bimodal peaks of receptivity, indicating an initial primary mating period followed by a secondary resurgence approximately four days later (Figures 14, 15, and 16). The first peak was immediate and lasted about eight days in each case. A relatively nonreceptive period of more than four days in different-aged, and nearly seven days in even-aged tests, followed. The second period was more prominent for young females than males, lasting six and four days, respectively. Even-aged moths produced a more pronounced second peak, but of only two days' duration. The basic patterns were essentially the same for all three groups, with minor differences in timing and magnitude.

Table 8. Effect of insect age on mating success in the pink bollworm moth indicated by the mean number of spermatophores transferred to females paired 1 day after emergence.

<u>Criteria For Analysis</u>				
Age of Males (Days)	Total Spermatophores Transferred	Spermatophores Transferred Per Female	Mated/Virgin Females	Multiple/Total Matings
2	13.40	1.34	5.00	0.25
4	12.40	1.24	5.33	0.22
6	10.80	1.08	3.17	0.25
8	10.00	1.20	3.70	0.35
10	9.80	0.90	1.23	0.22
12	5.80	0.58	1.62	0.06
14	10.20	1.02	3.50	0.23
16	7.00	0.70	1.30	0.21
18	2.80	0.28	0.44	0.03
20	2.40	0.24	0.36	0.00
d.f.	36	36	36	36
F-Value	7.95*	7.95*	3.07*	13.48*
\bar{Sx}	1.343	0.125	1.037	0.001

*Significantly different at the 5% level

Table 9. Effect of insect age on mating success in the pink bollworm moth indicated by the mean number of spermatophores transferred from males paired 1 day after emergence.

<u>Criteria For Analysis</u>				
Age of Males (Days)	Total Spermatophores Transferred	Spermatophores Transferred Per Female	Mated/Virgin Females	Multiple/Total Matings
2	12.60	1.26	4.20	0.19
4	7.20	0.72	1.34	0.23
6	15.00	1.50	3.73	0.29
8	11.00	1.10	3.28	0.25
10	6.80	0.68	1.58	0.17
12	4.60	0.46	0.74	0.12
14	6.40	0.64	2.32	0.19
16	1.80	0.18	0.17	0.07
18	0.40	0.04	0.02	0.10
20	4.00	0.40	0.39	0.27
d.f.	36	36	36	36
F-Value	7.14*	7.14*	1.95	0.99
\bar{Sx}	1.238	0.012	0.777	0.005

* Significantly different at the 5% level

Table 10. Effect of insect age on mating success in the pink bollworm moth. -- This was indicated by the mean number of spermatophores transferred in males and females paired on successive days after emergence.

Age of Males (Days)	<u>Criteria for Analysis</u>			
	Total Spermatophores Transferred	Spermatophores Transferred Per Female	Mated/Virgin Females	Multiple/Total Matings
2	7.40	0.74	1.92	0.19
4	9.69	0.96	1.72	0.19
6	4.00	0.40	0.61	0.09
8	7.80	0.78	2.40	0.10
10	5.80	0.58	1.10	0.15
12	5.00	0.50	1.13	0.00
14	2.40	0.24	0.23	0.15
16	1.80	0.18	0.14	0.27
18	7.40	0.74	1.37	0.31
20	2.00	0.20	0.22	0.34
d.f.	36	36	36	36
F-Value	4.86*	4.86*	2.65*	1.88
\bar{Sx}	0.876	0.888	0.340	0.005

* Significantly different at the 5% level

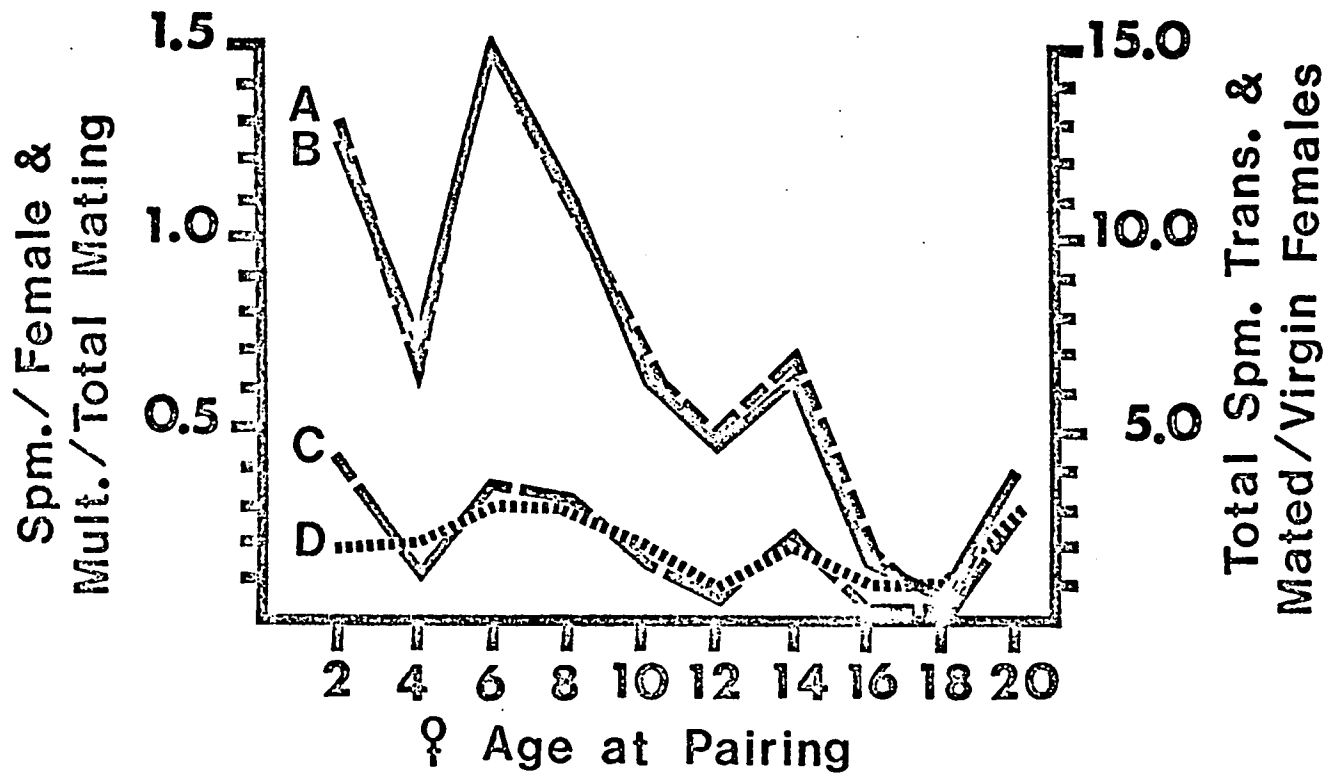


Figure 14. Frequency of spermatophore transfer from male pink bollworm moths paired 1 day after emergence with females 2-20 days of age, during the 20 days after mixing. -- Criteria for analysis include, (A) Total spermatophores transferred, (B) Spermatophores transferred per female, (C) Mated/virgin females, (D) Multiple/total mating.

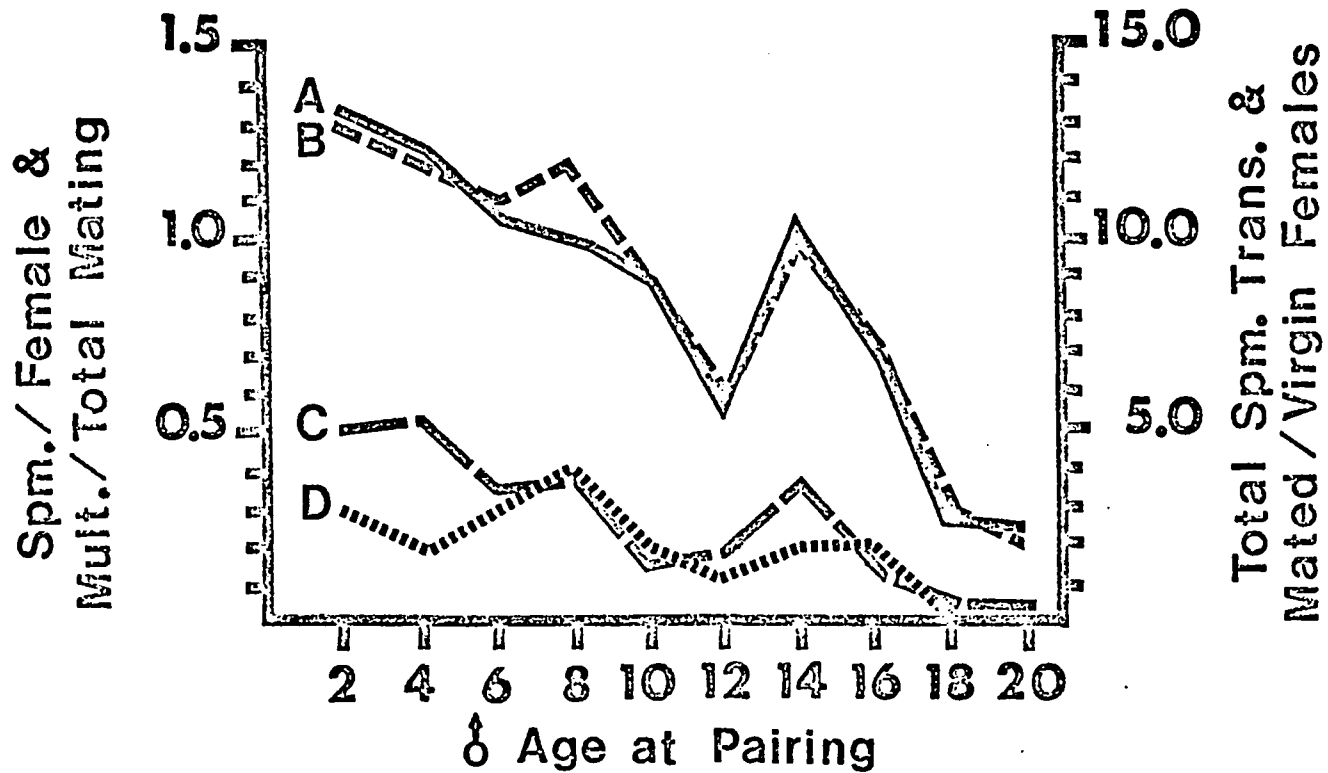


Figure 15. Frequency of spermatophore transfer to female pink bollworm moths paired 1 day after emergence with males 2-20 days of age, during the 20 days after mixing. -- Criteria for analysis include, (A) Spermatozoa transferred per female, (B) Total spermatozoa transferred, (C) Mated/virgin females, (D) Multiple/total mating.

Figure 16. Frequency of spermatophore transfer by male and female pink bollworm moths paired 2-20 days after emergence, during the 20 days after mixing. -- Criteria for analysis include, (A) Total spermatophores transferred, (B) Spermatophores transferred per female, (C) Multiple/total mating, (D) Mated/virgin females.

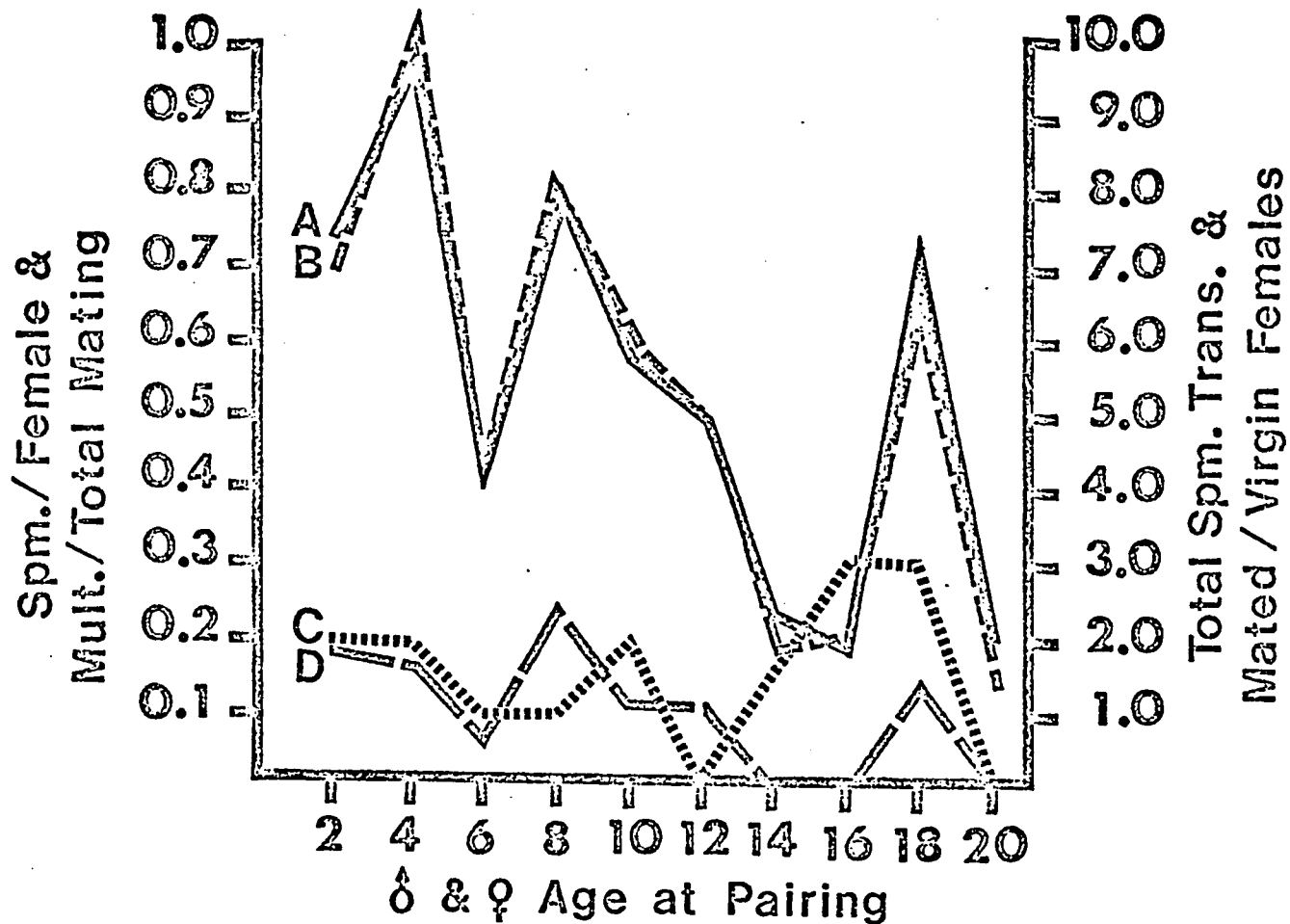


Figure 16. Frequency of spermatophore transfer by male and female pink bollworm moths paired 2-20 days after emergence, during the 20 days after mixing.

Spermatophores per female, or total transferred, provided the best estimates of mating frequency; however, all criteria indicated similar patterns. The only statistically significant deviation was in the level of multiple mating exhibited during the second period by even-aged moths (Figure 15). Apparently older moths are more capable of increased mating frequencies.

In addition to this cycle, a daily mating peak occurred between 8.5 and 9.5 hours after the onset of darkness (Table 11). The range, 6.5 to 10.5 hr approximated reported values and substantiated similarities between moths from different geographic localities. No further attempts were made to duplicate experiments previously conducted by others with the Texas Strain.

Oviposition Phenomena

Egg Deposition and the Environment

A wide variety of substrates was used for oviposition, at least to a limited extent, including dead insect bodies and pupal cases; however, nonporous sites yielded very low numbers of eggs. Counts from all but the cotton, cloth, and paper were essentially negligible. Oviposition followed a precise circadian rhythm with the phase set by photoperiod, but most of the other controlling factors remain to be investigated. Experimental evidence indicated that the onset of darkness was primary, with significant interactions from other environmental factors, tactile stimulation, conditions within the insects. Tests involving 960 moths indicated that peak oviposition followed maximum emergence by four to six days, and daily

Table 11. Circadian periodicity and duration of mating in the pink bollworm moth measured by direct observation.

<u>Periodicity of Matings</u>		<u>Duration of Matings</u>	
Time of Initiation	Number of Matings	Length of Time, Min.	Number of Matings
1230	2	30	3
1330	3	40	2
1400	3	60	3
1415	3	75	1
1430	5	80	1
1445	4	90	10
1500	7	100	2
1530	2	105	1
1600	1	110	2
		120	2
		150	1
		180	2

peaks occurred one to two hours after initiation of the scotophase (Table 12). Most eggs were deposited by ten days after emergence (Table 13). Embryos from viable eggs usually developed into very active larvae within 48 hours.

Table 12. Circadian periodicity of oviposition in pink bollworm moths. -- This was indicated by the number of eggs deposited on the first night after emergence. Insects were maintained as 10 pairs per 15 x 100 mm petri dish.

Trial	<u>Time of Day</u>						d.f.	Value	\bar{Sx}
	0600- 0700	0700- 0800	0800- 0900	0900- 1000	1000- 1100	1100- 1200			
1	5	3	1	2	1	0	-	-	-
2	63	46	18	7	1	6	-	-	-
3	17	7	2	6	0	0	-	-	-
Grand Means	28.3	18.7	7.0	5.0	0.67	2.0	15	4.17*	3.451

*Significantly different at the 5% level

Preference was established for cotton sites along the edge, as opposed to the middle of petri dish lids. Nine replications utilizing 900 moths indicated a significantly lower oviposition rate on the

Table 13. Daily oviposition rates for pink bollworm moths. -- This was indicated by the mean number of eggs deposited. These were conducted in 15 x 100 mm plastic petri dishes with the following site selection alternatives, (A) Cotton soaked in 10% sucrose solution center and dry cotton side choice, (B) Paper and sucrose-soaked cotton choice, (C) Two sucrose-soaked cotton sites, and (D) One sucrose-soaked cotton only.

Experimental Environments Tests
Oviposition Data

Days After Emergence	No. 5	No. 6	No. 7	No. 8	Sum
1	351	146	249	128	874
3	602	406	379	133	1520
5	968	670	1155	401	3194
7	869	429	627	350	2275
9	115	341	757	185	1398
11	179	229	2918	80	3406
13	272	301	345	55	973
15	163	64	380	7	614
17	52	177	155	-	384
19	28	128	91	-	247
21	11	-	-	-	11

center pad when both were soaked in sucrose (Table 14). The availability of sucrose solution enhanced oviposition, presumably due to its nutritive rather than stimulatory properties. When a dry cotton pad was substituted for one of the sucrose soaked sites, egg deposition increased significantly (Table 15). This occurred independently, without regard for location within the dish (Table 16). Apparently moths preferred the dry, pubescent, secluded arrangement near an abundant food supply. Results were similarly conclusive when analyzed in terms of total eggs and eggs per female.

Table 14. Effect of site location on oviposition by pink bollworm. -- This was indicated by the mean number of eggs deposited. Insects were maintained as 10 pairs per 15 x 100 mm petri dish.

Analysis Criteria	Site Location		d.f.	F-Value	\bar{Sx}
	Center	Side			
Total Eggs Deposited	121.72	235.78	32	18.068*	80.649
Eggs Deposited Per Female	10.94	21.18	32	20.032*	7.240

* Significantly different at the 5% level

Table 15. Effect of site condition on oviposition by pink bollworm moths. -- This was indicated by the mean number of eggs deposited. Insects were maintained as 10 pairs per 15 x 100 mm petri dish.

Analysis Criteria	Site Condition		d.f.	F-Value	\bar{S}_x
	Center & Side Sucrose Soaked	Center Soaked Side Dry			
Total Eggs Deposited	140.89	216.61	32	8.415*	53.544
Eggs Deposited Per Female	13.20	18.93	32	6.272*	4.051

* Significantly different at the 5% level

Table 16. Effects of site location or condition on oviposition by pink bollworm moths. -- This was indicated by the mean number of eggs deposited. Insects were maintained as 10 pairs per 15 x 100 mm petri dish.

Analysis Criteria	Center & Side Sucrose Soaked		Center Soaked Side Dry		d.f.	F-Value*	\bar{S}_x
	Center	Side	Center	Side			
Total Eggs Deposited	110.22	171.56	133.22	300.00	32	4.079	26.361
Eggs Deposited Per Female	10.25	16.15	11.64	26.22	32	3.592	2.168

* No significant difference at the 5% level

Dramatic and conclusive effects were produced by the experimental environment tests (Table 17). All four treatments which lacked a source of nutrition, reduced levels of egg deposition. All of the dry or water-soaked substrates were equivalent in their lack of suitability. Oviposition rates were significantly increased by treatments 5, 6, and 8. Under these conditions 10% sucrose solution was supplied at the site, with or without a dry paper in proximity. Highest levels were achieved in no. 7 which offered the choice between sucrose-soaked cotton pad in the center and a dry pad at the edge. Tests were replicated 12 times, using 2400 moths, and analyzed for total eggs deposited and eggs per female.

Modes of Oviposition

Total number of eggs, their distributions as scattered or massed, and the number of eggs per mass were recorded for 40 samples. No significant trends were indicated under laboratory situations; however, conditions were not optimum and sites were restrictive. In all, 1296 eggs were recorded with 698 scattered and 598 massed. This included 47 masses with a mean of 13 eggs, ranging from 5 to 30 eggs per mass. Gravitational effects were evident, with the upper surface preferred to the under side in every instance.

Influence of Male Moths

Forty female moths, isolated at 20 per container, produced no embryonated eggs and retained only 420 eggs when dissected at 20 days of age. No oviposition sites had been provided and dissections included

Table 17. Effect of experimental environments on oviposition in the pink bollworm moth indicated by the mean number of eggs deposited.

<u>Criteria For Analysis</u>		
Experimental Environments	Total Eggs Deposited	Eggs Deposited Per Female
1	1.50	0.13
2	4.42	0.50
3	44.33	4.87
4	56.92	7.41
5	300.75	31.12
6	248.50	24.22
7	432.08	38.54
8	281.83	26.85
d.f	77	77
F-Value	41.58*	41.12*
\bar{Sx}	25.470	2.360

*Significantly different at the 5% level

examination for transferred spermatophores as well as sex confirmation. Comparisons were made with tests conducted under identical conditions using an equal number of paired moths. In this case 365 eggs were retained, 235 from mated and 130 from virgin moths. Mated moths contained 110 embryonated and 125 unembryonated eggs. No significant differences were indicated in the number of retained eggs; therefore, it was assumed that females either deposited or resorbed their initial complement of eggs (Table 18). As an additional check 200 females were confined, at 25 per container, under optimum conditions with available oviposition sites. They deposited only 123 eggs and contained no spermatophores or embryonated eggs.

Table 18. Influence of site availability and male presence on oviposition by female pink bollworm moths indicated by the mean number of eggs deposited or retained.

Male and Female Pairs				
Site Availability	Mated		Virgin	
	Embryonated	Unembryonated	Embryonated	Unembryonated
Present	--	--	--	--
Absent	110	125	0	130

Females Only		
Site Availability	Retained	Deposited
Present	--	123
Absent	420	--

Sex ratio experiments revealed rather surprising results (Table 19). As expected, the first treatment, which included an excess of females, produced nearly twice as many eggs as the equal sex ratio. However, ten males with five females produced another significant increase. When the parameter of eggs per female was employed, the 2:1 male to female ratio was most productive even though only five females were contributing. The presence of excess males apparently produced the previously reported effects (Hussien et al., 1962).

Table 19. Effect of insect sex ratio on oviposition in the pink bollworm moth indicated by the mean number of eggs deposited.

Analysis Criteria	<u>Male : Female Sex Ratios</u>					\bar{Sx}	F- Value	d.f.
	1:4	1:2	1:1	2:1	4:1			
Total Eggs Deposited	267.00	142.00	44.00	155.00	76.33	20.580	10.51*	8
Eggs Deposited Per Female	13.35	14.20	8.80	31.00	15.27	3.370	3.80*	8

* Significantly different at the 5% level

DISCUSSION AND CONCLUSIONS

A basic periodicity was first established in the laboratory for locomotor activity, oviposition, emergence, feeding, mating, and general inactivity or diurnal aggregation. Flight and other movements were almost continuous throughout the scotophase (subjective night) with an initial peak and reduction near the end of each day. Emergence and feeding were engaged in during the early portion of each dark cycle with mating near the second one-third. Inactivity occurred just prior to lights-on and continued throughout the photophase, during which moths were observed to aggregate in the most secluded area available.

Field sampling techniques dependent upon phototaxis or light responsiveness would be influenced by this photoperiodic behavior (Corbet, 1966). Moths rapidly adapt to light; therefore, only newly emerged insects or those arriving in the area during the night would be affected. Movement to and from a shadowed area near the light could also produce a lights-on and off effect. Thus, nightly trap catches are potentially subject to ecological conditions within the habitat, proximity of moths to lights, and the duration of exposure (Graham et al., 1964). Valid comparison between nights or geographical areas, therefore, are practically impossible, since these ecological factors are not uniform and the actual field populations are not always sampled.

Circadian rhythms were directly related to a precise photo-periodic response. Instantaneous shifts from dark to light, or the reverse, elicited characteristic behavior from the entire population within a few minutes. Gradual changes in illumination provided by dusk and dawn also produced immediate rather than graded responses of increased or decreased activity. These thresholds, determined by a Leeds and Northrup Daylight Illuminometer, were at approximately 3 foot-candles as previously reported (Lukefahr and Griffin, 1957). As entrainment progressed, populations began to anticipate the approaching scoto-or photophases. Moths became conditioned to the subjective cycle, which was expressed on subsequent days. The extreme phase shift exhibited during free-run provided a measure of this ability which, for the pink bollworm, seemed to be poorly developed. The capacity to respond required periodic reinforcement, waning within a few days without the subjective light cycle.

Age typically reduced the amplitude or overall level of responsiveness, but not the periodicity. This could have important practical implications since subsequent handling, rather than treatments such as gamma-irradiation, reduces the vigor and life span of treated moths (Staton, 1972). The same predictable patterns were produced by different strains and uniform sex ratios. Arrhythmic behavior was always exhibited during free-run and this loss of periodicity was established by both activity measurement and determinations of mating frequency. In all cases, cyclic behavior was

expressed only following exposure to an initial phase-setting, photoperiodic cue.

Free-running patterns, characterized by extended periods of activity, represented breakdown of the entrained periodicity. The accompanying reduction in amplitudes resulted from an expected adjustment maintaining a relatively constant total amount of activity (Aschoff, 1960). The circadian oscillation, measured from the onset of daily activity, dissipated while frequency remained proportional to amplitude. Rhythmic behavior was therefore expressed even though its timing was no longer accurate.

Entrained patterns resulted in very precise expression of activity during the scotophase. Comparison with the free-running records suggests that light may be suppressing a normally plastic system. First, there was no latent period prior to activity at the initiation of each scotophase. Response was immediate. Secondly, termination of entrainment resulted in a rapid spread of daily movement which increased by a regular interval. Finally, in continuous light the situation was similar but adaptation was prolonged for several days before a normal level of arrhythmic behavior was displayed.

The adaptive significance of a potentially variable system is evidenced by the wide geographic distribution and broad seasonal occurrence of the pink bollworm. Photoperiod may synchronize each population but not restrictively; therefore, the endogenous circadian pacemaker is either poorly developed or extremely adaptable. During free-run, activity could be arrhythmic with specific behavioral events

in phase; however, direct observation indicated that mating, oviposition, and feeding were arrhythmic in both continuous dark and light. Coordinating stimuli such as pheromones and other cues must be important under these conditions since the insects are extremely successful in reproduction and survival.

The lack of transients during re-entrainment suggests that the system may not have broken down and that the scotophase was physiologically anticipated. The magnitude of the response to lights-on also supports this view. Evidence for some "carryover" from original entrainment to its subsequent re-establishment exists. However, identity of the system remains obscure and one may only speculate about simple conditioning, complex physiological cycles or, ultimately, an overriding endogenous circadian clock.

If these laboratory data could be extrapolated to field situations, important insight might be gained concerning the biology of this moth. Assuming that aggregation, facilitated by mutual biotic and abiotic environmental requirements, maintained the insects relatively close together during periods of inactivity, then they would be interacting in the immediate vicinity of each other at the onset of each scotophase. This would promote mutual stimulation exhibited as aggregate behavior. Actograms support this view, with minor individual variation interrupting the continuity of population response. Moths would require only local stimulation during the mating period to effect courtship. This could be achieved by "initial" calling at first and "overt" calling later if needed (Leppla, 1972).

One of the important ramifications of this hypothesis would be the local nature of chemical communication between moths. Aggregate behavior, closely linked to the microclimate, would favor the use of pheromones of relatively low volatility. Additional evidence for this rationale has been provided by the direct relationship between plant height and the number of moths captured in pheromone traps, indicating maximum activity near the terminals (Sharma et al., 1971). Pink bollworm moths, being weak fliers, short lived, and relatively fragile insects, are usually protected by their habitat. Environmental forces do disrupt these insects for short periods of time, however, but eventually, common requirements re-establish functional aggregations.

It has been repeatedly demonstrated that methylene chloride extracts of female abdomens contain substances which stimulate males to sexual behavior. This material is relatively nonvolatile at room temperatures even after being liberated from the moth. Exposed samples retained their potency for several days, and fractions refrigerated within sealed vials were effective after one month.

It appears that males rapidly lose their responsiveness in a graded fashion beyond 10 days of age; whereas, females retain their productive capability for at least 20 days. However, there are quantitative, and perhaps qualitative differences, in male stimulation associated with female age. Old males have been observed to mate almost immediately with fresh young females when they were added to the compliment of old females, possibly because young females

call more vigorously, for extended periods of time from more elevated positions.

Male antennae are essentials to the mating process and probably operate to receive the female sex pheromone. This was previously established using the premating dance as a bioassay (Berger et al., 1964), and further explored here with techniques for the analysis of mating frequency. Antennaless males were incapable of responding overtly or mating, even when confined in close contact with females. However, females exhibit normal levels of mating without their antennae. Alternative means of reception may exist, but antennae are probably the only mediators of male reception.

Additional information is provided by research concerning the mode of action of methylene chloride pheromone extracts (Guerra, 1968). Changes in concentration elicit quantitative differences in sexual behavior; therefore, it is postulated that the substance might act first as an attractant and then as an aphrodisiac. Male responsiveness is modified by temperature, humidity, wind velocity, moth age, moon phase, time of day, insect density, and similar criteria; however, these are secondary to the influence exerted by the microhabitat. Association of the insects in the region of cotton plant terminals seems to promote communication and, even in small petri dishes, only the males close to calling females may become excited.

Calling behavior is apparently adjusted for this dual role of the pheromone. Initial calling is seldom prolonged into overt calling in the presence of male moths and, under laboratory conditions

of enforced close contact, mating is almost immediate. Initially the gland and associated pheromone are applied to a substrate, thus effecting only localized dispersal of the substance.

Mating apparently eliminates the action of the scent, although it is still present and effective at termination. This may occur after a 2-hr mating, during which two sets of wings cover the three terminal abdominal segments of the female. Males have been observed to probe a mating pair before their wings were in place even though the ovipositor and associated gland were withdrawn. This may also occur during mating when the wings are elevated due to a disturbance. Thus, the material which has been postulated to be present before, during, and shortly after copulation is probably only released during calling. Residues of the relatively stable, nonvolatile substance remain active on the female genitalia which are not exposed in the copulatory position.

It has been demonstrated that increasing the opportunity for effective mating by the addition of excess males stimulates oviposition (Hussien et al., 1962). Egg production is also enhanced under conditions of continuous darkness. In these two situations, an increased female mating frequency (multiple/total) matings was the single significant factor involved in peak productivity. Thus, second and third matings may be the key events in producing high levels of fecundity. Although relatively few females mate more than once, they still could be responsible for a large proportion of the egg production. Typically, a few females demonstrated extremely high mating

frequencies, for example, 6, 8, and 10 or more times. Light trap data suggest that single mating might be the rule since captured females had mated an average of 1.1 times (H. M. Graham, P. A. Glick, M. T. Ouye, and D. F. Martin, 1965). Females may become satiated due to the presence of a viable spermatophore in the bursa. Experiments should be conducted to determine the relative numbers of viable eggs produced in relation to spermatophores retained.

Theoretically, an optimum level exists above which more males are of no benefit and, in fact, decrease the mating frequency. The significant decrease in mating frequency at a male to female ratio of 2:1 indicated the level at which excess males interfered with their own mating activity. The significance of this situation remains to be explained, but it could be an extremely important consideration in pink bollworm population dynamics.

The pink bollworm sterile moth release program presently involves the use of both male and female insects which have been treated with gamma radiation. This approach looks promising since field cage studies, using a 50:1 sterile to normal moth ratio, showed a 90.5% population reduction (Richmond and Graham, 1970). This was achieved by adding 5000 sterile moths to a normal population of 100 moths, 50% of each sex assumed in both cases. Thus, an effective 50:1 sterile to normal ratio of mixed male and female moths was provided. During these experiments the average sex ratios were probably within limits of noninterference; however, great excesses of males should be avoided,

since they would only add to the burden of mass culturing and may reduce the effectiveness of the program.

In my experiments, effective mating was limited by longevity to twenty days which is appreciably longer than previous observations of eight days (Lukefahr and Griffin, 1957), or sixteen days (Ouye et al., 1964). Their analyses were based on transferred spermatophores collected from isolated pairs rather than interacting groups. They also used different climatic conditions, experimental techniques, and moth strains. Philipp and Watson (1971) used the Tucson (Maricopa) Strain in a series of experiments, which included oviposition cycles under various temperature regimes. They also maintained moths together rather than isolated until given times, but their results coincide with those given here with surprising regularity. Moths maintained at twenty pairs per test exhibited initial oviposition peaks ranging from about four to ten days after emergence. A lesser resurgence then occurred from nine to fourteen days later. These data also delimit a two to four day latent or perhaps inductive period between peak mating (days 2-6) and maximum oviposition (Figure 17). During the reproductive life the second mating peak was particularly conducive to multiple mating in aged moths. If this occurs in nature, then important contributions to overall fecundity may be attributed to the second period. The importance of multiple mating has already been demonstrated but its influence on oviposition should be explored.

The reasoning and research reported here support the need for additional information relating pink bollworm behavior and population

Figure 17. Relationship between days after emergence and oviposition rate in female pink bollworm moths maintained in equal proportions with males. -- Egg counts are from tests involving experimental environments (5-8) which affect oviposition. These were conducted in 15 x 100 mm plastic petri dishes with the following site selection alternatives, (A) Cotton soaked in 10% sucrose solution center and dry cotton side choice, (B) Paper and sucrose-soaked cotton choice, (C) Two sucrose-soaked cotton sites, and (D) One sucrose-soaked cotton only.

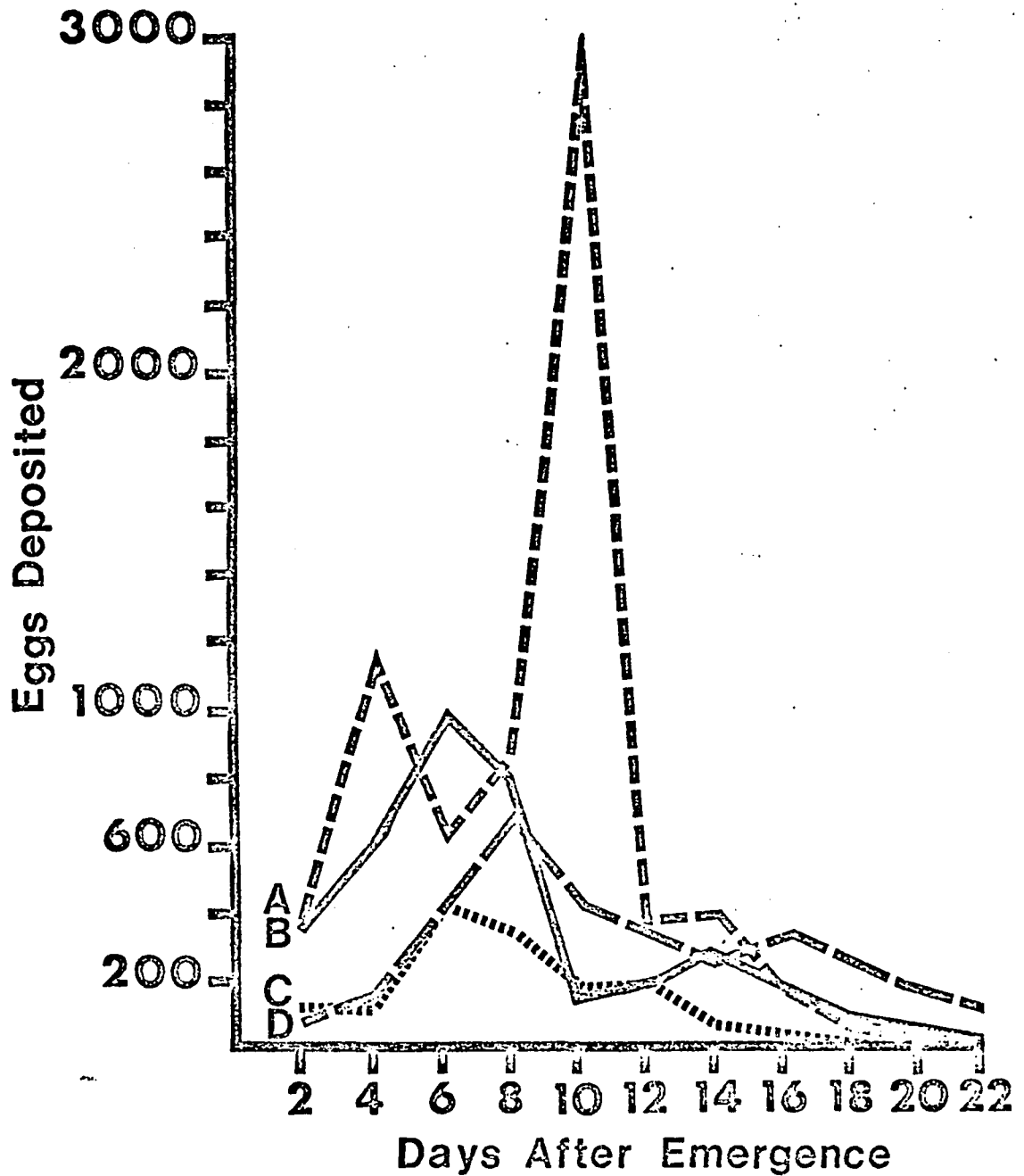


Figure 17. Relationship between days after emergence and oviposition rate in female pink bollworm moths maintained in equal proportions with males.

dynamics. Most of the parameters which affect mating frequency among normal moths have not previously been investigated or, at least, reported. For example the influence of sterile moths, as a single factor, needs to be evaluated. If the sex ratios and ages of the high numbers of moths necessary for control in the field, are actually primary variables, then success in reducing the field population will depend on effective sampling and manipulation of these parameters and the factors which affect them.

Several problems became apparent when attempts were made to resolve these behavioral data. Information from scattered sources was difficult to correlate because experiments had not been standardized in terms of environment or assay procedures. In laboratory studies alone, insects had been housed in 0.25-liter glass bottles, 1-pint glass jars, 1-gallon cardboard cartons, a variety of cages, and several types of petri dishes. Climatic conditions have been within reasonable limits for most behavioral work and this has facilitated comparison in many cases; however, field conditions have varied widely.

The use of specific bioassay criteria has often reduced the value of behavioral experiments. Typically, studies have been limited to a single parameter; thus, for oviposition eggs were counted, mating required spermatophore numbers, feeding was measured by orientation to food, and longevity was determined by recording the dead. The current value of these studies would be compounded several times if more data had been recorded. For example, population growth data could have

included measurements of mating, oviposition, longevity, hatching, length of instars, and eclosion, all taken from the same insects.

It has been repeatedly emphasized that parental searching for oviposition sites and the deposition of eggs are the most important acts contributing to larval survival and high population levels in subsequent generations (Dethier, 1959). Substrate availability is certainly critical and probably accounts for much of the population fluctuation observed in the field. Pink bollworm moths prefer oviposition sites which are pubescent, dry, and secluded from disruptive forces. Apparently, a carbohydrate source is essential for normal egg production, and water can not serve as a substitute. Glabrous plants and those with reduced extrafloral nectaries discourage oviposition and restrict adult survival. Cotton plants contain preferred sites only at specific intervals during their development and insects must be adapted to take advantage of the restrictive situations. In the absence of suitable sites, females retain their eggs, perhaps depositing a few or resorbing some, but rarely committing their progeny to unfavorable habitats. Without optimum location of the eggs, resulting larvae would be at a general disadvantage with respect to the climate, predation, and parasitism (Englemann, 1970).

Temporal patterns of oviposition have been manipulated in the laboratory by photoperiod, and they should be somewhat predictable in the field under natural conditions. Peak occurrence within a given generation may be correlated with age and mating frequency. Any population management based on egg production might make use of this

knowledge as well as specific information on oviposition habits. Larvicide applications could coincide with temporal patterns of hatching, and insecticides used against the adult would be most effective during the daily activity peak. The strategy is to become more specific and efficient, thus forcing the insect beyond a tolerance limit for an essential requirement during its life cycle. This is obviously difficult to achieve in practice.

Characteristic mating frequencies and oviposition rates are exhibited by moths maintained in male to female sex ratios ranging from 4:1 to 1:4. When graphed as percent response, the interrelationships between these variables provide an interesting analysis (Figure 18). In ratios of 2:1 (10 males to 5 females) the number of eggs deposited per female is increased over the response of a 1:1 ratio, but 20 males to 5 females produced a lesser response. The reverse occurs in mating frequency, with a reduction in the number of spermatophores transferred per female at 2:1 and a significant increase at 4:1. Thus, high levels of mating and oviposition are mutually exclusive depending on the preponderance of one sex over the other. At male to female ratios of 1:1, 1:2, and 1:4 values are equivalent for both mating and oviposition with the rates being proportional to the excess of females. Apparently, a small surplus of males reduces the level of mating but increases oviposition, whereas extra females enhance both.

Again, these experiments were intended to be preliminary examinations, hopefully providing insight and direction for further

Figure 18. Percent mating and oviposition at male:female pink bollworm moth sex ratios of 1:4, 1:2, 1:1, 2:1, and 4:1. -- Criteria for analysis include, (A) Total eggs deposited, (B) Total spermatophores transferred, (C) Eggs deposited per female, and (4) Spermatophores transferred per female.

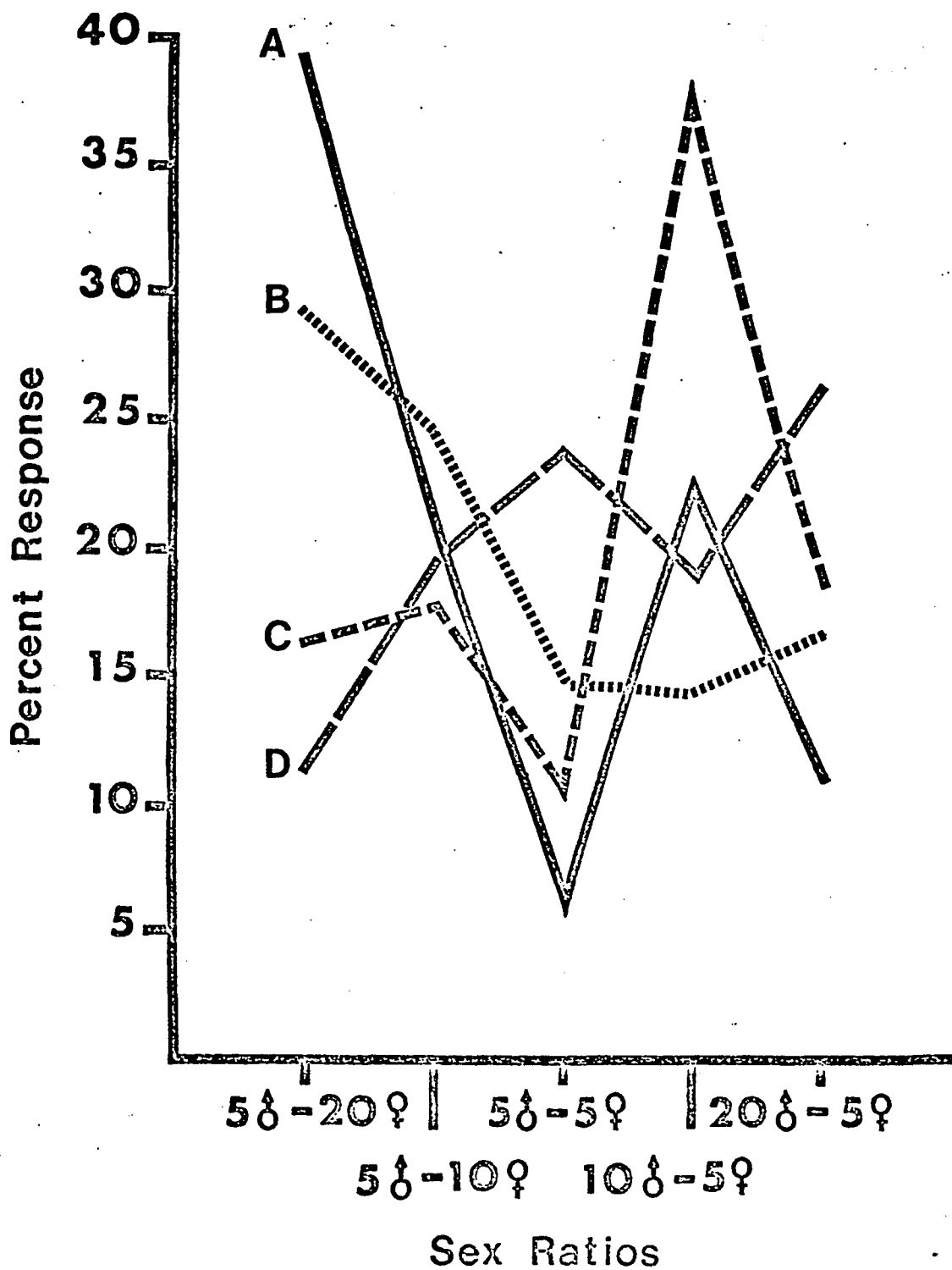


Figure 18. Percent mating and oviposition at male:female pink bollworm moth sex ratios of 1:4, 1:2, 1:1, 2:1, and 4:1.

research efforts. Under experimental conditions these moths are stressed and this might have produce somewhat abnormal results; however, the accumulation of information in the laboratory is fundamental to our understanding of this moth in the field.

SUMMARY

This study is an attempt to formulate scattered reports describing mating behavior, and experiments concerned with reproduction in the pink bollworm into a basic behavioral life history.

1. Adult pink bollworm moths are nocturnal, exhibiting circadian periodicity in their behavior. Nightly activity begins as the light intensity drops below 3 fc and females are continuously active throughout the scotophase, whereas males are active early and late in the night.
2. Typically, a biphasic response to lights-on is exhibited by these moths. There is an initial increase in locomotor activity which reaches peak levels within 15 min, after which the insects adapt rapidly to the photophase and cease moving within 30 min.
3. Reproductive behavior occurs from 6.5 to 10.5 hr after lights-off and is preceded by periods of oviposition and feeding. Rhythmicity is phase-set by photoperiodic induction and can be maintained under free-running conditions for about five days.
4. Timing of behavioral events may be significantly altered by manipulating the photoperiod. Individuals exposed to 10 or 25 kr of gamma irradiation produce normal actograph records.

Tucson and Brownsville moths exhibit practically identical rhythms which are maintained for at least 16 days after emergence. These insects habituate to continuous light or dark and are constantly active throughout each 24-hour period.

5. During the photophase, a period of inactivity, moths prefer to aggregate in resting sites which offer darkness and room for dorsal to ventral contact between individuals. Tactile stimulation from the environment and lateral contact with each other are not adequate substitutes even though preferred over situations exposing them to light.
6. Mating behavior is initiated and controlled by the release of sex pheromone from the female at the beginning of each nightly reproductive period. This involves two stereotyped postures, "initial" and "overt" calling.
7. Stimulation of male antennae by the pheromone presumably promotes searching and orientation flights. This insures encounters between males and females, and thus elicits the male pre-mating dance. Side to side probing by the male with his claspers leads to pairing, copulation, and spermatophore transfer.
8. Methylene chloride extracts from female sex pheromone glands of moths ten to twenty days of age elicit immediate and prolonged responses from day-old males. Males tested ten days after emergence exhibit latent but long lasting excitement; however, at twice this age they do not respond.

9. Removal of male antennae and subsequent pairing under various conditions result in no successful mating; whereas, antenna-less females are fully capable of mating in the laboratory.
10. Continuous light, dark, or L:D 14:10 photoperiods produce no statistically significant differences in levels of mating. Similarly, moth densities ranging from five to twenty pairs per 1-pint mason jar produce equivalent rates of spermatophore transfer. However, considerable variation occurs among male to female sex ratios of 1:4, 1:2, 1:1, 2:1, and 4:1.
11. Pairing of fresh young males one to three days old with females, two to twenty days after emergence, the reciprocal of this with fresh females, and tests with even-aged moths paired at two to twenty days of age, all produce a bimodal pattern of receptivity. The initial period, lasting eight days in each case, is followed by lowered receptivity for more than four days in different-aged, and nearly seven days in even-aged, insects. Finally, a second peak of four to six days duration occurs in all three groups.
12. Peak oviposition follows emergence by four to six days, and daily peaks occur one to two hours after initiation of each scotophase. The lifetime activity is bimodal and the initial oviposition peak ranges from approximately four to ten days after emergence, with a lesser resurgence nine to fourteen days later.

13. Pink bollworm moths prefer dry, pubescent, secluded substrates; however, they may deposit a few eggs on almost any object in the environment. They require a carbohydrate source, successful mating, and suitable substrate before depositing appreciable numbers of eggs under laboratory conditions.
14. Apparently eggs are developed in the absence of male moths but resorption rather than oviposition results. Under laboratory conditions eggs are scattered and massed in about equal numbers, and the upper surfaces of experimental substrates are preferred.
15. Groups containing excess females produce higher numbers of eggs. Excess males depress the rate except for the male to female ratio of 2:1. This effect is the reverse of the influence of sex ratio on spermatophore transfer.
16. Localized chemical communication is probably favored by these moths. Aggregation during each photophase, "initial" and "overt" calling postures in females, mating response in males, pheromone trap data and related observations all support this view.
17. Typically, behavior patterns involved in reproduction are stereotyped and relatively independent of minor fluctuations in the habitat. A wide range of tolerance to environmental factors, relatively plastic behavior, and extreme adaptability

have resulted in the remarkable biological success of the pink bollworm.

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