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CODLING MOTH FLIGHT AS INFLUENCED BY REPRODUCTIVE DEVELOPMENT AND LARVAL DIAPAUSE CONDITIONING

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CODLING MOTH FLIGHT AS INFLUENCED BY REPRODUCTIVE DEVELOPMENT AND LARVAL DIAPAUSE CONDITIONING

by

Richard Steven Cowles

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ABSTRACT

CODLING MOTH FLIGHT AS INFLUENCED BY REPRODUCTIVE DEVELOPMENT AND LARVAL DIAPAUSE CONDITIONING

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Effects of reproductive development and larval diapause conditioning on codling moth flight were investigated using circular and balancing flight mills. Distance of successive flights decreased (by 1.8% per flight) on circular mills, while velocity remained relatively constant (0.54 ± 0.04 m/s, mean ± SEM). Males and females aged zero to three days post eclosion did not differ in flight velocity or total distance flown. Older moths flew the same distance (12.5 ± 2.4 km, mean ± SEM) as younger moths. Weight loss differences between treatment combinations were dependent on initial weight. One day old males reared from non-diapause conditioned larvae flew less on balancing mills compared with diapause conditioned males (712 vs. 1570 seconds avg. for total flights, and 15.8 vs. 74.8 seconds/flight). This may reflect differences in the dispersive tendency for overwintering and summer generation moths. Moths on circular mills do not support their weight, lack tarsal contact and follow a circular path; balancing mills lack air movement

and a moving visual field. These physical factors make flight mill data difficult to compare with free flight.

Dedication

To my father, who has instilled in me a wonder and appreciation of Nature, and to Elizabeth for being so supportive during this endeavor.

Acknowledgements

My thanks go to those who offered guidance or stimulated thought for this work, Drs. Mark Whalon, Jim Miller, George Ayers and Guy Bush. My thanks also go to John Clarke III, of Iowa State University, and to Jamie Richardson and Dr. Jeremy McNeil of Université Laval in Québec for details of flight mills they had built. Thanks go to Dr. Carole McArthur for suggesting radioiodination of vitellogenin protein. I would lastly like to thank the National Science Foundation and Associate Dean Horne for help in obtaining a much needed computer printer.

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Introduction

Codling moth movement is a central concern in management of this fruit pest, since adults immigrating from wild or abandoned fruit trees provide pest pressure in commercial orchards. Literature on codling moth movement includes only field trapping and mark release recapture experiments (Worthley, 1932; Van Leeuwen 1940; Butt et. al., 1970; Howell and Clift, 1974). The findings from studies to date do not conclusively establish codling moth flight potential.

This thesis addresses laboratory experiments on codling moth flight biology. Two different designs of flight mills were constructed to measure the effects of sex, age and diapause conditioning of larvae on flight. Two main hypotheses were tested. The first hypothesis is that flight activity should be antagonistically related to reproductive development, especially as measured by vitellogenic activity in females. This hypothesis is based on Johnson's (1969) conclusion that migratory flight occurs in the newly eclosed adult before reproductive development has taken place. The second hypothesis is that flight activity differs between adults reared from diapause conditioned and non-diapause conditioned larvae. This question asks whether there are behavioral and physiological changes that cause differences in dispersal tendency in these two groups. If there are, then this may be related to differences in the adaptive value in dispersing from the host resources in the overwintering vs. the summer generations of moths.

Migration versus Trivial Flight

Throughout this work I shall be discussing Johnson's 1969 hypothesis regarding insect flight. Johnson's main contribution to the subject of dispersal and migration was to classify movement in two functional categories, "trivial" (or "appetitive") and migratory, based on behavioral and physiological characteristics. Migrating individuals have relatively straight, prolonged flights, and do not respond to stimuli such as potential mates or food. Migratory flight, Johnson says, always involves the female sex, usually in a prereproductive or recently mated state. It is important that migrants include females and be reproductively young, as this maximizes their value in colonizing a new habitat. Together these features indicate that migratory flight results in net displacement of individuals from their place of origin to a different breeding habitat.

Trivial flight, on the other hand, may involve males and females at various physiological ages. Appetitive movement is characterized by short flights with a high frequency of turns, and individuals are arrested by stimuli such as potential mates and food. Appetitive flight could consequently be summarized as activity that does not cause net displacement of individuals from the original breeding habitat.

This terminology has to be taken as a general concept rather than biological law. In Lepidoptera, prereproductive migratory flight and oogenesis may not be as distinct as in other orders. One distinction is that Lepidoptera are not known to undergo wing muscle histolysis following migratory flight, as has been noted in Orthoptera, Hemiptera, Homoptera, and Coleoptera (Johnson, 1969; Harrison, 1980). Changes in migratory tendency in Lepidoptera must be demonstrated by behavioral changes, rather than by histological changes as in these other groups. Seemingly accidental transport of many Lepidoptera by upward flight into fast moving winds blurs distinctions between migratory and trivial flight (Johnson, 1969). The problem is that chance displacement seems contrary to the definition of migrants as especially adapted physiologically and behaviorally for exodus flight. It is possible that in Lepidoptera, there may be a spectrum of dispersal behaviors, from vegetative to migratory, that rely upon a key movement (specifically, positive phototaxis), that governs the likelihood that an individual will be transported out of the breeding habitat.

Importance of Codling Moth Movement

Codling moth, Cydia pomonella (L.) (Lepidoptera: Olethreutidae) is a cosmopolitan pest of tree fruits. The primary hosts of codling moth are pome fruits, including pears, quince, large-fruited crabapples and domesticated apples. Other hosts include walnuts and larger stone fruits (Shel'Deshova, 1967). Damage is done by internal fruit feeding of larvae. Processors and fresh fruit buyers will not accept these damaged fruits, consequently codling moth is an economic pest throughout its range.

In commercial orchards there is generally a negligible population of codling moths, due to suppression with insecticides. In spite of low within-orchard populations, sprays are directed against moths immigrating from wild or abandoned hosts. The result of this spraying ultimately translates into economic loss, loss of beneficial insects and environmental contamination. Repeated spraying applies strong selective pressures on codling moths and other arthropod populations, and has led to pesticide resistance.

A key factor in the importance of codling moth management is the dispersal tendency of the adults. The population that is being sprayed originates not from within the orchard but consists of immigrants. The ability of codling moth to disperse affects both the ability of moths to colonize "clean" orchards from refugia and also governs the gene flow between populations. Gene flow may be of great importance in managing pesticide resistance (Tabashnik and Croft, 1982). Movement of susceptible individuals into resistant populations may dilute resistance genes, while the opposite movement.may bring resistance problems to areas where resistance has not occured. Resistance management requires an understanding of gene flow in the population; male movement thus could be at least as important as female movement.

General Biology

Codling moth females lay eggs on fruit or on leaves near fruit (Geier, 1963). These eggs hatch in 4 to 7 days, depending on temperature. First instar larvae locate nearby fruit, and enter the fruit by chewing through the skin. After passing through five larval instars, the mature larvae exit from the fruit and search for suitable sites for forming hibernacula. After spinning a silk cocoon, the larvae may either pupate or enter a resting (diapause) state, depending on the photoperiod and temperature conditions to which the larvae are exposed (Garcia Salazar, 1984). Pupae may develop into adults in ca. 11 to 20 days, again depending on temperature conditions. Mating generally takes place on the first or second evening following emergence (White, et. al., 1973).

Field Studies of Codling Moth Dispersal

Data have been collected in various manners in studying codling moth dispersal in the field. Some valuable observations were made by Borden (1931), by watching moth flight under artificial lighting. His conclusions were that codling moths tend to fly short individual flights (15 to 23 m), in zig-zag movements along the upper one-third of the tree. While most flights followed this description, some moths traveled in straight lines until lost to view. Flights mostly occur from 20 minutes prior to to 20 minutes following sunset. The minimum temperature for observed flight was 12^{o}C and the maximum 26^{o}C.

Early mark release recapture experiments (Worthley, 1932; Van Leeuwen, 1940) showed that the number of moths recaptured in bait pans declined quite regularly with distance from release site. These studies made releases from well within orchards since the principal objective of these investigations was to determine whether bait traps could be used to control codling moths. Van Leeuwen (1940) did place some traps in nearby orchards, and recovered moths that had flown 450 m from the release site. Worthley (1932), compared released male and female moths and found that they traveled the same distance. More recent studies conducted by USDA personnel in Washington State (Butt, et. al., 1970; White, et. al., 1973; Howell and Clift, 1974) have studied codling moth movement in relation to the sterile release approach for control. These studies mostly agreed with earlier work, and concluded that codling moth movement is limited. In two of these studies however, a small proportion of moths traveled much further than had previously been measured; most of this movement occured over areas lacking host plants. White, et. al., (1973), accidentally released fertile codling moths into a previously clean orchard. The resulting fruit damage indicated where the females had traveled. While 90% of the damage was restricted to within 300 m of the release site, the furthest damage was found 600 m from this site. Howell and Clift (1974) used traps baited with virgin females to study the movement of irradiated male moths. They concluded that a constant decrease in trap catches for each constant increase in distance from the point of release would only apply to the within-orchard habitat. Males leaving an orchard may travel long distances (8.7 km) while traversing non-host habitat.

These conclusions also agree with the work of Wildbolz and Baggiolini (1959) as interpreted by Geier (1963). Geier concluded from these and his own observations that codling moth dispersal follows a Bessel function. A Bessel function is derived from a theoretical movement model that assumes that organisms 1) move at random in uniform surroundings, 2) the rate of displacement varies with the rate of reproduction, and 3) individual life spans are distributed as a negative exponential function. In the simplest type of movement model, insects will not stop at borders, but will turn back, as if reflected, into the host habitat. Oviposition along borders in this model is the same as within the habitat. Codling moth oviposition occurs at a higher rate along orchard perimeters than predicted by reflective borders. The effect of the orchard border on moth movement implies that either flight is curtailed at orchard perimeters due to environmental factors (such as wind), or that there exists a migratory component in the population that is arrested at orchard borders following migratory flight.

Interestingly, these studies rely upon appetitive cues to trap moths; responses of female moths to bait or ovipositional sites, and of males to virgin females, would be considered appetitive activities (Johnson, 1969). These methods for capturing moths may be effective due to the attractiveness of the trap, however baits and virgin females may not be appropriate for sampling insects exhibiting truly migratory activity. A non-selective sampling method such as a Taylor suction trap (Johnson, 1969) would be preferable as it gives an absolute measure of the number of moths in a given volume of air. The boundary layer would be a particularly important region to sample in an orchard, considering that moth activity has been observed in this region (Borden, 1931). The boundary layer here is defined as the strata within which an organism may orient to surface features and may control its own displacement (Johnson, 1969). Vertical penetration of this layer of air is critical for accidental displacement of moths (Johnson, 1969), and is particularly rich in lepidopteran activity.

Another method that could be employed is using blacklight traps for recapturing moths. A study by Geier, (1960), compared the use of blacklights to bait pails, as related to the reproductive development of the captured females. His results indicated that there were different aged females caught by the two types of traps. Blacklight traps caught females at an earlier physiological age than the bait traps. Johnson (1969) interprets these data to mean that the females at an earlier physiological age were migratory, while the group responding to bait traps were non-migratory. The response of older females to bait pails has already been discussed, and would be considered trivial movement since they are responding to appetitive stimuli. Characterizing blacklight catches as migrants requires the assumption that strongly phototactic individuals be dispersive, since moving towards light means moving away from trees or moving upwards, which could bring the moth through the boundary layer into a region where dispersal would be dependent on wind velocity and direction.

Field studies conducted so far are prone to the bias introduced by the stimulus used to capture the moth. Studies using virgin females or baits may be inappropriate for assaying migratory behavior because the stimulus relies on appetitive behavior for moths to be captured. It may be no surprise that the field studies conducted so far have concluded that codling moths are by nature not apt to disperse from orchards. Data showing positive phototaxis in younger moths (Geier,1960) and the possibility of a migratory component in the population to explain border effects (Geier, 1963) are suggestive of a potential for migratory behavior, but have not been substantiated by field or laboratory data.

Flight Mills and Insect Movement

Field studies typically use the mark release recapture technique, which has many shortcomings. Some problems associated with mark release recapture studies include lack of environmental control, loss of most released individuals, and meaningful replication is difficult. Recapture without re-release may limit the total potential distance flown by the test insects. In a way the mark recapture method presents a paradox. In order to measure movement, a sample or measurement must be made; the process of measurement so affects that movement that simultaneous quantitation of location and velocity becomes impossible. There is the additional concern that the sampling method may bias the results by capturing only individuals responding to cues related to migratory or trivial movement.

Flight mills offer an alternative for studying flight biology in the laboratory. Laboratory experimentation allows environmental control and facilitates replication, eliminating the difficulties mentioned above for the mark release recapture method. These conditions allow sophisticated factorial experimentation that would be very difficult to conduct in the field.

Flight mills have been used by many workers to probe basic insect flight biology. Several groups have used flight mills to assay the effects of laboratory rearing, radiation, or application of pigments (Chambers and O'Connell, 1969; Remund, et. al., 1976; Crystal, 1977; Nakamori and Simizu, 1983) on flight ability. Often these studies are an end result in themselves, the study is used to decide whether treatments may affect flight performance of these insects compared to untreated controls. Such comparisons may be used for quality control of laboratory cultures, and is especially important for successful sterile male releases (Huettel, 1976). Other workers have used flight mills to assay flight metabolism (Rowley, 1970; Cooter, 1982), the influence of semiochemicals on flight initiation and arrestment (Roitberg, et. al., 1984; Bennett and Borden, 1971), control of flight velocity (Niehaus, 1981), and estimation of dispersal potential (Ito, 1980; Solbreck, 1980; Fasoranti, et. al., 1982; Foley, 1985).

Many varieties of flight mills have been designed, the most common involves attaching a test insect at the end of an arm that rotates on a low friction bearing. Test insects usually cannot land, although in some unusual designs (Ruzicka, 1984; Barfield and Gregory, 1985), insects are allowed to walk or crawl between flights by means of a flexible tether.

Chapter 1 - Circular Flight Mill Experiments

Introduction

Field studies of codling moth flight (Worthley, 1932; Van Leeuwen, 1940; Butt, et. al. 1970; White, et. al., 1973; Howell and Clift, 1974) have generally concluded that movement is limited except in unusual cases when moths traverse non-host habitat. Migratory flight may not have been noted in these studies because trapping methods relied on appetitive cues such as bait pans or virgin females. Borden's (1931) observation of straight flight, and Geier's (1960) discovery that moths of young reproductive age are preferentially caught at black-light traps suggest that some codling moths may exhibit migratory flight behavior.

Laboratory studies are appropriate for testing to see whether prolonged flight is possible in codling moths. Controlled environment and ease of replication allow specific hypotheses to be tested that otherwise would be difficult to study in the field. In this experiment two hypotheses were tested: 1) There are differences between flight activity (distance, number of flights, velocity and weight loss) as moths age; 2) There are differences between male and female flight. The first hypothesis is related to Johnson's hypothesis that migratory flight takes place early in the adult insect's life, in a post-teneral period before oogenesis takes place. Young moths would then be expected to fly more than old moths. The second hypothesis could reflect differences in the dispersal capability of the two sexes.

Materials and Methods

An eight-subunit circular flight mill was used to compare flight of moths reared from non-diapause conditioned larvae. The circular flight mill was used in a two by four factorial experiment comparing males and females aged <1, 1, 2, and 3 days post eclosion. A randomized complete block design was followed; blocks of eight treatment combinations were assigned randomly to the eight flight mills, the experiment was then replicated over days. A factorial arrangement was used so that possible interactions between age and gender would be detected.

Culture Maintenance

The codling moths used for all experiments were from a laboratory culture initiated in 1983 from larvae collected at the Trevor Nichols Research Complex, Douglas, Michigan (Garcia Salazar, 1984). All life stages were reared at 27°C, under 16L:8D photoperiod, in continuous culture. In addition to 16 hours of light supplied by cool white fluorescent bulbs, a microscope light was set on an appliance timer to simulate an hour of crepuscular conditions for both evening and morning.

Individual neonate larvae were transferred using a fine brush to the surface of artificial diet, prepared as described in Appendix A. Diet was poured immediately upon preparation into plastic 30 ml Dixie^R cups (#P012 BB). Corrugated cardboard was cut in 1 cm^2 pieces and glued to the inside of the Dixie^R cup lids (901P BB), providing a site for pupation. Mold contamination was effectively eliminated by: 1) Spraying the surface of artificial diet and lids with 16% methyl paraben (w/v in 95% ethanol), and 2) Exposing the surface of the diet to at least 20 minutes of UV light (GE G15T8 bulb, at a distance of 35 cm. from the diet surface) in a Microvoid^R dust hood.

Preliminary rearing experiments showed that survival for paired larvae was ca. 50% that for larvae reared individually, meaning that an increase in the number of larvae per cup would not improve efficiency in the rearing procedure. To reduce possible variation caused by larval competition, individual rearing of larvae was adopted.

Larvae were allowed to develop for 19 to 26 days. The cups were then cracked open and all pupae and hibernacula found in the cardboard or attached to the tops were collected and placed in an emergence cage. Any larvae forming hibernacula in the diet were discarded to prevent fungal growth on pupae.

Adults were collected daily from emergence cages. Cages were chilled for 3 to 4 minutes at -20°C, the cold anesthetized moths were then transferred with soft forceps to an ovipositional cage.

Ovipositional and emergence cages were made from acetate plastic sheets glued to form a 9 cm dia cylinder, 20 cm tall. The bottoms were disposable plastic petri dishes with a Whatman #1 or #2 filter paper lining; the tops were similar petri dishes with a 5 cm dia hole cut in the middle and covered with nylon mesh for ventilation. The sides of ovipositional cages were lined with wax paper to provide a suitable waxy surface for egg laying and 5% sucrose solution was provided in a 2 ml vial with an inserted dental wick.

As soon as the first eggs laid on the wax paper developed to the black-head stage, the wax paper was removed from the ovipositional cage, rinsed with distilled water, blotted with paper towels, and placed in a ziploc bag until larvae hatched.

Flight Mill Design

Circular flight mills (Figs. 1.1 and 1.2) were designed based on reports of several workers. Support for the flight mill arm, providing essentially a friction free bearing, was accomplished by suspending a sewing needle by its point from a stirring bar magnet (Fisher Cat. #14-511-65) (Cullis and Hargrove, 1972). Another stirring bar magnet, with the teflon coating removed, was used to keep the needle vertical; this magnet did not touch the needle and cause any friction. The needle passed half way through a flattened 2 cm section of a plastic drinking straw. A 0.4 mm diameter spring steel wire was then passed through the straw; this form of attachment allowed adjustment of the spring steel rotor so that the proper 15.9 cm radius would be obtained (giving a circumference of 1 meter).

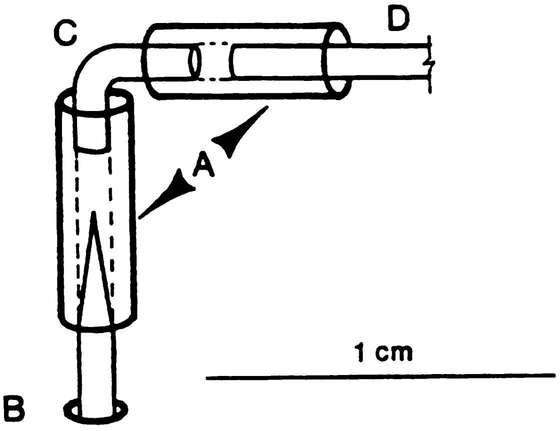

Figure 1.1. Harness used to attach moth to flight mill arm. (A) Medical teflon tubing, (B) Hand-shaped copper wire, (C) Elbow made from spring steel wire, (D) End of flight mill arm.

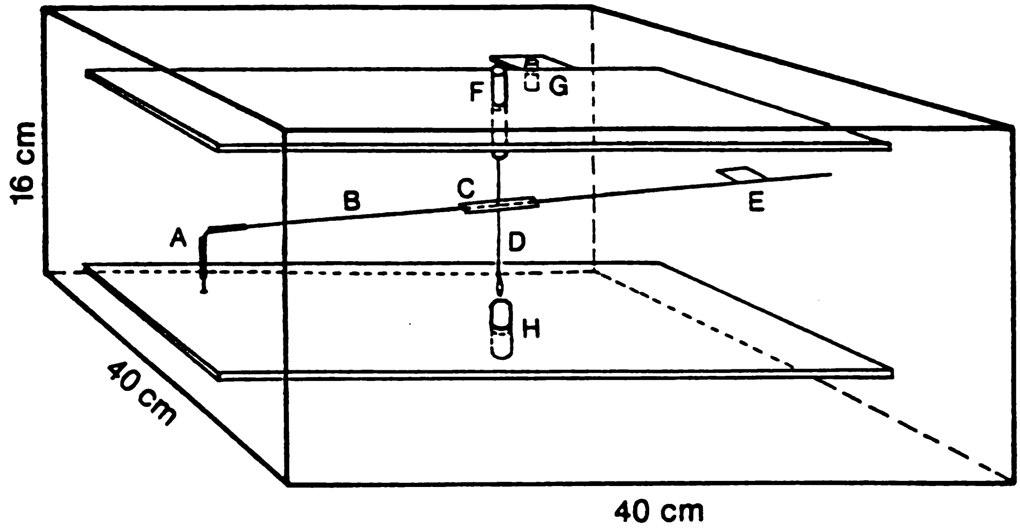

Figure 1.2. Single unit of circular flight mill. (A) Harness, (B) Spring steel rotor, (C) Flattened length of plastic straw, (D) Sewing needle, (E) Aluminum foil reflector, (F) Stirring bar magnet, (G) Light and photoconductor, (H) Stirring bar magnet.

At the end of the wire, a section of medical teflon tubing was fit, together with an elbowed wire and a hand shaped copper wire to form pressure fit joints for the insect harness (Fig. 1.1) (Chambers and O'Connell, 1969). On the opposite side of the flight mill rotor from the harness, a 2 X 1 cm piece of aluminum foil served as a reflector for the photoelectric sensor (Clarke, et. al., 1984). Each apparatus was fit into an open ended box made from 6 mm plexiglass. The lower two-thirds of the insides of each mill was spray painted with flat black paint to eliminate possible visual interactions between moths on adjacent mills. A cardboard platform was provided for support of each moth until all moths were connected and the experiment ready to begin.

Electronic Hardware

I decided that interfacing a computer to the flight mills would be the most effective manner to collect data. Previously used methods, relying on chart recorders (Cullis and Hargrove, 1972; Bennett and Borden, 1971) or multichannel event recorders (Chambers, et. al., 1976) are both very expensive and labor intensive. Microcomputers, on the other hand, can collect and summarize data, as well as store the data on a permanent medium (floppy diskette).

Designs published by Clarke, et. al., 1984, were used for building electronic hardware, including power supply and flight mill interface. The sensors for the flight mills consist of incandescent bulbs (Sylvania #327) paired with photoconductors (Clairex 603A). Voltage from the photoconductor changes the conductivity of a transistor. Current through this transistor causes a diode to emit light inside an optical isolator, allowing separation between the light and sensor circuitry (30 volt system) and the computer circuitry (5 volts). The resulting signal pulse is stored by a data selector chip until read by the user port (Clarke, et. al., 1984). The transistor, optical isolator, and the data selector chips produced a noise-free signal read by the computer.

Flight Mill Software

One concern in building the flight mill was matching the programming to the computer. A Commodore 64 was used rather than the Commodore Pet used by Clarke, et. al. (1984). It was discovered that there were distinctive hardware differences that prevented use of software developed by this group; the greatest problem being addresses in their assembly language routines that had no equivalent in the Commodore 64. A program was consequently written in BASIC language to collect data from the flight mill interface.

The computer continuously checks for any change in signal at the user port (which is connected to the interface). Whenever there is a change in this signal, the value in binary code can be used to identify which mill had a change in state. The interpretation of this signal is governed by software control, allowing flexibility in the analogue to digital conversion of data pulses coming from the mills.

The BASIC program, along with comments on how it operates for this type of mill, is included in Appendix B.

Experimental Procedure

Except for moths emerging the same day as an experiment, moths were collected each day by chilling for 3 min. at $-20^{\circ}C$ and sorting from pupae. Moths aged less than 1 day were collected from emergence cages without any chilling. This schedule was developed so that moths were not anesthetized within 24 hours prior to an experiment. Males and females were placed together in ovipositional cages, and kept at $27^{\circ}C$ with a 5% sucrose dispenser until ready to be used for an experiment.

Experiments were conducted in a walk-in growth chamber, with cool white fluorescent lighting (3000 lux) with the same conditions (16:8 L:D and 27 C) as the rearing chamber. Lights were scheduled to go off at 9:00 PM, meaning that the remainder of the night would have crepuscular conditions (44 lux) due to the television monitor screen and the sensor bulbs.

The same evening of the experiment, moths to be used in an experiment were placed in closed dixie cups and weighed. Vacuum was used to hold the moth (Rowley, et. al. 1968). A suction holder (Figure 1.3) made from a microfuge tube fit the moth abdomen closely, allowing manipulation of the thorax. Scales were removed from the pronotum, and the moth attached with Sanford's^{R} Rubber Cement (Bellwood, Ill.) to the flight mill harness. Following attachment, a cardboard platform was provided to allow tarsal contact and minimize pre-experiment flight activity.

At 8:00 PM, the experiment was begun by starting the flight mill program and removing the platform from under each moth. At ca. 8:00 the next morning, the replicate was terminated by turning on the printer and typing "s". Immediately after a hard copy was obtained, data were stored on floppy diskette.

Moths were then returned to a dixie cup, post-flight weights were taken, then moths were replaced into the breeding culture. Attachment with rubber cement and subsequent removal did not seem to affect moths adversely; they were capable of mating and laying eggs following flight experiments.

Results

Codling moth flight response on flight mills was highly variable, giving large coefficients of variation (Table 1.1). This same observation was made by Crystal (1977) working with the screwworm fly. High variation made treatment differences very difficult to detect. Another general observation was that all moths on circular flight mills flew to exhaustion. Exhausted moths were characterized by the splayed wing posture (Fig. 1.4B), as well as the trend for successive flights to decrease in distance (Figure 1.5). Velocity, on the other hand, tended to remain constant for each moth over successive flights (Fig. 1.6). It is clear that there was a regular decrease in flight

Table 1.1. Coefficients of variation for male and female moths aged 0 to 3 days post eclosion, flown on circular mills.

Response Variable	Coefficient of Variation
Distance	95 %
Weight loss	36
Velocity	41
Number of flights	125

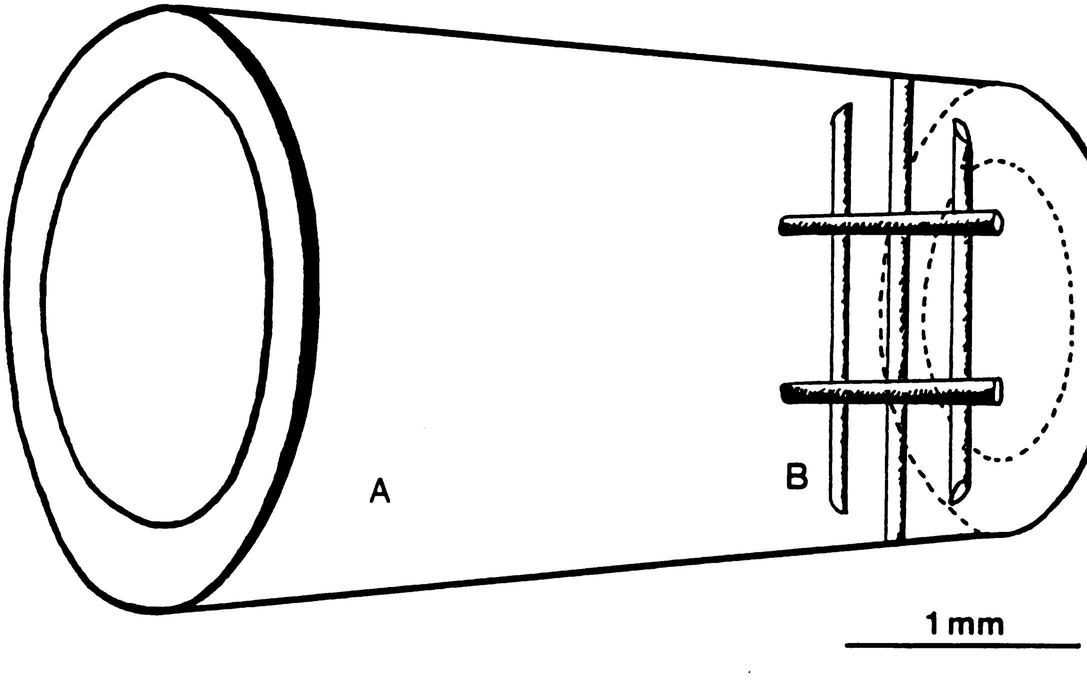

Figure 1.3. Suction apparatus used for holding moth while attaching to flight mill arm. (A) Section of plastic microcentrifuge tube, (B) _Minuten_ points inserted through plastic.

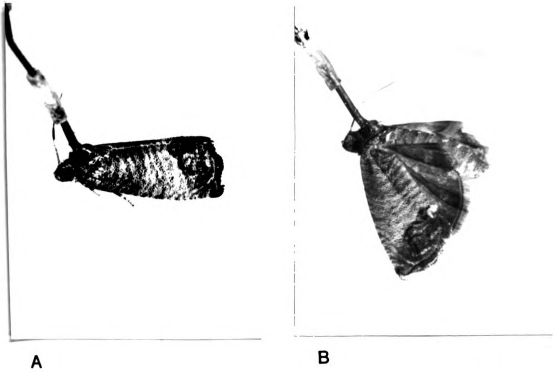

Figure 1.4. Characteristic postures of non-flown (A) and exhausted (B) moths.

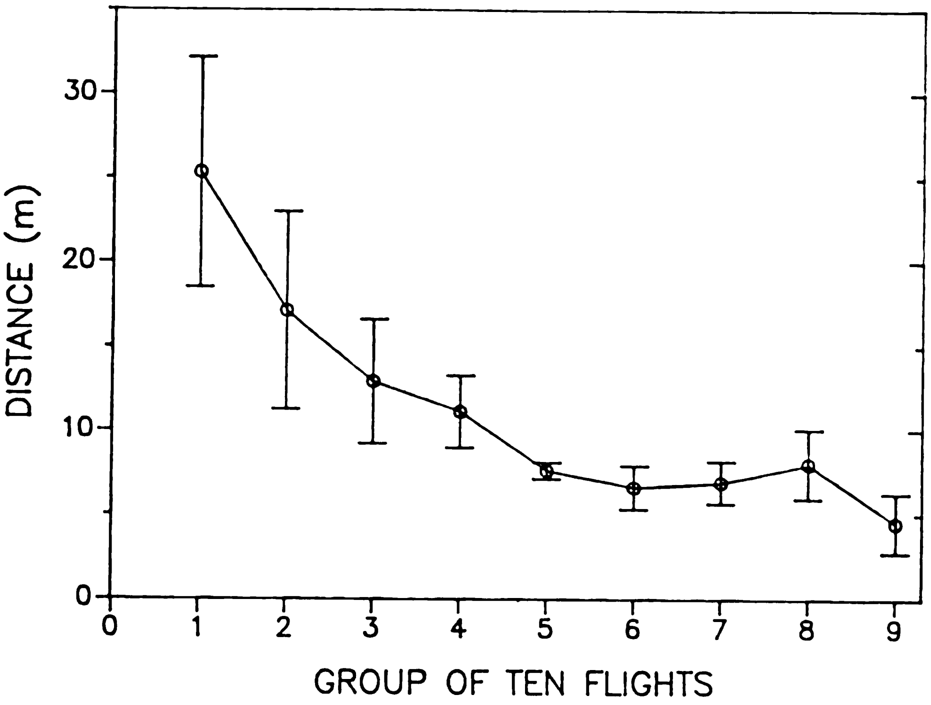

Figure 1.5. Average distance (mean ± SEM) for groups of ten flights, averaged for five moths flying ca. 100 flights on circular flight mills.

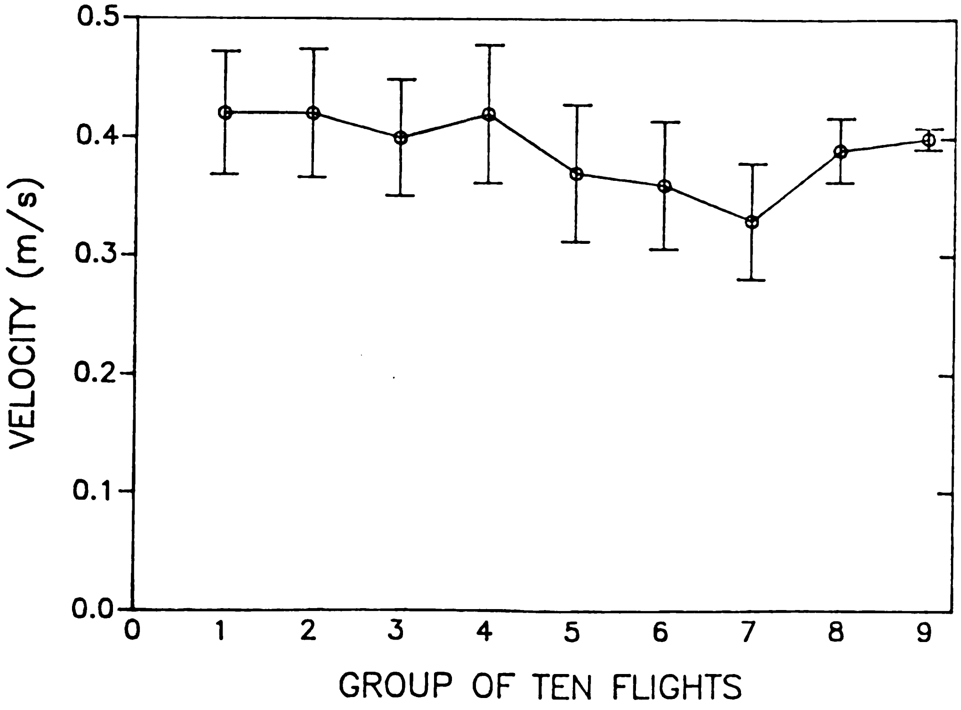

Figure 1.6. Average velocity (mean ± SEM) for groups of ten flights, averaged for five moths flying ca. 100 flights on circular flight mills.

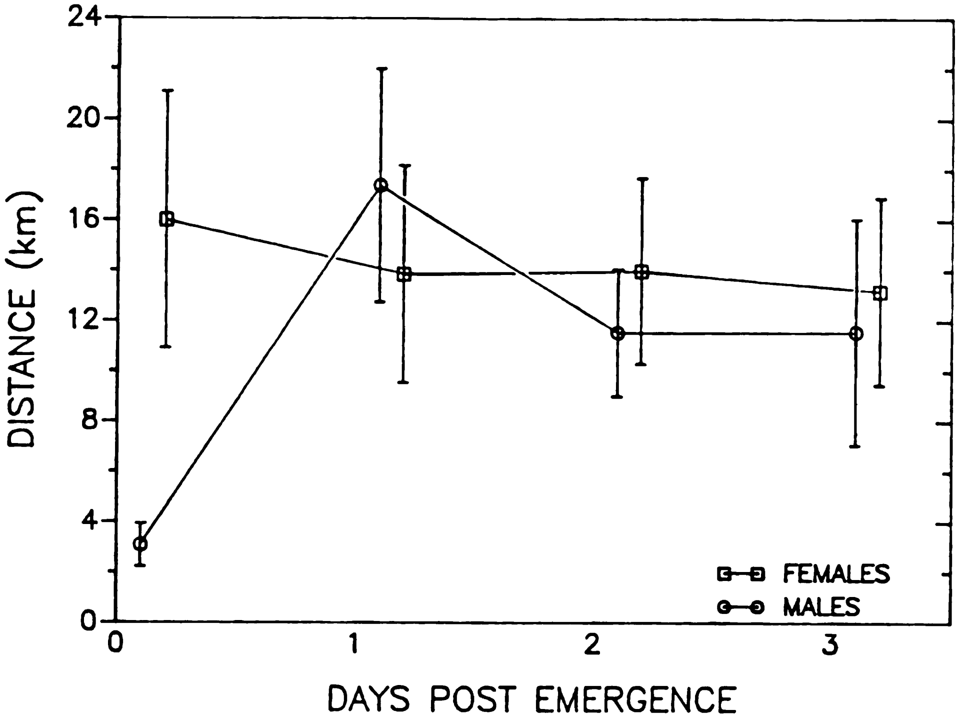

Figure 1.7. Distance (mean ± SEM) flown by male and female moths aged 0 to 3 days post eclosion on circular flight mills.

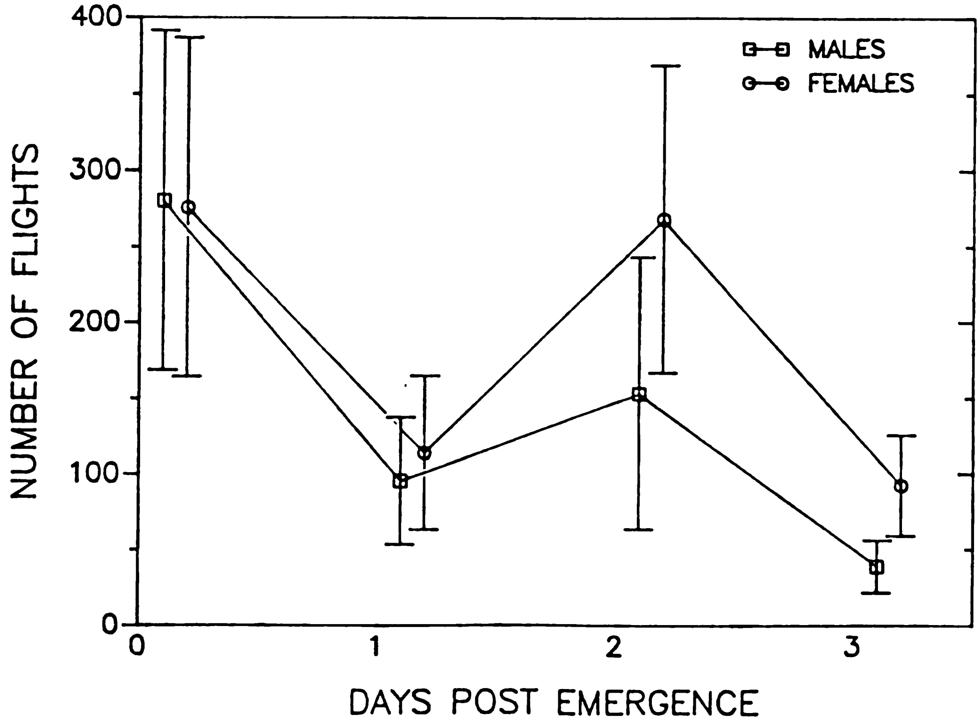

Figure 1.8. Number of flights (mean ± SEM) for male and female moths aged 0 to 3 days post eclosion on circular flight mills.

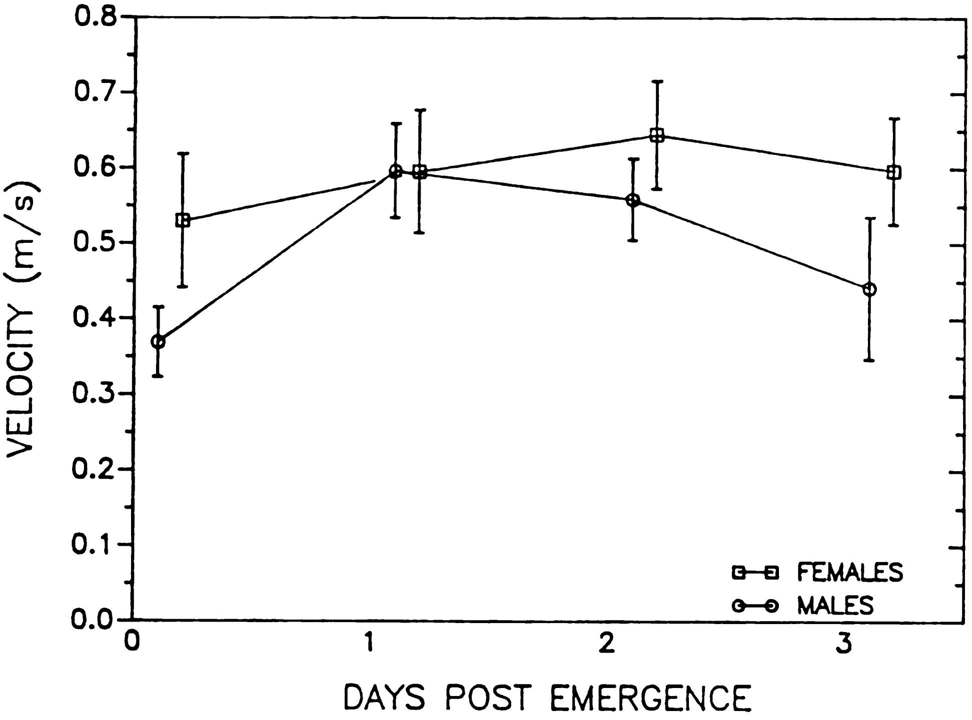

Figure 1.9. Velocity (mean ± SEM) for male and female moths aged 0 to 3 days post eclosion flying on circular mills.

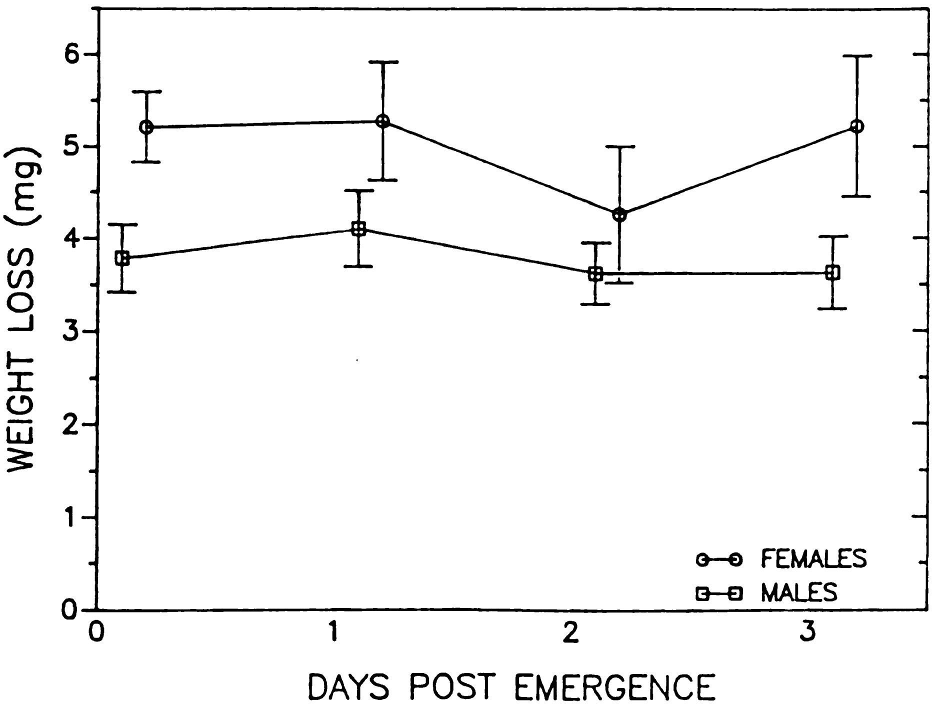

Figure 1.10. Weight loss (mean ± SEM) for male and female moths aged 0 to 3 days post eclosion flying on circular mills.

Table 1.2. Analysis of variance for male and female moths aged 0 to 3 days post eclosion flown overnight on circular mills.

Distance flown

Source	df	SS	MS	F	P > F
Replicate	8	722031676	9025396	0.64	0.74
Sex	1	176995227	176995227	1.26	0.26
Age	3	331320751	110440250	0.78	0.51
Sex X Age	3	650390777	216796922	1.54	0.21
Error	55	7745789794	140832541		

Weight Loss

Source	df	SS	MS	F	P > F
Replicate	8	63.90	7.98	4.88	0.0001
Sex	1	29.18	29.18	17.83	0.0001
Age	3	3.44	1.14	0.70	0.5561
Sex X Age	3	1.23	.41	0.25	0.8605
Error	55	90.02	1.64		

Number of Flights

Source	df	SS	MS	F	P > F
Replicate	8	343408	42926	0.78	0.621
Sex	1	29648	29648	0.54	0.466
Age	3	497241	165747	3.02	0.037
Sex X Age	3	29718	9906	0.18	0.909
Error	55	3021641			

Table 1.2 (cont'd.)

Velocity

Source	df	SS	MS	F	P > F
Replicate	8	0.2710	0.0338	0.68	0.703
Sex	1	0.1644	0.1644	3.32	0.074
Age	3	0.2679	0.0893	1.80	0.157
Sex X Age	3	0.0791	0.0026	0.53	0.662
Error	55	2.7232			

distance, in the case of moths flying ca. 90 flights as represented by Fig. 1.5, there was an average decrease in distance of 1.8% for successive flights, giving an exponential decay shaped curve.

Since it was not known a priori what response variables would be important, data were analyzed for number of flights, total distance, velocity and weight loss. This experiment showed that males and females of the same age flew approximately the same distance (12.6 km), at the same speed (0.55 m/s), and had the same number of flights (160 total flights per moth) (Figs. 1.7-1.10). Males flown on the same day as emergence appeared to fly much shorter distances than any other age of male or female moths (Fig. 1.7), however the analysis of variance for all eight treatment combinations indicated that this was not a significant difference. There was a slight but significant (P=.0375) trend for older moths to fly fewer flights than young moths. The analyses of variance for these factors were analyzed with SAS (SAS Institute Inc., Cary, NC) and are included in Table 1.2.

Weight loss depended on sex but not age differences (Figure 1.10 and Table 1.2). Weight loss differences were highly correlated with initial weight, which may explain differences based on sex. Females weighed much more than males of the same age (26.2 vs 16.6 mg average initial weight, averaged over all ages for females and males, respectively); consequently the females lost more weight than males. Stepwise linear regression analyses of response

Table 1.3. Results of stepwise linear multiple regression for male vs. female, 0 to 3 days post eclosion factorial flight experiment using circular mills.

Model is Distance Flown = f(weight, sex, age)

Source	df	MS	F	P > F
Regression	1	291661945	2.18	0.144
Error	69	135244438		

Parameter	B Value	Std Error
Intercept	5288	
Weight	340	230

Model is Velocity = f(weight, sex, age)

Source	df	MS	F	P > F
Regression	1	0.1847	3.84	0.054
Error	69	0.0481		

Parameter	B Value	Std Error
Intercept	0.3584	
Weight	0.0085	0.0043

Model is Weight Loss = f(weight, sex, age)

Source	df	MS	F	P > F
Regression	1	44.684	21.55	0.0001
Error	69	2.074		

Parameter	B Value	Std Error
Intercept	1.566	
Weight	0.132	0.028

variables showed that, with the exception of number of flights, initial weight, rather than any of the treatment combinations, explained most of the experimental variation (Table 1.3).

Discussion

High variability measured in this flight mill experiment could result from genetic heterogeneity within the laboratory population. It would be valuable to investigate this possibility through selection experiments so that: 1) more homogeneous populations could be tested in flight experimentation and 2) genetic components controlling flight behavior and physiology could be further explored.

Flight to exhaustion was probably an artifact of the circular flight mill design. Since moths could not land, tarsal contact never occurred; this may have prolonged wing beating when the moth otherwise would not have flown.

Other workers have observed the same trend of decreasing flight distance over consecutive flights (Foley, 1985; Clarke, et. al., 1984). Other insects known to be migratory show very little tendency to tire on extremely long flights. For example brown planthoppers, <u>Nilaparvata lugens</u> Stål, flew for an average of 4 hours per flight and flights as long as 10 hours are not uncommon (Ohkubo, 1973). It could be concluded that rapid fatigue, such as indicated in the present experiment may be characteristic of insects that are physiologically capable of short, "appetitive" movement whereas insects sustaining flight for long durations are adapted for true migratory flight.

Regression analysis and careful study of correlation coefficients point to a possible cause for the sex based weight loss differences. Initial weight of females was greater than that of males. This implies that females expended more energy to fly the same distance as males, since more work (work = force·distance) was accomplished. There were no differences in distances flown by males and females, so the observed differences in weight loss is the expected result.

An unexpected result was the constancy in the total distance traveled by all ages of moths. According to field data (Geier, 1960), as interpreted by Johnson (1969), younger moths should fly more than old moths. A possible explanation for this discrepancy is that as moths age, they lose weight; since other parameters influencing flight do not change (such as wing size), it is possible that on a per unit of work basis, an older moth will be carried further in a single flight.

There are two areas that may cause results from circular mills to differ from from moths in free flight: 1) Alteration of normal behavior of the moth, and 2) Physical differences between free flight and tethered flight. These considerations are discussed in greater detail below.

Behavioral Effects

Insect flight may be abnormal on circular mills. Suspended insects lack tarsal contact. Lack of tarsal contact may force a wing beating response when a moth is in an exhausted state and otherwise unlikely to initiate flight activity. Another possible effect is that of being forced to travel in a circular path. Codling moths were observed beating their inside wings more vigorously than the outside wing, a response that would normally turn the moth out of a circular path. The same response has been noted for bark beetles (Bennett and Borden, 1971). Whether the differential wing beating response is mediated by tactile stimuli or by an optomotor response (Niehaus, 1981), this response is an artifact introduced by the equipment design.

The insects' ability to sense their motion could affect the interpretation of flight distance measurements. It has been shown in Small Tortoiseshell (*Aglais urticae* L.) flight (Niehaus, 1981), that insects may regulate their speed on circular mills to match that of free flight. Since the tether support eliminates the requirement for lift, flight at the same velocity as free flight implies that the rate of energy expenditure on a flight mill could be much less than free flight. If the duration of flight were governed by energy expenditure, a flight mill would allow an insect to travel much greater distances than in free flight.

Tethered Flight Mechanics

Moths do not have to support their own weight while flying on a flight mill, since the rotor provides support. Component force analysis was conducted to determine thrust and lift vectors of moths in stationary flight on tethers (Table 1.4 and Fig. 1.11). About 82% of the energy expended in stationary tethered flight was thrust rather than lift. It appeared that in stationary tethered flight, the normal relationship between these forces was probably altered, since moths could only support a maximum of ca. 72% of their body weight (this is combined thrust and lift). In free flight a moth would have to supply lift equivalent to its own weight simply to stay aloft. This type of analysis should be considered only an approximation of flight while on a circular mill or in free flight, as the wing stroke is altered by stationary flight (Pringle, 1957), and forward motion may provide lift and reduce aerodynamic turbulence.

Conclusions

Some conclusions can be drawn from the component analysis and circular flight mill data: 1) moths do not support their own weight on the mill; 2) most of the force component in tethered flight is thrust; 3) moths without tarsal contact will fly to exhaustion. Together these facts imply that distance data collected from circular flight mills are biased towards overestimating potential free flight capability. Direct comparisons and conversions of flight mill data into equivalent free flight have been attempted (Chance,

Table 1.4. Components of force measured for stationary tethered moths.

Body weight	Lift	Thrust	Force vector	θ
25.9 mg	0.9 mg	18.7 mg	18.7 mg	2.7°
28.1	5.7	17.4	18.3	18.1
28.4	5.7	14.7	15.8	21.2
32.2	3.5	23.0	23.3	8.6

Explanation of Table 1.4. Female moths aged one day after emergence were tethered as for flight mill experiments. The flight mill rotor was replaced by a short wire which was fixed to a support. The support was then weighed on a Mettler balance while the moth was anaesthetized with CO_2. The change in weight was then recorded when the moth flew horizontally (lift) and vertically (thrust). These are force vectors which can then be added to give a composite vector that includes magnitude and direction (θ) in degrees above horizontal.

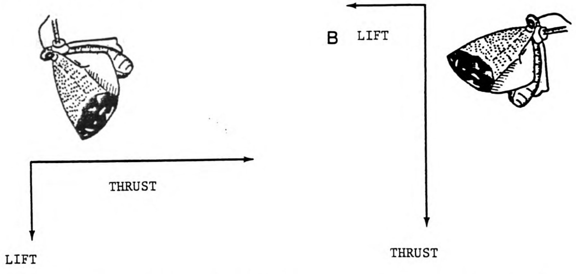

Figure 1.11. Components of force as measured in stationary tethered flight (A) orientation for measuring lift, (B) orientation for measuring thrust.

1971; Solbreck, 1980). These conversions assume that the only difference between tethered flight and free flight is flight mill resistance. This assumption is suspect due to both behavioral (lack of tarsal contact) and physical factors (support from the flight mill and presence of the tether attachment to the thorax). Distance data from flight mills would probably be better used for estimating relative distances that insects from different treatments would be capable of flying (Roitberg, et. al., 1984).

On circular mills it was demonstrated that non-diapause conditioned males and females aged 0 to 3 days post emergence flew approximately the same distance and at similar velocities. Older moths flew less flights than young moths, and females lost more weight in overnight flight than males. Caution must be used when comparing these results to field data, as physical and behavioral differences between moth flight on circular mills compared with free flight may affect the results.

Chapter 2 - Balancing Flight Mill Experiments

Introduction

Codling moths have two generations per season in Michigan conditions; where higher degree day accumulations are much greater (such as Israel and Mexico), three or four generations occur. Diapause conditioning of larvae are of interest as they reflect the differences in larval conditioning for first and second codling moth generations in one season. Comparisons of males and females were repeated because of the drawbacks (See p. 34) of using circular flight mills. Balancing mills could eliminate some of the difficulties in interpretation resulting from the lack of tarsal contact, the lack of ability for moths to land, and the circular path traveled by moths flown on circular flight mills.

Three hypothesis were tested in this experiment. The first hypothesis tested whether there were differences in flight tendency of the diapause conditioned vs. non-diapause conditioned moths. These differences could reflect the adaptive value of migration in the overwintering and summer generations of codling moths. A second hypothesis stated that differences in the flight tendency of males and females may exist. A difference would indicate the relative importance for each sex to disperse. The third hypothesis tested whether younger moths fly more than old (post-reproductive) moths, since there is no adaptive value for old moths to migrate.

Materials and Methods

Flight Mill Design

Balancing flight mill (Fig. 2.1) arms were made with 15.9 cm long (0.015 in diameter) spring steel wire passed perpendicularly through a 4 cm section of plastic drinking straw. The wire and straw can then rotate on a fulcrum made from a shorter piece (8.5 cm) of another spring steel wire run through the straw lengthwise. The same type of lights and photoelectric sensors (Clarke, et. al., 1984) were used as with the circular flight mills, however they were arranged differently so the light would shine directly at the sensor. The foil on the flight mill arm does not act to reflect light (as with the circular mill), but to interrupt the light beam.

The flight mill interface operated in the same manner as for the circular mill. A new program had to be written because the balancing flight mill does not act as an analog to digital converter. Instead, the program used for the balancing mill operated by matching the state of the moth's activity with the signal sent to the computer. If the moth took off, the flag on the mill arm no longer interrupted the light beam and the change of state was interpreted as being the start of a new flight. When the light beam was interrupted again for that mill, that flight was interpreted as having ended. Details regarding the BASIC program and how it operated with the balancing flight mill are included in Appendix C.

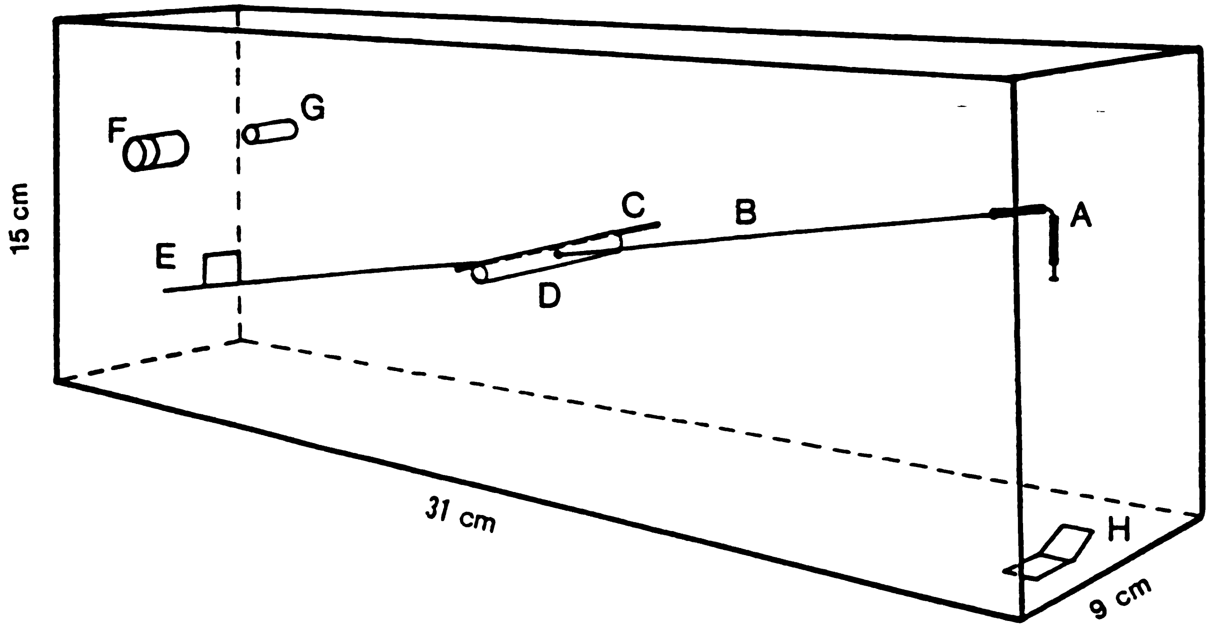

Figure 2.1. Single unit of balancing flight mill. (A) Harness, (B) Spring steel rotor, (C) Spring steel wire fulcrum, (D) Length of plastic straw, (E) Aluminum foil reflector, (F) Light, (G) Photoconductor, (H) Aluminum foil landing platform.

Table 2.1. Coefficients of variation for 2X2X2 factorial experiment with balancing flight mills.

Variable	Coefficient of Variation
Total time in flight	312 %
Number of flights	191
Weight loss	39

The arrangement of the flight mill arm and the plastic straw allowed fine adjustments of the effective weight of the moth at the end of the arm. Moths in all experiments using balancing mills were weighed prior to attachment and adjusted so that they would have to lift 40% of their body weight to become airborne. Forty percent of the moth's weight was chosen since stationary tethered flight trials showed that moths could not lift their entire weight. If moths were required to lift a smaller proportion (<25%) of their initial weight, they could lose sufficient weight during an experiment that the mill arm would become unbalanced and the moth would no longer land. In order for moths to be able to lift a significant proportion of their own weight, they had to be oriented at an angle of ca. 60° from the horizontal. This in turn required a landing surface at the same inclination. Aluminum foil was taped to the bottom of the mill floor providing a platform that could easily be adjusted to match the position of the landing moth.

Culture Maintenance

The same laboratory culture and techniques were used for culture maintenance as for the circular flight mill experiments (See pages 12-13).

Diapause Conditioning

Conditions determined to be ideal for induction and termination of diapause in Michigan strain codling moths (Garcia Salazar, 1984) were followed.

Larvae designated for diapause conditioning were placed in 12:12 L:D conditions at 27°C for 20 to 30 days, then sexes were separated and transferred from hibernacula formed in the diet cups to petri dishes containing heat-sterilized cardboard strips. These larvae readily spun new hibernacula within a few hours. These petri dishes were then replaced in the short day (12L:12D) growth chamber until the larvae had a minimum of 55 days conditioning as fifth instar larvae at 27°C. Petri dishes were then placed in plastic bags and kept in a walk-in cold room (4°C) for a minimum of 55 days. Following these diapause terminating conditions, larvae were placed in the long-day maintenance chamber (16:8 L:D, 27°C) to pupate and emerge as adults.

Experimental Procedure

Moths, except for those emerging the same day as a replicate, were collected each day by chilling for 3 min. at -20°C and sorting from pupae. Both sexes were placed together in ovipositional cages and kept at 27°C without feeding until ready for use. Experiments were conducted in a walk-in growth chamber, with cool white fluorescent lighting (900 lux) with the same conditions (16L:8D and 27°C) as the long-day rearing chamber. Lights off occurred at 9:00 PM; the remainder of the night had crepuscular light conditions (44 lux) due to the television monitor screen and the Sylvania #327 bulbs.

On the afternoon of the experiment, moths were placed in closed dixie cups and weighed. The same techniques for handling the moths was used as with the circular mill experiment (p. 18). Moths were attached with Scotch^R Contact Cement (St. Paul, MN), rather than rubber cement (as in the circular mill experiments) because contact cement set much more rapidly. Following attachment, if the moth showed pre-experimental flight activity, a small aluminum foil weight was placed on the flight mill arm to prevent take-off.

At 7:00 PM, the experiment was begun by starting the flight mill program and removing any weight from the flight mill arm. At ca. 8:00 the next morning, the replicate was terminated by turning on the printer and typing "s". Immediately after a hard copy was obtained, data were stored on a floppy diskette.

Moths were returned to a dixie cup to obtain post-flight weights, then were replaced into the breeding culture. Attachment with contact cement and subsequent removal did not seem to affect moths adversely, as they were capable of mating following flight experiments.

Experiment 1. Diapause, Age, and Sex Factorial Design

Experimental Design

The balancing flight mill was used in a randomized complete block, 2^3 factorial design, comparing moths aged <1 and 5 days post emergence, males versus females, and moths reared from diapause vs. non-diapause conditioned larvae. The eight treatment combinations were used in each replicate and randomized over the eight mills. Blocks of these eight treatment combinations were then replicated over days.

Results

Three response variables were analyzed from data collected using the balancing flight mill: time spent flying, the number of flights, and weight loss. There was high variability between individuals within treatments as indicated by the high coefficients of variation (Table 2.1).

Statistical analyses were made with MSTAT (Michigan State University, East Lansing, MI), and are given in Table 2.2. Older moths flew more (32.8) flights than younger moths (16.0 flights), but less total time (1141 vs. 3093 seconds, respectively). Weight loss was significantly different between the two ages ($P < 0.001$). Younger moths lost 3.9 mg, while older moths lost 2.3 mg. Another important result was that in none of the analyses was there any evidence of diapause X sex interactions. This finding means that the two sexes respond similarly to diapause conditioning with respect to the response variables measured, and implies that a simpler design testing one sex or the other would be sufficient to test hypotheses regarding the effect of diapause on flight tendency.

Experiment 2. Diapause vs. Non-diapause Conditioned Males

Experimental Design

The balancing flight mill was used in a randomized complete block design experiment comparing one day post

Table 2.2. Analyses of variance for 2X2X2 factorial experiment with balancing flight mills.

Time in Flight

Source	df	SS	MS	F	P > F
Replicate	6	288758011	48126335	1.10	0.37
Diapause	1	96446	96446	0.00	
Sex	1	5726722	5726722	0.13	
Diapause X Sex	1	26857920	26857920	0.61	
Age	1	53359873	53359873	1.22	0.27
Diapause X Age	1	6307400	6307400	0.14	
Sex X Age	1	75311845	75311845	1.72	0.19
Diapause X Sex X Age	1	2227218	2227218	0.05	
Error	41	1795366357	43789423		

Number of Flights

Source	df	SS	MS	F	P > F
Replicate	6	26474.6	4412.4	2.02	0.084
Diapause	1	8305.8	8305.8	3.81	0.057
Sex	1	120.1	120.1	0.06	
Diapause X Sex	1	0.3	0.3	0.00	
Age	1	3944.6	3944.6	1.81	0.186
Diapause X Age	1	4183.1	4183.1	1.92	0.173
Sex X Age	1	961.1	961.1	0.44	
Diapause X Sex X Age	1	12.1	12.1	0.01	
Error	41	89403.1	2180.6		

Table 2.2 (cont'd.)

Weight Loss

Source	df	SS	MS	F	P > F
Replicate	6	45.72	7.92	5.38	0.000
Diapause	1	0.27	0.27	0.18	
Sex	1	0.71	0.71	0.48	
Diapause X Sex	1	0.03	0.03	0.02	
Age	1	35.68	35.68	24.25	0.000
Diapause X Age	1	0.15	0.15	0.10	
Sex X Age	1	0.36	0.36	0.25	
Diapause X Sex X Age	1	0.05	0.05	0.04	
Error	41	60.34	1.47		

eclosion male moths reared from diapause vs. non-diapause conditioned larvae. Blocks consisted of four subsamples for each treatment randomly assigned to the eight mills, and replicated over days.

Results

Three response variables were analyzed from data collected using the balancing flight mill: time spent flying, the number of flights, and weight loss. There was high variability between individuals within treatments as may be seen from the high coefficients of variation (Table 2.3).

Statistical analyses were made with MSTAT (Michigan State University, East Lansing, MI), and are given in Table 2.4. In spite of the extreme variation among treatments, there appears to be a difference in the tendency of these two groups to fly. Diapause conditioning caused moths to fly for more prolonged time periods (1570 vs 712 seconds total flight time) (P=0.056). There was no difference in the number of flights, with means of 45.2 and 20.7 for non-diapause conditioned vs. diapause conditioned moths, respectively. When taken together, the average duration of flight for non-diapause conditioned moths was 15.7 seconds, while for diapause conditioned males this value was 75.8 seconds per flight. Although not statistically different, it is possible that initial weight could have affected these results, since the initial weights of the two groups were 17.1 and 15.9 mg for non-diapause conditioned and diapause conditioned moths, respectively.

Table 2.3. Coefficient of variation for diapause vs. non-diapause males flown on balancing mills.

Variable	Coefficient of Variation
Total time in flight	229 %
Number of flights	284
Weight loss	38

Table 2.4. Analyses of variance for diapause vs. non-diapause conditioned males flown on balancing mills.

Time in Flight

Source	df	SS	MS	F	P > F
Replicate	4	29701315	7425328	7.11	0.041
Diapause (Rep)	1	7366788	7366788	7.06	0.056
Error	4	4175170	1043792		
Subsamples	30	205126384	2837546		

Number of Flights

Source	df	SS	MS	F	P > F
Replicate	4	45843.6	11460.9	1.04	0.485
Diapause (Rep)	1	5978.0	5978.2	0.54	
Error	4	44147.8	11036.9		
Subsamples	30	262883.2	8762.8		

Weight Loss

Source	df	SS	MS	F	P > F
Replicate	4	45.39	11.35	3.68	0.117
Diapause (Rep)	1	15.25	15.25	4.95	0.090
Error	4	12.32	3.08		
Subsamples	30	146.65	4.89		

Discussion

Moths flying on balancing mills do not fly to exhaustion, therefore this design may be superior to the circular mill for measuring the tendency (rather than the physiological capacity) for flight. Moths usually did not fly for hours at a time (there were 3 exceptions, with 2 flights of 3 hours and one flight of 10 hours), in contrast to the circular mill where flights on the lasting several hours were not uncommon.

There are some problems when interpreting data collected from the balancing mills. Flight orientation of moths was adjusted to an inclination of ca. 60° above the horizontal; this steep orientation was necessary to enable the moths to lift their own weight on the balance. This is quite likely an artificially steep angle compared with free flight, and the effect on normal flight response is unknown. A second artifact in this flight mill design was the lack of wind flowing over the flying insect. Although an insect flying in place creates its own wind, the aerodynamics of flight may be affected enough to make a considerable difference in the lift component of flight. A wind flow over the moving insect has already been shown (Pringle, 1957) to affect the orientation of wings throughout the wing beat in free flying vs. stationary tethered insects. Another concern is the lack of stimuli that would indicate movement to the flying moth. It has been shown by Niehaus (1981) that tethered butterflies respond to optomotor stimuli by prolonging flight.

The results for these two experiments are consistent in showing that average flight duration is probably the best measure for comparing groups with the balancing flight mill apparatus. The use of average flight duration as a measure of dispersal tendency makes intuitive sense, as displacement requires prolonged flight. Short flights would be less likely to cause displacement even when they may add to the same total time in flight because: 1) change in direction between flights would reduce displacement and 2) prolonged flight would be necessary to attain altitude for penetrating the boundary layer. This second factor may be especially important for Lepidoptera, as it is common for moths to migrate by flying upward into fast moving air layers. There is some support from the field observations of Borden (1931) that occasionally codling moths have directed rather than zig-zag flight. Geier's data (1960) may also indicate directed flight. Since young moths are positively phototactic this could mean that they fly towards the brightest area in the sky (Johnson, 1969) or fly above the boundary layer, where their displacement would be largely governed by wind velocity.

Dispersal could be said to be an evolutionary adaptation that allows an organism to track changes in its host availability. Dispersal, and the benefit from finding unexploited resources, is offset with the risk of increased mortality while dispersing and energy expenditure that otherwise may have been used for provisioning eggs. Consequently it is clear that with these opposing forces, populations may have variations in the proportion of dispersing individuals, whether a result of genetic polymorphism or due to environmental cues governing dispersal tendency in genetically homogeneous populations (Harrison, 1980). Temporal instability in host availability implies a high risk that progeny will not survive in the habitat in which the parent developed. This could explain why codling moths that have passed through diapause conditioning have a greater tendency to disperse than moths that have not undergone diapause as larvae. Codling moths' preferred host is apple. Apples, especially non-cultivated apples under low nitrogen conditions (Westwood, 1978), tend to bear biennially; individual trees may be in a bearing or non-bearing state in one season depending on whether or not they bore fruit the previous season. It would be advantageous for codling moths to not disperse between generations within the same season; the successful development of a first generation implies that the trees are in a bearing year, and consequently there may be suitable fruits available to complete the second generation. Emergence from a diapause state, however, implies that the coming season is likely to be an "off" year for fruit production for the host tree on which that moth developed, and dispersal to another host tree would be highly advantageous. The control of expression of differences in flight tendency could be cued by environmental conditions, and may be closely related to physiological changes in the diapause process.

It would be expected from the results of Experiment 2 that codling moth populations developing from wild apple trees would tend to show greater differences in flight tendency between generations than codling moths reared from apple trees kept in a better nutritional state. This hypothesis for the underlying ecological reason for increased dispersal tendency following diapause could be tested in the following manner. Diapausing codling moth larvae could be trapped by wrapping corrugated cardboard strips around trunks, providing sites for these larvae to form hibernacula. Larvae could be collected from orchards with differing temporal patchiness. One site could be a recently abandoned orchard under high nitrogen conditions, containing cultivars with reduced tendencies for biennial bearing, such as McIntosh, Rome or Delicious. Another site could be an abandoned orchard with highly biennial bearing cultivars, such as Rhode Island Greening, Golden Delicious or Baldwin. The extreme case would be to collect larvae from seedling apples in woods. The last situation decreases the apple tree's ability to set fruit due to shading, as well as being in a low nitrogen surrounding. A large proportion of the larvae could be placed in a cold room, while the remaining larvae could be used to start a laboratory culture. The larvae in diapause could be brought out of diapause so that emergence would coincide with the emergence of the first generation in laboratory culture, thus minimizing the effects of laboratory selection. It would be expected that the groups would have decreasing flight tendency in the following order: diapause conditioned moths from wild apples, diapause conditioned moths from biennial bearing variety apples, diapause conditioned moths from annual bearing variety apples, and non-diapause conditioned moths.

Conclusions

The number of flights of old moths was greater than that of young moths in Experiment 1. There was also a threefold (though not statistically significant) difference in the means for total time spent flying between these groups, with older moths flying 1146 seconds and younger moths flying 3093.2 seconds. Average flight duration combines flight duration and total number of flights to give a new measure of flight tendency. The average flight duration of one day old moths was 193 seconds, while the average duration of flight for five day old moths was 34.8 seconds. Old moths flew for short bursts of activity, indicating that they would be very unlikely to be adapted for migratory flight. Younger moths showed a much greater capacity for sustained flight. This would indicate a greater dispersal tendency if this flight activity were directional. The conclusion drawn from Experiment 1 is that younger moths have an increased flight tendency compared to 5 day old moths.

These old moths are of declining reproductive value (Chapter 3), and consequently would not be expected to be migratory according to Johnson's (1969) hypothesis. Newly eclosed females do have some fully developed oocytes (Chapter 3), therefore the codling moths flown in these experiments do not fulfil Johnson's (1969) hypothesis that migratory flight precedes oogenesis.

The selective comparison of a single factor in Experiment 2 was shown to be extremely efficient at finding treatment differences, which is a special concern when experimental material is limited. With only five replicates possible due to a limited supply of moths, Experiment 2 was able to detect differences between diapause and non-diapause conditioned moths. While Experiment 1 was not as sensitive for detecting these differences, the two experiments complemented each other. The lack of sex by diapause interactions shown in Experiment 1 means that both sexes can be expected to respond in the same way to diapause conditioning when measuring flight tendency. The conclusion from Experiment 2 that diapause conditioned males had increased flight tendencies compared to non-diapause conditioned males can then be extrapolated to include female flight tendency as well. Unfortunately, female moths could not be tested further since all diapause material had emerged.

Chapter 3 - Female Reproductive Development

Introduction

Reproductive state has long been associated with migratory behavior; Johnson (1969) amassed extensive evidence that migratory flights occur in insects that have not yet reached reproductive maturity. Rankin and Rankin (1979) further conclude that though neuroendocrine control over life processes such as diapause, migration and reproduction may be complex, that migratory flight and oogenesis are coordinated in that reproductive development is generally antagonistic to migratory activity. Migratory flight is thus usually accompanied by a delay in reproductive development; when reproductive development does occur, migratory flight ceases. Several methods can be used to measure reproductive maturity, as this concept includes involvement of various tissues, behaviors, and physiological machinery. To better understand the possible relationship between flight activity and reproductive development, vitellogenesis and oocyte maturation were studied.

Experiment 1. Radioiodination Experiments

Introduction

Reproductive development in females can be considered to be a coordinated process involving neuroendocrine changes, production of vitellogenin by fat body, and the uptake of this protein by the ovaries. It was considered beyond the scope of this study to attempt measuring hormonal changes and vitellogenin production, so experiments were conducted to measure vitellogenic uptake by ovaries. Trial experiments with in vitro incubation of ovaries in 1% trypan blue containing saline, and microinjection of trypan blue solution for in vivo analysis, showed that these techniques, while effective for determining the vitellogenic state of oocytes (Telfor and Anderson, 1968), could not be used in quantitative assays. The dye apparently became covalently bound to cellular materials, making solubilization of trypan blue from homogenized ovary tissues impossible. The optical density (OD_{610}) of trypan blue consequently could not be read by a spectrophotometer.

Another approach to study vitellogenic uptake was followed. Since uptake of trypan blue could not be measured, radioactively labeled vitellin protein was used. Vitellin (yolk protein that is isolated from eggs) was used rather than vitellogenin (yolk protein isolated from hemolymph) due to simpler purification procedures (Gellissen, et. al., 1976). Some advantages to using isotopically labeled vitellin over the trypan blue method include: (1) the counts of radioactivity taken up by the ovaries could be standardized based on the actual number of counts injected, (2) the protein of interest could be used and (3) the efficiency of this method can be very high when using a gamma emitter (^{125}I) because of high penetrability and ease of detection.

Materials and Methods

The low ionic strength precipitation method for purifying vitellin (Gellissen, et. al., 1976), was used. Seventy whole ovaries were ground with an all glass homogenizer in 0.4 M NaCl (pH 7.2) at 0°C. The crude homogenate was centrifuged for 15 min. at 23,000 g and 4°C in a Sorvall ultracentrifuge. The supernatant was filtered through glass wool, and a sixfold dilution made with deionized distilled water at 0°C. Following this dilution, the solution was centrifuged at 23,000 g for 30 minutes. Vitellin is insoluble in these low ionic strength conditions, and precipitates. The supernatant was discarded and the pellet resuspended in 0.4 M NaCl. The process of sixfold dilution with deionized distilled water followed by centrifugation and resuspension in 0.4 M NaCl was repeated twice. The final sample was stored at -20°C in 0.4 M NaCl.

Iodination was carried out on this vitellin protein, following the Iodogen method (Markwell and Fox, 1978). A PD-10 desalting column (Pharmacia) was equilibrated with 3 column volumes of Dulbecco's phosphate buffered saline (PBS) at pH 7.2, 3 volumes of PBS plus 1 mg/ml bovine serum albumin (BSA), and one column volume of PBS. 50 ul of 1 mg/ml concentration vitellin protein was added to 0.5 mCi ^{125}I in an Iodogen coated 10 X 75 mm disposable test tube, shaken slightly, and allowed to react at room temperature for 10 minutes in a fume hood. The contents were then desalted to remove free ^{125}I on the PD-10 column, using PBS + 1 mg/ml BSA as the running buffer. Half milliliter fractions were collected, 20 ul from each fraction were then counted on a

Packard^R gamma counter. The 2 ml containing the iodinated vitellin were combined and frozen until ready for use.

A non-insect protein, isolated from Spiroplasma citri (a mycoplasma-like organism) and previously iodinated, was used as the negative control for an in vivo injection experiment.

One day old female moths were anesthetized for 4 min at -20°C and injected in the thorax with 1.5 ul of iodinated vitellin, iodinated control protein, or iodinated vitellin + trypan blue. The third treatment was included to investigate the effectiveness of the trypan blue to compete with the vitellin protein. The whole moths were counted immediately following injection to standardize for injection error and differences in dpm/ul. After a 4 hr incubation at room temperature, the moths were sacrificed, their ovaries dissected and washed twice in distilled water, and the ovaries counted on a gamma counter.

Results

As can be seen from Table 3.1, vitellin protein is indeed taken back up by ovaries when compared to control protein. Reduced counts were taken up in the presence of Trypan blue, although this difference is not statistically significant, increasing the number of replicates could possibly demonstrate competition with vitellin.

Conclusions

On further consideration, it was decided that the iodinated vitellin technique would yield information on the ca-

Table 3.1. Incorporation of [^{125}I]-vitellin into codling moth ovaries. Means sharing the same letter are not significantly different. (P< 0.01 Tukey's Test)

Treatment	Ovary CPM	% of Injected Counts	Mean ± S.E.(%)
Control	627	.54	.99a ± .27
	904	.49	
	448	1.56	
	707	1.38	
Vitellin	907	2.51	2.60b ± .25
	806	2.37	
	515	2.19	
	1155	3.31	
Vitellin + Trypan Blue	385	1.65	2.17b ± .28
	1295	2.87	
	1167	1.80	
	782	2.35	

pacity of ovaries to take up vitellin, which is a different level of process than what is of immediate concern for following female reproductive development. There are several complications when studying vitellin uptake. Competition with endogenous vitellogenin could obscure interpretation of *in vivo* studies of vitellin uptake, while hazards of working with ^{125}-Iodine and difficulties in purifying large quantities of protein preclude *in vitro* study. An additional concern is that although vitellin is readily purified, there could be alterations in its ability to be taken up again by ovaries compared to its vitellogenin precursor.

The methods described here would be of great use for investigating the molecular basis for vitellin membrane recognition of vitellogenin. Purified vitellogenin could be radioiodinated and injected with various substrates or antibodies to investigate *in vivo* competition.

Experiment 2. Whole Ovary and Ovipositional Studies

Introduction

As stated before, reproductive development entails coordinated hormonal, biochemical, and physiological processes. Investigation of each individual process may eventually lead to a complete understanding of how the reproductive machinery works; however end results such as ovary weight and oviposition are easily measured and immediately useful. Growth of ovaries, as measured by ovary weight or by the number of terminal oocytes, provides a direct measurement of reproductive development.

Materials and Methods

Codling moths used in these experiments were from the Michigan laboratory culture (see p. 12). Moths were kept in ovipositional cages under the same photoperiod and temperature conditions as for maintenance culture (see pp. 12-14). All adults were fed 5% sucrose as described on p. 14.

For the ovary studies, females aged from 0 to 7 days post emergence were weighed, dissected and their ovaries removed. The number of terminal (chorionated) oocytes per ovariole were averaged over the eight ovarioles per female. The pair of ovaries for each moth were then weighed on a Mettler balance. Ten females were dissected for each age.

For ovipositional studies, cohorts of 7 to 10 females were placed with 10 males in an oviposition cage and daily oviposition noted.

Results

Ovary weights and the number of terminal oocytes per ovariole are given in Figures 3.1 and 3.2. There are several results from this study which are of great interest. The first observation is that there is tremendous variation in the number of terminal oocytes for each age investigated, for example, newly eclosed moths had a range of 0 to 5.6 oocytes per ovariole. By the time moths were 1 day old, none of the moths had less than 3.7 oocytes per ovariole. Ovary weight and number of chorionated oocytes per ovariole rapidly reach a peak at 2 days post eclosion, then decline

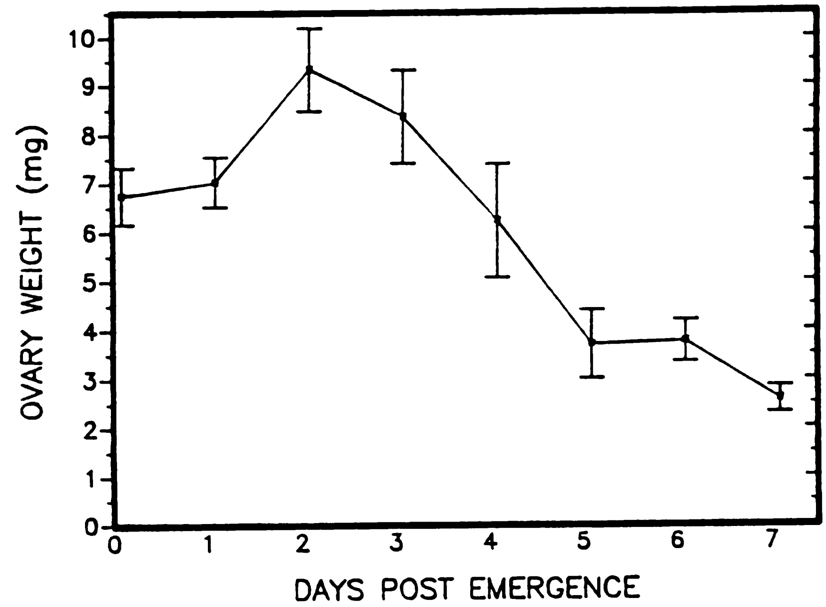

Figure 3.1. Ovary weight for moths aged 0 to 7 days post eclosion.

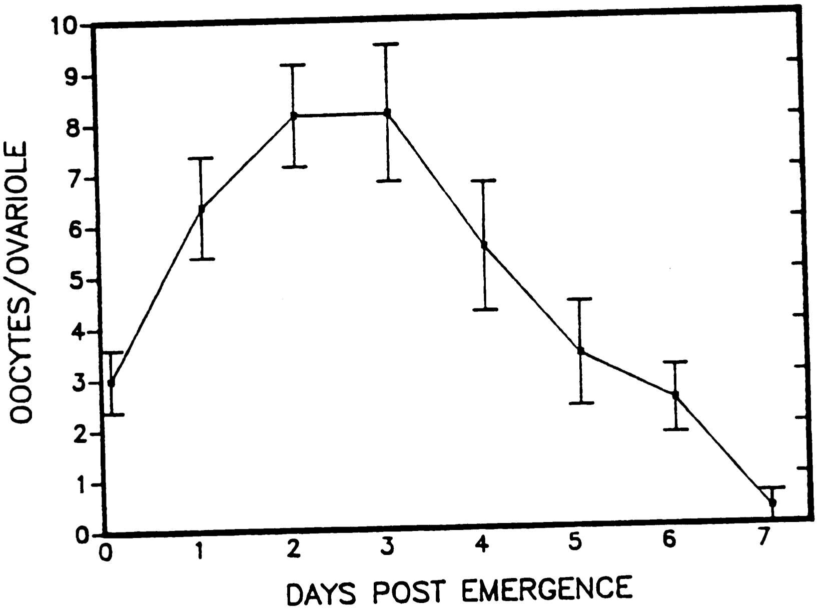

Figure 3.2. Number of terminal oocytes/ovariole in moths aged 0 to 7 days post eclosion.

rapidly following day 3. The two curves (Fig. 3.1 and 3.2) are very similar because ovary weight is closely dependent on the number of developed eggs. The greatest difference between these two curves is on the first day of emergence. This difference is due to the presence of more vitellogenic oocytes on this day than on following days. Completed yolk deposition in oocytes in days 1 to 7 post eclosion is more closely followed by chorionation, so the presence of fully yolk filled, non-chorionated oocytes is only seen the first day of adult emergence.

The data for the weighted average oviposition are included in Figure 3.3. Egg laying obviously depletes the ovaries, since the time of onset of oviposition coincides with the drop in ovary weight seen in Fig. 3.1, and the decline in oviposition coincides with the minimum weight reached by 7 days. Females lacked reserves to lay more eggs, since at the age of 7 days the fat bodies appeared depleted.

Discussion

The variation in ovary weight and number of terminal oocytes per ovariole was somewhat surprising, especially for moths on the first day of emergence. It was expected that the physiological state of moths would be synchronized at eclosion. Another surprise was the extent to which ovaries had developed at the time of eclosion. Presence of chorionated eggs and large yolk-filled eggs imply that there is no true pre-reproductive period and that the physiological

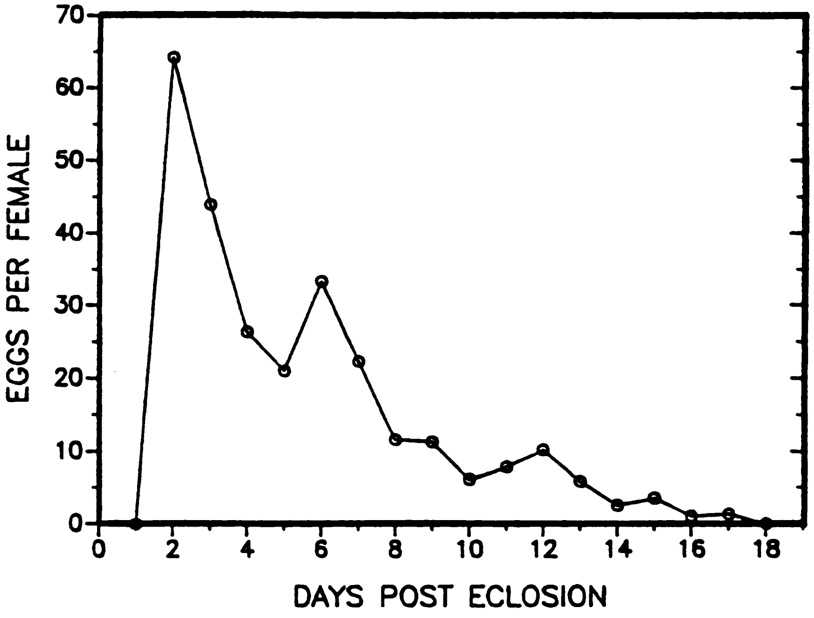

Figure 3.3. Average daily oviposition per female.

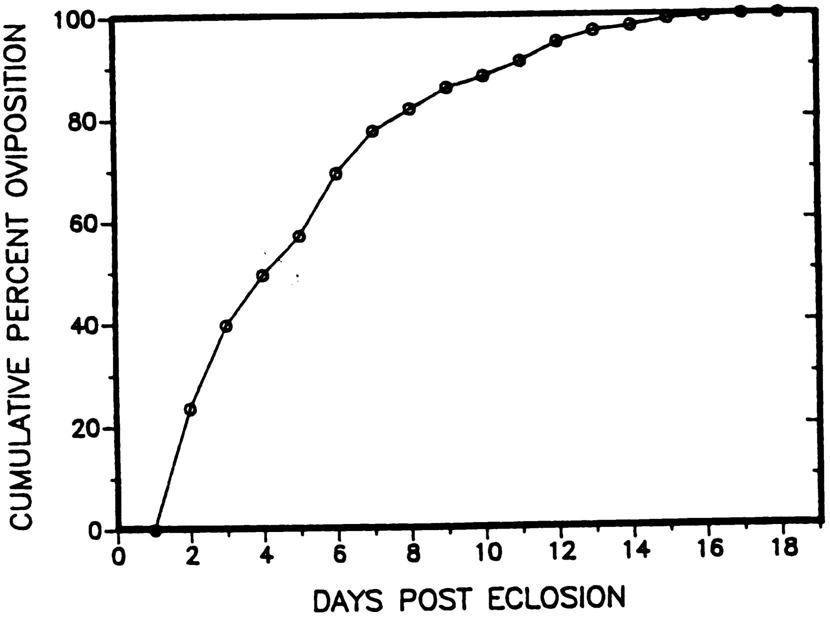

Figure 3.4. Average cumulative percent oviposition.

machinery for producing vitellogenin and sequestering this protein not only is fully functional at eclosion, but already has started operating.

The lack of a true pre-reproductive period is also supported by the ovipositional study. If it is assumed that the eggs laid on one day were fully chorionated eggs accumulated over the previous 24 hours (a valid assumption considering that a female can lay upwards of 100 eggs in one day, this means 12.5 terminal oocytes per ovariole, the maximum observed at any one time), then it is clear that the greatest rate of vitellogenesis occurs in the first two days of adult life. Vitellogenic rate could also be estimated relative to the slope of the cumulative percent oviposition curve (Figure 3.4). I would expect that if a pre-reproductive stage occurred in the adult that this cumulative percent oviposition curve would be sigmoidal.

These reproductive studies agree very closely with the review of codling moth reproductive development given by Mossa (1983). Other workers have also found that females contain mature oocytes at eclosion, that egg laying starts on the second or third day, and peak egg laying occurs on the third to fifth day following eclosion. Howell (1981) reported oviposition to be 90% complete by the fifth day following eclosion.

These data demonstrate that codling moths lack a clearly defined pre-reproductive period. There may be a pre-ovipositional period of one to two days, however the recorded 50% mating the first evening following emergence (White, et. al., 1973) and the intense vitellogenic activity starting at eclosion (possibly even before eclosion) may provide a better indication of moths' physiological state. The lack of a pre-reproductive state other than a brief teneral period (ca. 10 min.) following eclosion could explain why differences were not found when testing flight capacity of moths aged 0 to 3 days post emergence (Chapter 1). The only differences found over various ages of moths was when comparing newly emerged moths with 5 day old moths (Chapter 2). These five day old moths could be considered post-reproductive from the evidence given above, consequently their lack of flight ability may have been the result of depleted reserves.

Conclusions

Oogenesis in codling moths is initiated prior to adult eclosion. Moths on the day of eclosion have 0 to 5.6 chorionated oocytes per ovariole. By the first day following emergence, the minimum number of oocytes per ovariole is 3.7. This shows that even in the moths showing the most delayed development, vitellogenesis and oogenesis is occurring within the first day post eclosion. Egg development is most rapid within the first 4 days following eclosion, then declines rapidly as fat body is depleted.

Codling moths could not be considered a good model for Johnson's hypothesis (1969), that migratory flight occur in a post teneral, pre-reproductive period, since oogenesis is initiated prior to eclosion in codling moths.

Conclusions

The use of flight mills in the study of codling moth flight had both strengths and weaknesses. There are difficulties due to altered behavior and flight mechanics that do not allow comparisons to free flight. Circular mills affect moth behavior by not allowing tarsal contact and causing circular flight. In addition, circular flight is not appropriate for testing migratory behavior, as by definition migratory flight is adaptive for effecting displacement. Circular mills, however, do have their place in assaying physiological capacity for flight. The circular mill design made clear the underlying nature of flight in codling moths as "trivial" type movement since the moths appear to tire easily.

According to Johnson (1969), flight should be related to reproductive development (particularly in females), such that migratory flight takes place before reproductive development occurs. This is adaptive since the reproductive value of founding females will be maximized. Reproductive development studies in Chapter 3 showed that there does not appear to be a pre-reproductive state in the codling moths studied. This agrees with observations from Chapter 1 that the distances flown by moths aged the same day of emergence to three days post emergence showed no trend for a change in flight capacity.

Considering the conclusions above, it is perhaps somewhat surprising that males reared from diapause conditioned larvae flew further than moths reared from non-diapause conditioned larvae in the balancing flight mill experiments. It was expected that if there were differences in flight tendency, that they would be correlated with differences in reproductive development. Although male moths were flown, the same results would be expected from having flown females since the 2^3 factorial experiment showed no evidence of sex by diapause interactions. Since a measure of male reproductive development was not available, female ovarial development was compared in these two groups. There did not appear to be differences in ovarial development of diapause and non-diapause conditioned females (t-tests for ovary weights and oocytes per ovariole were not significant).

That diapause conditioning increases flight tendency does not come as a surprize from within the context of the codling moths' life system (Geier, 1963). Evaluation of flight within this context makes clear the adaptive value of differential migration between the two generations each growing season. Codling moths' preferred host is apple. Apples, especially non-cultivated apples under low nitrogen conditions (Westwood, 1979), tend to bear biennially; individual trees may be in a bearing or non-bearing state in one season depending on whether or not they bore fruit the previous season. It would be adaptive to codling moths to not disperse between generations within the same season; the successful development of a first generation implies that the trees are in a bearing year, and consequently there may be suitable fruits available to complete the second generation. Emergence from a diapause state, however, implies that the coming season is likely to be an "off" year for fruit production from the host tree that moth developed on, and dispersal to another host tree would be highly advantageous.

It would be expected from these results that codling moth populations developing from wild apple trees would tend to show greater differences in their diapause vs. non-diapause conditioned moths when compared to populations of codling moths reared from apple trees kept in a better nutritional state.

Some last considerations must be made regarding these experiments. First, the culture had been in continuous culture for approximately 20 to 30 generations. It is quite likely that field collected populations could yield further valuable information that could more readily be applied to field populations. Secondly, the perfect flight recording device has not yet been developed. At present, interpretation of data from flight mills is difficult because the mechanical and behavioral effects do not allow flight of the experimental insect to be "representative" of free flight or of flight under natural conditions (Stinner, et. al., 1983). Ideally, this device would simulate more of the behavioral stimuli that would be appropriate for the experimental animal. The balancing flight mill has made some advances towards solving this difficulty, inclusion of artificial wind and a moving visual field in response to take-off could greatly improve the relevance of the data collected to observed field behavior.

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Appendix A

213	g	Pinto Beans
32	g	Brewer's Yeast
3	g	Ascorbic Acid
2	g	Methyl Paraben
1	g	Sorbic Acid
1.75	g	Aureomycin
2	ml	Formaldehyde
13	g	Agar
25	ml	Vitamin Solution
650	ml	Distilled Water

Vitamin Solution - these ingredients are combined and brought up to 800 ml with distilled water, then frozen until ready to use in 25 ml aliquots.

16	g	Inositol
.8	g	Niacin
.8	g	Calcium panthotenate
.4	g	Riboflavin
.2	g	Pyridoxine HCl
.2	g	Thiamine HCl
.2	g	Folic Acid
.02	g	Biotin
.02	g	Vitamin B-12

Pinto beans are soaked overnight, boiled for 1 min in a fresh change of distilled water, then drained. Agar powder is added to 650 ml of boiling water and allowed to completely dissolve. All ingredients are then combined in a blender and mixed until only small fragments of beans remain.

Appendix B

Basic Program for Using the Circular Flight Mills

The circular flight mills act as analogue to digital converters, taking velocity information (a continuous variable) and converting this information into a stream of digital pulses. The time between pulses sent from a given mill indicates the velocity the moth on that mill has been travelling. Parameters have to be set to distinguish what minimum velocity is considered "flight". At one extreme, all flights would be grouped together and only averaged information for the entire night would be available. At the other extreme, each time a pulse is registered it could be individually recorded. Lines 5 to 25 allow these parameters to be properly set.

```
5 INPUT "DO YOU WISH TO USE DEFAULT VALUES (Y/N)";A$
7 IF A$="N" THEN 10
10 V=0.01 : SEC=10 : D=2 : GOTO 30
20 INPUT "MAXIMUM TIME FOR INTERRUPTS WITHIN FLIGHTS";SEC
25 INPUT "MINIMUM DISTANCE FOR FLIGHT";D
```

Lines 30 to 310 initialize variables used in the program. Information for each flight is stored in a two dimensional array, the information stored is starting time (STM), ending time (ETM), and total distance (DIST) for each flight. FTT and FCT are variables that store the time that current and previous interrupt signals occured from each mill. These variables are necessary to calculate the instantaneous velocity of the moth on that mill and to decide whether the moth has started a new flight or not.

```
30 INPUT "HOW MANY MILLS (1-8)";M
40 IF M<1 OR M>8 THEN 30
250 DIM FTT(M) : DIM FCT(M) : DIM DIST(M,99) :DIM G(M)
260 DIM STM(M,99) : DIM ETM(M,99)
270 FOR I=1 TO M
280 FTT(I)=0 : FCT(I)=0 : DIST(I,1)=0
290 STM(I,1)=0 : ETM(I,1)=0 : G(I)=0
291 N(I)=1
300 NEXT I
310 T2=PEEK(56577)
```

Line 320 checks the binary code at the user port to see if the value has changed from the initialized value (line 310). If this PEEK value changes, it means that a reflector has just entered or just left a light beam. Based on this binary code, the computer then calculates which mill sent the signal (line 360) and stores the time that the interrupt occured.

```
320 T1=PEEK(56577)
330 GET S$ : IF S$="S" THEN 470
```

```
340 IF T1=T2 THEN 320
350 L=ABS(T2-T1) : T2=T1
360 I=INT(LOG(L)*1.4427)+1
390 FTT(I)=INT(TI/60)
```

Line 400 determines whether a new flight has occured, then increments the distance for the appropriate flight (line 420 and 730), by 0.5 meter. The value 0.5 meter is used because for one revolution of the flight mill arm there are two changes of state, once when the reflector enters the light beam and once when it leaves the light beam.

```
400 IF FTT(I)-FCT(I)>SEC THEN 436
410 FCT(I)=FTT(I)
420 DIST(I,N(I))=DIST(I,N(I)) + 0.5
430 GOTO 320
436 IF DIST(I,N(I)) < D THEN 720
437 IF INT((DIST(I,N(I))/(FTT(I)-STM(I,N(I))))*10000/10000<V
THEN GOTO 720
```

When there have been 99 flights for one mill, the array is full. In order to not lose distance information for any further flights, all subsequent flight information is grouped under flight 99. This is accomplished in line 438 by simply not incrementing the flight counter for each mill once it reaches the value 99.

```
438 IF N(I)=99 THEN 410
440 ETM(I,N(I))=FCT(I) : N(I)=N(I)+1
450 STM(I,N(I))=FTT(I) : GOTO 410
```

Lines 470 to 605 provide a printout of flight mill data.

```
470 OPEN 4,4 : CMD4
472 FOR I=1 TO M
480 ETM(I,N(I))=FCT(I) : NEXT I
490 FOR I=1 TO M
495 PRINT#4 : TDIST=0
500 PRINT#4,"MILL NUMBER ";I
510 PRINT#4,"    BEGIN   END    FLIGHT"
520 PRINT#4,"FL# FLIGHT FLIGHT   TIME   DIST    VEL"
530 FOR J=1 TO N(I)
535 TDIST=TDIST+DIST(I,J)
540 TTM=ETM(I,J)-STM(I,J) : A=10000
545 IF TTM=0 THEN 590
550 VEL=INT(DIST(I,J)/TTM*A)/A
560 PRINT#4," ";J;"  ";STM(I,J);" ";
570 PRINT#4,ETM(I,J);"  ";TTM;"  ";
580 PRINT#4,DIST(I,J);"  ";VEL
585 IF J=99 THEN 595
590 NEXT J
595 PRINT#4,"TOTAL DISTANCE IS ";TDIST;" METERS."
600 NEXT I
```

```
605 CLOSE 4
```

Lines 610 to 710 allow data to be stored on a floppy disk in a sequential file. The carriage return symbol (CHR$(13)) is used to separate values, while -1 and -2 act as flags to indicate when new flight or new mill records start.

```
610 INPUT "DO YOU WISH TO STORE DATA ON DISK? (Y/N)";R$
615 IF R$="N" THEN 750
620 INPUT "FILE NAME";FI$
630 FI$="0:"+FI$+",S,W"
640 OPEN 2,8,2,FI$
645 C$=CHR$(13)
650 FOR I=1 TO 8
660 PRINT#2, "-1";C$;
670 FOR J=1 TO N(I)
680 PRINT#2,"-2";C$;STM(I,J);C$;ETM(I,J);C$;DIST(I,J);C$
690 NEXT J : IF J=99 THEN 700
700 NEXT I
705 PRINT#2,"-1"
710 CLOSE 2
```

This is a subroutine that closes one flight record and opens the next flight record.

```
720 STM(I,N(I))=FTT(I)
730 DIST(I,N(I))=0.5 : FCT(I)=FTT(I)
740 GOTO 320
750 END
```

Appendix C

Basic Program for Using the Balancing Flight Mills

Unlike the circular flight mills, the balancing flight mills do not act as analogue to digital converters. Instead, the signal is processed by interpreting whether a moth on a mill has taken off or landed.

Lines 250 to 310 initialize variables used in the program. Information for each flight is stored in a two dimensional array, the information, as before, and is stored as starting time (STM) and ending time (ETM). Total distance for each flight cannot be determined with this type of mill.

```
250 DIM FTT(8) : DIM FCT(8) : DIM TA(8)
260 DIM STM(8,99) : DIM ETM(8,99)
270 FOR I=0 TO 8
275 X=INT(TI/60)
280 FTT(I)=X : FCT(I)=X
290 STM(I,1)=X : ETM(I,1)=X : G(I)=X
291 N(I)=1 : G(I)=0 : TA(I)=0
300 NEXT I
310 T2=PEEK(56577)
```

Lines 320 to 390 have the same functions as in the previous program. Whether the change in PEEK value is negative or positive indicates whether a moth has taken off or landed, and is determined by line 370.

```
320 T1=PEEK(56577)
330 GET S$ : IF S$="S" THEN 470
340 IF T1=T2 THEN 320
350 L=T2-T1 : T2=T1
360 I=INT(LOG(ABS(L))*1.4427)+1
370 C=SGN(L)
390 FTT(I)=INT(TI/60)
```

"Bouncing" is a problem inherent in digital electronics and involves converting gradual transitions from one state to another into a digital signal. Instead of having one signal sent when a moth takes off or lands, it is usual to have a stream of 3 or 5 pulses. Since FTT and FCT are variables that store the time that current and previous interrupt signals occured from each mill, the time difference between them can be interpreted to indicate whether a change of state has actually occured. This processing of the signals is called "debouncing", and is carried out by line 400.

```
400 IF FTT(I)-FCT(I)<1 THEN 320
403 IF C<>-1 THEN 420
```

When there have been 99 flights for one mill, the array is full. In this program, all subsequent flight information is grouped under flight 99. This is accomplished with line 405, which doesn't increment the flight counter for each mill once it reaches the value 99. Also, the variables G and tA are used to count the number of grouped flights and to accumulate the time spent in flight.

```
405 IF N(I)=99 THEN 430
410 ETM(I,N(I))=FTT(I)
415 STM(I,N(I))=FCT(I)  : N(I)=N(I)+1
420 FCT(I)=FTT(I) : GOTO 320
430 G(I)=G(I)+1
440 TA(I)=TA(I)+(FTT(I)-FCT(I))
450 GOTO 420
```

Lines 470 to 605 provide a printout of flight mill data. The variable FA is used to provide a total of flight time for each mill for the entire night.

```
470 OPEN 4,4 : CMD4
471 X=INT(TI/60)
472 FOR I=1 TO 8
473 IF FCT(I)=ETM(I,N(I)-1) THEN GOTO 476
474 STM(I,N(I))=FCT(I)
475 ETM(I,N(I))=X
476 NEXT I
490 FOR I=1 TO 8
495 PRINT#4
500 PRINT#4,"MILL NUMBER ";I
505 PRINT#4,"THERE WERE ";G(I);"GROUPED FLIGHTS LASTING ";
TA(I);"SECONDS."
510 PRINT#4,"    BEGIN   END    FLIGHT"
520 PRINT#4,"FL# FLIGHT FLIGHT   TIME"
530 FOR J=1 TO N(I)
540 TTM=ETM(I,J)-STM(I,J)
545 IF TTM=0 THEN 590
560 PRINT#4," ";J;"  ";STM(I,J);" ";
570 PRINT#4,ETM(I,J);"  ";TTM;"  ";
580 FA=FA+TTM
585 IF J=99 THEN 595
590 NEXT J
595 PRINT#4,"TOTAL TIME IS ";FA;" SECONDS."
596 FA=0
600 NEXT I
605 CLOSE 4
```

Lines 610 to 710 allow data to be stored on a floppy disk in a sequential file, as in the previous program.

```
610 INPUT "DO YOU WISH TO STORE DATA ON DISK? (Y/N)";R$
615 IF R$="N" THEN 750
620 INPUT "FILE NAME";FI$
630 FI$="0:"+FI$+",S,W"
```

```
640 OPEN 2,8,2,FI$
645 C$=CHR$(13)
650 FOR I=1 TO 8
660 PRINT#2, "-1";C$;
670 FOR J=1 TO N(I)
680 PRINT#2,"-2";C$;STM(I,J);C$;ETM(I,J)
690 NEXT J : IF J=99 THEN 700
700 NEXT I
705 PRINT#2,"-1"
710 CLOSE 2
750 END
```