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The rhythms of emergence, oxygen consumption, and activity were studied in Megachile rotundata with the object of comparing the results to the major properties of the biological clock such as temperature independence, susceptibility to light synchronization, and entrainment. A further objective was to compare the three rhythms to determine if they were three aspects of the same rhythm or if they were indeed three different rhythms.

The emergence rhythm proved to possess a period of 23.7 h in DD and 23.0 h in LL. This difference was not statistically significant. The emergence period displayed a Q_{10} of 1.02 for a temperature rise of 25 to 30° C and a Q_{10} of 1.12 for a 30 to 35° C temperature rise. The rhythm was largely refractive to light but very responsive to temperature from the dark-eyed pupa stage through the adult stage. The temperature phase response curve displayed phase advances during the subjective high temperature period and phase

delays during the subjective low temperature period. No transients occurred in the response of M. rotundata to a temperature pulse.

The oxygen consumption rhythm responded to light and temperature in a manner similar to that of the emergence rhythms. However, no difference was detected in the length of the oxygen consumption period in LL as compared to DD. The rhythm did not respond to light entrainment (LD 12:12). The temperature phase response curve was similar to that of the emergence rhythm. The oxygen consumption rhythm was analyzed in the pre-adult and adult stages using power spectra analyses. The rhythm spectrum appeared to be less stable in the pre-adult stages than in the adult stage. The circadian component was very prominent in the adult while from two to four ultradian components appeared in the pre-adult stages.

The activity rhythm proved to be very similar to both the emergence and oxygen consumption rhythms in its response to light and temperature. Again the response to light was very slight and was only detected in the period length in LL compared to the period length in DD. In LL the activity period length was 22.34 h while in DD the period length was 22.86 h. This difference of 0.52 h was significant at the five percent level. The temperature response curve was almost identical to that of the emergence and oxygen consumption response curves.

A comparison of the three rhythms is drawn with the conclusion that the emergence and activity rhythms are probably the same basic rhythm. It is possible that the oxygen consumption rhythm is different from the other two but more experimentation is required before a more positive conclusion can be made.

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CIRCADIAN RHYTHMS IN MEGACHILE ROTUNDATA (FABRICUS)

INTRODUCTION

It is now generally accepted that organisms possess 24-hour rhythms and that these rhythms persist under constant conditions of light and temperature. Because of the feature of persistence, 24-h or circadian rhythms, have been described as endogenous, inferring that the organism is able to measure absolute time. Many workers are involved in studies with the ultimate aim of elucidating the mechanism of the endogenous clock (Aschoff, 1965; Pittendrigh, 1966).

It has been found by such workers as Aschoff and Pittendrigh, that insects possess rhythms and that they are a very convenient animal to use as experimental subjects for the study of rhythms. Bruce and Pittendrigh (1957) list several specific features of endogenous rhythms in insects which they feel are of general significance with respect to the clock mechanism. The ones pertinent to this study are listed below.

1. The demonstration of a natural period close to, but not exactly equal to 24 h.
2. Entrainability by light and temperature. Bruce and Pittendrigh (1957) state that in D. pseudoobscura Sturtevant, light has a stronger effect than temperature. Other workers

such as DeCoursey (1964) and Roberts (1962) working with the hamster and cockroach respectively, have also shown that light is the major entraining agent.

3. Circadian rhythms are temperature sensitive but they are temperature independent. Even though the clock may be phase shifted by temperature steps or pulses, the periods of circadian rhythms remain close to 24-h under different conditions of temperature. Zimmerman, Pittendrigh and Pavlidis (1968) reported a Q_{10} for Drosophila pseudo-obscura eclosion of 1.02 and Rawson (1960) measuring the activity of bats reported a Q_{10} of 1.02 and 1.07.

4. Circadian rhythms can be initiated by a single stimulus. The initiation of a rhythm by a single stimulus of light or temperature was shown by Pittendrigh (1954) and Brett (1955) to occur in D. pseudoobscura eclosion. Pittendrigh has suggested two alternative interpretations of this phenomenon. One interpretation postulates that the non-rhythmic population has not had their clocks set in motion and is non-rhythmic for this reason and the single stimulus "initiates" the population rhythm by initiating clock motion simultaneously in all individuals. The other interpretation postulates that the clocks in a non-rhythmic population are all running but are out of phase with each other. The

single stimulus "resets" and synchronizes the rhythm in all individuals to give an apparent "initiation" of a rhythm in the population (Bruce and Pittendrigh, 1957).

One more general feature of circadian clocks should be added to the above list. Aschoff (1960) suggested a connection between the nocturnal or diurnal habits of animals and the length of their circadian periods in different constant conditions. He formulated a general rule which postulates that: (1) light-active animals show a shorter circadian period in constant light than in constant dark, while the opposite holds for dark-active animals, and (2) in constant light the period decreases with increasing intensity of illumination in light-active animals, and increases in dark-active animals. Subsequently this rule has been called Aschoff's rule (Pittendrigh, 1960).

The present investigation was undertaken to study the rhythms of emergence, oxygen consumption, and activity in the leafcutter bee Megachile rotundata (F.) in relation to the above general features of rhythms. It was of interest to study these three different rhythms in the same animal and to determine their similarities and differences. It was intended to determine if the three rhythms were simply three expressions of the same rhythm, or, if they were fundamentally different. In experiments conducted by Tweedy (1967), it was indicated that the emergence rhythm of M. rotundata did not respond to light. If this was the case, it would make the leafcutter bee unique in

comparison to those animals whose rhythms have been reported in the literature to date.

M. rotundata females most commonly nest in existing holes that closely approximate their own size (Stephen and Torchio, 1961). These include hollow tubing, stems of pithy plants, in which the pith has been removed, or in cracks in boards. Bees used in this study were taken from cells that had been constructed in drinking straws. The cells, made from alfalfa leaf cuttings, were placed in a linear series in the straw which resulted in a totally opaque environment for the developing bee from egg deposition to adult emergence. Because of the opaque nesting medium it seemed probable that temperature would be the major phase-setter for the adult emergence rhythm. This was verified by Tweedy (1967). However, even though temperature was found to be the major phase setter for emergence it was thought that since M. rotundata is a light active animal, light would be the major phase setter of the activity rhythm. If the activity rhythm proved to be refractive to phase shifting by light this would provide strong evidence in favor of the hypothesis that the emergence of an individual bee takes place during the first activity period and that the emergence and activity rhythms are essentially the same rhythm.

LITERATURE REVIEW

Insect Emergence Rhythms

The literature on insect emergence rhythms was extensively reviewed by Tweedy (1967). Included in the following literature review are a few of the more relevant reports to this present study taken from the above work plus three reports that have appeared since the completion of the literature review for the above study.

Effect of Light and Temperature

Pittendrigh's (1954) work with Drosophila pseudoobscura is probably the most extensive single report on circadian rhythms in insects. He found that the period of the eclosion rhythm is temperature independent. Cultures under a normal light regime (LD 12:12) 12 h of light followed by 12 h of dark, showed emergence between 0600 and 0900 h. When these cultures were subjected to a temperature increase or decrease the subsequent emergence was advanced or retarded respectively, but the phase of the ultimate steady-state remained unchanged.

Pittendrigh also found that light can be used to reset the rhythm. The phase-setter was determined to be the dark-to-light transition in D. pseudoobscura and emergence was placed at any clock

hour simply by subjecting the culture to a single experience of light. The light stimulus used in these experiments was applied for a four hour period. As discussed above, although a temperature change cannot reset the emergence rhythm period, it can cause a perturbation of the period in the emergence subsequent to the temperature shock. Pittendrigh (1954) constructed a model for the clock system of D. pseudoobscura to account for the observed facts. He stated that the clock system of D. pseudoobscura consisted of a "terminal clock" comprising temperature sensitive events occupying, when properly "adjusted," the 24 h immediately prior to eclosion, and a temperature insensitive "primary clock" that measures, throughout the development of the fly, 24 h intervals between reference points in phase with the last seen dawn. In a later paper (Bruce and Pittendrigh, 1957) the above explanation was withdrawn. The departure from the natural period following a temperature stimulus was regarded as a "transient" imposed on the temperature-independent "primary clock." A transient was defined as the arhythmic response of an oscillator to a non-periodic stimulation (Pittendrigh, Bruce, and Kaus, 1958). Pittendrigh (1960) attempted to explain transients caused by light and temperature by proposing a 2-oscillator model of the D. pseudoobscura eclosion rhythm. The B-oscillation is the physiological control that immediately underlies eclosion and is autonomously oscillatory. The light sensitive A-oscillation serves as the pacemaker for

the organism. The light insensitive B-oscillation probably relies on this entrainment for the temperature compensation that characterizes the system as a whole. The B-oscillation can be directly entrained by temperature independently of its coupling to A. There is evidence of some feedback of B and A but it is slight.

The light or temperature-induced transients reflect the motion of the B-oscillator. The light-induced transients lead to a new steady-state whose phase is determined by the most recent light signal. This light signal resets the phase in the A-oscillation, and the observed transients marks the motion of B as it regains phase with the pacemaker (Pittendrigh, 1960). Following temperature-induced transients the new steady-state experiences a phase-shift of about two hours in D. pseudoobscura. Pittendrigh calls this shift "trivial" and points out that it bears no relation to the phase of the perturbation that induced the transients. The temperature shock which induced the transients affected the coupling of B to A. The temperature dependent period of B is temporarily manifested but disappears as B regains its coupling to A, which was almost insensitive to the temperature change.

A third characteristic of the D. pseudoobscura circadian emergence rhythm, verified by Pittendrigh (1954), is its persistence under DD (continuous dark) conditions. When cultures entrained to an LD regime were placed in DD conditions the previously established

rhythm was maintained.

A fourth characteristic confirmed by Pittendrigh (1954) is that a rhythm of emergence can be initiated in aperiodic cultures by a single light pulse. Cultures raised from eggs in DD, show no emergence rhythm, but larvae exposed to a single unrepeated light signal start measuring off intervals from the light cue. In a given culture all the eggs were laid within a two day period, yet emergence activity, commencing about 17 days later, was spread over a period of about eight days. Obviously some develop more slowly than others. However, when they emerge the population variance in development time is partitioned into "quantized packets" 24 h apart.

Myburgh (1963) found that there is a temperature-dependent diurnal rhythm in emergence of the larvae of the fruit flies Pterandrus rosa (Ksh.) and Ceratitis capitata (Wied.) from fruit. This daily periodicity is dependent on normal temperature decreases occurring overnight. Myburgh points out that the diurnal eclosion rhythm of the adults is temperature-independent. Since the pupae are in the soil it is assumed that fluctuation of atmospheric humidity and light could not be transmitted effectively to the soil. It is possible then that exposure to light in the larval stage sets in motion a time mechanism controlling the subsequent time of eclosion of the adults. Myburgh reports some experimental evidence indicating that the clock is set in the larval stage, as the bulk of adult eclosion occurred at the

corresponding time of day to that at which the larvae had been exposed to light.

Saunders and Knoke (1968) reported that the endogenous circadian rhythm of adult emergence in the ambrosia beetle, Xylegorus ferrugeneus (F.) possessed a period of 22.5 h in DD conditions at 24.5° C. After nine days in DD, emergence became aperiodic. In X. ferrugeneus, phase shifting and synchronization were both susceptible to temperature and light changes. However, the conditions of the experiments were not constant as they reported a fluctuation in temperature of up to 50C°. The illumination for the experiments under "constant" conditions was provided by four, 40-W incandescent bulbs. It is regrettable that fluorescent lights, which are the most commonly used lights in rhythm studies, were not used to reduce the risk of radiant heat masking the effect of light.

Effect of Light

Banerjee and Decker (1966), using field data, reported that the sod webworm, Crambus trisectus Walker, emerged between 2100 h and 0100 h. They did not report the period length of this rhythm. Both sexes emerged through the night with the males more predominant in the early hours of emergence and the females more numerous later.

It would appear that light was the main synchronizer. In

spring and summer, when the nights were shorter and darkness delayed, emergence began later in the evening than in the fall. The emergence peaks tended to occur between 2100 h and 2400 h even under different temperature conditions as 72 to 79° F in July and 55 to 72° F in September.

Fannia femoralis (Stein) and F. canicularis (L.) were shown to possess endogenous emergence rhythms with periods less than 24 h (Tauber, 1968). Light was the dominant time cue or Zeitgeber, being able to reverse the phase of the emergence rhythm.

Effect of Temperature

Scott (1936) showed that in the moth Anagasta (= Ephestia) küehniella Zell. a diurnal rhythm of emergence exists, with the maximum emergence occurring in the evening. He observed that the emergence rhythm exhibited a close relationship to the diurnal temperature rhythm, with maximum emergence occurring shortly after the beginning of temperature fall.

Verification of the temperature-dependent emergence rhythm of Anagasta (= Ephestia) küehniella Zell. was accomplished by Moriarity (1959). As well as verifying the temperature dependency of the rhythm, Moriarity showed that the emergence rhythm was not affected by a temperature increase but was set by a temperature drop of 5C°. This also agreed with Scott's work.

Insect Oxygen Consumption Rhythms

Effect of Temperature and Light

Rensing (1966) studied the patterns of the oxygen consumption rhythms in the larva, pupa and adult stages of Drosophila melanogaster Meigen. In reference to the ontogenesis of the oxygen rhythm, Rensing states that in the larvae and pupae the oxygen consumption maximum appears to be correlated with the dark-to-light change. He found this to be true for the volume of the nuclei also, although he did not report which nuclei he studied. During the third larvae instar and the prepupa, the phase of the rhythm is delayed (-). During pupation and emergence the phase moved in a positive direction. A decrease of positive phase angles ran parallel to a decrease in the rate of oxygen consumption, while the reverse phase movement was related to an increase in the rate. Rensing does not attempt to explain the differences in phase shifting, except to comment, that it might depend on a change in metabolism which is not clearly reflected in the rate of oxygen consumption.

A decrease in the amplitude of the oxygen consumption rhythm occurred in D. melanogaster from the third larval instar to the prepupa.

Rensing found a higher rate of metabolism in females but the

period length was similar for both sexes. He states that the period of the female was not significantly shorter.

With increasing light intensity up to 100 lux in LL (continuous light) conditions the length of the period increased. Above 100 lux there was little change in the period length with higher light intensities. The amplitude of oxygen consumption decreased with increasing light intensity.

At temperatures of 20 and 25° C the rhythm of oxygen consumption in adult D. melanogaster did not differ in their phase position in spite of varying consumption rate. It can be summarized that Aschoff's rule for the correlations of the level of the oscillation parameters, length of period and phase position in vertebrates, occurs in D. melanogaster only in single instances, while in other organisms, deviating correlations occur. Rensing states that genes, hormones, drugs, temperature, and light, probably influence the rhythm of oxygen consuming processes at different locations and have thereby differing effects on the parameters of the rhythm.

Phase difference between the synchronized endogenous oscillation and the Zeitgeber depends on the choice of the correlating points in both rhythms. The onset, middle or end of the light-dark time can be chosen, or the maximum or minimum phases of the circadian rhythm. Rensing states that since the maximum is susceptible to change due to exogenous factors, it is of advantage to

choose the point equidistant between two minima. Starting from that point and the middle of the light time, it can be shown that only small phase differences occur between the LD ratios of 4:20, 12:12 and 20:4. In each case, the phase difference is within three hours. This is also the amount of phase difference in the emergence rhythm. Rensing (1966) points out that, according to Pittendrigh's hypothesis, light directing depends on an equilibrium between acceleration and retardation effects of the light, the small deviation of the phase difference in relation to the middle of the light time is to be expected according to the response curve of D. melanogaster.

Effect of Light

Fingerman et al (1958) studied 39 lubber grasshoppers, Romalea microptera (Palisot de Beauvois). Analysis of their data revealed two types of oxygen consumption rhythms. Twenty-six individuals displayed a rhythm with a peak in the forenoon and 13 individuals showed a rhythm with a peak in the afternoon. The average interval between the peaks of the two groups was seven hours. Fingerman's data indicate a possibility of bimodality in the oxygen consumption curve. It would have been profitable to analyze this data using a spectral analysis (see Appendix) to determine if R. microptera possessed more than the one rhythm.

Moriarity (1959), while reporting an emergence rhythm for the

moth, Anagasta (= Ephestia) küehniella Zell., was not able to detect an associated oxygen consumption rhythm.

Campbell (1964), working with Tenebrio molitor L. larvae, found a multimodal curve of oxygen consumption. Peaks occurred at 0700, 1200, 1800, 2200, and 2400 h. The greatest rate of oxygen consumption occurred at 1800 h. The difference between the rate at 1800 and 0900 h, the time of lowest rate, was highly significant ($p > 0.001$).

Richards and Halberg (1964), using variance spectra analysis, showed three components to the oxygen consumption rhythm in male Periplaneta americana (L.) cockroaches. A circadian component of just under 24 hours was most prominent with two ultradian rhythms appearing, displaying periods of about 3.5 h and 0.8 or 0.9 h. These rhythms appeared when the cockroaches were kept in constant light following entrainment by a LD 12:12 cycle. The cockroaches were maintained at a temperature of 30° C with a light intensity of about 10 lux, and without food or water for 219 h. Beck (1964) found an eight hour oxygen consumption rhythm in diapausing larvae of the European corn borer, Ostrinia nubilalis (Hübner) under LD 12:12 conditions. The most prominent peak occurred at the onset of darkness. Beck concluded that the rhythm was phase set by the lights-off signal.

A similar rhythm was detected in the adult German cockroach,

Blatella germanica (L.). Again the most prominent peak occurred shortly after the onset of darkness and the rhythm was found to be phase set by the onset of darkness (Beck, 1964). It is of interest to note that the prominent peak of oxygen consumption coincided with the position of the circadian peaks of locomotor activity demonstrated in other roach species by other workers (Harker, 1956; Roberts, 1960).

Insect Activity Rhythms

In the review of the literature to follow, it should be pointed out that many of the workers did not report the effects of temperature on the parameters which they were studying.

Effect of Temperature and Light

Roberts (1960), working with three species of cockroaches, Periplaneta americana (Linneaus), Leucophaea maderae (Fabricius), Byrsotria fumigata (Guèrin), found that the activity patterns of female cockroaches were generally more erratic than those of males, consequently his subsequent studies and the results discussed below were with male cockroaches exclusively. In DD, the activity rhythm was circadian in nature for two typical roaches, one was 23 hours, 48 minutes and the other 24 hours, 14 minutes. The population studied ranged from 23.5 to 24.5 h in period length. Roberts also

found that the period in DD was labile--that is, it could change spontaneously or it could be induced to change in a predictable way in response to a perturbation of the environmental regime. Harker is quoted by Roberts stating that the locomotor rhythm was lost in the roach Periplaneta americana in LL, but in the three species studied by Roberts, this did not occur for up to 20 days. However, the period did change to become longer in LL. This was of an order of magnitude of about 20 minutes to one hour at intensities up to 25 fc (foot candles). Roberts did not analyze the data statistically but he stated that within the experimental range of intensities ("immeasurably low intensity" to 25 fc) there was no obvious correlation between the LL intensity and the degree of period lengthening. He concludes that the period lengthening in LL either saturates at a very low level, or is an "all-or-none" phenomenon in the cockroach. Under LL conditions, cockroach activity exhibited a secondary peak at a relatively fixed phase relation to the primary peak. In ten individuals tested the secondary peak occurred between 8 to 12 h after the primary peak. The free-running period was found to be temperature compensated. In L. maderae, DD periods for 20° C, 25° C, and 30° C were determined. A Q_{10} calculated for periods at 20° C and 25° C was 1.06, and for the periods at 25° C and 30° C the Q_{10} was 1.03. This extremely low value of the Q_{10} reveals the virtual independence of temperature which gives the circadian rhythm its functional

significance as a time measuring system.

Roberts attempted to phase shift the rhythms of the three roach species using light. A single 12 hour light signal, with a 200 fc intensity, succeeded in delaying the phase of the rhythm by about two h when it was applied in the middle of subjective day in a DD light regime. A similar light signal was applied beginning at the middle of the subjective night, and resulted in a one h phase advance. Roberts concludes that the three roach species that he studied are relatively refractive to phase-shifting by single discrete light signals, when compared to D. pseudoobscura whose rhythm of emergence can be shifted eight hours by a single 1/2000 of a second strobe-flash (Pittendrigh, 1954).

Roberts (1962) investigated the response of L. maderae to 12 h temperature pulses of from 25° C to 7° C and from 25° C to 12° C. He states that the results show: (1) the magnitude of the phase shift depends upon the timing of the temperature treatment--maximum and minimum responses occur when the exposure ends (temperature rise) at subjective roach hour 14:00 and 18:00 respectively; and (2) the phase dependence of the responses is nearly identical for the two temperatures studied--only the magnitude of the responses differ. Using 48 h exposure times instead of 12 h exposures, Roberts states that there was no evidence of a phase-dependent response, but rather a saturated response wherein the new phase was strictly

coincident with rising temperature.

Lohmann (1964) studied the effect of the intensity of illumination and of temperature on the circadian activity of dark active Tenebrio molitor. He measured four parameters: spontaneous frequency, phase-angle difference, amount of activity, and ratio of duration of activity to rest.

The spontaneous frequency in LL diminished with increasing intensity of illumination. When the intensity of illumination was increased by a factor of 100 in the range of 0.01 to 100 lux, the period was lengthened by 50 minutes, on the average. On the other hand, the period was decreased by only 30 minutes when intensity of illumination was lowered by the same factor. In DD conditions, temperature, between 20° C and 35° C, had no effect on spontaneous frequency. The negative phase-angle difference, which Lohmann states, was the time difference between the start of activity and the beginning of darkness, in LD 12:12 was on the average greater at 100 lux than at 400 lux. In regard to temperature, the negative phase-angle difference reached its maximum between 25° C and 30° C within the range of 20° C to 35° C.

The ratio of the duration of activity to rest, measured under constant conditions, was positively correlated both to temperature and to illumination intensity.

Lohmann compared the above findings to an oscillation model

proposed by Aschoff and Wever which is based on the above mentioned four parameters in studies on light-active birds. The hypothesis resulting from the model concerning dark-active animals was only partly confirmed in Lohmann's study. He states that the most obvious divergence from the model was that none of the four parameters, contrary to expectation, positively correlated with one or more of the others. For example, if measurements of two parameters showed similar behavior in varying light intensity, they were dissimilar in regard to temperature and vice versa.

In the beetle, Ptinus tectus Boie, a pest of stored products, light was shown to be a major phasing cue (Bentley, Gunn and Ewer, 1964). In LL, no 24-h rhythm was apparent, but in response to a single dark period, activity became practically confined to the dark period. The amount of activity seemed to vary with temperature. P. tectus showed greater activity at 10-20° C than at 25° C. In LL, they found that a temperature fluctuation of 6 C° was enough to phase set the activity rhythm.

Effect of Light

Fingerman, Lago and Lowe (1958) investigated the circadian rhythm of locomotor activity in the lubber grasshopper, Romalea microptera, in LD conditions, using an illumination of less than one fc. Two types of rhythms were revealed--one group of

grasshopper displayed an activity rhythm with peak activity occurring in the forenoon (1000 h), and a second group was maximally active in the afternoon (1600 h). No sex difference was detected in this study.

In the fly, Phormia regina Meigen, Green (1964) found a circadian activity period in DD but not in LL. The phase of the activity period in DD was not set by a dark-to-light experience.

According to Lewis and Taylor (1964) light is the major factor in controlling times of flight in the field. They studied diurnal flight periodicity in about one half a million individuals representing 400 taxa. They also found that the time of flight of males and females differed greatly in only two species.

Edwards (1964 and 1965) stated that the activity peaks of larval locomotion, adult emergence, and adult flight were closely associated with sunset time, in the silver-spotted tiger moth, Halisidota argentata Packard, and in the phantom hemlock looper, Nephytia phantasmaria Strecker. He also states that due to the correspondence, in time, of emergence and flight peaks, the possibility that emergence time might be a good indication of at least one major activity period is suggested. Edwards states that the activity times in both species of moth was governed primarily by light, with temperature only affecting amount of activity.

According to Nelson (1964), the amplitude of honey bee activity

was directly affected by temperature, whereas the activity period began with lights-on at 0800 h and ceased with lights-off at 2030 h with a peak just after 1200 h.

In the mosquito Anopheles gambiae Giles, Jones, Hill and Hope (1967), found a circadian activity period of about 23 h. Light was found to have a delaying effect if it was applied at the beginning of subjective night, and an advancing effect if applied at the end. In an LD 12:12 regime, peaks of flight activity followed both lights-off and lights-on. The peak following lights-off was found to persist in DD, but the peak following lights-on did not.

CIRCADIAN RHYTHM OF ADULT EMERGENCE

Methods and Materials

Cells of the leaf cutter bee M. rotundata containing diapausing prepupae were taken from soda straws that had been held at approximately 7° C for several months. The prepupae were placed in plastic petri dishes, covered with aluminum foil, and placed in an incubator set at 30° C. After the first day of emergence, the bees were placed in an emergence counting apparatus (Figure 1), which was designed specifically for these experiments. The apparatus was divided into four sections, each with its own counting assembly which consisted of a photocell connected to a channel on an Argus Event Recorder. Each section in the emergence apparatus contained 35 cells. During the study it became obvious that 35 bees was not a large enough sample to obtain accurate records of the emergence rhythms and therefore, in later experiments, the cells were ignored and the bees were placed in the cell region as well as in the cells. Each cell region could hold about 100 insects.

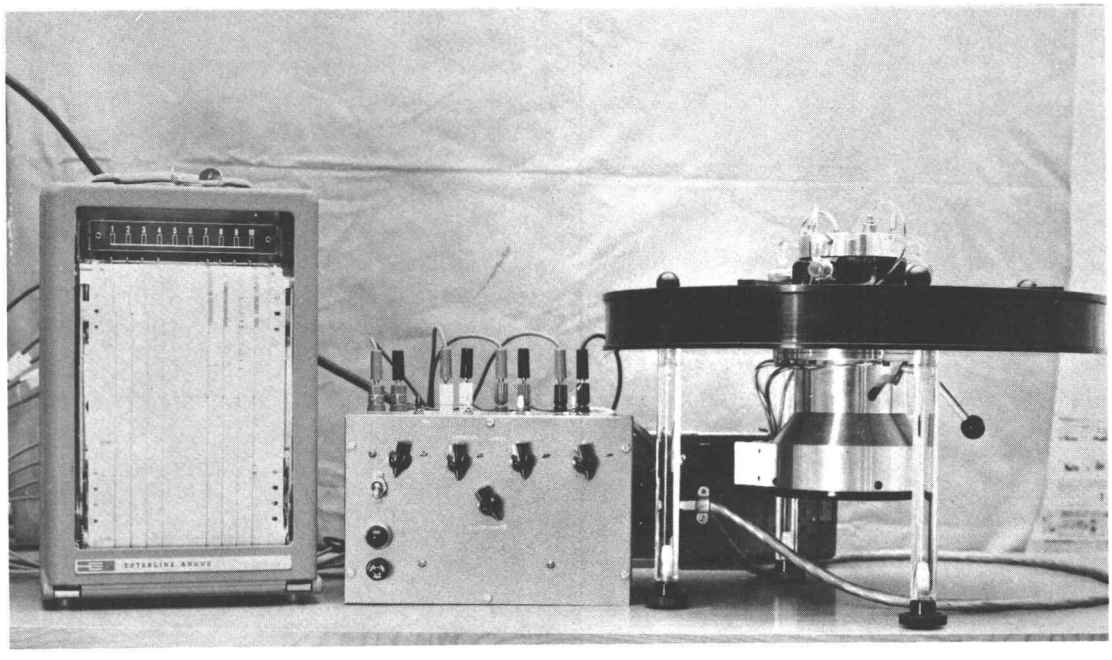
The purpose of individual cells was to reduce contact between emerged bees and preemerged bees as it was feared that the eclosed bees might influence the emergence time of later ones and produce a rhythm of emergence. However, the controls obtained from

Figure 1. Emergence counting apparatus.

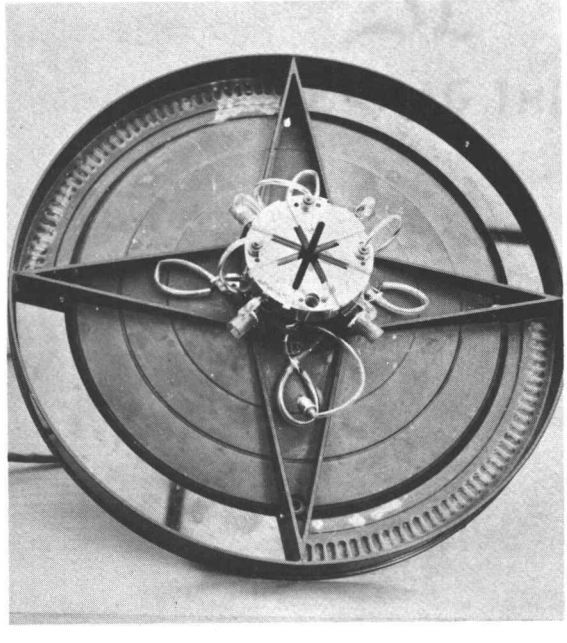
A. Arrangement of the control box, voltage regulator, event recorder, and emergence apparatus when it was in operation.

B. Emergence apparatus with cover off.

C. Emergence apparatus with cover on. Note the cell covers in C.



A



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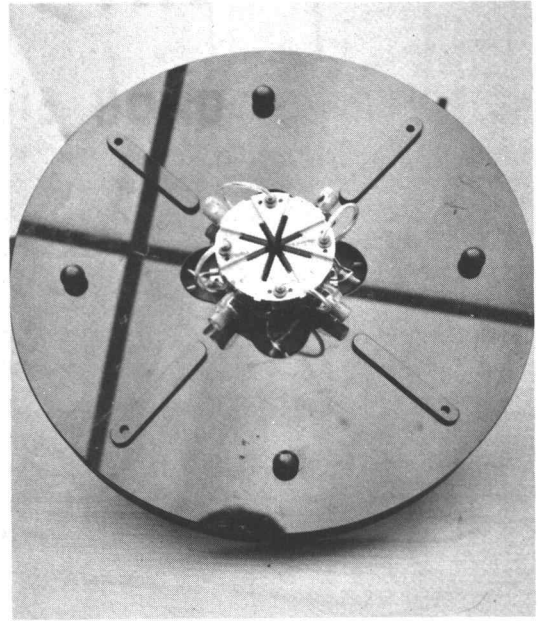


C

experiments which ignored the use of the individual cells did not substantiate this fear.

The apparatus was designed to stimulate the bees to leave the cell area, which was depressed from the apparatus floor, immediately upon emergence (Figure 2). The natural tendency for bees to climb was used to clear them from the emergence area. From observation after experiments were terminated, very few bees were found to have returned to their cells. After the bees climbed from the emergence area they were exposed to a light situated at the lower entrance to the emergence tunnel (Figure 2). From information gained from preliminary experiments, it was assumed that the bees would respond positively to the light and move to the tunnel entrance rather than back towards the emergence region. To reduce the chance of light entering the emergence region, it was covered with black acrylic leaving just enough space to allow the bees to escape. Once the bee entered the tunnel, it was encouraged to continue moving by the incline of the tunnel and the presence of light from the light tube situated at the upper entrance of the exit tunnel (Figure 2). The tunnel was roughened to facilitate the bees clinging to the surface. The photocell (clairex CL 603) used to count the bees was near the tunnel exit. It was hoped that once the bee reached the photocell it would be stimulated to emerge to the outside by the presence of light. However, after a number of experiments it was observed that this was

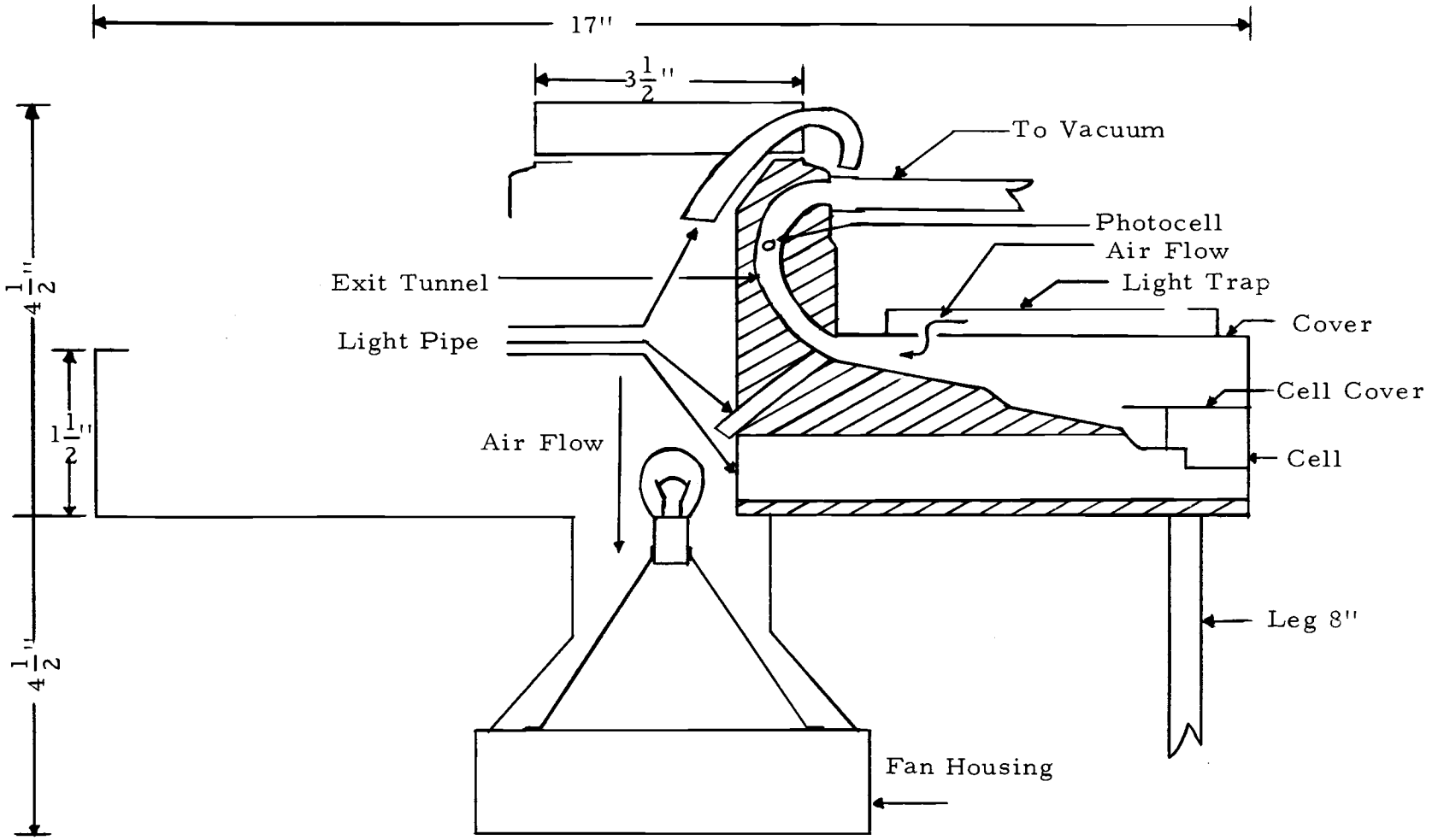


Figure 2. Schematic cross-section of emergence apparatus.

not the case for many bees. A vacuum arrangement was designed to remove the bees quickly once they reached the lower entrance of the tunnel. This was accomplished by causing air to flow quickly through the exit tunnel to a catch. The air flow was maintained by a Hoover industrial vacuum cleaner using a bypass motor to cause more effective cooling of the motor. Air was allowed into the exit tunnel through light baffled holes in the apparatus cover (Figure 2). The catch consisted of a glass tube fitted over the end of tygon tubing which led to the vacuum cleaner, and which was covered with a fine mesh screen. This was emptied at convenient intervals. The catch could hold at least ten insects with no noticeable effect on the efficiency of the counting process.

The emergence apparatus was equipped with a G.E. 93 high intensity incandescent light (Figure 2). A rheostat was installed in the control box to allow light intensity calibration. Much of the heat given off by the light was dissipated by a skipper fan (100-CFM) which ran continuously. The light had to pass through five inches of clear acrylic before it reached the insects. This reduced the amount of heat reaching the insects from the light. The apparatus was equipped with a light baffle system which made it possible to carry out experiments in the dark as well as in the light. The baffles were so constructed that for a given exposure two bees could be exposed to light while the other two remained in darkness. A constant voltage

transformer was connected to the apparatus via the control panel to reduce voltage fluctuation and thus eliminate short term fluctuations in light intensity.

The photocells were connected to a ten-channel Esterline-Angus event recorder via the control panel (Figure 1). The photocell caused a current to flow continuously to the event recorder. When the current was broken by the passage of a bee the pen shifted leaving a single line as a record on the event recorder chart. The circuit diagram for the control box is illustrated in Figure 3.

The emergence counting apparatus was placed in a controlled environment room which could be adjusted to the various temperatures required in the experiments conducted in this study. According to the Leeds and Northrup continuous recorder, which monitored the temperature in the apparatus, the temperature varied no more than $\pm$ 0.50 C^{o}.

Stage Susceptible to Synchronization by Light

For these experiments, diapausing M. rotundata prepupae were removed from their cells and placed in transparent no. 5 gelatin capsules. Prior to this, the tops of the gelatin capsules had been removed and the remaining openings were covered with paraffin to allow the bee to chew its way out. It was assumed that the cold diapausing prepupae would not be susceptible to a light stimulus and thus

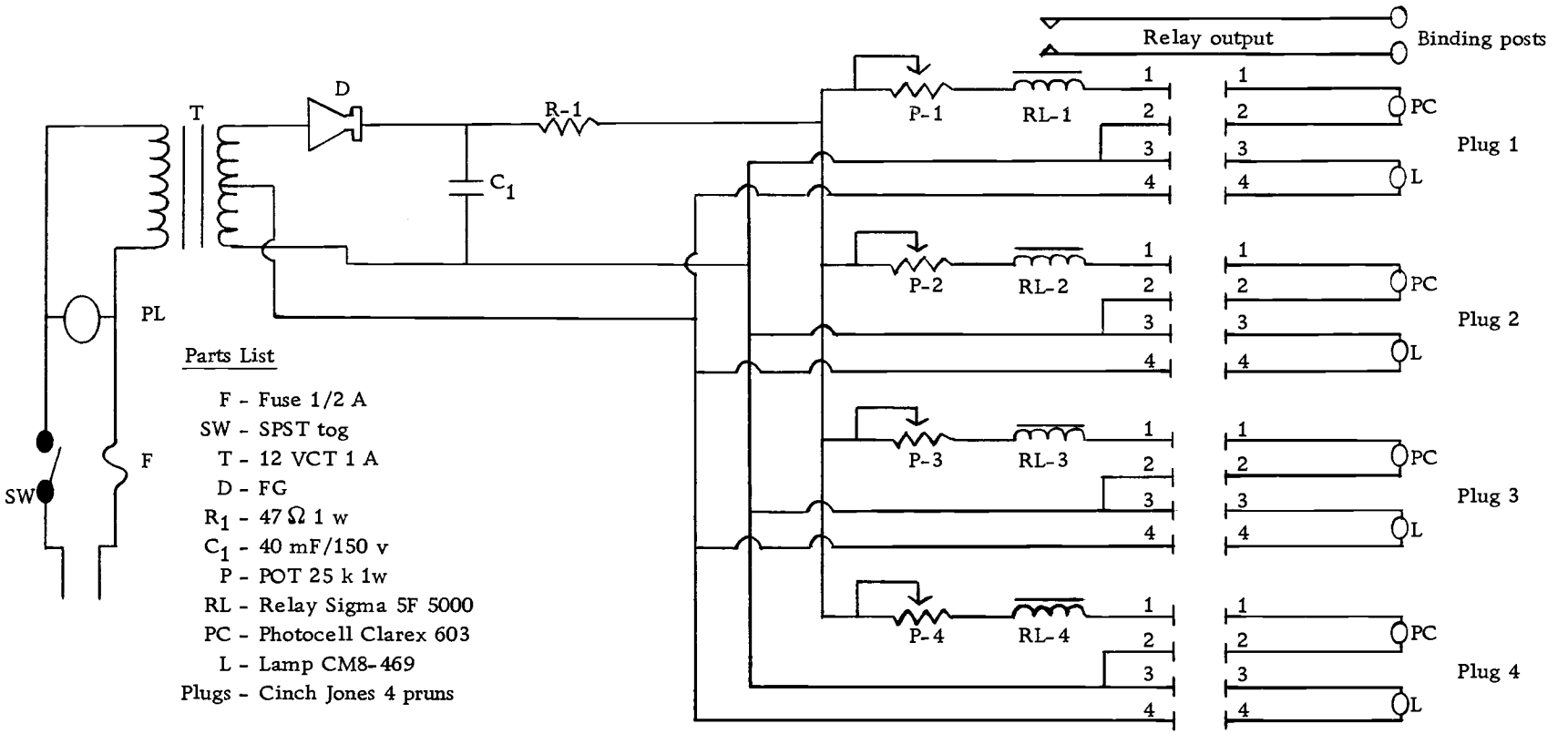


Figure 3. Photocell switch and power supply.

they were removed from their cells and placed in the capsules under room light conditions. This assumption was verified by the experimental data. The gelatin capsules containing diapausing prepupae were then placed in petri dishes and placed in an incubator set at 30° C. Four morphologically obvious stages occur in the development of the leafcutter bee from prepupa to adult--the prepupal (PP), the dark-eye pupa (DP), the dark-bodied pupa (BP), and the pre-emerge adult (A) stages. As the pupa develops pigmentation spreads from the eye to eventually cover the whole body. The dark-eye and dark-body stages are simply two easily identifiable stages in this process. As each stage was reached the bees were removed from the incubator and exposed to a 12-h light pulse of 32 fc and then returned to the incubator until the adult stage was reached. At this point they were placed in the emergence apparatus and emergence was recorded.

Four experiments were accomplished to determine the susceptibility of M. rotundata to light synchronization. A total of 170 PP, 150 DP, 150 BP, 100 A, and 100 controls were used. The resulting data were recorded and reported in the form of histograms.

Stage Susceptible to Synchronization by Temperature

The procedure for temperature studies was the same as that for light except that a temperature pulse instead of a light pulse was

applied. At the appropriate stage the bees were removed from the rearing incubator and placed in another incubator, which was maintained at 15° C for 12 h. They were then removed and placed in the holding incubator (30° C) until the adult stage was reached. Following the first day of emergence, the adult bees were placed in the emergence counting apparatus.

Three different experiments were used to determine the stages susceptible to synchronization by temperature in the leafcutter bee. The numbers of insects used were as follows: 103 PP, 144 DP, 70 BP, 35 A, and 70 controls. A greater number of prepupa and dark-eyed pupa were used than black-bodied pupa and adults as the earlier stages of pupal development proved difficult to obtain meaningful data from. Previous experiments had shown that the adult stage was susceptible to temperature synchronization (Tweedy, 1967).

Effect of Light on the Period Length

The preparatory procedures were similar to those for the other experiments. The baffles were opened to allow the light to reach the insects in the emergence area. It was originally planned to use three different light intensities to study the variable effect of light on the length of the emergence period. Using the highest light intensity possible in the emergence apparatus, and observing no effect due to light, it was decided not to pursue light intensity studies

further.

It would appear from the literature that low light intensities are effective as phase setters, and therefore, if no influence is detectable at low light intensities it is doubtful if higher ones will be any more effective. Roberts (1960), using the cockroach, Periplaneta americana, discovered that light, with such a low intensity that the human eye could only detect it after ten minutes of dark adaptation, was sufficient to phase-set the animal. Pittendrigh (1960) used light intensities from 75 to 100 fc to phase-set the emergence rhythm of Drosophila pseudoobscura. In D. melanogaster, the period length increased from DD to LL at 100 lux, but there was little change in the period length at intensities over 100 lux (Rensing, 1966).

One experiment was performed using 300 test insects and 100 controls exposed to LL for six days. The control insects were not synchronized but the 300 test insects were synchronized before their exposure to LL by a 15C°, 12-h temperature pulse. The results were graphed as histograms and the length of the emergence period was compared to that of other periods in DD conditions.

Effect of Temperature on the Period Length

Three different temperatures were used (25° C, 30° C and 35° C) in an effort to determine the effect of temperature on the length of the emergence period in M. rotundata. The temperature variations

were affected by changing the temperature of the controlled-temperature room in which the emergence apparatus was housed. The insects were prepared as in the previous experiments, temperature-pulsed (30° C to 15° C) for 12 hours and then placed in the emergence apparatus in DD conditions at the appropriate temperature.

To complete this study, two new experiments were required (25° C and 35° C). The data for 30° C was obtained from previous experiments in DD. For the experiment at 25° C, 300 test insects were used, which were temperature-pulsed before placing them in 25° C to make sure that they were synchronized. A group of 100 insects were not exposed to this 15C° temperature pulse, but of course did experience the temperature drop from 30° C to 25° C when they were placed in the emergence apparatus.

Three hundred test insects were used to study the effect of 35° C on the period length. The procedure was the same as that for the test group at 25° C.

The results were analyzed by determining the average period for each temperature (25° C and 35° C), and then comparing the resultant periods by a comparison of the means between groups using the t test.

Temperature Phase Response

The insects used in these tests were treated similarly to the

ones in the previous experiments except that the bees were not removed from their cells. On the second day of emergence, the bees were exposed to a 12-h temperature pulse, as in previous experiments, to synchronize the population. Following this original synchronization, the bees were temperature-pulsed again (12 hours at 15° C) at four hour intervals throughout the 24-hour period. Hour 0 was the hour of the beginning of the original synchronizing temperature pulse. This hour 0 becomes circadian time (CT-0). The resulting rhythms were analyzed to determine the relationship of phase shift to the phase of the free-running emergence rhythm.

Four experiments were accomplished, using 200 insects for each hour studied (CT-0, 4, 8, 12, 16, 20).

Phase Setting Ability of Temperature Rise and Temperature Drop

In this experiment, as in the temperature phase response curve experiments, the bees were not removed from their cells. On the second day of emergence, 200 bees were placed in an incubator held at 25° C for 12 hours, and 200 bees were placed in another incubator held at 35° C for 12 hours. The first group received a temperature pulse of +5 C° and the second group received a temperature pulse of -5 C°. As in other experiments, following the temperature pulses the bees were placed in the emergence apparatus for recording. The experiment was allowed to run for nine days.

Results and Discussion

Stage Susceptible to Synchronization by Light

Figure 4 records the pooled results from four different experiments. A 12-h light pulse was affected on the four developmental stages with no noticeable effect on the emergence rhythm. The pooled controls from the light and temperature experiments are shown in Figure 5.

It was hypothesized that light would most probably phase-set the rhythm of emergence in the later stages of development (dark and black-bodied pupa) as well as in the adult stage. However, the results, as recorded in Figure 4, suggest that M. rotundata is not susceptible to light synchronization at any of the stages examined in this study. The significance of this will be discussed later.

Stage Susceptible to Synchronization by Temperature

In previous work, it was shown that the adult, and most probably the completely pigmented stage (black-bodied pupa) of M. rotundata, was susceptible to temperature synchronization (Tweedy, 1967). The results shown in Figure 5 confirm this suspicion, and also show that the rhythm of emergence can be set as early as the dark-eye stage of the pupa. The prepupal stage did not show a response to the

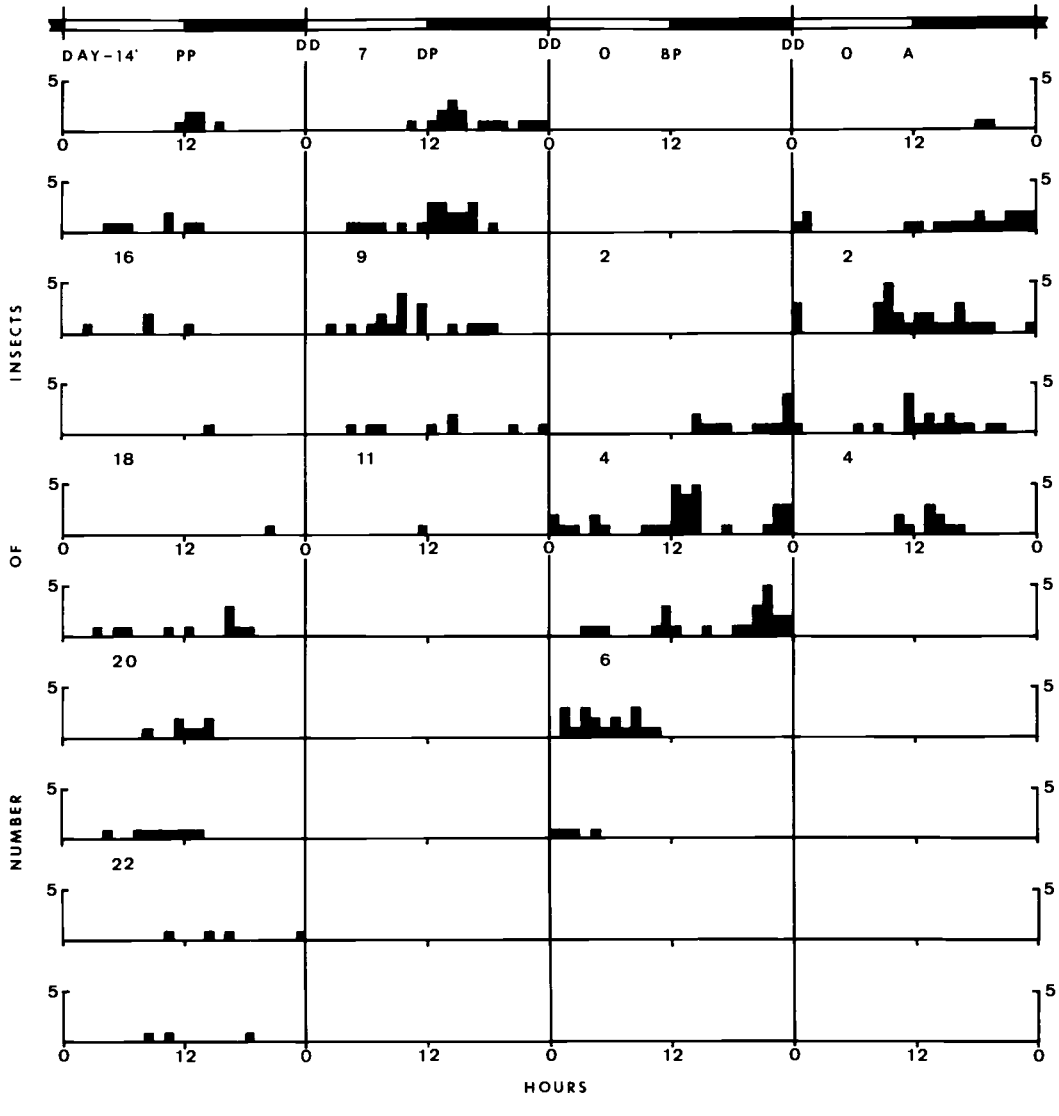


Figure 4. Stage susceptible to synchronization by light. Light regime at top of figure is the light regime that was applied on Day-0 only.

*Day-14. Fourteen days after the 12 h light pulse.

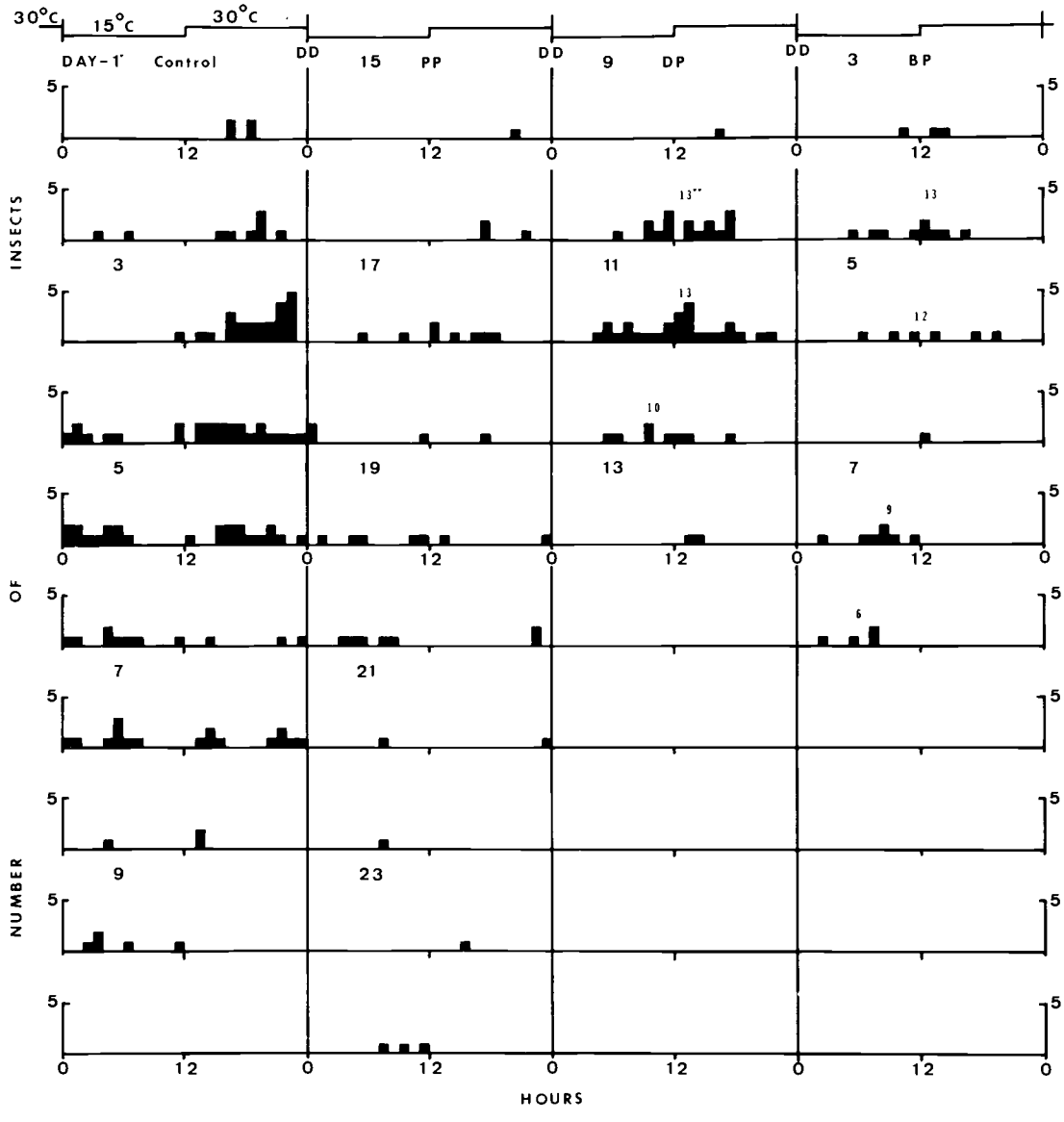


Figure 5. Stage susceptible to synchronization by temperature. Temperature regime at the top of the figure is the temperature regime that was applied on Day-0 only.

*Day-1. The first day (24 h) following the temperature pulse.

**Median hour of emergence

12-h temperature pulse.

Effect of Light on the Period Length

The average period of emergence in LL was 23.00 h as opposed to 23.70 h in DD (Figure 6). Although this is a difference of 0.70 h, it is not significant at the five percent level using the t test for a difference between means. This was probably due to the considerable variation in period length, particularly in the later days of the experiments. According to Aschoff's rule, one would expect that M. rotundata adults, being light active, would show a shorter period in LL than in DD. This is suggested by the results, and possibly more experimentation will show conclusively that the period is shorter in LL than in DD. However, it is also possible that the emergence rhythm is distinct from the activity rhythm and that it behaves in a similar manner to the activity rhythms of dark-active animals, which M. rotundata is at this stage of its life cycle. It could also be that the leafcutter bee simply does not follow Aschoff's rule. Other workers have found that a given organism will adhere to Aschoff's rule for certain parameters but not for others (Lohmann, 1964; Pittendrigh, 1960).

Effect of Temperature on the Period Length

Temperature showed a typical effect on the emergence rhythm

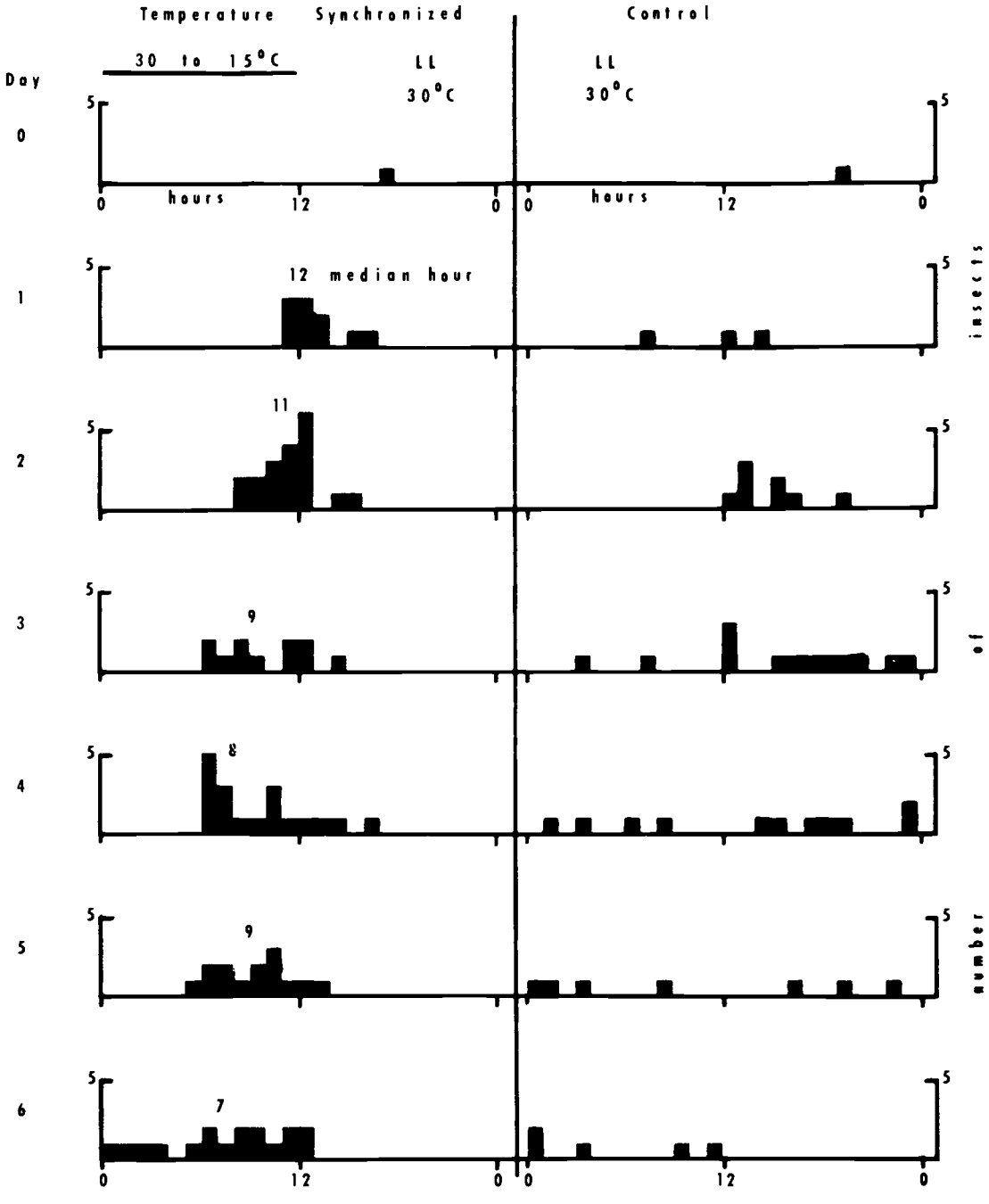


Figure 6. Effect of light on the emergence period length. A temperature pulse of 15 C^{o} was used to synchronize the bee sample.

in Megachile rotundata. Zimmerman, Pittendrigh and Pavlidis (1968) showed that the dependence of the period of the free-running oscillator in Drosophila pseudoobscura is extremely small: at 10° C it was 24.7 h, at 20° C it was 24.0 h, and at 28° C it was 23.7 h (Q_{10} = 1.02). Hastings and Sweeney (1957) found that a slight over-compensation occurred in the lumenescent period of Gonyaulax polyedra Stain. The Q_{10} between 16.5° and 26.8° C was equal to 0.86. In this leafcutter bee the period showed little change at 25° C, 30° C and 35° C (Table 1). But, since the populations under study were allowed to free-run, desynchronization occurred, which was probably due to the variation in individual rhythm periods (Figure 7). This desynchronization may have masked a period change due to temperature.

Table 1. Effect of temperature on period length of the emergence rhythm of Megachile rotundata.

Temperature ° C	Mean Period (hours)	Standard Deviation	Q_{10}
25	24.50	2.2173	
30	24.20	1.3449	1.02
35	22.83	2.8531	1.12

The differences between means at the three temperatures were not found to be not significant at the five percent level using the t test.

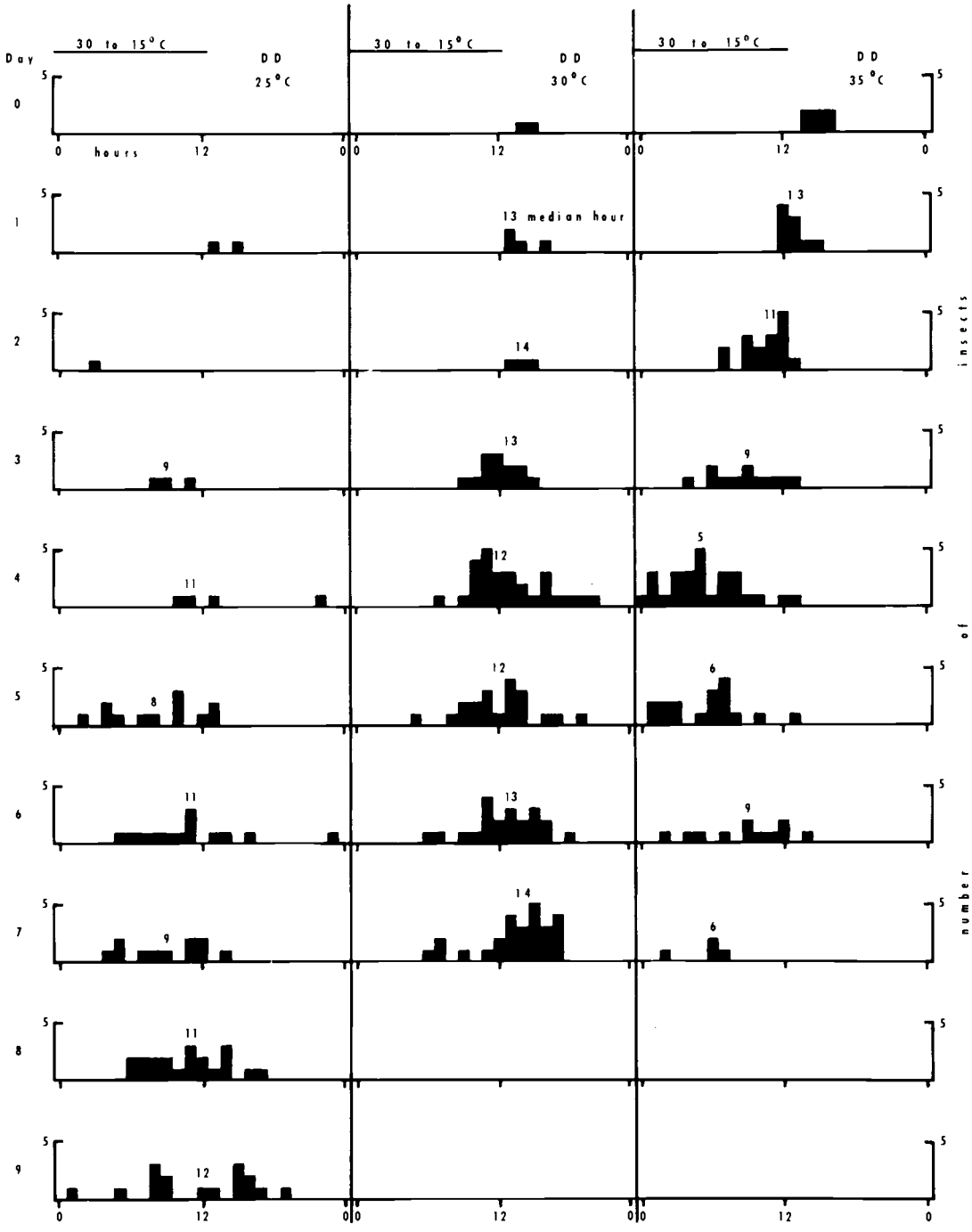


Figure 7. Effect of temperature on the emergence period length.

The formula used for the calculation of the Q_{10} was as follows:

$$Q_{10} = \frac{P_2}{P_1}^{\frac{10}{T_1 - T_2}}$$

P_1 = mean of periods at T_1

P_2 = mean of periods at T_2

Temperature Phase Response

Aschoff (1965) states that entrainment of a circadian rhythm by a Zeitgeber depends on phase control. One of the prerequisites for phase control is a periodically changing sensitivity of the organism to stimuli. In other words, the sensitivity of the organism to the Zeitgeber is a function of the phase of the circadian period. Pittendrigh (1954), working with D. pseudoobscura, was one of the first to show that single short perturbations could shift the phase of the eclosion rhythm.

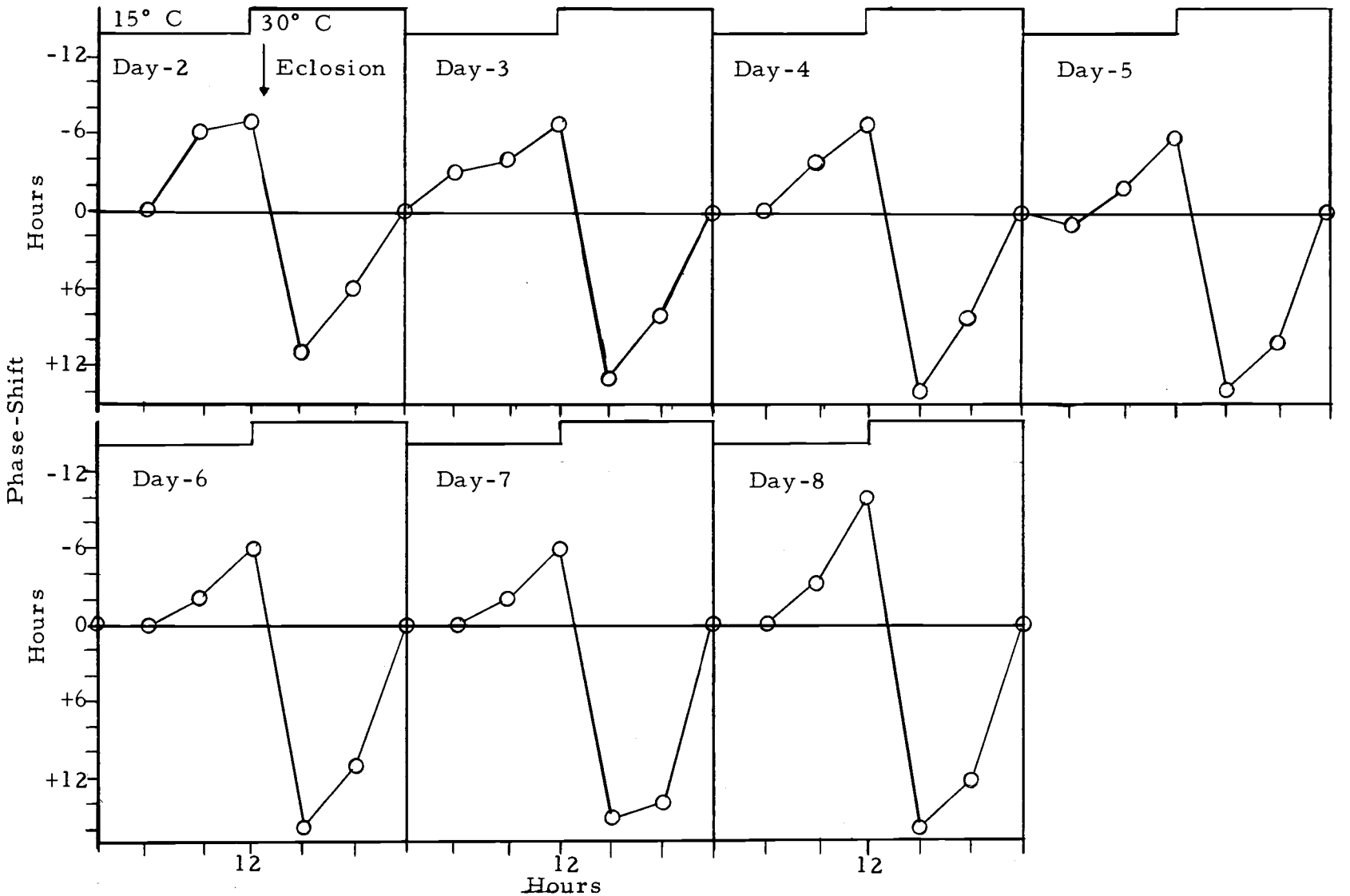
Two criteria, listed by Aschoff (1965), for detecting a phase shift in records of free-running rhythms are the time difference between last onset before and first onset after the signal, or the time difference between the slope of onsets before the signal and the steady state slope after the signal. The difference can be expressed either in time units of minutes or hours, or in angular units with one full period equal to 360 degrees.

Two types of signals can be used: The light or temperature pulse, with interruption of darkness in the case of light or a temperature increase or decrease for a certain span of time, and, the light or temperature step, with a change from constant illumination of one intensity to a higher one, or a change from one temperature to another.

A single stimulus can either delay (-) or advance (+) the phase. The method of graphing the results of the phase-response experiments used was similar to the widely accepted manner for graphing (Aschoff, 1965).

The results from the phase response experiments are graphed as phase response curves from day-2 to day-8 in Figure 8. Day-0 is the 24 h period following the first hour of the initial temperature pulse. No transients appeared following the various temperature pulses which were initiated at the six circadian hours. The graph of the phase shifts on day-2 is similar to that of day-8. It could be interpreted that the graphs from day-2 to day-8 represent the transients, and that, had the experiments not been terminated a different steady-state would have appeared. However, the plots of the phase response curve from day-2 to day-8 change very little and appear to be characteristic of a steady-state period. One would also assume that the steady-state period would be reached in a few days. DeCoursey (1964), studying the activity rhythm of a hamster,

Figure 8. Temperature phase response curve. Twelve-hour temperature pulse of 15 C°. Reference point is onset of temperature pulse.


15° C
30° C
Day-2
Eclosion
Day-3
Day-4
Day-5
Day-6
Day-7
Day-8
-12
-6
0
+6
+12
Hours
Phase-Shift
12
Hours

observed that the steady-state was reached in about three days following a phase shift.

The phase response curve described in Figure 8 is similar in shape to those of other organisms. Pittendrigh and Minis (1964), summarized the light phase response curves for six different organisms, Drosophila pseudoobscura Sturtevant (pupal eclosion), Glaucomys volans (L.) (activity), Mesocricetus auratus Waterhouse (activity), Gonyaulax polyedra Stein (luminescence), Phaseolus multiflorus Willd. (leaf movement), and Kalanchoë blossfeldiana v. Poelln (petal movement). He found that they were remarkably similar with phase advances occurring in the late subjective night and early subjective day, and phase delays occurring in the late subjective day and early subjective night. Pittendrigh's subjective day could correspond to the period of temperature drop in the temperature phase-response curve for M. rotundata. The subjective day and subjective night periods corresponded to the 12-h light and 12-h dark period that the organisms had previously been exposed to. In M. rotundata (Figure 8), the phase advances occurred in the 12-h period following the temperature pulse and the phase delays occurred in the 12-h period that had previously been exposed to the 12-h temperature drop.

A third characteristic of the temperature phase response curve of M. rotundata is that no significant change in the length of the emergence period occurred with a phase-shift. In the hamster

and finch, Pittendrigh (1960), showed that after a 12-h light signal, transients occurred followed by steady-state periods which were much shorter than the ones prior to the light pulse. Table 2 shows the resulting periods in DD following phase-shifts in the leafcutter bee.

Table 2. Emergence period lengths following temperature pulses at various circadian hours.

Time of 12-h Pulse	Period in DD (hours)
0	23.70
4	23.69
8	23.57
12	24.20
16	22.88
20	23.22

The phase-shift following the temperature pulse at CT-16 produced a 22.88-h period which appears to be shorter than the periods following the other phase-shifts. However, this difference was not significant at the five percent level using the t test for the difference between means.

The results of the phase-response experiments are plotted without reference to the cue's phase position. This probably does not lead to error as there was no noted concomitant change in period length with phase-shift. If a change in period length does occur, error can result with the magnitude of error being a function of the

magnitude of the period change and the distance between the points of measurement and the cue's phase position (Lohmann, 1966). This is, of course, assuming that the period change is effected immediately after the cue. It should be noted that the period length at CT-12 and CT-16 differs the most from the period length at CT-0, which is 23.70 (Table 2). The greatest phase shifts were also produced at these hours. This is to be expected if the phase-shifts cause period length changes (Enright, 1965). Thus, although statistically the period lengths are not different, from the point of view of circadian theory they are different.

Phase Setting Ability of Temperature Rise and Temperature Drop

Both a temperature rise of 5° C and a drop of 5° C phase set the M. rotundata emergence rhythm (Figure 9). These results differ from Moriarity (1959), who found that in Anagasta (= Ephestia) küehniella Zell. a temperature rise of 5° C (30 to 35° C) did not affect the existing emergence rhythm. It would appear from this experiment that the emergence rhythm locks onto the point of temperature rise. If this is the case, then both groups of bees experienced a similar temperature rise of 5C° but at different times and at different levels.

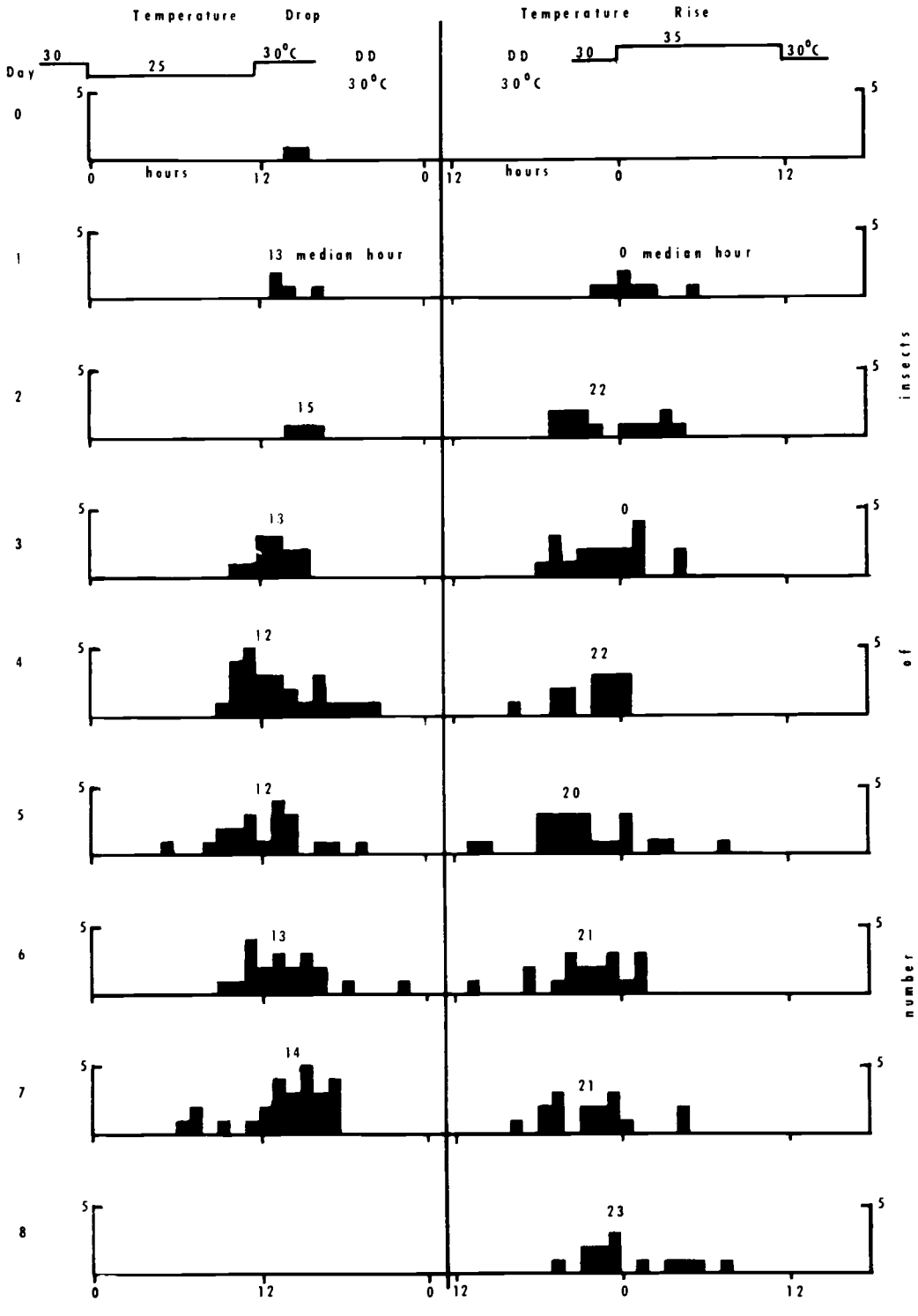


Figure 9. Phase setting ability of temperature drop and temperature rise.

CIRCADIAN RHYTHM OF OXYGEN CONSUMPTION

Methods and Materials

A Gilson Differential Respirometer was used for all the experiments on oxygen consumption. The respirometer was placed in a controlled-environment room where light conditions were controlled. Light was provided by two flourescent fixtures, each containing four lights. The intensity was varied by removing individual lights from the fixtures. Since all light intensity measurements were recorded at the surface of the water bath containing the respirometer vessels, the exact light intensity which the insects inside the vessels received was not determined. No apparatus was available to measure light intensity inside the vessels. For dark experiments, the lights were turned off and the respirometer was covered with black plastic. When transfer of insects in the dark was necessary a red light source was used.

For experiments involving adults, air was introduced into the respirometer vessels by means of evacuation of the old air while pulling in fresh air. The fresh air was held in a large erlynmeyer flask, emersed in water. The temperature of the air in the flask was maintained by a constant temperature circulator. This air was maintained at the same temperature as the air in the respirometer

vessels. The air was transferred from the reservoir to the respirometer vessels through tygon tubing.

Two sizes of respirometer vessels were used. A small 18 ml vessel was used for the prepupal and pupal stages and a larger 125 ml vessel was used for studies involving adults.

Preliminary experiments indicated that the adults lived longer if they were fed a solution of 100 ml of water +100 g of sugar + 0.025 g of nipigan, as opposed to tap water. This solution was placed in the center well of the vessel and covered with fine mesh copper screening to allow the bees access to the surface of the solution and yet prevent them from drowning.

Respirometer readings were recorded hourly. This involved a number of people, with no one person consistently reading the respirometer at the same time of day. No attempt was made to completely randomize the readings due to the problem it would present for the scheduling of personel. The respirometer readings were recorded in microliters of oxygen and then corrected to give microliters of dry gas at standard conditions of temperature and pressure using the following formula:

$$\frac{273\ (P_b - 3 - P_w)}{(t + 273)\ 760} = \text{multiplying factor}$$

t = water bath temperature in degrees C

P_b = operation pressure (barometric pressure) in mm of

mercury

P_w = pressure of water vapor.

All of the oxygen data was handled in a similar manner. It was first corrected to standard conditions of temperature and pressure as described above, and then computer-analyzed using the following power spectral analysis program: BMD 02T-autocovariance and power spectral analysis-version of August 18, 1964 Health Sciences Computing facility, UCLA. The rough data was plotted by the computer as differences from the mean. This data was studied to determine phase relationships to the cues of light and temperature that were administered to the insects. The power spectral data were plotted by the computer as frequency against percent variation. These data were analyzed to determine the number and frequency of oxygen rhythms that M. rotundata displayed. Data from experiments carried out under different conditions of light and temperature were compared to determine if these varying conditions affected the rhythms of oxygen consumption.

Stage Susceptible to Synchronization by Light

The insects were prepared as they had been in the emergence study. The procedure was described under Materials and Methods in the Emergence section. When the appropriate stage of development was reached, the insects were exposed to a light pulse (32 fc

for 12 h), then placed in the respirometer vessels and maintained at 30° C in DD. Four immature stages and the adult were studied for their response to light. The four immature stages were the prepupa (PP), white-eyed pupa (WP), dark-eyed pupa (DP), and black-bodied pupa (BP), reflecting the progression of pigmentation from early pupa to adult. Four experiments were accomplished using one experiment per stage. Three test and three control bees were used in each experiment. In all the experiments carried out in the respirometer, one vessel was kept empty to act as a respirometer control. The insects were sexed at the end of the experiments.

The data were computer analyzed and the power spectra plots of each stage were compared in an effort to determine if different stages of M. rotundata possessed different frequency spectra. The rough data plots were analyzed to determine if the light phase set the oxygen consumption rhythms at the various developmental stages.

Stage Susceptible to Synchronization by Temperature

The procedure for this study was identical to that of the previous one, except that the insects were exposed to a negative temperature pulse of 15C° at the appropriate stages. They were maintained in DD at 30° C in the respirometer. As before, four experiments were used to determine the effect of temperature on the various developmental stages. The resulting data were handled in the same

manner as that from the light experiments.

Effect of Light on the Rhythm

It was planned to use two different light intensities plus the data from a DD experiment to study the effect of light intensity on the oxygen consumption rhythm in Megachile rotundata. However, since an intensity of 120 fc did not appear to affect the period it did not seem profitable to study a higher intensity of light. Five insects (three males and two females) were exposed to a 15C° temperature pulse to synchronize their oxygen consumption rhythms. They were then placed in the respirometer and exposed to 120 fc of light for a period of four days.

The experiment in DD involved five adults (three females and two males) under the same conditions as above, except that the bees were never exposed to light. The results were analyzed as in the previous experiments.

Effect of Temperature on the Rhythm

Two different temperatures (25 and 35° C), as well as the data from DD experiments at 30° C were used to study the effect of temperature on the oxygen consumption rhythm. The insects were maintained at the appropriate temperature in DD. As in the previous experiments, the insects were synchronized using a temperature

pulse of 15° C.

Two new experiments were accomplished, one at 25° C and the other at 35° C. In the experiment at 25° C two females and two males were used. In the 35° C experiment, two females and two males were used but the females did not complete the experiment. As in the previous experiments the data were computer processed.

Temperature Phase Response

M. rotundata were reared at 30° C, then temperature pulsed to synchronize the test animals when they reached the adult stage. They were then placed in the respirometer at 30° C. Individuals were removed from the respirometer at the following intervals in an effort to scan the oxygen consumption period: CT-0, 4, 8, 12, 16, and 20. These test animals were removed, exposed to the 15C° shock for 12-h, and then returned to the respirometer.

Two experiments were carried out, each including the following arrangement. Of the six stations available in the respirometer, one was used for a control, one for an animal control, and four for the test animals. In the first experiment CT-0, 8, and 16 were tested. The insect used for CT-16 died. In the second experiment CT-4, 12, and 20 were successfully completed. One insect was used for each circadian hour. The insects were all females except for the bee used for CT-12. The results were computer analyzed and studied to

determine the amount of phase shift at each hour tested and to see if there was a concomitant period change with the phase shift.

Entrainment of the Oxygen Consumption Rhythm

Five experiments were accomplished in an effort to determine the effect of light entrainment on oxygen consumption in the leafcutter bee. The LD cycle for all entrainment experiments was the same--LD 12:12 (260:0 fc). Two experiments were completed which started with 24 prepupae. Although 24 insects were started not all of them completed the experiment, due to death and human error in handling the bees or in operating the respirometer. For adults, a total of six experiments were performed using five adults in each. Because of procedural problems the results of only three experiments are reported. In all the experiments, the bees were held at 30° C in an incubator until the appropriate stage was reached. They were then introduced into the respirometer and subjected to the LD 12:12 regime at 30° C. The results are reported in the form of plots of rough data to show the effect of light entrainment on phase, and as plots of frequency against variation to expose the effect of entrainment on the oscillatory spectrum of the oxygen consumption rhythm.

One experiment involving 12 pupae was performed using temperature as the entraining agent. Since a decrease in temperature is followed by a decrease in metabolic rate it was decided to study the

results following entrainment to determine if there was an after-effect. These results could then be compared to those of the light entrainment experiments, and also to the results of experiments in which the bees were subjected to only a single temperature pulse.

Results and Discussion

Many insects and sometimes whole experiments were lost due to missed readings as a result of alarm clock failure, death of the insects, misuse of the respirometer causing a blow through of the manometer fluid, or, temperature fluctuations in the respirometer due to electrical problems such as fuse failures. In many cases only a few insects are reported in the results for a given experiment rather than a larger more adequate sample. This was due to the variation in rates of development between individuals of M. rotundata and of the apparent high mortality rate of the young adults, as well as the procedural problems mentioned above. Thus one of the lessons learned in this study was that to insure an adequate sample of ten bees of the same age at the desired time, an original sample of at least 50 bees should be reared.

Stage Susceptible to Synchronization by Light

The raw data plots (Figures 10 and 11) were scanned to determine if there was any evidence of light synchronization from the

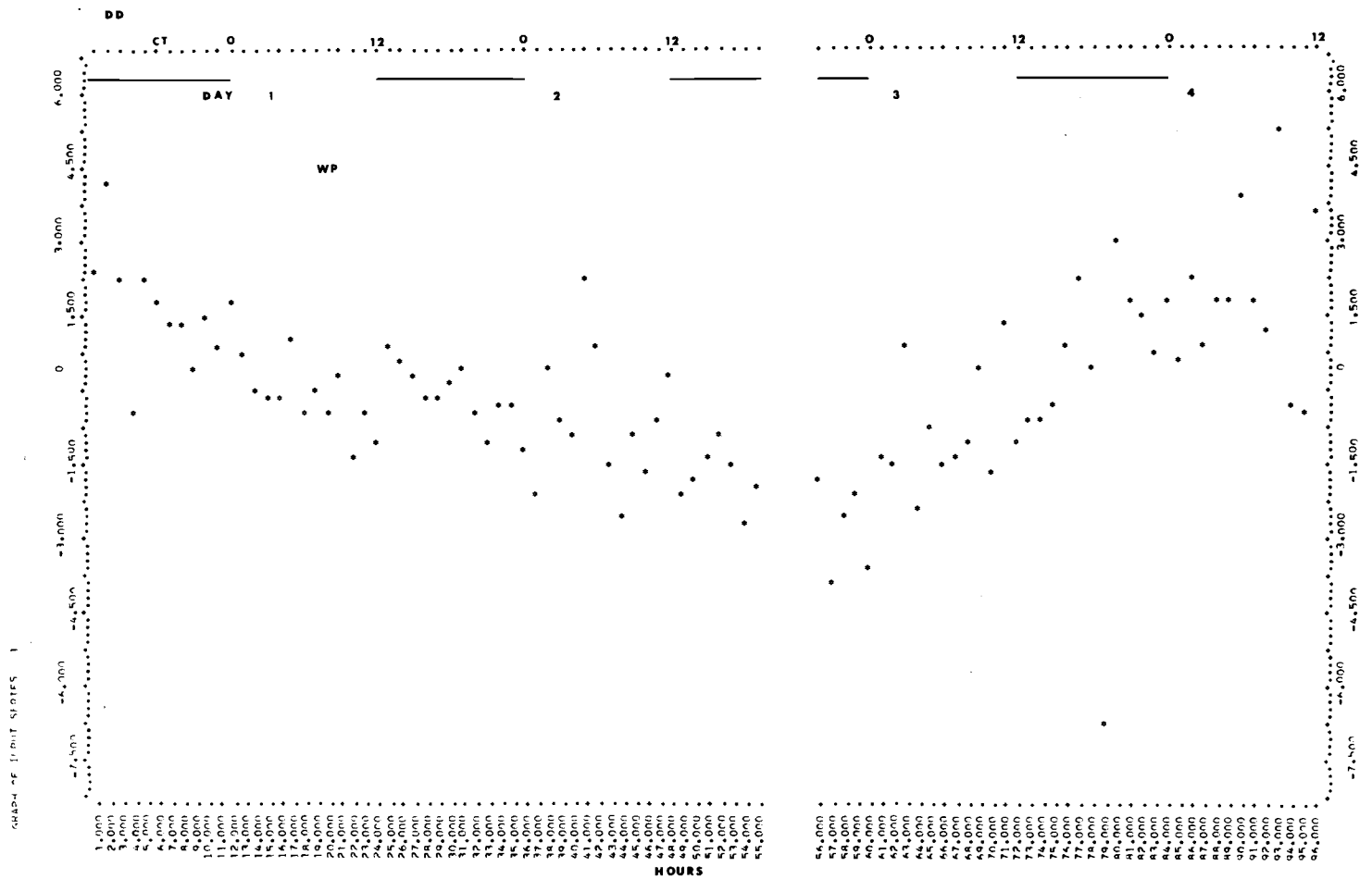


Figure 10. Plot of oxygen consumption as a difference from the mean (mean = 0) for a typical white-eyed pupa after exposure to a single light pulse. Light regime at top of figure represents the light regime applied on Day-0 only.

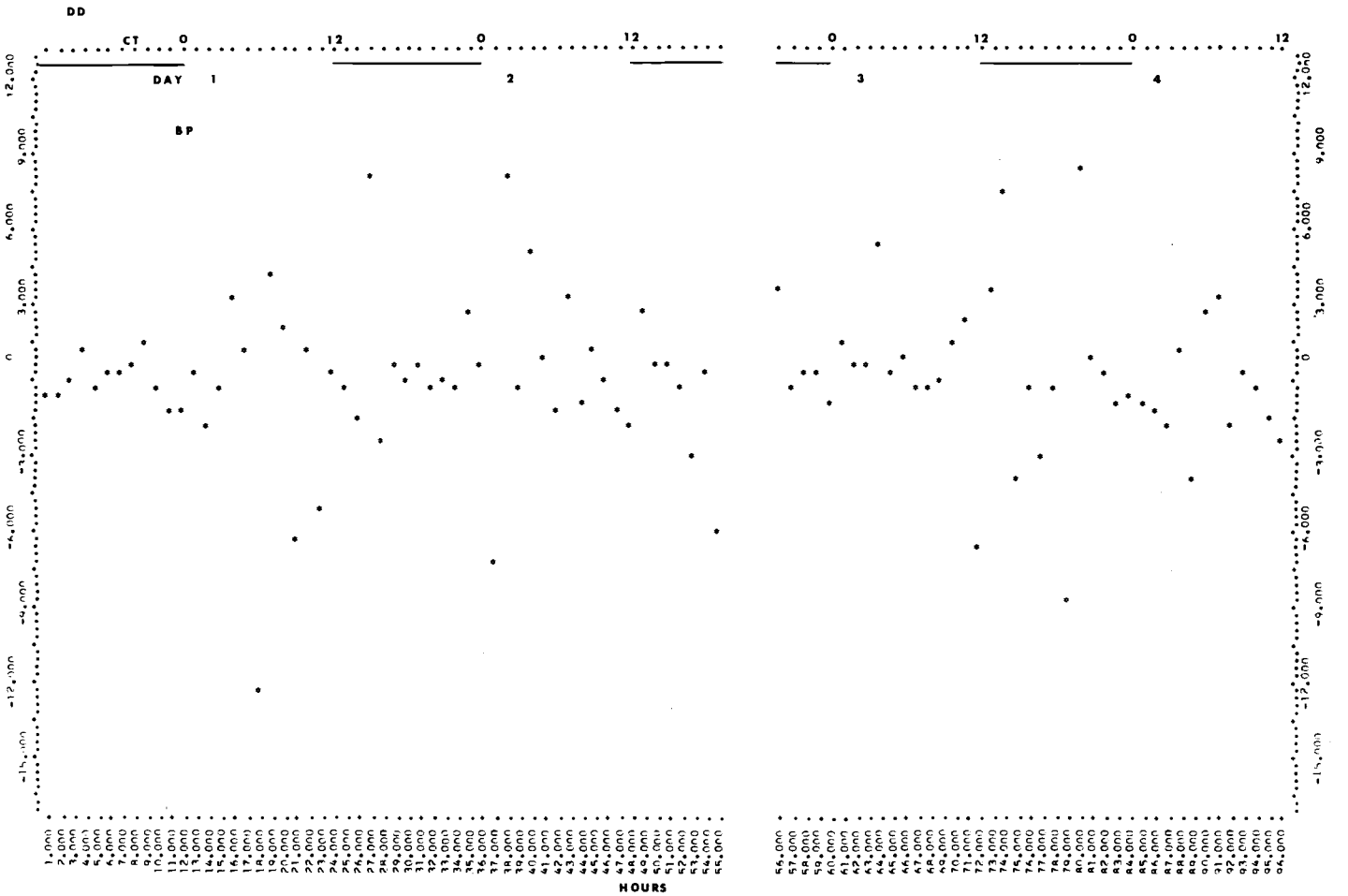


Figure 11. Plot of oxygen consumption as a difference from the mean (mean = 0) for a typical black-bodied pupa after exposure to a single light pulse. Light regime at top of figure represents the light regime applied on Day-0 only.

prepupal to the black-bodied pupal stages of M. rotundata. Because of the apparent randomness of the data to the human eye no definite conclusion can be made as to synchronization. From the results of the studies of the pre-adult emergence and activity rhythms it is most probable that the bees were not phase-set by light. The influence of light on the adult will be discussed in the section on entrainment. The number of insects used for analysis from each test is reported in the graph or table used to illustrate the results.

Spectral analysis of the data from the four stages reveals two or three ultradian frequencies in the prepupal and pupal stages. Figures 12 and 13 show the pooled spectral data from the prepupal stage. An ultradian bend occurs at three frequencies producing periods of 2.28 h, 3.69 h, and 6.85 h in the animals that were subjected to a light pulse (Figure 12). Figure 13 records the spectrum from the single control insect which showed distinct bands at 2.09 h, 2.82 h, and 4.0 h, as well as a circadian component of 23.81 h. It might be concluded that light suppressed the expression of the circadian frequency in the test animals but this conclusion is not supported by subsequent data.

The white-eyed pupal results are shown in Figures 14 and 15. In the test animals ultradian frequencies produced periods of 3.00 h, and 5.99 h. The results from the dark-eyed pupae are shown in Figure 16A. A strong circadian component appears as well as three

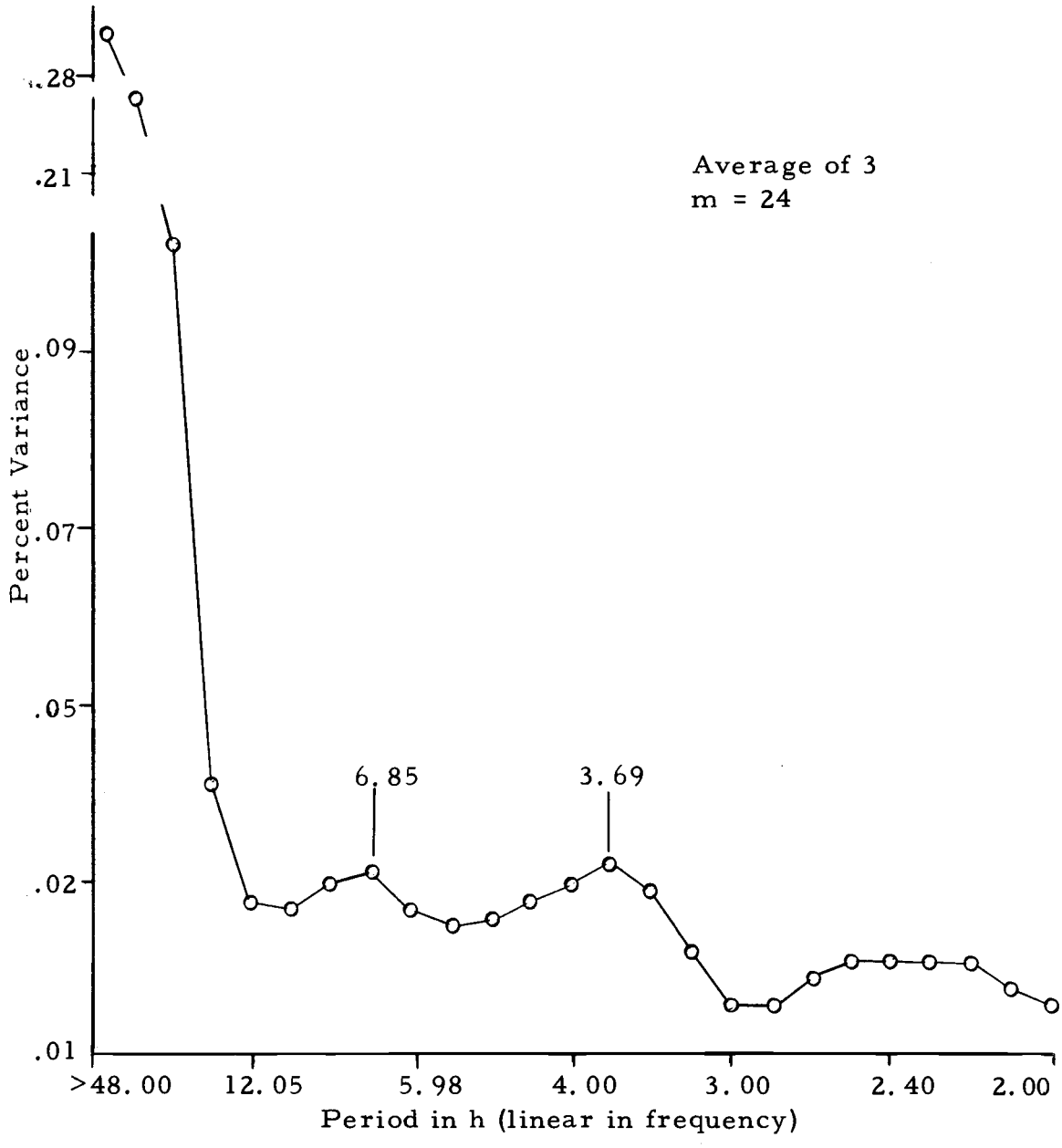


Figure 12. Average variance spectrum for the effect of a single light pulse on M. rotundata prepupae.

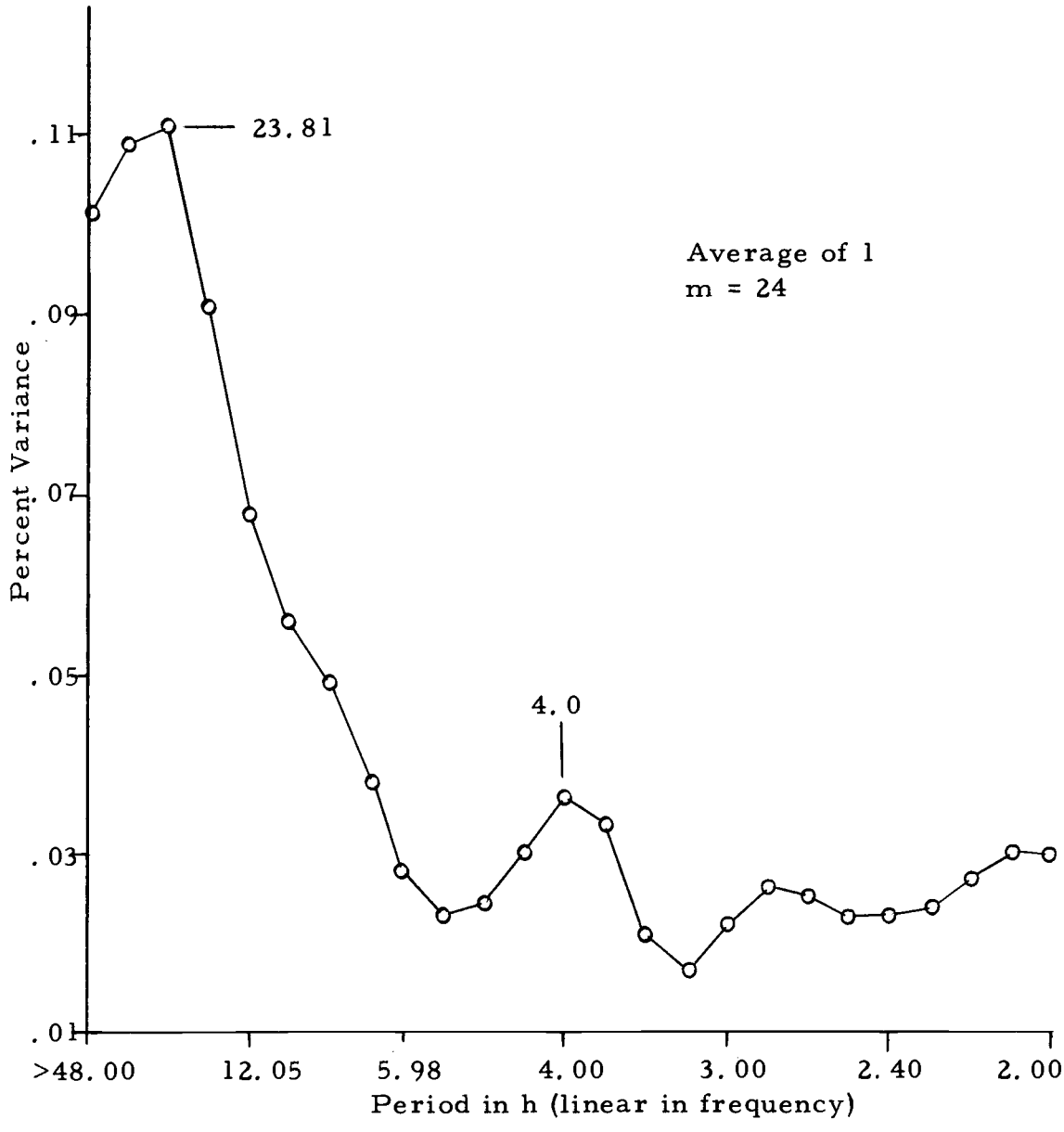


Figure 13. Control for the effect of a light pulse on the prepupal stage.

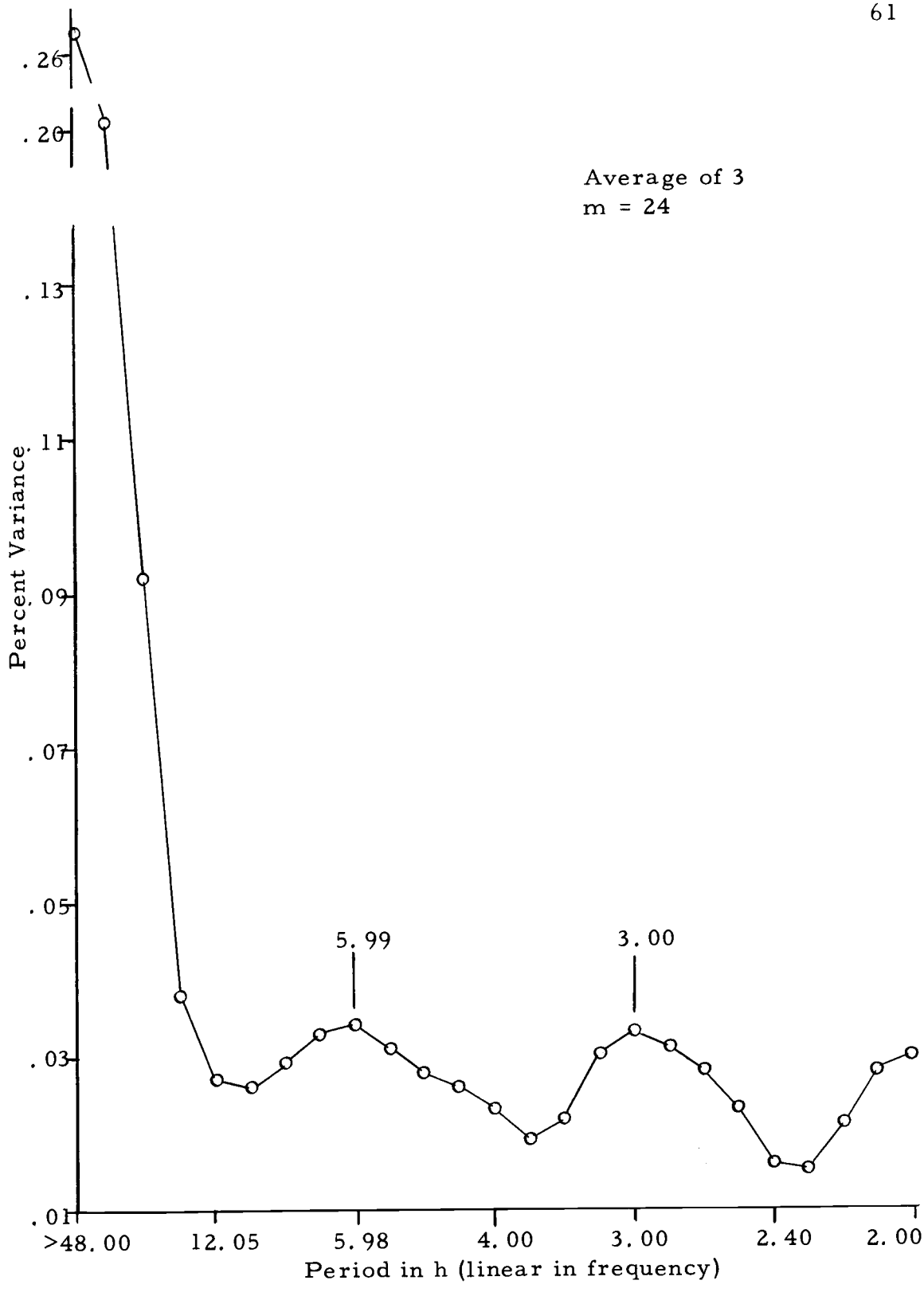


Figure 14. Average variance spectrum for the effect of a single light pulse on the white-eyed pupa.

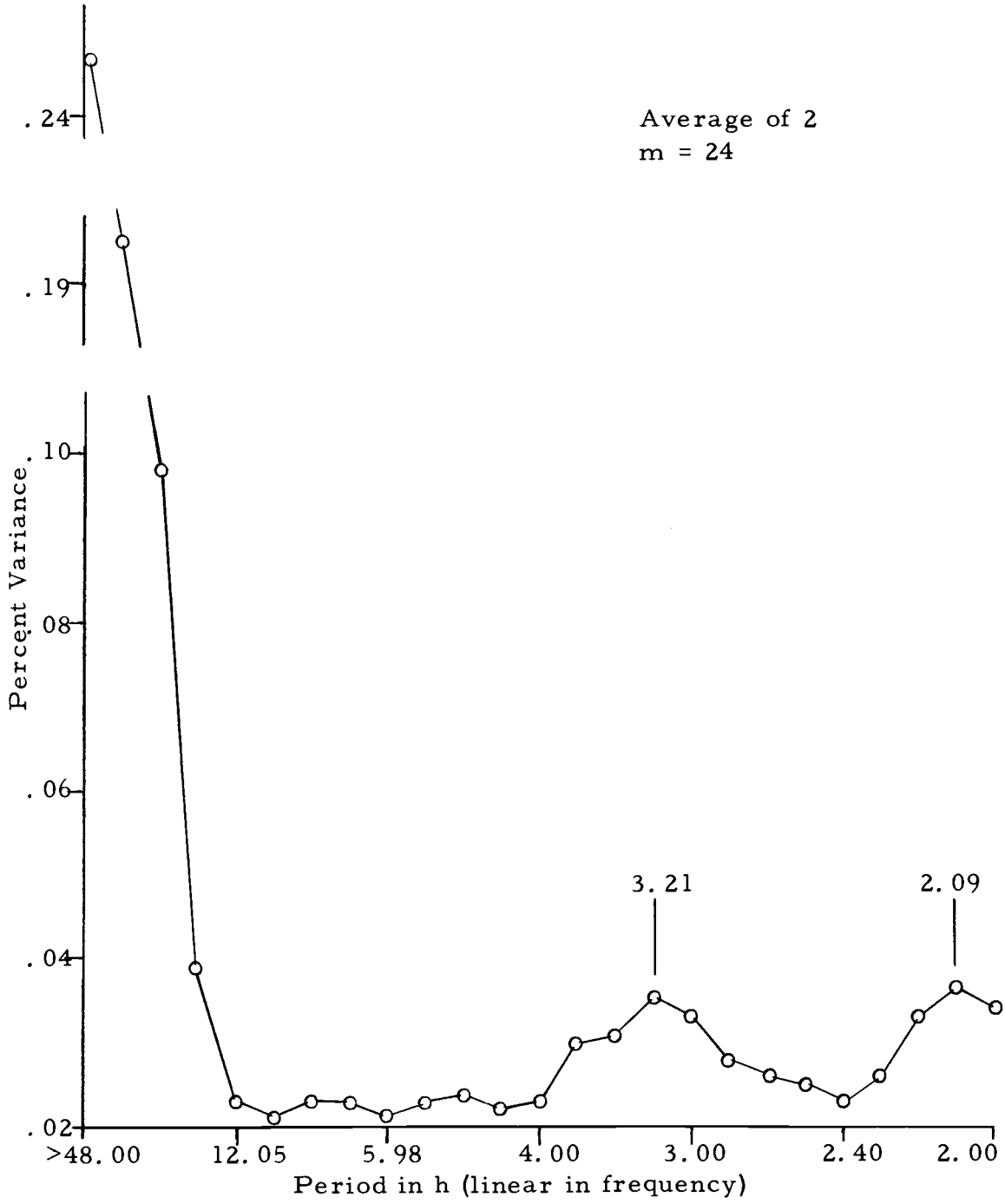


Figure 15. Average variance spectrum of the control for the effect of a single light pulse on the white-eyed pupa.

ultradian components with periods of 2. 18 h, 2. 82 h, and 5. 99 h. The strong circadian component appearing in the test animals which were exposed to 12 hours of light legislates against the tentative conclusion above, that light may have suppressed the appearance of a circadian period. The dark-eyed control, illustrated in Figure 16B, displayed a similar spectrum to that of the test insects except that the circadian component was not present.

The results from the black-bodied pupa are shown in Figure 17A. Here, four distinct ultradian bands appear with very little low frequency trend occurring. Considerable low frequency trend was present in the previous spectral plots. The control for the black-bodied pupa experiment is shown in Figure 17B. The controls show more low frequency trends which is quite common in non-stationary data, in contrast to the test animals which show little low frequency trend.

It is difficult to make a general conclusion regarding the effect of a 12-h light pulse on the oxygen spectra of the pre-adult stages of the leafcutter bee. It would appear from the data, especially from the variation in appearance of the ultradian and circadian frequencies, that there is much spectral variation between individuals.

Stage Susceptible to Synchronization by Temperature

Due to the lack of a definite circadian component,

Figure 16. A. Average variance spectrum for the effect of a single light pulse on the dark-eyed pupa.
B. Control.

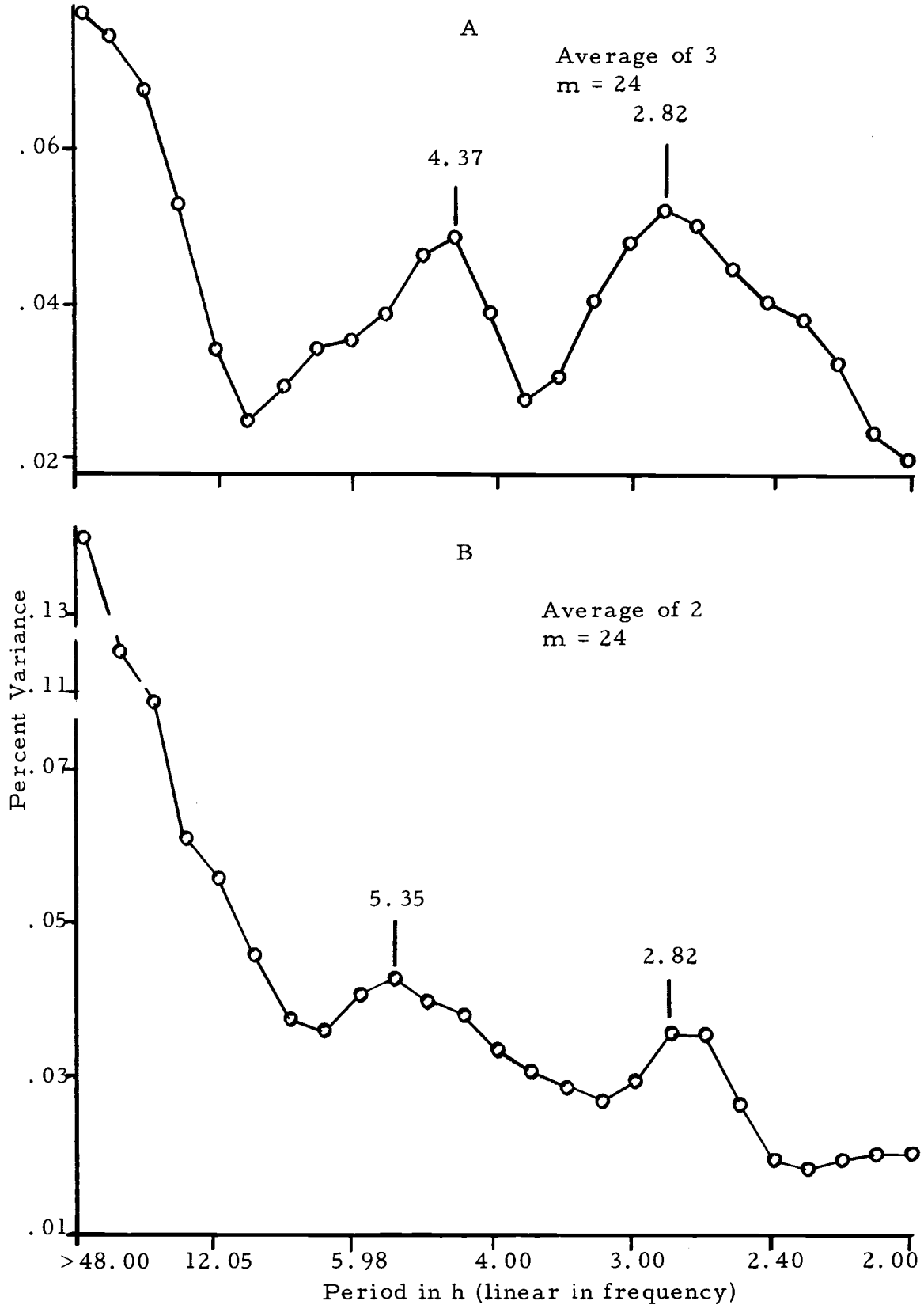
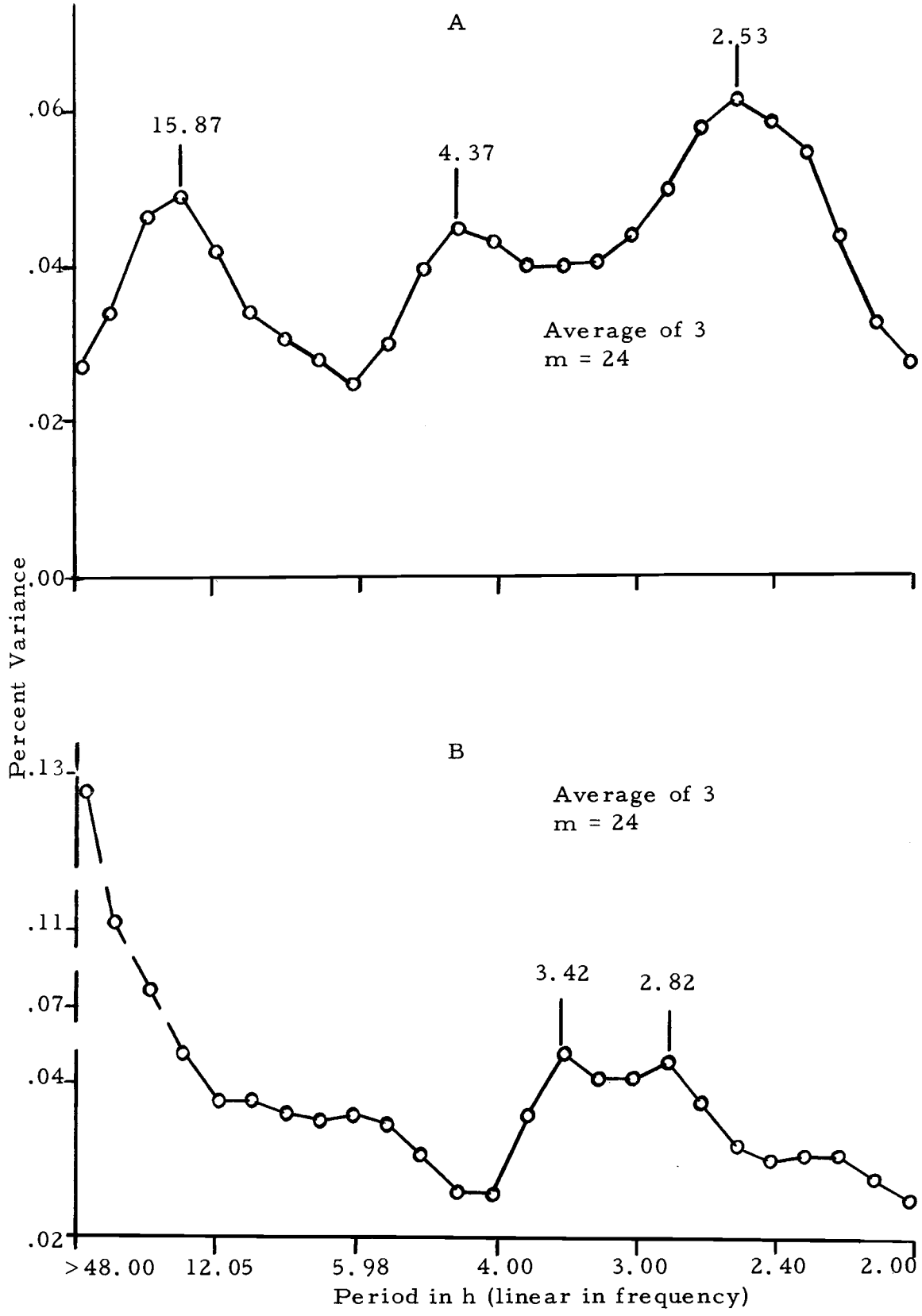

A
Average of 3
m = 24
2.82
4.37
.06
.04
.02
B
Average of 2
m = 24
Percent Variance
.13
.11
.07
5.35
2.82
.05
.03
.01
>48.00
12.05
5.98
4.00
3.00
2.40
2.00
Period in h (linear in frequency)

Figure 17. A. Average variance spectrum for the effect of a single light pulse on the black-bodied pupa.
B. Control.


A
2.53
15.87
4.37
.06
.04
.02
.00
Average of 3
m = 24
Percent Variance
B
.13
.11
.07
.04
.02
Average of 3
m = 24
3.42
2.82
>48.00
12.05
5.98
4.00
3.00
2.40
2.00
Period in h (linear in frequency)

synchronization was difficult to detect in the raw data (Figure 18). As in the light response study, spectral analysis of the data from the four pre-adult stages reveals two or three ultradian rhythms. The prepupal spectrum is shown in Figure 19. The control (Figure 20) differs very little from the test animals. Both groups show weak ultradian rhythms with most of the variation due to a low frequency trend. The test animals (Figure 19) showed ultradian periods of 3.0 h and 5.58 h. The controls showed a period of 3.00 h.

In the white-eyed stage a distinct 3.00 h component appeared (Figure 21A). In the control (Figure 21B) a definite 2.67 h component occurred as well as a 23.81 h period which appeared as a wobbling frequency.

In the dark-eyed pupal stage distinct ultradian rhythms again appeared with periods of 2.82 h, and 4.37 h (Figure 22). The control insects (Figure 22B) showed very little difference from the test animals (Figure 22A) except that the controls revealed a 15.07 h component. Again it would appear that a 12-h temperature pulse had no effect on the oxygen consumption rhythm spectrum.

The spectra from the black-eyed pupal experiment (Figure 23) were similar to the spectra of the previous stages studied. The temperature pulsed bees (Figure 23A) displayed distinct ultradian rhythms with periods of 2.08 h, 2.82 h, 5.98 h, and 15 h. The controls (Figure 23B) showed a spectrum similar to that of an adult with a

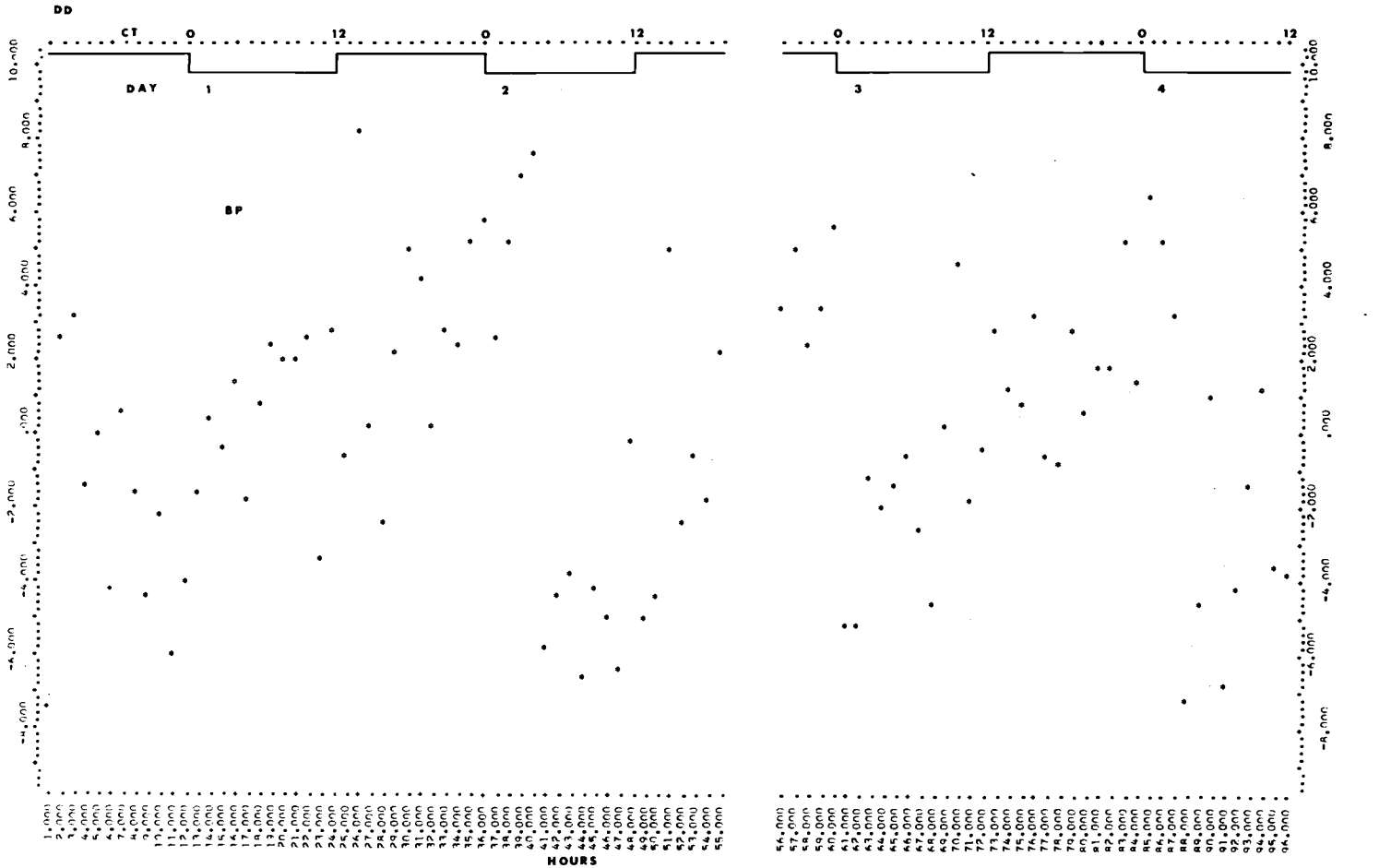


Figure 18. Plot of oxygen consumption as a difference from the mean (mean = 0) for a typical black-bodied pupa after exposure to a single temperature pulse. Temperature regime at top of figure represents the temperature regime applied on Day-0 only.

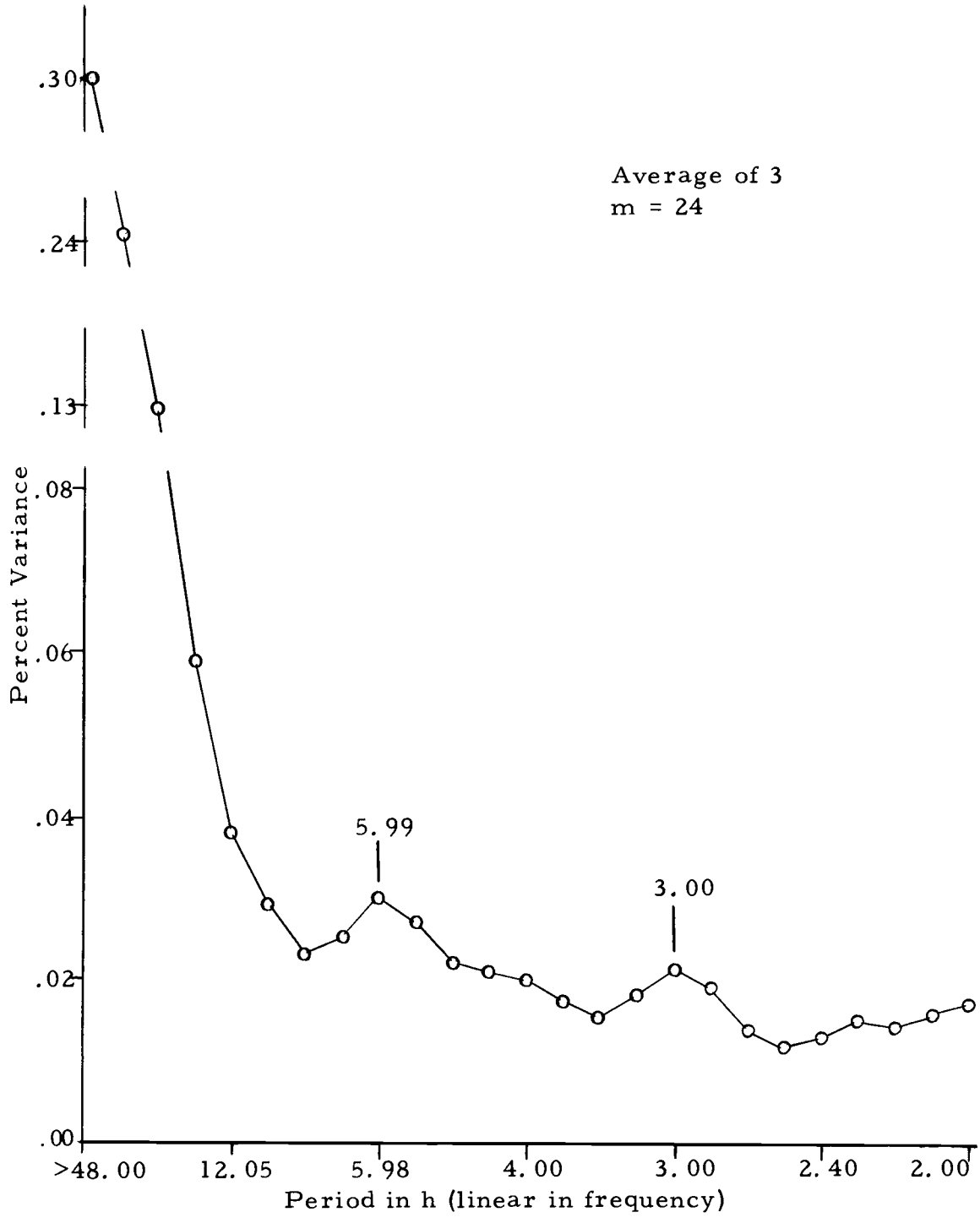


Figure 19. Average variance spectrum for the effect of a single temperature pulse on M. rotundata prepupae.

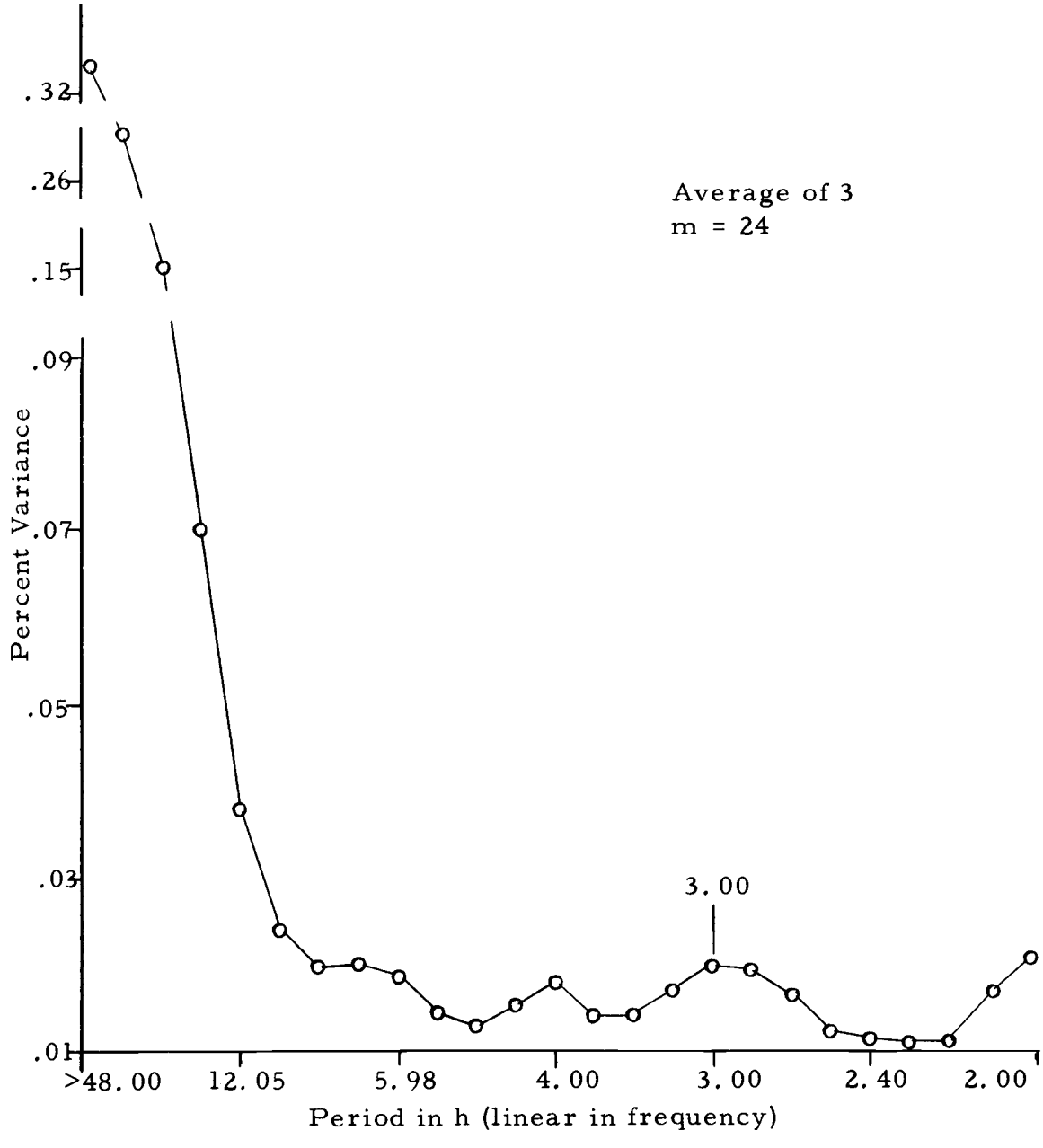


Figure 20. Average variance spectrum of the control for the effect of a single temperature pulse on M. rotundata prepupae.

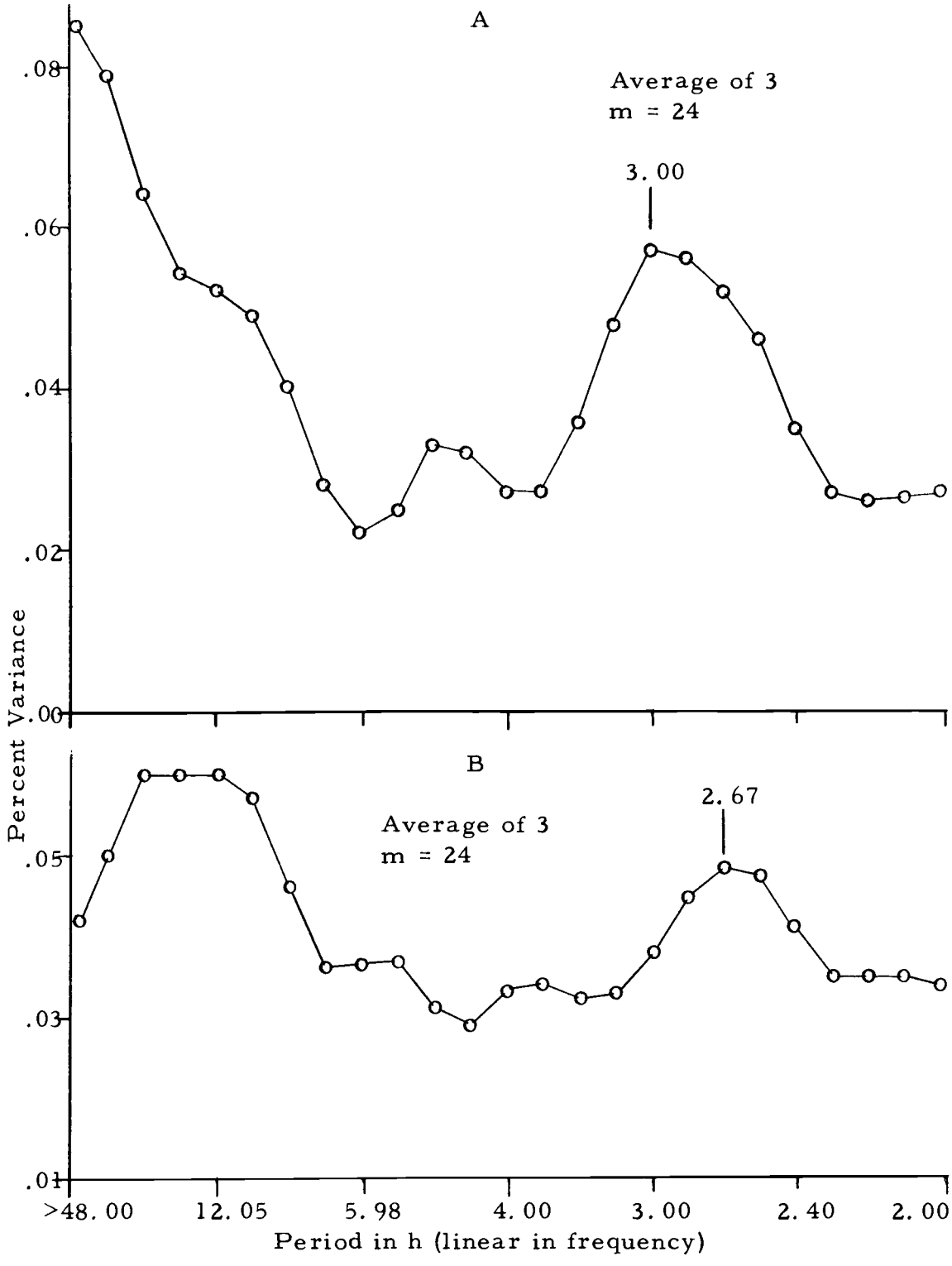


Figure 21. A. Average variance spectrum for the effect of a single temperature pulse on white-eyed pupae.
B. Control.

Figure 22. A. Average variance spectrum for the effect of a single temperature pulse on dark-eyed pupae.
B. Control.

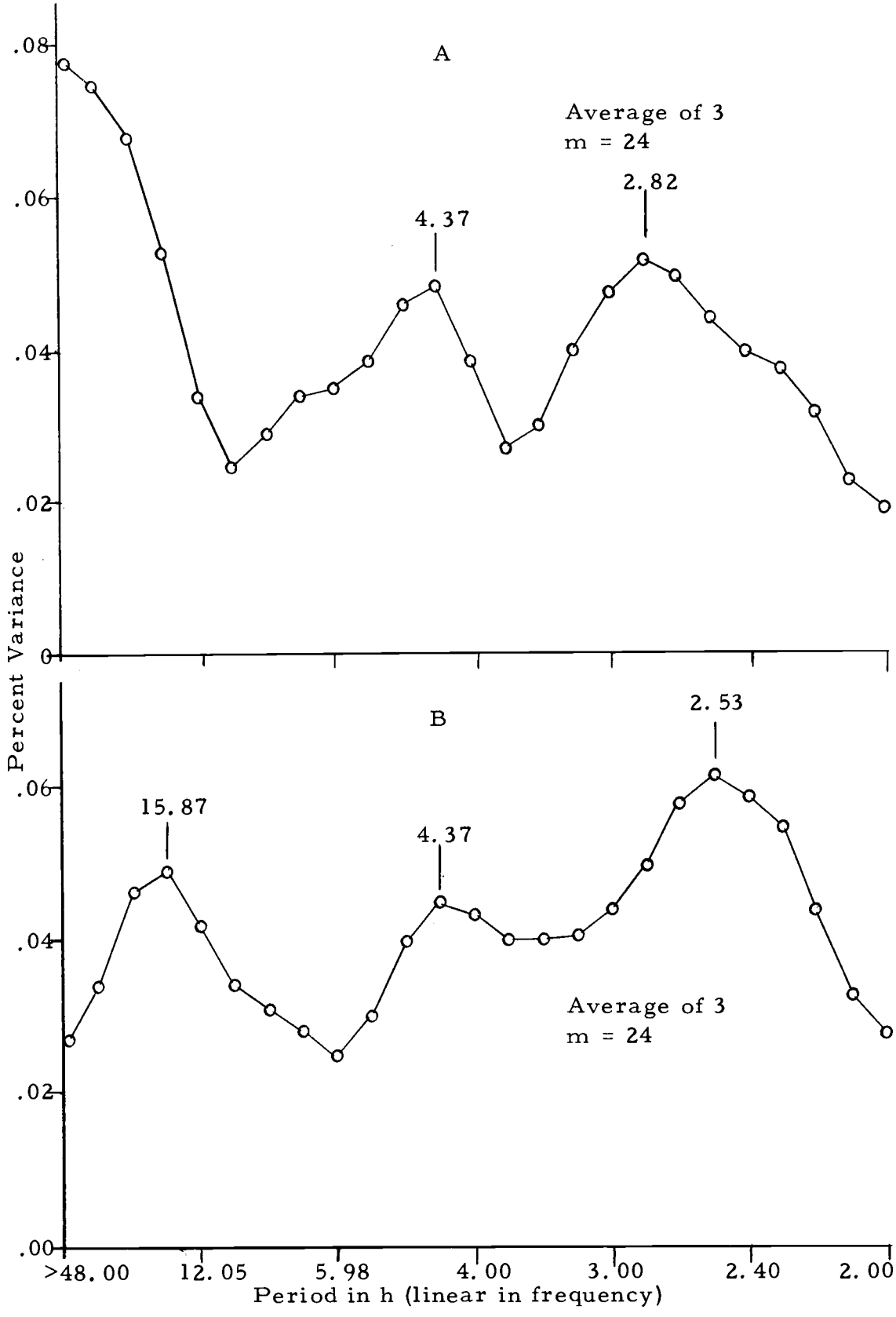
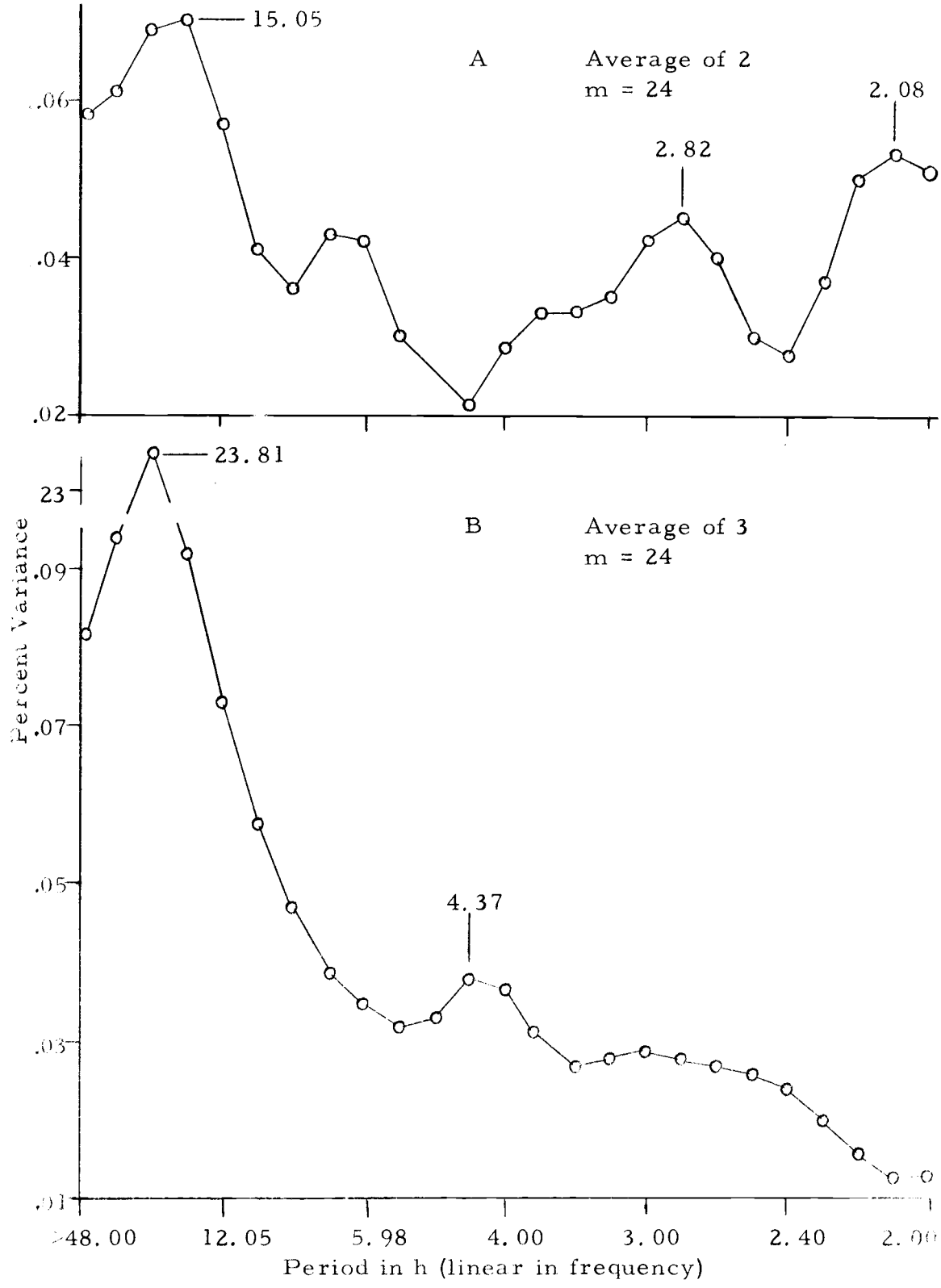

A
Average of 3
m = 24
2.82
4.37
.08
.06
.04
.02
0
B
2.53
15.87
4.37
Average of 3
m = 24
.06
.04
.02
.00
Percent Variance
>48.00
12.05
5.98
4.00
3.00
2.40
2.00
Period in h (linear in frequency)

Figure 23. A. Average variance spectrum of the effect of a single temperature pulse on black-bodied pupae.
B. Control.


15.05
A
Average of 2
m = 24
2.08
2.82
.06
.04
.02
23.81
23
B
Average of 3
m = 24
Percent Variance
.09
.07
4.37
.05
.03
.01
48.00
12.05
5.98
4.00
3.00
2.40
2.00
Period in h (linear in frequency)

distinct circadian component.

Synchronization to a 12-h temperature pulse of 15C° was attained in the adults. Table 3 records the results from three test bees and one control bee.

Table 3. Effect of temperature synchronization on the oxygen consumption rhythm in adult Megachile rotundata.

Number	Sex	Number of Days Following Temperature Pulse 1	2	3	4	
Test						
1	Female	15 h*(22)**	13 (24)	13	--	
2	Male	15 (23)	14 (24)	14	--	Average Period
3	Male	12 (23)	11 (23)	10	--	22.67 h
Control						
4	Male	8 (21)	5 (23)	4 (21)	1	

*Median hour of increased oxygen consumption (explanation in text).
**Emergence period in hours.

The median hour of increased oxygen consumption was determined by subjectively selecting the hours in which a distinct rise in oxygen consumption occurred and then calculating the median hour of this group. This median hour was used as the reference point for determining synchronization (Figure 24). Using the median hour as the reference point the test animals appear to be closely synchronized, especially the first two, showing similar median hours of day-1 through day-4. The control appears to be out of phase with the test

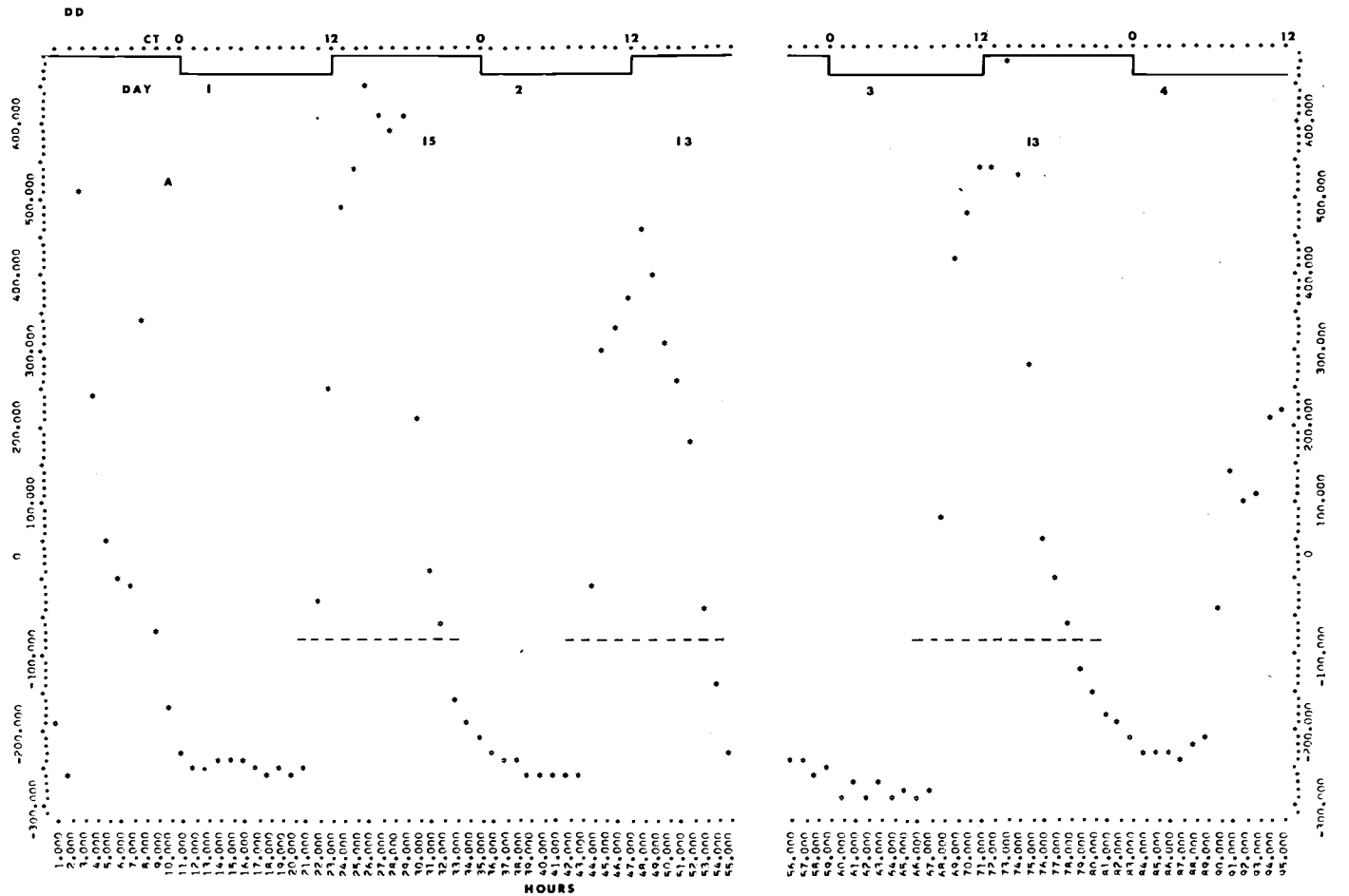


Figure 24. Plot of oxygen consumption as a difference from the mean (mean = 0) for a typical adult after exposure to a single temperature pulse. Temperature regime at top of figure represents the temperature regime applied on Day-0 only.

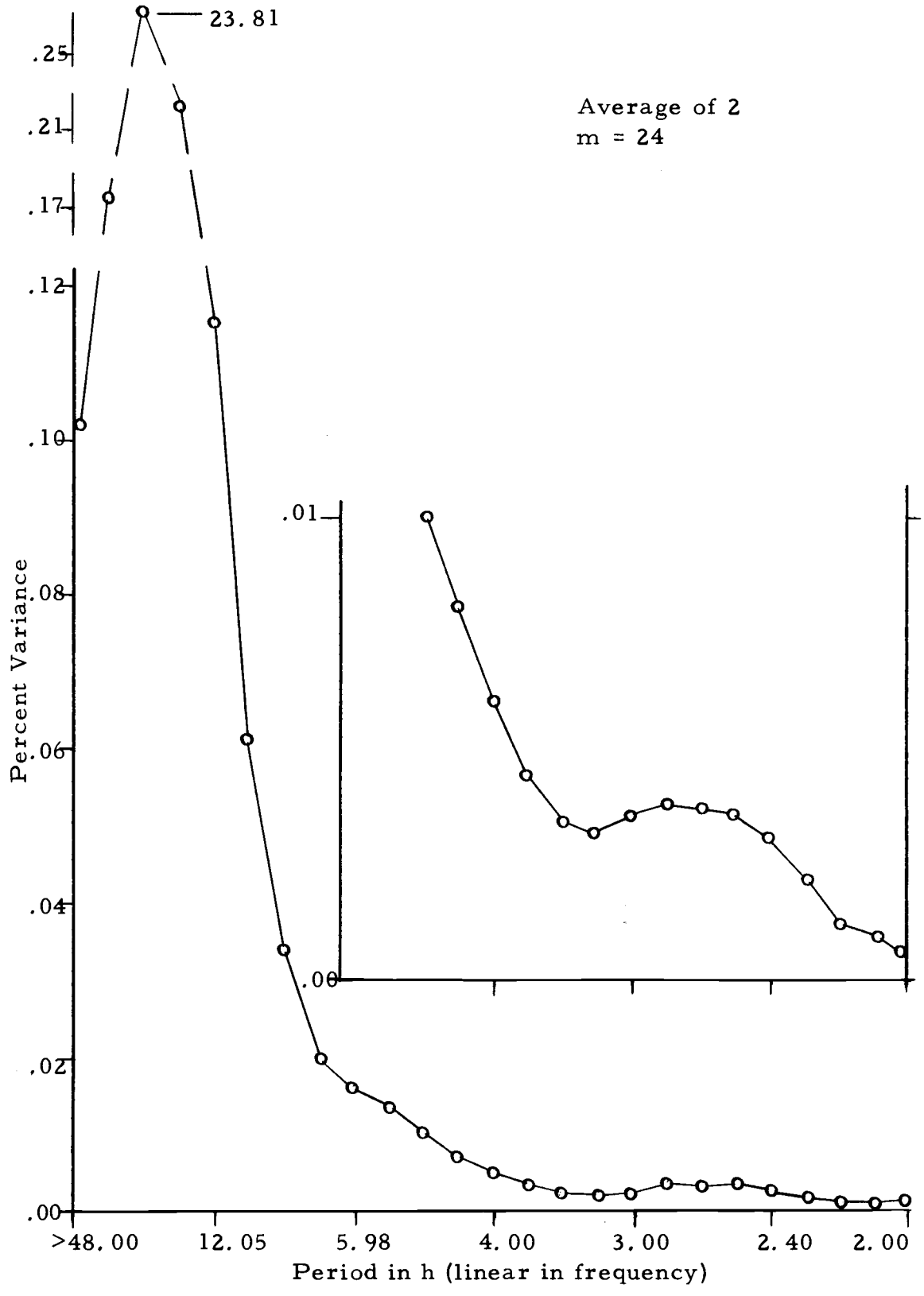
animals showing a median hour on day-1 at 8 h instead of at approximately 15 h. The average period calculated from the above table using the median hour of increased consumption was 22.67 which is very close to the 23.81 circadian component in the spectral plots (Figures 25 and 26). It would appear that the circadian oxygen consumption rhythm locks on to the point of temperature rise. On day-1 the point of temperature rise occurs at 12 h. The median hour of increased consumption occurs at hour 15, only three hours after the point of temperature rise.

Figure 25 and 26 record the spectral results from the adult study. The circadian component is very distinct in both the test animals (Figure 25) and in the control animals (Figure 26). A log plot of the higher frequency end of the spectrum is displayed, to illustrate the presence of the ultradian components. They are still there, but the circadian frequency is so dominant that a log plot is required to reveal the presence of the high frequency rhythms.

Effect of Light on the Rhythm

The results of the experiment conducted in LL are shown in Table 4. When these results are compared to those of an experiment conducted in DD (Table 3) no noticeable shortening of the period is detected in LL.

Figure 25. Average variance spectrum of the effect of a single temperature pulse on adult M. rotundata. Inset--log plot of high frequency variation.


23.81
Average of 2
m = 24
.25
.21
.17
.12
.10
.08
.06
.04
.02
.00
.01
.00
Percent Variance
>48.00
12.05
5.98
4.00
3.00
2.40
2.00
Period in h (linear in frequency)

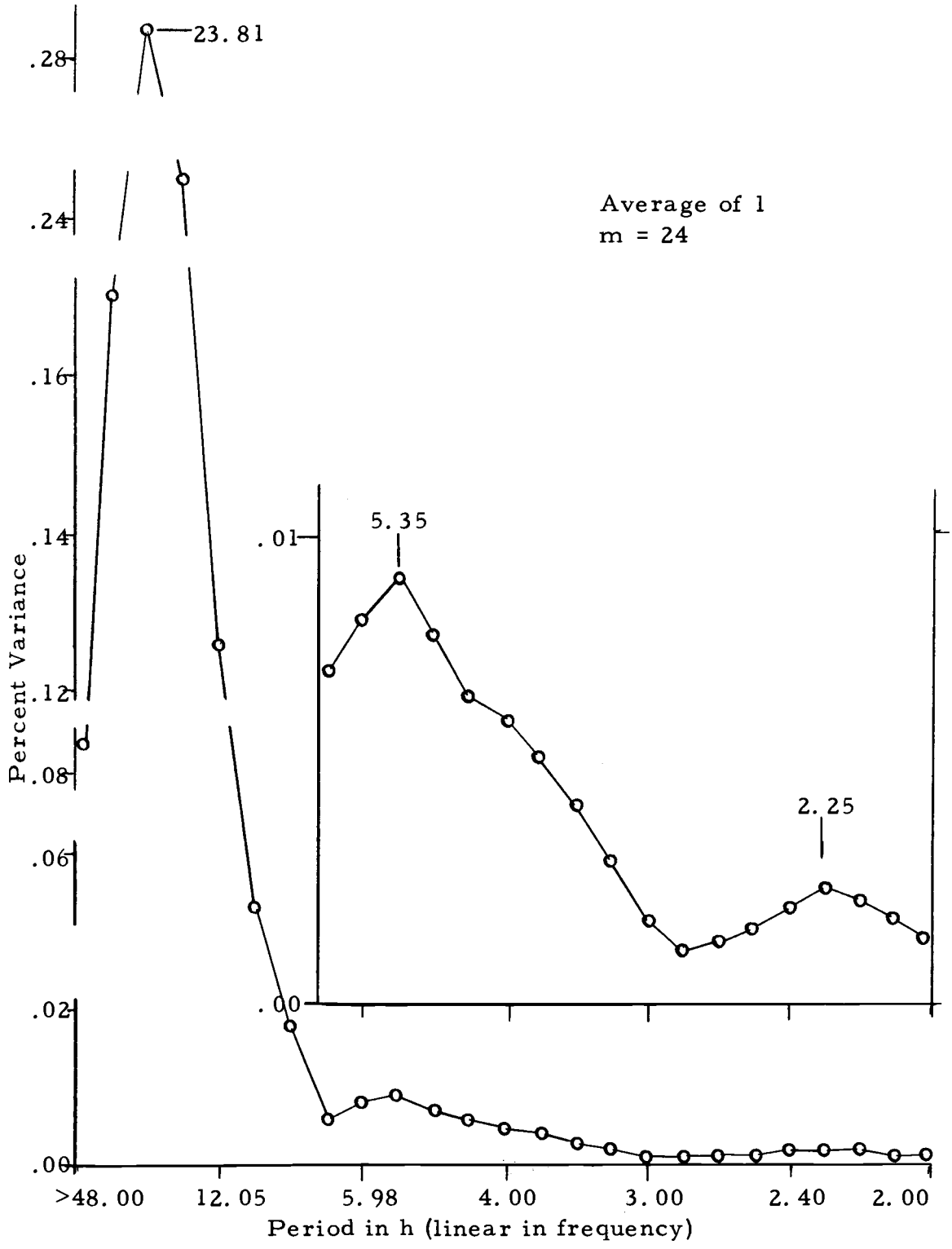


Figure 26. Control spectrum for the effect of a single temperature pulse on adult M. rotundata. Inset--log plot of high frequency variation.

Table 4. Effect of LL on the length of the oxygen consumption period in adult Megachile rotundata. Temperature pulsed to synchronize the insects.

Number	Sex	Number of Days Following Temperature Pulse 1	2	3	4	
Test						
1	Male	13 h*(22)**	11 (26)	13 (20)	9	
2	Male	15 (19)	10 (25)	11 (22)	9	Average Period
3	Male	15 (24)	15 (25)	16 (19)	11	22.50 h
Control						
4	Female	14 (23)	13	--	11	

*Median hour of increased oxygen consumption.
**Emergence period in hours.

Continuous light did not appear to affect the oxygen consumption spectrum. The spectrum from the insects exposed to continuous light (Figure 27) is very similar to that of the insects maintained in constant dark (Figure 28). The log plots are inserted to verify that the ultradian rhythms are still present.

Effect of Temperature on the Rhythm

The frequency plots of the results from experiments at the three different temperatures--30° C, 25° C, and 35° C (Figures 28, 29, 30) reveal little difference in the effect of temperature on the frequency spectrum. The bees, held at 25° C (Figure 29), showed a prominent peak at 3.12 h.

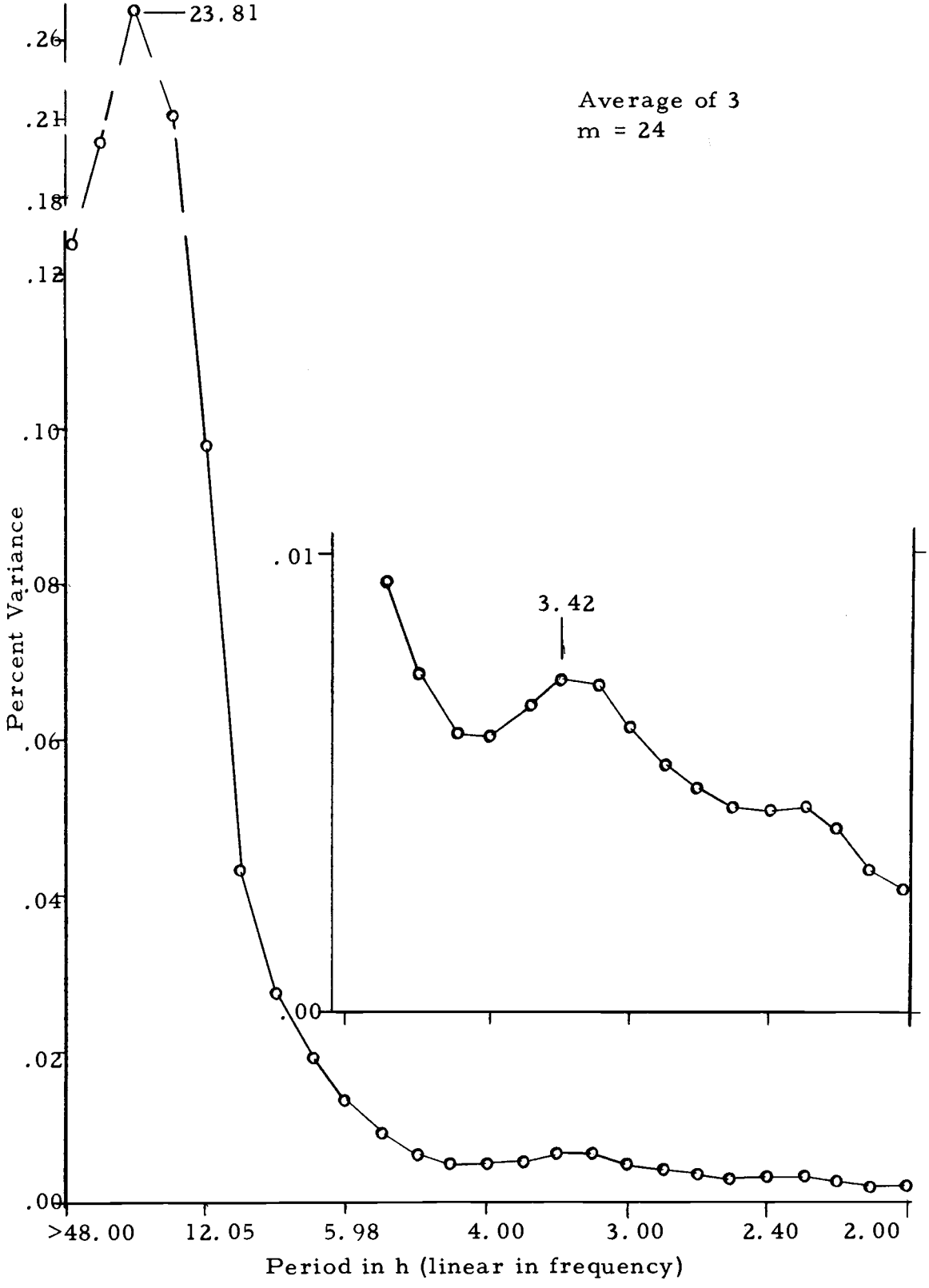


Figure 27. Average variance spectrum of the effect of LL on adult M. rotundata. Inset--log plot of high frequency variation.

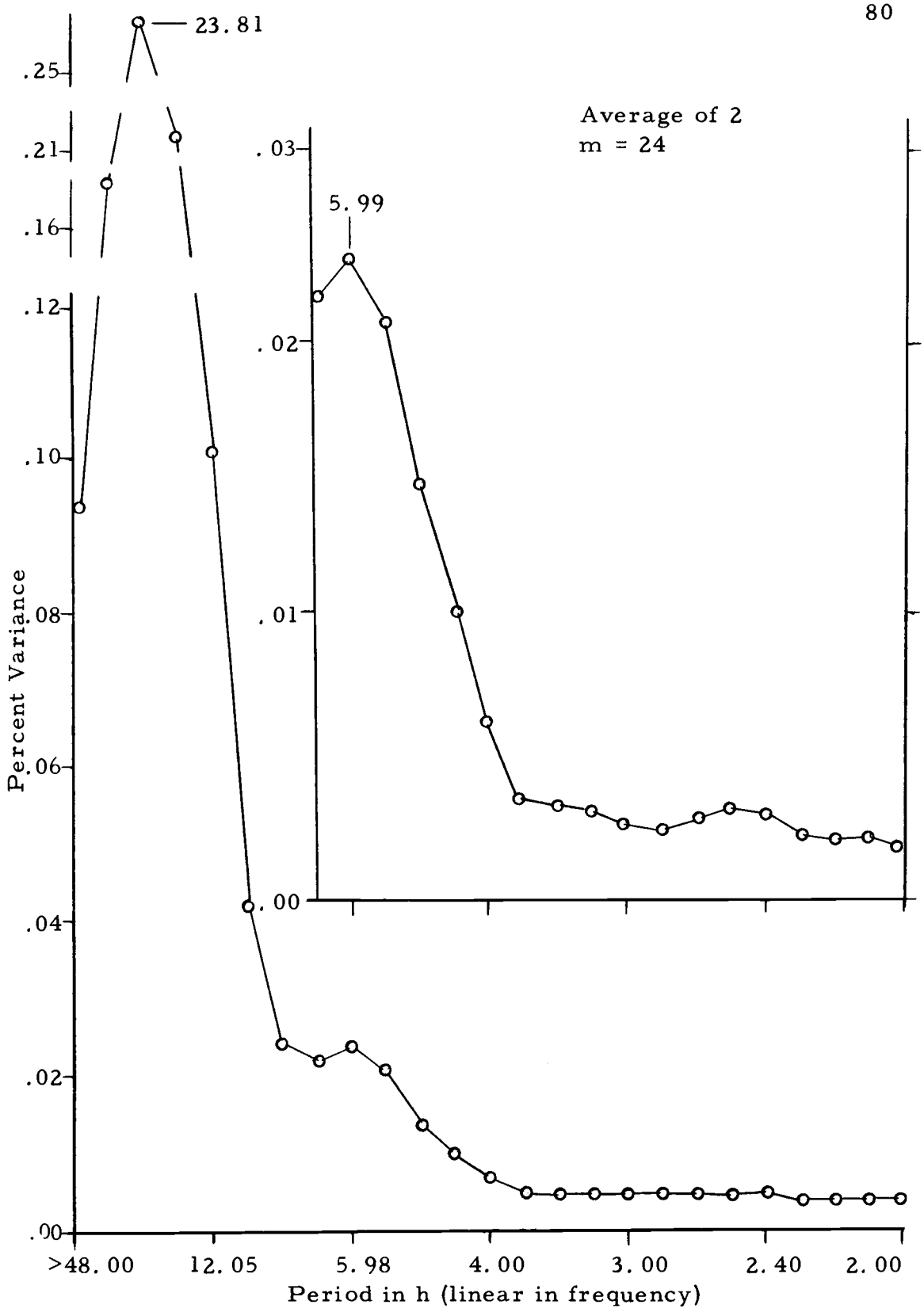


Figure 28. Average variance spectrum of the effect of DD on adult M. rotundata. Inset--log plot of high frequency variation.

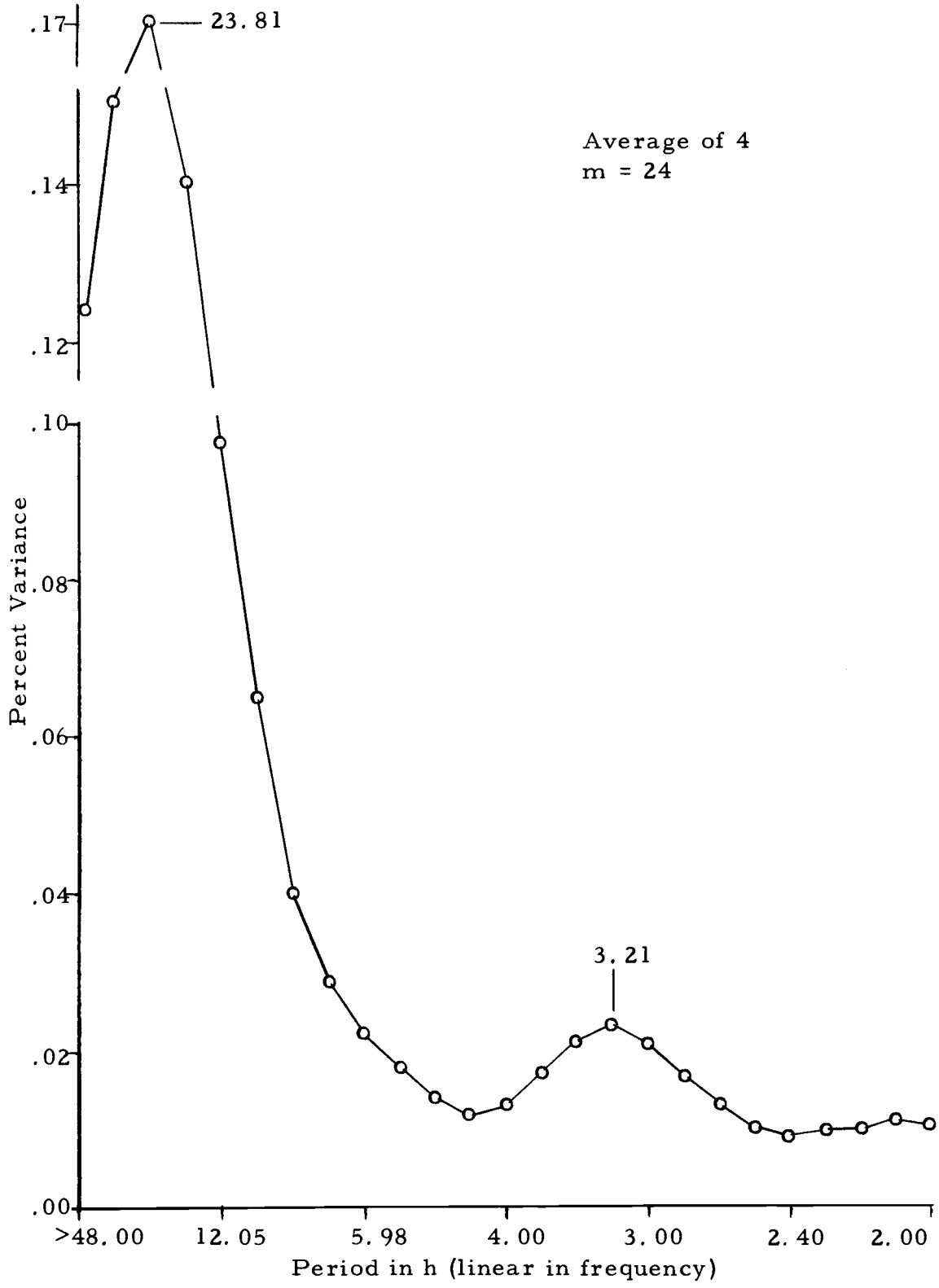


Figure 29. Average variance spectrum for the effect of a temperature of 25° C.

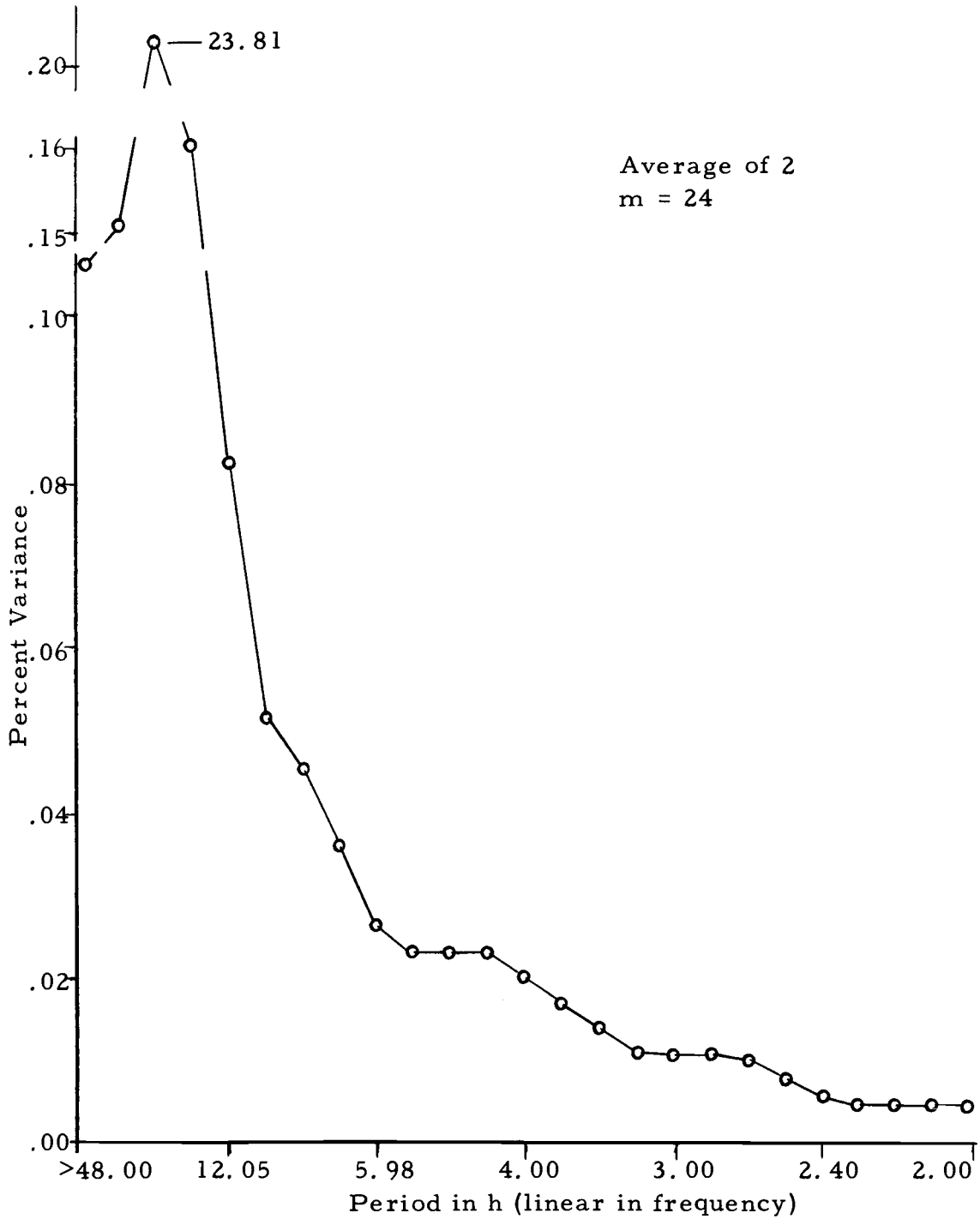


Figure 30. Average variance spectrum for the effect of a temperature of 35° C.

Temperature Phase Response

The phase response curve for the effect of temperature is recorded in Figure 31. The curve closely approximates the emergence response curve (Figure 8) and thus extensive discussion need not be repeated here. The CT-16 phase-shift was missed, but since the remaining points in the curve closely followed the emergence phase-shift points, CT-16 shifts were estimated and joined to the other points by dotted lines.

Entrainment of the Oxygen Consumption Rhythm

> Bruce (1960) defined entrainment as follows:
>
> Entrainment of a circadian rhythm is defined as the phenomenon whereby a periodic repetition of light and dark (a light cycle) or a periodic temperature cycle, or, more rarely, a periodically repeated stimulus of some other type causes an overt persistent rhythm to become periodic with the same period as the entraining cycle (Bruce, 1960).

No entrainment to light was obvious from the raw data plots of the prepupal and pupal stages. A plot of a typical individual is illustrated in Figure 32. Figures 33 and 34 show the spectral plots for the prepupal and pupal stages. The circadian frequency is absent and the ultradian rhythms are very similar to those of the prepupal and pupal stages that received only a single pulse of light (Figure 12). Figure 35 illustrates the spectral plot of a long series of data (531 h)

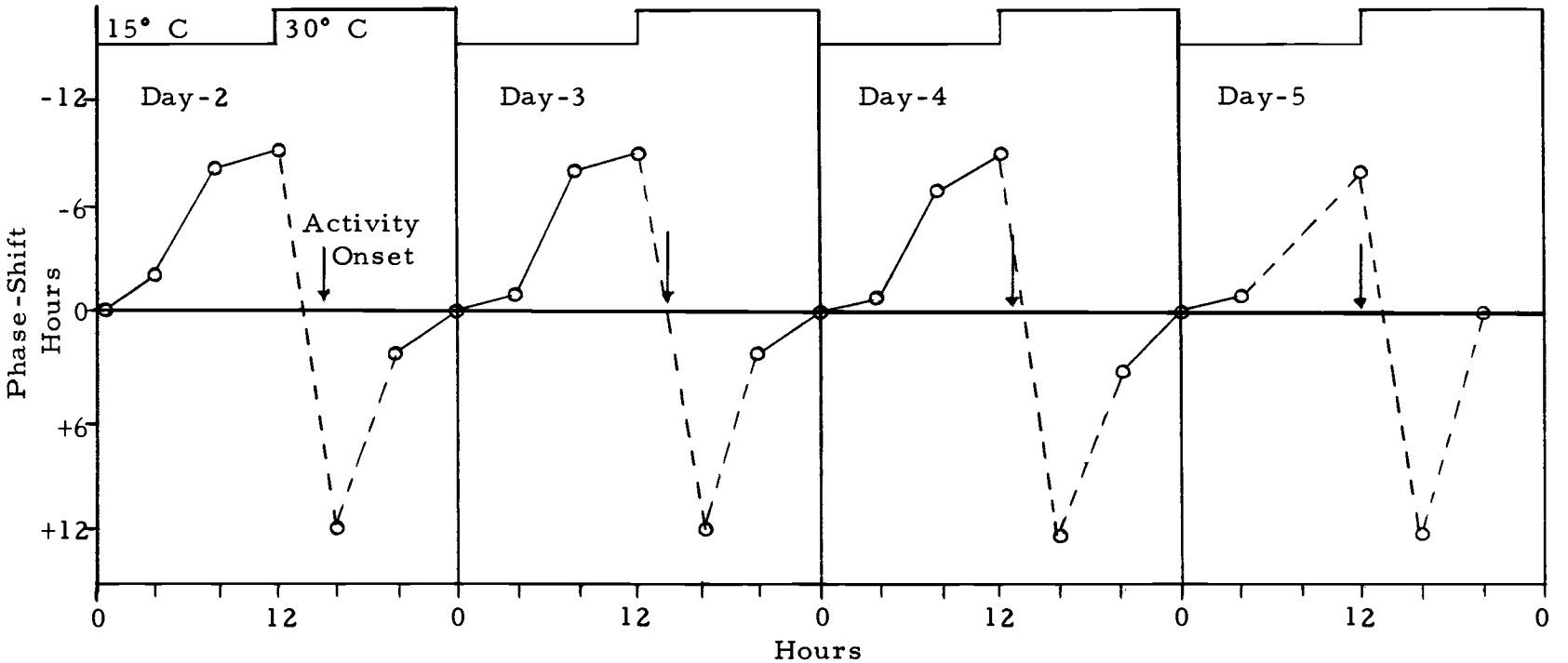


Figure 31. Oxygen consumption temperature phase response curve.

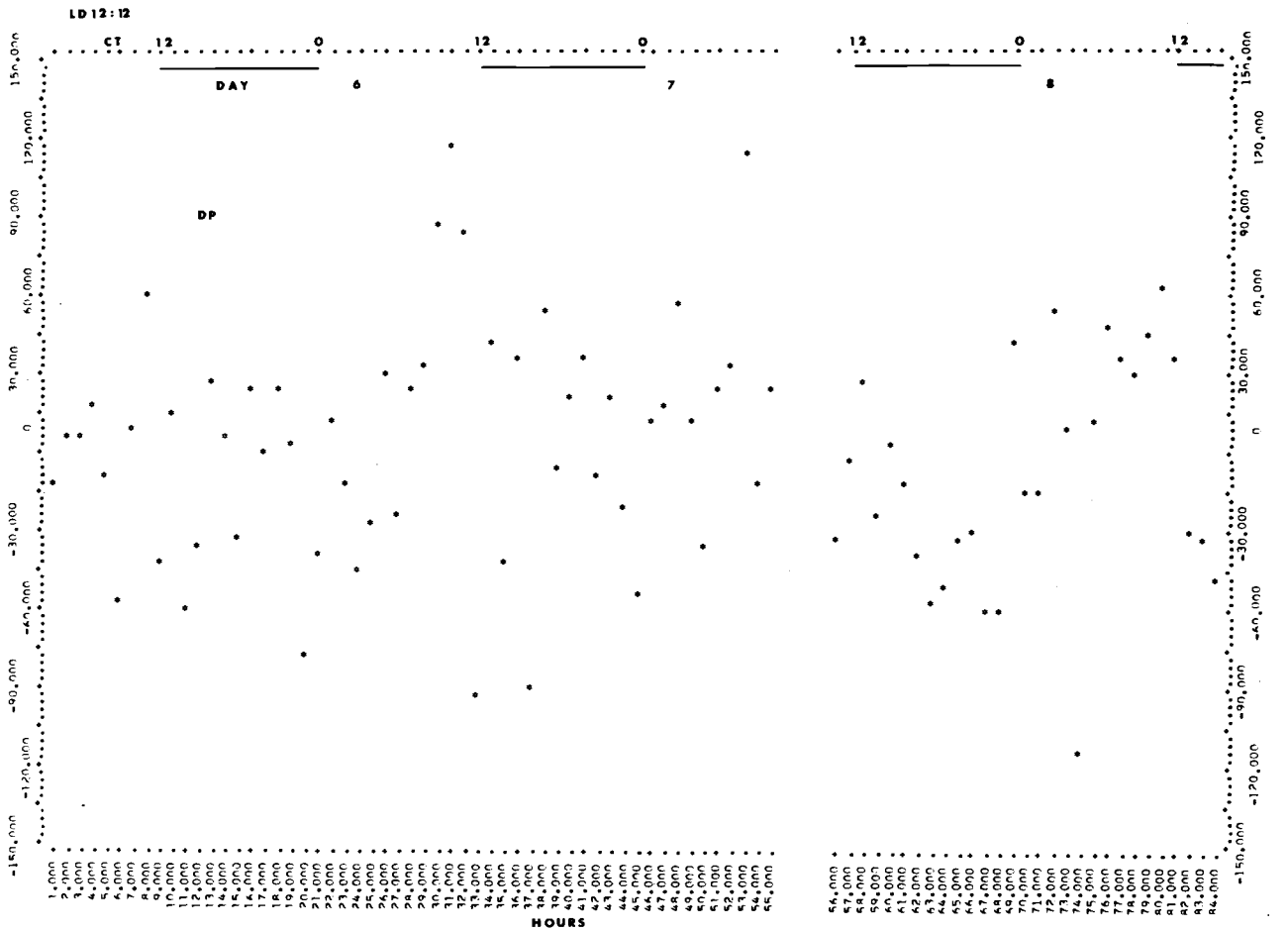


Figure 32. Plot of oxygen consumption as a difference from the mean (mean = 0) for a typical pre-adult under LD 12:12 entrainment. Light regime shown at top of figure.

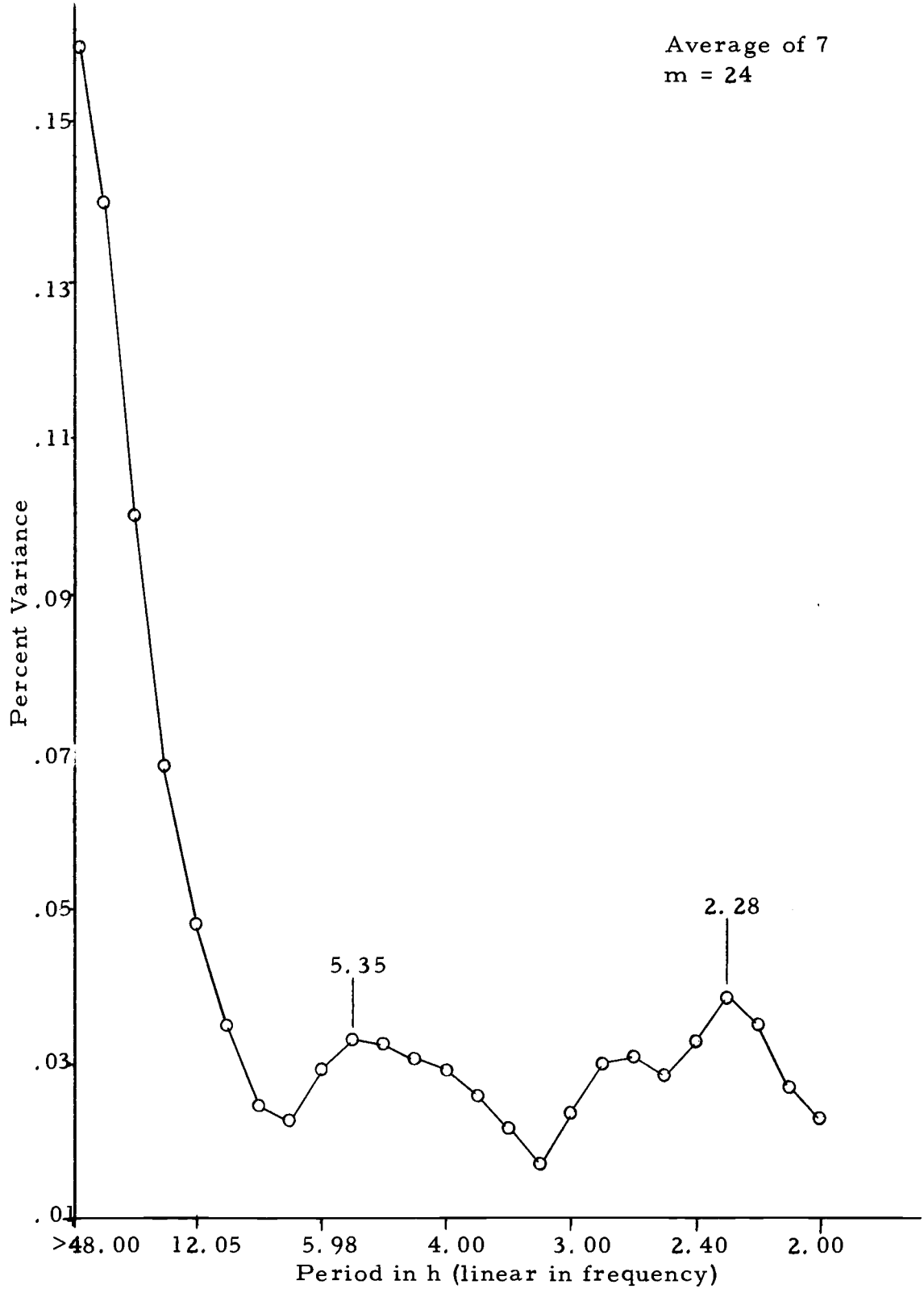


Figure 33. Average variance spectrum of LD 12:12 entrainment of prepupae.

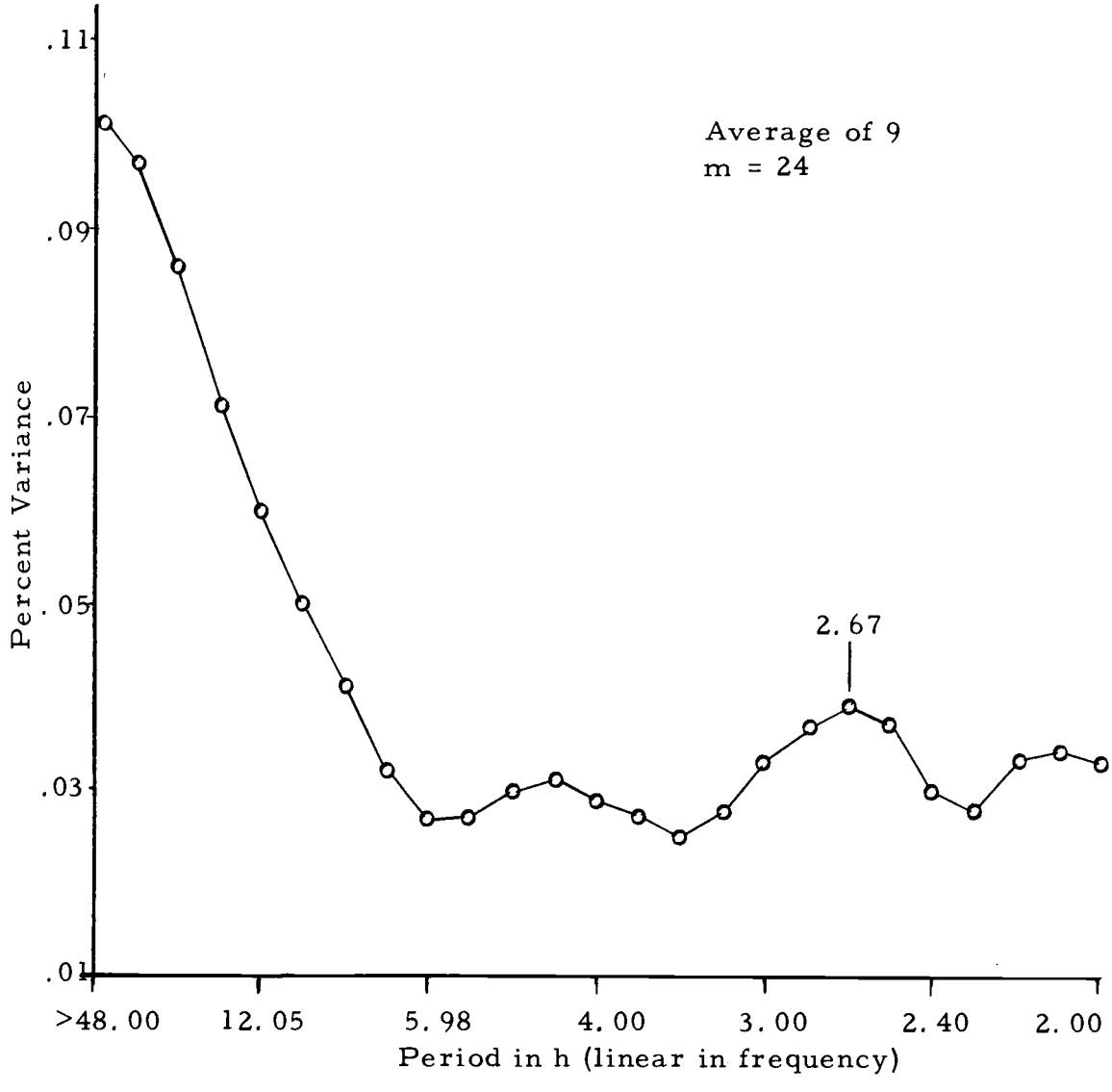


Figure 34. Average variance spectrum of LD 12:12 entrainment of pupae.

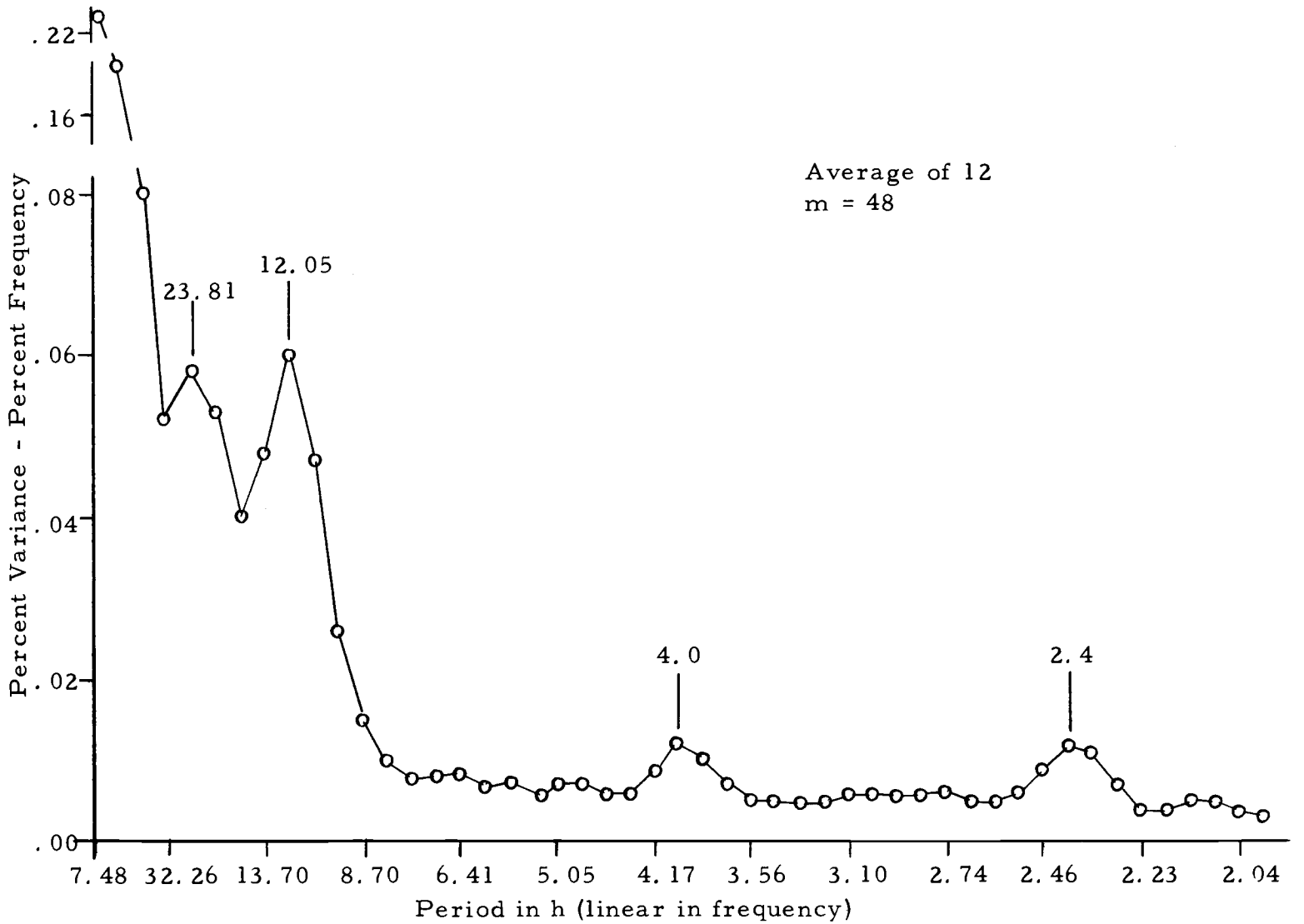


Figure 35. Average variance spectrum of the pre-adult stages of M. rotundata under LD 12:12 entrainment.

recorded from prepupal and pupal M. rotundata. Compared to the shorter series (average of 90 hours) that have been used to construct the previous graphs, Figure 35 represents a long series and facilitates the use of greater lag. For the analysis of shorter series the lag (m) was 24 but for the longer series a lag of 48 was used. In spectral analysis, resolution depends upon the number of lags at which the first (autocorrelation) transformation of the data is stopped. As lag is increased greater resolution is gained (Richards and Halberg, 1964). For the first time in the spectral records of the immature stages a definite 12.05 h component appeared. The appearance of the 12.05 h period could have been due to the better resolution attained using m = 48, or it could be that under a long period of entrainment a 12 hour rhythm develops.

The adults did not appear to be entrained by the light cycle (Table 5).

Insects 1, 2, and 6, were held in entrainment for four days before oxygen recordings were made, thus the first day that a record is available for them is day-5. If the insects were entrained by the light cycle their periods should be 24 h. If the insects were not entrained and remained free-running then their periods would not be exactly 24 h but would probably be between 22 and 24 hours as the emergence period was. Insects number 6 and 8, both males, showed consistent free-running periods while the rest proved to be quite

Table 5. Entrainment of adult Megachile rotundata to light.

Number	Sex	Number of Days Following Initiation of the LD Cycle							Entrainment
		3	4	5	6	7	8	9	
1	Female	--	--	3*(26)**	5 (24)	5 (25)	6	--	no
2	Female	--	--	3 (21)	0 (24)	0	--	--	no
3	Female	7 (25)	6 (28)	10	--	--	--	--	no
4	Female	--	2 (28)	6	--	--	--	--	no
5	Male	9 (20)	5	--	--	--	--	--	no
6	Male	--	--	3 (23)	2 (22)	0 (23)	23 (22)	21	no
7	Male	--	1 (28)	5 (19)	0	--	--	--	no
8	Male	5 (23)	4 (22)	2 (22)	0	--	--	--	no

*Median hour of increased oxygen consumption.
**Period in hours.

erratic in period length. The adults were separated by sex to discover if there was a difference due to sex in the rhythm spectra (Figures 36 and 37). The spectral plots are almost identical and therefore no sex difference was obtained.

Figure 38 records the spectral plot of the oxygen consumption of 11 adults immediately following five days of temperature entrainment. Similar ultradian rhythms occurred while the circadian component did not appear at all. It can be concluded from Figure 38, that temperature entrainment did not reveal any rhythms that were not exposed by a single temperature pulse.

Comparison of the Oxygen Consumption Rhythm Spectra in the Pre-adult and Adult Stages

The rhythms would appear to be less stable in the pre-adult stages of the leafcutter bee than in the adult stage. Many of the pre-adult stage records showed no circadian component (Figures 33 and 34) while others did (Figure 16A). The appearance of a circadian component in the immature stages could not be attributed to an increase in maturity alone, as the dark-eyed bees (Figure 16A) showed a definite circadian component while the more mature black-bodied bees did not (Figure 17A). The adult stage proved very stable under different conditions of light (Figures 27 and 28) and temperature

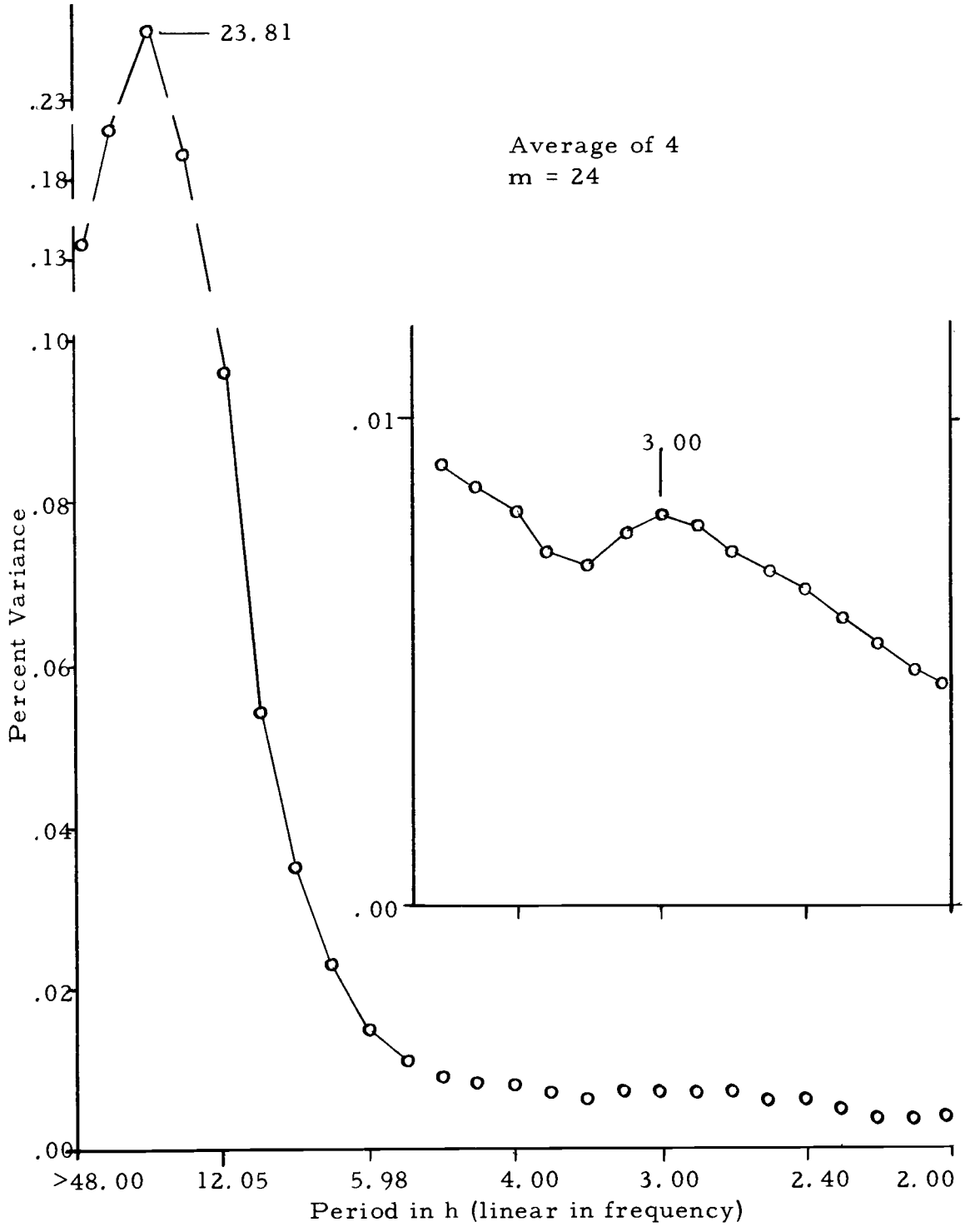


Figure 36. Average variance spectrum of adult female M. rotundata under LD 12:12 entrainment.

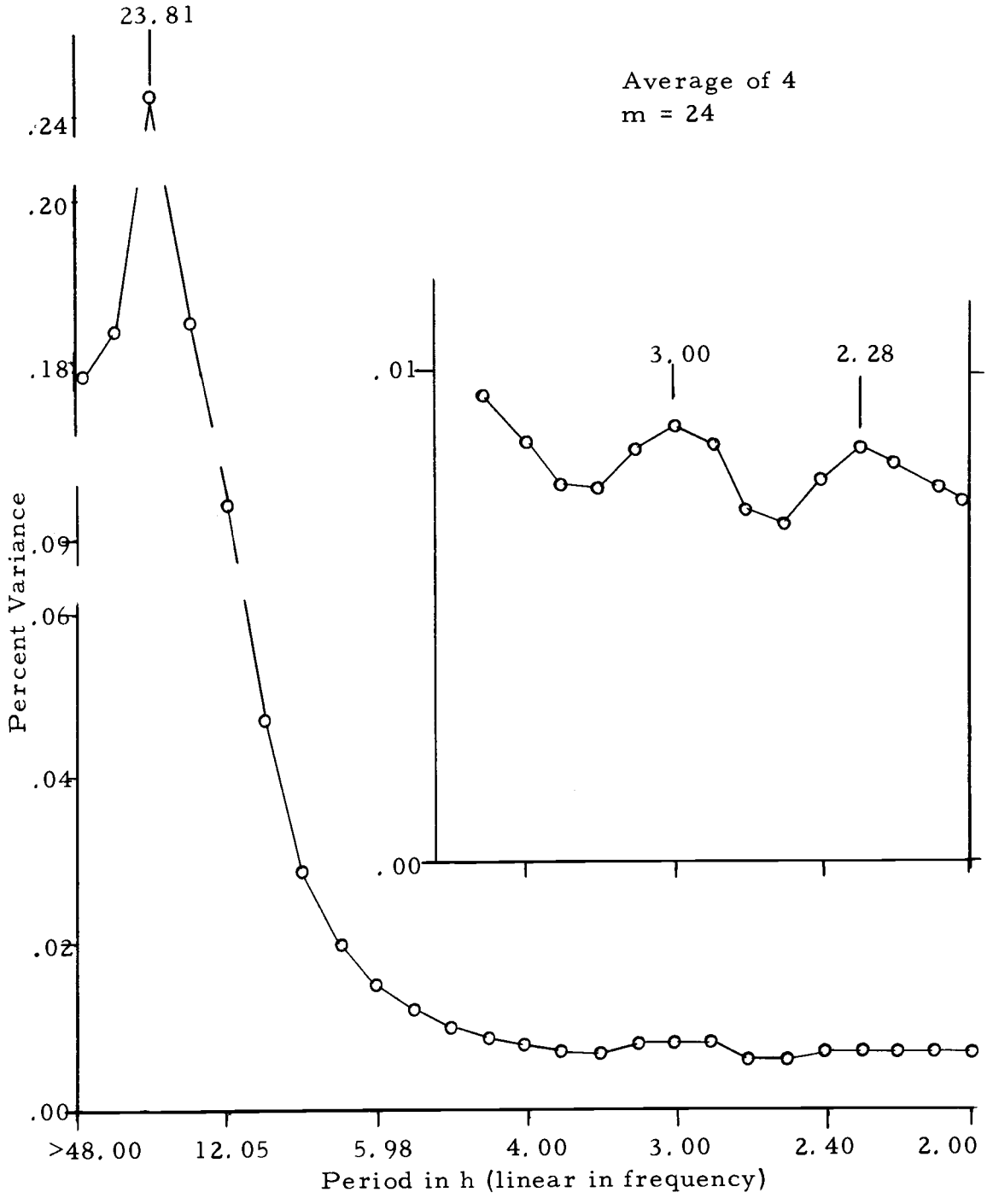


Figure 37. Average variance spectrum of adult male M. rotundata under LD 12:12 entrainment.

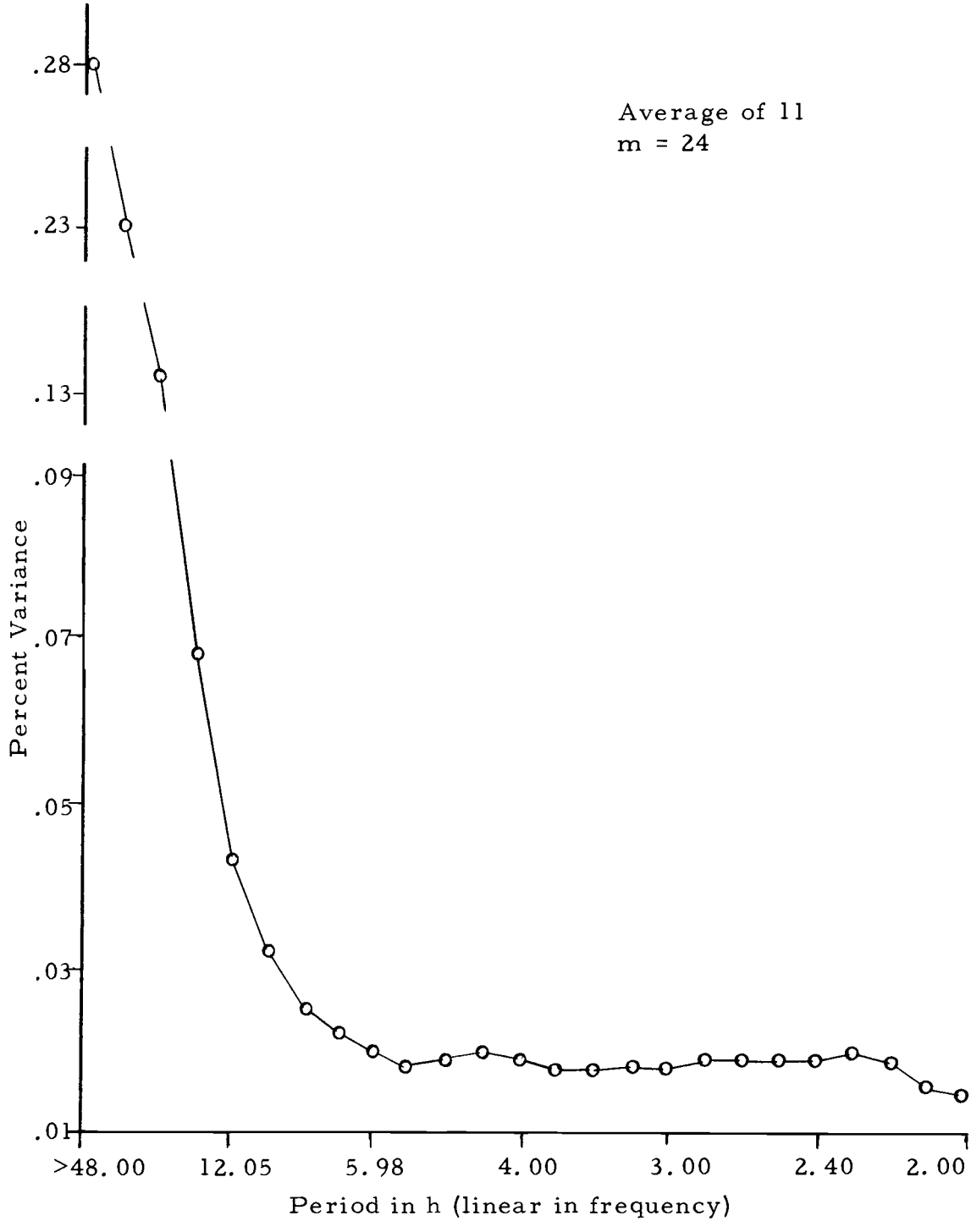


Figure 38. Average variance spectrum of pupae following temperature entrainment.

(Figures 25, 29, and 30) with the circadian component being very distinct.

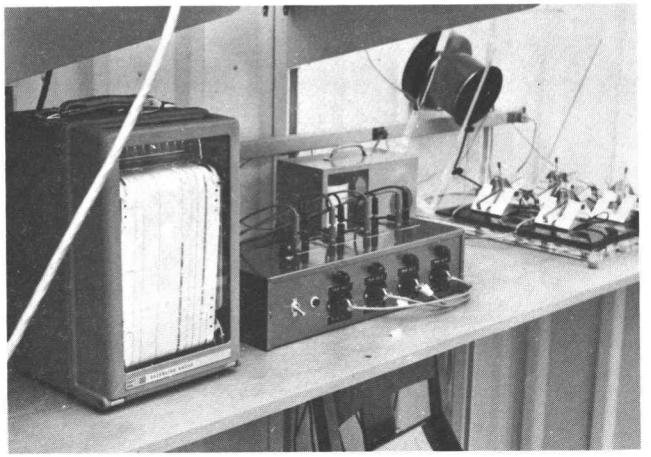
CIRCADIAN RHYTHM OF ADULT ACTIVITY

Methods and Materials

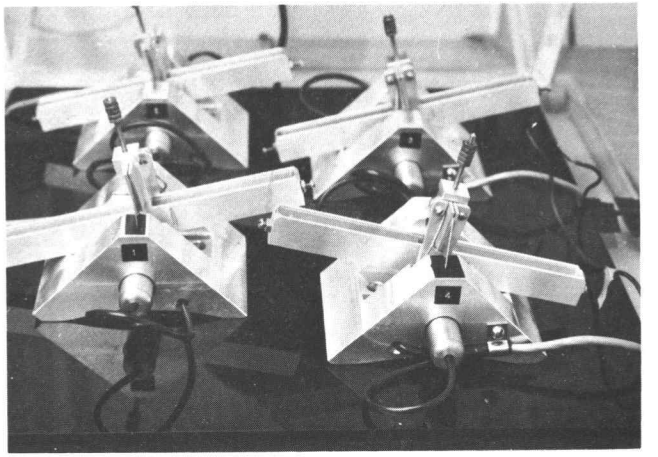
Preliminary handling of the bees was similar to that of the emergence and oxygen consumption studies. Newly emerged adult bees were light- and temperature-pulsed, then placed individually into the activity apparatus rockers (Figures 39 and 40). The rocker sides were aluminum and the top and bottom were plastic. The bottom was darkened to restrict the passage of light. A tongue projected from the center of the rocker which cut the path of light from the lamp to the photocell as the rocker moved. This in turn interrupted the flow of current to the event recorder causing the recorder pen to move. The average insect weight was about 25 mg. To reduce rapid movement of the rocker arm, which would cause it to bounce, an aluminum wire was attached to the center post with the free end curved and placed in an oil bath (STP, 100 percent pure petroleum). The sensitivity of the rocker could be regulated by adjusting the position of a series of nuts on the middle post. The rockers rested on knife blades (Stanley, heavy duty no. 1992-5) which in turn rested on a base covered with black electrical tape. When the rockers were placed in position, slight pressure was applied, causing the knife blades to make an impression in the tape. This provided a groove which kept

Figure 39. Activity apparatus.

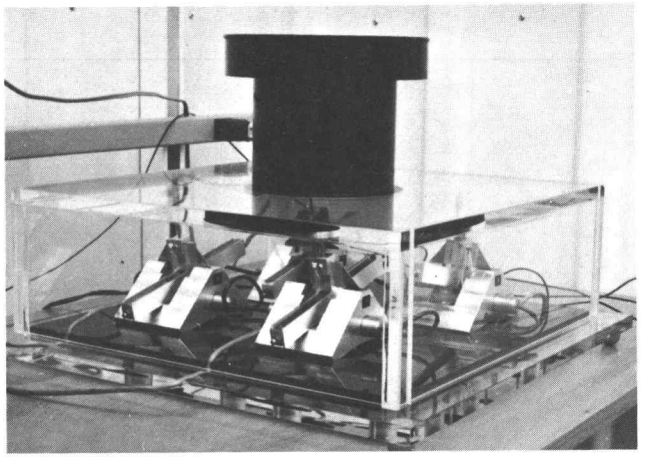
A. Arrangement of the event recorder, control box and activity chamber.
B. Close-up showing the four rockers.
C. Activity chamber with the black acrylic cover removed.
D. Activity chamber with the black cover in place.



A



B



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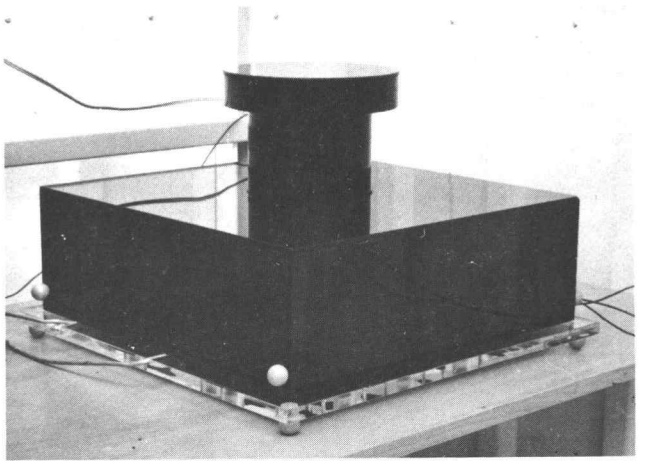

D

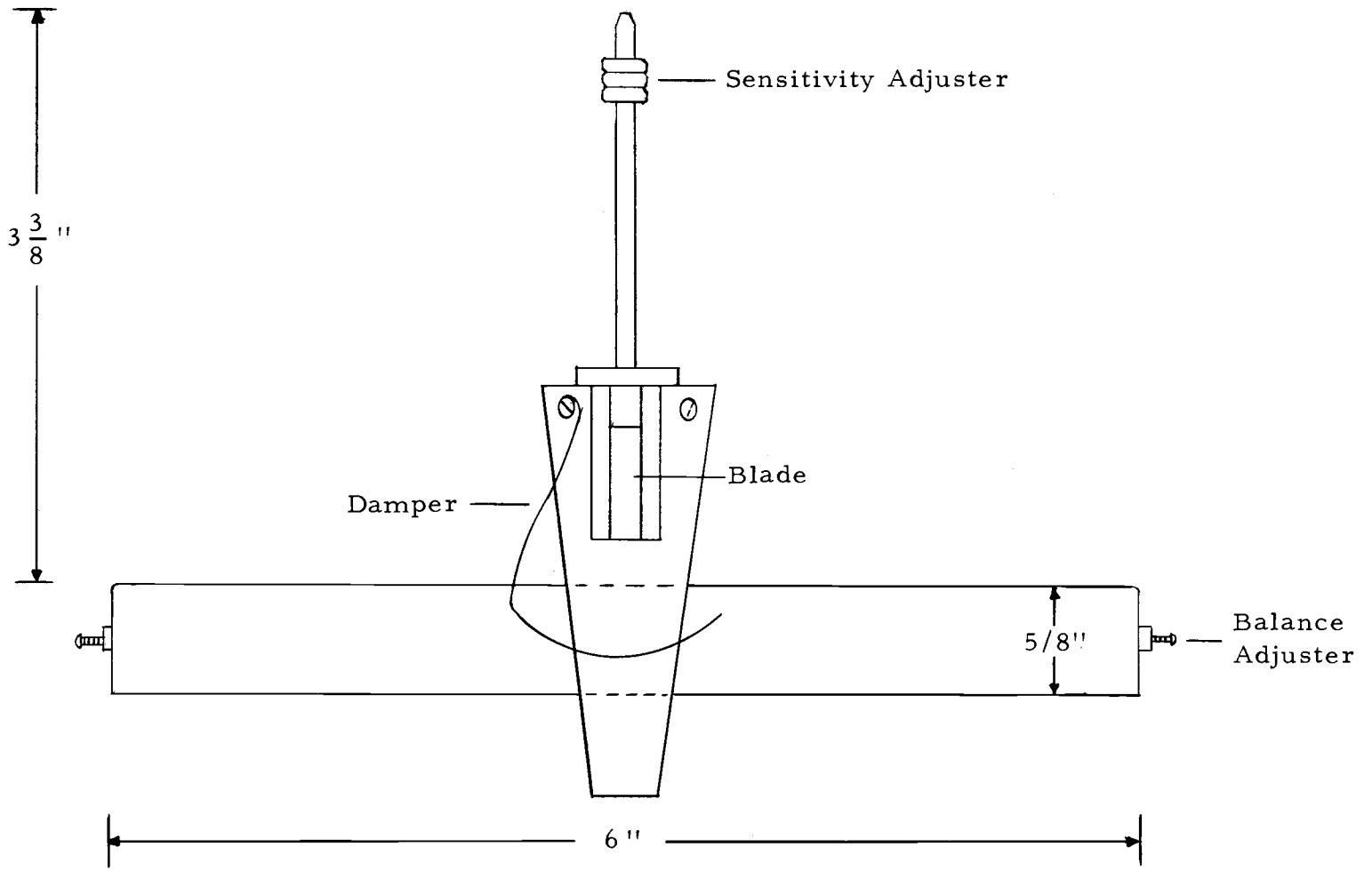

Figure 40. Side view of a rocker. Scale: 1" = 1".

the rockers centered. Usually the bees behaved well and during their activity period moved from end to end in the rocker. When an individual, who refused to move from end to end was discovered it was replaced. Food was provided for the insects by placing a small amount in the center of the rocker. This food was the same as that used in the oxygen experiments, except that it was more viscous.

A constant temperature was maintained in the rockers by a clear acrylic cover which fit tightly over a raised black acrylic base (Figure 39C). A skipper fan was installed in a black chamber in the center of the clear cover. The fan pulled air through the chamber; the air entered through four holes situated underneath the rocker bases. The fan was controlled by a rheostat which allowed adjustment of the air flow through the chamber. This provided a fine temperature control. The moving air also helped dissipate the heat produced by the lamps which operated the photocells.

The fan was baffled to restrict entrance of light during dark experiments. A black acrylic cover fit snugly over the clear cover (Figure 39D). For experiments conducted in LL conditions, or for light pulses the dark cover was removed exposing the clear cover.

The photocells, as in the emergence apparatus, were connected to the same ten-channel Esterline-Angus event recorder through a control box whose circuit diagram was similar to that of the emergence control box (Figure 5).

The activity apparatus was placed in the same controlled-environment room as the emergence apparatus. As with the emergence apparatus the continuous temperature recorder indicated that the temperature varied no more than ±0.5C°.

Stage Susceptible to Synchronization by Light

The preliminary procedures for these experiments were the same as those for the emergence studies and need not be repeated here.

Six different experiments, involving a total of approximately 25 insects, were performed in an effort to determine if light could phase-shift the activity rhythm in the prepupal, dark-eyed pupa, black-bodied pupa, and adult stages. Activity was recorded as pen strokes caused by the cutting of the path of light by the rocker tongue and resulted in an activity record such as is shown in Figure 41. The resulting periods of activity were recorded and analyzed statistically where possible.

Stage Susceptible to Synchronization by Temperature

The procedure for the temperature studies was the same as that for the light except that a temperature pulse instead of a light pulse was used in an attempt to phase-set activity in the various stages.

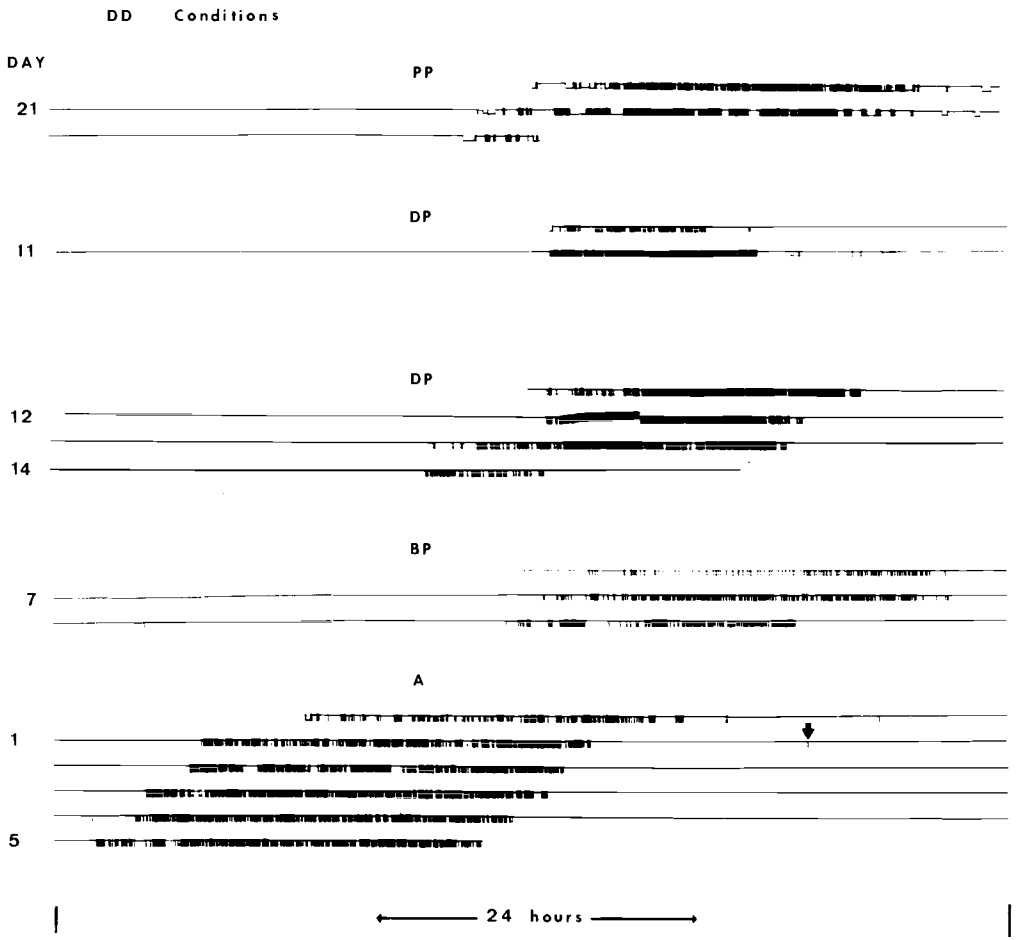

Figure 41. Effect of light on the various stages of M. rotundata. A single pulse applied on day-0 for the pre-adult stages. A single light pulse initiated in the latter part of day-1 for the adult

Three experiments were conducted using about 12 insects. The data were handled in the same way as they were for the light experiments.

Effect of Light on the Period Length

Adults, involving three different experiments, were studied to determine if M. rotundata displayed a difference in activity period length in LL as opposed to DD conditions. The resulting graphs were analyzed statistically using the t test to determine if there was a significant difference between means.

Temperature Phase Response

The procedures were similar in these experiments to those carried out in the emergence study. About 20 individuals in two separate experiments were used to scan the 24-h activity period at 4-h intervals. The results were analyzed to determine the magnitude of phase shift at the various 4-h intervals over the 24-h circadian period.

Light Phase Response

Five insects maintained in DD were light pulsed for 12-h during the early, middle, and late stages of the activity period. Observations were made to discover if a 12-h light pulse phase shifted

the activity rhythm.

Results and Discussion

Stage Susceptible to Synchronization by Light

Five typical activity records are illustrated in Figure 41. The numbers at the left of the record mark the days following the original 12-h light pulse. The criterion used to discover if the insects had been phase-set by light was to observe the presence or absence of synchronization of onset of activity. If the individuals, light pulsed in the black-bodied stage, were synchronous with those pulsed in the prepupal stage, then it could be concluded that light had had an effect. There is no indication in the results shown in Figure 41 that light had any influence on the resulting activity rhythms.

The bottom record of Figure 41 shows a five-day activity trace of an adult. A 12-h light pulse was initiated in day-1 (arrow) but no subsequent phase shift was noted. Other adults were light-pulsed at other times in the activity period and no observable phase shift resulted (Figure 43). It can be concluded that a 12-h light pulse of 32 fc does not phase-set the activity rhythm of Megachile rotundata either in the adult or preadult stages.

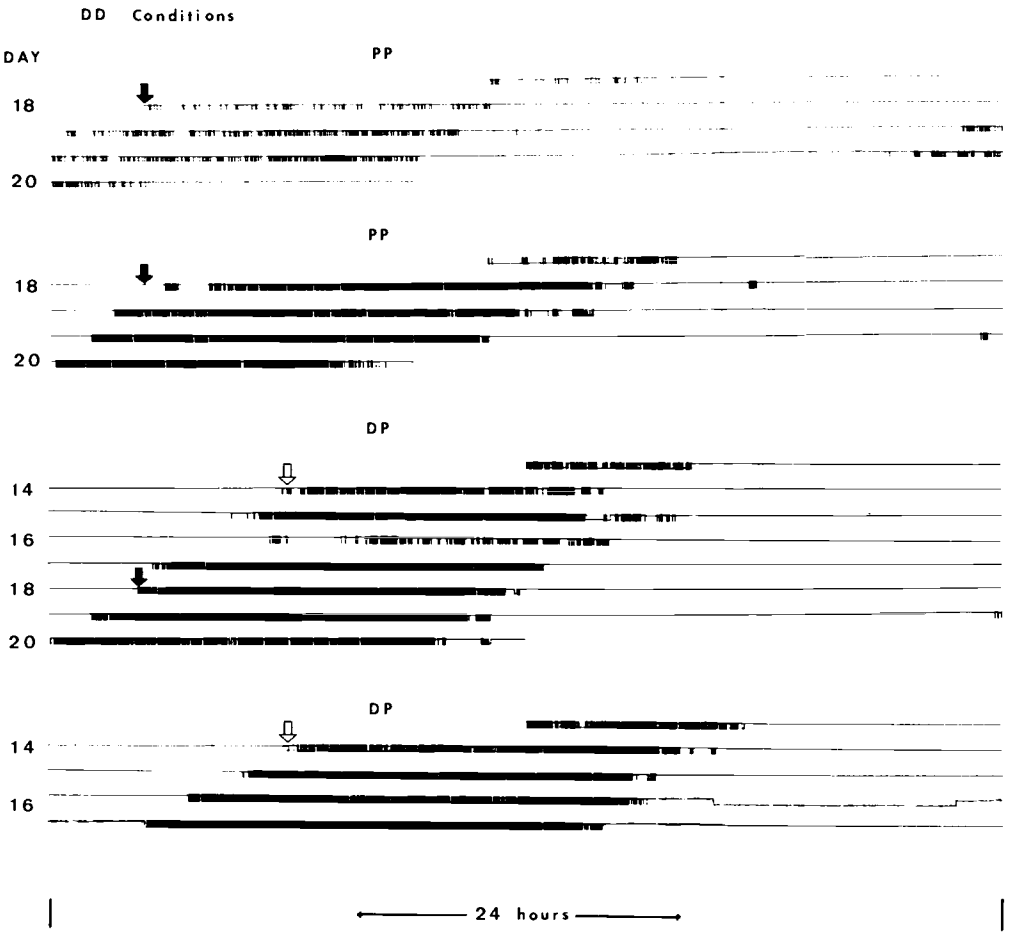

Figure 42. Effect of temperature on the various stages of M. rotundata. A single temperature pulse applied on day-0.

Stage Susceptible to Synchronization by Temperature

The activity records for four individuals are shown in Figure 42. The same criterion of synchronization was used for the effect of temperature as was used for the effect of light. The four individuals would appear to be synchronous. Eighteen days following the original temperature pulse, the two prepupal (PP) and the dark-eyed pupae (DP) appear to be almost exactly synchronous (solid arrows, day-18). The two DP individuals can be compared on day-14 through 17 (open arrow, day-14).

It is regrettable that the individuals used for the black-bodied pupal portion of the study did not survive. Starting with three bees, two died and the third did not cooperate in the rocker. However, if the prepupal and dark-eyed pupal stages were susceptible to temperature synchronization it is most likely that the black-bodied pupa would be also.

The adults also proved to be susceptible to temperature stimulation. The effect of a temperature pulse on adult M. rotundata will be discussed in the phase response section.

Effect of Light on the Period Length

The results of three experiments performed to determine the effect of light on period length are recorded in Figure 43 and Table 6.

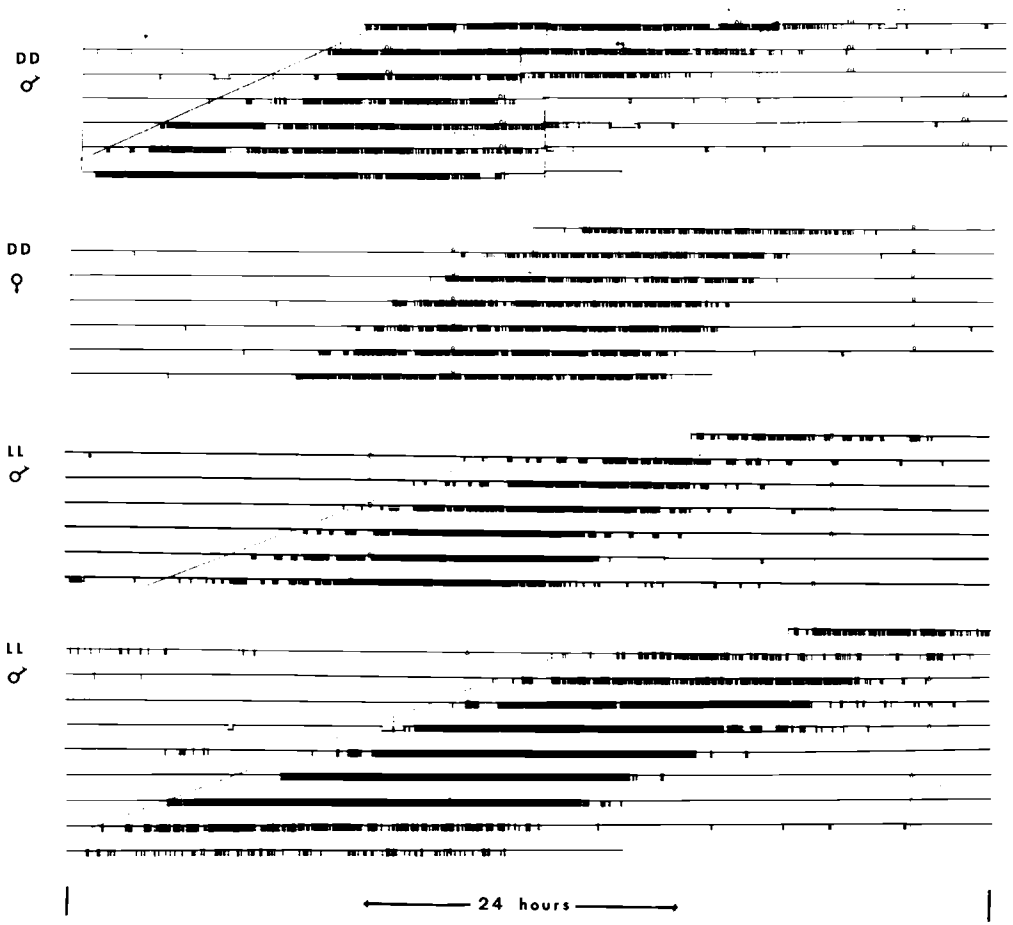

Figure 43. Effect of LL on the length of the activity period.

Table 6. Effect of LL on the free-running period of the M. rotundata eclosion rhythm.

Light Conditions	Number of Animals	Number of Periods	Mean Period (hours)	Standard Deviation
LL	3	18	22.34	0.6789
DD	6	30	22.86*	0.6843

*Significantly different at the five percent level.

The activity traces in Figure 43 are marked with a sloping line approximating the activity onsets. It can be observed that the slope of the line is greater for the DD activity traces than for the LL traces. This indicates that the period was shorter in LL than in DD. Using the t test for the difference between means the period of M. rotundata proved to be significantly shorter in LL than in DD (Table 6).

M. rotundata activity would appear to follow Aschoff's rule in its response to continuous light. Hoffman (1965), lists the results of studies on three species of lizards, one species of bird, and one mammal species, all of which displayed a shorter period in LL than in DD. The evidence from a few insect studies is not clear. Pittendrigh (1960), states that Drosophila melanogaster eclosion does not follow the rule. Hoffman also lists the results from studies of a number of dark active insects including cockroaches and crickets which followed Aschoff's rule and displayed a longer period in LL

than in DD.

Bruce (1960), hypothesized that one could explain the differences in period of free-running rhythms in the dark and in the light in terms of the coupling or uncoupling of separate oscillators with slightly different periods, or one could explain these period differences in terms of the lengthening or shortening of the period of a single oscillator. In the latter case, this lengthening or shortening might occur uniformly throughout the cycle or only during a portion of the cycle. These same considerations could also apply to the effect of different temperature levels on the free-running period.

Temperature Phase Response

The temperature phase response curve is drawn for day-2 only (Figure 44). It was difficult to maintain some individuals for an extended period of time in the activity apparatus, and thus the phase response data for the days following day-2 are meagre. As in the eclosion phase response curve, the M. rotundata activity response curve is very similar to the published results of other organisms. This similarity was discussed above with reference to the emergence phase response curve.

In an attempt to determine if the activity period length changed with a phase shift, the periods following the temperature pulse producing the phase shifts were analyzed. The results are listed in

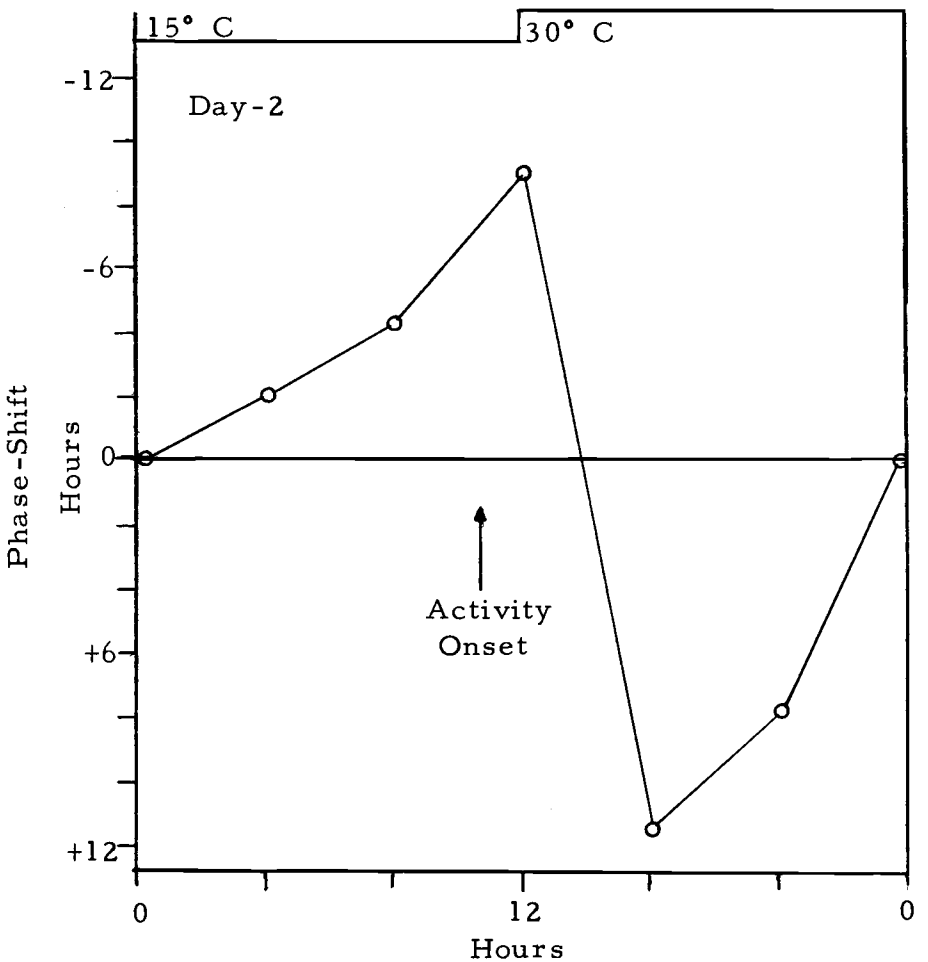

Figure 44. Temperature phase response curve for activity. Reference point is onset of activity.

Table 7.

Table 7. Period change resulting from phase shifts occurring at various circadian hours.

Time of 12-h Pulse (CT)	Period in LL (hours)	Number of Individuals	Number of Periods	Significantly Different from CT-0
0	22.34	3	18	--
4	22.48	1	4	no
8	23.01	1	6	yes
12	22.59	1	3	no
16	23.00	1	2	no
20	--	-	-	--

Because of the difficulty of maintaining individual bees in the activity apparatus, only one record for a single bee is recorded for each phase shift, except for circadian hour 20 for which no period lengths were available. The only insect showing a significant difference in period length was the one that was phase shifted at CT-8. According to circadian theory one would expect that the further away from CT-0 the phase shift occurred the greater would be the resulting change in period length. The phase response curve also supports this hypothesis (Figure 44). Thus, one would expect that if a significant difference in period length occurred at CT-8 it would also occur at CT-12 and 16. The fact that there was no significant change in period

length at CT-12 and 16 could possibly be due to the small number of periods available for analysis. This raises a point that applies to this study and to those reported in the literature--much variability in conclusions may be due to lack of sufficient data. Rhythm studies must be extended as long as it is practically possible, to insure accurate conclusions.

Light Phase Response

The results from the light response study are recorded in Figure 45. No obvious effect of light is revealed in the experiment. M. rotundata would appear to be unique in that it is the only animal studied so far whose rhythms are almost completely refractive to light shifting. Since continuous light shortens the activity period the leafcutter bee is not completely refractive to phase shifting by light. This shift is so small that long term experiments will have to be conducted in an effort to detect it. It should be noted that although the average period was shorter in LL than in DD it was shorter by only 0.52 hours (Table 6). DeCoursey (1964) experienced a similar problem in studying hamsters. She found that the daily scatter of onsets of activity from the mean was great enough in the majority of animals to mask most phase shifts smaller than 30 to 60 minutes.

Roberts (1960), studying the phase shifting ability of light on Leucophaea maderae found that the magnitude of phase shift varied

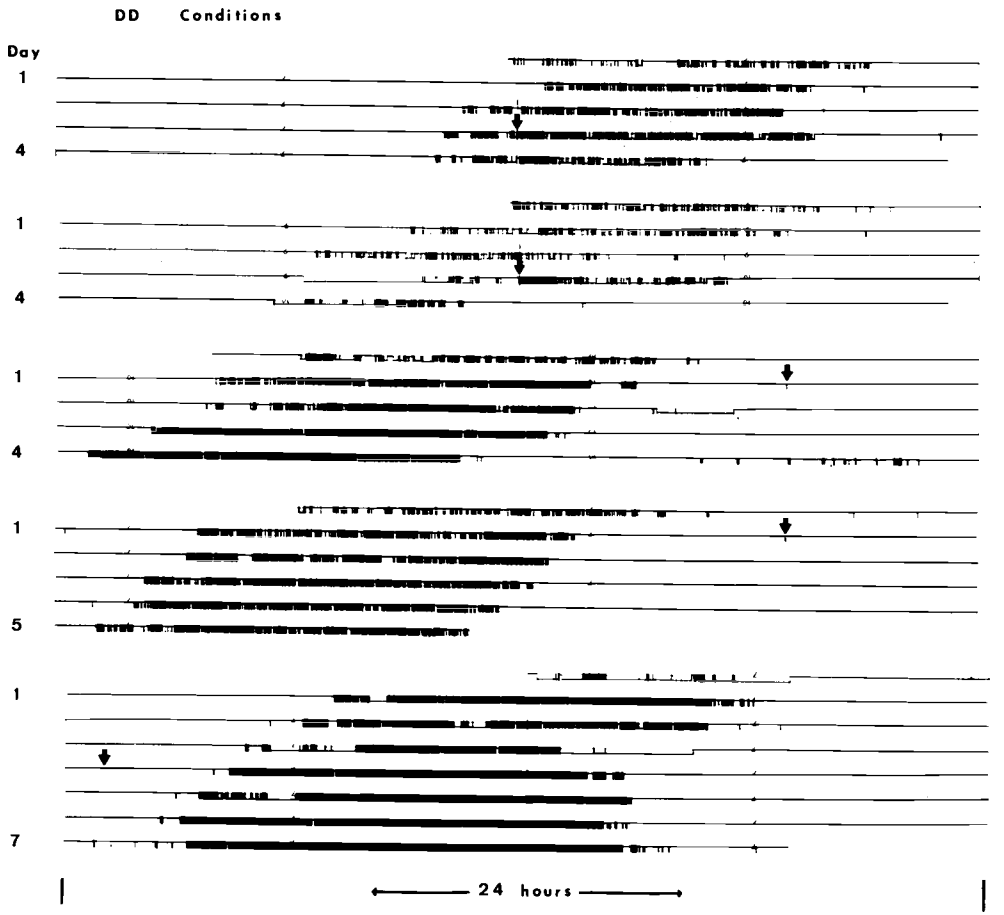

Figure 45. Light phase response for activity. A 12-h light pulse initiated at various times (arrow) throughout the 24-h period.

from one to two hours depending upon the circadian hour at which the light pulse was applied. In Drosophila pseudoobscura, the emergence rhythm was shifted eight hours by a single flash of light (Pittendrigh, 1954). These phase shifts correspond in magnitude to the phase shifts that occurred in M. rotundata in response to temperature pulses.

CONCLUSIONS

Emergence Rhythms

1. A 12-h light pulse does not synchronize emergence in the pre-adult stages of Megachile rotundata (F.).
2. The period of emergence is shorter in LL (23.00 h) than in DD (23.70 h) but this difference was not found to be significant.
3. A 12-h temperature pulse of 15C° synchronized the emergence rhythm in M. rotundata from the dark-eyed pupal stage through to the adult stage.
4. M. rotundata emergence responds to temperature in a manner similar to other organisms. The Q_{10} for the emergence period length is 1.02 for 25° C to 30° C and 1.12 for 30° C to 35° C. The differences in period lengths were not statistically significant.
5. The temperature phase response curve for M. rotundata is similar to the phase response curves of other organisms, both plant and animal. Phase advances occur during the subjective high temperature period and phase delays occur during the subjective low temperature period.
6. No transients occur in the response of M. rotundata to a temperature pulse.

7. No statistically significant change in the length of the emergence period occurred following phase shifting.
8. It would appear that the emergence rhythm locks on to the point of temperature rise when a temperature pulse is affected.

Oxygen Consumption Rhythm

1. A 12-h light pulse of 32 fc does not synchronize the oxygen consumption rhythm in the pre-adult or the adult stages of M. rotundata, nor does it affect the rhythm spectrum in these stages.
2. No difference was detected in the length of the oxygen consumption period in LL as compared to DD. The rhythm spectrum appears to be similar under both conditions.
3. Light (LD 12:12) does not appear to entrain the oxygen consumption rhythm in M. rotundata nor does entrainment effect the rhythm spectrum.
4. A temperature pulse of 15C° for 12-h does not appear to phase set the oxygen consumption rhythm in pre-adult M. rotundata, nor does it influence the rhythm spectrum.
5. A 12-h temperature pulse of 15C° does synchronize the oxygen consumption rhythm in the adult stage, but no after effect was detected in the rhythm spectrum.
6. Temperatures of 25° C, 30° C, and 35° C do not appear to

produce different oxygen consumption power spectra.

7. The temperature phase response curve was very similar to the emergence response curve.
8. The oxygen consumption rhythm spectrum seems to be less stable in the pre-adult stages than in the adult stage of the leafcutter bee. The circadian component is very prominent in the adult stage while from two to four ultradian components are most prominent in the pre-adult stages.

Activity Rhythms

1. Pre-adult and adult Megachile rotundata are not susceptible to light synchronization by a single light stimulus of 32 fc for 12-h.
2. M. rotundata activity appears to be almost completely refractive to phase shifting by light. The phase shifts are so small that they could not be measured.
3. The activity period conformed to Aschoff's rule and proved to be shorter in LL than in DD. This difference was significant at the five percent level.
4. The pre-adult and adult stages of M. rotundata proved to be susceptible to a 12-h temperature pulse at 15C°.
5. The temperature response curve is almost identical to that of the emergence and oxygen consumption response curves.

The Relationship of the Three Rhythms

The three rhythms responded similarly to light and temperature. Light had no measurable effect in the preadult stages and only a very slight effect in the adult stage in all three rhythms. Temperature had a similar effect in each rhythm with the temperature response curve being almost identical for the emergence, oxygen consumption, and activity rhythms. Figure 46 illustrates the relationship of the three rhythms to each other. The information displayed for the emergence rhythm was taken from four experiments involving approximately 100 insects per experiment. Results from six individual bees were used to construct the activity and oxygen consumption graphs--three for the activity rhythm and three for the oxygen consumption rhythm. The emergence period of day-1 fits into a segment of the activity rhythm for day-1. It is very possible that these two rhythms are fundamentally the same.

The oxygen consumption rhythm would appear to display a negative phase angle (phase lag) to the activity rhythm. However, there were too few replications involved in the experiments to draw definite conclusions as to whether these rhythms are coupled oscillations or whether they are simply two expressions of the same rhythm. The determination of the onset and termination of the activity rhythm was quite easy to establish but a highly subjective determination of

DD 30° C
12-h temperature pulse of 15C°

Day-1

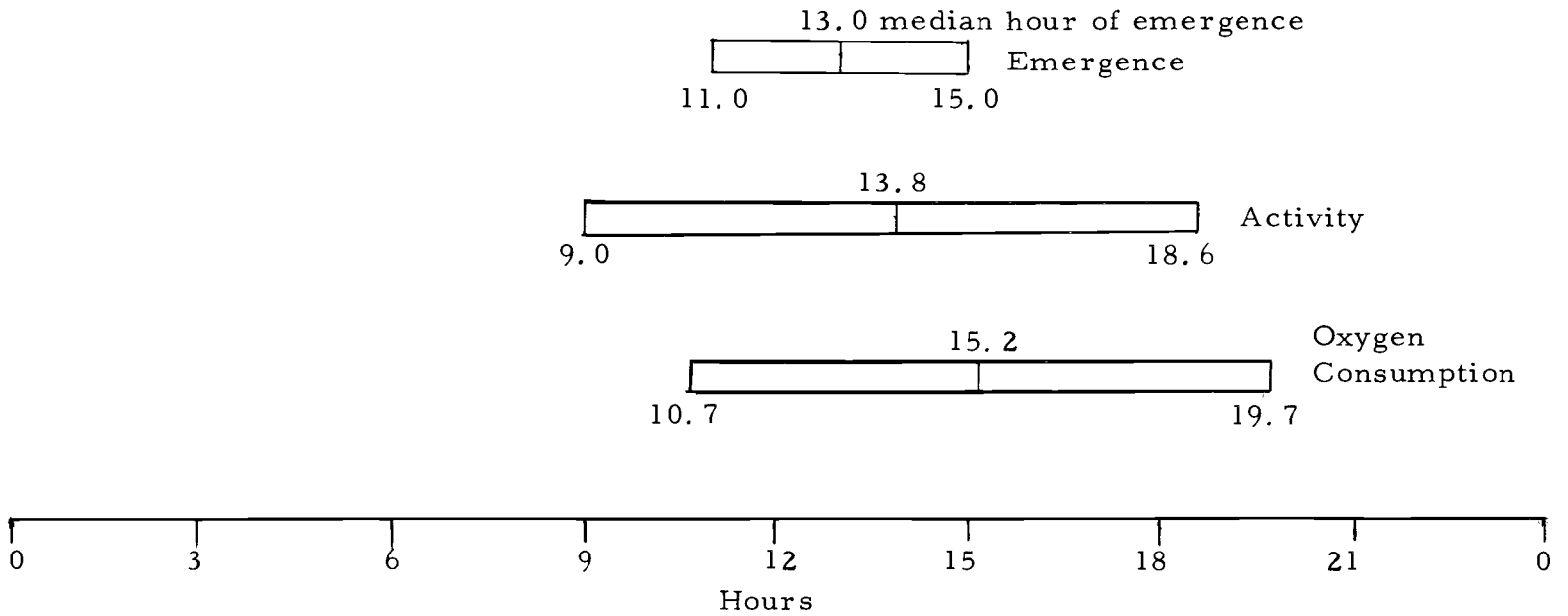

Figure 46. The relationship of the emergence, oxygen consumption, and activity rhythms.

increased oxygen consumption during the 24-h period was required. Rensing (1966) concluded that the oxygen consumption pattern of Drosophila melanogaster followed the locomotor activity pattern "closely" but he did not show the two in relation to each other nor did he comment on how "close" they were to each other. It is very possible that the M. rotundata oxygen consumption rate does lag the activity rhythm. Ralph (1957), working with the earthworm, discovered that the peak of oxygen consumption occurred when the animal had ceased burrowing and was at rest. However, the ability to experience an oxygen debt would be expected in the earthworm as its burrowing activity is performed in a relatively anaerobic environment. In contrast to the earthworm, the bee is active in an environment with an adequate oxygen supply and thus should not be forced to experience an oxygen debt. Chadwick (1947) found that the oxygen consumption rate of Drosophila melanogaster increased in proportion to the wing beat frequency.

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APPENDIX

APPENDIX 1

Steps taken in the spectral analysis of the oxygen consumption data.

For a more rigorous mathematical explanation reference should be made to Mercer (1960 and 1965) and Halberg and Panofsky (1961).

1. The first step was to correct the raw data by removing the mean (mean=o) and any trends. As Megachile rotundata develops from the prepupal to adult stage the rate of oxygen consumption increases. This produces a trend in the data that must be removed before the spectral analysis can have meaning.
2. The data was then analyzed by the method of autocorrelation. The procedure is to take the waveform, delay it by a time T (called lag) and compare it with the original waveform. These values are multiplied together to obtain a product curve. The average of the product wave form, over a long period of time is known as the autocorrelation function. In the oxygen data, lag was equal to 24. In other words, the values were multiplied together 25 times or lag +1 time (0 to 24). For one experiment, lag was increased to 48. This was done because of the length of the time series. Since greater lag produces greater resolution, a greater lag is commonly used with longer time series.
3. If the data were totally periodic, the autocorrelation analysis would be adequate to expose the periodicity. Due to random

frequencies which were present in the oxygen data, the periodicity did not stand out clearly following autocorrelation analysis. The autocorrelation function was then multiplied by a series of cosine waves. When this is accomplished, the resulting average product will differ only from 0 if there is a periodicity equal to that of the exploring cosine.

4. The results are then smoothed to produce the power spectrum where each point represents the average power over a frequency range of a specific width. The spectral points plotted in the graphs reported in the text are the mean points representing the average percent variation that occurred in the data at various frequencies in a given experiment.