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(FABRICIUS) AND NOMIA MELANDERI COCKERELL

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The purpose of the study was to determine an emergence rhythm, its period, and the developmental stages susceptible to emergence synchronization in the leafcutter bee Megachile rotundata (Fabricius). An attempt was made to determine if an oxygen consumption rhythm was present in the pupal and adult stages of the alkali bee Nomia melanderi Cockerell.

A circadian emergence rhythm with a 22 to 24 hour cycle was determined in Megachile rotundata. Temperature was found to be an effective emergence synchronizer. A temperature shock of 11 C° for 12 hours was sufficient to synchronize the emergence of M. rotundata cultures for at least six days. A temperature perturbation as low as six hours synchronized emergence in a culture of M. rotundata but a perturbation of one hour did not saturate the culture and thus a distinct emergence rhythm failed to develop.

Light intensity up to 14 ft-c. was found to have no effect on

the rhythm of emergence.

The phase of the emergence rhythm in M. rotundata is directly related to the time at which a culture is removed from the cold following a temperature shock rather than to the time it is placed in the cold. Cultures subjected to cold periods of 24, 12, and 6 hours displayed close phase relationship to each other when they were removed from the cold at the same time.

M. rotundata appears to be susceptible to emergence synchronization by temperature in the black-thorax and black-abdomen stages of the pupa and in the adult. It is impossible to draw definite conclusions from this study as to which stage is susceptible to emergence synchronization because of the high mortality rate in the insects used.

Various stages of pupal development were studied in an attempt to discover an oxygen consumption rhythm in the alkali bee, Nomia melanderi, but no rhythm was detected.

CIRCADIAN RHYTHMS IN MEGACHILE ROTUNDATA
(FABRICIUS) AND NOMIA MELANDERI COCKERELL

by

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CIRCADIAN RHYTHMS IN MEGACHILE ROTUNDATA
(FABRICIUS) AND NOMIA MELANDERI COCKERELL

INTRODUCTION

"All living organisms are set into the solid framework of the physical world, the structure of which pulses with an abundance of external rhythms: diurnal, tidal, seasonal, solar, sidereal." (Sollberger, 1965, p. 3). The capacity to oscillate with the environment is thus of evolutionary significance to the organic world. It is not surprising then that we find components endowed with rhythmic function in plants and animals.

Although the existence of biological rhythms had been known for many years fundamental investigations into the nature of the 'biological clock', or control of rhythms, began only about 30 years ago. Many of the more basic studies on biological rhythms have been done on insect activity and emergence. These studies have revealed that light is the most common synchronizer or phase-setter of biological rhythms. This is to be expected as of all the known environmental factors that entrain rhythms light is the most constant. Most animals live in environments that experience considerable daily temperature variation and thus a temperature-dependent 'clock' would guarantee only mistiming (Pittendrigh, 1954).

Both bee species used in this study, the leafcutter bee Megachile rotundata (Fabr.) and the alkali bee Nomia melanderi

Cockerell, are important alfalfa pollinators in the Northwestern States. They both develop in complete darkness from egg to adult and their rate of development as overwintering prepupae is temperature dependent (Stephen and Osgood, 1965; Stephen, 1965b). It had been reported that emergence in both bee species occurs only in the morning for a period of three to five hours (Stephen, 1959 and 1966). When held in darkness both M. rotundata and N. melanderi exhibit a circadian type activity rhythm (Stephen, 1966).

Megachile 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 emerged 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 phase-setter for the adult emergence rhythm. Essentially, this was established by Stephen (1965a) and Osgood (1964). Very few known insect emergence rhythms appear to be phase-set by temperature. The fact that M. rotundata emergence is phase-set by temperature and that it appears to be independent of a light:dark regime makes it relatively unique.

Nomia melanderi develops from egg to adult in cells carved in the earth at a depth of from five to ten inches (Stephen, 1959). Stephen (1965b) found that prepupae and pupae held at alternating temperatures (29-17° C, 32-17° C) developed at the same rate as those held constantly at the upper of each of the temperature pairs. No development was observed in prepupae held at 17° C for over a year and therefore it was anticipated that the cultures experiencing 12 hours of 17° C each day would not develop as quickly as those held constantly at 29 or 32° C. It may be that 'developmental zero' for diapause breakage is not identical with 'developmental zero' for growth or it could be that growth is rhythmic and thus does not take place for a certain period within the 24 hour day. If growth is rhythmic it was assumed that it would be reflected by an oxygen consumption rhythm. Again, since N. melanderi experiences its development from egg to adult below the surface of the ground, it was expected that light would not be the emergence rhythm phase-setter.

The objectives of the study were as follows: Megachile rotundata- (1) to determine an emergence rhythm and its period; (2) to determine the developmental stage or stages in which the 'clock' controlling the rhythm can be phase-set. Nomia melanderi- (1) to determine an oxygen consumption rhythm and its period; (2) to discover the developmental stage or stages in which the 'clock' controlling the rhythm can be phase-set.

LITERATURE REVIEW

Rhythms found in the organic world may be passive--entirely driven by some external agent, a synchronizer, or 'zeitgeber', or they may be active--representing spontaneously oscillating systems with natural periods. Active or endogenous oscillations that have periods corresponding to the external 24 hour day are called diurnal or circadian (circa = about, dies = a day) rhythms (Sollberger, 1965).

Pittendrigh (1960) lists 16 'empirical generalizations' about circadian rhythms but Harker's (1961) three main characteristics are most pertinent to this study. These three characteristics are: (1) circadian rhythms are persistent for at least two or three days in a constant environment; (2) they are little affected by temperature within normal biological limits; (3) the timing of the peaks or phases of the rhythms in relation to solar time is originally determined by an environmental change, such as a variation in light intensity or temperature.

Rhythm Synchronizers

Since most animals are nocturnal, diurnal, or crepuscular in their habits the observation is made that light is the most important of the environmental variables which affect the timing of the phases of a rhythm (Harker, 1964). However, it is difficult to determine

which of the features of the day-night cycle are responsible for the synchronization or setting of a rhythm. Chaffinches, which are day-active, were found by Aschoff (1960) to have their phase-setting at least partly determined by a change from darkness to light. Harker (1955) found that cockroaches, which are night-active, experience a burst of activity in the first hours of darkness followed by a decrease in activity. It would appear that the cockroach activity rhythm is set by a light to darkness change.

Light intensity has also been found to be a phase-setter. DeCoursey (1960) discovered a close correlation between the time of onset of activity in flying squirrels and a narrow range of light intensities at dusk.

An example of the the effect of photofraction on the eclosion of adult Drosophila is given by Brett (1955). He found that when the light fraction of the 24 hour cycle is close to that of natural conditions the flies emerge at the beginning of the light period, that is 'at dawn'. If, however, an LD4:20 cycle (light-4 hrs; dark-20 hrs) is operating the flies emerge during, although towards the end of, the dark period.

The majority of organisms are less sensitive to cycling temperature than to cycling light intensities (Harker, 1964). Functionally this is to be expected as temperature is a much more erratic environmental variable than light is. Temperature has been found

to be a phase-setter in *Euglena* (Pittendrigh and Bruce, 1957), the beetle Ptinus (Bentley, Gunn and Ewen, 1941), and in the two cockroaches Leucophaea and Periplaneta (Roberts, 1962; Cloudsley-Thompson, 1956). In plants the phase-setting of the sporulation rhythm of Pilobolus and Oedogonium has been found to be temperature sensitive (Harker, 1964).

Feeding time has also been found to be a phasing factor. This has been shown in a variety of animals such as bees and ants (Harker, 1964) and rats (Richter, 1927). Harker (1955) showed quite conclusively that food is not a phase-setter in the cockroach.

Other environmental factors such as barometric pressure and humidity have been investigated for their effect on endogenous rhythms. The results, however, are ambiguous and inconclusive (Harker, 1964).

Most of the workers in the field of circadian rhythms adhere to the theory that the organism is an independent, oscillating system with one or more of its own natural periods closely approximating a day. Entrainment of the organism's circadian periods is accomplished chiefly by the environmental light and temperature cycles. Perhaps the chief leaders and supporters of this theory of circadian rhythms are Aschoff (1965) and Pittendrigh (1960). An alternative hypothesis is advanced by Brown (1965) who states that the organism possesses no intrinsic daily rhythmicity under natural conditions

but is rhythmic as a consequence of responding simultaneously and continuously to such rhythmic geophysical factors as light and temperature on the one hand, and to 'subtle' pervasive geophysical factors on the other (Brown, 1965). In other words there is a 'duplicity of daily rhythmicity'. Brown (1965) draws on many illustrations from his own work to show that organisms respond to such geophysical factors as weak magnetic fields, weak electrostatic fields, and weak gamma fields.

The mechanism whereby the organism transforms a geophysical, periodic input into a 'circa'-periodic output Brown has termed autophasing. He has not attempted to explain this mechanism specifically whereby the organism receives stimulation of variable frequency from the environment and transforms it into rhythmic output.

Insect Emergence Rhythms

Periodic phenomena connected with adult emergence of holometabolic insects has not been intensively studied and the existing data are contradictory in many respects. No doubt there is diversity in the holometabola in their response to environmental stimuli but perhaps some of the discrepancies present in the literature are due to faulty experimental method. It is difficult to control conditions during an experiment--a flash of light or a slight alteration in temperature may phase-set the 'clock' in the organism under study and

yet go undetected by the investigator. However, studies on emergence rhythms of holometabolic insects afford some of the clearest evidence of temperature-independent clock systems in invertebrates (Cloudsley-Thompson, 1961). The literature on the subject has been reviewed by Palmen (1955) and Cloudsley-Thompson (1961).

Light as the Synchronizer

Bremer (1926) working with the moth Ephestia Kuehniella Zell., reported that a definite peak of emergence occurred between 1500 and 2000 hours when the cultures were kept under normal daily fluctuations of light. When he reversed the light regime under which the developing pupae were kept a partial reversal of the rhythm occurred, that is, 60 percent of the moths emerged in the morning instead of the afternoon. However, a high proportion of the insects still maintained their original rhythms in spite of the reversed conditions. Bremer (1926) quotes some observations by earlier writers stating that many butterflies and Sphingid moths emerge in the morning and Noctuid moths emerge in the evening.

While working with the fly Pegomyia hyoscyami Panz. Bremer (1926) found that adult emergence occurred in the morning between 0600 and 1200 hours in the normal photoperiod. When the light conditions were reversed under experimental conditions a corresponding change occurred in the time of emergence.

Phillipp (1938) working with the aquatic chironomid Chironomus thummi Kieff. suggested that the periodicity of adult emergence was correlated with diel changes of light intensity. He found that C. thummi under normal environmental illumination displayed maximum adult emergence during the light period of the day with very few emerging between 2000 and 0400 hours.

Mell (1939) reporting data on 202 species of Chinese Lepidoptera states that butterflies as a rule emerge after the change from darkness to light.

Suomalainen (1940) reported that a culture of the lepidopteran Leucodonta bicoloria Schiff. kept under normal photoperiod conditions displayed 54 percent adult emergence between 1200 and 1500 hours, with both sexes showing a distinct daily emergence peak at the same time. In Parasemia plantaginis L. the males emerged between 1800 and 2100 hours while the females showed no definite emergence peak. However, Suomalainen indicated that the number of females may have been too low to yield significant results.

Lewis and Bletchley (1943) working with Scopeuma (Scatophaga) stercocaria L. in the laboratory where the light intensity varied according to the normal daily cycle observed that the adults emerged mainly between 0900 and 1400 hours. Cultures that were kept under constant light or darkness still displayed emergence peaks but these peaks were less pronounced than in those that experienced the

normal light cycle. Lewis and Bletchley concluded that the normal photoperiod is at least contributory in controlling the emergence rhythm, and that alterations of periodic conditions of the environment may be able to modify the course of emergence.

The moth Sparganothis pilleriana Schiff. was shown by Gotz (1949) in a field experiment under normal photoperiod conditions to experience two definite peaks of emergence in a 24 hour period. The first occurred in the morning between 0600 and 1200 hours and the second in the evening between 1900 and 2300 hours. However, Gotz states that since the temperature and relative humidity of the air fluctuated daily from 14 to 28° C, and from 45 to 95 percent, respectively, it is difficult to determine the factors governing the periodicity of emergence.

In studying the marine midge Clunio marinus Halid. Caspers (1951) found that adult emergence on the Helgoland coast and at Varna, on the Black Sea, coincided according to local time--this being between 1630 and 1900 hours in mid-August. Animals transferred from Helgoland to Varna emerged about 80 minutes earlier than they would have if no adjustment to local time had taken place. Caspers concluded that since they adjusted to local time light was probably used as the 'clue'.

Working with nine species of chironomid midges in their natural habitats Palmén (1955) observed a definite peak of emergence

during the hours following sunset. Since there was a time difference of about two hours between the emergence peaks in June and August Palmén concluded that the timing of the mechanism governing periodic emergence is achieved by the day to night change in illumination.

Remmert (1955) showed that the emergence rhythm of the chironomid, Pseudosmittia arenaria Stenzke, is dependent on the length of day and specifically to the beginning of the light period. Under natural alternating light and dark conditions maximum emergence occurs six to eight hours after the beginning of the light period. In constant darkness there is no adult emergence and in constant light emergence is distributed evenly throughout a 24 hour period. Light intensity between 18 and 350 lux was shown to be of no importance.

Barnes (1930) studied emergence of Cecidomyiidae in some detail and found that the time of emergence varies according to the species. He suggested that the factors involved acted differentially on different species. Barnes also observed that the males usually emerged slightly before the females. In Dasyneura alopecuri Reut., the major emergence occurred between 0800 and 1100 hours, and nearly all the males had emerged by 1100 hours, while the females continued to emerge until 1600 hours. In Stictodiplosia geniculati Reut., 81 percent of the males and 58 percent of the females

emerged by 0900 hours, the bulk of the males, however, emerging by 0545 hours. In Contarinia merceri Barn., emergence occurred around noon, 65 percent of the males and 33 percent of the females emerging before noon, the rest emerging after noon. In Dosyneura pyri Bouche 36 percent of the males and 18 percent of the females emerged by noon and the others emerged after noon. In Rhabdophaga terminalis H. Lw., Barnes found emergence to begin slightly before 0800 hours, the peak of emergence of the males occurring at 1000 hours, and of the females at noon. Cultures of the above species were kept in constant darkness from December to April and from December to July. After this treatment midges from cultures that were transferred to normal photoperiod conditions before the onset of emergence displayed a daily peak of emergence corresponding to the normal daily cycle. It was also observed that the periodicity persisted even if emergence took place in total darkness.

Certainly no single insect genus had been used as extensively and yielded as much basic information about circadian rhythms as has Drosophila. A number of workers have studied circadian rhythms using Drosophila melanogaster L., and D. pseudoobscura Sturtevant (Kalmus, 1935, 1940; Bunning, 1935; Brett, 1955). However, Pittendrigh's (1954) work with D. pseudoobscura is probably the most extensive and precise. He found that the period of the eclosion rhythm is temperature independent. Cultures under a

normal light regime (LD 12:12) showed emergence between 0600 and 0900 hours. 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 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 Drosophila to account for the observed facts. He stated that the clock system of Drosophila consisted of a 'terminal clock' comprising temperature-sensitive events occupying, when properly 'adjusted', the 24 hours immediately prior to eclosion, and a temperature insensitive 'primary clock' that measures throughout the development of the fly 24 hour intervals between reference points in phase with the last seen dawn. In a later paper (Pittendrigh and Bruce, 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 arrhythmic 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 Drosophila 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 is a peripheral system coupled to and phased by the A-oscillation and 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 transient 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 Drosophila. 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 Drosophila circadian emergence rhythm, verified by Pittendrigh (1954), is its persistence under DD (continual darkness) conditions. When cultures entrained to an LD light 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 shock. 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 hours apart (Pittendrigh, 1954). Obviously then, the individual flies in a culture are not synchronized in development throughout their lives.

The shape of the daily emergence curve and the way this shape changes with the age of the culture suggests that synchronization of development is accomplished late in the history of the individual fly by the daily enforcement of a period that is forbidden for emergence itself or for the initiation of processes leading up to it (Pittendrigh, 1954).

Suppose the 24-hour period to be partitioned, by means of information from the endogenous clock, into a relatively short period (the data suggests 6 hours or less) and a long (18-hour) forbidden period. Flies that happen to be ready for emergence within the short allowed period emerge without further delay. However, flies becoming ready for emergence at random times within the forbidden period are required to wait onset of the next allowed period. Such a model, entirely formal as it is, explains three major facts: (1) how effective synchronization within daily periods of 6 hours is accomplished among individuals whose development rates show in other respects the variance of a nearly normal distribution spread over 8 days; (2) the way the peak is skewed heavily to the right within each allowed period (this is due to the fact that at the beginning of the short allowed period the bulk of the emerging flies have long since been ready and emerge immediately on removal of restraint); and, (3) most significantly, the way in which the shape of the daily peak is observed to change with culture age. Each emergence peak consists of two fractions; AP is the fraction composed of flies whose completed development falls by chance within the allowed period; FP is the fraction of flies whose completed development falls within the preceding forbidden period and were required to wait. For each culture there will be only one day when AP/FP is large, viz. the first day of emergence, containing the fastest flies in the culture. In this case the emergence peak should be either normally distributed within the allowed period or even skewed to the left, and this is precisely what is observed. As the culture becomes older, AP/FP should become progressively smaller and the skew to the right should increase which, again, is what is observed. (Pittendrigh, 1954).

Harker (1965) points out two problems in Pittendrigh's AP/FP concept for Drosophila eclosion. There is no evidence that pupal development is completed at all times of the night, and it is difficult to apply Pittendrigh's interpretation to strains of Drosophila that display dual emergence peaks such as the strain 'wild' of D. melanogaster (Harker, 1965). In studying the factors affecting the time of eclosion of Drosophila Harker (1965) divided pupal development into three stages for observation purposes. It was determined that the time of completion of a stage is dependent on the phasing of the time of entry relative to the light-darkness cycle, and is independent of the light-darkness condition at the end of the stage. The time-interval curves for all three stages take the same form, in spite of the fact that in some stages the majority of the pupae reach the end of the stage during the dark period and in others they reach the end during the light period (Harker, 1965).

The final eclosion rhythm is dependent on whatever processes control the developmental rate throughout the pupal stage. The eclosion rhythm is a population effect, and does not reflect the phasing of individuals to a dawn eclosion. Individuals can, and do, undergo eclosion at nearly all hours of the day and night, but the majority emerge at dawn because of the summation effect of circadian rhythms of development at earlier stages. (Harker, 1965).

Work showing that the eclosion rhythm of some insects can be set in the larval stage has been done by Bateman (1954) and Myburgh (1963). Bateman (1954) showed that the emergence rhythm of the

Queensland fruit-fly Dacus (Strumeta) tryone can be set in the larval stage or in the adult stage of the previous generation. He found that a rhythm could be induced by alternation of light and darkness in the larva or adult of the previous generation but not during the pupal stage. It was also shown that when the larvae were exposed to a small daily fluctuation in temperature the emergence rhythm was more pronounced with most of the flies emerging within a few hours after the initial increase in temperature.

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 (1963) 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.

Temperature as the Synchronizer

Scott (1936) showed that in the moth Ephestia kuehniella 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 Ephestia kuehniella 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 5 C°. This also agreed with Scott's work.

Insect Oxygen Consumption Rhythms

There is little information in the literature on insect oxygen consumption rhythms and the few insects that have been reported display considerable disparity. A carbon dioxide output rhythm has been shown to exist in the locust Schistocerca gregaria Forsk in which the period was 2.5 minutes in darkness at 32° C (Hamilton, 1959). Hamilton (1959) also reports a rhythm of carbon dioxide output in adult cockroaches.

Schneiderman (1956) and Levy and Schneiderman (1958) showed that the period of the rhythm of emission of carbon dioxide in the *Cecropia* silkworm pupae, Hyalophora cecropia, varied between 45 and 120 minutes at 25° C. The carbon dioxide output rhythm of Periplaneta americana was shown to vary according to the temperature and the amount of ambient carbon dioxide (Wilkins, 1960).

None of the above reported an oxygen rhythm, except that Levy and Schneiderman (1958) did comment to the effect that the *Cecropia* silkworm pupa appeared to take in oxygen almost continuously. Beck (1964) found an eight hour oxygen consumption rhythm in the diapausing larvae of the European corn borer Ostrinia nubilalis under LD12:12 conditions. The most prominent peak occurred at the onset of darkness and the subsequent peaks of the rhythm were much less prominent. Beck found that this rhythm was phase-set by the lights-off signal.

A similar rhythm was detected in the adult German cockroach, Blatella germanica. 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 also of interest to point out that the prominent peak of oxygen consumption coincides with the position of the circadian peak of locomotor activity demonstrated in other roach species by other workers (Harker, 1956; Roberts, 1960).

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

moth, Epeestia kuehniella, was not able to detect an associated oxygen consumption rhythm.

CIRCADIAN RHYTHM OF ADULT EMERGENCE
IN MEGACHILE ROTUNDATA

Materials and Methods

Cells of Megachile rotundata containing diapausing prepupae were taken from straws that had been held at 7° C for a number of months. The prepupae were placed in plastic petri dishes of 100 bees per dish (Figure 1), covered, and placed in the incubator. The bees were checked once a day for between 15 and 18 days until emergence took place. Following commencement of emergence observations were made hourly during the emergence periods.

Two incubators were used during the course of this study. One was a walk-in type incubator located in the Microbiology Department on the fourth floor of the Agricultural Building. The second incubator consisted of two boxes (Figure 2) which were placed in a room in the Horticulture cold storage on the fourth floor of Cordley Hall. To avoid needless repetition these incubators will be referred to as incubator-1 and incubator-2 respectively in the remainder of the text.

Incubator-1, which was used for the first few experiments, was discovered to be undergoing a temperature fluctuation of about 2.5 C°. Following this discovery the experiments were moved to incubator-2 in which it was possible to keep the temperature constant to within about 0.25 C°. Incubator-1 was held at a temperature of 31° C as



Figure 1. Petri dish containing 100 cells of Megachile rotundata.



Figure 2. Incubator boxes used for Megachile rotundata emergence studies. (Incubator-2).

the incubator was already set for this temperature when it was obtained, and it was within 1 C° of the optimum temperature for Megachile pupal development of 32° C.

The temperature in incubator-2 was maintained at 32° C. A continuous temperature recorder was used which showed that temperature varied, according to the instrument, no more than about 0.25 C°. However, following failure of the recorder, temperature readings using a potentiometer were taken at various intervals over a 24 hour period.

The temperature, or cold shock, was effected by placing the bee cells in a refrigerator registering a temperature of 18° C. Use had to be made of existing equipment and for the first few experiments the available refrigerator was set at 20° C.

In incubator-1 the light source consisted of two 25-watt fluorescent lights placed at a distance of two feet above the petri dishes containing Megachile rotundata. The resulting light intensity reaching the petri dish covers was 14 ft-c. In incubator-2 the light source was a clear 200 watt incandescent light situated six feet above the glass top of the box incubators (Figure 1). This resulted in a light intensity of 10 ft-c. reaching the top of the petri dishes containing the bee cells.

Constant air circulation was maintained in both incubators by means of a fan to reduce temperature gradients to a minimum.

A red light source was used to facilitate counting during dark periods.

Effect of Light

Two groups of Megachile rotundata each containing 200 prepupae were placed in incubator-1. One group was placed in an LD 12:12 light regime and the other in DD conditions. The dark conditions were obtained by placing the petri dishes containing Megachile into a cardboard box which in turn was covered with a black cloth. This box was placed at a similar distance from the heat source to that of the bees exposed to light, but the box was not exposed to direct light. The petri dishes were checked every hour when emergence was taking place and the emergents were removed and destroyed.

Effect of Temperature

Eight hundred prepupae, 400 in DD and 400 in LD 12:12 conditions were placed in incubator-1. An attempt was made to synchronize the emergence of the adults by exposing the bees to a temperature shock after emergence had begun. The temperature shock was accomplished at different times during the 24 hour period in the following manner: two dishes (100 insects per dish), one from each light regime, were given a two hour temperature shock from 0900

hours to 1100 hours; four dishes, two from each light regime, were placed in the cold for a 12 hour period--two dishes were temperature shocked from 1000 hours to 2200 hours and two dishes from 2200 hours to 1000 hours. Two dishes, one from each light regime, were used as controls. At the end of the cold period all the dishes were returned to incubator-1 and emergence was recorded. The LD 12:12 light regime operated with the light on from 2200 hours to 1000 hours.

In a second experiment the number of insects used was increased to 2400. All the insects were placed on an LD 12:12 light regime with the lights on from 0800 hours to 2000 hours. The temperature shock, effected after emergence was first noted, was the same as in previous experiments except that the insects were left in the cold for 24 hours instead of 12 hours. A temperature variation of about 2.5 C° was detected in incubator-1 and thus work was transferred to incubator-2 and the above experiment repeated. Twenty four hundred insects were again used but 400 of these did not undergo a temperature shock and were used as a control. Readings were taken hourly during the emergence of the test insects, and taken every two hours in the controls.

Effect of Duration of Temperature Shock

An attempt to determine the duration of the cold period, or temperature shock, required to induce an emergence rhythm in

Megachile rotundata was executed in incubator-2. Five hundred insects were used in each of four tests plus 400 insects used as a control. The insects were temperature shocked for 24, 12, 6, and 1 hours. The 24 hour temperature shock was initiated at 2300 hours on one day and terminated at 2300 hours the following day. The other three tests were temperature shocked at the appropriate hours to facilitate removal from the refrigerator together at 0300 hours on the second day. The temperature shock was effected the day after emergence was first noted. As before the control group was checked every two hours.

Stage Susceptible to Synchronization

Megachile prepupal cells were freed from their straws, cut open to expose the diapausing prepupae and placed individually into glass tubes which were sealed at both ends with paraffin (Figure 3). The tubes were then placed in plastic petri dishes which contained corrugated cardboard to restrict the movement of the tubes. Each petri dish contained a maximum of 30 cells or tubes.

The intent of the experiment was to divide the cells into six groups of 50 individuals each. However, approximately 70% mortality occurred in the prepupal stage and therefore 50 insects were not available for each test group. The stages tested for susceptibility to synchronization were the prepupal stage, the red-eye, the

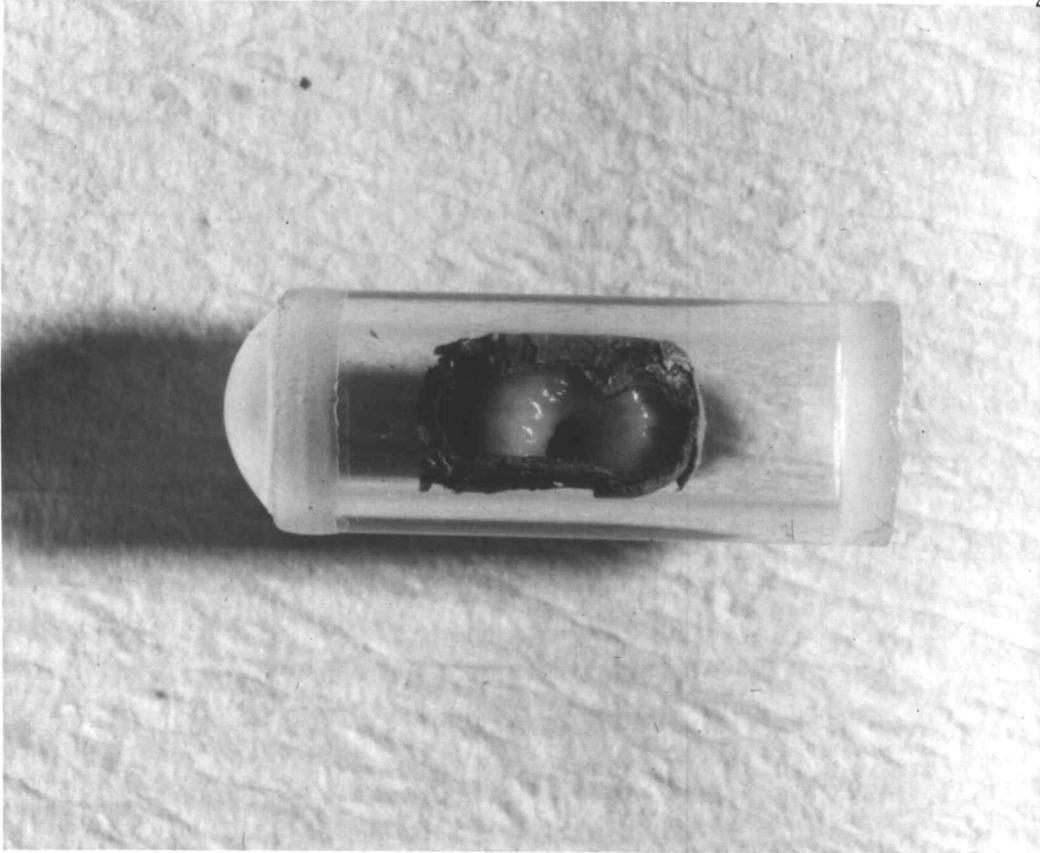


Figure 3. Glass tube containing an exposed Megachile rotundata prepupae.

black-thorax, and the black-abdomen stage of the pupa, and the adult stage. Fifty insects were reserved for a control. Since the mortality rate was so high and previous experiments indicated that the adult stage was susceptible to temperature synchronization it was decided to omit the adult test. The insects were temperature shocked for a 12 hour period, from 1100 hours to 2300 hours. The light regime and method of temperature shock were the same as in the previous experiments carried out in incubator-2. The emergence readings were taken every two hours between 0800 and 2400 hours with no readings taken from 0100 to 0700 hours.

Results and Discussion

Effect of Light

The effect of light on the Megachile emergence rhythm is recorded in Figure 4. The insects, being held in constant temperature, were not expected to show an emergence rhythm unless light acted as the synchronizer, nor were they expected to show distinct peaks of emergence. No rhythm appeared but both groups showed definite diurnal peaks of emergence. However, it is indicated that a temperature fluctuation may have been occurring which was resetting the clock each day because there were such distinct, arrhythmic emergence peaks and the two groups were not entirely

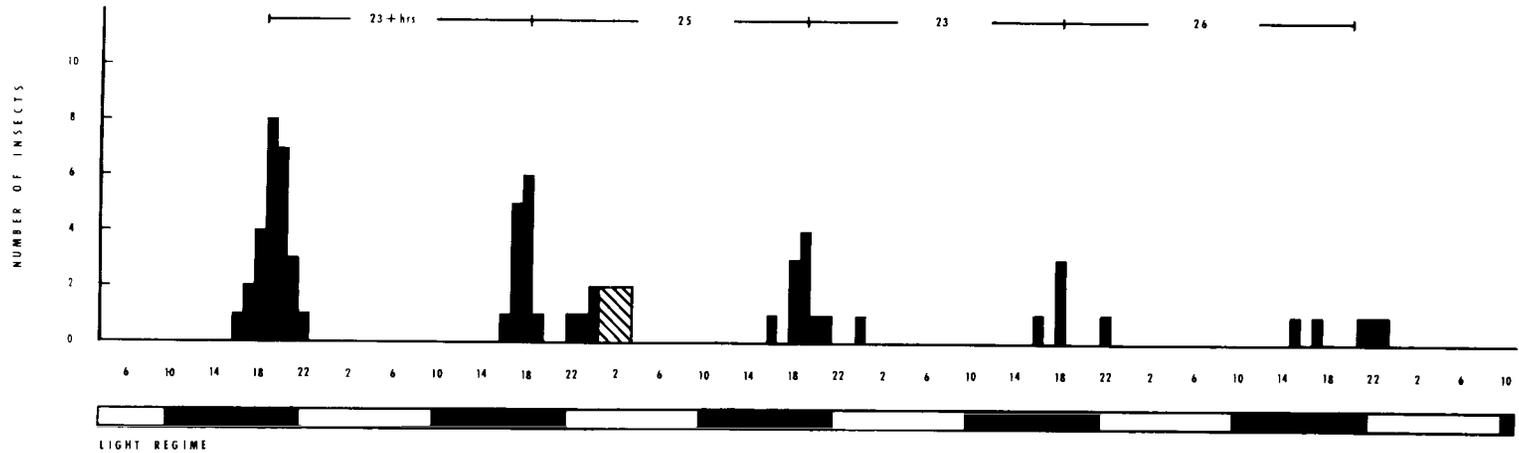
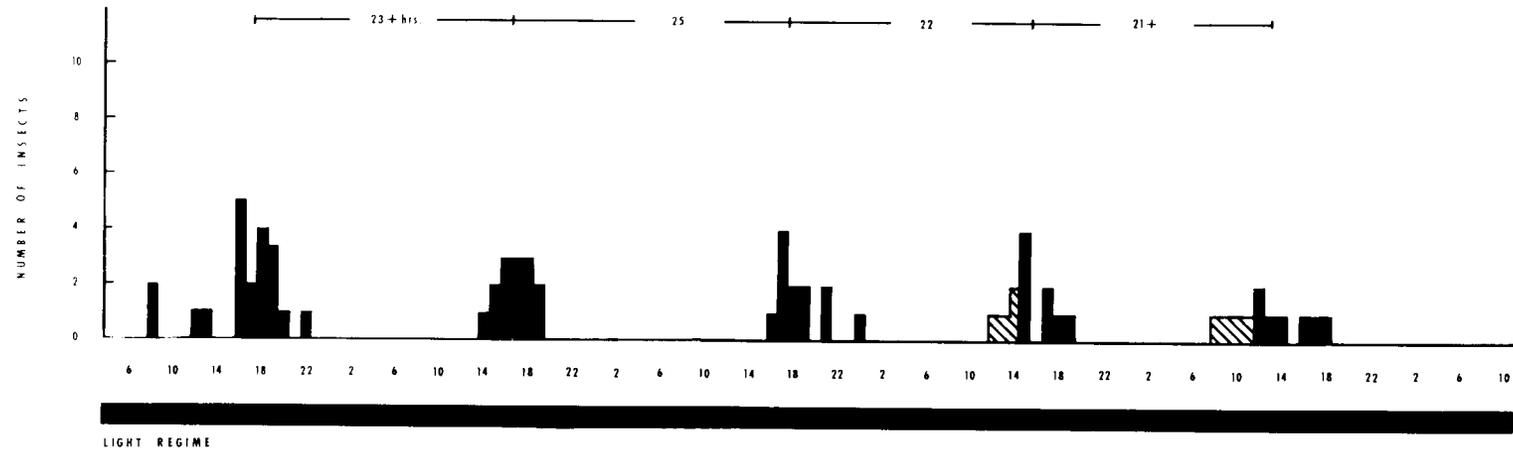


Figure 4. Effect of light on the emergence rhythm of *Megachile rotundata*. (Period determined by the number of hours between the mean emergence in each peak).

asynchronous. As has been mentioned above, a temperature fluctuation was detected in a later experiment but whether it occurred in this experiment is a matter of conjecture. It would seem that peaks may occur in emergence which do not reflect an emergence rhythm, as peaks occurred in a later experiment in a control group which displayed no emergence periodicity (Figure 4).

Apparently light (Figure 4), with an intensity of 14 ft-c., does not phase-set the emergence rhythm in Megachile rotundata. Although the emergence peaks of the LD and DD groups did not occur at exactly the same time each day they did remain approximately the same number of hours apart. If light was resetting the clock in the insects under the LD conditions the emergence peaks should have displayed a more definite period and they should have changed in their relationship to the emergence peaks of the insects in the DD light regime.

Effect of Temperature

The results were inconclusive in the first experiment using 800 bees in DD and LD light conditions. No emergence rhythm was detected and yet in future experiments a temperature shock of similar duration (12 hours) but with a degree difference (11 C°) initiated a definite rhythm (Figure 6). This lack of an overt rhythm could have been due to the fact that too few insects were used or that again

the temperature was fluctuating in incubator-1 and thus resetting emergence each day. The temperature shock may have been applied too late in the experiment, occurring after the bulk of the insects had emerged, thus not allowing enough time for a rhythm to develop.

The data from the two experiments using 2400 insects each (Figures 5 and 6) indicates that there is a definite rhythm of emergence in Megachile rotundata and that it is temperature sensitive. The data from Figures 5 and 6 indicates that M. rotundata has an emergence rhythm of between 22 and 23 hours under constant conditions. During the experiment performed in incubator-1 the temperature was observed to fluctuate from 30.2 to 32.5° C, a range of almost 2.5 C°. It is very possible then that the clock was reset during the fifth peak, and thereafter, after the temperature shock (Figure 5) which would account for the 24 hour period that followed. The fact that the culture returned to a 22 hour period towards the end of the experiment is difficult to explain except that it is possible that it was due to a temperature fluctuation occurring at a different time in the 24 hour day than it had occurred before.

Further evidence is displayed in this experiment (Figure 5) to show that light at 14 ft-c., does not affect the emergence rhythm of M. rotundata. Inception of light, 0800 hours, occurred near the beginning of emergence in the early part of the experiment but as the experiment progressed light onset occurred in the middle of the

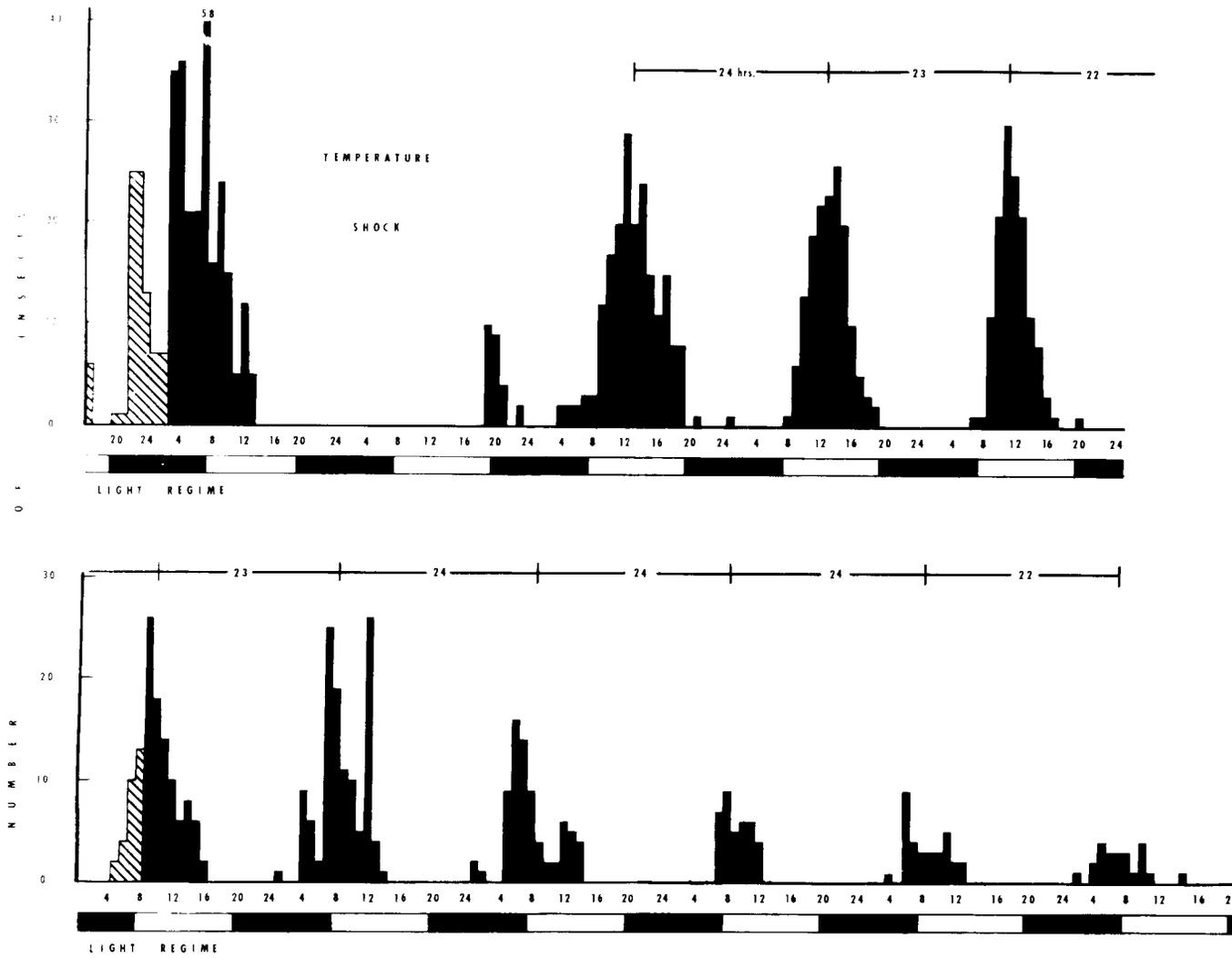


Figure 5. Effect of temperature on the emergence rhythm of *Megachile rotundata* under LD 12:12 light conditions.

emergence period.

In the second '2400-insect' experiment (Figure 6) the insects were exposed to an LL light regime and the emergence rhythm, 22+ hours, was the same as in the first. This provides further evidence that light does not affect the M. rotundata emergence rhythm. In this experiment the light intensity was 10 ft-c. The last peak (Figure 6) was spread over a 22 hour period whereas the earlier peaks showed emergence taking place within a ten to 12 hour period. This indicates that towards the end of the experiment many insects were emerging that had not been phase-set by the temperature shock.

Effect of Duration of Temperature Shock

The data (Figure 7) indicate that a temperature shock duration of 24, 12, and 6 hours will initiate an emergence rhythm in M. rotundata. The rhythm is not as well defined as it is in Figures 5 and 6 but this could have been due to the reduced number of insects. The one hour temperature shock appeared to initiate a rhythm which lasted for only one cycle. It is possible that the adults ready to emerge when the cold period was initiated were the only bees synchronized; it is also possible that the 22+ hour period in the first steady-state cycle following the transient was coincidental.

It would appear that the length of the cold, or temperature shock period is not important. There must be a minimum cold

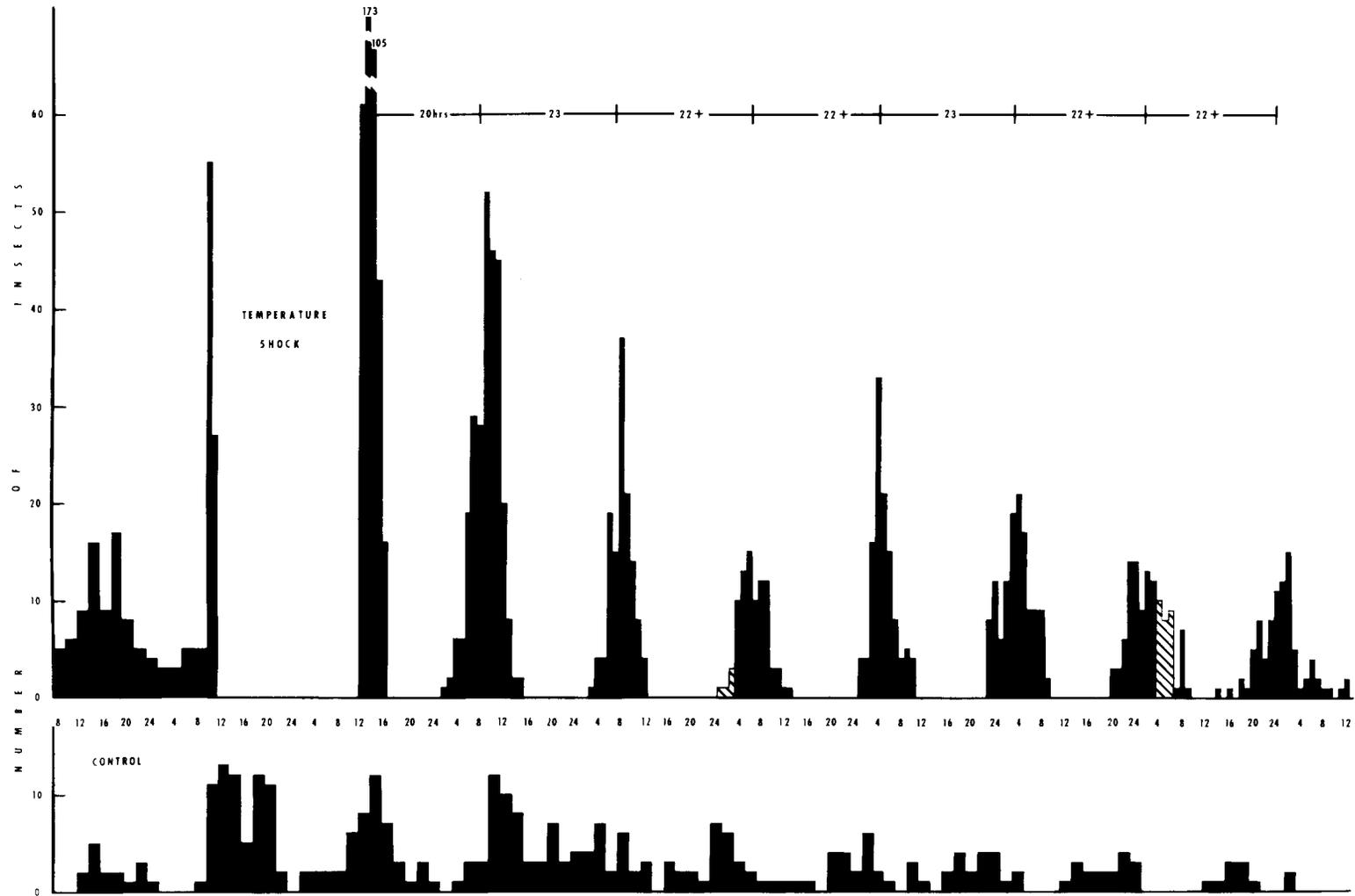


Figure 6. Effect of temperature on the emergence rhythm of *Megachile rotundata* under LL light conditions. (Control insects checked every 2 hours).

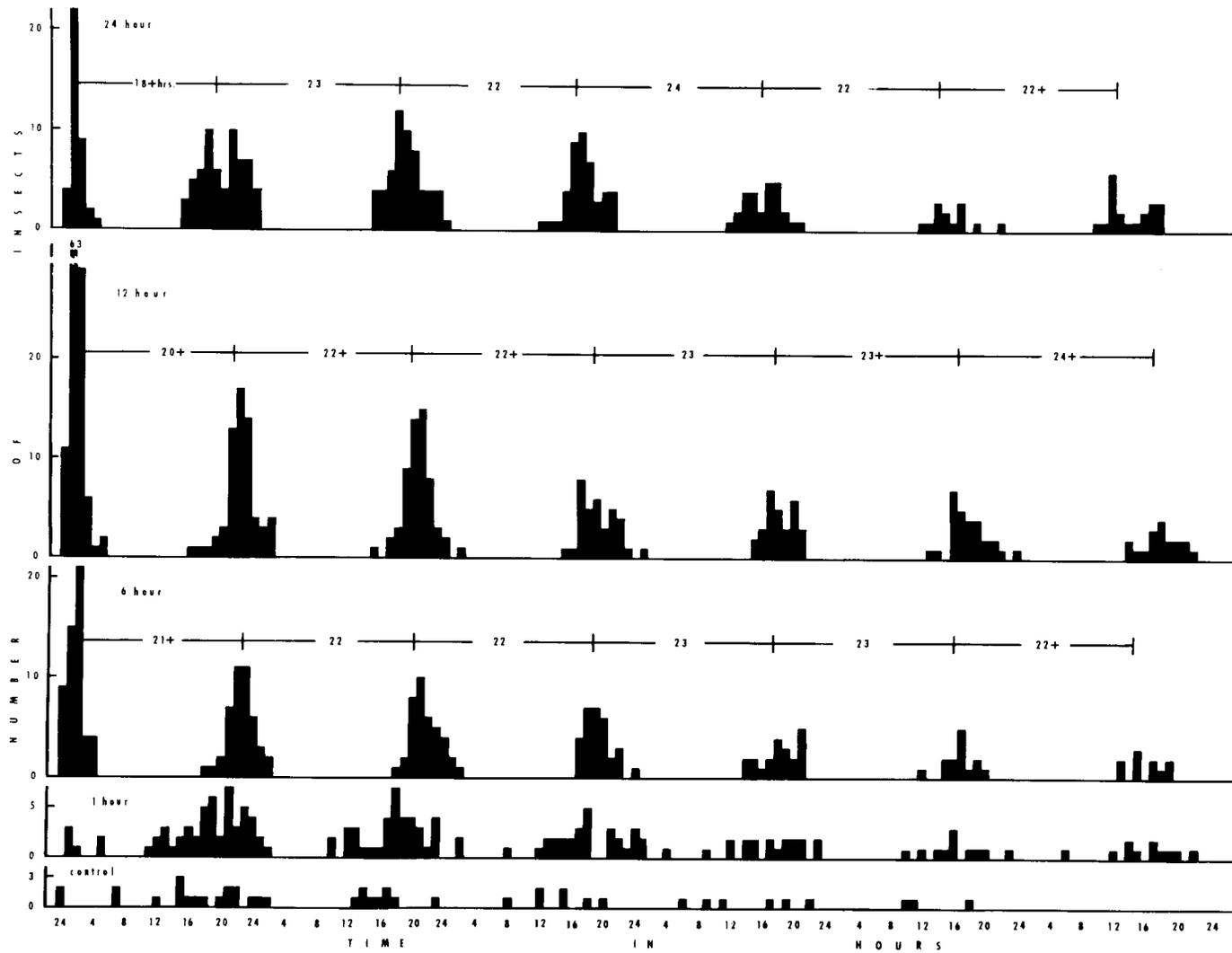


Figure 7. Duration of temperature shock required to phase-set the emergence rhythm of *Megachile rotundata*. (Control insects checked every 2 hours.)

period required to initiate a rhythm of emergence in M. rotundata but this was not determined in this experiment.

The single transient that followed each temperature shock, both in this experiment (Figure 7) and in the previous experiments (Figures 5 and 6) was to be expected. Most workers studying circadian rhythms report the occurrence of such transients which Pittendrigh (1960) defines as the interval between peaks of eclosion that is different from the recurrent interval that defines the period of the steady-state.

Stage Susceptible to Synchronization

Because of the high mortality of the prepupae, about 70%, only a few insects were available for testing. It is therefore recognized that the data (Table 1) from this experiment is not adequate for definite conclusions about the stage of development susceptible to synchronization in M. rotundata. Work is presently underway in an attempt to obtain a higher percentage of viability of the exposed prepupae. There are several possibilities as to why there was such a high mortality rate. Exposing the prepupae to continuous light may have inhibited development, although a preliminary experiment where the prepupae were held in continuous light displayed normal prepupal viability. In an attempt to overcome the continuous light problem about 100 prepupal cells were placed under LD 12:12

conditions. However, the prepupae were not placed in LD 12:12 conditions until after they had been exposed to LL conditions for 12 days. The alternating light-darkness light regime did not stimulate prepupal development. It is possible that the insects may have been damaged in handling.

Table 1. Effect of temperature shock on various developmental stages of M. rotundata. Twelve hour cold period terminated at 2300 hours.

Insect Stage	Time of Observation (hours)*								
	08	10	12	14	16	18	20	22	24
Control	6	--	--	1	3	--	1	1	--
Prepupae	2	1	--	--	--	--	--	--	--
Red Eye	--	4	3	5	1	3	2	1	3
Black Thorax	--	--	--	5	7	2	6	2	1
Black Abdomen	--	--	--	3	8	9	3	3	2

* No observations between 0100 and 0700 hours.

The data in Table 1 indicates that M. rotundata is susceptible to synchronization by temperature in the black-thorax and black-abdomen stages of pupal development. In other experiments with adults (Figures 6 and 7) using the same temperature shock time, the resulting rhythms possessed an allowed emergence between 1200 and 0200 hours. Insects receiving the temperature shock in the black-thorax and black-abdomen stages emerged between 1400 and 2400 hours, thus it appears that their biological clocks were phase-set. The results

for the red-eye stage are difficult to explain as emergence was limited to a period between 1000 and 2400 hours and yet it was not restricted to a 1200 to 0200 hour period. Obviously, the experiment needs to be repeated using a greater number of insects.

CIRCADIAN RHYTHM IN OXYGEN CONSUMPTION
IN NOMIA MELANDERI

Methods and Materials

The insects were taken from a sample of 30 diapausing prepupae that had been put in an incubator for ten days at 30° C. The optimum temperature for N. melanderi development is 29° C (Stephen, 1965b) but the only available incubator was set at 30° C. Fourteen insects were taken from the above sample and placed singly into 14 microvessels. These vessels were then placed in a Gilson Differential Respirometer which had been previously calibrated to 30° C. Care was taken to expose the insects as little as possible to the external environment in transferring them from the incubator to the respirometer. Readings were taken every two hours for a 127 hour period. After 73 hours the temperature was dropped 10 C° from 30° C to 20° C for a 12 hour period in an attempt to determine if an oxygen consumption rhythm could be established.

Oxygen consumption was read in microliters directly from the respirometer. No attempt was made to control the light conditions as the respirometer was located in a room used by a number of classes.

In a second experiment constant light was maintained using the fluorescent light attached to the respirometer. However, the light

intensity at the surface of the water bath underwent a daily variation between 0.2 and 6.5 ft-c. as the room, being used by classes, experienced varying light conditions. This was not the actual light intensity reaching the insects as they were in microvessels which were located immediately below the surface of the respirometer water bath. The temperature was maintained at 29° C. Twelve bees were used instead of 14 as this was the number of bees that had pupated when the respirometer became available. One of the 12 did not pupate until the second day of the experiment at which time it was introduced into the respirometer microvessel.

Of the 12 specimens five were taken from a group of prepupae that had been held for ten days to break diapause in uncontrolled conditions. The insects, contained in a petri dish, were placed in an incubator with a temperature between 25 and 29° C. The duration of light was constant as was the intensity at the surface of the petri dishes (23 ft-c.). The other seven insects were taken from a group of 30 N. melanderi that had been held in a glass jar in a water bath at a constant temperature of 29° C, with the duration of light constant but the intensity varying between 10 and 13 ft-c. at the water bath surface. The water bath was situated in a laboratory used by a number of individuals and thus it was difficult to control the light regime.

The method of temperature shock was the same as in the

previous experiment but the oxygen consumption readings were taken hourly instead of every two hours. Two 12 hour temperature shocks were effected in an attempt to phase-set the physiological clock.

Results and Discussion

This portion of the study was exploratory and the preliminary data here provided are intended to serve as a basis for future studies.

Of the 14 specimens placed in the respirometer eight prepupae apparently did not break diapause as they showed no oxygen consumption over the 127 hour period that the experiment was in progress. Therefore the results (Figure 8) discussed in this experiment are based on two prepupae, one late prepupa which pupated during the experiment, and three pupae which were in the red eye stage.

Nomia melanderi did not show an oxygen consumption rhythm at a constant temperature of 30° C. In both experiments the oxygen consumption of the bees appeared to coincide with each other although no rhythm appeared (Figures 8 and 9). After a 10 C° temperature shock and return to 30° C oxygen consumption fluctuation was reduced. There appeared to be a peak at 1400 hours and again at 1200 hours the following day which would constitute a cycle of 22 hours (Figure 8). This period appeared to be more pronounced in the prepupae than in the pupae. A possible explanation may be that the prepupae were approaching the pupal stage, a period of great tissue

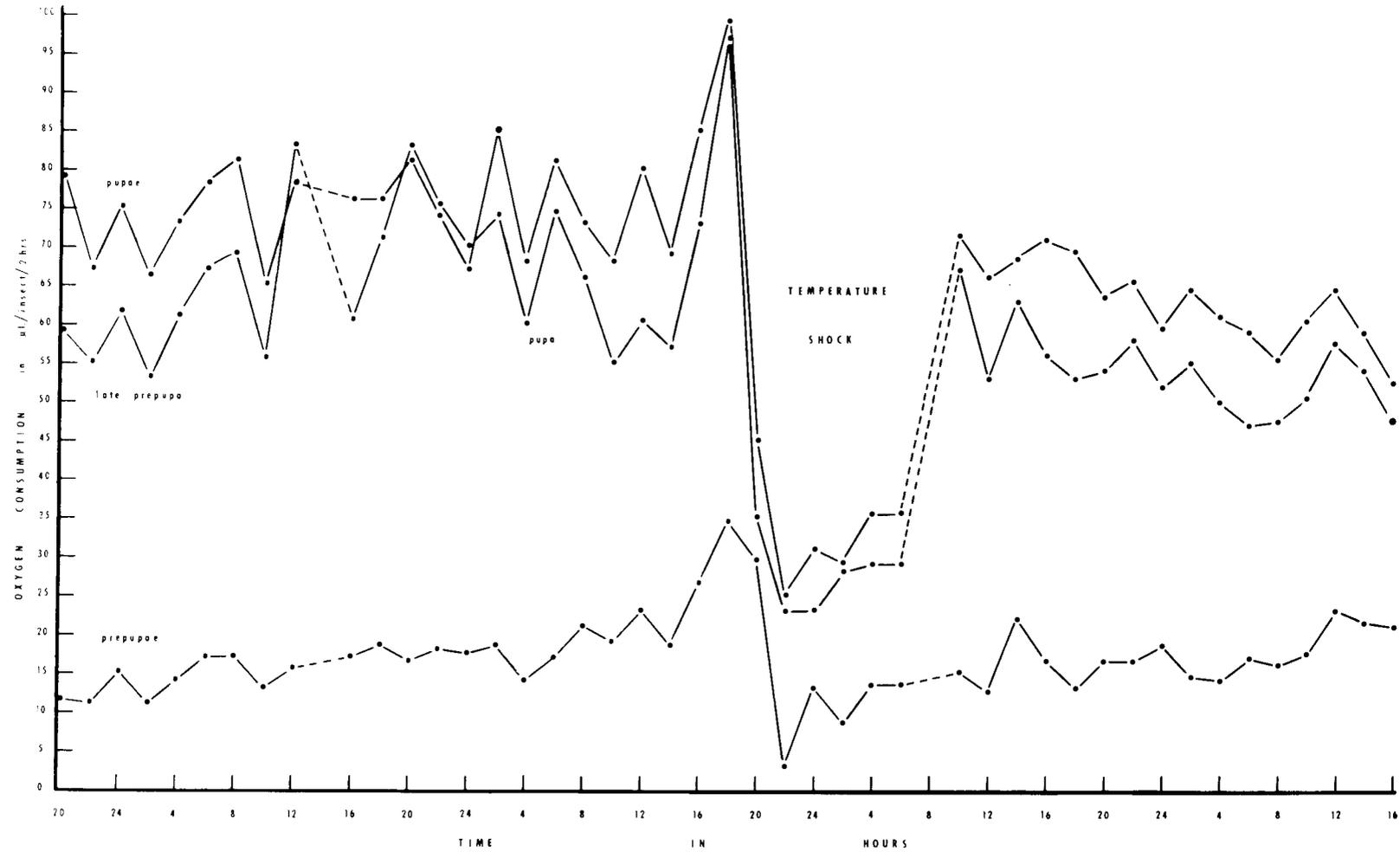


Figure 8. Effect of temperature shock on oxygen consumption in *Nomia melanderi*.

synthesis and therefore high oxygen consumption. Pupae, on the other hand, were completing their tissue synthesis and thus their oxygen consumption was decreasing. A more definite oxygen consumption rhythm may have developed if the temperature shock had been executed earlier in the experiment. It may be that a temperature shock greater than 10 C° is required to initiate an oxygen consumption rhythm in N. melanderi.

The results of the second experiment are recorded in Figure 9. Three pupae were selected as being representative--a white-eyed pupa, a new pupa, slightly younger than the white-eyed pupa, and a red-eyed pupa which was representative of the most mature pupae of the 12. The bottom curve in Figure 9 is the average oxygen consumption of the 12 insects. The initial intent was to study N. melanderi in various stages of development, such as the prepupa, the red-eye, the black-eye and black-thorax etc., up to and including the adult stage but the respirometer was available for very limited periods.

Here, as in the previous experiment, no definite oxygen consumption rhythm was evident. The assumption was made that a period, if it was to develop, would appear in the first 24 hours following the temperature shock, or, if not a distinct period, at least oxygen consumption peaks would occur that would indicate a response to the reduction in temperature. As no apparent change in oxygen

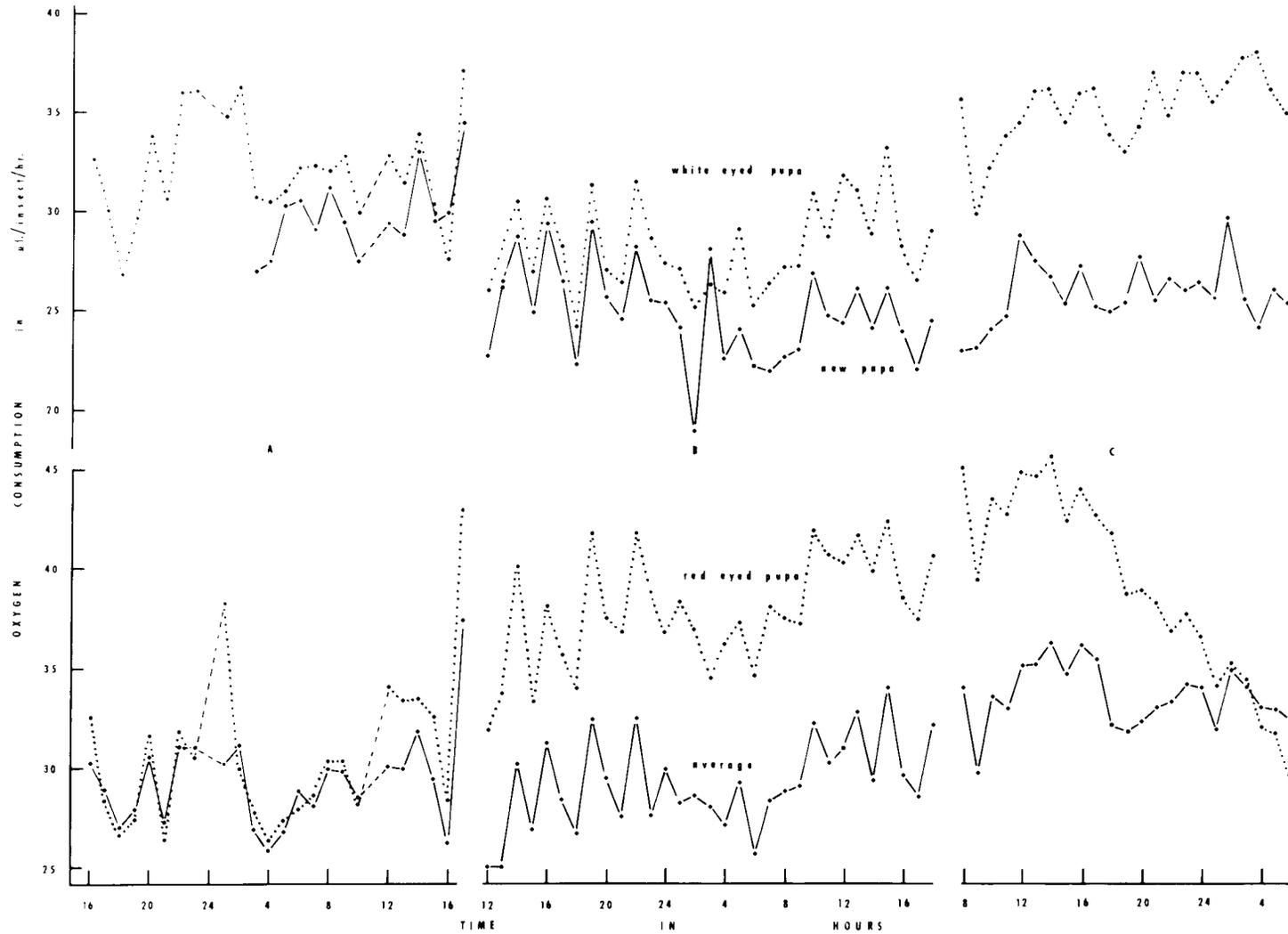


Figure 9. Effect of temperature shock on oxygen consumption in *Nomia melanderi*. (Temperature shocks of 12 hours duration were accomplished between sections A and B, and B and C.)

consumption was noted after the first temperature shock (Figure 9 B), a second temperature shock was performed. Again there were no peaks of oxygen consumption (Figure 9C) but it appeared to be less erratic in all but the red-eyed pupa. The experiment had to be terminated as the respirometer was required for other work. It is possible that a rhythm may have developed during the second 24 hour period following the second temperature shock but experiments of longer duration must be undertaken.

In addition studies must be extended to more mature pupae and to the early adult stage for it is possible that oxygen consumption rhythms do not develop until these later stages. The distinct possibility exists that Nomia melanderi does not possess an oxygen consumption rhythm, a conclusion that has been reached in other insects, including the moth Epeestia kuehniella (Moriarty, 1959).

CONCLUSIONS

1. Megachile rotundata exhibits an emergence rhythm with a 22 to 24 hour period. This rhythm, induced by a temperature shock, exists under constant temperature conditions.
2. Temperature is the phase-setter in the M. rotundata emergence rhythm, a temperature shock as small as 11 C° being sufficient.
3. A single temperature perturbation of 12 hours can synchronize emergence in a population of M. rotundata for at least six days.
4. Between one and six hours of cold is required to synchronize the emergence rhythm of a culture of M. rotundata. The exact minimum was not determined in this study.
5. The phase of the emergence rhythm is directly related to the time at which a culture of M. rotundata is removed from the cold following a temperature shock.
6. Light of an intensity of 14 ft-c. does not phase-set the emergence rhythm in M. rotundata.
7. Indications are that M. rotundata is susceptible to emergence synchronization by temperature in the adult stage and in the black-thorax and black-abdomen stages of the pupa.
8. No circadian oxygen consumption rhythm was discovered in Nomia melanderi.

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