ABSTRACT

Will R. Kiger, III. THE ROLE OF CYCLIC-AMP IN THE CIRCADIAN RHYTHM OF EGG-LAYING IN DROSOPHILA MELANOGASTER. (Under the direction of Dr. Patricia A. Daugherty). Department of Biology, June, 1982.

The role of cyclic-AMP as a possible factor in the control of the circadian rhythm of egg-laying in Drosophila melanogaster was examined. An oviposition rhythm in Drosophila occurs in which females lay the maximum number of eggs during the first few hours of darkness daily. There is evidence that cAMP is involved in the regulation of circadian rhythms, and that the biochemical model for the circadian clock involves cAMP.

Drosophila melanogaster treated with caffeine and theophylline were compared with untreated controls. Both caffeine and theophylline inhibit cAMP phosphodiesterase, and this inhibition leads to an increased level of cAMP. Cyclic-AMP levels were measured by radioimmunoassay.

The time of maximum egg deposition of treated flies was different from that of control flies. In both the control and treated groups, there were peaks in cAMP activity and peaks in egg deposition. In caffeine-treated flies, the inhibition of cAMP phosphodiesterase leads to an increase in cAMP, and this increase corresponds to an advance in time of maximum egg deposition. In theophylline-treated flies, the inhibition of cAMP phosphodiesterase also leads to an increase in cAMP; however, no consistency as to the time of maximum egg deposition, daily, was found. It also appears from synchrony between rises in maximum cAMP activity and rises in maximum egg deposition, that cAMP may exert a "stimulatory effect" on egg-laying in Drosophila. These results provide additional evidence that cAMP is involved in the control of circadian rhythms.

THE ROLE OF CYCLIC-AMP IN THE CIRCADIAN RHYTHM OF EGG-LAYING IN *DROSOPHILA MELANOGASTER*

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by

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THE ROLE OF CYCLIC-AMP

IN THE CIRCADIAN RHYTHM OF

EGG-LAYING IN *DROSOPHILA MELANOGASTER*

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Dedicated

to

My Parents

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INTRODUCTION

This study involved a series of experiments in which the circadian rhythm of egg-laying in Drosophila melanogaster was examined along with the role of cyclic-AMP as a possible factor in the control of this rhythm.

Ever since life first appeared on this planet, it has been exposed to strong and rhythmic environmental changes such as daily cycles of light and dark, and seasonal cycles of climatic change caused by the rotation of the earth in the universe. Marine and intertidal organisms have, in addition, been subjected to tidal and lunar periodicities. Only those organisms which have invaded the depths of the ocean or underground caves, have avoided this varying environment. Therefore, most organisms have developed mechanisms to counteract or to exploit these fluctuations. These mechanisms, that have evolved through time, constitute biological rhythms, including circadian rhythms.

A circadian rhythm is an activity of an organism that occurs with a periodicity close to 24 hours. The natural photoperiods of light and temperature serve to maintain these oscillations so that under natural conditions, their periods are in synchrony with the 24-hour solar day. The processes by which the coupling of an organismic rhythm to an external oscillating stimulus occurs are referred to as entrainment. However, in the absence of temporal cues from the environment, circadian rhythms may "free-run" and reveal their own natural periods which are generally close to, but not exactly, that of the solar day. Hence, the term circadian was derived from the Latin "circa diem" which means about a day.

The internal oscillations underlying circadian rhythms are now known to provide a temporal organization for physiological and behavioral activities in practically every group of organisms apart from the prokaryotes (Beck, 1980). It appears that twenty-four-hour rhythmicity is a fundamental property of eukaryotic life. It has, however, apparently been eliminated, or perhaps never been acquired by more primitive forms that lack nuclei, that is, the prokaryotes: bacteria and blue-green algae (Brady, 1979). Brady suggests that the reason prokaryotes appear to possess no kind of circadian rhythmicity is possibly that their life spans are frequently less than 24 hours so that day-long periodicity would be largely irrelevant to them.

Some of the first recorded evidence of biological rhythms goes back at least as far as 350 B.C. Here, an officer in the corps of Alexander the Great made the first observation of "sleep rhythms" in certain leguminous plants (Palmer, 1976). In more recent times, this "sleep rhythm" has been researched by E. Bünning ([1934] 1973). His work on the rhythmic motions of the leaves of seedling beans (Phaseolus multiflorus) in the 1930's sparked what is now considered an important integrated field of study -- chronobiology. Bünning observed that the seedling's primary leaves assumed an extended upright position during the daylight hours, but that a more drooping position prevailed during the night. These patterns continued even when the plants were held under constant regimes of temperature and continuous light or continuous darkness. In 1971, Bünning proposed that the photoperiodic receptors of plants are in the leaf epidermis; and that the upward movement of the leaves, during the hours of dawn, allows for the "precise perceptions of the photoperiodically decisive twilight intervals," and the downward

movements "lessens the disturbing effects of moonlight."

The easiest oscillations to measure are those involving mechanical displacements of some kind (Brady, 1979). In mammals, locomotor activity has been widely researched due to the above fact. The circadian rhythm of locomotion in small animals such as the hamster, Mesocricetus auratus, has been used extensively as a model for many research experiments geared to investigating the mechanisms behind the oscillation itself (Zucker and Stephan, 1973; Aschoff, et al. 1973; and Rusak, 1977). Under a 12:12 LD cycle (12 hours of light followed by 12 hours of darkness), activity in hamsters is almost exclusively nocturnal, beginning regularly soon after the beginning of the D phase. Rusak (1977) reported that the suprachiasmatic nuclei (SCN) of the hypothalmus is apparently responsible for the maintenance of normal circadian rhythmicity. It was found that destruction of the SCN eliminated completely the locomotor rhythms in the hamster. Another mammal, in which the destruction of the SCN results in the elimination of circadian rhythmicity, is the rat. Here, Stephan and Zucker (1972) found that lesions in the SCN eliminate circadian rhythms of locomotion and drinking behavior.

Circadian rhythms also occur in the Fungi. In Neurospora crassa, Brody and Harris (1973) reported that a circadian rhythm controls the alteration of conidiating and nonconidiating bands of growth. Neurospora also exhibits circadian rhythms in ribonucleic acid and deoxyribonucleic acid content in growth-front-hyphae of cultures grown on a solid medium (Martens and Sargent, 1972). Several other fungi possess rhythms in CO_2 output, with a clear circadian nature being seen in the case of Coniophora puteana (Smith, 1973)

Insects display individual and population behavioral patterns that recur on a predictable daily basis. These rhythms usually continue to be

manifested when the insects are experimently isolated from obvious environmental stimuli, such as light and temperature changes. There is now evidence of daily rhythms in insects of general locomotion, feeding, mating, pupation, oviposition, and pupal eclosion, in which these activities are restricted to a particular part of the day or night. One of the best known population rhythms is that of pupal eclosion in Drosophila. Pittendrigh (1954) found that there is a peak in pupal eclosion in D. pseudoobscura that occurs close to dawn. This peak at dawn is also seen in D. melanogaster (Brett, 1955; Clayton and Paietta, 1972).

Some of these circadian rhythms are direct responses to environmental change, but most are "overt manifestations of an endogenous periodicity" (Beck, 1980). Supporters of exogenously controlled rhythms, principally Brown (1960, 1965), believe that all observed periodicities are controlled by "subtle geophysical forces" associated with the solar day (such as air pressure, periodic fluctuations in gravity and cosmic ray intensity) which remain unaccounted for in laboratory experiments in which the obvious periodicities (light and temperature) have been controlled. There exists, however, much support for endogenously controlled systems. In a classic experiment, Hamner, et al. (1962) maintained a number of organisms at the South Pole on a turntable arranged to rotate once every 24 hours counter to the earth's own rotation. This procedure is assumed to eliminate most of the diurnal variables. Under these conditions, several rhythmic systems, including the pupal eclosion rhythm in D. pseudoobscura, continued to show a circadian periodicity apparently unaffected by either their location at the South Pole or by their rotation on the turntable.

Some investigators believe, however, that the mechanisms controlling activity rhythms have a mixture of endogenous and exogenous components.

Aschoff (1960) suggests that overt rhythms of activity are indeed controlled by an endogenous oscillation, and this oscillation is constantly modulated by the direct effects of the environment. This type of reasoning suffices for rhythms of a non-circadian nature; however, it fails to account for circadian rhythms that persist in the absence of temporal cues from the environment.

At the present time, it seems probable that circadian rhythms have evolved to match almost exactly the oscillations that occur in the physical environment. For example, the initiation of eclosion activity several hours prior to dawn is such that the emergence of adult Drosophila from pupae coincides with the optimal humidity level (Pittendrigh, 1960-61). It also appears that circadian rhythms are inheritable. Konopka and Benzer (1971) found evidence that the circadian rhythm of pupal eclosion in D. melanogaster is inherited, and that the gene responsible is located on the "X" chromosome. Therefore, circadian rhythms appear not to be imposed on organisms and also not learned.

There exist other circadian rhythms in Drosophila besides the population rhythm of pupal eclosion. Hardeland and Stange (1971) found that a courtship rhythm occurs in D. melanogaster in which activity is mainly exhibited during nocturnal conditions, and Fowler, et al. (1972) reported that the level of 5-HT (serotonin) peaks in these flies in the middle of the night.

Another important circadian rhythm that appears in Drosophila is that of oviposition. Gruwez, et al. (1972) found that the total number of eggs produced by Drosophila females from day three to day six post eclosion is equal in most cases. Even though the total number of eggs laid may be the same from day to day, this is not necessarily a reflection of a fly's

total productivity. McMillan and Fitz-Earle (1970) have shown that measurements in egg levels taken over a short time period are lower than measurements taken over a longer time period. Rensing and Hardeland (1967) found evidence of an oviposition rhythm in Drosophila in which females lay the maximum number of eggs during the first few hours of darkness, daily. Gruwez, et al. (1972) found that a period of 24 to 36 hours is necessary before a distinct rhythm of oviposition becomes apparent. They also reported that during this 24 to 36h time period, there must be a constant regime of light-dark cycles in order for entrainment of the rhythm to take place.

It is believed that in order for an organism to maintain a circadian rhythm, it has had to develop some sort of internal clock mechanism. This is, principally, based on the observation of free-running (not entrained) periods in organisms. The existence of organisms with free-running periods implies that they contain systems capable of continued endogenous oscillation. Most researchers refer to this internal controlling mechanism as the biological clock or the circadian pacemaker. Pittendrigh (1954, 1960) found that circadian rhythms are temperature-compensated; that is, they do not react to heat like most physiological processes in which there is, with every 10^{o}C rise in temperature, a doubling in the rate of activity. He, therefore, concludes that since rhythms are temperature-compensated, and that rhythms are used by organisms to measure the passage of time, there is justification for the use of the term "biological clock." Beck (1980) also adds that the biological clock is that part of the circadian system that confers upon the system the ability to persist in its rhythmicity without rhythmic environmental input. Without rhythmic input, the period of the rhythm expressed by the system will reflect the period of

the clock; and thus, its physio-chemical properties.

The expression of the biological clock can be accomplished by the mechanism of a single oscillator in organisms with only one rhythm or by many oscillators in organisms with more than one rhythm. It appears that single oscillators are only found in the Protista (Sweeney, 1969). Most authors agree that the circadian system in higher life consists of a larger number -- even a "population" of oscillators, each associated with a number of driven rhythms controlling behavioral or physiological phenomena (Saunders, 1976).

The evidence for such a multi-oscillator circadian system comes from a number of sources. One main source is from the laboratory of C.S. Pittendrigh. On the basis of the occurrence of transient phases in the adult emergence rhythm of D. pseudoobscura, and the differences between temperature and photoperiodic effects on the emergence rhythm, Pittendrigh, et al. (1958) found it necessary to postulate the existence of at least two endogenous oscillator systems. One oscillator was sensitive to photoperiodic stimuli, and the second oscillator was sensitive to temperature.

During the past decade, much research has been completed on the mechanism by which the oscillator controls the circadian rhythm. Most of this work has been performed on arthropods, rats, hamsters, and sparrows. The clearest story concerns the timing of emergence of silkmoths (Antheraea pernyi) from their pupal cases. Truman and Riddiford (1970) found that the control of this emergence was under hormonal action. They reported that the cerebral lobe area of the brain was necessary for emergence to occur; and therefore, must be the source of the timing mechanism (clock) for the rhythm. They also found that if a brain from one silkmoth

larva was removed and transplanted to the abdominal region of a brainless silkmoth larva, emergence would then occur in the recipient. They concluded that since there was no neural connection between the brain and the abdomen, the control of emergence must be exerted via a hormone that is secreted by the brain into the blood stream.

Another insect clock system that has been examined in depth is the one that controls the activity rhythms of cockroaches (Leucophaea maderae). It seems that the optic lobes of the brain are the crucial organs in the control of circadian rhythms here; and that this control is by neural means, rather than hormonal means. Here, Page (1978) created lesions in the optic tracts of cockroach brains without disturbing the neuroendocrine system and found that this abolishes rhythmicity. Considering this study on cockroaches and the study above on silkmoths, there appears to be the possibility of at least two types of clock systems in insects, one whose output is neural and the other whose output is hormonal.

In birds and mammals, the pineal organ plays an important role in the control of circadian rhythms. Menaker and Zimmerman (1976) suggest that the avian pineal contains a self-sustained oscillator, and as a consequence, produces a hormone that controls rhythmicity. This is based on an experiment in which the pineal organ from one sparrow was transplanted to a pinealectomized sparrow with the restoration of rhythmicity being observed within one day -- too soon for the transplanted organ to become reinnervated.

In the quest for finding a unifying link in the control of circadian rhythms in all organisms, Cummings (1975) has proposed a biochemical model for the control of circadian rhythms. He suggests that cAMP, a nucleotide found in practically all living matter, is the biochemical mechanism for the

circadian clock; and that the changes in the level of cAMP in an organism's system, at given times, is directly responsible for rhythmicity.

Cyclic-AMP has been actively investigated during the past 40 years. From work originating with E.W. Sutherland (1940's), it has been shown that cAMP plays an important key role in regulatory systems. Its most important attribute, stemming from Sutherland's work, is its function as a "second messenger" in hormonal action (Liddle and Hardman, 1971). A "first messenger," the hormone itself, travels to its target tissue where it then binds to specific receptor sites on the outside of the cell membrane. This binding of the hormone causes a increase in cAMP, the "second messenger," to occur. Cyclic-AMP then carries out the metabolic changes that are characteristic of the particular target tissue in response to the hormonal stimulation.

The regulatory role of cAMP is not limited to just hormonal action. It has been observed in the stimulation of the expression of genetic material where it seems to play a role in the control of the activity of genes. This is evident from work in Escherichia coli on the regulation of β-galactosidase mRNA synthesis by cAMP (Perlman and Pastan, 1969). Its operation, here, at the level of the gene suggests that cAMP acts in stimulating transcription (Pastan, 1972). Other activities of cAMP include its participation in synaptic transmission in the nervous system (Greengard, et al. 1972), and its function in the regulation of cell division (Pastan, 1972).

No doubt cAMP is a very versatile nucleotide, and it may play an important role in the control of circadian rhythms. According to Cumming's biochemical model, the level of cAMP in an organism is regulated via a dynamic equilibrium among cAMP and the enzymes, adenyl cyclase and cAMP

phosphodiesterase. Adenyl cyclase catalyzes the conversion of ATP to cAMP while cAMP phosphodiesterase is responsible for the breakdown of cAMP to AMP.

It is difficult to totally clarify the role of cAMP in circadian rhythms due to the fact that cAMP levels of activity are greatly influenced by many intracellular and extracellular factors. However, the first direct evidence of the involvement of cAMP in circadian rhythms comes from research conducted by Feldman (1975). Here, evidence was found that cAMP is involved in the circadian rhythm of conidiation in Neurospora crassa. Feldman altered the level of cAMP in Neurospora by the addition of methyl xanthines to Neurospora cultures and found that it led to a change in the period of conidiation. In addition, Gerisch (1975) reported that the aggregation of Dictyostelium discoideum cells in the formation of fruiting bodies is accomplished according to circadian rhythms that create "signals" for cells to come together. The cells respond to the cAMP signals by chemotaxis. It has also been found from research on the pineal gland in rats that the level of cAMP in pineal cells is affected by photoperiodic light stimuli and, therefore, could play a part in the biological clock (Axelrod, 1974).

Work by this author was done on the circadian rhythm of egg-laying in Drosophila melanogaster and its possible control by cAMP. There is evidence that one aspect of egg-laying, fecundity, is inherited (Keller and Mitchell, 1964; Chapco, 1968). Inheritance implies chromosomal activity; it has been established that fecundity in Drosophila is influenced by intrachromosomal effects and interactions, such as inversions, as well as by genetic background (Chapco, 1968). Since fecundity is inherited, one can hypothesize that a rhythm in egg-laying activity may

also be inherited. If this is true, it would reinforce the idea that circadian rhythms are inheritable. It has also been reported that oviposition site selection is inherited as a polygenic trait (del Solar, 1968; Pyle, 1976). The expression of any polygenic trait depends to a large extent upon environmental conditions, and this is no exception in Drosophila.

The investigation of cAMP as the controlling mechanism in this circadian rhythm of egg-laying was accomplished by the alteration of cAMP levels in D. melanogaster. Changes in cAMP levels were carried out by the inhibition of cAMP phosphodiesterase with the methyl xanthines, caffeine and theophylline. Comparisons with regard to cAMP levels, the time of maximum egg deposition, and the establishment of an egg-laying rhythm were then made between treated and controlled flies.

From the evidence outlined above, it is obvious that experimentation regarding the circadian rhythm of egg-laying in Drosophila melanogaster and its possible control by cAMP is certainly warranted. It is in this light that the following research has been conducted.

MATERIALS AND METHODS

Work on the circadian rhythm of egg-laying was done using wild-type Drosophila melanogaster. Eggs of flies treated with a methyl xanthine, either caffeine or theophylline, or water (control) were collected every six hours for 72-hour periods, and comparisons were made as to the time of maximum egg deposition by females. Treatment of flies was accomplished by soaking eggs in one of the methyl xanthine solution or water for two hours. This was followed by development of the eggs in a culture medium prepared with the addition of either a methyl xanthine solution or water. For example, eggs soaked in a caffeine solution continued development in a culture medium made up with a caffeine solution. This was done for eggs soaked in a theophylline solution or water as well. Once patterns in egg-laying were established, the levels of cAMP in adult treated and control flies were then measured at regular time intervals during a 24-hour cycle by radioimmunoassay.

Description of the procedure is divided into six sections: Initial Stock Cultures; Egg Collections; Treatment of Eggs; Experimental Runs; Cyclic-AMP Determinations by Radioimmunoassay; and Statistical Analysis.

Initial Stock Cultures

Stock cultures were set up of wild-type Drosophila melanogaster; flies were transferred to fresh culture medium every four weeks. Stocks were maintained in half-pint milk bottles containing medium prepared by adding 30ml of Instant Drosophila medium -- Formula 4-24 (Carolina Biological Supply, Burlington, NC) along with 30ml of distilled water to each culture bottle and a few granules of dry yeast. Twenty to thirty wild-type flies were introduced into the culture bottle and allowed to

mate and lay eggs. After one week, the flies were removed from the culture, and the flies which emerged later were collected for use.

Egg Collections

All egg collections were completed using a Drosophila Habitat™ (Fisher Scientific, Chicago, IL). Eggs were collected on yeasted spoons housed in three plastic vials that were inserted through openings in the habitat. A fourth vial, containing a spoon with water, was also inserted in the habitat. The preparation of the yeast solution and yeasted spoons used in collections were done in the following manner. The yeast solution was composed of 10g of dry yeast and 30ml of distilled water. One to two ml of this yeast solution was poured into each spoon, followed by the sprinkling of dry yeast granules on top of the solution. When this was completed, the solution was allowed to settle for ten minutes.

Treatment of Eggs

Flies obtained from the initial stock cultures were placed in a Drosophila Habitat.™ Yeasted spoons were prepared as described and were inserted into the habitat. Two overnight collections were made in order to obtain enough eggs for experimental and control stocks. A modification of a technique for handling Drosophila eggs developed by Shelton (1969) was employed.

The spoons were removed from the habitat, and by the use of a strong stream of water from a wash bottle, the contents of each spoon were washed into a buchner funnel lined with a black cloth. Filtration was started and was continued until the majority of the yeast particles was no longer visible on the cloth. The cloth was then removed and placed in a watch glass. At this point, the eggs on the cloth were washed into

a beaker, and the contents of the beaker were then divided into approximately equal portions and emptied into centrifuge tubes. The tubes were centrifuged for three minutes at an RCF of 1600 x g. The supernatant was carefully removed, and the resulting pellet was resuspended in about 5ml of 60 percent sucrose. A second centrifugation was then performed for one minute at an RCF of 1600 x g. This resulted in the accumulation of eggs at the surface of each tube; these then were removed easily by pipetting.

After the eggs were removed from all tubes, they were separated into three equal groups. One group of the eggs were placed in a beaker containing 30ml of a 5mM solution of caffeine. A second group of eggs was placed in a beaker containing 30ml of a 5mM solution of theophylline. A third group of eggs were placed in a beaker containing 30ml of distilled water. The eggs was allowed to remain in the beakers for two hours. During the soaking period, three culture bottles were prepared as described earlier except that in two of them, 30ml of a 5mM caffeine solution and 30ml of a 5mM theophylline solution, respectively, were substituted for distilled water. At the end of the soaking period, the eggs were transferred to the appropriate cultures. After approximately 10-14 days, the first adult flies appeared in the cultures and were removed. Two 24-hour collection periods for adult flies were initiated. The resulting flies were kept separate and were transferred to fresh culture medium prepared with distilled water and allowed to age for three days. Therefore, these flies, to be used in the experimental runs were four to five days old.

Experimental Runs

This part of the research consisted of seven control runs and four caffeine and four theophylline runs. For each run, the procedure described above for obtaining flies between the ages of four to five days old was carried out for each group used. All runs were for 72 hours and were conducted in a room in which the light cycle and temperature could be manipulated. The temperature was maintained between 23^{o} to 25^{o}C, and the light cycle employed was a 12:12 cycle (12h of light followed by 12h of darkness). The light was set to come on at 6 A.M. and go off at 6 P.M. daily. Eggs on yeasted spoons were collected every six hours during a 72-hour run at the following hours: 9 A.M., 3 P.M., 9 P.M., and 3 A.M. Eggs were collected during the dark period with the aid of a photographer's red light (Kodak filter designation Dark Red -- No. 2).

The procedure for each run was as follows. Habitats were set up with flies obtained from treated and control eggs. Initially, three yeasted spoons and one water spoon were prepared for each habitat used. The habitats were transferred to the experimental room where the light cycle, red light, and temperature were already adjusted. Each habitat was positioned at an angle to the surface of water in which it was placed. (This was done in order to avoid ant infestations.) At each collection time, the spoons were removed and replaced with freshly prepared ones. The contents of each spoon were washed into a buchner funnel lined with a black cloth as described before. The cloth was removed and placed in a watch glass. The watch glass was placed under a dissecting scope, and the number of eggs were counted. This was repeated for each spoon and the results were recorded.

This same procedure for the experimental runs (the determination of egg-laying activity) was carried out also in the second part of the research, the measurement of cAMP levels in controls and treated flies, with one change. During one of the assays for cAMP, the eggs laid in the experimental run were collected at 2h intervals instead of 6h intervals.

Cyclic-AMP Determinations by Radioimmunoassay

The radioimmunoassay was done in accordance with instructions from Becton-Dickinson for their cAMP RIA kit (Becton Dickinson Immunodiagnostics, Orangeburg, NY) with slight modifications. The principle of this assay involved the high affinity of cAMP standards and cAMP in samples to an antibody formed from rabbit gamma globulin. A tracer, 2-0 succinyl cAMP tyrosine methyl ester $\left[^{125}I\right]$, is displaced from the antibody by increasing amounts of cAMP in standards or samples. This results in a reduction in the amount of labeled cAMP bound to the antibody. Unlabeled cAMP competes with labeled cAMP for available antibody binding sites. The level of radioactivity bound is, therefore, inversely related to the concentration of cAMP in the sample or standard. In order to find the amount of radioactivity bound, a goat antiserum is added which precipitates bound cAMP. This precipitate is then counted in a scintillation counter adjusted for the measurement of $\left[^{125}I\right]$.

Two radioimmunoassays were performed. One assay was done using control flies and both caffeine and theophylline-treated flies. The other assay was done using control flies and two groups of theophylline-treated flies. Each radioimmunoassay was carried out in two steps: Tissue Preparation and The Assay.

Tissue Preparation -- Habitats were set up with treated and control flies, and rhythms in egg-laying activity were established. After 48

hours of the established rhythm, samples of flies were removed from the habitats according to the following schedule. In one of the assays, samples were removed from the habitats with caffeine and theophylline-treated flies at 12 A.M., 4 A.M., 6 A.M., 8 A.M., 10 A.M., 12 P.M., 2 P.M. and 6 P.M. Samples were removed from the habitat with control flies at 6 A.M., 10 A.M., 12 P.M., 2 P.M., 4 P.M., 6 P.M., 8 P.M., and 12 A.M. In this assay, egg-laying activity was examined in all three groups at 6h intervals. Therefore, in further discussions, this assay will be referred to as Assay A (6h). In the other assay, flies were removed from the control and theophylline habitats every two hours beginning at 6 A.M. and continuing until and including 6 A.M. the following day. Here, egg-laying activity was examined at 2h intervals. This assay will now be referred to as Assay B (2h). In Assay A (6h), four female flies were removed at each sample time, yielding 5mg of tissue. In Assay B (2h), eight female flies were removed at each sample time, yielding 10mg of tissue.

After the obtainment of a sample of flies, they were quickly anesthetized with "Fly Nap" (Carolina Biological Supply, Burlington, NC) and placed in a mesh cage and then submerged in liquid nitrogen for 15 seconds. The frozen flies were removed from the cage and transferred to a glass vial with a stopper. Then, the vial was placed in an ultra cold freezer set at -80^{o}C.

Once all samples were collected, the following procedure was conducted for each one. The flies from each sample were ground up in a glass homogenizer held in liquid nitrogen. Next, a six percent solution of trichloroacetic acid (500μl) was added to the ground up flies, and this mixture was then homogenized at 4^{o}C. The sample was centrifuged at +2^{o} to +8^{o}C for 15 minutes at an RCF of 1240 x g. The supernatant was then

recovered, and 5ml of ethyl ether saturated with water was added and mixed well. After the separation of the layers, the upper ether layer was removed and discarded. This ether extraction was repeated an additional two times. The resulting solution was evaporated to dryness in a constant stream of air. This evaporation process was carried out in a 60°-70°C water bath. The residue from the evaporation was dissolved in 1.0ml of sterile distilled water for use in the assay. A flow chart of this tissue preparation procedure appears in Figure 1.

The Assay -- The cAMP derivative $\left[^{125}I\right]$ (tracer) and the cAMP antiserum from the kit were reconstituted by the addition of 10ml of sterile distilled water to each of them. Both were then mixed well, avoiding foaming. The cAMP standards from the kit were reconstituted by the addition of 5ml of sterile distilled water to each one. All were then mixed well, avoiding foaming. Sixteen glass tubes were numbered for the standards along with two tubes for every sample assayed. The flow chart of the procedure for the assay that is shown in Figure 2 was then followed. After the final centrifugation, the supernatants were aspirated from all tubes except 1 and 2. (These tubes contained undiluted $\left[^{125}I\right]$ tracer.) All the tubes were then loaded into a scintillation gamma counter adjusted for the measurement of $\left[^{125}I\right]$. The radioactivity in each sample tube was counted for one minute. After the counts were obtained, the standard curve was plotted on three-cycle semi-log paper. From this graph, the levels of cAMP in pmoles for each sample were interpolated.

Statistical Analysis

Statistical evaluation of the data obtained for egg-laying activity was performed using one-way analysis of variance to determine significance of differences between control and treated flies as well as the differences among individual runs of control and treated flies. ($P<0.05$)

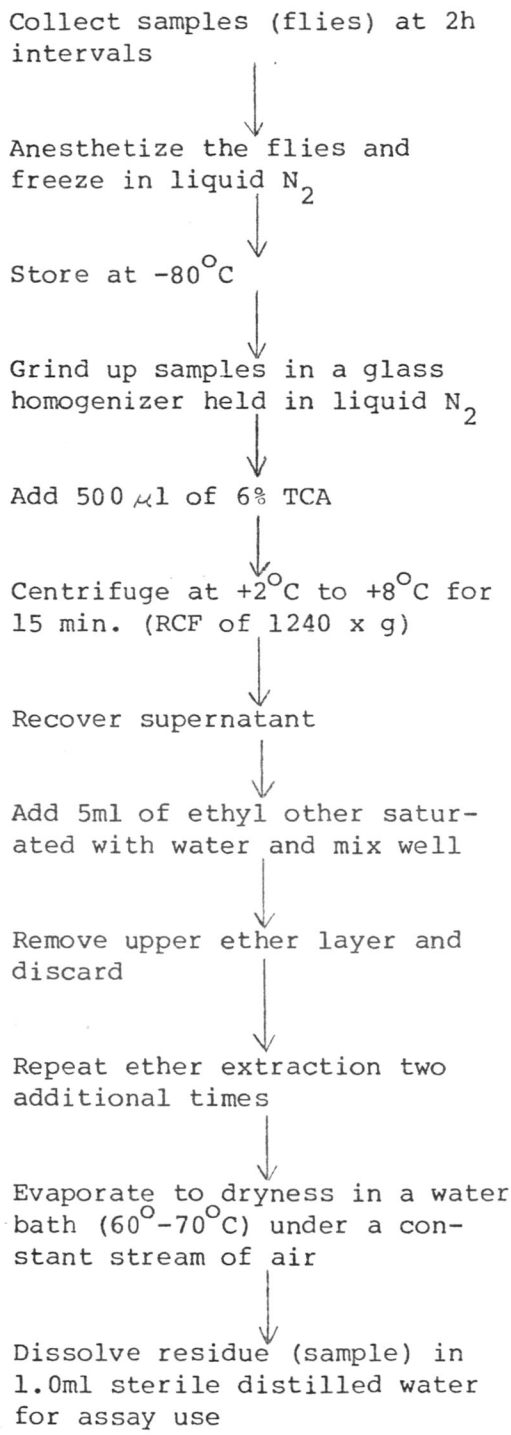

Figure 1. Flow chart of the procedure for tissue preparation used in the cAMP radioimmunoassays.

Tube No.	Acetate Buffer (μl)	Cyclic AMP Standard (μl)	Control or Unknown Sample (μl)	Cyclic AMP [^{125}I]	Cyclic AMP Antiserum (μl)	Incubation	Precipitating Antiserum-PEG (ml)	Centrifuge
1,2	---	----	---	100	---		---	Vortex and centrifuge tubes (except 1 & 2*) at 1000 x g for 10 min. in the cold.
3,4	400	----	---	100	---	Refrigerate tubes at +2 to +8^{o}C for 3 hrs. or 18-20 hrs.	1.0	
5,6	300	----	---	100	100		1.0	
7,8	200	100A	---	100	100		1.0	
9,10	200	100B	---	100	100		1.0	
11,12	200	100C	---	100	100		1.0	
13,14	200	100D	---	100	100		1.0	
15,16	200	100E	---	100	100		1.0	
17,18	200	----	100	100	100		1.0	
etc.								

* Tubes 1 and 2 contain undiluted [^{125}I] tracer.

Figure 2. Cyclic-AMP [^{125}I] radioimmunoassay flow chart.

RESULTS

Egg Collections Illustrating Egg-Laying Activity

The results of egg-laying are summarized in Figures 3-7. In these figures, the line connects the median values. The time of maximum egg deposition of flies treated with caffeine or theophylline was different from that of control flies. The results for the seven runs of control flies are given in Table 1 and Figure 3. For statistical purposes, in Figures 3-7, the number of eggs deposited is represented as the natural log of the percentage of total eggs collected in any given time period, in order to illustrate general trends and allow for the fact that the number of females in each run varied. The same approach is used for the treated groups of flies as well.

In the control flies, there are three distinct peaks of egg deposition occurring at the 9 P.M. collection times (Figure 3), which resulted in a significant time effect (Table 5). Thus, maximum numbers of eggs were laid by females between the hours of 3 P.M. and 9 P.M. daily. No significant difference was found among the seven control runs (Table 4).

In the four runs of the caffeine-treated flies, the maximum number of eggs were collected at the 3 P.M. collection time each day (Figure 4). Here, maximum oviposition occurred between the hours of 9 A.M. and 3 P.M., and resulted in a significant time effect (Table 5). (See Table 2 for the actual number of eggs laid by the caffeine-treated flies.) A comparison of the egg-laying activities of the caffeine-treated flies and the control flies appears in Figure 6. Again, from analysis of variance, no significant difference was found among the four caffeine runs (Table 4).

The time of maximum egg deposition in the theophylline-treated flies varied each day during the four 72h runs (Figure 5). (See Table 3 for the actual number of eggs laid by the theophylline-treated flies.) This was also evident from analysis of variance with respect to time and egg levels that was performed on both groups of treated flies as well as control flies. For the data, the effect of time was only significant in the control and caffeine runs and was not significant ($P<0.48$) in the theophylline runs (Table 5).

Due to this apparent variation as to the time of maximum egg deposition with theophylline-treated flies, it was necessary to examine the three days of the runs separately. In the first 24h period, the maximum number of eggs were collected at 3 A.M. During the second 24h period, the maximum number of eggs were collected at 3 P.M. while in the third 24h period, the maximum number of eggs were seen at 3 A.M. The peaks occurring at 3 A.M. are delayed with respect to the controls while the peak at 3 P.M. is advanced. The delayed and advanced peaks are evident in Figure 7 which compares controls and theophylline-treated flies. In addition, Table 4 shows overall that no significant difference existed between the four theophylline runs.

Table 6 represents the overall analysis of variance with respect to the effect of treatment on the time of maximum egg deposition. The null hypothesis tested is that there was no significant variation with respect to the time of maximum egg deposition between control and treated flies. It was found that the effect of treatment was highly significant. ($P<0.01$)

Besides the changes in the times of maximum egg deposition, it was also found that for the caffeine and theophylline-treated flies, there was a reduction in the number of eggs laid during peak hours. Eighteen percent of the total number of eggs were laid during the maximum peaks of egg-laying activity in the controls. In the caffeine-treated flies, 14 percent of the total number of eggs were laid at the peaks, and this constitutes a 23 percent reduction in the total number of eggs laid with respect to the controls. A 39 percent reduction in the total number of eggs laid was observed with the theophylline-treated flies in which only 11 percent of the total number of eggs were laid during the peaks.

Radioimmunoassays

Assay A (6h) (Egg collections at 6h intervals.) In this assay, cAMP levels were found in samples taken at 2h intervals while eggs were collected at 6h intervals. This was done for caffeine and theophylline-treated flies as well as controls.

The egg-laying activity of the control flies sampled for Assay A is shown in Figure 8. The first two peaks in egg deposition occurred within the interval of 3 P.M. and 9 P.M., as expected, based on the previous controls. However, the last two peaks occurred within the interval of 9 A.M. and 3 P.M. Therefore, this part of the control does not typically fit the pattern established earlier. During the last 18h of this run, the level of cAMP in female flies was measured every two hours. The results are shown in Figure 9. A small peak in cAMP activity (0.3 pmoles) occurred at 10 A.M. This small peak preceded the maximum peak of egg deposition at 12 P.M. (interval 9 A.M. - 3 P.M.) for this 18h period. A larger peak (0.45 pmoles) occurred between 4 P.M. and 8 P.M., and it is this peak that coincides with the egg deposition peak expected during the

time interval (3 P.M. - 9 P.M.) on the basis of previous controls.

Figure 10 represents the egg-laying activity of caffeine-treated flies for Assay A. Here, each peak in egg deposition occurred within the interval of 9 A.M. and 3 P.M. The results of the assay for cAMP in these flies is seen in Figure 11. A peak in cAMP activity (0.49 pmoles) was evident at 4 A.M. along with another peak at 12 P.M. (0.42 pmoles). The peak at 12 P.M. coincided exactly with the peak of maximum egg deposition for this interval.

The average of the maximum level of cAMP activity during the peaks of the controls was 0.38 pmoles, while the average of the peaks of the caffeine-treated flies was 0.46 pmoles. This constitutes an 18 percent increase in the level of cAMP present in the caffeine-treated flies as compared with controls.

The egg-laying activity of theophylline-treated flies in Assay A is shown in Figure 12. Four peaks of maximum egg deposition are found within the interval of 9 A.M. and 3 P.M. for this run. This egg deposition pattern is different from the results expected on the basis of the first experiments. (Refer to Figure 5.) Peaks of egg-laying activity occurred at the four 3 P.M. collection times. Again, as was done with the caffeine group and the control, the last 18h interval of this theophylline run was assayed for cAMP (Figure 13). A pronounced peak in cAMP activity occurred at 4 A.M. This peak is similar to the one observed at 4 A.M. with the caffeine-treated flies (Figure 11). For the remainder of the assay, there was a constant rise in cAMP activity starting at 10 A.M. and continuing till the end of the assay interval 6 P.M. The peak of maximum egg deposition occurred between 9 A.M. and 3 P.M.; and therefore, preceded the peak of maximum cAMP activity.

Considering the last constant rise in cAMP activity (Figure 13), as a possible peak along with the first distinct peak at 4 A.M., an average of 0.48 pmoles was obtained for maximum cAMP activity in theophylline-treated flies as compared with 0.38 pmoles for controls. This value for the theophylline-treated flies represents a 21 percent increase in the level of cAMP.

Assay B (2h) (Egg collections at 2h intervals.) In this assay, the level of cAMP in theophylline-treated flies along with controls was measured at 2h intervals simultaneously with collections of eggs. Two theophylline runs were executed in order to investigate the effects of one of the methyl xanthines more intensively. (The two theophylline groups of Assay B will be referred to as theophylline 1 and theophylline 2.)

The results of Assay B along with the distribution of eggs for the control appears in Figure 14. For control flies, the maximum number of eggs were laid between the hours of 6 P.M. and 8 P.M. The maximum peak of cAMP activity occurred within the interval of 2 P.M. to 6 P.M., and this peak preceded the peak of maximum egg deposition. A second peak in cAMP activity was evident at 2 A.M.; however, because the experiment was carried out for only 24h, it is not known whether this cAMP peak preceded a peak in maximum egg deposition.

The results of theophylline 1 (Figure 15) and theophylline 2 (Figure 16) of Assay B are similar in some respects and highly different in others. As for the peak of maximum egg deposition, it occurred within the interval (12 P.M. - 2 P.M.) for both of them. Also, they both exhibited a small peak in cAMP activity at 12 P.M., and these peaks preceded their peaks of maximum egg deposition.

As for the differences, there was a rise in cAMP activity in theophylline 1 (Figure 15) starting at 8 P.M. and continuing till 12 P.M., and then leveling off. In addition, as cAMP rose in theophylline 1, the number of eggs laid decreased (Figure 15). The maximum peak of cAMP activity in theophylline 2 (Figure 16) occurred at 4 P.M. (5 pmoles). The constant rise in cAMP activity that was evident in theophylline 1 was not present in theophylline 2; instead, there was a general trend of decreasing cAMP activity after the peak.

Again, as was evident in Assay A, a higher level of cAMP was observed with the treated flies, in this case theophylline-treated flies, as compared with the controls. The average of the maximum level of cAMP activity during the peaks of the controls was 1.5 pmoles as compared with 1.78 pmoles in theophylline 1 and 2.72 pmoles in theophylline 2. The value for theophylline 1 represents a 16 percent increase in the level of cAMP along with a 45 percent increase with theophylline 2 as compared with the control.

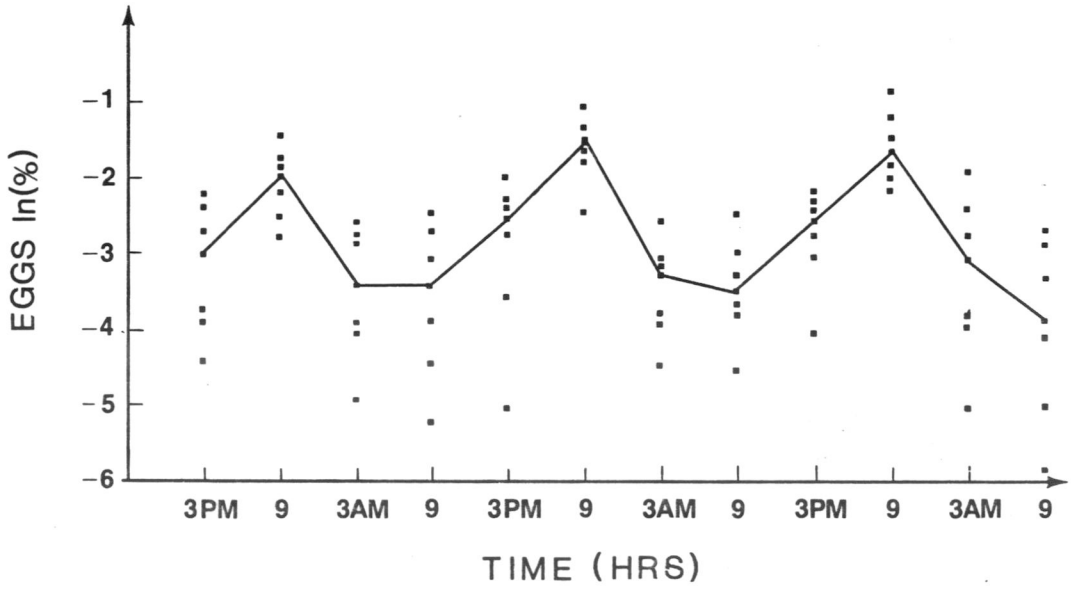

Fig. 3. The egg-laying activity of seven control groups of Drosophila melanogaster for 72 h periods.

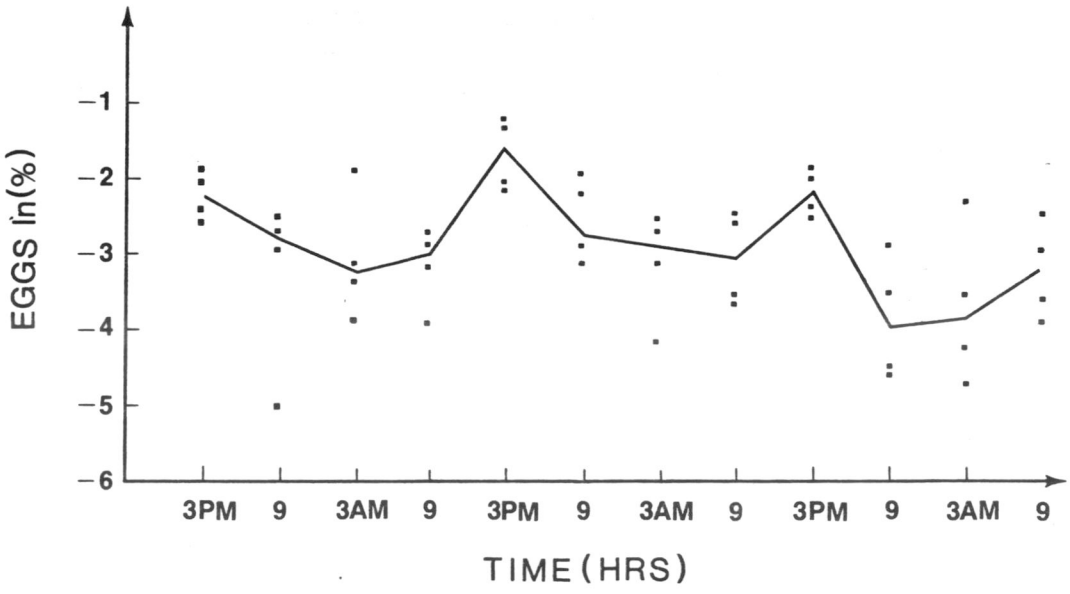

Fig. 4. The egg-laying activity of four caffeine-treated groups of Drosophila melanogaster for 72h periods.

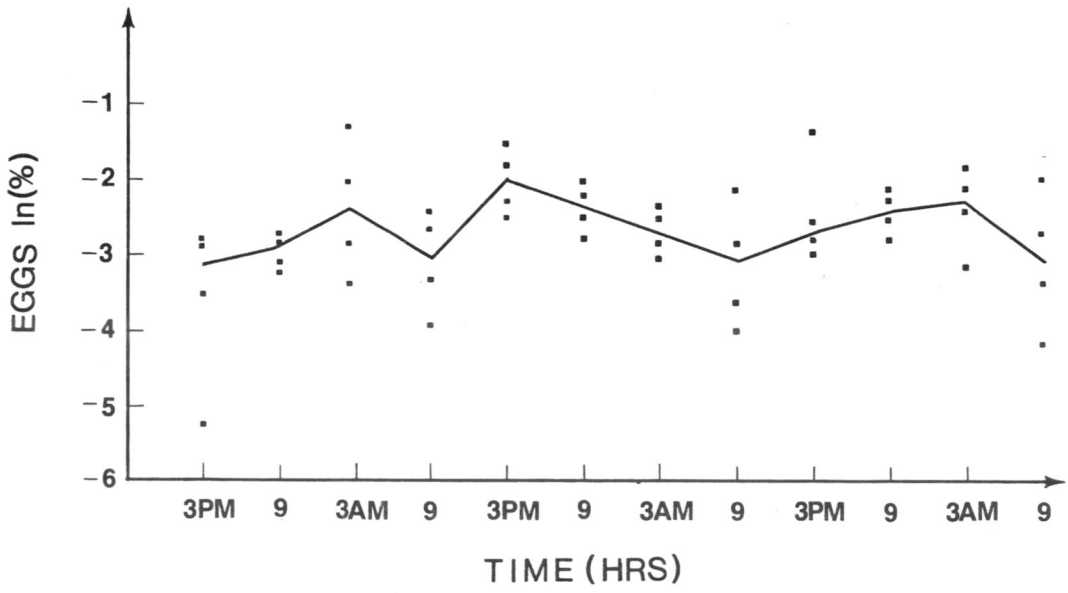

Fig. 5. The egg-laying activity of four theophylline-treated groups of Drosophila melanogaster for 72h periods.

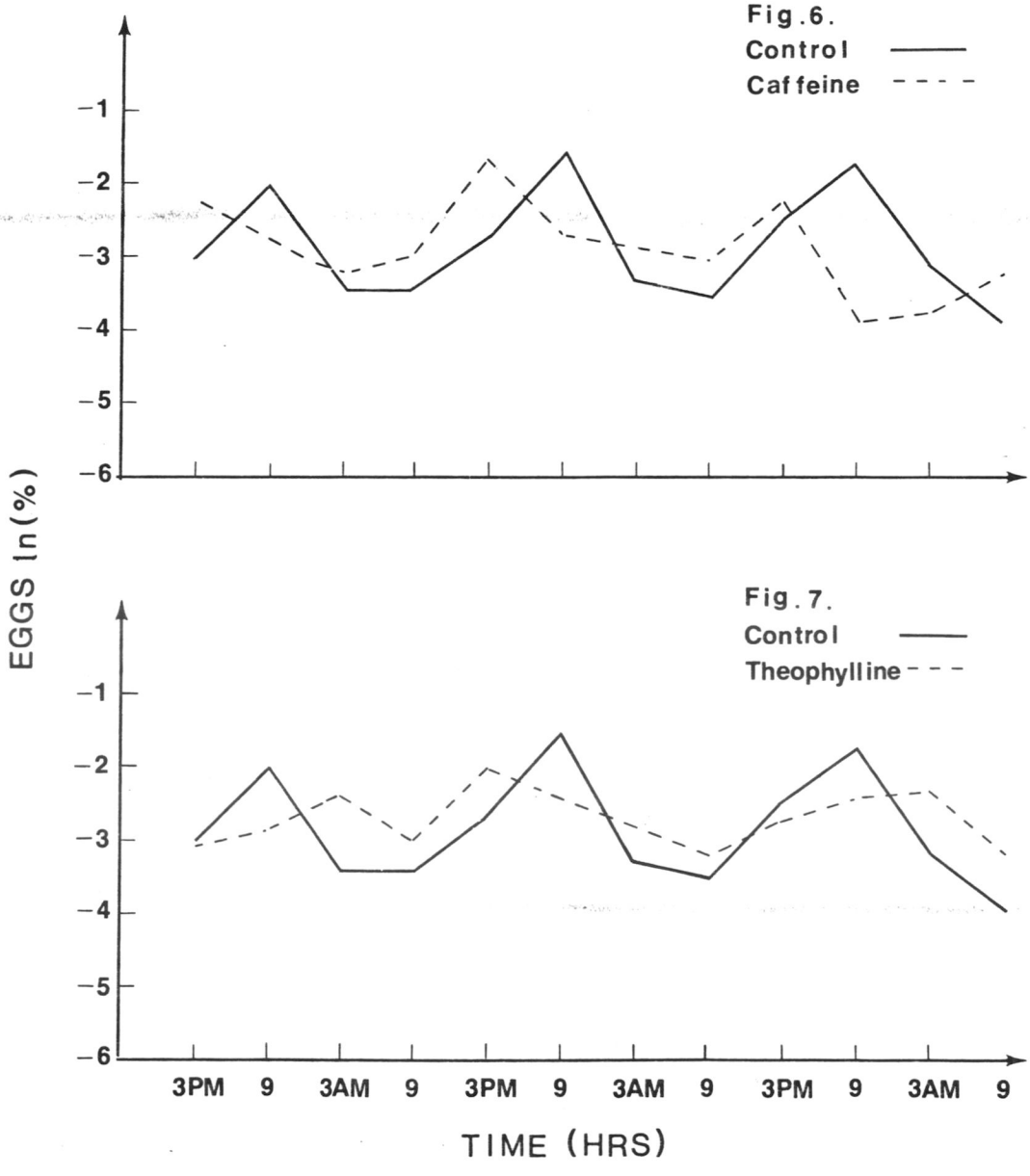

Fig.6. and 7. The effect of methyl xanthines on the circadian rhythm of egg-laying in Drosophila melanogaster.

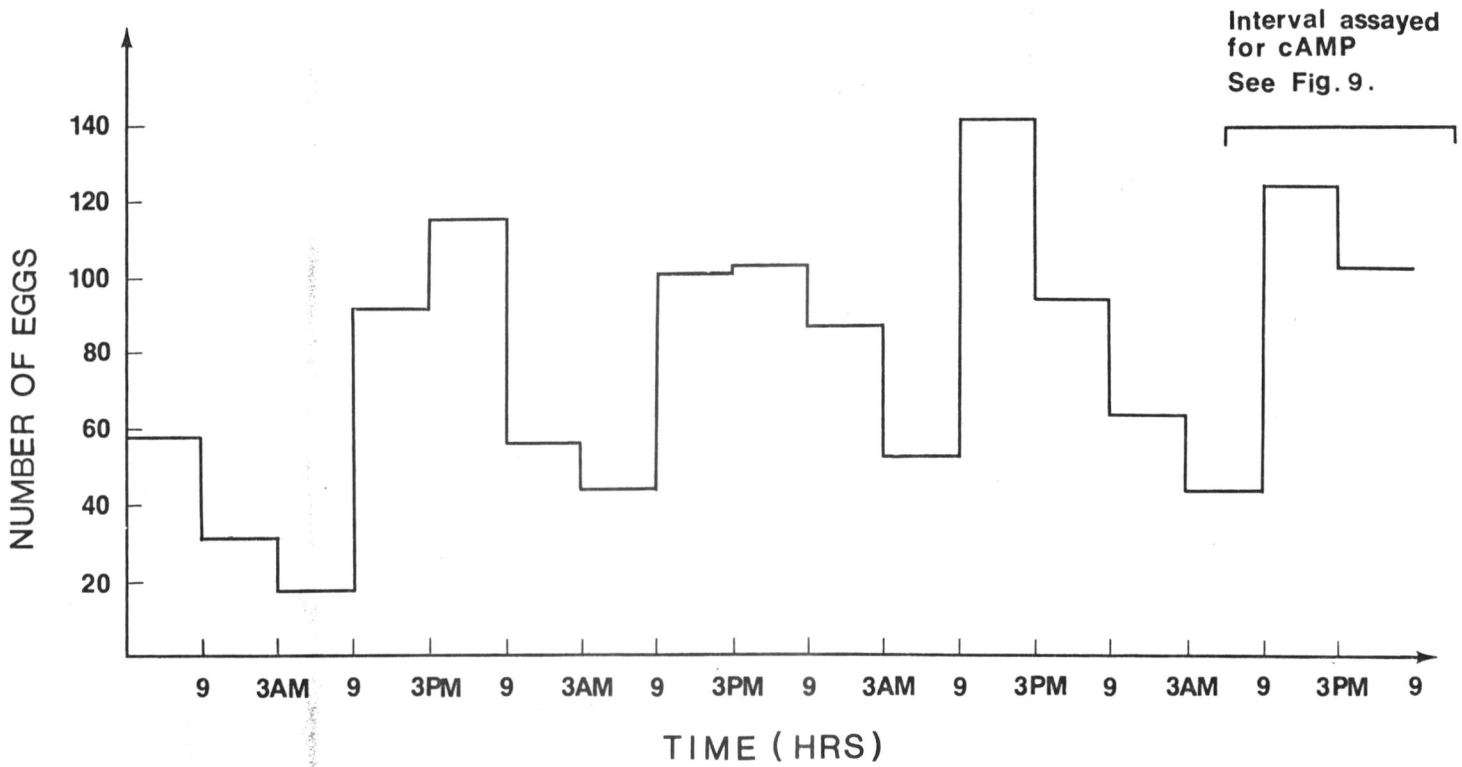

Fig. 8. The egg-laying activity of Drosophila melanogaster controls in cAMP Assay A.

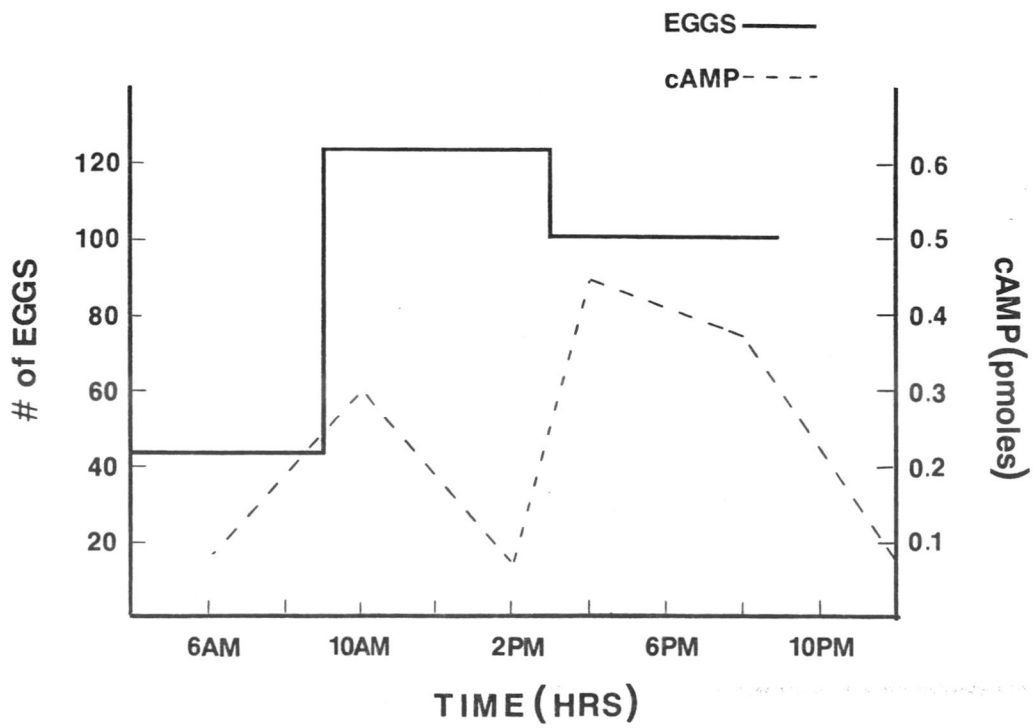

Fig 9. Control—Assay A. The level of cAMP in control flies at 2h intervals.

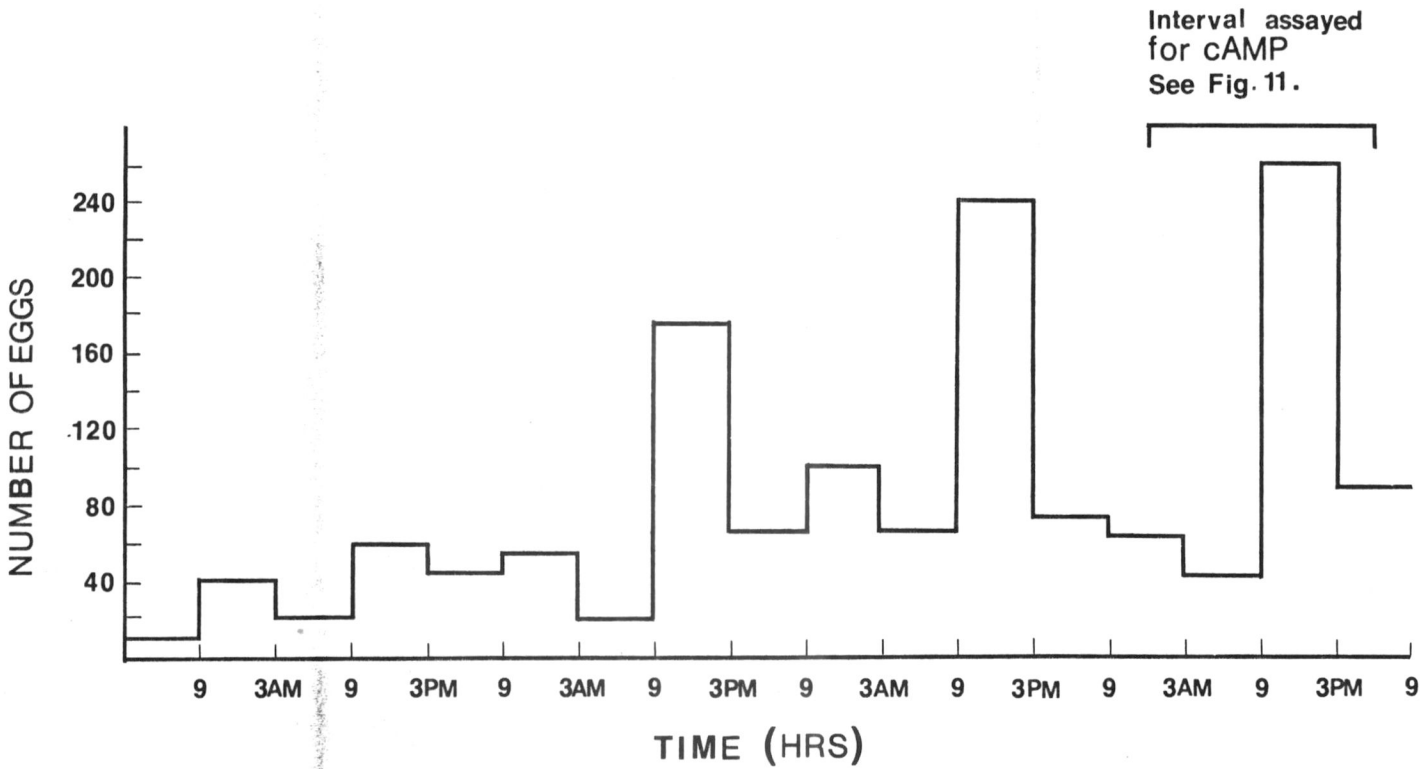

Fig. 10. The egg-laying activity of caffeine-treated Drosophila melanogaster in cAMP Assay A.

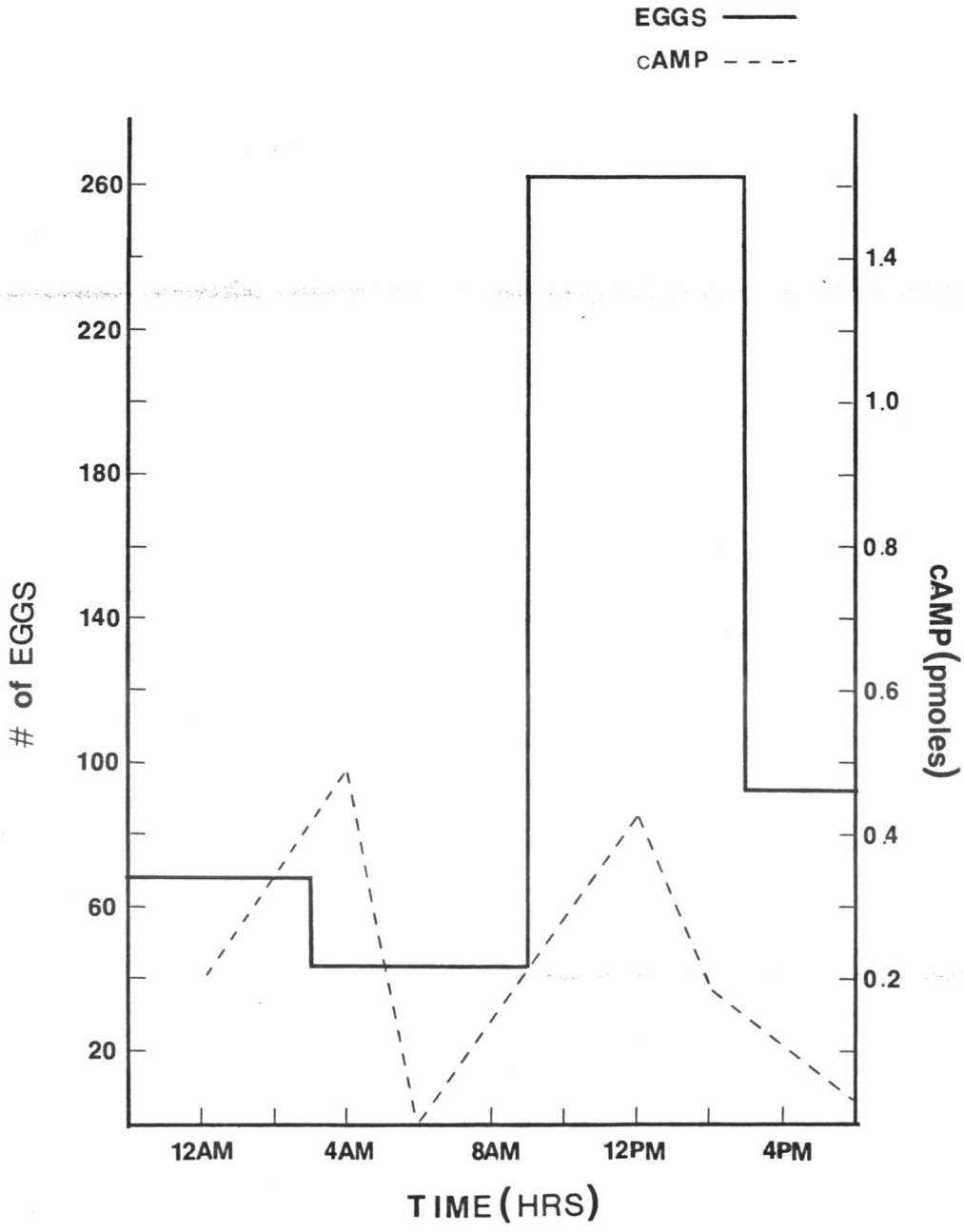

Fig. 11. Caffeine–Assay A. The level of cAMP in caffeine-treated flies at 2h intervals.

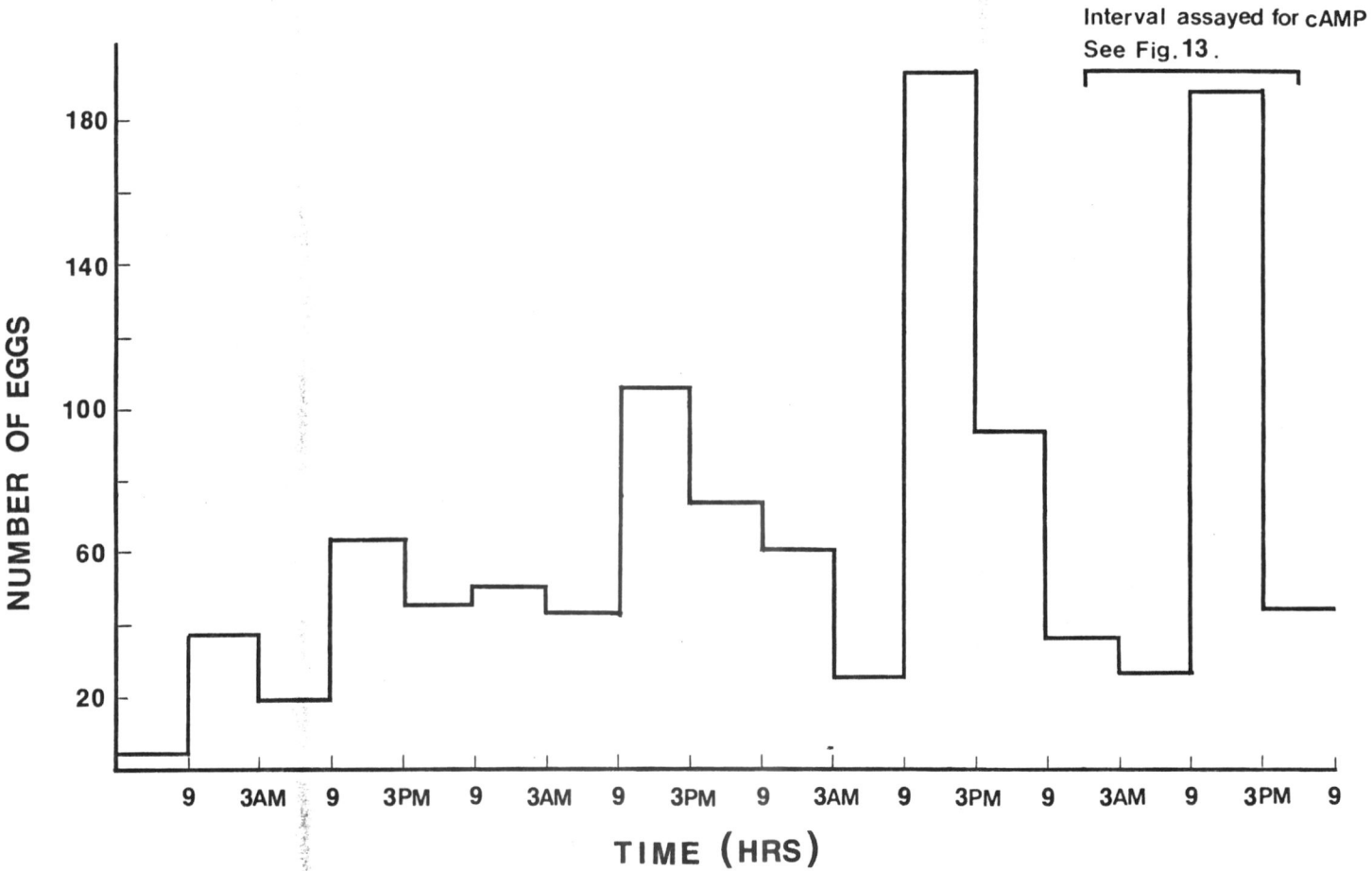

Fig. 12. The egg-laying activity of theophylline-treated Drosophila melanogaster in cAMP Assay A.

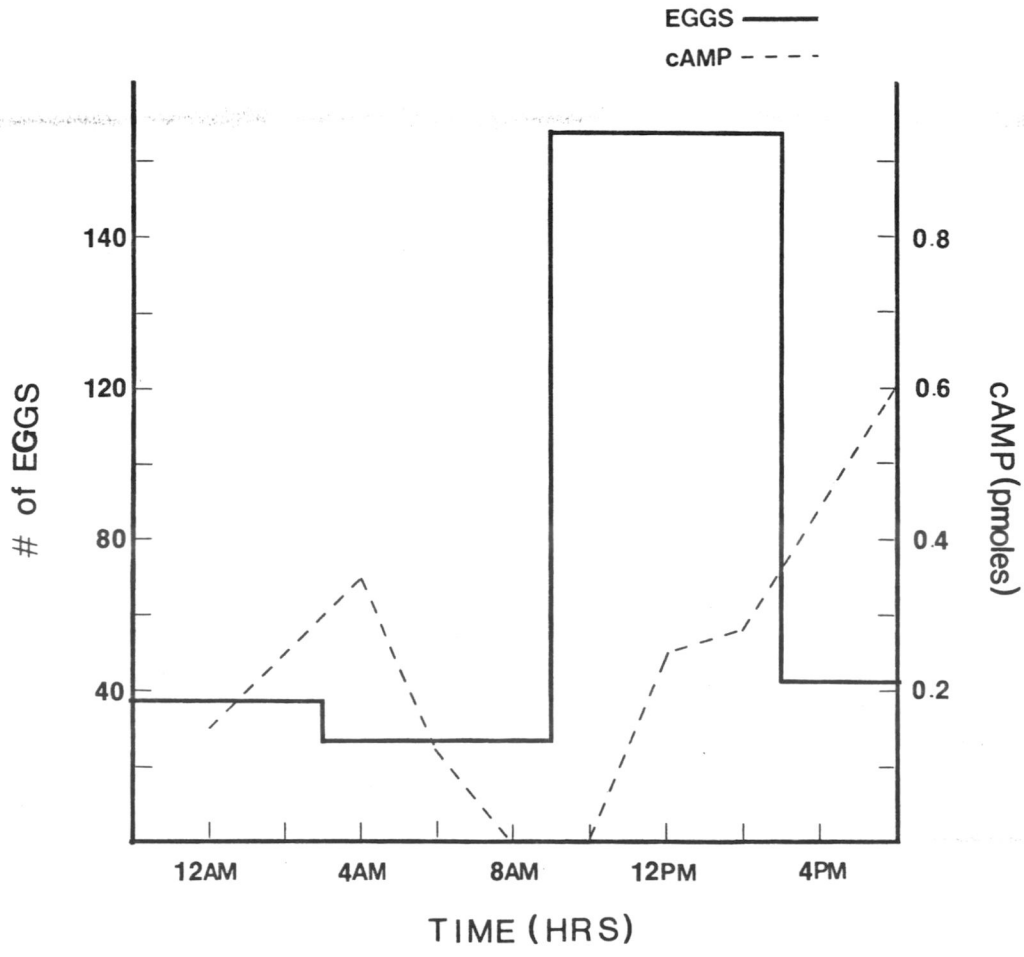

Fig. 13. Theophylline—Assay A. The level of cAMP in theophylline-treated flies at 2h intervals.

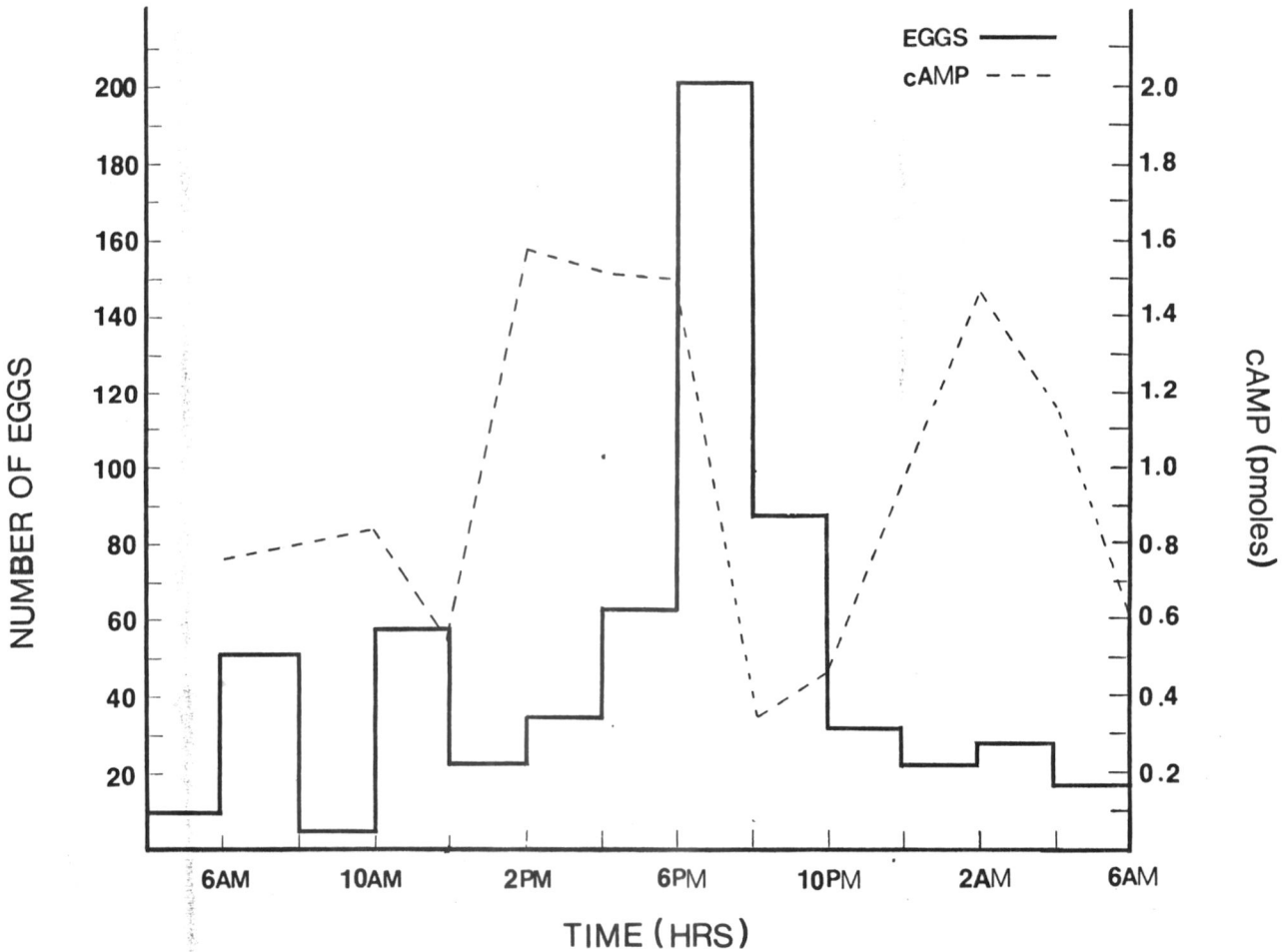

Fig. 14. Control-Assay B. The level of cAMP and the distribution of eggs in Drosophila melanogaster controls at 2h intervals.

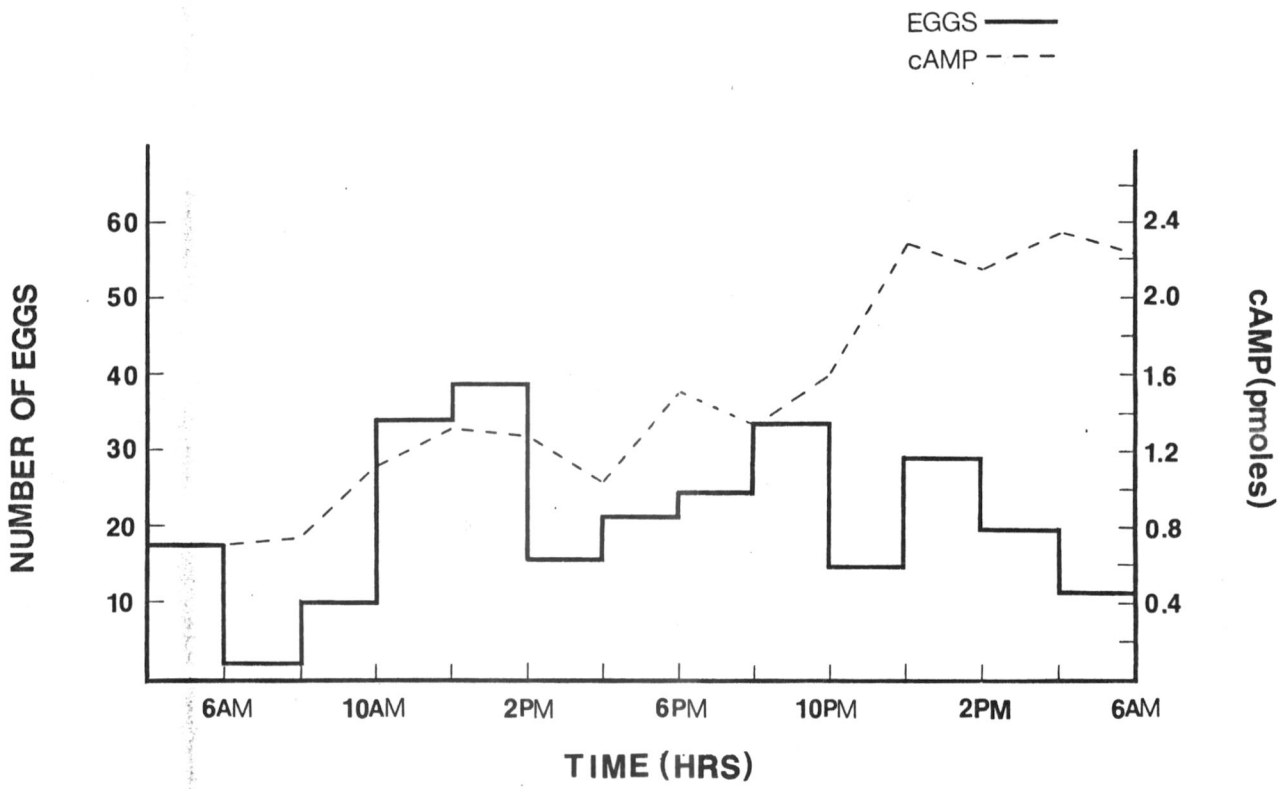

Fig 15. Theophylline 1—Assay B. The level of cAMP and the distribution of eggs in theophylline-treated Drosophila melanogaster at 2h intervals.

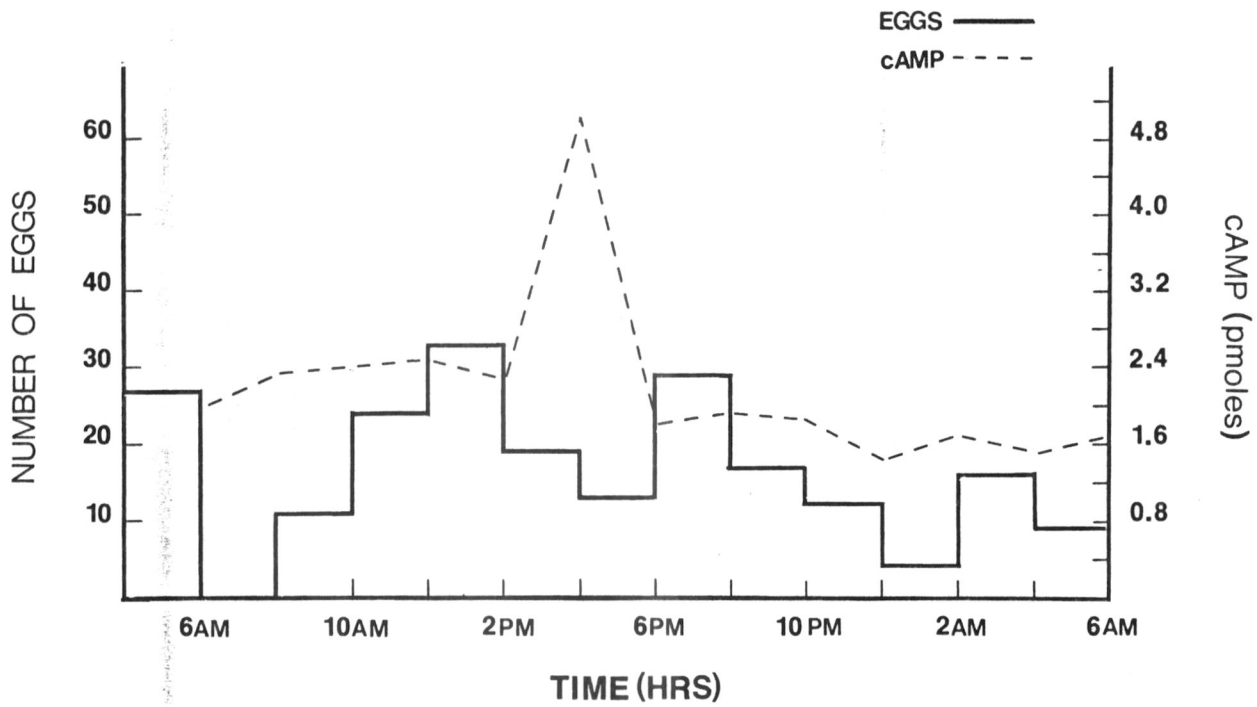

Fig.16. Theophylline 2 – Assay B. The level of cAMP and the distribution of eggs in theophylline-treated Drosophila melanogaster at 2h intervals.

Table 1. Egg deposition of seven groups of Drosophila melanogaster controls at 6h intervals during 72h runs.

	1 F 58	2 F 83	3 F 66	4 F 92	5 F 53	6 F 69	7 F 54
3 PM	550	74	152	364	11	25	71
9 PM	709*	845*	159*	708*	0	74*	255*
3 AM	352	67	87	158	9	20	91
9 AM	406	47	29	244	79*	5	44
3 PM	33	283	76	757*	33	95	90
9 PM	933*	670*	317*	433	252*	280*	491*
3 AM	404	81	47	206	11	24	59
9 AM	130	108	66	139	101	12	43
3 PM	82	368	106	610	89	102	69
9 PM	9	607*	207*	903*	316*	541*	123*
3 AM	764*	274	53	97	105	0	29
9 AM	331	124	2	102	58	0	23

Note: F = # of females in group
* - maximum number of eggs during a 24h interval

Table 2. Egg deposition of four groups of caffeine-treated Drosophila melanogaster at 6h intervals during 72h runs.

	1 F 54	2 F 34	3 F 31	4 F 24
3 PM	378	174	146*	116*
9 PM	289	12	68	74
3 AM	202	318*	42	19
9 AM	418*	208	75	35
3 PM	602*	243*	265*	292*
9 PM	444	138	64	130
3 AM	512	186	13	44
9 AM	405	173	26	26
3 PM	833*	157	162*	72*
9 PM	151	23	65	10
3 AM	70	186*	8	26
9 AM	447	121	23	25

Note: F = # of females in group
* - maximum number of eggs during a 24h interval.

Table 3. Egg deposition of four groups of theophylline-treated Drosophila melanogaster at 6h intervals during 72h runs.

	1 F 61	2 F 33	3 F 31	4 F 28
3 PM	247	46*	7	25
9 PM	184	0	107	56
3 AM	134	43	422*	111*
9 AM	321*	15	111	30
3 PM	385	150*	122	138*
9 PM	255	78	178*	83
3 AM	359	47	122	46
9 AM	450*	18	91	15
3 PM	914*	44	79	56
9 PM	261	72	165*	85
3 AM	147	73	126	117*
9 AM	130	95*	23	54

Note: F = # of females in group
* - maximum number of eggs during a 24h interval.

Table 4. Analysis of Variance: Variation among individual runs of each group.

Group	SS	df	F Value	(P)
Control	9.26	6	1.08	0.38
Caffeine	0.55	3	0.31	0.82
Theophylline	0.61	3	0.23	0.87

Table 5. Analysis of Variance: Effect of time on maximum egg deposition among individual runs of a group.

Group	SS	df	F Value	(P)
Control	30.3	3	7.05	0.01*
Caffeine	11.0	3	6.12	0.01*
Theophylline	2.22	3	0.84	0.48

* Significant

Table 6. Analysis of Variance: Effect of treatment on the time of maximum egg deposition.

Source	SS	df	MS	F	P
Treatment	113.1	59	1.92	2.06	0.01
Error	111.3	120	0.93		
Total	224.4	179			

DISCUSSION

Before the discussion of the actual results obtained, several aspects of the investigational procedure warrant further consideration at this time. One of these, of upmost importance to the study, is the mechanism of oviposition in Drosophila melanogaster. How does it occur?

Two modes of operation for oviposition in Drosophila were investigated by Grossfield and Sakri in 1972. In one of these modes of action, the brain was seen as the initial point of command for the response. Here, it is thought that stimuli that are received from courting males, or an oviposition substrate, may constitute brain inputs as well as the condition of the ovaries. This information is then processed, and nerve signals from the brain are sent to a thoracic ganglionic mass. From here, a signal is passed directly to the specific muscle group that is responsible for oviposition and, hence, oviposition occurs.

The other mode of action for oviposition behavior completely bypasses the brain as the initial point of command. In their work, Grossfield and Sakri (1972) removed the heads of D. melanogaster females and found that oviposition still occurred. They proposed that in the decapitated females, there may exist stretch receptors in the abdomen that move according to the degree of distension of the ovaries, and this causes a signal to be sent directly to the thoracic ganglionic mass which then causes oviposition to occur. This ability of decapitated females to lay eggs appears to be only evident in D. melanogaster. Grossfield and Sakri performed the same operation with D. tripunctata, D. pseudoobscura, D. virilis, and D. palustris and found that oviposition was eliminated.

Grossfield and Sakri finally concluded that since oviposition of all species of Drosophila examined did not occur after decapitation, the probable mode of operation for this behavior is a combination of these two mechanisms. The brain in Drosophila females may be responsible for handling certain external environmental stimuli while another pathway may handle intrinsic stimuli such as information from abdominal stretch receptors. At the present time, the latter conclusion seems to be more plausible than that of two entirely separate modes of activity present in the same genus.

Extensive investigations have been done in an effort to find the criteria (input signals) needed in order for oviposition to occur. Leahy (1966) found that the paragonial substance which Drosophila females receive at mating is a stimulant of oviposition. Chen and Buhler (1970) referred to this substance as the sex peptide. Leopold, et al. (1971) reported that the brain was the probable location for reception sites for the action of the male sex peptide; and that this sex peptide affected oviposition by facilitating brain output, possibly, by a hormonal or neurological pathway.

It also appears that the condition of the egg-laying substrate, along with the general surroundings, play a vital role in whether or not Drosophila females lay eggs. Maximum oviposition occurs on a scarified substrate (Ashburner and Wright, 1978); and in this study, this was accomplished by the addition of dry yeast granules to the top of the yeast solution used for collecting eggs. In addition, it was found that the egg-laying substrate must remain moist to facilitate egg-laying. This was supported by the "0" results obtained periodically for the number of eggs laid in an interval. (See Tables 1, 2, and 3.) The yeast solution

on the spoons, when they were removed for egg collections, was completely dry.

Besides the substrate, the environmental surroundings also affect egg-laying activity. A temperature of 25°C is optimal for egg deposition (Fogleman, 1979) along with a constant regime of light and dark cycles (Gruwez, et al. 1971). A 12:12 light-dark cycle was employed in this study because it has been reported to be most effective in the maintenance of circadian rhythms (Gruwez, et al. 1971; Ashburner and Wright, 1978).

In order not to interrupt the dark phase of the light cycle, a photographic safelight (Kodak filter designation Dark-Red, No. 2) was used during the removal of eggs at dark collection periods. This safelight transmits only those wavelengths of light greater than 650 Nanometers (Kodak, 1970), thus, excluding all but the red range of the visible spectrum. Frank and Zimmerman (1969) subjected D. pseudoobscura to various wavelengths of light and recorded the effects on the circadian rhythm of pupal eclosion. They found that wavelengths of light greater than 570 Nanometers had no effect on the circadian rhythm of pupal eclosion.

Another factor that needs consideration as having a possible affect on egg-laying activity is that of the actual movement of the habitat during egg collection times. Probably, this creates a certain state of "stress" in the flies in which egg deposition may be interrupted for a short period of time. Therefore, the overall effect would be a reduction in the number of eggs deposited during an interval. With this "stress" inflicted on the flies, there would probably be the need for a period of readjustment. The female flies would have to explore the new egg-laying

substrates introduced into the habitat, and signals would have to be sent back to the brain in order to elicit the response of oviposition. Nevertheless, since the same experimental procedures were carried out with all three groups, the control flies and the treated flies, the effects of the disturbances should be equal and should not affect the validity of the results.

From the results of the egg-laying activity experiments, a circadian rhythm of egg-laying is evident in the seven control runs with the maximum number of eggs being laid between the hours of 3 P.M. and 9 P.M. This is in agreement with the findings of Rensing and Hardeland (1967). The control flies laid the maximum number of eggs during a time interval which included the first few hours of darkness.

It also appears that the treatment of D. melanogaster, with caffeine or theophylline, has an effect on egg-laying activity. As is indicated in Table 6, the variation between the treated flies and the control flies with respect to time and treatment is highly significant ($P<0.01$). It was found that a circadian rhythm of egg-laying also occurred in the four runs of the caffeine-treated flies. However, this rhythm appears to be advanced with respect to the rhythm in control flies. Here, maximum oviposition occurred between 9 A.M. and 3 P.M. With the four runs of theophylline-treated flies, there appears to be no real consistency daily as to the time of maximum egg deposition; and therefore, there is no circadian rhythm of egg-laying. Referring back to Table 5, it was found that the effect of time on maximum egg deposition was significant ($P<0.01$) with control and caffeine-treated flies. It was concluded from this that a definite time of maximum egg deposition was established and was found to be statistically significant. As for theophylline-treated flies, the effect of

time on maximum egg deposition was not significant (Table 5); and therefore, no distinct time of maximum egg deposition was observed.

The radioimmunoassays A and B showed that the level of cAMP in caffeine and theophylline-treated flies was higher than that in the controls. It appears that treatment with both of the methyl xanthines, caffeine and theophylline, inhibited the breakdown of cAMP to AMP and, thus, caused a buildup of cAMP in the treated D. melanogaster. It should be noted that in Assay B, the number of flies that were sampled at each interval for cAMP was twice the number that were sampled in Assay A. This may account for the overall higher values of cAMP activity being observed in Assay B (Figures 14, 15, and 16).

Even though there is a definite increase in cAMP in the caffeine and theophylline-treated flies, it is not known if these two methyl xanthines are acting solely on cAMP phosphodiesterase. There was a reduction in the number of eggs laid by treated flies during the egg collections. It is possible that increased levels of cAMP may inhibit egg-laying activity directly.

Another important finding from the radioimmunoassay was that cAMP, itself, undergoes a cycling pattern with high and low periods of activity. In Assay A, the peaks of maximum cAMP activity almost coincide with, or slightly precede, the peaks of maximum egg deposition. This is seen clearly with the control flies and the caffeine-treated flies. The maximum peak of cAMP activity in the controls occurred between 4 P.M. and 8 P.M. with the highest level noted at 4 P.M. From the results of the seven previous control runs, it was established that maximum oviposition occurs within this interval also. Therefore, it can be deduced from this

that the peak in cAMP activity almost coincides or slightly precedes the peak in maximum egg deposition. The peak of maximum egg deposition for the controls during the assayed interval did not occur between 3 P.M. and 9 P.M. as expected with previous controls. Instead, it occurred between 9 A.M. and 3 P.M. This difference, as to the time of maximum egg deposition, is probably not significant here because the difference in the number of eggs collected between these two time intervals was only approximately 20 eggs.

The egg-laying activity of the caffeine-treated flies in Assay A appears to be typical of the previous caffeine runs in that the peaks of egg deposition daily (9 A.M. - 3 P.M.) were advanced with respect to the controls. Here, a peak at 12 P.M. in cAMP activity coincided exactly with the peak in maximum egg deposition. In the case of the theophylline-treated flies in Assay A, there was no correlation between the maximum in cAMP activity and the maximum in egg deposition.

In Assay B, the peak in maximum cAMP activity preceded the peak in maximum egg deposition in the controls (Figure 14). In addition, a slight peak in cAMP activity was observed at 10 A.M. followed by a rise in the number of eggs laid. Also, if the assayed interval had been extended beyond 24h, the rise in cAMP activity seen between 2 A.M. and 4 A.M. may have preceded a rise in egg deposition similar to the rise observed at the beginning of this control run (6 A.M. - 8 A.M.).

In theophylline 1 and 2 of Assay B, maximum egg deposition occurred between 12 P.M. and 8 P.M. There was in both cases a rise in cAMP activity before this peak of egg deposition; however, it was not the maximum peak in cAMP activity. The rise in cAMP activity in theophylline 1, that started at 8 P.M. and continued till 12 P.M., is characteristic of the

theophylline group of Assay A. However, the rise in Assay A was between the hours of 10 A.M. and 6 P.M. (Figure 13); and, thus, there is no correlation in this respect between theophylline 1 (Assay B) and the theophylline run in Assay A. In theophylline 2, the large value (5 pmoles) for the peak in cAMP activity at 4 P.M. may not be as accurate as the other values on the graph (Figure 12) due to the limitations of the standard curve from which it was interpreted. However, if this was the maximum peak of cAMP activity for this run, it occurred after the peak in egg deposition.

Thus, overall, it appears that the treatment of D. melanogaster with caffeine or theophylline causes a change in the time of maximum egg deposition; and, therefore, a change in the circadian rhythm of egg-laying. In addition, there is evidence that cAMP may have a stimulatory effect on egg deposition, at least in the case of the controls and the caffeine-treated flies, based on the observation of peaks in cAMP activity preceding or coinciding with peaks of egg deposition. The absence of synchrony between rises in cAMP activity and rises in egg deposition in theophylline-treated flies, along with the elimination of the circadian rhythm of egg-laying, may be due to the fact that theophylline is a more potent inhibitor of cAMP phosphodiesterase (Rall, 1969). This may also account for the greatest levels of cAMP being observed with theophylline-treated flies. If this is the case, then, the rhythm exhibited with caffeine-treated flies may be due to a weaker inhibition of cAMP phosphodiesterase. This would lead one to believe that a critical range in the level of cAMP is necessary for rhythmicity. A future investigation into this problem with lower concentrations of theophylline may prove to be helpful in determining if rhythmicity can be maintained with

theophylline-treated flies.

It is evident that increases in cAMP levels in D. melanogaster have a definite effect on the circadian rhythm of egg-laying. It would also be interesting to examine whether or not decreases in cAMP levels have the same effect on the circadian rhythm. This could be accomplished by a study in which the inhibition of adenyl cyclase is carried out instead of the inhibition of cAMP phosphodiesterase. One would expect that lower levels of cAMP would affect the circadian system; however, it is not known whether or not the same changed patterns resulting from higher levels of cAMP would be observed.

Now, the question remains in this investigation, how does cAMP affect the circadian system? In the search for the answer to this problem, one must examine the controlling mechanism behind the rhythm, that is, the circadian clock.

In 1979, Handler and Konopka found evidence for the location of the circadian clock for locomotor activity in Drosophila to be the brain. They concluded this because the removal of the brain resulted in the elimination of rhythmicity. They also reported that the action of the brain must be mediated by hormones. They transplanted Drosophila brains from one group of flies who exhibited 18-20h activity rhythms in constant environmental conditions (LD 12:12) into the abdomens of arrhythmic flies whose activity under these conditions is aperiodic. They then observed that rhythmicity was restored in the arrhythmic flies. They concluded that since the time period for rhythmicity to be restored was short, there was not enough time for neural connections to be formed. It appears from their research that the control of circadian rhythms in Drosophila is by hormonal means very similar to the controlling mechanisms

found in silkmoths (Riddiford, 1970) and in birds (Menaker and Zimmerman, 1976).

Considering the above studies, along with this investigation, it seems possible that cAMP may be acting as a "master controller" in circadian rhythmicity. No doubt, cAMP is a strong regulator in biological systems, and this role may also be exhibited here with circadian systems. In this particular study, cAMP levels may constitute another input signal for the brain like the signals from courting males, and substrate and environmental conditions. In this aspect, cAMP may be acting similarly to that of the sex peptide in that it facilitates brain output. Any alteration in cAMP levels may result in output behavior (oviposition rhythmicity) occurring earlier as was the case with caffeine, or may result in the complete elimination of rhythmicity as was seen with theophylline.

The possibility also exists that cAMP may be acting by way of its role as a second messenger in hormonal systems. A hormone secreted from the brain may be responsible for oviposition activity. Here, instead of stimulating output behavior, cAMP may be the chemical agent that, in response to the hormone, sets up activity in the abdominal central nervous system for oviposition.

As for evidence of a neurological control of circadian rhythms (Page, 1978), the same proposal of cAMP as a "master controller" may apply here also. It has been shown that cAMP participates in synaptic transmissions in the nervous system (Greengard, et al. 1972). From this line of reasoning, the level of cAMP, or its actual presence, may control whether or not nerve signals from the brain ever reach the muscles controlling oviposition. A study involving alterations of cAMP levels in organisms,

such as the cockroach in which a neurological control has been well established for circadian rhythms, would help to test this hypothesis.

In a different light, Njus, et al., (1974) proposed a membrane model for the circadian clock. They suggest that the biological clock may be a feedback system involving a network of membrane-bound ions and ion-transport channels. Here, oscillations are regulated by fluctuations in ion concentrations. It is possible that cAMP may be involved in this model due to its own association with membranes of hormonal target tissues.

At this time, one point of the procedure needs clarification. It was assumed that the acquirement of the methyl xanthines by Drosophila would be accomplished during early development. Of the four major stages of the life cycle of Drosophila (egg, larva, pupa, and imago or adult), it seems that the majority of the methyl xanthines would be absorbed during the larval stage. It is during this stage of the life cycle that the developing organism constantly feeds on culture medium; and in this study, the culture medium contained the methyl xanthines. A problem, however, arises in that it is not known when the mechanism of circadian rhythmicity is established in the life cycle. True, from Handler and Konopka (1979), it is known that the brain is the location of the circadian clock; and therefore, it would appear that the brain formed in the larval stage would be the origin of rhythmicity. However, sense organs, mouthparts, and bodily form present in larvae are entirely different from those in the adults (Highnam and Hill, 1969). It would be advantageous in a later study to subject normal adult Drosophila melanogaster to medium containing a methyl xanthine, and then study the effects on various circadian rhythms. This would allow one to conclude whether the rhythm was completely established before the adult form (no change in rhythmicity)

or if it could be altered at the adult stage.

Nevertheless, this investigation provides additional evidence for a role of cAMP in the control of circadian rhythms. The synchrony that appears between rises in cAMP activity and rises in egg deposition is certainly noteworthy. Some of the explanations suggested for the results will require further examination with other circadian systems. Once this is completed, a universal controlling mechanism of circadian rhythms in the form of the synthesis and degradation of cAMP may be evident.

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