SOME RHYTHMIC PHENOMENA IN *FUNDULUS HETEROCLITUS*

by

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ABSTRACT

Photoelectric and ultrasonic techniques were developed to measure and record colour change and locomotory activity, respectively, of Fundulus heteroclitus. The recordings obtained revealed endogenous rhythms of locomotory activity and colour change. Further studies were then carried out to determine some of the basic characteristics and control mechanisms of these rhythms.

Colour change had a free-running period of 23 hours 45 min $\pm$ 15 min. The length and phases of the rhythm were unentrained by light-dark, temperature, salinity or thermosaline changes. Illumination intensity, different background, and blinding also did not affect the rhythm. Hypophysectomy extinguished the rhythm of colour change but not the ability to adapt to different backgrounds. This observation was taken to indicate a hormonal role in the control of killifish colouration.

Locomotory activity of single fish and groups of 5 and 20 occurred in a diurnal pattern that could be entrained by photoperiod. Under constant conditions the period length of the circadian activity rhythm of single fish was dependent on the intensity of illumination (23 hrs 20 min $\pm$ 18 min - 24 hrs 5 min $\pm$ 15 min), whereas in the groups of fish the period was independent of the intensity of illumination.

Blinded fish maintained an endogenous activity rhythm that could be entrained by photoperiod. Hypophysectomy eliminated the endogenous rhythm but did not alter the responses to photoperiod.

The variations observed between the two rhythms suggest that a multi-oscillator system may be present in killifish.

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INTRODUCTION

(i) General Properties of Biological Rhythms

All living organisms inhabit an environment that has numerous rhythmic parameters: as a consequence the physiology and behaviour of many have rhythms with periods of the same length as those of the major alterations of their environment. The rhythms range from those of high frequencies, 10-100's of cycles per second, e.g. spontaneous discharge of nerve cells, beating of the heart; to those of low frequencies and long periodicities; hours to months, e.g. diurnal, tidal, lunar, seasonal, annual rhythms.

It is now generally believed that these organismic cycles are determined by "biological clocks" or oscillators within the plants and animals. The interpretation and analysis of "biological clocks" and rhythms is discussed in several reviews and symposia; Chovnik (1960); Cloudesely-Thompson (1961, 1971), Aschoff (1965), Sollberger (1965), Brahmarchy (1967), Bünning (1967), Brown and Hastings (1970), Menaker (1970). Studies of rhythms have now been made in almost all animal phyla. Investigations done on vertebrates have largely concentrated on birds and mammals: rhythms in fishes have not received any extensive analysis. The present study was carried out to investigate

some aspects of cyclic behaviour and physiology of Fundulus heteroclitus, the killifish or mummichog . Although there is obviously an intrinsic interest in such a study in an area where we have little basic knowledge, F. heteroclitus is also a species which is widely used as an experimental organism in general physiological research. In studies where rhythmicity is not specifically under investigation, the possibility of cyclic fluctuations in the value of a physiological variable is ignored - for example the time of day at which an experiment was carried out is rarely specified. If the physiological state of killifish shows cyclic variations, the outcome of this study could have much broader implications.

Biological rhythms or oscillators can be either exogenous or endogenous in origin. Exogenous rhythmicities are those variations in physiology and behaviour that are imposed upon an organism by external environmental cycles. The rhythmicity in the organism is directly caused by, and follows an environmental cycle. The external environmental oscillations are themselves referred to as being exogenous.

Endogenous biological rhythms are rhythmic oscillations in physiology and behaviour that are either inherent in the organism and continue(free-run) under constant conditions; or normally occur without any environmental correlates. Constant conditions exclude all light,

temperature, humidity and tidal cycles. Environmental cycles may affect the endogenous rhythmicities displayed by the organism but are not the cause of their occurrence.

Being kept under constant conditions often places organisms in an abnormal state in which extremes of physiological processes can no longer be attained (Bünning 1967). Many low frequency oscillations, i.e. period greater than 12 hrs.,may fade out after 4-5 cycles when the organism is placed in constant conditions. This fade-out or extinction does not indicate that the endogenous rhythmicity disappears, but rather that its externally shown pattern of variation is reduced below a detectable level. Therefore, according to an accepted convention (Bünning 1967),if a low frequency rhythmicity persists for at least 4-5 cycles under constant conditions it is termed endogenous. Endogenous rhythms that continue for long(er) periods of time are known as "self-sustaining" oscillations.

On one hand there is a group of investigators, notably Brown (1969), who state that organisms possess no endogenous rhythms. They contend that all living entities are able to sense and are influenced by subtle pervasive geophysical cycles, e.g. atmospheric tides, lunar gravitation, geomagnetic and electric fields, and cosmic radiation. These exogenous factors are presumed to produce

the cyclic changes in behaviour and physiology that other investigators have termed endogenous.

On the other hand it is more generally believed that most daily (diurnal) behavioural and physiological activities represent endogenous oscillations that are free running under constant conditions. Since these diurnal rhythmicities have their own inherent periodicities which approximate 24 hours, they are termed circadian (Halberg 1959).

Under either natural or laboratory conditions, circadian rhythms adopt the period of an exogenous environmental cycle (light, temperature, tidal), usually 24 hours but the period may vary between 22 to 26 hours. Outside these limits the rhythms run freely at their own inherent frequencies with superimposed environmentally determined variations. The phases of the endogenous rhythm are synchronized to occur with specific portions of the environmental cycle.

Environmental cycles that are able to synchronize or entrain endogenous rhythms, or cause the occurrence of exogenous variations, have been called synchronizing or entraining agents, cues, and 'Zeitgebers' (Bünning 1967). The process of obtaining synchrony is known as entrainment. Entrainment by biological oscillators entails the adoption of the Zeitgeber's period by the endogenous rhythm, accompanied by coupling of specific events or phases in the

internal and external cycles. When the phase of the Zeitgeber is shifted, the phases of the entrained cycle also shift to maintain a constant phase relationship with the external cycle. This process of entrainment has been observed for a number of Zeitgebers and a variety of rhythms.

(ii) Circadian Rhythms

For circadian rhythms, the most extensively utilized and studied Zeitgeber is photoperiod, with locomotory activity the behaviour usually investigated. Fish have been documented as being nocturnal, diurnal, or arrythmic in activity (Spencer 1939). Müller and Schreiber (1967) and Livingston (1971) have shown endogenous activity rhythms in two different fish species. There have been only limited demonstrations and studies of photoperiodically determined endogenous rhythms of locomotory activity or other physiological variations in fish, e.g. (Livingston 1971).

With killifish, Davis and Bardach (1965) demonstrated a "time-co-ordinated pre-feeding activity" determined by photoperiod. They suggested that this rhythm was coordinated or controlled by some endogenous clock that may be responsive to photoperiod. Davis (1962) also showed a daily rhythm in the reaction of fish to light, termed a 'light-shock' reaction.

Control of physiological reactions by day length is known as photoperiodic control and is discussed by Pittendrigh (1960). Many fish, including several species of Fundulus (Mast 1917, Goodyear 1970), are able to utilize the position of the sun or its azimuth as a reference point for orientation and navigation. To do so, they have to be able to compensate for daily and seasonal changes in day length and the position of the sun, suggesting the existence of time-keeping mechanism that can be entrained by photoperiod.

Continuous light and dark have different effects on period length and fade-out time of endogenous rhythms. The observation that period length varies with the light (L) intensity imposed under constant conditions is expressed as a general rule known as the "Circadian" or "Aschoff's Rule". (Aschoff 1960). According to this rule, the spontaneous frequency, which is the ratio of activity to rest time and the amount of total activity, should increase with increasing light intensity in light active animals, and should decrease in dark active animals. However, several organisms display either partial or complete exceptions to this rule (Hoffman 1965). With fish there has been only one investigation into the validity of the Circadian rule. Schwassmann (1970) showed that the period of the spontaneous discharge of electric fish was dependent on illumination intensity. In laboratory studies

the experimental design can often determine whether the animal is light or dark active. For instance Jones (1956) found that the minnow Phoxinus was light active in an empty tank but became dark active when shelter was provided.

There are several possible means by which a 24 hour (22-26 hours) light-dark (L-D) cycle can function to entrain circadian rhythms. These means also hold true for other Zeitgebers and rhythms. They can be listed as follows:

1) Through proportional effects: The rhythm is entrained by the ratio of L-D time. There are maximum and minimum ratios outside of which entrainment is not possible.
2) Through differential effects: Transition from one state to another is the effective synchronizer, e.g. dawn and dusk. In most laboratory studies the L-D transition is very abrupt without any gradual dawn-dusk transitions being considered. These gradual transitions may increase the limits for entrainment.
3) Through an impulse effect: One short signal, e.g. flash or occultation interrupting otherwise constant conditions.

From experimental and theoretical considerations (Bünning 1967), it appears the proportional effects are the most important sources of entrainment.

(iii) Tidal and Lunar Rhythms

Intertidal fish and other organisms, when maintained in constant conditions, often display an endogenous tidal rhythm of activity that rapidly loses synchrony with the tides, e.g. locomotory activity of the intertidal fish Blennius blennius (Gibson 1967), crabs (Naylor 1958). Under simple diurnal tides the period of this rhythm is 24.8 hours. Under constant conditions tidal rhythms appear very similar to, or actually revert to, a circadian form, e.g. activity of Blennius (Gibson 1970). However, upon exposure to the tides, tidal synchrony is rapidly reestablished.

It has been proposed that tidal rhythms are specialized circadian rhythms (Aschoff 1966). Organisms can simultaneously possess both tidal and circadian periodicities, each affecting different physiological processes or behaviours.

Although Fundulus heteroclitus is an intertidal fish, there have been no thorough demonstrations or investigations of possible tidal rhythms in behaviour and related physiological processes. Day (1968) suggested that Fundulus similis possesses a tidally phased rhythm of susceptibility to NaCl and endrin. He also proposed that under constant conditions this tidal susceptibility rephased to a more "basic rhythm".

The normal entraining factors for tidal rhythms include cycles of wave produced turbulence (Enright 1963), or cycles of hydrostatic pressure (Morgan 1965, Gibson 1971), rather than cyclic variations in temperature and light, to which tidal rhythms are relatively insensitive.

Lunar rhythms are also known to occur in several organisms (Fingerman 1960). The physiological and behavioural rhythms that occur, correspond either to the lunar cycle of 29.3 days, or half of it. Lunar rhythms are synchronized by either intensity of moonlight or lunar tides (Bünning 1967). Lunar variations are superimposed upon circadian or tidal rhythms. Lunar rhythms have also been interpreted as being the result of a "beat phenomena", that is, the result of interactions of endogenous circadian and tidal rhythm. Small differences in period length of the two rhythms would result in the coincidence and summation of phases at discrete intervals, i.e. at approximately 15 or 29 days. A detailed analysis by Enright (1972) showed that this "beat phenomena" was not an adequate explanation for most lunar rhythms. He emphasized that there is actually a lunar rhythmicity inherent in many organisms.

The best known lunar cycles are those associated with the reproduction of certain marine invertebrates (Bünning 1967). Lunar periodicities are also found in freshwater animals, e.g. lunar variation of the spectral

sensitivity in the guppy (Lang, 1965, 1967). There has been no investigation of lunar rhythms in killifish.

(iv) Hormonal and Oscillator Models for Biological Rhythms

Pittendrigh (1960) has proposed that organisms consist of "a population of quasi-autonomous oscillatory systems". These oscillators can entrain one another, giving the impression of being driven by a single master clock, or become synchronized to events occurring at high and lower frequencies.

Hoffman (1970) has experimentally demonstrated that a multioscillator system underlies an apparently simple circadian periodicity. He has shown that under constant conditions, the circadian activity pattern of the tree shrew split into several out of phase oscillations of different frequencies. After alterations in light intensity these oscillations became coupled in phase and frequency, appearing and functioning as a single circadian periodicity. Observations and studies of this type have not been carried out with fish.

The oscillators present may have different sensitivities to exogenous cycles. Through a variety of couplings they could produce tidal or circadian rhythms of different sensitivities. Experimentally observed and recorded rhythms demonstrate only an external function of

the biological clock. They do not reveal how the processess being studied are linked to the basic oscillator(s).

Hoffman (1970) suggests that endocrine influences, which can be modified by light, are involved in the internal synchronization of circadian oscillators and their behavioural and physiological counterparts. This involves the synchronization and integration of several different hormonal systems through a variety of feedback mechanisms.

The pineal, because it possesses an endogenous photoperiodically controlled hormonal (melatonin) and enzyme activity, has been postulated as an integrating or regulating agent for the activities of other hormonal systems; mainly those of the pituitary (Axelrod 1971, Quay 1971). The integration could take place through a number of direct (pituitary) and indirect (hypothalmic) feedback mechanisms. The pineal itself may also be affected by pituitary activity. Kastin et al. (1972) have demonstrated complex interrelationships in rats, in a study of interactions between pineal, hypothalmus and pituitary involving, melatonin, melanophore-stimulating hormone (MSH) and MSH release-inhibiting factor. In fish the nature of interactions between various hormonal systems is much less fully understood.

There is substantial evidence that the pineal gland and its light inhibited secretion of melatonin may

be the route for exogenous synchronization of locomotory activity in mammals, (Quay 1970). Melatonin can affect the amounts of locomotory activity exhibited by fish (Hafeez 1970). However, blinding and pinealectomy did not abolish the ability to entrain activity by varying photoperiod (Erikson 1972). This observation suggests that there are light-sensitive structures or organs and hormonal systems, in addition to the pineal, involved in the internal synchronization of activity rhythms in fish. There have been no extensive studies performed to determine characteristics and controls on locomotory activity of killifish.

In lampreys, the pineal has been shown to be necessary for the continuation of endogenous rhythms of colour change and background adaptation (Young 1935). The pineal has also been implicated as the controlling factor for the colour change rhythm of Pencil fish (Reed 1966). In killifish, in which a rhythm of colour change has not been previously shown, pinealectomy has not altered the ability to change colour (Abbott, unpublished).

Diurnal variations in the production and level of many diverse pituitary hormones have been shown (Curtis 1970), although direct relationships to physiological or behavioural rhythms have not been unequivocally established. Meier and Shivastra (1972) has shown diurnal variations in the levels of prolactin, ACTH, and adrenal steroids in Fundulus

grandis. He has also shown that hypophysectomy did not eliminate the diurnal variations in adrenal steroids. In Fundulus heteroclitus there have been several studies of the functioning of the pituitary and its hormones (Pickford 1957). There has not been any analysis done of the possible hormonal relationships of rhythmic physiological and behavioural processes.

It should be noted that even though some processes may be dependent on a certain level of a hormone, this does not imply that they respond to diurnal variations in the level of the hormone.

(v) Proposed Research

(i) Investigation of Activity and Colour Change Rhythms

There is relatively little information on the analytical and comparative aspects of basic rhythmic functions of fish (Schwassmann 1971). A central aim of the research reported here was the investigation of the occurrence of biological rhythms in fish.

In this study two behavioural and physiological functions of killifish were studied. These functions, locomotory activity and colour change, were established as being rhythmic and then analyzed for possible controlling mechanisms. Two different cycles were studied to obtain

a comparative analysis of rhythmic oscillatory systems in this fish.

The early work dealing with locomotory activity of fish was largely descriptive. No attempt was made to determine whether the observed periodicities were endogenous. Many of the early laboratory activity determinations gave inconclusive results because the methods and apparatus used were poorly designed and employed (Schwassman 1971). The activity of fish was inhibited, or the system was not sensitive enough to detect all motion, thus the records were inconclusive.

Killifish and other teleosts have not been investigated for the presence of endogenous rhythmic variations in their colour (melanophore activity). In a preliminary experiment, a variation in the colour of killifish at different times of day was observed that could not be attributed to changes in external conditions. Those observations indicated that there were daily variations in colour change which appeared to be systematic in occurrence. These determinations were not adequately controlled or conducted over long enough time intervals to determine the presence or absence of rhythmic components in killifish colour change. They did however, provide a basis for further investigation.

To carry out activity and colour change investigations, sensitive measuring and recording systems were designed. The systems developed were ultrasonic and photoelectric apparatus to record locomotory activity and colour change, respectively. These new apparatus were necessary in order to record satisfactorily all colour change and activity over extended time periods, i.e. 5 days or more. Recordings over an extensive time period were needed for reliable statistical analysis for the presence of rhythms in the data or time series.

(ii) Investigation of Colour Change

An additional purpose of this research was to study the mechanisms controlling colour change in killifish. This was carried out wherever possible and appropriate in the investigation.

Colour change and background adaptation of fish has been extensively studied, with the findings summarized in a number of reviews and monographs [Parker (1948), Odiorne (1957), Waring (1963), Fingerman (1963, 1965) and Fujii (1969)].

Colour change is carried out through the activities of integumentary pigment-containing cells called chromatophores. These cells are further classified according to their colour and the type of pigment they contain.

In killifish, melanophores containing the black pigment melanin, are the main cells by which background adaptation and colour change is carried out.

Placing a killifish on an illuminated black background causes melanin granules to disperse throughout the processes of the melanophores and the fish takes on a dark colour. On a white background melanin granules withdraw from the branches and aggregate in a tight cluster in the centers of the melanophores and the fish assumes a pale hue. These adaptations are complete in 5-10 minutes. The colour assumed by the fish is visually determined through the albedo (the ratio of the incident light to the reflected light). Under very low light levels the fish assumes an intermediate shade. Several different molecular mechanisms have been proposed to explain pigment migrations (Fujii 1969).

The chromatic background responses that take place very quickly and do not involve any alterations in the absolute quantity of melanin contained in the melanophores, are known as "physiological" colour changes. Physiological colour change involves both neural and hormonal controls, though the relative importance of each varies among different species and classes of fish. Generally those fish that respond rapidly (0-10 mins.) to different backgrounds have predominately neural control of melanophore activity,

e.g. elasmobranchs, cyclostomes, catfish (Parker 1948, Waring 1963).

Colour change of a more permanent nature, occurring after prolonged exposure to constant background and illumination and characterized by absolute gain or loss in the amount of pigment in cells and a corresponding increase or decrease in the total number of melanophores is known as "morphological" colour change. These changes are assumed to be almost entirely hormonally mediated (Fujii 1969).

Neural control of teleost physiological colour change has been conclusively proven several times (Waring 1963).

Recent experiments (Novales 1971) have shown that neural control of melanophore activity is sympathetically mediated through α (alpha) and β (beta) receptors. Stimulation of the α receptors, leads, through an increase of the melanophore cyclic AMP level, to melanin dispersion and darkening of the killifish. Stimulation of the β receptors decreases the melanophore cyclic AMP level, leading to melanin aggregation and paling of the killifish.

The presence of neurally mediated "physiological" colour change does not preclude the possession of hormonal controls. Hormones could act either directly on the melanophores or indirectly through alterations in nervous system activity. As the degree of neural control and

development is increaseddirect hormonal involvement is decreased (Fujii, 1969). Any hormonal influences present would be slowly demonstrated,possibly in a rhythmic manner.

The pituitary produces several hormones involved in pigmentary control; melanophore Stimulating Hormone (MSH) a substance causing melanin dispersion; and melanophore concentrating hormone (MCH) a postulated aggregating principle. The evidence for the presence and activities of these and other hormones in physiological colour change is at best contradictory (Pickford and Atz 1957, Fujii 1969). This investigation is an attempt to resolve the question of hormonal control mechanisms in physiological colour change of killifish.

Colour Measurement and Apparatus

Apparatus:

A photovoltaic apparatus, based on a method proposed by Hill *et al.* (1935), was designed to measure and record continuously the colour of live, intact, partially restrained killifish.

Fish colour was measured as the voltage produced by light reflected from a stationary killifish onto the receiving surface of a photovoltaic cell. The intensity of light reflected increased or decreased as the fish became light or dark respectively. The photocell produced a voltage output linearly proportional to the intensity of the light striking it. The energy of the reflected light remained the same since the reflecting surface itself was not altered. By constantly monitoring the voltage a continuous record of fish colour change was obtained. The apparatus used to make these measurements is shown in Figures 1 and 2.

A clear glass cell (0.32 cm. thick glass; 10 cms X 3.5 cms X 3.5 cms) served as a holding chamber for the fish whose colouration was being studied (Figure 1). A continuous flow of fresh or saltwater was maintained through the chamber.

During freshwater studies an open water supply system was used. A constant supply of aerated, dechlorinated water was maintained in a head tank and fed to the chamber.

through 0.79 cm. (I.D) Tygon tubing. Water temperature (10-25°C ± 1°C) was regulated by an immersion heater (Porta-Temp, Precision Scientific, New York) placed in the head tank (Figure 2).

In salt-water studies a closed system with continuous filtration, aeration, and temperature regulation was used. Compressed air was used to circulate and aerate the water. The total volume of water used was 1.0 liters and the time required to completely circulate and filter it was approximately 10 minutes. Fish were kept for a maximum period of one week in the same solution without the addition of water or a change of filter.

Filtration was carried out in a chamber [40 cm X 15 cm X 10 cm] containing glass wool and activated charcoal. The charcoal was used to absorb the nitrogenous wastes. Control of water temperature (10-25°C ± 2°C) was accomplished by a countercurrent flow of water through glass tubing (0.32 cm I.D) placed in the filtering chamber. The water was supplied from the head tank used in the freshwater studies.

With both supply systems the rate of flow to the chamber (40 ml/min) was controlled by a flowmeter (Monostat Corp., New York). The oxygen content of the water was maintained at saturation levels.

The holding chamber was placed on a background

(Number F2 photographic paper - see Appendix 1) chosen from the Ostwald Papers (Healey and Ross 1966). The Ostwald Papers are a series of shades of gray that range from white to absolute black and are numbered from 0 to 8.

A selenium photovoltaic cell (Megatron Type B, 9.7 cm X 3.5 cm, Megatron Ltd., London, England) with maximum spectral sensitivity of 0.6 μ was mounted 4.0 cms above the top of the holding chamber. During long term studies the cell was placed directly on top of the chamber. The positive and negative terminals of the photocell were connected to a chart recorder (E & M Physiograph Model DMP-4, Boston, Mass; or Servoriter II, Houston Texas) to record the millivolt output.

Illumination was provided by a pair of incandescent spotlights (General Electric Type R, 75W, 130V) symmetrically mounted 25 cm above the holding chamber (Figure 2). Their intensity was controlled and stabilized through a variable voltage transformer (Variac, Bristol, Conn.). The transformer and lights were calibrated by measuring the intensity of light falling on the chamber at different transformer voltage values. The voltage output of the cell was linearly proportional to the illumination intensities used in the experiments to be described. Light intensity was measured with a hand photometer (Sekonic Model L-28C, Japan). The heat produced was absorbed by a heat filter placed in

front of each light. These heat filters consisted of a thin wide glass cell (25 cm X 7 cm X 4 cm) through which water flowed at a rate of 175 ml/min.

The apparatus was checked after each experiment or at weekly intervals, to ensure that the operation of the lights, photocell, and recorder amplifier did not change with use. The spotlights were usually changed after each experiment. The photocell was checked with a hand photometer and voltmeter. There was some direct current (D.C.) drift present and caused mainly by the amplifier. According to the manufacturers specifications the photocell should show a slight but non-significant downward drift under the conditions that it was employed. The spot lights would also show a higher energy production as the colour temperature slightly rises. However, in the spectral ranges of the photocell this proved to be a negligible change. Over a week period the average drift was +15-20 millivolts.

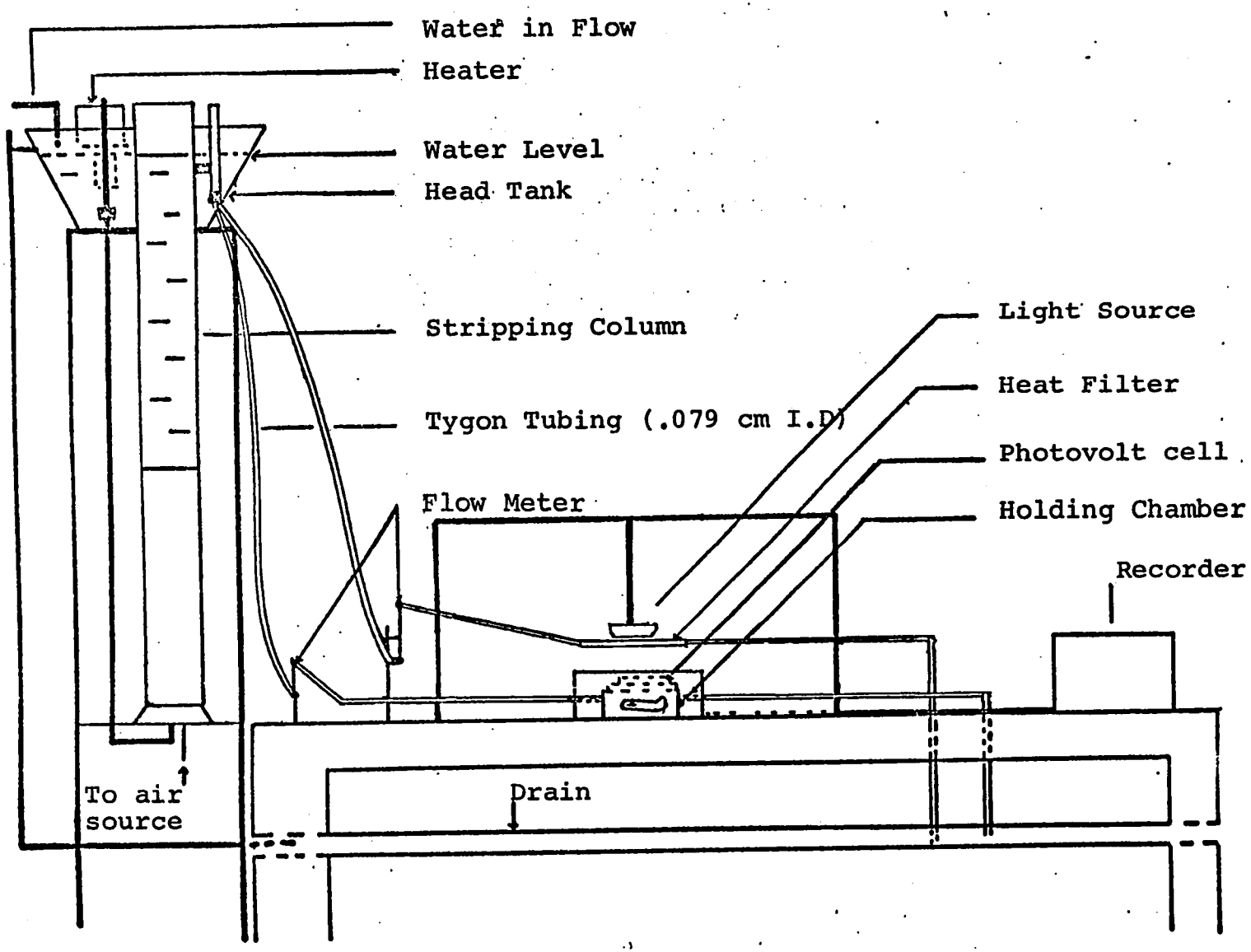


Figure 2: Apparatus for Determination of Colour Change of Killifish in Freshwater Studies.

Figure 1: Holding chamber, with killifish and photovoltaic cell.

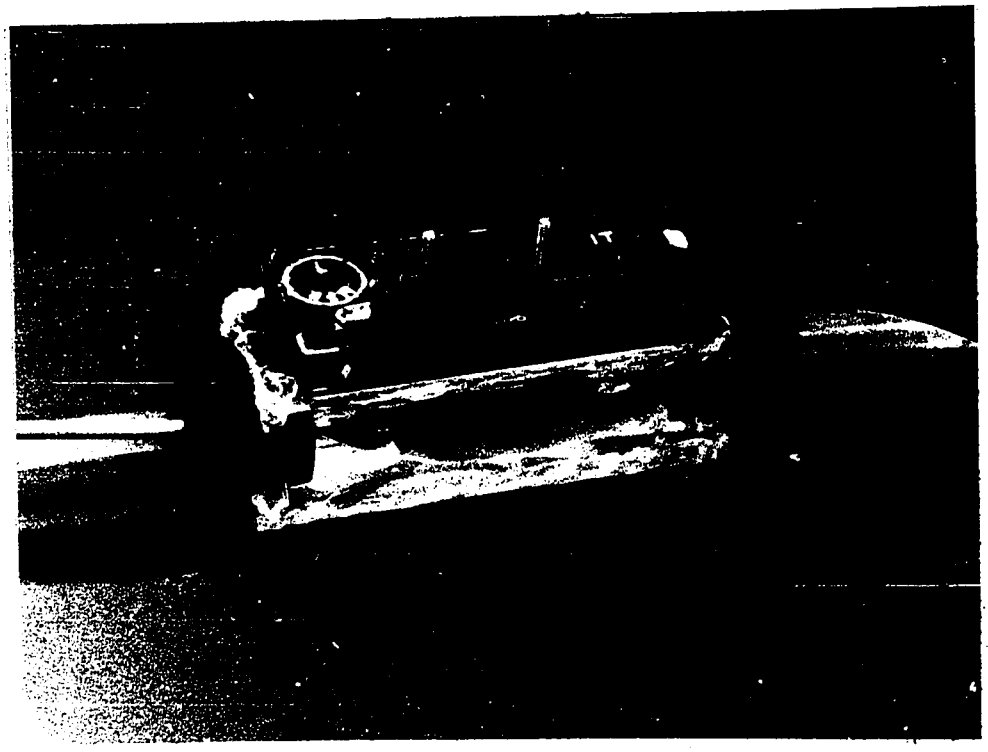
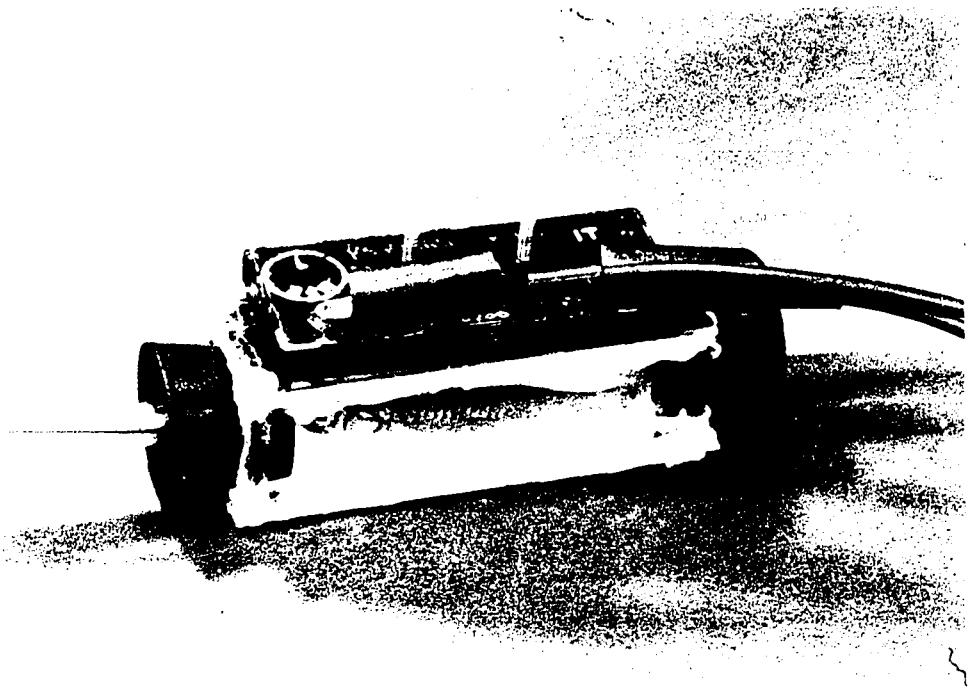


Figure 1: Holding chamber, with killifish and photovoltaic cell.



Colour Change: Determination of General Properties of Colour Change.

Methods (I):

Naive fish were placed in the holding chamber under different initial illuminations, backgrounds, and thermo-saline regimes. They were allowed to acclimate to these conditions for 48 hours. According to Fry (1971) and Sage (1971) 48 hours is an adequate length of time for acclim-ation.

After this period of acclimation temperature and salinity changes were made and their effects on fish colour-ation recorded. There were 5 different temperatures (5, 10, 15, 20, 25°C) X 4 salinities (0, 9, 18, 35‰) X 4 back-grounds (Numbers 2, 3, 4, 6 Ostwald Papers), all at 200 Lux, giving a total of 80 different conditions that were used. The same fish was used for a series of conditions. The series were replicated with at least 3 different fish.

While the intensity of the light reflected from the killifish,measured as millivolts was being recorded from the confined fishes; the Derived Ostwald Index (D.O.I.) was determined by visual comparison of the fish to the Ostwald Papers (Healy and Ross 1967). This permitted the establish-ment of a D.O.I. - millivolt relationship for each fish under a specific set of light, background, and water conditions. The different combinations of factors tested and their

effects on voltage output and fish colouration are shown in Figures 8 and 10.

Records were also taken of the voltage output from the empty chamber exposed to the test conditions. These were taken to eliminate the possibility of obtaining spurious records of colour change from alterations in the optical properties of the water. Fish motion also affected the records of colouration. Movement caused sharp peaks which were however, centered around the same base line. Colour change was determined according to the voltage alteration given by a changing base line.

Total testing times were of 30 - 120 minutes. The recording speeds of 0.0025 to 0.05 cm per sec, were varied with the parameters being studied; the degree of response, and the amount of physiological or behavioural disturbance shown by the fish. If the fish proved to be unusually restless, placing itself in awkward positions and orienting improperly to the inflowing current so that its ability to survive was impaired, recording was either done very rapidly or completely curtailed.

Rhythmic Colour Change Analysis

Methods (II)

A procedure was devised to determine and analyse the colour change displayed by Fundulus heteroclitus. By a preliminary recording of fish colour under constant conditions the existence of cyclic variations in colour change were established. Determinations were then made of the period, amplitude, and possible synchronizers (Zeitgebers) and hormonal controls of the rhythm.

The basic criteria that were used to test and define the colouration rhythm are listed below [modified after Aschoff (1960)].

1) Demonstration and measurement of the period of the colour change rhythm in constant conditions (Table 1).
2) Ability to establish and maintain a constant relationship with the rhythm to differently phased entraining cycles (Table 1).
3) Ability to establish and maintain a constant relationship to different frequencies of suspected Zeitgebers (Table 1).

Basically the experimental methods employed involved:

1) Maintaining fish for 30 days under a predetermined photoperiod, temperature, salinity and

background.

2) These conditions were used to try to entrain a rhythm of colour change. After being maintained, a continuous measurement of fish colour (5-14 days), was made under constant conditions of illumination, temperature, salinity, and background.

Constant conditions were used to test for the presence of a rhythm of colour change, and to see if the previous maintaining conditions had entrained an existing rhythm. All measurements described here were carried out with different fish supplies and occurred between May 1971 and February 1972. As far as possible equal sample sizes were maintained when tests were replicated.

Fish were taken from the holding tanks and placed under the maintaining conditions for 30 days. These maintaining conditions are listed in Table 1-5. Fish were held singly and in groups (20 per group). The constant conditions were maintained in a walk-in environmental chamber. The fish were held in the same type of tank and under identical filtration aeration, and feeding, conditions as after arrival (see Materials pg. 1 for a description of these conditions).

After the 30 day entraining period fish were selected at random and transferred to the holding chamber.

During the transfer times from maintaining tanks to the testing chamber fish were lightly anesthetized with a 1:10,000 dilution of tricane methane sulfonate (M.S. 222; Sandoz Chemicals, Montreal).

For the first 48 hours after transfer the previous maintaining conditions were continued. During this time period the colour of the fish was continuously recorded and a preliminary relationship between electrical output and colour, as measured by D.O.I., was established. Any fish displaying signs of physiological or behavioural distress, e.g. improper orientation, rapid thrashing, was rejected.

After the 48 hour acclimation time two types of test conditions were used. In one, temperature, salinity, background, and illumination were kept constant while colour was recorded. Each group of fish taken from the entraining conditions was measured under a different testing combination. The combinations of testing and entraining conditions used are listed in Table 1-5.

In the other testing conditions temperature and salinity were rhythmically varied with all other factors being held constant. Afterwards all conditions were held constant. During both testing conditions colouration (voltage output) was continuously recorded.

During the testing periods, of 5-21 days, the killifish were neither fed nor disturbed. At certain intervals their D.O.I. was obtained by visual comparison to the Ostwald papers.

The voltage output of the photovoltaic cell was continuously recorded at a paper speed of 0.0025 cm/sec.

For 7 days 20 fish were exposed to the testing conditions listed in Tables 4 and 5, then half were bilaterally blinded and the other half hypophysectomized. Testing was then resumed for a further 7 - 14 days.

Another 20 fish were operated, half bilaterally blinded and the other half hypophysectomized, reentrained for 7 days and then tested again for the presence of rhythmic colour change.

Bilateral blinding was accomplished by chemical cauterization: 0.5 ml of 100% ethanol was injected into the eye destroying the photoreceptors. The effectiveness of the operation was demonstrated by the loss of ability to adapt to extremes in background and loss of visual motor responses. The same tests were conducted on control fish.

Hypophysectomy was performed according to the method described by Abbott and Favreau (1970). Sham operations were performed as a control. These fish were tested in the same manner as the experimental group.

After colouration recordings and activity measurements, to be described later, were performed, fish were killed with an overdose of MS 222. The extent of pituitary removal and peripheral tissue was then verified by histological examinations.

The ability of several hormonal substances to alter fish colouration was also tested. The chemicals used were: melatonin (synthetic), total beef pituitary extract, prolactin (beef), thyroxine (beef), all supplied by Aldrich Chemicals (Wisconsin). All were injected intraperitoneally. The total volume injected was 0.25 ml with the concentrations of the active principle ranging between 0.01 - 5.0 µgm/gm wet body weight of fish. The hormones were dissolved in 0.15 N NaCl, which also served as a control injection. The chemicals were administered at different times and to fish kept under a variety of entraining-testing conditions.

Table 1: Maintaining and Test Conditions Used for Studying the Colour Change Rhythm in Killifish.

Maintaining Conditions				Testing Conditions			
Photoperiod Light:Dark L:D in hours LL = constant D y = intensity of illumination Lux	Temperature °C	Salinity o/oo FW = fresh water	Background Ostwald Index	Illumination intensity in Lux	Temperature °C	Salinity o/oo FW = fresh water	Background Ostwald Index
Natural (10:12)	20	9	2	100	15	9	2
				100	20	FW	3
				1	20	18	2
Natural (8:16)	20	0	2	100	15	FW	6
Natural (12:12)	20	9	2	100	20	9	3
				1	20	18	2
12:12[1] y = 100	20	9	2	100	15	FW	6
				1	20	9	2
12:12[2] y = 100	10	0	6	100	15	FW	6
				1	10	35	2
12:12[3] y = 100	20	35	2	100	20	35	2
				1	20	9	2
L:L y = 100	15	9	2	100	15	15	2
				1	10	FW	2

	Maintaining Conditions			Testing Conditions			
hotoperiod ight:Dark :D in hours L = constant D = intensity f illumination Lux	Temperature °C	Salinity o/oo FW = fresh water	Background Ostwald Index	Illumination intensity in Lux	Tempera- ture °C	Salinity o/oo FW = fresh water	Background Ostwald Index
L:L	10	18	2	100	10	18	6
y = 100				100	15	35	2
D:D	15	9	2	100	15	35	2
y = 1				1	15	9	2

1) L = 1200 - 2400 E.S.T.

2) L = 700 - 1400 E.S.T.

3) L = 2400 - 1200 E.S.T.

Table 2: Maintaining and Testing Conditions Used for Determining Effects of Different Photoperiods, Flashes and Occultations on the Rhythm of Colour Change in Killifish.

	Maintaining Conditions			Testing Conditions			
Photoperiod Light:Dark L:D in hours LL = constant D y = intensity of illumination Lux	Temperature °C	Salinity o/oo FW = fresh water	Background Ostwald Index	Illumination intensity in Lux	Tempera-ture °C	Salinity o/oo FW = fresh water	Background Ostwald Index
LL Occultations 2100 and 0900 y = 100	15	9	2	100 1	15 20	9 18	2 2
DD Flashes at 2100 and 0900 y = 1	15	0	2	100 1	15 20	9 35	2 2
08:16 y = 100	10	9	6	100 1	10 10	FW FW	2 2
16:08 y = 100	10	18	2	100 1	15 10	18 FW	6 2
08:08 y = 100	10	18	2	100 1	15 15	FW 35	6 2
15:05 y = 100	15	18	2	100 10	15 15	9 35	2 2

	Maintaining Conditions			Testing Conditions			
Photoperiod Light:Dark L:D in hours LL = constant D y = intensity of illumination Lux	Temperature °C	Salinity o/oo FW = fresh water	Background Ostwald Index	Illumination intensity in Lux	Tempera-ture °C	Salinity o/oo FW = fresh water	Background Ostwald Index
03:16	15	18	2	100	10	FW	4
y = 100				1	10	FW	2

Table 3: Maintaining and Test Conditions Used for Studying the Effects of Thermosaline Variations on the Rhythm of Colour Change in Killifish.

Maintaining Conditions				Testing Conditions			
Photoperiod Light:Dark L:D in hours LL = constant D y = intensity of illumination Lux	Temperature °C	Salinity o/oo FW = fresh water	Background Ostwald Index	Illumination intensity in Lux	Temperature °C	Salinity o/oo FW = fresh water	Background Ostwald Index
Natural (10:12)	20	9	2	100	10	FW for 12 hrs 35 for 12 hrs	2
12:12 y = 100	20	9	2	100	20	35 for 12 hrs FW for 12 hrs	6
12:12 y = 100	20	35	2	1	20° for 12 hrs 10° for 12 hrs	35	2
12:12	20	18	3	100	20° for 12 hrs 10° for 12 hrs	35 for 12 hrs FW for 12 hrs	4

Table 4: Maintaining and Testing Conditions Used for Studying the Effects of Blinding on Rhythm of Colour Change in Killifish.

	Maintaining Conditions			Testing Conditions			
Photoperiod Light:Dark L:D in hours LL = constant D y = intensity of illumination Lux	Temperature °C	Salinity o/oo FW = fresh water	Background Ostwald Index	Illumination intensity in Lux	Tempera-ture °C	Salinity o/oo FW = fresh water	Background Ostwald Index
12:12 y = 100	20	18	2	100	20	35	3
12:12 y = 100	15	0	6	1	15	9	2
13:12 y = 100	20	0	2	100	15	18	2
LL	15	9	2	100	15	9	2
LL	10	18	2	1	10	18	2
DD	15	18	2	1	15	18	2

Table 5: Maintaining and Testing Conditions Used in Studying the Effects of Hypophysectomy on the Rhythm of Colour Change in Killifish.

Photoperiod Light:Dark L:D in hours LL = constant D y = intensity of illumination Lux	Maintaining Conditions			Testing Conditions			
	Temperature °C	Salinity o/oo FW = fresh water	Background Ostwald Index	Illumination intensity in Lux	Tempera-ture °C	Salinity o/oo FW = fresh water	Background Ostwald Index
12:12.	20	9	2	100	20	35	4
12:12	15	18	2	100	15	35	3
12:12	20	18	2	1	15	35	2
LL	15	0	2	100	15	35	2
DD	15	18	2	1	15	35	2

Table 6: Substances Tested for Their Effects on Colour and Activity of Killifish.

Substance	Dose mgm/gm wet body weight of fish
Melatonin (synthetic)	0.1; 1.0; 10
Prolactin (mammalian)	1.0; 10
Pituitary extract (beef)	0.1; 1.0; 10
Thyroxine (mammalian)	1.0; 5.0

Table 7: Maintaining and Test Conditions Used in Studying the Effects of Various Substances on Colouration in Killifish.

Injection Time Eastern Standard Time (E.S.T.)	Testing Conditions				Previous Maintaining Conditions			
	Illumination y = Lux	Temperature °C	Salinity o/oo	Background Ostwald Index	Photoperiod Light:Dark L: Dim hours y = intensity of Illumination-Lux	Temperature °C	Salinity o/oo	Background Ostwald Index
1200 1800 2400 0600	100	20	18	2	12:12 y = 100	20	18	2
1200 1800 2400 0600	1	15	18	2	12:12 y = 100	15	18	2

Activity Determinations and Recording

Apparatus:

The apparatus used for activity determinations is shown in Figure 3 and Appendix 2. The method involved recording voltage changes, represented by peaks on a chart recording, caused by killifish locomotor activity. Basically these voltage alterations were caused by the fish motion altering an ultrasound pattern that had been set up in the tank.

The experiments were performed in a white, polyethylene, rectangular, tank (95 cm X 35 cm) provided with a glass stand pipe at a depth of 30 cm. A continuous flow of water, 50 ml/min, was provided from a head tank by two fine Tygon capillary tubes (0.1 cm I.D.). The tank, surrounded by heavy black plastic, was provided with overhead fluorescent lights (Sylvania Daylight) by which the photoperiod was controlled. During most experiments the tank was continuously illuminated by an overhead darkroom light (General Electric R-1).

A piezoelectric ultrasonic transducing element, 2 cm in diameter (Massa Model TR-7, Hingham, Mass.) was attached with silicone grease to the outside of each end of the tank (Figure 3). The associated circuit (Appendix 2) was mounted on rubber blocks to reduce vibration and shielded by a Faraday cage to minimize electrical disturbances.

The operating voltage, 6 volts at 250 ma, was drawn from a stabilized power supply (Sanyo Power Supply, Japan). Positive and negative leads from the circuit were connected to a chart recorder (E & M Physiograph) through a variable resistance (0 - 100 Ω) acting as a fine sensitivity control, and a 400 μfd capacitor functioning as an integrator. Some integration was necessary because the transducer responded much more rapidly than the recorder. The tanks, lights, Faraday cage, power supply, and recorder were all connected to a common ground.

One piezoelectric transducer served as a transmitter of ultra sound, at an operating frequency of 35 KHz, while the other element functioned as receiver. The transmitted sound set up a standing wave pattern in the tank. A portion of this pattern was detected by the receiving element and transformed into a recordable voltage output, at approximately 100 millivolts. With killifish in the tank the standing wave pattern was constant as long as the fish remained stationary. When the killifish moved, both the phase and frequency (by Doppler shift) of the received sound altered the standing wave pattern. These alterations were measured as a change in the voltage level and represented by a peak on the recording. Similar methods of using alterations in standing wave patterns to detect fish activity were used by Cummings (1963) and Meffert (1968).

The height and number of peaks was roughly proportional to the degree and total amount of locomotor activity. Prolonged activity gave a continuous set of peaks since the phase of the received pattern was continuously changed. A fish located directly in front of one of the transducers resulted in an off-scale tracing and one in the path directly connecting the two transducers gave a record of continuous activity. This path was in the mid-water region, an area where Fundulus were not observed to remain in a stationary state.

The relative positions of the two transducers were very important. Changing their positions altered the standing and received wave patterns. The two elements had to be placed in such a relationship that activity in any portion of the tank could be detected. These positions were determined by trial and error, and had to be altered each time water temperature or salinity was changed.

By adjusting the sensitivity of the circuit it was possible to measure the activity of one or more fish.

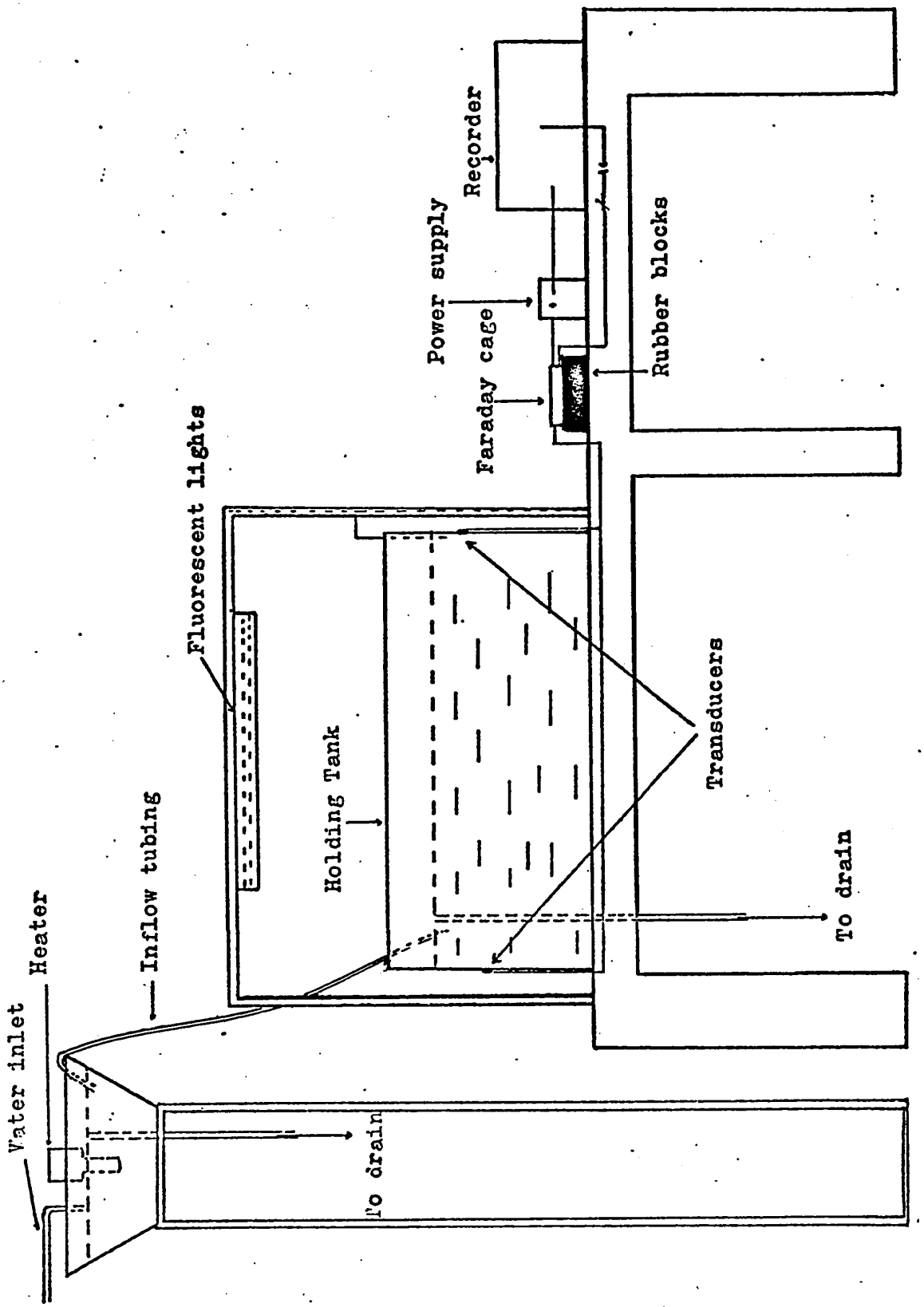


Figure 3: Diagram of Apparatus Used for Activity Determination.

Methods

Activity determinations

After 30 days in the environmental chamber under the maintaining conditions shown in Table 8, fish were taken, singly or in groups (group sizes of 5 and 20) and placed in the activity determination tank. Previous synchronizing conditions were maintained for a further 5-7 days, with a continuous recording of fish activity.

In order to test for the existence of a free-running rhythm of activity, fish were exposed to constant illumination levels, temperature, salinities and flow rates. Subsequently determinations were made of the ability of photoperiod to act as a Zeitgeber for rhythmic activity. These modifications and determinations were made according to the criteria described for the colouration study (page 21). The maintaining and testing conditions are listed in Table 8. Total testing times ranged from 5-14 days.

Because temperature and salinity changes altered the sensitivity of the detecting circuit, these factors could not be effectively studied as possible Zeitgebers for locomotory activity. However with continuous fine adjustments of sensitivity it was possible to make rough determinations of the effect of alterations in temperature and

salinity on locomotory behaviour. These determinations were of a very approximate nature and are mentioned where appropriate in the results.

Throughout all testing periods the fish were not fed. This was done to avoid external disturbances and the possibility of spurious synchronizations.

The effects of blinding and hypophysectomy on activity were tested. These operations were performed in the same manner as those described for the colouration determinations (pg. 30). Fish were entrained (maintained) and then; (1) operated on and tested or (2) tested and operated or (3) reentrained and tested. The various combinations of entraining and testing conditions are listed in the results as they are presented.

The effects of various hormonal substances on locomotor activity were studied. The substances were dissolved in 0.15 N NaCl and the volume of the solution injected intraperitoneally was 0.25 ml. The substances used (the same as in the colouration study), injection times, and entraining conditions used in this study are listed in the results.

Table 8: Maintaining and Testing Conditions Used for Studying Rhythmic Activity of Killifish.

(a) Intact Fish

Entraining Conditions			Testing Conditions			Group size
Illumination L:D y = intensity of L_1 = lux Y_2 = darkroom light (D) (not measured)	Temperature °C	Salinity parts per thousand p.p.t. F.W. = Freshwater	Illumination L.D. Y_1 Y_2 y_1= lux y_2= darkroom light not measured	Temperature °C	Salinity p.p.t. on FW	Number of Fish in group whose activity was recorded
12:12 y 100 y 200 y 50 L(From 1200-2400 E.S.T.)	10	F.W.	24:00 y = 100 y = 200 y = 50	15	F.W.	1,5,20
12:12 y 100 y 50	20	20	00:24	20	20	1,5,20
12:12 y 100 (L from 2400-1200 E.S.T.)	20	F.W.	24:00 y = 100 00:24	24	F.W.	1

Illumination L:D y_1= intensity of L = lux Y_2 = darkroom light (D) (not measured)	Temperature °C	Salinity parts per thousand p.p.t. F.W. = Freshwater	Illumination L.D. Y_1 Y_2 y = lux y_2= darkroom light not measured	Temperature °C	Salinity p.p.t. on F.W.	Number of Fish in group whose activity was recorded
12:12 y = 100 (L from 700-1400)	20	20	00:24 24:00	20	20	1
12:12 y = 100 (L from 1200-2400	15	F.W.	12:12 y 100 (L from 2400-1200)	15	F.W.	1,20
00:24 . Flashes 0600 Flashes 4500	10	F.W.	00:24	15	F.W.	1
24:00 y = 100 (Flashes 0600 (Flashes 0800	15	F.W.	24:00	15	F.W.	1
08:08 y = 100	20	40	24:00 y 100	20	40	
18:18 y = 100	25	F.W.	00:24	15	F.W.	

STATISTICAL METHODOLOGY

Colour change and locomotory activity records were quantified by listing millivolt values obtained at 10 minute intervals and total activity per half hour interval.

Before any detailed analyses were carried out, a simple "runs test" of the data was carried out to see if the time series could be due to random variation.

A sequence of data points that consecutively increase or decrease is termed a "run". The number of runs present in the data was counted and compared to the value expected if the data were purely random (Bendat and Piersol, 1968). A significant difference indicates that there may be a trend or cyclic component present in the time series. This is a very rough test, the reliability of which is minimized by the presence of serial correlation in the data.

Serial correlation arises if the value of one datum influences the value of the next datum. The sampling interval is so short that two or more sucessive data points will be recorded through the start and finish of the same process, inertia preventing an instantaneous change.

For example, when a fish is paling there is a certain neural and physical delay before this process can be stopped and the reverse starts. Sampling intervals occurring during such a delay would both include paling, causing

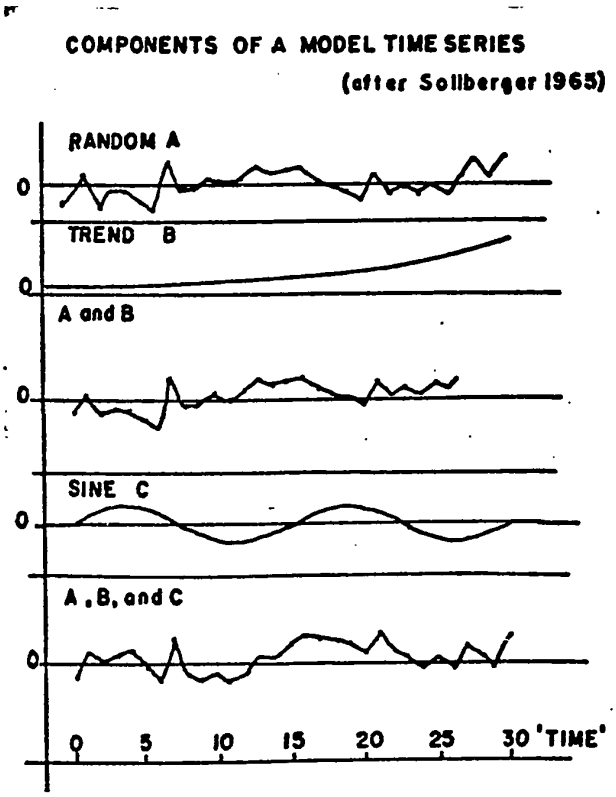
serial correlation. The sampling interval was selected so that the complete colour change and activity process could be completed in this interval, thus minimizing serial correlation effects in the time series. If too large a sampling interval is used short rhythmic processes in the time series may not be seen, or the basic underlying process may be obscured.

All analyses used are based on methods and formulae described by Halberg and Panofsky (1961), Panofsky and Halberg (1961), Mercer (1960),Strumwasser (1967), Bendat and Piersol (1968) and were carried out with a CDC (Control Data Corporation) 6400 computer.

Time series analysis consists of the description, separation, and study of three components of the series: (Fig. 4).

1) trend - A time series that possesses a trend is non-stationary.
2) cyclic fluctuations.
3) irregular and random fluctuations.

Figure 4:



Any trend present in the series was assumed to be linear and was determined through a regression analysis. After the trend line was estimated its value was subtracted from the time series leaving periodicity and all other aperiodic components. Linearity was not a completely valid assumption since the trend could be a slightly curvilinear portion of a long term cycle.

After removal of the linear trend the data was transformed to zero mean and unit standard deviation. These transformations were necessary because of fluctuations in the amplitude.

The autocorrelation function of the time series was then calculated. This substantially reduced aperiodic components in the data and revealed periodicies present in the time series.

The autocorrelation functions were determined by duplicating the time series and comparing it with the original series. The intensity of the relationship between adjacent values on the two curves was calculated. This relationship is expressed as the correlation coefficient R. Initially the correlation coefficient of a time series with itself is +1, since the two curves are identical and superimposed on one another. The duplicated series is then transposed a step k, called a lag value and equal to the original sampling interval of the data (h). This is the minimal lag

value that can be used. Correlation values for the points juxtaposed on the two curves were then calculated. The correlation coefficient decreased from the original value of +1 since the two series were no longer perfectly related. This step wise calculating process was repeated until a predetermined total lag value (m) was reached.

When the two curves are 180° out of phase, the correlation coefficient (R) approaches -1 and as they gain a proper phase relationship R approaches +1. If the original time series has a periodicity present, the calculated correlation coefficient will display this periodicity with most of the random variation removed. If the data possesses a trend, this will reduce the efficiency of the correlating process in eliminating random variation.

A plot of correlation coefficient R against lag k is called a correlogram, and graphically reveals the periodicity (p) present in the data. For example Fig. 5. Theoretical and actual time series, correlograms, and further spectral transformations are given in figures 5,6.

The autocorrelation functions were calculated by the following formula:

$$\hat{R}_r = R_{x(n)} = \frac{1}{N-r} \sum_{n=1}^{N-r} x_n \, x_{n+r}$$

N = total No. of data point

r = 0,1,2,3.....m

m = lag

x = datum point

$\hat{R}_r$ = correlation coefficient

To obtain the period length from the correlogram, the lag (k), in hours, at each cycle peak R(k) was graphed as a function of cycle number (Fig.6D) (Strumwasser 1967). A least squares regression line was computed through the data points. The slope of this line gave the mean period length and its 95% confidence interval could be calculated from the variance of the points about the line. Any slope discontinuties indicated changes in period length. Comparisons between slopes of regression lines obtained under different testing conditions, e.g. illumination intensities, were used to determine whether these factors significantly affected period length.

The lag (k) for each cycle peak could not be estimated with equal reliability. Whilst the majority of cycles were uniform in shape, (Figs. 6A), a few were

broader and irregular, and others were sharply pointed with a very distinct peak value.

These differences in the reliability with which the lag value was estimated were not taken into account in determining the regression line, because careful examination of the correlograms showed no systematic pattern in the occurrence of broad and narrow peaks. The estimation error was therefore assumed to be negligible and uniform throughout.

Determinations of the frequencies of fundamental sinusodial components and their harmonics present in the original data were obtained through a harmonic (Fourier) analysis of the correlogram. This yielded a variance or power spectrum; a measure of the contribution of oscillations of different periods to the total variance in the data. The spectrum is essentially a graph of the height (or amplitude, power, variance G[f]) of a series of sine curves of different fundamental frequencies. Any point in the line spectrum displaying considerably more power than its' neighbors indicates a periodic component at that frequency.

By a smoothing process, a line spectrum can be transformed into a continuous spectrum, allowing estimation of variance contributions by particular frequency ranges and the period range of possible cyclic components in the

time series. Examples of different types of autocorrelation functions and their corresponding power spectra are found in Figure 6H,E.

In power spectra the smallest frequency that can be resolved, F_c, the Nyquist frequency is determined by the sampling interval h.

$$F_c = \frac{1}{2h}$$

For colouration 3 cycles per hour
for activity 1 cycle per hour

The different frequencies for which power can be calculated are determined by the maximum lag value (m) of the autocorrelation value. This is dependent on the number of data points (n) and the length of the original time series.

$$F = \frac{kF_c}{m}$$

where F_c = smallest frequency obtained (Nyquist frequency).
m = maximum lag value of the autocorrelation.
k = 1,2,3,.....m.

. The limits (5 and 95%) of the power or variance (G) estimates are obtained from Chi-square (χ^2) values (Halberg and Panofsky 1961). The degrees of freedom (D.F.) are dependent on sample size and lag value (m),

decreasing as lag value is decreased.

$$D.F. = \frac{(2n - \frac{m}{2})}{m}$$

Confidence Limits

95% $\frac{\chi^2 .95/D.F.}{D.F.} G$ and 5% $\frac{\chi^2 .05/D.F.}{D.F.} G$

As can be seen the larger the lag value (m) that is used the finer the resolution of the period lengths but the poorer is the reliability of the estimate.

In this study the power spectra could not be used to make a satisfactory determination of period length because the total sampling time was too short. In the circadian-tidal range (23-25 hours), power spectrum analysis gave a much lower resolution than the regression method already described.

The duration of the experiments was not extended because it was thought that physiological and behavioural disturbances might develop that could obscure the results obtained in the earlier portions of the records.

The power spectra were used to indicate the presence and frequency ranges of obscured cyclic components that might be present in the correlograms and the original data. A significant power at zero frequency was taken as an indication of a non-linear trend being present in the original time series (Halberg and Panofsky 1961).

The power spectra were calculated from the auto-correlation function using the following formula:

$$\hat{G}_k = 2h[\hat{R}_o + 2 \sum_{r=1}^{n-1} \hat{R}_r \cos [\frac{\pi rh}{m}] + (-1)^k \hat{R}_m]$$

smoothed

$$\hat{G}_k = 0.25\ G_{k-1} + 0.5\ G_k + 0.25\ G_{k+1}$$

$\hat{G}_k$ = power

h = sampling interval

R_o = average correlation coefficient

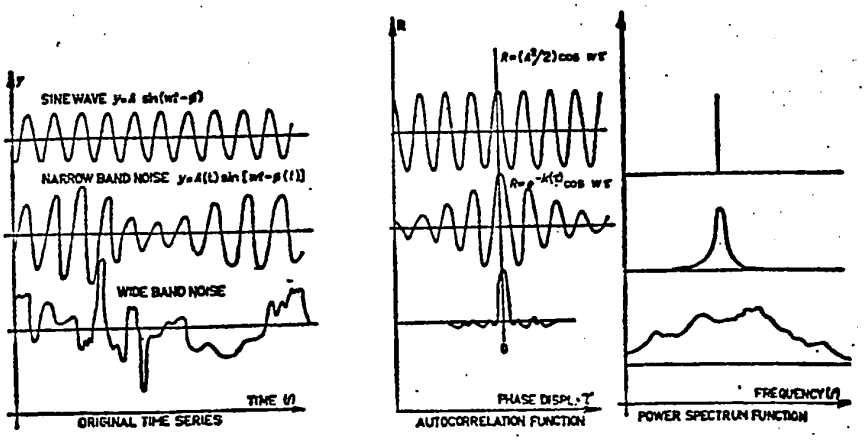
r = 0,1,2,3.....m

m = lag

k = 1,2,3,.....m

R_m = correlation coefficient

Fig. 5: Theoretical time series and their autocorrelation and power spectrum transformation.



From Sollberger 1965

Figure 6: Sample time series and their statistical transformations.

A. Sample colouration record.

B. Autocorrelation plot of the same colour record.

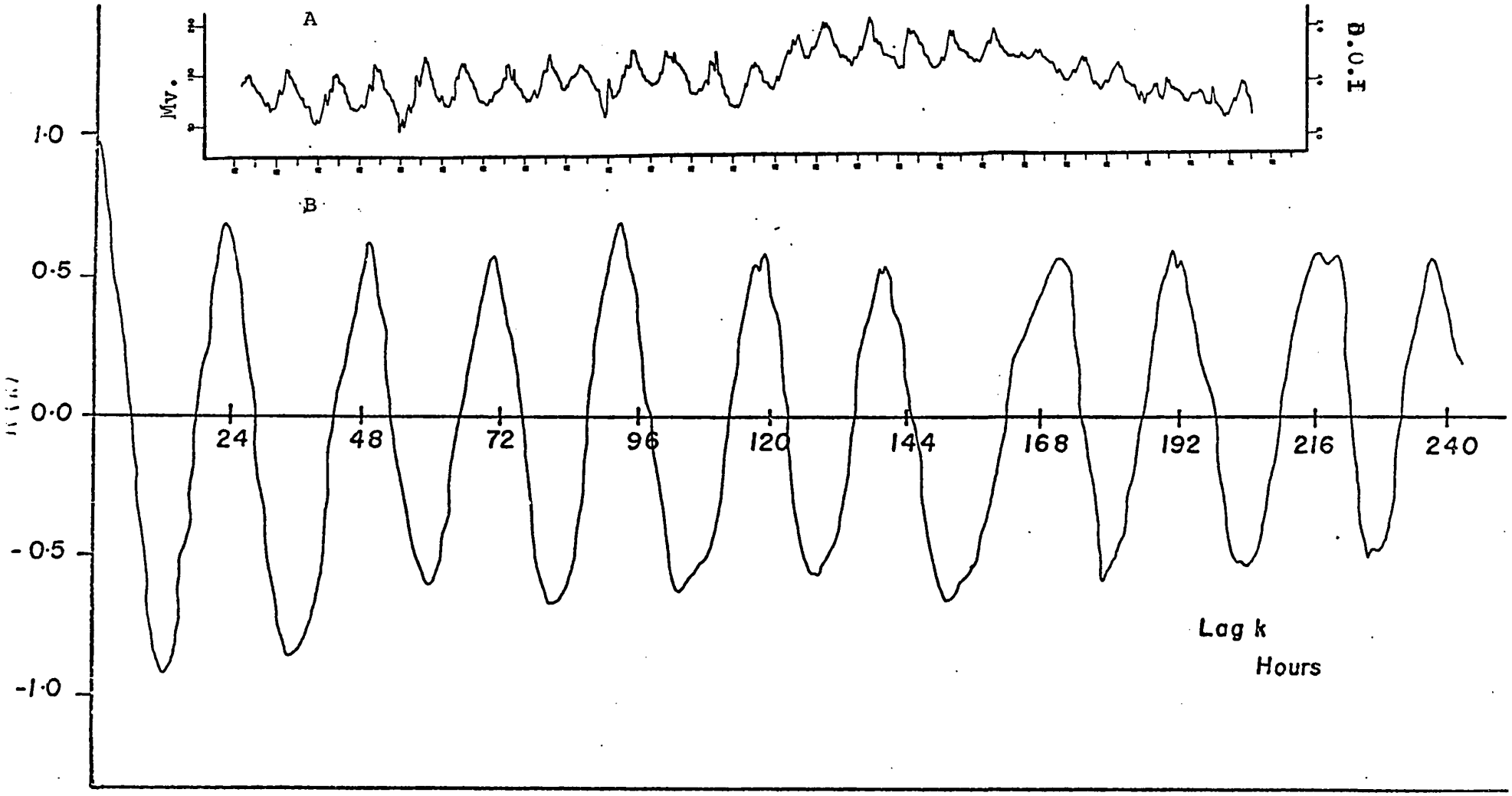
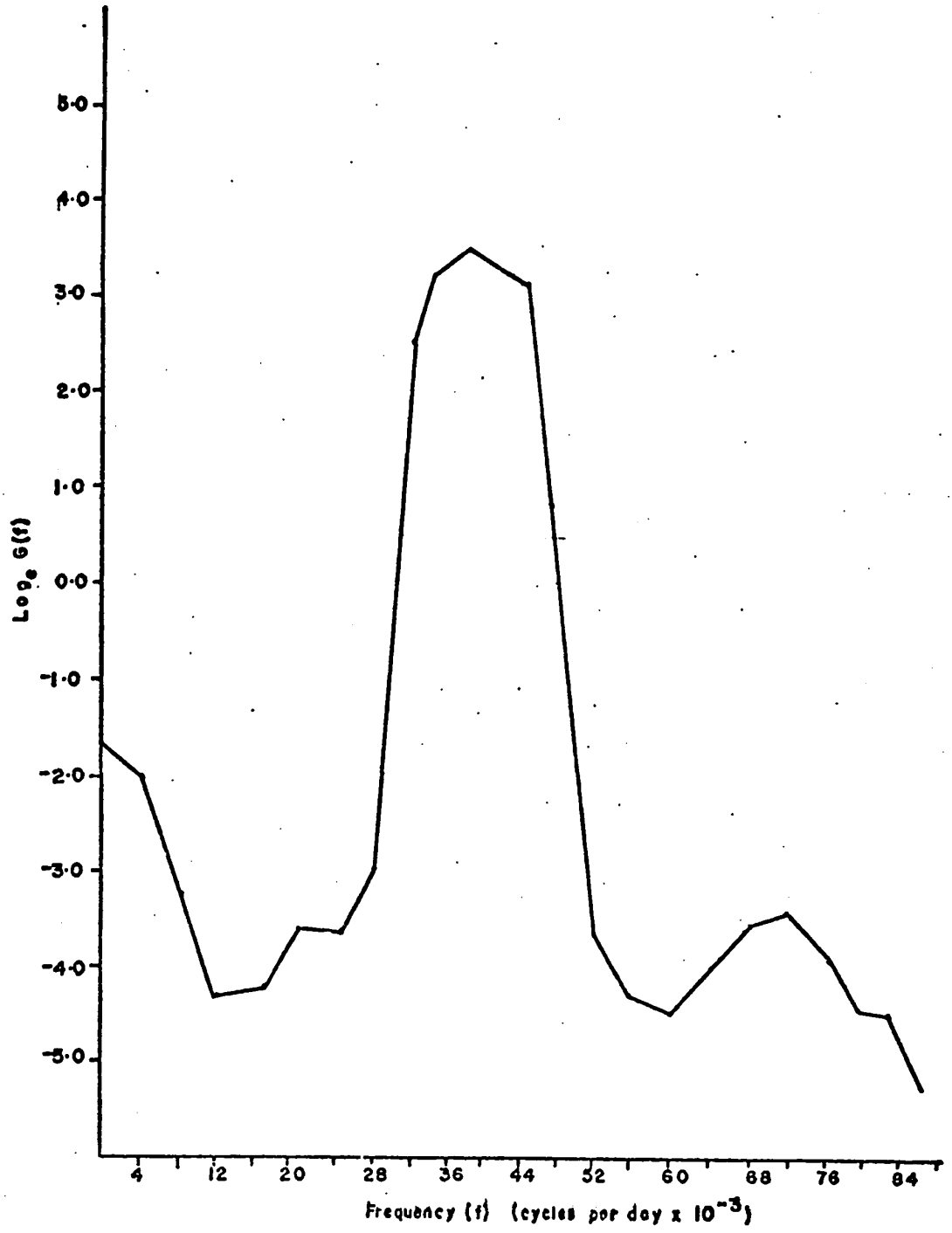


Figure 6C: Power spectrum transformation of autocorrelation function.



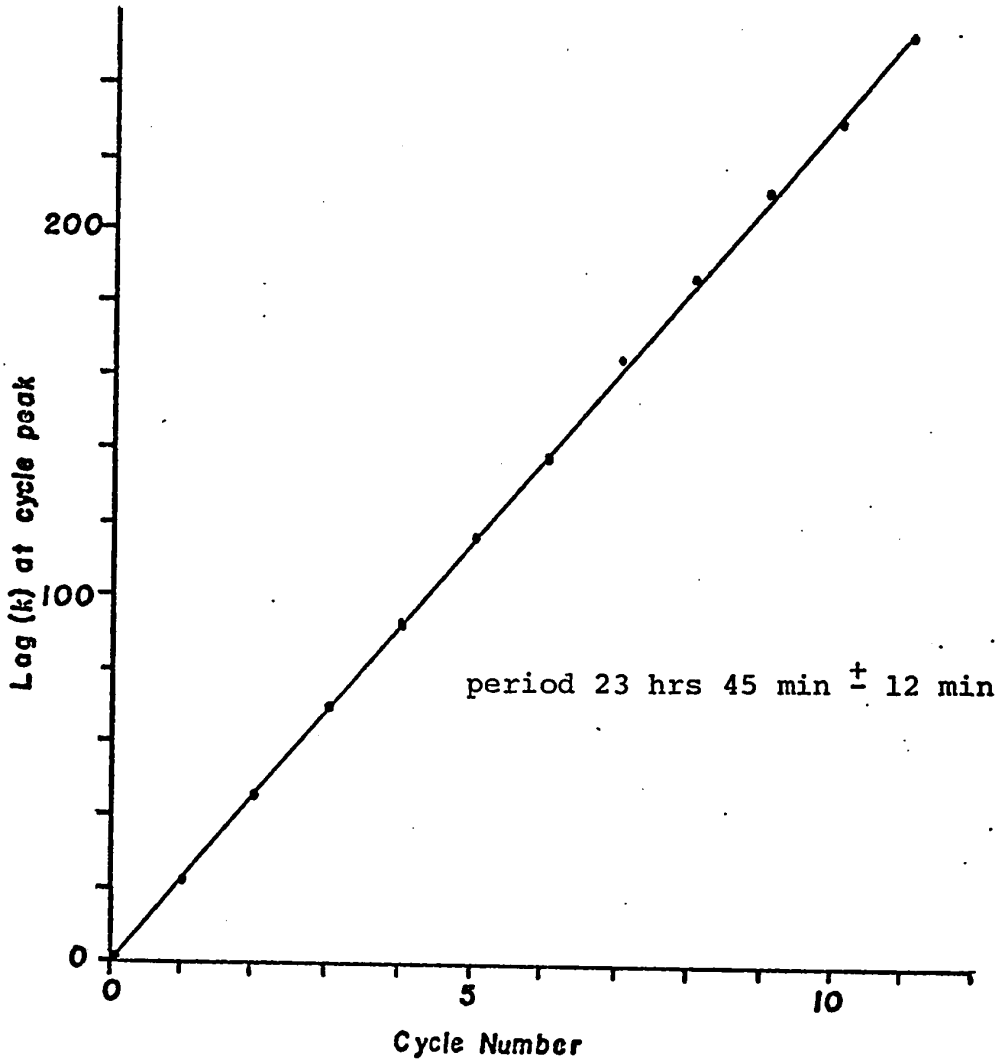


Figure 6D. Linear regression of lag(k) (hrs) measured at cycle peak of autocorrelation plot on cycle number - the slope of this line is an estimate of period length.

E. Sample locomotory activity record under 12-12 L-D, followed by constant D.

F. Autocorrelation plot of the constant dark portion of the above record after normalizing, satandardizing and detrending.

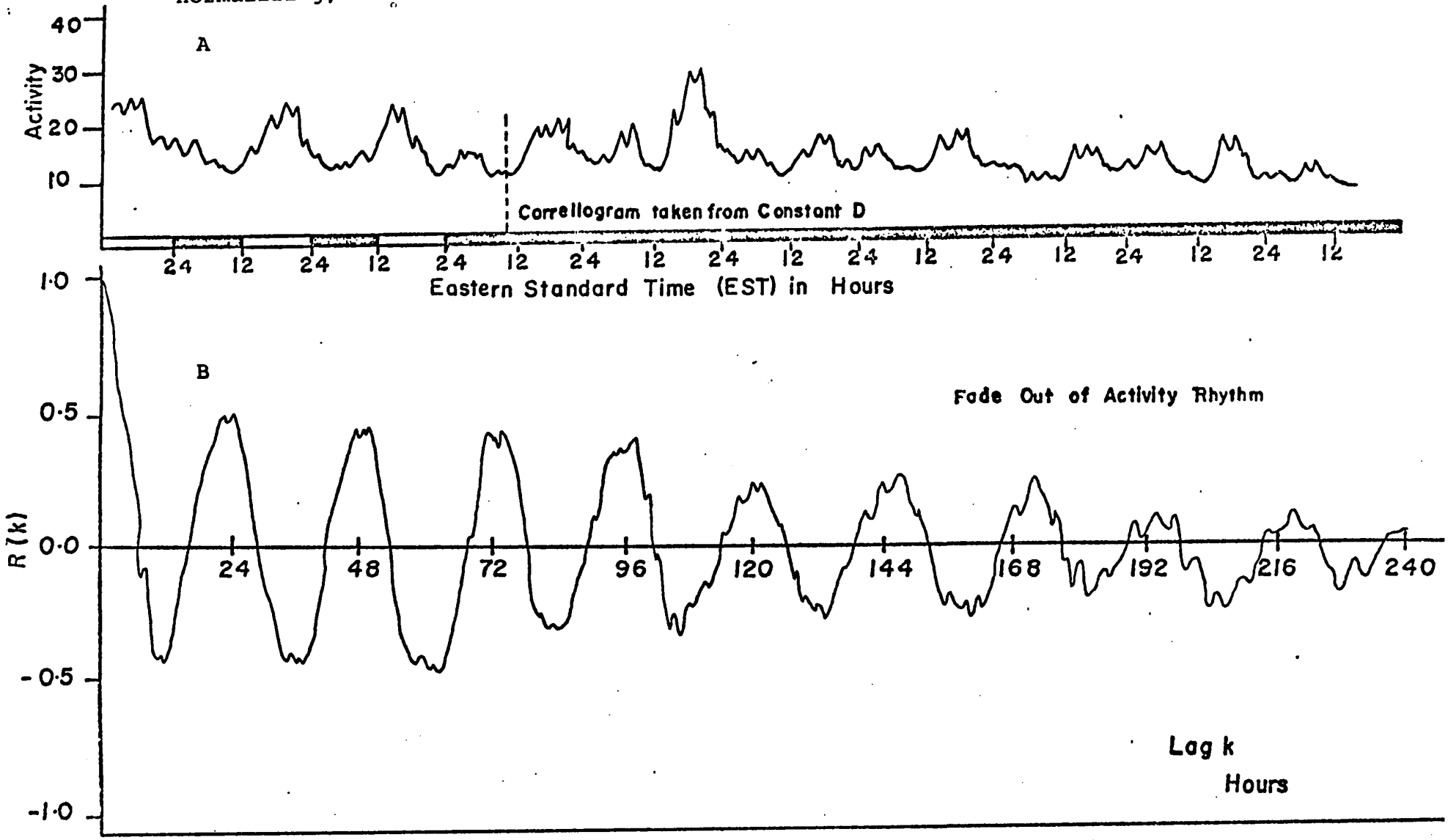
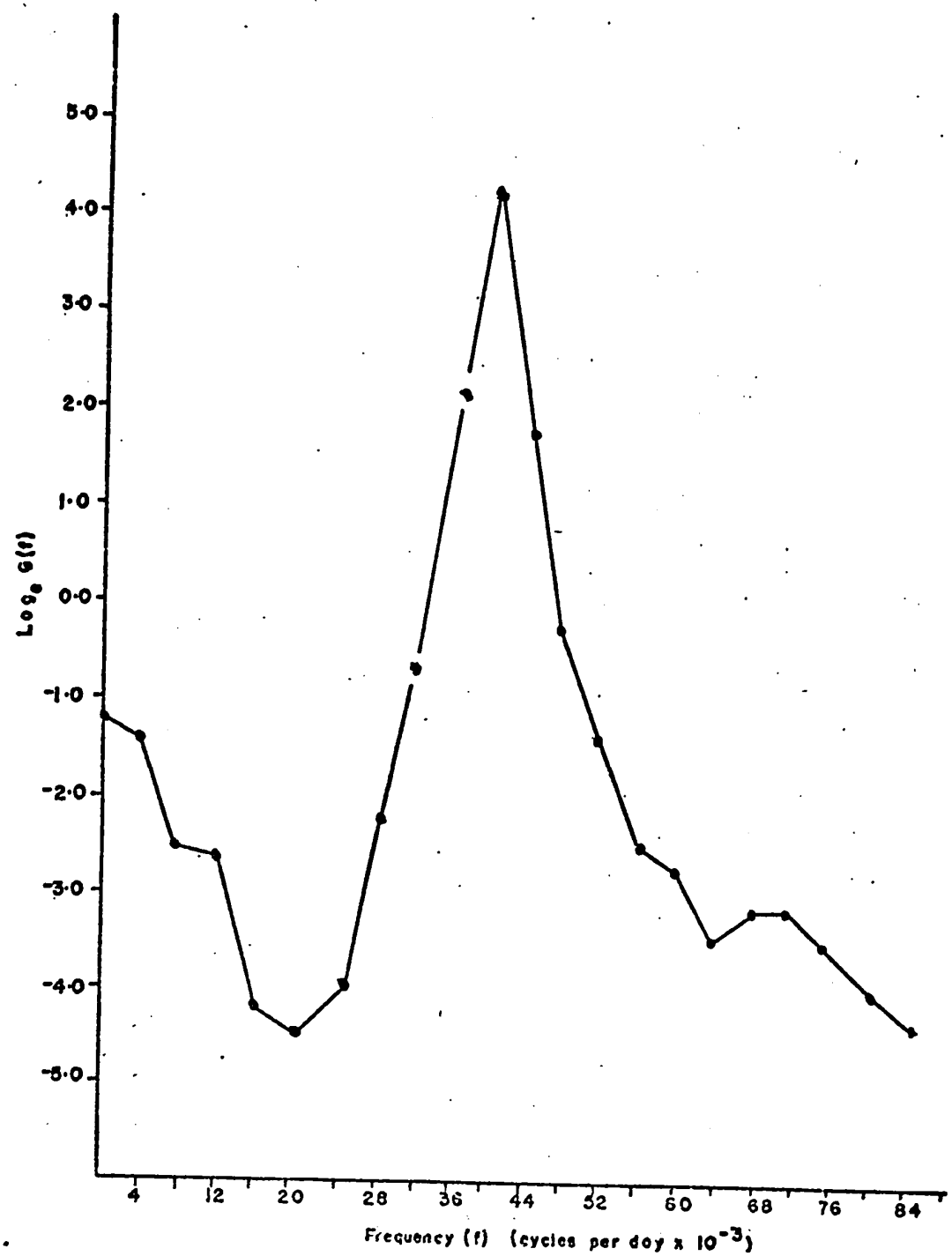


Figure 6H Power spectrum transformation of autocorrelation function.



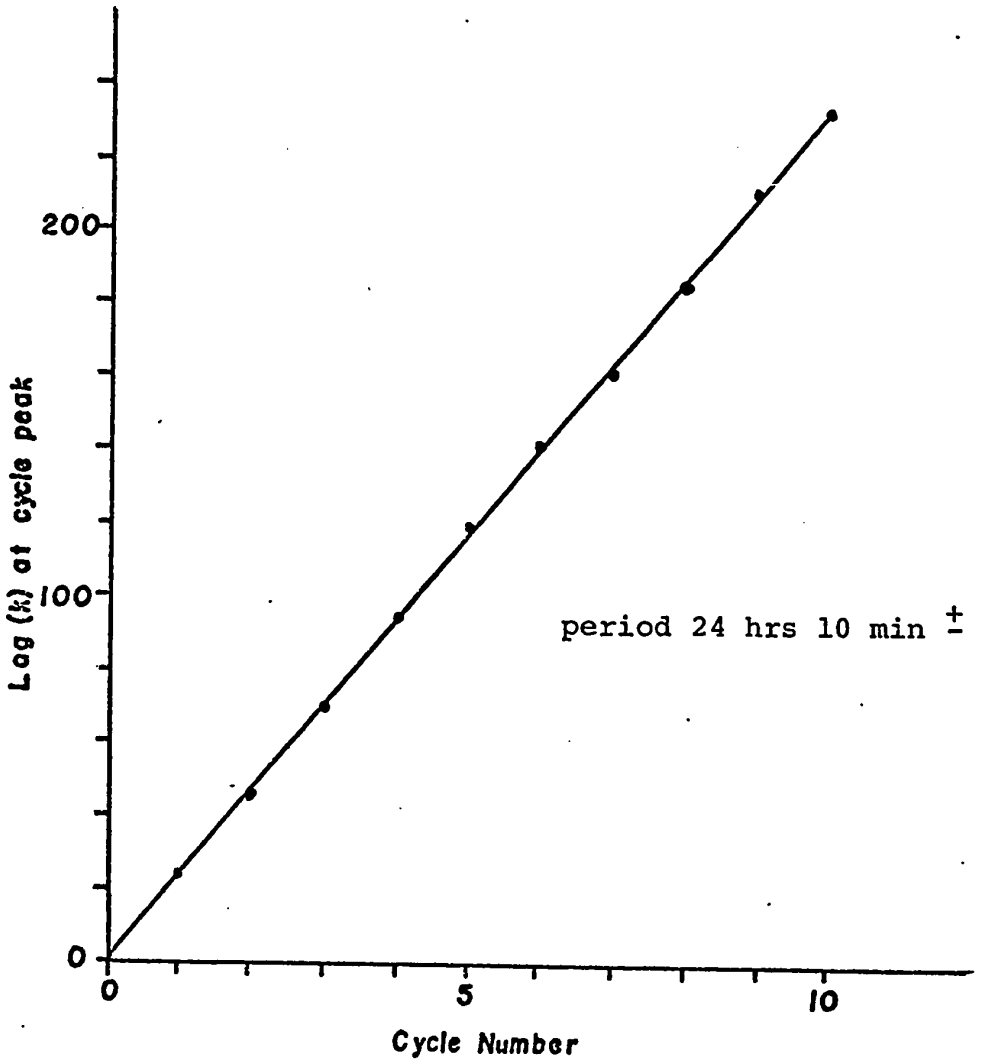


Figure 6I. Linear regression of lag(K) (hrs) measured at cycle peak of autocorrelation plot on cycle number - the slope of this line is an estimate of period length.

The final rhythm and period estimates are open to several sources of error:

1) recording and instrumentation error.
2) information loss and error during quantification.
3) non-linear trends.
4) information loss during statistical transformations of data.

Results:

Colour Change Determinations:

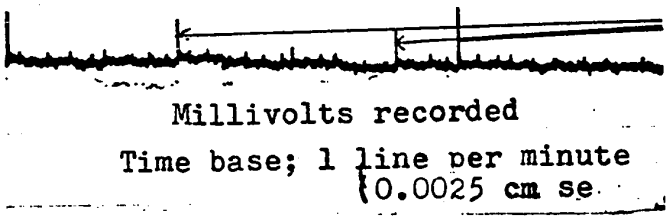
Operation of Apparatus and Effects of Salinity and Temperature on Colour Change

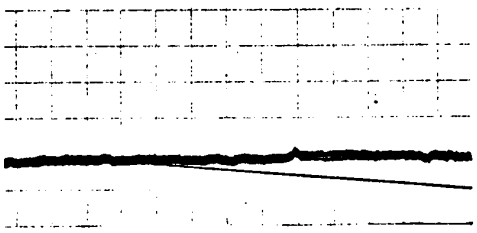
The fish usually remained quiescent, orienting against the water flowing through the holding chamber. Since the fish was not fully restrained a limited amount of activity was possible. This was represented by sharp peaks in the recording (Figure 7) and could be readily distinguished from colour changes. At low paper speeds (0.0025 cm/sec) respiratory movements were fused into a thickened tracing and presented no problem in the determination of colour changes.

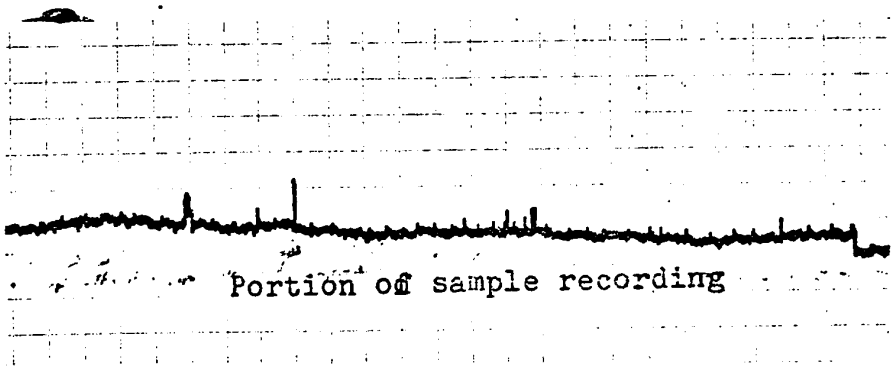
Light reflected from the dorsal surface and flanks of a restrained fish struck the receiving surface of the photovoltaic cell. Total voltage output varied with the size of the fish and background colour; but, since the background reflectance of each Ostwald shade was constant, changes in fish colour were the only sources of significant voltage variation (Fig. 8).

A relationship between D.O.I. and millivolt output was established for each fish by direct visual comparison of the confined individual to the Ostwald colour shades (Fig. 8).

Figure 7: Sample colour change records







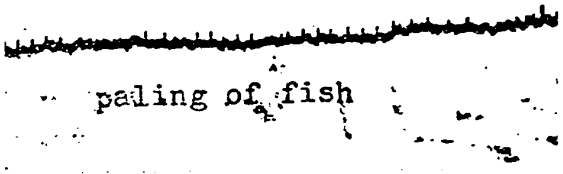


Figure 8: Effect of different background colours and fish sizes on output of photovoltaic cell (mv.) [at 200 Lux, 15°C freshwater] (Nos. 2,3,4,6 refer to Ostwald backgrounds.

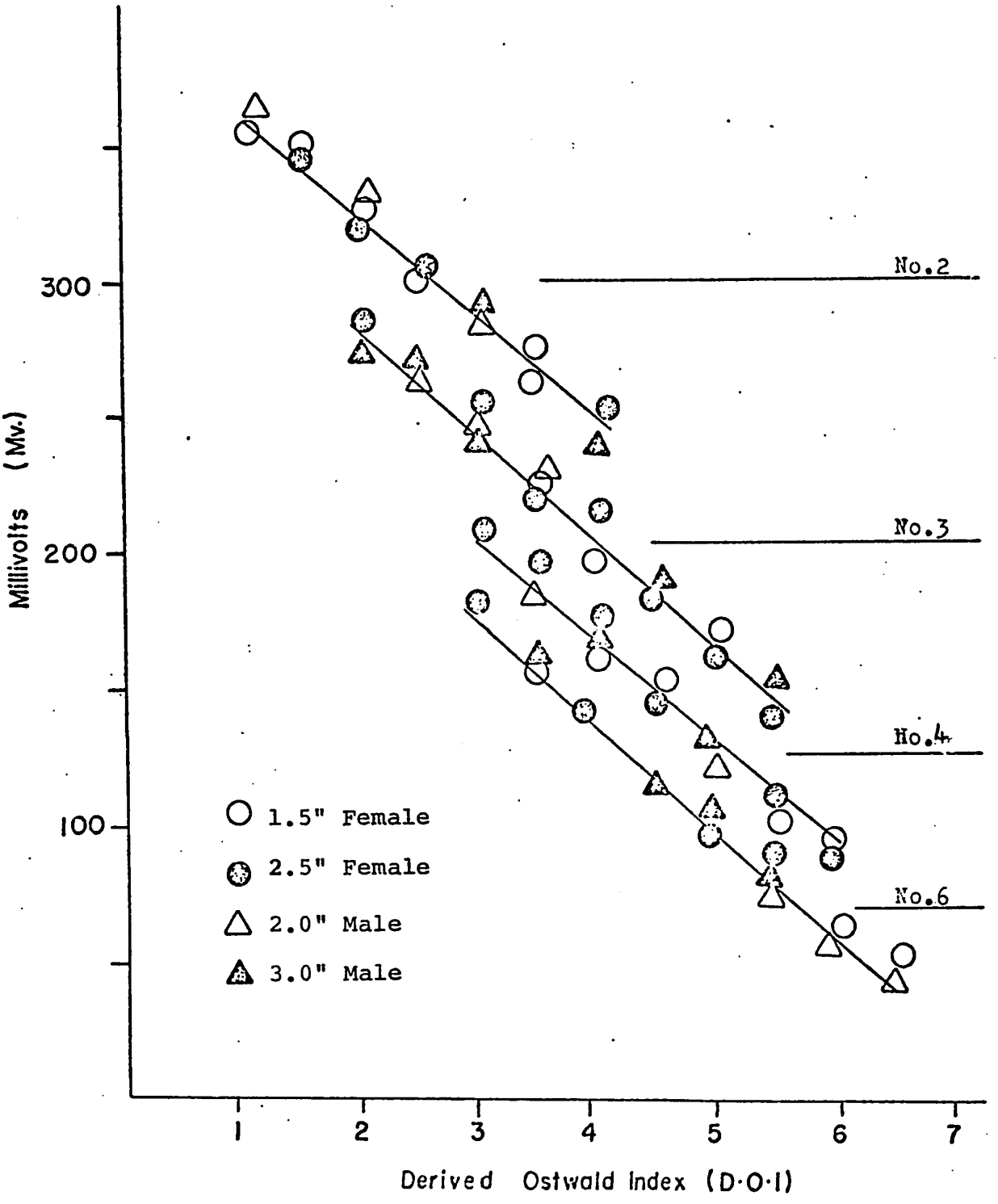
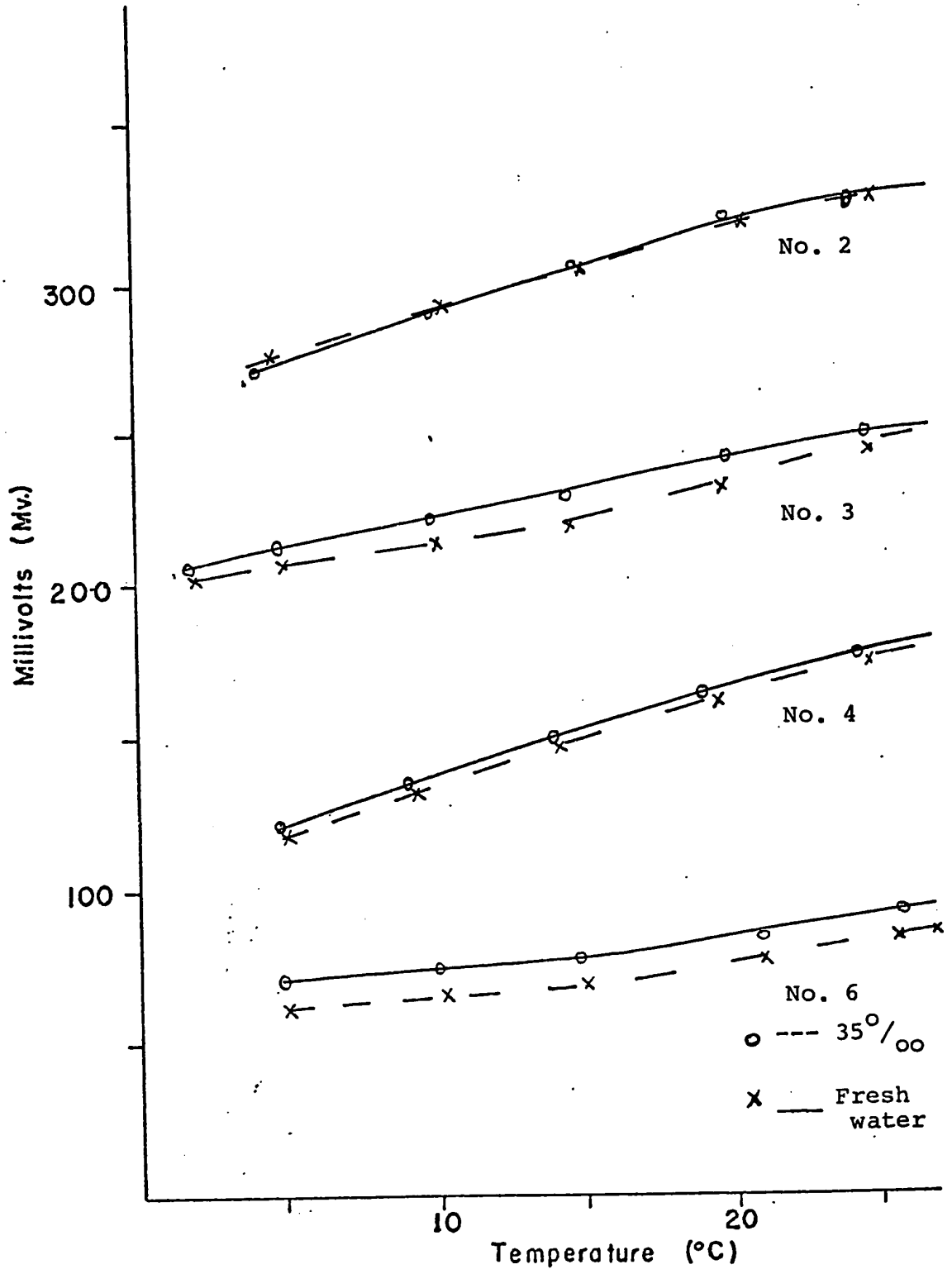


Figure 10: Effect of temperature and salinity on reflectance. Each point based on an average of at least 2 points (200 Lux, Nos. 2,3,4,6 denote Ostwald Colours).



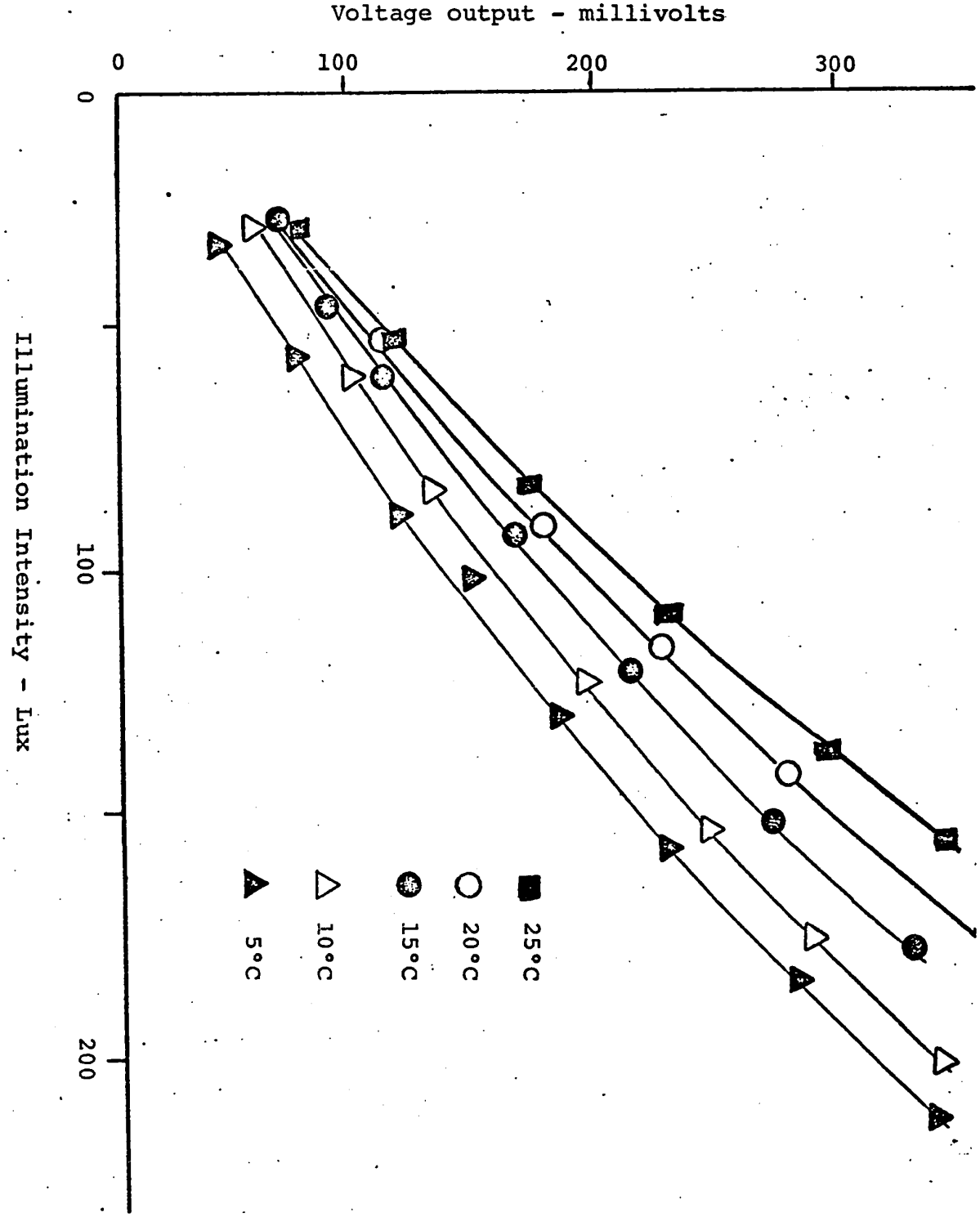
During continuous long-term recording the photocell was kept directly on the surface of the test chamber, reaching equilibrium with the chamber temperature. The output of the photocell was temperature dependent, increasing at higher temperatures, though not significantly in the temperature and salinity ranges (10-25°C; 0-35 p.p.t.) used in this study (Fig. 9). In the millivolt range (150-300; 50-100 mv.) that recordings were carried out the relation between light intensity and voltage output was substantially linear and of the same slope at different temperatures ($p > 0.15$) (Fig. 9).

To ensure that the melanophore system was responsible for the observed responses a red filter transmitting in the reflectance region of the melanophores (400-500 mμ) was placed on the receiving surface of the photocell. Comparison of records obtained with and without the filter indicated no significant differences in response. In order to gain greater overall sensitivity, especially at low light intensities (<10 Lux), further use of the filter was discontinued.

Temperature changes had a slight influence on fish colour, cold (10°C) causing melanophore dispersion and darkening, while heat (25°C) led to aggregation and paling (Fig. 10).

Salinity had no significant effect on colouration and the ability to adapt to different backgrounds (Fig. 10).

Figure 9: Relationship between light intensity and voltage output of the photovoltaic cell at various temperatures (F.W.).



Demonstration of Endogenous Rhythm of Colour Change

In this section sample colour change time-series (plotted at 10 minute intervals) along with their auto-correlation, linear regression and power spectrum transformations are shown. All other colour change records are given in Appendix 3.

Killifish were maintained under several different conditions for 30 days after which their colour was recorded under constant test conditions (i.e. constant illumination, temperature, salinity, background and flow-rate) for 5-21 days.

Under the test conditions rhythmic paling and darkening or, colour change, with a period of approximately 24 hours was recorded. This periodicity is displayed in the raw tracings and their autocorrelation functions up to the maximum testing time of 21 days and under a variety of different maintaining-testing conditions. The rhythm of colour change gave no evidence of fade-out. This persistence in the absence of all known Zeitgebers was used as a basis for terming the rhythm of colour change as being endogenous.

At all phases of the colour change rhythm killifish could still display rapid, neurally mediated, background adaptation. Even though rhythmic colour change was superimposed on these rapid neurally controlled responses, it did not inhibit or override neural controls.

The correlograms (Figs. 11-20) revealed that rhythmic colouration had a roughly sinusodial pattern with inter- and intra-fish variations in period length and precision.

Period length, determined from the slope of the linear regression plot of lag at cycle peak against cycle number, ranged between 23-25 hours depending on the intensity of illumination in the testing conditions (Fig. 11).

The period length under constant D (0.1 Lux), 24 hrs 5 min $\pm$ 25 mins (95% confidence interval) was greater than that under constant L (200 Lux), 23 hrs 45 mins $\pm$ 11 mins. Replicates of these determinations under different temperature, background, salinity conditions gave similar period lengths and 95% conficence intervals (Table 9). The differences in period between different conditions were not statistically significant.

The power spectra had a major power or variance at 22-25 hrs, indicating significant periodicity in this range. A smaller peak was found at first sub-harmonic (12 hrs) of the main spectral component. The spectrum had no further significant components of lower frequency till a minimum of 10 minutes set by the sampling interval (Fig. 11).

Colour change displayed inter- and intra-fish variations in amplitude. The amplitude of the free-running rhythm ranged between 1 and 2.5 D.O.I. units or approximately

100-200 millivolts when under illumination (Figs.11-20). Amplitude effects were minimized in the correlograms, these functions being based on normalized and standardized versions of the initial time series.

Amplitude of colour change was dependent on the background the fish were kept on during maintaining and testing. Extreme backgrounds (Ostwald Nos. 2 and 6) reduced the amount of physiological colour change possible (amplitude) and hastened morphological adaptations. Background effects can be seen by comparing the amplitude of colour change (D.O.I. and millivolts) of fish kept on No. 6 and 2 backgrounds to those kept on the intermediate ones (Nos. 3 and 4) (Figs. 16,17).

The intensity of illumination as determined by the albedo also affected the amplitude of colour change. Killifish utilize the albedo; the ratio between incident and reflected light, to match their colour to that of the background. At very low light intensities the D.O.I. of the fish could not be visually determined. At these low intensities killifish are known to assume an intermediate shade. They gave a fairly large millivolt amplitude of change relative to the maximum and minimum voltage values recorded. These ratios indicated that rhythmic melanophore movements and colour change continued. Blinded fish, who assumed an intermediate shade, and had most visual cues and

possible retinomotor rhythms eliminated, also displayed an endogenous rhythm of colour change (Figs. 20-21).

There were long term trends present in the colouration records (Figs. 11-12). All linear portions of these trends, caused either by instrument drift or morphological colour change,were removed before the auto-correlation and spectral functions were calculated. After this linear detrending the power spectra possessed a significant power at zero frequency,indicating the presence of a curvilinear component in the colour change series. However, because of the relatively short lengths of the records, the presence of long term rhythms of colour change could not be adequately tested for.

Figure 11: A Colour change record under constant light.

B Autocorrelation plot of same record after normalized, standardized and detrended.

	Illumination (Photoperiod-Lux)	Temperature °C	Salinity °/oo or F.W.	Background Ostwald number
Maintaing conditions	Natural 12/12(100)	20	9	2
Testing conditions	100	20	9	3

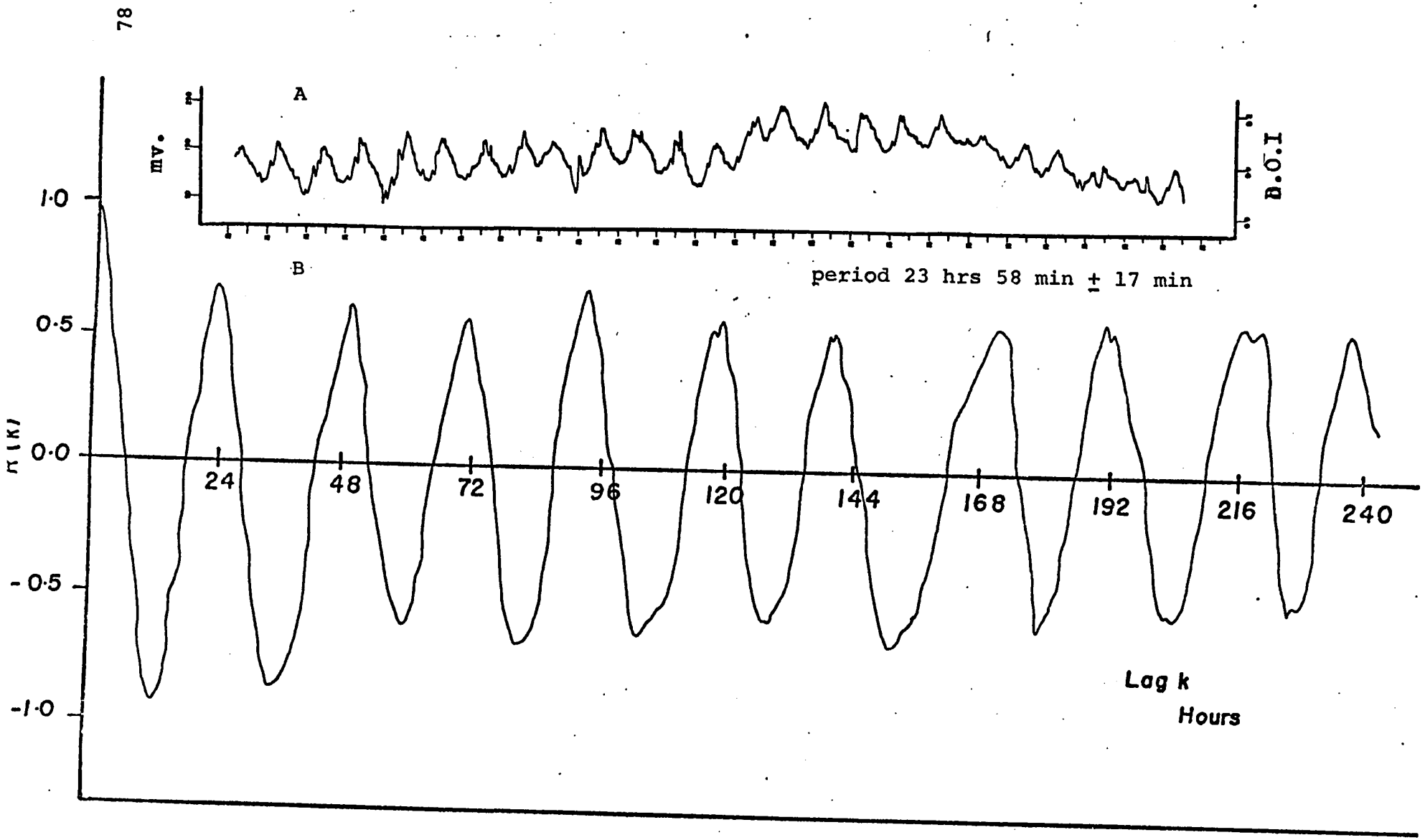
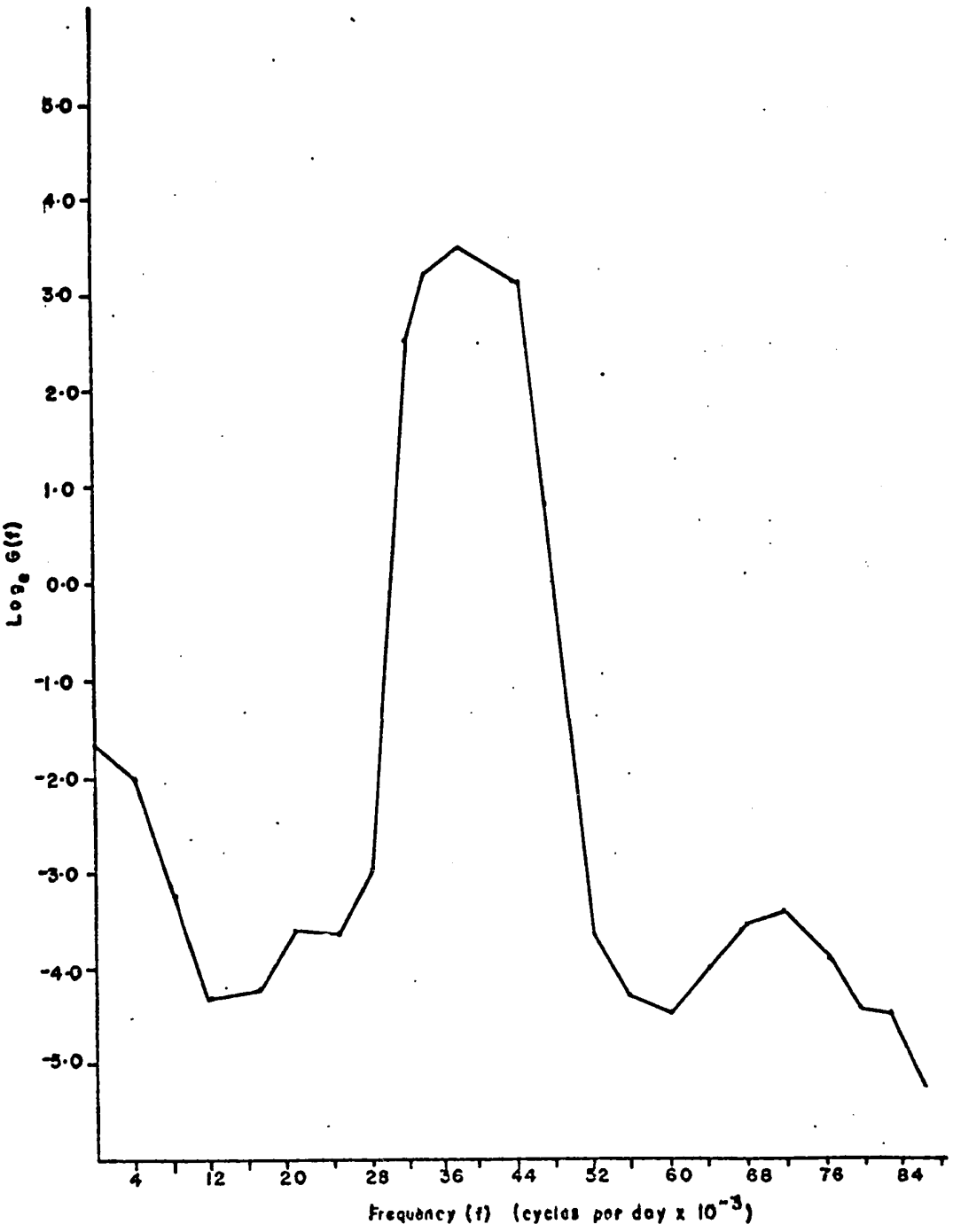

A
mv.
B
period 23 hrs 58 min ± 17 min
1·0
0·5
0·0
-0·5
-1·0
r (k)
24
48
72
96
120
144
168
192
216
240
Lag k
Hours

Figure 11(C): Power spectrum transformation of autocorrelation function.



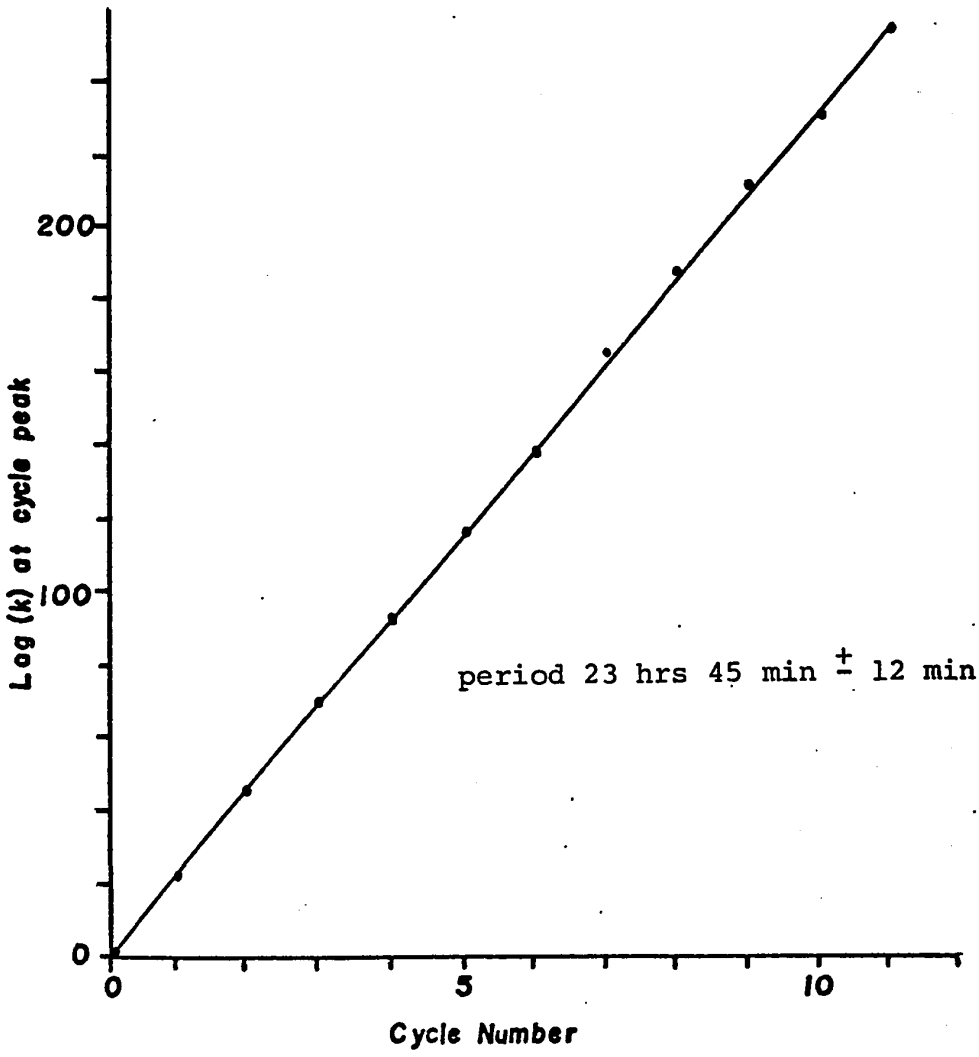


Figure 11D: Linear regression of lag(k) (hrs) measured at cycle peak of autocorrelation plot on cycle number - the slope of this line is an estimate of period length.

Figure 12: A Colour change record under constant dark.

B Autocorrelation plot of the same record after normalizing, standardizing and detrending.

	Illumination (Lux)	°C	$^{o}/_{oo}$	Bkgd.
Previous Maintaining Conditions	12/12 (100)	20	9	2
Testing Conditions	0.1 (D)	20	9	2

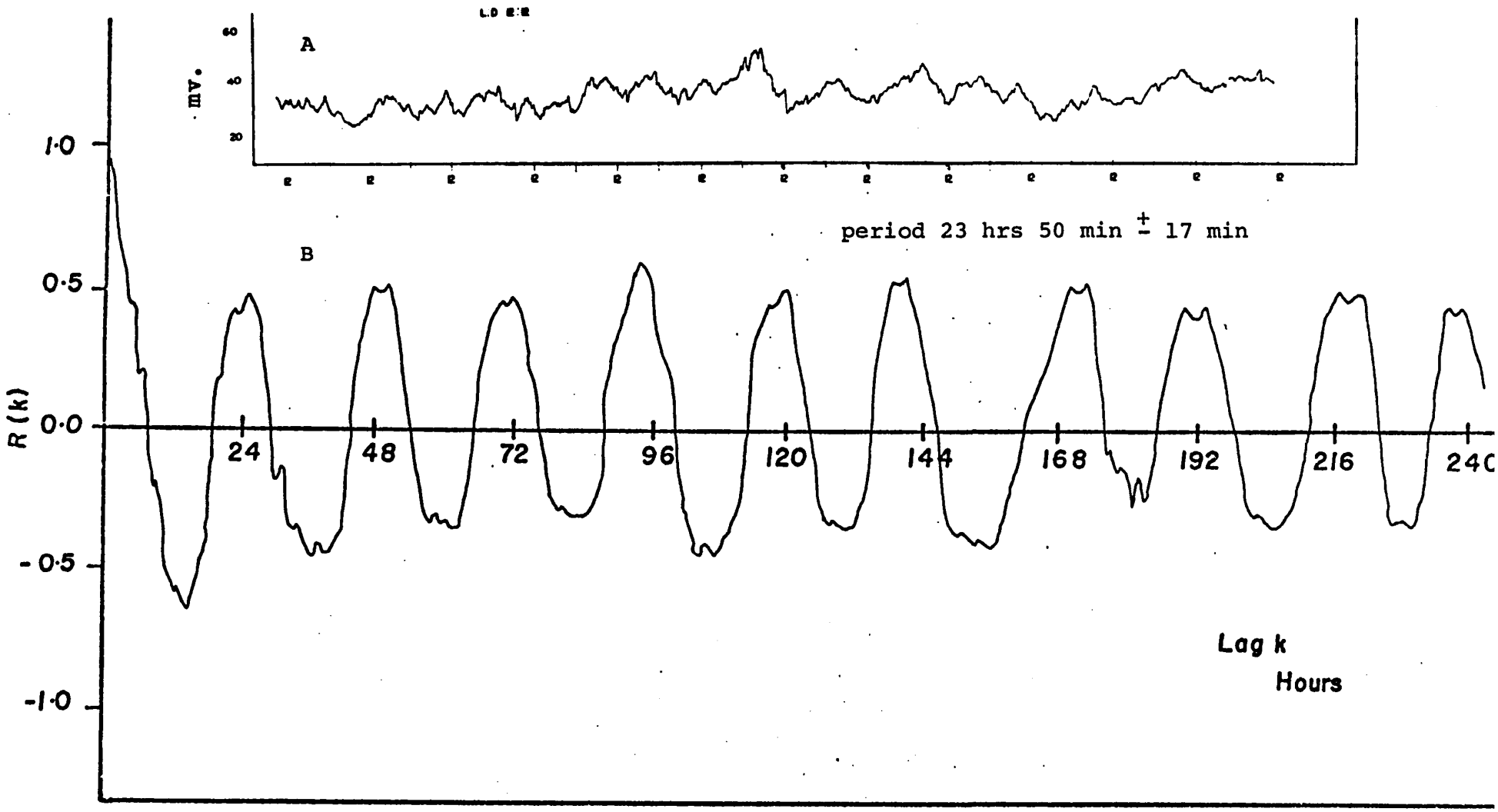

A
mV.
B
period 23 hrs 50 min ± 17 min
R (k)
1·0
0·5
0·0
-0·5
-1·0
24
48
72
96
120
144
168
192
216
240
Lag k
Hours

Figure 13: A Colour change record under constant dark.

B Autocorrelation plot of same record after normalized, standardized and detrended.

	Illumination (Lux)	°C	$^{o}/_{oo}$	Bkgd.
Maintaining Conditions	1(D)	15	9	2
Testing Conditions	1(D)	15	9	2

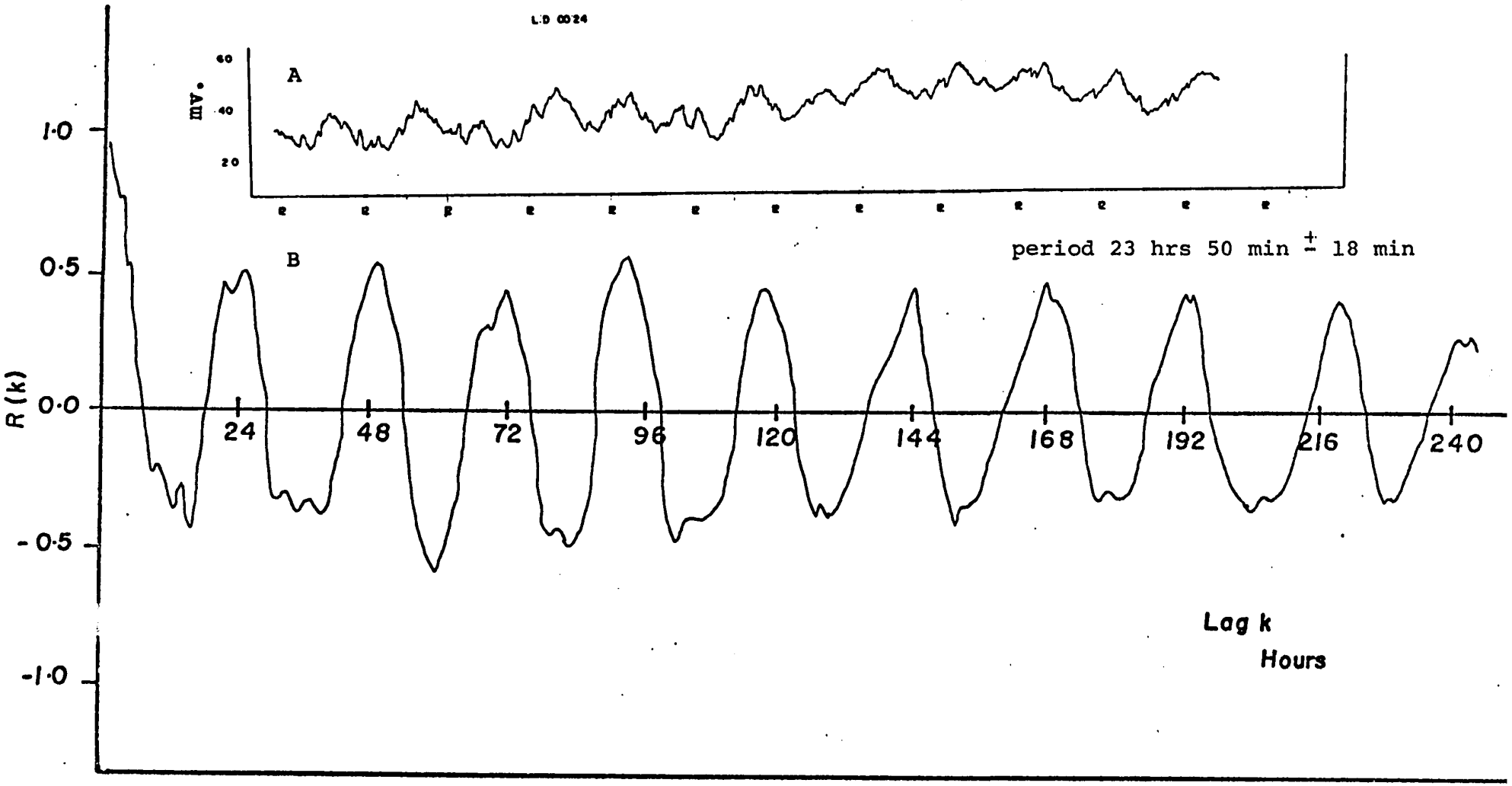
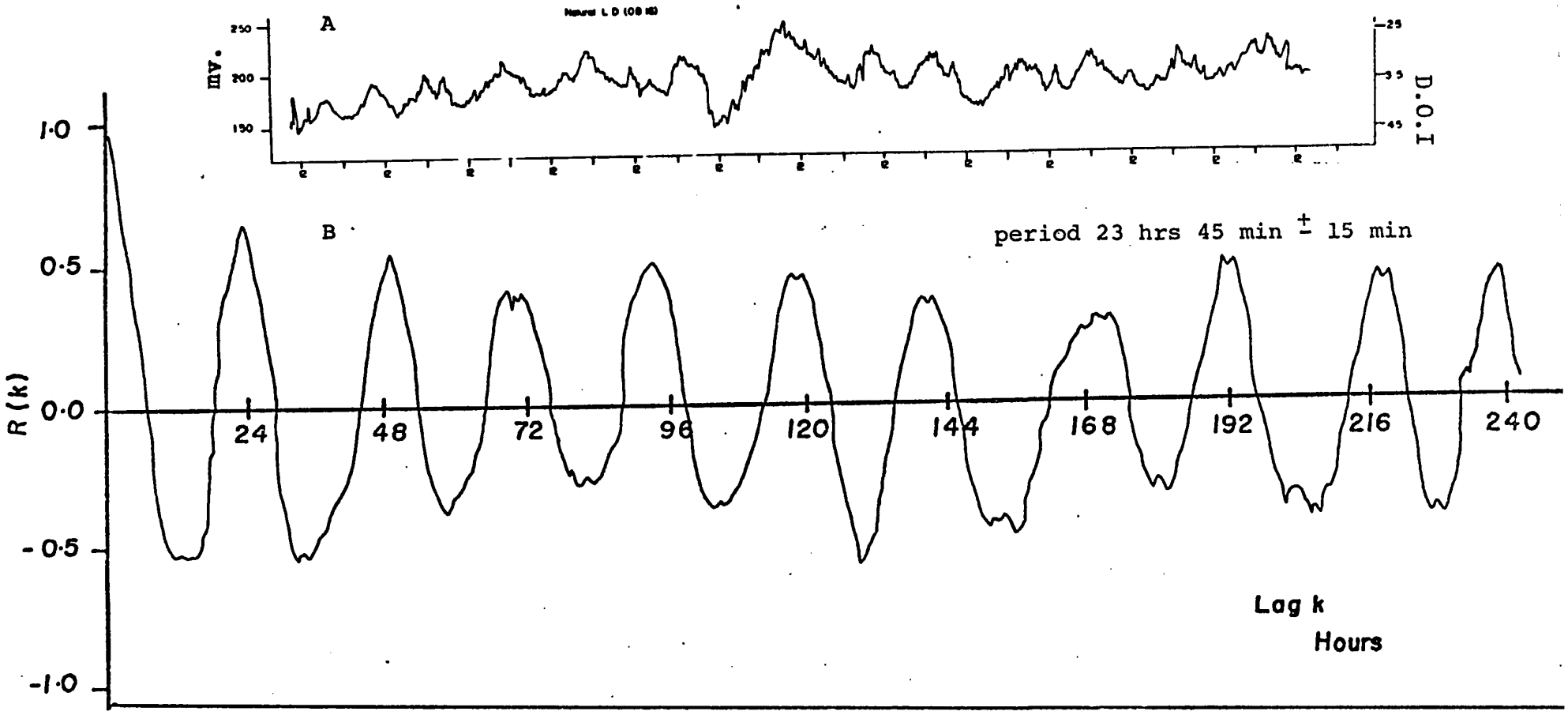

L.D 0024
A
mV.
60
40
20
B
period 23 hrs 50 min ± 18 min
R(k)
1·0
0·5
0·0
- 0·5
-1·0
24
48
72
96
120
144
168
192
216
240
Lag k
Hours

Figure 14: A Colour change record under constant light.

B Autocorrelation plot of same record after normalized, standardized, and detrended.

	Illumination (Lux)	°C	°/oo	Bkgd.
Maintaining Conditions	100	10	F.W.	3
Testing Conditions	100	10	F.W.	3


A
mV.
250
200
150
D.O.I
B
period 23 hrs 45 min ± 15 min
R(k)
1·0
0·5
0·0
-0·5
-1·0
24
48
72
96
120
144
168
192
216
240
Lag k
Hours

Effect of Photoperiod on the Rhythm of Colour Change

Killifish were maintained under a variety of different photoperiods and then placed under constant testing conditions of either Light or Dark.

If photoperiod entrained the endogenous rhythm of colour change then the first period(s) of the rhythm under constant conditions would show a consistent phase relationship to the previous Light-Dark cycle. Desynchronization would slowly occur through several cycles of the free-running rhythm.

No consistent initial phase relationships were found. In some of the records it did appear there were variable phase relationships present (Figs. 11-16). These variable and partial phase relationships could result from a partial co-ordination of colour change by photoperiod but not actual entrainment. They would indicate the exogenous effect of photoperiod on colour change, that may be superimposed on the free-running rhythm.

Extreme photoperiods (8-8, 16-16, 14-10, 10-14, L-D) did not significantly affect the correlograms of the free-running rhythms. Flashes and occultations imposed on fish maintained in otherwise constant conditions also did not cause any disturbance of the correlograms (Figs. 15,16).

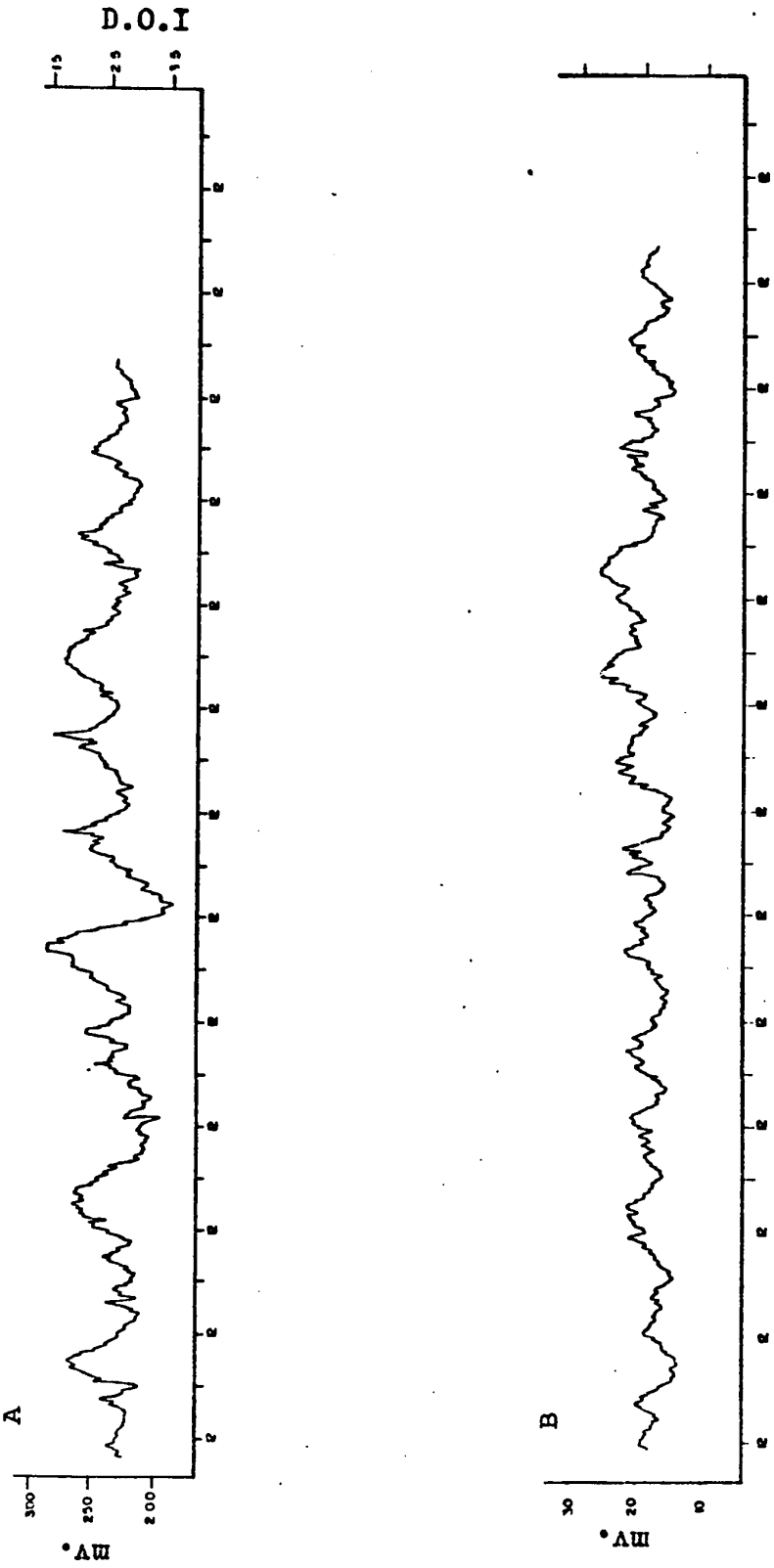
The results were taken as an indication that photoperiod was not by itself an effective Zeitgeber for rhythmic colour change of Fundulus heteroclitus.

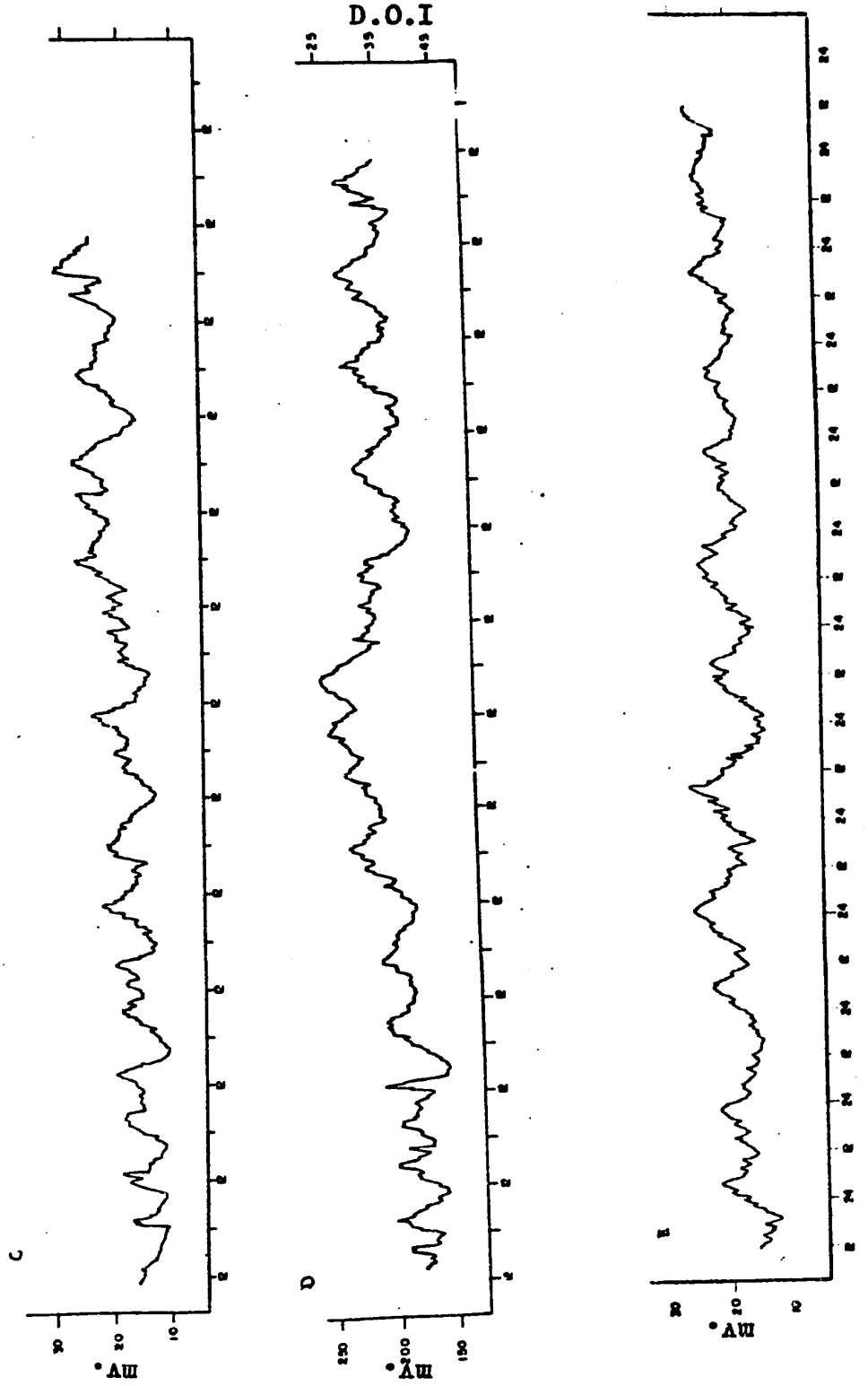
Figure 15: Colour change records under constant conditions. The fish were previously exposed to a variety of photoperiods.

Photoperiod Light:Dark L:D in hours LL = constant D y = intensity of illumination Lux	Temperature °C	Salinity o/oo FW = fresh water	Background Ostwald Index	Illumination intensity in Lux	Temperature °C	Salinity o/oo FW = fresh water	Background Ostwald Index
	Maintaining Conditions			Testing Conditions			
12:12[1] y = 100	20	9	2	A 100	15	FW	6
				B 1	20	9	2
12:12[2] y = 100	10	0	6	C 100	15	FW	6
				D 1	10	35	2
12:12[3] y = 100	20	35	2	E 100	20	35	2
				F 1	20	9	2
L:L y = 100	15	9	2	G 100	15	15	2
				H 1	10	FW	2

1) L = 1200 - 2400 E.S.T.
2) L = 700 - 1400 E.S.T.
3) L = 2400 - 1200 E.S.T.

(A-H refer to graphs on opposite page)


D.O.I
A
B
mV.
mV.


D.O.I
mV
mV
mV

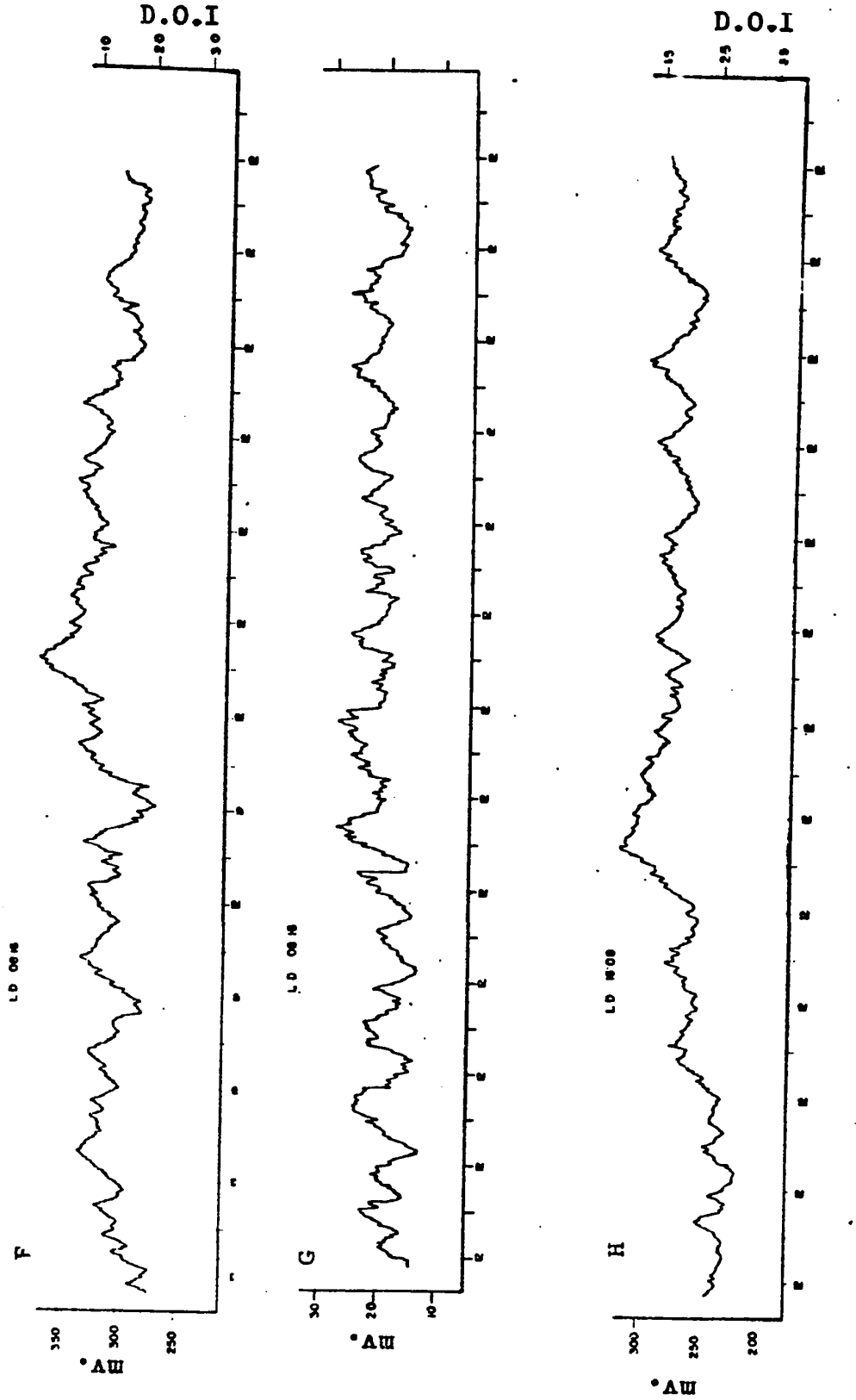
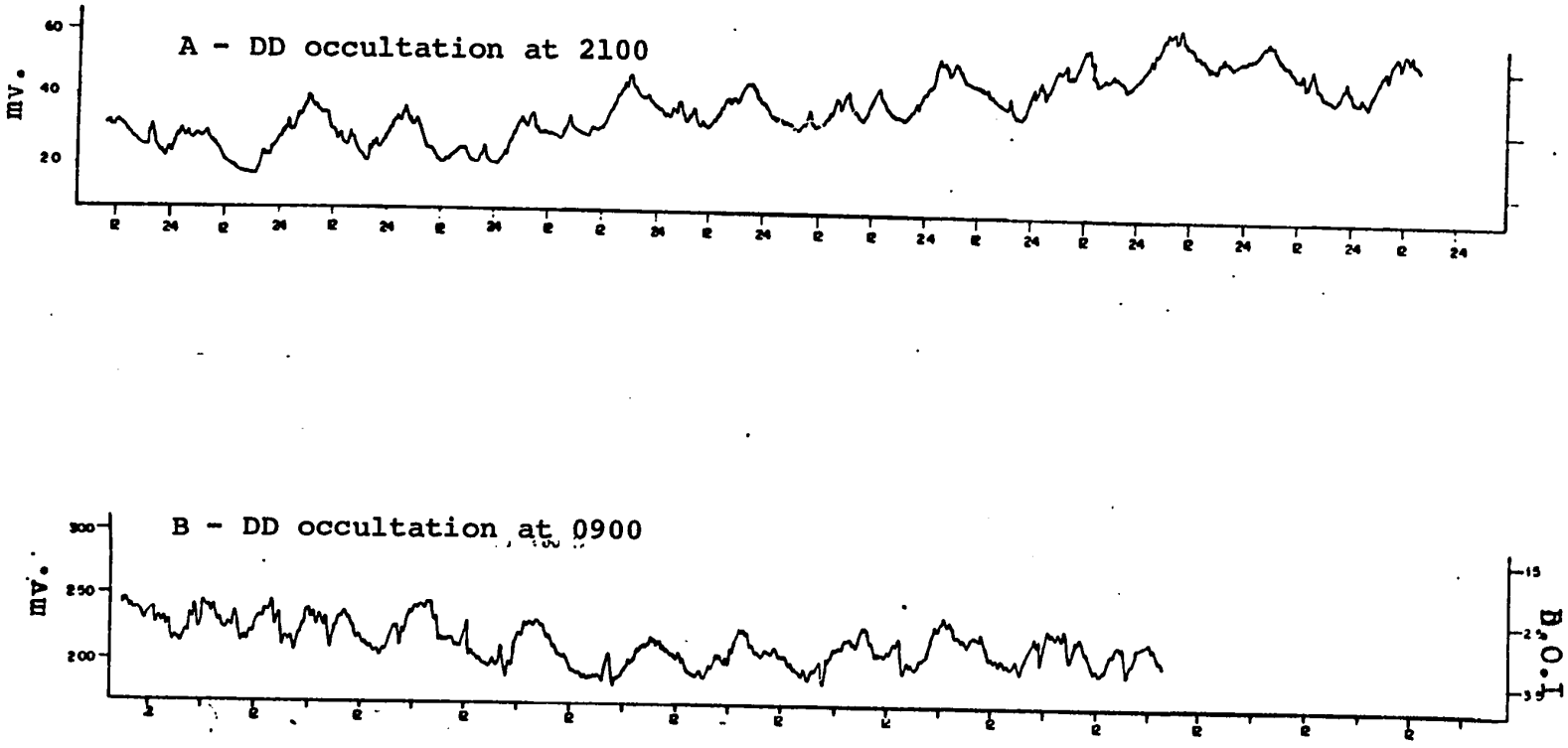
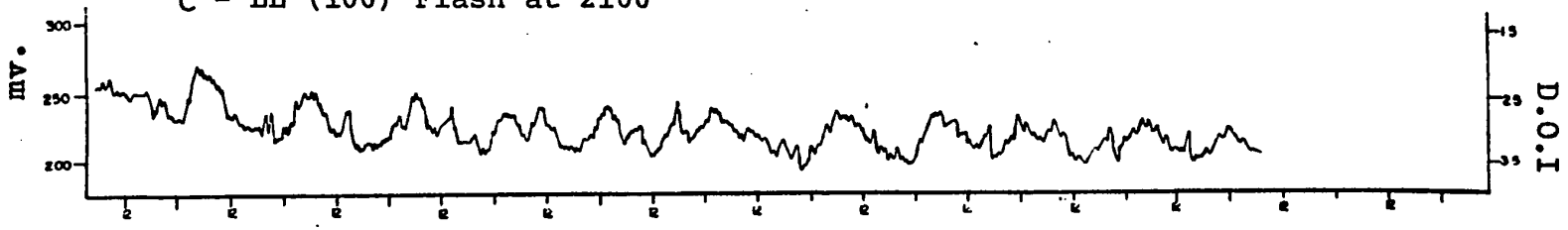

D.O.I
F
G
H
mV
AU

Figure 16: Colour change records under constant conditions. The fish were previously exposed to flashes and occultations.

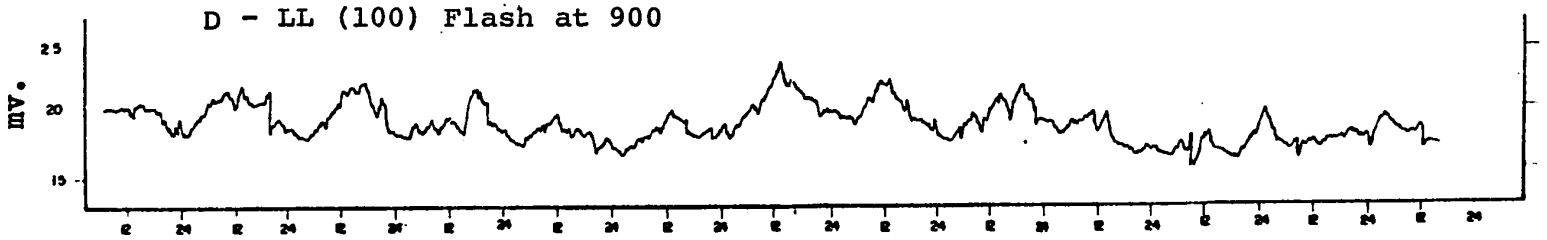
Photoperiod Light:Dark L:D in hours LL = constant D y = intensity of illumination Lux	Maintaining Conditions: Temperature °C	Salinity ‰ FW = fresh water	Background Ostwald Index	Testing Conditions: Illumination intensity in Lux	Temperature °C	Salinity ‰ FW = fresh water	Background Ostwald Index
Occultations 2100 and 0900 y = 100	15	9	2	100	15	9	2
				1	20	18	2
DD Flashes at 2100 and 0900 y = 1	15	0	2	100	15	9	2
				1	20	35	2


A - DD occultation at 2100
mv.
60
40
20
12
24
B - DD occultation at 0900
mv.
300
250
200
15
B.O.T.

C - LL (100) Flash at 2100



D - LL (100) Flash at 900



Effect of Temperature and Salinity on the Rhythm of Colour Change

Killifish were maintained and tested under a variety of different temperature and salinity conditions. In all cases the endogenous rhythm of colour change persisted giving no evidence of any temperature and salinity induced changes in period or amplitude. There were no significant differences between the correlograms and regression plots (Figs. 17-19).

While being held in the testing chamber a sample of fish was exposed to rhythmic, temperature, salinity, and thermosaline changes. These changes did not alter the period of the free-running rhythm or cause significant variation in the shape of the correlogram. Colour changes caused by extreme temperature changes (10° - 20°C) were superimposed on the free-running rhythm but did not affect the periodicity.

These findings indicate that temperature, salinity, thermosaline changes cannot, by themselves, function as synchronizers for rhythmic colour change.

Figure 17: A Colour change record under constant light and 12-12 (hr.) cycles of salinity change.

B Autocorrelation plot of same record after normalizing, standardizing, and detrending.

Testing Conditions	100 Lux 20°C	No 6 Bkgd.
35‰	1600 - 400	
F.W.	400 -1600	

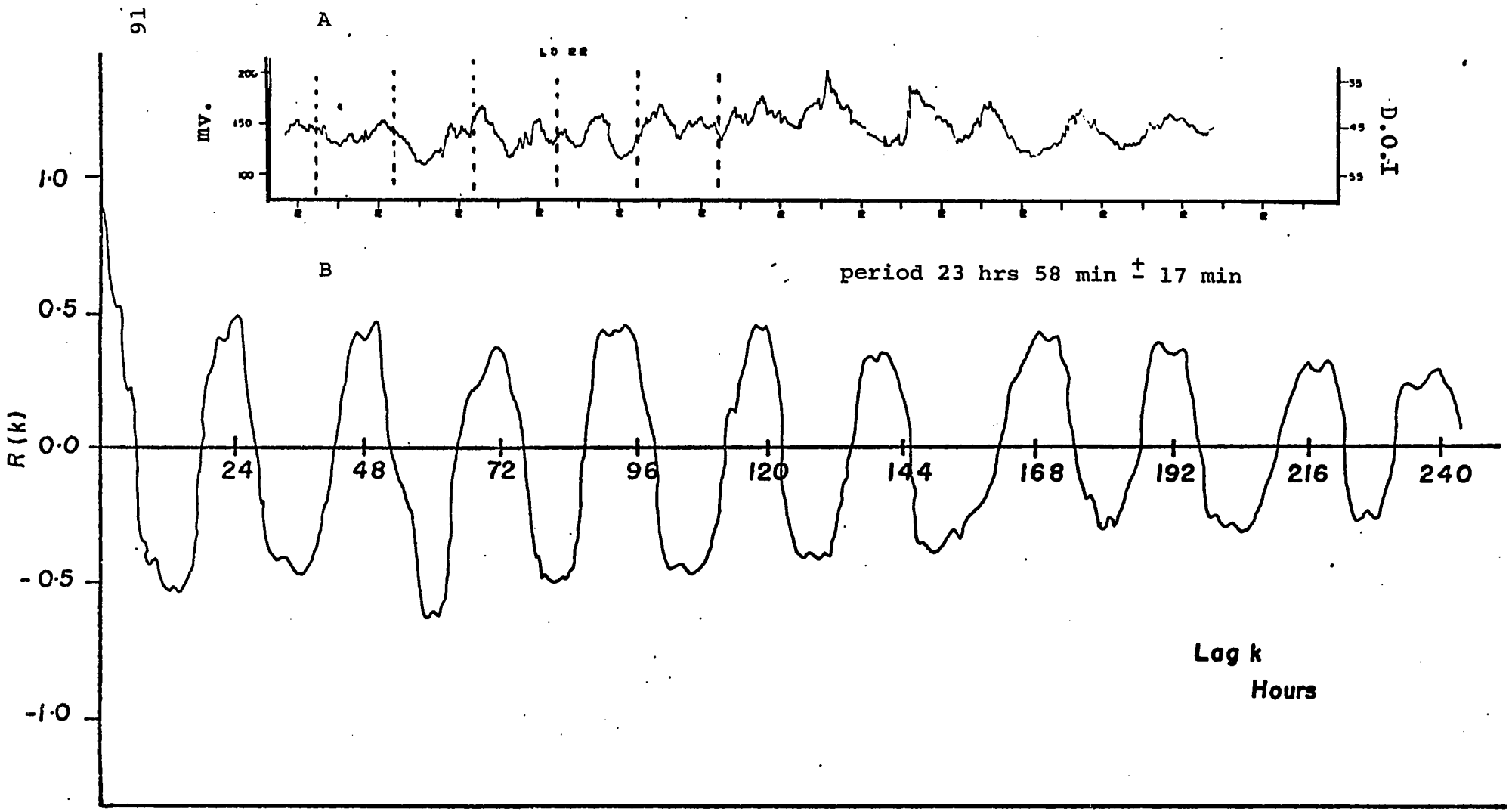

91
A
mv.
D.O.I
B
period 23 hrs 58 min ± 17 min
1·0
0·5
0·0
-0·5
-1·0
R(k)
24
48
72
96
120
144
168
192
216
240
Lag k
Hours

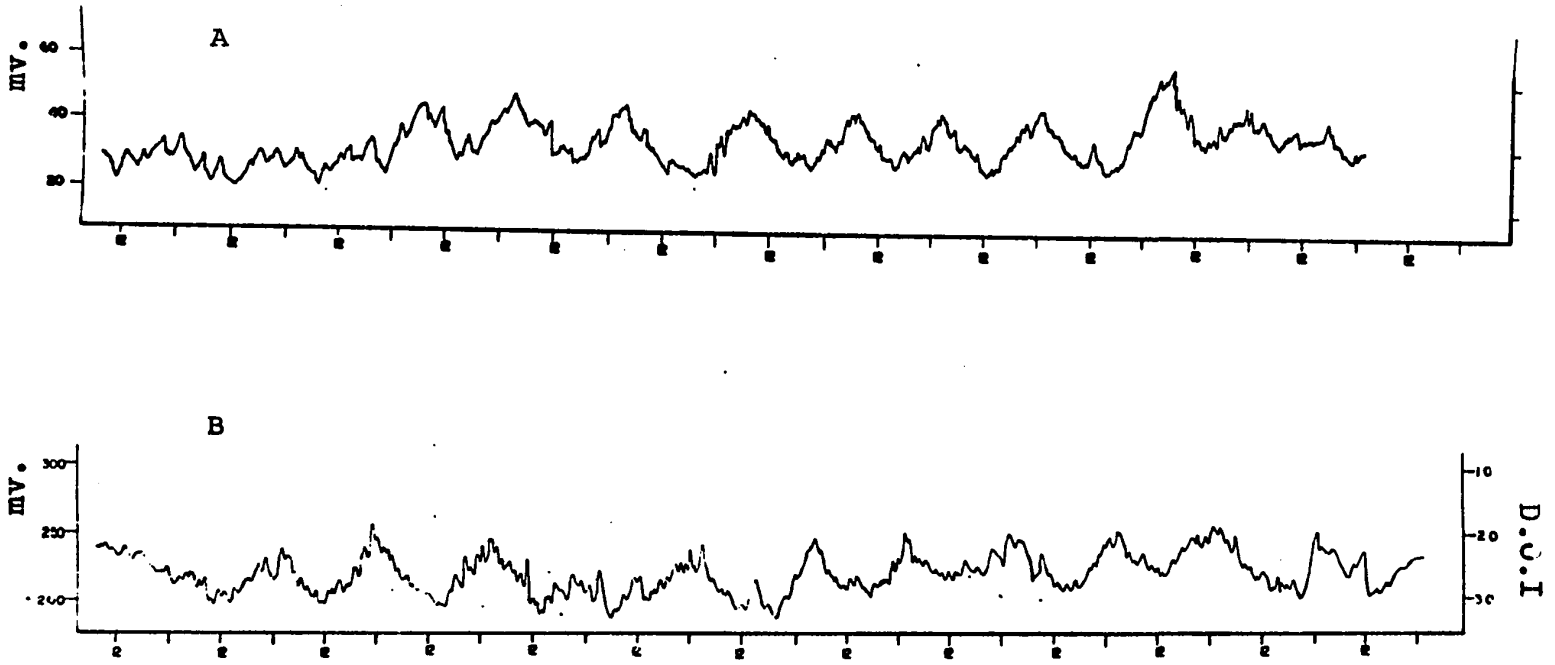
Figure 18: A Colour change record under constant Dark and 12-12 (hr) cycles of temperature change.

Testing Conditions:	[Lux(D)]	No. 4 Bkdg.
	20°C	0900 - 2100
	10°C	2100 - 900

B Colour change record under constant Light and 12-12 (hr) cycles of salinity.

Testing Conditions:	100 Lux	10°C	No 2 Bkgd.
	35 $^{o}/_{oo}$	0900 - 2100	
	F.W.	2100 - 900	

Autocorrelation plots of same records after normalizing, standardizing, and detrending.


A
MV.
B
MV.
D.C.I.

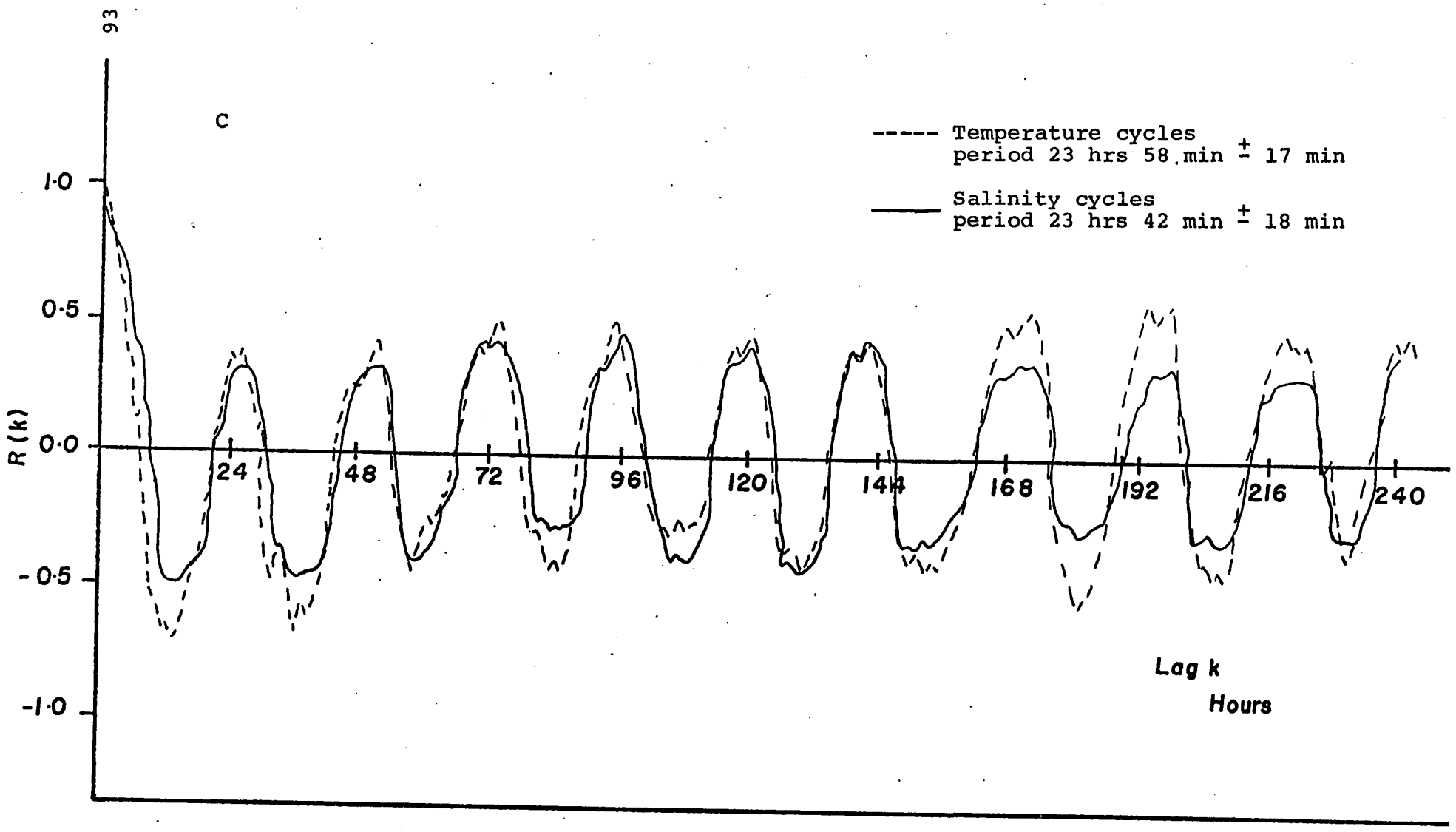
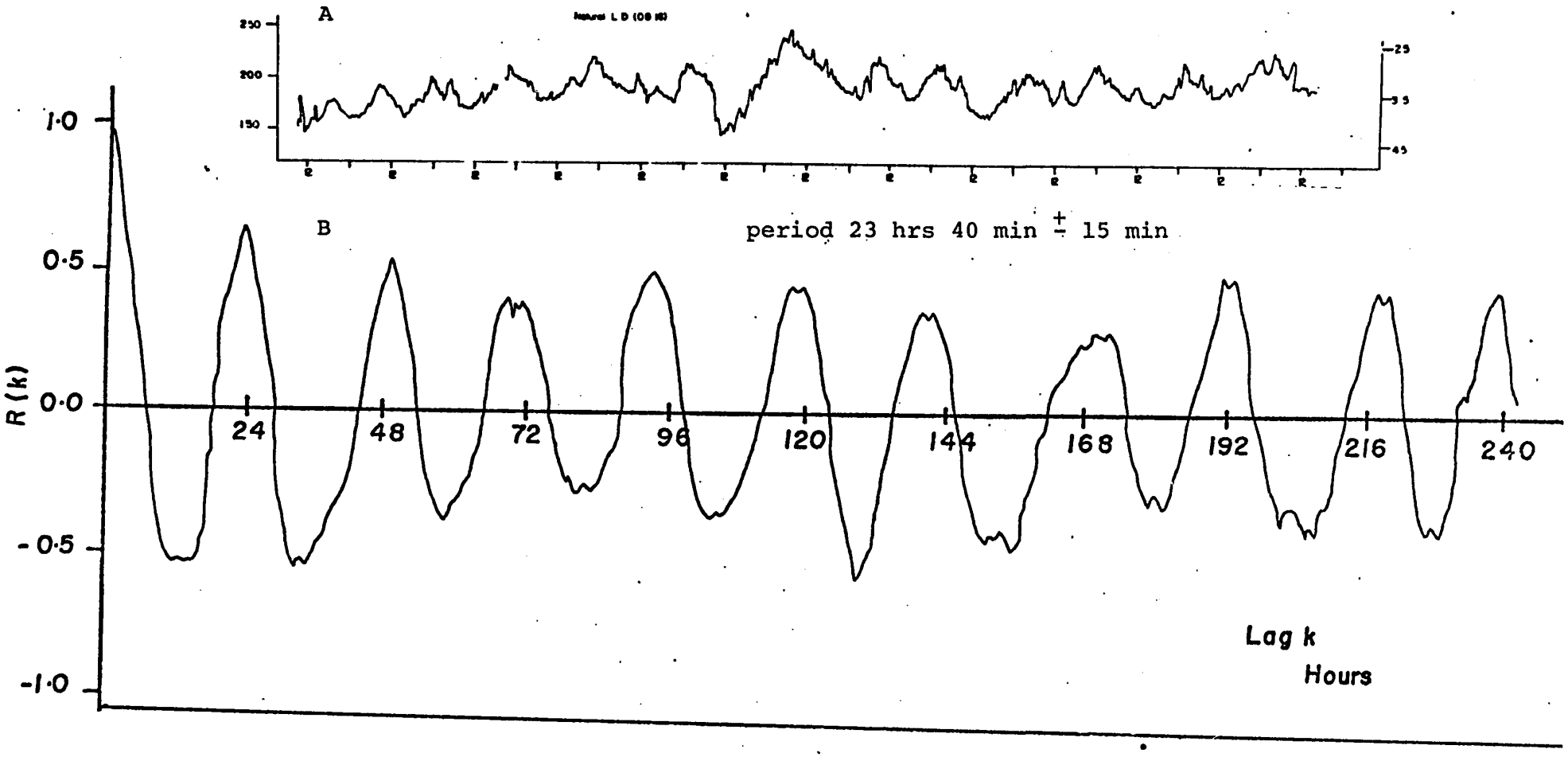

93
C
----- Temperature cycles
period 23 hrs 58 min ± 17 min
Salinity cycles
period 23 hrs 42 min ± 18 min
1·0
0·5
0·0
-0·5
-1·0
R (k)
24
48
72
96
120
144
168
192
216
240
Lag k
Hours

Figure 19: A Colour change record under constant dark and 12-12 (hrs) cycles of thermosaline changes.

B Autocorrelation plot of same record after normalizing, standardizing, and detrending.

Testing conditions 1 Lux (N)		
Temperature	20°C	1600 - 0400
	10°C	0400 - 1600
Salinity	35 $^{o}/_{oo}$	0900 - 2100
	F.W.	2100 - 0900


A
B
period 23 hrs 40 min ± 15 min
1·0
0·5
0·0
-0·5
-1·0
R(k)
24
48
72
96
120
144
168
192
216
240
Lag k
Hours

Effects of Blinding on the Endogenous Rhythm of Colour Change

Killifish were held under a variety of different maintaining conditions, bilaterally blinded and then retested. Another group was blinded, maintained and retested. In both cases the endogenous rhythm of colour change was maintained with the same period length and phasing as before blinding (Figs. 20,21), though the amplitude of change was reduced. The killifish assumed an intermediate basal colouration level immediately after blinding. Directly after blinding the rhythm was masked through 1-2 cycles (Fig 20,21) as the killifish assumed an intermediate colouration.

The precision of the rhythm of colour change was decreased, as indicated by the increased scatter and confidence interval in the regression determination of period length, and the more broadened peak in the power spectra (Fig 20(c)).

These results indicate that rhythmic colour change is not due to cyclic retinomotor variations. Blinding also reveals that vision is not necessary for the occurrence of the rhythmm. The increased variability of the rhythm indicate that light or vision plays a role in the precise occurrence of rhythmic colouration.

Figure 20: A Colour change record of blind fish under constant light.

B Autocorrelation plot of same record after normalizing, standardizing and detrending.

	Illumination	°C	$^{o}/_{oo}$	Bkgd.
Maintaining conditions	12:12 (10)	20	18	2
Testing conditions	100	20	35	3

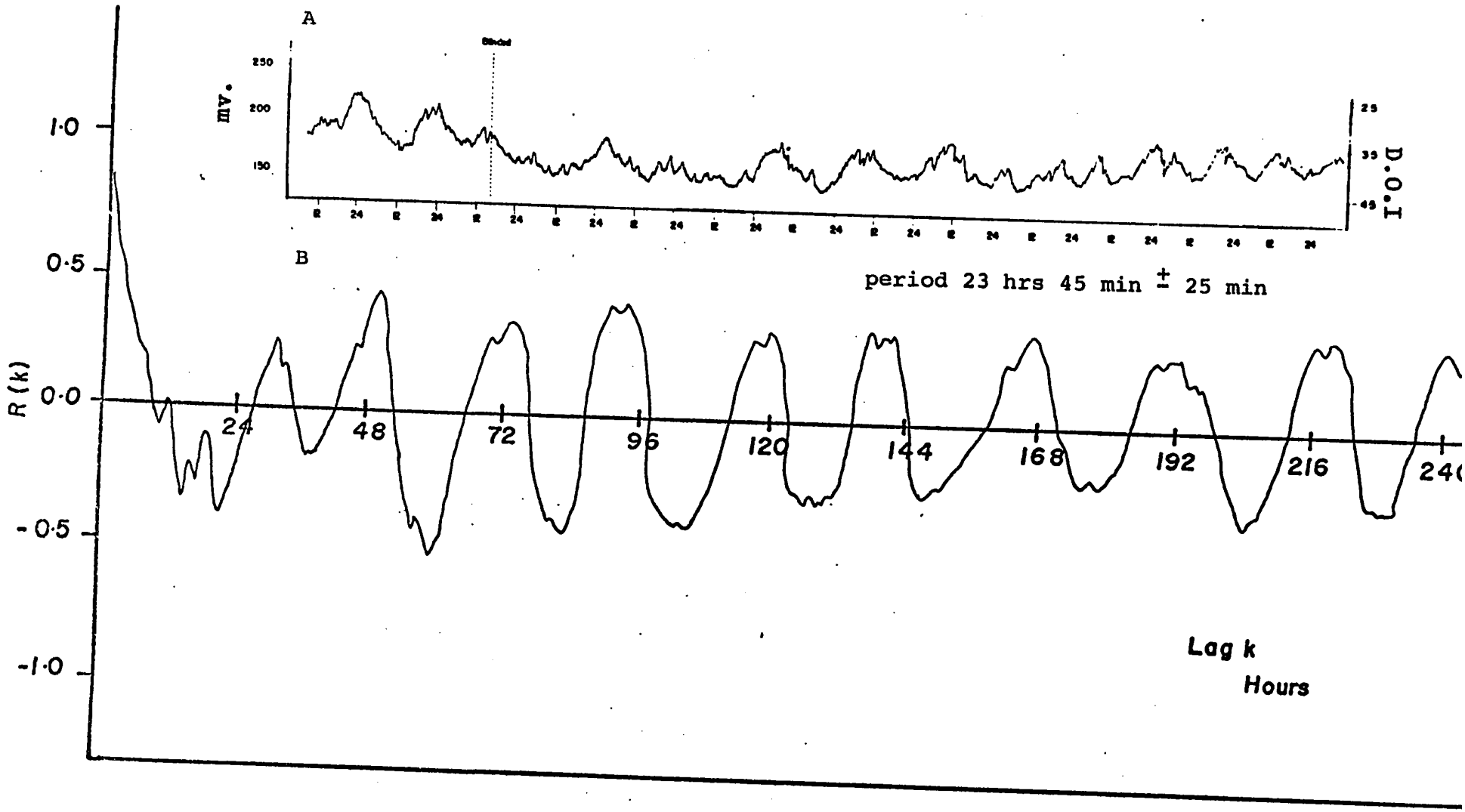

A
B
1·0
0·5
0·0
-0·5
-1·0
R(k)
24
48
72
96
120
144
168
192
216
period 23 hrs 45 min ± 25 min
Lag k
Hours

Figure 20(C): Power spectrum transformation of autocorrelation function.

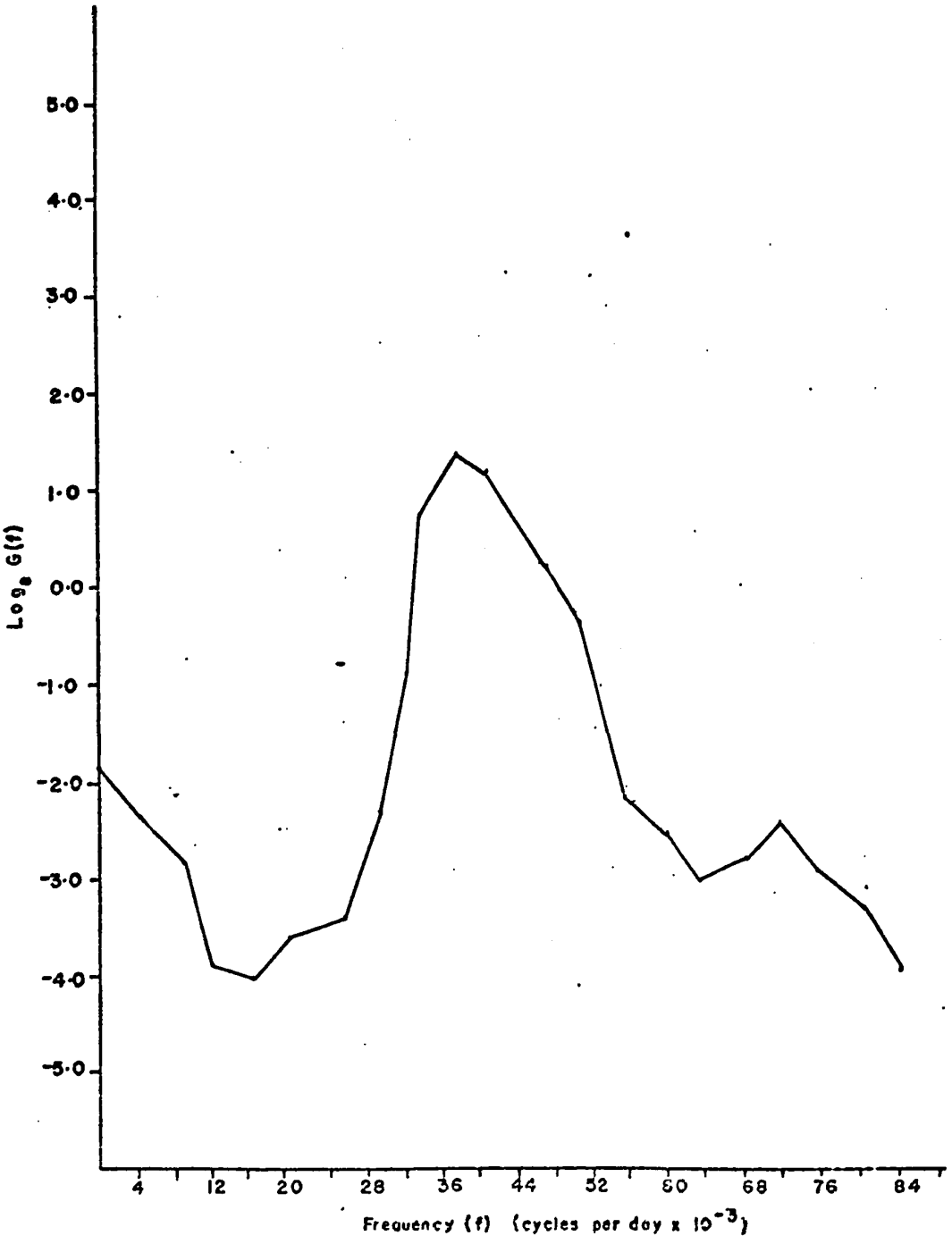
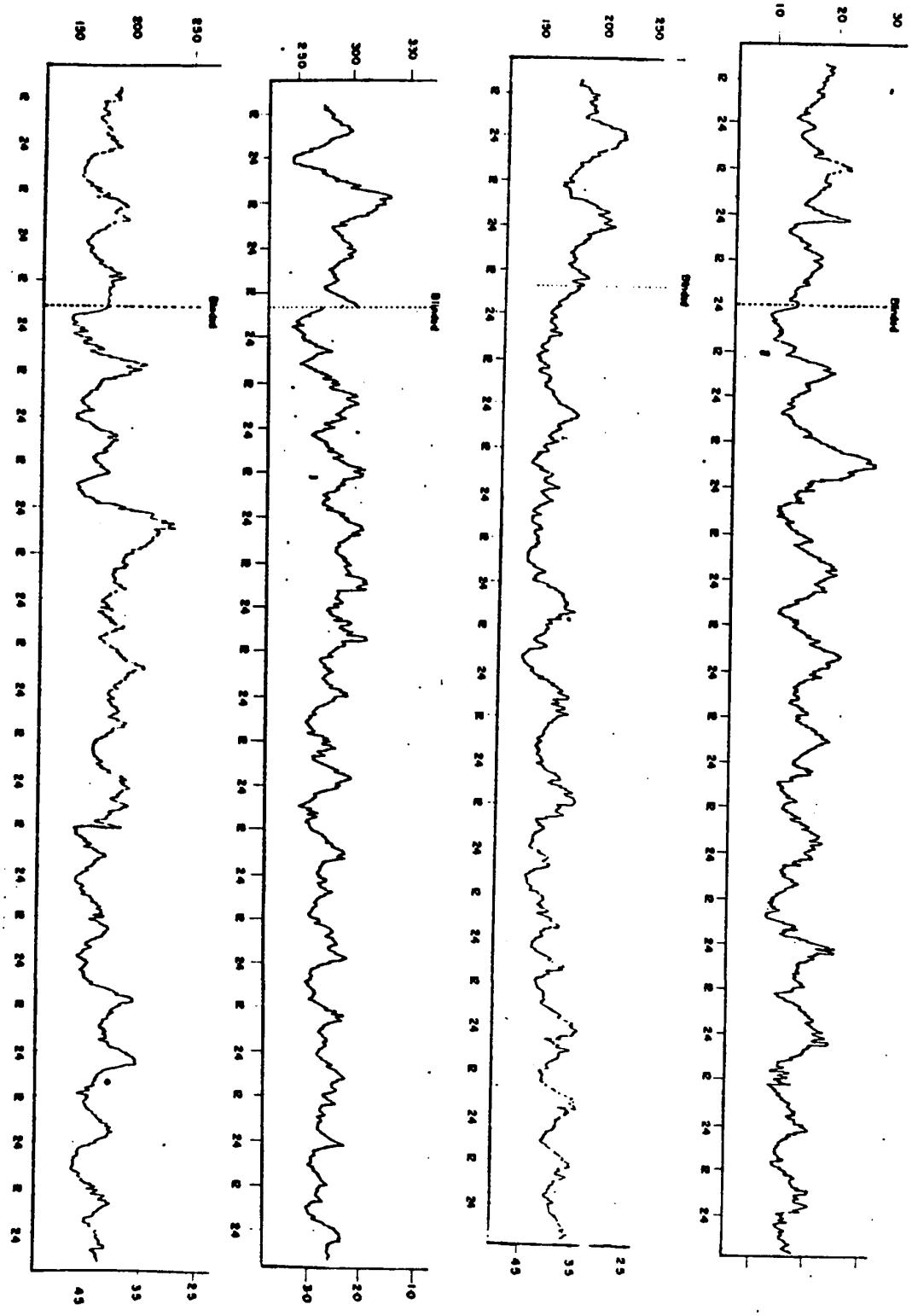


Figure 21: Colour change records of blinded fish under constant conditions.



Effect of Hypophysectomy on the Endogenous Rhythm of Colour Change

Killifish were either maintained, tested, hypophysectomized, and retested; or maintained, hypophysectomized, maintained, and retested. In both cases the rhythm of colour change, but not the ability to adapt to different backgrounds, was eliminated (Fig. 22-23). After sham hypophysectomy the rhythm of colour change remained relatively unaltered (Figs. 22B).

The loss of rhythmicity occurred through several cycles. This can be seen in the correlograms (Figs. 21C) where R(k), the correlation coefficient, quickly lost amplitude and oscillated around zero. This absence of periodicity can also be seen in the power spectra which indicate no significant periodicity present in the colouration of hypophysectomized fish (Fig. 22).

The elimination of rhythmic colour change by hypophysectomized fish also suggests that there are hormonal factors either directly or indirectly involved in controlling killifish colour change.

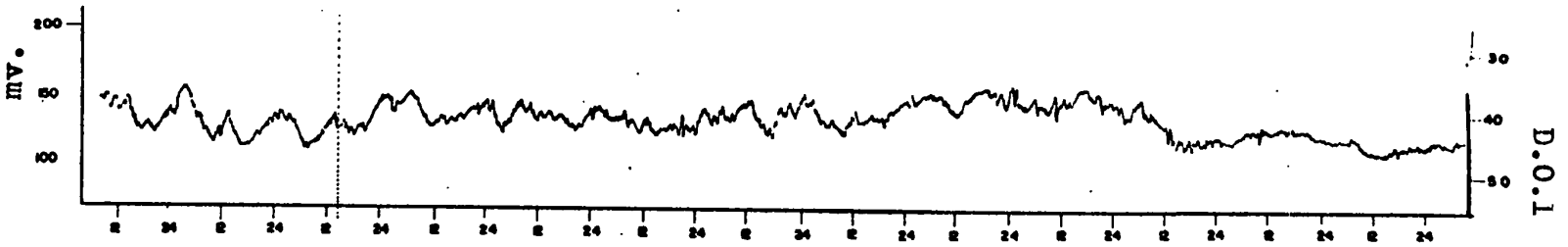
Figure 22: A Colour change record of hypophysectomized fish under constant light.

B Colour change record of sham hypophysectomized fish under constant light.

C Autocorrelation plots of same records after normalizing, standardizing, and detrending.

	Illumination (Lux)	°C	$^{o}/_{oo}$	Bkgd.
Maintaining Conditions	12:12 100	20	9	2
Testing Conditions	100	20	10	4


A - Sham hypophysectomy
mV.
200
150
100
35
45
55
D.O.I
12
24


B - Hypophysectomy
mV.
200
150
100
30
40
50
D.O.I
12
24

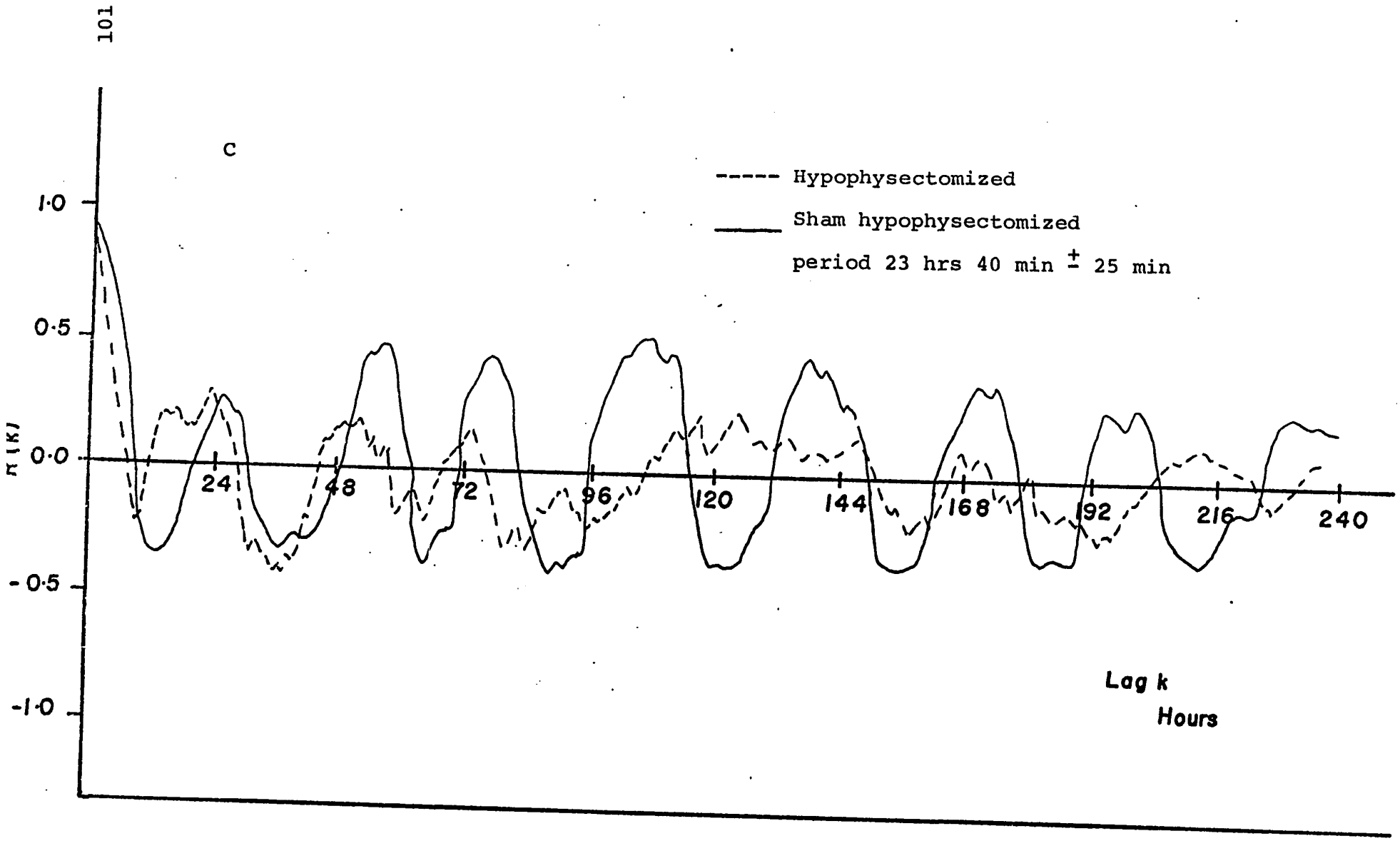

c
Hypophysectomized
Sham hypophysectomized
period 23 hrs 40 min ± 25 min
1·0
0·5
0·0
-0·5
-1·0
r (k)
24
48
72
96
120
144
168
192
216
240
Lag k
Hours

Figure 22D: Power spectrum transformation of autocorrelation functions.

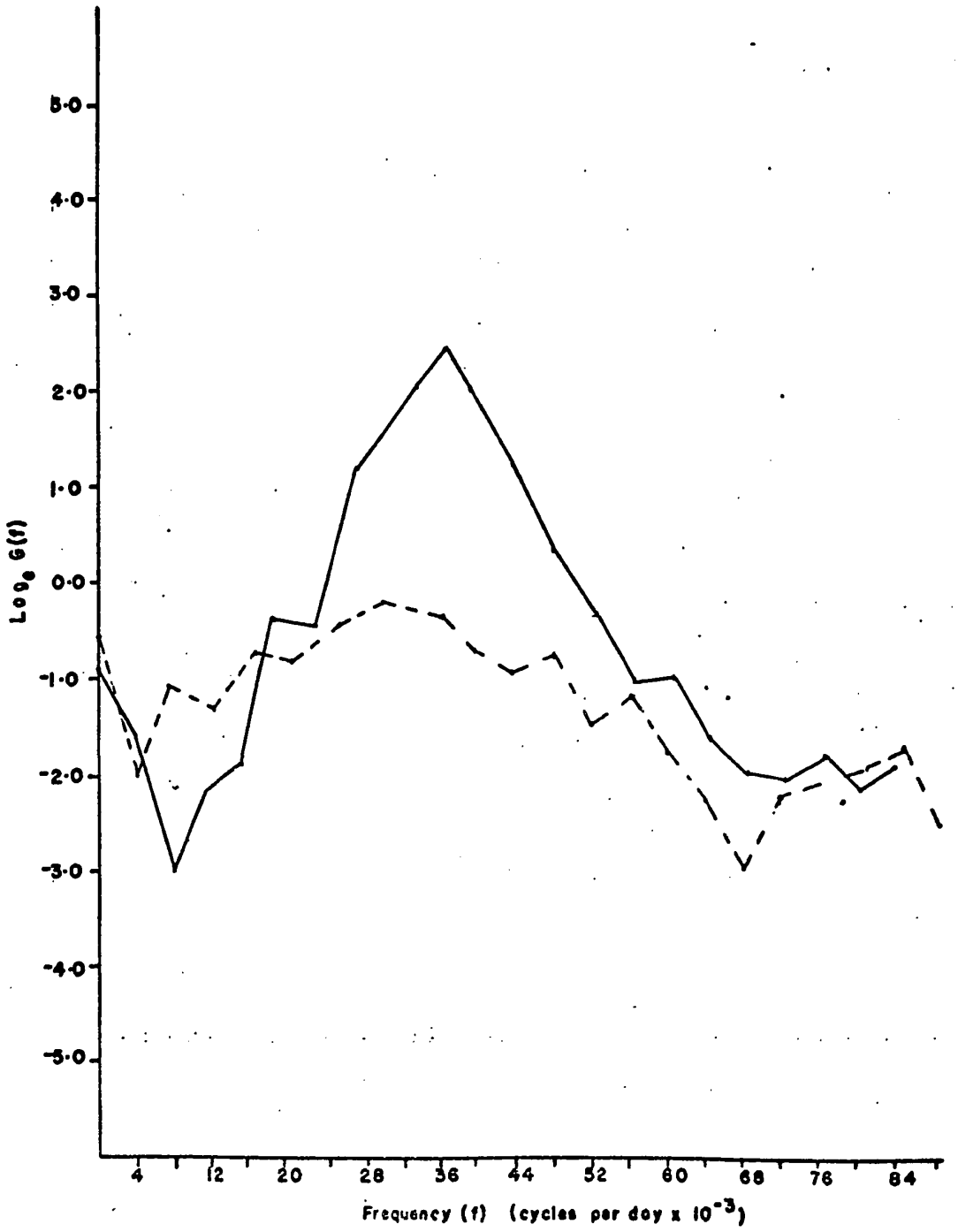
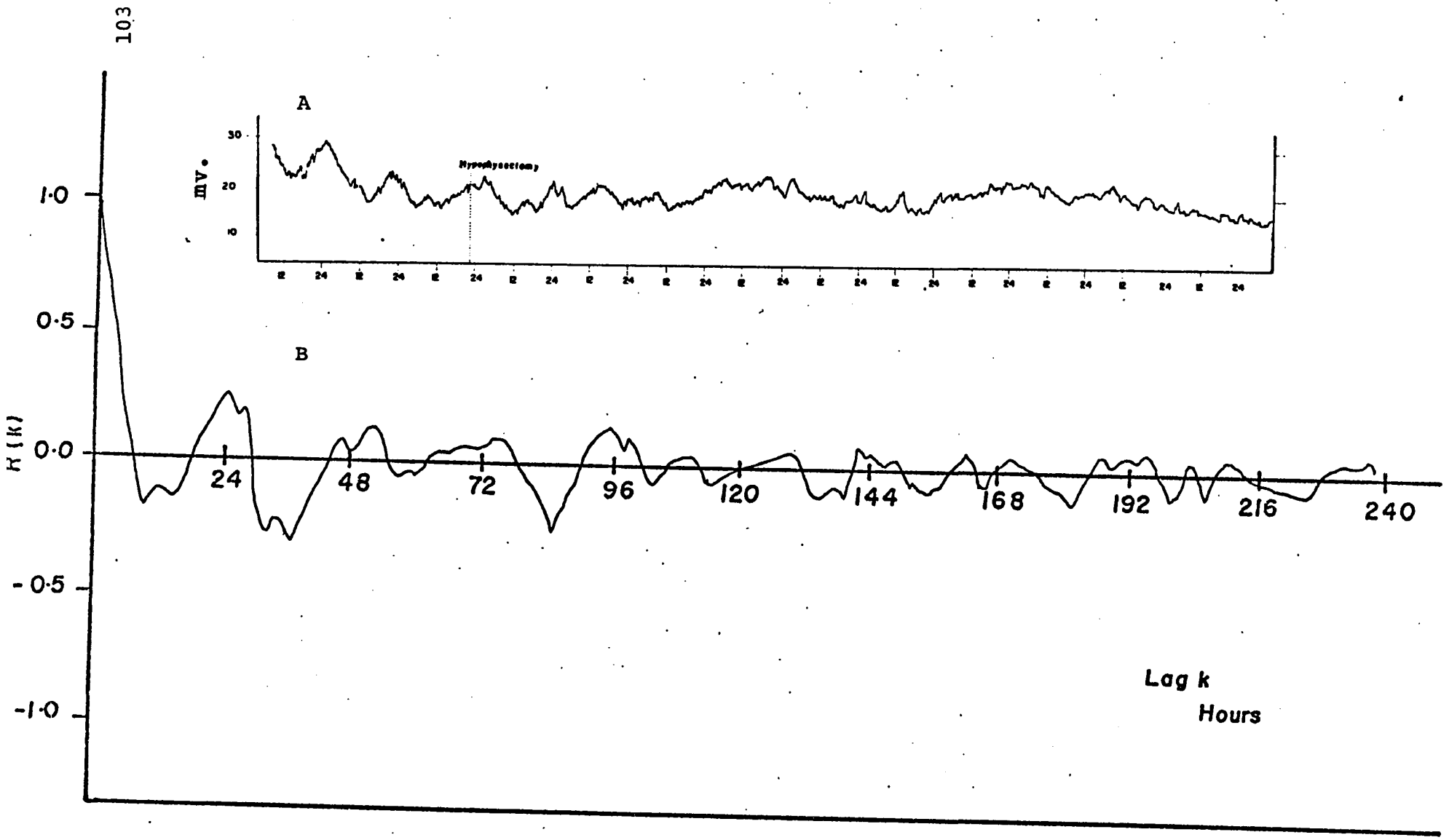


Figure 23: A Colour change record of hypophysectomized fish under constant dark.

B Autocorrelation plot of same record after normalizing, standardizing, and detrending.

	Illumination (Lux)	°C	$^{o}/_{oo}$	Bkgd.
Maintaining Conditions	12:12 (100)	20	18	2
Testing Conditions	1(D)	15	40	2


A
mV.
30
20
10
Hypophysectomy
B
1·0
0·5
0·0
- 0·5
-1·0
r (k)
24
48
72
96
120
144
168
192
216
240
Lag k
Hours

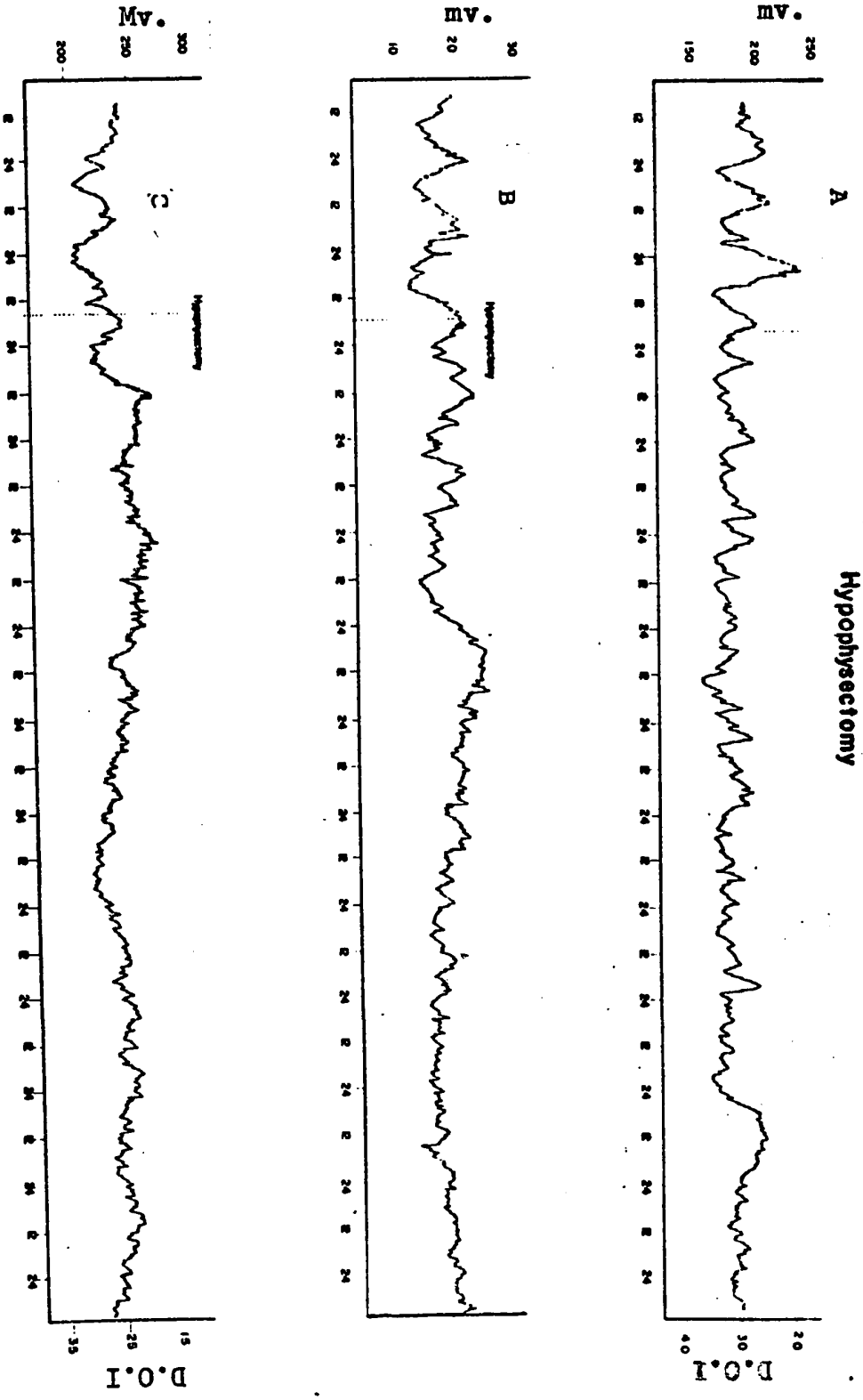

mv.
A
Hypophysectomy
D.O.I
mv.
B
D.O.I
Mv.
C
D.O.I

Figure 24: Colour change records of hypophysectomized fish kept under constant conditions.

Table 9:

Period Length of Rhythmic Colour Change Under Different Conditions.

Conditions	Period Length hrs. min ± 95% confidence interval
Constant L	
200 Lux	23 hrs 40 min ± 11 min
100 Lux	23 hrs 45 min ± 15 min
50 Lux	23 hrs 42 min ± 12 min
Constant D	24 hrs 10 min ± 25 min
	23 hrs 58 min ± 17 min
	23 hrs 50 min ± 18 min
Temperature cycles (12/12)	
100 Lux	24 hrs 6 min ± 23 min
D.D.	23 hrs 45 min ± 25 min
Saline cycles (12/12)	
100 Lux	23 hrs 58 min ± 17 min
D.D.	23 hrs 42 min ± 18 min
Thermo saline cycles (12/12)	
100 Lux	23 hrs 40 min ± 15 min
D.D.	24 hrs 20 min ± 13 min
Blinded	
100 Lux	23 hrs 45 min ± 25 min
D.D.	23 hrs 20 min ± 37 min

Hormonal Effects on Colour Change.

The hormonal injections did not have any immediate or long term effect on killifish colour or its' rhythm of change. Therefore these results are not presented. They are on deposit at Sir George Williams University.

Locomotory Activity Determinations

Operation of Apparatus

Locomotory activity of killifish was continuously recorded at a paper speed of 0.025 cm/sec. For ease of data analysis, visual interpretation, and presentation, activity was graphically presented as cumulative totals over half-hour intervals. These totals were designated by arbitrary units of activity.

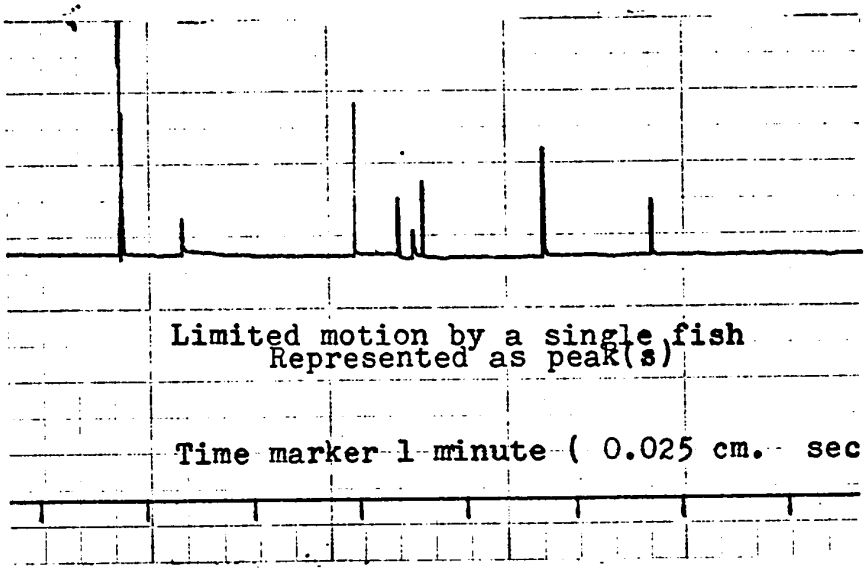
Killifish were never completely inactive. They engaged in limited vertical motion along the sides of the tank, represented by small single peaks in the tracing (Fig. 25).

When the fish engaged in more extensive activity, the peak height and number were increased. Overlapping peaks were counted as one, resulting in information loss. Activity occurred in sporadic bursts of several seconds duration (30-360 secs) interspersed with limited vertical motion. The graphical presentation did not permit discrimination between several large bursts accompanied by limited vertical locomotion, and a continuous number of smaller bursts. This is due to the use of activity totals taken over half-hour intervals.

When the fish moved directly in front of the transducer a large number of peaks were recorded. These could

however, be readily distinguished from the preceding and following activity that was recorded.

Figure 25: Examples of types of activity that were recorded.



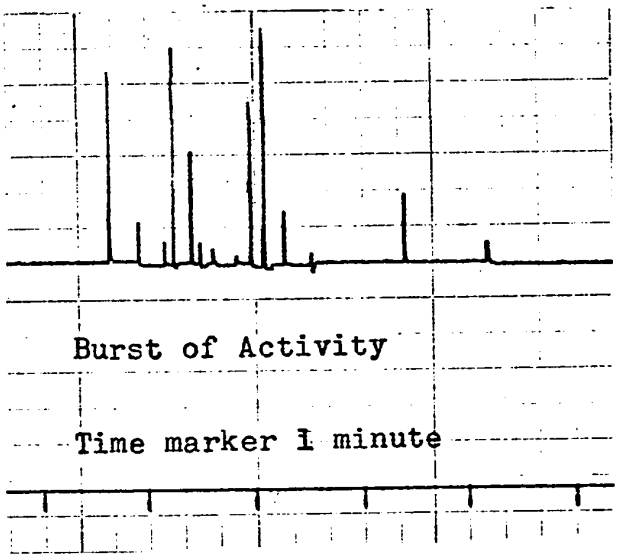
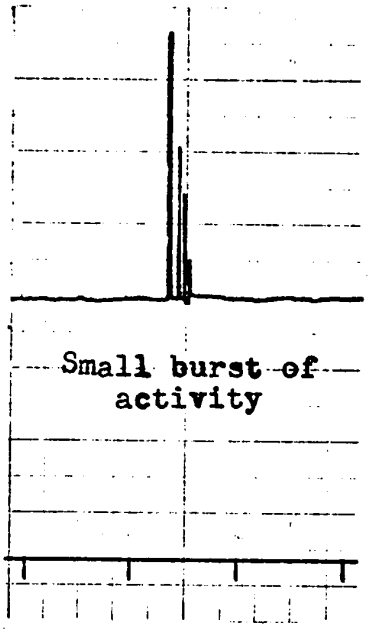
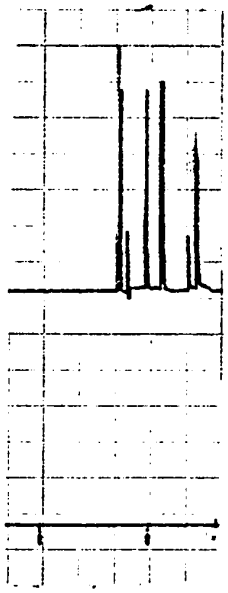
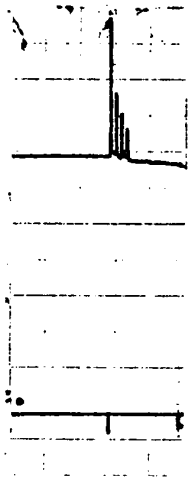


Figure 25 continued.





Burst of Activity



Limited motion in front of transducer

Rhythmic Locomotory Activity Analysis

In this section activity time series (plotted at 30 minute intervals) along with their corresponding autocorrelation, linear regression and power or variance spectrum transformations are shown. These results and their interpretation are given below. They describe the basic characteristics and properties of killifish locomotor activity.

Under controlled and natural photoperiods killifish are maximally active when illuminated. The activity maxima coincided with the photoperiod and complete inactivity was never observed (Fig. 26). The fish always maintained a basal amount of vertical and horizontal motion. This activity was confined to limited areas along the sides of the experimental tank.

Under the light portion of a Light-Dark (L-D) cycle activity became more concerted and increased in amount (amplitude) and duration above that of the basal level. Activity occurred in bursts of several seconds to minutes duration followed by extended periods of apparent immobility. Since the graphical representations of activity were based on total movements over half-hour intervals the sporadic, discontinuous nature of swimming was not revealed.

Killifish displayed a double peaked pattern of maximum activity (Figs.26-30). These activity maxima coincided with the late and early portions of the photoperiod

(Figs.20,27). This type of activity pattern is termed bimodal. There were intra- and inter-fish variations in peak height and the total amount of activity (Figs. 27-30).

Under constant conditions (Light or Dark) the rhythmic activity pattern continued showing a gradual extinction or fade-out to a mean level of continuous activity (Figs. 27, 38). The rhythmic nature of this activity was clearly shown in the correlograms and was indicated by the presence of a significant peak in the power spectra (Fig. 27). The persistence of the activity rhythm for more than 5 cycles under constant conditions was used as a basis for terming it endogenous.

Under the 24 hour Light-Dark Cycle (12-12 L-D) activity had a period equal to that of the imposed exogenous cycle. Under constant conditions the endogenous activity rhythm free ran at its natural oscillation frequency, losing phase synchrony with the previous L-D cycle. The length of the free running period depended on the intensity of constant illumination (Figs. 36, 37). The change in period length after imposition of constant conditions is shown by the discontinuity in the correlogram (Fig. 26) and change in shape and frequency of the autocorrelation function. Under constant conditions the rhythm lost its bimodal appearance and free ran with a single activity peak (unimodal). The power spectra obtained from the correlogram (Fig. 27C) indicated, through the presence of a peak, a significant

periodicity in the range of 23-25 hours. A more exact determination of period length was obtained from the regression plot. Period length varied between fish, though these differences did not approach significant values.

The period length was dependent on the intensity of constant illumination, thus following Aschoff's rule (Aschoff 1966). The period ranged from 23 hrs 20 min $\pm$ 15 mins under 200 lux to 24 hrs 20 min $\pm$15 mins under 0.1 Lux. The effects of illumination intensity on period length are described more fully in a later portion of the results.

Several of the spectra possessed significant power at zero frequency (Fig. 27C) indicating that activity may posses a curivilinear trend or long term rhythm.

Figure 26: A Locomotory activity record of single fish under 12-12 L-D followed by constant light (100 Lux, 15°C, F.W.).

B Autocorrelation plot of the constant light portion of the above record after normalizing, standardizing and detrending.

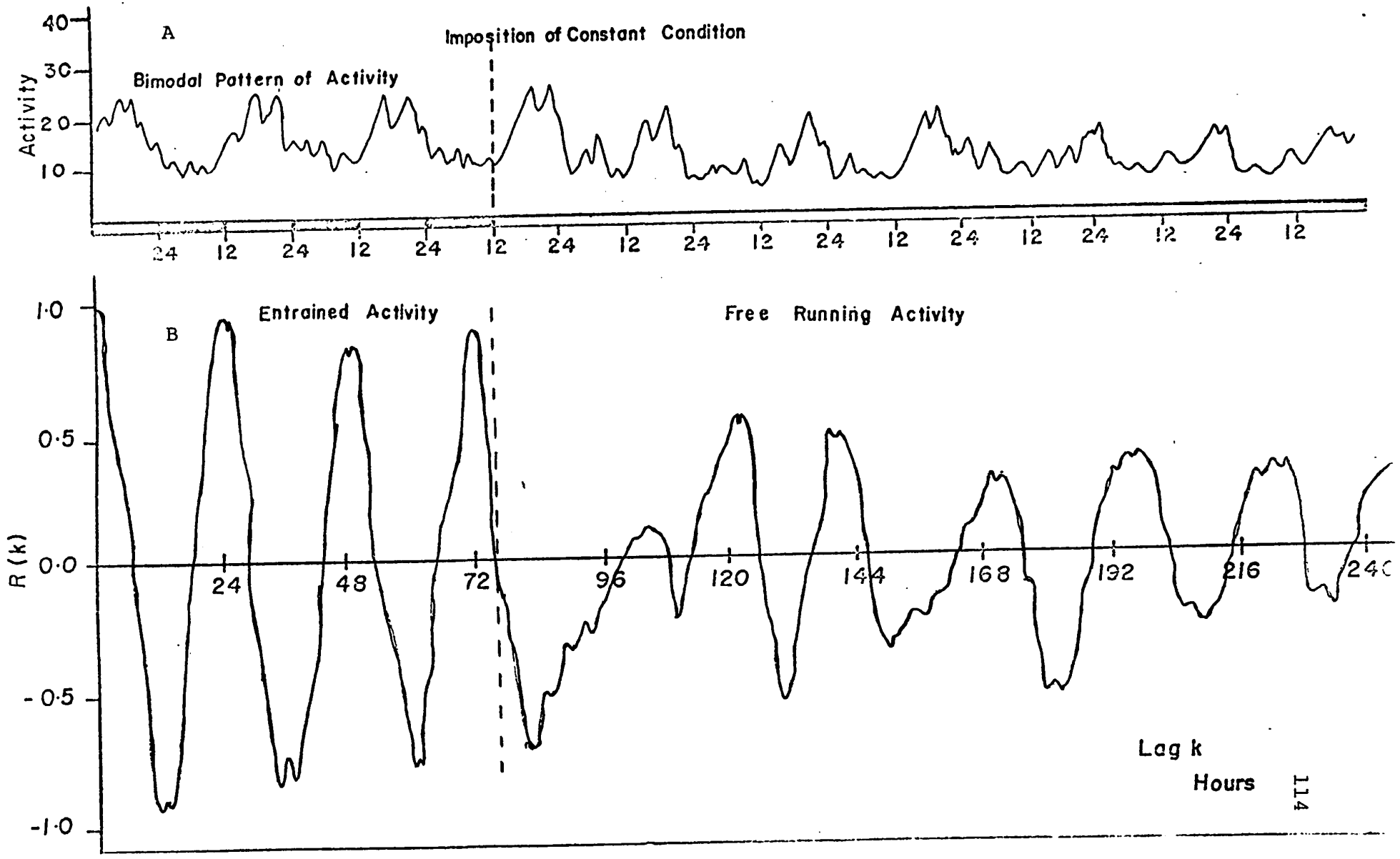

A
Imposition of Constant Condition
Bimodal Pattern of Activity
Activity
40
30
20
10
24
12
24
12
24
12
24
12
24
12
24
12
24
12
24
12
24
12
24
12
B
Entrained Activity
Free Running Activity
R (k)
1·0
0·5
0·0
-0·5
-1·0
24
48
72
96
120
144
168
192
216
240
Lag k
Hours

Figure 27: A Locomotory activity record of single fish under 12-12 L-D followed by constant dark (200 Lux; 20°C, 20°/$_{oo}$).

B Autocorrelation plot of the constant dark portion of the above record after normalizing, standardizing, and detrending.

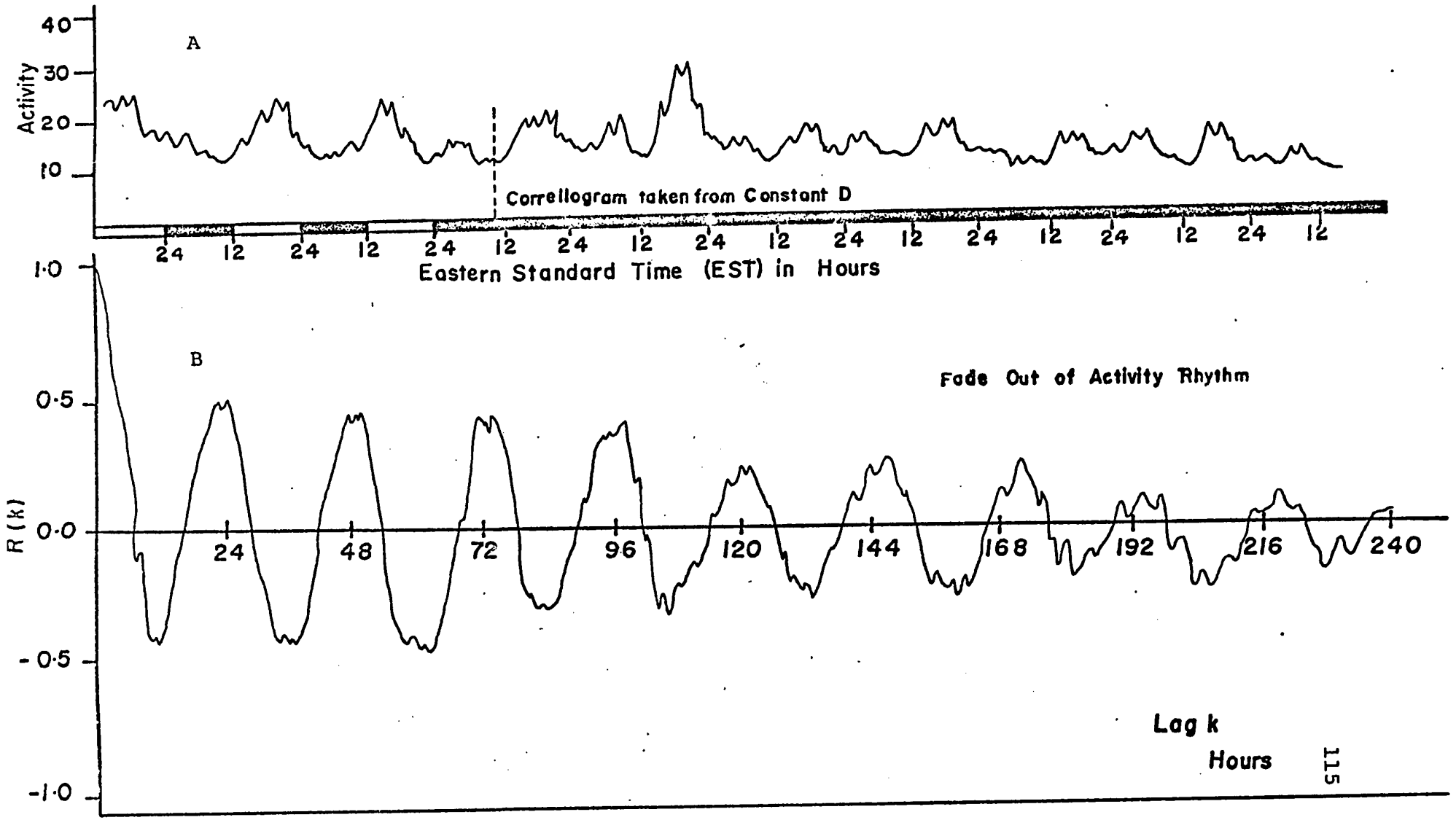
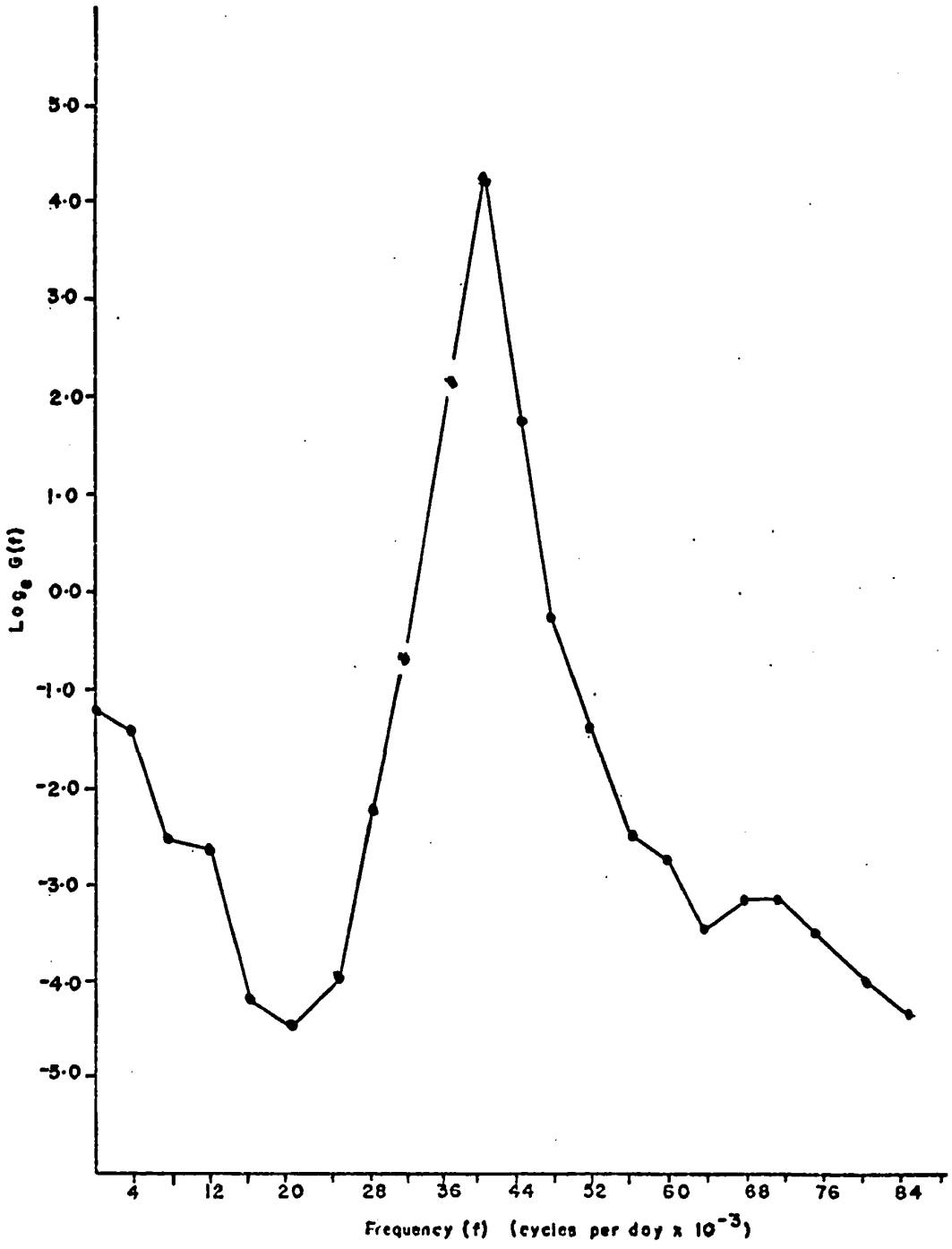

A
Activity
40
30
20
10
Correllogram taken from Constant D
24 12 24 12 24 12 24 12 24 12 24 12 24 12 24 12 24 12
Eastern Standard Time (EST) in Hours
B
Fade Out of Activity Rhythm
R(k)
1·0
0·5
0·0
-0·5
-1·0
24 48 72 96 120 144 168 192 216 240
Lag k
Hours

Figure 27C: Power spectrum transformation of auto-correlation function.



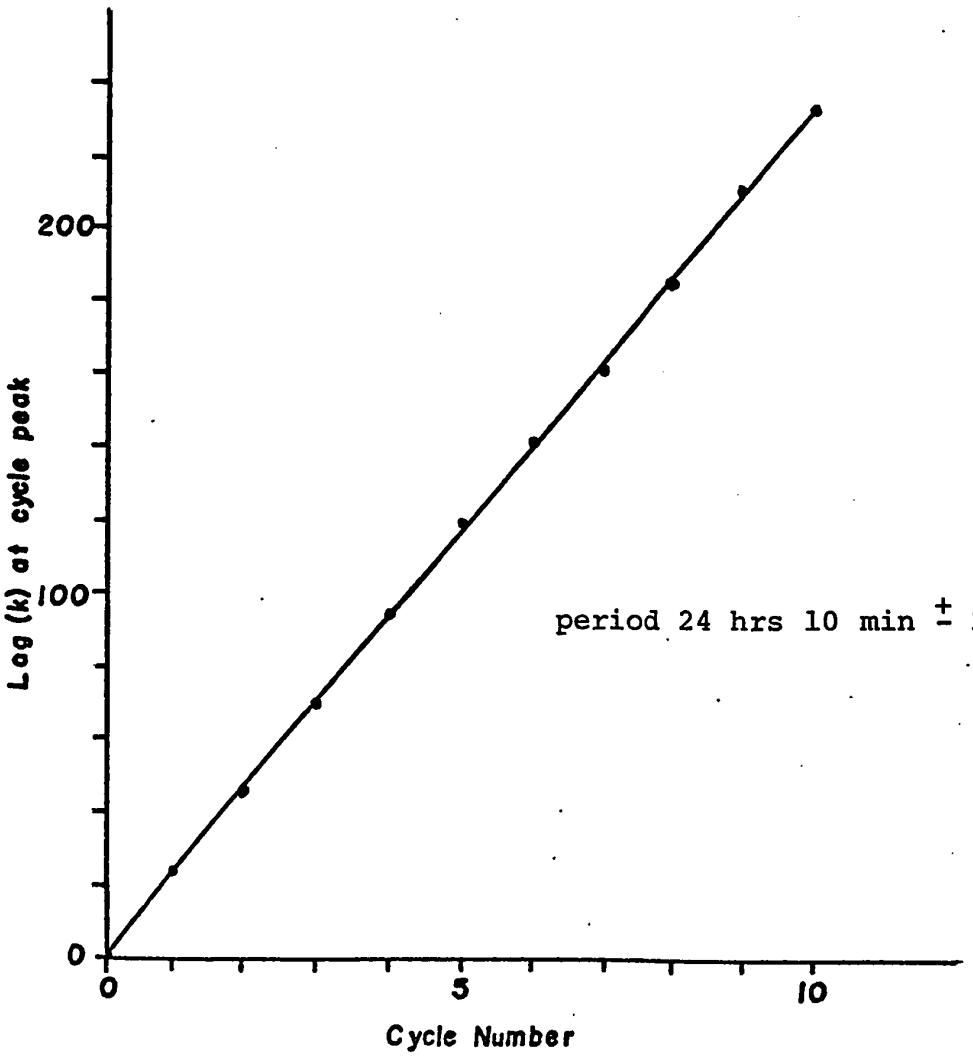


Figure 27D: Linear regression of lag(K) (hrs) measured at cycle peak of autocorrelation plot on cycle number - the slope of this line is an estimate of period length.

Effect of Photoperiod on the Endogenous Rhythm of Activity

Maximal locomotory activity occurred during the light (L) portion of an L-D cycle. Shifting the L-D cycle 90° caused activity to shift to maintain a constant phase relationship with the photoperiod. The activity rhythm required 3-4 days to shift and regain synchrony with photoperiod (Fig 28). Inverting the photoperiod (180° phase shift) caused activity to reverse through 1-2 cycles to the initial phase relationships. These reversals required 2-4 cycles (Fig. 29).

Placing a killifish under constant conditions led to desynchrony with the previous photoperiod. After several cycles of free running, the imposition of a 12-12 L-D cycle restored the initial 24 hour periodicity in activity and the phase relationships to photoperiod (Fig 30, 31).

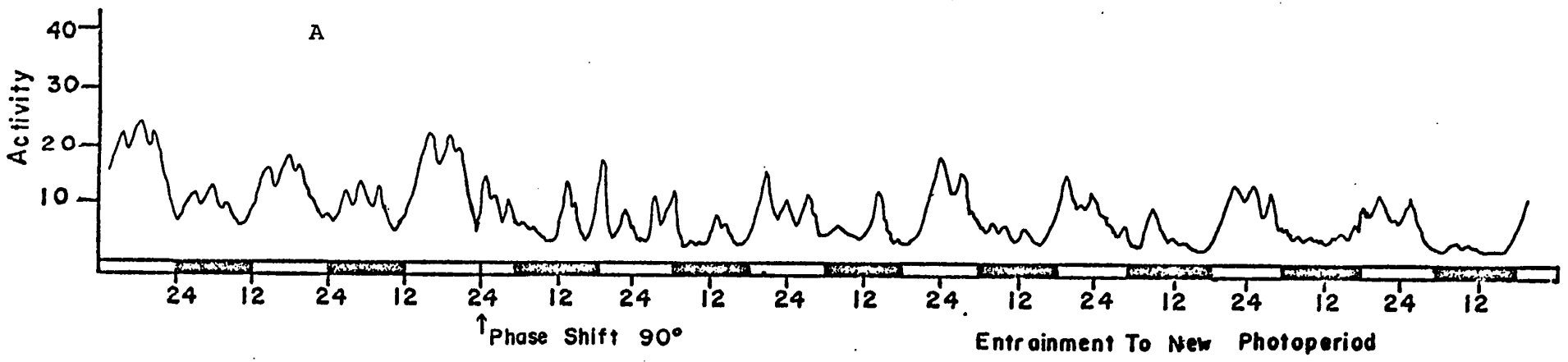
The synchronizing or entraining effects of a 12-12 L:D cycle were used as criteria to designate photoperiod as a Zeitgeber for killifish locomotory activity. Brief flashes at 24 hour intervals caused transient increases in activity (Fig 32). The flashes did have a slight affect on the endogenous rhythm, minimizing the amplitude decrease and fade-out under constant conditions.

Brief occultations at 24 hour intervals had a similar effect to that of the flashes. The occultations caused a less marked disturbance of the endogenous rhythm and were slightly less effective in preventing fade-out than flashes.

Photoperiods of 14-10 L-D and 10-14 L-D were able to entrain activity. (Fig 31). The times needed for entrainment were slightly increased as compared to that required for 12-12 L-D cycles. The bimodal nature of activity still persisted, though decreased in magnitide.

Light-Dark ratios of 16-16; 8-8, hrs were unable to entrain activity. The effects of the L-D cycles were superimposed upon the free running rhythm of locomotor activity. These superimpositions are illustrated in Figs. 34-35.

Figure 28: Locomotory activity records of single fish under 12-12 L-D, followed by 90° phase shift (200 Lux, 20°C, 20°/$_{oo}$).


A
Activity
40
30
20
10
24 12 24 12 24 12 24 12 24 12 24 12 24 12 24 12 24 12
Phase Shift 90°
Entrainment To New Photoperiod

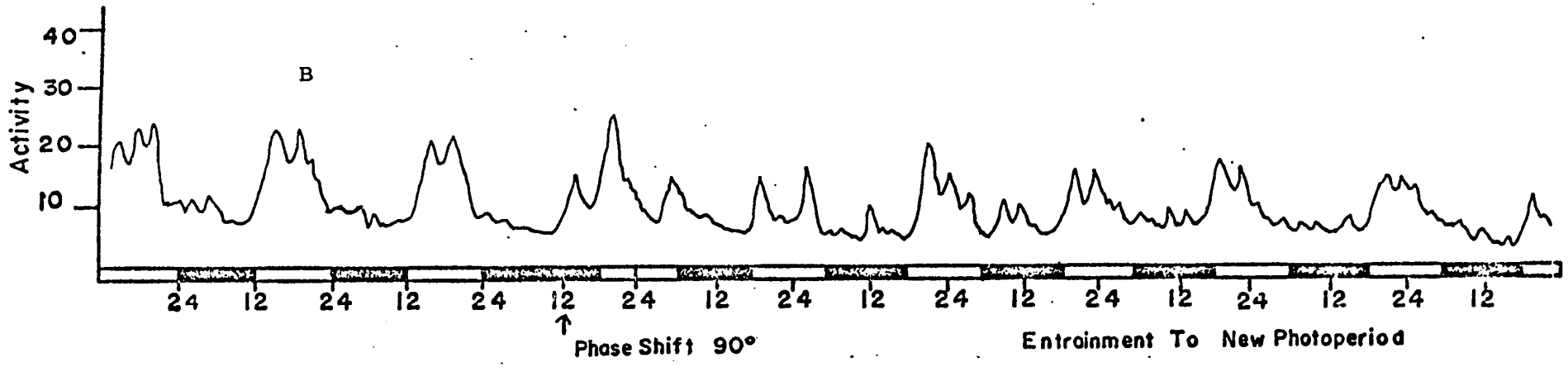

B
Activity
40
30
20
10
24 12 24 12 24 12 24 12 24 12 24 12 24 12 24 12 24 12
Phase Shift 90°
Entrainment To New Photoperiod

Figure 29: Locomotory activity records of single fish under 12-12 L-D followed by 180° phase shift (100 Lux, 15°C, 14 $^{o}/_{oo}$).

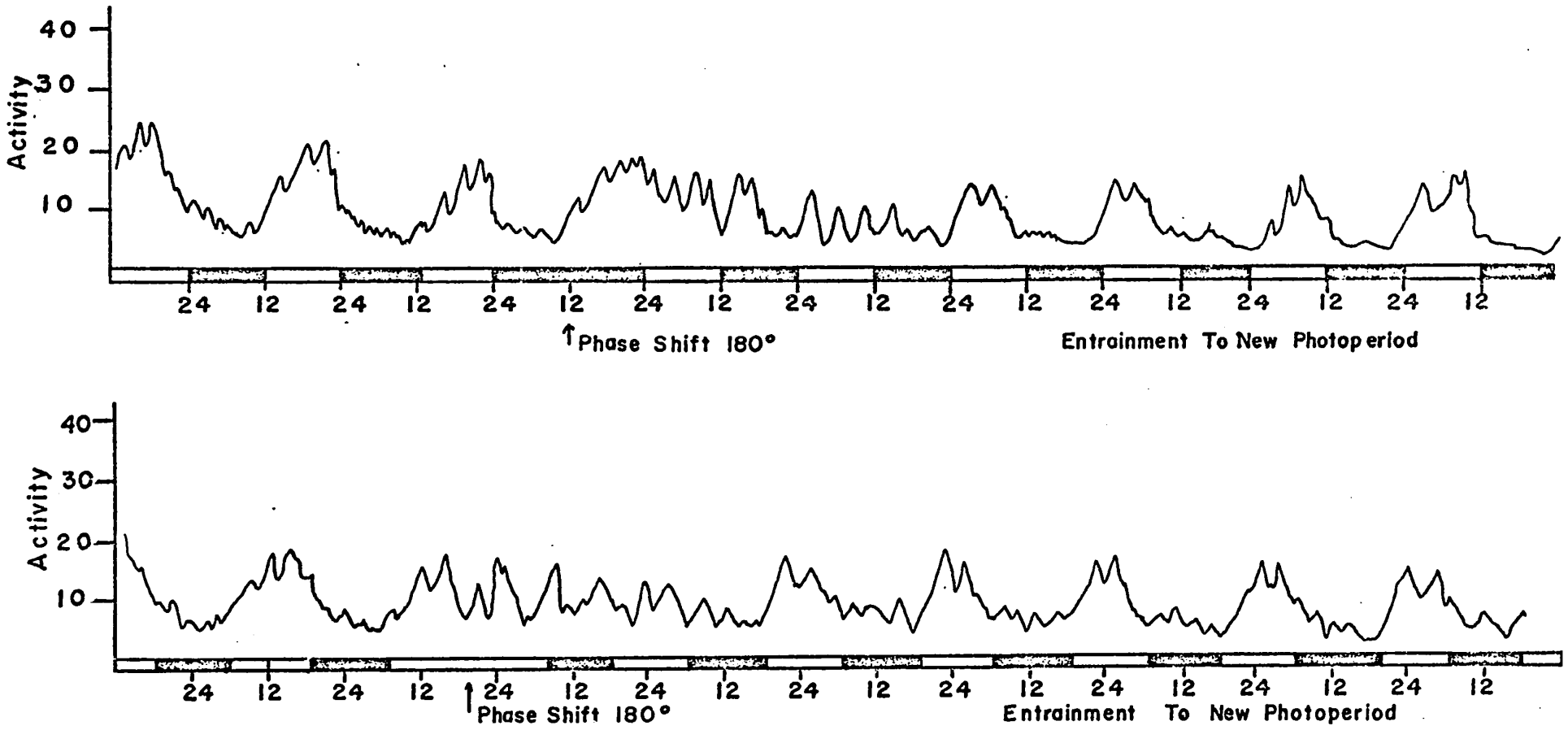


Figure 30: Locomotory activity records of single fish under 12-12 L-D, interposed by
(A) constant light (F.W.). (200 Lux)
(B) constant dark (F.W.).

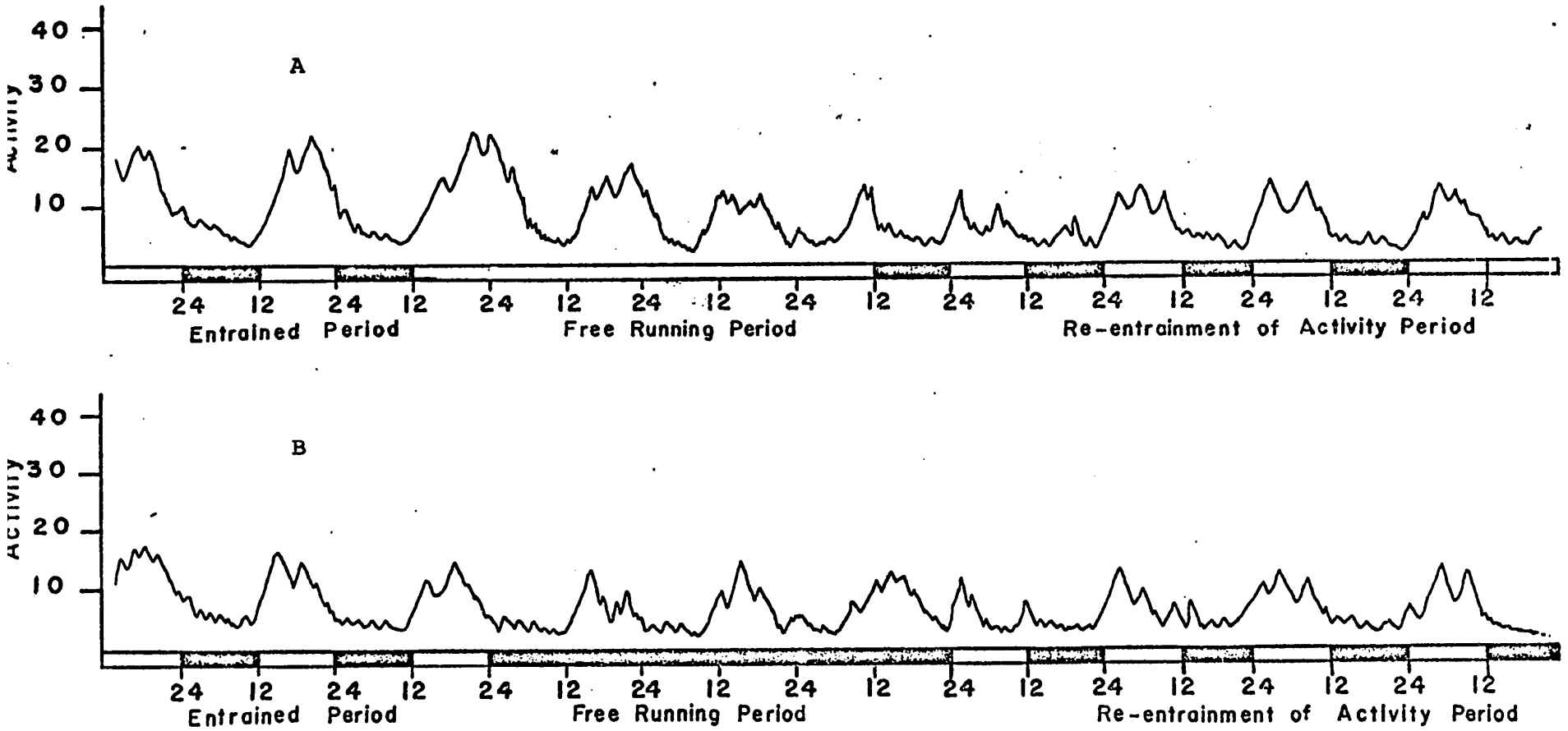


Figure 31: Locomotory activity records of single fish under 12-12 (L-D) followed by constant light and then followed by: (A) 10-14 L-D (100 Lux)
(B) 14-10 L-D (100 Lux)

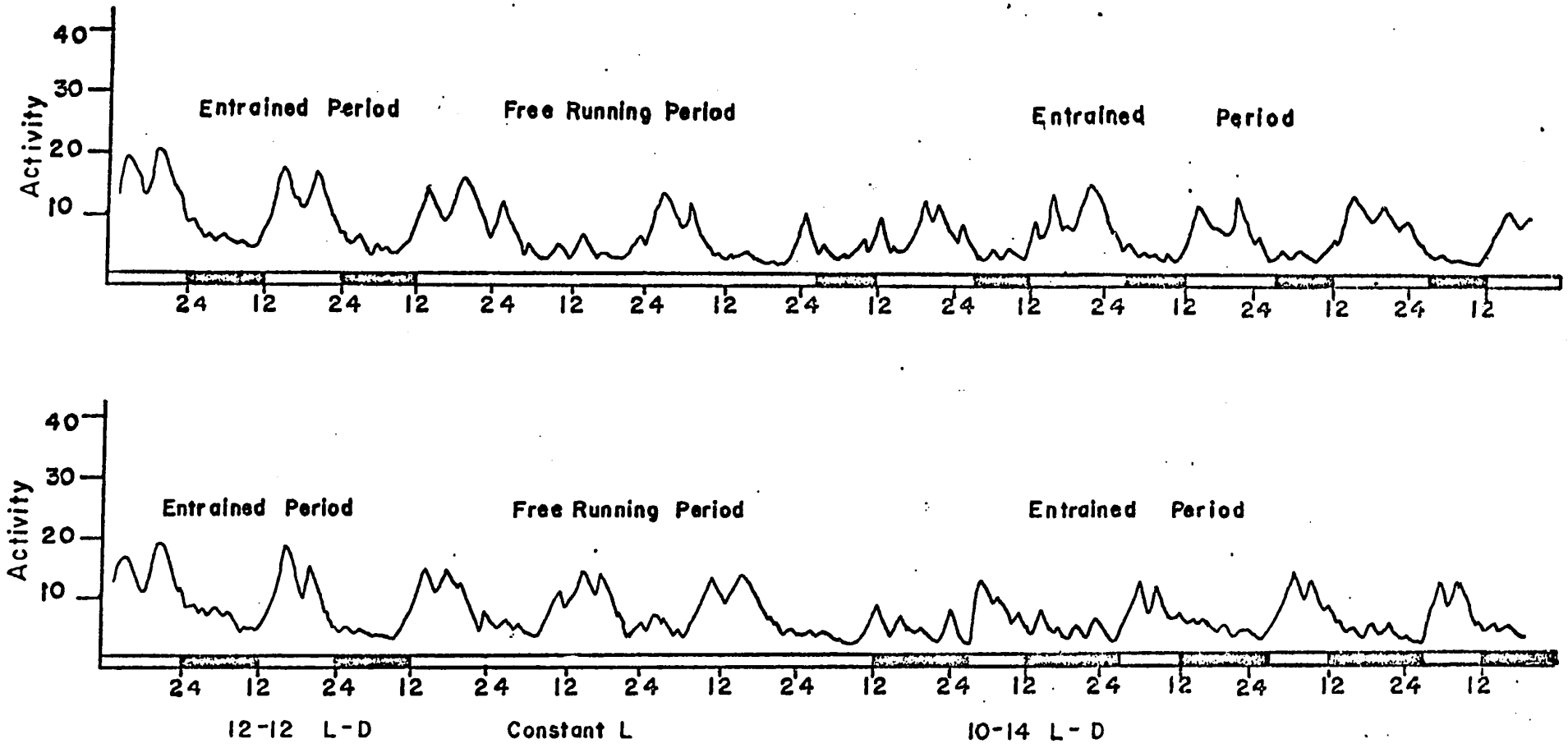


Figure 33: Locomotory activity records of single fish exposed to occultations followed by constant light.

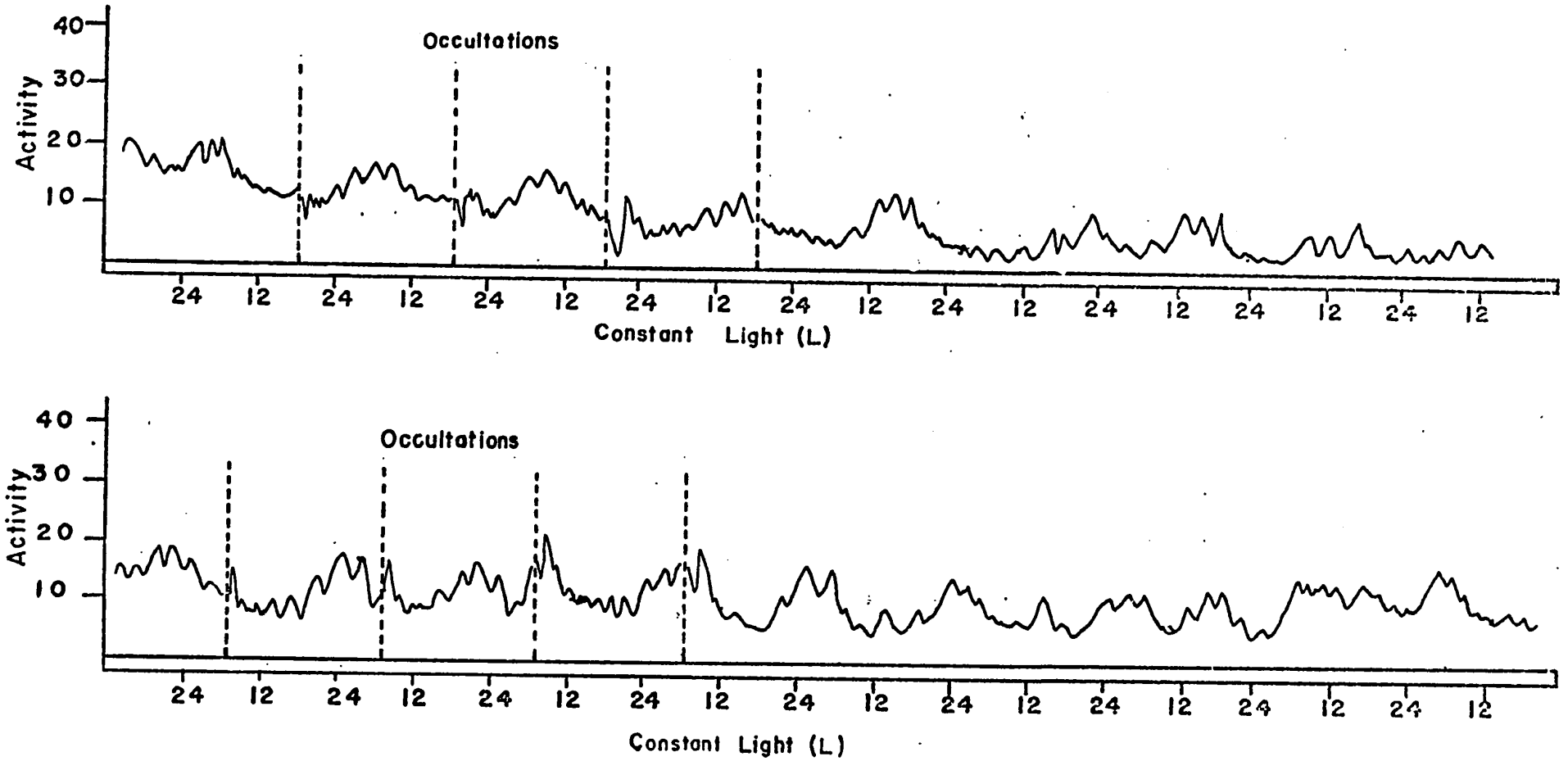


Figure 32: Locomotory activity records of single fish exposed to flashes followed by constant dark.

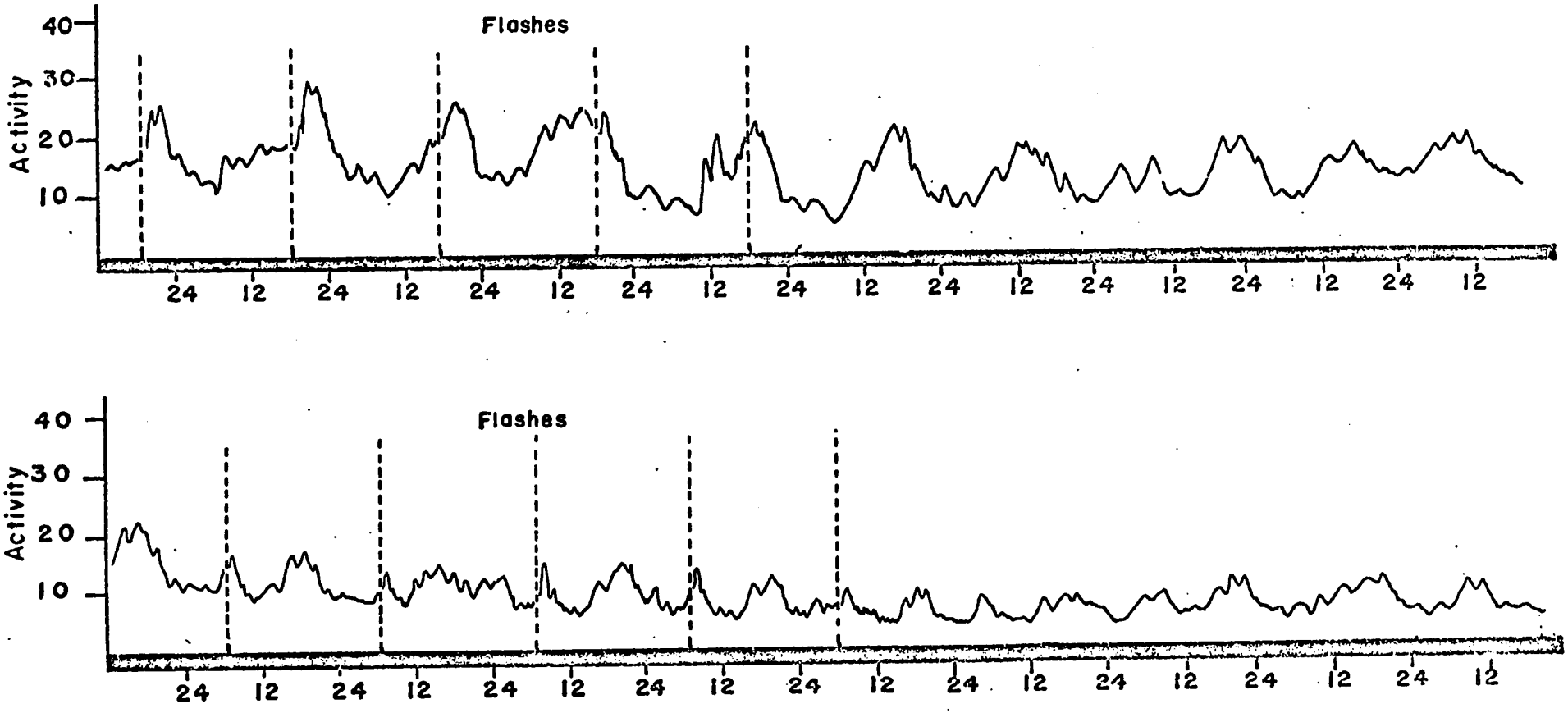


Figure 34: A Locomotory activity record of single fish exposed to 16-16 L-D followed by constant dark.

B Autocorrelation plot of the 16-16 L-D portion of the above record after normalizing, standardizing and detrending.

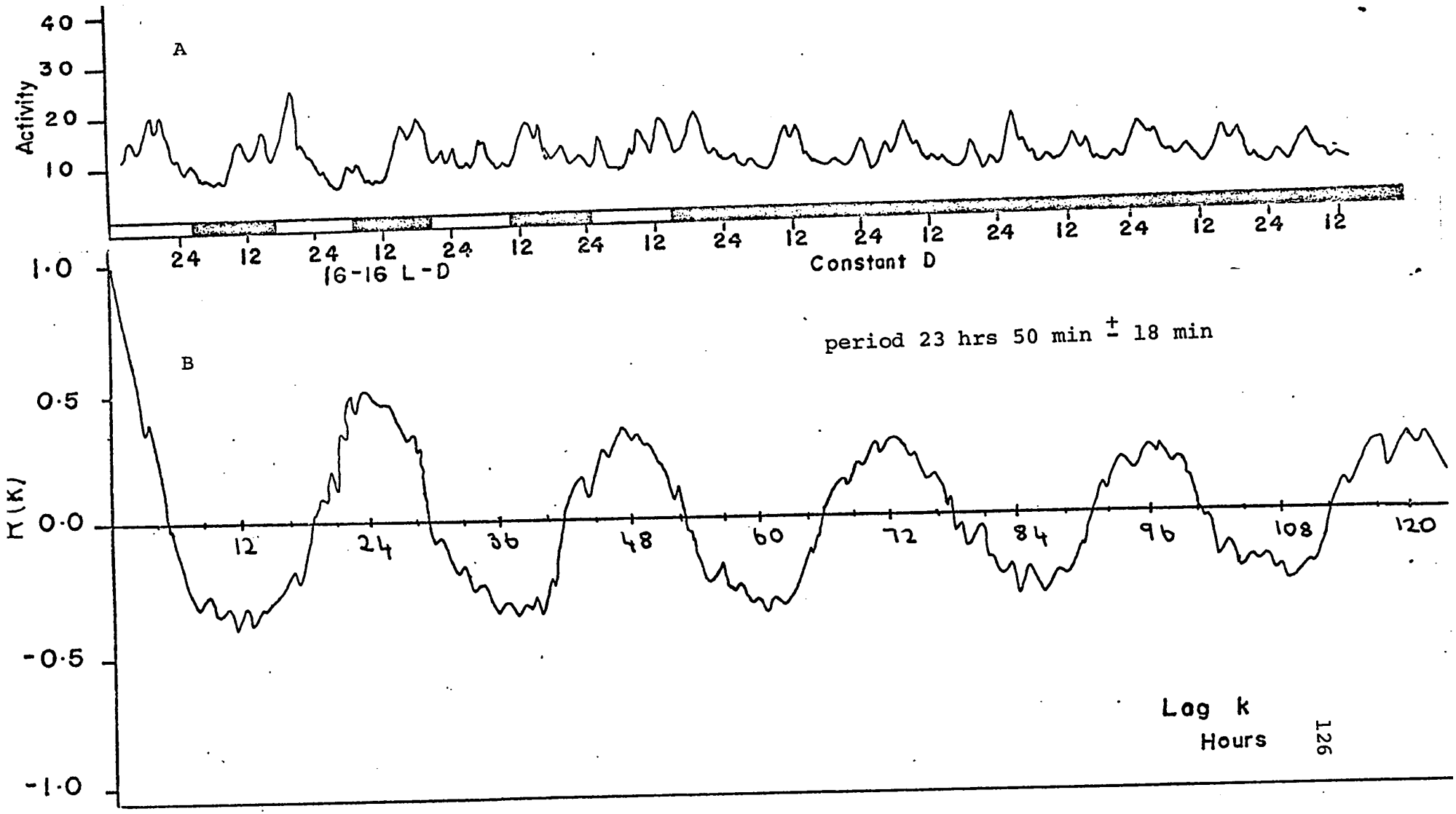
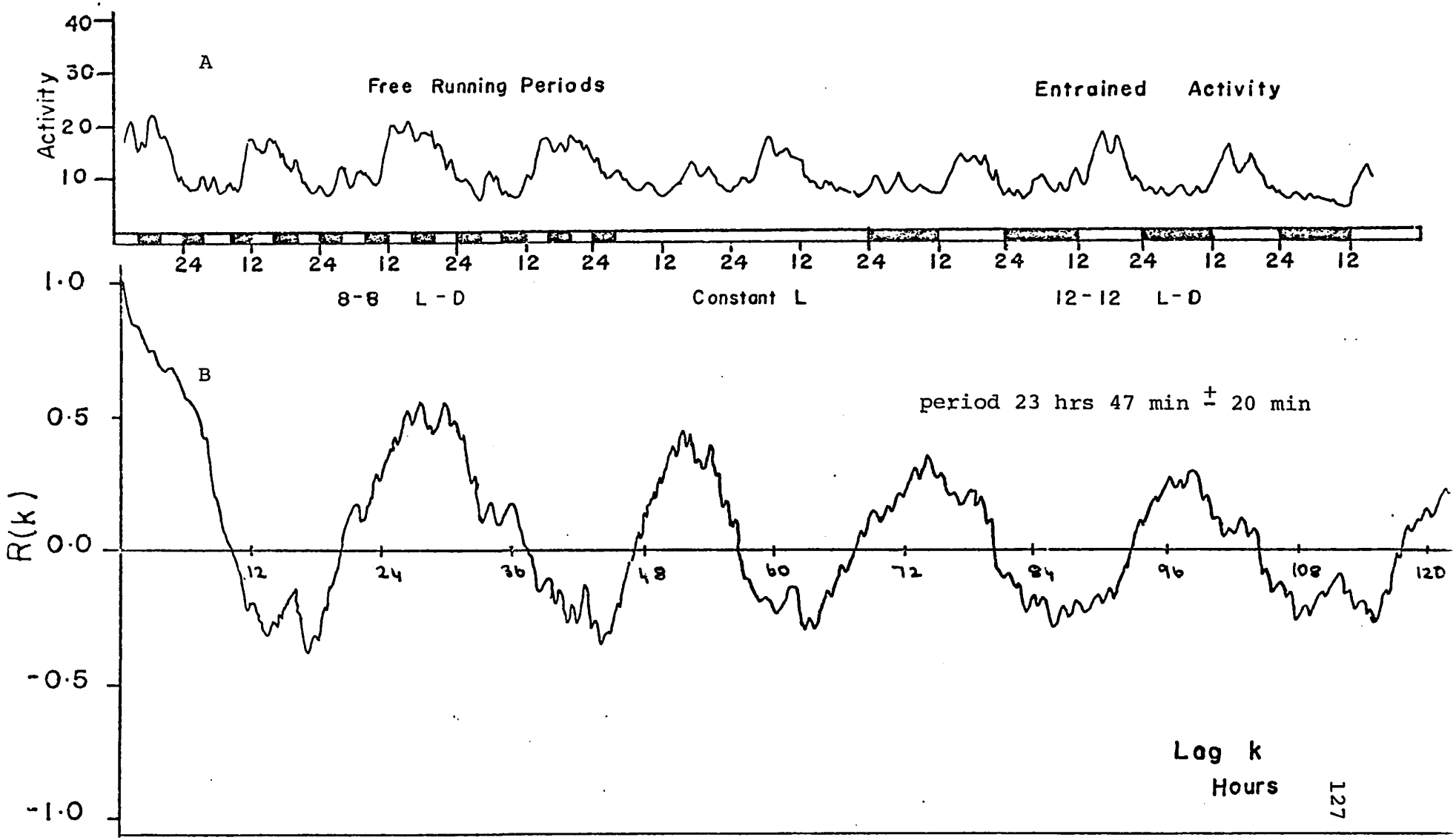

A
Activity
40
30
20
10
24 12 24 12 24 12 24 12 24 12 24 12 24 12 24 12 24 12 24 12
16-16 L-D
Constant D
B
period 23 hrs 50 min ± 18 min
r(k)
1·0
0·5
0·0
-0·5
-1·0
12 24 36 48 60 72 84 96 108 120
Log k
Hours

Figure 35: A Locomotory activity record of single fish exposed to 8-8 L-D followed by constant light, and then 12-12 L-D.

B Autocorrelation plot of the 8-8 L-D portion of the above record.


A
Free Running Periods
Entrained Activity
Activity
40
30
20
10
24 12 24 12 24 12 24 12 24 12 24 12 24 12 24 12 24 12
8-8 L-D
Constant L
12-12 L-D
B
period 23 hrs 47 min ± 20 min
R(k)
1·0
0·5
0·0
-0·5
-1·0
12
24
36
48
60
72
84
96
108
120
Lag k
Hours

Effect of Illumination Intensity on Endogenous Rhythm of Activity

Under constant conditions the length of the free-running period of activity was dependent on the intensity of illumination (Figs. 36,37).

Under a high L intensity (200 Lux) the period, 23 hrs 20 min $\pm$ 15 mins was significantly ($p < 0.001$) shorter than the period of 24 hours 20 minutes $\pm$ 15 mins found under constant dark (Figs. 36,37). All period lengths that were determined are listed in Table 10.

The total amount of activity, per 24 hr interval, as determined by the total number of peaks, was less under constant D than L. This difference was not statistically significant ($p > 0.05$). These results reflect a partial compliance to Aschoff's Rule by solitary fish.

The smoother, more acute correlograms, and sharper peaks in the power spectra obtained at higher L intensities indicate a less variable period length and slower fade out than those occurring at lower light intensities. The amplitude of maximum activity decreased more rapidly and approached a lower basal level under constant Dark than under constant Light (Fig. 38).

These activity decreases may reflect the fact that the fish were not fed during their testing periods. They may have minimized their metabolic expenditures through a reduction of locomotor activity.

Figure 36: Locomotory activity records of single fish exposed to 12-12 L-D followed by constant light at:

A) 200 Lux - 23 hrs 20 min $\pm$ 18 min
B) 100 Lux - 23 hrs 45 min $\pm$ 19 min
C) 50 Lux - 23 hrs 50 min $\pm$ 25 min

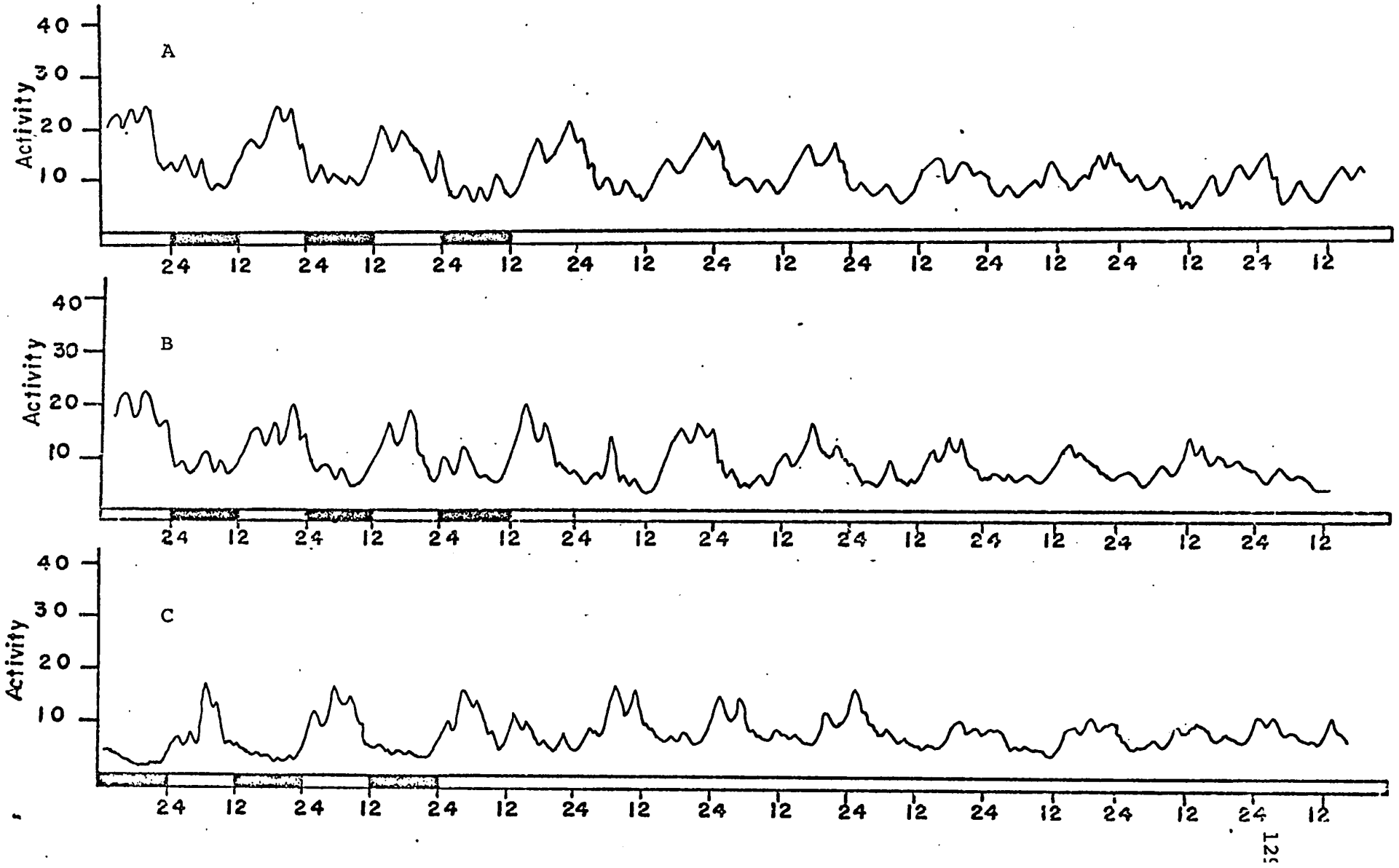

A
B
C
Activity
40
30
20
10
24
12

Figure 36(D) Autocorrelation plots of the constant light portions of the preceding records: 200 Lux - 23 hrs 20 min ± 18 min _____
100 Lux - 23 hrs 45 min ± 19 min ————
50 Lux - 23 hrs 50 min ± 25 min .....

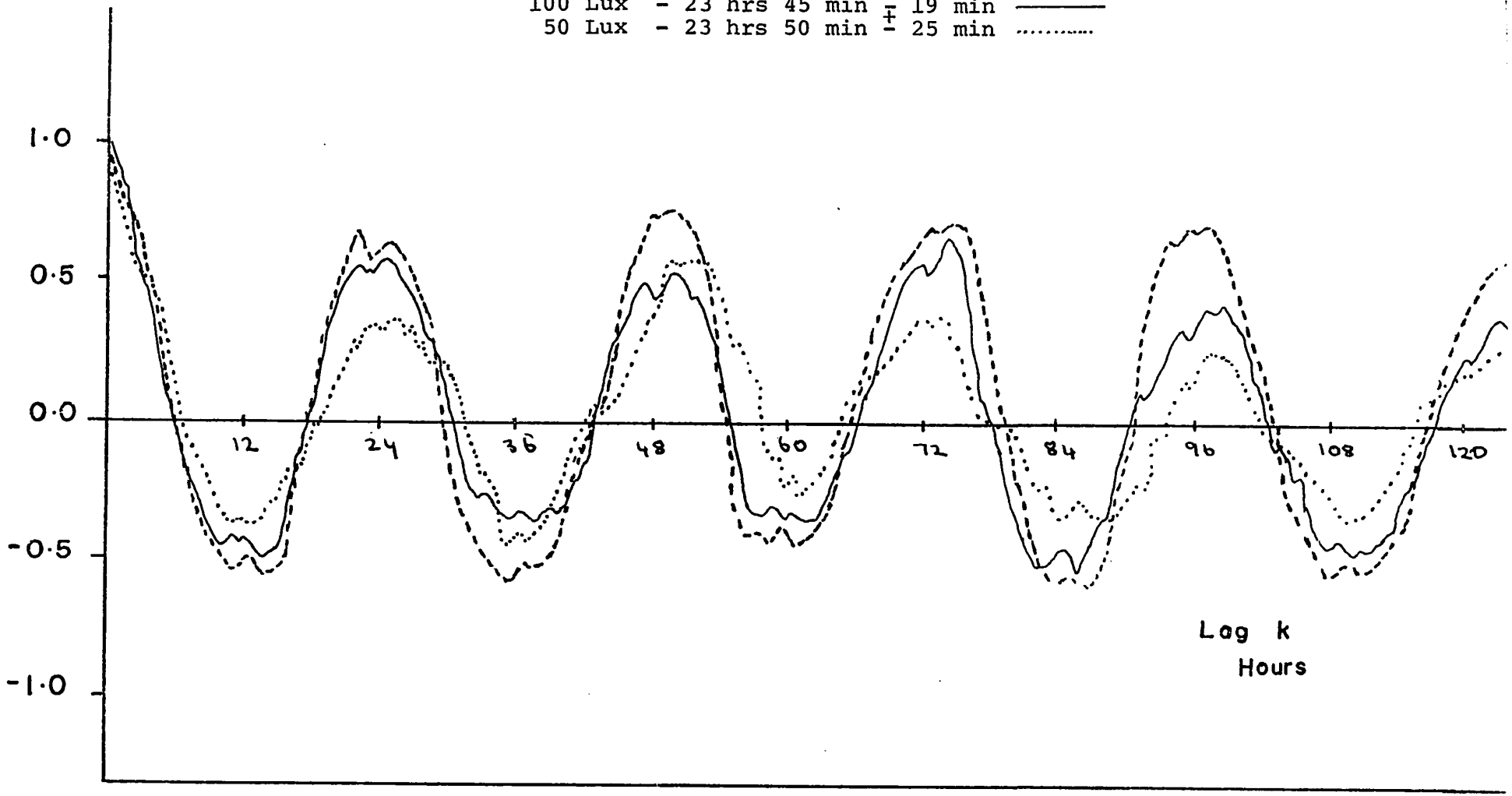
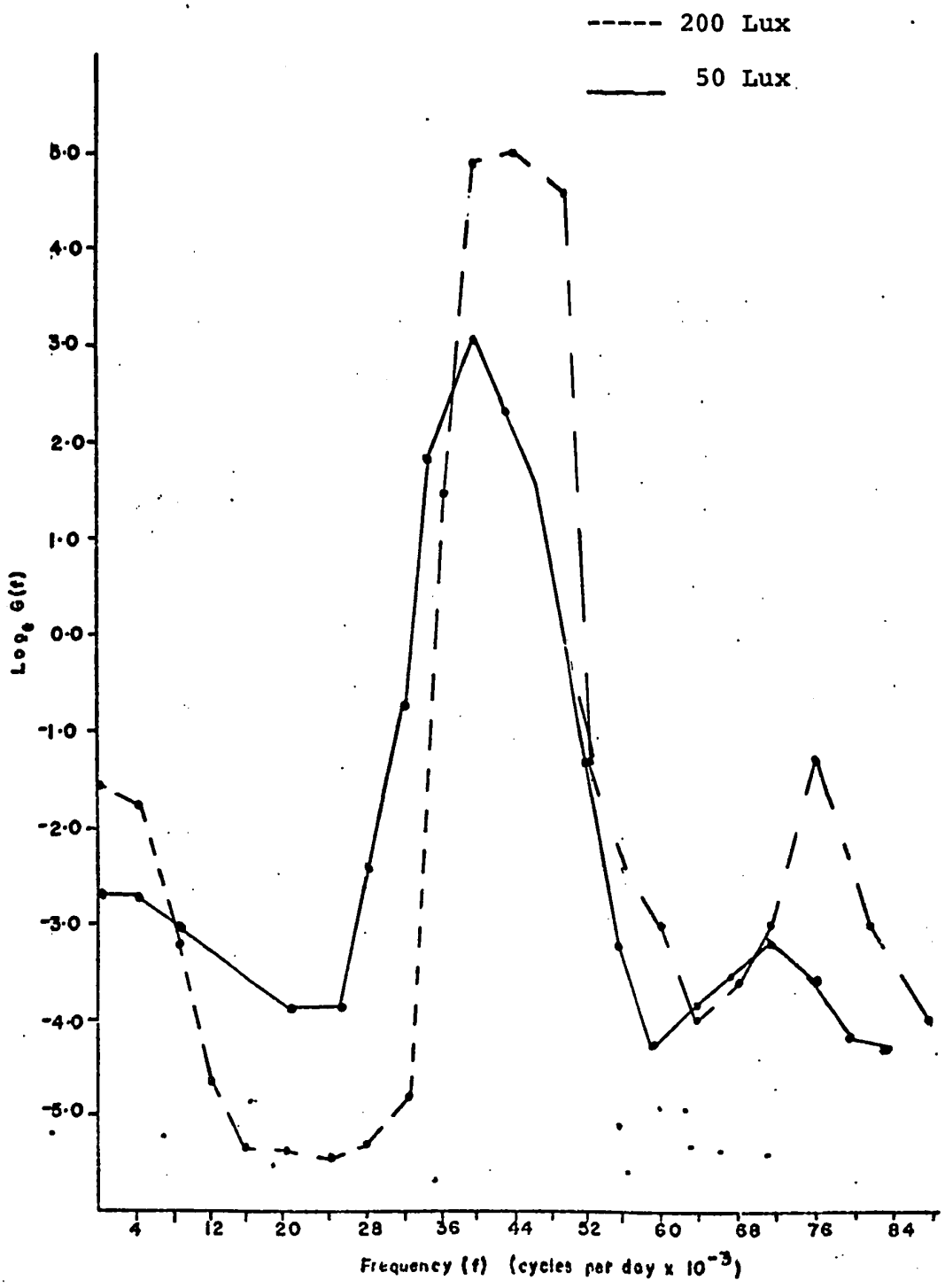


Figure 36E: Power spectrum transformation of autocorrelation functions.



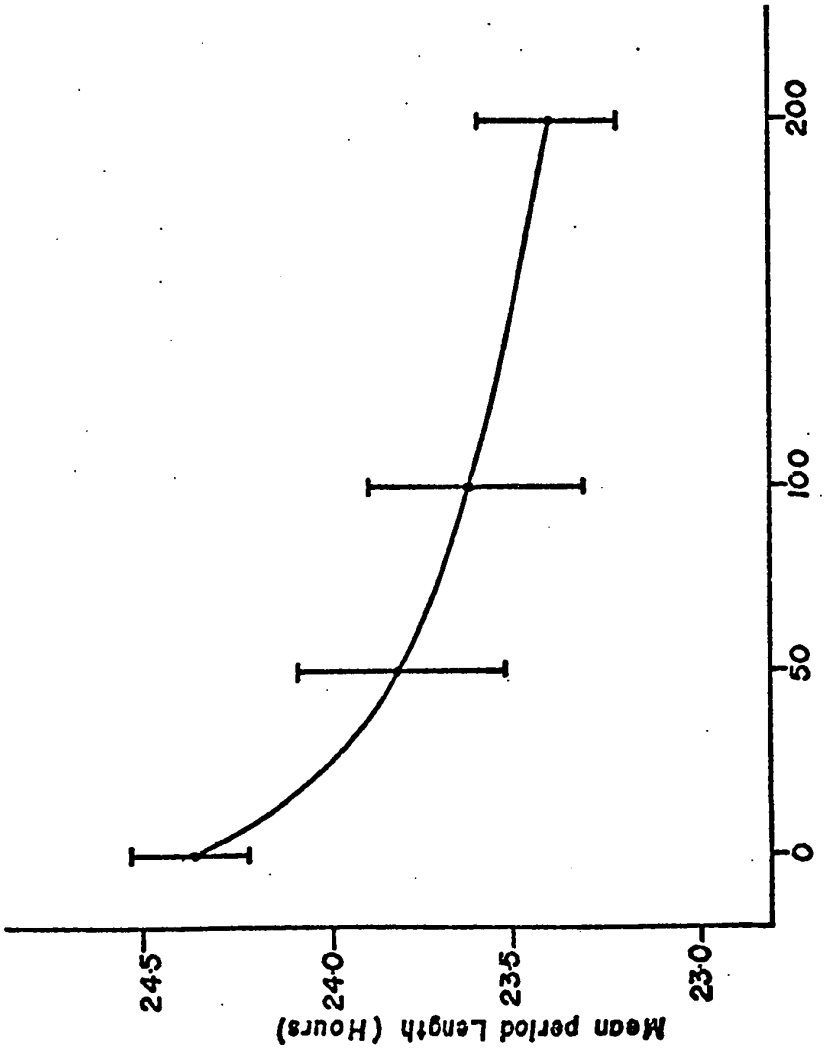
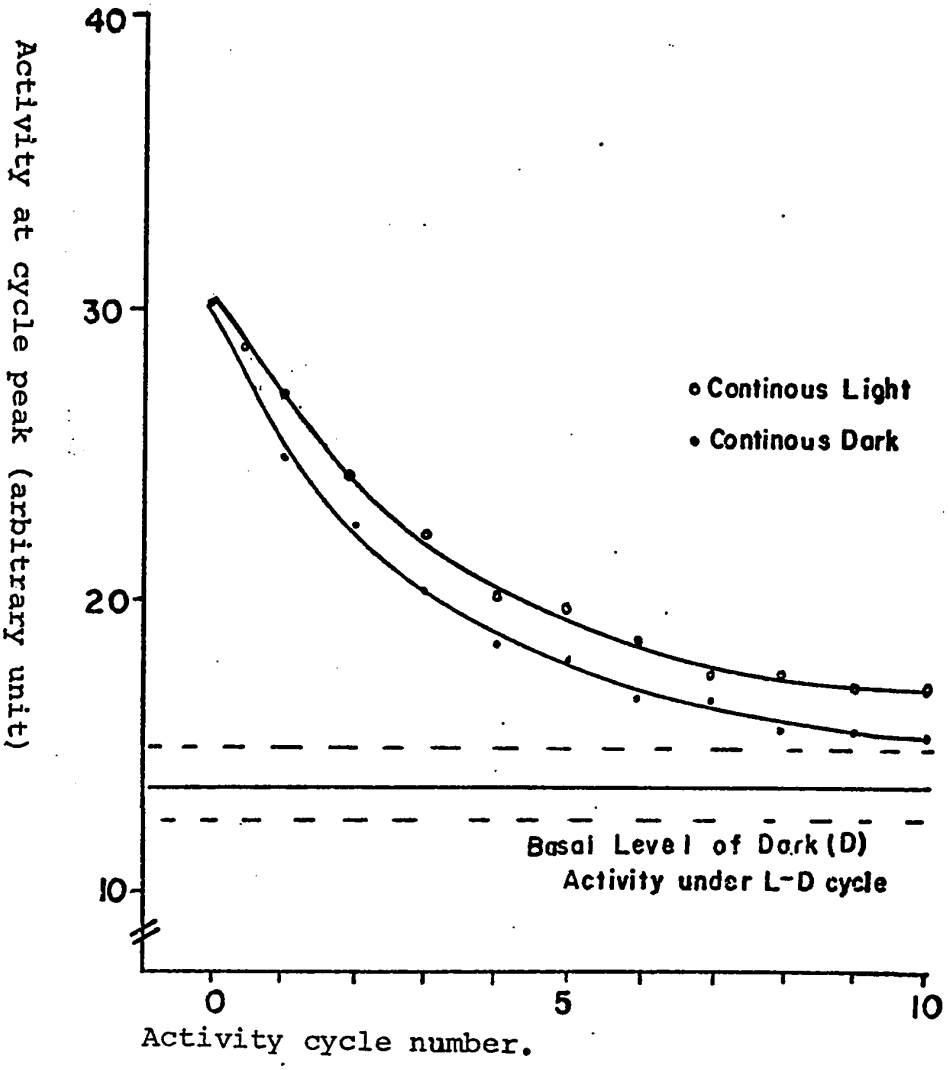


Figure 37: Relationship between free-running activity period and intensity of constant illumination (Lux).

The vertical lines represent 95% confidence interval of the period estimate as determined from the linear regression plot.

Figure 38: Relationship between activity amplitude at cycle peak (arbitrary units) and cycle number.

Dashed lines represent 95% confidence interval of basal activity.



Effect of Temperature and Salinity on Activity

The free-running and photoperiodically entrained rhythms of locomotory activity of killifish were unaffected by different temperatures and salinities. The period lengths (Table 10) and entrainment curves (Figs. 26-36). for the different temperatures (10-25°C) and salinity (0.35‰) combinations employed were essentially similar. Low temperature (10°C) decreased the amplitude and extent of activity recorded. These reductions were not consistent, varying between fish.

The role of exogenous thermosaline changes as Zeitgebers for locomotory activity could not be adequately studied because of the relatively large tanks and small fish reduced the sensitivity of the ultrasonic detecting system. In short term determinations salinity changes did not affect activity, while temperature changes slightly altered activity in the normal physiological manner, without affecting phasing. These changes were imposed on the fish over periods of 30-60 minutes, minimizing shock induced activity alterations.

Entrainment and testing under high temperatures [20-25°C and extended photoperiods (12-14 hrs L)] stimulated sexual development of the fish. This was evidenced by a heightened colouration and increased restlessness and activity. Sexual effects, and male and female differences in activity, were not considered in analysing the activity results, but undoubtedly contributed to the variation between fish.

Effect of Group Size on Activity

The activity of killifish was studied with single fish and groups of 5 and 20. Solitary fish displayed a distinct endogenous rhythm of activity that was readily synchronized by photoperiod. The period of the free-running rhythm, which was dependent on the intensity of constant illumination, showed little variation between fish (Figs 26-36 and Table 10), and a slow fade-out time.

The group of 5 fish dispersed throughout the tank and formed a loose aggregation. Spacing between the fish was not uniform. There were individual differences in orientation and behaviour that did not appear to be over ridden by any group behaviour except when the fish were frightened or startled.

Activity was photoperiodically entrainable (Figs. 38,39) with an endogenous rhythm of activity being displayed in constant conditions (Figs. 39,40). The period, 23 hrs 35 mins $\pm$ 45 mins (100 Lux), was more variable than that determined for solitary fish. This is also shown by the more irregular correlograms and broader peaks of less power in the spectrums (Figs. 39C,D).

The group of 20 fish were dispersed through the tank forming a group that functioned as a single unit, i.e. a school. Activity was photoperiodically entrained (Figs. 39,40) with a distinct free running rhythm apparent

under constant conditions.

In contrast to the smaller group of 5, this larger group had a more uniform correlogram with more acute, clearly defined turning points, and therefore less variable period length, 23 hrs 20 min $\pm$ 20 min (100 Lux). The spectra had sharper peak also indicating less variance in the period length (Fig. 39C).

The period length of the endogenous activity rhythm of the two groups was not influenced by the intensity of illumination (Fig. 40). This suggests that the activity of the two groups is not the summation of the activity of individual fish.

The total activity, per 24 hour interval, of a group member appeard to be less than that of a solitary fish. This may only reflect the reduced volume available to the fish. However, there was no significant difference in the activity of members of the two groups.

Figure 39A,B: Locomotory activity records of groups of fish (5 and 20 per group) exposed to 12-12 L-D followed by constant dark.
A) 20°C 40 °/oo
B) 10°C F.W.

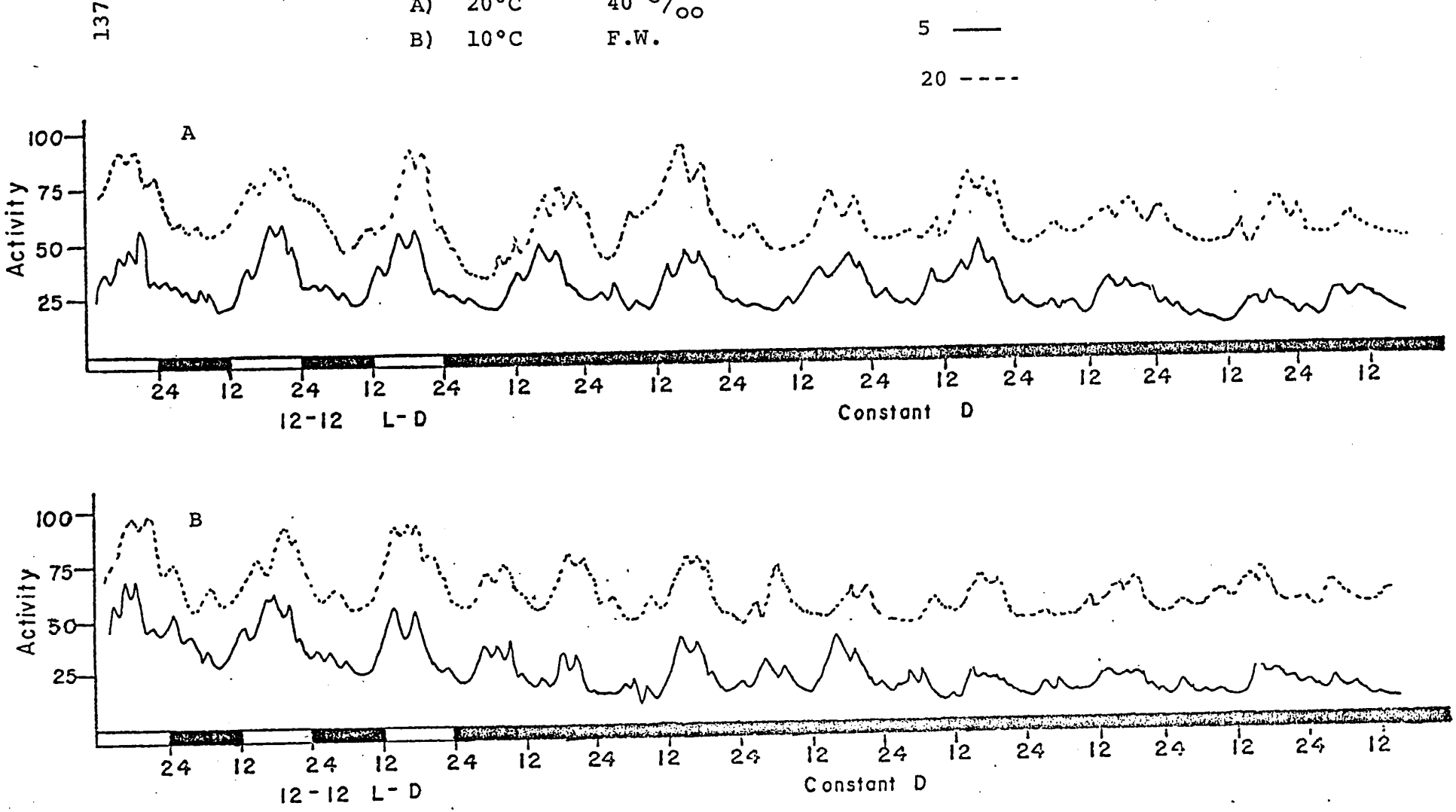


Figure 39(C): Autocorrelation plot of the dark portion of group activity records 20°C, 40°/oo.

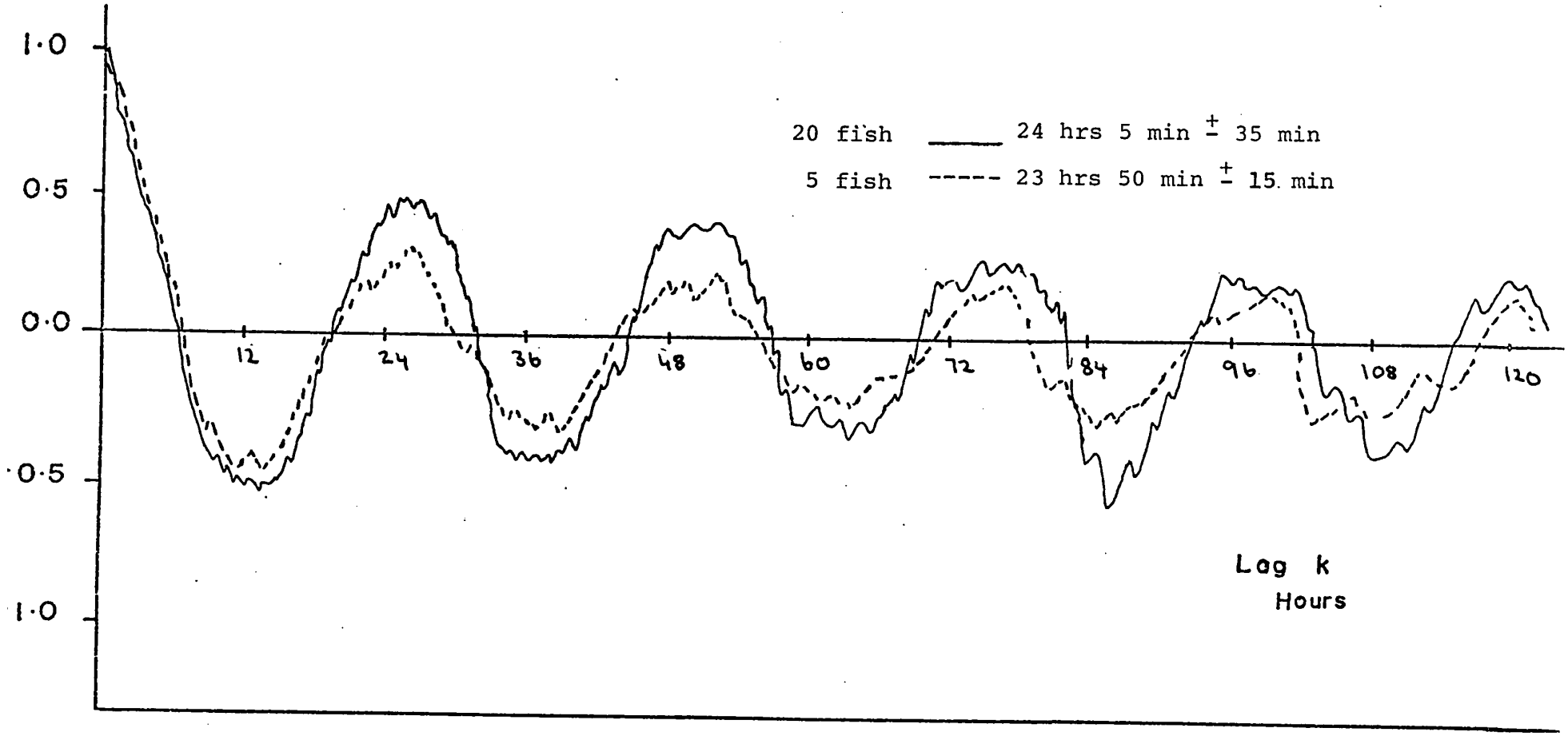


Figure 39 D: Power spectrum transformation of auto-correlation function.

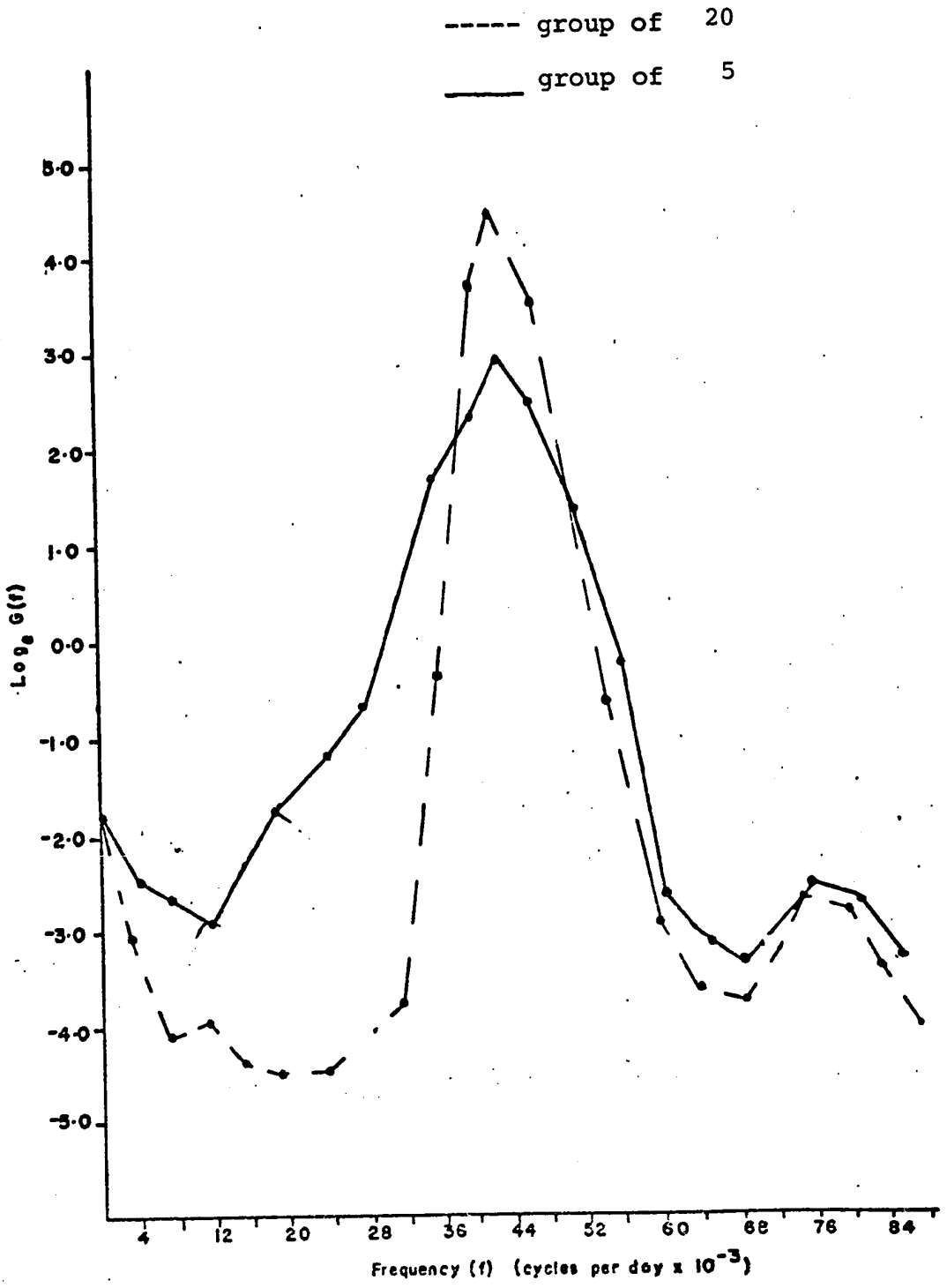


Figure 40(A,B,C): Locomotory activity records of groups of fish (5 and 20 per group) exposed to 12-12 L-D followed by constant light.
A) 200 Lux
B) 100 Lux
C) 50 Lux.

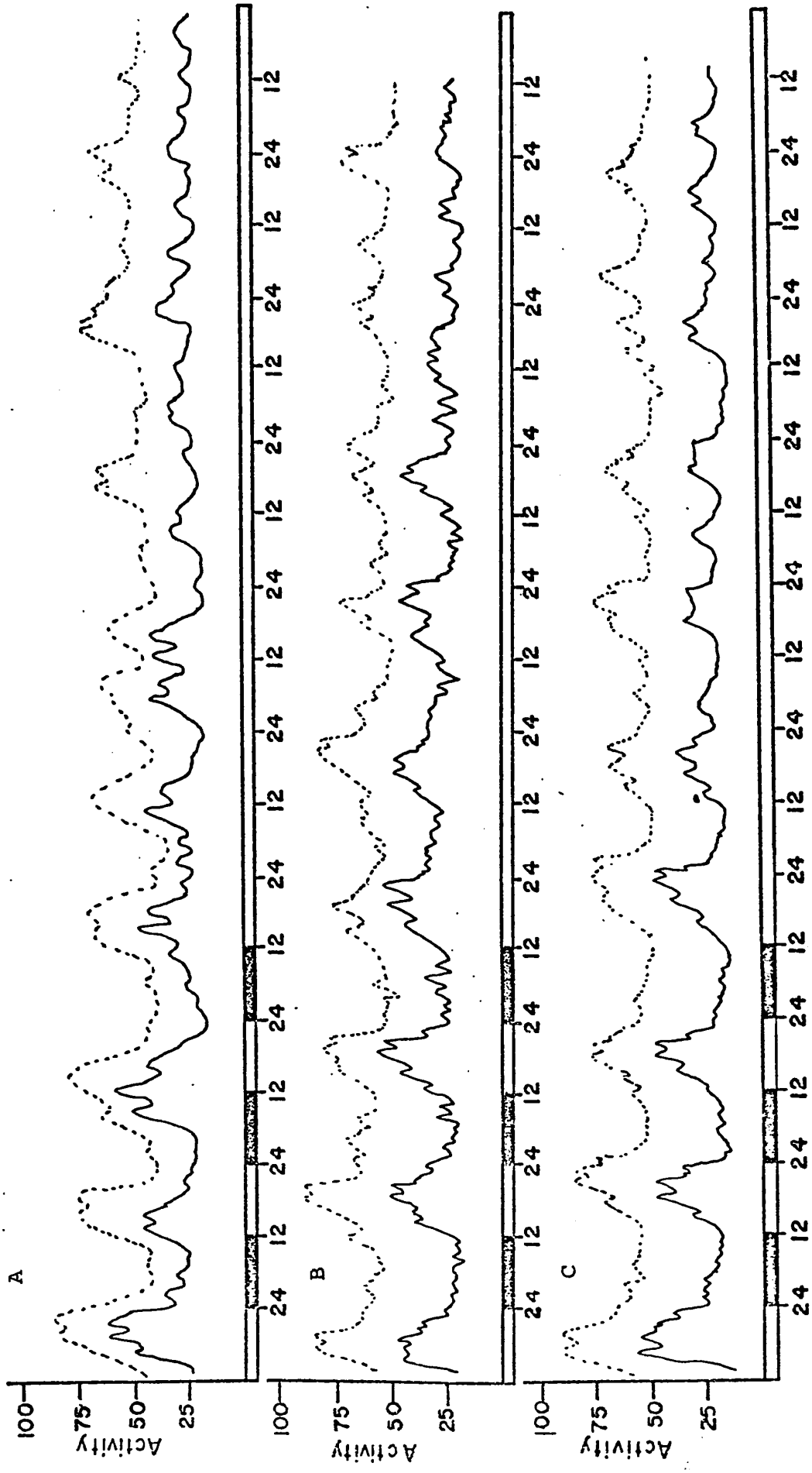

A
B
C
Activity
100
75
50
25
12
24

Figure 40(D): Autocorrelation plot of the constant light portion (200 Lux) of the preceding activity records .

----- group of 5 fish

_____ group of 20 fish

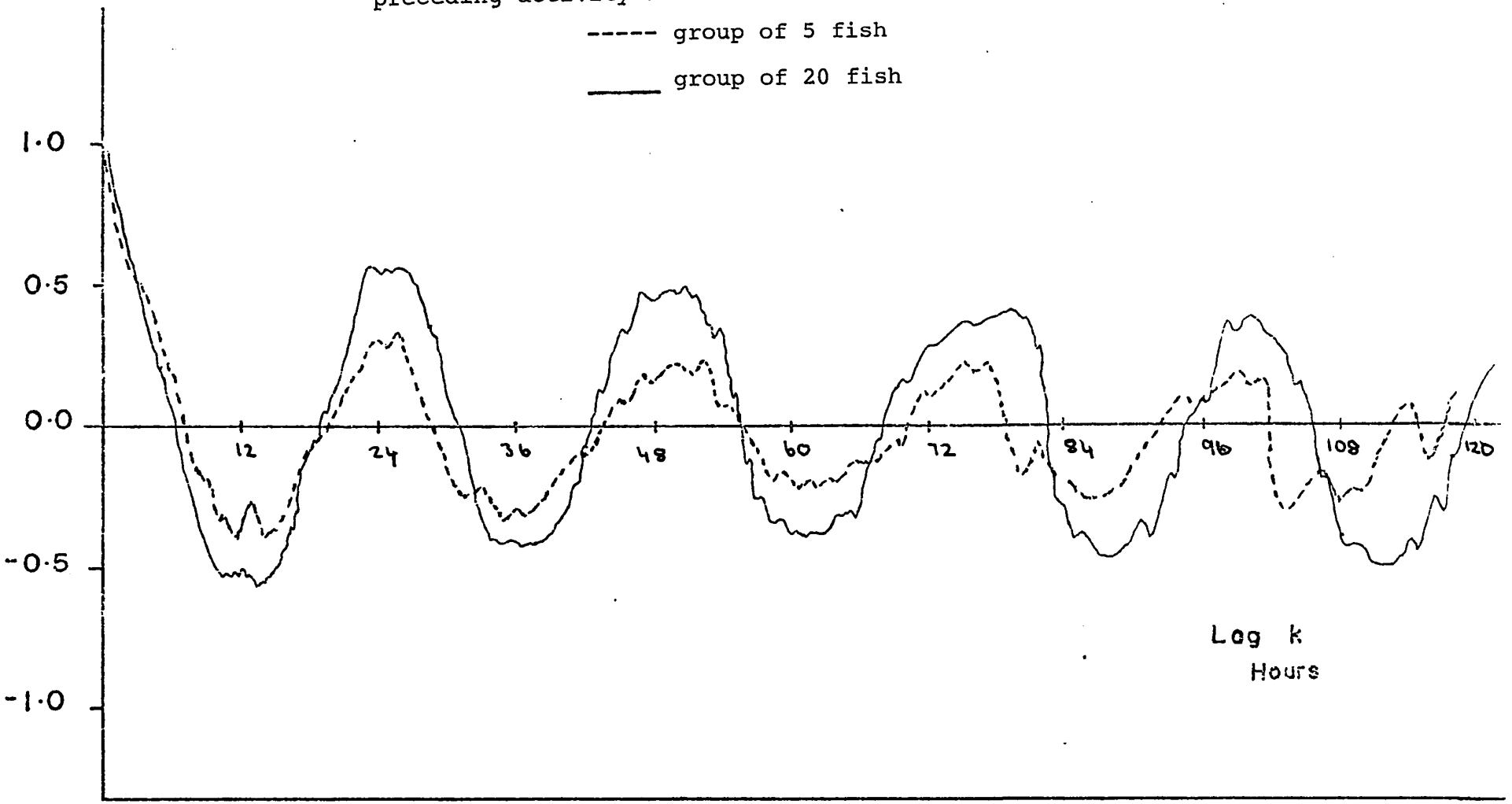
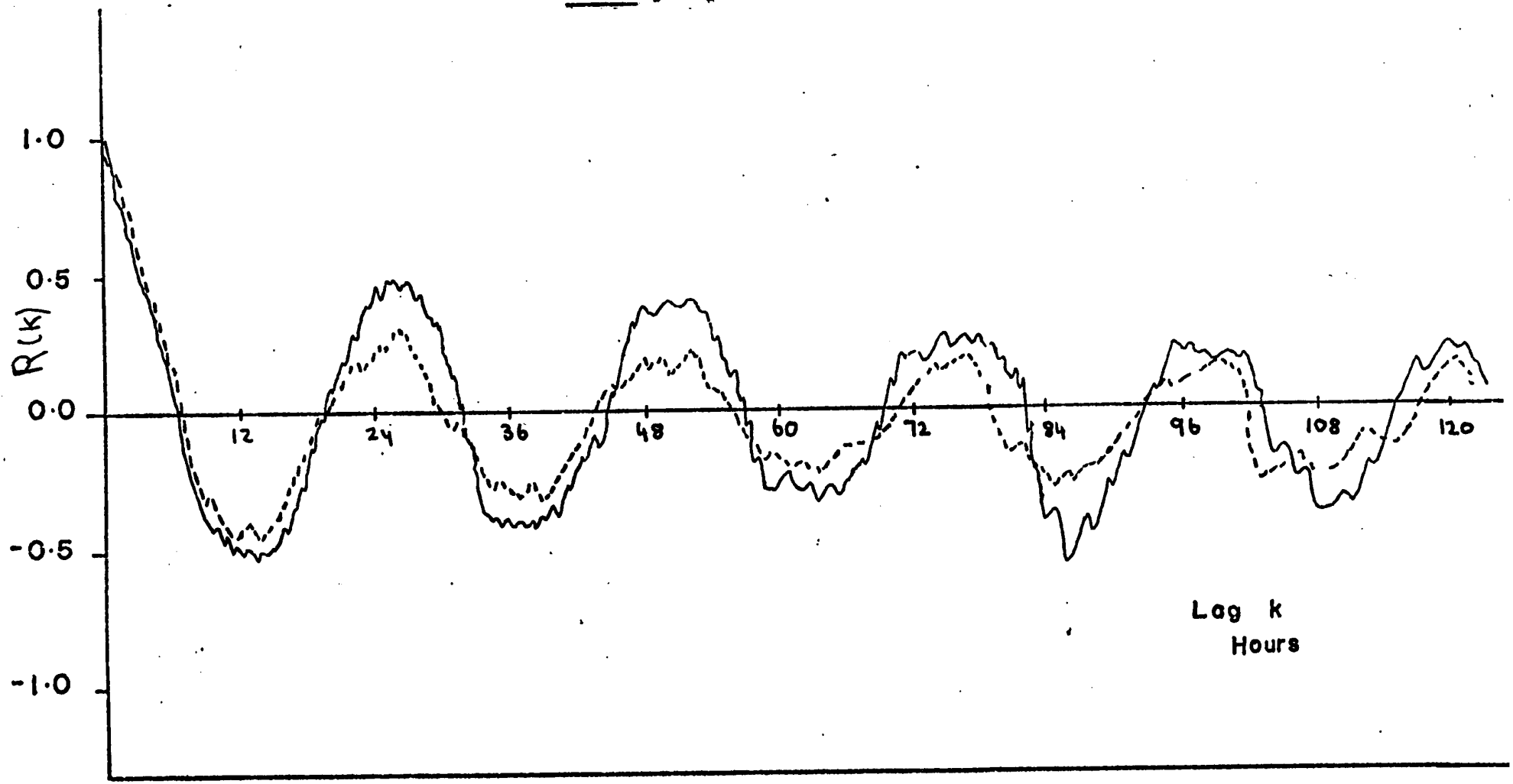


Figure 40(E): Autocorrelation plot of the constant light portion (50 Lux) of the preceding activity record.

----- group of 5 fish

group of 20 fish



Effect of Blinding on the Endogenous Rhythm of Activity

Immediately after bilateral blinding, locomotory activity of killifish was markedly reduced. After several hours their activity increased to a much lower basal level (Figs. 41, 42) than previously.

Blinded killifish still displayed an endogenous rhythm of activity with unaltered phasing (Figs. 41, 42).

The precision and amplitude of the endogenous rhythm was reduced. This reduction is shown by the variable correlograms, and obtuse power spectra. Asynchronous activity tended to occur as the endogenous rhythm faded out, obscuring the free running pattern (Fig 41C). The period length, 23 hrs $\pm$ 38 min $\pm$ 40 min was relatively unaffected by the intensity of constant illumination.

L-D cycles were still able to entrain activity in blinded fish, though the time required was slightly longer than for intact fish (Fig. 42).

Figure 41(A,B): Locomotory activity record of fish exposed to 12-12 L-D, blinded and then exposed to constant
A) constant light 200 Lux
B) constant light 100 Lux

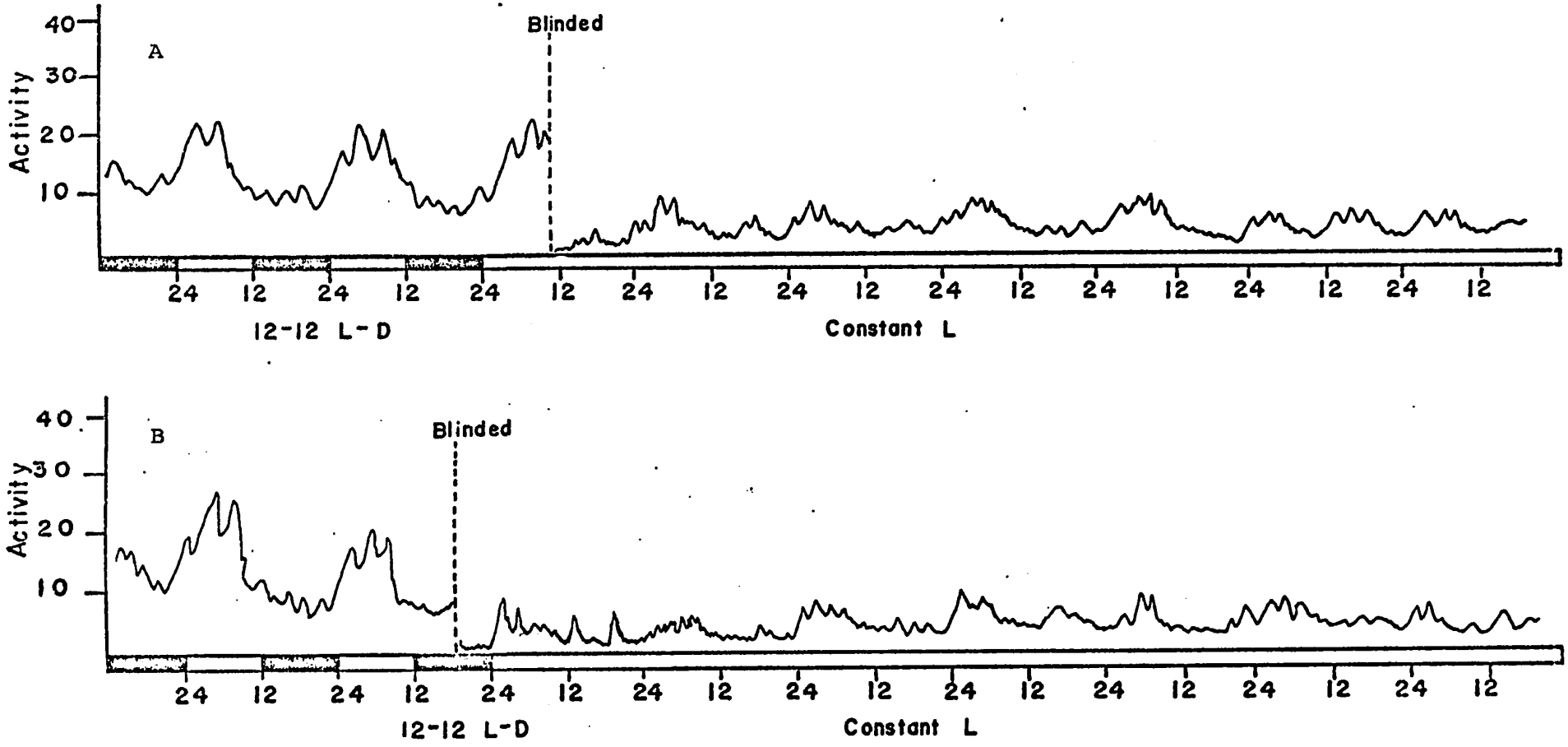

A
Blinded
Activity
40
30
20
10
24 12 24 12 24 12 24 12 24 12 24 12 24 12 24 12 24 12 24 12
12-12 L-D
Constant L
B
Blinded
Activity
40
30
20
10
24 12 24 12 24 12 24 12 24 12 24 12 24 12 24 12 24 12 24 12
12-12 L-D
Constant L

Figure 41(C): Locomotory activity record of fish exposed to 12-12 L-D, blinded and then exposed to constant dark.

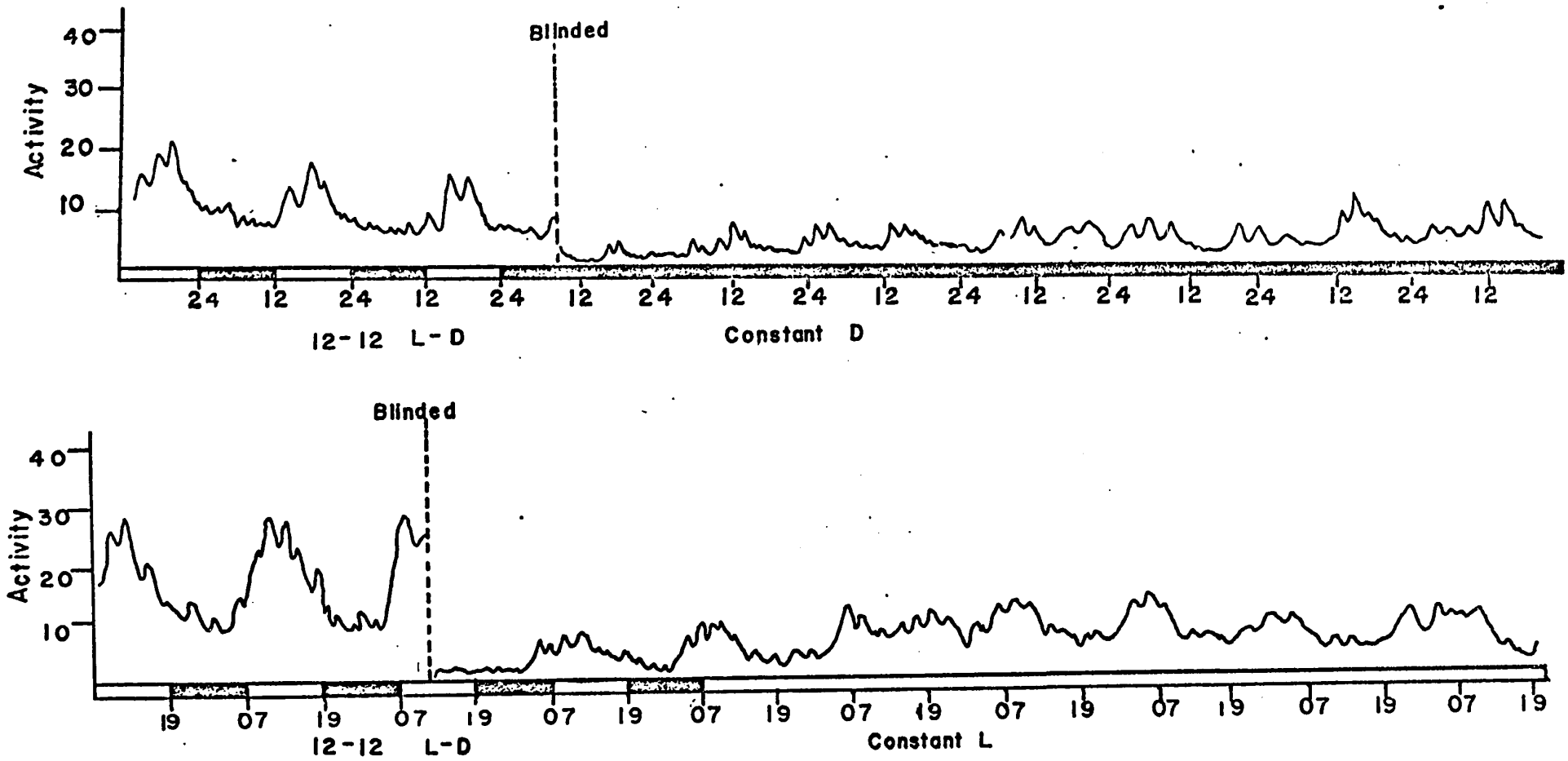


Figure 41(D): Autocorrelation plot of activity of blinded fish under constant D and constant L (200 Lux).

----- D period 23 hrs 41 min ± 30 min

_____ L period 23 hrs 35 min ± 27 min

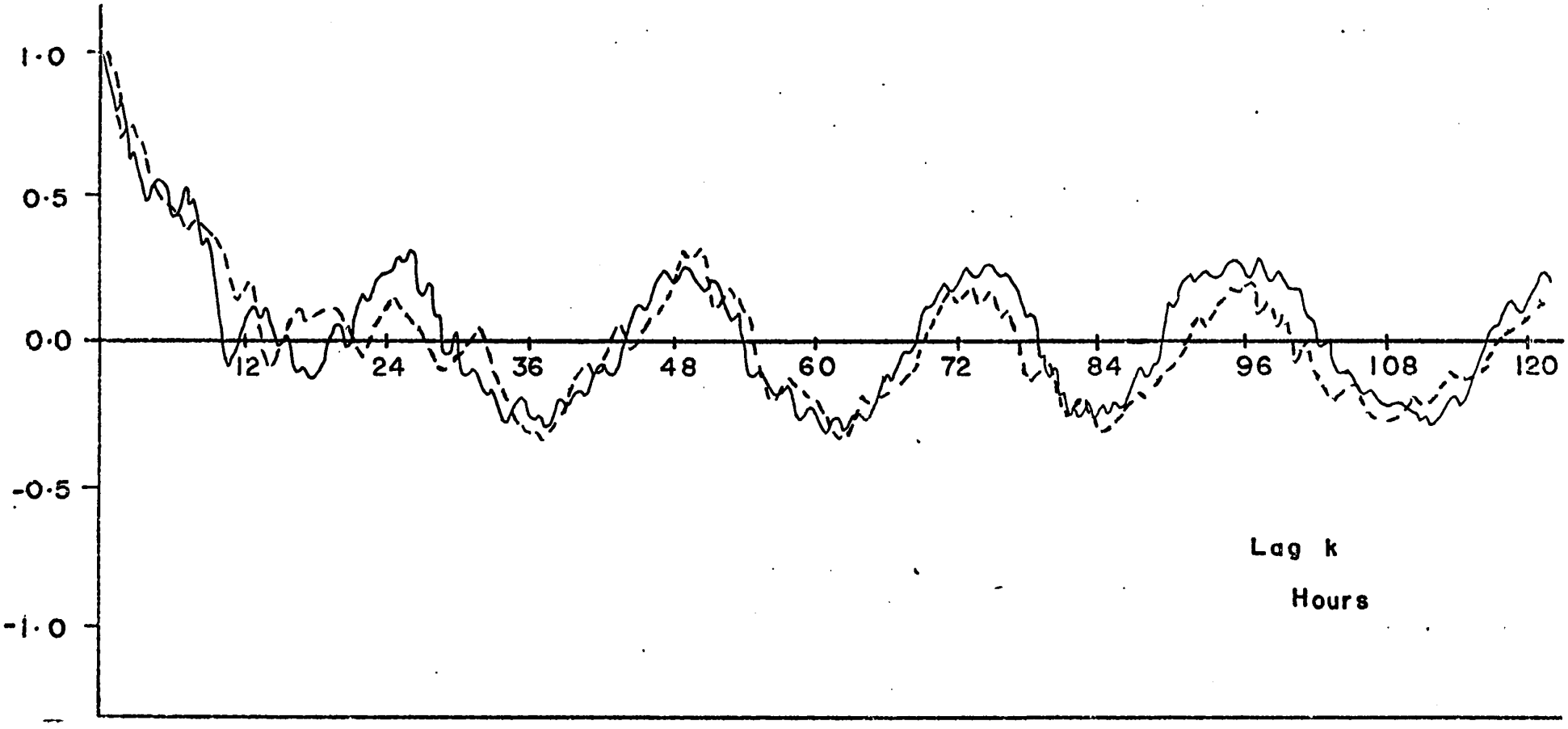
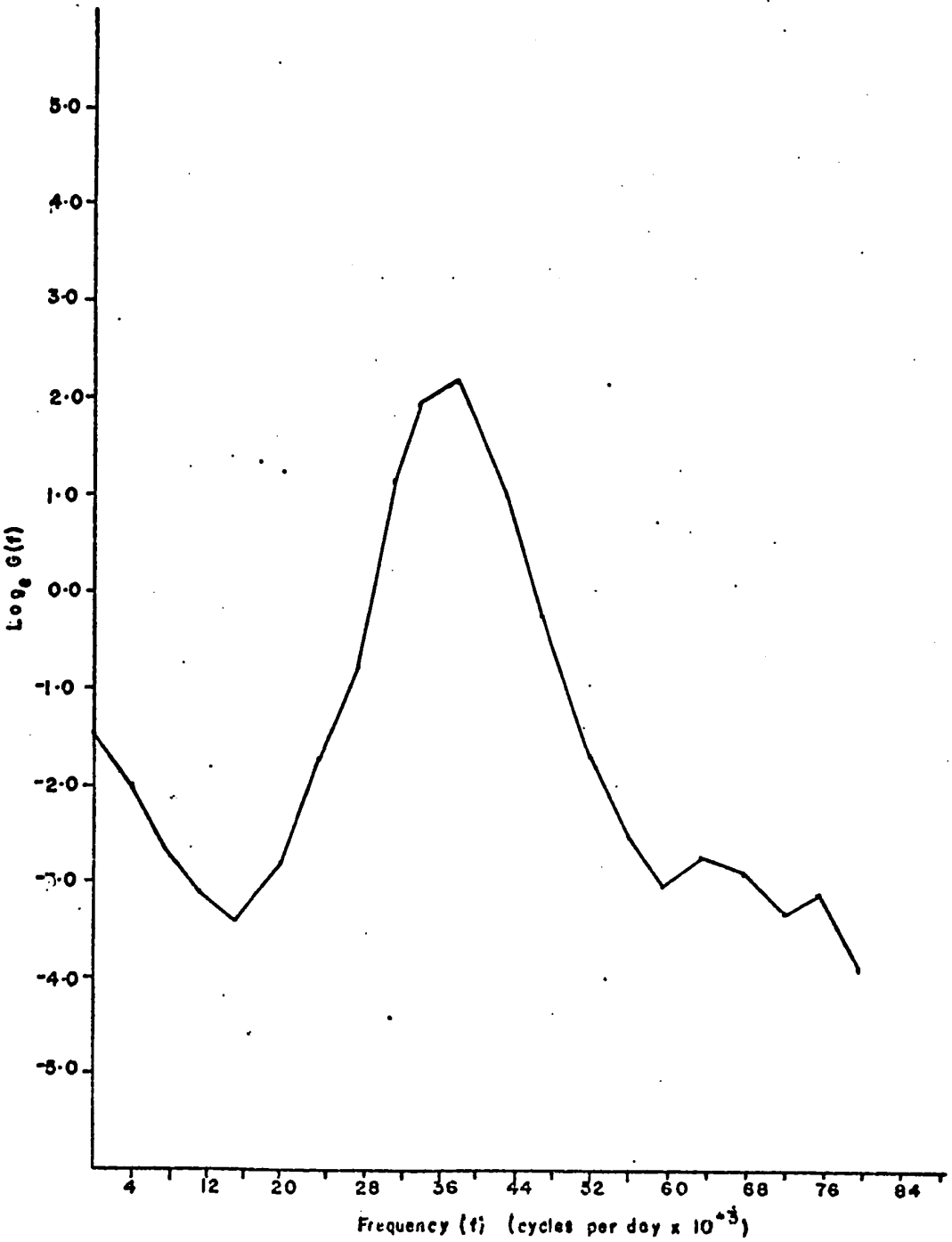


Figure 41E: Power spectrum transformation of auto-correlation function.



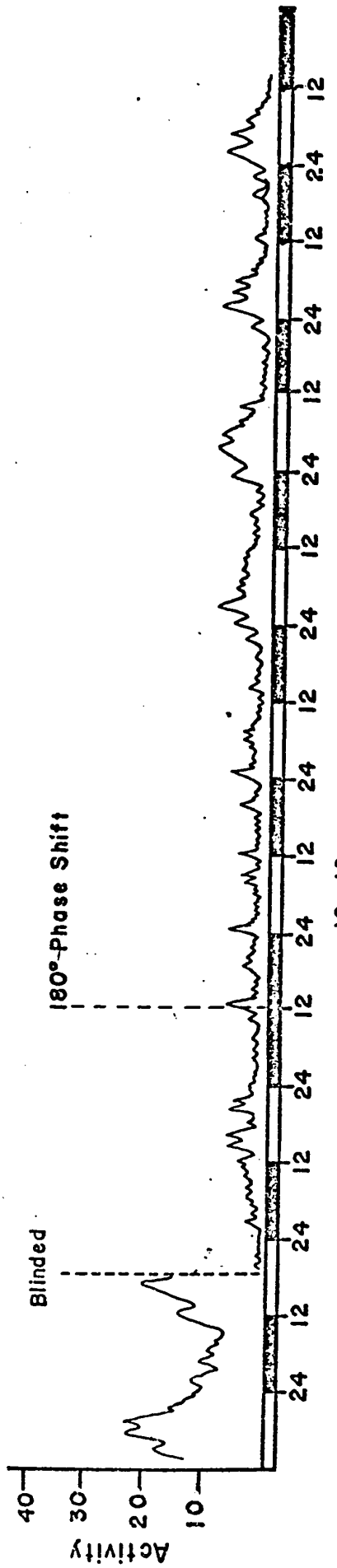


Figure 42: Locomotory activity record of blinded fish exposed to 12-12 L-D followed by 180° phase-shift.

Effect of Hypophysectomy on the Endogenous Rhythm of Activity

After hypophysectomy the locomotry activity of killifish was markedly reduced increasing afterwards to a lower basal level than previously under constant conditions. The endogenous rhythm faded out rhough 1-2 cycles after hypophysectomy. The elimination of the endogenous rhythm is shown in the correlograms, which oscillate around R=O, and their power spectra displaying no significant variance at any frequency. Sham hypophysectomy did not significantly alter the activity rhythm (Figs. 43, 44, 45).

Hypophysectomized killifish could have their activity entrained and phase shifted by different L-D cycles. The entrainment range and responses appeared to be similar to those displayed by intact fish. However, time required for entrainment was increased. After imposition of constant conditions, there was in hypophysectomized killifish, a rapid extinction of rhythmic activity (Fig. 46).

Figure 43(A,B): Locomotory activity of single fish exposed to 12-12 L-D A) hypophysectomized
B) sham-hypophysectomized
and then exposed to constant light.

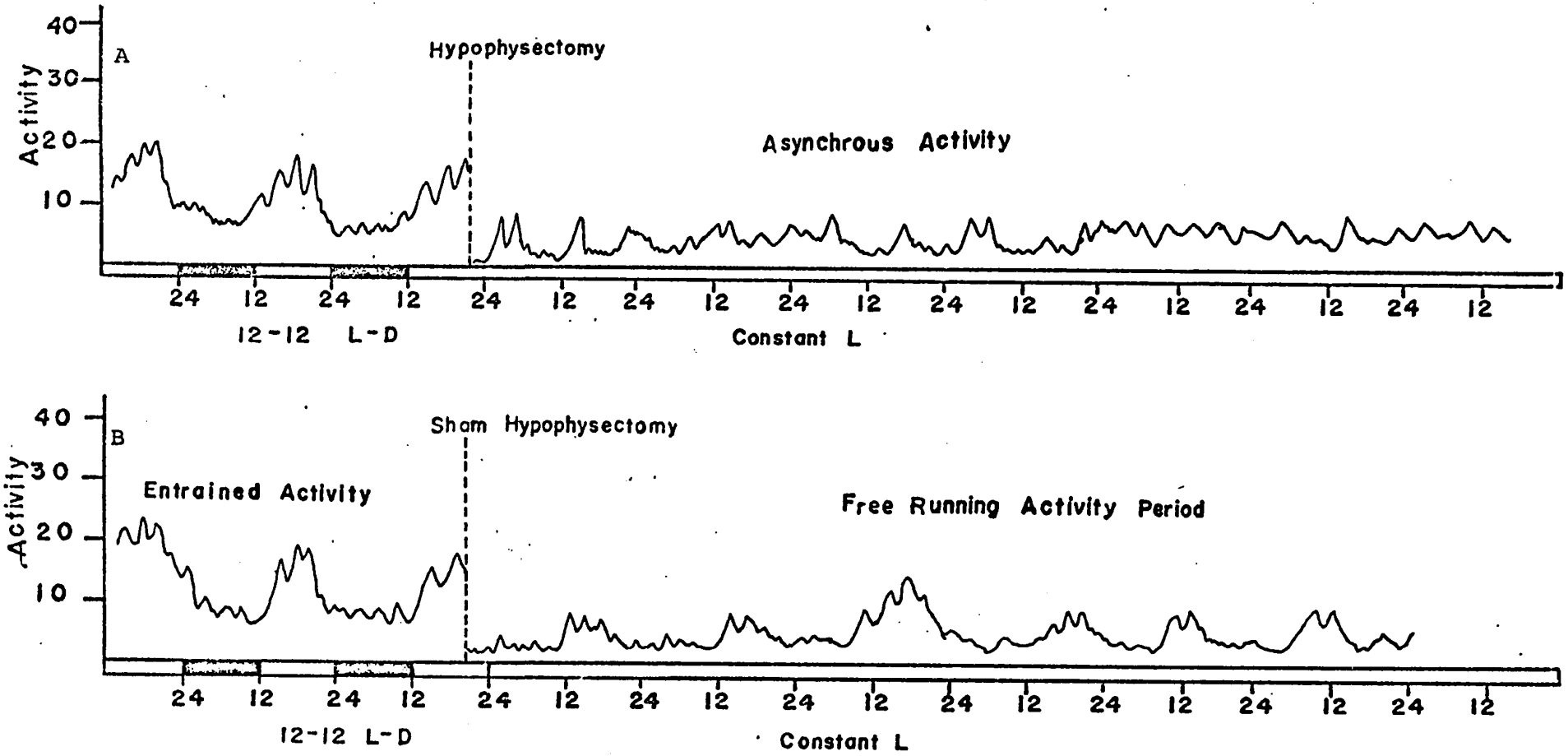

A
Activity
40
30
20
10
Hypophysectomy
Asynchrous Activity
24 12 24 12 24 12 24 12 24 12 24 12 24 12 24 12 24 12
12-12 L-D
Constant L
B
Activity
40
30
20
10
Sham Hypophysectomy
Entrained Activity
Free Running Activity Period
24 12 24 12 24 12 24 12 24 12 24 12 24 12 24 12 24 12
12-12 L-D
Constant L

Figure 43(C): Autocorrelation plot of locomotory activity of hypophysectomized and sham hypophysectomized fish.

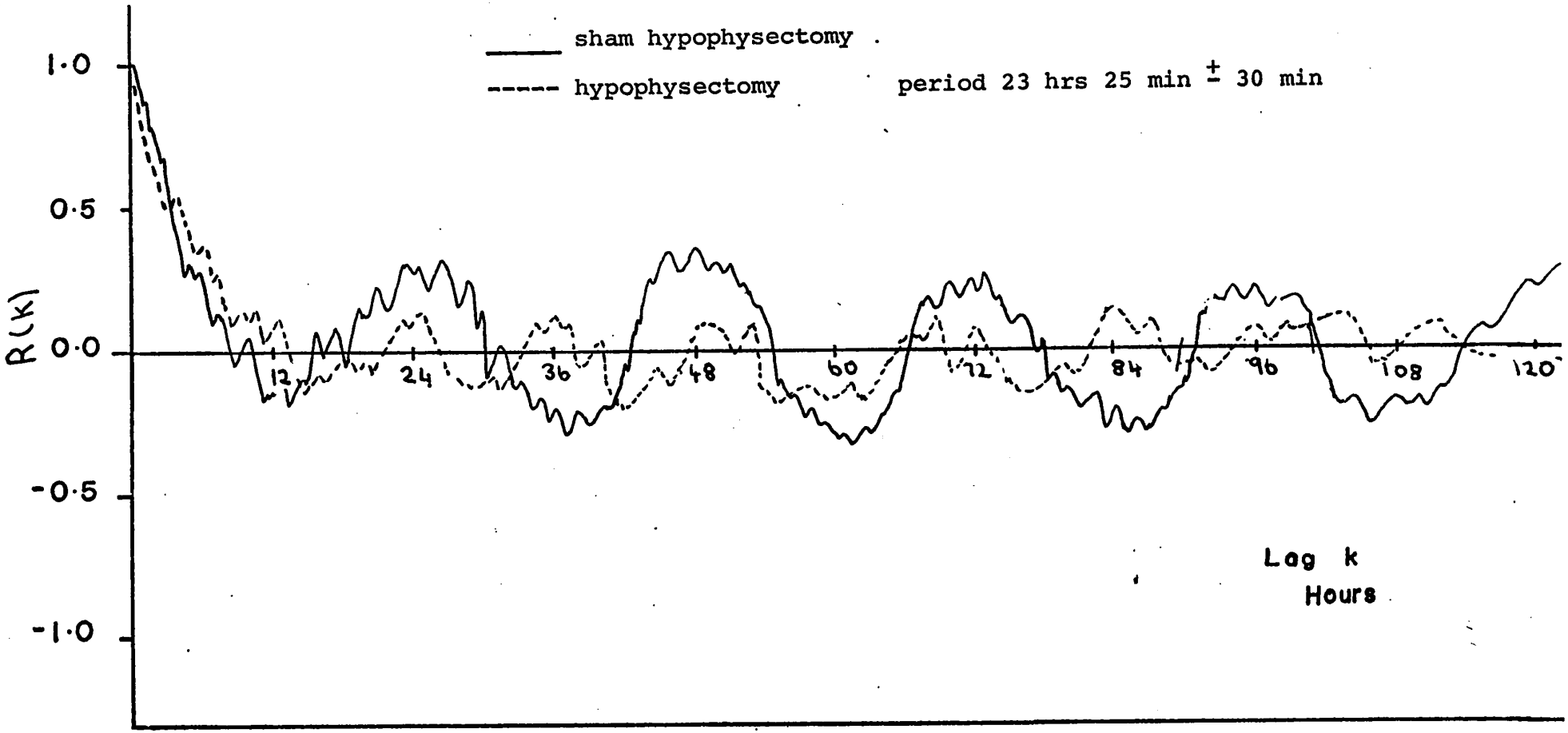


Figure 43D: Power spectrum transformation of autocorrelation functions.

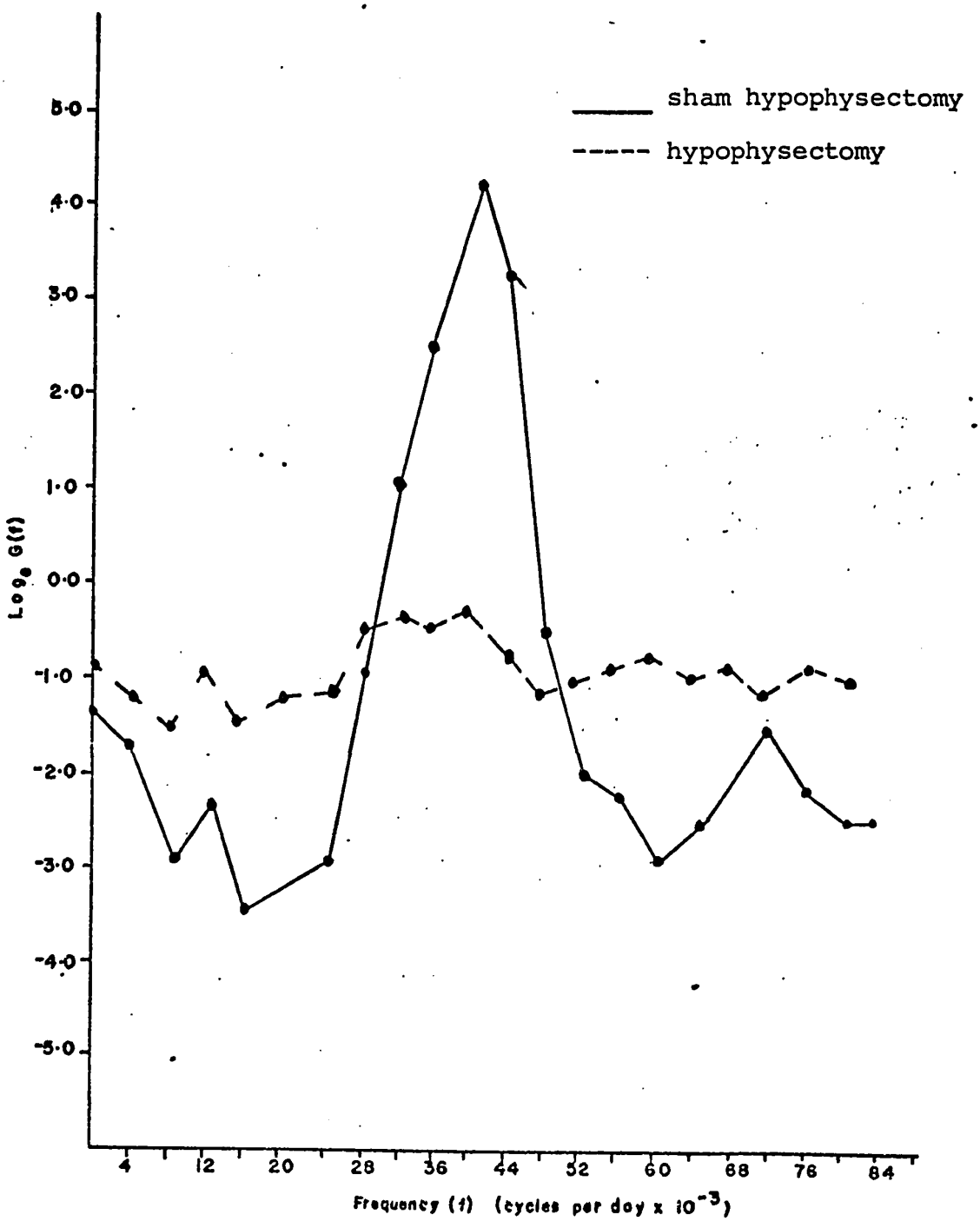


Figure 44: Locomotory activity record of hypophysectomized fish exposed to 12-12 L-D, followed by 180° phase shift and then constant light (100 Lux).

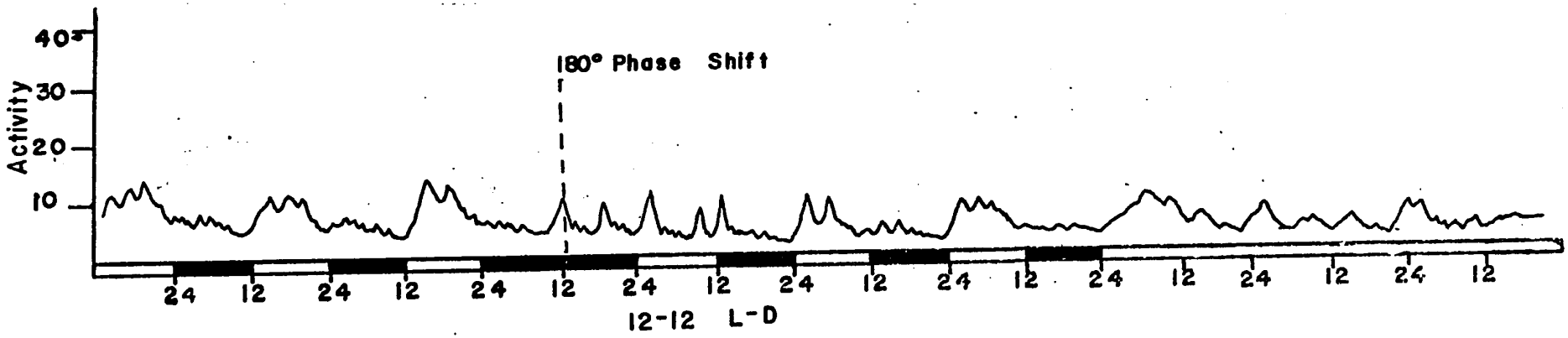


Table 10:

Period-lengh of free-running activity rhythm under different conditions.

Illumination Intensity	Period Length hr. mins ± 95% confidence intensity
Single Fish	
200	23 hrs 27 min ± 26 min
	23 hrs 30 min ± 22 min.
100	23 hrs 37 min ± 18 min
	23 hrs 45 min ± 19 min
50	23 hrs 55 min ± 30 min
	23 hrs 50 min ± 20 min
DD	24 hrs 22 min ± 7 min
	24 hrs 25 min ± 15 min
	24 hrs 21 min ± 13 min
Blinded Fish	
200	23 hrs 20 min ± 20 min
	23 hrs 45 min ± 35 min
DD	23 hrs 50 min ± 40 min
DD	24 hrs 20 min ± 35 min

Hormonal Effects on Locomotory Activity

Control injections of 0.15 N NaCl, the carrier solution used in hormonal studies, had no effect on locomotor activity of fish active during 6-1800 hrs. Locomotory activity was determined for a 24 hour period immediately after injection at 0600, 1200, 1800 or 2400 hours E.S.T. The activity of hormonally treated fish was compared to control and normal fish. Beef pituitary extract (1 and 10 µgm/gm wet body weight of fish) had no significant effect on locomotory activity at any of the injection times (Fig 45).

Mammalian prolactin, at a concentration of 1 µgm/gm wet body weight of fish, increased activity insignificantly when administered at 0600, 1200, or 2400 hrs (p's ≈ 0.05). At 1800 it significantly increased locomotory activity ($p > 0.05$), for a 24 hour period (Fig. 46).

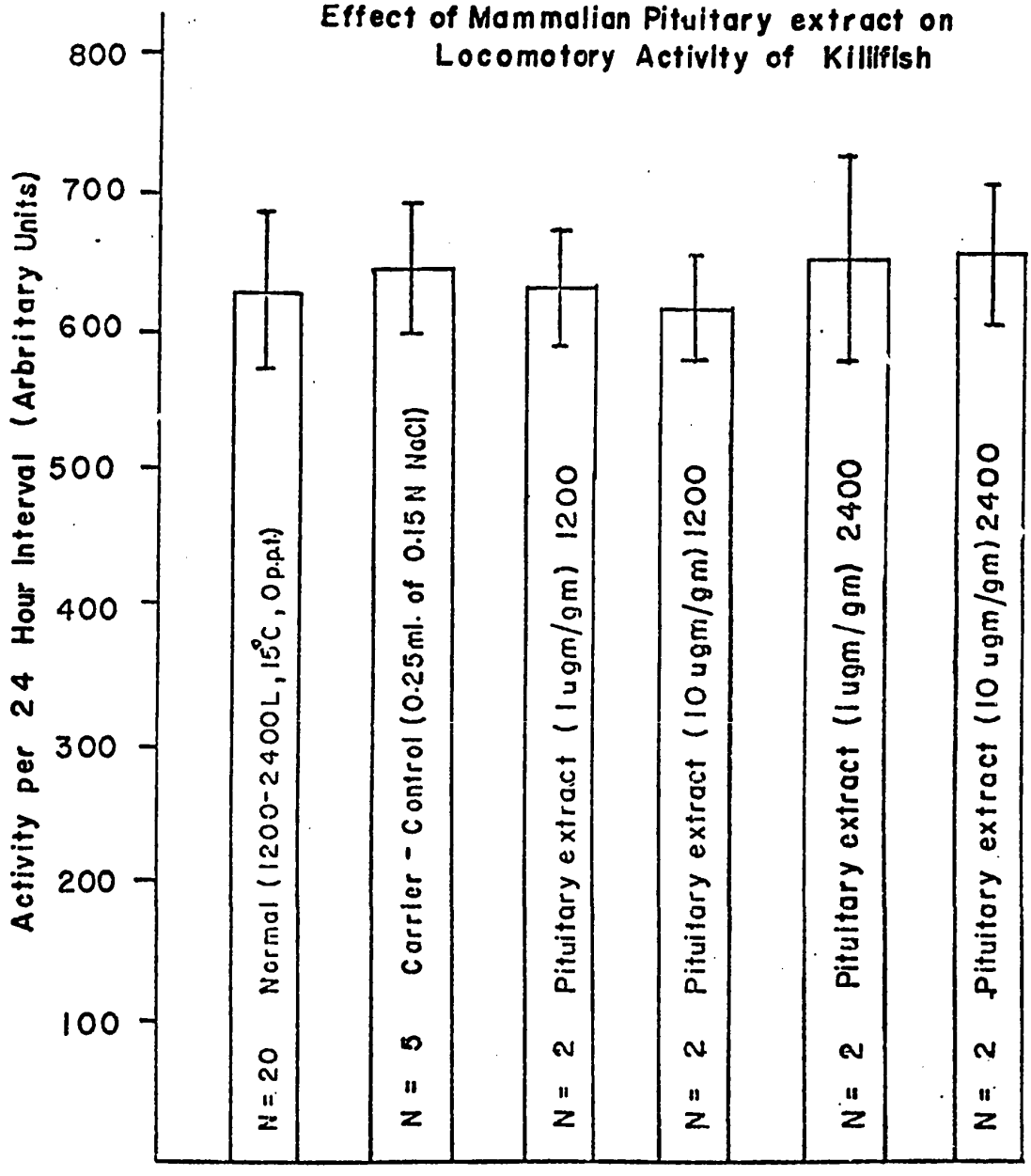
A concentration of 5 µgms/gm wet body weight of mammalian thyroxine significantly increased activity at 1200 ($p < 0.05$) but had insignificant effect at 2400 hrs. A concentration of 1 µgm/gm wet weight fish, of thyroxine did not significantly affect locomotory activity (Fig. 47).

A concentration of 0.01 µgm/gm wet body weight of synthetic melatonin administered at 0600 to 2400 significantly decreased locomotory activity (p's < 0.001). The melatonin injection at 2400 hrs had a significantly greater effect on activity than that administered at 0600 or 1200 hrs. (Fig. 48).

The activity of blinded fish was significantly less than that of normal fish. Melatonin decreased the activity of blinded fish, but this decrease was not statistically significant (Fig. 48).

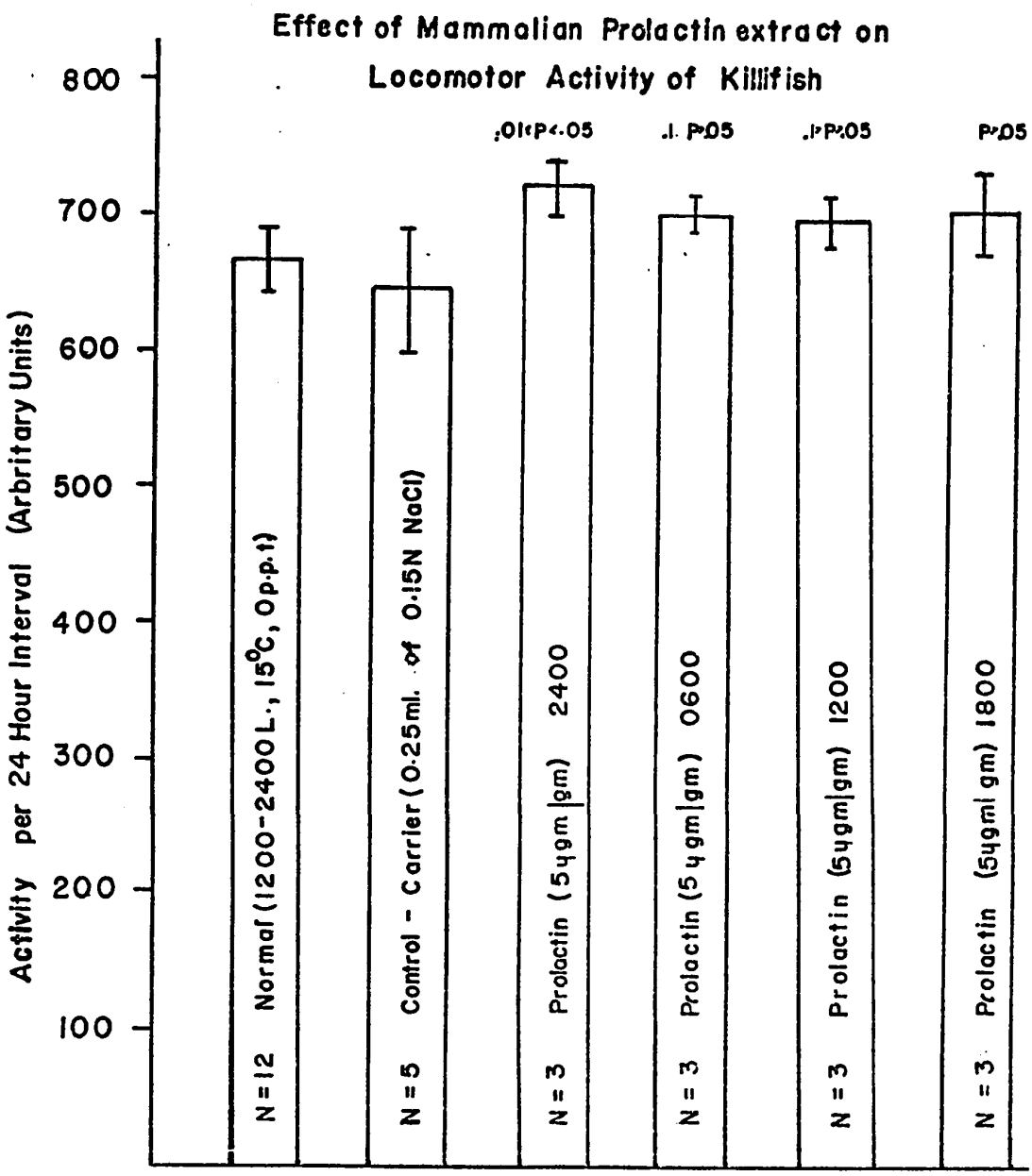
These results suggest that there were either diurnal variations, in the levels of hormones in intact fish, or diurnal variations in the responsiveness to hormones.

Figure 45:



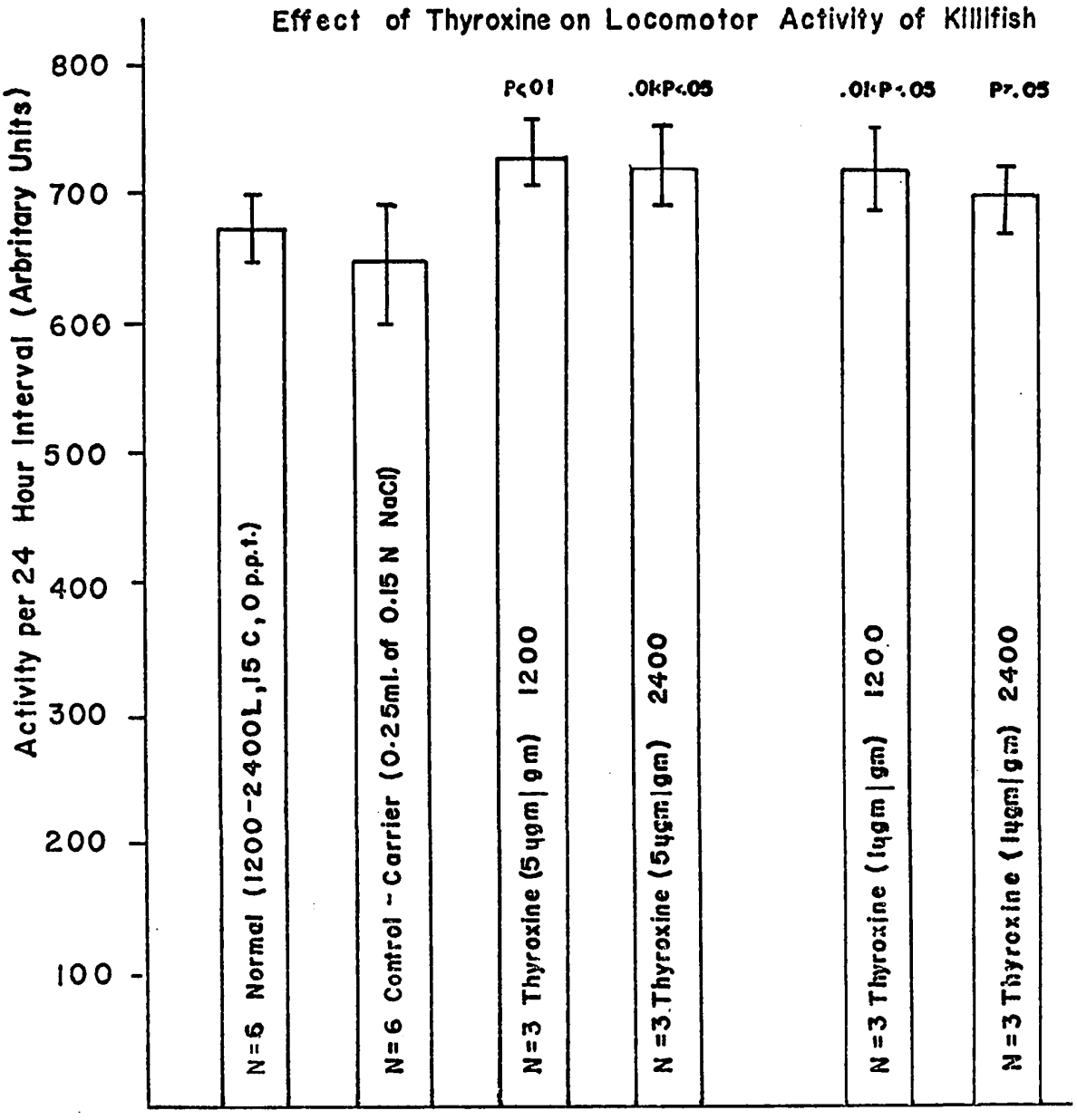
Vertical lines represent 95 % confidence intervals all comparisons made with control injections.

Figure 46:



Vertical lines represent 95% confidence intervals
all comparisons made with control injections

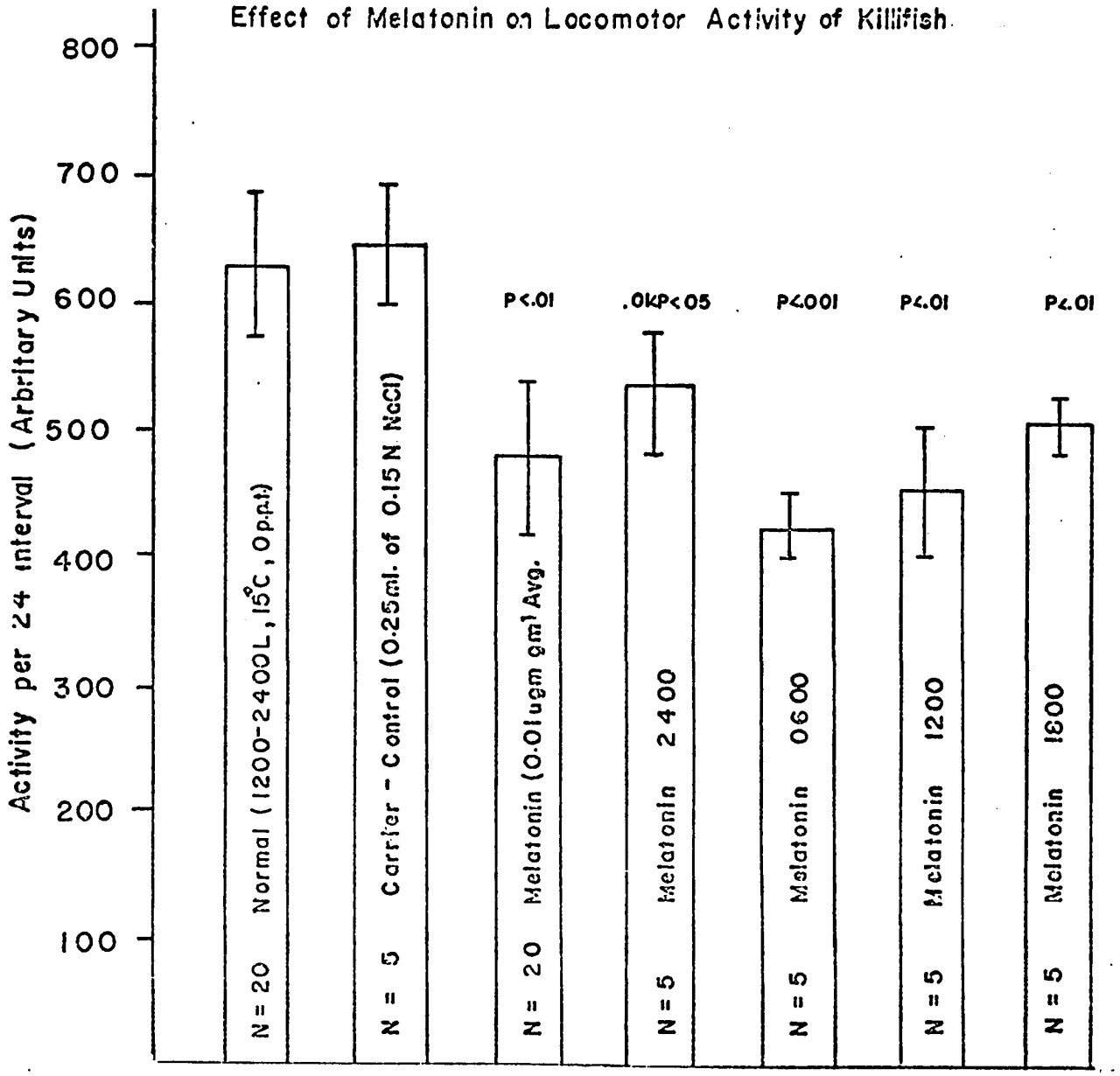
Figure 47:



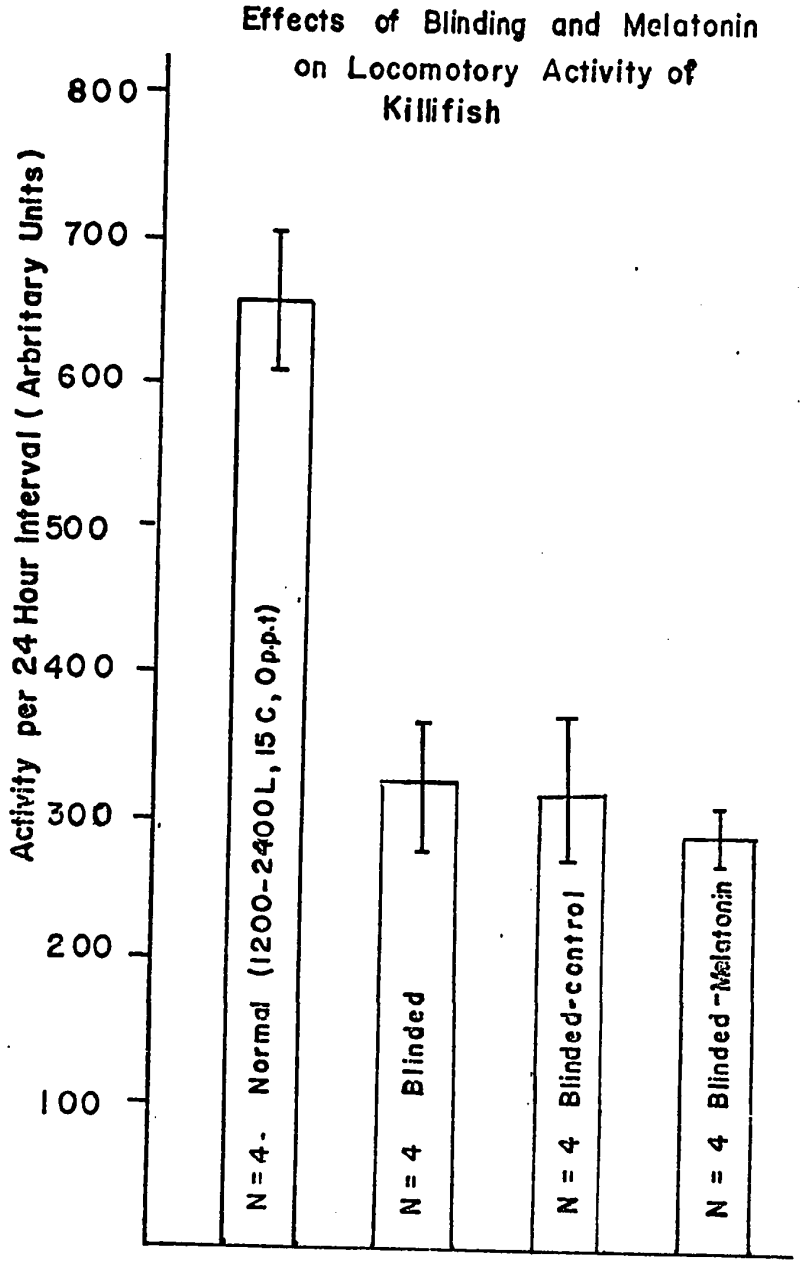
Vertical lines represent 95% confidence intervals

all comparisons made with control injections

Figure 48:



Vertical lines represent 95% confidence intervals
all comparisons made with control injections



Vertical lines represent 95% confidence intervals
all comparisons made with control injections

Techniques of Measuring Colour Change

Methods used to measure colour change in fish are described by Waring (1963) and Parker (1948). The Derived Ostwald Index (D.O.I) (Healy and Ross 1966) provided a subjective visual means of determining fish colour. The procedure used involved matching the colour of the entire fish to a standard series of grays chosen from the Ostwald Colour Series. The D.O.I. allowed comparison between fish exposed to similar and different experimental conditions but did not provide a practical or effective means of determining small and continuous changes in colour.

Photoelectric techniques, measuring either reflected or transmitted light, have been the most extensively used means of continuously determining and recording fish colour. (Hill et al., 1935, Fujii 1959, Fujii and Novales 1969, Wilhelm 1969, Finnin and Reed 1970). The majority of these photoelectric studies utilized light reflected or transmitted from a specific individual melanophore, or bands of melanophores. When colouration of the whole fish is being studied light reflected from the entire fish should be measured. Specific melanophore regions display different degrees of melanin aggregation and dispersion and are not necessarily representative of changes occurring in the whole fish.

In many of the procedures used to determine colouration, fish were surgically immobilized by spinal section. The resulting behavioural alterations and a gradual physical deterioration may modify fish colour (Finnin and Reed 1970). In killifish spinal sectioning is unsatisfactory, since the course of the nerves supplying the chromatophores is not known, with innervated melanophores displaying different physiological responses from denervated ones (Smith 1928). If unoperated, fish have to be given a certain degree of freedom of movement. In Phoxinus complete restriction of movement leads to a lowered ability to adapt to changes in the background (Gray 1956).

Many authors (Fujii 1969) have taken measurements or recordings of fish colour shortly after confinement of their fish. An acclimation time of 24-48 hours is necessary to stabilize hormonal and metabolic disturbances caused by restraint (Sage 1968). The previous experience of a fish, which can significantly affect its colour (Odiorne 1957), also has to be considered in any prolonged study of colour change.

The experimental photovoltaic measuring techniques and methods developed during this study have resolved most of the afore-mentioned difficulties.

Fish were placed in a holding chamber and all the light reflected from the dorsal surface was measured (Figs. 1, 2). The chamber imposed some behavioural

restrictions on the fish but did not completely inhibit their activity (Fig. 7) nor change the ability to respond to different backgrounds (Fig. 10). The limited activity still available to the fish caused some variation in the colour change records. During testing the fish could not be fed, promoting physiological and possible colour change.

Experimental variables such as temperature, salinity, background, and light intensity could be altered without affecting sensitivity of measurement. However, the fish could not be exposed to varying photoperiods without changing the precision of measurement. Therefore measurements were confined to fixed light intensities. The previous environmental history of the fish, photoperiods included, was experimentally determined.

The linear relationship between the Derived Ostwald Index (D.O.I), a subjective measure of colour, and light reflected from the entire fish (Lux transformed into millivolts), which was established (Fig. 8) allowed comparisons between fish subjected to different experimental conditions. Differences obtained between fish exposed to the same conditions were reduced but not completely eliminated.

Techniques of Measuring and Recording Activity

Locomotory activity of fish has been studied under laboratory conditions by several methods. Many of the studies have been of short duration and the methods of measurement have lacked sensitivity and accuracy. A major criticism of all laboratory techniques is the validity of extrapolation to field conditions. One attempted extension of laboratory techniques to the field was the use of fish cages placed in streams (Swift 1962, 1964). This method however, may have still unduly restricted free movement of the fish.

Direct visual observations of fish in tanks or aquaria have been widely relied upon in the past (Shaw 1970). Such observations have tended to be subjective and confined to limited time periods. Photographic recordings are more widely used now, but these are limited in effectiveness because of the need for continuous, and usually fairly high level of light.

The physical attachment of fish directly to recording instruments has been used, i.e. the "Ichthyometer" (Spencer 1929). The attachments encumbered the fish impeding and altering its locomotory behaviour.

One widely employed method is the use of mechanical devices to couple the fish and recorder. When a moving fish pushes or strikes a barrier (usually a metal or plas-

tic vane) placed in the tank, a circuit is completed and movement registered. (Spoor 1941; Jones 1956; Davis and Bardach 1965; Gibson 1965, 1967, 1971). This type of apparatus can detect only horizontal movements, and these only in limited portions of the tank. Different versions of a "circuit completion" type of recorder have been used for fish with specialized locomotory activity patterns e.g. flatfish (DeGroot and Schuye, 1967) and eels (Fohun and Winn 1966).

Oxygen consumption of fish has been used as an indirect measure of activity (Beamish and Mookherjii 1964, Livingston 1971). To carry this out it is necessary to confine fish in a respirometer and thus inhibit or alter its activity pattern.

Photoelectric devices have been extensively utilized (Chaston 1968; Hall 1971; Gibson 1967; Swift 1962, 1964; Müller 1968, 1969). These have been limited to the detection of activity in specific paths or bands. The one exception is the photoelectric grid designed by Kleerekoper (1969); however, this system is very expensive to install and operate.

Coupled thermoresistors detect water motion caused by fish activity (Beamish and Mookherjii 1964). These detectors appear to be limited in sensitivity and cannot be readily used with large groups of fish.

An electrode chamber described by Spoor et al. (1971) appears to be an efficient simple means of detecting activity. Use of this apparatus was not considered for this study because the detecting sensitivity is greatly reduced in saltwater.

To overcome many of the drawbacks mentioned, an ultrasonic system of detecting and recording activity was used. All fish movements, however limited in duration and extent, were detected in any portion of the tank (Fig. 25). A good approximation of the degree, extent, and direction of the activity exhibited was provided. The recorder, which is similar to the system used by Meffert (1968), detects the phase shifts caused by fish activity, which alter a standing wave pattern produced by the transmitting transducer.

A limitation of the determinations made in this study, was that the flow of water through the tank had to be kept very fine and diffuse. This was necessary to reduce the background water motion relative to that caused by the fish movement. This drawback arose because of the relatively large tank and small fish that were used. Another drawback, caused by the nature of the activity of killifish, was the need for continuous high speed recording (.025 cm/sec). This provided large records that were

unwieldy to handle. To overcome this difficulty activity was summated over half-hour periods, giving a distorted nature of its true sporadic nature.

Cummings (1963) appears to have been the first to use ultrasound to detect locomotory motion of fish. His system functioned on the detection of frequency (Doppler) shifts caused by alterations of the standing wave pattern. Only Meffert (1968) has reported any extended activity records obtained with ultrasonic apparatus functioning in a similar manner.

Muir et al. (1965) and Byrne (1970, 1971) used ultrasonic means to record continuously, fish activity for extended time periods. Because of the close proximity, and positions of their transducers, they were able to monitor activity in only limited portions of their circular tanks.

The ultrasound used is produced at a low energy and high frequency (35 KHz.). This frequency is well above the maximum detected capacity of killifish, their upper limit being approximately 2,000 Hz. (Tavolga 1971). Low energy, high frequency ultrasound has no reported, detectable,effects on fish (Tavolga 1971).

Basic Characteristics of Rhythms

Under constant conditions killifish possess persistent rhythms of colour change and slowly extinguishing cycles of locomotory activity. This can be seen in the raw activity and colouration records (Figs. 26-11) and their autocorrelation and spectral functions (Figs. 26-11).

A persistent, generally at least 5-7 cycles, free-running rhythm under constant conditions implies true spontaneity and the existence of an endogenous clock mechanism (Aschoff 1960). Some researchers, notably Brown (1967), (1969) contend that the organism is really responding to some unknown weak subtle synchronizer.

The two different rhythms studied will be separately described and discussed in terms of the following basic characteristics:

1) Frequency (1 /period)
2) Waveform
3) Basal level around which either colour or activity rhythm occurs
4) Amplitude
5) Phase, The temporal relation of rhythm to external (exogenous) cycles
6) Zeitgebers and other possible controlling agents

Characteristics of Colour Change Rhythem

Killifish display a persistent rhythm of colour change under constant testing conditions (Figs. 11-19). This rhythm, which appears to be endogenous, has a period that varies between 23 and 24 hours, average 23 hrs 45 min ± 15 min. The period is relatively independent of the intensity of constant illumination, thus not obeying the Circadian Rule (Aschoff 1965).

Under constant conditions the rhythm is roughly sinusodial in shape and shows no evidence of fade out. This characteristic shape is clearly visible in the oscillations shown in the correlograms, e.g. Fig. 11.

The level around which the oscillation occurs, the D.O.I., is dependent on the background colour and intensity of illumination or albedo (ratio of incident light to reflected light) see for example Figs. 11 compared to Figs. 12, 13. The range of colour change or amplitude also depends on background, and intensity of illumination. Background extremes, Ostwald Nos. 1 and 6, which force fish to adopt extreme colouration, reduce the magnitude of the amplitude (compare Fig. 11 and Fig. 16). There is still a great deal of inter- and intra-fish variation in amplitude (Figs. 11-19). As the time series cover a relatively short interval, rhythms with periods

of several days, or even weeks, which might be responsible for the variation in amplitude, could not be tested for.

During the maintaining conditions colour is neurally controlled through visual cues. In a high albedo, fish become pale, while under a lower albedo or reduced illumination they darken. These neurally mediated responses obscure rhythmic colour changes, since the basal oscillation level is altered. In the testing condition the external visual cues were markedly reduced in intact fish, and eliminated in blinded fish, minimizing direct neurally controlled colour change. The rhythm showed no evidence of fade out in blinded fish (Figs. 20,21), an indication that colour change was not due to rhythmic retinomotor changes.

If photoperiod entrained the endogenous rhythm of colour change then, under constant conditions, the first period(s) of the rhythm would show a constant phase relationship to the previous light-Dark (L-D) cycle. Loss of synchrony would occur through several free-running cycles, the number of cycles depending on the difference in period length of the endogenous rhythm from that of the imposed exogenous cycle (usually 24 hours).

This type of photoperiodic relationship was not seen. Continuous exposure to different photoperiods did not reveal any phase relationships indicative of previous

entrainment. Occasionally there were transient phase relationships (Figs.16,17) that may have been due to some form of oscillator interactions. Definite entrainment involves the formation of consistent phase relationship between the endogenous rhythms and exogenous environmental cycle, and the imposition of the exogenous periodicity on the endogenous rhythm. The results obtained indicated that the rhythm of colour change is not directly controlled by photoperiod.

Figures 17,18,19, showed that rhythmic salinity changes (freshwater-saltwater-freshwater) had no effect on colour. Temperature had a minor effect on colour, cold (10°C), causing a slight darkening. Any colour changes caused by cyclic temperatures were superimposed on the free running rhythms (Fig. 17). There was no evidence of entrainment by temperature, salinity, or thermosaline changes.

The colouration rhythm displays a trend, due to either gradual morphological colour changes, or possible instrumental drift. These trend components were assumed to be linear and were removed during the standardization and transformation of the original data. The power spectrum of the detrended data shows some power at zero frequency (Fig. 11d). This power is an indication of a curivilinear trend or additional component present in the original time series. Such a trend could result from

instrument drift, or from the presence of a superimposed long term cycle of colour change on the daily endogenous rhythm.

The marked persistence of the colour change rhythm along with its variable amplitude suggests that the rhythm may be responding to a very weak Zeitgeber. In the laboratory all well-known Zeitgebers were excluded. This leaves the possibility that very subtle geophysical or electromagnetic factors were acting as synchronizers. These, however, will not be considered here.

Characteristics of Locomotory Activity Rhythm

The results obtained show that killifish posses a bimodal, i.e. double peaked, circadian rhythm of locomotory activity, that persisted up to a maximum of 14 days under constant conditions. The time required for fade out varied between fish and conditions. Although the records sometimes appeared to show a single major activity peak, this was composed of two sub maxima the, first of which was higher than the second. This is a bimodal pattern of activity.

Most studies with fish show rapid (2-3 cycles), extinction of free-running activity patterns (Schwassman 1971b). The persistence obtained here with Fundulus heteroclitus may be partially attributed to the sensitivity of recording techniques used. All vertical and horizontal movements of the fish, even those of short duration or amplitude, were detected.

Fish are generally divided into rhythmic, nocturnally or diurnally active species (Spencer 1939). The extent, intensity, and rate of change of ambient light can markedly affect the behaviour patterns of fish. Woodhead (1956) working with minnows in a light gradient, demonstrated that the fish displayed marked changes in activity above and below a light intensity of 0.2 Ft-candles.

In the laboratory, experimental design can also affect the type of activity exhibited. The minnow Phoxinus phoxinus when placed in an open glass aquarium was diurnally active, but if shelter was provided the fish became nocturnally active (Jones 1956). However, in both cases the fish displayed circadian activity rhythms of similar period length (Müller 1968).

The endogenous activity rhythm of killifish was of varying period (23-25 hours) there being inter- and intra-fish variations (see Figs. 26-29). These variations were influenced by the environment.

The length of the free-running period of activity in the killifish was dependent on the intensity of constant illumination. (Fig 37). At an illumination of 200 Lux, the period was significantly shorter ($p < 0.01$), than at 50 Lux. The ratio of activity to "rest time" remained fairly constant throughout. At higher light intensities extinction occurred slightly more rapidly than in dark conditions.

These effects of different light intensities roughly follow Aschoff's Rule (Aschoff 1965). Complete agreement to the rule is rarely found, since in nature there are always several possible Zeitgebers influencing an organism. The biological meaning of Aschoff;s rule remains unclear. It may represent light intensity dependency of certain endogenous oscillations.

The bimodal activity pattern, which in the free-running condition tends toward unimodality, may represent interference between several oscillators, all not equally responsive to light (Hoffman 1971).

The killifish were never observed to be completely inactive, but rather there was a rhythmic change from a higher to a lower level of activity (Fig. 26). The extent and level around which this change occurred was temperature dependent, there being a decreased amount of activity as temperature was lowered (20° - 10°C) e.g. Figs. 26-30.

Salinity had no apparent affect on activity and the endogenous activity rhythm (Table 10). Since F. heteroclitus is a euryhaline fish it is exposed to thermosaline changes associated with tidal exchanges. These may interact to synchronize activity under natural conditions.

Activity was readily entrained by photoperiod. A constant phase relationship between activity and photoperiod was established with the double peaked activity maxima occurring during the light portion of a Light-Dark cycle (Fig. 20). Most efficient or complete entrainment was achieved at cylces of 12-12 L-D (Fig. 30A) though other cycles (14-10 and 10-14 L-D) did cause synchrony

but required longer entrainment times (Fig. 31). Phase shifting to establish temporal relationships between the L-D cycles and activity occurred very quickly, complete synchrony requiring 3-4 days (Figs. 28,29).

A circadian rhythm of light sensitivity has been postulated (Bünning 1960), and is now known as the "Bünning Hypothesis". According to this hypothesis a L-D cycle is most effective as an entraining agent when light is in phase with the photosensitive portion of the endogenous rhythm. In killifish the most light sensitive portion is the time of maximal activity.

The light portion of an L-D cycle can either synchronize an endogenous rhythm, or shift the phase of an already entrained rhythm. The time needed for these effects to become manifest varies, both with the phase of the endogenous rhythm present, and the length of light and dark portions in the L-D cycle. The effects of these variations can be observed in Figs. 28, 29 and are discussed on the following page.

Two types of entrainment schemes: impulse or occultations, and proportional Light-Dark were used. They were employed to determine whether the transition effects of Light or Dark were sufficient to explain photoperiodic synchronization, or the relative proportions of Light to Dark is the more important factor.

Within limits, different proportions of Light and Dark were able to entrain locomotory activity, while impulses and occultations had little effect. Ratios of 12-12, 14-10, 10-14 L-D entrained activity (Figs. 28,29). However, as the duration of either Light or Dark was decreased, or the exogenous period length significantly changed from 24 hours, entrainment no longer occurred. The Light-Dark effects of cycles differing from 24 hours (8-8, 16-8, 8-16 L-D) (Figs. 34,35) were superimposed on the free-running activity rhythm. From the results obtained with different L-D schedules, and under the thermosaline conditions tested, it appears that entrainment of killifish locomotor activity is dependent on mimimum amounts of Light and Dark.

Gradual light-dark (dawn or dusk) transitions were not considered in this study. These slow transitions could serve as additional synchronizing agents and extend the range of light ratios and intensities available for entrainment. Activity patterns can be altered through a reduction or elimination of pre-dawn peak(s) of locomotory activity

that are present in nature (Davis 1963). These pre-dawn peaks are generally entrained to feeding cycles. Feeding cycles were eliminated in this study.

The Light-Dark transitions used were not from absolute dark to light, a change never found in nature, but were rather from a higher to lower Light value (darkroom light). These transitions could have served to reduce the strong "light-shock" reaction that occurs in many fish upon abrupt light changes (Davis 1962). The "light-shock" reaction was displayed by F. heteroclitus when exposed to flashes, as a slight increase in locomotor activity. (Fig. 32).

Effect of Groups Size on Activity

Activity of Fundulus heteroclitus, normally a schooling fish, was studied using solitary fish and groups of 5 and 20 (Figs. 39,40).

Solitary fish displayed a circadian pattern of activity with a clearly defined period and prolonged fade out time (Figs. 38,39). The group of 5 had an indistinct activity rhythm with a greater variability in period length than solitary fish (see Fig. 39c) and consequently a, broad low peaked, power spectrum. The time required for fade out varied markedly between groups but was less than that required for solitary fish. According to the terminology of Breder (1959) these 5 fish formed an aggregation and acted as non-polarized individuals having very little interaction.

The group of 20 had a strong activity pattern with a gradual fade out (Fig. 38,39). These fish functioned as a school: a stable unit that minimizes individual differences in activity and functions as a mutually entraining agent (Breder 1959).

The intensity of illumination did not affect the length of the activity period, of schooling fish and only slightly modified that of the aggregates (Fig. 40). The circadian rule was not observed by the group while it was by solitary fish.

As the number of fish in the aggregation is increased, their density is augmented to a critical value at which mutual attraction and repulsion interact sufficiently, to lead to regular spacing and the formation of a school. For killifish this value has been estimated to be approximately 0.3 fish per fish length (Symons 1971). In laboratory studies the number of fish at which aggregation-school transitions occur is dependent on the size of the tank, light intensity, and photoperiod used (Breder 1959).

The ability to form, and the extent of school formation, is also dependent on light intensity. According to Breder (1959), school disruption occurrs when a certain minimum light intensity is reached. This minimum occurs before actual visual contact between all the fish is lost. School disruption would lead to the formation of aggregations, where there is less group synchrony and more individual behaviour.

The extent of school formation can also follow a diurnal periodicy (Hunter 1966), and the behaviour of the school itself can display a diurnal variation. Thines and Vandenbussche (1966) have shown the presence of diurnal fluctuations in the readiness of fish to school in response to external stimuli.

Schooling killifish, under the conditions tested,

appeared to display less activity per individual than when solitary (Figs. 26, 27, 38, 39,40). This decrease is probably due to a reduction in random behaviour and the imposition of the so-called "group effect" (Allee 1951). This "group effect", which also reduces the activity of aggregates, has been described in a variety of different fish species by Schuett (1934), Welty (1934), and Shaufer (1940). They showed that grouping of non-schooling fish led to a decrease in activity and average oxygen consumption per grouped fish as compared with an isolated fish.

The activity of a school of fish is not equivalent to the sum of that of the solitary fish. This is displayed by the different reactions to light and a decrease in total activity per member of a group as compared to a solitary fish (Figs. 38,39). The killifish school functions as a partially autonomous unit with different characteristics and properties than those of solitary fish.

Effect of Blinding on Colouration and Activity

The activity and colouration of blinded killifish were recorded (Figs. 20,41). These operations and tests were performed to eliminate the possibility that the colour rhythms observed were caused by cyclic changes in eye light sensitivity, or retinomotor activity.

Retinomotor rhythms consist of diurnal variations in the exposure of rods and cones to light. In the light adapted stage, the cones are aligned along the external limiting membrane while the rods are enveloped by pigment granules inside the pigment cell. In the dark adapted state, the positions of the rods and cones are reversed.

Under constant conditions photomechanical changes in the eyes of Ameiurus can persist for several days. John and Kaminstein,(1969) reported that retinomotor rhythms persisted in continuous darkness and below a certain threshold in continuous light.

Blinding, by destroying the photoreceptors, eliminates photomechanical changes. The locomotor and colouration rhythms were little altered following blinding (see Figs. 20, 41). The total amount of activity and base level of colour around which changes occurred were altered (Figs. 20, 21). Blinded fish

have a tendency to assume an intermediate colour regardless of background (Parker 1948).

This lack of effect by blinding on the rhythms, eliminated retinomotor changes as controlling agents. Immediately after blinding, the total amount of locomotor activity was greatly reduced (Figs. 41, 42) probably because of shock reaction.

The lack of alteration seen in the activity rhythm (Fig 41,42) is in agreement with the findings of Bertman and Müller (1970). They found that blinded rainbow trout had the same amount and phases of locomotory activity as unoperated fish.

After blinding, the killifish could not be as readily entrained to different L-D regimes. Although the pineal is photosensitive (Pang 1967), and serves as a light receptor, the complete phototactic response of fish depends on the presence of intact eyes (Fenwick 1970).

Effect of Starvation on Activity and Colouration

Throughout the testing times fish were not fed and during the maintaining periods they were given food only at randomly selected intervals. This procedure was used to eliminate the possibility of causing synchronization to feeding cycles. With killifish, feeding can act as a weak Zeitgeber for activity, leading to pre-feeding and pre-light pre-feeding activity maxima (Davis and Bardach 1965). These authors did not observe a diurnal activity pattern with their group of fish but did propose that pre-feeding activity was synchronized to an internal circadian clock.

The experimental procedures which Davis and Bardach (1965) used are open to several criticisms. The plastic vanes they placed in the tank to detect fish motion were maintained only in the feeding portion of the tank. The fish may still have displayed a diurnal activity in the rest of the tank after having learned that it was necessary to brush by the vanes and enter the feeding portion only at certain times.

Since killifish are surface feeders, their feeding times would presumably be closely linked to tidal exchanges when large amounts of food become more readily available at the surface.

Swift (1962, 1964) could not detect any pre-feeding activity in brown trout. Andrensson (1969) in a prolonged

study under artificial and natural conditions, showed that feeding does not affect the diel activity pattern of freshwater sculpins.

Starvation together with the elimination of Zeitgebers would tend toward the reduction of activity in free-running periods as is seen (Figure 27). Starved brook trout reduce their spontaneous activity during the first 2-3 days of starvation, after which activity remains constant (Beamish and Mookherjii 1964).

Colouration might be affected by a reduction in the metabolic expenditures that may be necessary for extreme colour changes. However, no consistent decreases in amplitude of colour changes were seen.

Effect of Sexual Phase on Activity and Colouration

The total amount of activity and colour change displayed by killifish can be affected by the sexual phase of the fish. In this study, sexual effects were not considered because experiments were conducted throughout the year, at a variety of temperatures, photoperiods, and consequently sexual phases.

Sexual differences probably contributed to the variation in period lengths, total activity, and colour change observed between different fish.

The degree of sexual development is dependent on the temperature and photoperiod, e.g. long photoperiods and warm temperatures inducing gonadal development. In the order Cyprinodontiformes the relative importance of temperature versus photoperiod has not been resolved (de Vlaming 1972).

In fully sexually responsive fish the increased gonadal steroid and thyroxine production increases the responsiveness of the central nervous system to external stimuli leading to greater overall activity (Hall 1969). The degree of aggregation and schooling is altered. Fish have a tendency to be more aggressive with increased activity per individual and less group synchrony.

In sexually mature fish the colour is enhanced, giving a larger amplitude in the daily rhythm. In males the development of colour is related to total activity and aggressiveness (Matthews 1939). Enhanced colouration is a factor used to demonstrate sexual maturity and responsiveness.

Relationship to Tidal Rhythms

Killifish are normally exposed to semi-daily tidal exchanges, of a period of 24.8 hrs. Tidal rhythmicities are reported to be insensitive to the intensity of constant illumination, or photoperiodic variations, as seen in the colour change records. The loss of the exogenous entraining cycle would result in the demonstration of a very persistent and seemingly unentrainable oscillation that appears to be circadian in length. There may be some transient sensitivity, or coordination to photoperiod. This may arise from a partial interaction with an oscillation controlling activity that is entrainable by photoperiod.

Tidal rhythmicities have been demonstrated in several species of marine fishes and invertebrates. Blennius pholis, an intertidal fish, displays a tidal activity rhythm that rapidly shifts to a unimodal pattern in constant conditions (Gibson 1970). Fundulus similis possesses a tidal rhythm of sensitivity to NaCl. Tidal synchrony is rapidly lost in constant conditions, being replaced through rephasing to a "more basic rhythm" (Day 1968).

The bimodal activity pattern of killifish would also seem to be ideally suited for synchronization with semi-daily tides. Intertidal invertebrates often show a bimodal pattern of activity repeated at circadian intervals

(Enright 1963). Aschoff (1966) suggested that bimodal activity patterns persist because of their ease of tidal synchronization. Bimodal patterns are also present in animals that in nature are never exposed to the tides and cannot be taken as direct indicators of tidal synchronization. The absence of tidal components (24.8 hrs) in the correlogram and spectra does not disprove their existence. The free tidal component(s) may be masked by photoperiodically entrainable components of approximately the same period length. The tidal oscillations may also manifest themselves only when exposed to their normal entraining agent. In this way tidal synchronization may occur in nature.

The normal entraining factors for tidal rhythms, include cycles of wave produced turbulence (Enright 1965) and, cycles of hydrostatic pressure (Morgan 1965; Gibson 1971). Salinity and temperature changes may prove to be too capricious to act as the sole synchronizers.

There are several possible means by which fish could detect changes in hydrostatic pressure. The swimbladder of fish is pressure sensitive and may serve as a detector for tidal changes (McCutheson 1969). At least two species of fish have otoliths that possess piezoelectric properties and could function as pressure or depth receptors (Morris and Kittleman 1967).

In rats the metabolism of serotonin and associated locomotory activity changes are affected by subtle changes in barometric pressure (Olivereau 1971). Presumably in fish,where serotonin plays a direct role in controlling activity (Fenwick 1970),and an indirect role through its pineal transformation product, melatonin, the metabolic relationship could be affected by hydrostatic pressure changes. Pressure changes would not be expected to be the sole synchronizing agent, for killifish live in surface waters and would presumably experience no, or very slight pressure changes.

Relationships to Multi-Oscillator Models

Solitary killifish possess endogenous rhythms of locomotory activity and colour change (Figs. 11,27). Both these rhythms have periods of approximately the same length, 23 hrs 45 min ± 18 min (approx.) for colour change, and 23 hrs 50 min ± 20 min (approx.) for locomotory activity. The length of the free-running activity period was significantly ($p < 0.050$) altered by different intensities of illumination, while the rhythm of colour change was relatively unaffected by L-D variations (Figs. 11-18). The entrained and initial free-running cycles of locomotory activity had bimodal maxima (Figs. 26-30) while the rhythm of colour change was unimodal (Figs. 11-18).

Under constant conditions colour change gave no evidence of fade out or reduction in amplitude, though there were periodic variations in the range of the amplitude. Over 7-14 days the free running rhythm of activity faded out to a uniform level of continuous activity (Figs. 27,38).

These differences in basic properties and photoperiodic control mechanisms of colour change and activity indicate that there are at least two different types of rhythmicities present in Fundulus heteroclitus. These rhythmicities are probably part of a multi-oscillator interacting system. The hypothesis that colour change and activity rhythms depend on some common factors present in a multi-

oscillator system is strengthened by the finding that hypophesectomy eliminates both free running rhythms (Figs. 22,43) There are several hormonal rhythmicities present in killifish and other teleosts [Meier and Shivastra (1972)] that may form part(s) of a more basic endogenous clock mechanism.

Hoffman (1970) reviewing experimental evidence concluded "..... that multi oscillator system underlies an overt circadian periodicity, even of a single function, and even if it appears as a single self-sustained oscillation, and that these oscillations are themselves circadian." Furthermore, he also states that, models based on single oscillators are not suitable for analysing the nature of the basic clock, although they have been useful in describing overall rhythmic behaviour and formulating some basic rules, e.g. Aschoff's Rule.

Killifish may possess several oscillator systems, each entrained by different factors. Wever (1972) has reported that varying amplitudes, as seen in the free running rhythm of colour change, can be the result of interaction between a circadian and another free-running rhythm of different period length, possibly tidal. In constant conditions the second rhythmicity may lose synchrony with the initial entraining conditions and run freely with a different period length. The period lengths may approach equality giving a single peak, as seen in the time series and correlograms of colour change.

Effect of Hypophysectomy and Hormones on Colour Change and Locomotory Activity

Hypophysectomy extinguished the free running rhythm of locomotory activity and led to a fade out of the endogenous rhythm of colour change (Figs. 23,42). These extinctions can be seen in the correlograms (Figs. 23,42) which oscillate around R=0, and their corresponding spectral transformations (Figs. 23,42), which show no significant power in any frequency range. (Figs. 42,10). The relative long fade-out times (1-2 cycles) may have been caused by the slow metabolism and elimination of residual pituitary hormones.

Elimination of the colour change rhythm by hypophysectomy reveals that, in addition to the formally demonstrated neural mechanisms controlling killifish colour change, there are other hormonal control factors involved. Hormonal oscillations may be required to produce the slow, rhythmic (24 hrs. approximately) rhythm of colour change that was observed. Hormonal effects may occur either, directly at the melanophore level, or indirectly through neural or other hormonal alterations. These hormonal mechanisms may alter the degree of neural response but cannot override it.

The rapid time course of background colour adaptation at all phases of the endogenous rhythm is a good indication of the predominance of neural control. Neural predominance is also revealed by the ability of hypophysectomized

fish to undergo rapid complete background adaptation, after hormonally induced oscillations are eliminated.

Cyclic AMP, whose level in the vicinity of melanophores is neurally controlled through alpha and beta receptors mechanisms, controls melanin aggregation and dispersion (Novales 1971). Since cyclic AMP is probably the second hormonal messenger, it is conceivable that it may be a factor that is either directly or indirectly affected by hormonal agents controlling colouration.

Locomotory activity, although significantly reduced and no longer free running after hypophysectomy (Fig 43), could still be entrained by photoperiod (Fig. 45). Hypophysectomy may have either eliminated endogenous oscillations that could be entrained by photoperiod, or prevented their proper coupling to locomotor activity. The coupling mechanism may be hormonal, neural, or a combination of the two.

Simultaneously hypophysectomy could also have disrupted non-photoperiodically synchronized endogenous oscillations involved in the production of rhythmic colour change. These effects would lead to the elimination of endogenous locomotory activity and colour change rhythms. However, photoperiodic induction and entrainment of exogenous oscillations and activity cycles could still occur.

Pituitary extract (5 µgm/gm wet weight fish) was tested at various portions of the cycles for its ability to alter either of the 2 rhythms. Intraperitoneal injections of beef pituitary extract into intact killifish did not significantly alter the locomotory behaviour or affect rhythmic colour change. This may have been due to the variable hormonal composition, and dissimilarity of the mammalian extracts to actual killifish principles.

Several hormones were investigated as possible links or controls in the production of rhythmic activity and colour change. The substances were; thyroxine, a thyroid hormone whose release is directly controlled by the pituitary; prolactin, a polypeptide hormone released by the adenohypophysis; and melatonin, the active pineal principle.

Locomotory activity of killifish was significantly increased by intra-peritoneal injections of thyroxine (1µgm/gm wet body weight of fish) (Fig. 47). The extent of the increase was time-dependent, being largest during the nocturnal portion of the activity cycle (Fig. 47), the time when killifish exhibited the least amount of activity (Fig 26).

There are several reports of thyroxine increasing activity and stimulating migratory restlessness in fish (Hasler 1970). Sage (1968) demonstrated that intraperitoneal

injections of thyroxine increased the activity and altered the swimming of Poecilia.

Thyroxine had no significant effect on the colour of intact killifish. This may partially be due to the fact that the endogenous level of thyroxine was high. Sage (1971) has pointed out that restriction or stressing of fish increases the release of thyroxine, and it is possible the fish, when confined in the chamber, were stressed.

The production of thyroxine by the thyroid is controlled by the amount of thyroid-stimulating-hormone (TSH) released by the adenohypophysis (Gorbman 1969). The hypothalamus exerts an inhibitory influence over the level of TSH, though there is always some basal level released (Baker 1969). Sage (1971) has shown a direct relationship between the release of TSH and activity of restricted fish.

The mechanisms of the action of thyroxine remain uncertain. It is generally believed to alter carbohydrate metabolism leading to a greater utilization of the pentose shunt (Gorbman, 1969).

Thyroxine acts to induce sexual behaviour, which in killifish is characterized by some increase in activity. The increased sexual behaviour is caused by a heightened olfactory sensitivity through stimulation of the olfactory centers (Gorbman 1969). Intraperitoneal injections of prolactin 1 µgm/gm wet weight of fish) significantly increased

the locomotory activity of killifish during the nocturnal portion of the activity cycle (Figs. 47).

Prolactin causes greater amounts of TSH and subsequently thyroxine to be released (Ball, 1969). Thyroxine has been shown to insignificantly increase activity (Figs. 48). The activity increase is not as large as that caused directly by thyroxine injections. This may be due to the fact that mammalian and fish prolactins are not wholly identical (Ball 1969).

Prolactin plays a prime role in osmoregulation (Pickford 1957). Hypophysectomized killifish can survive in freshwater,only if given prolactin injections (Pickford 1957). Prolactin prevents a rapid fall in plasma Na^+ and Cl^- by promoting the formation and maintenance of a stagnant mucosal layer at the integument and gills. Prolactin also induces parental behaviour and may in this manner directly alter swimming activity.

Through increasing dopa tyrosinase activity,prolactin promotes melanogenesis in fish (Gorbman 1969), though in this study no effect on colouration was seen.

A prolactin-inhibiting-factor (PIF) is produced by the hypothalamus (Ball 1969). Its release appears to be regulated by the brain catecholamine level. This factor is inhibited by serotonin and melatonin, the pineal product. The inhibition by serotonin is delayed and long lasting,

while that of melatonin is immediate (Kamberi et al. 1971).

Melatonin, injected at 0.001 µgm/gm had a significant inhibitory effect ($p > .005$) on killifish swimming activity (Fig. 49). This inhibition was maximum during the diurnal (L) portion of the activity cycle (Fig. 49), when normally activity and endogenous melatonin were at their minimum.

Hafeez (1970) has shown that melatonin inhibits the swimming activity of rainbow trout. Byrnes (quoted by Haffez 1970) reported that melatonin reduced the activity of juvenile sockeye salmon, with significant inhibition occurring only in the light portion of the photoperiodically determined activity cycle.

Ralph et al. (1971) have shown in rats, that there exists a rhythm of melatonin production in phase with the locomotory cycle. These rhythms persist in blinded rats but not ones exposed to continuous light. In killifish however, the endogenous activity rhythm persisted in continuous light, dark, in blinded fish. (Figs. 40-43).

The synthesis of melatonin from serotonin occurs in the pineal gland through a series of steps. The key conversion of N-acetylserotonin to melatonin, is controlled by hydroxyindole-o-methyltransferase (HIOMT), an enzyme whose activity is light inhibited. In constant conditions there is reported to be a diurnal rhythm in the production of HIOMT in rats.

Melatonin through its synthesis and release alters the pineal and hypothalmic serotonin levels. Melatonin induces pyridoxal kinase, an enzyme that leads to the formation of pyridoxal phosphate, a critical co-factor in serotonin synthesis. Fenwick (1970) showed with goldfish a relationship between decreased brain serotonin levels and increased activity.

The pineal of Fundulus heteroclitus is light sensitive (Pang 1965) and therefore may be directly affected by changes in ambient lighting. This may partially explain the ability of blinded killifish to have their endogenous rhythm entrained by different photoperiods.

Erikson (1972) has shown that blinded and pinealectomized rainbow trout can still have their activity synchronized by a variety of different photoperiods. They postulate that there exist photosensitive structures or organs other than the pineal and lateral eyes. These other light sensitive or detecting structures may be involved with the light induced oscillatory or hormonal systems.

SUMMARY

Fundulus heteroclitus shows endogenous rhythms of locomotory activity and colour change. Both of these rhythms are eliminated by hypophysectomy, indicating hormonal involvement in their control and origin. The different responses in these rhythms to changes in external variables suggests the killifish may possess several rhythmic hormonal or neural oscillators.

Rhythmic colour change had a slowly varying amplitude of 1.5 to 2.0 Derived Ostwald Index (D.O.I.) units. The rhythm had an average free-running period of 23 hours 45 min. ± 15 min., that was unentrained by cycles of light-dark, temperature, salinity, or thermosaline changes. Illumination intensity, different backgrounds and blinding were also without effect on the rhythm. The rhythm showed no evidence of fade-out in blinded or intact fish. In addition, there was a long-term cycle of colour change superimposed upon the daily rhythm.

In intact fish neurally-mediated colour change predominated over rhythmic affects. Hypophysectomy, while eliminating the endogenous rhythm of colour change, did not alter the ability to rapidly adapt to different backgrounds. This indicated that there are hormonal elements involved in the control of Fundulus heteroclitus colouration.

Killifish displayed a rhythm of diurnal activity that could be entrained by photoperiod. Under constant conditions single fish showed an endogenous circadian rhythm of activity with a period that was dependent on the intensity of illumination. Fade-out occurred very gradually. Blinding did not affect the endogenous rhythm of activity nor its entrainment by photoperiod.

Hypophysectomy eliminated the endogenous rhythm of locomotory activity, but not the entrainment of activity by photoperiod. This suggested that there may be several oscillators involved in controlling activity some of which, are dependent on hormonal control. The concept of hormonal involvement was further strengthened by the finding that injections of prolactin and thyroxine increased activity and melatonin decreased activity. The extent of these effects was dependent on the phase of the activity cycle at which the hormones were administered.

Groups of fish, 5 and 20 per group, also displayed an endogenous rhythm of activity that was entrained by photoperiod. However, the activity of a group differed from that of a single fish in the following respects:

1) The length of the free-running activity period of the groups was not dependent on the intensity of illumination.

2) The length of the activity period was more variable in grouped than single fish. This variation was less in the larger group than in the smaller group.
3) The grouped fish appeared to display less activity per individual than single fish. There was no significant difference between the activity per fish in the group of 20 as compared to the group of 5 fish.

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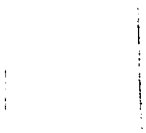
Appendix I: Ostwald Colours used to determine the Derived Ostwald Index D.O.I. of killifish (obtained after Healy and Ross 1966).

Number 1



Number 2

Number 3

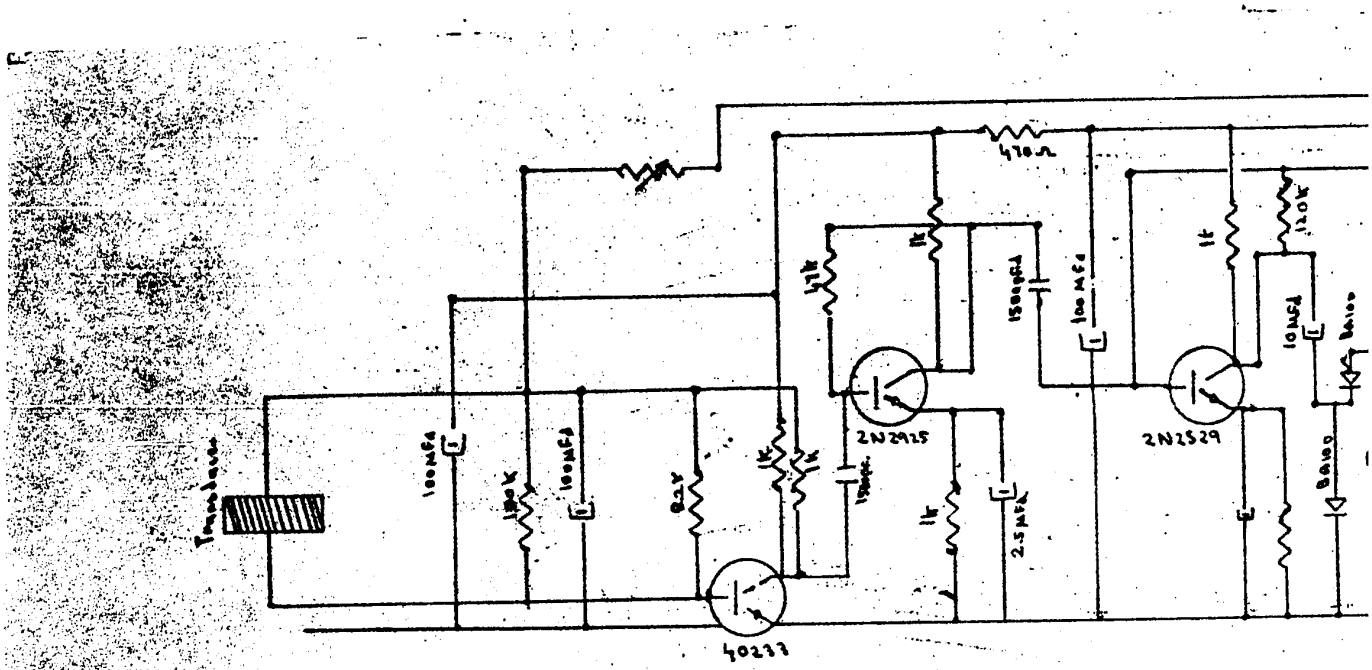
Number 4

Number 5

Number 6

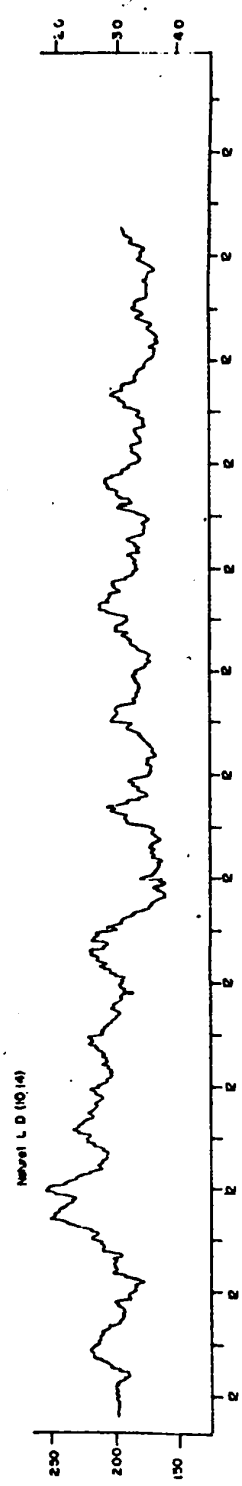
Number 7

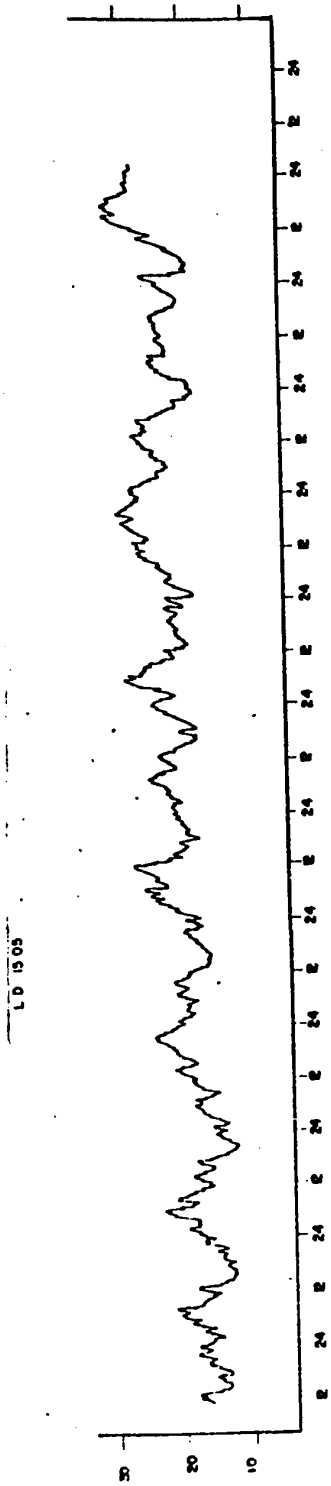
Appendix II: Circuit of ultrasonic activity detector.

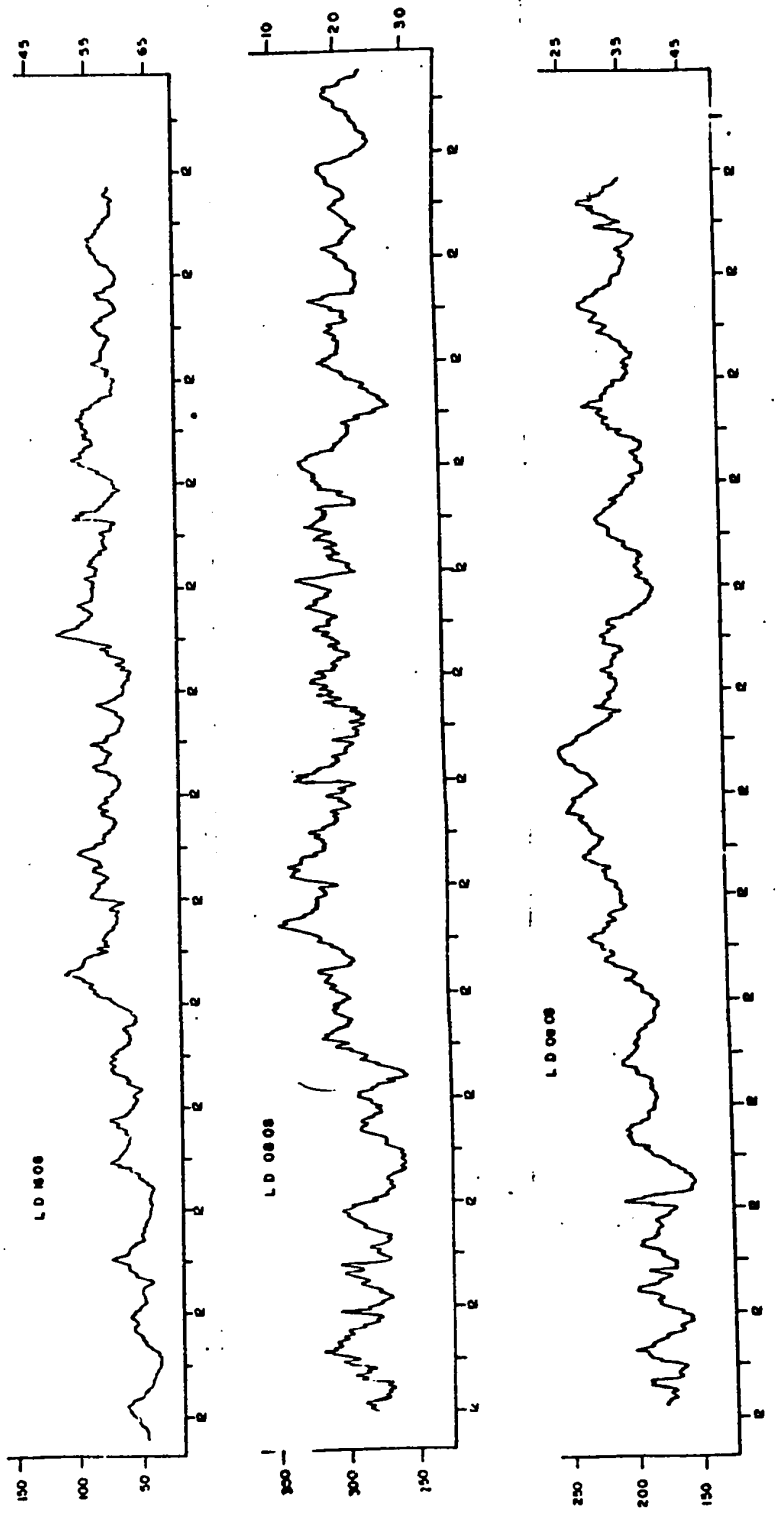


Appendix II: Circuit of ultrasonic activity detector.

Appendix III: Additional colour change records (Conditions as specified in tables).


Naval L D (10 14)
250
200
150
30
40


L D 1505
30
20
10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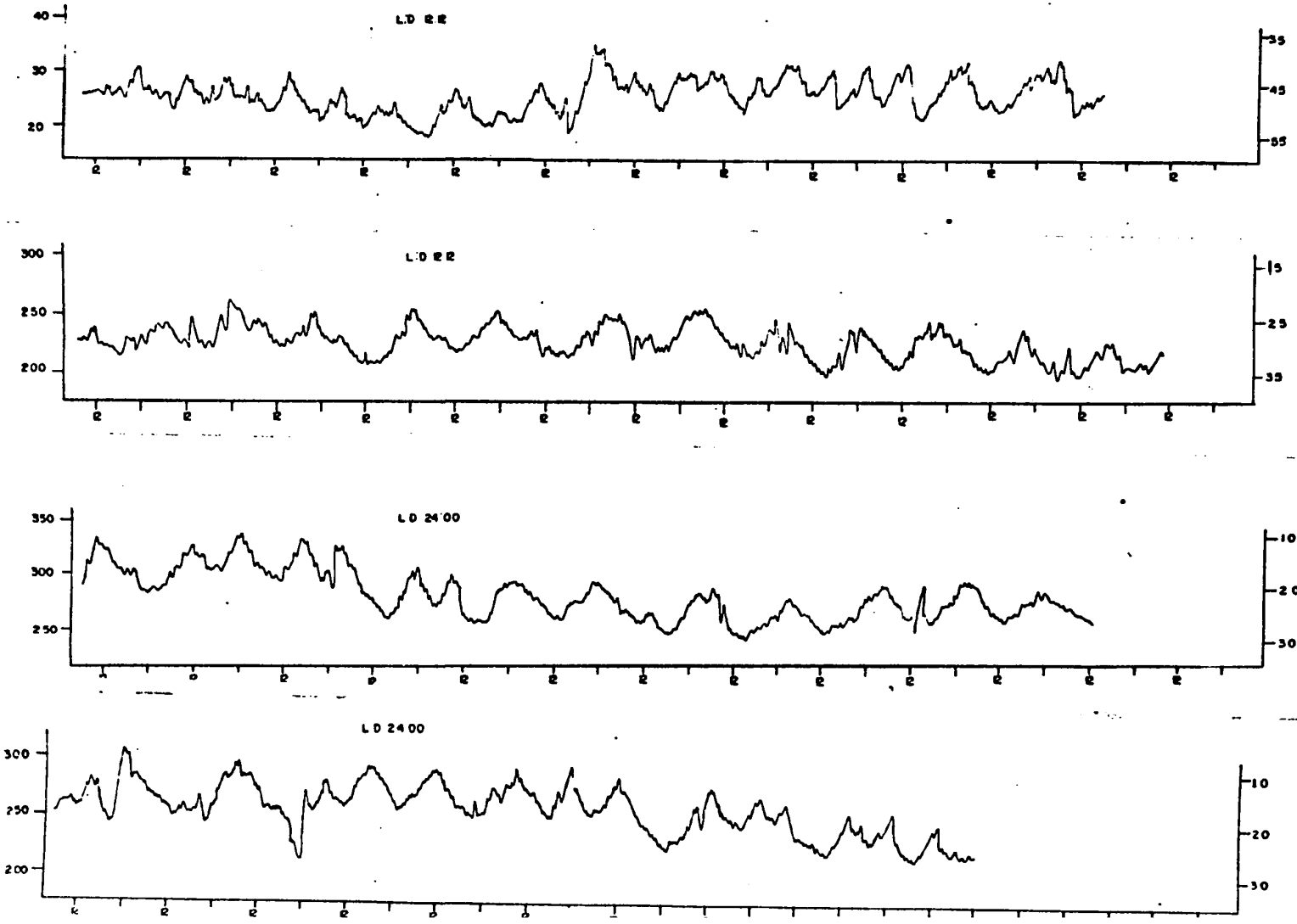
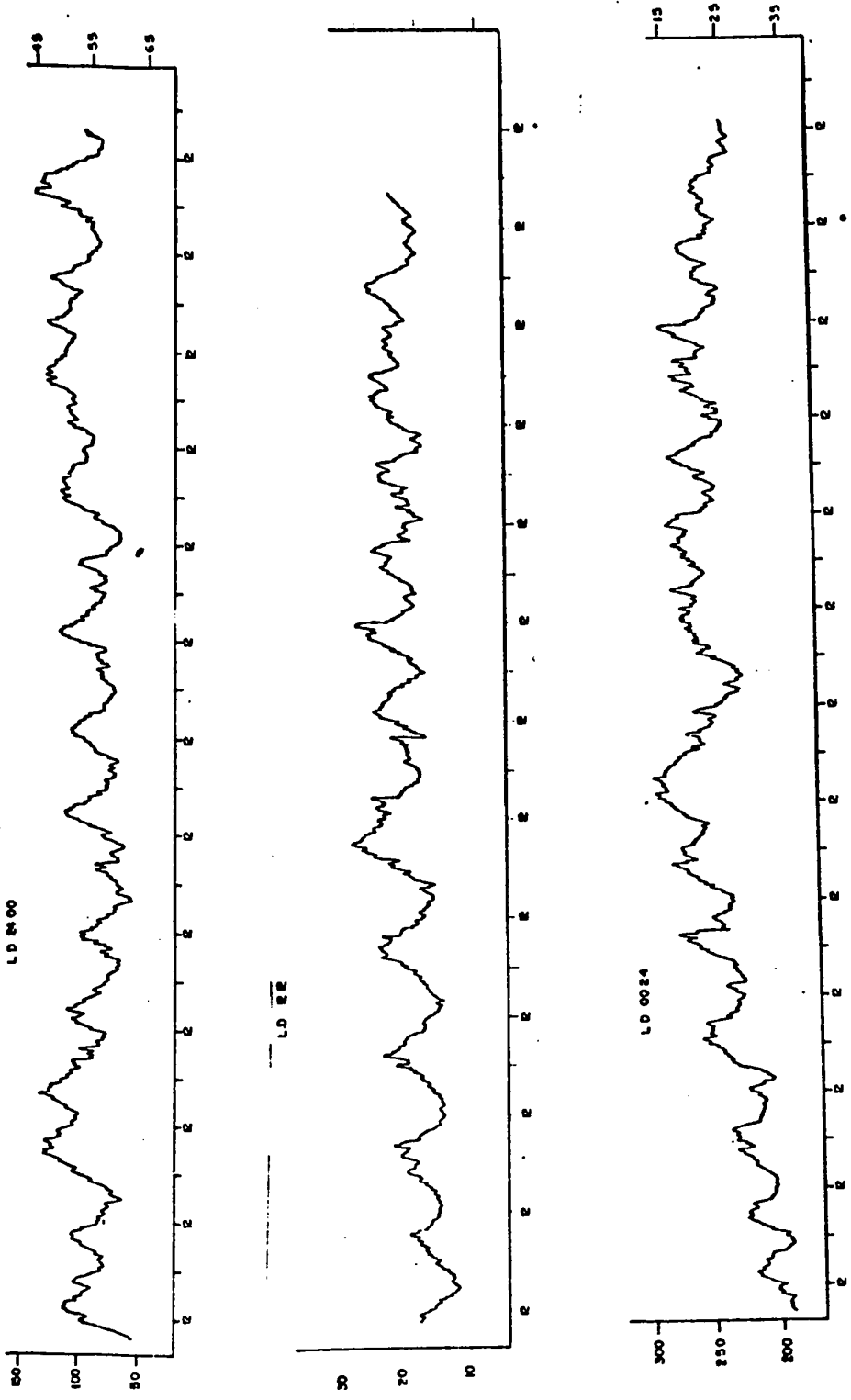
LD 0808
LD 0808


LD 22
LD 22
LD 24'00
LD 24:00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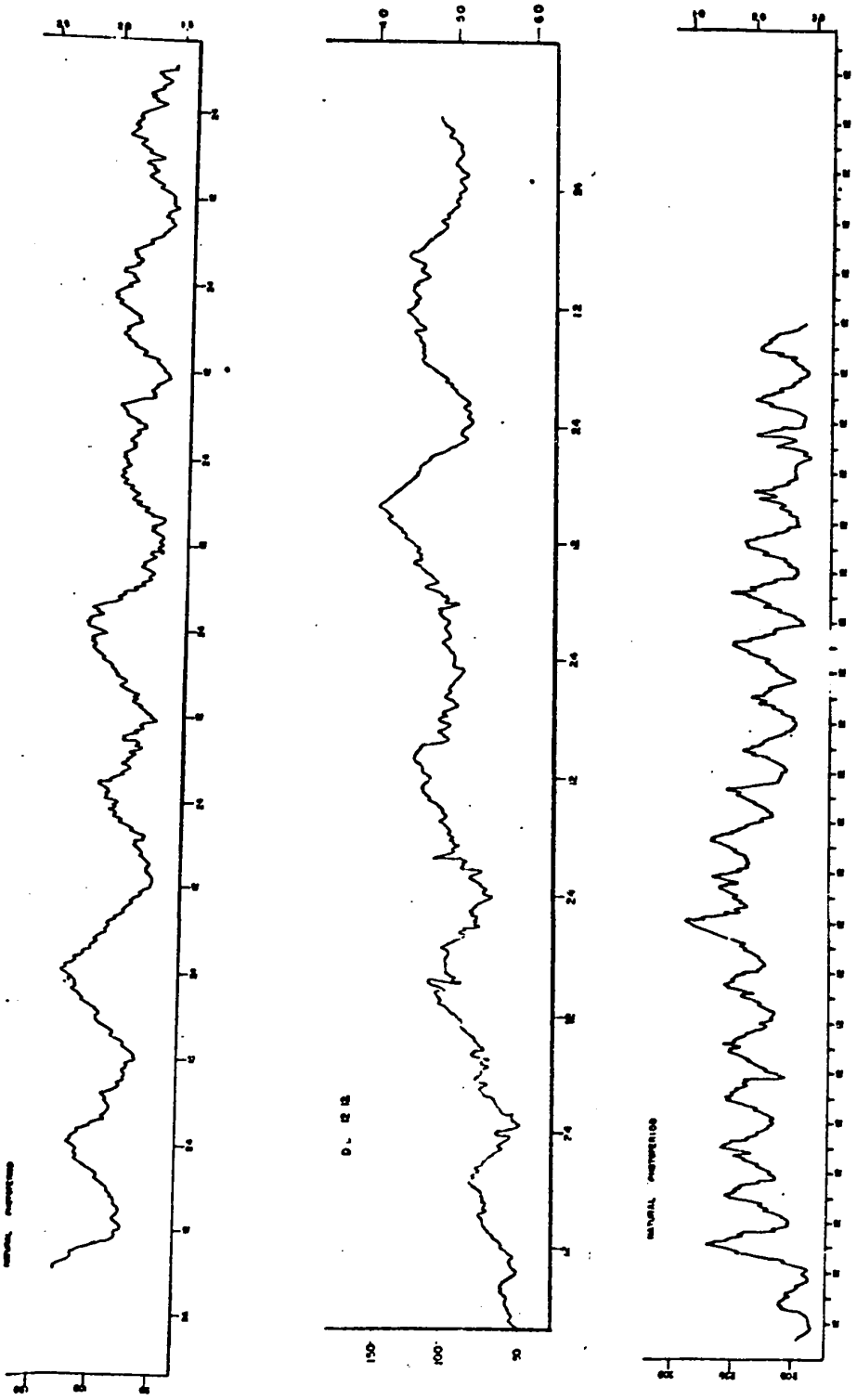
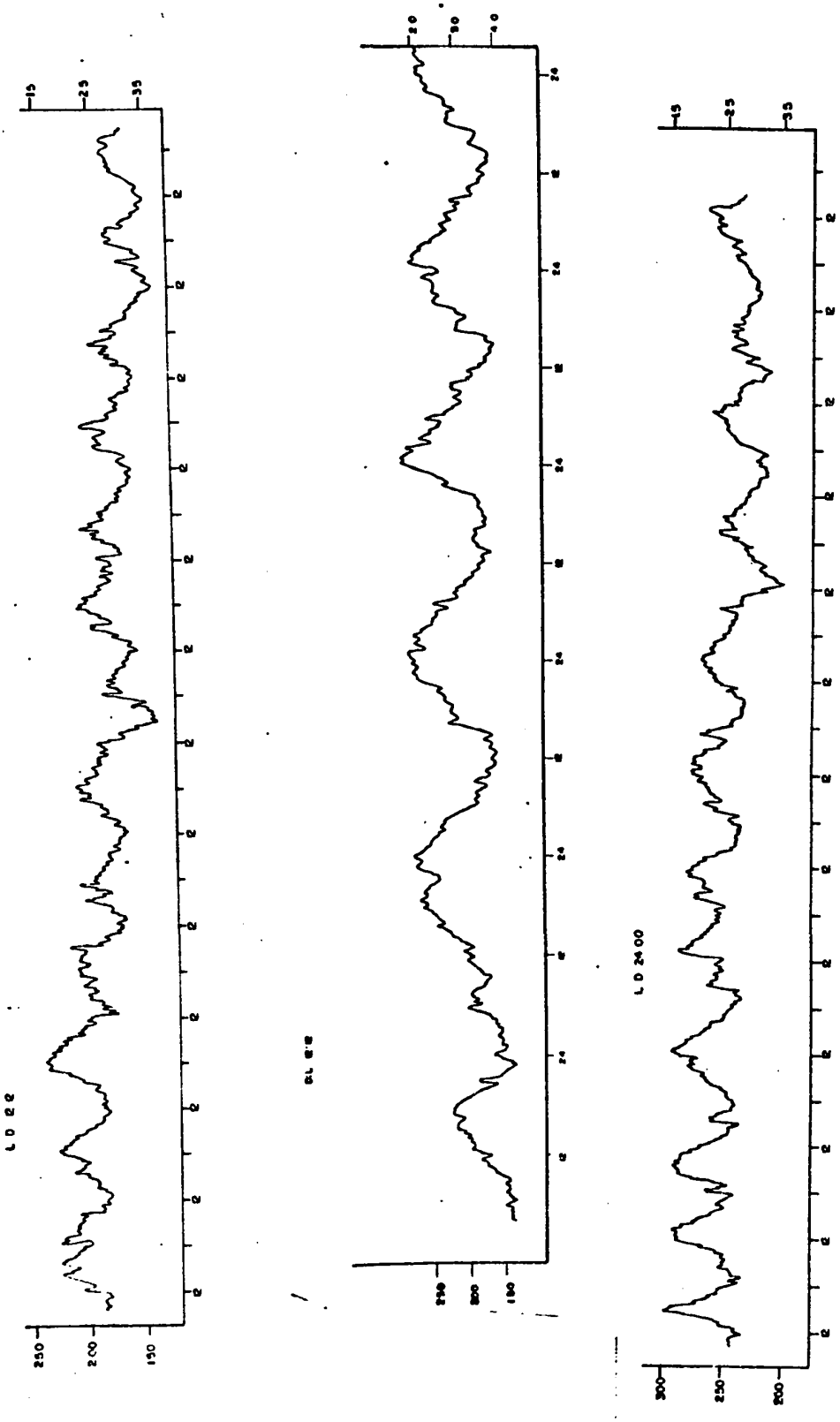
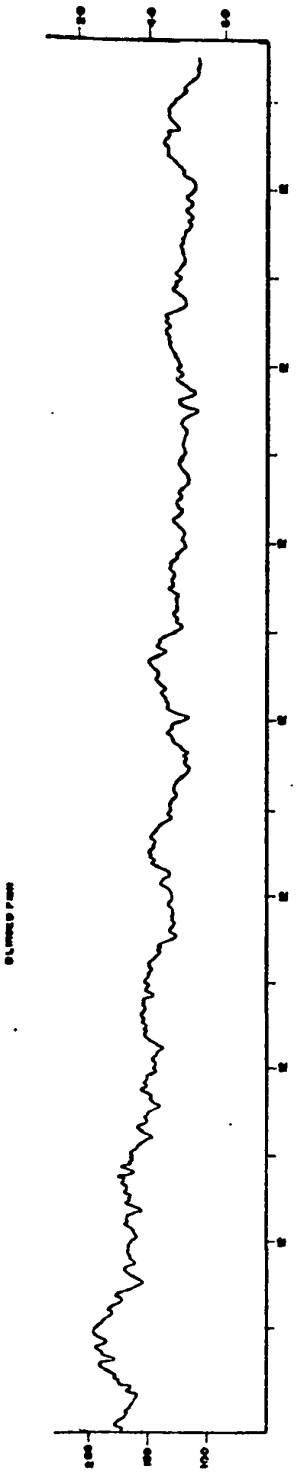
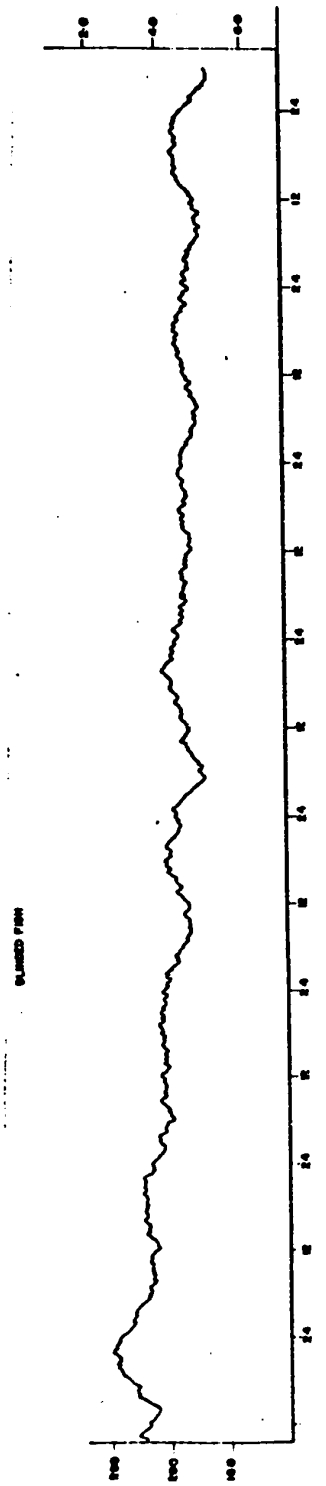
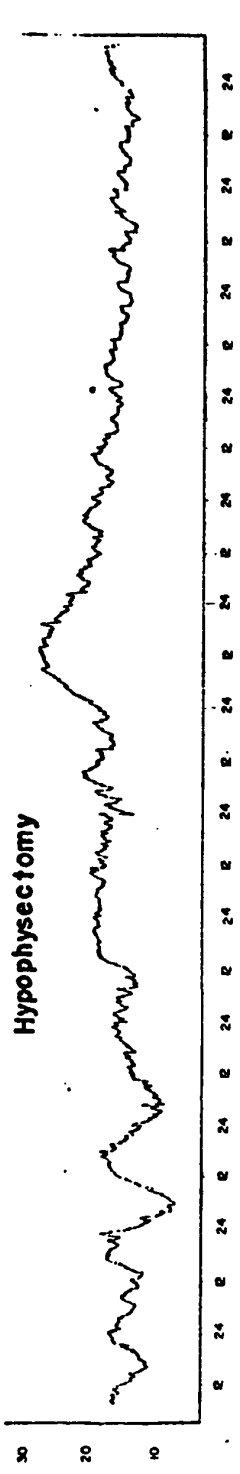
LD 22
LD 0024







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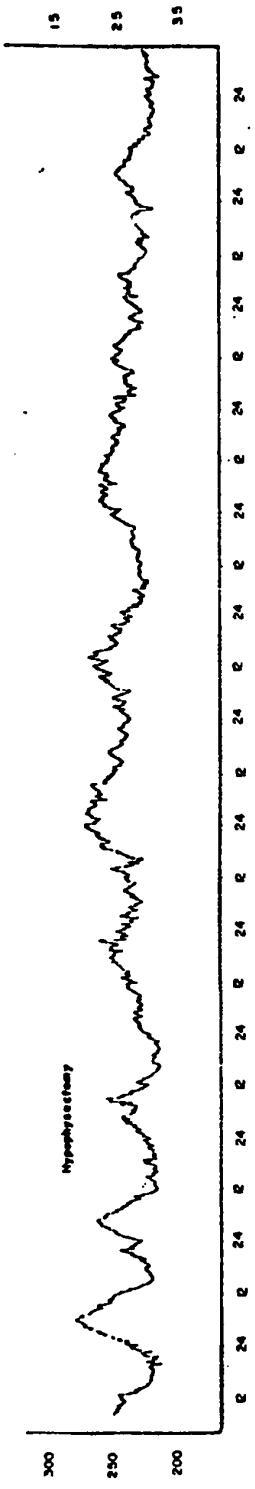
10
0
24
0
24


35
25
15
Hypophysectomy
300
250
200
0
24