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Review

Circadian Activity Rhythms in Crayfish and Some Pending Issues: A Historical Review

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Simple Summary

Many organisms possess internal biological rhythms, called circadian rhythms, by which they adjust their bodily functions and behavior to the daily cycle of light and temperature. In nocturnal crayfish, a rhythm of activity keeps them hidden during the day, thus avoiding excessive heat, and active at night, integrating into their ecological community at the opportune moment. Even under constant laboratory conditions, without changes in light or temperature, they maintain a nearly 24-hour freerunning rhythm. This review examines the main hypotheses, experiments, and results on how this activity rhythm works in crayfish. It begins with studies from the late 19th century and concludes by summarizing current knowledge and questions that remain open for future research.

Abstract

Among other adaptations, circadian rhythms provide crayfish with an appropriate timing to respond to daily environmental changes, such as fluctuations in light and temperature, and to maintain a coordinated temporal sequence of physiological and behavioral functions. In addition, the circadian rhythm of activity places them within their ecological communities at opportune times. In relation to this rhythm, some experiments have been re-examined, along with their results and interpretations, as well as the authors' proposals on the mechanisms responsible for generating the rhythm under either entrained or freerunning conditions. Among these proposals are the Schallek model and the Fingerman and Lago model. During this re-examination, some issues in need of reconsideration were identified, and information was found that may prove useful for addressing them. At the end of the review, the distinctive features of this circadian rhythm of activity in crayfish are summarized, and the design of experiments aimed at advancing the understanding of the mechanisms underlying these traits is outlined. These efforts aim to improve the understanding of the mechanisms that generate and regulate this rhythm.

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1. Introduction

To contextualize the following historical review of research on circadian rhythms of crayfish activity, it is necessary to recall three cognitive constructs that have been crucial to the development of science in general and physiology in particular: the definition of cause, the localization of functions, and the notion of mechanism. In the book The Assayer, Galileo (1623/1984) wrote his operational definition of cause: It is certain that only that thing should be called a cause which, when put in place, results in an effect, and when that thing is removed, the effect is also removed. When, in line with this postulate, chronobiologists removed the possible causes—the changes resulting from the Earth's rotation—of daily rhythms, placing organisms under constant conditions of light and temperature, they discovered spontaneous rhythms, now called circadian rhythms (CRs). It should be noted that this operation, which is fundamental to understanding these rhythms, rarely occurs naturally. Galen

(129-216 AD) was a pioneer in assigning specific functions to the different organs of the human body. He asks us to distinguish the actions of organs from their uses, understanding "use" as the indispensable contribution of the activity of each organ to the activities of the other organs and, therefore, of the body as a whole (Penella & Hall, 1973). Following this methodological consideration, some studies on CRs focused on determining the location where these rhythms are generated in organisms; however, it encountered difficulties in finding them. It wasn't until 1960, when Pittendrigh pointed out, that we concluded that the organism comprises a population of quasi-autonomous oscillatory systems. In "The Treatise on Man", Descartes (1664/2009) assumes that the human body is nothing more than a machine with all the necessary parts to perform its functions, plus many others we can imagine, which come solely from matter and depend solely on the arrangement of the organs. Thus, in line with Pittendrigh's conclusion and this methodological assumption of Descartes, a trend in research on RCs should be the search for explanations of the mechanisms by which these autonomous oscillatory systems are coordinated in entrainment and freerunning. In this work, we reconstruct the development of these inquiries regarding the rhythms of locomotor and motor activity in crayfish, from daily rhythms to CRs.

2. A Hypothesis for the Daily Rhythm of Activity of the Crayfish

According to Huxley (1880: 111), “the nervous system of the crayfish may be regarded as a system of coordinating mechanisms, each of which produces a certain action, or set of actions, on the receipt of an appropriate stimulus”. He continues with a differentiation of the internal or external origin of these stimuli, their perception, transmission and responses:

> A large proportion of these stimuli come from without through the organs of the senses. The greater or less readiness of each sense organ to receive impulses, of the nerves to transmit them, and of the ganglia to give rise to combined impulses, is dependent at any moment upon the physical conditions of these parts… On the other hand, a certain number of these stimuli are doubtless originated by changes within the various organs which compose the body, including the nerve centres themselves. [Huxley, 1880: 112].

Linked to this differentiation, Huxley (1880) referred to the concept of “spontaneous activity”: when an action arises from conditions developed within the body of an animal, because we cannot perceive the antecedent phenomenon, we call said action “spontaneous activity”. Within this theoretical framework, He formulated a hypothesis for the daily rhythm of activity of *Astacus astacus*, he supposes that crayfish “are intolerant of great heat and of much sunshine, they are therefore most active towards the evening, while they shelter themselves under the shade of stones and banks during the day [Huxley, 1880: 8]”. It should be noted that this refers to the adaptive value of variations in activity, not the adaptive value of its periodicity; the construction of cognitive assimilation schemes appropriates for understanding this function was just beginning.

3. First Recordings of Crayfish Activity on the Kymograph

Just as there were theoretical constructs that influenced the development of physiology, there were also material constructs that influenced it. Since a cause is that which produces an effect, and when that is removed the effect ceases to occur, both the effect and its absence must be recorded. One answer, among many others, to this need was given when, in 1846, Karl Ludwig introduced graphic recording in physiology using a kymograph with a continuous inscription surface and an ink marker, which years later would become the starting point of countless modifications (Rothschuh, 1974) to record the movements of tissues and organs of animals and plants, as well as of whole animals.

Szymanski (1918) considered that the first methodological requirement for addressing the question of activity and rest in animals and humans was to determine what objective characteristics distinguish these two states, and that, among them, the distinctive feature is movement. In accordance with this consideration, he used the kymograph to record the movements of blowflies, crayfish, worms, snails, rabbits, cats and a puppy. Regarding the crayfish, the recording conditions were like those experienced by this crustacean in its natural habitat. Each animal remained connected to the kymograph continuously for 24 hours. The crayfish recorded three types of tracks:

1) a straight line that occurs from time to time between the small peaks, this line, which was recorded during the day corresponded to the resting state; 2) a line with small points in a small number, this line, which was usually written during the night hours, corresponded to the state of low mobility and 3) a line with many large vertical strokes closely juxtaposed, this line which is usually observed during the night hours, corresponded to the state of lively movement (main phase of activity).[Szymanski, 1918: 437]

To make the processing of curves of periods of rest and activity more suitable, Szymanski (1918) translated the curves drawn by animals into diagrams that he called ‘Aktogramm’. These show that crayfish have only two main periods in a 24-hour cycle: a long rest period during the day and a period of intense activity at night. The period of lively movement usually occurs at the beginning of the main activity period, lasts on average about 50 minutes, and is located between 7 and 9 pm (Szymanski, 1918). This operation of dividing the recordings into only two periods, on the one hand, promoted the advancement of knowledge of the activity rhythms in crayfish, on the other, as will be seen later, it postponed the postulate of the participation of multiple oscillations in the CRs of these decapods. Were the three types of curves generated by three different functional states of one oscillator or by three coupled oscillators? Although not explicitly addressed, this is an underlying issue in research on CRs.

4. Nerves, Neurohumors and the Crayfishes' Locomotor Response

4.1. Simultaneous Recording of the Movements of Two Legs

To record the movements of the second legs on the right and left sides of *Procambarus clarkii*, Kropp and Enzmann (1933) connected these legs to a kymograph. Then they exposed both eyes to a light stimulus, covered one eye, covered both eyes, and cut one of the two optic nerves. Under illumination: a) when both eyes were exposed to light, there were no significant differences in either the frequency or amplitude of the movements; the leg movements on both sides were equal in number, but did not occur synchronously; b) when exposing one uncovered eye to light and covering the other eye, a marked difference was observed; the movements of the legs on the side of the exposed eye were more frequent and of greater amplitude; c) when the exposed eye is covered, thus covering both eyes, the leg movements become approximately equal in frequency and amplitude and a certain synchrony is established.

In the introduction to their article, Kropp and Enzmann (1933), report that the doctrine of animal tropisms postulates that the stimulus that acts asymmetrically on the sensory organs is mediated by the central and peripheral nervous system in such a way that it establishes a state of differential muscle tone on the two sides of the reacting animal. This was a hypothetical mechanism they intended to test, but they didn't strictly measure muscle tone; they measured the frequency, amplitude, and synchrony of the crayfish's leg movements. Although Kropp and Enzmann (1933) recorded short-term responses in their experiments, their results are presented here because a) they compare the movements of two bilateral effectors; b) they add a third variable to amplitude and frequency for the description of rhythmic movements, the phase relationship between two rhythms. and c) they hypothesized that the stimulus acting asymmetrically on the sensory organs is mediated by the central and peripheral nervous systems in such a way that it establishes an asynchrony between the movements of the two legs. However, given that a certain synchrony was established with both eyes covered, it is worth asking: if the organisms had been exposed to darkness instead of covering their eyestalks, would the asynchrony have been maintained?

4.2. Regulation of Daily Activity in Crayfish

Kalmus (1938) considered it worth investigating whether the regulatory centre of daily activity in crayfish is in the eyestalks. Unlike the experiment in the dark with a nocturnal maximum, the removal of the eyestalks would have to alter the regular daily distribution of motor activity; this is what happened. For his experiments with a European crayfish (possibly a species of the genus Potamobius -Shalleck, 1942-, currently Austropotamobius), Kalmus (1938) modified the recording method used by Szymanski (1918). Injected them with extracts of the crustaceans, of the stems, and of other substances. With uncovered eyes, a record was obtained with a precise nocturnal maximum, this distribution of activity was maintained for several weeks. In an record at 10 days after bilateral blindness, there is no peak activity within a rather low activity. On the second day after the removal of both eyestalks, there is an alternation of short periods of activity and rest, and an intermediate state of two to several days can be determined, in which short phases of increased and decreased activity may occur. In a recording, after 8 days with eyes covered + injections of 0.5 cm^3 of filtered extract of eyestalks of the brown shrimp, Crangon crangon, corresponding to 3 adult crayfish, a clear increase in activity is observed for several hours. In the control of the previous experiment, after an injection of 0.5 cm^3 of distilled water, no increase in activity was observed.

From the perspective of Kalmus (1938), with the positive results of his experiments, daily motor activity is regulated by a centre located in the eyestalk of the crayfish. In connection with the question, I posed as to whether Kropp and Enzmann (1933) had exposed the crayfish to darkness instead of covering their eyestalks, would the asynchrony of leg movements have been maintained? A comment is in order. Kalmus (1938) reports that a recording with a pronounced nocturnal maximum was obtained in the dark, and that in a recording after bilateral blindness, no maximum activity was observed within a rather low activity range. Although these recordings correspond to the movements of the entire individual, it should not be forgotten that when Kropp and Enzmann (1933) cover both eyes, the bilateral leg movements become approximately equal in frequency and amplitude, and a certain synchrony is established. Could it be that crayfish require background illumination, even in the dark, for the asynchrony of leg movements and the genesis of maximum peaks in circadian rhythmicity, which are lost when both eyestalks are covered?

4.3. The Schallek Model

Schallek (1942) attempted to demonstrate whether the effect of eyestalk removal on crayfish activity is due to nervous or endocrine elements of the eyestalk. Much of the work was done with *Faxonius virilis*, some with *P. clarkii* and *Lacunicambarus diogenes*. Activity was recorded on 24- or 48-hour kymographs using a method adapted from Kalmus (1938). Like this scientist, Schallek (1942) made basal recordings, removed the eye stalks and injected extracts, but as if that were not enough, he implanted the sinus gland and cut the optic nerves and the nerve cord in its portion anterior to the sixth abdominal ganglion.

Schallek (1942) observed a daily activity rhythm in all normal crayfish, which persisted for at least five weeks under constant darkness. In present-day terms this activity rhythm is a circadian rhythm (CR) under freerunning conditions. “About half of the animals showed a unimodal rhythm and half a bimodal rhythm. The activity peaks of unimodal animals generally occurred at noon or at midnight, while those of bimodal animals were usually at dawn and dusk… Although the type of rhythm is usually constant for any individual, the time and duration of the activity may vary from day to day, while one type of rhythm may change into the other [Schallek, 1942: 157 and 158]”. These variations should be noted, as they provide clues about a probabilistic structure in the circadian rhythm system of the activity of crayfish.

Schallek (1942: 161) observed that “when eyestalkless crayfish were exposed to normal daylight and darkness, they show a reversal of the activity of normal animals, becoming more active during the day than at night”. Control experiments in a room at constant temperature showed that this effect was due to light and not to temperature changes. He related these results to those obtained previously by Prosser (1934a) and Welsh (1934) and formulated a hypothesis. The first had discovered a photoreceptor in the sixth abdominal ganglion of the crayfish; the second had determined that illumination of this ganglion causes an increase in motor activity. The hypothesis was that "if this receptor was causing the increased activity of eyestalkless animals in daylight, it could be eliminated by cutting the nerve cord just anterior to it. This operation resulted in eliminating the difference [Schallek, 1942: 161]". He concluded that the "increased activity of the eyestalkless animal following illumination of the caudal photoreceptor is a typical photokinesis… and that, however, in the intact animal, this response is masked by the activity of the higher centres [Schallek, 1942: 162]". Although both statements are acceptable, it should not be forgotten that Huxley (1880), whose book is included in the references, considered the crayfish nervous system to be a system of coordinating mechanisms. It is therefore possible that, in normal specimens, the masking is a coordinated response between the sixth abdominal ganglion and the higher centres. Intertwined with this possibility, it can be recalled that the spontaneous and light-induced electrical activity of this caudal photoreceptor showed circadian variations, with its activity being more intense during the night than during the day (Rodríguez-Sosa et al., 2011).

In the discussion of his article, Schallek (1942) formulated a model, whose diagram is displayed in Figure 5 of his account. In it he integrates his results with the observations of other researchers. He wrote that: a) Kalmus (1938), using the European crayfish *Austropotamobius*, found that removal of the eyestalk resulted in irregular activity for several days, followed by continued inactivity; b) in contrast, his own work with other species shows that "the animals remain continually active as long as they live", and c) the differences in results are possibly due to physiological variations between the species used. There is more. Schallek (1942: 162) mentioned that his experiments "suggest that the crayfish is normally active and that its activity is inhibited during the day by the action of the nerve centers in the eyestalk". Schallek (1942: 163) noted that "the activity of the limbs is inhibited by fibers passing down the optic nerve and crossing the mid-line of the brain. These conditions are filled by the fibers which form the optic chiasma (…). Impulses from these fibers seem to inhibit the activity of the crayfish during the quiet phase of its [daily] rhythm". He recalls that Kropp and Enzmann (1933) covered one eye and exposed the uncovered eye to light, causing leg movements on the side of the exposed eye to be more frequent and of greater amplitude. According to their model, this can be explained based on the results of their experiments and the postulation that darkening one eye might decrease the activity of inhibitory fibers that cross the midline of the brain. "Such unilateral activity would be difficult to explain from an endocrine perspective. If impulses from inhibitory centers control the quiet phase of the activity rhythm, then these impulses should stop during the active phase [Schallek (1942: 164].

4.4. Background of the Schallek Model

To support his model, Schallek (1942) cites Vulpian (1866), Ward (1879), Bethe (1897), and Prosser (1934b). Vulpian (1866) made a transverse section of the ganglionic chain of *Astacus*, at the level of one of the intervals separating the abdominal ganglia. Some spontaneous movements could be observed in the false abdominal legs, simultaneous, regular, rhythmic, with normal characteristics. Ward (1879: 216-217) stated that "so long as the highest ganglia are connected with the rest of the (ganglionic) chain by a single commissure, there is no Iack of spontaneity and purpose in the movements of the crayfish". But with the section of the two supra-oesophageal commissures all this type of movement disappears, crayfish so treated were often found on their backs and perfectly still, or with only their swimmerets in motion, but still oftener with most of their limbs engaged in a peculiar and monotonous rhythmic swing. From Albrecht Bethe's (1897) summary of his experiments with *Astacus*, the following description is derived: after the removal of the brain, spontaneous movements, movements whose external causes are not demonstrable, not only do not disappear, but are even stronger than in normal animals. He also observed that "even by cutting the longitudinal commissures in a caudal position, spontaneous movements were not suppressed [Bethe, 1897: 484]" and asserted that "as a consequence of this one must look for an inhibitory centre in the brain [Bethe, 1897: 484]" of the crayfish. Prosser (1934b) discovered spontaneous activity in the nervous system of the crayfish.

4.5. Roberts' Contributions to the Understanding of Crayfish Activity Rhythms

As early as 1942, Roberts distinguished between what is now known as entrainment and freerunning, and referred to the endogenous nature of CRs, as well as their adaptive value. Several facts indicated to him that nocturnality is produced, at least in part, by an endogenous mechanism that tends to synchronize a locomotor rhythm with the natural occurrence of day and night: "Endogenous synchronization of rhythms with the occurrence of day and night can be acquired, while the endogenous tendency for activity to occur at 24-hour intervals is already present [Roberts, 1942: 366]". Regarding the adaptive function of rhythms, this scientist recalls that the possible advantages of nocturnal or diurnal behaviors had already been raised and were believed to explain the natural selection of the mechanisms that limited the appearance of these activities at an appropriate time in communities [Roberts, 1942: 387]". For example, the tendency of *F. virilis* to limit its locomotion outside its burrows to the nocturnal period is an adaptation that protects this species from potential predators (Roberts, 1944). Intertwined with the 24-hour period, prior to Halberg (1959), Roberts (1942) observed the distinctive quality of RCs: "The average interval in occurrence of locomotor activity of the crayfish, *F. virilis*, when kept in constant darkness, is about 24. hours (23.8 h) though the intervals between peaks measured to date vary from ten to forty hours… This indicates that the physiology of the crayfish, *F. virilis*… is probably in accord with a 24-h plan, even though no fixed endogenetic rhythm is present after animals remain under constant conditions for a few days [Roberts, 1942: 366]".

Roberts (1944) was interested in determining the extent to which external and internal factors regulate the locomotion rhythm of *F. virilis*. In figures three and four of his 1944 study, he presents two graphs of the average activity of 25 animals each. In both cases, the crayfish were under illumination for 12:12 (starting at 6:00 a.m.); the photoperiod in figure four was a fluctuation. In figure three, the temperature fluctuated naturally between 5 and 9 °C, and in figure four, it remained constant. A pronounced increase in locomotion immediately followed a marked reduction in illumination in each graph. This appears to be the peak that Page and Larimer (1972) termed the "lights-out" peak, whereas the other peak, the "lights-on" peak, was only observed under natural temperature fluctuations.

Regarding the activity of *F. virilis* under constant lighting conditions, the average activity of animals kept in constant darkness occurred mainly at night for the first few days and subsequently became irregular. Peaks occurred earlier or later, until half were diurnal; after six or seven days, half the individuals were nocturnal and the other half diurnal (Roberts, 1944). In my opinion, if the variation between peaks was ten to forty hours, these lags were predictable; what is surprising is the precision of the half-and-half. The average activity of crayfish under continuous illumination, without the use of shelters, showed virtually no significant activity peaks. It seems that, perhaps as in Viccon-Pale (2022), under this constant light condition, the number of ultradian oscillations increased, which in turn caused a flattening of the main peak.

According to Roberts (1944), there is an endogenous tendency in crayfish to move in continuous darkness at twenty-four-hour intervals, which persists for several days. However, this tendency disappears immediately after the removal of the eyestalks. Locomotion is no longer inhibited by exposure to light. Eyestalkless crayfish remain active almost continuously as long as food and fresh water are provided, either in darkness or under fluctuations in natural light. This indicates that light exerts its inhibitory influence through an ocular mechanism, that the initiation of locomotion in *F. virilis* is normally controlled by light, and that the internal mechanism regulating the daily locomotor rhythm of normal crayfish is dependent on the eyestalks, regardless of the presence of light.

To better support these indications, Roberts (1944) performed other surgical maneuvers. Severing the antennal nerves had no definite influence on the activity of *F. virilis*, demonstrating that the influence of eyestalk removal was not due to nerve stimulation. The Injury to the eyestalk tissue resulted in increased locomotion, although no nerves were severed, some of the increased intensity persisted indefinitely, but partial recovery usually occurred after the injury. Eyestalk ligation increased locomotion and generally maintained a high intensity level indefinitely, regardless of whether the crayfish were exposed to continuous darkness or natural light; this ligation was expected to prevent the release of a potential locomotion-inhibiting hormone. Roberts (1944) proposed a possible mechanism to explain these results: a) when crayfish are exposed to light, a locomotion-inhibiting hormone is released from the eyestalks into the body; b) locomotion inhibition in crayfish, in addition to being controlled by inhibitory nerve fibers extending from the brain to the legs, depends on such a chemical originating in the eyestalks; and c) other chemicals may be secreted by nerves at the site of action (neurohumors). To begin to complement Schalleck's (1942) model and Roberts' (1944) contributions and in line with what follows, it is worth highlighting that, in crustaceans, daily rhythms and CRs have been discovered in neurotransmitters, such as dopamine (Rodríguez-Sosa et al., 2011; Li et al., 2019), octopamine (Kass et al., 1983; Kass & Barlow, 1984 & 1992; Battelle et al., 1999; Bolbecker et al., 2009) and serotonin (Sandeman et al., 1990; Agapito et al., 1995; Escamilla-Chimal et al., 1998; García & Aréchiga, 1998; Castañón-Cervantes et al., 1999; Escamilla-Chimal et al., 2001; Fanjul-Moles & Prieto-Sagredo, 2003; Wildt et al., 2004; Calderón-Rosete et al., 2006; Rodríguez-Sosa et al., 2006; Rodríguez-Sosa et al., 2007; Rodríguez-Sosa et al., 2008; Strauss and Dircksen, 2010; Valdés-Fuentes et al., 2011), which, in the years in which Roberts (1942 and 1944) made his inquiries, were known as neurohumors.

4.6. The Fingerman and Lago Model

Fingerman and Lago (1957) undertook an investigation to determine whether *Faxonella clypeata* exhibits a CR in oxygen consumption and locomotor activity. Some results relating to the latter. In normal animals, under constant illumination two types of records were obtained. One type was produced by more active specimens between midnight and noon (TA). The second type was produced by exemplars more active between noon and midnight (TB). The population consisted of approximately equal numbers of two types of individuals. The amplitude of the activity rhythm gradually diminished in specimens keep in under constant illumination. Analysis of the records revealed that eyestalkless specimens possess a 24-hour locomotor activity rhythm, in spite of their hyperactivity. Eyestalkless crayfish exhibited the same two types, A and B, of rhythms of locomotor activity. To try to explain these results, Fingerman and Lago (1957) formulated a hypothesis based on the existence of two rhythmicity centers in *F. clypeata*. Each center would have its own rhythmicity throughout the 24 hours. One center would have an excitatory effect on locomotor activity, and the other an inhibitory effect:

Center I, the main center of rhythmicity, would be located in the supraesophageal ganglia and would activate the neurosecretory cells of the central nervous system. These neurosecretory cells would produce a hormone that would induce an increase… in general locomotor activity throughout the 24 hours of the day, with a peak at 6:00 a.m. or 6:00 p.m. Center II, located either in the central nervous system or in the nervous sues of the eyestalk, has an inherent 24-hour rhythmicity of its own. In contrast to center I, center II acts upon the sinus gland causing the gland to secrete a hormone which inhibits locomotor activity... The activity-inhibiting hormone acts as a "brake" on activity and is most active at times when center I is capable of inducing maximum locomotor activity prior to 6 A.M. in type A or 6 P.M. in type B crayfishes… After eyestalk removal, the target organ of center II, the sinus gland, is removed. and center I alone determines the form of the 24-hour locomotor activity rhythm. The "brake" has been removed. There-fore, activity is governed solely by center I and the activity-inducing hormone. Without the "braking" action of center II some crawfishes are so active at all times of day that their rhythmical behavior is not evident. Center I probably determines whether a crawfish will be type A or type B since the rhythm type is unaltered after eyestalk removal. [Fingerman and Lago, 1957: 392]

5. The Daily Rhythm in the Migration of Distal Pigments in the Eyestalk

When Roberts (1944) postulated that it was reasonable for eyestalk ligations to prevent the release of a hormone that inhibits locomotor function, he referred to the "Welsh method" (Welsh, 1930a). Indeed, this scientist found that in the eyes of other decapods, such as prawns, *Macrobrachium acanthurus* and *M. olfersii*, under constant illumination, the distal pigment cells “continue to show a daily movement, migrating distally at about 6: 00 P.M. and remaining in this position until about 5:00 A.M. the following morning [Welsh 1930b: 394]”. Similarly, Welsh (1930b: 394) found that "the distal pigment cells in excised eyes assume the position characteristic for the light whether they are kept in the light or in the dark [and that] ligation of the eyestalk prevents both distal migration in the dark and the diurnal migration of the distal pigment cells”. And since he knew that the daily movements of distal pigment cells parallel the changes in activity in animals under constant external conditions, it seemed to him that both types of movements could be controlled directly by the blood and indirectly by the nervous system. In the following, some results are given in this regard.

6. Entrainment and Generation of the Circadian Rhythm of the Crayfish

Under constant temperature and light cycles (LD 12:12), *P. clarkii* exhibits a bimodal locomotor activity rhythm. One peak of activity, the "lights-on" peak, is synchronized with the onset, whereas the other, the "lights-off" peak, occurs shortly after the end of the light (Page and Larimer, 1972). As will be recalled, in *F. virilis*, a marked reduction in illumination is followed by a pronounced increase in locomotion (Roberts, 1944). This appears to be the "lights-off" peak, whereas the other peak, the "lights-on" peak, was only observed under natural temperature fluctuations, not at constant temperature. Could this difference be since they are different species? Furthermore, placed in constant darkness (DD), *P. clarkii* maintains a freerunning, unimodal CR, involving only the "lights-off" peak of activity (Page and Larimer, 1972).

In *P. clarkii*, bilateral ablation of ommatidia of both eyes, or bilateral section of the optic lobes between the lamina ganglionaris and medulla externa, led to a several increase in daily activity, however, quantitative measurements of the locomotor activity revealed that a CR in the level of activity persists for 6-12 days following eyestalk ablation (Page and Larimer, 1972 & 1975). This surgical maneuver obliterates the "lights-on" peak but does not affect entrainment of the "lights-off" response. Thus [according to Page and Larimer, 1972: 107], the retina provides the pathway necessary to generate 'lights-on' activity but is not required for circadian rhythm entrainment". Furthermore, ablation or isolation of the sixth abdominal ganglion, the caudal photoreceptor, from the CNS has no observable effect on activity. In line with Page and Larimer (1972), this result may indicate that the caudal photoreceptor is not required for entrainment or the initiation of either of the two activity maxima. Therefore, if ablation of both the caudal ganglion and the retina does not eliminate rhythm entrainment, it can be assumed that *P. clarki* possess an extraretinal-extracaudal photoreceptor that provides a sufficient pathway for the entrainment signal (Page and Larimer, 1975).

Therefore, Page and Larimer (1975) attempted to determine whether the synchronization of two CRs, that of locomotor activity and ERG amplitude, in crayfish involved extraretinal photoreception. They monitored the movements of the first and fourth pairs of walking legs in intact and surgically altered *P. clarkii*. Sectioning the circumesophageal connectives (CEC) eliminated the “lights-on” and “lights-off” activity peaks from the right and left pereiopods. By severing the ventral nerve cord between the third and fourth thoracic ganglia, both peaks were preserved in the leg anterior to the severing and lost in the hind leg. “These results support the contention that the CR is mediated by axons in the ventral nerve cord and is not hormonally controlled [Page and Larimer, 1975: 64]”.

According to Page and Larimer (1975), the results of their experiments suggest that in *P. clarkii*: a) circadian oscillators for both the ERG amplitude rhythm and the locomotor rhythm are located within the supraesophageal ganglion and the latter is coupled to the thoracic locomotor centers through axons in the CEC; b) ERG amplitude rhythms and locomotor activity can be synchronized by an extraretinal pathway and c) the extraretinal photoreceptor may be located in the supraesophageal ganglion. They did not rule out the possibility of photoreceptive input from other regions of the CNS, particularly the optic lobe.

Regarding the CR of locomotor activity and photoreceptors in the brain of crayfish, Sullivan et al. (2009) investigated the ability of *Cherax destructor* and *P. clarkii* to adjust to 6-h phase shifts in the LD cycle, after surgical ablation of the two other known photoreceptors in these animals: the compound ocular photoreceptors and the caudal photoreceptors. The results of these experiments

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suggest that crayfish brain photoreceptors may function as extraretinal circadian photoreceptors, which are sufficient to synchronize locomotor activity rhythms with photic stimuli. To further investigate the potential contributions of the brain-photoreceptor pathway to circadian rhythmicity, they examined variations in the neuropeptide pigment-dispersing hormone (PDH) expression levels in the brain photoreceptor neuropils (BPNs) across the day/night cycle. In both *C. destructor* and *P. clarkii,* PDH levels in the BPN varied with time of day, reflecting the species-specific locomotor rhythm. Because the BPN is likely innervated by multiple PDH-expressing neurons, these results suggest that PDH levels in this neuronal population may cycle synchronously. In *C. destructor*, a significant diurnal rhythm in PDH levels was also observed in the somata of a pair of group 6 interneurons that were highly PDH-immunoreactive.

7. Relationships Between Circadian Rhythms of Activity and the Circadian Rhythm of ERG Amplitude in Crayfish

In isolated eyestalks of *Procambarus bouvieri*, under total darkness and constant temperature, Sánchez and Fuentes-Pardo (1977) measured the evoked electrical responses of visual photoreceptors, the electroretinogram (ERG), and observed the activity of pigmentary effectors. The amplitude of the ERG voltage showed circadian variations: the length of its period was close to 24 h and the oscillation damped and recovered. No circadian changes were noted in the activity of the pigmentary effectors. The ERG amplitude showed high-frequency oscillations that appeared to be related to the CR. From the authors' perspective, these high-frequency cycles likely result from multiple oscillators that, when coupled, give rise to CR variation.

Fuentes-Pardo and Inclán-Rubio (1981) recorded motor activity and ERG of *P. bouvieri*, for prolonged periods, under constant illumination and temperature. In simultaneous recordings, a 4-hour phase difference between these rhythms was generally observed. Locomotor activity reached its maximum value 4 hours after the ERG voltage peak. A change in the ERG-evoked flash frequency from 1/18' to 1/1800" of a second affected both rhythms. The durations of the Tr and circadian periods of the ERG rhythm were reduced to 12 h and 21.2 h, respectively. In the locomotor rhythm, the Tr was lengthened to 26 h, and the circadian period remained virtually unchanged, from 24.2 to 23.5 h. The change in stimulating flash frequency was followed by a brief transient and a prolongation of the ERG rhythm period, and a long transient with very little change in the locomotor rhythm period.

Figure 5 of the report by Fuentes-Pardo and Ramos-Carvajal (1983) shows a phase response curve (PRC) of the CR of the ERG amplitude of *P. bouvieri*. It shows phase advances when the stimulus was applied between 5 and 13 h circadian time (CT); null effects when the zeitgeber was applied between 13 and 20 h-CT; and delays when the stimulation was placed between 20 and 5 h-CT. At this point, the abrupt change from delays to advances occurred. CT = 0 was the point in the cycle at which the activity level exceeded 50% of the cycle amplitude, and CT = 24 occurred one full cycle later. For its part, Figure 4 of the article by Viccon-Pale and Fuentes-Pardo (1994) shows a PRC of the CR of motor activity of *Procambarus digueti* in response to a 15-minute light stimulus, measured on the fourth day after this, once the circadian system reached its steady state. The distribution of the advances and delays throughout the circadian cycle generates a bimodal curve with a very pronounced first peak around 5 h-CT and a second, less visible, peak at 15 h-CT. At this circadian time, $\Delta ø = 0$. This reflects a different response of the circadian system, since before and after 15 h-CT, $\Delta ø$ only represented delays. Although the leading peaks in the PRCs of the ERG amplitude and the motor activity of the crayfish coincide at 5 h-CT, there is a possible contradiction with the results of Fuentes-Pardo and Inclán-Rubio (1981), who found a phase difference of 4 h between these CRs in *P. bouvieri*. If there is no equivalent difference between the peaks of the two RPCs, is this because they belong to different species? Or because of differences in the protocol? For example, could it be that this difference also occurs at CT = 0, from which the phase difference calculations were made?

In the report by Viccon-Pale and Fuentes-Pardo (1994), regarding the synchronization of the CR of the activity of *P. digueti*, the phase transition curve (PTC) associated with the PRC of the activity and a graph of similarity between advances and delays produced by the light disturbance during the transient phase (abscissa) and the phase change produced by the same stimulus during the steady state (ordinate). The estimated slope of the PTC was -0.01, indicating that the rhythm resets to the same phase regardless of the CT of stimulus administration. In the graph, in Figure 5 of Viccon-Pale and Fuentes Pardo (1994), the transient-phase delays correspond only to the steady-state delays, whereas the transient-phase advances correspond to both steady-state advances and delays. These two sets of responses in that figure suggest the presence of at least two types of oscillators in the CR. In the advances-only quadrant, there are some points that are obviously separated from the rest. Could they represent the advances movement of ultradian oscillations?

8. Ultradian Oscillations in Circadian Rhythms of Crayfish Activity

In Figure 4 of the report by Park et al. (1941), there are graphs of ten recordings of the activity of the Mammoth Cave Crayfish, *Lacunicambarus pellucidus*, under constant darkness, in which ultradian oscillations can be observed. Also included in the figure is a graph showing the average of the data from these recordings, in which a bimodal rhythm can be seen, with a small peak, the "on" peak, at dawn (7 AM) and a more prominent peak at dusk (8 PM), the "off" peak. The researchers didn't normalize their data with CT. Since our understanding of CRs was just beginning, they lacked the analytical categories that would have allowed them to make the observations I'm making; they concluded that the activity was arrhythmic. Later, in 1961, Brown re-examined the data considering recent advances, and his results indicated that a statistically significant ($P<0.001$) 24-h rhythm is present in the activity of this species. With a minimum at approximately 9 AM and a maximum around 7 PM, there was also a statistically significant daily rhythm in the standard deviation. This contradiction between the original results, which suggest a bimodal circadian rhythm, and the re-examined data, which seem to indicate a unimodal pattern, places us in the dilemma of formulating a model that can accommodate either one or the other. A re-examination of the original data will be necessary.

In his report, Roberts (1944) notes that the average activity of *F. virilis* under continuous illumination, in the absence of shelters, showed virtually no significant peaks of activity. It is possible that these non-significant fluctuations correspond to ultradian oscillations.

When Viccon-Pale (2022) proposed that the zeitgeber (DL, 12:12 h) inhibits the prevalence of ultradian oscillations, the recording technique used did not allow for identifying which phase of the dark-light cycle produced this effect. Upon re-examining Figures 1–5 of Page and Larimer (1972), I now observe additional peaks—distinct from the 'lights-on' and 'lights-off' responses—that were not originally reported, and which likely correspond to ultradian oscillations. These peaks become evident during the dark phase and persist under constant darkness. This new observation suggests that it is specifically the photoperiod—the light phase of the DL cycle—that produces the inhibitory effect. In the third example in Figure 1, under DD, they even increase. Isolation of the caudal photoreceptor from the rest of the CNS by section of the ventral nerve cord between the fifth and sixth abdominal ganglia inhibited their incidence (Figure 2). In Figure 3, in the dark phase, there is a notable prevalence of these peaks, which decreases as the light cycle progresses, a decrease that is maintained after ablation of the caudal ganglion. In Figure 4, in the dark phase, after a few days these peaks become quite conspicuous, together with the light-off peaks, they synchronize with the delay, decrease with the ablation of the ommatidia and the lamina ganglionaris, and remain so after the cutting of the commissures and the advancement of the illuminated portion of the cycle (Figure 4). In Figure 5, during the dark phase of the light cycle, the peaks interpreted as ultradian oscillations are observed alongside the "lights-off" peak and the "lights-on" peak, and they disappear along with the latter as soon as the ommatidia and lamina ganglionaris are removed. They remain alongside the "lights-off" peak and increase significantly after the removal of the caudal ganglion. It is as if the ommatidia and lamina ganglionaris promote possible ultradian oscillations, while the caudal ganglion inhibits them.

9. A Summary of Some Observations and Some Pending Tasks

The CR of crayfish motor activity under entrainment is characterized by the presence of a "lights-on" peak, a "lights-off" peak, and ultradian oscillations. When placed in constant darkness (DD), this pattern shifts to a unimodal one, consisting only of the "lights-off" peak and ultradian oscillations. The main signals produced by Earth's rotation that entrain the CR are the daily variations in light and temperature. Regarding illumination, these signals are received, transduced, and relayed by the retinas of the eyestalks, the caudal ganglia, and the brain's photoreceptors. The results of the ablation experiments suggest that the ommatidia, the lamina ganglionaris, and the caudal ganglion are involved in the genesis of the "lights-on" peak, with the latter also playing a role in the inhibition of ultradian oscillations. The supraesophageal ganglion may be one of the sources of the driving oscillation that generates CR activity in crayfish. This oscillation would be transmitted to the thoracic locomotor centers via axons in the circumesophageal connectives. Without ruling out the possible participation of PDH in this process. However, most of the available results come from experiments conducted under entrainment, so the number of studies involving continuous recordings of the rhythm during both synchronized and freerunning conditions should be increased. In this context, eyestalk ablation could potentially be replaced with temporary blindness, a less invasive approach. Likewise, simultaneous recordings of other rhythms—such as ERG amplitude and distal pigment movement—could be incorporated. These recordings should be accompanied by simultaneous transcriptomic data, particularly on the expression of PDH and components of the dopaminergic, octopaminergic, and serotonergic systems, under the same experimental conditions. Activity rhythms can be recorded using idtracker.ai (Romero-Ferrero et al., 2019; Suryanto et al., 2025). The characterization of rhythm responses to both zeitgeber and freerunning conditions can be carried out by calculating the average and modal periods from the periodograms of the respective time series and fitting probability distribution models to the periodogram data (Viccon-Pale, 2022).

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Abbreviations

The following abbreviations are used in this manuscript:

CR	Circadian rhythm
CRs	Circadian rhythms

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