Entrainment of the Circadian Locomotor Activity Rhythm in Crayfish

The Role of the Eyes and Caudal Photoreceptor*

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Summary. 1. Crayfish (*Procambarus clarkii*) on a light cycle (LD 12:12) exhibit a bimodal locomotor activity rhythm. One activity maximum, the "lights-on" peak, is synchronized with the onset, while the other, the "lights-off" peak, occurs shortly after the offset of light (Figs. 1–5).

2. When placed in constant darkness (DD), these animals maintain a unimodal, free running circadian rhythm, involving only the "lights-off" peak of activity (Fig. 1).

3. Removal, or isolation from the CNS, of the sixth abdominal ganglion (the site of the caudal photoreceptor) has no observable effect on activity (Figs. 2, 3), indicating that the caudal photoreceptor is not necessary for entrainment or initiation of either activity maximum.

4. Removal of the ommatidia of both eyes, or bilateral section of the optic lobes between the lamina ganglionaris and medulla externa, obliterates the "lights-on" peak but does not affect entrainment of the "lights-off" response. Thus, the retina provides the necessary pathway for generating the "lights-on" activity, but is not required for entrainment of the circadian rhythm (Figs. 4, 5).

5. Finally, ablation of both the caudal ganglion and the retina does not abolish entrainment. It is assumed, therefore, that crayfish possess an extraretinal-extra-caudal photoreceptor which provides a sufficient pathway for the entraining signal (Figs. 4, 5).

Introduction

The involvement of extraretinal as well as retinal photoreceptors in the entrainment of circadian rhythms has become increasingly evident in recent years. Among the vertebrates, extraretinal pathways are present in birds (Menaker, 1968), reptiles (Underwood and Menaker, 1970) and amphibians (Adler, 1969a, b) while entrainment in mammals requires a retinal light input (Hunt and Schlosberg, 1969; Browman, 1943; Bruss et al., 1958; Richter, 1965, 1968). Attempts to locate the responsible CNS photoreceptors in vertebrates have, however, met with little success, perhaps in part because extraretinal receptors have not been described in vertebrates in general.

In contrast, several CNS photoreceptors are already known for invertebrates, and some are implicated in entrainment. But as in the vertebrates, both retinal and extraretinal pathways are involved in entrainment in these animals. Scorpions, for example, have been shown to have photoreceptors in one of their two terminal abdominal segments (Zwicky, 1968) which may provide a sufficient pathway for entrainment of locomotor activity (Zwicky, 1970). It has also been reported that illumination of the protocerebrum of aphids can affect photoperiodic induction of sexual and parthenogenic females (Lees, 1964), and it has been shown that photoreceptors in the brain of the silkworm, *Antheraea pernyi*, are involved in the photoperiodically controlled termination of pupal diapause (Williams, 1969) and in ecdysis (Truman, 1970). Zimmerman and Goldsmith (1971) have demonstrated entrainment of the emergence rhythm of carotenoid-depleted *Drosophila*, suggesting the involvement of an extraretinal photoreceptor. The requirement of a retinal input for entrainment of locomotor activity, however, has been demonstrated for cockroaches (Roberts, 1965; Nishiitsutsuji-Uwo and Pittendrigh, 1968).

One of the best known extraretinal photoreceptors in invertebrates is the caudal photoreceptor of crayfish (Prosser, 1934; Welsh, 1934; Kennedy, 1963) but its role in entrainment is not well understood. Chapple (1960) obtained data that implicated a retinal as well as a CNS photoreceptor in entrainment of circadian locomotor activity of crayfish, but was unable to establish whether the caudal photoreceptor was responsible. The locomotor rhythm of crayfish has already been thoroughly characterized as a result of a number of studies (Kalmus, 1938a; Schalleck, 1942; Roberts, 1944; Guyselman, 1957; and Chapple, 1960). When observed in a laboratory environment under a 24 hour photoperiod (LD 12:12), the activity rhythm characteristically has two peaks. One burst, generally of short duration, is synchronized with the onset of light, while a second somewhat longer burst of activity occurs shortly after the lights go off. Chapple (1960) concluded that the "lights-off" peak was circadian while the "lights-on" peak was not. He also obtained evidence that the input for driving this latter peak was exclusively via the retina.

The present study attempts to clarify the role of the eyes and caudal photoreceptor in crayfish locomotor rhythms, and further examines the nature of the two peaks of activity. Evidence will be presented from surgical interference experiments which supports the notion that the crayfish's circadian activity rhythm has a single "lights-off" peak, but suggests that it is entrained by an extraretinal-extracaudal photoreceptor. We will also suggest that the "lights-on" peak lacks certain characteristics of circadian rhythms and that it is activated via the

retina. Finally, we will conclude that the caudal photoreceptor does not function in either entrainment or in the "lights-on" peak of activity.

Materials and Methods

All the experiments presented here were performed on medium sized individuals of *Procambarus clarkii*. Prior to each experiment the animals were maintained in a holding tank under photoperiods of 12 hours light and 12 hours dark, and were fed dry dog food once weekly. During the experiments the animals were fed both dog food and earthworms at irregular intervals.

Activity Monitors. The activity monitors were constructed from light plastic pans 16.5 cm in diameter and 8.9 cm in depth. Numerous 2 mm holes were drilled through the bottom of an activity monitor for communication with the water in a large container. The monitor pan was floated inside the larger container with styrofoam, and was held in position by means of a tungsten wire which passed from an attachment on the bottom of the large pan through a small center hole in the monitor. Forward or backward movement of the animal caused the activity monitor to rotate, but the wire axis prevented any translational movement. A straight contact on the moving activity monitor bounced within a stationary circular contact on the wall of the large outer pan giving a measure of activity via switch closures. Each contact closure was recorded as a pen deflection on an Esterline-Angus Event Recorder (Mod. A620X). Discs of heavy black paper were placed between the animals and the switches to shield them from light generated by sparks which occasionally accompanied the switch closures.

The activity monitors were placed in light-tight wooden boxes, generally two per box. Each box was equipped with a 4-watt fluorescent bulb (Ken Rad F4T5/cs) which provided a light level of approximately 30 lux. The water temperature was maintained at $22 \pm 3°$ C. Some experiments were performed in which the light was covered with black plastic to show that the animals were not entraining to temperature variations or noise resulting from the lights being switched on and off.

To analyze the data, daily chart records were placed one below the other in chronological order. No attempt was made to quantify the data, and references to activity levels are only estimations.

Surgical Methods. Prior to surgery all animals were anesthetized by cooling in ice and rendered immobile by being attached to special lucite boards constructed for that purpose. No attempts were made to close wounds resulting from surgery since there was relatively little loss of blood in each operation. No deaths were recorded which could be attributed to surgery.

Two methods were used to isolate the caudal photoreceptor from the central nervous system. In one group of experiments the ventral nerve cord was severed between the fifth and sixth abdominal ganglia. In other animals the sixth abdominal ganglion was completely removed through an incision along the posterior edge of the sixth sternal rib. In all cases where these operations were performed, the animals, after observation, were sacrificed and dissected to verify that isolation or removal of the caudal ganglion was complete.

Two methods were also used to prevent photoreceptive input through the eyes. In the first, the cornea of the animal was surgically removed by cutting around the margins. After lifting the cornea off, fine forceps were used to remove the ommatidia. The second method involved cutting the eye stalk between the lamina ganglionaris and the medulla externa (i.e., the two distal ganglia of the optic lobe). The distal edge of the sinus gland, which just approaches the lamina ganglionaris,

was used as a reference point for the cut. Care was taken not to damage the sinus gland which was usually visible once the cornea and ommatidia had been removed.

Histological studies were made of eyestalks which had been surgically interfered with to determine the extent of damage and to verify the presence or absence of photoreceptive structures. After fixation in alcoholic Bouin's, longitudinal sections were cut at ten microns, and the preparations were stained with Mallory's Triple.

Results

Each experiment was begun by recording the activity pattern of an intact animal in a 24 hour light cycle (LD 12:12). In this way each animal served as its own control, and each record (Figs. 1–5) began with several days of the rhythmic activity of an intact crayfish. As has been previously reported (Chapple, 1960), crayfish exhibit marked individual variation in their relative and absolute levels of locomotor activity during a 24 hour cycle. In all individuals, activity occurs sporadically throughout the light and dark phases of the light cycle (Figs. 1–5). The locomotor rhythms described generally reflect a periodic increase in the level of activity from this "background" or "baseline" activity.

Crayfish placed in a light cycle (LD 12:12) characteristically exhibit a bimodal locomotor activity rhythm (Figs. 1–5). One of the activity peaks, the "lights-on" peak, has a duration of approximately 10–30 min and is synchronized with the onset of light. The second peak of activity, the "lights-off" peak, occurs about 10–30 min after the beginning of the dark phase of the photoperiod. There is much variation in the duration of this peak which involves an intense burst of activity lasting usually from one to five hours, often followed by a diminishing level of short, sporadic bursts (Figs. 1–5). Both the "lights-on" and "lights-off" peaks follow a shift in the phase of the light cycle (Figs. 3, 4). The phase of the "lights-on" peak is immediately reset while there are usually (but not invariably) one to two days of transients evident in the reentrainment of the "lights-off" peak (Fig. 4).

Free running rhythms are difficult to show in these animals (Chapple, 1960). An activity rhythm appears to be maintained intitially when the crayfish is placed in constant darkness (Fig. 1), but does not persist for more than a few days. The disappearance of the rhythm after nine or ten days is troublesome. If the initial drift in DD represents free run, as we think it does, then we can interpret the remaining data in the framework of current circadian rhythm literature. The remaining data and discussion assumes this. The rhythm exhibited by crayfish in constant darkness is unimodal and involves only the "lights-off" peak while the "lights-on" burst is immediately lost under these conditions. Endogenous activity free runs with a period of approximately 23.5 hours (periods of 23.3–23.6 hours were observed). These results indicate that the crayfish has an endogenously controlled, circadian locomotor
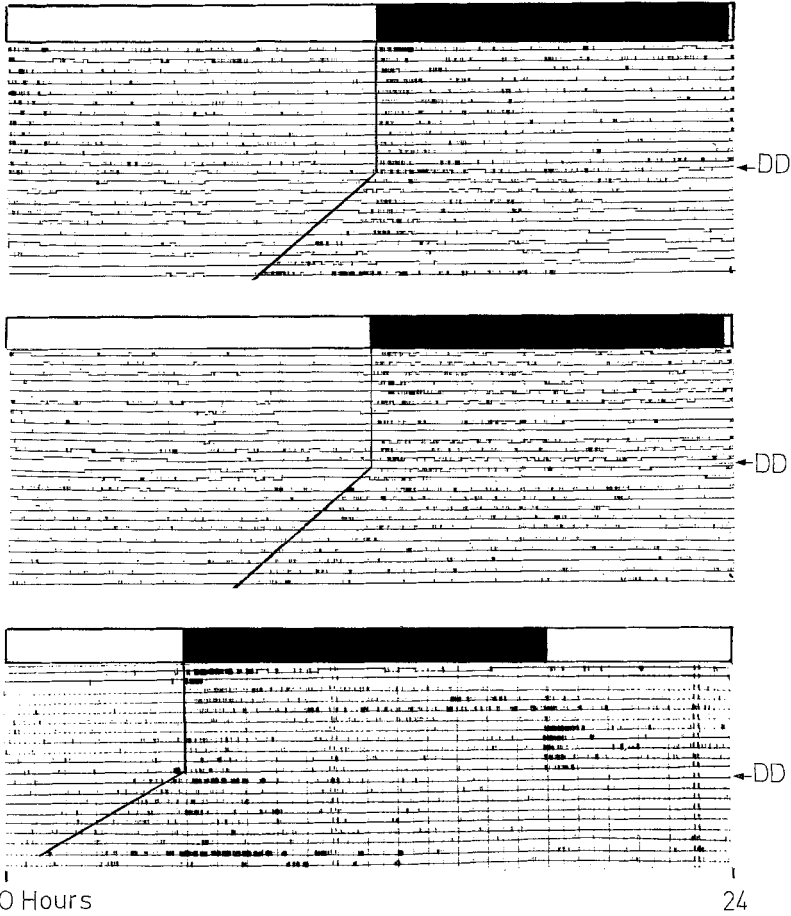
Fig. 1. Three examples of activity patterns of crayfish in a light cycle (LD 12:12) and in constant darkness (*DD*). Note the immediate loss of the "lights-on" peak of activity when the animals are placed in constant darkness. Typically a loss of rhythm is observed after about ten days in constant darkness. This may, however, be an artifact of the recording conditions. The bar at the top of the record designates the initial lighting regimen. The shaded area corresponds to the dark phase of the photoperiod; the white area indicates the light portion

rhythm. The loss of the "lights-on" peak implies that this aspect of the animal's activity is not part of that circadian rhythm. While this conclusion is not proven, it is also suggested by the observation above that transients of the "lights-off" peak appear during a phase advance, which is a characteristic of circadian rhythms (Pittendrigh, 1960), whereas transients of the "lights-on" peak are absent. Further evidence justifying this contention is presented below.
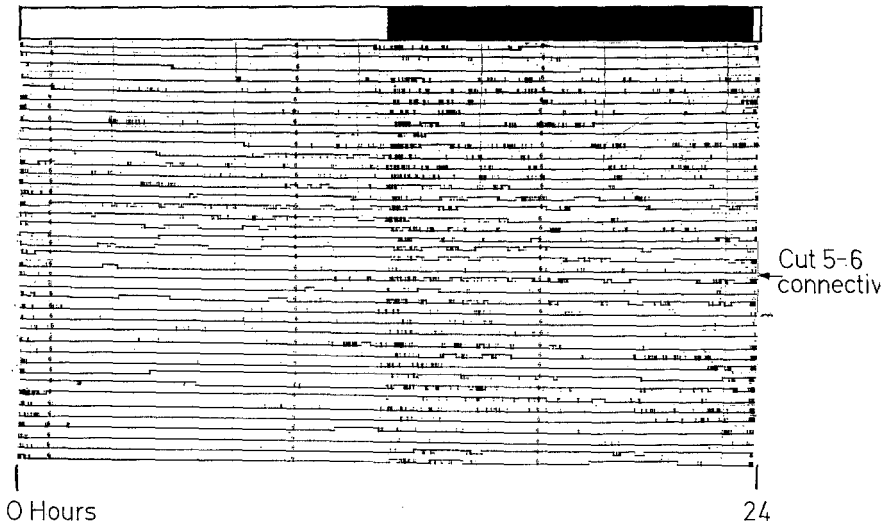
Fig. 2. This record illustrates the bimodal activity rhythm exhibited by an intact crayfish on a light cycle (LD 12:12). Severance of the ventral nerve cord between the fifth and sixth abdominal ganglia (cut 5–6 connective) has no observable effect on either activity maximum which remained entrained to the light cycle

The next group of experiments were involved with determining which pathways of light input were mediating entrainment of the locomotor rhythm. Thus photoreceptive input through the eyes and caudal photoreceptor was prevented by surgical ablation of these photoreceptive structures.

Ablation of the Caudal Photoreceptor. The caudal photoreceptor of six animals was isolated from the rest of the CNS by severing of the ventral nerve cord between the fifth and sixth abdominal ganglia. This procedure had no discernible effect on the activity rhythm (Fig. 2). Both peaks of activity were present in these animals and neither the intensity, duration, or time of occurrence of the activity maxima were altered. It was also shown that the phase of the activity rhythm followed a shift in the phase of the light cycle. Similar results were obtained with animals from which the caudal ganglion was completely removed (Fig. 3) usually in conjunction with ablation of the eyes (see below).

Ablation of the Eyes. Surgical removal of the ommatidia of both eyes was effected in nine animals, and both the ommatidia and lamina ganglionaris were removed in nine additional animals. In all 18 animals removal of these structures resulted in immediate loss of the "lights-on" peak. In contrast, all but two of the animals studied retained the "lights-off"
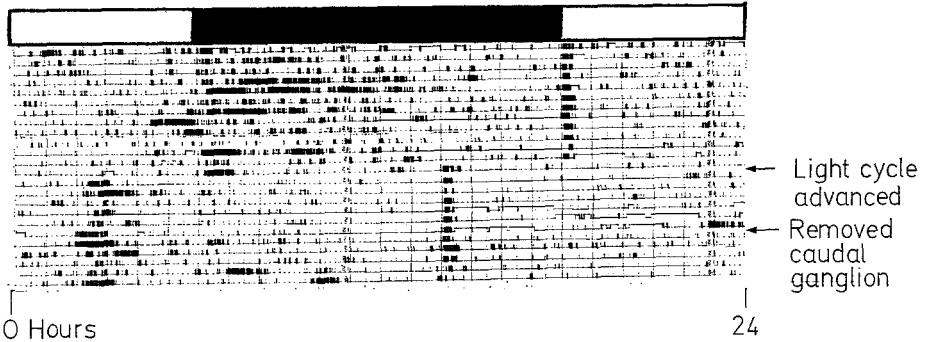
Fig. 3. An example of the response of an intact crayfish to an advance of four hours in the phase of the light cycle. Note that the "lights-on" peak of activity is immediately reset following the phase advance. Also shown is the effect of removal of the caudal ganglion. Both peaks of activity are retained and remain entrained to the light cycle
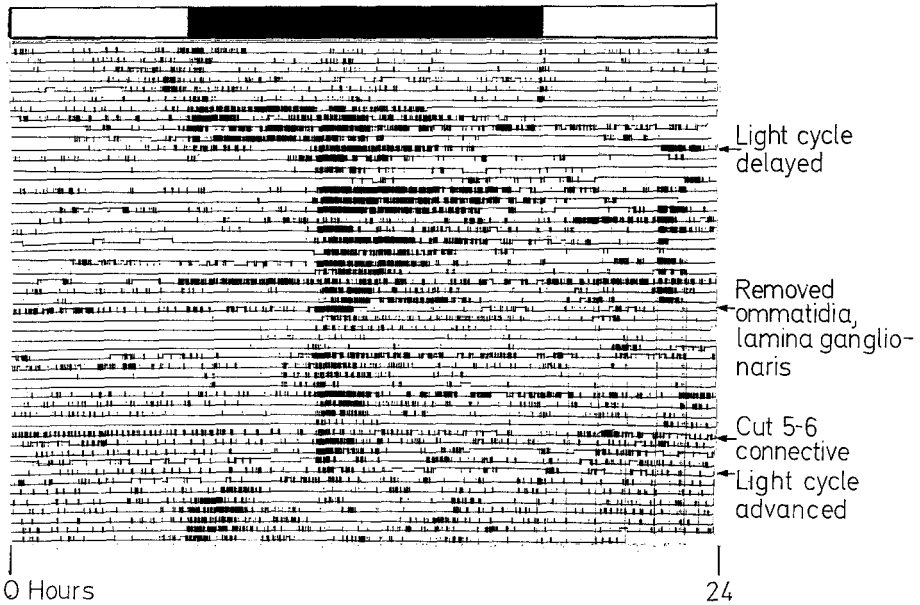

Fig. 4. An example of an animal whose rhythm alters phase to reentrain to a light cycle (LD 12:12) which has been delayed by four hours. An entrained rhythm is maintained subsequent to removal of the photoreceptors of the eyes. Note, however, the immediate loss of the "lights-on" peak of activity once the ommatidia have been removed. Isolation of the caudal photoreceptor has no effect and the animal is capable of entraining to the light cycle after it has been advanced by four hours.
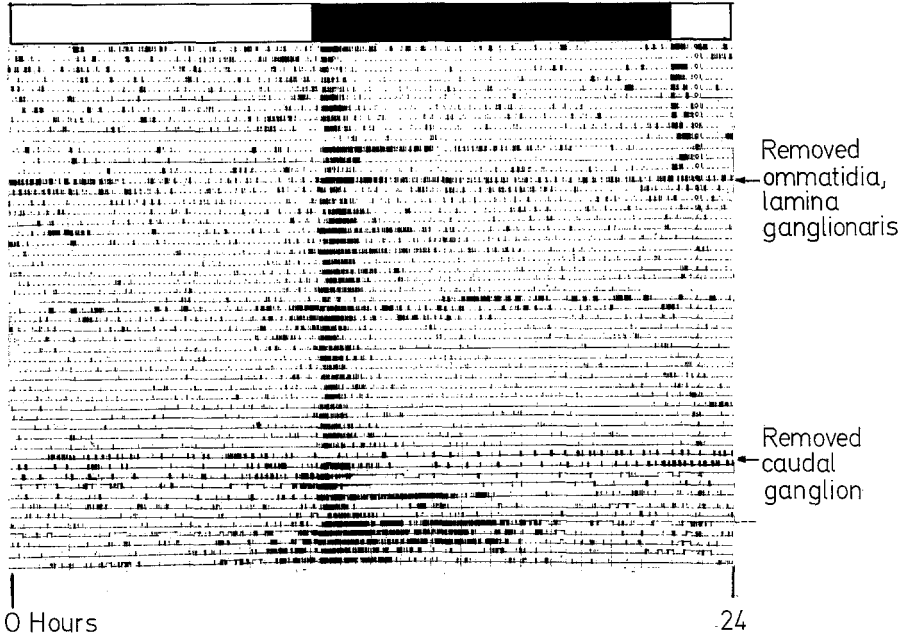Three cycles of transients appear as the phase of the rhythm is advanced

Fig. 5. Shows an animal which retains an entrained "lights-off" peak subsequent to removal of the ommatidia and lamina ganglionaris. The "lights-on" peak is lost immediately following the operation. (Note, the regular markings on the right side of the record are numbers printed on the chart paper and do not represent activity). Complete removal of the caudal ganglion does not abolish the entrained rhythm

peak of activity which remained entrained to the light cycle(Figs. 4, 5). The two animals which did not maintain an entrained rhythm were aperiodic.

Ablation of Both the Eyes and the Caudal Photoreceptor. In each of the experiments just described involving ablation of the eyes, the caudal ganglion of animals which retained an entrained locomotor rhythm was subsequently removed or isolated from the CNS. Isolation of the caudal photoreceptor (9 animals) had no discernible effect on the activity of the animals (Fig. 4), and seven of nine animals from which the caudal ganglion was completely removed also retained an entrained "lights-off" peak (Fig. 5). In both cases it was shown that the animals were capable of reentraining to a phase shift in the light cycle (Fig. 4).

The results of these experiments suggest the existence of a photoreceptor other than the eyes or caudal photoreceptor which is capable of mediating entrainment. Loss of the "lights-on" peak following removal of the retina also implies that light input through this structure is required for this peak.
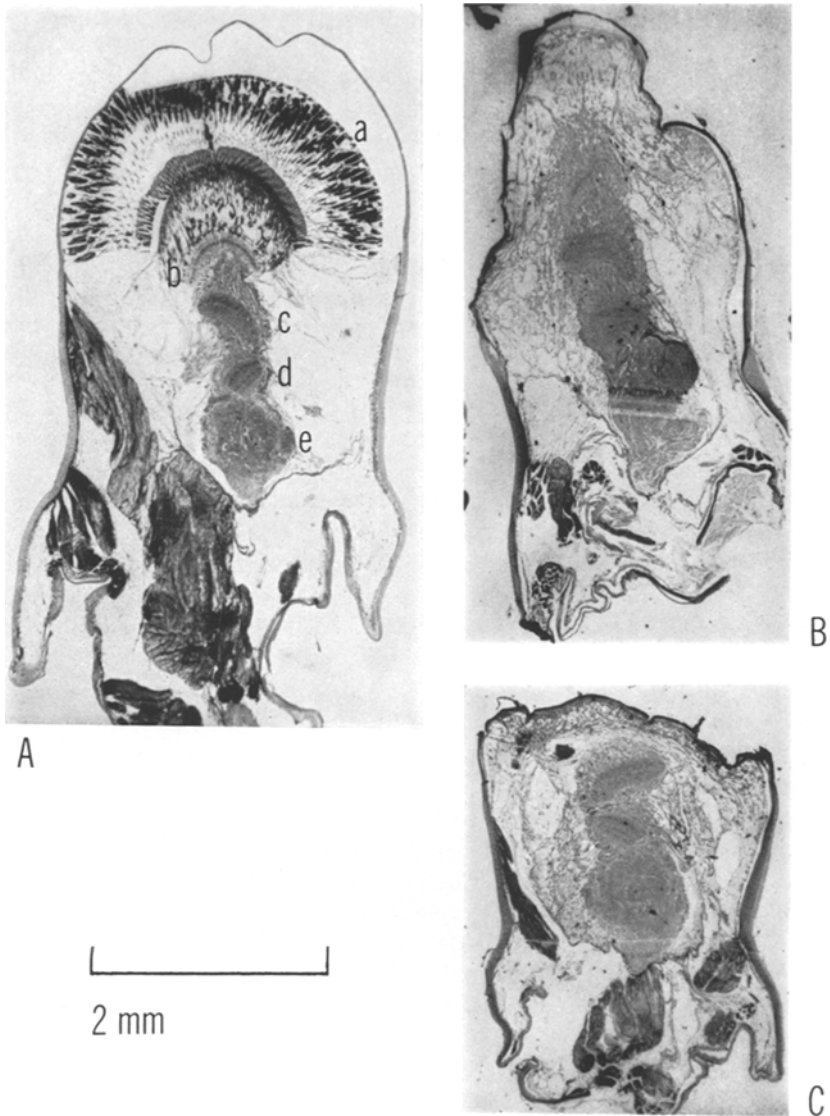
Fig. 6A—C. Longitudinal sections of crayfish eyestalks indicating the parts of the eye which were removed from various animals. A, section througha normal eyestalk (*a*, ommatidia; *b*, lamina ganglionaris; *c*, medulla externa; *d*, medulla interna; *e*, medulla terminalis). B, section through the eye of an animal whose ommatidia had been removed. C, section of an eyestalk of an animal whose ommatidia and lamina ganglionaris had been removed

Examples of the histological records showing the results of eyestalk surgery are presented in Fig. 6. It should be noted that in some instances histological examination of the eyes from which both the ommatidia and lamina ganglionaris were removed revealed that the medulla externa had been damaged. The locomotor behavior of these animals could not be distinguished from those in which no damage was found.

The aberrant behavior shown by two animals following removal of the sixth ganglion and two other animals following ablation of the eyes could not be explained from the results of autopsy or histological examination following the experiment. The arrhythmia observed in these cases is believed to be a result of surgical trauma rather than the disruption of some component utilized in the generation of the rhythm.

Discussion

When placed in a light cycle, crayfish have a bimodal activity rhythm which is synchronized with the photoperiod. In constant darkness a free running locomotor rhythm is maintained; however, this circadian rhythm appears to be unimodal involving only the "lights-off" peak of activity. This implies that an endogenous mechanism, a "biological clock", functions in the control of the expression of the "lights-off" response. The presence of transient cycles of activity during a shift in the phase of the rhythm is also consistent with this conclusion.

In contrast, there are three observations which suggest that the "lights-on" peak is exogenously generated via light input through the retina. First, the "lights-on" maxima are absent when the crayfish is in constant darkness; second, removal of the photoreceptive elements of the eyes abolishes this peak; and finally, the "lights-on" peak is reset immediately, without transients, when the phase of the entraining stimulus is either delayed or advanced. The implication of these results is that the "lights-on" activity is simply a manifestation of the avoidance response to light described by Welsh (1934) who showed that light shined on the eyes of a dark adapted animal resulted in locomotor activity. This conclusion was also suggested by Chapple (1960). No evidence was obtained that the "lights-on" activity is coupled to the circadian oscillator which controls the "lights-off" response; however, the observations do not necessarily eliminate this possibility, and some interdependence may occur between the activity maxima of an intact animal.

The Role of the Caudal Photoreceptor in the Locomotor Rhythm. The results of the ablation experiments indicate that the caudal photoreceptor has no influence on the rhythmic locomotor behavior of the animal. The observation that presence or absence of this photoreceptor has no effect on the "lights-on" peak of activity is particularly surprising

in view of behavioral studies which have shown that illumination of the
caudal photoreceptor of a dark adapted animal elicits a walking response
(Welsh, 1934). This conflict may, however, be explained by the fact
that Welsh was working with light intensities much higher than those
involved in this study. That the caudal photoreceptor is not necessary
for entrainment is also evident from the results of this study; and while
the possibility that this photoreceptor may be sufficient for entrainment
has not been eliminated, it is apparent that this is, at least, not its primary
function and that the animals need not rely on signals, neural or hormonal
from the caudal ganglion for maintenance of the rhythm.

   The Role of the Eyes in the Locomotor Rhythm. As previously stated,
the "lights-on" activity peak appears to be driven exclusively by photic
input to the retina. In contrast, these photoreceptive structures are not
required for activation or entrainment of the circadian "lights-off"
activity. As with the caudal photoreceptor, this conclusion does not
preclude the possibility that the eyes can mediate entrainment or exert
some influence on the circadian activity of an intact animal; however,
no evidence was obtained to suggest the eyes function in either process.
This observation is especially interesting in view of evidence that the
ganglia of the optic lobe are intimately involved with locomotor behavior
of decapod crustaceans (Schalleck, 1942; Aréchiga and Wiersma, 1969;
Hazlett, 1971) and that the sinus gland, which receives input from the
medulla terminalis, functions in other photoperiodic responses of crayfish
(Welsh, 1941; Kurup, 1963) and possibly in locomotion (Roberts, 1944).

   The Extraretinal-Extracaudal Photoreceptor. In view of the fact that
animals deprived of both the eyes and caudal photoreceptor are still
capable of maintaining an entrained locomotor rhythm, it must be
concluded that either the animal possesses still other photoreceptors
which are capable of mediating entrainment, or that the activity is
being entrained by some environmental parameter, other than light,
which is locked in phase with the light cycle. However, studies have
been done which suggest temperature is not particularly influential
on crayfish locomotor activity (Roberts, 1944) and in the present
investigation experiments which involved covering the light with black
plastic demonstrated that the animals were not entraining simply to heat
generated by the fluorescent lights. It has also been shown that locomotor
activity is not affected by normal ambient fluctuations in oxygen con-
centration or pH (Roberts, 1944), or barometric pressure (Guyselman,
1957). Consequently, it must be concluded that the crayfish possesses an
extraretinal-extracaudal photoreceptive structure which is involved with
maintaining an entrained circadian locomotor rhythm. It is interesting
to note that some evidence has been obtained (Jegla and Poulson,
1968) that the cave crayfish, *Orconectes pellucidus,* which is functionally

blind but still has its optic ganglia (Parker, 1890) has a circadian loco-motor activity rhythm which can be entrained to a light cycle.

The mechanisms coupling the extraretinal-extracaudal photoreceptor to locomotor activity and the circadian oscillator are obscure. Welsh (1934) demonstrated that dark adapted animals without eyes and with the caudal photoreceptor blocked gave no locomotor response to light stimulation. Thus it seems clear that the photoreceptor that is responsible for entrainment is not directly coupled to the locomotor structures in the manner of the eyes and caudal photoreceptor which are capable of meidating an avoidance response to light.

A number of observations indirectly suggest that hormonal elements located in the eyestalk are involved in the locomotor rhythm. Roberts (1944) demonstrated the existence of an eyestalk hormone which affects activity, and in light of the fact that the sinus gland has been shown to be the source of hormones controlling daily color change (Kalmus, 1938b) and daily retinal pigment migration (Kleinholz, 1936) in crayfish, it is feasible to speculate that the activity hormone might also originate in the X-organ-sinus gland complex. This complex is instrumental in molting behavior (Kurup, 1963) which is also photoperiodically con-trolled (Stephens, 1955; Aiken, 1969) and may be generally involved in rhythmic control mechanisms.

The medulla terminalis of the optic lobe may also be an important component of the circadian mechanism. For example, the optic ganglia have been shown to be important in various behavioral responses, including locomotion, of a number of decapod crustaceans (Maynard and Dingle, 1963; Maynard and Sallee, 1970; Maynard and Yager, 1968; Hazlett, 1971). In addition, Schalleck (1942) has shown that severance of the optic tract of crayfish proximal to the medulla terminalis results in hyperactivity and apparent loss of locomotor rhythm. This obser-vation has been confirmed in this laboratory, and further it has been demonstrated that ablation of the lamina ganglionaris or severe damage to the medulla externa do not result in this response. These observations suggest that either, or both, the medulla interna or the medulla termi-nalis are involved in inhibition of activity, and consequently may be important in the control of the circadian locomotor activity rhythm.

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