reverse phase high-pressure liquid chromatography which showed the absence of other ^3H-labeled components including ^3H_2O. Saccharin was the only ^3H compound detected (99.7 percent of activity) in urine samples (0 to 24 hours) from the rats that received 3-methylcholanthrene before saccharin.

These results demonstrate that significant metabolism is not induced by long-term administration of saccharin during the neonatal and weaning stages of two-generation studies. Because of the poorer quality of the chromatography of the pulsed-dose urine samples the limit of detection for any metabolite separated from saccharin by TLC was approximately 0.4 percent. In the low-dose studies the limits of detection were 0.2 percent by TLC in 2-methylcholanthrene-treated rats and 0.1 percent by TLC, 0.05 percent by high-pressure liquid chromatography and 0.01 percent of the dose as 2-sulfamoylbenzoic acid by reverse isotope dilution in normal rats. This last study sets the limit of detection of a saturable metabolic process at 0.06 μg per kilogram of body weight per 24 hours. Thus in animals fed a 5 percent saccharin diet this would represent < 0.015 μg of metabolite per gram of saccharin consumed (< 0.015 part per million), assuming each rat (250 g) consumes 20 g of diet per day. The total exposure of the bladder epithelium during long-term administration to any metabolite would be a maximum of 0.015 μg per day or approximately 5 μg per year. This is considerably below the concentration of organic impurities in commercial saccharin (up to 20 parts per million) that the recent report (*1*) concluded were present at such low levels that they would have to show "exquisite potency" to be responsible for the development of bladder tumors in rats fed diets high in saccharin.

The absence of detectable metabolism of saccharin at such low concentrations is important evidence that this anionic species cannot act as a classic electrophilic carcinogen. Thus we must conclude (*1*) that "saccharin is unusual in that its carcinogenic effects are due to the unmetabolized parent compound," and other possibilities, such as promotor effect or changes in physiological function or urine composition, must be investigated before the apparent mechanism of tumorigenicity and its specificity are understood.

T. W. SWEATMAN
A. G. RENWICK
Clinical Pharmacology Group, University of Southampton, Medical and Biological Sciences Building, Southampton SO9 3TU, England

References and Notes

1. *Saccharin: Technical Assessment of Risks and Benefits* (Institute of Medicine, National Research Council–National Academy of Sciences, Washington, D.C., 1978).
2. G. Kennedy, O. E. Fancher, J. C. Calandra, R. E. Keller, *Food Cosmet. Toxicol.* **10**, 143 (1972).
3. R. M. Pitkin, D. W. Andersen, W. A. Reynolds, L. J. Filer, *Proc. Soc. Exp. Biol. Med.* **137**, 803 (1971).
4. E. J. Lethco and W. C. Wallace, *Toxicology* **3**, 287 (1975).
5. J. L. Byard and L. Golberg, *Food Cosmet. Toxicol.* **11**, 391 (1973).
6. H. B. Matthews, M. Fields, L. Fishbein, *J. Agric. Food Chem.* **21**, 916 (1973).
7. L. M. Ball, A. G. Renwick, R. T. Williams, *Xenobiotica* **7**, 189 (1977).
8. A. G. Renwick and R. T. Williams, *Biochem. J.* **129**, 869 (1972).
9. A. G. Renwick, in *Drug Metabolism—From Microbe to Man*, D. V. Parke and R. L. Smith, Eds. (Taylor and Francis, London, 1978), pp. 169–189.
10. A. G. Renwick, L. M. Ball, D. L. Corina, R. T. Williams, *Xenobiotica* **8**, 461 (1978).
11. I. C. Munro and D. L. Arnold, in *Health and Sugar Substitutes*, B. Guggenheim, Ed. (Karger, Basel, 1978), pp. 76–81.
12. We thank C. F. George for comment and the Calorie Control Council, Atlanta, for support.

27 March 1979

Tidal Vertical Migration: An Endogenous Rhythm in Estuarine Crab Larvae

Abstract. *Field-caught larvae of the estuarine crab* Rhithropanopeus harrisii *have a tidal rhythm of vertical migration when maintained in constant conditions. Laboratory-reared larvae do not show this rhythm. Endogenous tidal vertical migrations aid the retention of these planktonic larvae in estuaries near the parent populations.*

Tidal fluctuations dramatically affect the lives of benthic intertidal organisms. It is not surprising that a number of these organisms have endogenous circatidal rhythms of behavior, physiology, and reproduction [see (*1–3*)]. Phasing of their internal clocks with respect to the tidal cycle is accomplished through synchronizers such as changes in hydrostatic pressure, wave agitation, and inundation, all of which occur at the land-water interface (*2*).

Because planktonic organisms remain in the water column, the above-mentioned synchronizers have little influence on them. However, in estuarine environments the tidal cycle is often accompanied by changes within the water column itself, brought about by flow-induced turbulence (*4*). Water quality factors likely to be thus affected are salinity, dissolved organic substances, and inorganic nutrients; temperature and turbidity cycles often occur as well. These changes could act to synchronize endogenous tidal clocks in some estuarine plankton. We now describe a tidal rhythm, presumably entrained by these water column synchronizers, in the planktonic larvae of the estuarine crab *Rhithropanopeus harrisii* (Gould).

We monitored vertical distributions of laboratory-reared or field-caught crab larvae under constant conditions for up to 8 days by means of an automated television system. To obtain laboratory-reared larvae, we collected ovigerous female crabs and maintained them at 25°C and 20 per mil salinity in a cycle of 12 hours of light alternating with 12 hours of darkness. Standard rearing techniques were used for the larvae (*5*), which were initially kept in the same conditions as the parent crabs, then changed to constant dark at the start of the experiment (*6*). Field-caught larvae were taken by towing a plankton net in water of 15 per mil salinity in the Newport River estuary near Beaufort, North Carolina. These samples were immediately returned to the laboratory for sorting of crab larvae into species and stage; no more than 5 hours elapsed between collection and the start of an experiment. Although tidal height records for the area of collection are not available, tides in the Beaufort area are of the semidiurnal type, with approximately equal amplitudes. The tidal cycle at the collection site has a period length of approximately 12.4 hours.

The general procedure was as follows. Between 60 and 100 larvae were added to the top of a Lucite column (1.9 m) (*7*) containing water of the same salinity as that in which the larvae had been living (*8*). Freshly hatched *Artemia salina* nauplii were added for food at the beginning of each experiment and again at random times every second day. Introduction of fresh food had no apparent effect on larval vertical distribution. After a brief period in which the larvae were allowed to distribute themselves, the monitoring system was automatically activated each half hour (*9*), so that an elevator could raise the television camera and scan the column against a dim red backlight in a period of 2 minutes. The scanned image was stored on videotape. Events were identical on the following cycle, but the camera was lowered to the initial position. Except for the time that the backlight was on, the larvae were in total

0036-8075/79/0907-1020$00.50/0

darkness with the room temperature maintained at 24.5° ± 1°C. Experiments were ended when there were fewer than 20 larvae remaining in the water column as a result of mortality and molting to the benthic juvenile crab.

Videotapes obtained from the monitoring device were replayed and the number of larvae in each 0.1-m vertical segment of the column were counted. The counts from the two scans at half-hour intervals were then summed in each segment, and the center of the larval distribution was calculated for each hour (*10*).

In laboratory-reared larvae, a weak circadian rhythm in vertical position was evident, initially showing a rise during the time of the original dark phase and a gradual fall during the original light phase (Fig. 1A). Fisher's periodogram test followed by use of Enright's periodogram (*11*) reveals a period length of 24.6 hours ($P < .01$). Since the period of this rhythm in constant conditions is longer than exactly 24 hours, the phase relationship to the original light-dark cycle drifted to later times during the course of the experiment. The amplitude of this weak migration was approximately 0.25 m. No tidal rhythm was apparent.

The results for larvae caught in the field at spring tides (Fig. 1B) were quite different. The larvae demonstrated a marked tidal migration, in which the maximum depths (troughs) were initially reached slightly after the time of naturally occurring low tides. Statistical analysis reveals a migration with a period length of 12.3 hours ($P < .01$). Alternate cycles differ in two ways: (i) successive cycles have alternating amplitudes of 0.4 and 0.75 m for the first 3 days; and (ii) the peaks of successive cycles fall alternately at early and late times in the cycle. This suggests that two 12.3-hour cycles produce a basic cycle of period length 24.6 hours, in agreement with hypotheses suggested separately by Palmer and by Enright (*3*) that the basic period length for tidal rhythms is 24.8 hours, not 12.4 hours. An alternative interpretation is that this pattern represents a circatidal rhythm in vertical migration with a circadian component superimposed, since the greater amplitude changes take place at night.

To further study the phase relationship between the endogenous larval migration and the tidal cycle, we made a second study with field-caught larvae obtained during neap tides (Fig. 1C). At this phase of the fortnightly cycle, tidal ranges are at a minimum, and the tidal phase times are approximately 6 hours later than at spring tide. Both the pattern of this migration and the phase relationship to the tidal cycle were similar to that of the spring tide experiment, in further support of the tidal basis of the rhythm; the period length was again 12.3 hours ($P < .01$). Although the alternating pattern of peak occurrence at early and late times in successive cycles was again apparent, the amplitude of the migration is reduced and the alternating amplitude pattern is not clearly expressed. These changes could result from the weaker synchronizing influences of the neap tides.

Although endogenous rhythmicity in diel (24-hour) vertical migration of plankton has been described (*12*), this is apparently the first report of an internally controlled migration on a tidal time scale. A tidal vertical migration is particularly adaptive for this organism. Estuaries are characterized by a net seaward flow of water due to the water's entering the estuary from its drainage basin. Estuarine plankton are continually at risk of being exported in this seaward flow. The situation is especially severe for the planktonic larvae of benthic estuarine animals, such as *Rhithropanopeus harrisii*; for unless at least some of the larvae are able to settle in the vicinity of the parent organisms, they will not contribute to maintenance of the population.

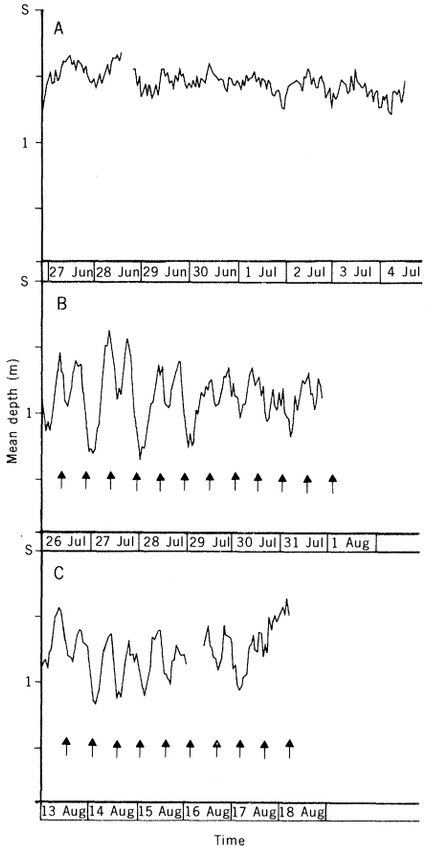

Fig. 1. Movement of the center of distribution of *Rhithropanopeus harrisii* larvae in a vertical column kept in constant dark and constant temperature. Dates are in 1978; *S*, surface. Gaps indicate missing data. (A) Movements by laboratory-reared larvae. Initially 100 larvae were introduced into the column. During entrainment, the light phase began at 0700 and the dark phase at 1900 hours. (B) Movements by larvae caught in the field at time of spring tides. Initially 62 larvae were introduced. The arrows indicate the predicted times of low tide at the collection site. Natural sunrise and sunset were at 0610 and 2005 hours, respectively. (C) Movements by larvae caught in the field at the time of neap tides. Initially 100 larvae were introduced. The arrows again indicate the predicted times of low tide at the collection site. Natural sunrise and sunset were at 0620 and 1945 hours, respectively.

Stratified, partially mixed estuaries have a layer of water of higher salinity, with a net landward flow that underlies the upper seaward-flowing water (*13*). Thus, it is possible for a planktonic organism to partition its time between the layers and minimize horizontal displacement from the parent population; this could conveniently be done by regular vertical migrations. Some field studies have revealed diel vertical migrations for estuarine zooplankton (*14*), while others have demonstrated tidal migrations between the substrate and water column for some estuarine larvae (*15*). The only unequivocal field observations to date of entirely planktonic tidal migrations have been for crab larvae; we have found a striking tidal migration in our fieldwork with *R. harrisii* larvae, and DeCoursey (*16*) has noted a similar migration for larvae of fiddler crabs (genus *Uca*).

For a short-lived planktonic larva, vertically migrating in synchrony with the tides would offer a more certain control over horizontal movements in the estuary than diel migrations. In the migration pattern illustrated by Fig. 1, B and C, the larvae would move high in the water column during the flood tide and thus be carried up the estuary. As the ebb flow began, the larvae would move to a lower position, and their seaward movement would be reduced by the slower seaward currents there and by boundary layer effects if the larvae move very near the bottom. Thus, the likelihood of retention within the upper part of the estuary near the parent populations would be increased, an obvious advantage to the species. What aspects of the estuarine tidal cycle entrain this endogenous tidal vertical migration rhythm remain to be determined.

THOMAS W. CRONIN
RICHARD B. FORWARD, JR.
Duke University Marine Laboratory, Beaufort, North Carolina 28516

References and Notes

1. *Biological Rhythms in the Marine Environment*, P. J. DeCoursey, Ed. (Univ. of South Carolina Press, Columbia, 1976); *13th European Marine Biological Symposium*, E. Naylor, Ed., in press.
2. J. T. Enright, *Science* **147**, 864 (1965); E. Naylor and R. J. A. Atkinson, *Symp. Soc. Exp. Biol.* **26**, 395 (1972); B. G. Williams and E. Naylor, *J. Exp. Biol.* **51**, 715 (1969).
3. J. D. Palmer, *Biol. Rev.* **48**, 311 (1973); J. T. Enright, in *Marine Ecology*, O. Kinne, Ed. (Wiley-Interscience, New York, 1974), vol. 2, pp. 467–494.
4. J. R. Schubel, *Science* **161**, 1013 (1968); E. N. Partch and J. D. Smith, *Estuarine Coastal Mar. Sci.* **6**, 3 (1978).
5. J. D. Costlow, C. G. Bookhout, R. J. Monroe, *Physiol. Zool.* **39**, 81 (1966).
6. *Rhithropaneus harrisii* passes through four zoeal stages and one postlarval stage (megalopa) before metamorphosing to the benthic juvenile crab. In our experiments, larvae were added to the vertical column either a few hours prior to the molt to zoeal stage 3 (laboratory-reared) or during stage 3 (field-caught). The record therefore includes data from late stage 2 (laboratory-reared), and stages 3 through the megalopa. Molting from one stage to the next occurred every 2 or 3 days.
7. The column was constructed of transparent Lucite (0.64 cm). The dimensions of the enclosed water column were 8.2 cm by 7.0 cm by 1.9 m, and the volume of water was 10.9 liters.
8. The desired salinity was obtained by diluting natural seawater, collected off the Beaufort Inlet, North Carolina, with distilled water. The salinity used for laboratory-reared larvae was 20 per mil, for field-caught larvae it was 15 per mil.
9. The monitoring system consisted of a television camera (Cohu model 4350) with a silicon intensified target vidicon, a videotape recorder (Panasonic model 30205D), and an elevator system to raise and lower the camera parallel to the vertical column. Light was provided by a pair of 8-foot (2.44-m) General Electric red fluorescent tubes further filtered and diffused by a red plastic screen and red cellophane to give only dim diffuse light of wavelengths greater than 600 nm. Larvae of *R. harrisii* are very insensitive to such a light source [R. B. Forward, Jr., and J. D. Costlow, Jr., *Mar. Biol.* **26**, 27 (1974)]. Since the light was mounted parallel to the vertical column, it presented no vertical orientation cues. A mechanical clock was used to show the time of each monitoring scan.
10. The center of the larval distribution was determined by multiplying the number of larvae in each segment by the depth of the center of that segment and dividing by the total number of larvae counted. This was a good descriptor of the larval depth distribution for two reasons; first, few or no larvae settled to the bottom of the chamber; and, second, the population vertical distribution was generally symmetrical about the center and concentrated near it.
11. J. T. Enright, *J. Theor. Biol.* **8**, 426 (1965); W. A. Fuller, *Introduction to Statistical Time Series* (Wiley, New York, 1976), p. 281.
12. J. E. Harris, *J. Mar. Biol. Assoc. U.K.* **43**, 153 (1963); J. T. Enright and W. M. Hamner, *Science* **157**, 937 (1967).
13. D. W. Pritchard, *J. Mar. Res.* **11**, 106 (1952); C. Officer, *Physical Oceanography of Estuaries* (Wiley, New York, 1976).
14. H. F. Bosch and W. R. Taylor, *Mar. Biol.* **19**, 172 (1973); A. I. Robertson and R. K. Howard, *Mar. Biol.* **48**, 207 (1978).
15. L. Wood and W. J. Hargis, in *Fourth European Marine Biological Symposium*, D. J. Crisp, Ed. (Cambridge Univ. Press, Cambridge, 1971), pp. 24–44; P. de Wolf, *Thalassia Jugosl.* **10**, 415 (1974).
16. P. J. DeCoursey, *Am. Zool.* **16**, 244 (1976).

17. We thank B. Hunnings for help with construction of the column and for design and construction of the elevator system. M. Swift and D. Stearns provided helpful comments on the manuscript. Supported in part by NSF grant OCE 77-26838.

15 January 1979; revised 11 June 1979

Subfornical Organ Efferents to Neural Systems for Control of Body Water

Abstract. *The subfornical organ, a circumventricular structure of the central nervous system, has efferent neural projections to sites within the brain known to be involved in drinking behavior and secretion of antidiuretic hormone. By using anterograde tracing techniques, it is shown that the subfornical organ projects to the nucleus medianus of the medial preoptic area, to the organum vasculosum of the lamina terminalis, and to the supraoptic nuclei bilaterally. Its efferent connectivity is confirmed by retrograde transport of horseradish peroxidase. The organum vasculosum of the lamina terminalis, another circumventricular organ and a suspected receptor site for angiotensin II, is involved in the circuitry of the subfornical organ and also has an efferent projection to the supraoptic nuclei.*

The circumventricular organs (CVO's) of the brain are unique structures that lack a blood-brain barrier (*1, 2*). They are highly vascular and lie in the midline of the brain at strategic positions on the surface of the cerebral ventricular system (*3–6*). Without a blood-brain barrier, they are ideally suited as receptor sites for blood-borne biologically active substances normally excluded by the brain (*7, 8*). Initially, their functional role was presumed to be neurosecretory on the basis of morphological evidence (*2, 4, 5, 9*). From the results of earlier experimental work it was speculated that the subformical organ (SFO) played a role in water balance (*10*). This has been supported by recent work showing that lesions of the SFO inhibit the natriuretic response to intracarotid hypertonic saline (*11*) and that extracts of the SFO given intracerebroventricularly affect salt-water balance (*12*). More significantly, Simpson and Routtenberg (*8*) and Simpson *et al.* (*13*) demonstrated that the SFO is an extremely sensitive site in the brain for the induction of drinking behavior by angiotensin II (AII). This was the first direct demonstration of a functional role for the SFO and firmly supported the contention that the SFO is involved in the regulation of water balance. However, it may not be the sole receptor site for AII-mediated drinking behavior. Other work implicates the periventricular tissue of the anteroventral third ventricle, which contains the organum vasculosum of the lamina terminalis (OVLT), another CVO (*14–16*). Both sites are very sensitive. The medial preoptic area is a third possible receptor site; however, it is a less likely candidate since it requires at least three to four orders of magnitude more AII to produce drinking (*17*) than does the SFO (*13*).

Since the SFO has been shown to be a receptor site for AII in the induction of drinking behavior, identifying its efferent projections offers the chance to trace the neural circuitry mediating a specific behavioral response elicited by a specific hormone at a specific site in the brain. Little is known about the efferent connections of the CVO's. The single exception is a projection from the area postrema to the neighboring nucleus of the solitary tract described in the cat (*18*). Nothing has been described for the SFO or OVLT. In this study we describe efferent connections of the SFO traced by labeling both anterograde and retrograde transport within the axons of SFO neurons.

The SFO is a small (0.4 by 1.0 mm) structure which lies in the anterior and dorsal part of the third ventricle below the ventral surface of the ventral hippocampal commissure and between the interventricular foramen of the lateral ventricles. Suspended from the fornix and separated from immediately adjacent nuclei by a fiber bundle, the SFO is ideally positioned for the application of anterograde autoradiographic tracing techniques (*19*). In this procedure tritiated amino acids in nanoliter volumes are locally injected at specific sites within the brain. They are taken up by neuronal perikarya, incorporated into protein, and carried down axons by axoplasmic transport mechanisms to their terminals, which are identified by autoradiography.

We examined the brains of 11 Sprague-Dawley male albino rats in which tritiated L-leucine ([3,4,5-^{3}H(N)], 79.8 Ci/mmole) in small volumes (10 or 20 nl, 50 or 25 μCi/μl, respectively) was injected through a 33-gauge beveled cannula (*20*).

Four rats had injections involving the SFO. In two of these rats the cannula terminated in the ventral hippocampal commissure just dorsal and anterior to the SFO and minimally involved the triangularis and septofimbrial nuclei of the septum (caudal septal nuclei). In the remaining two rats the cannula terminated more anteriorly and the injection site included the caudal septal nuclei. Of the

0036-8075/79/0907-1022$00.50/0