12. Chernega, A. N., Green, M. L. H., Haggitt, J. and Stephens, H. H., *J. Chem. Soc., Dalton Trans.*, 1998, 755.
13. Park, J. T., Song, H., Cho, J., Chung, M., Lee, J. and Suh, I., *Organometallics*, 1998, **17**, 227.
14. Maggini, M., Guldi, D. M., Mondini, S. and Scorrano, G., *Chem. Eur. J.*, 1998, **4**, 1992.
15. Roth, L. M., Huang, Y., Schwedler, J. T., Cassady, J. C., Ben-Amotz, D., Kahn, B. and Freiser, B. S., *J. Am. Chem. Soc.*, 1991, **113**, 6298.
16. Huang, Y. and Freiser, B. S., *J. Am. Chem. Soc.*, 1991, **113**, 8186.
17. Huang, Y. and Freiser, B. S., *J. Am. Chem. Soc.*, 1991, **113**, 9418.
18. Nagao, S., Kurikawa, T., Miyajima, K., Nakajima, A. and Kaya, K., *J. Phys. Chem.*, 1998, **A102**, 4495.
19. Koga, N. and Morokuma, K., *Chem. Phys. Lett.*, 1993, **202**, 330.
20. Haddon, R. C., *J. Comp. Chem.*, 1998, **19**, 139.
21. Rogers, J. R. and Marynick, D. S., *Chem. Phys. Lett.*, 1993, **205**, 197.
22. Seiders, T. J., Baldridge, K. K., O'Connor, J. M. and Siegel, J. S., *J. Am. Chem. Soc.*, 1997, **119**, 4781.
23. Billups, E. D. and Ciutolini, M. A., in *Buckminsterfullerene*, VCH Publishers, Weinheeim, 1993.
24. Hirsch, A., in *The Chemistry of the Fullerenes*, Thieme, Sttutgart, 1994.
25. Haddon, R. C., *Science*, 1993, **261**, 1545.
26. Jemmis, E. D., *J. Am. Chem. Soc.*, 1982, **104**, 7017 and references therein.
27. Sawamura, M., Iikura, H. and Nakamura, E., *J. Am. Chem. Soc.*, 1996, **118**, 12850.
28. Iikura, H., Mori, S., Sawamura, M. and Nakamura, E., *J. Org. Chem.*, 1997, **62**, 7912.
29. Yu, J. and Hehre, W. J., *J. Comp. Chem.*, in press.
30. SPARTAN 4.1, Wavefunction Inc., California, 1993.
31. Rees, B. and Coppens, P., *Acta Crystallogr.*, 1973, **B29**, 2515.
32. Berndt, A. F. and Marsh, R. E., *Acta Crystallogr.*, 1963, **16**, 118.
33. Dolge, R. P. and Schomaker, V., *Acta Crystallogr.*, 1965, **18**, 614.
34. Elian, M., Chen, M. M. L., Mingos, D. M. P. and Hoffmann, R., *Inorg. Chem.*, 1976, **15**, 1148.
35. Other ΔH_f values (kcal/mol) at PM3(tm) involved in the isodesmic equations are $C_6H_6 = 23.5$, $C_5H_5^- = 15.9$, $C_4H_4 = 99.1$, $C_5H_6Cl = 16.7$, $C_5H_6 = 31.8$, $C_2H_4 = 16.6$, $C_5H_5Li = 35.9$, $C_2H_4Ni(PH_3)_2 = -350.0$, $C_6H_6CrC_6H_6 = 158.1$, $C_6H_6MnC_5H_5 = -39.7$, $C_6H_6FeC_4H_4 = -117.2$, $C_6H_6CoC_3H_3 = -1531.2$, $C_6H_6RhC_3H_3 = -192.1$, $C_5H_5FeC_5H_5 = -166.0$, $C_5H_5CoC_4H_4 = -1568.0$, $C_5H_5NiC_3H_3 = -264.1$, $C_5H_5PdC_3H_3 = 54.6$, $C_6H_6Cr(CO)_3 = -45.2$, $C_6H_5ClCr(CO)_3 = -45.9$, $C_{60} = 811.8$, $C_{60}H_5^- = 642.5$, $C_{60}H_6 = 696.3$, $C_{60}Li = 707.3$, $C_{60}Ni(PH_3)_2 = 423.0$.
36. Muetterties, E. J., Bleeke, J. R. and Sievert, A. C., *J. Organomet. Chem.*, 1979, **178**, 197.
37. Hehre, W. J., Radom, L., Schleyer, P. V. R. and Pople, J. A., in *Ab initio Molecular Orbital Theory*, John Wiley & Sons, New York, 1986.
38. Burgi, H., Blanc, E., Schwarzenbach, D., Liu, S., Lu, Y., Kappes, M. M. and Ibe, J. A., *Angew. Chem. Int. Ed. Engl.*, 1992, **31**, 640.
39. Albright, T. A., Hoffmann, P. and Hoffmann, R., *J. Am. Chem. Soc.*, 1977, **99**, 7546.
40. Kroto, H. W., *Nature*, 1987, **329**, 529.
41. Curl, R. F. and Smalley, R. E., *Science*, 1988, **242**, 1017.
42. Kroto, H. W., *Science*, 1988, **242**, 1139.
43. Schmalz, T. G., Seitz, W. A., Klein, D. J. and Hite, G. E., *J. Am. Chem. Soc.*, 1988, **110**, 1113.
44. Kroto, H. W., *Angew. Chem. Int. Ed. Engl.*, 1997, **36**, 1578.
45. Smalley, R. E., *Angew. Chem. Int. Ed. Engl.*, 1997, **36**, 1594.
46. Haddon, R. C., *Nature*, 1995, **378**, 249.
47. Zanasi, R., Lazzeretti, P. and Fowler, P. W., *Chem. Phys. Lett.*, 1997, **278**, 251.
48. Ruud, K., Agren, H., Helgaker, T., Dahle, P., Kock, H. and Taylor, P. R., *Chem. Phys. Lett.*, 1998, **285**, 205.

ACKNOWLEDGEMENTS. Financial support from the Department of Science and Technology is gratefully acknowledged. The help of Mr Pankaz Kumar Sharma in the preparation of this manuscript is appreciated.

Received 26 March 1999; revised accepted 5 April 1999

A neurotransmitter role for methionine enkephalin in causing hyperglycemia in the freshwater crab, *Oziotelphusa senex senex*

P. Sreenivasula Reddy

Department of Biotechnology, S. V. University, Tirupati 517 512, India

Injection of methionine enkephalin caused a significant increase in the hemolymph glucose and total sugar level of intact crabs in a dose-dependent manner, apparently by triggering release of the hyperglycemic hormone.

EVER since the first report of presence of leucine enkephalin in the red swamp crayfish, *Procambarus clarkii* and in the spiny lobster, *Panulirus interruptus* by Mancillas *et al.*[1], there have been sporadic reports of occurrence of opioids in different crustacean species[2–6]. Though the status of opioid research in crustaceans is fragmentary, there are several interesting questions pertaining to demonstration of these peptides and the diverse functions they perform, which are very different from what they do in mammals[7,8].

Since the discovery of a 'diabetogenic factor' in the crustacean eyestalk[9], work has been carried out on its chemical nature, mode and site of action[10–14]. In view of the fact that opioid peptides act as neurotransmitters[6,15], it is conceivable that these peptides could help in the secretion of the hyperglycemic hormone from the neurosecretory cells that synthesize it. The present investigation was undertaken to determine, whether methionine enkephalin can indeed produce an increase in the hemolymph sugar level of the crab *Oziotelphusa senex senex* and if so whether it might involve stimulation of the release of hyperglycemic hormone.

Freshwater rice field crabs *Oziotelphusa (Paratelphusa) senex senex* Fabricius were collected

e-mail: pcjobs@mail.com

from local paddy fields in and around Tirupati and maintained at room temperature 27 ± 1°C in plastic tubs half submerged in tap water with a 12L:12D cycle. They were fed daily once *ad libitum* sheep meat and the medium was changed. Only adult, healthy, intermolt (stage C_4) male crabs with a carapace width of 30–32 mm and a body weight of 32–34 *g* were used in the present study.

One hundred crabs were divided into 10 groups of 10 animals each. Group 1 served as normal and this group of crabs which received no treatment were utilized on the first day of the experiment. Group 2 received 20 µl of crustacean saline through the arthrodial membrane of the coxa of the 3rd pair of walking legs and served as controls. Groups 3, 4 and 5 received 10^{-8}, 10^{-9} and 10^{-10} mol/crab of methionine enkephalin, respectively.

Eyestalks were removed from the remaining groups (groups 6–10) by cutting off the organs at the base without prior ligation but with cautery of wound after operation. After 24 h of eyestalk ablation, these groups were analysed for hemolymph sugar level. Crabs from group 6 were sacrificed without treatment while those from group 7 received only 20 µl crustacean saline. Groups 8–10 received 10^{-8}, 10^{-9} and 10^{-10} mol/crab of methionine enkephalin.

Methionine enkephalin purchased from the Sigma Chemical Company, St. Louis, Missouri, was dissolved in physiological saline and injected in 20 µl of volume. All hemolymph samples were removed two hours after injection and at the same time of the day to obviate any possible variation due to circadian rhythm in the hemolymph sugar level. The hemolymph sugar level was determined using anthrone reagent[16]. For the measurement of glucose, hemolymph (100 µl) was mixed with 300 µl of 95% ethanol. After deproteinization (4°C, 14,000 *g*, 10 min), the sample was mixed with a mixture of glucose enzyme reagent (glucose-6-phosphate dehydrogenase and NADP) and colour reagents (phenazine methosulfate and iodonitrotetrazolium chloride) (kit from Sigma). After 30 min, the intensity of the colour was measured at 490 nm and quantified with standards. One way ANOVA test was employed to analyse the data followed by Duncan's multiple range test to determine the level of significance.

The hemolymph total reducing sugar level in eyestalk-less crabs was significantly ($P < 0.001$) less (–25.98%) than in intact crabs. Methionine enkephalin significantly ($P < 0.001$) increased the hemolymph sugar level in intact crabs but not in eyestalk-less crabs (Table 1). These results revealed that not only did methionine enkephalin produce hyperglycemia in intact crabs, but also that this hyperglycemia was dose-dependent. Eyestalk ablation also resulted in hypoglucosemia (Table 1). Injection of methionine enkephalin elevated hemolymph glucose level in a dose-dependent manner in intact crabs. Administration of methionine enkephalin did not cause any change in hemolymph glucose level in eyestalk ablated crabs. Injection of physiological saline did not yield any significant change in hemolymph glucose and sugar level in intact as well as eyestalk-less crabs.

Table 1. Changes in the hemolymph glucose and total sugar level of *Oziotelphusa senex senex* after various treatments

Groups of crabs tested	Total sugar (mg/100 ml)	Glucose (mg/100 ml)
Intact crabs	8.43 ± 0.97	7.81 ± 0.82
Crabs injected with saline	8.51 ± 1.02[a]	7.83 ± 0.96[a]
Intact crabs injected with Met-enkephalin		
10^{-8} mol/crab	22.58 ± 1.81[b]	20.55 ± 2.16[b]
10^{-9} mol/crab	18.74 ± 1.41[b]	17.51 ± 1.92[b]
10^{-10} mol/crab	14.19 ± 1.06[b]	12.94 ± 1.66[b]
Eyestalk-less crabs (24 h post-ablation)	6.21 ± 0.62[b]	5.73 ± 0.67[b]
Eyestalk-less crabs injected with saline	6.37 ± 0.76[b,c]	5.72 ± 0.73[b,c]
Eyestalk-less crabs injected with Met-enkephalin		
10^{-8} mol/crab	6.42 ± 0.75[b,c]	5.74 ± 0.84[b,c]
10^{-9} mol/crab	6.53 ± 0.96[b,c]	5.72 ± 0.79[b,c]
10^{-10} mol/crab	6.45 ± 0.87[b,c]	5.72 ± 0.95[b,c]
F-ratio	435.3973	192.85
P	< 0.001	< 0.001

Values are mean ± SD of 10 individual crabs.
[a]Not significant compared with intact crabs.
[b]$P < 0.001$ compared to intact crabs.
[c]Not significant compared to eyestalk-less crabs.

The data indicate that eyestalk removal can lower both hemolymph sugar and glucose concentrations. Eyestalk extirpation is a classical operation of crustacean endocrinology; it removes the X-organ-sinus gland complex which is the source of an array of hormones[17], including the hyperglycemic hormone. Removal of eyestalks eliminates hyperglycemic hormone from circulation which results in significant decrease in hemolymph sugar level. We have reported earlier that hyperglycemic hormone is synthesized in and released from the sinus glands of eyestalks of the crab, *Oziotelphusa senex senex*[18].

Methionine enkephalin could have produced a rise in hemolymph sugar level in the intact crabs in several different ways such as by triggering release of hyperglycemic hormone, by mimicking the action of this hormone or even by directly stimulating glycogenolysis. However, because methionine enkephalin was not able to produce an increase in hemolymph sugar level in eyestalkless crabs, it seems most likely that methionine enkephalin exerted its hyperglycemic effect by triggering the release of hyperglycemic hormone from the sinus gland of the eyestalks. This also supports our earlier results[18] that the sinus glands in the eyestalks of this crab are the main release sites of hyperglycemic hormone. These results provide the first evidence that an opioid peptide is involved in the regulation of hemolymph sugar level in a crustacean and supports the hy-

pothesis that methionine enkephalin has a neurotransmitter role in decapod crustaceans[19,20].

1. Mancillas, J. R., McGinty, J. F., Silverston, A. I., Karten, H. and Bloom, F. E., *Nature*, 1981, **923**, 576–578.
2. Fingerman, M., Hanumanthe, M. M. and Vacca, L. L., *Soc. Neurosci. Abstr.*, 1983, **9**, 439–440.
3. Fingerman, M., Hanumanthe, M. M., Kulkarni, G. K., Ikeda, R. and Vacca, L. L., *Cell Tissue Res.*, 1985, **241**, 473–477.
4. Martinez, E. A., Vassell, D. and Stefano, G. B., *Comp. Biochem. Physiol.*, 1986, **83C**, 77–82.
5. Martinez, E. A., Murray, M., Leung, M. K. and Stefano, G. B., *Comp. Biochem. Physiol. C*, 1988, **90**, 89–93.
6. Sarojini, R., Nagabhushanam, R. and Fingerman, M., *Comp. Biochem. Physiol.*, 1995, **A111**, 279–282.
7. Leung, M. K. and Stefano, G. B., *Prog. Neurobiol.*, 1987, **28**, 131–142.
8. Nagabhushanam, R., Sarojini, R., Reddy, P. S. and Fingerman, M., *Curr. Sci.*, 1995, **69**, 659–671.
9. Abramowitz, A. A., Hisaw, F. L. and Papendrea, D. V., *Biol. Bull.*, 1944, **86**, 1–5.
10. Kleinholz, L. H., Kimball. F. and McGarvey M., *Gen. Comp. Endocrinol.*, 1967, **8**, 75–82.
11. Ramamurthi, R., Mumbach, M. W. and Scheer, B. T., *Comp. Biochem. Physiol.*, 1968, **26**, 311–319.
12. Keller, R., and Andrew, E. M., *Gen. Comp. Endocrinol.*, 1973, **20**, 572–578.
13. Kleinholz, L. H. and Keller, R., *Gen. Comp. Endocrinol.*, 1973, **21**, 554–560.
14. Telford, M., *Comp. Biochem. Physiol.*, 1975, **51**, 69–73.
15. Rothe, H., Lushen, W., Asken, A., Willig, A. and Jaros, P., *Comp. Biochem. Physiol.*, 1991, **C99**, 57–62.
16. Carroll, N. V., Longley, N. V. and Roe, J. H., *J. Biol. Chem.*, 1956, **220**, 583–593.
17. Kleinholz, L. H., *Am. Zool.*, 1966, **6**, 161–173.
18. Reddy, P. S. and Ramamurthi, R., *J. Reprod. Biol. Comp. Endocrinol.*, 1982, **2**, 49–57.
19. Quackenbush, L. S. and Fingerman M., *Comp. Biochem. Physiol.*, 1984, **C79**, 77–84.
20. Kulkarni, G. K. and Fingerman, M., *Pigm. Cell Res.*, 1987, **1**, 51–56.

ACKNOWLEDGEMENTS. I thank the Management, Pondicherry University, Pondicherry for granting sabbatical leave during which the present work was carried out. I also thank the Staff, Department of Zoology, S.V. University for their invaluable assistance. Portions of this work are a result of research supported by DST (SP/SO/CO4/96), New Delhi. Dr K.V.S. Sarma, Associate Professor, Department of Statistics helped in the analysis of the data.

Received 22 July 1998; revised accepted 9 February 1999

Temporal organization in population density of protozoans in septic tank sewage

Geeta Nair*, A. K. Pati and M. L. Naik

School of Studies in Life Sciences, Pt. Ravishankar Shukla University, Raipur 492 010, India

Variation in the population density of protozoans of a septic tank sewage from a boys' hostel located within the premises of Pt. Ravishankar Shukla University, Raipur, was studied over two consecutive years. Cosinor technique was used to analyse time series data to validate statistically significant annual rhythms in population density of protozoans. Results reveal that rhythmic patterns in population density of various species of sarcodines appear to be highly synchronized with peaks occuring in between mid-March and the first week of July. During the comparable time period at least 6 species of flagellates and 1 species of ciliates showed temporal synchrony with that of the sarcodines. Results of this study may help in optimizing sewage treatment practices involving protozoans.

SEWAGE is a complex ecological system with a rich abundance of organisms ranging from viruses to higher vertebrates. Among all the organisms, bacteria form the bulk and their role in treating sewage has been adequately investigated[1–4]. Apart from bacteria, protozoans also constitute one of the major components of sewage biodiversity. Attempts have also been made to analyse the role of protozoans in the treatment of sewage[5–11]. The sewage from activated sludge plant, oxidation ponds, etc. have been widely studied for various characteristics, but the sewage from septic tanks has been least studied. Furthermore, studies on rhythms in septic tank protozoans are meager.

In this study, therefore, attempts were made to examine temporal organization in the population density of large number of species belonging to three different classes of protozoans in septic tank sewage.

Thirty-six samples were collected over a period of 24 consecutive months at the rate of two equidistant samples per month in the first year and one sample per month in the following year, from a septic tank of a boys' hostel located in the premises of Pt. Ravishankar Shukla University, Raipur. The samples were brought to the laboratory in plastic cans for observing various protozoan types and their density. The types of protozoans were identified using appropriate keys[12–14]. For determining the population density of protozoans, drop count method was employed[15]. One drop of sample (0.05 ml) was placed on a glass slide and covered with a cover glass of 18 × 18 mm size. Protozoans, within the microscopic field were then counted. Simultaneously, the area under the field was measured. This procedure was repeated at several points on the slide. The population density was expressed as number of organisms per ml of

*For correspondence.