References and Notes

1. U. Varanasi and D. C. Malins, *Biochemistry* **9**, 3629 (1970).
2. ———, *ibid.*, p. 4576.
3. ———, *Biochim. Biophys. Acta* **231**, 415 (1971).
4. H. Jules, in *Handbook of Physiology: Adipose Tissue*, A. E. Renold and G. F. Cahill, Jr., Eds. (Williams & Wilkins, Baltimore, 1965), section 5, p. 181.
5. C. Litchfield, R. G. Ackman, J. C. Sipos, C. A. Eaton, *Lipids* **6**, 674 (1971).
6. K. S. Norris, in *Marine Bio-acoustics*, W. N. Tavolga, Ed. (Pergamon, New York, 1964), p. 181.
7. H. K. Mangold and D. C. Malins, *J. Amer. Oil Chem. Soc.* **37**, 383 (1960).
8. R. J. Komarek, R. G. Jensen, B. W. Pickett, *J. Lipid Res.* **5**, 268 (1964).
9. D. C. Malins and U. Varanasi, in *Proceedings of the 19th Colloquium on Protides of the Biological Fluids*, H. Peeters, Ed. (Pergamon, Oxford, 1971), p. 127.
10. H. Brockerhoff, *Biochim. Biophys. Acta* **42**, 624 (1965).
11. A. Dolev and H. S. Olcott, *J. Amer. Oil Chem. Soc.* **42**, 624 (1965).

12. This work was conducted under Office of Naval Research contract N00014-71-C-0439. We thank P. Robisch for assistance.

7 January 1972; revised 31 March 1972 ■

Call Types of the Rana pipiens Complex in Illinois

Abstract. *The presence of western, eastern, and northern call types of the* Rana pipiens *complex in Illinois supports evidence from the western United States that this complex can no longer be regarded as one species.*

Littlejohn and Oldham (*1*) found populations of the *Rana pipiens* complex (leopard frogs) in the western United States that differed markedly in mating calls (*2*). Four species were recognized (designated western, eastern, northern, and southern call types). Other work indicated a fifth call type in Arizona and New Mexico (*3, 4*). Sympatric records in Texas, Arizona, and Colorado (*1, 3–5*) indicate that the call types are maintaining reproductive isolation. The significance of this research is twofold. First, it casts doubt on the validity of frequently cited work (*6*) that regarded *"R. pipiens"* as a single species with populations from different regions showing varying degrees of genetic incompatibility. Second, previous experimental studies that revealed variation in *"R. pipiens"* may be unreliable because experimental material may have included two or more species. This is particularly important because of the extensive use of *"R. pipiens"* in experimental research.

Species names were not applied to the call types because topotypic calls of formerly described members of the complex were lacking, and the status of the complex in other areas of North America was unknown (*1*). We report the first records of the northern, eastern, and western call types in Illinois.

Calls were recorded with a Stancil-Hoffman Minitape M9 tape recorder and Electro-Voice 644 microphone. Temperatures were taken with a Schultheis quick reading thermometer (*7*). Recordings were analyzed with a Kay model 6061A Sona-Graph (*8*). Frog calls were recorded in the spring of 1970 and of 1971 at the following localities: northern call type, 9.0 km north of Grand Detour, and 9.1 km west of Ottawa; western call type, 14.2 km north of Normal; eastern call type, 11.1 km south of Bath.

The call types in Illinois have calls (Table 1) similar to those of the corresponding call types reported previously (*9*). Pulse durations of the northern call type from Illinois, however, are shorter than those previously reported (*1*). The distinctness of the call types from Illinois is most clearly seen in Table 2, because temperature variation is minimal (17.2° to 18.2°C). Presumed western call type males were heard (but not recorded) in sympatry with the northern call type at the Ottawa locality, and with the eastern call type at Bath. No hybrids were encountered. Only one call type was heard at the other localities.

Call types can often be distinguished by morphology, but overlapping character variation (*1, 4*) makes positive identification dependent on call analysis. Dorsolateral folds are usually displaced in the western call type and continuous in the northern and eastern call types. Vestigial oviducts are present in males of the northern call type but absent in males of the western and eastern call types. The three call types recorded in Illinois agree with these diagnostic characters. Another call type from New Mexico and Arizona (*4*) is similar to the western call type in that it has displaced dorsolateral folds and lacks male oviducts. However, the two call types have distinctive calls (*1, 4*).

Most previous work on the call types was carried out in the western United States. The Illinois records thus represent range extensions of considerable magnitude. The Ottawa record for the

Table 1. Some call characteristics of three call types of the *Rana pipiens* complex from Illinois compared with calls from other localities reported by Littlejohn and Oldham (*1*). Mean values are given above ranges which are in parentheses.

Localities	Individuals (No.)	Temperature (°C)	Pulse rate (No./sec)	Duration	
				Call (sec)	Pulse (msec)
			Western call type		
Illinois	5	22.1	5.1	0.68	24.0
		(21.8–22.5)	(4.9–5.3)	(0.57–0.77)	(19.8–29.0)
Texas*	7	. . .	5.6	0.66	27.0
		(20–25)	(4.6–6.8)	(0.48–0.89)	(23–35)
			Eastern call type		
Illinois	2	21.7	12.0	0.46	42.1
		(21.5–21.8)	(11.8–12.3)	(0.39–0.53)	(41.9–42.3)
Texas*	7	. . .	14.8	0.41	39.4
		(20–25)	(14.3–15.3)	(0.31–0.52)	(33–50)
			Northern call type		
Illinois	1	15.9	14.2	2.86	7.1
Colorado*	4	. . .	13.7	3.75	17.8
		(12–16)	(12.9–14.6)	(3.30–4.73)	(16–20)

* Data from Littlejohn and Oldham (*1*).

Table 2. Some call characteristics of three call types of the *Rana pipiens* complex from Illinois recorded at 17.2° to 18.2°C. Mean values are given above ranges which are in parentheses.

Call type	Individuals (No.)	Temperature (°C)	Pulse rate (No./sec)	Duration	
				Call (sec)	Pulse (msec)
Western	2	18.2	3.6	0.69	36.4
		(18.2)	(3.6–3.7)	(0.59–0.78)	(34.4–38.3)
Eastern	4	17.7	9.0	0.88	70.4
		(17.2–18.2)	(8.5–9.3)	(0.74–1.03)	(67.3–73.3)
Northern	5	17.9	19.4	2.90	7.3
		(17.6–18.2)	(17.0–21.2)	(2.48–3.39)	(6.9–7.5)

northern call type is the easternmost record for that call type and extends the range approximately 975 km from the nearest locality previously reported (*1*) in South Dakota. The Normal record for the western call type is the easternmost and northernmost record for that call type and extends the range approximately 580 km from the nearest record in Kansas (*1*). The Bath record for the eastern call type represents the northernmost and easternmost record for that call type and extends the range some 210 km from the nearest record in Missouri (*1*).

In conclusion, our finding of three call types of the *R. pipiens* complex in Illinois supports evidence from the western United States (*1, 3–5, 10*) which indicates that "*R. pipiens*" can no longer be regarded as a single, widely distributed species.

LAUREN E. BROWN
JILL R. BROWN
Department of Biological Sciences, Illinois State University, Normal 61761

References and Notes

1. M. J. Littlejohn and R. S. Oldham, *Science* **162**, 1003 (1968).
2. The mating call functions as the main premating isolating mechanism in anuran amphibians [W. F. Blair, *Quart. Rev. Biol.* **39**, 334 (1964)]. Call discrimination experiments have shown this to be true for hylids, but direct evidence is not available for the *R. pipiens* complex.
3. J. S. Mecham, *Southwest. Natur.* **13**, 35 (1968).
4. ———, *Year B. Amer. Phil. Soc.* **1968**, 314 (1968).
5. H. S. Cuellar, *Evolution* **25**, 399 (197 D. D. Post and D. Pettus, *Herpetologica* **23**, 323 (1967).
6. J. A. Moore, *Bull. Amer. Mus. Natur. Hist.* **82**, art. 8 (1944); *Genetics* **31**, 304 (1946).
7. All frogs recorded were calling in water, and little difference was found between water and cloacal temperatures. Therefore, either was used as the effective temperature in the tables.
8. When calls consisted of two or more series of pulses, we analyzed only the first series to allow comparison with the data of Littlejohn and Oldham (*1*).
9. Since temperature variation affects most call characteristics, our data in Table 1 were selected in regard to temperature to allow direct comparison with Littlejohn and Oldham's data (*1*).
10. D. D. Post and D. Pettus, *Southwest. Natur.* **11**, 476 (1966).
11. We thank M. J. Littlejohn, W. F. Blair, J. Bogart, R. Gray, and J. Loftus-Hills for reading the manuscript.

24 August 1971; revised 11 November 1971

Zinc in Entamoeba invadens

Abstract. *Atomic absorption spectroscopy, electron microprobe analysis, and dithizone staining of trophozoites and cysts of* Entamoeba invadens *demonstrate that these cells have a high concentration of zinc (approximately 10^{-6} microgram per cell or 1 percent of their dry weight). In the cysts of this organism, the zinc is confined to the chromatoid bodies, which previous work has shown to contain crystals of ribosomes. The chemical state and function of this zinc are unknown.*

Cysts of the intestinal parasite *Entamoeba invadens* have the remarkable ability to condense nearly all their ribosomes into crystals, the chromatoid bodies (*1, 2*). Attempts to isolate these crystals into conventional buffers were hampered by the fragility of the crystals and by the extreme sensitivity of their component ribosomes to nuclease (*3*). These experiments left the suspicion that magnesium-containing buffers, though usually sufficient for the isolation of ribosomes, were not reproducing the intracellular environment of *Entamoeba*. The demonstration that zinc was an essential element for the formation of ribosomes in *Euglena gracilis* (*4*) led us to investigate the role of this element in *Entamoeba invadens*. The results we present here allow us to conclude that zinc is a major constituent of chromatoid bodies. We will also describe the estimation of the amount of zinc per cyst by atomic absorption spectroscopy of the ash of counted numbers of cells, the demonstration that this zinc is actually in the cysts by electron microprobe analysis, and finally the localization of the zinc within the cysts by means of histochemical staining with dithizone.

Entamoeba invadens, strain TRM [see (*5*)], was cultured as described by Myer and Morgan (*6*). Cells were collected at room temperature by centrifugation, washed quickly three times with twice-distilled water, and resuspended in a known volume of water. The concentration of cells in this suspension was measured in a hemocytometer. (Although the fragile trophozoites are ultimately lysed by such a washing schedule, they will survive as recognizable objects long enough to be counted.) Water was then removed by drying at 95°C, and cells were ashed in an oven at 550°C for 12 hours. The ash was dissolved in a known volume of either 1*N* HCl or H_2O, and the concentration of zinc in this solution was measured with an atomic absorption spectrometer (Perkin-Elmer, model 303), by reference to a standard curve of the absorbancies of known solutions of $ZnCl_2$. The cells from a total of 11 cultures were prepared in this manner. In the four of these in which the number of cysts exceeded the number of trophozoites, the average zinc per cell was found to be 0.5×10^{-6} μg. In the remaining seven (which contained trophozoites almost exclusively), the average zinc per cell was found to be 0.8×10^{-6} μg. In view of the experimental uncertainties attending these measurements (which we estimate to be perhaps 20 percent), we do not feel that this apparent difference in zinc content between cyst and trophozoite is significant.

The most direct evidence that this zinc is in the cysts was provided by the electron microprobe. For these measurements, cysts were washed in water and then suspended in 0.1*M* ammonium acetate. A drop of this suspension was placed on a polished plug of beryllium, was frozen by immersing the end of the plug in a mixture of acetone and solid CO_2, and was evaporated to dryness under vacuum. The plug was then examined with an electron microprobe (Applied Research Laboratories, model 20 EMX), using a KAP crystal set for ZnLα x-rays (wavelength, 12.28 Å). We found, as have others (*7*), that the use of Lα x-rays improved the peak to background ratios by a factor of at least 6 compared to the ratios found with Kα x-rays. Figure 1 shows the zinc x-rays recorded during a scan across a cyst that was visualized in the instrument's microscope. Of 21 cysts randomly encountered on the surface of the plug, eight gave zinc x-ray signals that were appreciably above (that is, two to five times) background. This fraction corresponds to the fraction of cysts containing visible chromatoid bodies in this particular preparation. In order to quantitate these zinc signals, a series of known proportions (from 0.1 to 10 percent) of $ZnCl_2$ in gelatin were made and smeared on quartz glass slides. Under the same instrumental conditions, the ZnLα counting rates of these mixtures were recorded. These rates were proportional to the zinc content of the mixture, thus the maximum rate of Fig. 1 corresponds to a zinc content of 1 percent dry weight. The microprobe results leave no doubt that the metal we are concerned with here is zinc and that this zinc is within the cysts. The resolution of this instrument