6 Bosco, L., Filoni, S., and Cannata, S., J. exp. Zool. *209* (1979) 261.
7 Bosco, L., Filoni, S., and Cioni, C., J. exp. Zool. *213* (1980) 9.
8 Reeve, J.G., and Wild, A.E., J. Embryol. exp. Morph. *48* (1978) 205.
9 Filoni, S., Bosco, L., and Cioni, C., Proc. XXVII Riunione G.E.I., Genova-Nervi, 1981. Acta Embryol. exp., in press.
10 Reeve, J.G., and Wild, A.E., J. Embryol. exp. Morph. *64* (1981) 121.
11 Filoni, S., Bosco, L., and Cioni, C., Acta Embryol. exp. (1982) in press.
12 Nieuwkoop, P.D., and Faber, J., Normal table of *Xenopus laevis*. Daudin - North-Holland Publ., Amsterdam 1956.
13 Yamada, T., Embryologia *4* (1959) 175.

0014-4754/83/030315-03$1.50 + 0.20/0

Multiple insemination demonstrated experimentally in the kingsnake *(Lampropeltis getulus)*

R.G. Zweifel and H.C. Dessauer

Department of Herpetology, American Museum of Natural History, New York (New York 10024, USA), and Department of Biochemistry, Louisiana State University Medical Center, New Orleans (Louisiana 70012, USA), July 14, 1982

Summary. A female snake chosen on the basis of her previously determined blood protein genotype was mated on successive days to 2 males similarly selected. Electrophoretic analysis of 6 young showed that each male had sired 3 offspring, thereby providing an unequivocal demonstration of multiple insemination.

Multiple inseminations resulting in broods that have more than 1 male parent have been demonstrated in a number of kinds of animals. Among vertebrates, examples include fishes[1], salamanders[2], snakes[3], and rodents[4,5] (see the last reference for a more extensive bibliography). The subject is of interest from a least 2 standpoints: evolutionary theory regarding maximization of male fitness, and interpretation of the genetics of polymorphism. Here we report an unequivocal test of dual male parentage of a single brood of snakes.

A breeding colony of kingsnakes, *Lampropeltis getulus*, has been maintained at the American Museum of Natural History for several generations[6]. Starch gel electrophoresis of blood obtained by cardiac puncture (the snakes sampled remaining alive in the colony) showed that several proteins were polymorphic and that their presumptive genes were inherited in Mendelian fashion as codominant alleles[7]. Because it was known that a female snake would mate more than once within a relatively short time[6], it was possible to promote matings between snakes of known genotypes, chosen so that if more than 1 male fathered a particular brood this could be shown by means of protein phenotypes.

Materials and methods. Blood samples were drawn from adult snakes and analyzed according to standard techniques[7]. Procedures with the offspring differed only in that the snakes were sacrificed in order to assure adequate blood samples. 2 polymorphic proteins were involved: phosphoglucomutase (Pgm), with 2 alleles; transferrin (Tf), with 3 alleles.

Two females were mated each with 2 different males on successive days, the matings beginning approximately 24 h apart. Only 1 of the females oviposited, and this cross forms the basis of our report. Copulation took place on March 12 and 13, 1981; 8 eggs were laid on April 29, 6 of which hatched July 11–15. One egg evidently was infertile and the remaining one contained a dead embryo at or near term. Eggs were incubated at room temperature on moistened vermiculite in plastic bags[6]. Snakes are referred to by their American Museum catalog number if preserved (AMNH), or by a 'live book' number (AMNH-LB) if still alive at this writing.

Results and discussion. The female snake, AMNH-LB 1135E, is homozygous for the b allele of Pgm and heterozygous (a/b) for Tf. The male mated first, AMNH-LB 1042C, is heterozygous (a/b) for Pgm and homozygous for a 3rd (c/c) Tf allele. The male mated second, AMNH-LB 1135C (a sibling of the female) is identical to the female in Pgm and Tf genotypes. The critical diagnostic protein is Tf: appearance of the c allele in any offspring assures that 1042C was the father; absence of this allele confirms 1135C as the father. Presence of the a allele of Pgm would be additional evidence of 1042C as the father, but its absence would not be diagnostic for 1135C. The 6 viable offspring had the following genotypes:

AMNH	Pgm	Tf
122801	a/b	a/c
122802	a/b	b/c
122803	a/b	b/c
122804	b/b	b/b
122805	b/b	b/b
122806	b/b	a/b

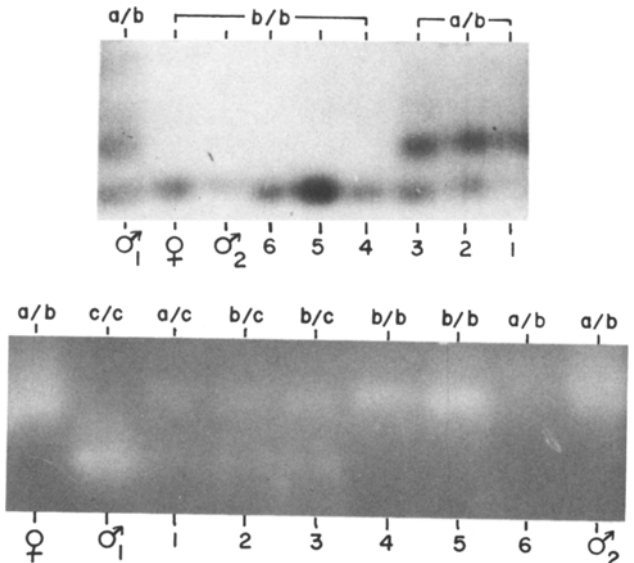

Figure 1. Electrophoretic phenotypes of blood proteins of parents and offspring; phenotypes are indicated by letters above the patterns, specimen identifications by letters beneath; anode is toward the top. Upper figure is Pgm, lower is Tf: δ_1, AMNH-LB 1042C; δ_2, AMNH-LB 1135C; ♀, AMNH-LB 1135E; numbers 1 to 6 are offspring AMNH 122801–122806.

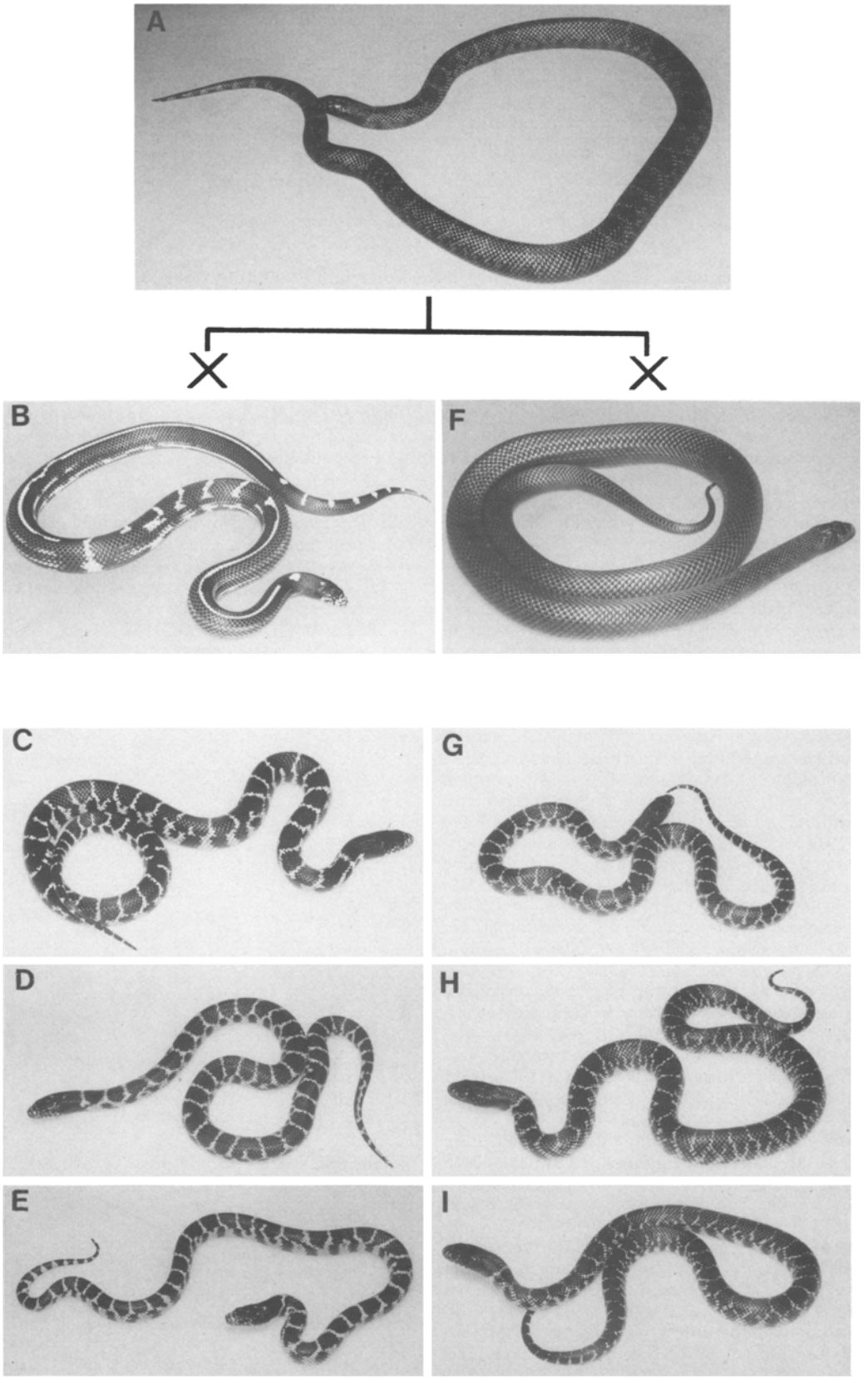

Figure 2. Parents and brood resulting from multiple insemination. *A* Mother, AMNH-LB 1135E. *B* Father, AMNH-LB 1042C. *C, D, E* Offspring fathered by 1042C, AMNH 122801-122803. *F* Father, AMNH-LB 1135C. *G, H, I* Offspring fathered by 1135C, AMNH 122804-122806.

It is clear from the Tf genotypes that 1042C was the father of numbers 122801-122803, whereas 1135C fathered numbers 122804-122806. The presence of the a allele of Pgm in numbers 122801-122803 independently establishes 1042C as their father. Photographs of the Tf and Pgm electrophoretic patterns of parents and offspring appear in figure 1.

The male snakes involved in the experimental cross have quite different color patterns, reflecting their descent in part from different subspecies of *Lampropeltis getulus*. Thus it was of interest to see if the young could be segregated and assigned the proper paternity on the basis of their color patterns.

The parents and offspring are shown in figure 2, arranged according to relationships determined electrophoretically. Differences in the 2 classes of young were sufficient to enable the correct prediction of their male parents in a 'blind' test before the protein phenotypes of the young were determined. Without the latter data, however, the assessment of parentage would have been inconclusive. The best color clue to the identity of the male parent was the greater amount of light pigmentation in the young of 1042C, especially evident on the head. On the basis of its color pattern, the individual that died before hatching appears to have been fathered by 1135C.

There is no reason to think that the propensity of captive female kingsnakes for multiple matings differs from the situation among snakes in the wild. Indeed, multiple inseminations among these snakes seem more likely than in garter snakes *(Thamnophis)*, where a cloacal plug formed at copulation inhibits remating for a time at least[8]. Yet multiple inseminations occur abundantly in garter snakes, as seen from the proportions of melanistic and normal patterns in the broods of melanistic females inseminated in the wild[3]. The potential for detecting multiple inseminations in wild *Lampropeltis getulus* seemingly exists, for there are polymorphic populations whose genetics are at least partly understood[9,10]. However, there are few appropriate published data on the morph ratios in broods of known mothers, and clutch sizes are mostly too small for skewed morph ratios to be demonstrated convincingly.

1 Leslie, J.F., and Vrijenhoek, R.C., J. Hered. *68* (1977) 301.
2 Tilley, S.G., and Hausman, J.S., Copeia *1976* 734.
3 Gibson, A.R., and Falls, J.B., Can. J. Zool. *53* (1975) 1362.
4 Birdsall, D.A., and Nash, A., Evolution *27* (1973) 106.
5 Hanken, J., and Sherman, P.W., Science *212* (1981) 351.
6 Zweifel, R.G., in: Reproductive Biology and Diseases of Captive Reptiles, p. 141. Ed. J.B. Murphy and J.T. Collins. Soc. Study Amphib. Reptiles, New York 1980.
7 Dessauer, H.C., and Zweifel, R.G., J. Hered. *72* (1981) 453.
8 Devine, M.C., Science *187* (1975) 844.
9 Zweifel, R.G., J. Hered. *72* (1981) 238.
10 Zweifel, R.G., Bull. Sth. Calif. Acad. Sci. *80* (1981/1982) 70.

0014-4754/83/030317-03$1.50 + 0.20/0

Morphogenetic effects of evans blue and of zinc ions in embryos of *Lytechinus variegatus*[1]

A.F. O'Melia

Department of Biology, George Mason University, 4400 University Drive, Fairfax (Virginia 22030, USA), July 19, 1982

Summary. Morphogenetic effects of evans blue and of Zn^{++} in *Lytechinus variegatus* embryos are described. Viable unhatched maximally-animalized embryos were induced with 4.2×10^{-5} M evans blue and with $4\text{–}8\times10^{-4}$ M $ZnCl_2$. Hatching inhibition was reversed only with $ZnCl_2$ suggesting that Zn^{++} is the preferred animalizing agent in this sea urchin species.

The application of certain chemicals to sea urchin embryos between early cleavage and the blastula stage produces either a hyperdevelopment of ectoderm (animalization) or exaggerated differentiation of endo-mesoderm (vegetalization)[2]. The polysulfonic acid dye, evans blue, and zinc ions (Zn^{++}) are two of the best chemical-inducers of animalization in whole sea urchin embryos[3-8]. When these animalizing agents are applied at weaker concentration, radialized (radially symmetrical) embryos are formed[2-6]. This report describes the morphogenetic effects of evans blue and of Zn^{++} in embryos of *Lytechinus variegatus* and the optimal conditions for induction of animalization and radialization in this sea urchin species.

Materials and methods. Methods for gamete collection, fertilization and culturing of *Lytechinus variegatus* embryos have been described[9]. The polysulfonic acid dye, evans blue, and Zn^{++}, as zinc chloride ($ZnCl_2$), each were applied to 2-cell *L. variegatus* embryos in both continuous- and temporary-treatment experiments. Experimental solutions of evans blue (EBS: evans blue solution) and of $ZnCl_2$ (ZnS: Zn^{++} solution) were prepared by dissolving the desired concentration of the respective chemical in SASW (artificial sea water containing appropriate concentrations of antibiotics)[9]. About 2×10^2 2-cell embryos were transferred to each Syracuse dish, containing a total 15 ml volume of solution. In continuous treatment experiments, 2-cell embryos were cultured continuously in experimental solutions. In temporary treatment experiments, 2-cell embryos were grown for 10–11 h in experimental solutions, washed 3 times in SASW and resuspended in 15 ml of SASW. Development proceeded at room temperature (23 °C). All experiments were performed at least in triplicate.

Results and discussion. Morphogenetic effects of evans blue in *L. variegatus* embryos are summarized in table 1. Animalization was induced by culturing 2-cell embryos continuously in 4.2×10^{-5} M, 2×10^{-5} M and 1.4×10^{-5} M EBS, while radialized larvae were found at 5.2×10^{-6} M EBS. At 4.2×10^{-5} M EBS, unhatched animalized embryos, ranging from type 3/4 to type 1/3 of Hörstadius[10] were observed, while at 2×10^{-5} M EBS, embryos showed a lesser degree of animalization. When control embryos were plutei, animalized larvae were unhatched, hyperciliated blastulae with laterally-extended and thickened ectodermal plates covered with long apical sterocilia, while radialized (radially symmetrical) embryos exhibited an ectodermal oral lobe,