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Lens forming transformations in larval *Xenopus laevis* induced by denatured eye-cup or its whole protein complement[1]

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Summary. Implants of lensectomized eye placed between the outer and inner cornea, denatured by ethanol treatment and implants of total protein pellets from homogenates of lensectomized eyes, induce lens-forming transformations of the outer cornea of larval *Xenopus laevis.*

In larval *Xenopus laevis* lens regeneration from the outer cornea is known to require the presence of the eye-cup[3,4]. It has been established by subsequent research that a necessary and sufficient condition for the outer cornea to produce a lens is for it to be in direct communication with the vitreous chamber environment. In order to achieve this, the 2 mechanical barriers consisting of the lens and the inner cornea, which in a normal eye lie between the outer cornea and the vitreous chamber, must be by-passed[5-7]. Damage to the outer cornea alone is not enough to trigger the regenerative process[6,8].

This suggests that a lens-inducing factor is probably present in the vitreous chamber and that lens regeneration is due to this factor spreading from the vitreous chamber to the outer cornea. Recent research indicates that the factor is produced in the neural retina[9-11]. Although we speak about a factor it cannot be excluded that there is more than 1 lens-inducing factor. The present work aims at providing more

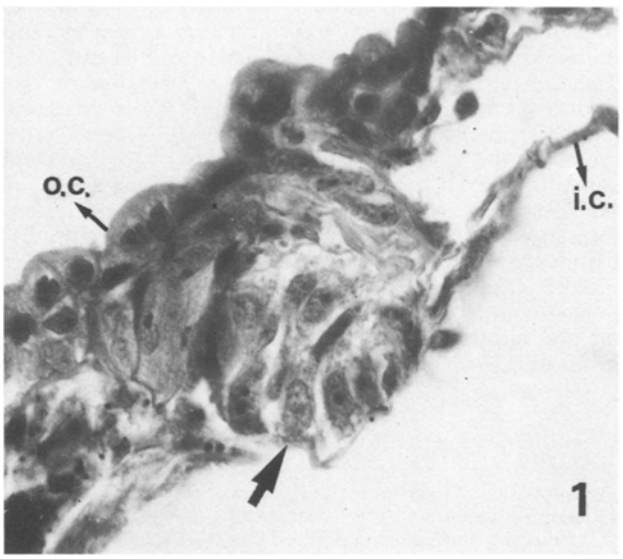

Figure 1. Lens vesicle at early stage 4 (arrow) developing between the inner (i.c.) and outer cornea (o.c.) 5 days after implantation of lensectomized eye devitalized 24 h after lensectomy (experiment 1). × 700.

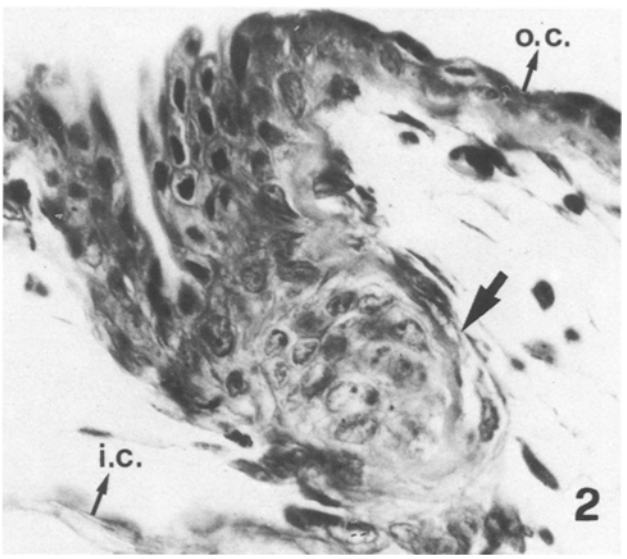

Figure 2. Lens vesicle at late stage 3 (arrow) developing between the inner (i.c.) and outer cornea (o.c.) 9 days after implantation of lensectomized eye devitalized immediately after lensectomy (experiment 2). × 550.

Summary of lens forming transformations of the outer cornea of larval *Xenopus laevis* induced by devitalized eye (experiment 1 and 2) or its whole protein complement (experiment 3)

Experiment	No. of operations	No. of dead/ discarded	No. of cases examined		Days after operation	No. of regenerates Stage 3	Stage 4	Stage 5	No. of lentoid like structures
1*	30	4	26	12	5	5	3	-	1
				14	7	5	2	-	-
				17	5	4	4	-	-
2**	55	2	53	16	7	3	-	-	-
				20	9	1	3	-	-
3***	35	4	31		7	2	7	1	2

* Graft of lensectomized eye denatured 24 h after lensectomy. ** Graft of lensectomized eye denatured immediately after lensectomy. *** Graft of a protein pellet from 3 days lensectomized eye.

direct evidence in support of the above hypothesis and represents the 1st step towards biochemical characterization of lens-inducing factor.

Materials and methods. *Xenopus laevis* larvae at stages 50 and 55-56 (according to Nieuwkoop and Faber[12]) were used. Animals were anesthetized with MS 222 Sandoz, at concentrations of 1:5000/1:2500 in 10% Holtfreter's solution, and the right eye operated on while immersed in full-strenght Holtfreter's solution. Cross-sections 7 µm thick were stained with hematoxylin-eosin.

Experiment 1. Graft of lensectomized eye, denatured 24 h after lensectomy (table). 24 h after lensectomy, the eyes of stage 50 larvae were denatured by immersion in ethanol 70% at −20 °C; 24 h later the eyes were cut into 4 equal quarters, each of which was washed in Holtfreter's solution to remove ethanol (according to Yamada, 1959[13] for chemical approach to the inducing agents) and then implanted between outer and inner cornea in the normal right eye of a same-stage host larva.

Experiment 2. Graft of lensectomized eye, denatured immediately after lensectomy (table). Larvae at stage 50 were used. Operated eye processing and implanting techniques were as in experiment 1.

Experiment 3. Graft of a protein pellet from 3 days lensectomized eye (table). 110 3 days lensectomized eyes of stage 55-56 larvae were homogenized in 5 mM phosphate buffer, pH 7.00, in a motor driven glass-Teflon potter homogenizer (Thomas, Philadelphia USA). The homogenate was centrifuged at 36,000 × g for 30 min in a Sorvall RC5B centrifuge. The supernatant was mixed slowly with ice-cold ethanol to a final concentration of 70% and, after standing in ice for 30 min, was again centrifuged at 36,000 × g for 30 min. 24 h later the pellet was rinsed in Holtfreter's solution and fragmented; each fragment was implanted between outer and inner cornea of a stage 50 larval eye.

Results and discussion. The results are shown in the table. The results obtained in experiment 1 show that eye denatured 24 h after lensectomy can induce lens forming transformations in the outer cornea, thus simulating the action of the living eye-cup. This suggests that a chemical factor takes part in the lens transformation process. The results of experiment 2 show that this inducing capacity is also present in eyes denatured immediately after lensectomy and thus indicate that the chemical factor is present also in a normal eye. However, the lens forming transformations obtained under these experimental conditions do not lead to the regeneration of completely differentiated lenses but stop short at the early stages of the lens forming process (stage 3, or early stage 4, according to Freeman's designation[3]) (figures 1 and 2).

This can be explained in terms of the rapid reabsorption of the graft (at day 9 only traces remain visible) since the eye-cup has already been shown to be important not only in triggering the lens forming transformation process but also in the subsequent development of newly formed structure[4]. The chemical factor involved in the regenerative process probably exerts a salient control over the appearance, growth and maintenance of the newly formed lens; moreover the living eye-cup presumably emits this agent continuously. This explains the difficulty encountered by the denatured eye-cup in maintaining the complete lens forming transformation process. The results of experiment 3 show that total protein extracts of eyes homogenized 3 days after lensectomy can induce a lens-forming transformation of the outer cornea (fig. 3). This opens up the possibility of isolating and purifying the chemical factor involved in the lens forming transformation process.

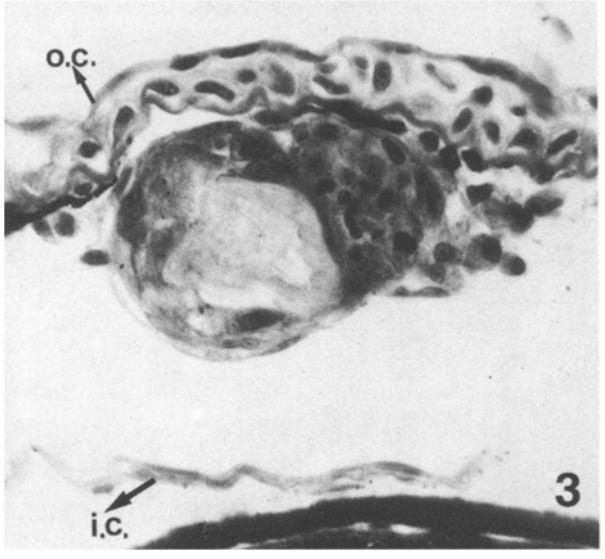

Figure 3. Newly formed lens at stage 5 developing between the inner (i.c.) and outer cornea (o.c.) 7 days after implantation of a proteic pellet (experiment 3). × 550.

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

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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

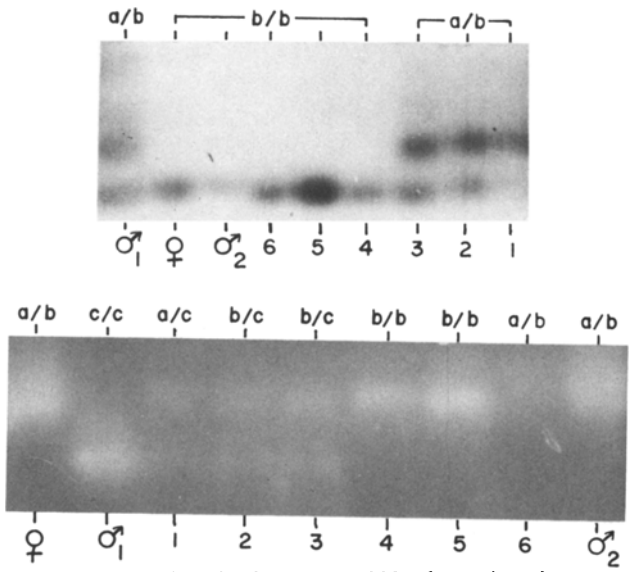

Figure 1. Electrophoretic phenotypes of blood proteins of parents and offspring; phenotypes are indicated by letters above the patterns, specimen identifications by letters beneath; anode is toward the top. Upper figure is Pgm, lower is Tf: δ_1, AMNH-LB 1042C; δ_2, AMNH-LB 1135C; ♀, AMNH-LB 1135E; numbers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