Embryonic Material for Laboratory Experimentation

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From many High Schools and Colleges have come requests for information regarding the technique for inducing egg-laying among the Amphibia during the academic year, which coincides with the normal period of hibernation for these animals. Qualified students, under appropriate guidance, can now conduct experiments along the lines of artificial parthenogenesis (Parmenter, '25) or can study the effect of hormones or of altered environmental conditions on normal development. Such experiments require but little equipment and the use of such objects as living frog or salamander embryos carries a tremendous stimulus to the beginnings of research interest. The rudiments of research technique can be acquired with real enthusiasm, and spectacular results can be achieved during the course of a single academic year. It is the purpose of this paper to describe the technique, now used, to secure fertilized amphibian eggs in the laboratory within 24 hours at any time during the academic year.

In 1929 two brief papers appeared (Houssay, Giusti & Lascano-Gonzalez and O. M. Wolf), one from Argentina and the other from Wisconsin, describing the effect of the anterior pituitary hormone on the ovaries of toads and frogs. In 1930 (Adams) the same technique was used successfully on salamanders. In 1935 (Rugh) an extended study of the pituitary-ovulation relation was reported and in 1937 (Rugh) the relationship was put on a quantitative basis. The following procedure is used almost daily throughout the academic year at the Laboratory of Experimental Morphology, Columbia University, and is being used in an increasing number of Colleges in connection with their courses in experimental zoology and in embryology.

There is reason to believe that any Amphibian, whether urodele or anura, will respond to the anterior pituitary hormone by ovulation if it is mature and has ovaries that contain mature eggs. Some of the more common forms that have been reported to respond to this hormone are:

Amblystoma tigrinum
Bufo: americanus, arenarum, d'Orbignyi, fowleri, and vulgaris
Desmognathus fuscus
Eurycea bislineata
Gyrinophilus porphyritus
Leptodactyllus ocellatus
Pseudobranchus
Rana: catesbiana, clamitans, palustris, pipiens, sphenocephala, temporaria, and vulgaris
Rhycatriton olympicus
Stereochilus marginatum
Triturus: pyrrhogaster and viridescens
Xenopus laevis

Since hibernating Amphibia are best to use, it is suggested that Rana pipiens be used from September until February; Rana clamitans from February to July; Rana catesbiana through the summer; and either Rana sphenocephala or Acris gryllus from July to October. These

periods precede the normal breeding seasor for each of the forms.

While the urodeles will keep well in the laboratory, and if properly fed, will produce eggs (under pituitary stimulation) throughout the winter, the frogs and toads (except Xenopus) should be fresh-caught while hibernating and used within two weeks. This is because laboratory temperatures and food supply are not appropriate; the animals starve and the ovaries degenerate. Until used, these anura are kept best either in a cold room (12–14° C.) or in the refrigerator (4° C.). Body size is a fair means of determining maturity, and the size ranges of North American Anura are given in the remarkably complete and useful Handbook by A. H. Wright. Rana pipiens, the leopard frog, is the most abundant and most easily procured American frog and the following description will apply particularly to this species. The same methods, however, with only slight modifications, may be used with other forms in the list above.

The body length of mature specimens of Rana pipiens is at least 73 mm. (snout to cloaca) and the best females usually measure 85–95 mm. If the frogs are secured from hibernation[1] and immediately placed in a little water in a cold environment, they can be kept healthy for several weeks. A copper-lined box or a copper pan within the container helps to reduce the probability of infection from red-leg.

Twenty-four to thirty hours before the eggs are desired, an obviously mature female is selected and is injected with from four to eight anterior pituitary glands recently removed from frogs. The pituitary is removed by cutting off the head of the frog 1 cm. posterior to the eyes cutting away the lower jaw; inserting the scissors along the side and beneath the medula so as to cut through the transverse processes of the parasphenoidal bone; deflecting this bone forward and exposing the kidney-shaped, pink, anterior pituitary gland. It is located slightly posterior to the level of the eyes, and is surrounded by white, flocculent endolymphatic tissue (see Proc. Soc. Exp. Biol. & Med. 1939, 40: 132). The gland is immediately placed in a very small amount of water.[2] After enough pituitaries have been accumulated (6–8 female glands in the Fall and 4–6 in early Spring) they are sucked up into the barrel of a hypodermic syringe; a #20 needle is applied and injected through the abdominal wall of the female frog. The soft and pliable glands will easily pass through such a needle into the coelomic cavity of the frog.

It has been found that female pituitaries are relatively about twice as potent as male pituitaries in inducing sexual reactions among the Amphibia, so that if males are used as donors the doses should be doubled. Pituitaries from other Amphibia are effective, but the dose varies with the source and generalizations cannot be made. Fish and mammalian pituitaries have been used with some success,

[1] Those who do not have the information may write the author who will be glad to supply the name and address (and the cost of the frogs) of a man who can supply excellent living animals at any time of the year.

[2] If the pituitaries are not to be used immediately, they may be kept in 100% alcohol for at least a year, if necessary, without loss of potency. Prior to injection, however, this solution must be reduced to at most 35% alcohol. Many pituitaries may be accumulated from physiology or morphology laboratories where they are generally discarded.

more particularly with the urodeles than with the anura. In fact, the human pituitary hormone has been used in such forms as Xenopus where urine from pregnant women will cause ovulation.

Another interesting point has very recently been discovered, namely that if the anterior pituitary gland is removed from the female to be injected, that the dose necessary to induce complete ovulation is very much reduced. In fact, if the pituitary is first removed and then injected into the same female twelve hours later, as many as 50% of the eggs will be released from the ovaries while if the host's pituitary is left intact the same response will require a total of four glands. It has long been known that ovulation among the Amphibia is not an all-or-none reaction, but that the number of eggs leaving the ovary depends in part upon the dose of the anterior pituitary hormone injected. The doses prescribed above should give almost 100% ovulation.

Following the injection the female is kept in enough water to be partially submerged, for normal ovulation involves a certain amount of imbibition. It is best to keep the temperature near 20° C., or lower, for the eggs within the uteri deteriorate quickly at the higher temperatures. However, it must be pointed out that the lower the temperature the longer period will elapse before there is response to the injected hormone.

To secure eggs and subsequently the tadpoles, the general practice has been to inject only the female since there are at all times during hibernation abundant mature spermatozoa within the testes of male frogs, which need only liberation in an appropriate medium for activation. However, it is well to demonstrate amplexus and normal egg-laying, and this can be done by simultaneously injecting a mature male with an equivalent dose of the anterior pituitary hormone. Amplexus, at room temperatures, will be achieved overnight, and eggs will be laid by the pair within twenty-four hours.

It is possible now to secure 100% fertilization by artificially controlling insemination in the laboratory, and as a result appropriate stages of development can be secured at any desired time. In this case the female is kept isolated. A sperm suspension is made by cutting up two or more pairs of testes in 10 cc. of a modification of amphibian Ringer's solution (Holtfreter, 1931), the formula of which is given below:

NaCl	0.35 gr.
KCl	0.005 gr.
$CaCl_2$	0.01 gr.
$NaHCO_3$	0.02 gr.
H_2O	100.00 cc.

This solution should be kept as stock and diluted with 9 parts of distilled water before using for sperm suspensions. In its concentrated form it is excellent for amphibian tissues. After a few minutes (1 minute to 6 hours) eggs may be stripped from the uteri of the ovulating female (into the sperm suspension) by gentle pressure around the body toward the cloaca. As many as 2,000 eggs may be secured from a single female. As the eggs drop into the sperm suspension, the finger bowl should be gently agitated so as to spread the eggs more or less evenly throughout the sperm suspension.

In about 15–30 minutes the egg mass should be flooded with the same water used for the sperm suspension, or with pond water in which Amphibia are known to live. This latter type of water may also be used for the sperm suspen-

sion, particularly if it is difficult to make up the more uniform Holtfreter's solution. Within an hour the eggs will have rotated and taken on their full quota of jelly and should be removed to a much larger container with abundant water. Ultimately the eggs should be cut into clusters of 25–50 and placed in finger bowls full of water. This number per finger bowl will grow quite normally. Development will proceed even at refrigerator temperatures, though slowly, but temperatures should be controlled at about 20–22° C. if possible. At higher temperatures the eggs should be further separated and given abundant water per egg.

Within 2½ to 3 hours at ordinary laboratory temperatures the first cleavage will be evident; within 8 hours the embryos will be blastulae; and within 24 hours they will be gastrulating. Care must be taken to use only such instruments as have never come in contact with any poisonous (killing) fluids; glassware that is thoroughly clean. It must be remembered that the highly pigmented amphibian egg is very receptive to radiant energy and must be kept away from either direct sunlight or prolonged artificial lighting.

The entire ovulation process may be observed if the female is anesthetized and the abdomen is opened within 14–16 hours after pituitary injection. The frog may be opened under Holtfreter's solution, which should be used abundantly to moisten all tissues. If the ventral body wall is cut away the entire ovulation process may be observed with the naked eye. Ovarian contractions will be seen; eggs will drop away from their follicles into the body cavity, to be picked up by the numerous coelomic cilia and carried to the ostia, into these openings, through the oviducts and into the uteri. It takes about four hours at ordinary laboratory temperatures for the eggs to travel from the ostium to the uterus, and the rotation within the oviducts is easily aparent.

There are few observations in Biology more fascinating than the development of an organism from the single egg, particularly when the observations begin with fertilization. It is, of course, necessary first to understand this normal development before experimental conditions are applied. And in every instance sufficient and appropriate controls must be carried, using the same animal material that is used for the experimental conditions. There are still an unlimited number of research problems which can be approached through the use of this accessible animal material.

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