AMERICAN MUSEUM NOVITATES

Number 513 | Published by The American Museum of Natural History New York City | Jan. 29, 1932

59.79: 15, 6

EXPERIMENTS ON THE EGG-LAYING OF SALAMANDERS

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The discovery that implants of the anterior lobe of the fresh pituitary gland will induce a normal egg-laying in a salamander several months before the usual season (Noble and Richards, 1930) has opened up the possibility of employing this technique in securing the previously undescribed eggs of other species. It has recently been emphasized (Noble, 1927) that the mode of life history of an amphibian often furnishes a valuable clue as to the relationships of the species. The pituitary technique, inducing a normal ovulation, should be of interest, therefore, not only to embryologists but to students of amphibian phylogeny who are able to secure the adults of the rarer species alive.

With a view to securing data of phylogenetic interest we have induced several salamanders whose eggs were previously unknown to lay in the laboratory. Before describing the eggs of these species under separate headings, we have reported our preliminary experiments with *Eurycea bislineata* in some detail, because these experiments form the basis of our assuming that a normal egg-laying will follow the pituitary treatment.

The Egg-Laying of *Eurycea bislineata bislineata*

In our first experiments with *Eurycea bislineata*, we implanted during November, December, and January, twenty-eight adult females with anterior pituitaries removed from others of the same species, while thirteen controls received muscle. Nineteen of the twenty-eight laid, but none of the controls. As previously reported (Noble and Richards, 1930), the females were maintained in separate crystallizing dishes 20 cm. in diameter. A few stones were arranged in each dish in such a way that they formed a bridge under which the salamander could freely move. The water in the dish barely covered the stones. The eggs were laid and attached by stalks to the under surface of the rocks. The average number of eggs laid was twenty-five, maximum number forty-one, minimum three. In only two of the nineteen cases were there any mature eggs left in the ovaries at the close of egg-laying. Hence, *E. bislineata* lays a variable number of eggs.

In this first series of experiments we varied the time between implants with rather striking results. In the case of seven females, only three of which laid, the implants were given a week apart. Of eight specimens, implanted on alternate days, six laid. The remaining thirteen females laid after a single implant. From November 17, 1930, to January 9, 1931, we implanted a second series of thirty adult *Eurycea bislineata* on alternate days, and in this case twenty-six laid. This second series was intended to determine if the thymus had an influence on egg-capsule formation and thymectomy was attempted before the pituitary treatment. The females in this second series also received the pituitaries of their own species. Whether or not all of the thymus was removed from each of the females has not been determined at the present time. In spite of the fact that a large part at least was extirpated, the egg-capsules of all the eggs had their usual form. Further, the eggs were attached to the under side of the stones in the dishes in the normal manner.

By combining the two series it may be said that we have induced forty-five *Eurycea bislineata* to lay out of season in the laboratory. All attached their eggs in the normal manner to the under side of stones although these were placed in crystallizing dishes with fresh but not running water. Our breeding dishes represented only an approximation to conditions found in the brooks where *E. bislineata* lays its eggs, but we could detect no deviation from the normal method of egg-laying.

The manner in which the female deposited her eggs was essentially the same in all cases. Our most detailed records were made November 22, 1929, on specimens G, J, and L. The female clings, upside down, to the under surface of a flat rock. The back and tail are arched so as to bring the cloacal lips in contact with the rock. A place is "felt out" on the stone by the slightly protruding cloaca and the egg deposited. The time between the deposition of each egg depends upon the amount of wandering about the salamander does. In one case the female moved, methodically, a few centimeters after depositing each egg. She required on an average three minutes for the attachment of an egg. This female, G, came to the edge of the rock once during the laying period and put her nose out of water. She remained upside down for a minute in this position and then backed down under the rock to resume her egg-laying. None of the animals observed were seen to lay eggs loose on the bottom of the dish. A few eggs were knocked off by the female swishing her tail as she located the next place to lay. Another female, L, continued to lay while maintaining a position with head out of water. In this position the body was, as usual, upside down, but the forelimbs did not make contact with the rock. They were thrust out awkwardly to either side. At frequent intervals the female left this position and climbed up on top of the rock out of water. Other females, also, came out of the water during egg-laying and then returned to approximately the original position to continue ovipositing. A single well-defined cluster of eggs is often not deposited at one time, as one might assume, but is laid by a female who has made several short excursions away from the breeding rock at intervals during the period of oviposition.

The eggs deposited by the females were for the most part fertile, showing that many female *Eurycea bislineata* carry sperm in their spermathecæ during the winter months. We reared some of the larvæ under a variety of conditions but were much more successful with running water than with still water.

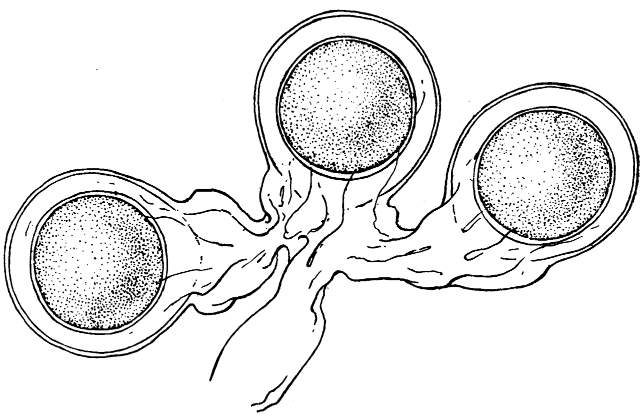


Fig. 1. Variation in egg-stalk of *Eurycea bislineata cirrigera* (Green). Fixed in formalin. ×7½.

Experiments with *Eurycea bislineata cirrigera*

In March 1930, we received from Mr. G. D. Ellis, in a collection of living salamanders from Winton, N. C., a lot of *Eurycea* eggs attached singly or in small clumps to water-weed and to bits of dead vegetation. The eggs were attached by short stalks and as many as eighteen were grouped on the same branch of water-weed with their bases in contact or very close together. It was apparent that the eggs had been laid in the field, for Mr. Ellis wrote of observing many. Some of these eggs were raised and the larvæ identified as *E. b. cirrigera.*

On April 9, 1931, a second collection of eggs laid in the field was received from Mr. Ellis, at Winton, N. C. The eggs were in eight lots, each lot having been laid presumably by a single female. As before, the eggs were attached by stalks to pieces of dead leaf, twigs, and moss. The number of eggs laid by one animal ranged from one to thirty-four, with an average of twenty. Several of the eggs were loose in the bottom of the container but, as they were stalked, it is probable that they were detached en route. In one case there were three eggs with a single common stalk (Fig. 1) and, on the same twig, two eggs with one stalk. This variation, the only one of its kind in this group of 159 eggs, shows a tendency toward the form of egg-capsule found in *Desmognathus fuscus*. The capsules of the remaining 154 eggs were essentially the same as those of *Eurycea bislineata*, although the stalks varied much more in length

The region near Winton, N. C., where the eggs were collected, lies in the coastal plain and, according to the unpublished observations of Mr. M. K. Brady, rocks do not occur in the streams. The question occurred to us, if *E. b. cirrigera* were given the choice between water-weed and a stone bridge, would it revert to the habits of *E. b. bislineata*. It is probable that many of the recorded differences in mode of life history of salamanders might be environmental and not hereditary. Would *Pseudotriton montanus*, for example, forced to breed in stony brooks, not lay its eggs on the under side of the stones in the manner of its close relative *P. ruber ruber* instead of scattering them among leaves in the way Brimley (1923) described? Here the testimony of induced egg-laying might yield interesting results.

We received from Mr. Ellis a large number of *E. b. cirrigera* swollen with eggs. We arranged a tank 30 cm.×75 cm. at the base, containing a stone bridge and several strands of water-weed in water of about 3 to 4 cm. in depth. A feeble current was maintained running the full length of the tank. Several females that had been previously kept in an ice box for several months were introduced into the tank. As indicated elsewhere (Noble, 1930), five of these laid their eggs attached to the under surface of the stone bridge. No eggs were attached to water-weed. It is possible that the water-weed was not of sufficient quantity or quality to attract the salamanders, but in view of the rather indiscriminate selection of vegetation in nature this suggestion does not appear likely. Apparently *E. b. cirrigera* would prefer to attach its eggs to the under side of stones but in the coastal plain habitat these are not available to the race. We could find no constant differences between the eggs of *E. b. cirrigera* when attached to stones and those of *E. b. bislineata* under the same condition.

Experiments with *Stereochilus*

The eggs of *Stereochilus marginatum* were unknown when we began our study. The species is not closely related to the other plethodontids of the coastal plain. Dunn (1926, p. 247), in his review of the family, remarks that the "species seems very isolated and it is scarcely profitable to speculate on its relationship" It seemed to us that the eggs of *Stereochilus* might throw some light on its relationship and hence we have endeavored to secure them under as natural a setting as possible. Since we had no information as to what this setting might be, we arranged a series of crystallizing dishes with various amounts of water, moss, sticks, and sand and introduced implanted females into these dishes where they could make a selection between a variety of "nesting sites."

For the work we selected twenty female *Stereochilus* collected December 14 to 19, 1929, at Winton, N. C., and maintained for nearly a year in large tanks of running water at the American Museum. From December 19, 1930, to January 11, 1931, we implanted these salamanders with fresh anterior pituitary glands secured from *Rana pipiens* before each operation. Nineteen of the twenty *Stereochilus* laid, the average number of eggs being fifty-seven, including the eggs eaten by the females during or immediately after oviposition. The females were maintained in separate dishes and four days after the last egg was laid they were preserved and the stomach contents noted. Six of the nineteen females ate some of their eggs. Examination of the ovaries and oviducts revealed that, had the females laid all the eggs mature at this time, the average number in each laying would have been seventy (maximum 121, minimum 16).

The females were divided into four groups of five individuals each, and the various groups subjected to different situations for egg-laying. The dishes in all cases were the same size, 25 cm. in diameter, but they were arranged with different material and amounts of water. The dishes of the first group included moss, sand, sticks, and water-weed, each with equal areas above and below water. The dishes of the second group included also moss, weed and sand but had only a fourth as much free water as sand and moss above the water. The third group was given a stone bridge and no water-weed. The bridge covered about half of each dish which was filled with water to the depth of 5 cm. The last group was given similar dishes but several strands of water-weed were allowed to float in the water. In this way the implanted *Stereochilus* were given the opportunity of laying their eggs under a wide variety of conditions.

Of the first group four out of five laid, the one that failed to do so having become infected. One laid after one implant, two after two implants, and the remaining one after four. Two laid at night, another under cover, and only one of this group permitted observations on the details of egg-laying. This individual (K in our records) received the first implant January 6 and another the following day. On January 10 she began to lay about 9 A.M. and continued throughout the day. It is uncertain when this individual finished laying because she was not observed again until January 12 when the laying was completed. The eggs were attached singly to water-weed in the water and confined to a limited portion of the weed where they tended to form a group. There were sixty-seven eggs in the lot and only one mature egg was later found in the ovaries.

The method of oviposition was of especial interest. Although the water-weed floated freely and could be approached from any side, the ovipositing female invariably turned over on her back before egg-laying. In some instances only the posterior part of the body was fully twisted, the anterior part being turned to one side and braced by the forelimb of that side (Fig. 2A). In such a position the body and tail were arched and the hind limbs free of support. In spite of the awkwardness of the position the female would "feel out" a suitable spot on a strand of submerged water-weed or moss with her slightly protruding cloacal lips and slowly push forth an egg from the cloaca until its very adhesive outer capsule came in contact with the selected spot. The time required for the extrusion of a single egg varied from approximately one to five minutes. Usually after laying an egg the salamander would regain her upright posture and walk about among the eggs and weed for a few minutes. Occasionally, after twisting the body and finding a suitable place for attaching the egg, there was a wait of from two to three minutes before the egg appeared between the lips of the cloaca. Although the salamander generally attempted to support the anterior part of the body with one forelimb while ovipositing, on several occasions this brace was withdrawn and the female turned completely over on her back (Fig. 2B).

During the day that this ovipositing female was under observation she was never seen to raise her head above the surface of the water. This is the more remarkable in that *Stereochilus* like other plethodontid salamanders fails to practice bucco-pharyngeal respiration when in the water. The nostrils are tightly closed and powdered carmine placed in the water shows no currents entering or leaving the mouth. Hence, during the period of egg-laying, *Stereochilus* may depend entirely on

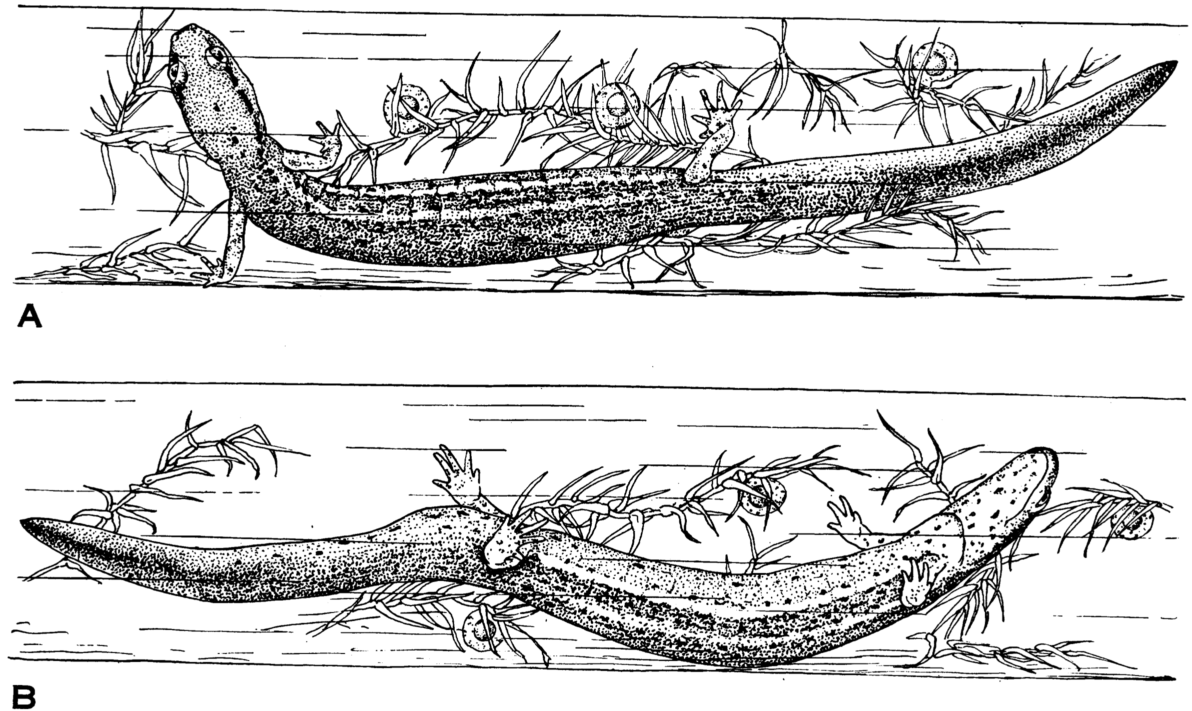


Fig. 2. *Stereochilus marginatum* (Hallowell). Specimen "K," in positions assumed during oviposition. ×1½.

cutaneous respiration. Subsequent observation, however, showed that this was not the rule, that usually ovipositing *Stereochilus* thrust its head above the surface and took air into the buccal cavity.

The three other salamanders of the first group did not all lay their eggs attached to water-weed in the water. Individual A of the series laid its eggs, seventy-nine in number, in the same manner as K. Individual N, however, laid thirty-one eggs on the underside of a sheet of moss which was only half submerged. Although the salamander's body was covered by the moss, her upside-down position was readily noted especially during the moments the head was thrust above the surface of the water. Later this female laid eleven unattached eggs free in the water. Possibly our examining the "nest" caused the animal to abandon her original site. The last salamander, C, of the first series laid a total of seventy-two eggs but only twelve were attached to water-weed as in "A" and "K," sixteen were loose in the water and forty-four were scattered on the wet sand just out of water. The egg-capsules of all the eggs laid out of water were swollen and slightly flattened, apparently due to their own weight. Although these eggs were left undisturbed, none developed, while those in the water began to develop normally.

In the second series of experiments the crystallizing dishes were partly filled with sand, moss, small stones, and leaves. A small pool was constructed in such a way that it occupied about a fourth of the total area of the bottom of the dish. The thought was that, if *Stereochilus* preferred a land to a water habitat for its eggs, ample opportunity would be available in these dishes. All five salamanders in this series laid: the first deposited thirty-three eggs after 12 implants, the second twenty-one eggs after two implants, the third fifty-seven eggs after one implant, the fourth nineteen eggs after one implant, and the last fifty-one eggs after one implant. Three of the five salamanders were observed while laying. Although the basin of water was very small, one salamander "T" laid in exactly the same manner as "K" described above. Another salamander "S" laid during the night, but since all the eggs were attached to water-weed in one portion of the basin we may assume that this individual also agreed with "K" in its manner of egg-laying. Two others, "Q" and "U" of the series, laid on the under surface of a piece of sheet moss lying partly submerged at the edge of the water. Aga n the observed females remained upside down during oviposition and frequently thrust their heads out until one or both nostrils were above the surface of the water. We have never seen any other species of salamander remain with only one widely open nostril above the surface of the water but we observed this behavior several times in these two females. We were able to determine by binocular examination that the valve closing the nostril is not opened until the surface is reached and is closed tightly the moment the nostril is drawn below the surface.

The last salamander, F, of the second series differed from the others in manner of egg-laying, but since it did not agree with "C" or with any in the first series its method is probably abnormal. The salamander began to lay at 2 P.M. and continued to lay at intervals throughout that afternoon and the following day. The first two eggs were merely extruded into the water. Ten minutes later the salamander assumed a position of rest on the edge of the pool with head and forelimbs out of water, but the hind limbs and posterior part of the body submerged. The tail was curved upward and bent slightly to the left but there was no other attempt to assume the inverted position. This was the female which had received twelve implants, and except for the first two eggs almost the entire batch of thirty-three eggs was laid in this one spot. It is possible that excessive chloretonizing or other feature of the treatment had affected this salamander, preventing a normal behavior. Nevertheless, the salamander wandered about considerably while not ovipositing, always returning to the same spot. The interval between the laying of successive eggs varied from thirty seconds to fifteen minutes.

The third group of *Stereochilus* was given the "*Eurycea bislineata* habitat" described above. Two or three flat stones were arranged to form a bridge under which the salamanders could easily crawl. The stones were covered with water but no water-weed was added to these dishes. All of the salamanders in this group laid: one depositing one hundred and twenty-one eggs after five implants, a second only sixteen eggs after seven implants, a third twenty eggs after three implants and the last seventy-four eggs after two implants. An average of 82 per cent of the eggs were laid attached to the stones. A few others were very probably broken loose accidentally by the female after they had been attached to the stones. Observations were made on three of the salamanders in this series while they were laying. *Stereochilus* lays its eggs in a manner very similar to *Eurycea bislineata bislineata* under the same conditions. The female clings upside down to the under surface of a flat stone. The rock is usually gripped by the female with her front limbs, although occasionally the hind limbs are also used for support. Her back and tail are arched, her cloacal lips slightly protruded and then cautiously brought forward to "feel out" a suitable place for egg attachment. At the moment the egg appears between the lips of the cloaca the latter is brought tightly against the rock and the egg, adhering firmly to the rock by its outer capsule, is pulled from the cloaca. The extrusion time is approximately as long as when water-weed formed the substratum (K). After laying one egg the salamander moves on to a new spot and again brings her protruding cloacal lips in contact with various parts of the surface before settling down to lay another egg. When walking the tail is switched slowly from side to side and while this tends to carry the salamander forward it sometimes knocks newly laid eggs from their attachment.

During the process of egg-laying the female frequently comes to the edge of the rock and thrusts her head out of water while maintaining the inverted position. In some cases the salamander may continue laying while the nostrils are above the surface. In order to test this oxygen requirement further, we raised the water in the dishes to a depth of 6 or 7 cm. after two salamanders (P and M) had begun to lay. Both salamanders continued to make efforts to reach the surface. Usually the hind legs and tail remained in contact with the under surface of the rock while the forelimbs assumed an awkward position at nearly right angles to the upwardly directed body. One salamander (M) was observed to lay an egg while her body was in this vertical position and the cloaca not in actual contact with the rock. It is clear that *Stereochilus* will make a considerable effort to breathe air at intervals during the period of egg-laying, although our observations on "K" would indicate that the female is also capable of remaining submerged for a long time during this period.

The fourth and last group of *Stereochilus* was placed in dishes provided with stone bridges as in the case of the third series but, in addition, water-weed was added. The weed was of the same species and arranged approximately as thickly as in the first series of experiments. All the salamanders in this group laid. One deposited seventy eggs after seven implants, another one hundred and ten eggs after six implants, a third fifty-seven eggs after two implants, a fourth sixty-three eggs after two implants and the last forty-eight eggs after one implant. In no case were the eggs attached to water-weed. With the choice of weed and stone 71 per cent of the eggs were attached to the stones, the remainder being free in the bottom of the dishes. As indicated above, many of these loose eggs may have been knocked from the rocks by accident while the female was switching her tail back and forth during the "feeling out" phase of egg-laying. Two salamanders of the series were observed in the act of egg-laying. Their behavior was exactly the same as in the case of the third group.

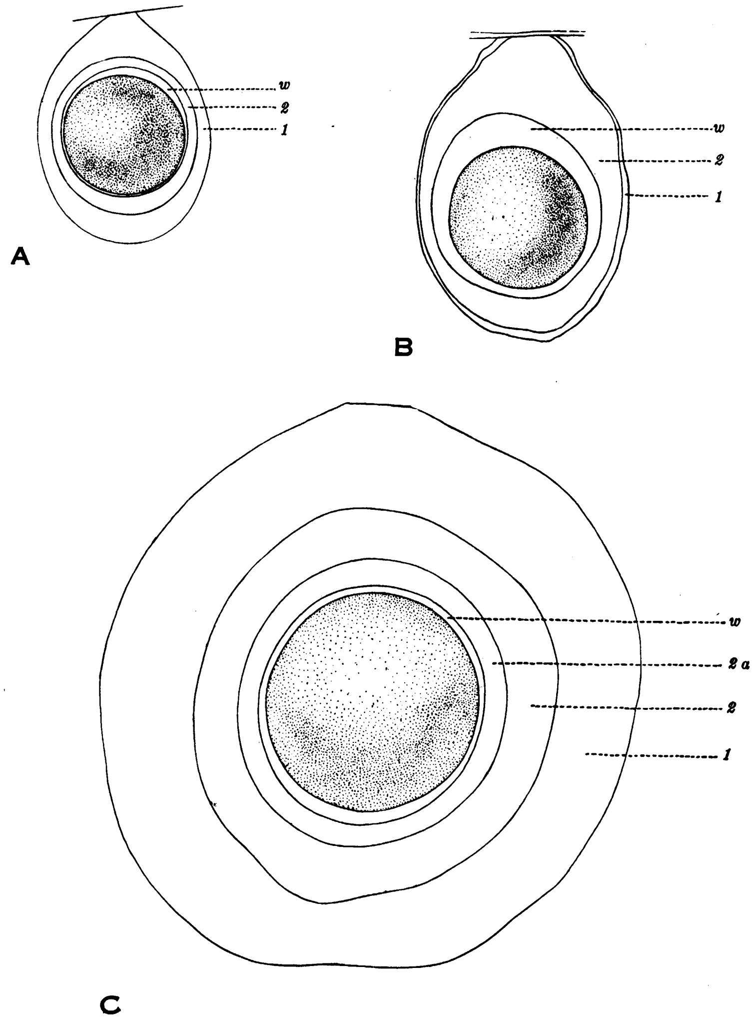


Fig. 3. A, Egg of *Stereochilus marginatum* (Hallowell). The outer capsule is elongated to form a slight stalk. Fixed in formalin. ×7½.
B, Egg of *Eurycea bislineata bislineata* (Green). Fixed in formalin. ×7½.
C, Egg of *Gyrinophilus porphyriticus* (Green). Fixed in formalin. ×7½.
1, Outer capsule; 2, inner capsule; 2*a*, second inner capsule; *w*, fluid space.

The Eggs of *Stereochilus* and Other Plethodontids

The eggs of *Stereochilus* as described above differ from those of *Eurycea bislineata* in having little or no stalk. On the average they are a little more than twice as numerous, although the adult female *Stereochilus* is only slightly larger than the adult female *Eurycea bislineata*. Correlated with this greater number, the eggs of *Stereochilus* are slightly smaller than those of *E. bislineata*. A more fundamental difference is to be found in the egg-capsules. Both *Stereochilus* and *Eurycea bislineata* have two egg-capsules surrounding a space filled with fluid. The egg covered by its very thin vitelline membrane floats in this space which under a lens might be mistaken for a capsule. The space is very much larger in *E. bislineata* than in *Stereochilus*, but in both the fluid is under such pressure that when a hole is made in the two outer capsules the egg, covered by its thin vitelline membrane, is forced out with strength enough to cause it to become ruptured.

An important difference between the eggs of *Stereochilus* and those of *E. bislineata* is to be found in the two outer capsules. In *Stereochilus* the outermost is thick, soft, and adhesive. In *E. bislineata* the outer capsule is very thin (Fig. 3B). The second, or inner capsule, is thin and very resistent in *Stereochilus*, while in *E. bislineata* it is thick and takes part in the formation of the stalk. The outer capsule of an egg of *Stereochilus* attached to a stone might become elongated to form a slight stalk (Fig. 3A), although this is not the rule (Fig. 4). The relative thickness of the capsules and their relation to the point of attachment will readily serve to distinguish any *Stereochilus* egg from that of *E. bislineata*. An average of five eggs of *Stereochilus* preserved in formalin compared with five preserved eggs of *E. bislineata* is as follows:

	Diameter of Egg	Diameter of Inner Capsule	Diameter of Outer Capsule
Stereochilus	2.25 mm. (2.5 max. 2.0 min.)	2.64 mm. (3.0 max. 2.5 min.)	3.1 mm. (3.5 max. 3.0 min.)
E. bislineata	2.60 mm. (2.75 max. 2.5 min.)	2.95 mm. (3.25 max. 2.5 min.)	3.8 mm. (4.5 max. 3.5 min.)

Stereochilus, in the retention of the parasphenoid teeth patches in a line continuous with the vomerine series, is one of the most primitive plethodontids. A comparison of its eggs with those of *Gyrinophilus*, the most primitive living plethodontid, should, therefore, be of interest. No egg-capsules of any species of *Gyrinophilus* have been described in detail. On March 6, by implanting a large female *G. porphyriticus* with three anterior pituitaries of *Rana pipiens*, we induced her to lay. The first seventeen eggs were laid on moss, before the salamander was transferred to a crystallizing dish provided with a stone bridge. On March 7, forty-six additional eggs were laid: twelve attached singly to the under side of a stone, eight attached to the edges of the same, and twenty-six were loose on the bottom (possibly some of these had been detached after laying). On March 9, eighteen more eggs were laid: nine on edge of rock, one on its under surface, and eight, when found, were loose on the bottom of the dish. A feature of special interest was the high percentage of "fused eggs." Ten of the eggs were in pairs enclosed in one or two capsules in common, and three formed a single group surrounded by

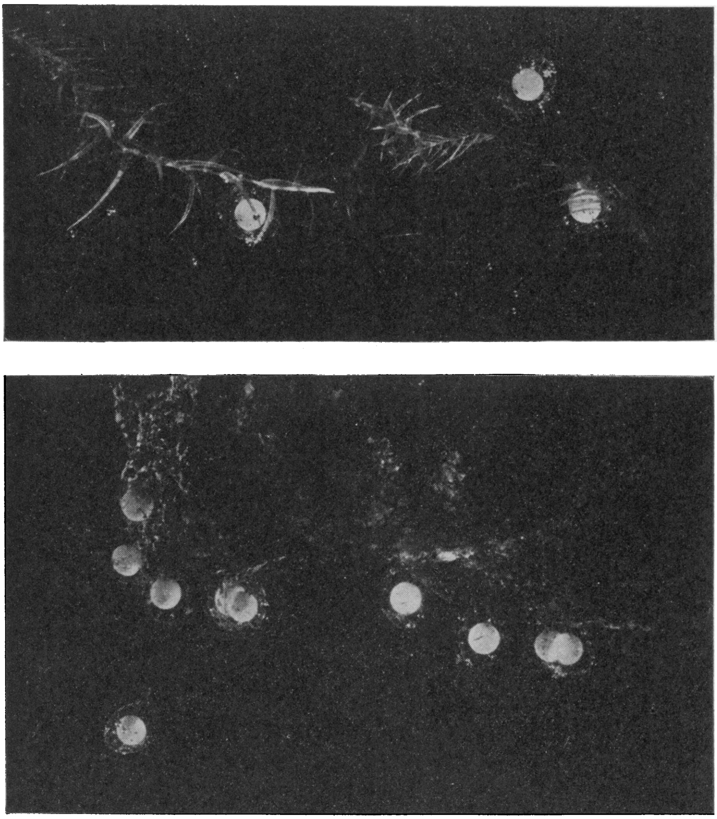


Fig. 4. Eggs of *Stereochilus marginatum* (Hallowell). Attached to stone and water-weed. Photographed a few hours after having been laid. ×2

a common outer capsule. Thus, this female laid ninety-three eggs in all. In spite of the fact that the adpressed surfaces of these eggs were considerably flattened, most of the double eggs developed to the neural plate stage, when one of each pair invariably died.

The eggs of *Gyrinophilus porphyriticus* agree with those of *Stereochilus* in lacking a definite stalk and in having a relatively thick, soft and adhesive outer capsule. The fluid-filled space is comparatively narrow as in *Stereochilus.* The outer capsule is more irregular than in that genus. The most distinctive difference between the eggs of *Gyrinophilus* and those of *Stereochilus* is to be found in the inner capsules. In *Stereochilus* the second or inner capsule has a thin, slightly opaque inner surface. In *Gyrinophilus* this lining membrane, if such it may be called, is swollen to form a distinct capsule. There is thus one more capsule in *Gyrinophilus porphyriticus* than in *Stereochilus*, but otherwise the egg-capsules are in close agreement (Fig. 3, A and C).

The eggs of *Gyrinophilus porphyriticus* are much larger than those of *Stereochilus.* The average dimensions of six eggs of the former are as follows:

Diameter of Egg	Diameter of Capsule 2A	Diameter of Capsule 2	Diameter of Capsule 1
3.8 mm. (4.0 max. 3.5 min.)	4.81 mm. (5.25 max. 4.25 min.)	6.5 mm. (7.00 max. 5.5 min.)	8.6 mm. (9.0 max. 7.5 min.)

It may be noted that the dimensions of living eggs and those preserved in formalin are not the same. In the case of the present species the capsules have slightly swollen in formalin. The above dimensions are of eggs that had been in formalin for five days. The same eggs were measured a few hours after they were laid and before they were put into formalin and the relative proportions have changed only slightly, the living eggs averaging 3.5 mm., 4.3 mm., 4.9 mm., and 6.2 mm., for the same parts as are listed above.

The Egg-Laying of Sirenids

Siren and *Pseudobranchus* are of especial interest to students of amphibian phylogeny for they are the only living representatives of a distinct suborder of salamanders, Meantes. No details were known concerning the eggs of either *Siren* or *Pseudobranchus* until recently, when one of us published a note concerning the eggs of the latter (Noble, 1930). Since then, February 1931, we received a series of *Pseudobranchus* from Mr. J. S. Alexander from Gainesville, Florida. By using pituitary implants we have found no difficulty in making ten of the largest females deposit eggs in crystallizing dishes half full of water and provided with abundant water-weed. The eggs are laid singly or in small groups. Each is provided with a thick, opaque, outer capsule which is very adhesive and usually forms a broad attachment to the bottom of the dish or to the more resistent weed. Closely adherent to the outer capsule is a transparent, much firmer, less adhesive inner capsule. An examination of a series of the eggs preserved in formalin has shown that this inner capsule which appears single in the fresh egg and was originally described as such is, in fact, duplex. Its inner portion is more resistent than the outer and the two parts may be distinguished with proper illumination of the preserved capsules. The inner capsule is separated from the egg and its closely adherent vitelline membrane by a wide fluid-filled space. The egg is very soft and when a small hole is made in the capsules the egg is driven out by the pressure of the intracapsular fluid. Although more than half the egg can go through a hole only a fraction of its diameter, the remainder usually ruptures before the egg is entirely free.

The eggs of *Pseudobranchus* undergo less change than the plethodontid eggs described above when fixed in the same strength formalin. Probably this is due to the resistent inner capsule and the large fluid-filled space around the egg. Five fresh eggs average as follows:

Diameter of Egg	Diameter of Inner Capsule	Diameter of Middle Capsule	Diameter of Outer Capsule
2.55 mm. (2.75 max.) 2.5 min.)	3 mm.	4.2 mm. (4.5 max. 4.0 min.)	5.6 mm. (6.0 max. 5.5 min.)

By way of comparison: an average egg fixed in formalin measures 2.6 mm., 3.1 mm., 4.1 mm., and 5.2 mm. for the same parts (Fig. 5A).

We experienced considerable difficulty in rearing the eggs of *Pseudobranchus*. Fertilization is apparently external in the Sirenidæ. Although we implanted males we never witnessed any sexual activity. Nevertheless, several of the eggs cleaved and one developed into a normal larva. Finally, in order to secure developmental material, we resorted to removing the eggs from the oviduct and artificially fertilizing them. A discussion of this material is reserved for a later report.

Having been successful in securing normal eggs of *Pseudobranchus* we were interested to compare these with those of *Siren*. We were fortunate in receiving, early in February 1931, a series of *Siren* from Mr. J. S. Alexander. Over the week-end of February 16, eight eggs were found in a large tank containing seventeen adult sirens. By isolating the larger specimens in different tanks we finally determined the female which was laying the eggs. The next day four more eggs were laid. Twenty eggs were laid the following day and nineteen more by February 21.

Since none of these eggs were apparently fertilized, although many were left in the tank with a large number of adults, we decided on February 25 to sacrifice the female and attempt artificial fertilization. The latter procedure presented some difficulties, since no external sexual difference is known in this species, and we could not be sure of the sex without dissection. Considering the possibility that the male would have a broad tail-fin, we selected the broadest tailed adult in the collection. Dissection revealed that this was a female. Our second choice, a narrow-tailed adult, proved to be a male. With this freshly prepared male we proceeded with the assistance of Mrs. E. R. Mason to examine the *Siren* which had been laying the eggs for several days previously.

When the female was opened the right oviduct was found to be greatly distended and no less than 265 eggs were removed from its caudal end. Many of these were swollen and all but fifty exhibited some creases or furrows on the surface of the egg proper. These furrowed eggs were placed in large dishes of water, but in no case did development proceed to gastrulation. The furrowing was so irregular that it appears extremely doubtful that this was a typical development.

The fifty eggs showing no evidence of cleavage, if such the furrowing may be called, were artificially fertilized by a sperm suspension made from the testis of the other *Siren*. None of these eggs developed normally or passed beyond gastrulation although many underwent some cleavage. Some of the furrowed eggs removed from the oviducts were preserved and sections of these may show whether or not fertilization had occurred. Since the eggs removed from the oviduct had the same number of capsules as those laid in the tank the capsules must be secreted by the more anterior portions of the oviduct.

The first eggs laid in the tank showed no evidence of furrows and we may assume these are normal, even though unfertilized, eggs. It was interesting to find how closely they agreed with the eggs of *Pseudobranchus*. Three egg-capsules were plainly visible even before fixation. The outer is opaque whitish in color, becoming whiter in time. The middle is much thinner than the outer. It is translucent and closely adherent to the latter. In dissecting out the egg the outer capsule may be separated only with difficulty from the middle. In striking contrast the middle capsule of the fresh egg does not adhere to the inner. When only a small section of the middle capsule has been cut the egg, surrounded by the inner capsule, slips out. In some of the fresh eggs a narrow fluid-filled space was found immediately surrounding the inner capsule. This is not demonstrable in eggs preserved in formalin and is not shown in the drawing (Fig. 5B). The inner capsule is much thinner than the middle but more resistent. Its firmness is due in part to the fluid surrounding the egg being under considerable pressure. A small hole made in the inner capsule releases the pressure but the egg forced

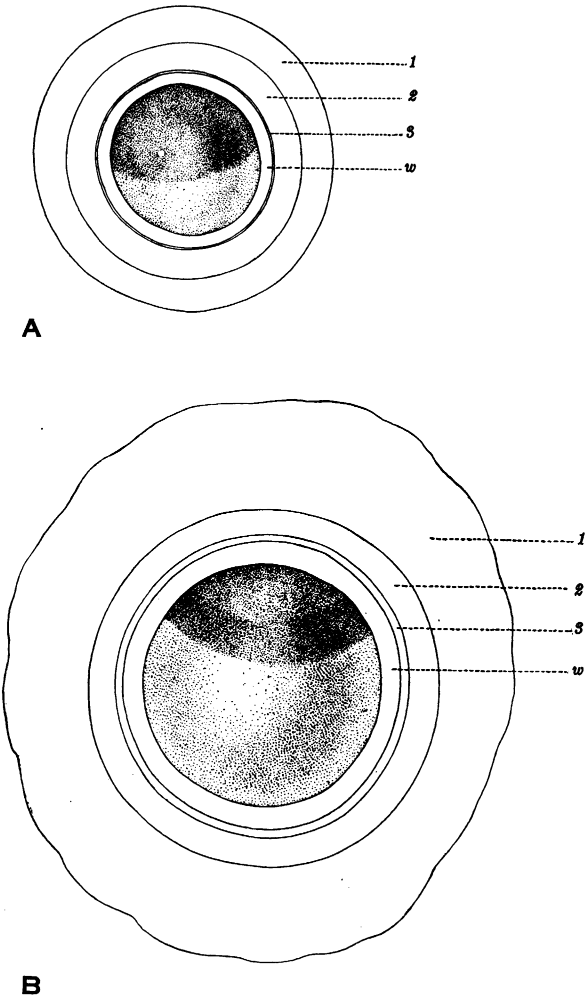


Fig. 5. Sirenid eggs. Fixed in formalin. ×7½.
A, *Pseudobranchus striatus* (Le Conte).
B, *Siren lacertina* Linné.
1, Outer capsule; 2, middle capsule; 3, inner capsule: *w*, fluid space.

into the opening usually bursts before the capsule may be opened wide enough to permit the egg's escape.

The eggs of *Siren* agree with those of *Pseudobranchus* in having the upper third or two-fifths heavily pigmented with brown. The polar bodies of *Siren* lie eccentrically in the pigmented cap and usually in a very small densely pigmented depression. The periphery of the pigmented cap of *Siren* is darker than the central portion. In some eggs the latter may be only a little darker than the vegetative pole.

To summarize, it may be noted that, while the eggs of *Siren* differ from those of *Pseudobranchus* in their larger size and unequally pigmented animal pole, they agree in all other essential particulars. In both: (1) the eggs are laid singly or in small groups; (2) the outer capsule is opaque and very adherent; (3) the middle capsule is translucent, adherent to outer capsule but free from an inner capsule; and (4) this thin inner capsule is well raised from the egg (and its vitelline membrane) by a fluid which is under considerable pressure.

In the above description we have compared the eggs of *Siren* and *Pseudobranchus* received from Florida. It is usually assumed that these two genera are represented by only a single species each. There is considerable evidence that the Arkansas *Siren* is referable to a distinct species. The egg-capsules of this western *Siren* do not agree in full detail with those of the Florida form. These differences will be discussed in the paper dealing with the description of the Arkansas material.

The Egg-Laying of *Rhyacotriton olympicus*

The family Ambystomidæ includes only three genera of salamanders: *Ambystoma*, *Dicamptodon* and *Rhyacotriton*. The Asiatic land salamanders, *Hynobius* and its allies, are not ambystomids as frequently stated but comprise a well-defined family, the Hynobiidæ (Cope, 1889; Dunn, 1923). Within the Ambystomidæ there appear to be two natural groups of genera, for *Dicamptodon* and *Rhyacotriton* are characterized by a lacrymal bone which is lacking in *Ambystoma*. The eggs of *Dicamp todon*, as shown by Storer (1925), differ remarkably from those of any species of *Ambystoma* so far as known and the question arose would the eggs of *Rhyacotriton* agree with those of *Dicamptodon*. Structurally *Rhyacotriton* appears to be a dwarf derivative of *Dicamptodon* but the question remained as to how far the life-history data gave evidence of this relationship.

Fortunately, Mr. Phillips G. Putnam was collecting *Ascaphus truei* in the Olympic Mountains, Washington, at the time we began this study and we urged him to collect gravid *Rhyacotriton* alive for us and to observe any details of life history which might be worked out in the field. Mr. Putnam collected in the Lake Cushman area from June 5 to September 9, 1930, and shipped, in chests partly filled with broken ice, a large series of living specimens. These were transferred to an ice box where they were available for the experimental work.

We began our experiments at once without waiting for Mr. Putnam's field notes, but his more significant observations may be recorded first since they have an important bearing on our findings. On June 5, in one of the small streams near Lake Cushman, *Rhyacotriton olympicus* was apparently paired. At least individuals of opposite sex were found together and the ovaries and testes appeared to be in a breeding condition. On June 7, in Elk Creek, a single egg was found attached to a tendril on the under side of a stone, and it was assumed to have been laid by *Rhyacotriton*. Another pair of *Rhyacotriton* was found together June 11 in Triple Trip Creek where the water temperature was 10° C. and the air temperature 14° C. Larvæ of *Rhyacotriton* were also collected in this stream. Paired *Rhyacotriton* were found in Madeline Creek on June 24 and Mr. Putnam writes in his field book under this date:

> In one section of Madeline Creek about one half mile above the trail junction the creek passes through a broken gorge with perpendicular walls up to 30 ft. in height. Here, little sunlight filters through and the spray from numerous waterfalls gives the effect of a constant drizzle. In this area I found *Rhyacotriton* numerous, especially in the cave-like recesses beneath large rocks. Here sometimes as many as eight *Rhyacotriton* would be seen together on a single mossy stone. They are very alert and most of them would plunge into the foaming water to escape.

Subsequent observation confirmed the conclusion that *Rhyacotriton* prefers those portions of the stream that are overhung by cliffs or trees and where little light penetrates. Mr. Putnam returned to Madeline Creek on July 6 and after considerable search found a single egg which he again assumed to have been laid by a *Rhyacotriton*. His previous dissection of gravid females had led him to conclude that a single individual must lay very few eggs and he writes in the field:

> The *Rhyacotriton* eggs are apparently deposited singly beneath stones and are without conspicuous gelatinous capsules. An adult *Rhyacotriton* deposits approximately only twelve eggs which are naturally difficult to find.

Subsequent search in this and many other creeks in the Lake Cushman area failed to disclose any additional eggs. No paired adults were seen again although a large series of adults and larvæ were collected. The two eggs secured by Mr. Putnam were destroyed in transit and no further notes concerning them were made. It is therefore fortunate that, due to Mr. Putnam's ingenuity, gravid specimens reached New York in good condition. For two evenings we allowed the adults of both sexes to wander about a tank supplied with running, iced water. Flat stones were added and the temperature of the water slowly raised and lowered. Although all observations were made at night with the aid of a red light, no definite courtship behavior was noted and no eggs were laid.

We, therefore, turned to the pituitary technique as a means of inducing ovulation.

On June 27, 1930, six gravid females and four adult males were implanted with anterior pituitary tissue secured from local *Desmognathus fuscus*, *Eurycea bislineata*, and *Rana catesbeiana*. The specimens were then returned to the tank mentioned above. The tank was provided with flat stones and the water cooled with ice to 6° C. The water was then allowed to warm to 16° C. Because of an intense heat wave the temperature was difficult to regulate in the tank and the specimens were transferred to a series of crystallizing dishes in the ice box. Here an approximate temperature of 7° C. was maintained and the dishes were provided with flat stones arranged as in the case of the *Eurycea bislineata* experiments reported above. On July 14, after eleven implants, one female laid. On the following day three others laid. The eggs were attached to the sides and upper surfaces of the rocks. On July 24, eight more females were implanted. After eleven implants, one laid. The remaining seven failed to lay even after thirty implants. The average number of eggs laid by the five females was five, the maximum number eight and the minimum number three.

From these experiments it would follow that five is the average number of eggs laid by *Rhyacotriton olympicus*. An examination of the ovaries of a female preserved in formalin in the field in 1922 shows four well-developed eggs in one ovary, and three in the other. The ovaries of a gravid female preserved in the field in 1930 show five eggs in all.

The egg of *Rhyacotriton olympicus* is large and pigmentless. Although a breeding female measures only 87 mm. in total length, the formalin-fixed eggs average 4.5 mm. in diameter, without the capsules. In the living egg there appear to be three capsules: a soft, gelatinous inner capsule, a firmer, more opaque middle capsule, and a thick transparent outer capsule. In the egg preserved in formalin (Fig. 6) the inner capsule is opaque and the middle capsule swollen. In the fresh egg the middle capsule is so thin that it might be considered merely a membrane covering the inner capsule. In the preserved egg the middle capsule is very distinct. For a few hours after laying the outer capsule exhibited a series of fine ridges. By the time the two-celled stage was reached these ridges had entirely disappeared.

By combining Mr. Putnam's field observations with the above description we may conclude that *Rhyacotriton* lays its eggs singly under or between stones in mountain streams. The eggs are very large, unpigmented and provided with three capsules. *Rhyacotriton* differs from

Dicamptodon in laying fewer eggs of very large size (as compared with the smaller body-size). There is also a difference in the mode of attachment of the eggs, *Dicamptodon* depositing its eggs in groups instead of singly. The eggs of the two species differ in that *Dicamptodon* has one instead of three capsules.

Small Amphibia usually lay fewer eggs than their larger relatives (Noble, 1927). The newts primitively laid their eggs in groups as in the case of *Pleurodeles waltl*, but many derived species such as *T. viridescens* lay their eggs singly. Hence the most important divergence of *Rhyacotriton* from *Dicamptodon* lies in the egg-capsules. In view of observations on the egg-capsules of the plethodontids and sirenids described above this difference would appear to be a fundamental one. The increase in the number of capsules is an approach to the condition in *Ambystoma.*

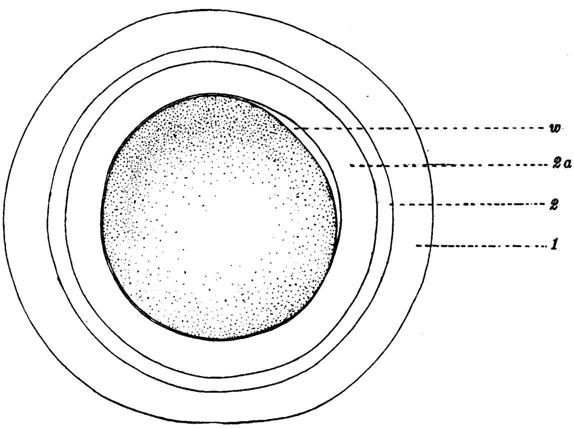


Fig. 6. Egg of *Rhyacotriton olympicus* (Gaige). Fixed in formalin. ×7½.
1, Outer capsule; 2, middle capsule; 2a, inner capsule; w, fluid space.

Experiments with *Ambystoma opacum*

From the above experiments it appears that implanting fresh anterior pituitary substance will induce salamanders to lay their eggs provided these have reached a certain stage of maturity. Although we implanted male *Gyrinophilus porphyriticus, Eurycea bislineata,* and *Rhyacotriton olympicus* in the same way as the females for extended periods, in not a single case did we obtain spermatophores which could be attributed to the treatment. This negative result could not be due to the fact that the breeding season was past and that sperm were no longer in the ducts. Several of the *Eurycea* and *Gyrinophilus* treated were individuals which were actively courting before we began the treatment. Houssay, Giusti and Gonzalez (1929) noted that the male toad required more implants than the female to induce sexual activity. Apparently male salamanders are even less sensitive than toads to the implants. The question should be tested by carefully planned experiments.

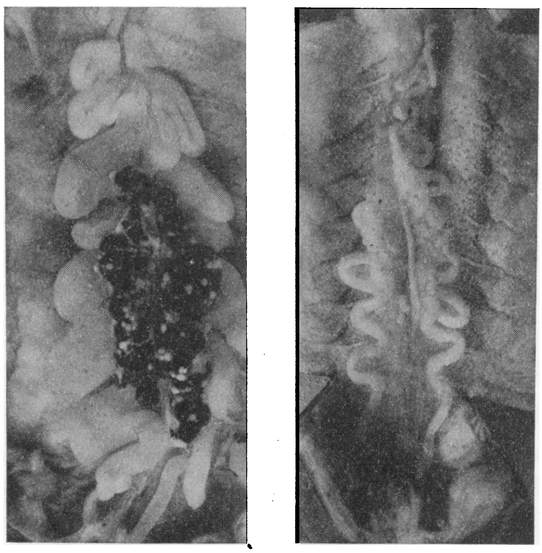
There is another question regarding the gonad stimulating influence of the anterior pituitary which the above experiments did not consider. Although implants of fresh anterior pituitary substance will induce a release of the eggs from the ovary several months before the normal season, the question remained as to what influence, if any, the pituitary implants would have in building up a spent ovary. Information in this direction might prove of practical value to the student who wished to obtain the maximum number of eggs from a limited number of females.

Ambystoma opacum appeared to be a favorable species for experimentation in this field, because the females after laying their eggs on land in the fall remain with them. Noble and Brady (in manuscript) have shown by dissection that brooding females are invariably spent.

In October 1929, a series of brooding *Ambystoma opacum* collected by Noble and Brady were set aside for experimentation. Four of the females were implanted with fresh anterior pituitary substance taken from a variety of salamanders and frogs. The implants were made approximately every other day. Two other females were implanted the same day with muscle. Each salamander was maintained in a separate crystallizing dish and adequately provided with moss and damp leaves. In order to be sure that both experimentals and controls secured the same amount of food, all the salamanders were force-fed on raw beef.

The salamanders did not stand the treatment well and eventually mold appeared on most of them. On March 27 one female, which had received twenty-nine implants and had been edematous for several weeks, died from the mold. On May 5 another female which had received thirty-nine implants died and one of the muscle-implanted controls was sacrified and preserved for comparison. The difference between the ovaries and oviducts of the second experimental and those of its control was very marked. The oviducts of the control specimen were less than a fifth as wide as those of the experimental and not nearly as convoluted. The ova were uniformly about .5 mm. in diameter, while the diameter of many of those of the experimental animal were from 1.5 mm. to 2 mm.

On May 6 and 7 the remaining salamanders of the series were sacrificed since forty implants had been given and none of the females appeared to be in a breeding condition. Again a very marked difference in the ovaries and associated ducts of the experimental specimens when compared with those of the control was noted. This difference is well shown in the photographs of one of the pituitary implanted females (Fig. 7, A and B), killed on May 7, and its control specimen. The oviducts, measured at the most posterior convolution, are twice as wide in the experimental as in the control and the ova of the pituitary implanted specimen measure from 1.5 to 2 mm. in diameter in contrast to those of the muscle-implanted control, which measure .5 mm. in diameter.



A *B*

Fig. 7. A, Ovaries and oviducts of *Ambystoma opacum* (Gravenhorst) implanted with anterior pituitary. ×2

B, Control specimen implanted with muscle. ×2

It is clear from the above experiments that anterior pituitary implants will build up the ovaries and hypertrophy the oviducts of a spent *Ambystoma opacum.* We did not succeed in inducing salamanders which had laid in the fall to lay again in the spring. This may have been due to the frequent infections of mold. It is possible that we did not implant enough pituitary substance at any one time. Judging from our experience with other salamanders, large frog pituitaries might have brought quicker results.

CONCLUSIONS

1.—*Eurycea bislineata bislineata* may be induced by fresh anterior pituitary implants to lay its eggs several months before the normal season. The eggs are attached to the under side of stones in the usual manner and develop normally.

2.—During oviposition the female may leave the breeding rock but she returns to continue laying in the immediate vicinity of the first-laid eggs.

3.—Although *Eurycea bislineata cirrigera* attaches its eggs singly in irregular groups to water-weed in its native habitat, it will attach them to the under side of stones in the manner of *E. b. bislineata* when it is given a choice between submerged water-weed or stones. The egg-capsules may show some variation but usually resemble those of *E. b. bislineata* closely.

4.—Implanted *Stereochilus marginatum* will lay its eggs under a variety of conditions, but the aquatic habitat appears to be the normal situation. When ovipositing in the water the female turns upside down. This appears to be the retention of the habits of mountain-brook ancestors which presumably attached their eggs to the under side of stones.

5.—The eggs of *Stereochilus* resemble those of *Eurycea bislineata* but are smaller, more numerous and lack stalks. They resemble those of *Gyrinophilus porphyriticus* most closely although these are larger and have an additional capsule.

6.—The eggs of *Pseudobranchus striatus* and *Siren lacertina* laid in the laboratory agree closely and differ from those of all other salamanders. The eggs are pigmented, laid singly or in small groups, and are provided with an opaque, adherent outer capsule, a translucent middle capsule, and a thin inner capsule separated by a wide fluid-space from the egg with its tightly fitting vitelline membrane.

7.—*Rhyacotriton olympicus* induced to lay in the laboratory by pituitary implants deposits on the average five eggs and this may be considered the average number of eggs for the species in nature. The eggs are large, unpigmented, and have three capsules. In nature *R. olympicus* breeds during June and July and attaches its eggs singly beneath stones in cold mountain-streams.

8.—Pituitary implants will build up the ovaries of a spent female salamander. A marked increase in size of both the ovaries and oviducts in spent *Ambystoma opacum* was obtained by pituitary implants.

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