THE EMBRYOLOGY OF CRYPTOBRANCHUS ALLEGHENIENSIS, INCLUDING COMPARISONS WITH SOME OTHER VERTEBRATES

I. INTRODUCTION; THE HISTORY OF THE EGG BEFORE CLEAVAGE

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FIFTY-SIX FIGURES

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V. The internal history of the egg before cleavage.

I. INTRODUCTION

For more than a generation zoologists have eagerly sought for the embryological material of the hellbender, Cryptobranchus allegheniensis Daudin. Until quite recently these efforts have been conspicuously lacking in success. It seems remarkable that the life history of an animal so large, so abundant in localities easy of access, and so important from a phylogenetic point of view, should so long remain shrouded in mystery. But the same difficulty has been encountered in attempts to work out the natural history of several nearly related forms. Eycleshymer ('06) says:

> After years of persistent and patient effort Professor Whitman finally discovered the nests and eggs of Necturus. Only those who have for years been baffled in their attempts to obtain the embryological material of other North American urodeles, such as Siren, Amphiuma, and Cryptobranchus can properly appreciate the enormity of the task.

In the case of Cryptobranchus the difficulty in finding embryological material seems to have been enhanced by the unusual breeding season of the animal; the eggs are laid in the fall, while most amphibia spawn in the spring. Townsend ('82) published a general description of some fertilized eggs which he states were deposited in August. McGregor ('96) described very briefly an embryo 16 mm. in length, and ('99) stated that the eggs are deposited in August and September. Yet the information thus acquired in regard to the time of spawning seems not to have become generally known to others who were searching for the eggs. A suggestion might have been obtained from Sasaki's ('87) observation that the Japanese 'giant salamander,' Cryptobranchus japonicus (Megalobatrachus maximus Schlegel), deposits its eggs in August; but this also seems to have been overlooked. Reese ('04) succeeded in obtaining some unfertilized eggs, of which he gave the first detailed description.

The embryological record for Cryptobranchus allegheniensis remained almost a blank until 1906, when I published a preliminary report containing, besides a description of the sexual elements, a brief account of the external development during the cleavage stages. A later contribution (Smith, '07), devoted chiefly to the habits, more particularly the breeding habits, included a very general account of the life history.

From a phylogenetic point of view great interest attaches to the amphibia; there is no doubt that they lie close to the extinct ancestral stock of the highest forms of vertebrate life. Concerning the origin of the amphibia themselves Kingsley ('99) says: "All the facts of structure and development go to show that the amphibia have arisen from the crossopterygian ganoids, and that existing groups have descended from the stegocephali, each by its own line of ancestry." But when we inquire further, and attempt to trace more particularly the origin of any group of existing amphibia from an extinct form exhibiting affinities to the crossopterygii, we are landed at once in the midst of uncertainties. Confining our attention to the urodeles, we are confronted with the difficult question of the phylogenetic relationships of the different members of this group. The problem will be more fully discussed in a later section; for the present it will be sufficient to call attention to one of its leading aspects. From existing urodeles we may select a series of forms illustrating all stages in a transition from an aquatic to a terrestrial mode of life, or vice versa. In which direction should the series be read? Or have we stated the question incorrectly, and have the urodeles reached their present condition, some from an aquatic, some from a terrestrial ancestry?

In studying this aspect of the phylogenetic problem our attention cannot fail to be attracted by Cryptobranchus. For here we have a urodele whose entire life is spent in the water, characterized by persistent gill slits, the most primitive brain (Osborn, '88), and external fertilization (Smith, '07). On the other hand Cryptobranchus is known to possess deciduous external gills, functional lungs, and a method of locomotion by crawling on the bottom which suggests a former terrestrial habit. Is Cryptobranchus primitively aquatic, or does it come down to us bearing evidence of a former land-living existence? An answer to this question would go far in advancing our knowledge of the phylogeny of the entire group.

In the solution of our phylogenetic problem comparative anatomy, paleontology and embryology must work together. It is the embryological evidence that has hitherto been most conspicuously lacking. Notwithstanding the important position of the aquatic urodeles, it is here that we find one of the widest gaps in our knowledge of comparative embryology. Not only has the development of Cryptobranchus allegheniensis remained undescribed, but little or nothing is known concerning the embryology of most of its near relatives. Very recently, it is true, considerable progress has been made in the study of the embryology of Cryptobranchus japonicus, but part of this work was done on very scanty material, and the field is by no means exhausted. Of the development of Amphiuma and Siren practically nothing is known. Some results have been obtained with special problems in the development of Necturus, but the life history has not been covered in a comprehensive manner. For a study of the phylogenetic relations of these forms a knowledge of the development in its general aspects as well as along special lines is imperative; and in no other form do the embryological data promise to shed greater light on phylogenetic problems than in the case of Cryptobranchus.

For the analysis of developmental processes from a morphogenetic point of view the eggs of Cryptobranchus present certain favorable features. One of these is the presence of a larger amount of yolk than is known in the egg of any other amphibian; they are thus favorable objects for the study of the influence of yolk on development. The eggs, moreover, are lacking in pigment, and the early segregation of the yolk gives a translucency to parts of the embryo even in the gastrula stage, enabling one to study satisfactorily in the living egg some of the internal features of development. The embryo is found to respond admirably to the influence of chemicals modifying the course of development; for certain experiments of this sort it gives results decidedly more definite than have been obtained with the embryo of the frog.

The present contribution is to be followed by other parts dealing with the embryonic and larval development.

This investigation has been pursued under a great variety of circumstances, and with many protracted interruptions due to the pressure of other work. Field work on the habits of Cryptobranchus, the collection and preservation of material, and the study of the living egg, have been carried on each autumn ('05–'11 inclusive) in northwestern Pennsylvania. For comparison with Cryptobranchus, I have collected embryological material of Necturus during the seasons of 1910 and 1911, from Lake Monona, Wisconsin. Laboratory work, principally on preserved material, begun in the Zoological Laboratory of the University of Michigan ('05–07), has been continued in the zoological laboratories of Lake Forest College ('07), Syracuse University ('08–09), the Bureau of Fisheries at Woods Hole (summer of 1908), the University of Wisconsin ('09–11), and Columbia University ('11–12). To the directors of these respective laboratories I wish to express my sincere thanks for uniform courtesy in placing the resources of each institution at my disposal. To Professor Bashford Dean, under whose direction the work is being carried on during the present year, I am profoundly indebted for his constant encouragement, kindly criticism, and valuable advice; for this I desire to record my grateful appreciation.

II. THE ADULTS

A. HABITAT

Cryptobranchus allegheniensis was found abundantly in the Brokenstraw Creek, a tributary to the Allegheny River, in northwestern Pennsylvania. The most favorable locality extends from the confluence with the Allegheny five or six miles upstream. The stream has a rather rapid descent, and a gravelly or rocky bottom. Shallow and rocky rapids make up the greater part of its course, alternating with areas of deeper and more quiet water.

As a rule, Cryptobranchus is found more abundantly in rather shallow and rapid water, where large flat rocks afford suitable cover. Usually the animals lie concealed in cavities under these rocks. As more than one individual is seldom found under a single rock, we conclude that its life is in general a solitary one. Cryptobranchus rarely comes out of its hiding place in the daytime, except in the spring and early summer and during the breeding season (the first two weeks of September). At night they venture abroad in large numbers; they are then seen by fishermen spearing by torchlight, who commonly report them in the deeper and more quiet water.

The cavity or cavern used for a more or less permanent dwelling-place has a rock for its roof and the gravelly bed of the stream for its floor. In perhaps the majority of cases, ready-made cavities are chosen as homes, and these are reached by a natural opening. But the cavity sometimes bears evidence of having been in part hollowed out by the animal, and is occasionally reached by a single tunnel-like entrance on the down-stream side of the rock; this is more often the case in cavities used for spawning purposes.

There is a striking similarity between the habitat of Cryptobranchus allegheniensis and that of the 'giant salmander' of Japan as described and figured by Ishikawa ('04).

B. GENERAL CHARACTERISTICS

1. Size

Out of the many hundreds of adults captured, the largest male (September 3, '08) measured 60 cm. (23½ inches) long and weighed 2½ pounds. The largest female captured (September 3, '09) weighed exactly 3 pounds. The latter animal unfortunately escaped from the aquarium in which it was confined and was not measured; probably it was no longer than the longest male, but heavier because distended with eggs. Professor McGregor reports a specimen 25 inches long, taken from the Scioto River.

The great majority of specimens captured by me were much smaller; specimens of about 30 to 50 cm. were most frequently taken. The smallest sexually mature male measured 30 cm.; the smallest mature female 35 cm.

2. Form

As compared with the young, the adult is more flattened dorsoventrally—an adaptation to life in shallow crevices. The head particularly shows this flattening: it is wedge-shaped as viewed from the side, a form which enables the animal to force its soft body into very shallow crevices.

Moreover, as compared with the young, the adult is distinguished by a general looseness and wrinkling of the skin at the sides of the body, forming broad lateral horizontal folds; and by similar flaps of skin on the posterior sides of the limbs. During locomotion these folds and flaps undulate in the water, contributing to the uncouth appearance of the animal.

3. Coloration

Young sexually mature individuals vary little in color or color pattern. The ground color is dull brown, with conspicuous black spots and less conspicuous yellow spots scattered over the dorsal and lateral surfaces. Both kinds of spots are irregular in size and form. The coloration of young adults is practically that of immature specimens from 16 cm. body length upwards; in these stages the spots are more conspicuous than in the larvae or the older adults. In the older, full-grown specimens the general color effect may vary in two ways: it may become either greenish-brown or decidedly reddish brown. As stated by Reese ('03) these variations in color occur in both sexes.

C. BREEDING HABITS

1. *Breeding season*

The following data indicate the beginning of the breeding season, as shown by the deposition of eggs, in northwestern Pennsylvania during a series of years:

1906	August 30	1909	August 29
1907	September 8	1910	September 1
1908	August 28	1911	September 4

The summer of 1907 was an unusually 'late season' as regards vegetation as well as the breeding season of Cryptobranchus. This indicates the probability that climatic conditions influence the time of spawning.

Egg-laying continues for a period of about two weeks. At the end of this time females have in a few instances been taken with the full complement of ripe eggs still in the ovary and showing signs of degeneration.

The occurrence of the breeding season of Cryptobranchus in the fall is in marked contrast to the habits of nearly all other urodeles. Some other urodeles which have a late breeding season are C. japonicus, which according to several authors (Sasaki, '87; Kerbert, '04; Ishikawa, '04; de Bussy '04 and '05) lays its eggs during the latter part of August and the early part of September; and Amphiuma, which according to McGregor ('99) breeds in midsummer. Among the anura, Scaphiopus holbrookii spawns during the summer, the time varying from June to August (Pike, '86; Hargitt, '88).

2. External sexual characteristics

The adult male may be recognized (Reese, '04) by the presence of a swollen ring about the cloaca, due to glands beneath the skin. This swelling is quite prominent during, and for a few weeks before the breeding season. I found it difficult to distinguish by external characteristics the sexes of a few specimens taken during the first week of July; during the latter part of July the males could easily be distinguished by the presence of the cloacal swelling. In a few males obtained and examined during the early part of November, the swelling was less pronounced than is usually the case during the breeding season. Females are characterized by the entire absence of the cloacal protuberance found in the male; also, the abdomen of the gravid female is slightly swollen.

3. Sex ratio and sex segregation

As a general rule, fewer females than males have been captured. The record of the sex of the great number of adults captured during the progress of the work is not complete, but the conclusion reached by later work is that the original ratio of 1:8 determined (Smith '07) during the fall of 1906 is much too large. In a series of years the proportion of females to males captured is about 1:2 or 1:3. These results are of course not conclusive as to the actual sex ratio; as will presently be explained, the sex ratio in the specimens captured varies for different times and places, and the true ratio may be disguised by the occurrence of seasonal segregation of the females from the more accessible localities.

In studying the distribution of the sexes throughout the year a distinction must be made between localities which experience has shown are chosen as breeding grounds, and other localities unsuited for breeding purposes. The breeding grounds are characterized by shallow water, a moderate current, and the presence of large flat rocks affording cover for cavities protected from the current. Elsewhere a swifter current, smaller rocks barely large enough to serve as cover, or deeper pools of quiet water, afford conditions in which Cryptobranchus can live, but which are not adapted for purposes of reproduction.

Studies of the sex ratio indicate a more or less perfect segregation of the sexes at certain seasons of the year. A dozen adults captured in June on the breeding grounds, by an assistant, proved to be all males. During the summer, search of the breeding grounds results in the capture of a few females and a much larger number of males; in localities unsuited for breeding one is more likely to find females, and males are seldom found in their immediate vicinity. Just before the breeding season one is more likely to find females on the breeding grounds, but the males are still considerably in excess, and there is apparently a tendency for the sexes to occur in groups: within a restricted area one may find only males, while within another area a short distance away one may find only females. At the height of the breeding season, both sexes are found on the breeding grounds in more nearly equal numbers.

For some days or weeks after the close of the breeding season the male remains in possession of the nest; females have never been found in nests containing eggs. At this time females have been found in considerable numbers in localities unsuitable for breeding, with no males in their immediate vicinity.

The general results of the studies of the sex ratio and the distribution of the sexes indicate that the males abound in localities suitable for breeding, throughout the year, and that they are less numerous elsewhere; it is positively established that the males alone are in possession of the nests after spawning takes place; and it is probable that there is a more or less perfect segregation of the females from the breeding grounds during a period extending from the close of the breeding season until the middle of the following summer.

In Necturus, segregation of the sexes at a certain season of the year seems to be more complete than is ever the case with Cryptobranchus allegheniensis. Eycleshymer ('06) says:

> In the autumn they are found in pairs or small groups. From this fact and others to be recorded later it is inferred that this is the mating season. . . . . During egg-laying [in the spring] the males are never found with the females, and where they remain is unknown.

4. The eggs

(*a*). *General history of the eggs and their envelopes before the time of spawning.* In an adult female of average size about 450 eggs are matured each season—225 from each ovary. In general the number is greater in the larger and presumably older females than in the smaller ones. At the approach of the breeding season the eggs which are about to become mature are readily distinguishable from the others by their much greater size and yolk content. The liberation of these eggs from the ovary and their passage down the oviduct takes place shortly before spawning. The exact date varies considerably in different individuals; for a week or ten days after the first cases of spawning, females may be found with mature eggs all still in place in the ovaries. The process of liberation of the eggs and their passage down the oviduct, once begun, must be accomplished with considerable rapidity; for out of more than a hundred females opened and examined during the breeding season in the course of several years, only four have been found in which the process was actually taking place. This state of affairs is in marked contrast to the condition in Bufo, where according to King ('05) the great majority of specimens collected soon after they had emerged from their hibernation contained eggs free in the body cavity. In three out of the four cases above mentioned for Cryptobranchus, eggs were found along the entire route: some still in place in the ovary; some free in the body cavity, for the most part collected at its anterior end, near the opening of the oviduct; others forming a procession down the oviduct; the remainder aggregated in the uterus. In the fourth case, the ripening eggs were found only in the body cavity, oviduct and uterus. The process takes place on the two sides of the body simultaneously.

During their passage down the oviduct the eggs receive their gelatinous outer envelopes, the product of the oviduct. At the upper end of the oviduct, the eggs collect in masses; a little further down, they are arranged in a solid row. In these parts of the oviduct the covering is absent or just beginning; the eggs are very soft, and elongated by pressure of the walls of the oviduct. In the middle and lower portions of the oviduct the eggs are distributed at fairly equal intervals; here the envelope is well formed, and consists of a capsule about each egg, and a slender connecting cord, giving a general resemblance to a string of beads.

After their descent through the oviduct, the eggs of each side of the body form a single string aggregated in a much twisted and tangled mass in the uterus. Considered as individuals without regard to their sequence in the string, the eggs display a striking regularity in their arrangment in the uterus, being packed in parallel spiral rows; but this is merely the result of mechanical pressure, as the string pursues a very sinuous and complicated course throughout the mass.

The egg capsules at the end of the uterus nearest the cloaca, hence those first formed, do not contain eggs; those nearest the oviduct, hence the last formed, are likewise devoid of eggs. These empty egg capsules are in general smaller than those that contain eggs, with a regular gradation in size from those at the extremity of the cord, which are scarcely more than a millimeter in diameter, up to those nearest the egg-containing capsules, where the diameter is only slightly less than normal. However small the size, these capsules are always perfectly formed, with a central spherical space; they are never solid. The 'empty' capsules contain a small amount of coelomic fluid in which are distinguishable under the microscope leucocytes, erythrocytes and yolk corpuscles. A cloudy mass of fluid with the same constituents occurs in the upper part of the uterus, outside of the egg envelopes. Similar capsules devoid of eggs are the 'wind eggs,' known in various vertebrates: birds, reptiles, sharks, chimaeroids.

As a result of experimental studies on the nature of the stimulus which causes the shell to be formed about the hen's egg, Pearl ('09) reached the following conclusions: (a) the stimulus which sets the shell-secreting glands of the fowl's oviduct into activity is mechanical rather than chemical in its nature; (b) the formation of a shell on the hen's egg is brought about by a strictly local reflex, and is not immediately dependent upon the activity of other portions of the reproductive system (nervous impulse or hormone formation). In this connection it is interesting to note that in Cryptobranchus the mechanical stimulus can hardly be the true cause of the formation of the capsule, since capsules are formed when only a small drop of coelomic fluid is present. Moreover it is here observed that coelomic fluid may pass down the oviduct without becoming enclosed in such capsules; on the other hand, every egg is provided with a capsule.

When distended with eggs, the uterus is spindle-shaped, about 10 cm. long, with a transverse diameter of about 4 cm. at its widest part. Its thin walls have a rich blood supply.

Apparently the eggs do not, as a rule, remain long in the uterus before spawning takes place. During the breeding season comparatively few females are found having eggs in the uteri; the majority of the females captured are either spent or with eggs still in place in the ovaries. Eggs taken from the uteri are, in the great majority of cases, capable of artificial fertilization; this subject will be more fully discussed later.

(*b*). *Oviposition, and nesting habits.* Under strictly natural conditions egg-laying takes place under cover of rocks in the bed of the stream; but in creek aquaria, arranged to afford conditions as natural as possible without too much cover, the process has been repeatedly observed.

Egg-laying begins slowly, a short string of eggs sometimes protruding from the cloaca for several hours before spawning begins in earnest. In the natural habitat, such short strings of eggs are often found in the open. Later, two long strings of eggs proceed slowly from the cloaca, one from each uterus; the majority of the eggs are then deposited more rapidly, in multiple strands, the process requiring less than five minutes. When egg-laying is completed, the strings are usually twisted together in a single tangled mass.

The 'nest' of Cryptobranchus allegheniensis has already been described as either a burrow or a natural cavity under a rock which is wholly or partially submerged. The eggs are not fastened in any way, but are protected by this sheltered position from being swept away by the current.

The nests of Cryptobranchus japonicus have been described by Ishikawa ('04), and closely resemble those of C. allegheniensis.

> Das Tier legte seine Eier in tiefe horizontal verlaufende Löcher, in denen das Wasser sehr ruhig ist. Manchmal is solch ein Loch 10 oder mehr Fuss tief und kaum für das Licht zugänglich. Die Brutstellen für die Eier sind aber nicht immer so tief. Oft fand ich Eier in einem Loch nicht tiefer als 3 oder 4 Fuss. Oeffnet man ein solches Loch, so findet man eine abgerundete Stelle, deren Boden ganz rein gehalten ist.

The nesting habits of Necturus have been described by Eycleshymer ('06), and the writer ('11). The eggs are attached singly by their gelatinous envelopes to the under side of a rock, board, or other object lying at the bottom of the water (figs. 55 and 56).

The eggs of Amphiuma found by Hay ('88 and '90) in an Arkansas swamp were in a comparatively dry situation, in a small excavation under a log several rods from the nearest water.

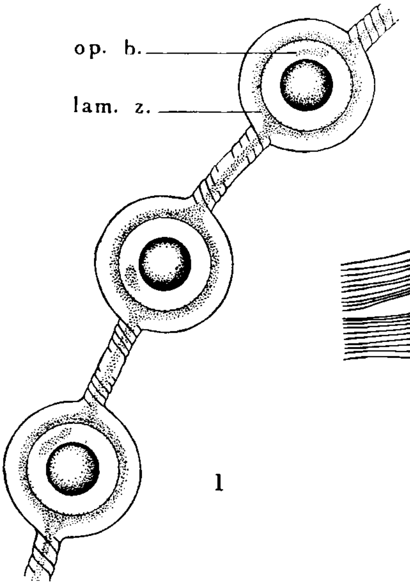
Brief reference to the nesting habits of some other amphibia has been made in previous papers (Smith, '06 and '07). Very remarkable are the nesting habits of the anuran Phyllomedusa, described by Budgett ('99); the eggs are deposited in a pocket made by bringing together the edges of a leaf overhanging the water.

Amongst the dipnoi, the nest of Protopterus (Budgett, '01 a and '01 b) is an oval hole filled with water and surrounded by swampy ground. The nest is at first entirely submerged, but by the partial drying up of the swamp it is left as an isolated pool. Lepidosiren (Kerr, '00) nests in a veritable burrow excavated in the black peaty soil of the swamp.

Nesting habits are well known in many teleosts, and in Amia (Dean, '96; Reighard '03). According to Budgett ('01 a) the crossopterygian Polypterus probably makes no nest, and certainly lays but few eggs at a time, these being scattered broadcast through the thick vegetation of the flooded grass lands. Comparison with Cryptobranchus suggests the possibility that these scattered eggs are but preliminary attempts at egg-laying.

(*c*). *The newly-laid egg and its envelopes.* (Figs. 54, 1 and 2.) In eggs taken from the uterus, the outer egg envelope or capsule fits closely about the egg proper; the envelopes are flaccid and much wrinkled. The capsule of the newly-laid egg takes up water rapidly; in the course of one or two hours a space, filled with fluid, appears between the egg and its capsule, sufficient to enable the egg to orient itself with the animal pole uppermost.

The egg proper is perfectly spherical when fresh, but it gradually becomes slightly oblate from the effects of gravity. It is about the size of a pea, and bright yellow in color—a rather deep yellow at the lower pole, grading to a very pale yellow at the upper. The general intensity of the yellow color varies considerably in eggs of different spawnings, but is quite uniform in eggs from the same female. The absence of black pigment is probably correlated with the fact that the eggs are laid in darkness: the



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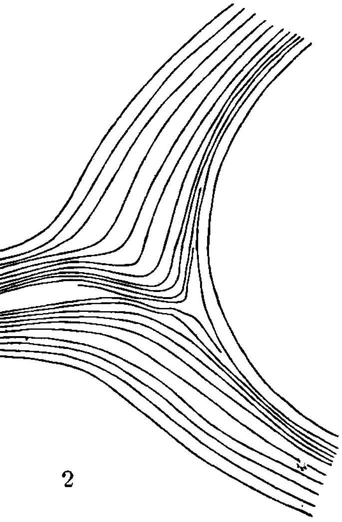
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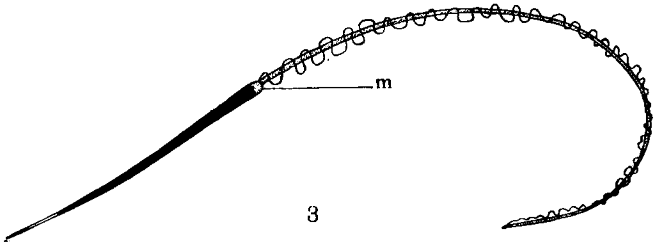

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Fig. 1 Eggs and egg envelopes of Cryptobranchus allegheniensis, natural size. *op. b.*, opaque body; *lam. z.*, lamellar zone of envelope.

Fig. 2 Optical longitudinal section through the lamellar zone of the envelope in the region of junction of the egg capsule with the connecting cord. × 13.

Fig. 3 Spermatozoon. × 500. *m.*, middle piece.

eggs of Necturus, Plethodon, Spelerpes and Desmognathus, which are also laid under cover, are likewise unpigmented.

A very thin and transparent 'vitelline membrane'—the zona pellucida of the ovocyte—closely invests the egg; it is quite inconspicuous in fresh material. This is not the true cell wall of the egg, which, as described in detail on page 112 lies immediately within the vitelline membrane and represents in a modified form the zona radiata of the ovocyte.

Proceeding from within outward, the coverings of the egg may be enumerated as follows: (a) the cell wall; (b) the vitelline membrane lying in close contact with the preceding; and (c) the capsule or thick gelatinous outer envelope, which is separated from the vitelline membrane by a space filled with fluid.

During the first few hours after fertilization the capsule gradually becomes turgid by osmosis, becoming in this way a much more efficient protection to the egg; the space between the egg and its capsule is increased by the absorption of water and in this the egg almost floats, resting lightly on the lower inner surface of the capsule. When the eggs are removed from water the egg proper looks much larger than it really is, because magnified by the spherical capsule.

For a day or two the envelopes are quite soft and somewhat viscous, making it rather difficult to cut them with scissors in order to remove the eggs. Gradually the material of the envelopes becomes firmer. The connecting cord is at first quite elastic, but it loses this quality to a considerable extent after prolonged immersion in water.

Until after the eggs have been in water for several days, the outer layers of the envelopes are still cast into wavy folds or wrinkles, usually extending spirally about the capsules and the connecting cord. As a rule the spiral is constant in the direction in which it extends about the axis of the string in all portions of the cord and capsule. These spiral folds are usually most strongly marked at the ends of the cord adjacent to the capsule, and here they often persist (fig. 1), suggesting the chalazae of the hen's egg.

The envelope is perfectly transparent when fresh, except that wherever viewed tangentially its inner layers have a misty appearance, represented by the shaded zone in fig. 1, and due to a fine lamellar structure sketched in optical section in fig. 2. The misty appearance is caused by the diffusion of light passing through these concentric layers in a direction tangential to their surfaces. The core or axis of the connecting cord has the same misty appearance, due to a continuation of the lamellar structure. The various layers or lamellae of the gelatinous envelope are in intimate contact; there is no fluid-filled space between them such as occurs between the capsule and the vitelline membrane.

The inner layer of the lamellar core of the cord in some cases exhibits a marked twisted or spiral arrangement, like that of the inner portion of the cord connecting the eggs of Ichthyophis as described by the Sarasins ('87–'93).

The eggs of a given spawning are fairly uniform in size, but there is considerable variation in the size of eggs from different parents. The average dimensions of the living egg and its envelopes, after two days' immersion in water, are as follows:

Diameter of egg proper	6.2 mm.
Diameter of egg with envelope	18 mm.
Diameter of connecting cord	5 mm.
Distance of one egg from another, measured from center to center along the cord, about	30 mm.

A few egg capsules, particularly among the empty ones, are double, formed by the union of two capsules without a connecting cord. In such cases the cavities of the two capsules are usually separated only by a thin gelatinous septum; but all gradations occur between this condition and that in which two capsules are connected by an unusually short cord. Rarely, three capsules are closely approximated.

I have found a few instances in which two eggs occurred in the cavity of one simple capsule, without any separation by a gelatinous membrane. It would seem possible that double embryos might be formed in this way, by the fusion of the yolk masses of two such eggs; but this could not account for the only double embryo that I have found in nature, for in this case, to be described later, each embryo is half the normal size.

After fertilization, numerous spermatozoa are found imbedded in the egg capsule, and floating in the liquid between the capsule and the egg; they also occur in capsules that do not contain eggs. The spermatozoa occur singly, not in masses, and they are entirely absent from eggs taken from the uterus. Fertilization occurs only after the eggs have been deposited in the water (Smith, '07).

An envelope so tough and thick as that of Cryptobranchus must exert a decided selective power with regard to the spermatozoa; of a considerable number of spermatozoa simultaneously coming in contact with the envelope, the most vigorous, as well as the ones structurally best adapted, would succeed in first entering the egg.

Floating in the liquid between each egg and its envelope, there occurs a fairly large irregular and slightly opaque mass, in appearance like a faint white cloud (see fig. 1; this mass is also faintly shown in the photograph, fig. 54). Under the microscope it is found to consist of a clear viscous matrix in which are imbedded numerous leucocytes and occasionally a few erythrocytes. In fertilized eggs, this mass, which I have called ('07) the 'opaque body' sometimes contains spermatozoa, but they are not restricted to it, nor especially numerous in it. The opaque body is uniformly present in eggs that do not contain spermatozoa.

A mass similar in general appearance and location to that described above as the opaque body, is figured by Ishikawa ('04) within the egg capsule of Cryptobranchus japonicus. In the text he refers to these masses as 'Samenhaufen,' and speaks of the presence of spermatozoa within the egg capsules as evidence of internal fertilization. He considers it improbable that the spermatozoa are able to penetrate the egg capsule, and supposes that they are taken up into the oviduct before the egg capsules are formed.

DeBussy ('04, p. 11) found a mass ('vlokje') of similar appearance within the capsules of unfertilized eggs of C. japonicus. Under the microscope he found the mass to consist of a slimy substance containing red blood corpuscles and yolk granules, but no spermatozoa. He concludes that the presence of spermatozoa is not essential to the formation of the mass, but that they may merely form an element of it; hence that the name 'Samenhaufen' is scarcely justified. This conclusion is in essential agreement with my results on C. allegheniensis; it seems therefore that the masses called 'Samenhaufen' in C. japonicus by Ishikawa are of the same nature as the 'opaque bodies' of C. allegheniensis, and like them of no significance in fertilization. The opaque body apparently consists of coelomic fluid that has escaped into the oviduct.

The egg strings of Cryptobranchus japonicus as described by Ishikawa ('04) and deBussy ('04) closely resemble in structure those of C. allegheniensis. Both eggs and capsules of the Japanese form are slightly larger; according to Ishikawa the egg proper is about 7 mm. in diameter, and the capsule varies from 20 to 25 mm. in diameter in different spawnings.

The egg capsules of Amphiuma as described by Hay ('88 and '90) have the same general structure as those of Cryptobranchus. For an opportunity to examine one of Hay's specimens of the embryological material of Amphiuma, I am indebted to Prof. C. W. Hargitt, to whom the specimen had been presented by the finder. The egg capsule has a glistening surface like isinglass; it is thinner and apparently tougher, and the connecting cord more slender, than in Cryptobranchus. These peculiarities may be due in part to preservation in alcohol, which tends to produce the same condition in the envelopes of Cryptobranchus; but my impression is that the egg capsules of Amphiuma are better adapted to retain moisture when exposed to the air.

Other amphibians whose egg capsules are fastened together like a string of beads are Alytes, Ichthyophis, and Hypogeophis (Brauer '97).

The general appearance of the egg capsules of Necturus is shown in figs. 55 and 56; some further details of structure are shown in fig. 4. There are three layers to the gelatinous envelope: (a) a comparatively thin but very dense inner layer, consisting of several lamellae; (b) a thicker median layer of moderate density, consisting of many lamellae; and (c) a very thick outer layer of homogeneous material, much less dense than either of the preceding. This outer layer is produced to form the stalk by which the capsule is attached to some solid object. As seen in optical section, the lamellae of the two inner layers have a somewhat sinuous or wavy outline. Leaving the stalk out of account, the entire structure bears a close resemblance to the gelatinous envelopes of the frog's egg. In the early stages of development of the embryo, the dense inner layer of the capsule fits so closely that it is not clearly differentiated from the embryo; this layer is best studied after the embryo has passed the gastrula stage, when a narrow space, filled with liquid, appears between the embryo and its capsule (see fig. 4). This space is not strictly homologous with the similar space that appears much earlier in the egg of Cryptobranchus; for in Cryptobranchus the space appears between the gelatinous envelope and the vitelline membrane (zona pellucida of the ovarian egg) which remains in close contact with the egg, while in Necturus the vitelline membrane apparently func-

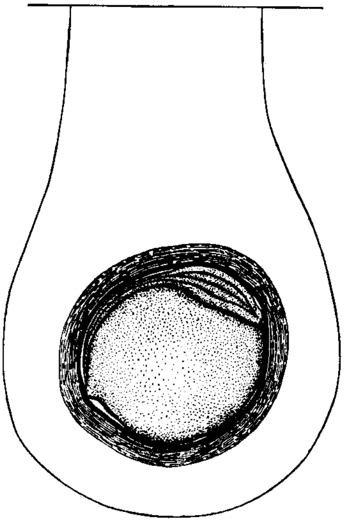


Fig. 4 Optical section through an egg capsule, and surface view of an embryo, of Necturus. The embryo is shown in a stage with neural folds, when the capsule is slightly separated from it by a space filled with water. × 4.

tions as the innermost lamella of the capsule. The entire inner layer of the capsule of Necturus has a tough consistency similar to that of the vitelline membrane of Cryptobranchus; this perhaps is the reason why it is so slow in enlarging.

In Necturus the egg proper is slightly smaller than that of Cryptobranchus; in the early cleavage stages it measures about 5.8 mm. in diameter.

A general description of the gelatinous envelopes of several species of Amblystoma has been given in a previous paper (Smith '11).

5. The sperm

The spermatozoon (fig. 3) has been figured by Reese ('04), who fails, however, to picture the middle-piece. The spermatozoon is about 225μ long, and stout in structure as compared with the spermatozoa of Amblystoma and Diemyctylus. The head, excepting the acrosome, stains deeply with Delafield's haematoxylin. The acrosome appears to be uniformly tapering, not spear-shaped as in Amphiuma (described by McGregor, '99). As stated by McGregor ('99) the middle-piece in Cryptobranchus is very short in comparison with that of other urodeles. The tail-piece is provided with an undulating membrane, bordered with a convoluted filament.

The ripe spermatozoon is motile, as regards both shaft and filament; but the spermatozoon as a whole is not so flexible as the more slender spermatozoa of Amblystoma and Diemyctylus. This greater rigidity of the spermatozoon is perhaps correlated with the method of fertilization: the spermatozoon must penetrate the tough and thick egg capsule after a brief exposure of the latter to the hardening effects of water.

In seminal fluid obtained from occasional individuals, an oval mass of granular protoplasm, about 13μ by 16μ, surrounds the posterior part of the head of the spermatozoon. The long axis of this bead-like mass coincides with that of the spermatozoon. I have found a similar mass of protoplasm present in spermatozoa from some individuals of Amblystoma punctatum, but here the oval mass usually occurs about the junction of the head and middle-piece. Probably the condition observed in the two species represents a late developmental stage of the spermatozoon—the metamorphosis of the spermatid into the spermatozoon is not quite complete.

The amount of seminal fluid present at one time in the vasa deferentia of a ripe male is very great in proportion to the size of the animal—a condition correlated, doubtless, with external fertilization. In one instance 20 cc. of seminal fluid was readily stripped from a single male.

6. *The method of fertilization*

The method of fertilization (external) has already been described (Smith, '07); subsequent observations have supplemented this account only in the fact, discussed later, that a single male may spawn with more than one female.

The method of external fertilization is well adapted to the normal breeding conditions. The 'nest' of Cryptobranchus consists of a hollow under rocks, a confined space protected from the current, and filled with very quiet water. As has been shown, the amount of milt that may be discharged at one time by a single male is considerable; in the case of a pair that spawned while being carried in a pail of water, it was sufficient to turn several quarts of water milky white. Such a quantity of sperm set free in the confined space of the nest would become diffused, especially when stirred about by the movements of the animals, so that every egg would be quickly reached and fertilized. As a matter of fact, few unfertilized eggs are found.

So far as I have been able to learn, this is the only case of external fertilization recorded for the urodeles. The inconclusive observations of Kerbert ('04) on Cryptobranchus japonicus, and Kunitomo ('10) on Hynobius, suggests to me that external fertilization may take place in these forms.

In Necturus, from the observations of Kingsbury ('95) it seems certain that internal fertilization takes place. A compound receptaculum seminis or spermatheca is present in the female; spermatozoa have been found in these spermathecae during the fall and winter, which suggests an autumnal fertilization, though it is possible that spermatozoa are left over from a spring fertilization. The method of transference of the seminal fluid from the male to the seminal receptacle of the female is unknown.

The breeding habits of some urodeles in which internal fertilization takes place by means of spermatophores (e. g., Amblystoma punctatum and Diemyctylus) have been considered by the writer in former papers (Smith, '07, '10 and '11). Fertilization is external in the anura, internal in the apoda.

In the elasmobranchs and holocephali, fertilization is internal. In the crossopterygian Polypterus (Harrington, '99; Kerr, '07 b), during the breeding season the anal fin of the male is modified in such a manner as to suggest internal fertilization; or possibly it serves to direct the sperm against the stream of eggs issuing from the female. Nothing conclusive is known regarding the method of fertilization in the dipnoi. In teleostean fishes, with a few exceptions, fertilization is external; e.g., as in Chrosomus (Smith, '08).

The question whether external fertilization in Cryptobranchus is primitive or secondarily acquired will be discussed under phylogenetic considerations in a later section.

7. *The brooding habit*

In a previous paper (Smith, '07) a paternal brooding habit was described for Cryptobranchus. This was observed in aquaria, and more extensively under natural conditions.

The data on the existence of a paternal brooding habit under natural conditions, while necessarily incomplete, are quite conclusive. In one case, a male occupying a nest containing eggs was observed to fight and drive away several males and a spent female which were attempting to enter the nest (Smith, '07); in another case, a male occupying a nest containing eggs was observed to oppose the attempt of a single male to enter the nest. It is not always possible to tell whether an adult is present in the nest; the rock may be too large to overturn, and while the eggs may be obtained by tilting the rock with a crow-bar, this method is not always successful in dislodging the adult. In cases where the rock is lifted and overturned, the water is discolored and the hellbender, aided by its protective coloration and the swift current, may escape. When seen, however, it may usually be captured. A record kept for six years ('06–'11) shows that from twenty-nine nests containing eggs a male was captured in ten cases, a female never.

The duration of the brooding habit has not been definitely determined, and perhaps varies greatly. In different nests in which a male was present, eggs were found in various stages of development up to about three weeks old; unfortunately I was obliged to discontinue field work at a date varying from two to four weeks after the beginning of the breeding season. In no case where the eggs were in an advanced stage of development can it be recorded that the male had been continuously present, or even that he was the same male that fertilized the eggs; but the entire absence of females from nests containing eggs is significant.

With regard to the origin of this paternal brooding instinct two suggestions (Smith, '07) were made: (*a*) the brooding instinct may have originated in connection with the feeding habit; or (*b*) in holding the nest the male may be primarily concerned in awaiting the coming of another ripe female. Both views assumed that in Cryptobranchus we have an example of the brooding habit in an incipient state. Further observations indicate that the brooding habit is well established and manifested as a distinct impulse from the moment of fertilization; its origin is thus thrown back into the remote past, and concerning it we can only speculate.

The evidence for the first interpretation may be briefly stated as follows: Both sexes are voracious eaters of the newly-laid eggs; during the spawning season the majority of the adults taken have the stomach filled with eggs. There is evidence that the females, when opportunity is afforded, gorge themselves with eggs more freely than the males. The number of eggs found in the stomach of a single adult usually ranges from fifteen to twenty-five, a number sometimes greatly exceeded in the stomachs of spent females. In one case, in which the body of a spent female appeared greatly swollen, the stomach was found to be greatly distended with eggs. When removed and measured by displacement of water, the stomach and its contents were found to have a bulk of over 200 cc. The mouth also was full of eggs, and strings of eggs protruded from the pharyngeal openings. The quantity of eggs present seemed to represent almost an entire spawning. The feat of swallowing such a quantity of eggs would seem possible only if they were taken before the swelling of their envelopes.

The digestive processes of the hellbender are extremely slow, and I have taken undigested eggs from the stomach a week after they were eaten. Under these conditions the presence of a single male hellbender in the nest is in the main protective. On account of the small number of eggs eaten at once, and the slowness of his digestive processes, fewer eggs are eaten than would be the case if other hellbenders, and especially the spent females, had free access to the nest.

As previously noted, a male has been observed to fight and drive away a spent female and several males that were attempting to enter the nest. The male in such cases has the advantage over the female because of the weakened condition of the latter; as regards the other males, he has the advantage of position.

The facts suggest that the male, in thus driving away others of his own kind, may be primarily concerned in guarding his own food supply; this guarding habit may become modified into a true brooding instinct. But it is difficult to believe that the male, after having filled his stomach with eggs, would any longer be concerned with the fate of the remaining eggs on account of their value as food.

According to the second interpretation, the male may hold the nest in expectation of the coming of another ripe female; an extension of this habit may give rise to the brooding instinct. According to this view, the brooding instinct has its origin in the breeding habit.

The reception of a ripe female by a male guarding eggs has not been directly observed, but the following data afford sufficient evidence on this subject: Out of twenty-nine nests examined during the seasons of 1906–1911 inclusive, eleven were found to contain eggs of at least two different spawnings, the product of different females. The eggs were in different stages of development, hence fertilized at different times. In two cases the number of eggs present in a single nest was sufficient to represent at least three spawnings by different females. It is possible to determine this with considerable certainty, for the number of eggs matured by a single female each season is limited, and these are all laid at one time. Moreover it is known that the intensity of the yellow color of the eggs is constant for all the eggs of a single female, but varies considerably in eggs from different individuals. In view of the observed vigilance and effectiveness of the male in possession of the nest in driving away other males, it is highly improbable that successive pairs of adults have occupied the nest; hence the facts indicate that the same male has spawned with successive females.

The second hypothesis seems supported by better evidence than the first; but while it is entirely possible that such may have been the origin of the habit in the remote past, there is evidence that at present the eggs are the object of paternal care from the time of fertilization, and this brooding instinct is only temporarily overcome by hunger or diverted by the breeding instinct. The behavior of males breeding in aquaria strongly suggests this: after fertilizing the eggs the male usually remains close beside them or crawls under or amongst them.

Concerning the brooding behavior of some specimens of Cryptobranchus japonicus in captivity Kerbert ('04) says:

> Nach Beendigung der Eiablage legte sich das Weibchen offenbar in grösster Ermattung in eine Ecke des Behälters hin und kümmerte sich um das Gelege gar nicht mehr. Das Männchen hingegen hat seitdem die Eiermasse nicht verlassen—ja sogar *die Brut fortwährend bewacht.* . . . . Denn sobald das Weibchen die Eiermasse zu nahe kam, stürzte das Männchen in sichtbarer Wut auf die Mutter los und vertrieb sie. . . . . kriecht der Männliche Riesensalamander zwischen den verschiedenen Strangen der Eiermasse hindurch und bleibt dan von der Eiermasse umhüllt liegen, oder er legt sich einfach neben die Eiermasse hin. In beiden Fällen aber hält er, hauptsächlich durch eine pendelartige Bewegung des ganzen Körpers, von Zeit zu Zeit die ganze Eiermasse in Bewegung. Durch diese Bewegung entsteht eine für den Atmungsprozess der Eier und Embryonen höchst wichtige Wasserströmung, wahrend die Lage der Eiermasse hierdurch gleichzeitig fortwährend wechselt.

It thus appears that the paternal brooding instinct in both species of Cryptobranchus is manifested from the moment the eggs are fertilized, though in C. allegheniensis at least it may be temporarily inhibited or overcome by hunger or by the breeding instinct. The brooding habit of Cryptobranchus is undoubtedly very old, and we must look to other forms to find examples of it in the incipient condition.

According to Whitman ('98) there are three distinct elements in brooding behavior: (*a*) the disposition to remain with or over the eggs; (*b*) the disposition to resist and to drive away enemies; and (*c*) periodicity. The first of these elements has its origin in the need for rest, protection to the offspring being at first incidental. The second element, pugnacity, is periodical and a part of the reproductive cycle. The third element, periodicity, is apparently an attribute of the two other elements, based on physiological conditions; its adaptiveness lies in correlating the other two elements with the hatching period of the eggs.

In Cryptobranchus, after spawning, the female is evidently much the weaker of the two; as a matter of observed fact, she is driven away by the stronger and more pugnacious male. It can scarcely be the need for rest that keeps the male in the nest, since he maintains exclusive possession at the cost of alert watchfulness and occasional combat. If the element of weakness were the important factor in initiating the brooding habit, we should expect the female rather than the male to remain in the nest. It may be that primitively the brooding impulse is a phase of the reproductive cycle that applies to both sexes, the female losing it on account of her hungry and exhausted condition due to the accumulation of a large amount of yolk in the egg. Perhaps (for this suggestion I am indebted to Professor S. J. Holmes) on the part of the male there is involved a proprietary interest in the nest, which he has chosen and in part excavated, and which he occupies as an advantageous breeding place and as a more or less permanent home.

To obtain conclusive evidence regarding the origin of the brooding habit one must study a series of closely related forms illustrating the habit in the making. In Cryptobranchus it appears that the habit is well established; it is improbable that the question can be settled by the study of this form alone, and the data here given are presented only in the hope that they may contribute something toward the final solution of the problem.

Concerning the brooding habit of C. japonicus in its natural habitat Ishikawa ('04) says: "Fast in jedem Loch, wo man von Ende August bis zu Anfang October ein weibliches Tier gefunden hat, findet man einen Eiklumpen. Dieser Umstand lässt schon vermuthen, dass das Tier eine Brutpflege hat wie Ichthyophis oder wie so viele andere Amphibien." Kerbert, however, asserts ('04) that it is the male that guards the eggs, and states that the sex of his specimens was carefully determined.

Other amphibia known to possess brooding habits are the urodeles Desmognathus and Plethodon; the caecilians Ichthyophis and Hypogeophis; Alytes and several other anura (Wiedersheim '00). In the cases of Desmognathus, Plethodon, Ichthyophis and Hypogeophis the female is said to care for the eggs; in the case of Alytes, the male.

The brooding habit seems to be lacking in Necturus. According to Eycleshymer ('06), Necturus sometimes eats the eggs of its own species.

The brooding habit is well known in many teleosts, and in Amia (Dean, '96; Reighard, '03); it is well developed in the lungfishes Protopterus (Budgett, '01 a and '01 b), and Lepidosiren (Kerr, '00). I can find no record of any observations pointing to the existence of a brooding habit in the crossopterygii.

D. SUMMARY

The breeding season of Cryptobranchus allegheniensis in northwestern Pennsylvania begins about the first of September and lasts about two weeks.

There is a tendency toward segregation of the females from the breeding grounds during a period extending from the close of the breeding season until the middle of the following summer.

About 450 eggs are matured each year by an adult female of average size. The egg capsules from each oviduct are fastened together in a single string. At the ends of each string are formed some small but perfect capsules which do not contain eggs.

The eggs are unpigmented, heavily yolk-laden and strongly telolecithal.

The nest consists of a submerged cavity under a rock in the bed of the stream. The cavity is sometimes in part the work of the animal.

Fertilization is external.

There is a paternal brooding habit, which is manifested from the moment of fertilization. The origin of this habit is problematical.

III. METHODS AND TECHNIQUE

A. COLLECTION AND CARE OF LIVING MATERIAL

To insure a convenient supply of adults for various purposes, these were collected before and during the breeding season and placed in a large creek aquarium, constructed of wire netting and placed in shallow water with a gentle current. This arrangement of the aquarium afforded abundant aëration; flat stones placed on the bottom provided cover; in general the conditions closely resembled those of the natural environment. The aquarium proved of great value as a means of insuring a supply of adults for use at frequent intervals in securing material for the study of ovogenesis, maturation, fertilization and the early cleavage stages.

Artificial fertilization was often resorted to in order to control the time of fertilization for the study of fertilization and early cleavage stages, and occasionally eggs were used that had been deposited and fertilized by specimens in captivity; but the greater part of the material used for the study of the development was obtained from the nests of the animals in their natural environment.

At first the problem of keeping the eggs alive in a favorable environment while studying their development promised some difficulty. Early attempts to keep the eggs in creek aquaria met with disastrous failure through the attacks of water-mould. The method finally employed was to keep the eggs in shallow earthenware dishes containing well water, in a cool cellar; a limited number of eggs were placed in each dish, and the water changed daily. Under these conditions they developed normally. During the early autumn all the laboratory work on the living egg, and the preservation of material, were carried on in this cellar, so that at no time were the eggs subjected to an unfavorable temperature. The eggs were in general shielded from the light; but for working purposes both direct and diffused sunlight, or a Welsbach light, were used.

On account of teaching duties observations in the field have never extended quite to the time of hatching, consequently it has been necessary to transport the living embryo for considerable distances. In the case of embryos taken after the closure of the neural folds, material shipped in cool weather by express, in a pail containing shallow water, did quite as well as material which was given personal care during transportation and for which the temperature was regulated with ice; in both cases the embryos developed normally. Younger embryos require much greater care in transportation; material in cleavage and gastrula stages shipped by express has usually died or developed abnormally, perhaps in the main because of untimely warm weather; all such material was discarded. Material kept in the laboratory thrives in shallow dishes containing well water, the dishes being partly immersed in cool running water; no artificial aëration is necessary. As a check on possible abnormalities in material that has been transported, I have had a series of late stages preserved from material kept without transportation.

B. FIXATION AND PRESERVATION OF MATERIAL

The envelopes may be removed in any stage without much difficulty, by means of scissors. This is very easily done after the eggs have been in water for several days, since the envelopes become inflated. For earlier stages, more care is necessary. Eggs from the uterus, and fertilization stages, may be handled more rapidly by fixing in Solution B (see below) before the removal of the envelopes; they may be preserved thus in formalin, but not in alcohol. After fixation the envelopes become brittle and may readily be removed with needles. Comparison with eggs fixed after the removal of the envelopes shows no essential difference in the results.

The fixation of such large and heavily yolk-laden holoblastic eggs presented a problem of considerable difficulty. A great variety of the usual fixing fluids were tried, but none of them succeeded without modification. After extensive experimentation, the mixture described below as Solution B was found to be very satisfactory for all the yolk-laden stages, for surface study, photography and for sectioning.

The following fixing solutions were found useful for the purposes indicated:

Solution A. Formalin, 10 per cent. Useful for preserving eggs in the envelopes for demonstration purposes, or for the study of the envelopes, as it leaves the envelopes clear and preserves the eggs in their natural color. Formalin is of some value for the surface study of cleavage, as it brings out the faint cleavage furrows of the lower hemisphere with great distinctness, and occasionally gives remarkably good preparations for the surface study of the cleavage of the upper hemisphere. In general the fixation of the micromeres is unsatisfactory, both for surface study and for sectioning. Formalin is unsurpassed for fixing larvae for museum purposes; for permanent preservation they should be changed to alcohol.

Solution B. Bichromate-acetic-formalin. The following proportions must be quite strictly adhered to:

Potassium bichromate	1	gram
Glacial acetic acid	2½	cc.
Schering's formalin, added at the time of using	5	cc.
Water	92	cc.

Fix about forty-eight hours in plenty of the solution, at a low temperature; change the solution once or twice.

Rinse in water and wash in 5 per cent formalin, in the dark, for at least two weeks, changing the formalin as often as it becomes discolored; preserve in 5 per cent formalin. Preservation in alcohol also gives good results for sectioning, but is not so good for surface study nor for photography.

During the process of washing in formalin the color changes from yellow to green. The yolk becomes dark green, while the blastodisc or embryo proper is much lighter in color, giving a sharp differentiation of the protoplasmic portions of the egg. The form of the egg is preserved perfectly, and remarkably good definition for surface study is secured. The eggs are easily sectioned by the paraffin method.

Not until after the closure of the neural folds is it possible to alter the proportions in the formula as given above without injury to the form of the embryo; an increase in the proportion of potassium bichromate results in the collapse of the embryo when in melted paraffin if not in an earlier stage of the process of preparation for imbedding. For later stages the proportion of potassium bichromate may be slightly increased (e.g., to 1½ per cent), without detriment to the surface features and perhaps with some gain in the histological results.

Solution C. Sublimate-acetic-formalin.

Saturated solution corrosive sublimate in 10 per cent formalin	97½ parts
Glacial acetic acid	2½ parts

Fix for a few hours, then transfer to formalin for a few days to insure thorough fixation of the yolk. Wash and preserve in either formalin or alcohol.

This is not so satisfactory a fixing solution as Solution B, but may be used for comparison. For surface study the results, especially in the early stages, are decidedly inferior to those secured with Solution B. For sectioning, good results are secured in the early cleavage stages and after the closure of the neural folds; the mercury crystals must be removed by prolonged treatment with iodin. In the blastula and gastrula stages the embryo usually collapses during the process of preparing for imbedding.

Solution D: Lavdowsky's.

Formalin	10 parts
Alcohol, 95 per cent	50 parts
Glacial acetic acid	2 parts
Water	40 parts

Fix for several days; preserve in 70 per cent or 80 per cent alcohol.

This mixture is especially useful for the yolk-laden ovarian eggs, and for maturation stages; it is not very satisfactory for embryonic stages. Envelopes, if present, must be removed before the eggs are fixed in this solution. The best results are obtained by sectioning the material soon after preservation.

Solution E: Zenker's. This mixture was found most useful for the early stages of ovogenesis, before the formation of any considerable amount of yolk. It is not good for embryonic stages, unless parts of the embryo are to be dissected off from the yolk before sectioning. It gives very inferior preparations for surface study in every stage.

For the early stages of ovogenesis, before the formation of yolk, both Flemming's and Bouin's solutions were used with fair results. For larvae after the disappearance of the yolk sac, Tellyesnicky's, Zenker's, or almost any good fixing solution may be used.

Of the various mixtures experimented with for the yolk-laden stages, those containing picric acid proved to be the very worst. The invariable result of the use of a solution containing picric acid was to cause the egg to disintegrate.

In preserving the embryological material of Necturus, Solution B was principally used. In the early stages of development, before the formation of the neural folds, the results are not so uniformly good as with Cryptobranchus; this is perhaps due to the fact that in these stages the eggs of Necturus are almost necessarily preserved before the removal of the very closely-fitting gelatinous envelopes. In successfully preserved eggs in the cleavage stages, the furrows of the upper hemisphere are more conspicuous and the contour of the micromeres more rounded, than in Cryptobranchus; they are thus, except for difficulties arising from the character of the envelopes, more favorable objects for photography. Late gastrula and neural groove stages of Necturus, preserved by this method, are rarely so favorable for surface study as the same stages in Cryptobranchus. After the formation of the neural folds, when a space has appeared between the envelope and the egg, the embryos of Necturus are preserved with very uniform success, whether fixed before or after the removal of their envelopes. In particular, stages after the closure of the neural folds give a sharpness of detail in the surface features rarely found in Cryptobranchus; these stages of Necturus are very favorable objects for photography.

C. SECTIONING AND STAINING

Kerr ('01), in describing the technique employed in studying the egg of Lepidosiren, has well said: "The investigation of a holoblastic egg 7 mm. in diameter and packed with yolk involves great technical difficulties, for the whole of each egg has to be converted into thin sections. The full extent of these difficulties will only be appreciated by embryologists who have essayed a similar task." In sectioning the heavily yolk-laden stages, Kerr used the celloidin method, and a combination of the celloidin and paraffin methods. DeBussy ('04) used the celloidin method in studying the cleavage stages of Cryptobranchus japonicus.

For sectioning the embryological material of Cryptobranchus allegheniensis and Necturus I have used the paraffin method exclusively; success with this method was found to be entirely a matter of careful attention to technique. The most important considerations are proper fixation and washing, and thorough infiltration with paraffin. In handling serial sections of large numbers of these eggs the advantage of the paraffin method is obvious.

With regard to staining, for general purposes the best results were obtained by staining *in toto* with Grenacher's borax carmine, and counterstaining on the slide with Lyons blue in absolute alcohol; to the Lyons blue solution sufficient picric acid was added to turn it green. By this method the effect of a triple stain, with excellent differentiation, is obtained. The chromatin is stained red, cell walls and cytoplasm blue; the yolk is first stained red by the borax carmine, but turns green in the counterstain. It is usually best to cut short the action of the counterstain at a time when the smaller yolk particles are stained green, while the larger ones are left red. The method has the advantage of rapidity, an important consideration when great series of large sections are to be handled in considerable numbers.

In sectioning and staining the early cleavage stages the exact mode of procedure is as follows:

From formalin pass the eggs to alcohol, 35 per cent, 50 per cent, two hours each.

Grenacher's borax carmine in 70 per cent alcohol, about two days.

Acid alcohol (0.25 per cent HCl in 70 per cent alcohol), about two hours.

Ninety-five per cent alcohol, two to twelve hours; 100 per cent alcohol, two to three hours.

Xylol, four to ten hours.

Paraffin with melting point 52° C. (at a temperature not exceeding 55° C.), two days. Change the paraffin at least once.

Imbed in a paper box, hardening the block under alcohol.

Cut sections 10μ to 15μ thick, using a Minot rotary microtome.

Counterstain on the slide with Lyons blue and picric acid mixture in absolute alcohol.

Wash in xylol long enough to destain slightly.

Mount in Canada balsam.

Early stages require longer for fluids (especially paraffin) to penetrate than do later stages. For the yolk-laden ovarian eggs, and maturation and fertilization stages, from two to three days in borax carmine, and about three days in melted paraffin, are necessary. I have found no serious ill effects in these stages from this prolonged immersion in paraffin at the temperature given.

Material fixed in Lavdowsky's solution stains and infiltrates more rapidly than with the other methods of fixation; also the yolk is less likely to crumble.

Late cleavage, and gastrula stages, are penetrated by the various fluids more rapidly than the early cleavage stages, so that the time may be reduced to two-thirds or one half. For still later stages, there is a further gradual reduction in the length of time required.

During the present year, at the suggestion of Professor Wilson, I have employed a slight modification of the method described above. After being cleared in xylol, the objects were left several days in a mixture of xylol and paraffin at about 38° C. By this preliminary treatment, the time required for infiltration with melted paraffin at a high temperature was reduced at least one-half, with some improvement in the quality of the preparations.

IV. THE EXTERNAL HISTORY OF THE EGG BEFORE CLEAVAGE

Some superficial aspects of the history of the egg before cleavage have already been considered in connection with the account of the breeding habits.

A. EXTERNAL CHANGES PRECEDING AND ACCOMPANYING MATURATION

Except where otherwise mentioned, the observations recorded under this heading were made on the living egg.

If the ovary of an adult Cryptobranchus be examined at any time during the summer, the eggs which are about to become mature are readily distinguishable by their much greater size and yolk content.

In the living ovaries of adults taken about the middle of August, the eggs show no positive surface indications of a telolecithal structure. The same eggs fixed by a variety of methods show a circular area or 'calotte' about 60° in diameter, which is somewhat lighter in color than the remaining surface of the egg. On account of its large size the egg now causes the ovarian wall to bulge strongly outward. In general the pale circular area is situated in the center of the more exposed hemisphere of the egg, and is not so profusely covered with ovarian blood-vessels as the remainder of this hemisphere, but this relation is not always exact.

Sections show that the calotte is the outward expression of a peripheral disc-shaped region richer in protoplasm and small yolk granules than the remainder of the egg; in the center of this disc lies the germinal vesicle. From its homologue in the teleostean egg I shall call this region the *germinal disc* or *blastodisc;* in surface views it may be referred to by the same names, or more strictly speaking, as the *germinal area.* Fixation serves to accentuate the optical differences between the germinal disc and the remainder of the egg, making the germinal area visible in preserved material at an earlier stage than in the living egg. The center of the germinal area defines the animal pole of the egg.

Shortly before the egg is ready to leave the ovary, the germinal vesicle appears at the very surface, at the center of the germinal area which is now visible in the living egg. After remaining here for a length of time that has not been accurately determined, the germinal vesicle disappears from view leaving only a faint dark spot to mark its former site.

In most ovaries obtained about the time of the beginning of the breeding season (the last week in August and the first week in September), all stages in the emergence of the germinal vesicle will be found; in some eggs the germinal vesicle has not yet reached the surface, but in a considerable proportion of cases it will be found exposed in varying degrees (see fig. 53).

The phenomena concerned with the appearance of the germinal vesicle at the surface are very striking, owing to the large size of the germinal vesicle, the sharp contrast between its transparent fluid contents and the surrounding opaque substance of the egg, and the distinct appearance of several opaque-white bodies, presumably nucleoli, within the germinal vesicle. All this may be seen even with the naked eye.

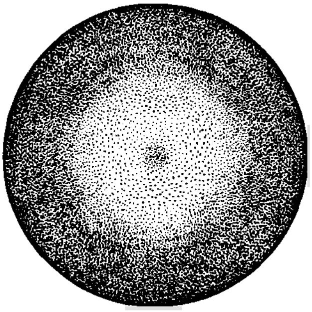
In order to obtain the sequence of the changes occurring in a single egg during this stage, many individual eggs were isolated in normal salt solution, or identified while in position in the ovary, and kept under observation for several hours. In the case of the first ovary studied during the fall of 1907, some of these eggs changed sufficiently before death ensued, to enable me, by combining several individual histories, to get a fairly complete idea of the normal course of events in a given egg. But in succeeding years, although several dozen ovaries containing eggs in this stage have been studied in females recently killed or anaesthetized with chloretone, no marked changes could be detected. Hence in the following account dependence is placed chiefly on a comparison of individual eggs in the same freshly-exposed ovary, and a comparison of ovaries in slightly different stages of development. In particular, incipient stages in the approach of the germinal vesicle to the surface could be distinguished from possible later stages in which it has disappeared from view, through a comparison of ovaries such as those described above, with others in which nearly all the eggs had been set free from the ovary.

The first indication of the approach of the germinal vesicle to the surface is the appearance of a faint dark spot, 1 to 2 mm. in diameter, in the center of the blastodisc. This dark spot grows more distinct; it is the optical effect of the scarcely-submerged germinal vesicle. Within this large dark area appears a small sharply defined much darker area, circular in outline, which grows at the expense of the larger and fainter dark area. At the time of its first appearance the small dark area is sometimes seen to pulsate slowly, quiver and change form, disappear and reappear. This small dark spot is a portion of the germinal vesicle which is actually in contact with the zona radiata (see section V). It may increase in size until almost an entire hemisphere of the germinal vesicle is exposed. In light of moderate intensity the germinal vesicle appears as a deep, dark well of transparent substance walled in by the opaque material of the blastodisc; in strong sunlight one may see within the germinal vesicle the reflection of the bright yellow yolk beneath. Several opaque-white bodies of various sizes appear within the germinal vesicle; these are probably nucleoli, though the largest ones are much larger than the nucleoli shown in sections.

An ovarian egg dissected out and immersed in water at the time of the appearance of the germinal vesicle at the surface orients itself with the animal pole upward.

The actual disappearance of the germinal vesicle from the surface, and the relation of this process to the rupture of the nuclear wall, have not been satisfactorily observed. Whether the germinal vesicle recedes slightly from the surface before or during the rupture of its wall, or disintegrates at the very surface, has not been positively established; it is possible that all three conditions occur in different eggs. In several cases there seemed to be a welling-up of material from the germinal vesicle which spread out to form a broad crater at the surface; in other cases the appearances favored the impression of a slight subsidence of the germinal vesicle. It is possible that the egg has never been observed at the exact time of the rupture of the nuclear wall, for though a large number of eggs from ovaries containing eggs with the germinal vesicle at the surface have been sectioned, in none of these eggs has the nuclear wall been found ruptured.

Several females have been taken in which only a few eggs remained in the ovary, the others being found in the body cavity, oviduct and uterus. The ovarian eggs of such specimens were invariably found to be in a later stage than those just described: sections showed that the dissolution of the germinal vesicle was complete, and in surface views these eggs showed a small faint dark spot or slight depression at the animal pole (see fig. 5).



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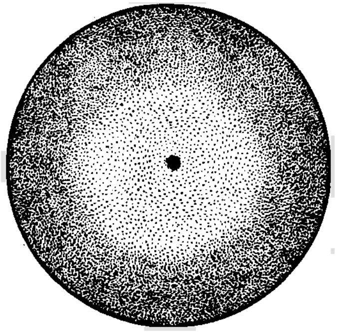

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Fig. 5 Surface view of the animal hemisphere of an egg of Cryptobranchus allegheniensis ready to leave the ovary, after the rupture of the germinal vesicle. The lightly stippled area indicates the blastodisc. Sketched from the living egg. × 7.

Fig. 6 Surface view of the animal hemisphere of an egg taken from the uterus, ready for fertilization, showing pit at the center of the blastodisc. Sketched from preserved material. × 7.

The dark spot is sometimes surrounded by a tumid ring, but this condition is probably pathological.

At the time of the escape of the egg from the ovary and its passage through the body cavity and upper oviduct, the egg seems softer in consistency than at other times. Some fixing solutions, particularly Lavdowsky's, which usually preserve perfectly the spherical form of the egg, now fix it as an irregularly-shaped mass. This plasticity may be of use to the egg in its escape from the ovary and passage down the oviduct.

In eggs taken from the lower oviduct there is found a slight extension of the blastodisc and a marked increase in the intensity of its differentiation, both in living and preserved material. Moreover, outside the rather indefinite limits of the blastodisc proper there seems to be a continuation of the same sort of material as an extremely thin whitish superficial layer extending beyond the equator and well into the lower hemisphere.

In eggs entering the uterus the blastodisc is well differentiated throughout an area about 90° in diameter, while the entire remaining surface of the egg shows a slight paleness as compared with earlier stages.

The dark spot or shallow depression at the animal pole persists, though often very faintly, up to about the time of fertilization, when its site is occupied by a minute but deep and sharply-defined pit (see fig. 6). The change usually does not take place until after the eggs have been for some time in the uterus. As shown by the study of sections, the appearance of this pit usually coincides with the time of formation of the second polar spindle.

B. CAPACITY OF UTERINE EGGS FOR FERTILIZATION

To test whether eggs newly arrived in the uterus are capable of fertilization, a female was taken in which only a small portion of the eggs had reached the uteri, the others being distributed all along the route from ovary to uterus. The eggs from one uterus—about 75 in number—were mixed with milt after the usual manner in artificial fertilization. Of the entire lot, not a single egg developed.

In another female nearly all the eggs had arrived in the uteri, a few remaining in the oviducts and body cavity, and none in the ovaries. All the eggs from the uteri were mixed with milt; about 5 per cent of them developed.

In a third female all the eggs were in the uteri, but none of them showed a distinct pit at the animal pole—evidence that they had only recently entered the uterus. All the eggs were mixed with milt; none of them developed. It should be noted that this female was evidently in the first year of sexual maturity and the eggs may have been slow in undergoing maturation changes, or defective in some way.

In the great majority of cases of females taken with all the eggs in the uteri, artificial fertilization has been successfully performed; a high percentage of fertilized eggs is reached when all the eggs show a distinct pit at the animal pole. In every case in which seminal fluid was examined under the microscope during the breeding season, the spermatozoa were motile; so it is not likely that any cases of failure in artificial fertilization were due to defective spermatozoa.

The evidence indicates that the eggs are incapable of fertilization at the time when the first eggs reach the uterus, but that about the time all the eggs reach the uterus the majority of them become capable of fertilization. This change in their potentiality coincides in time with, or slightly precedes, the formation of a distinct pit at the animal pole; it is probably correlated with the formation of the second polar spindle (see section V).

C. CHANGES VISIBLE FROM THE SURFACE DURING FERTILIZATION

The appearance of the blastodisc shortly after fertilization is shown in figs. 7 and 8. During the first eight hours after fertilization there is an increase in the extent of the blastodisc from a diameter of 90° to 130°–160°, with a corresponding increase in the intensity of its differentiation. From this time up to first cleavage there is no constant increase in the extent of the blastodisc, though the transition from the blastodisc to the darker region surrounding the vegetal pole becomes more gradual. The pit at the animal pole persists unchanged almost up to the time of first cleavage; it is sometimes double (see fig. 10). Shortly before first cleavage it becomes broader and shallower, and usually disappears before the beginning of the first cleavage furrow.

As early as fifteen minutes after artificial fertilization, pits or scars made by the actual or attempted entrance of a spermatozoon have been found on the surface of the egg. It seems remarkable that the spermatozoon can pierce through the thick and tough gelatinous capsule in so short a time. In living material, the point of actual or attempted entrance of a spermatozoon is often visible as a minute but sharply-defined pit, barely visible to the naked eye; hence the name 'sperm pit' will be used to designate the precise locality where the spermatozoon enters, though the word 'pit' does not always accurately describe the appearance in preserved material.

The sperm pits are best studied in material killed in the bichromate-acetic-formalin mixture and preserved in formalin. The 'pits' are not all alike, but readily fall into the following classes, which probably represent consecutive stages in the penetration of the egg by the spermatozoon (see fig. 8):

(a). A simple pit, deep and sharply defined, as observed in living material.

(b). The pit is surrounded by a very small circular opaque white spot.

(c). The pit has disappeared, and the white spot remains. This type is most numerous. (Rarely, the pit persists until much later—see fig. 10.)

(d). The white spot is surrounded and sharply limited by a dark circular line.

(e). The white spot is surrounded by two concentric circular lines separated by a narrow space which is darker than the general surface of the egg (best shown in fig. 7).

It is not always possible to tell from surface views whether the spermatozoon has actually entered the egg, but from the study of

Fig. 7 Equatorial view of an egg of Cryptobranchus allegheniensis, 15 minutes after fertilization, showing a single sperm pit. The lightly stippled area in the upper part of the figure indicates the extent of the blastodisc.

Fig. 8 Equatorial view of an egg 45 minutes after fertilization, showing numerous sperm pits.

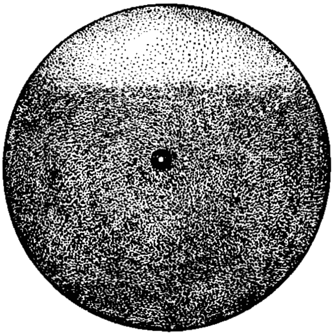
Fig. 9 View of the animal hemisphere of an egg 3¾ hours after fertilization, showing a sperm area near the edge of the blastodisc.

Fig. 10 View of the animal hemisphere of an egg 3¾ hours after fertilization, showing a later stage in the history of the sperm area. The boundary of the sperm area is a trifle too conspicuous in the figure.

Fig. 11 Equatorial view of an egg 6½ hours after fertilization, showing further extension of the sperm area.

Fig. 12 View of the animal hemisphere of an egg 7½ hours after fertilization, showing two sperm areas, on opposite sides of the blastodisc.

All the figures are drawn from preserved material. × 7.



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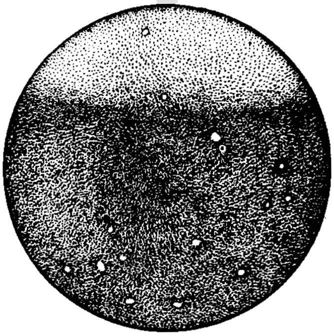

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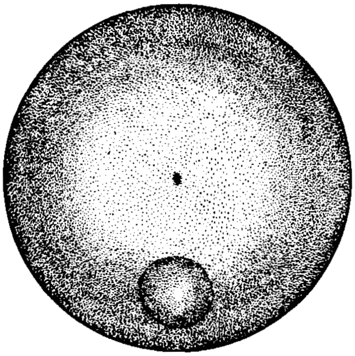

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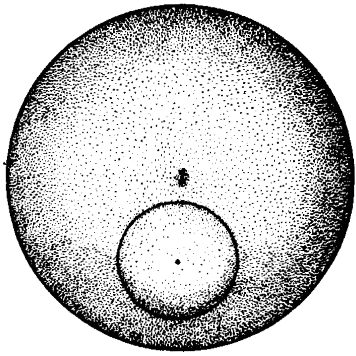

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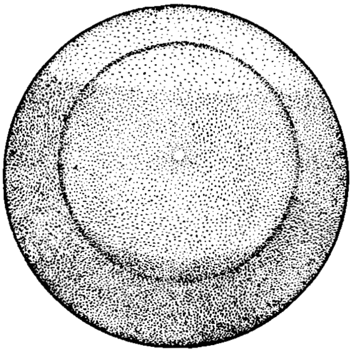

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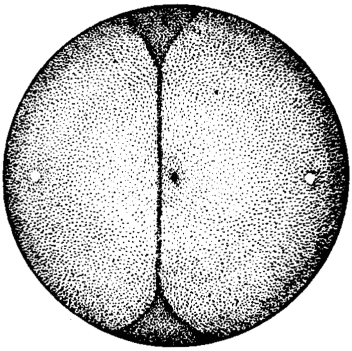

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sections it appears that the first three types of sperm pits indicate that the spermatozoon has barely penetrated through the cell wall, or that the attempt is an abortive one; the last two types indicate with considerable certainty that the spermatozoon has penetrated well into the egg.

Polyspermy is the rule. Cases of penetration by more than one spermatozoon have been found fifteen minutes after fertilization, while the surface of the egg may be scarred by a dozen or more wounds presumably made by other spermatozoa. An hour later, the majority of the eggs have been penetrated each by from one to ten spermatozoa, and sometimes scarred by as many as fifty more. In one case observed the entire number of sperm pits reached nearly a hundred.

About three hours after fertilization the small white spot representing the sperm pit is surrounded by a circular area about 10° to 15° in diameter, slightly darker than the general surface (see fig. 9). An hour later this area has increased in size, is whiter throughout its central portion, and is sharply bounded by a dark line which forms a perfect circle (see fig. 10). This dark line is, partly at least, due to a slight depression in the general surface of the egg. For convenience the area enclosed by this circle will be called the 'sperm area.'

During the next few hours the sperm area increases in size until it covers almost an entire hemisphere (figs. 11 and 12). Its surface is now in general a trifle paler than the remainder of the egg outside the blastodisc; its boundary may pass the animal pole without interruption. Two or even three sperm areas in this advanced stage may be present, their boundaries usually overlapping. Fig. 12 shows an egg fertilized from two opposite sides, the spermatozoa entering near the margin of the blastodisc; the two sperm areas meet at the animal pole, but remain widely separated in the lower hemisphere.

In monospermic eggs preserved and dissected in this stage, the sperm area is found to overlie a lenticular or disc-shaped mass, of firmer consistency than the remainder of the egg which may sometimes be shelled off in a few concentric layers like the fleshy part of an onion. Outside the boundaries of both sperm area and blastodisc there is left a crescentic area which retains the usual color of the heavily yolk-laden portions of the egg; this region often shows numerous fissures in the yolk, running parallel to the margin of the sperm area. These fissures separate the layers previously mentioned. In position and outline this area corresponds very nearly to the 'gray crescent' of the frog's egg (Roux, '83, '85, '87 and '03; Schultze, '00; see also Jenkinson, '09, p. 80 and fig. 43).

Within eight to twelve hours after fertilization the sperm pits have become indistinct and, as a rule, they all disappear before the first cleavage, though cases have been found as late as the fifth cleavage stage. Meanwhile the sperm areas also become indistinct, losing the dark line which serves as a boundary and gradually blending with the surrounding surface of the egg. Fifteen or twenty hours after fertilization, it is usually impossible to orient the egg with respect to the point of entrance of a spermatozoon; before the egg is ready for first cleavage it has resumed the general appearance of radial symmetry which it had before fertilization.

The sperm areas have not been observed in living material, but the examination was made without the aid of a binocular microscope, an instrument which has proved of great value in the surface study of the fertilization stage with preserved material.

In preserved material a space sometimes appears between the blastodisc and the vitelline membrane which elsewhere closely invests the egg. An examination of living eggs at intervals from fertilization to first cleavage shows that normally the vitelline membrane fits closely about the entire egg. The condition noted in preserved material is due to the subsidence of the blastodisc; the vitelline membrane does not spring away from the egg after fertilization, as occurs in some lower forms.

If one remove an unfertilized egg from its gelatinous envelope and immerse it in water, and place almost in contact with it a drop of seminal fluid, one observes that the spermatozoa by means of slow writhing movements disperse gradually in all directions. There is no evidence of attraction by the egg, but spermatozoa coming in chance contact with it adhere to its surface, so that in time there are more spermatozoa at the surface of the egg than at a little distance from it. As previously noted, spermatozoa are found in capsules that do not contain eggs; in this case there is no possibility of attraction by the egg.

In Cryptobranchus, as in other amphibian eggs, there is no preformed micropyle. In eggs fertilized in a natural manner, the spermatozoon may enter the egg at any point. More sperm pits have been found in the marginal region of the blastodisc, about midway between the equator and the animal pole, than elsewhere, indicating that this zone may be especially favorable to the entrance of the spermatozoon; but if any selective influence is at work, it cannot be a strong one, for spermatozoa have been found penetrating the egg close to the second polar spindle, and at various points in the lower hemisphere, even at the vegetal pole. Sperm areas are best developed about those sperm pits that occur near the margin of the blastodisc. In only one case has a sperm pit at the vegetal pole been found surrounded by a sperm area. Sperm pits are often more numerous on one side of the egg than on the opposite side, indicating a chance inequality in the exposure of the egg to the seminal fluid.

All the statements in this section regarding penetration of the egg by the spermatozoa have been confirmed by sectioning eggs which have first been carefully described externally.

D. SUMMARY

A germinal area is first visible in the ovarian egg taken about the middle of August. The germinal area is usually situated on the more exposed side of the egg, toward the periphery of the ovary; it has at first a diameter of about 60°, and increases gradually in size until about the time of first cleavage; it has then a diameter of about 145°.

In ovarian eggs examined about the first of September, the germinal vesicle is usually visible at the surface, in the center of the blastodisc; it disappears shortly before the egg leaves the ovary.

Soon after the eggs have reached the uterus, a sharply-defined pit appears at the animal pole; this pit persists up to the time of first cleavage. About the time of the appearance of the pit at the animal pole, the egg becomes capable of fertilization.

The point of entrance of a spermatozoon (the 'sperm pit') is easily recognizable in both living and preserved eggs. In preserved material, the influence of the spermatozoon on the egg substance is indicated in surface views by the differentiation of a large circular area (the 'sperm area') surrounding the sperm pit. This area is recognizable by a slight difference in color and by the presence of a bounding dark line; it increases in size until it covers nearly a hemisphere of the egg, then disappears.

In artificially fertilized eggs, and presumably in eggs fertilized in nature, polyspermy usually occurs.

There is no evidence of attraction of the spermatozoon by the egg.

The spermatozoon may enter the egg at any point, but sperm areas are best developed about those sperm pits that occur near the margin of the blastodisc.

V. THE INTERNAL HISTORY OF THE EGG BEFORE CLEAVAGE

The present section deals with a few features concerned in ovogenesis and maturation, and gives a more detailed account of the fertilization phenomena.

A. OVOGENESIS

The material for this study consists as follows:

(a). For the early stages it was found best to use larval and immature post-larval females, with a body-length ranging from 9 to 38 cm. (two years old and upward). Females with a body length of more than 38 cm. are almost always sexually mature.

(b). The residual eggs of spent females taken in September furnished ovocytes slightly older than those of the largest immature females taken in August and September.

(c). Mature females taken during July and August furnished material for the late stages of ovogenesis.

There remains a period during the last year of development, extending from October to June inclusive, which is not represented in the material. Since during this time, which includes the winter months, development is least active, the lack of these stages is of minor importance for the purposes of the present paper.

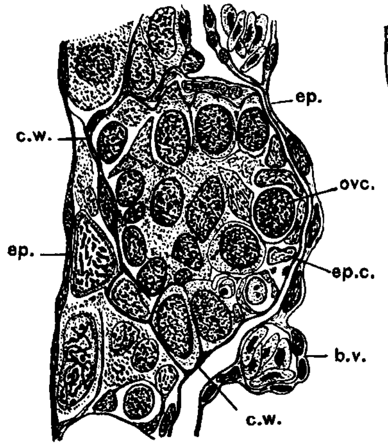
1. The formation of the follicle and the egg membranes

The young ovary of Cryptobranchus is essentially a sac with thick cellular walls. In a 9 cm. larva the ovarian wall (see figs. 13 to 17) shows structural differentiation as follows: (*a*) an inner and an outer limiting membrane of flattened epithelium; these membranes are connected by (*b*) a network of cells of a character similar to those comprising the limiting membranes, though usually not so greatly flattened; within the meshes of this network are found (*c*) young ovocytes in various stages of development.

In the ovary of a 9 cm. larva, more or less clearly defined groups or cysts of very young ovocytes (see fig. 13) may be found, each group surrounded by a thin epithelial membrane, the cyst membrane. All the ovocytes of each group or cyst are presumably the product of a single primary ovogonium. Epithelial cells also occur within the cyst. Within many of these cysts, development has gone further, and some or perhaps all the ovocytes have undergone an increase in size which involves both nucleus and cytoplasm (see fig. 14). Within each cyst, one ovocyte usually outstrips its fellows, and becomes surrounded by a layer of epithelial cells which form the follicle (fig. 15).

With a further increase in size of the ovocyte, the follicular layer assumes the character of a definite membrane with somewhat flattened cells, and that portion of the cyst membrane in contact with the ovarian membrane shows an increase in the number of its nuclei and is more clearly differentiated as a separate layer (see fig. 16).

With a still greater increase in size, as shown by the most advanced ovocytes of a 9 cm. larva and in later stages, the egg presses the overlying membranes into the central cavity of the ovary, so that the ovocyte comes to be suspended as in a sac, and is more nearly surrounded by the cyst and ovarian membranes (see figs. 17 to 21). In all three membranes, an increase in the



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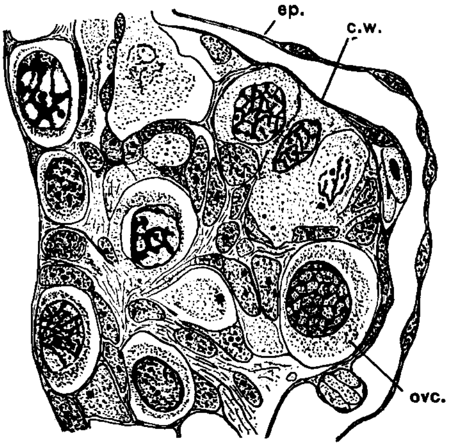

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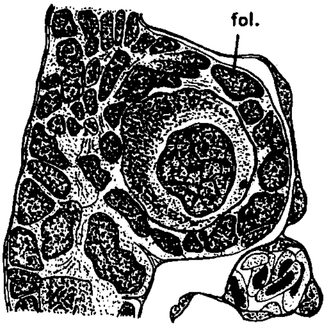

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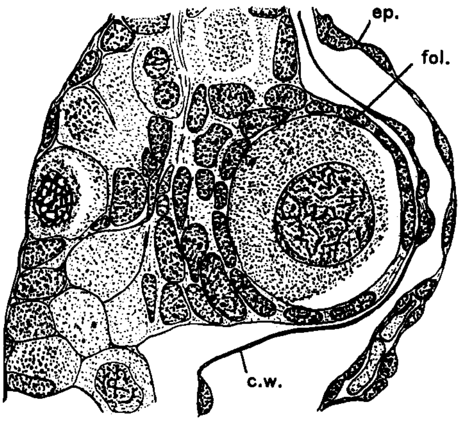

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Figs. 13 to 16 Cross-sections through the wall of the ovary of a 9 cm. larva of Cryptobranchus alleghenienesis. × 300. *b. v.*, blood vessel; *c. w.*, cyst wall; *ep.* (right), inner epithelial membrane of the ovarian wall; *ep.* (left), outer epithelial membrane of the ovarian wall; *ep. c.*, epithelial cell of the cyst; *fol.*, follicle cell; *ovc.*, ovocyte.

Fig. 13 A cyst containing young ovocytes and epithelial cells occupies the central part of the figure.

Fig. 14 A cyst containing slightly older ovocytes.

Fig. 15 An ovocyte surrounded by the newly-formed follicle.

Fig. 16 An ovocyte and follicle slightly more advanced than the one shown in preceding figure.

number of nuclei keeps pace with the increase in extent. In a 35 cm. female (see figs. 21 and 22), the nuclei of the follicular membrane are the most numerous and least flattened; those of the cyst membrane and inner ovarian membrane are both decidedly flattened. Somewhat rarely, the cyst membrane is ruptured by the expansion of the ovocyte. According to King ('08) in Bufo the rupture of the cyst membrane takes place regularly at an early stage.

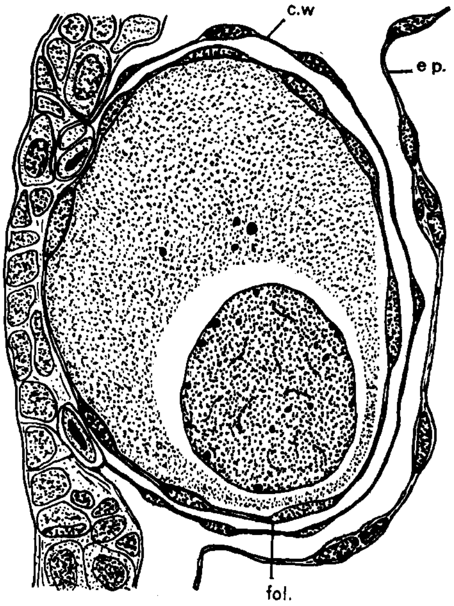


Fig. 17 Cross-section through the ovarian wall of a 9 cm. larva of Cryptobranchus alleghenensis, showing one of the most advanced ovocytes. × 300. Lettering as in the preceding figures.

The ovocyte in the advanced growth stage is thus surrounded by a single-layered follicle, suspended in a flask-shaped two-layered sac of which the inner layer is the cyst membrane, the outer layer is the inner epithelial membrane of the ovarian wall. In a broader sense, the entire three-layered structure may be called a follicle, and the neck of the flask-shaped sac may be called the stalk of the follicle. This triple-layered wall persists without any radical change in structure up to the time of maturation.

In the later stages of the development of the ovary, its walls anastomose by the formation of cross-walls or partitions, dividing the ovary into compartments or perhaps pockets; by these cross-walls the course of the inner ovarian membrane is greatly complicated.

The ovocyte of a female of 26 cm. and younger is apparently a naked cell, possessing no proper membrane. In females with a body length of from 30 to 35 cm. there occurs a rapid development of two non-cellular membranes closely investing the egg within the follicle. The inner of these two membranes exhibits a radial striation and is the zona radiata; at the time of maturation it becomes a simple cell wall to the egg. The outer membrane, clear and homogeneous, is the zona pellucida; it persists as the 'vitelline membrane' of the embryo.

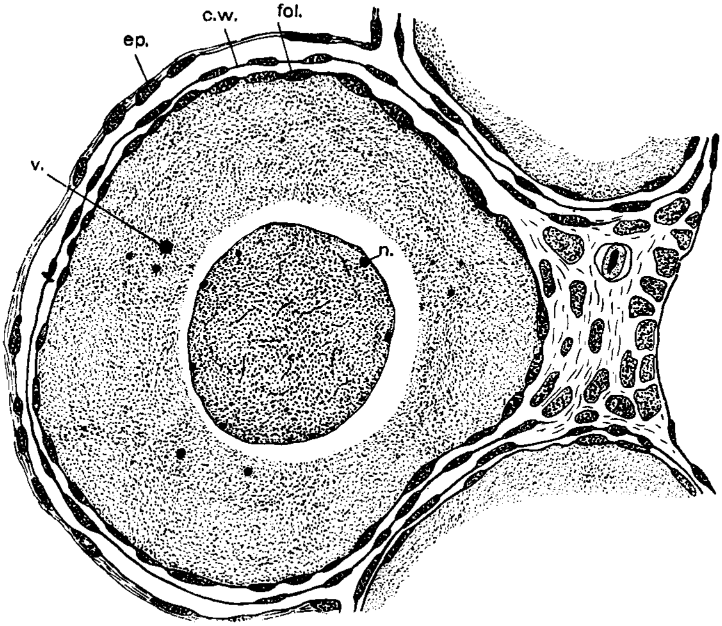


Fig. 18 Cross-section through the ovarian wall of a 26 cm. Cryptobranchus allegheniensis, showing one of the most advanced ovocytes. × 180. *n.*, nucleolus; *v.*, vitelline body; *ep.*, inner epithelial membrane of the ovarian wall. Other lettering as in figs. 13 to 16.

The zona radiata and the zona pellucida begin to form simultaneously, shortly before the appearance of yolk granules. In the most advanced ovocytes of a 35 cm. female, these membranes are well established and a narrow zone of yolk has appeared near the periphery of the ovocyte (see fig. 22).

The zona radiata arises from the peripheral cytoplasm of the ovocyte. In its early stages its inner boundary is not sharply defined; its staining reaction is like that of the egg cytoplasm; aside from its cross-striation its structure, like that of the egg cytoplasm, is finely granular. The zona pellucida, on the other hand, is formed *de novo* as a product of cellular activity. In the ovary of a 35 cm. female its staining reaction is different from that of any other structure present: with the borax-carmine Lyons-blue picric-acid mixture it becomes green, while the ground-substance of the follicular, cyst and ovarian membranes stains blue. Since, later, a membrane exactly resembling the zona pellucida in character sometimes, though not typically, forms between the cyst membrane and the follicle (see fig. 32), it seems reasonable to conclude that the zona pellucida is the product of the follicle rather than of the egg.

In the most advanced ovocytes of a spent female there is usually an increase in the thickness of the zona pellucida, while the zona radiata shows signs of degeneration—there is a slight loss in the distinctness of the radial striations. In adult females taken in July and August, there is a further loss in the distinctness of the striations of the zona radiata. In ovocytes taken just before maturation, with the germinal vesicle close to the surface, the zona radiata has in some cases almost lost its radial striation, is decreased in thickness, and is becoming a simple cell wall to the egg.

The literature on the zona pellucida and zona radiata of the amphibian egg has been reviewed by Waldeyer in Hertwig's ('06) Handbuch and needs no summary here.

The ovary of a young Necturus 20 cm. long, killed August 25, gives stages corresponding to those of a 35 cm. Cryptobranchus. The follicular layers and mode of attachment of the ovocyte to the ovarian wall are practically the same as in Cryptobranchus, with the exception that there is a marked difference in the appearance of the nuclei of the follicle proper: in Necturus these nuclei are more numerous, and in form are spherical or even elongated in a radial direction, instead of being flattened in the direction of the circumference of the egg as in Cryptobranchus. The follicle of Necturus more closely resembles that of the selachian egg in an early stage (see Hertwig's Handbuch, '06, figs. 105 and 195). The zona pellucida and zona radiata are much alike in the two urodeles; the striations of the latter membrane are rather more distinct in Necturus.

2. The establishment of polarity, and the progress of axial differentiation

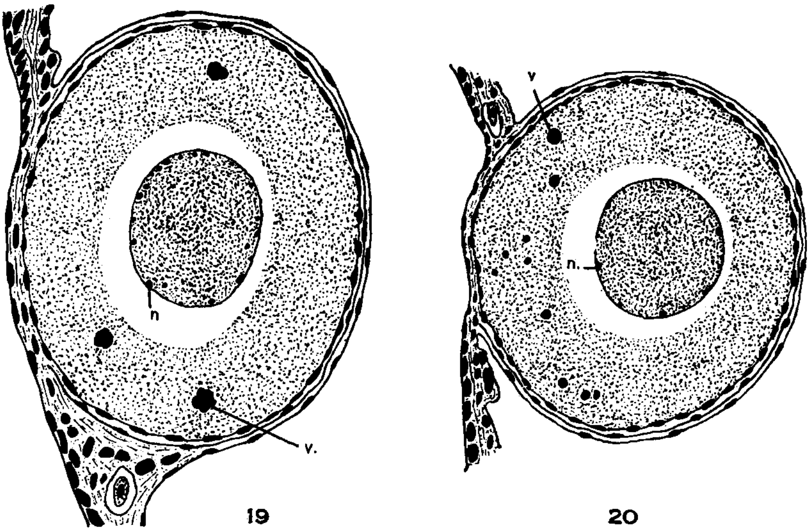
As already noted in the surface study of the ovarian egg, the ovocyte ready for maturation shows its telolecithal character in the presence of a superficial germinal area, in the center of which lies the germinal vesicle, while the remainder of the egg is heavily laden with yolk. It is the purpose of the present section to trace the changes by which this axial differentiation is brought about.

In the ovary of a 9 cm. larva, vitelline bodies (see King, '08) are recognizable in the cytoplasm of the ovocytes in all stages present, but are not very numerous nor conspicuous even in the most advanced ovocytes of such an ovary (see figs. 13 to 17). In the largest ovocytes, the germinal vesicle is usually somewhat excentrically situated, but with no constancy in the direction of excentricity. Faintly-staining nucleoli are distributed quite promiscuously thoughout the germinal vesicle, in the later stages with a slight tendency toward forming a ring at the periphery.

In the most advanced ovocytes of a 26 cm. female (see fig. 18) there is less excentricity in the position of the germinal vesicle; the nucleoli are most numerous at the periphery. There is an increase in the number and size of the vitelline bodies, which are more numerous on the side toward the central cavity of the ovary. After fixation in Zenker's fluid, both nucleoli and vitelline bodies take the nuclear stain, though faintly. In the ovary of a 27 cm.

female, fixed in Bouin's solution, the nucleoli take the nuclear stain very faintly; the vitelline bodies take the cytoplasmic stain.

In the most advanced ovocytes of a 30 cm. female (figs. 19 and 20) the germinal vesicle is quite centrally situated—a position which it retains until a very late stage of ovogenesis. The nucleoli, which still stain but faintly, are nearly all at the periphery, where they form a uniform ring. The vitelline bodies shown in the figures now stain brilliantly with borax carmine used after Zenker's fluid; in general they are much more numerous on the side toward the periphery of the ovary, in the region of the future animal pole. Some of the vitelline bodies are very large; these usually occupy an equatorial position, but are sometimes found on the inner side of the ovocyte. Comparison with the preceding stage suggests that the vitelline bodies originate on the inner side of the ovocyte and migrate to the outer side; that they reach their greatest development midway in the course of migration, and break up to form the smaller and more numerous vitelline bodies



Figs. 19 and 20 Sections through ovocytes and ovarian wall of a 30 cm. Cryptobranchus allegheniensis, showing the follicle and the distribution of vitelline bodies and nucleoli. × 90. *n.*, nucleolus; *v.*, vitelline bodies.

in the region of the future animal pole. But scattered throughout the cytoplasm are occasionally to be found other bodies, resembling the vitelline bodies but more irregular in form and staining very faintly. While it is possible that these bodies are different in kind from the brilliantly-staining vitelline bodies, their appearance suggests that they are stages in the degeneration of the latter. The faintly-staining bodies, though seldom numerous, are more frequently found in regions poor in deeply-staining vitelline bodies. These observations enable us to offer an explanation of the distribution of vitelline bodies, alternative to the theory of migration: a wave of development of vitelline bodies, followed by a wave of degeneration, may sweep from the inner to the outer hemisphere of the ovocyte. But whether migration is real or only apparent, the fact remains that the region of most abundant deeply staining vitelline bodies has shifted from the vicinity of the future vegetal to the future animal pole of the ovocyte. This change is perhaps an expression of polarity; if so, it is the first indication of polarity that I have observed. However, it is not at all certain that polarity is not present at an earlier period; in particular the history of the chromatin has not been sufficiently studied, moreover it is of course possible that a physiological polarity of the cell may precede its manifestation in a visible form.

In the ovary of a 34 cm. female, fixed in Flemming's solution, the distribution of vitelline bodies is much the same as noted in the 30 cm. female; in form the vitelline bodies are sometimes oval or irregular, but never mulberry-shaped as is sometimes the case with Zenker's.

In the ovary of a 35 cm. female, yolk granules are beginning to form in the most advanced ovocytes; other ovocytes nearly as large contain no yolk. In neither of these two stages are vitelline bodies in the typical condition present, but they are sometimes found undergoing a process of degeneration—they lose the intensity of their staining reaction, become irregular in form, and disappear. The disappearance of the vitelline bodies at the time of the formation of yolk suggests a correlation between the two phenomena; but so far as I have been able to observe, the final stages in the disappearance of the vitelline bodies are not closely associated with the formation of yolk granules, nor have I found any undoubted 'yolk nuclei,' such as have been described by King ('08) for Bufo. In view of the diversity in the methods of yolk-formation described for different amphibians, this result is not altogether surprising.

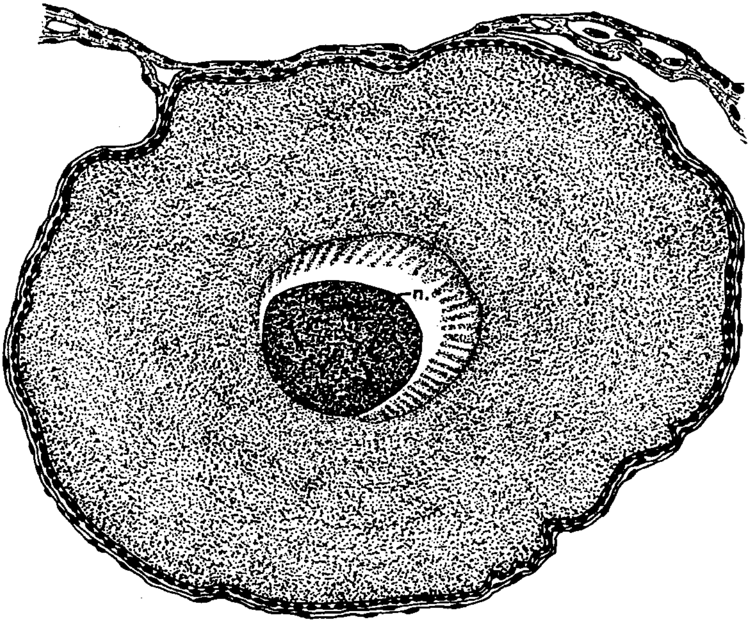


Fig. 21 Section through an ovocyte and ovarian wall of a 35 cm. Cryptobranchus alleghenienеsis, showing the follicle and the distribution of nucleoli. In this ovocyte the vitelline bodies have disappeared, but yolk-formation has not yet begun. × 60. *n.*, nucleolus.

In the more advanced ovocytes of a 35 cm. female (see fig. 21). the nucleoli stain deeply with borax carmine used after Zenker's fluid. There is usually a marked concentration of the nucleoli on the side of the germinal vesicle toward the periphery of the ovary. Account must be taken of the fact that shrinkage of the germinal vesicle also proceeds, as a rule, most extensively on this side, leaving a large space, while the opposite side remains in contact with the cytoplasm. This greater shrinkage on the outer side in part accounts for the greater concentration of nucleoli on this side, but it is inadequate to account for all of it; moreover the axis of excentricity in form due to shrinkage does not always correspond accurately to the axis of excentricity in the arrangement of the nucleoli.

This excentric distribution of material marks an axis which corresponds, roughly at least, to the polar axis at the time of maturation; the nucleoli accumulate on the side which is to become the animal pole, and thus perhaps afford a second indication of polarity. King ('08) found this condition in Bufo at the time when the nucleus was moving from the center of the egg to the animal pole, and suggested the possibility that the accumulation of most of the nucleoli in one part of the nucleus might have something to do with this movement. In Cryptobranchus this concentration of the nucleoli begins long before the migration of the germinal vesicle to the surface, and indeed before the formation of any yolk; it is most marked in the advanced ovocytes of a 35 cm. female, when the yolk is just beginning to form. As will appear from the study of later stages, this arrangement of the nucleoli does not persist during the actual migration of the germinal vesicle; nevertheless the early occurrence of axial concentration of nucleoli is significant.

In the ovary of a 35 cm. female, we find that occasionally, through the folding of the ovarian wall, an ovocyte has been thrust deep into the central cavity and has come in contact with the nutrient ovarian wall of the opposite side. The side opposite the stalk of the follicle now becomes the side best nourished, and here the nucleoli accumulate. Thus nature's experiment shows that the accumulation of nucleoli, and perhaps polarity, is not something predetermined in the egg, or even fixed by the relation of the egg to the ovarian wall within which it develops, but is a phenomenon depending upon larger environmental relations which probably have to do with nutrition; for as a consequence of the changed position of the egg the nucleoli accumulate on the opposite side from that favored by the original environment.

In the ovocytes of immature females with body lengths of from 35 to 38 cm., the yolk first appears in a narrow zone near the periphery and parallel to the newly-formed zona radiata, but separated from the latter by a narrow layer of clear cytoplasm (see fig. 22). At this time the ovocyte has a diameter of from 1.5 mm. to 2 mm. The yolk zone is divisible into two layers, an outer

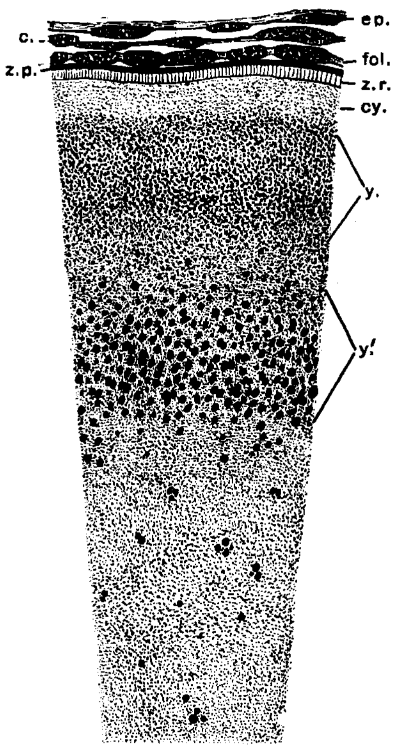


Fig. 22 Portion of a section through one of the most advanced ovocytes of a 35 cm. Cryptobranchus allegheniensis, showing structure of the membranes surrounding the egg and the distribution of yolk granules. × 340. The strip shown extends about half-way to the germinal vesicle. *c.*, cyst membrane; *cy.*, yolk-free peripheral zone of cytoplasm; *ep.*, inner epithelial membrane of the ovarian wall; *fol.*, follicular membrane proper; *z. p.*, zona pellucida; *z. r.*, zona radiata; *y.* and *y'.*, layers of fine and coarse yolk granules respectively.

layer of fine yolk particles and an inner layer of coarse yolk particles, separated by a narrow region poor in yolk.

In the largest residual eggs (2 to 3 mm. in diameter) of spent females, the yolk-laden zone has extended inward further than outward; a very narrow zone of clear cytoplasm persists at the periphery, and a much broader zone containing only a few scattering yolk granules surrounds the germinal vesicle. The middle portion of the yolk zone is now filled with coarse yolk granules; its margins consist of fine yolk particles. The germinal vesicle is still centrally situated, and the arrangement of yolk zones and cytoplasm is concentric. The nucleoli are still distributed at the periphery of the germinal vesicle, with only a slight tendency toward concentration at the outer side.

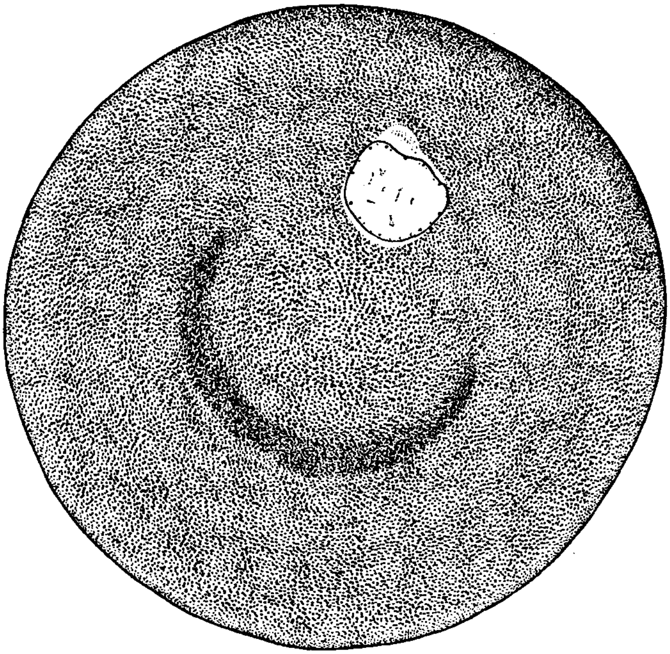


Fig. 23 Meridional section through one of the most advanced ovocytes of an adult Cryptobranchus allegheniensis killed July 6. The bounding line represents the zona radiata. × 20.

In the largest ovocytes of adults killed July 6 (see fig. 23), the germinal vesicle occupies a position midway between the center of the egg and the periphery, on the more exposed side of the egg, toward the stalk of the follicle. The animal pole is thus defined by a point on the surface, toward which the germinal vesicle is moving. The cytoplasm is now everywhere thickly interspersed with yolk granules; these granules are in general coarse throughout the central portion of the egg, finer and more densely packed at the periphery. Axial differentiation in the arrangement of yolk particles is now for the first time evident in a slight thickening of the peripheral layer of fine yolk particles in the region of the animal pole. This region is also somewhat richer in cytoplasm than the remainder of the egg. There is thus present the beginning of a germinal disc or blastodisc, which in later stages becomes visible in surface views as the germinal area.

In the vegetal hemisphere a region of particularly fine and dense yolk, crescent-shaped in meridional section, lies mid-way between the center of the egg and the periphery. This region I shall call the 'yolk cup.' Its appearance suggests that it may be a part of a once continuous zone completely enclosing the germinal vesicle, and that, in the animal hemisphere, this zone has been interrupted in consequence of the migration of the germinal vesicle toward the surface. Probably the yolk cup is the physiological equivalent of the concentric layers of dense fine yolk found in the egg of the hen and various other vertebrates. Riddle ('11) has shown that the alternate layers of yellow and white yolk in the hen's egg are due to a daily rhythm in nutrition; he has advanced the same principle in explanation of the concentric layers of yolk in the eggs of certain cyclostomes, selachians and reptiles. In Cryptobranchus, from comparison with ovarian eggs taken in the autumn after the close of the spawning season, it is evident that in the stage under consideration the yolk cup marks the limits of growth during the preceding winter; hence it seems very probable that the yolk cup is the result of a seasonal variation in nutrition, and represents a layer added during the winter months.

The nucleoli are still found mainly at the periphery of the germinal vesicle, but with no constant tendency toward concentration in an axial position.

It has been noted that the animal pole, as defined both by the center of the germinal disc and the point on the surface toward which the germinal vesicle is moving, lies in general on the more exposed side of the egg, within the stalk of the follicle. The animal pole thus lies in the opposite direction from that assumed in the ovarian egg of the hen (Lillie, '08, p. 29). According to King ('02) in the great majority of cases the egg of Bufo is attached in the equatorial region by the stalk of the follicle.

From a comparison of this stage with the preceding one (the ovocytes of a spent female), it is evident that yolk-formation proceeds concentrically about a centrally situated germinal vesicle until the egg is nearly or quite filled with yolk, and that axial differentiation in the arrangement of yolk particles does not appear until a very late stage of ovogenesis, two or three months before maturation. It is further apparent that the germinal vesicle attains its final position, not through unequal growth of the cytoplasm or excessive accumulation of yolk on the other side of the egg, but by a process of migration.

In the ovocytes of adults taken July 20, the germinal vesicle has migrated further toward the animal pole; it lies about one-third of the distance from the surface to the center of the egg. Both nucleoli and chromosomes are now aggregated at the center of the germinal vesicle. The yolk-cup persists, and there is an increase in the extent of the germinal disc. In some eggs a small cone-shaped mass of dense cytoplasm, with the apex of the cone pointing inward, lies immediately beneath the germinal vesicle.

In the ovary of an adult female killed August 17, the egg (fig. 24) has nearly reached its maximum size before fertilization; a meridional section cut in paraffin has a diameter of about 6 mm. (It should be noted that a yolk-laden egg does not shrink in paraffin to the same extent as ordinary tissues). The germinal vesicle lies only a short distance from the surface, and is bounded on the side toward the center of the egg by a large cone-shaped mass of cytoplasm. The apex of this cone is continuous with a slender meshwork of less dense but yolk-free cytoplasm extending half-way to the center of the egg. Owing to a slight obliquity of the slender cytoplasmic mass, it has not been found complete in any one section; in fig. 24 it has been added, from adjacent sections, to the one chosen for the remainder of the drawing.

Immediately beneath the zona radiata lies a peripheral layer of yolk-free cytoplasm, which from analogy with the teleost egg I shall call the 'protoplasmic mantle.' In the region of the vegetal pole this is so thin as to be barely recognizable with a magnification of 500 diameters; in the region of the animal pole it is thickened to form a disc which I shall call the 'cytodisc.' At the animal pole the cytodisc reaches its maximum thickness of about 15μ—a little thicker than the layer of follicle cells proper.

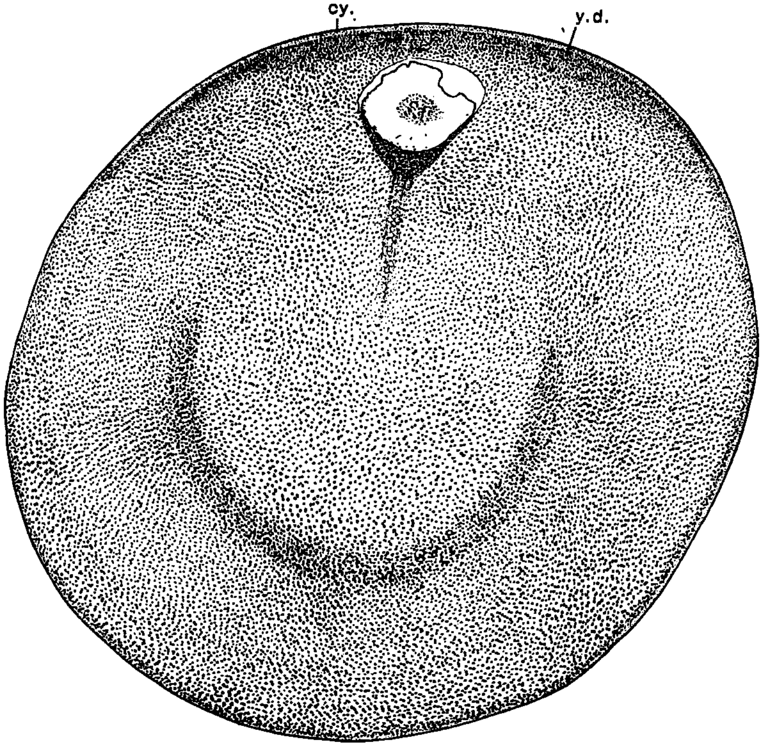


Fig. 24 Meridional section through an ovarian egg of an adult Cryptobranchus allegheniensis killed Aug. 17. × 20. *cy.*, cytodisc; *y. d.*, yolk disc.

The remainder of the egg is filled with yolk. Underlying the cytodisc and occupying an area about 100° in diameter surrounding the animal pole, is a thick layer of fine but dense yolk which I shall call the 'yolk disc.' The cytodisc and yolk disc combined represent the anlage of the germinal disc or blastodisc, which later comes to enclose the germinal vesicle and the cytoplasm accumulated beneath it. Elsewhere a very thin peripheral layer of fine yolk particles, continuous with the yolk disc, lies immediately beneath the protoplasmic mantle. The interior of the egg shows no particular change in the yolk.

The germinal vesicle is spherical when perfectly preserved; when flattened this is due to shrinkage. The ground-substance or nuclear sap appears homogeneous under a low power, but with a magnification of 500 diameters it exhibits an extremely fine but dense granular structure. The nucleoli are now nearly all aggregated at the center; some few persist at the periphery, particularly on the side toward the center of the egg. The chromosomes are for the most part confined to the central part of the area occupied by the nucleoli.

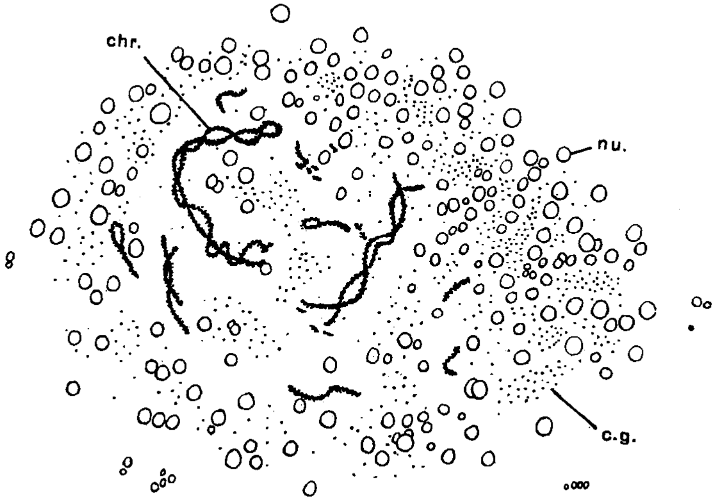


Fig. 25 Central portion of the germinal vesicle represented in the preceding figure, enlarged to show details. The finely granular ground-substance of the germinal vesicle is not shown. × 340. *c. g.*, chromatin granules; *chr.*, chromosomes; *nu.*, nucleolus.

Among the nucleoli, though not closely associated with them, there are now found very numerous and minute granules which stain like chromatin (see fig. 25). These are evidently formed in close association with the chromosomes; in earlier stages chromosomes have been found covered with these granules before the latter have appeared elsewhere.

In the ovarian eggs of an adult killed August 22, the most marked changes in the general topography as viewed in meridional sections are a slight advance in the migration of the germinal vesicle toward the surface, and an increased thickness of the peripheral zone of fine yolk particles, particularly in the yolk disc. In the vegetal hemisphere the protoplasmic mantle is no longer recognizable as a separate layer; its constituents have mingled with the peripheral layer of fine yolk particles. The cytodisc is reduced in thickness by the blending of its inner surface with the yolk disc.

The narrow path of cytoplasm leading toward the center of the egg from the apex of the cone of cytoplasm underlying the germinal vesicle has disappeared; likewise the yolk cup is, as a rule, no longer present. In this stage there is a slight increase in the number of chromatin granules dispersed amongst the nucleoli; otherwise the nuclear contents seem unchanged.

Ovaries taken during the last week in August and the first week in September usually contain some eggs with the germinal vesicle appearing at the surface. In the general organization of the egg before the germinal vesicle actually reaches the surface, there are few changes from the condition described for August 22. Fig. 26 shows the general topography of an egg with the germinal vesicle very close to the surface. The cone of cytoplasm underlying the germinal vesicle is beginning to mingle with the yolk; it is not present in the section figured. Within the germinal vesicle the nucleoli are massed more closely together at the center; there is an increase in the number of chromatin granules, and apparently a gradual disappearance of the chromosomes—in some eggs they could not be found.

At the close of the period considered, axial differentiation is evident in the following arrangement of material: (*a*) the excentric position of the germinal vesicle and the cone-shaped mass of cytoplasm underlying it; and (*b*) the formation about the animal pole of a germinal disc or blastodisc consisting of two layers, a very thin peripheral layer of yolk-free cytoplasm which has been called the cytodisc, and underlying this a thick lenticular layer of mingled cytoplasm and dense fine yolk which has been called the yolk disc. At any given level the egg is radially symmetrical about the axis of polarity. In general the egg has progressed from an alecithal through an isolecithal to a telolecithal stage.

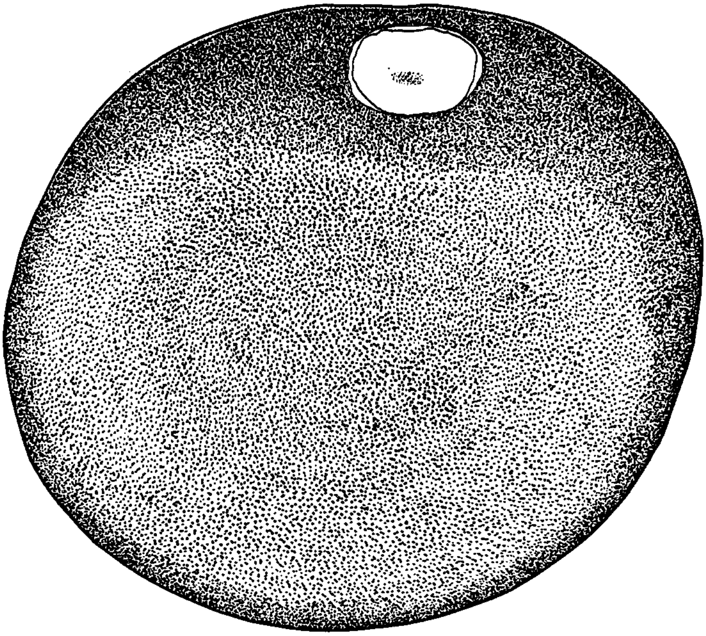


Fig. 26 Meridional section through an ovarian egg of an adult Cryptobranchus allegheniensis killed Sept. 6, 1910, showing organization just before the germinal vesicle reaches the surface. × 20.

The establishment of polarity, with axial differentiation, is an event of great morphogenetic importance, since the formative materials for the embryo are being segregated in the vicinity of the animal pole. Through later changes in the distribution of this material the animal pole comes to mark the anterior, the vegetal pole the posterior end of the future animal; hence the establishment of polarity defines the principal axis of the embryo.

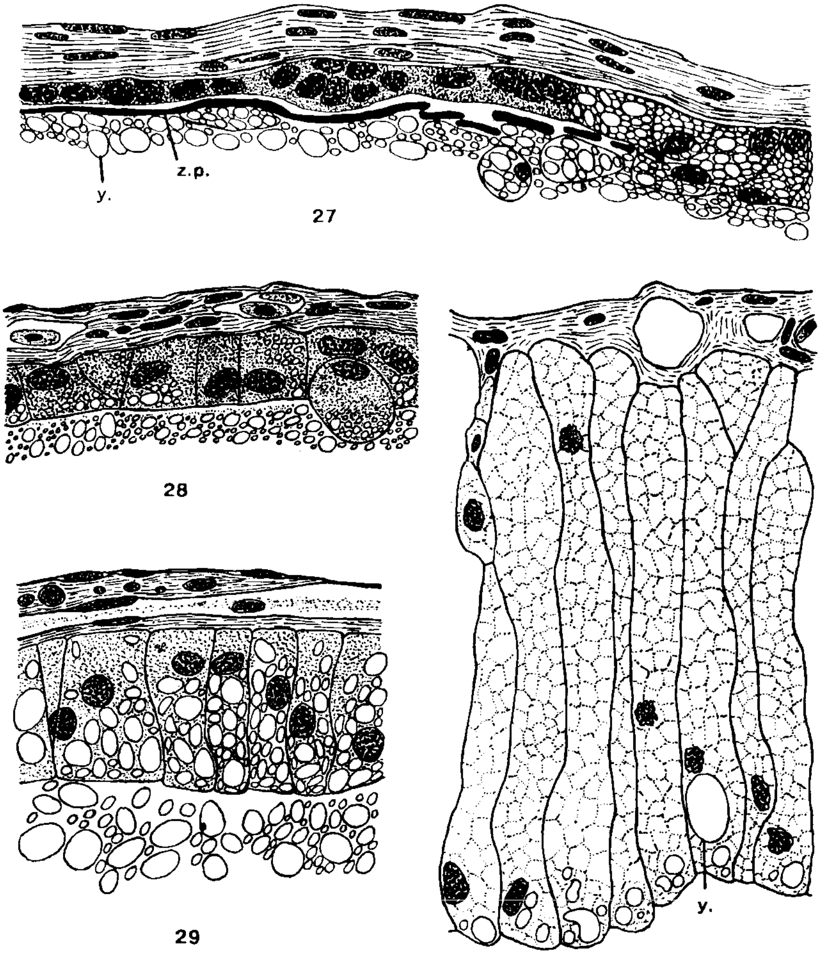
The changes that immediately follow—the appearance of the germinal vesicle at the surface, the rupture of its membrane, and the reorganization of the germinal disc with the incorporation of materials brought from the interior of the egg by the nucleus—lead up to maturation and will be considered in the account of that process.

3. Resorption of ovocytes; the follicle cells in a phagocytic rôle.

In young females nearing maturity (about 38 cm. body length), a few ovocytes reach an advanced stage of development, becoming filled with yolk and attaining a size nearly as great as the ovocytes of an adult. These precocious ovocytes fail to undergo maturation changes, and during the breeding season begin to degenerate, or rather to be resorbed, together with some of the less advanced ovocytes only partially filled with yolk. Viewed in the living ovary, these degenerating ovocytes are colored a very bright yellow or orange. Digestion and absorption of the yolk granules is accomplished through the medium of the cells of the follicular layer proper, which become greatly enlarged and function as phagocytes, thereby reversing their usual rôle as nurse cells to the egg.

The first step in the process of degeneration of the ovocyte is the disappearance of the zona radiata; the later stages are illustrated by figs. 27 to 30. The follicle cells enlarge, by increase both in the size of the nucleus and in the amount of cytoplasm. The zona pellucida is ruptured; at the same time it becomes irregularly thickened, a circumstance which may be interpreted either as a shortening of the fragments due to the release of tension, or as a step in the process of dissolution. The rupture of the zona pellucida allows the yolk to come in contact with the follicle cells; the latter engulf the yolk particles, and become surrounded by thin walls. About this time the zona pellucida disappears, and the follicle cells are left as large yolk-filled cells resembling columnar epithelium, forming a continuous layer around the egg.

Digestion of the yolk particles is completed first at the outer margin of the follicle cells, while the inner margin continues to engulf yolk. The included yolk granules stain less deeply with



Figs. 27–30 Changes at the periphery of an ovocyte in the process of resorption; the follicle cells are shown in a phagocytic rôle. Fig. 27, read from left to right, shows the beginning of the process (compare with fig. 22 showing the normal condition of the follicle). The remaining figures, taken from different ovocytes, show successively later stages. × 244. *y.*, yolk; *z. p.*, zona pellucida.

haemotoxylin than the others, and in sections stained with the borax-carmine Lyons-blue picric-acid mixture the included granules take the cytoplasmic stain more deeply.

The follicle cells later become greatly elongated, and the cytoplasm takes the form of a faint meshwork with large spaces. Ingestion of yolk continues at the inner ends of the cells, while the remainder of the cell functions as a long tube to convey the products of digestion to the periphery. The follicular layer remains one cell in thickness until the cells have reached a length of about 250 μ; with a further increase in thickness it becomes broken up into a meshwork of cells, amongst which are numerous capillaries. Ovocytes have been found in which this meshwork of cells reaches nearly to the center, and the remaining yolk is very small in amount.

In the adult female occasional eggs, though of full size, fail to escape from the ovary. Judging from their external appearance these ovocytes undergo resorption in the manner just described. A somewhat similar process of resorption has been described in the eggs of cyclostomes and fishes (Bühler, '02).

4. The organization of the egg shortly before the appearance of the germinal vesicle at the surface

At this point it may be well to summarize briefly the condition of the ovocyte in the stage immediately preceding the appearance of the germinal vesical at the surface (see fig. 26).

The egg lies within a triple-walled follicular sac whose cellular membranes have undergone little change since they first became well established. The stalk of the follicle, and in general the animal pole of the egg, lie toward the periphery of the ovary.

The zona pellucida persists unchanged, except for a slight increase in thickness; the zona radiata shows signs of atrophy, and in some cases is assuming the character of a simple cell wall.

The nucleus or germinal vesicle has migrated from the center of the egg to a position near the periphery, ordinarily on the side toward the stalk of the follicle. During the migration of the germinal vesicle a cone-shaped mass of dense cytoplasm has collected beneath it, and is now beginning to mingle with the surrounding yolk.

A germinal disc or blastodisc is evident in surface views of living material as a circular area, lighter in color than the rest of the egg, about 60° in diameter and situated on the more exposed side of the egg. In meridional sections it is shown to consist of two layers: a thin peripheral layer of cytoplasm, the cytodisc; underlying this a thick lenticular mass of mingled fine yolk particles and cytoplasm, the yolk disc. The germinal vesicle lies at the center of the yolk disc.

The yolk disc is continuous with a thin peripheral layer of fine yolk granules, mixed with cytoplasm, which lies in contact with the zona radiata everywhere except in the region of the cytodisc.

The remainder of the egg is filled with coarse yolk granules mingled with fine yolk granules and a small amount of cytoplasm.

The nucleoli are grouped at the center of the germinal vesicle, and amongst them are numerous chromatin granules. In some eggs chromosomes are found at the center of the group of nucleoli, in others the chromosomes have disappeared.

A point on the surface overlying the center of the germinal vesicle marks the animal pole. The general arrangement of materials is radially symmetrical about the axis of polarity, with differentiation proceeding in the direction of this axis.

B. MATURATION

1. *The germinal vesicle at the surface*

Meridional sections through ovocytes with the germinal vesicle at the surface show little change in the details of structure from the condition previously described. The germinal vesicle is usually somewhat flattened against the periphery, and a portion of its surface is in direct contact with the zona radiata. Masses of a wavy fibrous material are occasionally found in the nuclear sap. A few fragments of chromosomes are present in some eggs; in others no chromosomes have been found. The nucleoli and chromatin granules persist at the center of the germinal vesicle.

2. *The dissolution of the germinal vesicle, and the formation of the first polar spindle*

Material for the study of this stage was obtained from two females in which the majority of the ripening eggs had left the ovary, and were found distributed in the body cavity, oviduct and uterus. The nearly mature eggs left in these ovaries were found in every case investigated (nine eggs were sectioned) to have the germinal vesicle ruptured and its constituents well mixed with those of the blastodisc; in the majority of cases the first polar spindle had already formed.

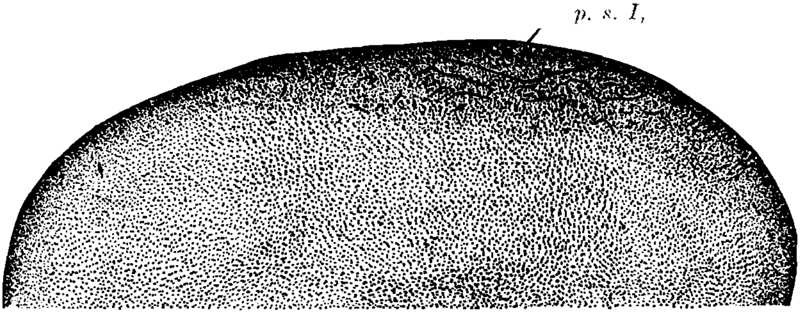


Fig. 31 Meridional section through an ovarian egg of Cryptobranchus allegheniensis, shortly after the rupture of the germinal vesicle. Fragments of the germinal vesicle are seen scattered throughout the blastodisc. × 18. *p. s. I,* first polar spindle.

The rupture of the germinal vesicle and the distribution of its materials throughout the blastodisc must take place with considerable rapidity, since in eggs sectioned only the beginning and the end of the process have been observed. Fragments of the nuclear membrane, together with the wavy fibrous material previously noted in the germinal vesicle and innumerable fine granules, probably derived from the cell sap, become widely scattered throughout the germinal disc (see fig. 31). During this process of disintegration of the germinal vesicle the nucleoli and chromatin granules are lost to view. It seems improbable that all the chromatin granules should again be segregated as nuclear material; at any rate the rupture of the germinal vesicle affords an opportunity for the contribution of important nuclear material to the cytoplasm.

The germinal disc or blastodisc no longer shows a division into two layers; the material of the cytodisc is intimately mingled with that of the yolk disc. The cone of cytoplasm following the germinal vesicle in its migration is likewise more or less thoroughly incorporated into the blastodisc.

The end result of the migration of the germinal vesicle to the surface and its disintegration in that situation is now apparent. All the material of the nucleus and a considerable amount of cytoplasm have been brought from the interior of the egg to the vicinity of the animal pole, fragmented, and the débris more or less scattered throughout the blastodisc. Out of this complex there soon emerges close to the surface at the animal pole the reconstructed nucleus in the form of the first polar spindle. One function of migration is doubtless to get this nuclear material to the periphery where a part of it may be disposed of in the maturation divisions. A further adaptation is found in the fact that, later, the egg-nucleus or female pronucleus is left in the center of the formative material of the blastodisc. A third end attained by migration is that the formative material of the blastodisc is added to by cytoplasm following the germinal vesicle, and also by substances derived from the germinal vesicle itself.

The zona radiata has become reduced in thickness, has lost its striation and no longer shows a distinct limiting inner surface—its inner margin is irregular or blends with the peripheral cytoplasm of the egg. When the egg is shrunken away from the zona pellucida the zona radiata usually remains organically connected with the egg. The character of the zona radiata has changed so radically that it will no longer be referred to by this name; it has become a simple cell wall to the egg, and as such takes part in the later process of cleavage.

The zona pellucida persists unchanged as the so-called vitelline membrane of the egg at the time of fertilization and during the early stages of embryonic development.

As in other amphibian eggs, only these two membranes, the zona pellucida and the cell wall formed from the zona radiata, accompany the egg in its escape from the ovary; the process of ovulation involves the rupture of the follicle which remains in the ovary.

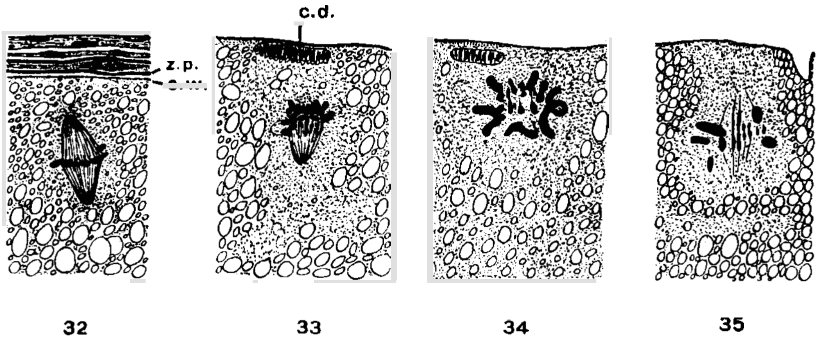
The occurrence of the first polar spindle was studied in two females, (*A*) and (*B*), in which the eggs were distributed from ovary to uterus inclusive. The first polar spindle was found in eggs taken from the following situations: ovary, body cavity, oviduct, and extreme upper part of the uterus; out of a total of twenty-eight eggs studied, the first polar spindle was found in thirteen cases. Five eggs taken from the lower uterus were studied; no first polar spindle was found.

In the case of another female (*C*), in which the eggs were all in the uteri, no first polar spindle was found in three eggs sectioned.

Allowance must be made for the fact that in some cases in which the first polar body is absent it may have been missed on account of imperfections in the series. The results are sufficient to justify the conclusion that the first polar spindle is usually present at the time the egg leaves the ovary and during its passage down the oviduct, and that it disappears about the time the egg reaches the uterus.

The first polar spindle (see figs. 32 to 35) is formed with its long axis either coinciding with the axis of polarity of the egg, or oblique to this axis. The number of chromosomes is probably twelve before any of them have divided. There is an outer ring of six large chromosomes, surrounding a central group of six small chromosomes usually found in a state of division; it is probable that these six small chromosomes are not all of equal size. These size differences of the chromosomes are interesting in the light of well-known recent work indicating individual differences in the chromosomes of many forms.

There is frequently present close to the cell wall overlying the spindle a disc-shaped body with an irregular cross-striated structure, which, from its probable mode of origin, I shall call the 'contact disc' (see figs. 33 and 34). This disc takes the cytoplasmic stain, and seems to be of the same composition as the cell wall. The adjacent cell wall is slightly thickened and sometimes shows a cross-striation, reminding one of the zona radiata (compare the effect on the cell wall of penetration by the spermatozoon, described later). The presence of the contact disc is uniformly accompanied by a deficiency of the spindle, which lacks an aster at the end nearest the disc. In a few cases there seems to be a small amount of sphere substance underlying the contact disc. The inference seems to be that the contact disc is the product of the aster of the first polar spindle modified by contact with the cell wall. The function of the disc, if it have any function, may be to anchor the spindle at the surface during the pulling-apart of the two sets of chromosomes. Unfortunately for this hypothesis the linin threads have not been traced from the chromosomes of the first polar spindle to the contact disc; but since the latter structure is never found except in conjunction with a polar spindle, there is no escape from the conclusion that it is in some way related to it.



Figs. 32–35 Meridional sections showing first polar spindle of Cryptobranchus allegheniensis. Figs. 32 to 34 are from ovarian eggs; fig. 35 is from an egg taken from the lower part of the oviduct. × 340.

Fig. 32 *c. w.*, cell wall, formed from the zona radiata. *z. p.*, zona pellucida.

Fig. 33 *c. d.*, contact disc.

Fig. 34 The section cuts the spindle obliquely and includes all the chromatin except one small chromosome belonging to the central part of the group, which is left in an adjacent section. There are probably six large chromosomes forming a ring, surrounding six small chromosomes in a state of division.

Fig. 35 A considerable part of the chromatin is left in an adjacent section. There are probably six large and six small chromosomes, arranged much as in the preceding figure.

Sections afford no explanation of the faint dark spot or shallow depression noted in surface views of the animal pole after the rupture of the germinal vesicle and before the formation of the second polar spindle. An actual depression overlying the first polar spindle is rarely found in sections; if present in the living egg it must be lost through shrinkage of the egg during the process of preparation for sectioning by the paraffin method.

The yolk granules immediately adjacent to the cytoplasm surrounding the spindle are distinctly larger than at the same level elsewhere; they are doubtless brought from a deeper situation by the migration of the nucleus.

The anaphase of the first polar spindle has not been observed, and the first polar body has been found only in a state of degeneration, in conjunction with the second polar spindle.

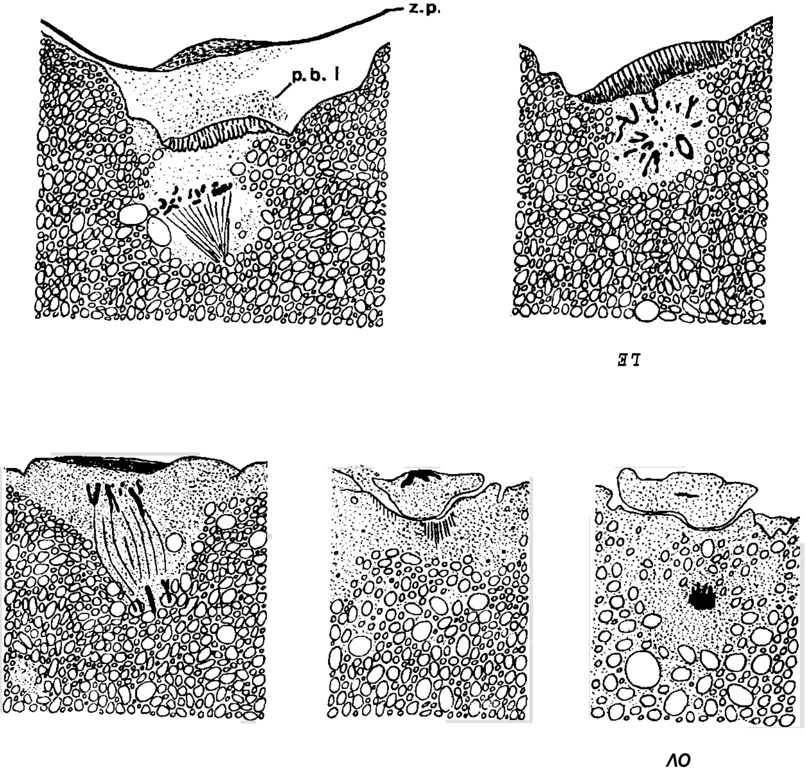
3. The second polar spindle

The second polar spindle (see figs. 36–38) may be distinguished from the first by the smaller amount of chromatin material, and by the fact that a well-defined pit already noted in surface views usually lies above it. This pit sometimes disappears in the late anaphase of the spindle.

The débris of the first polar body is usually found at the bottom or sides of the pit, outside of the cell wall; in some cases fragments of its chromatin are found mingled with the contact disc of the first polar spindle. The chromatin fragments stain but faintly with borax carmine.

The contact disc of the first polar spindle has fused with the thickening of the cuticle which overlies it. In the telophase of the second polar spindle a new contact disc is formed which soon fuses with the old. In some cases linin threads have been clearly traced from chromosomes to the contact disc of the early second cleavage spindle, thereby sustaining the view of the origin of the contact disc set forth in the account of the first cleavage spindle.

No second polar spindle has been found in any eggs of the two females, (*A*) and (*B*), for which the occurrence of the first polar spindle was tabulated. This indicates that the second polar spindle is not formed until after the eggs have been for some time



Figs. 36 to 40 Meridional sections showing the second polar spindle, and the formation of the second polar body and the egg-nucleus in Cryptobranchus allegheniensis. × 340.

Fig. 36 Section through the second polar spindle of an unfertilized egg taken from the uterus of a ripe female. *z. p.*, zona pellucida; *p. b. I*, débris of the degenerating first polar body.

Fig. 37 Section through the second polar spindle of an egg killed 15 minutes after fertilization. The section lies in the plane of the equator of the spindle.

Fig. 38 Late anaphase of the second polar spindle in an egg killed 2½ hours after fertilization. A considerable part of the chromatin is left in an adjacent section.

Figs. 39 and 40 Two consecutive sections through an egg killed 5 hours after fertilization; the first figure shows the second polar body, the second figure shows in addition the newly-formed egg-nucleus.

in the uterus. In the third female (*C*) considered, in which all the eggs were in the uterus, a second polar spindle was found in one out of the three eggs sectioned. This result is sufficient to show that sometimes, if not always, the second polar spindle is formed while the egg is still in the uterus, previous to fertilization; hence the penetration of the egg by the spermatozoon is not required as a stimulus to the formation of the second polar spindle.

The question arises whether the second polar spindle is normally or ever present after the penetration of the egg by the spermatozoon; in other words, do the processes of maturation and fertilization overlap? We must first take into consideration the possibility that eggs dissected from the uterus of a ripe female for purposes of artificial fertilization may not be quite so far advanced as eggs spawned and fertilized in a natural manner. Fortunately it has been possible to check results obtained through artificial fertilization by comparison with a case in which fertilization occurred in a more natural manner. For the study of fertilization three females, (*C*, *D*, and *E*), were principally used; eggs from the gravid uteri of the first two were artificially fertilized in the usual way; the third female spawned with a ripe male while the two were being carried in a pail of water.

Furthermore we must distinguish between what might be called potential fertilization, the mere contact of the seminal fluid with the gelatinous envelopes of the eggs, and actual fertilization, the penetration of the egg proper by the spermatozoon. While the act of fertilization is not consummated until the fusion of the germ-nuclei, the influence of the spermatozoon is felt in many ways as soon as it enters the egg cytoplasm, so that actual fertilization may be said to begin as soon as the spermatozoon pierces the cell wall of the egg. The time record is almost necessarily reckoned from the moment of mixing of the two sexual elements, or potential fertilization; actual fertilization follows after an interval necessary for the passage of the spermatozoon through the gelatinous envelope, which varies for the individual eggs and especially for eggs of different spawnings fertilized by different males, and which can be determined only by a careful microscopical examination of serial sections of each egg.

Out of twenty-one eggs from three females (*C*, *D* and *E*), preserved at intervals extending from fifteen minutes to two and one-half hours after fertilization, a second polar spindle was found in eighteen cases, and one or more spermatozoa were found in each of eleven eggs. The sections show that the spermatozoon may pierce the cell wall of the egg as early as fifteen minutes after contact with the outer envelopes, though a longer time is usually required.

Making allowance for faults of technique we may say that the second polar spindle is usually and probably always present from the time of fertilization up to two and one-half hours later, reckoned from the moment of mixing the sexual elements; there is no essential difference in this respect between eggs artificially and naturally fertilized.

Only early stages of the second polar spindle are found in eggs up to and including one and one-half hours after fertilization; exclusively anaphase stages are found in eggs taken one and three-quarters to two hours after fertilization; the formation of the second polar body and the egg-nucleus (see figs. 39 and 40) is confined to a period between 4 and 8 hours after fertilization. While a stimulus from the spermatozoon is not required to initiate the formation of the second polar spindle, it is evident that the later stages of this mitosis are passed through only after fertilization; in other words, the processes of maturation and fertilization overlap. Hertwig ('06) makes the general statement that in nature the time of fertilization of the amphibian egg falls between the formation of the first and second polar spindles.

4. *The organization of the egg immediately before fertilization*

At the time of spawning the egg is surrounded by the unchanged zona pellucida or vitelline membrane; within this is a thin cell wall, the transformed zona radiata, which is organically connected with the egg.

There are few changes in the general appearance of the blastodisc since the condition described shortly after the rupture of the germinal vesicle (see fig. 31). There is a more intimate incorporation of the materials of the germinal vesicle into the substance of the blastodisc; shreds of non-formative material, such as fragments of the nuclear wall and the fibrous material of the germinal vesicle, are each surrounded by a closely adherent film of cytoplasm and are being absorbed. In eggs ready for fertilization the second polar spindle is sometimes, though perhaps not always, fully formed; it lies beneath a sharply-defined pit at the bottom of which may be found the débris of the first polar body.

The peripheral zone of fine yolk particles in the vegetal hemisphere remains as described in the late ovarian egg.

C. FERTILIZATION

1. *The history of the egg-nucleus*

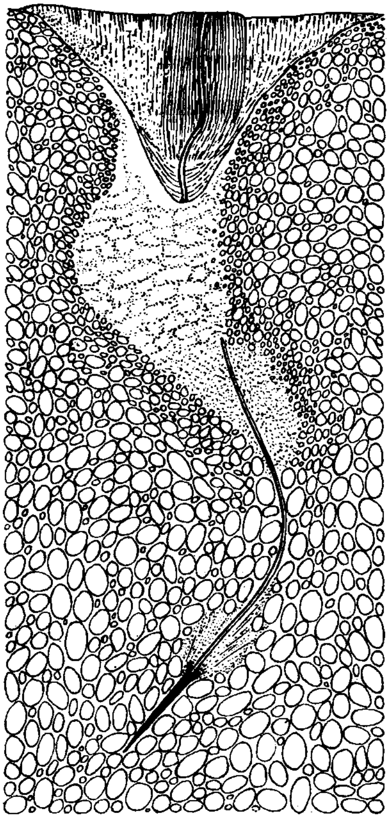
The formation of the egg-nucleus is shown in figures 38 to 40; the process is usually complete about five hours after fertilization. About ten and one-half hours after fertilization (see figs 47 and 48) the egg-nucleus has increased in size and sunk into the blastodisc to a point one-third as far from the surface as the position later occupied by the copulation-nucleus (see fig. 52). A yolk-free region, partly filled with cytoplasm, extends from the egg-nucleus for a short distance toward the surface, indicating the path of migration (fig. 48). At this time the egg-nucleus stains but faintly.

Figs. 41 to 43 Vertical sections of eggs of Cryptobranchus allegheniensis, showing penetration of the egg by the spermatozoon. × 240.

Fig. 41 From an egg killed $2\frac{1}{2}$ hours after fertilization. This figure is a reconstruction from two adjacent sections: the upper half of the figure is drawn from one section, the lower half from the other. The spermatozoon shown in the figure has entered the egg about 50° from the animal pole where the second polar spindle, shown in fig. 38, is in the late anaphase stage. Another spermatozoon in the same condition as the one figured has entered the opposite side of the egg a little below the equator.

Fig. 42 From an egg killed 3 hours after fertilization. The spermatozoon figured has entered the egg a little above the equator. This egg contains in all ten spermatozoa.

Fig. 43 From an egg killed 5 hours after fertilization. The arrow indicates the direction of the path of the spermatozoon which has entered the egg about 30° from the animal pole. The distance from the surface of the egg to the head of the spermatozoon is about one and one half times as great as in the preceding figures. The head of the spermatozoon is shown entire in this section; the tail persists in a somewhat abbreviated condition, but is not shown in the section figured. This egg contains another spermatozoon in the same condition.



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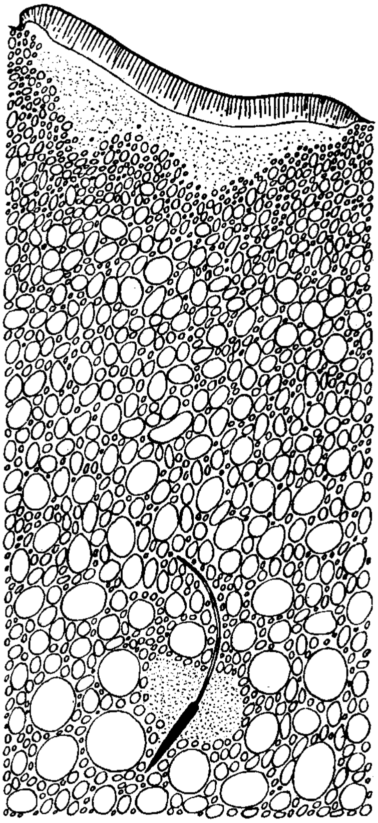

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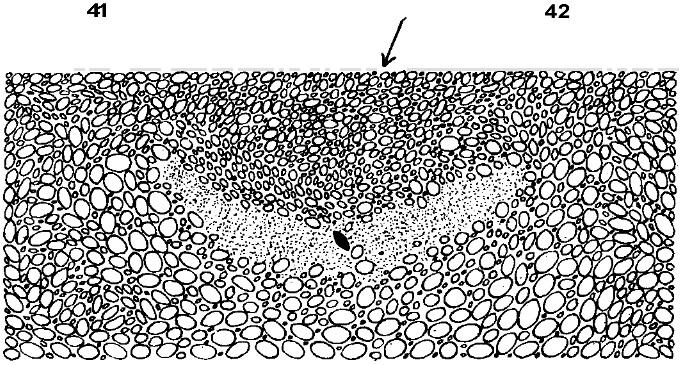


2. *History of the sperm-nucleus*

(*a*). *Penetration of the egg by the spermatozoon.* As previously noted, spermatozoa may be found entering an egg taken as early as fifteen minutes after fertilization. In describing the process, we may best begin with an egg taken about two and one-half hours after fertilization (see fig. 41).

The zona pellucida or vitelline membrane is not affected further than by the formation of a minute perforation which can only rarely be found in sections. The zona pellucida is omitted in the figures.

The cell wall of the egg becomes greatly thickened around the perforation made in it by the spermatozoon. The thickened region is conical in form, with the apex of the cone pointing inward; its outer and central portions are cross-striated. The perforation persists as a conspicuous pore lying in the axis of the cone. The entire structure greatly resembles a micropyle.

Beneath this pseudo-micropyle the path of the spermatozoon is clearly indicated by a yolk-free cytoplasmic region. The form of this region, and the attitude assumed by the spermatozoon itself, indicate that the course pursued by the spermatozoon is a spiral one, with the axis of the spiral lying in a radial direction.

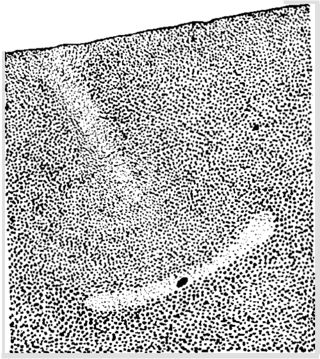
The spermatozoon at this time retains practically its normal form. As in Axolotl (Fick, '93) and Bufo (King, '01), the tail is not left behind at the surface; in Cryptobranchus it continues to serve as an efficient organ of propulsion. The undulating membrane persists, though it is not shown in the figure. The head at this time stains very faintly with the nuclear stain. The acrosome and middle-piece, always difficult to see with the magnification employed for the study of thick serial sections, have not been observed in this situation.

Surrounding the shaft of the spermatozoon for a short distance behind the head there is a spindle-shaped yolk-free region containing cytoplasm. This cytoplasm is particularly dense about the region of the middle-piece; from this locality as a center cytoplasmic strands, resembling linin threads, but finely granular, radiate in all directions, but those extending backward are more prominent. This phenomenon is much more marked in some other cases than in the one figured.

In eggs taken about three hours after fertilization (see fig. 42), the thickening of the cell wall has flattened to the form of a disc; it is strongly striated, recalling the zona radiata from which it takes its ultimate origin. The perforation made by the spermatozoon has disappeared. The cytoplasmic path of the spermatozoon has become filled with yolk, except for a broad shallow region underlying the thickening of the cell wall. The head of the spermatozoon has become shorter and thicker, and takes brilliantly the nuclear stain; the tail has become slightly shorter, perhaps by the degeneration of the posterior portion. The radiations of cytoplasm proceeding from the region of the middle-piece have disappeared, but in the same locality there is a somewhat larger spherical region of uniformly distributed yolk-free cytoplasm.

Five hours after fertilization (see fig. 43), the spermatozoon has penetrated only a little deeper into the egg. The thickening of the cell wall of the egg at the place of entrance of the spermatozoon has disappeared, but its site is marked by convolutions in the cell wall. The protoplasmic path leading from the surface of the egg to the spermatozoon has almost entirely disappeared, but traces of it persist at intervals along the route. The head of the spermatozoon is spindle-shaped and much shorter and thicker than before; the tail persists, but is somewhat abbreviated. The circular area of cytoplasm surrounding the head of the spermatozoon has expanded to form a large crescent, whose horns extend nearly at right angles to the path of the spermatozoon. The yolk granules underlying the crescent are decidedly coarser than those above it. This suggests a correlation of the internal structure with the 'sperm area' seen from the surface: the horns of the crescent produced would meet the margin of the sperm area (compare figs. 9 to 11 with figs 43 to 45).

Seven and one-half hours after fertilization (see fig. 44) the protoplasmic path is marked only by a region of sparsely distributed yolk granules extending from the surface for about two-thirds of the distance to the spermatozoon. The yolk granules are particularly dense in the region immediately above the crescent, and



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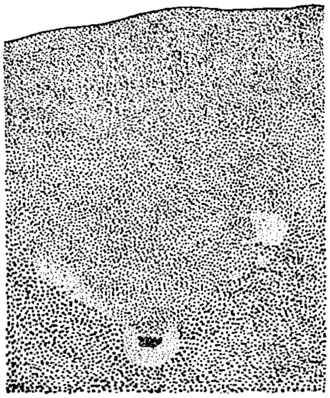

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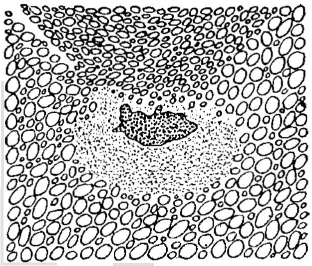

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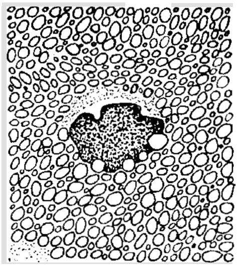

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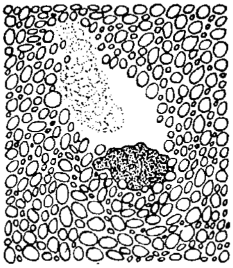

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Fig. 44 Vertical section through an egg of Cryptobranchus alleghenìensis killed 7½ hours after fertilization, showing a late stage in the penetration by the spermatozoon. × 80. This spermatozoon has entered the egg about 30° from the animal pole, and its path inclines toward the axis of polarity of the egg. The head of the spermatozoon is shown entire; the tail persists in an abbreviated and perhaps fragmented condition, but does not appear in the section figured. An aster is present in an adjacent section at a little higher level than the sperm head. Another spermatozoon in the same general condition is found in the same egg.

Fig. 45 Vertical section through an egg killed 10½ hours after fertilization, showing the sperm-nucleus. × 80. The spermatozoon has entered the egg about 25° from the animal pole. Fragments of the tail of the spermatozoon are to be found in the vicinity of the sperm-nucleus, but are not shown in this section.

Fig. 46 A portion of fig. 45 enlarged to show the sperm-nucleus. × 240.

Figs. 47 and 48. Two consecutive meridional sections through an egg of Cryptobranchus alleghenìensis, killed 10½ hours after fertilization, showing the egg-nucleus. This nucleus is situated about one-third as far from the surface as the copulation-nucleus shown in fig. 52. × 240.

are here finer than elsewhere at the same level. The crescent has become larger, and thicker at the ends than in the middle. The head of the spermatozoon is shortened to the form of a thick spindle and stains deeply; the tail persists in an abbreviated condition. In the case of the spermatozoon shown in figure 44, an aster is found at a slightly higher level than the sperm head and a little nearer to the egg-nucleus. A study of the protoplasmic paths of the aster and the sperm-head shows that they have separated at a point midway in the path of the latter.

Ten and one-half hours after fertilization (see figs. 45 and 46) the spermatozoon has become transformed into the sperm-nucleus, which is amoeboid in form; the tail of the spermatozoon is represented only by fragments. At this time the sperm-nucleus lies about half as far from the surface as the copulation-nucleus shown in fig. 52. Immediately beneath the sperm-nucleus lies a considerable mass of cytoplasm, perhaps formed at the expense of the crescent which is dwindling except at the extreme ends. The remains of the crescent, and the characteristic appearance of the surrounding yolk, enable one readily to distinguish the sperm-nucleus from the egg-nucleus. The sperm-nucleus is smaller than the egg-nucleus, and like the latter does not stain deeply at this time.

The cytoplasmic changes in the egg caused by the invasion of the spermatozoon may be tentatively interpreted as follows: Under the influence of the centrosome, whose seat appears in this case to be in the middle-piece, egg-cytoplasm collects about the neck of the spermatozoon. Here the centrosphere and eventually the entire aster is formed. As the spermatozoon invades the deeper region of coarser yolk particles, the resistance offered to the progress of the accompanying mass of cytoplasm causes it to flatten out like a bullet fired against a wall, assuming a form crescent-shaped in section. Presently the spermatozoon, during its transformation into the sperm-nucleus, comes almost to a full stop, allowing the mass of cytoplasm again to assume a spherical form.

Numerous observers have described in both invertebrates and vertebrates a rotation of the sperm head after it enters the egg, whereby the middle-piece is brought into position to precede during the further process of migration. Fick ('93) has described this process in Axolotl, and Dean ('06) has noted it in Chimaera. King ('01) found no indication of a rotation of the sperm head in Bufo; possibly this condition is correlated with the fact that in Bufo the centrosome appears to be located, not in the middle-piece, but in the head of the spermatozoon. In Cryptobranchus rotation of the sperm head apparently takes place at a rather late stage in the process of transformation into the sperm-nucleus. In the stages shown in figures 43 and 44, the greatly shortened sperm head is usually placed with its long axis oblique or at right angles to its former path, so that one end points toward the egg nucleus. But in these stages it has not been possible to trace any connection between the tail of the spermatozoon and its head, and since the aster has already separated from the sperm head, in no case can it be stated which end of the sperm head is the one pointed toward the egg-nucleus.

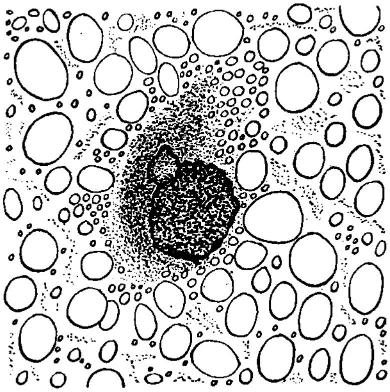
The spermatozoon ordinarily enters the blastodisc in a more or less centripetal direction, and continues in this direction for a considerable distance; sometimes its path inclines almost from the beginning in an oblique direction toward the point of future union with the egg-nucleus. In either case the axis of the spiral path is ordinarily straight up to the time of the transformation of the head of the spermatozoon into the sperm nucleus; the later course of migration has not been followed. In an egg preserved an hour and three-quarters after fertilization, a spermatozoon, which had entered the blastodisc unusually near the animal pole, described a path which proceeded in a centripetal direction only a very short distance, then curved sharply in a direction parallel to the surface, toward the second polar spindle which was in the late anaphase condition. The form of the spermatozoon remained unaltered, and rotation of the sperm head had not commenced. This case is instructive in showing that the factors tending to bring the germ-nuclei together are active at a very early stage of fertilization: the egg-nucleus was not fully formed, and the spermatozoon had not begun its process of transformation into the sperm-nucleus. Moreover it is evident that in this case at least the 'copulation path' (see Hertwig, '06, p. 529) is not dependent upon the rotation of the sperm head.

(*b*). *Polyspermy, and the fate of the supernumerary spermatozoa.* Brief data regarding the occurrence of polyspermy have already been given. It is possible that the method of artificial fertilization increases the number of spermatozoa entering the egg; but in nature the eggs are fertilized in a confined space, and I see no reason to doubt that polyspermy is a common occurrence under natural as well as artificial conditions. It is evident that we have here to deal with physiological, not induced or accidental polyspermy (see Brachet, '10), for the eggs develop in a normal manner.

While the distribution of spermatozoa entering the egg is largely if not entirely a matter of chance, the location in which a spermatozoon finds itself has much to do with its ultimate fate. Spermatozoa entering the lower hemisphere, especially in the region of the vegetal pole, never penetrate far, and since they are found in this hemisphere only during the first few hours after fertilization, must quickly degenerate. In the urodele Hynobius, Kunitomo ('10) found that a spermatozoon entering at the vegetal pole sometimes succeeds in reaching the egg-nucleus; but the careful study of many eggs has convinced me that this never occurs in the heavily yolk-laden and strongly telolecithal egg of Cryptobranchus.

Only in the blastodisc have spermatozoa been found in the stage characterized by the presence of the cytoplasmic crescent (figs. 43 and 44). Obviously, the conditions elsewhere are unfavorable for the formation of any considerable mass of cytoplasm about the spermatozoon. In the stage with a well-formed cytoplasmic crescent, not more than two spermatozoa have been found in a single egg. No accessory spermatozoa have been found in any situation after the formation of a sperm-nucleus. The supernumerary spermatozoa thus have but a transient existence, and the only advantage resulting from polyspermy is doubtless that, in an egg so large, penetration by several spermatozoa is of value in insuring fertilization.

The literature on the occurrence of polyspermy in the amphibian egg has recently been reviewed by Kunitomo ('10). As noted in a previous paper (Smith, '11), I have found polyspermy occurring in eggs of Amblystoma tigrinum fertilized under natural conditions; the material secured for the further investigation of this subject has not yet been worked up. Polyspermy seems to be characteristic of heavily yolk-laden eggs lacking a preformed micropyle.



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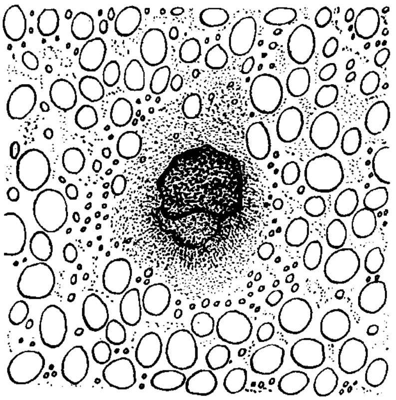


50.

Fig. 49 Meridional section through an egg of Cryptobranchus alleghenisis, killed 12 hours after fertilization, showing fusion of germ-nuclei. The sperm-nucleus is probably the smaller one. × 240.

Fig. 50 Meridional section showing fusion of germ-nuclei in another egg killed 12 hours after fertilization. The sperm-nucleus is probably the lower and smaller one. × 240. For the position of this copulation-nucleus in the blastodisc see fig. 52 which is drawn from the same section.

3. *Union of the germ-nuclei, and formation of the first cleavage spindle*

In two eggs killed twelve hours after fertilization, the germ-nuclei have been found in the process of uniting (figs. 49 and 50); in these two cases the copulation-nuclei are at approximately the same distance from the surface (see fig. 52), quite deeply situated in the blastodisc and a little to one side of the axis of polarity. The smaller germ-nucleus is probably the sperm-nucleus. The egg-nucleus stains brilliantly with borax-carmine; the sperm-nucleus takes the stain less deeply. The sperm-nucleus especially is surrounded with dense cytoplasm; in one case (fig. 50) this exhibits a tendency toward radial striation and probably represents the aster.

The study of the paths of migration of the germ-nuclei and the copulation-nucleus is not quite complete, but indicates that the germ-nuclei come together at a higher level than that occupied by the copulation-nuclei shown in the figures.

The first segmentation nucleus in a resting condition has been found in an egg killed eighteen hours after fertilization; the first cleavage spindle has been found in an egg killed seventeen hours after fertilization.

4. Changes in the blastodisc

In eggs taken from fifteen minutes to ten and one-half hours after fertilization, cytoplasm is accumulating in irregular patches underlying the animal pole (fig. 51). During this period, practically all traces of the débris of the germinal vesicle disappear. In places, the surface of the blastodisc is sometimes very irregular almost villous; this may be due to injuries resulting from the actual or attempted entrance of spermatozoa.

In eggs taken from twelve to eighteen hours after fertilization (copulation nucleus to first cleavage spindle) the cytoplasm is gathering in a broken layer close to the surface of the blastodisc. The beginning of this process is shown in figure 52. In Hynobius, Kunitomo ('10) has noted a somewhat similar condition. During the latter part of the period considered the layer of cytoplasm becomes much thicker than is shown in the figure, but retains its segmented character.

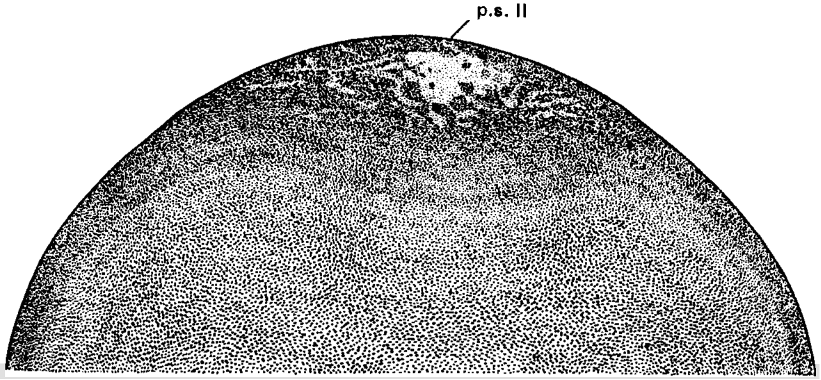
During the first two hours after fertilization there is a marked increase in the thickness and extent of the blastodisc as a whole (see especially fig. 51). Evidently the greater part of this change takes place before the egg has become oriented with the animal pole uppermost, hence it is independent of any possible sorting effect of gravity acting on the materials of the egg.

No marked changes have occurred in the lower hemisphere since the egg left the ovary.

The later changes in the blastodisc lead up to first cleavage and will be considered in that connection.

D. SUMMARY

The follicular layer proper of the ovarian egg of Cryptobranchus is formed from some of the deeper non-germinal cells of the ovarian wall which resemble the epithelial cells of the outer and inner limiting membranes. The follicular membrane proper completely surrounds the egg and is suspended in a two-layered flask-shaped sac which projects from the inner surface of the wall of the ovary



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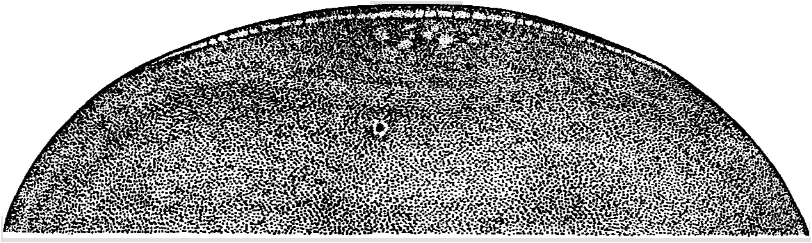

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Fig. 51 Meridional section through an egg of Cryptobranchus alleghenicnsis, killed 1¾ hours after fertilization, showing the condition of the blastodisc. The irregular faintly stippled areas near the animal pole contain yolk-free cytoplasm. × 18. *p. s. II*, second polar spindle.

Fig. 52 Meridional section through an egg of Cryptobranchus alleghenicnsis, killed 12 hours after fertilization, showing condition of the blastodisc and position of the copulation-nucleus. Yolk-free cytoplasm is segregated in a broken layer near the surface of the blastodisc. The copulation-nucleus is shown a little to the left of the center of the figure. × 18.

into the central cavity; in a broad sense, the entire three-layered structure may be called the follicle.

The zona radiata is formed from the peripheral substance of the egg proper; at the time of the rupture of the germinal vesicle it becomes transformed into a simple cell wall, in organic connection with the egg.

The zona pellucida is formed as a secretory product of the follicular layer proper; it persists unchanged as the 'vitelline membrane' of the embryo.

The earliest observed phenomena which may perhaps indicate polarity occur in the ovarian eggs of young females of a body length of 26 to 30 cm., as a shifting of the region of most abundant vitelline bodies from the future vegetal to the future animal hemisphere. In the ovarian eggs of young females of a body length of 35 cm there is a concentration of nucleoli on the side of the germinal vesicle toward the future animal pole; this may perhaps afford a second indication of polarity.

Yolk-formation begins in the most advanced ovocytes of young females with a body length of 35 cm.; the yolk is first laid down in concentric zones. With respect to the position of the germinal vesicle, the distribution of cytoplasm, and the size of the yolk particles in the different zones, the egg exhibits radial symmetry until after it is nearly or quite filled with yolk.

About the time when the egg becomes completely filled with yolk, the germinal vesicle migrates from its central position toward a point on the surface which is thus defined as the animal pole. Coincident with the migration of the germinal vesicle, axial differentiation of the cytoplasmic and yolk contents of the egg lead to the formation of a germinal disc in the region of the animal pole.

In general the animal pole of the egg lies within the stalk of the follicle and toward the periphery of the ovary.

In the late ovarian egg a structure called the yolk cup is interpreted as the physiological equivalent of the concentric layers of dense fine yolk found in the eggs of birds and various other vertebrates.

Shortly before maturation the germinal disc is temporarily differentiated into two layers: a thin peripheral layer of yolk-free cytoplasm, and underlying this a thicker layer of especially fine yolk particles rich in cytoplasm. Both layers are continuous with much thinner layers of the same character surrounding the remainder of the egg.

In the ovocyte ready for maturation, the germinal vesicle lies close to the surface at the animal pole, and is surrounded by the germinal disc. A mass of cytoplasm has accumulated beneath the germinal vesicle during the later stages of its migration. The arrangement of materials is now quite strongly telolecithal.

Shortly before the rupture of its wall, the germinal vesicle appears at the very surface at the animal pole. The rupture of the germinal vesicle takes place just before the egg leaves the ovary; the cytoplasmic and yolk layers of the blastodisc now mingle, and the materials of the germinal vesicle, together with the cytoplasm brought with it from the interior of the egg, are incorporated into the blastodisc.

Absorption of degenerating ovocytes is accomplished by means of the follicle cells, which reverse their usual rôle as nurse cells of the egg, and function as phagocytes.

The first polar spindle is formed about the time the egg leaves the ovary, and disappears about the time the egg enters the uterus. There are marked size differences in the chromosomes.

The second polar spindle is formed shortly after the egg enters the uterus; it lies beneath a deep pit readily visible from the surface.

The penetration of the egg by the spermatozoon is not required as a stimulus to the formation of the second polar spindle.

The late stages of the second maturation division, culminating in the formation of the second polar body and the egg-nucleus, are passed through only after the spermatozoon has entered the egg; in other words, the processes of maturation and fertilization overlap.

A structure resembling a micropyle is formed in the cell wall of the egg around the perforation made by the entrance of the spermatozoon. The influence of the entering spermatozoon upon the egg is shown by characteristic changes in the distribution of the yolk and cytoplasm.

Physiological polyspermy is of normal occurrence. The supernumerary spermatozoa lead but a transient existence.

Union of the germ-nuclei takes place at a point deeply situated near the center of the blastodisc, and is followed by the segregation of masses of cytoplasm forming a broken layer near its surface.

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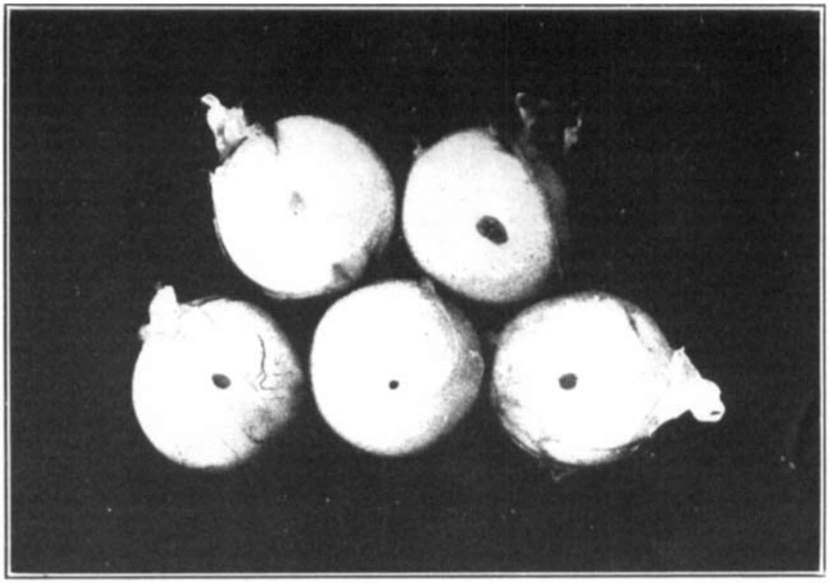
PLATE 1

EXPLANATION OF FIGURES

53 Cryptobranchus allegheniensis. Living eggs dissected from the ovary, showing the germinal vesicle at the surface. The small spots within the germinal vesicle are probably nucleoli. Ovarian membranes containing blood-vessels wholly or partially cover the eggs. × 4.

54 **Cryptobranchus allegheniensis.** Unfertilized eggs with their gelatinous envelopes, after two days' immersion in water. Natural size.



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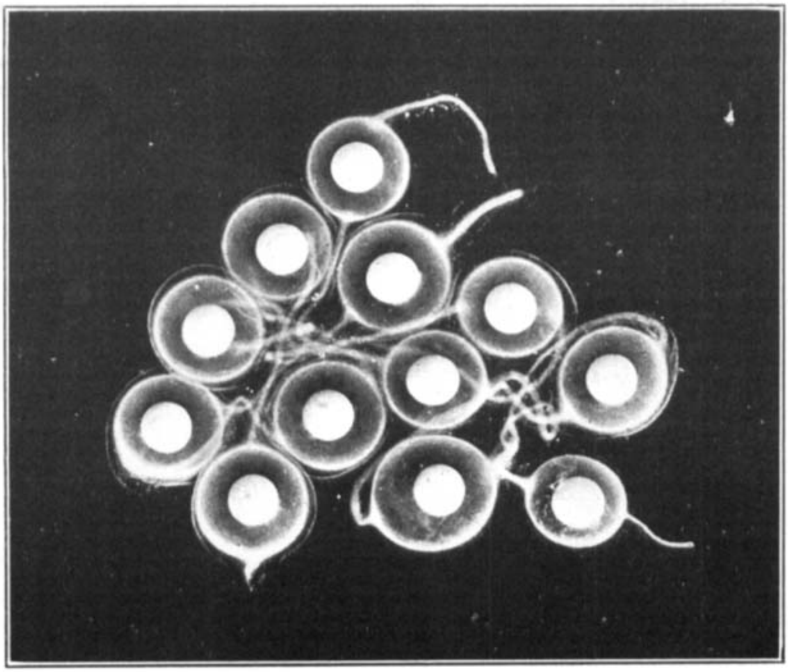

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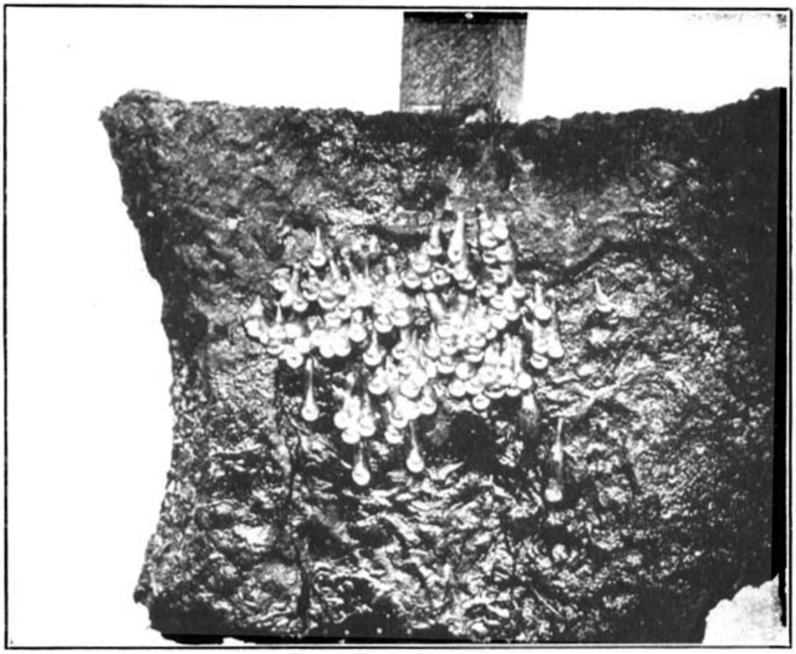
PLATE 2

EXPLANATION OF FIGURES

55 Necturus maculosus. 'Nest' of eggs of Necturus. The stone to which the eggs are attached has been removed from the water and set on edge on the wharf; it is about 16 inches in diameter. The embryos are in an advanced stage of development.

56 Necturus maculosus. Eggs and envelopes shown in their natural position in the water, attached to a piece of board; natural size. From a drawing by Professor Bashford Dean.



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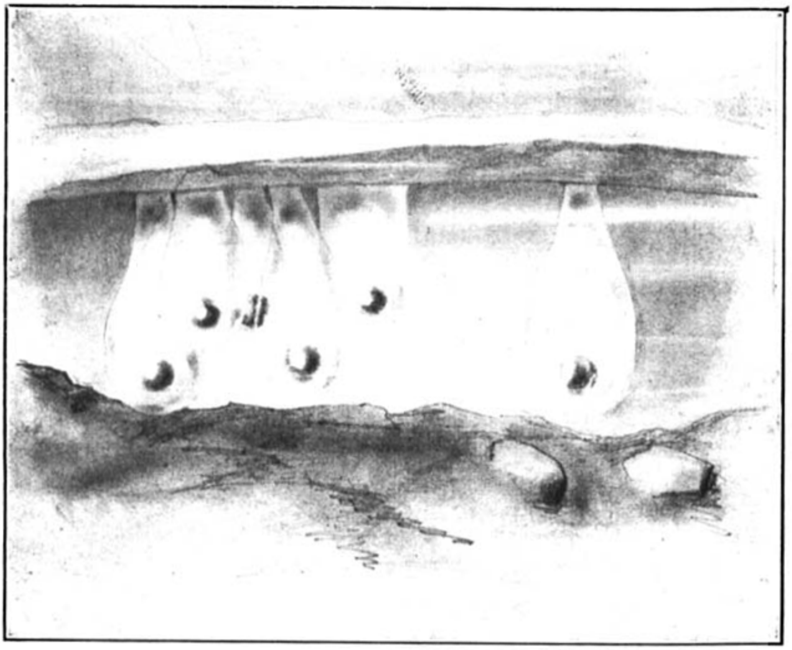

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