A Study of Early Development in Cumingia with Special Reference to Cytology.—By Margaret Elizabeth MacKay, B. A., M. A., Assistant in Histology and Embryology, Dalhousie University, Halifax, N. S.

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The purpose of this paper is a description of the changes which take place in the egg of *Cumingia* (Sowerby, 1833) from the primary oöcyte, through maturation, fertilization and segmentation up to the four celled stage. A cytological study of this egg was made by Jordan in 1910, in which particular attention was paid to the history of the chromosomes and the centrosomes. Since the publication of Jordan's paper, a great many workers have used the *Cumingia* egg for experimental purposes, but, in practically every instance the experimental work has not been checked up by cytological evidence. In order that future investigators working with this material experimentally, may have a clearer conception of the early development stages of the normal egg, the present paper was written with the idea of repeating Jordan's work and in addition, making a cytological analysis of fertilization.

The material was collected by Professor R. J. Bean at the Marine Biological Laboratory, Woods Hole, Mass., in the summer of 1923. The eggs were fixed in Bouin's fluid, sections cut 5μ in thickness, and stained by the iron-alum haematoxylin method. Starting with sections of ovary, the series contains the following material:

Eggs collected at the moment of shedding.

Eggs taken from sea water from 10-30 minutes after shedding.

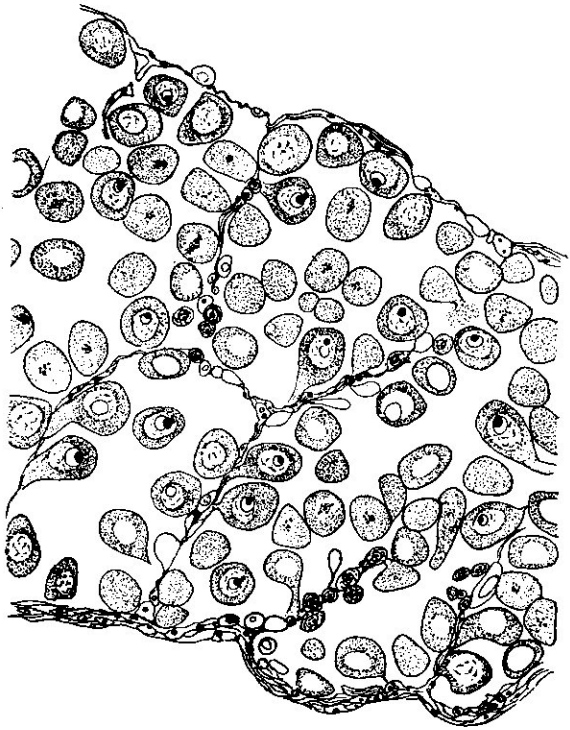
Eggs allowed to grow stale in sea water from 3-4 hours.

Inseminated eggs fixed 5 sec., 10 sec., 15 sec., 30 sec., 45 sec., respectively after exposure to a sperm suspension.

Inseminated eggs fixed at intervals one minute apart, from 60 secs. to 1 hour and forty-five minutes.

Acknowledgement is hereby made to Professor Bean who prepared most of the slides and whose advice has been very valuable in the preparation of this paper.

The ovaries of Cumingia are symmetrical, paired structures which occupy the posterior portion of the visceral mass (Fig. 1).



Ovaries with eggs in different stages from the primary oöcytes to the mesophase of the first maturation division.

[Figs. 2 to 14 at the end.]

Each is covered with a capsule from which trabeculae extend dividing the organ into acini. The latter are filled with eggs in varying stages of development, from the primary oöcytes to the mesophase of the first maturation division. The ova, which develop from cells in the ovarian epithelium, are irregular in shape, due partly to pressure conditions within the ovary. The larger ovals are coated with jelly and rest attached to the

ovarian wall at one point. This point of attachment represents the vegetal pole of the egg. Polarity is further marked by the tendency of the nucleus and nucleolus to take an eccentric position near the free surface of the egg, thereby establishing a true animal pole.

No signs of oögonial division could be found in the ovaries, though primary oöcytes were abundant. These oöcytes are spherical in shape and consist largely of nucleus, in the centre of which is an irregular mass of chromatin surrounded by a light network. As growth takes place the cytoplasm increases in volume more rapidly than the nucleus, becoming coarsely reticular and showing small black granules. Within the nucleus is a spherical nucleolus surrounded by several chromatic masses. When the oöcyte reaches a size about one half that of the full grown egg, the cytoplasm becomes finely granular, and the chromatic masses in the nucleus aggregate into an irregularly shaped body which is situated close to the nucleolus and lies between it and the nuclear membrane.

At a somewhat later stage in development, large spherules which stain very deeply with haematoxylin, appear scattered throughout the granular cytoplasm. The nucleus is large and well defined, showing a light achromatic reticulum and enclosing both the spherical nucleolus and the irregular outlined chromatin knot. The latter, now lies in contact with the nuclear membrane as well as the achromatic capsule of the nucleolus (Fig. 2). The nucleolar capsule presents a most striking figure at this stage of development. It is nearly twice the diameter of the nucleolus and because of this fact, the nucleolus assumes an eccentric position within its capsule.

Shortly before the breaking down of the germinal vesicle, the nucleolus is cast out bodily into the nucleus by rupture of the surrounding envelope (Fig. 3). This capsule disappears shortly after the nucleus breaks down and its further history cannot be traced. The nucleolus, spherical in shape and slightly decreased in size, retains its identity after the chromosomes are formed, though it is gradually absorbed by the cytoplasm and disappears before the anaphase (Fig. 4). This may indicate partial contribution of nucleolar substance to

the chromosomes as Jordan, 1910, suggests. The chromatin body from the nucleus breaks down immediately upon rupture of the germinal vesicle and its fragments appear to enter the spindle. This is in accordance with Jordan who found what he considered as positive evidence, that this body was distributed to the chromosomes.

The changes subsequent to the breaking down of the germinal vesicle take place rapidly. The out-flowing of the more fluid nuclear sap into the cytoplasm is followed by the immediate appearance of the chromosomes. The centrosome arises simultaneously with the chromosomes and the asters for the first maturation spindle lie near the centre of the egg. Each centrosome has a large, deep staining centriole, surrounded by a paler centrosphere (Fig. 5). As the spindle undergoes mitotic changes for the metaphase, the centriole gradually becomes smaller and the centrosphere increases in size until finally the whole centrosome is achromatic and oval in shape (Fig. 6). It is very probable that the centriole is changed into the pale staining centrosphere. Jordan (1910) describes a fragmentation of the centriole but such fragmentation was not observed in this investigation. Lillie in 1898 found a similar case in *Unio* in which he expresses an opinion that the centrosome granules are transformed into the substance of the centrosphere. The black granule or centriole does not reappear in any of the following mitotic figures, although centrosomes are indiscernable during each intermitosis and appear anew with each mitosis.

Coincident with shedding, the irregularly shaped ovarian egg becomes spherical, though in many cases there is a conspicuous evagination of cytoplasm in the region of the vegetal pole (Fig. 5). This protuberance marks the point of attachment of the egg to the ovarian wall and disappears about 30 seconds after the eggs are cast into sea water (Fig. 6.) A very thin membrane which is barely visible under oil immersion encloses the egg, and no cytological evidence for the discontinuity of this membrane at the vegetal pole could be found. Hielbrunn, 1915, was also able to demonstrate the existence of such a membrane in Cumingia by using micro-dis-

section methods. At the time of shedding, the first maturation spindle is in the mesophase of first polar body formation; but no polar bodies are given off unless the egg is activated parthenogenetically or fertilized with a spermatozoön. Exposure to parthenogenic agents or to a sperm suspension will cause maturation to proceed, though no morphological changes are visible for two or three minutes.

Sperm Penetration.

Sperm penetration is very rapid in Cumingia and the initial processes are obscure. About three minutes after insemination there appears in the vegetal pole of the egg a small cone of cytoplasm. This is one of the first morphological evidences of fertilization and the sperm may often be seen embedded in the jelly on the tip of this fertilization cone. The sperm shortly penetrates the vitelline membrane and comes to lie within the cytoplasmic cone (Fig. 7). As the spermatozoön enters the cone its long axis is parallel to the axis of the cone, but it soon rotates through an angle of 90 degrees. As the spermatozoön becomes withdrawn from the fertilization cone its identity is temporarily lost because of the profusion of black cytoplasmic spherules which lie in the cortex of the egg. Therefore it was impossible to determine whether or not further rotation occurred. The work of Lillie, 1912; Meves, 1912-14; Boveri, 1898; and Wilson, 1896; seems to indicate that a rotation of 180 degrees is a more or less general phenomenon, and it is very probable that Cumingia does not differ in this respect from the majority of other forms. Since the tail of the spermatozoön could not be found attached to the fertilization cone, it is assumed that the entire spermatozoön enters the egg even though the tail as such could not be distinguished.

The position of the fertilization cone is always in the vegetal hemisphere and with a few exceptions is directly below the central pole of the spindle, opposite the region where the polar bodies are given off. Its location corresponds to the point of attachment of the egg to the wall of the ovary and also

to the protuberance of the cytoplasm that appears after shedding. Though no micropile could be seen in this egg, it is certain that there is a restricted vulnerable area in the membrane, where the sperm enters, which is at the vegetal pole, and marks its point of attachment to the ovary. This view is permissible because in other Lamellibranchs where a micropile definitely exists, its position is approximately the same as the vulnerable area in Cumingia.

While the above process is taking place the vitelline membrane also undergoes certain changes. Occassionally after penetration of the sperm a tiny ragged marking remains in the jelly layer and vitelline membrane which indicates the point of sperm penetration, and in such cases a small quantity of cytoplasm flows out and the exudate together with the fertilization cone gradually flattens out and disappears. Before fertilization the membrane is almost indistinguishable, but after the entrance of the sperm it increases slightly in thickness and becomes clear and very slightly serrated (Fig. 6 and Fig. 7). No appreciable membrane elevation occurs under normal conditions, but Hielbrunn, 1915, demonstrated it experimentally by exposure of the egg to varying salt solutions.

The sperm, following penetration, undergoes certain definite changes in position and form. After its entrance into the egg, being temporarily obscured by the black cytoplasmic spherules, it appears between 5 and 6 minutes after fertilization as a spherical black chromatic mass slightly to one side of its point of entrance and close to the periphery of the egg (Fig. 8). It leaves no visible path in the cytoplasm but during maturation it changes its position with reference to the spindle, gradually moving toward the animal pole but remaining the same distance from the periphery of the egg. It usually comes to rest opposite the lower pole of the maturation spindle about ten minutes after fertilization, when the latter is in the anaphase of the first polar body.

During its migration the sperm undergoes the following morphological changes. Six minutes after penetration it appears considerably enlarged as a deep staining spherical body surrounded by a very narrow clear colorless zone of cytoplasm.

Two or three minutes later a new zone, pale grey in color, appears between the sperm and the clear zone of cytoplasm (Fig. 9). From this time up to a stage about 12 minutes after fertilization the greyish zone continually increases in width, while the deeply stained central portion diminishes until it is no longer distinguishable. From this stage on until the second polar body is formed the sperm may be seen at rest as a large grey spherule still surrounded by a zone of clear cytoplasm.

Following fertilization the spindle, which was resting in the mesophase, proceeds to maturation. The first noticeable change is a widening of the spindle and the arrangement of the chromosomes, thirty-six in number, in an equatorial plate. The form of the spindle changes distinctly upon insemination. Previous to insemination it is elongate and narrow and if the eggs are allowed to stale this condition becomes more and more pronounced. Within a very few seconds after insemination it becomes appreciably widened. Indeed the anaphase of the first polar body formation is quite definitely indicated before penetration of the sperm can be observed. It could not be determined whether the first polar division was reductional or equational. The polar bodies are given off at the chief axis of the egg, the elevation of the vitelline membrane is first apparent about six minutes after, though the first polar body is not actually formed for eleven or twelve minutes (Fig. 9). Eighteen chromosomes pass out in the first division and a surface view of the polar body shows them arranged radially in a ring. Those left behind, together with the centrosome, move up and lie under the first polar body.

The centrosome does not disappear during the formation of the polar bodies; but, like the chromosomes, retains its identity throughout the process of maturation, appearing as a pale central granule in the aster. The centrosome of the distal pole of the spindle passes out in the first polar body, while that left in the egg divides to form two asters and the second spindle. The axis of this second spindle is at first at right angles to that of the first spindle, but as it elongates a rotation of 90 degrees takes place. The two polar bodies remain attached to the egg for some time and may be seen through the early cleavages.

In the late telophase of the second maturation spindle, the chromosomes of the central pole become broken up, prior to forming the chromosomal vesicles (Fig. 11). There are several of these chromosomal vesicles, and Jordan suggests that there is one of the latter for each chromosome. These fuse later to form the kidney shaped female pronucleus.

Maturation is completed about 28 minutes after fertilization, although membrane elevation for the second polar body may be observed at a much earlier stage. Changes in the resting sperm closely follow the termination of maturation. It becomes larger and elongates until it assumes an arrow-head shape with the apex directed toward the center of the egg. At this stage the sperm changes its position moving a short distance toward the vegetal pole, leaving behind it a clear space which marks its former position. This clear vesicle in the cytoplasm only persists for a few moments and shortly becomes filled in with cytoplasm. About 30 minutes after fertilization, astral rays appear around the point of the arrow-head shaped sperm (Fig. 10). This is undoubtedly a typical sperm aster and may be seen actually attached to the sperm. This observation is in direct variance with the findings of Jordan who states that no sperm aster exists. At this stage the sperm shows a light chromatic network and is apparently a vesicular structure (Fig. 10). Three or four minutes later the arrow-head outline of the sperm is lost and it becomes first oval in shape and finally spherical.

About 33 minutes after fertilization both the male and female pronuclei are fully formed. They are spherical in shape and of the same size so that one cannot be distinguished from the other. Each shows a well defined nuclear membrane which encloses the delicately reticulated nuclear contents. As they approach the common meeting point, the fine reticulum of both appears to be knotted or beaded with chromatin material (Fig. 12). Just before the actual meeting of the pronuclei the reticulum breaks down and chromosal bodies are formed. At about the 38 minute stage the nuclear membranes come into contact with each other, fuse, and then break down along the line of fusion.

The origin of the asters for the first segmentation is somewhat doubtful. There is unquestionably a sperm aster, but, the central aster of the second maturation division also remains visible, near the female pronucleus, up until the time of fusion of the pronuclei. There is no evidence that either of these asters divide, neither do they disappear until after the anaphase of the first segmentation division. Therefore, it is probable that both asters are concerned with the formation of the first segmentation spindle.

The chromosomes which are formed from the pronuclei are much different in form from those observed in the early maturation stages. Each appears as a slender filament with a knot at one end. Upon fusion of the pronuclei, they become curled up in such a way as to resemble tiny signet rings. During the late anaphase of the first segmentation division they become compact and the shape of individual chromosomes varies considerably.

The axis of the first divisional spindle is at right angles to the polar axis of the egg and slightly eccentric so that the resulting cells are of definite size (Fig. 13). The nuclei of these cells resemble the male and female pronuclei, as they are oval in shape with no light chromatin network and no nucleolus or centrosome (Fig. 14). The latter arising suddenly with the spindles and chromosomes appears as a pale central granule of the aster. The plane of the second division is at right a ıgles to that of the first and is in line with the polar axis of the egg, separating it into one large and three small cells. The asters and chromosomes are very similar to those of the first divisional spindle.

Summary.

1. The ovaries of *Cumingia* during the breeding season, contain eggs in all stages of development from the primary oöcyte to the mesophase of the first polar body information.

2. The ovarian eggs are attached by the vegetal pole, the animal pole being free and containing the nucleus and nucleolus.

3. The eggs are shed in the mesophase of the first polar body formation, but the first polar body is not given off unless the eggs be inseminated or activated artificially.

4. The first morphological signs of fertilization are the appearance of a fertilization cone and a widening of the maturation spindle.

5. There is but one vulnerable area for sperm penetration in Cumingia, and this area lies at the vegetal pole. There is no well defined micropyle.

6. The vitelline membrane is very thin and does not become appreciably elevated until the first polar body is given off.

7. Sixteen chromosomes pass out in the first polar body and sixteen in the second.

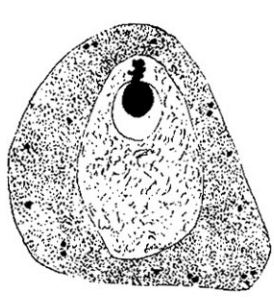
8. The sperm lies quiescent during the maturation divisions, after having migrated from the fertilization cones at the vegetal pole, to a position level with the equatorial plate of the first maturation spindle.

9. A large sperm aster is formed, but, the aster of the central polar spindle also remains visible and possible functions with the first segmentation spindle.

10. The position of the first segmentation spindle is such that the first segmentation is unequal and the resulting cells unequal in size.

11. It must be recognized that the time at which different morphological changes occur is variable for different batches of eggs, also, the speed at which any egg develops will depend a great deal upon the temperature of the water in which the egg is developing. On a very warm day development is much more rapid than on a cool day.

Description of Drawings.



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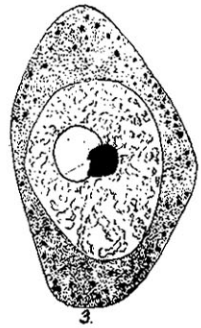
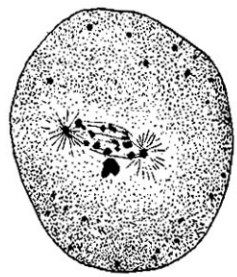


3.

2. Egg with intact germinal vesicle containing nucleolus in capsule with the chromatin knot outside.

3. Egg showing rupture of the nucleolar capsule.



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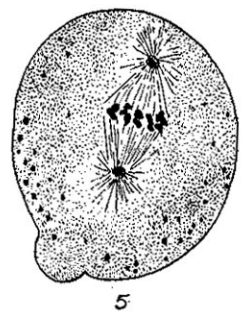

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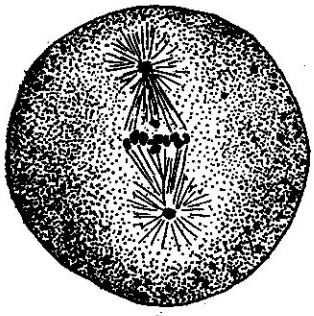
4. Egg after rupture of germinal vesicle showing formation of the spindle.

5. Egg immediately after shedding showing protuberance at the vegetal pole.



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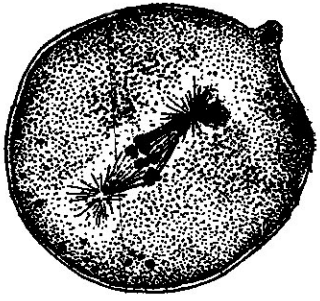

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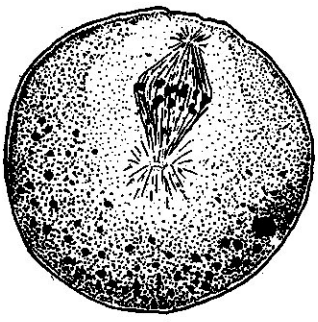
6. Egg thirty seconds after shedding, protuberance has disappeared and the centrosome is achromatic.

7. Egg with fertilization cone and sperm showing swelling of the vitelline membrane following fertilization.



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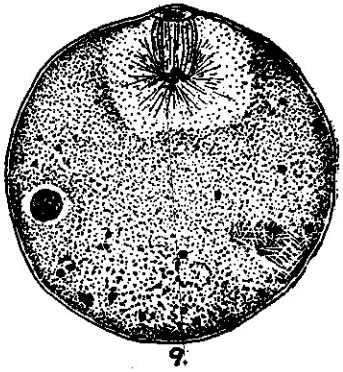

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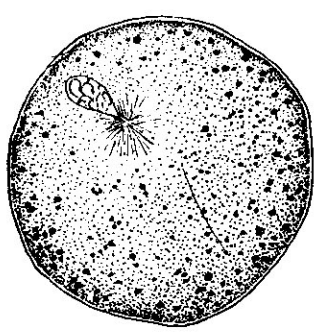
8. Egg 5-6 minutes after fertilization showing spherical black sperm.

9. Egg showing change in sperm and the first polar body.



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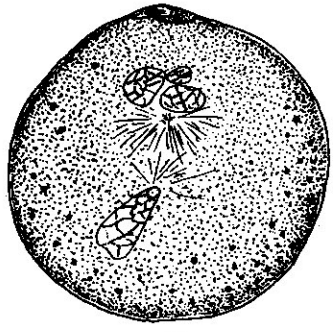

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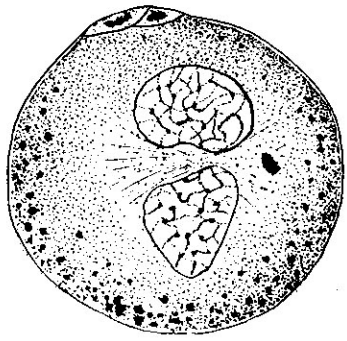
10. Shows male pronucleus and sperm aster.

11. Egg with chromosomal vesicles and arrow-head male pronucleus.



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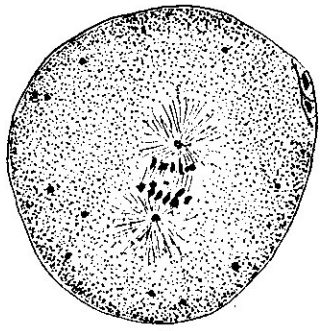

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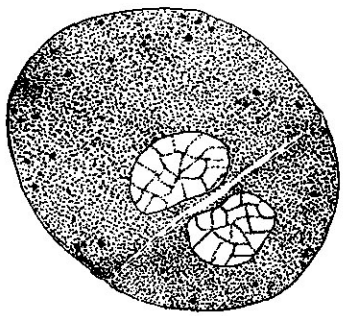
12. Shows two pronuclei before breaking down to form chromosomes for first divisional spindle.

13. Shows first divisional spindle.



14. Shows two celled stage.

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