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STUDIES ON THE CRITICAL PERIOD OF SUSCEPTIBILITY OF THE DEVELOPING FISH EGGS TO MECHANICAL DISTURBANCES

By

Shun OKADA

Institute of Zoology, Faculty of Agriculture
Hokkaido University

I. Introduction

Though fish generally lays numbers of eggs, comparatively few of them can survive to become adults, the rest perishing from various causes in the course of development.

Naturally, the mortality comes from unfavourable environment viz., abnormal temperature, unusual components of the water, mechanical shocks, pathogenetic bacteria, parasites, harmful enemies, etc. However, it should be remembered that in the course of the development of the egg there are physiologically susceptible periods at which it is readily affected by the environmental factors.

Since the problem how to promote the hatching rate of the fish eggs in the artificial propagation has become very serious, the writer has studied the susceptibility of the developing egg and its mechanism against mechanical disturbances which are considered to be one of the most important factors causing mortality. Chiefly the developing egg of dog-salmon, *Oncorhynchus keta* (WALBAUM) has been employed as the material and a series of experiments have been done.

Before going further, the writer sincerely wishes to acknowledge his indebtedness to Prof. T. INUKAI of the Faculty of Agriculture, Hokkaido University, for his kind suggestions and guidance, and to Prof. K. AOKI in the Institute of Low Temperature Science, Hokkaido University for his helpful criticisms. The writer's thanks are also due to Mr. T. ARAI, chief of Hokkaido Salmon Hatchery and the members of the hatchery, Mr. S. SANNO, Mr. K. SHIBATA and others who have supported the writer in various ways.

II. Historical Review of Studies on Resistance of the Developing Egg of Fishes to Mechanical Shock

So far as the writer knows, HEIN (1907), MORIWAKI (1910), NAKANO & KAWAZIRI (1924), HATA (1927), HATA (1929), ROLLEFSEN (1932), KOBAYASHI (1932), KISHIDA (1937), BATTLE (1944) and AFFLECK (1953) are the contributors of studies on the influence of mechanical disturbances on the developing egg of fishes.

They have made experiments in order to test the resistance of the developing egg against dropping, shaking or pushing shock. The eggs of *Salmo salar* (HEIN), *S. irideus* (KISHIDA), *S. gairdnerii* (AFFLECK), *Oncorhynchus keta* (MORIWAKI, KOBAYASHI), *O. masou* (NAKANO & KAWAZIRI, HATA), *O. nerka* (MORIWAKI), *Gadus* (ROLLEFSEN) or *Gasterosteus aculeatus*, *Fundulus heteroclitus* and *Enchelyopus cimbrius* (BATTLE) have been used for the experiments.

Most of them have agreed that the resistance of the developing egg is remarkably increased after the "eyeing" stage. ROLLEFSEN (1932) and KOBAYASHI (1932) have not referred to this stage.

The results so far reported concerning the resistance in pre-embryonal stage are not in accord. HEIN (1907) mentions that the egg is comparatively strong just after the insemination, but becomes weaker and weaker with the advance of the development until the 10~20th day of the rearing. There exists the susceptible period. Later the resistance recovers again toward the time of the hatching.

NAKANO & KAWAZIRI (1924) indicate that the susceptible period is at about the 10th day of the rearing. HATA (1929) sees it at about the 16th day and KISHIDA (1937) at the 10~15th day. According to them, the change of the resistance of eggs shows the same tendency as was reported by HEIN (1907).

MORIWAKI (1910) presents a little different conclusion in that the resistance of eggs is proved very weak during the period from the 1~2nd hour after the insemination to the 7~10th day before the "eyeing" stage. His result, however, might be brought about by a shock which was too strong. HATA (1927) also shows that there are two susceptible periods in the developmental stage, one being just after the insemination and the other at the 10~15th day of the rearing, though there is some probable failure in the experimental technique.

In most papers published by the above authors, the embryonal stage of the experimented egg has not been explained sufficiently, being only represented by the time after the insemination and without indicating even the rearing temperature in some papers. Needless to say, the hatching period varies with the rearing temperature as well as with the species. Therefore, the exact stage

of the development can not be determined in such papers.

None of the above authors has referred to the mechanism of the susceptibility except ROLLEFSEN (1932). ROLLEFSEN mentions as follows: The susceptibility of the cod egg increases up to the early stage of gastrula and decreases into the closing stage of the blastopore, remaining low afterwards. At early stages, the major part of the yolk of the egg is covered only with a thin layer of protoplasm, which easily bursts when the egg is exposed to mechanical disturbances and the egg is led to death. After the gastrula stage, the yolk is provided with a new covering of embryonal tissue which extends on the protoplasmic layer and completely envelops it at the closing stage of blastopore. This protects the protoplasmic layer from the mechanical disturbances and the resistance of the egg increases. ROLLEFSEN (1932), however, has not actually observed whether the protoplasmic layer bursts or not when the egg is exposed to mechanical disturbances.

BATTLE (1944) has obtained similar results with that of ROLLEFSEN (1932) in her experiments and agreed with his comments on the susceptibility.

III. General Observation on the Development in the Egg of Dog-Salmon

As mentioned above, most of the previous investigators have referred to the embryonal stage of experimented eggs as the time passed after the insemination. Naturally, this is not sufficient to determine the susceptible period and its mechanism. Therefore, for fundamental work in this field the present author has made general observation on the development of the egg of the dog-salmon, *Oncorhynchus keta* (WALBAUM). By this means the embryonal stages of experimented eggs may be indicated clearly.

Material and Method

In Hokkaido the dog-salmon ascends the river to spawn during autumn and winter, and their eggs are used for artificial hatching. The egg and sperm of the dog-salmon employed as the material in this observation were obtained from the Chitose Salmon Hatchery. Artificial insemination was performed and the eggs were reared in running water at a temperature of $9\pm 1^{\circ}\text{C}$ in the laboratory. The eggs were fixed in BOUIN's fixing reagent at short intervals and transferred into 70% alcohol after about twenty-four hours. Observations on the surface view of the embryo by binocular microscope were carried out after removing the chorion from the egg.

General Observation on the Development

In the freshly taken out egg before immersion into fresh water, the blastodisc of the uninseminated egg spreads thinly and widely over the animal pole, measuring about 3 mm in diameter (PLATE I, 1.). The egg diameter generally measures a little less than 7 mm.

When the egg is inseminated and immersed in fresh water, the blastodisc begins to condense gradually. The signs of condensation appear at one hour (PLATE I, 2.). Condensation continues until it measures finally about 1.5 mm in diameter at eight hours after the insemination (PLATE I, 4.).

The first segmentation of the blastodisc occurs at 9~11 hours (PLATE I, 5.), the second at 12~15 hours and the third at 16~19 hours (PLATE I, 6.).

After twenty-four hours, the development of the inseminated egg progressed as the following.

24 hours : Morula stage (PLATE I, 7.).

48 hours : Blastula stage. The blastoderm rises up from the yolk like a cap, measuring 1.2 mm in diameter (PLATE I, 8.).

72 hours : The blastula begins to flatten and expands on the yolk, measuring 1.5 mm in diameter.

5 days : The blastula flattens still more. Diameter is 1.8 mm (PLATE I, 9.).

6 days : The blastula flattens further and the central part becomes thin. The diameter is 2.2 mm.

7 days : Germ-ring stage. The central part becomes very thin. The diameter is 2.9 mm (PLATE I, 10.).

Until this stage, the blastoderm is located on the animal pole of the egg and the yolk is covered with a thin layer of protoplasm or vitelline membrane except the part of the blastoderm (PLATE I, 11.).

8 days : Gastrula stage. The embryonic shield appears at one part of the germ-ring. The diameter is 3.3 mm (PLATE I, 12.).

9 days : Neurula stage. The embryo begins to grow. The extra-embryonic part of the blastoderm or the ectodermal membrane is going to extend on the yolk sphere.

10 days : A pair of rudimental optic and auditory vesicles, and a number of somites are observable not clearly on the surface of the embryo. The diameter of blastoderm is 5 mm (PLATE I, 13.).

12 days : The optic and auditory vesicles are clearly observed. The anterior part of medullary canal differentiates into three parts i.e. fore-, mid- and hind-brain. Numerous somites are counted from outside. The blastoderm covers almost entire upper half of the yolk sphere (PLATE I, 14.).

14 days: The blastoderm envelops the surface of the yolk sphere except a small round part or blastopore behind the embryo. Five neuromeres of the hind-brain are clearly observed (PLATE I, 15.).

15 days: The blastopore decreases in size more and more until it disappears on that day. The yolk sphere is perfectly enveloped with the ectodermal membrane of the blastoderm (PLATE I, 16.).

17 days: Preceding the closing of the blastopore, the mesodermal membrane of lateral plates starts to develop under the blastoderm around the embryo. At this stage, this membrane extends to a certain area on either side of and behind the embryo (PLATE I, 17.).

20 days: The membrane extends further (PLATE I, 18.).

22 days: The membrane covers a half of the egg sphere. A pair of rudimental pectoral fins is clearly observed (PLATE I, 19.).

25 days: The membrane envelops almost whole surface of the egg sphere except the area in front of the embryo (PLATE I, 20.).

28±1 days: The membrane perfectly envelops the egg sphere under the blastoderm. At the same time, the egg enters into the "eyeing" stage in which a pair of black eyes is clearly observed through the chorion (PLATE I, 21.).

40 days: The embryo is growing larger (PLATE I, 22.).

50 days: The embryo continues growing (PLATE I, 23.).

58±2 days: The embryo hatches out. The alevin just after hatching measures about 22 mm in length, having a large yolk sac on the abdomen (PLATE I, 24.).

IV. Experiments to Determinate the Susceptible Period in the Developing Egg of Dog-Salmon

Most of previous investigators have discussed the problem on the basis of their experiments using the stages taken at intervals of about five days in the course of the development. The intervals of five days, however, are too wide to determine the susceptible period exactly. Though the experiment of MORIWAKI (1910) was performed using the eggs taken at very small intervals, the results obtained fail to determine the period as mentioned in chapter II.

The present experiments were carried out in order to determine the susceptible period of the developing egg as this is essential in order to solve the general problem of the susceptibility.

Material and Method

The materials of the present experiment were the same as mentioned in chapter III.

One hundred eggs at each stage of development were picked from the batch and transferred into a glass vessel, diameter 12 cm and depth 6 cm. containing 50 cc of water. Then the vessel was fixed on the board of "shaking-apparatus" which was devised to vibrate horizontally within the distance of 8.5 cm by means of a motor. The frequency of the shaking was regulated according to specifications of the experiment.

After the eggs were exposed to shaking for a certain number of minutes, the vessel was removed from the board and the eggs were transferred into running water. At the same time, one hundred eggs at the same stage were reared untreated for the control. Several eggs were fixed in *BOUIN's* reagent to ascertain the developmental stage. The dead eggs which turned opaque were counted and removed every day from both series.

A batch of eggs obtained from one female was employed in each experiment, as the susceptibility of eggs sometimes differs with the individual. There was no failure in the artificial insemination in the experiment and scarcely any unfertilized eggs were found.

Experiment I.

This experiment was performed to find out the general aspects of the resistance during the development. Therefore, a somewhat vigorous shaking was used for a comparatively long time for the eggs which were taken at intervals of about four days as the following:

Frequency of shaking: 70~76 per minute.

Time of shaking: 20 and 40 minutes.

Stages of eggs used: 4 hours, 24 hours, 4 days, 8 days, 10 days, 12 days, 15 days, 21 days and 26 days old.

Results of the experiment are given in Table 1. and Fig. 1. The highest rate of dead egg in the control as observed until the time of the hatching was only 6%, so it is considered that the experimented eggs were all sound and the treatment was of no apprehension.

Four hours old eggs are comparatively strong, showing resistance against 20 minutes shaking. At this stage the segmentation is not taking place.

On the contrary, the eggs aged twenty-four hours to fifteen days old were almost entirely destroyed. This period begins from morula stage and ends at just before the closing of the blastopore.

As there is remarkable decrease of the resistance in this period a question is presented whether that decrease is related with the segmentation of the blastodisc or not. This will be ascertained in a later experiment.

From sixteen days after insemination, the egg steadily recovers its resistance

TABLE 1. Percentage of dead eggs in Experiment I.

Stage		4 hours			24 hours			4 days			8 days			10 days		
Time		Cont	20	40	Cont	20	40	Cont	20	40	Cont	20	40	Cont	20	40
Days after shaking	1	0	27	46	0	81	97	0	93	100	0	94	100	0	91	100
	2	0	1	1	0	3	2	0	1		0	2		0	3	
	3	0	2	1	0	0	0	0	0		0	0		0	2	
	4	0	0	0	0	3	0	0	0		0	0		0	0	
	5	0	0	0	0	1	0	0	1		0	0		0	0	
	6	0	1	0	0	0	0	0	0		0	0		0	0	
	7	0	0	0	0	1	0	0	0		0	0		0	0	
Days after shaking	8-14	0	0	0	0	3	0	0	3		0	0		0	0	
	15-21	1	1	0	0	1	1	0	0		0	0		1	1	
	22-28	0	0	0	0	1	0	2	0		1	0		0	0	
	29-35	0	0	0	1	0	0	2	0		1	0		1	0	
	36-42	0	2	1	2	0	0	1	0		3	0		0	0	
	43-49	0	1	2	2	0	0	1	0		1	0		2	0	
	50-56	1	0	0	0	0	0	0	0		0	0		0	0	
Total (%)		2	35	51	5	94	100	6	98	100	6	96	100	4	97	100

Stage		12 days			15 days			17 days			21 days			26 days		
Time		Cont	20	40	Cont	20	40	Cont	20	40	Cont	20	40	Cont	20	40
Days after shaking	1	0	98	100	0	87	100	0	62	81	0	9	10	0	1	1
	2	0	1		0	4		0	4	2	0	0	3	0	0	0
	3	0	0		0	1		0	5	1	0	6	10	0	0	0
	4	0	0		2	2		0	1	3	0	4	16	0	0	0
	5	0	0		0	0		0	3	0	0	15	12	0	0	0
	6	0	0		0	0		0	1	0	0	3	8	0	0	0
	7	0	0		0	0		0	0	0	0	1	3	0	0	0
Days after shaking	8-14	0	0		0	1		0	0	1	0	3	4	0	0	0
	15-21	0	0		0	0		0	3	0	0	1	1	0	0	1
	22-28	0	0		1	0		1	0	0	2	2	0	4	0	0
	29-35	1	0		0	0		1	0	0	0	0	2	0	0	0
	36-42	0	0		0	0		0	0	0	0	0	0	0	0	0
	43-49	1	0		0	0		0	0	0	0	0	0	0	0	0
	50-56	0	0		0	0		0	0	0	0	0	0	0	0	0
Total (%)		2	99	100	1	95	100	2	79	88	2	44	69	4	1	2

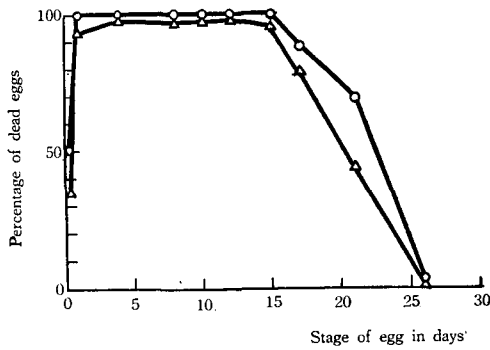


Fig. 1. Result of Experiment I.
 △ 20 minutes shaking,
 ○ 40 minutes shaking.

and the twenty-six days old egg shows almost no damage from the stimulus. The embryo is then at 2~3 days before "eyeing" stage.

In the 40 minutes shaking experiment, the egg at any stage shows the same tendency of resistance, though the mortality increases as compared with the case described above.

Experiment II.

Twenty-six days old egg is not effected by the given stimulus. Therefore, for the test of the resistance after the "eyeing" atage, the same intensity of the stimulus as that used before cannot be applicable. A stronger stimulus was employed in the present experiment for this period.

Frequency of shaking: 95~100 per minute.

Time of shaking: 40 minutes.

Stages of eggs used: 22 days, 29 days, 36 days, 49 days and 53 days old.

Results of the experiment are given in Table 2 and Fig. 2. In the controls, the highest rate of dead eggs as observed until the time of the hatching was only 8%.

In this experiment, the twenty-two days old egg shows comparatively high mortality while twenty-nine days old egg which is just after "eyeing" shows remarkable recovery of resistance, indicating that the resistance is continuously increasing as progress is made toward the "eyeing" stage.

After this stage, the egg is not influenced by such a strong stimulus. Therefore, the intensity of the resistance beyond the "eyeing" stage can not be clarified by this experiment.

The experiment of HEIN (1907), however, shows that the resistance of the developing egg of *Salmo salar* is continuously increasing in this period. In the

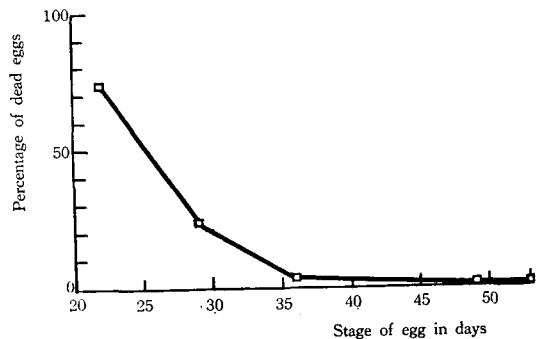


Fig. 2. Result of Experiment II.
 □ 40 minutes shaking.

TABLE 2. Percentage of dead eggs in Experiment II.

Stage		22 days		29 days		36 days		49 days		53 days	
Time		Cont	40	Cont	40	Cont	40	Cont	40	Cont	40
Days after shaking	1	0	34	0	1	0	1	0	1	0	1
	2	0	13	0	1	0	0	1	0	1	0
	3	0	9	0	0	0	0	0	0	0	0
	4	0	2	0	0	0	0	0	0	0	0
	5	0	1	0	0	0	0	0	0	2	0
	6	0	5	0	2	0	0	0	0	0	0
	7	0	1	0	4	0	1	0	0	0	0
	8-14	3	2	1	4	0	2	0	0	0	0
	15-21	0	2	1	9	1	0	0	0	0	0
	22-28	3	0	0	2	0	0	0	0	0	0
	29-35	1	1	0	1	0	0	0	0	0	0
	36-42	1	0	0	0	0	0	0	0	0	0
Total (%)		8	74	2	24	1	4	1	1	3	1

case of a practical artificial hatching, there is no need to pay any attention to the mechanical disturbances after the "eyeing" stage.

As shown in Tables 1 and 2, dead eggs generally appear within comparatively short time after the exposure to the stimulus. This means the shaking influence has set in immediately. However, the appearance of dead eggs in the seventeen, twenty-one, twenty-two and twenty-nine days old eggs extends over a comparatively long time respectively. The interpretation of this fact will be presented later.

Experiment III.

This experiment was carried out in order to ascertain the change of the resistance in the early stages using a milder stimulus, as in Experiment I the tendency was not made clear thoroughly.

Frequency of shaking: 56~60 per minute.

Time of shaking: 5 and 10 minutes.

Stages of eggs used: 1 hour, 7 hours, 18 hours, 26 hours, 72 hours and 6 days old.

Results of the experiment are given in Table 3 and Fig. 3. As already mentioned, dead eggs appear within a comparatively short time when exposed to the stimulus. Therefore, in the further experiment dead eggs found within

TABLE 3. Percentage of dead eggs in Experiment III.

Stage		1 hour			7 hours			18 hours		
Time		Cont	5	10	Cont	5	10	Cont	5	10
Days after shaking	1	0	0	0	0	1	12	0	13	24
	2	0	0	0	0	0	0	0	1	0
	3	0	0	0	0	0	0	0	0	0
	4	0	0	0	0	0	0	0	0	0
	5	0	0	0	0	0	0	0	0	0
	6	0	0	0	0	0	0	0	0	0
	7	0	0	0	0	0	0	0	0	0
Total (%)		0	0	0	0	1	12	0	13	24

Stage		26 hours			72 hours			6 days		
Time		Cont	5	10	Cont	5	10	Cont	5	10
Days after shaking	1	0	16	26	0	38	38	0	43	58
	2	0	0	0	0	1	0	0	7	5
	3	0	0	0	0	0	5	0	1	1
	4	0	0	0	0	0	0	0	1	0
	5	0	0	0	0	1	0	0	0	0
	6	0	0	0	0	0	0	0	0	0
	7	0	0	0	0	0	0	0	0	0
Total (%)		0	16	26	0	40	43	1	52	64

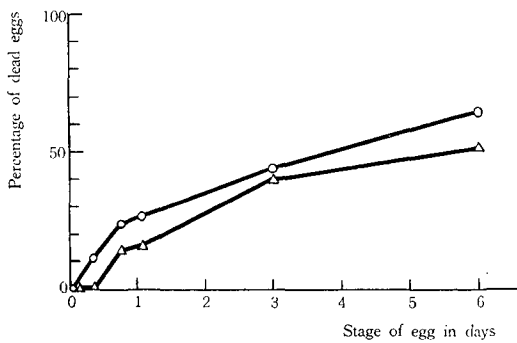


Fig. 3. Result of Experiment III.

△ 5 minutes shaking,
○ 10 minutes shaking.

seven days are considered to have resulted from the shaking.

Dead eggs which appeared within seven days in the control were only 1% in the six days old egg.

In the experiment of five minutes shaking, both one and seven hours old eggs are not affected by the stimulus. The later is at about two hours before the segmentation of blastodisc. However, eighteen

hours old eggs which are of morula stage show a little decrease of resistance,

and afterwards each stage shows more and more decrease in resistance with the advance of the development until six days old egg which is at germ-ring stage.

In ten minutes shaking, the tendency of resistance is almost the same with the case just mentioned above, though the mortality naturally increases to some extent. Only the resistance of seven hours old eggs on the contrary, is distinctly less in degree than that of one hour old eggs. This is probably due to the reason that the egg can endure five minutes shaking but cannot ten minutes shaking. From this fact, it is obvious that the resistance is continuously decreasing even before the segmentation of blastodisc begins.

Experiment IV.

This experiment was carried out to ascertain whether early gastrula is most susceptible, and at the same time to re-examine the result of the preceding

TABLE 4. Percentage of dead eggs in Experiment IV.

Stage		4 hours			8 hours			13 hours			24 hours			72 hours			
Time		Cont	5	10	Cont	5	10	Cont	5	10	Cont	5	10	Cont	5	10	
Days after shaking	1	0	2	8	1	2	18	0	10	21	0	10	21	0	29	56	
	2	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	
	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	6	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
	7	0	1	0	0	0	1	0	0	1	0	2	3	0	1	2	
Total (%)		0	3	8	1	2	19	0	10	22	0	12	26	0	31	58	

Stage		5 days			6 days			7 days			8 days		
Time		Cont	5	10	Cont	5	10	Cont	5	10	Cont	5	10
Days after shaking	1	0	46	74	1	53	74	0	57	79	0	63	81
	2	1	1	0	0	0	1	0	0	0	0	1	0
	3	0	1	0	0	0	0	0	0	0	0	0	0
	4	1	0	1	0	0	0	0	0	0	0	0	0
	5	0	0	0	0	0	0	0	0	0	0	0	0
	6	0	0	0	0	0	1	0	0	0	0	0	0
	7	0	1	0	0	0	0	0	0	0	1	0	0
Total (%)		2	49	75	1	53	76	0	57	79	1	64	84

experiment.

Frequency of shaking: 68~72 per minute.

Time of shaking: 5 and 10 minutes.

Stages of eggs used: 4 hours, 8 hours, 13 hours, 24 hours, 72 hours, 5 days, 6 days, 7 days and 8 days old.

Results of the experiment are given in Table 4 and Fig. 4. Dead eggs which appeared in the control within seven days were only 2% at highest.

In five minutes shaking, both four hours old and eight hours old eggs are scarcely influenced.

The stages after thirteen hours old, how-

ever, showed successively decreased resistance until eight days old.

In ten minutes shaking, the tendency of resistance is almost the same as the case described above, though the mortality naturally increases. Only eight hours old egg which is at one hour before the segmentation shows less resistance than the four hours old egg as observed in Experiment III. From these facts, it is sure that the resistance is continuously decreasing after the insemination.

In eight days old egg, the gastrulation is occurring and embryonic shield distinctly appears at a part of germ-ring. However, remarkable difference of resistance is not observed at all between the seven and the eight days old eggs, though a slow successive decrease is proceeding. Therefore, so far as the mechanical disturbances are concerned, there is no reason to maintain the view that the critical period is closely related to gastrulation.



Fig. 4. Result of Experiment IV.

\triangle 5 minutes shaking,

\circ 10 minutes shaking.

Experiment V.

This experiment was carried out to ascertain the change of the resistance in the period around closing stage of the blastopore and to re-examine the observations during gastrulation stage made in Experiment IV.

Frequency of shaking: 68~72 per minute.

Time of shaking: 5 and 10 minutes.

TABLE 5. Percentage of dead eggs in Experiment V.

Stage		6 days			7 days			8 days			9 days			11 days		
Time		Cont	5	10	Cont	5	10	Cont	5	10	Cont	5	10	Cont	5	10
Days after shaking	1	0	20	41	0	16	36	0	38	50	2	55	83	0	63	83
	2	0	0	2	0	2	2	0	0	4	0	1	0	0	0	0
	3	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0
	4	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	7	0	0	0	1	1	3	0	1	2	1	0	0	0	0	2
Total (%)		1	20	43	1	21	41	0	39	56	3	56	83	0	63	87

Stage		13 days			14 days			15 days			16 days			17 days		
Time		Cont	5	10	Cont	5	10	Cont	5	10	Cont	5	10	Cont	5	10
Days after shaking	1	0	28	42	0	51	93	1	51	72	0	4	24	1	3	10
	2	0	27	45	0	10	3	0	8	7	0	2	1	0	1	0
	3	0	3	4	0	4	2	0	2	0	0	2	0	0	0	1
	4	0	2	0	0	0	0	0	4	0	0	0	0	0	1	0
	5	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0
	6	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
	7	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Total (%)		0	61	91	0	66	100	1	65	79	0	8	25	1	5	11

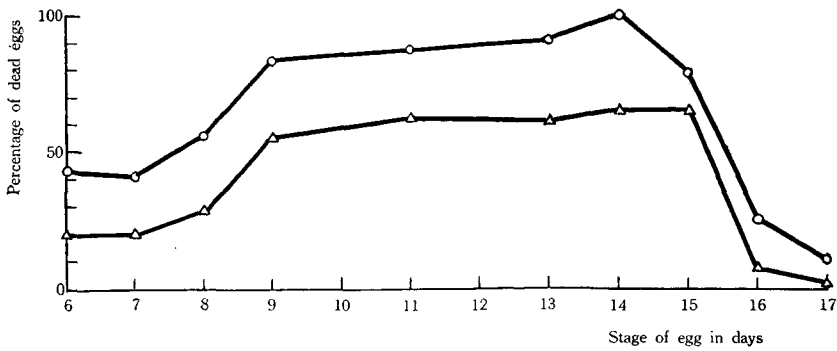


Fig. 5. Result of Experiment V.
 △ 5 minutes shaking,
 ○ 10 minutes shaking.

Stages of eggs used: 6 days, 7 days, 8 days, 9 days, 11 days, 13 days, 14 days, 15 days, 16 days and 17 days old.

Results of the experiment are given in Table 5 and Fig. 5. Dead eggs found in the control within seven days were at most 3%.

Against five minutes shaking, the resistance gradually decreases until nine days at which the embryo has reached early neurula stage. Remarkable difference of the resistance is not found between seven days and eight days old ones in the present experiment as was also observed in Experiment IV. The most susceptible period in the course of the development appears from the ninth day extending to the fifteenth day. In this period, extra-embryonic blastoderm extends over the yolk bit by bit until it entirely envelops the whole surface of the egg except a small area of blastopore behind the embryo on the fifteenth day.

Suddenly, the recovery of resistance appears in the sixteen days old egg in which the blastopore quite disappears; afterwards the egg becomes stronger and stronger in its resistance to mechanical shock.

In ten minutes shaking, the grade of the resistance is almost the same with the case mentioned above, though the mortality increases in every stage.

Experiment VI.

This experiment was performed to find out the delicate change of the resistance in the critical period found in Experiment V, using a milder stimulus.

Frequency of shaking: 53~57 per minute.

Time of shaking: 5 and 10 minutes.

Stages of eggs used: 10 days, 11 days, 12 days, 13 days, 14 days, 15 days, 16 days and 17 days old.

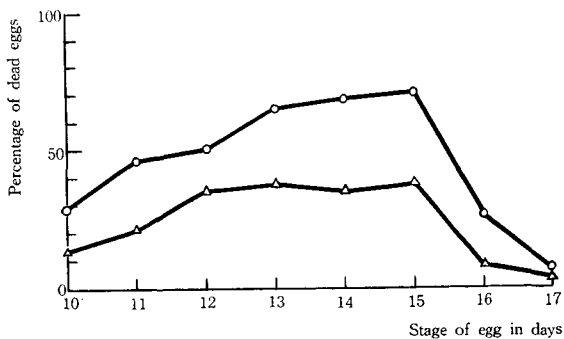


Fig. 6. Result of Experiment VI.

- △ 5 minutes shaking,
- 10 minutes shaking.

Results of the experiment are given in Table 6 and Fig. 6. Dead eggs counted in the control within seven days showed at most only 5%.

Against five minutes shaking, each stage extending from the twelve days old to the fifteen days old egg is particularly weak, having much narrower critical period than was found in Experiment V.

TABLE 6. Percentage of dead eggs in Experiment VI.

Stage		10 days			11 days			12 days			13 days		
Time		Cont	5	10	Cont	5	10	Cont	5	10	Cont	5	10
Days after shaking	1	0	14	25	0	21	38	1	31	46	2	34	60
	2	0	0	2	0	0	1	0	2	0	1	1	3
	3	1	0	0	0	0	0	0	0	1	0	1	0
	4	0	0	0	0	0	0	0	0	0	0	0	1
	5	1	0	2	0	0	4	0	0	0	1	1	0
	6	0	0	0	0	0	0	0	0	0	0	0	1
	7	0	0	0	0	1	3	0	2	3	0	0	0
Total (%)		2	14	29	0	22	46	1	35	50	4	37	65

Stage		14 days			15 days			16 days			17 days		
Time		Cont	5	10	Cont	5	10	Cont	5	10	Cont	5	10
Days after shaking	1	4	31	64	1	34	69	0	4	16	1	4	3
	2	1	3	2	0	4	0	0	3	7	0	1	2
	3	0	1	1	0	0	2	0	0	1	0	0	0
	4	0	0	1	0	0	0	0	1	1	0	0	0
	5	0	0	0	0	0	0	0	1	0	1	0	0
	6	0	0	0	0	0	0	0	0	0	0	0	0
	7	0	0	0	0	0	0	0	0	1	0	0	1
Total (%)		5	35	68	1	38	71	0	9	26	2	5	6

A sudden increase of the resistance occurs in the sixteen days old egg as was also observed in the preceding experiment.

In ten minutes shaking, the resistance is almost the same as in the case just described.

Discussion and Summarization

The results obtained in the above described experiments are summarized as follows.

The egg just after insemination is comparatively strong against mechanical disturbances. The resistance, however, decreases gradually until the fifteen days old stage, having no relation to the segmentation of blastodisc or to the gastrulation.

The observed tendency of the resistance in early period of the development is almost in agreement with the results of HEIN (1907), NAKANO and KAWAZIRI

(1924), HATA (1929), ROLLEFSEN (1932), KISHIDA (1937) and BATTLE (1944), though their results were mostly obtained from experiments performed with eggs taken at very rough intervals. The results of MORIWAKI (1910) and HATA (1927) which were a little different are out of consideration for the reasons explained already in chapter II above.

Generally the gastrulation stage is a critical period in the course of the development. As a matter of fact, the cod egg in this period shows the highest mortality under the influence of mechanical disturbance according to the experiments of ROLLEFSEN (1932). The egg of the dog-salmon, however, does not indicate any critical condition in gastrulation period which begins around the eighth day of the development, though the resistance of the egg is continuously decreasing from the preceding stage.

The most susceptible period extends from about the tenth day to the fifteenth day. In this period, the embryo grows more and more in size and various organs develop successively. Extra-embryonic blastoderm covers the yolk gradually until a small area is left as the blastopore behind the embryo. In most of previous papers, the critical period has not been exactly determined because the experiments were carried out at rough intervals by which embryonal stage could not be clearly shown. In sixteen days egg in which the blastopore is closed completely, there appears suddenly a remarkable increase of the resistance, showing that the most susceptible period has passed by. Hitherto, none of the investigators has noticed the recovery of the resistance in the closing stage of the blastopore, except ROLLEFSEN (1932) and BATTLE (1944).

Afterwards, the resistance of the egg continues to increase gradually until the time of hatching. Practically after approximately the "eyeing" stage, the egg furnishes strong resistance against almost any kind of mechanical disturbance.

V. The Mechanism of Killing by Mechanical Disturbances in the Egg of Dog-Salmon

The living egg of the dog-salmon is translucent and reddish in colour. When the egg turns white and opaque, it is generally recognized as dead.

On the mechanism of such change in appearance, GRAY (1932) has explained in the egg of *Salmo salar* as follows: The protoplasmic layer covering yolk sphere or the vitelline membrane has an impermeable property to water and salts. On account of this property, the globulin of the yolk is kept in dissolved condition for the existence of the salts retained in the yolk and the egg shows translucent appearance. Some mechanical stimulus to the egg causes a rupture of the vitelline membrane. The salts diffuse out of the rupture of the membrane

in the outside water and the concentration in the yolk becomes low. With these the globulin precipitates and the egg turns white and opaque inducing the death.

ROLLEFSEN (1932) has discovered that the resistance of cod egg to mechanical shock remarkably increases when extra-embryonic blastoderm envelops perfectly the protoplasmic layer of the yolk. According to him, the former protects the latter physically from rupture. However, he has not observed whether the protoplasmic layer actually bursts or not as a result of the mechanical shock.

AFFLECK (1953) has also discussed the mechanism of death introduced by mechanical stimulus, sunlight or electric light in the egg of *Salmo trutta* and *S. gairdnerii*, without any actual observation or experiment.

In the present chapter, the writer intends to describe his effort to ascertain whether visible rupture of vitelline membrane occurs and also whether embryonal membranes such as extra-embryonic blastoderm and mesodermal lateral plate actually play a part of protection for the vitelline membrane.

Observations on the Egg Immersed in RINGER's Solution

As the egg chorion of the dog-salmon is almost opaque, it is difficult to observe from outside in detail what change is occurring within the egg after the exposure to mechanical stimulus. However, when the egg is immersed instantly in RINGER's solution, the chorion becomes transparent and the change within the egg can be easily observed from outside.

The transparency of the chorion in the above case is not due to the direct effect of RINGER's solution because in the egg which is not treated with mechanical stimulus there appears no change in the medium. It seems to be due to the fact that zona radiata of the chorion are filled up with transparent yolk of which the globulin is prevented from precipitating by the presence of salts in the medium.

When the egg becomes gradually transparent in RINGER's solution after about ten minutes, a certain membrane which is shrinking within the chorion can be easily observed by means of a horizontal microscope.

Furthermore, the medium containing such eggs becomes cloudy by and by after about twenty hours and it appears reddish colour by application of BIURET's test, proving the presence of the protein.

From these facts, it is highly probable that through the rupture of the vitelline membrane the yolk flows out from its opening into the perivitelline space and the salts contained in the yolk diffuse to give the egg its white and opaque appearance before long.

However, it is difficult to indicate what kind of rupture occurs actually in

the vitelline membrane by mechanical stimulus.

Observations of the Egg Fixed in BOUIN's Solution

When the chorion of the egg is removed carefully after fixation by BOUIN's solution, vitelline membrane is seen perfectly enveloping the yolk sphere of the egg. This membrane is very thin and translucent in appearance, communicating directly with the blastodisc situated on the animal pole of the egg (Fig. 7; A). Oil drops are found scattered inside of the membrane and they aggregate especially under the blastodisc.

When the egg is fixed at about one minute after the stimulus which is given on a certain point of the egg and then the chorion is removed, a round wound is observed in the vitelline membrane and through it transparent yolk surface is revealed (Fig. 7; B, C and D). The chorion is removed with difficulty in this position because the yolk closely adheres to the chorion.

In a case when fixed after several minutes, the wound shrinks to a ring which is clearly visible through the transparent yolk which flows out from there (Fig. 7; E and F). Finally at about ten minutes after the stimulus, a great part of the yolk has flowed out and the membrane shrinks as an irregular mass at the animal pole enclosing oil globules (Fig. 7; G and H). This observation clearly indicates that mechanical stimulus actually causes a physical rupture in the vitelline membrane.

When an egg is immersed into tap water immediately after a point of the egg surface is struck with a blunt needle, a small opaque ring appears at the

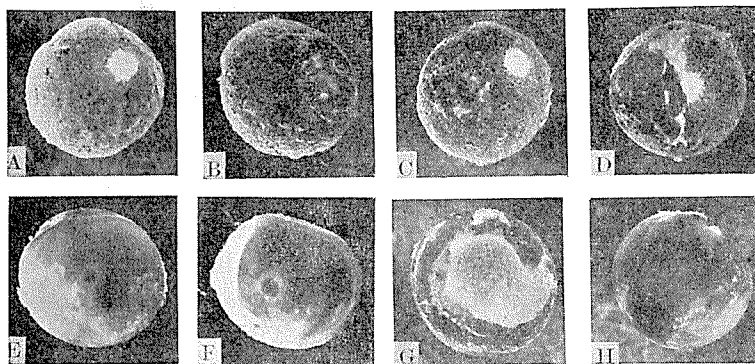


Fig. 7. Various states of vitelline membrane fixed with BOUIN's solution. A: normal, B, C and D: at about one minute, E and F: at several minutes, G and H: at about ten minutes after the exposure to mechanical stimulus, G: View of animal pole, H: View of vegetative pole.

point of shock after about twenty seconds. The ring enlarges in size gradually until it expands over the whole surface of the egg except the part of the blastodisc. Then the opaque ring thickens inwards gradually, and the whole area enclosed by the ring becomes perfectly opaque at last. With comparatively weak stimulus, the opaque ring sometimes ceases to expand at a certain state and a localized opaque area is formed there.

This phenomenon was demonstrated for the first time in the egg of *Salmo salar* by GRAY in 1932. He considered this ring at first to be an irreversible wave of permeability expanding over the vitelline membrane from the shocked point. But afterward he came to the conclusion that this was the margin of the yolk flowing out from within the opening of the rupture of vitelline membrane.

In order to ascertain the nature of the opaque ring, the eggs of the dog-salmon in which the ring had ceased to expand at various stages were fixed with BOUIN's solution without acetic acid. When the chorion was removed, the margin of the flowed out material from within the rupture of vitelline membrane appeared white, corresponding to the opaque ring observed from outside of the chorion. This region easily precipitates globulin and turns white because the yolk is very thin there and the salts diffuse rapidly, as GRAY reported.

Vitelline Membrane and the Resistance of the Egg to Mechanical Stimulus

When the egg is fixed with BOUIN's solution or other ordinary fixative reagents the yolk becomes extremely hard and preparation of microscopical sections is almost impossible. Therefore, the method of VIRCHOW was applied with success (BEHRENS, 1898).

As already mentioned above in chapter III, the yolk is enveloped only with a thin layer of protoplasm or vitelline membrane at early stages of development. The vitelline membrane is in contact directly with the blastodisc during the unsegmented period (PLATE IV; 1-a, 1-b). At first the blastodisc measures about 3 mm in diameter spreading thinly and widely on the animal pole. It continues to condense gradually until it measures about 1.5 mm in diameter at about nine hours after the immersion in fresh water. Then the segmentation occurs in the inseminated egg. The condensation of the blastodisc or bipolar differentiation has been already reported by BEHRENS (1898) in *Trutta iridea*, RUNSTROEM (1920) in *Salmo salvelinus* and YAMAMOTO, K (1947, '48, '51) in *Oncorhynchus keta*.

Moreover, YAMAMOTO, T. (1943' '44) has observed in *Oryzias latipes*, and KANO, Y. (1950, '52, '53) also in *Tribolodon hakuensis* that the protoplasm

is supplied from the surface and also from inside of the yolk to form the blastodisc. In the case of the dog-salmon, the movement of protoplasm toward the animal pole in the process of bipolar differentiation cannot be observed directly because the chorion is almost opaque. However, it seems true that the movement does occur in this case and the vitelline membrane decreases in thickness little by little in accordance with the advance of the condensation of the blastodisc.

This perhaps means that the vitelline membrane decreases the resistance to mechanical stimulus by and by. As already mentioned in chapter IV, the resistance of the egg against mechanical disturbances is comparatively strong just after the insemination and immersion in fresh water but it decreases gradually in accordance with the advance of the development until a certain later stage. The change of the resistance seems to be due to the reason mentioned above.

When the blastula begins to flatten after about three days of the development, the vitelline membrane loses the intimate connection with the blastodisc, only retaining loose connection with the marginal cells of periderm which make up the simple epithelium constructing the surface of the blastula (PLATE IV; 2-a, 2-b).

In this region, the vitelline membrane is comparatively thick and forms the periblast which contains many nuclei. Vitelline membrane under the blastodisc is also thick and after the late stage of the blastula it forms subgerminal periblast with many nuclei which invade from the periblast. The remaining part of the membrane is very thin as compared with the periblast and subgerminal periblast.

The surface of the yolk is covered only with vitelline membrane except the part of the blastodisc until the gastrulation which occurs on the eighth day of the development. It is highly probable that vitelline membrane becomes thin more or less during this period and thereby the endurance of the membrane against mechanical stimulus diminishes.

Development of Extra-Embryonic Blastoderm and Resistance of the Egg to Mechanical Stimulus

On the eighth day of development, gastrulation occurs and the embryonic shield appears on a corner of the germ-ring. On the tenth day, the egg enters neurula stage which begins to provide the figure of the embryo. At the same time, extra-embryonic blastoderm which is sent out from the ectoderm of the embryo begins to extend on the surface of the vitelline membrane.

Observations on the section of the egg on the tenth day of the development are as follows: In the region of the embryo, a simple epithelium of

endoderm is situated on the vitelline membrane, loading notochord, medullary chord, somites, and slightly developing lateral plates, and most external surface is covered with a simple epithelium of ectoderm (PLATE IV; 3-a). This ectodermal membrane directly extends over the vitelline membrane in extra-embryonic region and is in contact with it loosely at the marginal end which is constructed of multiple cells and looks somewhat heavy (PLATE IV; 3-b).

Afterwards, extra-embryonic blastoderm extends gradually in accordance with the development of the embryo, covering almost a half of the surface of the egg-sphere on the twelfth day and the whole surface, except the small area of the blastopore which is left behind the embryo, on the fifteenth day of the development.

Observations on the sections of the eggs on the fifteenth day of the development are as follows: The endoderm has transformed into a tube-like intestine. The lateral plates also differentiate into two layers at each side of the intestine, the splanchnic layer has extended on vitelline membrane and somatic layer now lines extra-embryonic blastoderm (PLATE V; 4-a). These two layers communicate with each other at the region which is a little apart from the embryo, showing the boundary of the vascular area which is visible from outside (PLATE V; 4-b). Outside of the vascular area, only extra-embryonic blastoderm extends on vitelline membrane (PLATE V; 4-c). The parallel section across the blastopore shows that vitelline membrane lies bare in the part of the blastopore (PLATE V; 4-d) while it is covered with three membranes in the remaining region viz., two membranes of vascular area and the extra-embryonic blastoderm (PLATE V; 4-e).

In brief, on the fifteenth day, vitelline membrane of the yolk is covered entirely with extra-embryonic blastoderm except a small area of the blastopore and between the two membranes there are two layers of lateral plate close to the embryo.

On the sixteenth day, the blastopore is closed up and the whole surface of the vitelline membrane is perfectly enveloped with extra-embryonic blastoderm.

The resistance of the egg to mechanical stimulus since the early stage of gastrula to the closing stage of blastopore has been already described in detail in chapter IV. Briefly speaking, the decrease of resistance since the stages preceding gastrulation goes on to the stage of the closing of the blastopore, showing point of lowest resistance after about the tenth day of the development. Then the resistance recovers suddenly on the sixteenth day and lasts until the time of hatching.

As extra-embryonic blastoderm goes on to extend over vitelline membrane which is protected by it, from the gastrulation until the fifteenth day the re-

sistance of the egg against mechanical stimulus should be increased gradually. However, the actual facts do not prove this. Extra-embryonic blastoderm might be burst as well as the vitelline membrane by mechanical stimulus in this case as the former is not strong enough for protection.

As a matter of fact, the two membranes rupture together in the part of extra-embryonic blastoderm in the dead egg to which mechanical stimulus has been applied in this period. This has been ascertained in the egg fixed with BOUIN's solution and naked after removal of the chorion.

Extra-embryonic blastoderm gets toughness soon after the blastopore is perfectly closed on the sixteenth day of development.

Development of the Vascular Area and Resistance of the Egg to Mechanical Stimulus

Before the closure of the blastopore, as already mentioned in chapter III, mesodermal vascular area which is composed of somatic and splanchnic layers is beginning to develop from the embryo between vitelline membrane and extra-embryonic blastoderm. This extends over the egg sphere by and by until it perfectly envelops the egg in the front part of the embryo at "eyeing" stage when the egg enters about the twenty-eighth day of the development.

From the observations on the section of the egg just before "eyeing" stage, it is clear that the yolk is covered perfectly with four membranes; vitelline membrane, splanchnic layer, somatic layer and extra-embryonic blastoderm. From the splanchnic layer, which sticks intimately to vitelline membrane, develop blood vessels which contain a number of blood cells (PLATE V; 5-a, 5-b).

The resistance of the egg which is increasing after the closing stage of the blastopore seems to be chiefly due to the extension and the growth of these mesodermal membranes.

After "eyeing" stage, the egg possesses very powerful resistance and practically it becomes almost indifferent to the influence of any mechanical disturbance.

When the egg at this stage receives an intensive mechanical stimulus, the death of the embryo occurs preceding the rupture of the coverings of the yolk. In this case the embryo becomes opaque and unmovable under the chorion at about twenty-four hours after the shock, but the whole egg retains normal reddish translucent appearance. However, such an egg turns opaque and white at length after three days or more. The coverings of the yolk are found remaining sound when the egg is fixed with BOUIN's solution and the chorion is removed. It is highly probable that the coverings of the yolk degenerate gradually after the death of the embryo and lose the property of impermeability to water and salts.

In chapter IV, the writer has especially spoken of the dead eggs which appeared within several days after exposure to shock and occurred only in the eggs of certain stages between the closing stage of the blastopore and the "eyeing" stage.

After the exposure of eggs in this period to mechanical shock, there appear a number of individuals which show reddish colour in the animal pole and pale in the rest among the normal living eggs and the opaque dead eggs. These unusual eggs turn white and become dead eggs with ordinary appearance one after another within several days.

When such an egg is fixed with BOUIN'S solution before death and the chorion is removed, one can find that extra-embryonic blastoderm exists as usual enveloping the egg. Physical rupture has occurred clearly on the exposed part of the vitelline membrane which is still not covered with vascular area and from there the yolk flows out in the space between extra-embryonic blastoderm and the membrane of the vascular area. The vascular area, consequently, shrinks under the embryo enveloping the mass of oil globules. This is the reason why the animal pole appears reddish and the remaining part pale.

When extra-embryonic blastoderm bursts together with vitelline membrane, the yolk flows out into perivitelline space and the egg turns white in a comparatively short time. However, when the rupture occurs only in the exposed area of the vitelline membrane, the death of the embryo seems to be introduced at first and then the degeneration of extra-embryonic blastoderm is resulted. From this the egg turns white and opaque. This is perhaps the reason why some dead eggs appear after several days.

The above phenomenon is not observed for two or three days immediately after the closing of the blastopore. Perhaps extra-embryonic blastoderm may not be able to endure the stimulus beyond some limit though it becomes remarkably strong after the closing stage of the blastopore and may burst together with vitelline membrane allowing the yolk to flow out into perivitelline space. Therefore, some dead eggs appear in a comparatively short time.

Afterwards, extra-embryonic blastoderm becomes strong enough to endure such grade of stimulus as will induce the rupture of vitelline membrane. In this period, some dead eggs appear after several days as mentioned above.

This phenomenon disappears again at several days before the "eyeing" stage. The reason may be that the exposed area of vitelline membrane is reduced extremely by development of the vascular area.

Discussion and Summarization

As mentioned in detail in the present chapter, a visible rupture actually

is caused in vitelline membrane by mechanical stimulus in the egg of the dog-salmon; the yolk flowing out from the rupture into perivitelline space precipitates globulins on account of the diffusion of contained salts and turns white and opaque causing the death of the egg, as interpreted by GRAY (1932).

The resistance of the egg to mechanical stimulus decreases by and by after the insemination in fresh water until the closing stage of the blastopore. Perhaps the layer of protoplasm covering the yolk or vitelline membrane becomes thin more or less with the advance of the development as the result of participation of the protoplasm in the growth of the blastodisc; then that layer becomes mechanically weak.

Though vitelline membrane is covered with extra-embryonic blastoderm by and by after the gastrulation on the eighth day of the development, the blastoderm in this period is not strong enough to protect vitelline membrane; by mechanical stimulus the blastoderm is burst together with vitelline membrane.

On the sixteenth day of development at which the blastopore is perfectly closed up, the resistance of the egg suddenly increases and afterwards continues to increase until the time of hatching.

Extra-embryonic blastoderm becomes remarkably strong after the closure of the blastopore and sometimes remains untouched even when vitelline membrane is ruptured. In addition to this, the vascular area which begins to develop, extends between vitelline membrane and extra-embryonic blastoderm gradually and perfectly envelops the vitelline membrane at "eyeing" stage at about the twenty-eighth day of development. Afterwards, the vitelline membrane is covered perfectly with three membranes, viz., mesodermal splanchnic layer, somatic layer and ectodermal extra-embryonic blastoderm. The increase of resistance in this period seems to come from the extension and growth of these membranes which protect vitelline membrane mechanically.

After "eyeing" stage, the death of the egg seldom results following the rupture of membranes covering the yolk even by a considerably strong stimulus. By a strong stimulus primarily the death of the embryo is induced and then the membranes covering the yolk degenerate losing their impermeable property. Next, the egg turns white and opaque after several days.

KROGH and USSING (1937) have reported that the vitelline membrane of the rainbow trout egg becomes somewhat permeable to water and salts after "eyeing" stage. AFFLECK (1953) has also mentioned that the "eyeing" egg of the rainbow trout with ruptured vitelline membrane continued to develop normally. In the egg of the dog-salmon, however, the vitelline membrane seems to retain impermeability before the hatching. The egg always turns opaque and dies when the membrane is harmed artificially even a little.

Since the report of KOPSH (1898), it has been well known that the egg surface is finally enveloped with the membrane of the extra-embryonic blastoderm. However, no one has demonstrated that the second membrane or the vascular area additional to extra-embryonic blastoderm covers the egg entirely at "eyeing" stage.

VI. Development of Organs in Dog-Salmon Egg in Period of Susceptibility to Mechanical Disturbances

As already mentioned, the developing egg of the dog-salmon is most susceptible to mechanical stimulus during the period from about the tenth day until just the fifteenth day of development. The writer has interpreted in the preceding chapter that the susceptibility is closely related to the strength of the vitelline membrane. However, as various important organs develop successively in this period, certain of them may possibly be affected by mechanical stimulus and induce the death of the egg. On the development of the salmonoid fishes numerous works have been published by many authors in former times. These have been summarized in the text-book by ZIEGLER (1902) including his own works. Comparatively recently, SAITO (1950) and MAHON & HOAR (1956) have studied also on the early development of the dog-salmon. However, the above authors have not demonstrated the facts in relation to the development during this period of susceptibility to mechanical shock. Therefore, the writer has investigated the development of organs especially during the period from the tenth day until the seventeenth day. (The surface views of embryos in every day from the eighth day to the seventeenth day are shown in PLATE II. The chief organs observed in the sections of the tenth, eleventh, thirteenth, fifteenth and seventeenth day embryos are drawn in PLATES VI-IX).

Central nervous system: On the tenth day, the central nervous system is at the stage of the medullary cord. Afterwards, it begins to differentiate to the medullary canal starting from the fore-brain and progressing backward. On the thirteenth day, the anterior half of the medullary canal is completed and on the fifteenth day the differentiation finishes.

Though on the tenth day fore-, mid- and hind-brain are scarcely distinguished, they become distinct with the advance of development. On the thirteenth day, the mid-brain increases the width remarkably. The hind-brain shows V-shape in section. Five neuromeres are distinct. After the fourteenth day, the hind-brain gives rise clearly to the cerebellum and the medulla oblongata.

Eye: On the tenth day the eye is rudimentary. This grows into the optic vesicle on the eleventh day. At the same time the ectoderm which is in contact

with the vesicle begins to thicken to become the rudiment of the lense. On the twelfth day, the optic vesicle differentiates into the optic cup and the lense is completed. Then the retinal and the pigment layers develop.

Ear: On the tenth day, the ear appears first as a thickening of the ectoderm on either side behind the hind-brain. This grows to a pot-like vesicle on the twelfth day.

Pituitary gland: The infundibulum appears at the base of the fore-brain on the thirteenth day. It protrudes towards the posterior end of the mid-brain and comes in contact with the anterior end of the endoderm on the sixteenth day.

Notochord: On the tenth day, the notochord rises under the medullary cord.

Intestine: On the tenth day, the rudiment of the intestine develops on vitelline membrane from the endoderm with a thickening of the rudimental branchial pouch at the part of the auditory vesicle. The latter protrudes and is in contact with the ectoderm on next day. The second pair of branchial pouches appears on the eleventh day, the third pair on the fourteenth day, the fourth and the fifth pairs on the sixteenth day. Finally on the seventeenth day, six pairs of branchial creft are observed. A half of the tube-like intestine differentiates on the thirteenth day. On the fifteenth day, the stomach rudiment appears. Next day the bending of the stomach becomes distinct. On the seventeenth day, the intestine is completed and the anus opens.

KUPFFER's vesicle: On the tenth day the KUPFFER's vesicle exists in the endoderm at the caudal end. This grows little by little at first, then decreases again and becomes entirely extinguished on the fifteenth day.

Somite: On the tenth day there exist several pairs of somites. Their number increases rapidly with the advance of development.

After the thirteenth day, the dermatome and the myotome differentiate from the somite. The somatic and the splanchnic layers develop from the lateral plate, forming the rudiment of the body cavity between them. After the sixteenth day, the blood vessels containing blood cells appear between vitelline membrane and the splanchnic layer.

Vascular system: The heart appears rudimentally under the branchial pouches on the thirteenth day. After the fifteenth day the dorsal aorta grows with blood cells.

A pair of intermediate cell masses appears on the eleventh day. After the fifteenth day they begin to differentiate into the cardinal vein and blood cells.

Pronephros: The pronephros appears and the pronephric duct runs backward on the thirteenth day. After the seventeenth day the pronephros forms

the glomerules and the duct opens to the distal end of the intestine.

As mentioned above, the important organs develop successively in this period. However, so far as the morphological observations are concerned, it can not be explained why the resistance begins to increase after the sixteenth day of development.

VII. Resistance to Mechanical Shock in the Developing Egg of River Lamprey

ROLLEFSEN (1932) has mentioned in his study on the resistance of the developing egg of the cod that the remarkable resistance against the mechanical shock appears when the blastopore is perfectly closed up and the protoplasmic layer of the yolk sphere is covered with the membrane of extra-embryonic blastoderm. The writer has also recognized the strong resistance at the same stage in the developing egg of the dog-salmon.

The present experiment has been undertaken to ascertain the feature of the resistance of the lamprey egg which is of another type of development showing the total cleavage.

Material and Method

The river lamprey, *Lampetra japonica japonica* (MARTENS)* ascends the rivers numerously for spawning in spring along the coast of the Sea of Japan in Hokkaido. Mature fishes caught in the Chitose R. were transported alive to the laboratory. The eggs were inseminated artificially and they were reared in flat vessels until hatching at room temperature (15–20°C). The water was renewed once a day.

The following stages were employed for the present experiments:

Stage 1. Five hours after insemination; unsegmented stage (PLATE III; 1).

Stage 2. Twelve hours; eight cells stage (PLATE III; 2).

Stage 3. Twenty hours; morula stage (PLATE III; 3).

Stage 4. Thirty hours; blastula stage (PLATE III; 4).

This stage continues for about twenty hours.

Stage 5. Seventy hours; early gastrula stage (PLATE III; 5).

The egg is translucent at the animal pole and the invagination begins to appear at the vegetative pole.

Stage 6. Ninety hours; gastrula stage (PLATE III; 6). The invagination progresses remarkably.

Stage 7. Four days and eighteen hours; neurula stage (PLATE III; 7). The

* The specific name follows the paper of SATO (1951).

medullary plate is formed distinctly.

Stage 8. Five days and twenty hours; also neurula stage (PLATE III; 8). The medullary canal is formed and the head is distinguished from the abdomen.

Stage 9. Six days and twenty hours; late neurula stage (PLATE III; 9). The head prolongs further and begins to move at times.

Stage 10. Seven days and eight hours; about one day before the hatching (PLATE III; 10).

The egg measures a little less than 1 mm in diameter. The chorion of the egg is transparent and the egg content is opaque presenting a pale gray or pale green appearance. The fresh egg is demersal and slightly adhesive. However the adhesive character is almost lost after one day or more. The embryo hatches out at the 9th~12th day at the water temperature of $17\pm 2^{\circ}\text{C}$ (Experiment I), at the 8th~11th day at $19\pm 1^{\circ}\text{C}$ (Experiment II).

In the experiment, the eggs of certain stage were picked up in a pipette with a little water and were dropped down from 30 cm height drop by drop on several different points of a dry glass dish which is 12 cm in diameter and 3 cm in depth. As about fifty eggs were contained in a drop, it will be understood that several hundreds of eggs were employed for one experiment.

Then the glass dish was filled with fresh water and the eggs were reared until hatching. The dead eggs were counted and removed every day.

For the histological observation, the eggs were fixed in BOUIN's fixative solution and sectioned by the ordinary procedure. The borax-carmin method was used for staining.

Result of Experiments

As shown in Tables 7 and 8, Figs. 9 and 10, Stages 2, 3, 4 and 5 are easily affected by the dropping shock. Stage 1 is comparatively strong as compared with other stages. At Stage 6 a remarkable increase of the resistance occurs and afterwards the mortality remains low.

The egg of Stage 1 is not segmented at all. The same susceptibility to the shock has been observed also in the unsegmented stage of the egg of the dog-salmon. At Stage 6 there is also a remarkable increase of the resistance and the invagination is promoted distinctly.

The histological differentiation of cells does not occur until the blastula of Stage 4. In the early gastrula of Stage 5, the ectodermal epithelium slightly differentiates on the animal pole (PLATE III; 11-a, 11-b, 11-c). In the gastrula of Stage 6, the surface of the egg is perfectly enveloped with highly differentiated epithelium of the primary ectoderm (PLATE III; 12-a, 12-b, 12-c). Of course, the differentiation of the ectodermal covering progresses further with the advance

TABLE 7. Experiment I.

Stage of eggs		1	2	3	4	5
After insemin.		5 hrs	12 hrs	20 hrs	30 hrs	70 hrs
Experiment	Number of eggs	348	298	286	336	287
	Number of dead eggs	205	250	266	295	226
	Percent. of dead eggs	59.0	84.0	93.0	87.8	79.0
Control	Number of eggs	350	302	321	267	204
	Number of dead eggs	14	67	123	23	4
	Percent. of dead eggs	4.0	22.2	38.3	8.6	1.0

Stage of eggs		6	7	8	9	10
After insemin.		90 hrs	4d, 18h	5d, 20h	6d, 20h	7d, 8h
Experiment	Number of eggs	287	278	262	255	274
	Number of dead eggs	54	46	39	22	30
	Percent. of dead eggs	18.8	16.5	14.8	8.6	11.0
Control	Number of eggs	246	223	208	168	183
	Number of dead eggs	1	1	1	3	3
	Percent. of dead eggs	0.4	0.4	0.5	1.8	1.6

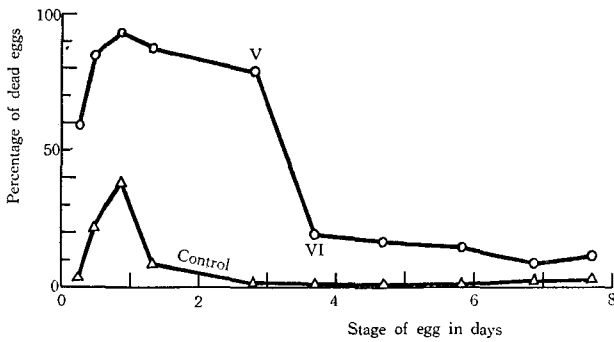


Fig. 8. Result of Experiment I.
 ○ Experiment,
 V: Stage 5, VI: Stage 6.

TABLE 8. Experiment II.

Stage of eggs		1	2	3	4	5
After insemin.		3 hrs	11 hrs	22 hrs	30 hrs	60 hrs
Experiment	Number of eggs	859	783	667	594	650
	Number of dead eggs	277	718	581	452	455
	Percent. of dead eggs	3.22	9.17	87.1	76.1	70.0
Control	Number of eggs	882	839	711	609	654
	Number of dead eggs	26	77	35	17	14
	Percent. of dead eggs	2.9	9.2	4.9	2.8	2.1

Stage of eggs		6	7	8	9	10
After insemin.		75 hrs	95 hrs	5d, 4h	6d, 0h	7d, 22h
Experiment	Number of eggs	547	597	638	624	575
	Number of dead eggs	174	107	187	128	68
	Percent. of dead eggs	31.8	17.9	29.3	20.5	11.8
Control	Number of eggs	645	598	690	634	622
	Number of dead eggs	7	11	20	13	30
	Percent. of dead eggs	1.1	1.8	2.9	2.0	4.8

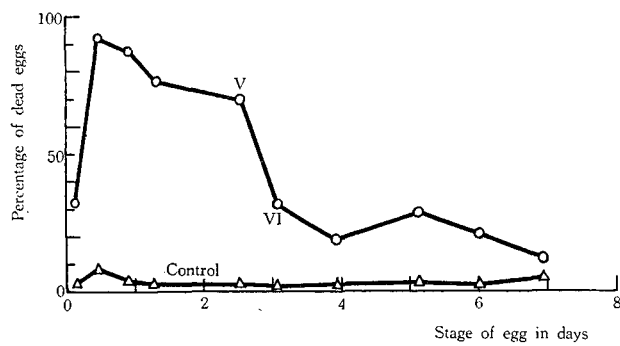


Fig. 9. Result of Experiment II.

○ Experiment,

V: Stage 5, VI: Stage 6.

of the development.

As mentioned above, the egg of the river lamprey increases its resistance remarkably at the period between Stages 5 and 6, when the egg is covered entirely with well differentiated epithelium of the ectoderm. This fact agrees with the case in the cod egg reported by ROLLEFSEN (1932) and the dog-salmon egg of the present writer.

However, the development of organs in the egg of the river lamprey progresses very slowly as compared with that of the cod and the dog-salmon and at the stage when the egg is covered by the ectodermal epithelium there is no differentiation of other organs, as may be seen in the sagittal section (PLATE III; 12-a) and the transverse section of Stage 6 (PLATE III; 12-d). In the dog-salmon, however, at the time when the ectodermal epithelium differentiates covering the whole surface of the egg, there are to be found well developed neural canal, notochord, intestine, somites, etc. It is obvious that the cod egg is also almost the same with this from the figure in the paper of ROLLEFSEN (1932).

From these facts, it may be concluded that the increase of the resistance has to do with the covering of the ectodermal epithelium but that the developmental stage of the embryo is little concerned.

The Rupture of the Surface of the Egg and the Death of the Egg

The consideration mentioned above is based on the assumption that the surface of the egg proper may rupture mechanically by the shock and thereby the egg may come to death.

Actually it can be observed under the low power microscope that various degrees of ruptures on the surface of an egg occur when it is dropped. To ascertain whether the rupture induces the death of the egg, the following experiments were carried out.

After dropping from 30 cm height, the eggs were carefully divided in two groups, "Non-ruptured eggs" and "Ruptured eggs" under the low power microscope and reared separately until hatching.

The results are shown in Table 9. In every stage before Stage 5, slightly damaged eggs which were difficult to distinguish exactly from "Non-ruptured eggs" appeared in large number. Some of them might be mingled in "Non-ruptured eggs" in Stages 1 and 2. Such doubtful eggs were all included in "Ruptured eggs" in Stages 3, 4 and 5, so "Non-ruptured eggs" were not found in these early stages. After Stage 6, even the eggs ruptured in the slightest degree could easily be distinguished from "Non-ruptured eggs", so there was

no likelihood to mingle such eggs in the group.

As shown in Table 9, dead eggs scarcely appeared in the group of "Non-ruptured eggs" except in stage 2 which might have contained some doubtful eggs, on the contrary, dead eggs occurred abundantly in the group of "Ruptured eggs". This fact suggests that shock does not directly affect the embryo but induces the death of the egg by rupturing the egg surface.

The ruptured eggs are observed abundantly in early stages reared under natural condition and such eggs die mostly. This tendency was observed also in the control of Experiment I. As the non-ruptured eggs were selected carefully, the mortality decreased in the control of Experiment II. When the wound is comparatively slight, sometimes the development advances normally leaving the pieces of torn-off yolk cells in the perivitelline space and finally the embryo hatches out. After gasturula stage, the ruptured eggs are found in comparatively small number and the embryo can develop in spite of considerably heavy wound,

TABLE 9.

Stage of eggs		1	2	3	4	5
Total number		348	298	286	336	287
Non-ruptured eggs	Number of eggs	113	69	0	0	0
	Percentage of eggs	32.5	23.2	0	0	0
	Number of dead eggs	9	29			
	Percentage of dead eggs	8.0	42.0			
Ruptured eggs	Number of eggs	235	229	286	336	287
	Percentage of eggs	67.5	76.8	100.0	100.0	100.0
	Number of dead eggs	196	221	266	295	226
	Percentage of dead eggs	83.4	96.5	93.0	87.8	79.0
Stage of eggs		6	7	8	9	10
Total number		287	278	262	255	274
Non-ruptured eggs	Number of eggs	73	29	57	85	188
	Percentage of eggs	25.5	10.5	21.8	33.4	31.5
	Number of dead eggs	0	0	0	0	4
	Percentage of dead eggs	0	0	0	0	2.1
Ruptured eggs	Number of eggs	214	249	205	170	86
	Percentage of eggs	74.5	89.5	78.2	66.6	68.5
	Number of dead eggs	54	46	39	22	26
	Percentage of dead eggs	25.5	18.5	19.0	13.0	30.2

though the hatching period is prolonged and deformed embryos are often born.

IX. Conclusion and Summary

The present study was performed in order to make clear the critical period of resistance of the developing fish egg against mechanical disturbance and its mechanism, chiefly artificially inseminated eggs of the dog-salmon, *Oncorhynchus keta* (WALBAUM) being used as materials.

(1) General observations on the development of the egg which was reared in running water at the temperature of $9\pm 1^{\circ}\text{C}$. were described at first in order to indicate the embryonal stages of experimented eggs clearly.

(2) The egg just after insemination is comparatively resistant to mechanical stimulus. The resistance, however, decreases gradually with the progress of development until the fifteenth day.

The decrease goes on regardless of the segmentation which begins at about nine hours after insemination.

The critical condition does not appear particularly in gastrulation which has been customarily accepted as a critical period in the course of development, but the resistance decreases successively from the preceding stage.

The most susceptible period exists during the stages which follow from ten days to fifteen days old, the eggs being easily induced to death even by means of the slightest mechanical stimulus.

In sixteen days old egg, a remarkable recovery of resistance appears suddenly and afterwards it continues to increase gradually until the time of hatching. Practically after the "eyeing" stage which corresponds to the twenty-eight day of development, the egg acquires strong resistance against almost any kind of artificial disturbance.

(3) When the eggs are exposed to mechanical stimulus, some of them turn white or opaque generally in a short time being recognized as dead eggs. This comes from the fact that the globulin of yolk precipitates for the protoplasmic layer covering the egg or vitelline membrane loses impermeability on account of the stimulus, as GRAY (1932) has explained in the egg of *Salmo salar*. However, it has not been made clear enough whether the mechanism consists in the invisible physiological degeneration of the membrane or in the visible physical rupture of it.

When the eggs are immersed in RINGER's solution instantly after the exposure to the stimulus, usually some of them become transparent. When the egg becomes gradually transparent after about ten minutes, a shrinking membrane can be easily observed from outside.

When the chorion of the eggs which are not treated with the stimulus is removed carefully after fixation by BOUIN's solution, vitelline membrane is revealed enveloping the egg perfectly. In the egg fixed at about one minute after treating with the stimulus, a round wound is seen in vitelline membrane and from there transparent yolk surface is exposed. In a case when fixed after several minutes, the wound shrinks to a ring which is clearly visible through the transparent yolk. In the egg at about ten minutes after treating, a great part of the yolk flows out and the membrane shrinks to an irregular mass at the animal pole enclosing the oil globules.

These facts clearly indicate that mechanical stimulus actually causes a physical rupture of vitelline membrane.

(4) Until the time of gastrulation, the surface of the egg is covered only with the protoplasmic layer or vitelline membrane. The membrane perhaps decreases in thickness with the advance of development, for the protoplasm both of the surface and of the inside of the egg must be moved toward the animal pole to perform the bipolar differentiation in the same manner as the case of most fishes, though it can not be observed directly in the egg of the dog-salmon on account of the almost opaque chorion.

This means that the membrane decreases resistance to mechanical stimulus gradually. So the resistance of the egg is comparatively strong just after the insemination but it decreases gradually with the advance of the development.

(5) After gastrulation, the extra-embryonic blastoderm begins to extend over the vitelline membrane until it covers the whole surface of the membrane except a small area of the blastopore in fifteen days old egg. In the cod egg, the resistance increases gradually with the development of extra-embryonic blastoderm. In the egg of the dog-salmon, it decreases successively from stage to stage. Perhaps the extra-embryonic blastoderm in this period is not strong enough to protect the vitelline membrane. As a matter of fact, it is often observed that with some stimulus the two membranes rupture together in the part of extra-embryonic blastoderm.

The blastopore is perfectly closed in the sixteenth day and the resistance of the egg begins to recover. The reason is that the extra-embryonic blastoderm increases in strength protecting the vitelline membrane. However, it bursts together with the membrane in case of an intense stimulus.

(6) Before the closure of the blastopore, the mesodermal vascular area which consists of somatic and splanchnic layers is proceeding to develop from the embryo between the vitelline membrane and extra-embryonic blastoderm. This extends to envelop the egg in front of the embryo at approximately the "eyeing" stage. The resistance of the egg which is increasing little by little

after the closure of the blastopore is chiefly due to the extension and the growth of the vascular area.

During this period, extra-embryonic blastoderm becomes strong gradually and sometimes it does not burst even when vitelline membrane is ruptured in the part which is not yet covered with the vascular area. In such a case, the eggs show unusual appearance being reddish in colour at the animal pole and pale in the remaining part, as the vascular area shrinks at the animal pole enclosing oil globules. These eggs turn opaque one by one after two or three days. Such eggs cease to appear just before the "eyeing" stage when the vascular area covers almost the whole vitelline membrane surface.

(7) After "eyeing" stage, the vitelline membrane is tightly covered with three membranes, namely splanchnic and somatic layers of vascular area and extra-embryonic blastoderm. Then the egg is seldom brought to death by the rupture of the membranes even by a considerably strong stimulus. With intense stimulus, primarily the death of the embryo is induced and then the membranes degenerate losing its impermeability to water and salts. The egg turns opaque after several days.

(8) As mentioned above, the susceptibility is related closely to the strength of the vitelline membrane. However, as various important organs develop one after another in the susceptible period, there is a possibility that certain organs may be affected by mechanical stimulus and that may lead to the death of the egg. Therefore, the development of organs in embryos especially during the stages from the tenth to seventeenth day were investigated in detail. However, so far as morphological observations are concerned, the writer has failed to explain why the resistance begins to recover suddenly in sixteen days old egg.

(9) The egg of river-lamprey, *Lampetra japonica japonica* (MARTENS) belongs to another type of development showing the total cleavage. The resistance of the egg was tested to compare with the case of the salmonoid and the cod eggs. The resistance gradually increases after the gastrula stage at which the egg surface is covered with well-differentiated epithelium of the ectoderm. This fact agrees with the case of the salmonoid and cod eggs. However, the developmental features of the lamprey embryo are different from those of the salmon; there are only primary ectoderm and endoderm, while in the latter the neural canal, notochord, intestine, etc. develop together with the covering.

The facts observed in the egg of the river-lamprey suggest that the resistance of fish eggs is based essentially on the protection afforded by the ectodermic tissue covering the egg surface, without any connection with the stage of development of the embryo.

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PLATE I.

Surface views of the developing embryo of the dog-salmon, *Oncorhynchus keta* (WALBAUM).

- Fig. 1. Blastodisc of the uniseminated egg before immersion into fresh water.
- Fig. 2. Blastodisc of the inseminated egg at one hour after the immersion into fresh water.
- Fig. 3. Blastodisc at five hours.
- Fig. 4. Blastodisc at eight hours.
- Fig. 5. Blastodisc at ten hours, two cells stage.
- Fig. 6. Blastodisc at sixteen hours, eight cells stage.
- Fig. 7. Blastodisc at twenty-four hours, morula stage.
- Fig. 8. Blastodisc at forty-eight hours, blastula stage.
- Fig. 9. Blastodisc at five days.
- Fig. 10. Blastodisc at seven days, germ-ring stage.
- Fig. 11. Same stage as Fig. 10.
- Fig. 12. Egg at eight days, early gastrula stage.
- Fig. 13. Egg at ten days.
- Fig. 14. Egg at twelve days.
- Fig. 15. Egg at fourteen days.
- Fig. 16. Egg at fifteen days.
- Fig. 17. Egg at seventeen days.
- Fig. 18. Egg at twenty days.
- Fig. 19. Egg at twenty-two days.
- Fig. 20. Egg at twenty-five days.
- Fig. 21. Egg at twenty-eight days, "eyeing" stage.
- Fig. 22. Egg at forty days.
- Fig. 23. Egg at fifty days.
- Fig. 24. Alevin just after hatching at sixty days.

(Figs. 1~10; ca. $\times 10$, Figs. 11~23; ca. $\times 4$, Fig. 24; ca. $\times 2.5$).

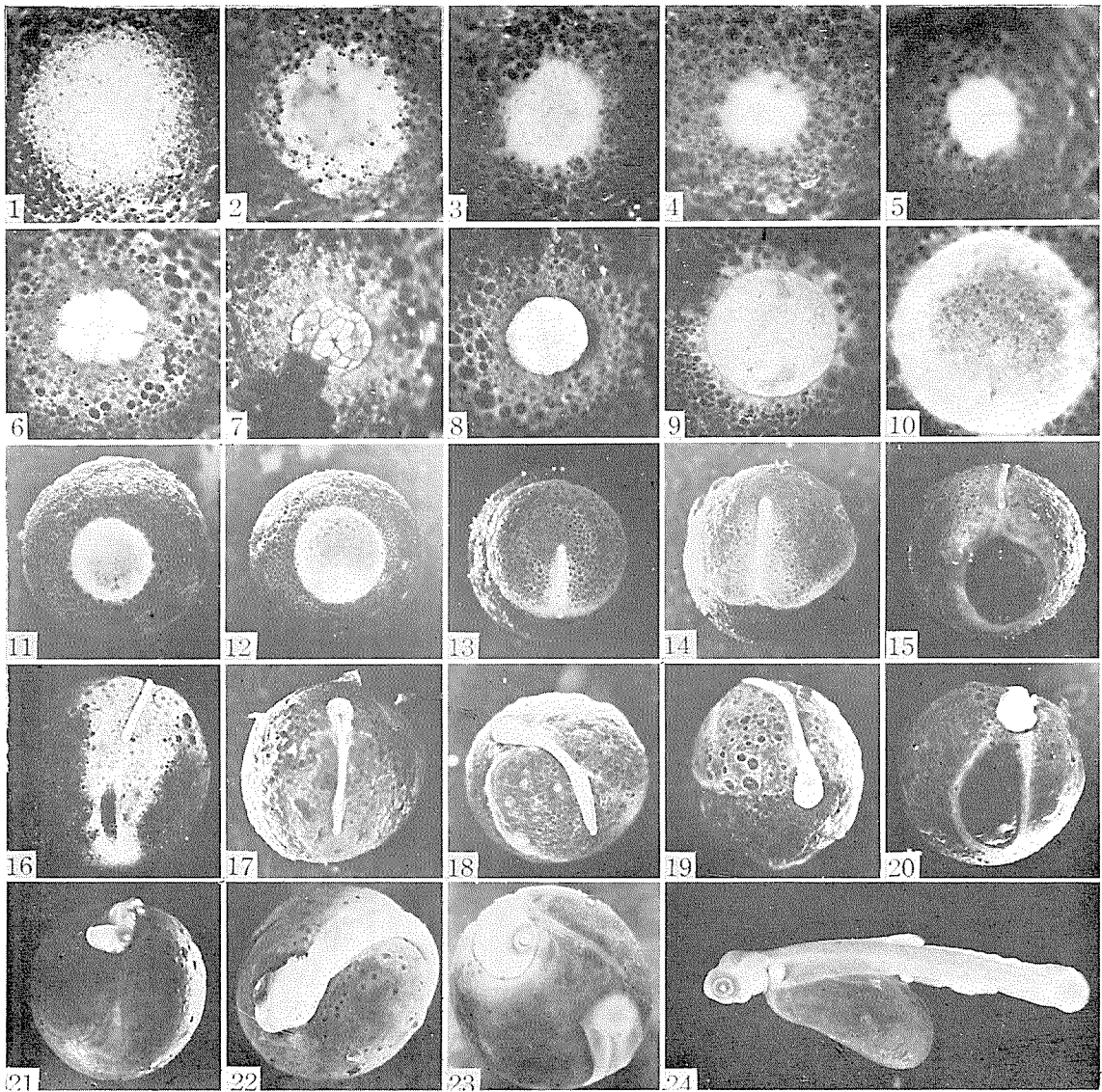


PLATE II.

Magnified views of the embryos of the dog-salmon during the period from the eighth day to the seventeenth day of development. ($\times 20$)

- Fig. 1. Eighth day embryo.
- Fig. 2. Ninth day embryo.
- Fig. 3. Tenth day embryo.
- Fig. 4. Eleventh day embryo.
- Fig. 5. Twelfth day embryo.
- Fig. 6. Thirteenth day embryo.
- Fig. 7. Fourteenth day embryo.
- Fig. 8. Fifteenth day embryo.
- Fig. 9. Sixteenth day embryo.
- Fig. 10. Seventeenth day embryo.

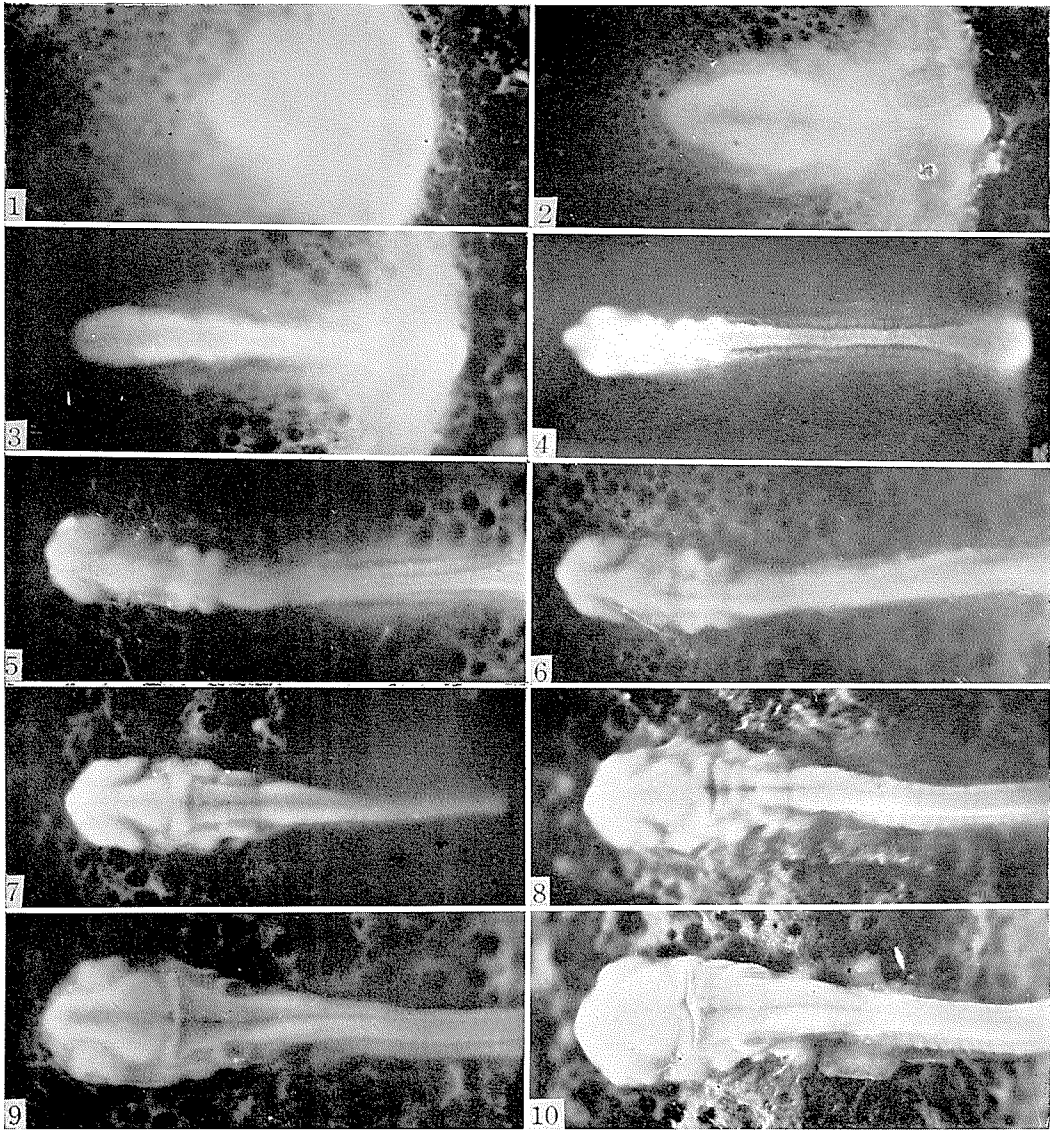


PLATE III.

Developing eggs of the river lamprey, *Lampetra japonica japonica*
(MARTENS).

- Figs. 1~10. Stages employed for dropping experiments.
- Fig. 11 a. Sagittal section of Stage 5.
 - 11 b. Magnified view of animal pole in Fig. 11a.
 - 11 c. Magnified view of vegetative pole in Fig. 11a.
- Fig. 12 a. Sagittal section of Stage 6.
 - 12 b. Magnified view of animal pole in Fig. 12a.
 - 12 c. Magnified view of vegetative pole in Fig. 12a.
 - 12 d. Cross section of Stage 6.

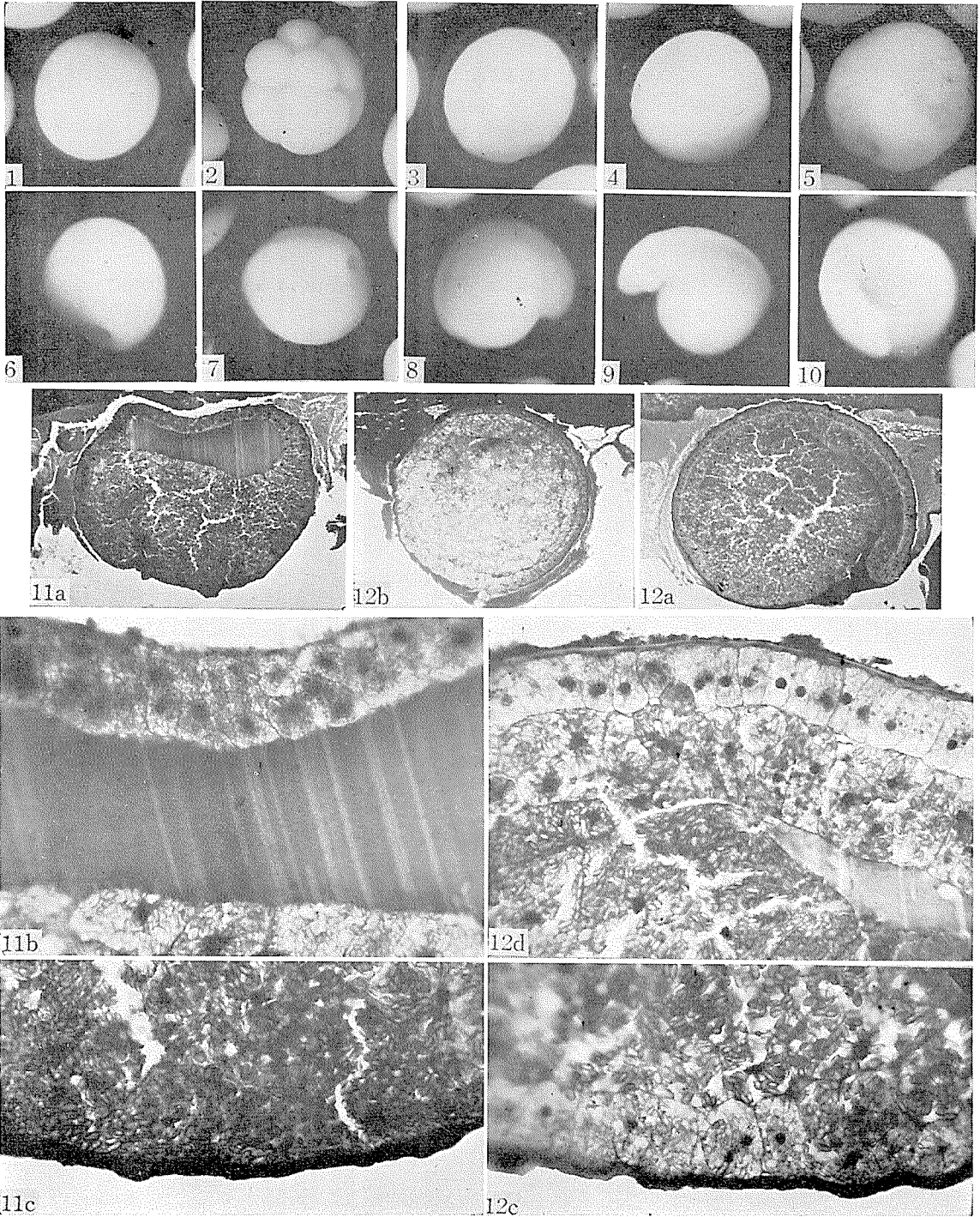


PLATE IV.

The structures of the coverings of yolk in the egg of the dog-salmon (1).

- Fig. 1 a. Cross section of unsegmented blastodisc with a part of vitelline membrane.
- 1 b. Magnified view showing direct communication between blastodisc and vitelline membrane.
- Fig. 2 a. Cross section of blastula at about three days of development with a part of vitelline membrane.
- 2 b. Magnified view showing loose connection between periderm of blastula and vitelline membrane.
- Fig. 3 a. Cross section in the region of embryo at neurula stage on the tenth day of development.
- 3 b. Cross section in marginal region of extra-embryonic blastoderm in neurula.

Abbreviations

- bl ... blastodisc or blastoderm
ch ... notochord
ect ... ectoderm, end ... endoderm, ep ... periderm
lat ... lateral plate
med ... medullary cord
n ... nucleus
per ... periblast
som ... somite, sub. per ... subgerminal periblast
vit ... vitelline membrane
y ... yolk granule

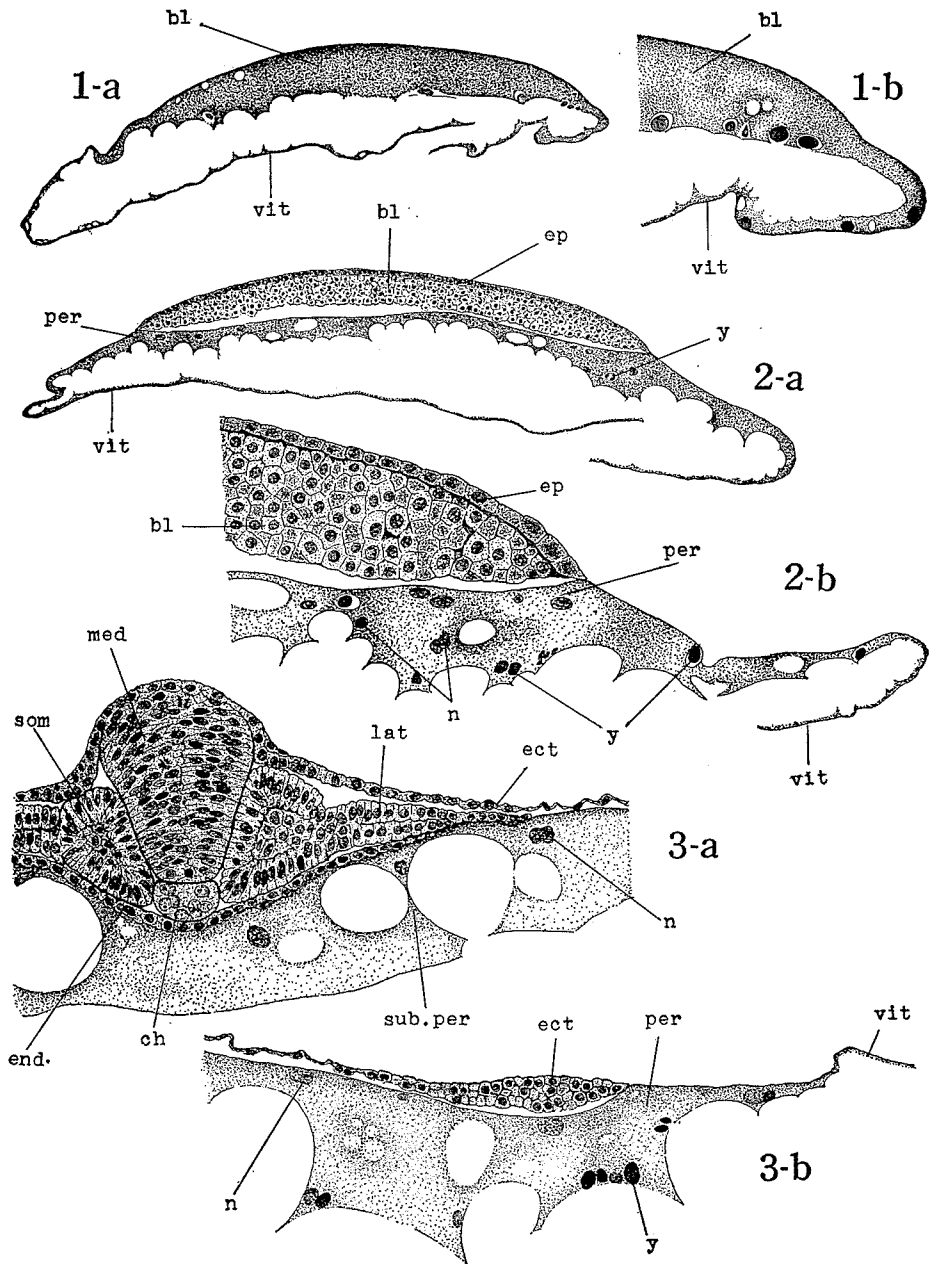


PLATE V.

The structures of the coverings of yolk in the egg of the dog-salmon (2).

- Fig. 4 a. Cross section of embryo on the fifteenth day of development.
4 b. Cross section in marginal region of vascular area in the same stage.
4 c. Cross section of outer region of vascular area in the same stage.
4 d. Cross section crossing blastopore in the same stage.
4 e. Cross section a little distant from the blastopore in the same stage.
Fig. 5 a. Cross section of embryo just before "eyeing" stage.
5 b. Cross section a little distant outside of the embryo in the same stage.

Abbreviations

- ao ... dorsal aorta
bl. c ... blood cells, bl. p ... blastopore
ch ... notochord
ect ... ectoderm, end ... endoderm
int ... intestine
med ... medullary tube, mes ... mesoderm
n ... nucleus
seg ... segmental duct, somat ... somatopleura,
 spl ... splanchnopleura
vit ... vitelline membrane
y ... yolk granule

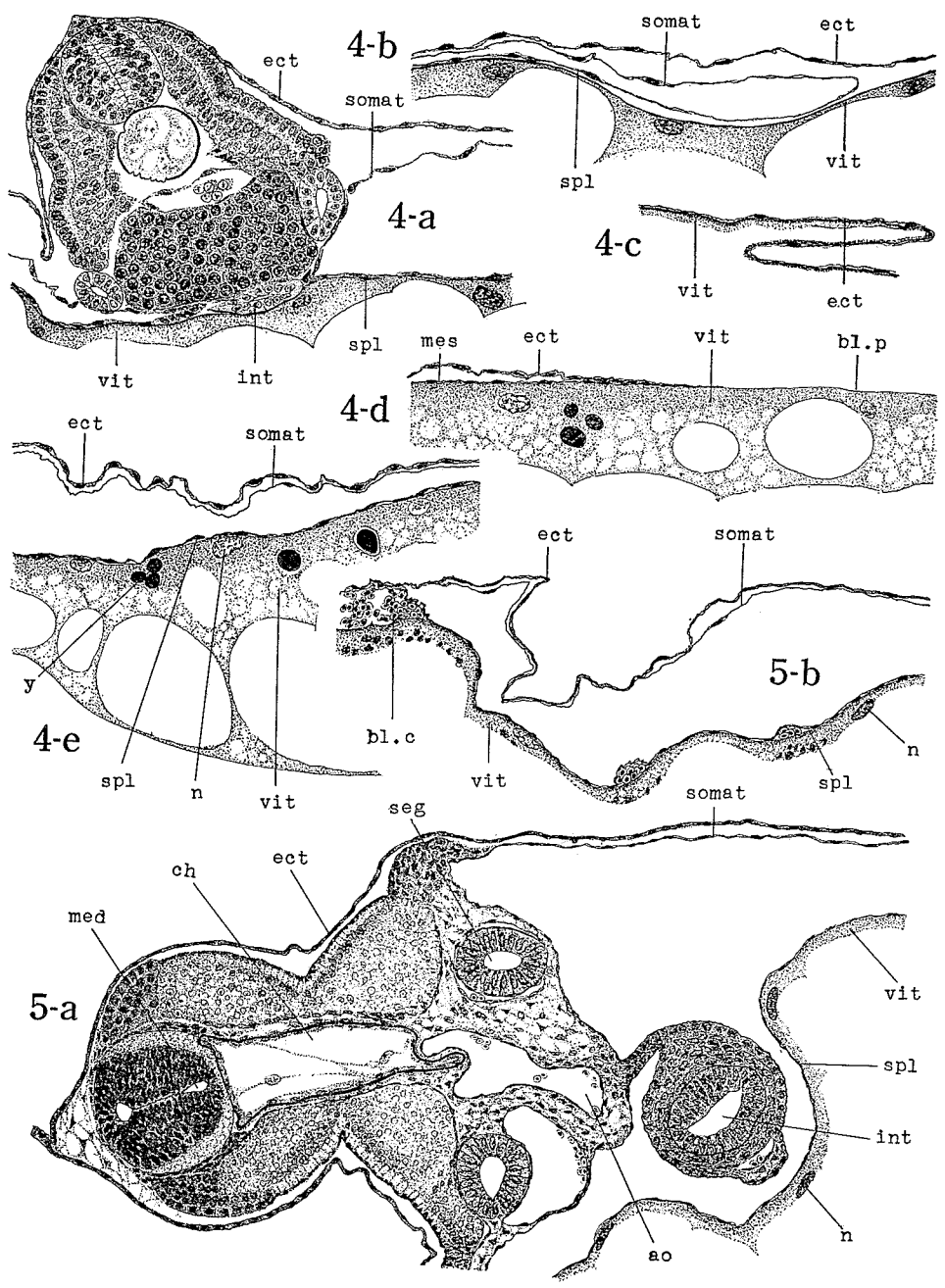


PLATE VI.

Development of organs in embryos of the dog-salmon on the tenth and eleventh days of development.

Fig. 6. Tenth day embryo.

- 6 a. Transverse section of forebrain.
- 6 b. Transverse section of hind-brain.
- 6 c. Transverse section of caudal end.

Fig. 7. Eleventh day embryo.

- 7 a. Transverse section of fore-brain.
- 7 b. Transverse section behind the preceding section.
- 7 c. Transverse section of hind-brain.
- 7 d. Transverse section crossing the seventh somite.
- 7 e. Transverse section crossing the thirteenth somite.

Abbreviations

aud ... auditory vesicle
bran ... branchial pouch
ch ... notochord
ect ... ectoderm, end ... endoderm
f. b ... fore-brain
h. b ... hind-brain
interm ... intermediate cell mass
K.v ... Kupffer's vesicle
lat ... lateral plate, le ... lense
m. b ... mid-brain, med ... medullary tube, mes ... mesoderm
n ... nucleus
op ... optic vesicle
peric ... pericardium
som ... somite, sub. per ... subgerminal periblast
y ... yolk granule

