THE CONDITIONS THAT LEAD TO NORMAL OR ABNORMAL DEVELOPMENT OF CIONA

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In earlier papers I have discussed the problem of normal versus abnormal development of cross-fertilized eggs of Ciona. It was shown that external conditions sometimes influence the result; at other times it was not apparent that the abnormal development was due to such conditions. However, by a critical series of experiments evidence was found that the eggs are very sensitive to what may be called the "cleanliness" of the dishes, i.e. to either chemical or organic contamination. Further experiments have been made to find out whether internal factors may also be concerned with abnormal development of the eggs.

Ciona is a particularly favorable type for study of this problem. The eggs, as soon as they are mature, leave the ovary and accumulate for 24 hours or longer in the oviduct. The germinal vesicle has disappeared and the polar spindle comes to lie at the pole of the egg. The polar bodies are not given off until a spermatozoon enters the egg, i.e. after the eggs have been set free in the sea water. This may happen every 24 hours or be delayed for several days. Spawning occurs early in the morning. If not ejected at the regular time the eggs may accumulate in the oviduct in very large numbers. When the animals are brought into the laboratory and kept in running sea water, or in sea water that is aerated by a stream of air bubbles, the Cionas may hold back their eggs for several days. It has been shown that these delayed eggs may give rise to normal embryos, but it has not been shown clearly that the percentage of abnormals may not be increased. Fresh eggs removed from the oviduct and kept for as long as 24 hours in covered dishes of shallow sea water can be cross fertilized and may give normal embryos.

The sperm are matured in the tubes of the testes that ramify over the walls of the intestine in the region of the outlet of the ovary. The ripe sperms pass into the long sperm duct that runs parallel to the oviduct. Only ripe sperms are present in the sperm duct, and some of them are set free when the eggs are ejected. When the eggs are collected by puncturing the oviduct, only mature eggs are set free, and likewise only ripe sperm comes out when the sperm duct is cut across. In most other marine animals, whose eggs are used for experimental purposes, one is apt to get ripe and unripe eggs when the ovary is opened. The uniformity of the eggs from the oviduct of Ciona simplifies the experiment.

In another respect, also, Ciona offers favorable material for a study of normal versus abnormal development. It is well known that polyspermy often plays a significant note in laboratory experiments, unless it is carefully guarded against. In Ciona, polyspermy also occurs after cross-fertilization, but is very rare and will account for only a small percentage of abnormality. It can easily be detected at the two-cell stage when the egg divides into four or more parts if polyspermic.

Even in cases where much abnormality occurs, I have found, by watching the cleavage, that polyspermy was not the main agent in the results.

Concerning the kind of abnormalities that occur, not much can be said. For convenience I have recognized three classes; (1) Normals, (2) Abnormals, and (3) Bent (or twisted) embryos. The abnormals are those that have not gone beyond an early stage of development. They are usually in the egg membrane and are spherical with one or two black "eye" specks. The twisted or bent are later stages in development, usually out of the egg membrane. The tail is present and crooked or bent, sometimes still coiled loosely or irregularly about the body. There are overlaps between the three different types. All three are generally found in the same dish, but the external or internal conditions that produce each kind are not known. It is safe to assume, however, that polyspermic eggs do not pass beyond the earliest stages of development, but external conditions may also stop the development in early stages.

Normal and Abnormal Development in Reciprocal Crosses

To test to what extent the ratios of normal to abnormal development are due to intrinsic (genetic?) factors or due to environmental conditions, further experiments were made in May 1944. Not only were reciprocal crosses made, but samples of the same eggs were crossed with three different sperm suspensions.

TABLE I

The percentage of normal embryos resulting from reciprocal crosses. The small numbers under the percentages represent, from left to right, the numbers of normal, bent, and abnormal embryos. The bent embryos have been left out of account in calculating the percentages.

c sp	*b sp*	*a sp*	
0	**93**	**8**	
0 0 148	126 0 8	12 42 132	*D eggs*
70	**89**	**99**	
67 9 28	49 2 6	128 0 1	*E eggs*
59	**96**	**49**	
73 33 49	310 1 12	51 7 54	*F eggs*
f sp	*e sp*	*d sp*	
0	**3**	**52**	
0 0 37	1 2 28	10 22 9	*A eggs*
14	**2**	**72**	
10 12 62	1 0 37	43 11 16	*B eggs*
16	**10**	**0**	
22 29 116	11 23 96	0 0 177	*C eggs*

Some of the eggs of A were distributed in three dishes (horizontal lines, Table I), also some of the eggs of B and C, each to three dishes. These were cross-fertilized with sperm of d, e and f. Two drops of sperm were added to each dish, and the normal, bent, and abnormal embryos recorded after 24 hours.

The eggs of D, E, and F had been put into three dishes each (horizontal lines) before the sperm of the same animals, d, e, and f, had been removed to cross-

fertilize the eggs of A, B, and C. Then two drops of the sperm suspension of a, b, and c were added to the D, E, and F eggs. These crosses are the reciprocals of the preceding ones.

In calculating the percentages, the bent embryos were left out of account. Most of them were so nearly fully developed that they might be classified with the normal, rather than with the abnormals. The bent embryos overlapped those abnormal embryos that were fairly well developed. The bent embryos would not have affected the percentages to any great extent if half of them had been combined with the normals and half with the abnormals.

As shown in Table I the percentages of normal embryos resulting from crossing A eggs by d sperm, e sperm and f sperm are very different (52, 3, 0%). This holds also for B eggs and C eggs by the same sperm. In other words, the results are different for the different combinations, and not consistent either for the eggs or for the sperm.

The reciprocal crosses are shown above in the same Table I. Comparing them, there is seen to be no agreement in the percentages. In other words, the reciprocals are no more likely to be alike than other crosses. Before discussing these results, another similar experiment will be described that was made the next day with Cionas brought to Pasadena (Table II).

This experiment was carried out in the same way as the last, except that in addition to the reciprocals, three more dishes of A, B, and C eggs were set out and crossed with sperm from three other Cionas, viz. g, h, and i. These serve to give further data for the same eggs with different sperm. The reciprocals of g, h, i were not made. Comparing the reciprocals of the first three tests, D, E, and F, each set are more like each other than are the sperm tests a, b, and c, and also more like each other than in the first experiment, but still not the same inter se. The F eggs were much fewer than the others and produced relatively much smaller numbers of normals.

A comparison of the three kinds of eggs, A, B, and C each with the six kinds of sperm, d–i, shows that there are wide differences in the percentages (Table II). Since the environment was as nearly the same as possible, it may seem to follow that the differences are due to internal factors of some kind, possibly genetic in origin.

Why are the reciprocals so often different? The diploid eggs are presumably all genetically alike in each Ciona before fertilization, but after the polar bodies are extruded, the eggs become haploid, and should give as many kinds of eggs as there are combinations of the six pairs of chromosomes (excluding the possibility of a larger number due to crossing over). If amongst these combinations there are certain combinations that might give abnormal development—lethals, in fact,—it might seem that the chances are about the same in the cross and in its reciprocal. In other words, even with many lethal factor combinations, any differences in reciprocal ratios would only be due to chance, unless the cytoplasm of the diploid egg is involved in the situation.

There are several more or less probable hypotheses that suggest themselves, and some of these may or may not have some connection with the theory of self-sterility in Ciona; for, if certain classes should be eliminated, those that remain and produce normal tadpoles might, theoretically, be the ones that carry factors for self-sterility.

An examination of the data from the dishes of the same eggs (horizontal lines; Table II), each fertilized by different sperm, show very great differences in most cases in the percentages of normals to abnormals. Occasionally, however, a set gives very low values (Table I, F eggs by (a), (b), (c) sperm) or very high ratios (Table II, E eggs by (a), (b), (c) sperm. Also an examination of the ratios of the same sperm used to fertilize three different lots of eggs shows sometimes high percentages in all three dishes (Table II, (d) sperm by A, B, C eggs) and at other times low percentages in all three dishes (Table II, (g) sperm by A, B, C eggs). On the other hand there are cases where each of the three sets of eggs, and also each of the three sets of sperm, show great differences in the ratios.

If the data are significant, i.e., if the differences are not due to infection in the

TABLE II

The percentages of normal embryos resulting from reciprocal and one way crosses. The small numbers under the percentages represent, from left to right, the numbers of normal, bent, and abnormal embryos. The bent embryos have been left out of account in calculating the percentages.

			c sp	*b sp*	*a sp*	
			97	**76**	**91**	
			71 2 1	287 8 35	152 3 14	*D eggs*
			98	**95**	**98**	
			303 12 6	256 3 9	289 11 5	*E eggs*
			3	**2**	**0**	
			1 0 32	1 0 48	0 0 34	*F eggs*
i sp	*h sp*	*g sp*	*f sp*	*e sp*	*d sp*	
99	**37**	**3**	**99**	**96**	**82**	
319 0 3	69 34 118	8 17 258	236 1 1	225 0 9	133 26 29	*A eggs*
2.8	**0**	**0.2**	**76**	**58**	**97**	
9 65 280	0 8 286	1 0 403	360 72 112	136 24 85	400 6 14	*B eggs*
0	**50**	**12**	**47**	**91**	**88**	
0 0 101	36 32 35	1 8 7	30 29 33	200 1 24	82 7 22	*C eggs*

dishes, these results show that the outcome is due neither to the eggs alone nor to the sperm alone, but to the combinations found at the time of fertilization.

In this connection there are some further considerations to be taken into account. The cytoplasm of the eggs has developed under the influence of the diploid set of chromosomes. If the constitution of this cytoplasm determines whether the development will be normal or abnormal, then the eggs of one individual might be expected to develop in the same way irrespective of what kind of sperm fertilizes the eggs. On the other hand, after the extrusion of the polar bodies, the haploid egg nucleus may have a very different make-up from the original diploid nucleus. But it is not likely that this haploid nucleus can affect or change the constitution of the cytoplasm in the very short time before it combines with the sperm nucleus to form a new diploid nucleus, whose products carry on throughout the following development. From the evidence discussed above it might seem, therefore, that whether the development is normal or abnormal will depend on whether the

combination of the two haploid genetic chromosome groups are harmonious or not in their action on the cytoplasm. If the cytoplasm is indifferent, then reciprocal crosses should give the same ratios, but since they do not do so, it may seem to follow that the cytoplasm, which has developed under the influence of the diploid egg nucleus, is one of the factors in determining whether normal or abnormal development takes place. This conclusion would mean that certain kinds of combinations are being continually eliminated. But it would not necessarily mean that these have anything to do with cross and self-fertilization of the combinations that survive. However, experiments that will be described later make it probable that the differences in the dishes reviewed in this experimnt are due to external factors, such as toxic infection, and are not due to inherited cytoplasmic differences in the eggs.

Test for Polyspermy and Abnormal Development

Five new sets of experiments were made (Table III) to test whether the abnormals that appear frequently are due to polyspermy, although it had often been recorded that the division of the eggs at once into four (or more) cells only very rarely occurs. Forty, 20 or 2 drops of sperm suspension were added to eggs in Syracuse dishes. After one hour the supernatant fluid was changed to fresh sea water. Reciprocal crosses were made in all cases. As the table shows, Table III, the relative number of normals to abnormals was no lower after 40 drops than after 2 drops of sperm suspension. Evidently polyspermy is not the cause of abnormal development. There are several striking exceptions to the general results. One (June 18–19, 1944), A^1–b^1, gave after 2 drops almost all abnormals (328), although after 40 or 20 drops nearly all were normal. Again (June 19–20, A^2–b^2, in one case after 20 drops all were abnormal (295); and in the reciprocal after 40 drops all were abnormal (199), although after 20 and 2 drops nearly all were normal. In another test (June 18–19), A^3–b^3, normals greatly predominated. In another (June 18–19), A^4–b^4, all were abnormal after 20 drops, but normal after 40 and after 2 drops sperm suspension. In another lot (June 17–18), A^5–b^5 and B^5–a^5, there were two cases where there were more abnormals than normals. These apparently contradictory results can only be explained on the assumption that the dishes were in some way responsible for the abnormal developments.

Delay in Fertilization and Abnormal Development

Whether or not a delay in fertilization causes abnormality after eggs and sperm have been kept in sea water was tested. The results are recorded in Table IV. The eggs of three individuals were distributed amongst six dishes. Then the eggs of A were fertilized by 10 drops of the sperm suspension of b and c at once, and after an hour and two hours. Similarly the eggs of B were fertilized by the sperm of a and c; and for the eggs of C by the sperm of a and b.

In A by b normals were produced by fertilizing at once, and after one hour, but nearly all abnormals after two hours. This is true also for A by c, but B by a and B by c and C by b gave more normals after two hours. C by a gave normals after one hour, but no fertilization after 2 hours. Perhaps the most striking results are A by b and A by c that gave normals at once and after a delay of one hour, but abnormals after two hours, but this did not happen in two other cases.

Either the sperm in the two latter cases was less affected by the delay, or there was some kind of difference in the dishes themselves. The irregularities in this experiment could only be accounted for on the assumption of infection in some of the dishes. The test was repeated (Oct. 14, 1944) with the eggs of new Cionas at

TABLE III

The effect of density of insemination in reciprocal crosses on the proportions of normal tadpoles, bent embryos, and abnormal embryos.

(June 18–19, 1944)

A^1 by b^1	Tad	Bent	Abn.	B^1 by a^1	Tad	Bent	Abn.
40 drops	405	0	0	40 drops	406	6	17
20 drops	443	0	2	20 drops	471	3	13
2 drops	3	40	328	2 drops	364	8	4

(June 19–20, 1944)

A^2 by b^2	Tad	Bent	Abn.	B^2 by a^2	Tad	Bent	Abn.
40 drops	122	21	39	40 drops	0	0	199
20 drops	0	0	295	20 drops	149	12	7
2 drops	351	0	0	2 drops	91	0	0

(June 18–19, 1944)

A^3 by b^3	Tad	Bent	Abn.	B^3 by a^3	Tad	Bent	Abn.
40 drops	211	5	0	40 drops	205	10	21
20 drops	268	7	17	20 drops	101	23	26
2 drops	148	14	7	2 drops	231	1	3

(June 18–19, 1944)

A^4 by b^4	Tad	Bent	Abn.	B^4 by a^4	Tad	Bent	Abn.
40 drops	230	0	0	40 drops	225	24	37
20 drops	0	0	263	20 drops	330	0	1
2 drops	266	0	0	2 drops	218	19	38

(June 17–18, 1944)

A^5 by b^5	Tad	Bent	Abn.	B^5 by a^5	Tad	Bent	Abn.
20 drops	377	6	7	20 drops	607	13	31
10 drops	293	3	1	10 drops	684	2	12
2 drops	45	43	116	2 drops	191	35	278

Corona Del Mar, and transferred to Pasadena in autoclaved flasks and stenders. Five of the lots fertilized at once, A by b, gave 99.9 per cent normals; one (B by c) gave 75 per cent normals. After 2 hours A by b gave 99.9; A by c, one normal and the rest unfertilized; A by c gave 0.5 per cent normal and the rest unfertilized.

TABLE IV

The effect of delaying insemination in reciprocal crosses on the proportions of normal, bent, and abnormal embryos, and unfertilized eggs.

		N	Bent	Abn.	Unfert.			N	Bent	Abn.	Unfert.
A by b	at once	504	0	7	6	B by a	at once	66	13	232	93
	1 hr.	343	0	1	0		1 hr.	131	2	84	98
	2 hrs.	3	5	390	25		2 hrs.	204	0	0	179
A by c	at once	428	0	20	27	C by a	at once	317	7	48	11
	1 hr.	464	0	9	27		1 hr.	304	1	11	12
	2 hrs.	14	30	364	4		2 hrs.	0	0	0	498
B by c	at once	763	8	39	20	C by b	at once	721	0	15	5
	1 hr.	483	14	63	4		1 hr.	870	0	8	6
	2 hrs.	888	2	10	7		2 hrs.	910	1	1	157

After 3 hours A by b gave 95 per cent normals and 5 per cent unfertilized; A by c gave 99 per cent normals and 1 per cent unfertilized; B by c were all unfertilized. Reciprocally B by a at once was, by oversight, not fertilized; but after 2 hours 99.8 per cent, and after 3 hours 100 per cent were normals. C by a at once gave 99.9 per cent normals; after 2 hours 99.9 per cent normals; and after 3 hours 100 per cent normals. C by b at once gave 100 per cent normals; after two hours 99.9; and after three hours none were fertilized.

Another test of the same sort gave much the same kind of result. The dishes had been boiled. The percentages of normals was not so high, but there was no evidence of more abnormals after one or 2 hours.

These tests make it quite clear that delay in fertilization up to 3 hours does not in itself cause abnormal development. The irregularities shown in Table IV may safely be ascribed to infection of the dishes. In fact other tests have shown that eggs may be kept in sea water for 24 hours, and, if fertilized with fresh sperm produce normal tadpoles.

Eggs Fertilized En Masse and Distributed at Two-Cell Stage to Separate Small Dishes

The exceptional cases, in which most or all of the eggs developed abnormally whereas others treated in the same way developed normally, suggested that some environmental conditions were responsible for the abnormals. The following experiments were made to further test this suggestion. Eggs were collected from the oviduct in large clusters, and then drawn up by a pipette and transferred to finger bowls with about 80 cc. sea water. They were cross-fertilized with 10 to 20 drops of sperm suspension. After about one hour, when the two-cell stage was reached, about 100 were picked out with a pipette and put into several small dishes or flasks where they remained for about 24 hours, and were then classified. The eggs in the two-cell stage should be nearly alike on the whole, and any difference observed should be environmental. As shown in Table V, there were found some dishes that gave a high percentage of abnormals. It would seem to follow that the differences are due to the environment rather than to internal factors. It is

especially to be noted that the eggs left in the finger bowls with more water generally gave nearly 100 per cent normals. The larger volume of water would be expected to dilute any contamination present in the larger dish or introduced with the eggs.

Again, a few eggs (about 100) in the two-cell stage were picked up with a pipette (2 or 3 drops) and each lot put into one of five small flasks, and into five jars with 10 cc. sea water. The flasks were stoppered and the jars had screw tops put on them to prevent evaporation. After twenty hours a few drops of formalin were added to each, and the condition of the embryos examined. Similarly the eggs of individual B were crossed by sperm of a. No counts were made

TABLE V

The effect of different glassware on the proportion of normal, bent, and abnormal embryos. Each of the two reciprocal crosses was made in a single finger bowl, and eggs in the two cell stage were pipetted into separate small dishes or flasks, in which they remained for about 24 hours.

A by b	N	Bent	Ab	B by a	N	Bent	Ab
1	101	0	3	1	0	0	271
2	103	0	0	2	150	0	0
3	88	2	6	3	0	0	176
4	164	2 short	2	4	125	1	1
5	29	62 bent	4	5	12	56	93
6	15	3	123	6	291	2	5
7	123	7	5	7	75	12	0
8	0	0	165	8	197	6 short	4
9	100	1	6	9	4	121 short	0 bent
10	148	0	4	10	8	68	14

of the 20 lots since practically all dishes contained either 99 or 100 per cent normal tadpoles, except one flask that had 38 tadpoles, 48 bent tadpoles, and 21 abnormal embryos. There was no evident differences between this dish and the others.

Another experiment, similar to the last, with fresh Cionas was made (Aug. 12, 1944). Five Syracuse dishes (12 cc.) were used for some eggs, 5 Stender dishes for other eggs, and 5 small flasks for others. The dishes were left at Corona del Mar, and formalin added to each after 20 hours. Reciprocal crosses B by a were made. The fertilized eggs, not used, were kept in finger bowls (covered) in about 80 cc. sea water. A by b in the finger bowls gave 95 per cent normals and 5 per cent abnormals. B by a gave 99 per cent normals. The records of the smaller dishes are given in Table VI. It is noticeable that while normals greatly predominated, especially in B by a, there are three striking exceptions where abnormals predominate, and other where a good many abnormals were present. Since the controls—the original larger finger bowls—gave 95 and 98 per cent normals, the exceptional cases in the smaller dishes must have been due to contamination of some kind.

TABLE VI

The effect of different glassware on the proportion of normal, bent, and abnormal embryos resulting from two reciprocal crosses.

		A by b			B by a		
		N	Bent	Ab	N	Bent	Abn
Syracuse	1	0	20	213	61	30	93
	2	121	9	12	115	22	10
	3	112	5	19	52	16	84
	4	100	24	6	157	0	7
	5	87	2	7	107	15	12
Stender	1	51	15	44	56	3	5
	2	80	26	24	188	0	1
	3	1	29	152	132	2	4
	4	81	2	15	134	1	3
	5	142	8	25	128	6	24
Flask	1	58	7	11	35	0	1
	2	89	2	14	77	—	6
	3	193	1	12	53	2	5
	4	72	3	17	86	1	0
	5	71	11	29	123	1	4

Another set of 20 tests was made with Syracuse dishes. The Cionas had been collected the day before and brought to Pasadena. They were in excellent condition with many stored eggs and much sperm. The eggs were inseminated with 30 drops of sperm in finger bowls of 80 cc. sea water. At the two-cell stage about 100 eggs were transferred to Syracuse dishes. Most of the dishes contained a high percentage of normals, but there were six dishes that contained nearly all abnormals, and one that contained a large excess of bent and short embryos.

In the same lot, there were three dishes of A by b left uncovered that gave 12 bent and 111 abnormal embryos; one half covered that gave 3 normals, and 15 bent; and one 7/8 covered that gave 153 normals and 1 abnormal. The three similar reciprocals, B by c, gave nearly all normals. Since evaporation must have been greater in those not covered, and partly covered dishes, than in the covered dishes, and since some of these gave normal, it does not seem probable that enough evaporation takes place in covered Syracuse dishes to cause abnormal development (see below). The large number of eggs kept in the finger bowl gave nearly all normals.

Another experiment was made in the same way (Aug. 21, 1944). Cross-fertilized in finger bowls, the eggs in the two-cell stage were distributed in eight Stender dishes. Five lots gave nearly all normals, but one gave all abnormals, and two gave more abnormals than normals. In the large number of eggs left over in the two finger bowls, one gave 100 per cent normals and the other 99.9 per cent normals. Obviously the conditions in three of the Stender dishes were unfavorable.

Effect of Evaporation of the Sea Water on Development

One of the possible influences that cause abnormal development might be evaporation of the sea water from the covered Syracuse dishes. To test this, 10

cc. of sea water was evaporated in the dishes to a slight extent before the eggs, that had been fertilized in a large volume of water, were transferred to the concentrated sea water (Oct. 16, 1944). As a control, two autoclaved dishes that had been covered, but their volumes reduced only to $9\frac{7}{10}$ and $9\frac{5}{10}$ cc. gave 100 per cent normals. Another dish, where the water had evaporated to $7\frac{9}{10}$ cc., gave all twisted and bent embryos. In another, evaporated to 7 cc., the eggs had remained in 2 cells after 24 hours. In general the water in a covered Syracuse dish is reduced during 24 hours by about $\frac{3}{10}$ to $\frac{5}{10}$ cc. As shown by these and other tests this amount of reduction does not interfere with normal development. Hence, the irregularities observed in the earlier experiments were not due to concentration of the sea water.

Sterilization of Dishes by HCl

Several tests in duplicate were made. In one (A by b, Sept. 3, 1944) the dishes had been sterilized with weak HCl and washed in distilled water and dried. There were eight that gave 95 to 100 normals, but two gave 100 per cent abnormals. Reciprocally, (B by a) there were six that gave normals (90 to 100 per cent) and four that gave mostly abnormals. The high frequency of dishes with abnormals may seem to be due to the acid, but if any was left after washing, it should have evaporated when the dishes were dried (24 hours). Many eggs were left in the finger bowls, and A by b and B by a each gave 100 per cent normals. Whatever the cause of occasional abnormal development, it appears that it was not removed from all the dishes by the HCl treatment. Either the HCl itself was not entirely removed by washing, or else something else was left behind after the treatment that was later the basis of contamination (infection) of the dishes. The result is in accord with earlier experiments of the same sort (*Biol. Bull.*, 80, 1941, pages 348–349).

Sterilization of Dishes by Heat

The records of the preceding experiments show almost without exception, one or more similarly treated dishes with abnormal embryos, while the rest contain only, or largely, normals. Such exceptions occur even when the eggs have been transferred to the smaller dishes at the two- or four-cell stage. The result suggests contamination or infection of some lots. Therefore, a new set of 10 duplicated lots and 10 reciprocals were tested in dishes that had been autoclaved (120° C.). The eggs were collected from the oviduct in such a dish and transferred to a large flask (also autoclaved) and there cross-fertilized. At the two-cell stage, 50 to 100 eggs were transferred to five flasks and five Stenders each, 20 in all. After 24 hours, A by b gave practically all normals, i.e. 85 to 100 per cent. The reciprocals all gave 100 per cent, except one lot that had 50 per cent somewhat bent tadpoles and 50 per cent normals. The eggs left over in the large flasks (A by b) gave 99 per cent normals and one per cent bent, about two thousand in all. In the reciprocal, there were about one thousand normal tadpoles.

Another experiment was made the next day with Cionas brought to Pasadena; 20 dishes were prepared as above (autoclaved). The eggs were kept in Syracuse dishes. They gave 99.9 per cent normals. The many eggs (about two thousand) left in a large beaker (sterilized) also gave 99.9 per cent normals.

A third experiment of the same kind in which the dishes had been boiled for a short time instead of autoclaved, gave nearly the same results. Most of the dishes, A by b, (Syracuse) gave from 95 to 100 per cent normals; only one had 75 per cent normals and 25 per cent abnormals. The reciprocals (B by a) gave five 90 to 98 per cent normals; four, 80 per cent, and one, 75 per cent normals. One (A by b) of the large finger bowls (80 cc.) gave 99.5 per cent normals and 0.5 per cent bent; the other gave only 5 normals, 1 bent and 94 abnormals. It is evident that this finger bowl (B by a) was contaminated, but the eggs that were removed from it at the two-cell stage and transferred to the ten smaller autoclaved dishes with 10 cc. fresh sea water gave 80 to 95 per cent normals.

These sixty tests are very convincing that the cause of abnormal development is due to some contaminating agent present in the dishes, or that develops there (bacterial action). Washing the dishes in tap water and even rinsing them in distilled water does not remove the source of the contamination, while autoclaving the dishes is effective. It should be recalled that in most of the earlier experiments the embryos after 24 hours were killed in weak formalin and the dishes allowed to stand for several hours or days, before washing them again in tap water. They were then dried for a day or longer. Whether the formalin combined with some organic matter in the dishes, or whether the organic matter alone is responsible for the contamination is not shown by these experiments.

Another test was made (September 30–October 1, 1944) with fresh Ciona at Corona del Mar. The eggs were brought to Pasadena in autoclaved flasks and Stenders. Eggs had been fertilized in large finger bowls (80 cc.), and transferred in an early cleavage stage to 5 flasks and 5 stenders, 20 in all. Only normal tadpoles developed. Again, a similar experiment was carried out at Pasadena (Oct. 1–2), but the eggs were transferred to 20 Syracuse dishes that had not been autoclaved since the last time they were used. They gave 99 per cent normals in all, but 5 dishes (out of 20) gave 95 per cent. These two tests corroborated the conclusion that in clean dishes nearly 100 per cent are normal tadpoles. The very small percentage of abnormals may be due to polyspermy, or to other defects in the cleavage.

A different test was carried out as follows (Oct. 1, 1944). A set of 20 dishes was made up as above, using Syracuse dishes that had been washed, but not autoclaved. There were 14 that gave 95 per cent to 100 per cent normals; 4 that gave only abnormals and one that gave 80 per cent normals. The 4 that gave abnormals were carried one step further. The water was drawn off and put into an autoclaved Stender. To the original Syracuse dishes, 10 cc. of fresh sea water was added. Eggs of another individual, in the two cell stage were added to each. After 22 hours all of the Stenders had only abnormal embryos. Evidently the water had been fouled in some way. The six original dishes with fresh water gave 95 per cent normals, 5 per cent abnormals; 50 per cent normals; 100 per cent abnormals; 100 per cent normals; 95 per cent normals; and 80 per cent normals. These eggs did much better in the old dishes with fresh sea water than in the old sea water in a fresh dish.

As a control test, six of the original (Syracuse) dishes that had given only normals were treated in the same way. The six original dishes with fresh sea water (10 cc.) gave all normals. The old sea water from 4 dishes transferred

to Stenders gave in one dish normals, another gave abnormals, and another 50 normals and 50 abnormals. More normals resulted than when the original dishes that gave only abnormals were tested. These results are consistent with the assumption that abnormal development is due to toxic material that develops in some of the dishes. It does not seem to be due to the eggs themselves, but to some foreign material present in some of the dishes. Ordinary washing does not remove the material, but sterilization of the dishes does remove it. If the toxic material is due to bacteria left in the dishes, the protein or other substance on which the bacteria grow may be due either to some substance left in the dishes, or to organic material that goes over with the eggs or the sperm.

Summary

Eggs of Ciona that develop in covered Syracuse dishes produce as a rule normal tadpoles in the course of 24 hours, but occasionally only abnormal embryos develop, or both normal and abnormal in the same dish.

Reciprocal crosses sometimes give very different proportions of normals and abnormals, but the relations are often very inconsistent. The results cannot be ascribed to the eggs alone or to the sperm or to their combinations. Even if an attempt is made to refer the outcome of the reciprocal crosses to the cytoplasm of the egg, that has developed under the influence of the diploid nucleus of the egg, still the differences cannot be satisfactorily accounted for.

Polyspermy can account for only a very small percentage of abnormal development, probably not more than half of one per cent.

Delay in fertilizing the eggs does not cause abnormal development. Delay in using the sperm suspension does not cause abnormals.

When the eggs of one individual are fertilized by the same amount of sperm suspension of six other individuals, the outcome may be normals in each dish or normals and abnormals, or occasionally, all are abnormals. When the sperm of one individual is used to fertilize the eggs of six others, the same kind of results may happen.

In order to make the samples of eggs as nearly alike as possible, they were first fertilized in a large volume of water, and, when in the two-cell stage, a few were transferred to several small Syracuse dishes. The same kind of irregularities appeared, which cannot be due to chance selection of different eggs, for the original eggs left in the large amount of water generally gave more than 95 per cent normals.

Therefore, several kinds of experiments were made to find out whether differences in the small dishes will account for the occasional, but persistent, appearance of abnormals. (1) Dirt or impurities in the sea water from the tap was excluded. (2) Evaporation of the sea water in the covered Syracuse dishes was not enough to cause abnormal development. (3) Ordinary washing of the dishes, even rinsing them in distilled water did not remove the cause of abnormal development in some of the dishes. (4) But sterilizing the dishes in an autoclave removed the cause of most of the irregularities.

It follows that the cause of the exceptional cases of abnormals is due to some contamination that remains in the dishes after washing them in fresh tap water. Since the contamination does not affect the early development of eggs (cleavage),

but only later stages, it must be putrefactive in origin; and since it is removed by autoclaving the dishes, it is probably bacterial or some sort of organic contamination.

The eggs come to rest on the bottom of the dishes in a few minutes where they remain until the tadpole emerges, hence local differences may affect the development and account for those cases where both normal and abnormal development takes place in the same dish.

The discovery of the cause of the occasional abnormal development removes the possibility that it is due to genetic factors, hence is not concerned with the self-sterility of Ciona.