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Experiments on the Gill Formation in the Urodelian *Triturus*

By

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With Plates VI—VII and 13 Text-figures

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Introduction

The ectoderm surrounding the branchial region of an amphibian embryo has the power of producing the gills, which fact has been well established by the experiments of EKMAN ('13), HARRISON ('21), DETWILER ('22) and SEVERINGHAUS ('30). EKMAN points out in *Bombinator pachypus* that the external gill can be derived from the ectoderm of the pronephric and cardiac regions, if it is transplanted to the branchial site, but not from that of the other parts of the body. EKMAN ('22) obtained also in *Rana esculenta* and *Rana fusca* the gill slits and inner gills from the other ectoderm up to the time when distinct gill plates appeared, though the formation was not quite perfect. HARRISON ('21) extended the results of EKMAN's experiments to the embryo of *Amblystoma punctatum* with closed medullary folds, and verified the gill-forming faculty of the ectoderm surrounding the branchial region, particularly that posterior to it, with diminishing intensity as the distance from the proper site increases. DETWILER ('22) reports nearly the same results as HARRISON in the same embryo in the tail-bud stage. Transplanting the fore-limb rudiment to the branchial region, he obtained in some cases abortive gills from the graft. SEVERINGHAUS' experiments ('30) on the same lines support the above-cited accounts of EKMAN, HARRISON and DETWILER. The ectoderm adjacent to the

branchial region can produce the gills on the proper site, but not ectoderm from the frank; nevertheless if a strip of the latter is grafted across the center of the branchial mound, it can be incorporated in the branchial ectoderm, without suppressing the development of the gills.

It is quite clear from the above citations that the gill-forming power resides not only in the gill region, but also extends beyond this to the surrounding area. The limits of this extension have not yet been defined. Generally, however, the ectoderm transplanted from the posterior parts of the embryo seems not to produce the gills, though EKMAN obtained the gill slits and inner gills in some anuran embryos. HARRISON, DETWILER and SEVERINGHAUS have worked on the tail-bud stage of *Amblystoma* embryo, so that it remains us to examine the gill-forming power of an embryo in an earlier stage than this, and if this power is distributed over the entire body, to show how in course of time it diminishes and becomes specific and is finally confined within the branchial region.

The self-differentiation of the branchial ectoderm, its polarity and the rôle played by the germ layers in the gill formation have been partly studied by EKMAN, HARRISON and SEVERINGHAUS. EKMAN believes that the ectoderm has the power of self-differentiation and definite orientation, while the endoderm is not concerned in the formation of the gills. As for the mesoderm, he states that it is necessary to form the blood vessels which are the essential factors in the subsequent development of the gills. However, HARRISON maintains that the gill pattern is not laid down in the ectoderm, but in some deeper layers, particularly in the mesoderm. HARRISON is of the opinion that the endoderm plays some important rôle and is not merely passive as EKMAN believes. Finally, according to SEVERINGHAUS the gill pattern is laid down in the endoderm which he considers the most important part in the development of the gills.

Under these circumstances, it is interesting and important to work out the nature of the germ layers with regard to gill formation.

The experiments to be described were carried out on the embryo of *Triturus pyrrhogaster* (BOIE), from the middle of April to the end of July in 1932, under the direction of Professor Yô K. OKADA, to whom I wish to express my indebtedness for his kindly suggestions and criticism.

Material and Methods

The adult animals were collected in the vicinity of Kyoto and made to lay eggs in an aquarium.

Embryos from stage 16 to 23 of KOYAMA'S normal plates of development (see Dobutsugaku Zasshi, vol. 42) were chosen as donors for most of the experiments with the ectoderm. At stage 16 the medullary folds are distinct, and not fused in any part of them (early neurula stage), they afterwards become partly closed in the middle (late neurula stage) and then completely (stage of the closed medullary folds). At stage 23 the gill plates become distinct (stage of the distinct gill plates). Embryos at stage 29 (just prior to the gill formation) were used in some cases as sources of the deeper layers, when the nature of these was examined.

The embryos chosen as recipients were always those from stage 23 to 25 where the gill plates were distinct.

In most cases the recipient embryos were previously stained *intra vitam* with Nile blue sulphate but in a few cases the donor embryos were contrarily stained. Such a treatment is indispensable, since the graft is apt to be obscured and it very often becomes difficult to say whether the gills are derived from the graft or from the recipient. In my experience, better results are always obtainable when the stained recipient is used. Moreover, with this staining it is easy to distinguish the epithelium from the deeper layers.

Operations were made with SPEMANN'S glass needle and hair-loop mostly on the right side of the embryo. (Unless otherwise stated, all descriptions apply to this side.) The pieces to be transplanted were cut in squares no more than 0.8 mm. in length in ¼ RINGER'S solution and quickly mounted on the required position in the recipient, the surface of which had been properly cut out before to receive it. After the wound was ascertained to have healed—generally in 2 or 3 hours after the operation, each individual was reared in a separate glass bowl filled with ordinary tap water, which was changed, day by day, by means of a pipette. No anaesthetics were used throughout the whole experiment.

Experiment I

The early neurula

Under this category come those embryos at stages 16 and 17 which correspond to HARRISON's stages 17 and 18 of *Amblystoma punctatum* respectively. In either case practically the same results are obtained.

For convenience, the outer surface of the embryo is divided into 4 regions; anterior, cardiac, pleural and abdominal, each of which is further subdivided into small sections as shown in Fig. 1.

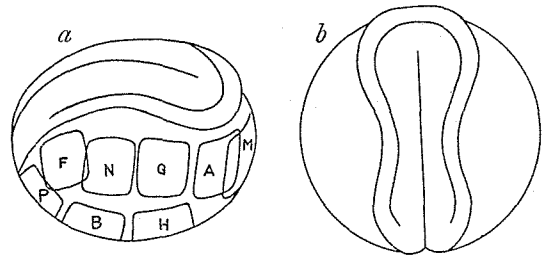

Fig. 1 *a* Profile of an early neurula, the parts used for transplantation being indicated by different squares. *b* Dorsal view of the same embryo. A, antero-lateral part; B, median abdominal part; F, postero-lateral part; G, branchial region; H, cardiac region; M, oral part; N, pronephric part; P, postero-abdominal part.

Pleural region

Just behind the branchial part (N): The ectoderm of this part is well proved to produce gills with great facility. One of the transplantations made on May 21 may be chosen for description. Five days after the operation 3 gill buds appeared on the graft, their development being somewhat retarded as compared with those on the unoperated side, but finally developed into typical gills (Fig. 2, *d*). The fact that the development of the gill is retarded on the operated side can apparently be attributed to the effect of the operation, for even if the branchial ectoderm is cut out *in situ* and placed again on the same site or if the wound is filled up with the branchial ectoderm from another embryo at the same stage, there is always some delay in the appearance of the gills on this side, although the gill-forming power of the ectoderm in question must be the same as on the other side. Another example of the same operation is shown in Photo. 1. In this specimen, however, the transplant is shifted somewhat posterior to the normal site, and accordingly the second and the third gills are derived from the graft, while the first is formed from the host.

Posterior part (F): Although it is said by EKMAN in *Bombinator* and by HARRISON and other American authors in *Amblystoma* that

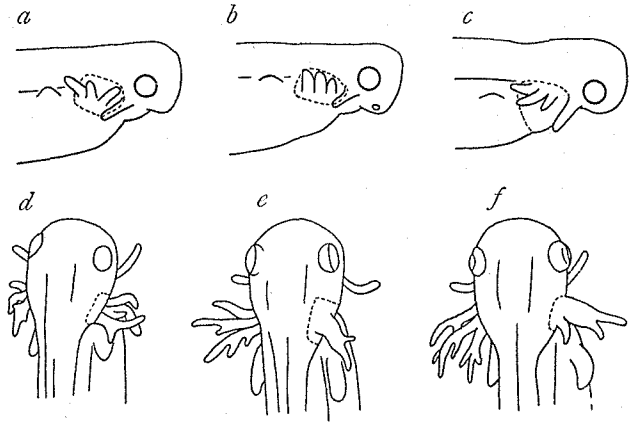

Fig. 2 Developed gills from the transplanted ectoderm at the early neurula stage, dotted line indicating the boundary of the grafted piece. (*a–c* profile, *d–f* dorsal view) *a* Showing 3 gills developing on the graft of the postero-abdominal part, the graft extending into the posterior part of the balancer; 7 days after the operation. *b* Showing 3 gills developing on the graft of the median abdominal part, the graft extending into the posterior part of the balancer; 5 days after the operation. *c* Showing 3 gills developing on the graft of the antero-lateral part; 6 days after the operation. *d* Showing 3 gills developing on the graft of the pronephric part; 7 days after the operation. *e* Showing an abnormal gill on the graft of the postero-lateral part; 8 days after the operation. *f* Showing 2 abnormal gills, perhaps fusion taking place in the first and the second gills on the graft of the oral parts; 8 days after the operation.

the ectoderm of this flank part has no gill-forming faculty, in our experiments gills could be distinctly derived from it. In one case shown in Fig. 2, *e* we find a large abnormal gill which is probably due to a fusion of the second and the third. There was considerable delay in the appearance of the abnormal gill. Abnormality in shape and delay in appearance of the gill are presumably to be attributed to the injury inflicted at the time of the operation as in the preceding case.

Abdominal region

Posterior part (P): The ectoderm of this part possesses the power of forming gills in a high degree. One of the experiments was performed on May 31. Four days after the operation 2 gill buds appeared on the graft with a slight retardation as compared with those on the other side. The third gill appeared later and remained in an abortive state. In this example the grafted ectoderm was farther extended forwards and produced a balancer, though it was not quite certain whether the graft had produced the balancer from its own tissue or

whether it was passively incorporated in the latter (Photo. 2). Fig. 2, *a* is another example, in which the normal number of gills appeared from the first on the graft.

Middle part (B): From the character of the preceding part it is to be supposed that the ectoderm of this part will produce gills more easily, and this expectation is confirmed by the experimental test. Fig. 2, *b* is one of the examples operated on on June 2, in which we observe the gill buds (4 days after the operation) both on the operated and the unoperated sides.

Cardiac region

The experimental cases, though few in number, confirm what EKMAN and HARRISON stated in regard to *Bombinator* and *Amblystoma*; the ectoderm of this part possesses a high power of forming gills. The experiments were made on May 30. Four days after the grafting 3 gill buds were formed in one case, and 2 in another, the second gill being undeveloped.

Anterior region

Antero-lateral part (A): Experimental tests prove that the ectoderm of this part produces the gills almost as well as that of the branchial region. Two embryos were operated on on May 22 but one died before giving any result. In the surviving embryo gills made their appearance simultaneously on both sides 5 days after the operation (Fig. 2, *c*), but the third gill on the graft side was not well developed (Photo. 3).

Oral part (M): This is the region where the mouth appears later, but at the time of the operation its opening is, of course, not yet formed. This part is proved to have also a high power of forming gills. Fig. 2, *f* shows one of the experimental examples, sketched 8 days after the operation. In this specimen 2 gills appeared on the graft, the anterior one being the larger. It is perhaps a fusion of the first and the second gills. Photo. 4 shows the same specimen 20 days later.

From these tests of the power of the ectoderm in the early neurula stage to produce gills, it may be concluded that the gill-forming faculty is still widely distributed in the entire surface of the body.

The neural ectoderm between 2 medullary folds was not tested. It has already morphologically differentiated even in the shape of the

cells and seems incapable of producing the gills under any conditions.

Table I

Grafts taken from the early neurula

		Number of experiments	Available cases	Shape and Size		Number of gills from the graft			
				Normal or nearly normal	Abnormal	3	2	1	0
Pleural region	Ant.	4	3	3	0	2	1	0	0
	Mid.	—	—	—	—	—	—	—	—
	Post.	4	2	0	2	0	1	1	0
Abdominal region	Mid.	3	2	2	0	2	0	0	0
	Post.	7	4	2	2	2	0	2	0
Cardiac region		2	2	1	1	1	1	0	0
Anterior region	Oral	2	1	0	1	0	1	0	0
	Ant.-Lat.	2	1	1	0	1	0	0	0

The late neurula

The embryos contained in this group are those from stage 19 to that a little prior to stage 20 of KOYAMA's normal plates and correspond to those in HARRISON's stages 19 and 20 of *Amblystoma* respectively; that is, the medullary folds are partly fused in the middle, while they are still open in other parts. Grafting experiments were made in various parts, as in the previous test, in order to examine whether the gill-producing faculty is still distributed all over the body or whether it is already partly restricted to certain regions.

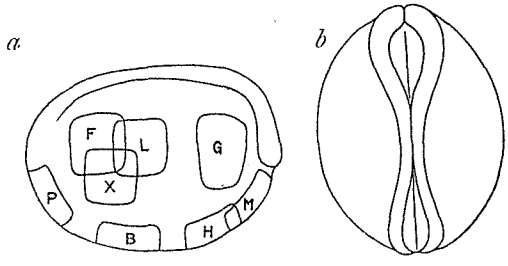

Fig. 3 *a* Profile of a late neurula. *b* Dorsal view of the same embryo, showing medullary folds fused in the median part. L, pleuro-median part; X, pleuro-ventral part; other letters as Fig. 1.

Pleural region

Median part (L): The tests started with the pleuro-median part, since if the gills are derived from the ectoderm of this middle part, the ectoderm anterior to it should possess the same faculty. A grafting of the ectoderm was made on June 9 on an embryo at stage 19, and 4 days after the operation 2 gill buds appeared on the graft, developing nearly at the same rate as those on the unoperated side. Fig. 4, *a* shows the same individual bearing the gills 2 days old. The embryos employed for the experiments of June 7 were slightly

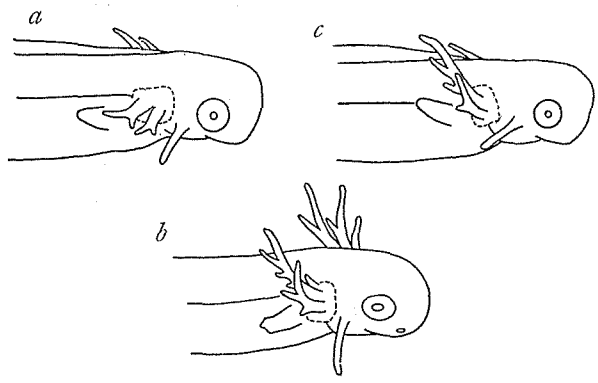

Fig. 4 Right side view of larvae with induced gills on the non-branchial ectoderm grafted at the late neurula stage. *a* Showing 2 gills developing on the graft of the pleuro-median part; 6 days after the operation. *b* Showing 3 gills developing on the graft of the cardiac region, the third being still rudimentary; 8 days after the operation. *c* Showing 2 gills developing on the graft of the oral part; 6 days after the operation.

older (stage 20) than the above mentioned case and quite different results were obtained. In none of these cases did the median part produce gills, though the graft swelled out a little; it seems, therefore, that the part in question represents the critical place for the production of the gills at this stage.

Posterior part (F): In order to confirm the above supposition, the part caudal to the preceding was next tested, and the result was affirmative. In all cases gills were not produced from the ectoderm of this part, except in one case where a rudimentary gill bud was formed. It was abortive and never developed into a gill.

Pleuro-ventral part (X): This part is found ventral to the preceding L and F parts, partly overlapping them as shown in Fig. 3. The tests of the ectoderm transplanted to the branchial site were

negative in all instances. The first transplantation was made on June 7, and on the third day gill buds began to be formed on the unoperated side, but not on the operated side. This specimen was kept for a long time but no gills were formed on the operated side.

Abdominal region

Posterior part (P): From the results of the foregoing experiment on the pleural region it can easily be supposed that there is no gill-forming faculty in this region. It was put to the test, however, and 7 embryos were operated on. One died before the gill formation started doubtless owing to mould, but in the rest we found well developed gills on the unoperated side, whereas there were none on the operated side.

Median part (B): This part is ventral and somewhat posterior to the median part of the pleural region which represents the critical boundary for the gill formation. It is, therefore, important to test whether this part of the abdominal region has the power of forming gills. The number of embryos experimented on was rather small, but in all cases gills were not produced on the graft.

Cardiac region

The experimental results indicate that this part possesses a very high power of producing gills. Fig. 4, *b* shows one of these specimens operated on on June 7 and sketched on June 15. Two anterior gills appeared 5 days after the operation and developed at the same rate as on the other side, while the third was formed 3 days later and remained in an abortive state (Photo. 5).

Oral region

The posterior boundary of this region slightly overlaps the cardiac region, as is shown in Fig. 3. The grafting experiment was tried in only one example, from which Fig. 4, *c* was made 6 days after the operation. Two gill rudiments appeared on the fifth day and developed into the typical form after 11 days. From this manner of development, it may be considered that the oral ectoderm has as great a power of forming gills as the cardiac ectoderm.

To summarize the results so far obtained, the gill-forming power of the ectoderm diminishes in the posterior half of the body in the

course of development from the early to the late neurula, and this diminution extends more anteriorly in the abdominal region than in the pleural region.

Table II

Grafts taken from the late neurula

		Number of experiments	Available cases	Shape and Size		Number of gills from the graft			
				Normal or nearly normal	Abnormal	3	2	1	0
Pleural region	Ant.	—	—	—	—	—	—	—	—
	Mid.	5	3	2	1	0	2	1	0
	Post.	12	9	0	1	0	0	1	8
	Pl.–vent.	10	9	0	0	0	0	0	9
Abdominal region	Mid.	3	3	0	0	0	0	0	3
	Post.	7	6	0	0	0	0	0	6
Cardiac region		3	3	3	0	2	1	0	0
Oral region		1	1	1	0	0	1	0	0

The embryo with closed medullary folds

The ectoderm of the posterior half of the body has lost its gill-forming power by the late neurula stage, so we shall next proceed to test the remaining half in more advanced embryos. For this purpose we choose KOYAMA's stages 21 and 22, which nearly correspond to HARRISON's stage 21 of *Amblystoma*, the medullary folds being completely fused and some 5 pairs of somites becoming distinct.

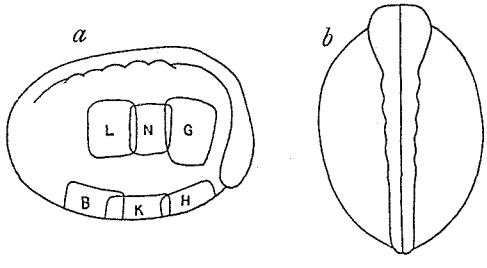

Fig. 5 *a* Profile of an embryo with closed medullary folds. *b* Dorsal view of the same, showing medullary folds closed completely. K, median abdominal part (anterior portion); other letters are the same as before.

Pleural region

Median part (L): The test made at the late neurula

stage gives positive proof that this part can form gills, but the same test at the present stage brought only negative results. Photo. 9 shows the only exception, in which a gill was formed on the boundary between the graft and the host. This gill appeared a week after the operation, but afterwards it did no develop well and remained in a rudimentary state.

Transplantation of parts anterior to the preceding median part was also tried. Since, however, the experiment was done at a later period of spawning, the operated embryos were not in good conditions perhaps owing to the rise of temperature, and no single example survived till the gill formation started.

Abdominal region

Median parts (B and K): Since the first part B has already lost the gill-forming faculty in the preceding stage, it is quite natural to find also negative results in the present case. The second part K was taken at a place more anterior to the first and yet no gill was formed.

Cardiac region

The transplanted ectoderm from this region still produced gills in a high percentage of the experiments. One of such operations was performed on June 22, and resulted in the production of 2 gill buds after 5 days (Fig. 6, *a*). In another example, which is shown in Fig.

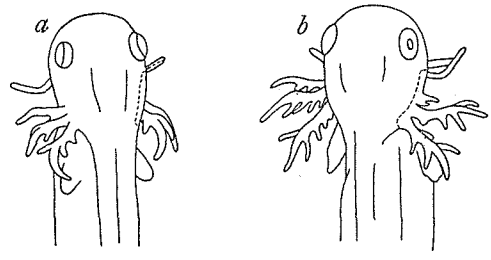

Fig. 6 Dorsal view of larvae with induced gills on the grafted ectoderm of the cardiac region from an embryo with closed medullary folds. *a* Showing 2 gills developed on the graft extending into the posterior part of the balancer; 6 days after the operation. *b* Showing 3 gills and a supernumerary balancer developing on the graft; 10 days after the operation.

6, *b*, the graft was so large as to include the balancer region, and a balancer was produced on the graft in addition to the gills. This fact may affirm the self-differentiation of the balancer ectoderm, as HARRISON ('25) maintains in *Amblystoma*.

In the embryo with closed medullary folds the gill-forming faculty is greatly reduced, being apparently confined within a small area around the branchial region, particularly that subjacent to it.

Table III

Grafts taken from embryos with closed medullary folds

		Number of experiments	Available cases	Shape and Size		Number of gills from the graft			
				Normal or nearly normal	Abnormal	3	2	1	0
Pleural region	Ant.	5	0	0	0	0	0	0	0
	Mid.	8	8	0	2	0	0	2	6
Abdominal region		5	4	0	0	0	0	0	4
Cardiac region		9	7	4	3	2	3	1	1

The embryo with distinct gill plates

Embryos of this kind are at stage 23 of KOYAMA's normal plates or at HARRISON's stages 22 and 23 of *Amblystoma*. The mesoblastic somites now count 7 or 8 pairs and the eye bulbs have become distinct. In the present stage the cardiac region alone was tested, since it is the only part which positively produced the gills in the previous stage. In 5 out of 7 cases, gills were still formed; they were, however, produced on the boundary between the graft and the host and never from the grafted ectoderm only. Photo. 10 shows one of such specimens, in which the third gill appears on the border of the graft. In other 2 negative cases gills failed to appear till the last on the operated side. From these facts it seems that the cardiac region is in the course of losing its gill-forming faculty in this stage.

From the foregoing data the gill-forming power of the ectoderm of *Triturus pyrrhogaster* may be tabulated as follows:

Table IV

Conclusion drawn from the above described results

Region / Stage	Pleural			Abdominal		Cardiac	Anterior	
	Ant.	Mid.	Post.	Mid.	Post.		Oral	Ant.-Lat.
Embryo at the early neurula stage	+	+?	+	+	+	+	+	+
Embryo at the late neurula stage	+?	+	−	−	−	+	+	
Embryo with closed medullary folds	±?	−	−	−	−	+		
Embryo with distinct gill plates	−	−	−	−	−	±		

+ : Produce, − : not Produce, ± : not Produce, but incorporate, ? : Probable results, not experimental.

Experiment II

Rotation of the branchial ectoderm

The branchial ectoderm of the embryo at the tail-bud stage was homopleurally transplanted to the proper position in another embryo at a slightly younger stage with inversion of the dv and ap axes. The result was the development of dorso-ventrally inverted gills. The characteristic non-pigmented tissue of the ventral side appeared on the dorsal side of the inverted gills, forming the fold-like "second gill plates" which were possibly homologous to the inner gills of the anuran tadpole, while on the ventral side the opercular formation was partly or completely disturbed and the tissue was deeply pigmented (Fig. 7, *a* and *b*). The inversion of the gills was most evidently shown after the development of filaments which were on the dorsal side, instead of ventral to the gill, directed upwards and anteriorly.

More operations of the same kind were made with somewhat younger embryos. Fig. 7, *c* shows one of such specimens. It was operated on on June 1. In this case the gill rudiments developed first in the antero-ventral direction in consequence of the inversion of the ap axis, but afterwards they rotated to the normal posture with respect to the median plane of the embryo. A part of the operculum was formed on the dorsal side of the head as is shown in the figure. Pigment appeared on the ventral (original dorsal) side, and not on the dorsal side.

Next the transplantation was made in embryos at stage 25 (beginning of the tail-bud), younger than the previous, of course. As a result of the experiment it was found that the dv axis was generally inverted, with some exceptions (2 out of 8 cases), while the inversion of the ap axis was found only in 3 out of 8 cases. The experimental results can be found in Fig. 7, *d* and in Photos. 11–13.

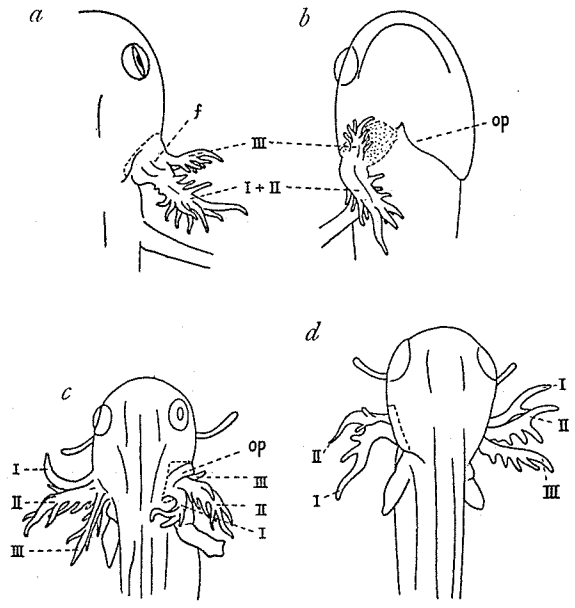

Fig. 7 Inversion of gill development due to 180° rotation of the branchial ectoderm at the tail-bud stage. *a* Showing the development of the so called "second gill plates" on the graft (stage 27–); 8 days after the operation, dorsal view. *b* The same larva in ventral view, showing the pigment development on the graft and incompleteness of the operculum on the operated side. *c* Showing the development of a part of the operculum on the operated side (stage 26–); 9 days after the operation, dorsal view. *d* Inverted gills developed from a graft taken at the beginning of the tail-bud stage (stage 25); 8 days after the operation, dorsal view. I–III, order of gills; op, operculum; f, second gill plate.

The polarity of the branchial ectoderm, especially the dv axis, so far as the foregoing experiments are concerned, seems to be nearly definitely determined by the time when the gill-forming faculty is restricted to the proper position and the gills cannot be formed by the ectoderm of the other parts of the body. However, the time when it is determined cannot yet be exactly given.

Table V

Rotation of 180 degrees

Stage	Number of experiments	Inversion of dv and ap axes	Inversion of dv axis	Inversion of ap axis	Non-inversion
25	8	3	3	0	2
26	4	2	1	0	1
27	5	3	2	0	0
Total	17	8	6	0	3

Experiment III

Removal of the branchial endoderm and mesoderm

The branchial endoderm and mesoderm were removed without disturbing the overlying ectoderm according to the instructions given by EKMAN; first a U-shaped incision was made around the gill plate, leaving the dorsal border only and the flap was turned upwards to expose the inner side to the view. The deeper layers were thus removed until the ectoderm and then the flap was turned back to heal around. If the ectoderm has the power of self-differentiation as EKMAN claims, the gill must appear even after such an operation. But the results were all negative. There was no single gill nodule formed on the operated side even 16 days after the operation. Photo. 14 shows one of these specimens.

Table VI

Removal of the branchial endoderm and mesoderm

Stage	Number of experiments	Results	
		Non-developmental cases	Developed cases
24	1	1	0
25	2	2	0
26	3	3	0

Heterotopic transplantation of the branchial ectoderm

This grafting was made to confirm the results of the previous experiment. If the results previously obtained are correct, no gills

should be formed in this case. In point of fact, the results were affirmative, showing no shingle instance of gill development. Fig. 9

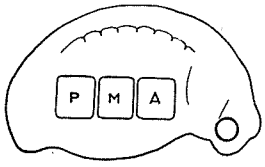

Fig. 8 Profile of an embryo at the beginning of the tail-bud stage (stage 25), squares indicating the place where the branchial piece is transplanted. A, antero-pleural part; M, mid-pleural part; P, postero-pleural part.

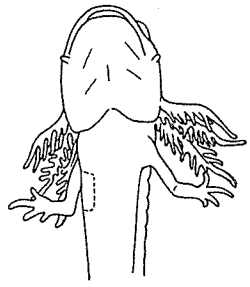

Fig. 9 Showing non-developmental state of the gills on the heterotopically grafted branchial ectoderm from another embryo just prior to the tail-bud appearance (stage 24); 16 days after the operation, ventral view.

shows one of such specimens. The experiment was further carried out on an embryo just prior to the gill development. Though the grafted ectoderm slightly swelled out in this case, yet it never developed into gill buds. Moreover, pigmentation took place in the graft and it became hardly distinguishable from the surrounding ectoderm. (It was especially difficult to find, if the piece was not previously stained with Nile blue sulphate.) The results of the experiment are generally in accord with those of SEVERINGHAUS in *Amblystoma*.

Table VII

Heterotopic transplantation of the branchial ectoderm

Stage	Number of experiments	Results	
		Non-developmental cases	Developed cases
23	2	2	0
24	4	4	0
25	2	1	0
26	1	1	0
29	1	1	0

Removal of the branchial endoderm

The isolated ectoderm has no power to form gills. It is therefore questionable whether they will be produced, if the mesoderm is added to, while the endoderm is separated. Four embryos were operated on for this test at stage 24 (just prior to the appearance of the tail-bud) and in only one case 2 typical gills developed but they were slightly smaller than normal (Fig. 10). Though the number of positive cases is very small, it may indicate that the mesoderm plays some part in the gill formation, to prove which, another experiment was attempted.

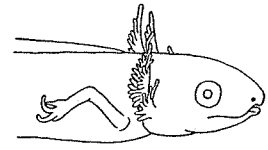

Fig. 10 Profile of a larva producing gills after the removal of the branchial endoderm; 30 days after the operation.

Heterotopic transplantation of the branchial ectoderm and mesoderm

A branchial piece consisting of ectoderm and mesoderm was transfered just ventrally to the pronephric region. Out of 9 cases 6 survived long enough to show any result. In 3 cases, the graft taken from an embryo at stage 25 formed, 4 days after the operation, a single small bud, projecting in the posterior direction (Fig. 11, *a*), but it gradually atrophied and completely disappeared in the course of the next 5 days, without producing gill filaments. This fact has already been observed by HARRISON and SEVERINGHAUS in *Amblystoma*. The blood cells were produced in the transplanted mesoderm and accumulated beneath the ectoderm. In the other cases, gill buds were

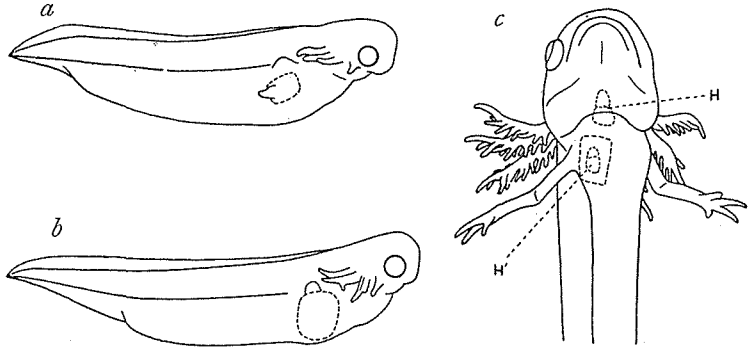

Fig. 11 Heterotopically grafted branchial ecto- and mesoderms. *a* Producing a small gill bud on the graft; 4 days after the operation. *b* No gill production on the graft; 4 days after the operation. *c* Ventral view of the same larva, showing a supernumerary heart in the graft; 18 days after the operation. H, normal heart; H', supernumerary heart.

not formed, but incidentally, an interesting fact was observed in one specimen (Fig. 11, *b*). Ten days after the grafting abundant blood cells were produced from the implanted tissue and another 7 days later a small heart was noticed, pulsating at the rate of 70 times per minute, whereas the normal heart beats about 100 times per minute. The graft was taken from the branchial region, but it may have included some parts possessing the faculty to produce the heart and thus they formed the small heart in the heterotopic position. This fact supports EKMAN'S ('21) and COPENHAVER'S ('24) statement that the mesoderm surrounding the heart and not normally contributing to its formation can still produce that organ if the organ proper is removed.

Heterotopic transplantation of the gill plate

For this experiment embryos at stages 22–29 were used, and the entire branchial layers were cut out and transplanted to a given posi-

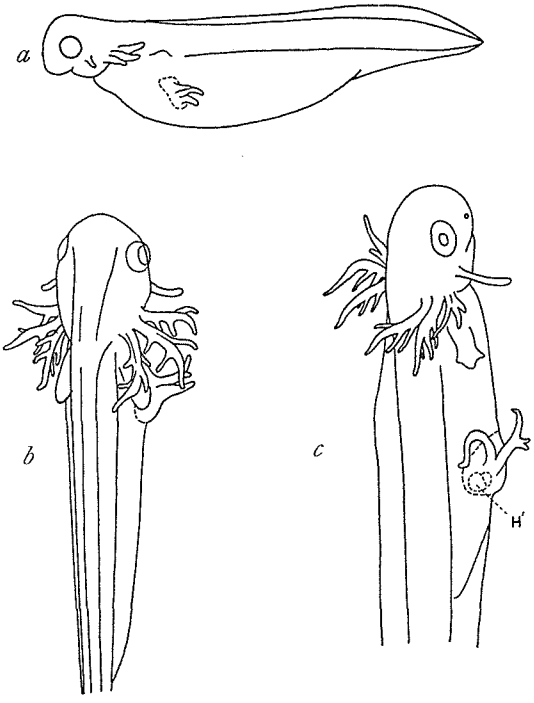

Fig. 12 Heterotopic transplantation of the gill plate, with the resulting development of gills in the abnormal position. *a* Graft taken at stage 25 and put in region A of Fig. 8, rotated 90°; 3 days after the operation. *b* Graft taken at stage 25 and put in region A of Fig. 8; 7 days after the operation. *c* Showing 2 inverted gills due to the inversion of the graft; graft taken at stage 22 and put in region P of Fig. 8; 10 days after the operation.

tion in the body. The graft developed in veery case with its own orientation and rate of growth as in the proper position (Fig. 12). In a transplantation of a piece from an embryo with closed medullary folds to the flank of another embryo at the early tail-bud stage in such a way that the graft was placed dorso-ventrally and homopleurally (made on June 27), 2 inverted gills were produced. Their filaments were plainly circulated by blood cells perhaps coming from the supernumerary heart produced in the grafted tissue, since the blood cells were not noticed before its pulsation. However, at the time when this specimen was preserved (August 4), the extra heart had stopped its pulsation and the gills had greatly reduced. From this fact it seems clear that the blood supply is not indispensable for the formation, but important for the growth of the gills.

Table VIII

Heterotopic transplantation of the entire gill plate

Stage	Number of experiments	Results	
		Non-developmental cases	Developed cases
22	1	0	1
25	3	0	3
26	1	0	1
29	1	0	1

Transplantation of the gill plate consisting of all 3 layers invariably causes perfect gills to develop, while, if the endoderm is excluded in such grafting, gills are sometimes produced and sometimes not, so that the presence of the endoderm seems also to be important for the formation of gills. To make this more evident, it is necessary to bring the endoderm directly in contact with the ectoderm by removing the mesoderm. Unfortunately, this experiment was fruitless, since the operated embryos all died before bringing any result, perhaps having been attacked by mould which grew abundantly at the time of operation owing to the rise of the atmospheric temperature.

Conclusion

EKMAN points out that in *Bombinator* embryos a non-branchial ectoderm can produce the gills in the branchial site, and this fact is confirmed by HARRISON ('21) and SEVERINGHAUS ('30) in *Amblystoma*.

According to these authors the faculty seems not to be very extensive; it is rather confined to a small area round the gill site, especially in the cardiac and pronephric region. The grafted ectoderm from the posterior part of the body always fails to produce the gills. However, these authors, except EKMAN, used more or less older embryos, namely embryos in the tail-bud stage, so that the above statement is not very conclusive. It is necessary to carry out a further experiment with younger embryos. Our experimental result in *Triturus* evidences that the entire ectoderm, with the exception of the medullary plate, shows a sufficient power of gill-formation at the early neurula stage; the ectoderm transferred from various parts to the proper site produces typical or nearly typical gills in most cases, but this condition gradually changes with the advance of development, so that by the stage of the late neurula the faculty is already reduced to the anterior half of the body as indicated in Fig. 13 by line G_3, and the experiments made at this stage agree with those recorded by the previous authors. As a matter of fact, as the development goes on, the faculty is farther reduced, being confined at the stage of the closed medullary folds to a small area, defined by line G_2 in Fig. 13. At the tail-bud stage the gill-forming faculty is finally restricted to the branchial region alone. Therefore, it is quite evident that the gill-forming faculty first distributed over the entire body surface gradually disappears from behind forwards and at the same time the branchial ectoderm is gradually differentiated from the remaining ectoderm.

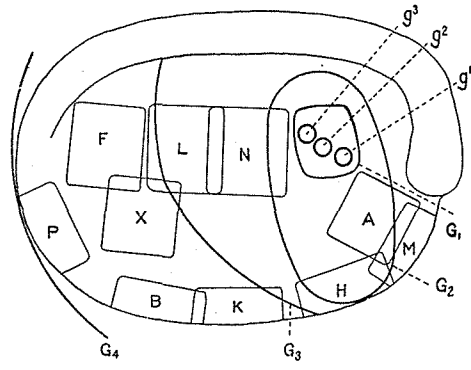

Fig. 13 Schema showing the positions of the grafted piece and the boundaries of the gill-forming faculty at various stages of development. G_1, the bonudary at the tail-bud stage; G_2, at the stage with closed medullary folds; G_3 and G_4, at the late and the early neurula stage respectively.

After the branchial ectoderm is definitely characterized, it can be rotated 180 degrees with the resulting production of inverted gills. In this case the determination of the dv axis is more marked than that of the ap axis. Among 17 experimental cases 14 individuals showed dv inversion, while ap inversion was found only in 8 animals. But at what stage this orientation is determined is not ascertained by the

present investigation. It seems, however, not to be so early as in *Bominator*, in which, according to EKMAN, it takes place when the medullary folds first become distinct.

That the ectoderm alone has no power to induce the gills is next proved by heterotopic transplantation of the branchial ectoderm or removal of the endoderm and mesoderm from the branchial region. Among 8 heterotopic transplantations from embryos just prior to or just after the appearance of the tail-bud, there was no single case where gills developed in alien surroundings To the data must be added one more case, where the graft was taken from an embryo just prior to the gill formation. In this case the graft swelled out to a slight extent, but without developing into anything which might be interpreted as a gill rudiment. This result confirms the finding of SEVERINGHAUS in *Amblystoma*, while it is not in accord with HARRISON'S statement that the gill formation in the abnormal position is due to the ectodermal layer. The power of the ectoderm to form gills has been more strongly emphasized by EKMAN in the anuran embryos, especially in *Rana fusca*. However, removing the branchial endoderm and mesoderm in 6 embryos, we observed no gill formation in all cases. This fact indicates that the branchial ectoderm, if it is alone, does not produce gills in the proper position.

A mesodermal element was next added to the heterotopic transplantation of the branchial ectoderm. Out of 9 experiments 6 were successful, 3 producing a gill bud, the others not. In another experiment in which the endoderm was removed, leaving the mesoderm and ectoderm, 2 gills were produced. Therefore, judging from these results of two kinds of experiments, the mesoderm plays the most important rôle in the gill formation. HARRISON is of the opinion that the mesoderm is an important factor not only in the formation of gills, but also in their subsequent development.

Finally when the entire gill plate is transplanted as a whole, it results in the production of nearly the typical gills with its own orientation and rate of growth. Therefore, the endoderm seems to be also important for the gill formation. This is especially emphasized by SEVERINGHAUS, who states that "the potency of the endoderm is further demonstrated by the failure of a gill to develop when the endodermal component is removed prior to gill formation" and that "transplantation of the branchial endoderm beneath the ectoderm and mesoderm of the region just posterior to the gills and ventral to the anterior limb gives rise to a structure which is permanent and which resembles somewhat an external gill".

Summary

1. The present work was carried out with embryos of *Triturus pyrrhogaster* (BOIE) to examine the gill-forming faculty with respect to its distribution in different stages of development and to the rôle of the germ layers in the formation of gills.

2. The faculty is distributed over the entire body surface in the early neurula stage, except the medullary plate, but with the advance of development it gradually disappears from behind forwards to the branchial ectoderm at the tail-bud stage.

3. The polarity of the branchial ectoderm, especially the dv axis becomes definite by the time the gill-forming faculty is limited to the proper position.

4. By heterotopic transplantation and by removal of underlying layers, the branchial ectoderm is shown to have no power of self-differentiation, while the mesoderm is important for the gill formation. The endoderm seems to play some part too, since the most typical gills are given rise to by the transplantation with all 3 layers.

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Explanation of Plates

Photos. 1-4 Replacement of the branchial ectoderm by non-branchial ectoderm of various parts at the early neurula stage.

1 Larva that has produced 3 gills, posterior 2 on the graft from the pronephric part, while the first is derived from the host in consequence of the grafted piece being slightly displaced to the posterior; 12 days after the operation.

2 Larva that has produced 3 gills on the graft from the postero-abdominal part, the third being in an abortive state, the grafted piece covers the posterior half of the balancer; 8 days after the operation.

3 Larva with 3 gills produced on the graft from the antero-lateral part, the third being in a finger-shaped state without filaments; 11 days after the operation. The early stage of this specimen is shown in text-fig. 2, *c*.

4 Larva with 2 gills produced on the graft from the oral part, the anterior being abnormal due to a fusion of the first and the second gills; 20 days after the operation. The early stage of this specimen is shown in text-fig. 2, *f*.

Photos. 5 and 6 Production of gills on the grafted ectoderm at the late neurula stage.

5 Larva with 3 gills produced on the graft from the cardiac region, the third being abortive; 13 days after the operation. The early stage of this specimen is shown in text-fig. 4, *b*.

6 Larva with 2 gills produced on the graft from the oral part; 11 days after the operation. The same individual is shown in text-fig. 4, *c*.

Photos. 7 and 8 Larvae that have produced gills on the graft from the cardiac region at the stage of closed medullary folds.

7 Larva with 2 gills; 7 days after the operation. The same individual is shown in text-fig. 6, *a*.

8 Larva with 3 gills, anterior 2 being produced on the graft, while the third is derived from the host owing to the shifting of the graft to the anterior; 6 days after the operation.

Photo. 9 Larva with an abortive gill on the boundary between the host and the grafted pleuro-median part of an embryo with closed medullary folds (stage 21); 13 days after the operation.

Photo. 10 Larva with an abnormal gill on the boundary between the host and the grafted cardiac region an of embryo with distinct gill plate (stage 23); 13 days after the operation.

Photo. 11 Larva with 2 inverted gills on the left side in consequence of the inverted grafting of the branchial ectoderm; 8 days after the operation. The same individual is shown in text-fig. 7, *d*.

Photo. 12 Dorsal view of a larva with 2 inverted gills developed from the inverted grafting of the branchial ectoderm. Non-pigmented ventral character is still distinctly visible on the dorsal side; 26 days after the operation. This specimen is shown in text-fig. 7, *a*.

Photo. 13 Ventral view of the same larva, showing the pigmentation on the ventral side.

Photo. 14 Dorsal view of a larva, showing no gill formation after the removal of the branchial meso- and endoderms, operated on at stage 25; 16 days after the operation.

Photo. 15 Dorsal view of a larva, showing no gill formation from the branchial ectoderm transplanted to region A of text-fig. 8. The graft being taken at stage 24; 12 days after the operation.

Photos. 16-20 Development of gills on the heterotopically transplanted gill plate consisting of all 3 layers.

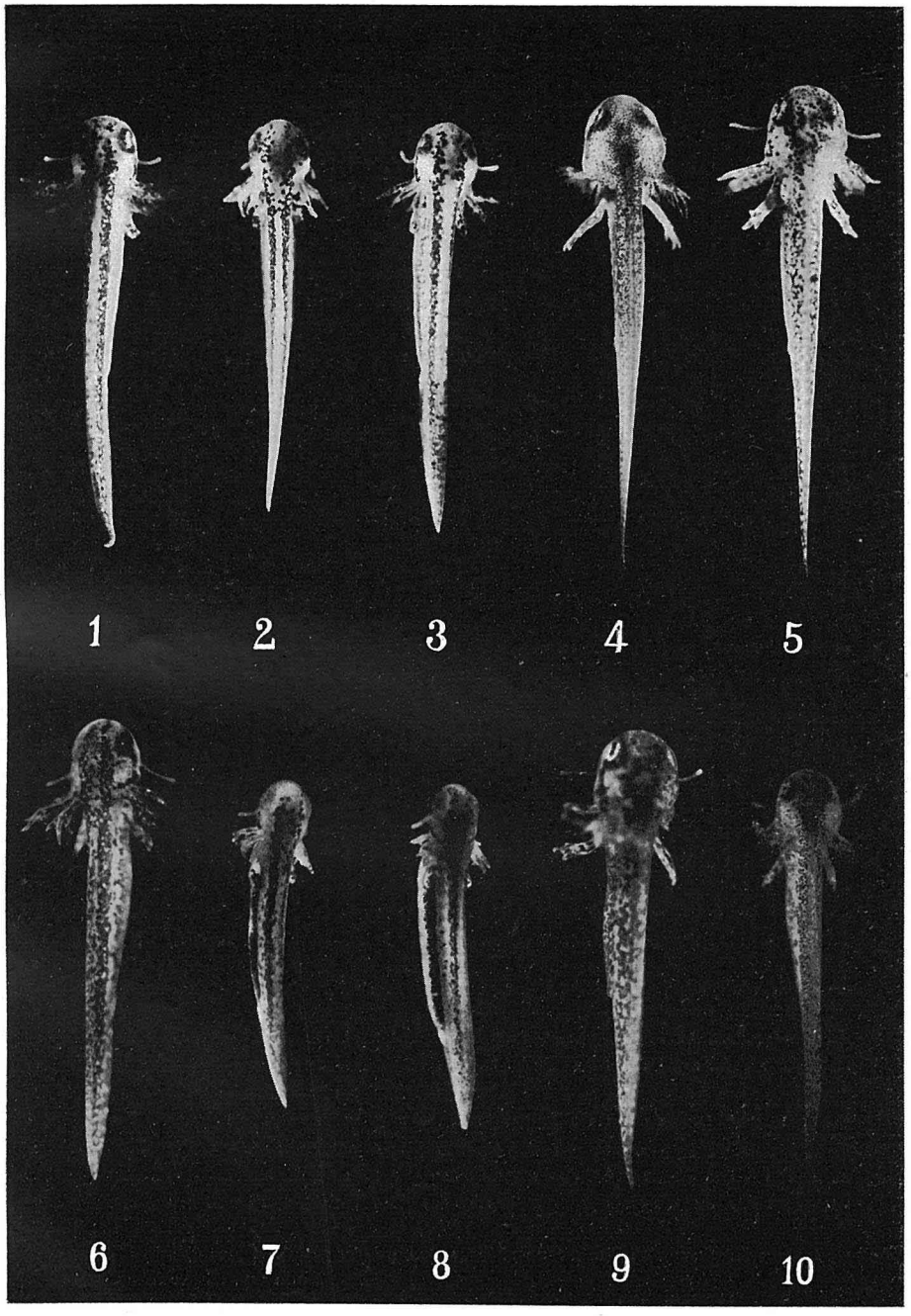
16 Dorsal view of a larva, having 2 inverted supernumerary gills on the side, the graft taken at stage 22 and put in region P of text-fig. 8 at stage 26; 11 days after the operation. This specimen is shown in text-fig. 12, *c*.

17 Dorsal view of a larva, having 3 supernumerary gills on the side, the graft taken at stage 26; 8 days after the operation.

18 Dorsal view of a larva, having 3 supernumerary gills slightly caudal to the left gills, the graft taken at stage 25; 7 days after the operation. The early stage of this specimen is shown in text-fig. 12, *a*.

19 Profile of a larva, having 3 supernumerary gills on the side, the graft taken at stage 29; 3 days after the operation.

20 Dorsal view of the same individual in further development, i. e., 10 days after the operation.



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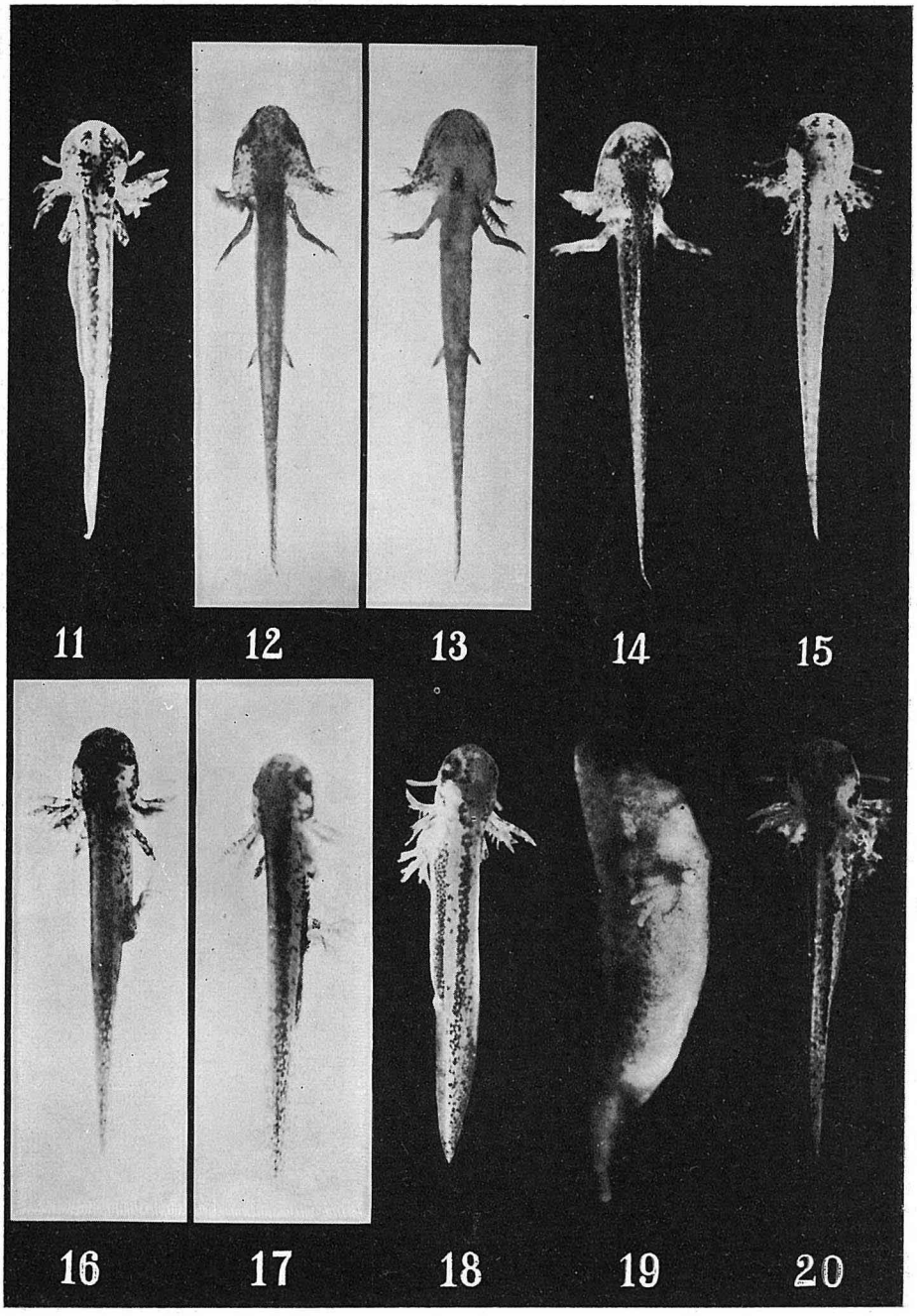

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