REGENERATION OF THE TAIL-FINS OF FUNDULUS EMBRYOS[1]

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That the fins of fishes possess the power of regeneration was first demonstrated by Broussonet (1786). From his experiments two observations are worthy of note in connection with this study. First he records that regeneration takes place more rapidly in young fish than in old and that the rate of regeneration may differ from one species to another. Secondly he states that the presence of the stumps of the fin-rays is necessary for regeneration and that in their absence regeneration will not take place.

Fraisse (1885) and Weismann (1892) state that there is very little power of regeneration in the fins of fishes. It is now a well-established fact, as pointed out by Morgan (1902), that many kinds of fish belonging to widely different families will regenerate their tail-fins.

The earliest recorded experiments on the tail-fins of fishes from the standpoint of morphogenesis were performed by Morgan (1900, 1902, and 1906). Later Morrill (1906), Scott (1907 and 1909), Beigel (1912), and Nabrit (1929 and 1931) performed experiments along this same line.

Broussonet (1786), Morgan (1906), and Morrill (1906) agreed that for regeneration to take place the ray stumps must be left. Morrill went so far as to suggest that regeneration does not take place even when the ray stumps are too small; on the other hand, Nabrit (1931) found that the ray stumps are not necessary for regeneration and that if the rays are entirely picked out new rays will appear under the influence of the articulating portion of the basal plate.

After comparing the findings of Harrison (1918) and Detwiler (1918) in *Amblystoma* with the results of his experiments on fish, Nabrit (1929) suggested that a possible similarity existed between the production of limbs in *Amblystoma* and of tail-fins in fishes. Both cases seem to be independently differentiating mesenchymal systems.

[1] I am particularly indebted to Professor S. M. Nabrit of Atlanta University for suggesting this problem and to Professor J. W. Wilson of Brown University for his valuable assistance in the preparation of this paper.

The experiments which form the basis of this paper were planned for three purposes:

1. To examine further the relationship of the ray stumps to regeneration of the fin-rays.

2. To obtain, if possible, new data on the rôle of the basal plate in regeneration of the fin-rays.

3. To compare the regeneration process in the embryo with facts already recorded for the adults.

These experiments were started at the Biological Laboratory at Cold Spring Harbor in the summer of 1931 and were completed there in the summer of 1933. Fixed preparations of the material were studied at Arnold Biological Laboratory, Brown University.

Material and Methods

The material chosen for these experiments was embryos of *Fundulus heteroclitus*. These embryos were obtained from the eggs before the time of hatching by the method devised by Nicholas (1927). The eggs were permitted to develop until the desired stage was reached and were then removed from the chorion. Great care was taken in this removal because the slightest pressure exerted on the eggs will cause injury to the embryo or yolk sac which will render the animal unfit for experimental purposes.

Embryos which are injured show the effect very soon after their removal from the chorion. After a few hours to see if the embryos had been injured in their removal they were ready for operation. Because of their small size the embryos must be operated on under the microscope and therefore it was necessary to employ a method whereby small bits of tissue might be removed. For this purpose Nicholas' (1927) modification of Spemann's technique was used. The cut was made by drawing the tissue to be removed into the lumen of a very fine pipette and carefully cutting the tissue against the tip of the pipette with a spear-point blade. In some cases, especially in embryos just about to hatch, it was found best to remove the parts by supporting the embryo with an ordinary dissecting needle and making the cut with a spear-point blade. In either case great care must be taken not to press the embryo against the bottom of the dish as this invariably results in death. The embryos lack movement and therefore anesthesia is unnecessary. In order to stop the bleeding which occurs when the cuts were made well up into the body, it was found best to transfer the operated embryos immediately to cold sea water or to a cold sodium chloride solution that was isotonic with sea water. The latter was found to be more satisfactory.

Throughout the course of the experiment the embryos were kept in small dishes and the water changed twice daily. No attempt was made to control the temperature as during the summer it was fairly constant in the laboratory, varying only from 18° to 21° C.

Observations were made mainly on living specimens which were placed in a small drop of water on depression slides and examined under a microscope with a 32 mm. objective. A few individuals were fixed in Bouin's fluid and preserved in alcohol for microscopic examination. These were stained in toto with alizarin and cleared in oil of wintergreen. Camera lucida was employed for recording the regeneration process.

Experimental

Preparatory to experimentation a study was made of the normal process of formation of the tail-fins and of the articulating plate. Nabrit (1929) describes the development of the tail-fins as follows: (1) there is a primitive natatory fold; (2) the mesenchymal mass from which the fin-rays and articulating plate develop forms between the end of the notochord and the extremity of the fold; (3) from this mesenchymal mass central rays are the first to differentiate; (4) at the time of differentiation of the first rays the natatory fold is further from the base of the notochord in the central region than in the dorsal and ventral regions; (5) in individuals that hatched in sixteen days the streaking of the rays begins on the seventh day; (6) the additional rays are added dorsally and ventrally but the blood vessels that pass between the rays may be seen to loop in the paths of the rays before the rays are vitally stainable with Nile blue sulphate or with alizarin after fixation. That the rays are present before they can be seen in stained preparations may be demonstrated by crushing the tail under a coverslip and observing the process of fragmentation. By the time the anlage of the first rays to develop are stainable, they are no longer connected with the mass which develops into the basal plate but form an articulation with it (Fig. 1). While my observations confirm Nabrit's in general, there is some doubt as to whether or not the rays and articulating plate develop from the same mesenchymal mass. Soon after hatching the most caudal of the vertebral spines enlarges and becomes incorporated in the plate (Fig. 2). Later the next spine anteriorly does the same and this process is continued until the adult pattern of the tail is formed (Fig. 3).

The experimental animals are divided into three groups dependent upon the mode of operation employed: (1) splitting of the tail-bud; (2) removal of the entire tail; (3) removal of part of the tail.

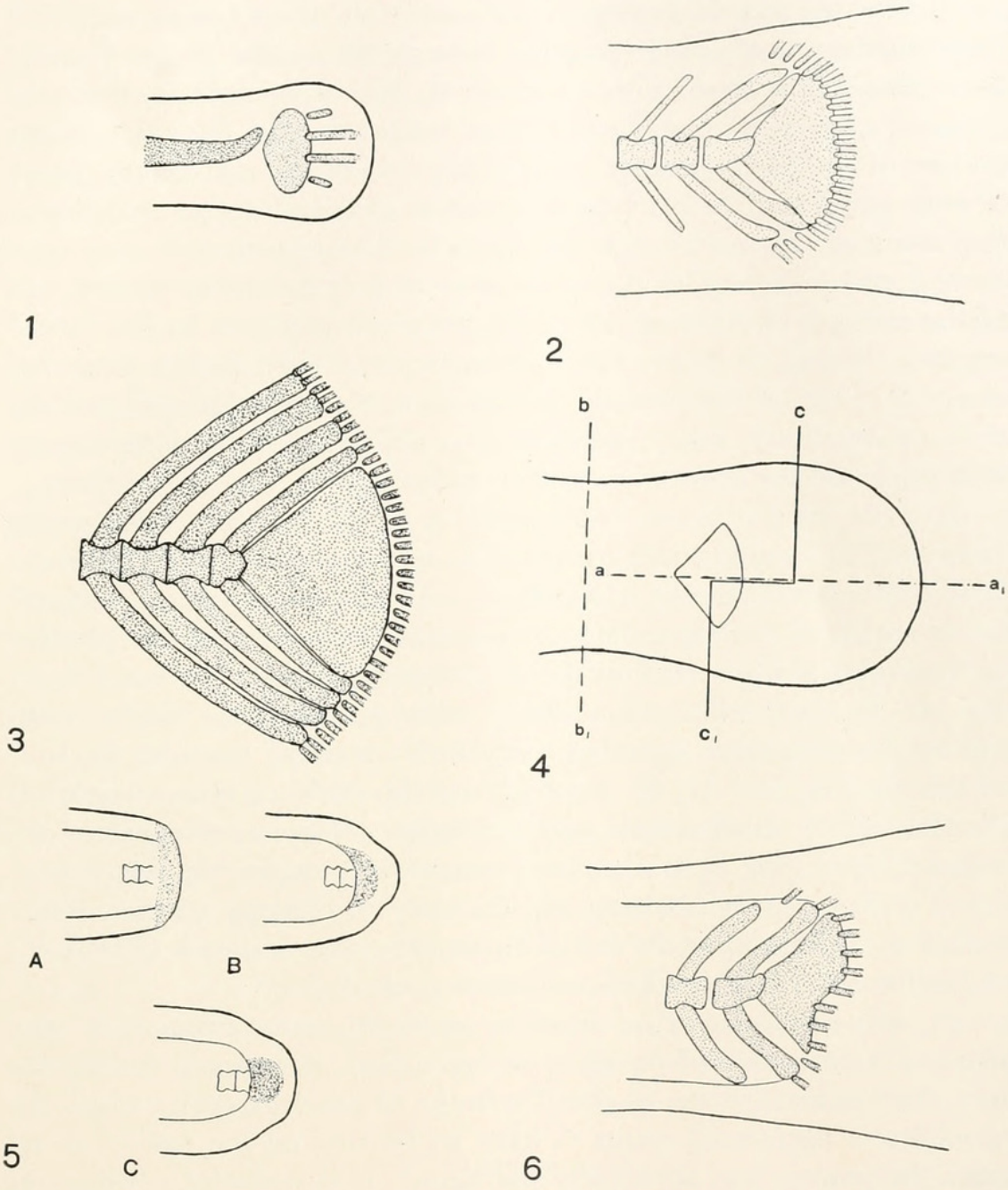

FIG. 1. Diagrammatic sketch of the tail-fin anlage just prior to the time it becomes stainable. The largest rays are the first to separate from the mass and articulate with the plate.

FIG. 2. A camera lucida drawing of the tail at the stage of development when the most posterior of the vertebral spines become incorporated in the piate. (Four days after hatching.)

FIG. 3. A camera lucida drawing of the basal plate of an adult showing the relationship of the vertebral spines to the articulating border of the plate.

FIG. 4. Diagrammatic sketch showing the relationship between the basal plate and the type of cuts made in the various experiments.

FIG. 5. Camera lucida drawings made at various stages after removal of the entire tail-fin anlage. (*A*) twenty-four hours after operation; (*B*) six days; (*C*) twenty-five days. See text for explanation.

FIG. 6. A camera lucida drawing showing the articulation of the regenerated rays with the exposed edge of the basal plate in the ventral region of the tail.

1. The tail-fins of embryos from seven to fifteen days old were deeply incised as nearly as possible through the middle of the fins and basal plate in a posterio-anterior direction, the cut being made along the line a–a_1 of Fig. 4. Such an incision divided the fin, the plate, and a portion of the posterior part of the body into dorsal and ventral parts. The operation is of such a drastic nature, so long an incision being made, that many of the animals operated upon in this manner died soon after the cut was made. The survivors were kept from ten to twenty days and at the end of this time all of the cut plates appeared to have fused together, though in some cases development of the tail was somewhat retarded. The wound usually left a distinct scar or groove running across the tail-bud, which, however, was obliterated after several days. In no case did the operation result in reduplication.

2. Tails were removed from embryos in all stages of development from the fifth day, at which time there is no visible differentiation of the fins or plate, until the time of hatching. The cut was made just anterior to the region of the basal plate and never as far anterior as the position of the dorsal and ventral fins, the cut being made along the line b–b_1 of Fig. 4. In about twenty-four hours after operation the wound heals and by this time there appeared some darkly staining tissue at the level of the cut (Fig. 5, A). By the sixth day this darkly staining tissue had rounded into a small nodule and had taken a place at the end of the spinal column (Fig. 5, B) where it remained as long as the fish was allowed to live without showing any tendency to undergo further differentiation. The mass at the end of twenty-five days is shown (Fig. 5, C) and is the same in appearance as on the sixth day.

In none of the embryos operated on in the above manner did any signs of regeneration of the plate or fins appear up to and including the sixty-fifth day after operation. The stage of development at which the operation is performed seems to have no bearing on the results, in all cases the process was apparently the same. It is doubtful whether regeneration of the plate or fins would ever occur after this type of operation but at present it can only be stated that regeneration fails to occur in the allotted time. Some experiments were carried out on young hatched *Fundulus* of unknown age (measuring from one to two centimeters in length) and the same results were obtained as above with the exception that no nodule was formed at the end of the spinal column. These older animals did not survive as well as the embryos, for death usually ended the experiment between the twentieth and thirtieth day after operation. The cause of death of these fish was apparently a sloughing-off of the tissue starting at the level of the cut, this sloughing process proceeding anteriorly until death took place.

3. Embryos twelve to fifteen days old, at which time the rays and plate are well differentiated, were operated upon so as to remove the ventral half of the plate and all of the rays distal to it, while in the dorsal half of the tail only the distal half of the rays were removed, the cut being made along the line c–c_1 of Fig. 4. At this time there were from six to ten rays articulating with the basal plate. The animals were allowed to regenerate and at the end of from fifteen to seventeen days some were fixed and stained with alizarin. In the ventral half of the tail a portion of the basal plate had been removed prior to differentiation of some of the rays and there was also removed a part of the basal plate with which some rays were articulating. Under the influence of the exposed edge of the basal plate, rays differentiated in the mass of regenerating tissue. These rays were similar in every respect to the other rays except for their more anterior articulation. These new rays articulate with the exposed edge of the basal plate (Fig. 6). This is true regardless of whether the part of the plate they articulate with is derived from spines or from the original central plate. In the latter case rays come out from the border of the plate even though it is not at the level of the unoperated portion of the plate. The shape of the plate may in this way influence the shape of the tail because, after reaching a definite size, the rays cease to grow in length and the tail remains somewhat shorter on the operated side. Regeneration of the plate is a relatively slow process as compared with the rays and in the length of time that the majority of the animals were under observation, usually seventeen days, the plate had not returned to its original size or shape (Fig. 7). In a much longer period, namely fifty days, it was found that the plate had almost completely replaced itself and at this time the tail ceased to be asymmetrical. So far as general observation reveals, the developmental processes of all other parts of the fish are normal.

Discussion

Concerning the extent of regeneration that will take place in the tails of fishes there seems to be, according to the literature, quite a diversity of opinions. All of the papers concerning this phase of the problem cannot be discussed but only the more outstanding ones will be mentioned.

Nussbaum and Sidoriak (1900) have demonstrated that *Salmo fario* operated on the day after hatching would regenerate the posterior part of the body in about ten weeks. If the cuts were made anterior to the anus a new posterior opening is established as well as a new opening for the urethra. After ten weeks fungus usually caused death of the culture.

Duncker (1905), using *Syngnathus,* demonstrated that when the entire tail is removed a perfectly normal one is regenerated. Also, if part of the vertebral column is cut out, accessory tails are produced at the point of injury. However, he was unable to obtain the same results on any other forms used.

The difference between the results obtained from the experiments recorded in this paper for *Fundulus* embryos and those recorded by the above investigators for *Salmo fario* and *Syngnathus* is not apparent at the present time but suggests an explanation on the basis that different species show quite different powers of regeneration.

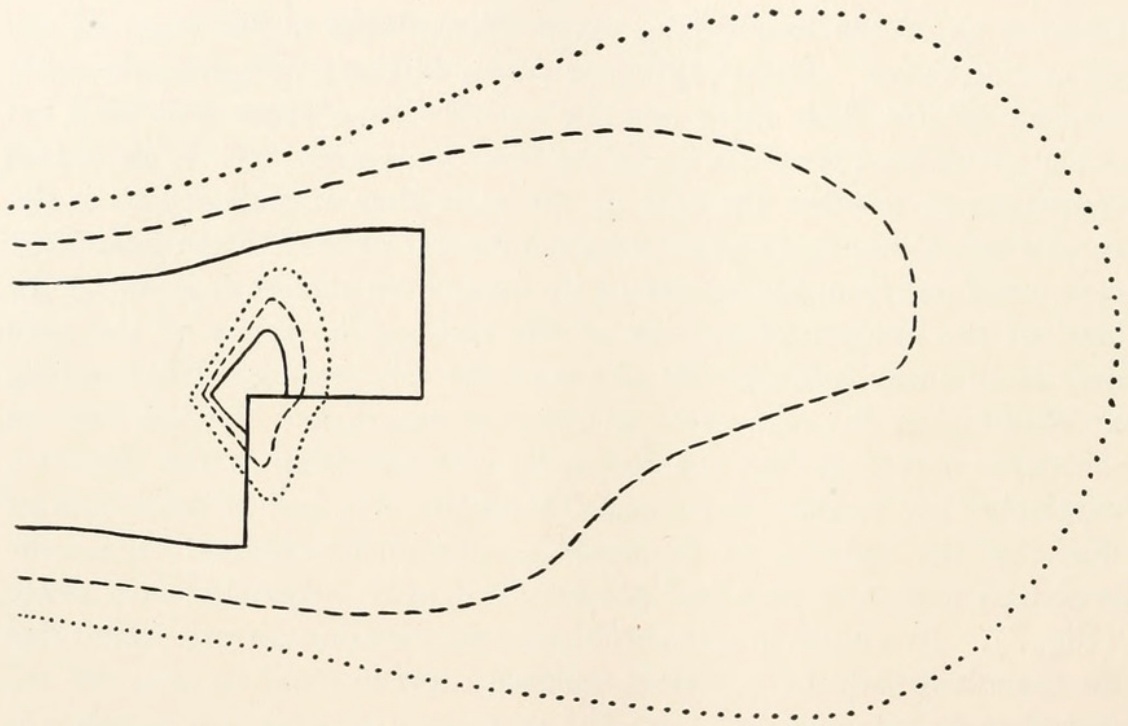

FIG. 7. A composite diagrammatic sketch showing the relative amounts of plate and fin material replaced at different stages after operation. The solid line represents twenty-four hours, the broken line twenty days and the dotted line thirty days after operation.

In a *Fundulus heteroclitus* embryo which was cauterized just posterior to the body cavity prior to tail-fin differentiation, Nabrit (unpublished results) has obtained an abnormal tail with four rays. The fish died soon after hatching. It was not possible to determine the exact origin of these rays. They appeared, however, in the center of the posterior surface. The fish did not regenerate the lost part of its body. There is no definite indication that regeneration will ever take place after such an operation but rather the undifferentiated mesenchymal bud of the dorsal and ventral fins may differentiate at the end of the animal and simulate a tail.

The observations recorded in this paper on removal of the entire tail-fin anlage indicate that the factors responsible for regeneration do not extend more anterior than the region of the basal plate. The experiments on removal of parts of the plate reveal that the rays can be regenerated even before the plate has completed itself. These results indicate that the regenerative factors, be they nervous system, distance from the base of the tail, axial gradient of metabolism, or other factors, are segregated in the plate-fin region early in the process of differentiation. In fact, this type of segregation apparently takes place before any visible differentiation has taken place. This observation confirms the work of Nicholas (1927), who has shown that *Fundulus* embryos undergo early in their development an extremely high degree of differentiation. Such a fact in itself may explain in part the lack of power of regeneration of certain parts. That splitting of the tail does not result in reduplication is a result to be expected considering the extremely high degree of differentiation in these embryos. Such results as reported above are not confined to *Fundulus* embryos as similar results have recently been obtained by using young sunfish and guppies.

It has been demonstrated that if all of the ray material and part of the basal plate is removed, the basal plate will replace itself and that rays will be differentiated in the regenerating tissue under the influence of the exposed edge of the basal plate. This fact indicates conclusively that the presence of ray stumps is not necessary for regeneration of the rays to take place.

Harrison (1918) and Detwiler (1918) have shown that in *Amblystoma* the fore-limb develops from a self-differentiating and equipotential system. The limb anlage therefore is an entity, which, except for its dependence for nourishment, is independent of its surroundings in the attainment of specific form. Detwiler showed that it was possible to initiate the development of a limb in almost complete absence of the shoulder girdle. They suggest that the limb anlage may be regarded not as a definitely circumscribed area, like a stone in a mosaic, but as a center of differentiation in which the intensity of the process diminishes as the distance from the center increases until it passes away into indifferent regions. This conception suggests that the growth of the limb may be controlled by the distance of the part from the center of the anlage. If the anlage is extirpated other mesenchyme could, however, in a restricted sense, simulate the extirpated anlage and produce an apparently normal limb. This explanation is based on the conception of the mesenchyme as a formative factor in growth and, according to Nabrit (1929), the same explanation may be offered for the type of regeneration obtained in the tails of fishes.

It is concluded, therefore, that regeneration of the tail-fins of fishes and the same process in the limbs of *Amblystoma* are similar. The basal plate is apparently like the shoulder girdle in regenerative capacity, since it may replace itself and give rise to or induce the development of rays. Hence the rays, like the limbs of *Amblystoma,* and the basal plate, like the girdle, belong to a self-differentiating mesenchymal system.

Summary

1. A study was made of regeneration of the tail-fins and basal plate of *Fundulus heteroclitus* embryos operated upon prior to the time of hatching.

2. Splitting of the tail-fin anlage does not result in reduplication of the tails.

3. Removal of the entire tail-fin anlage results in no regeneration up to and including the sixty-fifth day after operation.

4. If part of the basal plate is removed with the rays distal to it new rays will appear in seventeen days and the plate will regenerate in fifty days.

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