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University of Louisville
The Morphogenesis of the Pectoral Girdle
of Amblystoma Microstomum

A Dissertation
Submitted to the Faculty of the
Graduate School of the University of Louisville

In Partial Fulfillment of the
Requirements for the Degree
of Master of Science

Department of Biology

By

Raymond W. Lawrence

1939

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Title of Thesis: The Morphogenesis of the Pectoral
Girdle of Amblystoma Microstomum

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I. INTRODUCTION

Early experiments upon the structure of the shoulder-girdle of salamanders, as cited by Swett ('29 b), were made by Tornier ('06) who showed that a small portion of the shoulder-girdle of Triton will regenerate a complete girdle, including the free limb. Fritsch ('11) and Kurz ('12) substantiated these findings, according to Swett. They demonstrated that regeneration follows after complete removal of the free limb and shoulder-girdle in Triton.

Some of the earliest experiments on the limb anlage of Amblystoma were made by Harrison ('18). He found that there was a circular disc, three and one-half somites in diameter, in the embryo which contained almost all the cells of limb-forming potency. His results demonstrated that the free limb is an equipotential restitution system.

Detwiler ('18) on the other hand found that parts of the girdle rudiment of A. punctatum are already determined at the stage when the limb rudiment is present as a definite thickening of

the somatopleure (at the tail-bud stage). He reported that removal of a definite portion of the rudiment brings about a defect in that portion of the girdle corresponding to the part removed; that is, the pectoral girdle according to Detwiler's results is a mosaic rather than an equipotential system.

The location of the free limb itself within the disc was determined by Swett ('23), who grafted portions of the limb bud which had been previously stained with a vital stain.

Swett ('29 a), using well-developed, actively-feeding larvae of A. punctatum, found that girdles transplanted to orthotopic positions underwent some degree of size reduction, probably because of the interruption of the supply of nourishment available for their maintenance and growth. In all orthotopic series a normal free limb regenerated, irrespective of the normality of the girdle with which it articulated. Heterotopic transplantations were made by removing the girdle and placing it on the flank of the same animal. These transplants showed a marked degree of resorption. The speed of the resorption process was not dependent upon the length of time the

animal was permitted to live after receiving the graft. First, the girdle became reduced over the entire surface; later, the more outlying portions disappeared more rapidly, the scapula usually remaining the longest. Only two of these grafts produced free limbs, these limbs being small and irregular.

The results of Swett and Parsons ('29) appear to be contradictory to those of Detwiler, for they demonstrated that the cartilaginous shoulder girdle of Amblystoma will regenerate after its complete removal in older stages. They used A. punctatum larvae ranging from 15 to 40 millimeters. Series of operations included: (1) removal of the girdle and the free limb, (2) removal of the whole girdle leaving the free limb intact, and (3) removal of the girdle and humerus. The regeneration of the shoulder girdles in all three types of experiments was essentially complete. The process began by a condensation of cells in the girdle region and was quite similar to the embryological process which resulted in the production of the original girdle. Deficiencies were found largely in the most outlying parts of the girdle, chiefly in the suprascapula and

procoracoid.

Parsons ('29) has reported that the shoulder-girdle of A. punctatum will regenerate in a heterotopic position. The regeneration of the girdle took place in the presence or in the absence of the free limb. The limb also regenerated when removed. These transplants were from the right shoulder to the right flank and were of normal orientation. Girdles which developed from these grafts were removed under chlorethane anesthesia when the animals had been fed from two to seventy-five days. The larvae were then returned to water and fed daily for periods varying from forty to sixty-one days, at which time relatively complete restitution of the girdles had taken place.

Swett ('29 b) using A. punctatum larva which had just begun to feed reported that partial removal of the cartilaginous girdle was followed by its complete regeneration.

The combined experiments of Swett and Parsons show that in older stages the pectoral girdle behaves more like an equipotential system than a mosaic.

Stultz ('36) has shown that the pelvic girdle of A. punctatum is an equipotential system. When the hind-limb rudiment was extirpated, the regener-

ation of a complete free limb was accompanied by the regeneration of a normal girdle. In both the orthotopic and heterotopic transplants, in which an extremity regenerates, a topographically complete girdle results. In all his experiments both limb and girdle rudiments possessed regulatory ability and are regarded as self-differentiating, equipotential systems.

Why should not the pectoral girdle be like the pelvic girdle in this respect, since they are believed to be serially homologous? If the pectoral girdle is equipotential, any part or parts of the embryonic rudiment should produce a complete girdle, whereas if the limb anlage is a mosaic one, a number of centers of development should be present.

To compare A. microstomum¹ with A. punctatum, an attempt was made to transplant halves of the limb bud in a heterotopic, homopleural location. It was at first hoped that by feeding the animals for a longer period of time than had been done by Detwiler, a more complete girdle would be obtained.

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1. Although the name Amblystoma microstomum has been replaced by Amblystoma texanum by the systematists, the former name is still used by morphologists.

This, however, did not occur. In order to determine whether the pectoral girdle is an equipotential or a mosaic system, specific portions of the limb disc were extirpated and these portions transplanted to the flank of the animal. The rudimentary girdles which resulted have been studied both in the presence and absence of the free limb in order to determine the relationship, if any, between these two structures.

As will be shown in the body of this paper, the findings in these experiments upon A. microstomum agree very closely with the results of Detwiler ('18) upon A. punctatum. The present results show conclusively that the pectoral girdle of the former species is also a mosaic of at least four primordia. When any of these primordia were extirpated, corresponding defects occurred in the girdle, and when any of these rudiments were transplanted to a heterotopic location, only the actual rudiments included in the grafted tissue appeared in the transplant. An attempt will be made to explain the discrepancies between the present results and those of Swett and Parsons ('29) upon the bases of the difference in age of the larvae at the time the experiments were performed.

II. MATERIALS AND METHODS

Operations were performed at Harrison's stages 31-35 on embryos of Amblystoma microstomum obtained in ponds in the vicinity of Louisville, Kentucky.

The degree of development of the embryo has been indicated by the use of Harrison's (unpublished) numbered stages. A camera-lucida drawing of stage 31 is shown in figure 1. The somatopleure ventral to the pronephros has thickened, but there is as yet no appearance of the limb bud externally. During the next two stages the tail bud lengthens and the mesenchymal tissue in the limb bud region has gradually increased, making the limb bud a distinct rudiment. At stage 46 the animal begins to feed; the fore limbs have reached the three digit stage, and the hind limb buds are visible. Subsequent development is designated by the number of days after the operation or by the total length of the larva.

The operations on some of the older animals

were carried out under chloretone anesthesia¹. The effect of the anesthesia wore off rapidly as soon as the animals were returned to water and no ill effects were observable.

Dissection was done under the binocular microscope. All operations were performed on the right sides of the animals (homopleural). A wound was made about three somites posterior to the normal limb-bud to receive the excised portion of the limb anlage. Desired portions of the rudiment were excised with finely sharpened irredectomy scissors. This portion of the girdle anlage was superimposed over the exposed mesoderm and was placed in normal orientation (fig. 1, p. 13). The grafts were pressed firmly in place by a small glass straddle weight. The weight was removed in about an hour, at which time healing had occurred. All wounds were cleaned of loose mesoderm in order to insure the removal of all that portion of the girdle anlage. The animals were kept in tap water, only one animal to a finger bowl. They were fed on small worms (*Enchytraeus*) until they were about eight weeks old. Observations and sketches of

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1. Chloretone anesthetic was made by dissolving 1 gm. chloretone in 3,000 cc. of .04 percent NaCl.

developing buds were made at frequent intervals.

All the larvae were killed and fixed in a saturated solution of corrosive sublimate and five percent glacial acetic acid. Typical animals from each series were made into serial sections and studied histologically. Other experimental animals intended for whole mounts were stained in toto with a solution of one-half of one percent methylene blue dissolved in seventy-five percent alcohol. The solution stained the cartilages a faint blue, whereas the surrounding muscle tissue absorbed little or none of the color. The cartilages were dissected out and examined with the dissecting microscope.

An area, larger than is occupied by the girdle and which contained the girdle, was extirpated from the animal. Tissues which surrounded the girdle could now be easily removed with teasing needles.

Camera-lucida drawings were made of the grafted limb bud just after killing, of the serial sections, and of girdles taken from the whole mounts that had been stained with methylene blue. These girdles were stored in glycerine which allowed them to retain their color and kept them from deteriorating.

III. NORMAL ANATOMY OF THE PECTORAL GIRDLE

Each half of the shoulder-girdle of A. microstomum may be regarded as a single skeletal element which is incompletely divided into three regions, rather than as an aggregate of three fused segregates (Fig. 2, p. 13). The major portion of this arciferal girdle remains cartilaginous, becoming ossified only in the region of the glenoid cavity. The pectoral girdle of Salamandra maculosa has been described by Francis ('34) and a comparative account of the pectoral girdle in Amblystoma has been given by Noble ('31).

The coracoid is by far the largest portion of the pectoral girdle. It extends mesially from the glenoid cavity across nearly two-thirds the width of the body, so that the right side overlaps the left side. However, in one instance the left side was found to overlap the right side. These overlapping portions lie immediately ventral to the heart, serving as protection for it. The coracoid forms a deep, concave, cartilaginous plate with its convex surface ventral. Only a small part of the coracoid in the region of the glenoid cavity becomes

ossified. The peripheral free edges are exceedingly thin. The coracoid forms a faceted lip to the posterior border of the glenoid cavity.

The procoracoid forms the anterior portion of the ventral plate of the pectoral girdle. It is spatulate in shape and slightly curved with the convexity on the ventral side. The procoracoid is imperfectly demarcated from the coracoid by a deep notch, the incisura coracoidea which separates the cartilaginous portions of the two regions. It also is ossified in the region of the glenoid cavity, but remains cartilaginous anteriorly. This ossification is continuous with that of the coracoid except for the foramen supracoracoideum through which passes the N. supracoracoidiens and the corresponding artery and vein. A line drawn from the glenoid cavity through this foramen to the center of the incisura coracoidea represents the boundary between the procoracoid and coracoid. The procoracoid projects a conspicuous lip over the anterior face of the glenoid cavity which forms a definite facet to the head of the humerus. Thus the glenoid cavity is formed by lips of the coracoid and procoracoid.

The scapula regions form the dorsolateral portion of the pectoral girdle. The scapula proper

is completely ossified, whereas the distal portion of the scapula region comprises the suprascapula which is entirely cartilaginous. The whole structure is fan-shaped and so curved that the convexity is outwards. The scapula is elliptical in section and expands slightly toward its distal border, where it merges into the suprascapula. The suprascapula extends into a broad fan, being thick at its ventral border but thinning out dorsally.

Fig. 1. Camera-lucida drawing of an embryo of A. microstomum in the tail-bud stage showing areas of extirpations and locations of transplants.

Fig. 2. Right half of the pectoral girdle viewed from the ventrolateral aspect. Adult A. microstomum.

IV. EXPERIMENTAL RESULTS

A. Extirpations

1. Removal of the dorsal half of the fore-limb rudiment (Series $\frac{1}{2}$ DE). The symbols DE, VE, PE, and AE have been used to designate the four series of extirpation experiments, the first letters referring to the dorsal half, ventral half, posterior half, and anterior half of the limb bud respectively, and E, the second letter, indicating extirpation. In the series of transplants which have been designated by the symbols DG, VG, PG, and AG, the first letters again refer to the portion of the limb bud removed, and the G indicates graft. Individual animals in each series have been numbered from 1-10. These extirpation experiments consisted of the removal of the halves of the fore-limb bud as shown in figure 1. The dorsal half contained the area a-c x 1-2. In each case a wound was made on the flank of the animal to receive the excised portion of the limb rudiment. A further discussion of this phase of the experiment will be taken up in

connection with the development of the grafted limbs. In order to include all the limb-forming tissue, the excised dorsal half of the limb bud included also the rudiment of the pronephros. All wounds were cleaned of loose mesoderm cells and in most cases were completely healed within twenty-four hours. The wounds were not covered.

Thirty percent of these experiments regenerated complete limbs (table 1). In this series complete girdles were obtained with the presence of a regenerated limb. In case $\frac{1}{2}$ DE 1 each portion of the regenerated girdle (fig. 3 A) is somewhat larger than the corresponding parts of the normal with but one exception, that being the glenoid portion, which is slightly smaller than the normal.

On the other hand a regenerated girdle obtained in the absence of the free limb was not complete. This girdle was represented in three separate parts (fig. 4 A). The suprascapula is apparently complete, but remains separate from the other girdle parts. The anteroposterior length of the suprascapula is 360 microns as compared to 400 microns of the normal. There are two parts to the ventral portion of the girdle. The procoracoid and coracoid are rudimentary and are not joined at any place

Table 1

Showing results of extirpations

	Series	Total exp.	Lived	Regen.	Not Regen.
	$\frac{1}{2}$ DE	10	9	3	6
	$\frac{1}{2}$ VE	10	9	8	1
	$\frac{1}{2}$ PE	10	7	2	5
	$\frac{1}{2}$ AE	6	4	3	1

with each other or with the suprascapula. The scapula and glenoid fossa are completely missing.

Results of four additional cases studied in this series agree in general with the above conclusions. Animals that failed to regenerate a free limb also possessed an incomplete girdle, the central zone being absent, whereas in cases in which the limb regenerated, an apparently complete girdle was present. It is evident, therefore, that when both a girdle and limb regenerate, the girdle is normal in size and architecture as shown by a comparison with the normal girdle.

The presence of the suprascapula in all cases indicates that the primordium of this cartilage was located more dorsally than the limb disc excised. It would be necessary to extirpate the area dorsal to the limb bud in order to remove the primordium of the scapula.

2. Removal of the ventral half of the fore-limb rudiment (Series $\frac{1}{2}$ VE). The ventral half consisted of the area a-c x 2-3 (Fig. 1). Wounds were cleaned of loose mesoderm and left uncovered. Eighty percent of this series regenerated apparently normal limbs. In two cases not only was a normal limb regenerated, but the grafted tissue developed

into a pair of limbs. These were mirror images of each other. The humeri were ankylosed at their proximal ends and articulated in a single glenoid fossa, this fossa constituting the major portion of the grafted girdle (fig. 5 C). Here again in this series an orthotopic girdle with a regenerated free limb shows a more complete structure than an orthotopic girdle having no attachment with a free limb (figs. 5-6). The essentially complete girdle ($\frac{1}{2}$ VE 5) appears to be hypertrophied as compared to the normal.

Comparing the orthotopic girdle in cases in which a free limb regenerated also with the normal, a completely regenerated girdle is exhibited (fig. 5). Anteroposterior lengths of the normal and regenerated girdles are comparatively identical as shown in table 2.

Each portion of the regenerated girdle, with the exception of the suprascapula, is slightly larger than the normal; restitution has been accompanied by a super-regeneration. The fact that the suprascapula was apparently not disturbed during the operation is evidence of its extreme dorsal and deeply imbedded location.

Table 2
Case $\frac{1}{2}$ VE 5

Regenerated Girdle		Normal Girdle	
Suprascapula	640 microns	Suprascapula	660 microns
Scapula	390 microns	Scapula	380 microns
Procoracoid	510 microns	Procoracoid	470 microns
Coracoid	830 microns	Coracoid	770 microns

The regenerated girdle in the absence of the free limb is incomplete in every respect except for the suprascapula (fig. 6). The anteroposterior length of the regenerated girdle is 770 microns as compared to the normal of 640 microns. The two parts of the ventral portion of the girdle appear rudimentary and are not joined together. The scapula and glenoid fossa are missing.

Three other cases of this ventral series were sectioned. Four other cases were dissected, the girdle and free limb in the latter cases being removed in toto (fig. 7). Results in all cases studied agree in general with the above conclusions, namely, that in cases in which the limb failed to regenerate, the girdle is incomplete and usually in

separate parts, especially because of the absence of the scapula. In cases in which the limb regenerated, the girdle was found to have regenerated as an essentially complete structure.

3. Removal of the posterior half of the fore-limb rudiment (Series $\frac{1}{2}$ PE). The excised posterior half of the limb rudiment consisted of the area a-b x 1-3 (fig. 1). The orthotopic site was cleaned of any loose cells, especially under the pronephric tubules, which were also removed with the graft. Animals in this series were at stage 34. A slight movement was noted during some of the operations, although none of these animals was anesthetized. Only two of these animals regenerated limbs. In the case figured the regenerated limb was rudimentary, the radius and ulna were fused, and no digits were present. The girdle in this case was essentially complete in all dimensions (fig. 8).

Removal of the posterior half usually suppressed development of the external free limb, and in no case did these regenerate a completely normal free limb. Regenerated girdles were morphologically complete in this excised posterior series, even when there was not a free limb regenerated (figs. 9-10). Grafted girdles obtained in this series were incomplete, as

will be discussed later. In a case in which the limb failed to regenerate, it was found that all parts of the regenerated girdle were complete, including the glenoid fossa in which a rudimentary fragment of the humerus has developed, although there was no appearance of a regenerated limb externally (fig. 11 A). Similar results were obtained from a study of four other cases. Measurements of a typical case in which there was no visible free limb are given in table 3.

Table 3

Case $\frac{1}{2}$ PE 3

Regenerated Girdle		Normal Girdle	
Suprascapula	840 microns	Suprascapula	550 microns
Scapula	610 microns	Scapula	530 microns
Procoracoid	720 microns	Procoracoid	700 microns
Coracoid	820 microns	Coracoid	920 microns

The coracoid and glenoid fossa are slightly smaller than the normal. Limbs in these experiments failed to develop as in the dorsal and ventral series, although girdle formation is much more complete. These girdles were hypertrophied as antero-posterior measurements indicate.

Since a nearly perfect girdle was obtained in every case in which the posterior half of the disc was excised, with or without the presence of limb regeneration, it is evident that very little girdle-forming material is located in this portion of the limb bud.

4. Removal of the anterior half of the fore-limb rudiment (Series $\frac{1}{2}$ AE). This anterior half consisted of the area b-c x 1-3 (fig. 1). Animals used in this series were of stages 34-35. Since the mortality rate was high, only four animals were available for study. These, however, were good specimens, having regenerated limbs and grafted girdles. Wounds were cleaned of loose mesoderm and the same care was given these animals as in the previous experiments. The mesoderm of these older embryos was tough and sticky. Wounds did not heal as readily as those of stages 29-32.

There is remarkable similarity among regenerated girdles of this series and those of the dorsal and ventral series. Here again the ventral portions of the orthotopic girdle appear in two separate parts and are not articulated with each other or with the dorsal part of the girdle. Girdles which developed in the absence of a free-limb consist of the supra-

scapula, rudimentary coracoid and a procoracoid (fig. 12 A). Girdles regenerated with a free limb exhibit similar parts and in addition a rudimentary glenoid cavity which did not appear in the above mentioned girdle (fig. 13 A). Anteroposterior measurements of the latter case are given in table 4.

Table 4

Case $\frac{1}{2}$ AE 6

Regenerated Girdle		Normal Girdle	
Suprascapula	580 microns	Suprascapula	470 microns
Scapula	None	Scapula	260 microns
Procoracoid	290 microns	Procoracoid	320 microns
Coracoid	970 microns	Coracoid	1160 microns

Since in this series the suprascapula only is consistently present in the regenerated girdle, it is obvious that cells which form the suprascapula are not contained in the excised anterior half of the limb bud. As the scapula does not appear at all in this series, cells which form this portion of the girdle are apparently contained in the anterior half of the limb disc. The imperfect condition and reduced size of the coracoid and procoracoid indicate that these parts are also located

at least partially in the anterior half of the disc. In these experiments girdle formation seems to be independent of the presence or absence of the free limb.

B. Grafts

The excised portion of the right limb bud was grafted on the right flank of the same animal, all grafts therefore being autoplasmic, homopleural, and heterotopic. The size, shape, and location of the extirpated rudiments have been described previously (p. 8). Wounds somewhat larger than the excised portion of the limb bud were first prepared on the right flank of the animal and the excised portion of the limb bud was superimposed over the exposed mesoderm. All grafts were of normal orientation. Most of the grafts were completely healed in from twelve to twenty-four hours.

1. Transplantation of the dorsal half of the limb bud (Series $\frac{1}{2}$ DG). The area of tissue transplanted in these experiments is shown in fig. 1, as a-c x 1-2. The graft included part of the pronephros in order to obtain all the underlying mesoderm.

In two such transplants out of nine, the tissue was resorbed and no trace of a girdle was found

Table 5

Showing results of transplants

Series	Total exp.	Lived	Grafts Present	Grafts Resorbed	Re-dupli- cations
$\frac{1}{8}$ DG	10	9	7	2	0
$\frac{1}{8}$ VG	10	9	4	5	2
$\frac{1}{8}$ PG	10	7	5	2	0
$\frac{1}{8}$	6	4	3	1	0

(table 5). The other seven developed into limbs which were more or less abnormal. Most of these limbs were curved and atrophied; all lacked function.

The grafted girdle is rudimentary, consisting of an essentially complete scapula and glenoid fossa (fig. 4 C). The ventral portion of the coracoid is missing.

Table 6

Anteroposterior measurements $\frac{1}{2}$ DG 10

Graft (fig. 4 C)		Normal (fig. 4 B)	
Scapula	270 microns	Scapula	450 microns
Coracoid	530 microns	Coracoid	780 microns

The suprascapula and procoracoid are missing. The mid-portion of the girdle is well developed although somewhat reduced in size as shown in table 6. These missing parts are to be found in the regenerated girdle, namely, the suprascapula, procoracoid, and ventral portion of the coracoid (fig. 4 A). A comparison with the regenerated rudiment shows that a coracoid has developed in both. This might be explained on the supposition that this rudiment was divided into two parts by the operation, one part being grafted to the new location and one part being left in its

original position.

The presence of the suprascapula in an orthotopic position seems to indicate that this rudiment lies far dorsal on the flank, apparently above the point of extirpation. The presence of procoracoid in the orthotopic position and its absence in the graft is also very significant.

2. Transplantation of the ventral half of the limb bud (Series $\frac{1}{2}$ VG). The area of mesoderm transplanted in these experiments consisted of the area a-c x 2-3 (fig. 1). All grafts were autoplasmic, homopleural and of normal orientation. Four such transplants gave rise to deformed limbs, two of which showed reduplications of the entire free limb (table 5). In the cases of the reduplications, single girdles were found. The humeri were fused at their distal ends and articulated in a single glenoid fossa. One animal died before significant development had occurred. The remaining five developed imperfect grafts which were resorbed in from six to ten days.

Girdles which had developed in these experiments were composed of a central and ventral zone. These parts of the girdle are complete with respect to their elements, viz., scapula, procoracoid, and

coracoid. In both cases of reduplicated limbs, each girdle contained a glenoid fossa and a hypertrophied rudiment of the dorsal part of the coracoid (fig. 5 C). Girdles found in these experiments were similar to those of the dorsal series, that is, the suprascapula is missing entirely.

Table 7

Anteroposterior measurements $\frac{1}{2}$ VG 2

Grafted girdle (fig. 5C)		Normal girdle (fig. 5B)	
Scapula	470 microns	Scapula	270 microns
Procoracoid	70 microns	Procoracoid	440 microns
Coracoid	620 microns	Coracoid	1360 microns

The scapula is apparently complete although somewhat hypertrophied. The glenoid fossa and humerus (fig. 6 C) appear much the same as in the normal. The coracoid is essentially complete, although its anteroposterior measurement is about half that of the normal (table 7). The two parts of the girdle, scapula and coracoid, form a single piece of cartilage, but they are connected by only a narrow zone of junction. A deep glenoid fossa has formed.

Comparing the parts of this girdle with those

of the regenerated girdle, it is found that the grafted girdle is composed of a morphologically perfect scapula and coracoid; the former is missing entirely in the orthotopic position while the latter is represented by an extreme ventral rudiment. The procoracoid is found in both the grafted and the regenerated girdle. The procoracoid is situated in the mid line of the girdle.

Extirpating the dorsal or ventral half of the limb disc, it would be expected that a portion of the procoracoid would be included in the extirpation and a part would remain undisturbed in the orthotopic position. In the same way the extirpations did not include the extreme dorsal rudiment of the coracoid, hence, this portion developed in its original place. The entire scapula was included in these grafts, and therefore no rudiment of this scapula was to be found in the regenerated girdle. It is apparent that the suprascapula, due to its extreme dorsal location, would be absent in these grafts.

In three cases the transplanted girdles were stained in toto and dissected out. The results in general agreed with the case described in detail above.

3. Transplantation of the posterior half of the limb bud (Series $\frac{1}{2}$ PG). The area of mesoderm transplanted in these experiments consisted of the area a-b x 1-3 (fig. 1). In two cases out of ten the graft was resorbed after five days, and no traces of girdles were to be found. Three animals died shortly after the operation and were lost to the experiment (table 5). The other five developed limbs, all of which had abnormal hands. The forearm and hand of these grafted limbs were especially deformed. All limbs lacked function.

Girdles which developed in these experiments were rudimentary as compared with those formed in the other three series, yet a high percentage of free limbs were obtained. These grafted girdles were composed solely of the glenoid portion of the coracoid, other parts of the girdle being entirely missing. In fig. 10 C, the girdle is represented by a rudimentary coracoid with a well-formed glenoid fossa. In the case $\frac{1}{2}$ PG 8 (fig. 11 C) the girdle appears in two parts as dorsal and ventral rudiments, presumably both being parts of the coracoid. These parts are not joined at any place. The antero-posterior length of these two pieces of cartilage is 250 microns, a small rudiment as compared to 950

microns of the normal coracoid and 1020 microns of the same structure of the regenerated girdle. Although the glenoid fossa of the grafted girdle was very poorly developed in this case, in other cases (as $\frac{1}{2}$ PG 3, fig. 10 C) it was well formed. Regenerated girdles were completely formed except for poorly developed glenoid fossae.

The five transplants which developed grafts were found to possess complete external limbs but rudimentary girdles. A comparison of the grafted girdle rudiment with the orthotopic one shows clearly that these two parts combined would approximate a complete girdle. This shows that the suprascapula, scapula, and procoracoid portions of the girdle are not present in the posterior portion of the limb disc, but that abundant limb-forming cells are. A small part of the girdle rudiment was contained in the transplant along with limb-forming cells. The limb is an equipotential system as stated by Harrison ('18); therefore complete limbs were usually obtained.

Since only a small rudiment of the pectoral girdle appeared in the transplants, further evidence is obtained that it is a mosaic system. Detwiler's thesis, that when a portion of a rudiment is removed there appears a deficiency at that location and when a portion of a rudiment is transplanted only that

portion develops, is substantiated by these findings.

4. Transplantation of the anterior half of the limb bud (Series $\frac{1}{2}$ AG). The area transplanted in these experiments is shown in fig. 1, as b-c x 1-3. Six animals were used, two of which gave rise to deformed limbs. Each of these animals regenerated complete free limbs and girdles as well. In the case of $\frac{1}{2}$ AG 1 a complete grafted suprascapula was found in the region of the graft. The remaining embryos developed limb budiments that were resorbed from four to six days after the operation. Several days later these embryos died and were lost to the experiment (table 5).

The grafted girdles of this series are quite similar to those of the dorsal and ventral series. Two animals were studied, each possessing a grafted limb. The girdles were identical, consisting of a well developed scapula, procoracoid, glenoid fossa, and coracoid. No part of a suprascapula was to be found (figs. 12-13).

The measurements show the ventral half of the grafted girdle to be complete (table 8). The ventral portion of the grafted coracoid appears blunt in the case of each animal studied.

Table 8

Anteroposterior measurements $\frac{1}{2}$ AG 6

Grafted girdle (fig. 10C)		Normal girdle (fig. 10B)	
Scapula	290 microns	Scapula	250 microns
Procoracoid	210 microns	Procoracoid	420 microns
Coracoid	730 microns	Coracoid	730 microns

The most ventral part of the coracoid of these girdles appears in the orthotopic position. It then seems likely (figs. 12-13) that the ventral part of the coracoid was not included in the transplants. A small portion of the procoracoid also appears in the regenerated girdle. The scapula and glenoid part of the girdle are complete in the graft but entirely missing among the regenerated parts of the girdle.

Results of these experiments indicate that the parts of the grafted girdle would supplement those of the regenerated girdle to form a complete girdle. Girdle formation seems to be independent of the presence or absence of the free limb, as has been demonstrated in previous experiments. The anterior half of the girdle rudiment was transplanted with the anterior part of the limb rudiment. A limb

develops from the transplanted rudiment of the limb anlage but only that portion of the girdle develops which has been transplanted. Again these results agree with those of Detwiler ('18) that the pectoral girdle of Amblystoma is a mosaic.

In the case of $\frac{1}{2}$ AG 1, which was stained in toto, no part of the graft (limb or girdle) was visible externally. In the region of the graft a complete suprascapula could be seen after removal of the skin. This rudimentary girdle had no attachment and no other girdle parts were to be found. The regenerated girdle and limb of this specimen were complete, although the distal end of the suprascapula appears to be blunt. The suprascapula, as previously noted, lies so far dorsal that it was included in only this one transplant. Since this animal possessed such a perfectly regenerated limb and girdle, it is possible that the graft included only a small portion of the rudiment of the suprascapula (fig. 14).

V. GENERAL DISCUSSION

In all four series of experiments at least partial girdles developed in the absence of a free limb. Particularly when the posterior half of the limb bud was extirpated, complete orthotopic girdles developed in the absence of the free limb. This is in agreement with Detwiler ('18) and Swett and Parsons ('29) who obtained conclusive results showing that the pectoral girdle develops independently of the free limb. However, in the present experiments in the dorsal and ventral series girdles which developed in the presence of the free limb were more nearly complete than were girdles obtained in the absence of the free limb, showing that the girdles have some power to regenerate lost parts.

Orthotopic and grafted girdles in the dorsal and ventral series seem to supplement each other, as has been shown in this paper. The supposition that the posterior portion of the limb disc contains limb-forming cells would explain the absence of the free limb in the orthotopic position and the very rudimentary girdle in the heterotopic position.

In the dorsal and ventral series the orthotopic

girdle seemed more complete when the free limb developed along with the girdle. In one case ($\frac{1}{2}$ VE 5) a super-regeneration of the orthotopic girdle took place. All the parts of the girdle had not only regenerated, but the scapula and suprascapula were much thicker than in the control girdle. The grafted girdle in this case consisted of a rudimentary coracoid which was abnormally thick. When the posterior half was extirpated, morphologically complete girdles were obtained from the portion remaining even in the absence of the free limb. In two of the cases studied, orthotopic girdles were practically identical with the normal. A small distal end of the humerus was also found in each of these cases. As has been stated before, the posterior portion of the limb disc contains practically no girdle-forming elements, hence the girdle would not be greatly disturbed as results herein indicate. Schwind ('32) reports in his findings on the torsion of the forelimb that the entire girdle rudiment of the embryo (A. punctatum) lies anterior to a line formed by the projection of the fourth-fifth somite boundary. Grafted girdles in this series were very rudimentary, although free limbs accompanied these rudiments. Here also, only these parts of the rudiments which were

transplanted at the time of the operation developed from the grafts.

Cases studied in the anterior series present quite similar results, in that only the primordial segregates of the girdle which were included in the transplant developed. The remainder of the girdle was left in its original location and developed as isolated cartilaginous fragments.

In no case was the suprascapula absent in the normal position. Detwiler ('18) has reported that the suprascapula in A. punctatum develops from a separate rudiment of its own. Results in all these experiments on A. microstomum bear out his findings. He also reported that the suprascapula lies far dorsal and deeply imbedded so that excised parts of the limb disc, in order to contain the suprascapula, would necessarily cover a larger area and contain more deeply imbedded mesoderm. Swett and Parsons ('29), after removal of the whole girdle in A. punctatum at older stages, found deficiencies in all cases in the most outlying parts of the regenerated girdle, namely, the suprascapula and procoracoid.

In one case only ($\frac{1}{2}$ AE 1, fig. 14 C) has a suprascapula been found in a heterotopic location. This grafted suprascapula was the only segregate of the girdle to differentiate and can perhaps be ex-

plained in this way. The excised portion may have included tissue dorsal to the limb disc, but only the most anterior part of the limb disc proper.

The procoracoid develops later than the other parts of the girdle. It is small in larvae at the age sectioned, but could be recognized. Swett ('29) in extirpating the procoracoid (A. punctatum) found this segregate to be very small at stage 46. However, it seemed to be quite a distinct rudiment in A. microstomum since it was often left behind in the orthotopic position where it appeared as a separate piece of cartilage.

Development of a heterotopic scapula was obtained in all series of experiments except the posterior series. All results indicate that if the scapula is transplanted, development of this structure takes place in the graft and is entirely missing in the orthotopic position; not the slightest tendency of any restitution process was to be found in the case of this ossifying structure. The scapula was entirely absent in the graft from the posterior half of the limb disc, but fully developed in the orthotopic girdle. This agrees with our previous hypothesis that the posterior half of the limb disc contains largely limb-forming mesoderm, and that girdle-forming

mesoderm is present in extremely small amount.

The development of a rudimentary coracoid in every case in both the orthotopic and heterotopic positions is evidence that a part was transplanted and a part left undisturbed. Results in all cases studied show that orthotopic and heterotopic rudiments of the coracoid are largely supplementary. The fact that rudiments of the coracoid appeared in both the orthotopic girdles and in the grafts, and that these parts are supplementary may be evidence of at least two distinct segregates for this structure. More experiments would be necessary to prove this hypothesis.

There appears to be a slight restitution on the part of the coracoid. In cases where the major part of the coracoid was present, whether in the orthotopic girdle or graft, it underwent some restitution, especially by forming a narrow strip of cartilage ventrally.

Detwiler ('18) on A. punctatum has shown that operations used in extirpating the limb rudiment leave parts of the procoracoid and coracoid in the embryo. By a process of hyperplasia these cartilages gradually extend across the gap between them until they become united into a girdle which is topographically complete. This tendency of the coracoid to

undergo some restitution therefore bears out the results of Detwiler.

From the above results it is apparent that the pectoral girdle of A. microstomum is a mosaic and consists of at least four distinct primordia. It has, however, a strong tendency to regenerate missing parts in cases where the free limb regenerated.

Detwiler ('18) reports that by removal of the dorsal half of the limb bud together with the third, fourth, and fifth myotomes he was able to suppress development of the suprascapula, scapula, and the free limb so that only the ventral half of the girdle develops, whereas results in this paper show that removal of the dorsal half of the limb disc but not the myotomes has little or no effect on the suprascapula, but that development of the scapula and glenoid portions is suppressed, as well as development of the free limb. Detwiler found that heterotopic girdles, which developed from the dorsal transplants, consisted of a dorsal zone: the suprascapula, scapula, and the dorsal part of the coracoid. Results in this paper show a complete scapula, dorsal portion of the coracoid but not nearly as much of the suprascapula as is shown by Detwiler,

probably because the myotomes dorsal to the limb disc were not included in the transplant. The failure of the cells of either the ventral or dorsal zones to regenerate the missing zone indicates their lack of equipotentiality.

After removal of the pectoral girdle and the limb of A. punctatum at older stages (stage 46), Swett and Parsons ('29) supposed that a small bar of cartilage found just lateral to the pronephros was a fragment of the suprascapula which was not removed at the time of the operation. It is reasonable to assume, from the similarity of results in this paper and those mentioned above, that cells lying so close to the pronephros were also not removed in our extirpation experiments. In this way the repeated absence of the suprascapula in the grafted girdle and its consistent presence in the orthotopic position might be explained. They also reported that when they removed the girdle but not the free limb, a complete girdle developed. Microscopic study showed that a blastema of mesodermal tissue formed which resembled the limb primordium in earlier stages and from this differentiated a complete girdle.

In some cases Swett and Parsons ('29) reported that secondary limbs grew, finally fusing with the

primary limb at the distal end of the humerus. Regenerated girdles in these cases were single girdles. In the present study similar results were obtained when the ventral half of the limb disc was removed, namely, a single girdle associated with a pair of limbs. It is obvious that these results of Swett and Parsons ('29) are in complete disagreement with those of Detwiler ('18) who used the same species except at earlier stages, and of the results obtained in this paper. Apparently age of the larvae is the controlling factor.

When a part of the girdle rudiment is removed at such an early stage that the regions of the girdle are still preprimordial segregates, the regeneration processes do not manifest themselves in regard to the pectoral girdle. Therefore, whenever one or more of the four segregates of the girdle-mosaic is removed then that portion will be missing in the girdle which forms. This accounts for the partial girdles obtained by both Detwiler and the writer. In these early stages a regeneration blastema of the kind reported by Swett and Parsons fails to form. Healing is quick and complete and as a result no regeneration of missing girdle rudiments occurs.

1. The pectoral girdle of A. microstomum will develop in the presence or absence of the free limb although its power of restitution seems greater when the limb is present.

2. The embryonic shoulder-girdle is a mosaic composed of at least four primordial segregates in the tail-bud stage.

3. Each primordium develops independently in both orthotopic and heterotopic positions.

4. The posterior half of the limb-disc contains an abundance of limb-forming cells and very little girdle-forming tissue.

5. The procoracoid develops so late that it is very small in comparison to other girdle structures at stages studied in this paper.

6. The primordium of the suprascapula is located dorsal to the limb-disc, or dorsal to areas extirpated in the experiments reported here.

7. In cases in which the graft reduplicated, only a single shoulder-girdle was found.

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VIII. FIGURES

Abbreviations

cor,	coracoid	r.u,	radius, ulna
hum,	humerus	s.sc,	suprascapula
p.cor,	procoracoid	r g,	regenerated girdle
sc,	scapula	n g,	control left girdle

Fig. 3 Camera-lucida drawings of case $\frac{1}{2}$ DE 1.
x 40. A, orthotopic girdle developed after removal
of the dorsal half of the limb bud, the free limb
having regenerated. B, is control left girdle. The
graft was resorbed.

Fig. 4 Camera-lucida drawings of case $\frac{1}{2}$ DE 10.
x 40. A, orthotopic girdle developed after removal
of the dorsal half of the limb bud, the free limb
having failed to regenerate. B, control left girdle.
C, section of transplant.

Fig. 3

Fig. 4

Fig. 5 Camera-lucida drawings of case $\frac{1}{2}$ VE 5.
x 40. A, orthotopic girdle developed after removal
of the ventral half of the limb, the free limb having
regenerated. B, control left girdle. C, section of
transplant.

Fig. 6 Camera-lucida drawings of case $\frac{1}{2}$ VE 2.
x 40. A, orthotopic girdle developed after removal
of the ventral half of the limb bud, the free limb
having failed to regenerate. B, control left girdle.
C, section of transplant.

Fig. 5

Fig. 6

Fig. 7 Camera-lucida drawing of the regenerated girdle and free limb, case $\frac{1}{2}$ VE 8. x 40. Viewed from the mesio-dorsal aspect; shaded portion of the girdle - ossified. The graft in this case was resorbed.

Fig. 7. $\frac{1}{2}$ VE 8

Fig. 8 Camera-lucida drawings of the regenerated girdle and free limb, case $\frac{1}{2}$ PE 9. x 40.
A, viewed from the mesio-dorsal aspect. B, viewed from the ventrolateral aspect. Heavy stipple shows ossification. The graft in this case was resorbed.

Fig. 8. $\frac{1}{2}$ PE 9

Fig. 9 Camera-lucida sketch of cross-section of A. microstomum larva $\frac{1}{2}$ PE 3, passing through the region of the glenoid fossa. Coarse stipple, cartilage; hatching, muscle masses. r.g. normal girdle, n.g., regenerated girdle. x 40.

Fig. 10 Camera-lucida drawings of case $\frac{1}{2}$ PE 3.
x 40. A, orthotopic girdle developed after removal
of the posterior half of the limb bud, the free limb
having failed to regenerate. B, control left girdle.
C, two adjacent sections of the transplants.

Fig. 11 Camera-lucida drawings of case $\frac{1}{2}$ PE 8.
x 40. A, orthotopic girdle developed after removal
of the posterior half of the limb bud, the free limb
having failed to regenerate. B, control left girdle.
C, section of the transplant.

$\frac{1}{2}$ PE 3

Fig. 10

 $\frac{1}{2}$ PE 8

Fig. 11

Fig. 12 Camera-lucida drawings of case $\frac{1}{2}$ AE 5.
x 40. A, orthotopic girdle developed after removal
of the anterior half of the limb bud, the free limb
having failed to regenerate. B, control left girdle.
C, section of transplant.

Fig. 13 Camera-lucida drawings of case $\frac{1}{2}$ AE 6.
x 40. A, orthotopic girdle developed after removal
of the anterior half of the limb bud, the free limb
having regenerated also. B, control left girdle.
C, section of transplant.

Fig. 12

Fig. 13

Fig. 14 Camera-lucida drawings of the regenerated girdle, free limb, and graft, case $\frac{1}{2}$ AE 1. x 40, from the mesio-dorsal aspect. B, represents the entire transplant.