FURTHER STUDIES ON DIPLOPODIA

II. EMBRYOLOGICAL FEATURES

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INTRODUCTION

The diplopod phenotype (Taylor and Gunns, 1947) appears in embryos homozygous for an autosomal recessive gene, **dp,** which interferes with limb and beak differentiation. A gene-controlled alteration of this sort provides a tool similar to transplantation and extirpation methods for the causal analysis of embryonic processes. As well as providing information on the specific action of **dpdp,** the changes observed suggest the functions of the normal allele of **dp** during development. Selected lines of diplopod carriers having different incidences of diplopod embryos in their offspring (Taylor, Abbott and Gunns, 1959) and differing in the expressivity of diplopodia (Abbott, 1959) allow the study of interactions within different genetic milieu, as expressed in the developmental patterns of the individual lines and crosses.

The manifold phenotypic expression of diplopodia, involving as it does bones of the extremities, indicates a defect in either early cartilage or bone formation.

PROCEDURES AND RESULTS

Studies of the embryonic development of diplopod embryos fell into several categories. Each of these will be discussed separately.

1. *Early stages in the development of the limb of diplopod embryos*

Embryos from the normal-ratio line, I, were removed from eggs at six, eight and ten days of embryonic age, studied under a dissecting microscope and classified as either normal or diplopod. If they were diplopod, the numbers, sizes and positions of the foot digits (on eight- and ten-day embryos) or the shape and degree of digitation (on the six-day embryos) were recorded. From each stage, several diplopod and normal sibling control embryos were chosen for the following treatment; the left and right posterior limbs were severed from the body, flattened on a small rectangle of paper to orient the digits and placed in separate vials of Bouin's fixative. The tissues were then treated by the Celloidin method. Each block of tissue was divided into five layers and 15 or 20 sections saved for staining; this procedure provided a fairly accurate picture of the orientation of the long bones, which were often located on different planes. Sections were cut at an average of 8μ. They were stained for general detail with Haematoxylin and Eosin and with Iron Haematoxylin, Aniline Blue and Methyl

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Green and the Mallory Connective Tissue stain for detail on the development of hyaline cartilage, periosteal bone formation, fibroblast differentiation and tendon formation.

Compared with limbs from normal embryos, diplopod limbs were markedly retarded in (*a*) early cartilage differentiation, (*b*) rate of formation of cartilage bone models and (*c*) rate of osteogenesis. Within the limb of diplopod embryos, normal digits II, III and IV differentiated at a more rapid rate than the digits of the supernumerary group. Within the supernumerary group, the most proximal toe developed most rapidly.

In the normal fowl foot primitive mesenchyme condenses into four rod-like structures, the precursors of the four normal digits. The precursor of digit I (the hallux) is the last to appear. In the diplopod foot digit differentiation may follow one of two alternative courses. In diplopod embryos from the normal-ratio line, I, the mesenchymal condensations of tissue foreshadowing digits II, III and IV appear and eventually differentiate into the corresponding normal digits. However, the forerunner of digit I does not appear; instead new condensations of mesenchymal tissue appear in the pre-axial region of the foot. These condensations are evident slightly later than those in the post-axial region. They form a proximo-distal gradient in size. They do not appear to be mirror images of the normal organized mesenchyme. According to the second possible sequence, which is typical of embryos from the intermediate and low-ratio lines and from line crosses, the four normal condensations of tissue appear and differentiate into digits I, II, III and IV. Digit I is the last to appear. An additional condensation of mesenchyme, immediately proximal to the position of digit I, the hallux, forms almost immediately. It eventually differentiates into a normal appearing digit, which resembles the hallux very closely. Although this latter is the one of the most frequent sequences in low-ratio diplopod embryos, the numbers, sizes, and positions of supernumerary digits in such progenies are highly variable (Abbott, 1959).

At six days there was a marked difference in differentiation of the normal and diplopod limb. In the normal embryo three of the four metatarsal bone models and some of the tarsal elements could be distinguished. Accumulations of mesenchyme and differentiating cartilage marked the future positions of the bones. In the diplopod limbs only two cartilaginous metatarsal areas were apparent. The region of the normal digits was marked by accumulations of mesenchyme; no cartilage was observed. Additional areas of compact mesenchyme, proximal to the normal and more distinct mesenchymal accumulations, provided the only indication of the supernumerary digits. This mesenchymal tissue appeared normal apart from its tardy differentiation. Haematoxylin and Eosin stained sections of this stage gave little detail, whereas the Iron Haematoxylin, Aniline Blue and Methyl Green stained sections distinguished the developing cartilage bone models from the undifferentiated mesenchymal condensations. In the normal embryos these areas were larger and more deeply stained than in the diplopod embryos examined.

The tardy development of the diplopod limb was even more evident in the eight-day stages studied. Areas of maturing cartilage, which showed columnar alignment but not the characteristic hypertrophy of mature cartilage, were found in the larger bone models of the diplopod limb. Normal embryos of this age showed clear alignment and hypertrophy of cartilage cells in the central regions of all bone models. Four metatarsal bone models were evident; these were separate from each other and had not yet fused with the tarsal elements. In the diplopod embryos the metatarsal bone models corresponding to normal digits II, III and IV were not complete. Supernumerary metatarsal elements were more retarded than were those of the normal group.

Figure 1. A normal (center) and two diplopod limbs from ten-day embryos.

In Figure 1 (illustration prepared from a slide tracing), two views of diplopod limbs and one of a normal after ten days of embryonic development are shown. The cartilage models of both the metatarsal bones and the individual phalanges were well formed. Subperiosteal bone formation had commenced and was well underway in the medial region of all metatarsal bones in normal embryos. In this region a thick shell of developing bone could be seen (Figure 1). Metatarsal ossification was retarded in diplopod embryos of the same age. Fibroblast differentiation and tendon formation were retarded in the diplopod limbs. Supernumerary digits showed no bone formation, and cartilage differentiation in these was well behind that of the normal digits. Figure 1 compares also the position of the three extra digits with that of the hallux of a normal sibling. This section demonstrates the difference in orientation and stage of development of normal digit I and the supernumerary group. The position, form and connection of the extra digits with the metatarsal elements suggests that the extra digits in typical expression diplopodia are not mirror images of the normal group.

2. *Nine- to sixteen-day stages in the development of normal and typical[1] diplopod embryos*

Living normal and diplopod full-sib embryos of line I were sacrificed daily from nine to sixteen days of age. Embryo size, shape, position in the egg and the incidences of other abnormalities[2] were recorded. The embryos were than treated by the Alizarin bone-staining method as a means of studying the daily progress of osteogenesis and the replacement of the cartilage bone models by bone itself. The degree of ossification in the cranial and limb regions for each daily stage was recorded.

In normal embryos ossification begins in the limb before it does in the cranium. In the first stage studied, day 9, periosteal bone formation was only slightly advanced. In the posterior limb, the tibia and fibula were present as long hollow cylinders of bone; the second, third and fourth metatarsals were represented by small separate circular shells of bone. No bone was present in the first metatarsal. Tarsal elements were not apparent. In the wing, the radius and ulna had barely commenced ossification. Bone formation had just begun in the larger metacarpals but not in the wing digits. The major bones of the cranium were not visible. The only evidences of bone formation in the head region were two short ridges extending medially between the eyes, the maxillary and mandibular processes.

In the normal embryo the posterior limb ossified more rapidly than the anterior limb. The femur, tibia, fibula, humerus, radius and ulna showed bone formation first, followed by metatarsals II, III and IV and shortly thereafter by the metacarpals. Subsequently, metatarsal I and the larger proximal phalanges of the hind limb began ossification. Finally, the larger phalanges of the fore limb, the smaller distal phalanges of the hind limb and the cranium began bone formation. The bones of the mandibular and maxillary processes developed before and slightly more rapidly than those of the cranium proper. After the 11th day, bone formation proceeded rapidly in the cranial region.

At all stages diplopod embryos lagged behind their normal siblings in rate of development, particularly in bone formation in the extremities. The sequence of ossification in the normal bones of diplopod embryos was similar to that of normal embryos. However, the pre-axial supernumerary digits began ossification after the normal complement. The appearance of bone in the hallux (if present) was followed by its appearance in digits of the supernumerary group. In diplopod embryos of the normal-

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[1] Typical in this case refers to embryos with normal foot digits II, III and IV and three supernumerary digits replacing the hallux (normal digit I).

[2] Abnormalities not characteristic of diplopodia.

ratio line (lacking halluces) bone appeared even later in the extra digits and was first apparent in the large proximal digit.

Developmental retardation was most marked in the earliest stages studied. Ten-day diplopod embryos showed less bone formation than nine-day normal embryos. The femur appeared as a small hollow cylinder of bone; the tibia was represented by a dot and the second, third and fourth metatarsals were present as small faintly-staining rings of bone. In the wing, only the humerus, radius and ulna had begun ossification. The region of the metacarpals was indicated by a small dot of bone. In the head only a faint indication of bone in the proximal portion of the mandibular region was apparent. The eleven-day diplopod embryos studied were approximately equal in degree of ossification to the most advanced nine-day normal stage. In all stages studied, diplopod embryos were from 1-2 days behind normal embryos in ossification.

In diplopodia the inhibition of ossification is associated specifically with the type of osteogenesis, which is preceded by a stage of cartilage hypertrophy. Membrane bone forms in a more normal manner. A twelve-day embryo in this series, characterized by a syndrome of anomalies often found in embryos with particularly severe expressions of diplopodia (celosomia, hemorrhage and edema) showed an especially marked difference between rate of ossification in the limbs and the formation of membrane bone in the cranium. The head of this embryo was equivalent in bone formation to its normal sibling, whereas the limbs lacked bone entirely. In spite of the absence of bone, digitation was well advanced, suggesting that abnormalities in limb ossification are not necessarily associated with abnormalities in cartilage formation, and that ossification rates in different parts of the chick embryo can be independent of each other. These studies of bone development further indicated that bending of the limb bones in diplopod embryos commences during the twelfth day. After the twelfth day dystrophy of the limbs becomes increasingly pronounced in diplopod embryos developing severe expressions of the phenotype.

3. *Comparison of nine- to twenty-day stages in the development of normal and diplopod siblings from the normal-ratio and low-ratio lines*

Eggs containing living normal and diplopod full-sib embryos from the normal-ratio line and from low-ratio matings[1] were opened daily from 9-20 days of age and examined alive. The orientation of the embryo in the egg, the extent of feather development, the incidence of anomalies not characteristic of diplopodia, and the numbers of digits were recorded. Two measurements were obtained on each pair of normal and diplopod embryos. The distance from cranium to pygostyle (M I) estimated body length; the distance from the hock joint to the tip of digit III (M II) was considered as a measure of limb length. The embryos were then photographed and prepared for alizarin bone staining.

Diplopod embryos were retarded in such general features as time of assuming certain embryonic positions, rate of feather development and over-all growth rate. The

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[1] Lines II, III and low-ratio crosses.

degree of retardation was dependent on the source (line or cross) of the embryos. Embryos from low-ratio matings, having expressions involving only one supernumerary digit and with the hallux present, were least retarded.

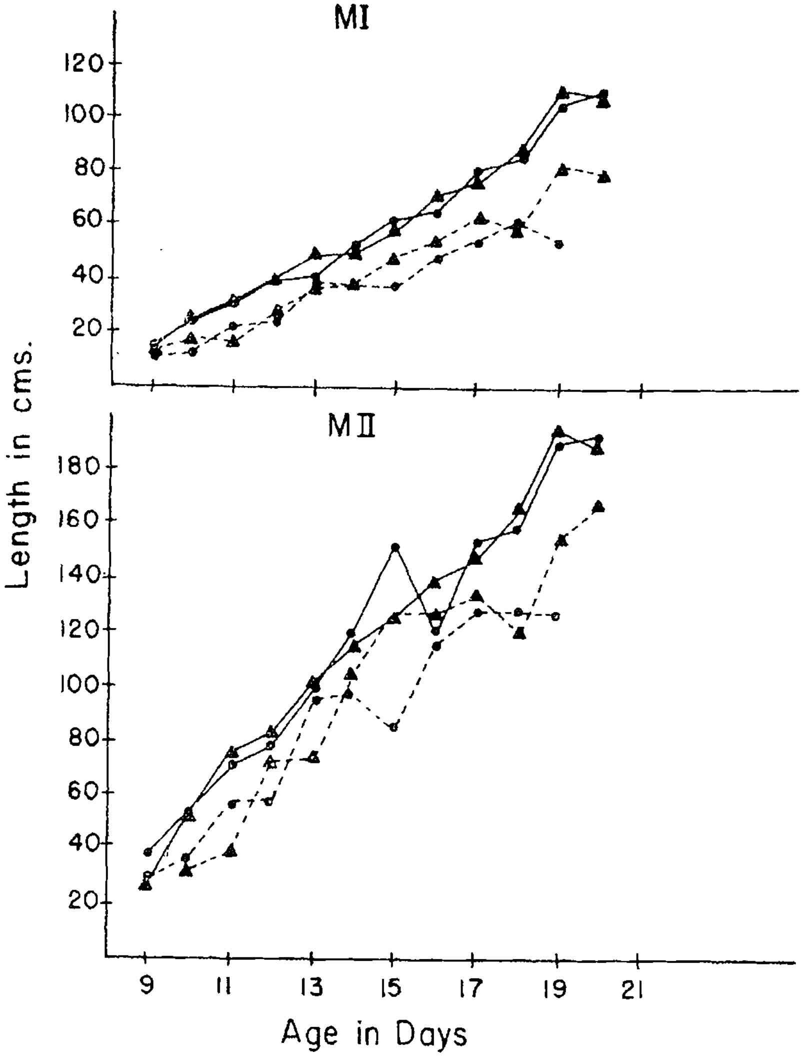

Figure 2. Development of normal and diplopod full-sib embryos of high and low-ratio matings from 9-20 days.

M I = Body length
M II = Limb length
Normal Embryos————
Diplopod Embryos - - - - - - -
High-Ratio. ●
Low-Ratio. △

The measurements of embryonic body length (M I) and limb length (M II) have been summarized in Figure 2 as growth curves. The marked effect of **dpdp** on over-all body size and limb length at all developmental stages examined is evident. There is a general (although not invariable) difference between diplopod embryos from the normal-ratio line and from the low-ratio matings; the latter resembling their normal controls more than the former.

The usual criteria for establishing the time of death of an embryo, based on stages of normal chick ontogeny, proved to be misleading in the case of diplopod embryos. These developmental studies revealed that actual and apparent ages at death of diplopod embryos were different. Diplopod embryos of line I, found alive at 20 or 21 days, resembled normal embryos of 18 days in apparent age. Had they died in shell at this stage they would have been routinely classified as earlier deads. In subsequent tests incubation of embryos for one additional day increased by 10% the incidence of diplopod embryos pipping the shell or hatching. The majority of these successful embryos were from the low-ratio matings. A larger number of phenotypically normal embryos hatched as well. The "normal" incubation period for diplopod embryos apparently exceeds 21 days.

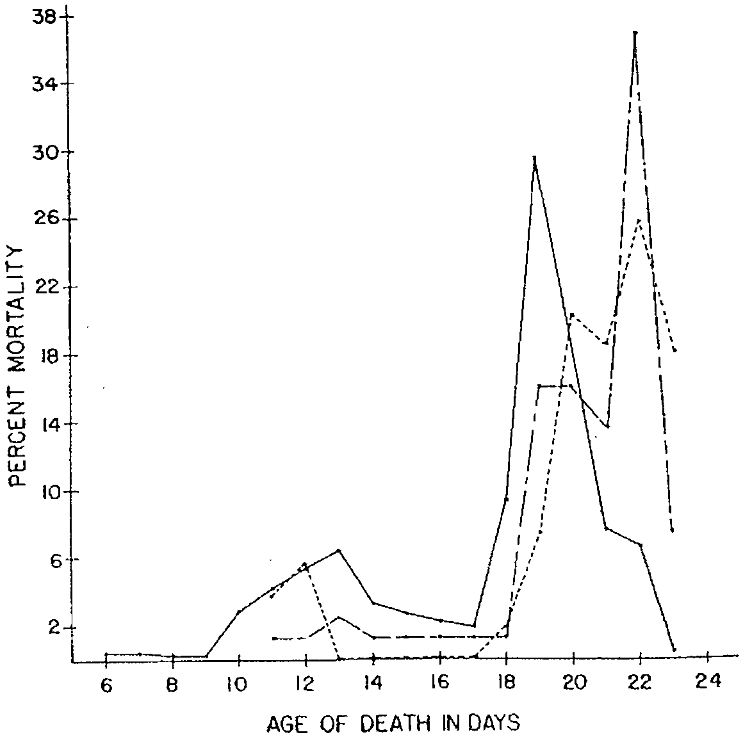

Figure 3. Relative survival of diplopod embryos from lines I———II - - - - and III——— - ——— - in 1954.

Age of death is expressed in days of incubation except that 22 represents embryos that have pipped the shell but not hatched and 23 represents hatched chicks.

4. *Distribution of embryo mortality in normal and diplopod embryos from the three selected lines and line crosses*

In 1953 length of survival was compared in normal and diplopod embryos from the three selected lines and from crosses of these lines. In 1954 data on survival of diplopod embryos from the same sources was collected. The mean age at death of diplopod embryos based on stages in normal ontogeny was computed for each type of mating. In evaluating these results it is important to bear in mind that the criteria employed in establishing the time of death of embryos, whether normal or diplopod, were based on stages of normal ontogeny where the most advanced (normal) structure is given most weight in age classification. Should there be intrinsic differences in developmental rate between diplopod and normal phenotypes, this procedure will be automatically biased by the extent of such differences.

In 1953 and especially in 1954 diplopod embryos from line I died earlier in incubation than those from lines II or III (Figure 3). A larger proportion died before the 14th day, and fewer were found alive-in-shell at the end of the incubation period or hatched. Line cross embryos, particularly those from the line I × III cross, died later than line I embryos, and more survived the incubation period (Tables 1, 2). Normal embryos produced by the three lines in 1953 had approximately the same death rate in the three periods considered. Data from 1954, when a larger number of progeny with the milder forms of diplopod expression occurred in low lines and in line crosses, indicated that line I embryos differed significantly from those of lines II and III in time of death (stage of development at death). Mean ages at death in terms of days of incubation in this year were: line I, 17·6±0·03; line II, 20·4±0·08; line III, 20·4±0·17.

Table 1. *Comparative embryo mortality in three lines and line crosses, 1953.*

Parental Genotype ♂♂ ♀♀	Phenotype†	Progeny Number	% Dead 1-14 days	% Dead 15-20 days	% Alive-in-shell at 21 days or hatched
I × I	D	356	28·7	65·2	6·2
	N	1205	5·1	13·2	81·7
II × II	D	119	10·9	63·9	25·2
	N	617	2·9	15·1	82·0
III × III	D	31	6·5	71·0	22·6
	N	320	4·7	10·9	84·4
I* × II	D	85	22·4	57·6	20·0
	N	504	4·4	15·9	79·8
I* × III	D	44	11·4	47·7	40·9
	N	394	4·1	10·9	85·0
II* × III	D	4	0·0	25·0	75·0
	N	92	2·2	27·2	70·7
NC** × NC	N	1639	2·1	9·1	88·8

* Data from reciprocal crosses.
** Data from matings of noncarriers of all lines.
† D=diplopod; N=normal.

Table 2. *Comparative diplopod embryo mortality in three lines and line crosses, 1954.*

Parental Genotype ♂♂ ♀♀	Number of Diplopod Progeny	% Dead 1-14 days	% Dead 15-20 days	% Alive-in-shell at 21 days or hatched
I × I	457	22·8	63·0	14·2
II × II	82	6·1	36·6	57·3
III × III	55	9·1	29·1	61·8
I* × II	136	18·4	44·8	36·8
I* × III	79	10·1	58·2	31·7
I × LC	24	25·0	50·0	25·0
III × LC	64	4·7	31·3	64·1

* Data from reciprocal crosses.

5. *Weight and bone measurements of sixteen-day normal and diplopod siblings from the three selected lines and from the cross III × LC*[1].

The body weight and seven bone measurements were obtained on a group of living embryos from each of lines I, II, III and from the cross III × LC. at sixteen days of age. In each group all diplopod embryos and one full-sib normal embryo from each family, serving as a control, were saved. The normal embryo chosen as a control was intermediate in size among normal embryos produced by each mating. The normal control and all diplopod embryos were weighed and the back length and six left limb bones measured. Duplicate measurements were made for the right limbs of diplopod embryos. The limb measurements recorded were as follows:

(*a*) length of femur,
(*b*) length of tibiotarsus,
(*c*) length of tarsometatarsus,
(*d*) length of foot digit III,
(*e*) length of carpometacarpus,
(*f*) length of principal normal wing digit (digit III according to Chamberlain, 1943). Paired normal and diplopod embryos of each line were photographed.

Plate 1 (*a*, *b*, *c*, *d*) illustrates characteristic sixteen-day normal and diplopod full-siblings of each of the three selected lines and of the cross III × LC. Differences in size and bone lengths were most marked in line I embryos (Figure 4). Here the outer perimeter of a polygon represents the mean normal measurement arbitrarily considered as 100 percent expression; the inner perimeter, the mean diplopod measurement expressed as a percentage of normal. Diplopod embryos from the normal-ratio line exhibited more extreme effects in all characters measured than did those from the other lines and from the line cross. The proportional effect on different bones is apparent from comparisons of the axes both within and between lines. The tibiotarsus was invariably more severely reduced than any of the other bones, contrary to Landauer's

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[1] LC—carrier female progeny from a cross between a line III male and line I females, which gave rise to progenies with a very low incidence of diplopod segregants (Taylor, Abbott, and Gunns, 1959).

1934 finding that in micromelia there is a proximo-distal gradient of increasing reduction in relative length of bones.

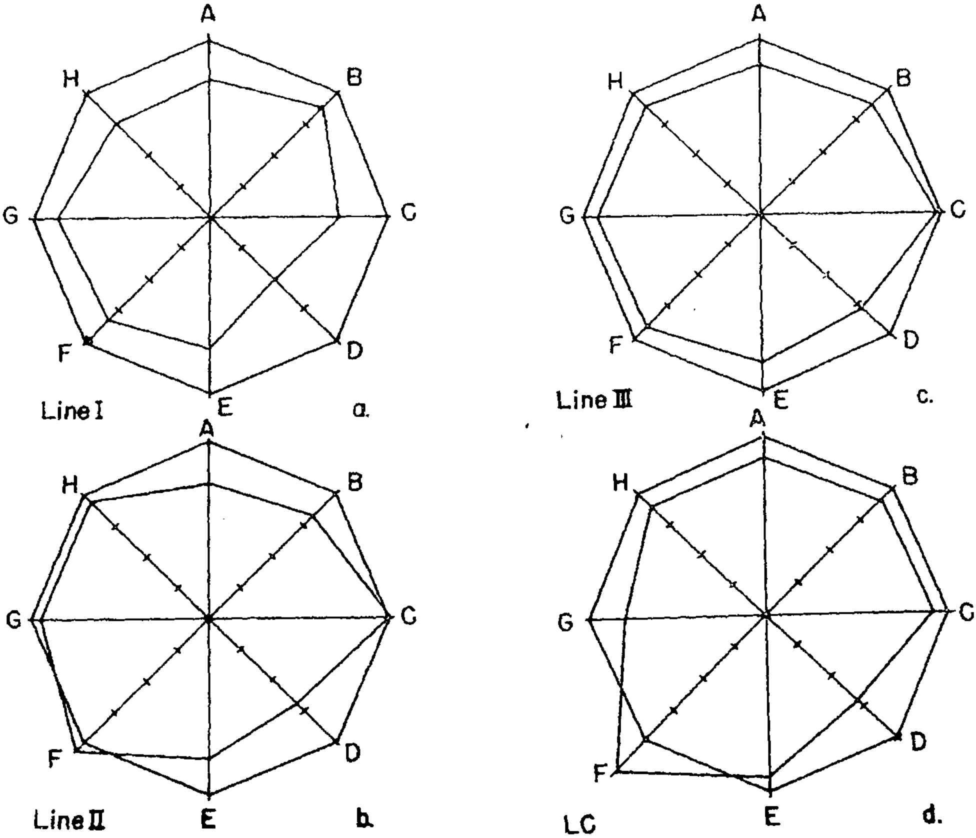

Figure 4. Comparison of body weight and seven bone measurements in diplopod and normal siblings of 16 days embryonic age of the three lines and the cross III × LC. The outer perimeter represents the mean normal measurement arbitrarily considered as 100% expression: the inner perimeter the diplopod measurement expressed as a percentage of normal.

Legend
A. Body Weight
B. Back Length
C. Femur Length
D. Tibiotarsus Length
E. Tarsometatarsus Length
F. Leg Digit III Length
G. Carpometacarpus Length
H. Wing Digit III Length

In each of the seven characters studied, the mean measurements of diplopod embryos from line I were significantly lower than those of their normal siblings, the difference being most marked in tibiotarsal length. In Plate 2 the relationships of foot and wing bones of sixteen-day old diplopod embryos are illustrated in pictures prepared from Alizarin transparencies.

6. *Arrangements of supernumerary digits in diplopod embryos*

Arrangements of extra digits on the feet and wings of diplopod embryos, including the absolute and relative sizes of digits and their location with respect to the normal digits were studied in all lines and crosses in 1953 and 1954. The arrangement of extra foot digits was recorded by a code (Abbott, 1955) which described the total number of digits, the presence or absence of the hallux and the relative sizes and positions of the supernumeraries. The maximum number of digits on a diplopod foot is seven. In the absence of the hallux as many as four supernumeraries may be present. In 1954 one exceptional specimen appeared with five supernumerary foot digits on one foot (a total of eight digits).

In the case of wing digit patterns the code described the total number of digits of the anterior field of a spread wing; the extra complement found in addition to or replacing the pollex of the normal wing which articulates with the radial-carpometacarpal junction. Two other digits are considered as normally present in the mature chicken wing by some authors (cf., Chamberlain, 1943). However, there exists disagreement as to the homology of these digits as well as that of the pollex with digits of the posterior limb. Chamberlain, for example, considers the pollex to be digit II although its involvement in diplopod expression does not appear to support this basis. Since the presence of these other digits is not affected by diplopodia and since adaptation of the avian wing for flight has so profoundly modified their form, all data on wing finger number in this and subsequent papers of this series exclude these from consideration. In Plate 2, 16-day digitation of several diplopod spread wings illustrates the field considered. On this basis the maximum number of digits on a diplopod wing is five (although in 1954 three embryos with six digits on one or both wings were described) and the minimum number is one. Since the pollex is generally absent in severe expressions of the syndrome, the maximum number of supernumeraries is five as well.

The positions and sizes of normal foot digits II, III and IV remain undisturbed in diplopod embryos, while the hallux, normal digit I, may be absent. If present, it is normal in size, shape and position. In 1953, among 925 diplopod embryos from all types of matings, 191 had a hallux on the right foot, and 188 had a hallux on the left foot, approximately 20 percent in each instance. In only 76 cases, eight percent, were halluces present on both feet. The incidence of diplopod embryos possessing halluces was related to parental line or cross (Table 3); 49·1 percent of recognized diplopods in line III possessed at least one hallux. In both line crosses the incidence of diplopod embryos with at least one hallux exceeded that of the mean incidence of the parental lines.

Supernumerary digits occur in a variety of positions proximal to the hallux. They may occur at the hock joint itself (Plate 2-1) or in any position between it and the position of the hallux. In mild forms of expression, characteristic of low-ratio matings, only one extra digit may be present, a "duplicate hallux", which is located immediately next to the normal hallux. This digit is similar in size and shape to the normal hallux. Embryos with this digit arrangement resemble those from breeds carrying the dominant gene for common polydactyly (Plate 1B).

Table 3. *Proportions of diplopod embryos with right and left halluces in lines I, II, III and in line crosses, 1953.*

	Right Foot			Left Foot	
Mating Type	No. dpdp Embryos	Hallux Present	Percent	Hallux Present	Percent
I × I	551	34	6·2	37	6·7
II × II	137	50	36·5	49	35·8
III × III	53	26	49·1	26	49·1
I × II*	136	49	36·0	45	33·1
I × III*	44	28	63.6	27	61·4
II × III*	4	4	100·0	4	100·0
Total	925	191	20·7	188	20·3

* Reciprocal cross data.

Table 4. *Frequency of most common right foot digit patterns involving six digits* (6011, 6009, 6008), *five digits* (5006, 5102, 5101) *and four normal digits* (4100) *in lines I, II, III and in line crosses, 1953.*

Mating type	% dpdp	% 6011	% 6009	% 6008	% 5006	% 5102	% 5101	% 4100
I × I	23·5	50·3	9·8	28·0	2·9	1·5	0·2	1·6
II × II	10·4	21·9	11·0	21·9	8·0	15·3	2·9	8·0
III × III	8·2	22·6	1·9	18·9	5·7	24·5	5·7	17·0
I* × II	15·7	30·2	7·3	22·8	3·7	17·7	4·4	8·1
I* × III	10·0	9·1	15·9	6·8	2·3	20·5	2·3	22·7
II* × III	4·2	0·0	0·0	0·0	0·0	25·0	0·0	50·0
Total		39·4	9·4	24·7	3·9	8·2	1·6	5·6

* Reciprocal cross data.

Twenty-nine patterns of foot digit expression and fourteen patterns of wing digit expression were studied. The right and left foot had approximately equivalent incidences of all patterns. Accordingly, differences between lines and line crosses in the frequencies of different patterns will be exemplified by data for the right foot. Line I was more constant in digit arrangement than either of lines II or III or line crosses, as is indicated in Table 4, which presents the frequency of the three most common patterns involving six foot digits (6011, 6009, 6008) and those with lower numbers of extra digits (5006, 5102, 5101 and 4100).

Wing digit arrangements varied more than foot digit arrangements. Line I was more consistent in wing digit arrangements than the other lines and crosses. In contrast to foot digit patterns, right and left wings differed in pattern frequency. The right wings had a higher representation of the more extreme patterns and the left wings were more heavily represented in the less severe classes (Table 5).

Extra toes were not distributed randomly; they formed distinct patterns. In the most common pattern the most proximal supernumerary digit, which was the largest, was equivalent in size to normal digit II; the medial digit was the smallest; and the most distal, which was intermediate in size between these two, was slightly shorter than the hallux. These extra digits did not correspond to the normal digits in size, numbers of phalanges or in general orientation. Digit patterns in diplopod embryos from the intermediate and low-ratio lines and line crosses were somewhat different. Here the digits present included, most often, digits I, II, III and IV of the normal complement and one or two supernumeraries. Frequently, only one extra digit was present, located immediately proximal to the hallux and resembling it almost exactly in size, shape and orientation. In other cases the single supernumerary was disposed in a completely different location, i.e., it could arise directly from the hock joint, in which case it tended to be longer than normal digits, or from any other position between the hock joint and the position of the normal hallux. A study of the variety of locations occupied by extra digits showed that the area on the posterior limb from the hock joint to the normal toes was competent to form digits. Digits of the supernumerary group were complete with phalanges and associated with extra tarso-metatarsi. Occasionally the medial digit of a group of three or the most proximal of a group of four consisted only of a hollow pouch of skin, lacking even a nail.

Table 5. *Frequency of most common wing digit patterns involving four digits* (49), *three digits* (35, 33), *two digits* (22, 21) *and one normal digit* (10) *in three lines and in line crosses, 1953.*

		Percentage of Pattern Type					
Mating Type	Wing	49	35	33	22	21	10
I × I	right	12	44	11	8	4	1
	left	6	41	15	9	5	2
II × II	right	3	21	20	15	23	5
	left	1	18	15	21	21	14
III × III	right	3	11	8	17	28	22
	left	3	8	11	25	19	31
I* × II	right	2	26	11	23	18	5
	left	3	14	7	20	25	15
I* × III	right	0	16	7	25	36	10
	left	0	11	16	21	30	23
II* × III	right	0	0	25	25	50	0
	left	0	0	25	25	25	25
Total	right	8	34	13	13	13	4
	left	4	29	14	15	13	9

* Reciprocal cross data.

Discussion

Extra digit formation in diplopodia may result from an alteration in the timing relationships of the processes controlling limb ontogeny. The largest numbers of supernumerary digits are present in the most retarded embryos. This relationship suggests that a limb inductor is active for a longer period than usual in diplopod embryos or that the ectoderm and mesoderm retain their competence to form digits for a longer period. The hallux and its associated metatarsal bone, which are the last to differentiate in normal embryos, are altered in typical expressions of **dpdp**, while normal digits II, III and IV and their associated metatarsal bones are not. The hallux, however, develops normally in the attenuated expressions of diplopodia characteristic of the low-ratio line. Where a greatly retarded developmental rate leads to a prolongation of the time of digit differentiation, the material for the hallux may be drawn into a field under the influence of a new evocation center. In typical line I embryos, which lack a hallux, the fact that the supernumerary digits are unequal in size with the most proximal digit larger than any others suggests a proximo-distal gradient in competence or in distribution of morphogenic substances. On the other hand, diplopod embryos from low-ratio matings, which are least retarded in developmental rate, possess normal-sized halluces; supernumerary digits occur close to the hallux and are either the same size or smaller than this digit. In this case the center of digit differentiation remains the same, and the gradient of morphogenic substances is highest in the normal post-axial site. These observations are in direct contrast to the idea (Sturkie, 1943) that a reduction in growth rate is antagonistic to the formation of a polydactylous limb. They find support from Gabriel's observation (1946) that growth inhibition occasionally exaggerates polydactyly.

There have been two general explanations of the presence of additional digits in polydactylous breeds or in other embryos following certain mechanical or chemical treatments: (1) the addition of extra digits to the pre-axial side of the normal foot (Harman and Alsop, 1938; Harman and Nelson, 1941; Warren, 1944), (2) the partial reduplication of the foot *via* the splitting of the limb field (Kaufmann-Wolf, 1908; Braus, 1908; Harrison (Amblystoma), 1921; Gabriel, 1946). The latter suggestion is based on the fact that a polydactylous foot is bilaterally symmetrical and presumes that the pre-axial region mirror images the post-axial area.

The arrangement of digits typical of diplopod embryos from line I and from lines II and III (as well as other polydactylous stocks) are basically different, suggesting that a threshold effect determines whether additional digits shall be added to the normal pattern or whether a shift in the center of pattern formation shall take place. If the pre-axial region can obtain its independence from the post-axial region, it may form its own pattern; if not, the extra digits appear as additions to the normal pattern. It seems evident that in none of the diplopod lines does mirror imaging form the basis of the duplication process. Mirror imaging implies dichotomous simultaneous events; in diplopodia digit differentiation is sequential.

The studies of diplopod ontogeny and digit arrangements indicate that at least two apparently different types of duplication can occur in lines selected from the same stock; one type of embryological process can be abandoned for another by appropriate selection. The form of embryological expression too, is strongly influenced by the residual genotype. It is probable that the impact of different genetic milieu on embryological behaviour may be mediated through an effect on developmental rate. Selection in the low-ratio lines may have favoured individuals producing progeny with genotypes able to overcome the effect of **dpdp** in slowing developmental rates and altering timing relationships in early limb differentiation.

SUMMARY

Studies of the comparative embryonic development of diplopod and normal embryos from selected lines differing in penetrance of **dpdp** have led to the following conclusions:

1. Diplopod limbs are markedly retarded in (a) early cartilage differentiation, (b) rate of formation of cartilage bone models, and (c) rate of osteogenesis.
2. The sequence of digit differentiation in the posterior limb of diplopod embryos is as follows: normal digits II, III and IV; normal digit I (if present); and supernumerary digits. If the hallux is absent and three supernumerary digits are present (the most frequent expression in line I), the most proximal supernumerary digit precedes the other two. If the hallux and two supernumerary digits are present, the more distal (closest to the hallux) precedes the proximal supernumerary digit.
3. In typical expressions of diplopodia, supernumerary digits are not mirror images of the three normal digits present.
4. When nine to sixteen-day stages of normal and diplopod siblings were compared, developmental retardation was most marked in the earliest stages studied. The rate of ossification in limb bones was from 1-2 days behind that of normal embryos. Inhibition of ossification was found to be associated specifically with the type of osteogenesis which is preceded by a stage of cartilage hypertrophy. Membrane bone appeared to form normally with respect to the developmental stage. Bending of the limb bones in diplopod embryos began during the 12th day of incubation and became more pronounced after that time in embryos with severe expressions of the phenotype.
5. Diplopod embryos were found to be retarded in general development. Actual and apparent ages (as based on stages in normal ontogeny) were different. The normal incubation period for diplopod embryos is in excess of 21 days.
6. Diplopod embryos from the low-penetrance lines (II and III) significantly exceeded those from line I in length of embryonic survival.
7. The tibiotarsus was more severely reduced than any of the other bones. The three lines differed in extent of reduction of lengths of long bones and in body size with line I showing the greatest effects in all characters studied.
8. Presence or absence of hallux and pollex was related to line of origin in diplopod embryos. Forty-nine percent of the recognized diplopods from line III matings possessed at least one hallux. The three lines also differed in incidence of several patterns of supernumerary digit expression studied.
9. Wing digit arrangements varied more than foot digit arrangements and the right and left wings differed in pattern frequency in contrast to the right and left legs.
10. The area on the posterior limb from the hock joint to the position of the normal hallux was competent to form digits.

11. Selection for low penetrance of **dpdp** appears to have altered the basic embryological expression of the genotype.

A discussion of the implications of the diplopod embryological studies is included.

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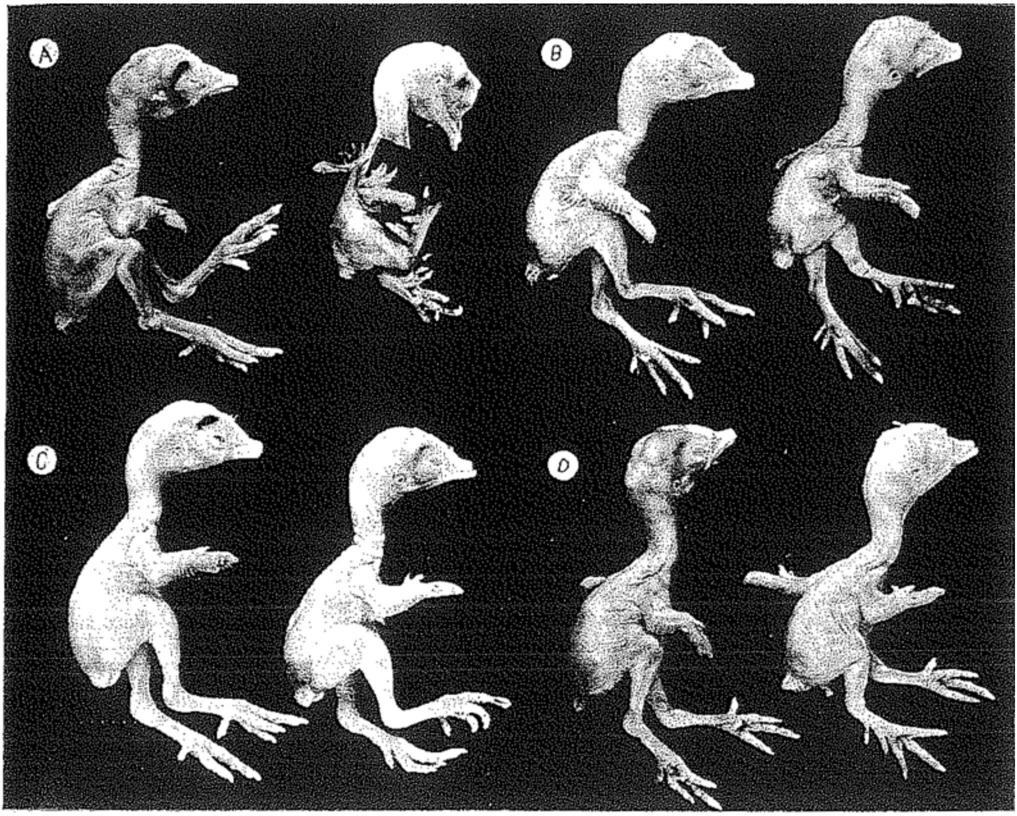

PLATE I

Normal and diplopod full-siblings of lines I, II, III and the cross III × LC.

(A) Normal embryo and diplopod sibling of line I. The diplopod embryo has 6 digits on each foot and four digits on each wing. The diplopod embryo shows the extreme forms of micromelia[1], limb curvature and a reduced body size. The pre-maxilla is reduced in length (grade 2).

(B) Normal embryo and diplopod sibling of line II. The diplopod embryo has five digits on each foot, four of the normal complement and one "duplicate hallux" and two digits on each wing, the pollex and one rudimentary supernumerary digit. The body size is nearly normal; the micromelia and limb curvature are of grade 1 (slight).

(C) Normal embryo and diplopod sibling of line III. This diplopod embryo is a wing-duplicate with normal numbers of foot digits, and extra digits on the wings. The right wing has three digits, the pollex and two small supernumeraries; the left wing has two digits, the pollex plus one supernumerary. The limbs are normal in size and shape. The pre-maxilla is reduced in length (grade 1).

(D) Normal embryo and diplopod sibling from a mating of a line III male with a LC female. The diplopod embryo has one foot with the normal complement of digits, four, and one with one extra digit. The wings each have two digits, the pollex and one supernumerary. Body size is almost normal and the embryo has grade 1 micromelia and limb curvature. The pre-maxilla is reduced in length (grade 1).

[1] Micromelia, limb-curvature and extent of reduction of pre-maxilla were classified according to severity grades as follows:

1. slight
2. intermediate
3. severe

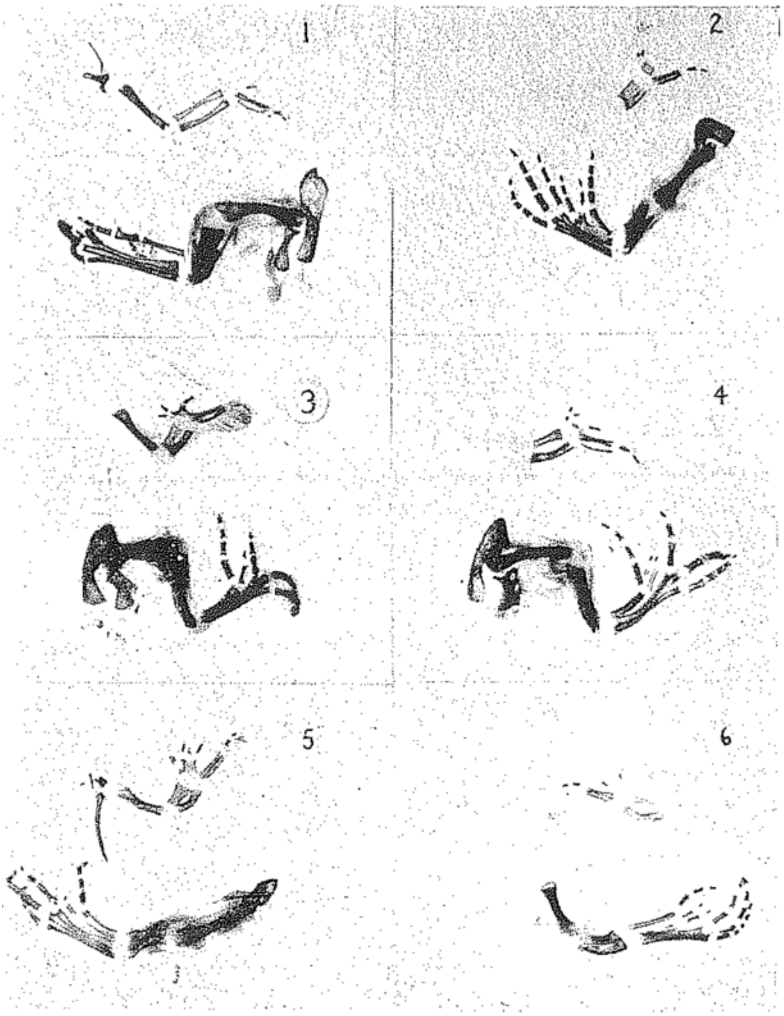

PLATE 2

Alizarin transparencies of leg and wing bones of 16-day diplopod embryos illustrating several digit patterns

(1) foot 6011 one long supernumerary metatarsal bone; note shape of femur and tibia. Wing
wing 33 bones almost normal in size;

(2) foot 6011 a typical diplopod foot; note thickness of metatarsal region and extreme shortening
wing 22 and curvature of tibia;

(3) foot 5006 2 supernumerary foot digits, extreme curvature of bones of both limbs, but almost
wing 35 normal length. Crooked toes.

(4) foot 7016 fourth supernumerary digit attached to long proximal digit which has 4 phalanges;
wing 48

(5) foot 6008 thick, shortened tibia, well developed supernumerary wing bones, crooked toes;
wing 58 pollex absent;

(6) foot 6009 disproportionate effect of **dpdp** on foot and wing; pollex present with one
wing 21 supernumerary.