ON THE IDENTIFICATION OF SEGREGATED PHENOTYPES IN PROGENY FROM CREEPER FOWL MATINGS

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IN 1930 LANDAUER and DUNN established that the genetic basis of the Creeper character in fowl is a single Mendelian factor pair, lethal in homozygous condition. The heterozygotes are viable and show the characteristic chondrodystrophy that has earned the designation 'Creeper.' The homozygotes as a rule show marked pathological changes on the third day of incubation and die shortly thereafter; occasionally they survive until near hatching time, in which case they are strikingly phokomelic (LANDAUER 1933). The present study has two general purposes: 1) to examine the time of phenogenetic segregation of the various genotypes from Creeper matings, since this information is fundamental to a study of the action of the *Cp*-factor; 2) to give practical indications for early identification of these genotypes, as a necessary basis for any experimental work on early stages.

LANDAUER (1932) has shown that at 72 hours' incubation approximately 25 percent of the embryos from a Creeper × Creeper mating ($Cp + \times Cp +$) are markedly retarded in body size and differentiation. He has further shown that at 48 and even at 36 hours' incubation approximately one-fourth of the embryos fall into a developmentally retarded group—as regards both somite number and dimensions—a group almost discontinuous from the main population (table 5). The obvious inference is that it is this retarded group that represents the homozygous lethal segregates, and this has until now been the basis for early identification of these segregates.

In order to establish this criterion on a firm experimental basis, and to find if it holds for stages earlier than 36 hours' incubation, we undertook independently a series of experiments in which somites were counted at definite periods of incubation, and the subsequent development of the individual embryos observed. All embryos came from the same stock, which has been maintained at Storrs; $Cp + \times Cp +$ matings were used, as well as one mating of $Cp +$ hens with a $++$ cock. Experiments were carried on at Storrs and at St. Louis. At Storrs a large forced-draft incubator was used, maintained at 36 degrees centigrade with very slight fluctuations. At St. Louis, a Buffalo incubator, without forced draft, was used at 38–39 degrees, with some slight fluctuations above and below this range.

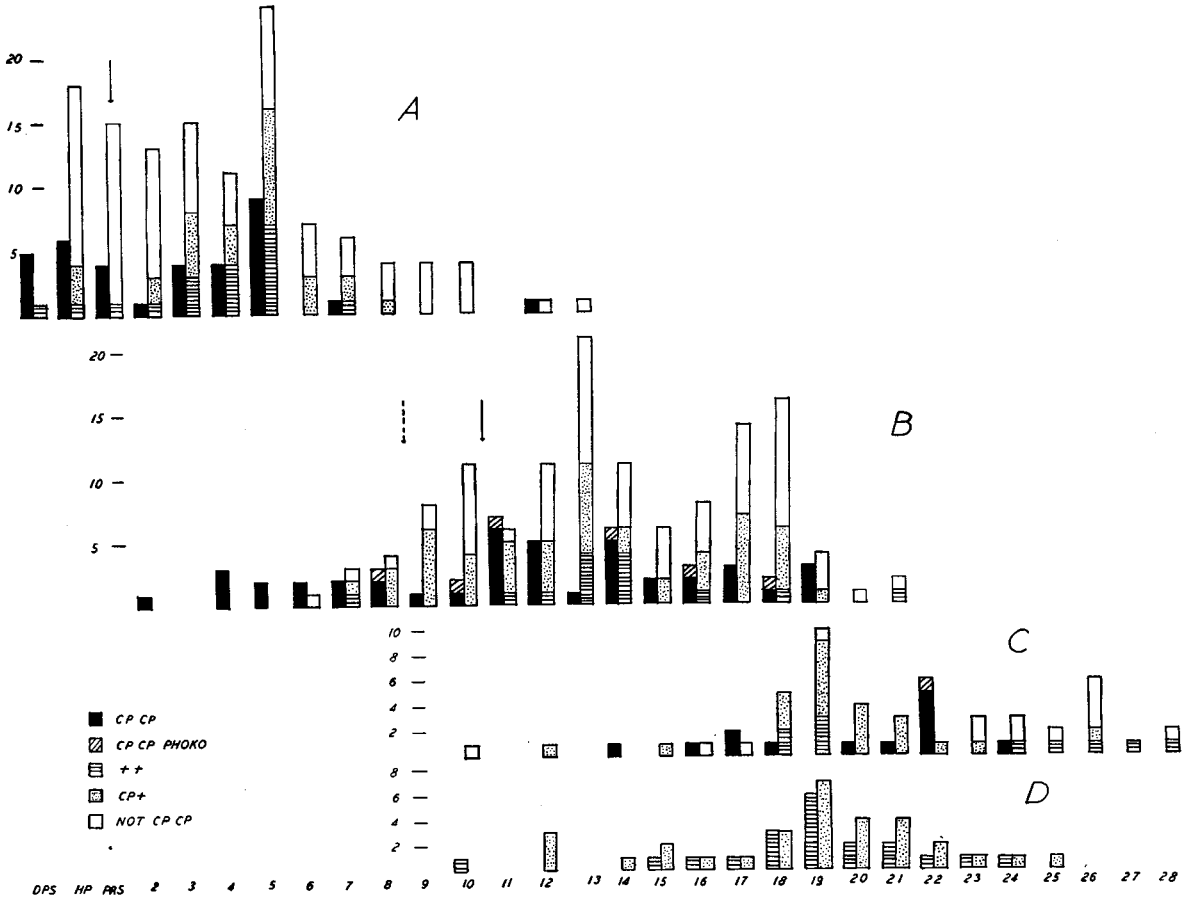

FIGURE 1.—Block diagrams showing distribution of somite stages among the segregates of $CP + \times CP +$ matings (A, B, C) and $CP + \times + +$ (D); A at 24–25 hours' incubation, B at 32–44 hours, C at 48–50 hours and D at 48 hours. DPS = Definitive primitive streak stage; HP = head-process; Pr S. = pre-somite; other stages defined by somite number.

For the somite counts, a window was made in the egg and the blastoderm was stained lightly with neutral red transferred from an impregnated agar plate. The somites were counted under a binocular microscope, and the egg closed and allowed to incubate further. Instruments and materials were of course sterilized. Final observations were made either at 3–4 days, which permits distinction between pathological and viable embryos, or at 8–10 days, which permits recognition of phokomelic, heterozygous and normal types respectively. The operative procedure clearly does not interfere with subsequent expression of these various characters. Only embryos showing the typical symptoms—early pathology or later phokomelia—were classified as *Cp Cp*; only typical chondrodystrophics as *Cp* +; doubtful cases have been eliminated from our tables.

RESULTS

Figure 1 is a block diagram summarizing the somite development of the various segregates at three stages of incubation. The first three distributions are for embryos from *Cp* + × *Cp* + matings. The first one (A) consists of embryos of 24–25 hours' incubation. The second one (B) groups cases examined at 32–44 hours' incubation. It was found that the range of somite variability in 36 hour embryos operated at various times in the spring and early summer was so great that additional cases both earlier and later than this standard stage could be added without increasing the spread. The third distribution (C) is of cases of 48–50 hours' incubation. Developmental stages are listed on the abscissae, number of cases on the ordinates, and at each stage two columns are shown, one of *Cp Cp* types (that is, early pathological and phokomelic forms) and a second including all viable segregates—*Cp* +, normal, and viable embryos opened too early to be classified. This latter category may include a small number of phokomelic homozygotes which look normal at 3–4 days.

Discrimination between Cp Cp and all other groups

a. Somite counts. In the 24–25 hour cases, the means have been calculated by simply assigning a number to each stage, beginning with the definitive primitive streak as stage 1, and later converting the mean back to the corresponding somite stage for the sake of uniformity. This procedure may be open to criticism as unduly distorting time-relations, but it must be recalled that the somite stages themselves are far from being evenly spaced on the developmental time-scale: to this unevenness we attribute, for example, the excessively large number of 5 somite stages. The means given here must be considered quite strictly as means of stages, not of time-classes. When calculated this way, the mean for *Cp Cp* embryos at 24–25 hours is 2.4 somites ± 2.8 and that for all non-*Cp Cp* em-

bryos is 3.7 somites ± 2.9. Hence the mean for homozygotes is slightly but far from significantly lower than that of normal embryos. The variance is much the same. There is then no natural break in distribution between the two groups.

The 32–44 hour cases show a similar result. Again the mean for *Cp Cp* embryos is lower (11.6 ± 4.5 somites) than that of non-*Cp Cp* ones (13.8 ± 3.4 somites) but the difference is clearly not significant. In this case the variance of the *Cp Cp* embryos is greater than that of the normal ones.

In both of the foregoing distributions it can be seen that the lower somite brackets have more than the expected 1:3 ratio of *Cp Cp* embryos. In the 48–50 hour group even this is not true. The *Cp Cp* mean is only slightly lower than that of the non-*Cp Cp* embryos (19.9 ± 2.9 as against 21.0 ± 4.0) and the variance is less, so that several cases of very low somite number are found to be non-*Cp Cp* embryos.

b. Other morphological symptoms. At this latter stage of incubation (48 hours and more) the morphological symptoms of the lethal effect begin to be observable; in a few of our cases we could predict from the first examination that an embryo would be of the *Cp Cp* type.

LANDAUER (1932, tables 7, 8) has described and analyzed by measurements the head-retardation in 72 hour *Cp Cp* embryos, and has shown that the posterior part of the body is less inhibited than is the head (his tables 1, 2). He has also described the abnormalities of the heart and circulatory system as well as of the limb regions. We have made some additional observations on the development of the lethal syndrome that may be of interest here.

Normal embryos undergo drastic morphogenetic changes in the 17–20 somite period (about 48 hours' incubation). The cephalic flexure increases from a slight bend to an angle of 90 and more degrees, and the forebrain elongates concurrently. Meanwhile the whole head rotates to lie on its left side, and the amnion closes over it. The vitelline arteries commence to consolidate, and rapidly become large conspicuous trunks. The heart becomes a loop. These changes show a certain independence of one another and of somite formation. For example, an embryo (from a normal mating) may have a completely rotated head without the amnion's covering it completely. Vitelline artery function may begin at any stage of this phase of head-morphogenesis and between the 18–20 somite stages. The establishment of the vitelline arteries is of course a conspicuous step in the transformation of the area vasculosa to a functional circulatory network, arising from the union of discrete blood islands.

The deficiencies of the *Cp Cp* embryos are primarily these: the head region begins showing retardation at the 17–20 somite stage; its flexure and rotation are delayed, although they eventually proceed, the flexure to at

least 90 degrees, the rotation to completion. The head does not grow in volume at anything comparable to the normal rate; by 72 hours' incubation microcephaly is striking. (See LANDAUER 1932, Plate XI figures 2–4; Plate XII figures 1, 8). Sections through the head at this stage show extreme thinning of the brain wall—sometimes to only two cell layers—as well as marked cellular degeneration in the nervous system. The head mesenchyme is also very deficient in quantity in the examples we have examined histologically.

Accompanying the diminished head growth is usually a striking asymmetry expressed in the eyes and the otocysts. At the time of rotation, the left side, that is, the side on which the embryo comes to lie, begins to be differentially retarded. The retardation may even precede rotation: in one case, a 20 somite embryo which had not yet undergone rotation and which showed other symptoms of the lethal effect, the left optic vesicle was seen to be slightly delayed as compared with the right, in making contact with the lateral head ectoderm. Following this stage, in *Cp Cp* embryos, the right eye continues development at about the same pace as the rest of the head, forming a small eye-cup and lens. The left eye, by 72 hours, usually has barely started to form a cup, and the lens remains a thickening continuous with the body wall, occupying a groove in the optic vesicle. In fixed preparations such lenses are apt to be everted and to project outside the body; this may be only an artifact due to the abnormal fluid pressures in the embryo. In the living state these left eyes appear as very small shrunken knots of tissue. The left otocyst may be similarly delayed in closure and retarded in growth.

The heart, as LANDAUER has pointed out, shows much variability, being retarded in form in a degree corresponding to the head retardation, but often being quite enlarged in size as compared to the normal heart of a similar morphological stage. This variability extends to the heart wall as well. Some cases we have examined histologically show a fairly good amount of myocardial tissue; others show absolutely no thickening even in the ventricle.

The thinning of tissues that is marked in the head-region at 60 or 72 hours is apparent also in the trunk, although so much more variable that posterior cross-sections in some examples look quite normal. The posterior neural tube is not necessarily thinned or degenerating. The somites are usually very deficient in cell mass; the process of segmentation itself is very little retarded by comparison, as LANDAUER shows and as the present results also indicate. The Wolffian ridge remains a thin layer, filled with mesenchyme, instead of being thickly packed with cells. Small limb buds may appear, delayed with reference to the rest of the body form.

While these events are occurring in the embryo, similar failures appear

in the vascular area. Although some attempt is made at forming vitelline arteries, and a feeble circulation may always be observed in the main channels, the peripheral blood islands never become incorporated in the network and no effective vitelline circulation is ever formed. The vascular area remains full of discrete blood clots, and the whole appearance becomes more and more anaemic as the blastoderm expands on the third and fourth days. In sections it is seen that endothelium is differentiated from the blood islands throughout the vascular area, but that these either do not interconnect, or connect only by very fine openings, so that the erythroblasts are not free to move, but remain in densely packed masses. Within the embryo too the circulation is defective; extravasation and clotting occur in the main channels, which are greatly distended; in sections various non-vascular cavities such as the amniotic space and the coelom are found full of erythrocytes.

During the fourth and fifth days of incubation, thinning and degeneration of tissues continues and fluid spaces enlarge progressively; the embryo becomes vesicular and shapeless. Hearts were found still beating as late as the fifth day. Complete necrosis probably ensues before the seventh day.

All these changes are foreshadowed in embryos of 48 or more hours which have reached the 20–21 somite stage without establishment of strong vitelline arteries. A few of our cases in the 48–50 hour group, as we have said, were such embryos; they subsequently developed the typical pathology. However, all *Cp Cp* embryos are not distinguishable at this stage, as can be judged from two cases of 22-somite embryos in which vitelline arteries were observed to be functioning. These two were subsequently found to be *Cp Cp* individuals. Hence the stage when all *Cp Cp* embryos are distinctly diagnosable must be put at the 24–25 somite period (48–54 hours' incubation). Lack of strong functional vitelline arteries is unmistakable at this stage of somite development, as is head retardation.

Discrimination between heterozygous and normal embryos

Figure 1 also presents somite stages for Creeper and normal embryos. Here the distribution is clearly parallel. In the 24–25 hour lot, the *Cp* + mean is 4.1 somites ± 2.0; that for normals is 3.6 somites ± 1.9. In the 32–44 hour group, the *Cp* + mean is 13.1 ± 3.4; the normal mean 13.8 ± 3.1. In the 48–50 hour group the *Cp* + mean is 19.5 ± 2.7 as against 22.3 ± 3.8 for the ++ embryos. The fourth distribution (D) on the table is the somite record for *Cp* + and ++ progeny in a mating of Creeper hens by a normal cock, from which the two sorts of offspring are expected in equal quantities. This series, incidentally, was made at approximately the same season (May-June) as most of the preceding 48–50 hour group, and it is

obvious that the spread of stages is approximately the same. In this lot the mean of the *Cp* + embryos is 19.9±2.9 somites, that of ++, 21.0±4.0 somites. There is possibly a slight tendency for the normal embryos to be more advanced than the heterozygous, but it is obvious that none of these differences in any way approaches significance.

To illustrate the reliability of the general table as indicating variability actually realized in small lots of eggs, we include summaries of two individual experiments. One (Storrs: May) consisted of 19 usable cases, examined at 24 hours. Of the 7 later found to be *Cp Cp*, one was in the definitive primitive streak stage, one pre-somite, one 2-, one 4- and three 5-somites respectively. One 5 somite embryo was found to be normal at 72 hours. *Cp* + embryos were found at 2, 3, 5, 6 somites, ++ ones at 3, 4 and 5 somites. The other experiment (St. Louis: February) was at 40 hours' incubation. *Cp Cp* embryos had 8 and 11 somites; one 9 somite embryo was found to be not *Cp Cp*; four 9 somite cases and one of 10 somites turned out to be *Cp* +. Thus in individual settings, as well as in the total population, *Cp Cp* embryos may be distributed throughout the whole range.

DISCUSSION

We conclude from the evidence presented above that in the stages and under the conditions employed, *heterozygous and normal* segregates from Creeper matings are not distinguishable on the basis of somite formation nor by other morphological criteria. Distinct macroscopic differences appear not earlier than on the 7th day (LANDAUER 1931).

Concerning the early segregation of *Cp Cp*-embryos and their viable sibs, LANDAUER (1932) states: 1) that homozygous embryos are smaller than normal ones, at least from the 36th hour of incubation on; 2) that "with regard to the number of somites the homozygous Creeper embryos lag most conspicuously behind the normal ones during the period when in normal development a rapid multiplication in somite number takes place (close of the 2nd day)" (p. 391). No striking difference in somite number was found at 72 hours of incubation.

Our data refer to somite numbers only. They show a slight shift toward the lower somite groups at 24–36 hours; this difference disappears at 48 hours. Thus we extend the continuous grouping of somite number shown by LANDAUER (1932, table 5) for 72 hour embryos back to the 48 hour stage, and we find that the artificial grouping in his 48 hour series is not in agreement with the constitution of the embryos. At 36 hours this grouping is not as strict a break as he suggests, but a statistical trend; the same is true at the 24 hour stage.

We have not collected data on size differences within our material. Some

of LANDAUER'S data on size differences may require revision in view of the present findings. The 36 and 48 hour embryos classified as "homozygous" (table 4) are identified as such by the criterion of somite number only and may therefore include a higher percentage of "normal" embryos than is assumed by the author (p. 368).

The size differences found by him at these stages are probably only the expression of differences in developmental stage, superimposed of course on great individual variability. If, for example, we examine head length (the dimension showing greatest reduction in the retarded individuals: p. 374) in those embryos of the 36 hour group which had 12 somites and were classified as normal (table 4, column 2, last 6 cases), we find a variation of from 1.60 to 1.83 mm, with a mean of 1.71 ± 0.09. In the 48 hour embryos classified as *Cp Cp*—that is, those with 12–15 somites—the head length varies from 1.20 to 2.04 mm with a mean at 1.49 mm $\pm$ 0.07. The probability that these groups belong to the same normal distribution is between .4 and .3 (FISHER 1936, p. 128 and table 4), and hence the difference of the means cannot be taken as statistically significant. There is no demonstration in this material—even though a number of individual cases in the tables may suggest it—that 12–15 somite embryos of 48 hours' incubation are absolutely or differentially smaller than embryos that have taken only 36 hours to reach the same stage.

A reinvestigation of the matter of size difference of the various genotypes as contrasted with developmental stage would be especially desirable since LANDAUER'S interpretation of the mode of action of the Creeper-factor as a primary growth retardation is partly based on these data on size differences in early *Cp Cp*-embryos. His data on 72 hour embryos (table 1 and 2) are not in doubt; however, they do not concern the essential point: whether or not growth differences *precede* the pathological abnormalities—both of which are strikingly apparent at 72 hours' incubation. This question cannot be considered as definitely settled until the search for micropathological changes has been extended to stages preceding the 17–20 somite stages using material of which the genetic constitution is checked.

For the practical problem of selecting *Cp Cp* embryos in early stages, counts of somite numbers are not reliable as far as the detecting of individual cases is concerned. Selecting in the lowest quarter of the population (the expected proportion of *Cp Cp*) in no instance gives even a 50–50 chance of obtaining a homozygote. From the table it can be seen that in the lowest one-fourth of the 24–25 hour group (marked off by the solid arrow) we have only about a 1:2 chance of selecting a *Cp Cp*. In the 32–44 hour group, the lowest one-fourth gives a similar ratio (16:27); if we select in the lowest one-eighth (to the left of the dotted arrow) the ratio rises to 13:8; embryos below 6 somites are all *Cp Cp*. This suggests that in a group

of approximately 36 hour embryos, averaging 13–14 somites, embryos that are greatly retarded (less than 6 somites) will probably be *Cp Cp*. In the 48–50 hour group, where one begins to find other morphological criteria of retardation, somite number ceases to be a differential in any sense.

As an example of the effect of selection, we may cite the ratios obtained by us individually. The expectation in the population is of course 1:3 for *Cp Cp*: non-*Cp Cp*; and 2:1 for *Cp* + : + + among those surviving till 8–9 days. The Storrs material (D. R.) was taken without any selection whatever. The 32–50 hour St. Louis material (V. H.) was partly used for other experiments and a small fraction of the highest somite groups discarded. The ratios in the various groups follow:

	STORRS				ST. LOUIS			
	Cp Cp: non-*Cp Cp*		*Cp* + : + +		*Cp Cp*:non-*Cp Cp*		*Cp* + : + +	
24–25 hours	27	76	28	19	8*	48*	—	—
32–44 hours	23	65	19	10	25	61	30	4
48–50 hours	9	32	11	8	5	12	10	2
Cp + × + + 48 hours	—	—	30	21	—	—	—	—

* We are indebted to MR. J. CAIRNS for placing these data at our disposal.

Thus selection of low somite embryos in practice selects against non-*Cp Cp* and evidently for homozygous embryos.

Differences in head length are useless for practical purposes; they are too slight in view of the great variability in length and developmental stage in a given age group. The only reliable criteria are the pathological symptoms listed above, particularly the asymmetry in eye and otocyst and the failure to establish a proper vitelline circulation. They become apparent at the end of the second day but show a certain variation in the time of their first manifestation.

SUMMARY

Embryos from Creeper × Creeper (*Cp* + × *Cp* +) matings were examined for somite number at three stages of incubation: 24–25 hours, 32–44 hours, 48–50 hours. Some cases from a *Cp* + × + + mating were also used at 48 hours. They were incubated farther and their phenotypic fate ascertained. The following conclusions are drawn:

1. There is no basis in somite number or morphology for distinction between the + + and *Cp* + segregates in these stages.

2. At the two earlier stages (24–25 hour, 32–44 hour) the *Cp Cp* segregates are on the average slightly less advanced in somite development than are their viable sibs, the mean somite number being 2.4 as compared with 3.7 at 24 hours; 11.6 as compared with 13.8 in the 32–44 hour group.

The distributions are entirely continuous, as is shown in figure 1. Selection in the lower quarter of these groups would give better than a 1:3 chance of obtaining a *Cp Cp* individual.

3. At 48–50 hours, somite number ceases to be a differential between the *Cp Cp* and the viable segregates. At this time, the first signs of pathological changes appear in the *Cp Cp* individuals. Some observations on the course of these pathological changes are offered in supplement of the descriptions of LANDAUER (1932).

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