Experimental analysis of control mechanisms in somite segmentation in avian embryos.

I. Reduction of material at the blastula stage in *Coturnix coturnix japonica*

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**SUMMARY**

The blastulae of unincubated eggs of the quail, *Coturnix coturnix japonica*, have been bisected *in ovo*, using the technique of Lutz (1949). Some embryos were harvested after 24 h and found to possess two primitive streaks. Most were fixed at 48 h or 72 h. Some were found to have regulated to form almost normal single axes, whilst others had developed into duplicitas anterior embryos, separate twins or collided axes. All three types of twinned embryos were smaller than the control embryos. The number of somites was not however reduced in the shorter embryos. This finding corresponds with a similar result obtained by Cooke (1975) who reported that if a *Xenopus* blastula is reduced in size, it nevertheless develops the correct number of somites.

The quail however adjusts the shape of the individual somites so that they fit into the reduced body length, whereas *Xenopus* reduces the size of somites. No miniaturized somites were ever seen in these quail embryos.

As a result of the present experiments, it was concluded that the length of incubation time does not directly control the rate of somite formation, because different numbers of somites were found in twins which possessed identical genomes and had developed in almost the same environment for identical periods. In addition, the size of the area pellucida does not appear to control somite formation. Probably, the most important influence is the regression of the node.

**INTRODUCTION**

The number of somites that form in an embryo determines the number of vertebrae that subsequently develop. It is essential therefore that only the appropriate number of somites should segment. (In the chick embryo this is about 52, although the last 10 pairs, which are in the tail region, soon disappear: Hamilton, 1952.)

One of the fundamental problems of Developmental Biology is that of how the embryo ensures that only the appropriate numbers of somites segment. Curiously, little attention has been paid to this problem in the chick, despite the fact that

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many papers have been published on other aspects of somitogenesis (e.g. Bellairs, 1979; Bellairs & Veini, 1980). It has received a little more analysis in the mouse (Flint, Ede, Wilby & Proctor, 1978; Tam, 1981) but has been tackled most thoroughly in amphibians.

A theoretical explanation, the 'Clock and Wavefront Model' was put forward for amphibian embryos by Cooke & Zeeman (1976). This model which will be discussed more fully in our second paper in this series was based to some extent on Cooke’s (1975) demonstration that if the amount of tissue was reduced in a *Xenopus* blastula, the appropriate number of somites developed though each was less than normal size. Thus in some way the embryo had been able to adjust the segmentation pattern to the reduced body length.

It has been sometimes suggested (Cooke, 1977) that a similar control system operates in avian embryos, especially since miniature somites have been reported as a result of experimental interference (Spratt, 1955, 1957). In our opinion however, these particular tiny somites are not valid evidence since they have without exception been obtained by interference with the embryo at a time when the segmentation pattern is already programmed even though the main morphological changes have yet to take place (Meier, 1979; Bellairs, 1980). The experiments described in the present paper have therefore been designed to test whether reduction of material in the blastula stages of birds leads to production of smaller somites as it does in amphibians. It should be noted however that although these experiments are similar to those performed on amphibians, they are not totally analogous, since a bird blastoderm contains some extraembryonic tissues in addition to the embryonic ones, whilst an amphibian blastula consists entirely of embryonic tissues.

Cooke & Zeeman's theory has received support from experiments in which the pattern of segmentation has been disrupted by treating amphibian embryos with heat shocks (Elsdale, Pearson & Whitehead, 1976; Pearson & Elsdale, 1979; Cooke, 1977). Similar experiments carried out on the chick embryo will be discussed in our second paper in this series.

MATERIALS AND METHODS

For technical reasons, *in ovo* operations proved to be simpler to carry out on quail embryos than on chicks. All the *in ovo* experiments described in this paper therefore utilised quails. A small additional series of experiments, not described here, demonstrated however that comparable results could be obtained with chick embryos. In staging the quails we have followed the chick normal tables of Hamburger & Hamilton (1951) and of Eyal-Giladi & Kochav (1976).

A window was cut in the shell of each unincubated quail egg and an assessment was made, if possible, of the stage of development. Usually, however unincubated quail embryos were found to be difficult to stage unless dissected from the yolk; most of them were about stage XII of Eyal-Giladi & Kochav (1976) so
that no clear-cut orientation was yet apparent. The embryos were therefore at the blastula stage.

Experiments consisted of bisecting the blastoderm in the manner described by Lutz (1949). The operation does not damage the vitelline membrane over the embryo. The knife cuts extended right across the area opaca as well as the area pellucida, so that each of the two pieces was about half the size of an unoperated embryo. After the operation, the shell was resealed with Scotch tape and the egg incubated.

No operation was performed on control embryos but the eggs were opened and resealed in the same way.

Most eggs were then incubated for either 48 h or 72 h before the embryos were removed for photography and fixation. A few eggs were however opened after 18–24 h of incubation so that the early stages of development could be inspected. Eight of these embryos were dissected from the yolk and explanted in culture by the technique of New (1955) so that their development could be followed.

The length of each embryo was measured after fixation, at 40× magnification, using an eyepiece graticule in a Nikon dissecting binocular microscope. The region measured was the maximum distance from the anteriormost tip of the embryo to the posterior limit of the area pellucida. Until about stage 13 (19 pairs of somites) this anterior point is the tip of the forebrain, but as cranial flexure takes place, it comes to be situated in the mesencephalon.

The sizes of the somites were measured on whole embryos after fixation, at 85× magnification, using an eyepiece graticule in a Wild binocular microscope. Only the last two pairs of somites were measured. The width and the length of each somite was recorded and the area of its dorsal surface was then calculated. The height was compared in a limited number of whole-mount embryos by measuring the shift in depth of focus from the lower to the upper surface of the somite on a calibrated microscope.

Serial sections were cut at 8 μm through seven pairs of twins and through six control embryos and these were stained with Harris’ haematoxylin and eosin. The numbers of cells were counted in the middle section of the last pair of somites in each series.

The area of the area pellucida was measured by means of a planimeter, from drawings made at 30× magnification with a Wild camera-lucida apparatus. Unfortunately, however, it is not always easy to define the precise border between area opaca and area pellucida in embryos after the extraembryonic circulation has begun, so that only a limited number of results (25), could be obtained.

RESULTS

I. Types of embryos

(a) Control embryos

Thirty-three quail eggs which had been opened and resealed gave rise to
normal embryos. Those examined after 48 h incubation had usually reached about stage 11 and possessed about 15 pairs of somites, whilst those examined after 72 h were usually at stages 15 or 16 and possessed about 20 pairs of somites. Two further embryos were found to be abnormal and were discarded. Twelve additional controls were fixed at times ranging from 24–84 h incubation. These were from eggs which had not been opened at all until the time of fixation.

(b) Operated embryos

Thirty-one embryos were examined 18–24 h after the operation, and in every case the cut was found to have healed. Five of the blastoderms each possessed two primitive streaks, whilst each of the remaining 26 had either regulated to form a single streak or had failed to form a streak at all. Of those embryos which were explanted in culture, one which possessed two primitive streaks anteriorly but only one posteriorly subsequently formed a duplicitas anterior embryo. Two others which each possessed a single primitive streak anteriorly but two primitive streaks posteriorly subsequently formed single axes. Those which had already regulated to form a single primitive streak developed a single axis.

Not all the embryos examined after 48 or 72 h had formed double axes. Twenty-three were discarded either because they were so distorted that they could not be interpreted, or because they had died. Thirty-six had each regulated to form what was principally a single axis, although abnormalities such as platyneuria and diplocardia were often present; a disorderly arrangement of the somites was also a feature of these embryos (Fig. 1).

Forty-two embryos had each formed well-defined double axes. These were of three main types:–

i) **Duplicitas anterior** (16 pairs of twins)

These are composed of two separate heads which share a common trunk. The level at which the two axes become united varies. Figure 2 illustrates a specimen in which the somites are not yet shared in the trunk.

ii) **Collided axes** (8 pairs of twins)

These appear to have resulted from two initially separate axes which have collided in the head region (Fig. 5). The heads are therefore often distorted.

iii) **Separate axes** (18 pairs of twins)

These may be orientated in a variety of ways in relation to one another. Those

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Fig. 1. Partially regulated embryo suffering from platyneuria and a disturbed arrangement of somites in the trunk. Fixed at 48 h. ×40.

Fig. 2. Duplicitas anterior embryo in which the two axes are not yet fully united. Each axis possesses the same number of somites. Fixed at 48 h. ×40.

Fig. 3. Separate twinned axes lying at right angles to one another. The left possesses 14 pairs somites, the right 22 pairs. Fixed at 72 h. ×20.

Fig. 4. Enlargement of the tail buds of the embryos illustrated in Fig. 3, to show that the two axes are separate from one another. ×60.
Somites in small avian embryos

Figs 1–4
Table 1. The table shows the range of stages (Hamburger & Hamilton) reached after 72 h incubation

<table>
<thead>
<tr>
<th>Type of embryo</th>
<th>Stages after 72 h</th>
<th>Pairs of somites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>13–15</td>
<td>19–25</td>
</tr>
<tr>
<td>Regulated single</td>
<td>11–16</td>
<td>13–27</td>
</tr>
<tr>
<td>Duplicitas anterior</td>
<td>8–13</td>
<td>4–19</td>
</tr>
<tr>
<td>Collided axes*</td>
<td>9–13</td>
<td>4–35</td>
</tr>
<tr>
<td>Separate axes</td>
<td>7–13</td>
<td>1–19</td>
</tr>
</tbody>
</table>

* It was not always possible to determine the precise stage of development of collided axes since the heads were frequently distorted. The figures are therefore based on three pairs only of collided axes.

shown in Figs 3 and 4 are at right angles to one another, whilst those in Figs 6, 7 and 8 are parallel to one another.

II. Comparison of experimental embryos and the controls

(a) Size of the embryos

Some of the experimental embryos examined after 18–24 h each possessed two full-length primitive streaks (stage 4 of Hamburger & Hamilton, 1951), but these were shorter than those of control embryos of the same stage. For example, they were 0-8 mm and 1 mm in one pair of twins and 0-75 mm and 1-0 mm in another pair. By contrast, the lengths in each of three control embryos were 1-25 mm, respectively.

When the twinned embryos were examined after 48 or 72 h, they were consistently found to be smaller than the unoperated controls. To some extent this could be attributed to the fact that they were a little retarded in development in comparison with the controls (see Table 1). It was decided therefore that any valid comparison between experimental and control embryos must be made between those individuals which had reached about the same stage of development, rather than between those which had undergone the same period of incubation.

The maximum length of each embryo was then plotted against the number of pairs of somites (see Fig. 9: Data for embryos with collided axes were not included because the heads were often distorted, and this led to difficulties in determining the anterior limits of the embryos).
Some variation in results was found within each group, but nevertheless it appears that a short embryo may have the same number of somites as a long one. We conclude therefore, that the number of somites which have developed at any given stage, does not depend on the length of the embryo.

(b) Size of the somites

In amniote embryos, the somites individually become bigger and change in morphology as development proceeds (Herrman, Schneider, Neukom & Moore, 1951). For this reason, measurements were confined in each embryo to the two
most-recently-formed pairs of somites (i.e. the two most-posteriorly-situated ones). The relative proportions of width to length were not however found to be consistent. In general, somites tended to be longer and narrower in single embryos than either in twinned ones, or in most regulated single embryos. In embryos suffering from platyneuria however the somites were consistently wider than long.

It was found however that provided the neural tube was closed the height of each newly formed somite varied little in the different experimental categories. It was considered therefore that the area of the dorsal surface could be used as a useful indicator of somite size. Furthermore, when the numbers of cells were counted in the middle section of each newly formed somite, they were found to be similar in both the twinned axes and the control embryos. Thus in the 14 twins, counts ranged from 60–84 (Mean 65 ± 7), whilst in the 6 controls, counts ranged from 58–84 (Mean 69 ± 9). The differences between the twins and the controls were not statistically significant when compared by the t-test, using a significance level of t < 0.1.

Table 2. This table shows the mean areas in sq μm of the dorsal sides of the last two pairs of somites, measured in embryos possessing between 5 and 20 pairs of somites

<table>
<thead>
<tr>
<th>Number of embryos</th>
<th>Mean dorsal area of somites in sq μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unoperated controls</td>
<td>14</td>
</tr>
<tr>
<td>Regulated singles</td>
<td>12</td>
</tr>
<tr>
<td>Duplicitas anteriors</td>
<td>8</td>
</tr>
<tr>
<td>Collided axes</td>
<td>9</td>
</tr>
<tr>
<td>Separate axes</td>
<td>18</td>
</tr>
</tbody>
</table>

Table 2 shows the sizes of the last-formed somites, as indicated by the dorsal surface area. Using these figures the size of the somites in the control group was compared with that in each of the experimental groups, using the formula:

\[ t = \frac{\bar{y} - \bar{z}}{\sqrt{\frac{sy^2}{ny} + \frac{sz^2}{nz}}} \]

where \( \bar{y} \) = mean from one group

\( sy = \) standard deviation of mean \( \bar{y} \)

\( ny = \) number of embryos in the group

and \( \bar{z}, sz \) and \( nz \) = comparable values for the other group.

This formula makes allowance for the fact that the standard deviations are not identical for every group.

Using a significance level of \( t < 0.1 \), no experimental group was found to have somites differing significantly in size from the control group. From this data, we
conclude that although the experimental embryos are often shorter in length than the controls, the somites remain within the same size range. No miniaturised somites were seen in any of the embryos.

Serial sections showed that the cells in the newly formed somites follow the pattern described for normal chick embryos by Bellairs (1980). Thus, each somite is composed of elongated, spindle-shaped cells arranged radially around a small central lumen, the myocoele. The diameter of each somite, measured in any direction is therefore two cells plus the myocoele.

(c) Size of the area pellucida

Table 3 shows the mean area of area pellucida of representative samples of the five categories.

<table>
<thead>
<tr>
<th>Type of embryo</th>
<th>Area in sq mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unoperated control</td>
<td>165 ± 70 (n = 5)</td>
</tr>
<tr>
<td>Regulated single</td>
<td>37 ± 16 (n = 8)</td>
</tr>
<tr>
<td>Duplicitas anterior</td>
<td>41 ± 17 (n = 4)</td>
</tr>
<tr>
<td>Collided axes</td>
<td>78 ± 20 (n = 3)</td>
</tr>
<tr>
<td>Separate axes</td>
<td>112 ± 50 (n = 5)</td>
</tr>
</tbody>
</table>

n = the number of embryos measured.

In those specimens consisting of two separate axes, the two parts of the area pellucida were sometimes completely separate, but in recording the measurements they were added together and treated as one. The results shown in Table 3 refer to those specimens only where one or both axes possessed between 11 and 20 pairs of somites. A great variation in size was found between individuals within the same category which possessed identical numbers of somites.

In no experimental group however were the mean areas found to be greater than the mean areas of the control embryos. Similarly, when comparisons were made between the areas of the experimental and control embryos at 72 h of incubation, irrespective of the developmental stages, the differences were even more pronounced.

It appears therefore that the area pellucida in an embryo with two axes, tends to be no greater in size than that in a control embryo, and indeed is often less.

III. Comparison of the two individuals in a pair of twins

(a) Duplicitas anterior twins

In the duplicitas anterior embryos, the two axes were separated anteriorly, so that each usually possessed its own double row of somites. The two axes were united into a single axis posteriorly however, which, provided development had
Somites in small avian embryos

Table 4. Shows the difference in number of somites between two axes in pairs of twins

<table>
<thead>
<tr>
<th>Types of twins</th>
<th>No. of pairs of twins</th>
<th>Twins with difference not more than 2</th>
<th>Twins with difference greater than 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duplicitas anterior</td>
<td>11</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>Separate axes</td>
<td>16</td>
<td>4</td>
<td>12</td>
</tr>
<tr>
<td>Collided axes</td>
<td>8</td>
<td>3</td>
<td>5</td>
</tr>
</tbody>
</table>

proceeded far enough, possessed one double row of somites. In only one out of eleven cases did the two anterior axes possess different numbers of somites from one another. In this specimen, one axis possessed three pairs of somites, whilst the other had five pairs.

(b) Separate twins and collided twins

In both these categories the twins had formed from separate primitive streaks. The main difference between them was in the orientation of the two axes. Thus separate twins might lie in any orientation in relation to one another (parallel with the same orientation, parallel with opposite orientation, or one at right angles to the other). By contrast, collided twins were always found to lie head to head in either a straight line, or at an angle of at least 150° (as in Fig. 5).

The two groups appeared to behave in a similar way however so far as somites were concerned (see Table 4). Thus, in about a third of the pairs of separate or collided twins, the two axes possessed a similar number of somites; for example in one set of collided twins, there were 18 pairs of somites in one axis, and 20 pairs in the other.

In the remaining pairs of separate or collided twins however, the two axes differed greatly. The following are examples of the different number of somites found between the two axes of representative pairs of twins: 18 and 28; 2 and 11; 4 and 12; 12 and 20; 14 and 22; 25 and 35. Where two axes possessed different numbers of somites, they usually appeared to be at different stages of development.

It was concluded therefore that where one axis appeared to be retarded, the two axes had developed at different rates and had therefore been subjected to separate controls.

DISCUSSION

I. Comparison with amphibian experiments

The main purpose of this investigation was to discover whether the development of somites was subjected to the same sorts of controls in bird embryos as in amphibians.

In his classical experiment performed on *Xenopus* embryos, Cooke (1975) removed a large proportion of cells from the blastula. Our present experiments
on the quail resemble those of Cooke, in that we too operated on the blastula to reduce the amount of material available.

The operated quail embryos resembled the operated amphibian embryos in being smaller than unoperated controls which possessed approximately the same number of somites. The quail embryos differed from the *Xenopus* embryos however in one important particular. In the amphibian embryos the individual somites were smaller in the experimental embryos than in the controls. In the quails however reduction in size of the embryos was not accompanied by reduction in size of the somites. It appeared rather that the appropriate number of somites was fitted into a reduced body length mainly by an adjustment of somite shape rather than of somite size. No miniaturised somites were ever seen in these embryos, although miniaturised somites may be obtained by operating at a later stage. We conclude therefore, that certain differences between amphibian and chick embryos exist in the control systems at the blastula stage of development. These will be discussed in more detail in our next paper.

II. Comparison between individuals of twin pairs

It might be expected that when two embryos are formed as a result of bisecting the original blastoderm, they would be almost identical, because they possess the same genome and the same environment and have been incubated for the same time. Moreover, both pieces of the original blastoderm have undergone similar trauma from the same operation. Differences were however found between the two partners and these were probably due to two factors either one or both of which might give one of a pair of twins an advantage over the other.

First, although we attempted to cut the original blastoderm into equal-sized pieces, it is unlikely that precisely the same number of cells was present in each part. Secondly, although most of the blastoderms showed no obvious sign of anteroposterior orientation at the time of operation, nevertheless it is likely that orientation was already established at least in a provisional way. Lutz & Lutz-Ostertag (1963) who first reported the effects of bisecting unincubated quail embryos reported that Köller's crescent was already visible in some embryos. It is possible however that most of our experiments on quail embryos were made at a slightly earlier stage than most of their experiments, and that greater regulation was therefore possible. This suggestion is supported by the fact that the proportion of relatively normal single embryos obtained after the operation was higher in our experiments than in theirs. A relatively normal single axis probably formed if the blastoderm developed a single primitive streak.

The development of two primitive streaks however does not guarantee the formation of two completely separate axes. We have shown that two potential axes may become united, forming partially single, partially duplicated embryos. If the two primitive streaks remain quite separate however, two separate axes develop. Although these axes may collide secondarily in the head region (collided axes), the trunks remain separate.
One of the major findings of the present experiments is that, provided the two trunk axes remain separate, (even if the heads collide) each appears to control its own number of somites. If however the trunks become united, as in the duplicitas anterior embryos, the two axes, almost without exception contain the same number of somites and are therefore probably under the same controls as one another.

III. Control of somite number during the first 72 h of incubation

The longer a young chick embryo is incubated, the bigger it grows and the more somites form. We do not know how the embryo controls the maximum number (52) which develop, but we will discuss here ideas on how the numbers may be controlled in the early stages. It might be expected that the number of somites is controlled directly by the length of incubation, or by the length of the embryo or by the size of the whole area pellucida. The following evidence however suggests that none of these factors is of direct importance:–

(1) The length of incubation time does not directly control the rate of somite formation because different numbers of somites were often found in twin axes which possessed identical genomes and had developed in the same environment for identical periods.

(2) The length of the embryo does not determine how many somites form, because the same number may be found in both short and long embryos.

(3) If there is some global control acting throughout the area pellucida this is overcome in the case of the separate (and collided) twins, since the two individuals may possess different numbers of somites even though they share the same area pellucida. It might be argued that the area pellucida had become too large in these cases to maintain its functional identity as a single global region and had therefore become two regions. Our evidence does not support this possibility either, since the total size of the area pellucida in blastoderms containing separate axes was no greater than that of control blastoderms containing one axis. We suggest therefore that if there is a global control it is not related to size of the tissue mass but to some other factor.

We suggest instead that the controlling factors are to be found in the morphogenetic movements, especially those associated with node regression. These are maximal along the primitive streak but also take place in the adjacent area pellucida, so that even where a duplicitas anterior embryo develops, the two anterior axes are subject to a unified regression. Where the two primitive streaks remain apart, then two separate regression movements take place and these are apparently not co-ordinated.

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