Growth and morphogenesis of the fibula in the chick embryo

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SUMMARY

The development of the avian fibula was studied both histologically and experimentally. It was found that from the onset of chondrogenesis, the fibula possessed a smaller diameter than the neighbouring tibia. The truncated growth of the fibula was a result of the loss of its distal epiphysis between stages 27–31. This epiphysis subsequently became fused to the tibia and formed the fibulare of the tibiotarsus. The experiment of Hampé (1960) was repeated by inserting tantalum barriers into the limb between stages 18 and 23: this sometimes prevented the separation of the fibula distal epiphysis, thus giving rise to an elongated element. A similar result was obtained from grafts of polarizing region into the leg bud at stages 18–20. It was concluded that there was no evidence for competitive interaction between the blastemata of the tibia and fibula. In addition, the differential growth in diameters between the tibia and fibula was largely a result of differential osteogenesis rather than chondrogenesis as previously thought.

INTRODUCTION

The development of the tibia and fibula in birds is arguably one of the most striking examples of differential growth in the avian embryo. Prior to chondrogenesis, the two condensations which represent these elements appear quite similar in dimensions, but by day 10 of incubation the fibula is only half the length and a quarter of the diameter of the adjacent tibia. In order to account for this, Wolff (1958) proposed the 'principle of competition' or the competitive interaction model. The essence of this idea is that the developing blastemata may compete for a definitive number of mesenchymal cells. Thus the blastema which exerts the greater 'field of influence' will recruit mesenchyme cells at the expense of a neighbour with a smaller 'field of influence'. He suggested that the tibial prechondrogenic condensation exerts a greater 'influence' than the fibular condensation and acquires mesenchyme cells which might originally have been designated to the fibula. Consequently, this early competition for mesenchyme gives rise to the subsequent differential growth seen in the chondrogenic elements.

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Experimental evidence for such a mechanism is, however, equivocal. For example, Wolff & Kieny (1962) showed that the fibula was more sensitive than the tibia to X-irradiation at the condensation stage. Thus they argued that depletion of mesenchyme would affect the element which exerted less 'influence' than its neighbour, in this case the fibula. Hampé (1959, 1960) showed that the insertion of a mica plate in the developing bud sometimes resulted in an elongated fibula. He argued that the mica plate blocked the acquisition of mesenchyme by the tibia from the fibula. Furthermore, the addition of extra mesenchyme to the young limb bud also resulted in an elongation of the fibula, whilst mesenchyme depletion often led to the loss of the entire element. Again, these results were interpreted in support of competition between these two elements for a definitive number of mesenchyme cells.

Such a view should be contrasted with one which suggests that each cartilaginous rudiment is non-equivalent (Lewis & Wolpert, 1976) and has an intrinsic programme of growth (Wolpert, 1978). Once each region has left the progress zone and has been specified (Summerbell, Lewis & Wolpert, 1973) its growth seems to be autonomous. Thus, when early limb buds are grafted between hosts of different ages, the growth of the separate cartilaginous elements is autonomous and there is no evidence for interaction between them (Summerbell & Lewis, 1975). Holder (1977a,b) for example, has shown that both the development of joints and the timing plus the site of ossification are autonomous. Thus, in this view the difference in both length and width between the tibia and fibula would be the result of the respective growth programmes laid down in the elements at the time of specification. Some evidence for such an interpretation has been provided by Hicks & Hinchliffe who are quoted in Hinchliffe & Johnson (1980) as having found that the growth of the tibia and fibula cultured after $6\frac{1}{2}$ days incubation is autonomous and they could find no evidence of interaction between them. However, this is later than the time at which any 'competitive' interaction could be expected.

In view of the importance of Hampé's experiments we did a comprehensive study of the histological events that are taking place in the zeugopod (foreleg) of the leg from stages 22–39 and we repeated the insertion of a barrier between the presumptive tibia and fibula.

Grafts of polarizing region to the wing are known to alter the pattern of the zeugopod (Tickle, 1980). For example if a polarizing region is grafted to the anterior margin, two ulnae or ulna–radius–ulna may develop in place of a radius and ulna. It is therefore possible that similar grafts to the leg bud can result in comparable morphological changes in the leg.

We also know from work of Cooke & Summerbell (1980) that cell proliferation is enhanced in most areas of the limb bud following grafts of polarizing regions; thereby increasing the mesenchyme cell number. Grafts of polarizing region to the leg may be looked at by way of comparison to the mesenchyme excess experiments of Hampé (1960).

Here we report the morphology of the zeugopod in which polarizing regions had been grafted to different positions along the anteroposterior axis of the leg bud. The digit patterns of these legs have previously been published by Summerbell & Tickle (1977) and these authors have kindly provided us with the original specimens for analysis of the zeugopod.

MATERIALS AND METHODS

Histological procedure

Fertilized White Leghorn eggs from a local breeder were incubated at 38 ± 1 °C and windowed on the fourth day of development. The embryos were staged according to Hamburger & Hamilton (1951). Embryos between the stages 22 and 39 inclusive were removed from the eggs and transferred to Bouin's fixative for a period of at least 24 h. After fixation, the hind limbs were dissected away from the body, dehydrated in a graded series of alcohols and embedded in fibrowax. Serial sections were cut at a thickness of 7 μm and stained in Harris' haematoxylin and eosin or in toluidine blue for metachromasia. Limbs were cut both longitudinally (through the anteroposterior plane) and transversely (through the dorsoventral plane).

Whole mounts

Embryos between the stages 24 and 36 were fixed in 5 % trichloroacetic acid, stained with 0·1 % Alcian green 2GX in 1 % acid alcohol, differentiated in acid alcohol (Summerbell & Wolpert, 1973), cleared in methyl salicylate and examined.

Growth measurements

Linear increments in growth of the tibia and fibula were made from the whole mounts prepared above. Each measurement was made from the limbs of two embryos at the respective sampling times.

Barrier insertion

Tantalum foil barriers 10 μm in thickness and approximately 200×400–500 μm were inserted into the hindlimb buds of stage 18–23 embryos as described by Hampé (1960). The barriers were placed in a proximodistal orientation along the bud adjacent to the apical ectodermal ridge. Limb buds of embryos (stage 18–20) could not accommodate such large barriers and smaller barriers (200 × 200 μm) were inserted. A few drops of Hanks balanced salt solution containing 1 % antibiotic/antimycotic (Gibco) was added to each egg before the windows were resealed with Sellotape and returned to the incubator. The embryos were sacrificed at 10 days of incubation and both legs were prepared as whole mounts. Linear measurements of the tibia and fibula were made in all cases.

Polarizing region grafts

Polarizing regions from wing and leg buds of donor embryos were grafted into host leg buds between stages 18–21 inclusive. For this purpose some of the data compiled by Summerbell & Tickle (1977) were re-examined in terms of tibia and fibula length and morphology.

RESULTS

Histogenesis and morphogenesis

At the histologically detectable onset of chondrogenesis in the hind limb (stage 24–25) the demarcation between cartilage synthesizing and non-chondrogenic cells is unclear (Fig. 1). By stage 26, sufficient metachromatic matrix has been deposited to make fairly clear distinctions between developing chondrogenic rudiments, the surrounding mesoderm and developing muscle blocks (Fig. 2). It is evident from transverse sections that whilst the fibula is rounded, the tibia is more oval. As a result, in longitudinal section the tibia appears to have a diameter of at least 50 % greater than the fibula (Table 1). By contrast, in transverse section the diameter along the dorsoventral axis of both elements is quite similar (Fig. 2). The disparity in tibial and fibular dimensions in the antero-posterior plane is reflected in the cell numbers across the two rudiments. Working from serial longitudinal sections (at stage 25+) it was found on average that the tibia diameter was 20 ± 3.5 cells across whilst the fibula only 14.4 ± 2.3 cells across which represents a 42 % difference. It is important to note that at this stage there is no evidence of a morphologically distinct perichondrium. By stage 27, the central cells in the tibial and fibial rudiments have begun to hypertrophy. A distinct multilayered perichondrium becomes apparent adjacent to the hypertrophying cells (Fig. 3) but is less well defined at the epiphyseal ends of the rudiments. At this stage, it is noticeable that the fibula is longer than the tibia

Table 1. *The diameters of the chick embryo tibia and fibula (mid-diaphyseal level) during successive stages of development*

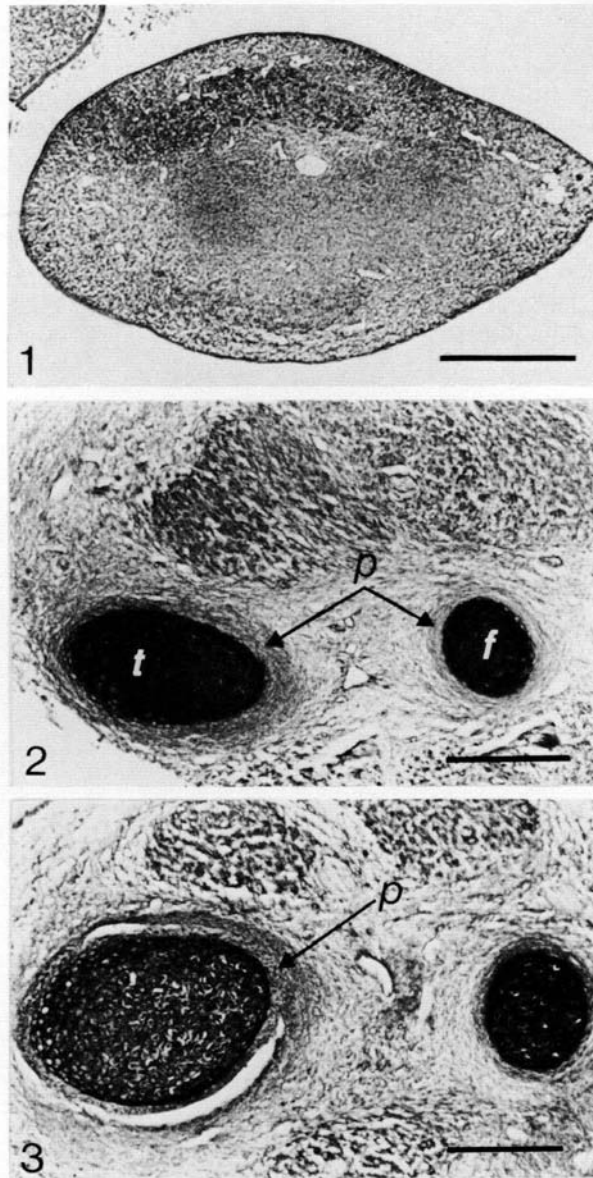| Stage	Tibia μm	Fibula μm	% difference of tibia over fibula
26	288	152	89
29	340	140	142
31	380	152	150
(Osteogenesis)			
34	552	180	206
36	832	212	292
38	1060	228	341
Total % increase	268	57	

Fig. 1. Transverse section of a stage-24+ hindlimb. There is no clear demarcation between cartilage secreting cells and non-chondrogenic mesenchyme. Toluidine blue.

Fig. 2. Transverse section of a stage-26 hindlimb. The tibia (t) and fibula (f) are clearly visible. The non-chondrogenic perichondrium (p), composed of flattened cells, can also be seen at this stage. Toluidine blue.

Fig. 3. Transverse section through the central region of the tibia and fibula at stage 27. Note the thick perichondrium (p) and hypertrophic chondrocytes. Toluidine blue. Scale bar in Figs 1–3 = 200 μm.

(Fig. 4). During the following 12 h of development (stage 28) a region of flattened cells becomes apparent between the diaphysis and distal epiphysis of the fibula (Fig. 5), and toluidine-blue staining shows that these cells, unlike normal flattened cells in long-bone rudiments, secrete little metachromatic matrix (Fig. 6). At about the same time, an asymmetric protrusion develops from the lateral aspect of the distal epiphysis of the tibia which enlarges (Figs 7B, 8B). During stages 28 to 33 this protrusion joins with the distal epiphysis of the fibula, which separates from the rest of the fibula in the region of the flattened cells (Figs 7, 8). Thus the distal epiphysis of the fibula becomes part of the distal tibia – it becomes the fibulare of the tibiotarsus.

In addition to the loss of the distal epiphysis there is a reduction in the number of cells across the diameter of the fibula whereas the cell number across the tibia remains fairly constant (Table 2). This occurs at the same time as an increase in the ratio of tibia/fibula diameters (stage 29) (Table 1).

The onset of osteogenesis (stage 31/32) occurs at the same time in both tibia
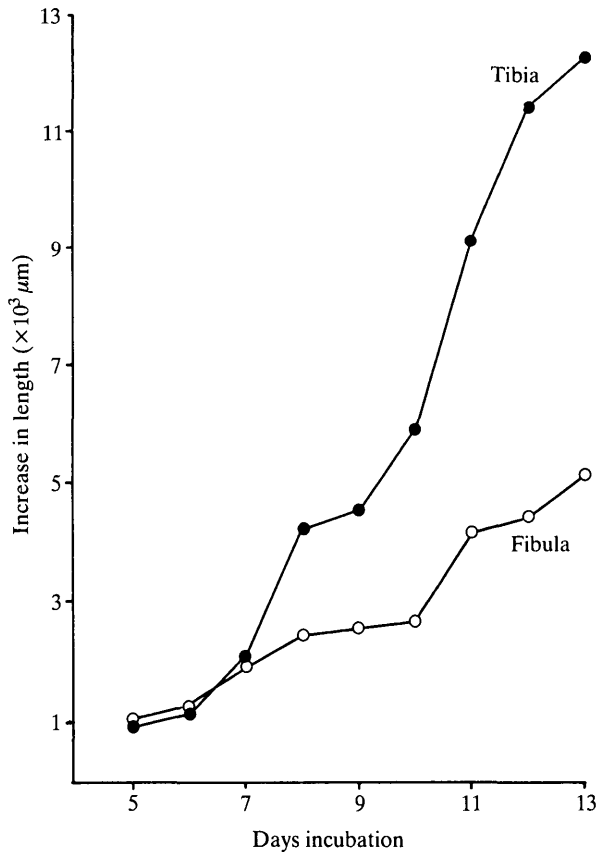

Fig. 4. Growth in length of the tibia and fibula. Measurements from early stages were made from histological sections. Later measurements (day 7 onwards) were made from whole mounts of two embryos at the respective stage.
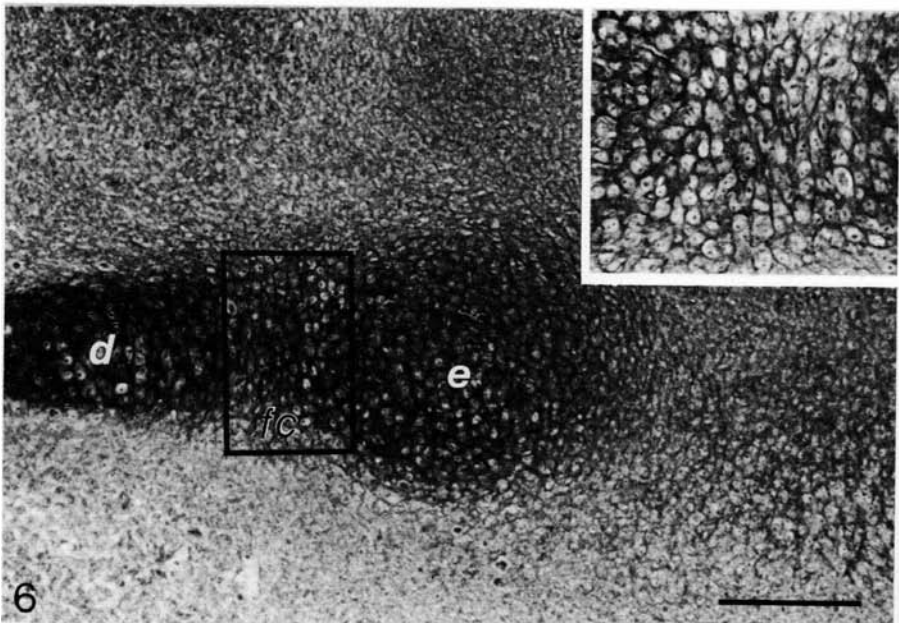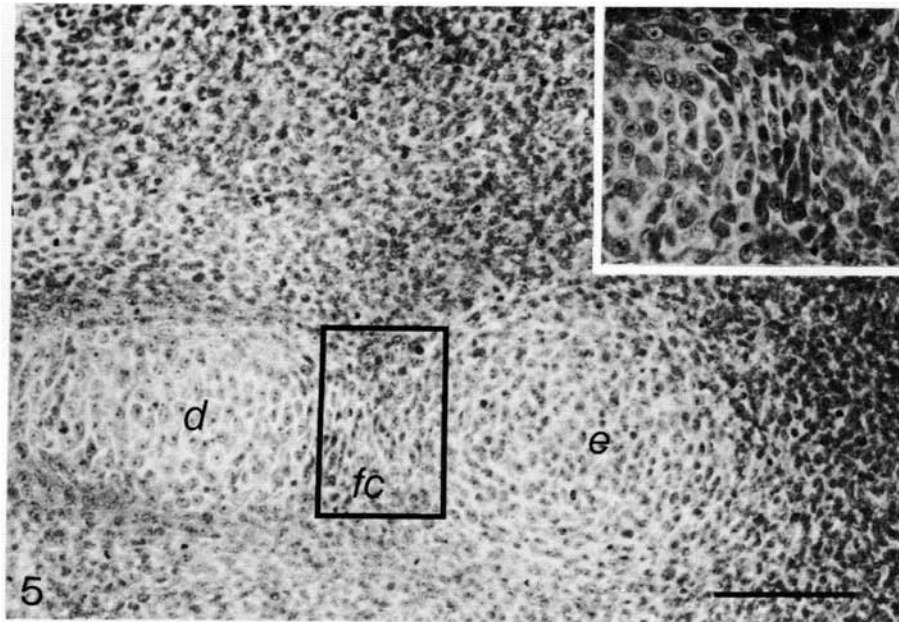
Fig. 5. Longitudinal section through the distal region of fibula (stage 27). A region of flattened cells (fc) can be seen separating the distal epiphysis (e) and diaphysis (d). Inset. High-power magnification of flattened cells. H & E.

Fig. 6. Similar section to that shown in Fig. 5, stained for metachromasia. Inset. High-power magnification showing the thin metachromatic partitions between the flattened cells (fc). Scale bar in Figs 5 & 6 = 100 μm.
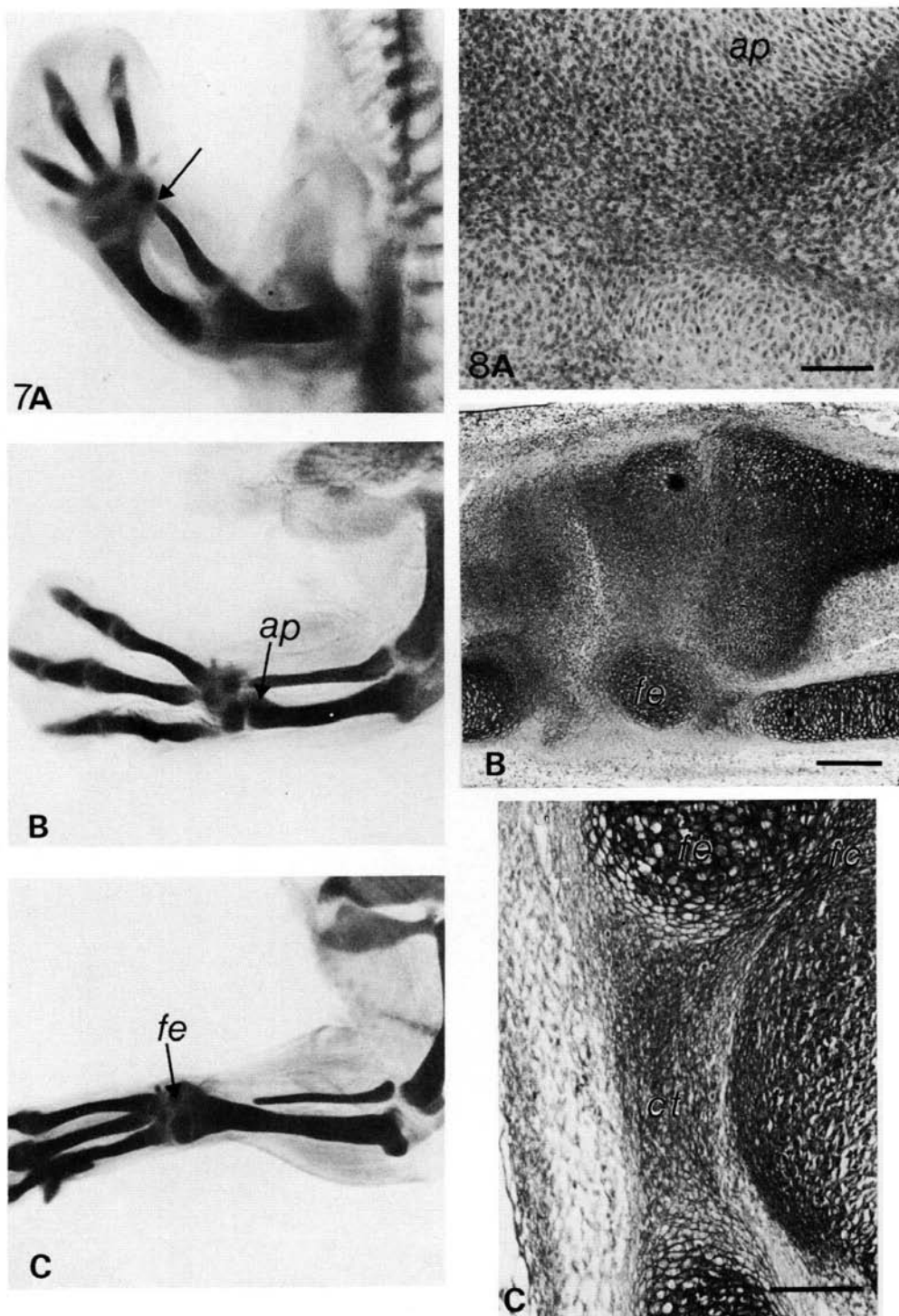
Figs 7–8

Table 2. *The number of cells across the mid-diaphysis (anteroposterior axis) of the tibia and fibula through successive stages up to the onset of osteogenesis*

Stage 25+	tibia	20·5 ± 3·5*
	fibula	14·4 ± 2·3
Stage 26+	tibia	19·4 ± 1·5
	fibula	14·2 ± 1·6
Stage 28	tibia	19·2 ± 1·5
	fibula	11·2 ± 1·3
Stage 31	tibia	19·3 ± 1·6
	fibula	10·0 ± 1·4

* Mean values calculated from five sections taken from each of three rudiments per stage.

and fibula, and the two subperiosteal osseous collars develop adjacent to one another (Holder, 1978). However, the bony collar in the fibula is eccentric since the distal epiphysis is missing. Also, the patterns of ossification differ markedly between the two elements. In the tibia the osteogenic process progresses in the manner described by Fell (1925). Vascular buds penetrate the hypertrophic cartilage in the central diaphysis (stage 33). These represent the initial sites of cartilage resorption which progress towards both epiphyses. At the same time, the osseous collar develops along the diaphysis, the extent of which invariably reflects the limit of adjacent cartilage hypertrophy. Both of these events appear to allow further sites of resorption to be established nearer the epiphyses, but these seem to play a secondary role to the rapid resorption originating at the centre of the rudiment. There is little or no endochondral ossification occurring at these sites of resorption. The initial bony collar of the tibia is succeeded by a

Fig. 7. Whole mounts of hind limbs between stages 28 and 32. (A) Stage-28 limb showing the region of flattened cells as a transparent zone between the distal epiphysis and diaphysis (arrowed). (B) Stage-30 limb. Note that the distal epiphysis has now separated from the diaphysis. The asymmetric protrusion (ap) which develops from the distal epiphysis of the tibia can be seen extending towards the separating fibula. (C) Stage-32 limb. The fibula has now completely separated from its distal epiphysis which has fused to the asymmetric protrusion of the tibia epiphysis, and becomes the fibulare (fe) of the tibiotarsus.

Fig. 8. Histological sequence showing the separation of the distal epiphysis from the fibula. (A) Stage 28. Separation has not yet begun and the region of flattened cells is clearly visible. However, the asymmetric protrusion (ap) of the tibia can be seen at the top of the figure. H & E Scale bar, 50 μm. (B) Stage 30. The asymmetric protrusion of the tibia can now be seen abutting the separation of the fibula epiphysis which now becomes the fibulare (fe). Toluidine blue. Scale bar, 100 μm. (C) Stage 31. Connective tissue (ct) joins the truncated end of the fibula with the fibulare (fe). This connective tissue persists throughout the life of the fowl. Note also that flattened cells (fc) separate the fibulare and distal epiphysis of the tibia and thus does not represent a true 'fusion' of cartilage matrix. Toluidine blue. Scale bar, 50 μm.

second, a number of transverse trabeculae separating the two, and by stage 37 a third osseous cylinder is evident (Fig. 9). Thus the diameter of the element is increased markedly.

In contrast, only a single bony collar is ever laid down in the fibula and cartilage resorption is retarded even though hypertrophy is extensive (Fig. 10). By stages 38/39 however, a number of resorption sites are present along the whole length of the hypertrophic zone (Fig. 11). These quickly occupy the diameter of the cartilage and extend longitudinally, eventually fusing to form a marrow cavity (Fig. 12). Endochondral ossification is occasionally observed. The failure of the fibula to form successive subperiosteal bony collars results in very little change in diameter after stages 35/36.

Barrier insertions

Sixty-eight barrier insertions were performed of which 15 embryos died of postoperative trauma, and 7 limbs had lost their barriers. The result of the remaining 46 embryos could be divided into three groups. 1) Barriers into stages 20 and younger, resulted in the division of the femur blastema. At 10 days of incubation the femora were split into two separate elements or were distally bifurcated each with its own epiphysis articulating with the tibia and fibula (5 out of 8 cases). Sometimes the femoral bifurcation displaced the fibula distally thus shortening this element. These elements also possessed a distal epiphysis (Fig. 14). 2) Insertions made in the anterior half of the leg bud (opposite somites 27 and 28) at stages 21 to 23 generally resulted in the retarded elongation of the tibia (7 out of 15 cases). 3) Insertions made more posteriorly (opposite somites 29/30) at stages 21 to 23 frequently resulted in an elongated split fibula which often possessed a distal epiphysis (Figs 15 and 16) (11 out of 23 cases).

Polarizing region grafts

The result from 120 successful grafts were analysed (Table 3). Thirty-seven percent of all grafted limbs showed fibulae that were longer than the contralateral controls irrespective of reduplication or of the embryonic stage at the time of grafting. Furthermore, of these elongated fibulae, some 55 % showed evidence of a retained distal epiphysis.


Fig. 9. Transverse section through a stage-37 tibia (t) and fibula (f). Note the three subperiosteal collars of bone (arrowed) which are separated by trabeculae in the tibia. In contrast, only a single collar of bone (b) is laid down in the fibula. H & E.

Fig. 10. Longitudinal section through stage-37 fibula. Observe the extensive hypertrophic cartilage and lack of resorption. H & E.

Fig. 11. Longitudinal section through stage-38 fibula. A number of resorption sites which develop laterally beneath the subperiosteal bony collar are now evident. H & E.

Fig. 12. Stage-39+ fibula showing the marrow cavity which has developed from the fusion of a number of resorption sites. Note also that still only a single collar of bone is evident. H & E. Scale bar in Figs 9–12 = 200 μm.

Grafts performed at stage 18 generally resulted in reduplication of zeugopod elements with the number of toe reduplicates depending on the position of the original graft (Summerbell & Tickle, 1977). The tibial elements in these limbs
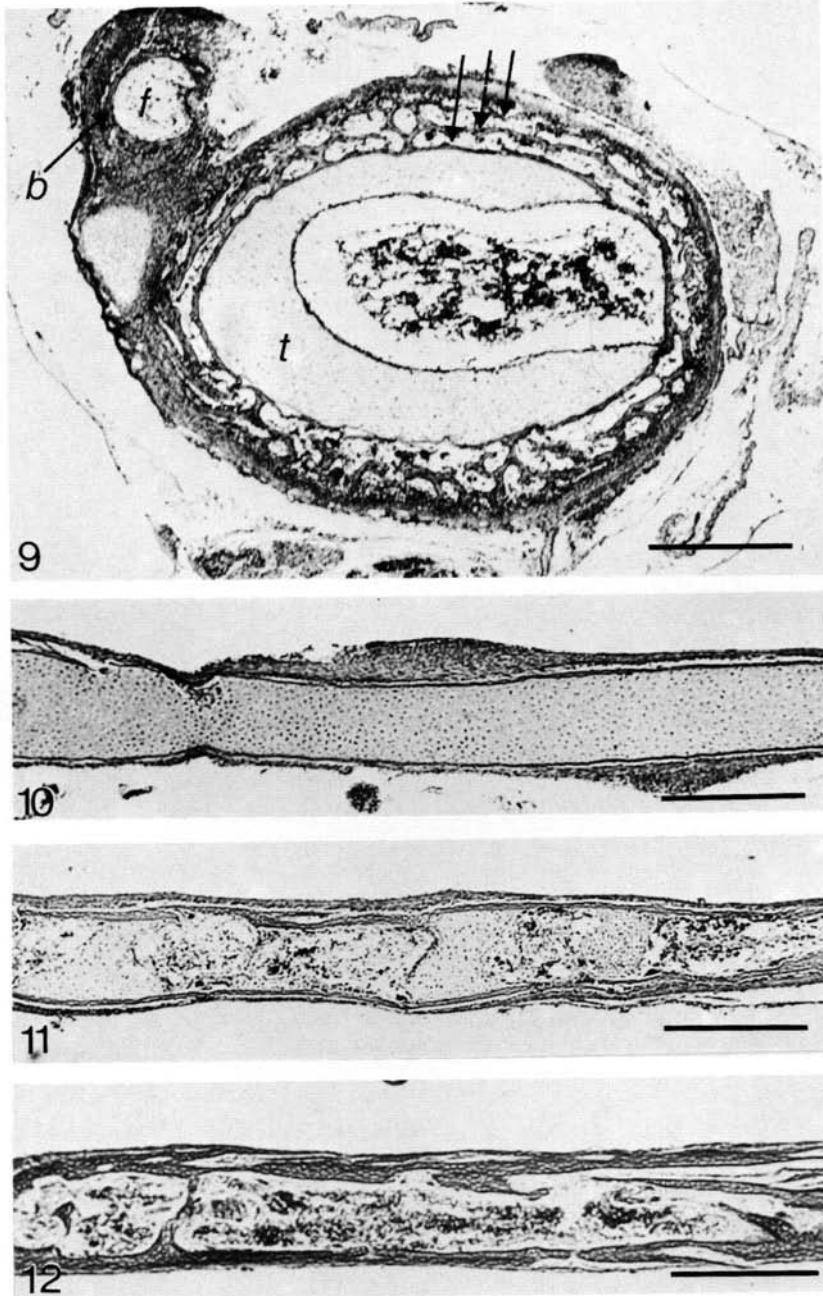
Figs 9–12

Table 3. *The effect of polarizing region grafts on fibula morphology*

Stage of host	No. of grafts	No. of elongated fibulae. Figures in parenthesis represent fibulae with distal epiphyses	percentage
18	72	21 (16)	29 (22)
19	13	5 (1)	38 (7)
20	35	21 (9)	60 (25)

were often shortened whilst the fibulae were elongated with distal epiphyses (Fig. 17). Reduplication of both the upper and lower limb was also apparent and again fibulae were often elongated and possessed distal epiphyses (Fig. 18). Generally grafts performed at stage 20 only resulted in reduplication of toes. However, in 60 % of these cases the fibulae were longer than the contralateral controls. Twenty-five percent of stage-20 operated limbs showed elongated fibulae with distinct distal epiphyses which articulated with reduplicate toes (Figs 19 and 20). No apparent differences in the tibia and fibula anomalies were noticed whether wing or leg polarizing tissue was grafted.

DISCUSSION

The results presented in this paper may contribute significantly to our understanding of the apparent differential growth of the fibula in comparison with the tibia. Three basic questions require to be answered. Firstly, why is the fibula

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Fig. 13. Whole mount of a control 10-day embryonic hindlimb.

Fig. 14. Whole mount of 10-day embryonic hindlimb after barrier insertion at stage 20. Note the split femur and the distally displaced fibula, which has still retained its distal epiphysis.

Fig. 15. Whole mount of 10-day embryonic hindlimb after barrier insertion at stage 22. Again the distal epiphysis (de) has failed to separate from the split fibula (f).

Fig. 16. Whole mount of 10-day embryonic hindlimb after barrier insertion at stage 22. The distal epiphysis of the fibula can be seen to articulate with the tarsal elements (arrow).

Fig. 17. Whole mount of 10-day embryonic hindlimb after polarizing region graft to the anterior margin performed at stage 18. Note the shortened appearance of the tibia (t). In contrast, the fibula (f) is elongated and possesses its distal epiphysis (de) which has also fused with the tibia to form the tibiotarsus.

Fig. 18. Whole mount of 10-day embryonic hindlimb after polarizing region graft to the anterior margin performed at stage 18. In the lower half of the limb the fibula has been reduplicated. Both reduplicates possess distal epiphyses which articulate with the toes. The upper half of the limb possesses a tibia and elongated fibula.

Figs 19 & 20. Whole mounts of 10-day embryonic limbs after polarizing region grafts to the anterior margin at stage 20. Only reduplication of the toes has occurred. However, the fibulae in both cases are elongated, the distal epiphyses of which appear to articulate with the reduplicate toes (arrowed).
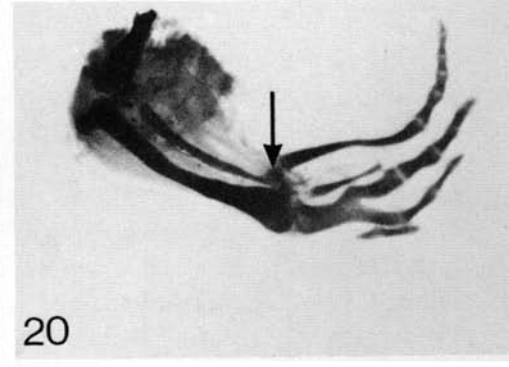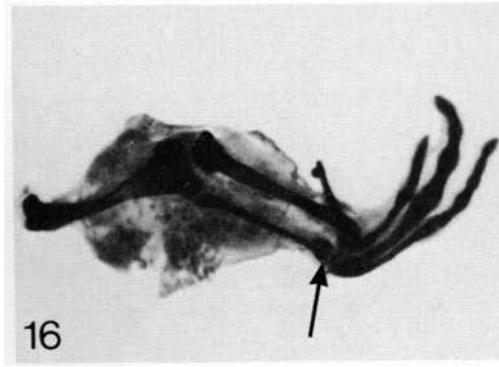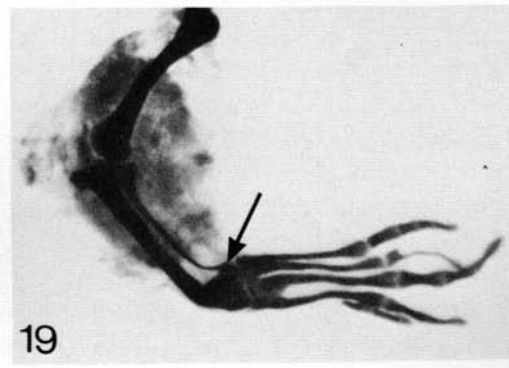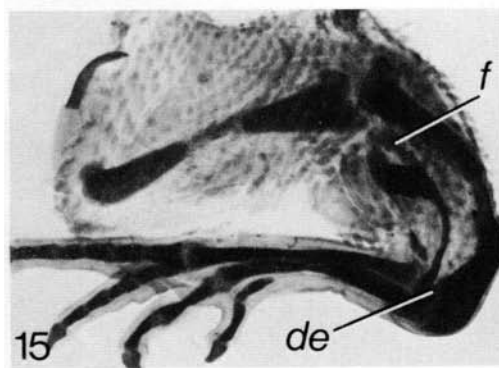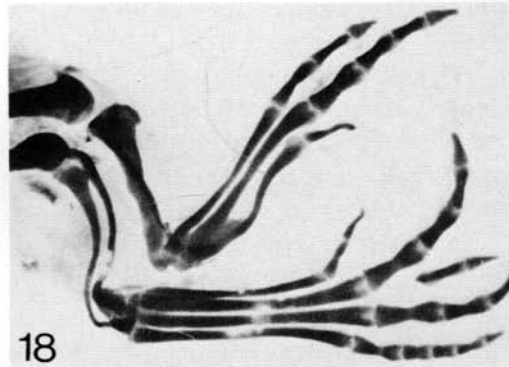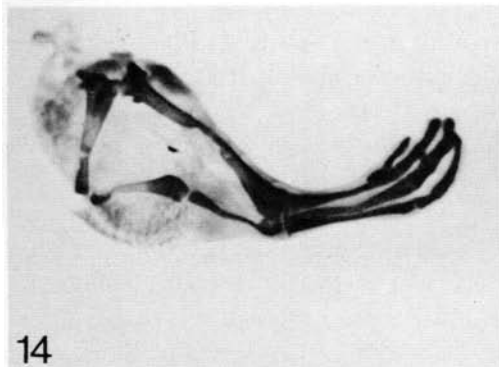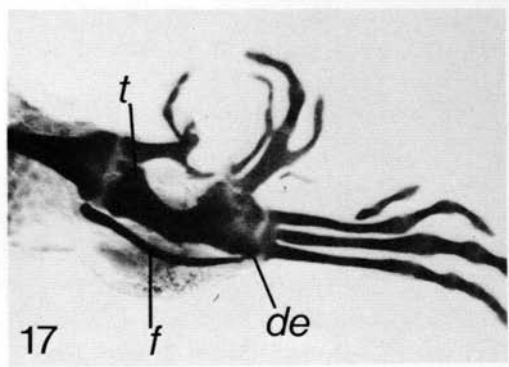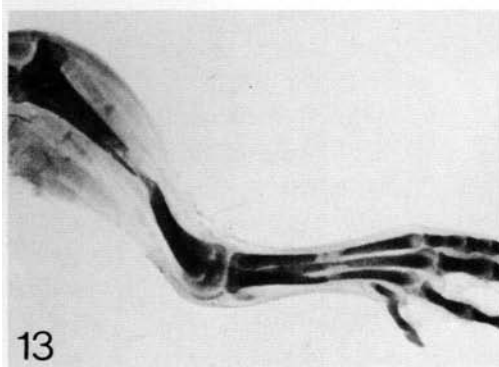
shorter than the tibia, secondly, why is there such a discrepancy in the diameters of the two elements in the young or adult fowl, and thirdly, why did Hampé obtain an elongated fibula?

From our results we would like to suggest that the failure of the fibula to grow to the same length as the tibia is due to a combination of intrinsically controlled events occurring in some cells of the fibula as well as the interaction of the tibia with the distal end of the fibula. Our histological observations show that initially the fibula possesses a 'normal' distal epiphysis which becomes detached and fuses with the tibia to form the fibulare of the tibiotarsus. Previously it has been regarded as a separate tarsal element (Hinchliffe, 1977). By stage 28 flattened cells in the fibula secreting little matrix are clearly visible and these demarcate the position where the epiphysis will separate from the diaphysis. The asymmetric outgrowth of the distal tibial epiphysis which joins with the distal epiphysis of the fibula may provide the mechanical stresses necessary for the separation. These would allow the subsequent fusion of the fibulal distal epiphysis (fibulare) to the tibia to form part of the tibiotarsus. This interpretation raises questions about the cellular basis of the protrusion from the tibia and its fusion with the epiphysis of the fibula.

Further evidence for such an interpretation comes from the position of the centres of ossification. In the tibia this is in the centre of the element. In the fibula, the centre of ossification is adjacent to that of the tibia but is eccentric – apparently displaced towards the distal end. However, its position is in fact central when viewed with respect to the element before detachment of the distal epiphysis.

It is noticeable that between days 6 and 7 (before separation) the fibula is longer than the tibia (Fig. 4). We do not know why this should be, but the relative increase in length occurs concomitantly with onset of the reduction in the number of cells across the diameter of the rudiment (Table 2). One possible reason for this is that the perichondrium may be acting as a constraining sheath around the cartilage, limiting circumferential expansion but favouring longitudinal growth (Wolpert, 1982). In the case of the fibula, the perichondrium may exert greater constraint than the perichondrium of the tibia and hence lead to enhanced longi-tudinal expansion via greater cell displacement or rearrangement.

Earlier work concerning the differential growth of the tibia and fibula (Hicks, unpublished data) concentrated solely on the synthetic, proliferative and cellular growth of the respective cartilage rudiments. He concluded that the only sig-nificant difference between the tibia and fibula was in the degree of chondrocyte hypertrophy (which could contribute to length and/or width of an element) but this difference was marginal compared with the 'differential growth' of the rudi-ments *in vivo* and *in vitro*. Therefore, it was clear that it was unlikely that these facets were major contributory factors towards the 'differential growth' process.

From our histological observations it appears that the variations in the diameter of the two skeletal elements is a result of differential osteogenesis rather than chondrogenesis. There are no obvious explanations why osteogenesis

should be so limited in the fibula, but the absence of appreciable cartilage resorption between stages 32–38 may be significant and possibly be related to the degree of periosteal vascularization. Differential vascularization may also explain why bony trabeculae fail to develop in the fibula although periosteal proliferation may also be very important but to date it is still not clear what factors promote or inhibit bone formation.

From the above account we would like to offer an alternative explanation to the classical experiment of Hampé (1960) and repeated by ourselves. Hampé proposed that insertion of a mica barrier into the hindlimb bud blocked the recruitment of mesenchyme cells from the fibula blastema to the tibia blastema. We would like to suggest that barrier insertion at the appropriate stages (22/23), prohibits morphogenesis and prevents the separation and subsequent fusion of the fibula distal epiphysis to the tibia. Often, the failure of the separation process is accompanied by the bifurcation of the femur which displaces the fibula distally. The resulting element is no longer than the contralateral control (Fig. 14). This phenomenon has also been described by other workers (Wilson, 1980). Occasionally, however, distal displacement does not take place and an elongated fibula results (Fig. 16).

A similar argument of impaired morphogenesis may also account for the elongated fibulae which can occur after polarizing region grafts. Fibula elongation and the retention of its distal epiphysis was most marked when the entire tibial element was missing, as in reduplicates of both the stylopod and zeugpod (Fig. 18) or when, tibial elongation was retarded. The failure of the tibiotarsus to elongate normally also occurs in the diplopodia mutant (dp^4) (Kieny & Abbott, 1962). These authors report the incident of fibulae which have the ability of accelerated growth *in vitro* and they also possess two epiphyses. Such a response would be expected in light of the proliferative centre which epiphyses represent in developing long-bone rudiments.

In summary, the discrepancy in size between the tibia and fibula in the mature chick is a result of two distinct processes. First, the difference in diameters of the two skeletal elements can be explained in terms of differential osteogenesis rather than chondrogenesis. Second, the truncation in growth of the fibula results from the loss of the distal epiphysis, which fuses with the distal end of the tibia to form the fibulare of the tibiotarsus. The specification of a region of cells which secrete little cartilage matrix in the fibula, and which subsequently represents a 'hinge' or 'weak spot' thus allowing the detachment of the distal epiphysis is an important step in the development of both the tibia and fibula. Failure of the distal epiphysis to separate allows the fibula to reach a comparable length to that of the tibia. Viewed in these terms the development in length of the tibia and fibula results from a combination of programmed chondrogenic growth and interactive morphogenesis between the distal portions of both elements. Such an interpretation discounts both competitive or differential chondrogenic growth programme mechanisms.

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