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Mechanisms of pigment pattern formation in the quail embryo

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Summary

One hypothesis to account for pigment patterning in birds is that neural crest cells migrate into all feather papillae. Local cues then act upon the differentiation of crest cells into melanocytes. This hypothesis is derived from a study of the quail-chick chimaera (Richardson *et al., Development* 107, 805–818, 1989). Another idea, derived from work on larval fish and amphibia, is that pigment patterns arise from the differential migration of crest cells. We want to know which of these mechanisms can best account for pigment pattern formation in the embryonic plumage of the quail wing. Most of the feather papillae on the dorsal surface of the wing are pigmented, while many on the ventral surface are white.

When ectoderm from unpigmented feather papillae is grown in culture, it gives rise to melanocytes. This indicates that neural crest cells are present in white feathers but that they fail to differentiate. If the wing tip is inverted experimentally then the pigment pattern is inverted also. This is difficult to explain in terms of a model based on migratory pathways, unless one assumes that the pathways became re-routed.

When an extra polarizing region is grafted to the anterior margin of the wing bud, a duplication develops in: (1) the pattern of skeletal elements; (2) the pattern of feather papillae; (3) the feather pigment pattern. The pigment pattern was not a precise mirror image although some groups of papillae showed a high degree of symmetry in their pigmentation. Both the tip inversions and the duplications produce discontinuities in the feather and pigment patterns. No evidence of intercalation was found in these cases. We conclude that pigment patterning in birds is determined by local cues acting on melanocyte differentiation, rather than by the differential migration of crest cells. Positional values along the anteroposterior axis of the pigment pattern are determined by a gradient of positional information. Thus the pigment patterns, feather patterns and cartilage patterns of the wing may all be specified by a similar mechanism.

Key words: quail, embryo, pigmentation, melanin, feather, pattern formation, positional information, polarizing region, limb.

Introduction

The pigment patterns of bird embryos are produced by melanocytes that arise from unpigmented precursor cells in the neural crest (reviewed by Le Douarin, 1982). We want to understand how the behaviour of these migratory cells can lead to a localized pattern of pigmented and unpigmented feathers. In particular, we are interested in whether crest cells are absent from white feathers, or whether they are present but fail to differentiate. The quail embryo is a good subject for this type of study because it has a well-defined pigment pattern in its wing plumage (Richardson *et al.* 1989). Most of the dorsal feather papillae are heavily pigmented, while most of the ventral ones are white (Fig. 1).

Some insight into how this pattern could arise has been provided by our study of the quail–chick chimaera (Richardson *et al.* 1989). We showed that a graft of quail neural crest cells produced a pigment pattern in the chick host that bore little resemblance to the normal quail pattern. This, together with other findings, led us to suggest that quail cells were responding to local cues in the feathers of the host. We showed that crest cells migrate into all the feathers of the chimaera, but in unpigmented feathers they fail to differentiate into melanocytes. The aim of this study is to see whether our model can account for pigment pattern formation in the normal quail embryo.

Our model assumes that local cues in the feathers are acting upon the differentiation of a uniformly distributed population of neural crest cells. Another view of pigment patterning is based upon the differential migration of crest cells rather than upon the spatial organization of their differentiation. It has been suggested (Watterson, 1942) that neural crest cells are unable

to migrate into those papillae that are destined to form unpigmented feathers. This suggestion was not supported by Cohen (1959), who found that the white feathers of the adult Light Sussex fowl contained melanoblasts; or by our study of the quail–chick chimaera (Richardson *et al.* 1989) in which we found crest cells in unpigmented papillae.

Differential migration could also act at the level of neural crest pathways. This idea comes from recent studies of larval fish and amphibians, where the early migratory pathways of crest cells are thought to be important in establishing local variations in pigmentation (Epperlein and Claviez, 1982; Epperlein and Löfberg, 1984; Löfberg *et al.* 1989; Trinkaus, 1988). These larval forms develop pigment at a very early stage of development: shortly after the onset of neural crest migration. Bird embryos do not show skin pigment as early in development as this. The crest cells in birds migrate from the neural tube at the wing level between embryonic stages 13 and 22 (Serbedzija *et al.* 1989), and enter the wing bud itself at stage 21 (Fox, 1949; Richardson *et al.* 1989). Yet pigment does not appear in the wing until much later – around stage 35 (Richardson *et al.* 1989).

According to our model, crest cells migrate into *all* regions of feather-bearing skin, and local variations in plumage pigmentation are not determined by the migratory pathways of crest cells. If this is true then any experimental procedure that disrupts the migratory pathway should have no effect on the pigment pattern. We examine this possibility by inverting experimentally the tip of wing bud.

We have no histological marker that could tell us whether or not neural crest cells migrate into the unpigmented feather papillae of the normal quail embryo. Antibodies that stain migratory neural crest cells in the early embryo do not stain mature pigment cells. For instance, HNK-1 immunoreactivity is lost from presumptive pigment cells at a relatively early stage in development (Serbedzija *et al.* 1989). In this study, therefore, we have cultured ectoderm from these unpigmented papillae in a medium that supports the differentiation of melanocytes. If neural crest cells are present in the tissue, we expect melanocytes to appear in the culture.

It is possible that local cues in the feather papillae are themselves specified by a positional information mechanism. This we have examined by grafting a polarizing region to the anterior margin of the wing bud. The polarizing region is thought to specify the positional values along the anteroposterior axis of the wing bud (Tickle *et al.* 1975, 1985); we want to see whether the pigment pattern is specified in a similar way.

Materials and methods

Tip inversions

All stages are according to Hamburger and Hamilton (1951). Japanese quail embryos (*Coturnix coturnix japonica*) were used for all experiments; they were incubated at 37°C±1°C, 100% relative humidity. Quail embryos cannot be staged accurately beyond stage 39. We therefore group the older quail together as stages 39–41. Donors were of stages 18–22, and hosts 18–24. Most of the wing bud was removed from the host, leaving behind a stump of proximal tissue (usually the prospective humerus). The tip of the wing bud from the opposite side of the donor was then grafted to this stump and held in place with platinum pins. The donor wing bud was attached so that it was inverted 180° with respect either to the dorsoventral or anteroposterior axis. Hosts were returned to the incubator and the experiment was stopped on the 11–13th day of incubation. Maps were prepared of the pigment pattern using the method of Richardson *et al.* (1989). We recorded the position of each papilla on a chart, together with a note of its pigmentation type: papillae were classified as either pigmented, unpigmented or trace of pigment.

Tissue culture

The growth medium was Ham's F-12 with glutamine, supplemented with 15% foetal calf serum and 1% antibiotic–antimycotic (all from Gibco). The medium also contained 5% embryo extract prepared from 9 to 10 day White Leghorn embryos. The complete medium was sterilised by membrane-filtration (Acrodisc, Gelman Sciences).

Wings were removed aseptically from 10 to 11 day quail embryos, and pinned out in a dish of phosphate-buffered saline (PBS). A fine tungsten needle was used to slit the longer feather papillae longitudinally, along one side, from base to tip. This helped the trypsin to penetrate the ectoderm. Smaller papillae were cut straight off the wing without being slit open. The papillae were then transferred to 2% trypsin (Gibco) in calcium- and magnesium-free PBS (Flow laboratories) at 4°C for 60 mins. The tissue was then transferred to a dish of fresh PBS. The ectoderm was freed of mesenchyme by manipulation with tungsten needles, followed by vigorous pipetting in several changes of fresh PBS. Pieces of tissue were cultured in 35 mm plastic culture dishes (Sterilin); for photography, some cultures were grown on glass coverslips placed in the bottom of the culture dish. To facilitate attachment to the substratum, Ham's F-12 with 50% foetal calf serum (both from Gibco) was used for the first 24 h, followed by growth medium changed every 2 days. Cultures were incubated at 37°C, 100% humidity, 5.5% CO_2; they were kept for 7 days.

For organ culture, feather papillae were cut from the wing and placed aseptically onto a piece of Millipore filter (0.8 μm pore size) supported by a stainless steel grid (Falcon). This assembly was placed in a 35 mm Petri dish (Sterilin) with 2–3 ml of growth medium which was changed every 2 days.

Grafts of polarizing region

Stage 18–22 quail embryos were used. Tissue from the polarizing region was removed from the posterior margin of a limb bud and grafted to the anterior margin of the wing bud of another embryo. The graft was held in place with platinum pins. Hosts were returned to the incubator, and the experiment was stopped on the 11th–13th day of incubation.

Results

Tip inversions

Twenty eight grafts were performed. Twelve of these survived and were of stages 37 and older when examined; two were severely malformed and are not included in the analysis. Detailed maps were prepared of the remaining ten cases, two of which had tips inverted

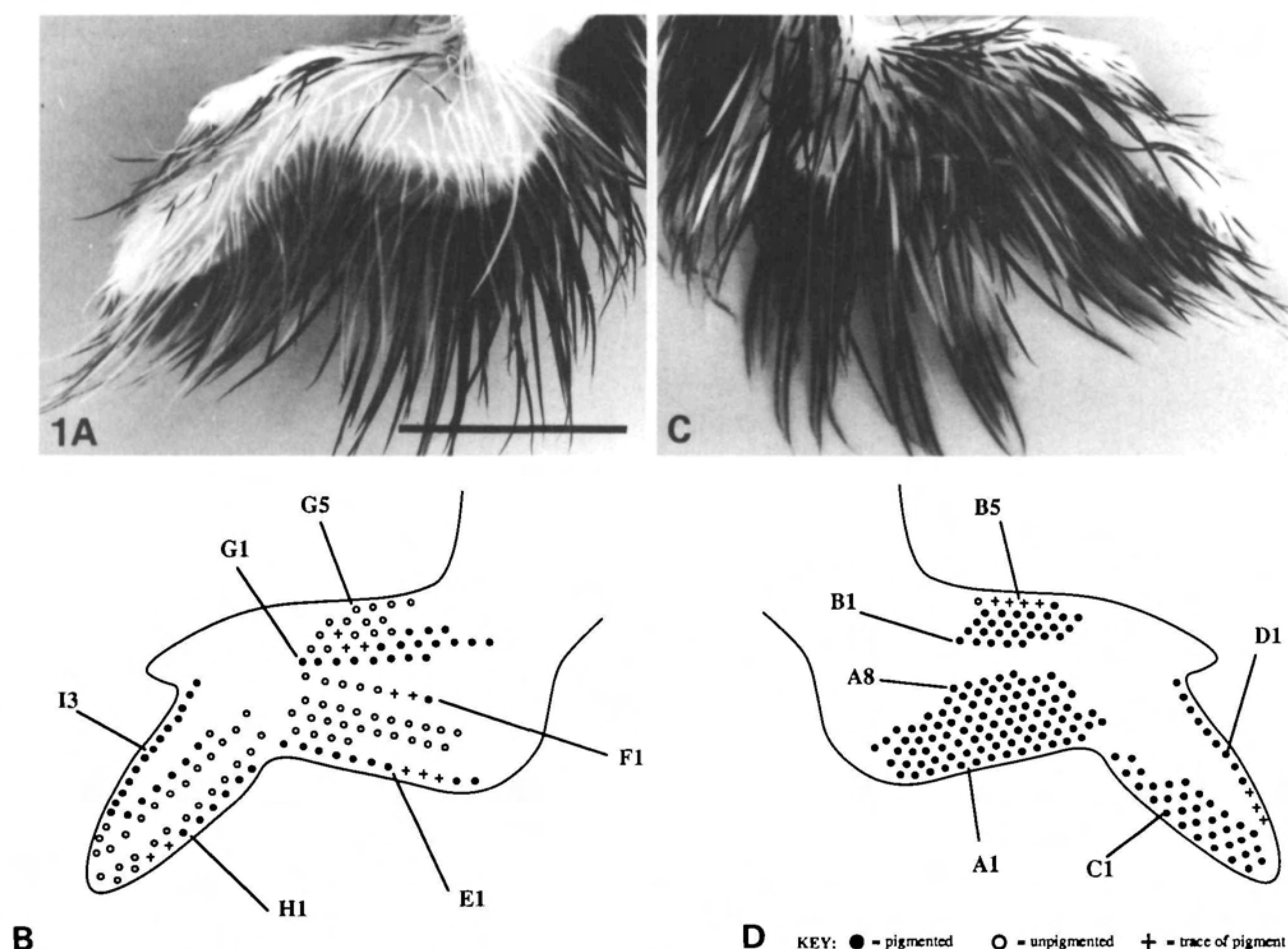


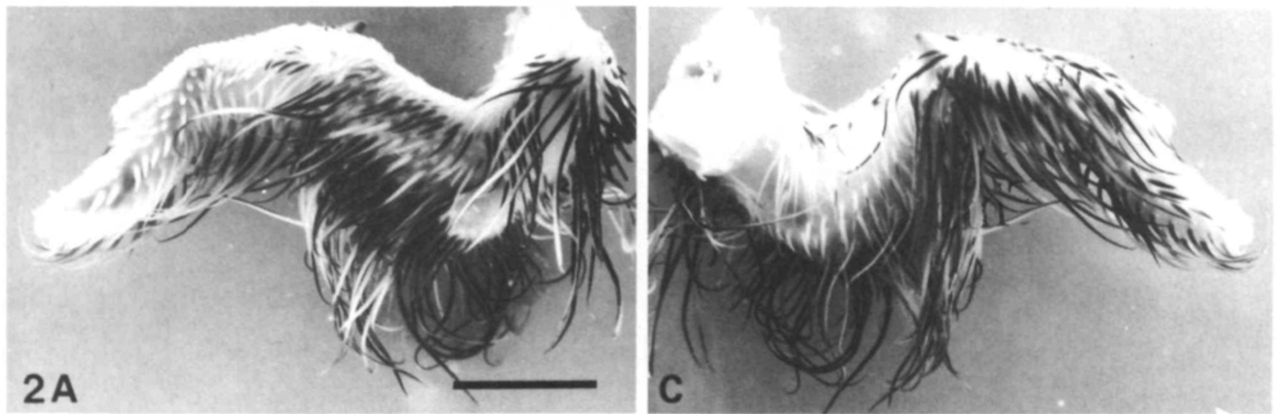
Fig. 1. Normal quail wing (about stage 40). (A and B) Ventral surface; (C and D) dorsal surface. Scale bar on A=5 mm.

about the anteroposterior axis, and eight about the dorsoventral axis. In most cases, the inverted hand was attached to the forearm of the host. In some cases, however, a hand and forearm developed from the graft and so the limb showed an extension as well as an inversion. In all cases, the inverted part developed autonomously with respect to the arrangement of the skeletal elements, the feathers and the pigment pattern. The orientation of these three features always conformed to the original orientation of the tip on the donor, and not its new orientation on the host. This can be seen quite clearly by comparing Fig. 1 (a normal quail wing) with Fig. 2 (a wing with a dorsoventral tip inversion and an extension). Striking discontinuities were often seen between the feather patterns of the donor and host parts. In Fig. 2 for example, dorsal feathers (A rows) are next to ventral ones (E, F and G rows). We found no evidence of intercalation across these discontinuities.

Tissue culture

Twenty two cultures of ectoderm from unpigmented feather papillae were established. The tissue came from rows E3 and H2 of stage 36–38 quail; these rows are always unpigmented in our strain of quail. In fifteen of these cultures, the explant became attached to the dish and formed an outgrowth within 24 h. The outgrowths were always sparse – around 10–20 cells in each. Melanocytes were seen in ten cultures, and they usually made their first appearance on the 3rd day. In many of the cultures, every cell in the outgrowth was a melanocyte by the 7th day (Fig. 3). This indicates that it is not ectodermal cells but crest cells that migrate from the explant. In seven cases, the ectoderm rounded up into a ball and failed to attach. These pieces of ectoderm remained unpigmented except in two cases where meanocytes appeared on the surface of the ball of tissue (Fig. 4).

When the mesenchyme from unpigmented feather papillae (stages 36–38) was cultured, it behaved quite differently from the ectoderm. It attached readily and formed a large outgrowth (Fig. 5); this consisted of densely packed, multilayered, fusiform cells in the centre, with large, polyhedral, migratory cells at the periphery. Twelve of these cultures were successfully established from unpigmented papillae in rows E2 and H2; none produced any melanocytes (the experiment was stopped after 7 days of culture). Four cultures of mesenchyme from pigmented papillae were obtained. Three remained unpigmented (Fig. 6) and resembled cultures of mesenchyme from white papillae while in



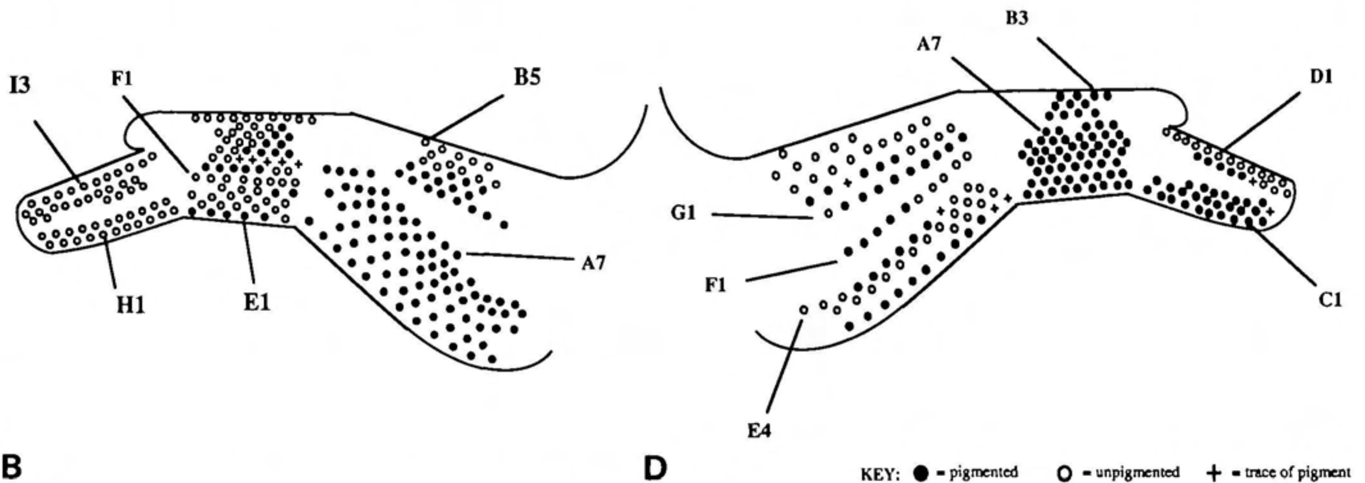


Fig. 2. Quail wing (about stage 39) with a tip inverted 180° about the dorsoventral axis. The host tissue has formed an upper arm and forearm, while the donor has formed a forearm and hand. (A and B) Host's dorsal surface; (C and D) host's ventral surface. Note that the pigment pattern of the inverted tip has developed autonomously. (E) Pattern of skeletal elements; dashed line indicates the junction between donor and host parts. Scale bar on 2 (A)=3.5 mm.

one culture, two melanocytes became visible on the 6th day of culture.

Twelve unpigmented, stage 38 quail papillae (E3, H2) were established in organ culture for a week. All were whole, untrypsinised papillae. None developed any pigmentation, although a few melanocytes were occasionally seen at the periphery of the mesenchyme that grew out from the cut end of the papilla.

Grafts of polarizing region

Of 20 grafts performed, 16 survived, and 10 of these showed duplications. All 16 wings, which were of stages 37 or older, were stained for cartilage and cleared. In no case was a duplication seen in the cartilage pattern without a corresponding duplication in the feather pattern, and *vice versa*. The arrangement of feather papillae on a duplicated part corresponds with the duplication of the underlying skeletal elements (c.f. Saunders and Gasseling, 1968; Iten *et al.* 1983). For instance, we find that when an extra ulna is present, an extra set of flight feathers (normally found on the skin covering the ulna) is also present; furthermore, the pigmentation of these extra feathers is appropriate to their regional type.

The pigment pattern of the duplicated part is always entirely quail-like; no novel patterns are seen. A few rows of feathers close to the midline of the wing may show a high degree of symmetry in their pigment patterns (Fig. 7F,G); most of the feathers, however, do not. We must distinguish at this point between primary patterns (made up of pigmented and unpigmented feathers) and secondary patterns (patterns within single feathers). Our grafts show that duplications in pigment patterns, both primary and secondary, are not perfect mirror images because differences can be found between the anterior and posterior patterns on the same wing. This variability is the same as the variability in pigment patterns between normal quail wings.

The wing shown in Fig. 7 has two hands. When the feathers on these two hands are mapped, it is quite clear

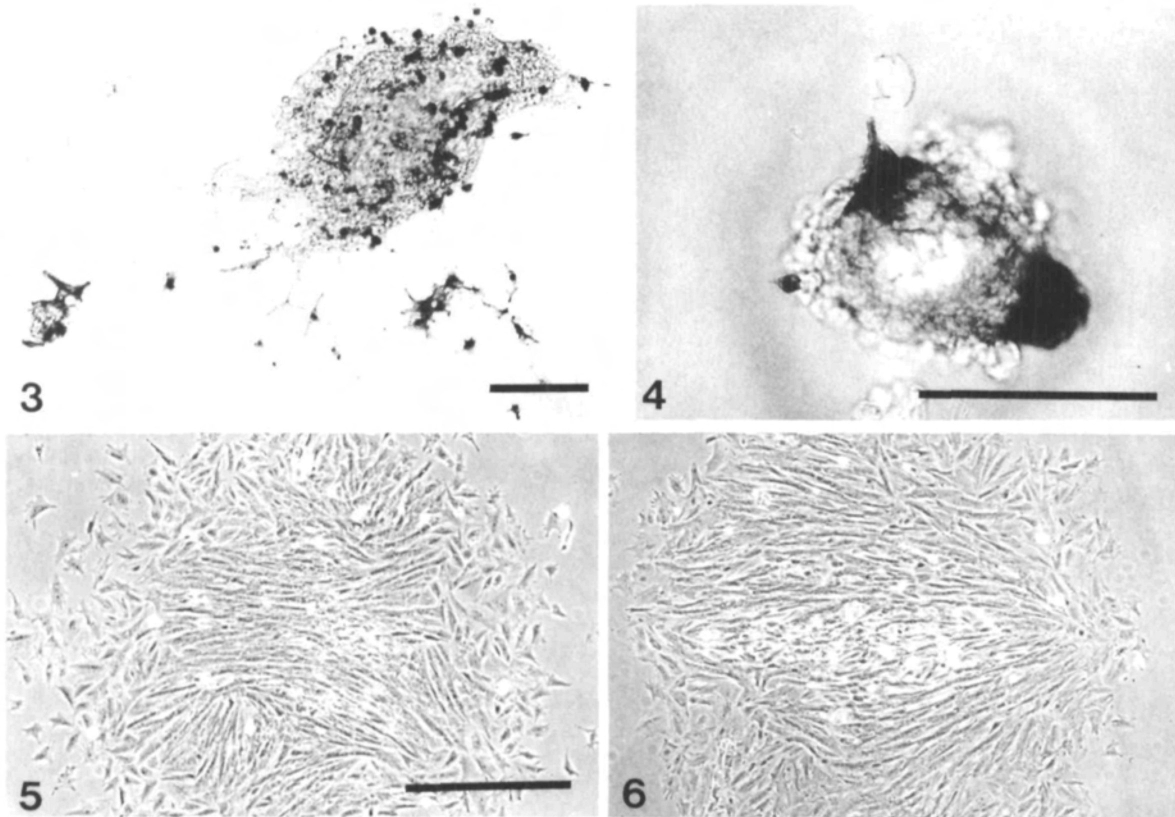


Fig. 3. Ectoderm, after 4 days in culture, from an unpigmented feather papilla (row E3, normal quail, stage 37). Scale bar=100 μm.
Fig. 4. Ball of ectoderm, which failed to attach to the dish, from an unpigmented papilla (row E3, normal quail, stage 37). Fourth day of culture; two large melanocytes have appeared on the surface together with one smaller one. Scale bar=100 μm.
Fig. 5. Mesenchyme from an unpigmented papilla (row E3, normal quail, stage 37) after 4 days in culture. No melanocytes have appeared. Scale bar=300 μm.
Fig. 6. Mesenchyme from a pigmented papilla (from the dorsal surface of a normal quail wing, stage 37) after 4 days in culture. No melanocytes have appeared. Scale as in Fig. 5.

that the primary pigment pattern is not a mirror image; compare, for instance, row I3 in these two hands (Fig. 7B). However, on the ventral surface of this wing, at the line of union between the two halves of the wing, a small group of feathers is seen in which the pigment patterns are a mirror image (Fig. 7F and 7G). Note also that the hexagonal packing of the feathers is preserved along the line of union.

Fig. 8 shows a hand which has the digit pattern **4334** (Fig. 8B); it is, structurally, a mirror image. However, when the *secondary* pigment patterns in the feather papillae of this part are examined (Fig. 8C) they lack mirror symmetry. In one wing from this series of grafts, there was no duplication. The wing was, however, interesting in another respect. The radius failed to develop. All the feathers normally associated with the radial margin of the wing – namely those of the web (prepatagium) – are missing. This results in a striking discontinuity whereby rows G1 and A7 lie next to each other. No intercalation has taken place between these two rows.

Discussion

Our findings do not support a model of pigment patterning based on the differential migration of neural crest cells. Such a model is suggested by studies on pigment patterns in larval fish and amphibia. If migratory pathways were important in pigment patterning in birds, then disrupting those pathways by inverting the wing tip ought to disrupt the pigment pattern. We find, however, that an inverted tip shows no disruption of its pigment pattern. Although this is not a stringent test for differential migration, it is difficult to explain in terms of pathways unless one assumes that the pathways become re-routed.

Our results provide persuasive evidence that the ectoderm of unpigmented feather papillae contains crest cells; when this ectoderm is grown in culture, it gives rise to melanocytes. This is contrary to the suggestion of Watterson (1942), but is in line with our study of the quail–chick chimaera, where we found undifferentiated quail crest cells at the apex of the barb

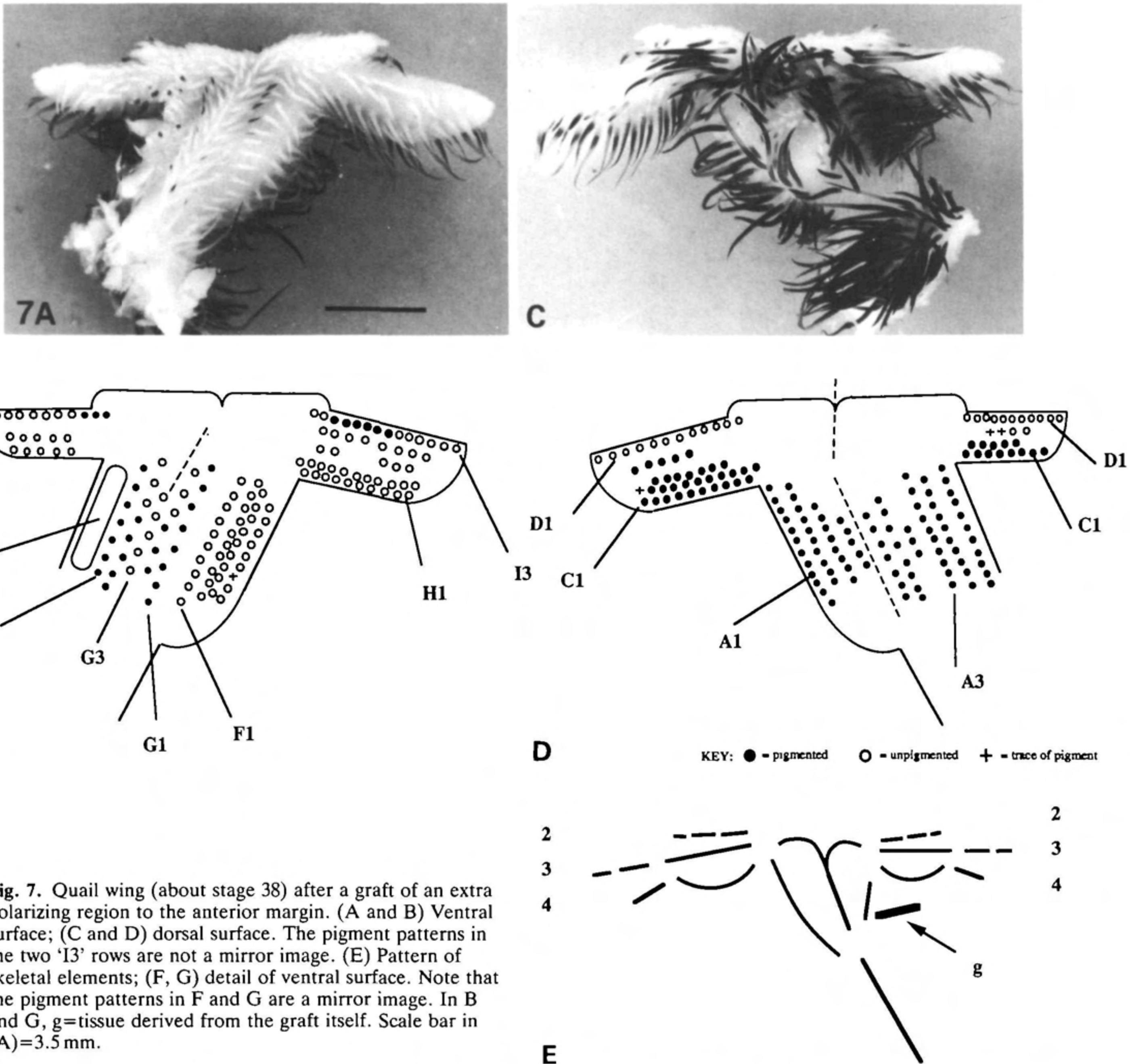
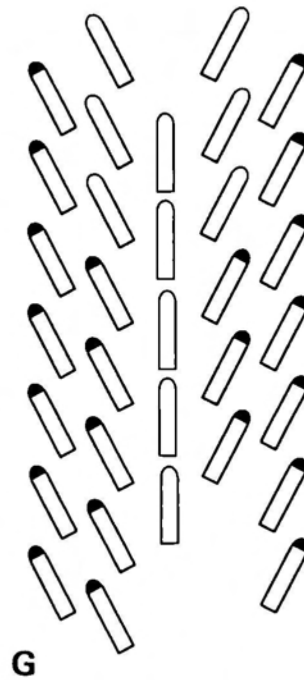


Fig. 7. Quail wing (about stage 38) after a graft of an extra polarizing region to the anterior margin. (A and B) Ventral surface; (C and D) dorsal surface. The pigment patterns in the two 'I3' rows are not a mirror image. (E) Pattern of skeletal elements; (F, G) detail of ventral surface. Note that the pigment patterns in F and G are a mirror image. In B and G, g=tissue derived from the graft itself. Scale bar in (A)=3.5 mm.

ridges of white feather papillae (Richardson *et al.* 1989). In that study we also found that white feather papillae from the normal quail did not contain melanoblasts. This was shown by their lack of DOPA-staining (DOPA or dihydroxyphenylalanine stains black any cell that contains tyrosinase, the enzyme of melanin synthesis). Together with the findings reported here, this suggests that the neural crest cells in white feathers cannot have differentiated very far along the melanocyte pathway. The fact that whole, unpigmented papillae remain unpigmented when grown in organ culture could indicate that either the mesenchyme or ectoderm of these feathers inhibits melanocyte differentiation.

In some of our cultures of ectoderm from white papillae, all of the cells that grow out from the explant become melanocytes. This suggests that those crest cells populating the ectoderm of unpigmented papillae are committed to the melanocyte lineage (to be sure of this we would have to show that our medium was capable of supporting the differentiation of other neural crest cell-types). This commitment may take place much earlier in development; Sieber-Blum (1989) has suggested that cells on the dorsolateral pathway in young embryos may already be committed to the melanocyte pathway. Indeed, these could be the cells that give rise to pure melanocyte colonies in clonal cultures of quail neural crest (Sieber-Blum, 1989).

Neural crest cells are absent from the mesenchymal core of all feather papillae in the quail–chick chimaera (Richardson *et al.* 1989). This is in accord with the findings in this paper; mesenchyme from unpigmented



papillae never produced melanocytes in culture; mesenchyme from pigmented papillae produced a few melanocytes in one culture out of four. Taken together, these results could indicate that crest cells are rare or absent in the feather mesenchyme. Whether this is because they fail to migrate into this tissue, or because of local cell death, remains to be determined. An alternative explanation could be that melanocytes failed to differentiate in our mesenchyme cultures because they require contact with the ectoderm.

Our model says that the special local properties of feathers control the differentiation of melanocytes. If this is the case, then the question arises as to how this pattern of local properties is specified. The grafts of polarizing region give rise to two sets of quail pigment patterns on the same wing, one of them having its anteroposterior axis reversed with respect to the other. There is a large amount of evidence that indicates that a result such as this, in which a duplication of pattern is present in a wing, can be explained by a positional information mechanism that specifies the anteroposterior axis of the pattern (Tickle *et al.* 1975, 1985). In most respects, the pigment pattern is not a precise mirror image because although each of the two patterns is entirely quail-like, they are not identical to each other. Only in a small group of feathers is the pigment pattern sometimes a perfect mirror image: those feathers that lie close to the line of union between duplicated and normal parts of the wing.

In our duplications, the feather pattern is always appropriate to the pattern of the underlying skeletal elements, and the pigment pattern is always appropriate to regional type of the feather. This indicates that the anteroposterior polarity of the feather and pigment patterns are determined by the same positional information mechanism that specifies the anteroposterior sequence of the skeletal elements. This does not mean that the gradient of positional information acts directly upon the pigment pattern; it could be that only the feather patterns are specified this way, and the pigment pattern then develops secondarily according to the feather pattern.

Three factors contribute to the lack of symmetry in the duplicated pigment patterns and it is important to consider them when looking at the data. Firstly, the duplicated parts are, in developmental terms, younger than the normal parts. This means that the duplicated feather papillae are slightly smaller than their normal counterparts and are therefore in a slightly earlier phase of pigmentation (compare the lengths of the feather germs on the two hands in Fig. 7 for instance). This age difference is considerably less than one Hamburger–Hamilton stage. Secondly, when two hands are present on the same wing, the duplicated hand is often shorter and thinner than its normal counterpart (Fig. 7). This effect is quite independent of the difference in developmental age between the two hands; the feathers on the duplicated hand are not simply shorter than normal; there are fewer of them indicating that the field is smaller. This confirms previous findings in which a reduction in the size of the field resulted in fewer rows of feathers, not in greater crowding (McLachlan, 1980).

There is another feature of the duplicated wings that could confuse us. No clear demarcation exists between the duplicated part and the more *proximal*, normal parts; there is instead an ill-defined transition. This

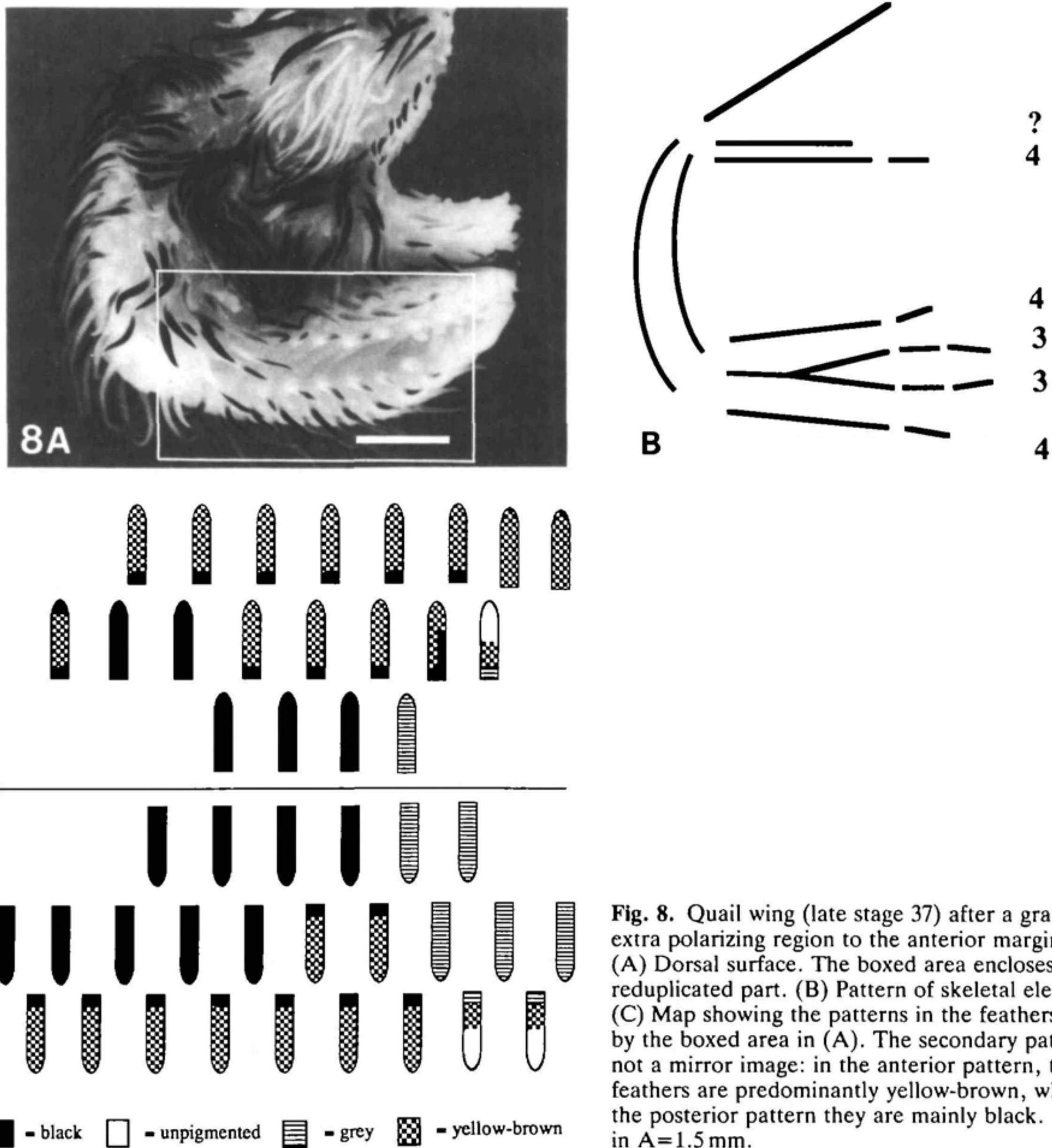


Fig. 8. Quail wing (late stage 37) after a graft of an extra polarizing region to the anterior margin. (A) Dorsal surface. The boxed area encloses the reduplicated part. (B) Pattern of skeletal elements. (C) Map showing the patterns in the feathers enclosed by the boxed area in (A). The secondary patterns are not a mirror image: in the anterior pattern, the feathers are predominantly yellow-brown, whereas in the posterior pattern they are mainly black. Scale bar in A=1.5 mm.

transitional zone has a very irregular arrangement of feather papillae which must not be thought of as belonging to the duplicated part.

When these three factors are allowed for, one still finds differences between the normal and duplicated pigment patterns, which can only be explained as being due to lack of precision. We believe that the differences between the two patterns in Fig. 8C are not due entirely to differences in developmental age or to a transition between the duplicated and normal, proximal parts.

We believe that two entirely different mechanisms may be involved in pigment patterning in vertebrate embryos. In larval amphibians and fish, it is thought that differential migration gives rise to local variations in pigmentation (Epperlein and Claviez, 1982; Epperlein and Löfberg, 1984; Trinkaus, 1988; Löfberg *et al.* 1989). In birds, we believe that local cues in the feather papillae act on a uniformly distributed population of crest cells to control their differentiation. These local cues constitute a prepattern and, in the wing, at least the anteroposterior axis of this prepattern is determined by positional information.

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