# Experimental analysis of the extensive pigmentation in the Silkie fowl embryo: evidence for an environmental regulatory process

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*Summary.* Heterospecific coelomic grafts, associated with the quail-chick marker system, showed that quail embryo melanoblasts exhibit the same invading behavior as Silkie fowl melanoblasts, when they came into contact with Silkie embryo organs. Thus the colonization or noncolonization of the organs of the Silkie fowl embryo by melanoblasts seems to depend on environmental cues.
*Key words.* Quail embryo; Silkie fowl; coelomic grafts; melanoblasts; quail-chick marker system; pigmentation; environmental regulation.

The factors and mechanisms involved in the migration of neural crest cells have focused the attention of numerous authors[1,2]. Whatever their pathway, neural crest cells migrate in cell free spaces, along or between the fibronectin-rich basement membranes of epithelia[3] and do not enter tissues, even though they are fibronectin-rich, until they reach their sites of arrest[4]. Such is the case for peripheral nervous progenitor cells and for premelanoblasts at least in numerous bird species (quail and different pigmented fowl strains, for example). In these species, melanocytes differentiate in muscle connective septa, in the mesenteries and peritoneum and are encountered in the vicinity of viscera and along nerves and blood vessels. However, in some species, premelanoblasts enter some tissues and organs and in Silkie fowl colonize most of them: muscle, different parts of the gut, lung, kidney, testis and ovary, for example. This pigmentation, which occurs in the connective component of most organs, has been named 'mesodermal pigmentation'[5]. This species offered a good opportunity to study the cues determining this extensive pigmentation, and to test the two following hypotheses. According to the first one this phenomenon depends on particular intrinsic invading capacities of Silkie melanoblasts, and according to the second, the seeding process of melanoblasts is determined by environmental cues.

*Materials and methods.* The experiments were performed in white Silkie fowl and Japanese quail embryos. The study of Teillet[6] showed that quail melanoblasts settle in great numbers in the epidermis on the 5th and 6th day of development and appear to migrate until the 11th day. As was expected, preliminary experiments proved that, by the 3rd day of incubation, quail internal melanoblasts which went up the ventral route[2,7,8] were still able to move and enter different kinds of tissue and organs grafted into the coelomic cavity.

Whole organ rudiments or fragments were excised from 21 Silkie fowl embryos, 5–5.5 days old, and grafted into the posterior part of the coelomic cavity of 3.5-day quail embryos. The excision was performed just prior to or at the onset of the seeding process of melanoblasts into the internal organs[9]. Whole post-umbilical intestine, caeca and spleen where grafted. Pre-umbilical intestine, rectum, lungs, mesonephros and gonads were divided into anterior and posterior fragments. Three or four fragments of gizzard, liver and heart were excised and grafted.

The grafts were recovered after 8–9 days, fixed by immersion in Bouin-Holland fluid and embedded in paraffin. Sections were cut at 5 µm and stained according to the Feulgen-Rossenbeck procedure, which makes it possible to distinguish quail and chick cells[10].

*Results and discussion.* The grafts corresponding to 8–10 organs were recovered and studied after Feulgen-Rossenbeck treatment.

All the anlagen of Silkie fowl embryo organs, which are normally populated by pigment cells: gizzard (fig. 1), post-umbilical intestine, rectum (fig. 2), mesonephros, caeca (fig. 3), lung (fig. 4), testis (fig. 5) and ovary were colonized by quail pigment cells when grafted into the quail embryo coelome. In the different organs, some cells exhibiting the quail nuclear marker, presumably melanoblasts, were devoid of pigment. In different quail embryos the grafted Silkie caecum developed in the vicinity of the host caeca. The grafted caecum which was populated and surrounded by melanocytes appeared black, in contrast to the host ones which remained white. Generally the grafts developed from the pre-umbilical intestine of Silkie fowl embryos exhibited quail melanocytes only at their periphery (fig. 6). However in some cases melanocytes were also observed within the submucosa. As reported by Kuklenski[11], and as we have also observed, the pigmentation of intestine developed in situ is irregular and in addition exhibits individual variations.

Those Silkie fowl organs which are devoid of pigmentation in situ (liver, spleen, heart) were never colonized by melanoblasts when grafted into the coelomic cavity of quail embryos (figs. 7, 8). Quail melanocytes were observed at the periphery of the grafted tissue (fig. 7). In some cases, they enter a short distance into the organ, following the perivascular tissue. These results are in agreement with previous findings obtained by means of grafting segments of quail neural tube including neural crest into white Silkie embryos[12].

Different authors[2,4] have underlined the particular migrating capacities of melanocytes and their progenitor cells. When injected into blood vessels, they migrate through the vessel wall and reach their definite localization in the skin of the host[13]. Kahn and Sieber-Blum[14] have shown that, among the progenitor cells of neural crest, those committed to the melanogenic pathway attain the competence for terminal differentiation prior to the adrenergic progenitors. Thus, competence seems to be acquired at the in vivo onset of migration. These data support the view that melanoblasts' migration capacities could be correlated with their commitment[4], uncommitted neural crest cells remaining only able to follow the well defined pathways of migration.

The studies of Le Douarin and Teillet[15] have shown that quail neural crest cells, when associated with hind gut mesoderm, gave rise to enteric ganglia and to numerous pigment cells distributed in two layers on each side of the circular muscle. Thus hind gut allows melanocyte colonization and differentiation when prospective pigment cells are experimentally introduced into this organ. However, these authors specified that in chick embryos isotopically grafted with quail neural crest primordium, quail pigment cells were never observed in the gut. Likewise, melanocytes do not enter the gut or other organs, despite the fact that they are encountered in their vicinity in pigmented chick species and the quail.

In situ, contrary to melanoblasts and melanocytes of most other species, those of Silkie fowl colonized many tissues and organs. The experiments we designed showed that Silkie fowl melanoblasts do not exhibit particular high invading capacities when compared to those of the quail. Quail melanoblasts, when they came into contact with grafted Silkie fowl embryo tissues, colonized the same tissues which were invaded in situ by the Silkie melanoblasts. So invasiveness seems to be a general characteristic of prepigment and pigment cells as a

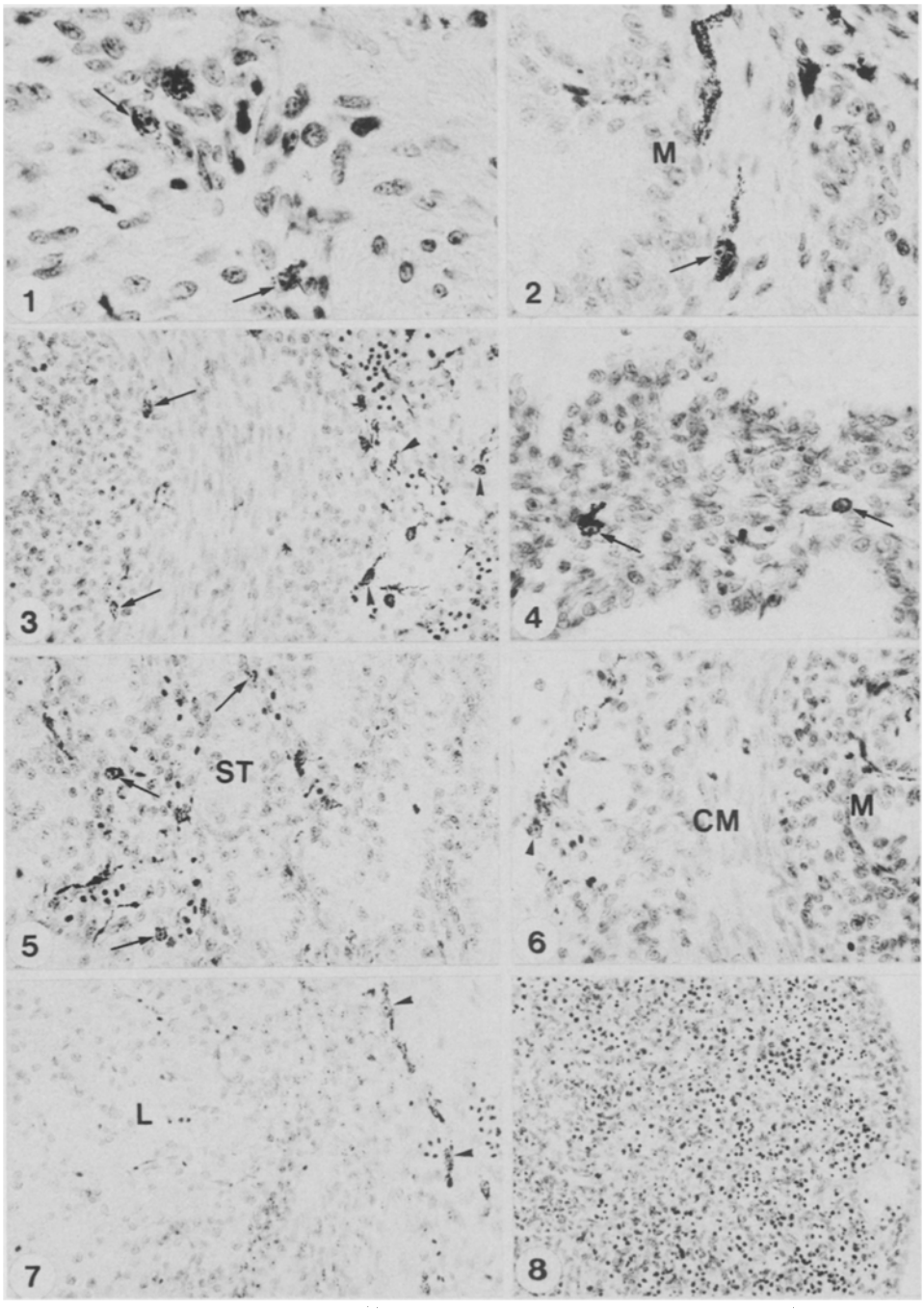


Figure 1. Section through a fragment of the gizzard of a 5.5-day Silkie fowl embryo grafted into the coelomic cavity of a 3.5-day quail embryo during 8 days. Quail melanocytes have populated the gizzard muscle and differentiated into melanocytes (arrows). Feulgen and Rossenbeck staining. × 550. Figure 2. Section through the anterior half of the rectum of a 5-day Silkie fowl embryo grafted into the coelomic cavity of a 3.5-day quail embryo during 9 days. Melanocytes are observed close to the mucosa (M). The arrow indicates a melanocyte exhibiting the quail nuclear marker. Feulgen and Rossenbeck staining. × 560. Figure 3. Section through a caecum developed from a caecum anlage provided by a 5-day Silkie fowl embryo and grafted into the coelomic cavity of a 3.5-day quail embryo. Quail melanocytes (arrow heads) are numerous at the periphery of the graft, but are also found on the inner side of the circular muscle (arrows). In their vincinity, quail cells do not exhibit melanin deposition. Feulgen and Rossenbeck staining. × 260. Figure 4. Section through lung tissue developed from a half-lung provided by a 5.5-day Silkie fowl embryo and grafted into the coelomic cavity of a 3.5-day quail embryo during 9 days. Quail melanocytes populated this organ (arrows). Feulgen and Rossenbeck staining. × 550. Figure 5. Section through a testis developed from the left gonad anlage provided by a 5-day Silkie fowl embryo and grafted into the coelomic cavity of a 3.5-day quail embryo during 8 days. The gonad rudiment differentiated into a testis. Quail melanocytes are observed between the seminiferous tubules (ST). Feulgen and Rossenbeck staining. × 260. Figure 6. Section through a fragment of duodenum differentiated from the pre-umbilical intestine of a 5-day Silkie fowl embryo grafted into the coelomic cavity of a 3.5-day quail embryo during 9 days. Quail melanocytes (arrow-head) settled only at the periphery of the small intestine. CM, circular muscle; M, mucosa. Feulgen and Rossenbeck staining. × 450. Figure 7. Section through a fragment of the liver rudiment of a 5.5-day Silkie fowl embryo grafted into the coelomic cavity of a 3.5-day quail embryo during 8 days. Quail melanoblasts did not populate the liver (L) and melanocytes (arrow-heads) only localized at the periphery. Feulgen and Rossenbeck staining. × 260. Figure 8. Section through the spleen of a 5.5-day Silkie fowl embryo grafted into the coelomic cavity of a 3.5-day quail embryo during 8 days. Quail melanoblasts did not populate the spleen. Hematopoietic cells exhibiting the quail nuclear marker are numerous. Feulgen and Rossenbeck staining. × 285.

whole and, in this respect, Silkie fowl melanoblasts do not differ from those of the quail. At this point, the question of the role played by the tissue itself arises. As those Silkie fowl organs that are not pigmented in situ were not invaded by quail pigment cells, it appears that the homing of melanoblasts is determined by environmental cues created in organs.

As for the environmental cues which regulate seeding of melanoblasts, one can ask the question whether they are permissive or attractive. The heavy accumulation of melanocytes in the depth but also at the periphery of some grafts, strongly suggests that they are not only permissive but also attractive for prepigment cells. Is the definite localization of pigment cells regulated by a chemo-attractant? The hypothesis of the production of a chemo-attractant by the primary lymphoid organs (thymus and bursa of Fabricius in birds) regulating the seeding of hemopoietic cells was proposed by Le Douarin[16]. This author also asks the question whether the colonization of epidermis by melanoblasts in bird embryos could be regulated by such a mechanism[2]. In any case, the chemo-attractant would be widely distributed in the Silkie fowl embryo. The question also arises whether fibronectin, which plays a major role in neural crest cell migration[4,16], is implicated in homing. As in quail embryo, neural crest derived cells, including prepigment ones, do not enter tissues even though they are fibronectin-rich[4]; the presence of fibronectin could be a prerequisite factor but not a sufficient one to ensure melanoblast seeding. Work is in progress showing that, by the stage of melanoblast homing, all the colonized organs are fibronectin-rich.

The broad but selective localization of melanoblasts in Silkie fowl could be of some help in studying the factor(s) playing a role in the seeding process and in the definite localization of neural crest cells, which remains a particularly challenging problem[2].

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## Effects of 2-β-D-glucosyl-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one on *Schizaphis graminum* (Rondani) (Insecta, Aphididae) feeding on artificial diets

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*Summary.* 2-β-Glucosyl-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA-Glc), the main hydroxamic acid from maize and wheat, and its aglucone, decreased survival of *Schizaphis graminum* reared on artificial diets. Both compounds were toxic for aphids and acted as feeding deterrents, at concentrations as low as 1 mM. The natural concentrations of glucosides of hydroxamic acids in wheat leaves reach up to 6 mmoles/kg fresh weight, thus falling within the range in which DIMBOA-Glc causes deleterious effects to diet-fed aphids.
*Key words.* Hydroxamic acid; cereals; maize; wheat; aphids; greenbug.

Hydroxamic acids from Poaceae (Gramineae) seem to play a role in plant resistance to *Ostrinia nubilalis*[2], *Rhopalosiphum maidis*[3], *Metopolophium dirhodum*[4] and *Schizaphis graminum*[5]. DIMBOA, the main hydroxamic acid from maize and wheat extracts, decreases feeding, survival and reproduction rate of aphids reared on artificial diets[6,7]. However, DIMBOA is present in tissues as a glucoside (DIMBOA-Glc). The purpose of this work is to describe the effects on aphids of DIMBOA-Glc and to compare them with those of DIMBOA.

*Experimental.* Isolation of DIMBOA-Glc. Seedlings (*Zea mays* L. cv T129s) were grown in a greenhouse under permanent light at 30 °C. 7-day-old seedlings (950 g) were slowly added to 1000 ml of boiling water, keeping the temperature above 90 °C. After 15 min the seedlings were cooled down, homogenized and filtered through cheesecloth. The pH of the extracts was adjusted to 10 and the sample centrifuged at 10,000 × g for 10 min. The supernatant fluid was acidified (pH 3, HCl) and washed three times with diethylether (2:1 v/v ether:extract). The volume of the aqueous phase was reduced to 25 ml and added to 600 ml of methanol. The resulting suspension was filtered and the solid (22.9 g) discarded. The volume of the filtrate was reduced to 20 ml to which 100 ml of methanol were added. The solid formed (1.2 g) was removed by filtration and discarded. Acetone (300 ml) was added to the solution and the precipitate formed (17.6 g) was also removed by filtration. The solution was evaporated to dryness and dissolved in 50 ml of water:methanol 3:1 v/v.

This solution was added to a column (300 × 35 mm i.d.) of SP-Sephadex C-25 (Pharmacia) in the Fe(III) form and equilibrated with water:methanol 3:1 v/v. This column binds hydroxamic acids, turning a deep blue[8]. The column was washed with 1700 ml of the same solvent (33 ml/h). The sample was then eluted with 500 ml of water:methanol 3:1 v/v saturated with NaCl. The blue fractions were collected (158 ml). The iron was displaced from its hydroxamic acid complex by addition of 70 ml of 0.83 M EDTA pH 8.5. The precipitate formed was removed by filtration. The solution was neutralized with NaOH, concentrated and filtered. Aliquots of 6.5 ml were added to a Sephadex G-10 column (720 × 45 mm i.d.) previously equilibrated with $CHCl_3$-saturated water and eluted with the same solvent as described[9], with a flow rate of 13 ml/h, collecting 2-ml fractions.

Elution profiles were made by measuring the absorbance of the fractions at 260 nm, by treating the fractions with $FeCl_3$ and