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***Bh* (black at hatch) Gene Appears to Cause Hemorrhage in the Homozygous Quail Embryo Lung**

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ABSTRACT—Abnormal development in *Bh* quail embryos was histologically examined. In addition to the abnormal plumage pigmentation in *Bh*-heterozygotes and homozygotes, and subcutaneous hemorrhage and liver degeneration in homozygotes as previously reported, the lungs in all 10-day homozygotes examined showed hemorrhage, suggesting that the *Bh* gene may also be expressed in this organ. Other organs, including the esophagus, the gizzard, the small intestine, the large intestine, the pancreas, the metanephros, the heart, the gonads and the hemopoietic organs in heterozygotes and homozygotes were histologically normal on days 7 and 10, though the development of homozygotes was slightly delayed. Primordial germ cells and hemopoietic cells were normally developed in gonads and hemopoietic organs such as the bursa of Fabricius and the spleen of the homozygote, respectively. These results suggest that *Bh* is a mutation inducing pleiotropic effects such as plumage pigmentation changes in both heterozygotes and homozygotes, and abnormal development of the blood vessels in the skin, the feather germs and the lung in homozygotes.

INTRODUCTION

The *Bh* mutation in the Japanese quail changes plumage pigmentation patterns: the longitudinal stripes of black and yellow seen in dorsal rows of the feather germs of wild-type embryos disappear in both heterozygotes and homozygotes (Minezawa and Wakasugi, 1977; Ono and Wakasugi, 1983; Nakamura and Kaneko, 1993; Kubota *et al.*, 1995). Heterozygotes have black plumage at hatching. Homozygous embryos die at an early stage of development due to subcutaneous hemorrhage and liver degeneration, and develop brown feather germs when they are alive on day 10 (Kubota *et al.*, 1995; Shiojiri *et al.*, 1996). Although the expression of the *Bh* gene in melanocytes might be responsible for the abnormal plumage pigmentation in this mutant, which was revealed using orthotopic transplantation of neural crest cells between *Bh* and imperfect albino embryos (Satoh *et al.*, unpublished data), it remains to be determined which cells express the *Bh* gene in the abnormal development of the skin and liver. Nor have there been any histological studies on other abnormalities in *Bh* embryos.

In the coat color of mice, many mutations have been reported, and some of them show pleiotropic defects in various tissues with lethal effects on homozygotes (Silvers, 1979; Jackson, 1985). Recent studies have also revealed molecular

mechanisms of gene action for some of the coat color mutations (Fleischman, 1993; Siracusa, 1994; Takeuchi *et al.*, 1996; Barsh, 1996). To study which mutation in mice is a counterpart of *Bh*, it might be helpful to examine other abnormalities in *Bh* embryos in detail.

In the present study, we histologically examined the development of *Bh* embryos, and report here that hemorrhage occurred in the lung of the homozygotes with very high incidence in addition to the subcutaneous hemorrhage.

MATERIALS AND METHODS

Animals

Embryos were obtained from matings of *Bh*/+ quails, and incubated in an incubator at 37.8°C with more than 70% humidity. Quail embryos of the wild-type, derived from matings of wild-type quails in intra-*Bh* stock, were also used. Chick embryos were obtained from a local breeder (Oohata Shever Co. Ltd., Yaizu, Japan).

Histological techniques

Bh embryos and wild-type embryos on days 5 to 10 were histologically examined (Table 1). Whole embryos on days 5 to 8, and 10-day organ tissues were fixed in Bouin's fluid and ordinary paraffin sections were prepared. Dewaxed sections were stained with Alcian blue-hematoxylin-eosin (AB-H-E) or azan.

Genotypes of 7-day *Bh* embryos were determined by chorioallantoic membrane grafting of the thigh skin (Kubota *et al.*, 1995). Thigh skin was transplanted onto the chorioallantoic membrane of 9-day chick embryos. The transplants were recovered 9–10 days after transplantation and examined under a dissection microscope. The genotypes were determined from the pigmentation patterns of the

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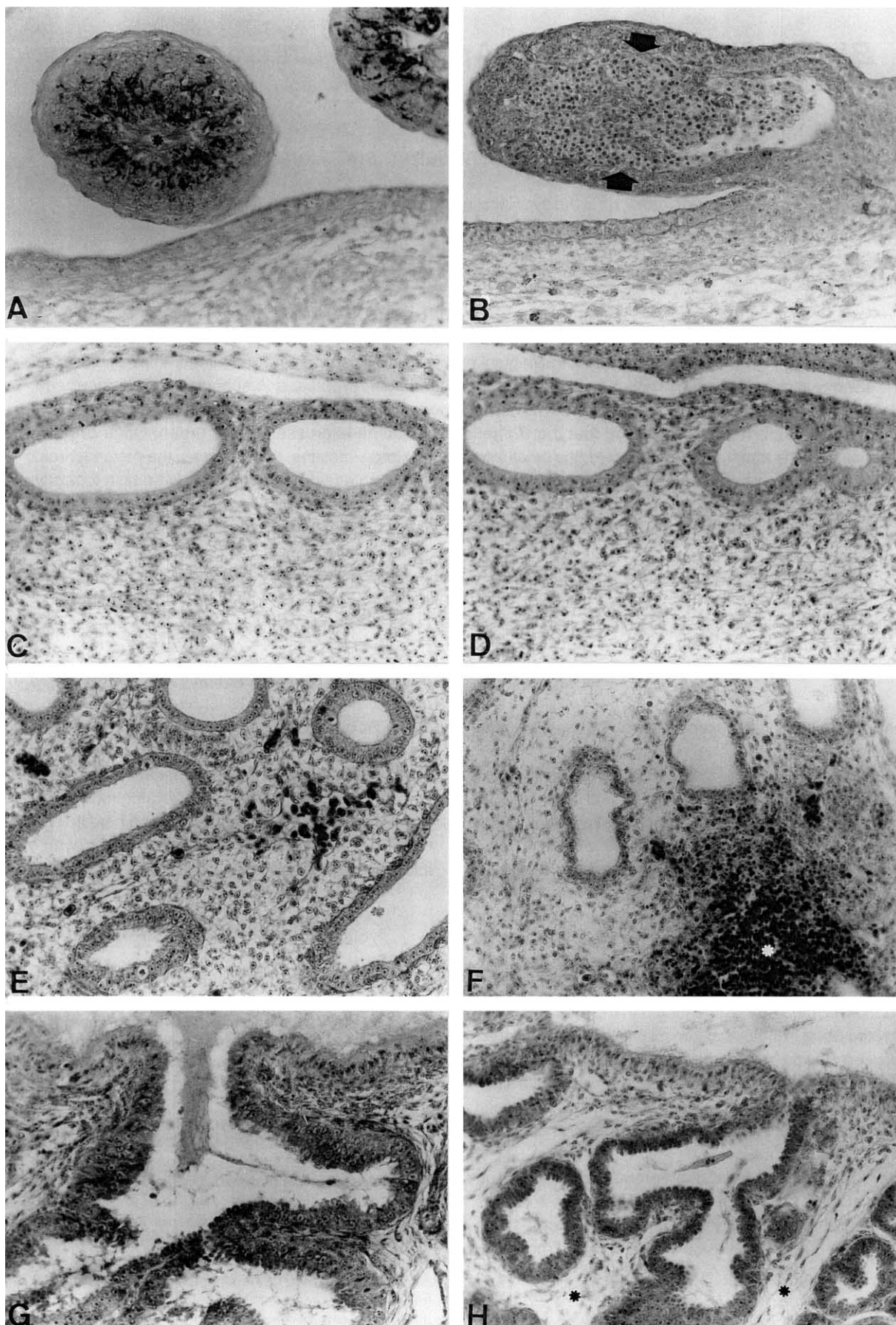


Table 1. Numbers of quail embryos used in the present histological study

days of incubation	5	6	7	8	10
<i>Bh</i> /+ × <i>Bh</i> /+	25	16	45 ^a	14	54 ^b
+/+ ^c	15	0	15	0	0

^a Fifteen embryos of each genotype were examined. Genotypes of 7-day *Bh* embryos were determined by grafting of their thigh skin onto the chorioallantoic membrane of chick embryos (see MATERIALS AND METHODS).

^b Eighteen embryos of each genotype were examined.

^c Obtained by mating +/+ quails.

developed feathers of the transplants. In wild-type feathers, black and yellow parts were clearly differentiated, while black pigment and yellow pigment were mixed in heterozygous feathers. *Bh* homozygous feathers were brown. Genotypes of 10-day *Bh* embryos could be determined by the pigmentation patterns of the feather germs (Minezawa and Wakasugi, 1977; Kubota *et al.*, 1995).

RESULTS

No abnormal development was histologically observed in *Bh* embryos on day 5, as in the wild-type embryos. From day 6, embryos with subcutaneous hemorrhage and liver degeneration started to be seen, though other abnormalities were not detected from days 6 through 8. In 7-day embryos whose genotypes were determined by the chorioallantoic membrane grafting of the thigh skin, homozygous embryos showed subcutaneous or whole body hemorrhage and liver degeneration with very high frequencies, as reported previously (Minezawa and Wakasugi, 1977; Kubota *et al.*, 1995). Primordial germ cells were developed in the ovary and testis, and basophilic hemopoietic cells existed in the spleen in the 7-day embryos of all genotypes. The 7-day lungs of both heterozygotes and homozygotes were histologically normal at this stage, and showed extensive epithelial branching (Fig. 1C, D). There were no differences in the gross morphology and histology of organs between wild-type embryos obtained by mating *Bh*/+ quails, and by mating wild-type quails.

On day 10, the blood vessels in the feather germs and the subcutaneous tissue of homozygotes were abnormal; they were often dilated, accumulated many blood cells, or showed hemorrhage (Table 2) (Fig. 1A, B). Development of the dermis appeared to be delayed in homozygotes, and its cell density was low. Focal necrotic figures were more often seen in the homozygous liver than in wild-type and heterozygous livers. The lungs (both lobes) of all homozygotes examined showed hemorrhage (Table 2) (Fig. 1E, F). The blood vessels of the

Table 2. Abnormal development of 10-day embryos of each genotype

genotype	+/+	<i>Bh</i> /+	<i>Bh</i> / <i>Bh</i> ^a
skin:			
low cell density in the dermis	0 ^b (18) ^c	0 (18)	60 (15)
dilation of subcutaneous blood vessels, accumulation of blood cells in the vessels or marked subcutaneous hemorrhage	0 (18)	0 (18)	100 (15)
degeneration of subcutaneous tissue	0 (18)	0 (18)	93 (15)
feather germs:			
dilation of the blood vessels or accumulation of blood cells in the vessels	0 (18)	22 (18)	100 (18)
proventriculus:			
low cell density in the connective tissue	0 (8)	0 (8)	63 (8)
liver ^d : focal cell death	17 (12)	24 (17)	100 (15)
lung:			
hemorrhage or accumulation of the blood cells in the vessels	0 (8)	0 (8)	100 (8)
mesonephros: cell death	0 (8)	0 (8)	38 (8)

^a Viable homozygotes.

^b Percentage of abnormal development.

^c Number of tissues examined.

^d Kubota *et al.* (1995).

homozygous lung also contained abundant blood cells, and the connective tissue had sparse cell density. Cell death was seen more frequently in the mesonephros of the homozygotes, and the connective tissue development of the proventriculus was sparse with a delay of glandular differentiation in the homozygote (Table 2) (Fig. 1G, H), compared to those of the wild-type and heterozygote. Other organs, including the esophagus, gizzard, small intestine, large intestine, pancreas, metanephros, heart, gonads and hemopoietic organs in homozygotes were histologically normal, though their development was delayed a little. Germ cells and basophilic hemopoietic cells existed in gonads and hemopoietic organs such as the bursa of Fabricius and the spleen in the homozygotes, respectively (Figs. 2 and 3). Granulopoiesis

Fig. 1. **A, B:** Feather germs of 10-day wild-type (**A**) and homozygote (**B**) quails. The connective tissue (*) in the wild-type feather germ has dense cell density. The blood vessel in the homozygous feather germ is dilated (arrows) and accumulates many blood cells. **C, D:** Lungs of 7-day wild-type (**C**) and homozygote (**D**) quails. **E, F:** Lungs of 10-day wild-type (**E**) and homozygote (**F**) quails. The lungs of the 7-day wild-type and homozygote exhibit similar histology whereas hemorrhage (*) occurs in the 10-day homozygous lung. **G, H:** the proventriculus of 10-day wild-type (**G**) and homozygote (**H**) quails. Glandular differentiation delays in the homozygous proventriculus. An asterisk indicates loose connective tissue of the homozygous proventriculus. **A-D, G, H:** AB-H-E staining. **E, F:** azan staining. × 240.

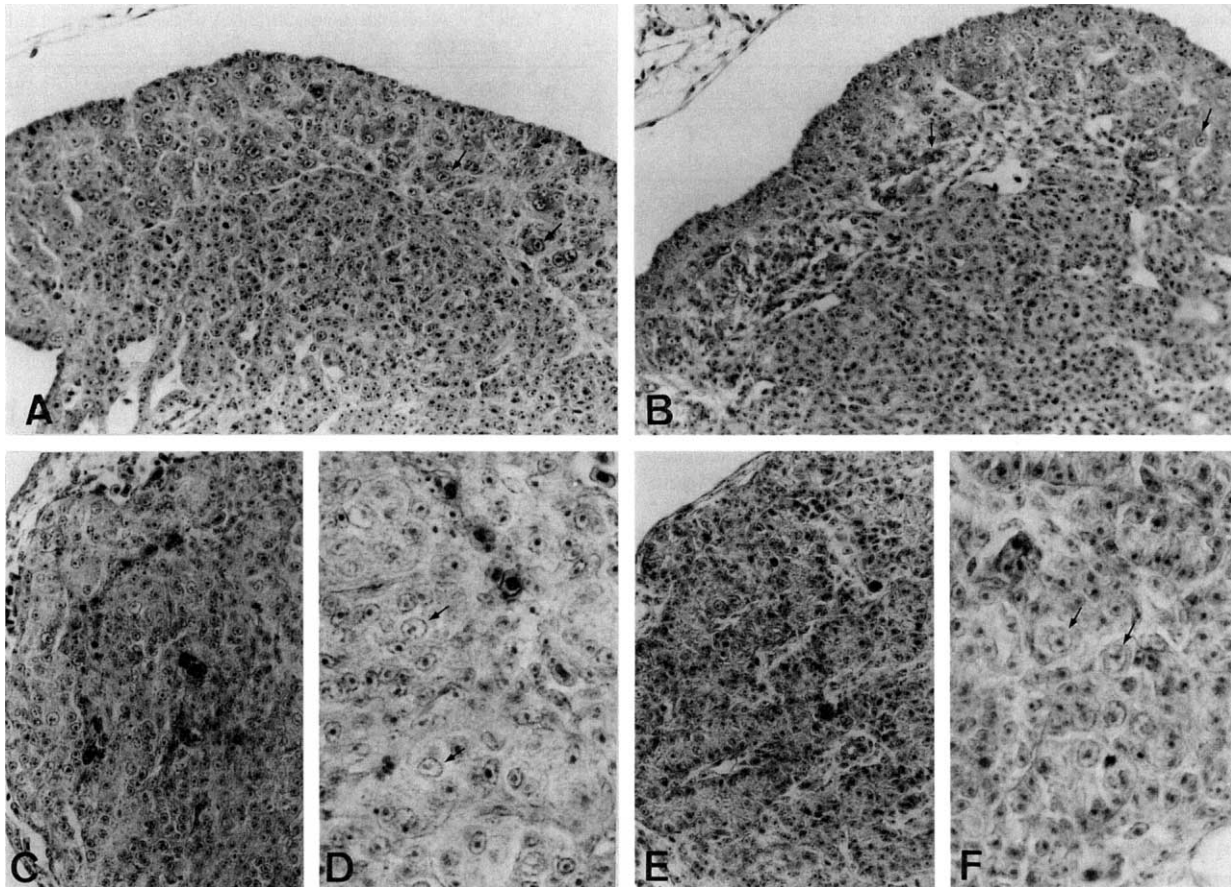


Fig. 2. **A, B:** Ovaries of 10-day wild-type (**A**) and homozygote (**B**) quails. Azan staining. **C-F:** Testes of 10-day wild-type (**C, D**) and homozygote (**E, F**) quails. AB-H-E staining. Germ cells (arrows) are as well developed in both ovaries and testes of the homozygotes as those in the wild-type. **A-C, E:** $\times 240$. **D, F:** $\times 500$.

occurred in the spleen and the bursa of Fabricius of the homozygotes as in the wild-type and heterozygote.

DISCUSSION

We have shown that the *Bh* mutation in quails not only affects plumage pigmentation patterns, but also causes subcutaneous hemorrhage and liver degeneration (Kubota *et al.*, 1995; Shiojiri *et al.*, 1996). In the present study, we demonstrated that, although the *Bh*-homozygous lung was histologically normal on day 7 in spite of the subcutaneous hemorrhage, it showed accumulation of blood cells in the blood vessels and hemorrhage with very high incidence on day 10. Considering that *Bh* mutation also causes dilation of plumage and subcutaneous blood vessels (Minezawa and Wakasugi, 1977; Kubota *et al.*, 1995; the present study), the *Bh* gene could be involved in the function or development of blood vessels, or the coagulation of blood cells. However, it is noteworthy that not all blood vessels in the homozygotes were dilated or hemorrhagic. Expression of the *Bh* gene in the blood vessels may vary with their types and developmental stages. In homozygotes, focal cell death in the liver, cell death in the mesonephros and loose connective tissue development in the

proventriculus were often observed. These abnormal developments may also be related to the expression of the *Bh* gene in the blood vessels. We have shown, using orthotopic transplantation of neural crest cells between *Bh* and imperfect albino embryos, that at least melanocytes might express the *Bh* gene involved in abnormal plumage pigmentation, and that the melanocytes expressing the *Bh* gene might not induce hemorrhage in the blood vessels in the feather germs and subcutaneous tissue, or cell death in the liver (Satoh *et al.*, unpublished data). Hemorrhage in the lung might originate from the expression of the *Bh* gene in the organ. However, further experiments should be carried out in the future such as cloning of the *Bh* gene and examination of the presence of *Bh* mRNA and protein in the tissues showing abnormal development.

Among coat color mutations of mice showing pleiotropic defects in various tissues (Silvers, 1979), *Steel* (*Sl*) mutation causes anemia and fetal death due to defective development of the liver for hemopoiesis in the homozygote condition (Chui and Russell, 1974; Chui and Loyer, 1975). *Bh*-homozygotes also showed abnormal liver development (Kubota *et al.*, 1995; the present study), but developed hemopoietic cells, primordial germ cells and melanocytes (Shiojiri *et al.*, 1996; the present

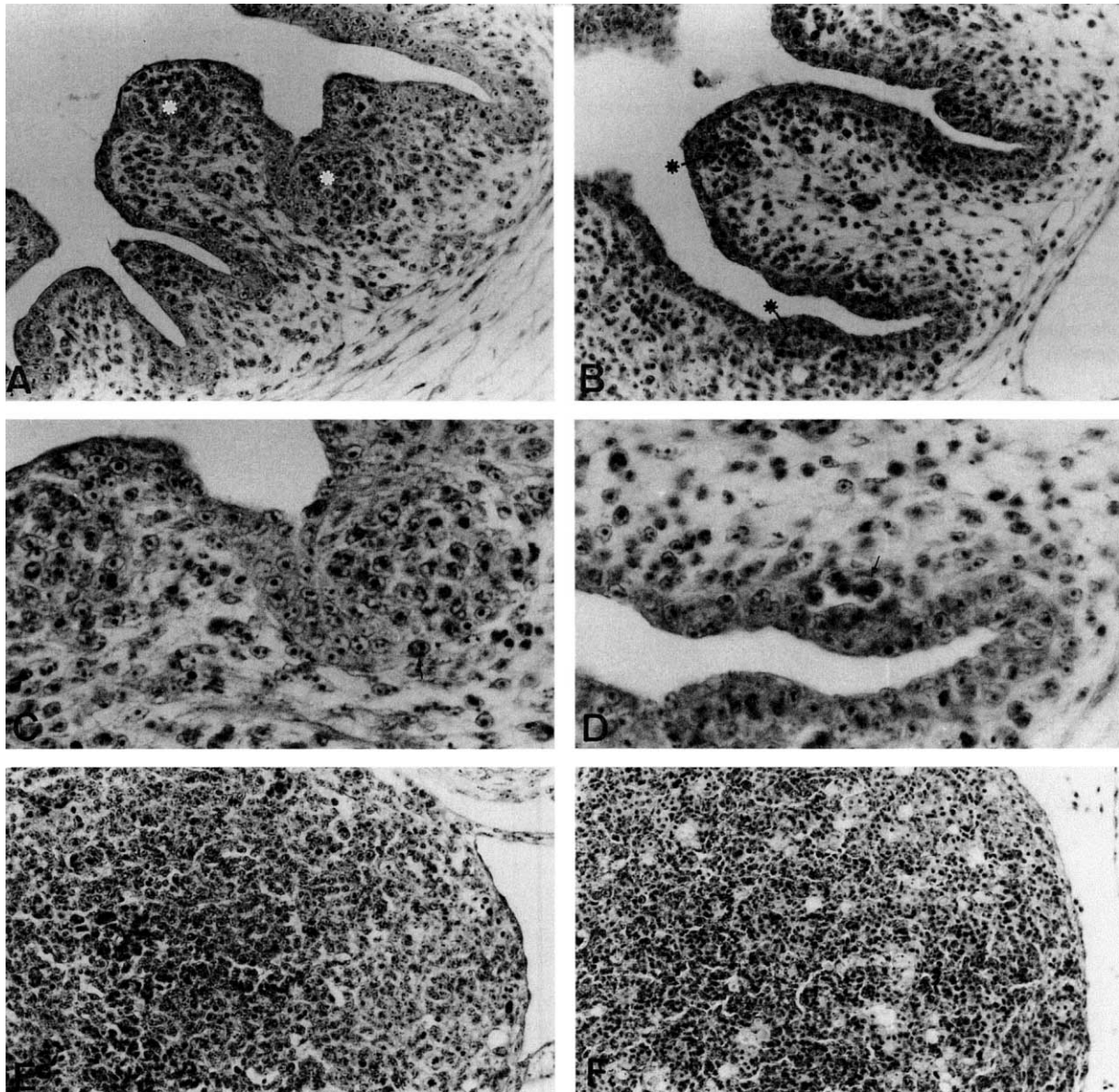


Fig. 3. **A-D:** The bursae of Fabricius of 10-day wild-type (**A, C**) and homozygote (**B, D**) quails. Basophilic hemopoietic cells (arrows) are invading the epithelial layer in the bursa of Fabricius. An asterisk shows the lymphoid follicles at early developmental stages. **E, F:** Spleens of 10-day wild-type (**E**) and homozygote (**F**) quails. Basophilic hemopoietic cells are present in the spleen in both wild-type and homozygote quails. AB-H-E staining. **A, B, E, F:** $\times 240$. **C, D:** $\times 500$.

study), which *Sl*-homozygotes lack (Bennett, 1956; Silvers, 1979). Subcutaneous hemorrhage and hemorrhage such as found in the *Bh*-homozygous lung in this study have not been reported in *Sl* mutation (Bennett, 1956; Silvers, 1979). Thus, the *Bh* mutation is not a counterpart of *Sl* mutation in mice.

In summary, the *Bh* gene might be expressed in melanocytes and other cell types such as cells of blood vessels at least, and might cause pleiotropic effects in various organs.

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