J. Embryol. exp. Morph. Vol. 56, pp. 125–137, 1980
Printed in Great Britain © Company of Biologist Limited 1980

Genetic and experimental studies on a pigment mutation, *Pale* (P^a), in the frog, *Bombina orientalis*

By MARK S. ELLINGER[1]

From the Department of Zoology, Southern Illinois University

SUMMARY

A mutant gene, *Pale*, (P^a) has been discovered in the discoglossid frog, *Bombina orientalis*. Breeding experiments indicate that the gene is recessive to the wild-type allele (P). Embryos, tadpoles and adults homozygous for the *Pale* gene are lighter in coloration than wild-type animals. Eggs produced by *Pale* females appear normally pigmented. Neural-retina defects were apparent in *Pale* tadpoles. Parabiosis experiments revealed that the *Pale* and wild-type phenotypes were unaffected by circulating factors from the opposite phenotype. Neural-crest-grafting experiments revealed that *Pale* melanophores retain the *Pale* phenotype when placed in a wild-type cellular environment. Likewise, wild-type chromatophores are unaffected by residence in *Pale* tissues. Melanophores of *Pale* tadpoles display reduced numbers of mature melanosomes. This is the primary morphological basis of the *Pale* phenotype. However, other chromatophore classes (xanthophores, iridophores) were also less intensely pigmented, demonstrating that a single gene may affect the pigmentation of all chromatophore classes in *B. orientalis*.

INTRODUCTION

Mutations that affect the distribution or synthesis of pigment are useful as models for the study of gene function during embryonic development (Markert & Ursprung, 1971). Amphibian embryos and tadpoles are well suited to studies of pigmentation; the epidermis is nearly transparent, and many embryonic tissues, including neural crest, are amenable to surgical manipulation. Though numerous melanistic variants in the amphibia have been reported (Hensley, 1959; Harris, 1968), relatively few have been studied extensively. Hypomelanistic mutations have been characterized in *Ambystoma mexicanum* and *A. tigrinum* (Dalton, 1949; Humphrey, 1967; Benjamin, 1970), *Rana pipiens* (Browder, 1972; Smith-Gill, Richards & Nace, 1972), *Rana temporaria* (Smallcombe, 1949) and *Xenopus laevis* (Bluemink & Hoperskaya, 1975; Hoperskaya, 1975; Tompkins, 1977).

In 1973, a pigment variant appeared spontaneously in the *Bombina orientalis* colony at the University of Minnesota. I designated the variant *Pale* (P^a), since affected tadpoles were much lighter in appearance than wild-type (P) tadpoles. Subsequently, the pigment variant was used as a nuclear marker in nuclear transplantation experiments with *B. orientalis* (Ellinger & Carlson, 1978).

[1] *Author's address:* Department of Zoology, Southern Illinois University, Carbondale, Illinois 62901, U.S.A.

In this paper, I report a series of observations and experiments providing information about the genetic basis and phenotypic expression of the Pale trait.

MATERIALS AND METHODS

The *Bombina orientalis* used in these studies are maintained as a colony in the Department of Zoology, Southern Illinois University at Carbondale. The colony was initiated in 1973 at the University of Minnesota with animals that had been obtained from Dr George Nace, The Amphibian Facility, University of Michigan, Ann Arbor, Michigan 48109.

Ovulation and amplexus were induced by dorsal subcutaneous injection of 250 i.u. of chorionic gonadotropin (Sigma). Fertilized eggs were reared at 20–22 °C in finger bowls containing 10 % Barth's Solution X (Barth & Barth, 1959). The finger bowls were kept on a beige background throughout the experiments. Melanophores were examined in whole tadpoles or tadpole tails fixed in Bouin's fluid. Fixed tissues were processed using standard paraffin procedures, sectioned at 10 μm, and stained in Harris' haematoxylin and eosin. Unstained whole-mount preparations of tail-fin epithelium were also examined. For comparison of melanosome numbers in *Pale* and wild-type melanophores, counts were confined to comparable-sized melanophore cell bodies in the dorsal tail fins of 13-day sibling tadpoles. A total of 100 cell bodies was scored under 900× magnification (ten in each of five *Pale* and ten in each of five wild-type tadpoles).

Parabiosis and neural-crest grafting experiments were done in Barth's Solution X, after the methods of Rugh (1962). Fused areas in the parabiosis experiments included the presumptive gill regions to ensure the establishment of cross circulation. For neural-crest grafting, *Pale* embryo neural folds were implanted into wild-type hosts, and vice versa. Graft beds were prepared in host embryos by removing a small area of ectoderm and underlying mesoderm from the ventral midline, midway between anterior and posterior ends. Approximately one mm of anterior neural fold was then removed from a donor embryo, flattened and placed within the graft bed (external surface remaining external). After 3 h, the grafted embryos were removed from Barth's Solution X and placed in 10 % Barth's Solution X for rearing.

RESULTS

Genetic analysis. A series of breeding experiments is summarized in Table 1. These studies establish that the *Pale* trait is due to a simple recessive gene.

Coloration. The P^a/P^a tadpoles were distinctly lighter than P/P tadpoles (Fig. 1). By examining embryos under a stereomicroscope, it was possible to identify the *Pale* phenotype after five days of development at 22 °C (gill circulation, pre-hatching). The eyes of P^a/P^a animals, under the stereomicroscope

Table 1. *Crosses Establishing the* Pale (P^a) *Mutation as a Mendelian Recessive*

Cross*	Results†		
	Pale	Normal	% *Pale*
(1) P^a/P ♀ × P^a/P ♂ (17 matings)	296	842	26·0
(2) P^a/P ♀ × P^a/P^a ♂ (7 matings)	178	182	49·4
(3) P^a/P^a ♀ × P^a/P^a ♂ (5 matings)	429	0	100·0
(4) P/P ♀ × P^a/P^a ♂ (2 matings)	0	48	0·0
(5) P^a/P^a ♀ × P/P ♂ (1 mating)	0	12	0·0
(6) P/P ♀ × P/P or P^a/P ♂ or: P/P or P^a/P ♀ × P/P ♂ (32 matings)	0	1,760	0·0

* Normally pigmented adults were determined to be homozygous or heterozygous on the basis of previous matings.

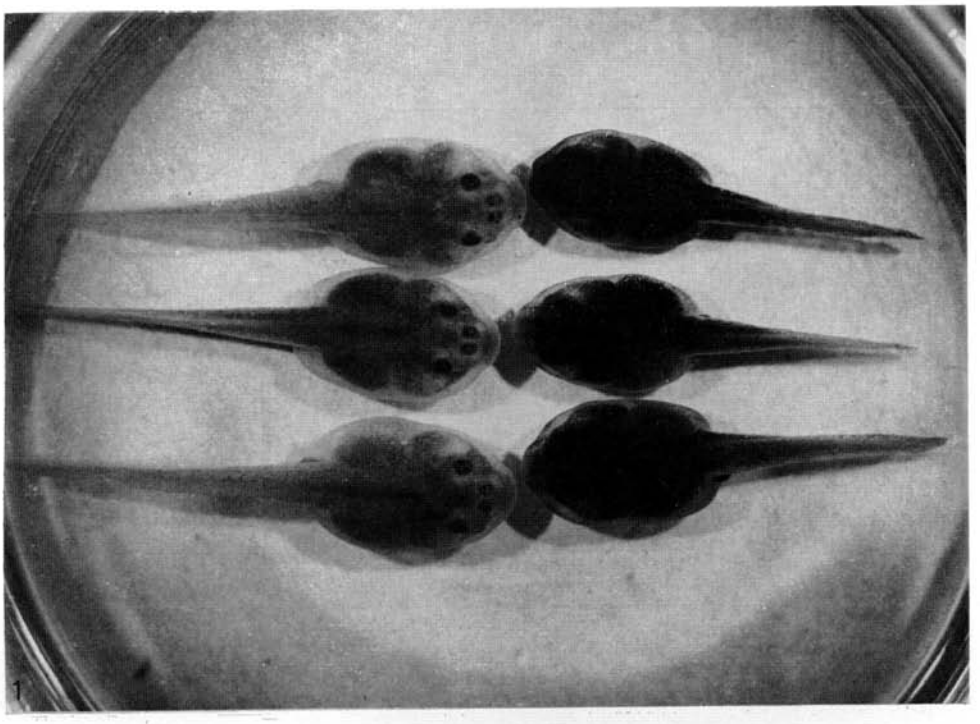
† Embryos were scored 48–72 h after hatching.

P = wild-type allele. P^a = *Pale* allele.

or with the unaided eye, appeared darkly pigmented (Fig. 1). Postmetamorphic P^a/P^a juveniles were light tan compared to the dark brown of P/P juveniles (Fig. 2). P/P adults were brown, green, or brown/green in dorsal coloration. In P^a/P^a adults, dorsal areas lacking green pigmentation were beige or light grey, as opposed to the usual brown. While green areas on P/P adults varied from light green to blue-green to dark green, only light green areas were seen on P^a/P^a adults. The eggs produced by P^a/P^a females appeared normally pigmented. Since the egg pigment is distributed in the epithelial cells of the early embryo, P^a/P^a and P/P embryos were indistinguishable until the first appearance of embryonic melanophores.

Histological analysis. The distinctive orthogonal geometry of epidermal melanophores seen in P/P tadpoles (Ellinger, 1979) was also observed in P^a/P^a tadpoles (Fig. 3). There did not seem to be a reduction in the number of melanophores; rather, individual melanophores seemed to possess reduced numbers of mature melanosomes (Figs. 4, 5). To substantiate this, epidermal melanophore cell bodies in the dorsal tail fin were scored for numbers of melanosomes. P^a/P^a cell bodies possessed approximately half the number of melanosomes ($\bar{x} \pm$ S.E.: 88 ± 2) of P/P cell bodies ($\bar{x} \pm$ S.E.: 178 ± 4). Melanosomes within the cell bodies and processes of both P^a/P^a and P/P melanophores appeared homogeneously distributed.

Histological sections revealed that the pigmented epithelium of P^a/P^a eyes was reduced in thickness, though the intensity of pigmentation within



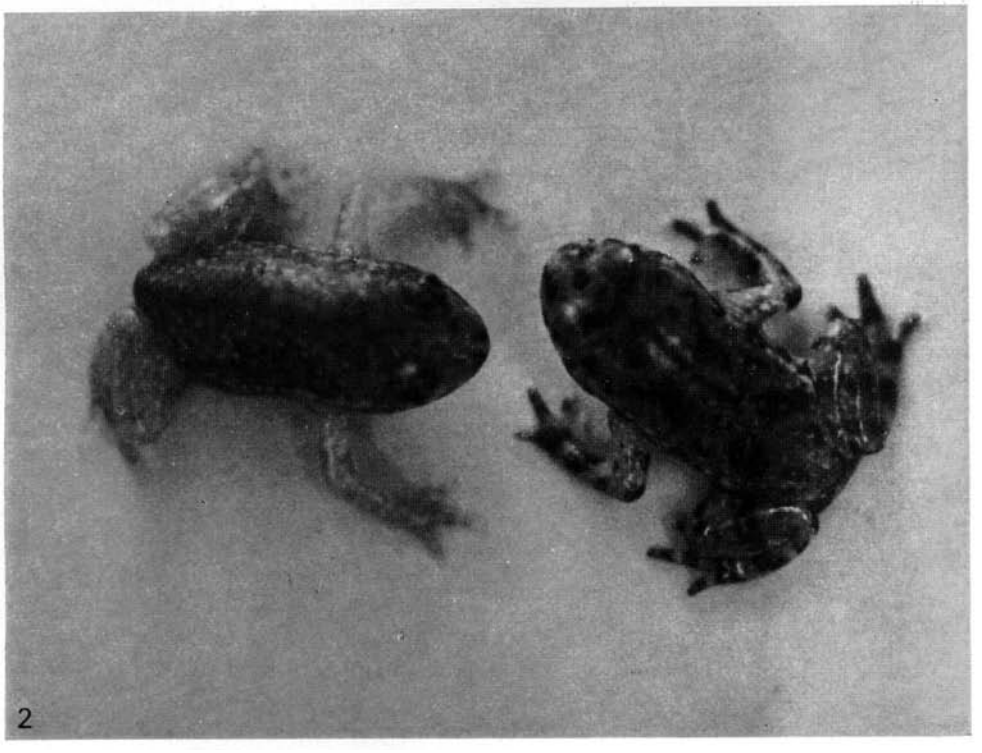


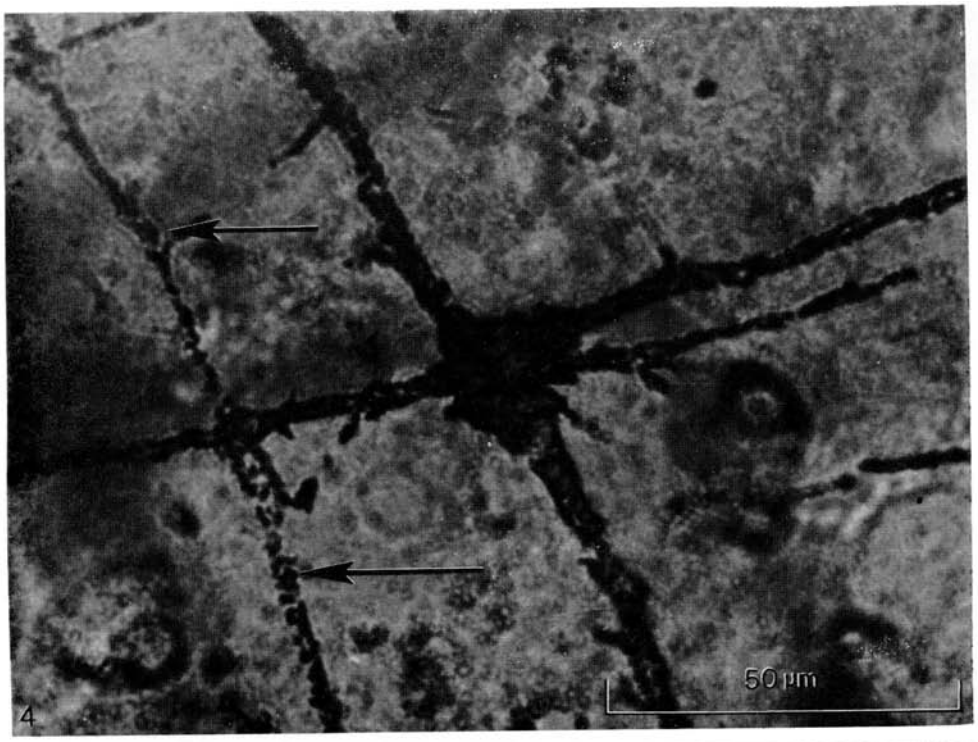
Fig. 1. *Pale* (left) and wild-type (right) *B. orientalis* tadpoles, 20 days post-fertilization.
Fig. 2. Newly-metamorphosed juveniles. Left – *Pale*; right – wild type.

Fig. 3. Unstained preparation of epidermal melanophores (large arrows) in P^a/P^a tail fin. The orthogonal network of epidermal melanophores, observed in P/P embyros, is also observed in P^a/P^a embryos. Irregular dark spots (small arrows) represent epithelial cells that have retained egg pigment.

the epithelium appeared comparable to P/P eyes (Fig. 6A, B). P^a/P^a neural retinas, from the inner plexiform layer to the outer segments of the rods and cones, appeared disordered. The severity of defects was somewhat variable between tadpoles; in general, segments of the rods and cones were shortened, irregular in outline, and/or reduced in number, and the nuclear layers appeared less compactly arranged (Fig. 6A, B).

Parabiosis. Eleven parabiotic twins (*Pale*/wild type) were reared through the feeding stage. In none was there an alteration in the pigment phenotype of either embryo (Fig. 7), though cross circulations were observed in each case. Limited migration of melanophores across the area of fusion was seen occasionally, involving the movement of P^a/P^a melanophores into P/P embryos, and vice versa.

Neural-crest grafts. Thirteen P^a/P^a embryos with P/P neural-crest implants were reared to the feeding stage. Normally-pigmented melanophores differentiated and migrated extensively within all of them. From the point of origin near the ventral midline, epidermal and dermal melanophores migrated anteriorly, laterally and dorsally (Fig. 8). The majority of epidermal melanophores



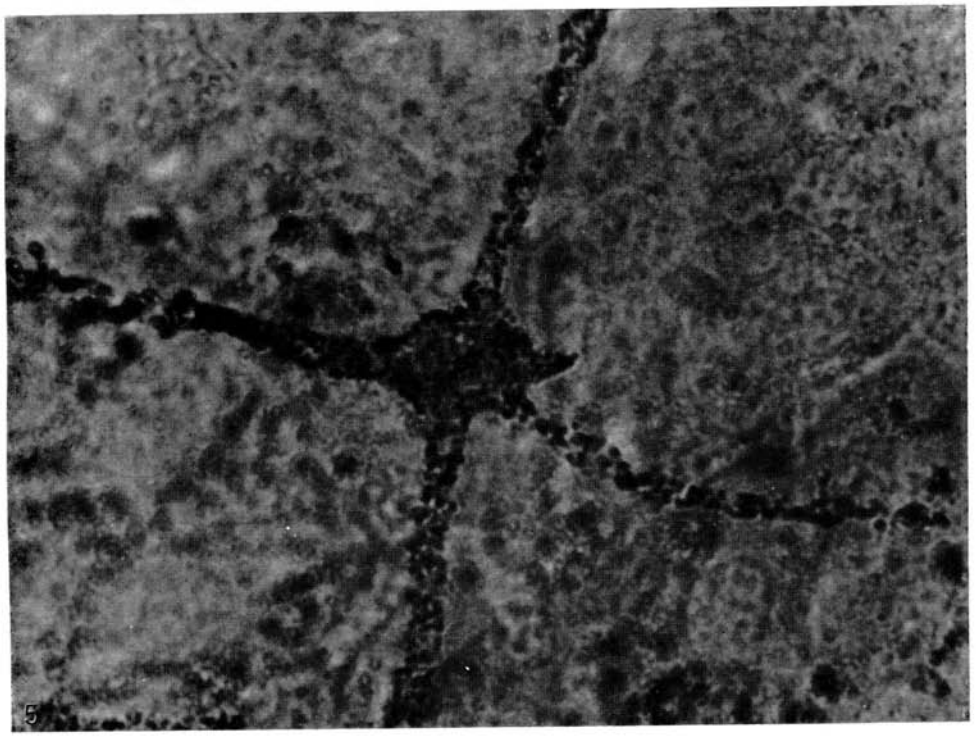


Fig. 4. P/P epidermal melanophore in the dorsal tail fin of a 14-day tadpole. Arrows indicate process from an adjacent melanophore.

Fig. 5. P^a/P^a melanophore in the dorsal tail fin of a 14-day tadpole.

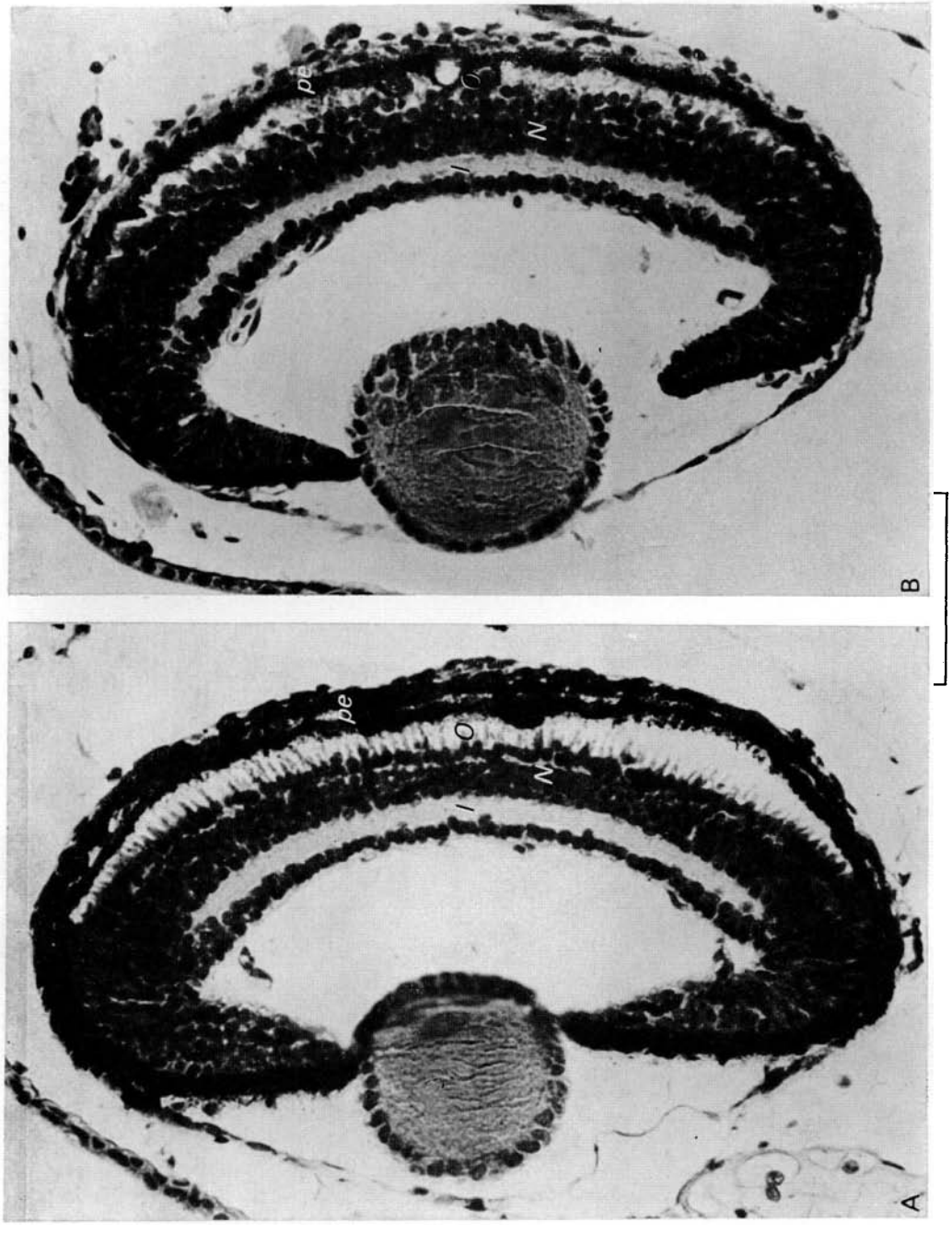


Fig. 6. Sections through the eyes of 14-day sibling tadpole. *I*, inner plexiform layer; *N*, nuclear layers; *O*, outer segments of rods and cones; *pe*, pigmented epithelium. (A) Wild-type eye. (B) *Pale* eye. Note reduction in thickness of the pigmented epithelium in comparison to (A). Also note abnormalities in the nuclear layers and the outer segments of the rods and cones.

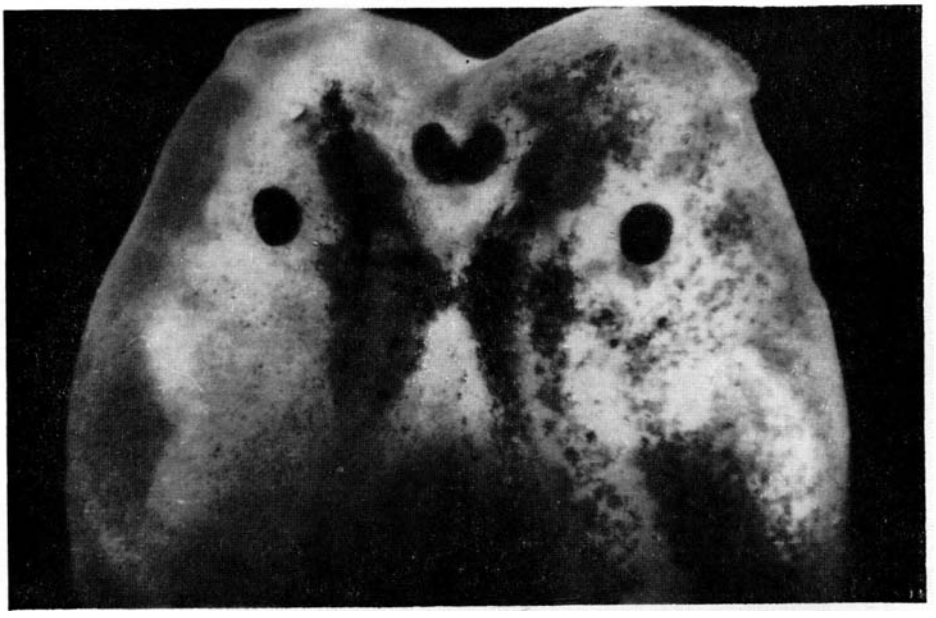


Fig. 7. Parabiotic twins, 10 days post-operation. The pigment phenotypes of the P^a/P^a (left) and P/P (right) tadpoles remain unchanged.

derived from neural-crest implants organized into the orthogonal networks characteristic of normal embryos. Dorsal migration of melanophores was consistently unilateral (either right or left); only a few dermal melanophores appeared occasionally on the contralateral side (Fig. 9). No melanophores originating from mid-ventral neural-crest implants were observed in the tail.

Xanthophores and iridophores also differentiated from the neural-crest implants. Compact aggregates usually appeared at the site of implantation, with smaller numbers of individual cells having migrated, predominantly on the side of the embryo that was also most heavily populated with melanophores (Fig. 10). Five of the grafted P^a/P^a embryos were reared through metamorphosis. Areas of wild-type pigmentation were still visible following tail resorption. No other neural-crest derivatives were seen in the host embryos, though a complete serial-section analysis was not performed in this study.

Ten P/P embryos with P^a/P^a neural-crest implants were also reared to the feeding stage. The differentiation, migration and persistence of P^a/P^a chromatophores in P/P hosts appeared similar to the behavior of P/P chromatophores in P^a/P^a hosts. The precise limits of P^a/P^a melanophore migration were difficult to ascertain, though the P^a/P^a implants maintained an extensive 'sphere of influence' into which host melanophores did not migrate (Fig. 11).

The close juxtaposition of P^a/P^a and P/P tissues resulting from neural-crest implants indicated that xanthophores and iridophores from P^a/P^a embryos were markedly 'pale' in comparison to their wild-type counterparts (Fig. 12). This had not been readily apparent from observations of unoperated embryos.

DISCUSSION

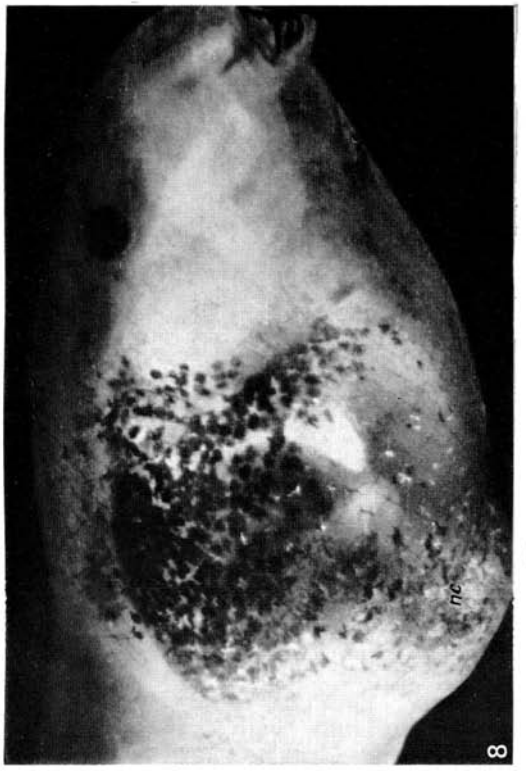
The *Pale* trait differs from a true albino where melanin synthesis is suppressed completely (Benjamin, 1970; Browder, 1972). The primary morphological basis of the *Pale* phenotype is a reduction in the density of melanosomes within melanophores. The *Pale* phenotype does not lead to an alteration in melanophore number, size or shape, and melanosomes were rather evenly dispersed throughout P^a/P^a melanophores as they were in P/P melanophores. Possible modes of action for the P^a allele might, therefore, include a defect in the enzyme tyrosinase or a defect in the maturation sequence of melanosomes from ER and Golgi elements.

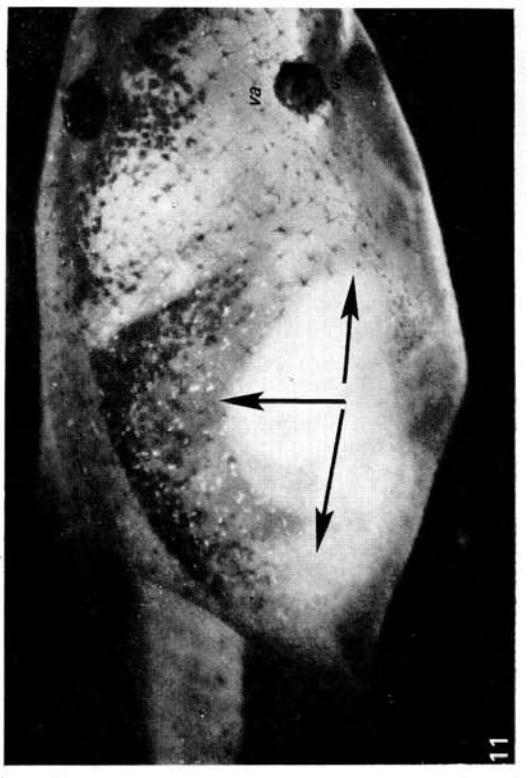
Initial observations of P^a/P^a embryos with the stereomicroscope suggested that melanin deposition in the pigmented retina was comparable to that seen in P/P embryos. However, histological examination of the eye indicated that the thickness of the pigmented epithelium was reduced in P^a/P^a tadpoles. The sections also revealed that homozygosity for P^a leads to defects in the organization of the neural retina. Further studies will be necessary to determine whether these defects are directly attributable to the P^a allele or whether they arise secondarily as a result of the reduced thickness of the pigmented epithelium.

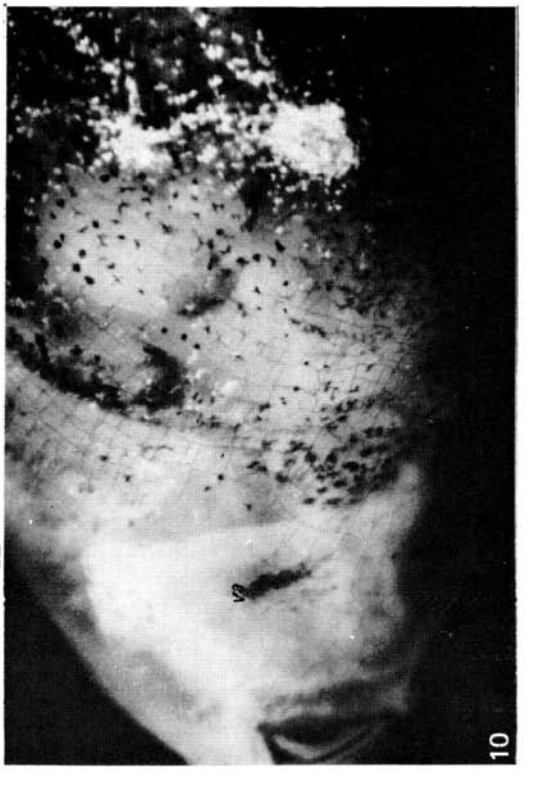
Parabiosis experiments were undertaken to determine whether the P^a allele influences chromatophores directly or through the action of a circulating factor such as an hypophyseal hormone. Though sharing a common circulation for several weeks with tadpoles of the opposite phenotype, neither P^a/P^a nor P/P tadpoles changed. This indicates that defects in hormonal or other circulating factors are not the basis of the *Pale* phenotype.

To test whether inductive interactions, possibly involving cell–cell contacts between melanophores and non-chromatophore cell types, were defective in P^a/P^a embryos, P^a/P^a neural crests were implanted into the bellies of P/P embryos and vice versa. These experiments indicated that P^a/P^a cellular environments did not affect the differentiation of P/P melanophores, nor did P/P environments enhance melanin deposition in P^a/P^a melanophores. Under these conditions, it was also clear that P^a/P^a xanthophores and iridophores were less intensely pigmented than their wild-type neighbors. Ultrastructural studies will be necessary to further clarify the nature of these differences.

Bagnara *et al.* (1979) have suggested that melanophores, xanthophores and iridophores are derived from a stem cell that contains a primordial organelle capable of differentiating into any of the pigmentary organelle classes – melanosomes, pterinosomes, or reflecting platelets. It is possible that the P^a allele is operating at the level of stem cell differentiation, since all three chromatophore types appear to be affected. Pigment mutations affecting more than one chromatophore class have also been reported in *Ambystoma mexicanum* (Dumeril, 1870; Humphrey & Bagnara, 1967; Lyerla & Dalton, 1971), *Rana pipiens*


no


va


va

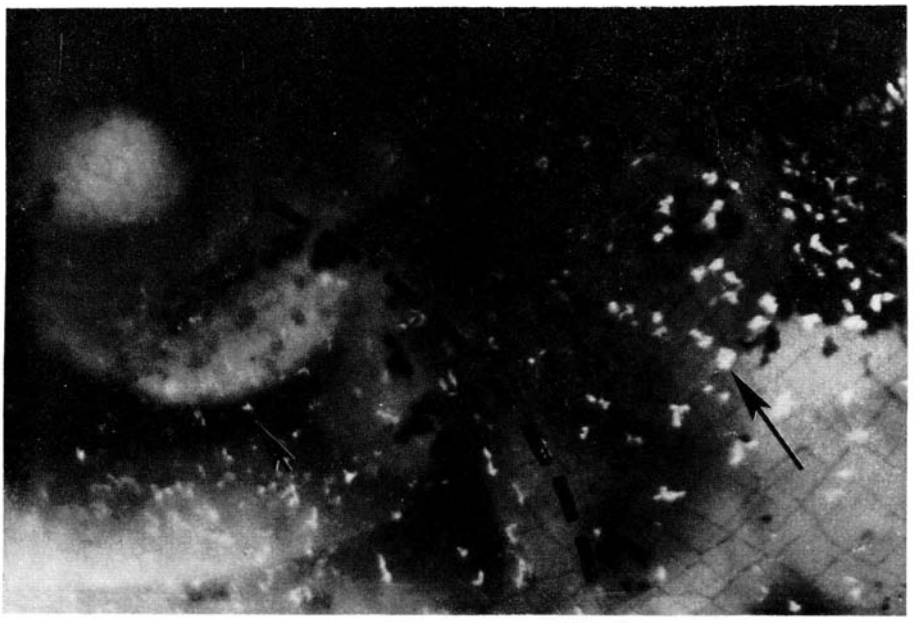


Fig. 12. Ventro-lateral view of P/P tadpole with mid-ventral P^a/P^a neural-crest implant. Dashed line indicates approximate boundary of *Pale* iridophore migration (lower left toward upper right). Short arrow on left indicates *Pale* iridophore; long arrow on right indicates wild-type iridophore.

(Richards, Tartof & Nace, 1969) and *Xenopus laevis* (MacMillan, 1979). Whether the P^a gene acts prior to or after migration from the neural crest, and whether other neural-crest derivatives (e.g. autonomic ganglia) are affected, remain to be determined.

Hoperskaya (1978) reported that, following transplants of wild-type neural folds into albino periodic mutants of *X. laevis*, skin melanophore migration was uniformly bilateral. Thus, the unilateral pattern of chromatophore migration in the present neural-crest transplant experiments was unexpected.

FIGURES 8–11

Fig. 8. Lateral view of P^a/P^a tadpole with P/P neural crest implant at the ventral midline. Epidermal and dermal melanophores have migrated extensively in the lateral and dorsal directions. *nc*, original site of neural-crest implant.

Fig. 9. Dorsal view of P^a/P^a tadpole with P/P neural-crest implant at ventral midline. Melanophore migration was predominantly on the left side, with only a few melanophores visible on the right.

Fig. 10. Ventral view of tadpole seen in Fig. 9, showing accumulations of xanthophores and iridophores. Migration of these cells was limited in comparison to melanophores, but also predominantly toward the left side. *va*, Remnants of ventral adhesive organs.

Fig. 11. Ventro-lateral view of P/P tadpole with mid-ventral P^a/P^a neural-crest implant. Area containing *Pale* melanophores (delineated by arrows) had excluded entry of melanophores from P/P host. *va*, Ventral adhesive organs.

The *B. orientalis* neural-fold implants were placed at the ventral midline, though they may have been slightly shifted to one side or the other. Hoperskaya (1978) did not report where the neural folds were positioned in the host embryos. In *B. orientalis*, it is possible that those cells which first migrate establish a tendency for other chromatophores of the implant to migrate in the same direction. Or, it is possible that the grafted neural folds, which were taken from either the right or left sides of the donor embryos, retained a right/left migration preference in the host embryos. This would need to be confirmed in a separate study.

Eggs produced by P^a/P^a females appear normally pigmented. Even when this egg pigment is dispersed within the epithelial cells of later embryos, such embryos retain their darkly-pigmented appearance. The *Pale* gene, therefore, while leading to a reduction in melanosome content of melanophores in embryos, tadpoles and adults, does not seem to affect the deposition of melanosomes during oogenesis. This may indicate that tyrosinase is unaffected by the mutation, and that some other aspect of melanosome differentiation is the target site. Another possibility is that embryonic tyrosinase is defective, but that an alternate form of tyrosinase, unaffected by the *Pale* gene, may be used for egg melanosome biogenesis. While multiple molecular variants of tyrosinase have been isolated from *R. pipiens*, the significance of these multiple forms remains obscure (Miller, Newcombe & Triplett, 1970; McGuire, Newman & Barisas, 1973; Mikkelsen & Triplett, 1975). Whether alternate forms of tyrosinase produce pigment in different tissues or in different phases of development remains to be demonstrated.

The *Pale* mutation may prove to be a useful model for further studies on the differential control of pigmentary organelle differentiation in oogenesis and embryogenesis, and for the manner in which a pigment mutation may alter the organization of the neural retina.

I would like to thank Dr Ronald A. Brandon and anonymous reviewers for reading the manuscript and making helpful suggestions. Helpful discussions with Dr Sally Frost are also gratefully acknowledged.

REFERENCES

BAGNARA, J. T., MATSUMOTO, J., FERRIS, W., FROST, S. K., TURNER, W. A. JR., TCHEN, T. T. & TAYLOR, J. A. (1979). Common origin of pigment cells. *Science, N.Y.* **203**, 410–415.

BARTH, L. G. & BARTH, L. J. (1959). Differentiation of cells of the *Rana pipiens* gastrula in unconditioned medium. *J. Embryol. exp. Morph.* **2**, 210–222.

BENJAMIN, C. P. (1970). The biochemical effects of the *d*, *m*, and *a* genes on pigment cell differentiation in the axolotl. *Devl Biol.* **23**, 62–85.

BLUEMINK, J. G. & HOPERSKAYA, O. A. (1975). Ultrastructural evidence for the absence of premelanosomes in the eggs of the albino mutant (a^p) of *Xenopus laevis*. *Wilhelm Roux, Archs devl Biol.* **177**, 75–79.

BROWDER, L. W. (1972). Genetic and experimental studies of albinism in *Rana pipiens*. *J. exp. Zool.* **180**, 149–155.

DALTON, H. C. (1949). Developmental analysis of genetic differences in pigmentation in the axolotl. *Proc. natn. Acad. Sci., U.S.A.* **35**, 277–283.

DUMERIL, A. (1870). Création d'une race blanch d'axolotl au museum. *C.r. hebd Séanc. Acad. Sci., Paris D* **70**, 782.

ELLINGER, M. S. (1979). Ontogeny of melanophore patterns in haploid and diploid embryos of the frog, *Bombina orientalis*. *J. Morph.* **162**, 77–91.

ELLINGER, M. S. & CARLSON, J. T. (1978). Nuclear transplantation in *Bombina orientalis* and utilization of the *Pale* mutation as a nuclear marker. *J. exp. Zool.* **205**, 353–359.

HARRIS, H. S. (1968). A survey of albinism in Maryland amphibians and reptiles. *Bull. Maryland Herpetol. Soc.* **4**, 57–59.

HENSLEY, M. (1959). Albinism in North American amphibians and reptiles. *Publ. Mich State Univ. Mus.* **1**, 135–159.

HOPERSKAYA, O. A. (1975). The development of animals homozygous for a mutation causing periodic albinism (a^p) in *Xenopus laevis*. *J. Embryol. exp. Morph.* **34**, 253–264.

HOPERSKAYA, O. A. (1978). Melanin synthesis activation dependent on inductive influences. *Wilhelm Roux' Archs devl Biol.* **184**, 15–28.

HUMPHREY, R. R. (1967). Albino axolotls from an albino tiger salamander through hybridization. *J. Hered.* **58**, 95–101.

HUMPHREY, R. R. & BAGNARA, J. T. (1967). A color variant in the Mexican axolotl. *J. Hered.* **58**, 251–256.

LYERLA, T. A. & DALTON, H. C. (1971). Genetic and developmental characteristics of a new color variant, axanthic, in the Mexican axolotl, *Ambystoma mexicanum* Shaw. *Devl Biol.* **24**, 1–18.

MACMILLAN, G. J. (1979). An analysis of pigment cell development in the periodic albino mutant of *Xenopus*. *J. Embryol. exp. Morph.* **52**, 165–170.

MARKERT, C. L. & URSPRUNG, H. (1971). *Developmental Genetics*. Englewood Cliffs: Prentice-Hall.

MCGUIRE, J., NEWMAN, D. & BARISAS, G. (1973). The tyrosinase–protyrosinase system in frog epidermis. *Yale J. Biol. Med.* **46**, 572–582.

MIKKELSEN, R. B. & TRIPLETT, E. L. (1975). Tyrosinases in *Rana pipiens:* Purification and physical properties. *J. biol. Chem.* **250**, 638–643.

MILLER, L. O., NEWCOMBE, R. & TRIPLETT, E. L. (1970). Isolation and partial characterization of amphibian tyrosinase oxidase. *Comp. Biochem. Physiol.* **32**, 555–567.

RICHARDS, C. M., TARTOF, D. T. & NACE, G. W. (1969). A melanoid variant in *Rana pipiens*. *Copeia*, pp. 850–852.

RUGH, R. (1962). *Experimental Embryology*, 3rd ed. Minneapolis: Burgess.

SMALLCOMBE, W. A. (1949). Albinism in *Rana temporaria*. *J. Genet.* **49**, 286–290.

SMITH-GILL, S. J., RICHARDS, C. M. & NACE, G. W. (1972). Genetic and metabolic bases of two 'albino' phenotypes in the leopar fdrog, *Rana pipiens*. *J. exp. Zool.* **180**, 157–168.

TOMPKINS, R. (1977). Grafting analysis of the periodic albino mutant of *Xenopus laevis*. *Devl Biol.* **57**, 460–464.

(Received 6 August 1979, revised 7 November 1979)