Bull. Shimane Univ. (Natural Sci.) No. 15, pp. 89–95. text-figs. 1–4 December, 1965.

Studies on melanomas induced by trematode infection on the skin of fresh-water teleosts

II. Formation of the dark spot

By

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A considerable number of descriptions concerning melanosis on the skin associated with infection of trematode larvae have been given in teleost fishes (Ishii 1915a, b, Smith 1935, Hsiao 1941, Caldwell and Caldwell 1962). Such melanosis resulted in excessive aggregations of melanin-containing cells in the tissue surrounding the skin parasite. Recently, the present author (Iga 1965) reported that, in *Oryzias latipes* and *Carassius auratus* with dark spots, *melanomas*, on the skin, these pathologically developed pigment cells were indistinguishable in responses to pigment-concentrating stimulants for the melanophores, such as adrenaline and potassium ion, and to electric current from usual melanophores in the adjacent areas of the skin. In spite of these investigations the process of the formation of these dark spots on the skin in fishes has remained an open question. Alternative possibilities are obvious; either the pigment cells in a parasitized region migrate to the site of infection from the other areas or are formed *in situ*. Such a question may be resolved by observations on the region of infection of a parasite *in vivo* during the formation of a dark spot.

The present papar described a course of the formation of the dark spot, *melanoma*, on the skin in *Oryzias latipes*.

Materials and Methods

Oryzias latipes possessing the dark spots, which in these fishes were induced by trematode infection, on the skin of their body and fins were used as materials. These fishes were caught in a stream near the Shimane University over a period of about a month from the middle part of October to November.

For histological studies small pieces of skin with the dark spot were fixed in Bouin's fluid, sectioned serially at 10 μ and stained with Heidenhain's azan stain or Heidenhain's haematoxylin and eosin.

Observations

(I) *Histological observations of the melanotic portion*

On some hundred fishes infected by the trematode larvae, the number, and the site of

the dark spots, which were attributable to the encysted parasite, on the skin of the body and fins in each of these animals was examined. The number of the dark spots varied with each individual ; it extended from one to several tens. A certain individual possessed on the skin so numerous dark spots as of seventy six in number. These spots were present on the tegumentary system from the tip of the snout to the caudal fin, including the cornea, as already stated in the previous papar (Iga 1965). They occurred also in parasitized areas on the lower jaw or the abdominal region, where the melanophores normally did not exist or a little.

When the melanotic portion with a dark spot was studied histologically, an encysted trematode larva was found above or below a scale, but not in the muscle tissue. The cyst was surrounded centrifugally by three or four layers of melanophores in the fully black skin. A dark spot on the skin concealed a cyst in a patch on the most case, but two cysts were rarely obsereved In this case, it may be the result that two parasites infected in the adjacent region of the skin. The connective tissues were hypertrophied so that the skin was more than three times as thick as the normal skin from a coresponding part of the body (Fig. 1).

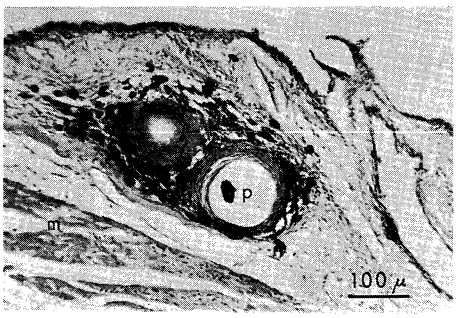

Fig. 1 Section of a severely melanotic portion, showing aggregation of the melanophores around the encysted parasite. m ; muscle tissue, p ; parasite, s ; scale.

(II) *Development of the melanophores in the parasitized region*

In order to elucidate the process of the aggregation of the melanophores in the region of the encysted skin parasite, direct observation of a parasitized area was made periodically *in vivo* for one month. Several fishes which possessed a small number of melanophores at the site of infection, in consequence the parasitized area could not be yet recognized as a dark spot with the naked eye, were chosen as materials. A portion of the caudal fin was exclusively used for ease of the microscopic observation. This portion was transparent and moreover flat, so it was suitable for the photographic researches. Being anesthetized by 1 % urethane solution, animals were photographed every day on the region of the encysted parasite on the caudal fin. In this way increases in the number, changes in size, and morphologies of the pigment cells were recorded photographically. A typical example of the formation of the dark spot was produced in figure 2. As shown in this figure, the pigment cells were newly formed in the area where the parasite infected, but not gathered there by migration of the melanophores which had existed normally in the adjacent region. No mitotic figures of the pigment cells were also observed. The melanin-containing cells appeared first in a small number in the region near the cyst and gradually increased in their numbers around it. Thus the infected area became dark more and more in color and developed into the recognizable dark spot.

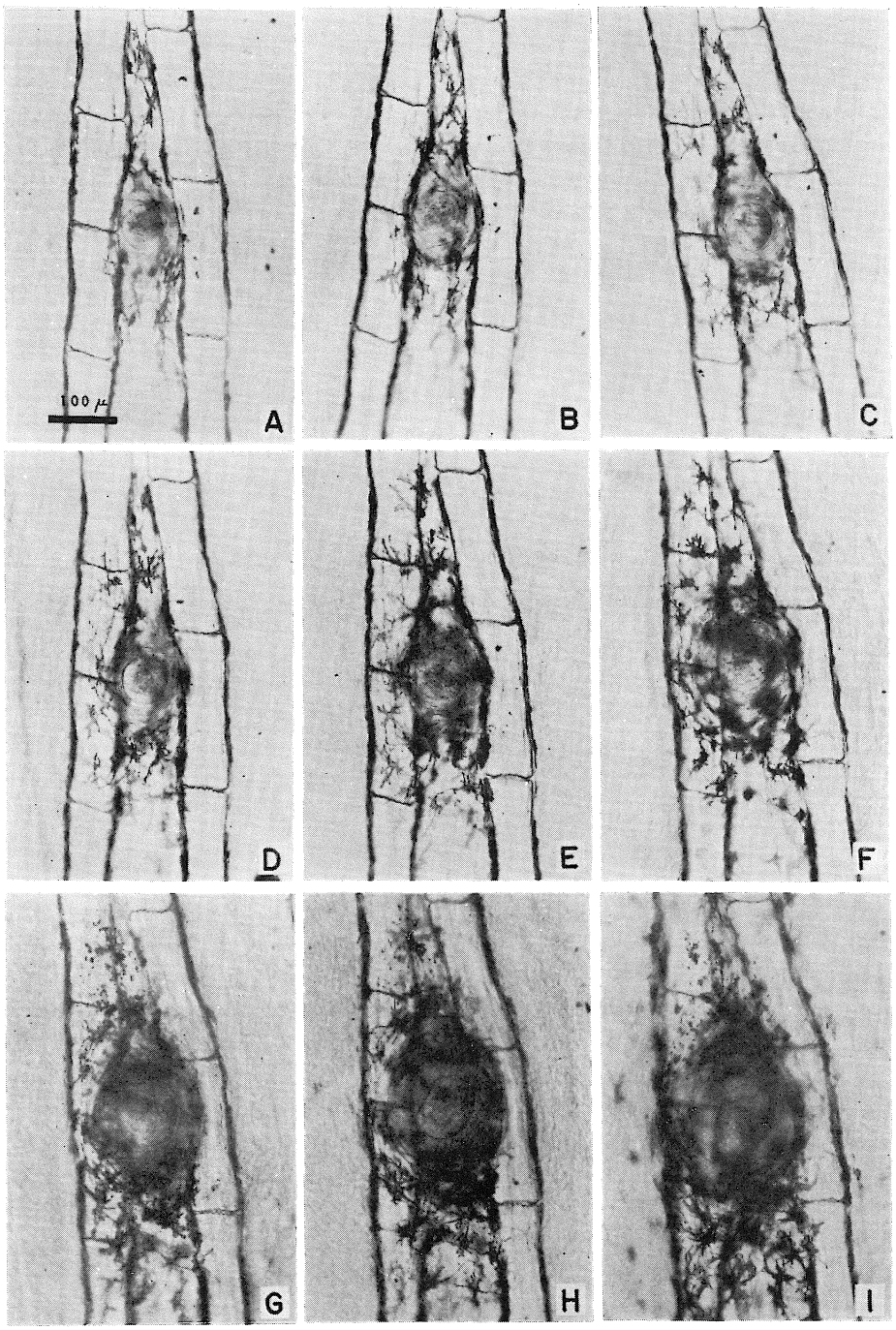

Fig. 2 Formation process of the dark spot on the caudal fin. A, At the beginning of observation. The eight subsequent photographs (B-I) were made after the following number of days : 1, 2, 3, 4, 5, 6, 9, 14. room temp. 17.0—19.5 °C.

Figure 3 illustrates the increase of melanophore's number and the change in size of the cyst. The axis of abscissa in this figure indicated the days after the beginning of this observation. Eighteen pigment cells in number were already produced in the region near the cyst on this preparation, when this investigation was begun. After 5 days the number of pigment cells increased in three times, and nine days after the examination the pigment cells in the region around the parasite were so numerous that they formed a continuous sheet, making it impossible to assertain their number.

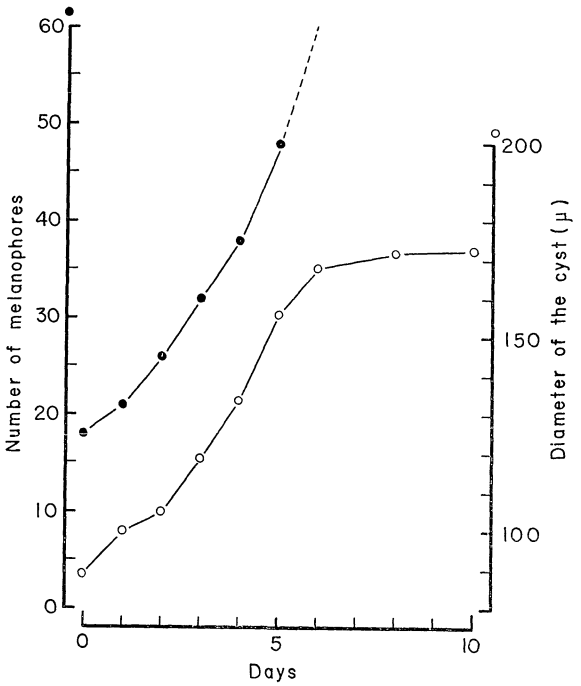

Fig. 3 Increase of the melanophore's number around the parasite and the change in size of the cyst.

The first recognizable pigment cells contained few melanin granules, they were small and somewhat dendritic. Then they increased in size and developed somewhat more pigment and dendritic processes. Few days after their appearance they differentiated into the melanophores of the well developed branched types. Figure 4 showed appearance and transformation of pigment cells at the site of the encysted parasite. These morphological differentiation of the pigment cells proceeded at the site of their appearance. However some of these pigment cells were obsereved to move slightly in the early stage of development. The direction of the migration of the pigment cells in these cases did not seem to be decided. Therefore, such a migration might play no part in the formation of the dark spot essentially.

In a parallel series of infected portions, a normal part of the caudal fin where the parasite was not contained was observed periodically during the course of experiments in the same procedures. In this part, an increase or a decrease of the melanophore did not occur.

(III) *Cessation of development of the pigment cells after the death of the parasite*

It became obvious from the observations in the previous section that the pigment cells

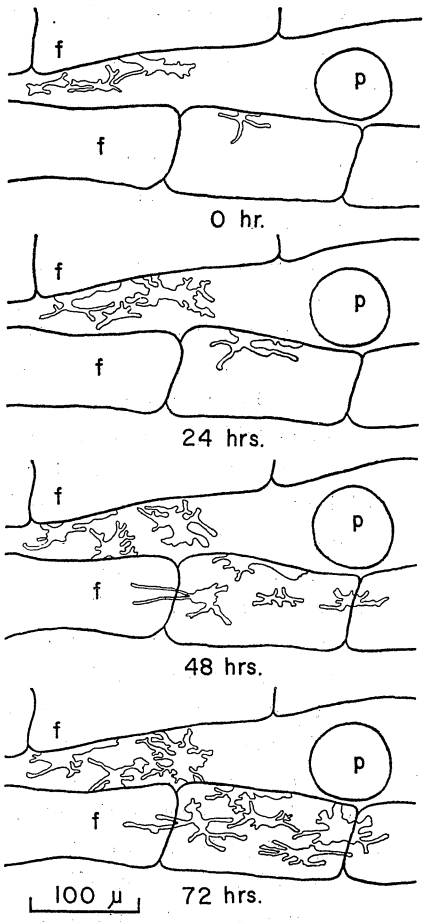

Fig. 4 Diagram of appearance and transformation of pigment cells around the encysted parasite. f; fin ray, p; parasite.

were formed in the site of infection of the parasite. In consequence, it may be interest to ascertain whether an occasion of development of these pigment cells depends on a mechanical stimulus, an invasion and a settlement of the parasite in the skin of a fish, or on a chemical one which the encysted parasite gives the host tissues. There was an individual which possessed in a region of the caudal fin an encysted parasite which might die soon after the invasion in the skin. There existed two melanophores in the area of infection at the time of the initial examination. On this fish, a periodic observation was continued for one month. However it was observed that no production, nor reduction in melanophores occurred in the area ; instead the number of these cells remained constant.

Discussion

Although *Oryzias latipes* which possessed on the skin the dark spots induced by the parasites were all the year round, they were collected in large numbers on and after October, when it began to decrease in atomospheric temperature. Further, the parasitized fishes which possessed the infected area in which aggregations of pigment cells did not yet proceed were sometimes found in the period of the late part of October. In the period of the middle part of November, the regions of infection were so dark in all the fishes observed. Sasaki (1935) pointed out, using *Carassius auratus* infected with trematode larvae, that the aggregations of melanophores concerned with the parasite might produced by a decrease in atmospheric temperature. It followed from the seasonal observations that the same was true of aggregations of pigment cells around the encysted parasites in the skin in *Oryzias latipes*.

Hsiao (1941) described a codfish displaying an unusual degree of melanosis associated with trematode infection. In this paticular cod the number of melanophores was so much in excess of that found in an ordinary cod and they so completely covered the whole body that migration of melanophores could not account for them. From these negative observations, he thought that the melanophores had to develop anew. A remarkable development of melanophores in an unusual region was reported by Osborn (1940), who demonstrated that functional melanophores were induced to form on the normally pale ventral surface of summer flounders either by placing the animals in black tancks

illuminated from below, or by blinding them and illuminated underneath. He (1941) further studied the source of these experimentally induced melanophores in the same fishes. He observed no subsequent migration of the melanophores in the scales of naturally unpigmented areas from originally pigmented surfaces. He concluded that the melanophores developed in the ventral scales *in situ* from potential melanophores (melanoblasts) whose presence was evidenced by the positive "Dopa" reacton, by direct observation of various stages of differentiation using direct and reflected light, by studies on regenerating scales.

It has been generally accepted that the locomotor activity of melanophores is limited only in their very early stages of growth. However, Caldwell and Caldwell (1962) indicated that, in an Atlantic goatfish, *Mullus auratus*, and in an Atlantic short bigeye, *Pseudopriacanthus altus*, melanophores continued to move well beyond the larval stage in these marine fishes that underwent metamorphosis with an environmental changes. They furthermore found that there were aggregations of melanin-containing cells in regions of encysted skin parasites in a Pacific goatfish, *Mulloidichthys xanthogrammus*. They thought that these aggregations of pigment cells raised the possibility of the melanophores' ability to migrate even when the fish had become adult and pointed out the generally accepted assumptions concerning melanophores in fishes had to be restricted to observed species. The present work from direct observation of the parasitized area *in vivo* indicated that, in *Oryzias latipes*, the characteristic dark spot was formed by the excessive development of the melanophores in the infected region of the skin parasite. In the previous papar, using *Oryzias latipes* and *Carassius auratus*, the present author (1965) pointed out that the pathologically induced pigment cells were indistinguishable in some physiological respects from the normal melanophores. When the transformation of newly developed pigment cells was traced in the present work, they showed the complete series of developmental stages from the small, somewhat dendritically, pigment cells to the large, dendritically fully pigment cells. These developmental stages of the pigment cells parallel those of the normal melanophores. From these observations it may be apparent that these pigment cells are indistinguishable also in embryological respects from the normal melanophores,

The occurrence of pigment cells in the skin of xanthic, melanin-free goldfish by X-ray irradiation has been described by several investigators (Smith 1935, Ellinger 1940). Chavin (1956), working with the goldfish, induced melanization by stress, the immersion of the fish in a salt solution, in intact fish and by implantation of goldfish or carp pituitaries into intact and hypophysectomized fish. He concluded that the release of ACTH from the pituitary is stimulated by exposing the fish to a salt solution (Chavin 1959). Recently, Egami *et al.* (1962) showed from the experiment of X-ray irradiation of the goldfish, that ACTH secretion from pituitary gland was stimulated by exposing the goldfish to X-rays, and the melanophores in the skin were produced by the oversecretion of the hormone. The development of the pigment cells around the parasite did not proceed after the death of the larval parasite. This observation suggests that, in melanization induced by the encysted parasite, a tropic influence for development of the pigment cells is probably effective in a chemical rather than in a physical stimulus.

Summary

The present papar described a course of formation of the dark spot, which comprised a mass of the melanophores and which was induced by the encysted larva, on the skin in *Oryzias latipes*. Developmental observations were carried out over a period from October to November.

Direct observation of a parasitized area showed that, in this fish, the pigment cells were newly formed in the area where the parasite infected, but not gathered there by migration of the melanophores which had existed in the adjacent region.

The process of differentiation of the newly developed pigment cells paralleled with that of the normal melanophores.

The development of the pigment cells around the encysted parasite did not proceed after the death of the larval parasite.

This work was done in cooperation with Miss Sumiko FUKUDA. The author wishes to express his cordial thanks to Prof. H. Nagahama of Hiroshima University for his encouragement.

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[MANUSCRIPT RECEIVED SEPTEMBER 6, 1965]