DYNAMICS OF LATERAL STRIPE FORMATION IN THE ZEBRA FISH, *BRACHYDANIO RERIO*

By

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DYNAMICS OF LATERAL STRIPE FORMATION IN THE ZEBRA FISH, *B. RERIO*

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This investigation was concerned with the formation of the lateral stripe in the zebra fish, *Brachydanio rerio*. This stripe, which consists of a row of anterior-posteriorly oriented melanophores, extends along the body in the horizontal skeletogenous septum over about a 25 somite span.

It was found that individual three day old fish showed differences in the numbers and arrangements of melanophores in the stripe while in eight day old fish the stripes were more nearly equivalent. Analysis of the regulatory mechanisms which control cell number and placement was done by extirpating the appropriate melanophores at defined stages in the first eight days

SCOPE & CONTENTS cont'd.

of development. When individual cells were removed at three days, the experimentally produced defect was regulated 85% of the time. When all the melanophores present at three days were extirpated in fish with large numbers of these cells, approximately 90% of the total number of eight day melanophores seen in control fish was present at eight days.

A scheme of pattern development involving two controls has been proposed for the early lateral stripe of the zebra fish. The first is the excluding effect that the three day cells have on the appearance of second wave melanophores (position control) and the second is the control of cell number, or numerical regulation. These two processes work together to ensure that the melanophores will be evenly distributed along the stripe in the appropriate numbers.

Observations were also made of later fates of the eight day lateral stripe melanophores. These cells can reorient out onto the flank to help contribute to the juvenile stripe or can remain stranded in the horizontal skeletogenous septum either as deep or more superficial cells in which reorientation is not complete. They can also undergo what appears to be a programmed type of cell death if they reorient onto the flank but

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do not successfully migrate into the forming juvenile stripe. This latter process would ensure that pattern aberrations do not occur.

This study shows that in the development of the zebra fish melanophore pattern there is a delicate interplay between cell position and number and that early lateral stripe melanophores may have important roles to play in further band genesis.

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CHAPTER I

INTRODUCTION

Pigment cells or chromatophores are the foundation for many biological phenomena. They control animal coloration by their arrangements, by internal redistributions of their pigmented organelles, or by transfer of the organelles into surrounding areas. In many instances chromatophores are arranged in specific patterns which enable the animal to attain some degree of camouflage in the environment. Many animals can furthermore modify their colour patterns in response to certain environmental stimuli and this affords them an even greater degree of protection.

There are two ways in which changes in pattern can occur. The first is called physiological colour change and is usually brought about by some rapid change in the environment, for instance a change in background. This type of colour change involves a modification of the original pattern so that it looks different. The numbers and arrangements of the cells do not change but granule rearrangement occurs within the pigment cells themselves. In many amphibians the vehicle for colour change is the dermal chromatophore unit consisting of three types of pigment cells arranged in three layers at the surface of

the animal (Bagnara, Taylor and Hadley, 1968). The numbers of each kind of chromatophore in the unit vary from species to species but the basic pattern is similar (Bagnara and Hadley, 1973). Yellow xanthophores comprise the outermost layer. These cells contain pteridine and carotenoid pigments in different proportions, depending on the species. In the middle layer are silvery iridophores, which contain purines (mostly guanine) deposited as reflecting platelets which act as light reflectors due to their orientation in the cell. The most basal layer consists of melanophores, black pigment cells containing the pigment melanin deposited in granules called melanosomes. Cytoplasmic arms of the melanophores extend up around the iridophores and terminate between the iridophores and the xanthophores. When a frog is on a dark background, the melanosomes of the melanophores migrate into the arms (expansion of melanophores) and thus obscure the iridophores. At the same time the opposite reaction occurs in the iridophores. Their reflecting platelets aggregate, thus reducing the reflecting surface even more. These opposite responses act to effectively darken the animal. On a light background the reverse occurs - aggregation of melanosomes (contraction) and dispersion of reflecting platelets. The animal then becomes much lighter in colour. Bagnara and Hadley (1973) point out that the actual colour seen by the observer depends on the orientations of the reflecting platelets, which determine which wavelengths will be scattered back

to the xanthophore layer. This layer then selectively absorbs some of these and transmits the rest. Physiological colour change is thus quite complicated and depends on the coordinated and opposite responses of two types of pigment cells. This kind of colour change is usually quite rapid and, naturally, the greater its rapidity, the greater is its benefit to the animal in question.

The second, slower type is called morphological colour change and is particularly interesting because it involves an actual alteration in the amount of pigment carried by the animal. The initial formation of a pigment pattern in the life cycle, which is a good example of this type of change, is brought about by the differentiation and arrangement into patterns of pigmented cells or their unpigmented precursors which subsequently become pigmented. Further morphological colour change can be brought about by the differentiation of more pigment cells, by mitoses of existing pigment cells, by a higher or lower rate of pigment deposition, or by the loss of cells. A combination of these processes can also occur. Morphological colour change can also be influenced by background and this will be discussed later.

Statement of the problem

Morphological colour change can thus occur in many ways. To study it a system is needed in which the different processes can be dissected from one another and

can be easily observed. The zebra fish, Brachydanio rerio, is such a system because much of the patterning process is carried out by pigmented cells. The zebra fish is a tropical Cyprinid native to southern Asia and Indochina. It is characterized by its distinctive pattern of alternating black and silvery-yellow stripes aligned parallel to the long axis of the body. There are usually five black stripes on each side of the body, though the most ventral may not always be well defined. When the melanophores are contracted, each black stripe is seen to consist of hundreds of these cells. The silvery-yellow stripes between the black stripes are more yellow in some fish than in others presumably because they carry different xanthophore complements. The stripes extend onto the tail and body fins (Fig. 1). The formation of this simple striped pattern was the initial interest in this study and attention has been focussed solely on the melanophores.

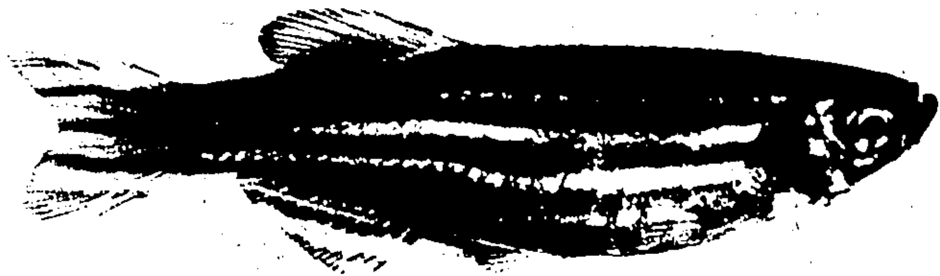
In this thesis, the terminology used to describe the melanophores of the zebra fish will be that of Fitzpatrick et al., (1966). While these terms are not usually applied to adult poikilotherms they are of value in distinguishing the developmental stages of the melanophores which form the early patterns.

Melanocyte: A cell which synthesizes the melanin-containing organelle, the melanosome. The melanocyte may

FIGURE 1

Adult female zebra fish, *Brachydanio rerio*

Magnification 4X



synthesize non-melanized, partly melanized or totally melanized pigment granules.

Melanophore: A melanocyte which is capable of taking part in physiological colour change by dispersing or aggregating its melanosomes. Any kind of melanocyte may undertake this reaction. Cells which do not behave as melanophores but carry any kind of melanosome (non-melanized or totally melanized) will be designated as melanocytes in this thesis.

Melanoblast: The cell which is the precursor of the melanocyte and melanophore.

Melanosome: The pigment granule in the melanocyte or melanophore in which melanization is complete.

Premelanosome: All stages in the development of the mature melanosome.

The first step in this developmental analysis of pattern formation was to examine the patterns of young fish to see if they in any way foreshadow the adult pattern. An eight day old fry has a much simpler melanophore pattern than does the adult. It consists of five stripes, one dorsal band, one lateral band on each side, one yolk sac band and one ventral band. The dorsal band is a double row of melanophores which extends from behind the head to the final somite, curving beneath it to a point on the ventral body wall (see Fig. 2). On the head, the melanophores are arranged in a diamond-shaped

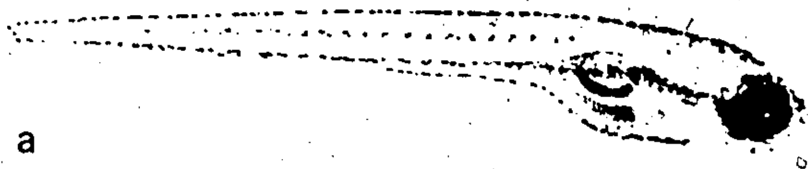
FIGURE 2

Eight and three day old fry

(a) Eight day old fry: Note that the melanophores of the fry contain differing amounts of melanin.

(b) Three day old fry: The stripes of the fry are still rather ragged. An early second wave melanophore is visible (arrow).

The diagrammatic representation and the photographs show the early melanophore bands and some of the body markers that are used in describing the pigmentation patterns. In neither of the photographs are the developing fins or somites visible. This is because the fry were photographed on sheets of white paper to make the pigment cells more visible. Somites are sketched in on the diagram of the three day fry which only shows body markers and the initial lateral stripe.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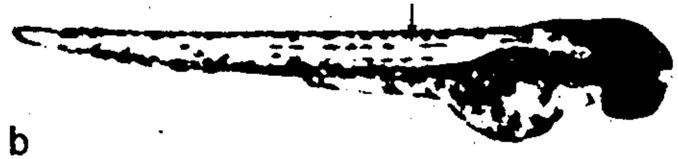
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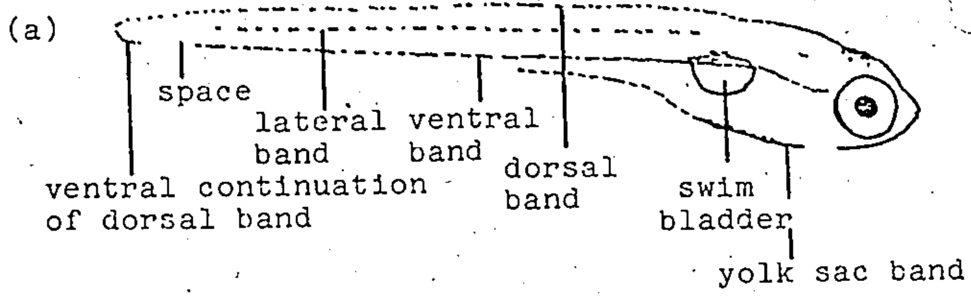

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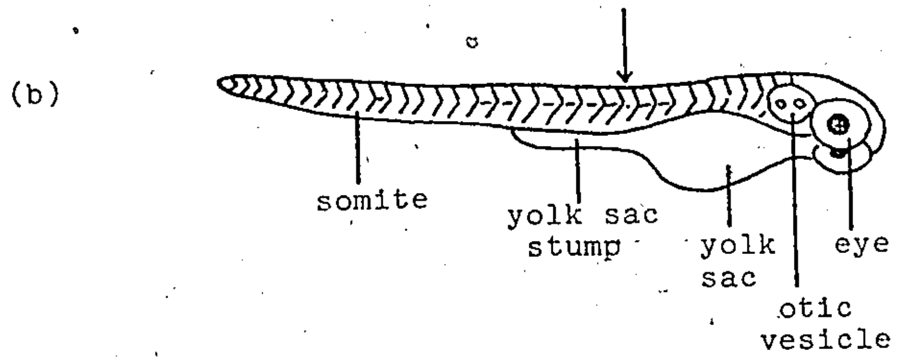


configuration while just posterior to the head a broad row exists which soon bifurcates to the double row of melanophore clusters, which merge again near the tail. Each lateral band consists of a single row of cells aligned in an antero-posterior direction, over about a 25 somite span. When viewed from the side these cells appear flat, but when viewed from above, each cell is seen to be irregular in shape. The ventral band extends from between the eyes over the yolk sac as a broad band and continues caudally, ventral to the somites as a double row of melanophore clusters which stops abruptly before the tail. It is followed by a space separating it from the ventral continuation of the dorsal band. The yolk sac band exists as a diamond-shaped configuration beneath the yolk sac and narrows to a band on the yolk sac stump where it terminates.

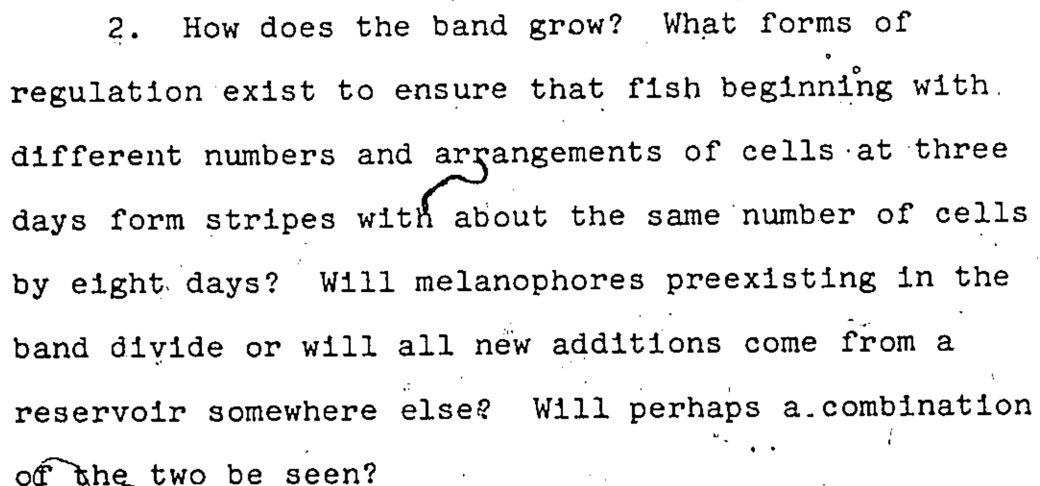
A three day fry has the same general whole body pigmentation pattern as an eight day fry. However, while the eight day lateral stripe consists of on the average 30 melanophores, the three day band may contain pigment cells varying in numbers from zero to 20 (Fig. 2b). Furthermore, right and left lateral stripes do not necessarily contain the same number of cells and the melanophore arrangements in the bands differ, not only between fish, but between the right and left flanks of one fish. In some cases the melanophores are

arranged as pairs in each somite while in other cases there is only one cell per somite. We speak of the number of cells per somite only to make their positions on the body easier to visualize. The cells of the lateral stripe are not located in the somites, but the somite boundaries offer a convenient way of mapping them on the body.

After these first observations had been made, the focus of interest changed from later to earlier stages in pattern genesis and the lateral stripe was selected for intensive study. The following three series of questions were asked about the lateral stripe of the zebra fish:

1. How is the three day lateral stripe formed? In the situation where two pigment cells occupy the area bounded by one somite, did they arise from separate melanoblast to melanophore transformations or from the division of a melanophore already in position?

2. How does the band grow? What forms of regulation exist to ensure that fish beginning with different numbers and arrangements of cells at three days form stripes with about the same number of cells by eight days? Will melanophores preexisting in the band divide or will all new additions come from a reservoir somewhere else? Will perhaps a combination of the two be seen?



3. Does the selective removal of melanophores in the stripe have any effects on further band development? Will the regulatory processes seen normally between days three to eight accelerate so that the defect is repaired, will some other novel mechanism of repair occur or will a permanent defect result? This latter result would perhaps have widespread ramifications for the patterns to follow and could, if the defect were great enough, have behavioral effects as well, for example, if the fish were not recognized by others of the same species. The ability to reform patterns would seem to be an important characteristic of an animal that lives against a changing background.

These questions thus formed the basis for this investigation. The study has been focussed on methods of pattern control in the lateral stripe of the zebra fish up to eight days of age. Specifically, what forms of regulation exist naturally and what forms can be called forth when the cells in the band are faced with the loss of some or all of their number. Results from experiments such as these should provide information concerning some of the fundamental pattern controls which exist in the young fish.

CHAPTER II

HISTORICAL PERSPECTIVES

1. The early experiments of Victor Twitty

Examination of some of the earlier work on the neural crest and its pigmented derivatives will provide a frame of reference not only for the general topic of pigmentation but for zebra fish pigment pattern development as well.

The neural crest, a transitory embryonic structure, arises early in development as a dorsal strand of cells between the neural tube and the overlying ectoderm. Soon after its formation its cells may migrate long distances through the body to give rise to a diverse array of tissue components including mesenchyme, odontoblasts, ganglia, parts of peripheral nervous structures, adrenal medulla tissue, some supporting elements of the nervous system and pigment cells (see Weston, 1970). In this discussion only the pigmented derivatives of the crest will be considered.

No discussion of pattern formation would be complete without an examination of some of the experiments of Victor Twitty. His contribution was enormous, not only because he developed what was at that time a workable theory of how and why patterns formed, but because his work was presented in such a way that the delight and excitement generated by these studies are immediately

sensed by the modern reader. Some of Twitty's hypotheses have since been proven wrong (for instance Youngs, 1957) but this in no way invalidates his experimental work. In fact, its relevance today stems from the fact that his ingenious experiments still do serve as the starting point for much experimentation and discussion.

The experimental animals used in Twitty's work were species of salamanders having different pigmentation patterns. Two species used throughout much of the work were Taricha torosus and Taricha rivularis. The pattern of T. torosus (Fig. 3) consists in part of a pair of black melanophore bands running antero-posteriorly near the dorsal midline, and connected by melanophore extensions to a deeper antero-posteriorly arranged group lying close to the neural tube yet visible through the dorsal skin. The remainder of the pattern consists of a few melanophores localized at the yolk-somite border. The pattern of T. rivularis (Fig. 3) consists of melanophores which are paler and more numerous than those of T. torosus and are spread uniformly over the back and sides of the animal. They extend further ventrally than the yolk-somite border. No cells are seen near the neural tube.

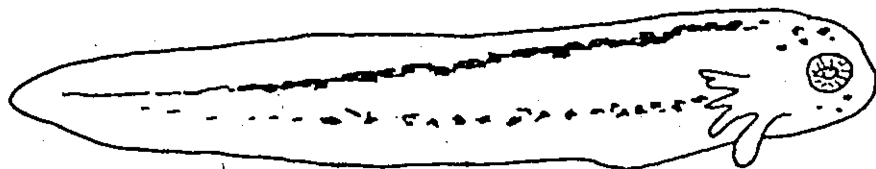
The discovery by DuShane (1935) that pigment cells are derived from the neural crest opened up a wide range of possibilities for experimentation. Since transplantation of neural crest, neural tube, somites and ectoderm was feasible, it was possible to discover

FIGURE 3

Sketches of the pigmentation patterns of (a) *Taricha torosus* and (b) *Taricha rivularis*.

(The melanophores of *T. rivularis* are stippled to indicate their lower content of melanin [redrawn from Twitty, 1966]).

(a)



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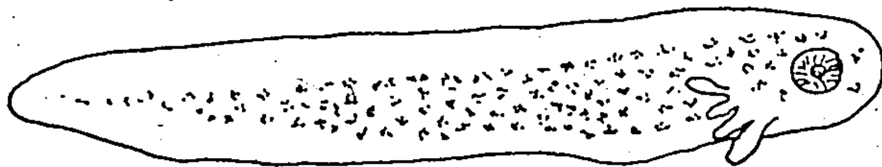


what caused these two patterns to be formed from cells which initially had the same topographical location in the embryo.

(a) Ectodermal effects on pattern formation

(i) Regional differences

Twitty's first experiments were done with *T. torosus* (Twitty, 1936). Stage 22-28 (early tail bud) back ectoderm was replaced with that from the flank or the belly. The crest cells which would begin to migrate out of the crest at around stages 29-30 would then encounter ectoderm from different regions of the body. In all experimental tests the bands which formed were normal. Therefore, at these early stages differences in ectoderm could be ruled out as far as their role in pattern formation was concerned.

(ii) Temporal differences

The next experiment consisted of transplanting flank ectoderm from older (stage 34-38) embryos dorsally onto younger (stage 25-28) embryos. With the oldest ectoderms used "the dorsal bands were absent beneath the graft in four, 'faint' in one and present only on one side in the remaining two " Twitty (1936). In one case this latter result was found to be due to slippage of the graft to one side while in the other case no note

was made if slippage had occurred. Twitty felt that either the pigment cells were prohibited from entering the graft or they relied on ectodermal substances for melanin production and the melanogenic potential of older ectoderm was not sufficient to allow melanization to proceed. On the basis of DuShane's (1935) work with the white (designated albino) axolotl, Twitty favoured the second interpretation. This animal had only a few dorsal melanophores but when a graft of black axolotl ectoderm was placed on the flank many melanophores were seen in it. No pigment was seen outside the graft. The black axolotl had many melanophores present on its body normally so DuShane postulated that its ectoderm thus had a "high melanogenic potential". Promelanophores were present in the white axolotl but supposedly could not elaborate melanin because of the low melanogenic potential of white axolotl ectoderm. When a piece of black axolotl ectoderm was substituted for it these latent or dependent pigment cells could be expressed. The melanophores that normally appeared on the white axolotl were thus not dependent or the white axolotl ectoderm could supply a small amount of the necessary melanogenic substances (see DeShane, 1943).

However, later there was a controversy as to whether this was the true explanation. H. C. Dalton (1950) believed that in the white axolotl propigment cells were

prohibited from reaching those places occupied by melanophores in the black axolotl because the environment prohibited their migration. Twitty, however, believed that the case of the white axolotl as propounded by DuShane could be applied to his experiments with old ectoderm. The cells were in place, but due to a lower melanogenic potential of older ectoderm, were unable to make melanin. Therefore, ectoderms of different age contributed to pattern formation in a quantitative way, influencing the number of cells seen but not the overall pattern.

In later studies, Lehman (1953) and Lehman and Youngs (1959) found that there was no difference at all in the bands formed under old ectoderm (stages 30-40) taken from dorsal regions of the embryo. Since Twitty's experimental animals were from stages 34-38, it is rather difficult to understand his experimental results. However, Lehman (1953) points out that after stage 27 when the crest cells begin to migrate out "there is much greater danger of removing a good portion of the host neural crest from the region in which the graft is to be placed." Lehman did find that lateral and ventral ectoderm, and ectoderm - mesoderm combinations did change temporally in their abilities to elicit the different kinds of chromatophore differentiation when transplanted dorsally. Also, he offered proof for Twitty's postulate that even though a

particular kind of chromatophore did not differentiate this did not mean that the cells were not present. Grafts of dorsal ectoderm from stage 37 'crestless' (the neural folds had been removed so that this ectoderm would not be 'contaminated' with pigment cells) embryos were grafted onto the flanks and bellies of stage 23 hosts (Lehman & Youngs, 1959). These grafts permitted melanophores and xanthophores to differentiate in numbers appropriate to the dorsal ectoderm covering them. Mid-ventral grafts were surrounded by pigment-free belly ectoderm. Therefore, the pigment cell precursors were located everywhere but only became pigmented where the environment permitted. An obvious criticism of this experiment is that the arrangement of pigmented cells gives no information about either the positions or the readiness for differentiation into chromatophores of unpigmented progenitors.

However, if we accept Lehman's theory that the pigment cells were everywhere and Twitty's that at even these stages they were prevented from making melanin we can proceed under the assumption that early regional differences are unimportant in determining band formation. The temporal differences observed by Twitty (but not by Lehman) influenced only the visible pattern, not the underlying pattern comprised of both visible and invisible cells.

(iii) Species differences

When stage 24-30 ectoderm from *Ambystoma punctatum*, *A. tigrinum*, *A. californiense*, *Taricha rivularis* and *T. pyrrogaster* were grafted onto *T. torosus* embryos of the same age there was no effect on the host pattern; it formed normally (Twitty, 1936). However, some increases in melanophore number (which were not counted) were seen at the yolk-somite border and possibly in the dorsal cells as well. The greatest increase in melanophore number was seen with *T. pyrrogaster*, a species where lateral flank melanophores are numerous. Twitty felt that these results showed once again that ectodermal effects on patterning were quantitative, influencing only the number of visible melanophores, not their patterns.

(b) Influence of other tissues on pattern formation

The next experiments consisted of transplants of neural crest alone or in combination with neural tube and somites (Twitty, 1936). When a block of tissue consisting of somites from both sides with associated neural crest, neural tube, and overlying ectoderm was transplanted from one *T. torosus* embryo to the flank of another, normal band formation was seen on the graft. When the somites of one side were excluded from the graft, the band formed only on the side with the somites (including the inner band near the neural tube). On the

side lacking somites most melanophores remained attached to the neural tube or scattered around it. When only neural tube and crest with overlying ectoderm were transplanted, even more cells were seen near the neural tube. In cases where small fragments of somite remained, clusters of melanophores were seen around them. When neural crest alone was transplanted, the melanophores were dispersed in and around the graft, but some clumping was still seen. Thus, in *T. torosus*, the neural tube and somites seem to be closely implicated in band formation. When these experiments were repeated with *T. rivularis*, the melanophores spread from the graft in all cases.

The next question asked was this: Where do the controlling factors lie in *T. torosus* and *T. rivularis*? Are they to be found in differences between the neural tube and somites of the two species or within the pigment cells themselves?

(c) Pattern controlling factors reside primarily within the pigment cells

When pieces of trunk neural crest were exchanged between *T. torosus* and *T. rivularis*, the donor crest cells faithfully reproduced their own pigmentation patterns on the host in the area carrying the donor crest (Fig. 4). The intensity of pigmentation of the grafted cells also matched that of the donors (Twitty, 1936).

FIGURE 4

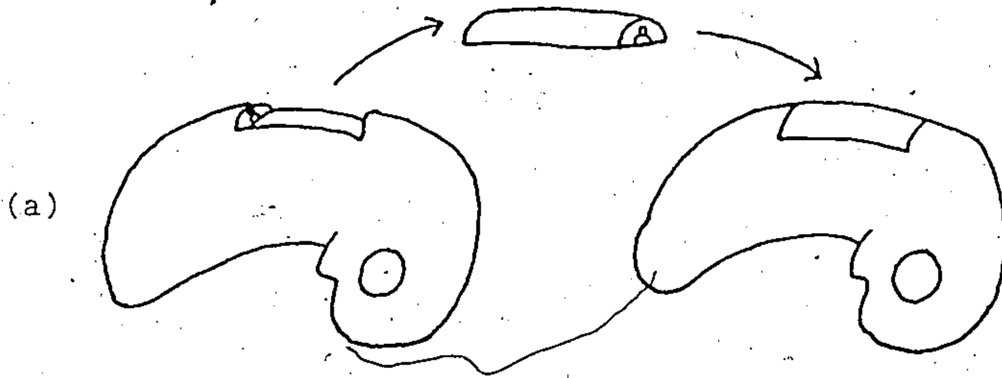
Pigment patterns formed after neural crest exchanges between *T. torosus* and *T. rivularis*

(a) A simplified diagram of the operation (from Twitty, 1966).

(b) *T. torosus* crest on *T. rivularis*.

(c) *T. rivularis* crest on *T. torosus*.

(a)



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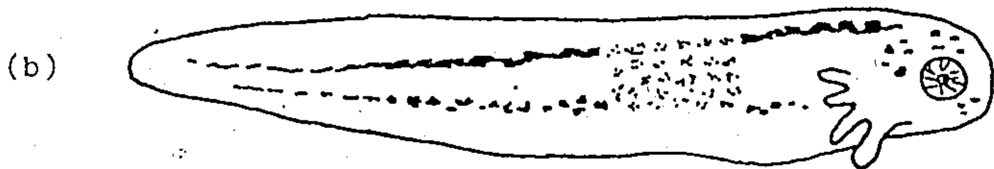

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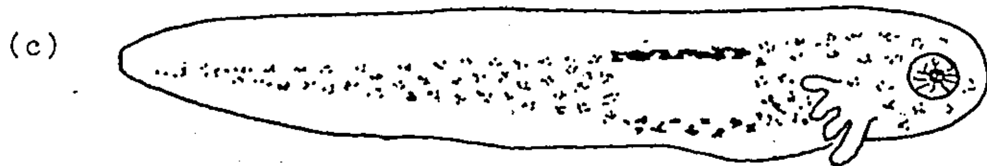


However, two host effects were seen. The first was a quantitative change in the number of cells in the donor pattern. T. rivularis crest on T. torosus had fewer cells present while T. torosus crest on T. rivularis had more cells present. Also, T. torosus cells extended further ventrally than they did on a T. torosus control and T. rivularis cells were halted further dorsally than they normally would be. Thus, there was a host effect on melanophore number and on the ventral migration of pigment cells. Otherwise, Twitty postulated, environmental factors affecting pattern formation were the same in the two species, but T. torosus reacted differently from T. rivularis. T. torosus cells were postulated to have a greater 'glutinosity' or 'adhesiveness' for themselves and for neural tube and somites than T. rivularis cells and thus did not disperse as far from these structures and from each other. During migration the T. torosus propigment cells were deposited near the neural tube and dorsal somite borders. When the somites rose above the neural tube during development the cells could not entirely resist this deformation and so pigment was seen near the neural tube as well as dorsal to the somites. That the cells were attracted to each other was evident from the melanophore interconnections observed between these two groups of cells. Species like T. rivularis were thus reflecting much lower levels of adhesiveness. Since their higher level of dispersion was correlated with their lower

load of melanin Twitty wondered if perhaps as the amount of melanin increased the 'glutinosity' did as well.

Evidence for the relationship between melanization and migration was found when Twitty and Bodenstein (1939) studied cellular migration and melanization in culture. Migration away from the explant was compared when T. torosus and T. rivularis neural tube plus neural crest was cultured in undiluted and diluted peritoneal fluid from adult salamanders and in Holtfreter's solution (a physiological salt solution). When T. torosus crest was cultured greater radii were seen when the peritoneal fluid was diluted 4X and 7X and when Holtfreter's solution was used as compared to undiluted peritoneal fluid. It was also noted that in Holtfreter's solution pigmentation appeared later and was in general less intense. Active migration was restricted mostly to the period before pigmentation began (see also Model and Dalton, 1968).

T. rivularis cells which were paler than T. torosus cells in controls approximated this condition in culture. In peritoneal fluid they became pigmented 24 hours later than did T. torosus cells. Their rate of melanization was slower and, though this was not measured exactly, they appeared to migrate farther away from the explant.

A relationship between melanization and migration was later found for _T. torosus_ cells developing _in vivo_ under different conditions (Twitty and Bodenstein, 1944). When older _T. torosus_ neural crest was grafted to the belly region of younger _A. tigrinum_ and _T. torosus_ hosts melanophores appeared earlier on the faster developing _A. tigrinum_ hosts. Though the appearance of pigmentation was delayed on the _T. torosus_ hosts melanophore migration was more widespread.

Thus, the conditions which influenced melanization _in vitro_ and _in vivo_ influenced the patterns of the cells. Twitty and Bodenstein postulated that band formation in _T. torosus_ therefore depended on the cells reaching the level of differentiation that they reached on normal _T. torosus_ controls. If migration was enhanced, band formation would be hindered simply because the cells would be too far apart to initiate it or because some other necessary prerequisite had not been fulfilled, (for instance, the correct degree of melanization).

Thus, a beautiful analysis of some of the factors that influenced early pattern formation was initiated by Twitty. In the next experiments, attention was focussed on the earlier stage of the process - the migration of cells out of the neural crest.

(d) Why do neural crest cells migrate?

In some of these experiments neural crest cells were allowed to grow on coverslips (Twitty and Niu, 1948). After the explant had been made, a second coverslip was placed near it but held up from the one on which the cells were growing. As the cells migrated out of the explant in all directions they would soon meet this low -ceilinged chamber and could migrate above and below it. The cells that migrated on top of the coverslip had the same cell densities as those that had not come in contact with it. The cells that migrated underneath it, however, quickly moved farther away from each other until they reached its far end, where they once more took up a more crowded arrangement. When the coverslip was shifted from a portion of the outgrowth which had been covered to one which had been uncovered, the cells now underneath it dispersed, and the new centre of dispersion was always from the centre of the coverslip. The cells which were at its near edge with respect to the explant reversed their direction of migration and moved back out underneath it in the direction of the explant! Twitty felt that the environmental effects that influenced the cells' behaviour were substances that they themselves produced and that the cells were responding to graded concentration differences of these substances. If they were responding to absolute levels of some excitatory

substance in the medium, the centre of dispersion in a covered explant would not be the centre of the coverslip, rather the cells would merely speed up their migrations in a direction away from the explant. No cells would be seen to reverse their direction of migration entirely.

Twitty felt that little net movement would be seen in the absence of other cells. This was elegantly shown when small numbers of cells were isolated in capillary tubes (Twitty and Niu, 1954). One cell in isolation shuttled back and forth with no net movement in either direction. Two cells which were initially close together moved farther apart. This result was seen to even greater extent when five cells were isolated together. They became evenly dispersed in the tube with the cells at either end having undertaken quite substantial migrations to get away from their initially close neighbours. Thus, cell-to-cell interactions were important, at least *in vitro*.

Proof that the same kind of spreading was occurring *in vivo* had been found when Twitty and Bodenstein (1944) grafted neural crest from *Ambystoma* to *Taricha*. *Ambystoma* has a much faster developmental rate than *T. torosus* and as pigmentation proceeded the *Ambystoma* melanophores were seen far anterior and posterior to the confines of the graft. However, if the theory was correct, spreading should be possible dorsally as well. Another experiment (Twitty, 1945) showed that this could occur. *T. rivularis* trunk neural

folds were excised at stage 16-17. After healing and closure of the neural plate, a T. torosus neural tube plus neural crest was grafted onto one flank at the yolk-somite border while a T. rivularis neural tube plus neural crest was similarly grafted onto the other side. Regardless of their altered topographical relationship on the embryo, the propigment cells formed the same patterns on each flank as they would normally. A sharp demarcation at the dorsal midline separated the two donor patterns, presumably because the two cell types met dorsally and were stopped from migrating in either direction by the cells in front of and behind them. There was thus no inherent ventral pull in neural crest migration. If the pathways were open, the cells could migrate dorsally, ventrally, anteriorly, and posteriorly. The authors postulated that what controlled the direction of migration were the interrelationships between the cells themselves.

(e) The formation of stripes on T. torosus

Though the pattern of T. rivularis could be explained by simple spreading of propigment cells in different directions, such a mechanism could not account for the stripes seen on T. torosus. To explain stripe formation Twitty (1945) introduced the concept of secondary reaggregation. He said that in normal development melanophores did look as if they were more widespread before band formation was complete but it was difficult to perceive

this because there were so few cells present. If the number of melanophores could be increased by the use of highly melanogenic ectoderm it would be perhaps easier to observe. To test this possibility, ectoderm from the black axolotl was grafted onto the flanks of T. torosus hosts. The number of melanophores did increase, especially at the yolk-somite border and they were more ventrally located than normal. This increased ventral migration should not have occurred if the ectoderm merely enhanced the number of visible cells. A gradual reaggregation process was seen and the melanophores which were initially spread out on the flank reorganized into two bands. Other experiments corroborate this. Clumping was seen both when neural crest was grafted onto T. torosus flanks and when it was cultured, where interconnecting melanophore processes were postulated to be shortening and drawing the cells closer together.

However, other explanations were offered for the stimulation of melanophore number. The first evidence for melanophore stimulation has already been discussed (DuShane, 1935). Black axolotl ectoderm caused more melanophores to appear by stimulating melanin synthesis in 'latent' melanophores which were already in existence. Delanney (1941) compared the increases in melanophore number when ectoderms of higher and higher melanogenic potential were grafted onto T. torosus. The stimulation

progressed in the order *rivularis*, *similans*, *pyrrogaster*, to *tigrinum*. Each ectoderm was said to allow the differentiation of more and more host melanophores which were already present *in situ*, but no actual counts were made. It was postulated that the promelanophore population had a range of thresholds to "X" the hypothetical limiting melanogen (DuShane, 1943). Promelanophores that responded to minute concentrations of "X" would react under weak ectoderm, that of *T. torosus*, while promelanophores with higher thresholds would receive only enough "X" under the more melanogenic ectoderms.

Youngs (1957) tested if the stimulation of melanophore number described by Twitty and DeLanney had occurred. She grafted *A. tigrinum* ectoderm onto *T. torosus* flanks and observed that the melanophores in the graft were more deeply pigmented and more expanded than those elsewhere on the host. When cell counts were made, there were almost the same number of cells on graft and non-graft sides of the animal but their distribution was different. On the graft side, the number of melanophores in the graft had been increased at the expense of the dorsal band above it. Youngs postulated that melanophore competition had occurred between the grafted *A. tigrinum* ectoderm and the dorsal somite borders; the *A. tigrinum* ectoderm had shifted the impetus for band formation ventrally but it had not increased the number of cells.

Another explanation for melanophore stimulation was offered by Dalton (1950). When neural crest exchanges were made between the black and white strains of the axolotl, host pigmentation patterns appeared unlike the case cited by Twitty for *T. torosus* and *T. rivularis*. When triangular pieces of ectoderm were grafted from black onto white embryos more melanophores were seen in the graft when the base of the triangle was oriented dorsally than when it was oriented ventrally (Fig. 5a). When rectangular shaped pieces were used, the graft became pigmented when it was oriented vertically so that it covered the dorsal as well as the ventral flank (Fig. 5b). When the graft was oriented horizontally, no pigment was seen if it was situated below the area normally covered with melanophores in white animals (Fig. 5c). Therefore, Dalton proposed that the positive melanogenic effect of black axolotl ectoderm was due to the better conditions for migration that it provided.

It should be recalled that in the crest exchange experiments between *T. torosus* and *T. rivularis*, Twitty (1936) reported that there was an effect on melanophore number and placement. This could be the same kind of effect. In future work, Dalton's experiments combined with cell counts could help to resolve whether Youngs, Twitty or Dalton is correct for ectodermal transplants onto *T. torosus*. It would also be interesting to do neural crest exchanges

FIGURE 5

Pigmentation patterns observed when differently shaped pieces of black axolotl ectoderm are grafted onto the white axolotl.

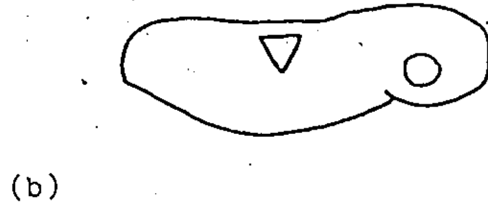
(a) Triangular ectodermal transplant where the base of the triangle is ventrally oriented.

(b) Triangular ectodermal transplant where the base of the triangle is dorsally oriented.

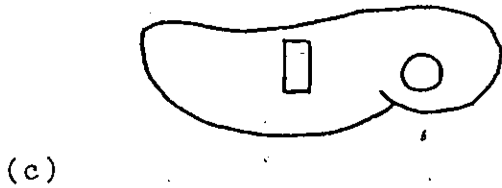
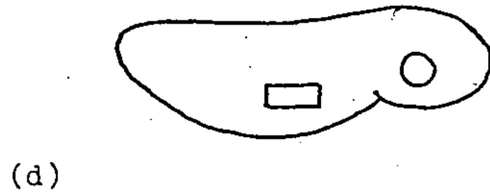
(c) Rectangular ectodermal transplants where the graft covers the dorsal and ventral flanks.

(d) Rectangular ectodermal transplants where the graft is located below the area normally covered with melanophores in white animals.

The diagrams were drawn from a description of the results (Dalton, 1950).



(a) (b)

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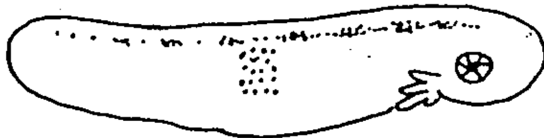
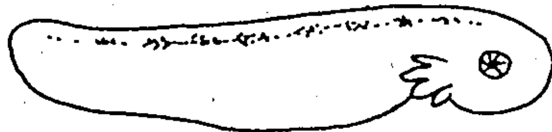
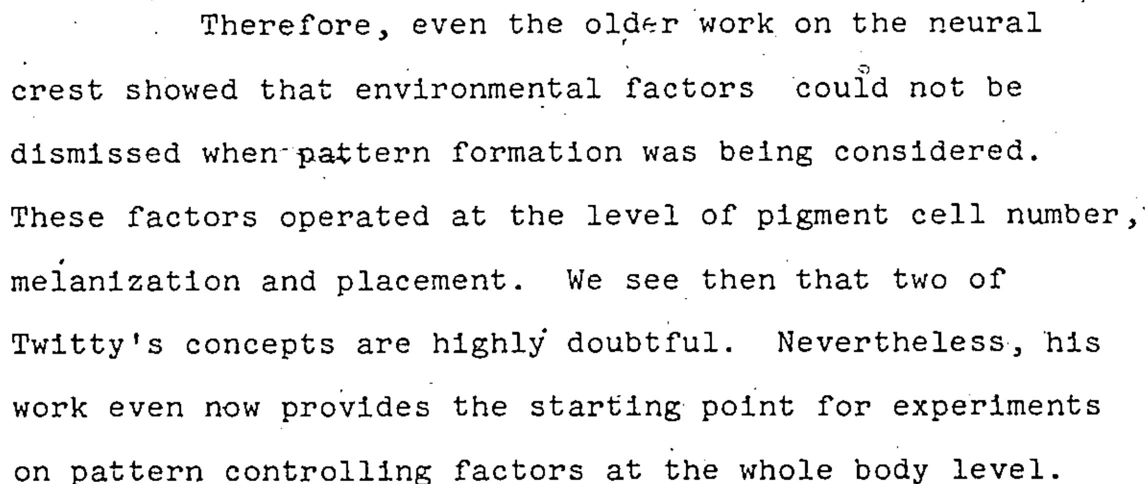



between T. torosus and the other species which were used because as the melanogenic potential of the ectoderm increased, the pattern made by the donor cells could come to resemble the pattern of the host more and more. Twitty's idea that the stimulation of melanophore number does not change the pattern is therefore called into question.

Twitty's (1936) postulate that the index of melanization is related to band formation has also been proven incorrect. Youngs (1957) raised early unpigmented T. torosus embryos in 0.003 - 0.004% phenylthiourea (an inhibitor of melanization). She found that when the inhibitor was removed the cells, as they appeared, were already localized in the bands: that is, band formation had been carried out by unpigmented, amelanotic melanophores.

Therefore, even the older work on the neural crest showed that environmental factors could not be dismissed when pattern formation was being considered. These factors operated at the level of pigment cell number, melanization and placement. We see then that two of Twitty's concepts are highly doubtful. Nevertheless, his work even now provides the starting point for experiments on pattern controlling factors at the whole body level.



2. Contemporary experiments on neural crest to pigment cell differentiation

(a) Neural crest cell migration and determination

Weston (1963) followed the time course and pathways of crest cell migration in the chick. There were two streams of cells, one in the superficial ectoderm and a more ventral one located in the mesenchyme between the neural tube and the myotomes. Once the cells entered the somitic mesenchyme they became segmentally arranged, with cells migrating further ventrally within than between somites. The direction and orientation of migration were the same with respect to the neural tube even if the tube was inverted on the flank or *in situ*. The cells were not therefore attracted specifically to certain areas in the embryo and the dorsal neural tube imposed the initial orientation on migration, which was then independent of the environment. To explain why the cells migrated away from their source Weston supported the suggestion of Abercrombie and Heaysman (1954), from the observations that fibroblasts in culture tended not to move over each other's surfaces = contact inhibition of movement of two cells which were touching. This would mean that crest cells would move away from each other because if they made contact their movements would cease. Twitty and Niu's (1948, 1954) theory of diffusible chemical substances was discarded because it was difficult for

Weston to imagine how these gradients would be maintained in the embryo.

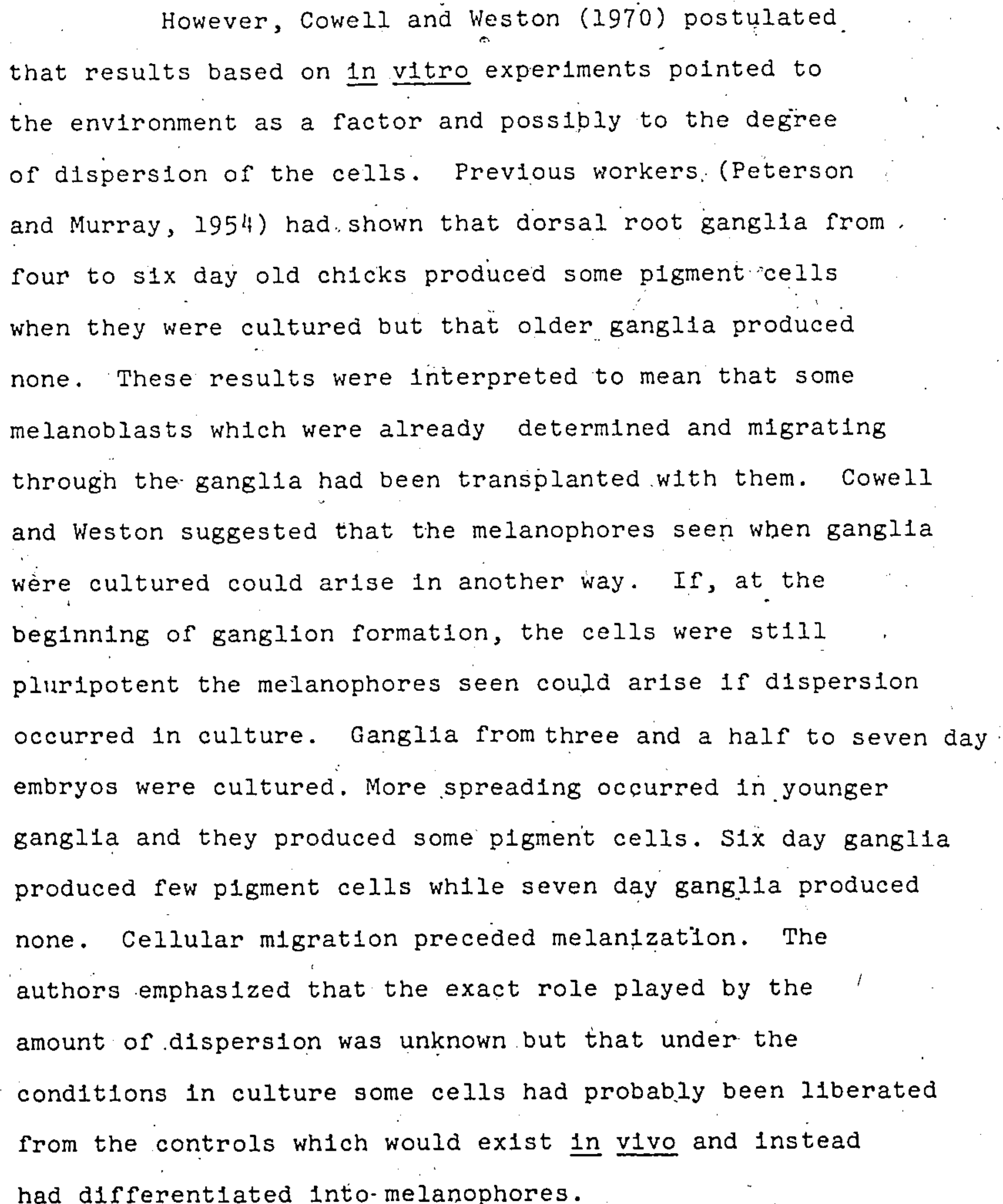
Crest cells which gave rise to integumental pigment patterns were found to migrate in the ectoderm contrary to the results of Teillet and LeDouarin (1970) for the bird and Mayer (1973b) for the mouse. These authors reported that melanoblasts migrate first within mesoderm and later invade the ectoderm. However, in the chick, internal pigmentation patterns arise as well and these cells, it was postulated arose from some crest cells which migrated beyond the area of the sympathetic complex. However, when did these pigment cells leave the crest? Did the cells that left the crest first migrate the farthest and become differentiated as pigment cells or sympathetic neuroblasts while those that moved out last remained closer to the neural tube and differentiated as ganglionic neuroblasts? This possibility was suggested because early in migration there were no accumulations of cells in the regions of the spinal ganglia. Therefore, Weston and Butler (1966) postulated, that 'linear' migration was occurring. This hypothesis would necessitate that the cells were predetermined before they left the crest and that the first cells to migrate out would be pigment cells or sympathetic neuroblasts. Then, they reasoned, if a piece of crest from which many cells had already emigrated was transplanted onto a 'young' host

and deficiencies were seen in the crest derivatives that formed, this would constitute proof that the cells remaining in the crest were developmentally restricted, and that the patterns of crest cell localization depended on characteristics residing within the cells themselves. However, crest of different ages did contribute to both sympathetic and spinal ganglia. Therefore, 'old' crest had the same range of capabilities as did 'young' crest, and the cells which left the crest first were not already determined as sympathetic ganglia cells.

However, the authors suggested that results could still be interpreted in two ways; either the crest cells were determined while in the crest but left it in random order and migrated in response to specific environmental cues, or the cells were pluripotent at the beginning of and during migration and their final locations and patterns of differentiation were under environmental control. Thus, if the first case were true, sympathetic ganglia should always be seen in grafts of 'young' crest to 'old' host. This was not found. With age, fewer labelled sympathetic ganglia were seen. Therefore, the environment did gradually change to restrict migration. The idea that distal migration could be stopped because the niches were already full was discarded for two reasons. The first was that labelled cells never seemed to reach the area and the second was that the authors could not see how the

information that the niche was filled could be transmitted. Aggregations of cells into ganglia would then result from a combination of three factors, "the loss of cell motility, selective adhesion, and proliferation of these cells".

However, it was still not known when pigment cell determination occurred. The older literature postulated that some determination occurred in the crest because a crest cell explant in culture still produced pigment cells. For instance, axolotl neural crest was cultured and the explant was detached at different stages in development without disturbing the original outgrowth. After pigmentation, the percentages of the different kinds of pigment cells were compared in the original outgrowths. There was an increase in the percentage of xanthophores and a decrease in the percentage of melanophores with time (Lehman and Youngs, 1959). It was postulated that prospective xanthophores migrated out of the crest later then did prospective melanophores. This result paralleled the appearance of pigmentation in control animals where xanthophores always appear later than melanophores. Early determination of crest cell elements was also postulated for the frog by Benson and Triplett (1974) who studied the appearance of tyrosinase (tyrosine - DOPA - oxidase) the enzyme that catalyzes the first two steps of the reaction which forms melanin from the amino acid tyrosine. They found that the enzyme makes its appearance at the neurula stage but is inactive until



hatching when overt pigmentation begins. Therefore, in amphibians, crest cell elements may be determined very early.

However, Cowell and Weston (1970) postulated that results based on _in vitro_ experiments pointed to the environment as a factor and possibly to the degree of dispersion of the cells. Previous workers (Peterson and Murray, 1954) had shown that dorsal root ganglia from four to six day old chicks produced some pigment cells when they were cultured but that older ganglia produced none. These results were interpreted to mean that some melanoblasts which were already determined and migrating through the ganglia had been transplanted with them. Cowell and Weston suggested that the melanophores seen when ganglia were cultured could arise in another way. If, at the beginning of ganglion formation, the cells were still pluripotent the melanophores seen could arise if dispersion occurred in culture. Ganglia from three and a half to seven day embryos were cultured. More spreading occurred in younger ganglia and they produced some pigment cells. Six day ganglia produced few pigment cells while seven day ganglia produced none. Cellular migration preceded melanization. The authors emphasized that the exact role played by the amount of dispersion was unknown but that under the conditions in culture some cells had probably been liberated from the controls which would exist _in vivo_ and instead had differentiated into melanophores.

(b) Pigment cell reservoirs

If it is the degree of dispersion coupled with local conditions in the embryo which determines the direction of differentiation of neural crest cells or even if determination occurs early, other intriguing questions are suggested. One of these is what is the size of the reservoir that becomes differentiated along pigment cell lines?

The construction of chimaeric mice has been a valuable tool in attempting to make these kinds of calculations. If cells of two different genotypes (X and 0) are combined in one animal, it is possible to calculate how many cells were set aside for each system that one is observing. For example, if two cells gave rise to a system, then based on randomness the following combinations would be seen: X0, 00, 0X, or XX. The chance of not being chimaeric would be 1/2. The chance of being chimaeric would be $[1 - (1/2)^{1}]$. Similarly, if Y cells were selected to form one system the chance of being chimaeric in that system would be $[1 - (1/2)^{Y-1}]$. If the calculation is now extended to include the total number of chimaeric systems (Ni) the equation becomes $[1 - (1/2)^{Y-1}]^{Ni}$ = the chance of being chimaeric.

Wegmann and Gilman (1970) fused eight cell mouse embryos which differed at three alleles; pigmentation, immunoglobulin, and haemoglobin. Out of 14 mice which survived,

nine were chimaeric to some extent for all three systems. The other five mice were not chimaeric in any system. Therefore, the incidence of chimaerism was 9/14 = 0.64. In this experiment the total number of chimaeric systems (N) was 27 - the number of independent systems (three) X the number of mice (nine). Therefore, the number of cells sampled (Y) was seven, ($[1 - (1/2)^6]^{27} = 0.65$). A minimum of seven cells was thus sampled to give rise to the melanocytes of the coat.

These kinds of studies should give insights into precursor cell population sizes in other animals as well.

(c) Specific chromatophore differentiation

The multiplication of the cells in the reservoirs and their subsequent stages in differentiation are also interesting problems. The factors that cause one type of pigment cell to differentiate instead of another are unknown.

There are two theories about the origin of different chromatophores which are not mutually exclusive. One is the stem cell theory of Alexander (1970). In the adult African chameleon, *Chamaeleo hohnelli* there exist three layers of chromatophores; xanthophores, iridophores and melanophores. In the young chameleon the three layers exist as well and Alexander found that in the iridophores and xanthophores there existed as well

as the characteristic organelles, bodies which were premelanosome-like. The melanophores of the young animal contained premelanosomes as well as melanosomes. However, in the young animal there existed a fourth cell type which contained many premelanosome-like granules but no mature melanosomes. Alexander designated this cell the "chromatoblast" and postulated that the three chromatophore types differentiated from it.

Bagnara (1972) and Bagnara and Hadley (1973) have suggested that during the course of pigment cell differentiation, primordial organelles capable of forming melanosomes, reflecting platelets or pterinosomes may exist in the developing chromatophores. This theory is based on observations that pigment cells often contain organelles not usually found in them. For instance, some of the erythrophores of the adult ribbon snake, <u>Thamnophis proximus</u> contain both pterinosomes and reflecting platelets (which are usually found in iridophores). The interesting thing about these erythrophores which contain more than one type of organelle is that they are found only where the two different cell types are in close contact. They therefore may be an example of chromatophores which are intergrades. Intergrade organelles have also been found. One such example is the iris of the Inca dove, <u>Scardafella inca</u> where some iridophores contain melanosomes and some of the reflecting platelets in these cells contain differing amounts of melanin (or an electron-dense material that appears to be melanin). Here as well, only those iridophores

located next to the tapetal cells (which contain melanosomes) contain more than one type of organelle. These pigment cells are thus also intergrade chromatophores, and, if the material in the reflecting platelets is melanin, they contain intergrade organelles as well. It has therefore been postulated that depending on the developmental stimuli received, the primordial organelle can form any kind of pigment cell organelle. The organelle has not yet been identified but Bagnara suggests that "unknown vesicular structures" which exist in these chromatophores may represent primordial organelles.

Thus, combining the tentative theories about pigment cell differentiation, we can arrive at a hypothesis of control. The pigment-forming cells could be determined in the crest or at some later stage in migration. These cells would make the primordial organelle or differentiate into the primordial stem cell. The specific kind of chromatophore which then differentiated would be under further developmental control. In mammals, the only pigment cell is the melanophore (Searle, 1968) and the progenitors would differentiate only in this direction. In other animals that contain more than one type of pigment cell, dissimilar developmental cues would cause different kinds of pigment cells to form. If as suggested (Bagnara, 1972; Bagnara and Hadley, 1973) primordial organelles exist and the developmental stimuli are not exactly coordinated to the cells on a cell to cell basis, gradations of pigment

cell organelles would result on the body. In fact, the existence of intergrade chromatophores does suggest a wave-like action for pigment cell differentiation, expecially for striped patterns. Thus, conditioned as well by the mitotic rates and migrations of both the precursor and the differentiated cells, different patterns in the shapes of stripes, spots or blotches could be produced.

While these ideas do not all have definitive experiments to back them up, it is unavoidable that we should speculate as to how patterns emerge. The topic, while appearing rather frustrating at the whole body level is an area of great excitement.

(d) Melanophores and genetic markers

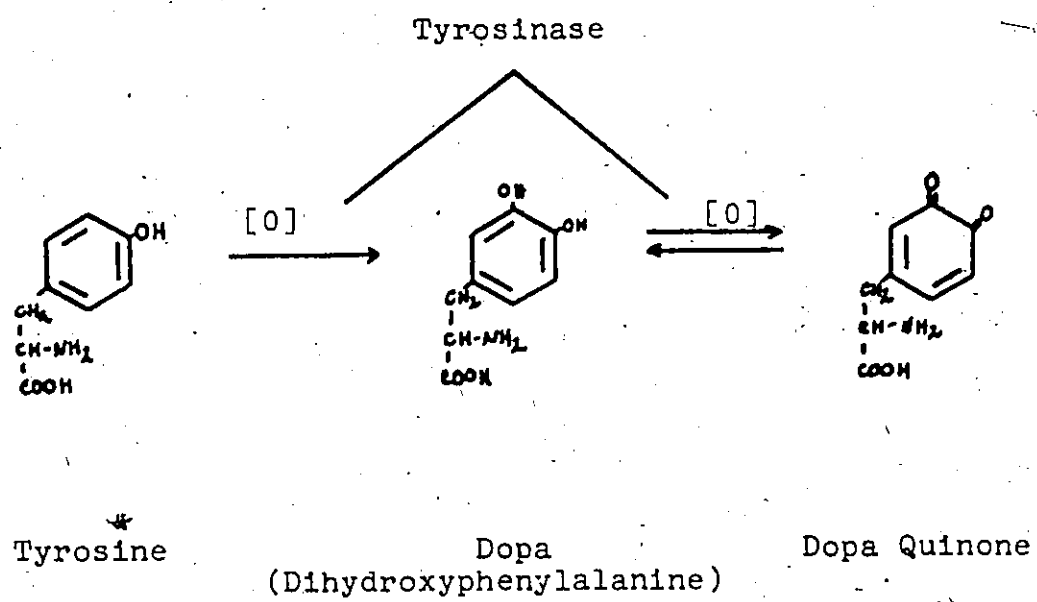
Melanophores differentiate from the neural crest and carry melanin deposited in small granules called melanosomes. Melanin, a complex polymer of high molecular weight, is derived from the amino acid tyrosine and is synthesized on the premelanosome via a complex series of reactions utilizing the enzyme tyrosinase (tyrosine - DOPA - oxidase). The enzyme is synthesized in the cytoplasm and transported to the Golgi complex where it is organized within small membranous vesicles into a fibrillar array (the premelanosome) consisting of the

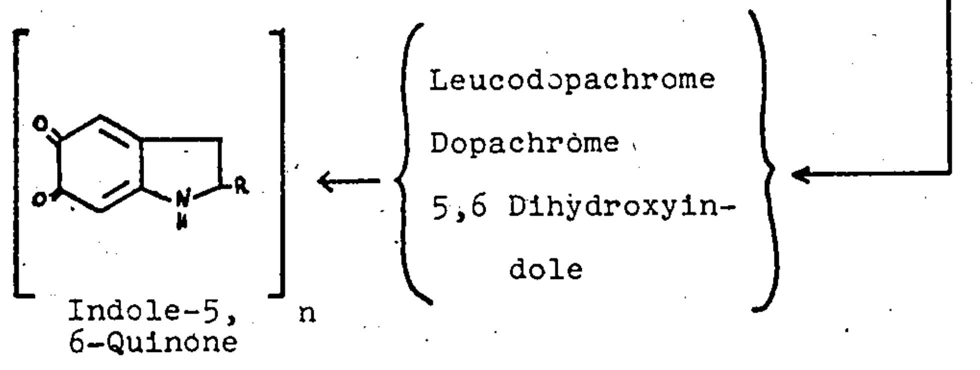
enzyme plus non-enzymic protein (Weston, 1970). Tyrosinase is used in the first two steps of melanin biosynthesis, first to catalyze the oxidation of tyrosine to dihydroxyphenylalanine (DOPA) and then to oxidize DOPA to DOPA-quinone. (There is now some evidence (Okun et al., 1973) that in mammalian melanogenesis the first reaction is catalyzed by a peroxidase and the second by DOPA oxidase). Further complex reactions occur until the complex molecule indole-5, 6-quinone is formed (Fig. 6). As Bagnara and Hadley (1973) point out there is now some evidence that melanin is not a homopolymer of indole-5, 6-quinone units but is a much more complicated structure consisting of indole units at different levels of oxidation and other tyrosine metabolites. The melanin is deposited on the fibres within the premelanosome until, depending on the amount of melanization that occurs, the structural details of the premelanosome can be obliterated. The mature granule is called a melanosome.

At the level of melanization, the genotype can act on the protein framework, shape of the granules and on melanization itself. For instance, tyrosinase may not be produced but the premelanosmes may appear perfectly normal (Searle, 1968). Even if tyrosinase is produced melanization may be controlled in another way - by environmental modification. A good example is the recessive

FIGURE 6

Biochemical steps leading to the formation of melanoprotein (from Bagnara and Hadley, 1973).





Eumelanin + protein ⟶ Melanoprotein

gene (Sl) of mice which leads to a white coat colour in the homozygous condition. The defect in pigment synthesis in this system was related to the skin rather than to the pigment cells by Mayer and Green (1968) when combinations of normal and mutant skin and neural crest were grafted onto White Leghorn chick embryos. When normal crest (to get crest material neural tubes were grafted) was combined with mutant Sl skin, the graft and the surrounding area of the host were pigment free in eight cases but in four cases the unpigmented grafts were surrounded by pigmented host tissues. Therefore, the melanoblasts must have been present (since they migrated out of the graft and into the surrounding skin in four cases) but were prevented from making melanin in the graft. The converse experiment, combining mutant Sl crest with normal skin gave pigment in all cases. Mayer (1973a) has extended this study and shown that the pigment inhibiting effect is located both in the dermis and epidermis, but the level at which it operates is not known.

Other genes act to determine the placing of cells in patterns. Searle (1968) points out that the crest itself may be deficient in some way, for instance it may be reduced or the daughter cells may be abnormal. Inhibition of migration can also occur. Recall the experiments of H. C. Dalton (1950) where the difference in pigmentation between the white and black axolotl was shown to be due

to the superior conditions for propigment cell migration that existed in the black axolotl.

Thus the genotype has profound effects on patterning. From the few examples that have been given it can be seen that the genotype operates at two levels; the level of the pattern-producing and pigment-forming cells and via the environment to also determine pigment cell placement and pigment production. For documentation of patterning genes in mammals, see Searle (1968).

3. Conclusions

The experiments of Victor Twitty thus formed the nucleus for later work on pattern formation. The concepts that were presented placed the impetus for the formation of patterns at the level of the chromatophores themselves subject to environmental modification under certain conditions. However, in looking only at differentiated pigment cells, the early literature attempted to draw conclusions about the events that preceded visible pattern formation. Since these authors were looking at pigmented products, their ideas that the unpigmented cells were already differentiated as chromatophores and that their arrangements as pigmented cells truly reflected their arrangements as unpigmented precursor cells had no experimental proof.

Experiments on the environmental cues that cause pigment cells to differentiate were not done until much

later. The theory developed by Cowell and Weston (1970) that the degree of dispersion coupled with local environmental conditions in the embryo caused pigment cell differentiation opposes the theory that some determination occurs in the crest (Lehman and Youngs, 1959; Benson and Triplett, 1974). The reason for these varying results may simply be that in the bird crest cell elements are determined later than they are in amphibians.

Ultrastructural work combined with cloning experiments on crest cell _in vitro_ under different conditions may be a way of discovering the conditions that cause certain kinds of chromatophores to develop. As Smith-Gill (1974) points out more work must also be done on the conformation of the environment through which the cells migrate. The problem is of course infinitely complex at the whole body level, but appropriate experiments in culture might enable us to at least make some accurate guesses about the factors that cause pigment cells to differentiate and patterns to form. The problem now is therefore one of deciding the level at which specific questions are to be asked and of dissecting out from that level questions which can be answered with the techniques that are now available.

CHAPTER III

MATERIALS AND METHODS

General Materials and Methods will be presented in this chapter and specific procedures will be given for each experiment in the Results section.

1. Maintenance of zebra fish at different stages in the life cycle

(a) Adults: Zebra fish were purchased from local pet stores and maintained in dechlorinated tap water. Room temperature was maintained at 28-30°C to ensure that the temperature of the water was between 26-28°C. Fish were fed jumbo brine shrimp and Tetramin Staple dry food four times a day.

(b) Eggs: Two methods of egg recovery were used. Both emphasized the necessity of separating the newly laid eggs from the adults, as the eggs would otherwise have been eaten. The preferred method of egg recovery utilized a ten gallon tank. A plastic frame was constructed which fit flush with the sides and milliner's net (mesh coarse enough to allow the eggs to drop through) was tied tightly over the frame with linen thread. The net was supported above the bottom of the tank by finger bowls beneath it, and rocks were arranged on it to create shallow

depressions which these fish seem to prefer for egg-laying. About four gallons of dechlorinated water were added to the tank and air was bubbled in gently. A plump female was selected from the holding tank and placed in the breeding tank in the evening. The next morning, after triggering of the light cycle (16 hours light:8 hours dark) and before feeding, an active male was added. Mating behaviour was usually initiated within an hour and 50-300 eggs were laid in bursts of ten to 15 until the female was exhausted. If no eggs were laid the first day the fish were kept together until the next day without feeding. This usually elicited mating behaviour the next day. At around 27° a female will lay eggs every two to three days.

After egg-laying ceased, the adults were taken out, the net and finger bowls removed, and the eggs collected simply by stirring the water and dipping them out with a fine mesh net. The eggs were washed twice in dechlorinated water to remove adhering debris, then placed in finger bowls of dechlorinated water and aerated. Two to three hundred eggs could be successfully raised together and would hatch under these conditions in three to four days.

The second method of egg recovery used one gallon tanks containing breeding traps but was less successful, probably because the fish were more constrained.

(c) Fry: The young fish begin to feed at around four to five days when the yolk supply is almost exhausted, but they are still too young to eat solid food. Wardley's Infusoria Medium (Wardley Products Co., Inc., Long Island City 1, N.Y.) was added to the water to cause an infusorial bloom upon which they could feed. The tanks were set up on day two of development to make sure that when the fish were added on day three the mild infusion was well underway. All batches of fry were raised in six litres of dechlorinated vigorously aerated water. Small quantities of the powder were mixed with water and squirted into the tanks three times a day. If the powder was merely spread on the surface it often did not submerge, resulting in a surface scum. On day three, the fry were added by pouring them out of the fingerbowl into the larger tank. Solid foods, ground to a fine powder with mortar and pestle and added in the same way as the Infusoria powder were gradually added beginning on day six. Wardley's Fryfare, Tetra-E Food for baby fish and Tetra Conditioning Vegetable Diet were the three foods used.

(d) Juvenile fish: Juvenile fish were fed ground brine shrimp and pulverized food three times a day.

2. Contraction of melanophores

To accurately count the number of melanophores, it is necessary to first 'contract' them by providing the

physiological conditions in which the melanosomes migrate to the centre of each cell. Each melanophore then appears as a punctate black dot. Zebra fish can undergo physiological colour change for the first time late in day three, paling on an illuminated white background and darkening on an illuminated black background. The normal illumination in the room is sufficient to elicit this response. Fry and juvenile fish were placed in watch glasses or fingerbowls in sufficient water so that they could swim unimpeded. If the fish was disturbed, that is if it couldn't swim freely, expansion and not contraction of melanophores resulted. The containers were placed on white sheets of paper and full contraction occurred in a few minutes. It was easy to tell by eye when contraction was complete because the reaction was so dramatic that the fish seemed almost to disappear against the white background.

3. Anaesthesia

Since these fish are very active, it is necessary to anaesthetize them before any observations are made. After melanophore contraction had occurred, the container was placed on a bed of crushed ice, also on a white background. As the water cooled, fry would swim more slowly and eventually sink to the bottom where they would twitch a few times before becoming still. Sometimes the heart

ceased to beat entirely but this was not deleterious because the fish were not kept on ice for long periods of time. Observations were then made quickly because as the water heated up, activity resumed almost immediately.

Juvenile fish did not survive this method of anaesthesia so drops of a 0.1% MS-222 (Methane-Tricane-Sulphate, Sandoz Ltd., Basle) solution were added until the fish turned over on its back. Anaesthesia was again carried out on a white background.

4. Inhibition of melanization with phenylthiourea

Phenylthiourea (PTU), (Eastman Organic Chemicals, Rochester 3, N.Y.) was used to block melanin synthesis. It is an inhibitor of tyrosinase, the first enzyme used in the production of melanin. At a concentration of 0.2 mM, PTU allows mapping of pre-existing melanophores, but prevents further melanoblast to melanophore transformations. This concentration of PTU has no deleterious effects on the fish. The PTU was dissolved in a small amount of boiling distilled water and then diluted to 0.2 mM with dechlorinated water. The solutions were changed every two days since the compound gradually decomposes in water. When the fish were removed to dechlorinated tap water minus PTU, repigmentation occurred within 24 to 48 hours.

5. Excision of selected melanophores

The removal of selected melanophores was accomplished with a simple technique requiring only a fine tungsten needle and an agar base to hold the fish. Tungsten wire (#18 gauge, Fisher Scientific) was cut into 2" lengths and fitted into insulated handles. The wire was heated in a bunsen flame and immersed while red-hot into molten sodium nitrite in a spun aluminium beaker. The speed of the reaction, in which tungsten is burned away, was controlled by rubbing the needle against the side of the beaker near the surface of the boiling liquid. After a 10-20 μm tip had been fashioned, the needle was rinsed gently in distilled water and it was bent slightly a short distance away from the tip. It was then trimmed to one inch in length and fitted into a small needle holder.

Three day fry were anaesthetized individually and the site for the operation determined. All extirpations were done on the right hand side and the eleventh somite behind the otic vesicle was the test site. Fish were deposited on a 5% agar base in a watch glass and all water was removed with a piece of absorbent tissue paper. Because the fish was out of water for only a short time this was not harmful and further anaesthetization was not necessary. The operation was done at a total magnification of 30 X. The tip of the needle (with the

curve pointed away from the fish) was applied directly over the melanophore causing the cell to burst. The watch glass was lowered into the recovery medium only low enough so that the fish floated off on top of the liquid. Surface tension acted to hold the fish straight until it recovered and swam down to the bottom.

The fish were raised in 0.1 mM PTU until melanophore fragments had disappeared. This concentration of PTU allowed gradual repigmentation to occur but the fragments of the excised cells could always be distinguished because they were black. The containers were placed on sheets of white paper two feet from fluorescent light sources. The light helped the infusion process and the white paper enabled observations to be made quickly because the melanophores were contracted when the fish were removed from the tanks. Two days after the operation all failures (where the melanophore had not been destroyed) were discarded and on day eight of development the melanophores were 'contracted', the fish anaesthetized and the experimentally produced defects examined.

6. Histological procedures

To visualize the positions of melanophores in relation to other tissues, 2 μm thick plastic sections of fish were made at different stages of the life cycle.

a) Processing and embedding

Fish were placed on white or black backgrounds to elicit contraction or expansion of melanophores.

i) Embryos were fixed 24 hours in Zenker's fixative (Zenker's stock solution: mercuric chloride, 50 gm., potassium dichromate, 25 gm., sodium sulphate, 10 gm., distilled water, 1000 ml.,) with glacial acetic acid (Zenker's stock solution, 95 ml., glacial acetic acid [99.7% acetic acid] 5 ml.).

ii) The fixative was gently removed with a Pasteur pipette, and the embryos rinsed repeatedly with tap water until no further yellow colour appeared in the solution. Further changes of tap water were made to make a total of 14 hours spent in it.

iii) The fish were dehydrated through a series of alcohols, (70%, 80%, 95%, and 2 X 100%), spending 30 minutes in each.

iv) The method for infiltration and plastic embedding in the Porter-Blum handbook (Ivan Sorvall, Inc., Norwalk, Conn.), was then followed. The embryos were infiltrated in solution A (glycol methacrylate, 2-butoxyethanol, and benzoyl peroxide, PolySciences Inc., Warrington, Pa.) for five hours at room temperature. The embedding medium was then mixed (42 parts A plus 1 part B [polyethylene

glycol 400 and N,N-dimethylaniline]). Two ml. of embedding medium were put in Plastic Block Molding Cups and one embryo placed in each. The block holder was inserted into the mold and hot paraffin was poured around the edge to seal it. The specimens were polymerized overnight at 30°C.

v) For transverse sections, the block was removed from the holder after polymerization and the tissue cut out in a square of plastic. It was then reoriented in the holder and step iv repeated without infiltration.

vi) When larger juvenile fish were processed they were fixed for 24 hours after which the required body segments were cut out. These segments were fixed for a further 24 hours before proceding to step iii.

b) Sectioning

Two µm sections were cut on a Porter-Blum ultramicrotome with glass knives, transferred to a 37°C distilled water bath and floated singly onto slides. After initial drying on an 85°C warming tray they were dried in an 85°C oven for 48 hours prior to staining.

c) Staining

Since these thin sections stained quite lightly and floated off the slides in alcohols, the conventional procedure for hematoxylin and eosin had to be modified

as follows. All steps were carried out in Copland jars at room temperature.

1. Distilled water — 1 min.
2. Alcoholic iodide, (iodine crystals, 1 gm., 95% alcohol, 100 ml.) — 5 min.
3. Tap water dip
4. Sodium thiosulphate, (sodium thiosulphate, 5 gm., distilled water, 100 ml.) — 5 min.
5. Gentle tap water wash — 10 min.
6. Erlich's Acid Alum Haematoxylin, (Haematoxylin, 4 gm., 95% alcohol, 200 ml., distilled water, 200 ml., glycerin, 200 ml., aluminium potassium sulphate, 6 gm., glacial acetic acid, 20 ml.; dissolve haematoxylin in alcohol and add remaining ingredients. Ripen immediately by adding 0.6 gm. sodium iodate). — 10 min.
7. Tap water rinse
8. Saturated lithium carbonate, (lithium carbonate, 1 gm., distilled water, 100 ml.), until nuclei and sections are bright blue.

9. Tap water rinse
10. Stock aqueous eosin, (water soluble eosin Y, 50 gm., distilled water, 1000 ml.; dissolve and add glacial acetic acid, 2.0 ml.) — 5 min.
11. Tap water dip — 2 X
12. Dry as much as possible
13. 100% alcohol — dip
14. Xylene — dip
15. Mount in Permount

7. Methods of observation

Observations were made with an A-O Spencer dissecting microscope. Antero-posterior, dorso-ventral, and medio-lateral melanophore positions were plotted on somite maps. Each map consists of a diagrammatic drawing of consecutively numbered somites, somite #1 being designated as the first somite behind the otic vesicle. This is not the first somite on the body but this numbering system was used for expediency since the lateral stripe extends back behind the otic vesicle. Antero-posterior and dorso-ventral positions were plotted by placing the cells along the base line (the horizontal skeletogenous septum) or up and down from it. To show the medio-lateral positions of melanophores, lines were projected down from

the base line and the cells mapped by their relation to the spinal cord, flank edge, and anterior and posterior edges of the somite.

These drawings were supplemented with photographs made at the appropriate stages in pattern development. Adult fish were photographed with a Zeiss Ikon camera fitted with a Pro-Tessar 1:1 lens. They were anaesthetized in MS-222. Whole-body photographs of fry were taken with an Asahi Spotmatic camera mounted on a Tessovar macroscopical optical system. To take close-ups of individual pigment cells, a Nikon EFMB camera mounted on a Leitz microscope was used. In all fry photography, a white plastic square was placed under the watch glass containing the fish to give better contrast. Stained specimens were also photographed with the Nikon EFMB camera mounted on the Leitz microscope.

Ilford Pan-F film (ASA 50) was used throughout this study. All films were developed at room temperature using Kodak D-19 developer diluted 1:4 with water. Where greater contrast was desired the developer was diluted 1.5:3.5. Films were developed for five min. at room temperature without agitation, rinsed briefly in tap water, and fixed for 5 min in Kodak Rapid Fixer. A 30 minute wash in running tap water followed plus a brief distilled water rinse. Pictures were printed on Ilford photographic papers.

CHAPTER IV

WHOLE-BODY MELANOPHORE PATTERN FORMATION - DAYS ONE TO THREE

The normal stages of development of Brachydanio rerio have been described by Hisaoka and Battle (1958). Before lateral stripe formation is described and analyzed in detail, a brief description of whole-body melanophore stripe formation will be presented.

To aid in the visualization of early pattern formation, stage 21 zebra fish (age approximately 24-30 hours) were dechorionated by grasping the chorion at one point with two pairs of watchmaker's forceps which were then quickly separated. The fish popped out of the slit. Stage 21 fish lashed their tails when stimulated but were not capable of any swimming movements. They could be manipulated and turned over with probes so that the melanocyte migrations could be observed from all angles.

The larva was placed in dechlorinated water in a watch glass which was put on a white plastic square to enable the first melanocytes to be seen. The body markers used in describing the early events are the eyes, the otic vesicle, the yolk sac and its stump, the notochord and the horizontal skeletogenous septum (refer to Fig. 2).

Results

The first indication of body pigmentation begins between 24 and 30 hours of age with the appearance of some very small, pale melanocytes over the dorsal and lateral body walls posterior to the otic vesicle and dorsal to the yolk sac (around somites #2 to #6). This pigmentation follows by a few hours the appearance of pigmentation in the eyes. (This was not studied here even though all the melanocytes of the eye, except for those of the retina are derived from the neural crest. Retinal melanocytes are derived from the embryonic optic cup [Searle, 1968]). Within half an hour, additional melanocytes appear here as well as anteriorly, up to the eyes. The cells gradually become darker, and as more pigment is synthesized in them, their amoeboid shapes are revealed.Their numbers are gradually augmented by the appearance of new cells over the dorsal and anterior flank area and soon on the dorsal yolk sac as well. The wave of melanoblast to melanocyte differentiations starts then above the yolk sac, progressing with time anteriorly, posteriorly and ventrally. In any area cells appear first in more dorsal locations. It takes only three hours for melanocytes to become visible as far posterior as somite #20.

About two hours after the first body melanocytes are visible, those in the yolk sac area are distributed over the flank and medially and laterally on the yolk sac. The flank melanocytes are mostly concentrated at its

dorsal and ventral extremities, being sparser over mid-flank regions and are distributed at two levels, a deep and a superficial one. Within five hours of their initial appearance these flank melanocytes are very large and deeply pigmented.

As the number of melanocytes increases in this antero-posterior, dorso-ventral wave, five ragged bands of pigment cells gradually emerge from the initial more scattered distribution; dorsal, ventral, yolk sac and dual lateral bands. Lateral flank melanocytes may move either dorsally or ventrally contributing some cells to the dorsal or ventral bands which are essentially complete by 48 hours. By 72 hours, the yolk sac band is almost complete as cells initially located dorsally on the yolk sac and its stump migrate ventrally. It is possible that lateral flank melanocytes may also contribute to the yolk sac band if they were early arrivals at its dorsal extremity. A few new melanocytes appear among the cells on the yolk sac and its stump as they migrate. The lateral stripe on each side arises between the dorsal and ventral extremities of the somites in the area of the horizontal skeletogenous septum. Its members are both deep and superficial melanocytes which fail to become incorporated into the dorsal or ventral bands. These cells, originally oriented dorso-ventrally, reorient antero-posteriorly and by 72 hours have essentially

established themselves in the horizontal skeletogenous septum, after which some shuttling may occur along the stripe site. The cells now in the bands can undergo physiological colour changes for the first time late in day three, and an accurate count can be made of them (see Fig. 2). They are now designated as melanophores.

To summarize, the following points emerge about initial whole-body pattern formation in the zebra fish:

1. Cells appear initially on the flank above the yolk sac, later more anteriorly on the head and posteriorly and ventrally on the flank.

2. From a scattered distribution on the body, the melanocytes become arranged into four bands: dorsal, dual lateral and ventral.

3. The yolk sac band forms as cells migrate ventrally from the dorsal areas of the yolk sac and its stump, their numbers being augmented by a few melanoblast to melanocyte differentiations during migration.

4. The lateral stripes form when some melanocytes initially scattered on the flank reorient into the horizontal skeletogenous septum. Not all cells present initially in mid-flank areas reorient. Most enter the dorsal and ventral bands (and possibly make some contribution to the yolk sac band as well).

Discussion

A neural crest source of chromatophores in teleosts was inferred (Lopashov, 1944; Orton, 1953), but not proven until 1961 when Shephard followed its condensation and dispersion in B. rerio. He observed the development of melanocytes from cells which migrated away from the crest and also described the stages of melanoblast differentiation immediately before melanogenesis. A great increase in cytoplasmic basophilia, nuclear volume and cell size was reported. The study was not, however, extended to earlier stages of melanoblast development.

Work on the origin of teleost pigment cells has, been complicated by the fact that in a few species, notably Fundulus heteroclitus, cells which become pigmented on the yolk sac migrate away from the extra-embryonic germ ring before migration out of the crest is seen. These wandering mesenchymal cells, first described by Stockard (1915) give rise to several derivatives including black and brown pigment cells, red blood cells, and endothelial cells. Shephard points out that the brown pigment cells may be of neural crest origin since they do arrive later. Thus, in some teleosts, there may be two sources of pigment cells and as Shephard points out this is an area where further work must be done since the source of these yolk sac melanophores has not been conclusively shown. However, in light of Ballard's (1973) studies which have necessitated a redrawing of the teleost fate map, it may be that cells

destined to become chromatophores all arise from the same place in the early gastrula. The phenomenon of the dual origin of chromatophores could thus be explained as follows. If, during gastrulation, similar inductive effects impinge on this group of cells, the fact that they are later found in different areas would only reflect subsequent post-induction movements. Since induction in teleosts has been little studied this is completely conjectural. The cells destined to form pigment could of course come from very different areas in the early gastrula and the origin of chromatophores in teleosts could thus be very complex.

Shephard (1961) found two streams of neural crest cells in the zebra fish, a dorsad one between the ectoderm and somites and a ventrad one which is seen in his photomicrographs to be migrating down the surface of the neural tube and notochord. Shephard studied only the formation of melanocytes, not of any other crest derivatives. He believed that the larval pigment of the zebra fish arose from the outer stream of neural crest cells. However, since the young fish are translucent even deeply located pigment cells are important in protective coloration. It is interesting that the lateral stripe receives contributions from cells which migrate via the deep and superficial streams of cells. This may be the usual mode of melanocyte arrival in species which contain melanophores in the horizontal skeletogenous septum.

The ultimate source of pigment cells in the zebra fish thus seems to be the neural crest (Shephard, 1961). Contributions from other areas are, of course, not ruled out.

By late three to four days of age, the young fry can adapt to a changing background by paling and darkening. It is interesting that physiological colour change is first seen in these fish at around the time of hatching. There are two possible mechanisms by which colour change may occur in B. rerio based on work with other species. The first may be the release of MSH (melanocyte-stimulating-hormone) from the pituitary when the fish is on a dark background (Bagnara and Hadley, 1973). Hormone release causes outward migration of melanosomes and aggregation of reflecting platelets, which darkens the animal.

The second may be a neural mode of control and could be the most important because it would be faster than hormonal control. The control is believed by some to be adrenergic in nature, the pigment cells containing alpha and beta receptors which regulate aggregation and contraction respectively (Reed and Finnan, 1972). However, others (Fujii and Novales, 1972) believe that there are separate pigment-aggregating and dispersing nerves and while the aggregating transmitter may be adrenergic, the dispersing transmitter may not be.

CHAPTER V

ESTABLISHMENT OF THE FIRST LATERAL STRIPE - DAYS ONE TO FOUR

Before the regulatory phenomena in the lateral stripe from days three to eight are described, the formation of the three day stripe will be presented in detail.

1) Arrival of initial melanocytes at the site and clearing of those melanocytes from the flank which are not incorporated into the stripe

Twenty individual dechorionated fish were observed for the first 96 hours of development as described in Chapter IV and sketches of three day pattern formation were made on melanocyte maps. At 48 hours of development the fish were placed in 0.2 mM phenylthiourea until the first contraction could be elicited and the melanophores were counted. This acted as a check to rule out early melanophore mitosis. Numbered cells already present on the flank near the stripe site were intensively studied. The time when the first pale melanocytes were seen on the fish was defined as T_0 (at around 30 hours of age).

Results

The first cells are scattered sparsely over the flank at two levels, deep and superficial. Figure 7

follows the events at the site over the next 72 hour period for one representative fish. In this figure, attention should be focussed on numbered cells only. It is assumed that the distribution of melanin in the cell reflects its true boundaries, although this may not be true, especially in the earliest pigmented cells.

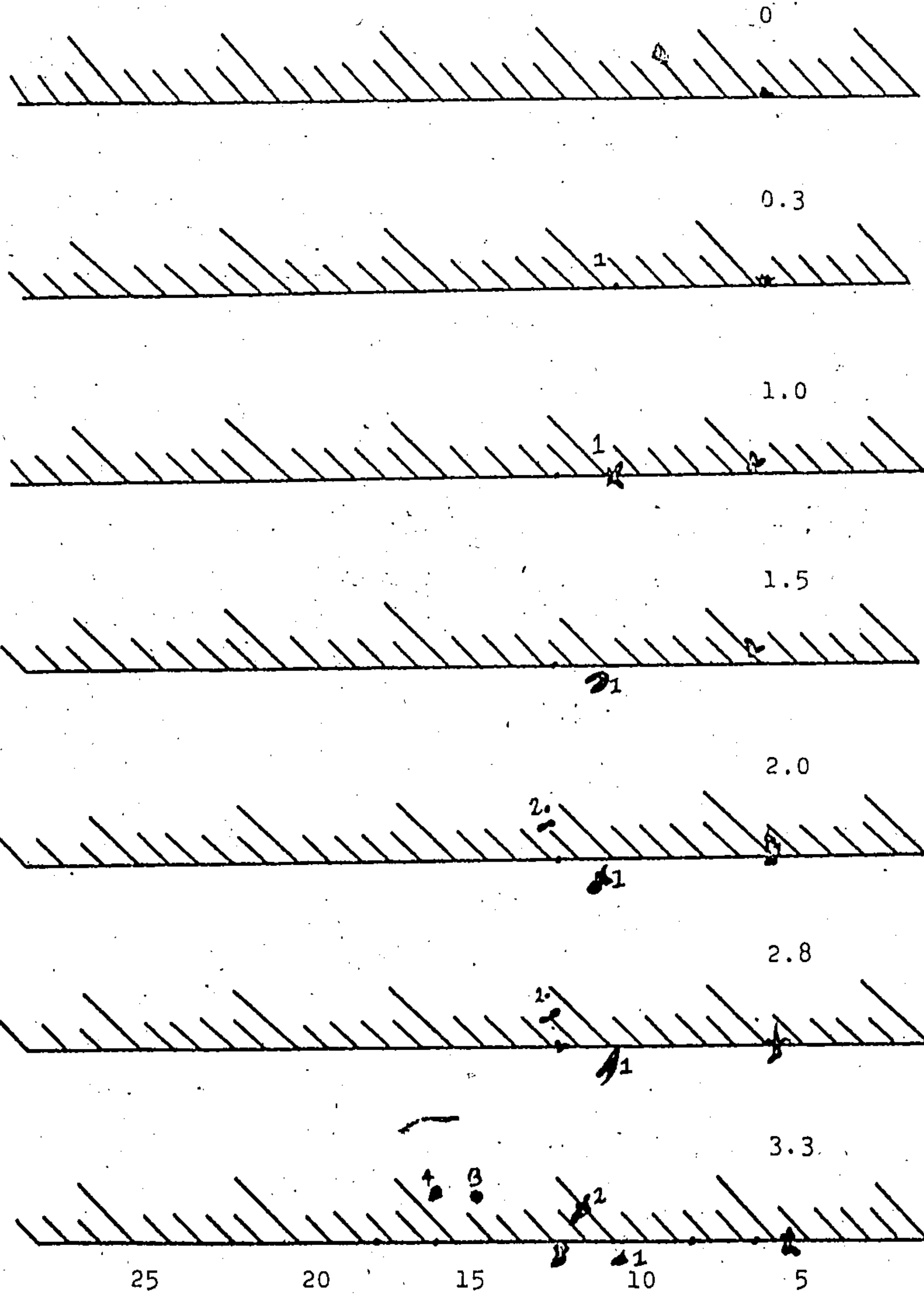
The history of cell #1 was traced over a period of 4.5 hours from its appearance near the stripe site to its incorporation into the ventral band. Cell #1, a deep cell, was first visible at T = 0.3 hours. In the next 45 minutes it elaborated enough melanin to enable its shape changes to be followed more easily. By T = 1.5 hours it had a U-shaped morphology which subsequently changed as it moved ventrally. By T = 2 hours a long arm of this cell was stretched dorsally which as the cell moved ventrally, became more and more attenuated until traces of it finally disappeared by 4.5 hours, when the cell was incorporated into the ventral band. This cell is designated a melanocyte since the pigment shunted around irregularly within it and there was no response to changes in background illumination.

Cell #2, a peripheral cell, appeared two somites caudally to cell #1 and was first observed at 2.0 hours. Starting from a position relatively high on the flank it moved ventrally and caudally undergoing extensive shape changes as its melanin shunted about. By T = 4.0 hours

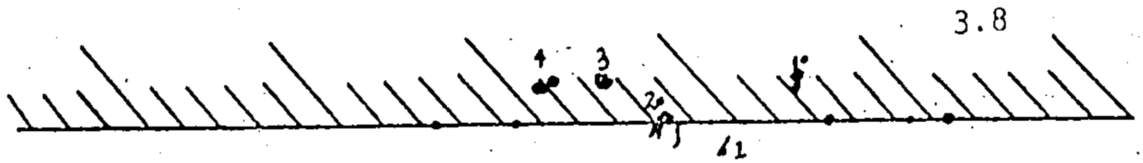
FIGURE 7

<u>Establishment of the initial lateral stripe in one representative fish to 102 hours</u>.

The cells which are numbered were intensively studied and those which were incorporated into lateral stripe are indicated by a dot over them. Time 0 is that time when the first melanocytes are seen (about 30 hours of age). The true age then of the fish for any given stage is 30 hours plus the time shown. The sketches also show the shapes of the cells and somite designations are given at the bottom of the 3.3 hour sketch.



Time (hours)
0
0.3
1
1.0
1
1.5
1
2.0
2
1
2.8
2
1
3.3
4
3
2
1
25
20
15
10
5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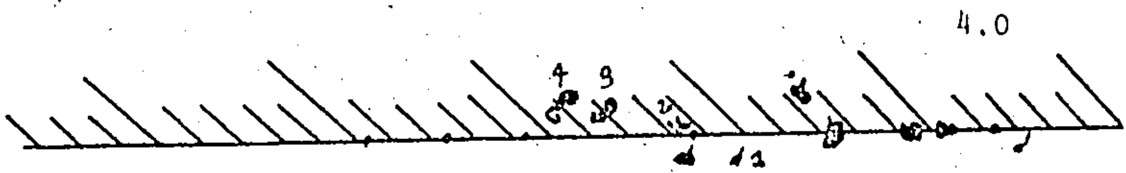
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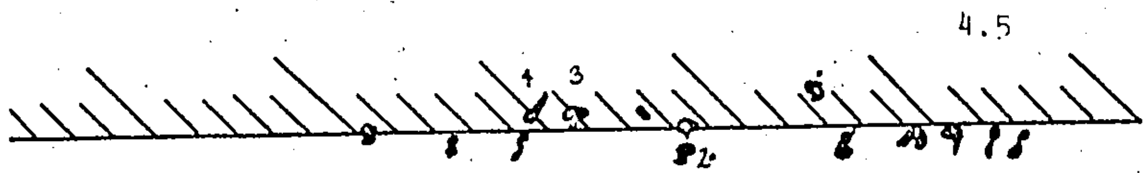
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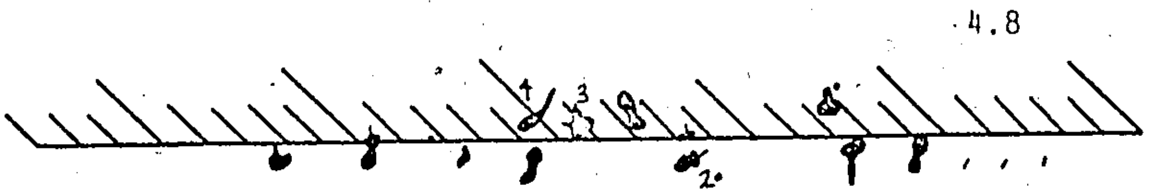
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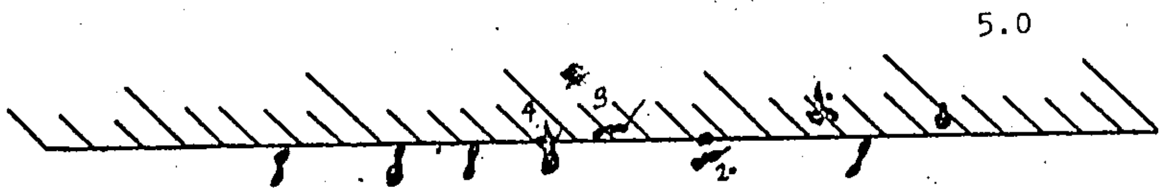
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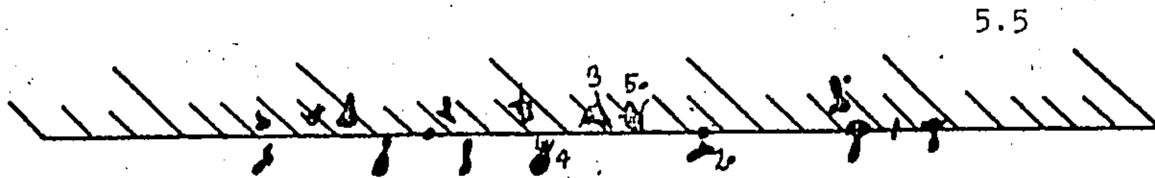
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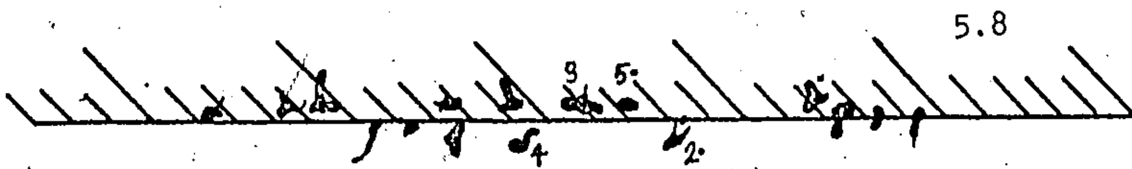
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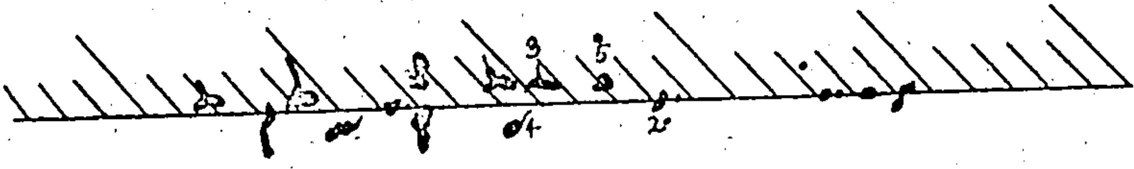
6.3



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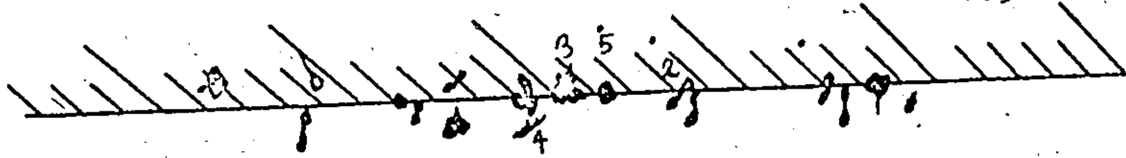

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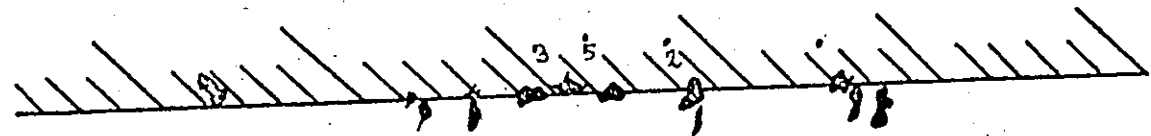

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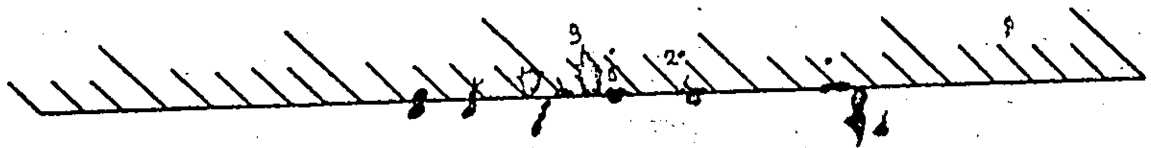

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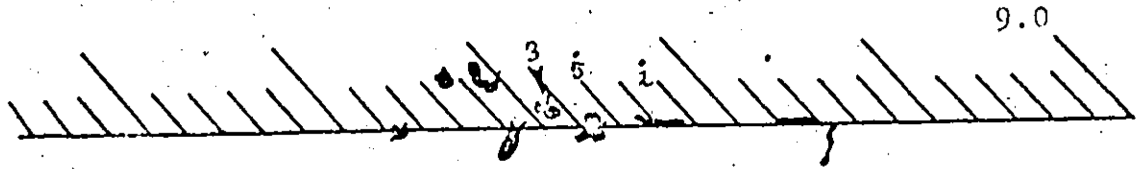
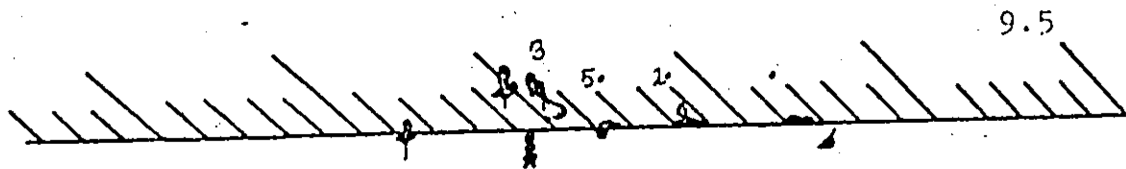
8.5


9.0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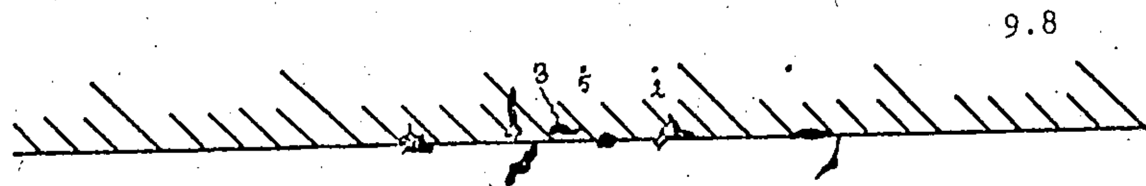
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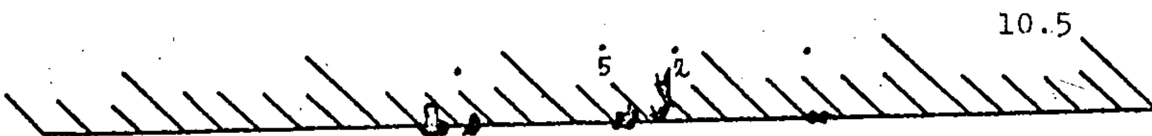
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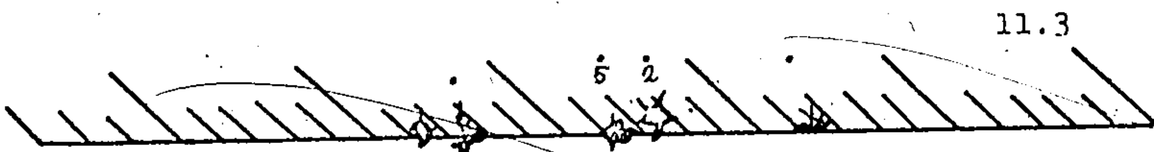
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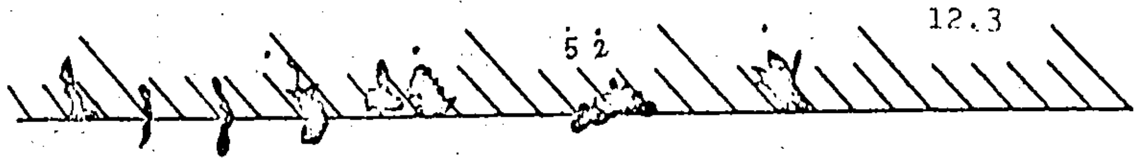
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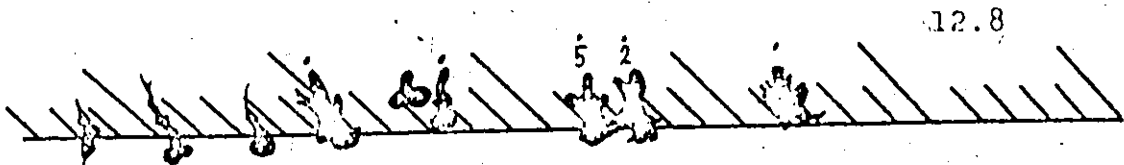
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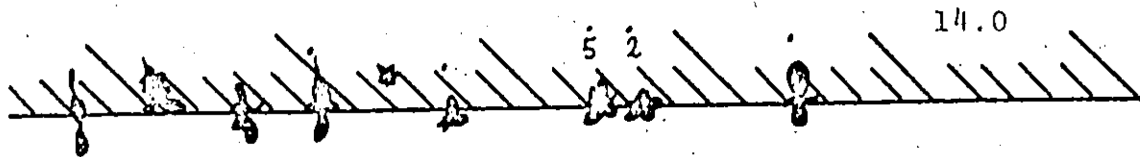
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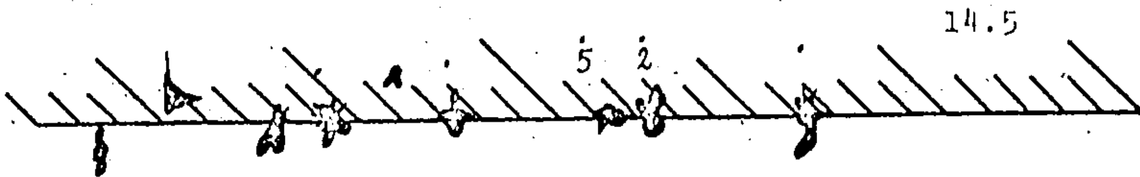
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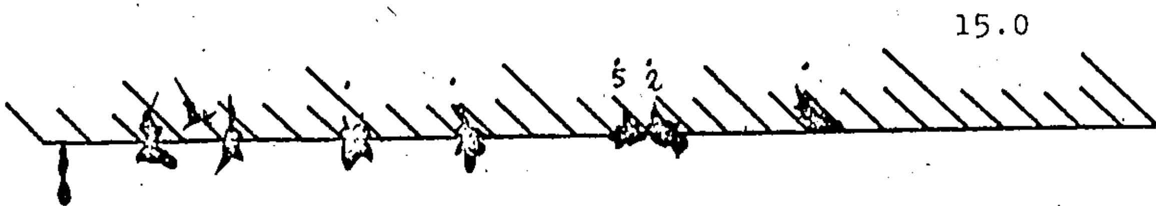
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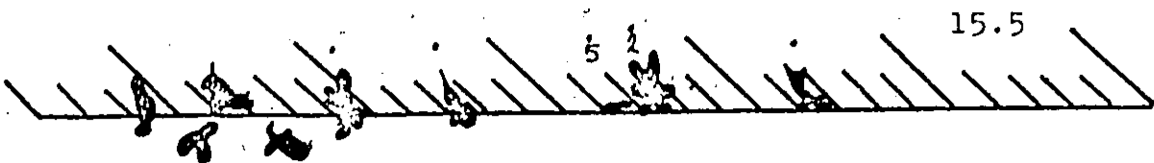
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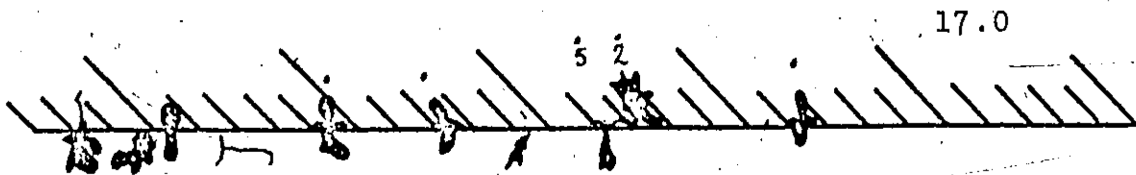
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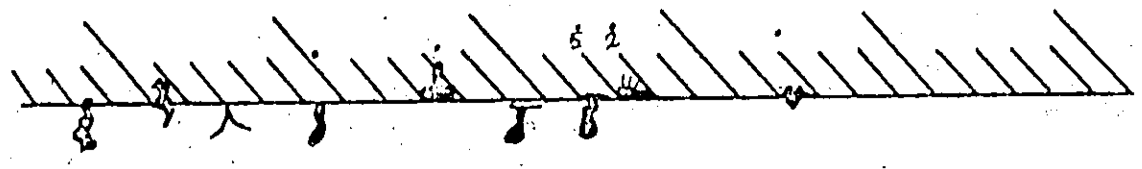

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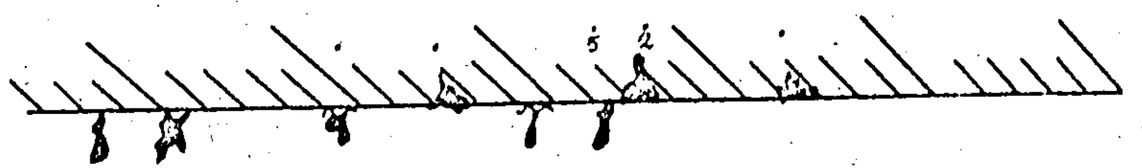

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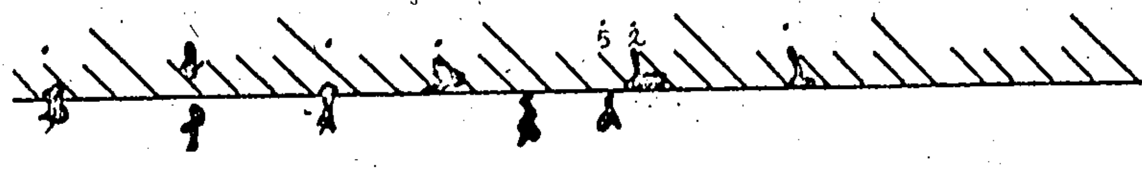

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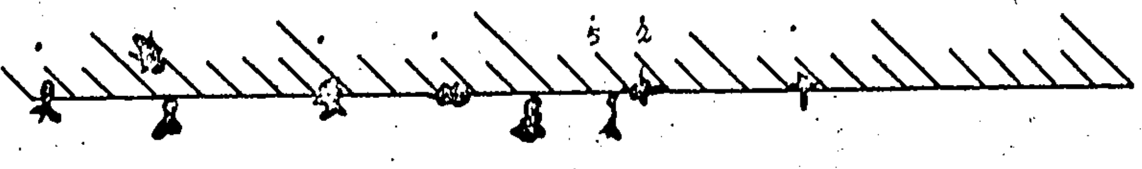

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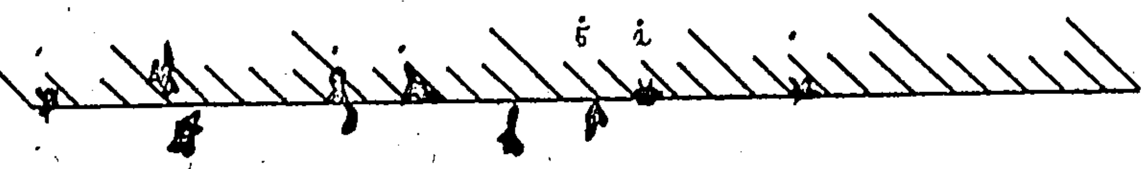

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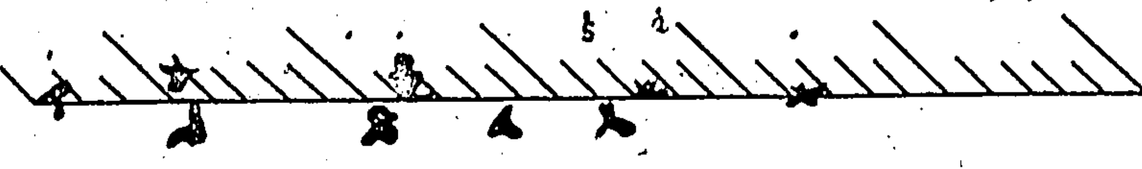

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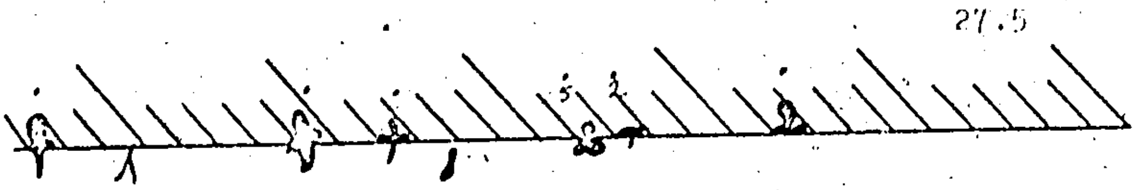
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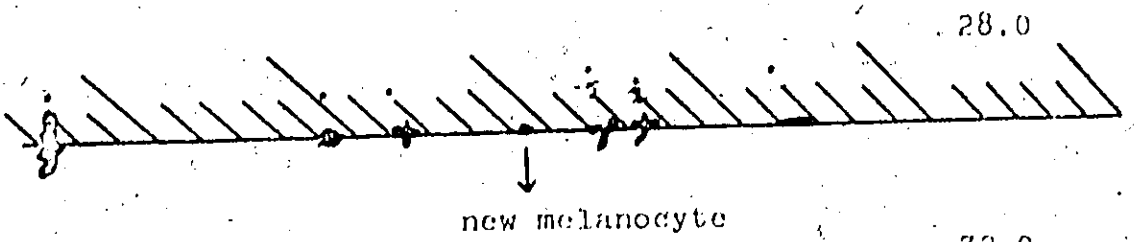
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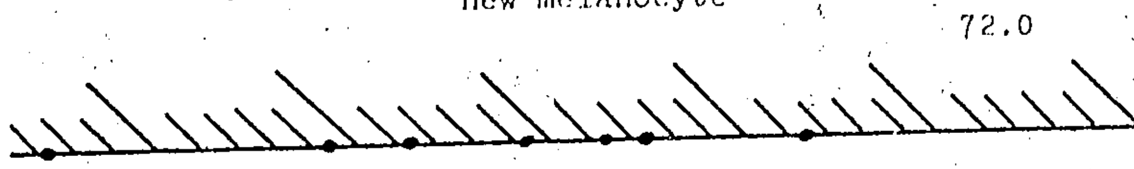
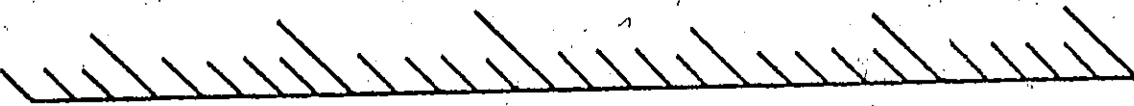
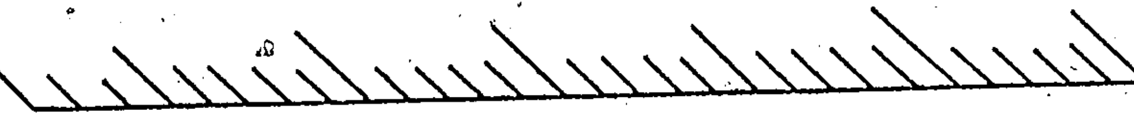
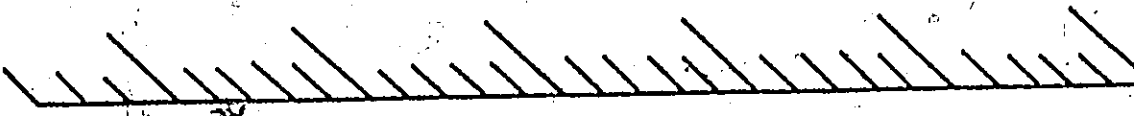
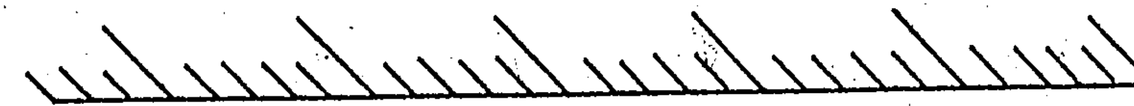
new melanocyte


72.0









this peripheral cell occupied a midflank position but by T = 4.5 hours it was located quite far ventrally. In its ventral migration it passed over a deep cell which had appeared at T = 4.0 hours and just when it looked as if it might be incorporated ventrally, it underwent another shape change, reversed its direction of migration and moved up laterally to the deep cell. The deep cell changed morphology several times between T = 4.0 hours and T = 7.0 hours and was incorporated into the ventral band between T = 7.0 and T = 7.25 hours. The shape and position changes of both these cells can be followed in (Fig. 7 - 2 hours to 7.25 hours).

Cells #3 and #4 are particularly interesting. They appeared in the same general location (in somites #13 and #14) at the same time (T = 3.25 hours) but had different fates. Cell #3 was elongated at T = 4.0 hours and became T-shaped 0.5 hours later. It continued to change morphology as it moved dorsally and was incorporated into the dorsal band at T = 10.5 hours. Cell #4 was also elongate at T = 4.0 hours but experienced numerous changes in shape as it migrated ventrally, being incorporated into the ventral band between 6.5 and 7.0 hours. At these early stages the cells do not seem to be constrained to any area of the flank. For about the first five hours they may move ventrally, but later they move dorsally as well.

The fish will now be dealt with in regions (somites #1-#8, #9-#12, and #13-#16) up to ten hours. In somites #1-#8 only one cell was present by 10 hours. It approached the midflank area as an amoeboid cell and then made an excursion into somite #9 (Fig. 7 - 7.3 hours). Later, part of it stretched into somite #8 again (Fig. 7 - 8.5 hours) but ultimately it returned to somite #9 before being incorporated into the stripe. All other cells moved ventrally, extending a pseudopod or two, which elongated and stretched down to the ragged ventral band. The rest of the cell then followed, leaving long faint pseudopods which eventually disappeared.

Somites #9-#12 remained empty except for the entry of cell #1 and cell #2 and the cells medial to it. Cell #1 was incorporated ventrally by 4.5 hours and cell #2 eventually became incorporated into the stripe but moved into somite #13 between 9.5 and 10 hours. Both of the deep cells migrated ventrally but were probably not displaced by cell #2. (In other experiments two cells - and in one case even three - have been seen to enter the area bounded by one somite; in some cases these cells all lie in the same plane.)

Of the eight melanocytes which appeared in somites #13-#16, seven were cleared from the flank by 10.5 hours, except cell #5 which was incorporated into the stripe. The cells that moved dorsally did not seem

to have pseudopods as attenuated as those which moved ventrally; however since pigmentation and not true cellular outline was used to determine shape changes, long arms could have been present in cells moving dorsally.

In summary, most of the cells which appeared in somites #1-#16 were cleared from the flank by 10 hours. Those which remained to be incorporated into the stripe shuttled back and forth but did not leave the stripe site except for cell #2 which returned to it. During the next 11.5 hours as the wave of pigmentation proceeded posteriorly, nine more large grey melanocytes were seen on the flank. Of these, two would remain and be incorporated.

The flank was eventually cleared (by T = 28 hours) of all but six cells. A seventh appeared later (Fig. 7 - 28 hours) but was already at the site when first observed. All the melanocytes which arrived were first amoeboid, then finely reticulate (between 11 and 17 hours) and later they became denser and bushier.

2) <u>Reorientation and shuttling of lateral stripe melanocytes</u>

The cells that are initially scattered on the flank are oriented dorso-ventrally, flattened parallel to the sides of the fish. Those in the lateral stripe are oriented perpendicularly to this, in the horizontal skeletogenous septum. The next step in formation of the three day stripe encompasses the change in orientation

of the melanocytes which remain at the site and has been designated lateral stripe reorientation. It begins anteriorly and gradually spreads to the most posterior flank melanocytes. About 36 hours is needed for each melanocyte to change its orientation. The process is shown in Fig. 8, photographs of four melanocytes, two of which are reorienting into one somite (cells #2 and #3).

The first indication that reorientation may be in progress is the appearance of slight thread-like concentrations of melanin at the septum (Fig. 8, cells #2 and #3, 48 hours). These pseudopodia-like structures gradually darken and thicken and become antero-posteriorly oriented. As more of the cell moves into the septum, reorientation can be seen from both dorsal and lateral aspects. A very small part of the cell sometimes does not migrate completely into the septum.

The original 'leading' edge does not always initiate reorientation. Assuming that all of these cells have come from the neural crest the term "leading" edge is taken to be the most ventral portion of the cell which in downward migration might be determining the direction of movement. There is a difference in reorientation depending on the location of the cells after initial migration (Fig. 9).

If the cell is located above the septum, the original cell front probably flows in first. If it is

FIGURE 8

Reorientation of lateral flank melanocytes in one zebra fish.

a) 48 hours of age: Note the bushiness of lateral flank melanocytes #2 and #3. While melanocyte #1 appears to have almost completely reoriented, these two cells are greatly spread out on the flank. The thread-like structures at their ventral extremities may be the first indications that a cell is about to begin reorientation.

b) 72 hours: Reorientation is now well underway. Note, however, that part of the melanocyte #1 has returned to the flank, suggesting that the reorienting cells are not completely limited to motion in one direction.

c) 84 hours: Melanocytes #1, #2 and #3 are now almost completely reoriented. Note that melanocyte #1 has retracted part of its surface up onto the flank. The pale grey cells are early new melanocytes (arrows).

Magnification 96X

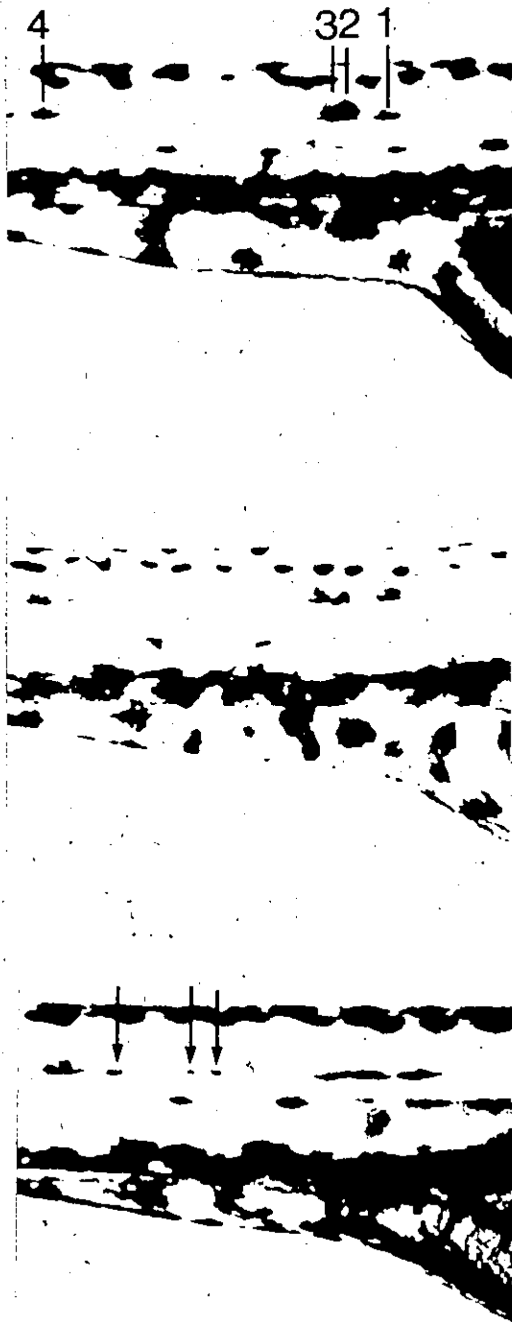

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FIGURE 9

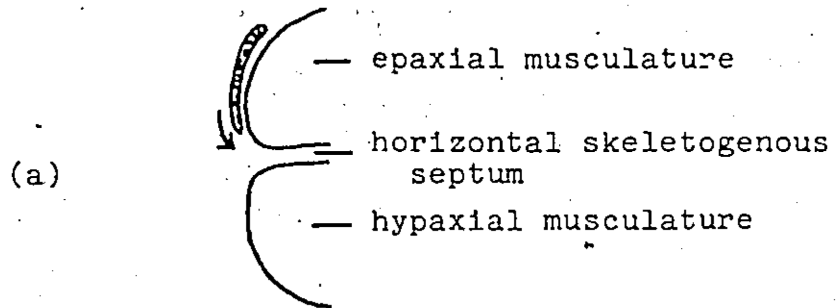
Diagrammatic representation of the possible positions of peripheral melanocytes after initial migration

a) Above the septum: The 'leading' edge initiates reorientation.

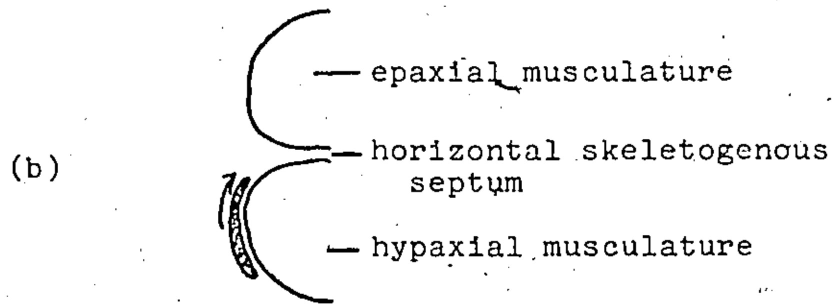
b) Below the septum: The 'trailing' edge initiates reorientation.

c) Over the septum: Reorientation is initiated from the middle of the cell.

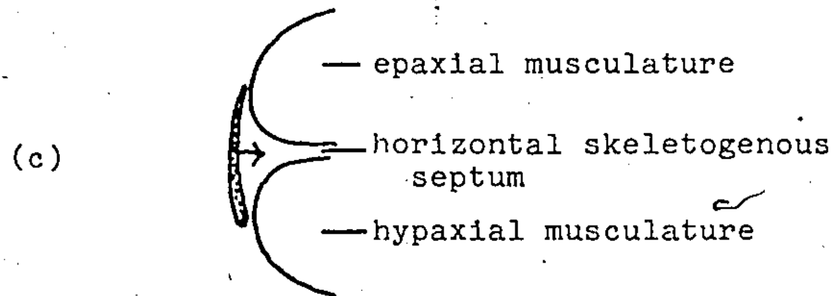
(a)



(b)



(c)



below the septum it is the 'trailing' edge of the melanocyte that does so. Deep cells undergo the same type of reorientation if they are similarly positioned with respect to the septum. However, deep and superficial cells that are located over the septum may initiate reorientation from the middle of the cell (Fig. 9, cell #3).

Figures 10 and 11 show the positions of cells considered to be incompletely and totally reoriented. Both deep and superficial cells are located in the connective tissue surrounding the notochord and the somites. After reorientation they are located in the horizontal skeletogenous septum. Note in Fig. 11 the small group of cells which looks like a developing lateral line organ. Before the histological work had been done, the initial stripe had been tentatively designated the lateral line band but since any relationship with the lateral line had not been shown it was called the lateral stripe With the observation that these melanocytes were seen in some cases to be so closely associated with the lateral line organs this designation became more meaningful and in the remainder of this thesis this stripe will be called the lateral line band.

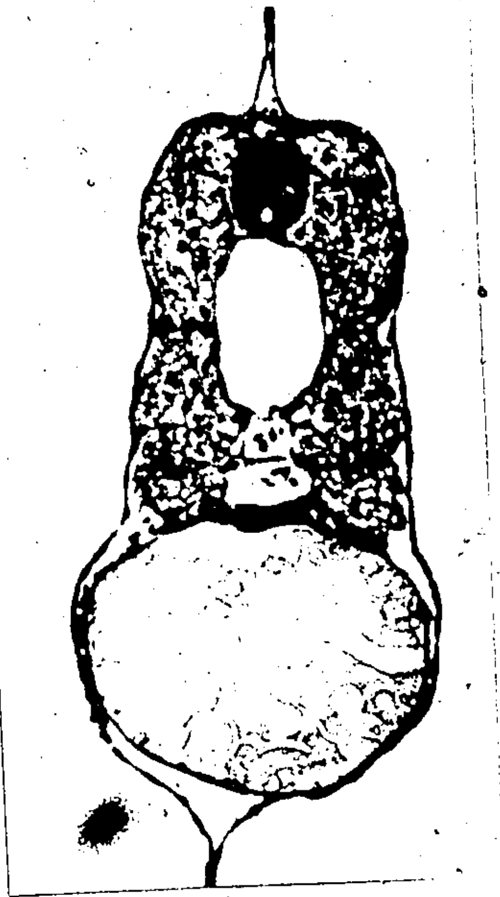
When reorientation is almost complete, the cells in the lateral line band become irregularly stellate in morphology. Shuttling movements along the line often occur over distances as great as one or two somites. In

FIGURE 10

Early positions of the initial melanocytes

Transverse 2 μm (plastic) section through a 36 hour old embryo in which the positions of the early melanocytes are visible. On the accompanying diagram melanocyte #1 (M#1) is peripheral, #2 is deep and appears to be reorienting out, while #3 appears to be moving ventrally. The aggregations of melanin (arrow) may be part of melanocyte #2. Other melanocytes seen are dorsal, ventral and yolk sac melanocytes (the latter are only midway down the yolk sac).

Haematoxylin and eosin stain. Magnification 356 X



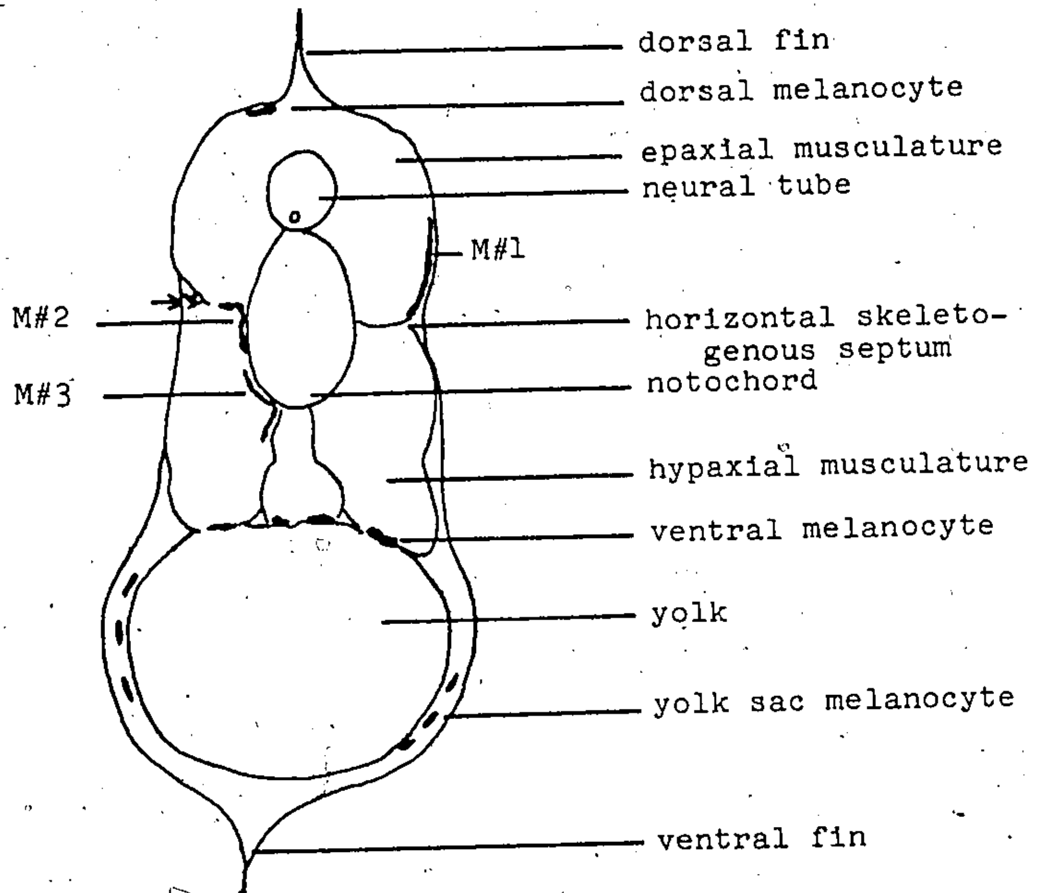

dorsal fin
dorsal melanocyte
epaxial musculature
neural tube
M#1
M#2
horizontal skeleto-
genous septum
M#3
notochord
hypaxial musculature
ventral melanocyte
yolk
yolk sac melanocyte
ventral fin

FIGURE 11

A completely reoriented melanophore in an eight day fry

A 2 µm plastic section through an eight day old fry showing a completely reoriented, expanded melanophore of the lateral line band. The pigment cell is contoured around the lateral line organ and extends over almost all of the horizontal skeletogenous septum.

Haematoxylin and eosin. Magnification 821 X

Symbols: n - nucleus, llo - lateral line organ.

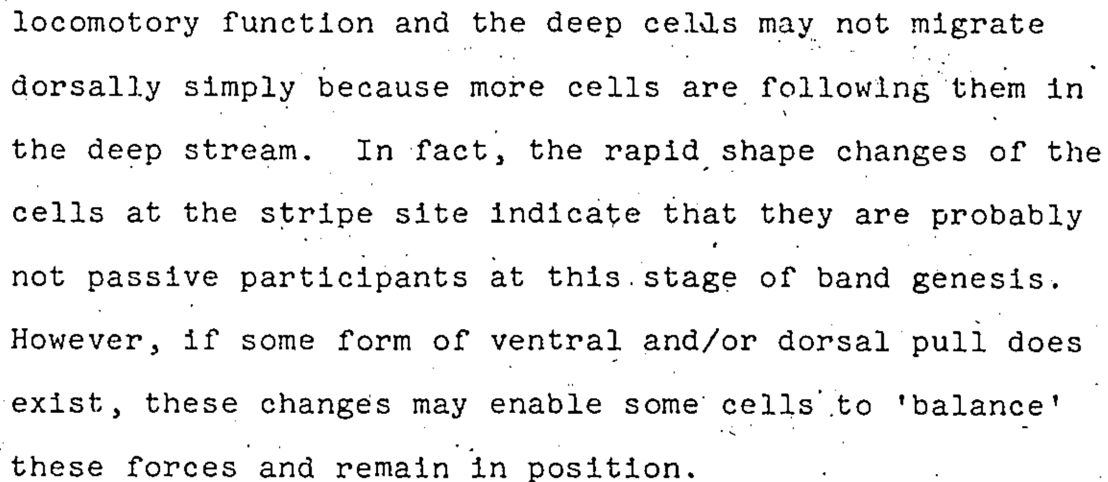
n
IIo

this way, pairs of melanocytes that resulted from two cells flowing into the area demarcated by one somite can break up and new pairs can form. The cells do not have to be completely reoriented for this movement to occur.

Discussion

The melanocytes that form the initial lateral line band are therefore those cells which remain in the vicinity of the stripe site and reorient into the horizontal skeletogenous septum. There are several possibilities why some cells remain at the site while others become incorporated into other bands. If intercellular connections are important in determining band formation (as Twitty, 1936, postulated) some cells may fail to make these with dorsal or ventral melanocytes and therefore may remain stranded on the flank. Alternatively, they may make connections with cells in both bands and thus not be subject to any net dorsal or ventral pull. There is, however, no evidence that in the zebra fish these connections exist.

It is interesting that deep cells have not been observed to migrate dorsally. If all cells in the dorsal band come from the superficial stream of neural crest cells interconnections may not extend from them into the deep stream. The pseudopodia may have only a



locomotory function and the deep cells may not migrate dorsally simply because more cells are following them in the deep stream. In fact, the rapid shape changes of the cells at the stripe site indicate that they are probably not passive participants at this stage of band genesis. However, if some form of ventral and/or dorsal pull does exist, these changes may enable some cells to 'balance' these forces and remain in position.

One possibility for the change in orientation of the cells at the site is that widespread changes in cell 'affinities' are occurring. These changes are not exactly coordinated with the initial appearance of the cells because cells that are located in the first few somites rarely reorient; they usually move either dorsally or ventrally before reorientation is visibly initiated in other cells remaining at the site. The reorientation process is likely graded in nature because posteriorly located cells usually take much longer to reorient. This discussion of changes in cellular affinities is purely speculative but some sort of change must occur to make the early melanocytes change their orientation so drastically. Perhaps at this time there is a conformational change in the environment (see Smith-Gill, 1974).

Another possibility for the change in orientation of the melanocytes which remain at the site is suggested from the work of Lehman and Youngs (1959).

These authors hypothesized that xanthophores are important in controlling melanophore pattern formation in the black axolotl. Recall that it was suggested that in the black axolotl prospective xanthophores migrated out of the crest later than did prospective melanophores (Lehman and Youngs, 1959). The dorsal pattern of alternating melanophore and xanthophore clusters was hypothesized to form because the melanoblasts, which at first evenly colonize the flank are only for a short time able to prevent xanthoblast invasion. When the xanthoblasts gain access to the flank they are able to 'repel' the differentiating melanophores, causing the black clusters to form.

In zebra fish fry, xanthophore pigmentation begins at around 72 hours of development and a diffuse yellow coloration appears on the body. The nature of the xanthophore precursors and their pathways of migration are not known in _B. rerio_. However, a similar mechanism of pattern formation can be postulated. The differentiating xanthophores may be able to 'repel' the melanocytes, causing band formation to occur. Melanocytes remaining at the site may then reorient because they are retreating from the invading xanthophore precursors.

Goodrich _et al._, (1954) have shown that xanthophores are important in controlling melanophore position in adults. Stripe regeneration in the anal fin

of *B. rerio* was described after the chromatophores had been destroyed by freezing. When only melanophores were destroyed new melanophores differentiated *in situ* after fragment clearing or migrated into the clear area from the remnants of the old stripe. When xanthophores or adjacent melanophores and xanthophores were destroyed, melanophores again invaded the clear area but did so only as long as no xanthophore barrier remained. Those melanophores which colonized the area where the yellow stripe would later form were subsequently destroyed and xanthophore differentiation occurred *in situ*. Xanthophores are thus important in controlling melanophore position, at least in fins. They may therefore also be important in controlling melanocyte pattern formation in young fry.

None of these theories for why some melanocytes reorient into the septum have any experimental proof. However, a pertinent observation was made in fry which had small or non-existent yolk sac stumps. The pigment of the yolk sac and its stump is derived for the most part from cells which have a prior location at the dorsal aspect of these structures. In those fish where the stump was reduced or absent, it was observed that at four days cells were scattered on the flank between the lateral line and ventral bands. It appeared that cells located ventrally (cells which had not migrated onto the stump) had effectively excluded the ventral cells on the

flank from joining the ventral band. These flank cells did not reorient into the lateral line band either, which would seem to be a reasonable alternative for them since several cells are often seen reorienting into the area bounded by one somite.

These observations may be interpreted in several ways. The melanocytes may have been prevented from reorienting into the septum because the differentiating xanthophores inhibited their migration. Cell destruction similar to that described by Goodrich et al., (1954) was not observed but as these fish died in the first week of development it may not yet have been initiated. Alternatively, the time for reorientation may be sharply limited (the stimulus may pass in a wave) with each cell getting one chance depending on its position and readiness or the surrounding environment may be appropriately oriented only for a short time. Both the factors governing reorientation and melanophore-xanthophore relationships must be investigated in future work.

Relevant to the foregoing observations that the cells which form the initial stripe migrate as individuals are observations that very occasionally initial wave melanocytes located posteriorly are seen not to be one cell, but two after shuttling. This could be due to a mitotic event at the line. However

Johnson (1966) observed that in the chick, cranial neural crest cells migrated "in a sheet-like fashion". Migration occurs later out of more posterior regions of the crest (Weston, 1970) and it is possible that in *B. rerio* melanization occurs before the cells migrating so closely together have separated. Melanization may therefore occur earlier in more posterior regions than it does anteriorly with respect to the time of migration of the cells out of the crest. This phenomenon was noted but not investigated further since one cannot predict in which fish it will occur. It must be studied in this system in future work.

The shuttling movements of the melanocytes after reorientation show that even after initial stripe formation cellular movements do not cease. Part of the population has therefore been very effectively shifted from the neural crest to the horizontal skeletogenous septum and the other bands.

3) Is melanization a necessary prerequisite for band formation in the zebra fish?

To see if there is a relationship between melanization and band formation in *B. rerio* as Twitty (1936) postulated for *T. torosus* the following experiment was done. Twenty-four hour unpigmented zebra fish were

dechorionated and divided into two groups. The control group (10 fish) was raised in dechlorinated tap water and the experimental group (10 fish) in 0.2 mM phenylthiourea (PTU) until four days of age when the fish were examined and the experimental group moved to dechlorinated tap water for repigmentation. The specific methods are given in the Materials and Methods section.

Results

Normal band formation was seen in the controls. Every experimental fish in which pigmentation had been completely inhibited, repigmented within 24 hours. The melanophores which gradually appeared were seen at the time of their first appearance to be already localized in the five stripes.

Discussion

This result corroborates that of Youngs (1957) for *T. torosus* where melanization was also inhibited with PTU. It shows that in the zebra fish there is no reason to assign a role to the index of melanization as being the factor which controls band formation.

CHAPTER VI

FORMATION OF THE EIGHT DAY BAND

1) Description of the normal eight day band

The eight day band of the zebra fish consists of a row of melanophores located in the horizontal skeletogenous septum and stretching posteriorly from the fourth to the eighth somite behind the otic vesicle over about a 20 to 25 somite span (Fig. 2a). Gaps are often seen at the anterior and posterior ends, and occasionally within the band. The number of cells in the band varies from 15 to 43 but there are usually 25 to 30. Therefore, just as there is a wide range in the numbers of cells in the three day band, there is also variation in the stripe formed by eight days of age. Some variation could perhaps be minimized by raising each fish in a separate container under the same rigorously defined conditions so that size differences (and therefore developmental age differences) are minimized. However, the purpose of this work was in part to study these differences between fish, so these kinds of individual raising experiments will have to wait until more work is done in this system.

The cells in the band have an irregularly stellate morphology. The major part of the cell is oriented parallel

to the long axis of the body but its external aspect may be positioned slightly differently, either contoured to the muscle or to the lateral line organ (as in Fig. 11). Figures 2a and 12 show eight day lateral line melanophores in the contracted state. Note that in Fig. 2a the cells do not all carry the same load of melanin. This will be discussed in the next section where the formation of the eight day band will be presented.

2) Formation of the eight day band

It was originally thought that the melanocytes which existed in the three day stripe (the initial cells), might divide to contribute to the band seen at eight days. This theory was tested even though in some three day fish a few melanoblast to melanocyte differentiations were observed (Fig. 2b).

To test this, twenty three day fish were selected from one batch of eggs. In none of these, had melanoblast to melanocyte differentiations occurred on day three. Ten were raised in dechlorinated tap water and ten in 0.2 mM PTU (see Materials and Methods). Every 24 hours the melanophores were contracted, the fish anaesthetized, and the cells in the right hand side lateral line band mapped and counted. Four days after the start of the experiment (day seven of development),

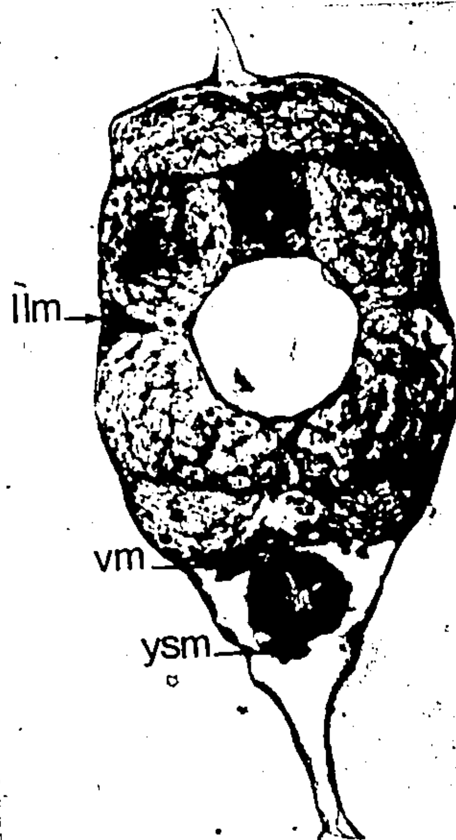
FIGURE 12

<u>Contracted melanophores in an eight day fry</u>

In this 2 μm plastic section the contracted melanophores of the lateral line, ventral and yolk sac bands are seen.

Haematoxylin and eosin. Magnification 269 X

Symbols: llm - lateral line band melanophore, vm - ventral band melanophore, ysm - yolk sac band melanophore.


llm
vm
ysm

the experimental fish in 0.2 mM PTU were transferred to dechlorinated tap water for repigmentation. Since melanogenesis had been inhibited in the experimental fish, the same number of cells should have been visible in these fish on day seven, unless melanophore division occurred after day three. At eight days of age the melanophores were counted and mapped in experimentals and controls. The long time that the experimental fish were in 0.2 mM PTU meant that all new arrivals were less deeply pigmented than the initial cells on day eight and could be easily distinguished from them.

Results

From Table 1 it can be seen that the numbers of melanophores present at three days (initial cells) varied from three to 19, averaging 10.7 for the experimentals and 11.1 for controls. On day eight, the range in melanophore numbers was 20 to 43, averaging 30.6 for experimentals and 29.6 for controls, indicating that the increase in cell numbers was due to the appearance of new cells in the band (second wave melanogenesis) and not to the mitosis of pre-existing initial cells. Figure 13 shows that the majority of cells in controls appeared in a wave between four to five days and that the number of new melanophores was constant but diminished after this time.

TABLE 1

Right-hand-side lateral line band melanophore counts from days three to eight of development for control and experimental fish (raised in 0.2 mM PTU from days three to seven of development) from days three to eight.

Experimental fish were raised in 0.2 mM PTU from days three to eight of development when they were removed to dechlorinated tap water for repigmentation. The number of melanophores were counted from days three to eight in control fish and experimental fish.

TABLE 1

Right-hand-side lateral line band melanophore counts from days three to eight of development for control and experimental fish (raised in 0.2 mM PTU from days three to seven of development) from days three to eight.

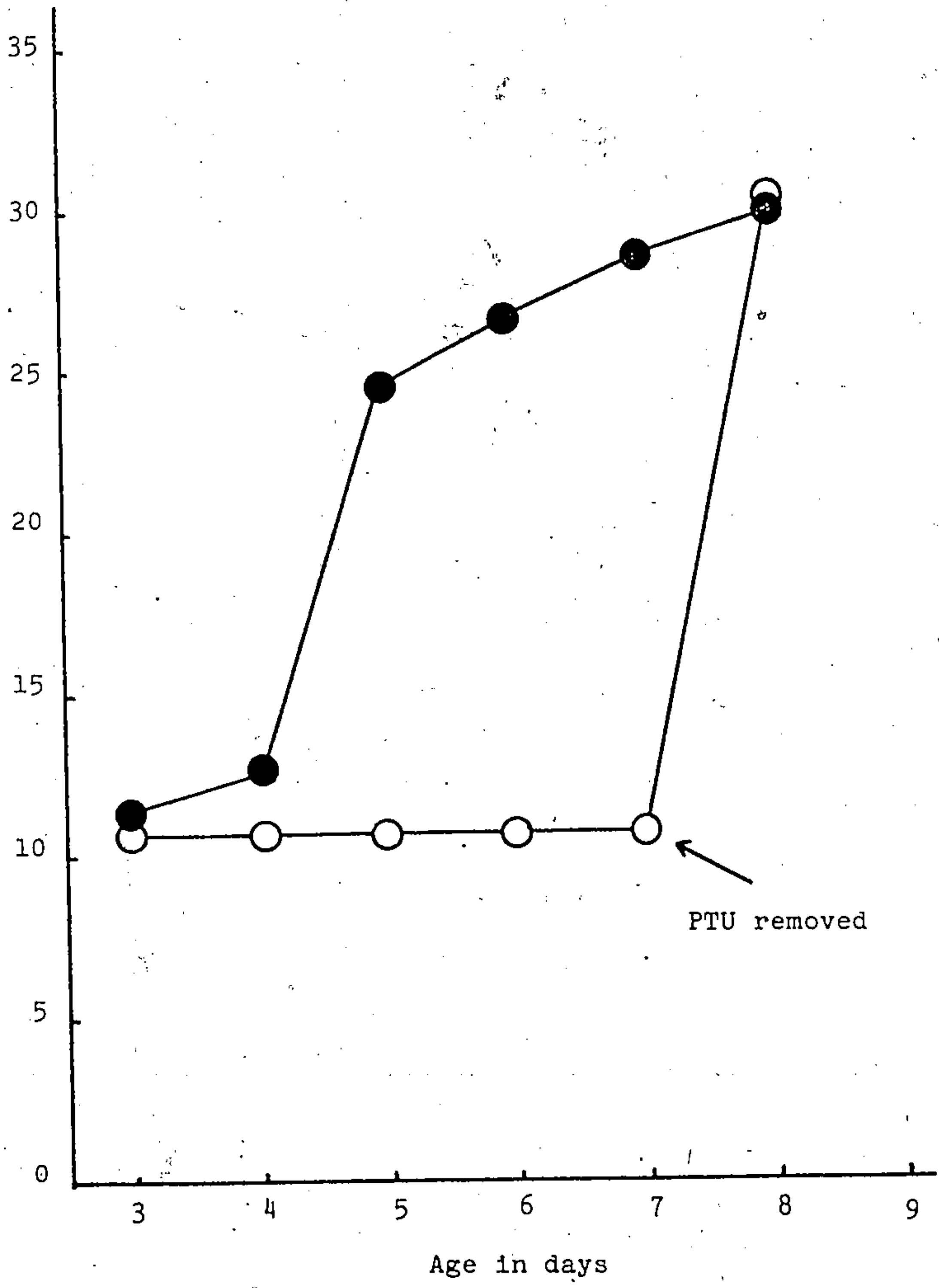
FISH NUMBER	MEDIUM	NUMBER OF CELLS IN THE LATERAL LINE OF DAY 3	4	5	6	7	8	NUMBER OF NEW CELLS	AVERAGE NUMBER OF SECOND WAVE CELLS PER INITIAL CELL
1	0.2 mM PTU	6	6	6	6	6	20	14	2.3
2	"	11	11	11	11	11	30	19	1.7
3	"	11	11	11	11	11	32	21	1.9
4	"	13	13	13	13	13	24	11	0.9
5	"	9	9	9	9	9	34	25	2.8
6	"	13	13	13	13	13	38	25	1.9
7	"	10	10	10	10	10	*	–	–
8	"	17	17	17	17	17	37	20	1.2
9	"	14	14	14	14	14	34	20	1.4
10	"	3	3	3	3	3	26	23	7.7
	Average	10.7					30.6	19.8	
11	H_2O alone	8	15	32	36	36	36	28	3.5
12	"	5	7	30	36	37	43	38	7.6
13	"	10	10	17	19	21	24	14	1.4
14	"	16	20	27	30	34	34	18	1.1
15	"	19	19	26	28	30	30	11	0.6
16	"	13	15	29	29	29	29	16	1.2
17	"	10	10	16	20	20	20	10	1.0
18	"	7	7	16	18	22	23	16	2.3
19	"	15	16	33	34	35	36	21	1.4
20	"	8	8	18	18	20	21	13	1.6
	Average	11.1	12.7	24.4	26.8	28.4	29.6	18.5	

* Fish Died

FIGURE 13

Average number of melanophores in the right-hand-side lateral line band for control and experimental fish (raised in 0.2 mM PTU from days three to seven of development) from days three to eight.

Experimental fish were raised in 0.2 mM PTU from day three to day seven of development when they were removed to dechlorinated water for repigmentation. The number of visible melanophores were counted from days three to eight for control (-●-) and experimental fish (-o-). The average number of melanophores visible each day was plotted (see Table 1).



Average number of melanophores in the right-hand-side lateral line band
35
30
25
20
15
10
5
0
3
4
5
6
7
8
9
Age in days
PTU removed

The new cells are already reoriented in the septum except for cells which appear early on day four. Cells which appear later are seen as completely reoriented cells which elaborate their melanin _in situ_. These cells can undergo physiological colour change even though they contain very small amounts of melanin.

There is a difference between the second wave of melanization and the initial wave which forms the three day stripe. Where the initial cells appear in an antero-posterior wave, the second wave cells appear more irregularly, filling in the spaces between the initial cells. In a representative fish (Fig. 14) the initial cells were spread out from one another. By day eight, somite #4 which originally contained two initial cells still did not have any second wave cells. Of the other ten somites which contained single initials, seven had new melanophores by day eight. The manner of cell appearance was as follows: on day four: four of the five cells which appeared did so in 'empty' somites; the remaining cell appeared in a somite already occupied by an initial cell (somite #15) but then moved into 'empty' somite #14 within another 24 hours. Day five: of 12 new cells, seven became visible in 'empty' somites, two (somites #20 and #22) appeared in the posterior aspects of somites containing initial cells (and in both cases the posterior somite was 'empty' and in somite #20 the new cell moved

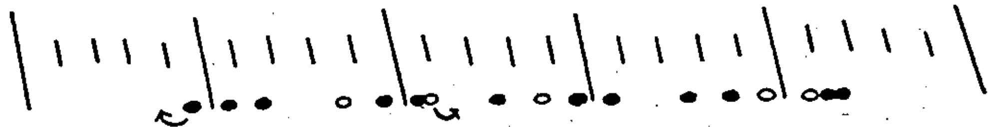
FIGURE 14

Mode of appearance of second wave melanophores in the right-hand-side lateral line band of one zebra fish from days three to eight of development.

Sketches were made from days three to eight of the right-hand-side lateral line melanophore band in one zebra fish. The aggregation of melanin into the centre of each cell after contraction was used to determine cell position. The initial melanophores are sketched as filled and the second wave melanophores as empty. The arrows denote melanophore shuttling. Age in days and the total number of cells are shown on each sketch. The numbers underneath the eight day sketch are the somite designations.

Day 3(12)

Day 4(17)



Day 5(29)

Day 6(34)

Day 8(34)

25 20 15 10 5

into that empty somite in another 24 hours) and three (somites #10, #13 and #20) were observed in somites containing initial cells where somites on either side were occupied. Day six: of five new cells, one joined an already visible new cell (somite #9) and the other four became visible in somites occupied by initials (somites #8, #11, #13 and #16). Two of these cells (in somites #13 and #16) shifted anteriorly within 24 hours. Day seven: there were no new melanophores. Day eight: three new cells appeared of which one did in a somite containing an initial cell (somite #13), and two (somites #6 and #20) did in somites containing second wave melanophores. The first second wave melanophores thus appeared in 'empty'somites . Those appearing later were seen in somites containing initial cells.

The number of new melanophores bears some relationship to the number of initial cells (Table 1 and Fig. 15). Figure 15 shows that as the number of initial cells increased, the number of new melanophores decreased.

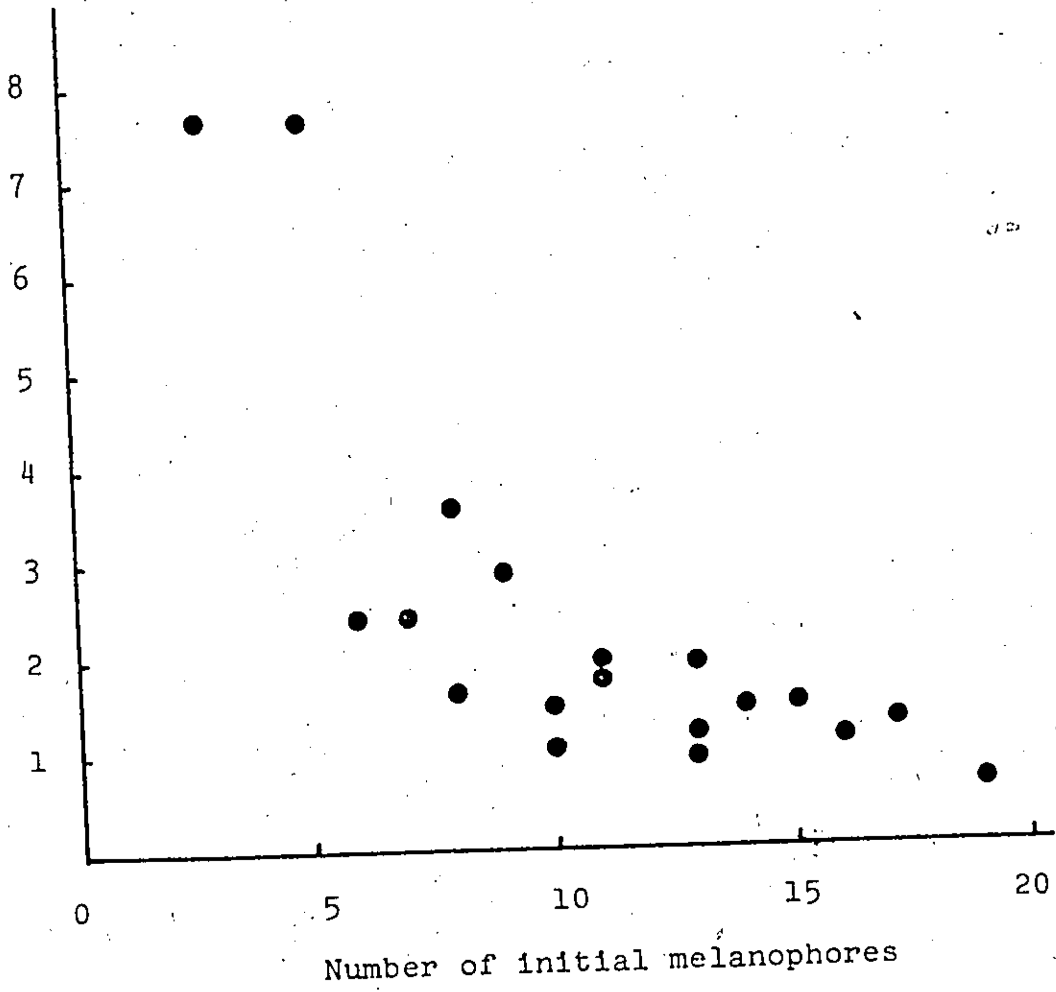
Discussion

The eight day lateral line band of the zebra fish is formed by the addition of new melanophores to the initial cells already present in the band at three days. No evidence was found for melanophore division. The possibility of a pigmented cell dividing to give

FIGURE 15

Effect of the number of initial cells on the number of second wave melanophores per initial cell.

The number of second wave melanophores which appeared in the right hand side lateral line band of each fish by day eight was divided by the number of initial melanophores in that fish to calculate the ratio of visible second wave melanophores to initial cells. The data from control and experimental fish were pooled.



Number of second wave melanophores per initial cell
8
7
6
5
4
3
2
1
0
5
10
15
20
Number of initial melanophores

rise to a pigmented and an unpigmented daughter has never been found although division of melanophores is documented, for instance in *Xenopus laevis* (Pehlmann, 1972). Further, the fact that fish are occasionally seen in which the three day lateral line band is absent although a normal eight day band forms argues as well against melanophore mitosis. It may be that mitosis would occur if the fish were raised on black backgrounds under conditions of continual illumination such as Pehlmann used. In *X. laevis* these conditions caused a great increase in the mitotic rate of the melanophores when compared to controls. This increase was attributed to higher levels of circulating MSH (melanocyte-stimulating hormone) under these conditions.

The phenomenon of addition to the stripe by new melanophore differentiation is designated here as second wave melanization. The melanocytes which appear during three day band formation probably all have melanoblasts as their immediate precursors. This is probably true as well for those melanocytes which appear before the fish can take part in orderly physiological colour change. However, it is suggested here that the cells which appear later in the eight day stripe have as their immediate precursors amelanotic melanophores. This suggestion is made because these cells take part in physiological colour change while they are melanizing.

A complete description of the stages in differentiation of both initial and second wave cells must wait until more work is done in this system.

It is very interesting that the second wave cells are already oriented similarly to the initial cells except very early on day four. It may be that all second wave cells have to reorient before entering the stripe site, and the process of melanization may occur in them at a later stage in migration than it does in the initial cells, making this reorientation for the most part invisible.

Also important is the fact that some shuttling of initial and second wave cells still occurs along the site during eight day band formation. The degree of shuttling may be controlled by the time of entry of the second wave melanophores into the stripe site.

While the initial cells did not completely prohibit the appearance of second wave melanophores into somites already occupied by them, it did seem from these initial observations as if the arrangements of initial cells had an effect on pattern formation by second wave melanophores. This effect, coupled with the facts that shuttling occurs before the second wave appears, that some shuttling occurs while the band is forming, and that early second wave cells are incompletely reoriented give at least indirect evidence that this wave of cells is

not present in the stripe site a long time before it becomes visible. It became important to study this further to determine the extent to which the presence of initial wave cells excludes the appearance of second wave melanophores.

However, there was also some correlation between the numbers of initial cells and the numbers of second wave cells (Fig. 15). As the number of initial cells increased, the number of second wave cells decreased. Could this be a second kind of regulatory effect? Thus, it seemed as if a numerical regulation mechanism operated along with the mechanism that determined cell placement. Much of the rest of the work was devoted to analyzing in greater depth these two possible regulatory controls of band formation by second wave melanophores.

CHAPTER VII

HOW DO INITIAL MELANOPHORE NUMBER AND PLACEMENT INFLUENCE SECOND WAVE MELANOPHORE PATTERN FORMATION?

This first study on numerical and positional regulation was done to see if these phenomena could be studied on a whole-body basis. Due to the complexity of the results a new method was developed for analyzing the phenomena observed.

The study was carried out on two batches of fry, from eggs laid and fertilized by the same pair of fish. Seventy eggs developed normally from batch #1 and fifty-one from batch #2. The eggs were maintained in dechlorinated tap water until three days of age at which time all fish that had not hatched were dechorionated. The young fry were placed in 0.2 mM PTU and on day six each fish was removed from the solution, its melanophores contracted, and after anaesthetization a right-hand-side lateral line band melanophore map was constructed. The fish were then grouped according to the number of cells that existed in the band and raised in dechlorinated tap water until day eight when the second wave cells were added to the initial cells on the maps. The second wave melanophores were again paler than the initial cells

and readily distinguishable from them.

The numbers and arrangements of initial cells, the number of second wave cells, and the number of pairs and triplets were counted. The numbers of fish with each number of initial cells were summed for batches #1 and #2. A calculation was made of the average number of pairs for each number of initial cells by dividing the total number of times that pairs arose for that number of initial cells by the number of fish. The number of second wave melanophores that appeared per initial cell was calculated for each fish as well as the average number of second wave melanophores that appeared for each batch, on a per fish and on a per initial cell basis.

Results

The number of initial cells in these two batches ranged from zero (2.5%) to 21 (0.8%) with half the population falling between six and ten (Table 2, Fig. 16). The average number of initial cells in each batch was 8.8 (batch #1, Table 2a) and 7.8 (batch #2, Table 2b). Figure 17 shows that as the number of initial cells increased the number of pairs did as well.

In batch #1 the average number of second wave cells that appeared in the line per fish was 14.9 and the

TABLE 2

Initial and second wave melanophore counts for batch #1 (a) and batch #2 (b).

Fry were raised in 0.2 mM PTU until day six of development when the initial melanophores were mapped. They were then grouped according to the number of initial cells and raised in dechlorinated tap water until day eight of development when the second wave melanophores were added to the initial cells on the maps. The melanophore maps were then used to calculate the number of initial cell pairs, the average number of pairs per fish, the number of initial and second wave melanophores and the ratio: number of second wave melanophores per initial cell. Calculations for each batch were also made of the average number of initial cells, the average number of second wave cells per fish and per initial cell, and the average total number of cells.

TABLE 2

Initial and second wave melanophore coun

(a)

NUMBER OF INITIALS	NUMBER OF FISH	INITIAL CELLS NUMBER OF PAIRS 0	1	2	3	4	5	6	AVERAGE NUMBER OF PAIRS /FISH	NUMBER OF SECOND WAVE CELLS AT 8 DAYS FOR EACH FISH	T
0	0				–				–	–	–
1	1	1	0	0	0	0	0	0	0	16	1
2	2	2	0	0	0	0	0	0	0	16,24	1
3	1	0	1	0	0	0	0	0	1.0	12	1.
4	3	1	2	0	0	0	0	0	0.7	23,16,19	2
5	5	1	3	1	0	0	0	0	1.0	22,13,16,19,21	2
6	5	2	3	0	0	0	0	0	0.6	14,14,15,16,13	2
7	11	3	8	0	0	0	0	0	0.7	14,18,15,11,20,15,18,16,11,11,15	2
8	5	0	3	1	1	0	0	0	1.6	16,9,15,15,13	2
9	9	0	4	4	1	0	0	0	1.7	12,17,12,9,20,8,15,19,17	2
10	7	1	2	2	2	0	0	0	1.7	10,15,14,8,13,16,17	2
11	5	0	2	1	2	0	0	0*	2.0	21,12,13,16,14	3
12	6	0	0	3	2	1	0	0	2.7	8,9,19,11,9,14	2
13	3	0	0	1	0	1	1	0	3.7	24,10,17	3
14	3	0	0	0	3	0	0	0	3.0	11,14,15	2
15	0				–				–	–	–
16	3	0	0	0	0	1	0	2	5.3	18,18,17	3
17	1	0	0	0	0	0	0	1	6.0	12	2
18	0				–				–	–	–
19	0				–				–	–	–
TOTAL		* ONE TRIPLET									
616	70	AVERAGE NUMBER OF INITIALS PER FISH = 616/70 = 8.8								1045	

AVERAGE NUMBER OF SECONDS/FISH = 10
AVERAGE NUMBER OF SECONDS/INITIAL =

2 of 2

:ABLE 2

ɔphore counts for batch #1(a) and batch #2(b).

..LLS AT	TOTAL NUMBER OF CELLS AT 8 DAYS FOR EACH FISH	NUMBER OF SECOND WAVE MELANOPHORES PER INITIAL CELL
	-	-
	17	16
	18,26	8.0,1.2
	15	4.0
	27,20,23	5.8,4.0,4.8
	27,18,21,24,26	4.4,2.6,3.2,3.8,4.2
	20,20.21,22,19	2.3,2.3,2.5,2.7,2.2
11,11,15	21,25,22,18,27,22,25,23,18,18,22	2.0,2.6,2.1,1.6,2.9,2.1,2.6,2.3,1.6,1.6,2.1
	24,17,23,23,21	2.0,1.1,1.9,1.9,1.6
	21,26,21,18,29,17,24,28,26	1.3,1.9,1.3,1.0,2.2,0.9,1.7,2.1,1.9
	20,25,24,18,23,26,27	1.0,1.5,1.4,0.8,1.3,1.6,1.7
	32,23,24,27,25	1.9,1.1,1.2,1.5,1.3
	20,21,31,23,21,26	0.7,0.8,1.6,0.9,0.8,1.2
	37,23,30	1.9,0.8,1.3
	25,28,29	0.8,1.0,1.1
	-	-
	34,34,33	1.1,1.1,1.1
	29	0.7
	-	-
	-	-
	1661	

ɔS/FISH = 1045/70 = 14.9
ɔS/INITIAL = 1045/616 = 1.7

TOTAL AVERAGE NUMBER OF CELLS ON DAY 8 = 1661/70 = 23.7

TABLE 2

(b)

NUMBER OF INITIALS	NUMBER OF FISH	INITIAL CELLS NUMBER OF PAIRS 0	1	2	3	4	5	6	AVERAGE NUMBER OF PAIRS /FISH	NUMBER OF SECOND WAVE CELLS AT 8 DAYS FOR EACH FISH	TO
0	3	0	0	0	0	0	0	0	0	34,27,24	34
1	0				-				-	-	-
2	2	2	0	0	0	0	0	0	0	31,32	33
3	3	2	1	0	0	0	0	0	0.3	33,23,29	36
4	5	0	5	0	0	0	0	0	1.0	39,33,24,12,20	43
5	1	1	0	0	0	0	0	0	0	37	42
6	9	2	1	5	1	0	0	0	1.6	26,22,16,30,25,28,7,23,11	32
7	9	0	5	4	0	0	0	0	1.4	28,21,23,25,23,26,13,3,10	35
8	3	0	2	1	0	0	0	0	1.0	22,18,21	30
9	1	0	0	0	1	0	0	0	3.0	19	28
10	1	0	0	0	1	0	0	0	3.0	18	28
11	3	0	1	2	0	0	0	0*	1.7	22,25,20	33
12	2	0	0	1	0	1	0	0	3.0	12,16	24
13	2	0	0	1	1	0	0	0	2.5	22,12	35
14	1	0	0	1	0	0	0	0	2.0	20	34
15	1	0	0	0	0	0	1	0	4.0	13	28
16	3	0	0	0	1	2	0	0	3.7	18,19,24	34
17	0				-				-	-	-
18	0				-				-	-	-
19	1	0	0	0	0	0	1	0	4.0	11	30
20	0				-				-	-	-
21	1	0	0	0	0	0	0	0	6.0	13	34
TOTAL											
398	51	* ONE TRIPLET								1113	

AVERAGE NUMBER OF INITIALS PER FISH = 398/51 = 7.8

AVERAGE NUMBER OF SECONDS PER FISH = 1113/
AVERAGE NUMBER OF SECONDS PER INITIAL = 11

2 of 2

TABLE 2

LLS AT	TOTAL NUMBER OF CELLS AT 8 DAYS FOR EACH FISH	NUMBER OF SECOND WAVE MELANOPHORES PER INITIAL CELL
	34,27,24	∞,∞,∞
	-	-
	33,34	16,16
	36,26,32	11,7.7,9.7
	43,37,28,16,24	9.8,8.3,6.0,5.0,4.0
	42	7.4
1	32,28,22,36,31,34,13,34,17	4.3,3.7,2.7,5.0,4.2,4.7,1.2,4.7,1.8
0	35,28,30,32,30,33,20,15,17	4.0,3.0,3.3,3.6,3.3,3.7,1.9,1.1,1.4
	30,26,29	2.8,2.3,2.6
	28	2.1
	28	1.8
	33,36,31	2.0,2.3,1.8
	24,28	1.0,1.3
	35,25	1.7,0.9
	34	1.4
	28	0.9
	34,35,40	1.1,1.2,1.5
	-	-
	-	-
	30	0.6
	-	-
	34	0.6
	1511	

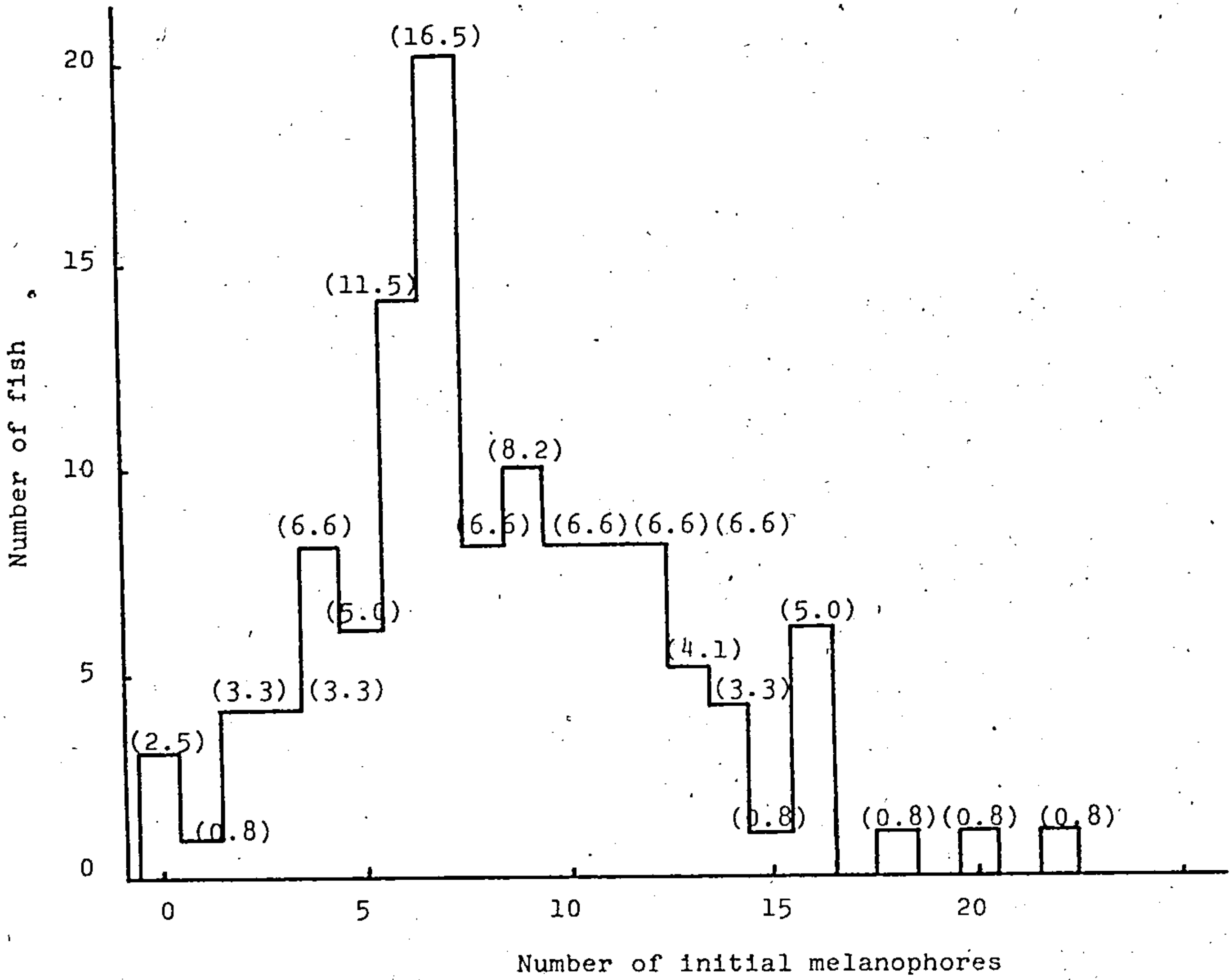
ISH = 1113/51 = 21.8
NITIAL = 1113/398 = 2.8

AVERAGE NUMBER OF CELLS PER FISH ON DAY 8 = 1511/51 = 29.6

FIGURE 16

Number of fish having a given number of initial wave melanophores.

Normalized values (percent of total fish) are given in brackets on the histogram. These data were collected from two separate batches, #1 and #2, of the same breeding pair.



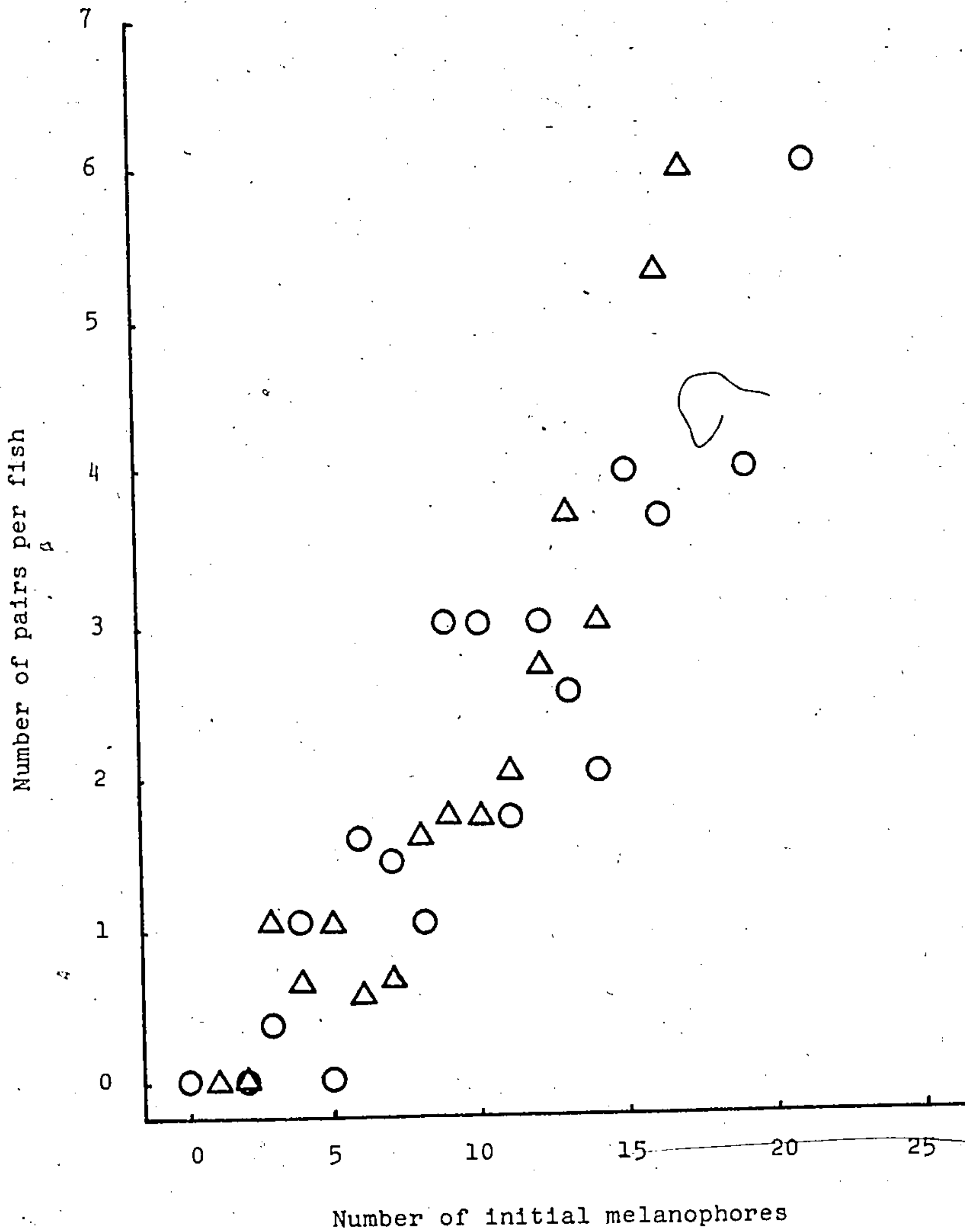
(16.5)
20
15
(11.5)
Number of fish
(8.2)
10
(6.6)
(6.6) (6.6)(6.6)(6.6)
(5.0)
(5.0)
(4.1)
5
(3.3) (3.3)
(3.3)
(2.5)
(0.8)
(0.8)
(0.8)(0.8)(0.8)
0
0
5
10
15
20
Number of initial melanophores

FIGURE 17

Increase in the number of paired melanophores per fish relative to the number of initial melanophores.

The average number of paired melanophores per fish was computed by dividing the number of times that pairs arose for each number of initial melanophores by that number of fish. A sample calculation would be as follows: in Table 2a three fish had four initial cells, and for those three fish there were two pairs. The average number of pairs in batch #1 for fish with four initial cells was therefore 2/3 or 0.7 (to one decimal place).

Batch #1 (-Δ-), batch #2 (-o-).


Number of pairs per fish
Number of initial melanophores
0
1
2
3
4
5
6
7
0
5
10
15
20
25

average number per initial cell was 1.7. The total average number of cells on day eight was 23.7 (Table 2a). For batch #2 the values were 21.8, 2.8 and 29.6 respectively (Table 2b). In both batches as the number of initial cells increased, the number of second wave cells decreased (Table 2a and b, Fig. 18).

Due to the fact that there was such variation in the number of second wave cells for each number of initial cells the data was analyzed using a three somite system. Somite #11 was chosen as the somite of inquiry and the results were analyzed on the basis of initial cell arrangements in #11 and in the somites on either side of it (#10 and #12). The possible arrangements were as follows: (x represents an initial cell):

12 11 10

| | | |, | | |x|, | | |xx|, |x| | |, |xx| | |, |x| |x|,
|xx| |x|, |x| |xx|, |xx| |xx|.

There were thus nine possible combinations in somites #10 and #12. Since somite #11 could either be 'empty' or 'full' that is containing one or more initials or containing no initials, there were eighteen possibilities.

Of the 121 fish, ten had either pairs or triplets in #11 and these were treated as being 'full' and were not separated from the rest of the population.

FIGURE 18

Effect of the number of initial cells on the number of second wave melanophores per initial cell.

The number of second wave melanophores which appeared in the right-hand-side lateral line band of each fish by day eight was divided by the number of initial melanophores in that fish to calculate the ratio of visible second wave melanophores to initial cells. The data from each batch are plotted separately; (a) batch #1, (b) batch #2.

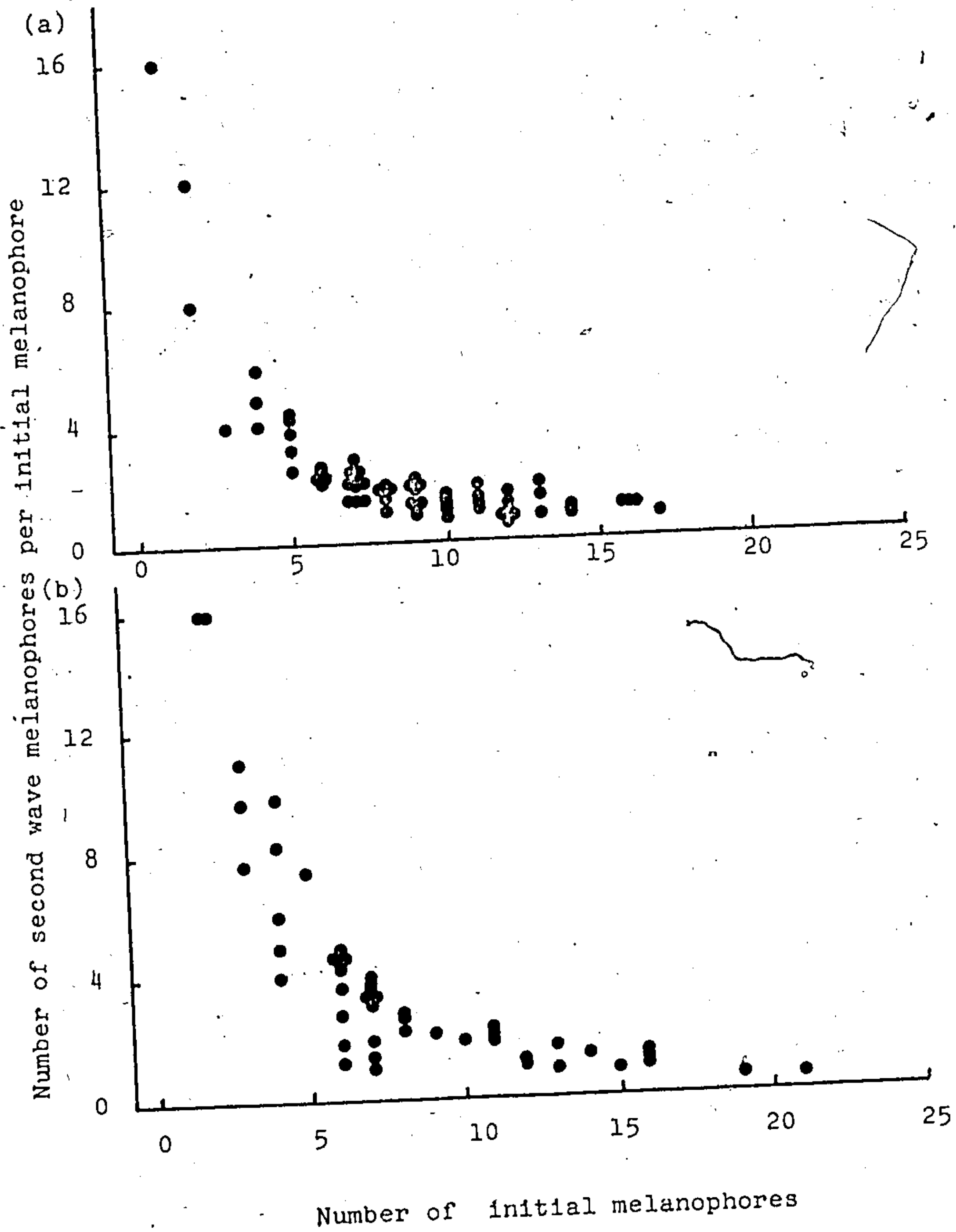

(a)
16
12
8
4
0
0
5
10
15
20
25
(b)
16
12
8
4
0
0
5
10
15
20
25
Number of second wave melanophores per initial melanophore
Number of initial melanophores

Table 3a shows for each batch the number of cases in which a given number of second wave cells became visible in somite #11 when it was 'empty' and when it was 'full'. In only one case did a second wave cell appear when there were two or more initials present. The summed results for batches #1 and #2 (Table 3b, Fig. 19) show that when an initial cell was present in somite #11 it excluded visible second wave cells in 46 out of 55 cases (83.6%). It should be noted that of the 66 fish with somite #11 'empty' initially, only one failed to repair the 'natural' three day defect (1.5% of cases). In all other fish where #11 was 'empty' initially, from one to four second wave cells became visible in that somite by day eight. Most of the 'natural' three day defects were filled by singles or pairs, but in four cases three cells were observed and in one case, four became visible.

Discussion

The results on whole eight day stripe formation show that there is an inverse correlation between initial (three day) and second wave melanophore numbers (numerical regulation). When a single somite is considered (three somite system analysis), if one or more initial melanophores are present, they tend to exclude the appearance of second wave cells (83.6%). On the other hand, rarely

TABLE 3

Three somite system analysis of second wave melanophore appearance in somite #11.

a) Three somite system analysis results on day eight for batches #1 and #2.

b) Summed three somite system analysis results on day eight for batches #1 and #2.

The 121 melanophore maps from batches #1 and #2 were examined on day eight of development using the three somite system of analysis (somites #10, #11 and #12). The fish were classified according to the arrangements of initial cells in these three somites and the number of second wave melanophores that appeared in somite #11 was counted for each fish. The data were then tabulated.

TABLE 3

(a) Three somite system analysis results on day eight for batches #1 and #2.

<table>
<tr><th rowspan="2">SOMITE SYSTEM</th><th colspan="5">NO INITIAL IN 11
NUMBER OF SECOND WAVE CELLS THAT ENTERED 11</th><th colspan="5">INITIAL CELL(S) IN 11
NUMBER OF SECOND WAVE CELLS THAT ENTERED 11</th></tr>
<tr><th>0</th><th>1</th><th>2</th><th>3</th><th>4</th><th>0</th><th>1</th><th>2</th><th>3</th><th>4</th></tr>
<tr><td>BATCH #1</td><td colspan="5">NUMBER OF CASES</td><td colspan="5">NUMBER OF CASES</td></tr>
<tr><td>| | | |</td><td>0</td><td>9</td><td>3</td><td>0</td><td>0</td><td>7</td><td>1</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>| | |x|</td><td>1</td><td>2</td><td>1</td><td>0</td><td>0</td><td>4</td><td>0</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>|x| | |</td><td>0</td><td>4</td><td>3</td><td>0</td><td>0</td><td>4</td><td>1</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>| | |xx|</td><td>0</td><td>1</td><td>0</td><td>0</td><td>0</td><td>2</td><td>0</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>|xx| | |</td><td>0</td><td>3</td><td>2</td><td>0</td><td>0</td><td>2</td><td>0</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>|x| |x|</td><td>0</td><td>1</td><td>2</td><td>0</td><td>0</td><td>4</td><td>1</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>|x| |xx|</td><td>0</td><td>3</td><td>0</td><td>0</td><td>0</td><td>3</td><td>1</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>|xx| |x|</td><td>0</td><td>0</td><td>1</td><td>0</td><td>0</td><td>4</td><td>0</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>|xx| |xx|</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td></td><td>1</td><td>23</td><td>12</td><td>0</td><td>0</td><td>30</td><td>4</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>BATCH #2</td><td colspan="5"></td><td colspan="5"></td></tr>
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<tr><td>| | |x|</td><td>0</td><td>2</td><td>1</td><td>2</td><td>0</td><td>1</td><td>1</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>|x| | |</td><td>0</td><td>1</td><td>3</td><td>0</td><td>0</td><td>1</td><td>0</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>| | |xx|</td><td>0</td><td>0</td><td>3</td><td>0</td><td>0</td><td>2</td><td>3</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>|xx| | |</td><td>0</td><td>1</td><td>1</td><td>0</td><td>0</td><td>2</td><td>0</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>|x| |x|</td><td>0</td><td>0</td><td>1</td><td>0</td><td>1</td><td>3</td><td>0</td><td>1</td><td>0</td><td>0</td></tr>
<tr><td>|x| |xx|</td><td>0</td><td>1</td><td>1</td><td>0</td><td>0</td><td>1</td><td>0</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>|xx| |x|</td><td>0</td><td>1</td><td>3</td><td>1</td><td>0</td><td>1</td><td>0</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>|xx| |xx|</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td></td><td>0</td><td>9</td><td>16</td><td>4</td><td>1</td><td>16</td><td>4</td><td>1</td><td>0</td><td>0</td></tr>
</table>

TABLE 3

(b) Summed three somite system analysis results on day eight for batches #1 and #2.

SOMITE SYSTEM	NO INITIAL IN 11 NUMBER OF SECOND WAVE CELLS THAT ENTERED 11					INITIAL CELL(S) IN 11 NUMBER OF SECOND WAVE CELLS THAT ENTERED 11				
	0	1	2	3	4	0	1	2	3	4
	NUMBER OF CASES					NUMBER OF CASES				
| | | |	0	12	6	1	0	12	1	0	0	0
| | |x|	1	4	2	2	0	5	1	0	0	0
|x| | |	0	5	6	0	0	5	1	0	0	0
| | |xx|	0	1	3	0	0	4	3	0	0	0
|xx| | |	0	4	3	0	0	4	0	0	0	0
|x| |x|	0	1	3	0	1	7	1	1	0	0
|x| |xx|	0	4	1	0	0	4	1	0	0	0
|xx| |x|	0	1	4	1	0	5	0	0	0	0
|xx| |xx|	0	0	0	0	0	0	0	0	0	0
	1	32	28	4	1	46	8	1	0	0

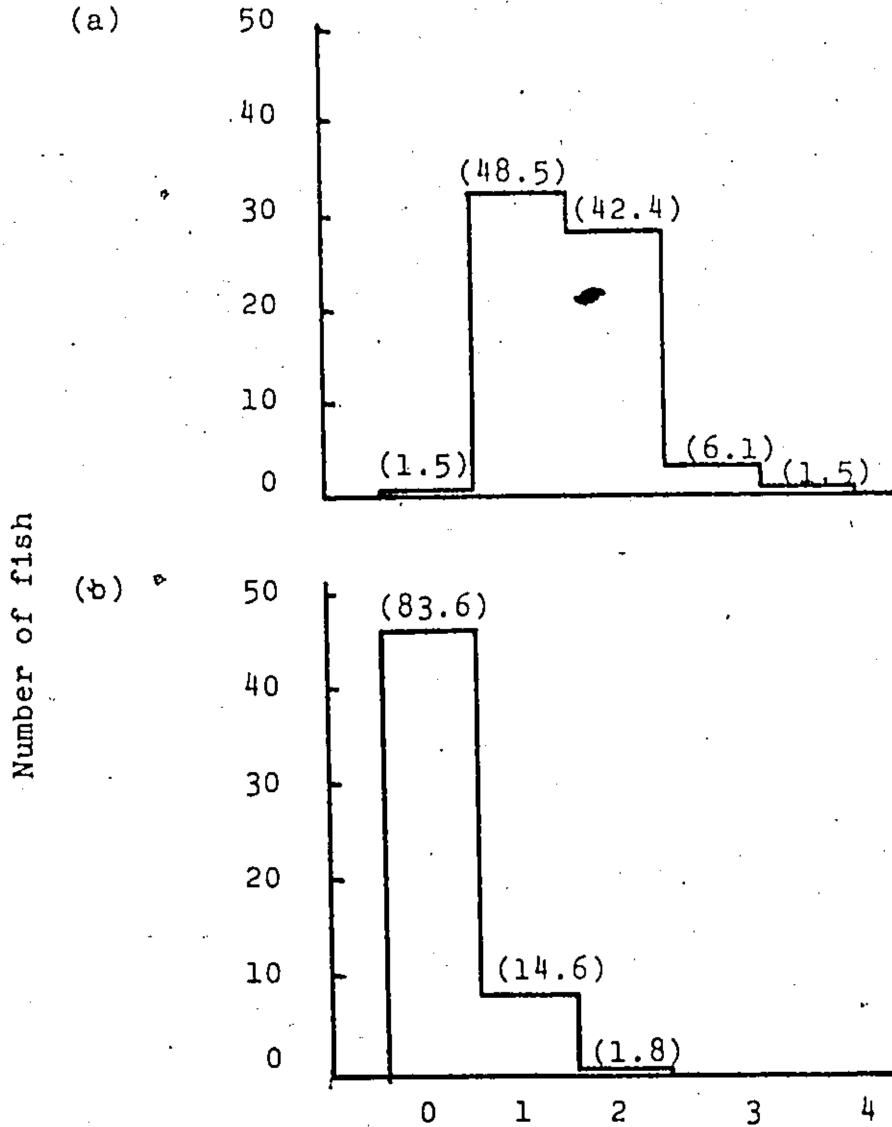
FIGURE 19

Second wave cell appearance in somite #11 when an initial cell is present or absent.

Fish were raised in 0.2 mM PTU from days three to six of development. On day six the initial visible melanophores of the right-hand-side lateral line band were mapped. The fish were then raised in dechlorinated tap water until day eight of development when the second wave melanophores were added to the initial cell maps.

The 121 melanophore maps from batches #1 and #2 were examined on day eight of development using the three somite system of analysis (somites #10, #11 and #12). The fish were classified according to the arrangements of initial cells in these three somites and the number of second wave melanophores that appeared in somite #11 was counted for each fish. The data were then tabulated.

This figure combines the data from batches #1 and #3 (Table 3b) for the two cases which existed in somite #11; (a) initial melanophore absent and (b) initial melanophore present. Percent of totals are shown in brackets above the histogram.


(a)
50
40
30
20
10
0
(48.5)
(42.4)
(1.5)
(6.1)
(1.5)
Number of fish
(b)
50
40
30
20
10
0
(83.6)
(14.6)
(1.8)
0
1
2
3
4
Number of visible second wave melanophores in somite #11 on day eight

does a somite remain 'empty' by day eight (1.5%). The melanophores appear in the spaces in the line preferentially and only rarely become visible in somites where initial cells are already present. This is designated here as the exclusion principle.

There are several possibilities for the mode of operation of the exclusion principle. The second wave cells may be already present at the site (as amelanotic melanophores, melanoblasts or their precursors). The exclusion principle would then operate to control melanogenesis of these cells or their differentiation into melanoblasts (which could then synthesize melanin). Alternatively, the second wave cells (amelanotic melanophores, melanoblasts or their precursors) may enter the stripe site from elsewhere (the pigment cell reservoir). They may sample the pigment cell niches and preferentially populate somites which contain no initial cells until all the available places in the line are filled. The observations of early second wave appearance suggest that the cells do enter from elsewhere since 1) the second wave cells which appear late on day three and early on day four are not completely reoriented and 2) some movement of initial and second wave melanophores occurs into 'empty' spaces in the band while it is forming.

The exclusion principle may not always operate with 100% efficiency. There are two possible explanations. One, the factors directly responsible for exclusion may

vary so that they operate less well in some fish than in others. If cell size contributes to the exclusion mechanism cells of different sizes could vary in their ability to prohibit the entry of second wave cells. Alternatively, melanogenesis of melanoblasts already at the site could be prohibited to different extents. On the other hand, another component of the system could vary; for instance increases in second wave cell population pressure (if the cells enter from elsewhere).

Whole right hand side melanophore maps were analyzed for the nine fish which did not obey the exclusion principle for somite #11. Table 4 shows the second wave to initial cell ratios for the five fish with five, six and seven initial cells which did not conform to the exclusion principle. The average ratio of second wave cells to initial cells was also calculated for each batch for all fish with five, six and seven initial cells even though the number of fish was small. Fishes #2 and #3 had ratios which were higher (4.5 and 4.7) than the average ratio (3.6). Thus, in these two fish second wave population pressure (if the cells enter from elsewhere) could result in second wave cells appearing in somites which already contained an initial cell. In fish #2 the cell appeared with a pair of initial cells while in fish #3 where somite #11 contained one initial cell and somite #12 was empty, the cell appeared in the posterior area of somite #11.

TABLE 4

Ratio of second wave to initial lateral line melanophores for fish with five, six and seven initial cells which did not obey the exclusion principle for somite #11.

The ratio of second wave to initial lateral line melanophores was calculated for each of these fish. The average ratio was also calculated for all the fish with that particular number of initial cells (individual batch calculations). The ratios were then compared.

TABLE 4

Ratio of second wave to initial lateral line melanophores for fish with five, six and seven initial cells which did not obey the <u>exclusion principle.</u>

FISH NUMBER (Batch number in brackets)	NUMBER OF INITIAL MELANOCYTES	NUMBER OF SECOND WAVE MELANOPHORES	RATIO OF SECOND WAVE CELLS TO INITIAL CELLS	
			FOR THE FISH WHICH DID NOT OBEY THE <u>EXCLUSION PRINCIPLE</u>	FOR ALL FISH FROM THE BATCH WITH THAT NUMBER OF INITIAL CELLS
1 (-1)	5	16	3.2	3.6
2 (-2)	6	27	4.5	3.6
3 (-2)	6	28	4.7	3.6
4 (-1)	7	15	2.1	2.0
5 (-2)	7	23	3.3	2.9

Fishes #1, #4 and #5 differed less from the average ratio and therefore no explanation can be offered for their differing results on this basis. However in fish #1 somite #10 was empty and the second wave cell appeared in the anterior area of somite #11, and in fish #4 both somites #10 and #12 were empty. In fish #5, where somites #10 to #12 each contained one initial cell, two second wave cells appeared in somite #11. In this fish the second wave to initial cell ratio (3.3) was slightly higher than the average ratio (2.9).

Thus, in three of these fish one mechanism can be postulated for why second wave cells intruded into occupied somites; namely a flexibility in the feedback mechanism which allowed proportionally more second wave cells to be delivered. The pattern in fishes #1 and #4 can then be explained by the fact that the initial cell in somite #11 was less able to prohibit second wave cell entry. Alternatively, if the second wave cells are already at the site, then in these five fish, the initial cell in somite #11 was less able to prohibit second wave cell differentiation or melanogenesis than were the other initial cells which did not allow second wave cells to appear. In all fish with five, six and seven initial cells, where somite #11 was occupied, the initial cell blocked second wave cell appearance in 14 of 19 cases (73.7%). In these fish

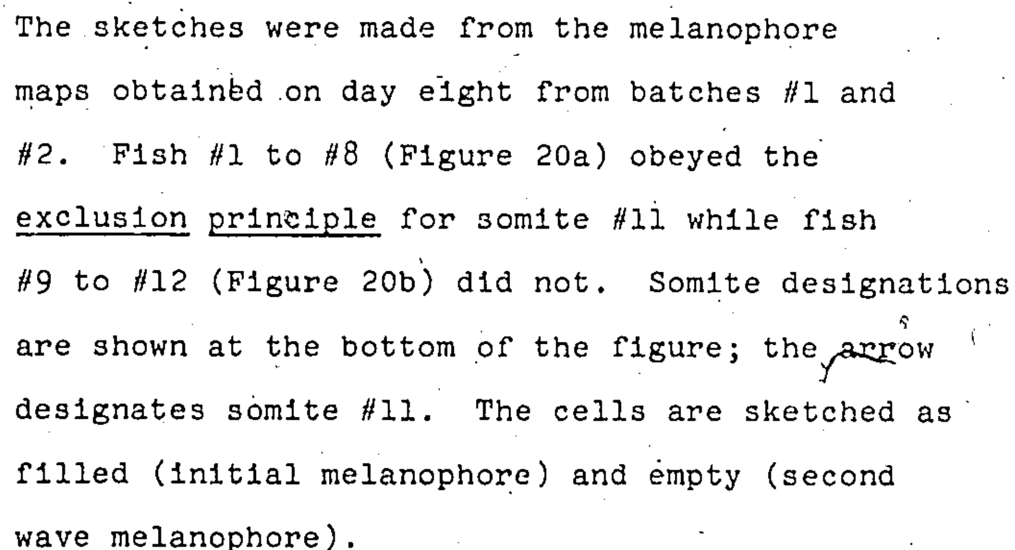
all pairs blocked second wave cell appearance in 12/16 (75%) of cases and single initial cells did in 74 of 91 cases (81.3%).

Four of the fish which did not obey the exclusion principle for somite #11 had large (14 or more) numbers of initial cells. Figure 20 is a diagrammatic representation of eight fish with thirteen or more initial cells which did obey the exclusion principle (Fig. 20a) and the four fish with similar numbers of initial cells which did not (Fig. 20b).

Initial and second wave melanophore numbers were counted and the ratio was determined. The number of pairs and the number of somites containing single initial cells were also calculated. The eight fish which obeyed the exclusion principle allowed second wave cells to appear in five of 31 (16.1%) somites containing pairs and eight of 60 (13.3%) somites containing initial cells. The exclusion principle therefore operated with 83.9% and 86.7% fidelity in occupied somites. Note that in fishes #3, #4 and #6 there were only four, five and five somites containing initial cells and in none of these did second wave cells appear. In the other five fish which obeyed the exclusion principle for somite #11 there were eight to ten somites containing single initial cells and either one or two of these allowed second wave cells

FIGURE 20

Some examples of the operation and the non-operation of the exclusion principle.



The sketches were made from the melanophore maps obtained on day eight from batches #1 and #2. Fish #1 to #8 (Figure 20a) obeyed the exclusion principle for somite #11 while fish #9 to #12 (Figure 20b) did not. Somite designations are shown at the bottom of the figure; the arrow designates somite #11. The cells are sketched as filled (initial melanophore) and empty (second wave melanophore).

FISH NUMBER AND BATCH (IN BRACKETS)

NU
INI
MEL

20(a)

1-(II)

2-(II)

3-(I)

4-(I)

5-(I)

6-(II)

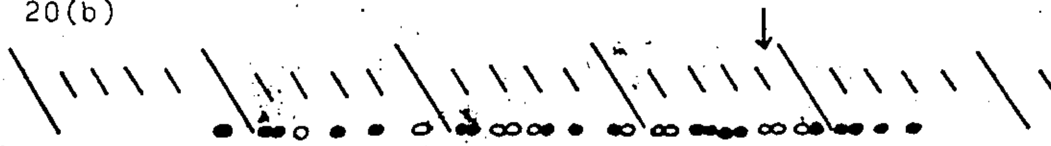
7-(II)

8-(I)

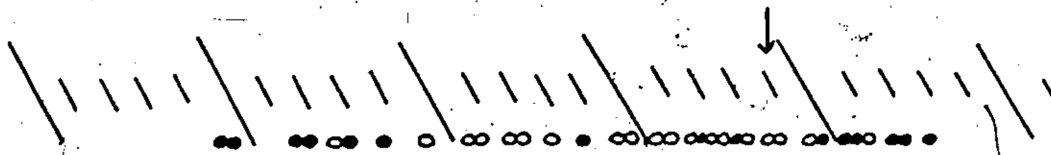
NUMBER OF INITIAL MELANOPHORES	NUMBER OF SECOND WAVE MELANOPHORES	RATIO OF SECOND WAVE MELANOPHORES TO INITIAL MELANOPHORES	NUMBER OF INITIAL MELANOPHORE PAIRS	NUMBER OF SOMITES CONTAINING ONE INITIAL MELANOPHORE	NUMBER OF SECOND WAVE MELANOPHORES WHICH ENTERED SOMITES CONTAINING ONE OR MORE INITIAL MELANOPHORES
16	18	1.1	3	10	2
21	13	0.6	6	9	5
16	18	1.1	6	4	0
13	10	0.8	4	5	0
14	11	0.8	3	8	1
15	13	0.9	5	5	0
14	20	1.4	2	10	3
13	17	1.3	2	9	4

FISH NUMBER AND BATCH (IN BRACKETS)	NUM INIT MEL

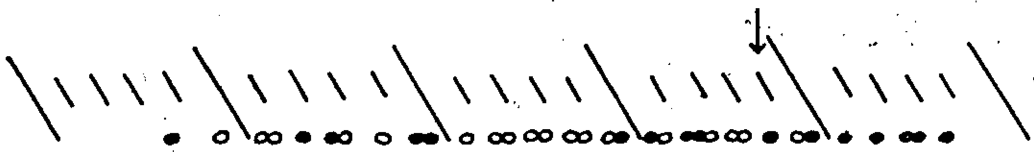
20(b)



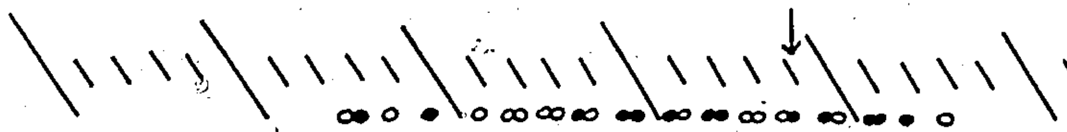
9-(II)



10-(II)



11-(I)



12-(I)

NUMBER OF INITIAL MELANOPHORES	NUMBER OF SECOND WAVE MELANOPHORES	RATIO OF SECOND WAVE MELANOPHORES TO INITIAL MELANOPHORES	NUMBER OF INITIAL MELANOPHORE PAIRS	NUMBER OF SOMITES CONTAINING ONE INITIAL MELANOPHORE	NUMBER OF SECOND WAVE MELANOPHORES WHICH ENTERED SOMITES CONTAINING ONE OR MORE INITIAL MELANOPHORES
19	11	0.6	5	9	3
16	19	1.2	4	7	7
16	18	1.1	3	10	5
14	14	1.0	3	8	5

to appear. Thus, as the number of somites occupied by single initial cells increased, so did second wave cell appearance in them.

The four fish which did not obey the exclusion principle for somite #11 allowed second wave cells to appear in two of 15 (13.3%) somites containing pairs and 16 of 34 (47.1%) somites containing single initial cells. The exclusion principle was therefore operative in 86.7% and 52.9% of cases. These fish similarly had eight to ten somites occupied by single initial cells, but these single cells were less able to prohibit second wave cell appearance. Assuming that the second wave cells enter the line from elsewhere, second wave to initial ratios cannot account for the discrepancy, since the ratios did not differ appreciably between these four fish and the other eight. The initial cells in these four fish were less able to prevent second wave cell entry, differentiation or melanogenesis either because of individual initial cell differences or because of the cumulative effect on second wave cell appearance of these particular arrangements of initial cells.

The observations of second wave cell appearance thus lead to a possible mechanism of band formation up to eight days. Initial cell number and arrangement control visible pattern formation at least partially. Initial cells arranged as close pairs are expected to be more

efficient at prohibiting second wave cell appearance than are initial cells located singly in somites. There are several possibilities why second wave cells may appear in initially occupied somites. The first mechanism of pattern formation postulates that the second wave cells are already at the site. Initial cells would then vary in their ability to suppress melanogenesis or differentiation. The analysis of 12 of the fish with large numbers of initial cells suggests that two initial cells are more efficient in this regard than is a single initial cell. The second mechanism of pattern formation postulates that the cells enter the site from elsewhere. Again, initial cells in pairs are more efficient at prohibiting second wave cell entry than are single initial cells, which may vary in their ability depending on a factor like cell size. However, in this mechanism feedback between the reservoir and the stripe site may not be exact and too many second wave cells may be delivered resulting in some of these entering 'full' somites. Similarly, if there are large numbers of initial cells, empty somites may be 'masked' and second wave cells may again enter somites containing initial cells. In both mechanisms of pattern formation the results seen may depend on the cumulative effect of all the initial cells at the line.

Pairs of melanophores were more effective in fish with many initial cells (83.9%) than they were in fish with five, six and seven initial cells (75%). In the fish with few initial cells they were all single pairs, that is either the somites on either side of the pair were 'empty' or they contained at the most one initial cell. Note that in fishes #2, #4, #5 and #6 there were two sets of triple pairs and five sets of double pairs (three or two pairs in a row). Both triple pairs (100%) and four of five double pairs (80%) prohibited second wave cell appearance. In fishes #1 to #8 the single pairs were effective in only eleven of fifteen cases (73.3%). Thus, the exclusion principle operates better when initial cells are located close together. It may operate with 100% efficiency at a certain ratio of second wave to initial melanophores (including a factor for initial cell arrangement) and at all ratios lower than this for each number of initial cells. Relevant to the observation that cells which are already present block second wave cell appearance is the work of Mayer (1970) which showed that there is a limit to the number of melanoblasts which can populate mouse skin. When amelanotic melanocytes were present in 13 to 18 day old mouse skin they prevented the invasion of normal melanoblasts and resulted in unpigmented hair.

One investigation that must therefore be pursued in future work is the mechanism governing exclusion and the stages in differentiation that the unpigmented cells pass through before they become pigmented. While the three somite system of analysis has been shown to be a valuable tool in understanding second wave cell appearance, it must be extended in future work to include more somites.

CHAPTER VIII

HOW LABILE IS EIGHT DAY BAND FORMATION? THREE DAY DEFECT EXPERIMENTS.

1) Extirpation of melanophores from somite #11.

The observations of whole body band formation, including the three somite system of analysis suggested that initial cell placement as well as initial cell number plays a role in visible second wave melanophore pattern formation.

On the basis of the exclusion principle an initial cell should, about 80% of the time, prohibit the appearance of second wave melanophores. What would happen if the initial cell was ablated? The defect could remain unrepaired with perhaps great consequences for further band genesis or could be repaired in several ways. First, the cells already visible in the line could redistribute themselves to fill in the defect. Second, either initial or second wave melanophores already in position might undergo mitosis to fill the gap. Third, defect repair might occur by new melanophore differentiation at the site, thus placing the onus for repair not on the pigmented cells already in the band, but on the unpigmented cells located at the site or in the pigment cell reservoir.

Three experiments were done on three separate batches of eggs laid and fertilized by one pair of fish. In each batch, one third of the fish with an initial cell(s) in somite #11 were raised as normal controls. Initial wave melanophores were extirpated from somite #11 in the remainder on day three of development. Fish which had no initial melanophore in somite #11 were raised as controls except for 11 which underwent sham operations (see Materials and Methods). All operated and unoperated fish were raised in 0.1 mM PTU to allow gradual repigmentation. The PTU solutions were not changed and were topped up with distilled water every day. Since the solutions were weak, the second wave cells were easily observed as pale grey melanophores once all the black fragments of initial cells had disappeared. Operated fish were examined at four and six days and those in which the initial cell had not been destroyed were discarded. On day eight, the melanophores were contracted, the fish anaesthetized and examined for the presence or absence of second wave cells in somite #11 (three somite system of analysis). Data were collected from 150 fish from batch #1, 85 from batch #2, and 194 from batch #3.

Results

The average number of second wave cells which appeared in an initially 'empty' and an operated somite

#11 were 1.6 and 1.4 for batch #1, 1.7 and 1.6 for batch #2, and 1.5 and 1.1 for batch #3 (Table 5 a,b, and c, and Fig. 21). Thus, in the three experiments, the ratio of defect repair to normal appearance in 'empty' somites was 87.5%, 94.1% and 73.3%.

In sham operations, 2.0 second wave cells appeared in somite #11 on a per fish basis (Table 5b). However, note that in one of these fish, four second wave cells became visible. This is a rare occurrence and was observed in only one other fish (also from batch #2, Table 5b).

Figure 22 shows for the summed results, that on a per fish basis, an initial cell blocked second wave melanophore appearance in 83.7% of cases. However, when the calculation is based on the number of second wave cells which appeared in initially 'full' somites, it is seen that the exclusion principle operated with only 81% fidelity (Table 5d).

In controls, where somite #11 was empty initially, a second wave melanophore failed to appear in only 3.1% of cases. In operated somites where the initial cell(s) were extirpated, the defects were not repaired in 15.8% of cases. However, extrapolating from the initially empty controls and assuming that 3.1% of these would have remained unrepaired anyway, the defect percentage becomes 15.3% (3.1% of 15.8%).

TABLE 5

Second wave melanophore appearance in somite #11 when an initial melanophore is present (control), absent or removed experimentally.

(a) Initial cell extirpation results for batch #1.

(b) Initial cell extirpation results for batch #2.

(c) Initial cell extirpation results for batch #3.

(d) Initial cell extirpation results for batches #1, #2, and #3.

Fish were classified according to the three somite system of analysis. Fish with no initial melanophore(s) in somite #11 were raised as controls except for 11 which underwent sham operations (batch #2). One third of the fish with an initial melanophore(s) in somite #11 formed the second control group and in the remainder the initial cell(s) were extirpated. All fish were raised in 0.1 mM PTU which was not changed to allow gradual repigmentation. On day eight the number of second wave melanophores which entered somite #11 was counted in controls and experimentals. The fidelity of operation of the exclusion principle was calculated here on the basis of the number of second wave cells which entered somite #11. In Table 5d, 16 second wave cells entered somite #11 when it was already occupied. The exclusion principle was therefore inoperational 16/86 x 100% = 19% of the time, and operational 100% - 19% = 81% of the time.

TABLE 5

(a) Initial cell extirpation results for Batch #1.

<table>
<tr><th rowspan="3">SOMITE SYSTEM</th><th colspan="15">NUMBER OF SECOND WAVE CELLS ENTERING SOMITE #11 BY 8 DAYS</th></tr>
<tr><th colspan="5">INITIAL CELL PRESENT</th><th colspan="5">INITIAL CELL ABSENT</th><th colspan="5">INITIAL CELL EXTIRPATED</th></tr>
<tr><th>0</th><th>1</th><th>2</th><th>3</th><th>4</th><th>0</th><th>1</th><th>2</th><th>3</th><th>4</th><th>0</th><th>1</th><th>2</th><th>3</th><th>4</th></tr>
<tr><td>12 11 10</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>| | | |</td><td>9</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>3</td><td>10</td><td>2</td><td>0</td><td>1</td><td>4</td><td>5</td><td>3</td><td>0</td></tr>
<tr><td>| | |x|</td><td>5</td><td>0</td><td>0</td><td>0</td><td>0</td><td>1</td><td>11</td><td>9</td><td>0</td><td>0</td><td>1</td><td>2</td><td>1</td><td>0</td><td>0</td></tr>
<tr><td>| | |xx|</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>3</td><td>5</td><td>1</td><td>0</td><td>0</td><td>2</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>|x| | |</td><td>4</td><td>1</td><td>0</td><td>0</td><td>0</td><td>0</td><td>2</td><td>6</td><td>0</td><td>0</td><td>1</td><td>1</td><td>3</td><td>0</td><td>0</td></tr>
<tr><td>|xx| | |</td><td>1</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>1</td><td>1</td><td>0</td><td>0</td><td>0</td><td>5</td><td>3</td><td>0</td><td>0</td></tr>
<tr><td>|x| |x|</td><td>2</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>6</td><td>3</td><td>1</td><td>0</td><td>0</td><td>6</td><td>4</td><td>0</td><td>0</td></tr>
<tr><td>|xx| |x|</td><td>1</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>3</td><td>0</td><td>0</td><td>0</td><td>2</td><td>4</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>|x| |xx|</td><td>1</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>1</td><td>1</td><td>0</td><td>0</td><td>0</td><td>0</td><td>1</td><td>0</td><td>0</td></tr>
<tr><td>|xx| |xx|</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>1</td><td>1</td><td>1</td><td>0</td><td>0</td><td>2</td><td>2</td><td>0</td><td>0</td></tr>
<tr><td>TOTALS</td><td>23</td><td>1</td><td>0</td><td>0</td><td>0</td><td>1</td><td>31</td><td>36</td><td>5</td><td>0</td><td>5</td><td>26</td><td>19</td><td>3</td><td>0</td></tr>
<tr><td>AVERAGE NUMBER OF SECOND WAVE MELANOPHORES</td><td colspan="5">1/24 = 0.04*</td><td colspan="5">118/73 = 1.6</td><td colspan="5">73/53 = 1.4</td></tr>
<tr><td>PERCENT OF FISH WITH DEFECTS ON DAY 8</td><td colspan="5"></td><td colspan="5">1/73 x 100% = 1.4%</td><td colspan="5">5/53 x 100% = 9.4%</td></tr>
</table>

* This calculation was made to two decimal places to permit the inclusion of these data in the table.

TABLE 5

(b) Initial cell extirpation results for Batch #2.

<table>
<tr><th>SOMITE SYSTEM</th><th colspan="15">NUMBER OF SECOND WAVE CELLS ENTERING SOMITE #11 BY 8 DAYS</th></tr>
<tr><th></th><th colspan="5">INITIAL CELL PRESENT</th><th colspan="5">INITIAL CELL ABSENT</th><th colspan="5">INITIAL CELL EXTIRPATED</th></tr>
<tr><td>12 11 10</td><td>0</td><td>1</td><td>2</td><td>3</td><td>4</td><td>0</td><td>1</td><td>2</td><td>3</td><td>4</td><td>0</td><td>1</td><td>2</td><td>3</td><td>4</td></tr>
<tr><td></td><td>5</td><td>0</td><td>0</td><td>0</td><td>0</td><td>1</td><td>3</td><td>6</td><td>4</td><td>0</td><td>1</td><td>2</td><td>4</td><td>0</td><td>0</td></tr>
<tr><td>| | |x|</td><td>2</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>5</td><td>1</td><td>1</td><td>0</td><td>1</td><td>1</td><td>3</td><td>1</td><td>0</td></tr>
<tr><td>| | |xx|</td><td>1</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>2</td><td>2</td><td>0</td><td>0</td><td>0</td><td>0</td><td>2</td><td>0</td><td>0</td></tr>
<tr><td>|x| | |</td><td>1</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>1</td><td>0</td><td>0</td><td>0</td><td>2</td><td>1</td><td>0</td><td>0</td></tr>
<tr><td>|xx| | |</td><td>1</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>2</td><td>2</td><td>1</td><td>0</td><td>0</td><td>0</td><td>0</td><td>1</td><td>0</td></tr>
<tr><td>|x| |x|</td><td>3</td><td>1</td><td>0</td><td>0</td><td>0</td><td>0</td><td>1</td><td>2</td><td>0</td><td>0</td><td>0</td><td>1</td><td>3</td><td>0</td><td>1</td></tr>
<tr><td>|xx| |x|</td><td>0</td><td>0</td><td>1</td><td>0</td><td>0</td><td>0</td><td>0</td><td>1</td><td>0</td><td>0</td><td>0</td><td>1</td><td>2</td><td>0</td><td>0</td></tr>
<tr><td>|x| |xx|</td><td>1</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>2</td><td>1</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>|xx| |xx|</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>1</td><td>0</td><td>0</td><td>2</td><td>1</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>TOTALS</td><td>14</td><td>1</td><td>1</td><td>0</td><td>0</td><td>1</td><td>15</td><td>17</td><td>6</td><td>0</td><td>4</td><td>8</td><td>15</td><td>2</td><td>1</td></tr>
<tr><td>SHAMS</td><td colspan="15"></td></tr>
<tr><td>|x| | |</td><td></td><td></td><td></td><td></td><td></td><td>0</td><td>1</td><td>0</td><td>0</td><td>0</td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>|xx| | |</td><td></td><td></td><td></td><td></td><td></td><td>0</td><td>4</td><td>4</td><td>1</td><td>1</td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>AVERAGE NUMBER OF SECOND WAVE MELANOPHORES</td><td colspan="5">3/16 = 0.19</td><td colspan="5">67/39 = 1.7
SHAM OPERATIONS
20/10 = 2.0</td><td colspan="5">48/30 = 1.6</td></tr>
<tr><td>PERCENT OF FISH WITH DEFECTS ON DAY 8</td><td colspan="5"></td><td colspan="5">1/39 x 100%
= 2.6%</td><td colspan="5">4/30 x 100%
= 13.3%</td></tr>
</table>

TABLE 5

(c) Initial cell extirpation results for Batch #3.

<table>
<tr><th rowspan="3">SOMITE SYSTEM</th><th colspan="15">NUMBER OF SECOND WAVE CELLS ENTERING SOMITE #11 BY 8 DAYS</th></tr>
<tr><th colspan="5">INITIAL CELL PRESENT</th><th colspan="5">INITIAL CELL ABSENT</th><th colspan="5">INITIAL CELL EXTIRPATED</th></tr>
<tr><th>0</th><th>1</th><th>2</th><th>3</th><th>4</th><th>0</th><th>1</th><th>2</th><th>3</th><th>4</th><th>0</th><th>1</th><th>2</th><th>3</th><th>4</th></tr>
<tr><td>| | | |</td><td>9</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>10</td><td>16</td><td>0</td><td>0</td><td>2</td><td>7</td><td>9</td><td>0</td><td>0</td></tr>
<tr><td>| | |x|</td><td>6</td><td>3</td><td>0</td><td>0</td><td>0</td><td>0</td><td>2</td><td>8</td><td>1</td><td>0</td><td>1</td><td>5</td><td>3</td><td>0</td><td>0</td></tr>
<tr><td>|xx| |xx|</td><td>1</td><td>1</td><td>0</td><td>0</td><td>0</td><td>1</td><td>2</td><td>3</td><td>2</td><td>0</td><td>1</td><td>2</td><td>1</td><td>1</td><td>0</td></tr>
<tr><td>|x| | |</td><td>6</td><td>1</td><td>0</td><td>0</td><td>0</td><td>2</td><td>4</td><td>5</td><td>0</td><td>0</td><td>0</td><td>5</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>|xx| | |</td><td>2</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>5</td><td>3</td><td>1</td><td>0</td><td>1</td><td>0</td><td>1</td><td>0</td><td>0</td></tr>
<tr><td>|x| |x|</td><td>6</td><td>3</td><td>1</td><td>0</td><td>0</td><td>1</td><td>8</td><td>4</td><td>0</td><td>0</td><td>4</td><td>2</td><td>4</td><td>0</td><td>0</td></tr>
<tr><td>|xx| |x|</td><td>2</td><td>2</td><td>0</td><td>0</td><td>0</td><td>0</td><td>1</td><td>3</td><td>0</td><td>0</td><td>4</td><td>6</td><td>1</td><td>0</td><td>0</td></tr>
<tr><td>|x| |xx|</td><td>2</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>2</td><td>0</td><td>0</td><td>0</td><td>1</td><td>0</td><td>1</td><td>0</td><td>0</td></tr>
<tr><td>|xx| |xx|</td><td>1</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>1</td><td>0</td><td>0</td><td>0</td><td>0</td><td>1</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>TOTALS</td><td>35</td><td>10</td><td>1</td><td>0</td><td>0</td><td>4</td><td>35</td><td>42</td><td>4</td><td>0</td><td>14</td><td>28</td><td>20</td><td>1</td><td>0</td></tr>
<tr><td>AVERAGE NUMBER OF SECOND WAVE MELANOPHORES</td><td colspan="5">12/46 = 0.26</td><td colspan="5">131/85 = 1.5</td><td colspan="5">71/63 = 1.1</td></tr>
<tr><td>PERCENT OF FISH WITH DEFECTS ON DAY 8</td><td colspan="5"></td><td colspan="5">4/85 x 100% = 4.7%</td><td colspan="5">14/63 x 100% = 22.2%</td></tr>
</table>

TABLE 5

(d) Initial cell extirpation results for Batches #1, #2, and #3.

SOMITE SYSTEM	NUMBER OF SECOND WAVE CELLS ENTERING SOMITE #11 BY 8 DAYS														
	INITIAL CELL PRESENT					INITIAL CELL ABSENT					INITIAL CELL EXTIRPATED				
	0	1	2	3	4	0	1	2	3	4	0	1	2	3	4
| | | |	23	0	0	0	0	1	16	32	6	0	4	13	18	3	0
| | |x|	13	3	0	0	0	1	18	18	2	0	3	8	7	1	0
| | |xx|	2	1	0	0	0	1	7	10	3	0	1	4	3	1	0
|x| | |	11	2	0	0	0	2	6	12	0	0	1	8	4	0	0
|xx| | |	4	0	0	0	0	0	8	6	2	0	1	5	4	1	0
|x| |x|	11	4	1	0	0	1	15	9	1	0	4	9	11	0	1
|xx| |x|	3	2	1	0	0	0	4	4	0	0	6	11	5	0	0
|x| |xx|	4	0	0	0	0	0	5	2	0	0	1	0	2	0	0
|xx| |xx|	1	0	0	0	0	0	2	2	1	0	2	4	2	0	0
TOTALS	72	12	2	0	0	6	81	95	15	0	23	62	54	6	1
AVERAGE NUMBER OF SECOND WAVE MELANOPHORES	16/86 = 0.19					316/197 = 1.6					192/146 = 1.3				
PERCENT OF FISH WITH DEFECTS ON DAY 8						6/197 x 100% = 3.1%					23/146 x 100% = 15.8%				
FIDELITY OF EXCLUSION PRINCIPLE	100% - (100-19)% = 81%														

FIGURE 21

Average number of second wave melanophores which appeared in somite #11 by day eight in experimentals (where the initial cell was extirpated on day three) and in controls where no initial cell was present.

Fish were classified according to the three somite system of analysis. An initial cell was extirpated on day three and control and experimental fish were raised in 0.1 mM PTU which was not changed to allow gradual repigmentation. The total number of second wave melanophores which entered somite #11 on day eight was counted in controls (initial cell absent) and experimentals (initial cell extirpated). This number was divided by the number of fish in that group to give the average number of second wave melanophores per fish. Individual calculations were made for each bath (Table 5a - batch #1, Table 5b - batch #2, and Table 5c - batch #3). Control fish - solid lines; experimental fish - hatched lines.

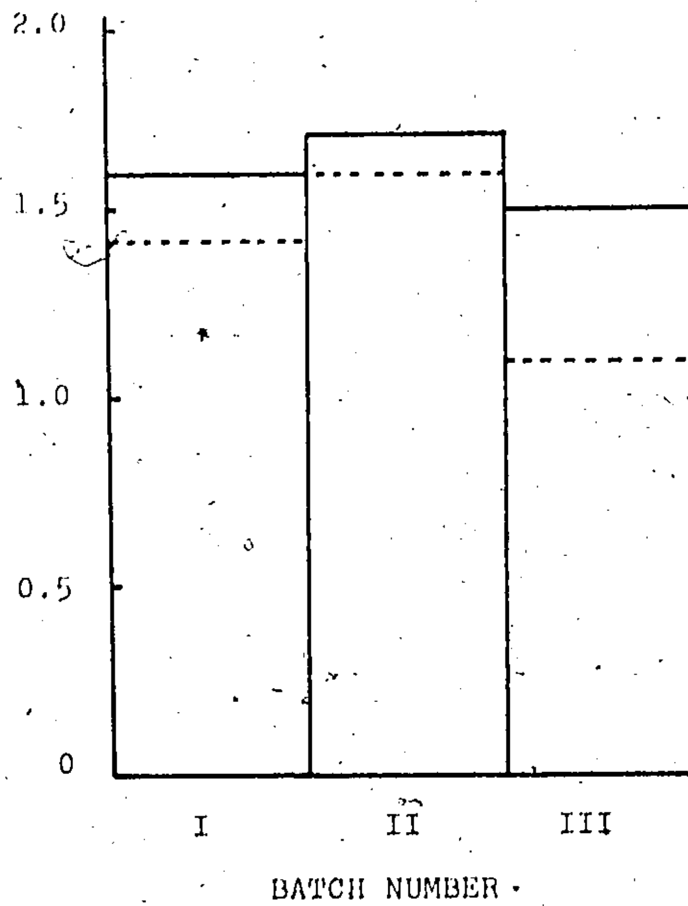
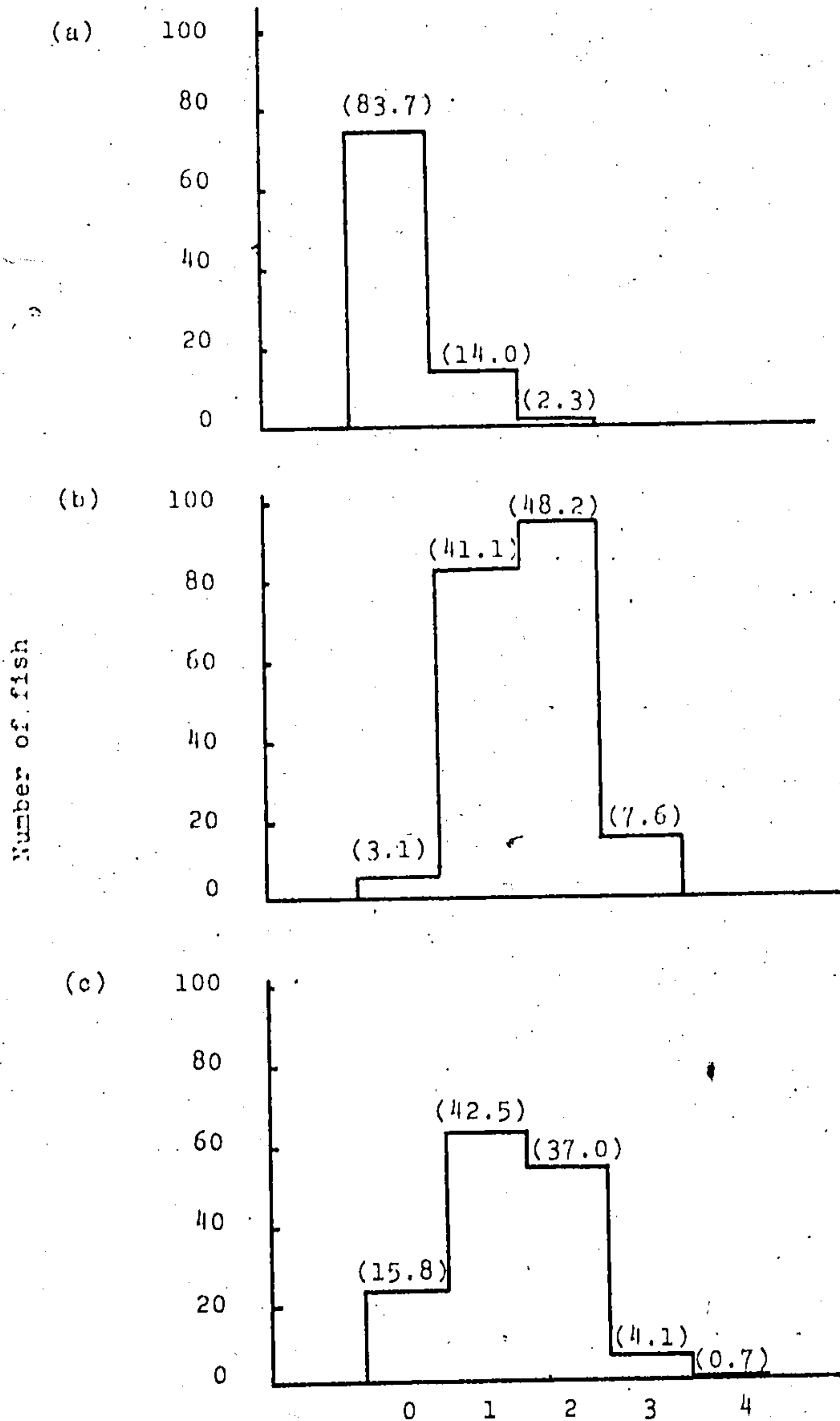

Average number of second-wave melanophores
in somite #11 on day eight
2.0
1.5
1.0
0.5
0
I
II
III
BATCH NUMBER

FIGURE 22

Second wave melanophore appearance in somite #11 when an initial cell is present, absent or extirpated.

Fish were classified according to the three somite system of analysis. An initial cell(s) was extirpated on day three and control and experimental fish were raised in 0.1 mM PTU which was not changed to allow gradual repigmentation. On day eight the number of second wave melanophores which entered somite #11 was counted in controls and experimentals. This figure shows the summed values (batches, #1, #2 and #3, Table 5d) based on whether an initial cell was (a) present (control), (b) absent or (c) removed experimentally. Percent of totals are shown in brackets.


(a)
100
80
60
40
20
0
(83.7)
(14.0)
(2.3)
(b)
100
80
60
40
20
0
(3.1)
(41.1)
(48.2)
(7.6)
(c)
100
80
60
40
20
0
(15.8)
(42.5)
(37.0)
(4.1)
(0.7)
0
1
2
3
4
Number of fish
Number of second wave melanophores in #11 on day eight

In controls where somite #11 was 'empty' initially, the following arrangements of second wave melanophores were observed on day eight of development: 42.4% singles, 49.7% pairs, and 7.9% triplets. In experimental fish the following arrangements existed: 50.4% singles, 43.9% pairs, 4.9% triplets and 0.8% as a group of four (Fig. 23). The pattern of appearance in control fish is virtually indistinguishable from that in operated fish.

Discussion

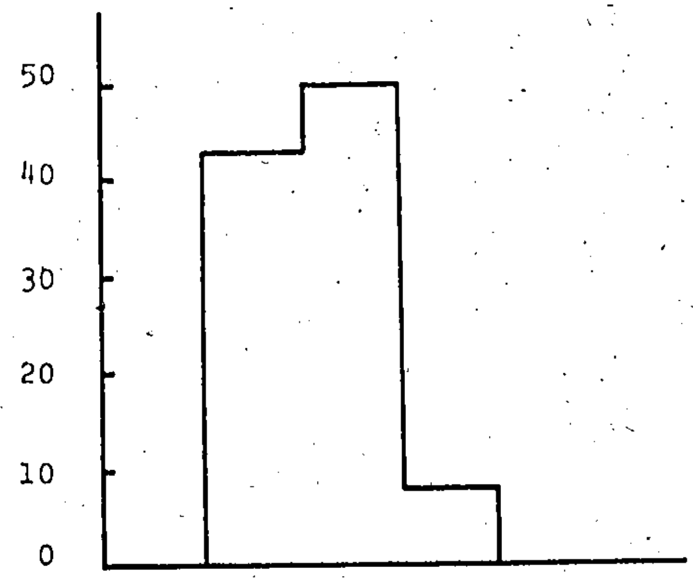
If all experimentally produced defects were repaired, the same number of second wave cells would appear in initially 'empty' and experimentally 'empty' somites by day eight of development (100% repair).

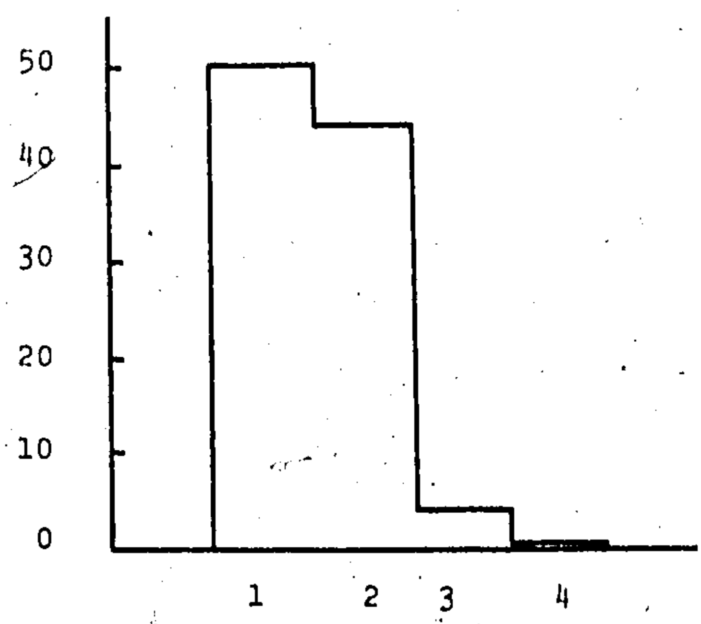
On a per fish basis, appearance of second wave cells in an occupied somite and the total number of defects were 4.0% and 9.4% (Batch #1, Table 5a), 19.0% and 13.3% (Batch #2, Table 5b), and 26.0% and 22.2% (Batch #3, Table 5c). Therefore, as the number of unregulated defects increased, so did the intrusion rate into somites containing an initial cell. One possible explanation is that the exclusion principle operated less well in the order; batch #1 to batch #2 to batch #3. However, if the principle did operate less well in batch #2 than batch #1 another possibility must be considered for why defect repair was high in batch #2. It was suggested in Chapter VII that the exclusion principle may operate best at certain arrangements of initial cells. Initial cell arrangements in some fish of batch #2 may have

FIGURE 23

Arrangements of visible second wave melanophores in somite #11 in initially 'unoccupied' somites and in somites with an experimentally produced defect.

The visible second wave melanophores on day eight (in somite #11) were counted in (a) initially 'empty' somites and (b) experimentally 'empty' somites. The values shown are based on the total number of arrangements; an arrangement being a single second wave melanophore, a pair, a triplet or a group of four.





Percentage of the total number of arrangements which entered somite #11 by eight days

Number of visible second wave melanophores which entered somite #11 by eight days

made defect 'perception' easier. High second wave cell population pressure may have been combined with this, resulting in a high rate of repair. Whole lateral line initial melanophore arrangements and second wave cell population effects must be investigated in future work. The factors directly responsible for exclusion (including the possible effects of cell fragments) must also be studied in this system.

Unrepaired defects and intrusion rates into somites containing initial cells were lower in fish where at least one somite on either side of somite #11 was 'empty'. In fish containing initial cell(s) in somite #11 and on either side of it in both somites #10 and #12, eight of 27 (29.6%) did not obey the exclusion principle while 13 of 56 (23.2%) experimentals showed defects. In fish where somites #10 and #12 were 'empty' these values were 0% and 10.5% respectively. (When somite #10 contained one or more initials the values were 21.1% and 14.3%, and when somite #12 contained one or more initials the values were 11.8% and 8.3%). This supports the proposal that if a defect is 'perceived' it is repaired. In many fish with cells on either side of somite #11 there were long rows of initial cells. The fact that 29.6% of control fish containing an initial cell in somite #11 did not conform to the exclusion principle and that 23.2% of experimental fish did not repair the defect could mean that even after the initial cell was extirpated it was harder for the second wave cells to 'perceive' the defect due to the cells present on either

side of it. The three somite system of analysis must therefore be extended to permit these finer distinctions.

Other evidence in favour of the theory that if a defect is 'perceived' it is repaired is the following. The average number of second wave melanophores that appeared in somite #11 on a per fish basis excluding defects was 1.7 for controls (where somite #11 was 'empty' initially) and 1.6 for fish with experimentally produced defects (controls 316/191 = 1.7, experimentals 192/123 = 1.6, Table 5d). When they include the defects these calculations become 1.6 (control) and 1.3 (experimental- 81% of the control value). Recognition that a somite was occupied also occurred 81% of the time. Thus, defect repair took place the same number of times as did operation of the <u>exclusion principle</u> suggesting that it is the recognition of 'full' and 'empty' somites that is an important factor governing the population of somites by the second wave of visible melanophores.

2) <u>Extirpation of the whole lateral line band</u>

The experiment (Chapter VII -1) in which melanophore extirpation was tested in one somite only allows one to define visible second wave cell position regulation. The entire three day lateral line band was extirpated to determine if true numerical regulation could occur, that is, would the appropriate number of

second wave cells become visible by eight days to make up the deficit?

Seventy-two three day old fish with initial cells in ten or more somites in the right-hand-side lateral line band were chosen from one batch. They were classified according to the number of somites which contained initial cells since the first contraction could not yet be elicited and it was sometimes hard to distinguish one cell from two. One third (24 fish) formed the control group and the remainder formed the experimental group (48 fish). In this group the lateral line band was totally extirpated. Even though the operation was done at three days, some second wave cells were already present. These cells were not touched but their presence was noted.

All fish were raised in 0.1 mM PTU and the PTU solutions were changed at four days of age. Since there were more fragments here (which took longer to clear) than when only one initial cell was removed the lower concentration of PTU was chosen to allow gradual repigmentation. This enabled all second wave cells to become gradually visible and distinguishable from any initial cell fragments. On day six, all experimental fish were observed, and 22 doubtful cases were discarded (cases in which the cells had not been destroyed or in which so many fragments remained at the site that further observations were obscured). At eight days, counts were made of the number of initial

and second wave cells in controls and the number of second wave cells in experimentals. Those cases in which fragments and second wave cells could not be distinguished were eliminated from the counts. This amounted at the most to two cells and occurred in eight of the 26 experimental fish.

Results

The average number of cells in the line at eight days was 31.4 for controls and 28.2 for experimentals (Table 6). The average number of second wave cells which entered was 16.7 and 28.2 respectively (Fig. 24).

Discussion

When the entire initial lateral line band was extirpated, true numerical regulation occurred to reconstitute 90% of the normal right hand side lateral line band. Thus, it seems that not only is sampling going on at the line to determine possible cell position but some sort of feed-back system is operating. Thus, in the lateral line band, initial cell placement and initial cell number both act to control visible second wave cell placement and number, perhaps by two different processes. One occurs at the line itself and is a sampling event concerned with visible pigment cell niches being either 'empty' or 'full'. The other may

TABLE 6

Initial and second wave melanophore counts at eight days for control fish and experimental fish in which the right-hand-side lateral line band was extirpated on day three.

Fish which had initial melanophores in ten or more somites on day three were chosen from one batch. One third formed the control group and in the remainder the entire right-hand-side lateral line band was extirpated. Control and experimental fish were raised in 0.1 mM PTU which was changed only at four days. On day eight the number of initial cells, the number of second wave cells and the total number of cells were counted in both groups.

TABLE 6

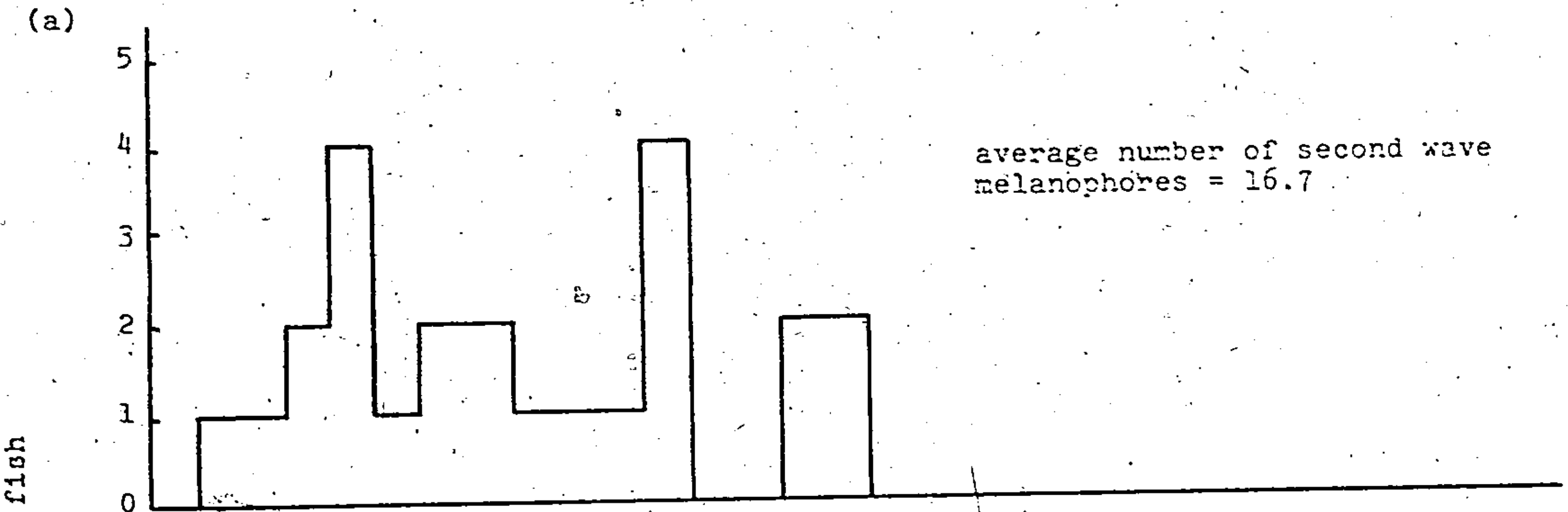
Initial and second wave melanophore counts at eight days for control fish and experimental fish in which the right hand side lateral line band was extirpated on day three. * Minimum estimate

NUMBER OF SOMITES WITH AN INITIAL CELL		NUMBER OF INITIAL CELLS AT 8 DAYS		TOTAL NUMBER OF CELLS AT 8 DAYS		NUMBER OF SECOND WAVE CELLS AT 8 DAYS	
CONTROL AT 8 DAYS	EXPERIMENTAL AT 3 DAYS	CONTROL	EXPERIMENTAL	CONTROL	EXPERIMENTAL	CONTROL	EXPERIMENTAL
10	10	12	0	25	25	13	25
10	10	13	0	28	24	15	24
10	10	13	0	27	28	14	28
10	10	11	0	24	25	13	25
10	10	11	0	23	29	12	29
10	10	15	0	31	26	16	26
10	10	11	0	22	27	11	27
10	11	16	0	28	27*	12	27
11	11	11	0	27	27*	16	27
11	11	14	0	32	33*	18	33
11	11	13	0	37	34	24	34
11	11	16	0	33	24	17	24
11	11	13	0	36	35	23	35
11	11	13	0	28	32	15	32
12	11	14	0	38	35*	24	35
12	11	16	0	36	36*	20	36
12	11	13	0	26	26	13	26
12	12	18	0	41	30*	23	30
13	12	19	0	39	20	20	20
13	12	14	0	34	30	20	30
14	12	17	0	27	28	10	28
14	13	17	0	36	29*	19	29
17	13	20	0	33	26	13	26
18	14	22	0	42	31	20	31
	14		0		24		24
	17		0		22*		22
			TOTAL	753	733	401	733
			MEAN	31.4	28.2	16.7	28.2

FIGURE 24

Effect of removal of the entire right-hand-side lateral line melanophore band on the number of second wave melanophores visible at eight days.

Fish which had initial melanophores in ten or more somites were chosen from one batch on day three. One third formed the control group and in the remainder the entire right-hand-side lateral line band was extirpated. Control and experimental fish were raised in 0.1 mM PTU which was changed only at four days. On day eight the number of second wave melanophores was counted in (a) controls and (b) experimentals.


(a)
average number of second wave
melanophores = 16.7
(b)
average number of second wave
melanophores = 28.2
Number of fish
0
1
2
3
4
5
10
20
30
40
Number of second wave melanophores in the right hand side lateral line band
on day eight

occur at the line or elsewhere and stimulates the pigment cell reservoir to deliver the appropriate number of cells or allows the differentiation of more visible melanophores from melanoblasts, amelanotic melanophores or progenitors already in position.

The phenomenon of numerical regulation was shown in Chapter VII (Fig. 18), but there was no information available about the time of determination of the visible second wave cells. This experiment indicates that the 'information' can reach the 'reservoir' at a time when second wave cells are appearing in some fish. Thus, it it obvious that at least until three days of age the feedback system is flexible and operative. A most interesting experiment would be to do the total initial cell ablations at later and later times, to see at what stage of development the 'reservoir' could no longer effectively respond to provide the regulated number of cells by eight days.

When this study was initiated, some thought was given as to how the pigment cell reservoir in the zebra fish could be located. Since migration from the neural crest probably occurs in an anterior-posterior wave as it does in other species (Weston, 1970) and since pigmentation and band formation also progress in this wave it was felt that the appropriate extirpations might give some information on where the reservoir(s)

is located. If whole-line extirpations were carried out on fish with many initials and only those fish were chosen which had melanophores at the anterior and posterior aspects of the line, a time lag in the appearance of melanophores posteriorly might suggest that the immigration of new cells into the line proceeded in an anterior to posterior wave. The fact that visible cell number and placement are governed by the positions and numbers of initial cells would suggest that second cell entry does progress in some sort of a wave perhaps from the front to the back of the fish. However, there is no proof for this. Evidence for this sort of entry into the line must wait until more experiments are attempted. If, in control fish where all initials are located at the front or at the back of the line, there is a corresponding delay in second wave arrival at the back of the fish with the initials anteriorly placed, some credence may be given to this theory.

Use of H^3-DOPA (Model and Dalton, 1968) may permit labelling of melanoblasts or amelanotic melanophores, provided that the tyrosinase system is active. Interference contrast microscopy has been used to study melanoblasts in Xiphophorus (Leuken and Kaeser, 1972) and this tool as well may prove to be useful in B. rerio. The time of arrival of the cells into the stripe site

could thus possibly be determined, provided that they can be observed before they enter the septum.

The production of chimaeric fish should be technically feasible. Band formation progresses similarly in the zebra danio and the spotted danio (B. nigrofasciatus) up to eight days. The pearl danio (B. albolineatus) has a similar striped pattern at the mid-flank area but the stripes are blue-violet and iridescent. It is possible that paler melanophores contribute to the coloration in adults and a pigment pattern similar to that of B. rerio may be formed by eight days. If chimaerism for pigmentation could be obtained by eight days the appropriate calculations (see Wegmann and Gilman, 1970) could perhaps give an indication of the number of cells set aside to form pigment cells. Perhaps chimaeric animals could be constructed where the anterior and posterior halves differed. Pigment cell extirpations in one half might then be repaired by the same kind of pigment cell or the different kind. However Volpe (1963) has found that when chimaeras are made between different species of frogs at the tailbud stage there is little passage of chromatophores across the line of fusion suggesting that there is not a reservoir located anteriorly or posteriorly but that the progenitors may more uniformly populate the body. This assumes, however, that if there was passage, donor

pigment cells from one species would still be recognizable in the other half of the chimaera.

A search for chromatophore precursors has never been done in *B. rerio*. Since three day lateral line melanophores migrate ventrally and eight day melanophores are seen first at the line at a time when xanthophore pigmentation is underway, it would be intriguing if eight day but not three day pigment cells contained pigmented organelles other than melanosomes. Chromatophores containing more than one type of organelle have been observed in other species of teleosts. For instance the tapetum lucidum of *Dasyatis sabina* contains melanophores and iridophores, and cells are occasionally seen which contain both reflecting platelets and melanosomes. In some cases these two different organelles are bounded by the same membrane (Arnott *et al.*, 1970). If intergrade chromatophores exist in *B. rerio* this could provide some proof that in this system a pigment cell precursor could differentiate along the appropriate cells lines that were perhaps governed by the environment. This kind of work, if coupled with the formation of chimaeric fish, the DOPA reaction and extirpations anteriorly and posteriorly could give some leads as to the nature of the precursors and the size of the population.

A different mechanism of repair might be seen if both xanthophores and melanophores were extirpated. If flank xanthophores were extirpated at the same time as the initial lateral line cells, melanophores in dorsal and

ventral stripes might migrate onto the flank and attempt to reorient into the septum. If such a mechanism of defect repair occurred, this would constitute proof that the time for reorientation is not limited and that xanthophores are important in controlling pattern formation by melanophores. The relationships between melanophores and xanthophores remain to be investigated in this system.

CHAPTER IX

CONTRIBUTIONS OF THE EIGHT DAY LATERAL LINE BAND TO ADULT PATTERN FORMATION

The main focus in this thesis has been pattern formation in the zebra fish up to eight days of development and specifically the formation of the lateral line band with its small number of melanophores. The stripes of adult fish, however, contain large numbers of these cells which are differently oriented with respect to those in the horizontal skeletogenous septum. While the lateral line melanophores are oriented for the most part perpendicular to the dorso-ventral axis of the fish, the cells in the adult stripes are oriented parallel to the body wall. The lateral line band has important protective functions in young fish, but what are its roles in later life? These cells may play a very active and important role in band genesis of older fish. Although this seemed doubtful in view of the different orientations of the cells in the lateral line band versus the adult bands, detailed observations were made of the further stages in band genesis to determine whether the eight day lateral line melanophores did play any role in adult pattern formation.

Ten three day old fish were raised in 0.2 mM PTU until five days of age when the initial cells were mapped (in somites #15-17). The fish were then placed in dechlorinated tap water and the positions of all further appearances at the site were sketched. The fish were observed for approximately six weeks until they were about 15 mm. in length and the juvenile stripe had formed.

Results

A brief description follows of the events seen around the lateral line (somite #16) of a representative fish (Fig. 25) which had two second wave melanophores present at eight days and was studied to 37 days of development. During the next four to five weeks there was a gradual addition of new melanophores to the band by new melanophore differentiations whichresulted in the cells taking up staggered arrays in the stripe. This was coupled with the gradual reorientation of lateral stripe melanophores located next to the edge of the flank. The exact beginning of reorientation was hard to pinpoint because the cells located directly at the edge were already partly reoriented anyway. A photomicrograph of a 2 µm thick plastic section of a reorienting cell is shown in Figure 26. The reorientation process is very gradual

FIGURE 25

Sketches of juvenile stripe formation in B. rerio.

Melanin aggregation into the centre of each cell after contraction has been used to define cell position. The size of each melanin aggregate is proportional to the length of time that the cell spent as a visible cell at the site. The sketches were made from days 12 to 37 in somite #16. The numbered cells are representative examples of cells with different fates. Sketches from 12 to 21 days show both dorsal and lateral views while sketches from 23 to 37 days show only the lateral view.

Cell #1: a second wave melanophore which becomes incorporated into the juvenile stripe

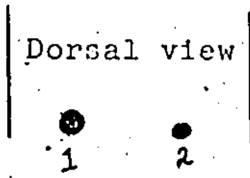
Cell #2: a second wave melanophore which remains in the horizontal skeletogenous septum

Cell #3: a later arrival (day 16) which unsuccessfully attempts reorientation and by day 25 is becoming hidden (star)

Cell #4: a later arrival (day 14) which does not successfully migrate dorsally, becomes fragmented and is cleared from the site

Cell #5: a late arrival (day 27) which appears in the area of the juvenile stripe and remains in the stripe.

Age in days



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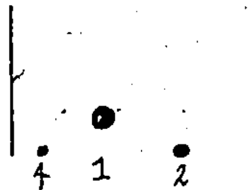

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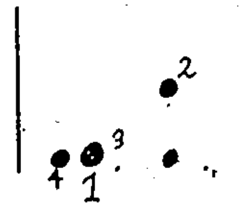

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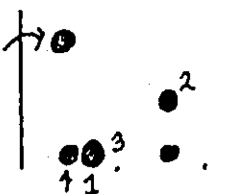

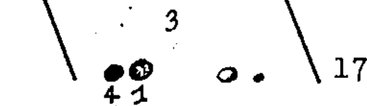

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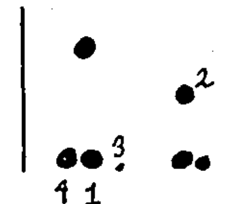

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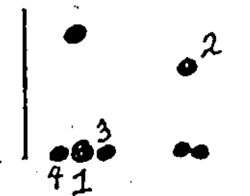

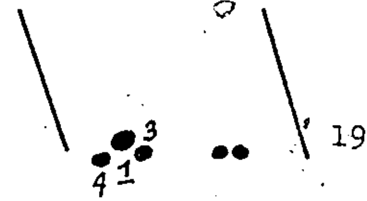

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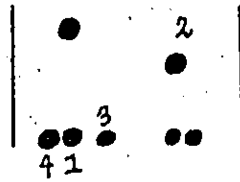
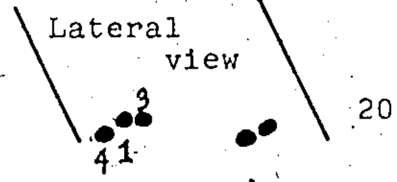
Age in days

Dorsal view

Lateral view





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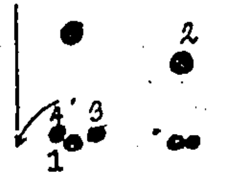

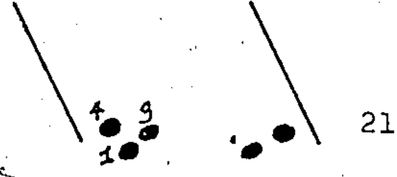

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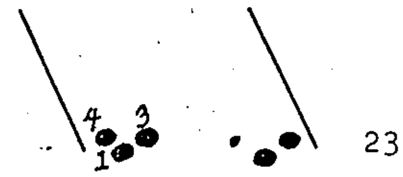

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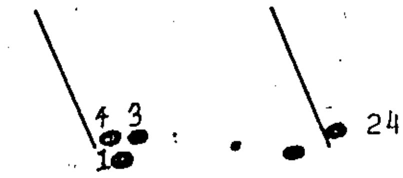

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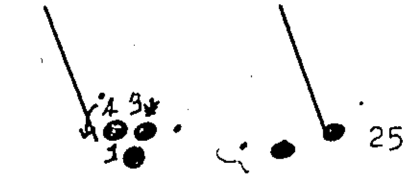

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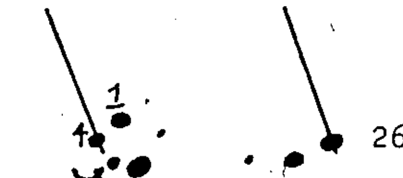

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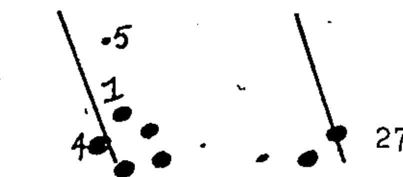

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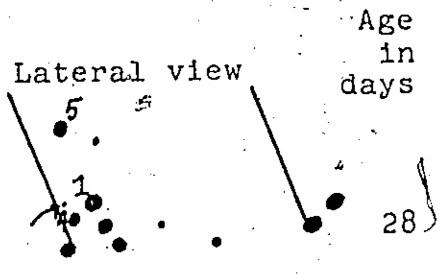
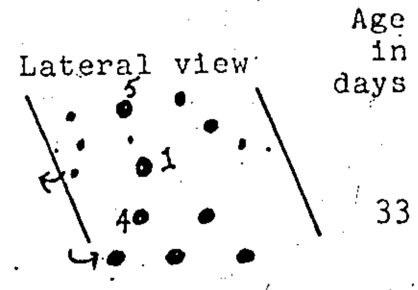
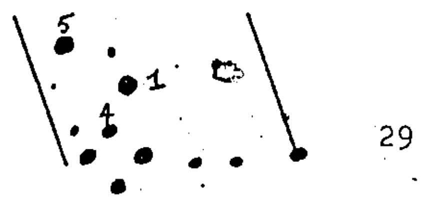
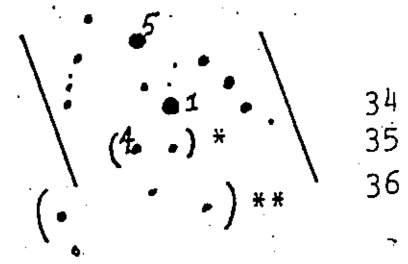
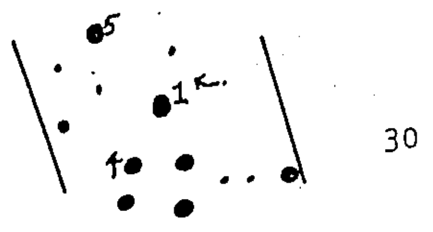
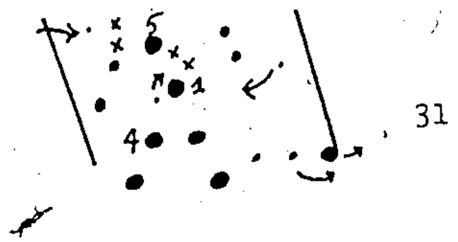


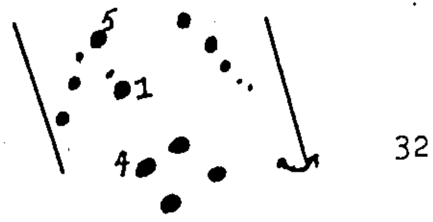












* clumps on day 35

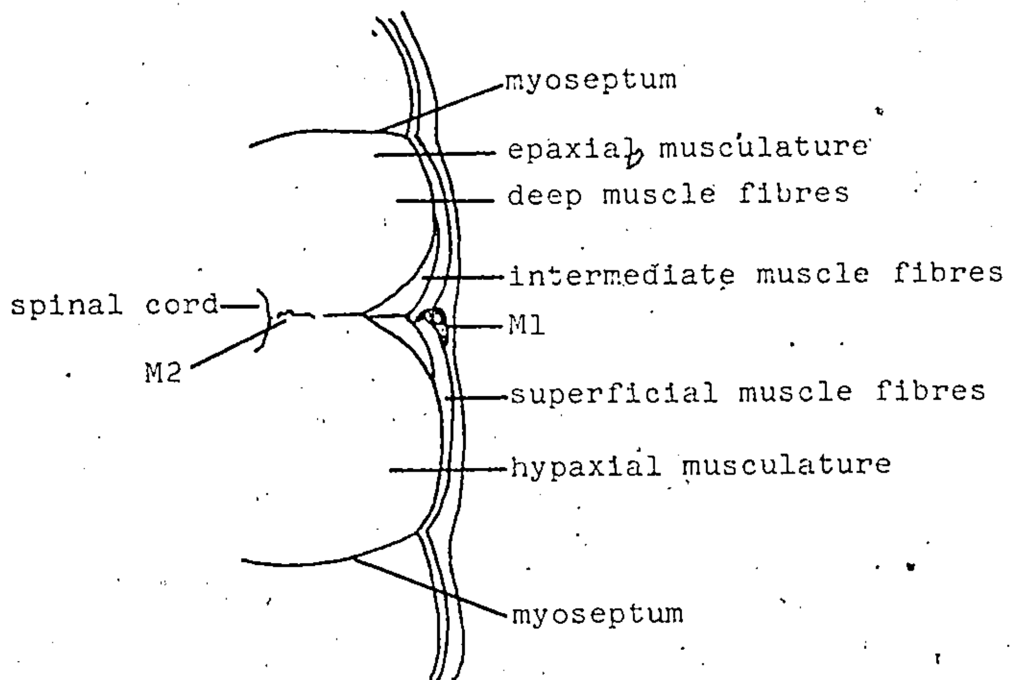
** clumps on day 36

FIGURE 26

The reorientation of lateral line band melanophores during juvenile stripe formation

A transverse 2 μm plastic section showing both a reorientating lateral line band melanophore (M1) and a second melanophore (M2) which remained stranded near the spinal cord. The clear area in M1 is the nucleus. The diagram accompanying the figure also shows the epaxial and hypaxial musculature, myosepta, and the deep, intermediate, and superficial muscle fibres (see Waterman, 1969).

Haematoxylin and eosin. Magnification 190 X


myoseptum
epaxial musculature
deep muscle fibres
intermediate muscle fibres
spinal cord
M1
M2
superficial muscle fibres
hypaxial musculature
myoseptum

sometimes taking up to two weeks for completion. There, is, however, a finite time in which it can occur because as the fish ages, the layers of tissue through which the outer cells migrate are thickening. Often some cells are seen which appear not to complete reorientation and remain stranded as deeper cells in the septum. The cells which do not move to the edge and begin reorientation are also gradually obliterated from view as the outer layers of the body thicken and become opaque. All cells take part in orderly physiological colour change, even those which are reorienting.

With time, many new cells appeared within the septum, at the edge of the septum, and as completely reoriented cells on the flank. Most early flank melanophores appeared near the septum, but later new melanophores were seen dorsal to this. The whole stripe gradually shifted dorsally and the second wave cell (Fig. 25, cell #1) seen at its ventral boundary was one which successfully reoriented and was joined there by others.

Discussion

There are many possible fates for a lateral line melanophore. The first is that it can remain in the septum and make no further contribution to the flank pattern (Fig. 25, cell #2).

The second is that it can not complete reorientation and remains stranded in the horizontal

skeletogenous septum (Fig. 25, cell #3). This implies that all cells near the flank receive the stimulus to begin reorientation. The third is that it can successfully migrate dorsally with the rest of the cells and thus make a contribution to the adult stripe (Fig. 25, cell #1). (What will be its further life history? Will it undergo mitosis or remain until its death without giving rise to any daughters?) The fourth, is possibly a programmed cell death. The cells in the area of these somites which reorient but do not migrate into the forming stripe, gradually undergo fragmentation and are cleared away. This melancphore destruction may be similar to that described by Goodrich and Nichols (1931) for stripe formation in the anal fin of B. rerio where the fin is initially evenly populated by melanophores which later are destroyed in the areas where the yellow stripes will form. This process in both cases could be a safeguard against pattern errors in adult fish. Melanophores that didn't migrate dorsally would remain in the silver stripe underneath the black one and cause a pattern aberration. It was perhaps fortuitous that the area studied was that where the stripe began to diverge dorsally. Lateral line melanophores and the melanophores accompanying them may have distinctly different fates elsewhere along the body.

Four new aggregates of melanin (Fig. 25; 31 days) which were considered tc be melanophores containing little

melanin appeared between days 30 and 31. However, on day 32 they were not observable. These 'melanophores' may have been only cellular fragments (melanophores which continued to make melanin even though they were being destroyed) or if they were whole cells, may have migrated out of the area or so close to other larger melanophores that they were indistinguishable from them. The fact that two initial cells located in the same somite often show adjoining punctate areas after contraction leads to this supposition and may mean that the cells are overlapping. The cellular relationships and positions of melanosomes after contraction must be studied in future work.

The process of juvenile stripe formation is very impressive. The sketches (Fig. 25) give a rough idea of the constant shifting of the cells within the boundaries of the forming stripe. Thus, in the three day stripe and the juvenile stripe cellular movements are not inhibited but the pathways along which the cells can move are changed. It appears that boundary states exist and changes in these boundaries determine where stripes will form.

Perhaps some lateral line melanophores reorient onto the flank because at that time new boundaries are forming. The wave of melanophore appearance on the flank would make it seem unneccesary for a few cells to reorient out from the septum, but the fact that this process occurs

points out how little is known about the factors that cause these stripes to form and the origins of the cells. If the external reorienting cells were removed before many new cells were seen, the deeper cells might then migrate out onto the flank. This is a real possibility, and if it occurred would point to a phenomenon operating at the level of the whole septum. The deep cells might not normally move to the edge of the septum simply because the melanophores already located there are blocking their outward migration.

In fact, the description of the appearance of new melanophores may mean that migration occurs from the horizontal skeletogenous septum out onto the flank, especially for the early cells which appear at the line already or partly reoriented. The first visible cells may be controlling dorsal migration of the cells which are added to the stripe. Alternatively, these new cells may be coming from dorsal areas of the fish and the first cells may be controlling ventral migration. The appropriate melanophore extirpations could perhaps test this and determine if the later waves of cells can form the stripe independently.

CHAPTER X

SUMMARY DISCUSSION

This experimental investigation is the first of its kind on pattern formation in teleosts. While others have studied the repair of fin patterns in B. *rerio* (Goodrich *et al.*, 1954) or mapped early melanophore pattern formation in other species (Orton, 1953), no studies have been reported in which a melanophore pattern consisting of only a few cells has been selectively destroyed to test the ability of the fish to recognize the defect. The zebra fish may be a good model system for pattern formation because so many of the steps in band genesis are observable and because some of the factors controlling band formation can be studied using the tool of melanophore extirpation.

A flow diagram (Fig. 27) has been constructed which shows the possible life history of a melanophore in B. *rerio*. The source of the initial cells is probably the neural crest but the source(s) of all later melanophores is not known at this time. A second wave lateral line melanophore seems to have a number of fates: 1) it can remain in the horizontal skeletogenous septum, 2) it can move to the edge of the septum and not complete reorientation, 3) it can successfully reorient onto the flank, but fail

FIGURE 27

Possible life history of a melanophore of *Brachydanio rerio*.

1. Migratory cells in inner and outer neural crest stream (other sources?)
   - ↓ incorp. into dorsal, ventral or yolk sac band
   - ↓ REORIENTATION I into the lateral line band
     - ↓ Initial lateral line cell

2. Cells from an unknown reservoir - second wave melanophores
   - ↓ REORIENTATION I (probably for early cells)
     - ↓ Second wave cell

Initial lateral line cell → Inner Cell; Initial lateral line cell → Outer cell

Second wave cell → Inner Cell; Second wave cell → Outer cell

Inner Cell ⟷ Outer cell (by defect formation?)

Inner Cell → Remains in the horizontal skeletogenous septum

Outer cell → REORIENTATION II → Juvenile stripe parallel to body wall

Juvenile stripe parallel to body wall → Incorp. into adult stripes → ?

Juvenile stripe parallel to body wall → Cell death

to move into the juvenile stripe and be destroyed or, 4) it can successfully migrate dorsally and perhaps make a major contribution to adult pattern formation. The further history of a lateral line melanophore which successfully contributes to the juvenile stripe must be investigated in future work.

What is seen in B. rerio is a continuum of events in which the initial population of cells, according to its distribution, has widespread effects on the positions and numbers of second wave melanophores, and with them contributes to juvenile stripe formation. Pattern formation is thus best understood as a whole pigment cell population phenomenon in which all cells can participate regardless of their 'age'.

From the questions posed and the experimental suggestions made in previous chapters of this thesis, it is obvious that this investigation forms only the basis for much more detailed work. Further studies of pattern formation in this fish should concentrate on the following questions:

What is the role of the visible cells in further pattern development and what are the controls on their migration? The visible cells may be the controlling

forces in pattern formation and might be able by themselves to form a rudimentary juvenile stripe (and perhaps an adult stripe). The appropriate extirpations could test this and could determine if later waves of cells are capable of forming stripes independently.

The initial (three day) lateral stripe may form because some relationship changes between the flank melanocytes and their environment, causing them to reorient into the septum. Superficial or deep melanocytes may reorient earlier or later under skin or somite transplants from 'older' or 'younger' fry. Alternatively, these cells may reorient because they do not make the appropriate connections with melanocytes in the dorsal or ventral bands. A study of melanocyte-melanocyte bonds and the possible effects of xanthophores must be made.

Where is the pigment cell reservoir(s) located? The progenitors may uniformly populate the body or they may exist in discrete groups. Analysis of many kinds of extirpation experiments (successively more ventral bands, combinations of bands, extirpations anteriorly and posteriorly, extirpations on chimaeric fish) might, if delays in repair are seen in certain cases enable the location(s) of the reservoir(s) to be pinpointed. Labelling experiments (H^3-DOPA) combined with electron microscopy at the stripe site might provide information about the

time of arrival of the second and later waves of cells. A study of pigment cell organelles should also be made. The organelles in melanophores which appear at different times could differ in different locations suggesting that pigment cell differentiation is labile and governed (at least in part) by the environment. Xanthophore pigmentation begins around 72 hours of development and iridophores first appear when the juvenile stripes are forming. Melanophores which appear close to xanthophores and iridophores could be intergrade chromatophores.

What is the mode of operation of the exclusion principle? Second wave cells may sample the lateral line stripe site and preferentially populate 'empty' somites. The exclusion principle may operate with 100% efficiency when initial cells are arranged in certain patterns, for instance twelve initial cells arranged as pairs are expected to be more effective at prohibiting second wave cell appearance than twelve initial cells arranged singly in somites. When many more whole eight day lateral line maps are analyzed it should be possible to predict when second wave cells will appear in somites that are already occupied on the basis of initial cell arrangements. This means that the three somite system of analysis must be extended to include all of the lateral line band. For each arrangement of initial cells the exclusion principle may operate 100% of the

time at a certain ratio of second wave cells to initial cells and at all values below this. The ability of an initial cell to prohibit second wave cell entry, melanogensis or differentiation, may also depend on a factor such as cell size. This means that the true limits and volume of melanophores as well as the source of the second wave cells in B. rerio must be defined.

What is the mechanism of melanophore destruction? The melanophore destruction observed during juvenile stripe formation is probably a safeguard to ensure that pattern aberrations do not occur. Why are only some cells destroyed? Does this destruction mechanism begin internally and are the products of destruction later phagocytized or does a wave of phagocytes pass down the flank to selectively remove those melanophores which would cause a pattern aberration? Similarly, how is fragment clearing accomplished when melanophores are extirpated? A careful histological and electron microscopical study of sections cut at different places in the body would provide a starting place to answer this question.

How is physiological colour change accomplished in B. rerio? Since migrating melanophores still participate in orderly physiological colour change, an investigation must be made of possible MSH and neural modes of control. If neural control exists are new

connections forming between the melanophore and the neuron or does the neuron 'grow' and innervate the same melanophore? Microtubules and microfilaments may be important in effecting colour change in B. rerio. Microtubules have been implicated in physiological colour change in other species of teleosts (Murphy and Tilney, 1974) but have not been studied in B. rerio. Both microfilaments and microtubules have been implicated in colour change in the frog (Malawista, 1971a & b). If either or both of these structures are important in the zebra fish are they present but not correctly arranged in the early melanocytes?

How does pattern formation occur when fish are raised in different environments? Different mechanisms of pattern formation and different patterns may form in response to different conditions of background and illumination (melanophore mitosis may occur). On black backgrounds under conditions of continual illumination would fish with no initial melanophores then fail to show any increase in cell number or would a new mechanism of eight day pattern formation be seen? Similarly, how would extirpations be repaired and how would the juvenile and adult stripes form? What behavioral effects would be seen?

How does whole body chromatophore pattern formation occur in B. rerio? In this thesis attention has been focussed solely on the melanophores. Xanthophores

and iridophores, however, contribute to the adult pattern. The investigation must therefore be extended to include the formation of the other chromatophore bands and to study these cells and their possible migrations. Does a functional chromatophore unit form in _B. rerio_?

The study of pattern formation in the zebra fish is just beginning. This thesis has provided a descriptive and experimental foundation for further analysis. Specifically, it has resulted in: (1) a detailed description of the establishment of a primary melanophore population and of the appearance of a second wave of melanophores, (2) evidence for the operation of interactions within the pigment cell population which lead to the integration of specific melanophores into the larval pattern, or their exclusion from the pattern, and (3) the identification of a series of questions and experimental approaches which will extend the analysis to the establishment of the adult pattern. In view of the great significance of pigmentation to the survival of animals, the study of pigment cell development is important. Moreover, such studies have widespread implications for many other developmental phenomena, including cell numerical regulation, tissue pattern formation, and cell - tissue interactions.

SUMMARY

1. Morphological observations of initial lateral line band formation in the zebra fish showed that the melanophores in the fist stripe did not migrate away from the stripe site during the period immediately before reorientation. These melanocytes reoriented into the horizontal skeletogenous septum and in some cases shuttled along the line before undergoing the first physiological contraction.

2. Fish differed in the numbers and arrangement of melanophores in the three day stripe while in the eight day fish, the stripes were more nearly equivalent.

3. The number of cells in the stripe increased in the period from three to eight days by the process of new melanophore differentiation (second wave melanogenesis) at the site. Early arrivals were often incompletely reoriented, suggesting that these cells, as well as the initial cells enter the site from elsewhere.

4. The number and positions of the melanophores in the eight day stripe are related to the numbers and arrangements of initial cells. As the number of initial cells increased, the number of second wave melanophores seen in the band at eight days decreased. The presence of an initial cell exerted an excluding effect on the appearance

of second wave melancphores and when the initial cell was removed experimentally, the defect was regulated 85% of the time. This experiment showed that the initial arrangement of melanophores controlled where the second wave of cells could appear, but did not give any information as to whether this repair process was true numerical regulation.

5. That true numerical regulation could occur was shown when whole band initial cell extirpations were done. Approximately 90% of the total number of eight day melanophores seen in control fish were present at eight days when the initial band had been extirpated at three days.

6. A scheme of pattern development involving two controls has been proposed for the early lateral line band of the zebra fish. The first is the excluding effect that the initial cells have on the appearance of second wave melanophores (position control), and the second is the control of cell number, or numerical regulation. These two processes work together to ensure that the visible melanophores will be evenly distributed along the line and present in the appropriate numbers to form a well-defined stripe by eight days.

7. The contributions of the eight day melanophores to juvenile pattern formation were also briefly investigated.

An eight day lateral line melanophore can: 1) reorient out onto the flank to help contribute to the forming juvenile stripe (and perhaps aid in stripe formation), 2) remain stranded in the horizontal skeletogenous septum either as a deep or as a more superficial cell in which reorientation is not complete, or 3) undergo what appears to be a programmed type of cell death if it does not successfully migrate into the juvenile stripe. This latter process would ensure that aberrations in patterns did not occur.

8. This study thus showed that in the development of the zebra fish pigment pattern, there is a delicate interplay between cell position and cell number in early band formation and that early lateral line melanophores may have important roles to play in further band genesis.

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