PIGMENT CELL RES 17: 326–336. 2004
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Review: Pigment Gene Focus

Genetics and Evolution of Pigment Patterns in Fish

ROBERT N. KELSH

Centre for Regenerative Medicine, Developmental Biology Programme, Department of Biology and Biochemistry, University of Bath, Bath, UK
**Address reprint requests to Dr Robert N. Kelsh, Centre for Regenerative Medicine, Developmental Biology Programme, Department of Biology and Biochemistry, University of Bath, Bath BA2 7AY, UK. E-mail: bssrnk@bath.ac.uk*

Received 20 April 2004; in final form 18 May 2004

Vertebrate pigment patterns are both beautiful and fascinating. In mammals and birds, pigment patterns are likely to reflect the spatial regulation of melanocyte physiology, via alteration of the colour-type of the melanin synthesized. In fish, however, pigment patterns predominantly result from positioning of differently coloured chromatophores. Theoretically, pigment cell patterning might result from long-range patterning mechanisms, from local environmental cues, or from interactions between neighbouring chromatophores. Recent studies in two fish genetic model systems have made progress in understanding pigment pattern formation. In embryos, the limited evidence to date implicates local cues and chromatophore interactions in pigment patterning. In adults, de novo generation of chromatophores and cell–cell interactions between chromatophore types play critical roles in generating striped patterns; orientation of the stripes may well depend upon environmental cues mediated by underlying tissues. Further genetic screens, coupled with the routine characterization of critical gene products, promises a quantitative understanding of how striped patterns are generated in the zebrafish system. Initial 'evo-devo' studies indicate how fish pigment patterns may evolve and will become more complete as the developmental genetics is integrated with theoretical modelling.

Key words: Zebrafish, Medaka, Genetics, Pigment pattern formation, Melanocyte, Xanthophore, Iridophore, Evolution

INTRODUCTION

The problem of pattern formation forms a key general theme within developmental biology: How are the correct cell-types arranged in the right relative positions to allow the organism to function? The most visual of these patterns are pigment patterns – the distribution of coloured pigments throughout the body and, especially, within the skin. Pigment pattern formation has long-fascinated biologists and has been a major area where theoreticians have also contributed to our thinking.

Pigment Cell Diversity, Origins and Migration

Mammals have only one pigment cell-type, the melanocyte (black, brown, red or yellow). In contrast, up to six different types of pigment cells have been described in fish (1, 2). Five cell-types, melanophores (black), xanthophores (yellow), erythrophores (red), iridophores (iridescent, blue, silver or gold) and leucophores (dull, whitish) are widespread; cyanophores (electric blue) are phylogenetically restricted in their distribution.[1]

Vertebrate pigment cells derive embryonically from the neural crest (3). In mammals, the neural crest origin of melanocytes was shown by Rawles (4). The origin of melanophores, xanthophores (and the related erythrophores) and iridophores from the neural crest has been shown in early extirpation and transplantation experiments by pioneers such as DuShane, although most work focused on amphibians (3, 5). In recent years, the neural crest origin of these three cell-types in zebrafish have all been definitively shown by iontophoretic labelling of single neural crest cells (6–9). The origin of the other types has not been studied,

Abbreviations – dpf, days postfertilization; EM, early metamorphic; hpf, hour postfertilization; LM, late metamorphic; Mc1r, Melanocortin receptor 1; MSH, melanin stimulating hormone

[1]Names of pigment cells, or chromatophores, in non-amniote vertebrates are usually given the suffix -phore, rather than -cyte. The distinction is often meant to refer to the ability of non-amniote vertebrate chromatophores to undergo rapid background adaptation, moving the constituent pigment organelles within the cell. That said, iridophores do not always show such behaviour, so the distinction is imperfect. We will use the standard terminology for fish chromatophores, if only because otherwise the term for leucophores becomes ambiguous. For further discussion, see (1).

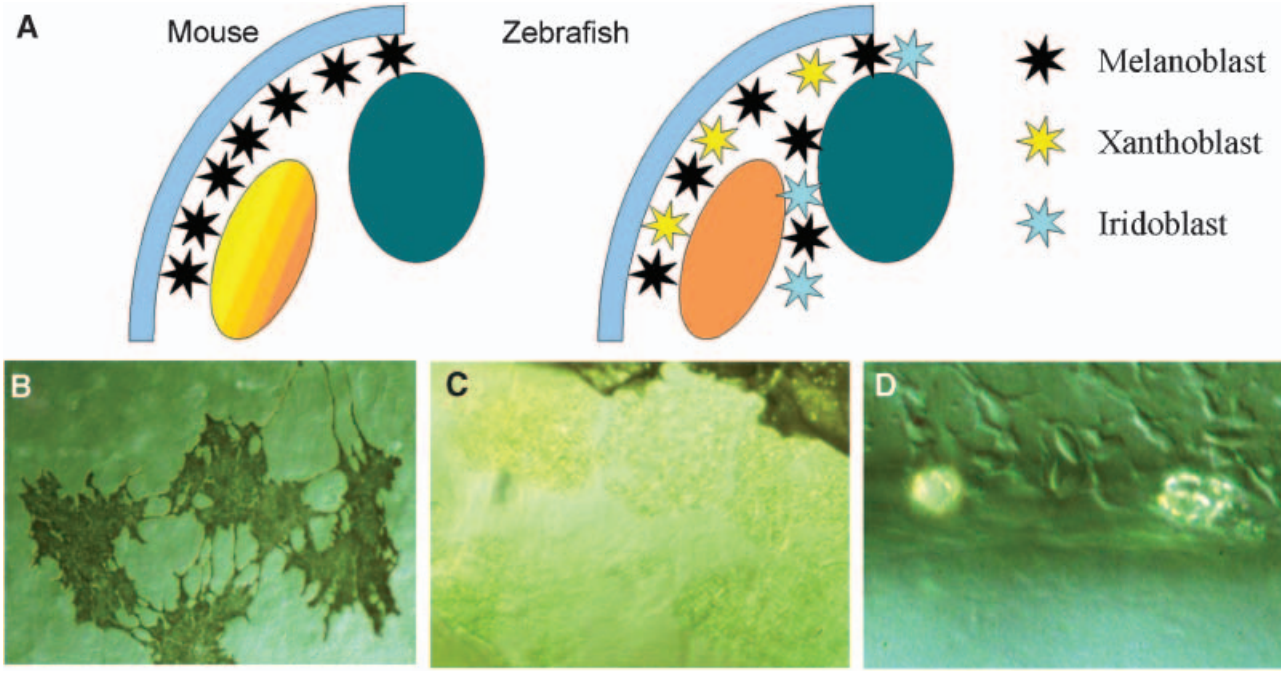

Fig. 1. Pigment cell migration routes. (**A**) Schematics of chromatoblast migration routes in trunk of mouse and zebrafish. Mouse embryos have only melanoblasts (black stars); these migrate exclusively on the dorsolateral pathway, between the epidermis (mid-blue) and the dermomyotome (yellow/salmon). Zebrafish embryos have both melanoblasts and xanthoblasts (yellow stars) on the equivalent lateral pathway. In addition, melanoblasts also migrate on the medial pathway between the neural tube (dark blue) and the myotome (salmon), where they are accompanied by iridoblasts (sky blue stars). (**B–D**) Typical zebrafish chromatophores in embryo, (**B**) melanophores on yolk sac, (**C**) xanthophores on dorsolateral head, (**D**) iridophores in dorsal tail.

although a neural crest origin seems most parsimonious; it would be of great interest to investigate whether leucophores and cyanophores are neural crest-derived.

Since pigment cells originate from the dorsal neural tube, they must reach their final locations by extensive migration. In mammals (and birds), migration of melanocyte precursors is normally restricted to a pathway under the developing epidermis (dorsolateral migration pathway), whereas cells using the ventral pathway, between neural tube and somites, are mostly neural in fate (Fig. 1). In zebrafish, the migration routes of specific chromatophores has been assessed. Unlike in mammals, melanophores migrate on both the lateral (under developing epidermis) and medial (between somites and neural tube) neural crest migration pathways (Fig. 1) (7). Xanthophores are restricted to the lateral pathway, and iridophores are restricted to the medial pathway (10–12; R.N. Kelsh, unpublished observations). In birds, migration on the dorsolateral pathway has been shown to depend upon specification to the melanocyte lineage (13); this remains to be tested for chromatophore migration in fish, but specification to pigment cell fates certainly begins very early, prior to migration (14). Note that in both mammals and fish, pigment cells migrate in an unpigmented state. These cells are, however, specified and are known as chromatoblasts (thus, melanoblast, xanthoblast, iridoblast). However, in fish, differentiation occurs relatively early, so that many migrating pigment precursors are partially pigmented.

Genetic studies of mammalian pigmentation have focused on two distinct mechanisms that can generate a pigment pattern. Firstly, mutants affecting 'white-spotting' genes (those involved in melanocyte specification, survival or migration) result in mutant animals in which patches of skin are devoid of melanocytes and the hair is thus white (15). These sites often lie in regions where melanoblast density is lower or in the more distal locations of the melanocyte migration pathways, e.g. ventral belly. In principle, pigment pattern evolution in mammals, many of which have a white ventrum, could involve the fixation of such white-spotting mutants. However, the pleiotrophic effects of these mutations in mouse and human (e.g. 16) make such a scenario intrinsically less likely.[2] An alternative mechanism focuses on patterned regulation of melanocyte physiology, in particular the Agouti signalling system (Fig. 2). Hairs can be cream/yellow or black/brown or alternately banded yellow and black (agouti). Their colour is dependent on the type of melanin that hair follicle melanocytes produce and this is regulated by Agouti protein produced locally in the hair follicle (17). Thus, melanin stimulating hormone (MSH) binding to Melanocortin receptor 1 (Mc1r) stimulates melanocytes to produce black or dark brown eumelanin, whereas Agouti-mediated inhibition of MSH/Mc1r signalling results in melanocytes producing pheomelanin, which has a characteristic yellow colour. Agouti signalling regulates the timing and distribution of pheomelanin production so that, for instance, alternating periods of active and inactive Agouti signalling in dorsal hair follicles results in the pheomelanin

[2]Interestingly, however, there are several examples of domestic breeds, e.g. of horse and dog, whose characteristic pigment pattern results from 'white-spotting' mutations.

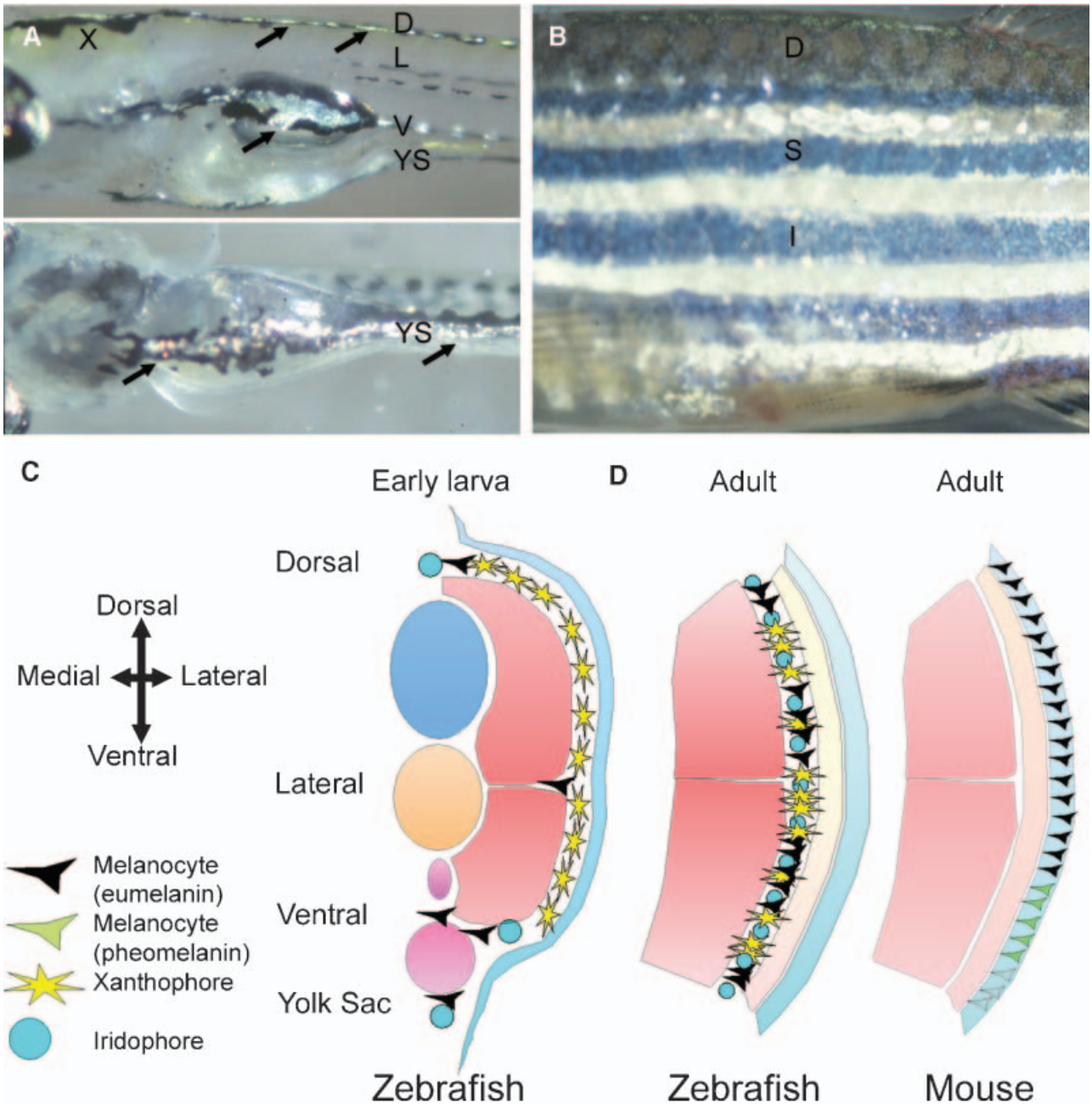

Fig. 2. Pigment pattern formation in fish and mammals depends on regulation of chromatophore distribution and physiology, respectively. (**A,B**) Zebrafish pigment pattern. (**A**) Lateral (upper panel) and ventral (lower) view of head and trunk of a 5 dpf larva to show early larval pattern. Melanophore stripes are indicated as follows: Dorsal (D), Lateral (L), Ventral (V) and Yolk Sac (YS) Stripes. Note iridophores (arrows) in Dorsal and Ventral Stripes form series of spots, whilst those in the Yolk Sac Stripe form a continuous sheet. Xanthophores (X) are visible as a yellow cast dorsally. (**B**) Lateral view of adult trunk zebrafish pigment pattern to show typical blue-black stripes (S) and silver interstripes (I); note the scale melanophores (**D**) in dorsum. (**C,D**) Schematic transverse sections showing chromatophore distribution in early larval zebrafish and adult zebrafish and mouse. (**C**) Early larval zebrafish pattern consists of differential distribution of three types of chromatophores. Melanophores (black, three-pointed stars) lie in four stripes, iridophores (sky blue circles) are arranged along three of the melanophores stripes, and xanthophores (yellow, seven-pointed stars) lie laterally between epidermis (blue) and myotome. Four melanophore stripes are: Dorsal, dorsal to dorsal myotome; Lateral, in horizontal myoseptum; Ventral, between dorsal aorta and gut (pink) and Yolk Sac, between gut and epidermis. (**D**) Adult pigment pattern in zebrafish and mouse. Shown are highly schematic impressions of part of flank to indicate distinctions in numbers of types of chromatophores and their location. Thus, zebrafish have three types of chromatophores arranged in the hypodermis. Iridophores are found throughout, melanophores aggregate in stripes, and xanthophores are found throughout but more concentrated in interstripes, giving yellow-silver interstripes and blue-black stripes. In mouse, melanocytes are distributed fairly evenly throughout the epidermis, but produce melanosomes containing eumelanin dorsally and pheomelanin ventrally (indicated by gradations in colour). Consequently, wild-type mice show a dark dorsum and pale ventrum, with a yellow flank band. Note that in many regions melanophores/-cytes are associated with skin structures (scales and hairs in zebrafish and mouse, respectively), although no attempt to portray this is made here. Likewise, no attempt is made here to portray the complex spatial arrangements of pigment cells within the mature hypodermis of zebrafish. Also note that in dorsal regions of mice, melanocytes briefly switch to pheomelanin production during a phase of the hair cycle to produce banded (agouti) hairs. Dermal melanocytes in adult mice are largely restricted to hairless skin (e.g. pinnae) (76).

and eumelanin banding of agouti hair. In ventral regions, Agouti is expressed constitutively, resulting in constant pheomelanin production by ventral melanocytes. Evidence to date suggests that the basic mechanism of mammalian (and avian) pigment patterning depends upon the Agouti-regulated physiological mechanism, although molecular confirmation of this is still necessary in most cases (18–23). Fish pigment patterns can be regulated by physiological mechanisms too. The intensity of red pigment in adult three-spined sticklebacks may be an example of this (24), as may the size, degree of melanosome dispersion or adundance of melanosomes that appears to underlie the spotted pattern of the catfish *Plecostomus* (25). However, in contrast to mammals, the characteristic patterns of both adult and larval[3] fish are primarily generated from the patterned distribution of chromatophores (Fig. 2).

Fish Diversity – A Plethora of Patterns

A casual glance at a coral reef, or even in a home aquarium, will make clear the astonishing array of pigment patterns and colours displayed by fish. Given that fish pigment patterns consist of different pigment cell-types distributed in an organized way, what might be the basis for these patterns?

Where are Fish Pigment Cells?

We will focus on the principal pattern elements of early larval and adult zebrafish, since most genetic studies to date utilize this model system (Fig. 2). The early larval pattern, complete by 5 days postfertilization (dpf), consists of four longitudinal stripes of melanophores, three of which are associated with iridophores in a characteristic pattern. Xanthophores, in contrast, are distributed rather homogeneously over the lateral face of the underlying myotome, in a slightly graded fashion (density higher dorsally). Formation of the larval pattern has been described (12, 26). Melanophore stripes, and (where appropriate) their accompanying iridophores, form in overlapping sequence essentially in order of their distance from the neural tube; thus, Dorsal, Ventral, Lateral and Yolk Sac. Melanophore accumulation to form the Dorsal Stripe is visible around 30 h postfertilization (hpf) and iridophores differentiate there from around 48 hpf. Xanthophores are first readily seen at 42 hpf in the trunk. Where molecular markers are used to examine chromatoblasts, they are seen to accumulate in position ahead of pigmented cells (10, 11, 27; S.S. Lopes, J. Mueller and R.N. Kelsh, unpublished observations). The embryonic pigment pattern in medaka is highly homologous to that of zebrafish and it is likely that many patterning mechanisms are shared (R.N. Kelsh, Y. Wakamatsu, K. Ozato, unpublished data).

The adult pattern is also primarily striped longitudinally, but these stripes are now distributed in the flanks. The blue-black stripes consist of melanophores and some associated iridophores and are separated by silver interstripes containing dense iridophores and prominent xanthophores. Full-grown adults characteristically have five body stripes in the trunk. The dorsalmost region of the body lacks stripe melanophores, but is invested with many melanophores associated with scales and giving a shaded dorsum. The opercula and ventral body and head have densely-distributed iridophores. The sequence of melanophore stripe formation has been carefully described by light microscopy (28–32). In an early phase (14–21 dpf), new (early metamorphic, EM) melanophores differentiate in the flanks of the fish, before aggregating immediately dorsal and ventral to the horizontal myoseptum to initiate the first two stripes. The first interstripe forms adjacent to the horizontal myoseptum during this phase. In a later phase (21–28 dpf), further (late metamorphic, LM) melanophore differentiation occurs in the stripes. Similarly, scale melanophores appear in the dorsum. Thus, the initial adult pattern of two melanophore stripes and a single interstripe is generated. The appearance of xanthophores during stripe generation has been rather harder to study, but they appear to differentiate first in the position of the first interstripe, adjacent to the horizontal myoseptum (33). Subsequent body stripes (and interstripes), ventral and dorsal to the original two, are added in postmetamorphic phases, as the body grows.

A recent ultrastructural study clarifies the cellular basis for the adult zebrafish pigment pattern and reveals a consistent radial layering of the pigment cells, which are positioned below the dermis and lateral to the body wall muscle, i.e. in the hypodermis (34). Thus, xanthophores overlie iridophores throughout the flank; their apparent prominence in interstripes may simply reflect the ease of seeing them with a bright rather than dark background. In the stripes, the iridophore layer is relatively thin and of lower density and lies over a layer of melanophores (backed by further iridophores). In interstripes, the iridophore layer is denser and thicker.[4] It is unclear when ubiquitous xanthophore distribution develops, nor when differences in iridophore distribution are established. It is likely that there are dramatic changes in the cellular arrangements within skin tissue during adult pigment pattern formation and thus similar ultrastructural studies during metamorphic stages will be vital to provide a context to the genetic studies described below; it is likely that such studies will help to explain the current inconsistencies between the ultrastructural studies of adult skin and the genetic studies of pigment pattern formation mechanisms, some of which are indicated below.

In addition to the body striping, the anal and tail fins show prominent alternating melanophore stripes and xanthophore interstripes, running parallel to the body stripes; iridophores are distributed independently along the fin rays. Stripe regeneration after damage has been demonstrated, most

[3]It should be noted that fish embryos are known after hatching as larvae. Thus, embryonic development results in a larval pigment pattern. This pattern is then stable for a period (around a week in zebrafish), before metamorphosing (from day 14) into the adult pattern (28).

[4]Iridophores are of two colour types, blue and silver, associated in wild-types with stripes and interstripes, respectively. Hirata and colleagues have proposed that the colour of iridophores may be dependent upon the presence or absence of underlying melanophores (34). However, blue and silver iridophores can be readily seen in both adult *albino* and *nacre* mutants, which lack melanin and melanophores, respectively. Furthermore, the patterned distribution of these cells is normal in *albino*, but abnormal in *nacre* mutants (R.N. Kelsh, unpublished observations). Thus, further study of the mechanism generating these colours is required.

extensively in the tail after resection (35–37). The source of the adult chromatophores formed during adult pattern formation and regeneration, presumably a widely-dispersed stem cell population, remains a subject of great interest. Melanocyte stem cells have recently been identified in mammals where they occupy a specific niche within the hair follicle (38). Identification of these cells and their niche in fish will be an exciting topic for future research.

Three Ways to Paint a Fish

Models of how pigment pattern formation might be regulated differ in terms of the degree of autonomy of the process to pigment cells and the distance over which organizing cues might act.

In one model, pigment cell distribution may be patterned by long-range signalling influences. One popular class of such models is based on reaction-diffusion mechanisms (39). Generation of diffusible activator and inhibitor molecules from a localized source may result in the formation of a striped or spotted pattern of activator concentration, with the precise pattern regulated by, for instance, size of the developmental field and diffusion coefficients of the activator and inhibitor molecules (40, 41). Pigment cells are proposed to respond to the local levels of activation, giving a visible readout of the activation pattern. Theoreticians have extensively explored these models and although no definitive examples have been demonstrated so far, some fish pigment patterning has been interpreted in the light of these models. Thus, a beautiful example of detailed correlations of stripe behaviour in a reaction-diffusion model with that of growing angelfish, *Pomacanthus sp*., is provided by Kondo and colleagues (42) and further improved by incorporating pigment cell movement parameters too (43, 44). Interestingly, the allelic series of pigment patterns seen in zebrafish *leopard* (see below) mutants has also been shown to correlate well with the predictions of a similar model (25). Changes in the field dimensions and in the diffusion parameters of the component molecules would then be the basis of both ontological and evolutionary changes in pigment pattern. One prediction of the mathematical models of pigment pattern formation is that changed growth parameters will result in changed patterns. A comparative study of growth patterns in *Danio spp*. does show such a correlation, with increased stripe number being correlated with size at the time of metamorphosis and with the presence of vertical elements to the pattern being correlated with a prolonged growth phase (45). The final test of these models will come from identification of the genes responsible for pigment pattern formation in a fish and examining whether the behaviour of their products corresponds to the components of a reaction-diffusion mechanism. With so few pigment patterning genes cloned to date, it is too early to begin to guess whether the correlations shown in fish, enticing as they are, have biological relevance.

Secondly, pigment cells might be patterned by interaction with local tissues, i.e. environmental cues. Clearly, melanoblast migration routes can be influenced by environmental cues, as is shown for example by the role of myotomal changes in permitting lateral pathway migration (46, 47). Mechanisms determining where migrating pigment cells stop have been little explored, yet must be fundamental, and may include interaction with specific organs in the periphery. For example, in mammals, melanocytes accumulate within the hair follicle, perhaps by a mechanism of trapping within the attractive adhesion conditions of the follicle environment (48, 49). Given the multiple chromatophore types of fish, it is important to consider the possibility of adhesive or attractive sites acting differentially for each cell-type. At its most extreme, the skin or underlying structures (or their extracellular matrix) might be prepatterned with sites adhesive or repulsive to individual pigment cell types, resulting in the patterned distribution of chromatophores. Evolution of pigment patterns would then result from the changed distribution of these patterning signals within the patterning tissue.

Thirdly, pigment cell patterning may depend predominantly on local interactions between different pigment cell-types. Differential cell affinity has been proposed as a general mechanism allowing cell sorting and has been shown experimentally (50, 51). Theoretical models of the mechano-chemical type incorporate parameters appropriate for this and the previous patterning mechanism (52), but have not yet been explored with respect to pigment pattern formation. Such mechanisms, acting between two or more distinct pigment cell-types, could generate striped or spotted patterns. For example, in larval salamanders an initially uniform distribution of melanophores is transformed into alternating vertical bars of melanophores and xanthophores. The mechanism proposed to explain this process is one of repulsive interactions (heterotypic) between the two pigment cell-types, coupled with prior aggregation of premigratory xanthophores via attractive homotypic interactions (53). Evolution of pigment pattern would result from changes in the adhesivities of interacting chromatophores. Clearly, such a mechanism is not readily available to the 'chromatophore-depleted' mammals. As we shall see, the experimental evidence in zebrafish has generally been interpreted in terms of these cell–cell interaction models.

The Genetics of Pigmentation in Fish

Fish-keepers around the world have identified and maintained strains of many species that show heritable pigmentation phenotypes. Particularly extensive collections of pigmentation mutants have been identified in two model fish. Thus, Tomita built up a collection of over 40 medaka *Oryzias latipes* pigmentation mutants from adults collected from wild populations (54, 55; see also http://biol1.bio.nagoya-u.ac.jp:8000/mutant.html). These mutants have recently been re-examined to investigate their effects on embryonic pigmentation (56). Likewise, more than 90 zebrafish pigmentation mutants were collected during mutagenesis screens in zebrafish *Danio rerio* (14, 30, 57–60). The current overview of the phenotypes in embryos and adults in the two species provides an interesting contrast. Although for both species the embryonic and adult phenotypes of most mutants are very similar, the effects are contrasting for mutants affecting chromatophore number (56). Thus, in medaka, mutants affecting melanophore

number clearly have related adult and embryonic phenotypes – both were scored as showing reduced melanophore numbers. In zebrafish, this is not always so clear, since mutants affecting embryonic melanophore numbers often have unexpected, dramatic effects on adult pigment pattern. It seems that, in zebrafish, mutations affecting pigment cell numbers disrupt their complex patterning mechanisms and thus have unexpected patterning defects. In contrast, medaka has a relatively simple, rather homogeneous distribution of adult chromatophores.

PATTERNING MECHANISMS IN EMBRYOS

Mechanisms generating the early larval pigment pattern of fish remain largely to be explored, although there are indications that both environmental cues and chromatophore interactions are important. Pigment cell distribution in wild-type and mutant larval zebrafish suggests that local tissues pattern chromatophores. Thus, in zebrafish, the association of melanophores in the Dorsal Stripe with the brain and with the dorsal myotomes is striking. Furthermore, the bilaterally paired arrangement of melanophores within the Dorsal Stripe in the trunk and tail correlates well with the underlying paired myotomes, and is evolutionarily conserved, being seen in medaka and even in amphibians (28, 53, 61; R.N. Kelsh, unpublished observations). In medaka, adult homozygous *Double anal fin* mutants have a mirror-symmetric dorsal duplication of ventral structures in the tail (54). In mutant embryos the dorsal pigment stripe is transformed into one with the morphology of a ventral stripe; furthermore, this correlates with an equivalent change in the myotomes (56, 61). Likewise, melanophores of the Lateral Stripe are associated with the horizontal myoseptum and, in mutants where this structure is absent, the Lateral Stripe never forms (58, 62). Finally, studies of a zebrafish pigment pattern mutant affecting the embryonic pattern, *choker* (58), suggest that myotomal cues prevent migration of melanophores away from the Dorsal and Ventral Stripes and thereby actively maintain embryonic melanophore stripes (V. Nikolic et al., unpublished data).

A role for cell–cell interactions has been deduced from observations of pigment pattern formation and mutant embryos. Early studies in zebrafish described melanophores populating the Lateral Stripe in two waves, first as pigmented melanophores, second as unpigmented melanoblasts (12, 63). Invasion of a specific myotome by a second wave melanophore was negatively influenced by the presence of a first wave melanophore in that position, suggesting cell–cell interactions influence melanoblast migration/differentiation (63). The tight association of melanophores and iridophores in zebrafish and melanophores and leucophores in medaka might suggest a role for cell–cell interactions between pigment cells (56, 58). However, iridophores and melanophores are respectively correctly patterned in mutants which entirely lack the melanophore and iridophore lineages, respectively (14, 58; S.S. Lopes and R.N. Kelsh, unpublished observations). Likewise, although leucophore numbers are reduced in three medaka mutants with reduced melanophore numbers, the locations of remaining chromatophores are not tightly correlated, again arguing against a strict requirement for one cell-type in patterning the other (56). A more convincing example of chromatophore interactions mediating their patterning comes from observations of xanthophore distribution in zebrafish mutants lacking melanophores. Wild-type zebrafish larvae show rather ubiquitous distribution of xanthophores over the outer face of the myotome, but exclusion from regions where melanophores are positioned; this is clearly shown in *albino* and other reduced melanin mutants (58). In contrast mutants lacking or with reduced melanophores (e.g. *sparse* mutants) show ectopic xanthophores in the positions of the Dorsal Stripe where melanophores are absent (58). Time-lapse studies of embryonic chromatophore patterning would allow direct testing of whether melanophore–xanthophore interactions regulate the latter's position.

PATTERNING MECHANISMS IN ADULTS

Several laboratories have investigated adult zebrafish pigment pattern formation using a set of pigment pattern mutants, the phenotypes of which are summarized in Table 1. These studies have used elegant combinations of genetics and cell transplantation to tease out cellular mechanisms of pigment pattern formation. They have highlighted the necessity of (a) de novo production of chromatophores, (b) chromatophore interactions, and (c) prepatterning cues

Table 1. Summary of pigmentation phenotypes of homozygous adult pigmentation mutants

Gene	Melanophore	Xanthophore	Iridophore	Cell-autonomy
sparse/kit	EM absent, LM normal; striped	Wild-type	Present	EM melanophore
rose/ednrb1	EM normal, LM absent; fewer stripes	Clustered, but poorly striped	Absent	–
puma	EM and LM absent (some embryonic melanophores persist abnormally)	Reduced	Present	Pigment cell lineages
nacre/mitfa	EM and LM absent	Clustered, but poorly striped	Present	Melanophore lineage
panther/fms	Reduced numbers, scattered distribution	Absent	Present	Xanthophore
obelix	Fewer, broader stripes	Similar to wild-type	Present	Melanophore
leopard	EM normal, LM absent; stripes replaced by spotted pattern	Concentrated in regions around melanophore spots	Present	Melanophore and xanthophore lineages

in generation of oriented, coordinated stripes and interstripes.

New Chromatophores are Required to Populate the Stripes

Mutations in two transmembrane receptor genes are required for production of normal numbers of melanophores in the adult stripes, but their mechanisms of action remain to be elucidated. Homozygous null *sparse*/*kit* mutants lose all melanophores prior to metamorphosis, yet still generate a normally striped pattern in the adults; however, scale melanophores are absent and melanophore number in the stripes is reduced (30, 64). Careful studies of the accumulation of melanophores suggest that *sparse*/*kit* mutants lack EM melanophores, but acquire LM melanophores normally (30). *Sparse*/*kit* function is required for melanophore lineage survival, suggesting that EM melanoblasts may be lost prior to differentiation in *sparse*/*kit* mutants (11, 37, 64). Conversely, LM melanophores are dependent on *rose*/*ednrb1* function, so that although *rose*/*ednrb1* mutants look normal during embryonic and early larval development, adults have only 50% of dermal melanophores and these are all of the EM population; scale melanophores are mostly present (10, 30). Iridophores are also essentially absent in adults. In the embryo, *rose*/*ednrb1* is initially expressed in all three pigment cell precursors, but later becomes restricted to the iridophore lineage. In metamorphosing fish *rose*/*ednrb1* expression in interstripes includes cells thought to be differentiating and differentiated iridophores; expression in melanophores is seen, but may only be transient (10). Thus, identification of the cell-type or types in which *rose*/*ednrb1* is required will be of great significance. Genetically, *sparse*/*kit*-dependent and *rose*/*ednrb1*-dependent melanophores are non-overlapping (37, 64). It will be of interest to establish whether the *kit*-dependent and *ednrb1*-dependent melanophore populations derive from different precursors.

In contrast to *sparse*/*kit* and *rose*/*ednrb1* mutants, *puma* mutants fail to develop both EM and LM melanophore populations and show reduced numbers of xanthophores (31, 32). Early markers for chromatophore lineages show that numbers of melanoblasts and, to a lesser extent, xanthoblasts are reduced in *puma* mutants, whilst examination of a glial marker, *sox10* (9, 65), shows that glial cell number is also reduced. Utilizing the temperature-sensitivity of the *puma* mutant allele, Parichy and colleagues demonstrate that *puma* functions throughout metamorphosis, but not in embryos, although its precise role remains to be characterized. The authors suggest that *puma* is critical for expansion of chromatophore (and glial lineages) in metamorphosis, perhaps via their recruitment from metamorphic stem cells or progenitors. Despite the extensive reduction of chromatophores, recovery of a semblance of the adult pattern occurs via late pattern regulation.

Local Cell–Cell Interactions Mediate Stripe Formation

The phenotypes of multiple mutants suggest that adult stripe formation is critically-dependent upon melanophore–xanthophore interactions. This is most clearly demonstrated by a beautiful series of studies of mutations in *panther*/*fms*, a receptor tyrosine kinase closely related to *sparse*/*kit*, which reveal a vital role for xanthophores in adult melanophore stripe formation and maintenance. In *panther*/*fms* (also previously known as *salz* and *pfeffer*; 33, 58, 59) mutants, few xanthophores form, but embryonic pigmentation is otherwise normal (10). Interestingly, however, in adults all xanthophore lineage cells are absent and hypodermal melanophores and iridophores are distributed in a very scattered fashion (scale melanophores are apparently undisturbed) (10, 33). In adults, consistent with the apparent restriction of *fms* expression to the xanthophore lineage, *fms* functions cell-autonomously for differentiation of xanthophore progenitors into xanthoblasts and survival of xanthophores (33, 66). Effects on melanophores are diverse, but importantly all are indirect; *fms* is required both for patterning of EM melanophores, which thus remain dispersed, as well as for survival of LM melanophores, which are mostly lost (10). Furthermore, loss of *fms* function at any metamorphic stage resulted in apoptotic loss of xanthophores and subsequent loss of melanophore stripe organization (i.e. melanophore dispersal) (66). Conversely, reinstatement of function at any stage up to and even during metamorphosis, resulted in the formation of xanthophores and the generation of melanophore stripes. Finally, cell-transplantation studies in *nacre*/*mitfa* mutants reveal that isolated patches of transplanted wild-type melanophores become organized into good stripes wherever they are in close proximity to xanthophores (33). Thus, *fms* function is required continuously for differentiation and survival of xanthophores from *fms*-independent progenitors and these xanthophores are themselves required for establishment and maintenance of the striped melanophore pattern.

Melanophores also appear to be important for xanthophore pattern, although difficulties of studying xanthophores mean that further clarification of this effect is necessary. Thus, *nacre*/*mitfa* encodes a transcription factor required for melanophore specification and differentiation (14, 67) and *nacre*/*mitfa* mutants entirely lack adult body melanophores, yet have many xanthophores and thus allow examination of the effect of lack of melanophores on xanthophore patterning (14, 33). In *nacre*/*mitfa* mutants, the first adult xanthophores form correctly in the vicinity of the horizontal myoseptum, but they are less abundant than in wild-types and fail to form a well-defined interstripe; other cells more ventrally form isolated clusters (33). This suggests that xanthophore clustering is independent of melanophores, but that correct *shaping* of the initial interstripes depends upon melanophore–xanthophore interactions. This clustered pattern of xanthophores appears to persist to maturity (33, 68), in contrast to the widespread distribution in wild-type adults (34), suggesting that xanthophore development remains affected throughout adult pigment patterning in *nacre*/*mitfa* mutants.

Melanophore aggregation may crucially influence the number of stripes. The *obelix* mutation affects stripe periodicity, with fewer, broader stripes, which are frequently interrupted, in heterozygotes, and only two broad stripes in homozygotes (33, 69). Larval pattern and initial stages of early adult pattern are normal in *obelix* mutants. However, unlike in wild-types, adult melanophores fail to migrate and to aggregate and so remain scattered. Transplantation

experiments arguing for a primary defect cell-autonomous to melanophores suggest a model in which reduced melanophore aggregation gives broader stripes. The authors argue that the aggregation defect is probably explained by loss of homotypic interactions between melanophores, rather than decreased melanophore motility.

Both homotypic and heterotypic chromatophore interactions may be defective in *leopard* mutants (originally known as the variant *frankei;* 70). Studies of weak and strong mutant alleles show that as *leopard* function is reduced, stripes become more irregular in form and increasingly disrupted, generating the spotted phenotype at its extreme (28–30, 33, 70). In all *leopard* mutants, larval and early adult patterning is normal, but LM melanophore formation fails. Also, similar to *obelix* mutants, EM melanophore aggregation fails. Curiously, *nacre; leopard* double mutants restore the widespread xanthophore pattern that is lost in *nacre* single mutants, arguing that the requirement for *nacre*/*mitfa* function to produce a normal number of xanthophores can be overcome by loss of *leopard* function (33). Extensive double mutant and cell-autonomy studies have been interpreted as suggesting that *leopard* function is required for homotypic interactions of both melanophores and xanthophores and for heterotypic interactions between these two cell-types (33).

In contrast to the necessity for xanthophores, melanophore stripe formation appears to be independent of iridophores. Thus, adults homozygous for presumed null *rose*/*ednrb1* alleles have reduced melanophores and almost lack iridophores, but remaining melanophores are organized into a largely complete dorsal stripe, and an incomplete ventral one, consisting of a row of spots (10, 30).

The genetics of fin striping are somewhat different from those of body striping, although it has been little studied (30). Work by Parichy and colleagues provide a dramatic indication that pigment cells can, at least under certain experimental conditions, self-organize into a striped pattern (66). Thus, as already noted, when *fms* activity is restored, even during late development, body stripes are rescued with normal orientation. However, in the fins, late restoration of *fms* activity rescues the formation of stripes of normal morphology, but with random orientation. Although an effect of *fms* on fin organization cannot currently be ruled out, it seems most likely that late recovery of xanthophores results in self-organization of melanophore stripes and xanthophore interstripes, independent of any spatial cues within the fin tissue itself.

Prepattern Cues Localize and Orient the Stripes

Although these studies have provided clear evidence for the primary role of melanophore–xanthophore interactions in generating and maintaining the adult body stripe and interstripes, the *nacre*/*mitfa* adult phenotype hints at an underlying prepatterning mechanism determining the position and orientation of the striped pattern (14, 33). Thus, despite the lack of melanophores, xanthophores are not randomly scattered throughout the flank of *nacre*/*mitfa* mutants, being instead concentrated in the region of the horizontal myoseptum in an approximation of the initial interstripe. Further, more ventral xanthophores are separated from this interstripe by a clear xanthophore-free zone approximately where the melanophore stripe would be. Even in *fms* mutants where melanophore distribution does approximate to random scattering in the flank, albeit with local clustering superimposed, nevertheless the melanophore clusters show a hint of a striped arrangement (10). Of course, this could reflect partial function of these loci, but melanophores and xanthophores are, respectively, absent from the mutant alleles described and, furthermore, the molecular lesions in each case give good grounds for assuming full loss of function (10, 14). Thus, investigation of the mechanisms allowing this residual patterning might reveal how the position and orientation of stripes is initiated. For the body stripes, the horizontal myoseptum, as well as the posterior lateral line nerve, are clearly candidates for environmental patterning cues (33). Unpublished data apparently consistent with this idea has been briefly described (33).

Data consistent with a separate mechanism being necessary for controlling the orientation of stripes, as opposed to stripe formation, comes from the abnormally oriented tail stripes generated by restoring *fms* activity late in development (66). Careful study of the ontogeny of fin stripes will help identify cues establishing the normal orientation. The tail fin stripes normally form an extension to the body stripes and thus may be oriented during normal development by the body stripe prepattern, as they do in regenerating fins (35–37). In contrast, in the anal fin stripes run parallel to those of the body and thus the anal fin may be an extension of the body field. Although recent studies have largely ignored the anal fin, it is interesting to note that pioneering studies of anal fin striping in zebrafish identified phases of widely-spread melanophores, subsequently refining into stripes as xanthophores differentiate (35, 71), indicating strong parallels between fin and body striping mechanisms.

A WORKING MODEL OF STRIPE FORMATION IN ZEBRAFISH

Studies of *nacre*/*mitfa*, *panther*/*fms*, *obelix* and *leopard* mutants have all usually been interpreted in terms of a cell–cell interaction model (10, 33, 66). Conclusions of these groups can be combined with work defining genetically-independent melanophore populations (30) to generate a largely coherent working model of how the initial interstripe and flanking stripes are established. Thus, *sparse*/*kit*-dependent EM melanophores first appear scattered throughout the flanks of the metamorphic larva. Xanthophores appear in a concentrated band associated with (and perhaps oriented by) the horizontal myoseptum and homotypic interactions between xanthophores, mediated in part by *leopard* function, maintain their clustering in the first interstripe. Concentration of melanophores into the first two stripes depends upon directed melanophore migration. This directed migration in turn requires the presence of xanthophores, perhaps because they provide a medium-range attractant to direct melanophore migration towards them. At the same time, homotypic interactions between melanophores, mediated directly or indirectly by *obelix* and *leopard* function, mediate the aggregation of these cells. Finally, *leopard*-dependent short-

range interactions between melanophores and xanthophores are such as to ensure separate melanophore and xanthophore stripes with straight borders, perhaps by minimizing the contact surface between melanophore and xanthophore aggregates. In a second (LM) phase, further melanophores differentiate within the stripe regions. These cells are dependent genetically upon *rose*/*ednrb1*, *panther*/*fms* and *leopard*, and fail to appear in null mutants of these loci.

At present, the proposed cellular mechanisms of establishment of the body stripes conflict somewhat with the pigment cell arrangements in the mature skin as described by ultrastructural studies. Further ultrastructural studies at early and intermediate stages of adult pigment pattern formation will be necessary to test whether in early stages melanophores and xanthophores are adjacent and thus are free to interact directly. Both establishment and maintenance of melanophore stripes are dependent upon formation and patterning of xanthophores, the survival of which is *fms* dependent throughout (66). However, it is likely that other aspects of the mechanisms of establishment and maintenance of stripes are different, since the hypodermal environment and arrangements of pigment cells must undergo significant changes during metamorphosis.

The major questions that remain around the model proposed concern (i) the molecular basis of *obelix* and *leopard* function; (ii) the precise effects on the LM melanophores of these mutations and the molecular basis for these effects; (iii) whether changes in chromatophore number or cell–cell interactions or both are primary, or just secondary, effects of *leopard*; (iv) identification of the molecular basis of homo- and heterotypic interactions mediating striping; and (v) the anatomical and molecular basis of the prepatterns controlling stripe orientation. As an alternative to this cell–cell interaction model of pattern formation, pigment patterns generated in *leopard* mutants show striking correlations with those predicted from a reaction-diffusion model (25). Thus, positional cloning and study of this gene offers a critical opportunity to test the predicted role of long-range signalling in zebrafish pattern formation. Genetic evidence argues for the origin of melanophores, iridophores and xanthophores in adults from a *kit* and *fms* independent progenitor population (30, 58, 64, 66, 69). Characterization of the nature of these cells, their number, location and niche requirements in the adult will be of major importance.

EVOLUTION OF PIGMENT PATTERN IN DANIOS

One goal of 'evo-devo' studies is the identification of genetic and cellular mechanisms responsible for the evolution of characteristics that vary between related species. Zebrafish are members of a genus containing both large and small species.[5] Larval pigment patterns in fish are often highly conserved; thus, five *Danio* sp. and mountain cloud minnow *Tanichthys albonubes* all show near identical larval patterns of four longitudinal melanophore stripes (29). Likewise in medaka homologous pattern elements are clearly recognizable (56; R.N. Kelsh, unpublished observations). In contrast, the diversity of *Danio* adult pigment patterns is testimony to the rapid evolution of pigment traits in fish and, excitingly, opens up the prospect of identification of the molecular basis for evolution of this trait. Patterns range from the even blue and silver striped pattern of *rerio*, through the broadly blue/narrowly silver striped pattern of *kerri*, to the mixed stripes and spots pattern of *nigrofasciatus* and *malabaricus*; in *albolineatus*, melanophore stripes and spots are suppressed and instead these cells are scattered at low density throughout the skin, whereas in *devario* silver patterning is restricted to reduced *vertical* barring (29, 72). The development and evolution of adult pigment patterns in *Danio* is discussed by McClure (29). In the context of the genetic analysis of zebrafish described above, it is notable that a common feature of adult pigment pattern formation in these varied *Danio spp*. is the organization of melanophore spots or stripes with respect to xanthophore clusters (29). Detailed analyses of pattern formation in these species will be necessary to test functionally which roles of specific chromatophores are evolutionarily conserved.

One elegant study has begun to identify the genetic basis for pigment pattern evolution in this genus. Thus, Parichy and colleagues initially compared the adult phenotypes of certain characterized zebrafish pigmentation mutants with the pigment patterns displayed in related *Danio* species (73). They then tested the idea that wild-type patterns in related species might have evolved by fixation of mutant variants of the patterning genes identified in zebrafish. Using interspecific matings the authors examined the phenotypes of surviving adult F1 hybrids between wild-type *rerio* and other related *Danio* sp. Hybrids showed a *rerio*-like striped pattern, suggesting dominance or semi-dominance of striping over other traits. They then used interspecific complementation tests to ask whether the changed pattern in various *Danio sp*. might result from mutations in the same gene or pathway. Now, since mutations in multiple genes can give the same pigment pattern defect it is not surprising that most tests were unsuccessful. However, one case provides a powerful example of how pigment patterns in adult *Danio* may have evolved. Thus, fms^{j4e1} *rerio* × *albolineatus* gave F1 hybrids with an *albolineatus*-like pattern, suggesting that a mutant variant of *fms* receptor tyrosine kinase may have become fixed in the *albolineatus* lineage. Testing of other *fms* mutant alleles did not give the same result, due perhaps to different genetic backgrounds or to allele-specific intergenic non-complementation, so further work is required to test the extent of involvement of *fms* in pigment pattern variation between these sister species. Nevertheless, this study indicates the potential of a genetic approach to pigment pattern evolution.

FUTURE PRIORITIES

To date, modelling studies have been most significant in showing that relatively minor changes of model parameters (in turn, reflecting physical parameters of the system, e.g. diffusion constants for signals) can generate an astonishing

[5]The small species were formerly classified as *Brachydanio*, but were reclassified on the basis of molecular phylogenies of rRNA sequences (74, 75).

array of different patterns, including the range of spotted and striped patterns seen so beautifully in fish. The mathematics of the reaction-diffusion and mechano-chemical models show an underlying similarity suggesting that the patterns they can generate are identical (52). Thus, in light of the superb correlations of in vivo pigment patterns with those generated by reaction-diffusion models (see above), critical testing of the role of cell interaction and long-range patterning mechanisms in pigment pattern formation is a priority. Significant progress in the study of adult pigment pattern formation has been made recently using genetic approaches, yet, surely, the majority of genes involved in fish pigment pattern formation remain to be discovered. Thus the most pressing need is the identification of pigment pattern genes through genetic screens and characterization of the cellular basis of their roles. At present, the molecular identities of components of fish pigment patterning systems remain unknown, but the near-complete genome projects for zebrafish and medaka are revolutionizing positional cloning projects. Likewise, interpretation of the genetic results is critically dependent upon accurate and complete knowledge of the precise spatial relationships between pigment cells throughout metamorphic stages. As the gene products of pigment pattern genes are identified, the next challenge will be to integrate the molecular genetics, anatomy and developmental biology into appropriate mathematical models, to test quantitatively the extent of our understanding. Parallel studies in other members of the genus will enable elucidation of the genetic basis for evolutionary changes. Although distant, the vision of a satisfying, quantitative model of adult *Danio* pigment pattern formation can just be discerned.

Acknowledgements – I wish to thank Prof. Ozato and Wakamatsu for hosting me during a sabbatical in Nagoya, when many of the thoughts presented here were crystallized. I thank D. Parichy for helpful discussions of adult pigment pattern formation. I thank J. Müller and D. Tosh for comments on a draft manuscript. The author is grateful for grants from the Wellcome Trust and Medical Research Council that have partially supported this work.

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