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Pigmentation development in hatchery-reared flatfishes

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Malpigmentation is common in hatchery-reared flatfishes, decreasing the market value of whole fish, and increasing the risk of predation for juveniles released to enhance wild stocks. Pigmentation development in flatfishes occurs in two phases. First, during embryonic and larval stages pigment cells differentiate on both sides of the body. Second, at metamorphosis larval melanophores disappear, and adult melanophores differentiate on the ocular but not on the blind side. Malpigmentation seems to result from disruptions of the second phase, and may take the form of albinism on the ocular side or darkening of the blind side. Both types of aberration may be related to aspects of the hatchery environment such as lighting, substratum, and diet. Larval nutrition appears to be a key factor and enrichment of larval diets with fatty acids and Vitamin A can greatly reduce malpigmentation rates; however, levels sufficient to prevent pigmentation defects frequently cause other abnormalities. Two developmental explanations for albinism have been proposed. The first is that differentiation of ocular-side skin follows the normal blind-side pathway and adult melanophores therefore fail to develop on the ocular side. The second hypothesis suggests that dietary deficiencies inhibit retinal development and the resulting visual defects lead to failure of a hormonal signal required for melanophore differentiation. These hypotheses may well be complementary; as yet neither has been thoroughly tested. Definitive tests will require a combination of manipulative techniques such as tissue transplantation and cell culture with nutritional, behavioural and hormonal assays. Such integrative studies will further the understanding both of normal pigmentation development and of the environmental factors that contribute to high rates of albinism in hatchery-reared flatfish.

Key words: flatfish; flounder; pigmentation; albinism; melanocytes; development.

INTRODUCTION

The depletion of flatfish fisheries as well as continuing strong consumer demand for high value flatfish have fostered aquaculture efforts world-wide. The success of aquaculture ventures requires the optimization of growth and the health of the fish at all life history stages (Waters, 1996). Substantial progress has been made in a number of areas, such as larval nutrition and the control of spawning (Dhert et al., 1994; Kanazawa, 1995; Rainuzzo et al., 1997; Sargent et al., 1997; Gara et al., 1998; Mangor et al., 1998; Masuda & Tsukamoto, 1998; Rønnestad et al., 1998a). Nevertheless, fundamental aspects of flatfish development, and developmental defects that commonly occur in hatcheries, are not well documented nor well understood.

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One of the commonest defects in hatchery-reared fish is malpigmentation, particularly albinism (hypomelanosis). Extreme albinism reduces the market value of hatchery-reared fish (Seikai, 1991; Seikai & Matsumoto, 1991; Naess et al., 1995). Moreover, albino juveniles lack cryptic colouration and are therefore easily seen by predators in natural environments. This contributes to poor survival rates when hatchery-raised fish are used to supplement wild stocks or enhance coastal fisheries (Seikai et al., 1987a; Seikai & Matsumoto, 1991; Howell, 1994; Furuta, 1998; Furuta et al., 1998). Two developmental explanations for malpigmentation have been proposed. Seikai and colleagues have suggested that failure of ocular-side pigmentation may be due to abnormalities in the histological environment of developing melanophores (Seikai, 1992; Kanazawa, 1993; Seikai & Matsumoto, 1994). Alternatively, Kanazawa (1993) has linked albinism to defects in the developing visual system. These hypotheses are not mutually exclusive: both may act in concert. To test the current hypotheses and further our understanding of the causes of malpigmentation requires both a thorough re-examination of existing aquaculture data, and new experiments incorporating developmental analyses (Estévez & Kanazawa, 1996; Venizelos & Benetti, 1999).

This review paper describes normal pigment development and the ways in which abnormal pigment development diverges from the normal pattern. A summary is presented of the major methods that have been used to reduce albinism in hatchery populations. Inferences are drawn regarding possible underlying developmental defects. Finally, key areas for future research, including ways to test existing hypotheses about the developmental causes of malpigmentation in flatfishes, are suggested.

NORMAL PIGMENTATION DEVELOPMENT

The design of rational and cost-effective solutions to the problem of defective pigmentation will require knowledge of how pigmentation develops normally, and at what point(s) in that process defects arise. Flatfish pigmentation development has been studied mainly in Japanese flounder (Paralichthys olivaceus Temminck and Schlegel) (Seikai, 1980; Seikai & Sinoda, 1981; Seikai et al., 1987a; Seikai & Matsumoto, 1991; Matsumoto & Seikai, 1992; Seikai, 1992; Seikai & Matsumoto, 1994; Suzuki, 1994). Although skin and pigment cell histology have been described in other flatfish species (Roberts et al., 1971; Burton, 1978, 1988; Burton & O’Driscoll, 1992; Gara et al., 1998), little is known of the underlying developmental processes.

PIGMENT CELL TYPES

All teleost skin pigment cells originate in the neural crest, a migratory population of ectodermal cells that forms alongside the neural keel during embryonic development (Hörstadius, 1950; Orton, 1953; Reedy et al., 1997). A subset of the neural crest forms chromatoblasts, which in turn differentiate into three types of pigment cells: melanophores, iridophores, and xanthophores. Melanophores synthesize tyrosine kinase, the enzyme responsible for melanin production and thus dark pigment. Iridophores appear white or silvery due to scattering of light by guanine inclusions, and xanthophores produce yellow to
orange pigments based on carotenoid compounds (Fujii, 1993). The adult pigment pattern is determined primarily by the number, size, and distribution of the different cell types.

The first skin pigment cells to develop in Japanese flounder are larval chromatoblasts. These stem cells initially give rise to larval melanophores, the primary type of pigment cell before metamorphosis, and to xanthophores and iridophores. Adult melanophores begin to differentiate from stem chromatoblasts during metamorphosis, while larval melanophores are still present. The two types of melanophores are readily distinguishable by size: dispersed larval melanophores are 70–100 μm in diameter, while adult melanophores are much smaller (20–50 μm) (Seikai et al., 1987a). Larval pigment cells occur symmetrically on both sides of the body, while adult melanophores develop predominantly on the ocular side.

Less is known about the development of iridophores and xanthophores that are of secondary importance in the overall pigmentation pattern. Both initially differentiate during larval stages in Japanese flounder and are retained through metamorphosis, although their distribution may shift. As in the case of melanophores, there may be distinct larval types of iridophores and xanthophores (T. Seikai, pers. comm.). During metamorphosis in Japanese flounder, iridophores occur in clusters along the fin folds on the ocular side (Seikai et al., 1987a) and are scattered on the blind side (Matsumoto & Seikai, 1992). Xanthophores in this species are densely distributed on the ocular side and absent on the blind side after metamorphosis (Matsumoto & Seikai, 1992). In plaice (Pleuronectes platessa L.), iridophores are the most prominent type of pigment cell on both sides of the fish after metamorphosis (Roberts et al., 1971).

DEVELOPMENTAL TIMING

Flatfish pigmentation patterns can change relatively rapidly in response to their environment, and also more slowly during the course of their ontogeny. The ability of flatfishes to adjust their upper-side colouration to match the substratum is well known (Burton & O’Driscoll, 1992; Ramachandran et al., 1996; Iwata & Kikuchi, 1998; Healey, 1999). This capacity is based on neurotransmitter-mediated changes in the melanophores. These cytological adjustments, which affect the concentration of pigment granules in different areas of the skin (Baker, 1991; Fujii, 1993; Vokey & Burton, 1998), are dynamic and reversible. In contrast, ontogenetic changes in flatfish pigmentation are based primarily on the differentiation or disappearance of chromatoblasts and are generally not reversible (though exceptions have been reported: Benetti, 1997; Denson & Smith, 1997).

The adult pigmentation pattern develops in two phases which may be regulated at least in part by an intrinsic clock (Matsumoto & Seikai, 1992). The first phase occurs during the embryonic stages, when prospective chromatoblasts migrate from the dorsal neural crest down both sides of the body to generate a predominantly symmetrical pattern. Some of these chromatoblasts differentiate into larval pigment cells during embryonic and larval stages, while others remain as undifferentiated stem cells. Although flatfish pigmentation is commonly bilateral before hatching (Norman, 1934), in some species asymmetries in
pre-hatch stages anticipate post-metamorphic changes (Norman, 1934; Burton, 1988).

The second phase of pigmentation development occurs during metamorphosis, when changes in many structures and tissues (eyes, skull, lateral line, skin, etc.) accompany a 90° rotation of the body and the asymmetric adult colouration becomes evident. On the ocular side of Japanese flounder, metamorphosis is marked by changes in skin histology, enzymatic activity, and pigment cell distribution (Seikai et al., 1987b). Adult melanophores first appear in clusters around larval pigment cells and are later seen in other areas, eventually becoming densely distributed over the ocular side (Seikai et al., 1987b). The development of this pattern may result either from the migration of differentiated cells (Seikai et al., 1987a) or from the differentiation of precursors already in place (T. Seikai, pers. comm.). Both mechanisms are used during establishment of the lateral line melanophore stripe in zebrafish (Danio rerio) (Milos & Dingle, 1978). At the end of metamorphosis larval melanophores disappear entirely and the final pattern is formed exclusively by differentiated adult pigment cells.

On the blind side of the fish, metamorphosis has opposite effects. In Japanese flounder there is no increase in tyrosine kinase activity on the blind side (Seikai et al., 1987b), and disappearing larval melanophores are not replaced by newly-differentiating adult pigment cells. Most stem cells on the blind side appear to undergo cytolysis (Seikai et al., 1987b; Seikai, 1992; Seikai & Matsumoto, 1994) while there is no direct evidence for apoptosis. The remainder of the stem cells become iridophores, and occupy the body regions populated by melanophores on the ocular side. Cycloid rather than ctenoid scales develop on the blind side of many species (Norman, 1934), and there may be more intraepithelial glands than on the ocular side (Andrew & Hickman, 1974).

There is some variation among flatfishes in the overall pattern in which adult pigmentation is established. In diamond turbot (Hypopsetta guttulata Girard) melanocytes first appear on the dorsal and dorsolateral surfaces of the body and tail (Orton, 1953). In Japanese flounder adult pigment cells appear first on the trunk, next along the dorsal fin fold, and finally over the rest of the body (Seikai et al., 1987b). Greenland halibut (Reinhardtius hippoglossoides Walbaum) are darkly pigmented on both sides as adults, but pass through a temporary stage during which blind-side pigmentation is lost. It is later re-established by the differentiation on the blind side of melanophores with a unique morphology (Norman, 1934; Burton, 1988).

**ABNORMAL PIGMENTATION DEVELOPMENT**

**DEFINITION AND CLASSIFICATION**
Malpigmented fish are characterized by either a deficiency of pigment cells on portions of the ocular side (albinism, pseudoalbinism, or hypomelanism), or excess pigmentation on the blind side (staining, spotting, or ambicolouration).

Patterns of pigment reduction in albinic fish are highly variable, but some generalizations are possible. The areas most likely to retain pigment on the ocular side of partially albinic Japanese flounder are from the tip of the snout to the margin of the eyes, and along the trunk (Seikai et al., 1987b). Similar patterns occur in Atlantic halibut (Hippoglossus hippoglossus L.; Gara et al.,
The opposite of albinism is ambi-colouration, the presence of dark pigmentation on the normally light-coloured underside of the fish. Norman (1934) divided ambi-colouration of flatfishes into three categories: staining, in which irregular patches of pigment develop gradually on the blind side; spotting, or presence of discrete dark areas; and true ambi-colouration, in which the blind side pigmentation mimics the normal pattern on the ocular side. True ambi-colouration and spotting appear at the end of metamorphosis. In contrast, staining develops after prolonged rearing of juveniles in tanks that lack sandy substrata (Stickney & White, 1975; Iwata & Kikuchi, 1998), and can also be induced by light (Seikai, 1991). As with albinism, spatial patterns of excess pigmentation may be highly variable (von Ubisch, 1951; Seikai, 1992).

This variability complicates attempts to establish classification schemes for pigmentation abnormalities. Norman (1934) developed a series of categories which, though not detailed, remain useful for describing overall types of malpigmentation in many flatfish species. Another approach is to measure the pigment index (PI, percentage of body area pigmented). This method is advantageous in studies that require a single quantitative measure of pigmentation success or melanophore expansion (e.g. Green & Baker, 1989), particularly if large numbers of individuals must be scored. Moreover, pigment indices may be less subjective than attempts to sort highly variable patterns into discrete categories. The drawback is that numerical PI measures make no distinction between types of pigmentation, such as staining and spotting, that are very likely distinct biological phenomena with different mechanisms and causes.

A recent study of malpigmentation in plaice (Dickey-Collas, 1993) introduced a PI measure calculated by subtracting percent pigment coverage on the blind side from that on the ocular side. This method has the advantage of including pigmentation on both sides of the fish in a single numerical measurement. However, it assigns the same score to highly divergent patterns: for example, a fish with no pigment on either side and one that is completely melanic on both sides would both have a PI of zero. Such an approach can confound the interpretation of results: in the nutritional study reported, one experimental treatment was either the most or the least effective at reducing malpigmentation, depending on whether results were measured by pigment index or by scoring per cent normal pigmentation on the ocular side alone (Dickey-Collas, 1993).

Classifications based on spatial patterns of pigment distribution incorporate much more biological information than do single measures of PI, although at the cost of greater complexity in scoring and analysis. Most such systems are ultimately derived from Seikai and Sinoda’s classification of eight major types of malpigmentation observed in hatchery populations of Japanese flounder (Seikai & Sinoda, 1981). Even with eight categories these authors found it necessary to list four combined patterns in their table of occurrence frequencies, as well as a small percentage of ‘others’. Denson & Smith (1997) used a simplified version of Seikai and Sinoda’s classification, with six categories instead of eight, in a study of southern flounder. Gara et al. (1998), working with Atlantic halibut,
reduced the scheme to five categories and then scored each side of the fish separately, assigning each individual a two-digit code (ocular: blind). This approach echoes the detailed but unwieldy system developed by von Ubisch for plaice, in which not only ocular and blind sides, but also four separate regions of each, were scored separately (von Ubisch, 1951).

Our ongoing attempts to establish a classification of pigmentation defects in summer flounder have reinforced the conclusion that it is difficult to sort malpigmented fish into a small number of discrete types. Any scheme simple enough to be useful is unlikely to reflect the reality of extensive variation. This dilemma is a practical inconvenience, but can offer a few biological insights. First, there is a great deal of developmental ‘noise’ in the system, though we do not yet know enough about pigmentation development to be able to interpret the patterns and extent of variation in terms of the generating mechanisms. Second, certain patterns (e.g. head pigmented differently than the rest of the body) are common in multiple species. Third, it is unwise to base a classification scheme on observations of a single batch of fish, as batches may differ significantly even when raised under apparently identical conditions. Finally, it is crucial to test a proposed classification, to assess how consistently the criteria can be applied.

OCCURRENCE AND SIGNIFICANCE IN THE WILD

Albinism is extremely rare in wild flounder populations (Norman, 1934; Benetti, 1997; Venizeles & Benetti, 1999), though isolated cases of wild-caught adults that showed partial or complete absence of ocular-side pigmentation have been reported from a range of species (Norman, 1934; von Ubisch, 1951; Eisler, 1963; Gartner, 1986; Hafsteinsson, 1994; Astarloa, 1995). One could argue that since adult flounders often bury in the substratum, ocular-side albinism might not matter in the wild. However, hatchery-reared Japanese flounder released into coastal waters spend more time swimming above the substratum than do wild juveniles (Furuta, 1998; Furuta et al., 1998; Masuda & Tsukamoto, 1998; Tsukamoto et al., 1997). Mortality due to this behaviour may be increased if the fish are albino, and therefore more visible to predators. Hatchery-reared juvenile flatfishes experience extremely high predation when released into the wild (Seikai et al., 1987a; Dickey-Collas, 1993; Furuta, 1998; Furuta et al., 1998; Masuda & Tsukamoto, 1998). Reduction of the rate of albinism among fish intended for stock enhancement programmes could help mitigate excessive losses to visual predators (Tominaga & Watanabe, 1998).

Excess blind-side pigmentation is probably of little or no adaptive significance in natural environments, and it has occasionally been observed in wild-caught flounder (Gartner, 1986; Astarloa, 1995) and halibut (Burton, 1988). However, blind-side pigmentation is uncommon in wild fish, in contrast to hatchery-reared populations in which up to 95% may have black areas on the underside (Tominaga & Watanabe, 1998).

PREVALENCE IN HATCHERIES

Although rare in the wild, albinism is commonplace in hatchery populations reared on particular diets. In early studies, 97–100% of Japanese flounder larvae fed Brazilian Artemia before metamorphosis developed as albinos, while fewer than 2% of larvae fed wild zooplankton were malpigmented (Seikai, 1985). High
rates of albinism associated with specific larval diets have been reported in Japanese flounder (Seikai et al., 1987c; Kanazawa, 1993), plaice (Dickey-Collas, 1993), turbot (Dhert et al., 1994) and Atlantic halibut (Gara et al., 1998). Since the 1970s, extensive research and careful control of culture conditions, particularly diet, have significantly decreased the rate of albinism in hatchery populations.

Excessive pigmentation on the blind side after metamorphosis has not been studied as thoroughly as albinism, but occurs frequently in hatchery-reared fish (Tominaga & Watanabe, 1998) and may be exacerbated by treatments designed to reduce the incidence of albinism (G. Nardi, pers. comm.). Hypermelanism of the blind side is so prevalent in Japanese hatcheries that it has been used as a marker in studies of dispersal and recapture rates of released Japanese flounder (Tominaga & Watanabe, 1998).

TIMING AND HISTOLOGY

Ocular-side abnormalities begin to appear around 3 weeks post-hatch (at lengths of 8–13 mm) in Japanese flounder (Seikai et al., 1987a). Albinic individuals can be positively identified by 40–50 days post-hatch in Japanese flounder (Seikai et al., 1987a; Kanazawa, 1993) and summer flounder (pers. obs.), though differences in developmental rates among flatfish species preclude broad generalizations about when the definitive ocular-side pigment pattern is established (Norman, 1934; Chambers & Leggett, 1987; Kvenseth et al., 1996).

Malpigmentation on the blind side, consisting of excess melanophores, can be detected in some flatfishes shortly after metamorphosis. However, pigmentation on this side is generally both slower to develop and more labile than that on the ocular side. Several authors reported development of blind-side pigmentation in wild-caught embryos and larvae of summer flounder and plaice reared in laboratory tanks (Houde, 1971; Stickney & White, 1975), and Seikai (1991) found that staining could be induced by fluorescent light exposure in juvenile Japanese flounder. Blind-side pigmentation developed gradually in Atlantic halibut juveniles held in smooth-bottomed tanks (Ottesen & Strand, 1996). Pigmentation of the blind side develops over the course of months in summer flounder (Stickney & White, 1975; G. Nardi, pers. comm.), and is partially reversible if juveniles are provided with a substratum in which they can bury themselves (Stickney & White, 1975).

Two studies have reported late development of normal pigmentation in albinic fish. Denson & Smith (1997) noted that partially albinic southern flounder raised in low light gained pigmentation after light levels were increased for 1 week after metamorphosis. This result suggests that partial albinos retained the ability to repair deficient pigmentation. One possibility is that the preliminary distribution of melanophores occurred normally, but their final differentiation required the stimulation of increased light levels. Benetti (1997) observed pigmentation recovery in two groups of speckled flounder (Paralichthys woolmani Jordan & Williams): wild-caught juveniles that had lost pigmentation after capture, and partially albinic hatchery-reared larvae that gained pigmentation after metamorphosis. The latter observation is difficult to interpret in the absence of detailed descriptions of rearing conditions.
Detailed histological studies of malpigmented skin in three species of flatfishes generally report comparable results. In Japanese flounder the ocular-side skin of albino fish closely resembles that normally seen on the blind side (Seikai et al., 1987b; Seikai, 1992; Seikai & Matsumoto, 1994). In albino plaice the ocular-side skin resembles blind-side skin in having few melanophores but a normal complement of xanthophores and iridophores (Roberts et al., 1971). The opposite pattern, typical ocular-side histology and pigmentation appearing on the blind side, occurs in amibicoloured winter flounder (Pseudopleuronectes americanus Walbaum; Burton, 1988). Diaz de Astarloa’s examination of a single ambicoloured specimen of Xystreuris rasile Jordan (Astarloa, 1995) also supports Norman’s original description of true ambicolouration as the presence of ocular-side patterns on the blind side (Norman, 1934). Suzuki, however, has argued that abnormally pigmented areas on the blind side of hatchery-reared Japanese flounder may follow a unique developmental pathway (Suzuki, 1994).

Ocular-side albino patches resemble normal blind-side skin in Japanese flounder and plaice (Roberts et al., 1971; Seikai et al., 1987b; Seikai, 1992; Seikai & Matsumoto, 1994), and blind-side dark patches are similar to normal ocular-side skin in winter flounder and Xystreuris rasile (Burton, 1988; Astarloa, 1995). Together, these observations suggest that patchy pigmentation defects may stem from patterning signals upstream of the mechanisms directly responsible for skin and chromatoblast differentiation.

POSSIBLE CAUSES AND REMEDIES FOR ABNORMAL PIGMENTATION DEVELOPMENT

REARING ENVIRONMENT

The contrast between the rarity of albinism in the wild and extremely high rates under commercial hatchery conditions indicates that some aspect of the hatchery environment contributes to abnormal pigment development. One interpretation of this disparity would be that albinism is common in newly-metamorphosed fish in the wild, but that albino individuals are rapidly removed from the population by predation. However, over time such strong selection against albino phenotypes would tend to drive any genetic basis for the defects to low frequencies. Studies of the genetic background of broodstock point to primarily nutritional, rather than intrinsic or genetic, causes for malpigmentation (Seikai & Matsumoto, 1994), and the choice and supplementation of larval diets can drastically reduce the incidence of albinism in hatcheries. Finally, studies in Japanese flounder (Seikai et al., 1987a) and in Atlantic halibut (Næs et al., 1995; Næs & Lie, 1998) have identified a specific premetamorphic stage as a critical period for dietary supplementation to reduce the later incidence of albinism. Together, these observations strongly implicate the hatchery environment as a direct or an indirect cause of malpigmentation.

Larval stocking density, water temperature and flow rate, the choice of substratum in rearing tanks, light intensity and direction, and UV irradiation have all been investigated as factors possibly related to the occurrence of albinism in hatchery production (Shelbourne, 1974; Stickney & White, 1975; Dhert et al., 1994; Seikai & Matsumoto, 1994; Ottesen & Strand, 1996; Denson & Smith, 1997; Iwata & Kikuchi, 1998; Venizelos & Benetti, 1999).
THE INFLUENCE OF LARVAL NUTRITION

The composition of the larval rearing diet is a critical factor in flatfish pigmentation development (Dhert et al., 1994; Kanazawa, 1995; Rainuzzo et al., 1997; Sargent et al., 1997; Mangor et al., 1998; Rønnestad et al., 1998a). Most dietary research has focused on two classes of nutrients: Vitamin A and its precursors (Miki et al., 1990; Kanazawa, 1993; Dedi et al., 1995; Estévez & Kanazawa, 1995; Takeuchi et al., 1995, 1998; Dedi et al., 1997; Rønnestad et al., 1998a,b), and fatty acids, particularly docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (Izquierdo et al., 1992; Watanabe, 1993; Dhert et al., 1994; Rainuzzo et al., 1994, 1997; Estévez & Kanazawa, 1995; Sargent et al., 1997).

Vitamin A is critical for a whole range of functions in larval fish, including pigmentation, stress tolerance, and formation of the retinal opsin needed for larvae to begin visual feeding (Dhert et al., 1994; Rønnestad et al., 1998a). In the wild, flatfish larvae acquire the biochemical precursors for Vitamin A in the form of carotenoid compounds in their zooplankton prey (Naess et al., 1995; Sargent et al., 1997; McEvoy et al., 1998).

Fatty acids also play many roles in embryos and larvae, and are particularly important for nervous system development (Izquierdo et al., 1992; Devresse et al., 1994; Dhert et al., 1994; Bell et al., 1995; Estévez & Kanazawa, 1995, 1996; Kanazawa, 1995; Estévez et al., 1997a; Rainuzzo et al., 1997; Sargent et al., 1997, 1999). A number of studies have suggested that a key factor in larval development is the ratio of DHA to EPA (Devresse et al., 1994; Dhert et al., 1994; Rainuzzo et al., 1994; Sargent et al., 1997), while others indicate that absolute levels of these compounds may be more important than their ratios (Naess & Lie, 1998).

In order to establish dietary requirements for essential nutrients, it is necessary first to understand the overall food requirements for normal growth and development (Sargent et al., 1997, 1999). Toward this end, both larval fish and their diets have been analysed (Rainuzzo et al., 1994; Naess et al., 1995; Estévez & Kanazawa, 1996; Sargent et al., 1997; McEvoy et al., 1998; Rønnestad et al., 1998b). Detailed examinations of the biochemical composition of Artemia and other cultured live diets reveal that the levels and types of fatty acids and free amino acids differ significantly from those characteristic of wild zooplankton (Naess et al., 1995; Sargent et al., 1997). These differences are probably important factors in reducing pigmentation success in larvae fed exclusively on cultured diets. Although wild-caught prey form the optimal larval diet from a nutritional standpoint, the cost, seasonal availability, and variation in species composition pose obstacles to their exclusive use in commercial hatchery production (Naess et al., 1995; Gara et al., 1998; Mangor et al., 1998). Instead, aquaculturists have sought to replace wild zooplankton in larval diets with cultured Artemia and rotifers. This approach offers greater reliability and control, but can also lead to very high rates of malpigmentation.

Three strategies have been used to minimize malpigmentation in flatfish larvae fed cultured prey. The first is to select the strains of Artemia that cause the least malpigmentation (Seikai et al., 1987c; Takeuchi et al., 1995). The second approach is to rear larvae on cultured prey except during a relatively short period leading up to metamorphosis (Seikai & Sinoda, 1981; Seikai et al., 1987a; Naess
et al., 1995; Næss & Lie, 1998). The third method is to enrich prey organisms with specific compounds required by developing larvae, or with precursors from which larvae can synthesize essential compounds (Watanabe, 1993; Dhert et al., 1994; Rainuzzo et al., 1994, 1997; Kanazawa, 1995; Sargent et al., 1997; Ronnestad et al., 1998a). The last two approaches seem to offer the greatest control of larval diets and the best chance of developing long-term strategies to overcome malpigmentation, and have thus received the most attention (Sargent et al., 1999).

Feeding zooplankton or copepods during the critical period for pigmentation development can avert the defects typical of Artemia-fed juveniles in both Japanese flounder (Seikai & Sinoda, 1981; Seikai et al., 1987a) and Atlantic halibut (Næss et al., 1995; Næss & Lie, 1998). The sensitive stage in cultured Japanese flounder occurs 2–3 weeks after fertilization, at an average length of approximately 8 mm (Seikai et al., 1987a). Næss & Lie (1998) located the ‘copepod window’ for Atlantic halibut between 2 and 3 weeks after first feeding, when larvae are about 16 mm (Ls). In Atlantic halibut, feeding copepods for only 7 days shortly before metamorphosis may be sufficient to prevent malpigmentation (Næss & Lie, 1998). Early diet apparently has no effect on pigmentation in this species; in fact, initial feeding on cultured Artemia yields better early growth and survival than feeding on copepods (Næss et al., 1995). In contrast, for plaice the duration of feeding on zooplankton, rather than the exact timing, may be the relevant factor (Dickey-Collas, 1993); however, the pigmentation scoring method used in this analysis makes the results difficult to interpret and to compare to data from other species.

Enriching cultured prey can significantly enhance larval nutrition and pigmentation, and this technique is now commonly used in the rearing of flatfish and other marine species (Dhert et al., 1994; Kanazawa, 1995; Rainuzzo et al., 1997; Sargent, et al., 1997, 1999). This method has been used both for fatty acids (Baker et al., 1998; Izquierdo et al., 1992; Kanazawa, 1993; Watanabe, 1993; Rainuzzo et al., 1994; Estévez & Kanazawa, 1995, 1996; Estévez et al., 1997a; Sargent et al., 1997, 1999; McEvoy et al., 1998) and for Vitamin A compounds (Miki et al., 1990; Dedi et al., 1995, 1997; Estévez & Kanazawa, 1995; Takeuchi et al., 1995, 1998; Ronnestad et al., 1998a,b).

Despite the widespread and generally successful use of prey enrichment, problems remain (Sargent et al., 1999). In particular, the determination of optimal levels for vitamin A supplementation is complicated by the multiple biological roles of vitamin A and its derivatives. Levels of vitamin A palmitate in Artemia sufficient to reduce rates of malpigmentation in Japanese flounder [about 1000 IU vitamin A g⁻¹ on a dry weight basis (Miki et al., 1990; Takeuchi et al., 1995)] far exceed those that cause skeletal abnormalities (50 IU g⁻¹; Dedi et al., 1995, 1997; Takeuchi et al., 1995). Similar findings have been reported in turbot (Estévez & Kanazawa, 1995).

Vitamin A (retinol) can be interconverted with retinal (retinaldehyde, vitamin A₁) by the action of specific enzymes. Retinal can in turn be oxidized to form retinoic acid, but the formation of retinoic acid is not reversible (Mayes, 1999). Thus, excess vitamin A may lead to retinoic acid overdoses, which can seriously disrupt gene expression and skeletal development (Dedi et al., 1995, 1997; Takeuchi et al., 1995, 1998; Takaki et al., 1996; Suzuki et al., 1998). Moreover,
compounds used to enrich Artemia or other live prey may be metabolized by those organisms, such that a very different set of compounds is delivered to fish larvae. Takeuchi et al. (1998) enriched Artemia with different forms of vitamin A, but found that retinol and retinol palmitate were both metabolized to retinoic acid within 22 h in Artemia, leading to retinoic acid overdoses in larvae fed enriched Artemia. Another chemical process, the autoxidation of fatty acids in enriched fish oils, can also confound attempts to supplement larval diets with measured levels of specific compounds (McEvoy et al., 1995). The development of artificial diets that avoid metabolic interconversion and autoxidation of enriching compounds may eventually allow better control of nutrition (Sargent et al., 1997, 1999) as well as more precise analyses of larval metabolism (Takeuchi et al., 1998).

Resolution of the persistent difficulties associated with the use of enriched cultured prey to provide correct levels of vitamin A to developing flatfish larvae will require integrating knowledge of metabolic processes in prey (Næss et al., 1995; Estévez & Kanazawa, 1996; Sargent et al., 1997) and larvae (Estévez & Kanazawa, 1996; McEvoy et al., 1998; Rønnestad et al., 1998b). Analyses of the tissue distribution and levels of vitamin-A-related compounds in normal fish (Estévez & Kanazawa, 1996; Rønnestad et al., 1998b) and a more complete understanding of the nutritional profiles of wild diets (Sargent et al., 1997, 1999; Gara et al., 1998) are also essential. Currently, feeding wild-caught prey during the sensitive period, though not always feasible, may be the most reliable nutritional approach to avoiding malpigmentation (Næss et al., 1995; McEvoy et al., 1998; Rønnestad et al., 1998b).

LIGHTING AND SUBSTRATUM

Most research on the effects of lighting and substratum type on malpigmentation concerns hypermelanosis on the blind side of the fish. Of Norman’s three categories staining appears most closely tied to environmental light and substratum type (Norman, 1934). Exposure to fluorescent light can cause the staining form of hypermelanosis in juvenile Japanese flounder (Seikai, 1991). Seikai (1991) found that hatchery-reared and albinic individuals were more prone to develop this form of malpigmentation than normal or wild-caught fish, and developed it more rapidly than did controls.

In the 1970s, observations of small numbers of wild-caught fish first suggested that substratum type might be an important factor in blind-side hypermelanosis. Stickney & White (1975) noted that wild-caught summer flounder postlarvae turned dark on the blind side after being maintained in the laboratory. However, providing a substratum into which fish could bury themselves prevented ambicolouration if fish had access to it during early juvenile stages, and could help reverse the condition in older individuals (Stickney & White, 1975). A small sample of juvenile sole (Achirus lineatus L.) showed similar responses to the provision of loose substrata (Houde, 1971).

More extensive studies have generally confirmed these earlier findings. Hatchery-reared juvenile Atlantic halibut had the highest rates of hyperpigmentation and skin lesions when reared in smooth-bottomed tanks (Ottesen & Strand, 1996). Fish in tanks provided with sandy substrata had the lowest occurrence of pigment abnormalities, but survival rates were poor, perhaps due
to the difficulty of maintaining water quality in these tanks (Ottesen & Strand, 1996). In an ingenious recent experiment, Iwata & Kikuchi (1998) separated the effects of light and substratum on hypermelanosis by rearing juvenile Japanese flounder on different substrata with varying light levels, including one condition in which light was transmitted through a glass ‘sand’ that allowed the fish to bury. Fish provided with the glass sand showed the least blind-side pigmentation despite high light exposure, while fish that were unexposed to light but could not burrow had the most pigmentation. Thus, it appears that in this species inability to bury is significantly more important than light exposure in causing blind-side hypermelanosis (Iwata & Kikuchi, 1998).

RELATIONSHIP TO VISUAL SYSTEM

Several lines of evidence suggest that the visual system may play a key role in the development of flatfish pigmentation. The dietary compounds required to forestall albinism (vitamin A and specific phospholipids) are components of retinal pigments and developing neural tissue (Estévez et al., 1997a), but not of skin pigments. The critical stage for dietary supplementation to reduce albinism corresponds to the period of retinal development in Japanese flounder (Seikai et al., 1987a; Seikai, 1992; Kanazawa, 1993). Abnormal retinal morphology results from diets that produce high rates of adult albinism in this species (Estévez & Kanazawa, 1995; Estévez et al., 1997a), and Estévez et al. (1997a) correlated retinal abnormalities with both reduced visual capabilities and suppression of normal pigment patterns. Hence it is not surprising that albino fish show aberrant visual behaviour (Kanazawa, 1993).

The primary hypothesis linking vision and pigmentation arose from a study that demonstrated that albino marbled sole (Limanda yokohamae Jordan & Snyder) failed to discriminate between dark and light environments (Kanazawa, 1993). Kanazawa suggested that both the abnormal responses to light and the lack of pigmentation stemmed from a common cause: retinal abnormalities caused by deficient experimental diets. Low levels of dietary vitamin A, DHA and phospholipids may interfere with rhodopsin synthesis in the retina. This defect could in turn disrupt transmission from the retina to the central nervous system of a signal required to trigger pituitary production of melanophore stimulating hormone (MSH). Without this endocrine signal, melanin synthesis cannot continue, so the end result is pigmentation deficiency (Table I).

The initial study that led to the hypothesis was not detailed, but subsequent work by Kanazawa and others has focused on the histological and behavioural predictions of the original model. Estévez et al. (1997b) observed abnormalities in the retinal epithelium of malpigmented Japanese flounder fed experimental diets, and suggested that normal pigment development had been suppressed as a result of a nutritionally-induced visual deficiency. However, the strong affinity of the developing visual and nervous system for critical compounds such as DHA means that diets that lead to measurable deficiencies in these tissues have even more severe effects elsewhere in the body. Thus, it is difficult to isolate effects due to retinal malformations from impacts on other systems and tissues (Bell et al., 1995). Moreover, at least some rhodopsin must be present at larval stages, even in albinos, for the fish to be able to see and capture live prey (T. Seikai, pers. comm.).
Feeding ability has been used to assess visual performance in larvae reared on different diets. Bell et al. (1995) reported that the feeding ability of juvenile herring (Clupea harengus L.) raised on a diet deficient in specific phospholipids was significantly impaired at low light. Interestingly, studies in several flatfish species have suggested that in bright light retinal defects associated with malpigmentation may be advantageous. Estévez & Kanazawa (1995) reported that malpigmented turbot tended to have excess retinal pigment, and suggested that this abnormality made albinos less subject to dazzling in very bright light. Albino fish were able to feed more effectively and grow faster than controls under hatchery conditions, which have much higher light intensities than the natural environment. Similar correlations between malpigmentation and high growth rates in culture were seen also in another study of turbot (Heap & Thorpe, 1987) and in Japanese flounder (Seikai & Sinoda, 1981; Seikai et al., 1987c).

We do not yet have the specific data required to assess the proposed endocrine linkage between retinal and pigment development, although the effects of hormones on metamorphosis and on pigment development have been studied in flatfishes (Inui & Miwa, 1985; Inui et al., 1994, 1995) and other teleosts (e.g. Bagnara & Hadley, 1973; Green & Baker, 1989; Baker, 1991; Suzuki et al., 1997; Hadley, 1999). Ambient light levels may serve as one cue for the onset of flatfish metamorphosis; both retinal and non-retinal pathways may be available to transduce this environmental information (Kvenseth et al., 1996). Metamorphosis itself may be mediated by thyroid hormone, as in amphibians (Inui & Miwa, 1985; Inui et al., 1994; Schreiber & Specker, 1998). MSH is secreted by the pituitary well before metamorphosis, and plays a key role in initiating melanin synthesis in ocular-side chromatophores (Kanazawa, 1993; Suzuki et al.,

**Table I. Hormones mentioned in the text that are involved in flatfish development, and in the formation of pigmentation patterns in other teleosts**

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Roles</th>
<th>Selected references</th>
</tr>
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<tbody>
<tr>
<td>Thyroid hormone (TH)</td>
<td>Timing of metamorphosis; stomach development and differentiation</td>
<td>Inui &amp; Miwa, 1985; Inui et al., 1994; Huang et al., 1998; Schreiber &amp; Specker, 1998</td>
</tr>
<tr>
<td>Melanocyte (melanophore) stimulating hormone (MSH)</td>
<td>Dispersion of pigment within melanophores; physiological regulation of colour; initiation of melanin synthesis in ocular-side chromatophores</td>
<td>Bagnara &amp; Hadley, 1973; Kanazawa, 1993; Hadley, 1999</td>
</tr>
<tr>
<td>Melanocyte (melanophore, melanin) concentrating hormone (MCH)</td>
<td>Aggregation (or in some cases dispersion) of pigment within melanophores; physiological regulation of colour</td>
<td>Green &amp; Baker, 1989; Baker, 1991 (review); Suzuki et al., 1997; Hadley, 1999</td>
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These observations are all consistent with the mechanism proposed by Kanazawa, but do not provide a direct test of the suggested hormonally-mediated linkage between vision and pigmentation development.

In a recent attempt to test Kanazawa’s 1993 hypothesis, Estévez & Kanazawa (1996) analysed fatty acids in neural tissues of normal and albino Japanese flounder raised on different diets, as well as the fatty acid composition of the diets themselves. The overall fatty acid profile was very similar in normal and albino fish, though some differences were apparent in neural tissues: neural tissues of albino fish had less EPA, and higher DHA : EPA ratios, than those of normal fish. (This last difference was not observed in a similar study of Atlantic halibut; McEvoy et al., 1998.) The only measure of visual capability in Estévez and Kanazawa’s study was growth rate, presumably based on ability to capture live prey, and in this respect albino fish significantly outperformed normal ones (Estévez & Kanazawa, 1996).

While Estévez and Kanazawa’s thorough analyses of lipid class composition add useful data to the discussion, the study as a whole fails to present a convincing test of the original hypothesis. Such a test will require integrating quantitative behavioural assays with measures of pigmentation success and analyses of retinal histology. It will be essential to compare data across a range of flatfish species, as the timing and details of retinal development can vary (Neave, 1984; Kawamura & Ishida, 1985; Huse, 1994; Kvenseth et al., 1996).

The overall relationship between larval nutrition and pigmentation is well documented. We know that specific dietary compounds are crucial to pigmentation success, and that pivotal events in pigmentation development occur during certain limited periods of larval growth and differentiation. Other external factors including lighting and substratum type can also apparently affect internal processes such as retinal and pigment cell differentiation. All of this knowledge has been immensely useful in optimizing diets and rearing conditions in hatcheries. We can now begin to apply it in a different way, to try to gain insights into why specific factors are important to pigmentation development, and what mechanisms link them to the internal developmental processes that are the proximate causes of pigmentation defects or success.

**DISCUSSION**

**INFERENCES ABOUT DEVELOPMENTAL MECHANISMS AND CAUSES**

Malpigmentation is the visible result of one or more defects in the normal developmental process; if we want to understand exactly what those defects are, we need to focus on the sources and roles of the signals that control differentiation. Our present goal is to consider what the existing data suggest about underlying developmental mechanisms and disruptions. Up to this point, applied aquaculture research has focused on reducing malpigmentation in hatchery-reared fish, and has made significant progress in identifying effective strategies. The next step is to see what current remedies can tell us about the underlying biology, in order to identify both outstanding questions and possible research strategies to help answer them. Ultimately, increased knowledge of the
biological mechanisms of pigment development will be a key element in further optimization of hatchery production.

We can examine abnormal pigment development at several levels which may lead to different hypotheses about its causes. Most research thus far has addressed larval diets. The resulting ability of aquaculturists to reduce malpigmentation rates significantly by supplementing the diet with particular nutrients and vitamins has focused attention on external or environmental causes of albinism. On another level, detailed histological work by Seikai and colleagues (Seikai et al., 1987b, 1993; Matsumoto et al., 1989; Seikai & Matsumoto, 1991, 1994; Matsumoto & Seikai, 1992; Seikai, 1992) comparing normally and abnormally pigmented skin from the ocular and blind sides of Japanese flounder has suggested that whether chromatoblasts differentiate or disappear at metamorphosis is controlled by their local tissue environment. This hypothesis raises the question of what establishes the histological differences between ocular-side and blind-side skin.

A third perspective is that of developmental biology. There is a substantial literature that addresses pigment cell development and differentiation in a range of vertebrate models (not including flounder) (Reedy et al., 1997). The approaches and techniques of developmental and cell biology offer a suite of new ways to examine the processes of normal pigment development in flatfishes and to test hypotheses about how and why malpigmented fish develop differently.

**CURRENT HYPOTHESES**

There are currently two major hypotheses about the developmental causes of malpigmentation in flatfishes. Kanazawa (1993) has proposed that dietary deficiencies lead to abnormal retinal development, which in turn derails the sequence of hormonal events necessary to trigger melanophore differentiation. Seikai (1992) and Seikai & Matsumoto (1994) ascribe the failure of melanophore differentiation in pseudo-albinic fish to the local tissue environment, and to the fact that ocular-side skin in albinos apparently follows a blind-side differentiation pathway. These hypotheses do not necessarily conflict, but they may have different implications for the level at which we should search for the proximate causes of malpigmentation.

Although a range of correlative evidence supports Kanazawa’s (1993) hypothesis of an endocrine link between the retina and the development of pigment, no study has yet integrated and tested more than two elements of the theory at once. Doing so would require examining retinal histology and correlating it directly with pigmentation patterns in individual fish, and carrying out specific tests of visual competence on albino and normally-pigmented fish. Assaying hormone levels in individual animals may show whether diets that induce retinal abnormalities lead to the predicted endocrinological disruptions.

Further tests will involve the examination of earlier stages of development, particularly in the interval identified as a critical period for dietary supplementation to reduce albinism (Seikai et al., 1987a; Næss et al., 1995; Næss & Lie, 1998). Comparison of the developing retinal histology in larvae fed on different diets at this sensitive stage would provide essential information about possible anomalies in early retinal development. Examination of visual behaviour and
then retinal morphology in fish whose diets were supplemented at different developmental stages would provide another strong test of the suggested correlation between retinal and pigment development. Moreover, a longitudinal study of retinal histology and skin pigmentation in groups of fish fed albinism-inducing diets (and in normal controls), beginning before the critical stage and continuing through to the end of metamorphosis, would allow correlation of structural changes occurring in both retina and skin during normal and abnormal development. One outstanding question is whether all albinic fish have abnormal retinas, and if not, whether patterns of malpigmentation differ between those with normal and those with defective retinas.

The second hypothesis, that ocular-side chromatophores in albino fish fail to develop because they find themselves in a ‘blind-side’ histological environment, is supported primarily by detailed descriptive data from Japanese flounder (Seikai, 1992; Seikai & Matsumoto, 1994). The next step is to borrow experimental techniques from developmental biology to try to identify the critical differences between ocular-side and blind-side environments. For example, regions of skin could be grafted between the ocular and blind sides of normal larvae, and between normal and (prospectively) albino individuals. These transplants would have to be done at premetamorphic stages, but the species-specific direction of body reorientation at metamorphosis makes it possible to predict, at least statistically, which side will be ocular and which blind. Such grafting techniques will offer a serious technical challenge, but precedents exist (Keller et al., 1982, 1992).

Cell culture techniques offer the additional possibility of examining in vitro the effects of hormone levels or explanted skin matrix on developing chromatoblasts: Seikai et al. (1993) showed that chromatoblasts can undergo key elements of their developmental program in culture. The tissue culture system provides a way to manipulate a greatly simplified physiological environment, and test the responses of isolated pigment cells to various stimuli. This might be a particularly useful approach for assaying effects of different hormones, but would need to be based on in-vivo studies that documented baseline concentrations of hormones in the histological environment in which pigment cell development normally occurs.

The two hypotheses about the causes of malpigmentation address patterning at different developmental stages and levels of organization, and they are not mutually exclusive. Rather, elements of each hypothesis may fit into a more inclusive explanation. The first hypothesis postulates a systemic problem (failure of hormonal signalling) that has differential effects on the ocular and blind sides. In contrast, the second theory suggests a mosaic patterning defect (areas of ocular-side skin following a blind-side pathway) that does not affect local differentiation processes: chromatoblasts develop appropriately for their histological environment, and defects are due to an error upstream. At this point, we need carefully-designed developmental studies to help determine whether abnormal skin is following a normal differentiation pathway in the wrong location, or whether it is doing something entirely different. This distinction is central to the formation of testable hypotheses about the mechanisms underlying malpigmentation: do defects result from the activation (or inactivation) of normal processes in the wrong places, or from aberrant processes that occur
nowhere in normal animals? Answering these questions will also shed light on whether patchy pigmentation patterns characteristic of malpigmented animals represent mosaics of normal and abnormal regions, or whether the patchiness reflects a more global effect.

THE QUESTION OF SIDEDNESS

Both hypotheses ultimately rest on the question of how ‘sidedness’ is established, and how systemic signals such as hormone levels are interpreted differentially at the local level. This is clearly a key issue if the histological identity (blind v. ocular) of the skin directly controls pigment cell differentiation. Alternatively, if pigment development requires a hormonal trigger, there must be some downstream mechanism that transduces the signal differently on the prospective ocular and blind sides of the fish. At some point in development a fundamental distinction is established between the right and left sides of the larva. The developmental basis of left-right asymmetry is the focus of research in a number of vertebrate ‘model’ systems (Yost, 1998a,b), but has not been addressed in flatfishes.

Regardless of the mechanism by which it is established, the left-right distinction is normally consistent within a species: there are right-eyed and left-eyed flatfishes. The existence of species-specific patterns indicates that there is a genetic basis for sidedness. However, the pattern can be reversed in individuals: ‘wrong-sided’ fish occur, and in fact this defect is often associated with albinism (G. Nardi, pers. comm.), though many fish with reversed morphology show normal pigmentation.

A secondary issue is whether defects of ocular- and blind-side pigmentation represent two ends of a continuum, or are distinct phenomena. In some studies (Dickey-Collas, 1993) different types of malpigmentation are not distinguished; however, we believe the available evidence suggests that defects on the ocular and blind sides are separate phenomena, and probably have different underlying causes. Ocular-side albinism is determined by the end of metamorphosis (or possibly much earlier), and appears to be permanent in most cases. In contrast, the ‘staining’ form of blind-side pigmentation can develop over several months, and is at least partially reversible in post-metamorphic juveniles (Stickney & White, 1975; Seikai, 1991). In Japanese flounder, diet seems to be the single most important factor in determining ocular-side pigmentation, while lighting and substratum are more significant than nutrition in controlling blind-side pigmentation (Seikai, 1991; Iwata & Kikuchi, 1998). Experiments that explicitly isolate and compare the effects of these factors in other species are yet to be undertaken.

It is not yet clear whether excess blind-side pigment consists of abnormally persistent larval melanophores, or of newly-differentiating adult melanophores. Persistence of larval pigment cells would suggest a failure of whatever mechanism normally causes them to disappear at metamorphosis. The presence of adult melanophores would suggest hyperstimulation of melanophore differentiation, or excessive sensitivity to differentiation signals on the blind side. Alternatively, late appearance of melanophores on the blind side could result from the abnormal persistence, and eventual differentiation, of stem chromatoblasts that normally self-destruct or are otherwise removed from the blind side at metamorphosis. Burton (1988) found that melanophores on the blind side of
ambicoloured winter flounder closely resemble those on the eyed side. In contrast, the unique melanophore morphology on the underside of Greenland halibut, which are normally dark on both sides, appears to be a secondarily evolved feature in adults of this species rather than a persistent larval characteristic (Burton, 1988).

CONTROL OF CHROMATOBLAST DIFFERENTIATION

At metamorphosis, chromatoblasts may either differentiate into adult melanocytes or disappear. Which path they follow depends on both intrinsic and extrinsic factors. The importance of intrinsic controls is underscored by in-vitro studies demonstrating the existence of an intrinsic ‘clock’ that triggers differentiation of melanocytes at appropriate developmental times in cultures of dissociated embryonic cells (Seikai et al., 1993). On the other hand, the effects of larval diet, light exposure of the skin, hormonal signals, and local tissue environment suggest a critical role for external information in guiding the differentiation of chromatoblasts.

Since prospective pigment cells migrate from the embryo’s dorsal midline out into the tissues in which they finally differentiate, it should be possible to transplant premigratory cells to new areas or to pigment-deficient individuals to assess the behaviour of the cells in a new environment. Such experiments can yield deep insights into the reasons cells fail to differentiate in albino or pigment-defective animals, by separating intrinsic from environmental factors.

One obstacle, however, is the difficulty of selecting donor cells from prospectively albino fish, as much evidence suggests that key controls of albinism occur later in development than migration of the neural crest cells. (Even if albinism were shown to be determined very early, one would need to perform surgical manipulations well before definitive pigment pattern becomes visible.) An alternative approach would be to make transplants between the prospective ocular and prospective blind sides of premetamorphic larvae: at premetamorphic stages chromatoblasts are distributed symmetrically on the right and left sides. After metamorphosis, the grafted skin could be examined to see whether its histological structure and pigmentation corresponded to its source or to its new location.

PROSPECTS FOR FUTURE WORK

Enormous progress has been made in minimizing malpigmentation in hatchery-reared flatfish, mainly through careful analysis and enhancement of larval nutrition. It is not always clear, however, how or why a successful treatment works: we can measure its efficacy at a statistical level, but remain ignorant of the mechanism of its effect at the developmental level.

Discovering the mechanisms of effective treatments will require integrative research strategies that enable us to connect environmental factors such as diet and substrate to internal mechanisms of cell and tissue differentiation. For example, nutritional studies that incorporate stage-specific sampling and histological analysis of the skin and retina can clarify the developmental effects of specific dietary components. In addition to employing classic aquaculture approaches such as feeding studies and statistical analyses, we need to add a developmental perspective, and make full use of the techniques that the field has to offer.
Developmental studies are underway for Japanese flounder, and can now be extended to other flatfishes, which will help us assess how widely we can apply findings from a single species. Extension of the breadth as well as the depth of interspecific comparisons is essential for several reasons. First, to date some of the most important developmental analyses have been carried out in only one species, and certain critical experiments have been done in other teleosts and the results extrapolated to flatfishes (e.g. Bell et al., 1995). Second, most species have not yet been thoroughly studied, and it is therefore particularly difficult to interpret results from them (e.g. Benetti, 1997). Finally, even among the most intensively studied flatfish species there are significant differences in patterns of malpigmentation, in dietary requirements (Watanabe, 1993), and in eye development (Neave, 1984; Huse, 1994).

Understanding the mechanistic basis of pigment development and defects in a range of flatfishes will enhance our ability to design specific and cost-effective interventions to reduce albinism in hatchery populations. Ultimately it will also broaden our understanding of vertebrate pigment development in general, an area with ecological, evolutionary, and medical significance as well as key importance to flatfish aquaculture.

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