How Zebrafish earn their Stripes: A paradigm for Pigmentation Patterning in Vertebrates

Devi SATARKAR

Institute of Bioinformatics & Biotechnology Savitribai Phule Pune University, Maharashtra State, India

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ABSTRACT

The world is a mosaic of delicate tints, intricate patterns and brilliant hues that have captured the eye of human beings from time immemorial. Scientists have spent decades trying to unravel the complexities of nature, and in the process, have uncovered revolutionary insights on the mechanisms governing coloration in animals. Zebrafish, a small minnow with distinct stripes on its body, has captivated the attention of researchers who wished to study pigmentation patterns in the animal world. The availability of a vast collection of pigment pattern mutants and closely related species of zebrafish allowed researchers to weave an incredible story of how pigment cells come together to form an elaborate pattern. This review comprises of the molecular and cellular mechanisms of stripe formation in zebrafish along with a sound mathematical basis for it. It also includes the ecological significance of a fish having stripes and the application of this knowledge of pigmentation in building robust zebrafish models for skin disorders in humans such as the invasive melanoma.

ABBREVIATIONS

TEM: Transmission Electron Microscopy
MITF: Microphthalmia-associated transcription factor
LTK: leukocyte tyrosine kinase
L-DOPA: L-3,4-dihydroxyphenylalanine
PKA: Protein kinase A

INTRODUCTION

From the patches on a leopard to the iridescent spots on a butterfly’s wing, the animal world is chock-full of tremendously diverse pigment patterns. These intricate patterns serve several essential purposes, such as kin recognition, sexual mating and camouflage. Since pigment patterns are a target for natural selection, they pave an evolutionary path.

The underlying mechanisms of pattern formation have captivated scientists for several years. Vertebrate colouration is due to pigment cells that originate from the neural crest during embryonic development [1]. Birds and mammals have melanocytes; specialized cells that produce a dark pigment called melanin. The melanin is secreted into the feathers of
birds and skin and hair of mammals. The patterns formed in these animals are due to variation in the amount of melanin within a single cell; the melanocyte [2], [3]. Fish, amphibians and reptiles, on the other hand, have several pigment producing cells called chromatophores.

One of the most well-studied animal for pigment patterning is the zebrafish (Danio rerio). The zebrafish is a small, freshwater fish that is marked by distinctive stripes on its body and fins, as a result of three types of chromatophores arranged in layers in the hypodermis. Black melanophores, yellow xanthophores and iridescent-blue iridophores containing guanine-rich platelets that reflect light to give the fish a silvery appearance (Figure 1) [4].

Decades of research have gone into investigating the biology of zebrafish patterning, which includes the development of chromatophores and their interaction with one another to give rise to a distinct pattern, the genes controlling this process and a possible extrapolation of these mechanisms in other animals. The need for exploring pigment pattern formation in zebrafish may have possibly stemmed from the fact that the same three chromatophores form a myriad of patterns in fish belonging to the Danio genus itself. While Danio rerio has horizontal dark stripes; Danio rerio has broader ones, Danio nighrofasciatus has stripes and spots on its abdomen, and Danio choprae has vertical stripes, to name just a few [5].

Moreover, the zebrafish is a relatively simple organism to study the pigmentation process in vertebrates at the developmental, genetic and cellular level. The larvae are transparent, the fertilization is external and they have high fecundity. The zebrafish genome shares 71.4% similarity with the human genome and at least 82% of human disease-related genes have a zebrafish orthologue [6].

Extensive genetic screens have been carried out to identify genes involved in pigmentation via mutants with altered pigmentation phenotypes. This review encompasses a few of these mutants and the subsequent deduction of the mechanisms of pigmentation in zebrafish. It also includes a possible mathematical reasoning for pattern formation in vertebrates using zebrafish as a model, the ecological significance of pigment patterns in fish, and the application of the extensive knowledge of pigmentation gathered in order to create zebrafish models for melanoma and other skin-diseases.

Mechanism of Stripe Formation

Zebrafish stripes are a construct of three chromatophores and the spatial arrangement as well as the morphology of these cells in the hypodermis of the fish has been elucidated using transmission electron microscopy (TEM). It was found that the dark stripe region contains dendritic melanophores with densely-packed melanosomes. The iridophores are of two types, based on the amount and size of crystalline platelets within them; L-type and S-type. The L-type iridophores form a layer beneath the melanophore layer giving the dark stripe a distinct blueish glimmer. The interstripe region houses xanthophores, which contain carotenoids and pteridines, lying on top of densely packed S-type iridophores that make the interstripe area appear golden [7], [8].

The pursuit of scrutinizing the process of stripe formation has led to the identification of numerous pigmentation mutants in large-scale mutagenesis screens. The genes responsible for melanocyte development, differentiation, and function have been subsequently elucidated. The isolated pigmentation mutants can be roughly segregated into three types; genes that affect formation of pigment, genes that affect formation of chromatophores and genes involved in the interactions between the chromatophores.

Table 1 lists a few well-known pigment mutants of zebrafish that have been instrumental in construing a process for stripe formation in the fish and subsequently shedding light on the mechanism of melanocyte development.

Mutants such as golden, albino, sandy and VA6 belong to the first class of genes that affect melanin synthesis. SLC24A5 and SLC45A2 are cation exchangers on the melanosome membrane, which is a lysosome-like vesicle that stores melanin. A disruption in the function of these proteins, causes an imbalance in the pH homeostasis of the vesicle which ultimately affects melanin synthesis from tyrosine. Both the golden and albino mutants are tyrosinase-positive, like the ocular-cutaneous albinism type 4 found in humans. An interesting aspect of this study was that the golden gene in zebrafish, SCL24A5, has been found to play a key role in human skin colour variation and the reason for freckles in Europeans. [9], [10].

The mutants sandy and VA6, coding for tyrosinase and Tryp1, have no melanin synthesis because the proteins are vital enzymes in the melanin formation pathway. Tyrosinase converts tyrosine to L-DOPA which is subsequently converted to dopaquinone and eumelanin by Tryp1 and Tryp2 respectively. [10], [11].

Genes that affect chromatophore formation have also been found via mutants such as colourless, shady, rose, nacre and panther. The colourless gene codes for Sox10 which has
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<table>
<thead>
<tr>
<th>Gene name (symbol)</th>
<th>Gene product</th>
<th>Adult phenotype</th>
<th>Visual Representation</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>golden (gol)</td>
<td>SLC24A5 (a cation exchanger)</td>
<td>Hypopigmentation of stripes, reduced melanin in melanophores, pale retina</td>
<td>![Fish Image]</td>
<td>[9]</td>
</tr>
<tr>
<td>albino (alb)</td>
<td>SLC45A2 (a cation exchanger on melanosome membrane)</td>
<td>No dark stripes, no melanin in melanophores, unpigmented retina</td>
<td>![Fish Image]</td>
<td>[10]</td>
</tr>
<tr>
<td>sandy (sdy)</td>
<td>Tyrosinase</td>
<td>No dark stripes, no melanin in melanophores, unpigmented retina</td>
<td>![Fish Image]</td>
<td>[10]</td>
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<tr>
<td>VA6</td>
<td>Tyrp1 (tyrosinase-related protein 1)</td>
<td>Melanophore death, unpigmented detritus left behind followed by regeneration of unpigmented melanophores, pale stripes</td>
<td>![Fish Image]</td>
<td>[11]</td>
</tr>
<tr>
<td>nacre (nac)</td>
<td>Mitf (Microphthalmia-associated transcription factor)</td>
<td>No melanophore development; more iridophores than wild-type, light stripes broken down into spots as iridophores spread out</td>
<td>![Fish Image]</td>
<td>[12]</td>
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<tr>
<td>shady (shd)</td>
<td>Leukocyte tyrosine kinase</td>
<td>No iridophore development, reduced melanophores, spotted stripes (less in number)</td>
<td>![Fish Image]</td>
<td>[13]</td>
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<tr>
<td>Mutant</td>
<td>Gene/Protein</td>
<td>Description</td>
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<tr>
<td>colourless (cls)</td>
<td>Sox10</td>
<td>Larval lethal, no pigment cells formed, apoptosis of all neural-crest derived cells</td>
<td>[14]</td>
<td></td>
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<tr>
<td>rose (ros)</td>
<td>Endothelin receptor b1</td>
<td>Disrupted stripe formation (spots), only 2 spotted stripes develop, no iridophores</td>
<td>[15]</td>
<td></td>
</tr>
<tr>
<td>panther (fms)</td>
<td>Colony-stimulating factor 1 receptor</td>
<td>Severely reduced xanthophores, melanophores reduced, impaired stripe development</td>
<td>[16]</td>
<td></td>
</tr>
<tr>
<td>leopard (leo)</td>
<td>Connexin 41.8</td>
<td>Spots instead of stripes, affects both melanophores and xanthophores</td>
<td>[17]</td>
<td></td>
</tr>
<tr>
<td>obelix (obe)</td>
<td>Kir7.1 (a potassium channel)</td>
<td>Wider stripes, no clear boundary between stripes and interstripes</td>
<td>[17]</td>
<td></td>
</tr>
<tr>
<td>idefix (ide)</td>
<td>Spermidine synthase</td>
<td>Fewer dark stripes, light stripe areas expanded</td>
<td>[18]</td>
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been associated with the Waardenburg-Shah syndrome in humans. The absence of this gene leads to an extensive loss of all chromatophores and the enteric nervous system. Thus, it plays a role in non-ectomesenchymal cell fates [14].

Melanophores require Mitf for differentiation [12], while iridophores require leukocyte tyrosine kinase (ltk). However, ltk is required by both L and S type iridophores while the absence of another protein, encoded by rose, affects only S-iridophores. Iridophores require endothelin signaling for proper distribution in the hypodermis [13], [15]. However, in most cases, a loss of iridophores unmistakably leads to a reduction in melanophores, which suggests that differentiated iridophores are required for the maintenance of melanophores in the skin. The panther mutant shows a defect in xanthophore formation. However, it is not required for establishing a set of precursor cells in the embryo, but rather for specifying the fate of pigment cells to xanthophores, as well as being responsible for melanocyte maintenance [16].

The leopard and obelix mutants are examples of genes affecting the interactions between two chromatophores; melanophores and xanthophores. In leo mutants, the stripes are replaced by spots while in obe mutants the boundaries between the stripes are almost abolished, leading to wider, diffused stripes. To understand the interactions between the two cell types, studies involving mosaic experiments of transplanted mutant cells was carried out [17], which revealed that the juxtaposition of melanophores and xanthophores was sufficient for the formation of a stripe. It was then deduced that obe is responsible for the separation of melanophores and xanthophores. Melanophores require obe to aggregate to a defined stripe width. Since this gene is lost in the mutant, the inherent affinity of xanthophores towards melanocytes overpowers their tendency to cluster, leading to xanthophores invading the melanophore domain. This results in intermingling of the stripe area [17].

In leo mutants, the boundary shape is disrupted and melanocytes cluster to form spots. Meanwhile, the cohesion between xanthophores and newly differentiating xanthophores weakens and they invade and subsequently expand their domain, ultimately resulting in xanthophores occupying the area between melanocyte clusters [17].

A study in 2016 by Frohnhöfer et al. described an idefix mutant which did not have spermidine synthase. They established the role of spermidine, a crucial polyamine for proliferation and differentiation of cells, in stripe formation in zebrafish. They found that spermidine in a wild-type zebrafish plays a role in regulating Connexin 41.8 and Kir7.1, proteins encoded by leopard and obelix respectively. The absence of spermidine synthase, and a consequent reduction in spermidine levels, leads to a phenotype very similar to the obelix mutant. The study also showed that the ide gene product did not autonomously act in the chromatophores, but indirectly affected their behavior. When mutant ide blastomeres were transplanted from a donor embryo to nacre or rose mutants, the mutant chromatophores were able to form the wild-type stripes despite the lack of one chromatophore [18].

The mutants listed above played a crucial role in forming the base upon which theories of pigmentation could be formulated. However, more molecular regulators are continually being discovered, adding to the plethora of information already available for the process of pigmentation patterning in zebrafish.

A gain-of-function mutation, mau encoding Aquaporin3a (aqp3a), was described to have a broken, rippling striped phenotype. However, transplantation experiments revealed that the pigment cells were not directly involved in the irregular pattern, but instead, it was the tissue environment that was affected in the mutant [19].

Cooper et al. elucidated the role of protein kinase A (PKA) signaling in iridophore development. Using, forskolin, an adenylyl cyclase activator, they used pnpt4a, an iridophore marker, and mitfa, a melanophore marker, to investigate the role of PKA signaling in chromatophore development. It was found that PKA acts like a switch by causing melanophores to differentiate whilst simultaneously decreasing iridophores [20].

In a recent study in 2019, a mutation in a protein called GTP cyclohydrolase2 was described which showed a defect in pteridine synthesis by larval xanthophores. In this camembert (cmm) mutant, the xanthophores appeared grey instead of yellow due to a lack of pteridine, a yellowish pigment found in xanthophores [21]. Subsequently, Saunders et al. showed that thyroid hormone activates carotenoid-associated genes and facilitates a switch from pteridine pigmentation in larval xanthophores to carotenoid pigment deposition in the adult cells [22].

Epigenetic control through modulation of intracellular pH was described by Natarajan et al. wherein carbonic anhydrase 14 (Ca14) was mutated in a zebrafish by a CRISPR-mediated mechanism. In the wild-type, it was found that Mitf in melanoblasts was able to elevate Ca14 levels, which led to a concomitant increase in the intracellular pH leading to an activation of histone acetyl transferases, causing the melanoblasts to differentiate to melanophores. In the mutant, due to a lack of Ca14, acidic immature melanoblasts were found in the fish, ultimately creating fainter stripes in the adult fish. Thus, along with Mitf, melanoblasts require a pH-dependent epigenetic control for melanophore differentiation [23].

Apart from epigenetic aspects of pigmentation, investigations are ongoing for post-transcriptional regulation as well. Recently, Dicer1-depleted zebrafish were shown to have increased Sox10 and mitfa expression and aberrant pigmentation. Dicer is a protein responsible for the activation of the RNA-induced silencing complex, which plays a crucial role in the functional maturation of miRNA. Based on the findings of this study, it was evident that Dicer1 was part of the gene regulatory network controlling melanocyte differentiation [24].

In conclusion, forward and reverse genetic screens have
identified numerous molecular regulators and led to the clarification of several pathways guiding stripe morphogenesis.

Development of the Stripe Pattern

Precursors of melanophores migrate along spinal nerves, whilst proliferating, during embryogenesis. They enter the hypodermis as melanoblasts, lying along the ventral and dorsal myotomes and the horizontal myoseptum. Once differentiated, they stop dividing and synthesize melanin in their melanosomes. The migration of proliferating melanoblasts was traced by GFP labeling of mitfa, a marker for melanocytes, in an experiment by Dooley et al. They also found that this migration is dependent on ErbB signals, however, it is no longer required once they differentiate and melanise, unlike glia cells which require ErbB signaling throughout their life [25].

On the other hand, iridophores continue proliferating even when they have migrated into the skin through the horizontal myoseptum. Singh et al. created labelled clones of pigment cells using Cre/loxP-mediated recombination, to track their movement over several weeks. They first emerge at the horizontal myoseptum and form the first interstripe and proliferate dramatically to spread along the dorsoventral axis. Eventually they aggregate to create a series of interstripes in between the stripes formed by melanophores [26].

Xanthophores remain undifferentiated throughout the larval stage, and at the onset of metamorphosis they begin proliferating, giving rise to adult xanthophores that cover the iridophores that have formed the interstripe. Using in vivo imaging and transplantation experiments, it has been shown that the xanthophores densely cover the interstripe-iridophores and form a loose stellate morphology over the melanophores of the dark stripe. Short-range interactions occur between the cells; the iridophores attract the xanthophores while the melanophores repel them [27].

The striped pattern on the flank of the fish, is a sequential process that involves the controlled migration, proliferation and differentiation of the three chromatophores. Figure 2 is a schematic representation of the process of stripe formation. At the onset of metamorphosis, larval xanthophores proliferate and cover the trunk of the fish. Iridophores appear through the horizontal myotome and form the first interstripe and continue dividing. The xanthophores overlying the interstripe region, become dense and compact, and accumulate pteridines. Meanwhile, melanoblasts appear through the dorsal and ventral myotomes, and differentiate into melanin-containing melanophores. They flank the first interstripe to form the dark stripes. The same process occurs as subsequent interstripes develop when more iridophores appear and xanthophores cover them. The xanthophores that were above the dark stripe, are loosely arranged with a stellate shape. This process gives rise to a stripe pattern formation on the body of the fish.

The horizontal myotome acts like a landmark for the formation of the first interstripe, after which interactions between the cells leads to the formation of subsequent stripes. A choker mutant, lacks the horizontal myotome and has a labyrinth of meandering stripes. They are parallel and of a normal width but have abnormal and random orientations. This is hypoth-

![Figure 2: Process of striped pattern formation in Danio rerio](image-url)
esized to occur due to random clusters of iridophores appearing on the flank due to the absence of the horizontal myotome [28].

Experiments that involved analysis of mutants lacking two chromatophores, revealed that the remaining pigment cells evenly covered the entire trunk. This implies that each chromatophore has the potential to evenly cover the skin but the stripe pattern emerges as a result of the presence of the other two chromatophores. Evidence from chimeric animals solidifies the theory that communication between the three cell types is vital for the stripe pattern formation in zebrafish [28].

**The Turing Mechanism to explain Stripe Formation**

Alan Turing, a mathematician, described a unique model for pattern formation in organisms in 1952, called the reaction-diffusion model. He explained that a set of mathematical equations could be used to measure the diffusion rates of two substances; an activator and an inhibitor. In a biological system, the activator compels the cells to behave a certain way and the inhibitor prevents that from happening. In case of pigment pattern formation, the activator molecule would diffuse over the cells, making them produce a pigment, while the inhibitor molecules would curb this diffusion. A difference in the diffusion rates of these two molecules would result in stripes, spots or labyrinths [29].

This theory was met with skepticism for several decades when it could not explain certain patterns in the biological world; for instance, the segmented embryo of *Drosophila* is a result of information contained in the genes of the organism rather than a set of chemicals controlling cell behavior. However, the interest of researchers was piqued when computer simulations could explain an underlying reaction-diffusion mechanism for various animal skin patterns.

In order to validate a possible Turing mechanism for pigmentation in zebrafish, it is vital to study the interactions between the three chromatophores. Mutants such as *leopard* and *obelix* could provide insights into the possible way by which melanocytes and xanthophores aligned themselves around and over each other during the formation of stripes. Further experiments were conducted to study the interactions between cells when stripes regenerated.

A study was conducted by Parichy et al. wherein xanthophores were ablated by a temperature sensitive *fms* (*panther*) mutation in developing embryos. They found that these fish failed to develop stripes properly due to a reduced number of melanophores, even though the *fms* gene is largely required for xanthophore specification. This implied that melanophores require signaling from xanthophores for survival. Since the mutation was temperature regulated, the xanthophores were induced to form by a shift in temperature and this led to an increase in melanophores as well. A striped pattern developed, albeit at random orientations to appear like a labyrinth. This study was instrumental in discovering that *de novo* stripes could be generated in adult zebrafish [30].

Interactions between neighboring melanophores and xanthophores were worked out by Nakamasu et al. They selectively ablated pigment cells in targeted regions of the skin, to study the effect of the loss of neighboring cells on xanthophore or melanophore survival and development. Their experiments revealed that xanthophores and melanophores compete for survival and inhibit each other locally. A combination of these two negative interactions results in a positive feedback loop. While melanophores show a local, inhibitory effect on xanthophores, they are distally activated by xanthophores, resulting in a long-distance positive interaction. Together, this creates a long-range negative feedback loop. This model of short-range activation and long-range inhibition is in conjunction with the conditions required for pigmentation patterning in the reaction-diffusion theory. Moreover, these experiments were shown to fit the reaction-diffusion model via computer simulations [31].

Turing’s reaction-diffusion model states that a single parameter change can affect the entire pattern. Watanabe and Kondo investigated this property by expressing the *leopard* gene ectopically using the *mitfa* promoter. They expressed various alleles of this gene at different levels, to essentially tune the melanophore-xanthophore interaction, in turn changing the parameters. They found that the *leopard* pattern could be generated exactly as predicted by Turing’s model and in concurrence with the parameter changes. This study proved that the reaction-diffusion model has potential to predict pigmentation patterns in various animals [32].

*In vitro* experiments were conducted to understand the dynamics of cell-cell interactions. Using the *jaguar* mutants, with a defective *Kir7.1* channel, the depolarization of melanophores was studied using a voltage-sensitive dye. When melanophores and xanthophores were isolated and examined *in vitro*, it was observed that wild-type melanophores depolarized transiently when they came in contact with the dendrites of a xanthophore. No depolarization occurred when they came in contact with fibroblasts, which suggested that a specific membrane-associated signal was present in xanthophores that caused it to depolarize a melanophore. Moreover, the wild-type melanophores tended to migrate away from the xanthophores, while *jaguar* mutants did not show this repulsive behavior. In conclusion, the *Kir7.1* potassium channel plays a role in contact-dependent negative interactions between melanophores and xanthophores that allows them to segregate during stripe formation [33]. This study was followed by experiments by Yamanaka et al. who modified the *in vitro* assay and found that xanthophores actively migrated towards the melanophores that were moving away. This “chase and run” behavior did not occur in *leopard* or *jaguar* mutants which indicates that this cell-cell interaction is important for pattern formation by the Turing model [34].

The role of Notch signaling in melanophores and xantho-
phores was studied to gather information about long-range interactions between these cells. It was found that Delta ligands were expressed by xanthophores and the melanophore membrane had Notch receptors. Upon ablation of xanthophores in the interstripes, melanophores in the stripe began to die and this process occurred when Delta/Notch signaling was inhibited. However, activation of Notch signaling ectopically, rescued the melanophores. Moreover, in vivo imaging showed that melanophores extend long projections towards xanthophores which aid in interactions between membrane-bound ligands and their receptors, like Delta/Notch. This long-range interaction was another validation for the Turing model albeit without a diffusing chemical relaying the signals [35].

The original Turing model described the presence of two chemicals reacting with each other and diffusing. However, experiments have shown that pattern formation in zebrafish is a consequence of pigment cells interacting with one another through dendrites and projections rather than a diffusible chemical substance. Regardless of this apparent inconsistency in the original claim that fish pigmentation pattern follows the Turing model, the biological system is mathematically analogous to the original reaction-diffusion theory and it can be concluded that the pigmentation patterning mechanism in zebrafish is equivalent to the reaction-diffusion model proposed by Alan Turing over 60 years ago.

Ecological Significance of Stripes on a Zebrafish

Pigmentation patterns in animals are often targets of natural as well as sexual selection, and thus play a tremendous role in evolution. Fish, in particular, have intricate patterns on their skin that play ecologically significant roles in mate selection, social aggregation, predator escape and camouflage.

The stripes on a zebrafish are distinct and unmistakable, and they have been implicated in various ecological roles. An important use for the stripes in zebrafish, is in the shoaling behavior that they exhibit. Shoaling occurs when fish aggregate to form a tight-knit group of individuals within a population. It provides a defense against predators by reducing their chances of being captured. It also enhances their ability to forage and find their own prey as well as look for a mate. Another advantage of shoaling is that being part of a large group increases the hydrodynamic efficiency [36].

Pigmentation patterns are believed to play a role in fish choosing their shoalmates. To investigate whether zebrafish show a shoaling preference for certain patterns, Engeszer et al. raised wild-type and nacre mutant zebrafish in different environments; with the same phenotype as theirs, with a different phenotype or in isolation. They found that the fish that were raised in isolation did not show any preference for a particular shoal. However, the fish that were raised in groups showed a strong preference for the fish phenotype it was reared with, irrespective of its own phenotype. These experiments suggest that zebrafish show learning and social preference very early in their development, based on the patterns they see while growing up [36].

Engeszer et al. later extended their study by changing the rearing conditions of the fish. Individuals were first reared with wild-type zebrafish and then cross-reared with nacre mutants. They found that the fish did not change their original shoal preference; fish that were raised with wild-type preferred wild-type even if they were reared with nacre mutants later in life. This shows that shoaling preference is attained during a critical time period of the organism’s life and this decision is immutable [37].

Rosenthal et al. performed experiments to see if the stripes act as a cue for the fish to shoal. They used computer animations to see the response to stripes in two striped species; Danio rerio and Danio nigrofasciatus and two unstriped mutants of Danio rerio; leopard and golden. They found that the fish showed stronger preferences for phenotypes that looked like their own. The striped fish went for striped shoal-mates while the lighter ones went with the light ones. The leopard mutant showed intermediate preference for the stripes due to the fact that it has an intermediate phenotype. In this way, selection acts strongly against “odd” phenotypes that are at a severe disadvantage in the wild due to their elevated vulnerability and reduced associations with mates [38].

Engeszer et al. also found that shoaling behavior was sex-specific. They investigated shoaling preference for pigment pattern variation by using 17 different pigment pattern mutants and closely related species. Surprisingly, while the wild and lab-grown zebrafish showed similar preferences, the males and females did not. Males preferred to shoal on the basis of species and stripe patterning while females did not have any such preferences for priori identifiable traits. This difference in preferences between the two sexes sheds light upon alternative motives for shoaling; it may correlate with access to mates or their avoidance in some cases [39].

Pigmentation in zebrafish also serves a purpose in camouflage. They can adapt their body pigmentation in accordance with their local environment, by a dispersion of melanosomes within the melanophores. This turns the zebrafish a colour similar to its environment and protects it from predators. However, Mueller and Neuhauss found a contradicting phenomenon wherein zebrafish larvae 2 days post fertilization turned darker when exposed to light. This made them more conspicuous and the camouflage function was lost. As the larvae grew, they got their camouflage property back but it led to scientists investigating an alternative UV-protection role for pigmentation in zebrafish. Experiments revealed that the melanophores accumulated melanin in the presence of UV light to protect the transparent larvae from its hazardous effects [40].

While the zebrafish adults are not sexually dimorphic, the males possess a brighter yellow coloration while mating. This implies that while stripes play a role in shoal selection, zebrafish rely on hormone-mediated coloration for mate selec-
tion and sexual activity [41]. Thus, the pigmentation patterning in zebrafish is extremely important for their survival in the wild and research in the pigmentation process can reveal new insights on animal behavior and evolution.

**Zebrafish Disease Models for Melanoma**

Melanoma is a type of skin cancer that stems from the malignant transformation of melanocytes. The risks associated with developing a melanoma include extreme exposure to the sun and UV radiation, a genetic predisposition towards melanoma, the number and types of nevi on the skin, and the pigmentation of the skin [42].

In order to diagnose, assess the risks associated, and develop new modes of therapy, it is extremely important to unravel the molecular and genetic processes that are involved in melanoma progression. Zebrafish has proven to be a robust and relatively simple vertebrate model for studying melanoma and its underlying mechanisms and discovering new therapeutic routes.

Apart from the highly useful properties of high fecundity, fast development outside the uterus, and the high transparency of the larvae in zebrafish, the genome of the organism shares 71.4% similarity with the human genome [6].

Over the last few years, information that has been collated for the pigmentation process in zebrafish using the various pattern mutants, has been applied to build several disease models for melanoma in zebrafish. It has helped identify vital genes that control melanoma progression and has provided revolutionary insights on chemoresistance that is prevalent with this disease.

Patton et al. in 2005 were able to create the first animal model for a melanoma caused by a mutant form of human BRAF (V600E) in zebrafish. They found that transgenic fish expressing BRAFV600E under the misfα promoter developed patches of ectopic melanocytes and large nevi. When p53-deficient zebrafish were used, BRAFV600E induced lesions developed into melanomas that resembled human melanomas in zebrafish. It has helped identify vital genes that control melanoma progression and has provided revolutionary insights on chemoresistance that is prevalent with this disease.

Patton et al. in 2005 were able to create the first animal model for a melanoma caused by a mutant form of human BRAF (V600E) in zebrafish. They found that transgenic fish expressing BRAFV600E under the mitfα promoter developed patches of ectopic melanocytes and large nevi. When p53-deficient zebrafish were used, BRAFV600E induced lesions developed into melanomas that resembled human melanomas histopathologically. This model was instrumental in establishing a cooperative interaction between p53 and BRAF pathways for melanoma progression [43].

Elizabeth Patton and her colleagues then found the role of MITF in melanoma development using their BRAFV600E transgenic fish. They used a temperature-sensitive mitfα splicing mutant, to regulate the endogenous MITF levels by simply changing the temperature of the water the fish were being reared in. Their results indicated that low levels of wild-type MITF activity along with BRAF activity is oncogenic and can develop melanomas in vivo. However, abolishing MITF activity led to a rapid regression of the tumors. Thus, while targeting MITF activity may have potential to be an effective anti-tumor therapy, it must be treated with caution since ineffective or partial targeting of the protein could be oncogenic in conjunction with BRAF-mediated melanomas [44].

Powerful melanoma models in zebrafish that can mimic human cancers have been created based on the ability to express numerous human melanoma causing oncogenes exclusively in melanocytes, along with the availability of an established, viable p53 mutant that is homozygous for both alleles [45].

In 2010, Santoriello et al. developed a transgenic zebrafish that could express the oncogene HRAS under the kita promoter which could develop human-like melanomas in 1-3 months without the need for any inactivating mutations in tumor suppressors such as p53. These melanomas were transplantable, had the same histological and molecular phenotypes as human melanomas, and could develop rapidly. Moreover, this p53-independent melanoma model had a visible larval phenotype of hyperpigmentation that allowed for specific and rapid screens for potential drugs or chemicals that could revert it [46].

A milestone in melanoma research using zebrafish was when White et al. described transparent adult zebrafish mutants called casper which were created by crossing nacre mutants with roy mutants. This conferred the optical advantages of the larvae to the opaque adults, providing an in vivo system to study tumor development, metastasis and angiogenesis [47]. To improve upon transplant experiments for tumors in zebrafish, Tang et al. created immune-deficient casper zebrafish using genome-editing that are deficient in T and B cells and could thus allow stable engraftment of fluorescently-labelled tumor cells from other strains and sources [48]. Another extremely useful mutant was created by Antinucci and Hindges, called crystal which was an extension of the casper mutant but with a transparent retinal pigmented epithelium, providing optical examination of the eyes as well as the body of the fish [49].

Heilmann et al. exploited the robustness of the casper mutant and created a high-throughput system for examining metastasis. They transplanted cells derived from stable melanoma cell lines into the casper mutant to quantitatively measure metastasis at a resolution of a single cell. Computer analysis was used to derive metastatic scores for different experimental conditions and measure the amount of affected cells via image analysis [50].

Zebrafish have also been widely used for large-scale chemical screens. Since the embryos are permeable and transparent, they can be merely placed in a 96-well plate and exposed to chemicals added to the water. Since they are highly fecund, they lay up to over 200 eggs per week. In order to test thousands of embryos rapidly, Adatto et al. developed a specialized breeding tank called iSpawn, which could collect over 10000 embryos in 10 minutes and test them against robotically distributed chemicals. Methods such as these are instrumental in discovering new therapeutic molecules as well as speed up the process of toxicological testing and safety regulations, in turn reducing the time required to reach clinical trials in humans [51].
Conclusion & Future Prospects

Pigmentation patterns are intricate and diverse across the animal kingdom. The striped pattern in zebrafish, in particular, has been studied extensively with the help of numerous pigmentation mutants with a visible phenotype of either stripes, spots or labyrinths. While these mutants have paved the way to understand the molecular mechanisms governing the pigmentation process, they have also helped validate classic theories such as the Turing model of reaction-diffusion which was considered obsolete for several years but can now be extrapolated to predict pigmentation patterning in other animals as well. With the advent of newer and more powerful gene-editing techniques, the mysteries of pigmentation in animals can be unraveled further by the engineering of specific mutants. Thus, exploring pigmentation in zebrafish will not only add to the ever-expanding knowledge base for the genetics of color patterning, but also the evolution of patterns in other vertebrates.

While the curiosity-driven exploration of the pigmentation mechanism has shed light on the ecology and evolution of teleost fishes like zebrafish, it has also helped create potent disease models for various skin diseases, including highly invasive melanomas. The inherent properties of zebrafish, like the transparent embryos, short life span and high fertility rate, along with the availability of a vast array of viable mutants, has turned the small minnow into a powerful drug discovery and validation platform. Zebrafish models are gaining traction as a faster and valid model for melanoma and other cancers, as compared to murine models.

Advancement in genome-editing methods has led to sophisticated in vivo modeling experiments and newer transplantation and drug administration methods have turned the zebrafish into a promising resource for pre-clinical research. However, zebrafish do have their fair share of challenges. There is a limited availability for antibodies, tools for conditional expression and a limited source of knowledge about the immunology of fish. It is vital to overcome these shortcomings in the future to turn the zebrafish into an even greater tool for research.

Despite these shortcomings, the zebrafish is indisputably versatile and has tremendous potential to be applied in personalized medicine; one can visualize zebrafish models for patient-specific mutations and rapid in vivo drug testing.

In conclusion, studying the stripes on a zebrafish has led to remarkable discoveries in aggressive cancers like melanoma, and their resourcefulness as a laboratory animal proves that they are not a just a pretty fish.

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