

PIGMENTATION PATTERN FORMATION IN SPOTTED AND STRIPED ZEBRAFISH

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Abstract

We examined the development of striped and spotted pigmentation by comparing and contrasting wildtype zebrafish (striped) with fish carrying the *leopard* mutation (spotted). We hypothesized that WT and *leopard* mutant fish would differ in pigmentation during larval and juvenile stages. Surprisingly, we found that the pigment formed similar complex but ordered patterns in both WT and *leopard* mutant zebrafish early in development. After seven to eight days of fertilization, both types of zebrafish had lines of dark pigment cells called melanocytes on their dorsal, ventral and lateral sides. Some zebrafish had two lines in the lateral side while others had one. Further, both WT and *leopard* mutant fish looked the same after four weeks of development. At week seven, the WT and leopard fish looked different. Both strains had two lines along the lateral sides. However, in the WT fish, the lines of melanocytes were contiguous and defined. In the *leopard* mutant fish, the lines had gaps with no black pigment between cells or groups of cells. Between week seven and adulthood (week eight and beyond), lines in the *leopard* mutants gradually disappeared and were replaced by spots.

INTRODUCTION

Pigmentation patterns serve multiple purposes to protect the animal including camouflage, mimicry, warning coloration, and protection of skin tissues. Pigments can also be used for environmental adaptation, courtship and reproductive behavior between animals (Spence *et al.* 2008). The tropical, freshwater zebrafish, *Danio rerio*, have pigmentation patterning in their skin used for **shoaling behavior**, mate recognition and mate choice (Parichy 2003). The striped and spotted patterns of closely related fish evolved due to natural selection and give fish camouflage in their environment (Kimmel 1995). Comparing the WT fish to *leopard* mutant fish can provide insight into how camouflage evolved in zebrafish.

Zebrafish as a Model Organism

The zebrafish is a popular model organism for studying regenerative abilities, vertebrate development, and pigment formation. A model organism is an organism that is widely studied because it has experimental advantages that can be applied and transferred to other organisms like humans. The zebrafish low cost, small size, and external development make it an excellent model for vertebrate development biology. Zebrafish are **oviparous**. They have transparent embryos, which allow researchers to observe what is happening during development. These zebrafish can be compared to other homologous vertebrates including humans (Spitsbergen *et al.* 2003). Zebrafish are used for studying pigment cell development which can be visualized in live embryos, larva and adult zebrafish (Kondo 2009). Zebrafish are used for the analysis of genetics, cellular behaviors, and ecological and evolutionary mechanisms (Parichy 2003).

Pigment Development

Zebrafish develop in several stages including the embryonic stage, larval stage, juvenile stage, and adult stage. A zebrafish embryo develops rapidly with major organs starting to appear 36 hours after fertilization. The embryonic stage lasts for two days. The larval stage occurs in the 72-hour period after the embryonic stage. The 72-hour period is when the zebrafish develops a protruding-mouth and is approximately 3.5 mm in total body length. After 30 to 44 days the zebrafish enters the juvenile stage and is approximately 10 mm in total body length. Adult fins and pigmentation are visible in the juvenile stage. All of the zebrafishes' pigmentation is clearly visible by day 90, when they reach the adult stage (Kimmel 1995).

Pigmentation starts to develop in the **neural crest cells** during the embryonic stage. The **neural tube** is the embryonic structure that forms the brain and spinal cord. Cells of the neural crest arise from the border between the neural tube and the adjacent **epidermal epithelium** during **neurulation**. When the neural tube closes, the neural crest cells migrate to regions of the embryo where they differentiate into pigment cells. The pigment cells

eventually spread throughout the body. Understanding cell migration can inform our understanding of how pigmentation cells are patterned and evolve through various stages of development (Spitsbergen *et al.* 2003). The neural crest is a temporary migratory population of stem cells. The neural crest results from the **dorsal** neural folds at the border between neural and non-neural **ectoderm**. Prospective neural crest cells are segregated from what is known as the **neuroepithelium**. The neural crest cells then delaminate or split from the neural tube and migrate into the **periphery**. In the periphery, the neural crest cells generate multiple differentiated cell types (Cheung 2003).

Types Of Pigments

Zebrafish pigment patterns are comprised of several different classes of pigment cells, or chromatophores. These pigment cells include black melanophores, yellow xanthophores, red erythrophores, and iridescent iridophores (Parichy 2003). Chromatophores produce pigment before starting migration. The black pigment cells that produce melanophores are the first to appear. The yellow pigment producing xanthophores appear after the melanophores, and the silvery pigment producing iridophores are the last cell to appear (Parichy *et al.* 2000). Erythrophores are red pigment cells that appear with xanthophores. Leucophores are white pigment cells that appear with iridophores (Parichy *et al.* 2003).

Development of stripes occurs in WT zebrafish because yellow producing xanthophores repel black producing melanophores resulting in two segregated cell types. In *leopard* mutants, groups of melanophores attract each other and help each other survive leading to a spotted pattern. Melanophores that are alone die off when the proportion of yellow xanthophores to melanophore is too high (Yamanaka *et al.* 2014).

Here we investigated the development of striped and spotted pigmentation by comparing and contrasting WT fish (striped) with fish carrying the *leopard* mutation (spotted) during different stages of development. We found that the pigment formed complex, ordered patterns in both striped and spotted zebrafish from weeks one through six. Furthermore, as the zebrafish approached adulthood, we found that the lines in the *leopard* mutants gradually disappeared and were replaced by spots. We hypothesized that between week seven and adulthood, the lines in the *leopard* mutants will gradually disappear and will be replaced by spots because it is at a stage where it will be transitioning from juvenile to adulthood.

We found that WT and *leopard* mutant zebrafish are indistinguishable until week six. They formed ordered but complex pigment patterns during the larval to juvenile stage. We noticed patterns of black melanophores arranged into stripes on the dorsal, ventral, and lateral side of their bodies. Differentiation of patterns occurred by week eight. This study is important because it helped us understand the cellular behaviors and evolutionary mechanisms underlying pigment pattern.

METHODS

Fish Husbandry

We used the following genotype fish, *leopard* mutant with long tail and spots (TL x TL) and WT Zebrafish *Danio rerio* with long tail (WT ZDR). Zebrafish were fed twice per day. Morning feedings were done with Paramecia and Green Food (Spirulina Microfine powder and Hatchfry Encapsulon) for young larva and Brine Shrimp for older larva and adults. Night feedings were done with Flake Food for older larva and adults, and Paramecia and Green Food for young larva. Zebrafish tanks were changed whenever the fish could not be easily seen through the front of the tank due to algae build-up. The tanks were checked for signs of growth and livelihood on a weekly basis. Fish were labeled with stock number, date of birth, and number of fish within a given stock. Nets were not used on zebrafish less than 3 months old, as netting young fish can cause serious injury and death. Pipettes were used to capture young fish.

Selection of Fish

Three WT (ZDR) and three mutant (TL x TL) fish were selected once they could be identified individually. WT and mutant zebrafish were placed in separate tanks throughout all development stages. The zebrafish were selected based off their known genotypes so we could predict and observe the gradual change in pigmentation

formation. After two weeks, fish with the genotype TL x ZDR (WT) died off due to low production of offspring. However, TL x ZDR were replaced with three similar zebrafish with similar genotype.

Image acquisition

To observe the progeny, pictures of fish were taken individually. Each fish was carefully selected using a pipette and fishing net. Zebrafish were placed in 0.017% MS-222 solution for a few seconds to anesthetize them. Methycellulose forms a clear viscous solution in water that limits the movement of the zebrafish. Methycellulose (3%) dissolved in aquatic system water was then used to keep the fish in place in a transparent depression microscope slide. Embryo loops were used to clear away unwanted debris. Pictures were taken approximately each week for 14 weeks using a Lecia dfc 400 microscope, a digital camera and the LAV 4.1 computer program. Microscope magnification 2.0 was used for the first four weeks (embryo stage) until the early juvenile stage. When the fish were in the late juvenile to adult stage from week eight to week 14, we used a constant magnification of 0.8 and a stitching program in our software LAV 4.1 to obtain full panorama pictures of the fish pigmentation. A picture of a ruler was taken to approximate the length of the fish. When done, the fish were quickly placed in their new tank, since they are colloquial. We assayed 3 WT and 3 TL (*leopard; long fin*) zebrafish weekly. Spots were counted in *leopard* fish. Observation of spots and stripe density and patterns were recorded. Spots were counted in *leopard* fish. Pigment placement and orientation were closely observed. We noted whether the pigments were in spots or stripes.

RESULTS

Wildtype and Mutant are Indistinguishable from Week 1 to Week 6

To better understand the cellular behaviors and evolutionary mechanisms that underlie pigment pattern, we compared and contrasted the formation of stripes and spots over a 10-week period from the larval to adult stage of WT and *leopard* mutant zebrafish. We predicted that there would be differences between the WT and *leopard* mutant fish during larval and juvenile stages.

Our prediction was not supported because WT and *leopard* mutant fish looked similar during the larval and juvenile stages of development. During seven to eight days post fertilization (week 1), both striped and spotted zebrafish had lines of **melanocytes** along the dorsal, ventral and lateral sides. Some zebrafish had two lines on the lateral side while others had one (Figures 1 and 2). WT and mutant larval stage zebrafish have similar pigment patterns through 6 weeks post fertilization (wpf) (Figure 1). WT and mutant larvae both exhibit several stripes of melanophores and iridophores, as well as widely scattered xanthophores in their bodies from week 1 to week 6. WT and mutant larvae had melanophores in the dorsal, ventral and lateral sides of their bodies. WT and mutant fish have similar “dotted” lines of melanocytes along the length of their bodies from week 1 to week 6. Pigments formed complex but ordered patterns in both WT and mutant zebrafish. Striped and spotted fish looked the same up to 6 weeks post-fertilization (Figure 1).

WT and Mutant pigment patterns are Distinguishable in Adulthood

After week 6, WT fish formed melanophore stripes along the body. Melanophore numbers increased sharply over time in both WT and mutant fish. Dark and light stripes in WT become evident after week 6. However, in *leopard* fish, melanocytes are dispersed all over the body. At week 8, the WT and *leopard* fish started to look different. Both strains had two lines along the lateral sides. However, in the WT fish, the lines of melanocytes were contiguous and defined. In the mutant fish, the lines had gaps with no black pigment between cells or groups of cells. We noticed that the lines in the *leopard* mutants gradually disappear and were replaced by spots at week 8 (Figure 2). In *leopard* fish, yellow cells (xanthophores), are more apparent in the fins and not in the body. In WT fish, stripes are added dorsally and ventrally as the fish continue to grow.

Pigment Differences in Adult WT and Mutant Zebrafish

The pattern of pigmentation is established by week ten in both WT and *leopard* fish (Figure 3). WT fish displayed a striped pattern. Mutant *leopard* fish displayed a dispersed spot pattern. We analyzed the pigment for

five additional weeks and found that WT and *leopard* fish looked different from week 10 to week 16. The *leopard* fish have fixed spotted patterns that gradually get darker as the fish age, while WT zebrafish have fixed stripe patterns that gradually get darker as the fish age. The pigments became more apparent after eight weeks and between weeks 10 to 16. *Leopard* pigments are more visible at week 16. As the fish age, the pigment in their bodies gradually darken. In future studies, we will further investigate why the pigments are getting darker as the fish age.

DISCUSSION

Pigment cell interaction is required for development of pigmentation in zebrafish and zebrafish are indistinguishable during the larval to juvenile stage

In this study, we tested the hypothesis that there would be differences between the WT and *leopard* fish during larval and juvenile stages. Our hypothesis was not supported. We analyzed both WT and *leopard* pigment as they gradually grew from larval to adult. We found that both WT and mutant zebrafish looked the same during the larval to juvenile stage. Striped and spotted zebrafish formed pigment with ordered but complex patterns during the larval to juvenile stage. Striped and spotted fish looked the same up to six weeks after fertilization. Larval melanophores are present along the dorsal, ventral and lateral side of the bodies of both the WT and *leopard* fish. During seven to eight days post-fertilization, both striped and spotted zebrafish had lines of dark pigment cells going from anterior to posterior region of their body. WT and *leopard* fish had melanocytes, on the dorsal, ventral and lateral sides. Some zebrafish had two lines in the lateral side while others had one. A magnified view of pigment formation in the bodies of the WT and mutant zebrafish showed us that both fish looked the same from week one to week two. We noticed that from week 1 to week 6, the larval and juvenile pigmentation pattern is composed of yellow iridophores and black melanophores arranged into dorsal, ventral, and lateral stripes on the sides of their bodies (Figure 1). We noticed that the pattern of pigmentation is indistinguishable up to six weeks after fertilization. A magnified view of pigment formation in the bodies of WT and *leopard* zebrafish in the larval to juvenile stage showed that WT and mutant fish have similar “dotted” lines of melanocytes along the length of their bodies (Figure 2). There are two different models for how pigmentation patterns develop.

Model 1: Development of stripes occurs in WT zebrafish because yellow producing xanthophores repel black producing melanophores resulting in two segregated cell types. The development of spots in mutant *leopard* zebrafish occur because groups of melanophores attract each other and help each other survive leading to a spotted pattern. Melanophores that are alone die off (Yamanaka 2014). Patterns in larval fish are different than WT because the cell-to-cell interactions are different. Our findings suggest that during the larval to juvenile stage, development of stripes did not occur in WT zebrafish because yellow producing xanthophores are not repelling black producing melanophores. Therefore the acquisition of two segregated cell types have not occurred. Our findings also suggest that in *leopard* zebrafish, during the larval to juvenile stage, the groups of melanophores are not yet attracting each other. The melanophores cannot help each other survive, which then lead to no spotted pattern formation. Through this finding, we were able to understand mechanisms that control pigmentation development. We were able to understand that the stage where melanophores are able to attract or repel each other to form stripes and spots occur at a later stage

Model 2: In 2006, Parichy explained that early larval pigment pattern arises from embryonic melanophores that differentiate directly from neural crest cells and these cells and the pattern they form persists through the early larval period. This supports our finding that the pigment pattern was indistinguishable between WT and *leopard* fish during the early larval to juvenile stage.

WT and leopard fish are distinguishable from week 8 and have fixed patterns that gradually get darker as the fish age.

Our results demonstrated that at week 8, the WT and *leopard* fish started to look different. At week 8, two lines along the lateral side of the fish were seen in both the WT and *leopard* fish strains. While both fish had two

lines, there were noticeable distinctions between them. WT fish had lines of melanocytes that were defined and adjoining. Mutant fish had gaped pigment lines with no black pigment between cells or groups of cells. WT and *leopard* fish have developed fixed patterns by week 10. These fixed patterns gradually got darker as the fish age. Our results show that WT and *leopard* fish patterns are established and looked the same from week 10 to week 16. WT fish stripes are defined and get darker from week 10 to week 16. *Leopard* fish spots are dispersed and are more apparent and darker as the fish ages (Figure3). In early larval stage, both WT and *leopard* have melanophores on their dorsal, ventral, and lateral sides of their body. Clusters of xanthophores are also beneath the middle of the stripe. Adult fish have alternating light and dark stripes containing xanthophores and melanophores. In wild-type larval fish, melanophores are present along the horizontal side of the fish while melanophores begin to appear in the middle of the body. This matches well with previous studies (Parichy 2003). This supports our finding that pigment formed complex but ordered patterns in both striped and spotted zebrafish and that lines in the *leopard* mutants gradually disappear and are replaced by spots as the fish age. Furthermore, our results suggest that during the adult stage, development of stripes occurred in WT zebrafish because yellow producing xanthophores are repelling black producing melanophores, therefore resulting in two segregated cell types. In adulthood, *leopard* zebrafish have reached the stage where groups of melanophores are attracting each other. This attraction forms spotted pattern formation. Previous experiments revealed that individual cells within the adult pigment pattern revealed a different mode of stripe development in WT (Yamanaka 2014). Early larval melanophores persist into the adult and leave their initial positions within the early larval stripes to join the developing adult stripes (Parichy *et al.* 2000; Quigley 2004). Our WT fish and *leopard* mutant fish pigment analysis provides us with useful insight as to how the development of pigment can help us understand the cellular behaviors that underlie pigment pattern. Our analysis of Danio pigment patterns reveals the mechanisms of pigment cell differentiation during the development of larval to adult forms.

Future Experiments

For future experiments, we plan to investigate why the pigments in the zebrafish are gradually getting darker as the fish age. We noticed that once both the WT and mutant zebrafish pigments are fixed in adulthood, their pigments are gradually getting darker as they age. We want to understand the role of melanophores in pigment development in both WT and mutant fish and how iridophores and melanophores influence stripe alignment and spot formation. We would also like to investigate the migration of the pigments as they transition from larval to adult. All of these investigations would be important for the field of developmental biology because it will give readers a better understanding of the process of pigmentation development as well as conceptual understanding of pigment interaction and growth.

Key terms

Shoaling Behavior: A behavior in which groups of fish are swimming closely together and follow a general direction.

Oviparous: An egg-laying organism. Produce eggs that hatch after leaving the body of the female.

Neural crest cell: group of embryonic cells that are pinched off during the formation of the neural tube (the precursor of the spinal cord).

Neural tube: A dorsal tubular structure in the vertebrate embryo that develops into the brain and spinal cord.

Epidermal: The protective outer layer of the skin. In vertebrates, it is made up of many layers of cells and overlies the dermis.

Epithelium: A membranous animal tissue made up of one or more layers of cells closely packed together and serves as a covering of internal and external surfaces of the body, including bodily cavities and vessels

Neurulation: formation in the early embryo of the neural plate, followed by its closure with development of the neural tube.

Dorsal: Dorsal is an anatomical term used to refer to the position of a body part in an organism. In vertebrates, the dorsal part of the animal usually refers to the location of the backbone.

Ectoderm: The outermost layer of cells or tissue of an embryo in early development, or the parts derived from this, which include the epidermis and nerve tissue

Neuroepithelium: The epithelium that is made up of cells specialized to serve as sensory cells for reception of external stimuli.

Periphery: The outermost part or region within a precise boundary; the part away from center.

Melanocytes: Melanocytes are cells located in the epidermis that are responsible for producing melanin, a brown pigment that helps screen against the harmful effects of UV light.

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Author Biography

Matilda Omoru is a senior studying Biology at the University of Minnesota Duluth. She is currently involved in the developmental research lab at UMD as an undergraduate researcher, studying pigmentation formation in stripes and spotted zebrafish. She was born in Nigeria and currently lives in Saint Paul. She someday hopes to earn her masters in public health. Her long term goal and biggest dream is to earn her MD MPH. Her hobbies include traveling, music, and cooking.

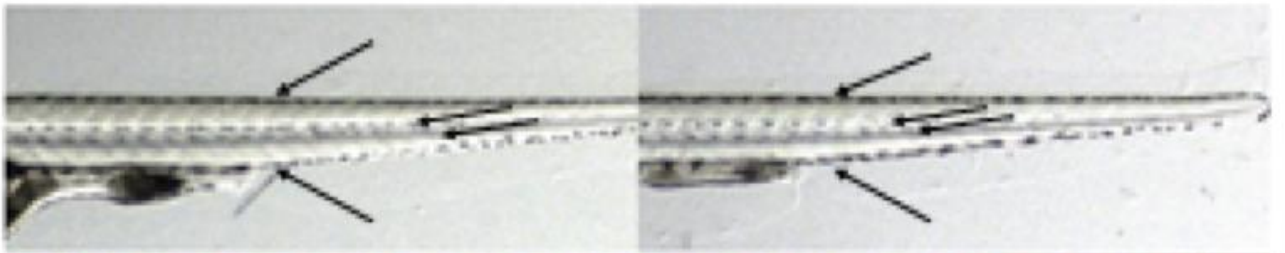
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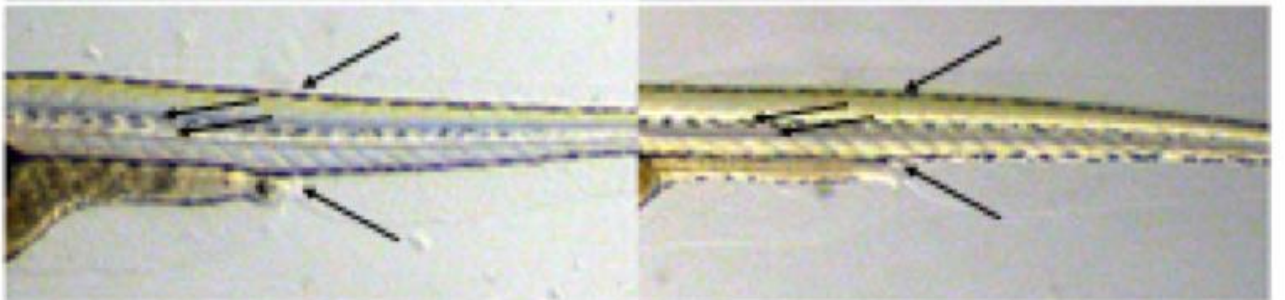
WT

leopard; long fin

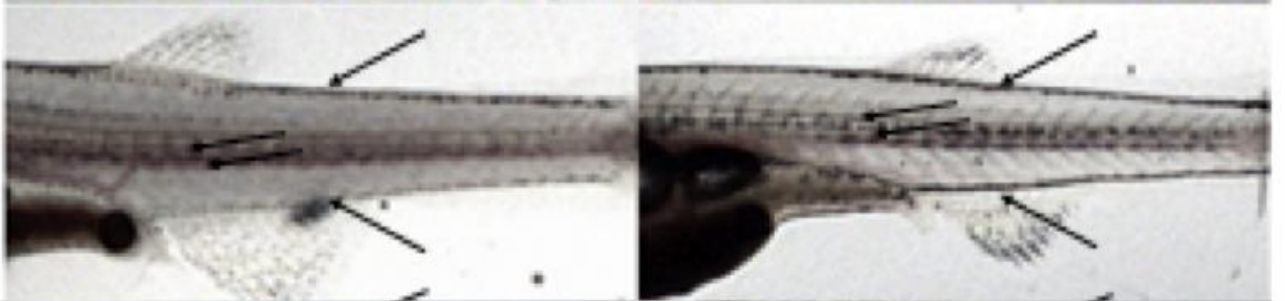
Week 1



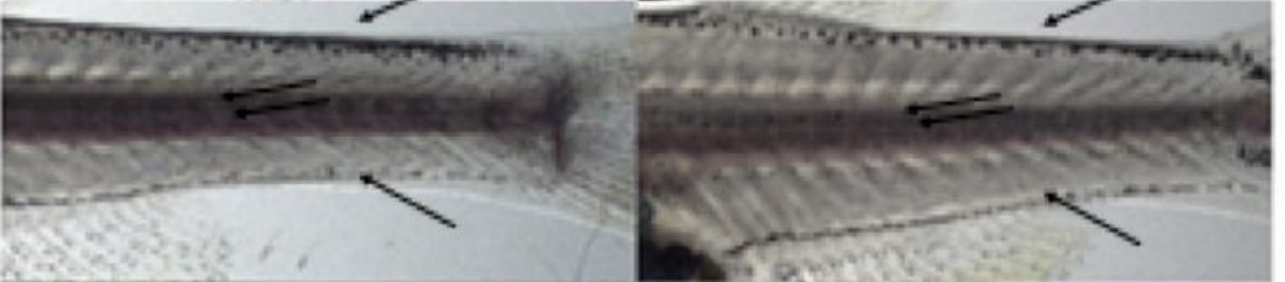
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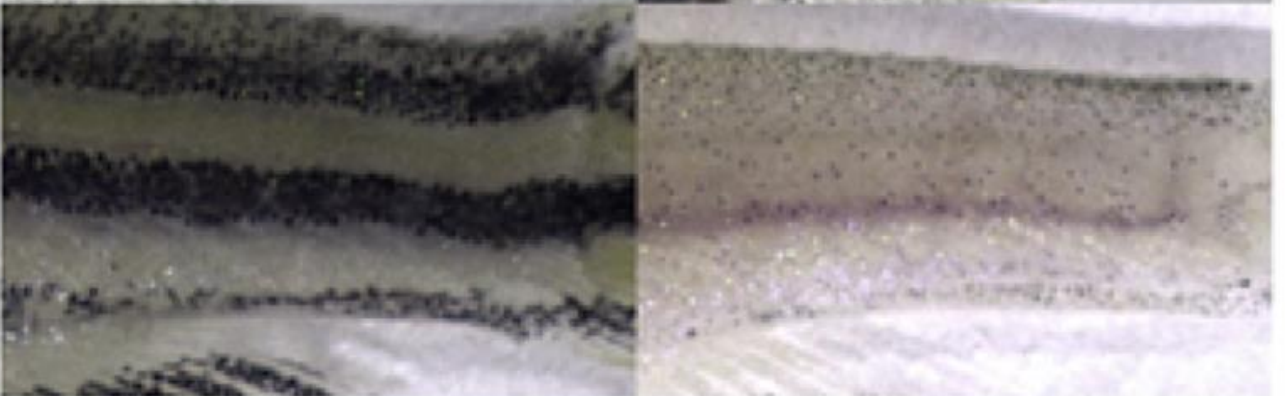
Week 5



Week 6



Week 8



WT

leopard; long fin

Week 1



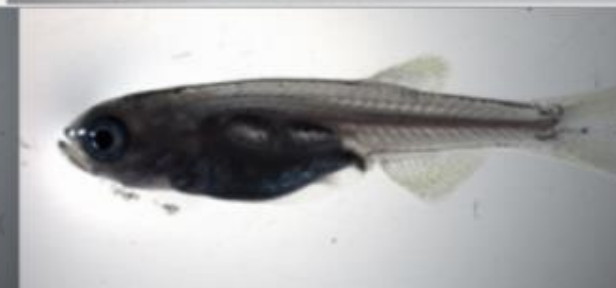
Week 2



Week 5



Week 6



Week 8



Wildtype: ZDR

Mutant: TL x TL

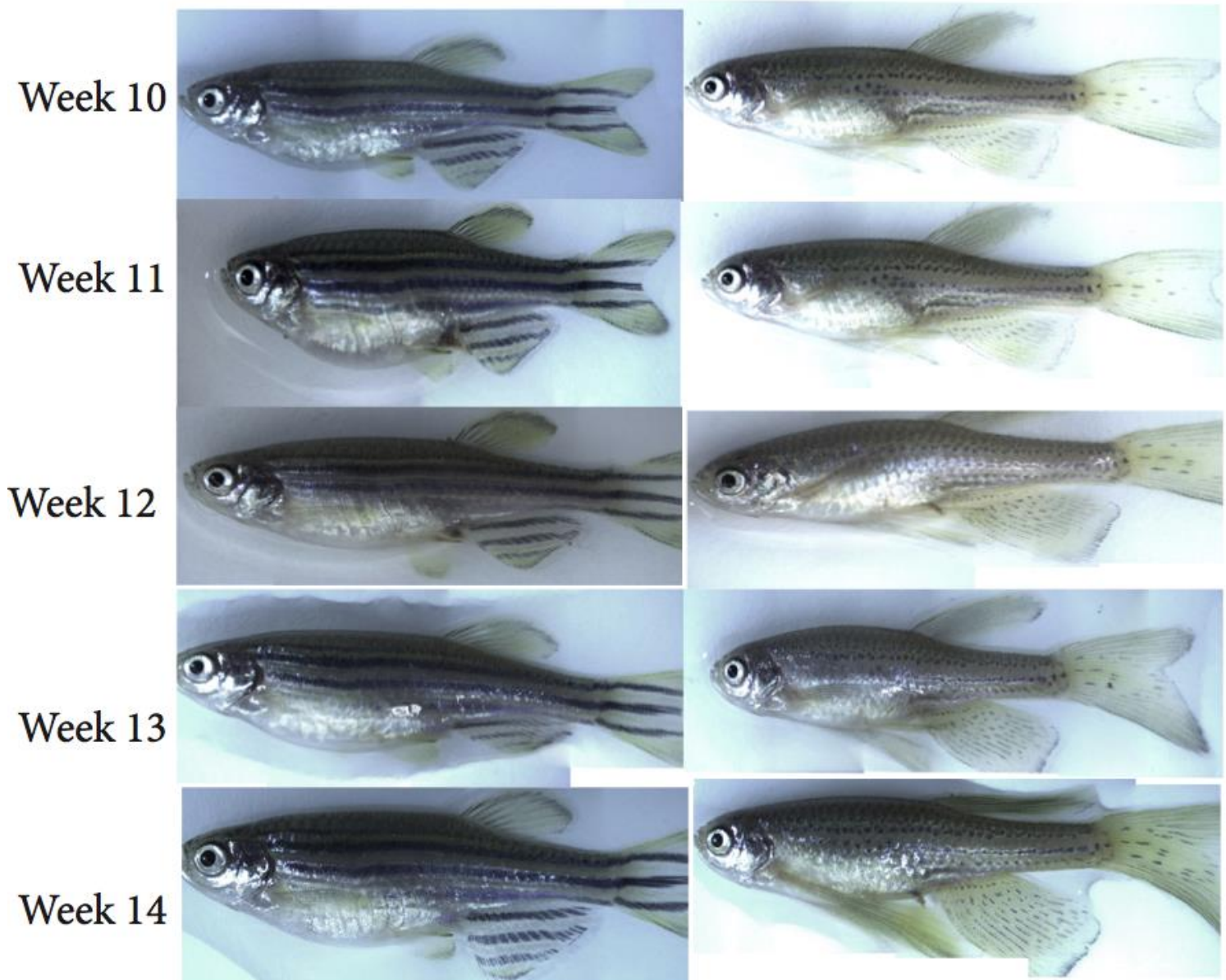


Figure 1: Pigment pattern in WT and *leopard* fish over a period of six weeks. The pattern of pigmentation is indistinguishable up to 6 weeks. From week 1 to week 6 larval and juvenile pigmentation patterns are composed of stripes of yellow iridophores and black melanophores arranged into stripes on the dorsal, ventral, and lateral sides of their bodies. At week 8, stripes have formed in WT and spots are gradually forming in *leopard*.

Figure 2: WT and spotted fish look the same up to six weeks. They start to look different after six weeks. Magnified view of pigment formation in the bodies of WT and *leopard* zebrafish. From week 1 to week 6 of development, WT and mutant fish have similar “dotted” lines of melanocytes along the length of their bodies (arrows). At week 8, melanocytes formed dense stripes in the WT body. In mutant fish, melanocytes are dispersed all over the body of the fish. Dispersed spots in *leopard* will gradually get darker as the fish age.

Figure 3: WT and *leopard* fish have fixed patterns that gradually get darker as the fish age. WT and *leopard* fish patterns are established by week 1 to week 6. WT pigments are established and looked the same from week

10 to week 16. Pigments are established and looked the same from week 10 to week 16. WT fish (left) stripes are defined and get darker from week 10 to week 16. *leopard* fish (right) spots are dispersed and become more apparent and darker as leopard fish ages from week 10 to week 16.