

ABSTRACT

Title of Document: MECHANISM AND EVOLUTIONARY
SIGNIFICANCE OF THE LOSS OF MELANIN
PIGMENTATION IN THE CAVE FISH
ASTYANAX MEXICANUS.

Ernest R. Hixon, Master of Science, 2008.

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The regressive evolution of traits such as eyes and pigmentation is common among cave organisms. As a model to study regressive evolution, I have used the teleost *Astyanax mexicanus*, which consists of eyed and pigmented epigeal forms and many populations of cave-dwelling forms that have lost those traits. This study investigates the mechanism for the loss of melanin production, from the origin of chromatophores from the neural crest to the synthesis of melanin within the melanocyte. I show that cavefish retain a migratory population of neural crest derived cells that are tyrosinase positive and respond to exogenous signals as expected of a melanocyte. I then propose that the regressive evolution of melanin pigmentation is a selectively evolved trait that provides for an excess of dopamine, supported by the near two-fold increase in dopamine in cavefish brains, quantified via HPLC analysis. This study suggests that regressive evolution sometimes occurs via selection.

MECHANISM AND EVOLUTIONARY SIGNIFICANCE OF THE LOSS OF
MELANIN PIGMENTATION IN THE CAVE FISH *ASTYANAX MEXICANUS*.

By

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Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
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Dedication

Dedicated to Nicole, without your support and inspiration this would not have been possible.

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Introduction

Most cave organisms share highly convergent phenotypes including loss or reduction of eyes and pigmentation. While pigmentation patterns are useful in epigeal environments for recognition (Parichy, 2001), they serve no purpose in a lightless cave environment. Body pigment cells originate from the neural crest and migrate throughout the body (DuShane, 1935). In cave populations of *Astyanax mexicanus*, there is a reduction or complete loss of melanin pigmentation. It is interesting that while all populations retain other pigment cell types, melanin containing chromophores are consistently affected. This raises several questions about the evolutionary mechanisms responsible for melanin pigmentation loss in *Astyanax* which require laboratory experiments for their solution. The purpose of this study is to first gain an understanding of the mechanism for melanin pigmentation loss and then consider that mechanism in an evolutionary context.

Astyanax mexicanus as a system to study evolution and development

The teleost *Astyanax mexicanus* is common among the streams of the Sierra de El Abra mountain range. These mountains are heavily cavernous, with many limestone caves having been populated with *Astyanax* within the last ten thousand years (Mitchell et al, 1977). Several populations of *Astyanax* trapped in the caves of this region are believed to have independently adapted to cave life to varying degrees. Some of these adaptations include the degeneration of the eye, changes in cranio-facial structure, changes in jaw shape, a number of behavioral changes, and loss or

reduction of melanin pigmentation. These fish are considered a single species and are interfertile. Additionally they are easily bred and raised in a laboratory setting. Because of these traits, *Astyanax* provides for a unique system for the study of development and evolution.

Evolution of the Neural Crest and Pigment cells

The neural crest is an ectodermally derived group of cells that originates from the dorsal ridge of the neural tube. In most organisms, the neural tube forms by a folding of the neural plate, and the neural crest is formed during this process of neurulation by cell delamination along the “seam.” In teleosts, however, the neural tube forms by hollowing of the neural keel, a solid mass of dorsal midline cells (Hall, 1999). Despite this difference in neurulation, the neural crest is largely unchanged, still forming along the dorsal ridge of the neural tube. There are a number of molecules, including Bone Morphogenic Protein (BMP) (Sela-Donnenfeld and Kacheim, 1999), several Wnt family genes (Dunn et al., 2000), and Fibroblast Growth Factor (FGF), which work to pattern the neural crest (Aybar and Mayor, 2002; Dorsky et al., 2000). It is believed that at this point or shortly after they have delaminated and prepare to migrate, that neural crest cells undergo terminal differentiation, though in some cell derivatives there remains a small amount of plasticity (Hall, 1999).

A short time after trunk neural crest formation, cells begin to delaminate and begin to migrate. This migratory process seems to be largely regulated by ephrin-B

ligands (Perris, 1997; Santiago and Erickson, 2002). Migrating cells of the trunk neural crest move within one of two pathways (Weston, 1963). The first, the medial pathway, proceeds from the dorsal neural tube and then ventrally into the body cavity between the neural tube and the somites. These cells produce a wide variety of cell types such as glia, smooth muscle, and neurons. The second pathway (the dorsolateral pathway) proceeds ventro-laterally from the neural tube. These cells pass between the somites and the epidermis. Cells in this pathway are far less diverse in their fates, and they are thought to exclusively form pigment cells. In fact, it is believed that all body pigment producing cells in vertebrates arise from the neural crest (Weston, 1970; LeDourain, 1982). These cells are believed to migrate to their destination, differentiate, and act as a stem cell population, producing the three pigment cell types (xanthophores, iridophores and melanophores) as the body of the animal increases in size.

*Melanophore development in *Astyanax mexicanus**

A neural crest derived stem cell population is believed to produce pigment forming cells throughout the life of the fish. The cells produced by these stem cells (to which I will refer to as chromatoblasts) have been shown to have some degree of plasticity (Hall, 1999). Though little is known about the genetic patterning mechanisms of iridophores, recent studies have shed some light on the relationship between xanthophores and melanophores. Melanoblast development is inhibited by the absence of kit and mitf gene products. Mitf, a helix-loop-helix transcription factor, has been shown to be crucial in the final stages of melanocyte maturation (Hou

et al., 2000; Tachibana, 2000). It is thought that these factors induce a somewhat plastic chromatoblast to differentiate into melanoblasts. Further development is triggered by dopachrome tautomerase (DCT) and up regulation of the tyrosinase gene by α -MSH (Aroca et al., 1993; Prota, 1992). The chromatoblasts can be induced into forming xanthoblasts by the presence of fms, a type-II receptor tyrosine kinase. Further development into mature xanthophores is mediated by several growth factors (Parichy and Turner, 2003). *Astyanax* cave forms have mature xanthophores and iridophores while exhibiting a reduction or complete loss of melanophores.

Melanin is produced in specialized vesicles within melanophores called melanosomes. These vesicles serve not only to sequester the toxic melanin from the rest of the cell but also to regulate pH as many of the reactions in the melanin metabolic pathway are highly sensitive to pH (Ancans et al, 2001). Once tyrosine is transported into the melanosome, it is catalyzed by tyrosinase into L-dopa and again by tyrosinase into dopaquinone (Prota, 1992). Because tyrosinase is exclusively found in the melanosomes of melanocytes, addition of exogenous L-dopa can be used to label and track melanoblasts in addition to assay for the presence of an active enzyme (Laidlaw, 1932).

Regressive evolution and pigmentation loss

While the study of evolution is typically focused on the selection for new traits, regressive evolutionary events are quite common. It has been proposed that for every single instance of progressive evolution, there exist up to ten instances of regressive evolution (Haldane, 1958). Regressive loss is typically either not thought

of as a major concern or considered to be the reduction of molecular “noise” (Regal, 1977). There are instances, though, of regressive traits that are viewed as compensatory for other advantageous traits. Examples include the loss of lungs in salamanders which ultimately allowed for a specialized projecting tongue (Lombard and Wake, 1986), or potentially the loss of eyes in *Astyanax* allowing for an increase in the number of taste buds (Yamamoto et al, 2004). Generally speaking, regressive evolution is often easily explained as a simple accommodation to energy conservation or accumulation of neutral mutations. While this is sometimes undoubtedly the case, few studies have been done to support this notion. Estimates of energy conservation, effects on fitness, and evolutionary rates are either rarely attempted or poorly understood (Fong et al, 1995).

It has been theorized that in the absence of light the eyes and pigment of *Astyanax* have no effect on fitness and are lost through the neutral accumulations of mutations in genes that affect their development (Wilkens, 1988). However, cavefish populations have all independently evolved a reduction or loss of melanin pigmentation while retaining xanthophores and iridophores (Figure 1). These cells should then be under the same evolutionary pressures that melanophores experience, yet they persist while melanophores do not. That melanophores are singled for regression provides a compelling argument that they could be under a unique set of selective pressures- perhaps the loss of pigmentation in some way confers greater fitness in the cave environment. Furthermore, because most cavefish populations have been isolated for as little as 10,000 generations it is unlikely that neutral mutations would have fixed in this relatively short time span (Barr, 1968).

Theories based on natural selection also exist to explain pigment loss, typically centering on the concept of energy conservation (Barr, 1968). However,

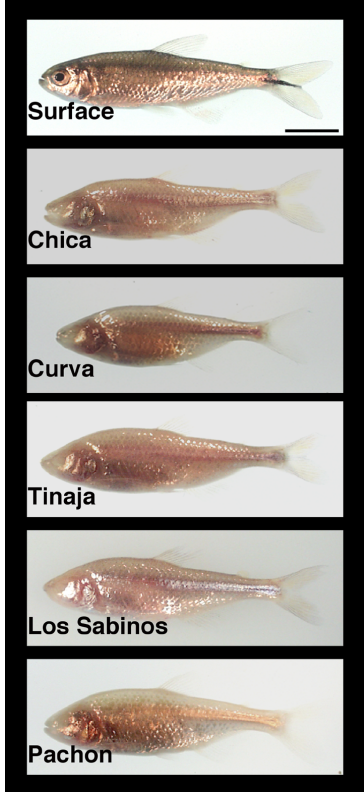


Figure 1. The surface form of *Astyanax mexicanus*, as well as several independent cave populations.

given that cavefish retain a population of cells that migrate and produce some of the tools for melanin synthesis, it is hard to imagine where energy is actually being conserved. I propose a novel hypothesis for the selective advantage of pigmentation loss. Disuse of the melanin metabolic precursor L-tyrosine could potentially produce an excess of the essential amino acid, allowing for other pathways that rely on L-tyrosine to be upregulated. Most notably, L-tyrosine is the metabolic precursor for the synthesis of dopamine, an important neurotransmitter involved in circadian rhythm (Andretic and Hirsh, 2000) and appetite regulation via the reward circuit (Hernandez and Hoebel, 1988). *Astyanax* maintains circadian rhythms,

although they are not as precisely regulated as in epigeal fish (Espinasa and Jeffery, 2006). Observations of cavefish in the laboratory show that they feed more than epigeal fish, a trait that would be advantageous in a nutrient poor cave environment (unpublished observations). It is therefore possible that dopamine is not regulated in the same manner in cavefish as in epigeal fish.

This study investigates the loss of melanin pigmentation, beginning at the development of the neural crest through the migration and maturation of

chromatophores, and ultimately to the synthesis of melanin within the melanosome. Based on the step in melanin production that I have found to be interrupted, a novel theory is proposed for the regressive evolution of melanin pigmentation via a selective mechanism that favors pigmentation loss to supply an increased demand for dopamine. Finally, the results of some experiments are described that test my hypothesis.

Materials and Methods

Biological Materials

Laboratory stocks of *Astyanax mexicanus* were founded by epigeal specimens collected from Balmorhea State Park, Texas, USA and hypogean forms collected from La Cueva de El Pachón in Tamaulipas, Mexico. Stocks were maintained on a 12:12h light:dark cycle at 25°C. Embryos were collected after natural spawning and raised to adults as described previously (Jeffery and Martasian, 1998; Jeffery et al, 2000). Methods were approved by University of Maryland animal care and use committee and followed National Institutes of Health guidelines.

DiI Injections

Embryos in Ringer's-1 solution (0.65% NaCl in double-distilled water; Westerfield 2000) were manually dechorionated with watchmaker forceps. CM-DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate, Cell Tracker, Molecular Probes, Eugene, OR, USA) was first dissolved in ethanol (2µg/µl) and then diluted 0.5µg/µl in 0.3M sucrose. Glass electrodes were backfilled with the solution. At 24hpf, embryos were placed in 3% methyl cellulose (Sigma Chemical Co, St. Louis, MO, USA) in Ringer's-1 solution and pressure injected with a bolus of DiI in the dorsal region of the neural keel at the 10th somite level. Embryos were reared for 24-72h at room temperature in the 3% methyl cellulose solution, fixed in 4% paraformaldehyde, and viewed with fluorescence microscopy.

Tyrosinase Assays

Tyrosinase assays were performed according to Laidlaw, 1932. Samples (embryos, hatched larvae, fin explants, or neural keel explants) were fixed for an hour in 5% formalin in PBS then washed several times with PBS. The PBS was replaced with 0.1% 3,4-dihydrophenylalanine (L-dopa, pH 7.4, Sigma) overnight at room temperature. After washing with PBS, some embryos were embedded in polyester wax and sectioned at 10 μ m. Sections were placed on a slide and viewed under a compound microscope. Some epigeal adults, embryos, hatched larvae and explants were incubated in a tyrosinase inhibitor (1mM 1-phenyl-2-thiourea in aquarium water, Sigma) (Bell, 1955).

Fin Explant Preparation

Adult fish were immobilized by brief immersion in iced aquarium water. Clips from the tail fin were excised by sharp scissors. Tissue samples were immediately immersed in 5% formalin in PBS and prepared for tyrosinase assay.

Neural Keel Explantation and Culturing

Embryos were raised to the 10 somite stage in Ringer's-2 solution and manually dechorionated with watchmaker forceps. Explantation was performed under a dissection microscope using two sharpened tungsten needles- one straight and one bent at a 90° angle. The explantation procedure was adapted from a similar study

(Sadaghani and Vielkind, 1990). First, the cranium and yolk mass were removed. The epidermis was then peeled off the mass of the embryo using the straight needle as an anchor and the bent needle to push. The mass of the embryo was transferred to a clean dish containing Ringer's-2 solution. Embryos were dissociated into parts by brief incubation (90-120 seconds) in 0.25% pancreatin (Sigma), while being gently agitated with a large bore plastic pipet. After incubation, the tissue was transferred to Ringer's-2 to stop enzyme activity and washed 4 times with further agitation. Somites and notochord generally separate from the neural keel with washing and agitation. Extraneous tissue still contaminating the neural keels was removed with tungsten needles.

Explants were cultured in Lab-Tek-4 chamber slides coated in 10 μ g/ml fibronectin (Roche, Indianapolis, IN, USA) in phosphate buffered saline (PBS). Culture media consisted of L-15 (Cellgro, Mediatech, Inc., Herndon, Va, USA) with 20% fetal bovine serum (Sigma), 1x gentamicin (Roche; 50mg/ml), and 1x penicillin-streptomycin (Roche; 1x is 100IU penicillin-0.1mg streptomycin-HCl). Some cultures included 12-O-Tetradecanoylphorbol 13-acetate (TPA; Sigma). The TPA was in DMSO and diluted to a concentration of 80nM in whole media. Other cultures contained 0.2 μ M α -MSH (Sigma) as noted.

Tyrosinase assays were performed on some cultures by first washing culture media with PBS, and then immersing in 5% formalin (pH 7.5) for 30min at RT. After washing several times in PBS for 20min, cultures were exposed to buffered L-dopa (pH 7.4) and incubated at 37°C for approximately 5 hours.

DiI-Tyrosinase Double Labeling

Embryos were injected with DiI as described above, then raised to 60hpf. Embryos were fixed in 5% formalin and assayed for tyrosinase activity as described above. Fixed embryos were viewed using both bright field and fluorescence microscopy. In these preparations, melanin containing cells are black and DiI labeled cells are red.

Detection of Macrophages and Melanoblasts by Neutral Red Staining

To distinguish between macrophages and melanoblasts, the neutral red vital staining method was used (Herbomel et al., 2001). Adult tail fin clips were obtained as described above. The clips were stained with 2.5ug/ml neutral red in the dark for 20hr at RT. Each clip was photographed before being fixed and processed for tyrosinase assay. Although neutral red staining was washed away by the fixation process, comparisons of double labeled cells were obtained by overlaying pre- and post-tyrosinase assayed images in Adobe Photoshop.

HPLC Analysis of Dopamine

High pressure liquid chromatography (HPLC) was used to detect dopamine levels in whole brains. Brain dissections were performed at the laboratory of Ed Kravitz, Department of Neurobiology, Harvard Medical School. The study consisted of a total of 27 fish (14 Pachon, 13 epigeon). Brains were either assayed whole or dissected into 3 parts: optic tecta, hypothalamus, and the remainder.

Fish were anesthetized by exposure to MS-222 (pH 7.0, Sigma) until unresponsive. Small scissors were used to first sever the spinal cord caudal to the skull. The top of the skull was cut open and removed, the brain lifted with a blunt probe, and the nerves connected to the brain were severed. Further dissection to remove the optic tecta and hypothalamus were done using a fine scalpel while brains were submerged in PBS. Samples were distributed evenly (alternating whole brain/dissected brain samples as well as epigeal/cave fish samples) over the course of the day to eliminate variation caused by circadian rhythms.

Tissue was immersed in 100ul ice cold 0.1M perchloric acid and homogenized using a plastic disposable pestle (Kontes Glass Company, Vineland, N.J., USA). The homogenate was incubated on ice for 1hr, then centrifuged at 16,100g for 2 minutes. Amine levels were measured with an ESA CoulArray Model 4600 HPLC with electrochemical detection equipped with a C18 column (ESA; 150 mM, 120Å), a 20- μ l loop (Rheodyne, Rohnert Park, Ca., USA), and a column heater (Eppendorf, Hamburg, Germany) held at 29°C. The flow rate was set at 500 μ l/min. The mobile phase was composed of 15% acetonitrile (Fisher, Pittsburgh, Pa., HPLC grade), 15% methanol (Fisher, HPLC grade), 75 mM NaH₂PO₄, and 1.5 mM SDS, pH 5.6 (Shulz and Robinson, 1999). The electrodes were set at 100, 425, 530, and 650 mV. Dopamine was detected on the 100mV channel. Forty microliters of each diluted homogenate was used for each injection. Retention times and concentrations of the amines were determined by comparison to a standard composed of 25nM dopamine in 0.1 M perchloric acid (Certel et al, 2007).

Results of HPLC were normalized by weight. Undiluted supernatant from homogenate was assayed for protein by using the Quick Start Bradford Protein Assay (Biorad, Hercules, Ca., USA) and a spectrophotometer. A protein standard was made using serial dilutions of a epigeal fish brain homogenate of known weight. Statistical analysis was performed using a two-tailed Student's T test for two samples of unequal variance.

Results

DiI Labeled Neural Crest Cell Migration in Pachón

DiI labeling experiments were performed to determine if cavefish neural crest cells are migratory. Cavefish from the Pachón population were used as they exhibit nearly complete albinism (although rarely pigmentation in the retinal pigmented epithelium is observed), typically exhibiting no body pigment. After injection of a DiI bolus in the region of the dorsal neural keel, migration of labeled cells was observed throughout the body in all directions (rostral, caudal and ventral) (Figure

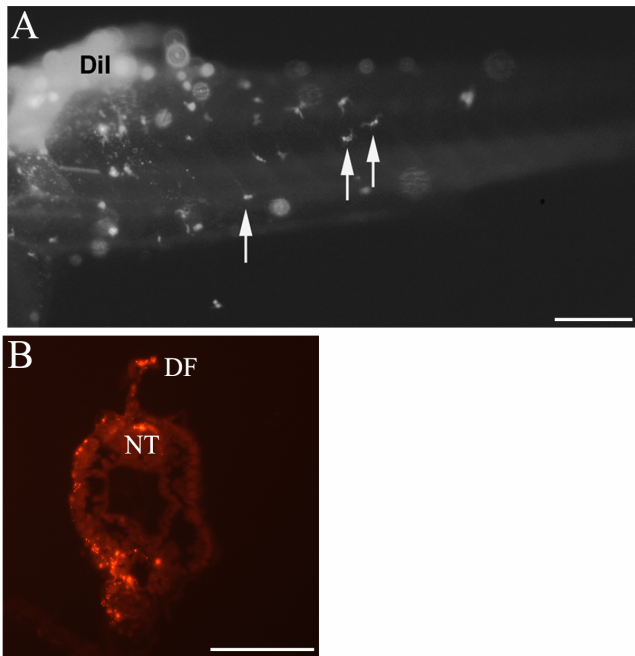


Figure 2. DiI marker used to trace migrating neural crest cells. DiI was injected into the dorsal neural tube at ~10 somites. After one day, whole mount embryos show a number of migrating cells (A). Cross sections show migrating cells to be primarily in the dorso-lateral pathway (B). DF, Dorsal fin. NT, Neural tube.

2A). Labeled cells were also observed in the yolk sack, one of the areas where melanophores are first observed in epigeal embryos. In addition, sections were made of DiI labeled embryos. Neural crest cells migrate through one of two pathways-medial and dorsolateral (Weston, 1963). Cells that migrate through the dorsolateral pathway exclusively become

chromatophores (LeDourarin, 1982; Erickson, 1986; Newgreen and Ericson, 1986). Sections of DiI labeled cells showed that many of the migrating cells were located just under the epidermis, indicating that they were migrating dorsolaterally and likely chromatophore precursors (Figure 2B). These experiments show that there is a migratory population of trunk neural crest cells, and some of these cells migrate through the dorso-lateral pathway, which is normally reserved for cells fated to become chromatophores.

Tyrosinase Positive Cells in Cavefish Embryos

Tyrosinase assays were used to look for the presence of cells capable of producing melanin. Embryos (epigean and Pachón) were assayed at 36hpf, the time at which body pigmentation is typically appearing in the eye, trunk, and yolk sac of epigean fish embryos (Figure 3A-B). Prior incubation of epigean embryos in 1-phenyl-2-thiourea (PTU, a tyrosinase inhibitor) prevented production of melanin (Bell, 1955). Tyrosinase assays after embryos were removed from PTU showed similar patterns of tyrosinase positive labeled cells among both epigean and cave fish embryos. Tyrosinase positive cells were first seen at about 24hpf in Pachón embryos, the same time at which melanoblasts can first be detected via tyrosinase assays in epigean embryos. Sections of assayed embryos show that labeled cells lie just below the epidermis in the trunk and fins (Figure 3D). These are areas where melanin producing cells typically are found in epigean embryos. Tyrosinase assays were also done throughout development. Tyrosinase positive cells were found in 24, 36, 60hpf

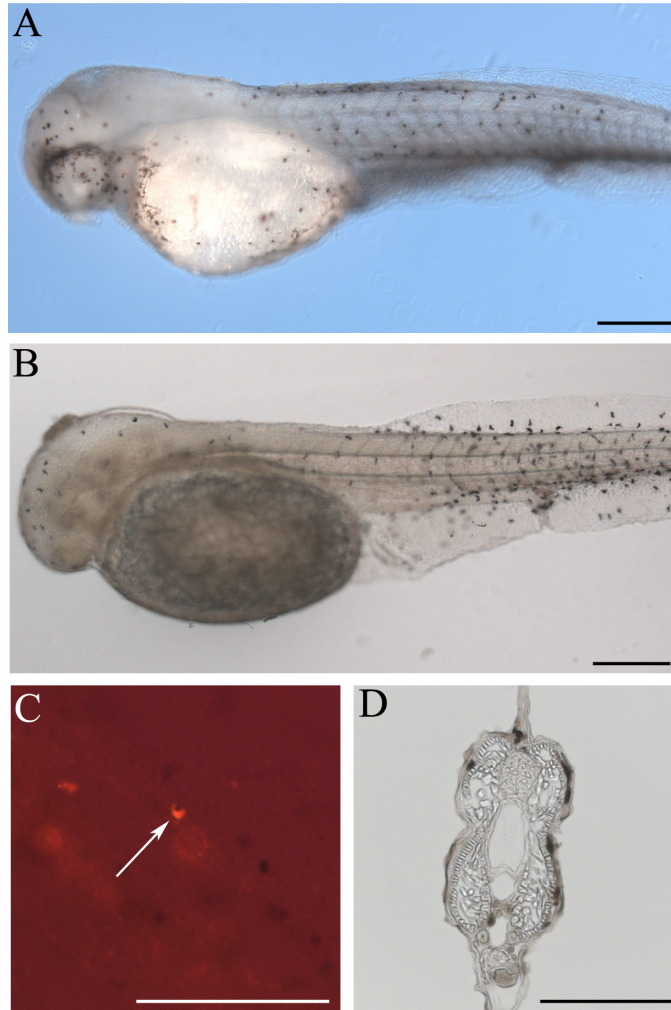


Figure 3. Tyrosinase assays in *Astyanax* embryos. Staining patterns of epigeal embryos raised in PTU (A) are similar to 30 hour cavefish embryos (B). Double staining of DiI and tyrosinase assays show some cells to be double labeled (arrow), showing these cells to be of neural crest origin (C). Tyrosinase positive cells are located between the somite and epidermis in cavefish, typical of melanoblasts or melanocytes (D). Bar, 100 μ m.

embryos and adult cave fish. Double labeling experiments using both DiI and the tyrosinase assay were performed to determine if neural crest derived migratory cells were fated to become pigment cells (Figure 3C). Some cells that migrated away from the injection site were found to be both tyrosinase and DiI positive, showing that Pachón possess a tyrosinase positive, migratory population of neural crest derived cells.

It has been reported that the tyrosinase assay may be non-specific in that macrophages, as well as pigment cells, are labeled (Laidlaw, 1932; Tucker and Erickson, 1986). In order to investigate that the tyrosinase assay was specific to melanoblasts, a double labeling experiment was performed (Figure 4). Fin clips from adult Pachón fish were first stained with Neutral Red, a macrophage specific marker, photographed, and then assayed for tyrosinase. Although the processing for the

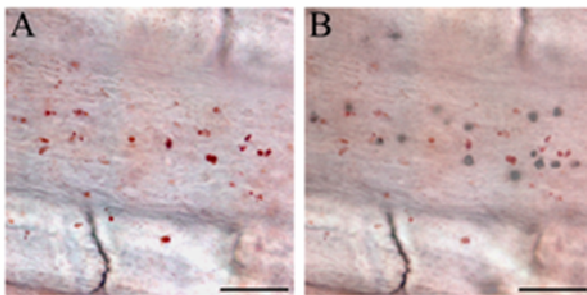


Figure 4. Neutral red staining of adult cavefish fin clips (A). Neutral red aggregates in macrophages, which have been reported to be tyrosinase positive in other vertebrates (Tucker and Erickson, 1986). In double labeling experiments with tyrosinase, no neutral red cells were found to be tyrosinase positive (B).

tyrosinase assay washed away the neutral red staining, photomicrographs of the same region of fin clip after each assay were overlaid to determine if any cells were labeled by both techniques. Analysis of the overlaid images showed no cells labeled by both techniques,

indicating that in *Astyanax mexicanus*, the tyrosinase assay is specific for melanoblasts.

These experiments show that although cave fish embryos do not have mature pigment producing melanophores, tyrosinase positive melanoblasts- cells that are able to produce melanin if provided L-DOPA as a substrate- still develop in a normal pattern similar to the epigeal embryos. These tyrosinase positive cells make up a portion of the migratory cell population that originates at the neural crest. Furthermore, tyrosinase positive melanoblasts are present into adulthood. Finally, double labeling experiments showed that the tyrosinase assay is specific to melanoblasts.

Tyrosinase Positive Cells are Neural Keel Derived Melanoblasts

Several approaches were used to confirm that the tyrosinase positive cells were of neural crest origin. First, double labeling with DiI and tyrosinase showed that cells migrating from the dorsal neural keel were tyrosinase positive. Second, I confirmed the ability for cells to migrate from explanted neural keel in culture as has been previously shown in other teleosts (Sadaghani and Vielkind, 1990) (Figure 5). In basic media epigeal and Pachón neural keel cultures did not develop melanoblasts, although a number of cells migrated away from the neural keel (Figure 5 A, D). Two different culture additives were used. Cultures were also given either α MSH, a hormone that induces melanin synthesis and a stellate morphology in melanoblasts (Aroca et al, 1993), or phorbol ester (TPA), which upregulates the MitF pathway (Prince et al, 2003). Mature melanocytes were visible in epigeal explant cultures containing both α MSH and TPA, though no melanin synthesis was induced in the Pachón explant cultures (Figure 5 B-C, E-F). Tyrosinase assays of Pachón explants

in normal and α MSH containing cultures showed that Pachón melanoblasts with α MSH were dendritic as opposed to punctuate, as those seen in cultures without α MSH (Figure 5 G-H). This result indicates that tyrosinase positive cells that migrate from the neural keel also respond to α MSH, exhibiting multiple melanoblast characteristics.

HPLC Quantification shows Pachón brains contain more dopamine than epigean fish brains

In addition to being a metabolic precursor to melanin, L-tyrosine is also the precursor to dopamine, epinephrine, and norepinephrine. In order to test whether an interruption of the melanin synthesis pathway had created a surplus of dopamine elsewhere, namely in the brain, HPLC was used to quantify the amount of dopamine present in the brains of both epigean fish and Pachón cave populations. Analysis of dopamine levels in whole brain homogenate showed nearly a two-fold increase in the amount of dopamine per tissue weight found in Pachón over epigean fish (40.24nM/g of tissue and 22.90nM/g of tissue respectively, $p < 0.05$) (Figure 6). Further analysis was done on individual portions of the brain to determine if there was a localized change in dopamine levels in two areas of the brain previously reported to have undergone morphological changes- specifically the optic tectum and the hypothalamus (Menuet et al., 2007) (Figure 7). Results from both specific regions showed higher levels of dopamine in Pachón brains, although probably due to the difficulty of the dissection, the variation was too high to achieve a suitable level of statistical significance. The remaining portion of the brains following dissection was

also assayed. Again, Pachón brains contained significantly more dopamine than seen in epigeal forms (19.151nM/g tissue and 12.44nM/g tissue respectively, $p < 0.05$). While a specific brain region was not identified as being responsible for the increased concentration of dopamine in Pachón brains, it was shown that there is a significant concentration disparity in an important neurotransmitter.

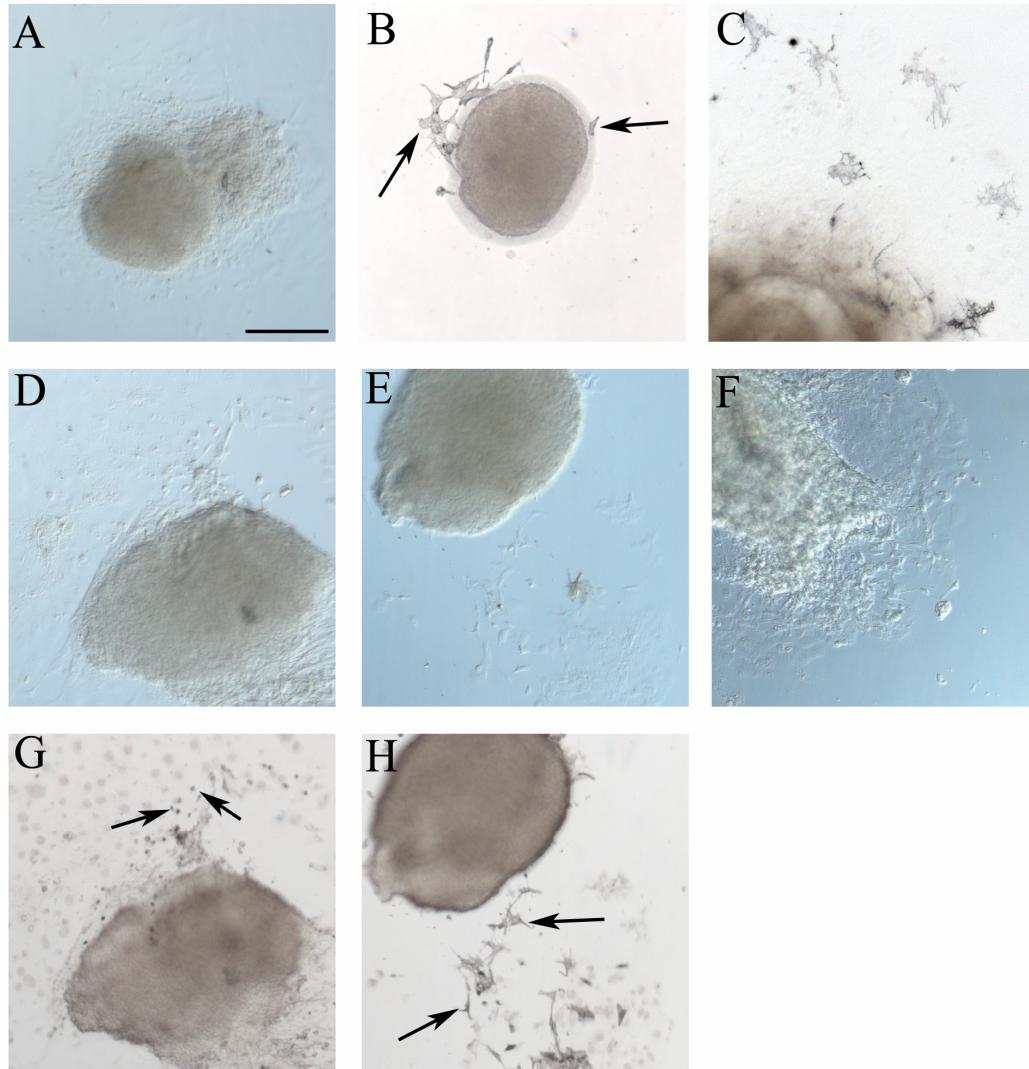


Figure 5. Cultured explants of *Astyanax* neural tubes. Surface fish cultures with normal media show no melanin (A) although addition of α MSH (B) or TPA (C) was sufficient to induce melanogenesis. Cavefish cultures in normal media (D), or with α MSH (E) or TPA (F) did not produced melanin. However, tyrosinase assays on cavefish normal media cultures and α MSH cultures showed tyrosinase positive cells with different morphologies, indicating cavefish melanoblasts respond to α MSH. Bar, 100 μ m.

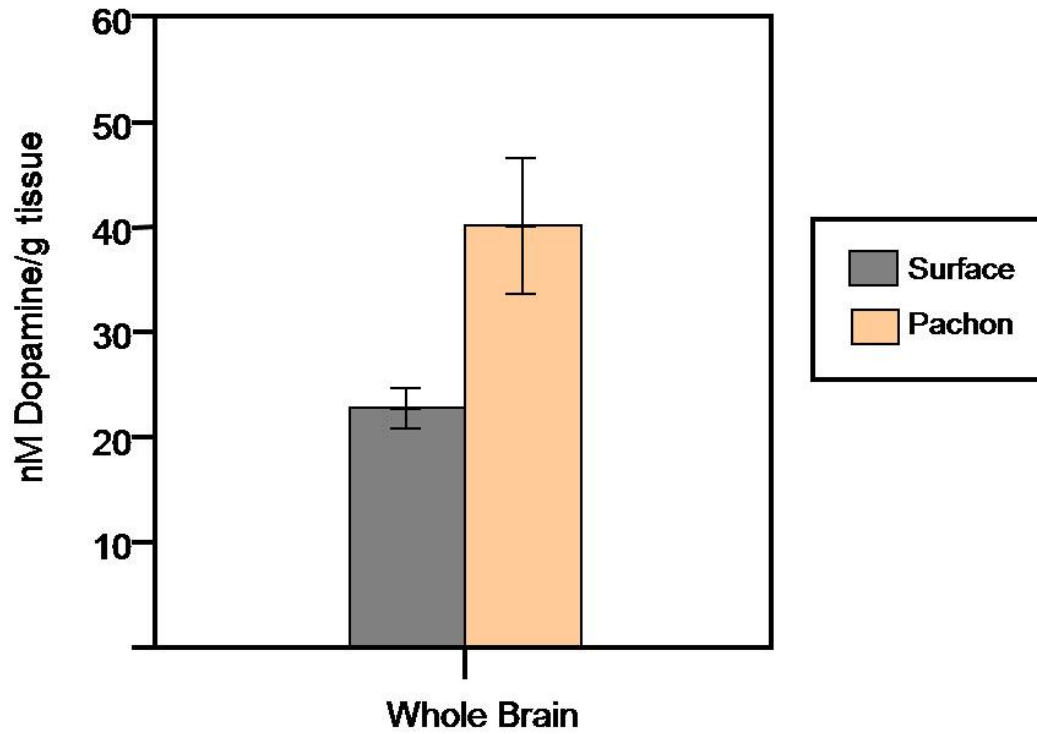


Figure 6. Histogram depicting the average amount of dopamine (nM) per gram of tissue in undissected brains of both surface dwelling *Astyanax* (n=5) and Pachon (n=4) populations as determined by HPLC. Pachon tissue contains almost twice as much dopamine per gram of tissue as surface fish populations. $p < 0.05$ (Student's t-test). Bars denote standard error.

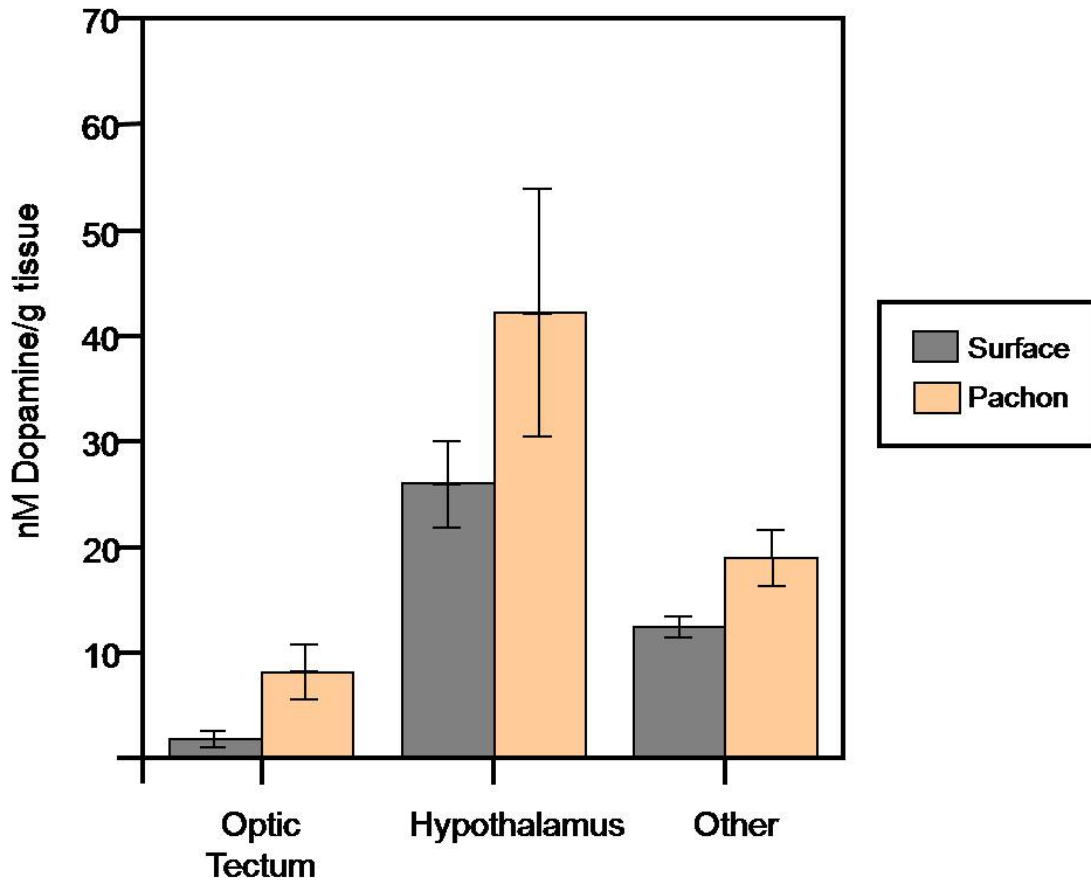


Figure R6. Histogram depicting the amount of dopamine in the optic tectum, hypothalamus, and remaining brain regions after dissection of both surface fish and Pachon. High variation in both optic tectum and hypothalamus groups prevent a statistically significant conclusion from being drawn. However, the remaining post-dissection portion of the brains showed a significantly higher concentration of dopamine in Pachon as compared to surface fish as determined by Student's t-test. Bars denote standard error. n= (Surface, Pachon). Optic tectum: n=(8, 7); $p < 0.05$. Hypothalamus: n=(6, 4); $p > 0.05$. Other: n=(8, 7); $p < 0.05$.

Discussion

Implications of a Functional Neural Crest in Cavefish

This study had two goals. First, to identify the step at which the maturation of melanin producing melanophores was interrupted, I used a number of techniques to compare the development of melanophores in cave and epigeal embryos of *Astyanax mexicanus*. Second, I proposed and tested a model for an evolutionary mechanism by which melanin pigmentation is lost due to selective forces. I determined that melanin synthesis is interrupted in the late stages of melanocyte development. Further, I provided evidence for a possible selective advantage to pigment loss, although it must be investigated more thoroughly.

In order to determine the point at which melanin pigmentation developed, I performed experiments to test each step of melanocyte development, from the origin of the neural crest, to migration, differentiation and ultimately maturation into a pigment producing melanocyte. Several experiments provide strong evidence that cavefish maintain a population of neural crest derived chromatophores that differentiate into chromatophores. DiI injections into the dorsal neural keel showed that cells migrate through both the medial and the dorsolateral pathway. Previous studies have shown that cells migrating through the dorsolateral pathway develop solely into chromatophores (LeDourarin, 1982; Erickson, 1986; Newgreen and Ericson, 1986). Cross-sections of DiI injected embryos also showed labeled cells migrating through the dorsolateral pathway, between the epidermis and lateral surface of the somites, suggesting these cells are fated to become chromatophores.

Histochemical assays using L-dopa as a substrate confirmed that the tyrosinase enzyme was functional. The assay also provided an additional label for unpigmented, migratory melanoblasts. Both epigeal and cave embryonic melanoblasts begin to produce tyrosinase early in their migration, soon after departing the neural crest (approximately 25-30hpf). Early observation of tyrosinase in unpigmented melanoblasts is not uncommon, having already been reported in several other organisms such as zebrafish (Camp and Lardelli, 2001) and ascidians (Whittaker, 1973). Tyrosinase assays confirmed that a subpopulation of chromatophores was beginning to differentiate into melanoblasts. Additionally, double labeling experiments using DiI along with tyrosinase assays demonstrated that the double labeled cells originated at the neural crest and were migrating. Finally, all tyrosinase positive cells were shown to be present just below the epidermis in the yolk, in the dorsal fin, or between the epidermis and somite in the trunk of the embryos- the expected locations for melanoblasts. Together, these experiments show conclusively that cavefish embryos maintain a population of neural crest derived melanoblasts which migrate dorsolaterally, and have differentiated to the degree that they have begun to produce an active tyrosinase enzyme. While this study focused on the Pachón population, tyrosinase positive cells have been observed in adult fin clips of four other populations (Chica, Los Sabinos, Tinaja, and Curva) in numbers substantially greater than those seen in epigeal fish (McCauley et al, 2004), indicating that this loss of mature melanocytes has evolved several times in separate populations.

Cell culture assays provided further evidence as to the origin and maturation of cavefish melanoblasts. First, cultures of isolated cavefish neural keels contained tyrosinase positive cells that had migrated away from the neural keel. This supports the conclusion that Pachón embryos maintain a migratory cell population fated to become melanoblasts. Culture additives provided insight into the degree of differentiation of these cells. While neither α MSH nor TPA proved to be sufficient to induce melanin synthesis in Pachón cultures, the cells did respond to α MSH by adopting a stellate morphology (Schwahn et al, 2001). These cells have therefore taken on a number of melanoblast characteristics- they arise from the neural crest, migrate through the dorsolateral pathway, produce tyrosinase, and change morphology in response to MSH as expected.

I conclude that while Pachón embryos and adults do not develop melanized body pigmentation, they do retain a population of melanoblasts, and therefore the interruption in melanin synthesis in melanogenesis must be at a late step. Tyrosinase assays exploit the presence of an endogenous, active enzyme proving that the enzyme function is unaffected. Tyrosinase catalyzes three concurrent reactions in the melanin synthesis pathway: the conversion of tyrosine to L-dopa, L-dopa to dopaquinone, and after several spontaneous reactions, 5,6-dihydroxyindole to indole-5,6 quinone (Korner and Pawelek, 1982). The assays demonstrate that all reactions beginning with the conversion of L-dopa to dopaquinone and all subsequent steps are intact and unaffected. Furthermore, it is unlikely that steps prior to the tyrosinase catalyzed reactions are affected, as an inability to convert L-phenylalanine into tyrosine would be lethal. Finally, a study showed tyrosinase assays on adult fin clips with tyrosine as

a substrate did not yield melanin in both Pachón and albino catfish (McCauley et al, 2004). It is therefore apparent that the interruption of the melanin synthesis pathway must lie in the conversion of tyrosine to L-dopa. In the cell cytoplasm, phenylalanine is converted to tyrosine. Tyrosine is then transported into melanosomes- membrane bound organelles that contain tyrosinase- where the rest of the melanin synthesis pathway occurs. It is likely that tyrosine and/or tyrosinase are sequestered in some manner, preventing the cavefish melanosomes from producing pigment.

The Role of OCA2 in Cavefish Hypopigmentation

Shortly after this research was completed, a study using a Quantitative Trait Loci (QTL) analysis revealed a strong correlation between pigmentation loss and mutations in a gene known to be responsible for tyrosinase-positive albinism (Protas et al, 2006). Oculocutaneous albinism, caused by mutations in the *Oca2* gene (referred to as the *p* gene in mice), is the most common form of albinism in humans. The *Oca2* gene codes for a large transmembrane protein that intergrates into the melanosomal membrane. The exact function of OCA2 is not well understood. Early studies suggested that the protein played a role in the transport of tyrosine across the melanosome membrane after observing that high concentrations of tyrosine could rescue pigmentation in *p*-deficient mouse retina cultures (Sidman and Pearlstein, 1965). However, recent experiments have shown the kinetics of tyrosine transport in *p*-gene mice melanocytes to be normal (Gahl et al, 1995; Potterf et al, 1998). Currently, it is believed that the OCA2 protein is involved in anion transport,

maintaining proper pH inside the melanosome (Brilliant et al, 1994; Brilliant, 2001) (Figure 8).

Sequencing analyses of Pachón and Molino, another population of cave fish, showed that Pachón OCA2 contained a unique deletion in exon 24 and that Molino OCA2 had a similar large deletion, though in exon 21 (Protas et al, 2006). Complementation experiments using Pachón and a third cavefish population, Japonés, failed to rescue pigmentation, suggesting that this population also has a mutation in OCA2 (Protas et al, 2006). Not only does this show the mutability of the OCA2 gene, but it also provides some evidence that the populations have evolved independently, a point that has been debated (Avisé and Selander, 1972; Mitchell et al, 1977; Wilkens, 1988; Espinasa and Borowsky, 2001; Strecker et al, 2003).

Melanocytes are typically divided into two major categories: cutaneous and extracutaneous (Prota, 1992). To this point, this study focused on cutaneous melanocytes that make up the body pigmentation seen on epigeal forms. Extracutaneous pigmentation does not arise from the neural crest. One example of extracutaneous pigmentation is the retinal pigmented epithelium, which is derived from the wall of the optic cup (Mann, 1964). While OCA2 is typically considered to be albinism of eye and cutaneous pigment, some populations produce individuals that have no or drastically reduced body pigmentation but a pigmented RPE. In fact, although Pachón typically has no melanin pigmentation at all, some individuals have been observed to have pigmented RPE (unpublished observation). While these individuals are extremely rare, it does bring to light some conflict within the OCA2 model of melanin loss in *Astyanax*. The differentiation of cutaneous melanocytes

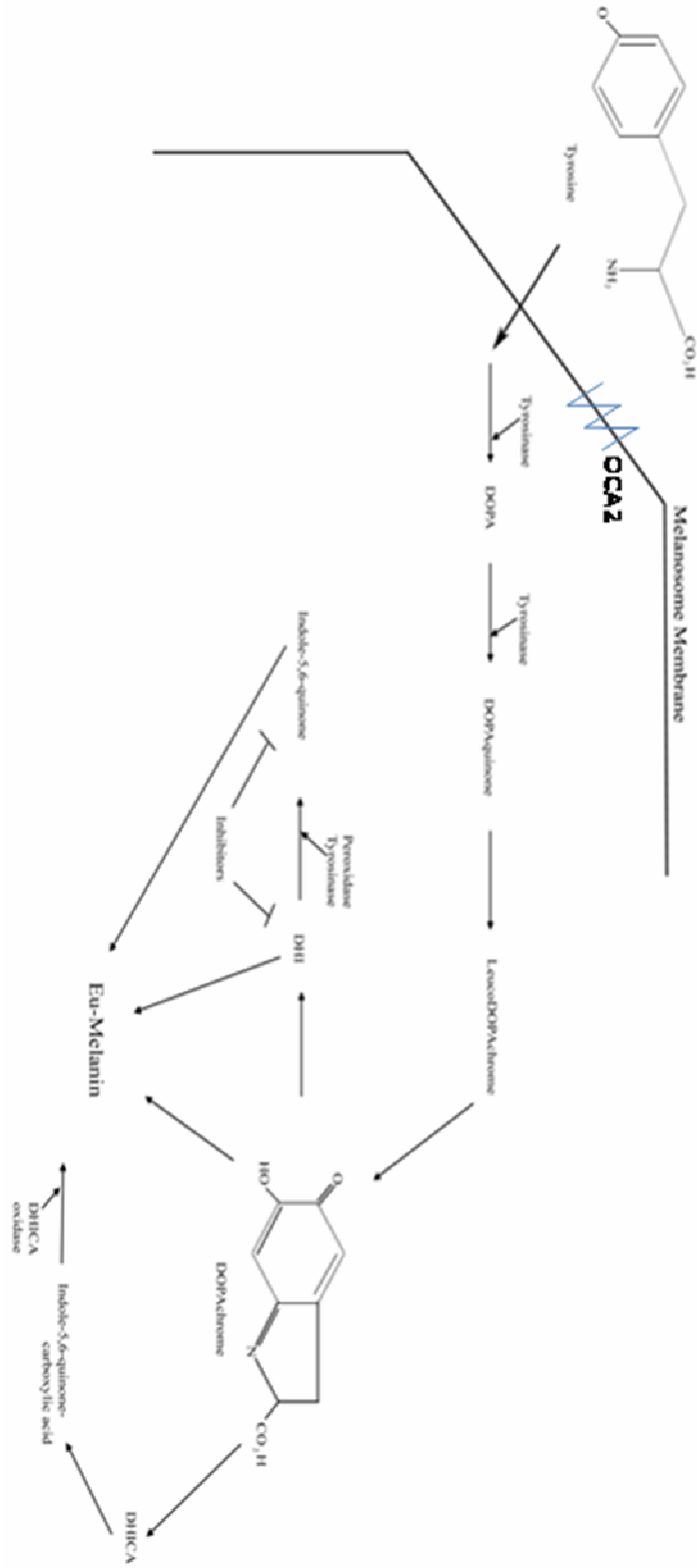


Figure 8. Melanin synthesis pathway. L-tyrosine crosses the melanosome membrane and is catalyzed by tyrosinase to form dopaquinone. The remaining steps can follow several pathways to create melanin, many of which are spontaneous. Note the presence of OCA2 on the melanosome membrane.

and RPE melanocytes is governed by slightly different mechanisms. RPE melanocytes are predominantly regulated by MitF, Otx2, and Pax6 (Martinez-Morales et al, 2004). It is worth noting that embryonic expression of Pax6 in cavefish is reduced, which may lead to smaller optic primordia (Strickler et al, 2001). However, expression in the retinal ganglion and amacrine cells later in development is unchanged (Strickler et al, 2001). In addition, there is evidence that growth factors released by the underlying endoderm have a role in RPE cell development (Nguyen and Arnheiter, 2000). It is therefore reasonable to conclude that there may be some ability for extracellular factors within the surrounding environment that are sufficient to override the mutations to OCA2, resulting in melanogenesis. This supports the proposed presence of two genes that regulate melanin pigmentation loss in cavefish (Sadogu and McKee, 1969, Wilkens, 1988).

*Regressive Evolution of Melanin Pigmentation in *Astyanax mexicanus* cavefish*

There is, historically, much debate as to the nature the regressive evolution of cave organism pigmentation. Pigment loss is seen in many cave organisms from arthropods and millipedes to salamanders and fish. Early theories about cave organism (specifically the African cavefish *Caecobarbus geertsii*) pigment loss centered on metabolic concerns, in which lower temperature or lower metabolic rates were responsible for retarded development of non-essential systems (Heuts, 1951). Today, many investigators subscribe to the neutral theory hypothesis- that mutations simply pile up in genes that are under no selective pressure- to explain pigmentation loss (Wilkens, 1988).

However, some flaws exist in the neutral theory explanation of *Astyanax* melanin loss. While all *Astyanax* populations show some degree of melanin loss, the other two chromatophores (xanthophores and iridophores) are retained. Secondly, neutral theory predicts a longer span of time for mutations to fix within a population than those mutations that are selected for (Kimura, 1983). Most cave populations of *Astyanax* are predicted to be as little as 10,000 generations old, an age that does not provide much opportunity for neutral mutations to fix within the population, though some argue that the small populations allow for a more rapid fixation of mutations (Barr, 1968; Mitchell et al, 1977; Avise and Selander, 1974; Kimura, 1983).

Due to the inability of the neutral theory to fully explain melanin pigment loss in *Astyanax*, I propose a model for a novel selective advantage to pigment loss. A mutation in OCA2 would result in quick saturation of L-tyrosine in melanosomes. I propose that it is possible that L-tyrosine would therefore be more available elsewhere in the body, namely the brain, where it could be converted into dopamine (Figure 9).

High performance liquid chromatography (HPLC) analysis of epigeal and Pachón adult brains showed nearly a two fold increase of dopamine in Pachón brains as compared to epigeal forms. In two brain regions known to have altered morphology in Pachón (optic tectum and hypothalamus), no change in dopamine expression could be determined with a suitable degree of statistical significance. Dissections of these regions proved difficult, and the experiment was further complicated by constraints in the number of animals that could be used. This resulted in high variation within the dissected samples. Despite this, I would expect that the

thalamic region of cavefish to be one of the regions of the brain that contains excess dopamine.

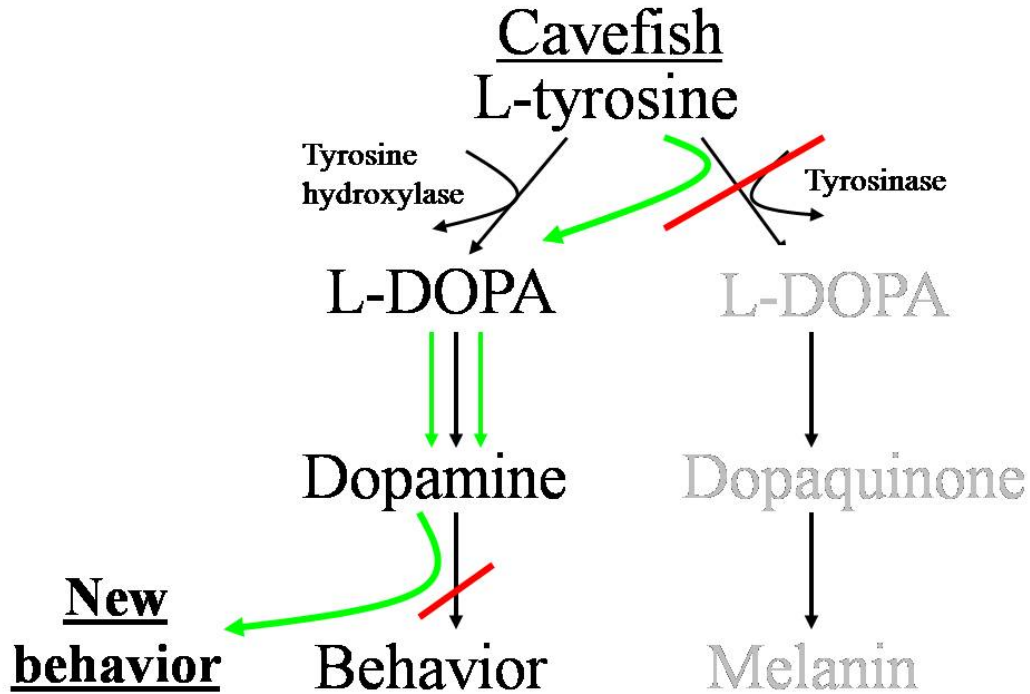


Figure 9. A model for a selective advantage for melanin pigmentation loss. Mutations of the OCA2 gene make the melanosome unsuitable for melanin synthesis. L-tyrosine is therefore free to be used elsewhere, in this case the dopamine synthesis pathway, resulting in changes to behavior.

Dopamine in the brain regulates a number of important processes, notably circadian rhythm, appetite and locomotion. Cave forms of *Astyanax* retain circadian rhythms, a process which regulates dopamine production in the hypothalamus, but they are not as tightly regulated as epigeal forms (Wilkins et al, 1993; Espinasa and Jeffery, 2006). In addition, experiments in *Drosophila* showed that dopamine receptors are more receptive to dopamine during night cycles (Andretic and Hirsch, 2000). Experiments on appetite regulation in humans showed dopamine agonists suppressed appetite while antagonists enhanced it (Comings et al, 1993). However, it is difficult to predict exactly what effect this excess of dopamine could have on the

behavior of cavefish without knowing its location in the brain. It should be noted, however, that concurrent with my HPLC analysis of dopamine, serotonin levels were also quantified. While cavefish had nearly twice the dopamine seen in epigeal forms, they had nearly half the serotonin than that of epigeal fish (unpublished). This is notable because one study has shown the ratio of dopamine and serotonin to be significant in the determination of food intake in humans (Meguid et al, 2000).

Conclusions

I have shown cavefish retain a population of neural crest-derived, migratory melanoblasts that exhibit typical melanocyte characteristics. These cells migrate through the dorsolateral pathway, an exclusive property of chromatophore precursors. Further, these cells produce an active tyrosinase enzyme and respond to α MSH and TPA in culture as one would expect a melanoblast to respond. Therefore, the mechanisms that oversee melanocyte development remain unchanged in cavefish. I showed the melanogenic processes is interrupted at a late step, prior to the conversion of L-dopa to dopaquinone, but after the synthesis of L-tyrosine. This model was consistent with the discovery of mutations in the OCA2 gene.

I believe that the neutral theory explanation of melanin pigmentation loss is unable to explain the speed with which the mutations were fixed in the population and the fact that other pigment cell types are conserved in all cave populations. I proposed a model to explain pigmentation loss by selection, hypothesizing that an interruption in pigmentation would result in excess tyrosine which could then be used for other metabolic pathways, namely the synthesis of dopamine. Cavefish brains

contained almost twice as much dopamine as epigean fish, which undoubtedly manifests in changed behaviors. Although this data is not conclusive, I believe it provides some justification for a selective advantage for melanin pigmentation loss.

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