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Melanin as an Oto-Protective Pigment in Two Fish Species: *Poecilia Latipinna* and *Cyprinus Carpio*

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MELANIN AS AN OTO-PROTECTIVE PIGMENT IN TWO FISH SPECIES: 
POECILIA LATIPINNA AND CYPRINUS CARPIO

A Thesis
Presented to
The Faculty of the Department of Biology
Western Kentucky University
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Of the Requirements for the Degree
Master of Science

By
Bethany N. Coffey

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MELANIN AS AN OTO-PROTECTIVE PIGMENT IN TWO FISH SPECIES:
POECILIA LATIPINNA AND CYPRINUS CARPIO

Date Recommended 7/28/2014

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Dean, Graduate Studies and Research  Date
I dedicate this thesis to my mother, who pushed me toward success even when I had lost faith in myself. Without her, I would not have had the drive, patience, or persistence that this project required. I would also like to dedicate this thesis to my nana, grandmother, and other members of my family who supported me during difficult times and helped me look forward to a bright future. Thank you all so much for giving me the encouragement that has led to this accomplishment.
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# CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Methods</td>
<td>9</td>
</tr>
<tr>
<td>Results</td>
<td>17</td>
</tr>
<tr>
<td>Discussion</td>
<td>27</td>
</tr>
<tr>
<td>Literature Cited</td>
<td>39</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Figure 1. Photographs of *P. latipinna* color morphs....................................................... 15

Figure 2. Photographs of *C. carpio* color morphs. ......................................................... 16

Figure 3. Light microscopy images of intact *P. latipinna* ears........................................... 21

Figure 4. Light microscopy images of intact *C. carpio* ears............................................. 22

Figure 5. TEM images of cross sections of *P. latipinna* crus commune ......................... 23

Figure 6. Melanin assay results......................................................................................... 24

Figure 7. Audiograms of experimental groups of *P. latipinna* and *C. carpio*............... 25

Figure 8. Audiograms of control groups of *P. latipinna* and *C. carpio*....................... 26
Melanin is the dark pigment found in most organisms that gives color to the skin, hair, feathers, and eyes of vertebrates. While melanin is known to also be present in the stria vascularis of the mammalian cochlea, its function in the inner ear is still unknown. Some previous studies have indicated that melanin may serve to protect the mammalian ear from hearing loss. Minimal previous research on melanin within the inner ears of fishes has been conducted. In this study, the melanin levels in the inner ears of different color morphs of two fish species (Poecilia latipinna and Cyprinus carpio) were examined, as well as the potential protective role of melanin from acoustical stress.

To identify the relationship between fish color morph and inner ear melanin, a spectrophotometric melanin assay was used for dissected inner ears, and transmission electron microscopy (TEM) was used to examine melanosome structure in the crus commune of the inner ears. For each color morph and species, hearing thresholds were quantified before and after sound exposure (150 Hz tone at 165 dB re 1 µPa for 48 h) by measuring auditory evoked potentials (AEPs). Melanin levels were associated with scale color, with black morphs having more inner ear melanin than white or golden morphs. TEM imaging showed that melanosome size varied among color morphs, with black $P.$ latipinna morphs having larger melanosomes than white morphs. Hearing thresholds did not differ significantly among color morphs before sound exposure in
either species. However, hearing thresholds post-sound exposure and the associated threshold shifts significantly differed between black morphs and other color morphs in the two species, with black morphs having lower thresholds and exhibiting less hearing loss than the other morphs. This suggests that melanin plays a protective role in the teleost inner ear, similar to what other researchers have found in mammalian models. Teleost fishes may be a new, more efficient model for testing melanin's function in the inner ear and how it interacts with stress from acoustical trauma and ototoxic drugs.
INTRODUCTION

Biomedical testing requires the use of animal models to emulate the conditions of the human body in order for the effects of new chemicals and treatments to be observed without causing harm to human subjects. Common animal models include invertebrates, such as flies and worms, and vertebrates such as mice, rats, and fish (Zon and Peterson, 2005; Lieschke and Currie, 2007). Mammalian vertebrates like mice and rats have physiologies more similar to humans; however, they are less amenable for large-scale mutagenic studies compared to invertebrate models such as *Drosophila* (Lieschke and Currie, 2007). Recently, the zebrafish (*Danio rerio*) genome was sequenced by the Sanger Center, which led to the creation of new technologies to manipulate and understand their DNA (Zon and Peterson, 2005). Advances in the understanding of zebrafish genetics have given rise to a model with a high reproduction rate (Tavares and Santos Lopes, 2013) and a plethora of known mutations that have advanced researchers’ understanding of how vertebrate genes function (Zon and Peterson, 2005). By using a model like zebrafish, researchers can address vertebrate questions with the time-efficient methods used for invertebrates (Lieschke and Currie, 2007).

Since the rise of the zebrafish as a biomedical model, they have been used in many experiments aimed at preventing or curing disease and otherwise improving the human condition. The similarities between mammalian and zebrafish models are numerous; mutations analogous to human mutations that cause polycystic kidney disease, heart disease, retinal degeneration, anemia, and cancers such as leukemia and melanoma have been found to affect zebrafish similarly (Zon and Peterson, 2005). In fact, many of the important genes and pathways involved in cancer formation are
conserved between fish and humans (Tavares and Santos Lopes, 2013). What is even more useful is the fact that there is also a high degree of similarity between zebrafish and humans when testing chemical and drug responses; some estimates reach as high as 95%, while others indicate 50-70% conservation of drug response when testing drugs that affect cell cycle (Zon and Peterson, 2005). Zebrafish are also being used as models to study various types of birth defects, infections, immunological diseases, endocrine diseases, and psychological and behavioral abnormalities such as addiction (Lieschke and Currie, 2007). While zebrafish will never completely replace mammalian models (Brittijn et al., 2009), the similarities between zebrafish and mammalian models allow for quick and cost effective experimentation (Zon and Peterson, 2005). Differences between mammalian and zebrafish models are not always a hindrance to biomedical testing. Fish are able to regenerate fins, scales, nerves and the spinal cord, and even their hearts (Brittijn et al., 2009), which has inspired the use of fish to study the pathways involved in regeneration. Zebrafish and other species of fish such as goldfish have become popular organisms of study in auditory experiments due to this regenerative power and the accessibility of hair cells in the inner ear and lateral line (Harris et al., 2003; Smith et al., 2006; Sun et al., 2011).

Teleost fish have been used as models for hearing disorders in humans due to the fact that the structures of their inner ears have been relatively conserved throughout evolutionary history. The inner ears of fishes are composed of three semicircular canals and three end organs called the utricle, saccule, and lagena (Popper and Schilt, 2008; Schulz-Mirbach et al., 2010). The semicircular canals are part of the vestibular system and detect the movement of the head in three directional planes – roll, pitch, and yaw
(Popper and Schilt, 2008); they serve the same function in other vertebrate lineages (Fritzsh, 1999; Carey and Amin, 2006). The end organs each contain an otolith, a dense mass of calcium carbonate in a protein matrix, which rests on top of the macula, the sensory epithelium which contains the sensory hair cells (Popper and Schilt, 2008; Schulz-Mirbach et al., 2010). The hair cells found within the sensory epithelium are mechanotransducers which convert a mechanical stimulus to an electrical signal that the brain can interpret (Popper and Schilt, 2008). While the utricle is generally thought to be part of the vestibular system (Schulz-Mirbach et al., 2011), the saccule and lagena detect particle motion caused by the vibrations of sound waves. When sound waves travel through the water, the waves pass through the fish’s tissues without much resistance due to the fish’s body having a very similar density to the water. Since the otoliths are more dense than the surrounding body, the particle motion travels through them at a different amplitude and phase, causing the stereociliary bundles of the hair cells to be deflected and produce receptor potentials (Popper and Schilt, 2008). This mechanotransduction is very similar to how other vertebrates hear. Mammals also have utricles and saccules within their inner ears, but over evolutionary time the lagena has been replaced by the coiled cochlea (Carey and Amin, 2006). Rather than otoliths, vibrations from airborne sounds are carried from the tympanic membrane by three small bones called the malleus, incus, and stapes in the middle ear to the cochlea. The bones vibrate the oval window of the cochlea, moving the fluids within the cochlea and deflecting of the stereociliary bundles of the hair cells (Schnupp et al., 2011).

While fish and humans have inner ears that function similarly, an important difference exists. In all vertebrate lineages but mammals, sensory hair cells can be
regenerated if damaged; however, mammals cannot regenerate hair cells that have been
lost due to acoustic or ototoxic trauma (Salvi, 2008), which is why deafness is a serious
problem for humans. Considerable research being has done on hearing loss, and while
technology like the cochlear implant helps some people cope with hearing loss, there is
currently no cure for sensorineural deafness. Some of the research conducted on
regenerating hair cells in mammals has proved ineffective, as mammalian hair cells grow
in a particular pattern which must be replicated by the new hair cells in order to function
(Salvi, 2008). Therefore, some researchers are moving away from attempting to find a
cure for deafness and are instead seeking ways to prevent deafness from occurring. One
avenue of such research is to study the factors and mechanisms associated with hearing
loss or its prevention. It has been observed that a lack of the pigment melanin is often
associated with deafness in mammals (Murillo-Cuesta et al., 2010), as white cats with
blue eyes are generally deaf (Darwin, 1859), one-fourth of Dalmatian dogs are born deaf
(Shelton et al., 1993), and fully albino cats occasionally exhibit deafness (Conlee et al.,
1984). This seems to indicate that the pigment melanin may play a role in hearing ability
or protection against deafness.

There are several types of melanin, including: neuromelanin, which is found in
the brain and produced differently than other melanins found in other areas of the body
(Zareba et al., 1995); pheomelanin, which is responsible for red hair and freckles; and
eumelanin, which is the most common form of melanin (Ohlemiller et al., 2009) and will
be the main melanin discussed in this paper. Eumelanin is a dark black/brown pigment
that is produced in specialized cells called melanocytes in mammals and melanophores in
fishes and amphibians (Cooper et al., 2009). Fishes and amphibians possess other
pigment producing cells that mammals do not – yellow xanthophores, iridescent iridophores (Matsumoto, 2002), and red erythrophores (Matsumoto and Obika, 1968). Pigment producing cells arise from the neural crest, the same area where neurons and glia of the peripheral nervous system originate during development of the organism (Gill and Salt, 1997; Le Douarin and Kalcheim, 1999). Within the melanocytes/melanophores are organelles called the melanosomes, where melanin is stored; one of the functions of melanocytes/melanophores is to transfer melanosomes to areas where they are needed (Cooper and Raible, 2009).

Melanin and other pigments are responsible for the coloration of the eyes, hair, feathers, and skin of animals. However, melanin’s function is greater than simply giving animals coloration; this pigment plays a multifaceted role within the body of a vertebrate. Without melanin, organisms could not undergo the critically important tanning response, which protects the body from DNA mutations caused by ultraviolet radiation (Yamaguchi et al., 2007). In this way, melanin in the skin protects against skin cancer. It is also thought that melanin may serve a protective role in the iris and retina (Ohlemiller et al., 2009) and a role in heavy metal scavenging, antioxidant activity, and regulating calcium homeostasis (Drager, 1985; Seagle et al., 2005; Bush and Simon, 2007; Ohlemiller, et al., 2009).

These aspects of melanin have made it a topic of interest for hearing research. In mammals, melanocytes are found in the membranous labyrinth and the cochlea (Gill and Salt, 1997); however, the main focus for research is the intermediate layer of the stria vascularis in the cochlea of the inner ear of mammals (Conlee et al., 1986; Conlee et al., 1989; Steel and Barkway, 1989; Gill and Salt, 1997; Murillo-Cuesta et al., 2010;
Ohlemiller et al., 2009). The stria vascularis is necessary for homeostasis of the fluids of
the inner ear, which is what allows the cochlea to function properly (Gottesberge, 1988;
Takeuchi et al., 2000). There is evidence that a lack of cochlear melanin is correlated
with a greater vulnerability to hearing loss (Conlee et al., 1986). There are also several
known pigmentation disorders that affect humans that are characterized by various
degrees of hearing loss, one of which is Waardenburg syndrome, which involves partial
albinism, bright blue eyes, a lock of white hair, and deafness (Cooper and Raible, 2009).
Melanin’s specific role in the mammalian inner ear is still unknown, but it is a topic that
several researchers are attempting to elucidate (Conlee et al., 1986; Conlee et al., 1989;
Steel and Barkway, 1989; Gill and Salt, 1997; Murillo-Cuesta et al., 2010; Ohlemiller et
al., 2009). If it can be found that the inner ears of fish respond similarly to those of
mammals due to pigmentation, new avenues for addressing this question will become
available to biomedical researchers.

Melanin in fish and melanin-lacking mutants has been discussed in several papers
which focused on pigmentation of the scales or larval melanophore generation
(Matsumoto and Obika, 1968; Kajishima, 1977; Cooper and Raible, 2009; Cooper et al.,
2009). Currently little attention has been paid to the role melanin plays in the inner ears
of fishes. One study by Lechner and Ladich (2011) found no differences in hearing
ability between normally pigmented and albino catfish. That study, a study that
compared cave-dwelling and normally pigmented surface-dwelling Astyanax (Popper,
1970), and photographs of the inner ears of fishes, where melanin can be clearly seen but
is not the focus of the respective paper (Deng et al., 2013), are the only previous
examples of teleost inner ear melanin being mentioned in the literature.
While Lechner and Ladich found no differences in hearing ability between normally-pigmented and albino catfish, they did not test the fish after their hearing had been compromised. It has already been shown that albino guinea pigs are just as sensitive, if not more so, than normally pigmented guinea pigs before a deafening treatment (Conlee et al., 1986; Conlee et al., 1989). Even though fish do not have a cochlea, which is the main focus of many mammalian inner ear melanin experiments, it is evident from my personal experience in dissecting fish ears and from published photographs of fish ears (Deng et al., 2013) that at least some species have melanin in their inner ears. This leads one to question the purpose of this inner ear melanin in fishes if it does not improve hearing. There is evidence to suggest that inner ear melanin may protect mammals from hearing loss (Conlee et al., 1986; Ohlemiller et al. 2009), and it is possible melanin plays a similar role in the inner ear of fishes as well.

This study examines the quantity and effects of melanin in the inner ears of two fish species, *Poecilia latipinna* (sailfin mollies) and *Cyprinus carpio* (koi), to test the potential protection role of melanin in the teleost inner ear. I hypothesized that there would be differences in inner ear melanin levels among different color morphs of each species, and that these differences would correspond with hearing ability after sound-induced hearing loss. I predicted that the most darkly pigmented fish would have the most inner ear melanin, and thus be more protected from hearing loss. To test this, images of the inner ears were taken, a melanin assay was performed, and hearing thresholds before and after sound exposure was quantified. If melanin does protect against hearing loss in fishes, it may reveal new possibilities for drug testing in the fight against hearing loss in humans.
*P. latipinna* and *C. carpio* were chosen because they are easily acquired in different color morphs from the aquarium trade. These species are also larger and harder than zebrafish, and therefore more likely to survive multiple hearing tests. Poeciliids such as guppies have been used in many experiments regarding the evolution of populations due to the high variability of their phenotypes and short generations times (Breden, 2006). While not as common in the biomedical field as zebrafish, some researchers are using poeciliid models to study melanoma and other cancers, and a unisex molly species, *Poecilia formosa*, has also caught the attention of biomedical researchers (Schartl, 2014). *C. carpio* belongs to the family Cyprinidae, along with zebrafish and goldfish, which have been used in many hearing experiments and have sensitive hearing thresholds (Fay, 1988; Smith et al., 2006; Uribe et al., 2013).

These two fish species perceive sound in two different ways. All fish can sense the particle motion aspect of sound through the movement of the otoliths, but many species lack the ability to sense the pressure component of sound directly and are less sensitive to sound. Some fishes have specialized structures that transmit the pressure component of sound to the inner ear, and are therefore highly sensitive to sound (Popper and Fay 1993; Popper and Schilt, 2008). *P. latipinna* is “motion sensitive” but is not known to have pressure detecting structures (Schulz-Mirbach et al., 2010) while *C. carpio* responds to both components of sound. *C. carpio*, as a cyprinid, have specialized Weberian ossicles that connect the swim bladder to the inner ear (Krumholz, 1943). As a result of the difference in hearing abilities between the two test species, I hypothesized that they would exhibit differential hearing loss following sound exposure, and that the protective effect of melanin in their inner ears might also differ.
METHODS

Test Subjects

Three color morphs of *P. latipinna* were used (Fig. 1): black; white, known in the aquarium trade as “silver”; and golden, which was assumed to be an albino morph. Golden mollies lack melanin in the scales and are orange in color; some are born with black eyes while others are born with red eyes. For this study, we used golden *P. latipinna* with red eyes, as the color of the eyes has been found to correlate positively with how much melanin can be found in the inner ear (Conlee et al., 1986). Two color morphs of *C. carpio* were used (Fig. 2): black and white, known in the aquarium trade as “platinum.” Each species was kept in its own aquarium with submersible heaters and charcoal filtration systems when not undergoing treatments or tests, with all color morphs of each species sharing the same space. Tanks were maintained at approximately 25°C.

Melanin Imaging

Information about melanin within the tissues of the inner ears of fishes is nearly non-existent, so first it had to be determined whether melanin is present in the inner ears of *P. latipinna* and *C. carpio* and whether the amount of melanin is associated with scale color. To visualize melanin in intact ears, fish of all target color morphs of both species were preserved in 4% paraformaldehyde for at least 24 hours. Following fixation, the intact inner ears were removed from the fish and imaged using a Leica MZ16 light microscope with an axial carrier. The images were produced using the Automontage software from Syncroscopy (Cambridge, U.K.), which combines images focused at different heights to create a perfectly focused image of a 3D object.

Subsequently, Transmission Electron Microscopy (TEM) was utilized to observe
the distribution and ultrastructure of the melanosomes within the tissues of the inner ears of *P. latipinna*. Due to time limitations, only black and white *P. latipinna* were used. Fish from both morphs were euthanized using an overdose of the fish anesthetic tricaine methanesulfonate (MS-222) dissolved in water. Inner ears were removed from freshly euthanized fish and otoliths were removed from the tissue. Tissues were fixed in 2% glutaraldehyde and then stained with 1% osmium tetroxide and preserved in a resin block. The block was then cut using a MT-X model ultra-microtome, creating cross-sections that were 100 nm or less in thickness. The cross-sections were taken from the crus commune of each ear, which was found in the light microscopy pictures to contain the most visible melanin. The crus commune of the poeciliid inner ear is the cylindrical structure between the saccule and the area where the anterior semicircular canal and the posterior semicircular canal meet (Schultz-Mirbach et al., 2011). The cross-sections were imaged using a JEOL100CX TEM at 1.3 kX, 20 kX, and 66 kX.

**Melanin Quantification**

A melanin assay (adapted from Xiao et al., 2007) was used to quantify differences in inner ear melanin levels among color morphs of each species. Inner ears were removed from freshly euthanized fish and otoliths were removed from the tissue. One ear from each fish was placed in one of five microtubes (black, white, or albino *P. latipinna* and black or white *C. carpio*) of PBS buffer depending on the species and color morph. Ears from nine black, seven white, and seven golden *P. latipinna* were used, and ears from ten black and eleven white *Cyprinus carpio* were used. As a single ear did not contain enough melanin to be detectable with the assay, tissues of each fish type were
pooled and homogenized using a Vortex mixer, washed with PBS, and suspended in 200 µl of nanopure H₂O and 1000 µl of ethanol-ether (1:1 volume) for 15 minutes. Samples were then centrifuged for 10 minutes at 3000 RPM and the supernatant discarded. The precipitates were air-dried overnight and then added to 1000 µl of 1 N NaOH. This mixture was heated at 80°C in a water bath for 1 h, mixed once more, and allowed to cool to room temperature. Using spectrophotometry, absorbance at 475 nm was used to quantify melanin levels. A standard curve was created to determine melanin levels from absorbance readings. Synthetic melanin (M8631, Sigma-Aldrich, St. Louis, Montana) was dissolved in 1 N NaOH at varying concentrations (0 µg/ml, 0.45 µg/ml, 1.13 µg/ml, 2.28 µg/ml, 7.04 µg/ml, and 35.2 µg/ml), which were analyzed for absorbance along with the experimental samples. The concentration of each tube was divided by the number of ears in each tube to get the mean melanin level per ear. The mean melanin level per ear was then divided by the mean mass of the individuals in order to obtain the mean inner ear melanin per body mass of each species.

Hearing Tests

To determine the hearing ability of the color morphs of each species, the physiological response in the form of auditory evoked potentials (AEP) were recorded for 23 *P. latipinna* (nine black, seven white, and seven golden) and 21 *C. carpio* (ten black and eleven white). This methodology is used commonly to perform hearing tests on fishes (Lechner and Ladich 2011; Smith et al., 2011; Ladich and Fay, 2013; Uribe et al., 2013). To perform these hearing tests, each fish was first anesthetized using MS-222 until it could no longer swim, but movement of the opercula was still observed. The fish
was then restrained using a mesh harness and kept suspended in a 19-L plastic chamber approximately 6 cm below the surface of the water and 22 cm above a University Sound UW-30 underwater speaker (Electro-Voice, Burnsville, MN). The water-filled chamber was held within a Faraday cage inside a sound isolation enclosure (WhisperRoom, Inc., Knoxville, TN) to reduce background sound and electrical noise. Stainless steel subdermal electrodes (27 gauge; Rochester Electro-Medical, Inc., Lutz, FL) were placed between the nares, in the muscle above the brainstem, and in the lateral muscle of the body to record AEPs. All electrophysiological equipment and software were produced by Tucker-Davis Technologies (Alachua, FL). The electrodes carried signals to an RA4LI Low Impedance Headstage, which transmitted the signal to an RX6 Processor; the software BioSig was used to analyze electrical impulses from the fish. During a hearing test, pure tone pips of one of nine frequencies (0.1, 0.25, 0.4, 0.6, 0.8, 1, 1.5, 2, and 3 kHz) were played through the underwater speaker at decreasing decibel levels in 5 dB steps until a visible AEP was no longer detected. The amplitude above that level was considered the hearing threshold for that individual at that frequency. By using the hearing thresholds for multiple frequencies, audiograms were created. *P. latipinna*, being the less sensitive species, was tested from 0.1 to 2 kHz. *C. carpio* was tested at these frequencies and at 3 kHz.

Each fish used in this experiment was tested using this method twice: once pre-deafening and once post-deafening. Pre-deafened audiograms were used to establish baseline hearing ability of the different groups of fish, whereas post-deafened audiograms were used to determine whether melanin could have a protective effect on hearing. Experimental fish were first placed in a 19-L plastic sound exposure chamber and
remained for 48 hours without sound exposure before undergoing AEP testing. This was to ensure no hearing differences among groups occurred due to simply being in the chamber for 48 hours. The fish were kept in a 16.5 by 12 by 13 cm nylon mesh box suspended 15 cm from an underwater speaker in the sound exposure chamber. This was to prevent fish from being exposed to different decibel levels of sound during an experiment. Within the chamber, there was also a submersible heater and an air-stone for proper aeration of the water that remained on during treatment. Water was changed in between testing, to prevent the buildup of ammonia due to the lack of a filter. After the fish were tested pre-sound exposure, the fish were placed back in the exposure chamber and the speaker was turned on. All groups were subjected to sound exposure for 48 hours (150 Hz tone at 165 dB re 1 μPa). After sound exposure, the fish again had their hearing tested using the same methodology as before.

Additional controls were also used to ensure that deafening occurred due to sound exposure and not due to the repeated AEP testing. The control groups were allowed to remain within the exposure chamber without sound for 48 hours for baseline data just as the experimental groups; however, when they were replaced in the deafening chamber, the sound remained off for the next 48 hours. This mimicked the treatment of the experimental groups, but did not include the sound exposure procedure. The control groups were pooled mixtures of morphs for each species. The *P. latipinna* control group consisted of five individuals (three black and two golden), and the *C. carpio* control group consisted of five individuals (two black and three white).
Statistical Analysis

Statistical analysis was performed using the SYSTAT 13 software (Systat Software, Inc., San Jose, CA). One-way Analysis of Variance (ANOVA) tests using type III sum of squares were used to determine whether masses, standard lengths, or times post-sound exposure varied among color morphs of each species. Paired t-tests were used to determine differences in hearing thresholds (averaged across all frequencies) between first and second AEP tests for control and experimental groups of each species. In the control groups, paired t-tests were used to determine whether repeated AEP tests affected subsequent hearing thresholds in later tests; in the experimental groups, paired t-tests were used to confirm that the fish had experienced deafening due to the sound exposure.

Two-way ANOVAs with frequency and color as the main effects were used to determine whether there were significant differences in hearing threshold or threshold shift among color morphs for experimental groups of each species. When differences among colors in overall ANOVAs were found, one-way ANOVAs with color as the main effect were performed for each frequency class that had visible gaps between standard error bars. P-values were adjusted using the Sequential Holm-Bonferroni method (Abdi, 2010). Only data for frequencies that displayed visible gaps between error bars were analyzed to retain statistical power; all frequencies where error bars crossed are assumed to not have significant differences. This was done because each additional test increased the likelihood of making a type I error. Alpha level for all tests was 0.05.
Figure 1- Photographs of the black (A), white (B), and golden (C) *P. latipinna* morphs used in this experiment.
Figure 2 - Photographs of black (A) and white (B) *C. carpio* morphs used in this experiment.
RESULTS

Melanin Imaging

Intact inner ears of the varying color morphs of *P. latipinna* (Fig. 3) and *C. carpio* (Fig. 4) indicated that the amount of visible melanin in the inner ear tissues varied by color morph. Black fish of both species had the most visible melanin, white fish of both species had little to no visible melanin, and golden *P. latipinna* had no visible melanin. However, the golden color morph had a large amount of yellow pigment in place of melanin.

While some electron microscopy has been used to examine the sensory epithelia of the inner ear of fishes (Lovell et al., 2006; Deng et al., 2013), TEM imaging had not previously been used to describe the histology of the semicircular canals. The low magnification TEM images of the crus commune (Fig. 5A & D) depict melanophores as the outermost layer of cells, followed by several layers of supporting cells. A layer of cells that are abnormally saturated with mitochondria make up the innermost layer separating the endolymph from the lumen of the semicircular canal; this is more evident in Fig. 5D. While this provides some insight into the ultrastructure of the crus commune, more imaging needs to be performed before all cell layers can be identified.

TEM images of black and white *P. latipinna* inner ear tissues revealed that there is not only a difference in the amount of melanin visible through light microscopy among morphs, but also a difference in the ultrastructure of the melanosomes within the melanophores (Fig. 5). When viewed at the same magnification, melanosomes from the white morph were smaller and more varied in shape than those of the black morph. In general, the melanosomes from the white morph were considerably smaller than those of
the black morph (Fig. 5B & E).

**Melanin Quantification**

Melanin levels per body mass were found to vary among color morphs of both *P. latipinna* and *C. carpio* (Fig. 6). Ears from black, white, and golden *P. latipinna* contained calculated means of 0.59 µg, 0.18 µg, and 0.10 µg of melanin per g of body mass, respectively. Ears from black and white *C. carpio* contained calculated means of 0.28 µg and 0.25 µg of melanin per g of body mass, respectively.

**Pre-Sound Exposure Hearing Tests**

In the overall ANOVA, no significant differences among color morphs were found in pre-sound exposure hearing thresholds for either *P. latipinna* or *C. carpio* (Fig. 7A & C). Separate ANOVAs confirmed that there were no significant differences in mass or standard length among color groups of either species.

While *C. carpio* had much lower hearing thresholds than *P. latipinna* for all tested frequencies, both species were found to be most sensitive at 400 Hz. Pre-sound exposure, the mean (± S.E.) hearing threshold for all experimental *C. carpio* at 400 Hz was 69.7 (±0.8) dB re 1 µPa, whereas the mean hearing threshold for all experimental *P. latipinna* at 400 Hz was 106.9 (±1.2) dB re 1 µPa. Differences in mean hearing thresholds between the two species varied by frequency, with the least difference at 100 Hz (16 dB) and the greatest difference at 600 Hz (44 dB).
Post-Sound Exposure Hearing Tests

After sound exposure, there was a significant hearing threshold shift in all color morphs of *P. latipinna*: black (t = 3.98, df = 8, p = 0.004), golden (t = 5.37, df = 6, p = 0.002) and white (t = 5.38, df = 6, p = 0.002). Significant differences in hearing ability post-sound exposure were found among color morphs (Fig. 7B) in an overall ANOVA (F2,160 = 7.33, p = 0.001). The Sequential Holm-Bonferroni method was used to compare hearing thresholds of *P. latipinna* at 400 Hz, as it was the only frequency where gaps between the standard error bars were observed when the data was graphed. Black morphs had significantly lower hearing thresholds post-sound exposure than both white (p = 0.012) and golden (p = 0.046) morphs at this frequency; however, the hearing ability of white and golden morphs did not differ significantly (Fig. 7B). There was an overall effect of color on temporary threshold shift (F2,160 = 6.26, p = 0.002), and black morphs experienced the least amount of shift due to sound exposure. All color groups experienced the greatest threshold shift at 400 Hz (Fig. 7).

Post-sound exposure, there was a significant hearing threshold shift in both white (t = 10.98, df = 10, p ≤ 0.001) and black (t = 17.85, df = 9, p ≤ 0.001) color morphs of *C. carpio* (Fig. 7). A significant difference in hearing threshold across tested frequencies was found between the two color morphs post-sound exposure (F1,171 =15.00, p ≤ 0.001) with the black morph having lower thresholds than the white morph (Fig. 7D). The Sequential Holm-Bonferroni method was used to compare black and white *C. carpio* at 100, 400, and 600 Hz, as these frequencies displayed visible gaps between the error bars when graphed. The black morph thresholds were significantly lower than those for the white morph (Fig. 7D) at these three frequencies (p = 0.03, 0.036, and 0.03, respectively).
It was also found that there was a significant effect of color on temporary threshold shift ($F_{1,171} = 11.60, p = 0.001$), with black morphs experiencing less threshold shift than white morphs. Similar to *P. latipinna*, *C. carpio* experienced the greatest threshold shift at 400 Hz (Fig. 7).

Hearing threshold shifts tended to decrease as time post-sound exposure increased. Since fish could only be tested one at a time, time-post sound exposure varied among fish. To combat this effect, time post-sound exposure was monitored closely. No significant differences in time post-sound exposure were found among color groups of either species.

*Control Hearing Tests*

No significant differences were found in hearing thresholds between the first and second control AEP hearing tests in either *P. latipinna* or *C. carpio* (Fig. 8). In other words, performing an AEP hearing test did not affect subsequent hearing test thresholds. Color morphs were pooled for the control groups, as no significant differences among color morphs were found for the control groups.
Figure 3 – Light microscopy images of intact left ears of black (A), white (B), and golden (C) *P. latipinna* color morphs. For A and B, scale markers indicate 200 µm; for C, scale marker indicates 500 µm. D = dorsal, R = rostral.
Figure 4 – Light microscopy images of intact left ears of black (A) and white (B) *C. carpio* color morphs. Scale markers indicate 2 mm. D = dorsal, R = rostral.
Figure 5 – TEM images of the crus commune of black (A, B, and C) and white (D, E, and F) color morphs of *P. latipinna* at 1.3 kX (A and D), 20 kX (B and E), and 66 kX (C and F). Scale markers indicate 1 µm (A and D), 0.5 µm (B and E), and 0.25 µm (C and F). Melanophore cell layers are marked with arrows. Melanosomes are the darkly pigmented spheres. Melanosomes pictured in image F are of the largest found in the white morph.
Figure 6 – Melanin assay results. (A) Standard curve using known melanin concentrations per ml of 1 N NaOH including 0 µg/ml, 0.45 µg/ml, 1.13 µg/ml, 2.28 µg/ml, 7.04 µg/ml, and 35.2 µg/ml. Calculated mean amount of melanin per g of body mass of the different color morphs of (B) *P. latipinna* and (C) *C. carpio*, respectively.
Figure 7 – Audiograms of *P. latipinna* (A and B) and *C. carpio* (C and D) in pre-sound exposure (A and C) and post-sound exposure AEP tests (B and D). * indicates significant $(P \leq 0.05)$ differences between black and other color morphs.
Figure 8 – Audiograms of control groups of *P. latipinna* (A) and *C. carpio* (B). AEP 1 lines represent hearing ability during the first hearing test; AEP 2 lines represent hearing ability 48 hours after first test with no other treatment.
DISCUSSION

No previous audiograms for *P. latipinna* could be found in the literature; however, a related species which has been recently examined, *Poecilia mexicana* (Atlantic mollies) can be used for comparison (Schulz-Mirbach et al., 2010). *P. latipinna* pre-sound exposure (baseline) AEPs found in this experiment were much higher than previously recorded AEPs of *P. mexicana* (Atlantic mollies). Schulz-Mirbach et al., (2010) found *P. mexicana* to be most sensitive at 200 Hz and 300 Hz when recording hearing thresholds based on sound pressure levels, whereas my study found *P. latipinna* to be most sensitive at 400 Hz. *P. mexicana* exhibited lower hearing thresholds than those for *P. latipinna* found in my study, with differences between 10 dB and 30 dB depending on frequency between the two studies (Schulz-Mirbach et. al., 2010). There are three possible reasons for this difference between the two audiograms. First, Schulz-Mirbach et al. (2010) used airborne sound and had the fish positioned with the nape of the neck above the surface of the water, whereas in my study, the sounds were emitted from an underwater speaker and the recording electrodes were placed underwater as well. This could have caused electrical interference, despite the precautions (such as the Faraday cage and sound isolation enclosure) taken to reduce background noise, and could have masked AEP signals. Another possibility is that the two species naturally have different hearing thresholds, as Schulz-Mirbach et al. (2010 and 2012) found differences in otolith morphology in different populations of the same species of *P. mexicana*. Although these otolithic differences didn’t correlate with changes in hearing ability between these populations, it is possible that the two species could differ drastically. *P. latipinna* are endemic to North America (Page and Burr, 1991) while *P. mexicana* is
found from Mexico to the lower countries of Central America (Proudlove, 1997), and it is possible that their different habitat ranges could have selected for differences in inner ear morphology. The final possible reason the two audiograms differ is that the fish used in my study were the product of the aquarium trade, whose lineages have been removed from the realm of natural selection long enough to drastically alter their appearance.

Schulz-Mirbach et al. (2010 and 2012) used a combination of wild-caught and captive-bred *P. mexicana* for their studies, but captive-bred populations were not artificially selected for color or other desirable traits. It is possible that the black, white, and golden morphs differ from their wild-type counterparts due to selective inbreeding or aquarium parameters at the breeding site.

*C. carpio* pre-sound exposure (baseline) AEPs found in this experiment were comparable to others previously discussed in literature. Kojima et al. (2005) found *C. carpio* to be most sensitive at 505 Hz, but they did not test at 400 Hz; my study found *C. carpio* to be most sensitive at 400 Hz, but did not test 500 Hz. It is possible that the frequency *C. carpio* are most sensitive to is between 400 Hz and 505 Hz. Both studies found the hearing threshold at the most sensitive frequency to be approximately 70 dB re 1 µPa. Kojima et al. (2005) also found the hearing threshold at 1000 and 3000 Hz was approximately 90 and 110 dB re 1 µPa respectively, which matches the current study. However, there are some discrepancies between these two findings; the thresholds recorded for 100 Hz and 200 Hz were much lower (approximately 80 dB re 1 µPa for both) than those found in my study. There are two potential reasons for this difference. The first is that the fish Kojima et al. (2005) used were much larger (79 - 115 g) than the ones I used (6.88 - 22.46 g). It is possible that the larger fish were simply more sensitive
at lower frequencies than my smaller fish. The second potential reason for the difference is that the method of acquiring the AEP varied between these two studies. As in the case with *P. mexicana*, Kojima et al. (2005) used airborne sound and had the fish positioned with the nape of the neck above the surface of the water. This could mean that true hearing thresholds were masked below background noise, but this is less likely to be the case for *C. carpio*, as the audiograms between the two studies match much more closely than those of *P. latipinna* and *P. mexicana*. Again, artificial selection for color could be a factor, but decorative *C. carpio* are often bred in ponds and not aquaria, and thus would not be as removed from natural selection as *P. latipinna*. Regardless of the reason behind the differences between past audiograms and current audiograms, pre-sound exposure thresholds did not differ significantly among color morphs of each species, meaning that differences between studies would not affect the findings of this study.

There were differences in hearing ability before and after sound exposure between the two species, as expected. *P. latipinna*, having poorer hearing in general, experienced less drastic threshold shifts than *C. carpio* in response to sound exposure (Fig. 7). *C. carpio* received more amplified acoustical stimuli through the vibrations of the Weberian ossicles, and therefore experienced greater threshold shifts. It has been shown in a previous study that fish species with better hearing experience greater threshold shifts in response to a given sound stimulus (Smith et al., 2004).

Hearing thresholds did not differ statistically among color morphs of either species before sound exposure, which is similar to some studies involving guinea pigs (Conlee et al., 1989; Gill and Salt, 1997). Post-sound exposure, there were statistically significant differences in hearing ability, with the black morphs of both species being
able to hear at lower sound pressure levels (SPL) than the other morphs (Fig. 7), and both species exhibited differences among morphs at their most sensitive frequencies, which has also been recorded in guinea pigs (Conlee et al., 1986). There were no significant differences in hearing ability between the golden and white *P. latipinna* color morphs.

The only frequency where the black *P. latipinna* morph differed significantly from the other morphs was at 400 Hz, the frequency *P. latipinna* was most sensitive to pre-sound exposure and experienced the greatest threshold shift post-sound exposure. This was different from the *C. carpio* results, where the black morph’s hearing ability differed significantly from the white morph’s at three frequencies: 100 Hz, 400 Hz, and 600 Hz. There were no significant size differences (either mass or SL) among groups that could cause this difference in hearing ability in either species. There was also no significant difference in time post-sound exposure the AEP tests were performed among the color morphs in either species. It was also indicated by control groups of both species that repeated AEP testing also did not significantly affect hearing ability in subsequent tests. Therefore, it can be assumed that the differences in hearing ability post-sound exposure among color morphs were due to pigmentation only. This indicates that melanin does, in some way, protect the hearing of *P. latipinna* and *C. carpio* at their most sensitive frequencies.

Following the melanin assay, there were several surprising pigmentation results that need to be addressed. First, the light microscopy image of the white *C. carpio* ear has very little visible melanin, yet the melanin assay indicated that the mean melanin per g of body mass was similar in both black and white morphs. While dissecting ears from the white morph, I observed variability in the amount of melanin found on the inner ears,
with some having very little and others having visible patches. It is possible that a few individuals had enough melanin present to raise the mean closer to the black morph. It should also be noted that both morphs of *C. carpio* had less inner ear melanin per g of body mass than the black morph of *P. latipinna*. However, *C. carpio* is a much larger fish than *P. latipinna*, and the average amount of melanin per ear was much higher in *C. carpio* before we accounted for body size. Lastly, the melanin assay indicated that there was melanin within the ear of the golden *P. latipinna* morph.

For this experiment, it was assumed that the golden *P. latipinna* morph was albino due to the red pigmentation of the eyes. It is true that there was no visible melanin to be seen during dissection; instead, the crus commune is covered in what appear to be xanthophores (Fig. 3C). However, the melanin assay indicated that there was a small amount of melanin within the inner ears of these fish (Fig. 6). There are several possible reasons for this finding. First, it is possible that the yellow pigment seen in Fig. 3C also absorbs light at 475 nm and caused a false positive. This is likely, as a study on frog skin showed that carotenoids found in xanthophores within frog skin had absorbance at 480 nm (Berns and Narayan, 1970). However, it should also be considered that the pigment producing cells on the crus commune of golden *P. latipinna* may not be xanthophores. Due to time limitations, no TEM images were collected of the inner ear of the golden morph, and thus identification of these cells is not certain.

There are different types of pigment mutations leading to white spotting and albinism. While white spotting causes a complete lack of melanocytes, which can cause the stria vascularis in mammals to improperly form (Conlee et al., 1986), the most common form of albinism causes melanocytes to form, but these melanocytes are unable
to produce melanin (Conlee et al., 1986; Gill and Salt, 1997). Therefore, it is possible that the yellow pigment producing cells found in golden \emph{P. latipinna} ears are actually melanophores, and the pigment found in their cytoplasm is not fully formed.

Alternatively, these pigment producing cells could be melanophores that contain a different type of melanin. Melanin is mainly found in two forms: eumelanin, responsible for the dark brown or black coloration associated with melanin, and less commonly pheomelanin, which appears yellow or red (Ohlemiller et al., 2009). While eumelanin is known to have protective properties in the skin and is being researched as a protective chemical in the inner ear, pheomelanin is thought by some researchers to be less effective and actually promote injury (Ohlemiller et al., 2009). It is also possible that these pigment cells are xanthophores that, instead of producing carotenoids, are producing a pigment similar to melanin. Within xanthophores are structures similar to melanosomes called pterinosomes, which have structures that highly resemble premelanosomes before the organelles begin synthesizing melanin (Matsumoto and Obika, 1968). In fact, melanosomes have been found in xanthophores in previous studies, and some researchers have suggested that melanophores are transformed xanthophores (Matsumoto and Obika, 1968). Without delving deeper into the histology and biochemistry of the golden \emph{P. latipinna} ear, it cannot be certain as to what pigment is present. But it can be stated that, whatever the pigment, its presence did not cause the golden morphs to be more or less susceptible to deafening than the white morphs.

Though no TEM images were collected of the golden \emph{P. latipinna} ear, images collected of the black and white morphs’ crus communes (Fig. 5) offer insight as to how skin pigmentation correlates with inner ear melanin levels. Not only is there a
quantitative difference in the amount of melanin in the ears among morphs (Fig. 6), but there is also a qualitative difference in the melanosomes that hold the melanin. Melanosomes in the white ear were mostly smaller and more varied in shape than those found in the black ear. Larger melanosomes in the white ear (Fig. 5F) were slightly smaller than the average melanosomes in the black ear. Similar differences in melanosome size have been found in studies of human skin, where darkly pigmented people had larger melanosomes than lightly pigmented people (Thong et al., 2003).

Melanin in the inner ears of mammals is most often discussed in relation to the stria vascularis of the cochlea (Conlee et al., 1986; Conlee et al., 1989; Steel and Barkway, 1989; Gill and Salt, 1997; Murillo-Cuesta et al., 2010; Ohlemiller et al., 2009), which is responsible for the production of the endolymph and the endocochlear potential (EP) which is necessary for the function of hair cells (Steel and Barkway, 1989; Gill and Salt, 1997). It has been determined in past experiments that most mice with white spotting (not common albinism) lacked melanocytes within the stria vascularis of the cochlea (Steel and Barkway, 1989). These mutants were found to have an EP of approximately 0 mV, which prevents proper hair cell function, whereas normally pigmented mice have an EP of around +100 mV (Steel and Barkway, 1989). It was also found that white spotting mutants that did possess a few melanocytes within the stria vascularis had higher EP than those that lacked melanocytes, with some having near normal EP at the basal turn of the cochlea (Steel and Barkway, 1989). However, this was thought to be due to morphological changes within the stria vascularis and not specifically due to a lack of melanin. It was hypothesized that the cells, with or without melanin, were needed for proper interdigitation between marginal and basal cells during
development (Steel and Barkway, 1989). This seems to be supported by a study by Gill and Salt (1997), which found albino (melanocytes present without melanin) guinea pig EP to be higher on average than the EP of pigmented guinea pigs, indicating that melanin is not required for the production of an EP.

This same study found that pigmented guinea pigs had higher concentrations of calcium ions within the endolymph than albino guinea pigs, and the concentration of calcium ions increased from base to apex within the cochlea as did pigmentation; albino guinea pigs with no pigmentation within the cochlea had one concentration of calcium ions throughout the cochlea (Gill and Salt, 1997). Melanin is known to have an affinity for calcium, and has been shown to be responsible in the maintenance of calcium homeostasis within cells (Gill and Salt 1997; Bush and Simon 2007). While calcium is important for the function of hair cells (Fettiplace and Ricci, 2006), this does not appear to be the only way melanin could serve to protect or maintain hearing.

Despite having higher EP and similar hearing ability to pigmented specimens before treatment, albino mammals are more susceptible to hearing loss via acoustical trauma (Conlee et al., 1986), ototoxic aminoglycosides (Conlee et al., 1989), and aging (Ohlemiller et al., 2009). Interestingly, it has been found that, in pigmented guinea pigs, the production of melanin within the stria vascularis increases after exposure to acoustical trauma or the introduction of ototoxic aminoglycosides (Gill and Salt, 1997). Some results indicate that one of the functions of melanocytes may be to absorb various constituents in order to maintain homeostasis of the endolymph (Conlee et al., 1986). Melanin has been shown to have high chemical affinity aminoglycoside antibiotics, which tend to accumulate within the pigmented epithelia of the inner ear; however, rather
than causing greater destruction of hair cells, once bound to melanin the drug is inactivated (Conlee et al., 1989). This is supported as the majority of hair cell loss in pigmented guinea pigs was at the base of the cochlea (Conlee et al., 1989), where there is naturally less melanin available to inactivate the drug (Gill and Salt, 1997).

It has also been found that melanosomes are able to function like lysosomes, and are able to inactivate many of the same enzymes (Ohlemiller et al., 2009). Most interestingly, in albino mammals, the cells that receive the most damage due to aging or other stressors are the marginal cells that pump potassium and other ions into the endolymph (Ohlemiller et al., 2009). While melanocytes only make up the intermediate layer of the stria vascularis, it has been noted that melanocytes may somehow influence the distribution of ion pumps or channels in the membranes of other, nearby cells (Steel and Barkway, 1989). Other research has shown that after treatment with an ototoxic aminoglycoside, melanocytes not only increase the production of melanosomes, but they also secrete their melanosomes into neighboring cells (Gratacap et al., 1989). In this way, it is possible that either the influence melanin has on calcium or melanin’s ability to inactivate certain enzymes or toxins could affect the cells directly responsible for the secretion of material into the endolymph.

Upon review of the TEM images of the crus commune of both black and white *P. latipinna*, the melanophores occur above a layer of mitochondria-rich cells that make up the innermost layer within the lumen (Fig. 5A & D). These cells may have a secretory function similar to that of the marginal cells within the stria vascularis of mammals, which would add evidence to support the idea that melanin serves to protect and maintain the marginal cells responsible for endolymph production. More research needs to be
conducted on this topic before conclusions are made, but it is possible that fish models
may open up new routes of studying this complex topic.

Though the exact function of melanin within the inner ear is still unknown, effects
of melanin on hearing thresholds among differently pigmented groups has not only been
studied in animal models, but has also been seen in humans as well. Several studies have
indicated that people of African descent, who naturally possess more melanin within their
eyes and skin, tend to have lower hearing thresholds as they age than their Caucasian
counterparts (Bunch and Raiford, 1931; Post, 1964; Roberts and Bayliss, 1967; Karsai et
al., 1972). It has also been shown that eye color in humans correlate with the
susceptibility to temporary hearing threshold shifts (TTS), but not all findings were
conclusive (Carter, 1980). A study performed in Sweden by Barrenas and Lindgren
(1990) found that differences in TTS susceptibility were not only applicable to race
differences, but were also found in differently pigmented Caucasian males. In this study,
test subjects were 16-17 years of age and were grouped based on skin tanning response
and hair and eye color (Barrenas and Lindgren, 1990). Due to ethical issues, studies of
hearing loss in humans related to melanin levels within the inner ear are restricted to use
of TTS or measurement of hearing ability after natural hearing damage such as age or
loud living conditions. As such, animal models are necessary for the testing of
pigmentation’s effect on the inner ear and of drug treatments that affect the inner ear.
Chemotherapy drugs, such as cisplatin, are providing new motivation to understand the
function melanin plays within the human inner ear.

Cisplatin is an anticancer drug that became clinically available in 1978 under the
name Platinol®, and is known as one of the most potent drugs used as chemotherapy
available today (Florea and Brusselberg, 2011). However, it is known to cause hearing
loss associated with hair cell degradation and alterations to the stria vascularis (Laurell et al., 2006). A recent study on guinea pigs has shed a little light on how cisplatin affects
the stria vascularis, particularly the marginal and intermediate layers (Laurell et al.,
2006). This study found that a single high dose of cisplatin caused a reduction of
melanin at the base of the cochlea along with some outer hair cell loss in this region when
compared to controls and hearing threshold shift at 30 kHz (Laurell et al., 2006). No cell
death was detected in the intermediate cells within this study, though they did appear
shrunken in size. Interestingly, the intermediate cells appeared to have been altered more
in response to the cisplatin than the marginal cells (Laurell et al., 2006). Some
researchers have hypothesized that cisplatin binds to melanosomes similarly to
aminoglycoside antibiotics, as albino guinea pigs appear more susceptible to hearing loss
following cisplatin treatment than pigmented guinea pigs (Schweitzer, 1993). This would
make sense, as the area that received the most damage within the pigmented guinea pig
cochlea was the base, which is already known to contain the least amount of melanin in
the base-to-apex gradient (Gill and Salt, 1997). Loss of visible melanin from affected
areas of the cochlea would seem to indicate that, if melanosomes do in fact bind or
interact with cisplatin to inactivate it, the melanosomes are degraded as a result. There is
some hope that hormone treatments could be used to boost melanosome synthesis to help
combat hearing damage in human cancer patients being treated with cisplatin (Laurell et
al., 2006).

There may still be many unanswered questions concerning melanin’s exact
function within the inner ear, but it does appear to have a protective effect within the
inner ears of *P. latipinna* and *C. carpio*, which supports my original hypothesis. Since *P. latipinna* and *C. carpio* exhibited post-sound exposure hearing differences among color morphs as found in mammalian models, it is possible that fish models could be used to efficiently investigate the relationship between drugs like cisplatin and inner ear melanin. There has already been some research into goldfish and zebrafish pigmentation mutations (Kajishima, 1997; Cooper and Raible, 2009; Cooper et al., 2009), and given the high fecundity of zebrafish (Tavares and Santos Lopes, 2013), it may be possible to examine melanin’s effects on hearing loss or drug ototoxicity within many genetically similar individuals in a cost-efficient manner. Future research involving melanin within the inner ears of fishes could include testing the potential protective effect of melanin when subjects are exposed to ototoxic drugs such as cisplatin or aminoglycoside antibiotics such as gentamicin.
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