

**The Effect of Environmental Contaminants on Hair Cell  
Regeneration in Larval Zebrafish**

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**As thesis advisor for \_\_\_ALEX YOUNG\_\_\_,**

**I have read this paper and find it satisfactory.**

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**Thesis Advisor**

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**Date**

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# **The Effect of Environmental Contaminants on Hair Cell Regeneration in Larval Zebrafish**

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## **A B S T R A C T**

Many contaminants, such as industrial pollutants, can be found in aquatic environments and may affect fish and other aquatic life. Some of these contaminants can also be found in trace amounts in water sources that are used for human consumption. Three of these contaminants are PCB-95, BPA, and fluoride. In this study we investigated the effect each of these contaminants has on hair cell regeneration in the larval zebrafish. Hair cells are the mechanosensory receptors that allow sound perception by transducing sound waves and vibrations into electrical impulses that are transmitted to the brain via stimulation of the auditory nerve. Unlike mammals, birds, fish, and amphibians have the ability to regenerate hair cells following a toxic insult. This study looks specifically at hair cells of the lateral line. The lateral line is a system of hair cells along the exterior of the fish that are homologous to the hair cells located in the mammalian inner ear. To test if a contaminant is having an effect on hair cell regeneration, we exposed fish to the hair cell toxin neomycin, and then let the fish recover for 24 or 48 hours in variable contaminant concentration. We saw no significant effect of PCB-95 and fluoride on hair cell regeneration, but observed that exposure to higher concentrations of BPA resulted in reduced hair cell regeneration. We then asked if hair cell regeneration occurs after recovery from BPA exposure, as BPA itself can kill hair cells. We observed reduced hair cell numbers 48 hrs post-BPA treatment, which suggests BPA continues to kill hair cells

post-exposure, similar to treatment with the ototoxic antibiotic gentamicin. These findings add to the growing list of harmful effects of BPA for both humans and aquatic life. Future experiments will distinguish if BPA continues to kill hair cells while they are trying to regenerate, or if BPA disrupts the proliferative regeneration process.

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### **Introduction:**

Over 30 million Americans live with moderate to severe hearing loss (Agrawal et al., 2008). Hearing loss can be genetic, chemically induced, as a result of aging, or from being exposed to loud noise or listening to excessively loud music (Henderson et al., 2008). Hearing loss is the direct result of sensory hair cells being damaged by any of the means mentioned previously. Hair cells are sensory receptors that allow us to perceive sound (Hudspeth, 1989). Hair cells are columnar shaped, with projecting hair-like structures called stereocilia (Figure 1). When the stereocilia of the hair cell are deflected by the mechanical energy of sound waves, it converts the energy into electrical energy that triggers stimulation of the auditory nerve. When the auditory nerve is stimulated, it transmits a signal to the brain that initiates auditory processing, leading to hearing.

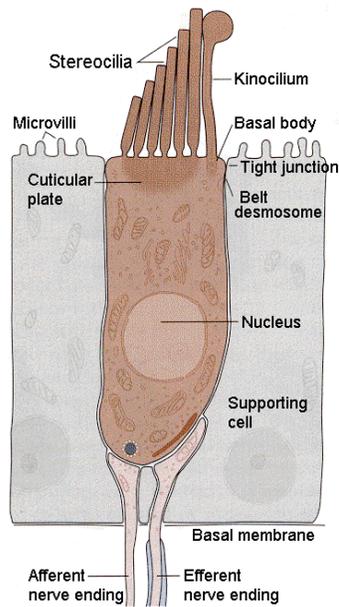


Figure 1: Hair cell morphology  
 Kandel, Schwartz & Jessell, *Principles of Neural Science*, 4th Edn.

Hair cells in all vertebrates are found in the inner ear, while in species of fish such as zebrafish, hair cells are also found externally along the body in a sensory system called the lateral line. Lateral line hair cells detect changes in fluid dynamics caused by the motion of other organisms and are used for predator/prey detection and navigation (Pohlmann et al., 2004). Zebrafish are an appropriate model for studying hearing loss and regeneration because the hair cells are located externally along the lateral line, which makes for easy observation (Ricci et al., 2012). The clusters of hair cells, called neuromasts, located on the lateral line are homologous to the hair cells located in the mammalian inner ear. Zebrafish have the ability to regenerate their hair cells, which makes them a prime model organism to study hair cell regeneration (Harris et al., 2003). Reptiles, birds, and other fish also have the ability to regenerate destroyed hair cells, while mammals cannot (Groves, 2010). In this research, we investigate if several

environmental contaminants could be affecting the zebrafish's ability to regenerate their hair cells.

The way hair cells regenerate in organisms that are capable of doing so, are by proliferation of supporting cells located basally of the hair cells. When a hair cell is killed, an identified signal is sent to the supporting cell that initiates cell division to create a new hair cell to replace the lost one. Once the supporting cell divides, the cell is able to differentiate into a new hair cell, which replaces the lost hair cells (Brignull et al., 2009).

### Environmental Contaminants Background and Rationale

Aquatic organisms are exposed to many different chemicals on a daily basis through environmental pollution. In this study, we examine the effect of exposure to three environmental chemicals, the polychlorinated biphenyl PCB-95, bisphenol A (BPA), and fluoride, on hair cell regeneration in the zebrafish lateral line. All three of these chemicals can cross the blood-brain barrier and directly interact with the nervous system, which includes sensory hair cells of the inner ear (Kania-Korwel et al., 2012; Pant & Deshpande, 2012; Valdez-Jimenez et al., 2011). In fish, hair cells of the lateral line are directly exposed to the aquatic environments, and may be more susceptible to aquatic contaminants.

PCB-95 is a toxin that results from industrial pollution that is found in low concentrations in our water (Fiedler, 1997). The US Environmental Protection Agency states that people that consume water containing PCB's over time may develop thymus gland problems, immune response deficiency, reproductive and nervous system disorders, and increased risk of cancer. We decided to look at PCB-95's effect on hair cell

regeneration due to evidence that suggests that exposure to PCB's during development correlate to hearing loss by cochlear dysfunction (Crofton et al., 2000; Powers et al., 2005; Poon et al., 2011).

BPA is a ubiquitous endocrine disrupting chemical that lines the inside of water pipes, is used in production of commercial plastics, and is found in thermal paper used to make receipts (US Environmental Protection Agency, 2010). BPA has been found to be toxic to hair cells in the lateral line (Sheth et al., 2013). BPA also is an estrogen disruptor, and higher estrogen levels are associated with improved hearing thresholds in humans and other species (Hulcrantz et al., 2006). If BPA is disrupting a hormone associated with hearing, it could potentially mean exposure to BPA could result in decreased hearing thresholds. BPA also disrupts Notch signaling, which is a signaling pathway found in most multicellular organisms that is crucial for cell proliferation and has been shown to play a role in regeneration of lateral line hair cells (Raya et al., 2003; Ma et al. 2008).

Fluoride is an anion with some toxic characteristics but is most widely known for its oral hygiene benefits and the ongoing debate on whether cities should fluorinate their water (Martin, 1989). We decided to investigate fluoride's effect on hair cell regeneration because there is little known on how fluoride may be affecting hair cells. With fluoride and the ongoing debate, people have a choice whether to fluorinate their water or not. By finding out if fluoride is negatively affecting hair cell regeneration, or harming hair cells, it could contribute additional information to the ongoing debate.

The implications of this research apply to both the environmental science and biomedical fields. Environmentally speaking, it is useful to know if a chemical in our water is negatively affecting wildlife and reducing the fitness of aquatic organisms in

their environment. This research furthers the understanding of how environmental contaminants affect hair cell regeneration.

### **Methods:**

Larval zebrafish: Fish that were 5-6 days post-fertilization (dpf) were used for these experiments. This age was used due to mature-like sensitivity to aminoglycosides (Murakami et al., 2003). AB wildtype fish were used for all experiments. Fish were incubated at 28.5° C and kept in embryo medium (EM) containing 994  $\mu\text{M}$   $\text{MgSO}_4$ , 150  $\mu\text{M}$   $\text{KH}_2\text{PO}_4$ , 42  $\mu\text{M}$   $\text{Na}_2\text{HPO}_4$ , 986  $\mu\text{M}$   $\text{CaCl}_2$ , 503  $\mu\text{M}$   $\text{KCl}$ , 14.9 mM  $\text{NaCl}$ , and 714  $\mu\text{M}$   $\text{NaHCO}_3$ , with the pH adjusted to 7.2 (Westerfield, 2000).

DASPEI scoring: Neuromasts were assessed using the mitochondrial dye 2-(4-(dimethylamino)styryl)-N-Ethylpyridinium iodide (DASPEI). For assessment, fish were incubated in EM containing DASPEI for 15 minutes, rinsed, then anesthetized with MS-222. For each fish, the same neuromasts since neuromasts develop in stereotyped locations, so we know where to expect them (Raible and Kruse, 2000). If a neuromast is absent, it is likely an effect of our drug treatment rather than due to development. 10 neuromasts were scored per fish, with each scored 0 (no labeling), 1 (moderate labeling), or 2 (bright labeling). Scores for each neuromast were added resulting in a score of 0-20 per fish (Harris et al., 2003).

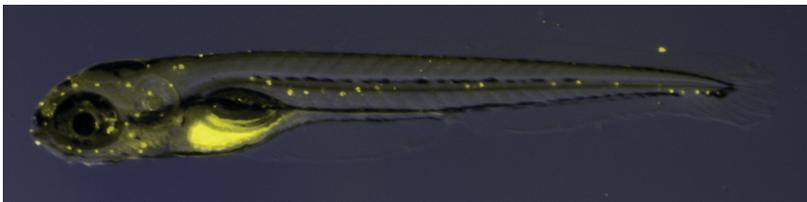


Figure 2: 5 dpf larval zebrafish neuromasts labeled with DASPEI (Coffin, 2010).

Immunocytochemistry (ICC): ICC using the anti-parvalbumin antibody allows for specific hair cell labeling (Heller et al., 2002). This allows for more precise neuromast assessment, allowing counts of how many individual hair cells comprise each neuromast. Fish were euthanized and fixed in 4% PFA, then blocked in phosphate buffered saline (PBS) solution with 0.1% Triton-X and 5% normal goat serum. Fish were then incubated in primary antibody to parvalbumin for 24 hours, followed by rinses in PBS and secondary antibody labeling with Alexa Fluor goat anti-mouse for 4 hours. Hair cells of 5 neuromasts, iO1, iO2, iO3, iO4 and iO5, were counted and summed for each fish.

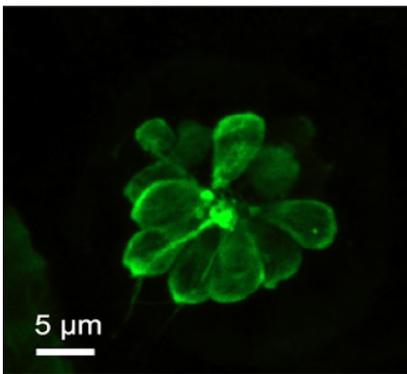


Figure 3: Immunocytochemistry labeling of a neuromast (Sheth, 2013).

### Regeneration in contaminants

In order to test how an environmental contaminant affects hair cell regeneration, we used an antibiotic to kill hair cells, and then let the fish regenerate in various concentrations of a contaminant. The hair cell toxin neomycin was used to kill existing hair cells in 5-dpf zebrafish (Harris et al., 2003). Once hair cells were killed, ~10 fish were assessed to determine how effective the neomycin was at killing hair cells. Another group of ~10 fish were untreated and also assessed at this time. These groups of fish were used as control groups. Fish were then put into experimental groups of various concentrations of PCB-

95, BPA, or hexafluorosilicic acid (fluoride) to recover and regenerate hair cells lost from the neomycin kill. Concentration ranges for each compound are shown in Table 1. After fish recovered for 24 hours, groups of fish from each experimental group were assessed, and the rest of the fish were assessed after 48 hours of recovery. Zebrafish typically regenerate almost all of their hair cells after 72 hours (Harris et al., 2003; Ma et al., 2008).

**Table 1: Environmental contaminant experimental concentrations.** Environmentally significant concentrations are concentrations that can be found in water samples of aquatic environments.

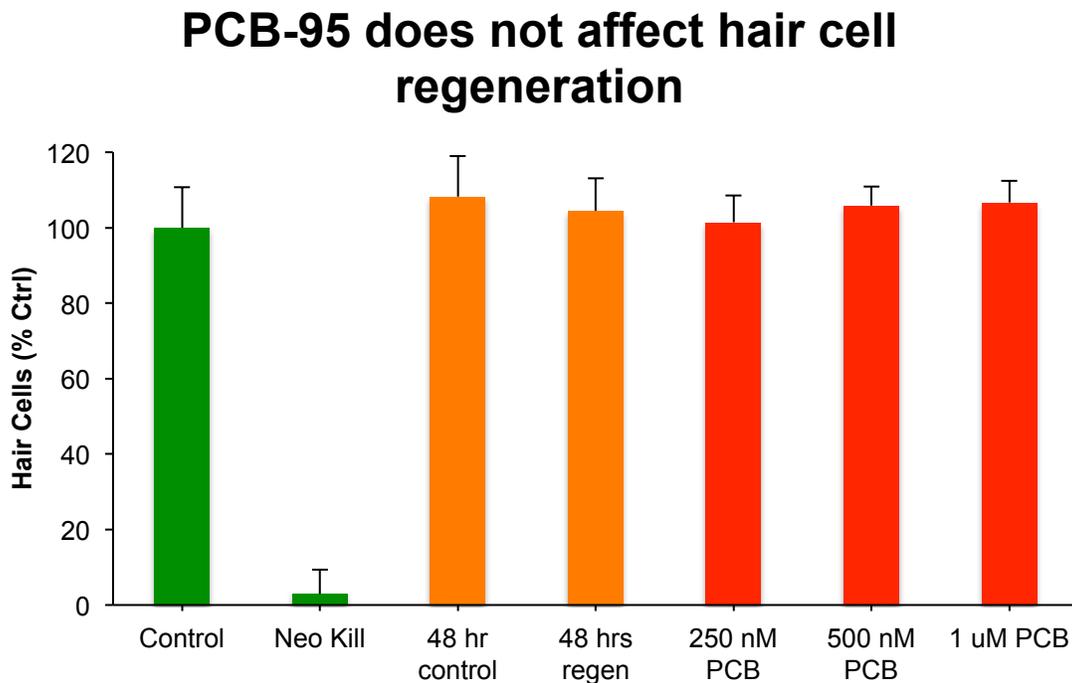
<b>Environmental contaminant</b>	<b>Concentration Range</b>	<b>Environmentally significant concentration:</b>
PCB-95	250 nM, 500 nM, 1 $\mu$ M	1 nM (United States EPA, 2013)
BPA	1 $\mu$ M, 10 $\mu$ M, 20 $\mu$ M	0.1 $\mu$ M (Flint et al, 2012)
Fluoride	25 $\mu$ M, 100 $\mu$ M 200 $\mu$ M	28 $\mu$ M (United States EPA, 2013)

Hair cell regeneration post-BPA exposure:

Sheth et al (2014) found that BPA is a hair cell toxin. We decided to examine hair cell regeneration after BPA induced hair cell death. 5-dpf zebrafish were exposed to 0.1  $\mu$ M or 20  $\mu$ M BPA for 24 hours, then removed and placed into fresh EM to be allowed to regenerate for 24 hours. A group of fish was immediately assessed after being removed from BPA as a control group, and an untreated group was also assessed for control. Hair cell regeneration was then assessed using either DASPEI scoring or ICC.

## Results:

**PCB-95 has no significant effect on hair cell regeneration.** Zebrafish were exposed to 250 nM, 500 nM, or 1  $\mu$ M PCB-95 for 48 hrs after hair cell kill, during the period of normal hair cell regeneration. We observed that at the concentrations used, which are well above the environmentally relevant concentration of 1 nM, that PCB-95 does not have any effect on the hair cells ability to regenerate. After 48 hours of PCB-95 exposure, there is no significant difference in amount of hair cells that regenerated with or without the presence of PCB-95.

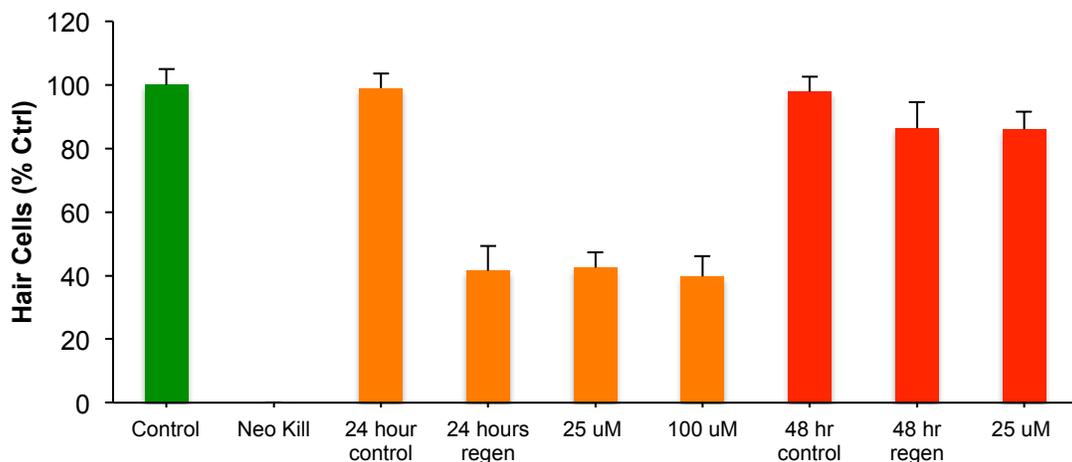


**Figure 4: PCB-95 has no significant effect on hair cell regeneration.** Hair cells were assessed with DASPEI. The green bars indicate the initial control groups. 300  $\mu$ M neomycin was used to kill hair cells. The orange bars indicate the control group 48 hours later, and 48 hours of regenerating in response to the neomycin kill. We observe complete

recovery. The red bars indicate 48 hours of regeneration in the respective concentrations of PCB-95. We see that PCB-95 concentration does not affect regenerative ability of hair cells in zebrafish (1 way ANOVA,  $p=0.93$ ).

**Fluoride has no significant effect on hair cell regeneration.** As with PCB-95, zebrafish were exposed to 25  $\mu\text{M}$ , 100  $\mu\text{M}$  and 200  $\mu\text{M}$  of fluoride while regenerating hair cells. We observed that at the concentrations used, which are at and well above the environmentally relevant concentration of 28  $\mu\text{M}$ , that fluoride does not have any effect on the hair cells ability to regenerate. After both 24 and 48 hours of regenerating in the presence of fluoride, there is no significant difference in amount of hair cells that regenerated with or without the presence of fluoride. 100  $\mu\text{M}$  and 200  $\mu\text{M}$  fluoride exposure were lethal to zebrafish.

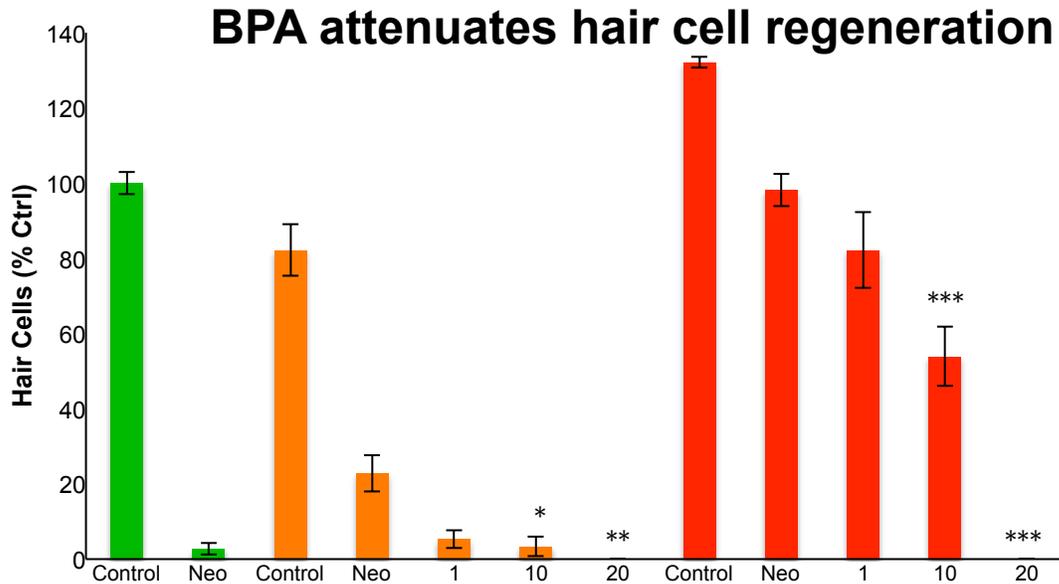
### Fluoride does not affect hair cell regeneration



**Figure 5: Fluoride has no significant effect on hair cell regeneration.** Hair cells were assessed with DASPEI. The green bars represent the initial control groups. 300  $\mu\text{M}$

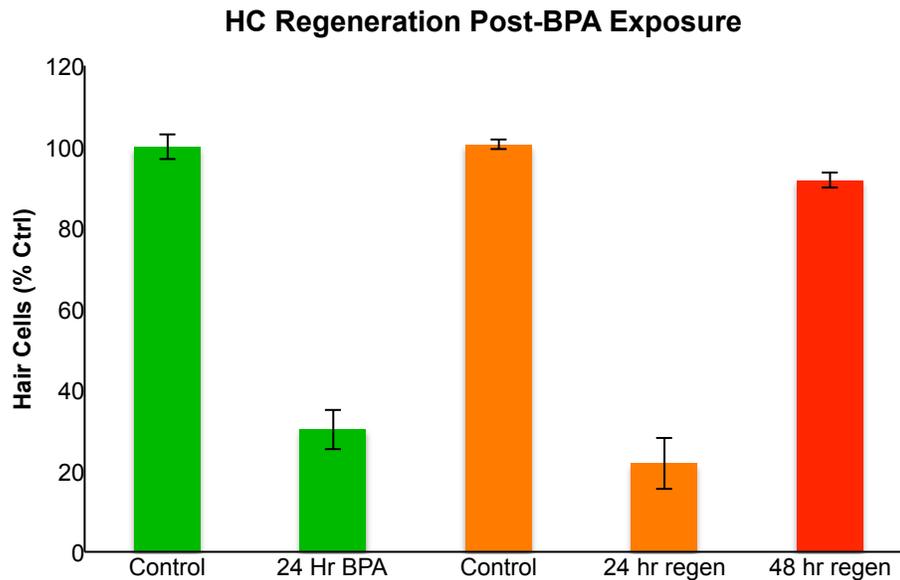
neomycin was used to kill hair cells. We saw complete hair cell kill with neomycin. The orange bars represent the fish assessed 24 hours later. We see no significant difference in regenerating hair cells in the presence or absence of fluoride at this point (1-way ANOVA,  $p=0.64$ ). Fish exposed to 200  $\mu\text{M}$  fluoride for 24 hours did not survive. The red bars represent fish assessed 48 hours later. Again, we see no significant difference between regenerating with or without fluoride (T-test,  $p=0.92$ ). Here, the 100  $\mu\text{M}$  and 200  $\mu\text{M}$  fluoride treated fish groups did not survive, which is why there is only a 25  $\mu\text{M}$  fluoride group shown at 48 hours.

**BPA attenuates hair cell regeneration.** We observed that BPA had a significant negative effect on hair cell regeneration in zebrafish. After 24 hours of regenerating in concentrations of BPA as low as 1  $\mu\text{M}$ , we observed that fewer hair cells as compared to the control group, which regenerated hair cells in the absence of BPA. We saw that when exposed to 20  $\mu\text{M}$  BPA, hair cells did not regenerate. This trend was true after 48 hours as well.



**Figure 6: BPA attenuates hair cell regeneration.** There is a significant effect of both BPA concentration and recovery time on hair cell survival, with more hair cells present after longer times or in lower BPA concentrations (2-way ANOVA,  $p < 0.001$ ). Green bars indicate fish not treated with BPA. There is a strong hair cell kill using 300  $\mu\text{M}$  neomycin. Orange bars represent controls, plus 24 hours of regeneration in BPA. The red bars represent 48 hours of regeneration in BPA. We observed no hair cell regeneration when fish were allowed to recover in 20  $\mu\text{M}$  BPA, suggesting that BPA is affecting the zebrafish's ability to regenerate their hair cells. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

**BPA exposure delays hair cell regeneration.** We saw that after 24 hours of being removed from BPA, the fish did not regenerate their hair cells, yet after 48 hours of recovery; fish seemed to regenerate their hair cells normally. This suggests that BPA may not be affecting supporting cells if the hair cells are able to regenerate, though the process is delayed.



**Figure 7: HC Regeneration after BPA exposure:** The green bars represent the initial control groups: untreated fish, and fish exposed to 20  $\mu$ M BPA for 24 hours. The orange bars represent 24 hours of regeneration in EM, and control fish. The red bar indicates 48 hours of regeneration in EM. We saw that there are decreased numbers of hair cells, or a delay of regeneration 24 hours later, even after removal from BPA. This data suggests that BPA may continue to kill hair cells post-exposure. After regenerating for 48 hours, we saw normal regeneration.

**Discussion:**

In this study we set out to test the effects of environmental contaminants PCB-95, BPA and fluoride on zebrafish ability to regenerate their hair cells. Fluoride and PCB-95 exposure showed no significant effect on hair cell regeneration, but BPA was observed to have a significant negative effect on hair cell regeneration. We also observed that after exposure to BPA, that hair cell regeneration was delayed. The main findings of this study

are that BPA contamination of aquatic environments may be having a detrimental effect on fish mechanosensory systems.

### BPA and Notch signaling

BPA is capable of disrupting cell signaling pathways such as Notch. Notch signaling is important for cellular differentiation and proliferation during development. In order for Notch to be functional as a transcription factor, a ligand such as  $\gamma$ -secretase must cleave the Notch intracellular domain (NICD) so it may move to the nucleus to modify gene expression. Baba et al. (2009) found that BPA acts as an inhibitor of  $\gamma$ -secretase, which therefore inhibits Notch signaling overall. BPA inhibits  $\gamma$ -secretase in a mechanism similar to DAPT, a known  $\gamma$ -secretase inhibitor commonly used to inhibit Notch signaling in experiments (Baba et al., 2009).

Notch signaling has been found to be important in hair cell regeneration for both fish and mammals. Young mice have the ability to regenerate hair cells when Notch signaling is inhibited, but this ability is quickly lost with age (Groves, 2010). Bramhall et al. (2014) has shown that pharmacological inhibition of Notch signaling in mice leads to hair cell regeneration. In zebrafish, a species that naturally regenerates hair cells, Notch signaling genes are upregulated following ototoxin exposure (Ma et al., 2008). Inhibiting  $\gamma$ -secretase with DAPT while hair cells are regenerating leads to excess hair cell regeneration, suggesting that Notch signaling limits hair cell regeneration by preventing supporting cells from over-proliferating (Ma et al., 2008). Therefore, Notch signaling plays a significant role in hair cell regeneration and differentiation from supporting cells to hair cells.

Since BPA has been shown to inhibit Notch signaling (Baba et al., 2009), and inhibiting Notch signaling results in increased hair cell regeneration (Ma et al., 2008; Groves, 2010) we would expect to see increased regeneration when fish are exposed to BPA. Interestingly we see the opposite. Sheth et al. (2013) found that BPA is toxic to hair cells, which may explain what we are seeing. If cells were trying to regenerate in a chemical toxic to them, it would make sense that less regeneration would occur, because BPA may kill the new hair cells as they regenerate. It could also be that BPA is toxic to supporting cells, the cells that proliferate and produce new hair cells. One way to test this hypothesis is to measure supporting cell proliferation in BPA-treated fish.

In zebrafish, the primary regenerative mechanism is supporting cell proliferation, followed by differentiation of the new cells into hair cells. Ma et al. (2008) found that there is a peak in supporting cell proliferation numbers that takes place 12-21 hours after ototoxin induced hair cell death. To tell if BPA is affecting the regenerative mechanism in zebrafish or killing newly differentiated hair cells, we can use a compound called BrdU. BrdU is used to label proliferating cells. We hypothesize that if BPA is affecting the proliferative process, we will see fewer BrdU labeled cells in fish regenerating in BPA compared to fish regenerating in fresh water. If we see no difference in BrdU labeled cells between fish regenerating in BPA versus fish regenerating in fresh water, we hypothesize that BPA is not affecting the regenerative process, but rather killing newly differentiated hair cells.

## BPA and Estrogen Signaling

BPA can act on pathways other than Notch. One of these pathways is the estrogen signaling pathway. BPA is an endocrine disruptor, which means it can directly compete with estrogen to bind to estrogen receptors (Gould et al., 2008). The role of estrogen in the ear is not fully understood. Sisneros et al. (2003) found that female midshipmen fish have better hearing in the summer compared to the winter. Implanting non-reproductive winter midshipmen fish with estrogen results in better hearing and more hair cells similar to the midshipmen fish in the summer (Sisneros et al. 2004; Coffin et al. 2012; Mohr, unpublished communication). This suggests that increased estrogen levels result in increased proliferation of supporting cells to produce more hair cells, allowing for better hearing.

BPA disrupts estrogen signaling, which is potentially involved in hair cell regeneration. This could mean that BPA, in addition to being toxic to hair cells, is reducing supporting cells proliferative ability via a different signaling pathway other than Notch. This could explain why even though BPA disrupts Notch, and Notch inhibition leads to increased regeneration, that we still see a reduction in hair cell regeneration with BPA treatment. Estrogen treatment has been shown to upregulate estrogen receptor expression as well as increase cell proliferation in mouse embryonic cells (Reviewed in McCullar et al., 2009). Estrogen treatment also increased proliferation of glial cells and neural stem cells in embryonic rats (Reviewed in McCullar et al., 2009). We hypothesize that estrogen treatment may increase regenerative proliferation of supporting cells in zebrafish. We also predict that BPA may outcompete estrogen's potential positive effect on supporting cell proliferation, therefore reducing hair cell regeneration.

Another question that will be addressed is if hair cells regenerated in the presence of an environmental contaminant are fully functional. This can be checked using an FM1-43 uptake assay. Functional hair cells will uptake the FM1-43 dye through the transduction channels and can then be visualized. If a newly regenerated hair cell is non-functional, it will not uptake FM1-43. Even though zebrafish are able to regenerate hair cells despite environmental contaminant presence, it does no benefit to the organism if the regenerated hair cells are not functional.

### Conclusion

We found that the environmental contaminant BPA may be having negative effects on the mechanosensory systems of fish. BPA exposure is linked to defective reproductive function, diabetes and cancer (Rezg et al., 2014). This research suggests BPA exposure also could possibly be affecting hearing. With this study, we offer further support for finding safer alternatives to BPA in industrial and commercial use to mitigate human and aquatic life exposure to this ubiquitous contaminant.

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