THE ROLE OF THE LATERAL LINE IN EARLY LARVAL ZEBRAFISH AND CONTRIBUTION TO MULTISENSORY BEHAVIORS

By

Alexandra Venuto

July 2023

Director of Dissertation: Timothy Erickson and Fadi Issa

Major Department: Biology

ABSTRACT

The lateral line is a hair cell-based sensory system that is important for multisensory behaviors like schooling, rheotaxis, and predator/prey detection in aquatic vertebrates. This dissertation work uses the first genetic model for the congenital loss of lateral line function in zebrafish. We have found zebrafish ohnologs of a gene required for hair cell function whose mRNA expression patterns are cleanly partitioned between inner ear and lateral line hair cell populations. Genetic disruption of each ohnolog results in specific loss of either auditory/vestibular function or lateral line function. Since lateral line mutants exhibit normal auditory and vestibular behaviors, we can investigate lateral line-mediated behaviors in developing larval zebrafish.

In my second chapter, I develop a repeated treatment timeline using neomycin sulfate to continually ablate the lateral line in early larval zebrafish to compare to the genetic lateral line mutant. I examine hair cell regeneration and proliferation rates, as well as associated toxicity of repeated neomycin treatments in larval zebrafish. In conclusion, I find that low doses of neomycin sulfate treatments every 12 hours in 3-4 day old zebrafish are sufficient to continuously disable lateral line function.

In my third chapter, I begin my analysis of these "lateral line-less" fish to investigate the role of the lateral line in modulating expression of the social hormone *parathyroid hormone 2*. Parathyroid hormone 2 (Pth2) is a vertebrate-specific neuropeptide for which thalamic expression is upregulated by social contact with conspecifics. In this project, I measure *pth2* levels in zebrafish mutants lacking hair cell function in either the lateral line only, or in both the inner ear and lateral line. Socially raised lateral line mutants express lower levels of *pth2* relative to wild-type siblings, but there is no further reduction when all sensory hair cells are nonfunctional. However, social isolation of hair cell mutants causes a further reduction in *pth2* expression, pointing to additional unidentified sensory cues that influence *pth2* production. Lastly, I report that social context modulates fluorescent transgenes driven by the *pth2* promoter. Altogether, these data suggest that lateral line mutants experience a form of isolation, even when raised socially.

In my fourth chapter, I characterize a novel swim bladder over-inflation phenotype associated with the lateral line mutants. Larval zebrafish achieve neutral buoyancy between 3-4 days post-fertilization by gulping air from the water's surface to inflate their swim bladders. We define this behavior of swimming to the air-water interface as "surfacing." Little is known about the sensory basis for this underappreciated behavior of larval fish. I observe that approximately half of lateral line mutants over-inflate their swim bladder during initial inflation and become positively buoyant. Thus, I hypothesize that larval zebrafish use their lateral line to sense the air-water interface during the surfacing behavior to regulate swim bladder inflation. I report that (i) over-inflation is caused by abnormal surfacing behaviors in lateral line mutants, (ii) lateral line defects are responsible for swim bladder over-inflation, and (iii) the lateral line is specifically responsible for surface detection during initial inflation. In summary, I reveal a novel sensory basis

for achieving neutral buoyancy where larval zebrafish use their lateral line to sense the air-water interface and regulate initial swim bladder inflation.

In my fifth chapter, I characterize surfacing as a multi-sensory behavior that involves photosensory and chemosensory cues in addition to mechanosensory cues. Only half of lateral line mutants over-inflate their swim bladder, so I hypothesize that other sensory systems, like photosensory cues, help larvae to properly perform the surfacing behavior for initial swim bladder inflation. In this chapter, I use a combination of genetic and environmental manipulations to investigate the roles of ocular and non-ocular photosensation, social isolation, and chemosensory cues on initial swim bladder inflation in the context of genetic loss of lateral line function. Overall, I find that initial swim bladder inflation in larval zebrafish is affected by non-ocular photosensory and chemosensory cues when larvae are genetically deprived of both visual and lateral line sensation.

THE ROLE OF THE LATERAL LINE IN EARLY LARVAL ZEBRAFISH AND CONTRIBUTION TO MULTISENSORY BEHAVIORS

A Dissertation

Presented To the Faculty of the Interdisciplinary Program in Biology, Biomedicine, and Chemistry, Harriot College of Arts and Sciences

East Carolina University

Submitted in Partial Fulfillment of the Requirements for the Degree

PhD in IDPBBC

by

Alexandra Venuto

July, 2023

Director of Thesis: Fadi Issa, PhD
Director of Thesis: Timothy Erickson, PhD
Thesis Committee Members:
Rebecca Asch, PhD
Josef Trapani, PhD
Ariane Peralta, PhD

ACKNOWLEDGEMENTS

I would like to thank my mom, Amy Venuto, for her overwhelming support, love, and encouragement. She is my role model and best friend, and she has sacrificed so much for me to live this wonderful life. I am immensely grateful for my grandparents, Maxine and Chaunce Venuto, for their support and being the greatest grandparents ever. I am also thankful for my partner, Albert Tucci. Not only did his vast understanding of statistics and data visualization enable me to complete many aspects of this dissertation, but his love and support has helped me enormously. I would also like to thank my dogs, Maggie (Moogs) and Woody for being the cutest pups and for their unyielding love. I am thankful for my friends, Dr. Katie Clements, Caitlin Dougher, Samantha Choo, Sophie Centi, Dr. Chris Anderson, Dr. Danielle Finger, Skyler Carrell, Chris Knuckles, and Jasper Leavitt for always being there for me and all the fun times we have shared! I also thank Taylor Swift for being the soundtrack to my life since I was 11.

I would like to thank my advisor, Dr. Timothy Erickson, for being an incredible mentor. His impact on my growth has been immeasurable. I also thank Dr. Fadi Issa for taking me in to his lab when Tim moved – I could not have completed this dissertation without his generosity. I am grateful to my committee members, Dr. Josef Trapani, Dr. Rebecca Asch, and Dr. Ariane Peralta for their tremendous guidance. I also thank my undergraduate research advisor, Dr. Angela DiBenedetto, for her support. I would like to thank co-authors on published chapters of this dissertation, Cameron Smith, Stacey Thibodeau-Beganny, and Marybelle Cameron-Pack, for their hard work. I thank Dr. Yong Zhu for his technical assistance. I also would like to thank the Nicolson, Schoppik, Prober, and Trapani labs for sharing fish lines used in this dissertation. Finally, I thank the members of the Issa and Erickson labs (both at ECU and UNB) and the ECU Biology Department for being amazingly helpful these last five years. Thank you.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Aanat2	arylalkylamine N-acetyltransferase 2
ANOVA	analysis of variance
Atoh7	atonal bHLH transcription factor 7
Cacna1da	calcium channel, voltage dependent, L type, alpha 1D subunit, a
cDNA	complementary DNA
CFP	cyan fluorescent protein
ChR2	Channelrhodopsin-2
CuSO4	copper sulfate
DAT	dopamine transporter
DNA	deoxyribonucleic acid
dpf	days post-fertilization
E3	embryo media
GFP	green fluorescent protein
hpf	hours post-fertilization
ISH	*in-situ* hybridization
L/D	light/dark
Lhfpl5	lipoma HMGIC fusion partner-like 5
MET	mechano-electrical transduction
MON	medial octavolateralis nucleus
mRNA	messenger RNA
myo6b	myosin VIb
ORC	olfacto-retinal centrifugal

OXT	oxytocinergic
Pcdh15a	protocadherin-related 15a
PCR	polymerase chain reaction
PPI	prepulse inhibition
Pth2	parathyroid hormone 2
PVL	periventricular layer
qPCR	quantitative PCR
RNA	ribonucleic acid
RFP	red fluorescent protein
Slc12a2	solute carrier family 12 member 2
Slc6a3	solute carrier family 6 member 3
Sst7	somatostatin 7
TIP39	Tuberoinfundibular peptide of 39 residues
Tomt	transmembrane O-methyltransferase
WT	wild type

CHAPTER 1: INTRODUCTION

Animals of all ages are constantly assessing their surroundings and using any sensory information available to make behavioral decisions. Sensory cues are often critical at young life stages to perform developmental behaviors that are necessary for continued growth and survival. Many of these behaviors are multi-sensory, involving combinations of various sensory inputs to react appropriately. For example, a baby learning to walk requires visual, vestibular, and tactile sensory capabilities. With practice and further development of recognizing various sensory cues, walking eventually feels like an innate ability. These foundations of movement can be further developed and joined with other sensory cues to achieve a plethora of behaviors, like dancing to a beat or running from a strange noise.

While most fish are unable to walk, they are no exception to using sensory cues to guide movement in the form of swimming. It is imperative to survival that fish from early larval stages through adulthood can enact behaviors like foraging for food, phototactic movement based on light, and escape responses to predators (Warburton 2003; Mueller and Neuhauss 2010; Domenici and Hale 2019). For larval zebrafish, their yolk sac full of nutrients is depleted by 5 days post-fertilization (dpf) and they must search for exogenous food (Wilson 2012). These behaviors require rapid development of both physical features and sensory systems for detection and appropriate reactions to their environment. For my dissertation, I aim to further our understanding of multisensory behaviors in early larval zebrafish, and I hypothesize that the *mechanosensory lateral line* is a significant contributor to social detection and initial swim bladder inflation.

An aquatic sixth sense: the mechanosensory lateral line

To help with navigating the hydrodynamic environment, fish and some amphibians possess a sixth sensory system called the *lateral line* (Dijkgraaf 1963). The lateral line is composed of

mechanosensory organs called neuromasts that are distributed along the body of the organism (Ghysen and Dambly-Chaudière 2007; Thomas et al. 2015) (Figure 1). In many adult fish species, there are two types of neuromasts – superficial and canal. The main differences are that superficial neuromasts occur on the skin and detect flow velocity, and canal neuromasts are sub-epidermal and detect acceleration (Montgomery et al., 1997).

Each neuromast contains many hair cells, which are the sensory receptors for the lateral line (Figure 1). At the apical end of the hair cell are stereocilia and a kinocilium which comprise the hair bundle (Hudspeth 1989). Hair cells convert mechanical force into electrical signals via mechano-electrical transduction (MET) channels at the tips of stereocilia in the hair bundle (Corwin and Warchol 1991). The MET complex is a multi-subunit ion channel, which allows potassium and calcium ions to enter the cell as the hair bundle leans toward the kinocilium. This deflection of the hair bundle leads to depolarization of the hair cell (Gillespie 1995).

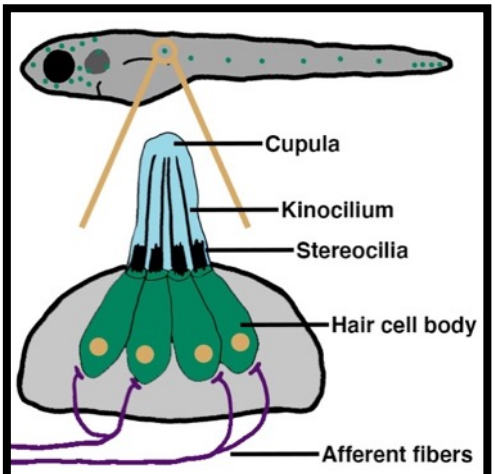


Figure 1. The mechanosensory lateral line. Larval zebrafish possess an anterior (head) and posterior (body) lateral line. Neuromasts contain the sensory hair cells responsible for detecting lateral line stimuli.

The lateral line influences behavior through transmission of mechanosensory stimuli from hair cells to afferent neurons (Ghysen and Dambly-Chaudière 2004; Nicolson et al. 1998). In larval zebrafish, the signal reaches an area in the hindbrain that corresponds to the medial octavolateralis nucleus (MON) in adult zebrafish (Liao and Haehnel 2012). This is where lateral line mechanoreceptive information is initially processed (McCormick 1981). Further processing occurs at higher-brain centers, which enables the nervous system to guide active body movements

(McCormick and Hernandez 1996). This neural circuit allows for fish to detect hydrodynamic information and respond with appropriate behaviors. Although behaviors reliant on lateral line sensory information have been under investigated in early larval fish, lateral line-mediated behaviors in juvenile and adult fish are well documented.

Multisensory behaviors in fish involving lateral line sensory input

The lateral line is integral to many multisensory behaviors in fish, including rheotaxis (or swimming against a current), schooling/shoaling, foraging, and predator avoidance (Suli et al. 2012; Bleckmann 2006; Montgomery, Baker, and Carton 1997; Baker and Montgomery 1999). Rheotaxis primarily combines visual and lateral line sensory inputs to orient and swim against oncoming water flow, but tactile and vestibular cues can also contribute (Coombs, Bak-Coleman, and Montgomery 2020). The lateral line is primarily responsible for water-motion cues, while the visual system (as well as proprioceptive and somatosensory systems) is responsible for body-motion cues where fish movement is compared to an external and fixed location (Arnold 1974; Montgomery, Baker, and Carton 1997; Bak-Coleman, Smith, and Coombs 2015; Baker and Montgomery 1999). Vestibular cues enable understanding of fish acceleration without the requirement of external fixtures (Pavlov and Tjurjukov 1995). Larval zebrafish exhibit rheotactic behaviors as early as 5 dpf, and it has been demonstrated that this behavior can persist despite the absence of any one of the aforementioned sensory systems (Baker and Montgomery 1999; Bak-Coleman et al. 2013; Coombs, Bak-Coleman, and Montgomery 2020). However, if fish are deprived of both visual and lateral line sensory systems, rheotaxis is significantly impaired (Liao and Haehnel 2012; Suli et al. 2012).

Schooling and shoaling are similar to rheotaxis where lateral line and visual inputs are primarily used to perform the behavior (Chaput et al. 2023; Peichel 2004; Pitcher, Partridge, and

Wardle 1976). Previous studies have determined that the lateral line is specifically implicated in body position while schooling or shoaling, as well as group startle response latency (Partridge and Pitcher 1980; Faucher et al. 2010). However, both sensory systems are required for maintaining appropriate distances between conspecifics (Partridge and Pitcher 1980; Chaput et al. 2023). The general consensus is that the ability to shoal or school decreases when either the visual or lateral line sensory systems are lost (Chaput et al. 2023).

During predator and prey detection, fish call upon the full gamut of sensory cues depending on context – mechanosensory, photosensory and chemosensory systems (Bianco, Kampff, and Engert 2011; Westphal and O'Malley 2013; Braubach et al. 2009; Carrillo and McHenry 2016; Kermen et al. 2013; Fischer et al. 2017; McHenry et al. 2009). There are instances where certain sensory cues take precedence, for instance, the lateral line is more heavily relied on for blind fish or those in dark or turbid environments (Lloyd et al. 2018; Carrillo and McHenry 2016). Even though it is a multisensory behavior, prey hunting is primarily driven by the visual system (Semmelhack et al. 2014; Förster et al. 2020). Therefore, processing for foraging in larval zebrafish largely occurs in the tectum, though many higher-processing brain centers contribute as well (Bianco and Engert 2015; Förster et al. 2020; Zhu and Goodhill 2023).

A heavily studied fish reaction to predators is the escape response, which is dependent on the direction of the predatory cue (Fetcho, Higashijima, and McLean 2008; Hatta and Korn 1998; Domenici 2010; Eaton and Emberley 1991). Auditory, visual, and tactile stimulation are the main triggers of this response, though other sensory inputs like the lateral line can contribute as well (Mirjany, Preuss, and Faber 2011; Domenici and Hale 2019). The neural circuit for this behavior is well defined where sensory cues, such as a loud noise, can go through a direct pathway from auditory hair cells to Mauthner cell to motor neurons (Korn and Faber 2005; Lacoste et al. 2015;

Medan and Preuss 2014). Sensory integration in the brain allows for fish to perform multisensory behaviors, like shoaling and foraging.

The cerebellum, tectum, and thalamus are major sensory integration centers in larval zebrafish

Primary processing of sensory information in zebrafish varies depending on the system. Some sensory inputs exhibit cross-modal sensory integration, like the visual and olfactory system with the olfacto-retinal centrifugal (ORC) pathway where olfactory stimulation can directly modulate visual sensitivity (Li 2019). However, most multisensory integration occurs at higher-processing centers in the brain, such as the cerebellum, tectum, and thalamus. The cerebellum is a sensorimotor processing region in the zebrafish brain (Ito 2006; Bell, Han, and Sawtell 2008). Many behaviors require knowledge of how the organism's body is generating movement in addition to stimuli generated from other organisms, and the cerebellum is responsible for managing this information (Knogler et al. 2017; Ito 2006). The lateral line exhibits a similar capacity for discriminating self-generated versus externally generated water movements (Perks, Krotinger, and Bodznick 2020). Known sensory inputs to the cerebellum include both visual and vestibular information, and the cerebellum likely mediates the vestibulo-ocular reflex during rolling movements (Knogler, Kist, and Portugues 2019; Blazquez et al. 2003; Laurens, Meng, and Angelaki 2013; Yakusheva et al. 2007; Favre-Bulle et al. 2018).

The tectum is another key processing center, especially for visually guided behaviors (Ingle 1973). The tectum receives inputs from the visual, auditory, vestibular, lateral line, and chemosensory systems (Thompson et al. 2016; Jundi et al. 2023; Migault et al. 2018; Favre-Bulle et al. 2018). The tectal neuropil is dense with multiple layers, and visual and non-visual responses seem to be segregated between layers with some exceptions (Thompson et al. 2016). As noted above, the tectum is directly implicated in prey capture and visual escape, and is hypothesized to

be involved with other multisensory behaviors like rheotaxis (Bianco and Engert 2015; Bianco, Kampff, and Engert 2011; Muto et al. 2013; Helmbrecht et al. 2018; Coombs, Bak-Coleman, and Montgomery 2020; Filosa et al. 2016).

Finally, the thalamus is a sensory integration and relay center where there is representation of lateral line, visual, and vestibular information (Mueller 2012; Anneser et al. 2020; Kappel et al. 2022; Favre-Bulle et al. 2018; Migault et al. 2018). The zebrafish thalamus has been shown to respond to looming stimuli resulting in an escape response through light detection, and it relays this information to the tectum (Heap et al. 2018). Another behavior linked to the zebrafish thalamus is social detection and attraction, which seems to be mediated through visual or lateral line cues (Anneser et al. 2020; Kappel et al. 2022).

Sensory detection of social context

Social detection, specifically of conspecifics, is necessary in zebrafish for large-scale behaviors like shoaling (Peichel 2004; Pitcher, Partridge, and Wardle 1976). Chemosensory, photosensory, and mechanosensory cues are all used in various contexts to identify conspecifics (Butler and Maruska 2016a; Santacà, Dadda, and Bisazza 2021; Gerlach and Wullimann 2021; Kappel et al. 2022). Social isolation often results in negative consequences in animals (Amaral 2002; Mumtaz et al. 2018; Weiss et al. 2004). Similarly, isolation of zebrafish has been shown to increase anxiety, stress, and startle sensitivity (Tunbak et al. 2020; Shams et al. 2017; Forsatkar, Safari, and Boiti 2017). In larval fish specifically, isolation has also been demonstrated to modulate social avoidance reactions and appetite (Wee et al. 2022; Groneberg et al. 2020). There is evidence to support that the neural basis for this occurrence is the activation of an oxytocinergic (OXT) circuit in the hypothalamus that is modulated by chemical cues from conspecific animals and regulates the avoidance and feeding behaviors (Wee et al. 2022).

Another example of hormonal modulation based on social isolation in larval zebrafish involves the neuropeptide *parathyroid hormone 2* (*pth2*) found in the vertebrate thalamus (Anneser et al. 2020; Bhattacharya et al. 2011; Dobolyi et al. 2012). PTH2 has been linked to social behaviors in other animals, including maternal care and social contact in rodents (Cservenák et al. 2013; Keller et al. 2022). In larval and adult zebrafish, *pth2* exhibits a significant decrease in expression following isolation (Anneser et al. 2020). In this study, the authors found evidence to suggest that the lateral line may be responsible for detecting conspecifics and subsequent neuromodulation of *pth2.* However, it is unclear how congenital loss of lateral line function affects the recognition of social context in larval zebrafish, especially before 5 dpf.

Tools to functionally disable the mechanosensory lateral line

Given the role of the lateral line in many juvenile and adult behaviors, it is important that the lateral line's involvement in early larval behaviors is also investigated. However, it has been difficult to assess behaviors dependent on lateral line function in larval zebrafish before 5 dpf due to lack of genetic tools. Many studies have sought to understand lateral line mediated behaviors by chemically ablating lateral line hair cells with ototoxic compounds (Coffin and Ramcharitar 2016; Harris et al. 2003; Montgomery et al. 1997; Williams and Holder 2000). Ototoxins include aminoglycosides (e.g., neomycin), certain heavy metals (e.g., copper), and various platinum derivatives (e.g., cisplatin) (Coffin and Ramcharitar 2016; Linbo et al. 2006; Mackenzie and Raible 2012; Ou et al. 2007). These compounds disrupt the lateral line by entering hair cells and causing oxidative stress until cell death occurs (Olivari, Hernández, and Allende 2008; Owens et al. 2007; Sheth et al. 2017). The various types of ototoxins can cause unique effects on regeneration rates of hair cells following treatment (Mackenzie and Raible 2012; Owens et al. 2007), as well as locomotive behaviors like rheotaxis (Han et al. 2020; Newton et al. 2023).

While ablation techniques have shown that certain behaviors are affected by temporary loss of the lateral line, these techniques are unable to determine long-term consequences of loss of lateral line function. Most studies do not treat larval zebrafish before 5 dpf due to the rapid regeneration and proliferation of hair cells in the zebrafish lateral line at 2-4 dpf (Murakami et al. 2003; Santos et al. 2006). The efficacy of repeated treatments with ototoxin to mimic a congenital loss of lateral line function at this stage is not well understood. Given that there are many behaviors that occur in larval zebrafish before 5 dpf, it is essential that we establish a way to disable the lateral line from 2-4 dpf to understand lateral line-mediated behaviors in early larval fish. While repeated treatments are useful for this endeavor, a genetic mutant is the preferable method to evaluate the effect of congenital loss of lateral line function on larval zebrafish behaviors.

Previously, it has been challenging to identify a genetic mutation that exclusively affects the lateral line, since hair cells of the inner ear are extremely similar to hair cells of the lateral line. Lipoma HMGIC fusion partner-like 5 (*LHFPL5*) is a subunit of the MET channel in vertebrate

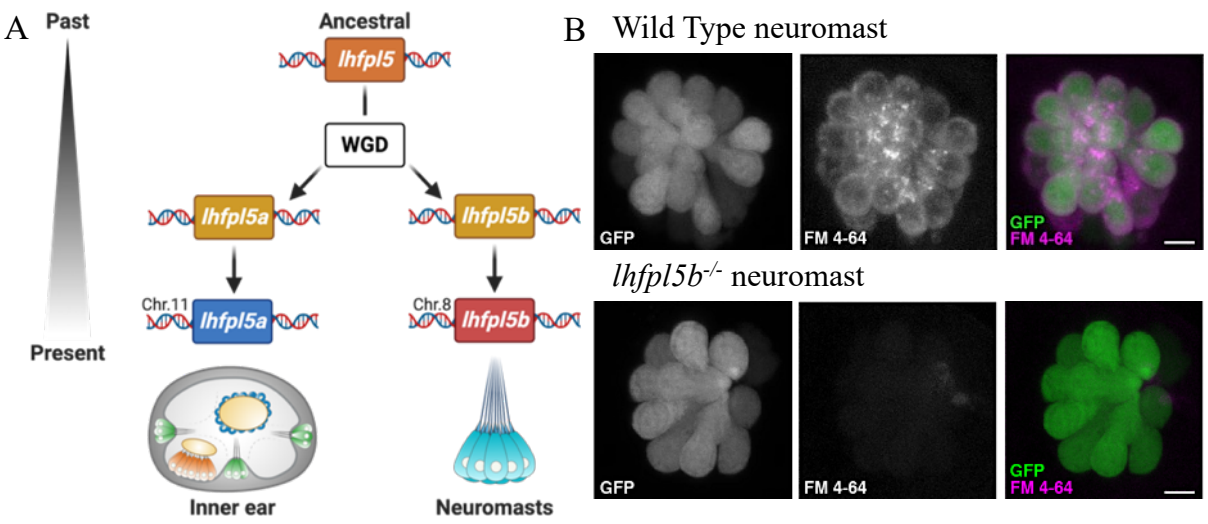


Figure 2. *lhfpl5* ohnologs in zebrafish. A) Diagram of whole genome duplication resulting in split-functionalization of the *lhfpl5* gene between the inner ear and lateral line. B) Confocal images of wild type and *lhfp5b* mutant larvae expressing the *Tg(myo6b:EGFP-pA)vo68* transgene in hair cells (green) and dyed in FM 4-64 (magenta) that only enters hair cells through functional MET channels. Mutant larvae possess non-functional hair cells as indicated through lack of FM dye uptake. Adapted from Erickson, et al., 2020.

hair cells, mutations in which cause deafness in humans, mice, and fish (Cunningham and Müller 2019; Xiong et al., 2012; Zhao et al. 2014; Erickson et al. 2020; Nicolson et al. 1998). Due to a whole genome duplication event, teleost fish possess ohnologous *lhfpl5* genes - *lhfpl5a* and *lhfpl5b*. In zebrafish, *lhfpl5a* is expressed solely in the auditory and vestibular cells of the inner ear and *lhfpl5b* is expressed only in hair cells of the lateral line (Erickson et al. 2020) (Figure 2). A CRISPR-Cas9 knockout of *lhfpl5b* (*lhfpl5b*^{vo35}) is the first genetic mutant where the lateral line is non-functional from birth but hearing and balance are normal. These zebrafish mutants provide an exciting opportunity to uncover novel roles for the lateral line, especially in behaviors that occur before 5 dpf like initial swim bladder inflation.

Swim bladder inflation in teleost fish

The swim bladder is a hydrostatic organ that gives fish neutral buoyancy in the water (Alexander 1966). Without a gas-filled swim bladder, the density of the fish causes it to sink, and continuously swimming upwards in the water column to counteract sinking exhausts energy (Alexander 1966). The swim bladder resides between the kidney and intestines in teleost fish, and development of the swim bladder involves an outpunching of the gut (Pelster 2004). There are two types of teleost fish with distinct swim bladders: physostomous and physoclistous. Physoclistous fish have a temporary connection from the swim bladder to the gut through the pneumatic duct, whereas physostomous fish retain a permanent connection (Bailey and Doroshov 1995).

There are grave consequences for fish that fail to inflate their swim bladders during the larval period, including disease, deformities, and ultimately death (Doroshev, Cornacchia, and Hogan 1981). Environmental factors such as water temperature and turbidity can have species-specific effects on swim bladder inflation (Battaglene and Talbot 1993; Trotter, Battaglene, and Pankhurst 2003; Martin-Robichaud and Peterson 1998). Non-inflation can occur when larvae lack

access to the air-water interface due to physical barriers, and often results in lordosis (Chapman et al. 1988; Kitajima et al. 1994). Swim bladder over-inflation is equally detrimental to fish survival and can occur when water is supersaturated with atmospheric gasses (Cornacchia and Colt 1984). In some fish species, swim bladder over-inflation presents as swim bladder disease causing the fish to swim sideways or upside down (Sirri et al. 2020). Both non-inflation and over-inflation are substantial issues in the fish farming industry (Woolley & Qin 2010), and it is important that researchers consider the behaviors necessary for fish to achieve appropriate swim bladder inflation.

Surfacing as a multisensory behavior – the climb to the surface

One of the most crucial actions that larval zebrafish execute at 3-4 dpf is a "swim up" or "surfacing" behavior for initial swim bladder inflation (Gisbert and Williot 1997; Lindsey, Smith, and Croll 2010) (Figure 3). To initially inflate the swim bladder, larval fish must identify which way is up, swim that direction, detect the surface, and then ingest air (Lindsey, Smith, and Croll 2010). That ingested air is then forced through the pneumatic duct that connects the swim bladder to the gut for initial inflation (Doroshev, Cornacchia, and Hogan 1981). Although the exact

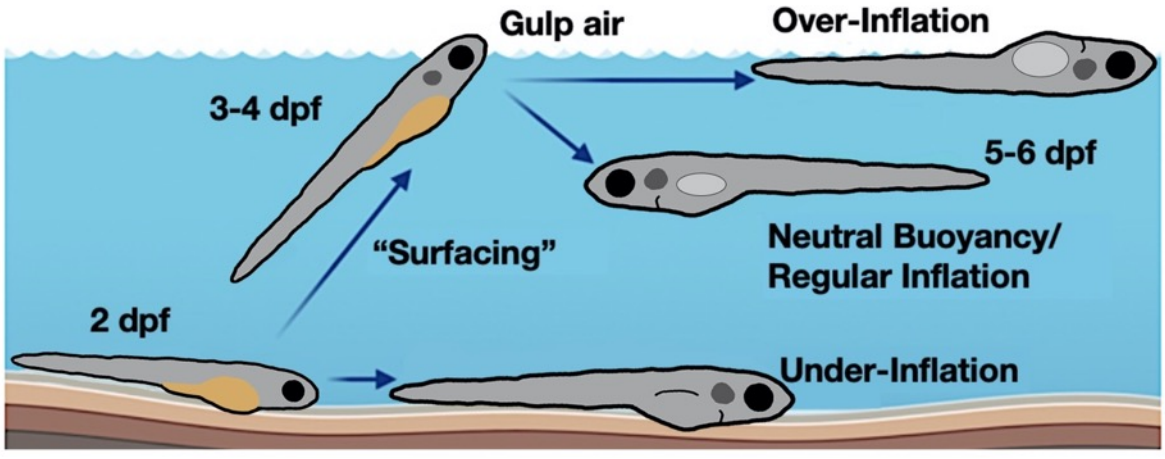


Figure 3. The surfacing behavior of larval zebrafish for initial swim bladder inflation. At 2 dpf, larvae hatch and test their swimming skills on the bottom of a tank or on the seafloor. By 3-4 dpf, they begin the vertical ascent to the surface for exogenous air intake to the swim bladder. By 5-6 dpf, initial swim bladder inflation is complete.

timeframe for initial swim bladder inflation varies depending on the species, the behavior occurs during a critical period of a few days where sensory systems and physical features mature simultaneously to allow for the transition from endogenous to exogenous feeding (Bailey and Doroshov 1995; Chatain 1989; Kimmel et al. 1995; Kitajima et al. 1994; Doroshev, Cornacchia, and Hogan 1981).

One sensory organ known to affect initial swim bladder inflation is the inner ear. There are two otolithic organs per ear in larval fish. These sensory organs are the saccule and utricle, where the former is primarily auditory, and the latter is primarily vestibular. The otoliths are attached and mechanically coupled to the hair cells responsible for auditory and vestibular information (Haddon and Lewis 1996). Mutations to the MET channel in hair cells result in compromised auditory and vestibular systems (Nicolson et al. 1998) and typically result in non-inflation of the swim bladder. The loss of the vestibular system, and specifically the inability to detect gravity by the utricular otolith, is responsible for the phenotype since larvae are unable to perform the surfacing behavior (Riley and Moorman 2000).

Gravitaxis refers to the movement of an organism in a specific direction in response to gravity. It has been studied in microorganisms (Häder 1999), flies (Kamikouchi et al. 2009; Hirsch 1959), and worms (Chen et al. 2021), but may be involved in the surfacing behavior in larval zebrafish as well. The vestibular system gives larvae an understanding of directionality based on gravity and has been demonstrated to help zebrafish larvae achieve coordinated vertical locomotion (Ehrlich and Schoppik 2017; Bagnall and Schoppik 2018; Ehrlich and Schoppik 2019). Additionally, when larval zebrafish are raised in a simulated microgravity chamber, they exhibit swim bladder non-inflation (Lindsey 2011). Video recordings show that the swim-up portion of surfacing is significantly slower and decreased in larvae subjected to the zero-gravity sensation

compared to larvae outside of the chamber (Lindsey 2011). Taken together, it appears that proper vestibular functioning and detection of gravity is necessary for the vertical climbing portion of the surfacing behavior to allow larvae to reach the surface for initial swim bladder inflation.

The effect of photoperiod on swim bladder inflation has been studied in many fish species to assist in fish farming efforts. Many studies have shown that constant light is associated with a decrease in swim bladder inflation rates and constant darkness with an increase in swim bladder inflation rates (Battaglene and Talbot 1990; Suchocki and Sepulveda-Villet 2019; Martin-Robichaud and Peterson 1998). The shift in swim bladder inflation based on photoperiod is due to larvae inflating primarily during dark hours (Trotter, Battaglene, and Pankhurst 2003), which is thought to be helpful for energy conservation and predator avoidance (Battaglene, McBride, and Talbot 1994). However, some species are unaffected by photoperiod (Hirata et al. 2009; Stuart and Drawbridge 2012), and others increase swim bladder inflation in full light conditions (Brüning, Hölker, and Wolter 2011) (Table 1).

Fish	**Manuscript**	**Dark**	**Light**	**No SD**
Australian Bass (*Macquaria novemaculeata*)	Battaglene and Talbot 1990	X		
California Yellowtail (*Seriola lalandi*)	Stuart and Drawbridge 2012			X
Bleak (*Albernus albernus*)	Brüning et al. 2011			X
Chub (*Leuciscus cephalis*)	Brüning et al. 2011		X	
Roach (*Rutilis rutilis*)	Brüning et al. 2011			X
European Seabass (*Dicentratchus labrax*)	Villamizar et al. 2009	X*		
Greater Amberjack (*Seriola dumerili*)	Hirata et. al. 2009			X

Pacific Blue Fin Tuna (*Thunnus orientalis*)	Kurata et. al. 2017	X		
Sand Whiting (*Sillago ciliata*)	Battaglene et al. 1994	X		
Striped Bass (*Morone saxatilis*)	Martin-Robachaud and Peterson 1998	X		
Striped Trumpeter (*Latris lineata*)	Trotter et al. 2003	X		
Yellow Perch (*Perca flavescens*)	Suchoki and Sepulveda-Villet 2019			X
Yellowfin Tuna (*Thunnus albacares*)	Partridge et. al. 2011	X		
White Seabass (*Atractoscion nobilis*)	Stuart & Drawbridge 2012			X

Table 1. The effect of photoperiod on initial swim bladder inflation in various fish species. An X in the "Dark" column indicates swim bladder inflation rates were highest in the photoperiod with the highest number of dark hours within the experimental conditions, and vice versa for the "Light" column. If changes to the photoperiod made no significant difference in swim bladder inflation rates, there is an X in the "No SD" column. The asterisk (X*) represents an unusual finding where there were more cases of swim bladder over-inflation in full light, but an overall increase in swim bladder inflation rates when the photoperiod included darkness.

While changes to photoperiod alter photosensory cues, the different types of photosensation (visual, pineal, and deep brain) have not been individually investigated for their effect on initial swim bladder inflation. Ocular photoreceptors provide visual acuity, while pineal and deep brain photoreceptors recognize light (Meissl and Yanez 1994). Fish can use non-ocular photoreceptors to perform light-driven behaviors and phototactic movement (Fernandes et al. 2012; Karpenko et al. 2020). Therefore, it is likely that non-ocular photoreceptors have an influence on the climbing portion of the surfacing behavior for species that exhibit higher rates of swim bladder inflation in darkness.

Pineal-based photoreception is responsible for driving circadian rhythm in zebrafish and controlling rhythms of locomotor activity (Ben-Moshe Livne et al. 2016; Ekström and Meissl 1997). For species that exhibit changes in swim bladder inflation when exposed to slight alterations to the photoperiod (while still having light and dark hours), it is plausible that pineal photoreceptors modulate the surfacing behavior. Deep brain photoreception has also been implicated in phototactic abilities, as determined through studies using eyeless zebrafish larvae with ablated pineal photoreceptors. These larvae that are only capable of deep brain photoreception perform a dive response and a brief period of hyperactivity upon loss of illumination (Fernandes et al. 2012). Zebrafish are generally positively phototactic (prefer light over darkness), although this status can change depending on the behavior (Chen and Engert 2014; Mueller and Neuhauss 2010). It is unclear whether zebrafish exhibit phototactic responses during initial swim bladder inflation since the effect of photosensory cues on the surfacing behavior has yet to be examined in larval zebrafish.

Chemosensation is another sensory input that may influence initial swim bladder inflation. Fish have three chemosensory systems responsible for detecting chemical stimuli in their environment: olfaction, taste, and solitary chemosensory cells (Hansen and Reutter 2004). In zebrafish, various chemostimulants have been shown to affect larval behavior, including pheromones, contaminants, and odorants where the chemical basis is amino acids or bile acids (Michel and Lubomudrov 1995; Ueda 2021; Lindsay and Vogt 2004). Some larval fish like lamprey use chemosensory cues for migration, wherein damage-released alarm cues increased the rate of downstream migration (Zielinski et al. 2005; Johnson et al. 2019). These findings suggest that larval fish could use chemotactic cues during the vertical ascent portion of initial swim bladder inflation. For example, larvae could release a pheromone or other chemical cue upon successfully reaching the surface that encourages conspecifics to do the same.

Surfacing as a multisensory behavior – detection of the surface

While vestibular, chemosensory, and non-ocular photosensory cues may aid in the vertical climb to the surface for initial swim bladder inflation, larval fish also require sensory cues once they reach the surface. Without surface detection, larvae are unable to intake exogenous air to their swim bladder. Visual acuity may aid in this part of the behavior. As noted previously, visual cues aid in multisensory behaviors like rheotaxis by providing body motion cues in reference to an identified external location (Arnold 1974; Coombs, Bak-Coleman, and Montgomery 2020). Additionally, visual photosensation becomes available and involved in other behaviors at 3 dpf in larval zebrafish, just in time for initial swim bladder inflation (Easter and Nicola 1996; Zimmermann et al. 2018). However, the specific involvement of vision has not been investigated for its role in surface detection during initial inflation.

The lateral line is also well-suited to detect the air water interface during initial swim bladder inflation. The surface possesses the unique quality of surface tension compared to the rest of the water column, which may be interpretable by the lateral line. The lateral line has been demonstrated to sense surface waves generated by predators, prey, and conspecifics (Bleckmann and Schwartz 1982; Wilcox 1988). Additionally, it is hypothesized that flying fish use their lateral line to detect entrance and exit from the water's surface (Gibbs 2004; Tsukamoto and Yoshino 1957). However, the link between lateral line and surfacing behaviors has not been identified, likely due to a lack of tools to disable the lateral line before 5 dpf. The *lhfpl5b* mutant provides the opportunity to investigate the lateral line's role in surface detection during initial swim bladder inflation.

Themes for this Dissertation

Multisensory behaviors, especially those involving lateral line sensation, have been sparsely investigated in early larval zebrafish despite being critical to fish development. In this dissertation, I tested the role of the lateral line in the multisensory behaviors of social detection and initial swim bladder inflation in larval zebrafish before 5 dpf (Figure 4). To assess the role of the lateral line in zebrafish before 5 dpf, it was necessary to develop tools that adequately disable the actively proliferating lateral line hair cells at this stage. In the second chapter, I developed the use of repeated ototoxic treatments to disable the lateral line from 2-5 dpf. I evaluated the toxicity and efficacy of repeated neomycin sulfate treatments at this stage and determined a repeated treatment timeline to effectively limit lateral line function in larval zebrafish before 5 dpf.

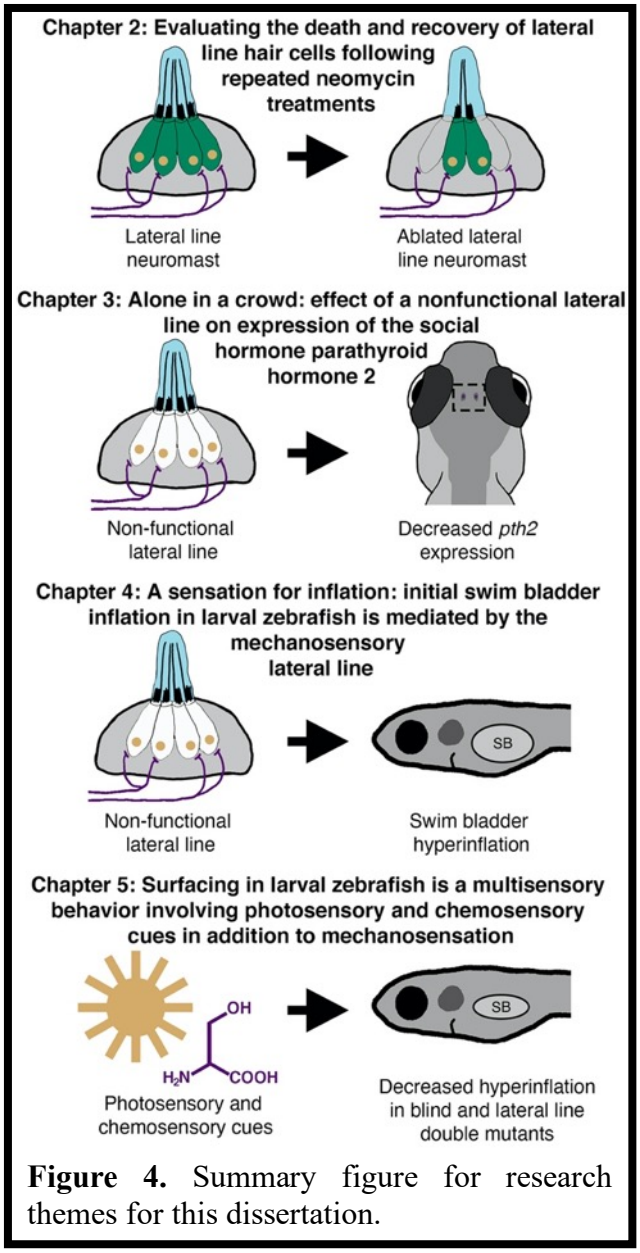


Figure 4. Summary figure for research themes for this dissertation.

In my third chapter, I used the repeated neomycin treatments as well as the lateral line mutant to investigate the role of the lateral line in detecting social context and modulating *pth2* expression. We knew from previous studies that *pth2* expression is modulated by social context,

but we were unsure how congenital loss of lateral line function would impact expression levels. I hypothesized that lateral line-less fish would exhibit a significant decrease in *pth2* expression. To test this hypothesis, I used both *in vitro* and *in vivo* techniques to find that larval zebrafish without lateral line function exhibited decreased *pth2* expression, similar to socially isolated larvae.

In my fourth chapter, I studied the surfacing behavior for initial swim bladder inflation in larval zebrafish that occurs between 3 and 5 dpf. While we knew that this complex behavior is required for fish survival and likely involves multiple sensory cues, the sensory basis for this behavior has been largely understudied. I hypothesized that the lateral line aids in the surfacing behavior for initial swim bladder inflation. In this study, I found that lateral line mutants exhibited hyperinflated swim bladders, which is an otherwise rare phenotype. I presented evidence to suggest that the lateral line is responsible for the surface detection portion of the surfacing behavior to mediate air intake to the swim bladder.

In my fifth and final research chapter, I examined the multisensory cues involved in the surfacing behavior for initial swim bladder inflation in larval zebrafish. We knew that photosensory cues play a role in surfacing for other species, so I hypothesized that ocular photosensory cues help lateral line mutants identify the surface and mediate initial swim bladder inflation. I tested this hypothesis by depriving lateral line mutant zebrafish larvae of ocular and non-ocular photosensory cues both genetically and environmentally. I found that visual cues alone do not aid initial swim bladder inflation, but that photoperiod has a moderate effect on swim bladder inflation in zebrafish larvae lacking visual and lateral line sensation. Through various forms of social isolation, I also found evidence to suggest a role for chemosensory cues in initial inflation when lateral line and visual sensory cues are absent. Overall, this dissertation offers novel findings on sensory detection

of the early larval zebrafish environment with particular focus on the role of the lateral line in multisensory behaviors (Figure 4).

CHAPTER 2: EVALUATING THE DEATH AND RECOVERY OF LATERAL LINE HAIR CELLS FOLLOWING REPEATED NEOMYCIN TREATMENTS

Published:

Alexandra Venuto and Timothy Erickson (2021) Evaluating the death and recovery of lateral line hair cells following repeated neomycin treatments. *Life* 2021, *11*, 1180. https://doi.org/10.3390/life11111180.

Abstract

Acute chemical ablation of lateral line hair cells is an important tool to understand lateral line-mediated behaviors in free-swimming fish larvae and adults. However, lateral line-mediated behaviors have not been described in fish larvae prior to swim bladder inflation, possibly because single doses of ototoxin do not effectively silence lateral line function at early developmental stages. To determine if ototoxins can disrupt lateral line hair cells during early development, we repeatedly expose zebrafish larvae to the ototoxin neomycin during a 36-hour period from 3-4 days post-fertilization (dpf). We use simultaneous transgenic and vital dye labeling of hair cells to compare 6- hour and 12-hour repeated treatment timelines and neomycin concentrations between 0–400 μM in terms of larval survival, hair cell death, regeneration, and functional recovery. Following exposure to neomycin, we find that the emergence of newly functional hair cells outpaces cellular regeneration, likely due to the maturation of ototoxin-resistant hair cells that survive treatment. Furthermore, hair cells of 4 dpf larvae exhibit faster recovery compared to 3 dpf larvae. Our data suggest that the rapid functional maturation of ototoxin-resistant hair cells limits the effectiveness of chemical-based methods to disrupt lateral line function. Furthermore, we show

that repeated neomycin treatments can continually ablate functional lateral line hair cells between 3–4 dpf in larval zebrafish.

Introduction

Fish and some amphibians possess a unique sensory system called the mechanosensory lateral line, which is comprised of clusters of mechanically-sensitive hair cells distributed all over the body in organs called neuromasts. The lateral line detects hydrodynamic information and contributes to several behaviors such as foraging, predator avoidance, and swimming coordination (Mogdans 2019; Montgomery and Baker 2020). For example, the lateral line is a critical sensory component for larval and adult fish to orient to water flow (rheotaxis) (Montgomery, Baker, and Carton 1997; Baker and Montgomery 1999; Suli et al. 2012). Additionally, aquatic organisms can identify locations of predators and prey by combining the direction and force of the water interacting with the neuromast pattern along the body (Coombs et al. 2000; Stewart et al. 2013).

To investigate these lateral line-mediated behaviors, researchers use a variety of physical and chemical strategies to functionally disrupt the lateral line (Coffin and Ramcharitar, 2016; Ton Parng 2005). The use of ototoxins to chemically ablate hair cells is the most popular technique due to their specificity, convenience, and applicability to a wide range of aquatic vertebrates. Common ototoxins include aminoglycoside antibiotics (e.g. neomycin), certain heavy metals (e.g. copper), and various platinum derivatives (e.g. cisplatin) (Harris et al. 2003; Linbo et al. 2006; Ou et al. 2007; Williams & Holder 2000). These compounds disrupt the lateral line by entering hair cells through functional mechanotransduction channels, causing oxidative stress, and triggering apoptotic cell death (Esterberg et al. 2016; Sha and Schacht 1999; Hirose, Hockenbery, and Rubel 1997; Olivari, Hernández, and Allende 2008; Owens et al. 2007). While useful for lateral line research, aminoglycosides are also major causes of irreversible hearing loss in humans (Forge and

Schacht 2000). Understanding how zebrafish hair cells respond to ototoxic assaults may lead to preventative or therapeutic strategies to combat human hearing loss (Kros and Steyger 2018).

The acute chemical ablation of hair cells is a valuable tool for studies on adult fish and free-swimming larvae. However, there is little information about how the lateral line influences behavior in early-stage larvae, likely because ototoxins are less effective at early developmental stages (Murakami et al. 2003; Santos et al. 2006). Previous research suggests two reasons why a single dose of ototoxin is less effective at younger stages compared to older larvae and adults: (i) the rapid proliferation of hair cells at early developmental stages, and (ii) the presence of immature hair cells that are resistant to chemical ablation. Regarding proliferation, initial neuromast deposition in developing zebrafish begins as early as 20 hours post-fertilization (hpf), and hair cells start to gain function by 34 hpf (Ledent 2002; Metcalfe, Kimmel, and Schabtach 1985; Raible and Kruse 2000; Ghysen and Dambly-Chaudière 2007). The number of hair cells per neuromast increases from 3-5 days post-fertilization (dpf) and results in the generation of over 300 functional hair cells (Ghysen and Dambly-Chaudière 2007; Harris et al. 2003; Mackenzie and Raible 2012; Raible and Kruse, 2000). Therefore, a single ototoxic treatment during this timeframe is unlikely to fully disable the lateral line, especially considering that approximately half of all hair cells are regenerated within 24 hours post-treatment (Harris et al. 2003). It has been noted that regeneration at this stage of development is difficult to track due to the rapid growth of the lateral line (Mackenzie and Raible 2012). Regarding ototoxin-resistance, susceptibility to chemical ablation requires that hair cells possess functional mechanotransduction channels (Alharazneh et al. 2011). Immature hair cells that have not yet developed mechanosensitivity are resistant to the effects of ototoxins (Murakami et al. 2003; Santos et al. 2006), like hair cells where mechanotransduction is disabled through chemical or genetic means (Owens et al. 2009; Seiler and Nicolson 1999).

Developing neuromasts may contain a relatively high proportion of immature hair cells (Hernández et al. 2007; Santos et al. 2006; Murakami et al. 2003) that can potentially gain mechanosensitivity after ototoxin treatment, allowing neuromasts to partially regain function without regenerating new hair cells. Indeed, following exposure to ototoxin, lateral line-mediated behaviors of zebrafish larvae recover before hair cell regeneration is complete (McHenry et al. 2009; Suli et al. 2012). However, whether functional maturation of immature hair cells precedes cellular regeneration has not been tested explicitly.

Recently, we reported the first genetic model for the specific loss of lateral line function in zebrafish (Erickson et al. 2020). To complement our studies with this mutant, here we aim to identify a timeline of repeated neomycin treatments in larval zebrafish aged 3-4 dpf that will mimic the congenital loss of lateral line function while minimizing off-target toxicity. We use two treatment timelines (every 6 or 12 hours) with neomycin concentrations from 50-400 μM. We compare these treatment regimens in terms of larval survival, efficacy of hair cell ablation, functional maturation of hair cells, and cellular regeneration of hair cells following repeated neomycin treatments. Our data supports the hypothesis that rapid functional maturation of ototoxin-resistant hair cells diminishes the effectiveness of ototoxins as a tool to study the lateral line. We also identify a set of treatment conditions that can be used in future studies along with the lateral line mutant to investigate how the lateral line contributes to the behaviors of larval zebrafish prior to 5 dpf.

Materials and Methods

2.1 Ethics statement and animal husbandry

All research involving animals was done at East Carolina University (ECU; Greenville, NC, United States) and complied with guidelines stipulated by the ECU Institutional Animal Care and

Use Committee. Zebrafish (*Danio rerio*) were maintained and bred using standard procedures (Westerfield 2000).

2.2 Neomycin Sulfate Treatments

A 1 mM Neomycin sulfate (EMD Millipore Corp., USA) stock solution was made in 25 mL of E3 (embryo medium) and diluted in 35 x 10 mm Petri dishes containing 30 mL E3 to make 50, 100, 200, 300 and 400 µM concentrations for each treatment round. These concentrations are consistent with those previously used as single treatments on larval zebrafish (Harris et al. 2003; Santos et al. 2006; Suli et al. 2012). Timeline One involved neomycin treatments every six hours for a total of seven treatments while Timeline Two entailed treatments every twelve hours for a total of four treatments (Figure 5A). Both timelines were repeated three times at all neomycin concentrations. All treatments were done in Petri dishes containing 18-25 larvae and incubated for 25 minutes at 28.5°C before being returned to Petri dishes with fresh E3.

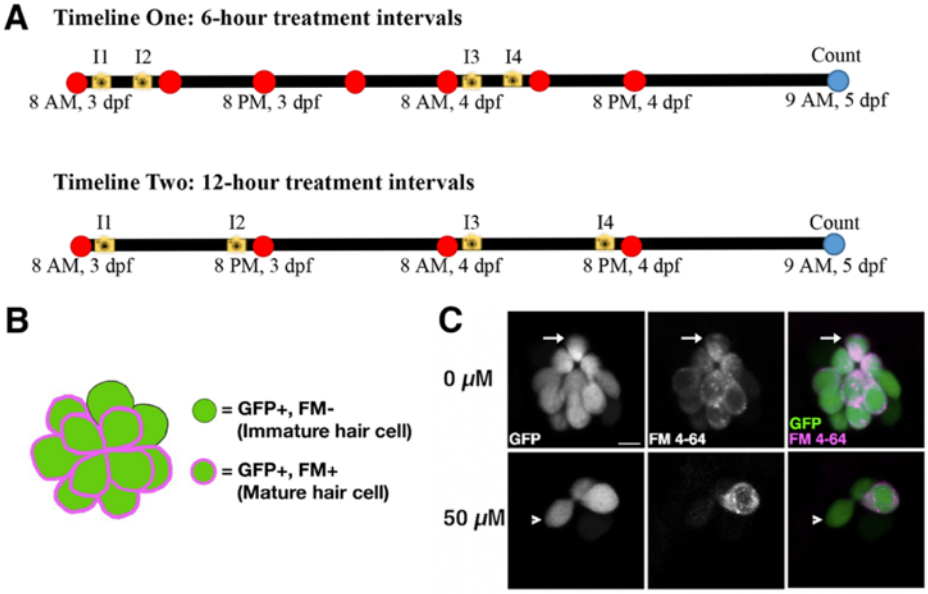


Figure 5. Experimental set-up. (**A**) The 6-hour and 12-hour treatment timelines with neomycin concentrations of 0, 50, 200, 300, and 400 µM beginning 8 AM at 3 days post-fertilization (dpf). Red dots indicate treatment timepoints, yellow cameras indicate imaging timepoints for hair cell

counts (I1-I4), and blue dots indicate final count of surviving larvae. (**B**) Representation of a neuromast showing the strategy used to differentiate between mature and immature hair cells. Mature cells are labelled with both GFP and FM 4-64 while immature cells are labelled with GFP, but not FM 4-64. (**C**) Representative confocal images of O2 neuromasts at imaging timepoint I1 in the 0 and 50 μM neomycin treatment groups. The *Tg(myo6b:EGFP-pA)vo68Tg* line is green and FM 4-64 is magenta in the merge of the two channels in the third column. The full arrow indicates a functional hair cell positive for both GFP and FM 4-64. The arrowhead indicates an ototoxin-resistant, immature hair cell that is positive for GFP only. Scale bar = 5 μm. Additional representative images from imaging timepoints I1-I4 are provided in Supplemental Figure 1.

2.3 Survivorship counts

Starting with the first treatment at 8 AM on 3 dpf, the number of surviving larvae in each treatment condition was recorded every 6 hours until the final treatment at 8 PM on 4 dpf. The final survivorship count was made the following morning at 9 AM on 5 dpf. In addition to the final survivorship count on 5 dpf, we quantified incidences of pericardial edema and imaged larvae with a ZEISS SteREO Discovery.V8 Microscope equipped with a Jenoptik Gryphax Arktur camera.

2.4 Hair cell counts and strategy to differentiate between mature and immature hair cells.

Figure 5A outlines the imaging time points for both neomycin treatment timelines. In all cases, the first round of imaging was done after an hour of recovery time following the first neomycin treatment (I1). The second round of imaging was done an hour before the second neomycin treatment (I2). The third round of imaging was done after an hour of recovery time following the neomycin treatment on the morning of 4 dpf (I3). The fourth and final round of imaging was done an hour before the next neomycin treatment on the afternoon/evening of 4 dpf (I4). Hair cell counts

were done at the 0, 50, 100 and 200 μM concentrations for Timeline One (6-hour intervals) and at all concentrations for Timeline Two (12-hour intervals). Timeline One counts did not include the 300 and 400 μM concentrations due to high larval mortality at later treatments. To quantify hair cell numbers, evaluate the efficacy of neomycin treatments, and quantify the functional and cellular regeneration of the lateral line following repeated neomycin treatments, we utilize simultaneous transgenic and vital dye labeling of lateral line hair cells to distinguish between mature, mechanically-sensitive hair cells and immature hair cells that have yet to develop a functional transduction apparatus (Figure 5B, C). We mark both immature and mature hair cells using the *Tg(myo6b:EGFP-pA)vo68Tg* line (Erickson et al. 2020) which uses the *myo6b* promoter to drive GFP in all hair cells beginning at an early stage of their development (Kindt, Finch, and Nicolson 2012). Simultaneously, functional hair cells were co-labeled with 3 μM FM 4-64 (Thermo Fisher Scientific) in E3 embryo media for 30 seconds, followed by rinses in E3. FM 4-64 is far-red fluorescent dye that enters hair cells through functional mechanotransduction channels (Seiler and Nicolson 1999; Meyers et al. 2003). In this study, we define mature hair cells as being positive for both GFP and FM 4-64 and immature hair cells as being GFP-positive but FM 4-64 negative (Figure 5B, C). For all experiments, 5-9 larvae from treated and untreated groups were randomly chosen for imaging. Live larvae were mounted in 1.2% low melting point agarose (IBI Scientific) dissolved in E3. The L1, MI1 and O2 neuromasts were imaged on a Zeiss LSM 800 confocal. After imaging, larvae were freed from the agarose and returned to their respective control or experimental groups. Hair cell counts were done using the Z-stack data.

2.5 Statistics

P-values of less than 0.05 were considered significant. Analyses were done in RStudio using the R stats and betareg packages (RStudio Team 2018; R Core Team 2021) and plots were made with ggplot2 (Wickham 2011).

Results

3.1. Toxicity of repeated neomycin treatments in 3-4 dpf zebrafish.

In the following experiments, we use repeated neomycin treatments delivered at either 6-hour or 12-hour intervals over the course of 36 hours in zebrafish at 3-4 dpf and at neomycin concentrations of 0, 50, 100, 200, 300, and 400 μM (Figure 5A). To assess general toxicity that may be associated with repeated neomycin treatments, we quantified survivorship across treatment schedules and concentrations. Neomycin concentrations of 100 μM or higher result in greater than 50% larval mortality when administered every 6 hours (Figure 6A). The two highest concentrations, 300 and 400 μM, result in 100% lethality and only the 50 μM concentration allows for greater than 50% survival using the 6-hour treatment timeline. 12-hour treatment intervals result in improved survival relative to the 6-hour intervals. While only 18% of larvae survive when exposed to 400 μM neomycin every 12 hours (n = 64), survival was almost 50% at 200 and 300 μM, 67% at the 100 μM, and 86% at 50 μM (Figure 6A). Despite increased survival using the 12-hour interval, many larvae from the 300 μM and 400 μM neomycin treatment groups exhibited pericardial edema and were smaller in size compared to untreated larvae at 5 dpf (n = 6/30 and 5/16 in the 300 and 400 μm groups, respectively) (Figure 6B-D). To determine which factors were culpable for the mortality rates, we ran a generalized linear model regressing percent survival after 24 hours against concentration of neomycin and the experimental timeline used (Table 2). Treatment concentrations 100 μM and greater are significantly associated with increased mortality

($p < 0.0001$), while less frequent treatments favors survival ($p < 0.0001$). Overall, we find that repeated neomycin treatments at 3-4 dpf at concentrations over 50 µM result in high levels of larval mortality.

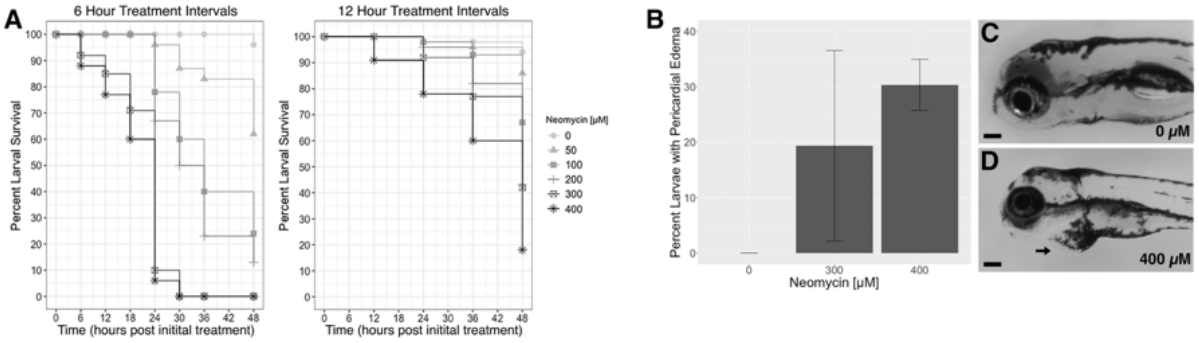


Figure 6. Toxicity of repeated neomycin treatments in zebrafish larvae. (**A**) Percent survival for Timeline One (6-hour treatment intervals) and Timeline Two (12-hour intervals). Final counts were done at 5 dpf, 48 hours following the first treatment. (**B**) Average percent of larvae with pericardial edema at 5 days post-fertilization (dpf) after four neomycin treatments given at 12-hour intervals. Error bars represent standard deviation. (**C**) Image of an untreated (0 µM neomycin) larva at 5 dpf. (**D**) Image of a 5 dpf larva exhibiting pericardial edema (arrow) after 4 treatments with 400 µM neomycin delivered every 12 hours. Scale bar = 0.1 mm.

Beta	Estimate	Standard Error	P value	Significance Level
Intercept (0 µM, 6 Hours)	4.1322996	0.4913248	4.08E-17	****
50 µM Neomycin	-0.9457874	0.5789734	0.102	ns
100 µM Neomycin	-2.6188895	0.5660887	3.72E-06	****
200 µM Neomycin	-3.2026104	0.5625068	1.24E-08	****
300 µM Neomycin	-5.2428102	0.6140916	1.37E-17	****
400 µM Neomycin	-5.8515287	0.6317583	2.00E-20	****

12 Hour Treatment Intervals	2.5645373	0.3409579	5.41E-14	****

Table 2. Results of a beta regression model regressing percent survival at 24 hours against neomycin concentration and treatment timeline to show which variables are significantly contributing to larval survival or mortality (**** = $p < 0.0001$, ns = not significant).

3.2. Hair cell proliferation and maturation in untreated larvae.

Chemical ablation of lateral line function may be less effective in larvae younger than 5 dpf due to the natural proliferation of hair cells at this developmental stage. To determine if neuromasts are still adding new hair cells between 3-4 dpf, we quantified the total number of hair cells (GFP+) and the number of functional hair cells (FM+) per neuromast in untreated larvae and expressed these numbers as a percent of the total number of GFP+ hair cells from the beginning of the experiment. There is continuous cell proliferation and functional maturation of lateral line hair cells during the 36-hour period between imaging timepoints I1 and I4 in untreated larvae (Figure 7). On average, neuromasts add 1.6 GFP-positive hair cells between imaging timepoints 1 and 4, representing a 12.0% increase during these 36 hours of development. Over the same period, neuromasts add an average of two FM-positive cells, a 18.9% increase (Figure 7). The number of functional, FM-positive hair cells is consistently less than the GFP-positive hair cells at all imaging timepoints, yet there is an overall increase in the the proportion of FM-positive cells relative to the total number of hair cells per neuromast between imaging timepoints I1 and I4 (FM / GFP = 0.849 for I1, 0.995 for I4; two-tailed z-score test for two proportions, $z = -2.31$, $p = 0.021$) (Figure 7). This indicates that a greater proportion of hair cells in each neuromast are mechanically-sensitive as development procedes. Together, these data suggest that, between days 3-4 of zebrafish

development, the number of hair cells per neuromast has not yet reached steady-state and both nascent and mature hair cells are still being added.

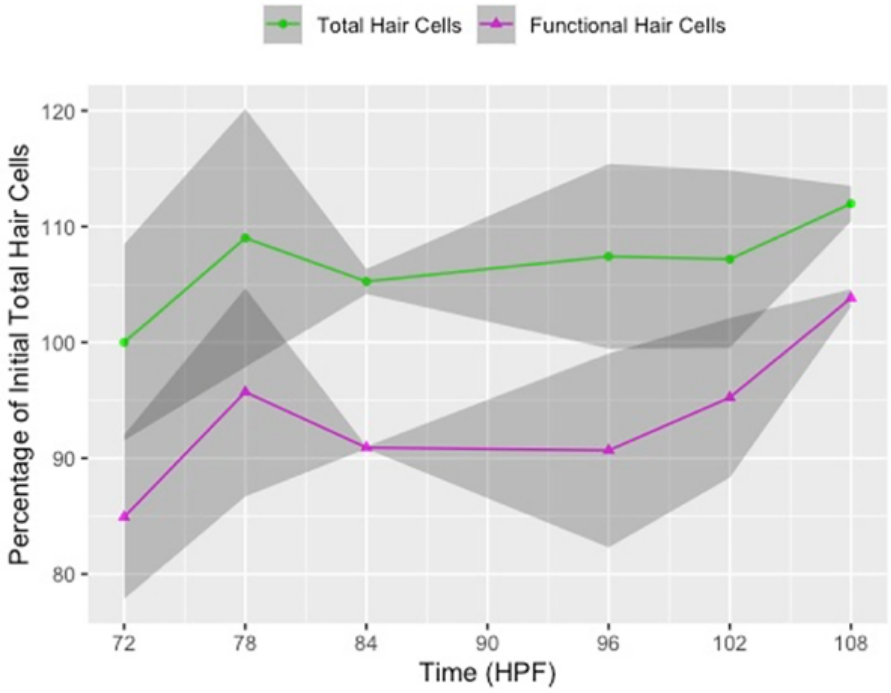


Figure 7. Hair cell proliferation and maturation in untreated (0 µM neomycin) larvae between 3-4 days post-fertilization (dpf). Line graph of the percentage of total (GFP+, green line) and functional (FM 4-64+, magenta line) hair cells in untreated larvae relative to the total number of GFP+ hair cells when larvae were 72 hpf (3 dpf) at the beginning of the experiment. Data points are from each imaging timepoint from all control group replicates of the 6- and 12-hour timelines combined. The x-axis represents hours post-fertilization (HPF). The shaded regions surrounding the line represent the 95% confidence intervals. Average counts from individual neuromasts in the 12-hour timeline are provided in Supplemental Figure 2.

3.3. Effect of neomycin concentration and treatment interval on hair cell death.

To measure the effect of neomycin concentration and treatment interval on the efficacy of hair cell ablation, we quantified the number of GFP-positive and FM-positive hair cells that remain

following neomycin exposure at imaging time points I1 and I3. We excluded the 300 and 400 µM neomycin treatments from these analyses due to high mortality and abnormal morphology observed at those concentrations (Figure 6). Consistently, we find that GFP-positive, FM-negative hair cells remain after a 25-minute exposure to neomycin at concentrations ranging from 50 – 200 µM (Figure 8A). At imaging timepoints 1 and 3, neomycin concentration did not significantly affect the number of remaining GFP-positive cells using either 6- or 12- hour treatment intervals (ANOVA with Dunn post-test, $\alpha = 0.05$) (Figure 8A). Next, looking only at mechanically-sensitive (FM-positive) hair cells, neomycin concentration did not have a significant effect on the number of FM-positive cells remaining after treatment at imaging timepoint 1 in either the 6- or 12- hour treatment intervals, with the exception of the comparison between the 50 and 200 µM neomycin concentrations (ANOVA with Dunn post-test, $p = 0.042$) (Figure 8B). At imaging timepoint 3 within both the 6- and 12-hour treatment experiments, neomycin concentration did not have a significant effect on the number of FM-positive cells remaining after treatment (ANOVA with Dunn post-test, $\alpha = 0.05$) (Figure 8B). In general, we observe a non-significant decrease in the numbers of total and functional hair cells as neomycin concentration increases (Figure 8).

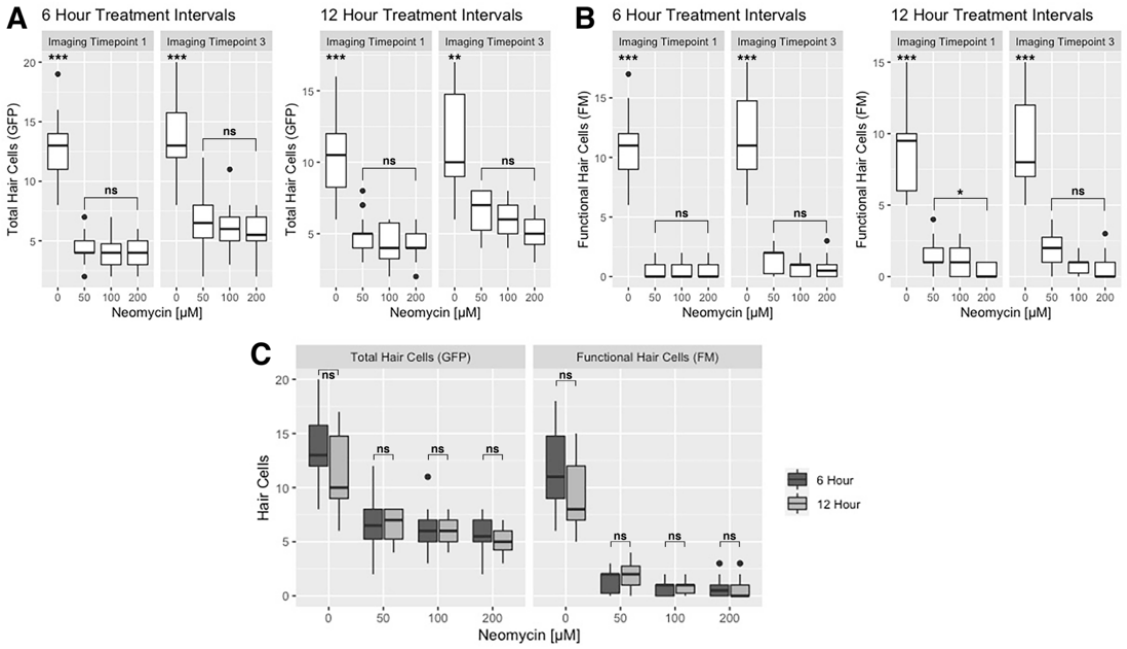


Figure 8. Effect of neomycin concentration and treatment intervals on hair cell death. Box plots represent the number of hair cells per neuromast for both the 6-hour and 12-hour treatment timelines. (**A**) Total (GFP+) and (**B**) functional (FM+) hair cells at imaging timepoints I1 and I3.. (**C**) Comparison of the 6-hour and 12-hour timelines in terms of the number of GFP+ and FM+ hair cells remaining after treatment at imaging timepoint I3 on 4 dpf. There is no significant difference in the hair cell counts between the two treatment timelines (Kruskal-Wallis ANOVA with Dunn post-test, $\alpha = 0.05$). Significance levels are as follows: *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$, ns = not significant.

Next, we analyzed whether the frequency of neomycin treatments had an effect on the level of hair cell ablation by comparing the 6- and 12-hour timelines in terms of the difference in the number of total and functional hair cells at imaging timepoint 3 for all neomycin concentrations. We find that more frequent treatments do not have a significant effect on cell death (ANOVA with Dunn post-test, $\alpha = 0.05$) (Figure 8C). Overall, we find that mechanically-sensitive hair cells in zebrafish at age 3-4 dpf are susceptible to neomycin exposure and can be continuously disrupted over 36 hours. Furthermore, neuromasts from 3-4 dpf larvae contain immature, neomycin-resistant hair cells that remain after treatment.

3.4. Cellular and functional regeneration in 3 dpf larval zebrafish.

We assessed cellular and functional regeneration following exposure to neomycin in 3 dpf larvae by analyzing the data from imaging timepoints I1 and I2. Given the high mortality rates associated with repeated neomycin treatments of 100 – 400 μM (Figure 6), we focused our analysis on the 50 μM condition. We observe a significant increase in the number of functional (FM-positive) hair cells between time points I1 and I2 for both the 6- and 12-hour treatment intervals

(Welch's t-test, 6-hour: M = 0.8, SD = 0.4, p = 0.028; 12-hour: M = 1.0, SD = 0.3, p = 0.002) (Figure 9A). There was no significant change in the number of GFP-positive cells between time points I1 and I2 in either the 6- or 12-hours experiments (6-hour: M = 0.39, SD = 0.71, p = 0.407; 12-hour: M = 0.56, SD = 0.16, p = 0.319) (Figure 9A). We conclude that the surviving immature hair cells start to acquire mechanosensitivity within 6 to 12 hours after neomycin treatment and that functional maturation of hair cells outpaces the regeneration of new hair cells at 3 dpf.

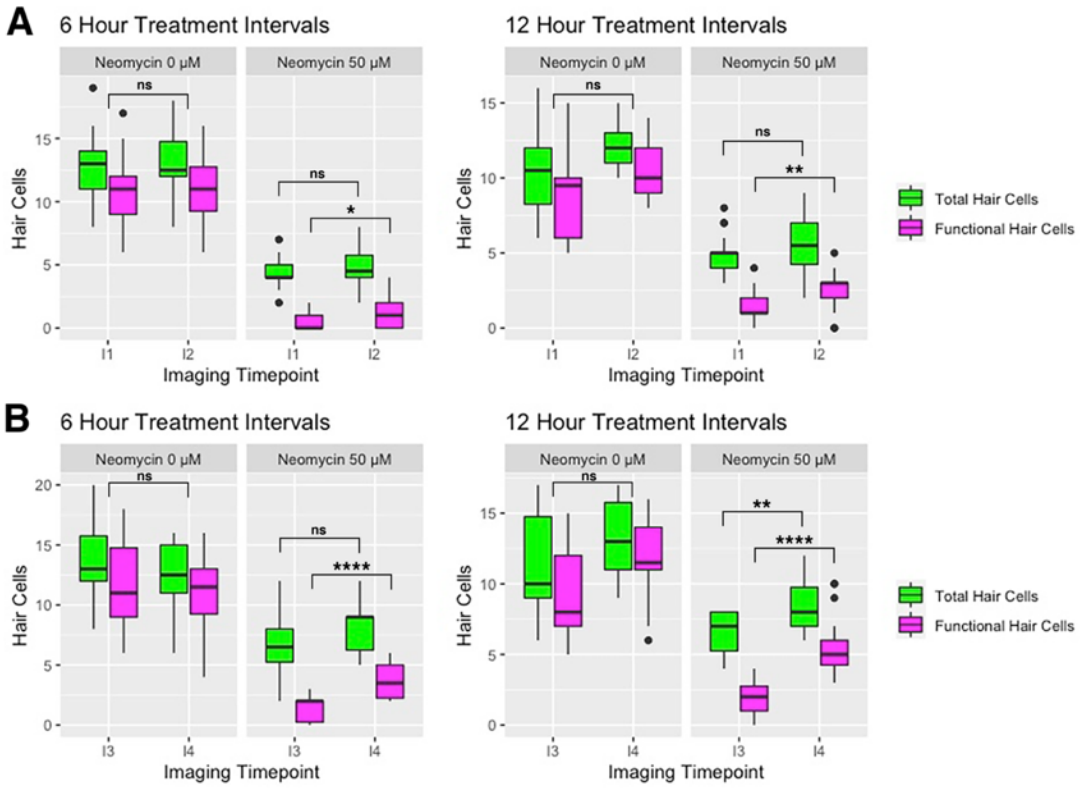


Figure 9. Comparison of hair cell counts in the 0 µM and 50 µM neomycin treatment groups at (**A**) 3 dpf and (**B**) 4 dpf. Green box plots indicate cell counts using the GFP transgene to mark both transducing and non-transducing hair cells and the magenta box plots indicate hair cell counts using FM 4-64 to label mechanically-sensitive hair cells. See Figure 5A for timing of imaging timepoints I1-I4. Performed Kruskal-Wallis ANOVA with Dunn post-test to find significance levels, which are as follows: **** = $p < 0.0001$, ** = $p < 0.01$, * = $p < 0.05$, ns = not significant.

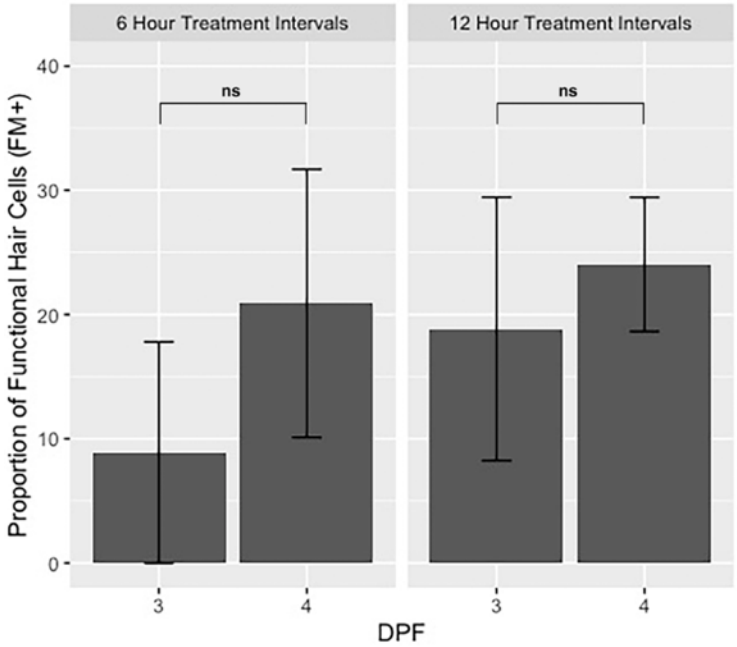
3.5. Cellular and functional regeneration in 4 dpf larval zebrafish.

Like the analyses done at 3 dpf, we assessed cellular and functional recovery at 4 dpf using 50 μM neomycin given every 6 or 12 hours. Between imaging time points I3 and I4, we observe a significant addition of FM-positive hair cells between 6-hour treatments and 12-hour treatments at 4 dpf (Welch's t-test, 6-hour: $M = 2.2$, $SD = 0.9$, $p = 0.000016$; 12-hour: $M = 3.9$, $SD = 0.2$, $p < 0.00001$) (Figure 9B). In the 6-hour treatment timeline, there is no significant increase in the number of GFP-positive hair cells between imaging timepoints at 4 dpf ($M = 1.3$, $SD = 1.1$, $p = 0.125$). However, in the 12-hour treatment interval, there is a significant increase in the number of GFP-positive hair cells between I3 and I4 at 4 dpf ($M = 1.6$, $SD = 0.5$, $p = 0.004$) (Figure 9B). These data suggest that, after neomycin treatment at 4 dpf, the remaining immature hair cells start to gain functionality within 6 hours while the regeneration of new hair cells begins within 12 hours.

3.6. Comparison of hair cell death and recovery between 3 and 4 dpf zebrafish.

Next, we compared the susceptibility of hair cells to 50 μM neomycin at 3 dpf and 4 dpf by quantifying the ratio of functional (FM+) to total (GFP+) hair cells from imaging timepoints I1 (3 dpf) and I3 (4 dpf) for both timelines. Comparing I1 and I3, we observe some variability in the proportions of functional hair cells per neuromast that remain following treatment, but the values are not significantly different in the 6-hour or 12-hour timelines (Two-tailed z-score test for two proportions; 6-hour: $z = -0.923$, $p = 0.356$; 12-hour: $z = -0.346$, $p = 0.731$) (Figure 10). Even though it is not statistically significant, there is a consistent increase in the ratio of FM:GFP from I1 to I3 in both timelines. To determine which factors were responsible for these results, we ran a generalized linear model regressing hair cell proportions against the days post fertilization and the number of treatments (Table 3). According to this beta-regression model, transitioning from 3 dpf to 4 dpf is associated with an increase in the proportion of mechanically-sensitive (FM+) hair cells

per neuromast ($p < 0.001$). Conversely, the number of neomycin treatments are significantly associated with a decrease in the proportion of mechanically-sensitive cells ($p < 0.001$). Overall,



we find that the proportions of functional hair cells that remain after neomycin treatment are statistically similar between 3 and 4 dpf, possibly due to the opposing effects of developmental time and repeated exposures to neomycin.

Figure 10. Bar graphs showing the proportion of functional hair cells (FM+) relative to total hair cells (GFP+) one hour after 50 µM neomycin treatments on 3 days post-fertilization (dpf) (I1) and 4 dpf (I3) for both 6-hour and 12-hour timelines. Error bars represent standard deviation, n = 15 for each group. Two-tailed Z-score test for proportions was used to evaluate the difference between the proportion of hair cells at 3 and 4 dpf. ns = not significant.

Beta	Estimate	Standard Error	P value	Significance Level
Intercept (0 µM, I1)	0.1024	0.1330	0.441	ns
4 dpf	1.1435	0.2106	5.68E-8	***

Number of Treatments	-0.6593	0.0655	< 2E-16	***

Table 3. Results of a beta regression model regressing FM:GFP hair cell proportions against the days post fertilization (dpf), treatment timeline, and number of treatments to show which factors were significant in contributing to the observed proportions of FM+:GFP+ hair cells between 3 and 4 dpf (*** = $p < 0.001$, ns = not significant).

To assess differences in hair cell proliferation and recovery between 3 and 4 dpf, we compared the change in the number of hair cells between imaging timepoints I1 and I2 (3 dpf) versus the change between I3 and I4 (4 dpf). There is a significant increase in the number of functional, FM+ hair cells added per neuromast after allowing for hair cell recovery between treatments at 4 dpf compared to 3 dpf in both the 6-hour and 12-hour treatment timelines (Welch's t-test, 6-hour: $p = 0.007$; 12-hour: $p = 0.00009$) (Figure 11A). However, there is no significant difference in the total number of GFP+ hair cells per neuromast added between treatments on 4 dpf compared to 3 dpf (6-hour: $p = 0.381$; 12-hour: $p = 0.113$) (Figure 11B). Looking at the ratio of functional (FM+) to total (GFP+) hair cells per neuromast on days 3 and 4, there is a significant increase in the proportion of functional hair cells after a 12-hour recovery on day 4 relative to day 3, but no significant increase after 6-hour recovery times (One-tailed z-score test for two proportions, 6-hour: $z = -0.845$, $p = 0.199$, 12-hour: $z = -1.792$, $p = 0.0363$) (Figure 11C). Overall, we find that functional recovery of lateral line hair cells occurs more quickly at 4 dpf compared to 3 dpf in zebrafish larvae.

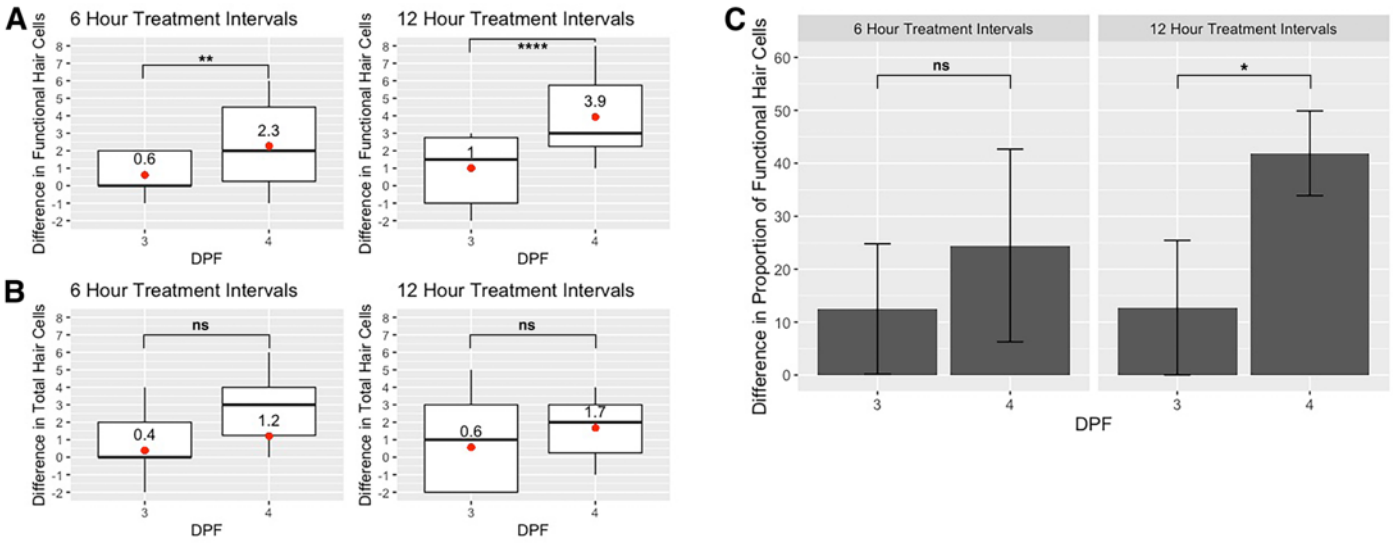


Figure 11. Hair cell recovery at 3 and 4 days post-fertilization (dpf). (**A**) Box plots showing the difference in functional (FM+) and (**B**) total (GFP+) hair cells between the first and second 50 µM neomycin treatments on 3 dpf (I2 minus I1) and 4 dpf (I4 minus I3) in 6- and 12-hour treatment timelines. Red dots and text indicate mean values. Statistal analysis was performed using Kruskal-Wallis ANOVA with Dunn post-test. (**C**) Bar graph showing the difference in the ratio of functional hair cells (FM+) to total hair cells (GFP+) between the first and second treatments on 3 dpf (I2 minus I1) and 4 dpf (I4 minus I3) for 50 µM treatments in 6-hour and 12-hour treatment timelines. Error bars represent standard deviation, n = 15 for each group. One-tailed Z-score tests for proportions were used to evaluate increases in the proportion of FM+ cells. Significance levels are as follows: **** = $p < 0.0001$, ** = $p < 0.01$, * = $p < 0.05$, ns = not significant.

Discussion

In this study, we use repeated neomycin treatments to continuously ablate sensory hair cells of the lateral line in 3-4 dpf zebrafish larvae. We find that immature, ototoxin-resistant hair cells remain after neomycin treatment, even after multiple exposures delivered every 6- or 12-hours. Our evidence suggests that these immature hair cells gain mechanosensitivity during the inter-

treatment intervals, and that functional maturation of nascent hair cells occurs more rapidly at 4 dpf compared to 3 dpf. To our knowledge, this is the first study to analyze hair cell death and recovery using repeated ototoxin treatments on 3-4 dpf zebrafish larvae. Overall, repeated neomycin treatments can be used to continuously disrupt mechanically-sensitive hair cells of the lateral line at this early stage of zebrafish development.

4.1. Toxicity associated with repeated neomycin treatments.

Single exposures to ototoxins are accepted to be well tolerated by larval and adult fish, thereby allowing their use in studies of lateral line-mediated behaviors (Coffin and Ramcharitar 2016). However, our data clearly demonstrates that repeated neomycin treatments are toxic to larval zebrafish. Survival was less than 50% in both the 100-400 μM neomycin groups treated every 6 hours and in the 200-400 μM neomycin groups treated every 12 hours. Furthermore, larval death occurred more rapidly with increased number and frequency of treatments (Figure 6). We consistently observe a major decline in larval survival between treatments four and five. This trend was apparent in both experimental timelines, despite differing treatment intervals. In the 300 μM and 400 μM neomycin treatment groups, surviving larvae often exhibit pericardial edema and developmental delays. Incidences of edema were variable between experimental replicates in the 12-hour, 300 μM group, suggesting that this condition is near the level where larvae begin to experience this toxic effect of neomycin. Our findings are consistent with other studies that have demonstrated off-target toxicity of compounds commonly used in lateral line research (Carreau an Pyle 2005; Han et al. 2020; Han et al., 2019; Hansen et al. 1999; Xu et al. 2017). Therefore, we conclude there is a toxicity threshold for larval zebrafish at 3-4 dpf that is surpassed after four treatments when using neomycin concentrations 100 μM and above.

4.2. Susceptibility of lateral line hair cells to repeated neomycin treatments at 3-4 dpf.

Hair cells of the lateral line are rapidly proliferating and maturing at 3-4 dpf (Harris et al. 2003; Mackenzie and Raible 2012; Raible and Kruse 2000). Mackenzie and Raible (2012) reported an increase in the number of functional hair cells per neuromast between 3-4 dpf and that the number of functional hair cells is significantly less than the total number of hair cells per neuromast at 5 dpf. At 3-4 dpf, we similarly find that the total complement of hair cells within a neuromast is always greater than the number of functional hair cells. Between 3-4 dpf, neuromasts in untreated larvae exhibit a 12% gain in total cells and 19% increase in functional cells over the same period. These results indicate that, as development proceeds, an increased proportion of hair cells per neuromast are mechanically-sensitive and therefore potentially susceptible to ototoxins. Furthermore, we find that these mechanically-sensitive hair cells are susceptible to neomycin in 3-4 dpf zebrafish and, in agreement with previous studies, that hair cell loss is incomplete due to functionally immature hair cells being resistant to the ototoxic effects of neomycin (Figures 8 and 9) (Hernández et al. 2007; Mackenzie and Raible 2012; Murakami et al. 2003; Santos et al. 2006).

4.3. Hair cell maturation and regeneration after repeated neomycin treatments at 3-4 dpf.

Previous studies have provided support for hair cell regeneration occurring more quickly in young larvae than in older larvae or adult fish (Cruz et al. 2015; Hardy et al. 2021; Mackenzie and Raible 2012). In our study, we observe that there is a significant addition of functional hair cells within 6 hours post-treatment at both 3 and 4 dpf (Figure 11). The increase in functional hair cells outpaces the addition of new GFP+ hair cells at both 3 and 4 dpf. This is likely due to the functional maturation of immature GFP+ hair cells remaining after treatment. However, we found that the addition of both functional and total hair cells occurred more quickly at 4 dpf than 3 dpf. Using the 50 μM neomycin treatments, the ratio of functional to total (FM+/GFP+) hair cells

increases by approximately 12% in 6 hours and 13% in 12 hours on 3 dpf. On 4 dpf, the proportion of functional to total (FM+/GFP+) hair cells increases by approximately 25% in 6 hours and 42% in 12 hours. We saw that in untreated larvae, the number of hair cells per neuromast increases from 3 dpf to 4 dpf. Therefore, the rate of functional and total hair cell addition following neomycin treatment at 4 dpf may be quicker than at 3 dpf due to a natural increased pace of hair cell proliferation. To confirm this, further studies investigating hair cell proliferation at 3 and 4 dpf are necessary.

4.4. Mimicking the chronic loss of lateral line function in larval zebrafish at 3-4 dpf.

To our knowledge, only one study has used repeated ototoxic treatments to study the long-term loss of lateral line-mediated behaviors (Carrillo and McHenry 2016). However, this study was conducted on fish older than 5 dpf, and there may be lateral line-mediated behaviors in younger larvae (prior to swim bladder inflation) that have not been investigated. Given that even partial regeneration can restore lateral line function and behaviors (Hardy et al. 2021; McHenry et al. 2009; Suli et al. 2012), a single exposure to ototoxin is not an effective way to assess such behaviors in early-stage larvae when hair cells are actively proliferating and maturing. Therefore, repeated treatments appear necessary, but the feasibility of multiple treatments during lateral line formation has not been previously tested.

Our results support using 50 μM neomycin every 12 hours to continuously disrupt hair cells of the lateral line in 3-4 dpf zebrafish larvae. Poor survivorship precludes the use of repeated neomycin treatments at concentrations of 100 μM and greater, particularly when delivered every 6 hours (Figure 6). Furthermore, neomycin concentrations between 50 – 400 μM are similar in their ability to kill mechanically-sensitive hair cells (Figure 8), suggesting there is little benefit to using higher concentrations. Although there is a statistically significant addition of functional hair

cells between treatments for both the 6- and 12-hour intervals, we observe little to no cellular regeneration (additional GFP+ hair cells) during the same intervals. Considering these results, and because rheotaxic behaviors begin to recover approximately 12 hours after exposure to neomycin at 5 dpf (McHenry, et al., 2009), we advise treating 3-4 dpf larvae with 50 μM neomycin every 12 hours to continuously disrupt lateral line hair cells while minimizing toxic side effects. This treatment regimen may complement the *lhfpl5b* zebrafish mutant in analysis of lateral line-mediated behaviors in early-stage larvae (Erickson et al. 2020).

CHAPTER 3: ALONE IN A CROWD: EFFECT OF A NONFUNCTIONAL LATERAL LINE ON THE SOCIAL HORMONE PARATHYROID HORMONE 2

Published:

Alexandra Venuto, Cameron P. Smith, Marybelle Cameron-Pack, Timothy Erickson; Alone in a crowd: effect of a nonfunctional lateral line on expression of the social hormone *parathyroid hormone 2*. *Biol Open* 15 October 2022; 11 (10): bio059432. https://doi.org/10.1242/bio.059432

ABSTRACT

Parathyroid hormone 2 (Pth2) is a vertebrate-specific neuropeptide whose thalamic expression is upregulated by social contact with conspecifics. However, social interactions fail to stimulate *pth2* expression in isolated zebrafish whose lateral line hair cells have been chemically ablated. These results suggest that modulation of *pth2* by social context is acutely dependent on mechanosensory information from the lateral line. However, it is unclear how a congenital loss of lateral line function influences the ability of zebrafish to interpret their social environment. In this study, we measure *pth2* levels in zebrafish mutants lacking hair cell function in either the lateral line only, or in both the inner ear and lateral line. Socially-raised lateral line mutants express lower levels of *pth2* relative to wild type siblings, but there is no further reduction when all sensory hair cells are nonfunctional. However, social isolation of hair cell mutants causes a further reduction in *pth2* expression, pointing to additional unidentified sensory cues that influence *pth2* production. Lastly, we report that social context modulates fluorescent transgenes driven by the *pth2* promoter. Altogether, these data suggest that lateral line mutants experience a form of isolation, even when raised in a social environment.

Introduction

The complex relationship between the brain and social behavior has been studied across animal species for decades (Chen and Hong 2018; Kravitz and Huber 2003; Brothers 1990; Robinson, Fernald, and Clayton 2008). Different social contexts can evoke a wide range of emotions, from joy and positivity (Burgdorf and Panksepp 2006; Panksepp and Burgdorf 2003) to fear and aggression (Amaral 2002; Neumann, Veenema, and Beiderbeck 2010) with a high level of plasticity (Chen and Hong 2018; Kolb and Whishaw 2003). In situations of social isolation, many animals exhibit anxious behaviors in addition to neurochemical changes in the brain (Weiss et al. 2004; Shams et al. 2017; Amaral 2002; Mumtaz et al. 2018).

Social behavior is modulated by a suite of neuropeptides (Oldfield and Hofmann 2011; Panksepp et al. 2007; Adkins-Regan 2009; O'Connell and Hofmann 2011; Schoofs, de Loof, and van Hiel 2017). Parathyroid hormone 2 (Pth2, formerly known as tuberoinfundibular peptide of 39 residues or TIP39) is a neuropeptide expressed in the vertebrate thalamus, which is a sensory integration and relay center (Anneser et al. 2020; Bhattacharya et al. 2011; Arpád Dobolyi et al. 2012; Mueller 2012). In rodents, the PTH2 receptor is expressed in several nuclei throughout the central nervous system (Dobolyi et al. 2012). Zebrafish have two *pth2r* paralogs, *pth2ra* and *pth2rb,* each with distinct expression patterns in the fish brain (Seitz 2019). Activation of PTH2R increases intracellular cAMP levels and can modulate neuroendocrine activity in the hypothalamic–pituitary–adrenal (HPA) axis (Dimitrov and Usdin 2010). PTH2-PTH2R signaling influences several behaviors, including maternal care (Cservenák et al. 2013), pain sensitivity (LaBuda and Usdin 2004; Dimitrov, Petrus, and Usdin 2010; Dimitrov et al. 2013), and anxiety-like responses (Anneser et al. 2022; Coutellier and Usdin 2011; LaBuda et al. 2004).

Recent work suggests that Pth2 signaling promotes social interactions between conspecifics. In zebrafish, *pth2* mutants exhibit decreased preference for conspecifics (Anneser et al. 2022), while chemogenetic activation of the Pth2-positive region of the rodent brain promotes social contact (Keller et al. 2022). Consistent with this, expression of *pth2* itself positively scales with the quantity of social interactions with conspecifics (Anneser et al. 2020; Keller et al. 2022). In rodents, physical touch from conspecifics activates *Pth2* expression (Cservenák et al. 2013; Keller et al. 2022). In addition to tactile touch sensation, fish also have another form of "distant touch" through the mechanosensory lateral line (Dijkgraaf 1963). The mechanosensory lateral line is a hair cell-based system in aquatic vertebrates that senses hydrodynamic information, such as that created by conspecifics (Butler and Maruska 2015, 2016a, 2016b; Groneberg et al., 2020; Montgomery et al. 2013). In zebrafish, chemical ablation of the mechanosensory lateral line prevents the rescue of *pth2* expression by a social environment following isolation (Anneser et al. 2020). Therefore, social "touch" (direct contact in rodents and hydrodynamic communication in zebrafish) seems to be a primary sensory activator of *pth2* expression.

Despite this knowledge, there remain several unanswered questions regarding the social regulation of *pth2* expression. First, how do zebrafish interpret social context when they are born without lateral line function? Secondly, do mechanosensory hair cells in the inner ear contribute to the ability of zebrafish larvae to respond to social context? Thirdly, is *pth2* expression regulated solely via mechanosensation, or can fish use additional sensory inputs to sense the presence of conspecifics? Here, we report that the congenital loss of mechanosensory hair cell function leads to chronically depressed levels of *pth2* expression in socially-housed larval zebrafish. Interestingly, social isolation of hair cell mutants further decreases *pth2* levels, suggesting that larval fish use additional sensory information to sense conspecifics and modulate *pth2* expression. Lastly, we

utilize fluorescent reporters driven by the *pth2* regulatory region to evaluate the morphology of *pth2*-expressing cells and visualize *pth2* dynamics *in vivo*. Overall, this study offers additional insight into the sensory basis for social interactions in zebrafish and introduces novel tools to track *pth2* expression in living fish.

Materials and Methods

Ethics statement

Animal research complied with guidelines stipulated by the Institutional Animal Care and Use Committees at East Carolina University (Greenville, NC, United States) and at University of New Brunswick (Fredericton, New Brunswick, Canada). Zebrafish (*Danio rerio*) were maintained and bred using standard procedures (Westerfield 2000). All experiments used larvae at 1–5 days post-fertilization (dpf), which are of indeterminate sex at this stage.

Mutant and transgenic fish lines

The following previously described zebrafish mutant and transgenic lines were used in this study: *lhfpl5b*^{vo35}, *pcdh15a*^{th263b}, *tomt*^{tk256c}, *cacna1da*^{tc323d}, and *Tg(slc6a3:EGFP)ot80* (Erickson et al. 2017, 2020; Maeda et al. 2017; Sidi et al. 2004; Xi et al. 2011). To make the *Tg(pth2:EGFP)unb2* and *Tg*(*pth2:TagRFP)unb3* transgenic lines, 2067 base pairs immediately upstream of the *pth2* coding region (including the 5' UTR and first intron) were PCR amplified with primers containing attB4-B1 recombination sites (*pth2*-specific sequence underlined: Fwd: GGGGACAACTTTGTATAGAAAAGTTG<u>GTAAAGGACACTCTTTGAGATCCT</u>, Rvs: GGGGACTGCTTTTTTGTACAAACTTGC<u>CTGCAGATGAATAAGTTGAATAATACA</u>. The PCR product was cloned into the Gateway pDONR-P4-P1R entry vector by a standard BP clonase reaction and a Multisite LR reaction was performed to create *pth2:EGFP-pA, cryaa:EGFP* and *pth2:tagRFP-pA, cryaa:mCherry* plasmids. pDestTol2pACryGFP and pDESTtol2pACrymCherry

plasmids were gifts from Joachim Berger & Peter Currie (Addgene plasmids # 64022, 64023). The expression constructs were co-injected with *tol2* transposase mRNA into 1-cell zebrafish embryos(Kwan et al. 2007). F0 founders were outcrossed to produce stable F1 transgenics. At a minimum, progeny from F2 transgenics were used in this study.

Mutant and social isolation experiments for in situ hybridization, qPCR, and transgenic lines

Zebrafish larvae were sorted for *lhfpl5b*^{vo35}, *pcdh15a*^{th263b}, *tomt*^{tk256c} homozygotes at 2 dpf with FM1-43 dye labelling for social isolation experiments. *cacna1da*^{tc323d} homozygotes were identified behaviorally by the "circler" phenotype at 4 dpf (Nicolson et al. 1998), as they cannot be identified by differential FM1-43 dye labeling and were not used in isolation experiments. *Tg*(*pth2:EGFP)* and *Tg*(*pth2:TagRFP)* transgenics were identified by their GFP or mCherry lens markers, respectively. *Tg(slc6a3:EGFP)* transgenics were identified by GFP expression in the brain. Identified larvae were divided into social groups of thirty, while another thirty larvae were placed in isolation, except for *cacna1da*^{tc323d} larvae which were only raised socially. Both social and isolated larvae were housed in 6-well tissue culture plates containing 5 ml of E3 embryo media. For the social condition, all 30 larvae were placed in one well with 5 ml of embryo media. For the isolated condition, one larva was placed per well with 5 ml of embryo media. Strips of paper were placed between each well to prevent visual access between wells. Larvae intended for mRNA *in situ* hybridization (ISH) were grown in E3 with 200 μM N-Phenylthiourea (PTU) (Alfa Aesar) to prevent pigment formation. Each group was incubated at 28.5ºC with a 14L:10D photoperiod from 2 to 4 dpf. At 4 dpf, larvae were either fixed for *in situ* hybridization, processed for qPCR, or imaged to visualize the transgene.

Repeated neomycin treatments

The repeated neomycin treatments were performed essentially as previously described (Venuto and Erickson 2021). At 24 hpf, WT embryos were divided into groups of 30 and grown continuously in the dark at 28.5ºC in media supplemented with 200 µM PTU to prevent pigment formation. Larvae were manually dechorionated at 2 dpf. Starting at 9:00 AM, 3 dpf larvae were exposed to either 0 or 50 µM neomycin sulfate hydrate (Alfa Aesar) for 30 minutes at 28.5ºC every 12 hours for a total of three treatments. At noon on 4 dpf, treated and untreated groups of larvae were anesthetized with 0.016% MS-222 and fixed in 4% PFA for mRNA ISH. The experiments were repeated three times, each with two technical replicates for the control and treatment groups. Three hours after treatment 1, ablation of lateral line hair cells was assessed by incubating 3 dpf larvae in 130 µM DASPEI (2-[4-(dimethylamino)styryl]-1-ethylpyridinium iodide) for 10 minutes. Following three washes with E3, larvae were anesthetized with 0.016% MS-222, positioned laterally on a depression slide in 1.2% low melting point agarose, and imaged with a Basler Ace 5.1 MP color camera on a Zeiss SV-11 microscope using an X-Cite 120Q illuminator and wideband GFP filter.

mRNA in situ hybridization

The *pth2* in situ probe sequence was amplified using the Invitrogen SuperScript IV One-Step RT-PCR kit (ThermoFisher) using the following forward and reverse primers: F: CATTGCATGGACGATTTACG; R: TGCCATGTCATTCAAAATCC. The RT-PCR product was purified by gel extraction and propagated in the pCR4-TOPO vector. The pCR4-*pth2* plasmid was linearized with NotI and the antisense DIG-labeled ISH probe was synthesized using the T3 RNA polymerase promoter. Probe synthesis and in situ hybridization were performed essentially as described (Erickson, French, and Waskiewicz 2010). Larvae were imaged at 20X magnification using either an Olympus BH-2 microscope outfitted with a Jenoptik Gryphax Arktur camera or a

Nikon Eclipse 50i microscope with a Basler Ace 5.1 MP color camera. ISH cell counts were conducted in a non-blinded manner by manually focusing through a mounted larvae prior to capturing a static image.

RNA isolation and real time quantitative PCR (qPCR)

For RNA isolation, ten larval heads were used per biological replicate. Samples were collected on ice, placed in 1.7 mL RNase-free microcentrifuge tubes containing 200 µl RNAzol (Molecular Research Center, Inc., OH. Catalog: RN 190) and homogenized immediately. This was repeated for a total of three biological replicates. Total RNA was extracted from homogenized solutions according to the manufacturer's protocol. For each sample, cDNAs was synthesized using 1 µg total RNA and a high-capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA, Catalog# 4368814) following the manufacturer's instructions.

The levels of *pth2* transcripts were determined by quantitative real-time PCR (qPCR) using SYBR green dye (Invitrogen) and a CFX Connect real-time thermal cycler (Bio-Rad Laboratories, Hercules, California, USA). The qPCR reaction was conducted with initial denaturation at 95 °C for 2 min, followed by 30 cycles of 30 s denaturation at 95 °C, 30 s annealing at 57 °C, and 30 s extension at 72 °C using the specific primers (Forward: CCACGCAACACACAGTCAAG, Reverse: GCAAGTTACTTTGCAGAGGTC) and GoTaq G2 DNA polymerase (Promega, Madison, Wisconsin, USA). Each PCR mixture (15 µl) consisted of 7.795 µl DNase free water, 3 µl 5X GoTaq buffer, 1.5 µl 25 mM MgCl2, 0.3 µl 10 mM dNTP mix, 0.15 ml 10µM forward or reverse primer, 2 µl cDNA, 0.03 µl 100X SYBR green dye (final concentration 0.2X), and 0.075 µl Taq. Two technical replicates per biological replicate were averaged together for analysis. Absolute values (copies/µg total RNA) were determined using Ct values of samples and a standard

curve generated from serial known concentrations of plasmid containing the target region of *pth2*. Relative expression was found by calculating the fold change compared to the mean value of the isolated, wild type condition. The efficiency of the PCR and authentic PCR products was further confirmed by analyses of melting curve and gel electrophoresis.

Transgenic imaging and quantification

Live zebrafish larvae were anesthetized with 0.016% MS-222 in E3 and mounted dorsally on a depression slide in 1.2% low melting point agarose in E3. EGFP was imaged using a Zeiss LSM800 laser-scanning confocal with a 40x water objective and Zeiss Zen software. To quantify results, cell counts were performed using Fiji/ImageJ image processing software (Schindelin et al. 2012). Cells were counted in a non-blinded manner by manually going through the z-stacks. The cell count process was repeated twice at a minimum to ensure accuracy. Images shown are maximum intensity projections of z-stacks. For the fluorescent intensity experiment, the intensity of each cell was collected from a sum slices z-projection in Fiji/ImageJ and the integrated density values were used for analysis. Depth encoded 2D and 3D projections were created in Fiji/ImageJ using the *Z-stack Depth Color Code 0.0.2* plugin. Figures were assembled in Adobe Photoshop and equally adjusted for brightness and contrast among corresponding treatment groups.

Statistical tests

All graphs and statistical tests were done using R (R Core Team 2021). For *in* situ hybridization cell counts, a one-tailed Welch's t-test was used (two biological replicates, n = 7-9 larvae per condition per replicate). For the qPCR experiment comparing the various hair cell mutants raised socially (Figure 13), a one-way ANOVA was conducted (three biological replicates, n = 10 per condition per replicate). For the same experiment comparing wild type versus mutant larvae raised socially, a one-tailed Welch's t-test was used to compare within mutant lines. For the

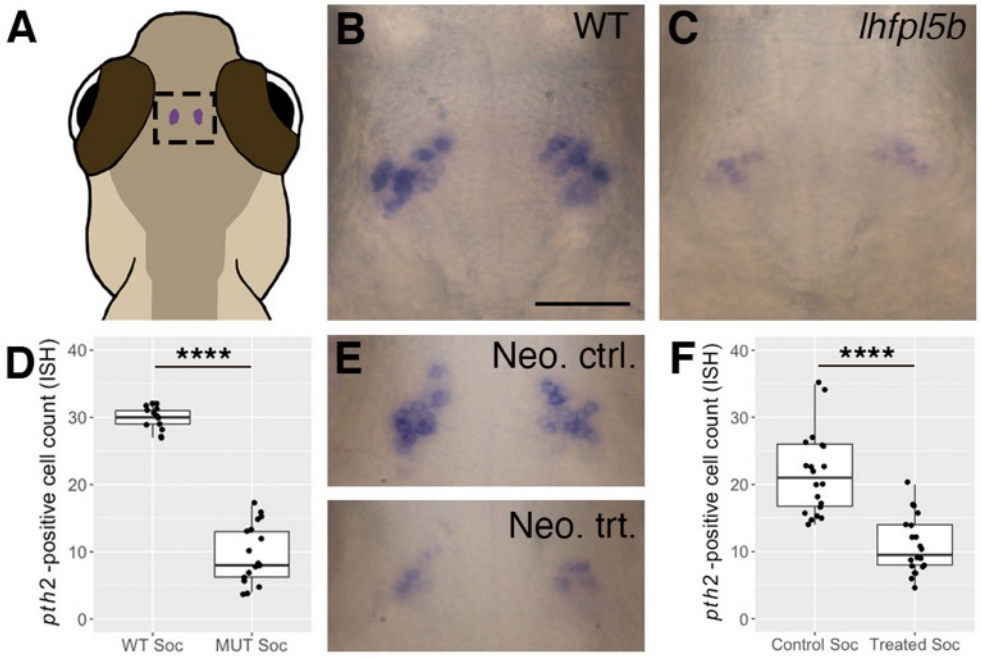
qPCR experiment comparing genotypes and social status (Figure 14), a two-way ANOVA was conducted (three biological replicates, n = 10 per condition per replicate). For the transgenic cell counts, a one-tailed Welch's t-test was used (three biological replicates, n = 5 per condition per replicate), except for the 1 dpf versus 2 dpf and GFP versus RFP cell counts where we used two-tailed Welch's t-test to test significance. A p-value of less than 0.05 was considered significant. The more stringent one-tailed t-tests were used due to only having an expectation for a decrease in *pth2* expression following isolation or loss of lateral line sensory information. Sample sizes were determined by the number of fish used in the experiment and no samples were excluded from the analysis. Pre-determined criteria to exclude samples included if the fish was not touch-responsive or displayed obvious developmental abnormalities.

Results

Decreased pth2 expression in lateral line-deficient larval zebrafish.

pth2 expression in the thalamus decreases when fish are raised in isolation but increases following a brief period of social interaction with conspecifics. However, when the mechanosensory lateral line is ablated in isolated fish prior to social exposure, *pth2* expression levels remain low (Anneser et al. 2020). This implicates the lateral line sensory system in recognizing conspecifics to stimulate *pth2* expression. To further evaluate the role of the lateral line in social recognition, we raised lateral line-deficient mutants (*lhfpl5b*$^{-/-}$) and their siblings in separate social groups and detected *pth2* expression by mRNA *in situ* hybridization (ISH) at 4 days post-fertilization (dpf). Socially-raised lateral line mutants exhibit a decrease in *pth2* expression (M = 9.7, SD = 4.3) compared to wild type (WT) siblings (M = 29.9, SD = 1.7) (Figure 12B, C), in terms of a qualitative decrease in the intensity of staining as well as a significant decrease in the number of detectable *pth2*-positive cells (Figure 12D; One-tailed Welch's t-test, t = 18.25, P =

$1.04x10^{-15}$, Cohen d = 6.18). We also ablated lateral line hair cells in socially-raised WT larvae using repeated neomycin treatments between 3 – 4 dpf (Venuto and Erickson 2021) (Figure 12E, Supplemental Figure 3). Even though these larvae had social experience prior to losing lateral line input, neomycin-treated larvae exhibit a significant decrease in the number of *pth2*-positive cells compared to untreated controls (Figure 12F; One-tailed Welch's t-test, t = 6.44, $P = 1.21x10^{-7}$, Cohen d = 3.22). These results confirm that a loss of lateral line function, whether genetically or



chemically-induced, impairs the ability of *pth2* cells to respond to social context.

Figure 12: Decreased *pth2* expression in lateral line-deficient zebrafish larvae. (A) Location of *pth2*-expressing cells in the thalamic region of a larval zebrafish. (B-C) Representative images of mRNA *in situ* hybridizations (ISH) showing *pth2*-positive cells in socially-reared wild type and lateral line mutant (*lhfpl5b*^{vo35}) larvae at 4 dpf. (D) Boxplots of *pth2*-positive cell counts from ISH images of *lhfpl5b* larvae at 4 dpf (WT = 14, MUT = 18 larvae from two replicates). (E) Representative mRNA ISH images from socially-reared control (Neo. ctrl) and neomycin-treated (Neo. trt.) larvae at 4 dpf showing reduced *pth2* expression following repeated chemical ablation of the lateral line hair cells. (F) Boxplots of *pth2*-positive cell counts from control and neomycin-treated larvae at 4 dpf (Control = 20, Treated = 20 larvae pooled from two replicates). Scale bar in

B is 50 μm and applies to all images. A one-tailed Welch's t-test was used to assess significance, **** = $p < 0.0001$.

Lack of evidence for modulation of pth2 expression by mechanosensory inputs from inner ear hair cells.

Low levels of *pth2* expression remain in socially-raised lateral line mutants (Figure 12B, C). To determine if mechanosensory input from the inner ear was responsible for this remaining *pth2* expression, we used three different types of mutations that disable hair cells of both the lateral line and inner ear to compare to the lateral line mutant (*lhfpl5b*$^{-/-}$) and wild type zebrafish. Two of these mutations (*pcdh15a*$^{-/-}$ and *tomt*$^{-/-}$) render the mechano-electrical transduction channel nonfunctional in all hair cells, similar to how the *lhfpl5b* mutation specifically disables lateral line hair cells (Erickson et al. 2017, 2020; Maeda et al. 2017). The third mutant disables the voltage-gated calcium channel in all hair cells (*cacna1da*$^{-/-}$), thereby blocking stimulus-dependent glutamate release (Sidi et al. 2004; Sheets, Kindt, and Nicolson 2012). Again, we measured *pth2* expression via mRNA ISH (Figure 13B-F) as well as quantitative PCR (qPCR) at 4 dpf (Figure 13G). Consistent with our ISH results from Figure 12, each class of hair cell mutant exhibits a significant decrease in *pth2* expression compared to their wild type siblings when raised socially (Supplemental Figure 4; One-tailed Welch's t-test; *lhfpl5b*: $t = 5.929$, $P = 0.011$, Cohen $d = 4.84$; *tomt*: $t = 3.857$, $P = 0.0305$, Cohen $d = 3.15$; *cacna1da*: $t = 5.154$, $P = 0.0044$, Cohen $d = 4.21$). However, the pan-hair cell mutants (*tomt*, *cacna1da*) did not differ in *pth2* expression levels compared to the lateral line-only mutant (*lhfpl5b*) (Figure 13G; One-way ANOVA; Mutant: $F = 2.425$, $P = 0.169$; Wild Type: $F = 0.791$, $P = 0.496$). Because pan-hair cell mutants and lateral line-only mutants are indistinguishable in terms of *pth2* expression levels, we conclude that inner ear

mechanosensory hair cells do not significantly contribute to the regulation of *pth2* expression in this context.

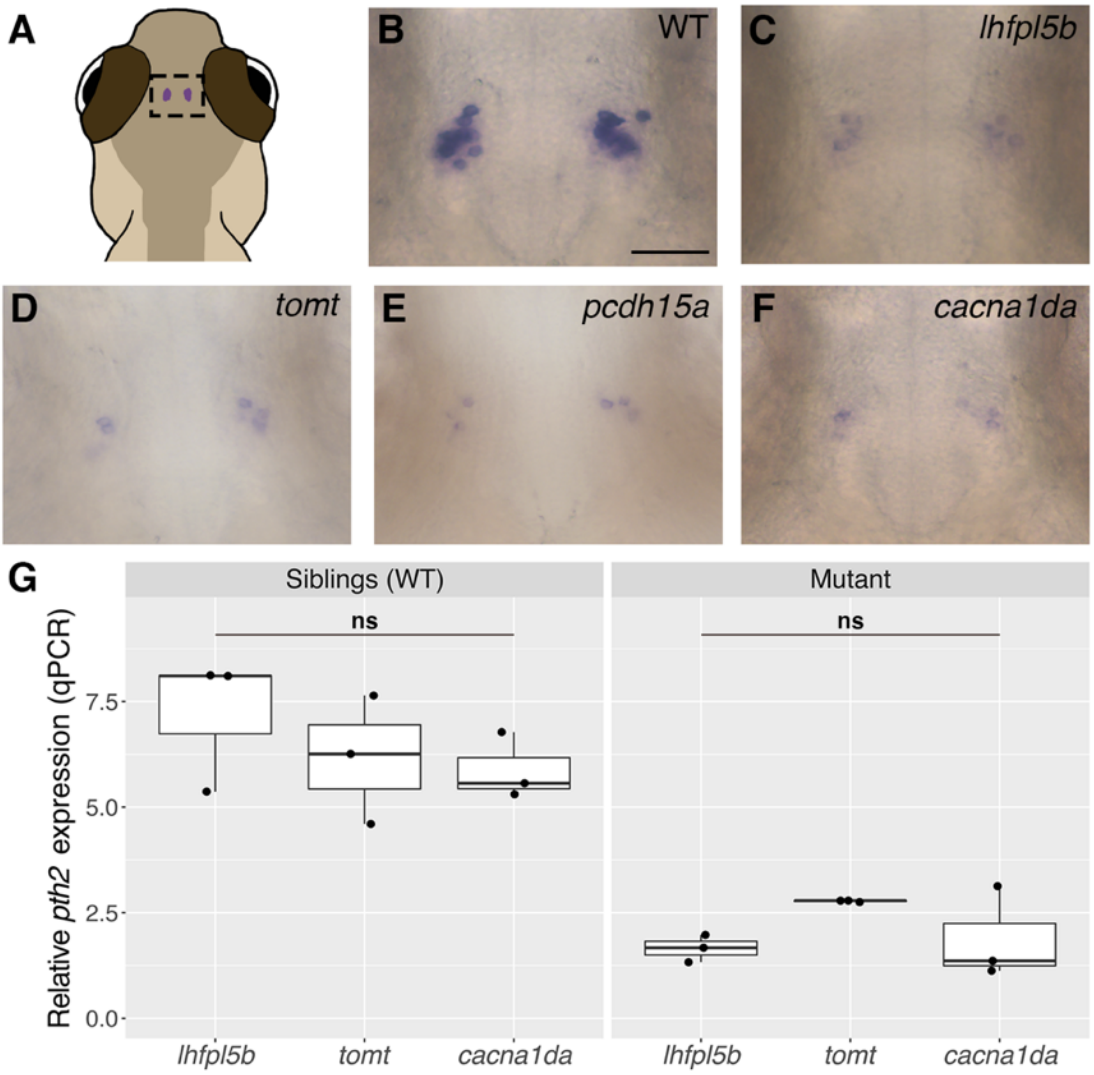


Figure 13: Lack of evidence for modulation of *pth2* expression by mechanosensory inputs from inner ear hair cells. (A) Location of *pth2*-expressing cells in the thalamic region of a larval zebrafish. (B-F) Representative images of mRNA *in situ* hybridizations (ISH) showing *pth2*-positive cells in socially-reared B) wild type (n = 14), C) *lhfpl5b*^{vo35} (n = 18), D) *tomt*^{tk256c}(n = 13), E) *pcdh15a*^{th263b} (n = 13), and F) *cacna1da*^{tc323d} (n = 18) larvae at 4 dpf. Scale bar = 50 µm, applies to all images. G: Boxplots of qPCR results showing *pth2* expression in socially-reared mutants and siblings at 4 dpf. Ten larval heads were pooled per condition to make one biological replicate, and three biological replicates were used to generate the data. Two technical replicates of the qPCR

experiment were conducted per biological replicate and averaged. One-way ANOVA to assess significance, ns = not significant.

Social isolation further reduces pth2 expression in sensory hair cell mutants.

Chemosensory and visual perception of conspecifics does not significantly rescue *pth2* expression in larval zebrafish raised in isolation (Anneser et al. 2020). However, since disabling both the lateral line and the inner ear does not completely eliminate *pth2* expression, other non-hair cell-based sensory systems may contribute to the modulation of *pth2* by social context. To test this, we compared *pth2* expression in social and isolated *lhfpl5b* and *tomt* mutants by both mRNA ISH (Figure 14A-C) and qPCR at 4 dpf (Figure 14D). If *pth2* responds to conspecific signals solely via the lateral line, we expect to see equivalently low expression in mutants raised in social and isolated environments. However, we find this not to be the case. Social environment has a significant effect on *pth2* expression, as isolated *lhfpl5b* and *tomt* mutants express less *pth2* than social mutant siblings (Two-way ANOVA; *lhfpl5b:* $F = 16.42$, $P = 0.007$; *tomt*: $F = 65.53$, $P = 0.00019$). Genotype does not have a significant effect on *pth2* levels when larvae are raised in isolation, as isolated mutants and isolated wild types are statistically indistinguishable (Two-way ANOVA; *lhfpl5b:* $F = 2.65$, $P = 0.154$; *tomt*: $F = 0.873$, $P = 0.386$). Because social isolation further reduces *pth2* expression in lateral line mutants, we conclude that *pth2* likely responds to multisensory input during social experience.

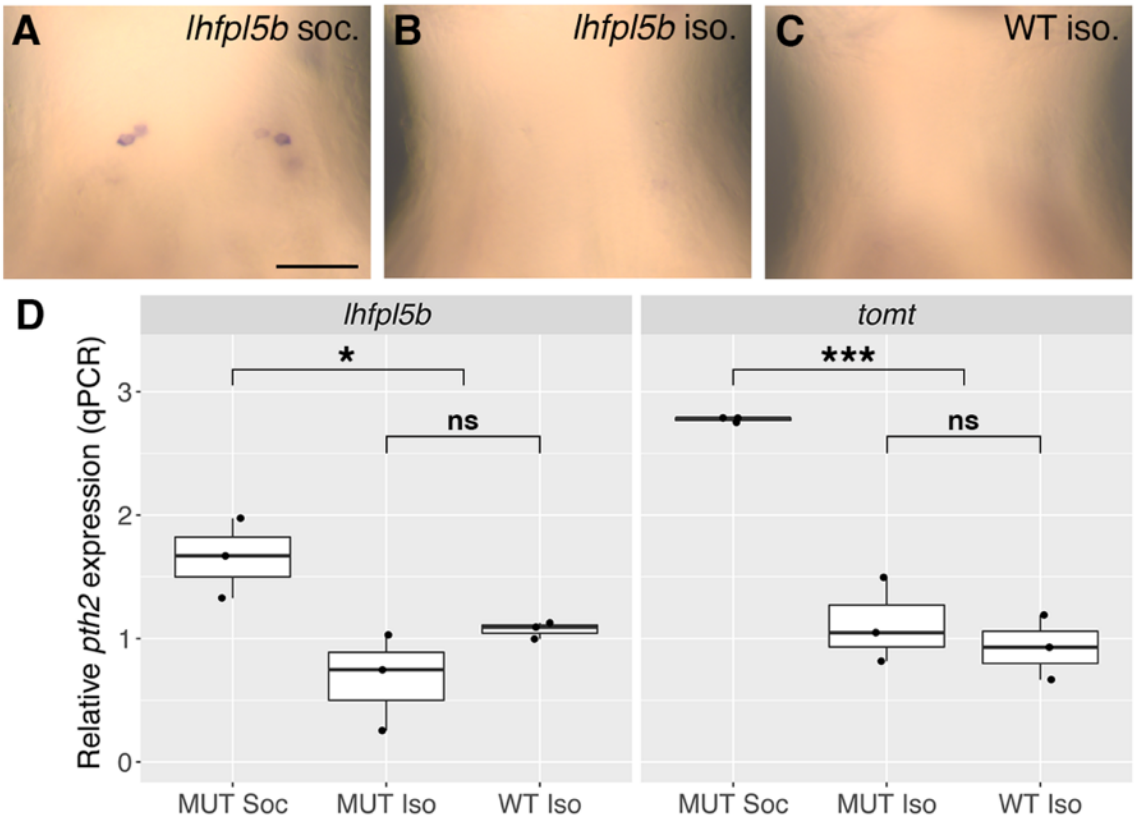


Figure 14: Social isolation further reduces *pth2* expression in sensory hair cell mutants. (A-C) mRNA *in situ* hybridization showing representative examples of *pth2*-positive cells in 4 dpf A) socially-reared *lhfpl5b*^{vo35} mutants (n = 48), B) isolated wild type larvae (n = 50), and C) isolated *lhfpl5b*^{vo35} mutants (n = 40). Scale bar = 50 µm, applies to all images. D: Boxplots of qPCR results comparing *pth2* expression in socially-reared *lhfpl5b*^{vo35} and *tomt*^{tk256c} mutants relative to isolated mutant and wild type siblings at 4 dpf. Ten larval heads were pooled per condition to make one biological replicate, and three biological replicates were used to generate the data. Two technical replicates of the qPCR experiment were conducted per biological replicate and averaged. Two-way ANOVA to assess significance, *** = $p < 0.001$, * = $p < 0.05$, ns = not significant.

Morphology of pth2-expressing cells using fluorescent reporters.

Fluorescent reporters provide information about the morphology and function of cells, along with the ability to quantify gene expression in living organisms (Choe et al. 2021; Soboleski, Oaks, and Halford 2005). Therefore, we created stable lines of transgenic zebrafish expressing

GFP or RFP under control of the presumptive *pth2* promoter (Figure 15). Imaging of socially-reared, transgenic larvae reveals the two bilateral cell clusters with complex projection patterns (Figure 15A, B). The anterior ipsilateral projections partially converge into a dense neuropil in the forebrain, while other fibers project ventrally and posteriorly toward the midbrain. We also find ventral projections that cross the midline between the two cell clusters. The projection patterns of the neurites and number of cells are similar for both the GFP and RFP transgenes (Supplemental Figure 5; GFP: M = 38.5, SD = 2.8; RFP: M = 37.1, SD = 2.6; Two-tailed Welch's t-test; $t = 1.422$, $P = 0.166$, Cohen $d = 0.519$). Combining counts from both clusters, there is an overall average of 40.3 cells per individual (SD = 2.27). The cells within each cluster exhibit a range in fluorescent intensity, and over half the cells fall within the two lowest intensity bins (Figure 15C, D; M = 27.9, SD = 3.42).

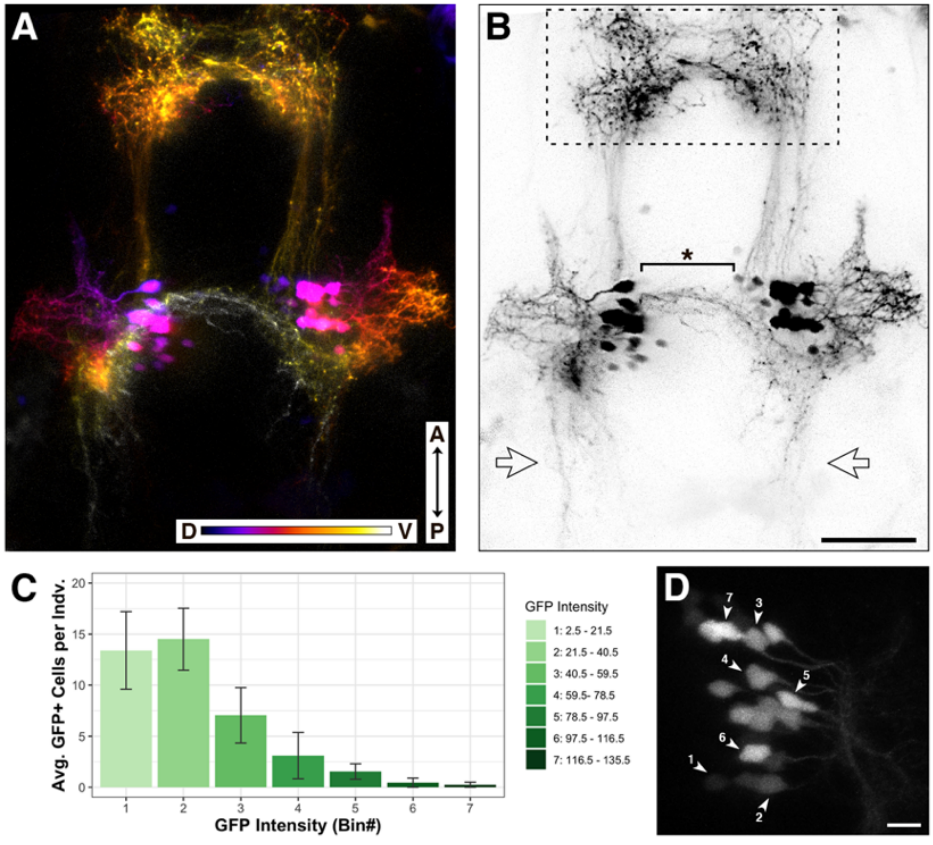


Figure 15: Morphology of *pth2*-expressing cells using fluorescent reporters. A: Depth-encoded representation of the *Tg(pth2:TagRFP)unb3* transgenic line at 5 dpf. The anterior-posterior (A-P) axis and dorsal-ventral (D-V) depth color scale are indicated at the bottom. Image depth is 152 μm. B: Black and white version of the image in A. From the top: the dashed box indicates the anterior neuropil, the starred bracket highlights the ventral neurites that cross the midline, and the bilateral open arrows point to posterior neurites. Scale bar in B is 50 μm, applies to both images. C: Bar graph showing binned fluorescent intensity of GFP-positive cells (n = 20 larvae from 2 different clutches at 4 dpf). D: Zoomed-in image of one cell cluster exhibiting the full range of cell fluorescent intensity, labelled according to bins outlined in the associated bar graph. Image depth is 32 μm. Scale bar is 10 μm.

The pth2 transgenes respond to social context and lateral line input.

Next, we tested if the *pth2* transgenes respond to social context and lateral line input like endogenous *pth2* (Figure 16). Consistent with our mRNA ISH and qPCR results, isolated *Tg(pth2:TagRFP)unb3* larvae exhibit a significant decrease in the average number of TagRFP-positive, *pth2* cells (false-colored green) (M=28.3, SD = 2.8) relative to socially-reared siblings (M = 37.3, SD = 2.6) at 4 dpf (Figure 16A-C: One-tailed Welch's t-test; $t = 8.949$, $P = 5.42\text{x}10^{-10}$, Cohen $d = 3.27$). Similarly, social *Tg(pth2:EGFP)unb2*; *lhfpl5b*$^{vo35/vo35}$ larvae have fewer fluorescent cells on average (M = 34.2, SD = 2.8) than their social, WT siblings (M = 43.2, SD = 3.9) (Figure 16D-F; One-tailed Welch's t-test; $t = 6.421$, $P = 1.55\text{x}10^{-6}$, Cohen $d = 2.62$). To gauge whether isolation specifically diminishes expression from the *pth2*-driven transgenes, we compared the effect of social isolation on transgene expression in *Tg(pth2:TagRFP)unb3; Tg(slc6a3:EGFP)ot80* double transgenic larvae. The *slc6a3:EGFP* transgene expresses GFP under control of the dopamine transporter (DAT) promoter in several discrete regions of the zebrafish brain (Xi et al. 2011), including a population of dopaminergic cells in the pretectum lying just dorsal to the *pth2*-expressing cells. If social context specifically modulates expression from the

pth2:TagRFP transgene, we expect that isolation will have no effect on the number of neighboring dopaminergic cells. Indeed, the number of GFP-positive, dopaminergic cells (false-colored magenta) in the pretectum do not change in response to social context (Figure 16A-C; WT Soc: M = 44.0, SD = 4.3; WT Iso: M = 45.1, SD = 4.7; One-tailed Welch's t-test, t = -0.654, P = 0.741, Cohen d = 0.239). These experiments demonstrate that the *pth2* transgenes specifically respond to both social context and lateral line input. However, we note that the effects of isolation and lateral line-deficiency on the *pth2* transgenes are not as pronounced as their effects on endogenous *pth2* mRNA expression (Figures 12 and 13). To test whether this discrepancy is due to the high stability of green and red fluorescent proteins (Verkhusha et al. 2003; Sacchetti et al. 2001), we compared the number of GFP-positive cells at 4 dpf when we begin isolation at 1 dpf vs. 2 dpf (Supplemental Figure 6). We find that increasing the duration of isolation does not significantly change the number of GFP-positive cells (1 dpf Iso: 29.8, SD = 2.6; 2 dpf Iso: M = 29.3, SD = 3.4; Two-tailed Welch's t-test; t = -0.371, P = 0.716, Cohen d = 0.166). This indicates that the discrepancy between transgenic and endogenous *pth2* expression is not due to insufficient isolation time.

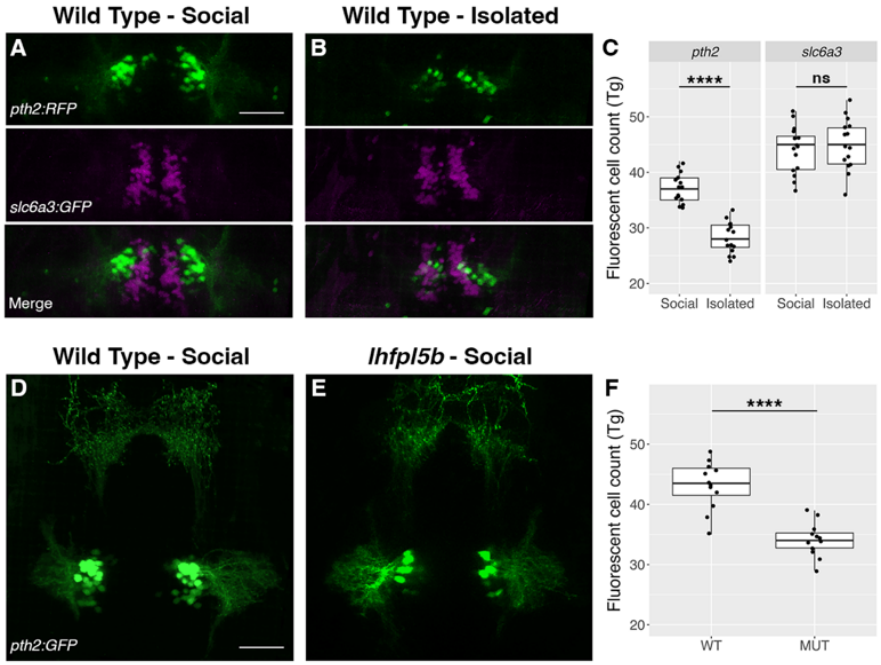


Figure 16: The *pth2* transgenes respond to social context and lateral line input. (A-B) Representative images of *Tg*(*pth2:TagRFP)unb3* (false-colored green) and *Tg(slc6a3:EGFP)ot80* (false-colored magenta) transgenes in socially-reared and isolated larvae at 4 dpf. Merges of the two channels show the close proximity of the *pth2* and *slc6a3*-positive cell clusters. (C) Boxplots of *pth2* and *slc6a3* cell counts (n = 15 per condition). (D-E) Representative images of *Tg*(*pth2:EGFP)unb2* transgene expression in socially-reared wild type and lateral line mutant (*lhfpl5b*^{vo35}) larvae at 4 dpf. (F) Boxplots of GFP-positive cell counts in wild type (WT) and *lhfpl5b* mutant (MUT) siblings (n = 12 per condition). Scale bar is 50 μm. For both experiments, a one-tailed Welch's t-test was used to assess significance, **** = $p < 0.0001$, ns = not significant.

Discussion

Previous work revealed that larval zebrafish use lateral line information to sense the presence of conspecifics, leading to upregulation of the social hormone Pth2. Here, we show that *pth2* levels are chronically depressed in socially-raised larvae born without lateral line function. However, social isolation further reduces *pth2* levels in lateral line mutants, suggesting that other socially-derived sensory information can modulate *pth2* expression.

The mechanosensory lateral line mediates pth2 expression.

Anneser et al. (2020) implicated hydrodynamic stimuli from conspecifics as a key social cue that upregulates production of the neuropeptide Pth2. Isolated larvae with chemically ablated lateral lines fail to upregulate *pth2* when returned to a social environment. In the present study, we use mutant zebrafish with a congenital loss of lateral line function to confirm that hydrodynamic information modulates *pth2* expression. Furthermore, we find that repeated chemical ablation of the lateral line also impairs the ability of social larvae to express *pth2*, even when exposed to conspecifics prior to loss of lateral line function. Our approach complements that of Anneser et al. (2020) by providing genetic evidence that *pth2* is modulated by lateral line sensory information

(Figure 12). We conclude that, in terms of Pth2 production, zebrafish born without a lateral line experience a form of isolation even when raised in a social context.

Auditory and vestibular stimuli do not modulate pth2 expression in larval zebrafish.

Inner ear hair cells are functionally and structurally similar to lateral line hair cells, and fish use auditory and vestibular sensory information in a variety of behaviors (Privat et al. 2019; Bagnall and Schoppik 2018; Kohashi, Nakata, and Oda 2012; Nicolson et al. 1998). Given that some *pth2* expression remains in lateral line mutants, we examined whether sensory information from the inner ear contributes to social modulation of *pth2.* Using zebrafish with mutations that disable both inner ear and the lateral line hair cells, we find that there is no additional loss of *pth2* expression compared to the lateral line-only mutant (Figure 13). In addition, we asked if different types of mutations to hair cells impact *pth2* expression. Three of the mutant lines used in this study, *lhfpl5b, tomt* and *pcdh15a,* render the mechanotransduction (MET) channel non-functional (Erickson et al. 2017, 2020; Maeda et al. 2017), and one mutant line, *cacna1da / cav1.3a,* affects the voltage-gated calcium channel (Sheets et al. 2012; Sidi et al. 2004). All mutant alleles are recognized as nulls since mutant hair cells show little to no residual mechanosensory activity, as demonstrated by the lack of evoked spikes in the posterior lateral line ganglion, the absence of microphonic potentials and mechanotransduction currents, or the absence of MET channel-dependent FM dye labeling of hair cells (Nicolson et al. 1998; Erickson et al. 2017; Erickson et al. 2020; Sidi et al. 2004). While the MET channel and calcium channel are both necessary components for proper hair cell signal transduction, there are key differences between the types of mutations. For mutants affecting the MET channel, potassium and calcium ions are unable to enter the cell in response to stimuli, but the potential for spontaneous calcium influx and neurotransmitter release remains (Trapani and Nicolson 2011). Whereas for the calcium channel

mutation, ions enter through the MET channel but there is no evoked or spontaneous neurotransmitter release. Regardless, all mutations that disrupt hair cell function result in the same decrease of *pth2* expression in socially raised fish compared to their wild type siblings (Figure 13). Therefore, we conclude that regulation of *pth2* by social context is primarily via the mechanosensory lateral line in larval zebrafish and that auditory and vestibular information likely does not contribute.

Additional socially-derived sensory cues modulate pth2 expression.

Anneser et al. (2020) demonstrated that neither chemical nor visual cues from conspecifics significantly restored *pth2* expression in isolated larvae. In our study, we found that isolation further reduced *pth2* expression in lateral line mutants to the same level as isolated wild types (Figure 14). This indicates that additional sensory modalities contribute to the regulation of *pth2* expression. Recent studies have identified thalamic neurons in the zebrafish that respond to biological motion and shown that these neurons are molecularly defined by the partially overlapping expression of *pth2* and *cortistatin* (*cort* / *sst7*) (Sherman, Kawakami, and Baier 2021; Kappel et al. 2022). It will be informative to test whether photo- or chemosensory systems can modulate *pth2* expression under sensitized circumstances in which hydrosensory information is compromised. Additionally, it is possible that tactile stimuli are contributing to *pth2* regulation in zebrafish, consistent with the effects of social touch in rodents. Future work is necessary to test this theory.

Visualizing pth2 expression in vivo.

We used *pth2* promoter-driven fluorescent transgenes to visualize *pth2*-expressing cells and their projections. Consistent with the Anneser et al. (2020) whole-mount immunostains, we see Pth2-positive cells located in bilateral clusters in the thalamus that feature lateral neurites,

anterior ipsilateral projections terminating as a loosely organized neuropil in the telencephalon, as well as posterior ipsilateral projections. The transgene reveals additional fibers that project ventrally from the cell bodies and cross the midline, potentially contacting the contralateral *pth2*-expressing cluster (Figure 15). Investigating the morphology of these cells will help us understand the cellular targets of Pth2 signaling and how activity of the two clusters is coordinated. We note an excess of putative *pth2*-expressing cells in the transgenics compared to our ISH experiments and the immunostain counts of Anneser et al. (2020). This discrepancy could be due, in part, to the increased sensitivity of using confocal microscopy to detect *pth2*-expressing cells in a live transgenic animal as opposed to imaging fixed tissue stained for mRNA or protein. Consistent with this idea, we find that the majority of GFP-positive cells in socially-reared *Tg(pth2:EGFP)* larvae exhibit low fluorescence intensity (Figure 15C, D). If these dim transgenic cells represent neurons that express *pth2* at a level below the detection limit of mRNA ISH or immunostain, this may account for the excess cell counts in our transgenics.

We also tested the *pth2:EGFP* and *pth2:TagRFP* transgenic lines as a proxy for *pth2* expression *in vivo* and found that both lateral line mutants and isolated wild type larvae exhibit fewer fluorescent cells than socially raised siblings (Figure 16). In contrast, the number of dopaminergic cells located dorsally to the *pth2* clusters exhibit no change to social context. Dopamine has been implicated in social interactions (Saif et al. 2013; Haehnel-Taguchi et al. 2018; Shams et al. 2018), so we used the nearby clusters of GFP-labeled dopaminergic cells in the *slc6a3:EGFP* transgenic line to test the specificity of the response of the *pth2* transgene to social context. Our results indicate that the social modulation of *pth2* is specific and distinct from the dopaminergic system in this brain region of larval zebrafish.

While our results with the *pth2* transgenes agree with our *in situ* hybridization and qPCR results (Figures 12-14), we note that the effects of isolation and lateral line deficiency on transgene expression are not as prominent as when assaying for endogenous *pth2* mRNA and protein. As mentioned above, the enhanced detectability of genetically-encoded reporters in live larvae may account for some of the residual fluorescence that remains following social isolation of transgenics. It could also be that the post-transcriptional and / or post-translational stability of the fluorescent transgenes differ from endogenous *pth2* mRNA and protein stability. We tested whether the long half-life of GFP accounts for the persistence of GFP-positive cells in isolated *Tg(pth2:EGFP)* larvae but found that extending the duration of isolation from 48 to 72 hours did not significantly affect the number of fluorescent cells (Supplemental Figure 6). Lastly, it is possible that additional regulatory elements in the *pth2* promoter or 3' UTR, along with the use of a destabilized fluorescent protein, are required to mimic the endogenous regulation of *pth2*. Future work will strive to distinguish between these possibilities and to optimize the transgene for more accurate reporting of *pth2* dynamics in vivo.

Overall, our results confirm that *pth2* expression is modulated by the mechanosensory lateral line (Figure 12). We also show that the lateral line is the only hair-cell based sensory organ regulating *pth2* expression (Figure 13). However, due to expression levels being slightly higher in social lateral line mutants compared to the same mutants or wild type larvae raised in isolation, we conclude that there must be unidentified sensory system(s) that contribute to the modulation of *pth2* (Figure 14). Finally, we created transgenic lines to visualize the morphology of *pth2*+ cells *in vivo* (Figure 15), and report that the transgenes respond to social context (Figure 16). These transgenic lines can be used in future studies to elucidate the role of *pth2* in zebrafish. Taken

together, this work suggests that zebrafish without a functioning lateral line experience a form of isolation, even in a social context.

CHAPTER 4: A SENSATION FOR INFLATION: INITIAL SWIM BLADDER INFLATION IN LARVAL ZEBRAFISH IS MEDIATED BY THE MECHANOSENSORY LATERAL LINE

Published:

Alexandra Venuto, Stacey Thibodeau-Beganny, Josef G. Trapani, Timothy Erickson; A sensation for inflation: initial swim bladder inflation in larval zebrafish is mediated by the mechanosensory lateral line. *J Exp Biol* 2023; jeb.245635. https://doi.org/10.1242/jeb.245635.

Abstract

Larval zebrafish achieve neutral buoyancy by swimming up to the surface and taking in air through their mouths to inflate their swim bladders. We define this behavior as 'surfacing'. Little is known about the sensory basis for this underappreciated behavior of larval fish. A strong candidate is the mechanosensory lateral line, a hair cell-based sensory system that detects hydrodynamic information from sources like water currents, predators, prey, and surface waves. However, a role for the lateral line in mediating initial inflation of the swim bladder has not been reported.

To explore the connection between the lateral line and surfacing, we utilized a genetic mutant (*lhfpl5b*$^{-/-}$) that renders the zebrafish lateral line insensitive to mechanical stimuli. We observe that approximately half of these lateral line mutants over-inflate their swim bladders during initial inflation and become positively buoyant. Thus, we hypothesize that larval zebrafish use their lateral line to moderate interactions with the air-water interface during surfacing to regulate swim bladder inflation. To test the hypothesis that lateral line defects are responsible for swim bladder over-inflation, we show exogenous air is required for the hyperinflation phenotype

and transgenic rescue of hair cell function restores normal inflation. We also find that chemical ablation of anterior lateral line hair cells in wild type larvae causes hyperinflation. Furthermore, we show that manipulation of lateral line sensory information results in abnormal inflation. Finally, we report spatial and temporal differences in the surfacing behavior between wild type and lateral line mutant larvae. In summary, we propose a novel sensory basis for achieving neutral buoyancy where larval zebrafish use their lateral line to sense the air-water interface and regulate initial swim bladder inflation.

Introduction

The swim bladder of teleost fish provides for neutral buoyancy in the water column, thereby minimizing the energy expenditure associated with locomotion (Alexander 1966). Larval teleosts initiate swim bladder inflation by swimming up to the surface to ingest air using a set of behaviors we refer to as "surfacing" (Lindsey, Smith, and Croll 2010; Evans and Damant 1928; Ledebur and Wunder 1937). Although surfacing poses a significant risk of predation, larval fish are highly motivated to reach the surface to intake air. For example, pre-inflated lake trout larvae will swim hundreds of feet at a constant rate in attempts to access the surface (Tait 1960). Surfacing is a complex task that requires a combination of newly developed physical features, sensory systems, and motor skills (Kitajima et al. 1994; Chatain 1989; Bailey and Doroshov 1995; Doroshev, Cornacchia, and Hogan 1981; Kimmel et al. 1995) – (i) Larvae must discriminate up from down and (ii) be able to move directionally towards the surface; (iii) they must appropriately sense the air-water interface, and (iv) be able to intake air through the mouth upon arrival at the surface. Ingested air is then moved by peristalsis into the swim bladder through the pneumatic duct that connects the gut and swim bladder (Rieger and Summerfelt 1998; Tait 1960; Doroshev,

Cornacchia, and Hogan 1981). Together, surfacing represents perhaps the most complex behavior that larvae perform prior to achieving neutral buoyancy (Fero, Yokogawa, and Burgess 2011).

Little is known about the sensory cues that instruct the surfacing behavior. Perturbations to the vestibular system typically result in hypoinflation (Schoppik et al. 2017; Nicolson et al. 1998). The inability to detect gravity by the utricular otolith is specifically responsible for the phenotype, presumably because larvae are unable to orient their movements upwards towards the surface (Riley and Moorman 2000). Photosensory cues may also play a role in directional orientation and surface detection. However, which photosensory systems are involved has not been defined and the effects of altering lighting conditions varies between species (Trotter, Battaglene, and Pankhurst 2003; Villamizar, García-Alcazar, and Sánchez-Vázquez 2009; Stuart and Drawbridge 2012). Even less is known about how larval fish detect the air-water interface itself. Fish and some amphibians possess an additional hair cell-based sensory system - the *mechanosensory lateral line* - that contributes to detection of near-field hydrodynamic information originating from water currents, predators and prey, conspecifics, abiotic objects, and surface waves (Anneser et al. 2020; Bleckmann 2006; Butler and Maruska 2016a; Carrillo and McHenry 2016; Mogdans 2019; Montgomery et al. 1997; Stewart et al. 2013; Venuto et al. 2022). Specific to surface detection, surface-feeding fish use their lateral line to detect surface waves created by their prey (Bleckmann and Schwartz 1982). Furthermore, Japanese flying fish are predicted to use their ventrally-located lateral line to sense transitions through the air-water interface (Gibbs 2004; Tsukamoto and Yoshino 1957). While the lateral line is a strong candidate to aid in surfacing behaviors that lead to initial swim bladder inflation in larval fish, its specific role in this important behavior has not been explored.

A CRISPR-Cas9 knockout of *LHFPL tetraspan subfamily member 5b* (*lhfpl5b*) is the first genetic zebrafish mutant where the lateral line is non-functional from birth but hearing and balance are normal (Erickson et al. 2020). These mutants provide an opportunity to uncover novel roles for the lateral line. Unexpectedly, homozygous mutant larvae present with a hyperinflation phenotype characterized by over-filled swim bladders and positive buoyancy, a very rare finding among swim bladder defects (McCune and Carlson 2004; Abbas and Whitfield 2009). Using several genetic and chemical techniques to manipulate lateral line function, we show that lateral line sensory information is used by larval zebrafish to appropriately fill their swim bladders, likely by detecting surface tension at the air-water interface using mechanosensory neuromasts located on their heads. Overall, our study uncovers a novel role for the mechanosensory lateral line mediating initial inflation of the swim bladder.

Materials and Methods

Animal husbandry and ethics statement

Adult zebrafish (*Danio rerio*) were maintained and bred using standard procedures (Westerfield 2000). All experiments used larvae at 2-6 days post-fertilization (dpf), which are of indeterminate sex at this stage. Except where otherwise stated, animals were placed in clear, plastic tubs (dimensions: 15 cm x 10 cm x 4 cm, E3 volume: 460 mL) at 2 dpf and maintained at 28.5°C on a 14:10 light/dark cycle at 500-700 lux in E3 embryo media (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgCl2, buffered with NaHCO3). Unhatched embryos were manually dechorionated before placing larvae in experimental conditions at 2 dpf. It is worth noting that there was no obvious difference in wild type versus *lhfpl5b* mutant hatching rate, though this was not explicitly studied. Animal research complied with guidelines stipulated by the Institutional

Animal Care and Use Committees at East Carolina University (protocol D372) and Amherst College (assurance number 3925-1 with the Office of Laboratory Animal Welfare).

Mutant and transgenic fish lines

The zebrafish mutant allele *lhfpl5b*^{vo35} and transgenic lines *Tg(myo6b:EGFP-lhfpl5a)vo23* and *Tg(myo6b:EGFP-PA)vo68* were used in this study (Erickson et al. 2017, 2020). For all experiments, *lhfpl5b*^{vo35} homozygotes were identified from their hetero- and homozygous wild type siblings by lack of FM 1-43 (ThermoFisher) labeling of the lateral line hair cells.

To create a fish line that expresses the Channelrhodopsin-2 (ChR2) optogenetic protein solely in the hair cells of the lateral line, approximately 2 kb of DNA upstream of the start codon of the *lhfpl5b* gene was cloned into the p5E plasmid of the recombination-based multisite Gateway cloning system (ThermoFisher). A Tol2 transposon backbone was then used to create the injectable expression plasmid *lhfpl5b2028:ChR2-EYFP-PA*. This plasmid was then co-injected with transposase mRNA into single-cell embryos (Kwan, et al., 2007) to create the *Tg(lhfpl5b2028:ChR2-EYFP-PA)* zebrafish line.

Buoyancy tests

To ascertain the buoyancy status of larval fish, larvae were anesthetized with MS-222 (Western Chemical Inc., Ferndale, WA) and placed at the vertical midway point of a 50 mL conical tube containing E3 media. At the expiration of a 30-second timer, the end position of the fish was recorded and the fish was removed from the tube for imaging under the microscope. If the fish reached the top or bottom of the tube before 30 seconds, then the time it took to reach that location was recorded. The total height of the water column was 8.9 cm, and the fish was placed half-way at 4.45 cm. The rate of buoyancy was determined by dividing the centimeters travelled by the seconds it took to travel that distance (cm/s). Over-inflated fish were categorized by larvae floating

to the top of the tube in 30 seconds, while under-inflated fish sunk to the bottom of the tube. Neutrally boutant or regular-inflated larvae remained near the middle of the water column. This test was conducted during the imaging portion at the end of each experiment, with the exception of the oil-water interface experiment where buoyancy could not be accurately determined due to the greater density of oil compared to air.

Imaging and swim bladder measurements

Swim bladders were imaged using a SteREO Discovery.V8 microscope (Zeiss) equipped with a Gryphax Arktur camera (Jenoptik). Lateral and dorsal images of the swim bladder were taken for each fish. Swim bladder measurements were conducted using the measurement tool in Adobe Photoshop where measurements were calibrated according to the image of the stage micrometer taken when imaging experiments. The major axis and minor axis of the lateral view of the swim bladder was measured as well as the minor axis of the dorsal view. All measurements were taken from numbered images so that the measurer was blinded to the genotype/condition. These meaurements were halved and used in the equation for the volume of an ellipsoid: $V = 4/3\pi abc$ to find the volume of the swim bladder (Lindsey, Smith, and Croll 2010).

Manipulation of photosensory cues during surfacing

At 0 dpf, zebrafish embryos were collected and immediately placed in plastic tubs filled with E3 (detailed above) in an unlit incubator. Larvae were not brought into light until 6 dpf, at which point lateral line mutants and wild type siblings were identified by FM 1-43 labeling. After sorting, larvae were analyzed for buoyancy. The experiment was repeated three times using 20-21 larvae (combined mutant and wild type) per trial.

Blocking access to the air-water interface

Mutant larvae and wild type siblings were sorted at 2 dpf into glass cylinders (diameter: 9.5 cm, E3 volume: 425 mL) equipped with fitted, wire mesh filter plungers (Bodum Inc., USA). Conditions were as follows: wild type with open access to the surface, wild type with blocked access to the surface, mutant with open access to the surface, and mutant with blocked access to the surface. All containers were filled half-way with E3 and in the experimental condition ("blocked access"), the filter was pressed down below the surface for both wild type and mutant larvae. The control condition used the same amount of E3 but the filter remained above the surface so that larvae could access the exogenous air. Conditions remained constant until 6 dpf when larvae were imaged and analyzed for swim bladder inflation. The experiment was repeated three times using 15-25 larvae per condition.

Analysis of surfacing behavior

Wild type and *lhfpl5b*^{vo35} larvae were were sorted at 2 dpf and blocked from having access to the surface until 9 AM on 4 dpf when individuals were immediately placed into clear rectangular containers (dimensions: 3 cm x 3 cm x 3 cm, E3 volume: 20 mL) for video recording. Recordings lasted 12 hours, from 9 AM to 9 PM on 4 dpf. At the end of filming, larvae were imaged and analyzed for buoyancy and swim bladder volume, as described. All surfacing videos were numbered for blind analysis. Surfacing analysis was conducted by manual observation of each individual larvae from the videos (30 frames per second) and manually counting visits made to the surface (counted as reaching the meniscus), the timeframe of the video at which the surfacing event occurred, and the duration of time spent at the surface per surfacing event. Recording was repeated six times with both mutant wild type larvae present in each recording. Larvae that did not inflate

their swim bladders by the end of filming (5 total larvae, 1 wild type and 4 mutants) were excluded from this analysis due to lack of surfacing events.

Lateral line hair cell ablations

For all experiments, wild type and *lhfpl5b* mutant larvae were treated with ototoxin with untreated siblings acting as controls (n = 7-15 larvae per condition per trial, 4 trials) For single treatments, surface accessed was blocked as described above for untreated and treated larvae until 9 AM on 4 dpf when treatment occurred. Single 30 minute treatments with neomycin sulfate (EMD Millipore Corp., USA) were done with a 100 µm solution in E3 while repeated neomycin treatments (Venuto and Erickson, 2021) were done as previously described with a concentration of 50 µM. For full ablation with copper sulfate (CuSO4; Ward's Science, Rochester, NY), the treatment concentration was 10 uM with a 30 minute exposure. For experiments where head and trunk neuromasts were selectively ablated with CuSO4, anesthetized larvae were moved to a rectangular plate (4.5 cm x 3 cm x 1 cm) coated with Sylgard (Electron Microscopy Sciences, Hatfield, PA). After removing excess water, a thin layer of petroleum jelly (Vaseline, Unilever USA) was placed horizontally at the back of the head. Next, 30 uM CuSO4 or E3 was pipetted onto the appropriate region depending on the experimental condition. Immediately following the 13 minute treatment, larvae were placed in fresh E3 in clear tubs detailed above for 24 hours until 9 AM on 5 dpf when larvae were imaged and analyzed for swim bladder inflation. For all experiments, larvae were imaged and analyzed for swim bladder inflation at 9 AM on 5 dpf. Each experiment was repeated three times using 10-24 larvae per condition.

Transgenic rescue of lateral line mutant

Larval zebrafish from crosses of *lhfpl5b*$^{+/-}$ and *Tg(myo6b:EGFP-lhfpl5a)vo23Tg; lhfpl5b*$^{+/-}$ were sorted at 2 dpf for transgene expression. At 6 dpf, larvae were numbered, imaged

for swim bladder inflation and genotyped for the *lhfpl5b*^{vo35} allele as previously described (Erickson et al., 2020). The experiment was repeated twice using 26-48 larvae per condition.

Optogenetic activation of lateral line hair cells with Channelrhodopsin-2 (ChR2)

After blocking access to the surface until 4 dpf, we exposed wild type and lateral line mutants, with or without the *lhfpl5b2028:ChR2-EYFP-PA* transgene, to LED blue light (470 nm) with an intensity of 730 lux over their holding tanks against a dark background. Light flashed for 25 milliseconds at one second intervals. Control larvae were kept in the same background, without blue light exposure. Conditions were held constant until 6 dpf when larvae were sorted for genotype, imaged and analyzed for swim bladder inflation. The experiment was repeated three times using 15-25 larvae per condition.

Oil-water interface experiments

Mutant larvae and wild type siblings were sorted at 2 dpf into clear cylindrical containers (diameter: 5 cm , volume: 60 mL) with embryo media only or embryo media with a 2 mm thick surface layer of lab grade mineral oil (Ward's Science, Rochester, NY). Oil on the surface decreases surface tension (Johansen 1924) and has been previously used in experiments involving the zebrafish swim bladder (Ehrlich and Schoppik 2017). Conditions remained constant until 6 dpf when larvae were imaged and analyzed for swim bladder volume. Because oil-filled larvae are negatively buoyant, we did not perform buoyancy tests and classified larvae as over-filled based on swim bladder volume alone. The experiment was repeated three times using 15-25 larvae per condition.

Graphs and statistical tests

All graphs and statistical tests were done using R (R Core Team 2021). One-way ANOVAs were used to compare the conditions as a whole (genotype independent variable plus environment

independent variable) with Tukey post-hoc test. Chi-squared tests were used to analyze proportion data for over and under-inflation. For the over-inflation comparisons, the two categories used in the comparison between conditions/genotypes were (i) over-inflation and (ii) all other inflation (regular and under), meaning that totals per category/genotype accounted for 100% of the population. Similarly, for the under-inflation comparisons, the two categories used in the comparison between conditions/genotypes were (i) under-inflation and (ii) all other inflation (regular and over). P-values less than 0.05 were considered significant.

Results

Lateral line mutants exhibit a swim bladder hyperinflation phenotype

The lateral line has not been previously implicated in initial inflation of the larval swim bladder. As such, it was unexpected to observe that lateral line mutants (*lhfpl5b*$^{-/-}$) exhibit hyperinflated swim bladders by 5-6 dpf (Figure 17A). Based on our swim bladder volume and buoyancy measurements, we categorized each larva in the mutant (n = 31) and wild type (n = 33) populations as over-, regular-, or under-inflated (Figure 17B). On average, 53.7% (SD = 6.4%, n = 41) of lateral line mutants over-inflated their swim bladders and 7.3% (SD = 7.1%, n = 41) under-inflated. For wild type larvae (including *lhfpl5b*^{vo35} heterozygotes), 0.0% (SD = 0.0%, n = 41) over-inflated and 4.9% (SD = 3.7%, n = 41) under-inflated their swim bladders during initial inflation on average (Figure 17C). The proportion of over-inflation was significantly different between mutant and wild type larvae (chi-square statistic: 70.45, $p < 0.00001$), while the proportion of under-inflation was not (chi-square statistic: 0.3546, $p = 0.552$). Average swim bladder volume of mutant fish was larger (M = 0.012 mm^3, SD = 0.002 mm^3) than wild type siblings (M = 0.0058 mm^3, SD = 0.00043 mm^3, $t(80) = 17.4$, $p < 0.0001$) (Figure 17D). The proportions of neutrally and positively buoyant larvae did not change when raised in total darkness (Supplemental Figure 7).

This unexpected hyperinflation phenotype in *lhfpl5b*^{vo35} homozygotes led us to investigate if the lateral line is contributing important sensory information during the surfacing behavior.

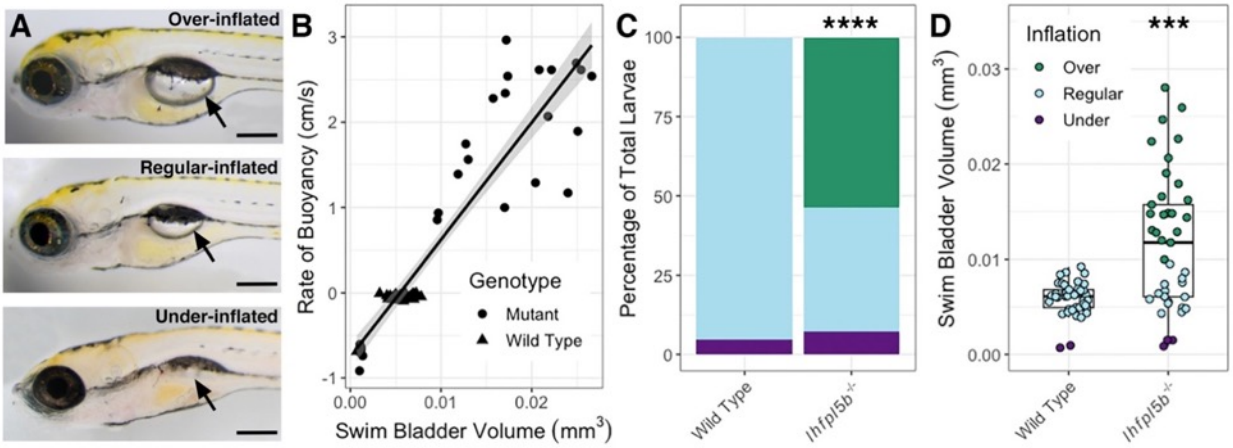


Figure 17. Hyperinflation of the swim bladder in lateral line mutants (*lhfpl5b*$^{-/-}$). A) Representative examples of over-, regular, and under-inflated swim bladders (arrows) in larval zebrafish at 6 dpf (top: *lhfpl5b*$^{-/-}$, others are wild type). B) Correlation between buoyancy and swim bladder volume. Line of best fit applied through the combination of mutant and wild type data (linear model, R-squared = 0.8574). C) Percentage of larvae that exhibit over-, regular, and under-inflation of the swim bladder, as defined by positive, neutral, and negative buoyancy. Legend from panel D applies to both panel C and D. A Chi-Squared Test was used to determine significance. D) Swim bladder volume (mm^3) in wild type and *lhfpl5b* mutant larvae at 6 dpf. A Welch's t-test was used to determine significance. *** = $p < 0.001$, **** = $p < 0.0001$. Scale bars = 0.2 mm.

Access to the air-water interface is required for the hyperinflation phenotype of lateral line mutants

For most teleosts, initial swim bladder inflation requires an intake of exogenous air, typically from the water's surface. To test if the hyperinflation phenotype of lateral line mutants requires access to surface air, we physically blocked access to the surface from 2-6 dpf (Figure 18A). As expected for wild type larvae, blocking access to the surface led to significantly more underinflated larvae (Figure 18B) and a significant decrease in the average swim bladder volume (Blocked: $M = 0.0037$ mm^3, $SD = 0.001$ mm^3, $n = 49$; Open: $M = 0.0066$ mm^3, $SD = 0.0009$ mm^3, $n = 63$) (One-way ANOVA with Tukey post-test, $p = 0.002$) (Figure 18C). For *lhfpl5b* mutant

siblings, blocking access to the surface completely prevented the hyperinflation phenotype, with 0% (*SD* = 0.0%, *n* = 43) of blocked *lhfpl5b* mutants exhibiting positive buoyancy, which is significantly less than mutants with access to the surface (chi-square statistic: 70.45, $p < 0.00001$; Figure 18B). Likewise, the average swim bladder volume of blocked mutants ($M = 0.0033$ mm^3, $SD = 0.0015$ mm^3) was significantly smaller than mutants with open access ($M = 0.014$ mm^3, $SD = 0.0027$ mm^3, $n = 58$) (One-way ANOVA with Tukey post-test, $p < 0.00001$). Blocked mutant and wild type siblings had statistically similar swim bladder volumes (One-way ANOVA with Tukey post-test, $p = 0.972$). From these data, we concluded that the *lhfpl5b* mutant hyperinflation phenotype requires intake of exogenous air and is not caused by abnormal gas exchange from surrounding media nor excess internal gas production.

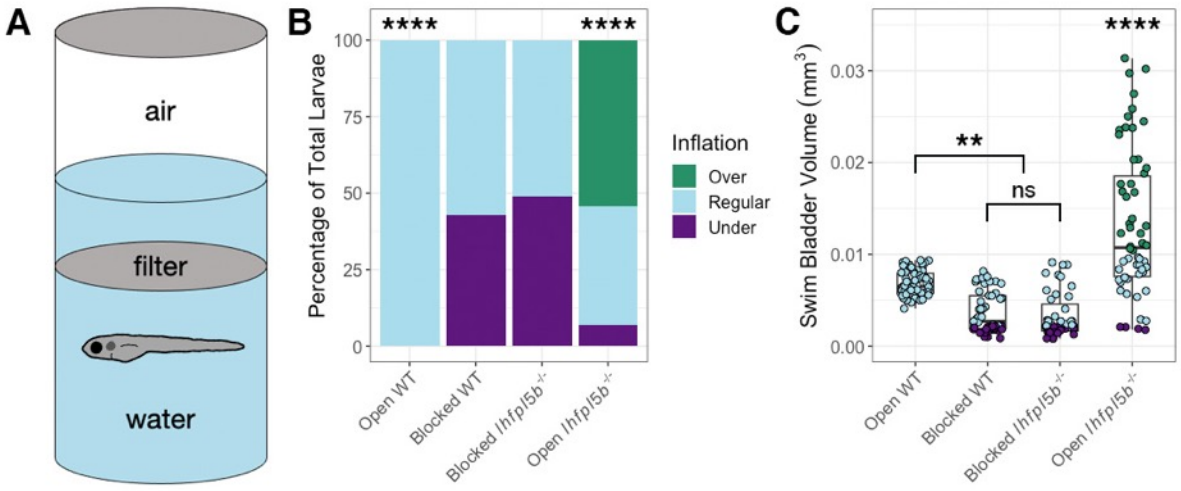


Figure 18: Blocking access to the surface eliminates the hyperinflation phenotype in lateral line mutants (*lhfpl5b*$^{-/-}$). A) Diagram of experimental set up with a filter blocking access to the surface. B) Percentage of larvae that exhibit over-, regular, and under-inflation of the swim bladder at 6 dpf. A Chi-Squared Test was used to determine significance. C) Swim bladder volume (mm^3) of larvae at 6 dpf. A One-Way ANOVA was used to determine significance. **** = $p < 0.0001$, ** = $p < 0.01$, ns = not significant.

Transgenic rescue of lateral line mutants restores normal inflation

We next examined if lateral line defects are responsible for the hyper-inflation phenotype of *lhfpl5b* mutants by restoring lateral line function in mutant larvae. We used the *Tg(myo6b:EGFP-lhfpl5a)vo23* transgene, which uses a hair cell specific promoter (*myo6b*) to drive a GFP-tagged *lhfpl5a* gene (Erickson et al. 2017). It has been shown that this *lhfpl5a* transgene rescues lateral line hair cell function in *lhfpl5b*$^{-/-}$ *(or* lateral line) mutant fish (Erickson et al., 2020). Using this rescue method, we observed no cases of hyperinflation in transgenic mutants (0%, *SD* = 0.0%, *n* = 17), which was significantly different from non-transgenic mutants (chi-square statistic: 82.12, $p < 0.00001$; Figure 19A). The average volume of the transgenic mutant swim bladder ($M = 0.0057$ mm^3, $SD = 0.00001$ mm^3) was significantly smaller than non-transgenic, *lhfpl5b* mutant siblings ($M = 0.0109$ mm^3, $SD = 0.0031$ mm^3, $n = 18$) (One-way ANOVA with Tukey post-test, $p = 0.000003$), but statistically similar to that of wild type transgenics ($M = 0.0057$ mm^3, $SD = 0.0005$ mm^3, $n = 57$) (One-way ANOVA with Tukey post-test, $p = 0.979$, Figure 19B). Because the restoration of *lhfpl5* function specifically in hair cells mitigates hyperinflation in *lhfpl5b* mutants, we concluded that the phenotype is due to defects in sensory hair cell function and not caused by an unrecognized role for *lhfpl5b* in another sensory organ or the swim bladder itself.

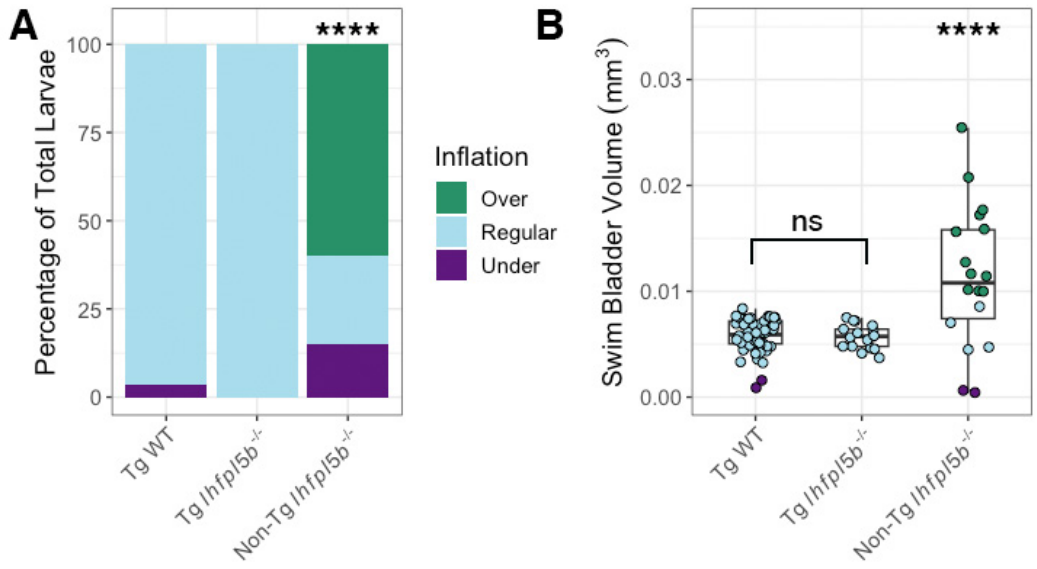


Figure 19: Rescue of lateral line mutants (*lhfpl5b*$^{-/-}$) with the *Tg(myo6b:EGFP-lhfpl5a)vo23* transgene. A) Percentage of larvae that exhibit swim bladder under, regular, and over-inflation at 6 dpf. A Chi-Squared Test was used to determine significance. B) Swim bladder volume (mm^3) of larvae at 6 dpf. A One-Way ANOVA was used to determine significance. **** = $p < 0.0001$, ns = not significant.

Selective ablation of head neuromasts produces a hyperinflation phenotype in wild type larvae

To further test if lateral line defects are responsible for the hyper-inflation phenotype seen in *lhfpl5b* mutants, we ablated lateral line hair cells in wild type larvae using either neomycin sulfate (neo) or copper sulfate (CuSO4) (Supplemental Figure 8 and Figure 20). Administering either single or repeated neomycin treatments during the initial swim bladder inflation period of 3 - 4 dpf resulted in a significant increase in the proportion of positively buoyant larvae (single neo chi-square statistic: 9.95, $p = 0.0016$, $n = 58$; repeated neo chi-square statistic: 34.88, $p < 0.0001$, $n = 47$; Supplemental Figure 8A), but a non-significant increase in the average swim bladder volume (One-way ANOVA with Tukey post-test, single neo $p = 1.0$, repeated neo $p = 0.578$; Supplemental Figure 8B). By comparison, a single CuSO4 treatment resulted in a hyperinflation

phenotype that was statistically indistinguishable from lateral line mutants. An average of 59.6% (*SD* = 7.7%, *n* = 72) of CuSO4-treated wild types over inflate their swim bladders, which was significantly different from wild type controls (chi-square statistic: 39.69, $p < 0.0001$, *n* = 44; Supplemental Figure 8A). The average swim bladder volume of the CuSO4-treated wild type larvae (*M* = 0.0126 mm^3, *SD* = 0.0027 mm^3) was statistically similar to the average swim bladder volume of untreated lateral line mutants (*M* = 0.0139 mm^3, *SD* = 0.0007 mm^3, *n* = 45) (One-way ANOVA with Tukey post-test, *p* = 0.813) (Supplemental Figure 8B).

Since the anterior lateral line is likely to be the only part of the sensory organ to interact with the air-water interface during surfacing, we predicted that disruption of the head neuromasts will result in hyperinflation and that trunk neuromasts will not play a role (Figure 20, Supplemental Figure 9). Selective ablation of the anterior region with CuSO4 resulted in an average of 35.9% (*SD* = 10.1%, *n* = 38) hyperinflated larvae, which was significantly different from unablated controls (chi-square statistic: 39.69, $p < 0.0001$; Figure 20B). Wild type larvae with head-specific ablations exhibited a significant increase in average swim bladder volume (*M* = 0.016 mm^3, *SD* = 0.0013 mm^3) compared to untreated wild type siblings (*M* = 0.0012 mm^3, *SD* = 0.0013 mm^3, *n* = 36) (One-way ANOVA with Tukey post-test, *p* = 0.0365) and statistically comparable swim bladder volumes to that of full CuSO4-treated larvae (*M* = 0.016 mm^3, *SD* = 0.0029 mm^3, *n* = 38) (One-way ANOVA with Tukey post-test, *p* = 0.987) (Figure 20C). Conversely, tail-specific CuSO4 treatments did not cause hyperinflation in wild type fish and resulted in statistically similar swim bladder volumes to untreated siblings (*M* = 0.012 mm^3, *SD* = 0.0031 mm^3, *n* = 34) (One-way ANOVA with Tukey post-test, *p* = 0.835) (Figure 20C). The *lhfpl5b* mutant hyperinflation phenotype was not affected by either neomycin or CuSO4 treatments (Supplemental Figure 8).

Taken together, we concluded that the anterior region of the lateral line system is critical to surface detection during initial swim bladder inflation.

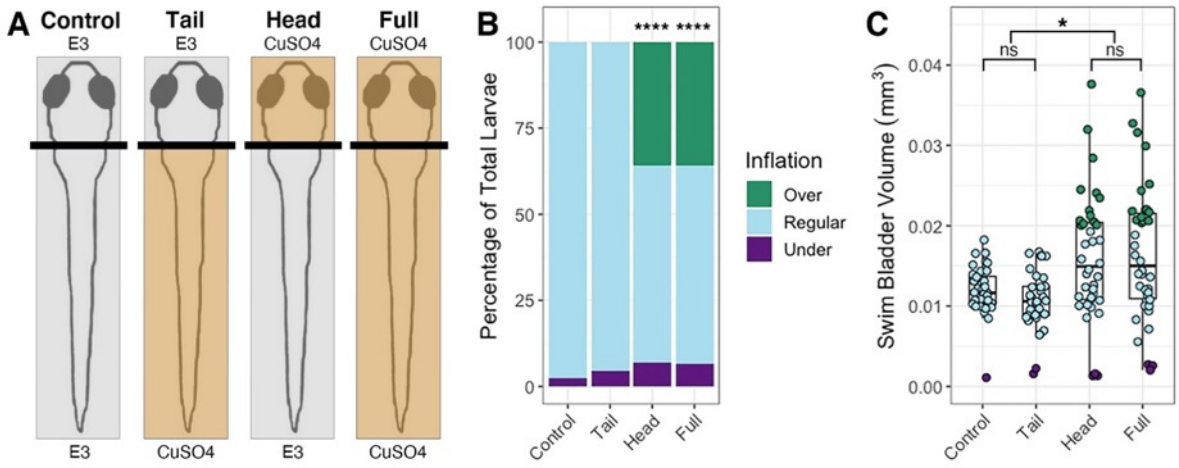


Figure 20: Head-specific ablations of the lateral line recapitulates the *lhfpl5b* mutant hyperinflation phenotype. A) Diagram of experiment. B) Percentage of larvae that exhibit swim bladder under, regular, and over-inflation at 5 dpf. A Chi-Squared Test was used to determine significance. C) Swim bladder volume (mm^3) of larvae at 5 dpf. A One-Way ANOVA was used to determine significance. * = $p < 0.05$, , **** = $p < 0.0001$, ns = not significant. Images of live larvae following treatment in Supplemental Figure 9.

Decreasing surface tension results in over-filling of the swim bladder

Surface tension is a characteristic that makes the air-water interface distinct from the rest of the water column. We predicted that larval fish sense this stimulus with their anterior lateral line neuromasts and that by decreasing the interfacial tension, we would observe abnormal swim bladder inflation in wild type larvae while lateral line mutants would be unaffected. The interfacial tension between oil and water is approximately half that of air and water (Johansen 1924). We layered oil onto the surface of the embryo media and allowed larvae to perform surfacing behaviors between 2-6 dpf (Figure 21). Using this experimental design, we found that the average swim bladder volume of wild types exposed to an oil-water interface (M = 0.016 mm^3, SD = 0.0029

mm^3, n = 32) was significantly greater than the control group with access to an air-water interface (M = 0.007 mm^3, SD = 0.001 mm^3, n = 28) (One-way ANOVA with Tukey post-test, $p < 0.0001$). Furthermore, oil-exposed wild types had statistically similar swim bladder volumes to that of *lhfpl5b* mutants (M = 0.014 mm^3, SD = 0.0038 mm^3, n = 24) (One-way ANOVA with Tukey post-test, p = 0.886) (Figure 21C). Lateral line mutants overfilled their swim bladders to statistically similar proportions when presented with either an air-water or oil-water interface (M = 0.01 mm^3, SD = 0.0026 mm^3, n = 28) (One-way ANOVA with Tukey post-test, p = 0.133). We concluded that surface tension is one of the surface characteristics the lateral line is capable of detecting during initial swim bladder inflation.

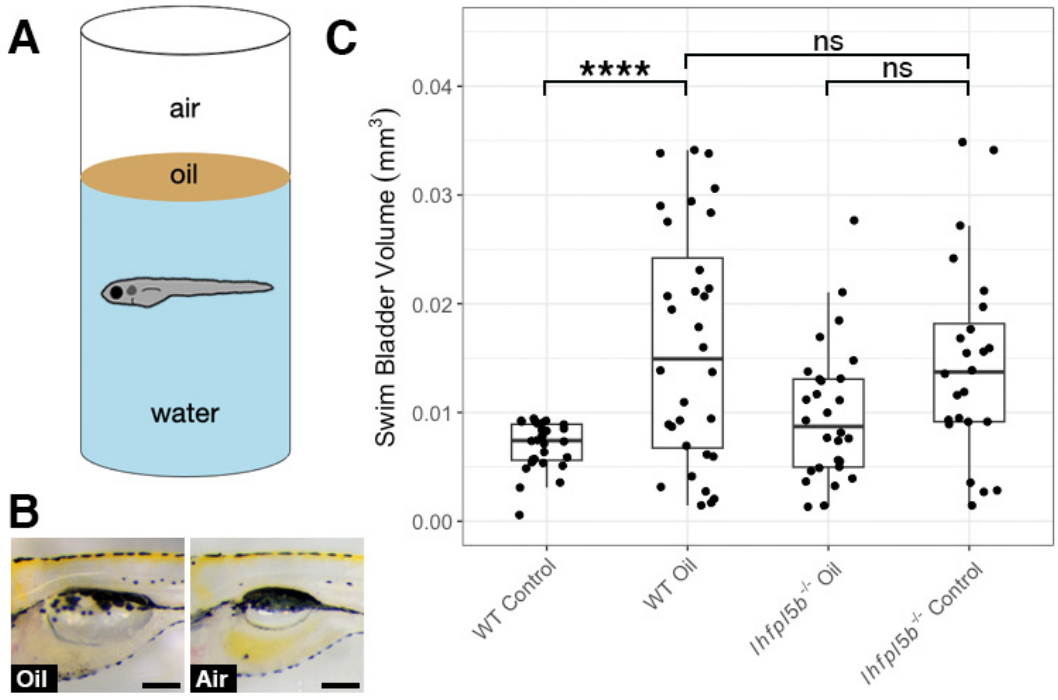


Figure 21: Decreasing interfacial surface tension with mineral oil results in over-filling of the swim bladder. A) Diagram of experiment with 2 mm layer of oil on surface. B) Images of swim bladder phenotypes resulting from surface oil and surface air exposure in wild type larvae at 6 dpf. C) Swim bladder volume (mm^3) at 6 dpf. A One-Way ANOVA was used to determine significance. * = $p < 0.05$, ns = not significant. Scale bars = 0.1 mm.

Optogenetic activation of lateral line hair cells prevents hyperinflation in lateral line mutant larvae

If hydrodynamic information is informing the surfacing behaviors of larval fish, then stimulus-independent manipulations of lateral line activity should alter their ability to correctly inflate their swim bladders. To test this, we created the *Tg(lhfpl5b2028:ChR2-EYFP-PA)* transgenic line that expresses the blue light activated channel, Channelrhodopsin-2, specifically in lateral line hair cells (See Methods, Supplemental Figure 10). We predicted that optogenetic stimulation of *lhfpl5b* mutants, whose lateral lines are insensitive to physical stimuli, would reduce the frequency of the hyperinflation phenotype. Consistent with these predictions, under blue-light stimulation, lateral line mutants with the *lhfpl5b:ChR2* transgene did not exhibit positive buoyancy and had a significant decrease in average swim bladder volume (M = 0.0046 mm^3, SD = 0.0034 mm^3) compared to unstimulated transgenic mutants (M = 0.012 mm^3, SD = 0.0031 mm^3) (One-way ANOVA with Tukey post-test, p = 0.00127) (Figure 22B). Transgenic wild types under blue light exhibited a non-significant decrease in swim bladder volume (M = 0.0052 mm^3, SD = 0.004 mm^3) compared to unstimulated transgenic siblings (M = 0.0079 mm^3, SD = 0.002 mm^3) (One-way ANOVA with Tukey post-test, p = 0.458). To demonstrate that blue light stimulation was reducing swim bladder volumes via activation of lateral line hair cells, we treated wild type *lhfpl5b:ChR2* larvae with CuSO4. As expected, we observed hyperinflation following CuSO4 treatment in stimulated wild type *lhfpl5b:ChR2* larvae, with an average swim bladder volume (WT: M = 0.012 mm^3, SD = 0.0037 mm^3) that was statistically similar to experimental non-transgenic *lhfpl5b* mutants (M = 0.011 mm^3, SD = 0.0024 mm^3) (One-way ANOVA with Tukey post-test, p = 0.998) (Supplemental Figure 10B). CuSO4 treatments in *lhfpl5b* mutants did not kill hair cells

due to the non-functional MET channel, but rather acted as a control for potential behavioral side effects of the CuSO4 treatments. Overall, optogenetic activation of mechanically-insensitive lateral line organs in *lhfpl5b* mutants prevented the hyperinflation phenotype. Together, these data support the hypothesis that the lateral line provides crucial sensory information during initial swim bladder inflation in larval zebrafish.

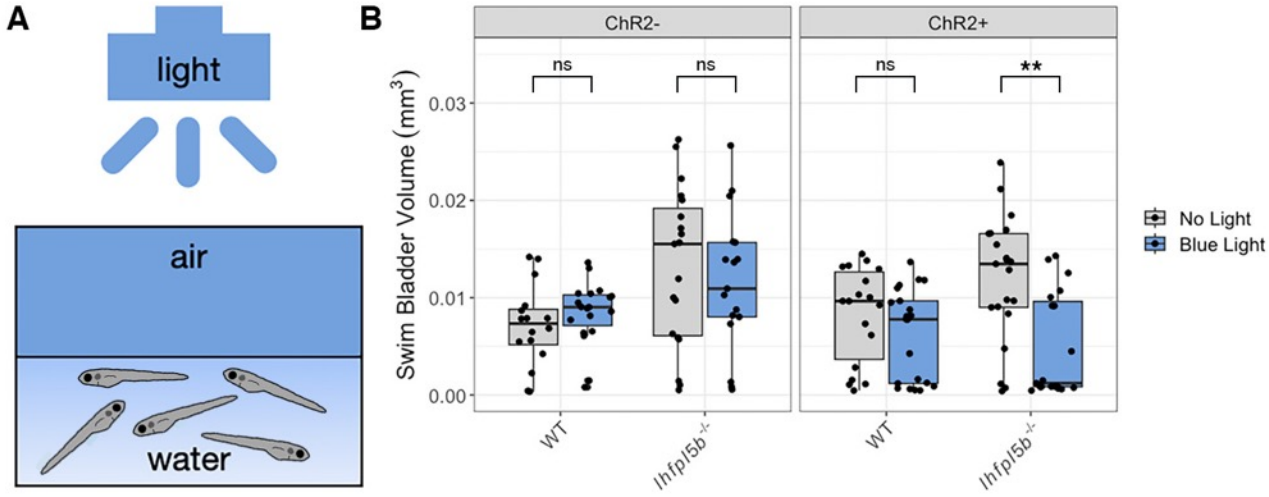


Figure 22: Optogenetic activation of lateral line hair cells with Channelrhodopsin-2 (ChR2) in wild type and *lhfpl5b* mutant larval zebrafish. A) Diagram of experiment with blue light stimuli delivered for 25 milliseconds at 1 second intervals. Treatments were started at 4 dpf and swim bladder volume was assessed at 6 dpf. B) Swim bladder volume (mm^3) of larvae at 6 dpf, n = 16-21 per condition. One-Way ANOVA was used to determine significance. ** = $p < 0.001$, ns = not significant.

Lateral line mutants exhibit abnormal surfacing behaviors

Our results suggested that larval fish are using their head neuromasts to sense the interfacial tension between air and water at the surface. Even in wild type zebrafish, the interaction with the surface during initial swim bladder inflation is not well characterized. To study this behavior in more detail, we quantified the number of unique visits to the surface and the amount of time per

visit spent within the meniscus region from videos of wild type (n = 7) and mutant (n = 14) larvae at 4 dpf. These counts excluded larvae (n = 5 total: 1 wild type and 4 mutant) that under-inflated due to lack of surfacing events. *lhfpl5b* mutants that over-inflate their swim bladders made more visits (M = 87.3, SD = 17.4) to the surface compared to both wild type (M = 42.0, SD = 16.3) and mutant siblings that end up inflating to normal proportions (M = 31.9, SD = 19.2) (One-way ANOVA with Tukey post-test, p = 0.0004) (Figure 23A). Mutants destined for hyperinflation also spent more time (M = 139.0 s, SD = 31.6 s) at the surface than normally-inflated wild type (M = 51.9 s, SD = 24.7 s) and mutant siblings (M = 42.3 s, SD = 29.5 s) (One-way ANOVA with Tukey post-test, p = 0.00006) (Figure 23B). Since excess surfacing could be due to some mutants initiating the behavior earlier, we also documented the time of the first surfacing event and found no significant difference between neutrally buoyant and over-inflated larvae (Figure 23C). We concluded there is a correlation between how larvae interact with the surface during the initial swim bladder inflation period and their resulting swim bladder volume and buoyancy status.

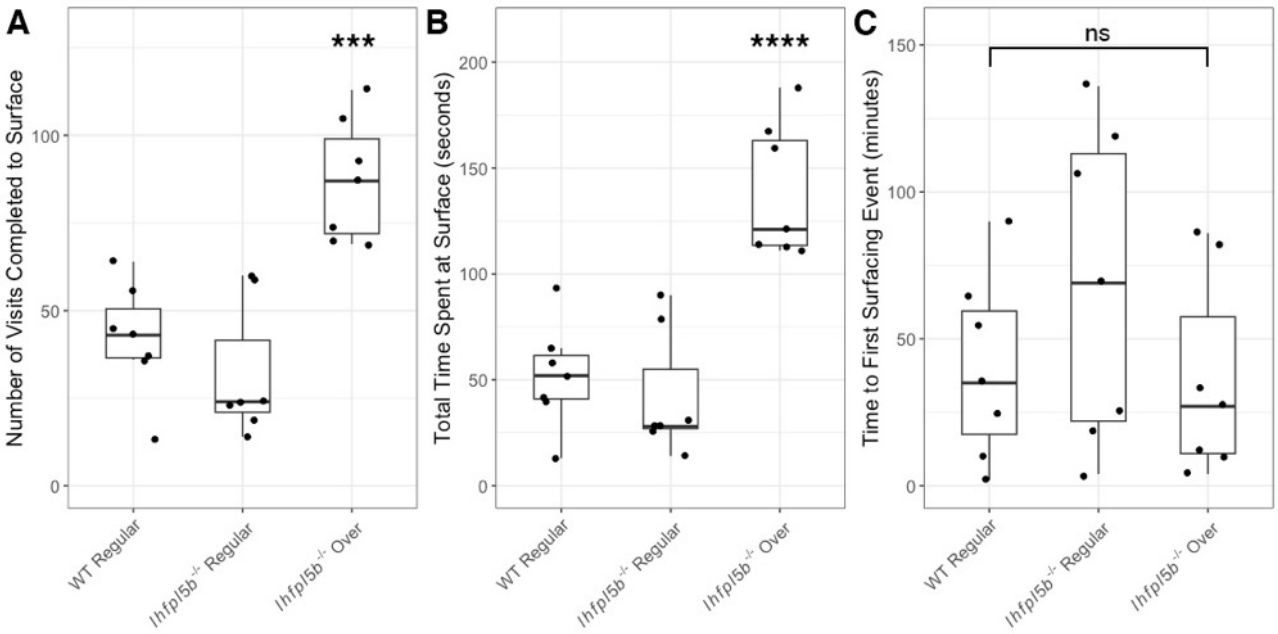


Figure 23: Quantification of the surfacing behaviors of wild type and *lhfpl5b* mutant zebrafish larvae during initial swim bladder inflation. A) Total number of individual visits taken by larval

fish to the air-water interface on 4 dpf for 12 hours (9 AM – 9 PM). B) Total amount of time larval fish spend at the surface for all visits combined on 4 dpf. C) Time to first surface visit after access was allowed. A One-Way ANOVA was used to determine significance. *** = $p < 0.001$, ns = not significant.

Discussion

The intake of exogenous air from the surface is a common feature for initial swim bladder inflation in many species of fish (Goolish and Okutake 1999; Ledebur and Wunder 1937; Jones and Marshall 1953; Furukawa et al. 2022; Korsøen et al. 2012). However, few studies have considered what sensory information is required to achieve neutral buoyancy. In this study, we describe a new role for the mechanosensory lateral line in mediating initial inflation of the swim bladder in zebrafish (Figure 24). Our data support a model in which larval zebrafish use their head neuromasts to detect the air-water interface in support of their transition to neutral buoyancy.

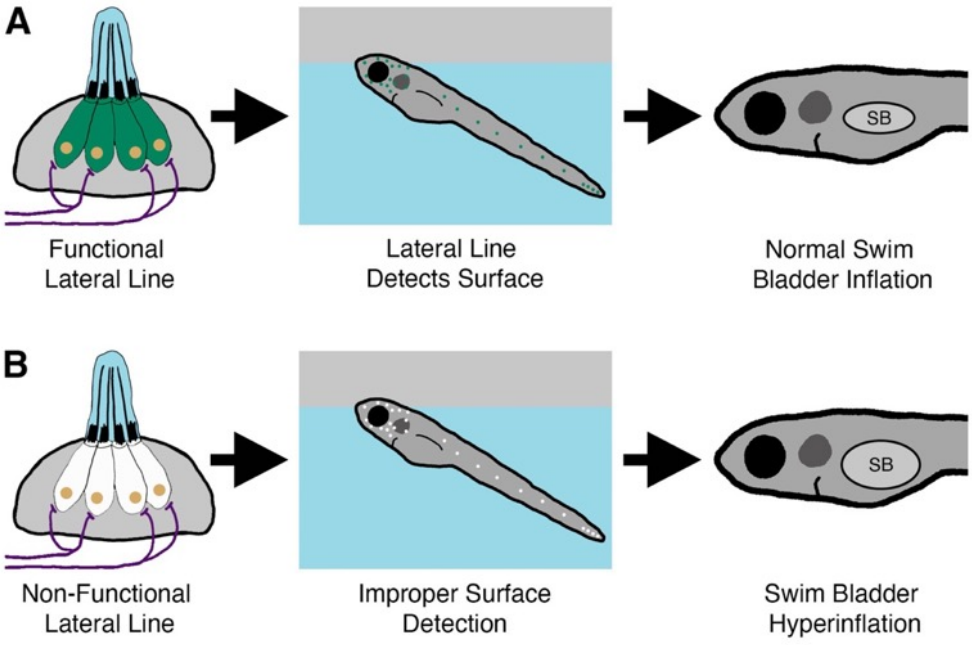


Figure 24. Summary of the surfacing behavior of wild type and lateral line-deficient zebrafish larvae. A) Wild type larvae use their lateral line hair cells (in green) to mediate interactions with

the air-water interface during surfacing. As a result of accurate surface detection, larvae take in an appropriate volume of air for swim bladder (SB) inflation and achieve neutral buoyancy. B) Larvae with a genetic loss of lateral line function (hair cells in white) misinterpret the air-water interface, leading to increased interactions with the surface. Consequently, lateral line mutants *(lhfpl5b*$^{-/-}$*)* take in an excess volume of air, resulting in hyperinflation of the swim bladder for approximately half of mutant larvae. Selective chemical ablations of the head neuromasts produce similar results, implicating the anterior lateral line as the primary sensory organ for interactions with the air-water interface during the surfacing behavior.

Lateral line defects in lhfpl5b mutants are responsible for hyperinflation phenotypes

lhfpl5b mutant zebrafish are the first genetic model for the specific loss of lateral line function in any organism (Erickson et al. 2020). These "lateral line mutants" exhibit an uncommon swim bladder hyperinflation phenotype that prompted us to investigate this novel connection between lateral line sensation and initial inflation of the swim bladder (Figure 17). Previous studies have demonstrated that restricting access to surface air during the initial inflation period prevents swim bladder inflation in larval zebrafish (Goolish and Okutake 1999; Lindsey 2011). In addition, one study showed that some *solute carrier family 12 member 2* (*slc12a2 / nkcc1)* mutant zebrafish over-fill their swim bladders and that blocking access to the surface prevents this phenotype (Abbas and Whitfield 2009). This gene is implicated in endolymph production in the inner ear, but further studies are required to determine the cause of over-inflation in *slc12a2* mutants. Likewise, we find that blocking access to the air-water interface eliminates over-inflation in lateral line mutants and greatly decreases average swim bladder volumes of both mutant and wild type larvae (Figure 18). Our method of blocking access to the air-water interface resulted in a small proportion of larvae (both wild type and mutant) inflating their swim bladder to normal proportions, though hyperinflation was never observed. Inflation was likely achieved by larvae accessing air bubbles

that remain in the water after submerging the filter (Goolish and Okutake 1999). Overall, the hyperinflation phenotype in lateral line mutants requires an intake of exogenous air, pointing to a sensory defect in *lhfpl5b* mutants rather than a physiological one.

In support of this sensory-deficit hypothesis, we show the hyperinflation phenotype is prevented by either transgenic rescue of *lhfpl5* function specifically in hair cells or optogenetic stimulation of mechanically-insensitive lateral line hair cells in *lhfpl5b* mutants (Figures 19 and 22). Furthermore, chemical ablation of lateral line hair cells in wild type larvae prior to initial inflation can phenocopy the *lhfpl5b* mutant. A single treatment with CuSO4 caused similar swim bladder over-inflation proportions to lateral line mutants (Supplemental Figure 8). Repeated neomycin treatments over 3-4 dpf caused the next highest percentage of swim bladder over-inflation and a single neomycin treatment only resulted in a few cases of wild type over-inflation. These results agree with previous work showing rapid hair cell regeneration following neomycin treatment and comparatively slower recovery (approximately 24 hours for full recovery) after CuSO4 exposure (Hernández et al. 2006; Mackenzie and Raible 2012; Murakami et al. 2003; Santos et al. 2006; Venuto and Erickson 2021). Thus, via multiple genetic and chemical approaches, our data show that lateral line hair cell activity can modulate swim bladder inflation in larval zebrafish.

Ototoxins have been extensively used to study lateral line-mediated behaviors in free-swimming larvae and adult fish (Baker and Montgomery 1999; Bleckmann 2006; Coffin and Ramcharitar 2016; Montgomery et al. 1997; Pavlov and Tyuryukov 1993). However, we are not aware of any behavioral studies where the lateral line was disrupted prior to larvae achieving neutral buoyancy, likely explaining why this connection between the lateral line and initial swim bladder inflation has not been noted in current literature. Interestingly, an 1896 study predicted a

relationship between the lateral line and swim bladder inflation, since disruption of the lateral line in adult goldfish resulted in "swelling of the swim bladder" and positive buoyancy (Richard 1896). However, it is not clear if the phenotypic similarities between these adult goldfish and our experimental larval zebrafish share the same underlying causes. Regarding roles for the lateral line in early life behaviors, a close parallel to our findings is the report that unhatched red-eyed treefrogs use their lateral line organ to mediate mechanosensory-cued hatching (Jung, Serrano-Rojas, and Warkentin 2020). In this case, the frog lateral line can sense vibrational stimuli, such as that produced by predators, and promote escape hatching behaviors even before the onset of vestibular function. Similarly, our data suggest that the lateral line is modulating early locomotor activities in zebrafish prior to the development of fully formed vestibular or rheotactic responses (Mo et al. 2010; Olive et al. 2016; Bagnall and Schoppik 2018). As such, the involvement of lateral line in the surfacing behavior may represent the earliest behavioral role for the lateral line yet described.

Head neuromasts are required for normal swim bladder inflation, likely by sensing surface tension

We expanded on the full-body lateral line ablation experiments with specific ablations of either the head or truck neuromasts with CuSO4. Previous studies have shown that head neuromasts are involved with detection of surface features and prey capture (Bleckmann 1988; Carrillo and McHenry 2016; Müller and Schwartz 1982; Schwartz and Hasler 1966). Indeed, ablation of the head neuromasts in wild type larvae yielded similar swim bladder hyperinflation levels to larvae that experienced full body treatments. While this specific experiment did not include *lhfpl5b* mutants, we find that head-treated larvae generally recapitulated the hyperinflation phenotype of *lhfpl5b* mutants. Ablation of the tail neuromasts had no effect on either swim bladder volume or buoyancy (Figure 20). These findings suggest that the head neuromasts interact with

the air-water interface during surfacing and that the posterior lateral line is dispensible for achieving neutral buoyancy. As such, our findings also provide additional support for the anterior lateral line as a central mediator of surface-related behaviors in fish.

The interfacial tension between air and water represents a unique hydrodynamic stimulus that may be detected by the lateral line. The tension between oil and water is half that of air and water (Johansen 1924). Thus, by adding a layer of oil to the surface, we predicted that surface detection would become increasingly difficult and result in over-filling of the swim bladder. We find that, when given access to an oil-water interface, wild type larvae over-fill their swim bladders with oil to a statistically similar volume as lateral line mutants who overinflate with air (Figure 22). We interpret this result as support for the hypothesis that the lateral line senses interfacial tension between air and water as larvae interact with the surface during initial swim bladder inflation. This interpretation is further supported by the observation that *lhfpl5b* mutants (who are already insensitive to interfacial tension) exhibit similar swim bladder volumes when exposed to either oil or air. However, a possible complementary interpretation for these data is that larvae who fill their swim bladders with oil may be deprived of the normal feedback on buoyancy levels as they intake from the surface. Since oil is denser than air, oil-filled larvae are negatively buoyant. In this scenario, larvae may continue to visit the surface for air intake in an attempt to achieve neutral buoyancy. However, future studies are required to examine if such a feedback mechanism exists in larval zebrafish.

Swim bladder over-inflation is correlated with excess time spent at the air-water interface

Since our data suggest that the air-water interface is providing a distinct stimulus to the lateral line, we investigated whether surface interactions differed between hyperinflated *lhfpl5b* mutants and their wild type counterparts. As predicted, over-inflated mutants took more visits *and*

spent more time at the surface than neutrally-buoyant mutant and wild type siblings (Figure 23). Excess breaching of the surface occurred despite over-inflated and neutrally buoyant larvae exhibiting similar timing for their first surfacing attempts. While it is unclear whether larvae ingest air during the entirety of the duration spent above the air-water interface, it is known that surface breaches are observed during initial inflation (Lindsey, Smith, and Croll 2010; Rieger and Summerfelt 1998; Goolish and Okutake 1999). Therefore, the increase in surface visits likely leads to an increase in air deposition to the swim bladder in lateral line mutants.

Multisensory integration during the surfacing behavior

Interestingly, approximately half of all lateral line mutants inflate their swim bladders to normal proportions and continue to develop into viable adults. To account for this fact, we hypothesize that additional somatosensory, photosensory, or mechanosensory systems may compensate for the loss of lateral line sensation and help mutant larvae achieve neutral buoyancy (Suchocki and Sepulveda-Villet 2019; Trotter, Battaglene, and Pankhurst 2003; Riley and Moorman 2000). The lateral line is involved several multi-sensory behaviors, including flow orientation (Liao 2006; Bak-Coleman et al. 2013; Coombs, Bak-Coleman, and Montgomery 2020), prey capture (Nelson, MacIver, and Coombs 2002; Carrillo and McHenry 2016; New 2002), and predator avoidance (Free et al. 2019; Jung et al. 2020; Stewart et al. 2013), and we predict that surfacing can be added to this list.

The photosensory and mechanosensory systems exhibit distinct organizational patterns for primary input to the central nervous system — even the anterior and posterior lateral line ganglia primary projections are somatotopically organized in zebrafish larvae (McCormick 1989; Mirjany and Faber 2011; Hale et al. 2016; Alexandre, Ghysen, and Ghysen 1999; Fame, Brajon, and Ghysen 2006; Liao and Haehnel 2012). The Mauthner neurons integrate multisensory input at the

level of the hindbrain, but the surfacing behavior is unlikely to use this circuit. For higher order processing however, information from the lateral line, vestibular, and photosensory systems are integrated in various brain regions. The thalamus is one such sensory integration center in zebrafish (Mueller 2012). Socially-relevant mechanosensory and photosensory information is represented in a thalamic region marked by the expression of *parathyroid hormone 2* (*pth2*) and *somatostatin 7* (*sst7*) (Kappel et al. 2022; Venuto et al. 2022; Anneser et al. 2020). Vestibular information is also represented in the thalamus (Migault et al. 2018; Favre-Bulle et al. 2018), though it is not clear if the exact same region is involved. Photosensory, lateral line, and vestibular/auditory information is also integrated in the tectum (Favre-Bulle et al. 2018; Migault et al. 2018; Isa et al. 2021; Filosa et al. 2016). In fact, neurons in the periventricular layer (PVL) of the tectum, which is similar to the superior colliculus in mammals, respond to visual, auditory, and lateral line inputs (Perrault, Stein, and Rowland 2011; King et al. 1996; Ingle 1973; Thompson et al. 2016). However, it is hypothesized that most of these modalities are processed in parallel at younger stages of development (Thompson et al. 2016). It is worth noting that these sensory systems are in developing stages during surfacing behaviors.

Photosensory cues did not alter the buoyancy status of either wild type or *lhfpl5b* mutant larvae (Supplemental Figure 6), suggesting that, at most, photosensory systems play a subtle modulatory role in the surfacing behavior of larval zebrafish. The vestibular system is likely required to coordinate the vertical orientation and climbing portions of surfacing (Ehrlich and Schoppik 2019; 2017; Bagnall and Schoppik 2018). Meanwhile, our data support the idea that the lateral line is responsible for the surface detection and breaching aspects of surfacing. However, given that both the vestibular and lateral line systems are providing spatial information in during the surfacing behavior, there may be crosstalk between them. For example, the vestibular system

must quickly adapt to a dynamic increase in buoyancy over the course of multiple breaching events, so feedback from the lateral line during surface interactions may help fine tune the vestibular sense. However, the link between these systems have not been well-investigated during deliberate behaviors like surfacing (Liu et al. 2021; Chagnaud et al. 2017). So, while sensory integration exists in zebrafish and seems necessary for larvae to achieve neutral buoyancy, the integration dynamics are unclear during the initial swim bladder inflation timeframe and require future investigation.

CHAPTER 5: SURFACING IN LARVAL ZEBRAFISH IS A MULTISENSORY BEHAVIOR INVOLVING PHOTOSENSORY AND CHEMOSENSORY CUES IN ADDITION TO MECHANOSENSATION

Abstract

Initial swim bladder inflation is a critical achievement for teleost fish, and it requires a complex set of behavioral tasks to reach neutral buoyancy. Larval fish must detect both directionality and the air-water interface at only 3-4 days post fertilization. Despite this challenging feat being necessary for fish survival, the sensory cues informing the surfacing behavior remain understudied. Past studies have determined the vestibular system to aid larvae in determining direction and vertical swimming. Additionally, the lateral line was recently implicated in surface detection for filling of the swim bladder. However, photosensory roles in surfacing remain a mystery in many fish species, including larval zebrafish. Finally, tactile and chemosensory cues require investigation for their potential involvement in initial swim bladder inflation.

In this study, we investigate the role of various photosensory systems in initial swim bladder inflation using larval zebrafish lacking visual and pineal photosensory input. We find no evidence for visual photosensation in surfacing behaviors, and instead find evidence for the influence of non-ocular photosensation in surfacing. We also find that, in specific sociogenetic contexts, chemosensory cues from conspecifics may play a role in initial swim bladder inflation. Overall, our data highlight novel sensory influences on the multisensory behavior of surfacing in larval zebrafish.

Introduction

Initial swim bladder inflation is a critical process that larval fish undergo to achieve neutral buoyancy (Alexander 1966; Evans and Damant 1928; Lindsey, Smith, and Croll 2010; Ledebur and Wunder 1937). This process involves a behavior called "surfacing" wherein larval fish must determine which way is up and swim upwards, similar to the behavior required for larval feeding (Doroshev, Cornacchia, and Hogan 1981; Kitajima et al. 1994; Friedmann and Shutty 1999). Then, larvae must detect the surface and intake exogenous air from the surface to inflate their swim bladder (Venuto et al. 2023; Lindsey, Smith, and Croll 2010; Rieger and Summerfelt 1998). In the wild, larvae are required to perform this complex behavior in dynamic environmental conditions, including changes in lighting, water turbidity and flow, and other animals in their surroundings. Despite the number of obstacles that these young larvae encounter during surfacing, they are highly motivated to perform the surfacing behavior and inflate their swim bladder (Tait 1960). If initial swim bladder inflation is unsuccessful, the result is disease, deformities, and ultimately death (Doroshev, Cornacchia, and Hogan 1981; Czesny, Graeb, and Dettmers 2005; Kitajima et al. 1994; Prestinicola, Boglione, and Cataudella 2014).

The surfacing behavior is an extremely complex behavior for which larvae must utilize a combination of sensory cues. For example, vestibular information provided by the inner ear is hypothesized to aid in directionality and vertical swimming (Riley and Moorman 2000; Schoppik et al. 2017; Nicolson et al. 1998). Additionally, we recently uncovered that the lateral line is utilized during the surface detection aspect of surfacing (Venuto et al. 2023). Approximately half of larvae with a non-functional lateral line exhibit swim bladder over-inflation, which is a rare phenomenon (Abbas and Whitfield, 2009; McCune and Carlson, 2004; Venuto et al. 2023).Given that this phenotype only affects a portion of the lateral line-deficient population, it is reasonable to assume

that other sensory systems are contributing to the surfacing behavior for successful swim bladder inflation.

Photosensory systems are leading candidates for aiding in surfacing. The effect of photoperiod on swim bladder inflation has been studied in many species to assist in fish farming efforts. Many studies in fish species such as Australian Bass and Pacific Bluefin Tuna have shown that environments with constant light are associated with a decrease in swim bladder inflation rates and constant darkness with an increase in swim bladder inflation proportions (Battaglene and Talbot, 1990; Kurata et al. 2017). This is due to larvae primarily inflating during dark hours (Trotter et al. 2003), which can be helpful for energy conservation and predator avoidance (Battaglene et al. 1994). However, some species like California Yellowtail are unaffected by photoperiod (Stuart and Drawbridge 2012), and only the common chub has exhibited an increase swim bladder inflation in full light conditions (Brüning et al. 2011). These studies have not been conducted in larval zebrafish, which offer useful genetic tools to understand the role of photosensation on initial swim bladder inflation.

Fish possess several populations of photoreceptive cells: ocular, pineal, deep brain, and spinal. Ocular photoreceptors in teleost fish include rod photoreceptors and four types of cone photoreceptors (Gestri, Link, and Neuhauss 2012). Retinal ganglion cells connect signals from the retina to the brain (Kay et al. 2001; Burrill and Easter 1994). Zebrafish develop visual acuity by 3-4 dpf, though visual acuity at this stage is unlikely to be matured enough to influence behavior (Easter and Nicola 1996; Haug et al. 2010). Non-ocular photoreceptors are potentially functional in zebrafish by 1 dpf and can inform light-driven behaviors (Meissl and Yanez 1994; Fernandes et al. 2012; Friedmann et al. 2015; Kokel et al. 2010; Vuilleumier et al. 2006). Pineal photoreceptors are similar to cone photoreceptors in the retina, and pineal photoreception is generally responsible

for locomotive behaviors based on circadian rhythm in teleost fish (Ben-Moshe Livne et al. 2016; Dekens et al. 2022; Mueller and Neuhauss 2012; Ekström and Meissl 1997). Additionally, deep brain photoreception can drive light-activated behaviors through various opsins and photosensitive cells (Fernandes et al., 2012, 2013). For example, larval zebrafish perform a dive response and brief period of hyperactivity upon loss of illumination that is dependent on deep brain photoreception (Fernandes et al. 2012). However, the various forms of photoreception and their potential influences on the surfacing behavior of larval zebrafish have not been investigated and may have an enhanced role in the absence of lateral line cues.

In addition to photosensory cues, it is likely that somatosensory cues contribute to the surfacing behavior. These cues may include environmental stimuli from substrates or the air-water interface. However, tactile cues are difficult to examine in larval zebrafish due to lack of molecular tools that solely affect somatosensation and not locomotion (Ribera and Nüsslein-Volhard 1998). A seemingly unlikely candidate to aid in surfacing is chemosensation. However, fish have been shown to rely on chemosensory cues to detect their social environment (Santacà, Dadda, and Bisazza 2021; Gerlach and Wullimann 2021). Additionally, some larval fish species use olfactory cues for migration and various chemostimulants have been shown to affect behavior in larval zebrafish (Zielinski et al. 2005; Johnson et al. 2019; Lindsay and Vogt 2004). Overall, the sensory cues necessary for initial swim bladder inflation require further investigation to better understand the critical surfacing behavior.

Given the partial hyperinflation phenotype exhibited by lateral line mutants, we hypothesize that other sensory systems mediate surfacing behaviors. In this study, we investigate surfacing as a multisensory behavior by using buoyancy measurements and swim bladder volume as experimental endpoints. We test how photosensory cues may influence initial swim bladder

inflation using zebrafish lacking ocular and/or pineal-based photoreception as well as environmental photoperiod manipulations. We also evaluate how social isolation affects initial swim bladder inflation. Finally, we investigate role of conspecific-derived chemosensory cues during surfacing in the absence of visual and lateral line sensation. Overall, we add to the current understanding of sensory cues involved in initial swim bladder inflation in larval zebrafish.

Materials and Methods

Animal husbandry and ethics statement

Adult zebrafish (*Danio rerio*) were maintained and bred using standard procedures (Westerfield 2000). All experiments used larvae at 2-6 days post-fertilization (dpf), which are of indeterminate sex at this stage. Except where otherwise stated, animals were placed in clear, plastic tubs (dimensions: 15 cm x 10 cm x 4 cm, E3 volume: 460 mL) at 2 dpf and maintained at 28.5°C on a 14:10 light/dark cycle at 500-700 lux in E3 embryo media (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgCl2, buffered with NaHCO3). Animal research complied with guidelines stipulated by the Institutional Animal Care and Use Committee at East Carolina University (protocol D372).

Mutant and transgenic fish lines

The zebrafish mutant alleles *lhfpl5b*^{vo35} (causes non-functional lateral line) and *atoh7*^{th241} (causes blindness) as well as transgenic lines *Tg(atoh7:gap43-RFP)*^{cu2} and *Tg(aanat2:CFP-NTR)*^{ct822} were used in this study (Kay et al. 2001; Erickson et al., 2020; Gandhi et al., 2015). For all experiments, *lhfpl5b*$^{vo35/vo35}$ homozygotes were identified from their hetero- and homozygous wild type siblings by lack of FM 1-43 (Thermo Fisher) labeling of the lateral line hair cells. *Tg(atoh7:gap43-RFP)*;*atoh7*$^{th241/th241}$ larvae were identified by a lack of RFP-positive retinal ganglion cell axons in the tectum. *Tg(aanat2:CFP-NTR)*^{ct822} were identified by CFP-positive cells

in the pineal. Unless otherwise stated, the wild type condition in all experiments is a mix of homozygous and heterozygous siblings.

Swim bladder inflation measurements

At the end of each experiment on 6 dpf, we ascertained the buoyancy status and swim bladder volume of all larvae in the experiment. First, larvae were anesthetized with MS-222 (Western Chemical Inc., Ferndale, WA) and placed at the vertical midway point of a 50 mL conical tube containing E3 media. At the expiration of a 30-second timer, the end position of the fish was recorded as previously described (Venuto et al. 2023). Over-inflated fish were categorized as larvae floating to the top of the tube in 30 seconds, while under-inflated larvae sunk to the bottom. Neutrally buoyant (regular-inflated) larvae remained near the middle of the water column. After buoyancy measurements, the fish was removed from the tube for imaging under the microscope.

Swim bladders were imaged using a SteREO Discovery.V8 microscope (Zeiss) equipped with a Gryphax Arktur camera (Jenoptik). Lateral and dorsal images of the swim bladder were taken for each fish. Swim bladder measurements were conducted using the measurement tool in Adobe Photoshop where measurements were calibrated according to the image of the stage micrometer taken when imaging experiments. The major axis and minor axis of the lateral view of the swim bladder was measured as well as the minor axis of the dorsal view. All measurements were taken from numbered images so that the measurer was blinded to the genotype/condition. These measurements were halved and used in the equation for the volume of an ellipsoid: $V = 4/3\pi abc$ to find the volume of the swim bladder (Lindsey, Smith, and Croll 2010).

Manipulation of photoperiod

Since lighting conditions have been shown to impact initial swim bladder inflation in some fish species, we investigated how full light versus full dark conditions affected swim bladder

inflation in wild type, blind, and lateral line-less zebrafish larvae. At 0 dpf, wild type, *atoh7* mutant, *lhfpl5b* mutant, and *atoh7; lhfpl5b* double mutant zebrafish embryos were collected and immediately placed in plastic tubs filled with E3 (detailed above) in an unlit incubator (28.5°C) for full darkness, an incubator with overhead light without a timer for full light, and an incubator with overhead light connected to a timer that switched the light off and on every 12 hours. For both full light and full dark conditions, the experiment was run simultaneously with the control 12:12 L/D experiment in a separate incubator. Incubators were of similar light intensity and dimensions. Larvae were not brought out of their respective incubators until 6 dpf, at which point lateral line mutants and wild type siblings were identified as detailed above. After sorting, larvae were analyzed for buoyancy and swim bladder volume. The experiment was repeated three times using 15-30 larvae (combined from all genotypes) per trial per treatment condition.

Pineal ablations

Pineal photoreceptors offer one form of non-ocular light detection, and we wanted to test the effect of loss of pineal photoreception on initial swim bladder inflation. For pineal ablations, wild type and *lhfpl5b* mutant larvae from the *Tg(aanat2:CFP-NTR)*; *lhfpl5b*^{vo35} line were split into two groups after sorting for transgene presence but remained in a heterogenic mix of wild type and *lhfpl5b* mutant larvae for both the control and experimental group. The experimental groups were treated with 15 mM metronidazole (Sigma-Aldrich, St. Louis, MO) at 60 hours post-fertilization (hpf) for 20 hours (until 80 hpf), then rinsed and placed in fresh embryo media for 24 hours (until 104 hpf) (Gandhi et al. 2015). A second round of treatment was done for another 20 hours (104 hpf-124 hpf) and then larvae were rinsed and placed in fresh embryo media until the end of the experiment. On 6 dpf, lateral line mutants and wild type siblings were identified as detailed above. After sorting, larvae were analyzed for buoyancy and swim bladder volume. Untreated controls

were placed in fresh embryo media with every media change that occurred in treated larvae. The experiment was repeated three times using 17-20 larvae (combined mutant and wild type) per trial per treatment condition. For pineal images, live zebrafish larvae were anesthetized with 0.016% MS-222 in E3 embryo media and mounted dorsally on a depression slide in 1.2% low melting point agarose in E3. CFP was imaged using a Zeiss LSM800 laser-scanning confocal with a 40x water objective and Zeiss Zen software.

Social isolation

Social isolation can affect larval behavior, so we tested the effect of social versus isolated conditions on initial swim bladder inflation in wild type, blind, and lateral line-less zebrafish larvae. At 2 dpf, presorted groups of wild type, *atoh7* mutant, *lhfpl5b* mutant, and *atoh7;lhfpl5b* double mutant larvae were each divided into two groups and placed in either isolated or social conditions. Both social and isolated larvae were housed in six-well tissue culture plates containing 5 ml of E3 embryo media with paper strips placed between the wells (Venuto et al. 2022). For the social condition, all larvae were placed in one well with 5 ml of embryo media. For the isolated condition, one larva was placed per well with 5 ml of embryo media. The experiment lasted until 6 dpf when larvae were imaged for swim bladder measurements The experiment was repeated three times using 15-30 larvae (combined from all genotypes) per trial per treatment condition. For social isolation in darkness to environmentally replicate the double mutant, conditions were similar except only wild type and *lhfpl5b* mutants were used and they were divided into social or isolated conditions at 24 hpf. Both social and isolated groups were placed in an unlit incubator until 6 dpf when larvae were imaged for swim bladder measurements. The experiment was repeated three times using 16-26 larvae (combined mutant and wild type) per trial per treatment condition.

Lateral line hair cell ablations

To environmentally replicate social isolation of the double mutant, wild type and *atoh7* mutant larvae were separated into isolated and social groups wherein half of the larvae in each group underwent treatment for lateral line ablations. Isolated and social larvae were placed in conditions as described above for the social isolation experiment. For lateral line ablations, larvae were treated with 10 μM copper sulfate (CuSO4; Ward's Science, Rochester, NY) for 30 minutes at 80 hpf. After 30 minutes, larvae were rinsed and placed in fresh embryo media for 24 hours until 104 hpf when another 30-minute treatment with 10 μM CuSO4 occurred. Larvae were rinsed and placed in fresh embryo media until the end of the experiment when imaging took place on 6 dpf. Untreated controls were placed in fresh embryo media at each treatment timepoint. The experiment was repeated three times using 6-10 larvae (combined mutant and wild type) per trial per condition.

Water motion experiments

We hypothesized that water flow may influence initial swim bladder inflation, so we subjected larvae to two different types of water motion (overhead fan and shaker environments). For both the fan and shaker experiments, wild type and *lhfpl5b* mutant larvae were randomly split into two groups at 2 dpf and placed into tubs containing E3 medium (described above). For the shaker experiment, the experimental group was placed on an orbital shaker set to constant rotation at 40 rpm while the control group was placed next to the shaker in the same incubator. For the fan experiment, the experimental group and was placed on a benchtop 4.5 feet below a Honeywell TurboForce Air Circulator Fan that was rotated 90° (facing parallel to the benchtop) to have the fan blow down onto tub surface. The fan was placed on the high-speed setting and was attached to a shelf above the benchtop. The second group of larvae were placed in a second tub, which was the control, and the control tub was placed on a second benchtop away from the fan. The experiment was conducted in a temperature controlled room with a 14:10 L/D cycle. For both the

shaker and fan experiments, larvae were sorted for the *lhfpl5b* mutation and assessed for buoyancy and swim bladder volume at 6 dpf. Both experiments were repeated three times using 14-30 larvae (combined mutant and wild type) per trial per condition.

Segregating by genotype

Other social conditions included a mix of mutant and wild type fish, but in this experiment we tested whether segregating by genotype had any effect on initial swim bladder inflation. At 2 dpf, wild type, *atoh7* mutant, *lhfpl5b* mutant, and *atoh7; lhfpl5b* double mutant larvae were sorted based on lateral line and visual function as detailed above. Half of the larvae in each genotype were raised socially but separated from other genotypes in six-well tissue culture plates containing 5 ml of E3 embryo media with paper strips placed between the wells (n = 3-8 per 5 mL well per trial). The other half were mixed with siblings from other genotypes and re-screened at 6 dpf when larvae were imaged for swim bladder measurements (n = 20-35 combined from all genotypes per 5 mL well per trial). The experiment was repeated three times.

Water exchanges (chemosensory cues experiment)

To evaluate if chemosensory cues influenced initial swim bladder inflation, we experimented with water conditioning. At 2 dpf, wild type, *atoh7* mutant, *lhfpl5b* mutant, and *atoh7; lhfpl5b* double mutant larvae were sorted based on lateral line and visual phenotypes as described above. Larvae from each phenotype were split into experimental and control groups, but all larvae remained socially raised and segregated by genotype. Larvae in the experimental condition were subjected to water exchanges where embryo media from homozygous wild type larvae (30 larvae in 5 mL of E3 in one six-well plate) that were 24 hours older was removed from the wild type larvae and put into the experimental segregated larval groups. When larvae were 3 dpf, water exchanges began in the morning and happened every 24 hours until 6 dpf when larvae

were imaged for swim bladder measurements. Control segregated larvae did not have water exchanges. This experiment was repeated four times with 3-7 larvae per genotype, condition, and trial. Water from experimental and control groups were measured for pH, ammonia, nitrate, and nitrite levels. pH ranged between 7.2 and 7.4, ammonia levels were at 1 ppm, and nitrite and nitrate levels were at 0 ppm for all conditions, indicating comparable water quality parameters between experimental and control groups.

Graphs and statistical tests

All graphs and statistical tests were done using R (R Core Team 2021). Two-way ANOVAs were used with Tukey post-hoc test, with the exception of the first two experiments (Figures 25 and 26) where only genotypes were compared so a one-way ANOVA was used with Tukey post-hoc test. Chi-squared tests were used to analyze proportion data for over and under-inflation. For the over-inflation comparisons, the two categories used in the comparison between conditions/genotypes were (i) over-inflation and (ii) all other inflation (regular and under), meaning that totals per category/genotype accounted for 100% of the population. Similarly, for the under-inflation comparisons, the two categories used in the comparison between conditions/genotypes were (i) under-inflation and (ii) all other inflation (regular and over). P-values less than 0.05 were considered significant.

Results

Ocular blindness does not affect initial swim bladder inflation in larval zebrafish

Several studies have investigated the role of photoperiod on initial swim bladder inflation in various species of fish (Stuart and Drawbridge 2012; Trotter et al. 2003; Villamizar et al. 2009). However, there have been no studies in larval zebrafish exploring the various photosensory cues that may mediate surfacing. In our first experiment, we investigated swim bladder inflation in a

vision-specific mutant (*atoh7*^{th241}) with either intact or genetically-disabled lateral line sensory function (lateral line mutant *lhfpl5b*^{vo35}) (Figure 25). *atoh7*$^{-/-}$ zebrafish larvae are blind due to the absence of retinal ganglion cells, though cone photoreceptors remain (Kay et al. 2001; Neuhauss et al. 1999). Like wild type larvae, blind (*atoh7*$^{-/-}$) larvae did not exhibit a hyperinflation phenotype (positive buoyancy - blind mutants: 0.0%, *SD* = 0.0%, *n* = 26; wild type: 0.0%, *SD* = 0.0%, *n* = 33; chi-square statistic: 0.0, *p* = 1.0; swim bladder volume - blind: *M* = 0.0059 mm^3, *SD* = 0.0007 mm^3; wild type: *M* = 0.0073 mm^3, *SD* = 0.0013 mm^3; One-way ANOVA with Tukey post-test, *p* = 0.735). When raised together in a 12:12 L/D photoperiod, *atoh7*$^{-/-}$ / *lhfpl5b*$^{-/-}$ double mutants exhibited statistically similar proportions of positive buoyancy to *lhfpl5b*$^{-/-}$-only larvae (double mutants: 58.3%, *SD* = 17.6%, *n* = 14; lateral line mutants: 68.6%, *SD* = 3.5%, *n* = 30; chi-square statistic: 2.61, *p* = 0.11), as well as similarly enlarged swim bladder volumes (double mutants: *M* = 0.012 mm^3, *SD* = 0.0036 mm^3; lateral line mutants: *M* = 0.013 mm^3, *SD* = 0.0035 mm^3; One-way ANOVA with Tukey post-test, *p* = 0.727). From this experiment, we conclude that, with or without lateral line function, visual photosensation does not play a significant role in zebrafish initial swim bladder inflation when larvae of mixed genotypes are reared socially under a 12:12 L/D photoperiod.

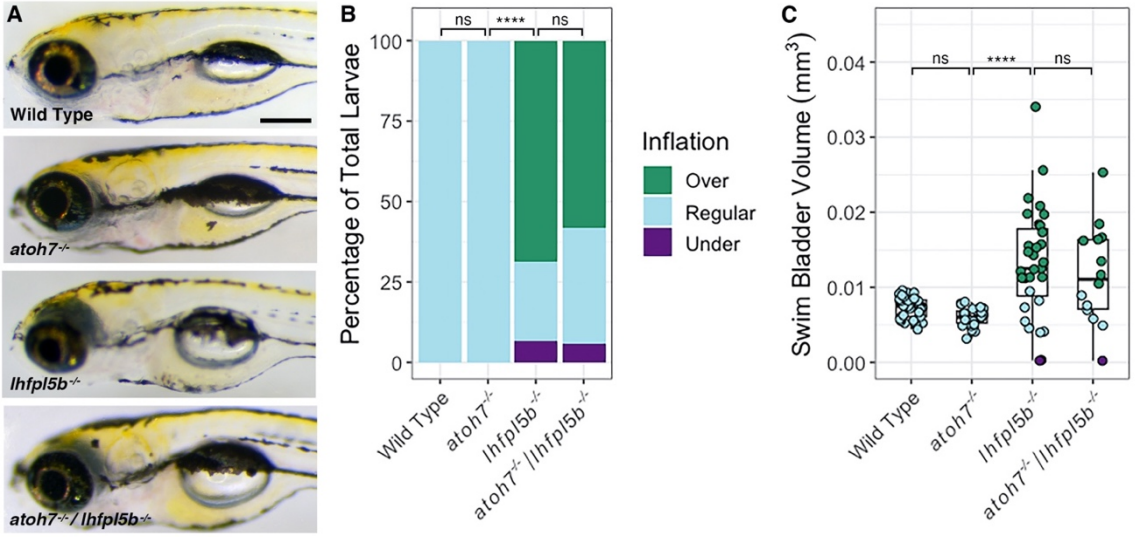


Figure 25. Blindness has no effect on initial swim bladder inflation in larval zebrafish. A) Representative examples swim bladder inflation in wild type, blind (*atoh7*$^{-/-}$), lateral line mutants (*lhfpl5b*$^{-/-}$), and double mutant (*atoh7*$^{-/-}$; *lhfpl5b*$^{-/-}$) larvae at 6 dpf. B) Percentage of larvae that exhibit over-, regular, and under-inflation of the swim bladder, as defined by positive, neutral, and negative buoyancy. A Chi-Squared Test was used to determine significance. C) Swim bladder volume (mm^3) in wild type and mutant larvae at 6 dpf. A one-way ANOVA was used to determine significance. ns = not significant, **** = $p < 0.0001$. Scale bar in top panel of A = 0.2 mm and is applicable to all images.

Darkness increases hyperinflation in blind and lateral line-less double mutants

Since some fish species exhibit swim bladder inflation changes with different photoperiods, we also wanted to test if swim bladder inflation in larval zebrafish was susceptible to lighting changes. Since our previous study showed that raising lateral line mutants in total darkness did not affect swim bladder inflation (Venuto et al. 2023), we predicted that lighting conditions would similarly have no effect on blind and lateral line-deficient double mutants. To test this, we measured swim bladder inflation in blind and lateral line-deficient double mutants raised socially with their siblings in full light (24:0L/D) and full dark (0:24 L/D) conditions (Figure 26). Eliminating photoperiod did not affect swim bladder inflation in wild type, lateral line mutants, or blind mutants individually. However, when raised in total darkness (0:24L/D), blind-lateral line double mutants exhibited a significant increase in the proportions of overinflated larvae compared to lateral line-only mutants (double mutants: 69.4%, SD = 4.8%, n = 10; lateral line-only mutants: 53.2%, SD = 12.2%, n = 14; chi-square statistic: 5.38, p = 0.02). There was a non-significant increase in average swim bladder volume of double mutants compared to lateral line mutants in total darkness (0:24 L/D) (double mutants: M = 0.015 mm^3, SD = 0.0018 mm^3; lateral line mutants: M = 0.011 mm^3, SD = 0.0027 mm^3; One-way ANOVA with Tukey post-test, p =

0.098). Conversely, under full light conditions (24:0 L/D), double mutants exhibited a significant *decrease* in proportions of swim bladder hyperinflation compared to lateral line-only mutants (double mutants: 36.1%, *SD* = 12.7%, *n* = 12; lateral line mutants: 56.7%, *SD* = 5.8%, *n* = 17; chi-square statistic: 8.86, *p* = 0.0029). Additionally, double mutants raised in full light (24:0 L/D) exhibited significantly smaller swim bladder volumes than lateral line mutants (double mutants: *M* = 0.0098 mm^3, *SD* = 0.0021 mm^3; lateral line mutants: *M* = 0.015 mm^3, *SD* = 0.0052 mm^3; One-way ANOVA with Tukey post-test, *p* = 0.0394). These data indicate a novel role for non-ocular photosensation wherein light dissuades initial swim bladder inflation when both lateral line and visual sensory information are unavailable to larval zebrafish.

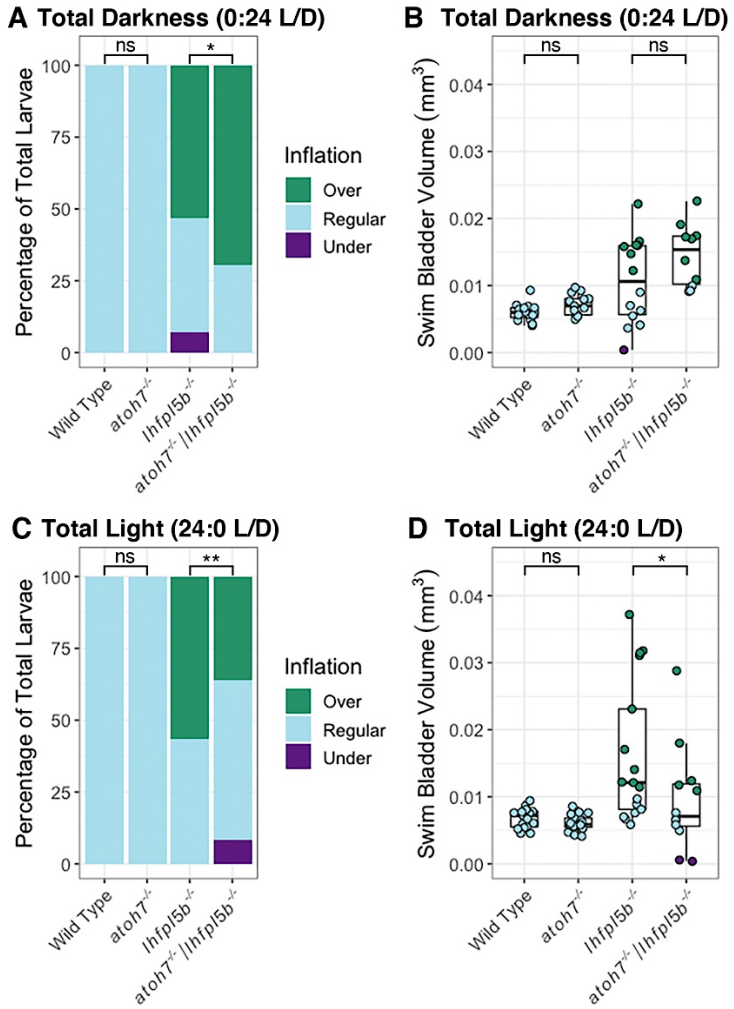


Figure 26: Alterations to the photoperiod affect swim bladder inflation in *atoh7*$^{-/-}$; *lhfpl5b*$^{-/-}$ double mutants. A) Percentage of wild type, blind (*atoh7*$^{-/-}$), lateral line mutants (*lhfpl5b*$^{-/-}$), and double mutant (*atoh7*$^{-/-}$; *lhfpl5b*$^{-/-}$) larvae that exhibit swim bladder under, regular, and over-inflation at 6 dpf in full darkness (0:24 L/D). A Chi-Squared Test was used to determine significance. B) Swim bladder volume (mm^3) of larvae at 6 dpf in full darkness (0:24 L/D). A One-Way ANOVA was used to determine significance. C) Percentage of larvae that exhibit swim bladder under, regular, and over-inflation at 6 dpf in full light (24:0 L/D). A Chi-Squared Test was used to determine significance. D) Swim bladder volume (mm^3) of larvae at 6 dpf in full light (24:0 L/D). A One-Way ANOVA was used to determine significance. ** = $p < 0.01$, * = $p < 0.05$, ns = not significant.

Ablation of the pineal gland has no effect on swim bladder inflation

Pineal, deep brain, and spinal photoreception have all been shown to modulate the behavior of larval zebrafish (Friedmann et al. 2015; Fernandes et al. 2012; Ben-Moshe Livne et al. 2016). To determine if photosensors in the pineal gland were responsible for the shifts in swim bladder hyperinflation exhibited by double mutants when photoperiod was eliminated (Figure 26), we used the *Tg(aanat2:CFP-NTR)* zebrafish line (Gandhi et al. 2015) to ablate pineal photoreceptors with metronidazole in *lhfpl5b*^{vo35} zebrafish (Figure 27). Metronidazole treatment of *Tg(aanat2:CFP-NTR); lhfpl5b*$^{-/-}$ zebrafish at 2-4 dpf produced no significant change in swim bladder over-inflation proportions compared to untreated siblings (untreated: 54.2%, SD = 9.1%, n = 26; treated: 61.5%, SD = 7.9%, n = 31; chi-square statistic: 1.003, p = 0.317). Similarly, there was no significant change in average swim bladder volume between untreated and treated mutant populations (untreated: M = 0.012 mm^3, SD = 0.0011 mm^3; treated: M = 0.013 mm^3, SD = 0.0007 mm^3; Two-way ANOVA with Tukey post-test, p = 0.998). These findings indicate that pineal photoreception is not used in surfacing behaviors when larvae are capable of visual sensation.

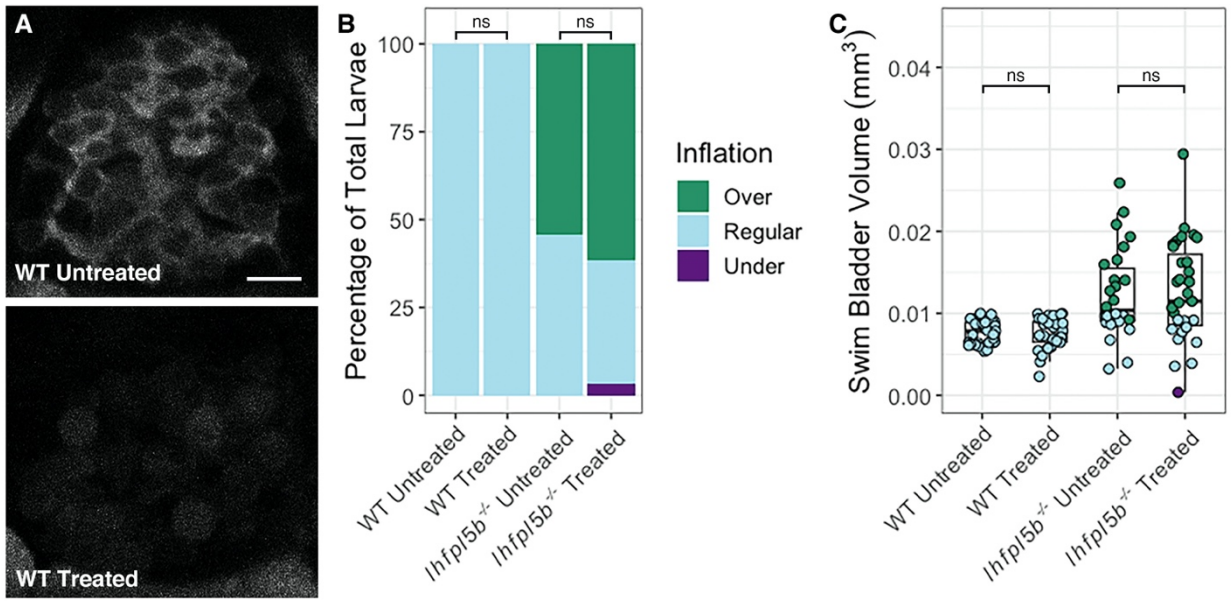


Figure 27: Ablation of pineal photoreceptors does not affect initial swim bladder inflation in lateral line (*lhfpl5b*$^{-/-}$) mutant zebrafish larvae. A) Representative images of CFP-positive pineal cells in untreated (top) and metronidazole-treated (bottom) *Tg(aanat2:CFP-NTR)* larvae at 4 dpf. Scale bar = 10 μM and is applicable to both images. B) Percentage of wild type (*lhfpl5b*$^{+/?}$) and lateral line mutant (*lhfpl5b*$^{-/-}$) larvae that exhibit swim bladder under, regular, and over-inflation at 6 dpf in treated and untreated conditions. A Chi-Squared Test was used to determine significance. C) Swim bladder volume (mm^3) of larvae at 6 dpf. A Two-Way ANOVA was used to determine significance. ns = not significant.

Social isolation decreases hyperinflation proportions in atoh7$^{-/-}$*; lhfpl5b*$^{-/-}$ *double mutants*

In addition to photosensation, tactile and chemosensory cues have not been investigated for their involvement in initial swim bladder inflation in larval zebrafish. In our laboratory setting, tactile cues involve stimuli from touching the sides and bottom of the arena as well as contact with conspecifics. Chemosensory cues involve chemicals in the water that may affect behavior via chemoreceptors like taste and olfactory receptors. We eliminated tactile and chemosensory cues from conspecifics by raising wild type, *atoh7*$^{-/-}$, *lhfpl5b*$^{-/-}$, and *atoh7*$^{-/-}$*; lhfpl5b*$^{-/-}$ zebrafish in isolation from 2-6 dpf (Figure 28). Since video experiments from our previous study involved

physical isolation, we did not anticipate for social isolation to have a significant effect on swim bladder inflation (Venuto et al. 2023). Indeed, swim bladder volumes and buoyancy proportions among wild type and single mutant *atoh7*$^{-/-}$ and *lhfpl5b*$^{-/-}$ larvae were unaffected by social isolation. However, we found a significant decrease in the proportion of hyperinflated double mutants (*atoh7*$^{-/-}$; *lhfpl5b*$^{-/-}$) when raised in isolation compared to double mutants raised socially under heterogenic conditions (Figure 28B; social: 44.4%, $SD = 9.6\%$, $n = 10$; isolated: 6.7%, $SD = 6.7\%$, $n = 12$; chi-square statistic: 36.03, $p < 0.00001$). There was a nonsignificant decrease in swim bladder volume (Figure 28C; social: $M = 0.009$ mm^3, $SD = 0.0047$ mm^3; isolated: $M = 0.0067$ mm^3, $SD = 0.0022$ mm^3; Two-way ANOVA with Tukey post-test, $p = 0.721$). These data suggest that social cues encourage surfacing behaviors when larvae are deprived of both ocular visual and lateral line input, warranting further investigation.

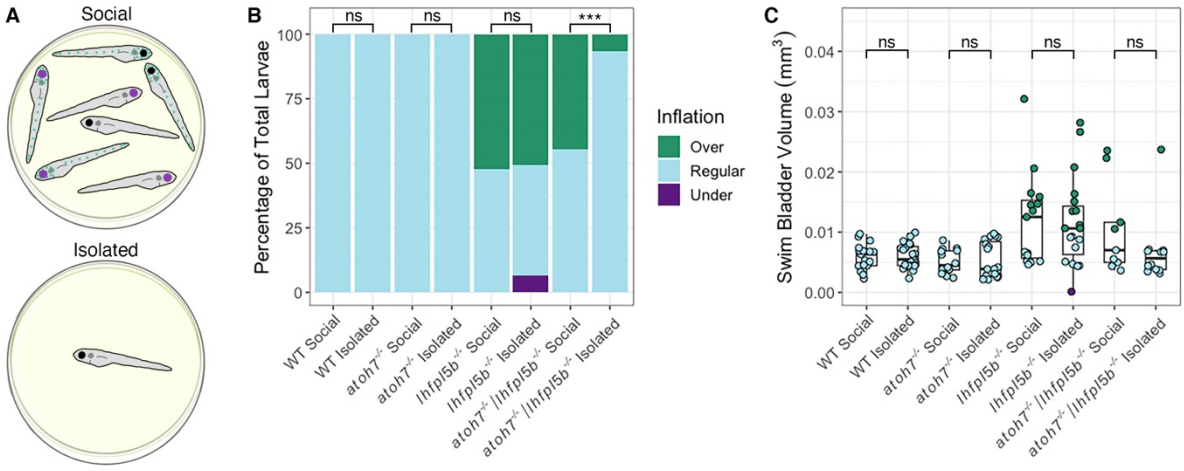


Figure 28: Social isolation decreases hyperinflation proportions in *atoh7*$^{-/-}$; *lhfpl5b*$^{-/-}$ double mutants. A) Diagram of experiment including a social, heterogenic condition and an isolated condition. Green neuromasts along the body of the larval fish indicate a functional lateral line and purple eyes on the larval fish indicate functional visual photoreception. B) Percentage of larvae that exhibit swim bladder under, regular, and over-inflation at 6 dpf. A Chi-Squared Test was used to determine significance. C) Swim bladder volume (mm^3) of larvae at 6 dpf. A Two-Way ANOVA was used to determine significance. *** = $p < 0.001$, ns = not significant.

Hyperinflation is reduced in blind, isolated zebrafish with chemically ablated lateral lines

We attempted to recreate the isolated double mutant phenotype by chemically ablating the lateral line in blind larvae with copper sulfate (Figure 29). We predict that chemical ablations of the lateral line in blind and isolated larvae will produce a decrease in hyperinflation, similar to the proportions exhibited by double mutants raised in isolation. Indeed, we find a decrease in the proportion of over-inflated blind larvae with chemically ablated lateral lines when raised in isolation compared to those raised socially (social: 47.8%, SD = 13.5%, n = 12; isolated: 30.0%, SD = 8.7%, n = 13; chi-square statistic: 6.81, p = 0.0091). However, the differences in average swim bladder volumes are insignificant (social: M = 0.012 mm^3, SD = 0.0028 mm^3; isolated: M = 0.010 mm^3, SD = 0.0039 mm^3; Two-way ANOVA with Tukey post-test, p = 0.743). This reduction in hyperinflation among isolated blind larvae with chemically ablated lateral line hair cells is similar to what is exhibited by double mutants raised in isolation.

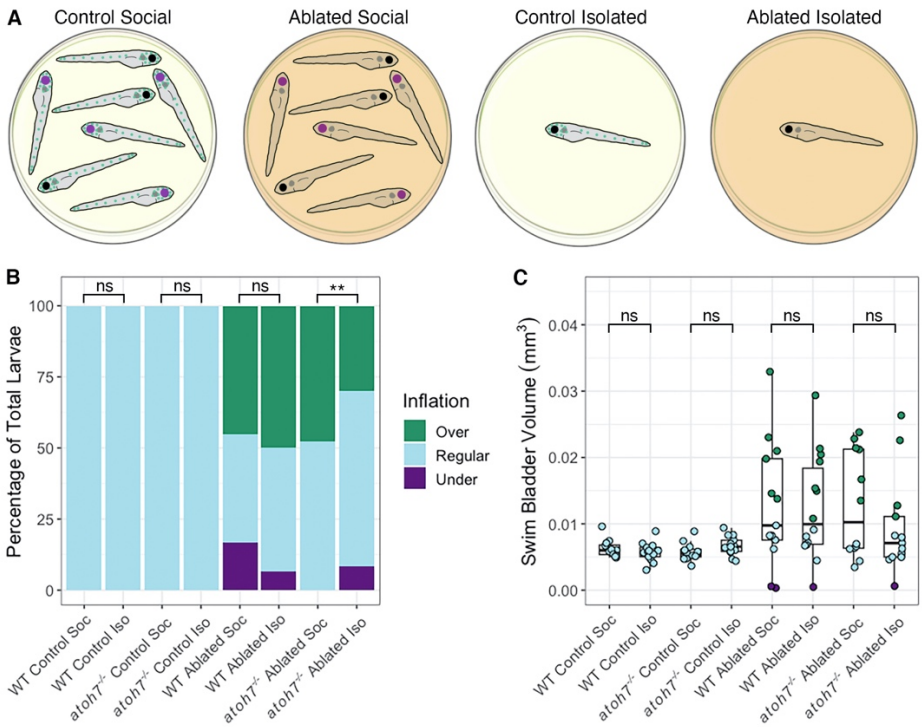


Figure 29: Ablation of the lateral line in blind (*atoh7*-/-) mutants raised in isolation decreases proportions of hyperinflation. A) Diagram of experiment. Green neuromasts along the body of the larval fish indicate a functional lateral line and purple eyes on the larval fish indicate functional visual photoreception. Orange media color represents copper sulfate treatments. B) Percentage of larvae that exhibit swim bladder under, regular, and over-inflation at 6 dpf. A Chi-Squared Test was used to determine significance. C) Swim bladder volume (mm^3) of larvae at 6 dpf. A Two-Way ANOVA was used to determine significance. ** = $p < 0.01$, ns = not significant.

Dark and isolated conditions cause hyperinflation in wild type larvae, but do not affect lhfpl5b mutants

We also attempted to recreate the decreased hyperinflation phenotype in isolated *atoh7*; *lhfpl5b* double mutants by raising isolated lateral line mutants in full darkness to eliminate visual cues independently of the *atoh7* mutation (Figure 30). We expected isolated lateral line mutants raised in darkness to also exhibit the decrease in proportions of swim bladder over-inflation. From this experiment, we found no significant difference in the proportions of hyperinflation between lateral line mutants raised socially or in isolation in total darkness (social: 66.1%, $SD = 4.3\%$, $n = 32$; isolated: 65.7%, $SD = 4.6\%$, $n = 33$; chi-square statistic: 0.0221, $p = 0.88$) or in swim bladder volumes (social: $M = 0.016$ mm^3, $SD = 0.0021$ mm^3; isolated: $M = 0.018$ mm^3, $SD = 0.0008$ mm^3; Two-way ANOVA with Tukey post-test, $p = 0.767$). It is worth noting that we observed cases of swim bladder over-inflation in isolated wild type fish raised in darkness in the 5 mL wells, and the proportions of hyperinflation in isolated wild type larvae were significantly greater than social wild type larvae (social: 0.0%, $SD = 0.0\%$, $n = 31$; isolated: 12%, $SD = 12\%$, $n = 30$; chi-square statistic: 9.95, $p = 0.0016$). However, there was no significant difference in swim bladder volume

(social: $M = 0.0071$ mm^3, $SD = 0.0008$ mm^3; isolated: $M = 0.0072$ mm^3, $SD = 0.0018$ mm^3; Two-way ANOVA with Tukey post-test, $p = 1.0$).

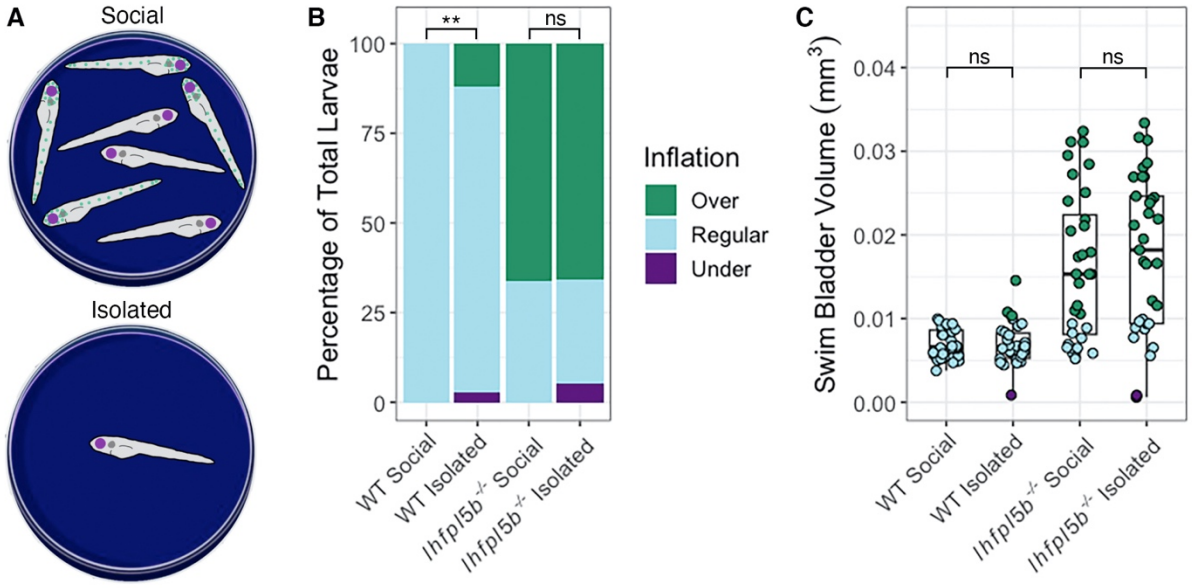


Figure 30: Raising lateral line (*lhfpl5b*$^{-/-}$) mutants in darkness in isolation does not affect initial swim bladder inflation. A) Diagram of experiment. Green neuromasts along the body of the larval fish indicate a functional lateral line and purple eyes on the larval fish indicate functional visual photoreception. Dark blue color represents being raised in total darkness. B) Percentage of larvae that exhibit swim bladder under, regular, and over-inflation at 6 dpf. A Chi-Squared Test was used to determine significance. C) Swim bladder volume (mm^3) of larvae at 6 dpf. A Two-Way ANOVA was used to determine significance. ** = $p < 0.01$, ns = not significant.

A recent preprint found similar results in wild type larvae raised in darkness and isolation and hypothesized that water motion (even slight perturbations from conspecifics) can affect initial swim bladder inflation in larval zebrafish (Lin et al. 2023). We further tested this theory by subjecting larvae to a shaker condition (rotates tub for continuous movement throughout the water column) and overhead fan (effect similar to surface waves) to create different forms of water motion (Supplemental Figures 11 and 12). Based on the study investigating the effect of water

flow on swim bladder inflation, we predicted that both types of water motion would decrease swim bladder over-inflation in wild type and lateral line mutant zebrafish. Overall, proportions of hyperinflation and swim bladder volume in lateral line mutants were significantly decreased in both the shaker and fan conditions compared to stagnant water conditions (Supplemental Figure 12). Wild type larvae were not significantly affected by the shaker or fan, leaving the effect of water motion on wild type swim bladder inflation unclear from our experiments.

Segregation by genotype yields similar swim bladder proportions to isolated conditions

The first set of experiments in Figure 25 involved double mutant larvae raised socially with the larvae of other genotypes (wild type, lateral line-less, and blind). The heterogenic social conditions resulted in double mutants exhibiting similar hyperinflation proportions to lateral line mutants (~50%). In this experiment, we investigated if segregating larvae by genotype at 2 dpf (before initial swim bladder inflation) would yield similar hyperinflation results as isolated double mutants (~7%) (Figure 31). If social isolation decreased hyperinflation rates due to cues from wild type larvae, then isogenic sorting of double mutants should yield similar hyperinflation rates to double mutants raised in isolation. Indeed, double mutants raised socially as an isogenic group exhibited a significant decrease in the proportion exhibiting the over-inflation phenotype (isogenic: 6.7%, SD = 6.7%, n = 16; heterogenic: 57.9%, SD = 8.3%, n = 12; chi-square statistic: 59.28, $p <$ 0.00001) and swim bladder volume (isogenic: M = 0.0068 mm^3, SD = 0.0045 mm^3; heterogenic: M = 0.016 mm^3, SD = 0.0031 mm^3; Two-way ANOVA with Tukey post-test, p = 0.000103). Wild type, lateral line-less, and blind larvae were unaffected by the segregation. These results indicate that isolation itself is not responsible for changes to initial swim bladder inflation, and instead potentially implicate chemosensory cues from wild type conspecifics.

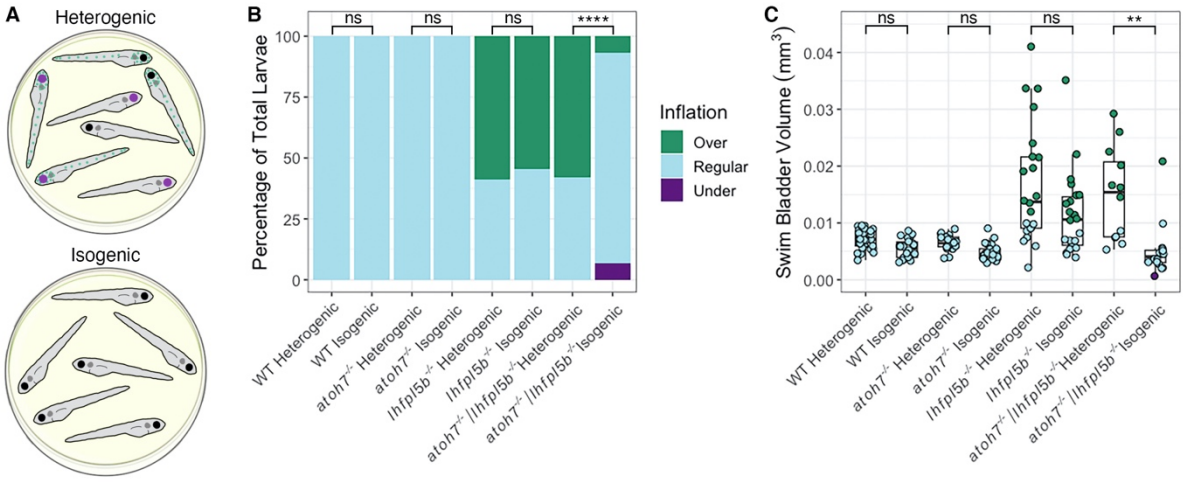


Figure 31: Isogenic rearing of *atoh7*$^{-/-}$; *lhfpl5b*$^{-/-}$ double mutants decreases the hyperinflation phenotype. A) Diagram of experiment. Green neuromasts along the body of the larval fish indicate a functional lateral line and purple eyes on the larval fish indicate functional visual photoreception. B) Percentage of larvae that exhibit swim bladder under, regular, and over-inflation at 6 dpf. A Chi-Squared Test was used to determine significance. C) Swim bladder volume (mm^3) of larvae at 6 dpf. A Two-Way ANOVA was used to determine significance. ** = $p < 0.01$, **** = $p < 0.0001$, ns = not significant.

Chemosensory cues from wild type larvae encourage swim bladder inflation in atoh7$^{-/-}$*; lhfpl5b*$^{-/-}$ *double mutants*

To assess the role of chemosensory cues on initial swim bladder inflation more directly, we devised an experiment where we exchanged water from wild type larvae into the group of segregated double mutants (Figure 32). This experimental design exposes segregated double mutants to water-borne chemicals produced by wild type larvae without the wild type larvae being physically present. We hypothesized that segregated double mutants exposed to the wild type water-borne chemicals would exhibit greater cases of hyperinflation (similar to double mutants raised in heterogenic social conditions) than double mutants left unexposed. From this experiment, we found that segregated double mutants that experienced the water change (experimental

condition) exhibited a significant increase in the proportion of over-inflation (54.2%, *SD* = 16%, *n* = 16) compared to segregated double mutants that were not exposed to water from wild type larvae (control condition) (10.4%, *SD* = 10.4%, *n* = 16; chi-square statistic: 44.49, $p < 0.00001$). Additionally, the experimental double mutants exhibited a significant increase in swim bladder volume (*M* = 0.0145 mm^3, *SD* = 0.0021 mm^3) compared to control double mutants (*M* = 0.0087 mm^3, *SD* = 0.00098 mm^3; Two-way ANOVA with Tukey post-test, $p = 0.0245$). No other genotypes were affected by water exchange, or lack thereof. This indicates a role for chemosensory cues derived from wild type conspecifics in initial swim bladder inflation when lateral line and visual sensory cues are unavailable in larval zebrafish.

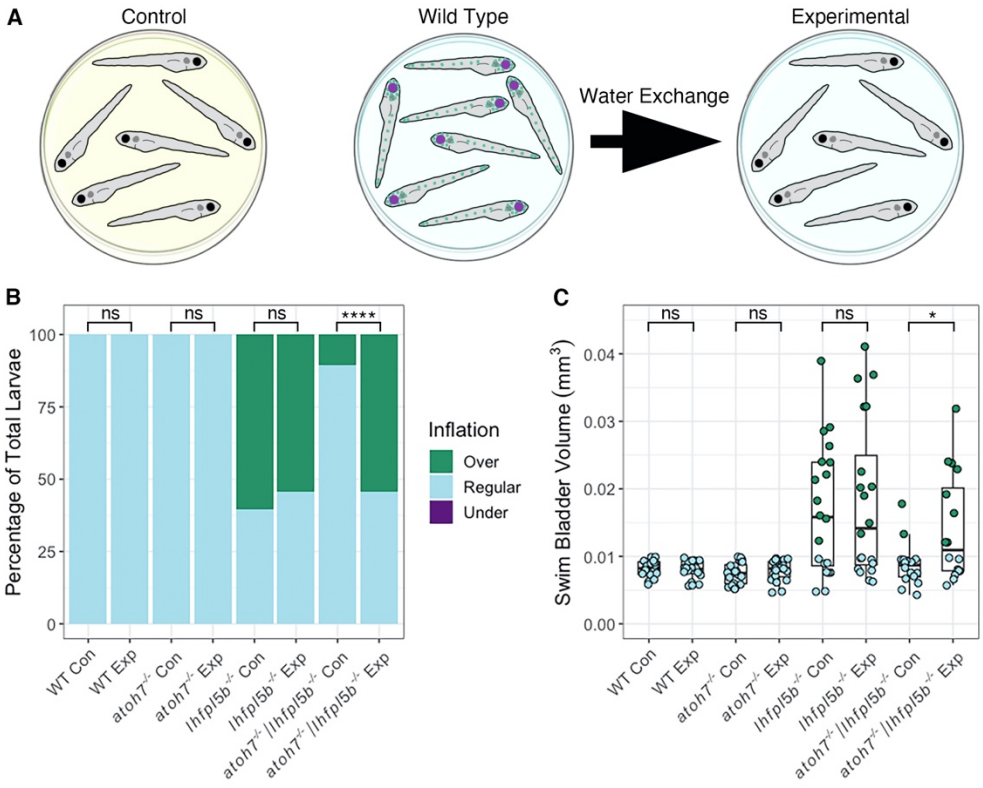


Figure 32: Chemosensory cues from wild type larvae restores the hyperinflation phenotype to isogenically-reared *atoh7*$^{-/-}$; *lhfpl5b*$^{-/-}$ double mutants. A) Diagram of experiment. Green neuromasts along the body of the larval fish indicate a functional lateral line and purple eyes on the larval fish indicate functional visual photoreception. Light blue media indicates water

exchange. B) Percentage of larvae that exhibit swim bladder under, regular, and over-inflation at 6 dpf. Experimental groups ("Exp") experienced water exchanges from wild type larvae, while control groups ("Con") did not. A Chi-Squared Test was used to determine significance. C) Swim bladder volume (mm^3) of larvae at 6 dpf. A Two-Way ANOVA was used to determine significance. * = $p < 0.05$, **** = $p < 0.0001$, ns = not significant.

Discussion

In this study, we hypothesized that sensory systems like ocular photosensation could help lateral line mutants recognize the surface and help modulate initial swim bladder inflation since approximately only half of lateral line mutants display the hyperinflation phenotype. From our experiments to test this hypothesis, we find that loss of vision does not affect initial swim bladder inflation in larval zebrafish (Figure 25). However, non-ocular photoreception subtly affects initial swim bladder inflation when lateral line and visual systems are non-functional (Figure 26). We see that full darkness increases proportions of swim bladder hyperinflation in double mutants, whereas full light decreases proportions of hyperinflation.

Additionally, we tested if sensory cues from conspecifics affected initial swim bladder inflation by subjecting larvae to various social conditions. We find evidence to suggest that chemosensation is used during surfacing behaviors when larvae are deprived of visual and lateral line sensation (*atoh7*$^{-/-}$; *lhfpl5b*$^{-/-}$ double mutants; Figures 28-32). When double mutants are raised separately from wild type larvae, hyperinflation is decreased, but when double mutants are exposed to the water that wild type larvae were raised in, hyperinflation levels are similar to intermixed larvae. Overall, we offer novel findings on photosensory and chemosensory cues utilized during surfacing in larval zebrafish for initial swim bladder inflation (Table 4).

Manipulation	Level of hyperinflation	Figure	Notes
Genetically blind	None	25	N/A
Genetic lateral line mutant	+ +	25	~50% Hyperinflation
Genetic blind and lateral line double mutant	+ +	25	~50% Hyperinflation, similar to lateral line mutant
0:24 L/D photoperiod in double mutants	+ + +	26	Increases hyperinflation rates in double mutants
24:0 L/D photoperiod in double mutants	+	26	Decreases hyperinflation rates in double mutants
Pineal photoreceptor ablation in lateral line mutant	+ +	27	~50% Hyperinflation, similar to untreated lateral line mutant
Social isolation of double mutants	+	28	Decreases hyperinflation rates in double mutants
Lateral line ablation in genetically blind and isolated	+	29	Slight decrease in hyperinflation rates
Genetic lateral line mutant in 0:24 L/D photoperiod and isolated	+ +	30	Wild type larvae exhibit mild cases of hyperinflation
Shaker-generated waves in lateral line mutant	+	S1	Decreases hyperinflation rates in lateral line mutants
Fan-generated waves in lateral line mutant	+	S2	Decreases hyperinflation rates in lateral line mutants
Isogenic raising of double mutants	+	31	Similar effect to isolation, decreases hyperinflation
Water exchange from wild type to double mutants	+ +	32	Restored ~50% hyperinflation in double mutants

Table 4. Summary table of experimental effects on swim bladder inflation. The number of plus signs (+) indicates the level of swim bladder hyperinflation relative to an ideal 100% neutrally buoyant wild type population. Each "+" indicates about a quarter of the population exhibiting hyperinflated swim bladders.

Non-ocular photosensation is responsible for light detection and modulation of surfacing

When larval zebrafish are without a functional lateral line, approximately half of the population routinely exhibits swim bladder hyperinflation due to improper surface detection during the surfacing behavior (Venuto et al. 2023). It is reasonable to assume that visual cues (or other sensory cues) may help larvae identify the surface and compensate for loss of lateral line sensation,

especially since visual and lateral line systems exhibit overlap in other multisensory behaviors, like rheotaxis (Suli et al. 2012; Montgomery et al. 1997). They also share downstream sensory integration regions in the thalamus and tectum (Mueller 2012; Thompson et al. 2016; Vanwalleghem et al. 2020).

If visual cues were utilized during the surfacing behavior, we may expect to see cases of swim bladder hyperinflation in blind larvae, and increased proportions of hyperinflation in double mutants deprived of lateral line and visual sensation. However, we instead saw blind (*atoh7*$^{-/-}$) larvae inflate to similar proportions as wild type siblings, and *atoh7*$^{-/-}$; *lhfpl5b*$^{-/-}$ double mutants inflate to similar proportions of lateral line (*lhfpl5b*$^{-/-}$) mutants (Figure 25). This indicates that ocular photosensation is not required for normal initial swim bladder inflation. However, we observe increases in hyperinflation when we remove additional photosensory cues (pineal and deep brain) by raising *atoh7*$^{-/-}$; *lhfpl5b*$^{-/-}$ double mutants in darkness (Figure 26). The opposite phenotype is exhibited by double mutants raised in full light. However, photoperiod manipulations have no effect on swim bladder inflation when larvae lack either lateral line or ocular sensory input alone. These results likely indicate that photoperiod is only used for surfacing in absence of both lateral line and visual sensation.

It is unclear why lateral line mutants raised in darkness do not exhibit a comparable increase in hyperinflation like double mutants in darkness, considering the dark condition eliminates visual sensation. These larvae were exposed to a normal photoperiod (14:10 L/D) for their first 24 hours of life, and visual photoreception does not develop until 2-3 dpf (Bollmann 2019; Natalia Villamizar et al. 2014). However, a study demonstrated that spinal opsins activated by light during the first 24 hours can suppress spontaneous locomotor behavior and neural activity (Friedmann et al. 2015). Therefore, the experiment could be improved upon by raising larvae in

darkness from 0 dpf. Additionally, there could be a difference in how genetic versus environmental sensory deprivation affects surfacing behaviors. For example, genetically blocking pineal function in larval zebrafish and subjecting them to full darkness (0:24L/D) was shown to decrease circadian rhythm-entrained location preference and locomotor activity compared to larvae in darkness that did not experience genetic blocking of pineal function (Ben-Moshe Livne et al. 2016).

Photoperiod-driven behaviors in larval zebrafish can be driven by pineal or deep brain photoreception in addition to ocular photosensation (Meissl and Yanez 1994; Fernandes et al. 2012). However, ablation of pineal photoreceptors in lateral line mutants produced no significant change in swim bladder inflation compared to lateral line mutants with intact pineal function (Figure 27). These findings suggest two non-mutually exclusive possibilities for how larvae use photosensation during initial swim bladder inflation: 1) the absence or presence of light is only recognized for surfacing purposes in larvae deprived of *both* lateral line and visual cues, or 2) deep brain photoreception is responsible for the shifts in swim bladder hyperinflation exhibited by double mutants raised in either full light or full dark conditions. To distinguish between these two possibilities, either ablation of pineal photoreceptors in double mutants and/or ablation of deep brain photoreceptors in lateral line mutants will be necessary.

Social isolation can deter initial swim bladder inflation

We also examined whether sensory cues from conspecifics affected initial swim bladder inflation by raising larvae in isolation (Figure 28). *atoh7*$^{-/-}$; *lhfpl5b*$^{-/-}$ double mutants exhibited a decrease in hyperinflation when raised in isolation, though lateral line mutants, blind mutants, and wild type larvae were unaffected. Therefore, it appears that social isolation only affects surfacing in larvae lacking both vision and lateral line sensation. We were able to recreate a similar phenotype by chemically ablating the lateral line in blind larvae raised in isolation (Figure 30).

However, there were more cases of hyperinflation exhibited by ablated blind larvae than when double mutants were raised in isolation. This is likely due to the transient and incomplete loss of lateral line function in CuSO4-treated larvae, which reflects findings of other studies using chemical ablation of the lateral line (Mackenzie and Raible 2012; Hernández et al. 2006; Santos et al. 2006).

Our attempt to recreate the double mutant phenotype by raising isolated lateral line mutants in darkness did not change the proportion of hyperinflated larvae (Figure 29). Again, this could be due to differences between congenital vs. environmental loss of vision, or the additional reduction of non-ocular photoreception that affected the surfacing behavior. Interestingly, isolated wild type larvae raised in darkness exhibited a few cases of hyperinflation. While the cases are mild in comparison to lateral line and double mutants, over-inflation is a rare phenomenon in wild type fish. By isolating wild type larvae and raising them in darkness and in still water, we rid larvae of all water perturbations. A recent preprint offers the hypothesis that decreased water flow correlates with an increase in swim bladder volume in larval zebrafish (Lin et al. 2023). These results align with this hypothesis, though further studies are necessary to determine if and how water motion affects initial swim bladder inflation.

Chemosensory cues from wild type larvae can encourage initial swim bladder inflation

With *atoh7*$^{-/-}$; *lhfpl5b*$^{-/-}$ double mutant larvae already being deprived of lateral line and visual information, chemosensory and tactile cues from conspecifics remain as potential sources of sensory information for use by larvae during surfacing. A recent study showed that mutant zebrafish can behave differently when they are raised with isogenic siblings compared to being raised with heterozygous and homozygous wild type siblings (Ribeiro et al. 2020). Therefore, to determine if chemosensory cues from siblings were responsible for encouraging surfacing, we

segregated larvae based on genotype at 2 dpf (Figure 31). Thus, larvae were raised socially, but only with conspecifics of identical genetic background, except for "wild type" larvae where there was a combination of homozygous wild-type and heterozygous mutant larvae. From this experiment, we saw minimal cases of hyperinflation in segregated double mutants, similar to proportions exhibited by isolated double mutants. This evidence suggests chemosensory cues from wild type larvae are influencing surfacing behaviors in double mutants, and possibly that double mutants fail to synthesize or release this chemical cue.

We specifically tested chemosensory cues by allowing segregated double mutants to inflate their swim bladders in water conditioned by wild type larvae (Figure 32). In isogenically-reared double mutants subjected to the water exchange, we restore swim bladder over-inflation proportions similar to that of lateral line mutants and double mutants that were raised socially with siblings of all genetic background. While this result was unexpected, it is not unheard of for larval zebrafish to use chemosensory cues to drive behavior, and chemosensory cues specifically affect migratory behavior in lamprey larvae (Zielinski et al. 2005; Johnson et al. 2019; Lindsay and Vogt 2004). A study in larval zebrafish showed evidence for larvae olfactory imprinting only at 6 dpf(Gerlach et al. 2008), which is after initial swim bladder inflation. However, our experiment used water from wild type larvae that were not siblings of the double mutants, so kin distinction is irrelevant in this scenario.

It is interesting that we only see the influence of chemosensation on initial swim bladder inflation when larvae lack both lateral line and visual sensory input. Therefore, it seems that *atoh7*$^{-/-}$; *lhfpl5b*$^{-/-}$ double mutants fail to produce a water-borne chemosensory cue that promotes surfacing, but they are still able to respond to this cue when it is supplied by wild type conspecifics. A recent preprint showed that lateral line hair cells integrate mechanical and chemical cues for

navigation in larval zebrafish (Desban et al. 2022). Since lateral line mutants still retain hair cells, it is possible that chemosensation is working directly through lateral line hair cells for surfacing. We know that the lateral line is responsible for modulating the social hormone *parathyroid hormone 2* (*pth2*) in larval zebrafish (Anneser et al. 2020; Venuto et al. 2022), and it is possible that there is sensory integration with visual stimuli in the thalamus that causes downstream effects on hormone release (Kappel et al. 2022).

A variety of chemical stimulants have been linked to zebrafish behavior and locomotion. In the study that investigated chemoreceptors in the lateral line, they found that larval zebrafish exposed to serotonin (released in the water by injured larvae) increases swimming speeds (Desban et al. 2022). Amino acids have also been shown to increase swimming activity in early larval zebrafish (Lindsay & Vogt 2004). Therefore, the chemosensory effect on swim bladder inflation exhibited by blind and lateral line double mutants could instead be a general effect on locomotion since surfacing involves vertical swimming. To rule this out, it is important to compare locomotive behavior of double mutants to wild type larvae, such as time spent actively swimming.

Surfacing as a multi-sensory behavior integrating vestibular, lateral line, photosensory, and chemosensory information

Overall, this study suggests that chemosensation and non-ocular photosensation can inform surfacing behaviors. Since both forms of sensation only exerted significant influence on swim bladder inflation in the absence of both visual and lateral line sensory cues, it is unlikely that either play extensive roles in initial swim bladder inflation in larval zebrafish. However, in their natural environment where obstacles and challenges are more variable, the chemosensory and non-ocular photosensory cues could become useful to inform surfacing behaviors. Perhaps these cues are subtle modulators of the behavior and can be used as compensatory systems for surfacing

behaviors if other sensory systems are unavailable. Alternatively, other fish species may have greater use for non-ocular photosensation and chemosensation for initial swim bladder inflation due to differences in their habitat, and zebrafish retain the ability to use these systems in unique or particularly challenging environmental circumstances. Evolutionary sensory compensation dependent on the species' environment has been studied in fish like Mexican cave fish, where multisensory behaviors rely more heavily on the lateral line sensory system due to the absence of visual photosensation (Lloyd et al. 2018; Baker and Montgomery 1999).

Overall, surfacing for initial swim bladder inflation is a complex behavior requiring multiple sensory systems. The vestibular system plays a primary role in the vertical climb to the surface (Bagnall and Schoppik 2018; Ehrlich and Schoppik 2017; Riley and Moorman 2000; Ehrlich and Schoppik 2019). We hypothesize from our results that non-ocular photosensation and chemosensation can be added to the list of cues that modulate the upward swimming for initial swim bladder inflation. For surface detection, our results do not support the hypothesis that the visual system is involved, and instead suggest that the lateral line may be primarily responsible for behaviors at the air-water interface (Venuto et al. 2023). The lateral line, photosensory, vestibular/auditory, and chemosensory systems all exhibit neural integration in the tectum (Favre-Bulle et al. 2018; Filosa et al. 2016; Isa et al. 2021; Migault et al. 2018; Jundi et al. 2023; Butler and Maruska 2016a). Therefore, while the surfacing behavior can be broken down into multiple components, it is perceivable that these sensory systems are working together to paint a picture of the larvae's environment via sensory integration in the tectum to enable surfacing and successful swim bladder inflation.

CHAPTER 6: DISCUSSION

In this dissertation, I investigated multisensory behaviors in early larval zebrafish. To aid in this process, I first established a repeated treatment protocol to continuously ablate the rapidly proliferating lateral line with neomycin sulfate at 2-5 dpf. Using this technique along with the lateral line mutant (*lhfpl5b*$^{-/-}$), I confirmed that zebrafish larvae use hydrodynamic information from conspecifics to modulate expression of the pro-social hormone *pth2*, but also found evidence that *pth2* expression is influenced by additional sensory systems. Finally, I explored the sensory basis of initial swim bladder inflation in larval zebrafish. I determined that the lateral line is responsible for surface detection during surfacing behaviors to moderate air intake to the swim bladder. Finally, I found that non-ocular photosensory cues as well as chemosensory cues play a role in initial swim bladder inflation when larval zebrafish are without lateral line and visual cues. Overall, this dissertation offers novel findings on the sensory basis for early life behaviors of larval zebrafish.

Repeated ototoxin treatments

In Chapter Two of this dissertation, I used repeated neomycin sulfate treatments to chemically ablate the lateral line. Neomycin-mediated hair cell ablation has been used in larval zebrafish on many occasions. However, only one study used repeated treatments to continuously ablate the lateral line over an extended period (Carrillo and McHenry 2016). Additionally, there is limited understanding of how the lateral line influences behavior before 5 dpf likely due to the lateral line's rapid cellular expansion between 2-5 dpf in larval zebrafish (Ghysen and Dambly-Chaudière 2007; Raible and Kruse 2000; Murakami et al. 2003). Despite knowing that cells are quickly proliferating at this stage, it was unclear whether repeated neomycin treatments could keep

up with the rate of proliferation. Also, if repeated treatments were able to continuously disable the lateral line, it was unknown if there would be any toxic side effects exhibited by the larvae.

I tested the hypothesis that repeated neomycin sulfate treatments could inhibit lateral line function for multiple days in larval zebrafish. I found that 50 μM neomycin sulfate delivered every 12 hours was sufficient to continuously ablate lateral line hair cells while avoiding unintended toxicity. While higher concentrations or more frequent treatments increased hair cell death, the larval deaths associated with these treatments indicated toxic side effects. In my studies investigating the effect of a nonfunctional lateral line on *pth2* expression, repeated neomycin treatments generated a similar decrease in *pth2* expression as observed in lateral line mutants. This indicates that although cell death was not complete under this treatment regimen, it was enough to phenocopy the social cognition of lateral line mutants in terms of *pth2* expression. Subsequently, when investigating the role of the lateral line in initial swim bladder inflation, I used the same treatment timeline to induce swim bladder over-inflation in wild type larvae (a phenotype I previously only saw in lateral line mutants). Once again, I was able to phenocopy the lateral line mutant using repeated neomycin sulfate treatments, indicating that repeated treatments are effective to disable lateral line function and affect behavior in larval zebrafish prior to swim bladder inflation.

Neomycin sulfate is only one form of ototoxin, and it has been shown that even different aminoglycosides can produce different effects on hair cell death, regeneration rates, and behavior (Han et al. 2020; Mackenzie and Raible 2012; Owens et al. 2009; Newton et al. 2023). It would be interesting to repeat this study using other ototoxins like gentamycin or copper sulfate. Other studies indicated that the regeneration time following copper sulfate treatments in larval zebrafish extends longer than neomycin sulfate treatments (Mackenzie and Raible 2012; Hernández et al.,

2006). Using this information for my swim bladder experiments, I used 10 μM copper sulfate treatments on the morning of 4 dpf after blocking larval zebrafish access to the surface. I found that this single copper sulfate treatment at 4 dpf resulted in nearly identical rates of swim bladder over-inflation as lateral line mutants. From this study, a single neomycin treatment caused the smallest proportion of hyperinflation cases, and a single copper sulfate treatment caused the largest proportion of hyperinflation cases, with repeated neomycin treatments roughly in-between. Consequently, it appeared that increased treatment intensity resulted in decreased lateral line function and caused increased swim bladder hyperinflation. However, treatment efficacy determined by hair cell counts would need to be evaluated to confirm this theory. It is likely that a similar phenomenon would occur in *pth2* expression, where less intense treatments yield greater expression than more severe treatments.

Lateral line-less fish experience a form of isolation

In Chapter Three of this dissertation, I test the hypothesis that a nonfunctional lateral line decreases expression of the social hormone *pth2.* In this study, I found that fish with a congenital loss of lateral line function express reduced levels of the prosocial hormone *pth2*. However, isolation further reduced *pth2* levels in lateral line mutants, suggesting that other sensory systems can facilitate *pth2* expression in social environments. A recent study reported no effect of chemosensory or visual cues on *pth2* expression in larval zebrafish with intact lateral line function (Anneser et al. 2020). However, in the absence of lateral line sensory cues, other sensory systems like vision could gain compensatory influence on *pth2* expression based on social context. It is perhaps most likely that somatosensory cues from conspecifics are the cause for the residual *pth2* expression that remains in social lateral line mutants compared to isolated larvae. To test this, touch

insensitive zebrafish larvae, such as *macho* mutants, could be used to see if there is any decrease in *pth2* expression (Ribera and Nüsslein-Volhard 1998).

In rodents, PTH2 (termed "TIP39") promotes parental care (Coutellier et al. 2011; Arpad Dobolyi, Cservenák, and Young 2018) as well as fear, anxiety, and pain processing (Coutellier and Usdin 2011; Dimitrov et al. 2013; Fegley et al., 2008). Specifically, TIP39 mutant mice exhibited were more active in displaying defensive burying following provocation compared to wild type mice, indicative of increased anxiety (Fegley et al., 2008). Similarly, a recent study found that *pth2* mutant zebrafish exhibit increased startle responses, which is also indicative of heightened anxiety (Anneser et al. 2022). As an extension of the startle response test in *pth2* mutant zebrafish, prepulse inhibition (PPI) tests could be performed. PPI uses a weak stimulus before a stronger startle stimulus that suppresses the startle response, and it is an important measure of sensorimotor gating mechanisms (Burgess and Granato 2007).

There is also evidence to suggest that PTH2 (TIP39) activates the oxytocin system for maternal care in female rats (Cservenak et al. 2017; Arpad Dobolyi, Cservenák, and Young 2018). In zebrafish, the oxytocin system is affected by social isolation (Gemmer et al. 2022; Wee et al. 2022). A recent study showed that chemosensory cues were most effective in modulating an oxytocinergic pathway (OXT), though visual cues also affected the circuit (Wee et al. 2022). Additionally, oxytocin receptor knockout zebrafish exhibit decreased shoaling abilities (Gemmer et al. 2022). Neither study tested the involvement of the lateral line. Since social cues are often multisensory in nature, it is plausible that the lateral line can also modulate oxytocinergic signaling. The concept of the lateral line modulating oxytocinergic signaling is especially interesting given that a recent preprint showed potential overlap between chemosensory and lateral line systems, wherein the lateral line hair cells integrate both stimuli (Desban et al. 2022).

Beyond increased startle responses, *pth2* mutant zebrafish also exhibit decreased social preference and looser shoaling formations compared to wild type zebrafish (Anneser et al. 2022). With shoaling being a multisensory behavior that relies heavily on lateral line sensory information, it could be interesting to test shoaling capabilities in *lhfpl5b-/-; pth2-/-* adult zebrafish. I hypothesize that zebrafish with the combination of lateral line and *pth2* mutations would exhibit similar shoaling behaviors to lateral line only mutants. If shoaling is worse in the lateral line/*pth2* mutants than zebrafish with only the lateral line mutation, it could indicate that the small amount of *pth2* expression remaining in lateral line mutants is sufficient to influence behavior. It could also indicate that *pth2* may have its own role in social feedback for shoaling purposes separate from the lateral line, which would be a novel finding on hormonal input for the shoaling behavior.

The lateral line's role in initial swim bladder inflation

In Chapter Four of this dissertation, I provide evidence for a novel role for the lateral line in initial swim bladder inflation, which is an early behavior critical to fish survival. Along with vestibular function, the lateral line is only the second sensory system to be explicitly required for surfacing in larval zebrafish. In this study, I found that the lateral line is likely detecting the air-water interface to mediate air intake to the swim bladder. This finding extends our understanding of how fish use the lateral line organ during interactions with the air-water interface. In one of the experiments, I use video recordings to show that, prior to over-inflating, lateral line mutants spend more time and take more visits to the surface than neutrally buoyant wild type and mutant siblings. However, it would be more informative to have high speed video recordings of air intake events. This way, it would be possible to measure the number of gulps to correlate it with swim bladder size. There may be a threshold amount of air taken in that once passed, the fish becomes positively buoyant with no route of return to neutral buoyancy. A more detailed analysis of behaviors at the

air-water interface may lend support to the hypothesis that lateral line mutants exhibit specific changes in surface interactions during initial swim bladder inflation.

This chapter points to surface detection as the lateral line's responsibility in initial swim bladder inflation. However, it remains unclear precisely how the lateral line is detecting the surface, and if other sensory systems contribute to surface detection. A proposed future experiment to further elucidate the lateral line's involvement in surface detection would involve the *lhfpl5b2028:ChR2-EYFP-PA* transgenic zebrafish line in combination with the *lhfpl5b* mutation. For example, by using a blue light laser beam strong enough to narrowly illuminate just under the surface of the water from the side of the tank, then channelrhodopsin activation in the lateral line would only occur near the surface. If lateral line activation specifically at the surface was enough to prevent hyperinflation, that would better indicate the lateral line's role in surface detection during initial swim bladder inflation.

To ensure that head neuromasts are necessary for surface detection, the same experiment activating the lateral line via channelrhodopsin could be repeated after ablating head neuromasts with copper sulfate. If hyperinflation remained in activated tail neuromasts only, then it would demonstrate that head neuromasts are specifically required for the behavior. This would likely indicate that the anterior lateral line exhibits a unique sensory processing pathway compared to the posterior lateral line during initial swim bladder inflation. Furthermore, select ablation of individual head neuromasts in wild type larvae prior to surfacing could clarify which neuromasts are necessary for detection of surface breaching during initial inflation. This would be done by identifying which subsets of neuromasts when ablated cause swim bladder hyperinflation. For this, it would be ideal to have an optogenetic line in hair cells which would allow for highly specific ablation of individual lateral line neuromasts.

The multisensory surfacing behavior

The most important question remaining from Chapter Four is why only half of the lateral line mutant population hyperinflates. It is possible that *lhfpl5b* could play an unrecognized role in other tissues beyond the lateral line, perhaps accounting for the mutant hyperinflation phenotype. However, I was able to recreate the mutant hyperinflation phenotype by chemically ablating the lateral line of wild type larvae. Additionally, I showed that driving expression of *lhfpl5a* (the inner ear ohnolog of *lhfpl5b*) with an established hair cell promoter that is independent of *lhfpl5* (*myo6b)* to restore hair cell function in lateral line mutants rescues the hyperinflation phenotype. If the loss of *lhfpl5b* in other tissues was responsible for the hyperinflation phenotype, then restoring only hair cell function would not have rescued swim bladder inflation in mutant larvae. Taken together, these results suggest that *lhfpl5b* does not affect other tissues in a way that causes the hyperinflation phenotype to only affect half of the mutant population.

In Chapter Five, I test the hypothesis that other sensory systems aid in initial swim bladder inflation. Since initial swim bladder inflation occurs at just 3-4 dpf, it does not leave much time for sensory compensation to occur. However, surfacing is a multisensory behavior involving at least vestibular and lateral line sensation, so there may be overlap in additional sensory cues utilized for the behavior. In Chapter Five, I conducted the first study on the role photosensory information during initial swim bladder inflation in zebrafish, and the first to combine it with loss of lateral line sensation.

Contrary to my hypothesis, I found that ocular photosensory cues do not contribute to surfacing behaviors for initial swim bladder inflation in larval zebrafish. However, non-ocular cues shifted hyperinflation proportions in blind and lateral line-less zebrafish larvae. The slight increase in hyperinflation in the blind/lateral line double mutants raised in darkness, combined with the

slight decrease in hyperinflation in double mutants raised in full light are similar to other studies conducted in various fish species where inflation rates are higher in photoperiods with increased darkness. While zebrafish generally exhibit positive phototaxis, this can change depending on the behavior (Chen and Engert 2014; Mueller and Neuhauss 2010). Therefore, increased swim bladder inflation in darkness is a relatively unsurprising finding, especially considering that many larval fish species exhibit various swim-up behaviors in the dark for predator avoidance and energy conservation (Battaglene et al. 1994).

Distinct from non-ocular phototaxis during swim-up, visual cues could help larvae identify the air-water interface. The lack of a role for visual cues in larval zebrafish emphasizes the importance of the lateral line in initial swim bladder inflation for its probable role in surface detection. Additionally, visual acuity in 3-4 dpf zebrafish is immature and may not offer useful insight to larval fish surroundings (Easter and Nicola 1996; Haug et al. 2010). However, other fish species might have more rapid visual development, or a delayed need for swim bladder inflation, that allows for use of ocular photosensation during surfacing. Furthermore, other fish species that spawn in regions with varying levels of water flow might have greater use for the visual system than zebrafish. Rheotaxis is a multisensory behavior largely dependent on lateral line and visual sensation, and studies have suggested that water currents impact initial swim bladder inflation (Clayton and Summerfelt, 2010; Lin et al., 2023). I found evidence of an influence of water motion on initial swim bladder inflation since surface waves deterred hyperinflation in lateral line mutants. Further supporting this theory, isolated wild type larvae in darkness devoid of water perturbations (socially isolated) exhibited mild cases of swim bladder hyperinflation.

However, the lack of an obvious role for visual acuity in larval zebrafish surfacing leaves unanswered questions about the hyperinflation phenotype only affecting roughly half of the lateral

line mutant population. Somatosensory (touch) cues may contribute to surface detection. There are three different sources of contact for larval zebrafish in our experimental arenas while surfacing: the tank walls, the air, and the water. From the video recordings in Chapter Four, some larvae approach the air-water interface near corners or sides of the tank, while others approach it near the center of the arena. There did not seem to be a preference for lateral line mutants to breach the surface near tank walls or vice versa for wild type larvae, though it was not quantified. The increased surface tension at a tank corner could potentially intensify the tactile stimulus of the air-water interface compared to those surfacing in the middle of the arena. Perhaps surfacing near a corner when the larva is reaching a neutral buoyancy threshold keeps some lateral line mutants in the neutrally buoyant category instead of reaching swim bladder over-inflation. Video recordings to track the number of surface visits near corners/edges versus mid-arena surfacing could be correlated to final swim bladder volume of lateral line mutants to test this theory.

An alternate hypothesis is that tactile cues are not involved in surface detection and that the lateral line is the sole sensory informant of air-water interface breaching. If this is the case, the hyperinflation phenotype only affecting half of the population could be due to a failure in interceptive feedback. Once again, if neutral buoyancy has a threshold and swim bladder over-inflation is the result of air intake passing that threshold, then lateral line mutants could be playing a guessing game at how much air they are taking in form the surface. The ones that "succeed" and become neutrally buoyant may happen to take a break from air intake and swim around enough for their vestibular system to catch up and inform them of their own specific gravity status (Bagnall and Schoppik 2018; Schoppik et al. 2017). According to this theory, the ones that hyperinflate may not realize they have taken in too much air until it is too late. By analyzing video recordings of lateral line mutants, I found that, for larvae that ended up hyperinflating, there would be a point

during a surfacing trip where body position relative to the surface would become more parallel instead of diagonal. After this, the larval fish would struggle to swim down past a few millimeters below the surface, indicating positive buoyancy.

The concept that vestibular feedback is used during initial swim bladder inflation could explain the incomplete penetrance of the swim bladder phenotype in lateral line mutants especially because the vestibular system plays a considerable role in surfacing (Riley and Moorman 2000). The vestibular system is involved with vertical swimming and gravity detection (Bagnall and Schoppik 2018; Ehrlich and Schoppik 2019; Liu et al. 2022). Therefore, feedback from the vestibular system upon changing specific gravity from swim bladder inflation could inform the fish that neutral buoyancy has been achieved and there is no longer a need for air intake. However, if that feedback does not occur before the neutral buoyancy threshold has been passed, the vestibular system can no longer assist in achieving neutral buoyancy. Unfortunately, there is no obvious way to test this theory since disruption of the vestibular system results in decreased surfacing attempts and swim bladder non-inflation.

Interoception is the ability to sense information about one's internal organs (Ceunen, Vlaeyen, and Van Diest 2016). As humans, we can understand when our lungs are expanding because of neurons with stretch activated sensors in the lungs (Kubin et al. 2006; Berntson and Khalsa 2021). Lungs are the terrestrial homolog of the swim bladder (Fänge 1983; Longo, Riccio, and McCune 2013). Little is known about the interoceptive capacity of fish and their swim bladder, though it is though that there are stretch receptors within the organ, similar to lungs .(Finney et al. 2006; Qutob 1963). It would make sense for fish to have a built-in feedback system from stretch receptors surrounding the swim bladder organ to regulate buoyancy. Some fish species can force out air from their swim bladder via a mechanism similar to burping (Shrimpton, Randall, and Fidler

2011; Smith and Croll 2011). However, there is no evidence to suggest that larval zebrafish can expel substantial excess air to regulate swim bladder volume.

The potential role for chemosensation in initial swim bladder inflation

Perhaps the most interesting finding from Chapter Five is the potential role for chemosensory cues in initial swim bladder inflation in larval zebrafish. Larvae genetically deprived of visual and lateral line sensory cues exhibit a significant decrease in hyperinflation proportions when raised separately from wild type larvae. The hyperinflation phenotype is fully restored when *atoh7-/-; lhfpl5b-/-* double mutants are raised with wild type siblings, and partially restored when segregated *atoh7-/-; lhfpl5b-/-* double mutants are allowed to inflate their swim bladders in water conditioned by non-sibling wild type larvae. It is important to note that kin detection is likely not involved with this form of chemosensation, since kin detection is not thought to occur in larval zebrafish until after initial swim bladder inflation (Hinz et al. 2013; Gerlach et al. 2008).

The potential involvement of chemosensory cues in initial swim bladder inflation was completely unexpected. However, between the vertical swimming aspect and surface detection aspect of the surfacing behavior, it is most likely that chemosensory cues play a role in vertical swimming towards the surface. While chemosensory cues have not been identified to play a role in directional swimming behaviors in larval zebrafish, chemosensory cues have been linked to migration in larval lamprey (Zielinski et al. 2005; Johnson et al. 2019). Furthermore, zebrafish are behaviorally susceptible to changes to chemosensory cues based on genetic background (Ribeiro et al. 2020).

Types of chemosensory cues potentially involved in initial swim bladder inflation

Zebrafish have demonstrated behavioral changes in response to chemostimulants by 4 dpf, which overlaps with surfacing behaviors (Lindsay and Vogt 2004). In this study, they found that 4 dpf zebrafish exposed to a mixture of 12 amino acids increased their swimming activity and swimming speed compared to zebrafish that did not encounter the amino acid mixture. It is possible that zebrafish larvae produce amino acids that influence vertical swimming during the surfacing behavior. This would imply that blind and lateral line double mutants are unable to produce the same amino acid mixture as wild type and lateral line only mutants or blind only mutants.

Returning to the preprint showing that lateral line hair cells are capable of chemoreceptive abilities (Desban et al. 2022), it is possible, given the lateral line's role in surfacing, that chemosensory cues during initial swim bladder inflation are being processed through lateral line hair cells. A variety of chemoreceptors were found in lateral line hair cells and can detect a diverse set of chemical cues including serotonin, hormones, and amino acids (Desban et al. 2022).They also found that serotonin released from injured larvae in the environment of a larval zebrafish activates lateral line-mediated swimming bouts (Desban et al. 2022). Despite loss of MET channel function, lateral line mutants still have hair cells in all neuromasts, albeit a reduced number. Therefore, it may be possible that lateral line mutants retain an ability to receive chemosensory information even though lateral line mechanosensation is impossible.

Ototoxic ablation of the lateral line should remove the capacity for the lateral line to process chemosensory information. Interestingly, ototoxic ablations to the lateral line in blind, isolated larvae resulted in a decrease in hyperinflation proportions, but not to the same extent as blind and lateral line double mutants raised in isolation. This could indicate partial involvement of lateral line chemoreceptors in the chemosensory basis for initial swim bladder inflation. However, it is

unlikely that lateral line-mediated chemosensation is fully responsible for the swim bladder phenotype in blind and lateral line double mutants.

As previously mentioned, larval zebrafish behaviorally responded to serotonin released by injured larvae in the lateral line chemoreceptor study (Desban et al. 2022). Injured fish release alarm cues that can alter larval zebrafish behavior (Lucon-Xiccato et al. 2020; Quadros et al. 2019). One study showed that larval zebrafish exhibit a decrease in activity upon exposure to alarm cues (Quadros et al. 2019). Considering that larvae remain unharmed during surfacing behaviors, it is unlikely that alarm cues accounted for the changes in swim bladder inflation in blind and lateral line double mutants. However, alarm cues contain a mixture of substances (Chivers et al. 2016), like serotonin, so it is possible that one or more of the substances involved in alarm cues was released and recognized by larval zebrafish in my swim bladder studies.

Serotonergic neuroepithelial cells are also found along the skin of zebrafish and are sensitive to oxygen (Coccimiglio and Jonz 2012). Perhaps surface breaching and access to air activates these oxygen receptor cells during initial swim bladder inflation in larval zebrafish. The trigger of these oxygen receptor cells could cause a release of serotonin and potentially moderate surfacing behaviors. Since serotonin was influential in lateral line chemoreception as well as oxygen-sensitive neuroepithelial cells, it may be worthwhile to investigate the effect of serotonin on initial swim bladder inflation by adding serotonin to the water of surfacing larval zebrafish.

Oxytocin is another neuropeptide that is heavily associated with social behaviors and chemosensory cues in zebrafish (Landin et al. 2020; Zimmermann et al. 2016). Oxytocin signaling has been identified as a stress indicator in adult zebrafish (Chuang et al. 2021). In larval zebrafish, a shift in behavior occurs in oxytocin receptor mutant zebrafish when they are raised isogenically versus heterogenically (Ribeiro et al. 2020). Additionally, social isolation affects appetite and

social avoidance in larval zebrafish, as kin-derived cues modulate an oxytocinergic circuit (Wee et al. 2022). Therefore, it is possible that the chemical cue from wild type conspecifics influencing initial swim bladder inflation in blind and lateral line double mutants involves oxytocin.

Alternatively, it is possible that the mystery chemical produced does not play a role in initial swim bladder inflation, and instead affects overall locomotor activity that happens to impact surfacing behaviors. Furthermore, it is possible that there are several chemicals that have similar stimulatory effects on locomotion, which could come from sources other than conspecifics. In the wild, a chemical from conspecifics is likely to be more dispersed than it is in a lab setting, and it would be mixed with chemosensory cues from other fish species or vegetation. However, if the mystery chemical cue has a general effect on locomotion, then a variety of sources could be influencing the surfacing behavior. Regardless of its potential involvement in initial swim bladder inflation, it is interesting that loss of visual and lateral line cues alters the release of chemical cues in larval zebrafish. This potentially indicates a pathway wherein visual and lateral line sensation work together to generate chemical cues. What is clear from this study is that further investigation is necessary into the locomotor behaviors of blind and lateral line double mutants, as well as the effect of photosensory and hydrosensory deprivation on sensitivity to chemical cues.

Sensory integration in the zebrafish brain

When we look to the zebrafish brain for potential regions of overlap for multisensory behaviors like surfacing, there are a few key sensory integration centers. Visual and olfactory information can be integrated in the retina by the olfacto-retinal centrifugal pathway (ORC) that goes from the terminalis nerve in the olfactory bulb to the retina (Wang et al. 2011; Li 2019). In this pathway, olfactory stimulation increases visual sensitivity in some behavioral contexts (Maaswinkel and Li 2003; Li and Dowling 2000). If the chemosensory cues from wild type larvae

worked through a similar pathway enhancing vision, I would not expect double mutants lacking vision and lateral line sensation to be affected, since there is no visual system to enhance. Instead, I would have expected to see a shift in lateral line only mutants in response to olfactory cues since vision was available to use for possible surface detection. Therefore, it is unlikely that olfactory sensory integration in the surfacing behavior in larval zebrafish is working through the ORC pathway.

As for higher processing centers in the zebrafish brain, the thalamus receives input from the visual, lateral line, and vestibular systems (Anneser et al. 2020, Kappel et al. 2022; Favre-Bulle et al. 2018; Migault et al. 2018). However, there is currently no evidence for chemosensory integration in the zebrafish thalamus. Most chemosensory information involves the olfactory bulb and/or the hypothalamus (Kermen et al. 2013; Gerlach and Wullimann 2021). However, the tectum is a brain region with known sensory integration from photosensory, mechanosensory, and chemosensory systems (Favre-Bulle et al. 2018; Filosa et al. 2016; Isa et al. 2021; Migault et al. 2018; Thompson et al. 2016). The tectum is a large brain region found in the larval zebrafish midbrain. It is mainly known for driving visually-guided behaviors in fish (Ingle 1973). However, the swim-up and surface detection aspects of initial swim bladder inflation combined with vestibular, lateral line, photosensory and chemosensory modulation make the tectum a main candidate for involvement in surfacing behaviors despite the behavior lacking obvious involvement of visual sensation. Since the lateral line and vestibular systems seem to be the primary sensory cues necessary for initial swim bladder inflation, it is possible that much of the relevant sensory processing occurs in regions like the thalamus despite not having chemosensory inputs. Extensive further studies are necessary to elucidate central processing regions for sensory integration during the surfacing behavior in larval zebrafish.

I studied both *pth2* expression and initial swim bladder inflation in the context of congenital sensory deprivation. Congenital sensory deprivation can have long-term effects on neural circuits as well as evolutionary changes that span generations (Chapman et al. 2010; Grubb and Thompson 2004; Pittman et al. 2013). For example, blind cavefish have developed greater reliance on the lateral line during prey capture than seeing cavefish (Lloyd et al. 2018). While many of the findings from congenital mutants were supported by additional experiments using environmental or chemical sensory deprivation, it is possible that lateral line-less fish would adapt and experience sensory compensation over time. Therefore, juvenile and adult lateral line mutants may rely more heavily on visual cues during multisensory behaviors like rheotaxis. We also do not understand how lack of a functional lateral line or visual system from birth might change the development of neural circuits.

Significance to the field and beyond

Chapter Two broadens our understanding of early lateral line proliferation and regeneration and offers a useful repeated ototoxic treatment tool to continuously disable the lateral line at young larval stages. Chapter Three elucidates a role for the lateral line in social detection and neuromodulation while providing a tool to track *pth2* dynamics *in vivo.* Chapter Four provides fundamental sensory context for the critical surfacing behavior to achieve neutral buoyancy. It also indicates a novel role for the lateral line in surface detection during initial swim bladder inflation. Finally, Chapter Five offers insight into the multisensory context of the surfacing behavior, surprisingly implicating non-ocular photosensory and chemosensory cues for roles in initial swim bladder inflation.

Ototoxins include a vast array of chemicals, including heavy metals and aminoglycosides. Some of these ototoxins could be prevalent in runoff to streams, rivers, and oceans and negatively

impact lateral line function (Young et al. 2018). My studies show that even partial disablement of the lateral line can significantly impact behavior and even fish survival, since swim bladder hyperinflation leads to death. Therefore, it is important that we monitor the use of metals and collection of waste to limit potential impact on fish sensory capabilities.

Initial swim bladder inflation is also a major issue in the aquaculture industry (Woolley and Qin 2010). Most of the studies surrounding initial swim bladder inflation are conducted to improve fish farming of specific species. Beyond understanding that certain metals or chemicals used in fish farming facilities could negatively impact lateral line function, it is also helpful to have better insight into the behavior to provide adequate resources and enable successful surfacing. For example, one of my experiments used oil to decrease the surface tension of the air-water interface, which caused hyperinflation in wild type larvae. Surface oils are very prevalent in fish farming conditions, and the awareness that excess oils could cause hyperinflation issues can help people in the industry to avoid the issue by clearing excess oil on the surface in advance of initial swim bladder inflation (Friedmann and Shutty 1999; Honryo et al. 2016; Kurata et al. 2012).

The potential roles for non-ocular photosensory and chemosensory cues displayed in larval zebrafish during initial swim bladder inflation may be of greater importance in other fish species. We see that fish can adapt their reliance on different types of sensory cues depending on their environment. It is possible that chemosensory and non-ocular photosensory cues are an evolutionarily conserved feature for sensory use in surfacing behaviors. Beyond zebrafish, other species might have robust needs chemosensory and photosensory systems as well as visual/lateral line integration for rheotactic capacity for larvae to achieve neutral buoyancy. Using zebrafish as a model organism to better understand the sensory basis and neural underpinning of the surfacing behavior is useful to our general understanding of initial swim bladder inflation. Overall, I provide

necessary framework for further understanding of the surfacing behavior for initial swim bladder inflation. Yet, surfacing remains an understudied phenomenon in many species, and this critical and complex behavior should continue to be investigated by researchers in fields that include neurobiology, behavioral ecology, and fisheries.

REFERENCES

Abbas, Leila, and Tanya T. Whitfield. 2009. “Nkcc1 (Slc12a2) Is Required for the Regulation of Endolymph Volume in the Otic Vesicle and Swim Bladder Volume in the Zebrafish Larva.” *Development* 136 (16): 2837–48. https://doi.org/10.1242/DEV.034215.

Adkins-Regan, Elizabeth. 2009. “Neuroendocrinology of Social Behavior.” *ILAR Journal* 50 (1): 5–14. https://doi.org/10.1093/ILAR.50.1.5.

Alexander, R. M. 1966. “Physical Aspects of Swimbladder Function.” *Biological Reviews of the Cambridge Philosophical Society*. John Wiley & Sons, Ltd. https://doi.org/10.1111/j.1469-185x.1966.tb01542.x.

Alexandre, D, A Ghysen, and Alain Ghysen. 1999. “Somatotopy of the Lateral Line Projection in Larval Zebrafish.” *Proceedings of the National Academy of Sciences of the United States of America* 96 (13): 7558–62. https://doi.org/10.1073/pnas.96.13.7558.

Alharazneh, Abdelrahman, Lauren Luk, Markus Huth, Ashkan Monfared, Peter S Steyger, Alan G Cheng, and Anthony J Ricci. 2011. “Functional Hair Cell Mechanotransducer Channels Are Required for Aminoglycoside Ototoxicity.” Edited by Michael N. Nitabach. *PloS One* 6 (7): e22347. https://doi.org/10.1371/journal.pone.0022347.

Amaral, David G. 2002. “The Primate Amygdala and the Neurobiology of Social Behavior: Implications for Understanding Social Anxiety.” *Biological Psychiatry* 51 (1): 11–17. https://doi.org/10.1016/S0006-3223(01)01307-5.

Anneser, Lukas, Ivan C. Alcantara, Anja Gemmer, Kristina Mirkes, Soojin Ryu, and Erin M. Schuman. 2020. “The Neuropeptide Pth2 Dynamically Senses Others via Mechanosensation.” *Nature 2020 588:7839* 588 (7839): 653–57. https://doi.org/10.1038/s41586-020-2988-z.

Anneser, Lukas, Anja Gemmer, Tim Eilers, Ivan C. Alcantara, Anett Yvonn Loos, Soojin Ryu, and Erin M. Schuman. 2022. “The Neuropeptide Pth2 Modulates Social Behavior and Anxiety in Zebrafish.” *IScience* 25 (3): 103868. https://doi.org/10.1016/J.ISCI.2022.103868.

Arnold, G. P. 1974. “Rheotropism in Fishes.” *Biological Reviews of the Cambridge Philosophical Society*. John Wiley & Sons, Ltd (10.1111). https://doi.org/10.1111/j.1469-185X.1974.tb01173.x.

Bagnall, Martha W., and David Schoppik. 2018. “Development of Vestibular Behaviors in Zebrafish.” *Current Opinion in Neurobiology* 53 (December): 83. https://doi.org/10.1016/J.CONB.2018.06.004.

Bailey, H. C., and S. I. Doroshov. 1995. “The Duration of the Interval Associated with Successful Inflation of the Swimbladder in Larval Striped Bass (Morone Saxatilis).” *Aquaculture* 131 (1–2): 135–43. https://doi.org/10.1016/0044-8486(94)00215-A.

Bak-Coleman, Joseph, Autumn Court, D A Paley, and S Coombs. 2013. “The Spatiotemporal Dynamics of Rheotactic Behavior Depends on Flow Speed and Available Sensory Information.” *The Journal of Experimental Biology* 216 (Pt 21): 4011–24. https://doi.org/10.1242/jeb.090480.

Bak-Coleman, Joseph, Derek Smith, and Sheryl Coombs. 2015. “Going with, Then against the Flow: Evidence against the Optomotor Hypothesis of Fish Rheotaxis.” *Animal Behaviour* 107 (September): 7–17. https://doi.org/10.1016/J.ANBEHAV.2015.06.007.

Baker, C. F., and J. C. Montgomery. 1999. “The Sensory Basis of Rheotaxis in the Blind Mexican Cave Fish, Astyanax Fasciatus.” *Journal of Comparative Physiology A: Sensory,*

Neural, and Behavioral Physiology 184 (5): 519–27. https://doi.org/10.1007/s003590050351.
Battaglene, S C, and R B Talbot. 1990. “Initial Swim Bladder Inflation in Intensively Reared Australian Bass Larvae, Mucquaria Nouemczculeutu (Steindachner) (Perciformes: Percichthyidae).” Vol. 86.
Battaglene, Stephen C., Shannon McBride, and R. Bill Talbot. 1994. “Swim Bladder Inflation in Larvae of Cultured Sand Whiting, Sillago Ciliata Cuvier (Sillaginidae).” *Aquaculture* 128 (1–2): 177–92. https://doi.org/10.1016/0044-8486(94)90112-0.
Battaglene, Stephen C., and R. Bill Talbot. 1993. “Effects of Salinity and Aeration on Survival of and Initial Swim Bladder Inflation in Larval Australian Bass.” *Progressive Fish-Culturist* 55 (1): 35–39. https://doi.org/10.1577/1548-8640(1993)055<0035:EOSAAO>2.3.CO;2.
Bell, Curtis C., Victor Han, and Nathaniel B. Sawtell. 2008. “Cerebellum-like Structures and Their Implications for Cerebellar Function.” *Annual Review of Neuroscience* 31: 1–24. https://doi.org/10.1146/ANNUREV.NEURO.30.051606.094225.
Ben-Moshe Livne, Zohar, Shahar Alon, Daniela Vallone, Yared Bayleyen, Adi Tovin, Inbal Shainer, Laura G. Nisembaum, et al. 2016. “Genetically Blocking the Zebrafish Pineal Clock Affects Circadian Behavior.” *PLoS Genetics* 12 (11): 1006445. https://doi.org/10.1371/JOURNAL.PGEN.1006445.
Berntson, Gary G., and Sahib S. Khalsa. 2021. “Neural Circuits of Interoception.” *Trends in Neurosciences* 44 (1): 17. https://doi.org/10.1016/J.TINS.2020.09.011.
Bhattacharya, Poulomi, Yi Lin Yan, John Postlethwait, and David A. Rubin. 2011. “Evolution of the Vertebrate Pth2 (Tip39) Gene Family and the Regulation of PTH Type 2 Receptor (Pth2r) and Its Endogenous Ligand Pth2 by Hedgehog Signaling in Zebrafish Development.” *Journal of Endocrinology* 211 (2): 187–200. https://doi.org/10.1530/JOE-10-0439.
Bianco, Isaac H., and Florian Engert. 2015. “Visuomotor Transformations Underlying Hunting Behavior in Zebrafish.” *Current Biology* 25 (7): 831–46. https://doi.org/10.1016/j.cub.2015.01.042.
Bianco, Isaac H., Adam R. Kampff, and Florian Engert. 2011. “Prey Capture Behavior Evoked by Simple Visual Stimuli in Larval Zebrafish.” *Frontiers in Systems Neuroscience* 5 (DECEMBER 2011): 101. https://doi.org/10.3389/FNSYS.2011.00101/ABSTRACT.
Blazquez, Pablo M., Yutaka Hirata, Shane A. Heiney, Andrea M. Green, and Stephen M. Highstein. 2003. “Cerebellar Signatures of Vestibulo-Ocular Reflex Motor Learning.” *The Journal of Neuroscience* 23 (30): 9742. https://doi.org/10.1523/JNEUROSCI.23-30-09742.2003.
Bleckmann, Horst. 1988. “Prey Identification and Prey Localization in Surface-Feeding Fish and Fishing Spiders.” In *Sensory Biology of Aquatic Animals*, 619–41. New York, NY: Springer New York. https://doi.org/10.1007/978-1-4612-3714-3_24.
Bleckmann, Horst. 2006. “The Lateral Line System of Fish.” *Fish Physiology*. https://doi.org/10.1016/S1546-5098(06)25010-6.
Bleckmann, Horst, and Erich Schwartz. 1982. “The Functional Significance of Frequency Modulation Within a Wave Train for Prey Localization in the Surface-Feeding Fish Aplocheilus Lineatus (Cyprinodontidae)*.” *Journal of Comparative Physiology, A*. Vol. 145.

Bollmann, Johann H. 2019. “The Zebrafish Visual System: From Circuits to Behavior.” *Https://Doi.Org/10.1146/Annurev-Vision-091718-014723* 5 (September): 269–93. https://doi.org/10.1146/ANNUREV-VISION-091718-014723.

Braubach, Oliver R., Heather Dawn Wood, Simon Gadbois, Alan Fine, and Roger P. Croll. 2009. “Olfactory Conditioning in the Zebrafish (Danio Rerio).” *Behavioural Brain Research* 198 (1): 190–98. https://doi.org/10.1016/J.BBR.2008.10.044.

Brothers, Leslie. 1990. “The Neural Basis of Primate Social Communication.” *Motivation and Emotion 1990 14:2* 14 (2): 81–91. https://doi.org/10.1007/BF00991637.

Brüning, Anika, Franz Hölker, and Christian Wolter. 2011. “Artificial Light at Night: Implications for Early Life Stages Development in Four Temperate Freshwater Fish Species.” *Aquatic Sciences* 73 (1): 143–52. https://doi.org/10.1007/s00027-010-0167-2.

Burgdorf, Jeffrey, and Jaak Panksepp. 2006. “The Neurobiology of Positive Emotions.” *Neuroscience & Biobehavioral Reviews* 30 (2): 173–87. https://doi.org/10.1016/J.NEUBIOREV.2005.06.001.

Burgess, Harold A., and Michael Granato. 2007. “Sensorimotor Gating in Larval Zebrafish.” *Journal of Neuroscience* 27 (18): 4984–94. https://doi.org/10.1523/JNEUROSCI.0615-07.2007.

Burrill, John D., and Stephen S. Easter. 1994. “Development of the Retinofugal Projections in the Embryonic and Larval Zebrafish (Brachydanio Rerio).” *Journal of Comparative Neurology* 346 (4): 583–600. https://doi.org/10.1002/CNE.903460410.

Butler, Julie M, and Karen P Maruska. 2015. “The Mechanosensory Lateral Line Is Used to Assess Opponents and Mediate Aggressive Behaviors during Territorial Interactions in an African Cichlid Fish.” *The Journal of Experimental Biology* 218 (Pt 20): 3284–94. https://doi.org/10.1242/jeb.125948.

Butler, Julie M., and Karen P. Maruska. 2016a. “The Mechanosensory Lateral Line System Mediates Activation of Socially-Relevant Brain Regions during Territorial Interactions.” *Frontiers in Behavioral Neuroscience* 10 (MAY): 93. https://doi.org/10.3389/FNBEH.2016.00093/BIBTEX.

Butler, Julie M., and Karen P. Maruska. 2016b. “Mechanosensory Signaling as a Potential Mode of Communication during Social Interactions in Fishes.” *The Journal of Experimental Biology* 219 (18): 2781–89. https://doi.org/10.1242/jeb.133801.

Carreau, Natalie D., and Greg G. Pyle. 2005. “Effect of Copper Exposure during Embryonic Development on Chemosensory Function of Juvenile Fathead Minnows (Pimephales Promelas).” *Ecotoxicology and Environmental Safety* 61 (1): 1–6. https://doi.org/10.1016/j.ecoenv.2004.10.008.

Carrillo, Andres, and Matthew J. McHenry. 2016. “Zebrafish Learn to Forage in the Dark.” *Journal of Experimental Biology* 219 (4): 582–89. https://doi.org/10.1242/jeb.128918.

Ceunen, Erik, Johan W.S. Vlaeyen, and Ilse Van Diest. 2016. “On the Origin of Interoception.” *Frontiers in Psychology* 7 (MAY). https://doi.org/10.3389/FPSYG.2016.00743.

Chagnaud, Boris P., Jacob Engelmann, Bernd Fritzsch, Joel C. Glover, and Hans Straka. 2017. “Sensing External and Self-Motion with Hair Cells: A Comparison of the Lateral Line and Vestibular Systems from a Developmental and Evolutionary Perspective.” *Brain, Behavior and Evolution* 90 (2): 98–116. https://doi.org/10.1159/000456646.

Chapman, Ben B., Lesley J. Morrell, Colin R. Tosh, and Jens Krause. 2010. “Behavioural Consequences of Sensory Plasticity in Guppies.” *Proceedings of the Royal Society B: Biological Sciences* 277 (1686): 1395. https://doi.org/10.1098/RSPB.2009.2055.

Chapman, Duane C., Wayne A. Hubert, and U. Thomas Jackson. 1988. “Influence of Access to Air and of Salinity on Gas Bladder Inflation in Striped Bass.” *The Progressive Fish-Culturist* 50 (1): 23–27. https://doi.org/10.1577/1548-8640(1988)050<0023:ioataa>2.3.co;2.

Chaput, Shayna Lee, Warren W. Burggren, Peter L. Hurd, and Trevor J. Hamilton. 2023. “Zebrafish (Danio Rerio) Shoaling in Light and Dark Conditions Involves a Complex Interplay between Vision and Lateral Line.” *Behavioural Brain Research* 439 (February): 114228. https://doi.org/10.1016/J.BBR.2022.114228.

Chatain, B. 1989. “Problems Related to the Lack of Functional Swimbladder in Intensive Rearing of Dicentrarchus Labrax and Sparus Auratus.” *Advances in Tropical Aquaculture, Workshop at Tahiti, French Polynesia, 20 Feb - 4 Mar 1989*, February. https://archimer.ifremer.fr/doc/00000/1432/.

Chen, Patrick, and Weizhe Hong. 2018. “Neural Circuit Mechanisms of Social Behavior.” *Neuron* 98 (1): 16–30. https://doi.org/10.1016/J.NEURON.2018.02.026.

Chen, Wei Long, Hungtang Ko, Han Sheng Chuang, David M. Raizen, and Haim H. Bau. 2021. “Caenorhabditis Elegans Exhibits Positive Gravitaxis.” *BMC Biology* 19 (1): 1–16. https://doi.org/10.1186/S12915-021-01119-9/FIGURES/6.

Chen, Xiuye, and Florian Engert. 2014. “Navigational Strategies Underlying Phototaxis in Larval Zebrafish.” *Frontiers in Systems Neuroscience* 8 (MAR): 39. https://doi.org/10.3389/fnsys.2014.00039.

Chivers, Douglas P., Matthew D. Mitchell, Tyrone Lucon-Xiccato, Grant E. Brown, and Maud C.O. Ferrari. 2016. “Background Risk Influences Learning but Not Generalization of Predators.” *Animal Behaviour* 121 (November): 185–89. https://doi.org/10.1016/J.ANBEHAV.2016.08.008.

Choe, Chong Pyo, Seok-Yong Choi, Yun Kee, Min Jung Kim, Seok-Hyung Kim, Yoonsung Lee, Hae-Chul Park, and Hyunju Ro. 2021. “Transgenic Fluorescent Zebrafish Lines That Have Revolutionized Biomedical Research.” *Laboratory Animal Research 2021 37:1* 37 (1): 1–29. https://doi.org/10.1186/S42826-021-00103-2.

Chuang, Hsin Ju, Chun Yung Chang, Huai Ping Ho, and Ming Yi Chou. 2021. “Oxytocin Signaling Acts as a Marker for Environmental Stressors in Zebrafish.” *International Journal of Molecular Sciences* 22 (14): 7459. https://doi.org/10.3390/IJMS22147459.

Clayton, Richard D., and Robert C. Summerfelt. 2010. “Gas Bladder Inflation in Walleye Fry Cultured in Turbid Water with and without a Surface Spray.” *North American Journal of Aquaculture* 72 (4): 338–42. https://doi.org/10.1577/A10-019.1.

Coccimiglio, Maria L., and Michael G. Jonz. 2012. “Serotonergic Neuroepithelial Cells of the Skin in Developing Zebrafish: Morphology, Innervation and Oxygen-Sensitive Properties.” *Journal of Experimental Biology* 215 (22): 3881–94. https://doi.org/10.1242/JEB.074575/258043/am/serotonergic-neuroepithelial-cells-of-the-skin-in.

Coffin, Allison B., and John Ramcharitar. 2016. “Chemical Ototoxicity of the Fish Inner Ear and Lateral Line.” In *Advances in Experimental Medicine and Biology*, 877:419–37. Springer, Cham. https://doi.org/10.1007/978-3-319-21059-9_18.

Coombs, Sheryl, Joe Bak-Coleman, and John Montgomery. 2020. “Rheotaxis Revisited: A Multi-Behavioral and Multisensory Perspective on How Fish Orient to Flow.” *Journal of Experimental Biology* 223 (23). https://doi.org/10.1242/jeb.223008.

Coombs, Sheryl, James J. Finneran, and Ruth A. Conley. 2000. “Hydrodynamic Image Formation by the Peripheral Lateral Line System of the Lake Michigan Mottled Sculpin,

Cottus Bairdi." *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences* 355 (1401): 1111–14. https://doi.org/10.1098/RSTB.2000.0649.
Cornacchia, J. W., And J. E. Colt. 1984. "The Effects of Dissolved Gas Supersaturation on Larval Striped Bass, Morone Saxatilis (Walbaum)." *Journal of Fish Diseases* 7 (1): 15–27. https://doi.org/10.1111/j.1365-2761.1984.tb00903.x.
Corwin, J T, and M E Warchol. 1991. "Auditory Hair Cells: Structure, Function, Development, and Regeneration." *Annual Review of Neuroscience* 14 (1): 301–33. https://doi.org/10.1146/annurev.ne.14.030191.001505.
Coutellier, L., A. Logemann, M. Rusnak, and T. B. Usdin. 2011. "Maternal Absence of the Parathyroid Hormone 2 Receptor Affects Postnatal Pup Development." *Journal of Neuroendocrinology* 23 (7): 612–19. https://doi.org/10.1111/J.1365-2826.2011.02149.X.
Coutellier, Laurence, and Ted B. Usdin. 2011. "Enhanced Long-Term Fear Memory and Increased Anxiety and Depression-like Behavior after Exposure to an Aversive Event in Mice Lacking TIP39 Signaling." *Behavioural Brain Research* 222 (1): 265–69. https://doi.org/10.1016/J.BBR.2011.02.043.
Cruz, Ivan A., Ryan Kappedal, Scott M. Mackenzie, Dale W. Hailey, Trevor L. Hoffman, Thomas F. Schilling, and David W. Raible. 2015. "Robust Regeneration of Adult Zebrafish Lateral Line Hair Cells Reflects Continued Precursor Pool Maintenance." *Developmental Biology* 402 (2): 229–38. https://doi.org/10.1016/J.YDBIO.2015.03.019.
Cservenak, Melinda, David Keller, Viktor Kis, Emese A. Fazekas, Hanna Ollos, Andras H. Leko, Eva R. Szabo, et al. 2017. "A Thalamo-Hypothalamic Pathway That Activates Oxytocin Neurons in Social Contexts in Female Rats." *Endocrinology* 158 (2): 335–48. https://doi.org/10.1210/EN.2016-1645.
Cservenák, Melinda, Éva R Szabó, Ibolya Bodnár, András Lékó, Miklós Palkovits, György M Nagy, Ted B Usdin, and Arpád Dobolyi. 2013. "Thalamic Neuropeptide Mediating the Effects of Nursing on Lactation and Maternal Motivation." *Psychoneuroendocrinology* 38 (12): 3070–84. https://doi.org/10.1016/j.psyneuen.2013.09.004.
Cunningham, Christopher L., and Ulrich Müller. 2019. "Molecular Structure of the Hair Cell Mechanoelectrical Transduction Complex." *Cold Spring Harbor Perspectives in Medicine* 9 (5): a033167. https://doi.org/10.1101/cshperspect.a033167.
Czesny, Sergiusz J., Brian D. S. Graeb, and John M. Dettmers. 2005. "Ecological Consequences of Swim Bladder Noninflation for Larval Yellow Perch." *Transactions of the American Fisheries Society* 134 (4): 1011–20. https://doi.org/10.1577/t04-016.1.
Dekens, Marcus P S, Bruno M Fontinha, Miguel Gallach, Sandra Pfl€ Ugler, and Kristin Tessmar-Raible. 2022. "Melanopsin Elevates Locomotor Activity during the Wake State of the Diurnal Zebrafish." *EMBO Reports* 23 (5): e51528. https://doi.org/10.15252/EMBR.202051528.
Desban, Laura, Julian Roussel, Olivier Mirat, François-Xavier Lejeune, Ludovic Keiser, Nicolas Antoine Michalski, and Claire Wyart. 2022. "Lateral Line Hair Cells Integrate Mechanical and Chemical Cues to Orient Navigation." *BioRxiv*, September, 2022.08.31.505989. https://doi.org/10.1101/2022.08.31.505989.
Dijkgraaf, S. 1963. "The Functioning and Significance of the Lateral-Line Organs." *Biological Reviews* 38 (1): 51–105. https://doi.org/10.1111/J.1469-185X.1963.TB00654.X.
Dimitrov, Eugene L., Jonathan Kuo, Kenji Kohno, and Ted B. Usdin. 2013. "Neuropathic and Inflammatory Pain Are Modulated by Tuberoinfundibular Peptide of 39 Residues."

Proceedings of the National Academy of Sciences of the United States of America 110 (32): 13156–61. https://doi.org/10.1073/PNAS.1306342110.
Dimitrov, Eugene L., Emily Petrus, and Ted B. Usdin. 2010. "Tuberoinfundibular Peptide of 39 Residues (TIP39) Signaling Modulates Acute and Tonic Nociception." *Experimental Neurology* 226 (1): 68–83. https://doi.org/10.1016/j.expneurol.2010.08.004.
Dimitrov, Eugene, and Ted Björn Usdin. 2010. "Tuberoinfundibular Peptide of 39 Residues Modulates the Mouse Hypothalamic-Pituitary-Adrenal Axis via Paraventricular Glutamatergic Neurons." *The Journal of Comparative Neurology* 518 (21): 4375–94. https://doi.org/10.1002/cne.22462.
Dobolyi, Arpad, Melinda Cservenák, and Larry J. Young. 2018. "Thalamic Integration of Social Stimuli Regulating Parental Behavior and the Oxytocin System." *Frontiers in Neuroendocrinology* 51 (October): 102–15. https://doi.org/10.1016/J.YFRNE.2018.05.002.
Dobolyi, Arpád, Eugene Dimitrov, Miklós Palkovits, and Ted B. Usdin. 2012. "The Neuroendocrine Functions of the Parathyroid Hormone 2 Receptor." *Frontiers in Endocrinology* 3 (OCT). https://doi.org/10.3389/FENDO.2012.00121.
Domenici, Paolo. 2010. "Context-Dependent Variability in the Components of Fish Escape Response: Integrating Locomotor Performance and Behavior." *Journal of Experimental Zoology. Part A, Ecological Genetics and Physiology* 313 (2): 59–79. https://doi.org/10.1002/JEZ.580.
Domenici, Paolo, and Melina E. Hale. 2019. "Escape Responses of Fish: A Review of the Diversity in Motor Control, Kinematics and Behaviour." *Journal of Experimental Biology* 222 (18). https://doi.org/10.1242/JEB.166009/223422.
Doroshev, SI, JW Cornacchia, and K Hogan. 1981. "Initial Swim Bladder Inflation in the Larvae of Physoclistous Fishes and Its Importance for Larval Culture." *Rapports et Proces-Verbaux Des Réunions Cons. Int. Explor. Mer* 178: 495–500. https://www.ices.dk/sites/pub/Publication Reports/Marine Science Symposia/Phase 2/Rapport et Proces-Verbaux des Reunions - Volume 178 - 1981 - Partie 114 de 148.pdf.
Easter, Stephen S., and Gregory N. Nicola. 1996. "The Development of Vision in the Zebrafish (Danio Rerio)." *Developmental Biology* 180 (2): 646–63. https://doi.org/10.1006/dbio.1996.0335.
Eaton, R. C., and D. S. Emberley. 1991. "How Stimulus Direction Determines the Trajectory of the Mauthner-Initiated Escape Response in a Teleost Fish." *The Journal of Experimental Biology* 161: 469–87. https://doi.org/10.1242/JEB.161.1.469.
Ehrlich, David E., and David Schoppik. 2017. "Control of Movement Initiation Underlies the Development of Balance." *Current Biology* 27 (3): 334–44. https://doi.org/10.1016/j.cub.2016.12.003.
Ehrlich, David E, and David Schoppik. 2019. "A Primal Role for the Vestibular Sense in the Development of Coordinated Locomotion." *ELife* 8 (October). https://doi.org/10.7554/eLife.45839.
Ekström, Peter, and Hilmar Meissl. 1997. "The Pineal Organ of Teleost Fishes." *Reviews in Fish Biology and Fisheries* 7 (2): 199–284. https://doi.org/10.1023/A:1018483627058/METRICS.
Erickson, Timothy, Curtis R French, and Andrew J Waskiewicz. 2010. "Meis1 Specifies Positional Information in the Retina and Tectum to Organize the Zebrafish Visual System." *Neural Development* 5 (1): 22. https://doi.org/10.1186/1749-8104-5-22.

Erickson, Timothy, Clive P Morgan, Jennifer Olt, Katherine Hardy, Elisabeth Busch-Nentwich, Reo Maeda, Rachel Clemens, et al. 2017. “Integration of Tmc1/2 into the Mechanotransduction Complex in Zebrafish Hair Cells Is Regulated by Transmembrane O-Methyltransferase (Tomt).” *ELife* 6 (May). https://doi.org/10.7554/eLife.28474.

Erickson, Timothy, Itallia V. Pacentine, Alexandra Venuto, Rachel Clemens, and Teresa Nicolson. 2020. “The Lhfpl5 Ohnologs Lhfpl5a and Lhfpl5b Are Required for Mechanotransduction in Distinct Populations of Sensory Hair Cells in Zebrafish.” *Frontiers in Molecular Neuroscience* 12 (January): 320. https://doi.org/10.3389/FNMOL.2019.00320/BIBTEX.

Esterberg, Robert, Tor Linbo, Sarah B. Pickett, Patricia Wu, Henry C. Ou, Edwin W. Rubel, and David W. Raible. 2016. “Mitochondrial Calcium Uptake Underlies ROS Generation during Aminoglycoside-Induced Hair Cell Death.” *Journal of Clinical Investigation* 126 (9): 3556–66. https://doi.org/10.1172/JCI84939.

Evans, H M, and G C C Damant. 1928. “Observations on the Physiology of the Swim Bladder in Cyprinoid Fishes.” *Journal of Experimental Biology* 6 (1): 42–55.

Fame, Ryann M, Carole Brajon, and Alain Ghysen. 2006. “Second-Order Projection from the Posterior Lateral Line in the Early Zebrafish Brain.” *Neural Development* 1 (1): 4. https://doi.org/10.1186/1749-8104-1-4.

Fänge, R. 1983. “Gas Exchange in Fish Swim Bladder.” *Reviews of Physiology, Biochemistry and Pharmacology* 97: 111–58. https://doi.org/10.1007/BFB0035347/COVER.

Faucher, Karine, Eric Parmentier, Christophe Becco, Nicolas Vandewalle, and Pierre Vandewalle. 2010. “Fish Lateral System Is Required for Accurate Control of Shoaling Behaviour.” *Animal Behaviour* 79 (3): 679–87. https://doi.org/10.1016/J.ANBEHAV.2009.12.020.

Favre-Bulle, Itia A., Gilles Vanwalleghem, Michael A. Taylor, Halina Rubinsztein-Dunlop, and Ethan K. Scott. 2018. “Cellular-Resolution Imaging of Vestibular Processing across the Larval Zebrafish Brain.” *Current Biology* 28 (23): 3711-3722.e3. https://doi.org/10.1016/j.cub.2018.09.060.

Fegley, D. B., A. Holmes, T. Riordan, C. A. Faber, J. R. Weiss, S. Ma, S. Batkai, et al. 2008. “Increased Fear- and Stress-Related Anxiety-like Behavior in Mice Lacking Tuberoinfundibular Peptide of 39 Residues.” *Genes, Brain and Behavior* 7 (8): 933–42. https://doi.org/10.1111/J.1601-183X.2008.00432.X.

Fernandes, António M., Kandice Fero, Aristides B. Arrenberg, Sadie A. Bergeron, Wolfgang Driever, and Harold A. Burgess. 2012. “Deep Brain Photoreceptors Control Light-Seeking Behavior in Zebrafish Larvae.” *Current Biology : CB* 22 (21): 2042–47. https://doi.org/10.1016/J.CUB.2012.08.016.

Fernandes, António M., Kandice Fero, Wolfgang Driever, and Harold A. Burgess. 2013. “Enlightening the Brain: Linking Deep Brain Photoreception with Behavior and Physiology.” *BioEssays : News and Reviews in Molecular, Cellular and Developmental Biology* 35 (9): 775. https://doi.org/10.1002/BIES.201300034.

Fero, Kandice, Tohei Yokogawa, and Harold A. Burgess. 2011. “The Behavioral Repertoire of Larval Zebrafish.” *Neuromethods* 52: 249–91. https://doi.org/10.1007/978-1-60761-922-2_12.

Fetcho, Joseph R., Shin ichi Higashijima, and David L. McLean. 2008. “Zebrafish and Motor Control over the Last Decade.” *Brain Research Reviews* 57 (1): 86. https://doi.org/10.1016/J.BRAINRESREV.2007.06.018.

Filosa, Alessandro, Alison J. Barker, Marco Dal Maschio, and Herwig Baier. 2016. “Feeding State Modulates Behavioral Choice and Processing of Prey Stimuli in the Zebrafish Tectum.” *Neuron* 90 (3): 596–608. https://doi.org/10.1016/J.NEURON.2016.03.014.

Finney, Jessica L., George N. Robertson, Chantelle A.S. McGee, Frank M. Smith, and Roger P. Croll. 2006. “Structure and Autonomic Innervation of the Swim Bladder in the Zebrafish (Danio Rerio).” *The Journal of Comparative Neurology* 495 (5): 587–606. https://doi.org/10.1002/cne.20948.

Fischer, Stefan, Evelyne Oberhummer, Filipa Cunha-Saraiva, Nina Gerber, and Barbara Taborsky. 2017. “Smell or Vision? The Use of Different Sensory Modalities in Predator Discrimination.” *Behavioral Ecology and Sociobiology* 71 (10): 1–10. https://doi.org/10.1007/S00265-017-2371-8/FIGURES/4.

Forge, A, and J Schacht. 2000. “Aminoglycoside Antibiotics.” *Audiology & Neuro-Otology* 5 (1): 3–22. https://doi.org/10.1159/000013861.

Forsatkar, Mohammad Navid, Omid Safari, and Cristiano Boiti. 2017. “Effects of Social Isolation on Growth, Stress Response, and Immunity of Zebrafish.” *Acta Ethologica* 20 (3): 255–61. https://doi.org/10.1007/S10211-017-0270-7/METRICS.

Förster, Dominique, Thomas O. Helmbrecht, Duncan S. Mearns, Linda Jordan, Nouwar Mokayes, and Herwig Baier. 2020. “Retinotectal Circuitry of Larval Zebrafish Is Adapted to Detection and Pursuit of Prey.” *ELife* 9 (October): 1–26. https://doi.org/10.7554/ELIFE.58596.

Free, Brian A., Matthew J. McHenry, and Derek A. Paley. 2019. “Probabilistic Analytical Modelling of Predator–Prey Interactions in Fishes.” *Journal of The Royal Society Interface* 16 (150): 20180873. https://doi.org/10.1098/rsif.2018.0873.

Friedmann, Bruce R., and Kimberly M. Shutty. 1999. “Effect of Timing of Oil Film Removal and First Feeding on Swim Bladder Inflation Success among Intensively Cultured Striped Bass Larvae.” *North American Journal of Aquaculture* 61 (1): 43–46. https://doi.org/10.1577/1548-8454(1999)061<0043:EOTOOF>2.0.CO;2.

Friedmann, Drew, Adam Hoagland, Shai Berlin, and Ehud Y. Isacoff. 2015. “A Spinal Opsin Controls Early Neural Activity and Drives a Behavioral Light Response.” *Current Biology : CB* 25 (1): 69. https://doi.org/10.1016/J.CUB.2014.10.055.

Furukawa, Satoshi, Yuichiro Machida, Kazuya Takeuchi, Yumiko Hoshikawa, and Kota Irie. 2022. “Failure to Gulp Surface Air Induces Swim Bladder Adenomas in Japanese Medaka (Oryzias Latipes).” *Journal of Toxicologic Pathology* 35 (3): 237–46. https://doi.org/10.1293/TOX.2022-0030.

Gandhi, Avni V, Eric A Mosser, Grigorios Oikonomou, and David A Prober Correspondence. 2015. “Melatonin Is Required for the Circadian Regulation of Sleep.” *Neuron* 85. https://doi.org/10.1016/j.neuron.2015.02.016.

Gemmer, Anja, Kristina Mirkes, Lukas Anneser, Tim Eilers, Caroline Kibat, Ajay Mathuru, Soojin Ryu, and Erin Schuman. 2022. “Oxytocin Receptors Influence the Development and Maintenance of Social Behavior in Zebrafish (Danio Rerio).” *Scientific Reports* 12 (1): 4322. https://doi.org/10.1038/S41598-022-07990-Y.

Gerlach, Gabriele, Andrea Hodgins-Davis, Carla Avolio, and Celia Schunter. 2008. “Kin Recognition in Zebrafish: A 24-Hour Window for Olfactory Imprinting.” *Proceedings of the Royal Society B: Biological Sciences* 275 (1647): 2165–70. https://doi.org/10.1098/RSPB.2008.0647.

Gerlach, Gabriele, and Mario F. Wullimann. 2021. “Neural Pathways of Olfactory Kin Imprinting and Kin Recognition in Zebrafish.” *Cell and Tissue Research* 383 (1): 273. https://doi.org/10.1007/S00441-020-03378-4.

Gestri, Gaia, Brian A. Link, and Stephan C.F. Neuhauss. 2012. “The Visual System of Zebrafish and Its Use to Model Human Ocular Diseases.” *Developmental Neurobiology* 72 (3): 302. https://doi.org/10.1002/DNEU.20919.

Ghysen, Alain, and Christine Dambly-Chaudière. 2004. “Development of the Zebrafish Lateral Line.” *Current Opinion in Neurobiology*. Elsevier Ltd. https://doi.org/10.1016/j.conb.2004.01.012.

Ghysen, Alain, and Christine Dambly-Chaudière. 2007. “The Lateral Line Microcosmos.” *Genes and Development*. Cold Spring Harbor Laboratory Press. https://doi.org/10.1101/gad.1568407.

Gibbs, Melissa Ann. 2004. “Lateral Line Receptors: Where Do They Come from Developmentally and Where Is Our Research Going?” *Brain, Behavior and Evolution* 64 (3): 163–81. https://doi.org/10.1159/000079745.

Gillespie, Peter G. 1995. “Molecular Machinery of Auditory and Vestibular Transduction.” *Current Opinion in Neurobiology* 5 (4): 449–55. https://doi.org/10.1016/0959-4388(95)80004-2.

Gisbert, Enric, and Patrick Williot. 1997. “Larval Behaviour and Effect of the Timing of Initial Feeding on Growth and Survival of Siberian Sturgeon (Acipenser Baeri) Larvae under Small Scale Hatchery Production.” *Aquaculture* 156 (1–2): 63–76. https://doi.org/10.1016/S0044-8486(97)00086-0.

Goolish, E. M., and K. Okutake. 1999. “Lack of Gas Bladder Inflation by the Larvae of Zebrafish in the Absence of an Air-Water Interface.” *Journal of Fish Biology* 55 (5): 1054–63. https://doi.org/10.1111/j.1095-8649.1999.tb00740.x.

Groneberg, Antonia H., João C. Marques, A. Lucas Martins, Ruth Diez del Corral, Gonzalo G. de Polavieja, and Michael B. Orger. 2020. “Early-Life Social Experience Shapes Social Avoidance Reactions in Larval Zebrafish.” *Current Biology* 30 (20): 4009-4021.e4. https://doi.org/10.1016/J.CUB.2020.07.088.

Grubb, Matthew S., and Ian D. Thompson. 2004. “The Influence of Early Experience on the Development of Sensory Systems.” *Current Opinion in Neurobiology* 14 (4): 503–12. https://doi.org/10.1016/J.CONB.2004.06.006.

Haddon, Catherine, and Julian Lewis. 1996. “Early Ear Development in the Embryo of the Zebrafish, Danio Rerio.” *Journal of Comparative Neurology* 365 (1): 113–28. https://doi.org/10.1002/(SICI)1096-9861(19960129)365:1<113::AID-CNE9>3.0.CO;2-6.

Häder, D. P. 1999. “Gravitaxis in Unicellular Microorganisms.” *Advances in Space Research : The Official Journal of the Committee on Space Research (COSPAR)* 24 (6): 843–50. https://doi.org/10.1016/S0273-1177(99)00965-5.

Haehnel-Taguchi, Melanie, António M. Fernandes, Margit Böhler, Ina Schmitt, Lena Tittel, and Wolfgang Driever. 2018. “Projections of the Diencephalospinal Dopaminergic System to Peripheral Sense Organs in Larval Zebrafish (Danio Rerio).” *Frontiers in Neuroanatomy* 12 (March): 20. https://doi.org/10.3389/FNANA.2018.00020/BIBTEX.

Hale, Melina E., Hilary R. Katz, Martin Y. Peek, and Rachel T. Fremont. 2016. “Neural Circuits That Drive Startle Behavior, with a Focus on the Mauthner Cells and Spiral Fiber Neurons of Fishes.” *Journal of Neurogenetics* 30 (2): 89–100. https://doi.org/10.1080/01677063.2016.1182526.

Han, Eunjung, Kyoung Ho Oh, Saemi Park, Yoon Chan Rah, Hae Chul Park, Soonil Koun, and June Choi. 2020. "Analysis of Behavioral Changes in Zebrafish (Danio Rerio) Larvae Caused by Aminoglycoside-Induced Damage to the Lateral Line and Muscles." *NeuroToxicology* 78 (May): 134–42. https://doi.org/10.1016/j.neuro.2020.03.005.

Han, Jian, Kechun Liu, Rongchun Wang, Yun Zhang, and Bingsheng Zhou. 2019. "Exposure to Cadmium Causes Inhibition of Otolith Development and Behavioral Impairment in Zebrafish Larvae." *Aquatic Toxicology* 214 (September): 105236. https://doi.org/10.1016/J.AQUATOX.2019.105236.

Hansen, Anne, and Klaus Reutter. 2004. "Chemosensory Systems in Fish: Structural, Functional and Ecological Aspects." *The Senses of Fish*, 55–89. https://doi.org/10.1007/978-94-007-1060-3_3.

Hansen, James A., James D. Rose, Robert A. Jenkins, Kenneth G. Gerow, and Harold L. Bergman. 1999. "Chinook Salmon *(Oncorhynchus Tshawytscha)* and Rainbow Trout *(Oncorhynchus Mykiss)* Exposed to Copper: Neurophysiological and Histological Effects on the Olfactory System." *Environmental Toxicology and Chemistry* 18 (9): 1979–91. https://doi.org/10.1002/etc.5620180917.

Hardy, Katherine, Ana E. Amariutei, Francesca de Faveri, Aenea Hendry, Walter Marcotti, and Federico Ceriani. 2021. "Functional Development and Regeneration of Hair Cells in the Zebrafish Lateral Line." *The Journal of Physiology* 599 (16): 3913–36. https://doi.org/10.1113/JP281522.

Harris, Julie A., Alan G. Cheng, Lisa L. Cunningham, Glen MacDonald, David W. Raible, and Edwin W. Rubel. 2003. "Neomycin-Induced Hair Cell Death and Rapid Regeneration in the Lateral Line of Zebrafish (Danio Rerio)." *JARO: Journal of the Association for Research in Otolaryngology* 4 (2): 219. https://doi.org/10.1007/S10162-002-3022-X.

Hatta, Kohei, and Henri Korn. 1998. "Physiological Properties of the Mauthner System in the Adult Zebrafish." *Journal of Comparative Neurology* 395 (4): 493–509. https://doi.org/10.1002/(SICI)1096-9861(19980615)395:4.

Haug, Marion F., Oliver Biehlmaier, Kaspar P. Mueller, and Stephan C.F. Neuhauss. 2010. "Visual Acuity in Larval Zebrafish: Behavior and Histology." *Frontiers in Zoology* 7 (March): 8. https://doi.org/10.1186/1742-9994-7-8.

Heap, Lucy A.L., Gilles Vanwalleghem, Andrew W. Thompson, Itia A. Favre-Bulle, and Ethan K. Scott. 2018. "Luminance Changes Drive Directional Startle through a Thalamic Pathway." *Neuron* 99 (2): 293-301.e4. https://doi.org/10.1016/J.NEURON.2018.06.013.

Helmbrecht, Thomas O., Marco dal Maschio, Joseph C. Donovan, Styliani Koutsouli, and Herwig Baier. 2018. "Topography of a Visuomotor Transformation." *Neuron* 100 (6): 1429-1445.e4. https://doi.org/10.1016/J.NEURON.2018.10.021.

Hernández, Pedro P., Virginia Moreno, Francisco A. Olivari, and Miguel L. Allende. 2006. "Sub-Lethal Concentrations of Waterborne Copper Are Toxic to Lateral Line Neuromasts in Zebrafish (Danio Rerio)." *Hearing Research* 213 (1–2): 1–10. https://doi.org/10.1016/j.heares.2005.10.015.

Hernández, Pedro P., Francisco A. Olivari, Andrés F. Sarrazin, Pablo C. Sandoval, and Miguel L. Allende. 2007. "Regeneration in Zebrafish Lateral Line Neuromasts: Expression of the Neural Progenitor Cell Marker Sox2 and Proliferation-Dependent and-Independent Mechanisms of Hair Cell Renewal." *Developmental Neurobiology* 67 (5): 637–54. https://doi.org/10.1002/dneu.20386.

Hinz, Cornelia, Simon Kobbenbring, Sigrid Kress, Lauren Sigman, Anke Müller, and Gabriele Gerlach. 2013. “Kin Recognition in Zebrafish, Danio Rerio, Is Based on Imprinting on Olfactory and Visual Stimuli.” *Animal Behaviour* 85 (5): 925–30. https://doi.org/10.1016/J.ANBEHAV.2013.02.010.

Hirata, Yoshiro, Katsuyuki Hamasaki, Akihiko Imai, Kazuhisa Teruya, Takashi Iwasaki, Kazuhisa Hamada, And Keiichi Mushiake. 2009. “Effects of Different Photoperiods and Water Temperatures on Survival, Growth, Feeding and Initial Swim Bladder Inflation of Greater Amberjack Seriola Dumerili Larvae.” *Nippon Suisan Gakkaishi* 75 (6): 995–1003. https://doi.org/10.2331/suisan.75.995.

Hirose, Keiko, David M. Hockenbery, and Edwin W. Rubel. 1997. “Reactive Oxygen Species in Chick Hair Cells after Gentamicin Exposure in Vitro.” *Hearing Research* 104 (1–2): 1–14. https://doi.org/10.1016/S0378-5955(96)00169-4.

Hirsch, Jerry. 1959. “Studies in Experimental Behavior Genetics: II. Individual Differences in Geotaxis as a Function of Chromosome Variations in Synthesized Drosophila Populations.” *Journal of Comparative and Physiological Psychology* 52 (3): 304–8. https://doi.org/10.1037/H0043498.

Honryo, Tomoki, Teruyoshi Tanaka, Angel Guillen, Jeanne B. Wexler, Amado Cano, Daniel Margulies, Vernon P. Scholey, Maria S. Stein, and Yoshifumi Sawada. 2016. “Effect of Water Surface Condition on Survival, Growth and Swim Bladder Inflation of Yellowfin Tuna, Thunnus Albacares (Temminck and Schlegel), Larvae.” *Aquaculture Research* 47 (6): 1832–40. https://doi.org/10.1111/ARE.12641.

Hudspeth, A. J. 1989. “Mechanoelectrical Transduction by Hair Cells of the Bullfrog’ Sacculus.” *Progress in Brain Research* 80 (C): 129–35. https://doi.org/10.1016/S0079-6123(08)62206-2.

Ingle, D. 1973. “Evolutionary Perspectives on the Function of the Optic Tectum.” *Brain, Behavior and Evolution* 8 (3): 211–37. https://doi.org/10.1159/000124355.

Isa, Tadashi, Emmanuel Marquez-Legorreta, Sten Grillner, and Ethan K. Scott. 2021. “The Tectum/Superior Colliculus as the Vertebrate Solution for Spatial Sensory Integration and Action.” *Current Biology* 31 (11): R741–62. https://doi.org/10.1016/j.cub.2021.04.001.

Ito, Masao. 2006. “Cerebellar Circuitry as a Neuronal Machine.” *Progress in Neurobiology* 78 (3–5): 272–303. https://doi.org/10.1016/J.PNEUROBIO.2006.02.006.

Johansen, E M. 1924. “The Interfacial Tension between Petroleum Products and Water.” *Industrial & Engineering Chemistry* 16 (2): 132–35.

Johnson, Nicholas S., Scott M. Miehls, Alex J. Haro, and C. Michael Wagner. 2019. “Push and Pull of Downstream Moving Juvenile Sea Lamprey (Petromyzon Marinus) Exposed to Chemosensory and Light Cues.” *Conservation Physiology* 7 (1). https://doi.org/10.1093/CONPHYS/COZ080.

Jones, F. R. Harden, and N. B. Marshall. 1953. “The Structure and Functions of the Teleostean Swimbladder.” *Biological Reviews* 28 (1): 16–82. https://doi.org/10.1111/j.1469-185X.1953.tb01370.x.

Jundi, Dania, Jean Pierre Coutanceau, Erika Bullier, Soumaiya Imarraine, Ziad Fajloun, and Elim Hong. 2023. “Expression of Olfactory Receptor Genes in Non-Olfactory Tissues in the Developing and Adult Zebrafish.” *Scientific Reports 2023 13:1* 13 (1): 1–10. https://doi.org/10.1038/s41598-023-30895-3.

Jung, Julie, Shirley J Serrano-Rojas, and Karen M Warkentin. 2020. “Multimodal Mechanosensing Enables Treefrog Embryos to Escape Egg-Predators.” *The Journal of Experimental Biology* 223 (Pt 24). https://doi.org/10.1242/jeb.236141.

Kamikouchi, Azusa, Hidehiko K Inagaki, Thomas Effertz, Oliver Hendrich, André Fiala, Martin C Göpfert, and Kei Ito. 2009. “The Neural Basis of Drosophila Gravity-Sensing and Hearing.” *Nature* 457. https://doi.org/10.1038/nature07810.

Kappel, Johannes M., Dominique Förster, Katja Slangewal, Inbal Shainer, Fabian Svara, Joseph C. Donovan, Shachar Sherman, Michał Januszewski, Herwig Baier, and Johannes Larsch. 2022. “Visual Recognition of Social Signals by a Tectothalamic Neural Circuit.” *Nature 2022 608:7921* 608 (7921): 146–52. https://doi.org/10.1038/s41586-022-04925-5.

Karpenko, Sophia, Sebastien Wolf, Julie Lafaye, Guillaume Le Goc, Thomas Panier, Volker Bormuth, Raphaël Candelier, and Georges Debrégeas. 2020. “From Behavior to Circuit Modeling of Light-Seeking Navigation in Zebrafish Larvae.” *ELife* 9 (January). https://doi.org/10.7554/ELIFE.52882.

Kay, Jeremy N., Karin C. Finger-Baier, Tobias Roeser, Wendy Staub, and Herwig Baier. 2001. “Retinal Ganglion Cell Genesis Requires Lakritz, a Zebrafish Atonal Homolog.” *Neuron* 30 (3): 725–36. https://doi.org/10.1016/S0896-6273(01)00312-9.

Keller, Dávid, Tamás Láng, Melinda Cservenák, Gina Puska, János Barna, Veronika Csillag, Imre Farkas, et al. 2022. “A Thalamo-Preoptic Pathway Promotes Social Grooming in Rodents.” *Current Biology : CB* 32 (21): 4593-4606.e8. https://doi.org/10.1016/j.cub.2022.08.062.

Kermen, Florence, Luis M. Franco, Cameron Wyatt, and Emre Yaksi. 2013. “Neural Circuits Mediating Olfactory-Driven Behavior in Fish.” *Frontiers in Neural Circuits* 7 (MAR): 62. https://doi.org/10.3389/FNCIR.2013.00062/BIBTEX.

Kimmel, Charles B., William W. Ballard, Seth R. Kimmel, Bonnie Ullmann, and Thomas F. Schilling. 1995. “Stages of Embryonic Development of the Zebrafish.” *Developmental Dynamics* 203 (3): 253–310. https://doi.org/10.1002/aja.1002030302.

Kindt, Katie S., Gabriel Finch, and Teresa Nicolson. 2012. “Kinocilia Mediate Mechanosensitivity in Developing Zebrafish Hair Cells.” *Developmental Cell* 23 (2): 329–41. https://doi.org/10.1016/j.devcel.2012.05.022.

King, A. J., J. W.H. Schnupp, S. Carlile, A. L. Smith, and I. D. Thompson. 1996. “The Development of Topographically-Aligned Maps of Visual and Auditory Space in the Superior Colliculus.” *Progress in Brain Research* 112: 335–50. https://doi.org/10.1016/S0079-6123(08)63340-3.

Kitajima, Chikara, Takeshi Watanabe, Yasuo Tsukashima, and Shiro Fujita. 1994. “Lordotic Deformation and Abnormal Development of Swim Bladders in Some Hatchery-Bred Marine Physoclistous Fish in Japan.” *Journal of the World Aquaculture Society* 25 (1): 64–77. https://doi.org/10.1111/j.1749-7345.1994.tb00806.x.

Knogler, Laura D., Andreas M. Kist, and Ruben Portugues. 2019. “Motor Context Dominates Output from Purkinje Cell Functional Regions during Reflexive Visuomotor Behaviours.” *ELife* 8. https://doi.org/10.7554/ELIFE.42138.

Knogler, Laura D., Daniil A. Markov, Elena I. Dragomir, Vilim Štih, and Ruben Portugues. 2017. “Sensorimotor Representations in Cerebellar Granule Cells in Larval Zebrafish Are Dense, Spatially Organized, and Non-Temporally Patterned.” *Current Biology* 27 (9): 1288–1302. https://doi.org/10.1016/j.cub.2017.03.029.

Kohashi, Tsunehiko, Natsuyo Nakata, and Yoichi Oda. 2012. “Effective Sensory Modality Activating an Escape Triggering Neuron Switches during Early Development in Zebrafish.” *The Journal of Neuroscience* 32 (17): 5810. https://doi.org/10.1523/JNEUROSCI.6169-11.2012.

Kokel, David, Jennifer Bryan, Christian Laggner, Rick White, Chung Yan J. Cheung, Rita Mateus, David Healey, et al. 2010. “Rapid Behavior—Based Identification of Neuroactive Small Molecules in the Zebrafish.” *Nature Chemical Biology* 6 (3): 231. https://doi.org/10.1038/NCHEMBIO.307.

Kolb, Bryan, and Ian Q. Whishaw. 2003. “Brain Plasticity and Behavior.” *Http://Dx.Doi.Org/10.1146/Annurev.Psych.49.1.43* 49 (November): 43–64. https://doi.org/10.1146/ANNUREV.PSYCH.49.1.43.

Korn, Henri, and Donald S. Faber. 2005. “The Mauthner Cell Half a Century Later: A Neurobiological Model for Decision-Making?” *Neuron* 47 (1): 13–28. https://doi.org/10.1016/J.NEURON.2005.05.019.

Korsøen, Øyvind J., Jan Erik Fosseidengen, Tore S. Kristiansen, Frode Oppedal, Samantha Bui, and Tim Dempster. 2012. “Atlantic Salmon (Salmo Salar L.) in a Submerged Sea-Cage Adapt Rapidly to Re-Fill Their Swim Bladders in an Underwater Air Filled Dome.” *Aquacultural Engineering* 51 (November): 1–6. https://doi.org/10.1016/J.AQUAENG.2012.04.001.

Kravitz, Edward A., and Robert Huber. 2003. “Aggression in Invertebrates.” *Current Opinion in Neurobiology* 13 (6): 736–43. https://doi.org/10.1016/J.CONB.2003.10.003.

Kros, Corné J., and Peter S. Steyger. 2018. “Aminoglycoside- and Cisplatin-Induced Ototoxicity: Mechanisms and Otoprotective Strategies.” *Cold Spring Harbor Perspectives in Medicine* 9 (11): a033548. https://doi.org/10.1101/CSHPERSPECT.A033548.

Kubin, Leszek, George F. Alheid, Edward J. Zuperku, and Donald R. McCrimmon. 2006. “Central Pathways of Pulmonary and Lower Airway Vagal Afferents.” *Journal of Applied Physiology (Bethesda, Md. : 1985)* 101 (2): 618. https://doi.org/10.1152/JAPPLPHYSIOL.00252.2006.

Kurata, Michio, Manabu Seoka, Yoshizumi Nakagawa, Yasunori Ishibashi, Hidemi Kumai, and Yoshifumi Sawada. 2012. “Promotion of Initial Swimbladder Inflation in Pacific Bluefin Tuna, Thunnus Orientalis (Temminck and Schlegel), Larvae.” *Aquaculture Research* 43 (9): 1296–1305. https://doi.org/10.1111/J.1365-2109.2011.02933.X.

Kurata, Michio, Yoshiki Tamura, Tomoki Honryo, Yasunori Ishibashi, and Yoshifumi Sawada. 2017. “Effects of Photoperiod and Night-Time Aeration Rate on Swim Bladder Inflation and Survival in Pacific Bluefin Tuna, Thunnus Orientalis (Temminck & Schlegel), Larvae.” *Aquaculture Research* 48 (8): 4486–4502. https://doi.org/10.1111/are.13274.

Kwan, Kristen M, Esther Fujimoto, Clemens Grabher, Benjamin D Mangum, Melissa E Hardy, Douglas S Campbell, John M Parant, H Joseph Yost, John P Kanki, and Chi-Bin Chien. 2007. “The Tol2kit: A Multisite Gateway-Based Construction Kit for Tol2 Transposon Transgenesis Constructs.” *Developmental Dynamics : An Official Publication of the American Association of Anatomists* 236 (11): 3088–99. https://doi.org/10.1002/dvdy.21343.

LaBuda, Christopher J, Arpad Dobolyi, and Ted B Usdin. 2004. “Tuberoinfundibular Peptide of 39 Residues Produces Anxiolytic and Antidepressant Actions.” *Neuroreport* 15 (5): 881–85. https://doi.org/10.1097/00001756-200404090-00030.

LaBuda, Christopher J., and Ted B. Usdin. 2004. “Tuberoinfundibular Peptide of 39 Residues Decreases Pain-Related Affective Behavior.” *NeuroReport* 15 (11): 1779–82. https://doi.org/10.1097/01.wnr.0000134849.63755.15.

Lacoste, Alix M.B., David Schoppik, Drew N. Robson, Martin Haesemeyer, Ruben Portugues, Jennifer M. Li, Owen Randlett, Caroline L. Wee, Florian Engert, and Alexander F. Schier. 2015. “A Convergent and Essential Interneuron Pathway for Mauthner-Cell-Mediated Escapes.” *Current Biology* 25 (11): 1526–34. https://doi.org/10.1016/J.CUB.2015.04.025.

Landin, Jenny, Daniel Hovey, Bo Xu, David Lagman, Anna Zettergren, Dan Larhammar, Petronella Kettunen, and Lars Westberg. 2020. “Oxytocin Receptors Regulate Social Preference in Zebrafish.” *Scientific Reports 2020 10:1* 10 (1): 1–12. https://doi.org/10.1038/s41598-020-61073-4.

Laurens, Jean, Hui Meng, and Dora E. Angelaki. 2013. “Neural Representation of Orientation Relative to Gravity in the Macaque Cerebellum.” *Neuron* 80 (6): 1508–18. https://doi.org/10.1016/J.NEURON.2013.09.029.

Ledebur, J. Frhr von., and W. Wunder. 1937. “Beiträge Zur Physiologie Der Schwimmblase Der Fische.” *Zeitschrift Für Vergleichende Physiologie 1937 25:2* 25 (2): 149–55. https://doi.org/10.1007/BF00339307.

Ledent, Valérie. 2002. “Postembryonic Development of the Posterior Lateral Line in Zebrafish.” *Development (Cambridge, England)* 129 (3): 597–604. http://www.ncbi.nlm.nih.gov/pubmed/11830561.

Li, Lei. 2019. “Sensory Integration: Cross-Modal Communication Between the Olfactory and Visual Systems in Zebrafish.” *Chemical Senses* 44 (6): 351–56. https://doi.org/10.1093/CHEMSE/BJZ022.

Li, Lei, and John E. Dowling. 2000. “Disruption of the Olfactoretinal Centrifugal Pathway May Relate to the Visual System Defect in Night Blindness BMutant Zebrafish.” *Journal of Neuroscience* 20 (5): 1883–92. https://doi.org/10.1523/JNEUROSCI.20-05-01883.2000.

Liao, James C. 2006. “The Role of the Lateral Line and Vision on Body Kinematics and Hydrodynamic Preference of Rainbow Trout in Turbulent Flow.” *The Journal of Experimental Biology* 209 (Pt 20): 4077–90. https://doi.org/10.1242/jeb.02487.

Liao, James C., and Melanie Haehnel. 2012. “Physiology of Afferent Neurons in Larval Zebrafish Provides a Functional Framework for Lateral Line Somatotopy.” *Journal of Neurophysiology* 107 (10). /pmc/articles/PMC3362281/?report=abstract%0Ahttps://www.ncbi.nlm.nih.gov/pmc/articles/PMC3362281/.

Lin, Allia, Efrén Efrén´ Efrén´alvarez-Salvado, Nikola Milicic, Nimish Pujara, and David E Ehrlich. 2023. “Hatchling Fish Disperse Using an Efficient Multisensory Strategy.” *BioRxiv*, January, 2023.01.24.525385. https://doi.org/10.1101/2023.01.24.525385.

Linbo, Tiffany L., Carla M. Stehr, John P. Incardona, and Nathaniel L. Scholz. 2006. “Dissolved Copper Triggers Cell Death in the Peripheral Mechanosensory System of Larval Fish.” *Environmental Toxicology and Chemistry* 25 (2): 597–603. https://doi.org/10.1897/05-241R.1.

Lindsay, Sara M., and Richard G. Vogt. 2004. “Behavioral Responses of Newly Hatched Zebrafish (Danio Rerio) to Amino Acid Chemostimulants.” *Chem. Senses* 29 (2): 93–100. https://doi.org/10.1093/chemse/bjh009.

Lindsey, Benjamin W., Tristan C. Dumbarton, Stephen J. Moorman, Frank M. Smith, and Roger P. Croll. 2011. “Effects of Simulated Microgravity on the Development of the Swimbladder

and Buoyancy Control in Larval Zebrafish (Danio Rerio).” *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology* 315A (5): 302–13. https://doi.org/10.1002/jez.677.

Lindsey, Benjamin W., Frank M. Smith, and Roger P. Croll. 2010. “From Inflation to Flotation: Contribution of the Swimbladder to Whole-Body Density and Swimming Depth during Development of the Zebrafish (Danio Rerio).” *Zebrafish* 7 (1): 85–96. https://doi.org/10.1089/zeb.2009.0616.

Liu, Zhikai, David G.C. Hildebrand, Joshua L. Morgan, Nicholas Slimmon, and Martha W. Bagnall. 2021. “The Organization of the Gravity-Sensing System in Zebrafish.” *BioRxiv*, July, 2021.07.09.451839. https://doi.org/10.1101/2021.07.09.451839.

Lloyd, Evan, Courtney Olive, Bethany A. Stahl, James B. Jaggard, Paloma Amaral, Erik R. Duboué, and Alex C. Keene. 2018. “Evolutionary Shift towards Lateral Line Dependent Prey Capture Behavior in the Blind Mexican Cavefish.” *Developmental Biology* 441 (2): 328–37. https://doi.org/10.1016/J.YDBIO.2018.04.027.

Longo, Sarah, Mark Riccio, and Amy R. McCune. 2013. “Homology of Lungs and Gas Bladders: Insights from Arterial Vasculature.” *Journal of Morphology* 274 (6): 687–703. https://doi.org/10.1002/JMOR.20128.

Lucon-Xiccato, Tyrone, Giuseppe Di Mauro, Angelo Bisazza, and Cristiano Bertolucci. 2020. “Alarm Cue-Mediated Response and Learning in Zebrafish Larvae.” *Behavioural Brain Research* 380 (February): 112446. https://doi.org/10.1016/J.BBR.2019.112446.

Maaswinkel, Hans, and Lei Li. 2003. “Olfactory Input Increases Visual Sensitivity in Zebrafish: A Possible Function for the Terminal Nerve and Dopaminergic Interplexiform Cells.” *Journal of Experimental Biology* 206 (13): 2201–9. https://doi.org/10.1242/JEB.00397.

Mackenzie, Scott M., and David W. Raible. 2012. “Proliferative Regeneration of Zebrafish Lateral Line Hair Cells after Different Ototoxic Insults.” *PLoS ONE* 7 (10): 47257. https://doi.org/10.1371/JOURNAL.PONE.0047257.

Maeda, Reo, Itallia v. Pacentine, Timothy Erickson, and Teresa Nicolson. 2017. “Functional Analysis of the Transmembrane and Cytoplasmic Domains of Pcdh15a in Zebrafish Hair Cells.” *The Journal of Neuroscience* 37 (12): 3231–45. https://doi.org/10.1523/JNEUROSCI.2216-16.2017.

Martin-Robichaud, D J, and R H Peterson. 1998. “Effects of Light Intensity, Tank Colour and Photoperiod on Swimbladder Inflation Success in Larval Striped Bass, Morone Saxatilis (Walbaum).” *Aquaculture Research* 29 (8): 539–47. https://doi.org/10.1046/j.1365-2109.1998.00234.x.

McCormick, Catherine A. 1981. “Central Projections of the Lateral Line and Eighth Nerves in the Bowfin,Amia Calva.” *The Journal of Comparative Neurology* 197 (1): 1–15. https://doi.org/10.1002/cne.901970102.

McCormick, Catherine A. 1989. “Central Lateral Line Mechanosensory Pathways in Bony Fish.” *The Mechanosensory Lateral Line*, 341–64. https://doi.org/10.1007/978-1-4612-3560-6_17.

McCormick, Catherine A., and Dorothy V. Hernandez. 1996. “Connections of Octaval and Lateral Line Nuclei of the Medulla in the Goldfish, Including the Cytoarchitecture of the Secondary Octaval Population in Goldfish and Catfish.” *Brain, Behavior and Evolution* 47 (3): 113–37. https://doi.org/10.1159/000113232.

McCune, Amy R., and Rose L. Carlson. 2004. “Twenty Ways to Lose Your Bladder: Common Natural Mutants in Zebrafish and Widespread Convergence of Swim Bladder Loss among

Teleost Fishes." *Evolution and Development* 6 (4): 246–59. https://doi.org/10.1111/J.1525-142X.2004.04030.X.
McHenry, M.J., K.E. Feitl, J.A. Strother, and W.J. Van Trump. 2009. "Larval Zebrafish Rapidly Sense the Water Flow of a Predator's Strike." *Biology Letters* 5 (4). https://doi.org/10.1098/rsbl.2009.0048.
Medan, Violeta, and Thomas Preuss. 2014. "The Mauthner-Cell Circuit of Fish as a Model System for Startle Plasticity." *Journal of Physiology, Paris* 108 (2–3): 129–40. https://doi.org/10.1016/J.JPHYSPARIS.2014.07.006.
Meissl, H, and J. Yanez. 1994. "Pineal Photosensitivity. A Comparison with Retinal Photoreception." In *Acta Neurobiologiae Experimentalis*, 54:19–29.
Metcalfe, Walter K., Charles B. Kimmel, and Eric Schabtach. 1985. "Anatomy of the Posterior Lateral Line System in Young Larvae of the Zebrafish." *Journal of Comparative Neurology* 233 (3): 377–89. https://doi.org/10.1002/cne.902330307.
Meyers, Jason R, Richard B MacDonald, Anne Duggan, David Lenzi, David G Standaert, Jeffrey T Corwin, and David P Corey. 2003. "Lighting up the Senses: FM1-43 Loading of Sensory Cells through Nonselective Ion Channels." *Journal of Neuroscience* 23 (10): 4054–65. https://doi.org/10.1523/jneurosci.23-10-04054.2003.
Michel, W. C., and L. M. Lubomudrov. 1995. "Specificity and Sensitivity of the Olfactory Organ of the Zebrafish, Danio Rerio." *Journal of Comparative Physiology A* 177 (2): 191–99. https://doi.org/10.1007/BF00225098/METRICS.
Migault, Geoffrey, Thijs L. van der Plas, Hugo Trentesaux, Thomas Panier, Raphaël Candelier, Rémi Proville, Bernhard Englitz, Georges Debrégeas, and Volker Bormuth. 2018. "Whole-Brain Calcium Imaging during Physiological Vestibular Stimulation in Larval Zebrafish." *Current Biology* 28 (23): 3723-3735.e6. https://doi.org/10.1016/J.CUB.2018.10.017.
Mirjany, Mana, and Donald S. Faber. 2011. "Characteristics of the Anterior Lateral Line Nerve Input to the Mauthner Cell." *Journal of Experimental Biology* 214 (20): 3368–77. https://doi.org/10.1242/JEB.056226.
Mirjany, Mana, Thomas Preuss, and Donald S. Faber. 2011. "Role of the Lateral Line Mechanosensory System in Directionality of Goldfish Auditory Evoked Escape Response." *Journal of Experimental Biology* 214 (20): 3358–67. https://doi.org/10.1242/JEB.052894.
Mo, Weike, Fangyi Chen, Alex Nechiporuk, and Teresa Nicolson. 2010. "Quantification of Vestibular-Induced Eye Movements in Zebrafish Larvae." *BMC Neuroscience* 11 (1): 110. https://doi.org/10.1186/1471-2202-11-110.
Mogdans, Joachim. 2019. "Sensory Ecology of the Fish Lateral-line System: Morphological and Physiological Adaptations for the Perception of Hydrodynamic Stimuli." *Journal of Fish Biology* 95 (1): jfb.13966. https://doi.org/10.1111/jfb.13966.
Montgomery, John, Horst Bleckmann, and Sheryl Coombs. 2013. "Sensory Ecology and Neuroethology of the Lateral Line," 121–50. https://doi.org/10.1007/2506_2013_17.
Montgomery, John C., and Cindy F. Baker. 2020. "Lateral Line and Fish Behavior." In *The Senses: A Comprehensive Reference*, 133–42. Elsevier. https://doi.org/10.1016/B978-0-12-809324-5.24169-2.
Montgomery, John C., Cindy F. Baker, and Alexander G. Carton. 1997. "The Lateral Line Can Mediate Rheotaxis in Fish." *Nature* 389 (6654): 960–63. https://doi.org/10.1038/40135.
Mueller, Kaspar P., and Stephan C.F. Neuhauss. 2012. "Light Perception: More Than Meets the Eyes." *Current Biology* 22 (21): R912–14. https://doi.org/10.1016/J.CUB.2012.08.038.

Mueller, Kaspar P., and Stephan C C.F. Neuhauss. 2010. “Behavioral Neurobiology: How Larval Fish Orient towards the Light.” *Current Biology*. Cell Press. https://doi.org/10.1016/j.cub.2009.12.028.

Mueller, Thomas. 2012. “What Is the Thalamus in Zebrafish?” *Frontiers in Neuroscience* 6 (MAY). https://doi.org/10.3389/FNINS.2012.00064.

Müller, U., and E. Schwartz. 1982. “Influence of Single Neuromasts on Prey Localizing Behavior of the Surface Feeding Fish, Aplocheilus Lineatus.” *Journal of Comparative Physiology A* 149 (3): 399–408. https://doi.org/10.1007/BF00619155.

Mumtaz, Faiza, Muhammad Imran Khan, Muhammad Zubair, and Ahmad Reza Dehpour. 2018. “Neurobiology and Consequences of Social Isolation Stress in Animal Model—A Comprehensive Review.” *Biomedicine & Pharmacotherapy* 105 (September): 1205–22. https://doi.org/10.1016/J.BIOPHA.2018.05.086.

Murakami, Setsuko L., Lisa L. Cunningham, Lynne A. Werner, Eric Bauer, Rémy Pujol, David W. Raible, and Edwin W. Rubel. 2003. “Developmental Differences in Susceptibility to Neomycin-Induced Hair Cell Death in the Lateral Line Neuromasts of Zebrafish (Danio Rerio).” *Hearing Research* 186 (1–2): 47–56. https://doi.org/10.1016/S0378-5955(03)00259-4.

Muto, Akira, Masamichi Ohkura, Gembu Abe, Junichi Nakai, and Koichi Kawakami. 2013. “Real-Time Visualization of Neuronal Activity during Perception.” *Current Biology* 23 (4): 307–11. https://doi.org/10.1016/j.cub.2012.12.040.

Nelson, Mark E., Malcolm A. MacIver, and Sheryl Coombs. 2002. “Modeling Electrosensory and Mechanosensory Images during the Predatory Behavior of Weakly Electric Fish.” *Brain, Behavior and Evolution* 59 (4): 199–210. https://doi.org/10.1159/000064907.

Neuhauss, Stephan C.F., Oliver Biehlmaier, Mathias W. Seeliger, Tilak Das, Konrad Kohler, William A. Harris, and Herwig Baier. 1999. “Genetic Disorders of Vision Revealed by a Behavioral Screen of 400 Essential Loci in Zebrafish.” *Journal of Neuroscience* 19 (19): 8603–15. https://doi.org/10.1523/JNEUROSCI.19-19-08603.1999.

Neumann, Inga D., Alexa H. Veenema, and Daniela I. Beiderbeck. 2010. “Aggression and Anxiety: Social Context and Neurobiological Links.” *Frontiers in Behavioral Neuroscience* 4 (MAR): 12. https://doi.org/10.3389/FNBEH.2010.00012/BIBTEX.

New, John G. 2002. “Multimodal Integration in the Feeding Behaviors of Predatory Teleost Fishes.” *Brain, Behavior and Evolution* 59 (4): 177–89. https://doi.org/10.1159/000064905.

Newton, Kyle C., Dovi Kacev, Simon R.O. Nilsson, Allison L. Saettele, Sam A. Golden, and Lavinia Sheets. 2023. “Lateral Line Ablation by Ototoxic Compounds Results in Distinct Rheotaxis Profiles in Larval Zebrafish.” *Communications Biology 2023 6:1* 6 (1): 1–15. https://doi.org/10.1038/s42003-023-04449-2.

Nicolson, Teresa, Alfons Rüsch, Rainer W. Friedrich, Michael Granato, Johann Peter Ruppersberg, and Christiane Nüsslein-Volhard. 1998. “Genetic Analysis of Vertebrate Sensory Hair Cell Mechanosensation: The Zebrafish Circler Mutants.” *Neuron* 20 (2): 271–83. https://doi.org/10.1016/S0896-6273(00)80455-9.

O’Connell, Lauren A., and Hans A. Hofmann. 2011. “Genes, Hormones, and Circuits: An Integrative Approach to Study the Evolution of Social Behavior.” *Frontiers in Neuroendocrinology* 32 (3): 320–35. https://doi.org/10.1016/J.YFRNE.2010.12.004.

Oldfield, Ronald G., and Hans A. Hofmann. 2011. “Neuropeptide Regulation of Social Behavior in a Monogamous Cichlid Fish.” *Physiology & Behavior* 102 (3–4): 296–303. https://doi.org/10.1016/J.PHYSBEH.2010.11.022.

Olivari, Francisco A., Pedro P. Hernández, and Miguel L. Allende. 2008. “Acute Copper Exposure Induces Oxidative Stress and Cell Death in Lateral Line Hair Cells of Zebrafish Larvae.” *Brain Research* 1244 (December): 1–12. https://doi.org/10.1016/j.brainres.2008.09.050.

Olive, Raphaël, Sébastien Wolf, Alexis Dubreuil, Volker Bormuth, Georges Debrégeas, and Raphaël Candelier. 2016. “Rheotaxis of Larval Zebrafish: Behavioral Study of a Multi-Sensory Process.” *Frontiers in Systems Neuroscience* 10 (FEB). https://doi.org/10.3389/fnsys.2016.00014.

Ou, Henry C., David W. Raible, and Edwin W. Rubel. 2007. “Cisplatin-Induced Hair Cell Loss in Zebrafish (Danio Rerio) Lateral Line.” *Hearing Research* 233 (1–2): 46–53. https://doi.org/10.1016/j.heares.2007.07.003.

Owens, Kelly N., Allison B. Coffin, Lisa S. Hong, Keri O’Connell Bennett, Edwin W Rubel, and David W. Raible. 2009. “Response of Mechanosensory Hair Cells of the Zebrafish Lateral Line to Aminoglycosides Reveals Distinct Cell Death Pathways.” *Hearing Research* 253 (1–2): 32–41. https://doi.org/10.1016/j.heares.2009.03.001.

Owens, Kelly N., Dale E. Cunningham, Glen Macdonald, Edwin W. Rubel, David W. Raible, and Remy Pujol. 2007. “Ultrastructural Analysis of Aminoglycoside-Induced Hair Cell Death in the Zebrafish Lateral Line Reveals an Early Mitochondrial Response.” *Journal of Comparative Neurology* 502 (4): 522–43. https://doi.org/10.1002/cne.21345.

Panksepp, Jaak, and Jeff Burgdorf. 2003. “‘Laughing’ Rats and the Evolutionary Antecedents of Human Joy?” *Physiology & Behavior* 79 (3): 533–47. https://doi.org/10.1016/S0031-9384(03)00159-8.

Panksepp, Jaak, Jeffrey Burgdorf, Margery C. Beinfeld, Roger A. Kroes, and Joseph R. Moskal. 2007. “Brain Regional Neuropeptide Changes Resulting From Social Defeat.” *Behavioral Neuroscience* 121 (6): 1364–71. https://doi.org/10.1037/0735-7044.121.6.1364.

Partridge, Brian L., and Tony J. Pitcher. 1980. “The Sensory Basis of Fish Schools: Relative Roles of Lateral Line and Vision.” *Journal of Comparative Physiology □ A* 135 (4): 315–25. https://doi.org/10.1007/BF00657647/METRICS.

Partridge, G.J., D.D. Benetti, J.D. Stieglitz, J. Hutapea, A. McIntyre, B. Chen, W. Hutchinson, and V.P. Scholey. 2011. “The Effect of a 24-Hour Photoperiod on the Survival, Growth and Swim Bladder Inflation of Pre-Flexion Yellowfin Tuna (Thunnus Albacares) Larvae.” *Aquaculture* 318 (3–4): 471–74. https://doi.org/10.1016/J.AQUACULTURE.2011.05.039.

Pavlov, D. S., and S. N. Tjurjukov. 1995. “Reactions of Dace to Linear Accelerations.” *Journal of Fish Biology* 46 (5): 768–74. https://doi.org/10.1111/J.1095-8649.1995.TB01600.X.

Pavlov, D S, and S N Tyuryukov. 1993. “The Role of Lateral-Line Organs and Equilibrium in the Behavior and Orientation of the Dace, Leuciscus Leuciscus, in a Turbulent Flow.” *Journal of Ichthyology* 33: 45–55.

Peichel, Catherine L. 2004. “Social Behavior: How Do Fish Find Their Shoal Mate?” *Current Biology* 14 (13): R503–4. https://doi.org/10.1016/J.CUB.2004.06.037.

Pelster, Bernd. 2004. “The Development of the Swim Bladder: Structure and Performance.” *American Fisheries Society Symposium* 2004 (January): 37–46.

Perks, Krista E., Anna Krotinger, and David Bodznick. 2020. “A Cerebellum-like Circuit in the Lateral Line System of Fish Cancels Mechanosensory Input Associated with Its Own Movements.” *Journal of Experimental Biology* 223 (4). https://doi.org/10.1242/jeb.204438/267452/am/a-cerebellum-like-circuit-in-the-lateral-line.

Perrault, Thomas J., Barry E. Stein, and Benjamin A. Rowland. 2011. “Non-Stationarity in Multisensory Neurons in the Superior Colliculus.” *Frontiers in Psychology* 2 (JUL). https://doi.org/10.3389/FPSYG.2011.00144.

Pitcher, T. J., B. L. Partridge, and C. S. Wardle. 1976. “A Blind Fish Can School.” *Science* 194 (4268): 963–65. https://doi.org/10.1126/SCIENCE.982056.

Pittman, Karin, Manuel Yúfera, Michail Pavlidis, Audrey J. Geffen, Willian Koven, Laura Ribeiro, José L. Zambonino-Infante, and Amos Tandler. 2013. “Fantastically Plastic: Fish Larvae Equipped for a New World.” *Reviews in Aquaculture* 5 (SUPPL.1): S224–67. https://doi.org/10.1111/RAQ.12034.

Prestinicola, Loredana, Clara Boglione, and Stefano Cataudella. 2014. “Relationship between Uninflated Swim Bladder and Skeletal Anomalies in Reared Gilthead Seabream (Sparus Aurata).” *Aquaculture* 432 (August): 462–69. https://doi.org/10.1016/J.AQUACULTURE.2014.06.020.

Privat, Martin, Sebastián A. Romano, Thomas Pietri, Adrien Jouary, Jonathan Boulanger-Weill, Nicolas Elbaz, Auriane Duchemin, Daphne Soares, and Germán Sumbre. 2019. “Sensorimotor Transformations in the Zebrafish Auditory System.” *Current Biology* 29 (23): 4010-4023.e4. https://doi.org/10.1016/J.CUB.2019.10.020.

Quadros, Vanessa A., Luiz V. Rosa, Fabiano V. Costa, Talise E. Müller, Flavia V. Stefanello, Vania L. Loro, and Denis B. Rosemberg. 2019. “Involvement of Anxiety-like Behaviors and Brain Oxidative Stress in the Chronic Effects of Alarm Reaction in Zebrafish Populations.” *Neurochemistry International* 129 (October): 104488. https://doi.org/10.1016/J.NEUINT.2019.104488.

Qutob, Ziad. 1963. “The Swimbladder of Fishes as a Pressure Receptor.” *Archives Néerlandaises de Zoologie* 15 (1): 1–67. https://doi.org/10.1163/036551662X00015.

R Core Team. 2021. “R: A Language and Environment for Statistical Computing.” Vienna, Austria. https://www.r-project.org/.

Raible, David W., and Gregory J. Kruse. 2000. “Organization of the Lateral Line System in Embryonic Zebrafish.” *Journal of Comparative Neurology* 421 (2): 189–98. https://doi.org/10.1002/(SICI)1096-9861(20000529)421:2<189::AID-CNE5>3.0.CO;2-K.

Ribeiro, Diogo, Ana Rita Nunes, Magda Teles, Savani Anbalagan, Janna Blechman, Gil Levkowitz, and Rui F. Oliveira. 2020. “Genetic Variation in the Social Environment Affects Behavioral Phenotypes of Oxytocin Receptor Mutants in Zebrafish.” *ELife* 9 (September): 1–10. https://doi.org/10.7554/ELIFE.56973.

Ribera, Angeles B., and Christiane Nüsslein-Volhard. 1998. “Zebrafish Touch-Insensitive Mutants Reveal an Essential Role for the Developmental Regulation of Sodium Current.” *Journal of Neuroscience* 18 (22): 9181–91. https://doi.org/10.1523/jneurosci.18-22-09181.1998.

Richard, M. Jules. 1896. *Comptes Rendus Des Séances de La Société de Biologie et de Ses Filiales.* Vol. ser.10:t.3. Paris,Masson [etc.]. https://www.biodiversitylibrary.org/item/100656.

Rieger, P. W., and R. C. Summerfelt. 1998. “Microvideography of Gas Bladder Inflation in Larval Walleye.” *Journal of Fish Biology* 53 (1): 93–99. https://doi.org/10.1111/J.1095-8649.1998.TB00112.X.

Riley, Bruce B., and Stephen J. Moorman. 2000. “Development of Utricular Otoliths, but Not Saccular Otoliths, Is Necessary for Vestibular Function and Survival in Zebrafish.” *Journal*

of Neurobiology 43 (4): 329–37. https://doi.org/10.1002/1097-4695(20000615)43:4<329::AID-NEU2>3.0.CO;2-H.
Robinson, Gene E, Russell D Fernald, and David F Clayton. 2008. "Genes and Social Behavior." *Science (New York, N.Y.)* 322 (5903): 896–900. https://doi.org/10.1126/science.1159277.
RStudio Team. 2018. "RStudio: Integrated Development Environment for R." Boston, MA. http://www.rstudio.com/.
Sacchetti, Andrea, Tarek el Sewedy, Ashraf F. Nasr, and Saverio Alberti. 2001. "Efficient GFP Mutations Profoundly Affect MRNA Transcription and Translation Rates." *FEBS Letters* 492 (1–2): 151–55. https://doi.org/10.1016/S0014-5793(01)02246-3.
Saif, Muhammed, Diptendu Chatterjee, Christine Buske, and Robert Gerlai. 2013. "Sight of Conspecific Images Induces Changes in Neurochemistry in Zebrafish." *Behavioural Brain Research* 243 (1): 294–99. https://doi.org/10.1016/J.BBR.2013.01.020.
Santacà, Maria, Marco Dadda, and Angelo Bisazza. 2021. "The Role of Visual and Olfactory Cues in Social Decisions of Guppies and Zebrafish." *Animal Behaviour* 180 (October): 209–17. https://doi.org/10.1016/J.ANBEHAV.2021.08.017.
Santos, Felipe, Glen MacDonald, Edwin W. Rubel, and David W. Raible. 2006. "Lateral Line Hair Cell Maturation Is a Determinant of Aminoglycoside Susceptibility in Zebrafish (Danio Rerio)." *Hearing Research* 213 (1–2): 25–33. https://doi.org/10.1016/j.heares.2005.12.009.
Schindelin, Johannes, Ignacio Arganda-Carreras, Erwin Frise, Verena Kaynig, Mark Longair, Tobias Pietzsch, Stephan Preibisch, et al. 2012. "Fiji: An Open-Source Platform for Biological-Image Analysis." *Nature Methods 2012 9:7* 9 (7): 676–82. https://doi.org/10.1038/nmeth.2019.
Schoofs, Liliane, Arnold de Loof, and Matthias Boris van Hiel. 2017. "Neuropeptides as Regulators of Behavior in Insects." *Http://Dx.Doi.Org/10.1146/Annurev-Ento-031616-035500* 62 (January): 35–52. https://doi.org/10.1146/ANNUREV-ENTO-031616-035500.
Schoppik, David, Isaac H. Bianco, David A. Prober, Adam D. Douglass, Drew N. Robson, Jennifer M.B. Li, Joel S.F. Greenwood, Edward Soucy, Florian Engert, and Alexander F. Schier. 2017. "Gaze-Stabilizing Central Vestibular Neurons Project Asymmetrically to Extraocular Motoneuron Pools." *Journal of Neuroscience* 37 (47): 11353–65. https://doi.org/10.1523/JNEUROSCI.1711-17.2017.
Schwartz, Erich, and Arthur D. Hasler. 1966. "Perception of Surface Waves by the Blackstripe Topminnow, *Fundulus Notatus*." *Journal of the Fisheries Research Board of Canada* 23 (9): 1331–52. https://doi.org/10.1139/f66-124.
Seiler, Christoph, and Teresa Nicolson. 1999. "Defective Calmodulin-Dependent Rapid Apical Endocytosis in Zebrafish Sensory Hair Cell Mutants." *Journal of Neurobiology* 41 (3): 424–34. https://doi.org/10.1002/(SICI)1097-4695(19991115)41:3<424::AID-NEU10>3.0.CO;2-G.
Seitz, Nicholas. 2019. "Characterization Of Pth Type-2 Receptor (Pth2rb) And Its Endogenous Ligand Pth2 In Zebrafish Development." *Theses and Dissertations*. Illinois State University. https://doi.org/10.30707/ETD2019.Seitz.N.
Semmelhack, Julia L., Joseph C. Donovan, Tod R. Thiele, Enrico Kuehn, Eva Laurell, and Herwig Baier. 2014. "A Dedicated Visual Pathway for Prey Detection in Larval Zebrafish." *ELife* 3. https://doi.org/10.7554/ELIFE.04878.

Sha, Su-Hua, and Jochen Schacht. 1999. “Stimulation of Free Radical Formation by Aminoglycoside Antibiotics.” *Hearing Research* 128 (1–2): 112–18. https://doi.org/10.1016/S0378-5955(98)00200-7.

Shams, Soaleha, Shahid Amlani, Christine Buske, Diptendu Chatterjee, and Robert Gerlai. 2018. “Developmental Social Isolation Affects Adult Behavior, Social Interaction, and Dopamine Metabolite Levels in Zebrafish.” *Developmental Psychobiology* 60 (1): 43–56. https://doi.org/10.1002/DEV.21581.

Shams, Soaleha, Diane Seguin, Amanda Facciol, Diptendu Chatterjee, and Robert Gerlai. 2017. “Effect of Social Isolation on Anxiety-Related Behaviors, Cortisol, and Monoamines in Adult Zebrafish.” *Behavioral Neuroscience* 131 (6): 492–504. https://doi.org/10.1037/BNE0000220.

Sheets, Lavinia, Katie S. Kindt, and Teresa Nicolson. 2012. “Presynaptic CaV1.3 Channels Regulate Synaptic Ribbon Size and Are Required for Synaptic Maintenance in Sensory Hair Cells.” *The Journal of Neuroscience* 32 (48): 17273. https://doi.org/10.1523/JNEUROSCI.3005-12.2012.

Sherman, Shachar, Koichi Kawakami, and Herwig Baier. 2021. “Retinal Input Influences Pace of Neurogenesis but Not Cell-Type Configuration of the Visual Forebrain.” *BioRxiv*, November, 2021.11.15.468630. https://doi.org/10.1101/2021.11.15.468630.

Sheth, Sandeep, Debashree Mukherjea, Leonard P. Rybak, and Vickram Ramkumar. 2017. “Mechanisms of Cisplatin-Induced Ototoxicity and Otoprotection.” *Frontiers in Cellular Neuroscience* 11 (October): 338. https://doi.org/10.3389/fncel.2017.00338.

Shrimpton, J. M., D. J. Randall, and L. E. Fidler. 2011. “Factors Affecting Swim Bladder Volume in Rainbow Trout (Oncorhynchus Mykiss) Held in Gas Supersaturated Water.” *Https://Doi.Org/10.1139/Z90-138* 68 (5): 962–68. https://doi.org/10.1139/Z90-138.

Sidi, Samuel, Elisabeth Busch-Nentwich, Rainer Friedrich, Ulrike Schoenberger, and Teresa Nicolson. 2004. “Gemini Encodes a Zebrafish L-Type Calcium Channel That Localizes at Sensory Hair Cell Ribbon Synapses.” *The Journal of Neuroscience* 24 (17): 4213. https://doi.org/10.1523/JNEUROSCI.0223-04.2004.

Sirri, Rubina, Luciana Mandrioli, Samuele Zamparo, Francesca Errani, Enrico Volpe, Giorgia Tura, Tim Barbé, and Sara Ciulli. 2020. “Swim Bladder Disorders in Koi Carp (Cyprinus Carpio).” *Animals 2020, Vol. 10, Page 1974* 10 (11): 1974. https://doi.org/10.3390/ANI10111974.

Smith, Frank M., and Roger P. Croll. 2011. “Autonomic Control of the Swimbladder.” *Autonomic Neuroscience* 165 (1): 140–48. https://doi.org/10.1016/J.AUTNEU.2010.08.002.

Soboleski, Mark R., Jason Oaks, and William P. Halford. 2005. “Green Fluorescent Protein Is a Quantitative Reporter of Gene Expression in Individual Eukaryotic Cells.” *The FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology* 19 (3): 440. https://doi.org/10.1096/FJ.04-3180FJE.

Stewart, William J., Gilberto S. Cardenas, and Matthew J. McHenry. 2013. “Zebrafish Larvae Evade Predators by Sensing Water Flow.” *Journal of Experimental Biology* 216 (3): 388–98. https://doi.org/10.1242/jeb.072751.

Stuart, Kevin R., and Mark Drawbridge. 2012. “The Effect of Photoperiod on Larval Culture Performance of Two Marine Finfish Species.” *Aquaculture* 360–361 (September): 54–57. https://doi.org/10.1016/j.aquaculture.2012.07.017.

Suchocki, Christopher R., and Osvaldo J. Sepulveda-Villet. 2019. “The Role of Phototaxis in the Initial Swim Bladder Inflation of Larval Yellow Perch (Perca Flavescens).” *International Aquatic Research* 11 (1): 33–42. https://doi.org/10.1007/s40071-019-0217-x.

Suli, Arminda, Glen M. Watson, Edwin W. Rubel, and David W. Raible. 2012. “Rheotaxis in Larval Zebrafish Is Mediated by Lateral Line Mechanosensory Hair Cells.” Edited by Bruce Riley. *PLoS ONE* 7 (2): e29727. https://doi.org/10.1371/journal.pone.0029727.

Tait, J. S. 1960. “The First Filling Of The Swim Bladder In Salmonoids.” *Canadian Journal of Zoology* 38 (1): 179–87. https://doi.org/10.1139/z60-021.

Thomas, Eric D., Ivan A. Cruz, Dale W. Hailey, and David W. Raible. 2015. “There and Back Again: Development and Regeneration of the Zebrafish Lateral Line System.” *Wiley Interdisciplinary Reviews: Developmental Biology* 4 (1): 1–16. https://doi.org/10.1002/WDEV.160.

Thompson, Andrew W., Gilles C. Vanwalleghem, Lucy A. Heap, and Ethan K. Scott. 2016. “Functional Profiles of Visual-, Auditory-, and Water Flow-Responsive Neurons in the Zebrafish Tectum.” *Current Biology* 26 (6): 743–54. https://doi.org/10.1016/J.CUB.2016.01.041.

Ton, Christopher, and Chuenlei Parng. 2005. “The Use of Zebrafish for Assessing Ototoxic and Otoprotective Agents.” *Hearing Research* 208 (1–2): 79–88. https://doi.org/10.1016/j.heares.2005.05.005.

Trapani, Josef G., and Teresa Nicolson. 2011. “Mechanism of Spontaneous Activity in Afferent Neurons of the Zebrafish Lateral-Line Organ.” *The Journal of Neuroscience* 31 (5): 1614. https://doi.org/10.1523/JNEUROSCI.3369-10.2011.

Trotter, A. J., S. C. Battaglene, and P. M. Pankhurst. 2003. “Effects of Photoperiod and Light Intensity on Initial Swim Bladder Inflation, Growth and Post-Inflation Viability in Cultured Striped Trumpeter (Latris Lineata) Larvae.” *Aquaculture* 224 (1–4): 141–58. https://doi.org/10.1016/S0044-8486(03)00212-6.

Tsukamoto, Yoshikazu, and Shizuo Yoshino. 1957. “A Study of the Lateral-Line System in Fish II. On the Relation between the Function of the Lateral-Line Organ and the Habits of Fish.” *Japanese Journal of Ichthyology* 6 (4–6): 131–35. https://doi.org/10.11369/jji1950.6.131.

Tunbak, Hande, Mireya Vazquez-Prada, Thomas Ryan, Adam R. Kampff, and Elena Dreosti. 2020. “Whole-Brain Mapping of Socially Isolated Zebrafish Reveals That Lonely Fish Are Not Loners.” *ELife* 9 (May). https://doi.org/10.7554/ELIFE.55863.

Ueda, Hiroshi. 2021. “Chemoreception in Fishes.” *Oxford Research Encyclopedia of Neuroscience*, March. https://doi.org/10.1093/ACREFORE/9780190264086.013.333.

Vanwalleghem, Gilles, Kevin Schuster, Michael A. Taylor, Itia A. Favre-Bulle, and Ethan K. Scott. 2020. “Brain-Wide Mapping of Water Flow Perception in Zebrafish.” *Journal of Neuroscience* 40 (21): 4130–44. https://doi.org/10.1523/JNEUROSCI.0049-20.2020.

Venuto, Alexandra, and Timothy Erickson. 2021. “Evaluating the Death and Recovery of Lateral Line Hair Cells Following Repeated Neomycin Treatments.” *Life* 11 (11): 1180. https://doi.org/10.3390/life11111180.

Venuto, Alexandra, Cameron P Smith, Marybelle Cameron-Pack, and Timothy Erickson. 2022. “Alone in a Crowd: Effect of a Nonfunctional Lateral Line on Expression of the Social Hormone Parathyroid Hormone 2.” *Biology Open* 11 (10). https://doi.org/10.1242/bio.059432.

Venuto, Alexandra, Stacey Thibodeau-Beganny, Josef G. Trapani, and Timothy Erickson. 2023. “A Sensation for Inflation: Initial Swim Bladder Inflation in Larval Zebrafish Is Mediated

by the Mechanosensory Lateral Line." *Journal of Experimental Biology*, May. https://doi.org/10.1242/JEB.245635.

Verkhusha, Vladislav v., Irina M. Kuznetsova, Olesia v. Stepanenko, Andrey G. Zaraisky, Michail M. Shavlovsky, Konstantin K. Turoverov, and Vladimir N. Uversky. 2003. "High Stability of Discosoma DsRed as Compared to Aequorea EGFP." *Biochemistry* 42 (26): 7879–84. https://doi.org/10.1021/BI034555T/ASSET/IMAGES/LARGE/BI034555TF00003.JPEG.

Villamizar, N., A. García-Alcazar, and F. J. Sánchez-Vázquez. 2009. "Effect of Light Spectrum and Photoperiod on the Growth, Development and Survival of European Sea Bass (Dicentrarchus Labrax) Larvae." *Aquaculture* 292 (1–2): 80–86. https://doi.org/10.1016/j.aquaculture.2009.03.045.

Villamizar, Natalia, Luisa María Vera, Nicholas Simon Foulkes, and Francisco Javier Sánchez-Vázquez. 2014. "Effect of Lighting Conditions on Zebrafish Growth and Development." *Zebrafish* 11 (2): 173. https://doi.org/10.1089/ZEB.2013.0926.

Vuilleumier, Robin, Laurence Besseau, Gilles Boeuf, Aurélien Piparelli, Yoav Gothilf, Walter G. Gehring, David C. Klein, and Jack Falcón. 2006. "Starting the Zebrafish Pineal Circadian Clock with a Single Photic Transition." *Endocrinology* 147 (5): 2273–79. https://doi.org/10.1210/EN.2005-1565.

Wang, Xiaokai, Luoxiu Huang, Yue Li, Xinle Li, Ping Li, John Ray, and Lei Li. 2011. "Characterization of GFP-Tagged GnRH-Containing Terminalis Neurons in Transgenic Zebrafish." *Journal of Cellular Physiology* 226 (3): 608–15. https://doi.org/10.1002/JCP.22369.

Warburton, Kevin. 2003. "Learning of Foraging Skills by Fish." *Fish and Fisheries* 4 (3): 203–15. https://doi.org/10.1046/J.1467-2979.2003.00125.X.

Wee, Caroline L., Erin Song, Maxim Nikitchenko, Kristian J. Herrera, Sandy Wong, Florian Engert, and Samuel Kunes. 2022. "Social Isolation Modulates Appetite and Avoidance Behavior via a Common Oxytocinergic Circuit in Larval Zebrafish." *Nature Communications 2022 13:1* 13 (1): 1–17. https://doi.org/10.1038/s41467-022-29765-9.

Weiss, Isabelle C., Christopher R. Pryce, Ana L. Jongen-Rêlo, Nina I. Nanz-Bahr, and Joram Feldon. 2004. "Effect of Social Isolation on Stress-Related Behavioural and Neuroendocrine State in the Rat." *Behavioural Brain Research* 152 (2): 279–95. https://doi.org/10.1016/J.BBR.2003.10.015.

Westerfield, Monte. 2000. *The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (Danio Rerio).* 4th ed. Eugene OR: University of Oregon. http://zfin.org/zf_info/zfbook/zfbk.html.

Westphal, Rebecca E., and Donald M. O'Malley. 2013. "Fusion of Locomotor Maneuvers, and Improving Sensory Capabilities, Give Rise to the Flexible Homing Strikes of Juvenile Zebrafish." *Frontiers in Neural Circuits* 7 (MAY): 108. https://doi.org/10.3389/FNCIR.2013.00108/BIBTEX.

Wickham, Hadley. 2011. "Ggplot2." *Wiley Interdisciplinary Reviews: Computational Statistics* 3 (2): 180–85. https://doi.org/10.1002/wics.147.

Wilcox, R. Stimson. 1988. "Surface Wave Reception in Invertebrates and Vertebrates." In *Sensory Biology of Aquatic Animals*, 643–63. New York, NY: Springer New York. https://doi.org/10.1007/978-1-4612-3714-3_25.

Williams, J. A., and N. Holder. 2000. "Cell Turnover in Neuromasts of Zebrafish Larvae." *Hearing Research* 143 (1–2): 171–81. https://doi.org/10.1016/S0378-5955(00)00039-3.

Wilson, Carole. 2012. “Aspects of Larval Rearing.” *ILAR Journal* 53 (2): 169–78. https://doi.org/10.1093/ILAR.53.2.169.

Woolley, Lindsey D., and Jian G. Qin. 2010. “Swimbladder Inflation and Its Implication to the Culture of Marine Finfish Larvae.” *Reviews in Aquaculture* 2 (4): 181–90. https://doi.org/10.1111/j.1753-5131.2010.01035.x.

Xi, Yanwei, Man Yu, Rafael Godoy, Gary Hatch, Luc Poitras, and Marc Ekker. 2011. “Transgenic Zebrafish Expressing Green Fluorescent Protein in Dopaminergic Neurons of the Ventral Diencephalon.” *Developmental Dynamics* 240 (11): 2539–47. https://doi.org/10.1002/DVDY.22742.

Xiong, Wei, Nicolas Grillet, Heather M. Elledge, Thomas F J Wagner, Bo Zhao, Kenneth R. Johnson, Piotr Kazmierczak, and Ulrich Müller. 2012. “TMHS Is an Integral Component of the Mechanotransduction Machinery of Cochlear Hair Cells.” *Cell* 151 (6): 1283–95. https://doi.org/10.1016/j.cell.2012.10.041.

Xu, JiangPing, RuiTao Zhang, Ting Zhang, Guang Zhao, Yan Huang, HuanLing Wang, and Jing-Xia Liu. 2017. “Copper Impairs Zebrafish Swimbladder Development by Down-Regulating Wnt Signaling.” *Aquatic Toxicology* 192 (November): 155–64. https://doi.org/10.1016/J.AQUATOX.2017.09.018.

Yakusheva, Tatyana A., Aasef G. Shaikh, Andrea M. Green, Pablo M. Blazquez, J. David Dickman, and Dora E E. Angelaki. 2007. “Purkinje Cells in Posterior Cerebellar Vermis Encode Motion in an Inertial Reference Frame.” *Neuron* 54 (6): 973–85. https://doi.org/10.1016/J.NEURON.2007.06.003.

Young, Alexander, Valentin Kochenkov, Jenifer K. McIntyre, John D. Stark, and Allison B. Coffin. 2018. “Urban Stormwater Runoff Negatively Impacts Lateral Line Development in Larval Zebrafish and Salmon Embryos.” *Scientific Reports* 8 (1). https://doi.org/10.1038/S41598-018-21209-Z.

Zhao, Bo, Zizhen Wu, Nicolas Grillet, Linxuan Yan, Wei Xiong, Sarah Harkins-Perry, and Ulrich Müller. 2014. “TMIE Is an Essential Component of the Mechanotransduction Machinery of Cochlear Hair Cells.” *Neuron* 84 (5): 954–67. https://doi.org/10.1016/j.neuron.2014.10.041.

Zhu, Shuyu I., and Geoffrey J. Goodhill. 2023. “From Perception to Behavior: The Neural Circuits Underlying Prey Hunting in Larval Zebrafish.” *Frontiers in Neural Circuits* 17 (February): 2. https://doi.org/10.3389/FNCIR.2023.1087993/BIBTEX.

Zielinski, Barbara S., Kim Fredricks, Rod McDonald, and Aliya U. Zaidi. 2005. “Morphological and Electrophysiological Examination of Olfactory Sensory Neurons during the Early Developmental Prolarval Stage of the Sea Lamprey Petromyzon Marinus L.” *Journal of Neurocytology* 34 (3–5): 209–16. https://doi.org/10.1007/S11068-005-8354-0/METRICS.

Zimmermann, Fernanda Francine, Karina Vidarte Gaspary, Anna Maria Siebel, and Carla Denise Bonan. 2016. “Oxytocin Reversed MK-801-Induced Social Interaction and Aggression Deficits in Zebrafish.” *Behavioural Brain Research* 311 (September): 368–74. https://doi.org/10.1016/J.BBR.2016.05.059.

Zimmermann, Maxime J.Y., Noora E. Nevala, Takeshi Yoshimatsu, Daniel Osorio, Dan Eric Nilsson, Philipp Berens, and Tom Baden. 2018. “Zebrafish Differentially Process Color across Visual Space to Match Natural Scenes.” *Current Biology* 28 (13): 2018-2032.e5. https://doi.org/10.1016/J.CUB.2018.04.075.

APPENDIX A: ANIMAL USE PROTOCOL APPROVAL LETTER

ECU

Animal Care and Use Committee
003 Ed Warren Life Sciences Building | East Carolina University | Greenville NC 27834 - 4354
252-744-2436 office | 252-744-2355 fax

March 12, 2021

Fadi Issa, Ph.D.
Department of Biology, ECU

Dear Dr. Issa:

Your Animal Use Protocol entitled, "The sensory biology of swim bladder inflation in larval zebrafish" (AUP #D372) was reviewed by this institution's Animal Care and Use Committee on 03/12/2021. The following action was taken by the Committee:

"Approved as submitted"

****Please contact Aaron Hinkle prior to any hazard use****

A copy of the protocols is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. **Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP/Amendment and are familiar with its contents.**

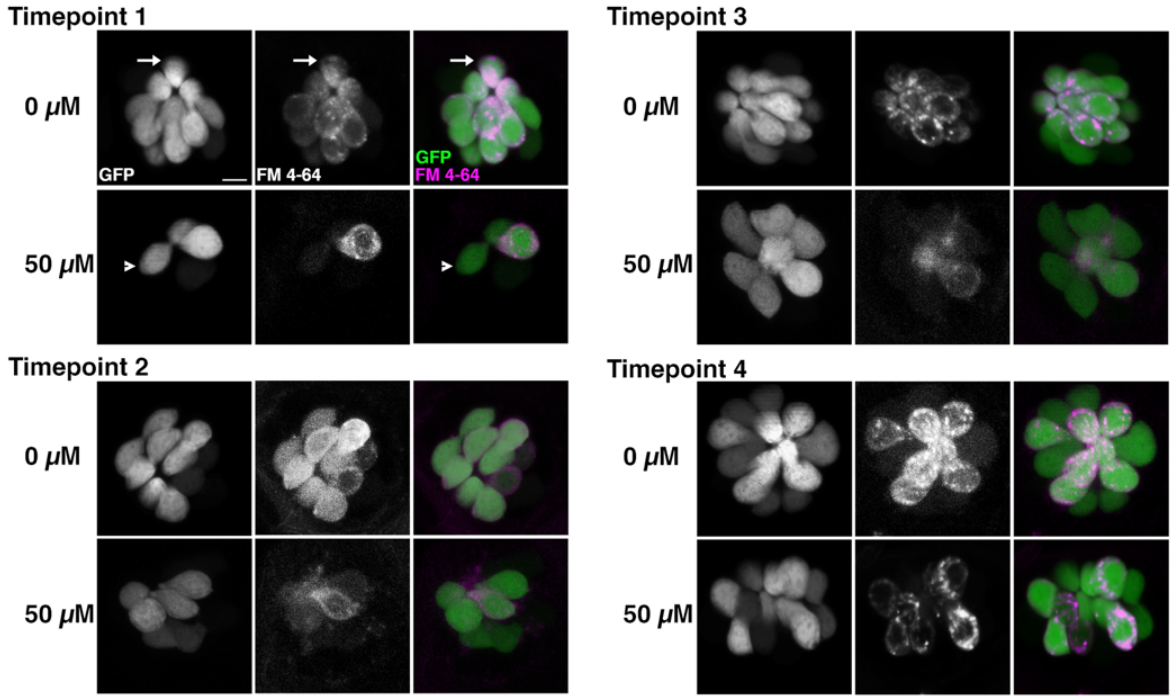
Sincerely yours,

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/GD

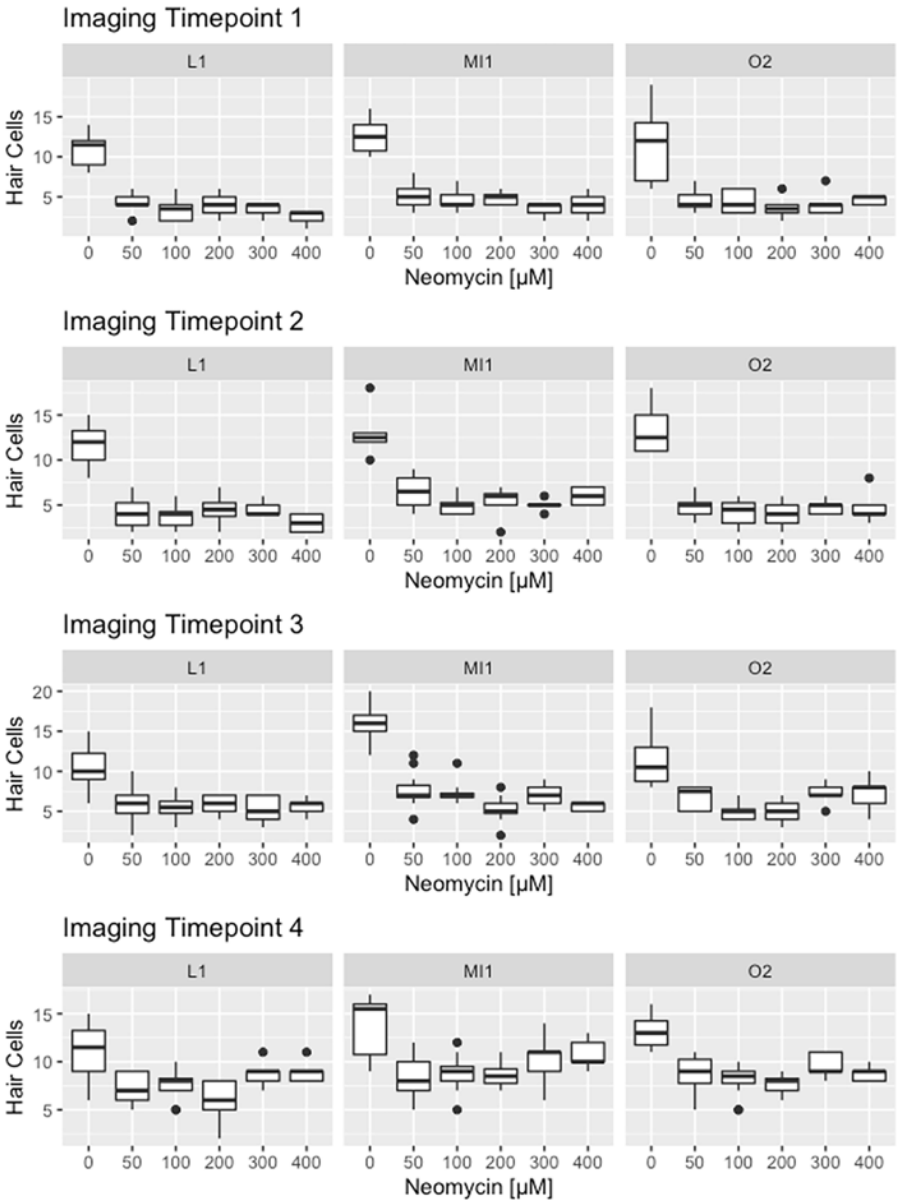
enclosure

APPENDIX B: SUPPLEMENTAL FIGURE 1



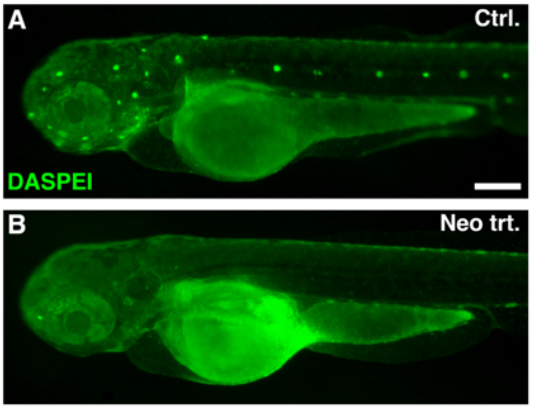
Supplemental Figure 1: Representative confocal images of O2 neuromasts at imaging timepoints I1-I4 in the 0 and 50 µM neomycin treatment groups. The *Tg(myo6b:EGFP-pA)vo68Tg* line is green and FM 4-64 is magenta in the merge of the two channels in the third column. Scale bar = 5 µm for all images.

APPENDIX C: SUPPLEMENTAL FIGURE 2



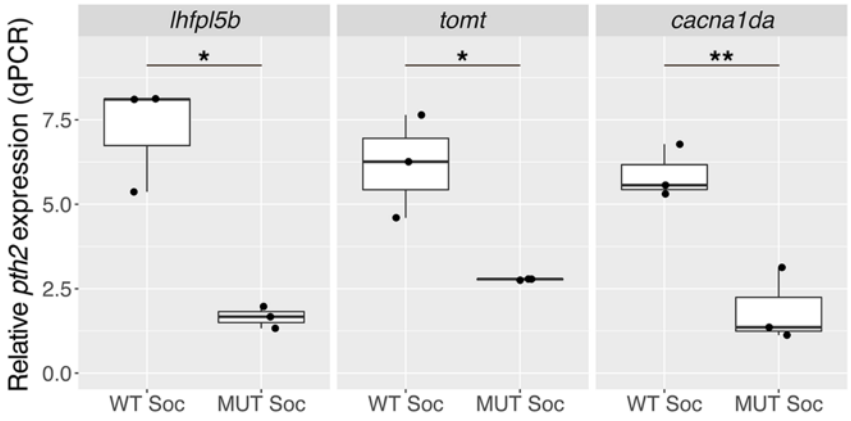
Supplemental Figure 2: Box plot of total hair cell counts (GFP-positive) from individual neuromasts L1, MI1, and O2 at each imaging timepoint in the 12-hour treatment timeline.

APPENDIX D: SUPPLEMENTAL FIGURE 3



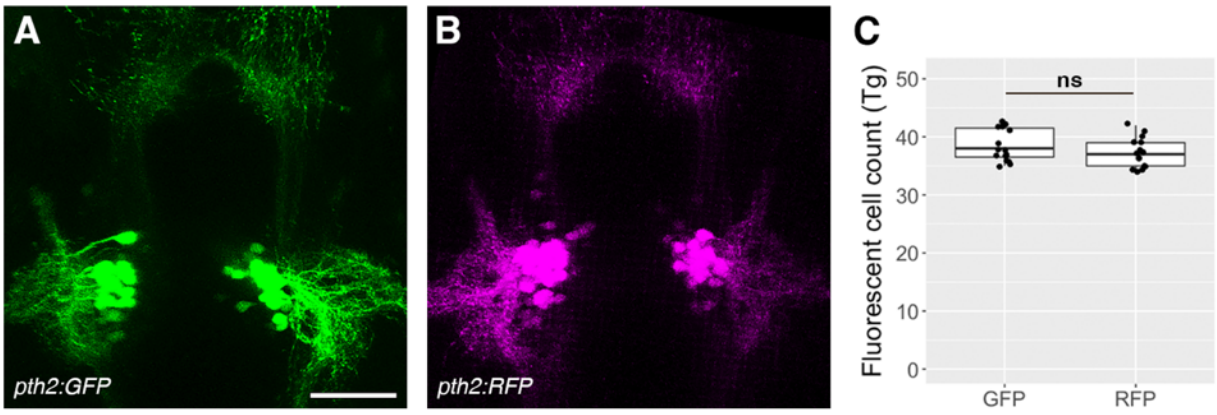
Supplemental Figure 3: Vital dye staining of lateral line neuromasts in control and neomycin-treated larvae. Representative 3 dpf larvae from control (A) and neomycin-treated (B) groups stained with DASPEI three hours following a 30-minute treatment with either 0 µM or 50 µM neomycin. Note the near absence of punctate neuromast staining in B, indicating successful ablation of the lateral line hair cells. Scale bar = 200 µm.

APPENDIX E: SUPPLEMENTAL FIGURE 4



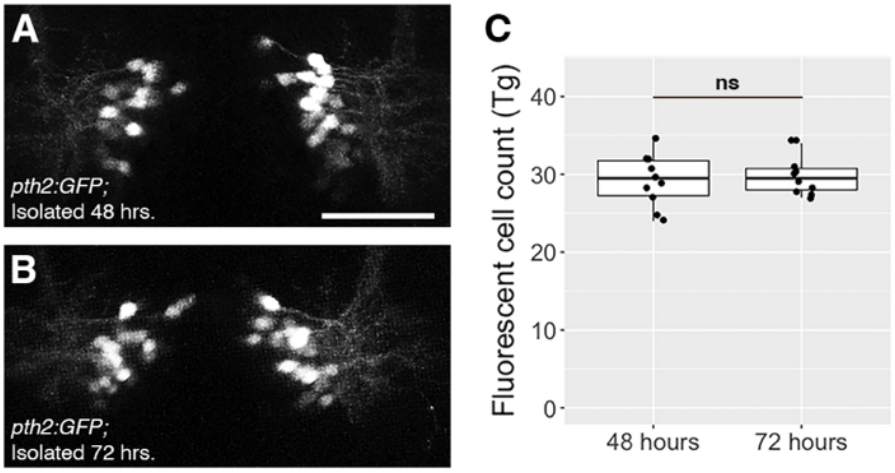
Supplemental Figure 4: Boxplots of qPCR results showing *pth2* expression in socially-raised hair cell mutants and wild type siblings at 4 dpf. Ten larval heads were pooled per condition to make one biological replicate, and three biological replicates were used to generate the data. Two technical replicates of the qPCR experiment were conducted per biological replicate and averaged. A one-tailed Welch's t-test was used to assess significance, * = $p < 0.05$, ** = $p < 0.01$.

APPENDIX F: SUPPLEMENTAL FIGURE 5



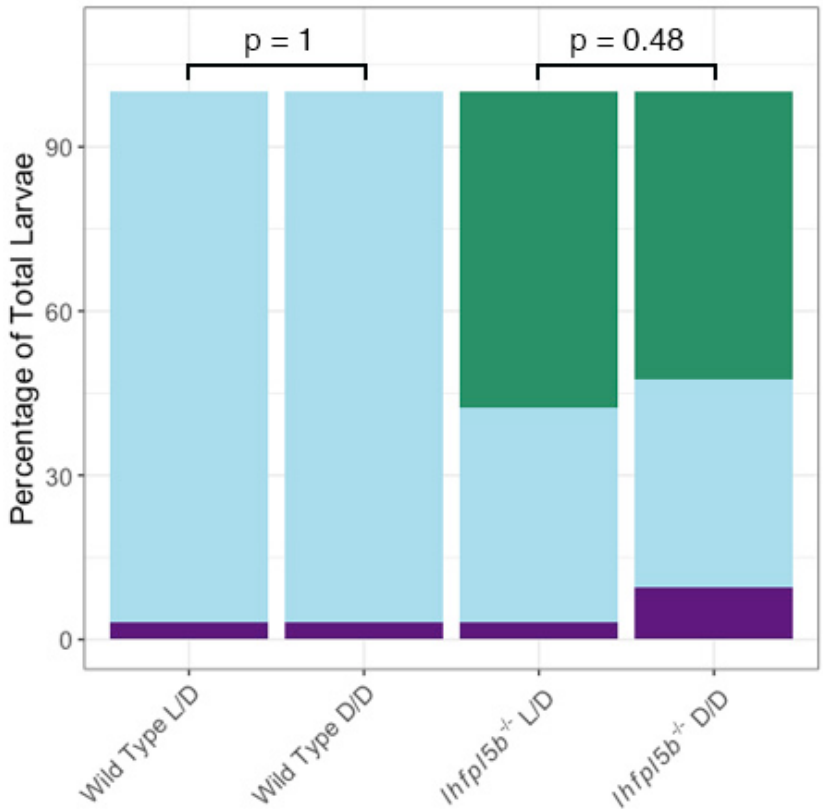
Supplemental Figure 5: *Tg*(*pth2:EGFP)unb2* and *Tg*(*pth2:TagRFP)unb3* transgenes exhibit similar morphology and number of cells. (A-B) Representative images of socially-reared 4 dpf larva possessing either the A) *Tg(pth2:EGFP)unb2* transgene (green) or B) *Tg(pth2:TagRFP)unb3* transgene (magenta). (C) Boxplot of GFP and RFP cell counts (n = 15 larvae per condition). Scale bar is 50 μm. A two-tailed Welch's t-test was used to assess significance, ns = not significant.

APPENDIX G: SUPPLEMENTAL FIGURE 6



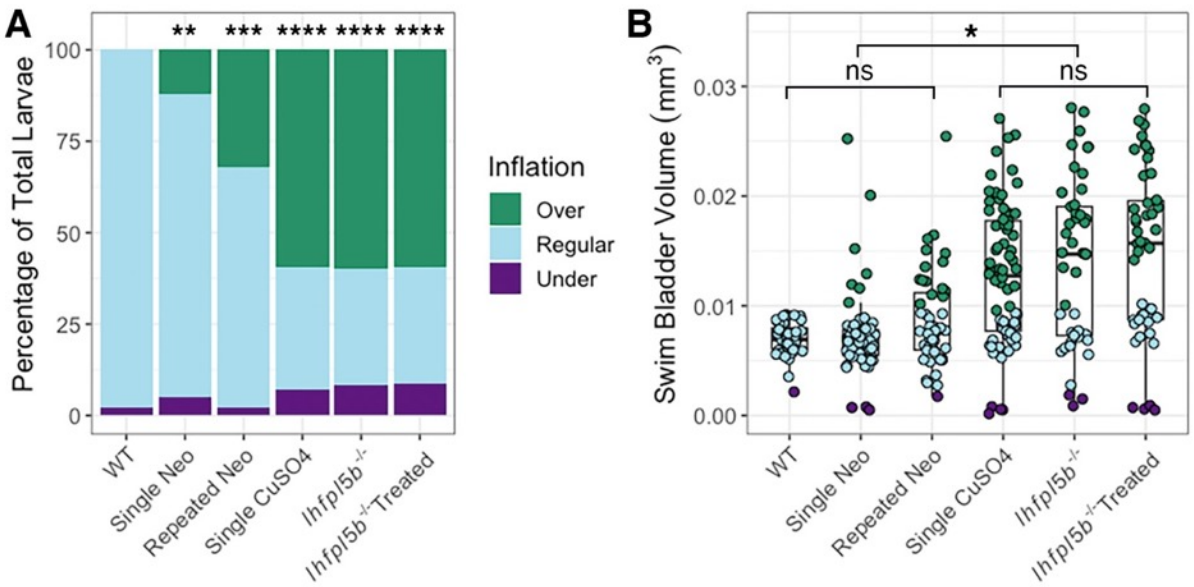
Supplemental Figure 6: Increasing the isolation period from 48 to 72 hours does not affect the number of GFP-positive cells in *pth2:GFP* transgenics. (A-B) Representative images of 4 dpf *Tg*(*pth2:EGFP)unb2* larva raised in isolation for A) 48 hours or B) 72 hours. (C) Boxplots of GFP-positive cell counts for *Tg(pth2:EGFP)* larvae raised in isolation for 48 or 72 hours (*n* = 10 per condition). Scale bar is 50 μm. A two-tailed Welch's t-test was used to assess significance, ns = not significant.

APPENDIX H: SUPPLEMENTAL FIGURE 7



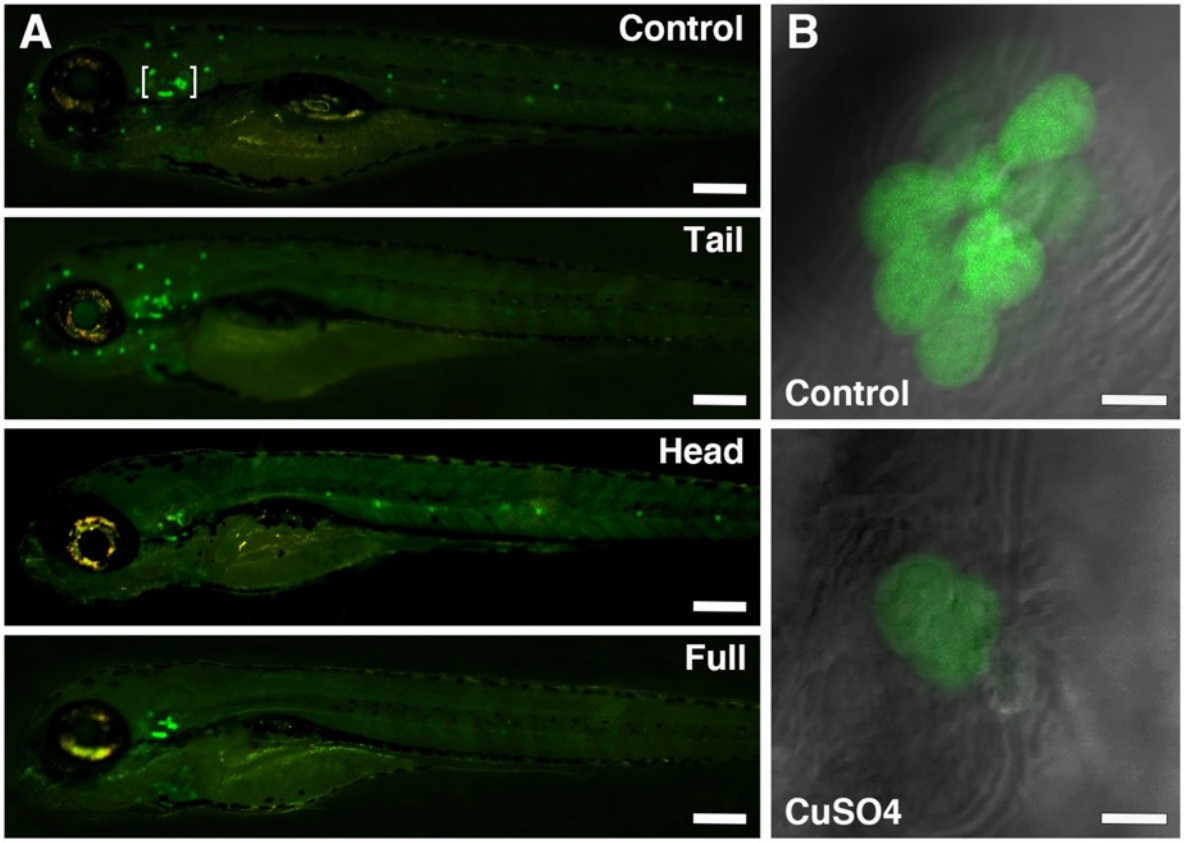
Supplemental Figure 7: Buoyancy proportions of larvae raised in total darkness. Percentage of larvae that exhibit swim bladder under, regular, and over-inflation raised in either a 14:10 light/dark cycle (L/D) or total darkness (D/D). A Chi-Squared Test was used to assess significance. Both Wild Type L/D and Wild Type D/D over-inflation proportions: $M = 0.0\%$, $SD = 0.0\%$, $n = 32$. *lhfpl5b*$^{-/-}$ L/D over-inflation proportion: $M = 57.5\%$, $SD = 6.6\%$, $n = 30$. *lhfpl5b*$^{-/-}$ D/D over-inflation proportion: $M = 52.4\%$, $SD = 4.1\%$, $n = 30$.

APPENDIX I: SUPPLEMENTAL FIGURE 8



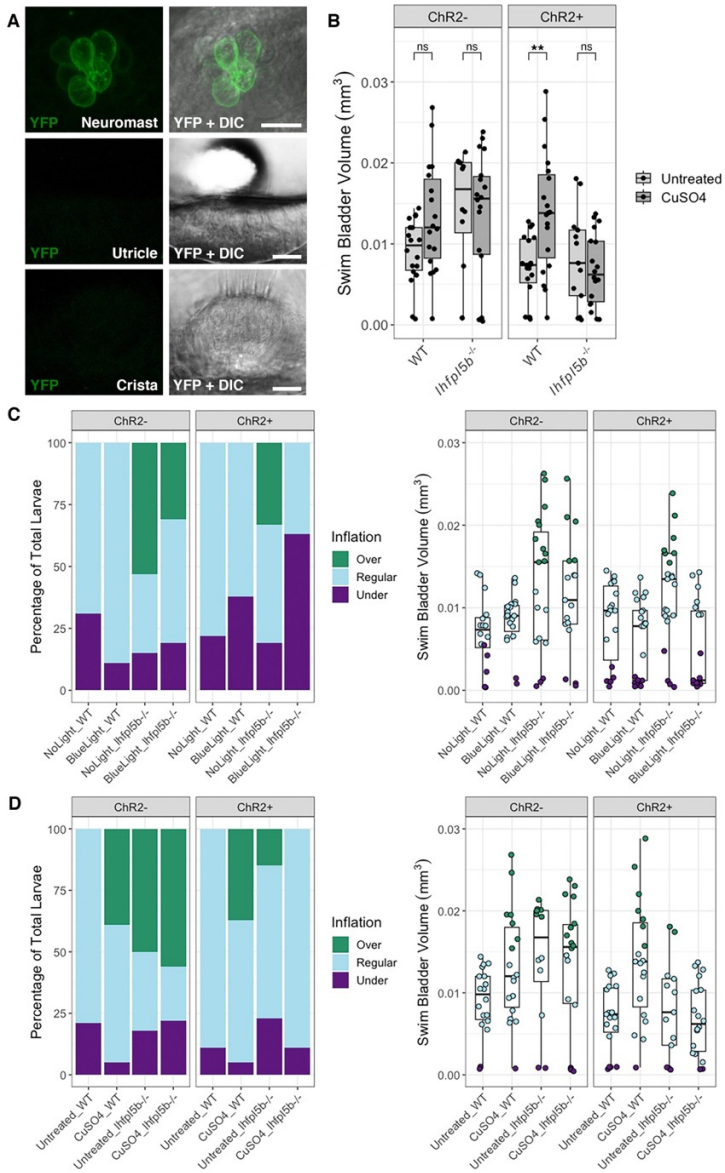
Supplemental Figure 8: Ototoxic ablations of the lateral line. A) Percentage of larvae that exhibit swim bladder under, regular, and over-inflation. A Chi-Squared Test was used to assess significance. B) Swim bladder volume (mm^3) in 5 dpf larvae. A One-Way ANOVA was used to determine significance. **** = $p < 0.0001$, *** = $p < 0.001$, ** = $p < 0.01$, ns = no significance.

APPENDIX J: SUPPLEMENTAL FIGURE 9



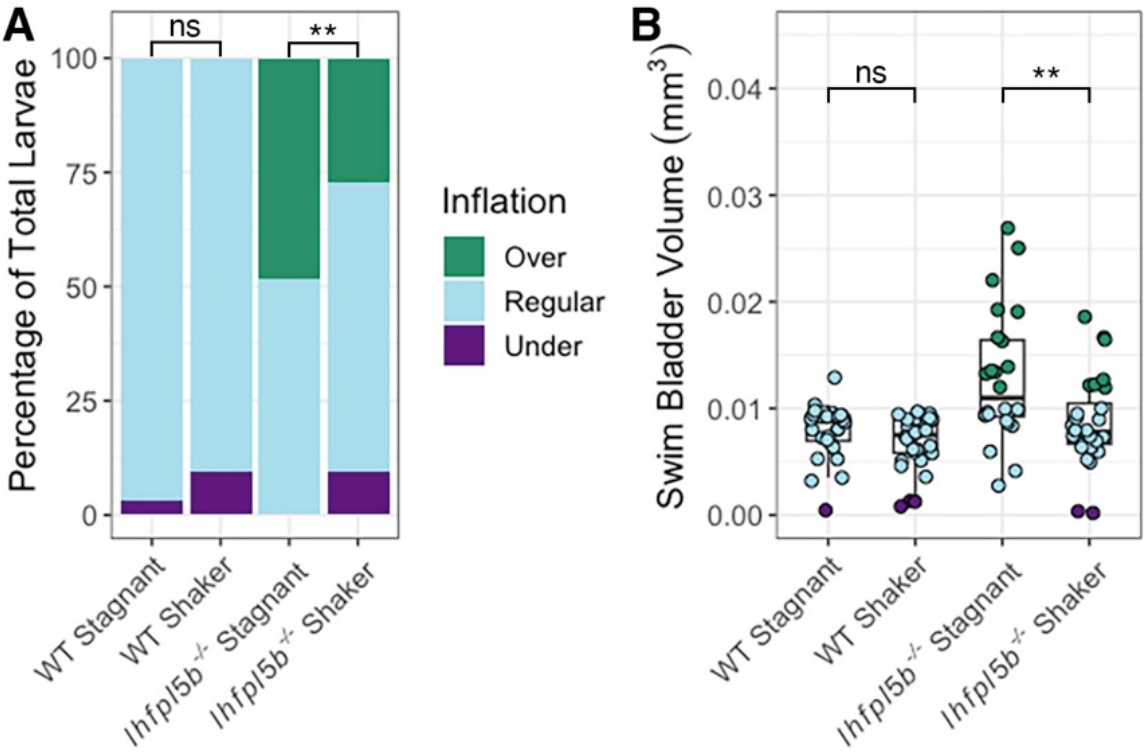
Supplemental Figure 9: Anterior and posterior-specific lateral line ablations with CuSO4. A) Representative images of live *Tg(myo6b:EGFP-pA)vo68* transgenics at 4 dpf, two hours after CuSO4 treatment. This transgene labels hair cells of the lateral line and inner ear (indicated by white brackets on the Control image), and treatment only affects lateral line hair cells so inner ear expression remains post-treatment. Scale bars = 0.2 mm. B) Representative images from untreated Control and CuSO4-treated L1 neuromasts. Scale bars = 5 uM.

APPENDIX K: SUPPLEMENTAL FIGURE 10



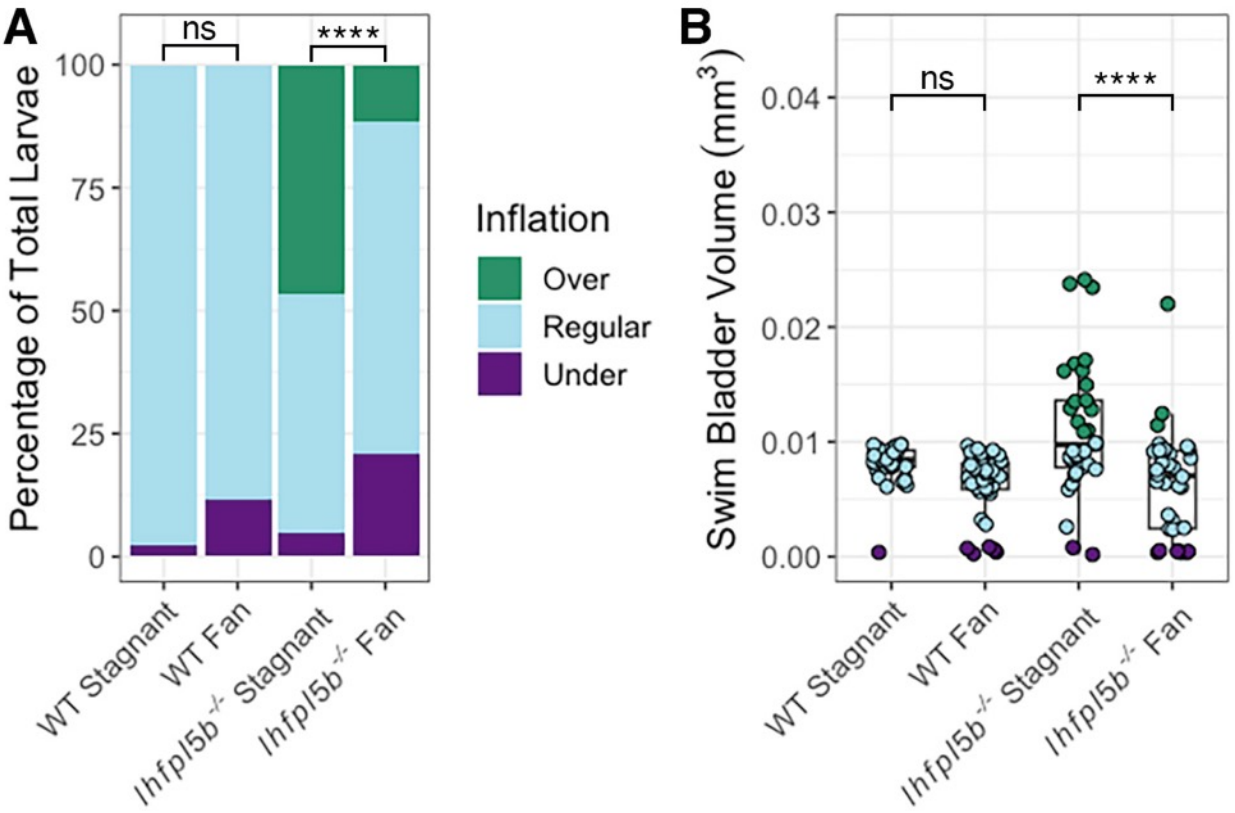
Supplemental Figure 10: Channelrhodopsin-2 (ChR2) expression in *Tg(lhfpl5b2028:ChR2-EYFP-pA)* transgenics. A) Images of ChR2-YFP in hair cells in a 2 dpf neuromast, utricle, and crista where fluorescence expression is only seen in the neuromasts of the lateral line. First panel is YFP only (false-colored green) and second panel is merge of YFP and DIC. Scale bars = 10 uM. B) Swim bladder volume (mm^3) of 6 dpf larvae from experimental condition (blue light overhead). A One-Way ANOVA was used to determine significance. ** = $p < 0.00724$, ns = no significance. C) Percentages and box plots of experiment in Figure 22 to show inflation categories. D) Percentages and box plots of experiment in Supplemental Figure 10B to show inflation categories.

APPENDIX L: SUPPLEMENTAL FIGURE 11



Supplemental Figure 11: Rocking waters decreases swim bladder hyperinflation in lateral line mutants (*lhfpl5b*$^{-/-}$). A) Percentage of larvae that exhibit swim bladder under, regular, and over-inflation in wild type and lateral line mutants in shaking or stagnant water conditions. A Chi-Squared Test was used to determine significance. B) Swim bladder volume (mm^3) of larvae at 6 dpf in wild type and lateral line mutants in shaking or stagnant water conditions. A Two-Way ANOVA was used to determine significance. ** = $p < 0.01$, ns = not significant.

APPENDIX M: SUPPLEMENTAL FIGURE 12



Supplemental Figure 12: Surface waves decrease swim bladder hyperinflation in lateral line mutants (*lhfpl5b*$^{-/-}$). A) Percentage of larvae that exhibit swim bladder under, regular, and over-inflation in wild type and lateral line mutants in fanned or stagnant water conditions. A Chi-Squared Test was used to determine significance. B) Swim bladder volume (mm^3) of larvae at 6 dpf in wild type and lateral line mutants in fanned or stagnant water conditions. A Two-Way ANOVA was used to determine significance. **** = $p < 0.0001$, ns = not significant.