A sensation for inflation: initial swim bladder inflation in larval zebrafish is mediated by the mechanosensory lateral line.

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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.

Keywords: behavior, buoyancy, hair cells, hydrodynamics, lateral line, lhfpl5, swim bladder, sensory system, zebrafish

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 initate swim bladder inflation by swimming up to the surface to ingest air using a set of behaviors we refer to as "surfacing" (Gisbert & Williot, 1997; Lindsey, et al., 2010). 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 find the surface (Tait, 1960). Surfacing is a complex task that requires a combination of newly developed physical features, sensory systems, and motor skills (Bailey & Doroshov, 1995; Chatain, 1989; Doroshev et al., 1981; Kimmel, et al., 1995; Kitajima, et al., 1994) – (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 (Doroshev et al., 1981; Rieger and Summerfelt, 1998; Tait, 1960). Together, surfacing represents perhaps the most complex behavior that larvae perform prior to achieving neutral buoyancy.

Little is known about the sensory cues that instruct the surfacing behavior. Mutations that render the hair cells of the inner ear non-functional typically result in hypoinflation (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 (Stuart and Drawbridge, 2012; Trotter et al., 2003; Villamizar et al., 2009). 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 hydrodynamic information including water currents, predators and prey, objects, and surface waves (Mogdans, 2019). 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 lateral line to sense their transition through the air-water interface (Tsukamoto and Yoshino, 1957). However, while the lateral line is a strong

candidate to aid in larval surfacing behaviors that lead to initial swim bladder inflation, its specific role in this important behavior has not been explored.

A CRISPR-Cas9 knockout of *lhfpl5b* (*lhfpl5b*^{vo35}) 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. Homozygous mutant larvae present with a rare hyperinflation phenotype characterized by over-filled swim bladders and positive buoyancy. In this study, we show that anterior lateral line sensory defects are responsible for the hyperinflation phenotype. We also demonstrate that manipulating lateral line sensory information alters swim bladder inflation. Finally, we find that abnormal surfacing behaviors are correlated with swim bladder over-inflation. Overall, our study uncovers a novel role for the lateral line as a necessary sensory system for initial swim bladder inflation in larval zebrafish.

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 (recipe: 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 Committees at East Carolina University (Greenville, NC, United States).

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., 2020, Erickson et al., 2017). 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 Channelrhodopsin2 (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)* 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 in the middle 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).

Imaging and swim bladder measurements

All experiments 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 et al., 2010).

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 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 5 larvae (3 mutant and 2 wild type) recorded per recording. Larvae that did not inflate their swim bladders by the end of filming were excluded from this analysis due to lack of surfacing events.

Lateral line hair cell ablations

For all experiments, half of the wild type and mutant larvae were treated with ototoxin with untreated larvae 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 & 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, we anesthetized the larvae and pipetted them on agarose gel. Excess water was removed and 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 follow 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(-6myo6b: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.

Channelrhodopsin2 (ChR2) experiments

After blocking access to the surface until 4 dpf, we exposed wild type and lateral line mutants, with or without the *lhfpl5b:ChR2-EYFP* 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 with a one second delay between each pulse. Control larvae were kept in the same background, without blue light exposure. Conditions were held 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.

Oil experiments

Mutant larvae and wild type siblings were sorted into clear cylindrical containers (diameter: 5 cm , volume: 60 mL) with embryo media only or embryo media with a layer of lab grade mineral oil (Ward's Science, Rochester, NY) on the surface (thickness: 2 mm). 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. 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 1A). We measured swim bladder volume and buoyancy for each larva in the mutant and wild type populations (Figure 1B) and found that, on average, 53.7% (SD = 6.4%, n = 41) of lateral line mutants over-inflate their swim bladders and 7.3% (SD = 7.1%, n = 41) under-inflate their swim bladders during initial inflation. In wild type fish (including *lhfpl5b*^{vo35} heterozygotes), 0.0% (SD = 0.0%, n = 41) over-inflate and 4.9% (SD = 3.7%, n = 41) under-inflate their swim bladders during initial inflation on average (Figure 1C). The proportion of over-inflation is significantly different between mutant and wild type larvae (chi-square statistic: 70.45, $p < 0.00001$), while the proportion of under-inflation is not (chi-square statistic: 0.3546, $p = 0.552$). Average swim bladder volume of mutant fish is 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 1D). 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.

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

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 2). On average, 0% (SD = 0.0%, n = 43) of blocked mutant fish over-inflate their swim bladders, which is significantly different from mutants with access to the surface (chi-square statistic: 70.45, $p < 0.00001$; Figure 2B). The average volume of the blocked mutant swim bladder (M = 0.0033 mm^3, SD = 0.0015 mm^3) was significantly smaller than open access mutants (M = 0.014 mm^3, SD = 0.0027 mm^3) (One-way ANOVA with Tukey post-test, $p < 0.00001$), and blocked mutant larvae had statistically similar swim bladder volumes to blocked wild type larvae (M = 0.0037 mm^3, SD = 0.001 mm^3)

(One-way ANOVA with Tukey post-test, p = 0.927) (Figure 2C). From these data, we conclude that the *lhfpl5b* mutant hyperinflation phenotype requires the intake of exogenous air and is not caused by abnormal gas exchange from the surrounding media nor excess internal gas production.

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(-6myo6b:eGFP-lhfpl5a)vo23Tg* transgene, which uses a hair cell specific promoter (*myo6b*) to drive a GFP-tagged *lhfpl5a* gene (Erickson et al., 2017). It has been shown through FM dye uptake 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 observe no cases of hyperinflation in transgenic mutants (0%, SD = 0.0%, n = 17), which is significantly different from non-transgenic mutants (chi-square statistic: 82.12, p < 0.00001; Figure 3A). The average volume of the transgenic mutant swim bladder (M = 0.0057 mm^3, SD = 0.00001 mm^3) is significantly smaller than non-transgenic mutant siblings (M = 0.0109 mm^3, SD = 0.0031 mm^3) (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) (One-way ANOVA with Tukey post-test, p = 0.979, Figure 3B). Because the restoration of *lhfpl5* function specifically in hair cells mitigates hyperinflation in *lhfpl5b* mutants, we conclude 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 enteric system.

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 1 and Figure 4). Administering either single or repeated neomycin treatments during the initial swim bladder inflation period of 3 - 4 dpf results in a significant increase in the proportion of positively buoyant larvae (single neo chi-square statistic: 9.95, p = 0.0016, repeated neo chi-square statistic: 34.88, p < 0.0001; Supplemental Figure 1A) 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 1B). By comparison, a single CuSO4 treatment results in a hyperinflation phenotype that is statistically indistinguishable from lateral line mutants, consistent with a previous report that CuSO4 exposure slows hair cell regeneration compared to neomycin (Mackenzie and Raible,

2012). An average of 59.6% (*SD* = 7.7%, *n* = 72) of CuSO4-treated wild type larvae exhibit over-inflation, which is significantly different from wild type larvae (chi-square statistic: 39.69, $p < 0.0001$; Supplemental Figure 1A). The average swim bladder volume of the CuSO4-treated wild type larvae (*M* = 0.0126 mm^3, *SD* = 0.0027 mm^3) is statistically similar to the average swim bladder volume of untreated lateral line mutants (*M* = 0.0139 mm^3, *SD* = 0.0007 mm^3) (One-way ANOVA with Tukey post-test, $p = 0.813$) (Supplemental Figure 1B).

Since the anterior lateral line is likely to be the only part of the sensory organ to breach the air-water interface during surfacing, we predict that selective ablation of the head neuromasts will result in hyperinflation and that the trunk neuromasts will not play a role (Figure 4, Supplemental Figure 2). Ablation of the anterior region with CuSO4 results in an average of 35.9% (*SD* = 10.1%, *n* = 38) hyperinflated larvae, which is significantly different from unablated controls (chi-square statistic: 39.69, $p < 0.0001$; Figure 4B). Wild type larvae with head-specific ablations exhibit 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) (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) (One-way ANOVA with Tukey post-test, $p = 0.987$) (Figure 4C). Conversely, tail-specific CuSO4 treatments do not cause swim bladder over-inflation in wild type fish and result in statistically similar swim bladder volumes to untreated larvae (*M* = 0.012 mm^3, *SD* = 0.0031 mm^3) (One-way ANOVA with Tukey post-test, $p = 0.835$) (Figure 4C). The *lhfpl5b* mutant hyperinflation phenotype was not affected by either neomycin or CuSO4 treatments (data not shown). Taken together, we conclude that the anterior region of the lateral line system is critical to surface detection during initial swim bladder inflation.

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 predict 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. 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 5). Using this experimental design, we find that the average swim bladder volume of oil-exposed wild

type larvae (M = 0.016 mm^3, SD = 0.0029 mm^3) is significantly greater than control wild types (M = 0.007 mm^3, SD = 0.001 mm^3) (One-way ANOVA with Tukey post-test, $p < 0.0001$). Furthermore, oil-exposed wild types have statistically similar swim bladder volumes to that of *lhfpl5b* mutants (M = 0.014 mm^3, SD = 0.0038 mm^3) (One-way ANOVA with Tukey post-test, p = 0.886) (Figure 5C). We conclude that surface tension is one of the surface characteristics the lateral line is capable of detecting during initial swim bladder inflation.

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 the ability of larval fish to correctly inflate their swim bladders. To test this, we created a *lhfpl5b:ChR2-EYFP* transgenic line that expresses the blue light activated channel, Channelrhodopsin-2, specifically in lateral line hair cells (See Methods, Supplemental Figure 3). 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 do not exhibit positive buoyancy and have 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 6B). Transgenic wild types under blue light exhibit 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). As expected, we observe hyperinflation following CuSO4 treatment in stimulated wild type and mutant *lhfpl5b:ChR2* larvae, with average swim bladder volumes (WT: M = 0.012 mm^3, SD = 0.0037 mm^3, MUT: M = 0.011 mm^3, SD = 0.0023 mm^3) that are statistically similar to each other as well as experimental non-transgenic mutants (M = 0.011 mm^3, SD = 0.0024 mm^3) (One-way ANOVA with Tukey post-test, p = 0.998) (Supplemental Figure 3B). Overall, optogenetic activation of mechanically-insensitive lateral line organs in *lhfpl5b* mutants prevents the hyperinflation phenotype. Together, these data support the hypothesis that the lateral line provides critical sensory information during initial swim bladder inflation in larval zebrafish.

Lateral line mutants exhibit abnormal surfacing behaviors

Our results suggest that larval fish are using their head neuromasts to sense the unique stimulus located at the surface air-water interface. Even in wild type zebrafish, the interaction with the surface during initial swim bladder inflation is not well understood. To study this behavior in more detail, we analysed videos of the surfacing behavior from wild type (n = 7) and mutant (n = 14) larvae at 4 dpf. Mutant fish that will over-inflate their swim bladders make 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 will inflate to normal proportions (M = 31.9, SD = 19.2) (One-way ANOVA with Tukey post-test, p = 0.0004) (Figure 7A). Mutants destined for hyperinflation also spend 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 7B). 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 7C). We conclude that there is a correlation between time spent at the surface during the initial swim bladder inflation period and the resulting buoyancy status of larval zebrafish.

Discussion

Previous studies have shown that the surfacing behavior is necessary for intitial swim bladder inflation in many species of larval fish (Goolish and Okutake, 1999; Lindsey et al., 2010; Riley and Moorman, 2000; Ledebur and Wunder, 1937), but few have considered what sensory information is required to achieve neutral buoyancy. In this study, we show that lateral line mutants exhibit a rare swim bladder hyperinflation phenotype (Figure 1). We conclude that the mechanosensory lateral line detects the air-water interface ("surface"), which is critical during the surfacing behavior in larval fish for achieving neutral buoyancy.

Lateral line defects in lhfpl5b mutants are responsible for hyperinflation phenotypes

Lateral line mutants exhibit a rare swim bladder over-inflation phenotype that led us to investigate the lateral line's involvement in surfacing (Figure 1). A previous study demonstrated that lack of surface air during the critical inflation period causes swim bladder under-inflation in larval zebrafish (Goolish and Okutake, 1999). Likewise, we find that blocking access to the air-water interface eradicates over-inflation in lateral line mutants and greatly decreases the average swim bladder volume of mutant and wild type zebrafish populations (Figure 2). This method of

blocking access to the air-water interface resulted in some larvae (both wild type and mutant) inflating their swim bladder to normal proportions, though hyperinflation was never observed. Inflation was likely due to 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 behavioral defect in *lhfpl5b* mutants rather than a physiological one.

In support of this hypothesis, we show that transgenic rescue of hair cell function also restores normal swim bladder inflation in lateral line mutants (Figure 3). 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 1). Repeated neomycin sulfate 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 more rapid hair cell regeneration following neomycin treatment compared to CuSO4 (Hernández et al., 2006; Mackenzie and Raible, 2012; Murakami et al., 2003; Santos et al., 2006; Venuto and Erickson, 2021). Ototoxins have been extensively used to study lateral line-mediated behaviors (Baker and Montgomery, 1999; Bleckmann, 2006; Montgomery et al., 1997; Pavlov and Tyuryukov, 1993). However, we are not aware of any studies disrupting the lateral line before 4 dpf to study larval behavior, which is likely why this connection between the lateral line and swim bladder inflation has not been noted in current literature. However, 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).

The lateral line likely detects surface tension for initial swim bladder inflation

We expanded on the full-body lateral line ablation experiments by specificly ablating the head and / 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, our results implicate the head neuromasts in surface detection during surfacing. Ablation of the head neuromasts caused over-inflation while ablation of the tail neuromasts had no effect on either swim bladder volume nor buoyancy (Figure 4). These findings suggess that the posterior lateral

line is dispensible for the surfacing behavior, and only the head region that breaches the surface is critical for achieving neutral buoyancy. Our conclusions also provide further support for the anterior lateral line as a central mediator of surface-related behaviors.

It has been hypothesized that flying fish can recognize entrance and exit from the water through the lateral line (Tsukamoto and Yoshino, 1957). One of the reasons that the lateral line may be used for surface detection is that the interfacial tension between air and water represents a unique hydrodynamic stimulus compared to the rest of the water column. The surface tension of oil and water is half of the surface tension 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. We find that when given access to an oil-water interface, wild type larvae over-fill their swim bladders with oil to a volume comparable to lateral line mutants with air (Figure 6). Since oil is denser than air, we were unable to perform a buoyancy test on these larvae and categorized over-inflation based solely on swim bladder volume. Regardless, the abundance of wild type larvae exhibiting increased swim bladder volume when exposed to an oily surface is evidence that the lateral line is utilized for surface detection during initial swim bladder inflation. Furthermore, our findings predict that surface tension might be the main stimulus detected by the lateral line as larvae breach the surface.

Optogenetic stimulation of lateral line hair cells eliminates over-inflation in lhfpl5b mutants

The *lhfpl5b* mutant lacks lateral line function due to non-functional mechano-electrical transduction channels in the stereocilia of neuromast hair cells (Erickson, et al., 2020). Channelrhodopsin-2 expressed exclusively in lateral line hair cells provides the opportunity to elicit excitation of hair cells independently of mechanotransduction channel function. We found that reactivation of hair cells in this manner supresses the over-inflation phenotype of lateral line mutants, indicating that sensory information from the lateral line is indeed modulating the surfacing behavior (Figure 5). This experiment strengthens the argument for lateral line hair cell signal transduction being necessary for proper swim bladder inflation. Furthermore, our data suggest that the lateral line may be modulating early locomotor activity, but future studies investigating locomotion specifically are necessary to test this prediciton.

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 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 7). 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 (Goolish and Okutake, 1999; Lindsey et al., 2010; Rieger and Summerfelt, 1998). Therefore, the increase in breaching events likely leads to an increase in air deposition to the swim bladder in lateral line mutants.

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 photosensory (Suchocki and Sepulveda-Villet, 2019; Trotter et al., 2003) and somatosensory information may compensate for the loss of lateral line sensation and help mutant larvae achieve neutral buoyancy. The lateral line is involved several multi-sensory behaviors, including flow orientation (Bak-Coleman et al., 2013; Coombs et al., 2020; Liao, 2006), prey capture (Carrillo and McHenry, 2016; Nelson et al., 2002; 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. For example, the vestibular system guides vertical movement and is critical for coordinated locomotion in developing larvae (Bagnall and Schoppik, 2018; Ehrlich and Schoppik, 2017; Ehrlich and Schoppik, 2019). The lack of inflation when the vestibular sense is compromised indicates that the initial vertical climb of the surfacing behavior is largely dependent on vestibular sensation (Riley and Moorman, 2000). Meanwhile, our data support the idea that the lateral line is responsible for the surface detection and breaching aspects of surfacing. However, there are two main qualities of the surfacing behavior where the vestibular and lateral line sensory systems may overlap. The first is the change in buoyancy that could affect vertical swimming coordination (vestibular) for the larval fish as the swim bladder develops via multiple visits to the surface (lateral line) for air intake. The second is the requirement for a dynamic understanding of body position relative to the water column (vestibular) and hydrodynamics (lateral line) during surface breaches to regulate air intake. In conclusion, the lateral line and vestibular systems, and potentially other sensory systems mentioned above, are likely working together during surfacing to help larvae achieve neutral buoyancy.

Acknowledgements

The authors would like to thank Dr. Fadi Issa (ECU) for his support and technical advice. The authors also thank the Issa, Trapani, and Erickson lab members for fish care and maintenance.

Funding

This work was funded by East Carolina University's Research, Economic Development and Engagement Council (REDE) funding provided to T.E. and the East Carolina Department of Biology funding provided to A.V. This work was also supported in part by a National Sciences and Engineering Research Council of Canada (NSERC) award provided to T.E. and a National Institute on Deafness and Other Communication Disorders (NIDCD) award to J.G.T. (NIH R15DC014843).

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Figures

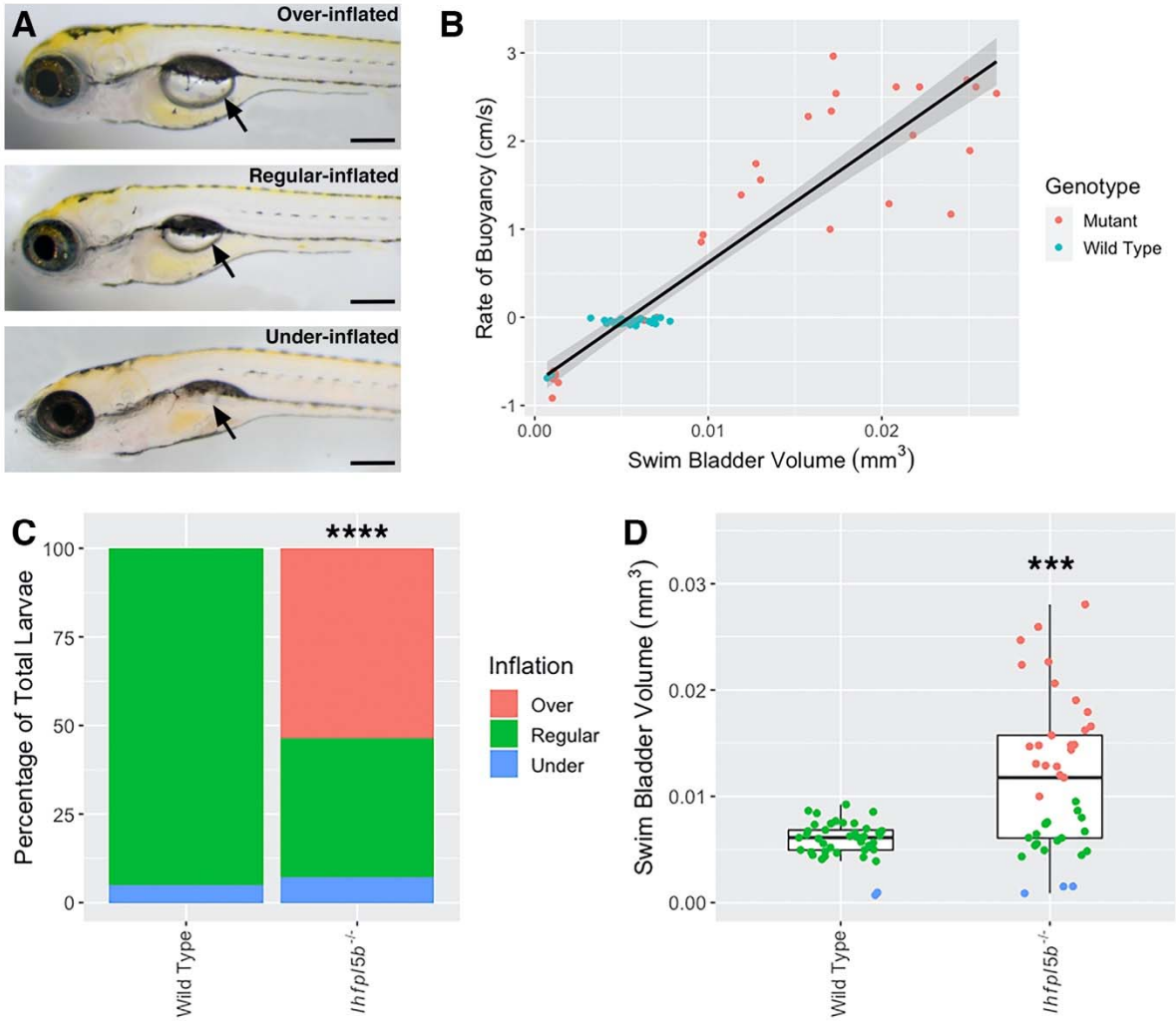

Figure 1. Hyperinflation of the swim bladder in lateral line mutants (*lhfpl5b*$^{-/-}$). A) Examples of over, regular, and under-inflated swim bladders (arrows) in larval zebrafish at 6 dpf (top: *lhfpl5b*$^{-/-}$, others are wild type). B) Correlation between buoyancy and swim bladder volume. Line 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. 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 bar = 0.2 mm.

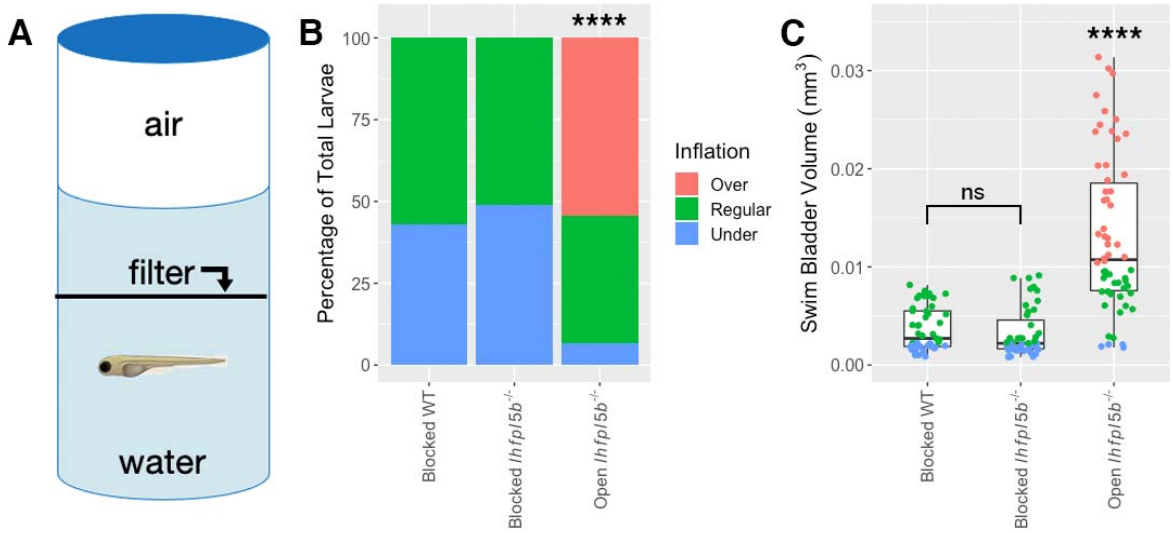

Figure 2: Blocking access to the surface eliminates the hyperinflation phenotype in lateral line mutants (*lhfpl5b*$^{-/-}$). A) Diagram of experimental set up with a filter blocking access to the surface. B) Percentage of larvae that exhibit over-, regular, and under-inflation of the swim bladder at 6 dpf. C) Swim bladder volume (mm^3) of larvae at 6 dpf. A One-Way ANOVA was used to determine significance. **** = $p < 0.0001$, , **** = $p < 0.0001$, ns = no significance. Full ANOVA table in supplemental tables.

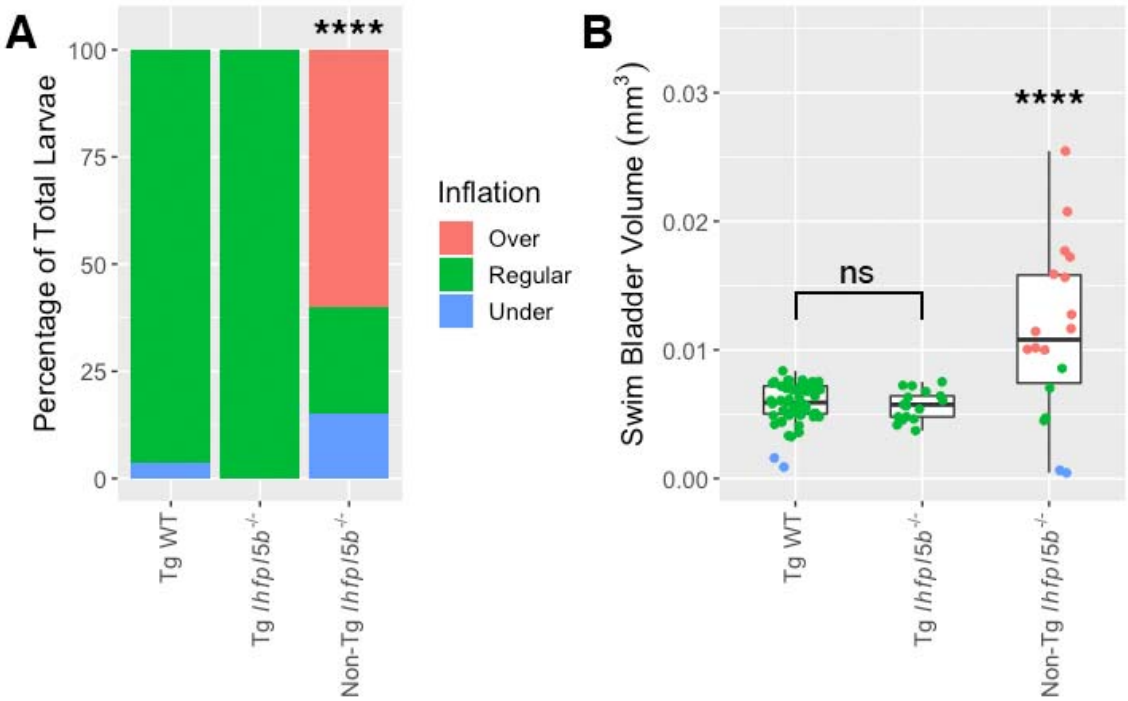

Figure 3: Transgenic rescue (*Tg(myo6b:eGFP-lhfpl5a)vo23)* of lateral line mutants (*lhfpl5b*$^{-/-}$). A) Percentage of larvae that exhibit swim bladder under, regular, and over-inflation at 6 dpf. B) Swim bladder volume (mm^3) of larvae at 6 dpf. A One-Way ANOVA was used to determine significance. **** = $p < 0.0001$, ns = no significance. Full ANOVA table in supplemental tables.

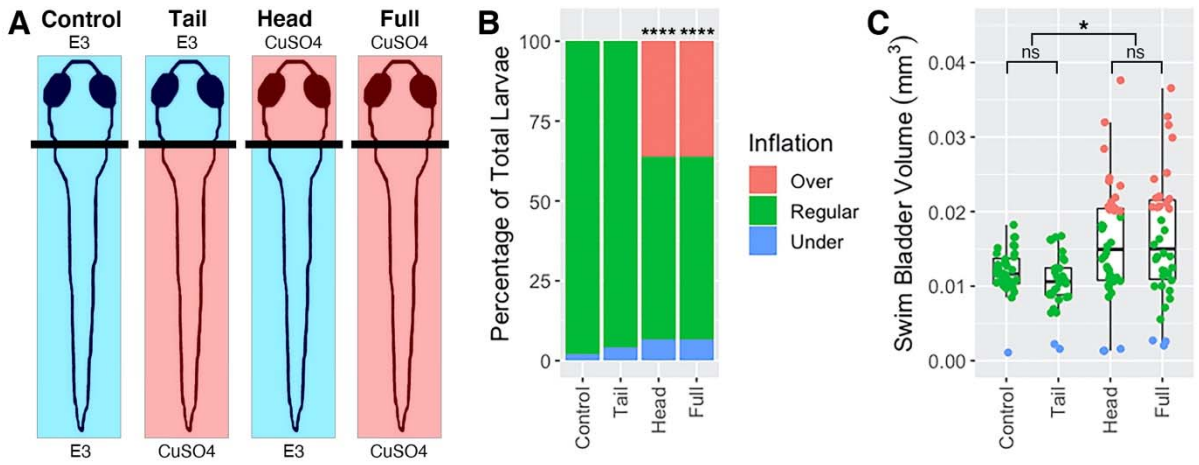

Figure 4: Head and tail specific ablations of the lateral line. A) Diagram of experiment. B) Percentage of larvae that exhibit swim bladder under, regular, and over-inflation at 5 dpf. C) Swim bladder volume (mm^3) of larvae at 5 dpf. A One-Way ANOVA was used to determine significance. * = $p < 0.05$, , **** = $p < 0.0001$, ns = no significance. Full ANOVA table in supplemental tables. Images of live larvae following treatment in Supplemental Figure 2.

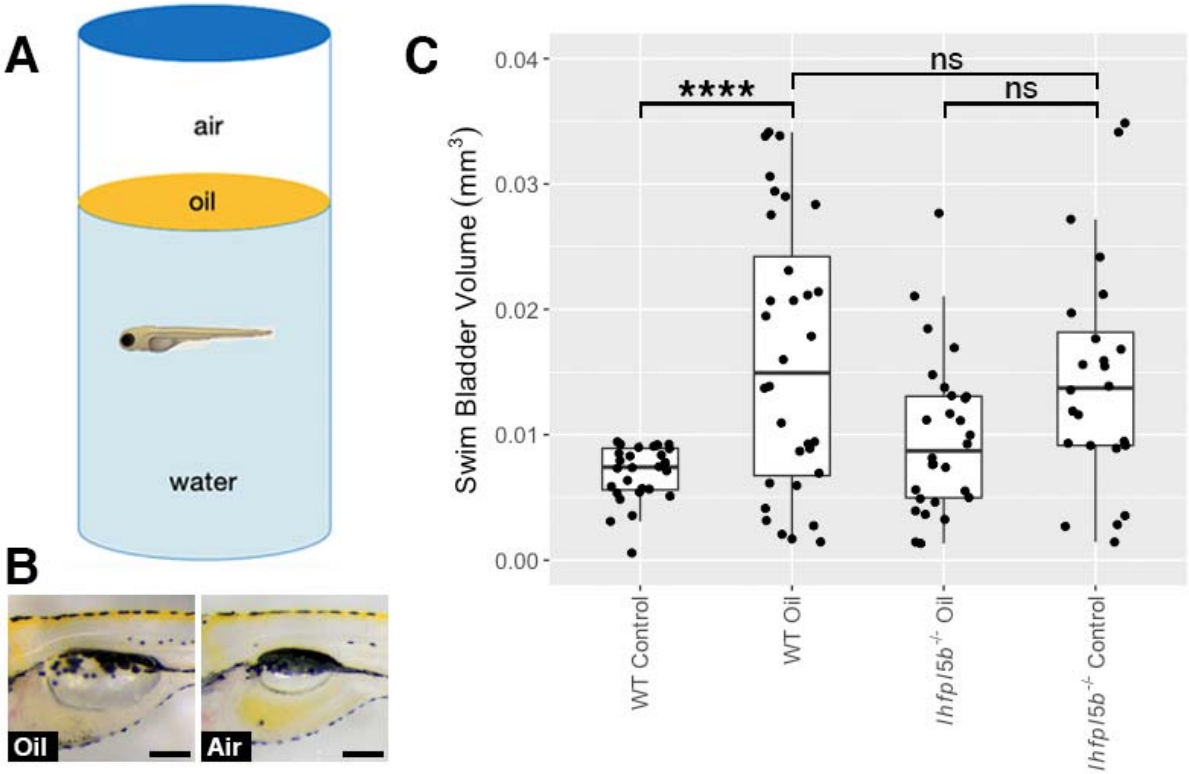

Figure 5: Decreasing interfacial surface tension with mineral oil results in over-filling of the swim bladder. A) Diagram of experiment with 2 mm layer of oil on surface. B) Images of swim bladder phenotypes resulting from surface oil and surface air exposure in wild type larvae at 6 dpf. C) Swim bladder volume (mm^3) at 6 dpf. A One-Way ANOVA was used to determine significance. * = $p < 0.05$, ns = no significance. Scale bar = 0.1 mm. Full ANOVA table in supplemental tables.

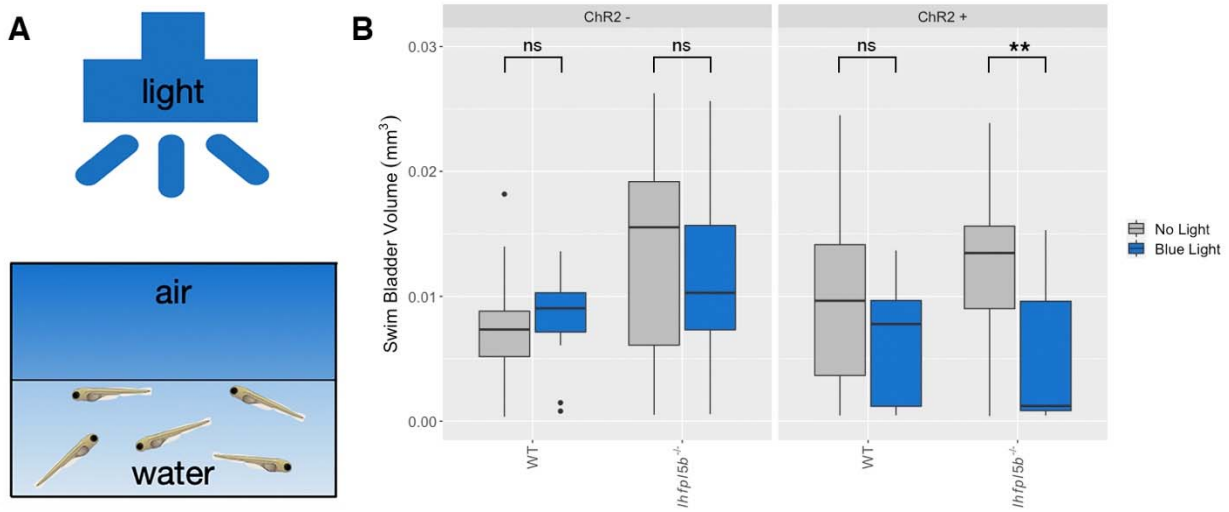

Figure 6: Channelrhodopsin-2 (ChR2) activated in lateral line hair cells during 4-6 dpf using the *Tg(lhfpl5b2028:ChR2-EYFP-pA)* transgene. A) Diagram of experiment with blue light overhead flashing for 25 ms with 1 s intervals. B) Swim bladder volume (mm^3) of larvae at 6 dpf, n = 16-21 per condition. One-Way ANOVA was used to determine significance. ** = $p < 0.001$, ns = no significance. Full ANOVA table in supplemental tables.

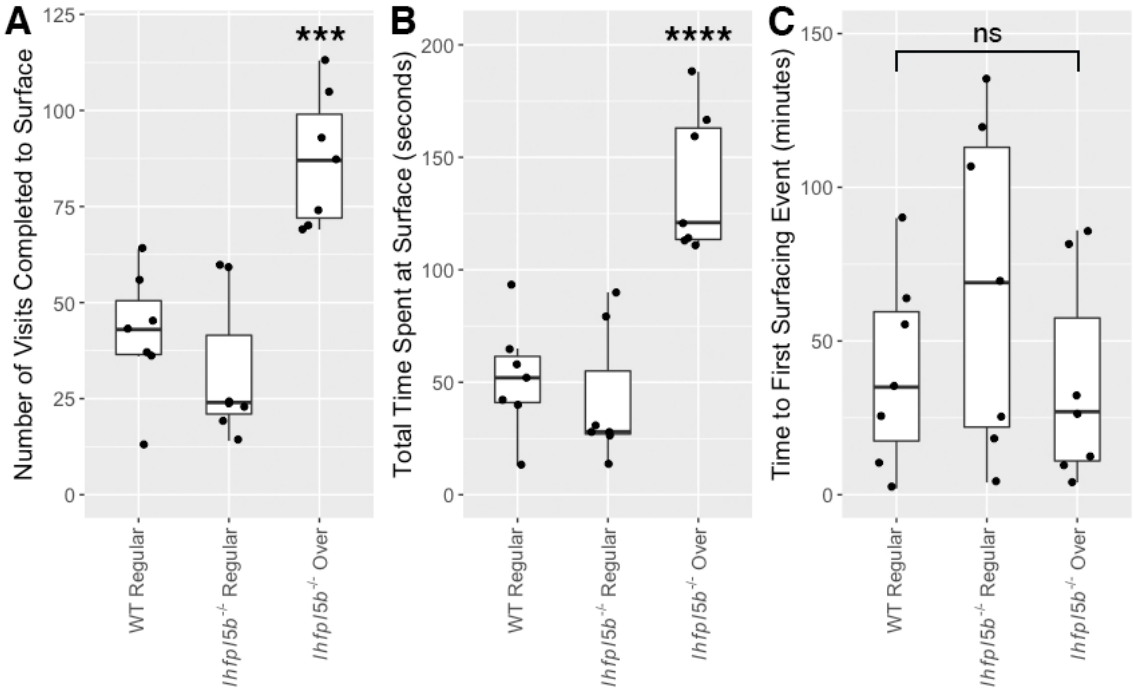

Figure 7: Quantification of the surfacing behaviors of wild type and *lhfpl5b* mutant zebrafish larvae during initial swim bladder inflation. A) Total number of individual visits taken by larval fish to the air-water interface on 4 dpf for 12 hours (9 AM – 9 PM). B) Total amount of time larval fish spend 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 = no significance.