

EVOLUTIONARY CONVERGENCE OF A NEURAL MECHANISM IN THE CAVEFISH LATERAL LINE SYSTEM

Sensory afferents are more active in blind cavefish

Elias T. Lunsford¹, Alexandra Paz², Alex C. Keene^{2,3}, and James C. Liao¹

¹The Whitney Laboratory for Marine Bioscience

Department of Biology

University of Florida

Saint Augustine, FL USA 32080

²Department of Biological Sciences

Florida Atlantic University

Jupiter, FL USA 33458

³Department of Biology

Texas A&M University

College Station, TX USA 77843

Phone: 904-201-8404

Email: jliao@whitney.ufl.edu

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34

35 **Abstract (163/200)**

36 Animals can evolve dramatic sensory functions in response to environmental constraints,
37 but little is known about the neural mechanisms underlying these changes. The Mexican tetra,
38 *Astyanax mexicanus*, is a leading model to study genetic, behavioral, and physiological evolution
39 by comparing eyed surface populations and blind cave populations. We compared
40 neurophysiological responses of posterior lateral line afferent neurons and motor neurons across
41 *A. mexicanus* populations to reveal how shifts in sensory function may shape behavioral diversity.
42 These studies indicate differences in intrinsic afferent signaling and gain control across
43 populations. Elevated endogenous afferent activity identified a lower response threshold in the
44 lateral line of blind cavefish relative to surface fish. We next measured the effect of inhibitory
45 corollary discharges from hindbrain efferent neurons onto afferents during locomotion. We
46 discovered that three independently-derived cavefish populations have evolved persistent afferent
47 activity during locomotion, suggesting for the first time that regression of the efferent system can
48 be an evolutionary mechanism for neural adaptation of a vertebrate sensory system.

49

50 **Introduction**

51 Our understanding of the sensory systems and behavior of animals is challenging to
52 contextualize within the framework of evolution. Anatomical comparisons between species have
53 allowed us to infer sensory capabilities, but this approach cannot directly reveal neural function.
54 Discovery of neural mechanisms that underlie behavior are often constrained to a limited number
55 of model species (Jourjine & Hoekstra, 2021). Like morphology, neural circuits can adapt to the
56 environment. Of these, many circuits are sensory and regulate essential behaviors such as foraging,
57 navigation, and escapes (Blin et al., 2018; Hoke et al., 2012; Hüppop, 1987; Paz et al., 2020).

58 The Mexican blind cavefish, *Astyanax mexicanus*, is a powerful model to understand the
59 evolution of physiological and molecular traits that contribute to behaviors such as sleep (Duboué
60 et al., 2011; J. B. Jaggard et al., 2018) and prey capture (Lloyd et al., 2018; Yoshizawa et al.,
61 2014). *Astyanax mexicanus* exists in two morphs; 1) eyed surface-dwelling populations and 2)
62 blind cave populations. There are at least 30 independently evolved cavefish populations in the
63 caves of the Sierra de El Abra region of Northeast Mexico (Herman et al., 2018; McGaugh et al.,
64 2020; RW Mitchell et al., 1997). *Astyanax mexicanus* populations are interfertile, and this attribute
65 has allowed investigators to demonstrate independent convergence of numerous behavioral,
66 developmental, and physiological traits (Chin et al., 2018; Kowalko, 2020; Riddle et al., 2018;
67 Stockdale et al., 2018; Varatharasan et al., 2009). Our goal is to apply a neurophysiological
68 approach across multiple *A. mexicanus* populations to examine the functional evolution of the
69 lateral line sensory system.

70 The mechanoreceptive hair cells of the lateral line system detects fluid motion relative to
71 the body and play an important role in essential behaviors (McHenry et al., 2009; Mekdara et al.,
72 2018; Olszewski et al., 2012; Oteiza et al., 2017; Stewart et al., 2013). Cavefish have evolved

73 anatomical enhancements of the lateral line, presumably to compensate for the loss of vision
74 (Kowalko, 2020; McGaugh et al., 2020; Teyke, 1990; Yoshizawa et al., 2014). These anatomical
75 alterations have been linked to substantial changes in behavior (Lloyd et al., 2018; Yoshizawa et
76 al., 2010). However, almost nothing is known about physiological changes that accompany
77 evolution, despite the fact that the response of peripheral senses to environmental change has been
78 well documented (Kelley et al., 2018; McBride, 2007).

79 Endogenous depolarizations within sensory cells are transmitted to afferent neurons
80 (hereafter “afferents”) as spontaneous action potentials (hereafter “spikes”), and are essential for
81 maintaining a state of responsiveness and sensitivity (Dey et al., 2021; Douglass et al., 1993;
82 Köppl, 1997) (Kiang, 1965; Manley & Robertson, 1976). This is true in the lateral line, where
83 spontaneous depolarizing currents within the hair cell maintain a resting potential within the
84 critical activation range of channels. This range is required to ensure transmitter release; thus these
85 currents decrease the detection threshold of the system (Trapani & Nicolson, 2011). Spontaneous
86 afferent activity is an established and reliable target for probing the neurophysiological basis of
87 sensitivity across taxa (Hedwig, 2006; Krasne & Bryan, 1973; Mohr et al., 2003). Here, we use
88 spontaneous afferent activity as an entry point into understanding the neural mechanism
89 underlying lateral line function in cavefish.

90 Another important mechanism that determines lateral line sensitivity is an inhibitory
91 feedback effect of the efferent system during swimming. Feedback mechanisms in general sculpt
92 sensory systems in important ways; for example, by changing detection thresholds by altering the
93 transmission frequency of afferent spikes (Crapse & Sommer, 2008; Straka et al., 2018). The
94 efferent system of hair cells in particular has repeatedly evolved to modulate sensory processing
95 (Köppl, 2011). More specifically, hindbrain efferent neurons (hereafter “efferents”) issue

96 predictive signals that transmit in parallel to locomotor commands, termed corollary discharge
 97 (CD). CDs inhibit afferent activity to mitigate sensor fatigue that can result from self-generated
 98 stimuli (Russell & Roberts, 1972). CD is an important mechanism for sensitivity enhancement but
 99 has rarely been implicated for ecologically-relevant behaviors. For example, active-flow sensing
 100 by cavefish depends on detecting reafferent signals while swimming (Tan et al., 2011). Increased
 101 reliance on self-generated fluid motion (Odstreil et al., 2021; Patton et al., 2010; Teyke, 1985) is
 102 divergent from our current understanding of the CD's role in predictive motor signaling in the
 103 lateral line (Lunsford et al., 2019; Pichler & Lagnado, 2020).

104 For the first time, we identify a neurophysiological mechanism that has convergently
 105 evolved across *A. mexicanus* populations to increase hair cell sensitivity after eye loss. By
 106 investigating how differences in afferent and efferent signaling contribute to sensory enhancement
 107 in a comparative model, we provide insight into a potentially ubiquitous mechanism for sensory
 108 evolution.

Results

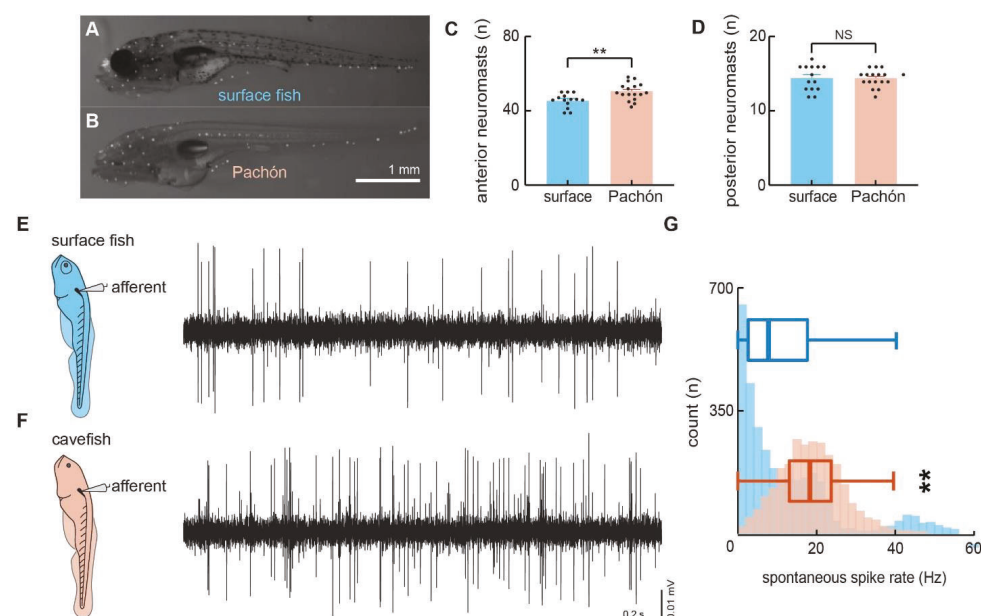


Figure 1. Spontaneous afferent neuron activity is elevated in blind cavefish. DASPEI staining of 6 dpf surface (A) and cavefish (B) show significantly different quantities of anterior lateral line neuromasts (C) and similar quantities of posterior neuromasts (D). Extracellular recordings were made in posterior lateral line afferents where the neuromast densities were similar in order to resolve the differences observed in afferent activity between larval surface fish (E) and Pachón cavefish (F) between 4-7 dpf. Number of occurrences and median intrinsic spike rates in both surface (blue; 12.4 Hz, n=10 fish) and Pachón (red; 18.6 Hz, n=5 fish) fish suggests that lateral line response thresholds in cavefish are lower than those of surface fish (G). Error bars are $\pm$ SE.

Neuromasts of surface fish and Pachón cavefish larvae (6 days post fertilization; dpf) were labeled via 2-[4-(Dimethylamino)styryl]-1-ethylpyridinium iodide (DASPEI) staining and subsequently imaged (Figure 1A-B). Anterior lateral line neuromasts had previously been shown to differ in quantities and morphology as early as 2 months post fertilization between surface and cave fish (Yoshizawa et al., 2010). Here we show that Pachón cavefish exhibit this significant increase in anterior neuromast quantity as early as 6 dpf ($p < 0.01$, $t = 3.168$, $df = 29$; Figure 1C).

118 In contrast, both populations exhibit a similar number of posterior lateral line neuromasts ($p =$
119 0.77 , $U = 111.5$; Figure 1D). Therefore, to investigate differences in underlying neurophysiology,
120 we concentrated on the posterior lateral line system where neuromast density is similar across
121 blind and surface morphs. We exclusively probed the posterior lateral line afferent neurons to
122 establish whether sensory systems that are anatomically similar exhibit neurophysiological
123 differences that contribute to enhanced sensitivity.

124 To examine the physiological basis of differences in lateral line function across surface and
125 cavefish we used extracellular lateral line recordings adapted from protocols used in zebrafish
126 (Figure 1E-F). Extracellular recordings of posterior lateral line afferents revealed intrinsic
127 spontaneous activity was higher in Pachón cavefish (18.6 ± 0.2 Hz) while the animal was at rest,
128 relative to surface fish (12.4 ± 0.3 Hz; $p < 0.01$, $t = 15.97$, $df = 5,795$; Figure 1G). Instantaneous
129 afferent spike rate demonstrates substantial decreases during swimming in surface fish (surface =
130 $3,167$ swim bouts) and little effect in Pachón cavefish (Pachón = $2,612$ swim bouts; Figure 2 A-
131 B). We quantified and compared spike rates during swimming relative to the pre-swim period to
132 examine patterns of the inhibitory effect between populations. During most surface fish swim
133 bouts there was a reduction in afferent activity ($n = 1,966/2,291$, 85.8%), many of which resulted
134 in complete quiescence of transmissions ($n = 1,112/2,291$, 48.5%). Conversely, afferent activity
135 partially reduced during many swim bouts in Pachón cavefish ($n = 1,303/2,439$, 53.4%), but very
136 few instances led to complete inhibition ($n = 275/2,439$, 11.3%). The distributions of relative spike
137 rates during swimming reveal surface fish have a higher likelihood of experiencing no afferent
138 activity during swimming while cavefish experience afferent activity during swimming similar to
139 that of pre-swim activity levels (Figure 2 C). Therefore, surface fish experience significantly
140 higher levels of inhibition ($68.5 \pm 0.01\%$) compared to cavefish ($28.9 \pm 0.01\%$; $p < 0.01$, $t = 36.5$,

141 df = 4449; Figure 2 D). Surface fish demonstrate lateral line inhibition during swimming
142 comparable to other fishes with intact visual systems (Flock & Russell, 1973; Lunsford et al.,
143 2019; Pichler & Lagnado, 2020; Russell & Roberts, 1972) suggesting cavefish have evolved a
144 unique functional phenotype for sensory gain control.

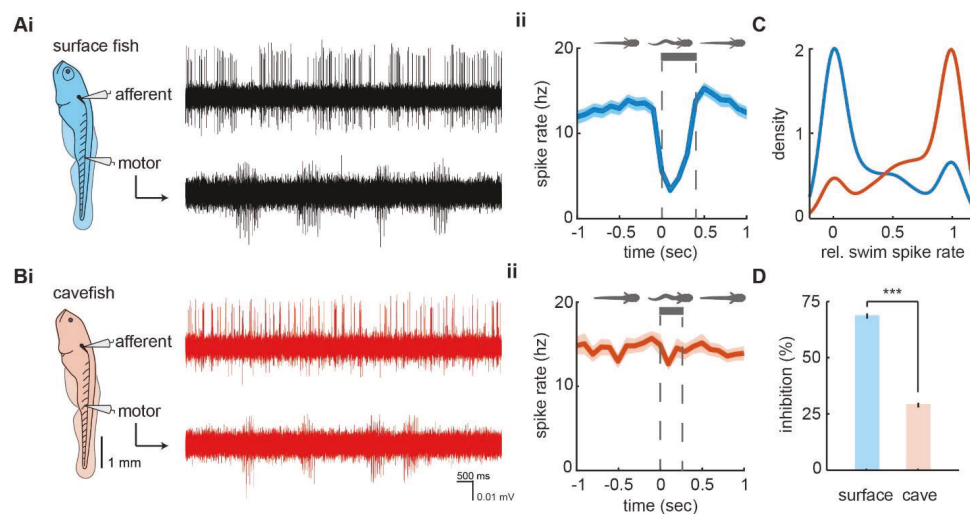


Figure 2. Afferent spike rate decreases during swimming in surface fish but not cavefish. Simultaneous recordings from afferent neurons from the posterior lateral line afferent ganglion and ventral motor roots along the body in (Ai) surface fish and (Bi) Pachón cavefish. Afferent spike rate decreases at the onset of swimming (time = 0) in (Aii) surface fish while (Bii) Pachón cavefish spike rate remained relatively constant during swimming. Bars represent average swim duration for surface fish (0.39 sec, n = 2,272 swims) and Pachón cavefish (0.27 sec, n = 2,429 swims). C. Kernel density estimate of spike rate during swimming relative to the pre-swim interval in both surface fish (blue) and cavefish (red). D. Surface fish experience greater levels of inhibition during swimming than cavefish. Significance level indicated by '***'. Error bars are $\pm$ SE.

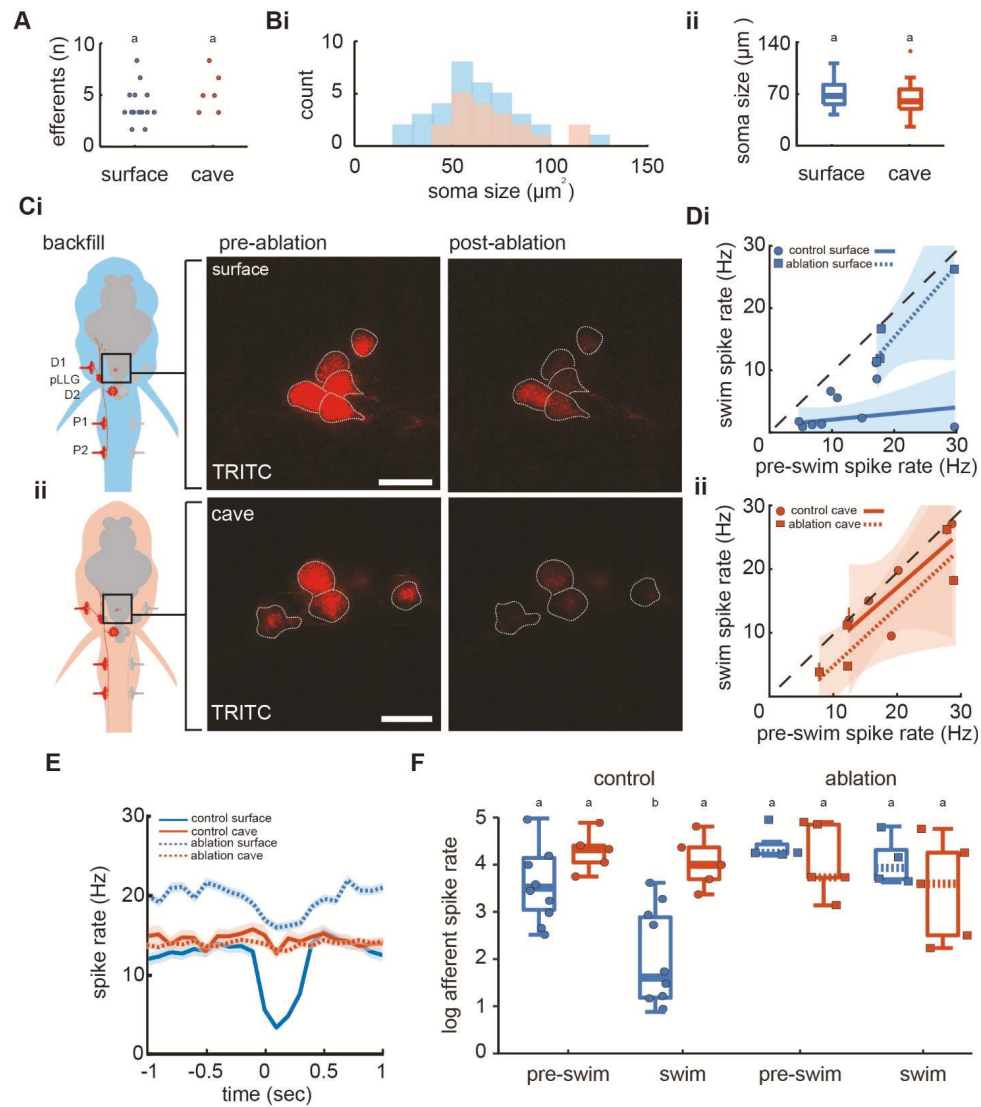
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146 We imaged hindbrain cholinergic efferent neurons to determine anatomical and functional
147 connectivity. Between populations, backfilled efferents revealed similar soma quantities (surface:
148 2.4 ± 0.3 ; cave: 3.2 ± 0.5 ; $p = 0.2$, $t = 1.36$, $df = 18$) and size (surface: $62.1 \pm 3.6 \mu\text{m}^2$; cave: 70.9
149 $\pm 4.5 \mu\text{m}^2$; $p = 0.1$, $t = 1.50$, $df = 51$; Figure 3 A-C). From electrophysiological recordings, we

150 observed average spike rates during and prior to a swim were not positively correlated in control
 151 surface fish ($r^2 = 0.15$, $F_{1,13} = 2.3$, $p = 0.2$), but the slope of the line indicates a fractional
 152 suppression of 78% that is significantly less than unity (slope 0.1, confidence interval (CI): -0.2-
 153 0.4; Figure 3 Di). After efferent ablation, surface fish average spike rates during and prior to a
 154 swim were not distinguishable from unity (slope 1.2, CI = -0.1-2.4) indicating that spike rates
 155 during swimming intervals differ from non-swimming intervals no differently than chance and
 156 therefore there is no detectable inhibitory effect. While swimming, surface fish without
 157 functioning efferent neurons demonstrated a signaling phenotype similar to both intact cavefish
 158 (slope 0.88, CI = -0.5-2.2) and cavefish with ablated efferents (slope 0.9, CI = 0.1-1.8; Figure 3
 159 Dii). Therefore, putative cholinergic hindbrain efferents are necessary for afferent inhibition in
 160 surface fish, but do not demonstrate modulatory control of afferents in cavefish.

161 We compared pre-swim and swim spike rates across populations and treatments to
 162 determine efferent contribution to inhibition (Figure 3 E). We found significant differences in
 163 afferent activity among pre-swim and swim intervals ($F_{7,40} = 7.6$, $p < 0.01$; Figure 3 F). Surface
 164 fish afferent spike rates during swimming (3.9 ± 0.1 Hz) were 71% lower than the immediate pre-
 165 swim period in control fish (13.7 ± 0.2 Hz; Tukey's post hoc test, $p < 0.01$). Post-swim spike rate
 166 (13.7 ± 0.3 Hz) recovered to pre-swim spike rate. In control Pachón cavefish, we observed some
 167 decrease in afferent spike rate during swimming (16.8 ± 0.2 Hz) but it was not statistically
 168 discriminated from pre-swim (20.5 ± 0.2 Hz; Tukey's post hoc test, $p = 0.94$). Efferent ablation in
 169 surface fish resulted in afferent activity during swimming (17.4 ± 0.3 Hz) to increase to pre-
 170 swim levels (20.7 ± 0.3 Hz; Tukey's post hoc test, $p = 0.99$). Ablated surface fish also demonstrated
 171 spike dynamics during swimming comparable to ablated and control cavefish (Pachón ablated,
 172 pre-swim: 18.6 ± 0.3 Hz; Pachón ablated, swimming: 14.7 ± 0.3 Hz). These findings indicate that

173 efferents are necessary for inhibition of afferents during swimming in surface fish, but not
174 cavefish.



175

Figure 3. Efferent neurons are necessary for inhibition observed in afferents during swimming in surface fish but not cavefish. **A.** Backfilled hindbrain cholinergic efferent neurons were present in comparable numbers (2-3 cells) in both surface fish (n = 14) and cavefish (n = 6). **Bi.** Efferent soma size in surface (blue) and cavefish (red) is similar in both populations (**ii**). **C.** Efferent cell bodies were identified by backfilling rhodamine through posterior lateral line neuromasts in both surface fish (**i**) and cavefish (**ii**) and were ablated with a 30-s UV pulse. Scale bar: 20 μ m. **D.** The line of best fit of spike rates before compared to during the swim significantly excludes unity in non-ablated, control surface fish (circle), but not in ablated surface fish (square), implying spike rate suppression in the former but not the latter (**i**). The slope of the line for control fish suggests the inhibition is not correlated to the spontaneous afferent activity preceding the swim. The line of best fit of Pachón cavefish pre-swim and swim spike rates did not exclude unity in both control (circle) and ablated (square) treatments (**ii**). Dashed line indicates the line of unity, corresponding to no average difference of spike rate during swimming. **E.** Instantaneous spike rates of Pachón cavefish were unaffected by ablating the lateral line whereas the inhibitory effect was eliminated in ablated surface fish. Time is relative to the onset of motor activity. **F.** Surface fish (blue) display reduced spike rates during swimming compared to before swimming in control fish. Pachón cavefish (red) did not display reduced spike rate during swimming in neither control nor ablated treatments. Ablated surface individuals also did not display reduced spike rate during swimming resulting in a signaling phenotype comparable to cavefish. Groupings of statistical similarity are denoted by ‘a’ and ‘b’, whereas a is significantly different from b. All error bars represent $\pm$ SE.

176

177 We compared lateral line activity between three different cave populations (Figure 4 A); the Tinaja
178 and Pachón populations, which are derived from similarly timed colonization events, and the
179 Molino population that is derived from a more recent colonization event (Brdic et al., 2012;
180 Dowling et al., 2002; Herman et al., 2018). Blind cavefish populations exhibited similar
181 spontaneous spike rates across populations ($F_{2,17} = 0.68$, $p = 0.52$), and swimming showed little
182 effect on lateral line activity across blind cavefish populations (Figure 4 B). We observed a minor
183 decrease in afferent spike rates across cavefish populations and the relative change (i.e. inhibition)
184 was similar in Pachón and Tinaja (Tukey’s post hoc test; Pachón: 0.28 ± 0.01 ; Tinaja: 0.32 ± 0.02 ;
185 Figure 4 C). Molino demonstrated an intermediate phenotype compared to Pachón and Tinaja
186 (Tukey’s post hoc test; Molino: 0.23 ± 0.01 ; $p < 0.01$). The Molino population (new lineage) is
187 most distantly related to the Pachón and Tinaja populations (old lineage), originating from a more
188 recent surface fish colonization of caves thus providing phylogenetic evidence that

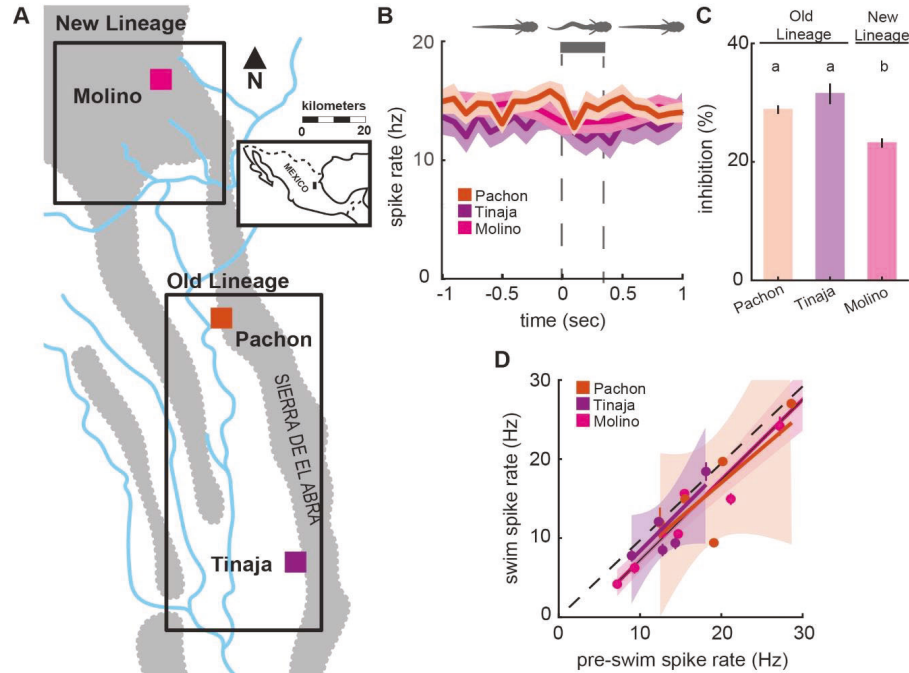


Figure 4. Enhanced lateral line sensitivity during swimming convergently evolved across three blind populations. **A.** Molino cave populations (pink; New Lineage) have evolved more recently relative to Pachón (red) and Tinaja (purple; Old Lineage) cave populations. Lineage delineations inferred from phylogenetic data (Herman et al. 2018). **B.** Mean spontaneous afferent spike rate remains constant at the onset of fictive swimming (time = 0) in Pachón (n = 5), Molino (n = 8), and Tinaja (n = 5) populations. Bar represents average swim duration for Pachón (0.27 sec, n = 2,429 swim bouts), Molino (0.42 sec, n = 1,474 swim bouts), and Tinaja (0.35 sec, n = 464). **C.** Percent change in spike rate from pre-swim to swim intervals (i.e. inhibition) was small, but significantly different between blind cave populations. Post-hoc Tukey test revealed that Molino cavefish experienced significantly less reduction in spike rate when compared to Pachón and Tinaja populations. Statistically similar groups are indicated by 'a' and 'b'. **D.** The line of best fit of pre-swim and swim spike rates does not significantly exclude unity in any of the blind cavefish populations implying there is no detectable inhibitory effect. Dashed line indicates the line of unity, corresponding to no average difference of spike rate during swimming. All values represent mean $\pm$ SE.

coincides with statistically similar groupings (Herman et al., 2018). We examined the correlation

192 between non-swimming and swimming spike rates across cave populations to determine whether
193 the inhibitory effect was significant. Spike rates during swimming and pre-swim intervals were
194 not positively correlated in all cave populations, and afferent spike rates during swimming were
195 not statistically distinguishable from unity across populations (Pachón: slope = 0.88, CI = -0.5 –
196 2.2; Tinaja: slope = 1.03, CI = -0.2 - 2.2; Molino: slope = 1.02, CI = 0.8 - 1.2; Figure 4 D),
197 indicating there was no significant decrease in afferent activity during swimming within blind
198 cavefish populations.

199 **Discussion**

200 Our principle finding indicates that elevated afferent activity underlies the increased
201 responsiveness of cavefish to flow stimuli. The increased afferent activity in cavefish is a likely
202 consequence of eye-loss, which has robust effects on other physiological systems (Duboué et al.,
203 2011; Varatharasan et al., 2009). Heightened lateral line sensitivity in adults has been previously
204 attributed to increased neuromast density in the anterior lateral line (ALL) (J. Jaggard et al., 2017;
205 Lloyd et al., 2018; McHenry et al., 2008; Patton et al., 2010; Teyke, 1990; Yoffe et al., 2020, 2020;
206 Yoshizawa et al., 2010). This difference in neuromast quantity does not exist in the posterior lateral
207 line at the larval stage, allowing us a unique opportunity to investigate the neural mechanisms that
208 can enhance flow sensing in a strong model for evolution. Our discovery of increased spontaneous
209 activity in cavefish is a powerful addition for flow sensing, and we predict that this in combination
210 with the increased density of neuromasts in the ALL is what ultimately enables cavefish to perform
211 active flow sensing (Yoshizawa et al., 2010).

212 Higher spontaneous activity is one of two mechanisms that are responsible for higher
213 lateral line sensitivity. The other involves the inhibitory effects of the efferent system during
214 swimming, a feedback mechanism that is conserved across the diversity of fishes (Flock & Russell,
215 1973; Lunsford et al., 2019; J. Montgomery et al., 1996; J. C. Montgomery & Bodznick, 1994;

216 Roberts & Russell, 1972; Tricas & Highstein, 1991). This is true in swimming surface fish but not
 217 in cavefish. We found that three blind populations of cavefish (i.e. Pachón, Molino, Tinaja) have
 218 repeatedly lost the capability for efferent modulation during swimming. When one considers that
 219 lateral line efferent activity can be driven by direct inputs from the visual system in zebrafish
 220 (Reinig et al., 2017), it seems possible that eye degeneration induces the loss of efferent function.
 221 This interpretation is consistent with the idea that lateral line efferents are thought to have
 222 undergone regressive loss before in the ancestral lamprey and hagfish (Kishida et al., 1987; Köppl,
 223 2011; Koyama et al., 1990), both of which are nearly or completely blind during development
 224 (Dickson & Collard, 1979; Fernholm & Holmberg, 1975). However, our results illustrate that
 225 cholinergic efferent system is still present in cavefish, having lost functionality rather than
 226 disappearing (the efferent system is functional in surface fish). Exploring pre- and postsynaptic
 227 differences such as acetylcholine release or the density of nicotinic acetylcholine receptors
 228 (nAChR) may explain the reduced inhibitory efficacy and reveal molecular targets that could
 229 disrupt efferent function over the course of evolution (Dawkins et al., 2005).

230 Our demonstration of CD inactivity in cavefish provides an alternative mechanism by
 231 which evolution can enhance sensitivity, one that proceeds by decreasing inhibition rather than
 232 augmenting sensor morphology or density (Yamamoto et al., 2009; Yoshizawa et al., 2010, 2014).
 233 The impact of increasing sensitivity through a lack of inhibition is apparent during active-flow
 234 sensing in adult *A. mexicanus*. Active-flow sensing occurs during swimming and involves using
 235 the reflection of self-generated flow fields (Bleckmann et al., 1991) to follow walls (Patton et al.,
 236 2010), avoid obstacles (Teyke, 1985; Windsor et al., 2008), and discrimination between shapes
 237 (De Perera, 2004; Hassan, 1989; Von Campenhausen et al., 1981). The repeated loss of inhibitory

238 feedback across blind cavefish populations suggests that it is easier to cease function than to
239 develop more neuromasts or other additive alternatives.

240 Cavefish swim by using different body motions than surface fish. This finding has been
241 interpreted as a mechanism to enhance wall following, which occurs when the bow wake of a
242 swimming cavefish is reflected off of a solid surface and then detected (Patton et al., 2010; Sharma
243 et al., 2009). Altered swimming kinematics is also thought to have arisen from a general increase
244 in sensitivity to flow (Tan et al., 2011; Windsor et al., 2008). Our results provide an alternate
245 suggestion; the lack of efferent function in cavefish precludes the sensory feedback necessary for
246 sensing self-movement and body position in water (proprioception). Corollary discharge, a parallel
247 motor command that decreases the afferent activity of fishes when swimming, has recently been
248 found to play a critical role in swimming efficiency by enabling the tracking of the traveling body
249 wave during undulation (Skandalis et al., 2021). We hypothesize that the evolved loss of efferent
250 function that enables cavefish to successfully avoid collisions in subterranean habitats is likely
251 favored over optimizing swimming efficiency (Nakamura, 1997; Uysal et al., 2010). We predict
252 that loss of efferent function will be found in other blind hypogean species (Costa Sampaio et al.,
253 2012) and that their respective surface populations will possess intact efferent functionality, as we
254 have found in *A. mexicanus*. Neurophysiological recordings across a wider diversity of species
255 would provide valuable insight into how efferent function may be sculpted by environmental
256 selection and phylogenetic membership.

257 By employing neurophysiological approaches in the lateral line system of *A. mexicanus* for
258 the first time, we show that elevated lateral line afferent activity and loss of efferent function have
259 repeatedly evolved together across cavefish populations. Our findings come at a time when genetic
260 tools in *A. mexicanus* enable brain-wide imaging and gene-editing based screening to identify

261 candidate neural circuits and genes critical in the evolution of sensory systems (Jaggard et al.,
262 2020; Warren et al., 2021). Going forward, applying genetic and electrophysiological tools in well-
263 characterized neural circuits promises to inform our understanding of the evolution of neural
264 systems and behavior more broadly.

265 **Materials and Methods**

266 *Animals:* Fish were progeny of pure-bred stocks originally collected in Mexico (Duboué et
267 al., 2011) that have been maintained at the Florida Atlantic University core facilities. Larvae were
268 raised in 10% Hank's solution (137 mM NaCl, 5.4 mM KCl, 0.25 mM Na₂HPO₄, 0.44 mM
269 KH₂PO₄, 1.3 mM CaCl₂, 1.0 mM MgSO₄, 4.2 mM NaHCO₃; pH 7.3) at 26°C. All experiments
270 were performed according to protocols approved by the University of Florida or Florida Atlantic
271 University Institutional Animal Care and Use Committee. Animal health was assessed by
272 monitoring blood flow throughout each experiment.

273 *Neuromast imaging:* To assess neuromast quantities, larvae aged six dpf were submerged in 5
274 µg/ml DASPEI dissolved in embryo medium for 15 minutes as previously described (Van Trump
275 et al., 2010). Larvae were then transferred to ice-cold water for 30-45 seconds then immersed in
276 8% methylcellulose for imaging. Images were taken using a Nikon DS-Qi2 monochrome
277 microscope camera mounted on a Nikon SMZ25 Stereo microscope (Nikon; Tokyo, Japan).
278 Neuromasts innervated by posterior lateral line afferents and anterior lateral line afferents were
279 tabulated separately.

280 *Electrophysiology:* Prior to recordings, *A. mexicanus* larvae (4-7 dpf) were paralyzed using
281 0.1% α -bungarotoxin (Lunsford & Liao, 2021). Once paralyzed, larvae were then transferred into
282 extracellular solution (134 mM NaCl, 2.9 mM KCl, 1.2 mM MgCl₂, 2.1 mM CaCl₂, 10 mM

283 glucose, 10 mM HEPES buffer; pH 7.8, adjusted with NaOH) and pinned with etched tungsten
284 pins through their dorsal notochord and otic vesicle into a Sylgard-bottom dish.

285 Multiunit extracellular recordings of the posterior lateral line afferent ganglion were made
286 in surface fish (n = 10) and Pachón cave fish (n = 5). Prior to recording from the afferent neurons,
287 a bore pipette was used to break through the skin to expose the afferent soma. Recording electrodes
288 (~30 μ m tip diameter) were pulled from borosilicate glass (model G150F-3, inner diameter: 0.86,
289 outer diameter: 1.50; Warner Instruments, Hamden, CT) on a model P-97 Flaming/Brown
290 micropipette puller (Sutter Instruments, Novato, CA) and filled with extracellular solution. Once
291 contact with afferent somata was achieved, gentle negative pressure was applied (20-50 mmHg;
292 pneumatic transducer tester, model DPM1B, Fluke Biomedical Instruments, Everett, WA).
293 Pressure was adjusted to atmospheric (0 mmHg) once a stable recording was achieved.
294 Simultaneously, ventral root (VR) recordings were made through the skin (Masino & Fetcho,
295 2005) to detect voluntary fictive swimming. All recordings were sampled at 20 kHz and amplified
296 with a gain of 1000 in Axoclamp 700B, digitized with Digidata 1440A and saved in pClamp10
297 (Molecular Devices).

298 All recordings were analyzed in Matlab (vR2016b) using custom written scripts. Both
299 spontaneous afferent spikes and swimming motor activity identified using a combination of spike
300 parameters previously described (Lunsford et al., 2019). Afferent neuron activity within a time
301 interval equal to the subsequent fictive swim bout, hereafter termed “pre-swim”, was quantified
302 and compared to afferent activity during swimming to measure relative changes in spontaneous
303 firing.

304 *Efferent Ablations:* Hindbrain efferent neurons were backfilled with tetramethylrhodamine
305 (TRITC, 3 kDa; Molecular Probes, Eugene, OR). *A. mexicanus* larvae (4 dpf) were anesthetized

in MS-222 (Tricaine, Western Chemical Inc. Ferndale, WA) and embedded in agar. To selectively label the hindbrain cholinergic efferent neurons, we systematically electroporated (Axoporation 800A Single Cell Electroporator, Axon CNS Systems, Molecular Devices LLC, San Jose, CA) TRITC into the efferent terminals that innervate the D1, D2, L1, and L2 neuromasts of the lateral line in surface fish (n = 14) and Pachón cavefish (n = 7). Electroporation does not ensure labelling of all efferent neurons so we standardized parameters (30 V, 50 Hz, 500 ms, square pulse) and targeted the same neuromasts across populations to minimize variation in labelling efficacy. Larvae were then gently freed from the agar and allowed to swim freely and recover overnight. Larvae (5 dpf) were then paralyzed via α -bungarotoxin immersion, remounted in agar dorsal surface down, and imaged on a Leica SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany). Efferent soma size and quantity was measured within identified TRITC-labelled cells in ImageJ (v1.48; U. S. National Institutes of Health, Bethesda, MD). To perform targeted ablations of surface fish (n = 5) and cavefish (n = 6) efferent neurons, a near-ultraviolet laser was focused at a depth corresponding to the maximum fluorescence intensity of each soma, to ensure we were targeting its centre. We applied the FRAP Wizard tool in Leica application software to target individual cells. We ablated target cells with a 30 s exposure to the near-ultraviolet laser line (458 nm), and successful targeting was confirmed by quenching of the backfilled dye. This method has been successfully applied and validated in similar systems (Liu & Fetcho, 1999; Soustelle et al., 2008). Fish were again freed from agar and allowed to swim freely and recover overnight. Electrophysiological recordings were performed on ablated surface fish (n = 4) and cavefish (n = 5) at 6 dpf to simultaneously monitor afferent activity and motor activity.

Statistical analysis: Neuromast data were analyzed using GraphPad Prism 8.4.3. Normality was assessed via Shapiro-Wilk test. Anterior lateral line neuromast count data were found to be

329 normally distributed. Anterior lateral line neuromast quantities in surface and cave larvae were
330 compared using an unpaired t-test. Posterior lateral line neuromast count was found to not be
331 normally distributed and was subsequently analyzed via Mann-Whitney U-test

332 Analyses of electrophysiological data were performed using custom written models in the
333 R language (R development core team, vR2016b) using packages car, visreg, reshape2, plyr, dplyr,
334 ggplot2, gridExtra, minpack.lm, nlstools, investr, and cowplot (Aguie et al., 2017; Baty et al.,
335 2015; Breheny & Burchett, 2017; Fox & Weisberg, 2018; Wickham, 2007, 2009; Wickham et al.,
336 2019; Wilke, 2019). Spontaneous afferent spike rate was calculated by taking the number of spikes
337 over the duration of time where the larva was inactive. Instantaneous afferent spike rate was
338 calculated using a moving average filter and a 100 ms sampling window. Pre-swim and swim spike
339 rate were calculated by taking the number of spikes within the respective period over its duration.
340 Pre-swim periods of inactivity made it challenging to interpret changes in afferent activity, so we
341 restricted the dataset to only include swim bouts that were preceded by a minimum of one afferent
342 spike within the pre-swim interval (surface = 2,291 swim bouts; Pachón = 2,429 swim bouts;
343 Molino = 1.474 swim bouts; Tinaja = 464 swim bouts). Swim frequency was calculated by taking
344 the number of bursts within a swim bout over the duration of the swim bout. Relative spike rate
345 was calculated by taking the swim spike rate over the pre-swim spike rate. All variables were
346 averaged for each individual fish. The precision of estimates for each individual is a function of
347 the number of swims, so we analyzed variable relationships using weighted regressions, with
348 individual weights equal to the square root of the number of swims. We log transformed variables
349 in which the mean and the variance were correlated. To quantify the inhibition of the afferent spike
350 frequency during swimming we tested for a significant difference in afferent spike frequency
351 during swimming as compared to non-swimming periods using a paired sample student's t-test.

352 Differences in afferent spike rates between the periods of interest (pre-swim and swim) across
 353 populations and treatments were tested by N-way analysis of variance (ANOVA) followed by
 354 Tukey's post-hoc test to detect significant differences in spike rates between swim periods or
 355 treatments. Linear models were used to detect relationships between spike rate during swimming
 356 and other independent variables (e.g. spike rate pre-swim). Data is shown throughout the
 357 manuscript as mean $\pm$ standard error. Statistical significance is reported at $\alpha = 0.05$.

358 **Author Contributions:**

359 E.T.L. and J.C.L. conception and design of research; E.T.L. performed electrophysiology and
 360 hindbrain imaging experiments; A.P. performed lateral line imaging; E.T.L. and A.P. analyzed
 361 data; E.T.L., A.P., A.C.K. and J.C.L. interpreted results of experiments; E.T.L. prepared figures;
 362 E.T.L. drafted manuscript; E.T.L., A.P., A.C.K. and J.C.L. edited and revised manuscript; E.T.L.,
 363 A.P., A.C.K. and J.C.L. approved final version of manuscript.

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523 [0publications%20the%20museum%20Texas%20Tech%20University&author=R.W.%20Mitchell&publicati](https://scholar.google.com/scholar_lookup?title=Mexican%20eyeless%20characin%20fishes%2C%20ge)
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