MURAJ
Minnesota Undergraduate Research & Academic Journal

Analyzing the laterality of superficial facial neuromasts in Astyanax mexicanus

By Scarlett Anderson[1], Maya Enriquz[2], Suzanne McGaugh[2]

Abstract

Astyanax mexicanus are a species of fish that populate northeastern Mexico with both river-dwelling surface populations and cave populations. The cave fish have no eyes or pigmentation, and a variety of other altered behavioral traits, which allow them to adapt to the extreme darkness of the cave environment. Cave fish have also independently evolved enhanced lateral line systems to compensate for their lack of sight. This project aimed to determine if three populations of cave fish (Molino, Pachon, and Tinaja) differ from surface fish regarding the number of superficial neuromasts found in the suborbital 3 (SO3) region, and whether there are different degrees of asymmetry of neuromasts in the left versus right side. ImageJ was used to outline the SO3 region on each side of a fish, and the number of neuromasts was recorded. Neuromasts (left, right, and total) were averaged for each population using R. The absolute difference in neuromasts between each side for each fish was also analyzed. It was found that all cave fish populations contained more neuromasts than surface fish, both when separately comparing the left and right sides, and when combined. Pachon and Tinaja populations contained greater differences in the neuromast asymmetry than their surface counterparts. These results give further insight into the utility of asymmetry within the lateral line system in A. mexicanus populations and the mechanisms that drive the evolutionary selection of traits.

Keywords

Neuromast, Asymmetry, Laterality, Astyanax mexicanus

1 Introduction

Environments with extreme conditions, such as deserts, caves, and mountains, help us better understand how natural selection favors specific traits; allowing certain species to thrive in harsh conditions where most cannot survive.[1] *Astyanax mexicanus* is a species of fish widely used as a model organism for studying to extreme environments. Some populations live in surface rivers, while others inhabit dark caves,[2] yet all can interbreed

[1] College of Biological Sciences, University of Minnesota
[2] Department of Ecology, Evolution and Behavior, University of Minnesota

and create fertile offspring, indicating they remain a part of the same species.[3]

There are more than 30 cave populations across Texas and northeastern Mexico, [2,4] each of which exhibits physiological and morphological differences compared to their surface fish counterparts, such as eye loss[5,6] and a lack of pigmentation[6] (Figure 1). Each cave population has undergone an independent evolution of traits, creating slight variance between the populations of cave fish.[7] Due to eye regression and a loss of vision, cave fish have also developed increased sensory systems to compensate for their impaired vision.[8,9]

Figure 1. Astyanax mexicanus cave and surface fish. *Image of an adult Astyanax mexicanus cave fish (top right) compared to an adult surface fish (bottom left). Image from Stowers Institute.*

One example of this compensation is the lateral line system, which allows fish to detect movement and obstacles in their surroundings.[9] The lateral line system is composed of different types of neuromasts, which are sensory organs containing hair cells that detect adjacent water displacement.[10] In fish, neuromasts are located both within canals and superficially on the skin.[9] Past studies have shown that cave fish have larger neuromasts and a larger sensory area than surface fish, likely used to increase lateral line sensitivity.[9]

Cave fish populations have also evolved fragmentation of the suborbital 3 (SO3) bone region beneath the eye orbit.[11] This often leads to asymmetries in the shape and pattern of cave fish's facial bones, which are not observed in surface fish.[12] Gross et al. found an asymmetric pattern distribution of SO3 neuromasts in all *A. mexicanus* populations, but the greatest asymmetry was found within cave fish populations.[13] They compared positions of neuromasts on the left and right side, but did not specifically quantify the number of neuromasts on each side of the *A. mexicanus* populations analyzed. This project aims to address this gap and determine how the number of neuromasts and degrees of asymmetry differ between and within surface and cave *A. mexicanus* populations to help gain insight into the evolutionary mechanisms behind asymmetry in cave fish.[14] Based on previous research, it is hypothesized that cave fish populations will have more neuromasts, and a greater degree of neuromast asymmetry in the SO3 region, better allowing them to detect differences in the water movement around them and navigate their environment without sight.

2 Methods

2.1 Data Collection

Images were collected by collaborators in the Kowalko Lab at Lehigh University, who bred surface *A. mexicanus* fish,

along with Pachon, Molino, and Tinaja cave populations.

They collected data on 63 surface fish, 42 Pachon, 33 Molino, and 30 Tinaja cave fish. The neuromasts were stained using DASPEI, a fluorescent dye that accumulates in neuromasts,[15] and images were captured of the SO3 area with a fluorescent dissecting scope (Figure 2). Then, neuromasts were quantified using a custom ImageJ code using background subtraction to count visible neuromasts. All values were reviewed and verified. The number of neuromasts on each side of each fish was also recorded.

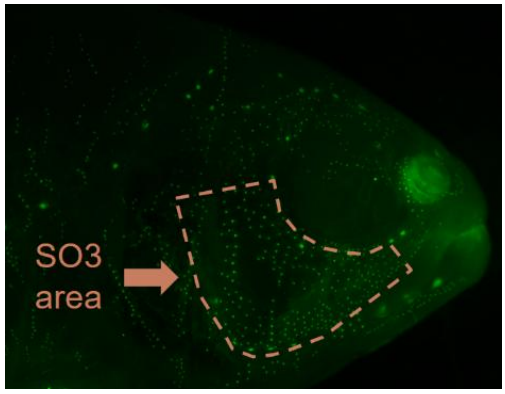

Figure 2. Outline of the suborbital 3 area*. Processed image of a right-facing A. mexicanus cave fish. Superficial neuromasts appear highlighted in light green. The suborbital 3 (SO3) area, where neuromasts are counted, is outlined. Image created by Maya Enriquez, University of Minnesota*

2.2 Statistical Analysis

To determine if there were significant differences in the number of neuromasts between cave and surface *A. mexicanus* populations, the number of neuromasts found on the right side, left side, and in total for each population was compared through a series of statistical tests run in R (Figure 3). A Kruskal-Wallis non-parametric ANOVA, a Shapiro-Wilk test, and a Levene test were first run to assess for significant differences, normal distribution, and equal variance within the data respectively. If the assumptions were not met, a Bonferroni non-parametric post-hoc test was performed. In contrast, if the assumptions were met, a parametric ANOVA and a Tukey's Honest Significant Difference (HSD) post-hoc analysis was run to determine which populations were significantly different.

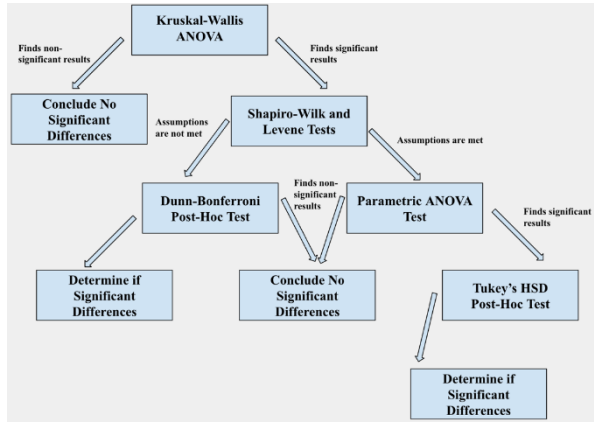

Figure 3. Flowchart of statistical processing*. Shows the flowchart of statistical tests performed for each data set in R. Ends with either concluding no significant differences between groups or determining if there are any significant differences.*

To understand if there were significant asymmetrical differences in superficial neuromasts between different populations, the absolute value of the number of neuromasts on the left side of each fish subtracted from the number of neuromasts on the right side of each fish was calculated. This metric was chosen because similar to Gross et al., the aim was

to evaluate asymmetry of neuromast distribution, independent of its direction. This approach prevents opposing directional differences from cancelling out when comparing the different populations. The same statistical analysis steps described above were then performed on these values, comparing the averages for each population and analyzing for significant differences.

3 Results

The average number of superficial SO3 neuromasts on the right side for each population of fish was found to be 101 for surface populations, 210 for Molino, 278 for Pachon, and 224 for Tinaja (Figure 4). For each comparison (left, right, and total), the Kruskal-Wallis ANOVA found significant results ($p<0.05$). All populations met both assumptions for normal distribution and equal variance, except Molino, whose data was not normally distributed ($p=0.000126$). An ANOVA test was then performed, and its p-value was found to be $<2.0*10^{-16}$. A Tukey HSD test was performed, and Pachon and Tinaja cave fish were found to have significantly more right-sided superficial SO3 neuromasts than surface fish, with Pachon fish also having significantly more than Tinaja individuals. Molino populations were found to have significantly more right-sided neuromasts than surface fish, and significantly less than Pachon, while Molino and Tinaja were not significantly different ($p=1.0$). Overall, all cave populations were found to have significantly more right-sided SO3 neuromasts than the surface populations, and Pachon cave fish were found to have significantly more right side SO3 neuromasts than Molino and Tinaja populations ($p<0.05$).

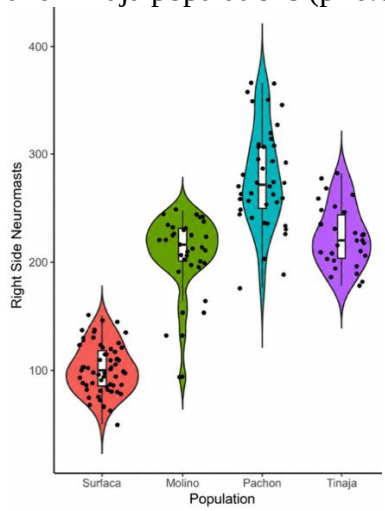

Figure 4. Right side neuromasts in surface vs. cave populations*. The violin plots show the numerical distribution and probability density of the number of neuromasts on the right side of the A. mexicanus surface populations, and Molino, Pachon, and Tinaja cave populations. The figure was created using ggplot2 in R.*

On average, there were 100 superficial SO3 neuromasts on the left side of surface fish, 212 for Molino, 271 for Pachon, and 222 for Tinaja cave fish (Figure 5). Similar to the right side, the Molino population was found to not meet the normality assumption ($p=0.0420$), but an ANOVA test recorded a p-value of $<2.0*10^{-16}$. When a Tukey HSD test was performed, both Pachon and Tinaja cave fish were found to have significantly more left-sided superficial facial neuromasts than surface fish, with Pachon fish also exhibiting significantly

higher counts than Tinaja populations. The Dunn-Bonferroni test found that Molino and Tinaja populations were the only two that did not differ significantly, while Molino had significantly more left-sided SO3 neuromasts than surface fish and significantly fewer than Pachon cave fish. Overall, all cave populations were found to have significantly more left-sided SO3 neuromasts than the surface populations, and Pachon cave fish were found to have significantly more SO3 neuromasts than Molino and Tinaja populations ($p<0.05$).

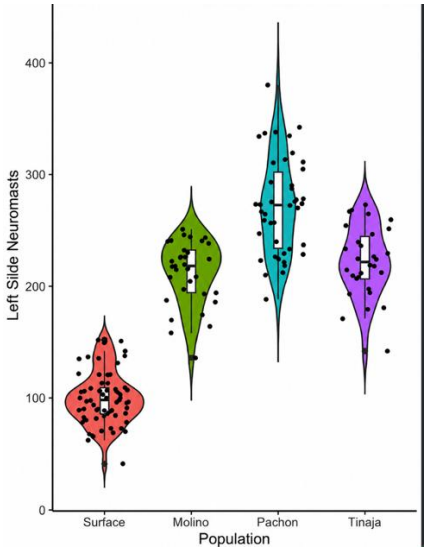

Figure 5. Left side neuromasts in surface vs. cave populations*. The violin plots show the numerical distribution and probability density of the number of neuromasts on the left side of the A. mexicanus surface populations, and Molino, Pachon, and Tinaja cave populations. The figure was created using ggplot2 in R.*

For total superficial facial neuromasts, there were an average of 201 for surface, 422 for Molino, 549 for Pachon, and 445 for Tinaja (Figure 6). An ANOVA p-value of $<2.0*10^{-16}$ and a Tukey HSD test found that Pachon and Tinaja cave fish had significantly more superficial SO3 neuromasts than surface fish, and Pachon individuals had significantly more than Tinaja fish. Molino fish data was not normally distributed ($p=0.00171$) but had significantly more SO3 neuromasts than surface fish, fewer than Pachon cave and no significant difference from Tinaja cave fish ($p=1.0$). Overall, all cave populations were found to have significantly more SO3 neuromasts than the surface populations, and Pachon cave fish were found to have significantly more SO3 neuromasts than Molino and Tinaja populations ($p<0.05$).

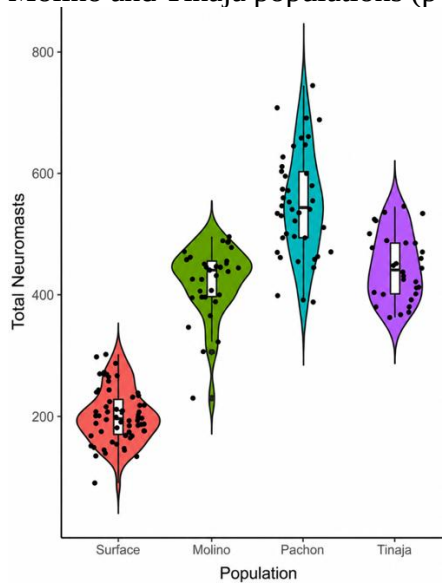

Figure 6. Total neuromasts in surface vs. cave *populations. The violin plots show the numerical distribution and probability density of the total number of neuromasts in the A. mexicanus surface populations, and Molino, Pachon, and Tinaja cave populations. The figure was created using ggplot2 in R.*

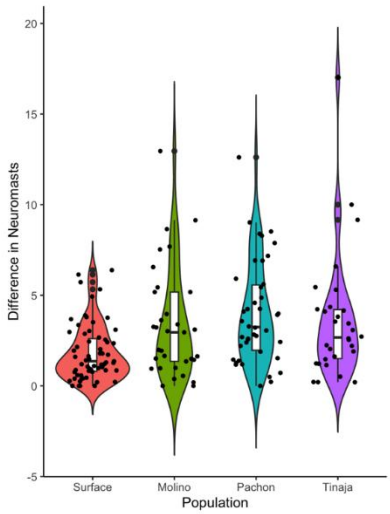

Figure 7. Neuromast asymmetry in surface vs. cave populations. *The violin plots show the numerical distribution and probability density of the absolute difference in neuromasts between the right and left side of the A. mexicanus surface populations, and Molino Pachon, and Tinaja cave populations. The figure was created using ggplot2 in R.*

When comparing the average absolute differences between the number of superficial facial neuromasts on each side for every *A. mexicanus* population studied, there was an average difference of 1.85 neuromasts for surface populations, 3.39 for Molino, 3.99 for Pachon, and 3.61 for Tinaja (Figure 7). None of the populations were found to have a normal distribution, so a Dunn-Bonferroni test found that Pachon and Tinaja cave fish had significantly more numerical asymmetry than surface fish with a p-value <0.05, but Molino populations did not differ significantly from surface fish populations. Overall, Pachon and Tinaja cave populations had significantly greater levels of asymmetry than surface populations, but none of the cave populations varied significantly from each other.

Cavefish populations have many more neuromasts than surface fish, so larger neuromast asymmetries may be more likely due to high neuromast counts rather than increased biological asymmetry. To account for this, the equation |L-R| / [(L+R)/2] was used to normalize the bilateral asymmetry and find how large the left-right difference is relative to the individuals total neuromast abundance. The values were found to be 0.0197 for surface fish, 0.0147 for Pachon, 0.0178 for Molino, and 0.0171 for Tinaja. T tests found that none of the populations normalized values significantly differed. Surface and Pachon populations differed the most (p=0.059, while the p values was 0.637 for Surface vs. Molino and p=0.516 for Surface vs. Tinaja. Overall, there were no significant differences in neuromast asymmetries when accounting for total number of neuromasts in each population.

4 Discussion

The results showed that cave fish *A. mexicanus* populations have significantly more neuromasts on each side and in total than surface fish populations, and that Pachon cave fish have significantly more neuromasts than both Molino and Tinaja populations. The differences in neuromast quantities between Pachon individuals and the other cave fish populations

could be related to the evolutionary history of *A. mexicanus*, as all three cave fish populations share the same common ancestor but evolved through split lineages,[4] meaning some of their traits evolved independently. Molino cave fish are a part of lineage 1, while Pachon and Tinaja are a part of lineage 2 (Figure 8). Since both Pachon and Tinaja cave fish populations were found to have more neuromast asymmetries than surface fish while Molino did not, it is possible that the development of increased numerical asymmetry in neuromasts may have evolved in only lineage 2. Although Pachon cave fish are a part of the same linages as Tinaja populations, the two populations differ in the number of superficial neuromasts. It is possible that Pachon cave fish had evolved more superficial neuromasts after their lineage had split from Tinaja populations. There may also be differences in the environment where Pachon cave fish live in that are leading to the increase in neuromasts.

Overall, the findings that cave fish have more neuromasts than their surface counterparts are consistent with the work by Lunsford et al., that found Pachon cave fish developed a greater quantity of neuromasts when compared to surface fish.[16] From previous findings, it has been hypothesized that cave fish populations have developed an increased number of neuromasts in order to better detect movement in the water and to compensate for their loss of vision.[9] Since all cave fish populations in this study have seemingly developed and maintained an increased number of neuromasts compared to surface fish, the hypothesis that having more neuromasts is advantageous to cave fish survival is supported. However, while this is one popular hypothesis, these findings may be the result of sampling variation, and more investigation is necessary.

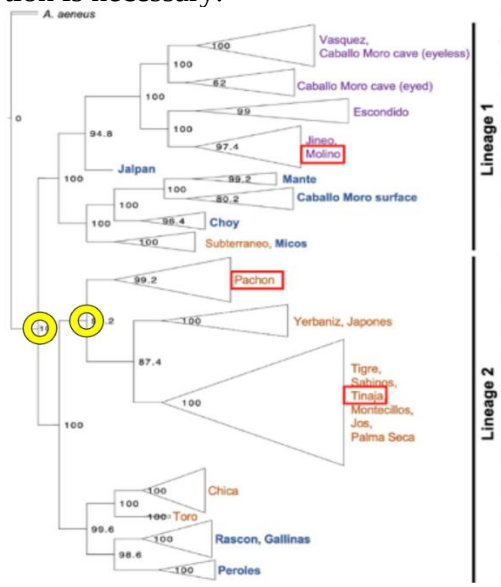

Figure 8. Split lineages of Astyanax mexicanus cave fish*. Shows lineage 1 and lineage 2 of cave fish, originating from an A. aeneus ancestor population. Molino cave fish are in a red box in lineage 1, while Pachon and Tinaja are in red boxes and a part of lineage 2. The yellow circles symbolize when the lineages split apart. Original image from Moran et al.[18]*

The analysis of the average difference in neuromasts between the left and right side for the different *A. mexicanus* populations also found that the amount of numerical asymmetry was significantly greater in the Pachon and Tinaja cave populations when compared to the surface populations, and that there were no significant differences between the cave

populations. This shows that, along with cave fish having a more asymmetric bone fragmentation and neuromast pattern,[13] they also generally have greater degrees of lateral asymmetries regarding numerical differences in neuromasts. Because these results were consistent for the majority of the cave fish populations, there may be a genetic link regarding the asymmetrical development of the SO3 region and the number of superficial neuromasts that develop on each side of a cave fish. It also suggests that there may be a benefit to having different numbers of neuromasts on the left versus right side, which could contribute to the evolution of this trait in cave fish populations. One possible explanation for this phenomenon is that having different amounts of neuromasts on each side allows the cave fish to receive different levels of vibrations and a greater variety of sensory input, making them better able to sense their surroundings. Fernandes et al.[17] found that cave fish populations expressed more vibration attraction behavior (VAB) when they received more sensory input from neuromasts on the left side. VAB allows cave fish to navigate their environments without visual sensory input, and neuromasts provide crucial information related to VAB. But, neuromast asymmetrical differences between populations disappeared after normalization with total neuromast abundance. This suggests that many cave fish populations may have had greater asymmetry values only because they have greater neuromast abundance rather than actual differences in bilateral asymmetry. The asymmetrical neuromast distribution may aid the cave fish when navigating waters without sight, but the lack of significant normalized results suggests that there may not be a strong evolutionary force behind the asymmetrical findings. In further research, Fernandes et al.[14] found no detectable L-R asymmetries in superficial neuromast numbers in surface and Pachon cavefish, although they found instead that Pachon fish had higher VAB sensitivity. So, cavefish may have more VAB due to the sensitivity of their neuromasts rather than their asymmetrical distribution.

5 Conclusion

This analysis demonstrated that cave fish populations of *A. mexicanus*, compared to surface populations, have more neuromasts on each side and in total, and have a greater magnitude of asymmetry between the number of neuromasts on each side, but no significant difference when normalized for the total number of neuromasts. This supports the hypothesis that there is greater asymmetry in cave fish. The normalized results suggest that the asymmetrical findings may be confounded due to the differences in total neuromasts rather than an evolutionary force driving this phenomenon. Despite conflicitng results, this data provides a better understanding of how asymmetries in neuromast bone fragmentation and neuromast patterns affect asymmetries in neuromast quantities. Further research on how SO3 bone fragmentation is

linked to neuromast development could help explain these results, and accounting for factors such as fish body length could further standardize results. Furthermore, a better understanding of VAB could help conclude if cavefish neuromasts are more sensitive to water movements, or if asymmetries are leading to the enhanced detection. One limitation of this study is that it was conducted with small sample sizes for each population, leading to higher variability and potentially less accurate results. The statistical measure should account for sample size, but this study could also be repeated with a larger sample size and more cave fish populations to better understand the significance of the results and their potential implications on evolutionary biology.

.

Analyzing the laterality of superficial facial neuromasts in Astyanax mexicanus

References

1. Azua-Bustos, A., González-Silva, C., Arenas-Fajardo, C., Vicuña, R. Extreme environments as potential drivers of convergent evolution by exaptation: the Atacama Desert Coastal Range case. (2012). Front. Microbiol. 3. https://doi.org/10.3389/fmicb.2012.00426
2. Jeffrey, W. R. *Astyanax* surface and cave fish morphs. (2020). Evo-Devo. 11. https://doi.org/10.1186/s13227-020-00159-6
3. Borowsky, R. *Astyanax mexicanus*, the blind mexican cave fish: A model for studies in development and morphology. (2008). Cold Spring Harb. Protoc. https://doi.org/10.1101/pdb.emo107
4. Swaminathan, A., Xia, F., Rohner, N. From darkness to discovery: Evolutionary, adaptive, and translational genetic insights from cavefish. (2024). TiG. 40, 24-38. https://doi.org/10.1016/j.tig.2023.10.002
5. Krishnan, J., Rohner, N. Cavefish and the basis for eye loss. (2017). Phil. Trans.R. Soc. B. 372. https://doi.org/10.1098/rstb.2015.0487
6. Maldonado, E., Rangel-Huerta, E., Rodriguez-Salazar, E., Pereida-Jaramillo, E., Martínez-Torres, A. Subterranean life: behavior, metabolic, and some other adaptations of *Astyanax* cavefish. (2020). J. Exp. Zool. B. Mol. Dev. Evol. 334, 463-473. https://doi.org/10.1002/jez.b.22948
7. Kowalko, J. E., Rohner, N., Linden, T. A., Rompani, S. B., Warren, W. C., Borowsky, R., Tabin, C. J., Jeffery, W. R., Yoshizawa, M. Convergence in feeding posture occurs through different genetic loci in independently evolved cave populations of *Astyanax mexicanus.* (2013). Biosci. 110, 16933-13938. https://doi.org/10.1073/pnas.1317192110
8. Kowlako, J. Utilizing the blind cavefish *Astyanax mexicanus* to understand the genetic basis of behavioral evolution. (2020). Behav. Biol. 223. https://doi.org/10.1242/jeb.208835
9. Teyke, T. Morphological differences in neuromasts of the blind cave fish *Astyanax hubbsi* and the sighted river fish *Astyanax mexicanus*. (1990). Brain Behav. Evol. 35, 23-30. https://doi.org/10.1159/000115853
10. Gibbs, M. A. Lateral line receptors: Where do they come from developmentally and where is our research going? (2004). Brain Behav. Evol. 64, 163-181. https://doi.org/10.1159/000079745
11. Gross, J. B., Stahl, B. A., Powers,A. K., Carlson, B. M. Natural bone fragmentation in blind cave-dwelling fish, *Astyanax mexicanus*: Candidate gene identification through integrative comparative genomics.

(2016). Evol. Dev. 18, 7-18. https://doi.org/10.1111/ede.12131

12. Powers, A. K., Davis, E. M., Kaplan, S. A., Gross, J. B. Cranial asymmetry arises later in the life history of the blind mexican cavefish, *Astyanax mexicanus.* (2017). PLoS One 12. https://doi.org/10.1371/journal.pone.0177419
13. Gross, J. B., Gangidine, A., Powers, A. K. Asymmetric facial bone fragmentation mirrors asymmetric distribution of cranial neuromasts in blind mexican cavefish. (2016). Symmetry 8, 188. https://doi.org/10.3390/sym8110118
14. Fernandez, V. F. L., Glaser, Y., Iwashita, M., Yoshizawa, M. Evolution of left-right asymmetry in the sensory system and foraging behavior during adaptation to food-sparse cave environments. (2022). BMC Biol. 20. https://doi.org/10.1186/s12915-022-01501-1
15. Nakae, M., Asaoka, R., Wada, H., Sasaki, K. Fluorescent dye staining of neuromasts in live fishes: An aid to systemic studies. (2012). Ichthyol. Res. 59, 286-290. https://doi.org/10.1007/s10228-012-0274-2
16. Lunsford, E. T., Paz, A., Keene, A. C., Liao, J. C. Evolutionary convergence of a neural mechanism in the cavefish lateral line system. (2022). eLife 11. https://doi.org/10.7554/eLife.77387
17. Fernandes, V. F. L., Macaspac, C., Lu, L., Yoshizawa, M. Evolution of the developmental plasticity and a coupling between left mechanosensory neuromasts and an adaptive foraging behavior. (2018). Dev. Evol. 441, 262-271. https://doi.org/10.1016/j.ydbio.2018.05.012
18. Moran, R. L., Richards, E. J., Ornelas-Garcia, C. P., Gross, J. B., Donny, A., Wiese, J., Keene, A. C., Kowalko, J. E., Rohner, N., McGaugh, S. E. Selection-driven trait loss in independently evolved cavefish populations. (2023). Nat. Commun. 14. https://doi.org/10.1038/s41467-023-37909-8