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Proposal for Comparative Morphometrics of Tübingen (TU) and Tübingen longfin (TL) Zebrafish (*Danio rerio*)

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Disclaimer: Due to the impacts of COVID-19, all on-site research within the Department of Biology at Portland State University has been suspended indefinitely, including the laboratory of Dr. Kim H. Brown in which this study was to be conducted. As a result, the scope of this project has been altered to a summary of the research that was intended to be carried out. All data represented within this paper are hypothetical in order to illustrate data analysis methods, and do not represent novel findings.

Abstract

The TL strain of zebrafish (*Danio rerio*) originated from the TU strain, and differs in fin pattern with a phenotype predominated by increased fin length. Two genes are known to affect the phenotype: *leo* and *lof.* The pattern-affecting *leo* gene is not believed to affect the growth rate, development, or proportionality of the body (van Eeden, 1996). The *lof* gene does however result in significant increase in fin length compared to the wild-type phenotype of TU. Using populations from both TL and TU genetic strains, development was observed from 48 hours post fertilization through sexual maturity at 90 days post fertilization (dpf) (n=100). Results found isometric growth up until 60 dpf, when significant hyperallometric growth in the fins began, resulting in roughly one and a half times the length observed in the TU population for both anal and caudal fins ($\alpha = 1.55$, $\alpha = 1.63$). Similar ratios were observed for width ($\alpha = 1.38$, $\alpha = 1.31$), with length accounting for 84% of overall variance. No significant difference in body shape was found.

Introduction

Zebrafish (*Danio rerio*) are named after their prominent zebra-like striping along the side of their bodies and fins. These small shoaling fish are native to southern Asia and breed readily in captivity, producing as many as 300 eggs every 1-3 days (Reed and Jennings, 2010). Zebrafish

take between 60-90 days from the time of egg fertilization to fully sexually mature, making them ideal for observation of development (Reed and Jennings, 2010). The Tübingen zebrafish (TU), a strain originating from a mixed population of wild-type phenotype fish from the pet trade in Tübingen, Germany, is commonly used as a model organism for wild-type zebrafish in research (Pannia 2013, Guryev 2006). In the last decade, the entire TU genome was sequenced by the Sanger Institute (Howe, 2013). Wide use and good understanding of TU genomics with the existence of a reference genome makes the TU population ideal for comparison of related strains expressing genetic mutations.

The Tübingen long fin mutation (TL) strain originates from the TU population and expresses the recessive leopard (*leo)* gene, and is homozygous for the dominant longfin (*lof)* gene (Zebrafish Information Network, n.d.). These genes affect fin pattern and length respectively. While *leo* has been observed to correlate with an increase in body depth and faster growth rate in one notable study (McClure and McCune, 2003), it is not widely supported, and replication is required to rule out the possibility of inadvertent or meaningful selection for growth rate in the original stock obtained from the pet trade. The *lof* gene expresses the long fin phenotype in all fins of the adult fish, but is not known to affect juveniles (van Eeden, 1996). This extra length is created by the continued addition of fin ray segments after maturity in AB *lof* strain populations (Iovine and Johnson, 2000). Wild-phenotype AB populations without this gene slow growth and maintain isometric growth after roughly 10 weeks or maturity. TL fin growth and overall morphometrics, especially during development, are not well understood with current literature. Anecdotally, the caudal fin length of TL fish are observed to be longer than in TU, AB, or AB mutations expressing only the *lof* gene, but this has yet to be quantified.

Iovine and Johnson (2000) propose that each fin ray may have a different proportionality constant. Commonly, *D. rerio* morphometrics focus on the caudal fin, with very little imaging and data currently available for the anal fin. There is possibility that proportionality may differ between caudal and anal fins, as well as between individual rays, in a given strain. This investigation aims to provide a set of proportionality values for overall fin length and size on the TU population and use this data to comparatively quantify growth rate and morphometric differences observed in the TL strain. By observing the development of the TU and TL strains, and identifying where and when isometric growth becomes allometric, evidence may help understand if the proposed mechanisms for longfin growth in AB populations may also apply to the TL strain. Further research may use data collected in this investigation to compare TU strains bred to express only *leo* or *lof* genes respectively to identify any possible additive effects in TL strains, or propose a novel mechanism if appropriate. Studies including AB strains bred for both genes should also be conducted for comparative analysis.

Methods

Eggs are estimated to be roughly two days post-fertilization (dpf) upon arrival after purchase from the Zebrafish International Resource Center. Methods were based on established standard operating procedures of Kim H. Brown Laboratory at Portland State University. Upon arrival, the received eggs were transferred into pre-prepared clean, embryo media (50-100 mg/L CaCO3) at 82-85°F (roughly 27-29°C) and pH between 6.8 and 7.5 (Avdesh et al., 2012) until embryos are brought up to temperature (under 15 minutes). A small, soft nylon strainer was used to remove them from packaging, and the embryos were rinsed in tank water prior to placing them into a 500mL beaker of the embryo media. The eggs were maintained in the media of the

aforementioned parameters for an additional 24-36 hours until all larvae are hatched (roughly 72 hours since fertilization) and free-swimming (roughly 5-6 dpf) (Avdesh et al., 2012). A 50% media change should be performed on the 500mL beakers every 48-72 hours to maintain water quality and to monitor survival rate of larvae until large enough to be put into the multi-tank system. At that time, the fish are placed into 1 gallon (approximately 3.8L) clear glass tanks at a density not exceeding 20 fish per liter, and fed live rotifers from an established culture as needed so that they remain in the water column to be constantly available to the fish (Brown, personal correspondence, 2019). Both TU and TL larvae were placed in the flow-through system fed from the same sump to ensure consistent water parameters between populations. Waste water was collected in a separate bin fed by an overflow within each holding tank, triple filtered, and disposed of. This created the effect of a 50% water change per 1 gallon container per day. After 1 week, desalinated artemia were offered in place of rotifers 2 to 3 times daily. After 8 days, a small drip flow at a rate of approximately 0.5mL per minute (Brown, personal correspondence, 2019) was used in each tank.

After fish reached 7mm in size, flow rates in the tanks were brought up to 25mL per minute. At this time, tank stocking of 5 fish per liter was supplied with artificial plants to provide coverage and environmental enrichment. Fish were measured and growth tracked independently when possible. Individuals were marked by dorsal fin clipping to identify fish in each tank at close proximity or under the dissecting microscope. Fin clippings persisted for approximately 30 days before being re-grown completely (Delcourt, 2018), and were re-clipped as necessary while under anesthesia to maintain discernibility. These fish live in shoals and are known to nip each others' fins in both aggressive and breeding behaviors; fin clipping is understood to be both a commonly accepted method of marking, and virtually painless (Delcourt, 2018; Brown personal

correspondence, 2019). Measurements and imaging of overall anal and caudal fin length at the farthest point, standard length, and fin maximum height were taken as soon as fins were fully developed and distinct (21-30 dpf) every week through sexual maturity (90 dpf). These measurements as well as digital landmark measurements were applied for shape analysis.

For data collection, fish were netted with soft nylon netting into a container of 100mL of clean, dechlorinated water with 4mL of 4mg/mL tricaine methanesulfonate (MS-222) already thoroughly mixed into solution (Brown, personal correspondence, 2019). Once in solution, the fish was removed for measurement as soon as it failed to maintain an upright position in the water column, given operculum movement had declined significantly in developed specimens. The fish were removed from solution and laid laterally on a wet surface with the head facing left under a dissecting microscope and accompanying camera, with a millimeter marker for scale for measurement and imaging. After imaging, the fish were moved into a new container with enough clean tank water to submerge the entirety of the fish to recover. Time from removal from anesthetic solution to being placed in the recovery solution remained under one minute to avoid risk of suffocation or prolonged bradycardia. Fish that are subject to anesthesia were not returned to stock tanks until normal swimming behavior had been observed in recovery for at least 1 min (Brown, personal correspondence, 2019).

Data analysis methods

Digital landmarks were used to minimize the amount of time spent out of the water for the fish as well as efficiency of data processing. Standard length and total length are commonly used as representations of fish length for non-mutated specimens of known freshwater species, with insignificant difference in replicability (Onsoy et al. 2011). For this study, standard length

was used due to the fin length variance in question. The midpoint of the fin to the point of the tissue connection with the body along the midline was recorded as a distinct landmark, and therefore total length may be inferred from the raw data (Figure 1). Maximum girth was also recorded as a measurement of fish size. These landmarks allowed for analyses to compare fin and body morphometrics to understand levels of within-group variation and provide representative values of the TU strain control. Landmarks were placed after safe return of the fish to their tanks using ImageJ software (Schneider, 2012). Distances between landmarks as well as x and y coordinates of the landmarks are both measured and recorded by the program. These values and images can be converted to tps files using tpsDig (Rohlf, F.J., 2013) for further analysis using MorphoJ or excel format for SPSS software. The landmarks selected for this project were chosen specifically for the data desired and are illustrated below (Figure 1).

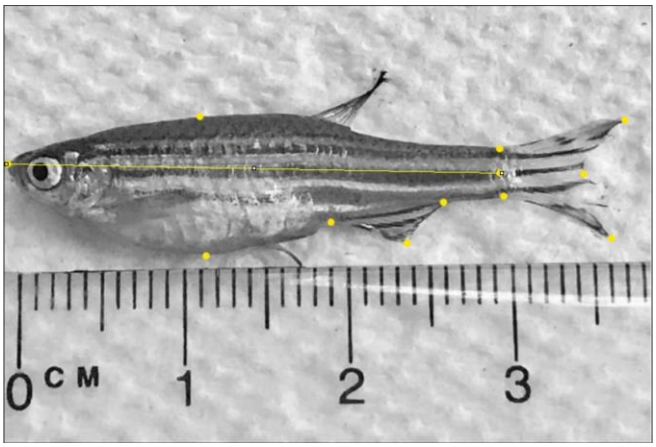

Figure 1. Example of proposed landmarks using a modified example photo from De Leon J. et al. (2019). Landmarks are represented by yellow dots, and the yellow line represents proposed standard length measurement as performed in ImageJ.

Using the data from ImageJ, the standard length (SL) of each group (approximately n=30) was graphed over time in weeks to provide a representation of growth rate. Chi-squared analysis was used to eliminate outlying data each week. Standard length and depth values are not

expected to demonstrate statistically significant ($p<0.05$) body size differences between TL and TU strains upon analysis (van Eeden, 1996). If significant difference in body size is found, this could support additional effects of the *lof* gene as observed by McClure and McCune (2003). Assuming no statistical difference in body size during development, this data was used to isolate tail and anal fin measurements along with growth rate as unique variables resulting from TL genetics. The rate of growth (SL/time) was then compared to the growth of each fin (fin length/time) for an understanding of if and when the relationship becomes allometric using the equation $log\ y = \alpha\ log\ x + log\ b$ to compare them proportionally (Murphy, personal correspondence, 2018). In this equation, y represents the length of the fin being analyzed, x represents the overall body length, $log\ b$ represents the y-intercept and α represents the growth coefficient. The experimental fin growth rate of the TU strain was used as the "expected" slope, where the value of the growth coefficient $\alpha = 1$. Evidence predicts positive allometric growth ($\alpha > 1$) of the tail and anal fins of TL around 60 days or maturity (Iovine and Johnson, 2000).

Labelled tps or Excel files with raw data for each individual can be fed into multivariate and bivariate principal component analysis (PCA) to understand growth allometry using MorphoJ software. A Procrustes superimposition for 2D data was performed using the program to align landmarks (C. P. Klingenberg, 2011). This removed differences in overall size and rotation in order to measure shape, hence the analysis for significant fish size differences was performed separately. From here, a covariance matrix can be created for PCA. PCA can then be conducted, and the program will automatically generate graphs illustrating the axes of greatest variation for the first principal component. Pooled within-group covariances are accounted for within the software.

Example Results

Example data were created using a random number generator given a normal distribution and a standard deviation of 1, based on total length mean values based on Zebrafish Information Network's Zebrafish Developmental Staging Series values (Westerfield, 2000) from fertilization through 90 days. These examples were created for illustration purposes only and do not represent laboratory observations.

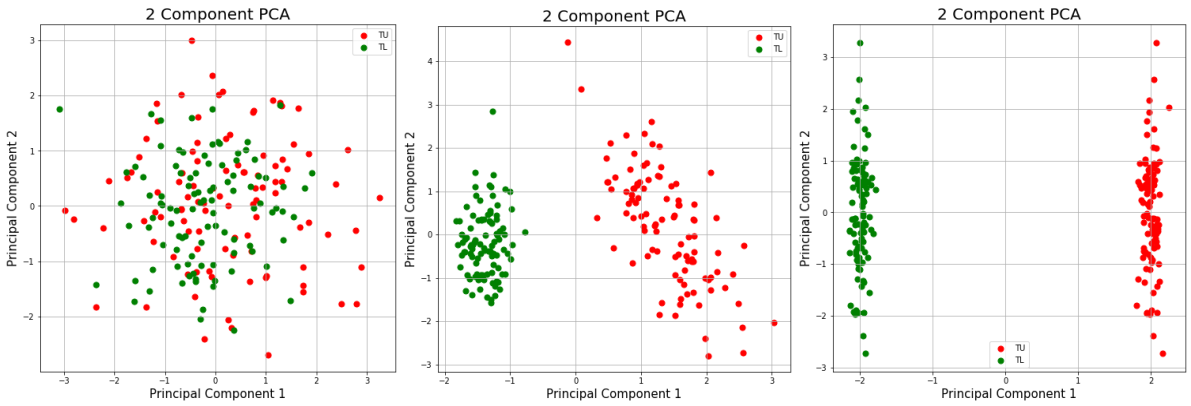

Figure 2. Using principal component analysis and data from each week, this example image demonstrates relative correlation of length and width of the caudal fins using the landmarks described in Figure 1. The leftmost graph describes the larvae at 7-14 days, the middle graph describes the data at 60 days, and the rightmost graph describes observations at 90 days and full sexual maturity. TU fish are illustrated in red, and TL fish in green.

The average ratio of anal fin length to the length of the caudal fin showed no significant difference depending on the genetic strain using the Student T-test at 95% significance ($p<.05$), with similar results for the widths (p=0.69, p=0.56) at 90 days. It follows that every week prior was also insignificant in this regard. This supports the idea of while the TL fins are longer than the TU strain, the anal and caudal fins remain proportional to each other. This may make support for a single mechanism for the growth of all affected fins more likely. Similarly, the standard lengths and depths of body for each strain were insignificantly different (p=0.978, p=0.961),

indicating that body size as represented by these measurements are identical between TU and TL fishes. This supports previous findings that the genes unique in the TL strain are only expressed in the fins. Furthermore, when setting up the appropriate allometric equation the length and depth of the body do not have to be included for analysis of growth coefficient.

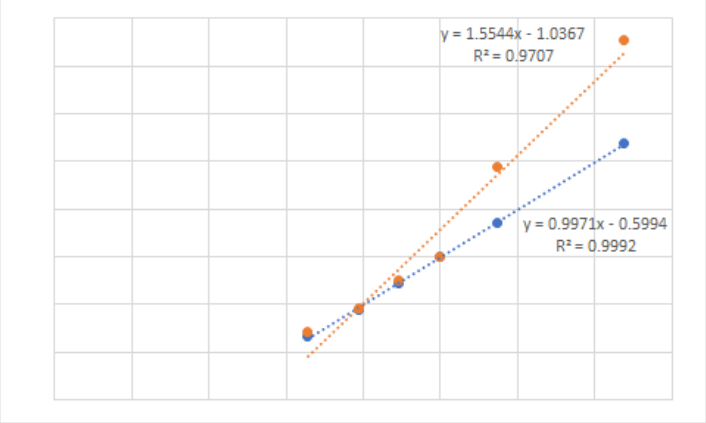

Figure 3. Linear representation of caudal length in TU and TL populations over standard growth rate. Equations are shown, demonstrating isometric relationship between the TU group (α = 0.99) and allometric relation with TL (α = 1.55).

The standard length and the TU fin length was represented logarithmically in order to set the wildtype as the expected α=1 from which distance was compared (Figure 3). The length of fin growth displayed a hyperallometric relationship in both caudal (α = 1.55) and anal fins (α = 1.63) with non-significant difference. Fin width in the TL population showed slightly less dramatic increase in growth (α = 1.38, α =1.31), but did not reach a level of significance (p=0.51).

At the 60 day mark, the difference in length and width become significant (p=0.01) between the TU and TL populations as seen in Figure 2. This significance increases as the fish continue to sexual maturity (p=0.001 at 90 days). At a mature length around 30mm, the average caudal fin of the TL population was on average 2.7 times longer than its TU counterpart, but only roughly

twice the width. The anal fins on the TL population averaged 2.46 times longer and about twice as wide. PCA results indicated that length accounted for approximately 84% of the overall variance in shape observed, while width accounted for over 15% of overall variance, (Figure 4).

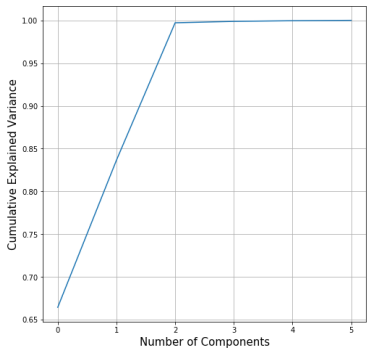

Figure 4. Percentage of explained variance by components.

Discussion

Given the generated data, the null hypothesis of proportional effect in the caudal and anal fin cannot be rejected. Any disproportionate change in body depth or body shape over all by the genes unique to the TL strain are unsupported. The most significant result of this data set would be the presentation of the growth coefficient of $\alpha \geq 1.5$ observed in the TL population. This would be one of the first investigations quantifying just how much longer the *lof* phenotype is within this population. Comparative ratios of the body and fins, quantifying just how much longer TL fins are compared to TU, and understanding the directions in deviations from isometric growth help to convey exactly what TL genetics change about the phenotype of the TU strain. In this case, that length accounted for more variance (84%) in the TL population than width (approx. 15%), which was only roughly twice that of the TU population. These quantified phenotypic changes may aid in supporting proposed mechanisms for the observed extra *lof* fin growth. If significant differences in fin growth were noticed well before sexual maturity, or if

significant differences in other body ratios during development were observed, it may be hypothesized that the long fins of the TL strain come by different and/or additional mechanisms to the AB *lof* strain. However, initial significance of shape difference at 60 days using the example data supports findings by Iovine and Johnson (2000) for correlation with sexual maturity. Were the example data to be observed, this would represent support for similar or identical mechanisms responsible for the extra length in fins within the TL population as compared to the AB *lof* population. Were this to be the case, radiographic imaging of the fin rays in the mature specimens for confirmation of additional fin ray segments would be highly recommended. Additionally, comparative genetic sequencing may also provide support or refute apparent correlation in mutation origin. More research is required to see if similarities are sustained between other genetic morphs expressing longfin phenotypes. Currently, literature citing growth coefficients and quantifying average difference in fin length, width, and shape are not readily available for comparison in AB and other morphs. Collecting that data would be a natural continuation of this research. Further investigation into longfin phenotypes and their mechanisms are required to have the best understanding of how these mutations come to be in not only *Danio rerio*, but also in vertebrate evolutionary history, vertebrate mutations, and development.

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Work cited

Avdesh et al., (2012). *Regular care and maintenance of a zebrafish (*Danio rerio*) laboratory: An introduction*. J. Vis. Exp. (69). e4196, doi: 10.3791/4196

Brown, (2019). *Zebrafish embryo handling and care standard operating procedure*. Kim H Brown Laboratory, Portland State University. Personal correspondence.

Brown, (2019). *Zebrafish larval rearing standard operating procedure.* Kim H Brown Laboratory, Portland State University. Personal correspondence.

Brown, (2019). *Zebrafish anesthesia standard operating procedure.* Kim H Brown Laboratory, Portland State University. Personal correspondence.

C. P. Klingenberg. 2011. MorphoJ: an integrated software package for geometric morphometrics. *Molecular Ecology Resources* 11: 353-357. doi: 10.1111/j.1755-0998.2010.02924.x

Delcourt et al., (2018). *Individual identification and marking techniques for zebrafish.* Rev. Fish Biol. Fisheries 28 (2018): 839-864. DOI: 10.1007/s11160-018-9537-y .

De Leon J. et al. (2019) *Toxicology of nanomaterials on zebrafish.* American Journal of Engineering and Applied Sciences 12 (2): 193.203. DOI: 10.3844/ajeassp.2019.193.203

Eeden, Freek & Granato, M & Schach, U & Brand, Michael & Furutani-Seiki, Makoto & Haffter, P & Hammerschmidt, Matthias & Heisenberg, C & Jiang, Yun-Jin & Kane, Don & Kelsh, Robert & Mullins, M & Odenthal, Joerg & Warga, Rachel & Nüsslein-Volhard, C. (1997). *Genetic analysis of fin formation in the zebrafish,* Danio rerio. Development (Cambridge, England). 123. 255-62.

Guryev V. , Koudijs M. J., Berezikov E., Johnson S. L., Plasterk R. H. A., van Eeden F., Cuppen E. (2006). *Genetic variation in the zebrafish.* Genome Research. 16: 491-497. doi: 10.1101/gr.4791006

Howe K, Clark MD, Torroja CF, Torrance J, Berthelot C et al., (2013). *The zebrafish reference genome sequence and its relationship to the human genome.* Nature 2013; 496; 7446; 498-503. DOI: 10.1038/nature12111.

IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.

Iovine M. K. and Johnson S. L., (2000). *Genetic Analysis of Isometric Growth Control Mechanisms in the Zebrafish Caudal Fin.* Department of Genetics, Washington University School of Medicine, Saint Louis, Missouri 63110. Genetics vol. 155 no. 3 1321-1329.

McClure M. and McCune A. , (2003). *Evidence for developmental linkage of pigment patterns with body size and shape in danios (teleostei: cyprinidae).* The Society for the Study of Evolution. Evolution 57(8): 1863-1875.

Murphy M. (2018), *Physiology and allometry* [Personal correspondence and PowerPoint lecture]. Vertebrate Zoology Fall 2018. Retrieved from personal notes. Portland State University, Oregon. BIO387.

Onsoy, Bahadir & Tarkan, Ali & Filiz, Halit & Bilge, Gökçen. (2011). *Determination of the best length measurement of fish.* North-Western Journal of Zoology. 7. 178-180.

Pannia E., Tran S., Rampersad M., Gerlai R., (2013). *Acute ethanol exposure induces behavioral differences in two zebrafish* (Danio rerio) *strains: A time course analysis*. Behavioral Brain Research 259 (2014) 174-185.

Schneider, C. A.; Rasband, W. S. & Eliceiri, K. W. (2012), "NIH Image to ImageJ: 25 years of image analysis", *Nature methods* **9(7)**: 671-675, PMID 22930834

Reed and Jennings, (2010). *Guidance on the housing and care of zebrafish* Danio rerio. Research Animals Department, Science Group. Royal Society for the Prevention of Cruelty to Animals, pp 9-50.

Rohlf, F.J. (2013). TpsDig version 2.32 (Tps_Digitize). http:// life.bio.sunysb.edu/morph/.

Ruzicka L, Howe DG, Ramachandran S, Toro S, Van Slyke CE, Bradford YM, Eagle A, Fashena D, Frazer K, Kalita P, Mani P, Martin R, Moxon ST, Paddock H, Pich C, Schaper K, Shao X, Singer A, Westerfield M. (2019). *The Zebrafish Information Network: new support for non-coding genes, richer Gene Ontology annotations and the Alliance of Genome Resources*. Nucleic Acids Res. Jan 8;47(D1):D867-D873. doi: 10.1093/nar/gky1090.

Van Eeden et al., 1996. *Genetic analysis of fin formation in the zebrafish*, Danio rerio. Development 123, 255-262.

Westerfield, M. (2000). *The zebrafish book. A guide for the laboratory use of zebrafish (Danio rerio). 4th ed*., Univ. of Oregon Press, Eugene.

Zebrafish Information Network, The (ZFIN), n.d. *Genotype: Tüpfel long fin.* ZFIN ID: ZDB-GENO-990623-2. University of Oregon. Retrieved from https://zfin.org/ZDB-GENO-990623-2