

Short Communication

Sex and size do not influence gastrointestinal transit time in zebrafish (*Danio rerio*): A computer vision approach

João Rafael Vilela Possani Santos¹ , Marlise Teresinha Mauerwerk¹ 

Joel Rodrigues dos Santos¹ , Lilian Carolina Rosa da Silva¹ 

André Rodrigues da Cunha Barreto-Vianna¹  & Álvaro José de Almeida Bicudo¹ 

¹Department of Animal Sciences, Federal University of Paraná, Palotina, PR, Brazil

Corresponding author: Álvaro J.A. Bicudo (alvaro.bicudo@ufpr.br)

ABSTRACT. Zebrafish (*Danio rerio*) are a vertebrate model used in biomedical and ecological research that requires standardized feeding protocols to ensure the reproducibility and accuracy of trials across laboratories. This study evaluated gastrointestinal transit time (GITT) in zebrafish using a computational red, green, and blue (RGB)-based method to analyze the transition of fecal color. While sex and size did not significantly influence GITT, relative fecal production varied ($P < 0.05$) by sex-size interactions: small males produced 29.7% of their average body weight, whereas large females and males yielded 6.1 and 5.2%, respectively. The RGB method demonstrated superior precision (coefficient of variation - CV < 18%) compared to human observation (CV = 70.83%), with strong inter-channel correlations (red-green: $r = 0.971$, red-blue: $r = 0.944$; $P < 0.05$). Human vision estimated GITT at 4:00 h, while the RGB channels indicated a range of 3:40-3:48 h. These findings validate computer vision as a standardized tool for digestive studies and highlight the need for sex-specific feeding protocols in zebrafish aquaculture to optimize nutrient utilization and welfare.

Keywords: feeding strategies; cyprinid; model organism; *Danio rerio*; fish nutrition

In recent years, the use of zebrafish (*Danio rerio*) as a vertebrate model in various research areas has significantly increased, leading to a rapid expansion of understanding in many aspects of the species' physiology. However, studies on the nutritional aspects of the species have lagged. Initially, *D. rerio* was kept in captivity as an ornamental fish, which explains the use of feeding protocols similar to those of other ornamental species, without considering specific biological characteristics, such as the absence of a stomach (Cassar et al. 2018). Thus, implementing standardized protocols for the rearing and feeding of zebrafish is crucial to ensure the reliability and comparability of results across different studies (Watts & D'Abramo 2021).

The absence of hunger, one of the five principles of laboratory animal welfare (Canedo et al. 2022), necessitates the development of species-specific feeding protocols. For instance, a strong correlation exists between hunger and the degree of depletion in the gastrointestinal system, which is influenced by gut motility (Mock et al. 2022). Therefore, information on the gastrointestinal transit time (GITT) is crucial to ensure an adequate frequency of feed supply to fish. The GITT can be influenced by several aspects of feeds, such as crude fiber content and chemical composition (Welker et al. 2020), biological features of species, such as size and feeding habit (Handeland et al. 2008, Miegel et al. 2010), or rearing conditions, such as temperature (Fauconneau et al. 1983, Mock et al. 2022).

Various methodologies are available for measuring the GITT in fish; however, some, such as serial x-ray or stable isotope measurements (Sandre et al. 2016), may have limited application due to their high cost or the need for specialized equipment. Others present a significant limitation due to ethical guidelines for animal use in research, which necessitate the sacrifice of multiple animals (Kitagima & Fracalossi 2010). The most used methodology for measuring GITT in fish is based on the study by Storebakken (1985). This methodology involves the use of two inert markers in feeds, typically titanium dioxide (a whitish color) and chromium oxide (a greenish color), to contrast the color of feces. A gradual change in the color of feces allows for the establishment of a gradual scale (0, 0.5, and 1.0). The GITT is determined when the feces reach 1.0 degree. Although human color perception is versatile, it can be subjective and influenced by individual differences or environmental conditions, such as lighting, which may make it difficult to detect subtle color differences.

To address this limitation, an objective red, green, and blue (RGB)-based computational approach is proposed to standardize fecal color analysis by reducing observer-dependent variability and enhancing reproducibility in GITT assessment. All methods described have been approved by the Committee for Ethics in Animal Experimentation of the Federal University of Paraná, Palotina, PR, Brazil (Protocol 11/2022).

The experiment was conducted using 20 polycarbonate aquariums (3 L) within a water recirculation system. Each aquarium was equipped with a supplementary aeration system (dissolved oxygen: 6.2 ± 1.2 mg L⁻¹), provided by air diffuser stones. The water temperature was maintained at $28 \pm 1^\circ\text{C}$ using an air conditioner in the room. A photoperiod of 12:12 h of light:darkness was established.

A powdered commercial fish diet (Raguife®) was used as a basal diet (90.9% of dry matter, 45.0% of crude protein, 5.7% of ether extract, 6.1% of crude fiber, 17.9% of ash, and 4.0 kcal g⁻¹ of gross energy). In this diet, 1% of titanium dioxide or chromium oxide markers were added to observe changes in fecal color. The diets were moistened, granulated to a diameter of 1 mm, dried in a forced-air oven at 55°C , broken, and sieved to retain crumbles between 300 and 425 μm , which were used to feed the fish.

The zebrafish originated from a pooled collection of eggs from multiple breeding pairs, incubated and raised to approximately 90 ± 10 days post-fertilization, and were distributed into each aquarium based on sex and

body weight, categorizing them as small (females = 800 ± 210 mg; males = 330 ± 40 mg) and large (females = $1,830 \pm 180$ mg; males = $1,370 \pm 370$ mg) in a completely randomized 2×2 factorial design with five replicates per treatment.

The gastrointestinal transit was evaluated using an adapted methodology applied to juvenile rainbow trout (*Oncorhynchus mykiss*) (Storebakken 1985). In the present study, fish were fed a diet containing titanium dioxide to apparent satiety daily from 7:00 to 19:00 h at 2-h intervals over a period of five days. On the fifth day, additional feedings of the titanium dioxide diet were provided from 21:00 to 23:00 h at 30 min intervals. The following day, from 7:00 to 10:00 h, the fish were fed until apparent satiety with a diet containing chromium oxide every 30 min. After the final feeding, water input was finished, and only a slightly supplemental aeration was maintained through air stones. Each aquarium was then carefully siphoned to remove any feces or leftover feed before fecal sampling began, which started 1 h after the last feeding. At hourly intervals, all feces from each aquarium were meticulously pipetted and placed onto a pre-weighed white filter paper, which was then observed and photographed under a stereo microscope. A visual assessment of the greenish color of each sample was conducted using a scale ranging from 0 to 1, with increments of 0.25, which is a modification of the original methodology used by Storebakken (1985), as referenced by Sandre et al. (2016) and Fabregat et al. (2015). A single, previously trained observer determined the greenish hue of the feces to avoid interspecific error discrepancies. Following this, the filter papers were dried in a forced-air oven until a constant weight was achieved. The mass of the feces in each experimental unit was determined by the gravimetric method. The relative fecal production (%) was determined by dividing the mass of feces produced by the biomass present in each aquarium.

The images of fecal samples collected hourly from each aquarium were analyzed using ImageJ® software. Ten random points on the feces were selected using the software's multi-point tool to measure the values of the RGB color spaces. The mean of these 10 points was used to establish the RGB values for each sample (group of feces).

The Shapiro-Wilk and Bartlett's tests were utilized to confirm the normal distribution and homoscedasticity of all data, respectively. Consequently, a two-way ANOVA was conducted to examine the effects of sex, size, and their interaction. If a significant effect was detected ($P < 0.05$), a Tukey's test was performed to compare the means. Additionally, regression analysis

was employed to evaluate feces production over time. The Pearson correlation test was used to verify the correspondence between the color transition of feces, from whitish to greenish, as observed by human and computational vision (Bate & Clark 2014).

Strong negative correlations were observed between human vision and RGB channels (R: $r = -0.676$, G: $r = -0.654$, B: $r = -0.690$; all $P < 0.05$), whereas inter-channel correlations were highly positive (R-G: $r = 0.971$, R-B: $r = 0.944$, G-B: $r = 0.969$; $P < 0.05$).

The regression analysis revealed a minor discrepancy between the GITT estimated by human vision and the values obtained from the RGB channels. Visual analysis estimated the transit time to occur after 4:00h ($Y = -48.5714 + 61.3244x - 7.6935x^2$; $R^2 = 0.5403$), while the red, green, and blue channels estimated it at 3:43 h ($Y = 201.2257 - 36.0740x + 4.8460x^2$; $R^2 = 0.3482$), 3:48 h ($Y = 211.6986 - 35.4743x + 4.6649x^2$; $R^2 = 0.3384$), and 3:40 h ($Y = 197.4114 - 51.7524x + 7.0733x^2$; $R^2 = 0.4317$), respectively. Additionally, human vision showed high variability (70.83% coefficient of variation) in recording values, compared to computer vision's red (11.92%), green (10.6%), and blue (17.6%) channels.

The influence of sex and size, as well as their interaction, did not significantly affect the gastrointestinal transit time of zebrafish, regardless of whether fecal color was measured by human observation or computer vision. However, the relative fecal production showed a significant interaction ($P < 0.05$) between the sex and size of the fish. However, the mass of feces produced was similar across all groups (Table 1).

The production of fecal matter in zebrafish peaked at 2:10 h post-collection (Fig. 1). By the seventh hour, fecal production was registered in 15 out of 20 aquariums (75%). At the eighth hour, only four experimental units (20%) showed fecal production; thus, this data was excluded from the analysis.

The RGB color space is a three-dimensional rectangular coordinate system used to represent color images. Each pixel in an RGB image is composed of three components (red, green, and blue) with intensities ranging from 0 to 255, which are electronically combined to create a digital color picture (Afshari-Jouybari & Farahnaky 2011). The color scale, formed using the RGB color space, illustrates the gradual change in fecal pigmentation observed over a 7 h period (Fig. 1).

The GITT of zebrafish was shorter (3 h and 48 min) compared to values reported for other fish species, such as 11 h and 30 min for channel catfish (*Ictalurus*

punctatus) by Kitagima & Fracalossi (2010), 14 h for pacu (*Piaractus mesopotamicus*) by Dias-Koberstein et al. (2005), 10 h for oscar (*Astronotus ocellatus*), and 12 h for angelfish (*Pterophyllum scalare*) by Fabregat et al. (2015). Indeed, stomachless species, such as zebrafish, generally exhibit shorter GITTs compared to carnivorous or omnivorous species with stomachs (Aréchiga-Palomera et al. 2023). For example, the marine, stomachless species, topsmelt silverside (*Atherinops affinis*) initiated defecation 2.1 h after feeding on algae (Logothetis et al. 2001). Determining GITT enables the optimization of feeding regimes in cultured fish, which aids in estimating appetite recovery (Gao et al. 2022).

By aligning feeding frequency with the return of appetite, which is associated with the emptying of the gastrointestinal tract, it is possible to improve growth, enhance feed conversion, and reduce water pollution (Liu et al. 2022). *D. rerio* is usually fed *ad libitum* (Millington et al. 2024), but frequency across laboratories shows high variability. Two or three meals per day are the most popular choice, despite facilities using only one meal daily (Lidster et al. 2017, Licitra et al. 2024). The shorter GITT in zebrafish (3 h and 48 min) compared to stomach-bearing species (e.g. pacu: 14 h) underscores the need for species-specific feeding protocols. Frequent meals (≥ 3 per day) are recommended to align with their rapid digestive physiology and ensure welfare (Canedo et al. 2022).

In fish, distinct metabolic strategies are observed: a locomotor metabolic profile prioritizes mobility at the expense of digestive efficiency, whereas a visceral metabolic profile enhances digestion during reproductive phases (Liu et al. 2024). While males typically exhibit higher mass-specific metabolic rates (energy turnover per unit body mass) (Jerde et al. 2019), which may be associated with accelerated intestinal transit, the zebrafish study did not detect any sex-based differences in GITT. Fecal output variations may reflect sex-specific digestive efficiency. In zebrafish, intestinal estrogen receptors have been found to regulate motility and enzymatic secretion directly (Chandrasekar et al. 2010). During vitellogenesis, resource allocation to yolk synthesis was associated with a 20-40% reduction in digestive enzyme activity (trypsin, lipase) in zebrafish females, indicating a trade-off between reproduction and nutrient processing (Levi et al. 2009).

The high coefficient of variation in human assessments (70.83%) highlights the subjectivity of traditional methods. In contrast, the RGB channels provided consistent measurements (CV < 18%), align-

Table 1. Fecal production of zebrafish (*Danio rerio*) by sex and size after 7 h. Different superscript letters within columns indicate significant differences (Tukey's test, $P < 0.05$).

Size: sex	Fecal mass (mg)	Relative fecal production (% biomass)
Small female	100.0 ± 8.0 ^a	13.3 ± 4.0 ^b
Large female	111.0 ± 19.0 ^a	6.1 ± 1.4 ^c
Small male	97.0 ± 6.0 ^a	29.7 ± 3.8 ^a
Large male	100.0 ± 13.0 ^a	5.2 ± 1.1 ^c
Two-way ANOVA (P -values)		
Sex	0.2222	0.0001
Size	0.2551	0.0001
Sex × size	0.4435	0.0001

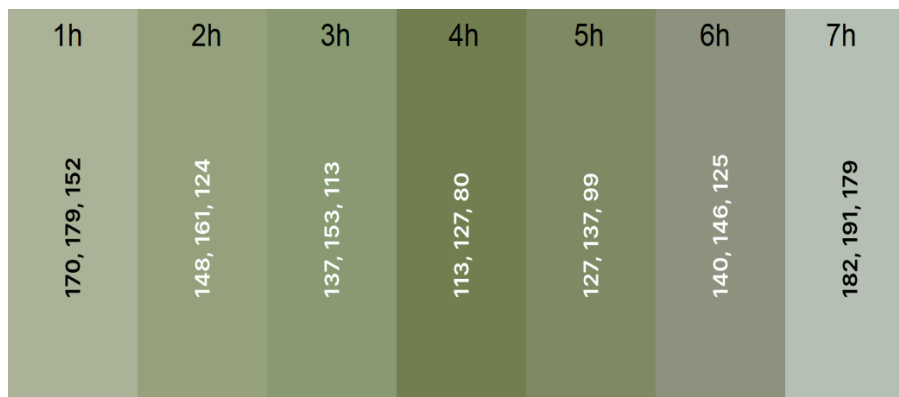


Figure 1. Hourly chromatic progression of zebrafish feces post-ingestion of chromium oxide-enriched feed. The three values displayed alongside each hourly interval correspond, respectively, to red (R), green (G), and blue (B).

ing with digital colorimetry standards (Afshari-Jouybari & Farahnaky 2011).

This study highlights the importance of evidence-based feeding protocols in zebrafish aquaculture, demonstrating that GITT remains consistent across both sexes and sizes. Additionally, the computational RGB analysis method demonstrated greater precision compared to human observation, providing an objective standard for digestive studies.

Credit author contribution:

J.R. Vilela Possani Santos: investigation, writing and original draft; M. Teresinha Mauerwerk: investigation, methodology, writing and original draft; J. Rodrigues dos Santos: investigation and visualization; L.C. Rosa da Silva: resources, writing, review and editing; A. Rodrigues da Cunha Barreto-Vianna: funding acquisition, resources, methodology and validation; Á.J. de

Almeida Bicudo: conceptualization, funding acquisition, project administration, formal analysis, writing, review and editing.

Conflict of interest

The authors declare that they have no conflict of interest.

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REFERENCES

- Afshari-Jouybari, H. & Farahnaky, A. 2011. Evaluation of Photoshop software potential for food colorimetry. *Journal of Food Engineering*, 106: 170-175. doi: 10.1016/j.jfoodeng.2011.02.034
- Aréchiga-Palomera, M.A., Nolasco-Soria, H., Carrillo-Farnés, O., et al. 2023. Contribution to some aspects of the digestive tract anatomy and food evacuation of the Pacific fat sleeper *Dormitator latrifrons*. *Latin American Journal of Aquatic Research*, 51: 521-529. doi: 10.3856/vol51-issue4-fulltext-3038
- Bate, S.T. & Clark, R.A. 2014. The design and statistical analysis of animal experiments. Cambridge University Press, London.
- Canedo, A., Saiki, P., Santos, A.L., et al. 2022. Zebrafish (*Danio rerio*) meets bioethics: the 10Rs ethical principles in research. *Ciência Animal Brasileira*, 23: 70884. doi: 10.1590/1809-6891v22e-70884
- Cassar, S., Huang, X. & Cole, T. 2018. High-throughput measurement of gut transit time using larval zebrafish. *Journal of Visualized Experiments*, 140: 58497. doi: 10.3791/58497
- Chandrasekar, G., Archer, A., Gustafsson, J.Å., et al. 2010. Levels of 17 β -estradiol receptors expressed in embryonic and adult zebrafish following *in vivo* treatment of natural or synthetic ligands. *Plos One*, 5: 0009678. doi: 10.1371/journal.pone.0009678
- Dias-Koberstein, T.C.R., Carneiro, D.J. & Criscuolo-Urbinati, E. 2005. Tempo de trânsito gastrintestinal e esvaziamento gástrico do pacu (*Piaractus mesopotamicus*) em diferentes temperaturas de cultivo. *Acta Scientiarum*, 27: 413-417. doi: 10.4025/actascianimsci.v27i3.1219
- Fabregat, T.E.H.P., do Nascimento, T.M.T., Pereira, T.S., et al. 2015. Caracterização das proporções anatômicas e tempo de trânsito gastrointestinal do apaiari e do acará-bandeira. *Boletim do Instituto de Pesca*, 41: 671-676.
- Fauconneau, B., Choubert, G., Blanc, D., et al. 1983. Influence of environmental temperature on flow rate of foodstuffs through the gastrointestinal tract of rainbow trout. *Aquaculture*, 34: 27-39. doi: 10.1016/0044-8486(83)90289-2
- Gao, X.Q., Wang, X., Wang, X.Y., et al. 2022. Effects of different feeding frequencies on the growth, plasma biochemical parameters, stress status, and gastric evacuation of juvenile tiger puffer fish (*Takifugu rubripes*). *Aquaculture*, 548: 737718. doi: 10.1016/j.aquaculture.2021.737718
- Handeland, S.O., Imsland, A.K. & Stefansson, S.O. 2008. The effect of temperature and fish size on growth, feed intake, food conversion efficiency and stomach evacuation rate of Atlantic salmon post-smolts. *Aquaculture*, 283: 36-42. doi: 10.1016/j.aquaculture.2008.06.042
- Jerde, C.L., Kraskura, K., Eliason, E.J., et al. 2019. Strong evidence for an intraspecific metabolic scaling coefficient near 0.89 in fish. *Frontiers in Physiology*, 10: 01166. doi: 10.3389/fphys.2019.01166
- Kitagima, R.E. & Fracalossi, D.M. 2010. Validation of a methodology for measuring nutrient digestibility and evaluation of commercial feeds for channel catfish. *Scientia Agricola*, 67: 611-615. doi: 10.1590/s0103-90162010000500016
- Levi, L., Pekarski, I., Gutman, E., et al. 2009. Revealing genes associated with vitellogenesis in the liver of the zebrafish (*Danio rerio*) by transcriptome profiling. *BMC Genomics*, 10: 141. doi: 10.1186/1471-2164-10-141
- Licitra, R., Fronte, B., Verri, T., et al. 2024. Zebrafish feed intake: a systematic review for standardizing feeding management in laboratory conditions. *Biology*, 13: 209. doi: 10.3390/biology13040209
- Lidster, K., Readman, G.D., Prescott, M.J., et al. 2017. International survey on the use and welfare of zebrafish *Danio rerio* in research. *Journal of Fish Biology*, 90: 1891-1905. doi: 10.1111/jfb.13278
- Liu, Y., Kou, C., Chen, J., et al. 2024. The response of the gut physiological function and microbiome of a wild freshwater fish (*Megalobrama terminalis*) to alterations in reproductive behavior. *International Journal of Molecular Sciences*, 25: 137425. doi: 10.3390/ijms25137425
- Liu, R., Zhou, Y., Li, Z., et al. 2022. Evaluation of the effects of temperature on gastric evacuation and the associated mathematical models in different sizes steelhead trout (*Oncorhynchus mykiss*). *Aquaculture*, 549: 737815. doi: 10.1016/j.aquaculture.2021.737815
- Logothetis, E.A., Horn, M.H. & Dickson, K.A. 2001. Gut morphology and function in *Atherinops affinis* (Teleostei: Atherinopsidae), a stomachless omnivore feeding on macroalgae. *Journal of Fish Biology*, 59: 1298-1312. doi: 10.1006/jfbi.2001.1740
- Miegel, R.P., Pain, S.J., van Wettère, W.H.E.J., et al. 2010. Effect of water temperature on gut transit time, digestive enzyme activity and nutrient digestibility in yellowtail kingfish (*Seriola lalandi*). *Aquaculture*, 308: 145-151. doi: 10.1016/j.aquaculture.2010.07.036

- Millington, M.E., Lawrence, C., Sneddon, L.U., et al. 2024. Environmental enrichment for zebrafish. In: Allen, C. & Mocho, J.P. (Eds.). Zebrafish: A practical guide to husbandry, welfare and research methodology. CABI, Wallingford, pp. 6-52. doi: 10.1079/9781800629431.0002
- Mock, T.S., Alkhabbaz, Z.H.R.A.A., Rocker, M.M., et al. 2022. Gut transit rate in Atlantic salmon (*Salmo salar*) exposed to optimal and suboptimally high water temperatures. Aquaculture Research, 53: 4858-4868. doi: 10.1111/are.15979
- Sandre, L.C.G., Buzollo, H., do Nascimento, T.M.T., et al. 2016. Natural stable isotopes for the determination of gastrointestinal transit time in fish. Journal of the World Aquaculture Society, 47: 113-122. doi: 10.1111/jwas.12244
- Storebakken, T. 1985. Binders in fish feeds. I. Effect of alginate and guar gum on growth, digestibility, feed intake, and passage through the gastrointestinal tract of rainbow trout. Aquaculture, 47: 11-26. doi: 10.1016/0044-8486(85)90004-3
- Watts, S.A. & D'Abramo, L.R. 2021. Standardized reference diets for zebrafish: addressing nutritional control in experimental methodology. Annual Review of Nutrition, 41: 511-527. doi: 10.1146/annurev-nutr-120420-034809
- Welker, T.L., Overturf, K. & Barrows, F. 2020. Development and evaluation of a volumetric quantification method for fecal particle size classification in rainbow trout fed different diets. North American Journal of Aquaculture, 82: 159-168. doi: 10.1002/naaq.10138

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