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A Standardized Structural Environmental Enrichment for Zebrafish Kept Research Facilities

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Abstract

Environmental enrichment for research animals does not only promote animal welfare but is also essential for obtaining scientifically relevant and reproducible results. Currently, all available "enrichments" for zebrafish aquaria are either not standardized, pose a biosafety risk, or may lead to increased aggression due to their monopolization by the dominant individual. We have developed the environmental enrichment divider which provides standardized enrichment for zebrafish aquaria mimicking hanging vegetation found in the natural habitat of zebrafish. The divider cannot be monopolized and thereby does not lead to increased aggression. Furthermore, it can easily be implemented in the workflow of any zebrafish research facility and is made of inert, autoclavable plastic, which does not pose a biosafety risk.

Keywords: zebrafish, environmental enrichment, standardization, husbandry

Introduction

Zebrafish is a commonly used laboratory model for both basic and translational research and represents the second-most used animal for scientific research in the European Union. Besides its numerous scientific benefits, the zebrafish is an integral part of implementing the 3Rs in animal research as it can partly replace traditional murine models, reduce the number of these required and refine experiments that must be carried out in mice and rats.

During the last years, the zebrafish community has started many initiatives to harmonize husbandry and care of zebrafish and developed standardized protocols, for example for health monitoring[1] or import/export and shipping of zebrafish.[2] However, important husbandry procedures and details such as the number of fish per volume, the chemical composition of the water, rearing protocols and similar are often barely standardized.[3–6] Especially structural environmental enrichment for zebrafish aquaria is intensively discussed in the zebrafish community. Often, zebrafish are kept in barren tanks which poorly reflects their natural habitat where they are predominantly found in water bodies rich in vegetation.[7] The arguments for keeping zebrafish in barren tanks such as increased cost and labor as well as the potential negative effect on standardization are essentially the same that have been put forward against enrichment in mouse cages some 20 years ago. However, there is an overwhelming body of evidence available that proper enrichment is essential for obtaining reproducible and significant high-quality data.[8]

Even though enrichment in zebrafish aquaria is essential for animal welfare reasons and legally required in many countries, special care needs to be taken when increasing the complexity in the aquaria. Ideally, the enrichment should have the following characteristics: (1) it should provide a stimulating environment with suitable hiding places, (2) it must not obstruct the possibility to inspect the fish, (3) it must be made of inert material that does not leach chemicals into the water, (4) it should not be monopolizable by a dominant individual, (5) it should not obstruct the schooling behavior of the animals, and (6) it should be easy and fast to handle so that even large facilities with thousands of tanks can implement it with minimal impact on labor time and cost.

We have developed an environmental enrichment divider and tested it in a large aquatic facility at Karolinska Institutet. We believe that this structural enrichment provides superior characteristics to available enrichments and that it can easily be implemented even in large zebrafish facilities.

Material and Methods

Husbandry

Zebrafish were housed in self-cleaning 3.5 L tanks with a density of circa 5.7 fish per liter in a centralized recirculatory aquatic system (Tecniplast). Basic water parameters were continuously surveilled and automatically adjusted to a temperature of 28°C; conductivity 1200 μS/cm, pH 7.5. Other chemical water parameters were checked minimum monthly using

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colorimetric tests, see also Supplementary Table S1. Reef base salt (Tropical) was used for adjusting conductivity, Sodium bicarbonate (Merck) was used to adjust the pH, autoclaved oystershells were used to adjust general hardness and carbonate hardness. The illumination scheme was set to 14 h light/10 h dark with a 20 min dawn and dusk period. All fish younger than 11 weeks were fed standardized amounts of dryfeed (Zebrafeed, Sparos) by a feeding robot (Tritone, Tecniplast) and standardized volumes of rotifers using a Multipette (Eppendorf) according to the facility standard protocol. All fish older than 11 weeks were fed twice a day standardized amounts of dryfeed (Zebrafeed, Sparos mixed 1:1 with O-range Grow, OceanNutrition) using a hand-held feeder and standardized volumes of rotifers using a Multipette (Eppendorf). Zebrafish are euthanized either by hypothermic chock or by an overdose of lidocaine.[9] All husbandry is performed by staff according to standardized procedures which have been evaluated and approved by the animal welfare body of Karolinska Instituted. All procedures have been evaluated and approved by the Stockholm's ethical committee, section 2 (Dnr 14049-2019 and 15591-2023). All standard operation procedures are available on request.

Fishlines used

For this study, the AB line was used. The AB line was originally imported by ZIRC and is bred in-house with a mating technique following the "round-robin" protocol developed by ZIRC (see zfin.org for details).

Rotifer culture

Rotifers were kept in a continuous culture at a concentration of ca 0.5×10^6 rotifers per liter. The culture was fed by an automatic feeding system once an hour with Rotigrow Plus (Reef Nutrition). The rotifer culture was submitted for health monitoring every quarter.

Health monitoring

The daily health check was performed by facility staff following the Karolinska Institutet template for determination of zebrafish health (available on request). Animals with a score above 0.3 were terminated. The routine health monitoring of the colony was performed according to the Federation of European Laboratory Animal Science Associations guidelines.[1] *Mycobacteroides chelonae* and *Mycobacterium fortuitum* have been detected in sludge samples, ZfPV-1 has been detected in sentinels, randomly sampled animals and sludge samples. Any strains are imported to a physically separate quarantine unit from which only surface-disinfected eggs are transferred to the breeding colony barrier. The biosafety plan and health monitoring protocols of the facility are available on request.

Breeding

The hometank of the zebrafish is removed from the system and transported on a trolley to the breeding area. Breeding tanks (Aquaneering) are filled with system water and plastic plants are added. A divider is used to separate male and female fish. 6.5 h after beginning of the dusk period, the animals are transferred into the breeding tank, the breeding tank is enriched with a plastic plant on each side and put on a black surface. The following day, 30 min after beginning of the dusk period, the dividers are removed and the water exchanged to start the spawning behavior. Eggs are collected with a tea-sieve and the mating is terminated 4.5 h after the dividers were removed.

Experimental procedure: analysis of breeding performance

Two independent groups of AB fish were analyzed. The breeding performance of group A was analyzed from May to November; the breeding performance of group B was analyzed from October to April. At 11 weeks of age, fish are sexed and counted and 3.5 L tanks (Tecniplast) are populated with 10 randomly selected males and 10 randomly selected females. The tank is either equipped with an artificial plastic plant ("Spawning wadd", AquaSchwarz, Germany) or an environmental enrichment divider. The tanks are subsequently placed on alternating positions into the same row of a standard rack (Tecniplast). Weekly for a total of 27 weeks, matings are set up as described above. The position of the different groups on the mating shelves is alternated weekly. Approximately two hours after spawning, the fertilization rate and number of successful breeding pairs is determined. 822 matings of group A and 636 matings of group B have been analyzed.

Experimental procedure: counting midline passages

Minimum 1 hour after feeding, the tank to be analyzed was removed from the system, placed on a gravel image with a camera positioned above the area and the fish were allowed to acclimatize for minimum 20 min. After 20 min, the tank was filmed from above for 20 min. No personnel was present in the room during the filming to prevent disturbing the fish. The number midline passages were manually counted 5 times for 60 s each (minute 1–2, 4–5, 10–11, 13–14, and 16–17). The total number of midline passages was calculated. Two independent sets of tanks with either artificial plant or environmental enrichment divider were analyzed.

Experimental procedure: feeding, determining body weight and standard length of adult fish

Fish of all groups were fed a standardized amount of feed either by a Tritone feeding robot (<11 weeks) or by using a manual feeding gun as described above. Fish were terminated by hypothermic chock, gently dried off with a paper towel and placed on a millimeter paper on a balance. The weight and the standard length (tip of the nose to caudal peduncle) was recorded.

Experimental procedure: environmental enrichment

The environmental enrichment divider was modified from the available divider (ZB30DV, Tecniplast) which is made of polycarbonate. Holes and cutouts were done using manual tools and the edges were sanded off. As control, plastic plants were used ("Spawning wadd," AquaSchwarz, Germany).

Statistics

Statistics for analysis of midline passages, weight and size were calculated with R either based on the ANOVA or *t-test*. $p > 0.05$: not significant; $p < 0.05$: *; $p < 0.005$:**; $p < 0.0005$:***.

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Results

We developed the design of the structural enrichment based on the following requirements: (1) It should be made of inert plastic that does not pose a biosafety risk and which can easily be disinfected; (2) it should not be monopolizable by one individual which would lead to increased stress and aggression; (3) it should not block the fish from foraging food; and (4) it should be easy to implement it in large research facilities and not increase the workload for staff. Based on these prerequisites, we developed the enrichment divider.

The enrichment divider is made of a flat sheet of polycarbonate plastic, which fits into the divider slits in a 3.5 L Tecniplast tank (Fig. 1A and B). The divider has recesses on the top side to allow the fish to freely feed and on the lower side to allow for the self-cleaning function of the tank. In the center of the divider holes with a diameter of ca 4 cm are cut out which easily allow animals to pass (Fig. 1A and C). We reasoned that this design allowed the animals to seek hiding places if needed, but at the same time, it allowed them to form schools and freely feed.

We feed standardized amounts of dryfeed to our animals by using a "feeding gun" (see material and methods section) and the dryfeed is supplied through a hole sitting on the right-hand side of the lid. We analyzed both how the dryfeed is distributed on the water surface and how long the animals need to consume the dryfeed in tanks supplied with a plastic plant compared in tanks equipped with an environmental enrichment divider.

Indeed, we neither observed that the distribution of the feed on the water surface was obstructed by the enrichment divider (Fig. 1D and E), nor that time until all feed is consumed increases in a tank equipped with the enrichment divider compared to a control tank with an artificial plant. (Supplementary Video).

We next determined if the environmental enrichment divider affects the growth of the animals during their lifetime in the facility. Animals were reared in barren tanks for 11 weeks according to our standardized rearing protocol followed by sexing and distributing them into groups of 20 fish consisting of 10 males and 10 females. The groups were randomly assigned to tanks which were either equipped with an environmental enrichment divider or an artificial plant and placed into alternating slots of the same row of a Tecniplast multi-link rack system. The animals were kept according to the description in the material and methods section, fed a standardized amount of feed during their whole lifetime and bred with regular intervals for animal welfare reasons. The animals were kept for 46 weeks in tanks either equipped with an enrichment divider or a plastic plant before they were euthanized by hypothermic shock and their weight and standard length was measured. We neither observed a significant weight difference nor a significant difference of the standard length of animals reared in tanks equipped with an artificial plant compared to animals reared in tanks equipped with the environmental enrichment divider (Fig. 2A and B).

Besides determining the effect of the environmental enrichment divider on the foraging behavior and consequently on their growth, we sought to investigate its effect on the swimming and schooling behavior outside feeding times. For that, we manually counted the number of animals passing the midline of the tank, the position in which the environmental enrichment divider is installed. We analyzed two independent biological replicates with 40 individuals per replicate and observed that in tanks equipped with an environmental enrichment divider the number of midline passages is significantly smaller compared to tanks that contained an artificial plant (Fig. 3).

We proceeded to investigate if the environmental enrichment divider affects the breeding performance of animals. For

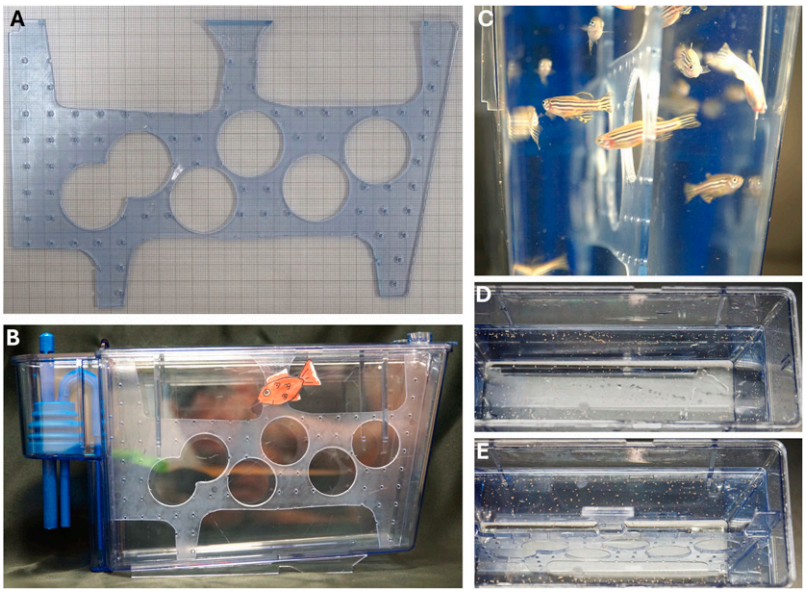


FIG. 1. The design and measurements of the enrichment divider **(A)**. The recesses on the top allow for free forging behavior; the recesses on the bottom allow for the self-cleaning function of the tank **(A, B)**. The enrichment divider fits into the slots positioned in the midline of a 3.5 L tank manufactured by Tecniplast **(B)**. Animals passing the holes in the enrichment divider **(C)**. The distribution of feed is not obstructed by the enrichment divider compared to the tank enriched with a plastic plant **(D, E)** (see also Supplementary Video).

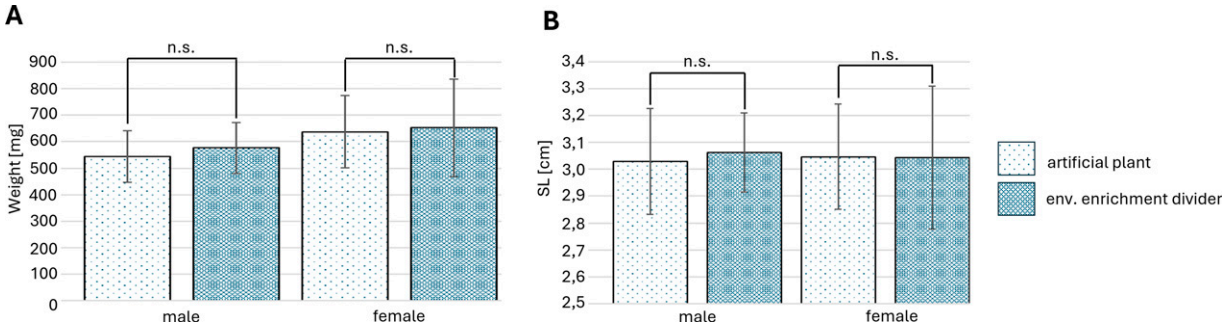


FIG. 2. Weight distribution of animals kept for 46 weeks in tanks either equipped with an artificial plant or with an enrichment divider. No significant difference was observed. N = 27 (male/enrichment divider); N = 30 (female/enrichment divider); N = 27 (male/plant); N = 30 (female/plant) **(A)**. Standard length distribution of animals kept for 46 weeks in tanks either equipped with an artificial plant or with an enrichment divider. No significant difference was observed. N = 27 (male/enrichment divider); N = 30 (female/enrichment divider); N = 27 (male/plant); N = 30 (female/plant) **(B)**.

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that, we analyzed the number of successful breeding pairs and the percentage of fertilized eggs of two independent groups of AB wildtype fish (the two independent breeding groups are termed "group A" and group "B"). After rearing the animals in barren tanks for 11 weeks according to our standardized protocol, the fish were sexed and distributed into groups of 20 fish consisting of 10 males and 10 females. The groups were randomly assigned to tanks which were either equipped with an environmental enrichment divider or an artificial plant and placed into alternating slots of the same row of a Tecniplast multi-link rack system. Matings of group A were set up with regular intervals according to the description in the material and methods section and the number of successful breeding pairs and fertilization rate of the clutches was recorded for 27 weeks. After the analysis of group A was finished, the breeding performance of group B was analyzed in the same way to account for potential seasonal or other environmental effects.

A total of 822 matings for group A and 636 matings of group B were analyzed. We did not observe any significant difference of the number of successful breeding pairs of animals housed in tanks equipped with an artificial plastic plant compared to breeding pairs of animals housed in tanks equipped with the environmental enrichment divider (Fig. 4A and B). We did not observe a significant difference in fertilization rate of the spawned eggs (Fig. 4A and B).

Discussion

The welfare of animals used for research purposes is essential, primarily for ethical reasons and for obtaining reproducible and scientifically significant data, but also to promote acceptance of the general society for carrying out research in animals. Environmental enrichment, hence providing research animals with a stimulating environment that mimics their natural habitat, is central for increasing animal welfare.[10,11] The

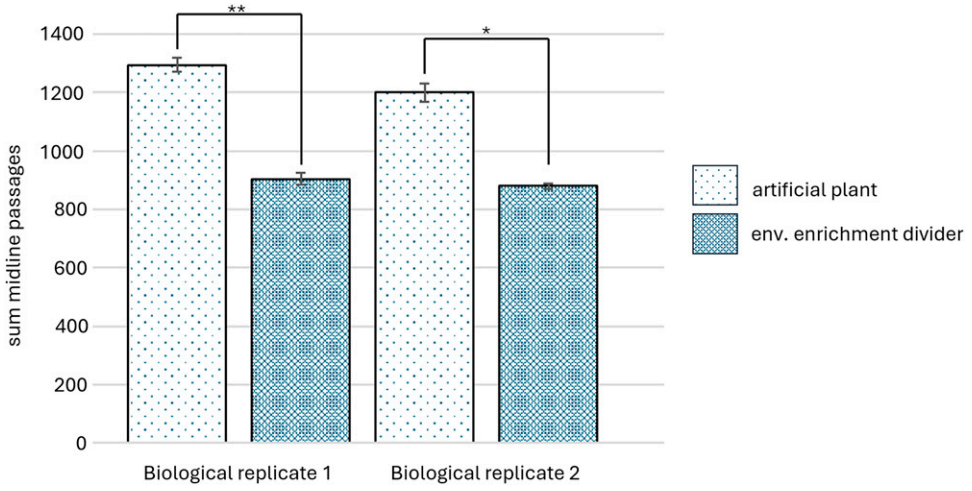


FIG. 3. The number of animals passing the midline of the tank has manually been counted six times for one minute each during a 20 min period. Two tanks equipped with an environmental enrichment divider and two tanks equipped with an artificial plant were analyzed. In tanks containing an artificial plant, 1294 fish (tank 1) and 1200 fish (tank 2) have crossed the midline. In tanks containing the environmental enrichment divider, 904 fish (tank 1) and 880 fish (tank 2) have crossed the midline. The standard deviation shown in the chart represents the differential number of animals crossing the midline during the six-time windows analyzed. The standard deviation shown in the chart represents the differential number of animals crossing the midline during the six-time windows analyzed.

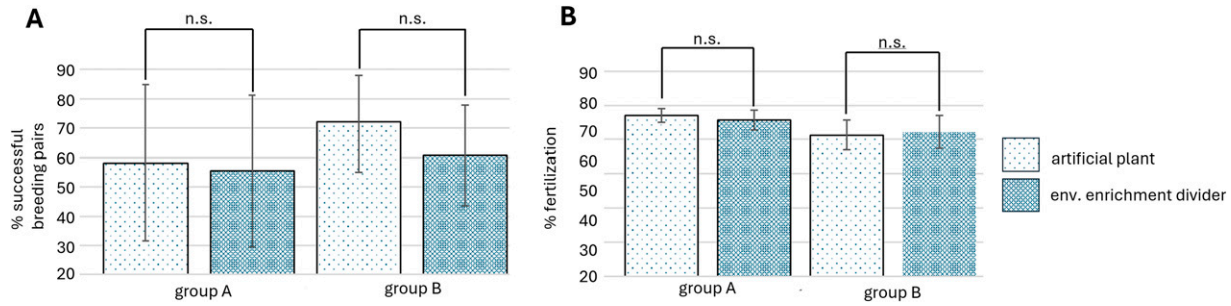


FIG. 4. The breeding performance and clutch quality was analyzed for two independent groups of fish (group A and group B). No significant difference in the number of successful matings **(A)** or fertilization rate **(B)** was observed. N = 822 (group A); N = 636 (group B).

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concept of environmental enrichment has since long been implemented in the husbandry of traditional laboratory animals such as rodents and non-human primates. Also for zebrafish, there is a large body of literature available discussing the positive effects of social, sensory, occupational, nutritional and physical enrichment (reviewed in,[10,11] however zebrafish are still often kept in barren tanks.[3] The latter is a welfare problem as it has previously been shown that zebrafish prefer enriched aquaria if given a choice.[12] Furthermore, a systematic review by Gallas-Lopes has recently been shown that environmental enrichment for laboratory zebrafish has clear benefits for science and animal welfare, however that standardization of the enrichment is urgently required.[13]

To address this problem, we have developed the environmental enrichment divider and tested it extensively in the largest zebrafish core facility of Sweden. The core facility delivers husbandry and experimental services to more than 60 research groups which not only allowed us to study the effect of the enrichment divider on large cohorts of animals using standardized and highly controlled husbandry procedures, but also to optimize the enrichment divider for the usage in large research facilities with thousands of tanks.

The design of the enrichment divider mimics hanging vegetation found in the natural habitat of zebrafish, hence providing hiding places for individual animals but at the same time not limit the schooling or foraging behavior of the animals nor the self-cleaning function of the aquaria.

When designing environmental enrichment for zebrafish, resource guarding must be considered. Resource guarding is common in fish and refers to an individual defending a territory, shelter, mates or food resources. Zebrafish can become highly territorial over objects such as structural enrichment, spawning sites or similar,[14] and that it has been suggested that "enriching" the tank with an object such as a plastic plant which can easily be monopolized by a dominant individual may in fact increase stress and aggression levels.[8,12,15,16] In contrast to objects such as plastic plants, the enrichment divider cannot be monopolized by a dominant individual due to its size and shape. Another fact to take into consideration when designing structural enrichment is that zebrafish tend to nibble on softer plastics which plastic plants are often made of. The plastic may be ingested and leach unknown chemicals into the digestive tract with potentially disastrous effects on scientific outcomes.

Zebrafish are known to form shoals when kept in larger groups and shoals are believed to be a sign of decreased fearfulness.[17] We wanted to ensure that the environmental enrichment divider does not block the animals to form shoals and restrict the volume the fish are exploring. For that, we have determined the number of fish passing through the environmental enrichment divider and compared it to the number of animals passing the midline of tanks equipped with a plastic plant. The enrichment divider leads to significantly fewer number of passages between the two halves of the tank which indicates that individuals may hide behind the divider if desired. However, based on the large number of animals passing through the environmental enrichment divider we conclude that free passage for individual animals that wish to pass is possible and, as consequence, the formation of shoals is not hindered. In essence, the enrichment divider gives the animals the possibility to actively choose on which side of the environmental enrichment divider they wish to be.

We carefully followed two independent generations of animals in our facility during their whole lifetime and could neither see that the enrichment divider negatively affects breeding success or the quality of the spawned eggs, nor the size and weight of the animals. We have not observed increased levels of aggression or an increased number of sick, injured or dead animals in groups housed in tanks equipped with the environmental enrichment divider.

The published findings on structural environmental enrichment for zebrafish are complex and it becomes apparent that finding a suitable structural enrichment for zebrafish tanks is a multi-factorial problem involving many variables such as tank size, density of fish, group composition (single sex vs mixed sex), water flow and more[[16] and for example discussed in review[10,16]]. Hence, implementing structural enrichment should be embedded in a larger standardization approach where the factors mentioned above are addressed at the same time.

We have designed a structural enrichment which is standardized and which can be easily implemented in most zebrafish research facilities independent of their size. The design of the enrichment makes it impossible to be monopolized by a dominant individual and the plastic the enrichment is made of (polycarbonate) does not pose any biosafety concerns and can be disinfected by cage washers or sterilized by autoclaves. Importantly, the enrichment is standardized in size, shape and position within the tank which provides an essential base for increasing scientific reproducibility and relevance of animal experiments carried out in zebrafish.

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Authors' Contributions

A.W. developed the initial design of the environmental enrichment divider. W.V., K.P., and L.B. performed experiments and analyzed data. K.P. and L.B. designed all the experiments. All the authors have read and agreed to the published version of the article.

Disclosure Statement

No competing financial interests exist.

Disclaimer

The small-scale series of the environmental enrichment divider was produced by Tecniplast (Buguggiate, Italy). Tecniplast had no influence on the initial design of the enrichment divider, data acquisition, data analysis of the article.

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Supplementary Material

Supplementary Data
Supplementary Video

References

1. Mocho JP, Collymore C, Farmer SC, Leguay E, Murray KN, Pereira N. FELASA-AALAS recommendations for monitoring and reporting of laboratory fish diseases and health status, with an emphasis on zebrafish (Danio Rerio). Comp Med 2022;72(3):127–148.
2. The Zebrafish in Biomedical Research. In: (Cartner SC, Eisen JS, Farmer SC, Guillemin KJ, Kent ML, Sanders GE., eds). The Zebrafish in Biomedical Research: Academic Press; 2020. p iii.
3. Lidster K, Readman GD, Prescott MJ, Owen SF. International survey on the use and welfare of zebrafish Danio rerio in research. J Fish Biol 2017;90(5):1891–1905.
4. Tsang B, Zahid H, Ansari R, Lee RC, Partap A, Gerlai R. Breeding Zebrafish: A review of different methods and a discussion on standardization. Zebrafish 2017;14(6): 561–573.
5. Stevens CH, Reed BT, Hawkins P. Enrichment for laboratory Zebrafish-A review of the evidence and the challenges. Animals (Basel) 2021;11(3):698.
6. Paull GC, Lee CJ, Tyler CR. Beyond compliance: Harmonising research and husbandry practices to improve experimental reproducibility using fish models. Biol Rev Camb Philos Soc 2024;99(1):253–264.
7. Lee CJ, Tyler CR, Paull GC. Chapter 4 - Geographic Range and Natural Distribution. In: (Cartner SC, Eisen JS, Farmer SC, Guillemin KJ, Kent ML, Sanders GE., eds). The Zebrafish in Biomedical Research: Academic Press; 2020. pp. 41–56.
8. Lee CJ, Paull GC, Tyler CR. Effects of environmental enrichment on survivorship, growth, sex ratio and behaviour in laboratory maintained zebrafish Danio rerio. J Fish Biol 2019;94(1):86–95.
9. Mocho J-P, Lang F, Valentin G, et al. A multi-site assessment of anesthetic overdose, hypothermic shock, and electrical stunning as methods of euthanasia for Zebrafish (Danio rerio) embryos and larvae. Biology (Basel) 2022; 11(4):546.
10. Lieggi C, Kalueff AV, Lawrence C, Collymore C. The influence of behavioral, social, and environmental factors on reproducibility and replicability in aquatic animal models. Ilar J 2020;60(2):270–288.
11. Stevens CH, Reed BT, Hawkins P. Enrichment for Laboratory Zebrafish—A Review of the Evidence and the Challenges. Animals 2021;11(3):698.
12. Schroeder P, Jones S, Young IS, Sneddon LU. What do zebrafish want? Impact of social grouping, dominance and gender on preference for enrichment. Lab Anim 2014;48(4): 328–337.
13. Gallas-Lopes M, Benvenutti R, Donzelli NIZ, Marcon M. A systematic review of the impact of environmental enrichment in zebrafish. Lab Anim (NY) 2023;52(12):332–343.
14. Spence R, Smith C. Male territoriality mediates density and sex ratio effects on oviposition in the zebrafish, Danio rerio. Animal Behaviour 2005;69(6):1317–1323.
15. Woodward MA, Winder LA, Watt PJ. Enrichment increases aggression in Zebrafish. Fishes 2019;4(1):22.
16. Sen Sarma O, Frymus N, Axling F, Thörnqvist P-O, Roman E, Winberg S. Optimizing zebrafish rearing-Effects of fish density and environmental enrichment. Front Behav Neurosci 2023;17:1204021.
17. Miller N, Gerlai R. From schooling to shoaling: Patterns of collective motion in zebrafish (Danio rerio). PLoS One 2012;7(11):e48865.

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