Journal of the American Association for Laboratory Animal Science
Copyright 2016
by the American Association for Laboratory Animal Science

Vol 55, No 3
May 2016
Pages 291–294

Effects of Environmental Enrichment on the Fertility and Fecundity of Zebrafish (*Danio rerio*)

Lemnique N Wafer,[1,*] V Behrana Jensen,[2] Jesse C Whitney,[1] Thomas H Gomez,[1] Rene Flores,[1] and Bradford S Goodwin[1]

Zebrafish (*Danio rerio*) are a popular vertebrate model in biomedical research, but information describing the effects of environmental enrichment on fertility and fecundity of zebrafish is sparse. In the current study, 18 breeding pairs were placed in divided 1.5-L breeding tanks containing 1 of 3 enrichment conditions: plastic grass (n = 6), plastic leaves (n = 6), or no enrichment (n = 6, control). The pairs were allowed to spawn for 3 h the next day, after which eggs were counted and breeding pairs were returned to holding tanks for use in subsequent sessions. Spawning sessions were repeated at 7-d intervals until the completion of 9 trials, with pairs rotating to a different condition at each interval. Total egg count (mean ± SEM) after 3 h was greater for zebrafish spawning in the grass environment (48.0 ± 7.7 eggs) than in the leaf or control environments (29.4 ± 5.3 and 20.4 ± 3.7 eggs, respectively). An interaction emerged between enrichment type and the age of the spawning pair on the number of fry at 6 d postfertilization (dpf). Initially, more fry were obtained from 110- and 160-dpf pairs with the grass enrichment, but from 173- and 180-dpf pairs there were more obtained with leaf enrichment than grass. A separate experiment showed that enrichment type did not have an effect on fry survivability. Overall, our data indicates that, under certain conditions, zebrafish fertility and fecundity are greater in a breeding tank containing environmental enrichment than in a bare tank.

Abbreviation: dpf, days postfertilization

Zebrafish (*Danio rerio*) are one of the most important vertebrate model organisms in the areas of developmental biology, genetics, neurophysiology and biomedicine.[10] Many favorable characteristics, including high fecundity, small size, rapid generation time, and optical transparency during early embryogenesis, contribute to their popularity as a model of human disease and development.[4,10]

Institutions receiving Public Health Service funding within the United States are required to follow the guidelines and recommendations of the *Guide for the Care and Use of Laboratory Animals*,[2] an internationally accepted primary reference on laboratory animal care, including its specific sections on the environmental enrichment, care, and management of aquatic species.[10] The use of environmental enrichment is not well established for aquatic species in research settings, but the *Guide* notes that substrates can provide enrichment for aquatic animals by promoting species-appropriate behaviors, such as burrowing, foraging, and enhanced spawning.[2] Because of the evolving guidelines and the wide use of zebrafish in various types of studies, institutions are now starting to explore improvements regarding the wellbeing of the fish in laboratory animal programs.[10]

Zebrafish are native to South Asia and are found in freshwater habitats where the waters are often cloudy, with low visibility and abundant submerged vegetation.[4,5,9] In nature, zebrafish prefer to spawn in sites replete with aquatic vegetation.[9,10] In controlled environments, zebrafish spawn preferentially in vegetated compared with nonvegetated sites and in shallow water.[1,8] However, in laboratory settings, zebrafish typically are housed within a barren microenvironment. In addition, an environment lacking enrichment may restrict the natural behavioral repertoire of zebrafish and compromise their wellbeing if the animal is highly motivated to perform a particular behavior.[11]

To develop housing and environmental enrichment strategies in research and production facilities using zebrafish, increased understanding of the effect of the breeding tank environment on their reproductive performance is needed. Few studies on zebrafish reproduction have focused on larval growth in natural habitats and the influence of environmental enrichment on oviposition. The objective of the current study was to evaluate the effect of environmental enrichment in the breeding tank on egg and fry production across multiple weekly spawning sessions. We hypothesized that the fertility and fecundity of zebrafish in a breeding tank with environmental enrichment would be greater than those of fish in a bare tank.

Materials and Methods

Life-support system, husbandry, and feeding. Zebrafish (*Danio rerio*) used in this experiment were of the wild-type AB strain and were spawned from a colony held in our vivarium. The AB fish used in this experiment were from a line maintained at our institution for at least 6 y. To generate fry for the experiment, a group of AB zebrafish was group-spawned, and the resulting fry were separated into 3 barren tanks of approximately 30 fry per tank. At 90 d postfertilization (dpf), 18 pairs were selected at random from the 90 fish and housed in designated barren 2.8-L tanks for use in sequential spawning sessions throughout the duration of the study. The self-cleaning housing tanks were kept on racks on a recirculating aquatic housing system (Aquaneering, San Diego, CA) in a holding room maintained on a 12:12-h light:dark cycle (lights on, 0800). The temperature of the system water was maintained at 27 °C, pH between 7.2 to 7.4, conductivity between 600 and 700 µS/cm, alkalinity at 120 mg/L as $CaCO_3$, nitrite (NO_2^-) undetectable, and nitrate (NO_3^-) at 0 to 40 mg/L. Temperature, conductivity, and pH

Received: 15 Apr 2015. Revision requested: 11 May 2015. Accepted: 17 Aug 2015.
[1]Center for Laboratory Animal Medicine and Care, The University of Texas Health Science Center at Houston, Houston, Texas, and [2]Department of Veterinary Medicine and Surgery, The University of Texas MD Anderson Cancer Center, Houston, Texas.
[*]Corresponding author. Email: lwaferdvm@gmail.com

were measured continuously, and the other water parameters were measured weekly. A 5% water change was performed daily by using an automated controller delivering water purified by reverse osmosis. Zebrafish fry were fed microencapsulated fry food (Hatchfry Encapsulon, Argent Laboratories, Redmond, WA) 3 times daily until 10 dpf. Between 11 and 21 dpf, fry received microencapsulated fry food and *Artemia* nauplii (JEHM, Lambertville, NJ) as much as they could consume within 5 min twice daily. After 21 dpf, the juvenile fish received *Artemia* nauplii and freeze-dried *Artemia* replacement (Cyclop-eeze, Argent Laboratories) as much as they could consume within 5 min twice daily. All experiments were approved by the IACUC of The University of Texas Health Science Center at Houston, and zebrafish were maintained in an AAALAC-I-accredited facility.

Effects of enrichment on egg production: spawning sessions and counting of eggs. Breeder pairs were identified and housed together in designated 2.8-L tanks for 1 wk. The designated tanks were labeled 1 through 18 to track the breeding pairs throughout the experiment. At 110 dpf, each pair was placed in a 1.5-L breeding tank (Aquaneering) in the afternoon. The breeding tank included 4 main components: an outer tank, inner tank, divider, and lid. The removable central divider was placed in the inner tank, to allow for the separation of sexes until breeding was desired. Each 1.5-L breeding tank contained 1 of 3 environmental enrichment conditions (n = 6 per condition): no environmental enrichment (control), plastic *Hygrophilia* leaves (height, 9 cm; diameter, 6 cm; Top Fin, PetSmart, Phoenix, AZ), or plastic grass (height, 7 cm; diameter, 6 cm; Top Fin, PetSmart; Figure 1). Approximately 25% to 30% of the surface of the tank was covered with the enrichment item.

Because zebrafish typically spawn at dawn,[10] tank dividers were removed within 5 min of the start of the light phase on the morning after placement, to initiate spawning. The design of the breeding tanks allowed for oviposited eggs to drop through slits in the inner tank to the bottom of the outer tank, to prevent the eggs from being consumed by the breeder fish. Pairs were maintained in the breeder tanks until 1100 (3 h), at which time, each spawning pair was returned to its labeled 2.8-L holding tank without enrichment, and all ova were collected and counted. Spawning sessions were repeated at weekly intervals over a 3-wk period by using the same breeding pairs, with each pair exposed to a different enrichment condition after each interval (Figure 2); this pattern was repeated for 2 additional cycles. Thus, over the 9-wk experiment, each pair was exposed to each condition 3 times. Immediately after each session, the enrichment devices were immersed in 10% bleach solution overnight, rinsed thoroughly with water purified by reverse osmosis, and allowed to air dry for approximately 5 d until needed again.

Rearing and counting of fry. Collected ova were transferred to culture dishes containing E3 media (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, and 0.1% methylene blue), incubated at 28.5 °C for 6 d, and then visually counted and recorded. Culture dishes were labeled to indicate the parents, date of breeding, and type of environmental enrichment for spawning. At 6 dpf, the culture dishes were photographed (model D90, Nikon, Tokyo, Japan) and the images uploaded onto a computer. Individual fry on uploaded images were marked and numbered in black by using Paint software (Windows 7, Microsoft, Redmond, WA) to facilitate counting of all surviving fry.

Effect of enrichment on the survivability of zebrafish fry. To determine the direct effect of enrichment on the survivability of zebrafish fry, a separate experiment was conducted. After the completion of the first experiment, the same 18 pairs of breeders were grouped together in a single breeder tank to generate embryos for the calculation of fry survivability rates. After the eggs were collected, the 3 environmental enrichment conditions used in the first experiment were replicated in 30 individual culture dishes (n = 10 dishes per condition). To enable us to determine whether nitrification occurred during incubation, the enrichment devices used in this experiment were not bleached as they had been in the previous experiment. Eggs (n = 20 per dish) were placed in dishes containing E3 media and the designated enrichment devices (Figure 3) and were incubated at 28.5 °C for 6 d. Surviving 6-dpf fry were counted, as done previously.

Figure 1. Plastic leaves (left) and grass (right) used in breeder tanks.

Figure 2. Breeder pairs underwent weekly spawning sessions in tanks with 1 of 3 enrichment conditions. Each breeder pair was tested in each condition over a 3-wk cycle; all pairs completed a total of 3 cycles for the first experiment. Breeder pairs were started at 1 of the 3 enrichment conditions and were tested weekly under a different condition of no enrichment (left), plastic grass enrichment (middle), and plastic leaf enrichment (right). Once a cycle was completed, pairs were tested again under the same conditions in the same sequence.

Statistical analysis. The numbers of eggs and 6-dpf fry were analyzed as randomized complete block designs, with the spawning pairs representing the experimental blocks. Factors influencing fertility and fecundity were analyzed as a 3 × 9 factorial arrangement of environment type and age of spawning pair by using the MIXED procedure of SAS (SAS Institute, Cary, NC). Least-squares means were compared by using the PDIFF function of SAS, when protected by a significant (P < 0.05) enrichment type or age of spawning pair effect. When an interaction of enrichment type × age of spawning pair interaction was significant (P < 0.05), those comparisons were evaluated at each age.

The influence of the enrichment type on fry survivability was determined by χ^2 analysis, according to the FREQ procedure of SAS (SAS Institute).

Figure 3. Modified enrichment used for the second experiment: culture dishes (n = 10 dishes per treatment) with no enrichment (left), 3 blades of plastic grass clipped from the larger units used in the first experiment (middle), and 1 segment of plastic *Hygrophilia* leaves separated from the larger strands (right).

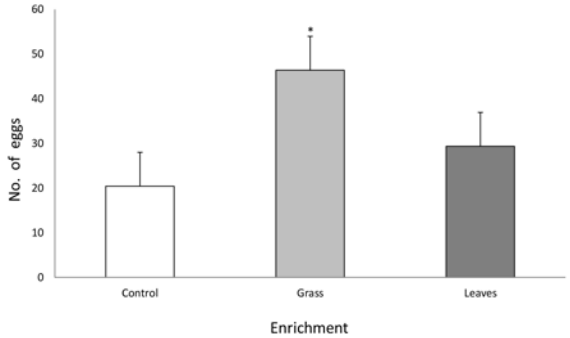

Figure 4. Total egg count (mean ± SEM) at 3 h produced by zebrafish breeding pairs for each enrichment condition averaged across all 3 testing cycles in the first experiment. The mean egg count was greater (*, $P < 0.05$) in the grass environment than in the other enrichment conditions.

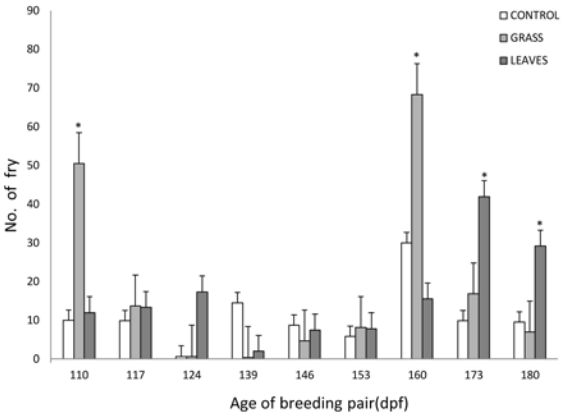

Figure 5. Fry count (least-squares mean ± SEM) at 6 dpf averaged across all spawning sessions. The number of fry at 6 dpf was influenced by an enrichment type × age of breeding pair interaction (*, $P < 0.05$).

Results

Effects of enrichment on zebrafish egg and fry counts. The total number of eggs counted per spawning event for the 9-wk experiment (mean ± SEM) was significantly ($P < 0.05$) greater for zebrafish spawning in the grass-enriched environment (48.0 ± 7.7 eggs) than in the leaf-enriched environment (29.4 ± 5.3 eggs) or unenriched (control) environment (20.4 ± 3.7 eggs; Figure 4). The absolute number of eggs counted for all breeding pairs for each condition was 2591 for grass enrichment, 1587 for leaf enrichment, and 1102 for no enrichment. The rate of successful spawning events (that is, spawnings resulting in 1 or more ova) averaged 58.6% across all enrichment conditions for the 9-wk experiment (grass, 64.8%; leaf, 61.1%; control, 50.0%).

The number of 6-dpf fry per spawning event for the 9-wk experiment (mean ± SEM) did not differ significantly as a function of enrichment condition: grass, 18.0 ± 4.7 fry; leaf, 16.3 ± 3.7 fry, and no enrichment, 11.0 ± 2.4 fry ($P = 0.18$). Because the number of 6-dpf fry appeared to vary as a function of the age of the spawning pair for each enrichment condition, we analyzed the interaction of environment type and age of spawning pair. Across the duration of the experiment, the number of 6-dpf fry was influenced ($P = 0.0088$) by the interaction of environment type and age of the spawning pair. Zebrafish spawning in a grass environment produced more fry ($P < 0.05$) when breeders were 110 and 160 dpf compared with other ages. However, fry counts in the leaf environment were greatest ($P < 0.05$) on the last 2 sampling dates of the experiment, when breeders were 173 and 180 dpf (Figure 5).

Effect of enrichment on the survivability of zebrafish fry. The enrichment type did not influence ($P = 0.32$) fry survivability. The mean fry survivability was 72% for the control condition, 76% for the leaf enrichment, and 79% for the grass enrichment condition.

Discussion

Limited data exist that describes the influence of environmental enrichment on reproductive performance of zebrafish.[4] In the current study, zebrafish spawning in breeding tanks containing plastic grass for environmental enrichment produced more eggs than did zebrafish spawning in breeding tanks containing plastic leaves or no enrichment. However, when averaged across all spawning sessions, there were no significant differences in the number of fry at 6 dpf as a function of enrichment condition. Nevertheless, when tracking the same breeding sets over the duration of the experiment—that is, looking at the effects of environmental type and age of spawning pair on the production of 6-dpf fry—an interaction of environment type × age of spawning pair was determined to be statistically significant. At the start of the study, zebrafish that spawned in the environment enriched with the plastic grass had a higher ovular production rate than did zebrafish in the leaf-enriched environment. Toward the end of the study, however, the ovular production rate for the leaf-enriched environment tended to be higher, particularly on the last 2 sampling dates. This pattern might be explained by the fact that both reproductive maturity and spawning efficiency depend on the size of the fish.[6] When zebrafish reach sexual maturity and first start to spawn, they are small in size and may find more hiding opportunities and therefore demonstrate a preference for the smaller grass units. As zebrafish increase in size and become more experienced, the larger leaf units may provide better hiding opportunities and a preferred location to spawn, compared with the plastic grass.

In our study, zebrafish seemed to have a preference for spawning in a grass-enriched environment, given that pairs spawning in this environment had a higher fertility rate in terms of number of larvated eggs produced than did those in the other environments. When we inspected the data across spawning sessions, we noted different trends as the pairs rotated from one environment to another. When pairs moved from the control environment to the one enriched with plastic grass, the total egg count increased for 52 of the 54 total rotation periods. In addition, for each rotation period, egg counts decreased as pairs were shifted from the grass-enriched to the leaf-enriched and control environments. Given the higher egg counts in the

grass and leaf environments compared with the control (Figure 5), we believe that the plastic vegetation in the breeder tanks provided an additional benefit by preventing the adults from consuming their embryos.[7] In their natural setting, zebrafish reside and spawn among the grass stems and vegetation at the flooded margins of ponds.[9] Therefore, the higher counts of eggs that we observed also might reflect the breeders' preference for simulated vegetation, especially grass, given its similarity to the natural breeding habitat of these fish.

Adding complexity, such as plastic grass or plastic leaves, to the microenvironment of zebrafish offers other benefits as well. Zebrafish are shoaling species and are usually observed swimming in mixed-sex groups of 10 to 20 fish, with the composition of groups determined in large part by social status.[5] At low densities, zebrafish can be aggressive and may bite or chase subordinate fish, resulting in increased stress and reduced fertility when subsequently used for breeding.[7] By adding environmental enrichment, such as plastic grass, to the divided breeding tank, the fertility rate might increase due to the reduction of aggression after removal of the divider, because plants can provide a place of refuge from aggressive zebrafish partners.[7,12]

We performed a subsequent study to evaluate the direct influence of enrichment type on fry survivability. Fry were raised in E3 media containing the same environmental-enrichment devices as used in the first experiment, to determine the possible effect of the devices on the survivability. Previously used plastic plants might harbor nitrifying bacteria, which could have neutralized the waste produced by the embryos during incubation,[4] thereby improving water quality and reducing mortality. However, fry survivability did not differ as a function of enrichment type.

Overall, our data supported our hypothesis that zebrafish in a breeding tank with plastic plant enrichment will show greater fertility and fecundity than those in a barren tank. Introducing artificial aquatic vegetation to zebrafish breeder pairs or groups requires careful planning and consideration. Animal care oversight is required to observe and assess the wellbeing of the fish. Furthermore, standard operating procedures for the method and frequency of cleaning of the objects should be developed.[7] Currently, few studies on environmental enrichment for zebrafish have been published,[3] and the current study may heuristically lead to further refinements. Measuring the cortisol levels of the fish exposed to enrichment devices and other changes to breeding environment, such as reductions in water levels, would provide additional insight. Additional future studies might examine keeping breeding pairs in the same tanks over several weeks instead of performing weekly spawning sessions in different tanks. This scheme might possibly reduce the stress of the zebrafish breeders, resulting in an increased reproductive rate. Furthermore, continued research on zebrafish in their native environment, including their habitat preferences and reproductive behavior, is necessary for both the refinement of husbandry standards and the optimization of their use in various research studies.[4] Finally, future studies on the influence of novel types of environmental enrichment on the reproductive performance of zebrafish would contribute to this evolving topic.

References

1. **Harper C, Lawrence C.** 2011. The laboratory zebrafish. Boca Raton (FL): CRC Press.
2. **Institute for Laboratory Animal Research**. 2011. Guide for the care of laboratory animals, 8th ed. Washington (DC): National Academies Press.
3. **Kinth P, Mahesh G, Panwar Y.** 2013. Mapping of zebrafish research: a global outlook. Zebrafish **10:**510–517.
4. **Lawrence C.** 2007. The husbandry of zebrafish (*Danio rerio*): a review. Aquaculture **269:**1–20.
5. **Lawrence C.** 2012. Environmental enrichment and the laboratory zebrafish. The Enrichment Record **11:**11–15.
6. **Nasiadka A, Clark MD.** 2012. Zebrafish breeding in the laboratory environment. ILAR J **53:**161–168.
7. **Reed B, Jennings M.** [Internet]. 2011. Guidance on the housing and care of zebrafish, *Danio rerio.* [Cited 04 August 2014]Available at: http://science.rspca.org.uk/ImageLocator/LocateAsset?asset=document&assetId=1232723034494&mode=prd
8. **Schroeder P, Jones S, Young IS, Sneddon LU.** 2014. What do zebrafish want? Impact of social grouping, dominance, and gender on preference for enrichment. Lab Anim **48:**328–337.
9. **Spence R, Ashton R, Smith C.** 2007. Oviposition decisions are mediated by spawning site quality in wild and domesticated zebrafish, *Danio rerio.* Behaviour **144:**953–966.
10. **Spence R, Gerlach G, Lawrence C, Smith C.** 2007. The behaviour and ecology of the zebrafish, *Danio rerio.* Biol Rev Camb Philos Soc **83:**13–34.
11. **Williams TD, Readman GD, Owen SF.** 2009. Key issues concerning environmental enrichment for laboratory-held fish species. Lab Anim **43:**107–120.
12. **Wolfensohn S, Lloyd M.** 2013. Handbook of laboratory animal management and welfare, 4th ed. Ames (IA): Wiley–Blackwell.