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An Inexpensive, Efficient Method for Regular Egg Collection from Zebrafish in a Recirculating System

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Abstract

Zebrafish in our laboratory are usually bred by removing the fish from the recirculating aquatic system and placing them into 1–2 L spawning tanks. These spawning tanks consist of a bottom reservoir, a lid, and an insert that fits in closely into the bottom reservoir. When the fish breed, the eggs fall through holes of the insert and into the reservoir, thus preventing them from being cannibalized. Because fish in these spawning tanks are not fed and do not get fresh water, they are bred only once a week. During a period where we had high demand for embryos, we instead tried breeding the fish for multiple consecutive days on the recirculating system. Fish were placed into the spawning insert as usual, but the insert was placed into the home tank instead of into the bottom reservoir. We found that there was no significant difference in the number of fertilized eggs produced between the spawning tank and home tank breeding methods. Further, the fish in the home tanks regularly produced fertile embryos over a 28-day time course, with the highest number of eggs per pair produced by the tank with only one pair of adult fish. This method is time-saving as fish bred in home tanks only require to be set up once. It is also an effective way to collect embryos over long periods from the same pair or group of fish and to more easily obtain embryos from stocks with low spawning frequency.

Introduction

Zebrafish are capable of breeding all year round, with a single pair capable of producing more than a hundred embryos in one day.[1–5] This is one of the characteristics that make the zebrafish a powerful model organism for studying the mechanisms that control vertebrate development, and has led to its growing use in the study of disease, behavior, and a number of other fields. Several factors have been found to improve the rate of spawning in laboratory conditions. The frequency of spawning increases with rising temperature, with a minimum of 5 days between spawning for fish at 26°C and a minimum of 2 days at 30°C.[1] Keeping males and females together has a positive effect on the number of eggs produced per day and is required for the release of oocytes in the female.[2,5] In addition, a depth gradient within the spawning tank increases the number of embryos produced, and there is a corresponding increase in residency of the fish at the shallow end of the tank.[6] These findings match well with what is known about the natural history of zebrafish, which are river fish native to India, Bangladesh, and Nepal.[4]

A number of methods have been used to breed zebrafish in laboratory settings. One of the first methods was to include marbles in a region of the tank as a spawning site.[1,7–9] Eggs fall into the marble layer, and a siphon is used for egg collection.[9] As the number of zebrafish laboratories has expanded, commercially available spawning tanks have become available. These tanks consist of a lower reservoir holding 1–2 L of water and an insert with holes in the bottom. The adult fish are placed within the insert, and the eggs fall through the holes into the bottom reservoir, protecting them from cannibalism by the parental fish. These spawning tanks do not allow continuous spawning, as there is no circulation or filtration of the water. Breeding fish are typically placed in the spawning tanks from the evening through the following morning. More recently, several methods have been generated for obtaining large quantities of embryos at the same stage of development. For instance, Adatto and colleagues invented a specialized breeding vessel that enables over 100 fish to be bred at once over short periods of time (10 minutes).[10] The fertilized eggs are easily collected as they fall into a narrow opening at the bottom of the vessel.[10]

We have built on these earlier techniques to develop a breeding method with a unique set of advantages. In this method, the inserts that come with commercially available spawning tanks are placed directly into 10 L or 3 L tanks on a recirculating aquatic system. The adult fish are placed into the insert, and embryos are collected from the bottom of the tank by pouring or using a self-starting siphon. Comparison of this method with breeding in commercial spawning tanks found

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no significant difference between the numbers of fertile embryos produced. Further, because the fish are maintained on the recirculating system and can be fed regularly, we found this method can be used over many days or weeks. Fish maintained in this spawning system for 28 days continued to produce fertilized embryos every 1–3 days. Setting up the fish for breeding in the home tanks took much less time than for the commercial spawning tanks. Thus, the home tank method would be especially useful for gathering embryos to be raised, expanding a new stock, or working with sensitive fish that might be harmed by removal from the recirculating system. Since gathering embryos from 3 L home tanks took a similar amount of time to traditional spawning tanks, breeding in these smaller home tanks would be suitable for techniques that require rapid harvesting of embryos, such as embryo injections. The home tank method offers an alternative approach that can be used for many purposes and requires only equipment that is already available in most laboratories, or easily and inexpensively purchased from commercial sources.

Materials and Methods

Aquatic system

All fish were maintained in their respective 10 L and 3 L home tanks in an aquatic facility equipped with an automatic recirculation system. Water in this facility was maintained at 450–800 μS conductivity, pH 7.4, and ~28°C. The fish were maintained in a 14:10 h daily light:dark cycle. Zeitgeber (ZT) was used to indicate time within this cycle, with ZT0 corresponding to when the lights turned on in the morning and ZT14 to when the lights turned off in the evening. Fish were fed on weekday mornings with live brine shrimp and on weekday afternoons and weekends with commercial flake food (a 1:1 mixture of Freshwater Aquarium Flake Food from Ocean Star International and Tetramin Tropical Flakes from Tetra, Inc.). During the breeding experiments, the fish were fed with commercial flake food or with commercial flake food and a fine powder consisting of a 1:1 mixture of Argent Technology's Spirulina Microfine Powder and Hatchfry Encapsulon.

Breeding in spawning tanks

For the experiments in spawning tanks, fish were removed from their home tank into temporary 3 L tanks for sorting by gender. The fish were initially placed into one central tank and then sorted into one tank for females and another for males. Females were sorted based on their rounder, whiter abdomen. Males were identified based on their slightly more yellow coloring and their flat abdomen. The male and female fish were then evenly distributed between 2 L spawning tanks (Supplementary Table S1; Supplementary Material is available online at www.liebertonline.com/zeb). These spawning tanks consisted of a lower reservoir that held the water and a spawning insert with holes in the bottom that fit into this reservoir (Aquatic Habitats). These tanks were filled to approximately 2 cm from the top with aquatic system water, and plastic plants were placed into the insert to provide refuge.

The following day, the adult fish were removed from the spawning insert and placed back into their home tank on the recirculating system. The bottom reservoir was kept still for a few minutes to allow the eggs to settle to the bottom. Most of the water was then poured out slowly from one corner. When there was approximately 30 mL of water left in the reservoir, the water was agitated to suspend the eggs, and then the remaining water and eggs were poured quickly into a deep Petri dish (100 mm × 25 mm).

Breeding in home tanks of the recirculating aquatic system

Males and female fish were sorted and set up in a 2 L spawning as described above for breeding in spawning tanks. However, after completion of this set up, the spawning insert, the adult fish, plastic plants, and the cover to the spawning tank were placed into a 3 or 10 L tank on the recirculating system (Fig. 1). The water entering into the tank was adjusted to ~200 mL/min (fast drips) so the eggs would not be washed out of the tank. The fish were not fed brine shrimp during the experiment, as we found it would quickly fall through the bottom of the insert and contaminate the eggs. The following day the fish, insert, plastic plants, and spawning tank cover were temporarily moved to the bottom reservoir of a spawning tank to enable the eggs to be collected (Fig. 1).

Eggs were collected from the 3 L home tank by pouring, similar to egg collection from the traditional spawning tanks (Fig. 1). For the 10 L home tanks, the eggs and all of the water in the home tank were moved into the collection tank using a self-starting siphon (Small 15/8″ × 9″ UltraGravelVac from Lee's Aquarium and Pet Products; Fig. 1). The nozzle of the siphon was placed into the 10 L home tank, and the end of the siphon tubing was clamped to the inside of a collection tank that consisted of a 10 L tank and a 400 μm screen baffle (Fig. 1) (Aquatic Habitats). The collection tank was then placed on a secure surface several feet below the home tank (Fig. 1). The water flow was started by quickly moving the siphon nozzle up and down until the tubing was completely filled with water and no bubbles were present. The nozzle was kept submerged during the whole siphoning process.

Typically, there was about 1 L of water left in the home tank after water flow through the siphon had stopped. This last 1 L often contained many eggs, and so was poured manually into the 10 L collection tank. The home tank was rinsed with about 0.5 L of aquatic system water, and this rinse water was also poured into the 10 L collection tank. Before this final rinse was added to the protocol, we occasionally collected embryos that were over 24 hpf, suggesting eggs had been missed during the previous day's collection.

Statistical analysis and modeling

Data was analyzed by Repeated Measures Analysis of Variance (MANOVA) using JMP 9.0 software. Three dimensional models were created using SketchUp 8.0.

Results

Comparable levels of embryo production when fish are bred in spawning tanks and home tanks

Our first goal was to compare embryo production between fish bred within the recirculating system and fish bred in regular spawning tanks. To make this test as realistic as possible, we used the WT fish in four existing tanks. These tanks each had fish of different ages, levels of prior breeding, and numbers of males and females (Supplementary Table S1).

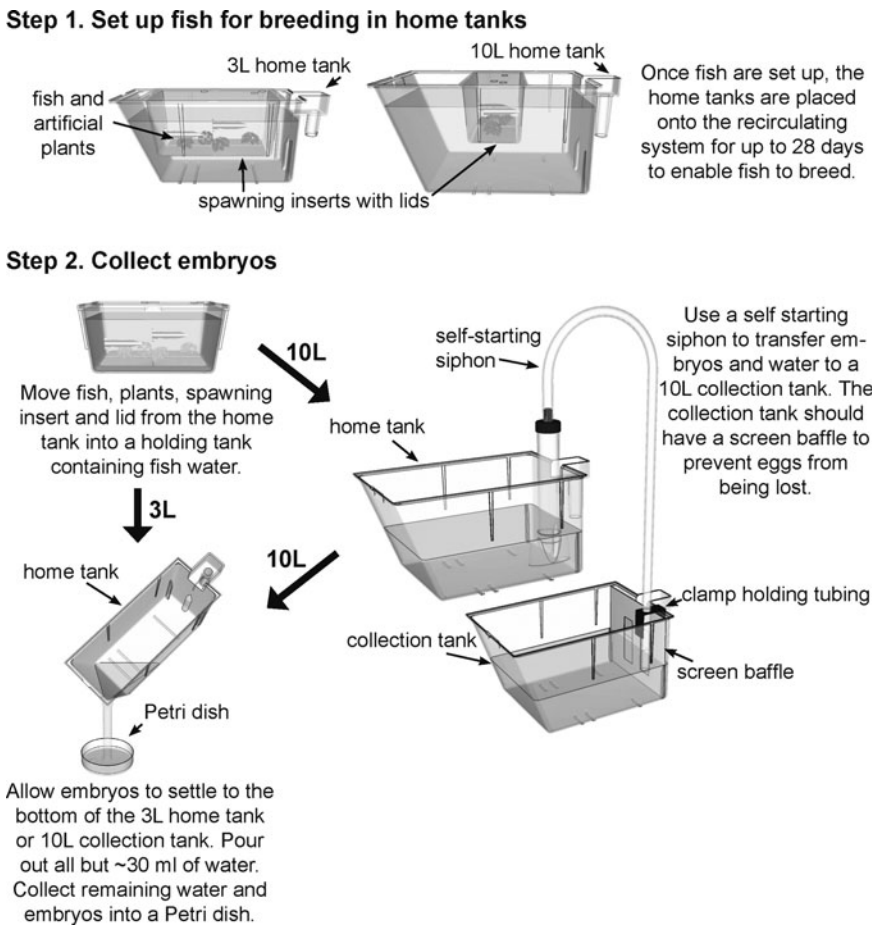

FIG. 1. Outline of home tank breeding procedure.

The fish in each tank were set up four times: twice in spawning tanks and twice in their home tanks. In each case, the week of daily breeding started on Monday and continued to Friday, so the number of eggs produced was counted for four consecutive days (Fig. 2). The numbers of fish in each "spawning insert" were kept as constant as possible, and each tank of fish was given a week of rest between each week of breeding.

For the weeks when the fish were in spawning tanks, the males and females were put together at ZT6.5 each day from Monday-Thursday, and then left together until ZT3.5 the following day. Between ZT3.5 and ZT6.5 they were placed back into their home tank and fed once or twice with flake food before being moved back into the spawning tanks for the next round of breeding. The number of infertile embryos, fertile embryos, and their stages of development were scored between ZT4 and ZT4.5 on the day the eggs were collected.

For the weeks when fish were in their home tanks, the fish were sorted by gender and placed into spawning inserts in their home tank at ZT6.5 on Monday. The adults remained in the spawning insert in their home tank until ZT3.5 on Friday, except for short periods when they were placed into a temporary tank so that eggs could be collected. As with the breeding in spawning tanks, the eggs were collected at ZT3.5, and counted and staged between ZT4.5 and ZT5.

We found that there was a large variation in the number of fertile embryos produced each day, with a low of 0 embryos and a high of 225 (Fig. 2A). The number of nonviable embryos was similar and low for each method ($n = 76/1175$ for spawning tanks, $n = 31/1306$ for home tanks). These results are quite consistent with our previous experiences. There was also not a clear trend of change in the number of embryos from Tuesday to Friday, likely because there were multiple females in each breeding tank, increasing the chance of producing eggs on multiple days during the week (Fig. 2). The data were analyzed using MANOVA, which took into account that each group of breeding fish was assayed several times over the time course of the experiment. This analysis produced a p value of 0.70, suggesting that there was no significant difference in the number of eggs produced between the two methods of breeding.

Fish in home tanks continued to breed throughout 28-day time course

One potential advantage of the home tank breeding method was it could be done continuously over a long period of time, as the fish do not miss any feedings and are continuously supplied with fresh water. To gain insight into long-term patterns of breeding, we kept groups of fish in spawning inserts in 3 L and 10 L home tanks for 28 days. As in the earlier experiment, the eggs were collected at ZT3.5 each day and the embryos were counted and staged between ZT4.5 and ZT5 on the day they were collected.

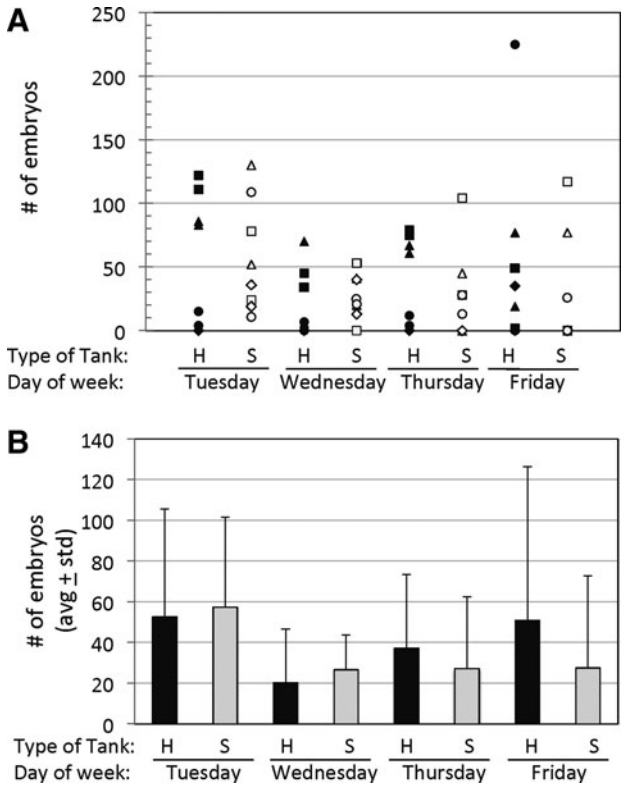

FIG. 2. Comparison of embryos produced by fish in spawning tanks and home tanks. **(A)** The number of embryos produced in the previous 24 hours is reported for each spawning of the fish in four different tanks (Table 1). Half were in spawning tanks (S) outside of the recirculating system and half in home tanks (H) within the recirculating system. **(B)** Presentation of the data in A as average ± std for each time point.

The highest number of embryos was typically produced during the first week (n = 11/12 home tanks; Fig. 3). Interestingly, examination of the time line by day instead of by week revealed that the large number of embryos during the first week was primarily due to a large number of eggs being

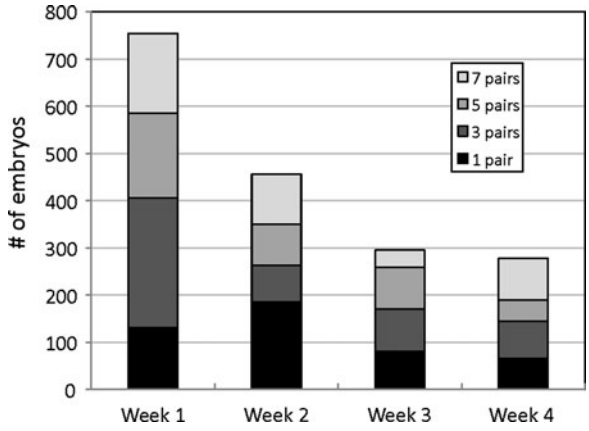

FIG. 3. Embryos produced each week in 28-day time course in 10 L home breeding tanks. The number of fertile embryos produced each week by each of four tanks during a 28-day time course. The tanks containing 1, 3, 5, and 7 pairs were assayed in parallel over the same 28-day period. This experiment was repeated two times, with the first trial shown here.

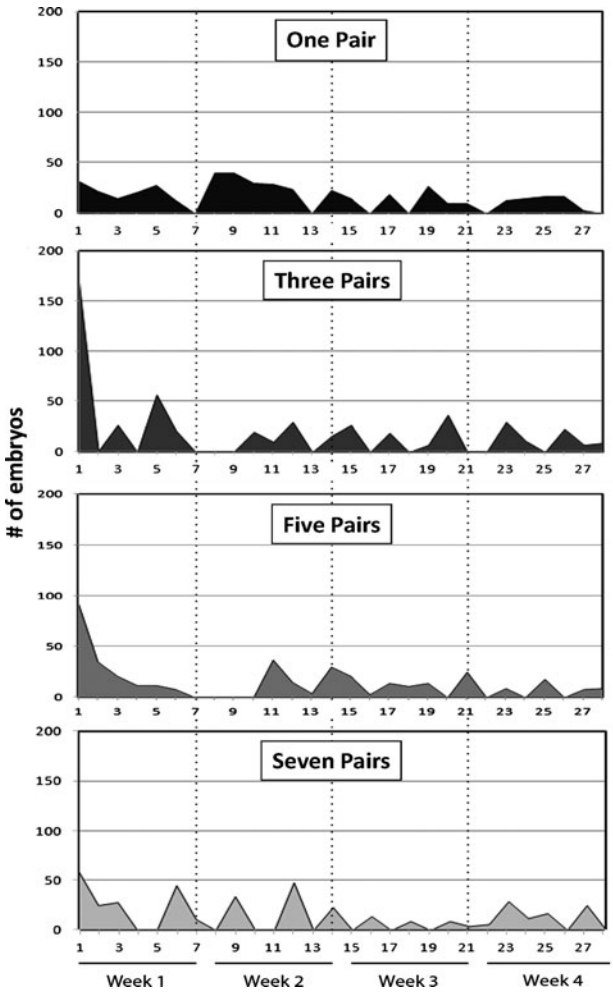

FIG. 4. Daily spawning in a 28-day time course in 10 L tanks. Embryos collected each day for the same experiment summarized in Figure 3. *Dotted lines* mark embryos collected on Mondays, and each week of the experiment is marked by a set of underlined days. The combined analysis of daily spawning for this experiment and one repetition is included in Table 1.

produced on the first day (Fig. 4). This suggests that the fish had many mature gametes ready to release right away, but that gamete production persisted for several weeks at lower levels. Statistical analysis indicated that there was a significant difference between the 10 L and 3 L tanks when 3 pairs were present ($p = 0.05$) but not when one pair was present ($p = 0.38$). However, when only 3 pair trials done in parallel were compared $p = 0.24$, suggesting the significant difference was in part due to factors other than tank size.

To determine if embryo production would increase if fish were bred for fewer days at a time, we carried out 3-day experiments with a minimum of 3 days rest in between each trial. The fish in this less intense breeding scheme produced similar numbers of embryos per day as the fish bred continuously for 28 days, suggesting that the rests did not have a positive effect (Table 1).

As part of these experiments, we also gained insight into the effect of fish density. Each of the tanks had between one and seven male/female pairs (Supplementary Table S1). Surprisingly, the number of embryos did not increase linearly

Table 1. Daily Embryo Production for Breeding in Home Tanks

	Daily embryo production (avg ± std)	*Daily embryo production per pair (avg)*
28-day time courses 10 L home tanks[a]		
1 pair	19.5 ± 19.5	19.5
3 pairs	14.9 ± 27.8	5.0
5 pairs	13.4 ± 18.2	2.7
7 pairs	12.3 ± 16.1	1.8
28-day time course in 3 L home tanks[b]		
1 pair	7.3 ± 11.5	7.3
3 pairs	7.8 ± 18.0	2.6
3-day time courses in 3 L home tanks[c]		
1 pair	16.7 ± 15.1	16.7
3 pairs	8.6 ± 17.7	2.9

[a] $n = 54$ days; [b] $n = 28$ days; [c] $n = 18$ days.

with the number of pairs (Table 1). In both the 3 L and the 10 L home tanks, the single pair mating produced larger number of embryos per pair than higher fish densities (Table 1). Further, as the number of fish increased, the number of embryo per pair decreased (Table 1). The difference in embryo production between 1 pair and 3 pairs reached significance in the 3-day experiments ($p = 0.004$), but not in the 28-day experiments ($p = 0.17$).

Discussion

We have developed an economical and time efficient method of breeding fish within a recirculating aquatic system. This method requires very little handling of the adult fish, makes it possible to breed the same fish continuously over a long period of time, and has similar levels of embryo production as the common method of breeding in spawning tanks. Overall, breeding in the 10 L and the 3 L home tanks took less hands-on time because the fish did not have to be netted and sorted by gender except on the first day. We suggest that breeding on the recirculating system would be very useful for producing new stocks of fish, producing eggs from the same fish multiple times in one week, breeding fish that are sensitive to being netted, and studies on spawning behavior. The biggest potential deficit is that this method could potentially take away from space needed for other purposes, as the fish in their home tanks produced embryos most efficiently when at low densities.

Because harvesting embryos from 10 L home tanks took much longer than other methods, breeding in 10 L tanks would only be useful for experiments that require low fish densities and infrequent egg collection. In contrast, a similar amount of time was needed for collection of embryos from 3 L home tanks and 2 L spawning tanks. Therefore, breeding in 3 L home tanks could be used for single pair matings to identify fish carrying a specific mutation, crosses to produce embryos for injection, and to generate mutant and transgenic embryos for experiments. This would be especially useful for fish that are breeding poorly as they would have a longer opportunity to spawn.

We found that zebrafish spawning continued periodically during 28 days in their home tanks, with a large peak of embryo production on the morning after the fish were placed into the inserts. Our male and female fish were housed together in between breeding experiments. Based on earlier studies, this would have stimulated the constant production of oocytes by the females.[2,5] Thus, when the females were placed into the more conducive breeding environment of the spawning insert, there could have been a large store of gametes ready to be released the following morning. After this large peak of embryo production, the fish began to produce lower numbers of embryos every 1–3 days. This closely matched the periodicity of embryo production in other experiments where zebrafish had continuous opportunity to spawn.[5]

Interestingly, our comparison of embryo production by fish at different densities found the highest number of eggs per pair occurred in the tank with only one pair of fish. Since zebrafish have complex spawning behaviors that require interactions between males and females, it might be anticipated that embryo production would increase at higher densities. However, several other studies have also found decreasing embryo production with increasing density.[7] Male to male aggression and competition among females for limited spawning space may limit egg production in crowded tanks.[7]

There are several ways that this method could potentially be improved. Most of our tanks contained fish from the same clutch. As sexually mature zebrafish females have a preference to avoid siblings, improvements in embryo production could come from using a more genetically diverse group of parental fish.[11] We found that using live brine shrimp was problematic in this breeding method, as the shrimp would fall through the bottom of the spawning inserts and contaminate the embryos. Thus, addition of another rich food would likely improve embryo production and the overall health of the breeding adults, and extend the time fish can kept be in this breeding scheme to several months. Finally, using different tank topologies could also bring improvements. For instance, adding a depth gradient to the spawning insert containing the adult fish has been shown to increase embryo production by approximately 20%.[6]

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Disclosure Statement

No competing financial interests exist.

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