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# A Rotifer-Based Technique to Rear Zebrafish Larvae in Small Academic Settings

Raymond L. Allen,[1,2] Robert L. Wallace,[1] and Barbara E. Sisson[1]

## Abstract

Raising zebrafish from larvae to juveniles can be laborious, requiring frequent water exchanges and continuous culturing of live feed. This task becomes even more difficult for small institutions that do not have access to the necessary funding, equipment, or personnel to maintain large-scale systems usually employed in zebrafish husbandry. To open this opportunity to smaller institutions, a cost-efficient protocol was developed to culture *Nannochloropsis* to feed the halophilic, planktonic rotifer *Brachionus plicatilis*; the rotifers were then used to raise larval zebrafish to juveniles. By using these methods, small institutions can easily raise zebrafish embryos in a cost-efficient manner without the need to establish an extensive fish-raising facility. In addition, culturing rotifers provides a micrometazoan that serves as a model organism for teaching and undergraduate research studies for a variety of topics, including aging, toxicology, and predator–prey dynamics.

## Introduction

As a model organism, zebrafish has become increasingly popular, with uses in fields ranging from developmental biology to toxicology. With this increased status, researchers at small academic institutions, including K-12 schools and small colleges and universities, have turned to this organism to use in research and teaching.[1–5] However, to maintain zebrafish colonies, researchers must be able to raise zebrafish, an effort that for practical and financial reasons may be unrealistic.

From fertilization to the larval stage (~5 days), zebrafish embryos feed on stored yolk; after exhausting most of their yolk, they must begin to feed. Two typical feeding protocols are employed: a processed diet or live prey. The use of live prey is most commonly employed within the research community. Larval zebrafish normally ingest their prey whole, but prey size is limited by their gape.[6] This characteristic severely limits live prey to paramecium, brine shrimp, or rotifers. Paramecia are slow moving, making them easy prey for the larvae, but they have the disadvantage of having limited nutritional value.[6,7] Brine shrimp, *Artemia* nauplii, on the other hand, have good nutritional value,[8,9] but are quick swimmers and are sometimes too large for a larval zebrafish to consume. Rotifers also have good nutritional value, but they are smaller than brine shrimp and are slower swimmers; these characteristics make them an appealing intermediate food source.[7,9,10] One of the more popular species used in the culturing of fish fry is the halophilic rotifer *Brachionus plicatilis*[11] (Fig. 1A).

Use of live feed in the husbandry of zebrafish at large research institutions is common, but to create and maintain large systems require extensive investment. Small educational and research institutions often lack sufficient resources to raise zebrafish embryos on a large scale. To overcome the problems associated with raising zebrafish until they could consume a dry fish flake diet or diet of *Artemia,* a simple inexpensive protocol was developed that allows continuous culture of *B. plicatilis*.

## Materials and Methods

### *Zebrafish maintenance*

Adult zebrafish lines of *Tg(hsp70l:dnfgfr1a-EGFP)*[12] and *5D*[13] were maintained in 9.5 or 18.9 L glass aquariums with tap water aged 3 days, a heater set to 28°C, and a sponge filter (Bio-Filter 9; Drs. Foster and Smith). Approximately, 70% of the water was exchanged once a week and the tanks cleaned of debris and algae using a 20-mL pipet and an algae scrubber (Drs. Foster and Smith), respectively. Embryos were collected from natural spawning in 2-L breeder tank (Aquatic Habitats) and staged according to a standard scheme.[14] The Ripon College IACUC approved the fish protocols described in this article.

[1]Department of Biology, Ripon College, Ripon, Wisconsin.
[2]Developmental and Stem Cell Biology Graduate Program, Duke University, Durham, North Carolina.

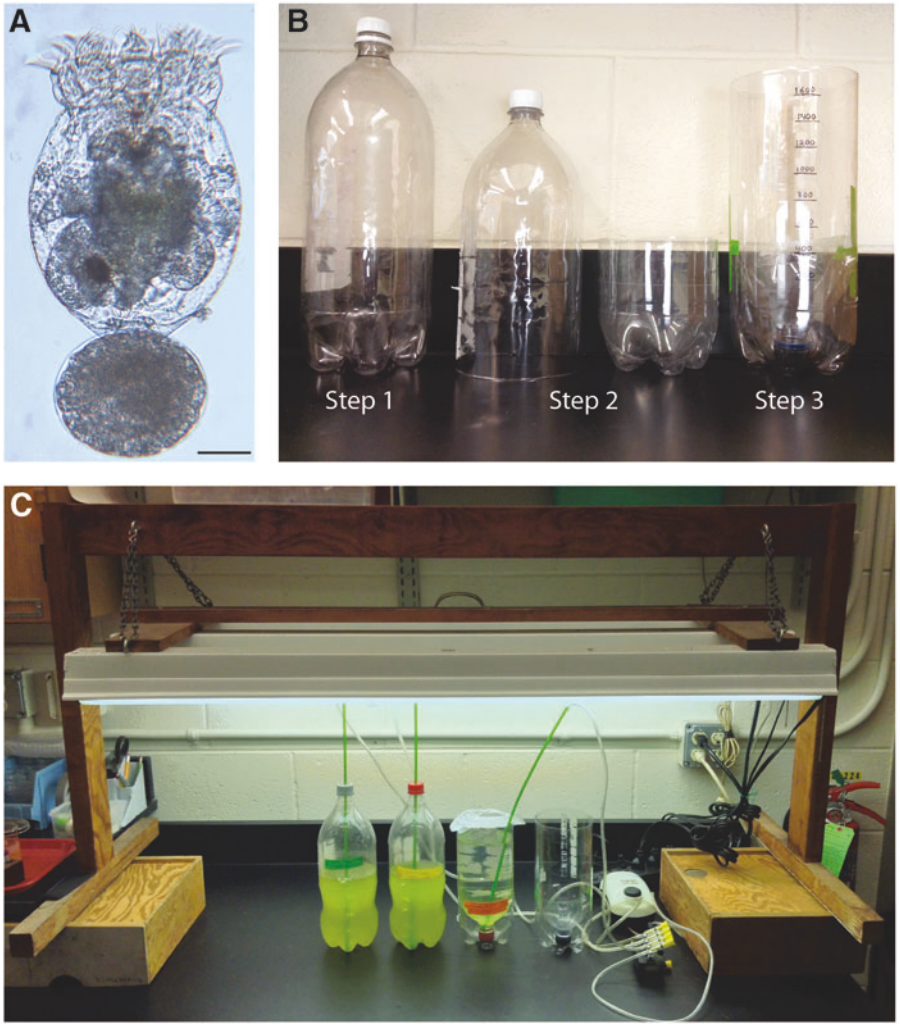


**FIG. 1.** Simple rotifer culture apparatus. **(A)** *Brachionus plicatilis* and a mictic egg. Scale bar = 50 $\mu$m. **(B)** Rotifer culture apparatus assembly. Step 1: clean and rinse two clear 2-L soda bottles. Step 2: cut one bottle 11 cm from the bottom and the other 24 cm from the top. Step 3: invert the top of the second bottle into the bottom portion of the first bottle. Tape the sections together and mark the appropriate volumes of the bottle. **(C)** Two aerated cultures of *Nannochloropsis* grown in 18 parts per thousand (ppt) salt+MBL media,[15] an aerated culture of *B. plicatilis* (15 ppt salt water), and an empty rotifer culture bottle at room temperature under fluorescent lights (*left* to *right*, respectively).

### *Algae culture*

A Micro Algae Disk of *Nannochloropsis* (Florida Aqua Farms) was used to inoculate 1 L algae cultures, which were maintained in 2-L soda bottles (Fig. 1B, step 1).[15] The cultures were grown at room temperature, in a modified MBL medium[16] supplemented with thrice the amount of vitamins and 18 parts per thousand (ppt) Instant Ocean (Drs. Foster and Smith) (Supplementary Data; Supplementary Data are available online at www.liebertpub.com/zeb) under constant fluorescent light and with constant aeration (Fig. 1C). Under the described conditions, cultures were ready for use in 1–2 weeks when they were bright green in color (Fig. 1C). If the algae did not reach a bright green color, we doubled or tripled the amount of vitamins added to the medium because they lose their potency over time even when refrigerated. When algae cultures were bright green, they were split or replenished; if the cultures are not replenished, they may reach a shade of yellow or white, indicating that the culture crashed. Once cultures were depleted to around 500 mL, they were replenished with the previously described modified MBL medium. While the vendor reports that *Nannochloropsis* can be grown at lower salinities, we experienced frequent contamination by an unidentified protozoan that caused the cultures to crash at ca. 6 ppt.

### *Assembly of 1.6-L rotifer culture bottles*

To assemble a rotifer culture bottle (RCB), two, 2-L plastic soda bottles were used. The labels on the bottles were removed; the bottles were washed and rinsed with reverse osmosis water (ROW) (Fig. 1B, step 1). After washing the bottles, one bottle was used to create the base of the apparatus by cutting along the horizontal axis 11 cm from the bottom. The second bottle was cut to make the top portion, which houses the rotifers (Fig. 1B, step 2). To create the top portion, a bottle was measured 24 cm from the top of the bottle and cut along the horizontal axis. The top of the apparatus was positioned upside down into the base and the two units were taped together using labeling tape. A volumetric scale was approximated on the side of the RCB in increments of 200 mL up to a volume of 1.6 L (Fig. 1B, step 3).

### *Rotifer culture*

Following the vendor's instructions, a vial of Resting Rotifer Culture (Saltwater, Laboratory cultured L strain Rotifer Cysts, 3000–5000 cysts; Florida Aqua Farms) was used to start a culture of *B. plicatilis*. The cultures were maintained in the aerated 1.6-L RCB at the highest recommended salinity (15 ppt) at room temperature under constant fluorescent light

(Fig. 1C). The rotifers were fed once daily with ~25 mL of *Nannochloropsis* per 1 L culture, adjusted down to 15 ppt with ROW, and 1–2 drops of Roti-rich® (Florida Aqua Farms) until the culture was slightly green and cloudy. Approximately 50% of each culture was harvested daily to restrict sexual reproduction.[17,18] Backup *B. plicatilis* cultures were maintained as per Lawrence *et al.*[19]

Over time (1–2 weeks), we found that the rotifer cultures accumulated algae on the sides of the bottles. When this occurred, the culture was transferred to a new RCB and the older bottle was rinsed thoroughly in ROW as needed. To avoid the culture bottles from tipping over, we recommend constructing a simple wooden frame in which the culture bottles are held.

### *Zebrafish feeding*

At 6 days post fertilization (dpf), embryos were transferred to a 2-L breeder tank (Aquatic Habitats), filled with egg water,[20] with the breeding liner removed (Fig. 2A). Clutches were fed rotifers raised in 15 ppt seawater per above. To harvest the rotifers, slightly less than half of two rotifer cultures was poured into a 53-$\mu$m sieve (Brine Shrimp Direct) (Fig. 2B). They were rinsed with 6 ppt Instant Ocean and concentrated into a beaker in 100 mL of 6 ppt Instant Ocean; creating a rotifer feeding suspension that lasts for a morning and afternoon feeding. Because the rotifers are temporarily osmotically shocked during this process and begin to settle to the bottom of the beaker, for their immediate use, they were resuspended in the water column by stirring the solution by hand before feeding them to the zebrafish. Subsequent feedings from the same feeding solution were stirred to equally distribute the rotifers. We estimated the number of rotifers per mL by placing 1.0 mL of the rinsed, concentrated, and resuspended rotifers into a Bogorov Counting Chamber (Wildco). The embryos were fed 25 mL of ~250 rotifers/mL twice daily.

Between 18 and 20 dpf, larval zebrafish were also fed a few drops of ground 1-day-old Brine shrimp (Brine Shrimp Direct), cultured in large hatchery cones (Brine Shrimp Direct) in 18 ppt Instant Ocean and ground in a glass tissue grinder twice daily. At 20–31 dpf, larval zebrafish were then fed whole, live 1-day-old brine shrimp and rotifers. After being weaned to brine shrimp, the juvenile zebrafish were then fed brine shrimp twice daily or a combination of brine shrimp and dry food (TetraMin® Tropical Flakes) (Fig. 3). Some researchers also use finely ground TetraMin Tropical Flake food to feed their 14–28 dpf larval zebrafish.

### *Water and tank changes*

In parallel to feeding, changes in tank size and water exchanges were also needed to raise zebrafish clutches. As the fish grew, they were moved to larger containers. Given that the larvae were fed rotifers at 6 ppt twice daily, the salt concentration of the previous tank gradually increased and therefore the salinity of the first water exchange was adjusted accordingly. At 21 dpf, the fish were moved to 3-L containers (Petco Pet Keeper, Small; Petco) (Fig. 2C) containing 5 ppt water; at this point, the tank was held at 28°C with constant aeration using an air stone. At this time in their development, the larvae were fed a combination of brine shrimp and rotifers (see *Zebrafish feeding*). Accumulating debris was removed as needed. To maintain water quality, the water was exchanged each week, gradually decreasing the salinity at each exchange.

Adult zebrafish in our facility are in tap water aged at least 3 days or treated with Tetra AquaSafe Plus® (Drs. Foster and Smith) (Fig. 2D). To transition the fish to the aged tap water, 200 mL of the tank water was removed each day for a week

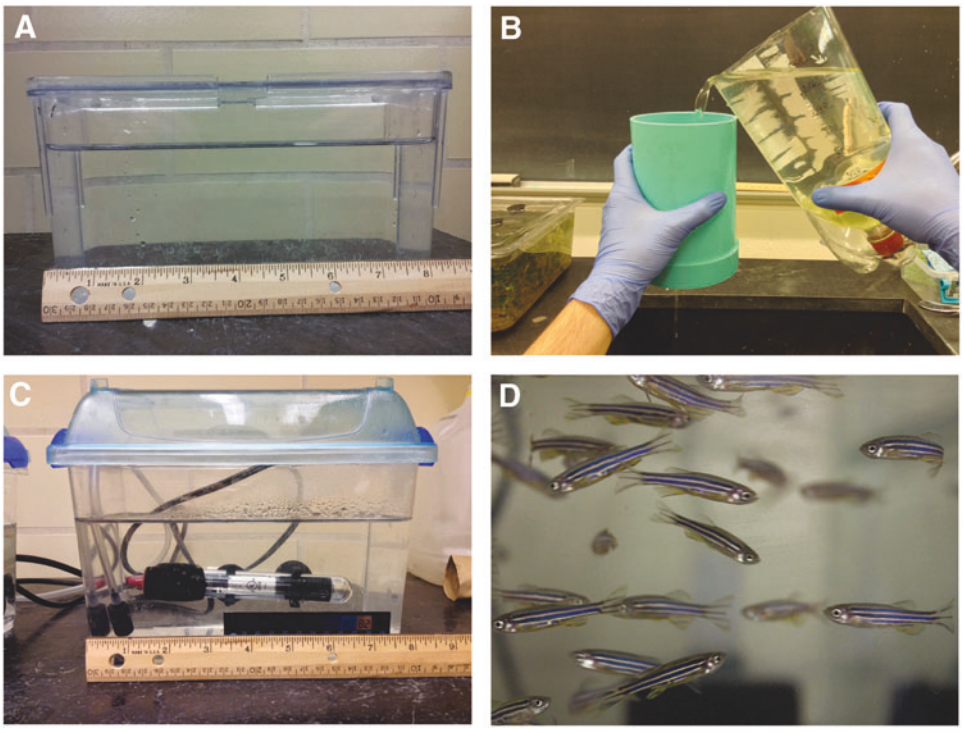


**FIG. 2.** Raising zebrafish larvae. **(A)** Zebrafish larvae are first raised in a 2-L crossing tank at 28°C. **(B)** To feed the larvae, half of a *B. plicatilis* culture is poured through a sieve to concentrate the rotifers. **(C)** At 21 days post fertilization (dpf), the larvae are moved to a 3-L tank equipped with a tank heater and an air stone. **(D)** Adult zebrafish, which were raised using these techniques.

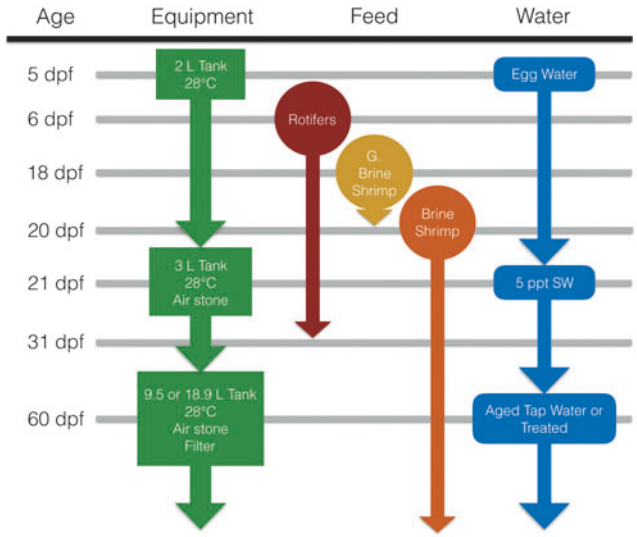


**FIG. 3.** Schematic of the feeding and water change strategy. Feed regiment, tank size, saline concentration (ppt), water temperature, and ancillary equipment needed for the larvae are adjusted depending on dpf. G. Brine Shrimp, Ground Brine Shrimp; SW, salt water.

and substituted with aged tap water. At 60 dpf, or earlier, depending on the size of the population, juvenile zebrafish were transferred into 9.5- or 18.9-L tanks containing aged tap water, a tank heater at 28°C, and a sponge filter (Bio-Filter 9; Drs. Foster and Smith) (Fig. 3).

## Results

### *Embryo to juvenile survival rates*

All of our zebrafish clutches have been successfully raised and transitioned to a diet of brine shrimp or a combination of brine shrimp and flakes in tanks containing aged tap water. Early clutches using this technique started with 100 embryos per 700-mL tank and resulted in a $45\% \pm 12$ (standard deviation [SD]) survival rate to the juvenile stage ($n = 2$ clutches). The technique was adjusted and the subsequent clutches were raised by placing between 39 and 60 embryos in a 2-L crossing tank as noted in the methods. This adjustment yielded a survival rate of $82\% \pm 7.7$ (SD) ($n = 4$ clutches), slightly lower than the previously published survival rate of $\sim 90\%$ when using *B. plicatilis* as live feed.[11,21]

While not a part of this study, adult zebrafish raised using this technique (Fig. 2D) have been crossed on a regular basis and produce embryos for use in the classroom and in developmental biology research.

## Discussion

The methods described in this study allow for small institutions to raise zebrafish for use in education and research. In combination, these methods allow the temporary or continuous culture of <2 L volumes of algae and rotifers. These techniques allow students to gain a variety of skill sets from making solutions to husbandry and provide two model organisms available for teaching and research.

### *Culturing rotifers as a source of live food for zebrafish embryos*

While this is not the first time *B. plicatilis* has been described as a source of live food for zebrafish larvae,[7,11,22] previous studies focused on raising large amounts of rotifers for fish facilities. Our study adjusts these methods for use on a small scale using inexpensive materials. While the survival rates are lower than previous studies, 82% versus 90%,[11,21] this is not surprising as large research institutions can afford personnel dedicated to zebrafish husbandry. If a sturdier culture apparatus is desired, small cultures of rotifers can be maintained in commercially made hatchery cones (Brine Shrimp Direct or Florida Aqua Farms) or using Imhoff cones (Wildlife Supply Company). Another option might be an apparatus marketed to culture brine shrimp called the Brine Shrimp Hatchery Kit (Drs. Foster and Smith). While we have not tried this apparatus, it is similar to our RCBs, except the soda bottle screws into a base in which airline tubing can be attached. Using a sturdier culture apparatus is logical when rotifers are used as a sole food source for larvae and adults.[22] If an institution does not desire to continually spend funds on brine shrimp cysts or processed feed, our protocol can be modified to raise a larger number of rotifers. Our protocol could also be adapted for use with commercially available algal paste to feed the rotifers (e.g., RotigrowPlus or Nanno3600 from Reed Mariculture, Inc.). These pastes have successfully been used in larger rotifer cultures maintained for zebrafish husbandry.[11,19,22]

Recently, Aoyama *et al.*[23] demonstrated a method to rear zebrafish larvae using the freshwater rotifer *Brachionus calyciflorus*. We believe that our protocol could be adjusted to grow these rotifers and raise a continuous culture of *Chlorella* (Florida Aqua Farms) to use as their food source. The *Chlorella* could be maintained in MBL media[16] supplemented with thrice the amount of vitamins or cultured in COMBO media.[24] While raising freshwater rotifers allows for uneaten rotifers to survive in the water longer than saltwater rotifers, currently published protocols for raising freshwater rotifers as a source of larval zebrafish feed require the rotifer cultures to be kept at $\sim 20$ rotifers/mL.[23] In a small academic environment, it is more convenient to maintain a culture of saltwater rotifers because they can be kept in smaller volumes at higher densities.

### *Educational uses for rotifers*

Maintaining cultures of rotifers not only provides a food source for developing zebrafish larvae but also can serve as a model organism for undergraduate teaching and research. Rotifers have been used for a variety of topics suitable for undergraduates, including feeding studies using latex microspheres,[25–27] visualizing rotifer behaviors,[28–33] food preferences in rotifers,[34,35] predator–prey studies,[36–39] competition,[40,41] and toxicological studies.[42,43]

## Conclusions

The techniques described in this study allow researchers and educators to easily raise zebrafish embryos to juveniles without the use of expensive fish facilities. Raising zebrafish reduces the cost of obtaining adult fish from laboratories or stock centers. Maintaining a larval food source, *B. plicatilis*,

also enables students to conduct studies on all stages of zebrafish development. Furthermore, maintaining a culture of rotifers provides a micrometazoan for use in teaching and undergraduate research.

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### Authors' Contributions

R.L.A. graduated from Ripon College in 2015 with an AB degree in Chemistry-Biology. While at Ripon College, he helped design the technique, completed the experiments, and wrote the first draft of the article. He is currently a first year graduate student at Duke University in the Developmental and Stem Cell Biology program where he helped edit the article.

R.L.W. received his BS in Zoology from the University of Rhode Island in 1970 and a PhD from Dartmouth College in Aquatic Ecology in 1975. After completing a postdoctoral fellowship at the University of Washington from 1975 to 1977 with E.T. Edmondson, he joined the faculty at Ripon College where he is professor of Biology. He helped with culturing the rotifers and in editing the article.

B.E.S. received her BA in Biology from Lake Forest College in 1998 and a PhD from Northwestern University in Developmental Biology in 2006. After completing a postdoctoral fellowship at Feinberg School of Medicine–Northwestern University from 2007 to 2011 with Jacek Topczewski, she joined the faculty at Ripon College where she is an assistant professor of Biology. She helped design the technique and edit the article.

### Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
*Barbara Sisson, PhD*
*Department of Biology*
*Ripon College*
*300 West Seward Street, PO Box 248*
*Ripon, WI 54971*

*E-mail:* sissonb@ripon.edu