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Larval Rearing Methods for Small-scale Production of Healthy Zebrafish

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Abstract - Raising larvae of *Danio rerio* Hamilton (zebrafish) is a challenging task that requires skill and a significant daily time investment. We have developed a simple nursery and a husbandry regimen that streamlines procedures and is feasible for small laboratories to carry out in the absence of support staff. The nursery is inexpensive to build and easy to maintain. The regimen uses a simple benchtop nursery that houses up to 300 larvae. Feeding is simplified by using defined volumes of microencapsulated feeds with only 1 type of live prey as a dietary supplement, *Artemia franciscana* Leach (brine shrimp). Tests of the regimen using wild-type lines showed that it supports timely entry into the metamorphic period and supports survival rates of at least 75%. Further, inexperienced users were able to raise larvae successfully. Here, we describe how to assemble the nursery and how to carry out the feeding and care regimen.

Introduction

The daily husbandry of *Danio rerio* Hamilton (zebrafish) is relatively easy compared to that of many other aquatic vertebrates. This is due, at least in part, to the availability of commercial housing systems, the multiple print resources that provide detailed husbandry guidelines (e.g., Harper and Lawrence 2011; Nüsslein-Volhard and Dahm 2002; Varga 2011, 2016; Westerfield 2007) and the various services provided by the Zebrafish International Resource Center (ZIRC). Additionally, zebrafish researchers may have access to core facilities with full-time husbandry staff, or they may have the benefit of laboratory research technicians who can devote considerable attention to husbandry. However, in small laboratories with no support staff, zebrafish husbandry can consume a significant portion of each day for principal investigators and students. The most significant time challenge is larval rearing, as zebrafish are vulnerable at this stage and require intensive care.

To raise larval zebrafish, relatively elaborate nurseries can be built as a do-it-yourself project (Cattin and Crosier 2004). Nurseries may provide features such as recirculating water and water filtration. However, the cost, space requirements, and maintenance of such systems can be serious drawbacks for small laboratories. To avoid these problems, we designed a benchtop nursery that is inexpensive, takes up little space, and requires minimal maintenance. The nursery is essentially a water bath that maintains static-water fish tanks at an appropriate temperature. One nursery can house 300 larvae. Setting up multiple nurseries increases the capacity and, when not needed, the nurseries can be stacked and stored off the bench. Here, we describe how to build and maintain this nursery system.

The nursery system is a key component of a streamlined larval husbandry regimen that we developed in the course of training inexperienced users. Our goals were to develop foolproof practices where possible, ensure the growth of healthy zebrafish, and save time while reducing costs. The regimen was influenced by "Hoff's rules", quoted here (Hoff 1996:107):

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1. Provide a simple, adequate stable environment that can be easily manipulated and maintained.
2. Provide adequate, quality foods on a consistent basis.
3. Provide strict maintenance procedures on a daily basis.
4. Develop a good wet thumb, be observant, and keep records.

The regimen described here incorporates these general principles in combination with zebrafish-specific practices, especially those found on the ZIRC website, and innovations developed from our own experience. The regimen was tested across multiple clutches and generations for its ability to support zebrafish growth and health. We report here that the regimen allows most larvae to enter the metamorphic period by 15 dpf and that survival rates of populations are high. Further, the husbandry methods are relatively easy. Much of the testing reported here was carried out by students with no prior zebrafish experience, including AP biology high school students in their classroom and undergraduate biology majors in a research lab. The students built nurseries, implemented the husbandry regimen, and collected growth and survival data. Their results for growth and survival rates were consistent with results obtained by more-experienced zebrafish researchers. Therefore, we conclude that the regimen is easy for first-time users to learn and implement and, most importantly, that first-time users can support larval zebrafish growth successfully. We believe that the simple methods presented here will be of general use for laboratories interested in small-scale larval rearing and for which a high survival rate is essential.

Methods

General husbandry

Adult zebrafish were maintained following standard procedures (Westerfield 2007). Most adults were housed in research animal facilities using commercial stand-alone recirculating systems (Aquaneering, Aquatic Habitats) and a regulated daily light cycle of 14 hours (hrs) light:10 hrs dark. For the recirculating system, water was generally maintained as follows: temperature 28–29, pH 7.50–7.60, conductivity 480–500 microSiemens (μS), general hardness (GH) 71 ppm, carbonate hardness (KH) 18 ppm. Ammonia, nitrite, and nitrate were generally kept at 0 ppm. One population of adults was maintained in a laboratory in a glass aquarium tank on a natural light cycle. For the aquarium tank, water parameters at the start of the study (when adults were crossed) were pH 7.4, conductivity 111 μS, GH 35 ppm, KH 0 ppm. Ammonia, nitrite, and nitrate were 0.25 ppm, 0.8 ppm, and 80 ppm, respectively.

Fertilized wild-type eggs were obtained by crossing either AB or a line obtained from local pet stores. Fertilized AB eggs were also obtained from ZIRC. Embryos were bleached following a standard protocol then cultured at a density of 50 embryos in approximately 120 mL E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, Brand et al. 2002) supplemented with 120 μl/L 0.1% methylene blue. The embryo density followed ZIRC guidelines (Basic Nursery Instructions, http://zebrafish.org/documents/protocols.php) and guidelines from Varga (2011). Embryos were maintained in 4.5" diameter soda-lime glass culture dishes (Carolina Biological) and held at 28.5ºC using an incubator (Fisherbrand, Heratherm). When an incubator was not available, dishes were held in the larval nursery to maintain temperature. In either case, dishes were stacked or covered with a Petri dish lid to minimize evaporation. Dishes were checked daily to remove unfertilized eggs and nonviable embryos. Any removed eggs were replaced from a spare bowl, when possible, in order to maintain the starting density. Chorion debris was removed daily. At 3 days post-fertilization (dpf), any larvae that had not hatched were dechorionated manually.

At 5 dpf, larvae were transferred to 0.8 L tanks (Aquaneering) at a density of 50 larvae per 250 mL medium and moved to the nursery. Tanks were fitted with a baffle, a 400 μm screen, and a tank lid (Aquaneering). Tank medium was 0.5X E3 medium, 1X E3 medium, or facility water filtered with a 0.2 μm filter. Nurseries were maintained either on a regulated daily light cycle of 14 hrs light:10 hrs dark or on a natural light cycle. At 11 dpf, larvae were transferred 1:1 to 1.8 L tanks and moved to a recirculating rack system. Water flow on the rack system was initially maintained as a slow laminar flow down the wall of the tank by positioning the water supply tubing on the gap between the lid and tank side rather than using the central tubing hole of the tank lid. For some experiments, 11 dpf larvae were split 1:2 and maintained in the nursery until they reached 20–22 dpf, then transferred to the rack system. Procedures were approved by the Institutional Animal Care and Use Committees of Appalachian State University, Sanford Research/University of South Dakota, and Augustana College.

Nursery components

Each nursery consisted of a translucent polypropylene tote bin, 23" L x 16¼" W x 6" H (Sterilite) and a stack of black opaque polypropylene support grating, 36" L x 18 ¾" W x ½" H (#SPG1, Pentair) that was cut equally lengthwise into three 12" pieces. Each nursery held a 100-watt submersible aquarium heater (Marineland) or two 50-watt heaters (ViaAqua, Aqueon). An aquarium thermometer was used to monitor temperature (Lifegard, Coralife).

Nursery tank cleaning supplies

The siphon was assembled by attaching a 100 μL pipet tip onto the end of 3/16" flexible aquarium tubing (e.g., Top Fin, Penn-Plax) that was 24–31" long. The siphon was started by filling the tubing using a squeeze bottle of either E3 medium or filtered facility water (depending on the tank medium). Pasteur pipets (5¾", Fisherbrand) were prepared by trimming the end with a glass scribe to create the desired bore, then fire-polishing the cut end. A 2 mL rubber bulb (Fisherbrand) was attached to the Pasteur pipet.

Larval diets and general feeding

Diets consisted of mixes of microencapsulated formulated feeds plus various dry supplements. Formulated feeds were Golden Pearls (GP) Reef and Larval Diet (Brine Shrimp Direct). Food Mix 1 consisted of equal parts by weight of GP 5–50 μm, GP 50–100 μm, and artificial plankton Rotifer Formula V (Ocean Star International). In some cases, freeze-dried rotifers (Brine Shrimp Direct) were substituted for artificial plankton Rotifer Formula V. Food Mix 2 was formulated as described for Food Mix 1 except that GP 5–50 μm was replaced by GP 100–200 μm. All formulated feeds and food mixes were stored dry at -20°C for long-term storage. Prior to feeding larvae, the food mix was suspended in the same medium as in the larval tank, at a concentration of 1g/L, following ZIRC guidelines (Detailed Nursery and Food Instructions, http://zebrafish.org/documents/protocols.php). Suspended food mix was stored in a media bottle with a stir bar at 4°C, and kept for no longer than 1 week.

Twice daily, at 9 am and 3 pm, zebrafish were fed suspended food mix. For these feedings, the food mix bottle was briefly agitated to suspend the food particles evenly and the mix was delivered to each tank using a serological pipet and automatic pipettor or other pipet aid. Once daily, at noon, newly-hatched *Artemia franciscana* Leach (brine shrimp) were fed to each tank. Brine shrimp were either the Great Salt Lake strain or the San Francisco Bay strain (Brine Shrimp Direct). Shrimp were cultured specifically for

larvae using a Hobby hatchery (Dohse Aquaristik). The hatchery used approximately 725 mL of 30% sea salt (Oceanic or Instant Ocean) dissolved in RO/DI water and shrimp were incubated either at ambient temperature or at 26–27ºC. After 24 hours of culture, shrimp were collected, rinsed thoroughly in E3 medium or filtered facility water (for larvae in a nursery) or rinsed in unfiltered facility water (for larvae on a recirculating rack system), and immediately fed to larvae using a Pasteur pipet.

Length and survival measurement

Larvae were anesthetized in tricaine solution (Westerfield 2007), or by gradual addition of crushed ice made from the same medium as the tank water (E3 medium or filtered facility H_2O) as previously described (Eames Nalle et al. 2017). Anesthetized larvae were transferred to a stage micrometer (Peak glass scale 50) using a wide-bore fire-polished Pasteur pipet. Larvae were oriented by gentle manipulation with a short loop of fishing line (6 lb test) attached to a wooden applicator stick (Puritan Medical Products) and imaged with a digital camera. Length was determined as standard length, defined as the distance from the tip of the snout to the posterior extent of the caudal peduncle, where the fleshy region of the body meets the caudal fin (Carlander and Smith 1945). For immature larvae in which the notochord was straight rather than flexed (with no distinct caudal peduncle), we recognized standard length as the distance from the tip of the snout to the posterior end of the notochord (Parichy et al. 2009). Length was measured using ImageJ (Schneider et al. 2012). Mortality was tracked twice daily for each tank and dead larvae were promptly removed as part of the regular tank cleaning regimen.

Statistical Analyses

Length data were analyzed by ANCOVA using GraphPad Prism version 7.03. Because of heteroscedasticity, logarithmic transformation was applied to the data prior to calculating linear regression lines (Zar 1999). Heteroscedasticity was confirmed by the D'Agostino-Pearson normality test.

Results

Assembling a nursery and tank-cleaning supplies

The nursery is a tote bin that holds RO water heated by 1 or 2 submersible aquarium heaters. The larval fish tanks are bathed in the heated RO water and this maintains the tank medium at a stable temperature. To accommodate the heaters inside the nursery tote bin, plastic grating is stacked in the bin to make a platform that raises the water level. The heaters are positioned adjacent to the platform and the larval tanks rest on top of the platform. The tank medium is refreshed twice daily while the nursery's RO water is merely topped up as needed.

How to assemble the nursery. The materials for the nursery, described in the Methods section, include a tote bin and a support grid cut into 3 equal pieces. To make the tank platform, stack the 3 cut pieces of support grid inside the tote bin, as shown in Figure 1A. Attach two 50-watt submersible aquarium heaters (or one 100-watt heater) to one of the longer walls of the tote bin, in the gap adjacent to the platform. Add enough RO water to cover the heaters to a depth of ~2". Use lab tape on the front of the tote bin to indicate the fill line (Fig. 1B). Insert an aquarium thermometer probe in the center of the bin and run the heaters for at least a few days prior to adding fish so that the temperature can stabilize. Each day, monitor the temperature and add RO water to replace evaporated water. If pre-warmed water is used, the temperature will stabilize quickly.

How to assemble the larval tanks. The larval tanks (including a baffle, 400 µm screen, and lid) should have the baffle in the slot closest to the tank wall, as recommended by the manufacturer. Fit the 400 µm screen in the slot farthest from the baffle, as shown in Figure 2A. Push down firmly on the screen to ensure there is no gap between the screen and the tank bottom. This will ensure that fish cannot pass under the screen and creates a fish-free chamber between the screen and the baffle. This chamber will be used for siphoning dirty tank water without disturbing the fish, as described below.

How to assemble a tank-cleaning siphon. The siphon requires a ~24" length of flexible aquarium tubing with a trimmed 100 µL pipet tip and a trimmed 1 mL pipet tip attached at one end. These materials and the assembled siphon are shown in Figure 3. To make the siphon, trim the wide end of the 100 µL pipet tip so that it can be inserted into the tubing. Applying some aquarium silicone lubricant may be necessary. Trim the narrow end of the 100 µL pipet tip to widen it slightly and also trim the narrow end of the 1 mL pipet tip. Fit the larger pipet tip over the smaller tip. The larger tip adds weight that prevents the siphon from falling out of the tank during cleaning. Additionally, it allows the siphon to "break" if left unattended so that the tank cannot be siphoned dry.

A streamlined feeding and care regimen

The husbandry regimen calls for feeding on a strict schedule and using pre-mixed dry diets (Food Mix 1 and Food Mix 2) that combine different particle sizes. The schedule and food mixes, together, eliminate the need for judging when to transition larvae to the next diet. The schedule additionally specifies a date for splitting larvae and for transferring them to a recirculating system (if available). Daily tasks are stream-lined in multiple

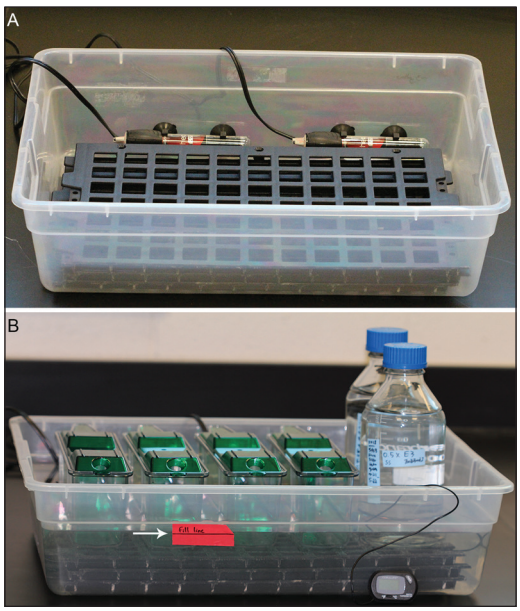

Figure 1. Larval nursery. A) Tote bin holding three stacked support grids and two heaters. B) Nursery in operation, with larval tanks surrounded by heated RO H_2O. The nursery can hold up to six tanks. With fewer tanks, media bottles can be accommodated as shown. Arrow: adjustable label indicating the RO fill line.

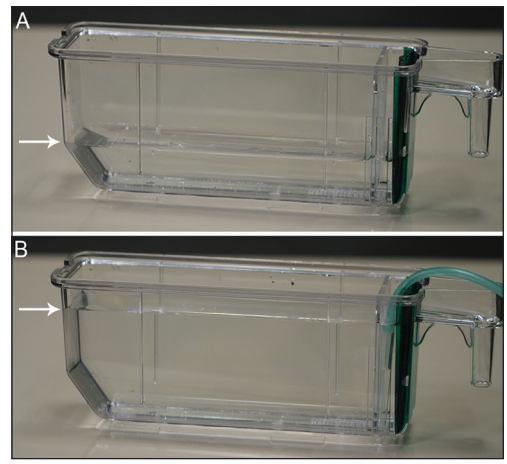

Figure 2. Larval tank assembly. A) The green baffle is inserted in the slot at the back of the tank. The 400 µm screen is inserted in the front-most slot, farthest from the green baffle. The tank is filled with ~250 mL medium so that the volume is level with the front angle of the tank (arrow). B) To refresh the tank medium, an additional volume (~400 mL) is added to the tank, raising the medium level as indicated by the arrow. To restore the starting tank volume, the siphon is inserted in the chamber between the screen and the baffle.

ways including culturing only 1 type of live prey (brine shrimp) and cleaning tanks by a relatively fast siphoning method that prevents accidentally removing larvae in the process. The daily regimen is summarized in Table 1.

How to transfer 5 dpf larvae from bowls to tanks. At 5 dpf, transfer larvae from fish bowls to tanks. Tank assembly is described above. Have on hand a squeeze bottle of tank medium such as the 0.5X E3 medium used here. First, add ~400 mL of pre-warmed E3 medium to the tank. Then, carefully pour larvae from the bowl into the tank. The medium that is already in the tank will prevent the larvae from hitting the bottom and being stunned. Towards the end of pouring, use the squeeze bottle to rinse out any larvae that may otherwise be left behind in the bowl. Label the tank with lab tape to indicate the fish line, birthdate, and number of larvae. Use the siphon, as described below, to remove excess volume so that approximately 250 mL remains. It is important to note that 250 mL is level with the front angle of the tank (arrow, Fig. 2A). This angle conveniently eliminates the need to directly measure the volume. Next, add the tank lid and transfer the tank to the nursery. After all tanks have been transferred, check the nursery's RO fill line. This fill line should match the fill line of the E3 medium inside the tanks. Adjust the RO water level if necessary and adjust the nursery's fill line label (Fig. 1B). Begin the regular feeding and care regimen as described below.

How to maintain clean tanks. Clean the tanks twice daily, at 9 am and 3 pm, prior to delivering the suspended food mix. Have on hand a fire-polished Pasteur pipet fitted with a rubber bulb, fresh E3 medium, a siphon, and a waste container. Cleaning is a 2-step process. First, any large, loose debris is removed from the tank bottom. Second, the tank's E3 medium is changed. The fresh E3 medium is ideally pre-warmed to 28ºC by storing it in the nursery or in an incubator. During cleaning, keep the tank on the counter rather than in the nursery, so that the tank contents can be monitored.

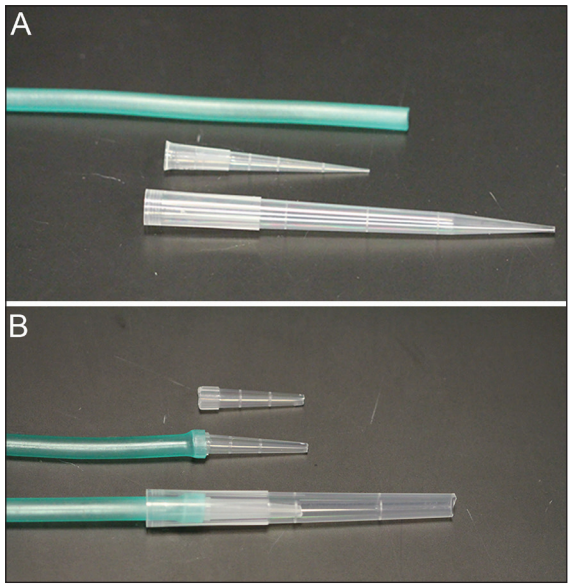

Figure 3. Tank-cleaning siphon. A) Starting materials, uncut. From top to bottom: flexible aquarium tubing, 100 µL pipet tip, 1 mL pipet tip. B) Assembly of the siphon, from top to bottom: 100 µL pipet tip trimmed at both ends, trimmed 100 µL pipet tip inserted into the flexible tubing, trimmed 1 mL pipet tip inserted over the smaller tip and tubing.

To begin cleaning, start by lifting the tank out of the nursery and inspecting the tank bottom for food debris and dead larvae. Then, use the fire-polished pipet to remove any loose debris that is too large to pass through the tank's screen. Next, dilute the tank's old E3 medium by adding approximately 400 mL of fresh medium (arrow, Fig. 2B). Get the siphon started (see Methods) and insert the pipet tip end into the fish-free chamber at the back of the tank, as shown in Figure 2B. Direct the siphon outflow into the waste container. This works best when the waste container is directly below the tank, resting on the floor. Continue siphoning until the starting volume of ~250 mL is

restored. The action of the siphon appears to be gentle as it does not pull larvae towards the screen and does not cause an observable change in swimming behavior.

Periodically, the tank bottom may be too dirty to clean with the Pasteur pipet. In this case, transfer the fish to a clean tank. This is typically necessary at 6 or 7 dpf as a large amount of uneaten food will settle on the tank bottom during the first day or so of exogenous feeding. To save supplies and time, the baffle, screen, and tank lid from the original tank can be transferred to the new tank, as none of these tank components become fouled even when used through 22 dpf. Fill the new tank with ~250 mL of fresh tank medium. Carefully pour the fish into the new tank, top up with additional tank medium, and siphon as described above to restore the starting volume of ~250 mL.

How to deliver food to the tank. For the scheduled 9 am and 3 pm feedings of dry food, the tanks are cleaned and siphoned before adding more food to them. Prior to the scheduled feeding, a bottle of food mix should be prepared ahead of time to avoid delays. Details regarding preparation of the dry food mix are given in the Methods section. To feed, have on hand a bottle of prepared food mix, a reusable serological pipet, and a pipet-aid. Resuspend the food mix by manually shaking the bottle. If a stir bar is kept in the bottle, a brief shake by hand will be sufficient. Use the pipet to deliver the appropriate volume to each tank, as indicated in Table 1.

For the noon feeding of live brine shrimp, have on hand a squeeze bottle of E3 medium, a small beaker for collecting shrimp, and a Pasteur pipet fitted with a rubber bulb. Collect newly-hatched nauplii from the hatchery dish after 18–24 hours of culture, following the manufacturer's protocol. Briefly, the free-swimming shrimp will be in the center of the dish, above the removable sieve. If the shrimp density is sparse, a light can be shined directly above the sieve to attract shrimp more efficiently. Lift the sieve and rinse the shrimp thoroughly with E3 medium. (It is important to thoroughly remove the shrimp culture water to avoid delivering salt water and hatching metabolites to the zebrafish larvae.) Turn the sieve over the beaker and rinse from the opposite side to transfer the shrimp to the beaker. Use the Pasteur pipet to deliver shrimp to the fish tank. Figure 4 shows a suggested density of shrimp that would be suitable for feeding several tanks of larval zebrafish.

How to adjust care as larvae grow. As the larvae grow, they must be graduated to different husbandry conditions. Changes include introducing progressively larger food particles, decreasing the density of individuals within tanks, moving larvae from static tanks to a recirculating system, and progressively increasing the water flow rate. The timing of

Table 1. Larval feeding and care regimen.

Age	Time	Procedures
5–10 dpf	9:00 am	Clean and siphon tanks Deliver food mix 1, 5 mL per 50 fish
	Noon	Deliver *Artemia* to each tank
	3:00 pm	Clean and siphon tanks Deliver food mix 1, 5 mL per 50 fish
11 dpf	Anytime	Transfer 1:1 to 1.8 L tanks Move tanks to recirculating system
11–21 dpf	9:00 am	Deliver food mix 2, 7 mL per 25 fish
	Noon	Deliver *Artemia* to each tank
	3:00 pm	Deliver food mix 2, 7 mL per 25 fish

these changes can be determined by using keen observation and good judgment, or they can be scheduled as part of the regimen, as described here for young larvae.

Food particle size is increased at 11 dpf by switching from Food Mix 1 (5–100 μm) to Food Mix 2 (50–200 μm). The overlapping particle size ranges are intended to decrease competition. The larvae are maintained on Food Mix 2 until at least 21 dpf (Table 1). Subsequently, fish should begin receiving a larger-particle dry food mix, twice daily, delivered to the surface of the tank water, along with live brine shrimp, once daily.

The larval density per tank is adjusted at 11 dpf. This is done by transferring fish 1:1 from the 0.8 L tanks to fresh 1.8 L tanks and moving them to a recirculating system. The initial water flow rate should be a slow, laminar flow down the side of the tank (see Methods). As larvae grow, the tubing should be moved to the central tank lid hole and the water flow rate should be increased. The fish should continue to be divided into additional tanks, as needed, to accommodate their growth. Large adults are typically kept at ~5 fish per liter in 2.8 L tanks.

Record keeping. Diligent record keeping is essential for successfully raising zebrafish. Keeping records ensures that daily tasks are accomplished, allows long-term trends to be detected, and provides documentation for institutional animal care committees. Our approach includes using a dry-erase board (whiteboard) mounted on a wall near the nursery. The whiteboard is used as a calendar and checklist for daily husbandry tasks. Prior to populating the nursery, and ideally prior to setting up mating crosses, the calendar should be filled in with dates and times. This will allow lab personnel to plan the schedule for all husbandry tasks so that there are no surprises with conflicting schedules. The whiteboard should include a checkbox for each daily task and should include the age (in dpf) of the larvae as well as the calendar date. An example is shown in Figure 5. In addition to being a daily checklist for cleaning and feeding, the whiteboard also includes boxes to record both the daily (morning) nursery temperature and the daily check of the RO fill line. Overall, the checklist serves as a prompt that reminds lab personnel of the tasks that need to be performed and when to perform them. Further, it allows personnel to see at a glance whether specific tasks have been completed or not. After larvae are moved to the recirculating system, the whiteboard can be photographed as a permanent record.

In addition to the daily checklist, it is often useful to track larval mortality. This allows lab personnel to monitor the success or failure of their husbandry efforts. Mortality tracking is conveniently done as part of the 9 am and 3 pm tank cleanings. Dead larvae can be indicated by tallying on lab tape on the side of each tank. Mortality tracking is most useful if it is done on a tank-by-tank basis, as this allows assessment of whether there is a system-wide problem versus 1 unhealthy clutch.

General methods for streamlining and preventing mistakes. Having enough tank medium on hand on a daily basis is the major bottleneck for this regimen. If there is a convenient source of facility water, then it may be best to raise larvae in filtered facility

Figure 4. Shrimp density at 24 hours of culture. The Pasteur pipet typically draws up just over 1 mL of the culture. A representative result is shown in which 668 brine shrimp are held in approximately 1.2 mL. One culture can typically feed 12 tanks or more by filling the pipet multiple times.

water. If using E3 medium, then a key strategy is to have a series of stocks, including 50X, 1X, and 0.5X stocks. First, keep a 1 L stock of filter-sterilized 50X E3 medium at 4ºC. Prior to 5 dpf, and as needed, prepare a large stock of 1X E3 medium from the 50X stock and store it in a dedicated benchtop carboy. Use the 1X stock to make enough 0.5X E3 medium ahead of each tank cleaning session. The 0.5X E3 medium should be stored in media bottles held in an incubator or in the nursery at 28.5ºC.

To further streamline E3 medium handling, maintain a dedicated, reusable 25 mL serological pipet for diluting the 50X stock, and also maintain a dedicated non-breakable, 1 L graduated cylinder for diluting stocks. To help lab personnel identify supplies and limit confusion, all of these supplies (50X E3 stock bottle, 0.5X E3 bottle, 1X E3 carboy, serological pipet, and graduated cylinder) should be labeled with the same color of lab tape. The color-coding can additionally signal to users that these items can be simply rinsed thoroughly in RO water before reusing, rather than washing each time.

Using lab tape to color-code food supplies is also useful for limiting feeding errors. For example, Food Mix 1 should be labeled with the same color whether it is in the freezer or suspended in a media bottle. Regardless of which food mix is used, the nursery tote bin or tanks should be labeled with the same lab tape color as the food mix containers. This visual indicator of which food to deliver to the tank is a simple way for lab personnel to recognize whether they are using the correct food mix. Finally, as the suspended food mix may start to go rancid after about 1 week, it is important to indicate on the label not just the date of preparation but also the expiration date. This will promote consistent feeding of fresh food.

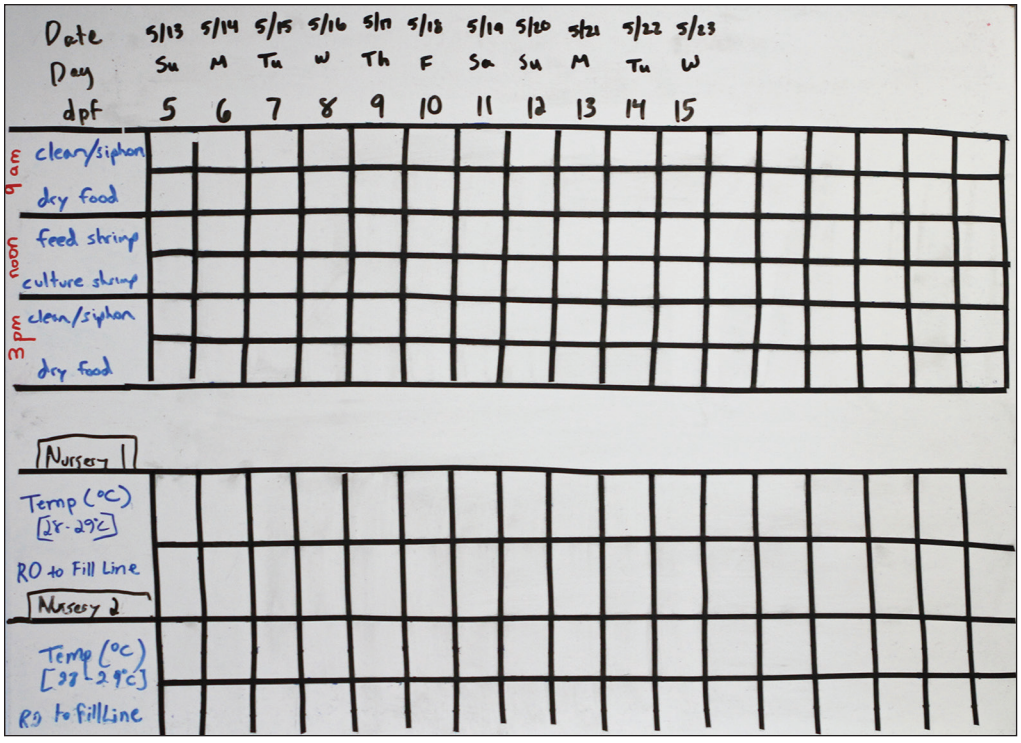

Figure 5. Daily husbandry checklist. A whiteboard mounted near the nursery provides space for maintaining a checklist and record of daily tasks: tank cleaning, fish feeding, shrimp culturing, and topping up the nursery RO H_2O to the fill line. Space is also provided for recording the daily temperature of the nursery.

Have on hand a supply of food mix aliquots, stored dry at -20ºC so that fresh food mix can be prepared quickly, as needed.

The regimen supports larval growth and metamorphosis

We tested our regimen for its ability to support larval health. Larvae were assessed by tracking survival, visually assessing whether larvae had entered metamorphosis in a timely manner, and measuring standard length across 3 time-points. For survival, we tracked mortality for dozens of wild-type clutches and found that we could expect a typical survival rate of at least 80%, with a range of 75–100% (Fig. 6).

Our daily observations suggested that most larvae were well into the metamorphic period by 21 dpf and that mortality was rare after that milestone was reached. To test when larvae entered the metamorphic period, we randomly sampled larvae (AB and a wild-type pet store variety) at 14 dpf and 15 dpf for closer analysis. Live fish were anesthetized, imaged, and scored as to whether caudal fin rays had begun to appear (Parichy et al. 2009). The analysis was performed 3 independent times. We found that, on average, 76% (41 out of 54) of larvae were metamorphic by 15 dpf. We repeated this analysis at 20 dpf and 21 dpf, on new samples of larvae and found that 84% (63 out of 75) were metamorphic. Thus, we concluded that the regimen supports timely entry into the metamorphic period.

Next, we tested how well the regimen supported growth in length. We measured standard length across 3 development time points: immature larvae that were newly-reliant on exogenous feeding, sampled at 9 dpf; then more mature larvae that were from populations expected to contain a mix of non-metamorphic and metamorphic larvae, sampled at 15 dpf; and finally older larvae from populations that were expected to contain mostly "postlarvae" (Hubbs 1943) that had entered the metamorphic period, sampled at 21 dpf. For this test, beginning at 11 dpf we maintained approximately half of the larvae in the nursery and half were transferred to a recirculating water system. This allowed us to ask whether larvae transferred to a recirculating system would show similar growth compared to larvae in the nursery. We found that the larvae showed steady growth in either condition and that growth was not enhanced on the recirculating system (Fig. 7A).

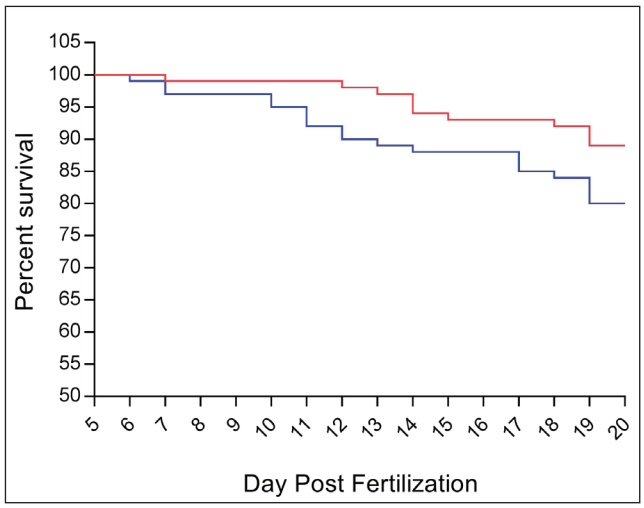

Figure 6. Survival curves. Wild-type larvae (AB line) were raised in the nursery from 5–20 dpf and mortality was tracked daily. All larvae were from eggs fertilized on the same day, from an unknown number of parents, and were raised simultaneously in two separate nurseries (Group A and Group B) by novices.

Finally, we shared our larval husbandry regimen with student groups at 3 institutions who had no prior experience with zebrafish. We asked them to follow a detailed protocol for setting up a nursery and raising larvae. The students performed all husbandry tasks, imaged larvae at 3 time-points, and then

measured standard length following our protocol. As shown in Figure 7B, the growth in length over time appeared to be similar between the test groups. Statistical analyses of the 4 regression lines demonstrated no significant differences between the slopes ($F(3, 4) = 0.3216$, $P = 0.8108$) and no significant differences between the elevations ($F(3, 7) = 3.92$, $P = 0.0622$). Therefore, we concluded that the regimen performs similarly whether carried out by experienced personnel or not.

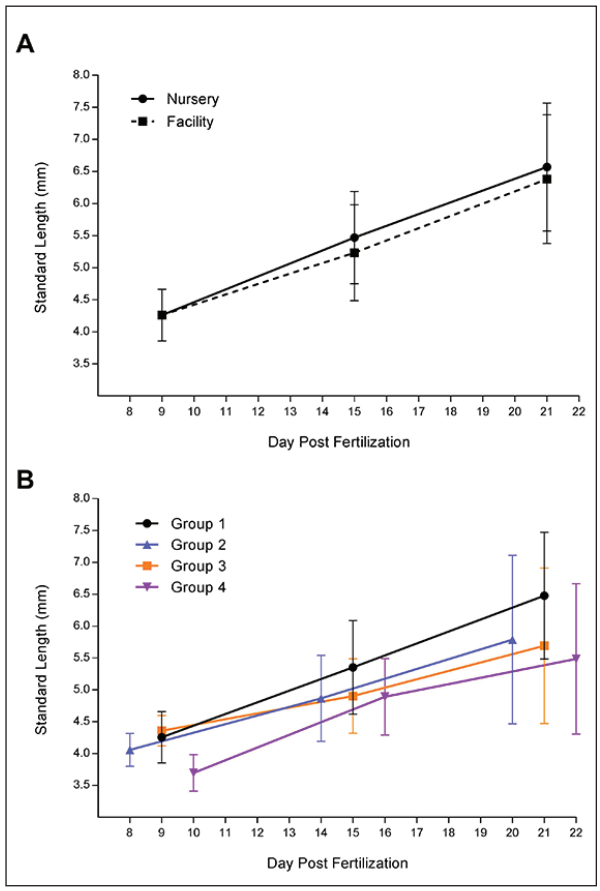

Figure 7. Growth in length. A) The husbandry regimen was tested by raising larvae from 5–21 dpf in the nursery versus transferring them to a recirculating system at 11 dpf. B) The regimen was tested by students with no previous Zebrafish experience at three institutions. Group 1: Undergraduate biology majors and Master's students. These data are the same as shown in panel A (solid line), repeated here for comparison. Group 2: Middle school and high school teachers. Group 3: Undergraduate biology majors. Group 4: AP biology high school students. Mean and standard deviation. Sample sizes are shown in Supplemental Table 1 (see Supplemental File 1, available online at https://eaglehill.us/ebioonline/suppl-files/ebio-spec01-kinkel-s1.pdf)

Discussion

Multiple successful larval husbandry regimens exist for zebrafish, as summarized in surveys and methods papers (e.g., Cattin and Crosier 2004, Dabrowski and Miller 2018, Lawrence 2011, McNabb et al. 2012, Osborne et al. 2016, Trevarrow 2004, Wilson 2012). While these regimens differ in their specific details, a common theme is a strategy for transitioning larvae, as they grow, to different foods, different containers, and different water flow rates until adulthood is reached. Typically, transitioning larvae in a timely and appropriate manner requires a keen judgment gained by experience. In the absence of experienced caregivers who can recognize and respond to the changing needs of larvae, growth rates and survival may be negatively impacted. Here, we have presented an example of a larval zebrafish husbandry regimen that provides a clear guide that allows inexperienced people to successfully transition larvae to new feeds and housing to accommodate larval growth. Further, the regimen relies on streamlined procedures that allow lab personnel to provide a high quality of husbandry in the absence of support staff.

Tests of our regimen support the conclusion that 3 daily feedings adequately support larval survival and growth. Although several authors report feeding 4 or more times daily (Dabrowski and Miller 2018, Farias and Certal 2016, Kaushik et al.

2011), our survival rates at 20 dpf of 80% and 89% seem satisfactory (Trevarrow 2004) and are consistent with, or higher than, survival rates at similar ages reported by others (Carvalho et al. 2006, Goolish et al. 1999, Onal and Langdon 2000). We have periodically achieved survival rates as low as 75% and as high as 100%. The lower rate was observed when raising the first generation of offspring from wild-type fish on a new recirculating system. We hypothesize that the parents were less healthy than zebrafish on a mature system and that this impacted the health of the offspring.

Our analyses of growth in length from first-feeding larvae to the metamorphic period showed that growth was steady. Growth rates are difficult to compare with published studies, as authors variously report standard length, fork length, or total length. While our growth results appear to be similar to those of Eaton and Farley (1974) who reported standard length, our larvae grew slower than those of Best et al. (2010). When various inexperienced student groups tested the regimen, the larvae showed similar patterns of growth in length between the groups. However, scrutiny of the data suggests that growth was initially slow for larvae raised in a high school classroom (Group 4, Fig. 7B). This group initially had difficulty culturing brine shrimp and, as a result, brine shrimp were unavailable until 9 dpf. This suggests that an early nutritional deficit may contribute to a delay in growth.

Because rearing larval zebrafish, by its nature, requires diligence and is time-intensive, there can be daily opportunities for mistakes. We found that implementing routine safeguards into daily procedures can have a positive effect on growth and survival. Safeguards include the use of checklists and color-coding as part of an organizational system that follows the principles of 5S that are used in lean manufacturing (see e.g., Gawande 2009, Michalska and Szewieczek 2007, Peterson and Smith 1998). Additionally, we found that reducing the number of daily tasks was important, since the fewer the tasks, the less opportunity for errors. Reduction of daily tasks includes using frozen aliquots of pre-mixed dry foods to avoid having to prepare foods daily.

Another significant time reduction is achieved by culturing only brine shrimp rather than supplying *Paramecium* spp. Müller (Paramecia) or *Branchionus* spp. Pallas (Rotifers) as a first feed as commonly recommended (e.g., Best et al. 2010, Westerfield 2007). Additionally, the brine shrimp cultures require minimal effort to set up and harvest. Since small culture volumes are used, no aeration is required. Also, the hatched shrimp self-sort by swimming into a sieve that is free of unhatched cysts. Self-sorting has the distinct advantage of avoiding lengthy decapsulation or manual sorting procedures. A minor drawback is the possibility of a greater proportion of unhatched shrimp than in more elaborate culture systems. However, in our experience, this potential drawback is outweighed by the time-saving aspects.

We recognize that this regimen may not be best for all labs. Indeed, if fish care staff are available, then this regimen might be improved by adding another feeding of food mix in the evening. With a third feeding of food mix, we casually observed that size disparities seem to be reduced when the larvae become juveniles and adults. While we have not tested this directly, this observation is consistent with studies showing that feeding more frequently can reduce size disparities (Eaton and Farley 1974). However, an additional feeding may not be feasible for small labs and this was our motivation for testing whether 2 daily feedings of suspended dry food, with 1 feeding of brine shrimp, is sufficient. On this briefer regimen, size disparities nevertheless seem to be reduced compared to our previous practice of sprinkling dry food on the water surface. Because the suspended food disperses throughout the tank, competition may be reduced and therefore size disparities are reduced.

We believe that this regimen is valuable because it relies on core principles but with room for adapting to specific needs of different researchers. In recent years, there have been some calls for standardizing diets and methods across labs (e.g., Kent and Varga 2012, Lawrence 2007, Penglase et al. 2012, Tsang et al. 2017). While standardization may allow labs to collaborate more easily, we propose that the flexibility to modify procedures is quite valuable. Indeed, recent work by Dabrowski and Miller (2018) emphasizes the importance of continuing to test new approaches. In our own work, we tested husbandry approaches over the course of more than 13 years across multiple institutions to develop our current regimen. We repeatedly found the need to modify our practices based on local conditions as well as the changing availability of commercial feeds. This flexibility allowed us to continually adapt and improve the regimen and we expect to continue to make improvements. Indeed, it is unlikely that a single regimen would suit all zebrafish researchers. Critically, good husbandry relies on not only the regimen, but on diligence, so that daily tasks are carried out consistently. In fact, we suspect that consistent care may be at least as important as the regimen. As noted in a slightly different context, "system and discipline [are] both essential (Grant 1982:364).

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