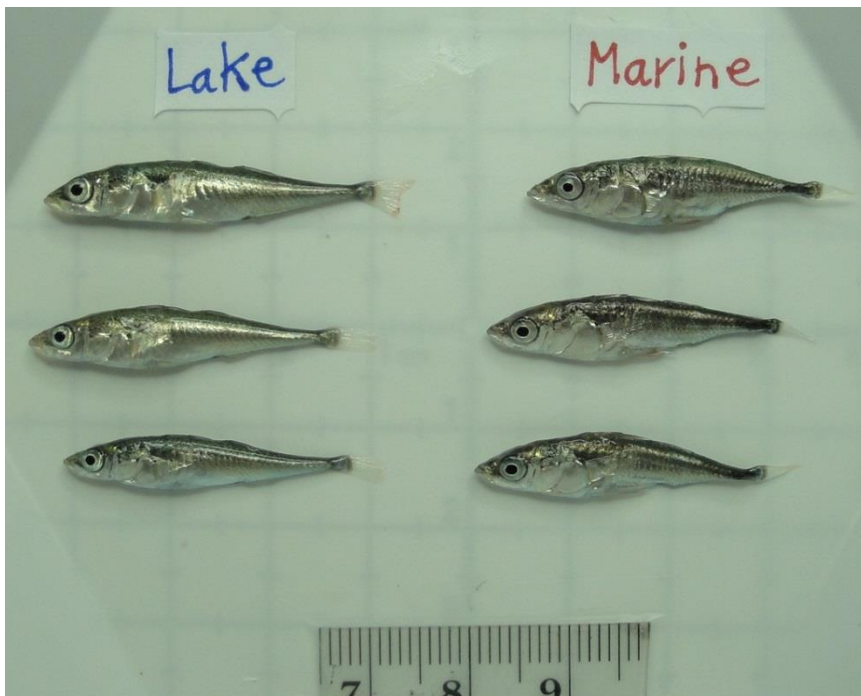
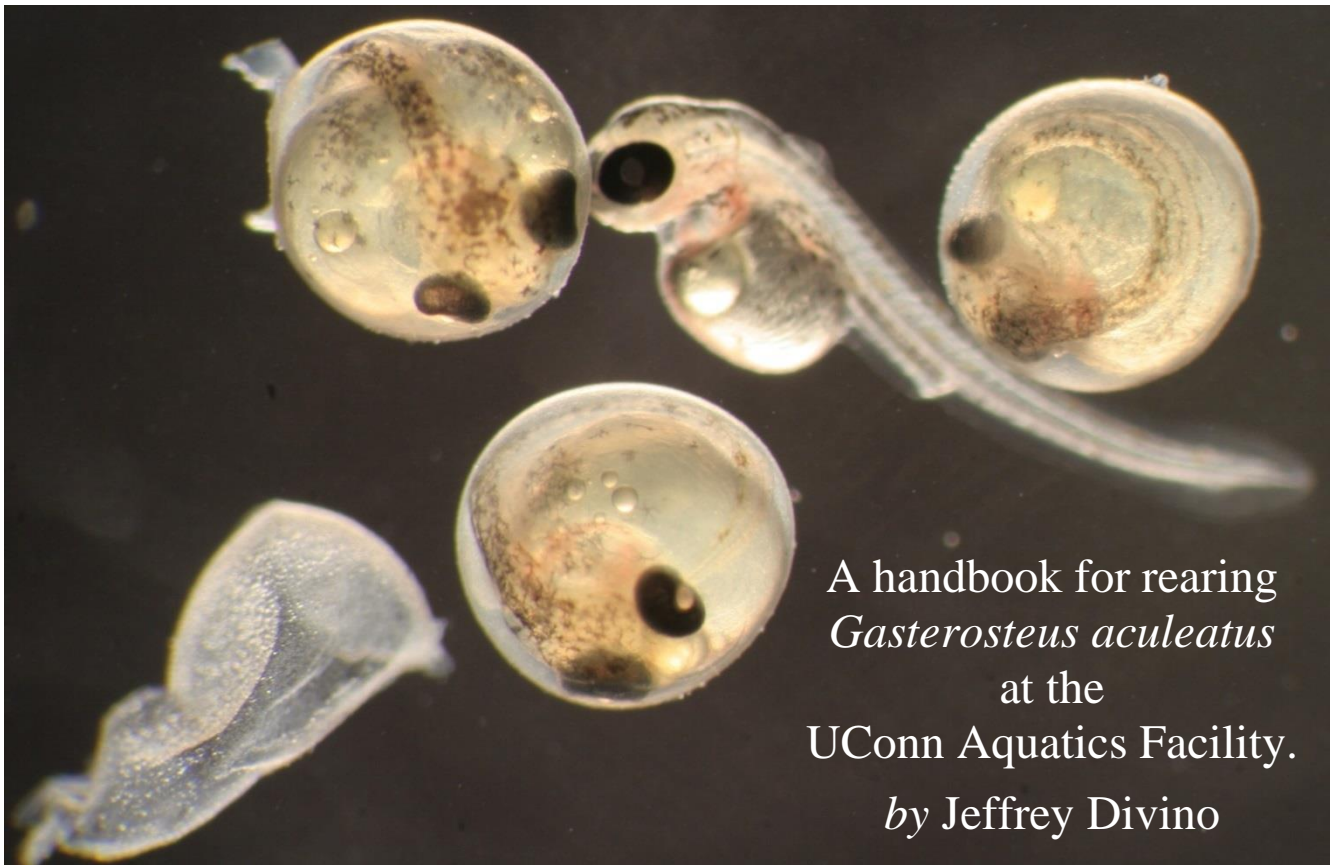


# Juvenile Threespine Stickleback Husbandry: Standard Operating Procedures of the Schultz Lab



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# Juvenile stickleback husbandry

by Jeffrey Divino

Revised: 30 July 2014

This Threespine Stickleback rearing handbook used by the Schultz lab has been adapted mainly from protocols developed by the Baker-Foster (Clark University) and Bell (SUNY) labs, our gracious stickleback suppliers. Departures from their methods, primarily with respect to fish housing and feeding regimen, reflects the unique set-up of our on-campus Aquatics Facility as well as our lab's research emphasis on osmoregulatory physiology of juvenile life stages, which necessitates maximizing energetic condition and growth rates. As such, our detailed feeding protocol is discussed separately. The following sections provide guidance for establishing appropriate housing conditions in preparation to receive clutches of stickleback embryos, and subsequently pre- and post-hatch husbandry of the colony. Maintenance routines are prescribed as the growing fish are transferred from Petri dishes to jars to aquaria.

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## I. Preparing to keep sticklebacks in the UConn Aquatics Facility

The necessary accommodations for housing sticklebacks should be prepared long before clutches are shipped. Consult the Checklist of Supplies (Appendix A) to gather all of the materials you will need to furnish the fish room. Maintaining adequate water quality and food supplies are the key considerations. Some of the climatic settings described below may be specific to UConn's Aquatic Facility, but serve as a general reference for establishing appropriate rearing conditions.

*Temperature & photoperiod.*— The ideal incubation and rearing temperature is 17-19°C. Developmental abnormalities can arise if eggs reach 23°C or if larvae reach 25°C. The common Aquatics Facility temperature of **20°C** is suitable since ambient room temperature is almost always 1-2°C cooler. Photoperiod conditions vary from lab to lab and will not impact larval development so long as there is enough daylight to not impede feeding times. We have adopted a **14 hour light : 10 hour dark** cycle. For example, the timer for the fish room can be set to turn the fluorescent lights on from 7:00-21:00. Recently, the Aquatics Facility has installed a “sunrise/sunset” dimmers that gradually turn the lights on and off. Approximate full-strength light intensity is 1100 Lux. These constant thermal and lighting settings obviously do not conform to those experienced by Alaskan stickleback in the wild and differ slightly from optimal laboratory breeding conditions.

*Relative humidity & evaporation.*— Like temperature, relative humidity of each fish room in the Aquatics Facility is monitored by a portable, battery-powered device. Temperature is rather stable throughout the year, but relative humidity (RH) fluctuates seasonally. RH is much higher in the warmer months, easily rising above 60%, and is also influenced by the number of operating aquaria in the room. In the winter, RH drops to ~20% and contributes to much more noticeable evaporative cooling of aquaria that have continually running filters. Water temperature can drop to 15-17°C, even though room temperature remains stable at 18-19°C.

*Preparing fish water.*— You will need to have clean water ready for arrival of fish, as well as for periodic water changes and salinity treatments. Our Aquatics Facility is outfitted with a Reverse Osmosis (RO) and UV sterilization water treatment system. Alternatively, water piped in from natural sources or dechlorinated tap water could be used.

1. Fill a 44-gallon Brute barrel with RO water from the hose to a desired volume. These barrels screw onto a wheeled trolley for easy movement around the fish room. *Note:* smaller volumes of water can be prepared in 5 gallon buckets.
2. Check the temperature of the RO water. If it is several degrees warmer or cooler than ambient, then as a precaution against temperature-shocking the fish, make water in advance and let the barrels stand overnight to equilibrate to room temperature. *Note:* Sticklebacks are hardy in this respect.
3. Add Instant Ocean aquarium salt (IO) with a sturdy plastic scoop. *Note:* One 15-lb bag of IO fits into a large 1.5 gal Rubbermaid “Lock-Its” container, which will prevent the salt from absorbing moisture after opening.
4. Mix the salt by stirring the solution with a 5', capped PVC pipe (1.25" dia).
5. Check salinity periodically using a YSI-85 meter and adjust as needed.
6. Before exposing fish to the new water, recheck temperature and salinity of the barrel.

## II. Caring for embryos

After the *in vitro* crossing of stickleback gametes, fertilized embryos are treated with one or more disinfectants (e.g., iodine, methylene blue, or Furan) and then washed with new “embryo medium”, which is typically sterile, oxygen-saturated, distilled water at ~3 ppt salinity. Clutches are shipped from the facility at Anchorage, Alaska overnight in separate 50 mL centrifuge tubes brimmed with medium to minimize sloshing during transport. Similarly, the screw-cap lids of the tubes are sealed with duct tape to prevent leaks. They are also layered in bubble tape or other packaging material within a Styrofoam box. The tubes of embryos are chilled with ice packs to keep metabolism (i.e., oxygen consumption) low and to keep them below 20°C throughout the duration of the shipment.

*Disinfection of embryos using iodine.*— Chemical disinfection of embryos is a safeguard against introduction of potential pathogens into the lab colony, but it can potentially *cause* egg mortality if performed improperly. If embryos are rush-shipped without being first disinfected, this step can be done when the clutches arrive. The Foster-Baker lab uses dilute iodine, which is not as risky as disinfecting with dilute bleach, and is a simple procedure:

1. First make the iodine solution:

**1% iodine solution** – orange color; makes 50 mL

- a. Pipet 0.5 mL of iodine stock solution (1 N) into a 50 mL conical tube.
  - b. Top up to 50 mL with RO water. Invert mix.
  - c. Foil wrap tube to protect from light & store at room temperature.
2. Open a tube containing a clutch, discard at least half of the medium into a waste beaker and then carefully pour the remaining contents of the tube into a 100 mm dia Petri dish. Swirling the water in the tube helps dislodge any stuck eggs. Flush out any residual eggs with a transfer pipet.
  3. Tilt the dish on angle and aspirate as much of the embryo medium as possible with a transfer pipet. If necessary, tap down any floating eggs with probe before tilting the plate.
  4. Refill the Petri dish with the iodine solution until the embryos are submerged. Swirl plate, then let incubate for **3 min**.
  5. Following treatment, tap down any floating eggs before draining the iodine waste out of the dish.
  6. Completely remove the residual iodine by changing the medium of the plate a total of **3x** before incubating the eggs in new embryo medium.

*Salinity.*— Addition of artificial sea salt to the water is another way to stave off infection. Because stickleback are euryhaline, standard rearing salinity varies from lab to lab. Baker-Foster embryo medium is 0.5 ppt and is then gradually increased to a “larval medium” concentration of 3 ppt, at which the stickleback will be raised throughout their life. The Bell and Kingsley labs, in contrast, incubate embryos and subsequent life stages at the same concentration of 3.5 ppt (referred to as “10% seawater”). In our four years of experience raising larval stickleback in the lab, the presence of salt greatly decreases the likelihood of infection-related die-offs of fish within containers and eliminates the presence of globulous growths found on some larvae caused by unidentified microbes. Indeed, it is for this reason that some labs (e.g., Cresko) rear stickleback in 5 or 6 ppt to better ward off the potential for pathogens. Because of the potential effects of acclimation salinity on tolerance, we have adopted a standard rearing (control) salinity of **3 ppt**.

Additional benefits of adding sea salt are that it increases the pH (which helps reduce ammonium concentration) as well as buffers the water, and is more tolerable to live brine shrimp nauplii, which

translates into extended feeding periods for the sticklebacks. The saltier the water, the more actively the brine shrimp swim in the water column. In contrast, brine shrimp in distilled water die quickly if they are not consumed and will sink, decompose, and foul the water. This asymmetry in nauplii performance is why it is preferable to introduce the fish to artificial pellets prior to salinity challenge experimentation (see Feeding Protocol).

Our lab is one of many that uses Instant Ocean brand sea salt for all our rearing and challenge water; others, like Stephen McCormick's lab, use Crystal Sea. We analyzed six dissolved ions and found comparable, though not identical, ionic composition between brands.

In this handbook, the general term "rearing medium" may refer to either freshwater, standard, or seawater conditions, depending on the particular study design. Because our lab is interested in the evolution of stickleback osmoregulatory physiology, we sometimes split clutches and rear them at different salinities, thus producing "population-acclimation" groups. We achieve target salinities gradually, by daily increasing (or decreasing) the salinity level by 2-7 ppt. Stickleback embryos and larvae can easily acclimate to such small osmotic changes with no mortality.

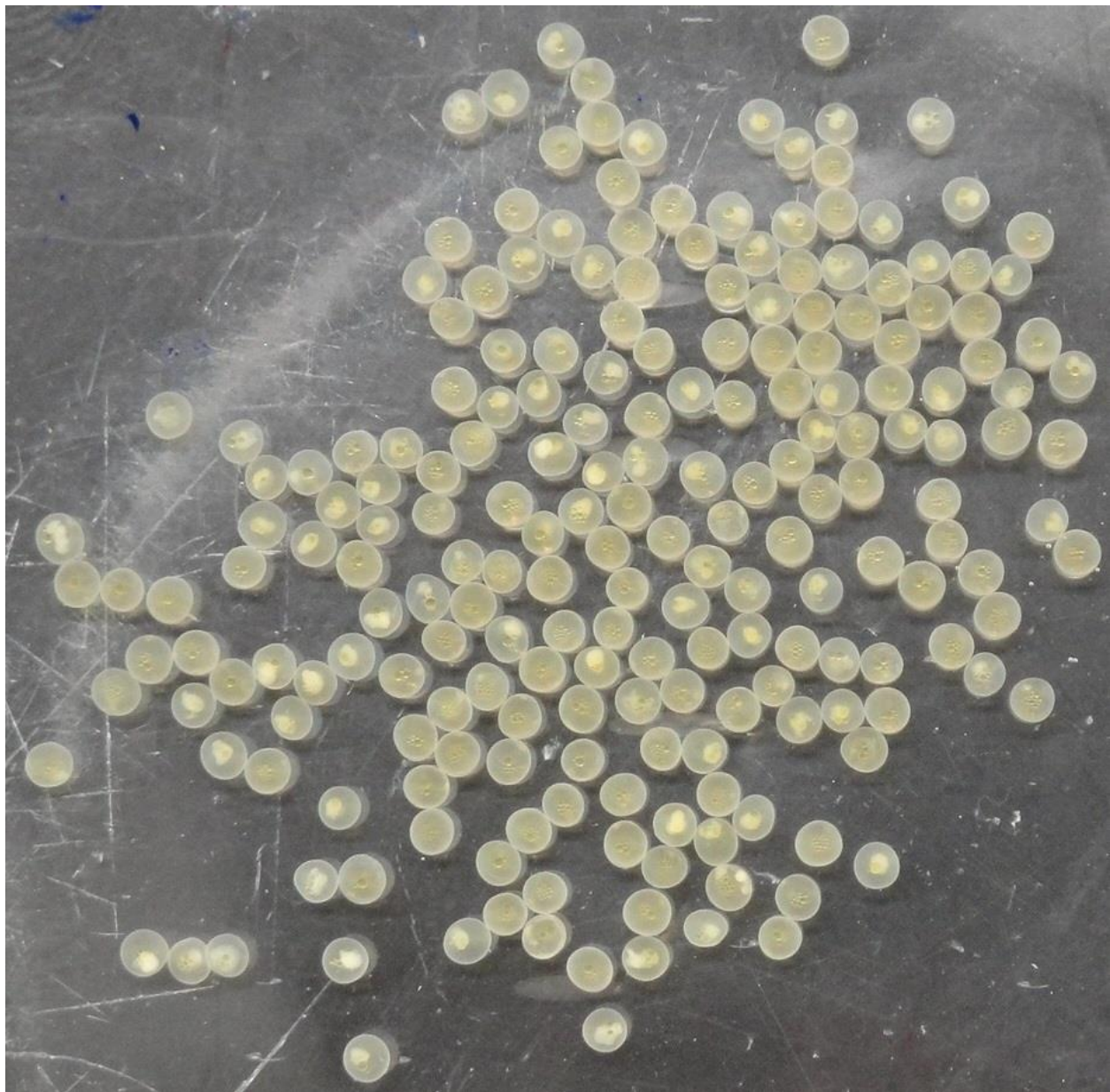
*Types of egg mortality.*— Embryo mortality can be detected macro and microscopically, depending on the source. Eggs should be inspected at low magnification under a dissecting microscope upon arrival, and all nonviable embryos should be removed as soon as possible to prevent spreading of infection and oxygen depletion from egg decomposition. Likewise, unfertilized eggs should also be removed, which are soft and almost transparent. It is common to have a small number of nonviable eggs in each clutch that aborted development, due a number of possible stressors prior to or during shipment. Nonviable embryos can be most readily distinguished by a large, bright smear of tan-colored protein near the center of the egg, making it look fried (Fig. 1). This mark is often flanked by yolk globules and should not be confused with the hazy animal pole disc or, if in later stages of development, an elongate outline of a developing embryo, which is why it is important to examine the clutches microscopically for an assessment of egg viability based on other characters.

At low magnification, dead embryos can be identified by the absence of a clear perivitelline space, which lies between the chorion and vitelline membrane. Another characteristic that differs between viable and nonviable embryos is the appearance of yolk globules. All eggs will contain a few yellow yolk globules adjacent the embryo, but they are more prominent in nonviable eggs from having coalesced into a large sphere. The speed and confidence at which you can pick out the bad eggs from the clutch comes with experience viewing them under the microscope. A collection of micrographs that I have taken of living versus dead eggs is available upon request.

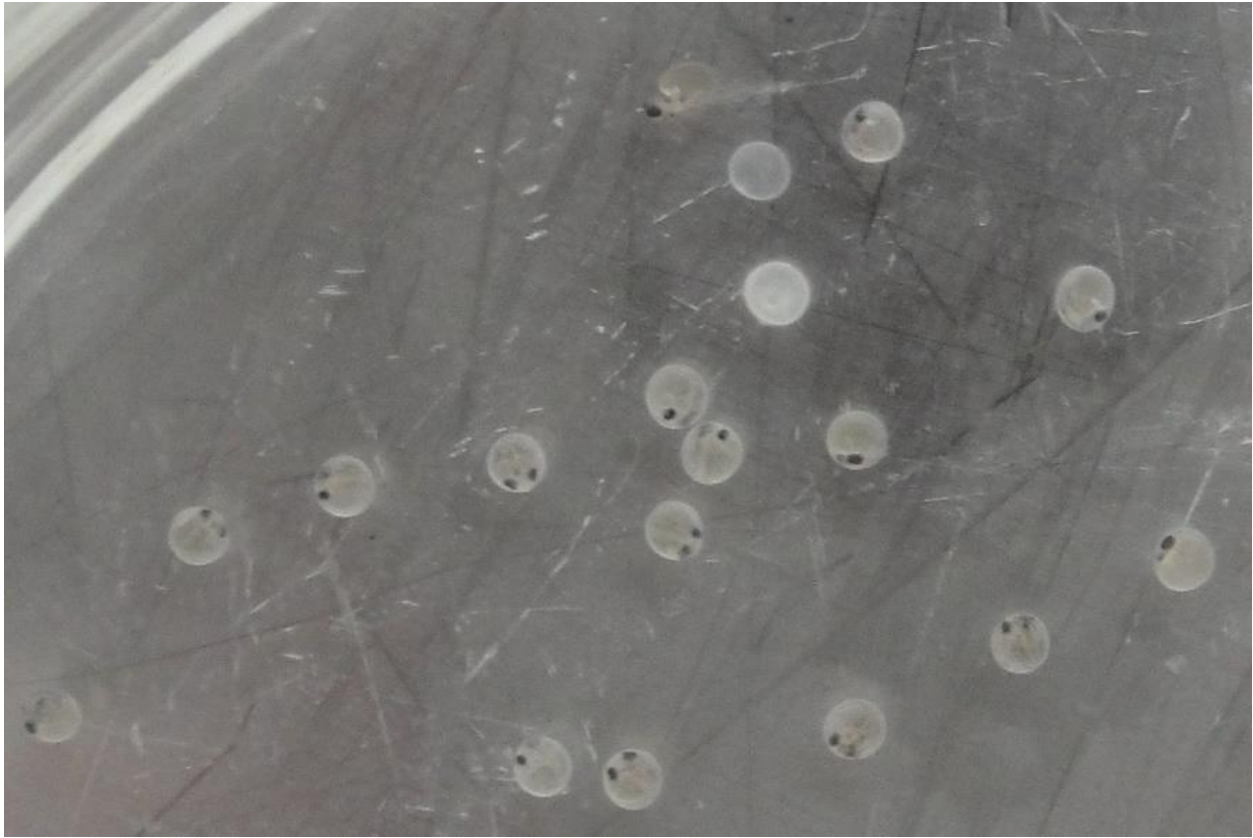
Some eggs appear viable, but have a greatly reduced yolk sac, which will be incapable of supporting embryonic development to hatching. Monitor these closely or remove outright. Developmental abnormalities of embryos, e.g., reduced eyes, are readily detectable in later stages. Contamination can also be detected with microscopy. Inspect dead eggs for microorganisms attached to or swimming near the chorion. Remove these egg mortalities, and water change and quarantine such dishes. Also check that salinity of the embryo medium is sufficient; increasing salinity level should combat outbreaks.

Usually, mortality is spotted easily macroscopically during routine inspections (Figs. 1, 2), and they become increasingly easier to spot over time as the surrounding viable embryos develop, making for a greater contrast between live and dead eggs. Embryos that die during more advanced "eyed" stages turn opaque white (Fig. 2). If you are unsure about the status of certain eggs, you can quarantine them and check them in the next day or two to confirm viability.





**Figure 1.** In this uncommonly poor cross, nonviable embryos are evidenced by the ‘fried’ look with an opaque patch of protein near their center, whereas viable eggs have numerous yolk globules and are translucently yellow throughout. (This clutch had just arrived ~1 d post-fertilization and had not been completely separated.)



**Figure 2.** Stickleback embryos in the eyed stage and a yolk-sac larva after about 5 d of development. Can you also identify the unfertilized egg and the nonviable egg?

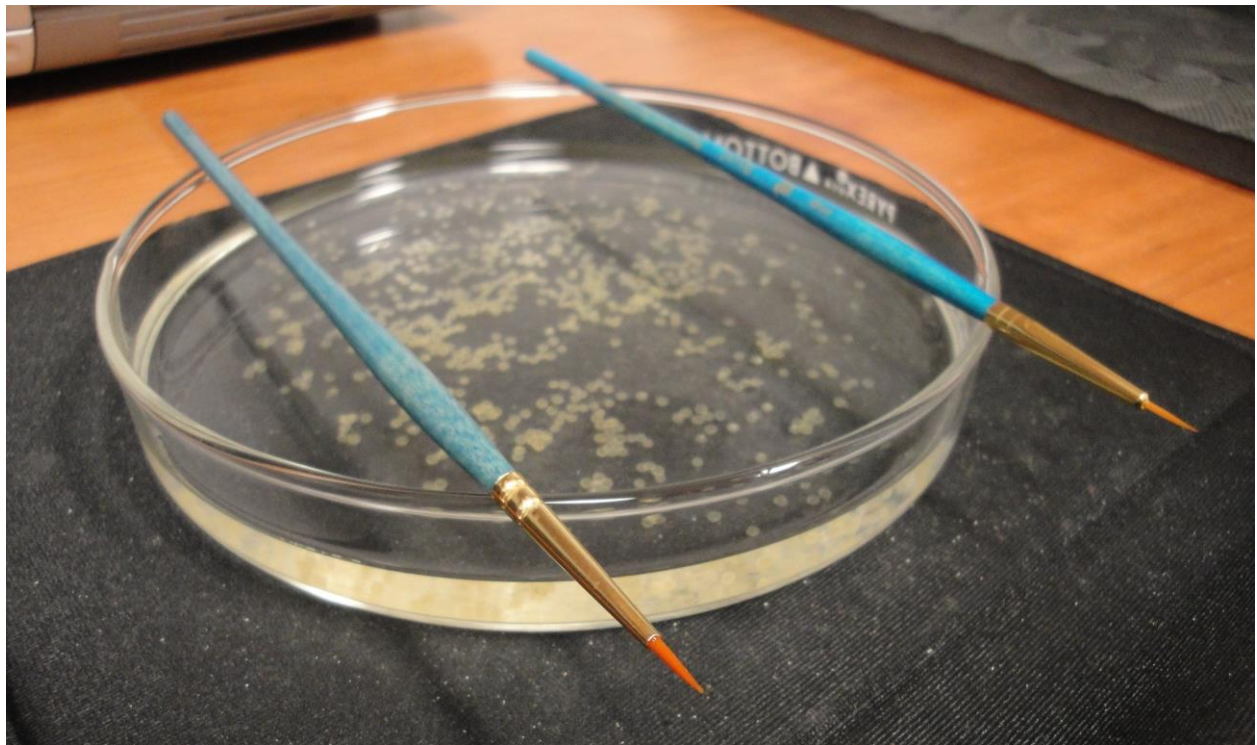
*Separating clutches.*— Although the embryos can survive for several days in the shipping container – as has been proven when packages have been mistakenly rerouted to other cities! – temperature changes and decomposition of dead embryos (which consumes oxygen) puts viable eggs at risk. To prevent likelihood of “skunking”, it is best to air out the clutches immediately upon arrival, even if you cannot separate and count the eggs until a later time.

1. Depending on the size of the cross, partially fill a sterile, 100 or 200 mm dia Petri dish with new medium. The larger dish provides more medium for mass crosses and lowers egg density.
2. Wearing gloves, remove the sealing tape and cap from a tube of embryos. A foul odor is an indicator of nonviable embryos in the clutch and/or old medium.
3. Have additional medium readily available to have on standby and fill a clean transfer pipet with some for squirt-flushing any eggs that might remain in the original clutch tube following transfer.
4. Carefully decant ~80% of the shipping medium into a wastewater container (e.g., a beaker or jar). Alternatively, you may wish to save this water for future analysis, such as salinity, dissolved oxygen, or pH. You can pool the shipping medium from multiple clutch tubes for these tests.
5. Gently swirl and empty the tube of embryos into the Petri dish. Flush out any stuck eggs using the transfer pipet.
6. Swirl the Petri dish to spread out the eggs. If separating the embryos at a later time, be sure to submerge the entire clump and place a lid on the dish to reduce evaporation, but remember that the



eggs must respire, so make sure not to seal the dish airtight! Alternatively, if you are ready to process the clutch, then proceed to the next step.

7. There are several tools that can be used to separate the water-hardened eggs from each other. Members of the Baker-Foster lab use a pair of professional dental picks to immobilize and tug eggs apart. Traditionally, we have made our own picks by bending the points of dissecting needles 90°. Still other researchers prefer to press the widened opening of a transfer pipet tip down between two eggs, cookie-cutter style. I recommend following the Bell lab for this job, which I was taught in 2012. They use detailing paint brushes (size 3/0) for separating adhered eggs (Fig. 3). The technique is similar to using metal picks, but is far gentler and reduces the chance of puncturing eggs. With a brush in each hand, apply slight downward pressure with the bristles on a clump of eggs and attempt to pull the eggs apart by pushing/rolling them in opposite directions.



**Figure 3.** Detailing paint brushes used to gently separate water-hardened stickleback embryos.

8. After the main clump of embryos has been broken up, you will be left with mostly egg doublets and triplets. Usually a doublet can be freed with just one hand by pressing the bristles down between the two eggs. Simultaneously rotating the brush while pressing will cause the bristles to twist and slide deeper through the juncture. If the eggs are well adhered, each brush can be inserted between the embryos from opposite directions and then pushed apart. Separating egg triplets are more difficult; try inserting the tip of a brush through the center of the triplet and flossing it between one of the fusions.
9. Use a modified transfer pipet (e.g., 3 mL) to relocate individual embryos or several at once. The bore of the pipet tip should first be cut so that its diameter is about 50% larger than the diameter of the egg. In other words, you want to be able to keep the eggs from scraping against the inner wall of the pipet as you transfer them.
10. While it is faster to separate eggs without the aid of the microscope, it is better in the long run to remove the nonviable eggs prior to plating the clutch, so you should examine each egg under

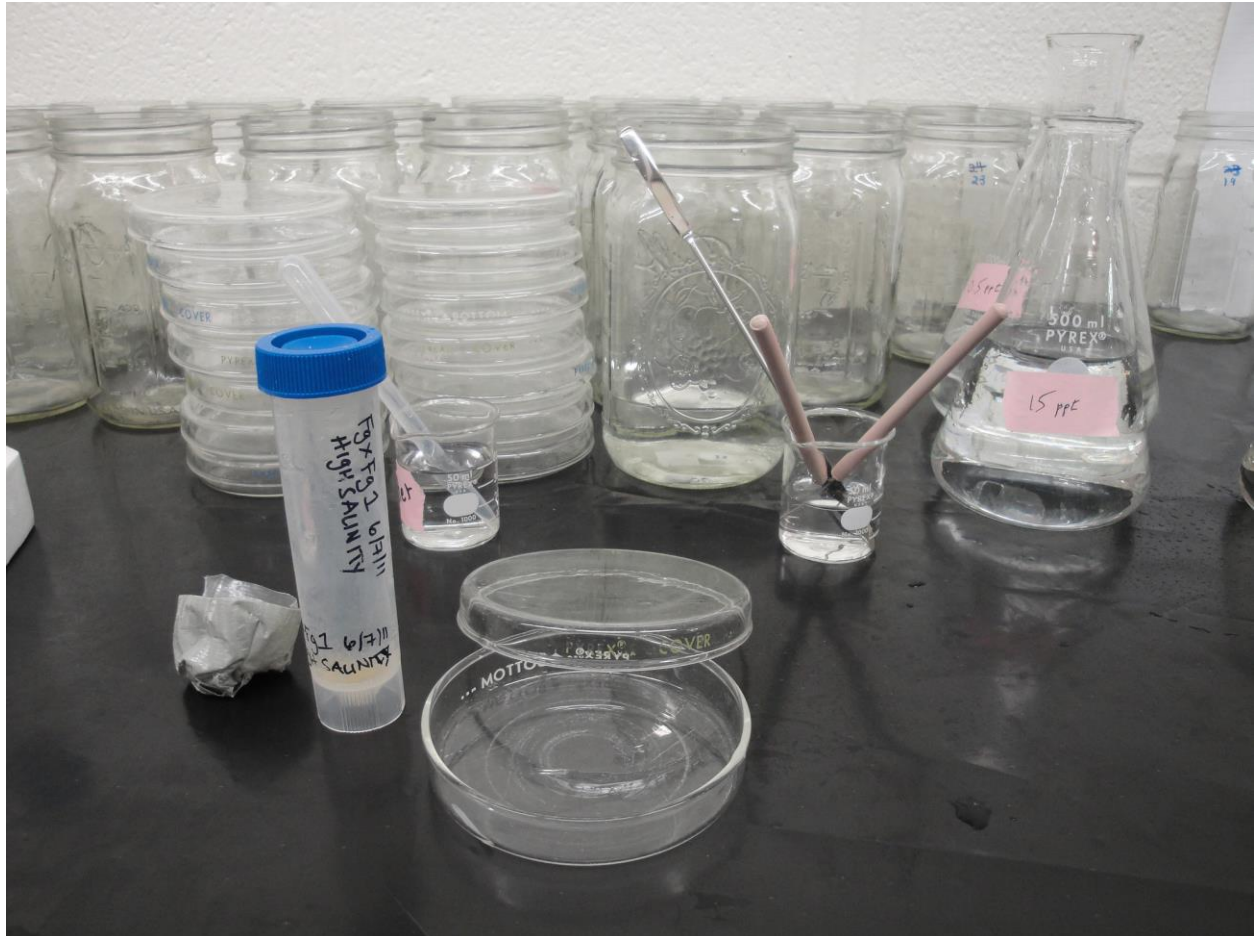
magnification while you are separating them and segregate the dead eggs into a corner of the dish. (A larger dish allows more room for picking clumps apart and dividing the clutch.)

11. Suck up nonviable eggs with a transfer pipet and discard them in the wastewater container. Transfer pipets carrying dead eggs should be sanitized regularly by flushing in 70% ethanol. After purging the pipet by squeezing the bulb several times in a small beaker of ethanol, rinse thoroughly by flushing the pipet in a large beaker of RO water so as to not expose the eggs to any ethanol! Paint brushes should similarly be cleaned between clutches to prevent the spread of any disease among families or populations. Instruments may be stored temporarily in the ethanol. Dump and replace the RO periodically with use.

*Plating embryos.*— Once all of the eggs have been separated, you must “plate them”, i.e., divide the clutch into low-density subpopulations of no more than 30 embryos per 100-mm dia Petri dishes. Splitting up the clutch improves water quality and hedges against epidemic losses. Most labs use disposable plastic Petri dishes, but we have six aluminum boxes of glass ones that we autoclave and reuse.

1. Acquire a stack of sterile glass Petri dishes and an Erlenmeyer flask of 3 ppt water (or custom salinity). Prefill a Petri dish with rearing medium to about 50% capacity, allowing for the additional pipetsful of water that will be added along with the transferred embryos. A typical egg-plating station is shown in Figure 4.
2. Add a total of **25 viable embryos** into the Petri dish using a clean transfer pipet (no more than 30). The pipet can hold a string of eggs, enabling you to move several at a time. You can expel groups of embryos in different locations of the plate to keep track.
3. Depending on the experimental design, you may wish to enumerate and pre-assign all of the embryos in a clutch destined for a particular salinity treatment or trial by first arranging the eggs in clusters within the plate and then dispensing them.
4. Following egg transfer, examine the final water level of the dish, which should be not more than 75% full. Overfilling the Petri dishes will lead to water sticking to the underside rim of the lid when jostled or tilted, which seals off the plate from the air, preventing gas exchange. Remove excess water as needed.
5. With plate lidded, gently swirl in a “figure-8” to spread out the embryos.
6. Affix a clutch and dish ID sticker to the glass lid and tape over it to (partially) waterproof. Fold one edge of the tape so that you can more easily remove the label in the future. (Alternatively, you could affix the sticker label to the underside of the bottom of the Petri dish, but these easily get wet after water changes.) If using disposable plastic Petri dishes, simply label the bottom and the lid with marker.
7. For each plate, write the number of embryos on the glass lid using a dry erase marker, so that the tally can be easily erased and updated.
8. Repeat Steps 1-7 until the initial clutch plate has been emptied. The final plate(s) in the series may be shorthanded.
9. For each clutch, record fertilization date, number of Petri dishes and viable embryos, as well as additional information on the Clutch Inventory form (Appendix B1).

10. Neatly arrange all of the Petri dishes on the plastic tables in the fish room, usually around the perimeter for easier inspection. Leave enough space between each for lid removal during inspections.
11. If personnel are available, the plating operation can be streamlined through task specialization. Seated egg “pickers” can make the plates, while being supported by egg “runners” who refill flasks with embryo medium, label the plates, and arrange them on the tables.



**Figure 4.** Stickleback egg plating station. The duct tape seal has been removed from the skirted clutch tube (embryos are the tan mass on bottom of tube) and a sterile glass Petri dish is ready to receive the clutch. Bent dissecting probes and a steel spatula are sanitizing in ethanol, while the shortened transfer pipet sits in RO water. Erlenmeyer flasks contain embryo medium of various salinities.

*Water-changing Petri dishes.*— Taking no chances with water quality, embryos should have their medium replaced daily. The water change also provides an opportunity to inspect each dish for developmental progress or new mortalities. Daily water changes should continue post-hatch until the larvae are transferred to jars.

As mortality reduces egg density, you may pipet-transfer embryos occupying dishes with few remaining survivors into similar Petri dishes of low density to restore uniform groups of ~25 eggs/plate, which will prevent future density-dependent variability in growth among hatchlings and also reduce workload. Only reallocate embryos if you do not suspect disease. Otherwise, quarantine the eggs and monitor their development closely.

1. Before refreshing medium, scan all of the Petri dishes. If embryo mortality is high, it is easier to first remove all of the dead eggs with a transfer pipet during an initial pass through the dishes, discarding them in a wastewater container, such as a large glass bowl or 2-gallon bucket. To prevent cross-contamination, sterilize the pipet periodically in ethanol and then rinse it in RO water.
2. Consolidate plates of low-density embryos as needed (within treatments).
3. In preparation of the water change, situate the wastewater container in a centralized location on the table of embryo dishes. Also make ready a 0.5 or 1 L Erlenmeyer flask with new 3 ppt (or custom) medium and a modified turkey baster for removing the old medium (Fig. 5).
4. Remove the lid from a Petri dish containing embryos and set it down adjacent to the dish. During this step, be sure not to confuse the labeled lids in the array of dishes. To help stay organized, all or blocks of dishes designated for a water change can be subtly distinguished by partially removing their lids; close the lids of all dishes that have been media-refreshed (Fig. 6).
5. Grasp the outside of the plate bottom with one hand and hold it on edge slightly. With your other hand, use the modified baster to withdraw any dead eggs along with the old medium. As the water level drops, increase the angle of the dish to pool the water in the bottom corner of the plate, allowing you to remove more water with the baster, ~80-90% in all. Alternatively, if space is cramped, then you can pick up the plate to about chest height and remove the medium with the baster in a similar manner (Fig. 6). This method is easier on your back and also brings your eyes closer to the embryos, making them easier to observe and avoid with the baster.
6. Most of the embryos will be sunk on the bottom, but watch out for floating embryos; tap these down to prevent spillage. As the plate is tilted, many eggs will roll downhill and remain submerged, but it is okay if some of the embryos remain stuck in-place on the bottom of the plate and are temporarily exposed to the air during the water change.
7. Check baster to ensure no viable embryos were accidentally taken up, then expel the baster's contents into the wastewater container.
8. Refill the dish to ~75% capacity by gently pouring in new medium from the Erlenmeyer flask. The pouring action will further aerate the water.
9. Return the lid, gently swirl to spread out the eggs, and update the embryo count for the plate by changing the tally written on the lid with a dry erase marker.
10. Proceed to the next dishes. Discard wastewater and refill flask(s) with new medium as needed.
11. After hatching, use a baster or transfer pipet to remove all egg shells (chorions) from the plates, as they can decompose and can become substrate for microbes. As with dead embryo removal, it may

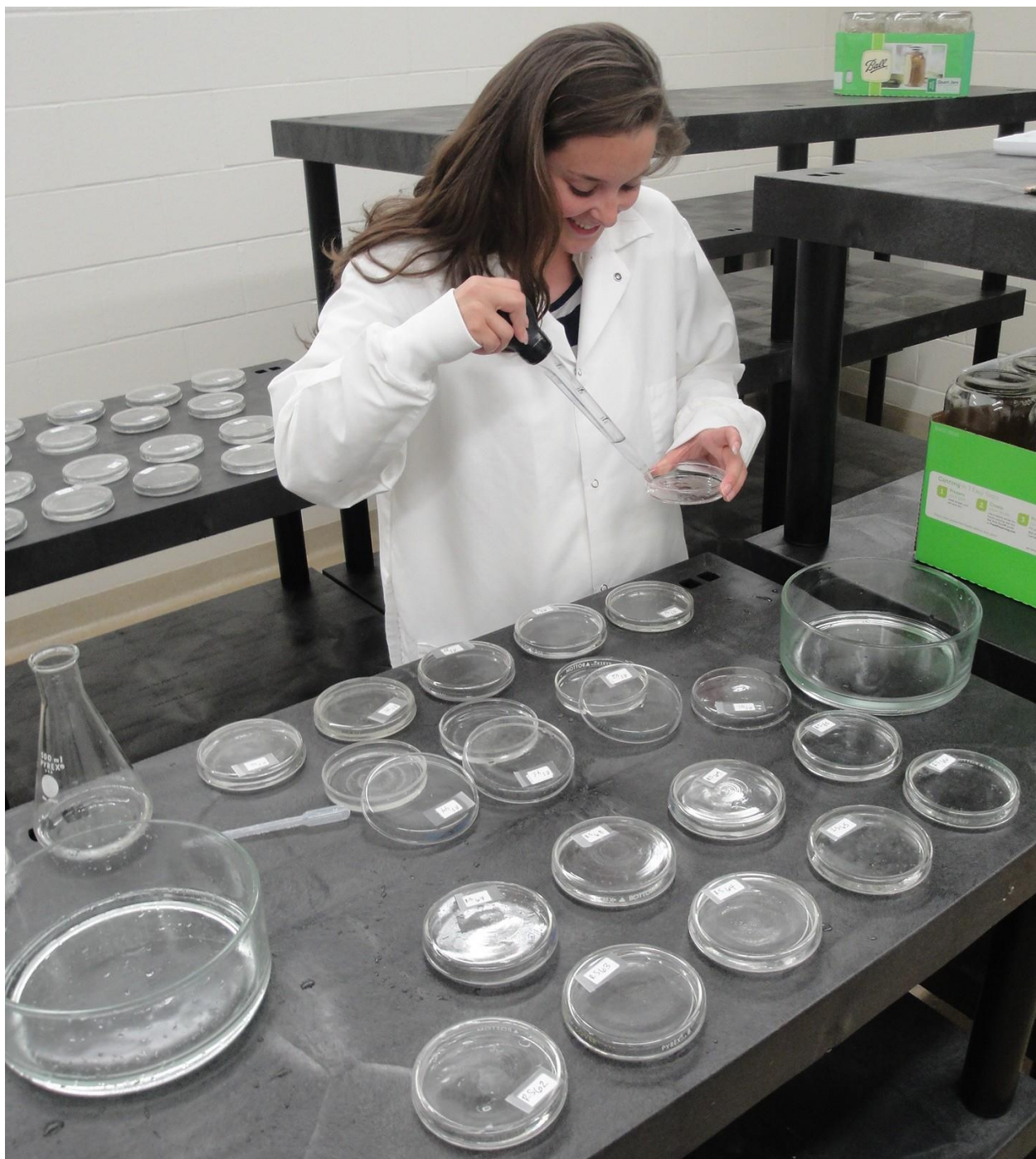
be easier to take out all of the grey shells before the next round of water changes. *Note:* If working in a team, one assistant can remove the shells while the other follows, changing the water.

12. A small percentage of healthy-looking hatchlings cannot break free from their shells, which remain attached to the yolk sac, or worse, the head and pectoral fins. Assisted shell removal can be performed at low magnification using the 3/0 paint brush. Usually the larvae can wriggle free once the shell is held fast by the bristles. However, “helmet heads” are harder to deal with. Try pressing down on a flap of broken chorion with the brush in one hand, and with a transfer pipet in the other hand placed by its head, gently encourage the stickleback to swim away from the brush. *Note:* bent dissecting needles can be used to remove chorions, but these can damage the fish.
13. Exercise care to not suck up fast-darting larvae into the baster during water changes. It is best to navigate around them by moving the flexible tubing tip of the baster along the bottom of the dish as you draw up water. The larvae will often stay out of the way of the baster by settling down at the lower, wet corner of the dish. Nonetheless, you will not be able to remove exactly the same amount water from each dish due to differences in cooperativeness of the larvae with this process.
14. Record when water changes are performed on the Fish Room Feeding & Maintenance log (Appendix B2).



**Figure 5.** Turkey baster with electrical tape wrap (red) and segment of 1/8” soft aquarium tubing.





**Figure 6.** Nicolle Murphy demonstrates how to use a baster to change the water in glass Petri dishes containing stickleback embryos and yolk-sac larvae. The large glass bowl collects the waste water and the flask contains replacement medium. A transfer pipet can be used for more targeted removal of dead eggs or shells. Note the row of dishes that have yet to be refreshed are partially uncovered.

### III. Caring for larvae

*The first feeding.*— Stickleback hatchlings possess a sizeable yolk sac that will be absorbed over the next 2-3 d. Although they will not actively hunt for food before that time, John Baker and others argue that it is nonetheless beneficial to expose them early to live prey, typically brine shrimp nauplii (see Feeding Protocol for nauseatingly in-depth instructions on culturing brine shrimp).

1. Prior to the day's water change, scatter a few drops of brine shrimp suspension into a Petri dish of larvae and observe how the stickleback larvae respond.
2. Return after a few minutes and see if larvae are chasing or feeding. If nauplii have been consumed, add a few more drops of the suspension.
3. If no feeding has been witnessed after additional exposure time, water change the plates, removing as many uneaten nauplii as possible.
4. When the larvae readily eat, administer 1-2 small doses of nauplii per day and prepare to transfer the sticklebacks to jars.

*Transferring larvae from plates to jars.*— After one or two days of successful feeding, the stickleback larvae can be transferred into glass mason jars (Fig. 7). Because this is such a larger volume of water, the medium will remain fresher longer and does not need to be changed out on that day. Do not feed the larvae the morning of the transfer; wait until after they have gone into the jars. Observing fish feeding immediately following the transfer is a sign that they are acclimating well.

1. Line up clean quart-sized Ball mason jars, one jar per Petri dish of larvae, and prefill to at least 400 mL with rearing water. *Note:* The larger, ½ gallon jars can be used to house the fish, though these are typically reserved for salinity challenges (Fig. 7). We have >130 jars in both sizes.
2. Fill another jar with medium for flushing larvae from plates. You can rest the baster in this jar.
3. Remove lid of a Petri dish, grasp the outside of the dish with one hand, and carry it over the mouth of the jar. With your other hand, fill the modified baster with 3 ppt.
4. Tilt the plate to begin to pour out the water and larvae. If needed, swirl the plate to knock any stranded larvae to the lower corner. Pour with increasing acceleration as you increase the pouring angle.
5. When the plate nears a vertical orientation and beyond, begin to flush out remaining larvae with the baster. Flush in a zig-zag pattern from top to bottom. Continue to invert the plate during flushing.
6. Give the plate a final drip-shake and inspect it to make sure all larvae transferred.
7. Remove ID sticker from the lid of the Petri dish and affix it to the upper portion of the jar. Write the census on the jar with dry erase marker. The side of the jar with graduation lines should be facing forward.
8. **OPTIONAL:** Add habitat enrichment to the jars. Common types include a plastic plant sprig and/or a bio-brick. However, I do not recommend adding objects to jars: the plants can hide mortalities of young fish and block consumption of floating food, leading to decomposition, while sunken objects shift when the jar is handled and can spook or even strike fish.
9. Clean and stack the empty Petri dishes. *Note:* Be a good lab mate and autoclave the glass Petri dishes after use. Stack them into aluminum boxes and place a piece of autoclave tape over the lid of each box. Run the autoclave basic dry glassware settings. For the third floor autoclave in the Pharmacy-Biology Building, use "Cycle:02-Unwrap", which sterilizes for 10 min at 250°F and

dries fast (exhaust mode: 1) for 20 min, which helps dry the inside of the dishes. For additional drying back in the lab, remove the aluminum lids of the boxes for a few days.

10. **FEEDING:** Administer small amounts of concentrated brine shrimp nauplii to the jars 1-3x per day and monitor consumption rates. Add more if fish “clear” the food. Record mealtimes on the Appendix B2 form. *Note:* Do not overfeed in this small volume of water, but there is a trade-off here because that these fish are still relatively poor swimmers and may have difficulty hunting low-density nauplii - underfeeding may be worse.



**Figure 7.** Wide-mouth glass mason jars are the most widely used item in stickleback husbandry. Pint jars are used to prepare food, quart jars house larvae, while the half-gallon (pictured) is the best size for salinity challenge experiments on juveniles.

*Removing dead fish.*— As with most organisms, stickleback mortality rate will decrease with size/age. Once the fish reach 2-3 cm standard length, daily mortality should be negligible. Laboratory-raised sticklebacks have high survival, but egg and juvenile mortalities will nonetheless occur and should be dealt with promptly. Keep an accurate census and watch for spikes in mortality rate as a possible indicator of poor water quality or the onset of an epidemic, which you want to prevent from spreading throughout the colony.

To reduce density-dependent differences in stickleback growth rates, jars containing less than ~10 total individuals should be combined with other low density jars to restore approximate initial densities of 25 larvae per container. This will also decrease your workload by reducing the number of fish containers to inspect, feed, and water change.



1. Remove all stickleback mortalities and record them on our fish room log by treatment-population (Appendix B3). For reared fish not used in an experiment, family-wise mortality records are not essential for this form. Small larvae can be sucked into the modified baster; remove larger fish with 12" forceps.
2. Do not forget to also mark the total mortalities for the day as "deductions" on the OAC (Office of Animal Care) form.
3. Keep a working census by writing the current number of individuals on the glass of each container of fish with a dry erase marker.
4. Reallocate fish as needed to achieve approximately uniform densities among containers.
5. Dead embryos and hatchlings can be pooled in a waste container, bleached, and sinked. However, juveniles that are bony and large enough to be picked up with forceps should be collected and placed in a large, labeled re-sealable plastic bag, to be kept in the Aquatic Facility's community chest freezer.
6. When the project is over and you are moving out of the Aquatics Facility, temporarily store the bag of dead fish in the Schultz lab freezer. Then, sign out this biological waste and get a key in the Biology Central Services Office (Torrey Life Sciences 161) to unlock the shed on the ground level on the south side of TLS, between the elevator and the greenhouse. Deposit the bag in one of the trash receptacles for later pick-up and incineration.

*Water changing fish jars.*— Rearing jars are more convenient to work with than are Petri dishes, and their greater water volume makes a daily water change less critical. However, uneaten food and excrement is still a problem, so it is best to minimize the amount of time in this container type regardless, especially in the case of mass crosses, where families of sticklebacks do not need to be kept segregated. In short, quart jars provide 4-7 day interim housing until 10-gallon aquaria are made ready to house juveniles from multiple jars.

1. Prepare new fish water in barrels to desired salinity(ies) and make sure that it is near ambient temperature before proceeding.
2. Inspect the jars and remove mortalities with the baster or long forceps (and update the census). If necessary, combine low-density, intra-cohort jars to reduce density-dependent size variation among sticklebacks.
3. Pour out at least half of the water in the jar into a 5-gallon wastewater bucket or 20-gallon barrel (also on a trolley), as determined by following the graduations on jar. Fish *should* settle to the bottom of the jar, but taking no chances, pour over a 4" dip net to catch any escapees. Reduce the pocket of the dip net by wrapping it partially around the frame and securing it there with a rubber band on the left and right side. A flattened pocket greatly facilitates return of the fish. *Note:* the best instrument to pick-up a fish that has flopped onto the table or floor is a plastic spoon.
4. Return the drawn-down jar to its original place on the table and repeat the above steps for all jars.
5. Insert submersible pump into a water barrel (Fig. 8). Turn on the submersible pump and refill the jars, preferably in the same order that they were drained. Adjust the valve to create a low flow rate and aim the hose at the side wall of the jar so that fish are minimally disturbed by an indirect stream. Increase the water level in the jars with successive water changes to accommodate the growing fish. Doubling the water volume can count as a water change. *Note:* if no pump is available, fill jars by slowly pouring from an Erlenmeyer flask or beaker.

6. When finished, disconnect power to the submersible pump, open the valve completely to drain it after removing it from the barrel to let dry. *Notes:* Pumps can be cleaned by running them in a barrel of RO. One pump can be reserved for seawater; the other fresh. If changing salinities, purge the pump by discarding the first few seconds-worth of new water.
7. Record when water changes are performed on the Fish Room Feeding & Maintenance log (Appendix B2).



**Figure 8.** One of two 350 gph submersible pumps attached to ½” barbed fittings and vinyl tubing. The shorter intake hose sucks water from the bottom of the barrel, which will then exit through the long hose, regulated by a ball valve.

*Transferring larvae from jars to tanks.*— We move stickleback larvae into 10-gallon aquaria after only 4-7 d in the jars. Compared to the Baker lab, this is at a very young age, but we do so because our fish room has limited space for holding dozens of rearing jars plus all of the experimental, salinity challenge jars. Secondly, water-changing and feeding jars is also time-consuming and there is always the risk of water quality quickly deteriorating; freshwater treatments (i.e.,  $\leq 0.5$  ppt) are especially susceptible. Even though a partially-filled 10-gallon aquarium provides an exceedingly large volume of water for 1-cm long larvae, they are able swim in larger groups and can successfully forage brine shrimp.

1. Plan a tank layout for housing the colony. A randomized design controls for possible confounding effects of room-orientation, but may be prone to costly mistakes. Consider grouping clutches/families by population and/or by common rearing salinities to prevent accidental cross-contamination.
2. Position rows of ‘double-stacked’ black plastic modular tables along the wall(s) of the room. The long sides of 10-gallon aquaria fit the width of these tables, each of which can hold six.
3. To test for leaks, fill aquaria with rearing water and mark the water line with dry erase marker. Let stand overnight and check for a drop in water level the following day. Small cracks can be repaired with aquarium-safe silicone. I recommend 10-oz caulking gun tubes of Marineland Aquarium Sealant. Seepage through joints in the frame is difficult to pinpoint and may require complete recaulking. *Note:* Apply caulking bead to dry glass and shape using a gloved finger or spatula



dipped in isopropanol. Allow silicone to cure, preferably in a fume hood, for at least 24 hrs before wet-testing.

4. Once the tanks have proven they can hold water and are ready to receive larvae, remove excess water from the aquaria so that they are about one third full. More water will be added from several quart jars during fish transfer (see next section).
5. **OPTIONAL:** Add a few cobblestones to the tanks for habitat enrichment. (The rocks make loud noises when they hit the bottom glass, so it is better to add them prior to stocking.)
6. Affix a vinyl, static-cling ID sticker to the upper corner of the glass. The bottom of these 1.5" tall stickers can be used to eventually mark the maximum water level for the tank (Fig. 9). For now, the aquaria can be 1/3-1/2 full for these small larvae; gradually increase the water level as the fish grow in step with water changes.
7. Inspect jars, remove all mortalities, and update the census. Then with a clean hand or long forceps, remove any structures from inside the jar.
8. Arrange the jars by roughly equivalent numbers into groups that will share aquaria. Initial stocking densities of larval stickleback can be 100-200 individuals per tank. Again, do not feed the fish just prior to transfer; wait to feed until after they are in tanks and then monitor feeding behavior.
9. Pick up a jar of stickleback and partially submerge it in the destination tank. Slowly turn jar 90° so that the mouth is halfway submerged and the waters mix. Gently invert the jar an additional 45° while pulling back so that the entire contents of the jar are emptied (the jar water will help condition the tank). *Note:* Completely submerging the mouth of the jar will trap air and disturb the water when you lift the jar out.
10. Record the total number of stocked fish on the front aquarium glass with a dry erase marker. Continue to update this census.
11. **FEEDING:** With filters turned off, add food to tanks 2-3x per day, sensu Feeding Protocol. Record mealtimes and amount fed on the Appendix B2 form.
12. After several weeks of growth in the aquaria, when the water is at full height, add additional structure for the fish. For example, I designed a 4" diameter black, ABS pipe with plastic plant sprigs inserted into the top through holes (Fig. 9). *Note:* immature sticklebacks will swim through the black pipe, but are not territorial and do not seek cover inside of it.

*Redistributing fish among tanks.*— Decrease tank density in proportion with fish growth. Removing fish for experiments or growth rate is a form of culling that will prevent overcrowding and improve water quality. However, if growth outpaces removal, the fish should be split between two aquaria. Again, do not feed the fish hours prior to any container transfer (although fasting the sticklebacks matters less when they are large).

1. Prepare additional 10-gallon aquaria to receive fish. Lay a clipboard or bucket lid over adjacent tanks to prevent accidental introduction of fish into the wrong tanks. Mixing stickleback populations destroys any prospects of experimentation and many populations share phenotype, so you will not be able to tell which is which!
2. Remove approximately half of the tank's population using two dip nets. Capture fish by herding them with a smaller dip net into a large, blue Z-Mod net. If the destination tank is nearby, directly release the catch into it the new tank by net; if it is further away, then you may instead fill a 2-gallon bucket with water *from that tank* and pour out the bucket of fish (otherwise you would

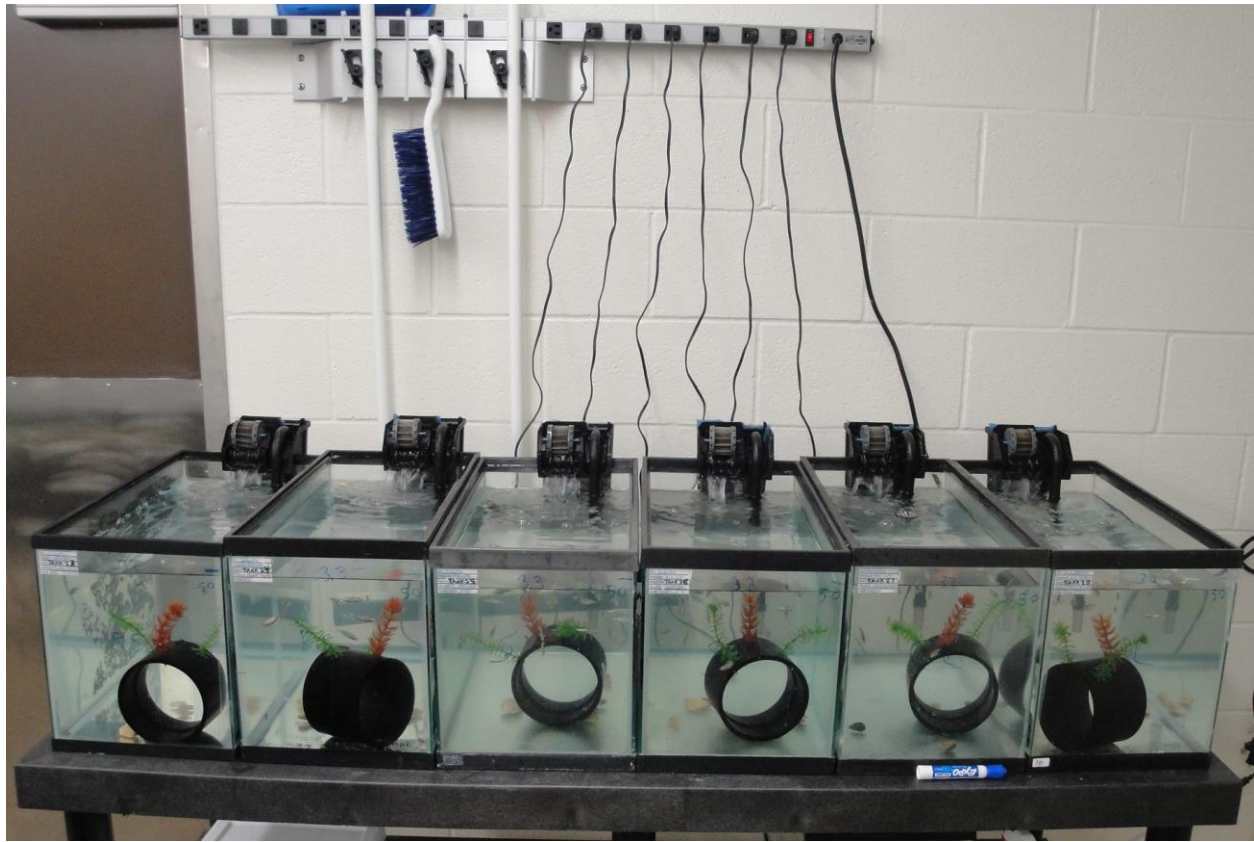
increase the water level). *Note:* Wheeled carts are helpful for transporting buckets of fish across the room. You should try to keep a running tally of how many fish you transfer, but this slows down the job and may be unnecessary if fish are abundant. Keeping exact counts of larval fish is almost impossible.

*Aquarium filtration.*— The purpose of the clip-on aquarium pump is to filter the water as well as to aerate, which it does by disturbing the water's surface by its waterfall outflow (Fig. 9). Continually running filters is nonessential for larval fish, but dissolved oxygen can be depleted by larger juveniles. Once the fish have demonstrated that they can escape the pull of the pump's intake, we typically leave the filters on by default as a safeguard to maintain oxygen saturation.

In general, the rate of tank evaporation is a function of RH and the duration of running time of the filter. Filters will increase rate of evaporation, which in turn decreases water level and lowers temperature of the tank. Noticeable evaporative cooling occurs in the winter because the room's low relative humidity at that time. Be aware that the evaporative decrease in water level concomitantly increases tank salinity, which should be corrected during the regularly scheduled water changes by refilling the tank with water that is slightly less than 3 ppt (or other target) salinity.

In cases where sticklebacks are reared at different acclimation salinities, the spray from the filters can affect the salinity of neighboring tanks. Salt spray, in particular, can "contaminate" freshwater treatments, which is why you should not position the two salinities adjacent to one another.

1. Hang a Penguin or Whisper filter on the rear, short-side of each aquaria. Filter media includes Fluval bio-bricks, a fragment of clam shell (to increase calcium), and the wool pad insert, which is filled with activated charcoal. Plug the filters into power strips so that they are easier to turn off. Power strips must be fastened (e.g., with cable ties) at a position higher than the aquaria (Fig. 9).
2. Use small cable ties to secure a sleeve of white, 2-mm mesh netting over the intake strainer as a precaution to prevent larval fish from getting sucked up the filter. *Note:* this mesh screen will get clogged with tank particulates and should be removed along with the pipe and cleaned periodically.
3. Prime the reservoirs and run the pumps to ensure their proper working order. It is best to test them in the absence of fish. *Note:* Common pump failures are a jammed impeller and a loose intake pipe that sucks air. Bio-wheel axles may also be broken, which prevents them from spinning.
4. Filters do not need to be turned on at all for the first 7-10 days after the tiny larvae are introduced to the aquaria. Afterwards, check that the fry can easily avoid the filter. If mortalities are found on the intake mesh, then it may be too early to run the pumps - though cause of death cannot be certain because dead fish could be sucked into the screen after the fact.



**Figure 9.** Six aquaria arranged on a single, two-tier plastic table. Each tank has an ID sticker and a waterfall filter. A dry erase marker can mark number of fish, water level, salinity, etc., on the front glass. Rocks, plant, and a pipe comprise the habitat enrichment. No lids are needed because the sticklebacks will not jump.

*Water quality monitoring.*— The typical room that we rent in the Aquatics Facility does not have a filtration-circulation system. Thus, manual water changes are required maintain water quality in the aquaria. Ammonia levels will build despite filter media or the presence of nitrifying cultures, especially as the sticklebacks grow and consume more food. (I have not seen a reduction in ammonia concentration in tanks treated with EasyStart compared to control tanks.) Sticklebacks are tolerant of moderate-high concentrations of ammonia, so long as the pH is neutral or basic (another benefit of adding Instant Ocean to rearing medium). However, older water tends to become more acidic as wastes accumulate, which increases ammonium ( $\text{NH}_4^+$ ) levels.

Water quality tests are to be performed on a periodic basis on randomly selected jars or tanks or used to diagnose any signs of fish stress. The YSI-85 meter monitors salinity, conductivity, temperature and dissolved oxygen. For fresh and brackish aquaria, pH, ammonia/ammonium, nitrite, and nitrate can be colorimetrically tested with the Freshwater Master Test Kit by Aquarium Pharmaceuticals, Inc, following their instructions. This kit also works for saltwater tanks, except you must read the ammonia value from a different card and use the High Range pH bottle to measure the more basic pH. The pH can be more precisely measured with the digital handheld pH meter (by HM). Enter container ID along with the values on the Water Quality data sheet (Appendix B4).

*Water changing aquaria.*— To ensure sticklebacks used in experiments are coming from clean water, regularly schedule weekly tank water changes. Always replace water after, rather than before, a feeding so that no food will be removed and the new water will remain cleaner. If there are too many aquaria than can be water-changed at one time, divide them into blocks and establish a maintenance schedule where each block is cleaned in rotation, which can be documented in Appendix B2.

1. Calculate approximately how much new water is needed. If fish are small, wastes are low, and water is clean, a 50% water change is adequate. However, if ammonia is high and much solid wastes are visible, an 80% water change will be needed to effectively decrease ammonia levels. The number of gallons replaced in each 10-gallon aquarium obviously affects how much new water must be mixed. It also will determine the salinity of this new water, which must be adjusted to achieve a target treatment salinity, as explained in the next step.
2. Measure the salinity of a few representative tanks with the YSI-85 meter. All tanks should be at or slightly greater than the set-point, due to evaporation. If tanks differ in salinity, you can keep them straight by marking the salinity on the glass with a dry erase marker. The higher the salinity, the lower the salt concentration needed for the replacement water. Similarly, the lower the percentage of tank water you replace, the lower the replacement water's salinity needs to be to achieve target salt concentration. For example in a 3 ppt rearing tanks, a 50% water change of a 3.2 ppt tank requires ~5 gal of 2.8 ppt.
3. Prepare new rearing medium in a 44-gallon Brute barrel(s) by mixing Instant Ocean with RO water as previously described. The salinity will vary depending on the results from Step 2 above (e.g., 2.5 - 3 ppt for standard rearing conditions). The barrels can differ in salinity as needed for different types of tanks. Check that the water temperature in the barrel is nearly equivalent to that of the tanks before proceeding.
4. Turn off filters and remove the 4" pipe structures, which can roll during the drawdown and interfere with vacuuming.
5. Remove the wool filter pad cartridges and clean them in the sink by spraying them with pressurized RO water in the sink. (Press your thumb over the end of the RO tubing.) Shake the pads dry and return them.
6. OPTIONAL: If mesh screens on the strainer heads of the filters appear clogged, remove intake pipe with the mesh, check for any mortalities, and pressure wash with RO water in the sink. You can also flush debris by running RO through the pipe towards the screen, in reverse of the natural direction of flow. *Note:* When the fish are much too large to fit in the strainer head, remove the mesh covers, clean, and store them.
7. OPTIONAL: If aquarium glass appears to be coated in algae/biofilm, clean the inside glass walls of the aquarium with the long-handled algae scrubbing brush. Rinse brush with RO periodically between tanks. *Note:* Kitchen scouring pads or sponges may contain antimicrobial chemicals that are dangerous to fish!
8. Prime a gravel vacuum in the aquarium by squeezing the bulb several times while the long cylindrical neck is fully submerged. This disturbs the water, so prime in a corner away from the fish. Once the gravel vac is primed, water automatically will begin to flow out of the tank. It is easiest to let the outflow hose hang down onto the floor and let the wastewater run out towards the gutter in the corner of the room. *Note:* Due to the risk of sucking poor-swimming larvae, cover the head of the gravel vac with a layer of 2-mm mesh nylon using a rubber band (Fig. 10).

9. The powerful suction of the vacuum allows you to remove solid wastes during the water removal. Suck up all fecal matter, uneaten food items, and sloughed algae, which can pollute the water. This should take at least the first third of the tank's water to complete. *Note:* Do not use the nylon cover once the fish are large enough to escape the pull of the vacuum, since the mesh impedes removal of large debris.
10. When all particulates have been cleared, continue to siphon out water from the aquarium down to desired level by angling the mouth of the cylinder at a bottom corner of the tank. The more vertical the position of the cylinder, the more water that can be drained before the siphon is lost when it sucks air.
11. When the draw-down is almost complete, deftly lift the head of the gravel vacuum and out of the tank so that all of its water will not empty back into the aquarium once the siphon breaks.
12. Flush the gravel vacuum with RO water periodically to keep it clean.
13. Refill aquaria to high-water mark with barrel water by means of the submersible pump. Aim the flow at a side wall away from the fish to reduce disturbance.
14. OPTIONAL: If additional personnel are available, establish specialized roles for a tank 'Drainer' and a 'Filler'. The Filler, who must wait for the Drainer, can also assist by removing habitat structures, cleaning filter pads, and scrubbing the glass.
15. When finished, return the black pipe structures to the tanks and turn the filters on again.
16. OPTIONAL: Perform a quality control check on the final salinity of a few representative tanks with the YSI-85 meter.



**Figure 10.** Lee's Ultra Gravel Vac<sup>®</sup> fitted with a 2-mm nylon mesh cover to prevent the inadvertent suction of stickleback larvae. The cover impedes removal of large debris and should be removed when fish are large enough to avoid the vacuum.



## Feeding juvenile stickleback a complete diet

by Jeffrey Divino

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The main staple of a stickleback's diet is live brine shrimp nauplii. Unlike zebrafish, which can be first-fed granulated micro-capsules (e.g., Zeigler's Larval AP50), we have been unsuccessful at getting newly-hatched stickleback fry to eat any artificial foods. Consequently, it is imperative to have reliable, daily nauplii harvests to sustain stickleback larvae once their yolk reserves run out. Despite this labor intensive regimen, there are benefits that come with a live diet. John Baker, project collaborator and stickleback supplier from Clark University, speaks of the merits that hunting for brine shrimp nauplii has on larval swimming performance, coordination, and sensory development. But eventually, it becomes unsustainable to feed large colonies of growing fish exclusively on the tiny nauplii, and most labs transition age-1 fish from a diet of live brine shrimp to frozen or freeze-dried organisms, such as bloodworms or copepods (e.g., Argent's Cyclop-eeze).

Because the Schultz lab focuses on osmoregulatory physiology of juvenile threespine sticklebacks, we wanted to enhance the nutritional value of brine shrimp to maximize fish growth and energetic condition. We accomplished this by gut-enriching the nauplii prior to feeding them to the fish. Second, we sought to hasten the fish's acceptance of artificial food so that we would not need to introduce brine shrimp suspensions into our experimental salinity treatments, which could potentially confound results. In July 2012, we succeeded at weaning four week-old sticklebacks, training them to eat a variety of artificial pellets and flake food. The next summer, we began weaning fry at two weeks. The three sections below elaborate on culturing brine shrimp, weaning, and use of artificial feeds.

### I. Live brine shrimp nauplii

*Purchasing brine shrimp cysts.*— Brine shrimp nauplii are commonly used in aquaculture and by hobbyists for feeding larval fishes. There are many vendors and the price of cysts varies widely. Hatchability mainly determines price and is the most important factor to consider when purchasing. Avoid any brand with less than a 90% hatch rate: unhatched cysts, beyond simply lacking nutritional value, are often fatal to the smallest larvae because they cannot be passed through the gut. Argent sells 'Platinum Grade' *Argentemia* (origin: San Francisco Bay, CA) in a 16 oz can for \$250, which boasts a minimum 95% hatch out rate. Their 'Gold Grade' cysts (Great Salt Lake, UT) cost half as much and have a 90% hatch out rate, and yield nauplii that have a slightly lower HUFA (highly unsaturated fatty acid) content than Platinum cysts. Cheaper grades of brine shrimp also hatch at larger sizes (500 versus 450  $\mu\text{m}$ ), but larval sticklebacks have large gapes and seem to handle them without difficulty.

In an effort to reduce costs of stickleback feed, in 2011 we began to purchase 'Premium Grade' cysts from Brine Shrimp Direct (BSD) at \$50 per 16 oz can (90% hatch out). Although hatching was noticeably reduced compared to that of the *Argentemia*, the cost savings of the BSD cysts more than compensated for their decreased viability. Moreover, in 2012 and 2013, we began to nutritionally fortify the nauplii and wean them onto pellet feed anyway.

*Cyst storage.*— Recommended storage conditions for brine shrimp cysts will vary, depending on the brand. BSD recommends 4°C, whereas *Argentemia* has a wider range of storage temperatures, up to 30°C. We store working aliquots of cysts in our lab fridge or the common fridge in the Aquatics Facility. Due to the risks associated with storing precious fish food in a community refrigerator, we prefer to store stickleback food in a mini-fridge that we bring into our fish room. Cysts can also be frozen for long term storage.

Regardless of storage temperature, it is essential that the cysts remain dry. Moisture will cause cysts to decompose, so do not leave cyst containers uncovered. Because of the large quantity and repeated opening of a 16 oz can of cysts, it is safest to apportion it into aliquots, as described below.

1. Label and date several 50 mL centrifuge tubes.
2. OPTIONAL: Wrap the tubes in opaque tape to protect from light. Alternatively, make a snug, plastic sleeve that can be reused on other tubes.
3. Use a small measuring spoon and a funnel to fill each tube with cysts. You can also make a few larger aliquots or leave a fraction of the cysts in the can for higher-throughput use when the fish are larger and consume more nauplii.
4. Parafilm around the threads of the conical tubes (and the lid of the can of the cysts).
5. Screw the caps back on tightly and freeze aliquots at -20°C indefinitely.
6. Prior to an aliquot's first use in a hatchery, it is recommended to slowly thaw the frozen cysts by placing the tube in a fridge overnight. This minimizes the temperature differential.

*Preparing the hatchery for first use.*— Brine shrimp cysts require salt, agitated air, light, and warmth to hatch. Because continuous daylight is required to hatch brine shrimp cysts, we set up the hatchery in a different room in the Aquatics Facility (typically the oyster tank room A-226) than where the sticklebacks are kept (rooms A-220 or A-222). Room temperature and lights are not critical to the hatchery operation since the hatchery cone is separately lit and heated, but these conditions may still influence brine shrimp hatching success. Because our hatchery is radiant-heated, if room temperature climbs, so will the water temperature, and vice versa. Having the ceiling fluorescent lighting continuously on (which it has been in the past) may increase hatch-out by augmenting light levels.

1. Gather two Aquatic Habitats hatchery systems, each containing a stand, a cone with lid, a drain spigot and collection tube, and a stiff length of airline tubing. Figure 1 shows the entire rig.
2. If using a new cone, you should check for leaks, mark 1 L graduations, and clean it. Place the cone and stand near a sink, and with the spigot closed (handle perpendicular to the drain flow), partially fill with distilled water. If the lower drain tube or spigot is dripping, then drain the cone and replace spigot with one from another cone to hopefully get a tighter fit. Repeat leak test until it passes.
3. To make graduations, stand the cone on a level surface and turn the spigot to the “off” position. Fill the cone with 1 L of distilled water. Once the water has calmed, mark a dash at the water line with a permanent marker at several places around its outer circumference. Add a second liter of RO water and repeat until a total of 6 L have been marked.
4. Clean the cone by filling it with 1-2 L of RO water and a small amount of Alconox. Dissolve soap powder by vigorously mixing with long scrub brush. Rub suds up entire length of cone and also scrub the lid. Standing on a stepstool increases your reach with the brush. Drain cone and rinse multiple times to completely remove soap residue.
5. The stiff airline tubing can also be cleaned by flushing it with water. If the tubing has a clog, then first try to break it up by inserting long bristles from a brush or with a dissecting probe. Next, flush with hot tap water before a final RO water rinse. Whip the tube through the air to clear out the residual water.
6. If necessary, repeat Steps 2-5 for the second hatchery cone.



**Figure 1.** The Aquatic Habitats brine shrimp hatchery. The many additional components include lights, ring stand, air pump, tubing and valves, buckets, ½ gallon jars for seawater and nauplii suspension, measuring spoons for cysts, collecting sieve, baster for dispensing, and thermometer.

7. Fill cone with 4 L of 28-30 ppt “brine shrimp water”, made by mixing Crystal Sea “junk salt” (to conserve Instant Ocean) into RO water in 5 gallon bucket. Check salinity with YSI 85 meter. Small deviations in brine shrimp water salinity will not affect hatch out, but we set 28 ppt as a minimum.
8. Test aeration system by attaching the stiff airline tubing to flexible 1/8” aquarium tubing, which in turn, attaches to a 10-gallon aquarium air pump (larger pumps may be used). Somewhere along the airline connection should be a regulator for adjusting flow. Twin splitter valves are preferred so that a second container of hatched nauplii can be aerated by the same air pump (Fig. 1).
9. For this second airline, attach a ~8” segment cut from a 1 mL polystyrene serological pipet to flexible airline tubing that will be connected to the valve. Insert the straight plastic into a ½ gallon jar and submerge in water.
10. Place round lid on the cone and feed the stiff airline tubing through the center hole on the lid down to the drain spigot. Plug in air pump and adjust the valves until the cone has moderate bubbling - strong enough agitation to send waves across the surface of the water. The jar should also be well aerated, so it can be tough to balance air flow. Switch to a larger air pump, e.g., Sweetwater if needed (Fig. 1).
11. Set up two clamp lights, one mounted overhead with a 100 Watt outdoor floodlight bulb shining downward at the top of the cone and a horizontally-directed 50 W outdoor light shining at the side of the cone. The former light can be clamped to an electrical conduit; the latter can be positioned on a ring stand (Fig. 1).
12. Insert a partial immersion alcohol thermometer with twisted wire line and counterweight (Fig. 1) through larger hole in the lid of the cone. Suspend the end of the thermometer at proper depth so that its black line is submerged in the seawater. Return later to check temperature as the seawater warms in the radiant heat of the two floodlights.
13. After rechecking the cone’s water temperature, move lights closer or further away as needed to achieve target temperature: optimal water temperature for hatching is 28°C, and setting a target of **29 ± 1°C** will achieve this, although it takes hours. Once this temperature is reached, mark boundaries on the table for the hatchery and the light stand with lab tape, so that they will be placed in the same spot every day.
14. OPTIONAL: Alternatively, a submersible heater can be inserted into the hatchery cone (Fig. 2). However, cysts stick to the heater, hands touch the culture while positioning it under the water, and it requires a cool-down period so that it will not shatter during cleaning.
15. Once the hatchery unit has been tested for aeration and temperature, test brine shrimp cyst viability by adding a ½ tsp or so of cysts to the seawater. This is especially important if you are trying a new lot or the cysts have been stored for a while.
16. OPTIONAL: To improve hatchability and digestibility, brine shrimp cysts can be decapsulated prior to being added to the hatchery cone. This time intensive procedure involves many steps and solutions. In short, the cysts are first hydrated and then the tough shells carefully dissolved using bleach, after which they are neutralized and rinsed. Alternatively, the decapsulated, orange naups can be fed directly to the sticklebacks unhatched. Decapsulated cysts can be stored at 4°C in a brine solution (e.g., 2-10x artificial seawater) to preserve them in a dehydrated state.
17. Use the baster to wash any cysts that get stuck on the wall of the cone above the water; the static cling of cysts can be a frustrating problem that you will just have to accept.

18. Allow the brine shrimp cysts to incubate for at least **16 hr**, although longer incubations of 24 hr or more) will yield higher hatch rates. This is why it is better to start a new batch in the second cone before harvesting the previous batch.

*Switching cones and starting a new batch.*— Although only one hatchery cone is necessary, it is more efficient to alternate between two cones so that one can be harvested and thoroughly cleaned without delaying the start time of the next incubation. The second cone also gives you a ready back-up.

1. When revisiting the brine shrimp hatchery following the incubation period, first verify brine shrimp hatch-out and inspect cone temperature and aeration conditions. The water should be turbid and appear orange from the hatched nauplii.
2. Check temperature by inserting the white thermometer through larger hole in the lid. Both floodlights must be shining on the cone at predetermined distances to achieve this temperature. If a bulb blew overnight, then replace it and allow the cysts to incubate longer until the optimal temperature has been restored.
3. The air line should also be bubbling from the lowest point above the drain spigot. If for some reason airflow had stopped during the night, the unhatched cysts and many of the naups will settle to the narrow bottom of the cone, which would quickly become anoxic; the batch may be lost.
4. After the conditions and hatch-out have been checked, first dismantle the hatchery cone by disconnecting and removing the rigid airline tube from the flexible hose and move the cone and stand to a new location - typically the sink countertop on the opposite wall of the room - to make room for setting up the next batch in the second cone.
5. Set up a new hatchery using a 2nd, previously cleaned, cone by placing it where yesterday's cone was under the two lights. Be sure spigot is closed before filling the cone and remove the lid.
6. Retrieve an aliquot tube of chilled brine shrimp cysts from the 4°C fridge.
7. To ensure a steady supply of live food, especially for young larvae, culture excess brine shrimp. The chart below serves as a general guide for feeding 2,000 sticklebacks. Volumes of seawater are approximate. Using more water decreases nauplii density, but will take longer to warm.

Weeks Post-Hatch	Container	Volume of Cysts	Approx. Mass	Volume of SW
0	Petri dish	⅛ Tsp	0.4 g	1 L
1	Quart jar	¼ Tsp	0.7 g	2 L
2	Aquarium	½ Tsp	1.3 g	3 L
4	Aquarium	1 Tsp	2.5 g	4 L
6	Aquarium	2 Tsp	5 g	5 L
8	Aquarium, split	1 Tbsp (3 Tsp)	8 g	6 L

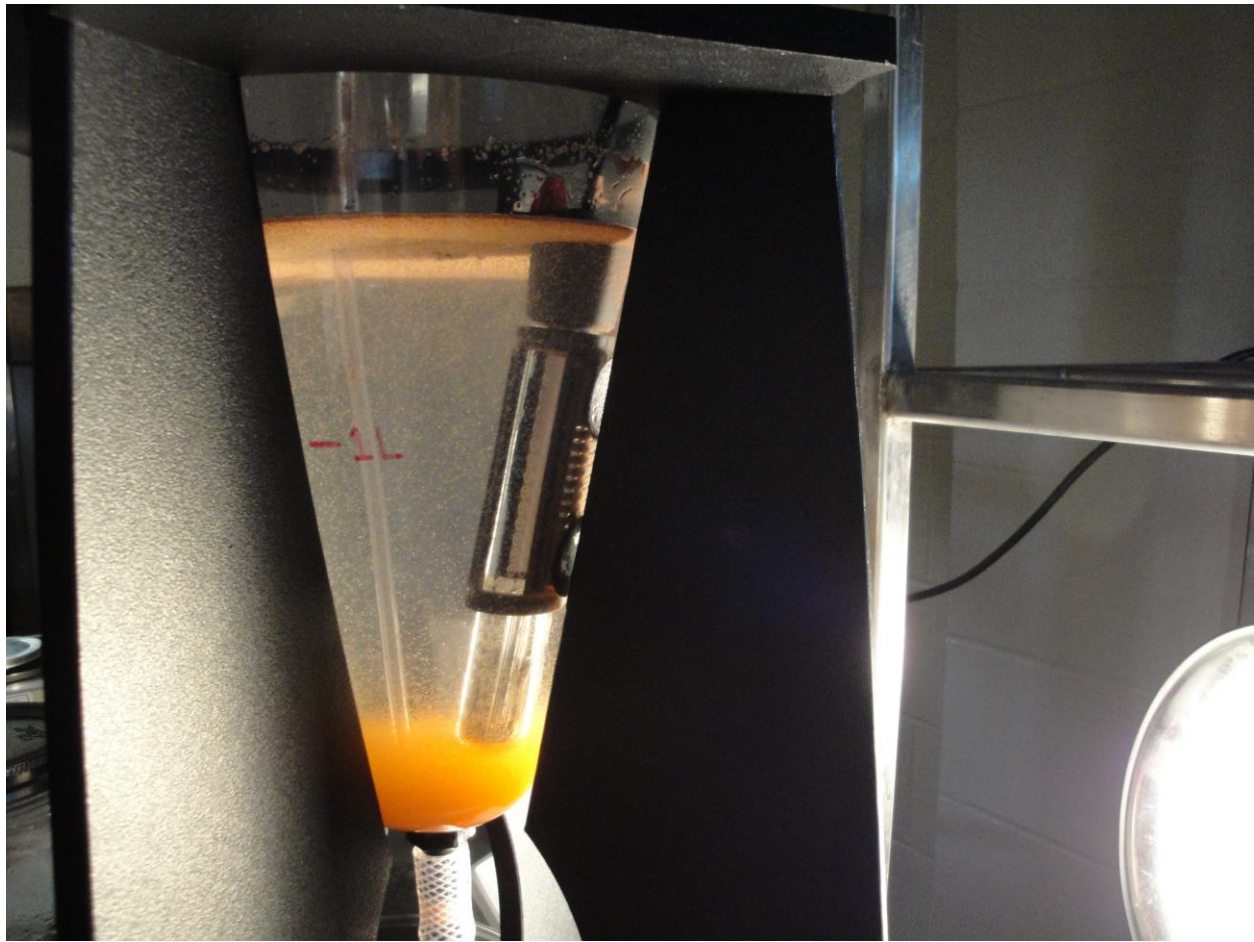
8. Because dry cysts float and also have a static cling to surfaces, first add the cysts to the bottom of the cone prior to adding seawater. Sanding on a step stool, scoop out brine shrimp cysts from the tube using a measuring spoon, reach deep into the cone, and add them to the empty cone.
9. Next, pour on top of the cysts 28-30 ppt brine shrimp seawater using a dedicated ½ gallon jar or 2-gallon bucket to achieve a *maximum* density of **2 g cysts / L** (BSD recommends 1 g/L). There is no minimum density, except the benefit of conserving the seawater.
10. If cone appears a solid brown color, then cyst density is too high. Add brine shrimp water to dilute.
11. Connect a clean airline tube to hose and feed through small center hole in the circular lid.



12. Turn on the air pump and bubble deep down into the lowest point of the cone at the spigot, which keeps the cysts from settling and prevents an anoxic zone.
13. Stuck cysts can be flushed from the wall of the cone with a baster filled with brine shrimp water, even during aeration. But again, cysts will often re-stick, so we rely on the steady turbulence of the water, caused by moderate aeration to keep the cysts hydrated and knock them down.
14. Finally, after set-up return later to check hatchery rig for optimal conditions: temperature should be rising to 29°C and aeration should be strong enough to make waves on the surface: too much air causes foaming and stranded cysts; too little...nauplii mass grave. This is usually the final task of the day.

*Basic nauplii harvest.*— The inverted shape of the hatchery cone is designed to concentrate brine shrimp nauplii down at the drain spigot and to separate them from the cysts they have emerged from, which usually float. Since nauplii are attracted to light, shining a light at the narrow end of the cone is meant to accelerate the vertical migration downward and further increase nauplii density. However, they will eventually settle down to the bottom of the hatchery cone regardless, so it is optional to turn the fluorescent ceiling lights off in the room during the harvest. In fact, without aeration, the nauplii will settle in any container they are introduced: collection jars, fish jars, or aquaria. (This is why they should be regularly stirred when administering them to the stickleback.)

1. Place a small LED flashlight on a test tube rack and shine it upwards at the bottom of the cone to attract and condense the nauplii near the drain spigot. You can also drape a black polymer sheet over the top of the cone to block out light from above, but this does not seem to help much because the LED is so bright and the film of floating cysts stops light penetration as well.
2. Allow at least 5 min for the bottom of the hatchery cone to turn an opaque orange with concentrated nauplii (Fig. 2).
3. Collect the dense clump of orange nauplii by holding a large glass jar under the drain tube with one hand and opening the spigot valve with the other. Close the spigot as soon as the naups have been drained or if you are about to overfill the jar. Avoid harvesting large amounts of unhatched cysts and shells (which is noticeably higher for BSD cysts than for *Argentemia*).
4. The best practice is to periodically drain dense nauplii suspension from the cone as they continue to settle over time. This way you are collecting the greatest number of nauplii with the least amount of hatchery water; you should not have to drain much more than 1 L of culture. *Note:* Hatchery water contains unhatched cysts that sticklebacks will consume along with the live nauplii. I have found cysts in fish feces, gills, and throughout the gut. At best, the digested cysts will pass through the gut undigested - a kind of insoluble fiber. But cysts can lead to bowel impaction in the smallest fry that have just begun to feed and should thus be avoided when harvesting.
5. If you have filled the collection jar and really want to collect the nauplii that will not come down, you can open the spigot partially and drain the cone directly over a 74-µm mesh sieve (Fig. 3). Wash the naups with RO water (see Step 11) and flush them into the collection jar.
6. Discard the remainder of the cone's contents by fully opening the spigot and let drain into the sink.
7. When time permits, usually following fish feeding, thoroughly clean out the hatchery cone and its components as described above. Flush out all of the residual cysts from the cone with RO water and then partially refill it with RO water mixed with Alconox. Scrub-clean the cone and lid to remove any biofilm or microbial growth. Because bubbling has a mild suction force, plaque can slowly build up inside the open end of the stiff airline tubing. To prevent a possible blockage of air flow over time, periodically scrub the tube out and flush it with water.



**Figure 2.** Concentrating newly hatched nauplii near the drain of the hatchery cone prior to harvest. The cysts from which the emerged nauplii are floating at the surface of the water and will be discarded along with much of the water after brine shrimp collection. Note the submersible heater, which is one method for maintaining optimal hatchery temperature.

8. Unfortunately, unhatched cysts are heavy and will rapidly sink, so many will be harvested along with the nauplii. If there are significant numbers of unhatched cysts, you can remove them from the nauplii by allowing them to sink in the collection jar and then steadily pour the suspension into a new jar, leaving the dregs behind.
9. Prepare one pint jar to hold the final brine shrimp resuspension and another with clean water of desired salinity, e.g., 3 ppt for standard rearing conditions. To feed sticklebacks kept in other salinities, nauplii can be reconstituted at matching salinities in additional pint jars. To facilitate this process, maintain 5-gallon carboy stocks of treatment water. Keep in mind when feeding stickleback juveniles in 10-gallon rearing aquaria that the water volume of the tank is so great that pipet or baster-fuls of concentrated brine shrimp suspension will not appreciably affect tank salinity. *Note:* If feeding nauplii to experimental sticklebacks in freshwater challenge experiments, the nauplii should be reconstituted in RO water to prevent an increase in treatment salinity.
10. Swirl the jar of collected nauplii and slowly decant the brine shrimp suspension through the blue, **74- $\mu$ m** mesh sieve (Fig. 3) over the sink basin. The sieve filters slowly and catches all of the naups, so there is no need to collect the filtrate in a jar with this fine mesh size. *Note:* You can strain nauplii through larger mesh sizes, such as a 180  $\mu$ m sieve (green plastic frame) or a basic brine

shrimp net, but many will flow through, so you will have lower yield even if you save the filtrate in a jar and re-strain.

11. If there is a large number of nauplii to be strained, the fine mesh sieve can become clogged. A thick layer of nauplii is also more difficult to wash and a higher percentage of them may become damaged. Stop pouring if/when the surface area of the sieve becomes completely covered (Fig. 3).



**Figure 3.** The 74  $\mu\text{m}$  brine shrimp sieve teeming with nauplii after straining a collection jar. The nauplii can be rinsed and then reconstituted to any salinity. Here, there are two pint jars: the one on the left contains new water of a given salinity, e.g., 3 ppt, some of which had been poured into the jar on the right to catch the naups. After inverting the sieve over the right jar, the mesh will be flushed with a baster using additional water from the left jar.



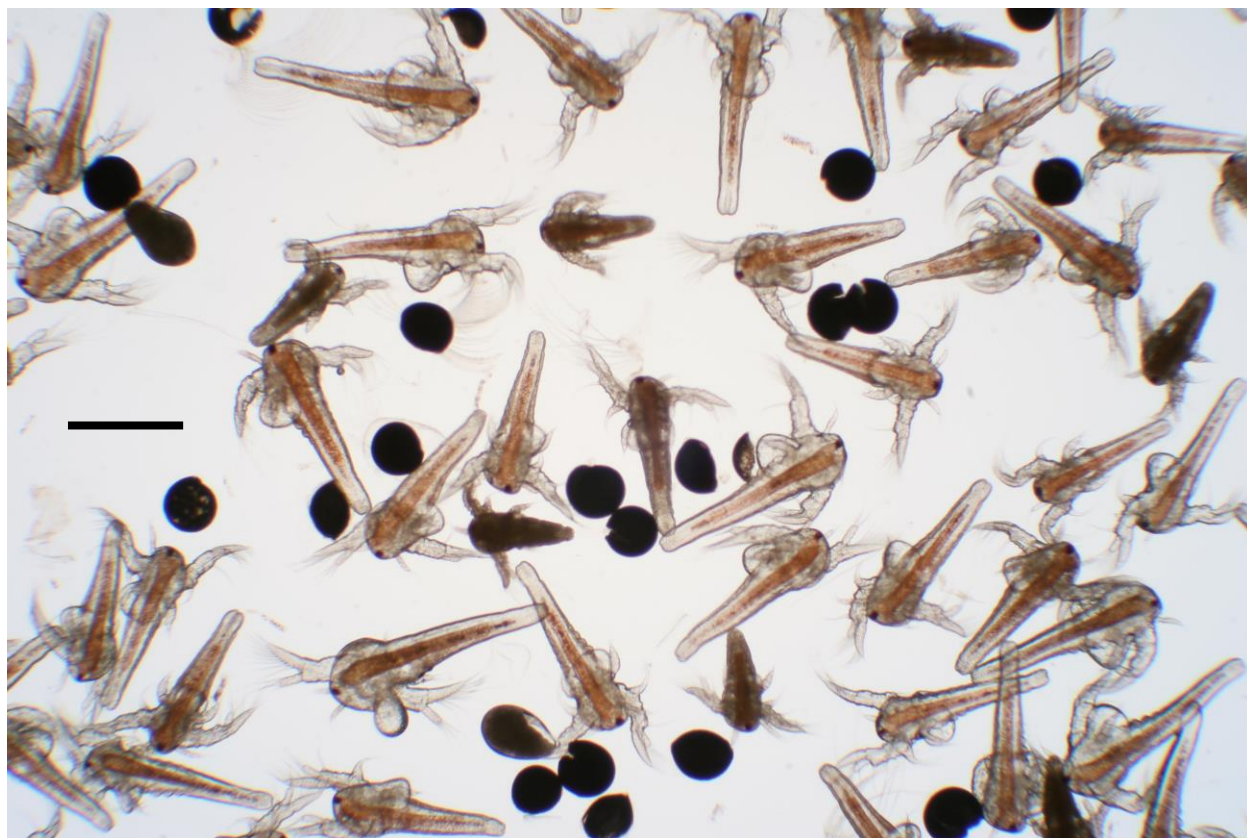
12. Rinse nauplii gently but thoroughly with RO water. Turn on the RO valve to a soft flow. Then grasp the RO hose in one hand and the handle of the sieve in the other. Over the sink, run the water over the nauplii while slowly stirring the sieve. It is common for the sieve to begin to fill with water, so allow ample drain time.
13. Pour some of the new suspension water into the pint jar to break the fall of the brine shrimp being poured into it (Fig. 3).
14. Next, empty the sieve by inverting it over the pint jar(s) and then flushing out the residual naups using blasts from a regular baster (i.e., no tubing tip) filled with the new suspension water. You want to minimize the water used in this process so that the brine shrimp are highly concentrated. As a general guideline, the resuspension should resemble pulpy orange juice. Therefore, adjust the volume the water used for resuspending the nauplii in proportion to the hatch yield.
15. If you wish to add more nauplii, swirl the ½ gallon collection jar again and strain, wash, and flush additional nauplii into the same resuspension, or into a different pint jar.
16. If there are still many unhatched cysts, allow them to settle to the bottom of the jar. If administering nauplii to Petri dishes or jars of larval stickleback, then simply avoid pipetting them. Alternatively, decant the suspension into another jar, leaving the settled cysts behind. (There is no need to go through this trouble for large fish.)
17. Aerate whatever brine shrimp culture remains in the jar for later use. Refresh the suspension with new brine shrimp water to improve water quality and place jar on the lighted hatchery table for warmth. At first, when the fry are still small, it is common to have many extra nauplii. These “leftovers”, or alternatively the entire batch, can be nutritionally enriched, as described below.

*Gut-enriching nauplii.*— Brine shrimp nauplii possess a small yolk reserve when they hatch and will undergo their first molt first instar transformation around 24 hours post-hatch, and will then begin filter feeding. You can readily see the morphological differences between a newly-hatched 1<sup>st</sup> instar and a 2<sup>nd</sup> instar larvae under the microscope (Fig. 4). Sticklebacks will, of course, consume both life stages, but the benefit of feeding with the more developed nauplii is that these can be gut-enriched to increase their nutritional value for the fish.

There are several nutritional supplements that can be administered to brine shrimp cultures that make them more nutritious for fish. Various algae pastes such as *Spirulina* can be fed to sustain growing brine shrimp meant to reach the adult stage before being fed to larger fish. Other, more digestible supplements improve fish growth through a process called gut-enrichment, where the nauplii accumulate nutritive particles in their intestinal tract as they filter feed (Fig. 5). (They are surface-coated by the particles as well.) In this case, brine shrimp cannot be cultured long-term because they do not directly benefit from the substance they have ingested. However, the small nauplii will contain high amounts of lipids, such as HUFAs (highly unsaturated fatty acids), which noticeably enhances growth in larval sticklebacks. One such additive is **SELCO** (self-emulsifying lipid concentrate) from Brine Shrimp Direct, which resembles orange acrylic paint. More information about SELCO can be found here: <http://www.brineshrimpdirect.com/SELCO-c47.html>.

1. As described in the previous section, you may retain all or some portion of the nauplii harvest and aerate them in a ½ gallon collection jar topped-up with new seawater for future feedings. These “leftovers” can be enriched overnight in SELCO. *Note:* If the harvest is not immediately needed because there are other nauplii available for the day’s feedings, then SELCO the entire harvest for use over the next 1-2 d.

2. Clean the culture by straining the nauplii through the brine shrimp sieve, wash them in RO water, and then reconstitute them in 1-1.5 L of new brine shrimp seawater in a clean ½ gallon jar. Do not fill the jar up to the neck.
3. Place the jar of harvested nauplii on the hatchery cone table and vigorously aerate with the second airline coming off the air pump (Fig. 1). Avoid splash-out caused by excessive bubbling.
4. Remove the SELCO bottle from the mini-fridge, remove parafilm, and open the nozzle. Invert bottle and squeeze 4 “**short drops**” per liter of brine shrimp suspension (5 drops for a full jar). SELCO comes out like a paste and requires several minutes of aeration to dissolve.
5. When finished, parafilm the nozzle of SELCO bottle to prevent the paste from hardening and clogging and store 4°C.
6. During the SELCO incubation, the jar of naups should be kept warm by facing direct light from the side lamp of the hatchery. Since only a few unhatched cysts remain in the suspension, temperature is not critical, but can be checked with a thermometer.
7. The SELCO can be added immediately to the suspension or later in the day if you are busy, since the nauplii only need to be immersed in the nutritional bath of emulsified lipids for a minimum of 12 hr. Longer incubations, e.g., 18 hours are fine and may even improve the enrichment if more nauplii achieve Instar II during that additional time.
8. Following the SELCO incubation, the entire batch should be fed to the fish within 48 hr. In other words, batch sizes should be scaled appropriately to be consumed within 72 hr of harvest.



**Figure 4.** Micrograph of a strained brine shrimp hatch, showing nauplii interspersed with unhatched cysts and shells. The two oblong cysts are decapsulated, revealing not yet emerged nauplii. Instar I nauplii are short, dark brown, and have less developed appendages than Instar II. Bar = 500  $\mu$ m



9. **OPTIONAL:** For longer periods, nauplii can be chilled at 4°C to increase their shelf-life. Aeration is not needed because metabolism will be mostly arrested at this low temperature.



**Figure 5.** Instar I nauplii (upper right) are nourished by yolk reserves and do not begin to filter feed until their first molt 18-24 hours post-hatch. Notice the orange SELCO droplets throughout the lumen of the gut of the two Instar II nauplii. (The dark structures are eyespots.) Bar = 100  $\mu$ m

*Brine shrimp feeding regimens.*— Juvenile sticklebacks should be fed live cultures at least twice per day, usually thrice on weekdays. Newly hatched nauplii may be immediately given to the fish, or they can be fed exclusively SELCO-enriched nauplii, or meals can alternate between “raw” and enriched nauplii. The following feeding regimens (weekly schedules depicted in Figures 6-8) assume that all or at least a portion of the brine shrimp harvest is reserved and incubated for a second night in SELCO before being fed to the fish:

**Regimen A:** Daily hatchery cones; all meals (breakfast, lunch, and dinner) enriched

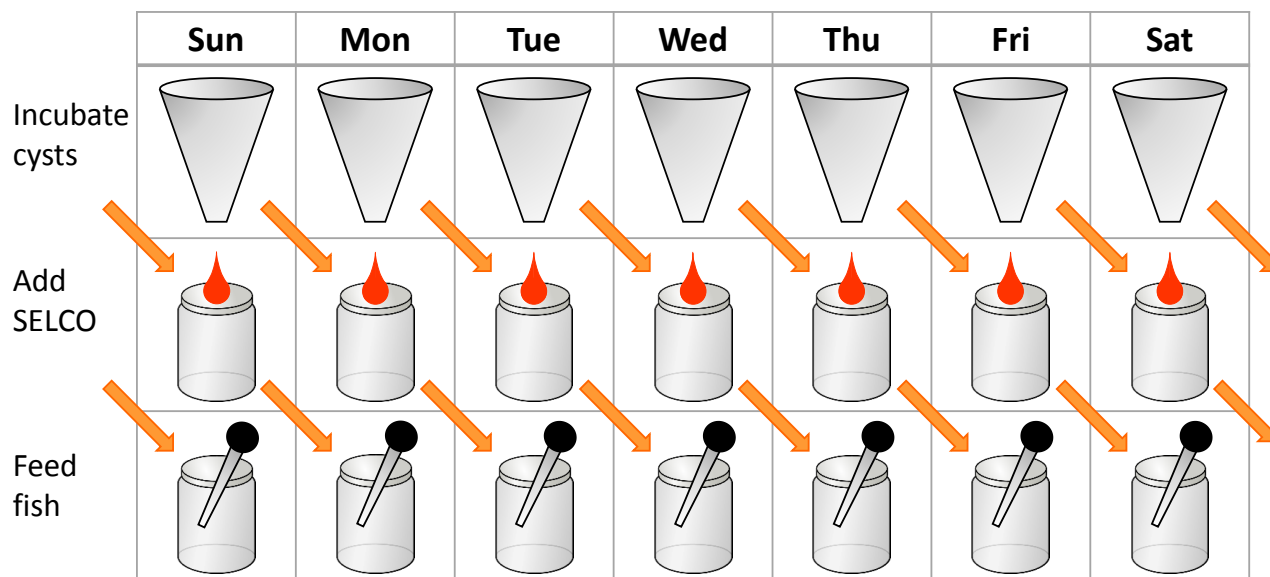
**Regimen B:** Cones set-up Monday, Wednesday, and Friday; all meals enriched

**Regimen C:** Daily hatchery cones; both enriched and raw meals

Regimen A is preferred for very large stickleback colonies where high demand for naups causes the entire harvest to be fed the day following enrichment. Regimen B bears less of a workload because only three hatchery cones are set-up each week, which is suitable for rearing a smaller fish colony. In 2012, we followed Regimen C, cautiously testing gut-enrichment on only a portion of the brine shrimp harvest. This method bet-hedges against failure of the enriched culture, such as through loss of the air supply. But after consistent survival of enriched naups, even for three days in aerated jars, we scaled up enrichment to the entire harvest the following year, which was more efficient.

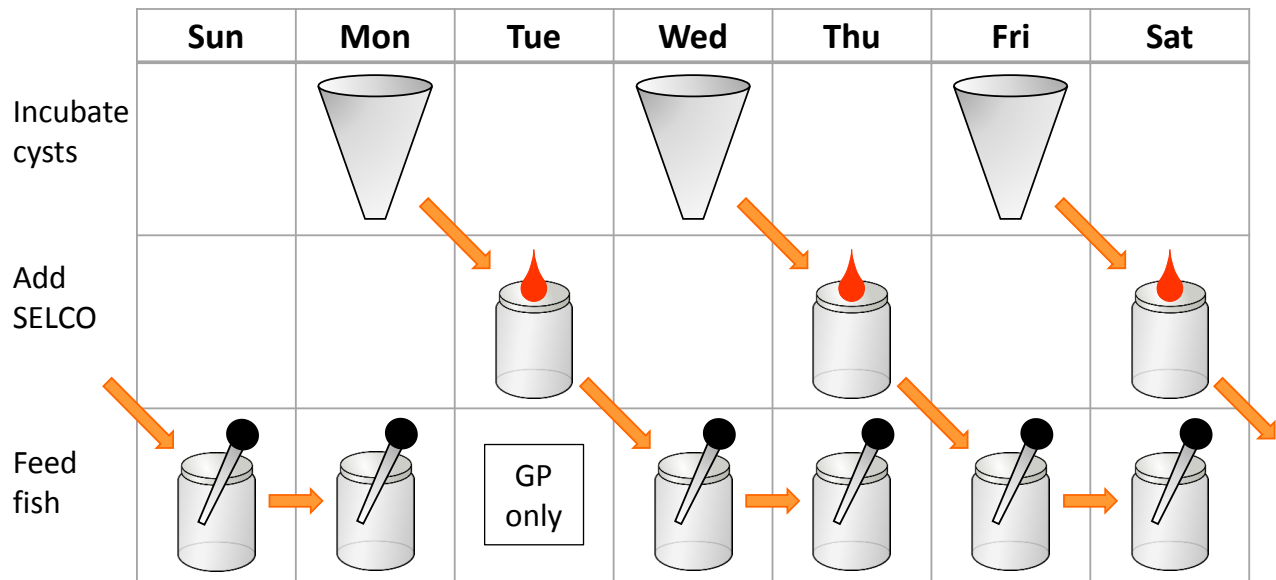
Because a daily brine shrimp feeding regimen will entail timing the growth of cultures to partially overlap, or involve splitting harvested nauplii into subcultures, it is critical to remain organized. Record on the Brine Shrimp Hatchery log (Appendix B5) dates that cysts were added to a hatchery cone, the size of the culture, when nauplii were harvested and enriched, as well as when they were fed to the fish.

### Regimen A: Daily hatcheries, all nauplii enriched



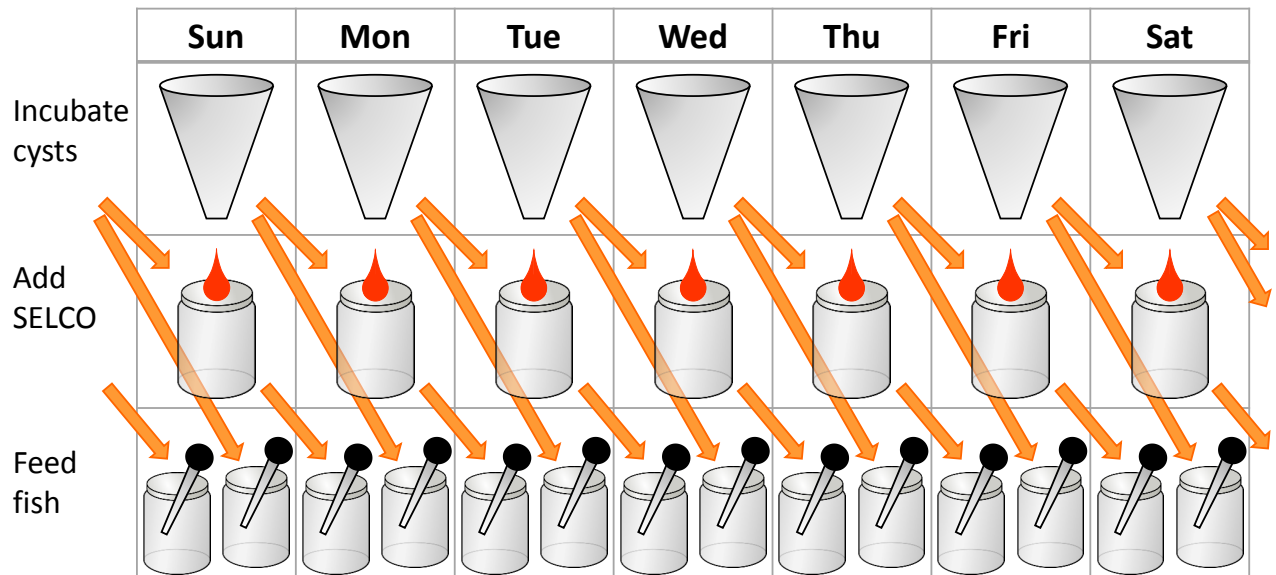
**Figure 6.** Regimen A is geared for culturing enriched nauplii for feeding several thousand larval sticklebacks and involves daily incubation and harvesting of brine shrimp cysts. The SELCO-enriched nauplii are then fed to the fish from the batch that was prepared 48 hr prior.

## Regimen B: M-W-F hatcheries, all nauplii enriched



**Figure 7.** Under Regimen B, hatchery cones are only set-up three times per week and the same batch of enriched nauplii are fed to the sticklebacks for two consecutive days. This alternative format requires far less work, but may not be able to sustain large colonies. There is also one day when no nauplii are available for feeding. Here, artificial foods such as Golden Pearls (GP) must be administered on Tuesdays. (Incubating a half-batch of cysts on Sundays would fill this gap.)

## Regimen C: Daily hatcheries, raw and enriched nauplii



**Figure 8.** Regimen C splits daily harvests of brine shrimp nauplii into two jars. The first portion can be rinsed and served “raw” to the sticklebacks. The remainder of the batch are SELCO-enriched overnight and fed to the fish the following day. Dividing the harvest protects against food shortages due to inadvertent loss of the enrichment culture.

*Serving a meal of brine shrimp nauplii.*— The following generalized procedure is compatible with either preparations of raw or enriched nauplii. The precise timing of each feeding is not critical - especially for *ad libitum* feeding regimens where live, uneaten nauplii are always present in the container - but never schedule water changes immediately after a feeding.

1. For larger fish held in aquaria, turn off all filters by turning off their power strips.
2. To feed with *enriched* nauplii, carry to the fish room the ½ gallon jar of SELCO-enriched leftover nauplii, which had been bubbling overnight next to the hatchery cone. These nauplii are gut-loaded and surface-coated with fatty acids and are especially nutritious.
3. To feed with *raw* nauplii (Regimen C only), harvest approximately half of the day's hatch and reserve the rest for overnight enrichment, adding new seawater and SELCO to the collection jar (see the "Gut-enriching nauplii" section above).
4. Follow Steps 9-16 of the "Basic nauplii harvest" section above to strain, rinse, and reconstitute the SELCO-enriched brine shrimp suspensions at the desired salinity(ies).
5. Dispense nauplii to sticklebacks, avoiding unhatched cysts. Use a transfer pipet for fish in Petri dishes or jars and a turkey baster (without tubing tip) for fish in aquaria. Gently stir nauplii up off the bottom of the jar with the pipet or baster before filling it to ensure a consistent concentration going into each container.
6. Spread the brine shrimp over a large surface area to maximize their availability to all of the fish.
7. Rearing tanks are typically fed *ad libitum*, so for each scheduled feed, pipet nauplii into the containers of sticklebacks in multiple passes, or rounds, to ensure satiation.
8. To prevent over-feeding, it is best to administer small amounts of nauplii to each container and then make one to two additional rounds. The table below serves as a general guideline:

Container	Approx. # of Age-0 Fish	Dispenser	Vol. Nauplii / Round	# Rounds
Petri Dish	25	Pipet	3 drops	1-2
Jar	25	Pipet	1 mL	1-3
Aquarium	200 small to 50 large	Baster	¼ - ½ oz.	2-3

9. Check each container for nauplii clearance and spot-feed as necessary. *Note:* smaller sticklebacks in dishes or jars are poor swimmers and inexperienced hunters, so it is beneficial to allow exposure to the nauplii for extended periods to give the fish more time to feed. During early ontogeny, fish rarely clear their food and it is common to find uneaten nauplii swimming the following morning.
10. Aim to use up the entire SELCO-enriched nauplii to avoid extended carry-over of older cultures. However, surplus nauplii can be aerated between meals and even last another day.
11. If tank sticklebacks have completely cleared the naups, then you may turn the aquarium filters back on. The filters often need to first be primed by filling their reservoirs with water from their respective tanks using a qt jar. (Office of Animal Care staffers worry if the filters are not running when they check the room!) *Note:* If filter does not work, the reservoir likely needs more water, the joints of the intake pipe could be loose and are sucking air, or the impeller could be stuck.
12. Document mealtimes and amount fed on Feeding form (Appendix B2).
13. Make sure to leave the Aquatics Facility to take a break between feedings, especially if you start to feel ill, lethargic, or crazy. Young sticklebacks will feed more actively, especially at the surface, when the room is devoid of caretakers. (And take the stairs so that you are forced to be exposed to natural light.)

## II. Weaning

Stickleback juveniles voraciously feed on live brine shrimp and older juveniles in aquaria can each potentially eat hundreds of the 450  $\mu\text{m}$  nauplii, so demand will eventually outstrip supply. Maintaining active cultures of brine shrimp is extremely time-consuming, wearisome, and becomes progressively futile and financially costly as the fish's appetite grows insatiable. Furthermore, as stated previously, brine shrimp are not recommended for use in salinity challenge experiments due to their reduced conspicuous swimming and lower survival in freshwater, which in turn potentially leads to unequal decomposition and water quality among treatments. For these reasons, it is beneficial to wean the stickleback fry as soon as possible, switching them to an exclusively artificial diet.

Weaning requires some patience, but the pay-off of shutting down brine shrimp production is worth it in the end. In 2012, after failed attempts to get hatchlings to accept all sizes of Zeigler flakes, we were first successful with a special pellet feed called Golden Pearls (GP) given to four week-old sticklebacks residing in aquaria. In 2013, we began the transitional diet on just over two week-old fry.

Once the sticklebacks begin to accept artificial foods, you can begin to phase out brine shrimp production. However, young fry will always prefer nauplii to substitutes, so removal of nauplii from the diet altogether could potentially decrease consumption rate, and in turn, growth. Consequently, we usually continue serving one meal of nauplii per day, as outlined below.

*The weaning progression.*— This procedure describes the transitional sequence from SELCO-enriched nauplii to artificial pellets. The fish are gradually introduced to greater amounts of GP, while still being fed brine shrimp to maintain high growth trajectories. Fry are more prone to ingest GP when they are hungry, so pellets are always fed at the first meal of the day, which ensures that juvenile sticklebacks are fully trained to eat all of their 'vegetables'.

1. For the initial weaning, pre-mix pinches of 100-300  $\mu\text{m}$  diameter GP in the enriched brine shrimp suspension. Baste the solution into the aquaria as usual. The goal is for the sticklebacks to accidentally ingest pellets as they forage for similarly-looking nauplii and then associate both as equally palatable. If the GP are uneaten, wait another day before trying again. (You may need to remove excess GP from the tanks.)
2. Once the fish begin to consume GP, i.e., they are not spitting them back out, feed GP-nauplii mixtures for both the AM and PM feedings.
3. After the sticklebacks have demonstrated that they readily target GP, you can administer GP to the tanks separately (see next section), then follow with squirts of brine shrimp. Do this for both the AM and PM feedings.
4. After a few days, begin to exclusively feed the fish GP pellets for the AM meal. Later on in the day, fish can be fed servings of GP followed by enriched nauplii (saving the 'dessert' for last).
5. Keep track of the weaning progress by recording food mixtures and quantities in the Feeding form (Appendix B2) and make adjustments based on what is being completely consumed.



### III. Artificial foods

Post-weaning, Golden Pearls (GP), sold by Brine Shrimp Direct, is our standard artificial feed. They are orange and designed to mimic nauplii in appearance. GP are essentially neutrally buoyant. The small pellets float for some time as they hydrate and then sink slowly, at a rate proportional to their diameter. Golden Pearls come in a suite of sizes, with pellets ranging from 5-1000  $\mu\text{m}$  in diameter to accommodate a variety of larval stages. A 12 oz jug costs ~\$28. More information on Golden Pearls can be found here:

<http://www.brineshrimpdirect.com/Original-Golden-Pearl-Diets-Active-Spheres-cl1.html>.

A varied diet helps ensure adequate nutrition for maximal growth and can be achieved once experienced and emboldened fish have learned to surface-feed. Once they rise for GP, they will accept other artificial foods as well, such as crushed flakes, freeze-dried copepods, and small sinking pellets, such as Thera (New Life Spectrum). Always test-feed a new food item on a small number of fish to make sure it is accepted before adding it to the menu. Dry food types can also be mixed together in a clicker feeder (Fig. 9). You can also add variety to the fish's diet by alternating food items.

Dried foods are easiest to prepare, but we have also fed juvenile stickleback several frozen foods, such as bloodworms (chironomid larvae), packets of Sally's brine shrimp nauplii, and cubes of Spirulina-fed adult brine shrimp (chopped while frozen with a razor blade and then rinsed). The fish are highly attracted to blood worms and adult brine shrimp. However, frozen foods are problematic because they all smell bad and discolor the water to some degree. They also have the potential to foul the water from the decomposition of unconsumed material (e.g., bloodworm cuticle or brine shrimp appendages). Moreover, bloodworms are not very nutritious and serving them is a messy operation. Because the worms are too large to be swallowed whole, for small fish at least, they need to be chopped into pieces (while frozen) and then rinsed with RO water in a brine shrimp net, to minimize the amount of blood diffusing into the aquarium water.

Artificial pellet or flake foods circumvent some of these problems, but if uneaten, they too will sink, soften, and decompose. Thus, the fish must be fully weaned off live brine shrimp so that they will readily consume the pellets while they are still in the water column.



**Figure 9.** The adjustable clicker feeder is the most efficient means of dispensing dry foods across aquaria. Multiple food types can be mixed: here are GP and freeze-dried copepods (which tend to clump into balls). The plastic spout is detachable and can be used as a funnel for refilling the chamber.

*Serving a meal of Golden Pearls.*— These steps outline our basic pellet-feeding technique and may hold true for other types of artificial foods.

1. Remove large 12 oz. jar of appropriately-sized Golden Pearls (GP) from mini-fridge. (Alternatively, aliquots can be made in 50 mL conical tubes.) We typically purchase the following pellet sizes: 200-300, 300-500 “Active Spheres” (greatest resemblance to nauplii), and 500-800  $\mu\text{m}$ . Increase the size of the GP pellet as the fish grow. Always test-feed a new pellet size to make sure the fish will go for it.
2. Fill the Aquatic Habitats hand clicker feeder (Fig. 9) with GP by turning it upside down and twisting the black funnel attachment counterclockwise until it detaches. Carefully add GP into the reservoir with a plastic spoon or small measuring spoon, then screw the funnel back in place. Work over a tray to catch spillage.
3. Lift and turn the dispenser cap back on the feeder and set it to the desired dispensing volume: “pinch”, “1/8 tsp”, or “1/4 tsp” (Fig. 9). There is a large difference between each setting, so to feed greater amounts of GP, it is often better to click two or three times at a lower setting.
4. As with the brine shrimp nauplii, turn off aquarium filters before feeding fish.
5. Hold the clicker feeder vertically over the rearing tank and fully depress the dispenser cap with your thumb. For multiple clicks, dispense GP to different parts of the tank.
6. Invert the clicker feeder to mix the GP before dispensing them into the next stickleback tank in line. I prefer the ‘tomahawk chop’ action to empty the collecting spout every time.
7. Repeat the above step for each aquarium and observe how many GP are being consumed. If the fish “clear” the food right away, then round through again and feed fish a second time. Record the number of feeding passes, and if necessary, increase dispensing volume for the next day. *Note:* larger pellets will occupy more space inside the fish’s gut; scale servings accordingly.
8. Step out of the room to give the fish more time to eat and then return later to check that all the food has been consumed. Uneaten pellets, and flake diets as well, will sink to the bottom of the aquarium, become soggy, and rot. The larger the pellet, the faster it sinks. If you over-fed a tank, or observe that the sticklebacks are not bottom-feeding, remove the uneaten food with a dip net or by sucking it up with a baster.
9. When feeding time is over, turn the aquarium filters back on.
10. Record mealtimes, type(s) of food, and amount fed on Feeding form (Appendix B2).
11. Store GP at 4°C. The clicker feeder may be stored in a plastic cup upside-down in the mini-fridge to keep the GP cold between feedings.
12. OPTIONAL: For feeding sticklebacks in experimental jars, dispense GP “manually” from an aliquot using a flat spatula. Again, when feeding to approximately satiation, add small amounts GP in multiple rounds so that you can make sure the fish clear what you give them after each pass. Spot-feed as needed and remove any never-to-be food on the bottom of the jars.

*Effect of enhanced feeding regimens on growth.*— We have observed substantial size increases in juvenile sticklebacks fed the enhanced diet of enriched brine shrimp nauplii and GP when compared to similarly aged fish from prior years when SELCO and artificial pellets were not used. For example, in 2012 we only began to implement Regimen C, with nauplii enrichment and GP feeding, mid-summer when the fish were approaching one month of age. At six months of age on this diet, mean standard length (SL) and wet mass of anadromous juveniles from Rabbit Slough were 42 mm and 0.79 g, respectively. According to Clark University researchers, these measurements exceed those of similarly-aged fish that are raised on the traditional diet of plain brine shrimp.

In 2013, we further refined our feeding technique on new stickleback clutches, following Regimen B and feeding the fry SELCO-enriched nauplii from first-feed onward. We also began supplementing meals of GP by two weeks of age. At the end of our briefer experimentation that summer, average SL and wet mass of 10 week-old Rabbit Slough juveniles were 32 mm and 0.34 g. Comparisons of size-at-age measurements to our previous year-classes revealed a large disparity in growth rate: 2013 fish were 20-25% longer and 78% heavier than their counterparts raised on mostly non-enriched nauplii.



## Checklist of Supplies for Fish Room

*Revised: 31 July 2014*

### Embryo Care

- Plastic folding table
- Plastic chairs
- Transfer Pipettes, 3 mL
- Razor blades
- Stereo microscope
- Paintbrushes, size 3/0
- 50 mL centrifuge tubes

### Glassware

- Autoclaved glass Petri dishes, 10 & 20 cm dia.
- Erlenmeyer flasks
- Small and large beakers
- Wastewater bowls
- Glass mason jars (pint, quart, and ½ gallon)

### Aquarium supplies

- 10-gallon rearing aquaria
- Waterfall filters
- Filter media (wool pads, Bio-bricks, shells)
- Power strips
- Cable ties
- Habitat enrichment (4" plastic pipes and plants)
- 3"-5" dip nets
- Vinyl tank labels
- Dry erase markers
- Submersible pumps (350 gph) & ½" tubing
- Extension cords (outdoor)
- Algae scrubber
- Water quality test kit
- Hand-held pH meter

### Water storage & salt mixing

- YSI-85 meter
- 44-gallon barrels with lids and trolleys
- 2 & 5-gallon buckets with lids
- Carboys, 5 gal

- Large plastic "powder" funnels
- Fisher labeling tape (multiple colors)
- ½" tubing for faucet
- ½" barbed adaptors for RO faucet
- Instant Ocean sea salt
- Plastic scoops
- Rubbermaid plastic "Lock-Its" (1.5 gal)
- PVC mixing poles

### Salinity Challenge Set-up

- Large weigh boats with taped metric ruler
- Camera and tripod
- Desk light
- Metric grid
- Air pumps, stones, & rubber bands
- Plastic spoons
- MS-222 aliquot

### Salinity Preference

#### Experiments

- Digital refractometer
- Kimwipes
- Labels
- Rack for draining carboys
- Small, medium, or large Staaland tanks
- Blinds
- Binder clips or clamps

### Feeding

- Mini-fridge
- Brine shrimp cyst can / aliquots
- Measuring spoons
- SELCO
- 74 µm brine shrimp sieve
- Basters (modified)
- Artificial fish food (Golden Pearls)
- Clicker feeder

- Spatula

### Brine shrimp hatchery

- Air pump
- Airline tubing and valves
- Aquatic Habitats Hatchery cone rig (cone, spigot, airline, lid)
- Clip lamps with outdoor floodlights
- Ring stand
- Thermometer
- Flashlight

### Office Supplies

- Pencils, pens and permanent markers
- Scissors
- Roll of transparent tape
- Sticker labels
- Plastic totes

### Cleaning

- Bleach spray bottle
- Ethanol spray bottle
- Parafilm
- Trash can (red barrel and black bags)
- Cleaning brushes
- Scouring pad
- Soap (Alconox)
- Broken glass/sharps bin

### Paperwork

- Clipboards
- Fish room Maintenance, Inspection, and Mortality logs
- Brine Shrimp sheet
- Water quality data sheets
- IACUC protocol approval letter in sheet protector
- OAC forms

### Stickleback Clutch Inventory

1 tally = 25 eggs

<b>Total</b>	100%
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# eggs

*p.*

[illegible]



[illegible]

[illegible]

[illegible]

[illegible]