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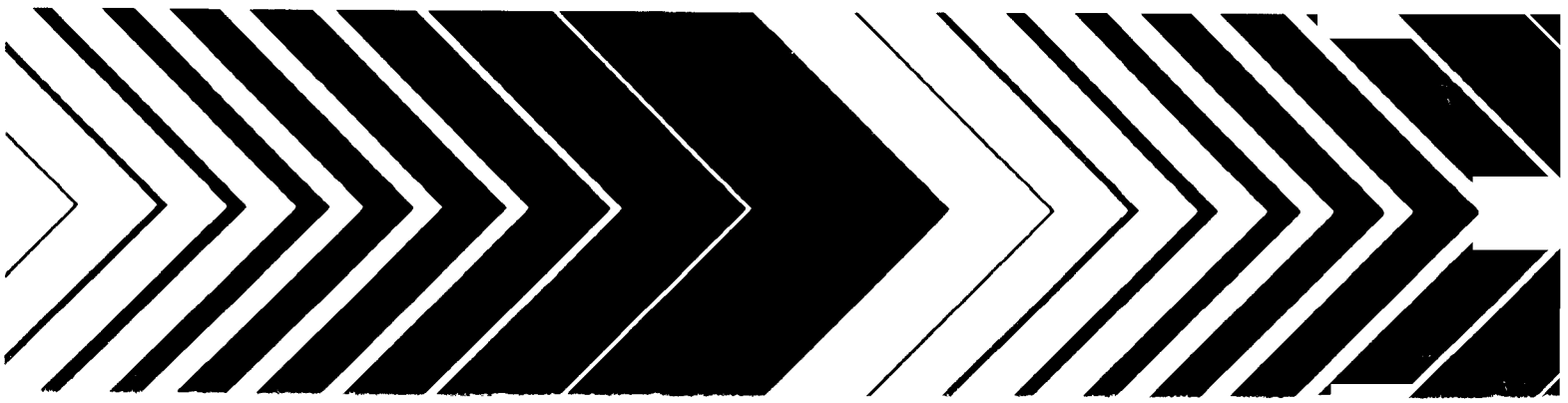
Guidelines for the Culture of Fathead Minnows *Pimephales Promelas* for Use in Toxicity Tests



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GUIDELINES FOR THE CULTURE OF FATHEAD MINNOWS PIMEPHALES PROMELAS FOR USE IN TOXICITY TESTS

by

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NOTICE

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FOREWORD

Fathead minnows, Pimephales promelas Rafinesque, have been cultured at the Environmental Research Laboratory - Duluth for use in aquatic toxicity tests since the establishment of the laboratory in 1967. The techniques and apparatus described in this report were developed over the years by many researchers.

This paper sets forth the conditions and procedures now being used to produce research quality fathead minnow embryos, larvae, juveniles, and adults. These guidelines can be modified to adapt to different circumstances and needs.

ABSTRACT

This paper describes the mechanical apparatus and biological techniques now in use to culture fathead minnows (*Pimephales promelas*), at the US Environmental Protection Agency's Environmental Research Laboratory in Duluth, Minnesota. Physical system information includes water supply, construction materials, water temperature, photoperiod, and the water delivery system. The biological section addresses the selection of spawning fish, incubation of embryos, larval and adult feeding, disease, and gene pool considerations. This document is meant to be a guide for those interested in culturing fathead minnows for use in fish toxicology research.

ACKNOWLEDGEMENTS

For continual help throughout the development of this guide I would like to thank Larry Herman, Barb Halligan, Jodi Collins, and Andy Peterson. For expert advice on the culture of fatheads I would like to thank Barb Halligan, Armond Lemke, Duane Benoit, Dick Carlson, and Don Mount. For manuscript review I am indebted to all of the above mentioned people, as well as to Richard Siefert, Quentin Pickering, J. Howard McCormick, Teresa Norberg-King, Scott Heinritz, John Brazner, and Jim Taraldsen. I extend special thanks to Jodi Collins for illustrations and Eric Peterson for photographic support.

Much of the credit for this document is due to present and former staff members of the Environmental Research Laboratory - Duluth, who have, over the years, developed this system of fathead minnow culture.

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PURPOSE

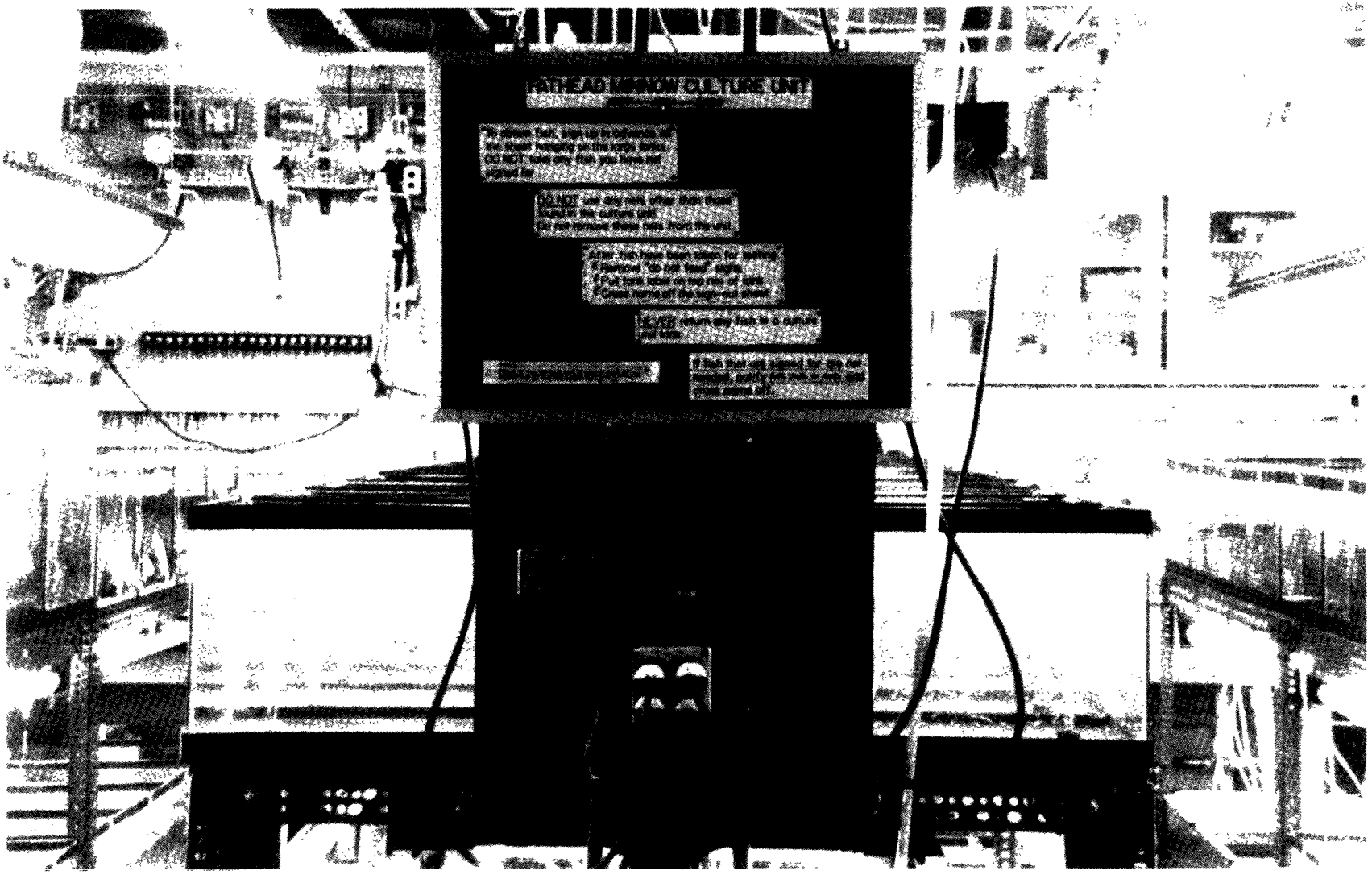
A fathead minnow culture facility provides a continuous supply of embryos or fish of known age, raised under known conditions, for aquatic toxicity testing. The use of laboratory reared animals is advantageous since age and genetic background are known, diet is controlled, fish are free from disease, and are available year-round.

This document describes in detail fathead minnow culture techniques used at the Environmental Protection Agency's Environmental Research Laboratory in Duluth, Minnesota. Present practices are based on years of expertise developed by staff scientists. Local conditions and specific needs of researchers may require modification of these guidelines.

DESIGN

The life stages of fish in greatest demand for testing are less than 24 hr old embryos, 0 - 24 hr old larvae, and 30 day old juveniles. This system is designed to best produce these three life stages.

The fathead culture unit at ERL-D has 54 m^2 of floor space in two adjacent rooms. The main room (36 m^2) contains 24 spawning tanks, 60 juvenile rearing tanks, 20 brood stock tanks, 6 hatching trays for eggs, and 20 more tanks for experimental/emergency use (See Photograph #1). This provides ample space for 96 pairs of adult spawners, 400 - 500 maturing



Photograph 1. View of main fathead minnow culture laboratory.

fish for use as future spawning stock, and about 15,000 juveniles being reared to 30 days of age. This system produces from 1000 - 2000 eggs/day, and provides 400 - 500 30 day old juveniles each day for toxicity testing. The adjacent room (18 m^2) provides space to quarantine and breed incoming wild fish used to intermittently diversify the gene pool.

Production now supplies about 15 - 20 researchers engaged in full time toxicity testing , and fills occasional requests from other scientists. A slight over - production is necessary to allow for variations in egg production and for recycling spare fish into new brood stock.

PHYSICAL SYSTEM

3.1 Tanks

The tanks are 57 liter (15 gallon) glass aquaria, 31 cm x 61 cm x 32 cm deep, with standpipe drains adjusted to provide 20 cm of water depth. These contain approximately 40 liters of water per tank. The tanks, supported on racks made of slotted angle iron and 1.9 cm (3/4 inch) plywood, are arranged in 2 tiers, in rows of 12 tanks. Two rows placed back to back form a bank of 48 tanks. Three such banks make up the entire culture system.

When starting a culture program, new tanks must be acid (10% nitric), and acetone rinsed before using. Used tanks must be disinfected with hypochlorite. Allow culture water to flow

through the tanks for a day or two before adding fish.

For spawning fish, divide the tanks into four sections with stainless steel screen (eg: 5 mm mesh, 0.89 mm wire), glued in place with silicon glue. The screen material must be acid leached before use to remove machine oil. A single water inlet, drain, and air stone can service each tank.

White plastic dishpans, commonly available in department stores (eg: 53 cm x 40 cm x 12 cm deep) make good hatching trays for embryo incubation. White trays provide the best background color for seeing the newly hatched larvae. Place six hatching trays in a temperature controlled water bath (eg: 130 cm x 125 cm x 8 cm deep, constructed from 1.9 cm (3/4 inch) plywood and sealed on the inside with epoxy paint). If this bath is placed in a rack below a bank of fish tanks, drain water from the fish tanks will provide an inexpensive heating source for embryo hatching. Fish tanks at 25°C will warm hatching pans to 23°C.

3.2 Water Supply

Unfiltered Lake Superior water is the water supply for the ERL - D fathead culture unit. It has a pH range of 7.4 - 8.2, alkalinity (as $CaCO_3$) of 42 mg/l, and total hardness of 45 mg/l. For more detailed chemical characterization of Lake Superior water, see Glass (1977).

Water supply must be the most important consideration in establishing a fathead culture facility. Use a natural supply such as a spring, well, or controlled surface water with consistent water quality, if possible. Culture water should be similar to water used in testing. Examine the source for contamination by pesticides, heavy metals, sulfides, disease vectors, or any other suspected contaminants. Make checks of water quality periodically. Filtration may be necessary if well water is used (Mount, 1971). Dechlorinated tap water from a municipal water supply should be used only as a last resort (Benoit, 1982). If the water supply is contaminated with fish pathogens, pass the water through an ultraviolet or similar sterilizer immediately before it enters the system (Allison and Hermanutz, 1977).

Water quality parameters such as hardness, alkalinity, and anions should fall within the following limits: hardness - 40 - 300 mg/l (as $CaCO_3$), and alkalinity slightly less than the hardness. The anions should be those found in a normal stream or lake. Avoid well or spring waters which have high iron, silica, sulfides, or chlorides not found in surface waters.

The quantity of water necessary depends on the size of the intended culture unit. The ERL - D system of over 150 tanks consumes 15 - 20 liters/minute when in full operation. Smaller systems, or systems in areas of limited water supply could operate on a reduced flow. Though less desirable, static renewal or recirculating systems may be effective.

3.3 Water Delivery System

The ERL - D water delivery system is a constant temperature, flow-through system. It is gravity fed, with custom welded stainless steel mixing boxes (46 cm x 28 cm x 40 cm deep) positioned in an open ceiling approximately 3.5 m overhead. Lake Superior water warmed to 20°C flows through a toilet tank valve[1] to maintain water level in the headbox. Water heated to 30°C flows through a solenoid valve[2] positioned over the headbox. To achieve the desired constant temperature, a temperature probe[3] suspended in the headbox controls a solid state temperature controller (Syrett and Dawson, 1975). This controller opens and closes the solenoid valve when resistance of the temperature probe changes due to change in temperature. When the headbox temperature falls below 25°, the solenoid valve activates, adding 30° water until the headbox temperature reaches 25°, when the probe/relay system closes the solenoid valve. Headbox temperature must be maintained at 26- 27° to provide 25° at the tanks. This is due to heat loss to ambient temperature. This basic water delivery system can supply most experimental water

[1] Numbers refer to suppliers that manufacture or distribute the type of product described. A list of suppliers is appended. Mention of trade names or commercial products does not constitute endorsement or recommendation for use by the U.S. Environmental Protection Agency.

temperatures, depending only on the temperature of the water supply to the float and solenoid valves. (See Figure #1).

Air stones or some other type of agitator[4] must be used in the headbox to assure complete mixing and oxygenation, and to prevent supersaturation of gases caused by heating water. Water flows from the headbox through a 1.27 cm (1/2 inch) threadable, polyvinyl chloride (PVC) pipe (schedule 80, available from plumbing supply houses). The outlet is on the side of the headbox, about 2 -3 cm up from the bottom. Detailed instructions on the design of the mixing boxes, electronic relays, etc., are available in Syrett and Dawson, 1972, 1975, and McCormick and Syrett, ms, 1970.

Water flows into the tanks through a 1.27 cm (1/2 inch) PVC pipe manifold. Above each tank there is a T with a 1.27 cm (1/2 inch) to .95 cm (3/8 inch) reducer attached. Into the reducer a 3 ml disposable syringe barrel[5] is glued with silicone glue. This allows the use of different sizes of hypodermic needles[5] to control flow rates. Seventeen gauge needles provide 100 - 150 ml/min with 1 - 2 m head pressure.

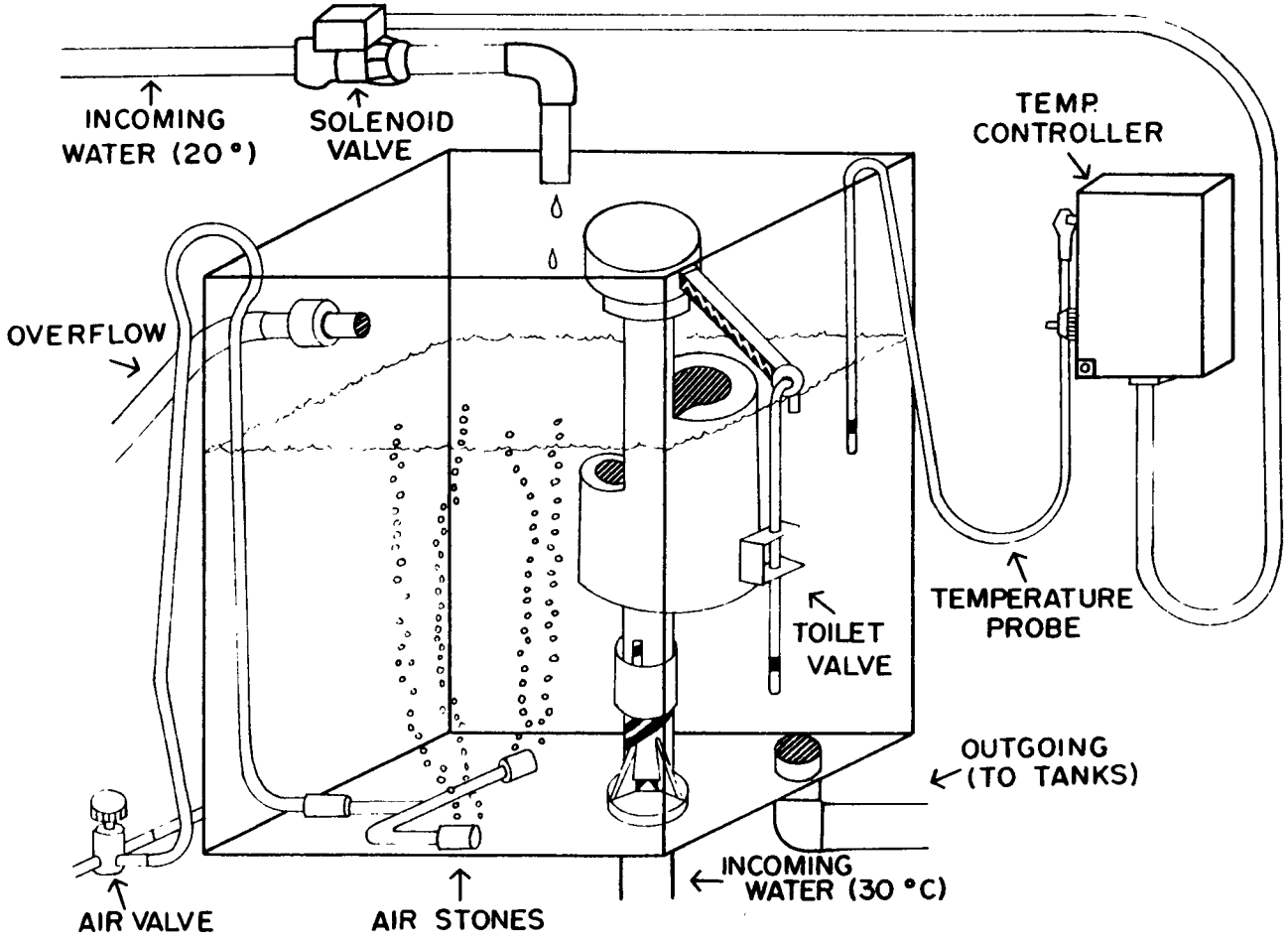


Figure 1. Headbox configuration.

3.4 Construction Materials

Construction materials which come in contact with the water must not contain leachable substances. Rubber, copper, brass, or plastics containing fillers, additives, stabilizers, plasticizers, etc, must not be used (Mount,1971). Glass, stainless steel, teflon,and PVC are the preferred construction materials. All piping should be of rigid PVC. Schedule 80, threadable PVC must be used to avoid the danger of toxicity from PVC glue. Silicone[6] glue is safe to use as long as enough curing time is allowed. (Follow manufacturers instructions for complete curing.) Check all batches of neoprene stoppers for toxicity prior to use. Recent static tests at ERL - D show that certain types of neoprene stoppers are acutely toxic to fathead minnow larvae. A simple 24 hr static test exposing fathead larvae to the stoppers in a beaker of culture water will indicate whether the stoppers are safe.

Ground fault interruptors[7] are necessary on all electrical components because of the close proximity of electricity and water in these systems. Electrical equipment must be three pronged and well grounded. Avoid hanging extension cords, or extension cords on the floor, where tank overflow could cause them to get wet.

3.5 Temperature

Water temperature in all tanks in the ERL - D fathead culture unit is maintained at 25°C ± 1°C for maximum egg production and growth. Brungs (1971a) found that temperatures below 22°C or above 26°C reduced fathead minnow reproduction. The described temperature control system will maintain 25 ± 1°C. Culture systems will function down to 22°C.

3.6 Aeration

Provide continuous gentle aeration to the tanks to maintain dissolved oxygen concentrations above 5.0 mg/l at all times, but avoid vigorous aeration, especially with newly hatched larvae. If a level of 5.0 mg/l cannot be maintained, remove some fish from the tank (Mount, 1971). Brungs (1971b), found that larval growth was the most sensitive indicator of sublethal effects of lowered dissolved oxygen, and significant effects were seen at 5 mg/l. Provide an oil free air supply of at least 150 cc/min per tank. An oil trap or filter may be necessary on some systems. Check the location of air intakes and efficient operation of laboratory air compressors to avoid introducing contaminants.

Arrange air tubing, brass air valves, and air stones, (obtainable in aquarium stores) to provide an airline to each tank. Incorporate a pressure regulator into the system and feed each group of 6 or 12 tanks from a central, larger diameter

manifold. A pressure regulator on the main manifold will allow use of valves with slip-on tubing, without blowing the tubing off.

3.7 Photoperiod

Use lights that simulate the wavelength spectra of sunlight. A combination of Durotest Optima FS[8] and wide spectrum Sylvania Gro - Lux[9] fluorescent tubes has proven satisfactory in the ERL - D system. Light intensities at the water surface should average 400 - 500 lux. The photoperiod should be constant at 16 hours light/8 hours dark (Mount, 1971). Gradual changes in light intensity at dawn and dusk may be included within the photoperiod if desired (see Drummond and Dawson, 1970). Paragon Model #4001 timers[10] control photoperiod in the ERL - D system. Lighting warms the culture water, and this effect must be considered as part of the overall design.

3.8 Spawning Substrates

Since fathead minnows deposit their adhesive eggs on the underside of submerged or floating objects, the spawning substrate provided must yield a similar surface. The ERL - D culture system uses fiber cement water pipe, 7.6 to 10.2 cm in diameter, cut into 7 - 10 cm long sections. When halved lengthwise and inverted, these pipe sections form a semicircular

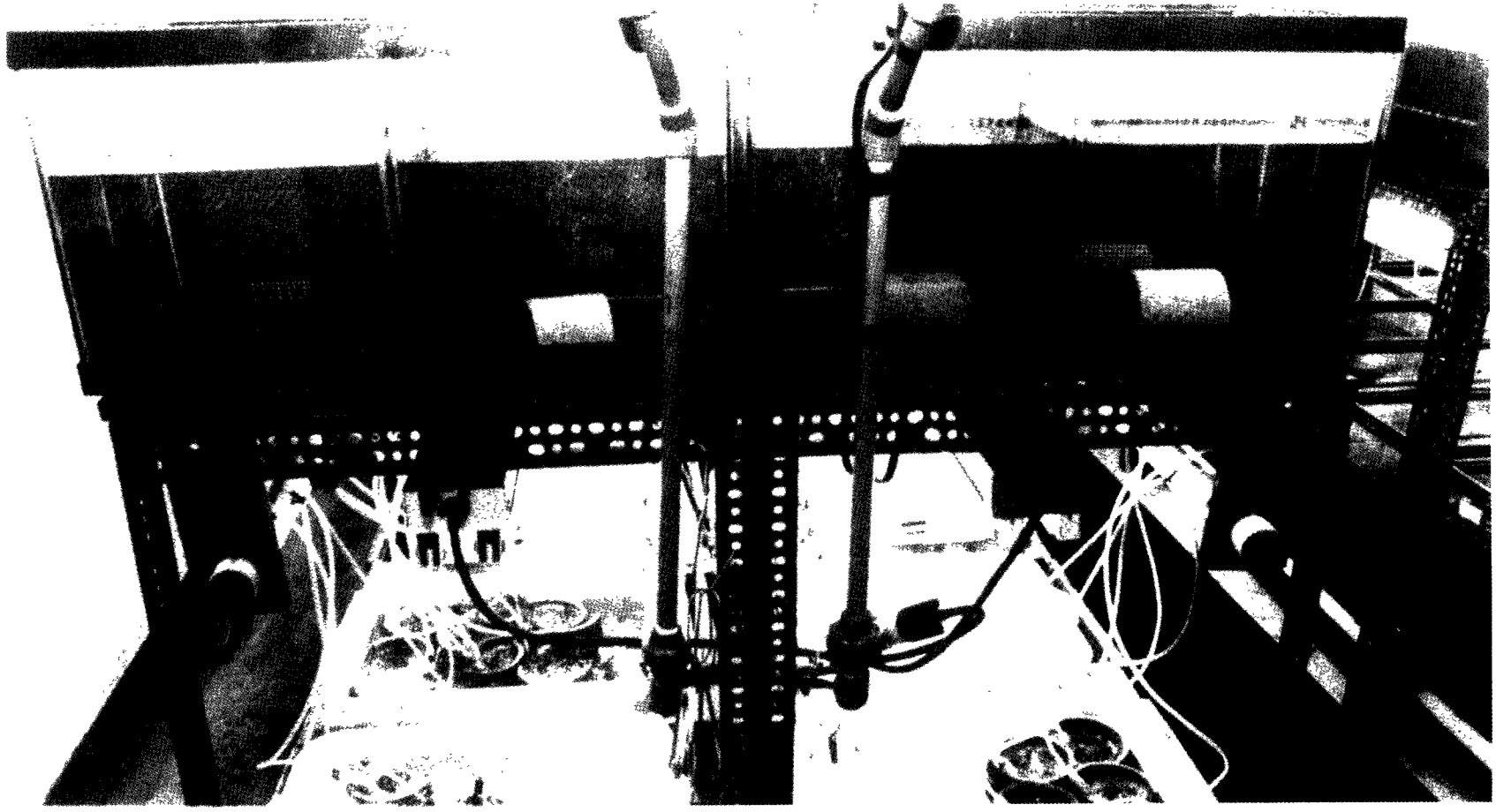
arch. Benoit (1982), used stainless steel (16 - 18 gauge), cut and bent into the same size and shape with a thin layer of quartz sand glued on the underside with silicone glue. This type of substrate can come unglued after prolonged use, however. Gale and Bunyak (1982), successfully used PVC pipe in the same configuration. (See Photograph #2 for general view of spawning and egg incubation apparatus).

3.9 Fail Safe Devices

An alarm system or fail-safe device will protect against temperature control or water flow failure. Temperature recorders[11] monitored hourly by security personnel are in use at ERL - D. Alternatively, use high - low alarm systems, or solenoid valves to shut off water to the tanks if the temperature rises above 28^{o} or falls below 20^{o}C.

3.10 Tank Cleaning

ERL - D personnel scrape tanks and siphon residue on a biweekly, rotating schedule. Some tanks are siphoned between cleanings if necessary. Most culturists allow light growths of algae, rotifers, etc, to remain, for these provide a dietary supplement for fish. Excessive growths of blue-green algae or fungus must be removed. Test cleaning tools for possible toxicity before use.



Photograph 2. View of spawning tanks, showing screen dividers and spawning substrates. Also shown are the water delivery system and the hatching trays with embryos incubating on the spawning tiles.

3.11 Disturbance

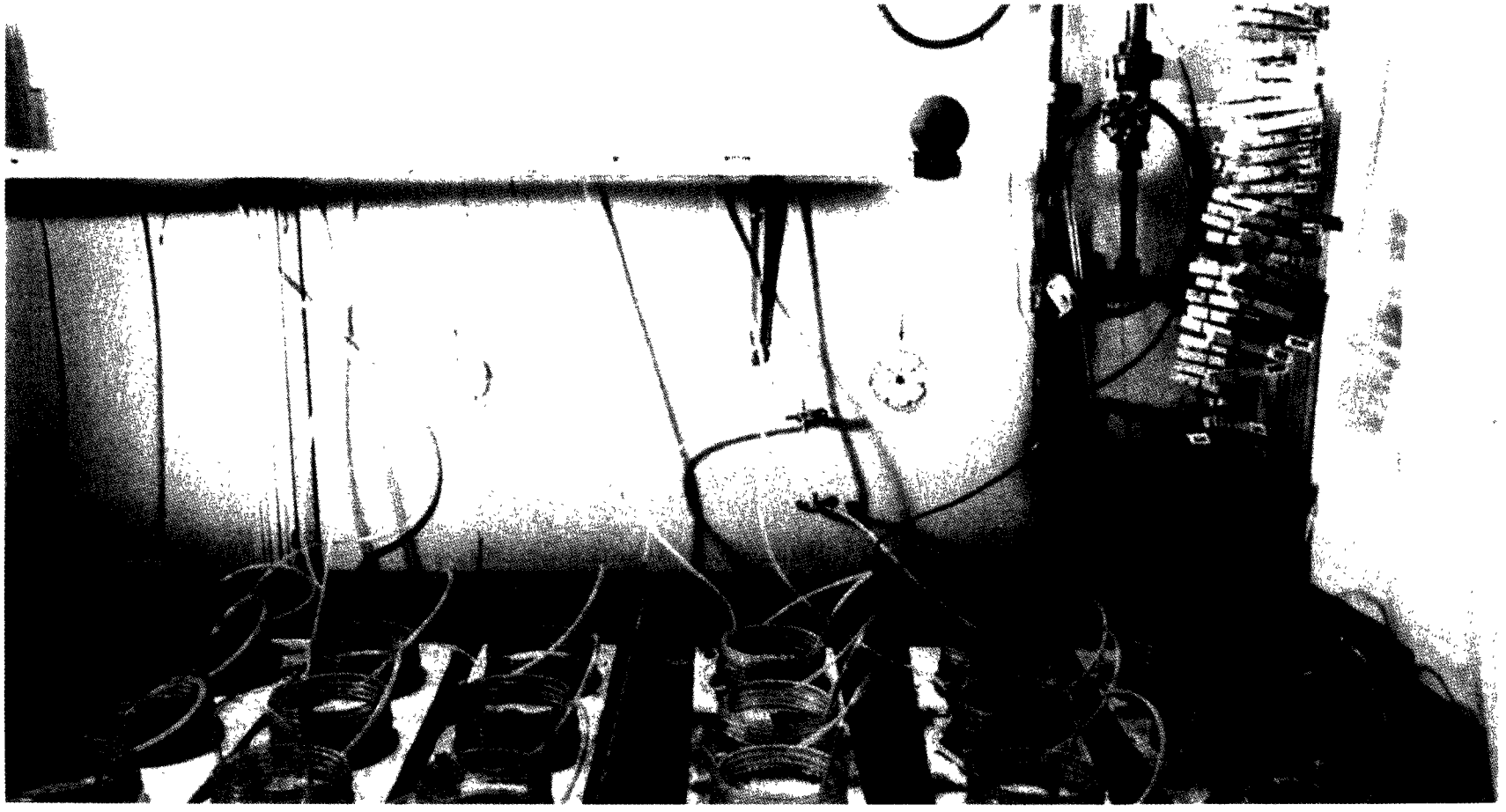
Shield the fish from continual or drastic disturbance. Avoid construction noises, continual human presence, and extraneous lights that might alter the photoperiod.

3.12 Brine Shrimp (Artemia spp.) Hatchery

The brine shrimp hatchery at ERL - D consists of a rectangular fiberglass tank, 2.1 m x 0.6 m x 0.5 m deep, that serves as a water bath. (See Photograph #3). Rails mounted on the inside, 9 cm down from the top, support 10 to 12 movable plywood racks (53 cm x 15 cm). Each of these racks has three 11.5 cm diameter holes that hold brine shrimp hatching jars. A manifold of airline tubing provides an air supply to each hatching jar.

Brine shrimp will hatch in any container with a conical bottom, as long as air bubbled at the bottom of the cone keeps the eggs in constant motion. Some culturists use separatory funnels, and others use inverted 1 liter plastic soda bottles with the bottoms cut out.

The hatching jars in use at ERL - D are made from round bottom glass light fixtures[12], about 11 cm in diameter and 24 cm long. A ring of airline tubing glued around the jar 5 cm from the open end suspends each jar through a hole in the plywood



Photograph 3. Brine shrimp hatchery.

rack, partially submerged in the water bath. The capacity of each jar is about 1.5 liters. This system provides for up to 36 hatching jars. A temperature probe and controller system similar to the headboxes feeding the fish tanks maintains the bath at 25°C.

BIOLOGICAL SYSTEM

4.1 Obtaining Brood Stock

Fish that are free of disease and adapted to laboratory conditions make the best initial brood stock. For the least risk of disease, and greater ease of shipment, begin with embryos. Use of embryos also avoids any bioaccumulation of toxicants that may occur with adults. Less desirable is the use of fish captured in the wild or purchased from a bait dealer. Take care to verify that the animals are Pimephales promelas, and not a related species. Examine all fish, and especially wild caught or bait dealer fish, for signs of disease. Use prophylactic treatment (described in section 4.7) to insure good health. These fish should then be bred through one full generation to determine vigor, fecundity, and freedom from disease before use in toxicity testing.

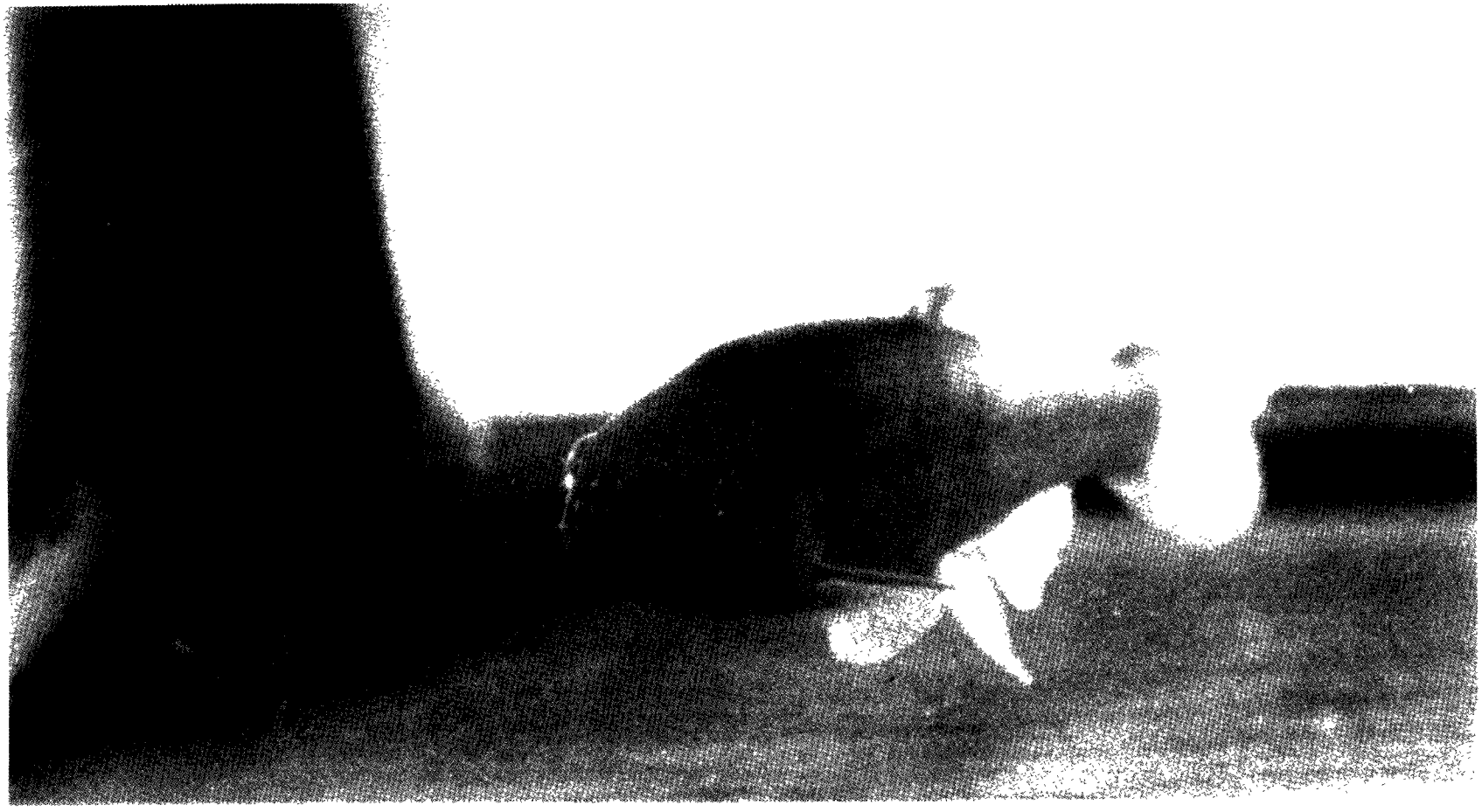
4.2 Selection of spawning fish

Determination of the sex of immature fathead minnows is nearly impossible, which makes it difficult to select mating pairs before the fish actually begin breeding. Breeding males develop a conspicuous gray pad of spongy tubercles on the dorsal surface anterior to the dorsal fin, and two rows of strong tubercles across the snout. The sides of the body become almost black except for two wide vertical bars which are light colored. Another characteristic of the breeding male is the presence of a dark spot at the anterior insertion of the dorsal fin. Females remain quite drab (Eddy and Underhill, 1974). The female fathead minnow exhibits an ovipositor at least a month before spawning (Flickinger, 1969). (See Photographs #4 & 5, and Figure #2).

Breeder fish are selected as follows: juvenile fish 3 - 4 months old are stocked at a density of 30 - 40 fish per 15 gallon tank, and provided with 2 or 3 spawning substrates as previously described. The presence of spawning substrates hastens the maturation process. In 1 - 2 weeks some males in the tank will show signs of maturing. Females will begin to become gravid soon after the males exhibit spawning color. For observation, net fish from the tank and place individually into a 400 ml beaker with approximately 3 cm of water. Sexual maturity can then be determined as previously described. Backlighting makes the female ovipositor easier to see.

Removal of the mature fish for service as spawners will

Photograph 4. Sexually mature female fathead minnow. Note presence of ovipositor between pelvic and anal fins.



Photograph 5. Sexually mature male fathead minnow. Note presence of breeding tubercles on snout, and black spot at anterior margin of dorsal fin.

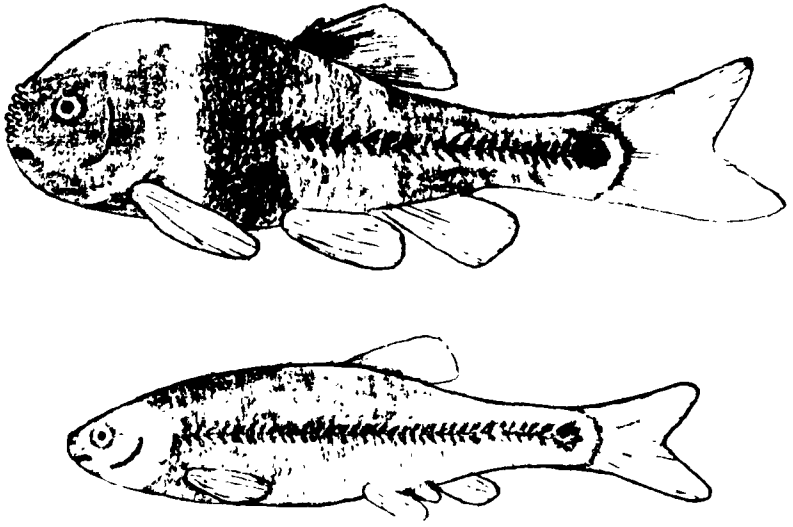


Figure 2. Male (top), and female fathead minnows. Note breeding tubercles, dorsal pad, and black spot on dorsal fin of male, and ovipositor of female. Length of average male is approximately 7.5 cm, and average female 5.5 cm.

stimulate the subordinate juveniles in the brood stock tanks to ripen, and take the places of the dominant fish that were removed from the hierarchy. This method will provide a continuous source of mature fish. Some of each month's leftover larvae or juveniles not used in testing must be set up in brood stock tanks to begin the maturation process. The ERL - D system uses 16 brood tanks, along with 8 larger holding tanks (208 liters) for bringing fish to maturity.

4.3 Spawning

The fathead minnow is an intermittent, multiple spawning species with an extended breeding season, possibly spawning intermittently all summer (Hasler, 1946; Radcliff, 1931). Under controlled culture conditions fathead minnows will spawn throughout the year.

Fathead minnows usually spawn beneath objects, even under such transient objects as old maple and oak leaves (Isaak, 1961). Males clean the underside of the object selected for the nest site using the head tubercles in a scraping action and pulling pieces of algae and associated debris off the nest surface with the mouth (Andrews and Flickinger, 1973). The male and female swim back and forth beneath the prepared overhead site and roll on their sides to emit the sex products. Close lateral contact and body vibration characterize the act of spawning. McMillan (1972) describes the spawning act as follows: "Finally, when a

sufficient degree of vibratory stimulation has been reached, the male lifts and presses the female's ventral surface against the object's underside. In doing so, he turns so that he is beneath the female and can use the posterior part of his body to manipulate her upward. The tubercules on his large pectoral fins also help him to grip the female tightly. As the fishes' bodies are taut and strained in this position, the female emits one or perhaps several eggs, and the male probably releases sperm at this instant. Then they abruptly separate, although a new bout of vibrating may begin only seconds later."

The buoyant, adhesive embryos stick to each other and to the undersurface of the nesting object. After deposition is complete, the male remains at the nest site tending and defending the embryos until hatching occurs (Andrews and Flickinger, 1973). More than one female may spawn in the male's nest, and up to 12,000 embryos have been found in one nest (Markus, 1934), indicating that several females contribute to the embryo mass.

Egg counts in wild mature females have ranged from 800 to 9,000 eggs per female, depending on size of female, water temperature, geographic strain, etc. Unpublished data indicate an average of 258 eggs/spawn and an average of 3095 total eggs/female during 100 days of spawning activity (Olson, 1974).

For spawning tanks, a standard tank is divided into 4 chambers as previously described. Each spawning chamber is then approximately 15 cm x 30 cm. A spawning substrate, and a pair of sexually mature fish is placed in each section, for a total of

four pairs per spawning tank. There are 24 spawning tanks in the ERL - D system, for a total of 96 spawning pairs.

Separation into spawning pairs is not essential. Other investigators have reported success with male to female ratios of 2/4 (Olson, 1974), 3/6 (Benoit and Carlson, 1977), and 4/10 - 15 (Mount, 1971). The males are territorial, so at least as many spawning substrates must be provided as there are males in the tank to achieve optimum egg production.

Paired spawning reduces fighting and competition between males. When ERL - D began to use the paired method, the number of embryos produced in the culture unit almost doubled (Benoit, 1982). Paired spawning also allows the culturist to follow the fecundity of individual fish. Sterile or "spawned out" fish identified through daily spawning records can then be replaced to maintain egg production. A good procedure is to review daily spawning records every three weeks. If a pair has not spawned during that period, discard both male and female and replace with new spawners.

4.4 Embryo Incubation

Each morning at approximately 10:30 am (7 days/week), all spawning tiles are checked for the presence of embryos. It is not necessary to remove the tile from the tank to see the embryos. Gently running the fingertips on the undersurface of the spawning substrates will reveal their presence. If no

embryos are present, the spawning substrate is left in the tank. This creates less disturbance than daily removal, inspection, and replacement of each substrate (See Photograph #6).

When checking for embryos, estimate and record the number deposited by each spawning pair. Provide fresh tiles for each chamber from which embryos were removed. Researchers requiring embryos less than 24 hrs old for testing can take charge of the embryos at this point. If the embryos are to be incubated, leave them on the spawning tiles, and place the tiles in the dated hatching tray. Wipe the tops of the tiles clean with a sponge to reduce transfer of sediment to the hatching pan. Place tiles on end, with two tiles pushed together to form a circle, and place an airstone between them to circulate water. (See Photograph #7). Inspect embryos daily, and remove any dead ones with tweezers to decrease the spread of fungus. (Nonviable embryos are opaque or clear with a white "dot" inside where the yolk has precipitated. Viable embryos will be clear for the first 36 - 48 hrs until they reach the eyed stage). If fungus has attacked more than 50% of the embryos on a particular tile, discard the entire spawn on that tile.

At 22^{o}C embryos will begin to hatch in 5 days. If embryos have been removed from spawning chambers each day, so that the age of each group is known to within 24 hours, then 95% should hatch within a 24 hour period. If a toxicity test is planned that calls for less than 24 hr. old larvae, provide the fish to the researchers at this point. If larvae are to be

Photograph 6. Fathead minnow embryos on the underside of a spawning substrate.

Photograph 7. Spawning substrates containing embryos, incubating in hatching trays.

reared to 30 days old for testing as juveniles, or to adulthood for future brood stock, remove them from the hatching tray using a large bore, 50 ml volumetric pipette. Count larvae into lots of 250, and stock into rearing tanks (15 gal tanks with appx 20 cm water depth). Label each rearing tank with the date of hatch. Thirty day old juveniles that weigh approximately 150 mg each (wet weight) are the desired goal, although fish in the range of 100 to 300 mg per fish are acceptable (EPA, 1982).

Clean spawning tiles after each use by soaking them in a mild HTH[13] bath (sodium hypochlorite, appx 12 g HTH/liter) for 1 hour. Follow with a tap water rinse, and then soak tiles in appx. 5×10^{-3} M sodium thiosulfate for at least 10 minutes to remove residual chlorine. After a final rinse in culture water, set tiles aside to dry and store for reuse.

4.5 Larval Feeding

All fathead minnow larvae less than 30 days old are fed live brine shrimp twice each day. Since larvae begin feeding during the first day of hatch, feeding must start immediately. It is important that the brine shrimp nauplii be small enough for the fathead larvae to ingest (Norberg and Mount, 1985). Gape size of larval fathead minnows is in the range of .24 - .28 mm, so the "width" of the nauplii when offered must be slightly less than this for the nauplii to be ingested. Growth rates depend on the brine shrimp nauplii used, since different strains of the

cysts vary in nutritional adequacy (ASTM Draft No. 2, 1984). The brand now in use by the ERL - D fathead culture unit is Biomarine[14] which has yielded more uniform growth than some others. Any strain that offers a good hatching percentage, small size of nauplii upon hatch, nutritional adequacy, and freedom from contaminants will yield good results. Some culturists also supplement the larval diet with powdered or flake foods to insure a more rounded diet. Opinions vary as to the efficacy of this supplementary feeding. Some trout chow formulations have proven to be inadequate fathead minnow diets in experiments in our laboratories.

Conditions for hatching each strain of cysts are usually provided by the supplier. Live nauplii are harvested from 24 - 48 hrs after set-up at 25°C. To harvest, remove the air tube from the hatching jars approximately 15 minutes before feeding, to allow the live shrimp to settle. Unhatched cysts will form a brown layer at the bottom of the hatching jar, while the live shrimp will form a bright orange layer just above the unhatched cysts. The layer of live shrimp is then siphoned from the hatching jars into a 1 liter beaker. Rinsing the brine shrimp is not necessary in flow-through systems with large tanks. In static systems rinsing is desirable to avoid salt build up. Exact quantification of shrimp fed to each tank is not necessary as long as all fish receive an adequate amount. Inspect tanks 10 - 15 minutes after feeding. If all shrimp have been eaten in this short time, provide more. It is important that larvae be

feed ad libitum, especially during the first few weeks of life. Approximately 50 ml of the concentrate is fed to each tank of 250 larvae, twice per day. Larvae less than 1 week old require slightly less. Nine 1.5 liter jars started with approximately 25 cc of eggs each, provide two feedings per day for 60 tanks of 250 larvae each. Different brands, feeding schedules, and stocking densities may alter the amount required.

Adult Feeding

Feed spawning pairs, replacement brood stock, and any other fish over 30 days old frozen brine shrimp[15] twice per day. Allow frozen brine shrimp to thaw slightly (not fully) for ease of handling. Feed fish ad libitum, with each spawning pair receiving 1/8 - 1/4 teaspoon, and other tanks according to number of fish. A rule of thumb is that the right amount of food will be consumed in about 10 minutes.

4.7 Disease

Discard any diseased lots of fish and disinfect the tanks with hypochlorite. To minimize the risk of spreading disease between tanks, disinfect all nets, siphons, brushes, and other tools if disease is thought to be present. Adding fifty ml formalin[16], and 10 ml Roccal II[17] to 50 liters of water in a plastic garbage can makes a handy disinfectant bath.

Fungus on eggs is not considered a disease (Mount, 1971). Fungus is ubiquitous in the aquatic environment, and its presence should not be considered a valid reason to discard all associated embryos unless the labor involved in picking and sorting out the bad embryos is not worth the return at hatch.

Examine fins, skin, and gills of fish from random tanks monthly for parasites. Perform other disease checks sufficient to assure that disease is not a problem. If present, it is best to destroy fish from infected tanks. Treatment is in order only if the fish cannot be sacrificed.

Treated fish are rarely, if ever, used in bioassays. Disease treatment does have a place, however, for fish brought in from the wild. When bringing in wild brood stock the following prophylactic treatments may be useful. First, bring fish to 25°C from their arrival temperature, at a rate not to exceed 2°C per day. After an extra day at 25° for acclimation, disease treatments begin, following the methods of ORSANCO (1974). Perform disease treatments on incoming fish in a quarantined area, away from the main culture unit, if possible.

Treat fish with potassium permanganate for external parasites. Using a 1% $KMnO_4$ solution, add 1.0 ml per liter of water. The water will turn dark purple, and must be aerated vigorously during the treatment. After 1/2 hour, neutralize the $KMnO_4$ with .01 ml/liter of 0.1N sodium thiosulfate. Within 15 - 20 minutes the solution will turn yellow - brown. Siphon the tank or drain it down as far as possible without stressing the

fish, and then refill. Give this treatment for two consecutive days, and curtail feedings during this time.

Once a day for the next four consecutive days use a tetracycline bath, at the rate of 25 mg/liter, for bacterial control. After a one hour static bath turn the water on, so that the initial dose is being slowly diluted. Do not drain the tanks after this treatment, and feed the fish during the treatment. Tetracycline can also be added to the feed as an antibacterial drug against Aeromonas, Hemophilus, and Pseudomonas. The recommended dose is 2.5 - 3.75 g/45 kg fish/day for 10 days, in feed (Schnick, et al, 1986).

4.8 Gene pool

The Environmental Research Laboratory - Duluth periodically (every 2 years) mixes existing brood stock with healthy wild minnows to eliminate the risk of developing a homogenous strain (Benoit, 1982). Homogenous genetic stock may provide smaller variance in test results, but it correspondingly reduces the strength of possible inferences that can be made. Excessive inbreeding leads to increased rate of genetic deformities. When bringing in fish for the infusion of new genes make every attempt to obtain the healthiest fish possible. Fish that show any sign of disease, or that have a known history of exposure to disease, dissolved oxygen stress, or exposure to other environmental perturbations must be rejected. Fish that are selected for the

outbreeding process must be subjected to the previously described temperature acclimation and disease treatment measures.

After acclimation and treatment, set up the new brood stock in an area separate from the main culture unit. Maintain these fish in quarantine, and breed through the second generation using the same procedures as in the main culture unit. When second generation fish are sexually mature, mix in spawning pairs with original brood stock, in combinations of new male/existing female and new female/existing male. The generation resulting from these crossings will be the new brood stock for the main culture unit. Replace one fourth of the spawning pairs in the culture unit every three weeks until all pairs have been changed. Consult researchers using the fish in toxicity tests so that they are aware that new genes will be entering the pool.

4.9 Record keeping

Maintain records on the estimated number of eggs spawned each day by each spawning pair. Also record the date that each spawning pair was introduced, and the dates on which each holding tank was stocked with larvae. Measure and record routine water chemistries (pH and D.O.) at least twice weekly on random tanks. Records on source of new brood stock, date of arrival, disease treatments, and procedures used for mixing into the gene pool are also important. Record all mortalities in the culture unit, and observe fish daily for abnormal appearance or behavior.

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