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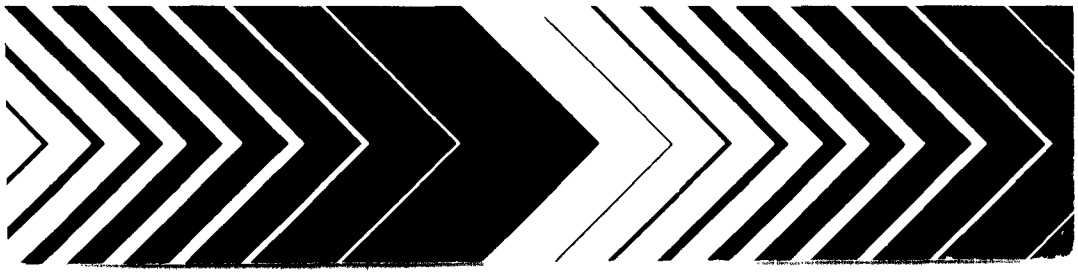
Methods for Spawning, Culturing and Conducting Toxicity-Tests with Early Life Stages of Four Atherinid Fishes:

The Inland Silverside, *Menidia beryllina,* Atlantic silverside, *M. menidia,* Tidewater Silverside, *M. peninsulae* and California grunion, *Leuresthes tenuis*



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Methods for Spawning, Culturing and Conducting Toxicity-Tests with Early Life Stages of Four Atherinid Fishes: The inland silverside, *Menidia beryllina*, Atlantic silverside, *M. menidia*, tidewater silverside, *M. peninsulae* and California grunion, *Leuresthes tenuis*

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FOREWORD

Timely assessment of the environmental risks of pesticides and toxic substances to fish requires that test species be readily available. Methods for the acquisition, spawning, culture and testing of the early life stages of marine and estuarine fishes should be formulated and presented in a format that will enable the experienced aquatic biologist to conduct tests with minimal difficulty. Moreover, a compilation of methods should provide for utilization of fishes from all coastal regions of the United States.

This manual presents methods for field and/or laboratory spawning of four species of atherinid fishes including:

- Inland silverside, *Menidia beryllina*

  Estuarine populations - Cape Cod, Massachusetts to Texas. Freshwater populations - States adjacent to Mississippi River Basin; and Texas, Oklahoma, California.

- Atlantic silverside, *Menidia menidia*

  Estuarine populations - Maine to N.E. Florida.

- Tidewater silverside, *Menidia peninsulae*

  Estuarine populations - N.E. Florida to Texas.

- California grunion, *Leuresthes tenuis*

  Coastal populations - San Diego to Los Angeles, California.

Procedures are also presented for culturing and conducting acute and early-life stage toxicity tests with each of the species. All of the methods have been used extensively by investigators at the Gulf Breeze Environmental Research Laboratory. Guidelines provided in this manual are based upon a compilation of studies that have been published in the peer-reviewed literature.

ABSTRACT

Procedures are presented for spawning, culturing and conducting acute and chronic toxicity tests with four atherinid fishes: the inland silverside, *Menidia beryllina*, Atlantic silverside, *M. menidia*, tidewater silverside, *M. peninsulae*, and California grunion, *Leuresthes tenuis*. Guidelines also are provided for growing of food organisms (*Chlorella* sp., *Brachionus plicatilis*, and *Artemia* sp.) that are required for successful culture and testing of the atherinid fishes.

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CONTENTS

I. INTRODUCTION

This methods manual provides guidelines for conducting toxicity tests with four atherinid fishes: the inland silverside, *Menidia beryllina*; Atlantic silverside, *Menidia menidia*; tidewater silverside, *Menidia peninsulae*; and California grunion, *Leuresthes tenuis*.

We have conducted research to determine optimal conditions for collecting, handling and transport of the three species of silversides and for field stripping, fertilization and shipment of embryos of the California grunion. Methods have been developed for laboratory spawning of silversides and for incubation of embryos and culture of larvae of all four atherinids. Toxicity test methods also were developed. The methods described include acute static and flow-through procedures as well as early life-stage (ELS) test methods for each species.

An important aspect of fish culture is the availability of adequate food resources. This manual provides information required for growth of the mixohaline rotifer, *Brachionus plicatilis*, and *Artemia* sp. nauplii. These two food items are essential for successful culture and testing of the larval atherinid fishes.

The manual has been formatted to provide a complete synopsis of culture and testing procedures for individual species. To avoid redundancy and save space, sections describing procedures for *M. menidia* and *M. peninsulae* at times refer the reader to methods previously described for *M. beryllina*.

Other procedures for fish transport, culture of embryos and larvae, and exposure to toxicants may work equally well. Our recommended methods have been used repeatedly with success at the Gulf Breeze Environmental Research Laboratory.

II. Biology of the Atherinids

A. The inland silverside, *Menidia beryllina*, and the Mississippi silverside, *Menidia audens*, are now considered conspecific (Johnson, 1975; Chernoff et al., 1981).

1. Geographical distribution -- *M. beryllina* is a ubiquitous resident of estuaries, coastal rivers and numerous lakes from Massachusetts to Texas and is also found in the Mississippi River Basin (Sisk and Stephens, 1964; Gomez and Lindsay, 1972; Clay, 1975; Johnson, 1975; Chernoff et al., 1981). Moreover, it occurs in Clear Lake, California where it was introduced in 1967 (Cook and Moore, 1970) and has since moved to the Sacramento-San Joaquin River System (Moyle et al., 1974), and the Lexington Reservoir (Fisher, 1973). The general biogeographic distribution of *M. beryllina* is shown in Figure 1 and a compilation of selected biogeographical data are provided in Appendix A. These data, along with the literature citation for each entry, provide a detailed listing of potential collection locations for each of the species described in this manual.

2. Ecology and reproduction -- *M. beryllina* is euryhaline, living in freshwater lakes, rivers and reservoirs, and in coastal areas at salinities from 0 to 35 ‰ (Robbins, 1969; Hubbs et al., 1971; Echelle and Mosier, 1982). Although estuarine forms seem to prefer salinities of 19 ‰ or less (Johnson, 1975; Middaugh et al., 1986); in the Laguna Madre, Texas, *M. beryllina* has been found at a salinity of 75 ‰ and reported as abundant at 45 ‰ (Simmons, 1957). The duration of reproductive activity varies according to geographic location and, apparently, water temperature. Sexually mature *M. beryllina* are found in June and July at Woods Hole, Massachusetts (Rubinoff and Shaw, 1960). Although the spawning period is also brief in Rhode Island, lasting for only several weeks (Bengtson, 1984) it has been noted that if *M. beryllina* from these latitudes are maintained in the laboratory, they will continue to spawn throughout much of the year (pers. comm., Bengtson, Dept. of Food Science and Nutrition, Univ. of Rhode Island, Kingston, 02881). In the Chesapeake Bay, spawning occurs from early April to late September (Hildebrand and Schroeder, 1928), at Beaufort, North Carolina from March to September (Hildebrand, 1922) and in Tampa Bay, Florida, throughout the year with the

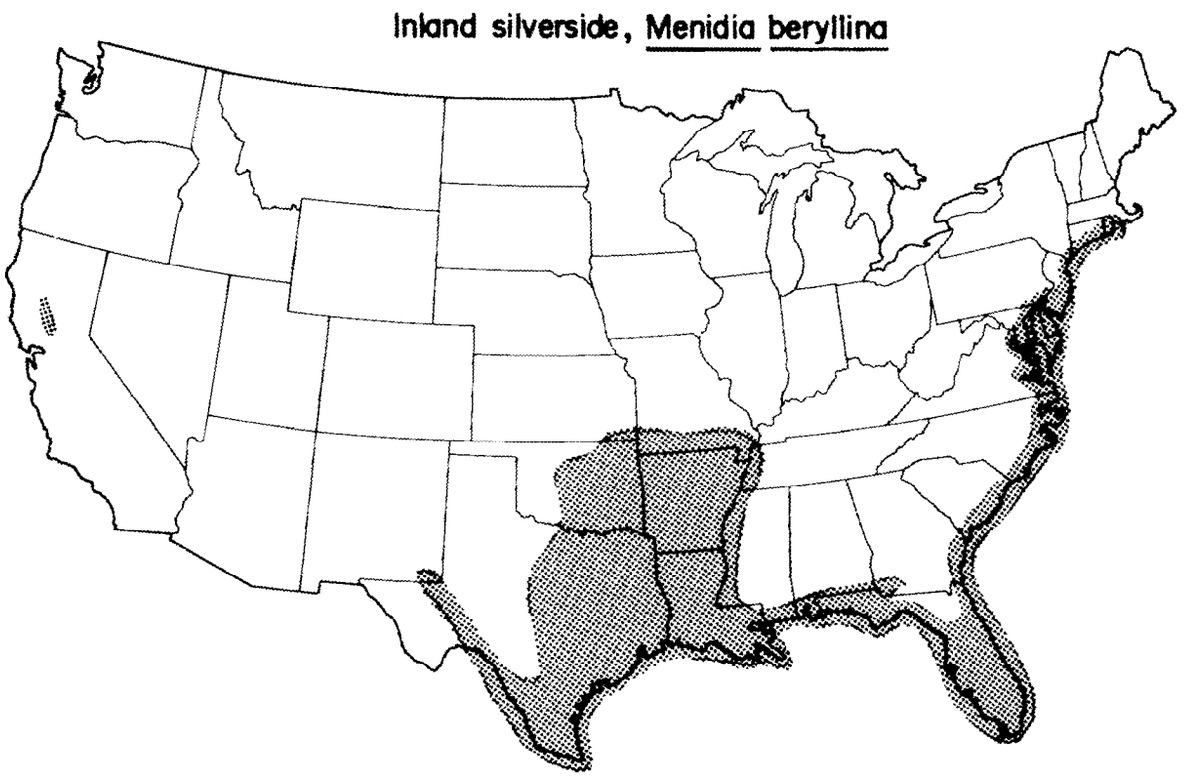

Figure 1. Biogeographical distribution of the inland silverside, *Menidia beryllina*.

exception of January and August (Springer and Woodburn, 1960). In coastal Texas, Gunter (1950) reported gravid females in February and March at respective water temperatures of 20 and 25°C. In Lake Texoma, Oklahoma, reproduction generally occurs from late March through mid-July at water temperatures of approximately 15 to 30°C (Mense, 1967; Hubbs et al., 1971; Hubbs, 1982). Populations in Lexington Reservoir, California, spawn from early May until mid-September (Fisher, 1973). Clear Lake populations spawn from about late March through early July (Cook and Moore, 1970).

3. Identification -- The inland silverside, *Menidia beryllina*, and the Mississippi silverside, *Menidia audens*, are now considered conspecific (Johnson, 1975; Chernoff et al., 1981). The largest inland silverside, *Menidia beryllina*, examined by Robbins (1969) was a female 90.7 mm standard length (SL). Females attain a larger size than males. Scales are large, usually well imbricated, and with well developed circulii and radii. Usually there are not more than 37-50 scales in the lateral series (Robbins, 1969; Chernoff et al., 1981). The first dorsal fin has 2-7, usually 4 or 5 spines, with the origin well in advance of the anal fin origin and lying over a point above the anterior edge of the anus. The second dorsal fin has one spine and 7-11, usually 9 or 10, rays. The anal fin has one spine and 13-20, usually 15-17, rays (Robbins, 1969). The gas bladder in *M. beryllina* is long and translucent (Echelle and Mosier, 1982), and extends to a position approximately above the fourth anal fin ray (Robbins, 1969). It is bluntly rounded posteriorly and is a good characteristic for use in the field to quickly identify *M. beryllina* and separate this species from *Menidia menidia* and *M. peninsulae* which have a truncated opaque gas bladder. Another diagnostic characteristic for separating *M. beryllina* and *M. peninsulae* is measurement of the horizontal distance between the origins of the first spinous dorsal fin and anal fin (Chernoff et al., 1981). This measurement is ≤7% of SL in *M. beryllina* and ≥7% of SL in *M. peninsulae*. Moreover, mature and hydrated *M. beryllina* eggs, 0.9 to 1.1 mm diameter, possess 1 or 2 long-thick filaments (length usually is equal to 15 to 30 egg diameters) and 1 to 15 short-thin filaments. In contrast, fully hydrated *Menidia menidia* and *M. peninsulae* eggs possess 15 to 50 short-thin filaments and no long-thick filaments. *M. beryllina* also may be taken in freshwater areas where the brook silverside, *Labidesthes sicculus*, is present. *M. beryllina* is more robust than *L. sicculus*. Viewed from above, the premaxillary of *L. sicculus* is pointed, forming a cone shape while that of *M. beryllina* is crescent shaped, not forming a

pointed beak. *L. sicculus* scales are also smaller than those of *M. beryllina* (Blair et al., 1968).

4. Collection, handling, spawning and transport.

   a. Where -- Sexually mature fish generally will be available in lakes, rivers and estuarine habitats from March or April through August at water temperatures of 15 to 30°C (Hubbs, 1982; Hubbs, 1976; Rubinoff and Shaw, 1960; Hildebrand and Schroeder, 1928; Springer and Woodburn, 1960; Gunter, 1945). Certain locales such as the Upper Laguna Madre, Texas have populations that spawn throughout the year (Simmons, 1957) while at northern latitudes, such as in Rhode Island estuaries, the breeding season may be as short as two or three weeks in late June and early July (Bengtson, 1982). *M. beryllina* frequents shallow waters along shorelines where sandy to partially vegetated substrates occur. Beaches, bordering open but protected waters, are preferable for collecting. In Lake Texoma, *M. beryllina* is found in areas with a sandy bottom (Mense, 1967; Hubbs, 1982). They are found in a similar habitat in Clear Lake, California (Cook and Moore, 1970). Elston and Bachen (1976) made collections in the Lexington Reservoir, California in a shallow sandy area with sparse growth of rooted aquatic plants and a border of tule beds, *Scirpus* spp.

   b. When -- The optimal time to collect *M. beryllina* is during early to mid-morning between 0800 and 1200 hrs. This time is recommended because of the diel reproductive pattern noted for *M. beryllina* in Lake Texoma where most mature fish were ripe between 0800 and 1200 hrs (Hubbs, 1976). Similar reproductive timing was noted in Lexington Reservoir, California where spawning occurred during mid-morning over a vegetated gentle slope at water depths of 2.5 to 60 cm (Fisher, 1973). This same reproductive timing was noted by Robbins (1969) in Lake Eustis, Florida. Moreover, Middaugh et al., (1986) were able to determine the sex of individual *M. beryllina* collected during mid-morning from Blackwater Bay, Florida in mid-April. Extrusion of ripe eggs and sperm was possible, suggesting that estuarine populations also may be ready to spawn during early to mid-morning.

   c. How -- A 1 x 10-m bag seine with knotless 5-mm mesh is ideal for collecting. Since *M. beryllina* typically resides in shallow water (<1.5 m deep), they are easily captured by seining close to shore. It is important to avoid total beaching of the bag seine when collecting *M. beryllina*. These fish will quickly die if removed from water and ripe females often abort their eggs if stranded. Ideally, the bag

portion of the seine containing captured adults should remain in water 5 to 15 cm deep (Middaugh et al., 1986).

d. Spawning in the field.

1. Refer to Figure 2 for a diagrammatic explanation of the procedure outlined below.

2. Immediately after seining (while still on the beach) -- Three to five ripe females should be dipped into a bucket of water from the collection site to remove sand and detritus.

3. Eggs -- Females with hydrated eggs are stripped into a 20 cm diameter glass culture dish containing ambient temperature water (Middaugh and Lempesis, 1976) or directly onto a nylon screen (0.60 to 1.0 mm mesh) which is then gently lowered into the culture dish of ambient temperature water with the eggs on the upper surface of the screen (Barkman and Beck, 1976). If excessive pressure on the abdomen is required to strip eggs, the female should be discarded. Mature eggs are clear with an amber-green hue.

4. Milt -- Several males should then be stripped into a seperate culture dish containing ambient temperature water. Eggs are then fertilized by pouring water from the dish containing sperm into the dish containing eggs. Upon contact with water, adhesive threads on mature eggs uncoil, making enumeration and separation difficult. If eggs are stripped directly into the culture dish, one end of a nylon string may be dipped into the dish and gently rolled so the embryos adhere (Middaugh and Lempesis, 1976). The Barkman and Beck (1976) technique for attaching the eggs to nylon screening minimizes the natural clumping tendency due to entanglement of the filaments on *Menidia* eggs and is recommended.

5. Strings of embryos or embryos on screens -- These are transported to the laboratory by placing in an insulated glass or stainless steel container half-filled with water from the collection site.

6. In the laboratory -- Embryos should be suspended in a 10 to 20 l all-glass container. The temperature and salinity of the water at the collection site should be used as guidelines for holding conditions in the laboratory. If convenient, water from the collection site should be used for holding embryos in the lab. Since *M. beryllina* embryos are euryhaline and eurythermal, slight changes in salinity and incubation temperatures (to a new constant salinity and temperature) are acceptable,

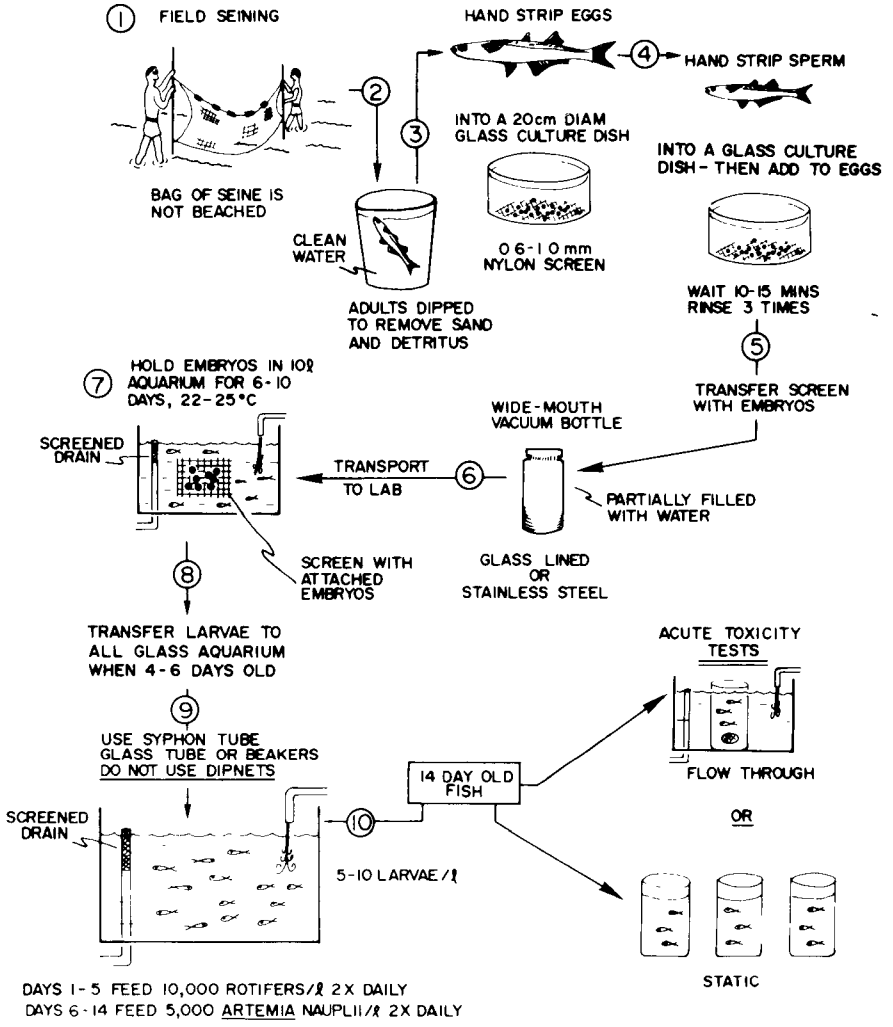

Figure 2. Diagrammatic explanation of procedure for collection, fertilization, transport, culture and testing of embryonic and larval stages of *Menidia*.

provided the constant laboratory regime is not likely to result in salinity or thermal stress. The optimal temperature for survival of embryos and growth of young larvae is 25°C (Hubbs et al., 1971; Middaugh et al., 1986). A salinity of 15 ‰ produced the best survival and growth of larval *M. beryllina* from parental stock taken from a brackish water (1-5 ‰) habitat (Middaugh et al., 1986).

e. Transport of adults to the laboratory.

1. Handling -- Adults should be removed from the bag end of the seine by hand and placed immediately in a bucket containing 12 to 15 l of ambient temperature water from the collection site. Not more than 25 adults should be placed in a bucket at any one time and these should be quickly and gently transferred to the transport tank.

2. Transport tank -- A container of 100 to 350 l volume with smooth sides (fiberglass, rigid styrofoam or stainless steel) should be used to transport adults to the laboratory. A loading capacity of 5.0 g l^{-1} (2 adults of ~2.5 g each) should not be exceeded. The transport tanks should be partially filled with water before addition of adult fish. Vigorous aeration from 2 to 3 airstones should be provided continuously using a battery-operated portable aerator.

3. Before transport -- Care should be taken to prevent the introduction of sand, mud, clay or any other abrasive materials to the transport tank while loading of ambient water or fish.

4. At the laboratory -- Adult fish should be dipnetted with fine mesh nets and transferred quickly from the transport tank to buckets containing 12 to 15 l of water, then carried to the laboratory spawning system and divided among the brood tanks as described in the next section.

f. Laboratory spawning.

1. Spawning system -- Inland silversides, *M. beryllina* may be spawned in the laboratory on a year-round basis. Procedures described by Middaugh et al., (1986) provide for maintenance of a brood stock of 50 individuals, sex ratio 1:1 (i.e., 25 males + 25 females) in each of 2 circular fiberglass tanks, diameter 1.3 m, water depth 38 cm. A filter reservoir system is employed to maintain water quality and a March TE5C MD pump (March Manufacturing Co., Glenview, Illinois) circulates water from the filter-reservoir system to each spawning tank (Figure 3). A once-through system without the filter-

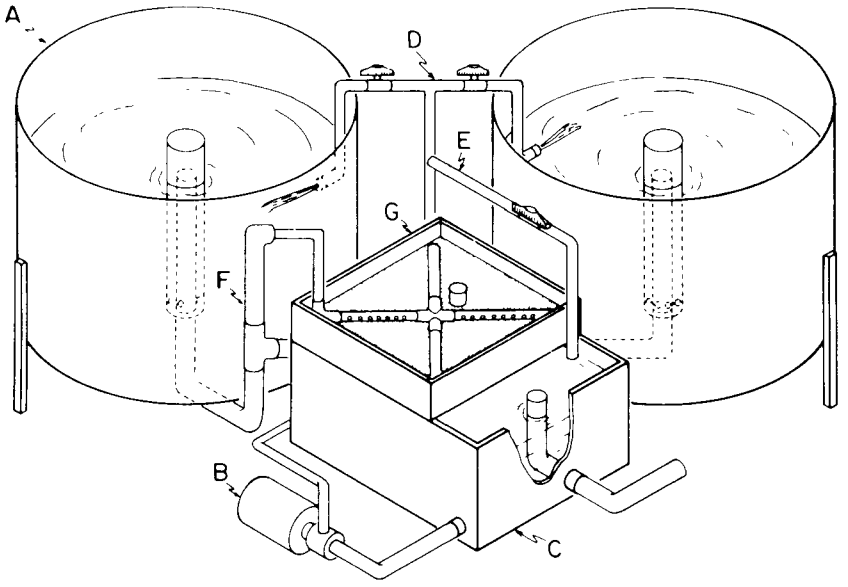

Figure 3. Laboratory spawning system utilized with *Menidia beryllina*, *M. peninsulae* and *M. menidia*, A. 1.3 m diameter holding tanks; B. seawater circulation pump; C. filter-reservoir tank; D. seawater discharge lines from circulating pump; E. shunt-return line to filter-reservoir; F. drain-return line from holding tanks routed into reservoir tray; G. reservoir filter-tray. The tray contains several strata of filter material including (from top) aquarium filter fiber (2-3 cm deep), activated charcoal (1-2 cm deep), coarse stone gravel (2-3 cm deep) and crushed oyster shell (1-2 cm deep).

reservoir can also be used if the incoming water is of sufficiently good quality. Flow rates are adjusted to provide a surface current velocity of 4 to 8 cm sec^{-1} in each holding tank. Water flows out of the bottom of each tank through an inverted standpipe. A perforated air tube positioned at the outer bottom edge of each inverted standpipe maintains dissolved oxygen at > 5.0 mg l^{-1} and produces a gentle upwelling current.

2. Feeding schedule -- Fish in each holding tank are fed 8.0 g of Tetramin (Standard Mix-large flake) food each morning, 0800 to 0900, 4.0 g at 1000 to 1100, 4.0 g at 1400 to 1500 and a final 8.0 g feeding at 1500 to 1700 hrs. Daily feedings may be supplemented between 1100 and 1200 hrs with ~150,000 *Artemia* nauplii. At least once each week, excess food is siphoned from the bottom of each tank, and aquarium filter fiber and activated charcoal in the filter-reservoir system changed. Approximately 20% of the water in each system is removed weekly and replaced with temperature and salinity adjusted water.

3. Water quality -- The spawning system should have water quality similar to that at the collection site. *M. beryllina* adults from freshwater habitats such as Lake Texoma, Oklahoma; Clear Lake, California; or Lake Eustis, Florida should be maintained in water with a hardness, pH, alkalinity, and total organic carbon, similar to the water from which they were collected. In contrast, fish collected from estuarine locales should be maintained at salinities similar to those measured at the collection location.

4. Water temperature and salinity -- These environmental variables should resemble values encountered at the collection location. It appears that reproduction in *M. beryllina* occurs over a temperature range of ~ 15 to 30°C, with 25°C considered optimal for embryo development and survival. Thus adult brood stock should be held at 25°C after an appropriate acclimation period if collections are made early in the reproductive season (i.e., when water temperatures may be low). *M. beryllina* taken from Blackwater Bay, Florida, temperature 23.7 to 26.3°C, salinity 3 to 5 ‰ are routinely held at 25°C and 5 ‰ while in brood tanks (Middaugh et al., 1986).

5. Photoperiod and light intensity -- A photoperiod of 13L:11D is recommended for *Menidia* brood stock with a light intensity of 300 lux provided by two banks of 16 cm long 40 watt "cool white" fluorescent tubes mounted 1.5 m above holding tanks. Timer controlled lights are

turned on at 0600 and off at 1900 hrs. Pairs of spawning tanks must be isolated from outside ambient light and general disturbances by light-tight curtains. These curtains remain partially open to facilitate easy feeding of adult fish during daytime, but are tightly closed at night. A small exhaust fan is mounted on the plywood ceiling of our enclosure to prevent a buildup of excess heat (from the water circulation pump) and humidity from the holding tanks.

6. Combinations of spawning signals -- We recommend running the circulation pump continuously. Introduction of a 13L:11D photoperiod (lights on 0600 and off 1900 hrs) is used to mimic the natural photoperiod during spring-early summer when reproductive activity is evident throughout the geographical range of *M. beryllina*. *M. beryllina* tend to spawn throughout the day and night. However, in our studies most egg release appears to occur between 0800-1200 hrs each day for populations collected from freshwater lakes and reservoirs, and 1800-2300 hrs for estuarine populations. The laboratory spawning time for freshwater populations is similar to times of spawning in nature (Hubbs, 1976; Robbins, 1969). Daily egg production from a tank containing 25 females and 25 males (from an estuarine locale), during 13 days when eggs were enumerated, ranged from 659 to 4,649 ($\bar{x}$=2,316)(Middaugh et al., 1985). Egg production was similar for a population of fish taken from Lake Chicot, Arkansas (Middaugh and Hemmer, unpublished).

7. Spawning substrates -- Polyester aquarium filter-fiber substrates, size ≃15 cm long x 10 cm wide x 10 cm thick are suspended just below the water's surface and in contact with the side of each holding tank. A synopsis of recommended environmental variables for spawning *M. beryllina* and other silversides in the laboratory is provided in Appendix B.

g. Culture of laboratory spawned *Menidia beryllina*.

1. Fertilized eggs -- They should be removed from the surface of the spawning substrate, generally between 1300 and 1400 hrs daily. No effort should be made to tease individual eggs from the substrate, rather, concentrations of eggs and accompanying substrate are removed from the main body of the spawning substrate with forceps. It should be noted, however, that minimization of the amount of spawning substrate removed with the embryos is desirable.

2. Embryos -- Developing embryos that are attached to polyester substrates are suspended in a 10 to 20 l glass aquarium containing 8 to 18 l of water adjusted to the temperature (25°C) and salinity (freshwater to ~20 ‰) at which adult brood fish were held and eggs spawned and fertilized in the laboratory.

3. Water temperature and salinity -- Utilization of a single temperature and salinity regime for spawning adults, embryo incubation, and larval culture and testing eliminates questions of acclimation and is often helpful because the same source of water can be used in the various production and testing systems.

4. Transfer -- Newly hatched larval *M. beryllina* should be maintained and fed in the 10 to 20 l glass culture container for 4 to 6 days after hatching, with water temperature and salinity appropriately adjusted. A density of 5 to 10 larvae l^{-1} of water is desirable, thus a maximum of 180 larvae should be placed in a tank containing 18 l of water. A 1.5 cm inside diameter (I.D.) glass tube, approximately 45 cm in length, equipped with a rubber squeeze bulb is used to make transfers of 4 to 6 day old *Menidia*. Alternatively a small siphon tube, 3 cm I.D. x 1.5 m in length may be used to siphon fish from the hatching aquarium to the grow out tank. We prefer to transfer larvae to 200 ml beakers for enumeration and then pour the contents into a 20 to 40 l tank. Under no circumstances should a dipnet be used to transfer larval *M. beryllina. Mass mortalities will occur if a dipnet is used.*

5. Feeding -- The mixohaline rotifer, *Brachionus plicatilis*, must be provided *on the day* that *M. beryllina* hatch. Two feedings, one between 0800 and 0900 hrs and a second at 1400 to 1500 hrs are required. At each feeding, rotifers are added at a rate of 10,000 l^{-1} of water in the holding aquarium. Thus an 18 l volume would require the addition of 180,000 rotifers, twice daily. This regime is continued through the fifth day after larval *M. beryllina* hatch. On days six through fourteen, 5,000 newly hatched (<8 hr old) *Artemia* nauplii l^{-1} are added each morning and afternoon. A synopsis of recommended environmental variables for laboratory incubation of embryos and larval culture is provided in Appendix C.

B. Atlantic silverside, *Menidia menidia.*

1. Geographical distribution -- *M. menidia* ranges as far north as the Magdalen Islands, Province of Quebec, Canada (Cox, 1921). The southern range is limited to the Atlantic coast of

northern Florida (Gosline, 1948; Robbins, 1969). Johnson (1975) collected a few *M. menidia* as far south as New Smyrna Beach, Florida where it seems to intergrade with the tidewater silverside, *M. peninsulae*, which is generally found from Daytona Beach, southward (Chernoff et al., 1981). *M. menidia* has been taken from Scarborough Harbor and off Todd Point in southern Maine (Robbins, 1969). It is a resident of Essex Bay, Massachusetts (Conover and Kynard, 1981), southern Rhode Island estuaries (Bengtson et al., 1986), and is common at Woods Hole, Massachusetts (Kendall, 1902). It is also found in southern Connecticut estuaries opening into Long Island Sound (Cadigan and Fell, 1985). Moreover, *M. menidia* occurs in the lower regions of Chesapeake Bay tributaries (Bayliff 1950; Robbins, 1969). Massman (1954) collected adults approximately 54 km upstream from brackish water in the James, Rappahannock and Pamunkey Rivers, Virginia. An offshore migration of *M. menidia* north of Cape Hatteras occurs in fall and winter as the estuarine and nearshore water temperatures drop to about 6 to 8°C (Conover and Murawski, 1982). South of Cape Hatteras, *M. menidia* was extremely abundant and the only fish occurring in shallow water throughout the winter (Hildebrand, 1922). In South Carolina, *M. menidia* is a year-round resident of intertidal creeks (Cain and Dean, 1976; Shenker and Dean, 1979) and is found in the surf zone of barrier beaches throughout the winter (Anderson et al., 1977). The general distribution of *M. menidia* is shown in Figure 4 and a list of selected biogeographical data, that should aid in selection of collection sites, is provided in Appendix D.

2. Ecology and reproduction -- *M. menidia* is euryhaline and eurythermal, living in the upper reaches of rivers in the Chesapeake Bay (Massman, 1954) and migrating offshore some 50 km during the colder months at northern latitudes (Conover and Murawski, 1982). De Sylva et al., (1962) collected *M. menidia* at salinities ranging from 2 to 35 ‰ in the Delaware Bay and found the greatest abundance at water temperatures from 12 to 30°C. Bayliff (1950) observed that adults were less numerous in shallow waters of the Chesapeake Bay during fall as water temperatures declined to less than 12°C; at 6°C or lower, few *M. menidia* were to be found in the shallows. Hildebrand and Schroeder (1928) were able to collect specimens at a depth of approximately 50 m during the winter in Chesapeake Bay. A decline in abundance was noted at temperatures below 10°C in the North Edisto River estuary of South Carolina (Middaugh, unpublished). Moreover, Dahlberg (1972) reported that *M. menidia* occurred in a Georgia estuary at water temperatures from 7 to 31.5°C but observed that adults became scarce when water temperatures dropped below 12°C. *M. menidia* is an

Figure 4. Biogeographical distribution of the Atlantic silverside, *Menidia menidia*.

opportunistic omnivore, feeding on mysids, copepods, molluscan larvae, annelid worms, amphipods, young gastropods, crab larvae, diatoms and other fishes including its own young (Kendall, 1902; Bayliff, 1950; De Sylva et al., 1962; Mulkana, 1966). Sexually mature adults spawn from March through August depending upon the latitude. Ripe fish have been found in June at Prince Edward Island, Canada (Leim and Scott, 1966), and in June and July at Woods Hole, Massachusetts (Kuntz and Radcliffe, 1918; Rubinoff and Shaw, 1960; Kendall, 1902). In Salem Harbor, Massachusetts, Conover and Kynard (1984) noted that *M. menidia* had a spawning periodicity that coincided with new and full moons. Intensity and frequency of spawning was correlated with the height of daytime high tides. Breeding occurred from late-April through June at water temperatues of 9 to 21°C. Daytime spawners deposited eggs on mats of intertidal, filamentous algae. At the Pataguanset River estuary in eastern Connecticut, Cadigan and Fell (1985) reported reproductive activity in *M. menidia* from early-May until late-July and observed that water temperature was an important factor influencing reproductive activity. An apparently critical lower temperature for reproduction, 16°C, was reached in mid-late May. Interestingly, Middaugh (1981) reported that 16°C was required for spawning to occur in *M. menidia* from the North Edisto River estuary in South Carolina. Spawning generally began in early to mid-March when the critical minimum water temperature was reached and ended in June or July as estuarine water temperatures exceeded 30°C. In South Carolina estuaries, *M. menidia* primarily spawns during a 3 to 4 day period on daytime high tides following new and full moons (Middaugh, 1981; Middaugh et al., 1981). Eggs are deposited on a variety of upper intertidal substrates including, *Spartina alterniflora*, detrital mats and in abandoned crab burrows along erosional scarps (Middaugh et al., 1981).

3. Identification -- The Atlantic silverside, *Menidia menidia* is the largest species in the genus. The largest specimen examined by Robbins (1969) was a 117 mm SL female. Males are generally smaller than females. Scales are small to moderate in size, well inbricated, and usually with well developed circulii. Branchial lateral line scales are usually 41-47, post pectoral lateral line scales usually 42-46; predorsal scales 18-22. The first dorsal fin has 3-7, usually 4 or 5 spines; origin is over the posterior edge of the anus or anal fin origin. The second dorsal fin has one spine and 7-11, most frequently 8 or 9, rays. The anal fin is long with one spine and 19-29, usually 21 to 26, rays. The air bladder scarcely reaches a point above the anal fin origin and is opaque and abruptly truncate in shape posteriorly. The snout

is moderately blunt or sub-conic to angular and is usually greater than eye length. Jaws are equal. The distal tip of mandible does not project beyond tip of premaxillary, and the premaxillary is rounded anteriorly (Robbins, 1969). Mature and hydrated *M. menidia* eggs are 1.0 to 1.2 mm in diameter and bear a cluster of 15 to 50 thin filaments and no thick filaments.

4. Collection, handling, spawning and transport.

   a. Where -- North of Cape Cod sexually mature fish will generally be available in estuaries during late April through June or July, at water temperatures of 2 to 21°C (Kendall, 1902; Kuntz and Radcliff, 1918; Rubinoff and Shaw, 1960; Bengtson, 1985). At Salem Harbor, Massachusetts sexually mature adults were taken from late April through June at water temperatures of 9 to 21°C (Conover and Kynard, 1981). From Cape Cod southward to Florida, it appears that water temperatures ≥16°C are required for spawning in spring (Cadigan and Fell, 1985) and that reproduction ceases as water temperatures approach 30°C (Middaugh, 1981).

   b. When -- *Menidia menidia* should be collected just prior to natural spawning runs. These runs at all latitudes appear to occur 25 during daytime and are timed at, or just after, high tides (Middaugh, 1981; Conover and Kynard, 1984). Generally, the 1 to 4 day period after new or full moons is best for collecting. High tides will occur between ~0800 and 1200 hrs on these days.

   c. How -- Refer to section II. A. 4. c. for *Menidia beryllina.*

   d. Spawning in the field.
      1. Immediately after seining -- Refer to sections II. A. 4. d. 1-6. for *M. beryllina.*

   e. Transport of adults to the laboratory.
      1. Adults -- Refer to section II. A. 4. e. 1-4. for *M. beryllina.*

   f. Laboratory spawning.

      1. Spawning system -- Atlantic silversides, *Menidia menidia,* may be spawned in the laboratory during the natural reproductive season. Procedures described by Middaugh and Takita (1983) should be utilized. The system described by Middaugh and Hemmer (1984) and Middaugh et al., (1986) for spawning *M. peninsulae* and *M. beryllina* (Fig. 3) is also suitable for laboratory spawning of *Menidia menidia* (Middaugh and Hemmer, unpublished).

2. Feeding schedule -- Refer to section II. A. 4. f. 2. for *M. beryllina*; and Appendix B.

3. Water quality -- The pH, total organic carbon, and ammonia in the spawning system should be maintained at levels similar to the collection location.

4. Water temperature and salinity -- *M. menidia* from northern latitudes, north of Cape Cod, should be held at 18 to 24°C (Conover and Kynard, 1981) while those from south of Cape Cod should be held at 20 to 25°C (Cadigan and Fell, 1985; Middaugh, 1981). Reproductively active *M. menidia* occur in freshwater (Massman, 1954) and in nearly full strength seawater (Anderson et al., 1977; Middaugh 1981). The salinity in spawning tanks should be similar to that at the collection site, provided there is evidence that *M. menidia* is reproductively active where collected. Middaugh and Takita (1983) utilized temperatures ranging from 16 to 25°C and a salinity of 30 ± 2 ‰ in laboratory spawning studies with *M. menidia*. In contrast, Conover and Kynard (1984) reported laboratory spawning at 13 to 24°C during May through July for adults collected from Essex Bay, Massachusetts.

5. Photoperiod and light intensity -- Refer to section II. A. 4. f. 5. for *M. beryllina*.

6. Combinations of spawning signals -- "Tidal" signals, interruptions of the current velocity, are accomplished by using an electrical timer to turn off the water circulation pump for 1 hr at the specified times of 1200 to 1300 and 2400 to 0100 hrs. *M. menidia* is sensitive to interruptions in current velocity that mimic conditions during slack high tides in nature. They will spawn in the labortory during daytime 1200 to 1300 hrs in response to the interruption in current velocity, but not at night, between 2400 and 0100 hrs when the circulation pump is also turned off (Middaugh and Takita, 1983; Middaugh, 1981). A synopsis of recommended environmental variables for spawning *M. menidia* in the laboratory is provided in Appendix B.

7. Spawning substrates -- Refer to section II. A. 4. f. 7.

g. Culture of young *Menidia menidia*.

1. Fertilized eggs and embryos -- Refer to section II. A. 4. g. 1-2. for *M. beryllina*.

2. Water temperature and salinity -- Utilization of a single temperature and salinity regime for spawning adult *M. menidia*; and for embryo incubation, larval culture and

testing is advisable. This procedure minimizes questions of acclimation and is often helpful because the same source of water can be used in the various production and testing systems. For northern latitudes we recommend a culture temperature of 22°C and salinities of 25 to 30 ‰, unless environmental variables vary widely from these parameters at the locale where fish are collected. For southern latitudes (south of Cape Cod) we recommend a temperature of 25°C and salinities of 25 to 30 ‰, again with the caveat for environmental variables.

3. Transfer -- Refer to section II. A. 4. g. 4. for *M. beryllina.*

4. Feeding -- Refer to section II. A. 4. g. 5. for *M. beryllina.* However, Middaugh and Lempesis (1976) were able to obtain acceptable (70%) survival of *M. menidia* larvae by feeding them on the day-of-hatch and daily thereafter with very young (≤8 hr old) *Artemia* nauplii. Similar results were reported by Barkman and Beck (1976) and Conover and Kynard (1981). *The reader is cautioned:* It is absolutely essential to begin feeding *M. menidia* larvae with young *Artemia* nauplii on the day that *Menidia* hatch. A one day delay in adding *Artemia* nauplii will result in markedly reduced survival of larval *M. menidia* (Middaugh and Lempesis, 1976). If the use of *Artemia* nauplii alone (without initial feeding of *Brachionus* sp.) is unsuccessful then procedures outlined in section II. A. 4. g. 5. should be utilized. A synopsis of recommended environmental variables for laboratory incubation of embryos and larval culture is provided in Appendix C.

C. **Tidewater silverside**, *Menidia peninsulae*, recently recognized as a distinct species (Johnson, 1975; Chernoff et al., 1981) was once considered conspecific with *Menidia beryllina* (Gosline, 1948; Robbins, 1969). These two atherinids are often found in close proximity in estuaries along the southeastern coast of Florida and throughout the Gulf of Mexico (Echelle and Mosier, 1982; Lucas, 1982). The general distribution of *M. peninsulae* is shown in Figure 5 and a list of selected biogeographical data are provided in Appendix E. This list should aid in the identification of appropriate collection sites.

1. Geographical distribution -- *M. peninsulae* has a disjunct distribution extending from Daytona Beach, Florida to Horn Island, Mississippi and Galveston Bay, Texas to Tamiahua, northern Veracruz, Mexico (Johnson 1975; Chernoff et al., 1981).

2. Ecology and reproduction -- *Menidia peninsulae* along the northern part of its range intergrades with *M. menidia.* At

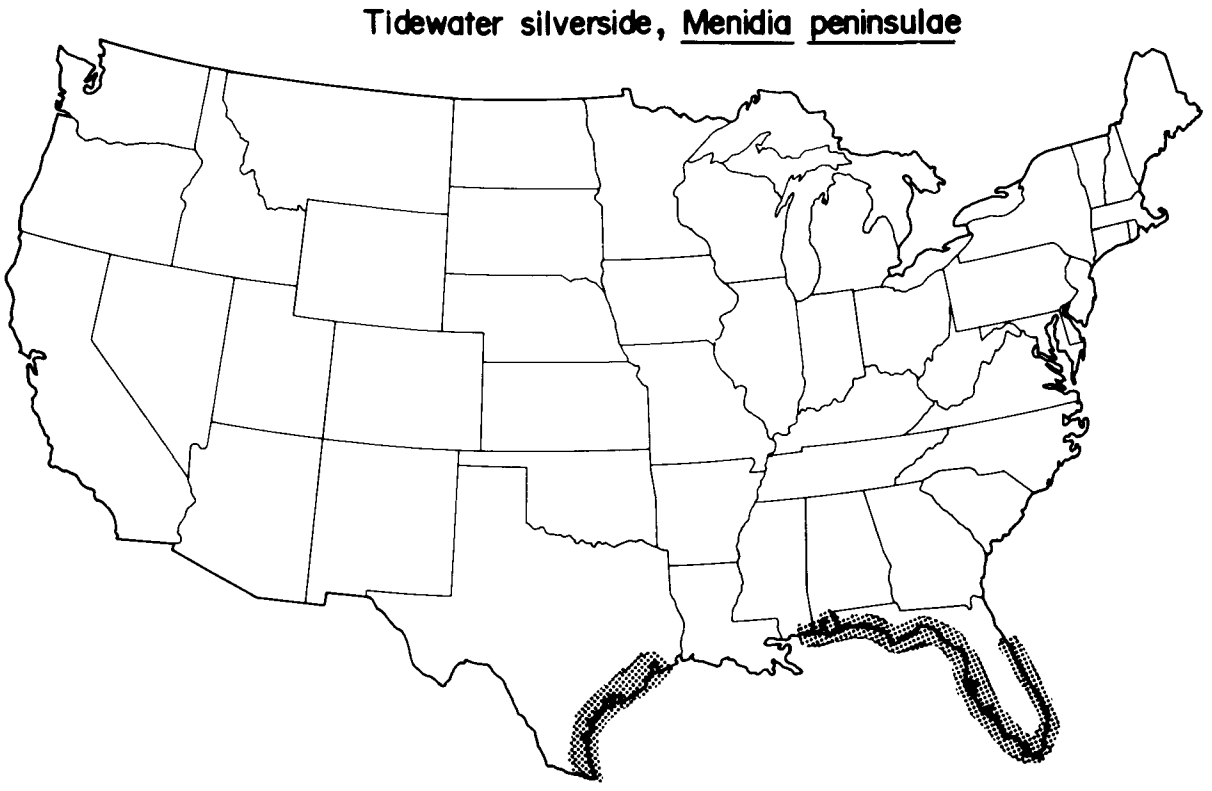

Figure 5. Biogeographical distribution of the tidewater silverside, *Menidia peninsulae*.

Fort George Inlet, Duvall Co., Florida, collections yielded 99% *M. menidia*. However, at Flagler Beach, Flagler Co., Florida, the ratio was 63% *M. menidia*, 20% hybrids and 19% *M. peninsulae*. In contrast, a more southerly location, Melbourne, Brevard Co., yielded no *M. menidia* or hybrids and 100% *M. peninsulae* (Johnson, 1975). Moreover, *M. peninsulae* and *M. beryllina* are often found in close proximity in estuaries along the southeastern coast of Florida and in the Gulf of Mexico (Echelle and Mosier, 1982; Lucas, 1982). Despite sympatric occurrence and collection of *M. beryllina* and *M. peninsulae* in the same seine haul at four localities; two in Florida and two in Texas, Johnson (1975) observed that the species were discrete in these areas. Collections yielded a very low frequency of hybridization. Although *M. peninsulae* has been collected at salinities of less than 5 ‰ to greater than 35 ‰; Johnson (1975), Echelle and Mosier (1982) and Middaugh et al., (1986) observed that *M. peninsulae* typically resided at salinities of 15 ‰ or greater while *M. beryllina* generally occurred at salinities of 19 ‰ or less. In the Crystal River, Florida locale, Lucas (1982) observed that *M. peninsulae* was present throughout much of the year, but disappeared during December and January, temperature 6.0 to 17.0°C, salinity 22 to 24 ‰ and in July, water temperature 30.7°C, salinity 28 ‰. In contrast *M. peninsulae* from the Pensacola, Florida locale were present in most months but disappeared from the shoreline in December and January when water temperatures were below 12°C (Middaugh and Hemmer, 1986a). Lucas (1982) described three feeding stages for *M. peninsulae* from Crystal River, Florida. In early spring, young-of-the-year fed on tychoplankton and detritus. During late spring through winter, calanoid copepods and cypris larvae were selectively eaten. Reproductively active *M. peninsulae* fed primarily on amphipods and larval silversides during February and March. A reproductive peak was observed only in the spring, no such peak was apparent during fall. Middaugh and Hemmer (1986a) observed spawning during March at low tide on a red alga, *Ceramium byssoideum*, which was growing in the cracks and crevices of a rocky substrate just below the low tide line. The annual reproductive cycle of *Menidia peninsulae* from Santa Rosa Island, Florida extends from February through July or August with the greatest spawning activity during March through June at water temperatures of 16.7 to 30.8°C.

3. Identification -- While differences in *M. peninsulae* and *M. beryllina* are diagnostically clear, these differences are difficult to express in terms of individuals (Johnson, 1975). One of the best diagnostic characters for *M. peninsulae* is the horizontal distance between the origins of the first spinous dorsal and anal fins. In *M. peninsulae*, this distance is $\geq 7\%$

of SL while in *M. beryllina* the distance is ≤7% of SL (Chernoff et al., 1981; Middaugh et al., 1986). Chernoff et al. (1981) reported measurements of this distance in *M. peninsulae* primary type material of 9.3% and 10.7% SL; in *M. beryllina* 5.7% and in *M. audens* (now considered *M. beryllina*) 4.4% SL. The least destructive way to identify live *M. peninsulae* and *M. beryllina* is by the relative posterior extension of the gas bladder. In *M. peninsulae* the opaque gas bladder is truncated and extends to the first to third soft anal ray (Johnson, 1975; Echelle and Mosier, 1982). In contrast, the translucent gas bladder of *M. beryllina* is bluntly rounded and extends to a position approximately above the fourth or fifth soft anal fin ray. The fully hydrated eggs of *M. peninsulae* are 0.9 to 1.1 mm in diameter and possess 15 to 50 short-thin filaments and no long-thick filaments.

4. Collection, handling, spawning and transport.

   a. Where -- Sexually mature fish will generally be available from early March through late June or early July (Middaugh et al., 1986). *M. peninsulae* frequents shallow waters along shorelines where sandy to partially vegetated substrates occur and prefers locales where salinities are ≥15 ‰ (Johnson, 1975).

   b. When -- On several occassions, *M. peninsulae* has been observed spawning on morning (0730 to 1130 hrs) slack low tides.

   c. How -- Refer to section II. A. 4. c. for *M. beryllina*.

   d. Spawning in the field.

      1. Immediatedly after seining -- Refer to section II. A. 4. d. 1-6. for *M. beryllina*.

   e. Transport of adults to the laboratory.

      1. Adults -- Refer to section II. A. 4. e. 1-4. for *M. beryllina*.

   f. Laboratory spawning.

      1. Spawning system -- Tidewater silversides, *Menidia peninsulae*, may be spawned in the laboratory throughout the year. The system described in Figure 3 and section II. A. 4. f. 1. should be utilized.

      2. Feeding schedule -- Refer to section II. A. 4. f. 2. for *M. beryllina*.

      3. Water quality -- Refer to section II. A. 4. f. 3. for *M. beryllina*.

4. Water temperature and salinity -- These variables should be similar to that from the collection site. *M. peninsulae* from the the Santa Rosa Island locale, collected at 22°C and 23 ‰ were successfully maintained and spawned in the laboratory at 24°C (22.1 to 25.4°C) and 26 ‰ (23 to 28 ‰) Middaugh et al., (1986).

5. Photoperiod and light intensity -- Refer to section II. A. 4. f. 5. for *M. beryllina.*

6. Combinations of spawning signals -- "Tidal" signals, interruption of the 8 cm sec^{-1} current velocity in holding tanks should occur at 1200 to 1300 and 2400 to 0100 hrs. These interruptions in current velocity are accomplished by using an electrical timer to turn off the circulation pump for 1 hr at the specified times of 1200 to 1300 and 2400 to 0100 hrs. *Menidia peninsulae* apparently is sensitive to interruptions in current velocity that mimic conditions during slack low tides in nature (Middaugh et al., 1986). They will spawn in the laboratory during nightime (2400 to 0100 hrs) in response to the interruption in current velocity, but not during daytime from 1200 to 1300 hrs when the circulation pump is also turned off (Middaugh and Hemmer, 1984; Middaugh et al., 1986).

7. Spawning substrates -- Refer to section II. A. 4. f. 7. for *M. beryllina.* A synopsis of recommended environmental variables for spawning *M. peninsulae* in the laboratory is provided in Appendix B.

g. Culture of young *Menidia peninsulae.*

1. Fertilized eggs -- Refer to section II. A. 4. g. 1. for *M. beryllina.*

2. Embryos -- McMullen and Middaugh (1985) learned that *M. peninsulae* embryos from Santa Rosa Island, Florida, incubated at 20°C, showed poor hatchability and post-hatch survival and growth. They concluded that optimal conditions for embryo incubation and subsequent larval growth and survival are 25°C and 30 ‰.

3. Water temperature and salinity -- Utilization of a single temperature and salinity regime for spawning adult *M. peninsulae*; and for embryo incubation, larval culture and testing is advisable. This procedure minimizes questions of acclimation and is often helpful because the same source of water can be used in the various production and testing systems. For *M. peninsulae* we recommend a culture temperature of 25°C and salinity of 30 ‰. This recommendation is based upon the work of McMullen and Middaugh (1985) who found this

combination to be optimal for larval survival and growth, and upon the successful spawning of *M. peninsulae* at a similar temperature and salinity (Middaugh and Hemmer, 1984; Middaugh et al., 1986).

4. Transfer -- Refer to section II. A. 4. g. 4. for *M. beryllina*.

5. Feeding -- Refer to section II. A. 4. g. 5. A synopsis of recommended environmental variables for laboratory incubation of embryos and larval culture is provided in Appendix C.

D. **California grunion**, *Leuresthes tenuis*.

1. Geographical distribution -- *L. tenuis* ranges from Monterey Bay, California to Bahia Magdalena on the outer coast of Baja California Sur (Moffatt and Thomson, 1975). The general distribution of *L. tenuis* is shown in Figure 6 and a list of selected biogeographical data are provided in Appendix F. This list identifies locations where *L. tenuis* has been collected.

2. Ecology and reproduction -- *L. tenuis* is perhaps the best known of the four atherinid fishes described in this manual because of its unique reproductive behavior. Adult *L. tenuis* reside in near-shore waters of southern California where annual water temperatures range from 12 to 28°C (Moffatt and Thomson, 1975). They are surface-dwelling fishes (Reynolds et al., 1977) that attain a maximum length of 150 to 180 mm (Walker, 1952). Spawning generally occurs from late February through August. *L. tenuis* spawns in a sand substrate at the approximate time of new and full moons (Middaugh et al., 1983). Spawning runs take place at night and are timed just after the highest high tides during each semilunar period, subsequent high tides and wave action result in depositon of sand over the incubating embryos (Moffatt and Thomson, 1978; Middaugh et al., 1983). Approximately 2 weeks after deposition, developed embryos are washed out of the sand by the next series of high tides of the same or greater height (Shepard and LaFond, 1940). The buried embryos are protected from thermal stress and remain relatively moist even though they usually are not inundated for a week or more during incubation (Walker, 1949; Middaugh et al., 1983).

3. Identification -- The California grunion, *Leuresthes tenuis*, grows to 143 mm SL or larger (Moffatt and Thomson, 1975; Clark, 1925). It is greenish above and silver on the sides with a dark lateral stripe. Scales along the mid lateral band are highly and irregularly crenulate. Lateral scale range is 69-80, $\bar{x}$ = 75 (Moffatt and Thomson, 1975) with 7-9 scales between the first and second dorsal fins (Miller and Lea,

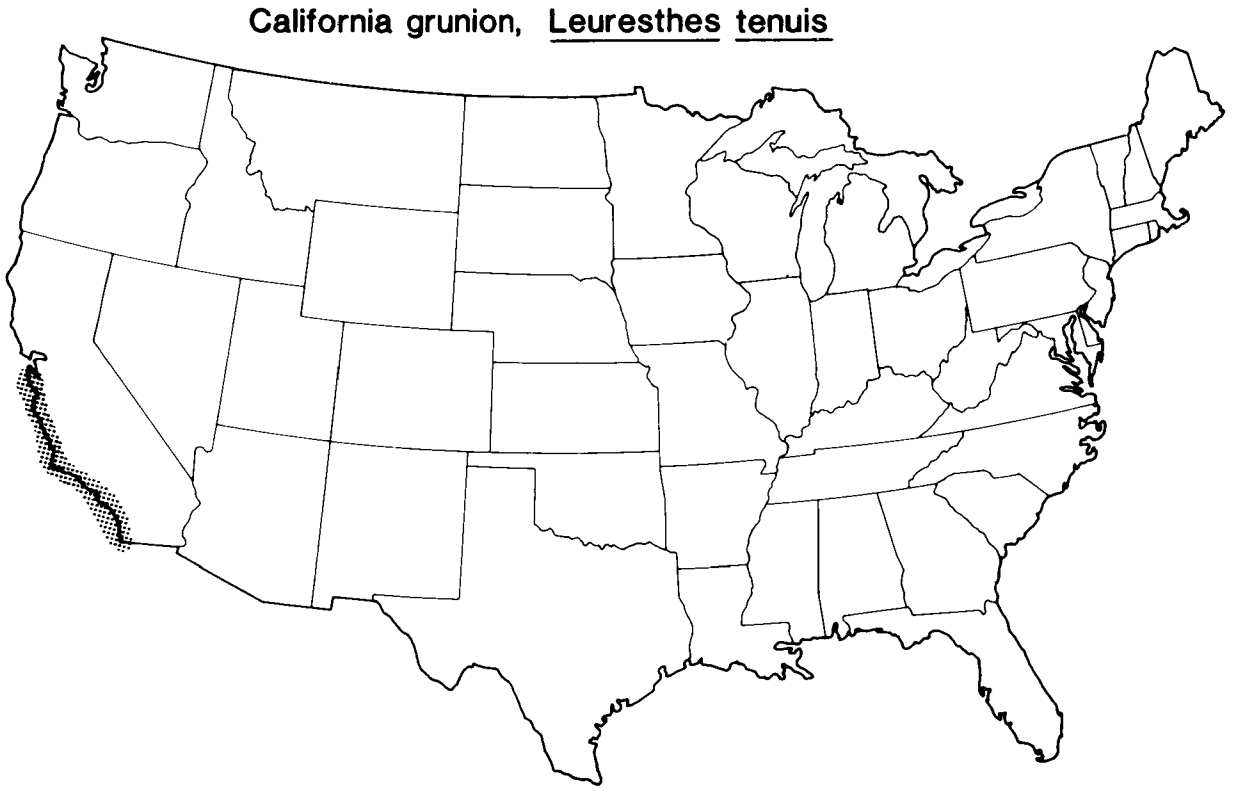

Figure 6. Biogeographical distribution of the California grunion, *Leuresthes tenuis*.

1972). The premaxillary extends over the mandible. The first dorsal fin has 5-7 spines, second dorsal fin 1 spine and 9-10 rays, and anal fin 1 spine plus 21-24 rays.

4. Collection handling, spawning and transport of embryos.

   a. Where -- California grunion eggs are collected along beaches of southern California during natural spawning runs which occur from February through August, with a peak in spawning activity during April and May (Walker, 1949).

   b. When -- Runs occur at night and are timed just after the highest high tides during each semilunar cycle. Generally, the 1 to 4 day period just after new and full moons is best for collecting. Runs usually occur as the high tide is receding (Walker, 1952).

   c. How -- California grunion are collected by hand as they are stranded on the beach during natural runs. Use of nets is forbidden by the California Department of Conservation. A scientific collection permit is required and is available from the Department of Conservation, Sacramento, CA.

   d. Spawning in the field.

      1. Refer to Figure 7 for a diagrammatic explanation of the following procedure.

      2. Eggs -- Twenty to 25 females, captured just prior to natural spawning, should be stripped into a 20 cm diameter glass culture dish containing clean seawater (depth 1 to 2 cm) from the spawning locale.

      3. Milt -- Five to 8 males are then stripped into a separate dish containing clean seawater. Water containing sperm is then poured into the dish containing eggs and mixed by gently stirring. After 10 minutes, the fertilization water is carefully decanted from the culture dish which is then refilled with clean seawater. This refill-rinse procedure should be repeated at least 2 additional times.

      4. Fertilized embryos -- Grunion embryos are carefully pipetted, using a 1.5 cm I.D. x 45 cm glass tube equipped with a rubber squeeze bulb, into all-glass or stainless steel vacuum bottles. Several thousand embryos may be transported in a vacuum bottle containing approximately 500 ml of seawater from the collection site. Water temperatures along the California coast will usually range from 16 to 20°C during the height of the reproductive season and salinities are generally ≥ 32 ‰ (Middaugh et al., 1983). *Note:* It is absolutely essential

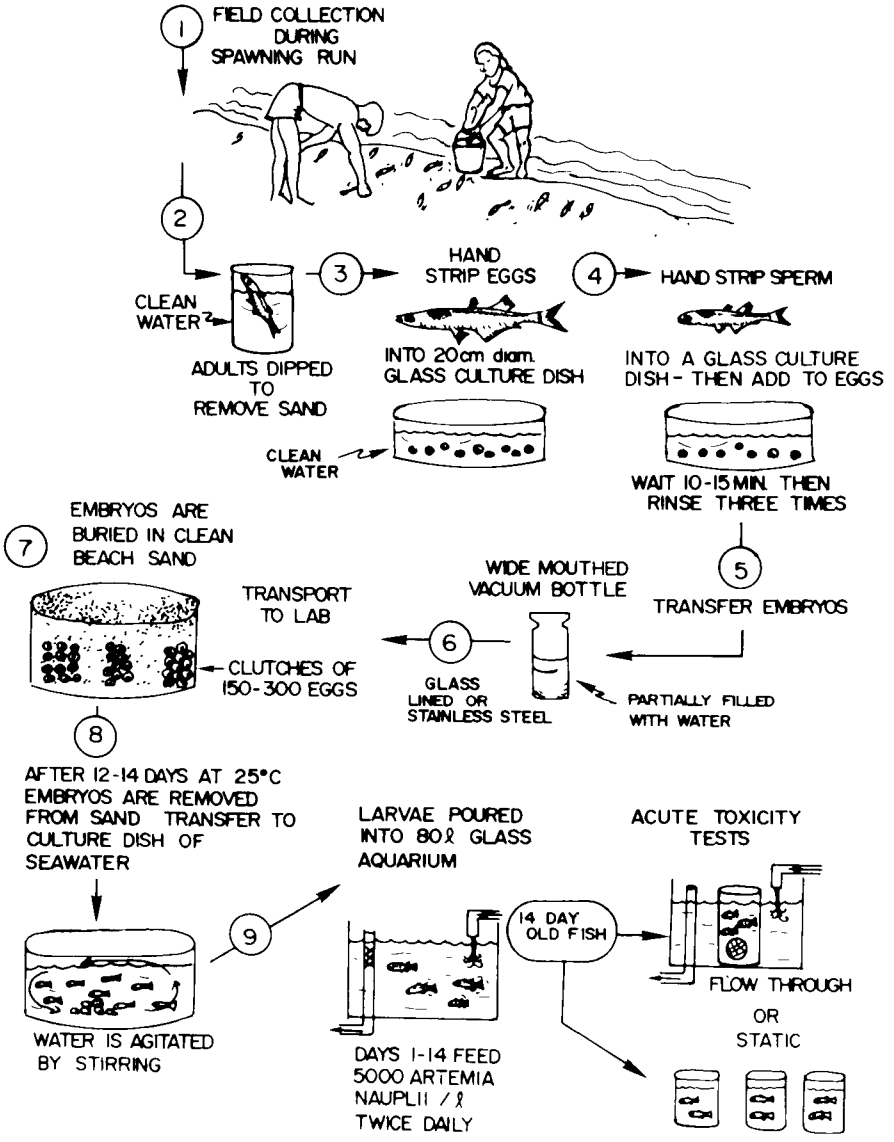

Figure 7. Diagrammatic explanation of procedure for collecting, fertilizing, incubating and testing of California grunion, *Leuresthes tenuis*, embryos.

that sand not be pipetted into the vacuum bottles with fertilized embryos.

5. Sealed vacuum bottles -- Embryos from the collection site should be transported to the laboratory in vacuum bottles. Borthwick et al. (1985) and Goodman et al. (1985a) were able to successfully ship fertilized eggs from southern California to Florida in vacuum bottles. The transport time ranged from 24 to 36 hrs.

e. Embryo incubation in the laboratory.

1. Upon arrival at the laboratory -- Embryos are pipetted into clean, dry beach sand (2.0 cm deep) in a 20 cm diameter glass culture dish. Small depressions (1.0 cm deep) are formed at 3 or 4 equally spaced locations within the sand and 150 to 300 embryos are then pipetted into each of these shallow depressions.

2. Embryos -- Dry sand is used to cover the embryos so that they are buried 0.5 to 1.0 cm deep. Thirty ml of seawater (from the vacuum bottle) is then poured into the sand at each location where embryos were buried. The culture dish is then covered to prevent evaporation and placed in an incubator at 25°C. This incubation temperature is based upon field measurements taken at locations where grunion embryos incubate naturally, 19 to 32°C, $\bar{x}$ = 25°C (Middaugh et al., 1983). Moreover, Hubbs (1965) reported that hatching only occurred between 14.8 and 26.8°C. Similarly, Ehrlich and Farris (1971) observed that grunion embryos hatched when maintained at 14.0 to 28.5°C. Optimal hatching, close to 100%, occurred between 16 and 27°C. Hubbs (1965) pointed out that embryos incubated at temperatures of 19°C or above would be able to hatch on the next series of highest tides in nature (approximately 10 to 14 days after they were fertilized).

3. After 12 to 14 days -- Embryos are carefully removed from the sand substrate with a stainless steel spatula or spoon.

4. To initiate hatching -- Approximately 500 to 1000 embryos and surrounding sand are placed in a 20 cm glass culture dish containing $\simeq$1.5 l of 25°C, 28 to 35 ‰ salinity seawater. A spatula or a glass rod moved around the inner edge of the culture dish will create a circular current. This agitation of embryos and suspension of sand grains induces hatching.

5. Hatched larvae -- Should be transferred to 160 l (or larger) glass aquaria by pouring them from the 20 cm diameter hatching dish, or by using a 1.5 cm I.D. x 45 cm

glass pipette equipped with a rubber squeeze bulb. Care should be used to minimize the amount of sand and nonviable embryos transferred to the grow-out aquaria.

6. Density -- The number of larval grunion in the grow-out aquaria should not exceed 10 l^{-1}. A water temperature of 25°C and salinities of 28 to 35 ‰ should be maintained (Borthwick et al., 1985).

   a. In a flow-through (dynamic) system -- The holding aquarium (160 l) should receive 20 l hr^{-1} of temperature and salinity adjusted seawater.

   b. In a recirculating (static) system -- The holding aquarium (160 l) should be equipped with a Dyna-Flo Model 425 Aquarium Filter (or equivalent). This filtration system should be charged with spun polyester aquarium filter-fiber and activated charcoal. It is essential to cover the siphon intake with 300-400 mm mesh plankton netting so that grunion larvae are not impinged on the syphons or pulled into the filtration unit. Units should be turned off between 0700-1600 hrs (during the twice daily feedings). Filter media should be changed at least twice weekly and the syphon screens checked and cleaned daily if necessary.

7. Feeding -- On the day of hatching and until used in toxicity test, grunion should be fed 5,000 *Artemia* nauplii l^{-1}, twice daily (i.e., 0800 to 0900 and 1500 to 1600 hrs). For a 160 l holding aquarium, 800,000 *Artemia* nauplii should be added at each feeding.

III. Acute toxicity tests, *M. beryllina*, *M. menidia*, *M. peninsulae* and *L. tenuis*

A. Static tests (96 hours).

1. Conducted -- In 4 l wide mouth glass jars containing 3 l of filtered (5 µm) seawater maintained at 25 ± 1°C and 0 to 35 ‰ salinity (depending upon the species used). It is convenient to utilize a salinity similar to that in the grow-out aquaria. Refer to Appendix G for recommended test conditions.

2. Age of fish -- Fourteen day-old atherinids are recommended for testing. Because hatching of larvae is synchronous, all individuals should be of identical age.

3. Transfer of larvae -- Under no circumstances should larvae be dip-netted. *Mass mortalities will occur.* Transfer from the grow-out aquaria is accomplished by siphoning atherinids (use a 3.0 cm I.D. x 1.5 m long plastic tube) into a 12 to 15 l glass or plastic container. Individuals are then pipetted (1.5 cm x 45 cm glass pipette equipped with a rubber squeeze bulb) or dipped into a 200 ml beaker. It is easy to enumerate 5 larvae in each beaker. Volume is then reduced to 10 ml in the beakers and the contents are poured into test jars. A squeeze bottle of uncontaminated test media should be available to wash larvae from beakers if they are stranded.

4. General guidelines -- Procedures provided by ASTM (1980) should serve as a guide in conducting acute toxicity tests with the atherinids, however, *it is essential to feed* all species with *Artemia* nauplii during 96 hr tests. Live *Artemia* (20 to 30 nauplii per fish) are provided 2 to 3 times daily during exposure. This amount of food should provide minimal nutrition without confounding results due to dissolved oxygen or loading problems (Borthwick et al., 1985).

5. The toxicant -- If the chemical being tested results in rapid mortality of *Artemia* nauplii, it may be necessary to remove the dead nauplii from the bottom of test containers prior to each subsequent feeding with live *Artemia*. It is also important not to over-feed if fish will not eat available *Artemia*.

6. Test containers -- No aeration should be required. Dissolved oxygen concentrations should be ≥60% of saturation. A

photoperiod of 14L:10D with a light intensity of 11,000 lux is recommended.

7. Temperatures and salinities utilized -- These variables should be similar to conditions in adult spawning and larval rearing tanks. The values summarized in Appendix G are recommended.

B. Flow-through tests (96 hours).

1. Conducted in 60 l glass aquaria supplied with ≈20 to 60 l hr^{-1} of 20 µm filtered seawater.

   a. Toxicant metering system -- Should be selected on the basis of the amount of toxicant to be metered, the flow rate of dilution water and the series of toxicant concentrations under consideration (ASTM, 1980; Clark et al., 1985).

   b. Toxicant carrier concentrations -- These concentrations should be constant for all exposures within a test. A seawater control and carrier control should be maintained along with the 4 to 7 exposure concentrations of toxicant.

   c. To facilitate observations and enumerate mortalities -- Fish may be confined to 4 l wide-mouth glass jars with two screened (100 -140 mm mesh nylon screen) openings, 3 cm diameter, 1 cm above the base.

      1. Each 60 l aquaria may contain 2 to 4 of the 4 l wide mouth glass jars with 10 fish jar^{-1}.

      2. A self-starting syphon (6 cm I.D.) in the 60 l aquarium provides a 10-15 cm draw-down of water approximately once every 15 min., thus ensuring adequate water exchange between the 4 l holding jars and larger 60 l aquaria.

      3. Other operational parameters are identical to those provided for static tests. Refer to section III. A. 2-7.

IV. Early Life-stage toxicity tests, silversides, *Menidia beryllina*, *M. menidia*, and *M. peninsulae*.

A. **Seawater** -- Dilution water for this 28 day test should be passed through a sand filter and 20 µm polypropylene filter. Water temperature should be continuously maintained at 25 ± 1°C for all three species. Salinity should be maintained at approximately 4 to 6 ‰ for *M. beryllina* (or at the collection site-laboratory spawning salinity if higher or lower) and 18 to 30 ‰ for *M. menidia* and *M. peninsulae* (Goodman et al., 1985b).

B. **Exposure system** -- A glass proportional diluter similar to that described by Schimmel et al., (1974) is used in each test. The diluter delivers 1 l of water to each control and treatment at each cycle (approximately every 15 min). Glass splitter boxes equipped with two 2 mm I.D. glass capillary tubes slowly deliver the seawater from the diluter to each of the two exposure aquaria for each treatment. A 4 mm I.D. self-starting siphon varies the water depth in each glass aquarium (21 cm long by 22 cm wide by 10 cm high) between approximately 4 and 7 cm. The draw-down time is 1.5 to 3.0 min and helps to ensure adequate exchange of toxicant between the exposure cups and aquarium. Exposure aquaria are partially immersed in a freshwater bath that maintains the respective tests at 25 ± 1°C. The photoperiod during all testing is 14L:10D and light intensity is approximately 1400 lux.

1. Embryos --The embryos of each species have numerous chorionic fibrils that must be gently teased apart and their fibrils clipped before selecting individuals to be used in the toxicity test. Embryos are examined microscopically; groups of four or five viable and synchronous individuals are then randomly placed in each incubation cup until the cup contains 16 embryos. Two incubation cups are then placed in each of the two duplicate aquaria for each of the seven treatments.

2. Incubation cups -- These containers should be constructed of glass petri dish bottoms (9 cm I.D.) with 10-cm-high cylinders of 363 µm nylon mesh attached to the inside walls with silicone adhesive. These cups are used in tests with all three species. At test initiation, embryos should be approximately 32 to 36 hrs post-fertilization. If held at 25°C, these embryos

should be in stages 18 to 20 of development (Lagler et al., 1977).

3. Observations -- Daily observations are made for deaths and signs of poisoning. Dead animals are removed and recorded when observed. Larvae are usually first observed on day 4 in tests with *M. menidia* and *M. peninsulae* and on day 5 in the *M. beryllina* test. Silverside larvae are fragile and susceptible to injury or stranding on the mesh of incubation cups. Therefore, during intervals after hatching begins, the incubation cups should not be removed from the exposure aquaria. Larvae are counted in place. Intervals are two days for *M. menidia*, five days for *M. peninsulae*, and ten days for *M. beryllina*. Before incubation cups containing hatched fish are removed from the aquaria, the water level should be lowered slowly to below the top of the petri dish using a 4 mm (I.D.) glass siphon.

4. Feeding -- Rotifers, *Brachionus plicatilis*; and *Artemia* sp. nauplii (≤8 hours old) are used as food for *Menidia* spp. Each *M. menidia* incubation cup is provided a 1:1 mixture of rotifers (2,500) and *Artemia* (2,500) twice daily (0800 to 0900 and 1400 to 1500 hrs) for the first eight days after hatching begins, and thereafter, only *Artemia* (5,000) twice daily until test termination. *Menidia peninsulae* and *M. beryllina* are fed rotifers three times (0800 to 0900, 1200 to 1300 and 1400 to 1500 hrs) daily for the first 8 days after hatching begins at a rate of 5,000 feeding^{-1} followed by a three-day transition period in which both rotifers (2,500) and *Artemia* (2,500) are provided in 3 feedings day^{-1} and then *Artemia* (5,000) 3 times day^{-1} until test termination. In each test, equal portions of food are dispensed to all cups containing hatched fish except those with few survivors, which should received approximately one half as much if considerable food remains from previous feedings (Goodman et al., 1985b). Fish are not fed on the last day of the test.

5. Test completion -- At termination of the 28-day experiments, fish are killed by immersion in ice water, drained on paper towels, weighed individually, and may be combined within exposure concentrations and frozen for subsequent chemical residue analysis.

6. Statistical treatment -- Analysis of variance and Duncan's multiple range test may be used to analyze survival and weight data. Individual fish weights are used in weight analyses. Percentages should be arcsine transformed; a minimum significance level of $\alpha = 0.05$ is used for all statistical purposes.

V. Early life-stage toxicity tests, *L. tenuis*

A. **Seawater** -- Dilution water for this 35-day test should be passed through a sand filter and 20 µm polypropylene filter. Water temperature should be continuously recorded and maintained at 25 ± 1°C. Salinity should be maintained at 25 to 30 ‰ (Goodman et al., 1985a)

B. **Exposure system** -- A glass proportional diluter similar to that described by Schimmel et al., (1974) delivers 1.0 l of water to each treatment at each cycle (approximately every 15 min). Glass splitter boxes equipped with two 2 mm I.D. glass capillary drains delivered the seawater from the diluter to the two glass exposure aquaria per treatment. Exposure aquaria (I.D. = 21 cm long x 22 cm wide x 10 cm high) should be partially immersed in a freshwater bath that will maintain test water temperature at 25 ± 1°C. During embryo exposure, the aquaria are equipped with a 5-mm I.D. self starting siphon that fluctuates the water depth between 2 and 7 cm. The only carrier-solvent used successfully in California grunion ELS tests is triethylene glycol at a concentration of 0.4 µl l^{-1}. Attempted ELS tests at higher triethylene glycol concentrations failed because embryonic development in treatments receiving the carrier-solvent was slower than in the seawater-control treatment (Goodman et al., 1985a).

1. Embryos -- During the test, embryos should be immersed in seawater and kept in darkness prior to testing. The test should be started within 48 hrs after embryos are fertilized. Embryos should be in developmental stages 22 to 24 (Lagler et al., 1977) or younger. The embryos are examined microscopically and groups of four viable specimens placed randomly in each of 28 or more incubation cups. This process is repeated until all cups contain 16 embryos. Treatment and duplicate numbers are assigned by using a random number table. Two incubation cups are then placed in each of the two duplicate aquaria per treatment except that at least two additional cups should be placed in the carrier-control treatment for hatching trials.

2. Incubation cups -- Embryos are maintained in containers constructed by replacing the bottom of a 250-ml glass beaker with 450-mm nylon mesh secured with silicone adhesive. Two 7.5 cm long glass tubes (8-mm I.D.) attached horizontally on

opposite edges support the cup above the aquarium bottom. Embryos are incubated in darkness until hatching is stimulated. During this pre-hatch interval, a flashlight is used when taking water samples or checking viability of embryos. During the later stages of embryonic development care must be taken to prevent jostling the embryos because this could stimulate hatching.

3. On exposure day 9 -- (embryos ~12 days old) Embryos from one of the extra cups in the carrier-control treatment should be gently rinsed into a larval incubation cup, constructed by attaching 9-cm-high cylinders of 450 µm nylon mesh to the inside walls of 100 x 15 mm glass Petri dish bottoms with silicone adhesive. The embryos are placed on a laboratory shaker and then alternately shaken (approximately 68 cycles min^{-1}) and kept stationary for 2-min intervals until no additional fish hatch in two consecutive 2-min periods; two or three intervals of shaking are required. If a large percentage ($\simeq$75%) of the embryos hatch readily, then hatch those in all treatments, but discard those from the extra carrier-control cup. If a good hatch is not obtained from the first cup attempted, wait another day and try the second extra carrier-control cup. After removing cups with larvae from the shaker, dead embryos are removed, and after replacing the siphons in the aquaria with 2 mm I.D. siphons that vary the water depth from 5 to 7 cm, the incubation cups are placed in their respective aquaria. The photoperiod is changed from OL:24D to 12L:12D.

4. Fish -- In this test are fed <24-hr-old *Artemia* nauplii twice daily, except for the last 24 hrs of the experiment when no food is provided. Equal volumes of a suspension of nauplii are dispensed to each incubation cup. The suspension should be sampled frequently and the number of *Artemia* in a volume equal to that dispensed per cup at each feeding should average 5,000 (Goodman et al., 1985a). Dead animals are removed and recorded daily, and qualitative observations of signs of poisoning are noted.

5. At termination of the experiment -- Fish are killed by immersion in ice water, then drained on paper towels, individually weighed, and may be frozen for subsequent analysis. Survival and growth data may be analyzed by one way analysis of variance. Percentage survival data should be arcsine transformed before analyses. Treatment means may be compared by using Duncan's multiple-range test. A significance level of $a = 0.05$ is used for all analyses.

VI. Batch culture of the alga, *Chlorella* sp. and mixohaline rotifer, *Brachionus plicatilis*.

A. Apparatus used.

1. Six to eight, 13 to 20 l pyrex glass carboys and stoppers to fit.
2. Four to six, 4 l pyrex glass flasks with cotton plugs.
3. High wattage thermostatically controlled immersion heater, Thelco-Thermajust Model 15094 1000W-110V or equivalent.
4. Laboratory timer, up to 120 mins elapsed time and able to accomodate immersion heater of 1000W-110V capacity.
5. Source of clean compressed air.
6. Analytical balance-top loader.
7. Microscope and hemacytometer.
8. Automatic pipettor - 0.01 to 1.0 ml capacity, preferably with disposable tips.
9. Plankton netting ~60 µm mesh to collect rotifers.
10. Tygon or equivalent tubing 3 and 10 mm I.D.
11. Glass tubing 3 and 10 mm O.D.

B. Media preparation and *Chlorella* sp. culture.

1. Refer to Figure 8 for a diagrammatic explanation of the following procedure.
2. Clean carboys -- Wash thoroughly with hot soapy water, rinse with tapwater, then triple rinse with deionized or distilled water.
3. Fill carboy -- Filtered (20 µm) natural seawater, salinity 25 to 30 ‰ should be used. If an artificial seawater mix is used, the reconstituted media should be handled according to manufacturer's instructions and allowed to age for at least 7 days prior to pasteurization. The carboy is filled to the top lip.
4. Immersion heater -- Should be placed in carboy with thermostat set at 70 to 80°C. Heater power supply is always routed through an automatic timer as a safety precaution.

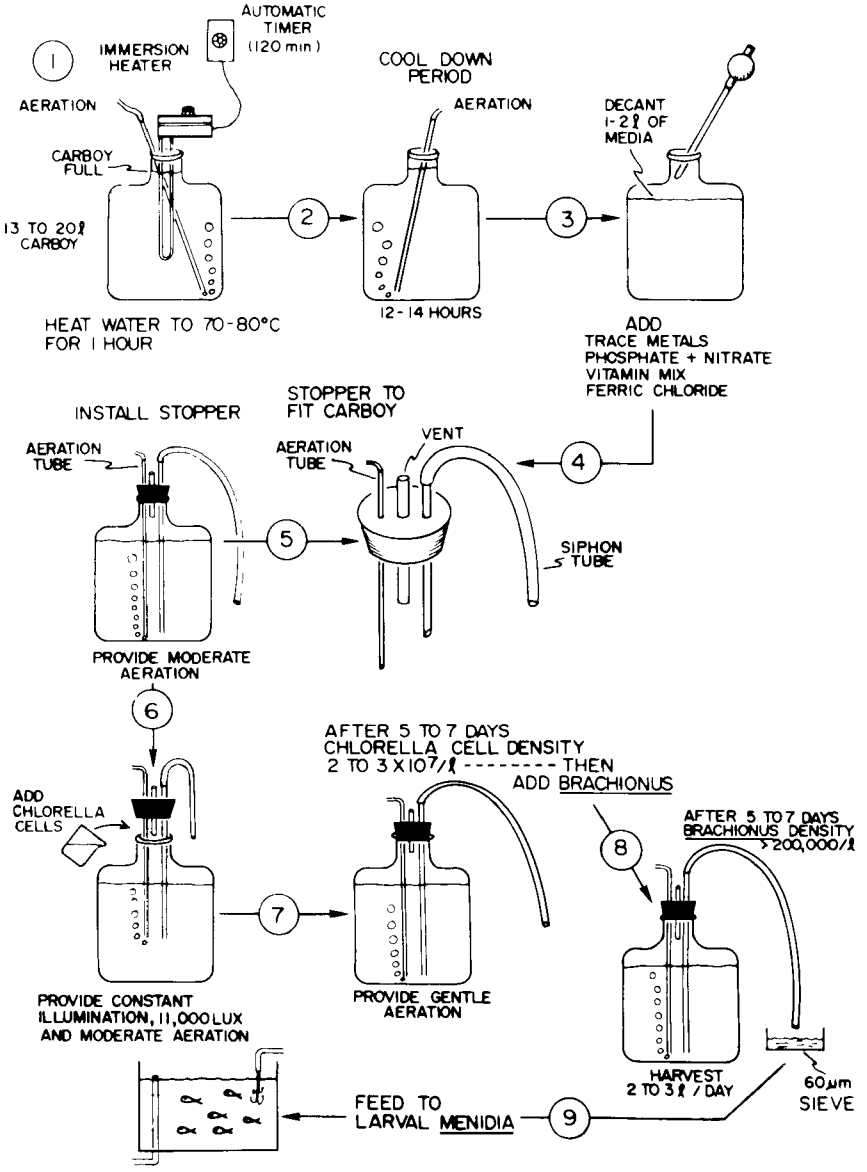

Figure 8. Diagrammatic explanation of procedure for culturing the mixohaline rotifer, *Brachionus plicatilis*.

5. Seawater -- Should be heated to a thermostatically set temperature of ~70 to 80°C. This temperature should be maintained for 1 hr. A glass tube (3 mm O.D.) inserted into the carboy (before heating begins) and attached to an air line provides moderate aeration/circulation of the water during the pasteurization process. This aeration/circulation is essential to ensure even heating of media and to prevent a thermocline from forming in the carboy.

6. After maintaining ~70 to 80°C for 1 hr -- Turn off the immersion heater, let it cool down for 5 mins, then remove it. The automatic timer system can be set to the proper elapsed time to accomplish heat-up, pasteurization and cool-down on an automatic basis after heating dynamics of the carboy-immersion heater system are determined on the first few carboys of seawater. *Note:* If a large autoclave is available it may be used to sterilize culture media thus avoiding the procedures outlined above in B. 4-6.

7. Gentle aeration -- Should be maintained for 12 to 14 hrs until the seawater has cooled to ambient temperature. The carboy should then be moved to a convenient location where high intensity lighting (> 11,000 lux) is continuously provided.

8. The aeration tube-siphon tube-vent system -- This is wrapped in clean aluminum foil and maintained in a drying oven at ~50 to 60°C, it is then removed from the oven and installed in the carboy of pasteurized seawater.

9. Appropriate enrichment media -- To grow *Chlorella*, nutrients are then added to the carboy and allowed to mix for 5 mins. Enrichment media will differ according to the type of seawater used, natural or artificial, and the locale where collected. One media enrichment formulation is summarized in Appendix H. This formulation may need to be modified for optimal growth of *Chlorella* sp. under local conditions. Additional formulations are available in Walsh and Alexander (1980), Theilacker and McMaster (1971) and the American Public Health Association, Standard Methods (1985).

10. Moderate aeration -- Should be provided to mix nutrients and to keep *Chlorella* sp. cells in suspension.

11. Carboy -- It should then be inoculated with 4 to 5 x 10^5 *Chlorella* sp. cells.

12. *Chlorella* sp. cells -- They should be allowed to grow for 5 to 7 days depending upon the initial density of starter cells added to the 13 to 20 l carboy.

    a. 5 ml samples of *Chlorella* sp. should be removed and cell density determined using the hemacytometer until the

culture becomes a bright green and contains 2 to 3 x 10^7 cells l^{-1}. We generally take replicate 5 ml samples from the carboy and make 4 to 8 counts per sample to determine cell density.

C. Culture of *B. plicatilis*.

1. Starter cultures -- *Brachionus plicatilis* cultures are maintained in static 4 l glass flasks containing 2 to 3 l of *Chlorella* sp. with an initial cells density of 2-3 x 10^7 cells l^{-1}.

2. Cultures -- Should be renewed at 7 to 10 day intervals by innoculation of new pasteurized (sterilized) and nutrient enriched culture media with *Chlorella* sp. When *Chlorella* sp. cell densities grow to 2 to 3 x 10^7 cells l^{-1}, *Brachionus* is added.

   a. To determine *Brachionus* densities -- The 4 l glass flask is shaken to ensure an even distribution of *Brachionus*. The density of *Brachionus* may be determined by pouring 1 l of the media from the flask through ~60 mm plankton netting. This sample is then washed into a 100 ml beaker (using a squeeze bottle containing pasteurized seawater) and pasteurized water is then added to the beaker to bring the volume to 20, 50 or 100 ml, depending upon the concentration of *Brachionus* present.

   b. Five, 10 µl subsamples are then removed from the 100 ml beaker. Each sample is placed in a deep well depression slide or in the center of a clear glass crystallization dish. One drop of a 10% neutral buffered formalin solution is then added to each 10 µl sample of *Brachionus*.

   c. Counts of *Brachionus* -- Should be conducted at 15 to 30x magnification using a dissecting microscope, and the mean number of *Brachionus* determined for the five samples.

   d. Back calculate -- The number of *Brachionus* 100 ml^{-1} (or to 50 or 20 ml of concentrate) is calculated from the original 1 l sample.

      1. For example in five samples the $\bar{x}$ number of *Brachionus* = 80 per 10 µl x 100 = 8000 ml^{-1}

         8000 ml^{-1} x 100 ml = 800,000 l^{-1}
         8000 ml^{-1} x 50 ml = 400,000 l^{-1}
         8000 ml^{-1} x 20 ml = 160,000 l^{-1}

   e. Use 10,000 to 20,000 *Brachionus* l^{-1} to innoculate each 13 to 20 l carboy of *Chlorella* sp. when the alga cell density has reached 2 to 3 x 10^7 cells l^{-1}.

3. *Brachionus* densities -- The densities should reach 150,000 to 300,000 l^{-1} by the 5th to 7th day after a carboy is innoculated.

The procedure explained in VI. C. 2. a. through e. is used to quantify *Brachionus* in carboys.

a. When densities are $\geq$200,000 l^{-1} we recommend that the entire volume of the carboy be used over a 4 to 5 day period.

   1. Daily use of 2 to 3 l of *Brachionus* is recommended.

   2. Daily counts of *Brachionus* l^{-1} are required to maintain a record so that feeding rates can be adjusted (by dilution or concentration of 1 l samples). Recall that a feeding rate of 10,000 *Brachionus* l^{-1} volume of holding aquaria for *Menidia* sp. is recommended.

Caution:

4. *Brachionus* is not capable of synthesizing essential fatty acids (EFA's) for marine fish in the quantities required by those fish (Lubzens et al., 1985). The rotifers must therefore be fed on algae that contain high levels of EFA's for marine fish (e.g., *Skeletonema, Tetraselmis, Chaetoceros, Isochrysis,* or marine *Chlorella,* but not *Dunaliella* or *Phaeodactylum*).

VII. Hatching of brine shrimp, *Artemia* sp.

A. **Chemical analyses**--The strain of *Artemia* used should be analyzed for chemical residues including heavy metals and pesticides.

B. **Apparatus required.**

1. Four to six, 2 l separatory funnels.
2. Several 500 ml beakers.

C. **Hatching procedure.**

1. Fill -- A 2 l separatory funnel with approximately 1,800 ml of 20 µm filtered seawater or reconstituted artificial seawater that has been aged for at least 7 days. The salinity should be adjusted to 25 to 30 ‰.
2. Add -- *Artemia* cysts, 15 to 20 ml (dry measure) to the separatory funnel.
3. After adding *Artemia* cysts -- Clean air is vigorously bubbled through a 1-ml pipette which is lowered through the neck of the funnel until the tip rests on the bottom. Aeration keeps the cysts and newly hatched *Artemia* nauplii in suspension. Cysts should hatch in 24 to 36 hrs when the temperature is 27°C.
4. After 24 hrs -- The pipette supplying air is removed. Allow *Artemia* nauplii to settle to the bottom of the separatory funnel. A light source placed near the bottom of the separatory funnel will enhance the settling process. Empty cysts will rise to the surface.
5. After approximately five mins, using the stopcock, collect the nauplii into a 500 ml beaker with a 100 mm mesh screen bottom. Discard the hatching water and rinse the nauplii into a 500 ml beaker.
6. After another five mins, again collect the nauplii and rinse into the beaker.
7. The nauplii are further concentrated by pouring the suspension into a small cylinder which has one end closed with 100 mm plankton netting.

8. The concentrate is resuspended in 50 ml of appropriate culture water, mixed well, and dispensed with a pipette.

9. Discard the remaining contents of the hatching vessel, wash the vessel with hot soapy water, and rinse thoroughly.

10. Prepare fresh seawater for each new batch culture of *Artemia* nauplii.

11. To have a fresh supply of *Artemia* nauplii daily, several hatching vessels must be set up and harvested on alternate days.

D. Enumeration of *Artemia* nauplii.

1. Suspensions of nauplii should be well mixed.

   a. draw off five 1.0 ml aliquots of the suspension.

   b. the *Artemia* nauplii in each 1.0 aliquot may then be slowly pipetted onto white filter paper so that the 1.0 ml volume is dispersed over a wide area of the paper.

   c. *Artemia* nauplii are then enumerated by placing the filter paper on a dissecting microscope. The mean number of nauplii in five samples is then used in back calculations to determine the volume of original suspension to be fed to *Menidia* sp. or *Leuresthes tenuis*. Recall that a feeding rate of 5,000 *Artemia* nauplii l^{-1} volume of holding aquaria is recommended.

E. Nutritional quality of *Artemia*.

1. To the extent that the atherinid species discussed here are marine, they require a marine diet. Marine and freshwater fish have different nutritional requirements, especially with regard to essential fatty acids. Watanabe et al., (1978) pointed out that *Artemia* can be divided in two categories, those that are adequate for marine organisms and those that are not, based on their fatty acid composition. Different geographical strains of *Artemia* vary in their nutritional quality for *Menidia* (Beck et al., 1980; Beck and Bengtson, 1982) and a single geographical strain can also vary over time (Leger et al., in press).

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Appendix A

Selected biogeographical data for occurrence of the inland silverside, *Menidia beryllina*

State	Locale	Habitat	Citation
Rhode Island	Pattaquamscutt R.	Estuarine	Bengtson, 1984
Rhode Island	Point Judith Pond	Estuarine	Bengtson, 1984
Massachusetts	Massachusetts Bay	Estuarine	Kendall, 1902
Massachusetts	Quincy Bay	Estuarine	Robbins, 1969
Connecticut	Mystic R. (lower)	Estuarine	Pearcy and Richards, 1962
Connecticut	Mill R.	Estuarine	Johnson, 1975
Delaware	Delaware R. (lower)	Estuarine	DeSylva et al., 1962
Virginia	Potomoc R. (lower)	Estuarine	Kendall, 1902
Virginia	James R.	Estuarine	Raney, 1950
Virginia	Rappahannock R. (45 mi upstream)	Riverine	Massman, 1954
North Carolina	Pasquotank R.	Estuarine	Smith, 1893
North Carolina	Edenton Bay	Estuarine	Smith, 1893
North Carolina	Perquiman's R.	Estuarine	Johnson, 1975
North Carolina	Neuse R.	Estuarine	Tagatz and Dudley, 1961
Florida	Palatka	Lake	Johnson, 1975
Florida	L. Eustis	Lake	Chernoff et al., 1981
Florida	L. Weir	Lake	Chernoff et al., 1981
Florida	St. Johns R.	Riverine	Chernoff et al., 1981
Florida	South Lake	Lake	Kendall, 1902
Florida	L. Monroe	Lake	Kendall, 1902
Florida	L. Jessup	Lake	Kendall, 1902
Florida	Escambia Bay	Estuarine	Chernoff et al., 1981
Florida	Blackwater Bay	Estuarine	Middaugh et al., 1986
Florida	Perdido Bay	Estuarine	Chernoff et al., 1981
Alabama	Gulf Shores	Estuarine	Johnson, 1975
Mississippi	Escatawpa R.	Riverine	Chernoff et al., 1981
Mississippi	Moon Lake	Lake	Chernoff et al., 1981
Louisiana	L. Pontchartrain	Estuarine	Chernoff et al., 1981
Louisiana	L. Angola	Lake	Chernoff et al., 1981
Louisiana	Wax Lake	Lake	Chernoff et al., 1981
Louisiana	Mississippi Delta	Riverine/ Estuarine	Chernoff et al., 1981
Louisiana	Chandeleur Islands	Estuarine	Chernoff et al., 1981
Louisiana	L. St. John	Lake	Johnson, 1975
Arkansas	L. Chicot	Lake	Chernoff et al., 1981
Tennessee	Reelfoot Lake	Lake	Johnson, 1975
Kentucky	Hamby Pond	Pond	Sisk, 1973
Illinois	Mississippi R.	Riverine	Smith, 1979
Texas	L. Marble Falls	Lake	Tilton and White, 1964
Texas	L. Buchanan	Lake	Tilton and White, 1964
Texas	L. Inks	Lake	Tilton and White, 1964
Texas	L. Brownwood	Lake	Tilton and White, 1964
Texas	L. Texoma	Lake	Mense, 1967
Texas	Colorado R.	Riverine	Tilton and White, 1964
Texas	Laguna Madre	Estuarine	Simmons, 1957
Oklahoma	L. Texoma	Lake	Hubbs, 1982
Oklahoma	Keystone Reservoir	Lake	Gomez and Lindsay, 1972
Oklahoma	Arkansas R.	Riverine	Gomez and Lindsay, 1972
Oklahoma	Boomer Lake	Lake	Sisk and Stephens, 1964
California	Clear Lake	Lake	Cook and Moore, 1970
California	Sacramento River	Riverine	Moyle et al., 1974
California	Lexington Reservoir	Lake	Moyle et al., 1974

Appendix B

Recommended environmental variables and feeding regimes for laboratory spawning of three atherinid fishes

Environmental Variable	Atherinid species *M. beryllina*	*M. menidia*	*M. peninsulae*
Adult holding/spawning			
Photoperiod	13L:11D	13L:11D	13L:11D
Intensity (lux)	175-300	175-300	175-300
Tidal signals/ interrupted current velocity	none required	1200-1300 hrs 2400-0100 hrs	1200-1300 hrs 2400-0100 hrs
Salinity (‰)	0 - 5	25 - 30	25 - 30
Water Temp. (°C)	25	22 (N. lat.) 25 (S. lat.)	25
Food required			
Tetramin Flakes	8 g at 0800-0900 hr 4 g at 1000-1100 hr 4 g at 1300-1400 hr 8 g at 1500-1700 hr	8 g at 0800-0900 hr 4 g at 1000-1100 hr 4 g at 1300-1400 hr 8 g at 1500-1700 hr	8 g at 0800-0900 hr 4 g at 1000-1100 hr 4g at 1300-1400 hr 8 g at 1500-1700 hr
Artemia sp. nauplii (optional) ca. 150,000 l [1]	1 liter at 1130 hr	1 liter at 1130 hr	1 liter at 1130 hr

Appendix C

Recommended environmental variables and feeding regimes for laboratory incubation and larval rearing of four atherinid fishes

Environmental Variable	Atherinid species			
	M. beryllina	*M. menidia*	*M. peninsulae*	*L. tenuis*
Embryo-larval rearing				
Photoperiod	14L:10D	14L:10D	14L:10D	14L:10D
Intensity (lux)	11,000	11,000	11,000	11,000
Salinity (‰)	0-15	25-30	25-30	25-30
Water Temp. (°C)	25	22 (N. lat.) 25 (S. lat.)	25	25
Larval density l^{-1} (grow-out tanks)	5-10	5-10	5-10	5-10
Food required				
Brachionus sp.	days 1-5	days 1-5	days 1-5	not required
amount l^{-1} vol. of grow-out tanks	10,000 twice a day	10,000 twice a day	10,000 twice a day	not required
Artemia sp.	day 6 through end of holding	day 6 through end of holding	day 6 through end of holding	day 1 through end of holding
amount l^{-1} vol. of grow-out tanks	5,000 twice a day	5,000 twice a day	5,000 twice a day	5,000 twice a day

Appendix D

Selected biogeographical data for occurrence of the Atlantic silverside, *Menidia menidia*

State	Locale	Habitat	Citation
Maine	S. Portland	Estuarine	Johnson, 1975
Maine	Scarborough Harbor	Estuarine	Robbins, 1969
Maine	Todd Point	Estuarine	Robbins, 1969
Maine	Casco Bay	Estuarine	Kendall, 1902
Rhode Island	Point Judith Pond	Estuarine	Bengtson, 1984
Rhode Island	Pettaquamscutt R.	Estuarine	Bengtson, 1982
Rhode Island	Bissell Cove	Estuarine	Bengtson, 1982
Massachusetts	Massachusetts Bay	Estuarine	Bigelow and Schroeder, 1953
Massachusetts	Vineyard Sound	Estuarine	Kendall, 1902
Massachusetts	Buzzard Bay	Estuarine	Kendall, 1902
Massachusetts	Nantucket Sound	Estuarine	Kendall, 1902
Massachusetts	Eel Pond	Estuarine	Kendall, 1902
Massachusetts	Great Harbor	Estuarine	Kendall, 1902
Massachusetts	Hadley Harbor	Estuarine	Kendall, 1902
Massachusetts	Katama Bay	Estuarine	Kendall, 1902
Massachusetts	Woods Hole	Estuarine	Kendall, 1902
Massachusetts	Salem Harbor	Estuarine	Conover and Kynard, 1984
Connecticut	Noank	Estuarine	Johnson, 1975
Connecticut	Pataguanset R.	Estuarine	Cadigan and Fell, 1985
New York	Fire Island Inlet	Estuarine	Briggs, 1975
Delaware	Delaware R. (lower)	Estuarine	DeSylva et al., 1962
Virginia	James R.	Estuarine	Massman, 1954
Virginia	Rappahannock R.	Estuarine	Massman, 1954
Virginia	Pamunkey R.	Estuarine	Massman, 1954
Virginia	Oyster	Estuarine	Johnson, 1975
Maryland	Solomons Island	Estuarine	Bayliff, 1950
Maryland	Drew Point	Estuarine	Bayliff, 1950
Maryland	Green Holly Creek	Estuarine	Bayliff, 1950
Maryland	Molly's Island	Estuarine	Bayliff, 1950
North Carolina	Moorehead City	Estuarine	Kendall, 1902
North Carolina	River's Island	Estuarine	Hildebrand, 1922
North Carolina	Neuse River (lower)	Estuarine	Tagatz and Dudley, 1961
South Carolina	Cape Romain	Estuarine	Fowler, 1945
South Carolina	Magnolia Beach	Estuarine	Fowler, 1945
South Carolina	Cape Island	Estuarine	Fowler, 1945
South Carolina	North Edisto R.	Estuarine	Middaugh, 1981
South Carolina	Edisto R. (lower)	Estuarine	Fowler, 1945
South Carolina	Battery Island	Estuarine	Johnson, 1975
Georgia	Sea Island	Estuarine	Fowler, 1945
Georgia	Ogeechee R.	Estuarine	Fowler, 1945
Georgia	Sapelo Island	Estuarine	Fowler, 1945
Georgia	St. Simons Island	Estuarine	Robbins, 1969
Georgia	Jekyl Island	Estuarine	Robbins, 1969
Florida	Ft. George Inlet	Estuarine	Johnson, 1975
Florida	Matangos R.	Estuarine	Johnson, 1975
Florida	Flagler Beach	Estuarine	Johnson, 1975
Florida	New Smyrna Beach	Estuarine	Johnson, 1975
Florida	Mosquito Lagoon	Estuarine	Gosline, 1948

Appendix E

Selected biogeographical data for occurrence of the tidewater silverside, *Menidia peninsulae*

State	Locale	Habitat	Citation
Florida	Melbourne	Estuarine	Johnson, 1975
Florida	Ft. Meyers	Estuarine	Johnson, 1975
Florida	Shell Point	Estuarine	Johnson, 1975
Florida	Southport	Estuarine	Johnson, 1975
Florida	Pass-A-Grille	Estuarine	Chernoff et al., 1981
Florida	Mosquito Lagoon	Estuarine	Chernoff et al., 1981
Florida	Crystal R.	Estuarine	Chernoff et al., 1981
Florida	Cedar Key	Estuarine	Chernoff et al., 1981
Florida	Santa Rosa Island	Estuarine	Middaugh and Hemmer, 1984
Florida	Escambia Bay	Estuarine	Johnson, 1975
Mississippi	Pascagoula	Estuarine	Chernoff et al., 1981
Mississippi	Horn Island	Estuarine	Chernoff et al., 1981
Texas	Galveston Bay	Estuarine	Johnson, 1975
Texas	Aransas Pass	Estuarine	Chernoff et al., 1981
Texas	Leffy Ann	Estuarine	Chernoff et al., 1981
Texas	Capano Bay	Estuarine	Johnson, 1975

Appendix F

Selected biogeographical data for occurrence of the California grunion, *Leuresthes tenuis*

State	Locale	Habitat	Citation
California	Morrow Bay to Cayucos	Beach	Walker, 1952
California	Ismo Beach	Beach	Walker, 1952
California	Santa Barbara	Beach	Walker, 1952
California	Malibu	Beach	Walker, 1952
California	Santa Monica	Beach	Walker, 1952
California	Venice	Beach	Walker, 1952
California	Hermosa Beach	Beach	Walker, 1952
California	Cabrillo Beach	Beach	Walker, 1952
California	Long Beach	Beach	Walker, 1952
California	Belmont	Beach	Walker, 1952
California	Huntington Beach	Beach	Walker, 1952
California	Newport Beach	Beach	Walker, 1952
California	Corona del Mar	Beach	Walker, 1952
California	Doheny Beach	Beach	Walker, 1952
California	Del Mar	Beach	Walker, 1952
California	Black's Beach	Beach	Walker, 1952
California	La Jolla	Beach	Walker, 1952
California	Mission Beach	Beach	Walker, 1952
California	Coronado Strand	Beach	Walker, 1952

Appendix G

Recommended test parameters and feeding regimes for conducting static or flow-through 96 hr acute toxicity tests with 14 day-old atherinid fishes

Environmental Variable	Atherinid species *M. beryllina*	*M. menidia*	*M. peninsulae*	*L. tenuis*
Photoperiod	14L:10D	14L:10D	14L:10D	14L:10D
Intensity (lux)	11,000	11,000	11,000	11,000
Salinity (‰)	0-15	25-30	25-30	25-30
Water Temp. (°C)	25	22 (N. lat.) 25 (S. lat.)	25	25
Feeding Requirements				
Artemia sp nauplii	20-30 fish^{-1} 2 to 3 times daily	20-30 fish^{-1} 2 to 3 times daily	20-30 fish^{-1} 2 to 3 times daily	20-30 fish^{-1} 2 to 3 times daily

Appendix H

Enrichment media for seawater used to grow *Chlorella* sp.

Nutrient Mixes	Amount
TRACE METALS [a]	
$MnCl_2 \cdot 4H_2O$	361 mg
$ZnCl_2$	42 mg
$CuSO_4 \cdot 5H_2O$	8 mg
$Na_2MoO_4 \cdot 2H_2O$	5 mg
$CoCl_2 \cdot 6H_2O$	8 mg
Glass distilled or deionized water	1.0 liter
IRON [b]	
$FeCl \cdot 6H_2O$	480 mg
Glass distilled or deionized water	100 ml
NITRATES AND PHOSPHATES [c]	
$NaNO_3$	75 mg
NaH_2PO_4	6 mg
Glass distilled or deionized water	1.0 liter
Vitamin mix [d]	
Thiamine hydrochloride	0.4 g
Biotin	1.0 mg
B12	1.0 mg
Glass distilled or deionized water	1.0 liter

[a] add 1.0 ml l^{-1} of seawater media
[b] add 0.1 ml l^{-1} of seawater media
[c] add 1.0 ml l^{-1} of seawater media
[d] add 1.0 ml l^{-1} of seawater media