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Methods for Conducting Bioassays Using Embryos and Larvae of Pacific Herring, *Clupea pallasi*

Paul A. Dinnel · Douglas P. Middaugh · Nathan T. Schwarck · Heather M. Farren · Richard K. Haley · Richard A. Hoover · James Elphick · Karen Tobiason · Randall R. Marshall

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Abstract The rapid decrease of several stocks of Pacific herring, *Clupea pallasi*, in Puget Sound, Washington, has led to concerns about the effects of industrial and nonpoint source contamination on the embryo and larval stages of this and related forage fish species. To address these concerns, the state of Washington and several industries have funded efforts to develop embryo and larval bioassay protocols that can be used by commercial laboratories for routine effluent testing. This article presents the results of research to develop herring embryo and larval bioassay protocols. Factors evaluated during protocol development included temperature, salinity, dissolved oxygen (DO), light intensity, photoperiod, larval feeding regimes, use of brine and artificial sea salts, gonad sources, collection methods, and egg quality.

Pacific herring (*Clupea pallasi*) is an important forage and commercial fishery species throughout its range on the Pacific Coast of the United States and Canada, including the Puget Sound, Washington State region (Penttila 2007). Some Puget Sound stocks of Pacific herring have suffered precipitous decreases in abundance and spawning biomass during the last several decades. Indeed, the largest stock of herring in Washington State, the Cherry Point stock, is now in critical condition, with only approximately 10% of the spawning biomass present compared with the mid-1970s (Lemberg et al. 1998; EVS 1999; Stout et al. 2001; G. Bargmann, Washington Department of Fisheries, personal communication 2009), and the fishery at Cherry Point has been closed since 1997. Although much of this decrease may be due to natural factors (e.g., long- and short-term temperature increases, predation by fish and marine mammals, changes in zooplankton populations) and/or excessive fishing pressure, neither point nor nonpoint sources of pollution can be ruled out as potentially significant stressors that may be acting in concert with natural stressors (EVS 1999; Stout et al. 2001).

Evidence exists that herring populations have been adversely affected by pollution. The strongest evidence is the conclusion by Carls et al. (2002) that the *Exxon Valdez* oil spill in Prince Williams Sound (PWS), Alaska, damaged 25–32% of Pacific herring embryos spawned in PWS in the year of the spill and that the effects of the oil spill cannot be ruled out as a partial cause of the virtual collapse of PWS herring stocks in 1992–1993. In Puget Sound, herring embryo in situ exposure studies conducted by Kocan et al. (Kocan and MacKay 1991; Kocan 1991, 1992, 1998) at Cherry Point, Washington, led to the conclusion that embryos suffered adverse effects in near-shore spawning areas adjacent to industrial plants. Two of their four in situ studies suggested that there were adverse impacts on embryo survival, degree of normality at hatch, and size at hatch at several stations offshore of two oil refineries and one aluminum smelter (EVS 1999). However, similar out-planting studies by Hershberger et al. (2005) failed to find site-specific effects, and they

P. A. Dinnel (✉) · N. T. Schwarck · H. M. Farren · R. K. Haley · R. A. Hoover
Shannon Point Marine Center, Western Washington University, Anacortes, WA 98221, USA
e-mail: padinnel@aol.com

D. P. Middaugh
Belle W. Baruch Institute, Pensacola, FL 32503, USA

J. Elphick · K. Tobiason
Nautilus Environmental, LLC, Tacoma, WA 98124, USA

R. R. Marshall
Washington Department of Ecology, Olympia, WA 98504, USA

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concluded that impaired embryo survival and development was a result of a generally stressed herring stock perhaps related to high adult mortality and severely constricted age class structure (i.e., young spawners that produce poor quality eggs relative to older spawners).

Another study by O'Neill and West (2001) sampled adult herring and herring eggs from various locations in Puget Sound. They found that the highest adult body burdens of polychlorinated biphenyls and pesticides generally occurred in the southern and central portions of Puget Sound but that biliary fluorescent aromatic compounds, which are polycyclic aromatic hydrocarbon metabolites, were detected at low concentrations (1.0–3.5 μg/kg wet weight) at two of three Cherry Point stations, whereas none were detected in eggs from the Port Gamble (central Puget Sound) area. The investigators concluded that the presence of some of these compounds was consistent with exposure to Alaska North Slope crude oil (which is refined nearby); however, concentrations were lower than a published lowest–observable effects concentration (LOEC) (O'Neill and West 2001).

The sensitivity of herring embryos to some environmental contaminants, especially PAHs, has been previously documented by a number of other researchers (e.g., Blaxter 1977; Carls et al. 1999, 2002; Eldridge et al. 1977; Kocan et al. 1987; Kinne and Rosenthal 1967; Lindén 1978; Norcross et al. 1996; Struhsaker et al. 1974; von Westerhagen et al. 1979; Vines et al. 2000), although side-by-side comparisons between herring and other fish species are generally lacking.

Embryonic and larval herring are known to be especially sensitive to PAHs at concentrations as low as 1 μg/L (Carls et al. 1999; Brown et al. 1996; Kocan et al. 1996; Laur and Haldorson 1996). Sediments in the Cherry Point near shore have been contaminated by up to 73 reported refinery spills, creosote-treated pier pilings, and possible point and nonpoint effluent sources. PAHs from the Cherry Point region are known to accumulate in marine biota, as documented by Salazar (Stout et al. 2001). However, Stout et al. (2001) concluded that although Puget Sound herring are exposed to, and accumulate, various contaminants known to affect embryo and larval herring in laboratory tests, there were no conclusive cause and effect data to show that toxic chemicals have been a significant factor in the decrease of Cherry Point and other stocks of herring in Puget Sound.

Because of a need for cause-and-effect data on chemical and temperature stresses to herring early life stages, Washington resource agencies and local industries initiated an effort to develop standardized herring embryo and larval bioassay protocols that may be used to test effluent discharges (i.e., whole effluent toxicity [WET] testing [Marshall 1998; Washington Department of Ecology [WDOE; 2008]), ambient water quality, and possibly sediments in laboratory tests. Presently, the only marine fish embryo or larval toxicity tests approved by the United States Environmental Protection Agency (USEPA) or the state of Washington are the topsmelt (*Atherinops affinis*) and inland silverside (*Menidia beryllina*) survival and growth tests and the sheepshead minnow (*Cyprinodon variegatus)* embryo and survival and growth tests (ASTM 2009; USEPA 1991, 1995; WDOE 2008). However, none of these test species is native to Washington State. *M. beryllina* is a United States East Coast species and *C. variegatus* is native to the East and Gulf Coasts. *A. affinis* is a Pacific Coast species that ranges as far north as Vancouver Island, British Columbia (Gotshall 1981) but is generally rare north of California. Thus, development of Pacific herring embryo and larval bioassays would allow for testing in Washington State, British Columbia, and Alaska using a local species of significant economic and food chain importance.

Although toxicity testing with herring of several species has been conducted by various investigators (e.g., Vigers et al. 1978; Struhsaker 1977; Lindén 1978; Smith and Cameron 1979; von Westerhagen et al. 1979; Kocan et al. 1987, 1996; Hose et al. 1996; Norcross et al. 1996), no one has developed a standardized and validated laboratory bioassay protocol specific to Pacific herring in the Pacific Northwest. The effort that comes closest to development of a routine West Coast laboratory protocol for herring embryos and larvae is work by Middaugh et al. (1998). Middaugh et al. developed a "Petri-dish method" for testing Pacific herring embryos and larvae while at the USEPA Gulf Ecology Division (GED). They used herring gonads collected from San Francisco Bay and shipped by way of air express to GED to test the toxicity of biodegraded Alaska petroleum fractions on developing embryos and larvae. Based on studies by Boudreau et al. (2009), the potential also exists for parallel development of an East Coast Atlantic herring (*C. harengus*) test protocol.

The Pacific herring embryo bioassay protocol developed at the Shannon Point Marine Center in Anacortes, Washington, used the work of Middaugh et al. (1998) as well as provisions recommended by the USEPA for developing new WET test protocols (USEPA 2000) as a starting point. The objective of our protocol research was to develop embryo and larval test methods that are easy to conduct, cost-effective, easy to quantify, and that use small volumes of effluent or ambient waters.

Protocol Development

Three herring test protocols were developed in parallel: (1) an embryo development test, (2) a 96-hour acute larval test, and (3) a 7-day larval survival and growth test. Variables assessed during test development included

temperature, salinity, light intensity, photoperiod, DO, gamete quality, fertilization dynamics, test chamber types and volumes, and types of assessment end points. Additional variables assessed for larval testing included feeding dynamics (food type and density), larval loading rates, effects of larval culling, and use of brines and artificial sea salts.

Embryo and Larval Sources

Embryo tests are initiated with freshly fertilized eggs, which are then incubated in control and test solutions until hatching occurs. Sperm and eggs must be obtained from gonads of freshly caught (or cultured) mature herring and the eggs fertilized just before test initiation. Gonads can be stored in sealed containers for up to several days before use.

Larval tests may be accomplished using eggs that are fertilized and hatched in the laboratory or by field-collecting natural spawn on eelgrass or algae and hatching in laboratory tanks.

Embryo Test Development

The basic method of Middaugh et al. (1998), which used embryo exposures in Petri dishes, was used as a starting point for test method development. We found the Petri-dish exposure method to be satisfactory and investigated the effects of the following testing variables to arrive at a final recommended test protocol.

Gamete Quality and Fertilization

Although many factors can affect the quality of the gametes used for embryo tests, we found that carefully collected, shipped, and handled gonads can remain viable for approximately 5 days. One experiment that evaluated gonad longevity found that gametes shipped from California to Washington and stored at 4°C retained high (>80%) fertilization success and normal hatch rates after 5 days of storage before fertilization.

Early in the protocol development process, we conducted tests designed to determine possible limitations when fertilizing eggs in the laboratory before initiating embryo tests. Two tests assessed time delays in adding either sperm or eggs to Petri dishes, whereas the opposite gametes were being exposed to control seawater. In the first test, we found that herring eggs could be fertilized for at least 60 min after their addition to seawater and that sperm would remain viable for at least 32 h in 16‰ salinity at 10°C. In the second test, there were no significant differences in fertilization success when the eggs were added to seawater first or vice versa.

For some species, the density of sperm used to fertilize eggs can affect test results. Too few sperm can decrease fertilization rates, whereas too many sperm might have the potential for polyspermy (multiple sperm penetration), which can result in abnormal development. We ran two tests to determine the minimum density of sperm necessary to obtain good fertilization success in 30 mL control seawater in Petri dishes and two tests to determine if high sperm densities had any adverse effects on the proper development of embryos.

Results of the first two tests showed that there was no evidence of decreasing fertilization success over a range of sperm densities from 3.4×10^8 to 6.7×10^5 per Petri dish. Results of the last two tests indicated that sperm densities of 5, 10, and 100 times our normally used density (approximately 1×10^8 sperm resulting from 1-mL additions of a sperm suspension prepared by macerating a 1 cm^2 piece of testis in 100 mL control seawater) did not cause a significant decrease in the percentages of normally hatched larvae that might have resulted from polyspermy. Thus, our tests indicate that any sperm density from approximately 1×10^6 to 1×10^{10}/Petri dish is likely satisfactory for fertilizing herring eggs.

The quality of the gametes used for embryo tests can vary depending on such factors as egg and sperm ripeness, age of the spawning fish, stresses induced during collection and shipping, and time after collection. From 2001 through 2007, we conducted 72 embryo tests for which we have data on % fertilization versus % normal larval hatch. During these tests, the criterion for a successful embryo test was that the normal hatch rate must be ≥70%. Based on this criterion, 72.2% of the embryo tests conducted from 2001 through 2007 were successful tests. However, these data included results during the early stages of the project. For tests conducted from 2004 through 2007, the success rate improved to 82.4%.

It is always best to initiate embryo tests using good-quality gametes that produce fertilization of ≥90%. However, for reasons described previously, this is not always possible. There is a good direct correlation between fertilization success and embryo survival/normal hatch (Fig. 1). However, even batches of embryos that had moderate fertilization rates (50–80%) can still produce tests that are acceptable based on the ≥70% normal hatch criterion (Fig. 1, area A). The reason for this is that unfertilized and poorly developing embryos are removed from the test dishes 48 h after fertilization, and the remaining fertilized eggs have a good chance of surviving and hatching normally. Although rare, the reverse of this can also be true, i.e., batches of eggs showing high

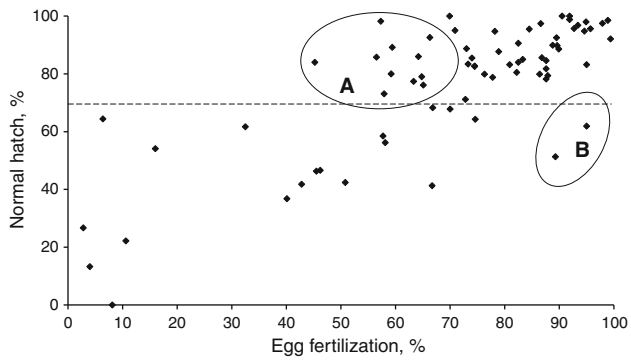

Fig. 1 Percentages of average normal hatch in control samples compared with starting egg fertilization percentages in embryo tests conducted during a 7-year period. Tests must have at least a 70% control normal hatch (area above dotted line) to be considered valid. Some tests having relatively low fertilization success were successful (circle A), whereas a few tests with high initial fertilization failed to satisfy the 70% control normal survival criterion (circle B)

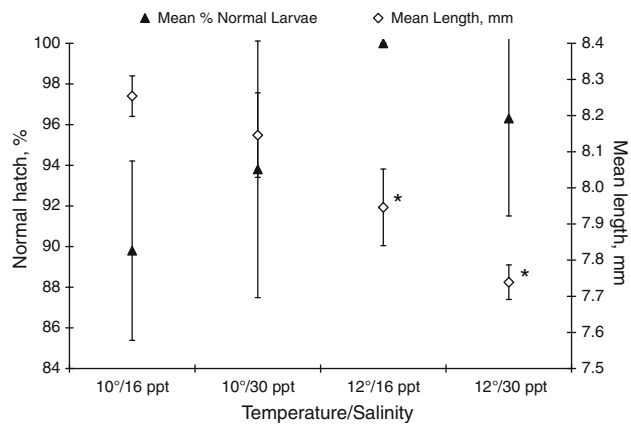

Fig. 2 Mean percentages of normal larvae and their mean lengths at hatch after exposures to control seawater at two temperature/salinity combinations. The asterisks indicate the two samples that had significantly ($p = 0.05$) lower average lengths at hatch compared with the control sample (10°/16 ppt)

fertilization rates can fail to survive or hatch normally (Fig. 1, area B), for reasons presently unknown.

Glass Versus Plastic Petri Dishes

Based on the results of a previous study (Middaugh et al. 1998), glass Petri dishes were chosen as the exposure chamber of choice for our protocol development work. However, because plastic dishes can be cheaper and easier to use, disposable plastic (polystyrene) dishes were tested in a side-by-side test with glass dishes to see if plastic could be substituted for glass dishes. This test was conducted by running a "standard" embryo test in glass and plastic Petri dishes using 6-fold replication. The nominal size for each Petri dish was 100 × 15 mm. The plastic dishes were slightly smaller, with a volume of 80–85 mL versus 95–100 mL for the glass dishes.

Average normal hatch rates for glass and plastic dishes were not significantly different, indicating that either glass or polystyrene dishes can be used. However, our experience was that plastic dishes proved more difficult to use (fluid spillover) due to their slightly smaller volume. If plastics other than polystyrene are to be used, these should also be tested before routine use because some plastics may leach potentially toxic compounds into test solutions.

Temperature

Previous investigations, as summarized by Alderdice and Hourston (1985) and EVS (1999), found that the optimal temperature range for Pacific herring development is generally in the range of 6°C to 10°C, and Alderdice and Velsen (1971) reported that high larval mortality occurred at temperatures >13.3°C. Middaugh et al. (1998) used 12°C for their Pacific herring embryo tests, which were the prototype for the present protocol, but they used eggs from San Francisco, California, fish. Based on the previous information and on the fact that we were using gonads collected from San Francisco to Alaska, we decided in 2000 to standardize the temperature for embryo and larval tests to 10°C. Because 12°C later proved to be optimum for larval testing, the effects of running embryo tests at 10°C *vs*. 12°C or 14°C were investigated in 2007.

The first of two embryo tests compared percentages of normal hatch and larval lengths at hatch at 10°C and 12°C and two salinities (16‰ and 30‰). Results of this test showed that normal hatch rates were highest, but not significantly so, at 12°C and that there was a significant ($p \leq 0.05$) decrease in larval lengths at hatch at 12°C (Fig. 2). Results of a second test that compared embryo development at 12°C *versus* 14°C showed that at 14°C embryo survival was only 51%, normal hatch was only 29%, and larval lengths at hatch were even less. Each of these measures was significantly less compared with the 12°C group. Results of these two tests showed that embryo tests should not be run at 14°C but that 10°C and 12°C are satisfactory.

Salinity

Some previous tests of the effects of salinity on development of Pacific herring embryos have shown that development is optimum in approximately half-strength seawater (approximately 15‰ to 18‰ salinity) (e.g., Griffin et al. 1998; Vines et al. 2000). One early test in our laboratory confirmed that normal hatch rates were unaffected at a range of salinity from 8‰ to 24‰. Hence, subsequent

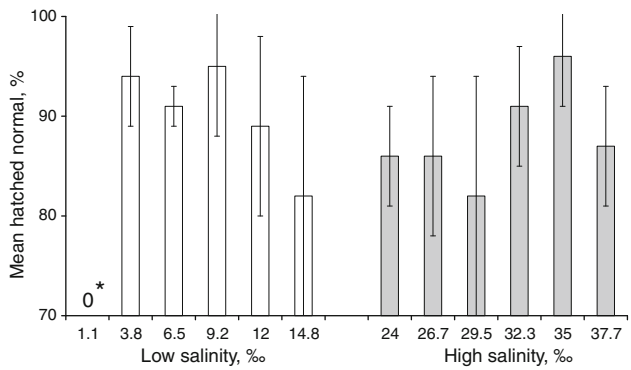

Fig. 3 Percentages of normal survival at hatch for embryos incubated in a range of salinities after fertilization in two parallel embryo tests. The 14.8‰ sample was considered the control for the low-salinity test, and the 24‰ sample was the control for the high-salinity test. The asterisk indicates the one sample that was significantly ($p = 0.05$) different from the 14.8‰ control in the low-salinity test

protocol development tests were routinely conducted at 16‰ salinity.

Once the embryo test protocol was essentially completed, we ran two additional tests to define the upper and lower salinity limits for the new protocol. These two tests were a low-salinity test, which assessed the effects of salinities from 1.1‰ to 14.8‰, and a parallel test at 24‰ to 37.7‰. The mid-range salinities (15‰ to 24‰) were not tested because previous studies had shown these to be optimal for good development. The results of these two tests showed that there were no significant differences in normal hatch rates at all salinities except that none of the embryos developed normally at 1.1‰ salinity (Fig. 3). Likewise, heart rates at days 6 or 7 were not significantly different

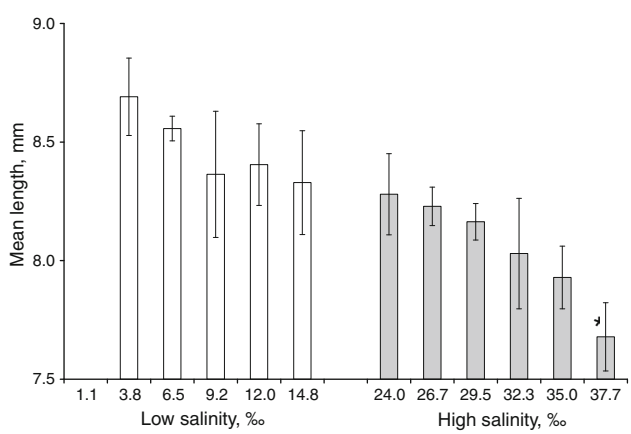

Fig. 4 Average mean lengths at hatch for embryos incubated in a range of salinities after fertilization in two parallel embryo tests. The 14.8‰ sample was considered the control for the low-salinity test, and the 24‰ sample was the control for the high-salinity test. The asterisk indicates the one sample that was significantly ($p = 0.05$) different from the 24‰ control in the high-salinity test. No embryos survived in the 1.1‰ sample

across the whole range of salinities except for the 1.1‰ group, which had heart rates approximately 20% lower than the low-salinity controls (14.8‰). However, there was a gradual decrease in larval lengths at hatch that were in inverse relation to salinity, with the 37.7‰ group being significantly smaller within the high-salinity group compared with the high-salinity control (24‰) (Fig. 4). These results indicate that embryo tests can be conducted using a wide range of salinities, although larval lengths at hatch will gradually decrease with increasing salinity >24 ‰.

Dissolved Oxygen

Kocan and Landolt (1990) found that developing herring embryos maintained in a small test system might suffer from O_2 starvation and/or CO_2 suffocation during the later stages of development, although Braum (1973) (discussed in Alderdice and Hourston 1985) found that Atlantic herring eggs could hatch successfully at DO concentrations approaching only 20% saturation.

Although we did not run experiments that measured DO in the shallow Petri dishes, one test did assess frequency of opening the Petri dishes to ambient air on varying schedules. Five replicate dishes were opened on a daily basis to replenish oxygen and release accumulated CO_2, and five replicates each were opened on a 2-, 3-, 4-, or 6-day schedule. Results of this test showed that there were no significant differences in average normal hatch rates between the five groups. Indeed, the two groups with the highest average normal hatch were the 1- and 6-day groups, which strongly suggests that embryo development was not affected by a lack of O_2 (or increase in CO_2) in the 6-day group.

Light Intensity

One embryo test was conducted to evaluate the effects of light intensity on embryo survival and normal hatch rate. Test light intensities were dark (0 lux), shaded light (170 lux [approximately 17 foot candles]), and strong light (2,650 lux [approximately 265 foot candles]) using cool white fluorescent bulbs. Replication was 5-fold for each light intensity. Results of this test showed that embryo normal hatch rates were significantly higher ($p = 0.020$) in the dark group (71.2% ± 15.4%) versus the shaded group (37.0% ± 21.2%) and the strong-light group (43.9% ± 14.0%). Results of this single test suggest that embryo tests should be conducted under dark or dim light conditions.

Brine and Artificial Sea Salts

Some commercial testing laboratories do not have access to good-quality natural seawater and must rely on artificial

sea salts for control and dilution waters. In addition, effluent samples, which are usually fresh water, must be salinity-adjusted using either brines or artificial sea salts. Thus, we conducted one herring embryo test using freeze-prepared brine (of laboratory seawater) and four commercial brands of artificial sea salts (Crystal Sea [Marine Enterprises International, Baltimore, MD, USA], Red Sea [Red Sea Fish Pharm, Eilat, Israel], Instant Ocean [Aquarium Systems, Mentor, OH, USA], and Ocean Pure [ViaAqua, Camarillo, CA, USA]). In each case, brine or sea salts were added to deionized freshwater to make test solutions of 30‰ salinity.

Results of this test showed that there were no significant differences in embryo heart rates or percentages of normal hatch for the brine or sea salt preparations compared with the natural seawater control. In addition, average larval lengths at hatch were all greater for the brine and sea salt samples compared with the natural seawater control, although not significantly so. Although herring embryo development and growth appear to be robust in a variety of brine and artificial sea salt formulae, laboratories should evaluate their specific brine or sea salt of choice for any negative effects before using for routine herring testing.

Recommended Embryo Test Protocol

Gonad Sources, Collection, Shipping, and Holding

Herring embryo tests ideally are conducted using freshly collected ovaries and testis from locally caught fish that are in a "running ripe" (late stage V to VI) condition. However, due to spawn timing and limits on local availability, gonads may need to be collected from remote locations and shipped to the laboratory. Sufficiently ripe herring are caught on or adjacent to their normal spawning grounds (intertidal and shallow subtidal eelgrass (*Zostera marina*) and kelp of various species) using trawls, small gill nets, or a fishing pole with herring jigs. Running ripe fish (eggs and sperm easily expressed from the gonopores) are killed and opened in the field, and the gonads of each sex are extracted and put into separate Petri dishes (or similar containers). Care must be used to avoid egg contact with water (fresh or salt), which can water-harden the eggs and interfere with subsequent fertilization. Likewise, an excessive amount of air may also dry out the eggs. Thus, if ovaries are small, it might be best to put multiple ovaries in a single Petri dish. Another alternative is use of Petri dishes that are segmented into two to four chambers. The respective dishes are then taped shut and put on ice (or a packaged "ice" product) in an ice chest for transport to the laboratory. If collections are from remote locations, then the ice chest should be shipped by way of an overnight express service or other means such that the elapsed transit time is kept as short as possible. Care must be taken to ensure that the gonads are maintained at <8°C but do not freeze during shipping. On receipt of the gonads in the laboratory, the arrival temperature should be measured and the gonads stored unopened in a refrigerator at 2°C to 6°C until use.

Embryo tests are initiated with the most robust eggs available. Variables to look for in assessing fitness are (1) eggs that have flowed out of the ovary and are free in the dish, (2) eggs that are "fluid" and translucent rather than rubbery or sticky, and (3) eggs that show the best fertilization success during initial trial fertilizations. Avoid using eggs that are difficult to remove from the ovary because these eggs may not be fully ripe. If using eggs from inside the ovary, first use eggs from the posterior end of the ovary.

Test fertilizations can be accomplished by adding samples of eggs from each ovary to approximately 30 mL control seawater in Petri dishes and adding a quantity of sperm. After approximately 30–45 min, fertilization success in each test batch can be evaluated by noting the presence or absence of the raised fertilization membrane (Fig. 5).

Embryo Test Initiation

Embryo tests are initiated by selecting eggs from the ripest and freshest ovary (or eggs from the ovary showing the best fertilization success) and fertilizing the eggs in 100 mm (diameter) × 15 mm (depth) glass Petri dishes (see Table 1 for a summary of key protocol specifications). The outside bottoms of these dishes should have three parallel lines inked on them such that each dish is divided into four equal height rows. These parallel lines will

Fig. 5 Examples of fertilized Pacific herring eggs and one unfertilized egg (*lower left*). Note the presence of the raised fertilization membranes

Table 1 Summary of test conditions for Pacific herring embryo tests

Test organism	Pacific herring, *C. pallasi*
Test type	Static renewal, but flow-through should also work
Test organism source	Eggs fertilized in the laboratory using gametes from freshly caught ripe herring
Test organism age	Approximately 30–60 min after fertilization
Test duration	Until all eggs have hatched or been determined to be dead (approximately 12–15 d)
Feeding	None
Test chamber	100-mm diameter × 15-mm deep glass Petri dish
Test solution volume	30 mL
Test solution renewal	100% every 2 days (or daily if toxicant dynamics so dictate)
Test temperature	10°C to 12°C ± 1°C, although lower temperatures will work but will prolong time to hatch
Dilution water	Natural filtered seawater
Salinity	6‰ to 35‰, although slightly lower and higher salinities may also be used
Salinity adjustments	Freeze-prepared brines and some artificial sea salts
Number of organisms/chamber	Approximately 100 initially and then culled to approximately 20 healthy developing embryos on day 2
Number of replicates	Minimum of 4
Light intensity	0–50 lux (approximately 0–5 foot candles)
Photoperiod	16:8-hour light-to-dark photoperiod or total darkness
Aeration	None
Control acceptability criteria	≥70% average control normal survival
Test end points	Percent normal survival and length at hatch; optional end points include heart rate and/or embryo movements at approximately day 7 and presence/absence of yolk sac edema at hatch

facilitate later evaluations under a dissecting microscope. The fertilization process is accomplished by macerating 1 × 1-cm pieces of testis from several male fish in 100 mL control seawater in a culture dish to release the sperm. Sperm from two or more male fish is mixed, and 1 mL of the resulting sperm solution is added to the test and control Petri dishes, which contain approximately 30 mL control seawater at the test temperature (ideally 10°C to 12°C). Small aliquots of eggs (approximately 15–30) are then added to the Petri dishes until approximately 100 eggs have been added to each dish. Eggs are best added with a small Teflon spatula using a gentle "sweeping" motion as the spatula is lowered into the water. This motion will help to distribute the eggs in the dishes and minimize egg clumping. Some clumping of eggs is unavoidable. The eggs will adhere firmly to the glass Petri dish. Allow the eggs to rest for 30–60 min to ensure fertilization and water hardening of the chorion. Thereafter, assess for % fertilization by observing the presence or absence of the raised fertilization membrane (Fig. 5). Fertilization should be ≥80%, although tests with lower fertilization rates may still result in satisfactory tests. After determining the percentage fertilization, decant the control water and sperm suspension from each Petri dish and gently wash the eggs with control seawater from a wash bottle. The eggs will remain in the dishes because they are solidly adhered to the dish surfaces. Finally, refill the dishes with 30 mL of the appropriate control or test solution and add covers. Put all dishes in an incubator at the appropriate temperature at a dark to dim light level (0–50 lux [approximately 0–5 foot candles]) with a 16:8-hour light-to-dark (or total dark) photoperiod.

On day 2 of the embryo test, the number of eggs in each Petri dish are decreased to approximately 20. The goal of this step is to remove any unfertilized or poorly developing eggs and any eggs that are in clumps such that approximately 20 single, well-spaced healthy eggs remain in each dish. This removal operation is best accomplished with forceps while working under a dissection microscope at approximately 50 × to 100 × magnification and in a temperature environment the same as the incubation temperature. If desired, average % fertilization success can be assessed by counting the number of fertilized and unfertilized eggs in a subset of dishes before the selection step described previously.

Because this is a static test, control and test solutions need to be replaced at 1- or 2-day intervals through day 8. During the protocol development period, solution changes were standardized to every 2 days, but the frequency may differ based on specific experimental needs and the volatility and degradation rates of the toxicant. Solution changes are easily accomplished by decanting the old solutions and replacing with 30 mL control or test solutions. These replacement solutions should be prechilled to the test temperature. Before this step, the embryos in each dish should be monitored for mortality before solution changes. Dead embryos are removed and noted on the test bench sheets.

Assessing Heart Rates and Embryo Movements

During development, embryo heart rates and frequency of movement may be assessed as two optional sublethal end points. These measurements are made at approximately day 6 or 7 after fertilization by viewing the embryos at a proper magnification under a dissection microscope in a constant-temperature room at the test temperature. Three to five embryos are randomly selected from each of the replicate dishes, thus yielding 12–20 measurements/toxicant concentration. If a heartbeat is not observed in a selected embryo (due to embryo position), then another embryo is randomly selected.

Heart rates are measured by counting the number of heartbeats in 1 min using a stopwatch with a countdown feature and an audible alarm when 1 min has elapsed. Simultaneous counts of embryo movements (from twitches to complete rotations within the chorion) can usually be made unless the movements are too frequent. In this case, separate counts of heartbeats and movements may need to be made. Simultaneous movement counts can be made by using a hand counter to record movements while heartbeats are counted. Some toxicants (e.g., crude oil [Incardona et al. 2009]) may also cause cardiac arrhythmia, which can also be assessed during heart-rate monitoring.

Test Termination

The embryo test is terminated when either the embryos have all hatched or the remaining eggs are considered dead. Hatching of herring larvae occurs during a several-day interval, generally commencing at approximately day 10 (at 12°C [lower test temperatures will prolong time to hatch]), although some toxicants may cause earlier or later hatching. When hatching is detected in daily checks starting at approximately day 9, hatched larvae are evaluated for normality and any dead eggs removed and tabulated. The number of normal larvae (Fig. 6) are recorded, removed from the Petri dishes, anesthetized in an appropriate fish anesthetic, and put into labeled vials to which concentrated neutral buffered formalin (final concentration 5–10%) or other appropriate fixative is added for preservation. The abnormal larvae (Fig. 7) are also counted and recorded and then discarded or preserved in an appropriate fixative if subsequent observations are planned (e.g., microscopy or histopathology). The preserved normal larvae are later measured for standard length using a computer-based optical imaging system with associated measuring and recording software. An optional sublethal measure of the amount of yolk sac edema may also be made at this time (Carls et al. 1999). Step-by-step methods for conducting herring embryo tests and solution changes are provided in greater detail in Dinnel (2008).

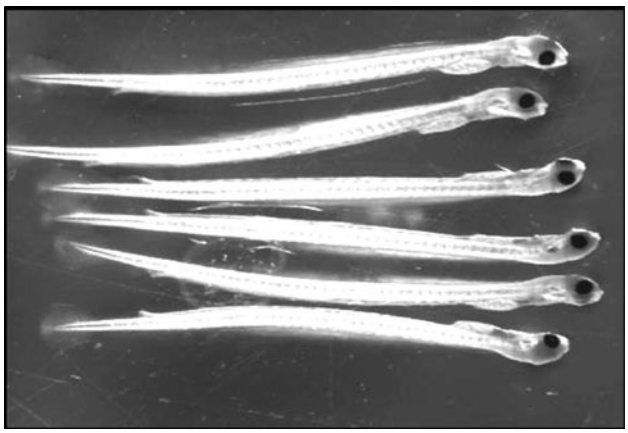

Fig. 6 Examples of normal Pacific herring larvae early after hatching

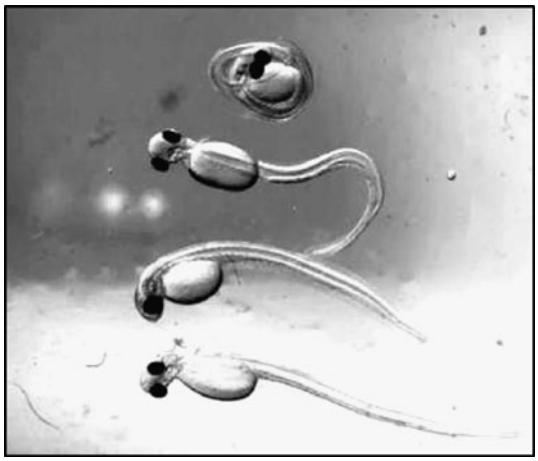

Fig. 7 Examples of varying degrees of abnormal herring larvae early after hatching

Data Analyses

The two primary embryo test end points are percentage normal hatch and larval length at hatch. Optional test end points may include embryo heart rates, embryo movements, time to 50% hatch, and presence or absence of yolk sac edema (Carls et al. 1999). Commercially available bioassay software programs may be used to calculate 50% lethal (LC_{50}) or effective (EC_{50}) concentrations, lowest observed–effect concentrations (LOECs), no observed–effect concentrations (NOECs), 50% inhibitory concentrations (IC_{50}s), or other similar effect measures and percentage effect levels as desired.

Acute Larval Test Development

The larval acute and survival and growth tests were developed in parallel. Most protocol parameters for both

tests were set using the results of survival and growth tests because the acute test was the more robust of the two. Younger, nonfeeding larvae used in the acute test proved substantially less sensitive to handling stresses compared with older feeding larvae.

Larval Sources

Larval herring may be obtained in one of two ways: (1) by fertilizing herring eggs in the laboratory using freshly collected herring gonads or (2) by incubating natural spawn on eelgrass or algae in either a flow-through or a static holding system. If method 1 is used, follow the collection, shipping, and handling methods described under the Embryo Protocol section. A convenient hatching method is to disperse 400–500 eggs in 20-cm glass culture bowls containing seawater and herring sperm to a depth of approximately 1 cm. After fertilization, wash the eggs and add fresh seawater close to the desired test salinity and incubate at 8°C to 12°C until hatch. Change the water at 1- to 2-day intervals.

If natural spawn on eelgrass or algae is used, the eggs are field-collected, and the eggs on vegetation are held in a flow-through seawater system (ideally) or a static system with aeration and frequent (2- to 3-day) water changes until hatching. If using a flow-through hatching system, use a large screened water outlet that does not impinge the hatching larvae. Transfer the hatched larvae to 20-cm culture bowls (density of 300–500 larvae/bowl) and incubate at the desired test temperature until use. If using a static hatching system, seawater must be changed several times daily to maintain water quality.

Survival of Unfed Larvae

A set of 20 tests evaluated survival of unfed larvae from five different regions of the United States and Canada (San Francisco = 3, Puget Sound = 5, Cherry Point, North Puget Sound = 4, British Columbia = 3, SE Alaska = 5). Cherry Point survival data are identified separately from other Puget Sound locations because this stock spawns unusually late during the reproductive season. Previous investigations (EVS 1999; Hershberger and Kocan 2000) have suggested that Cherry Point embryos and larvae are smaller and have higher embryo/larval mortality and abnormality rates. The survival tests were conducted during a several-month period because the various herring stocks have different spawning times. Each test was conducted in 30-mL volumes of control seawater (16‰ salinity) in glass Petri dishes at 10°C with no solution changes. Replication was 4- fold, and survival was monitored daily.

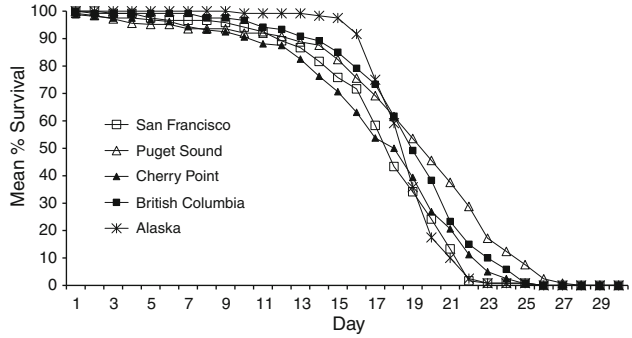

Fig. 8 Mean posthatch survival of unfed herring larvae across a set of 20 tests using larvae from five areas along the United States and Canadian west coasts

Survival curves showing the means of all tests from each of the five regions indicated that survival rates were similar for larvae from all regions (Fig. 8). All tests had a mean survival rate >90% for the first 10 days after hatch with steadily decreasing survival after that. Statistical analyses of these results found no significant differences in regional mean survival on days 5, 10, 15, 20, or 25. Thus, 96-hour acute larval tests using unfed individuals from any region and initiated 24–48 h after hatch should be acceptable in terms of control survival.

Acute Test Control Survival

Since 2001, 74 acute larval tests were conducted for which 96-hour mean control survival rates could be tabulated. Mean control survival rates ranged from a low of 80.0% to a high of 100%. Mean control survival for all 74 tests was 95.6%. The overall test success rate (based on a protocol criterion of ≥80% mean control survival) for these 5 years was 100%, and 83.8% of all tests had ≥90% mean control survival at 96 h.

Recommended Acute Larval Test Protocol

Acute larval tests are initiated by distributing 24- to 48-hour old unfed larvae to control and treatment beakers containing 200 mL solution in 400-mL glass beakers at the desired salinity (10‰ to 35‰, although higher and lower salinities may be possible) (see Table 2 for a summary of key protocol specifications). A fire-polished glass tube (10 mm o.d., approximately 10–15 cm long) with a pipette filler bulb (with suction and evacuation valves) is recommended for transferring larvae from the culture bowls into the test beakers. Larvae should be transferred in groups of approximately 5–10 until 10 larvae are in each beaker. The transfer is completed as delicately as possible. After the larvae have been slowly sucked into the pipette, the end of

Table 2 Summary of test conditions for the 4-day acute and 7-day survival and growth Pacific herring larval tests

Test organism	Pacific herring, *C. pallasi*
Test type	Static renewal
Test organism source	Larvae hatched from eggs fertilized in the laboratory or from natural spawn on eelgrass and/or algae
Test organism age	24–48 h after hatch for the acute test; 6–8 d after hatch for the survival and growth test
Test duration	96 h (acute) or 7 d (survival and growth)
Feeding	None for the acute test, *Artemia* nauplii daily for the survival and growth test (2000/beaker at test initiation and 1000/beaker on days 1–6)
Test chamber	400-mL beaker
Test solution volume	200 mL
Test solution renewal	Three-fourths solution renewal every 2 days (or daily if toxicant dynamics so dictate)
Test temperature	10°C to 12°C ± 1°C (lower temperatures may be acceptable, but growth may be affected)
Dilution water	Natural filtered seawater
Salinity	10‰ to 30‰, although slightly lower and higher salinities may also be used; developing embryos should be incubated at or near the test salinity
Salinity adjustments	Freeze-prepared brines and some artificial sea salts
Number of organisms/chamber	10 for the acute test; 15–20 initially for the survival and growth test culled to 10 healthy feeding larvae at 24 h
Number of replicates	Minimum of 4
Light intensity	Acute test: 20–100 lux (approximately 2–10 foot candles). Survival and growth test: 600–1200 lux (approximately 60–120 foot candles)
Photoperiod	16:8-hour light-to-dark photoperiod
Aeration	None unless DO concentrations decrease <5.0 mg/L, then aerate all beakers using approximately 100 bubbles/min
Control acceptability criteria	≥80% mean survival for both tests; in addition, for the survival and growth test, mean dry weight of the controls should be 1.3 times greater than initial control dry weights
Test end points	Acute: % survival; survival and growth: % survival and growth in terms of dry weight (biomass may also be used)

the pipette is touched to the solution in the beaker and the fish are allowed to swim out on their own (do not expel the water in the pipette into the beaker). This method minimizes stress to the larvae and essentially eliminates addition of diluting water into the toxicant concentrations.

Replication per treatment should be as dictated by your specific experimental design. A minimum replication of four times is suggested at this time. Beakers are conveniently held in batches of a dozen on cafeteria trays (lined with white paper towels), with beaker placements randomized. Place beakers in an incubator at 12°C (or lower temperatures as necessary) with a lighting schedule of 16:8-hour light-to-dark photoperiod and a light intensity of 20 to100 lux (or 2–10 foot candles [cool white fluorescent dim light]). Larvae are not fed during acute tests. No aeration is necessary unless DO concentrations decrease <5.0 mg/L, in which case all beakers must be aerated using <100 bubbles/min.

On test days 1, 2, and 3, count the number of live and dead larvae, remove dead larvae, and note any larvae with signs of disease (e.g., fungal growths). Larvae that are swimming erratically or are on the bottom of the beaker and still alive should be counted as alive and should not be removed from the beaker. Death is defined as the lack of any movement. Check for lack of movement by gently squirting water on larvae with a small pipette. If no movement is still detected, gently touch the caudal end of motionless larvae with the pipette and/or gently aspirate the caudal end into the pipette a few times. The test ends at 96 h, although exposure periods ≤10 days have been used.

A 75% (150 mL) toxicant and control solution change is conducted at 1- or 2-day intervals (depending on the stability of the toxicant) throughout the 4-day test period. Solution changes should take place in a temperature-controlled room using temperature adjusted toxicant and control solutions.

Old test solutions can be removed from test beakers with a 10-mL macropipette or small siphon, or solutions can be carefully decanted into another beaker after initially cleaning the bottom of debris with the macropipette. Avoid sucking up or decanting larvae; if this happens carefully replace the larvae and note this event on the data sheet. Renew test and control solutions by gently pouring 150 mL of new solution into the beakers. The test end point is survival relative to controls. Mean control survival at the end of the test should be ≥80%.

Larval Survival and Growth Test Development

Larval Quality from Eggs with Poor Fertilization Success

Occasionally, batches of eggs and/or sperm obtained from gonads appear to be of "poor quality" based on poor fertilization success and/or poor embryo survival. Situations like this negate their use for embryo tests, but the question remains as to whether or not the resulting larvae can be used successfully in survival and growth tests. During protocol development testing, we hatched larvae from two such "compromised" batches of eggs (fertilization success of only 10–15%) and tested the larvae under control conditions (laboratory seawater only) using a 10-day survival and growth test. Results of both tests showed that these potentially compromised larvae were able to perform in a satisfactory way. Larval survival in these two tests was excellent (93.0 and 98.3%), and growth was good (mean growth in terms of dry weight 53.7 and 88.5%). Although data are limited, it appears that larvae derived from batches of eggs showing poor fertilization or embryonic development success can be used successfully for larval tests once abnormal and weak larvae are culled.

Survival of Fed Larvae

One test compared survival of unfed larvae with individuals fed cultured rotifers, *Artemia* nauplii or a combination of *Artemia* and rotifers. Survival was >80% for all treatments through day 16 and then decreased thereafter (Fig. 9). All larvae in the unfed group died by day 24. Mortality was >90% at the test end (day 31) for the rotifer treatment. Survival for the *Artemia* and *Artemia*/rotifer groups was best and essentially equal, with approximately 50% survival at day 31.

A second test that explored the amount of growth of larvae on an *Artemia* nauplii diet during a 30-day period found that larvae doubled in length (0.85 to 1.53 cm) and increased in dry weight by 4.5 times (0.116 to 0.519 mg). A subsequent 34-day test showed similar results, with a doubling in larval length (0.78 to 1.57 cm) and a 5.4 fold increase (0.136 to 0.731 mg) in larval dry weight.

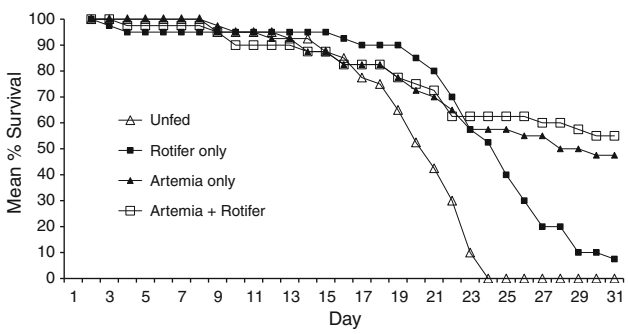

Fig. 9 Mean daily survival of herring raised in control seawater for 31 days on three different diets compared with unfed larvae

Two additional tests assessed the effects of feeding *Artemia* diets differing in highly unsaturated fatty acid content. No significant differences in growth were detected when fed three grades of Argentemia [Argent Chemical Laboratories, Redmond, WA, USA] *Artemia* nauplii or *Artemia* hatched from USEPA Reference Cysts (RAC-III).

Food Type

Food types other than live rotifers and *Artemia* nauplii were periodically tested during protocol development. Several types of potential food were ineffective as nourishment either due to their immobility (herring larvae like motile food) or their lack of ingestion by larvae. These foods included commercially available decapsulated *Artemia* cysts, Otohime [Reed Mariculture, Campbell, CA, USA] (a larval fish food), and green sea urchin (*Strongylocentrotus droebachiensis*) pluteus larvae. Early tests with wild-caught copepods (a mixture of species) proved inconclusive, and this source was dropped for logistical reasons. A cultivated copepod, *Acartia tonsa*, was ineffective as food because it was virtually immobile at the routine test temperatures of 10°C to 12°C. Another copepod, the harpacticoid, *Tigriopus californicus*, attached themselves to the larvae in large numbers, resulting in high larval mortality.

One other feeding experiment compared survival and growth on a diet of *Artemia* and the same *Artemia* raised in a commercial "enhancement" product (A1 Super Selco; Premix Inve France Nord, SA). There was no significant difference in either survival or growth between these two treatments. Hence, many grades of commercially available *Artemia* cysts, which can be easily hatched in 24 h, should serve as good sources of nutrition for herring larvae for the first several weeks after hatch.

Food Density

Two 10-day survival and growth tests were conducted to determine optimum *Artemia* density in the 400-ml test beakers. Densities of *Artemia* in the test beakers ranged from 300 to 3600 for the two tests. There were no significant differences in survival or growth in either of these two tests, and survival remained high (>90%) at most *Artemia* densities, including the highest. However, growth in the lowest food density (300 *Artemia*/beaker/d) was the least. The conclusion from these tests was that food densities should probably be >300 *Artemia*/beaker/d and that the selected food density should minimize excess food to

avoid poor water quality. Thus, for final protocol specifications, we recommend a density of 1000 *Artemia*/beaker/d (except for the initial feeding for which 2000 *Artemia*/beaker are used because the test is initiated with 15–20 larvae [see later text]).

Test Chamber Design and Size

Use of Petri dishes as test chambers (as described by Middaugh et al. 1998) proved to be quite satisfactory for embryo tests, but their utility for the larval tests (especially the survival and growth test) was questionable because of problems with the larval survival and growth test end points. A preliminary test that compared survival in Petri dishes and beakers suggested that feeding larvae might fare better in 300- to 600-mL beakers.

A subsequent set of four tests that compared 10-day survival and growth in Petri dishes and in 400-mL beakers containing 200 mL control seawater showed that survival was always higher in the beakers and averaged approximately 8% higher across all tests. Daily feeding success was approximately the same in both containers, but overall mean growth across all tests favored beakers by approximately 11%.

Given the results noted previously, we conducted all future larval tests in glass beakers. Yet to be decided was the most efficient size of beaker to be used. Thus, two tests were run to compare herring larval survival and growth in three beaker sizes: (1) 250-mL beakers with 100 mL test solution, (2) 400-mL beakers with 200 mL test solution, and (3) 600-mL beakers with 400 mL test solution. Tests were initiated using 7-day posthatch larvae and *Artemia* nauplii at the following feeding rates (*Artemia*/beaker/d): 250-mL beaker = 600, 400-mL beaker = 1,000, and 600-mL beaker = 2400. We found that there were no significant differences in either larval survival or growth between the three beaker sizes.

Based on the results of these tests and the need to standardize the beaker size, we selected the 400-mL beaker using 200 mL test solution as the best alternative. Although various sizes of beakers may be acceptable, the 400-ml size was selected because of ease of handling and cleaning, cost, and space required. While conducting the beaker size tests, we especially noted that use of beakers <400 mL caused crowding of larvae, which made it more difficult to count larvae accurately and increased difficulty in cleaning dead *Artemia* and debris from the bottom of the beakers.

Stock Culture Culling and Larval Transfer Method

When larvae are being held and fed in 20-cm diameter glass culture bowls before use in toxicity tests, some larvae will fail to initiate feeding. If these larvae are used for survival and growth tests, the survival and growth end points will be compromised. Thus, it has become common practice to cull these larvae from the cultures before test initiation.

In addition, the act of transferring larvae from the culture dishes to the test beakers results in stresses that can cause some larval mortality and cause other larvae to cease feeding. We experimented with a variety of transfer methods but rejected some because they required the transfer of substantial amounts of seawater to the test beakers, thus diluting test solutions. Our eventual preference was use of a fire-polished 10-mm diameter glass tube (with autopipettor bulb) to transfer larvae to test beakers. We chose the glass tube method of transfer because there is virtually no addition of culture solution to the test beakers.

Culling Larvae from the Test Beakers

Although many nonfeeding larvae are culled from the stock cultures before test initiation, it is possible for 10–30% of the larvae to die or cease feeding after transfer to the test beakers. To resolve this issue, we conducted a second culling step at approximately 24 h after test initiation.

Tests are now initiated by transferring 15–20 feeding larvae (removing any larvae that do not have *Artemia* nauplii in their transparent guts) and followed-up at 24 h by removal of larvae (with priority given to dead or nonfeeding larvae) until only 10 feeding larvae remain. These larvae then constitute the test animals for that beaker. Tests conducted by Nautilus Environmental Laboratory (2006) compared larval survival and growth with and without this culling step. They found that 10-day survival in control seawater improved by ≤30% when culling was used, although there did not appear to be any significant differences in final dry weights.

Larval Loading Weights

Loading weights for fish tests should be limited so that concentrations of DO in the test chambers do not become unacceptably low and test organisms are not stressed because of aggression or crowding. The ASTM (2009) Standard Guide for fish tests recommends that loading in each test chamber be no more than 800 mg/L at any time.

Larval herring used in survival and growth tests are approximately 6–8 days after hatch and weigh approximately 2 mg each (wet weight). The larvae can then grow to a maximum of approximately 5 mg/fish at the end of a 7-day test. If the test starts with 20 larvae/beaker in 200 mL solution (to be culled to 10 larvae at 24 h), the loading weight is equal to approximately 200 mg/L test solution. At test termination, larvae can weigh as much as

5.3 mg each, with a maximum total loading rate of approximately 265 mg/L. In addition, the wet weight of 2000 *Artemia* used for food (maximum number present at any one time) is approximately 16 mg, which equals a loading rate of approximately 80 mg/L. Thus, the maximum loading weight of fish + *Artemia* in any one beaker is approximately 345 mg/L. This loading rate is less than half of the ASTM (2009) recommended maximum loading of 800 mg/L.

Light Intensity

Because light intensity might affect larval activity, survival, and ability to feed and grow on an *Artemia* diet, one experiment assessed the effects of three light intensities (8, 40 and 1300 lux [very dim, moderate, and full light, respectively) on these parameters. Light intensity had little effect on larval survival between the three groups during the 13-day test period. However, the percentage of larvae feeding on any given day increased with increasing light, especially for the 1300-lux group. Growth measurements performed on the survivors at the end of the 13-day exposure period showed that growth, in terms of both larval length and dry weight, increased in direct proportion to the light intensity. Based on the results of this test, a range of 600–1200 lux (approximately 60–120 foot candles) is recommended for the larval test protocol.

Light Photoperiod

Because early results for the larval growth end point had been poor to marginal during early development of the protocols, we assessed the effect of conducting the survival and growth test under continuous 24-hour light to see if this might improve feeding and growth. Two tests compared the standard 16:8-hour light-to-dark photoperiod *versus* 24 h of light. No significant differences in survival or growth were detected in these two tests. However, results of two other tests that used a 24-hour light photoperiod had the highest control growth ever observed (>100%) for a survival and growth test. Because a 16:8-hour light-to-dark photoperiod is common for toxicity testing, our protocol recommends that this photoperiod be used. However, use of other photoperiods, including 24 h of light, remains an option for specialized testing.

Temperature

As explained in the Embryo Temperature section above, we initially standardized the temperature for embryo and larval tests at 10°C. Subsequent to this decision, several additional temperature tests were conducted using the survival and growth test to re-examine the optimum larval protocol temperature. Use of a test temperature >10°C was of particular interest if it could help improve the amount of growth in the survival and growth test.

One experiment tested larvae at four temperatures (10°C, 12°C, 14°C, and 16°C) at 16‰ salinity. Survival was lower at 14°C and 16°C, but growth steadily increased with temperature, dramatically so for larvae at 16°C (Fig. 10).

A subsequent test re-examined growth of larvae at 12°C but at a salinity of 30‰ instead of 16‰. Newly post-hatch larvae were raised for 18 days in a 20-cm culture bowl with an excess of *Artemia* as food. There was little mortality during the 18 days, essentially no growth (in terms of dry weights) for the first 6 days, and steadily increasing growth after day 7, with a 349% increase in dry weight by day 18. Larval dry weight for the typical 7-day survival and growth test period (day 6–13 after hatch) more than doubled (125%). Because the results of these temperature tests indicated good survival and better growth at 12°C, the current protocol now recommends the routine use of 12°C for both the acute and survival and growth larval tests.

Salinity

Previous tests of the effects of salinity on development of Pacific herring embryos showed that development is euryhaline (e.g., McMynn and Hoar 1953; Dushkina 1973), with optimum development occurring in approximately half-strength seawater (e.g., Griffin et al. 1998; Vines et al. 2000; Alderdice and Velsen 1971). Thus, our early larval testing was conducted at a salinity of 16‰ on the assumption that early stage larval survival and growth would be best at that salinity. However, future testing of ambient seawaters and effluents that are diluted with ambient salinity seawaters may require that herring tests be run at salinities as high as approximately 35‰ salinity or,

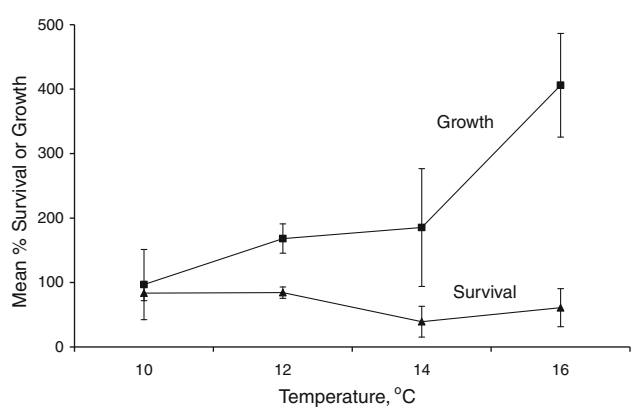

Fig. 10 Mean larval herring survival and growth (final dry weights) and SDs at the end of a 10-day survival and growth test at four test temperatures

perhaps, salinities <16‰. Thus, two tests were conducted that assessed the effects of both lower and higher salinity on the survival and growth test.

The first test compared five salinities (12‰ to 28‰) at 10°C using larvae that had been fed and acclimated in 16‰ seawater for 8 days before testing. Larvae in test salinities other than 16‰ were acclimated to their respective salinity during a 24-h period just before test initiation. There were no significant differences in survival or growth between the various salinities. However, the highest survival and growth occurred in the lowest salinities (12‰ and 16‰), with a gradual downward trend in both indices with increasing salinity.

The second test also compared six salinities (13‰ to 28‰) at 10°C. However, this test differed from the first in this respect: All larvae were acclimated to their respective test salinity starting at hatch and throughout the 9-day pretest period (after hatch, larvae were slowly adjusted to their respective salinity during a 1- to 3-day period). There were no significant differences in survival, but there was a significant increase in growth for larvae in the two *highest* salinities (25‰ and 28‰) (Fig. 11).

Results of these salinity tests suggested that larvae acclimated to low salinity after hatch are adversely stressed when exposed to higher salinities but that early acclimation to high salinities does not result in this same stress. Indeed, the results of the second test suggest that 5- to 20-day old larvae may be less stressed by salinities of 25‰ to 30‰ than lower salinities as long as they are acclimated to higher salinities from hatch.

Because the larval survival and growth protocol had undergone gradual refinements during development and a new test temperature (12°C) had been adopted, there was a need to redefine the salinity tolerance of the protocol using the refined conditions. Thus, two additional tests evaluated the effects of a range of low- and high salinities surrounding the previous standardized test salinity of 16‰.

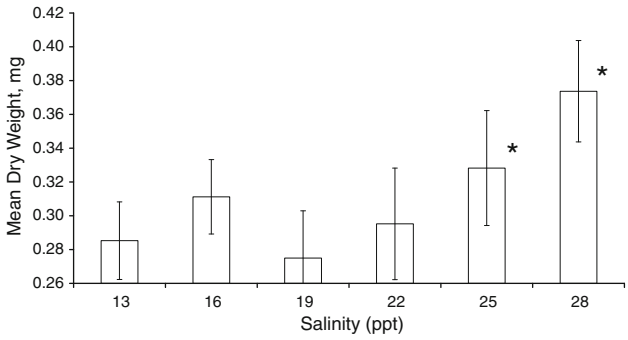

Fig. 11 Mean and SDs of dry weights of herring larvae exposed for 10 days to six salinities. All larvae were acclimated to their respective salinity shortly after hatch. * Significantly greater growth ($p = 0.05$) compared with 16‰ salinity

The first test assessed a low range of salinities (1.1‰ to 14.8‰ [embryos hatched in 16‰ salinity]), and the second test used a high range of salinities (24‰ to 37.3‰ [embryos hatched in 30‰ salinity]).

For the low-salinity range test, larval survival was generally satisfactory at salinities as low as 6.5‰, and survival was significantly lower at 3.8‰ and 1.1‰ (compared with the 14.8‰ "control" treatment [Fig. 12]). Regarding larval dry weights, there was a downward trend in weights in salinities lower than approximately 12‰ and significantly decreased dry weights at 3.8‰ and 1.1‰ salinity (Fig. 13). For the high-salinity range test, there were slight downward trends in survival and dry weights at 35‰ and 37.5‰ salinity, but none of these decreases were significantly different compared with survival and dry weights in the 24‰ "control" treatment (Figs. 12, 13).

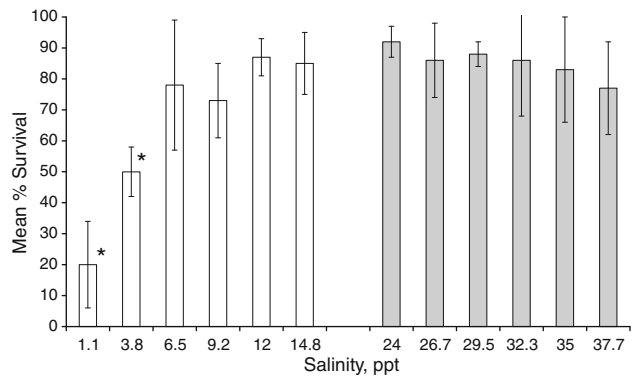

Fig. 12 Mean herring larval survival and SDs after 10-day exposures to a range of low- and high-salinity treatments. Low-salinity test larvae were raised in 16‰ salinity, and high-salinity larvae raised in 30‰ salinity. * Significant decrease in survival ($p = 0.05$) for low-salinity treatments compared with 14.8‰ salinity. There were no significant differences for the high-salinity test (compared with 24‰ salinity)

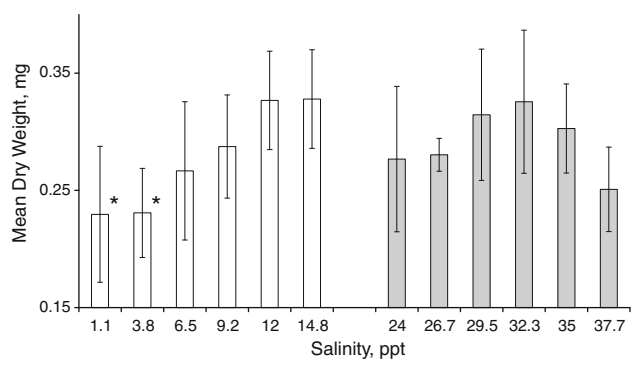

Fig. 13 Mean herring larval dry weights and SDs after 10-day exposures to a range of low- and high-salinity treatments. Low-salinity test larvae were raised in 16‰ salinity, and high-salinity larvae were raised in 30‰ salinity. * Significant decrease in weights ($p = 0.05$) for low-salinity treatments compared with 14.8‰ salinity. There were no significant differences for the high-salinity test (compared with 24‰ salinity)

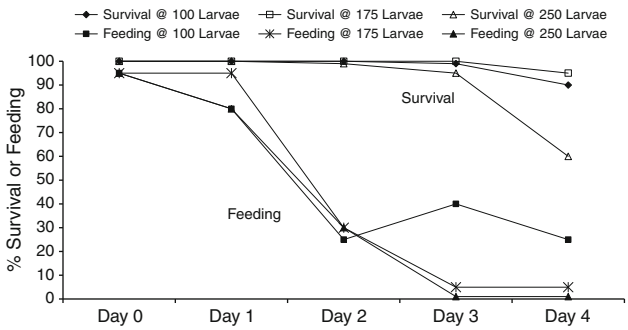

Fig. 14 Effects of decreasing DO concentrations on survival and feeding rates of larval herring at loading densities of 100, 175, and 250 larvae/beaker in 200 mL control seawater during a 4-day period

Dissolved Oxygen

High loading densities of herring larvae and food (*Artemia* nauplii) were used to depress DO concentrations in a set of three tests that attempted to define the lower limits of DO necessary to conduct a successful herring survival and growth test. The first two tests used densities ranging from 10 to 200 larvae in 200 mL control seawater in 400-ml beakers for 15–18 h. DO concentrations during these two tests were in the range of 4.1–6.4 mg/L, and larval survival was slightly decreased to 81% in the beakers with the highest fish densities.

The third experiment tested fish densities of 100, 175, and 250 in 200 mL control seawater in 400-mL beakers. DO, survival, and feeding were monitored during a 4-day period. During this test, DO concentrations gradually decreased to a range of 2.4–3.5 mg/L by day 4. Survival remained high (90–95%) in the 100 and 175 larvae/beaker treatments but was only 60% in the 250 larvae/beaker treatment beaker at day 4 (Fig. 14). Feeding success remained high at day 1 (≥80%) but decreased after that. However, there was still an approximately 25% feeding rate in the 100 larvae/beaker treatment at day 4. Results of these three DO tests suggest that larvae were stressed at DO concentrations lower then approximately 4.5 mg/L.

Brine and Artificial Sea Salts

As for the embryo protocol, we conducted one survival and growth test using a freeze-prepared brine and the four artificial sea salts (adjusted to 30‰ salinity with deionized water) as mentioned in the Embryo Brine and Artificial Sea Salts section above. This 10-day test, conducted at 12°C with larvae continuously maintained at 30‰ salinity, resulted in no significant differences in survival (all averaged 70–80%) or final dry weights (all averaged 0.21–0.26 mg) for any of the sea salts or brine compared with the natural seawater control. Thus, freeze-prepared brines and most artificial sea salt formulations should be satisfactory for conducting larval tests. Any sea salt formulations not mentioned previously should be tested against natural seawater before being used for routine testing.

Survival and Growth Test Control Survival

During the first 3 years of survival and growth testing (2003–2005), only 76% of the 10-day survival and growth tests achieved the minimum required 70% control survival that was deemed necessary to validate a larval fish test, and some tests resulted in poor growth of the control larvae. One problem was that tests were often started using some larvae that were feeding poorly or not feeding at all. During 2006, added effort was made to insure that tests were initiated with a high proportion of actively feeding larvae. This was performed in two ways: (1) nonfeeding larvae were actively culled from the pretest 20-cm diameter glass culture bowls for several days before test initiation, and (2) 15–20 larvae were added to beakers at test initiation and then culled to 10 *feeding* fish at day 1. These two steps helped to ensure that most or all of the larvae would continue feeding throughout the 10-day test period.

Partially because of strict adherence to the two steps previously outlined, control survival in the 2006 tests improved substantially. Only one of nine survival and growth tests failed a new ≥80% control survival criterion, this being one test that used larvae from Cherry Point. Of the other eight tests, all but one had control survival >90%, with the remaining one having 83% survival. The mean control survival was 90.8% for all nine 10-day tests.

Fifteen herring larval survival and growth tests were conducted in 2007; 8 tests had test durations of 10 days, and the other 7 tests were shortened to a duration of 7 days. In addition, 5 parallel topsmelt (*A. affinis*) survival and growth tests (USEPA 1995) were also completed, all 7 days in duration. Mean control survival for the herring tests was 89.7 and 95.9% for the 10- and 7-day–duration tests, respectively, whereas survival was 100% for the topsmelt tests.

Survival and Growth Test Control Growth

Early in protocol development, the herring growth end point for the survival and growth test was problematic. Growth of the control larvae for tests conducted from 2003 to 2005 averaged only approximately 24% (range –22% to +84%). The lower the growth in a test, the more difficult it is to discern differences between the control and treatments for the growth end point. With the greater emphasis on culling nonfeeding fish from test beakers in 2006, eight of nine survival and growth tests in 2006 resulted in >60%

control growth. The ninth test (Cherry Point larvae with control survival <70%) had 34% growth. The 2006 mean control growth was 82.2% for the nine herring tests (all 10-day tests).

Control larval growth for tests conducted in 2007 was excellent. It averaged 76.0% for 10-day larval herring tests and 60.9% for 7-day tests. In contrast, % growth for five 7-day topsmelt tests was only 37.5%. One possible factor contributing to better larval herring growth in 2007 was the change in test temperature from 10°C to 12°C.

Recommended Larval Survival and Growth Test Protocol

Larval survival and growth tests are initiated with 6- to 8-day old feeding larvae (see Table 2 for a summary of key protocol specifications). On the day of test initiation, after a final culling of nonfeeders, feeding larvae are transferred from the 20-cm diameter culture bowls to 400-mL glass beakers containing 200 mL test or control solutions at 12°C and the desired salinity (10‰ to 35‰, although higher and lower salinities may be possible). Transfer 15–20 larvae to each control and treatment beaker using the large-diameter pipette method described previously for the acute test (rejecting any larvae that do not have food in their stomachs). Replication per treatment should be as dictated by your specific experimental design. A minimum replication of 4 × is suggested at this time. Beakers are conveniently held in batches of a dozen on paper towel–lined cafeteria trays with beaker placements randomized.

At test initiation, and after any final culling step, collect a subsample of at least 30 larvae from the 20-cm glass culture dishes. All of the fish collected for this sample will be used to establish the mean starting dry weight. All of the fish in this sample should be feeding. Place the live larvae into a labeled vial containing 5–10 mL 200 mg/L MS-222 (a fish anesthetic) dissolved in control seawater and, after a few minutes, add concentrated formalin solution to give a final concentration of 5–10% formalin in the vials.

Finally, add 2000 freshly hatched *Artemia* nauplii to each test beaker and place the beakers in an incubator at 12°C with a 16:8-hour light-to-dark photoperiod and a light intensity of 600–1200 lux (approximately 60–120 foot candles [cool white fluorescent bulbs]). No aeration is necessary unless DO concentrations decrease <5.0 mg/L, in which case all beakers should be aerated using <100 bubbles/min. (Note: the effects of aeration on larvae are currently unknown.)

At day 1, cull out all dead or damaged larvae, larvae that do not have food in their guts, plus any others until 10 healthy *feeding* larvae remain in each beaker. These larvae then constitute the test organisms. In the higher test concentrations, many larvae may have already died or may not be feeding. If so, then retain as many live larvae as possible, with priority given to feeding fish.

On an every-other-day schedule (days 2, 4, and 6), enumerate the number of larvae alive and dead. This step is most easily accomplished after removal of 75% (300 mL) of the test solution and most old *Artemia* (see later text). If desired, note the percentage of larvae that have *Artemia* in their stomachs (this information will provide an optional index of feeding success). Keep careful records of all dead larvae removed during the test so that dead larvae plus ending number of larvae can be reconciled with larvae counted on day 1 (which can sometimes be difficult to count accurately).

A 75% (150 mL) toxicant (and control) solution change is conducted at 2-day intervals (daily if the stability of the toxicant/effluent is low) throughout the 7-day test period (i.e., days 2, 4, and 6). Solution changes should take place in a temperature-controlled room (12°C ± 1°C). For the solution changes, use a 10-mL macropipette to carefully remove as many of the old *Artemia* nauplii as reasonably possible and discard. Some old *Artemia* will remain in the beakers. Avoid accidental decanting or aspiration of larvae. If aspiration of individual larvae happens, carefully return them to their beaker and note this event on the data sheet. After *Artemia* nauplii are removed from the bottom of a beaker, remaining solution can also be carefully decanted into another beaker. Renew test and control solutions by gently pouring 150 mL new solution into the beakers.

On day 1 and on each day through day 6, add 1000 freshly hatched *Artemia* nauplii to each test beaker in 0.5–1 mL solution. On the solution-change days, add newly hatched *Artemia* nauplii after the solution-change step.

On test termination at day 7, count the number of live and dead larvae and put all live larvae into small labeled vials containing 5–10 mL 200 mg/L MS-222 dissolved in control seawater, and after a few minutes add concentrated formalin solution to give a final concentration of 5–10% formalin in the vials. These larvae will subsequently be assessed for dry weight. For a test to be considered successful, mean control survival should be ≥80% and mean dry weight of the controls should be at least 1.3 times greater than mean dry weight at test initiation. Step-by-step methods for conducting herring larval tests and solution changes are provided in greater detail in Dinnel (2008).

Data Analyses

The survival and growth test has two primary end points: (1) survival and (2) growth relative to the controls in terms of dry weight. Either weight (dry weight per surviving larva) or biomass (total dry weight divided by start count)

can be used as an end point. An optional end point is daily feeding success based on the percentage of fish with *Artemia* in their guts. Commercially available bioassay software programs may be used to calculate LC_{50}, EC_{50}, LOEC, NOEC, and IC50 concentrations, or other similar effect measures and percentage effect levels, as desired.

Discussion

The goal of our research was to define herring embryo and larval test protocols that can be used by research and commercial testing laboratories for testing effluents and ambient marine water quality. To accomplish this goal, we adapted herring test methods developed by previous researchers (e.g., Kocan and Landolt 1990; Middaugh et al. 1998; Vines et al. 2000) and investigated the effects of test variables (temperature, salinity, DO, light intensity, photoperiod, *etc*.) so as to select robust test conditions.

The decision to develop Pacific herring test protocols was stimulated by the need to protect a valuable Puget Sound forage fish, whose early life stages are potentially at risk from a multitude of near-shore pollution sources. These sources include both point discharges (e.g., industrial and sewage effluents, oil spills) and nonpoint sources (e.g., farm/agriculture runoff, urban storm water, creosote pilings and timbers, contaminated sediments, groundwater). Herring, surf smelt (*Hypomesus pretiosus*), and sand lance (*Ammodytes hexapterus*) all spawn in intertidal and shallow subtidal zones. They are important forage fish that feed salmon, marine fishes, marine birds, marine mammals, and humans. Efforts to protect these species must focus on vulnerable near-shore spawning habitats in terms of both the physical environment and contaminants.

Although Pacific herring are a valuable food web and fishery resource, the decision to develop herring bioassay protocols for routine WET testing must also be based on other considerations, including sensitivity to contaminants, availability of test animals, and ease of use in testing laboratories. McKim (1977) reviewed the results of 56 life cycle tests with four fish species and 34 organic and inorganic chemicals. He concluded that fish "embryo-larval and early juvenile life stages were the most, or among the most, sensitive. Tests with these stages can be used to estimate the maximum acceptable toxicant concentration (MATC) within a factor of two in most cases."

The protocols presented in this article recommend methods that have worked best for us during test development. Use of standardized methods is critical for repeated testing, for comparison of results between laboratories, and for regulatory testing programs. Other methods can and have been used successfully by others, including use of flow-through exposure systems, distributing eggs on glass slides instead of in Petri dishes, exposures at lower temperatures, larger larval test chambers, larval transfer methods other than our recommended large-bore pipette, and alternate larval diets. Use of some of these alternate methods may affect test results, whereas some alternative methods may not. Care should be used when deviating from the recommended protocols when testing is directed at interlaboratory comparison or regulatory testing programs. Methods, results, and discussions more detailed than those presented in this article may be found in a series of project reports to the WDOE (Dinnel et al. 2002, 2003, 2005, 2006, 2007).

One significant limitation of these protocols is that test materials (gametes and embryos) are only available for approximately 6 months of the year (late December to May); hence, efforts to improve gamete availability using laboratory-raised and conditioned herring would be valuable. A second limitation for the embryo test (less so for the larval tests) is the issue of gamete quality. Embryo tests must be initiated with reasonably ripe eggs that have been collected, shipped, and handled as carefully as possible. Selection of the best gametes can be aided by pretest fertilization trials. In addition, embryo tests with less than perfect eggs can still be accomplished because unfertilized and poorly developing eggs are culled from the test chambers at day 2, leaving only approximately 20 good-quality developing embryos in the test dishes.

The larval tests are more robust in terms of gamete/embryo quality. Any abnormal larvae can be culled at hatch, and for the survival and growth test, poorly developing and nonfeeding larvae can be culled from culture bowls and further culled from the test chambers one day after test initiation.

Some future tasks to be accomplished in support of the developed herring protocols include (1) establishing a more reliable source of gonads for the embryo test, (2) training commercial laboratories in the use of herring tests, (3) developing an interlaboratory validation study using reference toxicants, and (4) exploration of a sediment bioassay using herring embryos.

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