Laboratory Demonstration of Environmental Factors And Their Effects on Early Stage Development of Clupea pallasi

Final Report

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Adverse biological responses can result from many different types of stressors. Abnormal development has been observed in response to naturally occurring changes in water quality (e.g., salinity, temperature, dissolved oxygen, and pH), exposure to ultraviolet light and to air during tidal extremes, as well as to anthropogenic stressors such as oil and chemicals in urban runoff; in addition, reduced viability of fertilized embryos has been related to the condition of gametes at spawn. The experimental studies conducted on the development of Pacific herring embryos in 2008, approximately three to four months after the Cosco Busan Oil (CBO) Spill did not show a CBO chemical exposure. In 2009, CBO experimental exposure arrays resulted in a diagnostic chemical signature in the developing embryos at all exposure concentrations ranging from high to low that was not observed in the embryos collected in 2008. Thus the observation of adverse responses made in 2008 for the natural spawning assessments occurred in the absence of CBO exposure; the artificially spawned larvae exhibited no adverse effects. The 2009 laboratory studies were however, also unable to demonstrate a relationship between other potential stressors and the adverse effects because of the presence of laboratory and measurement artifacts. In 2010, an experiment was developed to evaluate the potential impact of 2008 environmental stressors on developing embryos of Pacific herring.

This study was designed to provide a controlled laboratory assessment where the environment mimicked the 2008 field conditions. The environmental stressors evaluated were 1) donor fish condition and health of gametes, 2) fluctuating temperature and salinity defined by data collected by NOAA/BML to match two locations within the range of spawning activities, 3) the presence of UV light, and 4) the influence of aerial exposure that occurs during periods of intertidal stranding. In the 2010 laboratory studies there was no oil exposure. These conditions were effectively mimicked except the intertidal stranding which probably underrepresented the actual conditions during the 2008 development of herring eggs.

The 2010 experiments demonstrated that all of the abnormalities observed in the 2008 assessment were also found in 2010 under exposure to environmental variables but not to oil. We developed a repeatable method of distinguishing and measuring pericardial and yolk sac edema which also matched our qualitative assessments. These measurements and assessments of edema were developed on the 2010 data and then applied on the 2009 and 2008 images provided by NOAA/BML. All measurements were conducted on randomized sample designations so that assessment and measurement staff were blind to any experimental treatment information. Note that all larvae assessed hatched naturally (were not dechorionated) and therefore these descriptions refer to apparent development at hatch.

This quantitative assessment of types of edema demonstrated that pericardial edema, while present to a large degree in the 2009 experimental oiling experiments was only incidentally present in the 2008 and the 2010 assessments. Pericardial edema was only observed in larvae that had gross body axis defects where separation of pericardial and yolk sac edema was not feasible or appropriate.

Adverse effects were identified in association with each of the potential contributing factors that were examined. Significant yolk sac edema, body axis defects, and disruption of early

development occurred in the absence of petroleum contamination and in the presence of non-chemical based stressors. We also determined that there are significant problems with the assessment of dechorionation of developing embryos, especially on organisms that have undergone natural spawning and that represent very different developmental ages.

The combination of observations from the 2008, 2009, and 2010 studies provides a petroleum based chemical signature that demonstrates exposure as well as a biological signature of the type of stressor that appears to be involved with the creation of the defect in development. These conclusions are summarized in the following table.

Chemical Signature	Stressor Type	Biological Response
Without Exposure to Oil (CBO)	Population	Low percentage of body axis defects (~2% major and <5% less obvious); combination of pericardial and yolk sac edema only in major body axis defect individuals
	Gamete Condition	High early mortality (<48h); those that survive have a high percentage of normal hatch
	UV	Higher incidence of body axis defects (BAD)
	Increased Temperature	Increased incidence and intensity of yolk sac edema
	Intertidal/estuarine fluctuating stressors (salinity/air exposure)	Increased incidence and intensity of yolk sac edema; no pericardial edema except in individuals with major body axis defects
With Exposure to Oil (CBO). Relative contribution of chemical components of complex mixtures create tissue signature of exposure – 2009 Oil Generator Column Studies	Chemical Stressors (petroleum, TBT, Cu, algal)	Pericardial edema often combined with yolk sac edema and other adverse responses as exposure increases

The examination of observations from the 2008, 2009, and 2010 studies led to the conclusion that combination of fluctuating temperature and salinity, with UV exposure and some degree of aerial exposure of the developing embryos and the developmental condition of the adult spawning fish and their gametes were the cause of abnormalities observed in 2008. The adverse biological effects, particularly a higher incidence of yolk sac edema and a low incidence of pericardial edema for naturally spawned larvae in 2008 were the same as the response by larvae exposed to multiple environmental stressors in the absence of petroleum contamination during the 2010 environmental stressor laboratory study.

A companion report "Framework for Assessment of Causal Relationships between Early Life Stage Developmental Anomalies of Clupea pallasi and Cosco Busan Oil" provides an assessment framework for evaluating the body work for conducted relative to the CB Oil Spill (NewFields 2010c). This report uses an inference framework to compare all data collected relative to CBO and larval development with effects-based literature summaries to make conclusions.

1 INTRODUCTION

A program was initiated cooperatively between the state and federal trustees and the responsible party in 2008 to assess the injury to Pacific herring (Clupea pallasi) derived from the aftermath of the Cosco Busan Oil Spill (CBOS). Because of the known toxicity of crude and refined oils to herring eggs (Linden et al. 1978, 1980, Smith and Cameron 1979, Pearson et al. 1985, 1995, 1999, Carls et al. 1999, 2002) and the intertidal and shallow subtidal distribution of San Francisco Bay herring eggs, herring eggs in the central San Francisco Bay were considered at risk for injury derived from the oil spill. Two lines of investigation were initiated in the 2008 herring spawning season: 1) Artificially spawned herring embryos were deployed in cages at six locations within the central Bay to test effects that occurred during embryogenesis as a result of potential exposure to oil residuals from CBOS; and, 2) Naturally spawned embryos were collected from four central bay sites and subsequently incubated under laboratory conditions to examine direct effects caused by the CBOS that had stranded or impacted those locations prior to herring spawning activity (NOAA/BML 2008, Incardona 2008). Two additional studies were conducted following the NOAA/BML investigations. The first focused on the analytical chemical data generated by collection of water, egg tissues, and sediment from the designated sites of concern as well as reference sites with the Bay (NewFields 2009a). This examination of analytical chemistry produced a data quality assurance assessment, a chemical fingerprint assessment, and statistical analyses of the chemistry data associated with the NOAA/BML study (Douglas 2008). The second study re-examined a subset of the digital imagery taken during the NOAA/BML study of artificially spawned and naturally spawned embryos (NewFields 2009b and 2010) to verify the biological effects observed.

The salient outcomes concerning injury to herring culminating from these studies are the following:

- The pattern of the biological effects observed in the naturally spawned embryos does not match that expected from exposure to petrogenic PAHs.
- The levels of PAHs in intertidal eggs in 2008 were quite low compared to those in other studies that did elicit abnormalities and the intertidal eggs had no CBO signature.

Without a demonstrated exposure pathway, the adverse effects could not be attributed to CBO. A series of experiments were conducted in 2009 to test the hypothesis that exposure to oil or a combination of oil and UV would induce the same lethality and abnormalities evident in naturally spawned embryos collected from several sites in the central Bay in 2008 without a recognizable CBO chemical signature. An array of treatment chambers directly receiving seawater from three concentrations of CBO laden gravel was set up outdoors with and without UV exposure. Four experiments were conducted using the same oil generator columns representing increased oil weathering over time, but under somewhat varying experimental conditions. Alaska North Slope oil was tested during the first three experiments. The results of these laboratory experiments demonstrated that CBO oil exposure provides an identifiable chemical signature in both water and egg tissues even though the data were compromised by other identified contributing factors (NF 2010). However, the CBO signature demonstrated by 2009 analytical chemistry was not evident in chemical profiles from 2008. After an assessment of causal relationships, several potential causes to the observed 2008 effects were discounted (Pearson 2009), and only two hypotheses remained:

1) An unmeasured CBO component combined with ultraviolet (UV) radiation may have caused the decreased hatching success in 2008, and

2) A combination of environmental stressors including variable salinity, temperature, UV transmittance, and donor fish condition may have contributed to the effects.

Hypothesis 1 was discounted because the absence of a chemical signature, diagnostic of CBO exposure also negates the idea that an unmeasured chemical in CBO was available and influenced the development of the embryos. A laboratory study was designed to test the second hypothesis. To evaluate the potential contribution of environmental factors on disruptions to normal development of Pacific herring embryos, laboratory experiments were set up to mimic fluctuating field conditions encountered during the 2008 spawning season. Two locations were selected within San Francisco Bay: Point San Quentin was chosen to represent average conditions in the north central part of the San Francisco Bay, and Peninsula Point was selected to represent conditions in the south central part of the bay. The experiment was conducted at the NewFields Environmental Laboratory at Port Gamble WA. This facility was selected because it can provide clean seawater for continuous flow experiments and has the technical capability to precisely achieve the variations in temperature and salinity conditions required for this experiment. The following report details the objectives, laboratory and data analytical methods, statistical evaluation and results of this testing effort.

The conclusions regarding the impacts of CBO oil to developing herring embryo are provided in a companion report prepared by NewFields (2010c). This report is a decision framework with the rationale for interpreting causal relationships between developing herring larvae and any adverse effects related to CBO or other contributing environmental factors.

2 STUDY DESIGN BASED ON 2008 FIELD CONDITIONS

This laboratory study was designed to demonstrate the extent to which factors such as temperature, salinity, ultra-violet (UV) light, aerial exposure, and age of the donor fish and condition of gametes contribute to abnormal embryonic development of Pacific herring. The temperature and salinity regimes in San Francisco Bay relevant to the 2008 spawning events were examined and the laboratory experiments were designed to reproduce the patterns of temperature and salinity fluctuations observed in the field during the spawn incubation to evaluate effects caused during embryogenesis. Additionally, possible effects of UV and aerial exposure were also considered.

2.1 2008 TEMPERATURE, SALINITY, AND TIDAL DATA SOURCES

Data loggers deployed by the NOAA/BML team provided information on the temperature and salinity in the shallow subtidal zones at Sites Point San Quentin (PSQ), Horseshoe Cove (HC), Peninsula Point (PP), Sausalito (SA), and Keil Cove (KC) (Appendix A). The data logger at San Rafael Bay failed to return data (NOAA/BML 2008). Verified water levels at the Crissy Field Wharf in San Francisco provided information on the tidal levels present during the spawning events (Appendix A). The time series of plots were adjusted using the predicted times of tides nearest the site of interest. Meteorological records provided information on air temperatures in San Francisco during the 2008 spawning event (Appendix A). Some limited data were available on the seawater temperature and salinity from water quality studies of San Francisco Bay (USGS 2010). The tidal level records provided estimates of the time periods for aerial exposure. Because the NOAA/BML team collected eggs on macroalgae from approximately +1 ft MLLW to 0 ft MLLW, time of aerial exposure was estimated from the time when the tidal level fell below 1+ ft MLLW to the time when the level returned to +1 ft MLLW. Plots of temperature, salinity, and tidal level were examined to discern similarities and differences in regimes among the sites.

2.2 TEMPERATURE AND SALINITY PATTERNS AT THE SITES FOR THE 2008 COLLECTION OF NATURAL SPAWN

The sites from the west side of Richardson Bay (SA) to the east side of Richardson Bay (PP) to Keil Cove (KC) to the reference site near San Rafael Bay (PSQ) show a gradient from Richardson Bay where salinity varies from 22 to 30 psu with the tides to the upper bay site where salinity varies from 11 to 26 psu under the influence of riverine flow from the Sacramento River.

The temperature and salinity regime at PSQ differed substantially from those at SA, PP, and KC (Figure 2-1). The PSQ regime showed not only some influence of tidal cycles but also influence from variations in river flow into the upper bay.

At the Sausalito site, the data record clearly shows that salinities of about 24 psu prevailed at low tides and then increase to salinities of 26 to 27 psu at high tides. Water temperatures were about 11°C but warmed to around 12°C at the lower low tides when the water column was approximately 2 or 3 feet over the sensor. Peninsula Point salinity cycles were more heavily coupled to the tidal height, and the variation in salinity was much wider. At higher high tides, salinity approached or slightly exceeded 30 psu, and at low tides salinity was approximately 24 to 25 psu. Water temperature was fairly steady at around 11°C, but at the lower low tide the water warmed up by about 3°C in a few hours. Keil Cove cycles of salinity and temperature were similar in pattern to those at PP but showed sharp drops in salinity down to 20 to 22 psu at lower low water levels. At higher high water levels, salinities were about 28 to almost 30 psu. Water temperature ranged from 10.5 to 11°C and warmed at the lower low tide to about 12°C. At the

northern-most bay site, Point San Quentin, the salinity regime was dominated by river flow and switched between low salinity (10 to 15 psu) and higher salinity (20 to 25 psu). The salinity at PSQ did not rise above 25 psu as seen in the sites in and near Richardson Bay. Water temperature was fairly constant at about 11°C and warmed slightly at lower low tide.

2.3 ESTIMATION OF AIR EXPOSURE AND AIR TEMPERATURE AT TIME OF EXPOSURE

Due to the tidal cycles and the variation in elevation of natural spawn transects compared to sea level, it is likely that the substrates for some of the eggs were out of the water for periods of time. Unseasonably high temperatures were also recorded in the few days prior to and during field collection and transport of spawned eggs to the laboratory (Table 2-1, Figure 2-1). The high temperatures began the day the spawned eggs were collected at San Rafael. Thus, these eggs were not subjected to the same extremes as the other sites. The high air temperatures combined with low tides indicated that eggs were exposed to extreme conditions for 0.5 to 5.5 hours per day.

Table 2-1. Air Temperatures for 2008 Spawning Period.

Date	Herring Spawn or Sampling	Location	Air Temperature (°F)[1]			Air Temperature (°C)		
			Max	Min	Mean	Max	Min	Mean
16-Feb-08			61	46	54	16.1	7.8	12.2
17-Feb-08	Spawn	SRB	55	45	50	12.8	7.2	10.0
18-Feb-08	Spawn	SRB	56	47	52	13.3	8.3	11.1
19-Feb-08	Spawn	SRB	53	48	51	11.7	8.9	10.6
20-Feb-08	Spawn	SA, PP, KC	59	48	54	15.0	8.9	12.2
21-Feb-08	Spawn	SA, PP, KC	58	49	54	14.4	9.4	12.2
22-Feb-08	Spawn	SA, PP, KC	55	47	51	12.8	8.3	10.6
23-Feb-08			54	45	50	12.2	7.2	10.0
24-Feb-08			58	45	52	14.4	7.2	11.1
25-Feb-08			58	49	54	14.4	9.4	12.2
26-Feb-08	Sampling	SRB	66	48	57	18.9	8.9	13.9
27-Feb-08	Sampling	SA	67	51	59	19.4	10.6	15.0
28-Feb-08	Sampling	KC	70	48	59	21.1	8.9	15.0
29-Feb-08	Sampling	PP	58	48	53	14.4	8.9	11.7

[1]Air Temperatures (°F) from NOAA Climatological Data (NOAA) for San Francisco Downtown

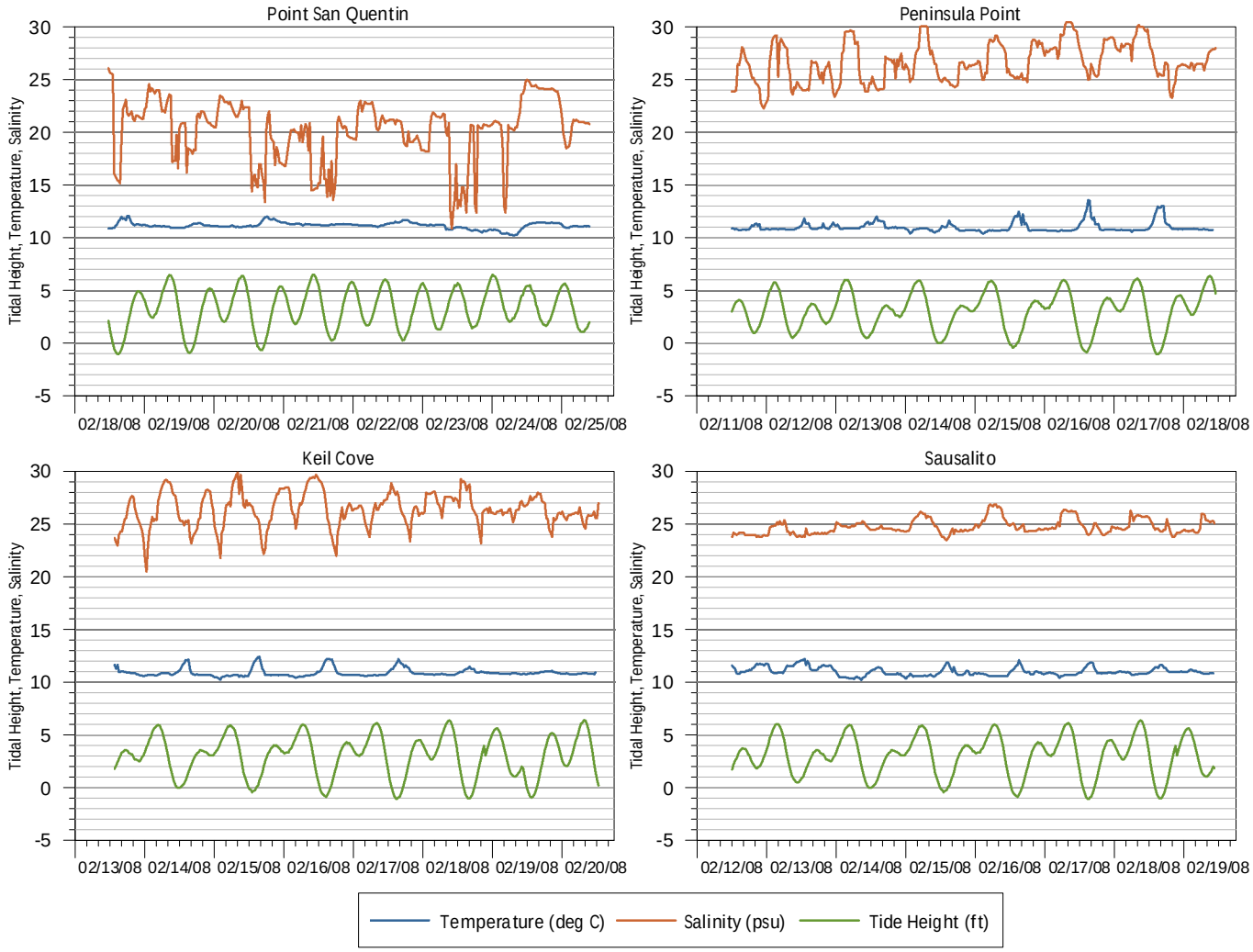


Figure 2-1. Temperature, Salinity, and Tidal Data from 2008 Spawning Period.

2.4 IDENTIFICATION OF SCENARIOS FOR THE 2010 EXPERIMENTAL STUDIES

The conditions at the various natural spawn locations were used as a basis for the design of the 2010 laboratory study. The salinity regimes at Point San Quentin and at Peninsula Point were used to structure the salinity and temperature changes in the laboratory. The scenario based on Peninsula Point (Treatment A) had one set of replicates also exposed to higher temperatures and air exposure (Treatment A-TS) to mimic beach stranding. Treatment B mirrored field conditions at Point San Quentin. The laboratory conditions and schedule of salinity and temperature changes are described in detail in Section 3.6.

2.5 UNCERTAINTIES ASSOCIATED WITH THE LABORATORY SCENARIOS RELATIVE TO ACTUAL CONDITIONS IN 2008

The data from the loggers were not ideally matched to the actual exposure dates but were sufficient to discern that the temperature and salinity at PSQ in the reference area was substantially different than those at the three potentially oiled sites (SA, PP, and KC). There was some uncertainty about precise tidal height sampled in the collection of natural spawn in 2008; however, examination of macro-algal species collected as egg substrate showed macro-algal species that are characteristic of the low intertidal zone. To take this into account, Treatment A included both an exposure simulating complete submersion and the thermal shock and air exposure (A-TS). Some of the conditions in the 2010 laboratory design may have been less harsh than those in the field. For example, the duration of exposure of Treatment A-TS to air and higher temperatures was likely shorter than actual field conditions. It is unlikely from the data records that actual conditions were more benign than in the study design.

3 FIELD SAMPLING AND LABORATORY METHODS

3.1 COLLECTION OF CLUPEA PALLASI

Pacific Herring were collected from San Francisco Bay between February 19 and 24, 2010 by NewFields staff on a small vessel owned and operated by John Brezina. Collection of fish and subsequent harvesting of gonads followed a protocol adapted from Dr. Paul Dinnel (Dinnel et al. 2007; pers comm. 2010). The modified protocol is provided in Appendix B. Gonads were obtained from adult donor fish in two different size groups: approximately 160 mm in length (smaller fish) and 180 mm in length (larger fish).

Two previous spawning events had occurred in central San Francisco Bay in 2010 before the NewFields collection of spawning adults. At the time of collection, adult herring were visible in large numbers and eggs could be seen on the shorelines in Richardson Bay and along the Tiburon peninsula, extending north to the San Rafael Bridge. A general search was conducted throughout central San Francisco Bay, and adult herring were located by visual observation of the shoreline, bird and mammal feeding activity, and by using an electronic Fish Finder on the boat. Figure 3-1 illustrates birds gathering to feed which usually indicates the location of schooling fish.

Figure 3-1. Feeding Birds Indicating Potential Herring Spawning Site.

This third spawning event was smaller and no eggs were visible on shorelines. Adults were only detected between Angel Island and Sausalito, where they gathered in a large school at approximately 60' below the surface. A trickle spawn event occurred in Richardson Bay in which small groups of herring sporadically came into the shallow bay to reproduce (Figure 3-2). Adult herring were collected in Richardson Bay as they came in to spawn. A search of central San Francisco Bay was conducted daily, but adult herring were only observed in the large school just outside Richardson Bay. The school remained at a depth of about 60' throughout the collection period and continued to trickle spawn in Richardson Bay throughout the week. All adults were collected from this site.

The site fell between the following GPS coordinates: 37° 52.315' N, 122° 28.672' W and 37° 52.172' N, 122° 28.710' W (Figure 3-2); the square on the map marks the site. Water depth ranged from 5.5 – 12.5 feet MLLW at the site, and salinity and water temperature were measured daily (Table 3-1).

Figure 3-2. Map of Richardson Bay.

Individual fish were caught from the site using a fishing rod with a Sabiki rig lure. Each adult herring was tagged with a unique collection number and photographed on a measuring board (Figure 3-3). The collection number referenced the collection site (RB = Richardson Bay), the collection date, and the fish number by day. Gender, standard length (snout to caudal peduncle), total length (snout to posterior tip of tail fin), and time of collection were recorded in the field collection notebook.

Table 3-1. Salinity and Temperature at Richardson Bay Collection Site.

Date	Salinity (ppt)	Temperature (°C)
2/19/10	23.0	12.0
2/20/10	24.0	13.0
2/22/10	22.0	11.5
2/23/10	22.5	12.0
2/24/10	25.5	11.5

Gonads from a total of 61 adult herring were collected from San Francisco Bay during the sampling period. Gonads were removed immediately. An incision was made with a scalpel starting at the ovipositor and extending to the gills; gonads were located and gently placed into individual glass Petri dishes and labeled with their unique collection numbers (Figure 3-4). The circumference of each Petri dish was wrapped with parafilm and secured with clear tape to seal in moisture. Dishes were then wrapped in bubble wrap and placed in an ice chest with frozen "blue ice" packets with newspaper layered between the ice

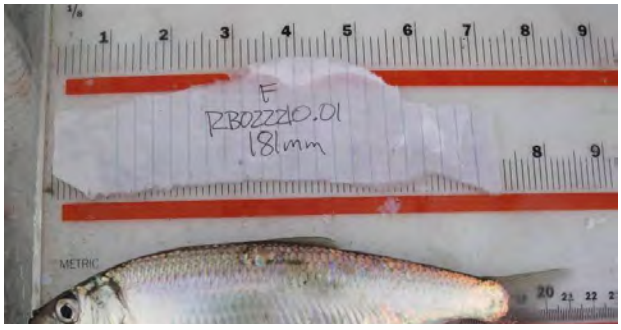


Figure 3-3. Photographic record of fish collected with gender (F = female), unique sample number, and standard length.

and the petri dishes to protect the gonads from becoming too cold. When possible, gonads were shipped on the collection day via FedEx for overnight delivery. Some gonads were held in a refrigerator over the weekend which ranged from 2 – 5 °C. A chain of custody was included with each shipment.

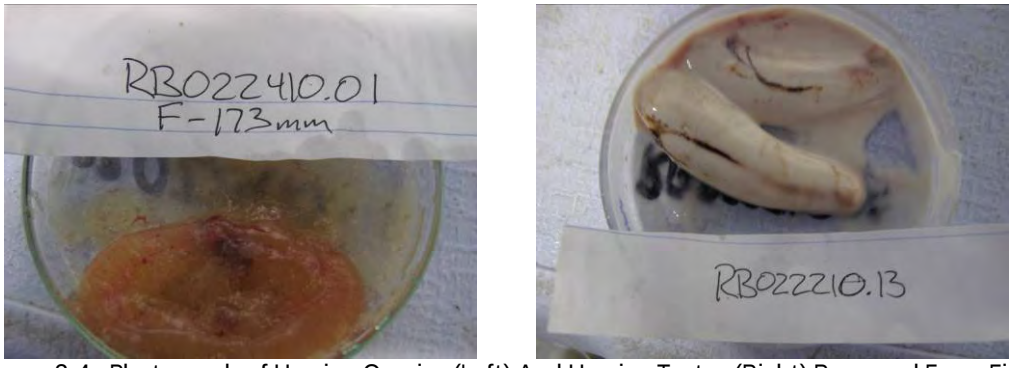


Figure 3-4. Photograph of Herring Ovaries (Left) And Herring Testes (Right) Removed From Fish.

Fish were received at the NewFields laboratory between February 22–24, 2010, a subset were shipped to Alpha Analytical in Mansfield, Massachusetts for background PAH tissue analysis (data provided in Section 0 of this report). Gonads were stored in a walk-in cold room at approximately 4 °C until test initiation. Table 3-2 provides a summary of the fish collection information and subsequent use of gametes for testing.

Table 3-2. Summary of Fish Used for Chemical Analysis or Fertilization

Fish ID	Collection Date	Gender/Donor Size Group	Standard Length (mm)	Total Length (mm)	Subsample Submitted for Chemical Analysis	Gametes Used for Fertilization
RB022210.18	2/22/10	Male/Larger	170	196	2/24/10	2/26/10
RB022210.10	2/22/10	Male/Larger	173	201	2/24/10	2/26/10
RB022310.08	2/23/10	Male/Larger	172	193	2/24/10	2/26/10
RB022310.07	2/23/10	Female/Larger	177	202	2/24/10	2/26/10
RB022410.01	2/24/10	Female/Larger	173	200	2/24/10	2/26/10
RB022210.04	2/22/10	Male/Smaller	158	182	2/24/10	2/26/10
RB022210.13	2/22/10	Male/Smaller	169	192	2/24/10	2/26/10
RB022210.12	2/22/10	Male/Smaller	155	176	2/24/10	2/26/10
RB022210.06	2/22/10	Male/Smaller	157	179	2/24/10	2/26/10
RB022210.02	2/22/10	Female/Smaller	146	167	2/24/10	2/26/10
RB022210.11	2/22/10	Female/Smaller	168	194	2/24/10	2/26/10
RB022210.21	2/22/10	Female/Smaller	160	181	2/24/10	2/26/10
RB022010.10	2/20/10	Female/Smaller	152	173	2/24/10	2/26/10
RB022010.22	2/20/10	Female/Smaller	159	180	2/24/10	2/26/10

3.2 SIZE OF DONOR FISH

Two different sized groups of donor fish were used for this study in order to compare relative hatching success of two age classes. Other researchers have shown that differences in overall

spawning success may be related to health or size of the spawning adults (Hershberger et al 2005). Size of the spawning adults was also implicated as a causative factor of observed adverse effects from the 2009 laboratory study conducted by NOAA (NewFields 2010a). Therefore, size was added as one of the treatment variable within this study. In the larger size group, testes from three males and eggs from two females were included; in the smaller group, gametes from four males and five females were used in the experiment (Table 3-2).

3.3 FERTILIZATION AND TEST INITIATION

The experiment was initiated on February 26, 2010. Dr. Paul Dinnel, noted herring expert from Western Washington University, aided NewFields staff in identifying healthy, viable gonads for use in testing. General procedures for fertilization followed the methods developed by Dr. Dinnel for herring larvae (Dinnel, 2002 – 2010). The protocol used for this experiment is provided in Appendix C.

The success of herring embryo tests is influenced by the condition of the gonads, and late stage V to VI adults, termed as "running ripe," are considered ideal (Dinnel 2010, in prep). Running ripe fish have gametes that are easily expressed from the oviducts. Testes and ovaries were chosen based on size; larger gonads were considered more mature and viable. Ovaries were selected based on apparent egg quality. A general fluidity of eggs is associated with more viable eggs, while ovaries with rubbery masses of eggs are considered less viable. Eggs should be fluid enough to separate them from each other during fertilization. Eggs that have flowed out of the ovary are considered healthy, while those that are hard to extract are most likely not fully ripe. When possible, gonads were selected based on these criteria.

Control test water was prepared with 0.45 µm filtered seawater from Hood Canal Bay, Washington and diluted with deionized water to create test water at fertilization test conditions of 16 ppt and 12°C, noted by other researchers to be optimal conditions for herring embryos in San Francisco Bay (salinity: Cherr and Pillai 1994; Griffin et al. 2004; temperature: Dinnel et al.

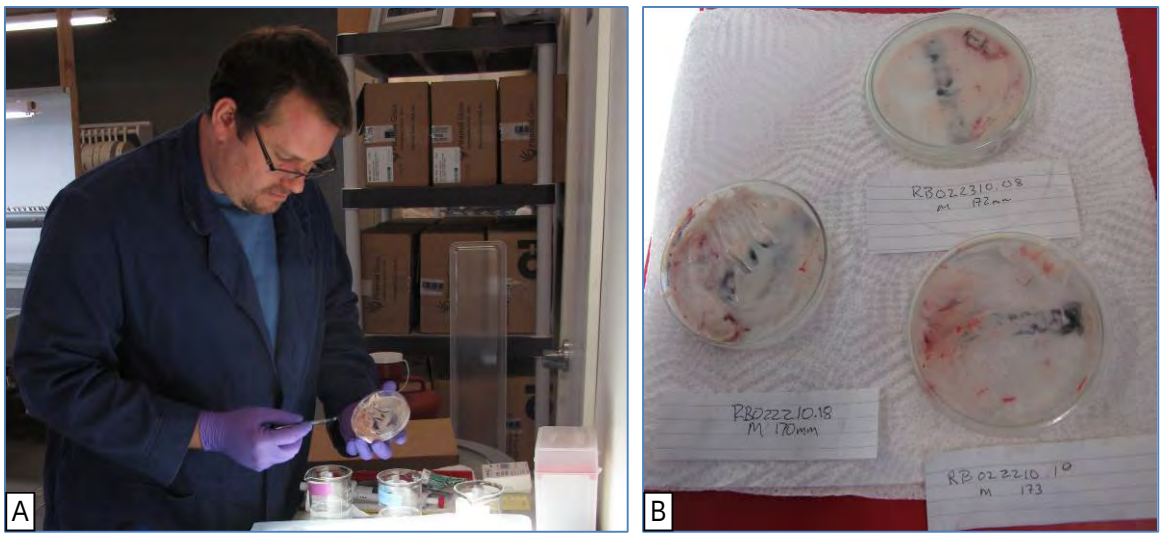


Figure 3-5. Researcher Introducing Macerated Tissue to Beaker with Clean Seawater (A); Close-Up of Macerated Testes (B).

2008; Vines et al. 2000; Alderdice and Velsen 1971). For each age class 100 mL of control test water was poured into a clean 250 mL beaker. A new, clean razor blade was used to cut a 4 cm^2 piece of testis from the posterior portion of the organ (Figure 3-5). Each section was placed in a Petri dish containing control test water and macerated with the razor blade until a milky sperm solution was achieved. This water was added to the 250 mL beaker, and any leftover solid tissue still remaining in the beaker was removed. This step was repeated for each testis in the given donor size group, and all sperm for each size group was pooled together in the 250 mL beakers.

Approximately 500 mL of test water was subsequently poured into Pyrex® baking dishes for each test at the appropriate fertilization conditions (16 ppt, 22 ppt, or 28 ppt at 12 °C). Glass slides were placed on the bottom of the baking dishes. The sperm solution was homogenized by gently swirling the beaker, and approximately 20 mL of solution was added to each dish to provide sufficient gametes for fertilization.

Eggs were then extracted by cutting the mesovarium of each ovary open with fine tipped scissors to uncover the eggs. A Teflon™-coated spatula was used to scoop eggs from the ovaries. Eggs were transferred to the baking dishes by using a gentle, side-to-side sweeping motion to evenly distribute single eggs onto the glass slides. The spatula was wiped with a Kimwipe™ after each submersion into the dishes containing sperm. Care was taken to avoid allowing clusters of eggs to gather on the slides, and the ultimate goal was to collect approximately 100 single eggs on each slide. Four replicate slides were prepared for the tests as indicated in Table 3-3; three replicates for the test design while the fourth replicate was used for daily assessments to minimize handling of the test replicates.

Table 3-3. Number of Replicates Fertilized per Test Treatment.

Treatment	Larger Donor Size Group Slides (L)			Smaller Donor Size Group Slides (S)		
	Salinity			Salinity		
	16 ppt	22 ppt	28 ppt	16 ppt	22 ppt	28 ppt
A	4		4	4		4
A-TS			4			4
B		4			4	
Control	4			4		
CUV	4			4		

Once distribution of eggs onto slides was complete, baking dishes with slides were placed in water baths at 12 °C, covered, and fertilization was allowed to occur over a 30 minute period. The fertilization water was then carefully decanted, and slides were gently rinsed with water at the appropriate test salinity. A qualitative review of the eggs for a distinct fertilization membrane was conducted by Dr. Dinnel. Based on this cursory review it was determined that the overall fertilization rate was acceptable for continuing with the experiment. The slides were then placed into test chambers using forceps (Figure 3-6).

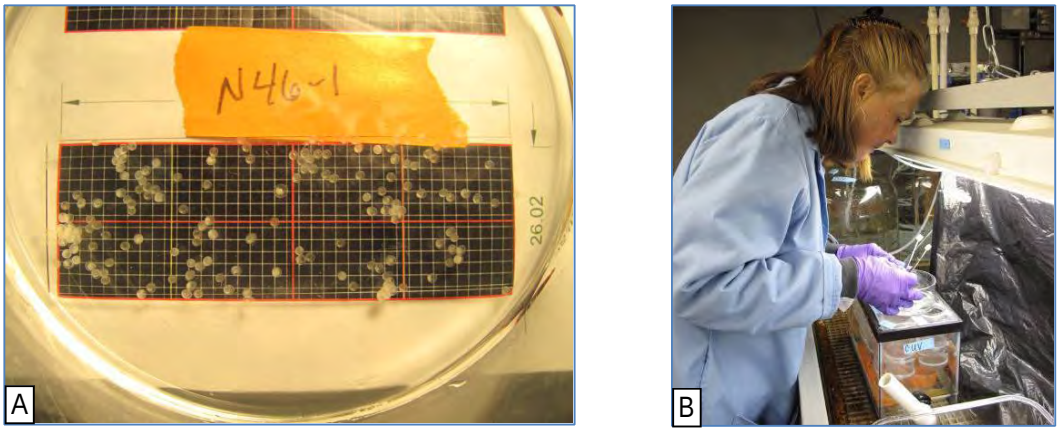


Figure 3-6. Slide Containing Fertilized Eggs (A); Researcher Placing Slide into Screen Tube (B) Chamber.

3.4 TEST SETUP

Test chambers consisted of eight screen tubes suspended from a plexiglass frame mounted on the top of a 10 L aquarium. The screen tubes were made from polycarbonate tubes with one end capped with a 1 millmeter mesh screen. The tubes were approximately 5 to 6 in. long with a 2 in diameter. Screen tubes were constructed of two tubes and connected with a mesh screen collar and zip ties. The mesh screen was secured to the bottom of one end of the individual tube and secured with a fitted sleeve. Screen tubes were suspended above the bottom surface of the aquarium at a depth which ensured 150 mL (85 mm depth) of water remained in each tube. The water passed through an entry port on one end of the aquarium and exited through a port located on the opposite side of the tank. Figure 3-7 shows the test setup.

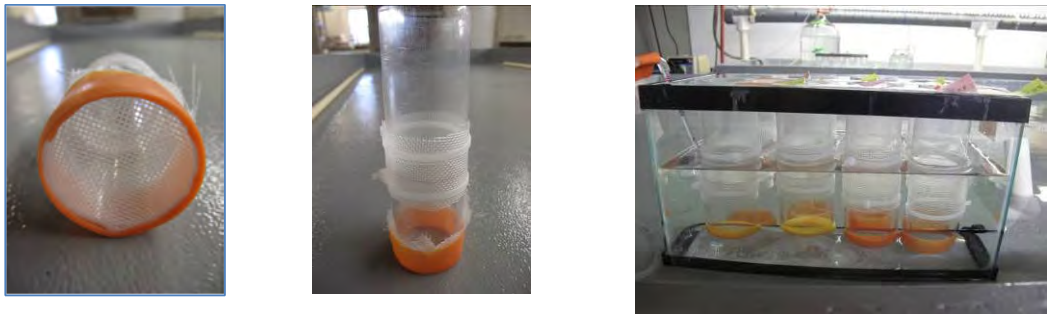


Figure 3-7. Test Chamber with Nytex Screen and Aquaria Housing Slide Chambers.

One glass slide with fertilized eggs was placed into each screen tube. Tubes in each aquarium were labeled in a pre-determined random order mixing the two size classes to avoid a placement effect. Samples of Treatment A-16 (16 ppt fertilization) and A-28 (28 ppt fertilization) were randomly placed in two aquaria while all other treatments occupied one aquarium each (A-TS, B-22, Control, and CUV).

Aquaria were placed in water bath tables to prevent ambient room temperature from affecting test temperatures. Trickle flow aeration was provided to each tank. A Hobo temperature

monitor was placed in each aquarium to monitor temperatures continuously throughout the test. Additionally, YSI water quality probes placed in each aquarium recorded temperatures and salinities during the test. Data collected from the Hobo and YSI monitors were downloaded and the data was used to ensure salinity and temperature profiles during testing followed the study design. Water quality including dissolved oxygen, pH, temperature, and salinity was measured in each aquarium daily. Target measurements are presented in Table 3-4.

Table 3-4. Water Quality Target Limits.

Treatment	Dissolved Oxygen (mg/L)	pH	Salinity (ppt)	Temperature (°C)
A-16	> 4.8	6 - 9	Per treatment protocol (see Section 3.6)	
A-28				
A-TS				
B-22	> 4.8	6 - 9	Per treatment protocol (see Section 3.6)	
Control	> 4.8	6 - 9	16	12
CUV	> 4.8	6 - 9	16	12

3.5 SAMPLE IDENTIFICATION

This was a blind study in which the assessment teams during and after the experiment did not know from which treatment any given slide, hatched larvae, or image originated. To achieve this, each replicate slide was assigned a unique number code. Colored tape with the number codes were placed on the top of the plexiglass frames next to their respective screen tubes at test initiation. A label with the corresponding number code was placed on a transportation dish for identification when a slide was moved for viewing under the microscope. The conversion of the coded numbers to treatment was only made after all measurements were obtained and when the data were analyzed; this information was not available to researchers evaluating the larval images.

3.6 SALINITY AND TEMPERATURE FLUCTUATIONS

This experiment required changes in salinity and temperature to simulate fluctuating site conditions that occurred during the 2008 spawning events in San Francisco Bay. Table 3-5 summarizes the salinity and temperature regimes targeted for this study; Figure 3-8 and Figure 3-9 summarize the detailed schedule for the salinity and temperature changes.

Table 3-5. Sampling Design for Treatments A and B, and Controls.

Treatment	Fertilization Salinity	Salinity Regime	Temperature Regime
A-16, A-28	16 ppt or 28 ppt	Varying between 24.5 ppt and ambient salinity (28+ ppt) (Figure 3-8)	11 °C with periodic increases (Figure 3-8)
A-TS	28 ppt		11 °C with periodic increases (Figure 3-8)
B-22	22 ppt	Varying between 14 and 24 ppt (Figure 3-9)	11 °C (Figure 3-9)
Control, CUV	16 ppt	16 ppt	12 °C

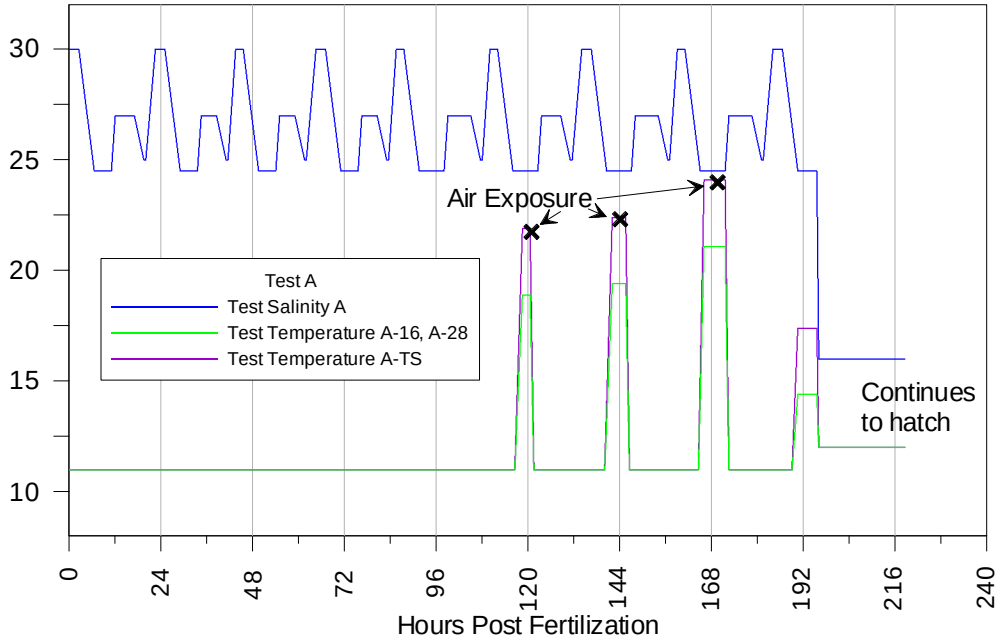


Figure 3-8. Plan Temperature and Salinity Profile for Treatment A-16, A-28, and A-TS.

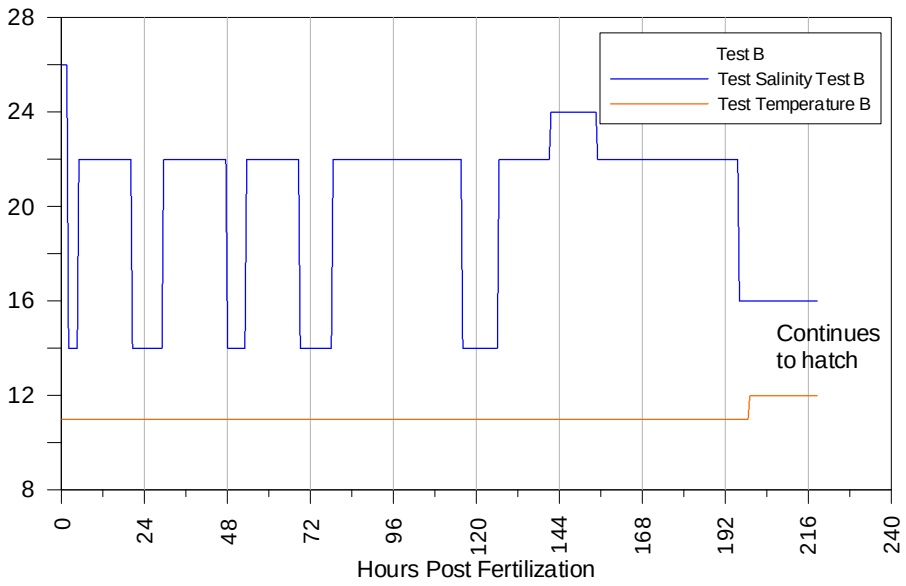


Figure 3-9. Plan Temperature and Salinity Profile for Treatment B-22.

3.6.1 SALINITY ADJUSTMENT SYSTEM

Aquaria were setup with a constant renewal of flowing seawater. Test water was prepared in 20 L glass carboys and consisted of 0.45 µm filtered seawater collected from Hood Canal, Washington diluted with deionized water to the appropriate test salinities. Water was delivered directly to the aquaria from the carboys using peristaltic pumps and tubing. Figure 3-10 shows the system for one treatment.

Figure 3-10. Salinity Adjustment System, Carboy Contains Test Water at Appropriate Salinity.

Each aquarium was maintained at a volume of 6 L. A line was drawn on the outside of the tanks as a visual cue to mark the 6 L line. It was determined that 16 L of water were required to completely renew the 6 L of water in each aquarium. The flow rate on the peristaltic pumps was adjusted to deliver the 16 L over the desired time period of the salinity change. For example, if the desired salinity change was to occur over 2 hours, the flow rate was set to deliver 135 mL/min. When no salinity change was occurring flow rates were set at 22 mL/min to ensure complete renewal in the aquaria over a 24 hour period.

Two different techniques to change the salinity were used during this experiment. One method was performed when the salinity change was to be gradual over time. The other method was performed when the salinity change was designed to be more immediate. When gradual

increases or decreases in salinity were required, a 20 L carboy was prepared with the appropriate test water as described above. The water in the carboy was allowed to acclimate to test temperature and water quality was measured to ensure proper test conditions. At the designated time for a salinity change the peristaltic pump tubing was placed in the new carboy. The flow rate was adjusted if necessary and measured.

When the salinity change was to occur rapidly, a 10 L tub and a 20 L carboy were prepared with the appropriate test water as described above. Both vessels were placed into the temperature controlled bath and allowed to acclimate to the test temperature. Water quality measurements were recorded prior to the water change. At the appropriate time the peristaltic pumps were shut down and the tubing was transferred to the new 20 L carboy. The overflow spout on the side of the aquarium was then rotated horizontally, and water in the test chamber was allowed to flow out of the tank until the water level was just above the top of the slides inside the screen tubes. A clean funnel was placed inside the tank on the opposite side of the overflow spout, and water from the 10 L tub was carefully poured into the funnel. The overflow spout was then moved back to its original position, and the water level in the tank was brought back to the 6 L line. The peristaltic pump was turned back on, and the flow rate was adjusted if necessary and measured. The control treatments did not require any salinity fluctuations and carboys were replaced as they were depleted (twice daily).

3.6.2 TEMPERATURE CONTROL SYSTEM

The laboratory seawater delivery system is equipped with Fuji Electric Micro-controller (Figure 3-11) automated temperature controllers. A hot water line and an ambient temperature water line are piped into each controller unit. A sensor measures the temperature of the mixed water and adjusts a gate valve to allow either cold or hot water to flow to achieve the desired temperature of water delivered to the water bath tables.

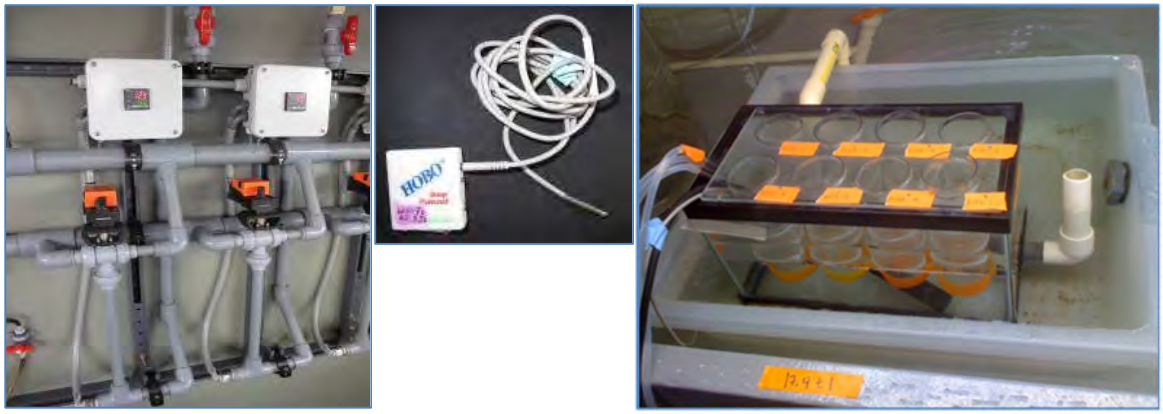


Figure 3-11. Wall-mounted Temperature Control Units, Continuous Temperature Recorder, and Tub with Flowing Temperature Controlled Water.

Gradual temperature changes occurring over a period of time were programmed into the controller as dictated by the study design. In this case, water flowed to the water bath tables, and temperatures inside the tanks (and screen tubes) came to equilibration over a course of hours. Rapid temperature changes were manually dialed up or down on the temperature controller. Aquaria were placed in a plastic tub in the water bath and some water was diverted from the

incoming water bath line and flowed directly into the tubs to achieve the sudden temperature change.

To adhere to the fluctuating temperature and salinity regime researchers continuously monitored and renewed the test systems. This constant observation period continued up to Day 8 post fertilization when the test chambers were moved to a constant temperature and salinity incubation room.

3.6.3 Air Exposure

To model field conditions the study design took into account tidal fluctuations. Eggs deposited in the intertidal zone in San Francisco Bay are exposed to air during low tides. To test the effect this may have on developing embryos, one treatment included several air exposure periods in which the embryos were removed from the water and exposed to air for preset periods of time.

Air exposure took place in an incubator equipped with UV lighting and set at the appropriate test temperature. The plexiglass frame with screen tubes was carefully removed from its water environment and placed in a clean 10 L aquarium with a small amount of test water on the bottom to maintain humidity. The screen tubes did not have contact with the water. The aquarium was then placed in the incubator for the allotted amount of time. At the end of the air exposure phase, the plexiglass frame was placed back into the treatment tank in the water bath.

3.6.4 UV Light Exposure

UV lighting simulated ambient lighting conditions in San Francisco Bay in February 2008, which called for an 11 hour light, 13 hour dark cycle. All test treatments except the Control were placed under overhead UV lighting positioned approximately 12" from the top of the aquaria. UV light was provided by fluorescent light ballasts containing one Duro-Test Vita-Lite® (40W, 5500°K, 91 CRI) fluorescent bulb and one standard fluorescent bulb (Phillips F40CW). The Control treatment was exposed to fluorescent lighting only. A replicate control treatment was setup (CUV) that received UV lighting for compare potential effects of UV in the absence of any other stressor. Light intensity was measured daily at the top of the screen tubes and next to the aquaria at the top of the slides.

3.7 Experimental Observations

Fertilization success was assessed at 48 h post fertilization (hpf). At this stage development should be mid-blastula to early stage epiboly as described by Hill and Johnston (1997). Most of the embryos in this experiment were in transition from blastula stage to epiboly as shown in Figure 3-12. For enumeration, slides were carefully removed from test chambers and placed into glass crystallization dishes filled with water at appropriate test conditions. Each slide was photographed with a Canon G10 PowerShot digital camera. The camera was mounted on a tripod to

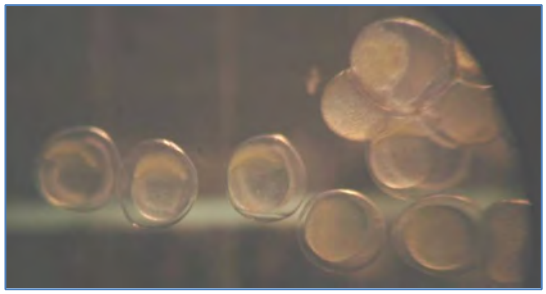


Figure 3-12. Embryos at Approximately 48 hpf. Transition to epiboly is clearly evident in the three embryos on the left side of slide.

give the photographer a stable and consistent platform. A dark background with a grid pattern was aligned with the glass slide to provide sharp contrast and to aid in enumerating the eggs.

After imaging, slides were viewed using a Nikon dissecting microscope. Eggs on each slide were enumerated and classified as fertilized, unfertilized, or dead. Eggs were considered fertilized and viable if there was a membrane surrounding the egg indicating cell division. Unfertilized eggs were those with no apparent membrane surrounding the egg (Figure 3-13). Eggs that were not affixed to the slide were not included in the counts. Unfertilized and dead eggs as well as clusters of eggs were removed from the slides with forceps when possible without damaging fertilized, developing eggs while also retaining 80–100 eggs on each slide (Figure 3-14). Care was taken to minimize the amount of time the slides were in crystallization dishes to prevent temperatures in the dishes from rising above test conditions. After enumeration and culling, slides were returned to their respective test chambers using forceps. Replicate 4 slides for all treatments were photographed every other day from this point forward.

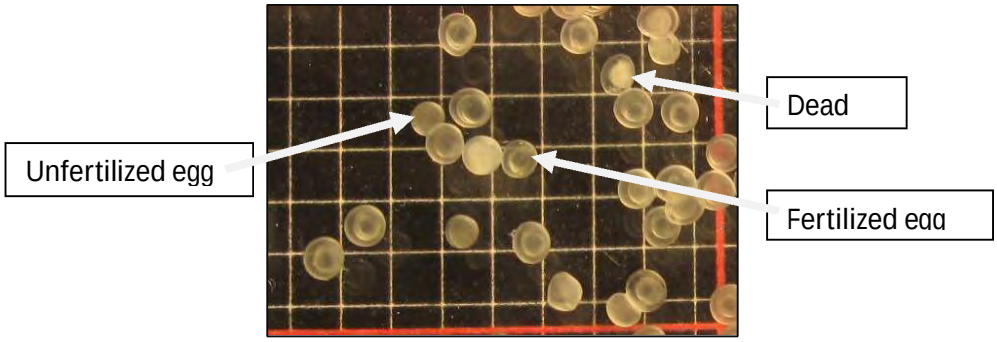


Figure 3-13. Examples of Fertilized, Unfertilized, and Dead Eggs at 48 hpf.

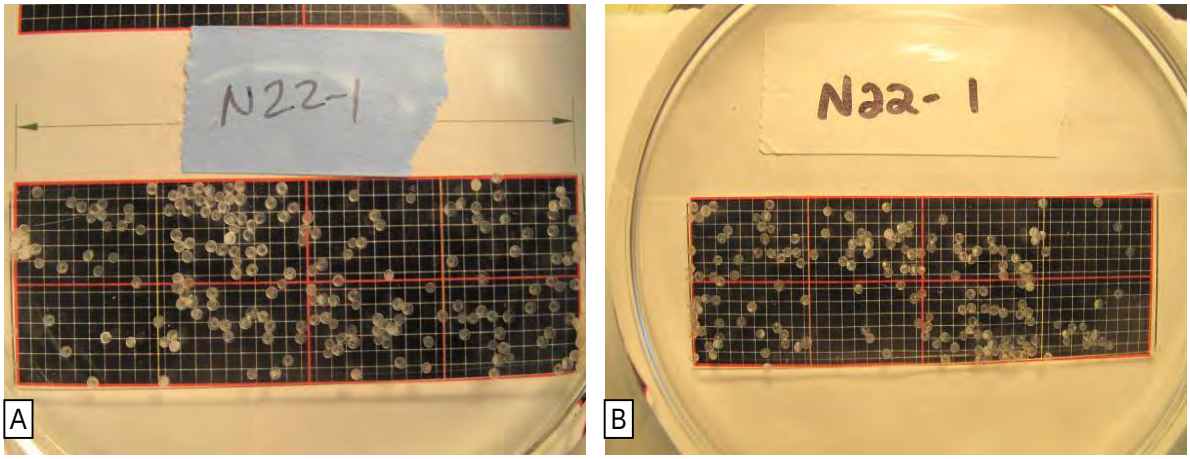


Figure 3-14. Slide Before Removal of Unfertilized Eggs (A); After Slide Was Culled (B) - slide is rotated 180°.

3.8 DAY 8 DPF: INCUBATION OF TEST SLIDES AT CONSTANT TEMPERATURE AND SALINITY

At 8 days post fertilization (dpf) all slides were transferred from the continuous flow test system to a static renewal regime staged in crystallization dishes at 16 ppt salinity and 12 °C. All slides were photographed at this time with the Canon camera. Additionally, slides were viewed on an Olympus SZX7 microscope fitted with a camera attachment (Olympus DP72) and eggs were enumerated and classified as unfertilized, live normal, live abnormal, or dead (Figure 3-15).

Live eggs were considered normal if they had no apparent opacity and had two distinct eyes. Abnormal live eggs were embryos with a heartbeat but that appeared abnormal or had disrupted physical features. Some were underdeveloped or were opaque; partially hatched larvae were also included in this group. Dead embryos included those with no beating heart or a lack of movement. Dead eggs were grouped into two categories; those that had achieved later stage development indicated by the presence of eyes were categorized as "dead - eyed." Those with no visible eyes, suggesting early mortality, were counted as "dead - other."

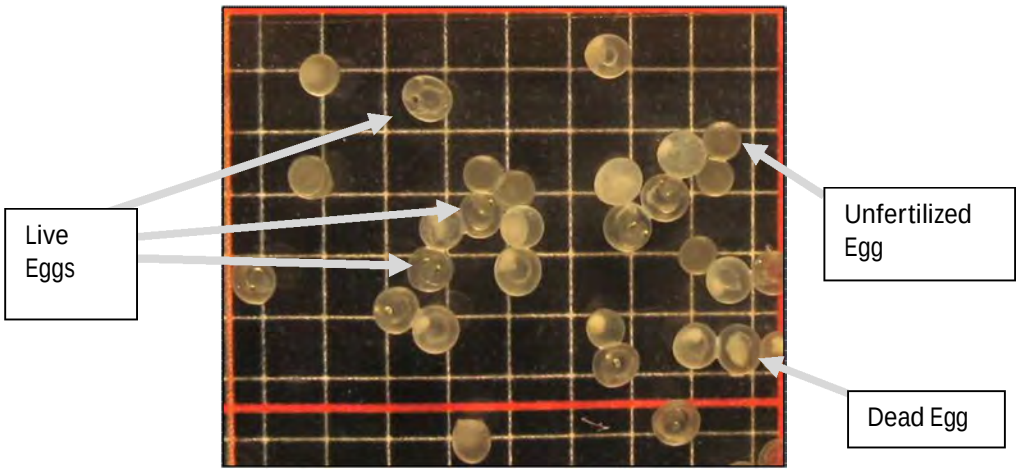


Figure 3-15. Images of Live, Dead, and Unfertilized Eggs at 8 dpf.

After slides were photographed they were placed back into the crystallization dishes containing water at 16 ppt and 12°C. Dishes were contained in plastic bags with a vent for aeration and moved into a temperature controlled room maintained at 12°C where they remained until hatch. Trickle flow aeration was provided (Figure 3-16).

A daily water renewal was performed until all eggs in a dish had either hatched or were assumed dead. The renewal was performed by transferring the slide with forceps into clean pre-labeled crystallization dishes containing the renewal treatment water (16 ppt and 12°C). Hatched larvae remained in the original dish for enumeration. Note that all larvae assessed hatched naturally (were not dechorionated) and therefore these descriptions refer to apparent development at hatch.

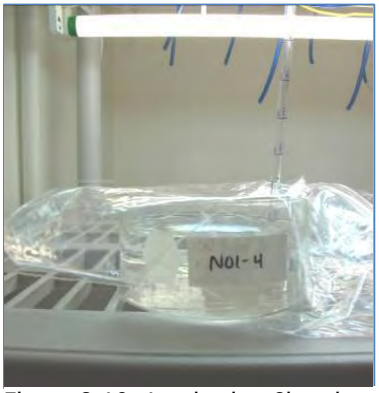


Figure 3-16. Incubation Chamber at 12°C and 16 ppt with Continuous Aeration.

3.9 YOLK SAC ABSORPTION EXPERIMENT

An additional experiment was performed prior to the image analysis to gain a clearer understanding of normal yolk sac absorption over time. Newly hatched herring larvae were observed and photographed over a two day period. Herring eggs were provided by Dr. Paul Dinnel collected from Puget Sound, Washington. Eggs were held in seawater from Hood Canal, Washington (ambient salinity) at 12°C until they hatched.

Test chambers consisted of Mason jars with holes drilled in the sides. Five replicate jars were filled with seawater from Hood Canal, Washington (ambient salinity) at 12°C; a constant trickle of unfiltered seawater provided a flow-through system. Screen tubes fitted with fine screen mesh were placed in the Mason jars and zip ties were used to suspend screen tubes above the bottom of the jars. Trickle flow aeration was placed outside of screen tubes.

Ten newly hatched larvae were placed in each screen tube using a glass Pasteur pipette with the tip cut off. Larvae were not fed during the experiment. During the two day test period, three fish were randomly selected from the test chambers twice daily (am and pm). At each observation event larvae were placed in crystallization dishes and anesthetized with 50 mg/L MS-222. Anesthetized larvae were photographed on an Olympus SZX7 microscope with a camera attachment (Olympus DP72). Figure 3-17 illustrates the change in the pericardial and yolk sac regions over time as the yolk is absorbed.

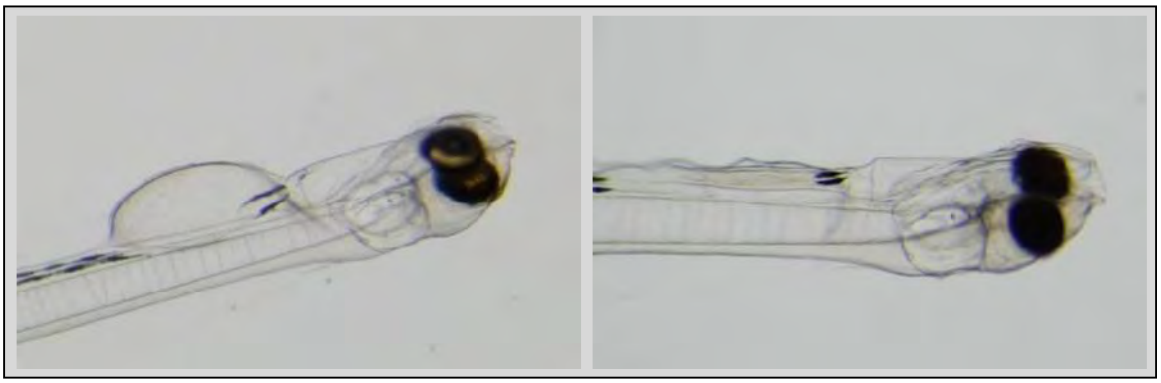


Figure 3-17. Changes in Yolk Sac over Time (Post Hatch).

4 POST HATCH IMAGING PROCESS

A preliminary experiment was conducted to determine the appropriate anesthetic level to administer to the larval samples. The method and results relative to the anesthetizing agent chosen for this study are summarized in Appendix D. As a result of this evaluation, MS-222 at a concentration of 50 mg/L was chosen to sedate the larval samples prior to imaging with the caveat that time under anesthesia did not exceed 60 minutes before the specimen was examined. Care was taken to minimize the amount of time fish remained in MS-222 to avoid producing any potential physical effects or anomalies due to prolonged exposure to MS-222.

Each crystallization dish was checked daily for hatched larvae. As larvae hatched they were photographed using the Olympus microscope and digital camera system. Hatched larvae were placed in petri dishes with a Pasteur pipette with the tip cut off and anesthetized in 50 mg/L MS-222. Larvae were then transferred to a Sedgewick Rafter cell with enough water so they were completely submerged. Individual fish were oriented into a lateral position when possible. Sometimes due to more advanced fin development, it was impossible to orient the fish laterally, and only a dorsal or ventral position could be attained. A clear, full length image of each fish was captured using different zoom levels, fine zoom controls, and lighting, with the focus on the pericardial and yolk sac regions. Images were saved as TIF files and later converted to JPEG files for analysis. The flow of the procedure is shown in Figure 4-1.

Because images were captured at different magnifications, metadata associated with each image was imbedded and saved with the original TIF files. For statistical analysis, this metadata was used to transform measurements into millimeters so the entire dataset could be compared.

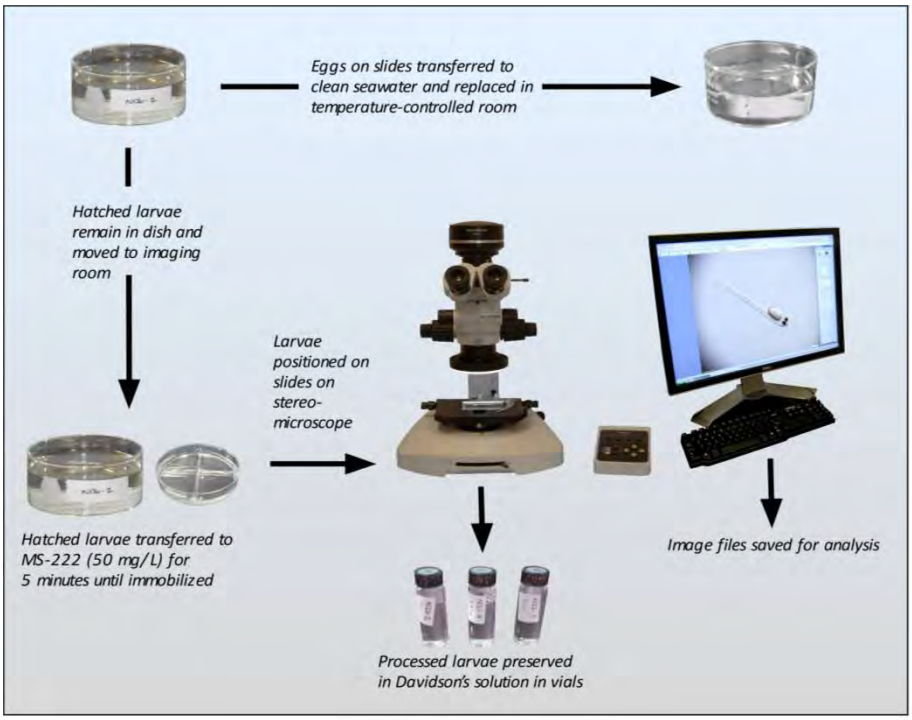


Figure 4-1. Procedure for Handling and Imaging of Hatched Larvae.

4.1 PHOTOGRAPHIC DOCUMENTATION AND PRESERVATION OF SLIDES AND LARVAE

Photographs of slides were taken at test termination using the Canon PowerShot camera. All slides containing unhatched embryos were subsequently preserved in Davidson's solution and archived by sample identification code. Larvae were euthanized with a stronger dose of MS-222 and then preserved in glass vials with Davidson's solution following examination and imaging; labels included hatch date and unique identification code. Chain-of-custodies documents provide a record of the slides and vials which were stored in a secure, metal fireproof cabinet.

4.2 MEASUREMENT PROCESS AND DATA MANAGEMENT

Images of larvae photographed at hatch after test terminations were subsequently assessed for occurrences of several categories of abnormalities. ImageJ software (http://rsbweb.nih.gov/ij) was used to make the quantitative measurements. Images were assessed by hatch date (3/7/10 – 3/12/10). A combination of the quantitative measurements and qualitative scoring was used to assess the health of each larva. Herring larvae are mostly transparent, at times making distinctions between membrane layers as well as the separation of peritoneal and pericardial cavities difficult. Indeed, often in previous studies judgments of edema were based on qualitative assessments only (Incardona et al. 2009; Hill et al. 2004; Wassenberg and Di Giulio 2004; Pearson et al. 1995, Kocan et al. 1996). We amplified the number of quantitative assessments made with ImageJ software by paying particular attention to the pericardial and yolk sac areas. We found that alternating the image from light to dark fields often increased our ability to detect important membrane differences (Figure 4-2).

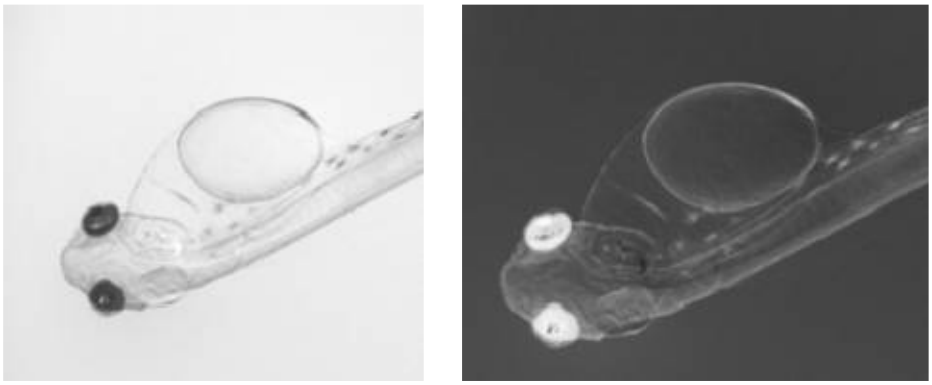


Figure 4-2. Increased Optical Clarity with Alternation between Light and Dark Fields

Figure 4-3 illustrates the various quantitative measurements made on the images. Measurements were recorded from ImageJ software results in pixels; metadata associated with each image was used to convert the measurements from pixels to millimeters. Table 4-1 and Table 4-2 summarize the quantitative and qualitative assessments made on each image analyzed. Image files were saved as "final" images with the quantitative measurements. Spreadsheets and final images were stored on a Western Digital storage device in a secure location.

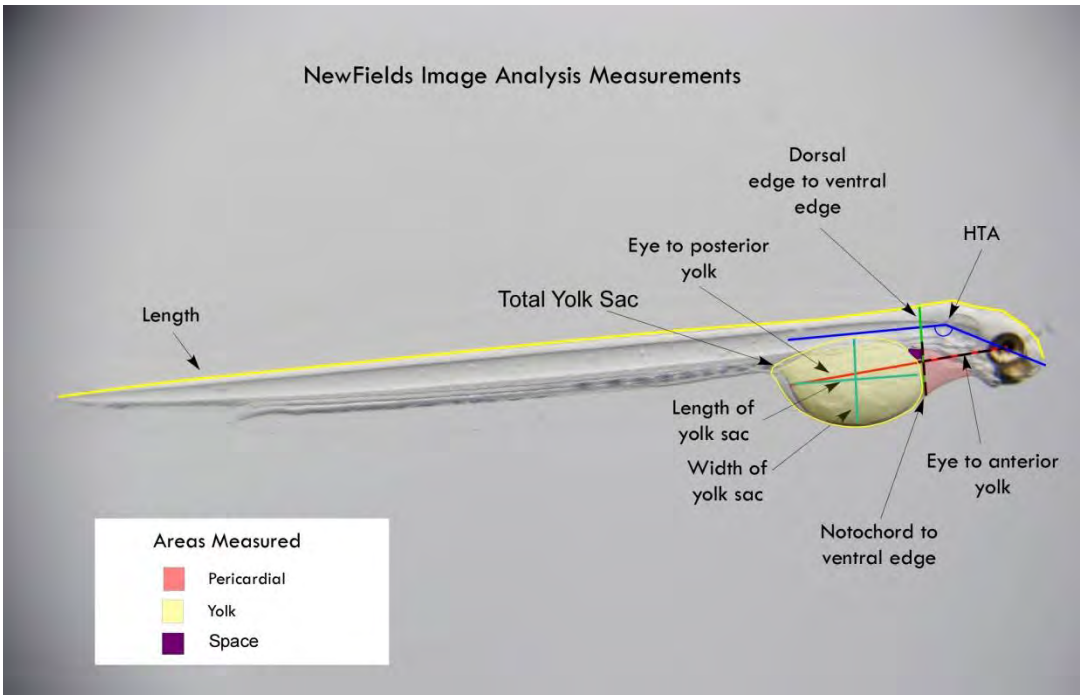


Figure 4-3. Diagram Indicating Measurements on Herring Larval Specimen in the Lateral View.

Table 4-1. Quantitative Measurements Assessed for 2010 Laboratory Data Using ImageJ Software.

Measured Parameter	Units	Data Base Abbreviation	Description	Notes
Standard Length	px/mm	StLength	Measured from snout along body line ending at caudal peduncle	
Head-to-Trunk Angle	degrees	HTA	Head to trunk angle	Parallel line drawn from in middle of notochord running perpendicular to the body line through the head regions. Second line drawn crossing through middle of closest eye and middle of otic vesicle intersecting first line. Measured the angle at the intersection on ventral side of the fish.
Yolk Area	px/mm	YolkArea	Yolk area	Area of space occupied by yolk only.
Total Yolk Sac Area	px/mm	TYSA	Total yolk sac area	Includes any space surrounding yolk.
Pericardial Area	px/mm	PA	Pericardial area	Drawn at the junction of the total yolk sac area and the pericardium as noted by a membrane line (shown best when image was inverted using ImageJ), following PA along bony jaw structure to attachment point in the middle of the head between the two otic vesicles and back towards yolk sac.
Space	px/mm	ExtraSpace	Area between PA and TYSA if present	Sometimes additional space occurred between the pericardium and total yolk sac areas. Most often observed in older "late stage" larval samples.
Length from eye to posterior edge of yolk	px/mm	EyePostYS	Measured from middle of closest eye to posterior side of yolk.	These 2 measurements were analyzed in relation to each other as a possible indication of PA or yolk sac edema.
Length from eye to anterior edge of yolk	px/mm	EyeAntYS	Measured from middle of closest eye to the anterior side of yolk following same trajectory as prior measurement.	
Length from dorsal edge to pericardium	px/mm	DorsalPA	Perpendicular line drawn tangent to the anterior edge of the total yolk sac from the dorsal edge of the body line to ventral edge of pericardium.	These 2 measurements were considered in relation to each other and indicated depth of the PA and potential PA edema.
Length from bottom of notochord to edge of pericardium	px/mm	NotoPA	Line drawn following same trajectory from ventral edge of notochord to ventral edge of pericardium next to total yolk sac.	
Length of Yolk	px/mm	YolkLength	Measured across the middle of the yolk from anterior to posterior edges.	Together these measurements showed provided some indication of yolk shape which may imply an abnormal yolk.
Depth of Yolk	px/mm	YolkWidth	The height measured through the middle of the yolk.	

Table 4-2. Qualitative Measurements Assessed for the 2010 Laboratory Data.

Measured Parameter	Data Base Abbreviation	Description	Notes
BAD Score	BADScore	Body Axis Defects Score: 0=None, 1=moderate, 2 =severe	Deviation from a straight linear body axis.
BAD Type	BADType	Description of defect	Lordosis, kyphosis, bent tail, scoliosis, excessive coiling
Opacity	OPScore	Score: 0=None, 1=moderate, 2=severe	Non transparent area anywhere in body or head.
Trunk Thickness	TTScore	Score: 0=None, 1=moderate, 2=severe	Thicker body appearance towards the posterior end of larvae rather than the tapered appearance or a normal larval sample
Tissue Disruption	TDScore	Score: 0=None, 1=moderate, 2=severe	Disrupted tissue material along dorsal or ventral edges of larvae
YS Deformity	YSANScore	Score: 0=None, 1=moderate, 2=severe	Included pitting or vacuolization of yolk and irregularly shaped yolks.
YS Edema	YSEdScore	Score: 0=None, 1=mild, 2=moderate, 3 =severe	Space within or around the total yolk sac area.
PA Edema	PAEdScore	Score: 0=None, 1=moderate, 2=severe	Extra space of PA next to total yolk sac area or anterior edge of PA is convex rather than concave.
Flattened YS	FlYSScore	Score: 0=None, 1=moderate, 2=severe	Flattening on anterior or posterior side of yolk.
Melanophore Development	MDScore	Score: 0=None or underdeveloped, 1=moderately developed, 2=well developed	Bands of black pigmented areas running along ventral side of larvae.

4.2.1 Body Axis Defects and Developmental Abnormalities

A body axis defect was defined as a body line deviating from linear. Body axis defects were scored to indicate the degree to which the larvae were impaired. Anomalies included kyphosis, lordosis, scoliosis, coiling, and bends in the head and/or tail regions (Figure 4-4). Definitions are provided below.

- Kyphosis: the body is curving towards the yolk sac
- Lordosis: the body is curving away from the yolk sac
- Scoliosis: lateral (side-to-side) curvature or pinching of the notochord
- Coiling: body tightly curled around yolk sac and head
- Bent Head: head is bent at an angle closer to 90° than 180°
- Bent Tail: a distinct bend in tail at an angle other than 180°

Other developmental abnormalities included opacity, trunk thickening, and tissue disruption; these were given a score to indicate degree of impact. Standard length was measured to partially assess growth and development was further evaluated by scoring the presence of melanophores. Melanophores are pigment bands running along the ventral edge of the body line and provide some indication of the developmental stage of the specimen.

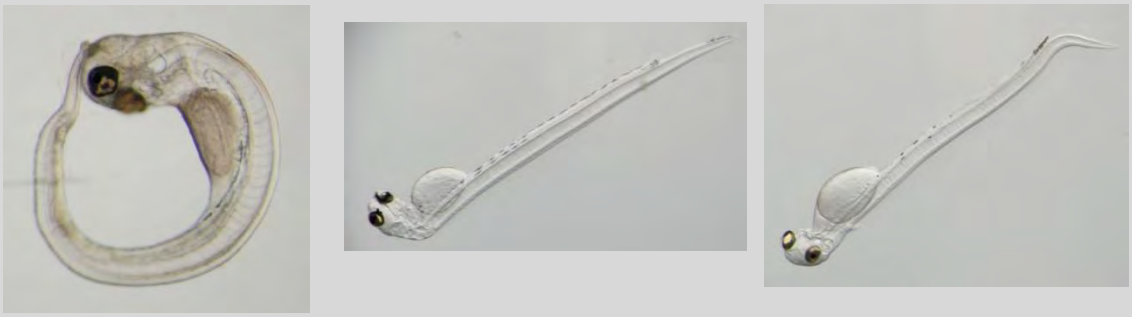
4.2.2 PERICARDIUM AND YOLK SAC ABNORMALITIES

The physical condition of the pericardial and yolk sac regions was assessed by numerous quantitative measurements and qualitative scoring. These regions influence each other and also change over time as the yolk-sac larvae develop. The first step in the evaluation process was to

A. Kyphosis, opacity, trunk thickening, tissue disruption, underdeveloped melanophores
(Image 3.12.10 N47-01-001a)

B. Lordosis, trunk thickening, tissue disruption, underdeveloped melanophores
(Image 3.11.10 N47-01-001a)

C. Scoliosis, opacity, trunk thickening, underdeveloped melanophores
(Image 3.8.10 N06-02-011a)



D. Coiled, opaque, trunk thickening, tissue disruption, underdeveloped melanophores
(Image 3.11.10 N30-02-001a)

E. Bent head, developed melanophores
(Image 3.8.10 N08-03-001a)

F. Bent tail, underdeveloped melanophores
(Image 3.8.10 N09-03-020a)

Figure 4-4. Examples of Body Axis Defects and Developmental Abnormalities.

determine and measure the area each region was occupying. The junction of the total yolk sac area and the pericardium is separated by the transverse septum (shown best when the image light field is inverted to dark field using ImageJ software). In some instances there was no space between the two regions, while in other cases space is evident (Figure 4-5). Areas measured included pericardium, yolk, total yolk sac, and any space if present.

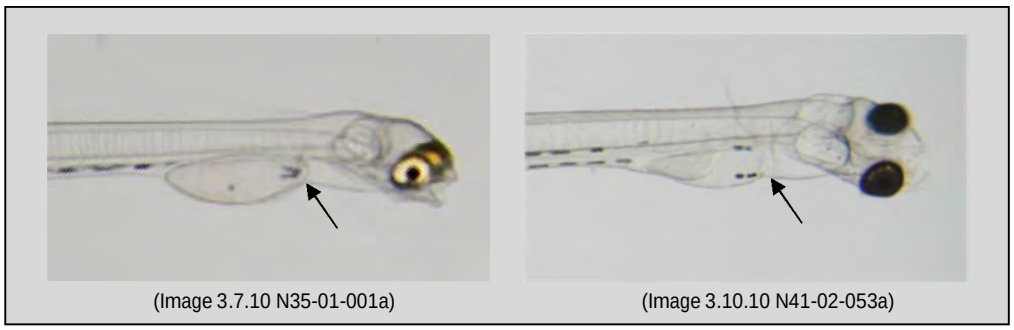


Figure 4-5. Examples of Varying Space between Pericardial and Yolk Sac Areas.

4.2.2.1 Quantitative Assessment Using ImageJ Software

To quantitatively evaluate the pericardial region a line was drawn to measure the depth of the pericardial region, extending from the ventral edge of the notochord to the ventral body margin. Another measurement was made of the distance from the dorsal body margin to the ventral body margin approximating the location of the transverse membrane. When feasible, the pericardial area was measured with a polygon dimensioning tool. The pericardial and yolk sac region was quantitatively assessed using four sets of measurements:

1) The distance from the midpoint of the closest eye to the anterior edge of the yolk sac and the distance from the midpoint of the closest eye to the posterior edge of the yolk;
2) The length and width of the yolk, indicating overall roundness of the yolk;
3) The areal measure of yolk area and total yolk sac area was made with the polygon dimensioning tool; and
4) The areal measure of the pericardium was also made with the polygon dimensioning tool.

4.2.2.2 Qualitative Scoring

Qualitative scores were given for yolk sac edema and pericardial edema to indicate degree of swelling. Other yolk sac abnormalities were scored to classify the level of impact observed. These abnormalities included pitting or vacuolization of the yolk and irregularly shaped yolk sac. Also, flattening on either the anterior or posterior edge of the yolk was scored.

Yolk sac edema was defined as space on the anterior and/or posterior edges of the yolk or space completely surrounding the yolk material and possible flattening of anterior yolk material. For the assessment of pericardial edema, the developmental stage and the view of the specimen (lateral, ventral, or dorsal) were taken into consideration. Note that all larvae assessed hatched naturally (were not dechorionated) and therefore these descriptions refer to apparent development at hatch.

- Hatch: hatching glands absent, yolk sac changing to an elliptical shape, jaw structure formed
- Post-Hatch: same as hatch but yolk either completely gone or very small

5 EXPERIMENTAL RESULTS

5.1 PHYSICAL AND CHEMICAL RESULTS

5.1.1 WATER QUALITY MEASUREMENTS

Water quality parameters measured included dissolved oxygen (DO), pH, temperature, and salinity. For the control treatments, temperature and salinity were targeted to remain constant throughout the test. Water quality was measured daily in aquaria and in carboys prior to use. Additionally, a Hobo monitoring device constantly recorded temperature in the water bath where both controls were located. Table 5-1 summarizes water quality measured during the test. All measurements remained within target parameters throughout the test period.

For the test treatments, the study design called for DO and pH to remain within targeted ranges considered ideal for Pacific herring, while temperature and salinity intentionally fluctuated outside of optimal ranges to determine effects. DO and pH were measured in test aquaria daily and in carboy holding tanks before water exchanges occurred. Table 5-2 summarizes DO and pH values measured in the test aquaria and in the carboys prior to use. DO ranged from 5.5 to 9.6 mg/L in carboys and from 5.7 to 9.2 mg/L in test aquaria. These values met the criteria of > 4.8 mg/L. pH ranged from 7.1 to 8.3 in carboys and 7.0 to 8.1 in test aquaria, which were within the acceptable range of 6.0 to 9.0.

Table 5-1. Summary of Water Quality Conditions in Renewal Carboys and Test Chambers for Control and CUV Samples.

Treatment	Dissolved Oxygen (mg/L)		pH		Salinity (ppt)		Temperature (ºC)	
	Min	Max	Min	Max	Min	Max	Min	Max
Measured in Carboy (prior to use)								
Control	5.6	9.6	7.3	8.2	15.0	16.5	11.7	13.8
CUV	5.5	9.6	7.1	8.3	15.9	16.5	11.8	13.4
Measured Daily in Test Aquaria								
Control	5.9	8.8	7.5	8.0	15.0	16.3	12.1	13.4
CUV	6.0	9.3	7.4	8.0	15.7	16.5	12.3	13.3
Measured by Hobo in Water Bath								
Control, CUV	NA	NA	NA	NA	NA	NA	11.8	13.3

Table 5-2. Dissolved Oxygen and pH Measured in Test Treatment Renewal Carboys and Aquaria.

Treatment	Dissolved Oxygen (mg/L)		pH	
	Min	Max	Min	Max
Measured in Carboy (prior to use)				
Control	5.6	9.6	7.3	8.2
CUV	5.5	9.6	7.1	8.3
A-16, A-28	6.1	9.3	7.3	8.2
A-TS	6.3	9.0	7.1	8.2
B-22	6.5	9.6	7.3	8.1
Measured Daily in Test Aquaria				
Control	5.9	8.8	7.5	8.0
CUV	6.0	9.3	7.4	8.0
A-16, A-28	5.8	7.8	7.5	8.1
A-TS	5.7	8.0	7.0	8.0
B-22	5.8	9.2	7.5	8.0

Salinity and temperature were measured by continuously recording meters and verified with water quality probes in test aquaria daily and in carboys prior to use. Salinity and temperature were intentionally fluctuated in Test A, while only salinity was varied in Test B. These fluctuations occurred from 0 - 8 dpf when all treatments were transferred to control conditions (16 ppt, 12°C). Table 5-3 illustrates targeted and actual salinity and temperature ranges for the test.

Figure 5-1, Figure 5-2, and Figure 5-3 show the detailed salinity and temperature ranges by treatment during the test. As demonstrated in these figures, the observed temperatures and salinities matched the test design very closely.

Table 5-3. Comparison of Planned versus Measured Salinity and Temperature in Test Treatments.

Salinity and Temperature Results				
Treatment	Target		Actual	
	Salinity (ppt)	Temperature (ºC)	Salinity (ppt)	Temperature (°C)
A-16	16 – 30	11.0 – 21.1	16.0 – 30.0	10.3 – 22.0
A-28				
A-TS		11.0 – 24.1	16.0 – 29.2	10.3 – 24.1
B-22	14 – 26	11.0	13.4 – 28.4	10.7 – 12.0

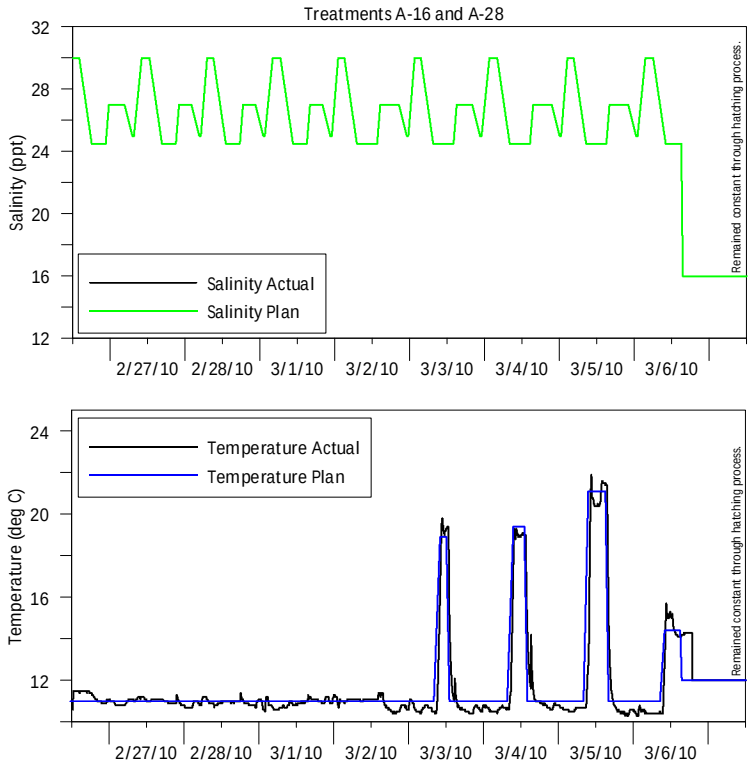


Figure 5-1. Planned versus Actual Salinity and Temperature for Treatments A-16 and A-28.

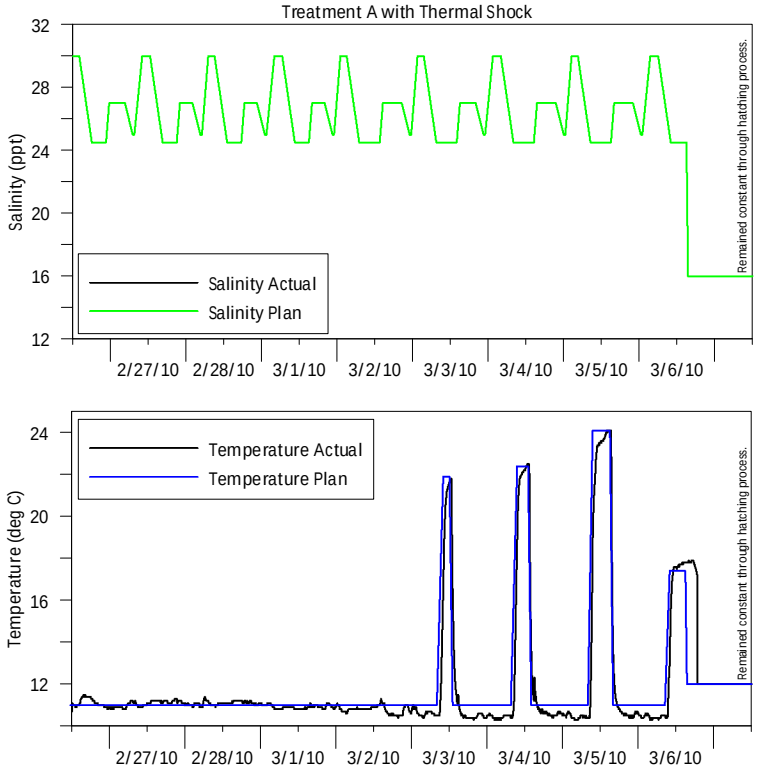


Figure 5-2. Planned versus Actual Salinity and Temperature for Treatment A-TS.

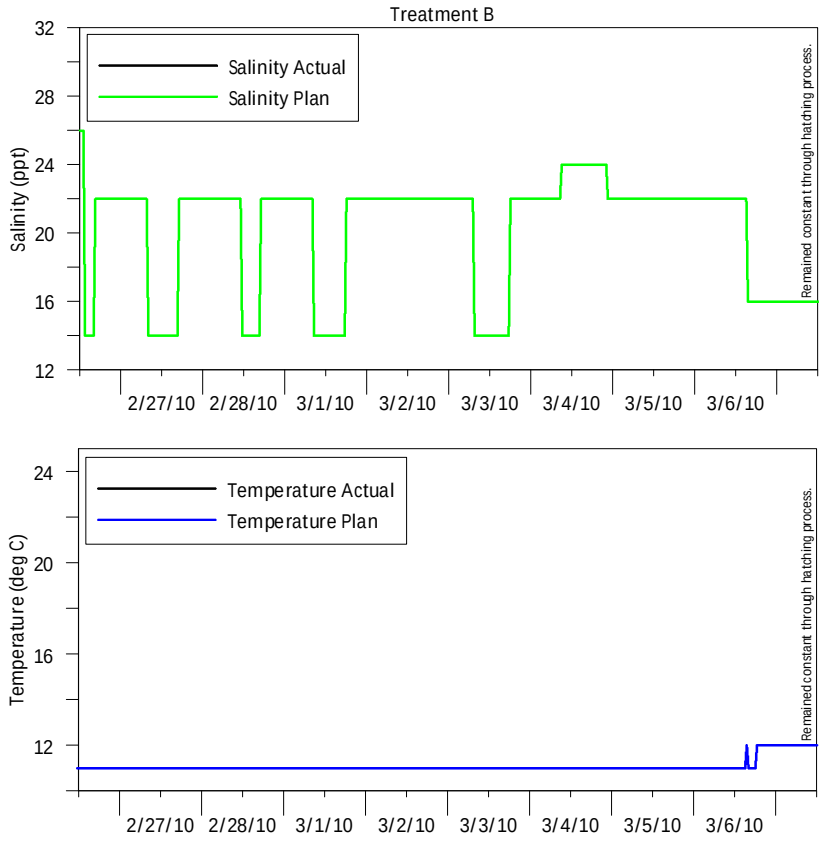


Figure 5-3. Planned versus Actual Salinity and Temperature for Treatment B.

All treatments were exposed to UV lighting with the exception of the Control; fluorescent lighting was used for the Control. Light levels were measured at the top of the test aquaria on Days 1 – 3 and on the side of the aquaria approximately at the same level as the top of the slides on Day 4. Both measurements were taken on the subsequent days. The results are summarized in Table 5-4. As expected, irradiance was very low in the Control, and the levels in the CUV treatment corresponded to those measured in the test treatments.

Table 5-4. Summary of UV Light Conditions by Treatment.

Irradiance (µmol / m^2s^1)										
	Control		CUV		A-16 and A-28		A-TS		B-22	
Location	Min	Max	Min	Max	Min	Max	Min	Max	Min	Max
Aquaria Top	1	2	59	64	47	63	36	51	47	57
Aquaria Side	2	4	39	47	25	44	30	41	36	39

5.1.2 TEST WATER CHEMISTRY

Chemical analysis of test water used in this study was performed by Alpha Analytical and Analytical Resources, Inc (ARI). PAHs were analyzed specifically for this study; metals, pesticides, and PCBs were measured as a part of NewFields annual water testing protocol. Test water

included 0.45 µm filtered seawater from Hood Canal, WA and deionized water. The complete laboratory reports are provided as Appendix E; the results are summarized in this section.

Alpha Analytical analyzed PAHs. Water samples were collected on April 20, 2010 and shipped on ice via UPS overnight. Results are shown in Table 5-5. No PAHs were detected above reporting limits in either water sample; numbers shown are estimates between the minimum detection limit and the reporting limit. Total detectable PAHs were estimated at 5.8 ng/L in the seawater sample and 1.4 ng/L in the deionized water sample. Laboratory QC procedures fell within approved criteria and therefore the results of the chemical analysis were accepted.

ARI analyzed total metals, pesticides, and low-level PCBs. Water samples were collected on April 22, 2010 and shipped on ice via UPS overnight to ARI. Results are summarized in Table 5-6. Arsenic, copper, and nickel were found at concentrations just above reporting levels; calcium, magnesium, potassium, and sodium were present at levels typical of seawater. No pesticides or PCBs were detected in either the filtered seawater or deionized water samples. Laboratory QC tests met the required criteria, thereby validating the chemical analysis.

Table 5-5. PAH Concentrations in Laboratory Test Water.

Analytes	Filtered Seawater			Deionized Water		
	Result (ng/L)	Qualifier Code*	Reporting Limit (ng/L)	Result (ng/L)	Qualifier Code*	Reporting Limit (ng/L)
cis/trans-Decalin		U	10		U	10
C1-Decalins		U	10		U	10
C2-Decalins		U	10		U	10
C3-Decalins		U	10		U	10
C4-Decalins		U	10		U	10
Benzothiophene		U	10		U	10
C1-Benzo(b)thiophenes		U	10		U	10
C2-Benzo(b)thiophenes		U	10		U	10
C3-Benzo(b)thiophenes		U	10		U	10
C4-Benzo(b)thiophenes		U	10		U	10
Naphthalene	1.42	J	10		U	10
C1-Naphthalenes	0.778	J	10	0.701	J	10
C2-Naphthalenes		U	10		U	10
C3-Naphthalenes		U	10		U	10
C4-Naphthalenes		U	10		U	10
Biphenyl		U	10		U	10
Dibenzofuran		U	10		U	10
Acenaphthylene		U	10		U	10
Acenaphthene		U	10		U	10
Fluorene	0.965	J	10		U	10
C1-Fluorenes		U	10		U	10
C2-Fluorenes		U	10		U	10
C3-Fluorenes		U	10		U	10
Anthracene		U	10		U	10
Phenanthrene	2.01	J	10	0.744	J	10
C1-Phenanthrenes/Anthracenes		U	10		U	10
C2-Phenanthrenes/Anthracenes		U	10		U	10
C3-Phenanthrenes/Anthracenes		U	10		U	10
C4-Phenanthrenes/Anthracenes		U	10		U	10

Table 5 5. PAH Concentrations in Laboratory Test Water (Continued)

Analytes	Filtered Seawater			Deionized Water		
	Result (ng/L)	Qualifier Code*	Reporting Limit (ng/L)	Result (ng/L)	Qualifier Code*	Reporting Limit (ng/L)
Retene		U	10		U	10
Dibenzothiophene		U	10		U	10
C1-Dibenzothiophenes		U	10		U	10
C2-Dibenzothiophenes		U	10		U	10
C3-Dibenzothiophenes		U	10		U	10
C4-Dibenzothiophenes		U	10		U	10
Benzo(b)fluorene		U	10		U	10
Fluoranthene		U	10		U	10
Pyrene	0.652	J	10		U	10
C1-Fluoranthenes/Pyrenes		U	10		U	10
C2-Fluoranthenes/Pyrenes		U	10		U	10
C3-Fluoranthenes/Pyrenes		U	10		U	10
C4-Fluoranthenes/Pyrenes		U	10		U	10
Naphthobenzothiophenes		U	10		U	10
C1-Naphthobenzothiophenes		U	10		U	10
C2-Naphthobenzothiophenes		U	10		U	10
C3-Naphthobenzothiophenes		U	10		U	10
C4-Naphthobenzothiophenes		U	10		U	10
Benz[a]anthracene		U	10		U	10
Chrysene/Triphenylene		U	10		U	10
C1-Chrysenes		U	10		U	10
C2-Chrysenes		U	10		U	10
C3-Chrysenes		U	10		U	10
C4-Chrysenes		U	10		U	10
Benzo[b]fluoranthene		U	10		U	10
Benzo[k]fluoranthene		U	10		U	10
Benzo[a]fluoranthene		U	10		U	10
Benzo[e]pyrene		U	10		U	10
Benzo[a]pyrene		U	10		U	10
Perylene		U	10		U	10
Indeno[1,2,3-cd]pyrene		U	10		U	10
Dibenz[a,h]anthracene		U	10		U	10
Benzo[g,h,i]perylene		U	10		U	10
4-Methyldibenzothiophene		U	10		U	10
2/3-Methyldibenzothiophene		U	10		U	10
1-Methyldibenzothiophene		U	10		U	10
3-Methylphenanthrene		U	10		U	10
2/4-Methylphenanthrene		U	10		U	10
2-Methylanthracene		U	10		U	10
9-Methylphenanthrene		U	10		U	10
1-Methylphenanthrene		U	10		U	10
Total Detectable PAHs	5.825	J		1.445	J	

* U = undetected; J = estimated value, below reporting limit

Table 5-6. Metal, Pesticide, and PCB Concentrations in Test Water.

Analytes	Filtered Seawater			Deionized Water		
	Result	Qualifier Code*	Reporting Limit	Result	Qualifier Code*	Reporting Limit
Total Metals (ug/L)						
Aluminum		U	250		U	50
Arsenic	4		2		U	0.2
Calcium	329,000		250		U	50
Chromium		U	5		U	2
Cobalt		U	2		U	1
Copper	8		5		U	0.5
Iron		U	250		U	50
Lead		U	10		U	1
Magnesium	1,040,000		250		U	50
Nickel	13		5		U	0.5
Potassium	329,000		2,500		U	500
Selenium		U	5		U	0.5
Sodium	8,730,000		2,500		U	500
Zinc		U	40		U	4
Pesticides (ug/L)						
alpha-BHC		U	0.0050		U	0.0050
gamma-BHC (Lindane)		U	0.0050		U	0.0050
Heptachlor		U	0.0050		U	0.0050
Aldrin		U	0.0050		U	0.0050
Dieldrin		U	0.010		U	0.010
4,4' - DDT		U	0.010		U	0.010
gamma Chlordane		U	0.0050		U	0.0050
alpha Chlordane		U	0.0050		U	0.0050
Hexachlorobenzene		U	0.0050		U	0.0050
Hexachlorobutadiene		U	0.0050		U	0.0050
PCBs (ug/L)						
Aroclor 1016		U	0.010		U	0.010
Aroclor 1242		U	0.010		U	0.010
Aroclor 1248		U	0.010		U	0.010
Aroclor 1254		U	0.010		U	0.010
Aroclor 1260		U	0.010		U	0.010
Aroclor 1221		U	0.010		U	0.010
Aroclor 1232		U	0.010		U	0.010
Aroclor 1262		U	0.010		U	0.010
Aroclor 1268		U	0.010		U	0.010

* U = undetected; J = estimated value, below reporting limit

5.1.3 Chemical Analysis of Adult Donor Tissue

Portions of the gonads used for fertilization of the eggs used in the 2010 laboratory study were sent to Alpha Analytical for PAH analysis (Figure 5-4, laboratory report is included as Appendix F). PAH analytes were detected in the gonads, although most concentrations above detection limits were also below reporting limits (qualifier code J). Relative proportions of the PAHs showed a

signature similar to that of eggs exposed to background urban gravel from the 2009 NOAA/BML laboratory study, shown as a filled area in Figure 5-4.

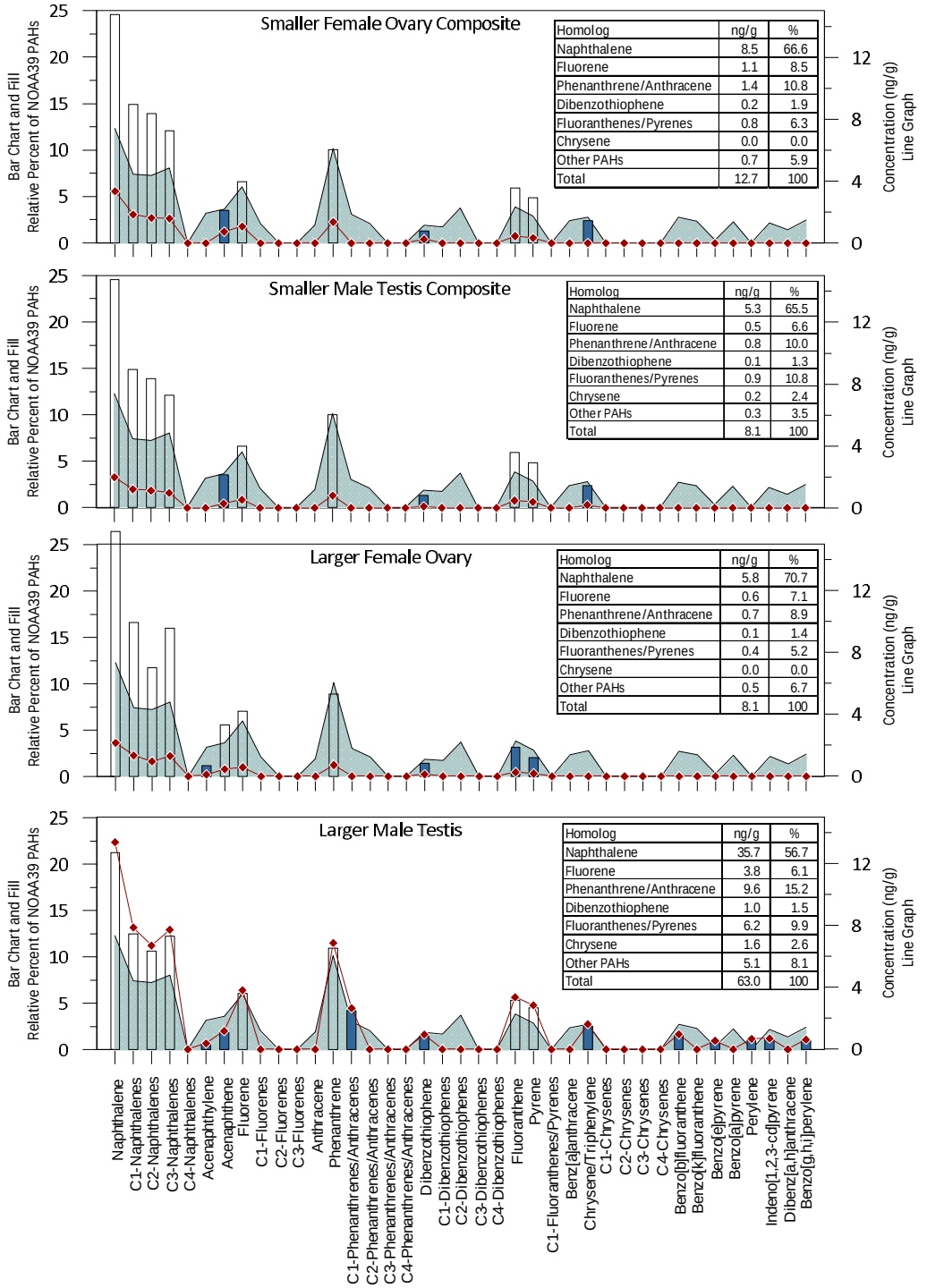


Figure 5-4. Relative (Bars) and Actual (Line) Concentrations of PAHs in Donor Fish Gonads Compared to Urban Background Signature (Filled Area).

5.2 EMBRYO ENDPOINTS

5.2.1 FERTILIZATION RATES

Fertilization rates were estimated by treatment at 48 hours post fertilization; the results are shown in Table 5-7. The fertilization rates ranged from 80.5% to 97.8%. Dinnel (2010 in prep) suggests fertilization rates of ≥90% are preferable but found moderate fertilization rates from 50% to 80% can lead to acceptable hatching rates of ≥70%. Hatching success for this study is described in Section 5.3. The percentage fertilization was calculated by subtracting the unfertilized and unhardened eggs from the total eggs counted.

Four of the six treatments from each donor spawning group had fertilization rates between 80% and 90%; the remaining treatments had fertilization above 90%. Although the larger spawning group had somewhat higher fertilization rates, the occurrence of early death of embryos was more frequent for these treatments as described below.

Table 5-7. Fertilization Rates at 48 Hours.

Donor Size	Treatment	Unfertilized or Unhardened (%Total Eggs)	Fertilized (%Total Eggs)	Dead Embryos at 48 hours (% Fertilized Eggs)	Viable Fertilized Embryos (% Fertilized Eggs)
Smaller	Control	18.6	81.4	29.7	70.3
	CUV	14.1	85.9	30.1	69.9
	A-16	2.9	97.1	56.6	43.4
	A-28	19.5	80.5	27.2	72.8
	A-TS	2.7	97.3	30.6	69.4
	B-22	14.5	85.5	19.7	80.3
Larger	Control	11.3	88.7	73.9	26.1
	CUV	13.7	86.3	75.0	25.0
	A-16	10.9	89.1	46.8	53.2
	A-28	2.2	97.8	51.9	48.1
	A-TS	7.1	92.9	53.8	46.2
	B-22	10.5	89.5	47.7	52.3

5.2.1 EARLY DEATH DURING EMBRYOGENESIS AND VIABLE EMBRYOS

Early death of embryos was classified based on opacity of the embryo or dense white tissue mass within the chorion often associated with arrested development during the blastula stage (Table 5-7). Only one treatment from the smaller spawning group (A-16) had greater than 50% dead embryos at 48 hours; the remaining treatments showed 30% or fewer dead embryos. In contrast, for the larger spawning group all of the treatment groups and the control ranged from 47 to 75% dead embryos. This high incidence of early death, particularly with the eggs from the larger donor fish, suggests that either the quality of the gametes used for testing was compromised or other environmental stressors were involved.

The percentage of viable eggs (Table 5-7) was calculated by dividing the viable eggs by the number of fertilized eggs. The larger spawning group had a smaller percentage of viable eggs than the smaller spawning group with a range of viable eyed embryos from 25 to 53%. In comparison, the smaller spawning group, with one exception (A-16, 43%), had a range of viable eyed embryos from 69 to 80%.

5.2.2 EMBRYO HEART RATE

One indication of embryo health and probable development to hatch is heart rate. Heart rate was measured for Control embryos following protocol developed by Dinnel (2010 in prep). Heart rate was obtained by examining the embryo under a dissecting microscope and counting the number of heartbeats in 30 seconds. Initial measurement of heart rates was assessed on replicate 4 Control samples (non-UV treatment) for the smaller and larger size groups. Six embryos were assessed on each slide. Table 5-8 provides a summary of the results. The baseline heart rates established by Dr. Dinnel for herring embryos from other studies ranged from 72.4 to 82.2 beats per minute for control embryos (Dinnel, pers comm.). In all but one instance, the heart rates of the experimental control embryos fell within this range. In the one exception and for one other embryo, the embryos were observed to exhibit abnormal characteristics.

Table 5-8. Heart Rate Data Collected from Control Embryos.

Treatment	Slide Quadrant	Specimen	Heart Rate Beats per Minute	Comments
Control Smaller Donor Fish	6	1	88	
	2	2	94	
	3	3	94	
	7	4	76	Observer noted abnormal embryo
	1	5	90	
	8	6	70	Observer noted abnormal embryo
Control Larger Donor Fish	3	1	84	
	2	2	86	
	8	3	88	
	1	4	82	
	6	5	90	
	6	6	80	

5.3 LARVAL ENDPOINT: HATCHING RATES

Over 2800 larvae hatched during the period from 9 to 14 dpf. Larvae observed swimming were tallied in addition to the total number hatched. Hatch rates were determined by the number of fertilized eggs remaining on the slide at 8 dpf. As shown in Figure 5-5, hatch rates were higher overall for the larvae from the smaller donor size group. The A-16 and A-28 treatments showed the most variability for both size groups. Lower hatching rates were observed for all of the samples from the larger donor size group with the lowest hatching rate in the Control sample. This may indicate that the donor eggs from this group were less healthy or ripe at the time of fertilization. It is interesting to note that even with the reduced hatch in the larger spawning class, most of the larvae that did hatch from this spawning class were classified as swimming larvae (Figure 5-5). In addition, in both Control samples the viable embryos at 2 dpf (Table 5-7; Smaller 70%, Larger 26%) are essentially the same as the swimming hatch (Smaller 70%, Larger 25%), indicating that those embryos that survived the first two days of embryo development proceeded to hatch with apparent normality.

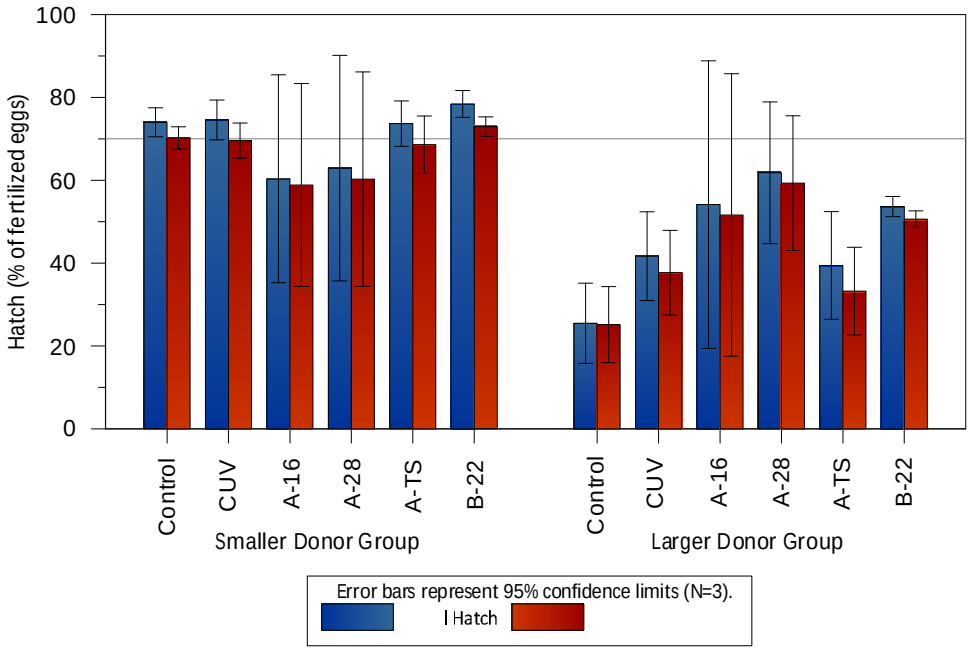


Figure 5-5. Hatch Rates by Treatment for Total and Swimming Larvae.

Larvae from the different treatments exhibited variations in hatching rates as a function of days post fertilization as shown in Figure 5-6. In the Control samples, both from the smaller and larger donor size groups, about 90% of the larvae hatched at 9-10 dpf. In contrast, larvae in the B-22 treatment hatched in similar numbers on 10 and 12 dpf. Larvae in the A-16 treatment from smaller donor fish showed a pattern similar to the B-22 larvae while all other treatments had a majority of the hatch on Day 10 with the remainder of the hatch spread over 9, 11, and 12 dpf. Only a few fish hatched after 12 dpf.

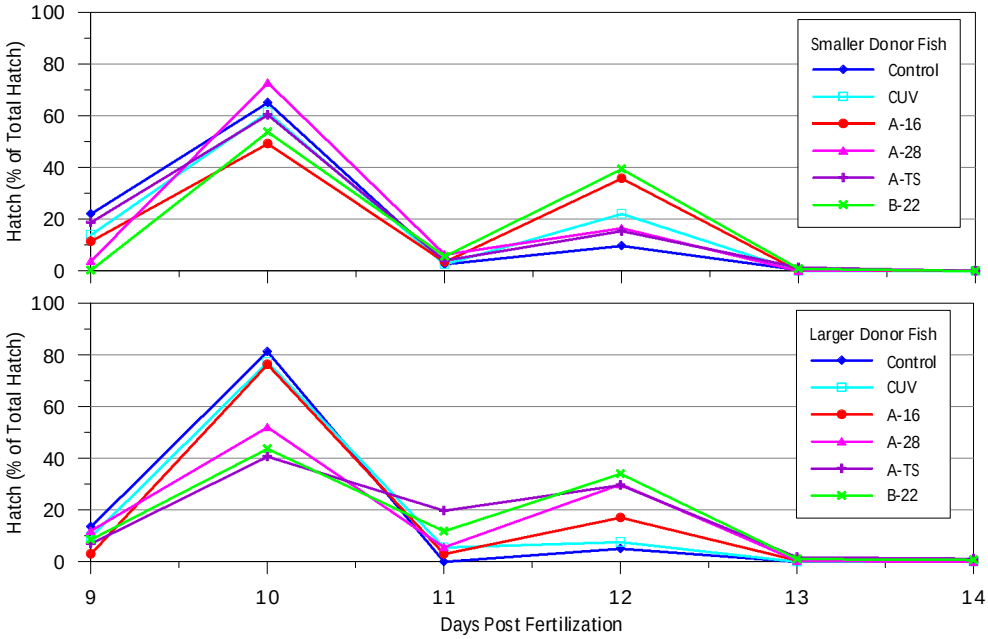


Figure 5-6. Percent of Larvae Hatched Per Day.

6 IMAGE ANALYSIS RESULTS

6.1 DATA PROCESSING

Data from measurements and scoring of images were entered directly into Excel spreadsheets. Separate sets of spreadsheets were maintained for measurements and scores; data for each day of hatch resided in individual spreadsheets. Each fish assessed was uniquely identified by hatch date, laboratory sample number, and image number. Measurement and scoring data were merged together and combined with the key to treatment and information on magnification for each image. All length and area measurements were maintained as pixels and also converted to millimeters for comparisons of larvae imaged at differing magnifications. The number of fish imaged for each sample was recorded. Due to the large numbers of hatch on 3/8/2010 (10 dpf) and 3/10/2010 (12 dpf), not all larvae were imaged. All fish that indicated gross abnormality at hatch (e.g., not swimming or coiled) were imaged; a subset of those that were swimming were imaged. The subset was assumed to be representative of all swimming larvae hatched on the day of observation. For all statistical evaluations, the measurements and scores for the imaged larvae were weighted for the proportion they represented of the total hatch per sample on the day of hatch.

6.2 DEVELOPMENTAL IMPACT ON LARVAL ATTRIBUTES

Different degrees of development at the time of larval imaging were noted in addition to obviously abnormal development; many larvae were observed to be in a later stage of yolk sac absorption. Examples of both of these are shown in Figure 6-1. While the overall percentages of larvae in the abnormal and late stage categories were relatively small (6 and 7% respectively), they were not evenly dispersed across the treatments (Table 6-1). The differences in standard length and size of yolk for abnormal, late stage, and all other larvae are summarized in Figure 6-2 where it can be seen that significant differences in size were observed. Because of these differences, and to increase the ability to detect differences between treatments smaller than the differences between the developmental categories, analyses were run on the full dataset and also with these two categories excluded.

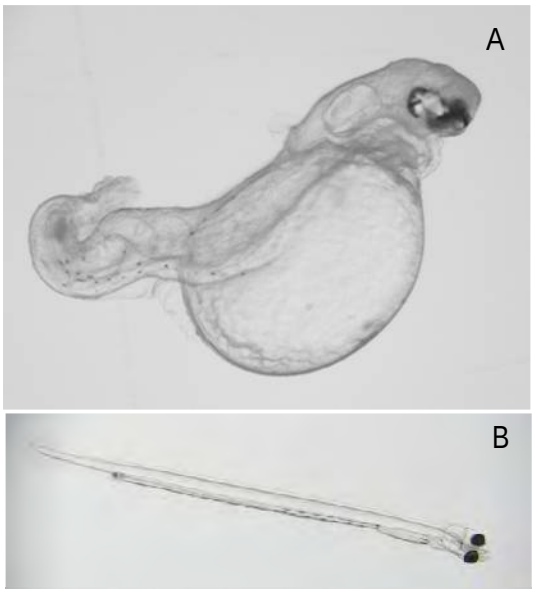
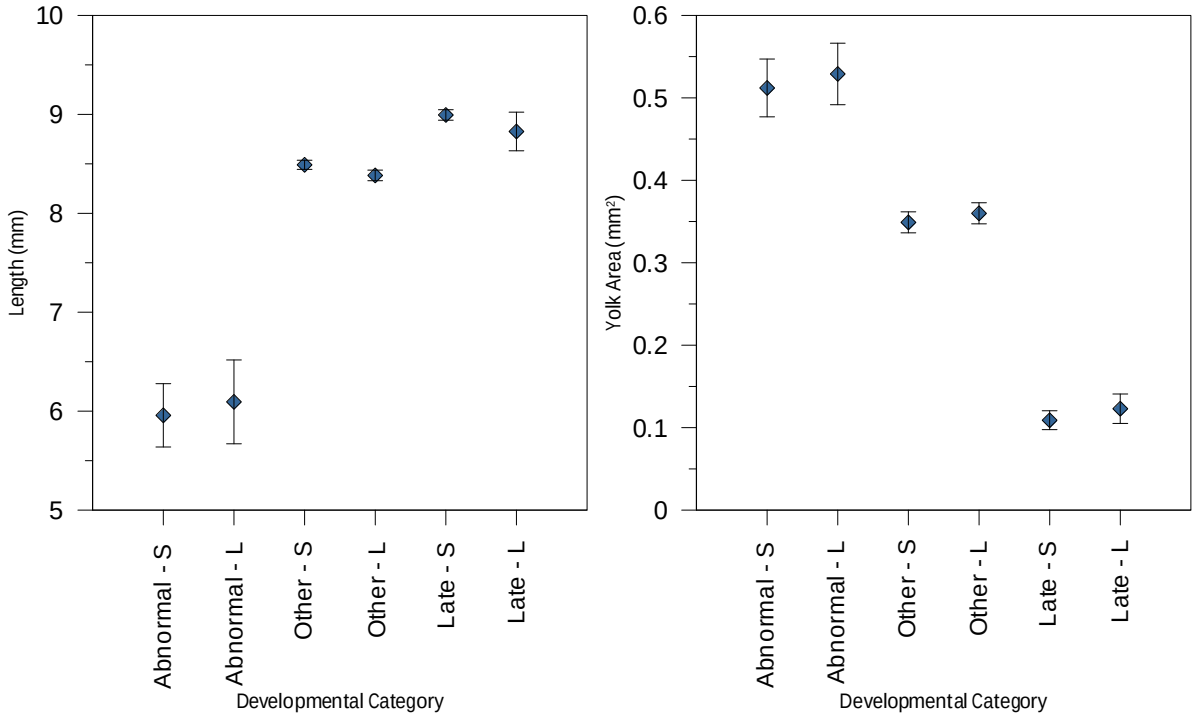


Figure 6-1. Examples of Abnormal Development (A) and Late Stage Yolk Sac Absorption (B).

Table 6-1. Number and Percent of Larvae Observed by

Developmental Category.

Donor Size	Label	Total Hatch (sum of 3 reps)	Abnormal (Body Axis Defect Score = 2)		Late Stage Yolk Sac Absorption		Other (Not Categorized)	
			Larvae	Percent of Hatch	Larvae	Percent of Hatch	Larvae	Percent of Hatch
Smaller	Control	269	16	5.9	14	5.3	242	89.9
	CUV	307	18	5.9	20	6.4	269	87.7
	A-16	201	5	2.5	39	19.6	157	77.9
	A-28	284	13	4.6	11	3.7	260	91.7
	A-TS	298	22	7.4	17	5.7	266	89.1
	B-22	355	22	6.2	82	23.0	250	70.3
Larger	Control	59	1	1.7	1	1.7	57	96.6
	CUV	145	15	10.4	3	1.7	129	89.2
	A-16	198	10	5.2	5	2.7	182	92.1
	A-28	268	9	3.4	20	7.4	239	89.3
	A-TS	172	16	9.0	9	5.2	149	86.4
	B-22	282	16	5.7	17	6.1	249	88.2



Error bars represent 95% confidence limits.

Figure 6-2. Mean Measurements by Developmental Category (S = Smaller Donor Size, L = Larger Donor Size).

6.3 TREATMENT RELATED EFFECTS

6.3.1 STATISTICAL ANALYSES

Analysis of Variance (ANOVA) was used to compare the control samples to the various treatments. Data for larvae from each donor size group (larger and smaller) were analyzed separately. The comparisons were made on the scored assessments and on the measurements collected on each larval fish. In each case, the mean of the measurement or score was calculated for each laboratory replicate; the replicate means were used in the ANOVA (n=3 for each treatment). Statistical testing was performed with SAS/STAT® software (SAS 2008). ANOVA was performed with the General Linear Models procedure (PROC GLM) with a Dunnett's test to compare treatment means to the Control mean (Dunnett 1955). Note that an ANOVA that produces significant differences amongst treatments only implies there are differences between at least two of the treatments; the comparison test determines which treatments differ from the Control. The ANOVAs were run twice; first using data for all larvae, and secondly excluding the extreme abnormal fish (BAD Score = 2) and those observed in late stage yolk sac absorption. The second analysis was performed to determine whether variability introduced with the small number of noticeably different fish was masking subtler differences between treatments. Complete statistical results are provided in Appendix G.

6.3.2 ANOVA RESULTS ON SCORES

Scores given to the observed abnormalities in each imaged larva were averaged for each sample, adjusting for the number of larvae photographed out of the total hatched per day. The mean scores for the three replicate samples in each treatment were used in the ANOVA. For the scores, a one-tailed Dunnett's test was used to determine any treatments with significantly higher scores than in the Control; data from each donor size group were kept separate. Table 6-2 shows the results based on all larvae. For both donor size groups, only yolk sac edema demonstrated significantly higher scores in treatments (A-16, A-28, A-TS for smaller donors; A-TS for larger donors).

Similar results were obtained when the obviously abnormal larvae and those in late stage yolk sac absorption were excluded as shown in Table 6-3. The only change was the ability to discern a difference between Control and the B-22 treatment for pericardial edema in larvae from the small donor size group. This result, however, was based on only two larvae with pericardial edema; the test on the larger larvae was based on three fish.

Table 6-2. ANOVA Results on Scores for All Larvae.

Donor Size	Measure	Prob>F	Min. Sig. Difference	Control	CUV	A-16	A-28	A-TS	B-22
Smaller	Melanophore Dev. Score	0.284	0.18	1.83	1.75	1.88	1.84	1.72	1.81
	Body Axis Defect Score	0.456	0.12	0.16	0.19	0.16	0.13	0.18	0.23
	Opacity Score	0.554	0.23	0.21	0.25	0.19	0.29	0.25	0.35
	Trunk Thickness Score	0.520	0.05	0.08	0.09	0.06	0.05	0.09	0.08
	Tissue Disruption Score	0.704	0.12	0.13	0.14	0.14	0.07	0.15	0.14
	Pericardial Edema Score	0.451	0.04	0.03	0.01	0.01	0.00	0.01	0.02
	Yolk Sac Edema Score	<0.001	0.26	0.23	0.28	0.81	0.69	0.85	0.31
	Yolk Sac Anomaly Score	0.892	0.35	0.57	0.54	0.64	0.52	0.57	0.47
Larger	Melanophore Dev. Score	0.844	0.26	1.79	1.79	1.75	1.88	1.76	1.80
	Body Axis Defect Score	0.270	0.27	0.05	0.26	0.28	0.13	0.26	0.16
	Opacity Score	0.417	0.26	0.12	0.14	0.30	0.29	0.27	0.21
	Trunk Thickness Score	0.137	0.11	0.02	0.09	0.14	0.07	0.12	0.05
	Tissue Disruption Score	0.432	0.23	0.02	0.21	0.18	0.11	0.12	0.13
	Pericardial Edema Score	0.542	0.03	0.00	0.00	0.02	0.00	0.01	0.02
	Yolk Sac Edema Score	0.008	0.42	0.54	0.40	0.69	0.65	1.20	0.55
	Yolk Sac Anomaly Score	0.296	0.34	0.59	0.80	0.64	0.52	0.51	0.52

Shading shows significant ANOVA results, purple shaded cells indicate significantly larger mean scores based on Dunnett's one-tailed test.

Table 6-3. ANOVA Results on Scores Excluding Larvae with Gross Abnormalities and Late Stage Yolk Absorption.

Donor Size	Measure	Prob>F	Min. Sig. Difference	Control	CUV	A-16	A-28	A-TS	B-22
Smaller	Melanophore Dev. Score	0.373	0.17	1.91	1.83	1.87	1.94	1.80	1.88
	Body Axis Defect Score	0.174	0.09	0.05	0.07	0.13	0.03	0.05	0.10
	Opacity Score	0.930	0.24	0.18	0.17	0.18	0.23	0.16	0.24
	Trunk Thickness Score	0.770	0.04	0.01	0.02	0.03	0.01	0.01	0.02
	Tissue Disruption Score	0.393	0.14	0.05	0.05	0.14	0.02	0.05	0.08
	Pericardial Edema Score	0.034	0.01	0.00	0.00	0.00	0.00	0.00	0.01
	Yolk Sac Edema Score	0.000	0.30	0.16	0.26	0.93	0.72	0.89	0.36
	Yolk Sac Anomaly Score	0.787	0.31	0.51	0.53	0.67	0.50	0.53	0.53
Larger	Melanophore Dev. Score	0.687	0.24	1.81	1.93	1.82	1.93	1.86	1.89
	Body Axis Defect Score	0.574	0.17	0.03	0.06	0.15	0.07	0.11	0.04
	Opacity Score	0.525	0.25	0.12	0.08	0.24	0.24	0.20	0.13
	Trunk Thickness Score	0.920	0.05	0.02	0.01	0.02	0.03	0.03	0.01
	Tissue Disruption Score	0.687	0.12	0.02	0.09	0.04	0.07	0.05	0.08
	Pericardial Edema Score	0.523	0.03	0.00	0.00	0.02	0.00	0.00	0.01
	Yolk Sac Edema Score	0.009	0.49	0.57	0.37	0.76	0.67	1.29	0.56
	Yolk Sac Anomaly Score	0.285	0.32	0.57	0.76	0.58	0.50	0.44	0.53

Shading shows significant ANOVA results, purple shaded cells indicate significantly larger mean scores based on Dunnett's one-tailed test.

6.3.3 ANOVA Results on Larval Measurements

ANOVA was used to determine differences in measurements made on variously treated larvae from those in the Control samples. Comparisons to the Control were made with a Dunnett's two-tailed test. Results of the analysis on all larvae are shown in Table 6-4. Differences were noted in the measurement from the eye to the posterior of the yolk in both donor size groups in the A treatments. In the larger donor size group the mean lengths from the notochord and the dorsal edge to the pericardium were longer in the A-TS treatment than in the Control. While not significantly different from the Control, 7 of the remaining 11 measures showed the largest

difference from Control in the A-TS treatment; indicating that this treatment may have induced more anomalies on larvae than the other treatments.

Table 6-4. ANOVA Results on Measurements for All Larvae.

Donor Size	Measure	Prob>F	Min. Sig. Difference	Control	CUV	A-16	A-28	A-TS	B-22
Smaller	Head to trunk angle	0.890	27	160	158	149	154	153	158
	Standard length (mm)	0.339	0.41	8.46	8.42	8.34	8.51	8.21	8.49
	Yolk area (sq mm)	0.602	0.16	0.35	0.37	0.37	0.29	0.36	0.30
	Yolk sac area (sq mm)	0.633	0.16	0.40	0.42	0.44	0.37	0.42	0.36
	Yolk:Yolk sac ratio	0.345	0.11	0.86	0.85	0.80	0.78	0.83	0.80
	Pericardial area (sq mm)	0.171	0.03	0.07	0.07	0.07	0.08	0.06	0.08
	Notochord to pericardial edge (mm)	0.554	0.06	0.25	0.26	0.26	0.25	0.28	0.23
	Dorsal edge to pericardial edge (mm)	0.565	0.09	0.49	0.50	0.49	0.47	0.50	0.46
	Notochord:Dorsal measure ratio	0.540	0.06	0.52	0.52	0.53	0.53	0.54	0.51
	Eye to anterior of yolk (mm)	0.537	0.20	0.95	0.94	0.86	0.92	0.85	0.95
	Eye to posterior of yolk (mm)	0.013	0.17	1.85	1.83	1.68	1.66	1.68	1.84
	Yolk length (mm)	0.100	0.14	0.95	0.94	0.86	0.81	0.86	0.86
	Yolk width (mm)	0.650	0.17	0.46	0.49	0.51	0.44	0.50	0.43
	Yolk width:Yolk length ratio	0.191	0.14	0.48	0.52	0.58	0.54	0.59	0.49
Larger	Head to trunk angle	0.820	15	154	158	152	159	159	159
	Standard length (mm)	0.494	0.46	8.43	8.47	8.34	8.29	8.19	8.24
	Yolk area (sq mm)	0.816	0.14	0.33	0.32	0.35	0.35	0.35	0.39
	Yolk sac area (sq mm)	0.532	0.12	0.39	0.38	0.42	0.42	0.44	0.45
	Yolk:Yolk sac ratio	0.532	0.11	0.84	0.85	0.83	0.83	0.78	0.85
	Pericardial area (sq mm)	0.723	0.04	0.05	0.07	0.07	0.07	0.06	0.07
	Notochord to pericardial edge (mm)	0.020	0.06	0.21	0.22	0.26	0.26	0.29	0.27
	Dorsal edge to pericardial edge (mm)	0.044	0.10	0.42	0.44	0.49	0.49	0.54	0.50
	Notochord:Dorsal measure ratio	0.027	0.04	0.50	0.49	0.53	0.53	0.54	0.53
	Eye to anterior of yolk (mm)	0.006	0.16	0.95	1.04	0.84	0.90	0.80	0.88
	Eye to posterior of yolk (mm)	0.001	0.13	1.87	1.88	1.67	1.77	1.58	1.78
	Yolk length (mm)	0.524	0.14	0.89	0.90	0.86	0.88	0.82	0.91
	Yolk width (mm)	0.714	0.14	0.47	0.45	0.50	0.48	0.51	0.52
	Yolk width:Yolk length ratio	0.043	0.10	0.53	0.50	0.58	0.55	0.62	0.56

Shading shows significant ANOVA results with significant differences from Control, purple shaded cells indicate significantly different mean scores based on Dunnett's two-tailed test.

To determine whether the abnormal larvae may have masked differences between treatments by increasing the variability, the ANOVA was also run excluding the obviously abnormal larvae and those with late stage yolk sac absorption. As can be seen in Table 6-5, more significant differences are observed when these larvae are excluded, particularly for the larvae from the larger donor size group. As seen with all larvae, the treatment that appears most impacted is A-TS.

Table 6-5. ANOVA Results on Measurements Excluding Larvae with Gross Abnormalities and Late Stage Yolk Absorption.

Donor Size	Measure	Prob> F	Min. Sig. Difference	Control	CUV	A-16	A-28	A-TS	B-22
Smaller	Head to trunk angle	0.235	7	160	160	164	156	157	159
	Standard length (mm)	0.123	0.38	8.56	8.56	8.31	8.58	8.29	8.55
	Yolk area (sq mm)	0.455	0.14	0.35	0.38	0.39	0.30	0.36	0.33
	Yolk sac area (sq mm)	0.461	0.14	0.40	0.43	0.47	0.37	0.43	0.39
	Yolk:Yolk sac ratio	0.065	0.06	0.86	0.86	0.82	0.79	0.83	0.84
	Pericardial area (sq mm)	0.217	0.03	0.07	0.07	0.07	0.08	0.06	0.08
	Notochord to pericardial edge (mm)	0.479	0.06	0.25	0.26	0.26	0.25	0.28	0.23
	Dorsal edge to pericardial edge (mm)	0.450	0.08	0.48	0.50	0.49	0.46	0.50	0.46
	Notochord:Dorsal measure ratio	0.528	0.06	0.52	0.52	0.53	0.53	0.54	0.51
	Eye to anterior of yolk (mm)	0.693	0.15	0.94	0.93	0.91	0.90	0.85	0.94
	Eye to posterior of yolk (mm)	0.031	0.17	1.85	1.84	1.73	1.64	1.69	1.84
	Yolk length (mm)	0.010	0.09	0.95	0.95	0.89	0.83	0.87	0.91
	Yolk width (mm)	0.342	0.14	0.46	0.50	0.54	0.44	0.51	0.46
	Yolk width:Yolk length ratio	0.052	0.11	0.48	0.53	0.61	0.54	0.59	0.50
Larger	Head to trunk angle	0.944	12	154	158	159	159	157	159
	Standard length (mm)	0.145	0.43	8.57	8.63	8.44	8.38	8.25	8.28
	Yolk area (sq mm)	0.446	0.11	0.34	0.31	0.35	0.36	0.34	0.40
	Yolk sac area (sq mm)	0.172	0.09	0.40	0.37	0.42	0.43	0.43	0.46
	Yolk:Yolk sac ratio	0.559	0.10	0.84	0.84	0.83	0.83	0.79	0.85
	Pericardial area (sq mm)	0.694	0.04	0.05	0.07	0.07	0.07	0.06	0.07
	Notochord to pericardial edge (mm)	0.010	0.06	0.22	0.22	0.26	0.26	0.30	0.27
	Dorsal edge to pericardial egde (mm)	0.031	0.08	0.44	0.44	0.49	0.48	0.54	0.50
	Notochord:Dorsal measure ratio	0.025	0.05	0.49	0.49	0.53	0.53	0.54	0.53
	Eye to anterior of yolk (mm)	0.004	0.12	0.95	1.04	0.84	0.91	0.79	0.88
	Eye to posterior of yolk (mm)	0.001	0.13	1.87	1.88	1.66	1.79	1.57	1.79
	Yolk length (mm)	0.123	0.12	0.90	0.90	0.86	0.89	0.81	0.93
	Yolk width (mm)	0.326	0.11	0.47	0.43	0.49	0.49	0.51	0.53
	Yolk width:Yolk length ratio	0.005	0.08	0.52	0.48	0.57	0.55	0.63	0.57

Shading shows significant ANOVA results with significant differences from Control, purple shaded cells indicate significantly different mean scores based on Dunnett's two-tailed test.

6.3.4 ANOVA ON FREQUENCY OF OCCURRENCE OF EDEMA

The ANOVAs performed on the scores used the mean score per sample to determine differences between treatments and the Control. The frequency of occurrence of edema was also calculated per sample and tested by ANOVA. For this test, yolk sac edema scores of 2 or 3 were counted as presence of edema; scores of 1 (area to the anterior or posterior of the yolk, but not surrounding the yolk) were counted as a normal stage of development. Scores of 1 and 2 for pericardial edema were considered to be presence of edema. There were no larvae scored with either pericardial or yolk sac edema in the late stage yolk absorption larvae. The results of the ANOVAs with all larvae and with the obviously abnormal larvae (BAD score = 2) and those with late stage yolk sac absorption excluded are shown in Table 6-6. Larvae in the A-TS treatment had significantly higher occurrence of yolk sac edema in all four tests and frequencies of yolk sac

edema decreased when the abnormal fish were excluded. Frequencies of pericardial edema also decreased; of the 22 larvae noted with pericardial edema in the complete dataset only 6 larvae were included from the second ANOVA. The highest occurrences of yolk sac edema were found in the A-16, A-28, and A-TS treatments which experienced changing temperature in addition to changing salinities. Highest occurrence as noted above was found in the A-TS treatment where the incubating embryos were exposed to more extreme temperature changes in addition to three episodes of air exposure.

Table 6-6. ANOVA Results on Frequency of Occurrence of Edema.

Donor Size	Measure	Prob>F	Min. Sig. Difference	Control	CUV	A-16	A-28	A-TS	B-22
All Larvae									
Smaller	Pericardial Edema (%)	0.671	2.4	1.5	0.6	0.6	0.0	0.6	1.3
	Yolk Sac Edema (%)	0.042	10.7	4.6	4.6	8.8	14.0	16.7	3.7
Larger	Pericardial Edema (%)	0.526	2.3	0.0	0.0	1.0	0.2	0.9	1.5
	Yolk Sac Edema (%)	0.006	16.7	0.0	4.0	15.3	14.3	32.9	10.7
Larvae with BAD Score = 2 and Late Stage Yolk Sac Absorption Excluded									
Smaller	Pericardial Edema (%)	0.023	0.4	0.0	0.0	0.0	0.0	0.0	0.5
	Yolk Sac Edema (%)	0.024	10.1	1.9	3.3	8.3	12.8	15.1	2.5
Larger	Pericardial Edema (%)	0.491	1.6	0.0	0.0	1.0	0.2	0.0	0.7
	Yolk Sac Edema (%)	0.011	17.1	0.0	2.8	15.3	13.1	29.9	8.7

Shading shows treatment mean comparisons to Control based on Dunnett's one-tailed test, purple shaded cells indicate significantly larger means.

6.3.5 Overall Responses to Treatments

The analyses discussed previously have looked at results for the two size groups of donor fish separately, with similar results. As noted in Section 5.3, the percentage of swimming hatch of both size groups was essentially the same as the percentage of viable eyed embryos at 48 hours. To determine the overall effects of the exposure treatments, a two-way ANOVA was performed on key responses (severe BAD, yolk sac edema, and pericardial edema) to determine whether the size groups differed in their response to the different treatments. The results are shown in Table 6-7; no response showed a significant interaction between size group and treatment, and no differences were found between size groups. Differences between treatments and the Control were found for BAD and yolk sac edema occurrence but not for occurrence of pericardial edema. BAD occurrence was higher than in the Control sample for all treatments indicating a UV light effect and significantly higher in the CUV and A-TS treatments. Occurrence of yolk sac edema was also higher in all treatments than in the Control but dramatically and significantly higher in the three treatments that experienced increased temperatures (A-16, A-28, A-TS). Additionally, yolk sac edema occurrence in the A-TS (higher temperature and air exposure) was approximately double (25%) that observed in the other two A treatments (A-16 12%; A-28 14%) and also had significantly higher BAD.

Table 6-7. Results of Two-Way ANOVA on Key Responses.

Response (% Occurrence)	Size p > F	Larger	Smaller	Treatment p > F	Control	CUV	A-16	A-28	A-TS	B-22
Severe BAD	0.386	6.17	5.40	0.018	3.46	8.37	4.95	4.13	7.65	6.14
Yolk Sac Edema	0.101	12.87	8.73	<0.001	2.31	4.27	12.1	14.1	24.8	7.23
Pericardial Edema	0.692	0.60	0.76	0.519	0.75	0.31	0.77	0.12	0.75	1.39

6.3.6 A Quantitative Measurement of Yolk Sac Edema

Yolk sac edema has been identified as an abnormality in developing embryos. To date the presence of edema has typically been noted using qualitative assessments, sometimes termed best professional judgment. While a knowledgeable researcher can likely make an accurate assessment of the presence of yolk sac edema, a quantitative procedure for determining the presence of the condition would be preferable and repeatable amongst observers. To this end, multiple measurements of hatched larvae were made to determine whether a quantitative method of determining the presence of this condition could be established. Measurements of yolk area, yolk sac area, yolk width, and yolk length were made as described in Section 4.2. The ratio of the yolk area to the total yolk sac area was hypothesized to be most likely to predict yolk sac edema, followed by the ratio of the yolk width to the yolk length.

To test this hypothesis, the obviously abnormal larvae (BAD Score = 2) and larvae identified as late stage yolk absorption were excluded from the dataset. The mean yolk area to yolk sac area ratio was calculated for each category of yolk sac edema score in each treatment (e.g., mean of all ratios for larvae scored as YS Edema = 1 in Control – Smaller). These mean values were used in a linear regression with the scores to establish a proportion that quantitatively indicates yolk sac edema. The regression showed a statistically significant slope with an R^2 of 0.892 and visually indicates that a ratio of about 0.75 separates those fish with no yolk sac edema or with a score of 1 from those fish with edema scores of 2 and 3 (Figure 6-3). As discussed in Section 4.2.2, scores of 1 were given when a small area of the yolk sac was void of yolk either to the anterior or posterior of the yolk. This condition was considered to be part of the normal process of yolk sac absorption and not indicative of edema within the yolk sac area. Scores of 2 and 3 were

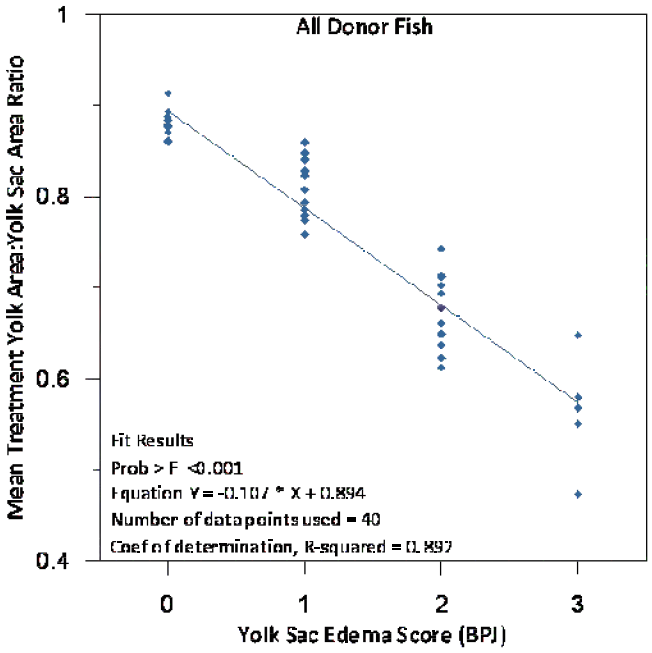


Figure 6-3. Regression of Yolk Area Ratio to Yolk Sac Edema Score.

given when space was observed surrounding the yolk (2 indicating the presence of space and 3 indicating severe edema).

The regression run on the mean treatment ratios gives a clean representation of the relationship between the ratio and the qualitative scores. To determine the strength of this relationship when assessing individual fish, a frequency distribution was calculated based on all fish (N=1217). Fish were categorized in a two-way cross-tabulation grouped by No Edema (Score = 0 or 1) and Edema (Score = 2 or 3) in addition to ratio greater than 0.75 or less than 0.75 (see Table 6-8). For a ratio of 0.75, this resulted in agreement with qualitative scoring (true positive and true negative) of 84.7%. The 15.3% disagreement was weighted towards false positive assessments as seen in Figure 6-4. Determination of the best fit between the ratio and the yolk sac edema score was made by adjusting the ratio downward to find the highest level of agreement with the true results along with the most even distribution of false positives and false negatives. This juncture occurred at a ratio of 0.71 yolk area to yolk sac area where agreement was 86.5% with a false positive rate of 6.8% and a false negative rate of 6.7%.

Table 6-8. Cross-Tabulation of Edema Scores and Ratios.

Ratio	No Edema	Edema
≥ 0.75	True Negative 72.9%	False Negative 4.3%
< 0.75	False Positive 11.0%	True Positive 11.8%
≥ 0.71	True Negative 77.2%	False Negative 6.8%
< 0.71	False Positive 6.7%	True Positive 9.3%

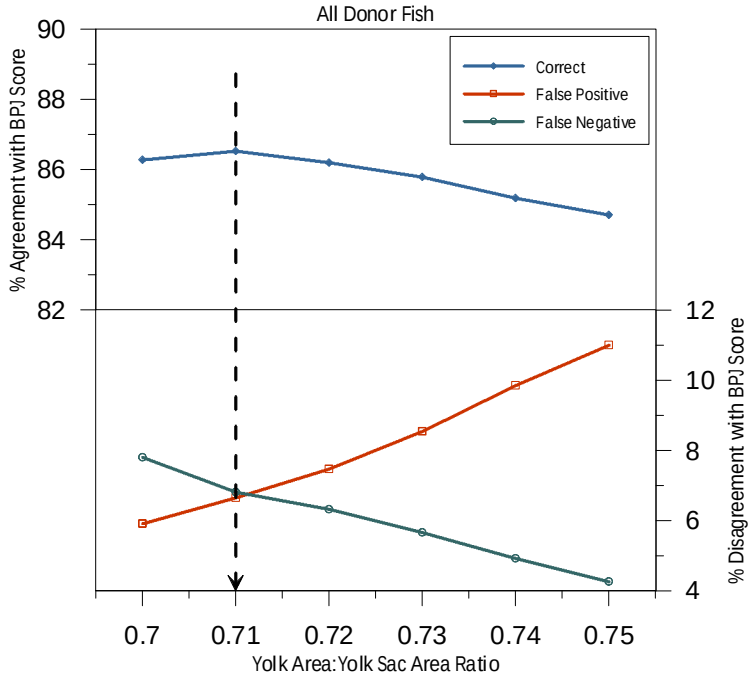


Figure 6-4. Agreement Between Yolk Area Ratio and Yolk Sac Edema Score.

7 DISCUSSION

This study was designed to evaluate the potential impacts of non-petroleum based stressors on the hatching success and development of Pacific herring embryos. It is one of the three primary assessments that have been performed since the COSCO Busan Oil Spill. They consist of 1) a field and laboratory assessment of exposure and effects on Pacific herring conducted in 2008, 2) a laboratory assessment in 2009 that exposed developing herring embryos to known sources of contaminants (3 concentrations of CBO, 3 concentrations of ANS, and an urban and clean control) and 3) the non-petroleum stressor experiment conducted in 2010. This report discusses the conclusions of the laboratory study (NewFields 2010b) focused on non-petroleum based stressors conducted in 2010. A final summation report (NewFields 2010c) includes results from all of these studies and provides an assessment framework for determination of adverse impacts to herring development associated with petroleum based and also non-petroleum based exposure. The non-petroleum based stressors identified as candidates for the 2010 laboratory study included those environmental variables that may have influenced the 2008 development within San Francisco Bay. Those stressors included size and health of donor fish, fluctuating temperatures, differences in salinity during fertilization and during development, exposure to UV light, and the influence of air exposure during intertidal stranding episodes. The experimental design for the 2010 study mimicked the environmental conditions experienced by developing herring embryos during 2008 prior to collection of the eggs by NOAA and BML and during the laboratory incubation period.

There were two basic patterns of variation in salinity and temperature that occurred within the spawning habitat in 2008. The two patterns represented conditions encountered during the natural spawning and development period that occurred at Peninsula Point and Point San Quentin in central San Francisco Bay in 2008. We used the available information on tides and measured temperature and salinity data to develop the 2008 exposure conditions that were then replicated in the laboratory. An additional factor was the influence of intertidal aerial exposure during day time low tides. The air exposure was limited to one hour which is much lower than the organisms experienced in 2008. This compromise did not match the time the embryos would have been exposed because we were unsure how to adjust that exposure to the other potential controllers associated with wind or maintenance of moisture content and temperature control by surrounding plants that would have also been encountered during this intertidal period. We believe that the experimental intertidal exposure under represents the potential stress that may have occurred in 2008.

Quantitative (15) and qualitative (9) measurements were made on the developing herring embryos (1606 individuals). The measurements made on each of the embryos were documented on images captured by electronic media and summarized in spreadsheets. The measurements included assessments of edema (pericardial, yolk sac and coelomic), body axis defects (lordosis, kyphosis, scoliosis, arrested development, thickened trunk, head/trunk angle), percent normal hatch, early developmental mortality, stage of development, craniofacial abnormalities and opacity of various parts of the organism. Once the methods were developed to precisely make each of these measurements a random subset of the samples from 2009 and 2008 (representing each treatment or location) were also assessed using the same procedures.

We also developed a repeatable method of distinguishing and measuring pericardial and yolk sac edema which also matched our qualitative assessments (BPJ). These measurements and assessments of edema were developed on the 2010 data and then applied to the 2009 and 2008 images provided by NOAA/BML. All measurements were conducted on randomized sample designations so that staff performing the assessments and measurements were blind to any experimental treatment information.

Adverse effects were identified in association with each of the potential contributing factors that were examined as summarized in Table 7-1. Natural population variability as evidenced by the number of abnormal larvae in control samples was observed in 2 to 5% of the samples; similar to levels of abnormal development cited for control samples in other studies (Incardona and Vines 2009; Griffin et al. 2009; Vines et al. 2000).

Table 7-1. Biological Response to Stressors and Chemical Signatures.

Chemical Signature	Stressor Type	Biological Response
Without Exposure to Oil (CBO)	Population	Low percentage of body axis defects (~2% major and <5% less obvious); combination of pericardial and yolk sac edema only in major body axis defect individuals
	Gamete Condition	High early mortality (<48h); those that survive have a high percentage of normal hatch
	UV	Higher incidence of body axis defects (BAD)
	Increased Temperature	Increased incidence and intensity of yolk sac edema
	Intertidal/estuarine fluctuating stressors (salinity/air exposure)	Increased incidence and intensity of yolk sac edema; no pericardial edema except in individuals with major body axis defects
With Exposure to Oil (CBO). Relative contribution of chemical components of complex mixtures create tissue signature of exposure	Chemical Stressors (petroleum, TBT, copper, algal)	Pericardial edema often combined with yolk sac edema and other adverse responses as exposure increases

We also observed a high percentage of early developmental egg mortality in the embryos from the larger donor fish. This finding was somewhat contrary to our original hypothesis that the larger and possibly older spawning adults would produce gametes with higher numbers of surviving normal larvae while the smaller, and potentially first time spawners, would produce gametes of lower overall quality with lower numbers of surviving normal larvae. This indicates that the health or developmental stage of those eggs at fertilization was a potential problem. An additional line of evidence supporting this observation was that the appearance of the eggs was sticky and not runny when egg sac was opened. These eggs were evaluated during our preparation for fertilization and concern for their potential viability was indicated (Dinnel, pers comm.). Additionally, there were relatively few large fish that were captured during sampling therefore the eggs were taken from only two females. We suspect that these larger fish were not representative of the larger fish that staged and spawned in January. However after 48 hpf, those embryos that survived had a high percentage of normal hatch. The overall performance of the

gametes from the larger spawning group may be explained by Dinnel et al. (2010) that indicates that poor gamete health or undeveloped gametes and early stage mortality are related but that those organisms surviving past 48h generally have a high percentage of normal development.

Embryos exposed to UV light in this study exhibited a higher occurrence of severe body axis defects compared to the control sample. Occurrences varied in the UV treatments, but all treatments showed higher occurrence than in the control varying from 5% to 8% of the hatched larvae. This finding is similar to that of Strähle and Jesuthasan (1993) in their study on zebrafish. The occurrence of pericardial edema in this study was limited to those larvae exhibiting severe body axis defects, but only about 1% of hatched larvae exhibited pericardial edema.

Significant yolk sac edema was observed in all treatments exposed to fluctuating temperatures, both in intensity and incidence of the response. Yolk sac edema was identified in 12 to 14% of hatched larvae in the two treatments exposed both temperature and salinity changes and in 25% of larvae that were subjected to the aerial exposure (stranding). The treatment with only salinity fluctuations (B-22) showed occurrence of yolk sac edema in 7% of the larvae, higher than the control (2%) or the control with UV (4%) but well below the occurrence in the A treatments. Therefore it appears that the incidence of yolk sac edema was more closely related to the temperature changes and air exposure than to the changes in salinity.

The quantitative assessment of types of edema demonstrated that pericardial edema, while present in the 2009 experimental oiling experiments, was not present in the 2008 or the 2010 assessments except in larvae that had gross body axis defects. This conclusion is demonstrated in the companion report to this one "Framework for Assessment of Causal Relationships between Early Life Stage Developmental Anomalies of Clupea pallasi and Cosco Busan Oil" (NewFields 2010c) which is an interpretive summary of the studies on herring following the CBO spill.

8 BIBLIOGRAPHY

This list contains literature cited in the report as well as all literature reviewed for this project.

Adema-Hannes R and J Shenker. 2008. Acute Lethal and Teratogenic Effects of Tributyltin Chloride and Copper Chloride on Mahi Mahi (Coryphaena hippurus) Eggs and Larvae. Environ Toxicol Chem, 27(10): 2131-2135.

Alderdice DF and AS Hourston. 1985. Factors Influencing Development and Survival of Pacific Herring (Clupea harengus pallasi) Eggs and Larvae to Beginning of Exogenous Feeding. Canadian Journal of Fisheries and Aquatic Science, 42(1): 56-58.

Alderdice DF, H Rosenthal and FPJ Velsen. 1979. Influence of Salinity and Cadmium on the Volume of Pacific Herring Eggs. Prepared for the Canadian-German Scientific and Technical Cooperation Agreement (Contribution No. 7).

Alderdice DF and FPJ Velsen. 1971. Some Effects of Salinity and Temperature on Early Development of Pacific Herring (Clupea pallasi). Journal Fisheries Research Board of Canada, 28: 1545-1562.

Alpharma Animal Health Ltd. 2001. MS222 (Tricaine Methane Sulphonate). Technical Bulletin. 6pp.

ASTM. 1998. Standard Guide for Conducting Early Life-Stage Toxicity Tests with Fishes. ASTM E 1241-98.

Barron MG, MG Carls, R Heintz, and SD Rice. 2004. Evaluation of Fish Early Life-stage Toxicity Models of Chronic Embryonic Exposures to Complex Polycyclic Aromatic Hydrocarbons Mixtures. Toxicological Sciences, 78: 60 -67.

Barron M, M Carls, J Short and S Rice. 2003. Photoenhanced Toxicity of Aqueous Phase and Chemically-dispersed Weathered Alaska North Slope Crude Oil to Pacific Herring Eggs and Larvae. Environmental Toxicology and Chemistry, 22(3): 650-660.

Barron M, M Carls, J Short and S Rice. 2002. Photoenhanced Toxicity of Aqueous Phase and Chemically-dispersed Weathered Alaska North Slope Crude Oil to Pacific Herring Eggs and Larvae. Report prepared for Prince William Sound Regional Citizens' Advisory Council. Anchorage, AK.

Bauder MB, VP Palace, and PV Hodson. 2005. Is Oxidative Stress the Mechanism of Blue Sac Disease in Retene-exposed Trout Larvae? Environ Toxicol Chem, 24(3): 694-702.

Bento MP, FNS De Medeiros, GLB Ramalho and LCL Medeiros. 2004. Image Processing Techniques to Monitor Atmospheric Corrosion. COTEQ-093.

Berry JP, M Gantar, PDL Gibbs and MC Schmale. 2007. The Zebrafish (Danio rerio) Embryo as a Model System for Identification and Characterization of Developmental Toxins from Marine and Freshwater Microalgae. Comp Biochem Physiol C Toxicol Pharmacol, 145(1): 61-72.

Blaxter JHS. 1977. The Effect of Copper on the Eggs and Larvae of Plaice and Herring. Journal of Marine Biology, 57: 849-858.

Blaxter JHS and G Hempel. 1963. The Influence of Egg Size on Herring Larvae (Clupea harengus L.).

Blaxter JHS. 1963. The Feeding of Herring Larvae and Their Ecology in Relation to Feeding. California Cooperative Oceanic Fisheries Investigations Report Volume X, July 1962 to June 1963.

Brannon EL, KM Collins, JS Brown, JM Neff, KR Parker, and WA Stubblefield. 2006. Toxicity of Weathered Exxon Valdez Crude Oil to Pink Salmon Embryos. Environ Toxicol Chem, 25(4): 962-972.

Braunbeck T and E Lammer. 2006. Fish Embryo Toxicity Assays. University of Heidelberg, Aquatic Ecology & Toxicology, Heidelberg, Germany.

Bray JF and JT Curtis. 1957. An Ordination of the Upland Forest Communities of Southern Wisconsin. Ecological Monographs, 27: 325-349.

Boudreau M, MJ Sweezey, K Lee, PV Hodson, SC Courtenay. 2009. Toxicity of Orimulsion-400 to early life stages of Atlantic herring (Clupea harengus) and mummichog (Fundulus heteroclitus). Environ Toxicol Chem. 28(6):1206-17.

Brinkman SF, JD Woodling, AM Vajda, and DO Norris. 2009. Chronic Toxicity of Ammonia to Early Life Stage Rainbow Trout. Trans Amer Fish Soc, 138: 433-440.

Brinkworth LC, PV Hodson and S Tabash. 2003. CYP1A Induction and Blue Sac Disease in Early Developmental Stages of Rainbow Trout (Oncorhynchus mykiss) Exposed to Retene. Journal of Toxicology and Environmental Health, 66: 627 – 646.

Brown ED and M Carls. 1998. Pacific Herring, Clupea pallasi. In Restoration Notebook. Exxon Valdez Oil Spill Trustee Council.

Brown ED, TT Baker, JE Hose, RM Kocan, GD Marty, MD McGurk, BL Norcross and J Short. I996. Injury to the Early Life History Stages of Pacific Herring in Prince William Sound After the Exxon Valdez Oil Spill. In Proceedings of the Exxon Valdez Oil Spill Symposium, Anchorage, AK on 25 Feb 1993.

Bunn NA, CJ Fox, and T Webb. 2000. A Literature Review of Studies on Fish Egg Mortality: Implications for the Estimation of Spawning Stock Biomass by the Annual Egg Production Method. Center for Environment, Fisheries and Aquaculture Science, Technical Report Number 111.

California Department of Fish and Game. 2010. San Francisco Bay Pacific Herring Spawning Population Assessment for the 2009-10 Season Informational Handouts. Prepared for the Director's Herring Advisory Committee Meeting, Wednesday, April 21, 2010. San Rafael, CA.

California Department of Fish and Game. 2008. Pacific Herring Commercial Fishing Regulations. Sections 163, 163.1, 163.5, and 164. Title 14: California Code of Regulations, Final Supplemental Environmental Document.

California Department of Fish and Game. 2001. California's Living Marine Resources, A Status Report.

California Regional Water Quality Control Board, San Francisco Bay Region. 2008. Proposed Basin Plan Amendment for a Total Maximum Daily Load (TMDL) for Pathogens in Richardson Bay. Marin County, California.

Camp Dresser & McKee Inc. and the Bay Institute of San Francisco. 2000. San Pablo Bay Watershed Restoration Framework Program. Prepared for the Coastal Conservancy and the US Army Corp of Engineers.

Carls MG, L Holland, M Larsen, T Collier, N Scholtz and J Incardona. 2008. Fish Embryos are Damaged by Dissolved PAHs, Not Particles. Aquatic Toxicology, 88: 121-127.

Carls MG, R Heintz, G Marty and S Rice. 2005. Cytochrome P450 1A Induction in Oil-exposed Pink Salmon Embryos Predicts Reduced Survival Potential. Marine Ecology. Progress Series, 301: 253-265.

Carls MG, D Marty and JE Hose. 2002. Synthesis of the Toxicological Impacts of the Exxon Valdez Oil Spill on Pacific Herring (Clupea pallasi) in Prince William Sound, Alaska, U.S.A. Canadian Journal of Fisheries and Aquatic Science, 59: 153-172.

Carls MG, D Marty and JE Hose. 2001. Synthesis of the Toxicological and Epidemiological Impacts of the Exxon Valdez Oil Spill on Pacific Herring in Prince William Sound, Alaska. Restoration Project Final Report.

Carls MG, JE Hose, R Thomas and S Rice. 2000. Exposure of Pacific Herring to Weathered Crude Oil: Assessing Effects on Ova. Environmental Toxicology and Chemisty, 19 (6): 1649-1659.

Carls MG, JS Rice and JE Hose. 1999. Sensitivity of Fish Embryos to Weathered Crude Oil: Part I. Low Level Exposure During Incubation Causes Malformations, Genetic Damage, and Mortality in Larval Pacific Herring (Clupea pallasi). Environmental Toxicology and Chemistry. 18: 481-493.

Carls, MG, L Holland, J Short, R Heintz and S Rice. 2004. Monitoring Polynuclear Aromatic Hydrocarbons in Aqueous Environments with Passive Low-density Polyethylene Membrane Devices. Environmental Toxicology and Chemistry, 23(6): 1416-1424.

Cherr GN, M Morisawa, CA Vines, K Yoshida, EH Smith, T Matsubara, MC Pillai, FJ Griffin and R Yanagimachi. 2008. Two Egg-derived Molecules in Sperm Motility Initiation and Fertilization in the Pacific Herring (Clupea pallasi). International Journal of Developmental Biology, 52: 743-742.

Cherr GN and MC Pillai. 1994. Progress Report: Environmental Factors Affecting Reproduction and Recruitment of Pacific Herring in the San Francisco Estuary. Interagency Ecological Program for the Sacramento-San Joaquin Estuary.

Connor M, JH Hunt and C Werme. 2005. White Paper: Potential Impacts of Dredging on Pacific Herring in San Francisco Bay. Prepared for South Pacific Division of US Army Corp of Engineers and Long-Term Assessment Strategty Data Gaps Workgroup.

Costello MJ and JC Gamble. 1992. Effects of Sewage Sludge on Marine Fish Embryos and Larvae. Marine Environmental Research, 30: 49 - 74.

Couillard CM, K Lee, B Legare, TL King. 2005. Effect of dispersant on the composition of the water-accomodated fraction of crude oil and its toxicity to larval marine fish. Environ Toxicol Chem 24:1496-1504.

Davis J, M Sedlak and M Connor. 2008. The Pulse of the Estuary, Monitoring and Managing Water Quality in the San Francisco Estuary. The San Francisco Estuary Institute.

Deththlefsen V, H von Westernhagen, H Tug, PD Hansen, and H Dizer. 2001. Influence of Solar Ultraviolet-B on Pelagic Fish Embryos: Osmolality, Mortality and Viable Hatch. Helgo Mar Res, 25: 45-55.

Dietrich HW III, M Westerfield and LI Zon. 2009. Essential Zebrafish Methods: Cell and Developmental Biology. Elsevier-Academic Press: Burlington, MD. 546 pp.

Dietrich, E. 1938. Die Hydrocoele Embryonalis (Dotterblasenwassesucht) der Salmoniden. Zeitschrift fur Fischerei, XXXVI(4): 605-642.

Dinnel PA, D Middaugh, NT Schwarck, HM Farren, RK Haley, RA Hoover, and RR Marshall. 2010. Methods for Conducting Bioassays Using Embryos of Pacific Herring, Clupea pallasi. Arch Environ Contam Toxicol DOI 10.1007/s00244-010-9600-8.

Dinnel PA. 2010. Clarification on Methods for Conducting Bioassays Using Embryos of Pacific Herring, Clupea pallasi. Oral communication during NewFields 2010 Laboratory Study, February 26, 2010.

Dinnel PA, R Hoover, L Lechuga, K Tobiason and J Elphick. 2008. Development of Larval Pacific Herring, Clupea pallasi, Bioassay Protocols: Refinement, Validation, Refinery Effluent and Cherry Point Ambient Water Testing During 2007. Final Report for Washington Department of Ecology by Shannon Point Marine Center, Western Washington University, Anacortes, WA. 81 pp.

Dinnel PA, C Montanez, K Bergmann, and J Elphick. 2007. Refinement of the Larval Pacific Herring, Clupea pallasi, Survival and Growth Bioassay Protocol. Final Report for Washington Department of Ecology by Shannon Point Marine Center, Western Washington University, Anacortes, WA. 40 pp.

Dinnel PA, HM Farren, L Marko and SA Morales. 2005. Development of Embryo and Larval Pacific Herring, Clupea pallasi, Bioassay Protocols: Phase IV. Final Report for Washington Department of Ecology by Shannon Point Marine Center, Western Washington University, Anacortes, WA. 82 pp.

Dinnel PA and L Marko. 2004. Toxicity of a Proposed Ballast Water Biocide, SeaKleen®, to Embryo and Larval Stages of Pacific Herring, Clupea pallasi. Final Report for Garnett, Inc., Watkinsville, GA. 25pp.

Dinnel PA, NT Schwarck, A Balderas, and M Cotter. 2002. Development of Embryo and Larval Pacific Herring, Clupea pallasi, Bioassay Protocols: Phase II. Final Report for Washington Department of Ecology by Shannon Point Marine Center, Western Washington University, Anacortes, WA. 56 pp.

Douglas G. 2009a. Cosco Busan Herring Studies Data Analysis. PowerPoint presentation, August 2009.

Douglas G. 2009b. Preliminary Evaluation of Water and Egg Chemistry Results from the NOAA 2009 Herring Egg Study. Draft Report. August 2, 2009.

Dunnett CW. 1955. A Multiple Comparisons Procedure for Comparing Several Treatments with a Control. Journal of the American Statistical Association, 50: 1096–1121.

Eisler R and T Backiel. 1960. Narcotization of Chinook Salmon Fingerlings with Tricaine Methanesulfonate (M.S. 222). Transactions of the American Fisheries Society, 89: 164-167.

EVS Environment Consultants, Inc. 1999. Cherry Point Screening Level Ecological Risk Assessment. Prepared for Washington State Department of Natural Resources, Aquatic Resources Division.

Exxon Valdez Oil Spill Trustees Council. 2008. Prince William Sound Integrated Herring Restoration Program.

Farwell A, V Nero, M Croft, P Bal and D Dixon. 2006. Modified Japanese Medaka Embryo Larval Bioassay for Rapid Determination of Developmental Abnormalities. Archived Environmental Contamination and Toxicology, 51: 600-607.

Feyrer F, LR Brown, RL Brown and JJ Orsi. 2004. Early Life History of Fishes and the San Francisco Estuary and Watershed. American Fisheries Society, Symposium 39: Bethesda, MD.

Finn RN and BG Kapoor. 2008. Fish Larval Physiology. Science Publishers: Enfield, NH. 714 pp.

Fox GA. 1991. Practical Causal Inference for Ecoepidemiologists. Journal of Toxicology and Environmental Health, Part A, 33(4): 359-373.

French-McCay DP, JJ Rowe, N Whittier and S Sankaranarayanan. 2005. Evaluation of the Consequences of Various Response Options Using Modeling of Fate Effects and NRDA Costs of Oil Spills into Washington Waters. Proceedings of the International Oil Spill Conference, Paper 395, May 15-19, 2005: Miami, FL. Sponsored by American Petroleum Institute, Washington DC.

French-McCay DP, N Whittier, S Sankaranaarayalnan and H Sook Kim. 2005. Use of Probabilistic Trajectory and Impact Modeling to Assess Consequences of Oil Spills with Various Response Strategies. Proceedings of the 28th Arctic and Marine Oil Spill Program (AMOP).

Technical Seminar, Emergencies Science Division, Environment Canada: Ottawa, ON Canada. pp. 253-271.

French-McCay DP. 2004. Oil Spill Impact Modeling: Development and Validation. Environmental Toxicology and Chemistry, 23: 2241-2256.

Galkina LA. 1971. Survival of Spawn of the Pacific Herring (Clupea harengus pallasii val.) Related to the Abundance of the Spawning Stock. Murmansk Marine Biological Institute of the Academy of Sciences of the USSR, U.S.S.R.

Galkina LA. 1968. Survival of Herring Eggs and Larvae on the White Sea Spawning Grounds During a Period of Massive Spawning. Murmansk Marine Biological Institute, Kola Branch Academy of Sciences of the USSR, Dal'nie Zelentsy.

Garcia KL, JJ Delfino, and DH Powell. 1993. Non-regulated Organic Compounds in Florida Sediments. Water Res, 27:2347-2353.

González-Doncel M, González L, Fernández-Torija C, Navas JM, and Tarazona JV. 2008. Toxic Effects of an Oil Spill on Fish Early Life Stages May Not Be Exclusively Associated to PAHs: Studies With Prestige Oil and Medaka (Oryzias latipes). Aquatic Toxicology, 87: 280-288.

Griffin FJ, EH Smith, CA Vines and GN Cherr. 2009. Impacts of Suspended Sediments on Fertilization, Embryonic Development, and Early Larval Life Stages of the Pacific Herring, Clupea pallasi. Biological Bulletin, 216: 175- 187.

Griffin FJ, MR Brenner, HM Brown, EH Smith, CA Vines and GN Cherr. 2004. Survival of Pacific Herring Larvae is a Function of External Salinity. In Early Life History of Fishes in the San Francisco Estuary and Watershed, F Feyrer, LR Brown, RL Brown and JJ Orsi, eds. Proceedings of the Symposium, Early Life History of Fishes in the San Francisco Estuary and Watershed, Santa Cruz, California on August 20-23, 2003. American Fisheries Society: Bethesda, MD. pp 37-46.

Griffin FJ, MC Pillai, CA Vines, J Daaria, T Hibbard-Robbins, R Yanagimachi and GN Cherr. 1998. Effects of Salinity on Sperm Motility, Fertilization, and Development in the Pacific Herring, Clupea pallasi. Biological Bulletin, 194:25-35.

Griffin FJ, CA Vines, MC Pillai, R Yamagimachi and GN Cherr. 1996. Sperm Motility Initiation Factor is a Minor Component of the Pacific Herring Egg Chorion. Develop Growth Differ, 38:193-202.

Haegele CW and DN Outram. 1978. The Effects of Diet and Ration on the Growth and Survival of Pacific Herring (Clupea harengus pallasi) Larvae. Department of Fisheries and the Environment, Fisheries and Marine Service, Resources Services Branch, Pacific Biological Station, Nanaimo, British Columbia.

Hansell DA and CA Carlson. 2002. Biogeochemistry of Marine Dissolved Organic Matter. Academic Press: San Diego, CA. 774 pp.

Haralick RM, K Shanmugam, and I Dinstein. 1973. Textural Features for Image Classification. Ifee Transactions on Systems, Man, and Cybernetics. SMC-3 (6).

Heintz R, S Rice, A Wetheimer, R Bradshaw, F Thrower, J Joyce and J Short. 2000. Delayed Effects on Growth and Marine Survival of Pink Salmon After Exposure to Crude Oil During Embryonic Development. Marine Ecology Progress Series, 208: 205-216.

Heintz R, J Short and S Rice. 1999. Sensitivity of Fish Embryos to Weathered Crude Oil: Part II. Increased Mortality of Pink Salmon Embryos Incubating Downstream from Weathered Exxon Valdez Crude Oil. Environl Toxicol Chem, 18(3):481-493.

Helfman GS, BB Collette and DE Facey. 1997. The Diversity of Fishes. Blackwell Sciences: Malden MA. 528 pp.

Hershberger PK, NE Elder, J Wittouck, K Stick and RM Kocan. 2005. Abnormalities in Larvae from the Once-Largest Pacific Herring Population in Washington State Results Primarily from Factors Independent of Spawning Location. Transactions of the American Fisheries Society, 134: 326-337.

Hielsher R, H Schaeben and D Chateigner. 2007. On the Entropy to Texture Index Relationship in Quantitative Texture Analysis. Journal of Applied Crystallography, 40: 371-375.

Hill AJ, SM Bello, AL Prasch, RE Peterson and W Heideman. 2004. Water Permeability and TCDD-Induced Edema in Zebrafish Early-Life Stages. Toxicol Sci, 78: 78-87.

Hill J and IA Johnston. 1997. Temperature and Neural Development of the Atlantic Herring (Clupea harengus L.). Comp Biochem Physiol, 117A(4): 457-462.

Hill J and IA Johnston. 1997. Photomicrographic Atlas of Atlantic Herring Embryonic Development. Journal of Fish Biology, 51: 960-977.

Hodson PV, CW Khan, G Saravanabhavan, L Clarke, RS Brown, B Hollebone, Z Wang, J Short, K Lee, T King. 2007. Alkyl PAH in Crude Oil Cause Chronic Toxicity to Early Life Stages of Fish. Proc. 28th Arctic and Marine Oilspill Prog; Env Sci & Tech Div; Environment Canada. 10 p.

Hofer, B. 1896. Uber Fischkrankheiten. Zeitschrift fur Fischerei, 7(VI): 313-325.

Hoie H, A Folkvord and A Johannessen. 2000. A Multivariate Analysis of Condition of Herring Larvae from Different Environmental Conditions. University of Bergen, Department of Fisheries and Marine Biology. CM2000/R:04.

Hose JE, MD McGurk, GD Marty, DE Hinton, ED Brown and TT Baker. 1996. Sublethal Effects of the Exxon Valdez Oil Spill on Herring Embryos and Larvae: Morphological, Cytogenetic, and Histopathological Assessments, 1989-1991. Can Jnl Fish Aquat Sci, 53: 2355-2365.

Hourston AS, H Rosenthal, and H von Westerhagen. 1984. Viable Hatch from Eggs of Pacific Herring (Clupea Harengus pallasi) Deposited at Different Intensities on a Variety of Substrates. Can Tech Rept Fish & Aquat Sci No. 1274.

Hornung MW, JM Spitsbergen, and RE Peterson. 1999. 2,3,7,8-Tetrachlorodibenzo-p-dioxin Alters Cardiovascular and Craniofacial Development and Function in Sac Fry of Rainbow Trout. Toxicol Sci, 47: 40-51.

Incardona JP and CA Vines. 2009. Cosco Busan Oil Spill Natural Resource Damage Assessment: Data Report of Laboratory and Field Herring injury Studies Performed 2008-2009. Draft; NOAA NWFSC and Bodega Marine Lab.

Incardona JP, MG Carls, HL Day, CA Sloan, JL Bolton, TK Collier and NL Scholz. 2009. Cardiac Arrhythmia Is the Primary Response of Embryonic Pacific Herring (Clupea pallasi) Exposed to Crude Oil during Weathering. Environ Sci Technol, 43:201-207.

Incardona J, H Day, T Collier, and N Scholz. 2006. Developmental Toxicity of 4-ring Polycyclic Aromatic Hydrocarbons in Zebrafish is Differentially Dependent on AH Receptor Isoforms and Hepatic Cytochrome P450 1A Metabolism. Toxicol & Appl Pharm, 217: 308-321.

Incardona J, M Carls, H Teraoka, C Sloan, T Collier and N Scholz. 2005. Aryl Hydrocarbon Receptor-independent Toxicity of Weathered Crude Oil during Fish Development. Environ Health Pers, 113: 1755-1762.

Incardona JP and N Scholz. 2005. Proposal Year 3 Mechanisms of Petroleum Hydrocarbon Toxicity in Fish Early Life History Stages. Environmental Conservation Division, NOAA/Northwest Fisheries Science Center.

Incardona J, T Collier and N Scholz. 2004. Defects in Cardiac Function Precede Morphological Abnormalities in Fish Embryos Exposed to Polycyclic Aromatic Hydrocarbons. Toxicology and Applied Pharmacology, 196(2): 191-205.

Johnston IA. 2006. Review: Environment and Plasticity of Myogenesis in Teleost Fish. Journal of Experimental Biology, 209: 2249-2264.

Johnston IA, Vieira VLA, Temple GK. 2001. Functional consequences and population differences in the developmental plasticity of muscle to temperature in Atlantic herring Clupea harengus. Marine Ecology Progress Series 213:285–300.

Johnston IA, NJ Cole, M Abercromby, and VLA Vieira. 1998. Embryonic Temperature Modulates Muscle Growth Characteristics in Larval and Juvenile Herring. Journal of Experimental Biology, 201: 623-646.

Kashiwada S, H Tatsuta, M Kameshiro, Y Sugaya, T Sabo-Attwood, GT Chandler, PL Ferguson and K Goka. 2008. Stage-Dependent Differences in Effects of Carbaryl on Population Growth Rate in Japanese Medaka (Oryzias latipes). Environmental Toxicology and Chemistry, 27(11):2397–2402.

Kimmel CB, WW Ballard, SR Kimmel, B Ullmann and TF Schilling. 1995. Stages of Embryonic Development of the Zebrafish. Developmental Dynamics. 203: 253-310.

Kinne O and H Rosenthal. 1967. Effects of Sulfuric Water Pollutants on Fertilization, Embryonic Development, and Larvae of the Herring, Clupea harengus. Marine Biology, 1: 65-83.

Kiparissis Y, P Akhtarr, P Hodson and R Brown. 2003. Partition-controlled Delivery of Toxicants: A Novel In Vivo Approach for Embryo Toxicity Testing. Environ Sci Technol, 37:2262-2266.

Kioerboe T, P Munk, and JG Stoettrup. 1985. First Feeding by Larval Herring Clupea harengus L. Dana, 5: 95-107.

Kocan RM, JE Hose, ED Brown, TT Baker. 1996. Pacific Herring (Clupea pallasi) Embryo Sensitivity to Prudhoe Bay Petroleum Hydrocarbons: Laboratory Evaluation and In Situ Exposure at Oiled and Unoiled Sites in Prince William Sound. Can J Fish Aquat Sci, 53:2366-2375.

Kocan RM. 1993. Prince William Sound Herring Embryo Study: Sublethal Effects in Situ and In Vitro 1191 – 1992. Progress Report to Alaska Department of Fish and Game. Fisheries Research Institute: Seattle, WA. 30 pp.

Kocan RM and ML Landolt. 1990. Use of Herring Embryos for In Situ and In Vitro Monitoring of Marine Pollution. In Situ Evaluation of Biological Hazards of Environmental Pollutants. Springer Publications, New York, NY.

Kocan RM, H von Westernhagen, ML Landholt, and G Furstenberg. 1987. Toxicity of Sea-surface Microlayer: Effects of Hexane Extract on Baltic Herring (Clupea harengus) and Atlantic Cod (Gadus morhua) Embryos. Marine Environmental Research, 23: 291-305.

Korn S, DA Moles, and SD Rice. 1979. Effects of Temperature on the Median Tolerance Limit of Pink Salmon and Shrimp Exposed to Toluene, Naphthalene, and Cook Inlet Crude Oil. Bull Env Contam Toxicol, 21: 521-525.

Kristoffersen BA and RN Finn. 2008. Major Osmolyte Changes During Oocyte Hydration of a Clupeocephalan Marine Benthophil Atlantic Herring (Clupea harengus). Marine Biology, 154: 683-692.

Kunz YW. 2004. Developmental Biology of Teleost Fishes. Springer: Norwell, MD. 636 pp.

Laine P and M Rajasilta. 1999. The Hatching Success of Baltic Herring Eggs and Its Relation to Female Condition. Journal of Experimental Marine Biology and Encology, 237: 61-73.

Lankford JF and I Zelo. 2008. A System for Integrated SCAT Data Collection and Management: eSCAT, SCATDB, and Photologger. International Oil Spill Conference, Savannah International Trade & Convention Center, Savannah, GA.

Lassuy DR. 1989. Species Profiles: Life Histories and Environmental Requirements for Coastal Fishes and Invertebrates (Pacific Northwest) Pacific Herring. Report for Coastal Ecology Group of the US Army Corp of Engineers and the Research and Development of the US Fish and Wildlife Service. Biological Report 82 (11.126). TR EL-82-4.

Leatherbarrow JE, LJ McKee, DH Schoellhamer, NK Ganju and AR Flegal. 2005. Concentrations and Loads of Organic Contaminants and Mercury Associated with Suspended Sediment Discharged to San Francisco Bay from the Sacramento-San Joaquin River Delta. San Francisco Estuary Institute SFEI Contribution 405.

Lecoz N, M Malecot, C Quiblier, S Puiseux-Dao, C Bernard, F Crespeau and M Edery. 2007. Effects of Cyanobacterial Crude Extracts from Plaktothrix agardhii on Embryo-larval Development of Medaka Fish, Oryzias latipes. Toxicology, 51(2):262-269.

Lefebvre KA, VL Trainer and N Scholz. 2003. Morphological Abnormalities and Sensorimotor Deficits in Larval Fish Exposed to Dissolved Saxitoxin. Aquatic Toxicology, 66(2):159-170.

Levene H. 1960. Robust Tests for the Equality of Variance. In Contributions to Probability and Statistics, I. Olkin, ed. Stanford University Press, Palo Alto, CA. pp 278–292.

Lieschke GJ, AC Oates and K Kawakami. 2009. Zebrafish Methods and Protocols. Humana Press: New York, NY. 335 pp.

Linden O, R Laughlin Jr., JR Sharp, and JM Neff. 1980. The Combined Effect of Salinity, Temperature, and Oil on the Growth Pattern of Embryos of the Killifish, Fundulus heteroclitus. Marine Environmental Research, 3: 129-144.

Linden O. 1978. Biological Effects of Oil on Early Development of the Baltic Herring Clupea harengus membras. Marine Biology, 45: 273-283.

Linden O. 1975. Acute Effects of Oil and Oil/Dispersant Mixture on Larvae of Baltic Herring. Swedish Water and Air Pollution Laboratory (IVL), Nykoping, Sweden.

Longwell AC, and JB Hughes. 1980. Cytologic, Cytogenetic and Developmental State of the Atlantic Mackerel Eggs from Sea Surface Waters of the New York Bight and Prospects for Biological Effects Monitoring with Ichthyoplankton. Rapp P-V Reun Cons Int Explor Mer, 179:275-291.

Longwell AC. 1977. A Genetic Look at Fish Eggs and Oil. Oceanus, 20(4): 4658.

Lougee LA, SM Bollens and SR Avent. 2002. The Effects of Haloclines on the Vertical Distribution and Migration of Zooplankton. Journal of Experimental Marine Biology and Ecology, 282: 111-134.

Macqueen DJ, DHF Robb, T Olsen, L Melstveit, CGM Paxton, and IA Johnston. 2008. Temperature Until the 'Eyed Stage' of Embryogenesis Programmes the Growth Trajectory and Muscle Phenotype of Adult Atlantic Salmon Biol Lett, 4: 294-298.

Marinkovich M. 2000. SF Herring Spawn Doubts. Pacific Fishing, March 2000. pp 89-93.

Marty G, J Hose, M McGurk, E Brown, D Hinton. 1997. Histopathology and Cytogenetic Evaluation of Pacific Herring Larvae Exposed to Petroleum Hydrocarbons in the Laboratory or in Prince William Sound, Alaska, After the Exxon Valdez Oil Spill. Canadian Journal Fisheries Aquatic Sciences, 54(8): 1846-1857.

McGrath, J. 2005. Progress Report: Impacts of Low Level Residual Oils on Toxicity Assessment of Oil Spills. CRRC. http://pubpages.unh.edu/~jell/mcgrath.htm.

McGurk, MD. 1985. Multivariate Analysis of Morphometry and Dry Weight of Pacific herring Larvae. Marine Biology, 86: 1-11.

McGurk, MD and ED Brown. 1996. Egg-larval Mortality of Pacific Herring in Prince William Sound, Alaska, After the Exxon Valdez Oil Spill. Canadian Journal of Fisheries and Aquatic Sciences, 53: 2343-2354.

McKim JM. 1977. Evaluation of Tests with Early Life Stages of Fish for Predicting Long-term Toxicity. J Fish Res Board Can, 34: 1148-1154.

Merten, Amy. 2005. Sublethal Narcotic Impacts of Dietary Polycyclic Aromatic Hydrocarbons on the Bioenergetics of and Polychlorinated Biphenyl (PCB) Bioaccumulation in Fundulus heteroclitus. PHD Dissertation. University of Maryland, Chesapeake Biological Laboratory, MD. 440 pp.

Middaugh DP, ME Shelton, CL McKenney Jr., G Cherr, PJ Chapman and LA Courtney. 1998. Preliminary Observations on Responses of Embryonic and Larval Pacific Herring Clupea pallasi, to Neutral Fraction Biodegradation Products of Weathered Alaska North Slope Oil Archives of Environmental Contamination and Toxicology, 34:188-196.

Milan D, A Giokas, F Serluca, R Peterson and C Macrae. 2006. Notch 1b and Neuregulin are Required for Specification of Central Cardiac Conduction Tissue. Development, 133: 1125-1132.

Millero F. 1996. Chemical Oceanography-second Edition. CRC Press. Boca Raton, FL. 469 pages.

Morgan JD and CD Levings. 1989. Effects of Suspended Sediments on Eggs and Larvae of Lingcod (Ophidon elangatus) Pacific Herring (Clupea harangus pallasi) and Surf Smelt (Hypomesus pretiosus). Canadian Technical Report of Fisheries and Aquatic Sciences. No. 1729.

Morley SA and RS Batty. 1996. The Effects of Temperature on 'S-strike' Feeding of Larval Herring, Clupea harengus L. Mar Freshw Behav Physiol, 28:123-136.

Morrison JA, IR Napier and JC Gamble. 1991. Mass Mortality of Herring Eggs Associated with a Sedimenting Diatom Bloom. ICES Marine Sciences, 48:237-245.

Neave DA, and RS Batty. 1982. A Simple Method for Measuring Fish Larvae Using Silhouette Photography. Aquaculture, 29: 165-168.

Neff, JM. 1979. Polycyclic Aromatic Hydrocarbons: Evaluations of Sources and Effect. National Academy Press, Washington, D.C.

Nelson TA, DJ Lee, and B Smith. 2003. Are "Green Tides" Harmful Algal Blooms? Toxic Properties of Water-Soluble Extracts From Two Bloom-forming Macroalgae, Ulva fenestrate and Ulvaria obscura (Ulvophyceae). Journal of Phycology, 39: 874–879.

NewFields. 2009a. Evaluation of Measurement Endpoints for the Assessment of Potential Petroleum Related Responses by Pacific Herring: Early Development. Prepared for Polaris Applied Sciences, Inc.

NewFields. 2009b. Biological Review of 2009 Experimental Data on Cosco Busan Oil Effects on Herring, Part 1: Test and Data Validation. Prepared for Polaris Applied Sciences, Inc.

NewFields. 2010a. Biological Review of 2009 Experimental Data on Cosco Busan Oil Effects on Herring, Part 2: Evaluation of Measured Endpoints and Relationship to Potential Causes. Prepared for Polaris Applied Sciences, Inc.

NewFields. 2010b. Laboratory Demonstration of Environmental Factors And Their Effects on Early Stage Development of Clupea pallasi. Prepared for Polaris Applied Sciences, Inc.

NewFields. 2010c. Framework for Assessment of Causal Relationships between Early Life Stage Developmental Anomalies of Clupea pallasi and Cosco Busan Oil. An interpretive summary of 2007, 2008, 2009, and 2010 datasets. Prepared for Polaris Applied Sciences, Inc.

Ninness MM, ED Stevens and PA Wright. 2006. Removal of the Chorion Before Hatching Results in Increased Movement and Accelerated Growth in Rainbow Trout (Oncorhynchus mykiss) embryos. The Journal of Experimental Biology, 209:1874–1882.

NOAA. 2007. The 2007 Cosco Busan Oil Spill: Assessing Toxic Injury to Pacific Herring Embryos and Larvae in the San Francisco Estuary. Draft Proposal. NOAA Fisheries. Northwest Fisheries Science Center; Environmental Conservation Division; Ecotoxicology and Environmental Assessment Programs.

NOAA and BML. 2008. The 2007 Cosco Busan Oil Spill: Assessing Toxic Injury to Pacific Herring Embryos and Larvae in the San Francisco Estuary. Northwest Fisheries Science Center, National Marine Fisheries Service, and National Ocean and Atmosphere Administrations: Environmental Conservation Division; and Ecotoxicology and Environmental Science and Policy and Aquatic Resources Group, Bodega Marine Laboratory.

NOAA and BML. 2007. Standard Operating Procedures for 2007 Cosco Busan Oil Spill: Assessing Toxic Injury to Pacific Herring Embryos and Larvae in the San Francisco Bay Estuary. Northwest Fisheries Science Center, National Marine Fisheries Service, and National Ocean and Atmosphere Administrations: Environmental Conservation Division; and Ecotoxicology and Environmental Science and Policy and Aquatic Resources Group, Bodega Marine Laboratory.

NOAA. 2007. Data Standard Model. NOAA Data Structure Overview. CRRC Workshop. September 2007.

Norcross BL, JE Hose, M Frandsen, and ED Brown. 1996. Distribution, Abundance, Morphological Condition, and Cytogenetic Abnormalities of Larval Herring in Prince William Sound, Alaska, Following the Exxon Valdez Oil Spill. Can Jnl Fish Aq Sci, 53: 2376-2387.

Nusslien-Volhard C and R Dahm. 2002. Zebrafish. Oxford University Press: Oxford, UK. 303 pp.

Ogle S. 2005. A Review of Scientific Information on the Effects of Suspended Sediments on Pacific Herring (Clupea pallasi) Reproductive Success. Prepared for South Pacific Division of the US Army Corp of Engineers and the Long Term Management Strategy Science Assessment and Data Gaps Workgroups.

Ogle S. 2004. Pacific Herring (Clupea pallasi Valenciennes 1847): a Bibliography of Scientific Literature of Pacific Herring (Clupea pallasi), and Additional Selected References for Baltic Herring (Clupea harengus). Prepared for South Pacific Division of the US Army Corp of Engineers and the Long Term Management Strategy Science Assessment and Data Gaps Workgroups.

O'Neill SM and JE West. 2001. Exposure of Pacific Herring (Clupea pallasi) to Persistant Organic Pollutants in Puget Sound and the Georgia Basin. Washington State Department of Fish and Wildlife, Puget Sound Research.

Oros DR and N David. 2002. Identification and Evaluation of Unidentified Organic Contaminants in the San Francisco Estuary. San Francisco Estuary Institute, SFEI Contribution 45. 119 pp.

Parichy, DM, MR Elizondo, MG Mills, TN Gordon, and RE Engeszer. 2009. Normal Table of Postembryonic Zebrafish Development: Staging by Externally Visible Anatomy of the Living Fish. Developmental Dynamics 238: 2975-3015.

Pearson WH. 2005. PAHs and Other Contaminants in Effluents from Artificially Weathered Oil on Gravel. 2005 International Oil Spill Conference: Miami, FL.

Pearson WH. 2009. Technical Note Review of Assessment of Injury to Pacific Herring from the Cosco Busan Oil Spill, 25 pages.

Pearson WH, E Mokness and JR Skalski. 1995. A Field and Laboratory Assessment of Oil Spill Effects on Survival and Reproduction of Pacific Herring Following the Exxon Valdez Spill. In: Exxon Valdez Oil Spill: Fate and Effects in Alaska Waters; ASTM STP 1219. PG Well, JN Butler and JS Hughes, Eds. American Society for Testing and Materials: Philadelphia, PA.

Pearson WH, DL Woodruff, SL Kiesser, GW Fellingham and RA Elston. 1985. Oil Effects on Spawning Behavior and Reproduction in Pacific Herring (Clupea harengus pallasi). Final report to Environmental Affairs Department of the American Petroleum Institute. Washington DC.

Pearson W H., Elston R A, Bienert R W, Drum A S, Antrim L D Why did the Prince William Sound, Alaska, Pacific herring (Clupea pallasi) collapse in 1993 and 1994? Review of hypotheses. Canadian Journal of Fisheries and Aquatic Sciences (1999) 56:711–737.

Prince William Sound Herring Data Portal. 2010. Herring Data and Information Portal. http://www.pwsherringportal.org/Visualizations/index.cfm.

Rajasilta M, P Laine and J Eklund. 2006. Mortality of Herring Eggs on Different Algal Substrates (Furcellaira spp. and Cladophora spp.) in the Baltic Sea – An Experimental Study. Hydrobiologia, 554(1): 1573-5117.

Ramachandran SD, MJ Sweezey, PV Hodson, M Boudreau, SC Courtney, K Lee, T King, JA Dixon. 2006. Influence of salinity and fish species on PAH uptake from dispersed crude oil. Mar Pollut Bull 52:1182-1189.

Ramdahl T. 1983. Retene – a molecular marker wood combustion in ambient air. Nature, 306: 580-582.

Rees WG. 2001. Physical Principles of Remote Sensing. Cambridge University Press: Cambridge, UK. 343 pp.

Rhodes S, A Farwell, L Hewitt, M MacKInnon and D Dixon. 2005. The Effects of Dimethylated and Alkylated Polycyclic Aromatic Hydrocarbons on the Embryonic Development of the Japanese Medaka. Ecotoxicology and Environmental Safety, 60(3): 247-258.

Rice SD, RB Spies, DA Wolfe and BA Wright, Eds. 1996. Proceedings of the Exxon Valdez Oil Spill Syposium. American Fisheries Society; Anchorage, AK. 18: 448-462.

Sanders AM, SM Bollens and TM Johnson. 2000. Condition Indices of Larval Pacific Herring (Clupea pallasi) in the San Francisco Estuary. Http://userwww.sfsu.edu/~bioocean/research/epaherring/epaherring.html.

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Sanders AM, SM Bollens and TM Johnson. 2000. Condition Indices of Larval Pacific Herring (Clupea pallasi) in the San Francisco Estuary. Http://userwww.sfsu.edu/~bioocean/research/epaherring/epaherring.html.

Schein A, JA Scott, L Mos and PV Hodson. 2009. Oil Dispersion Increases the Apparent Bioavailability and Toxicity of Diesel to Rainbow Trout (Oncorhynchus mykiss). Environmental Toxicology and Chemistry, 28(3): 592-603.

Schereschewsky, H. 1935. Die "Kinderkrankheiten" der Salmoniden (Dotterblasenwassersucht und Dotterblaseneinschnurung). Zeitschrift fur Fischerei, 33(3): 474-501.

Schoellhamer DH, TE Mumley and JE Leatherbarrow. 2007. Suspended Sediment and Sediment-associated Contaminants in San Francisco Bay. Environmental Research, 105: 119-131.

Sherrill J, ES Weber III, GD Marty, and S Hernandez-Divers. 2009. Fish Cardiovascular Physiology and Disease. Vet Clin Exot Anim, 12: 11-38.

Simoneit, BRT. 1984. Organic Matter of the Troposphere – III: Characterization and Sources of Petroleum and Pyrogenic Residues in Aerosols Over the Western United States. Atmospheric Environment, 18: 51-67.

Smith RL and JA Cameron. 1979. Effect of Water Soluble Fraction of Prudhoe Bay Crude Oil on Embryonic Development of Pacific Herring. Transactions of the American Fisheries Society, 108: 70-75.

Sokal RR and FJ Rohlf. 1981. Biometry: The Principles and Practice of Statistics in Biological Research. WH Freeman and Co. San Francisco, CA. 859 pp.

Standard Methods. 1998. Standard Methods for the Examination of Water and Wastewater. Part 8000. American Public Health Association: Washington DC.

Stouthart XJHX, MAJ Huijbregts, PM Balm, RAC Lock and SE Wendelaar Bonga. 1998. Endocrine Stress Response and Abnormal Development in Carp (Cyprinus carpio) Larvae after Exposure of the Embryos to PCB 126. Fish Physiology and Biochemistry, 18: 321–329.

Strähle J and S Jesuthasan. 1993. Ultraviolet Irradiation Impairs Epiboly in Zebrafish embryos: Evidence for a Microtubule-dependent Mechanism of Epiboly. Development, 119: 909-919.

Struhsaker JW. 1977. Effects of Benzene (A Toxic Component of Petroleum) on Spawning Pacific Herring Clupea harengus pallasi. Fishery Bulletin, 76(1): 43-48.

Struhsaker JW, MB Eldridge, and T Echeverria. 1974. Effects of Benzene (A Water-Soluble Component of Crude Oil) on Eggs and Larvae of Pacific Herring and Northern Anchovy. Pollution and Physiology of Marine Organisms, 253-283.

Talbot GB and SI Johnson. 1972. Rearing Pacific Herring in the Laboratory. The Progressive Fish Culturist, 34(1): 2-7.

Tiedeken JA, JS Ramsdell and AF Ramsdell. 2005. Developmental Toxicity of Domoic Acid in Zebrafish (Danio rerio). Neurotoxicology and Teratology, 27: 711-717.

Tillitt DE, PH Cook, JP Giesy, W Heideman, and RE Peterson. 2008. Reproductive Impairment of Great Lakes Lake Trout by Dioxin-Like Chemicals. The Toxicology of Fishes. RT Di Giulio and DE Hinton, eds. Chapter 21, pp. 819-875.

Tytler P and J Ireland. 2000. The Influence of Salinity and Temperature Change on the Functioning of the Urinary Bladder in the Early Larval Stages of the Atlantic Herring Clupea harengus L. The Journal of Experimental Biology, 203: 415–422.

Tytler P, J Ireland, and E Fitches. 1996. A Study of the Structure and Function of the Pronephros in the Larvae of the Turbot (Scophthalmus maximus) and the Herring (Clupea harengus). Mar Fresh Behav Physiol, 28: 3-18.

United States Coast Guard U.S. Department of Homeland Security. 2008. Incident Specific Preparedness Review (ISPR). M/V Cosco Busan Oil Spill in San Francisco Bay.

United States Department of the Interior and United States Army Corps of Engineers. 1989. Pacific Herring. Species Profiles: Life Histories and Environmental Requirements of Coastal Fishes and Invertebrates (Pacific Northwest). Biological Report; 82 (11.126).

USEPA. 1995. Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to West Coast Marine and Estuarine Organisms. EPA/600/R-95-136.

Van Trump WJ and MJ McHenry. 2008. The Morphology and Mechanical Sensitivity of Lateral Line Receptors in Zebrafish Larvae (Danio rerio). The Journal of Experimental Biology, 211: 2105-2115.

Vigers GA, JB Marliave, RG Janssen, and P Borgmann. 1977. Use of Larval Herring in Bioassays. Proceedings of the Fourth Annual Aquatic Toxicity Workshop. November 8-10, 1977, Vancouver, British Columbia.

Vines C, T Robbins, F Griffin and G Cherr. 2000. The Effects of Diffusible Creosote-derived Compounds on Development in Pacific Herring (Clupea pallasi). Aquatic Toxicology, 51: 225-239.

Vines CA, K Yoshida, FJ Griffen, MC Pillai, M Morisawa, R Yanagimachi and GN Cherr. 2001. Motility initiation in herring sperm is regulated by reverse sodium-calcium exchange. PNAS, 99(4): 2026-2031.

Washington Department of Fish and Wildlife. 2001. WDFW Studies Causes of Cherry Point Herring Decline. Fish and Wildlife Science, an Online Science Magazine. http://wdfw.wa.gov. Posted July 2001.

Wassenberg DM, Di Giulio, RT. 2004. Synergistic embryotoxicity of polycyclic aromatic hydrocarbon aryl hydrocarbon receptor agonists with cytochrome P4501A inhibitors in Fundulus heterolitus. Environ Health Perspect 112:1658-1664.

Watters DL, HM Brown, FJ Griffin, EJ Larson, and GN Cherr. 2004. Pacific Herring Spawning Grounds in San Francisco Bay: 1973-2000. American Fisheries Society Symposium, 39: 3-14.

Wendelaar SE and RAC Lock. 2008. Osmoregulatory System. The Toxicology of Fishes. RT Di Giulio and DE Hinton, eds. Chapter 8, pp. 401-415.

Westernhagen Hv, V Dethlefsen, P Cameron, J Berg, and G Furstenberg. 1988. Developmental Defects in Pelagic Fish Embryos From the Western Baltic. Helgolander Meeresuntersuchungen, 42: 13-36.

Westernhagen Hv, V Dethlefsen, and H Rosenthal. 1979. Combined Effects of Cadmium, Copper, and Lead on Developing Herring Eggs and Larvae. Helgolander wiss. Meeresunters, 32: 257-278.

Wolf K. 1956. Experimental Induction of Blue Sac Disease. Trans Am Fish Soc, 86: 61-70.

Wolf K. 1954. Progress Report on Blue-sac Disease. Prog Fish-Cult, 16(2): 51-59.

Yamagami K. 1996. Studies on the Hatching Enzyme (Choiolysin) and Its Substrate, Egg Envelope, Constructed of the Precursors (Choriogenins) in Oryzias latipes: A Sequel to the Information in 1991/1992. Zoological Science, 13: 331-340.

Zhang Z, H Hu, H Zhen, X Wu, and C Huang. 2008. Reproductive Inhibition and Transgenerational Toxicity of Triphenyltin on Medaka (Oryzias latipes) at Environmentally Relevant Levels. Environ Sci Technol, 42: 8133-8139.

Zotin AI. 1958. The Mechanism of Hardening of the Salmonid Egg Membrane after Fertilization or Spontaneous Activation. Jnl Embryology and Experimental Morphology, 60(4): 546-568.