ORIGINAL ARTICLE

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OBJECTIVE: A natural resource damage assessment (NRDA) was conducted by federal and state trustees after the 2007 Cosco Busan oil spill (CBOS) in San Francisco Bay. Pacific herring embryos were collected from selected central bay sites and examined for early-life stage developmental injury; reported anomalies were thought to have derived from exposure to CBOS fuel oil. Chemical analyses of embryonic tissues did not demonstrate a clear pathway of CBOS exposure. Since the herring embryos were collected from shallow, dynamic estuarine waters, we conducted a controlled laboratory study to further investigate the possible effects on developing herring embryos caused by the fluctuating environmental stressors that naturally occurred in these shallow intertidal spawning areas (especially shifts in temperature, salinity, and ultraviolet light exposure that is intensified as a consequence of stranding in shallow water).

METHODS: This unique laboratory study evaluated effects on herring development caused by a suite of estuarine environ-mental stressors in the absence of CBOS by replicating near shore environmental conditions occurring at two sites within the central bay during the 2008 post-spill spawning. In addition to assessing developmental response endpoints such as body axis defects following a qualitative scoring process, we developed a new approach for assessment of pericardial and yolk-sac edema based on quantified measurements.

RESULTS: A significantly higher incidence and intensity of yolk-sac edema was observed in the embryos exposed to temperature and salinity changes whereas pericardial edema did not occur in larval specimens at statistically significant levels compared to controls. Embryos exposed to ultraviolet light were associated with a higher incidence of body axis defects in larval specimens relative to the control. These experimental results indicate that fluctuating environmental conditions contributed to the abnormalities observed in naturally spawned herring collected during the post-spill spawning event.

Key Words: Pacific herring; ELS development; Environmental stress; Peri... and yolk-sac edema; Quantitative assessment

ABBREVIATIONS: ANS Alaska north slope crude oil; ANOVA Analysis of variance; BD Body depth (vertical axis); BML Bodega marine laboratory; ... Cosco busan fuel oil; CBOS Cosco busan oil spill; DPF Days post fertilization; ... Days post hatch; ELS Early-life stage; NOAA National oceanic and atmo... administration; NRDA Natural resource damage assessment; PAH Polyc... aromatic hydrocarbons; PD Pericardial depth (vertical axis); PP Peninsula ... Francisco; PSQ Pt san quentin San Francisco; SCAT Shoreline clean... assessment technique; SL Standard length; YD Yolk-sac depth (vertical axis); YL Yolk-sac length (horizontal axis); UV Ultraviolet irradiance

After the container ship Cosco Busan struck the Bay Bridge in San Francisco Bay in November 2007 and spilled 58,000 gallons of IFO 0380 fuel oil (US Department of Homeland Security, NOAA Incidentnews. gov), the oil was observed by Joint Shoreline Cleanup Assessment Teams (SCAT teams) in other areas of the bay historically used as spawning habitat by Pacific herring (1). Larval Pacific herring (*Clupea pallasi*) were selected as a biological indicator species to determine potential impact to near shore fish populations created by exposure to Cosco Busan oil (CBO). Assessment of the potential impact to early life stage (ELS) Pacific herring (*Clupea pallasi*) caused by the Cosco Busan oil spill (CBOS) launched three separate investigations and provided an opportunity to examine a suite of biological response patterns occurring as a result of three scenarios:

- The CBOS spill event in estuarine conditions [investigation conducted in 2008] (1-3)
- Fuel oil exposure in mesocosm experiments [investigation conducted in 2009] (4-6)
- The present study, conducted in 2010 in the absence of CBO with fluctuating environmental stressors replicating the conditions recorded at two locations of field investigations after the spill (7).

Naturally spawned eggs were collected from the intertidal zone in February 2008 by the NRDA team at locations within the bay where CBO had been observed by the SCAT team as well as from potentially non-oiled locations. Eggs and hatched larvae were examined by members of the damage assessment team for multiple biological endpoints representative of the health and/or abnormalities in the development of early-life stages of herring. The team reported assessment of hatching success and developmental abnormalities indicating that several of the sites were severely impacted (1). Many of the reported abnormalities were similar to anomalies associated with Alaska North Slope (ANS) syndrome (and blue sac disease) suggesting a potential cause and effect relationship between CBO and the observed anomalies (8-12). However, chemical analysis of eggs collected from the impacted areas failed to demonstrate the presence of the characteristic chemical signature indicative of exposure to CBO (1), therefore, a clear linkage of CBO exposure and the associated anomalies observed in the naturally spawned eggs collected in the 2008 post spill investigation could not be established.

In 2009, a series of mesocosm studies were conducted to document that CBO exposure would produce anomalies similar to those reported for the naturally spawned herring larvae collected in 2008. These experiments documented that morphological, physiological and developmental abnormalities occurred in the presence of CBO (13). Chemical analyses of water and larval tissue samples documented exposure of embryos to CBO, uptake of PAHs into tissues, and that a unique chemical signature of CBO was present in the water and within the tissues (14). While these experiments were severely compromised by numerous experimental artifacts such as gamete quality, extreme shifts in temperature, pH, algal fouling, and fluctuations in UV exposure, the chemical signature of exposure to CBO was present even at the lowest exposure concentration (14). Because these mesocosm experiments were so severely compromised by extreme natural fluctuations, definitive determinations of cause and effect relationships for CBO exposure comparable to the field observations and herring development could not

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be established from the 2009 study. We hypothesized that fluctuating environmental stressors may have contributed to decreased herring hatching success and normal development during the field evaluations in 2008 and the mesocosm observations in 2009. The variable and fluctuating environmental conditions occurring at the intertidal spawning locations in 2008 may have been a contributing feature of the nearshore environment causing reduced hatch rates and compromised early development and physiological health of larvae.

The present study was undertaken to explore the influence of fluctuating environmental stressors on developing herring embryos and post-hatch larvae in the absence of oil. Common metrics of growth (standard length, and hatching success), morphological development (body axis deformities) and physiological functioning (particularly the onset of pericardial and/ or yolk-sac edema) were examined as endpoints. Natural environmental fluctuations were recorded in oceanographic data collected from near shore locations within the central portion of San Francisco Bay, and upon detailed examination, we observed that the same oscillations were not evident at all sites. Two of six locations investigated during the post-CBOS resource damage assessment were selected for further study under laboratory conditions mirroring those recorded in the field: Peninsula Point (PP) represented an area potentially impacted by the CBOS, while Point San Quentin (PSQ) represented an area to the north furthest away from the CBOS and was designated as a reference site for the NRDA investigation (Figure 1). Although the two field locations selected for the present laboratory study are situated within the central bay region of San Francisco Bay, different salinity and temperature profiles were recorded at PP compared with PSQ during the collections by NRDA team members of naturally spawned herring embryos in 2008. The PSQ site salinity records demonstrate a large variation in salinity profiles responding to the relative dominance of riverine influence from the Sacramento River or oceanic influence from the Pacific Ocean. Collections at PSQ were made during a sequence of high tides, which effectively drives more saltwater in from the Pacific Ocean into the central bay. In contrast, low tides and unseasonably warm temperatures were noted to occur during daylight hours during the post-spill spawning events in the vicinity of Peninsula Pt. during the collections, which would have exposed incubating embryos to shallow water stranding and increased thermal and UV exposure. Previous studies have been performed to determine the effects of different temperatures and salinities on developing embryos (15-18); however, experimental treatments in these studies were performed at static temperatures and salinities. To our knowledge a laboratory study designed to simulate real-time changing estuarine conditions and consequent onset of developmental anomalies such as pericardial and yolk-sac edema had not been conducted.

MATERIALS AND METHODS

Field collected environmental data from Peninsula Pt. and Pt. San Quentin

This laboratory study was designed to demonstrate the extent to which fluctuating environmental conditions such as temperature, salinity, increased UV intensity, and transient air exposure occurring in shallow intertidal waters may contribute to abnormal embryonic development of Pacific herring. Data for salinity and temperature cycles were collected from continuously recording water quality instruments placed in the two study locations. The site records were reviewed and laboratory seawater exposu regimes were adjusted to replicate these specific parameters during an 8 d continuous flow experiment with different exposure scenarios.

Production of live embryos from field-collected herring

Eggs from mature fish captured in Richardson Bay were artificially spawned and fertilized in the laboratory. Spawning adult male and female Pacific herring were collected between February 19 and 24, 2010 from shallow Richardson Bay spawning grounds (centered at 37.87°N latitude, 122.48°W longitude) in the San Francisco estuary. Fish were captured using fishing rods rigged with Sabiki lures; gonads were removed immediately on board the vessel and placed into individual glass Petri dishes, sealed with parafilm and tape, labeled and placed over paper-covered ice in coolers for transpo to the laboratory. Upon arrival at the laboratory, the gonads were composited from adult male and female fish. Initially, the study design included parallel testing of two age classes of gravid female herring. However, eggs collect from older, larger females (3 year and older; ≥ 180 mm standard length) were not of sufficient quality and did not achieve minimal control hatch rates to ensure a successful test. Therefore, the data brought forward represen results from embryos from females in the younger age class (2 year old fis 160 mm standard length).

The fish gametes were artificially spawned and fertilized within 4 days of collection in San Francisco Bay following methods outlined in Dinnel et al. (19). Test water was prepared with 0.45 µm filtered seawater from Hood Canal Bay, Washington diluted with deionized water to create fertilization water at test conditions of 12°C and 16, 22 and 28 ppt salinity. Testes were chosen based on size; larger gonads were considered more mature and viab Eggs were fertilized following protocols in Dinnel et al. (19) and extruded onto glass microscope slides (25 mm × 75 mm). Approximately 100 egg were distributed onto each slide and the slides were placed in glass dishe containing 500 ml of sperm solution. After a 30 min exposure, slides were assessed for relative percent fertilization and then removed, rinsed gentl

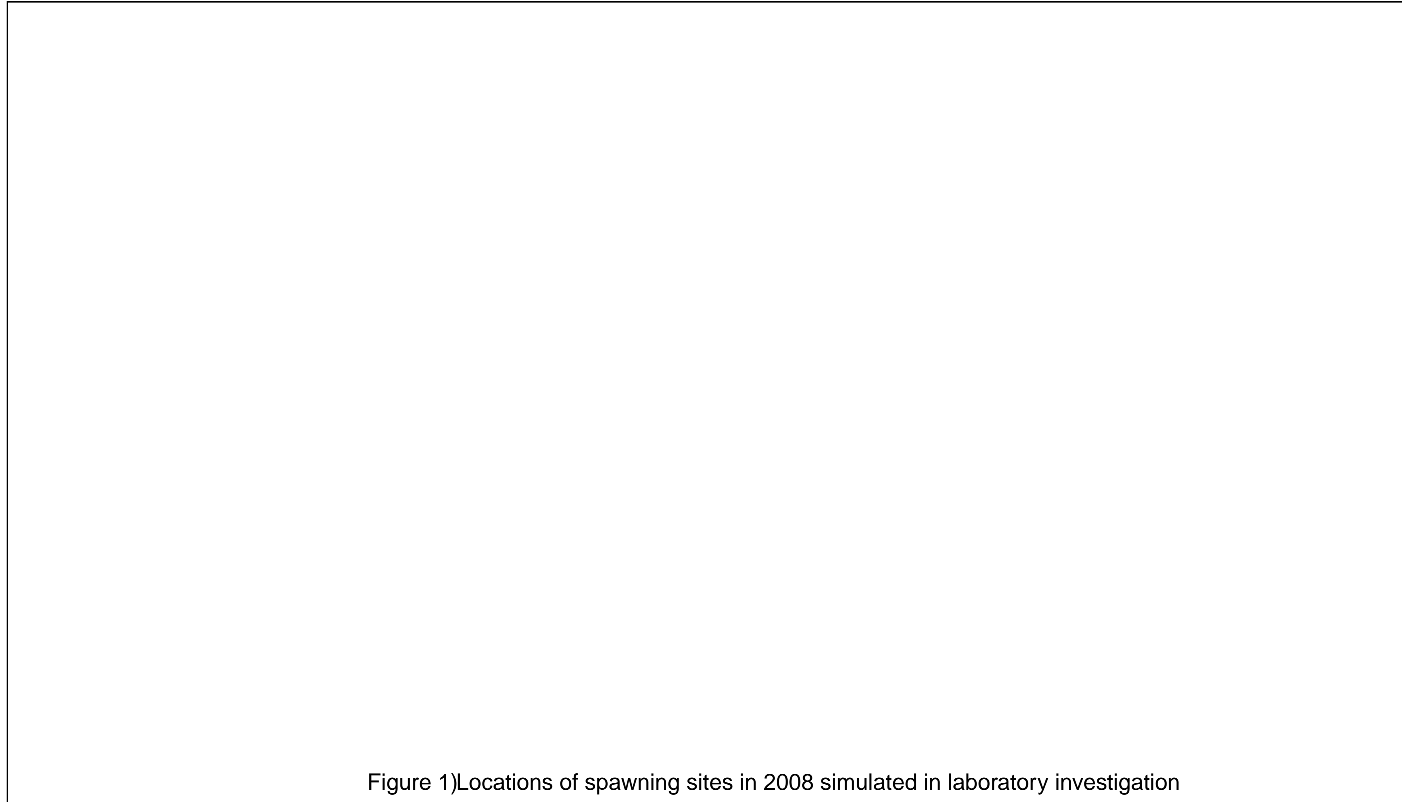

Figure 1)Locations of spawning sites in 2008 simulated in laboratory investigation

with control seawater at the appropriate test salinity, and placed in separate incubation chambers within individual 10 L aquaria.

Test treatments

Table 1 summarizes test conditions for each treatment. Two control treatments (Control, Control-2) were run under optimal conditions for herring embryos from San Francisco Bay, i.e. temperature of 12 ± 1°C and salinity of 16 ± 1 ppt (18,20) with different levels of UV irradiance. The test treatments required temperature and salinity to fluctuate outside of optimal quality control ranges in order to simulate those conditions experienced by developing eggs after the oil spill. One test treatment (PSQ) simulated the fluctuations in salinity that had occurred at Point San Quentin during the time period of egg collections by the NRDA team. Three test treatments (PP-1, PP-2 and PP-3) approximated the complexity of variations in environmental conditions at Peninsula Point (i.e., water temperature, salinities and tidal height fluctuations). The first two treatments examined embryo exposures that may have occurred while submerged within the water column: PP-1 tested variable salinity and temperature exposure from point of fertilization through embryonic development as if embryos were submerged in the water column, while the PP-2 treatment examined the effect of fluctuating temperatures only. Variations in exposure conditions for PP-3 incorporated periods of shallow water stranding, which was expected to induce thermal shock and possible aerial exposure based on recorded extreme tidal cycles and coincident unseasonably warm air temperatures during the collection of eggs by the NRDA team from the PP site (NOAA weather service).

Experimental array

Four replicate glass slides containing the fertilized eggs were placed within individual incubation chambers suspended from a Plexiglas frame into a 10 L glass aquarium. Three replicates were used for experimental results; a fourth replicate was used for daily observations to minimize laboratory impacts on the test replicates. The incubation chambers were created from polycarbonate cylinders fitted with a 1 mm Nytex mesh screen covering the bottom end of each chamber with an additional opening in the center which was also covered with a secured Nytex sleeve. The Nytex screens provided a conduit for water exchange within each incubation chamber. The chambers were suspended above the bottom surface of the aquarium at a depth which provided 150 ml (85 mm depth) of water volume for each chamber (Figure 2). The 24 samples (6 treatments × 4 replicate slides suspended in each aquaria) were identified as sample numbers 1 to 24 with only enough information about the treatment regime provided to allow accurate testing conditions.

Test aquaria received a constant renewal of flowing source water maintaining a volume of 6 L. Seawater was prepared in a 20 L glass carboy; 0.45 µm filtered seawater was diluted with deionized water to the appropriate test salinity. Water was delivered directly to the aquaria from the carboys using peristaltic pumps. Test water flowed into the aquaria via Tygon tubing connected to the carboys and exited the aquaria through an adjustable port located on the opposite side of the tank. Aquaria and carboy reservoirs were placed in water-bath tables with flowing seawater. Temperature control was automated by Fuji Electric PXR4 Micro-controllers) to maintain to control test temperatures (12 ± 1°C) whereas water-bath temperatures for the test treatments were individually modified to mirror onsite buoy data recorded for the post-spill period. The controller was programmed to change temperatures according to the testing plan. Trickle-flow aeration (roughly two

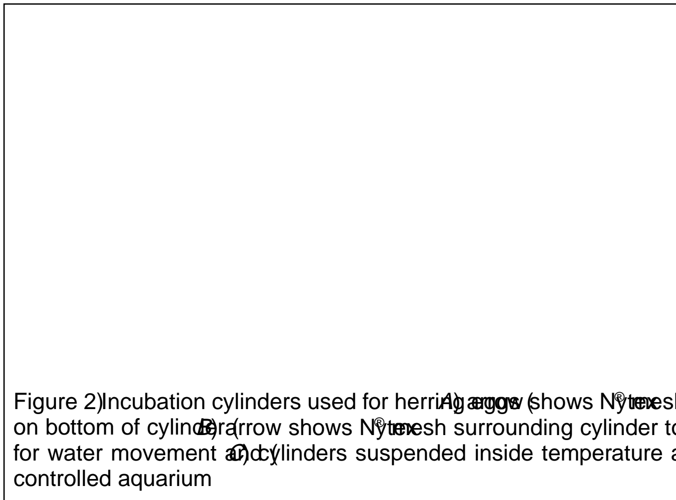

Figure 2) Incubation cylinders used for herring eggs (A) arrow shows Nytex® mesh on bottom of cylinder (B) arrow shows Nytex® mesh surrounding cylinder to allow for water movement and (C) cylinders suspended inside temperature and salinity controlled aquarium

bubbles per second) was provided to each tank; temperature and salinity we monitored continuously throughout the test with recording YSI Professional Plus water quality meters. Dissolved oxygen, pH, temperature, and salinity were measured in each aquarium daily using Orion 5 Star meters. Salinities fluctuated according to a pre-determined schedule by altering the incoming water supply to freshly prepared water of the desired temperature and salinity, and adjusting the flow rate of the pump to supply the water flow over the scheduled time period. When no salinity changes were scheduled for a given treatment, and for the control aquaria, water flow was set such that a complete renewal of water occurred every 24 h. (One test treatment PP-3, received increased thermal and aerial exposure to simulate exposu during a low-tide cycle. These conditions were achieved by moving the tes array containing the PP-3 embryos to a separate aquarium without seawat and placing it inside a temperature-controlled incubator equipped with UV light for 3 periods of ~1 h duration, simulating the expected effects of the low tide cycle noted in records for the PP site. Dissolved oxygen and pH wer measured daily and remained within target limits throughout the test period in all samples (Table 1). Temperature and salinity in the control and CUV treatments remained constant throughout the test as noted by a log created by continuously recording salinity and temperature measurements collected by a monitoring device positioned in the water bath where both treatments were located.

The experimental photoperiod mirrored the hours of daylight during February 2008 (NOAA, Greg Baker pers. comm); treatments received 13 h light and 11 h darkness daily matching conditions in San Francisco Bay during the 2008 post-spill spawning period. Lighting was provided by fluorescent light ballast containing one fluorescent bulb (Duro-Test Vita-Lite; 40W, 5500°K, 91 CRI) and one standard fluorescent bulb (Phillips F40CW) placed within 12” above the incubation chambers. All incubation chambers were left uncovered to prevent UV loss. Light intensity was measured at the water level of test chambers with a Reed LM-811X meter. The control treatment was exposed a low level of UV from the standard fluorescent light bulb (measured as <5 µW/cm²) only. All other test treatments including the Control-2 treatment were exposed to higher UV irradiance, ranging from 115 to 135 µW/cm².

Determination of fertilization success at 48 h

Fertilization success was assessed at 48 h post fertilization; the results a shown in Table 2. Most of the embryos were in transition from blastula stage to epiboly as described by Hill and Johnston (21). The fertilization rates ranged from 80.5% to 97.3%. Percentage fertilization was calculated by subtracting the unfertilized and unhardened eggs from the total eggs counted. At 48 h, the percentage of fertilized and viable embryos ranged from 43 to 80.3%. The control treatment at 48 h had 70.3% fertilized and viable embryos validating the test (22).

Slides were carefully removed from the test chambers and placed into glas crystallization dishes filled with water at appropriate test conditions. Each slide was photographed with a Canon G10 Power Shot digital camera mounted on a tripod to provide a stable and consistent platform and then the slides were viewed using a Nikon dissecting microscope. Eggs on ea slide were enumerated and classified as fertilized, unfertilized, or dead; th slide was placed over a grid matrix to facilitate accurate counts. Eggs we considered fertilized and viable if there was a membrane surrounding the egg indicating water uptake post-fertilization had occurred. Unfertilized eggs were those with no apparent yolk membrane. Unfertilized and dead eggs as well as densely clustered eggs, were removed from the slides with force without damaging developing eggs in order to retain 80 to 100 eggs on eac slide. Care was taken to minimize the amount of time the slides were in crystallization dishes to prevent temperatures in the dishes from rising abov test conditions.

Assessment of hatch success at 8 dpf

The fertilized eggs were incubated in the flow-through test aquaria at tes exposures for 8 days after which time all slides were transferred to individua 100 × 50 mm crystallization dishes maintained at a constant temperature of 12°C and salinity of 16 ppt water; the dishes were used to secure the emerging hatchlings from escape or entrapment on the mesh screen that wa used as part of the flow-through incubation chamber. Water was renewed in the dishes daily until hatch completion. At 8 days post fertilization (dpf) the 24 slides were photographed again with a Canon G10 Power Shot digita camera. Eggs were classified and enumerated as unfertilized, live normal, li abnormal or dead. Live eggs were considered normal if they had no apparen opacity and had two distinct eyes. Abnormal eggs were embryos with a viab heartbeat but with noticeable abnormalities or distorted physical features.

TABLE 1

Experimental test conditions

Treatment	Primary variables	Fertilization salinity (%)	Salinity regime (Target ± 1%)	Temperature regime (Target ± 1°C)	UV (µW/cm²)	Aerial stranding	Water quality	
							Dissolved oxygen (mg/L)	pH
&RQWURO	2SWLPXP ORZ 89						!	$PELHQW “
&RQWURO	2SWLPXP PRGHUDWH 89							
364	6DOLQLW\							
33	6DOLQLW\ WHPSHUDWXUH			SHULRGLF LQFUHDVHV WR				
33	WHPSHUDWXUH			SHULRGLF LQFUHDVHV WR				
33	7KHUPDO VKRFN DHULDO H[SRVXUH			SHULRGLF LQFUHDVHV WR		<HV		

Change in salinity from spawning/fertilization to treatment

TABLE 2

Fertilization rates determined at 48 h post fertilization

Treatment	Fertilized (% Total Eggs)	Unfertilized or unhardened (% Total Eggs)	Dead embryos at 48 h (% Fertilized Eggs)	Viable fertilized embryos (% fertilized Eggs)
&RQWURO				
&RQWURO				
364				
33				
33				
33				

Some abnormal eggs were underdeveloped or 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).” After slides were digitally recorded, those with unhatched eggs were placed back into the crystallization dishes containing water at 12 ± 1°C and 16 ± 1 ppt salinity; forceps were used to transfer each slide into a clean pre- labeled crystallization dish containing the renewal treatment water (16 ±1 ppt and 12 ± 1°C). When hatch was complete (no viable eggs remaining on the slides), final photographs of slides were taken using the Canon Power Shot camera.

Test termination and preparation of larvae for image processing

Hatched larvae were retained in respective crystallization dishes until processing. Within 12-24 h of hatch, herring larvae were briefly anesthetized for viewing purposes (typically for 5 min) using a low dosage of tricaine methanesulfonate (MS-222; 50 mg/L). The 50 mg/L MS-222 concentration was selected as optimum based on anesthetic trials conducted using a series of concentrations ranging from 25 to 200 mg/L MS-222. This concentration was the lowest dosage which immobilized the larvae for imagery and showed no visible adverse effects to the larvae. Higher concentrations of MS-222 showed signs of edema. No fixatives or preservatives were used prior to assessment of normal development, since the associated chemicals can compromise the tissue and skeletal integrity of the specimen. Larvae were not anesthetized for an extended period of time (~5 min) prior to obtaining digital image records. Each specimen was viewed within a crystalline dish using a high-powered microscope (Olympus SZX7 with a DP72 high performance Peltier cooled stage) integrated with a 12.8 MP digital color camera with digital recording software to record each specimen in a lateral plane for archived digital records. Whole-body photo records for each specimen were captured at 25x magnification, and close up views of the peritoneal area were captured at 56x; subsequent image analysis using Image J software magnified each digital image up to 400% so that the specific yolk and pericardial membranes could be closely examined. Once newly hatched larvae were imaged, they were humanly euthanized using a lethal dose of MS-222 (50 mg/L) according to laboratory protocols and then preserved in Davidson’s solution for archival purposes.

Measurement of endpoints

Quantitative measurements of standard length and pericardial and yolk-sac attributes of each larva were made using analytical tools for linear measurements available in Image J software (http://rsbweb.nih.gov/ij were in pixels). The measurements performed on each larva--standard lengt length and depth of the yolk-sac and depth of pericardial region--are shown in Figure 3. Herring larvae are mostly transparent, making the distinction between pericardium and yolk sac difficult at times. Additionally, the post-hatch development of herring larvae proceeds extremely rapidly, and the changing relationships of these cavities make it imperative to properly identify the pericardial boundary prior to assessment of edema. A membrane, the transverse septum, defines the posterior end of the pericardium, securing cardinal and hepatic veins and stabilizing future organ growth regions (e.g. liver) as illustrated in Figure 3. Recognizing the functional aspect of the transverse septum is an important refinement to methods used previously to describe pericardial edema (23-25). Identification of the septum is a crucia step in accurately delineating the pericardial and yolk-sac regions and makin proper assessment of the respective sites of edema. While the pericardi and yolk cavities are adjoined in the early stages of larval development, afte hatch the pericardial and yolk-sac regions of the larvae become spatiall separated by the developing organs in the pectoral fin girdle. Further illustrations of allometric changes in the pericardial and yolk sac and co-locating the transverse septum during these accelerated development stag are presented in the Supplemental Figures 1 and 2. While the yolk sac is ultimately resorbed into the body cavity approximately 4 to 6 days post hatch (dph), the location of the transverse septum remains constant relative to the anterior edge of the dorsal fin where it joins the body wall and appears to be co-located with the anterior edge of the base of the pectoral fin. These two visual reference points were helpful in ascertaining the position of the septum in images where it is not as clearly visible. In these cases, it was a helpful to alternate between light and dark fields, a tool available with Image J software.

The measurement of the depth of the pericardium (PD) and total body depth (BD) were made at the location of the transverse septum; the ratio of these two measurements (PD: BD) is indicative of the relative amount of distention of the pericardium and is unit less. The ratio of the length of the yolk sac to the depth of the yolk at midpoint demonstrates a relative enlargement of the yolk-sac area when it occurs; this ratio is also unit less. The use of th ratios rather than dimensional measurements allows for comparisons among datasets having different magnifications of the images or measurement unit (pixels or millimeters).

For each larva, body axis defects were scored semi-quantitatively; th deviation of the body lateral line from the horizontal plane were assigned an indexed degree of anomaly ranging from 0 (no deformity) to 3 (severe deformity), following the approach presented in McIntosh et al. (24). The associated type of dysfunction were noted: lordosis (convex arched spina curvature), kyphosis (concave arched spinal curvature), scoliosis (sidewa

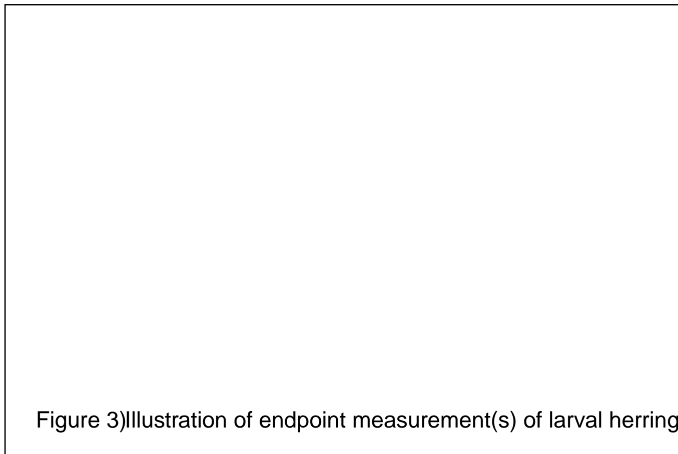

Figure 3)Illustration of endpoint measurement(s) of larval herring specimen

spinal curvature), stunted and thickened trunk (short trunk, thick body mass) and craniofacial (jaw) abnormalities.

Data analyses

All statistical analyses of hatch success and quantitative and semi-qualitative endpoints were performed on the mean of the measurement or score for all larvae in each replicate sample. Frequency of occurrence of edema and body axis defects was calculated by the number of larvae in each replicate exhibiting the abnormality divided by the total hatched larvae in the sample. Larvae with multiple defects were scored in this way for each of the defect types. Treatments were compared with a one-way analysis of variance (ANOVA) with significance set at 0.05. All treatments were then compared to the control sample using a Turkey's HSD statistical test (25), a one-tailed test was used for scores and frequencies and two-tailed tests were used for measurements. All statistical tests were performed with SAS/STAT® software (26).

During the imaging of live specimens, many larvae were observed to be in a later stage of yolk-sac absorption (Figure 4). The larvae in the late stage yolk-sac absorption stage were encountered on days 10 and 12 post-fertilization when the majority of hatch occurred. Because of the large number of larva at hatch there was an increase in the time required for processing the specimens. The numbers of larvae found in this more advanced stage are attributed to their continued development between hatching and the time at which processing actually occurs. On the high hatch dates 9 to 11 days after fertilization, up to 20 randomly selected larvae were imaged from each sample. When more than 20 larvae hatched from a sample in a day, the remaining larvae were photographed after the entire set of laboratory samples was processed. While the overall percentage of larvae in the late-stage category was relatively small (7%), they were not evenly dispersed across the treatments; those treatments observed later in the day had more time for the hatched larvae to develop before imaging occurred. Consequently, the larvae with visually evident resorption of the yolk sac were excluded from assessment of yolk sac and pericardial edema to avoid biasing the results.

RESULTS

Attainment of test experimental conditions

Salinity and temperature were intentionally fluctuated for three test treatments representing a potentially oiled site with multiple stressors, Peninsula Point (PP-1, PP-2 and PP-3), while only salinity was varied in the treatment representing Point San Quentin (PSQ). Changes in laboratory temperature and salinity regimes simulating conditions naturally occurring during February 2008 were successfully achieved as shown in Figure 5. The targeted thermal spikes in all PP treatments were intentional; differences in targeted laboratory temperature exposure and field recordings for PP is due to a perceived margin of temperature elevation not accounted for by the position of the field recordings made by the loggers at deeper water depths, which did not address more extreme temperature shifts and potential for aerial exposure in shallower intertidal water depths resulting from low-tide cycles. Exposure to air in the thermal shock treatment was maintained for a briefer length of time than likely occurred during these low-tide cycles in PP during the 2008 collections.

Pre-hatch development and hatch success

Determination of early death of embryos was based on opacity of the embryo or a disrupted white tissue mass within the chorion often associated with arrested development during the blastula stage (Table 2). Only one treatmen (PP-1) simulating fluctuating salinity conditions from fertilization through the remaining developmental states plus temperature variation had greate than 50% dead embryos at 48 h; the remaining treatments showed 30% or fewer dead embryos. The percentage of viable eggs at a later stage development was calculated by dividing the viable eyed eggs by the number fertilized eggs. Results were different among treatments: PP-1 had the lowe percent viable embryos (43%) while PSQ had the highest percent (80%), th remaining treatments fell within this range (Table 2).

Over 600 larvae hatched during the period from 9 to 14 dpf. Larvae observed swimming in the incubation chambers were tallied in addition to the total number hatched from the glass slides into the glass dishes. Hatch rates we determined by the number of fertilized eggs remaining on the slide at 8 dpf. As shown in Figure 6, PP-16 and PP-28 treatments showed the most variabili in hatch rates. In addition, in the control samples the viable embryos at 2 dpf (70%; Table 2) were essentially the same as the swimming hatchling (70%) indicating, in general, those embryos surviving past 2 dpf in control treatments preceded to normal hatch.

Natural population variability was observed in the control treatments as evidenced by the number of abnormal larvae (2 to 5%), similar to incidence of abnormal development cited for control samples in other studies with San Francisco Bay herring (1,4,27,28).

Growth, morphological and physiological endpoints

In comparison to the control, there were no significant differences in Standard Length (SL). The shortest lengths were found in the temperature stressed development (PP-3) while the largest were in treatments PP-28 a PSQ. The frequency of body axis defects was calculated based on ser qualitative assessment. There were no significant differences among statior compared with the controls (Figures 7 and 8).

The ability to quantitatively measure deviations from normally developing larval fish in assessment of pericardial and yolk-sac edema has importar implications for establishing cause and effect relationships producing the specific edema syndromes. Baseline ranges for normal metrics ar represented by over 90 control measurements. Ratios of relevant allometri yolk and pericardial measurements were computed and tested for difference from control treatments with ANOVA and Turkey's HSD statistical tests. Results of ANOVA tests on the quantitative measurements and examination of the PD: BD ratio summary data indicated that occurrences of pericardial edema were significantly different. Turkey's HSD evaluation revealed no significant differences in PD from control, and only PSQ-22 showed a significant difference from control for BD. The ratio of BD: PD showed no significant differences from control, (Table 3). ANOVA revealed significant differences among treatments for YD, YL, and YD: YL. The differences from the control treatments include PP-28 and PSQ-22 for YL; PSQ-22, PP-TS PP-16 and PP-28 for YD, and CUV, PP-28, PP-TS and PP-16 for the ratio YD YL. Treatments simulating the environmental stressor sites (PP-28, CUV, PP 16 and PP-TS) were found to have significantly higher occurrences of yolk-sa edema as defined by these measurements (Table 3).

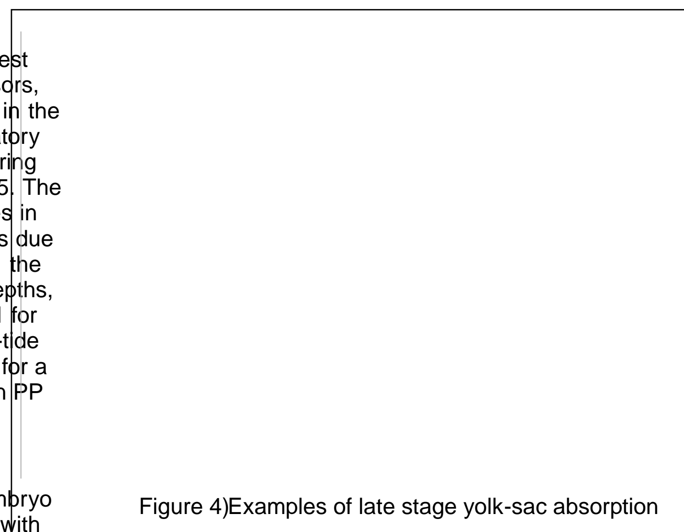

Figure 4)Examples of late stage yolk-sac absorption

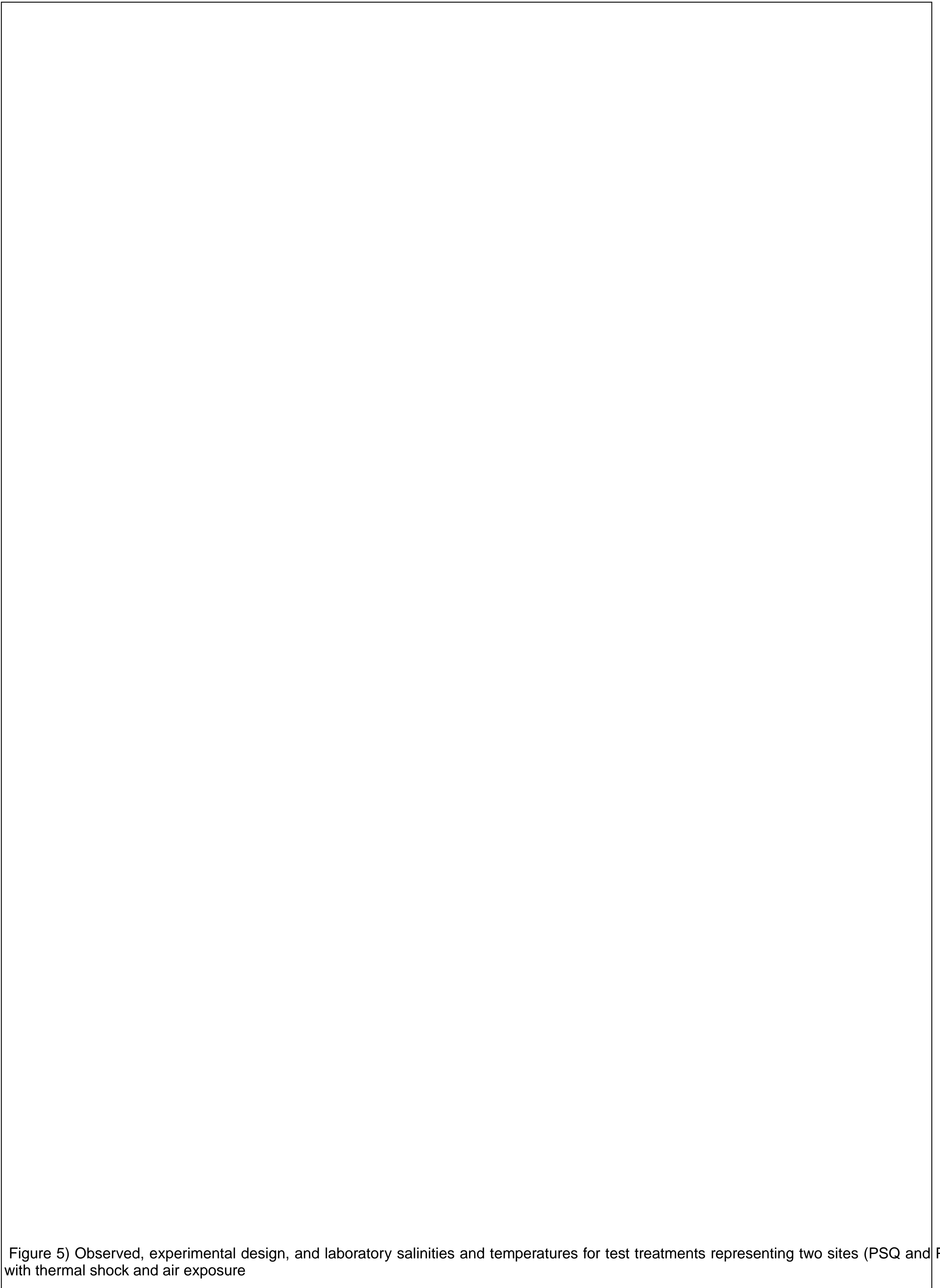

Figure 5) Observed, experimental design, and laboratory salinities and temperatures for test treatments representing two sites (PSQ and PP) an with thermal shock and air exposure

TABLE 3

Results of ANOVA and Turkey test results from quantitative larval measurements, values significantly different from control are in bold. Refer to Figure 3 for description of measurements

Measurement	P	MSDa	Control	Control-2	PSQ	PP-1	PP-2	PP-3
6WDQGDUG OHQJWK PP								
3HULFDUGLDO GHSWK 3' 1RWRFKRUG WR YHQWUDO HGJH PP								
%RG\ GHSWK %' 'RUVDO WR YHQWUDO HGJH PP								
5DWLR 3' %'								
<' <RON GHSWK PP								
</ <RON OHQJWK PP								
5DWLR <' </								

MSDa Minimum significant difference

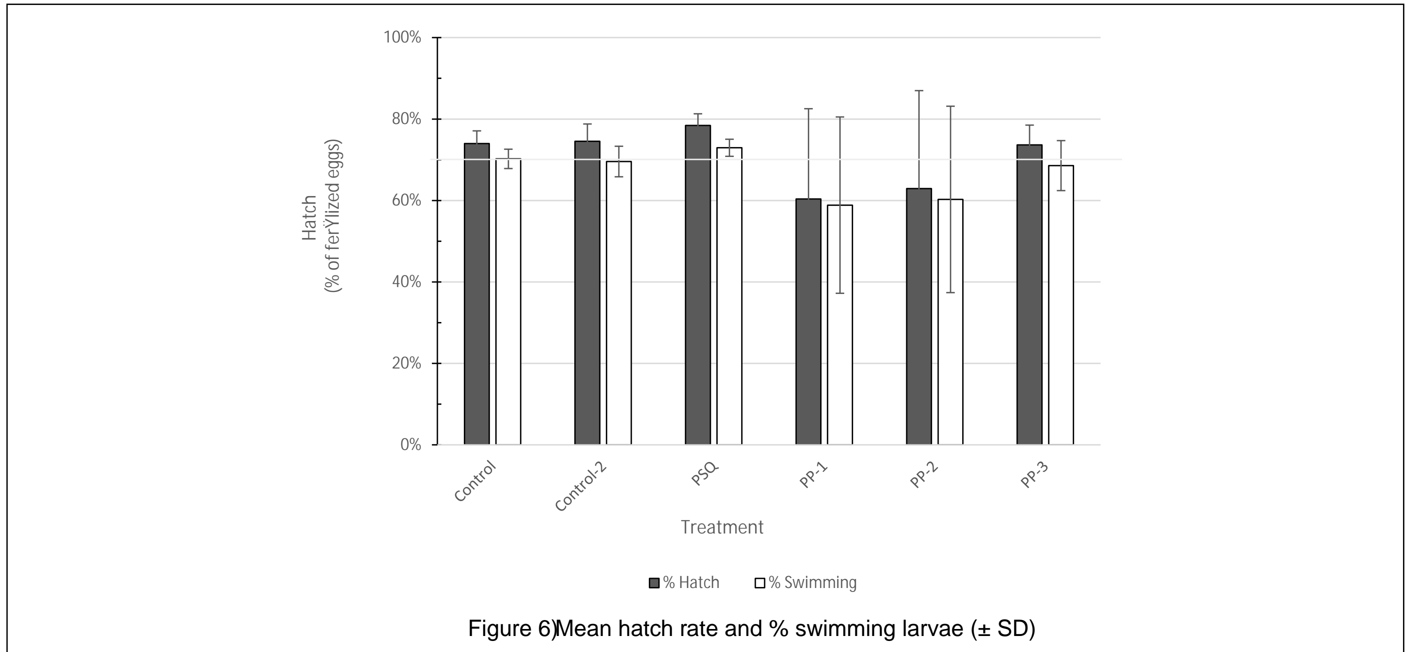

Figure 6)Mean hatch rate and % swimming larvae (± SD)

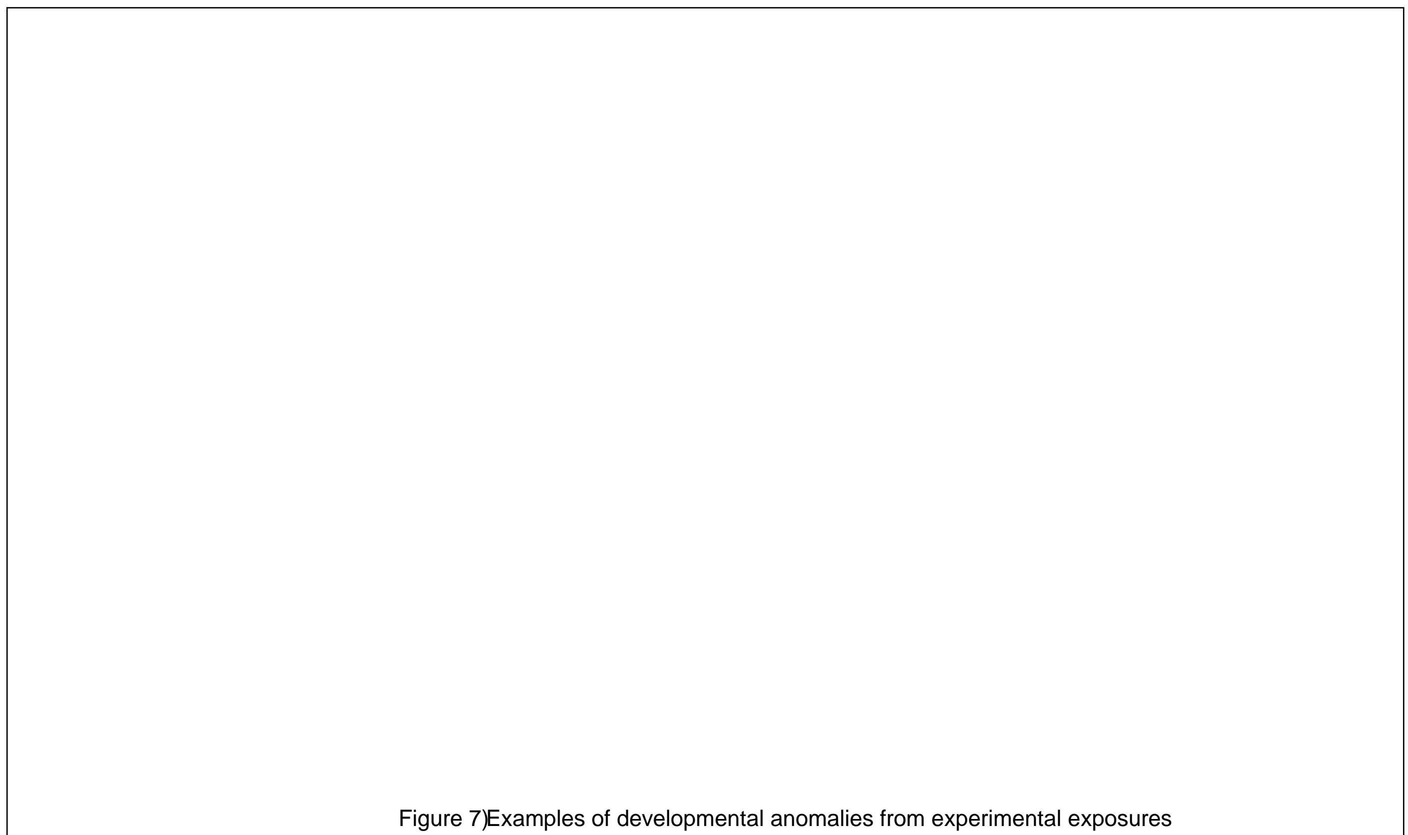

Figure 7)Examples of developmental anomalies from experimental exposures

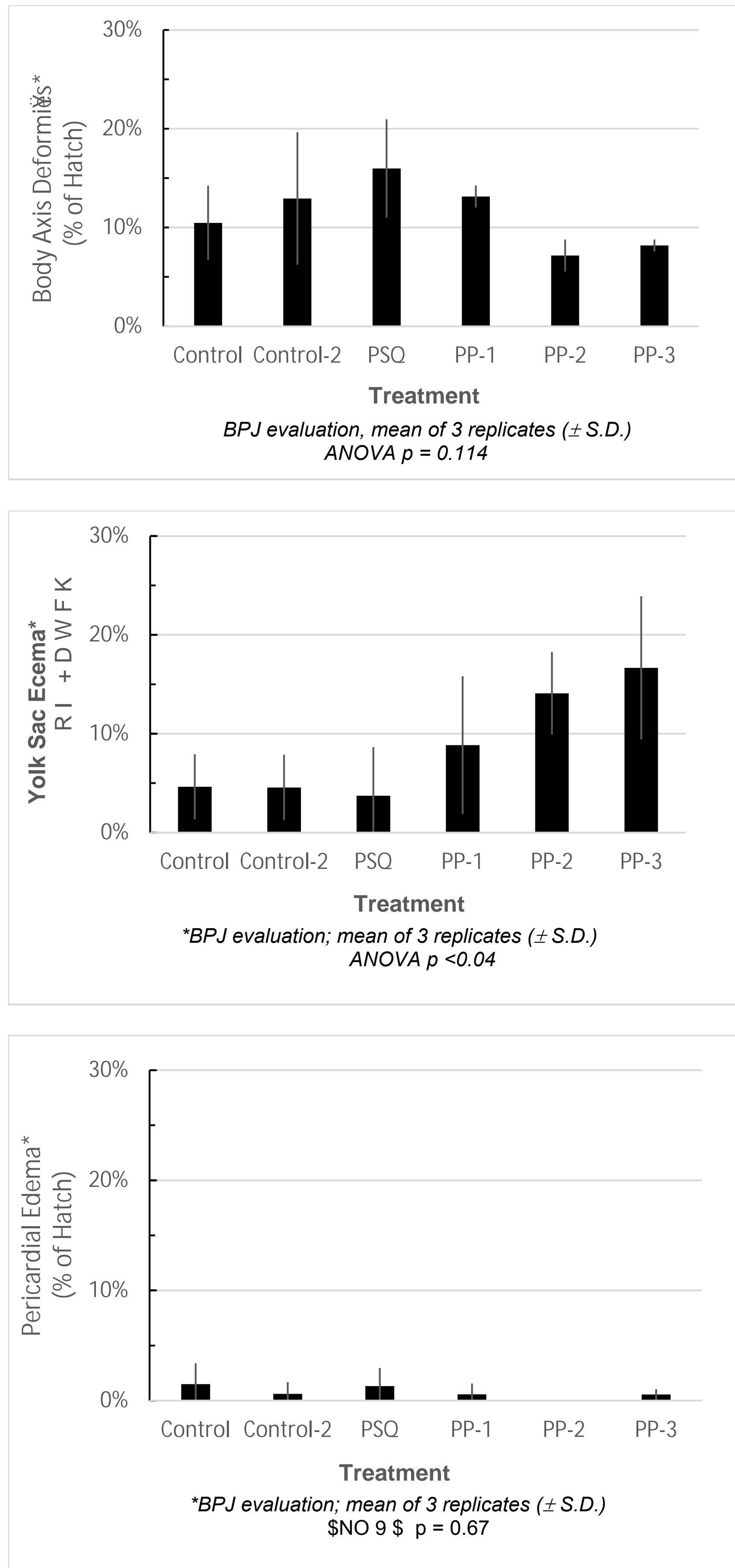

Figure 8) Mean occurrence of (A) severe body axis defects (B) moderate and severe yolk-sac edema and (C) pericardial edema

DISCUSSION

The use of larval herring has an established precedent in evaluations of petroleum impact; published literature has been augmented by resource damage assessments using the Exxon Valdez oil spill (EVOS) in 1989 (9,23,24,29-35). A standardized bioassay method for examination of early-life-stage herring recently published includes a similar suite of biological endpoints such as hatch success, growth, pericardial irregularities (heart rate), and presence/absence of yolk-sac edema (21). The present study evaluated similar endpoints with laboratory exposures based on non-chemical environmental stressors.

Environmental stressors examined during an 8 days laboratory experiment simulating conditions present at Peninsula Point and Point San Quentin during the herring spawn following the CBOS were: fluctuating salinity and temperatures, exposure to UV light, and the influence of air exposure during intertidal stranding episodes during embryonic development. A comparison of targeted salinity and temperature regimes versus measured salinity and temperature data collected during the experiment indicated that we successfully matched the salinity and temperature profile records for the two sites, and achieved a sequencing of elevated water temperature and exposure to warm air temperatures to reflect the more extreme conditions which likely prevail during low tide events. The air exposure in the PP-3 treatment was limited to 1 h sessions in a temperature-controlled incubator in the laboratory to incorporate a representation of slack tidal periods which is likely less than the embryos would have experienced in 2008; consequently, the experimental exposure likely underrepresents the potential stress that may have occurred in 2008 in the shallow intertidal habitat. Test results were based on similar developmental stages of post-hatch larvae. For herring, embryonic development occurs rapidly during organogenesis and larval development is accelerated over the first few days post-hatch (21). This rapid growth results in a narrow time window for making comparable developmental assessments; allowing variability in developmental age severely handicaps the quality of data assessment. Dechorionation disrupts the very rapid growth that occurs during organogenesis, and it is more difficult for the investigator to accurately determine the boundary between the pericardium and yolk sac, especially in very early developmental stages (o and p). Additionally, the transverse septum has a curvilinear aspect during embryonic development that differs from the perpendicular orientation relative to the lateral body plane in naturally hatched larvae (Image 2B, SI). Therefore, our examinations of developmental and physiological anomalies were conducted on naturally hatched larvae to eliminate potential bias that may be induced by using DE chlorinated larvae. Previously, assessment of edema has been problematic because transparency of herring larva makes distinctions between membranes and cavities, such as the peritoneal and pericardial cavities, difficult to determine. Recent advancements in microscopy and digital imagery enable precise delineation of pericardial and yolk areas, if the examiner identifies the transverse septum which clearly separates the pericardium from the peritoneal cavity. The presence of body axis defects is visually apparent and becoming standardized in reporting. A refined approach using a severity index to assign relative degree of observed biological stress has been employed (24), but this approach is also semi-qualitative. Edema has been reported in the scientific community as a significant biomarker of PAH exposure, however previous evaluations have largely been based on subjective and qualitative observations that reflect the experience of the investigator (i.e., best professional judgment). Incardona et al. (36) conducted laboratory studies with marine and freshwater fish that examined effects of Alaska North Slope crude oil on embryogenesis, focusing on edema and cardiac dysfunction and the correlation of toxicity responses to tricyclic (three-ringed PAH) compounds (4,36,37). Although there is mounting evidence of specific etiology of stress created by petroleum hydrocarbons, the limitations of qualitative evaluations compromise verification of these important biological assessments (38). The incidence of abnormally developed embryos at hatch from the control treatment of the present study (10%) was within the range reported from other investigations (≤ 10%) (23,33). Photographs of typical examples of abnormal development taken during the experiment are shown in Figure 7. Adverse effects were identified in association with the environmental factors that were examined (UV exposure, and the combination of temperature and salinity changes). A summary of semi-quantitative evaluations of body axis deformities, yolk sac edema, and pericardial edema is shown in Figure 8. Occurrences of developmental abnormalities varied in the UV treatments and appear to have increased in response to the added stressor of fluctuating salinity (PSQ). Three treatments were higher than the control treatment (Control-2, PP-1 and PSQ) whereas two treatments had lower incidence (PP-2 and PP-3). The trend of developmental skeletal abnormalities keyed to light conditions is supported by observations reported in Strähle et al. (39) in their study on zebra fish (Danio rerio) where exposure to UV in the absence of othe contaminants caused injury during several phases of embryogenesis. In o example offered by Strähle et al. (39), separation of the blastoderm from the yolk cell was noted which formed a vesicle resting on the disintegrating yolk mass. Further UV exposure may disrupt the epiboly processes, which ma lead to grossly retarded embryo development particularly in the trunk and tail regions. More recently, Dinnel et al. (19) evaluated the effects of the visible light spectra on embryo survival and normal hatch rate and concluded that embryo normal hatch rates were significantly reduced under shaded (170 lux and strong-lighting (2650 lux), reducing normal hatch rates to 37% and 44% respectively, compared to 71% normal in dark (0 lux) conditions. Although these authors found that normal hatch rates were not affected by a range o salinity test conditions from 8% to 24%, these test results do not reflect the potential for the incidence of developmental anomalies to be magnified by the additive effect of multiple-stressor exposures.

Fluctuating temperatures and salinities were strongly associated with significant yolk-sac edema, both in intensity and incidence of the response The treatment representing salinity only fluctuations (PSQ) showed occurrence of yolk-sac edema in 4% of the larvae, similar to the Control (5%) or the Control-2 treatment (5%) but well below the occurrence in the treatments representing multiple stressors under simulated Peninsula Poin conditions. Yolk-sac edema was identified in 9 to 14% of hatched larvae in two treatments (PP-1 and PP-2) exposed to both temperature and salinit changes and in 17% of larvae that were additionally subjected to thermal shock simulating the low tide cycles (PP-3). Therefore it appears that th incidence of yolk-sac edema was more closely related to the temperatu changes, air exposure, and possibly salinities between 24 to 28 ppt tha to the changes in salinity in the lower range of 14-24 ppt with constant temperature. Research conducted by others indicates that physical stresso (temperature, salinity, pH, UV, dissolved oxygen in the absence of chemica contaminants) can create biological responses consistent with edema (1 19,35). The occurrence of yolk-sac edema associated with the non-chemic environmental stressors in the present study was comparable to reporte incidence and intensity of edema at the sites potentially impacted by the oil spill in 2007 and investigated during the NRDA assessment in 2008 (NOAA/BML 2008).

In contrast to the elevated incidence of yolk-sac edema resulting from exposure to non-chemical environmental stressors, there was no relationshi between any combination of non-contaminant environmental stressors and the onset of pericardial edema. All specimens exhibited a low incidence of pericardial edema when exposed to non-chemical environmental stressors ranging from 0% to 1% (Figure 8C).

CONCLUSION

Peninsula Point was considered by the NRDA team to be an oiled site, and this location was reported to have large numbers of abnormal larvae (4). A demonstrated by our test scenarios that closely mirrored field conditions at PP and PSQ, PP experienced larger fluctuations of temperature than PSQ While the SCAT teams qualitatively documented the presence of CBO in the water column and at shoreline sites in 2007 (40), the herring eggs tha were spawned in 2008 did not have a CBO chemical signature (1). Incardona and Vines (4) demonstrated that herring embryos exposed to CBO using flow through mesocosm exposures resulted in PAH uptake into egg tissu that matched the chemical signature of CBO obtained from analysis of waterborne profiles. The diagnostic chemical signature of CBO in egg tissue from mesocosm studies and the dose responsive incidence of pericardia edema to elevated concentrations of CBO support the conclusion that CBO exposure can result in the manifestation of pericardial edema (14).

The absence of a CBO chemical signature in the tissues of the naturally spawned 2008 herring eggs indicates that these organisms were not likely have been exposed to CBO. Additionally, our review of images of larvae from eggs collected during February 2008 provided by the damage assessme team using the same quantitative assessment approach presented in th present study indicated that similar rates of yolk-sac and pericardial edem were found in the present laboratory study and the 2008 field investigation. We conclude from the present study that the occurrence of multiple environmental stressors including rapidly changing temperatures and salinity plus thermal shock and air exposure can induce yolk-sac edema in Pacif herring from San Francisco Bay and the frequency of occurrence was simila to assessment of field collected embryos reported elsewhere (3,5,11,17,39-4 These studies highlight the complexity of establishing biological response patterns associated with a particular contaminant or environmental

condition. McDonald et al. (38) recommended that a quantitative or semi-quantitative method be developed to improve the reproducibility of experimental results among various investigators. McIntosh et al. (24) developed a semi-quantitative scoring method based on a severity index, but this is also based on subjective interpretations made by individual researchers. Although pericardial and yolk-sac edema have been widely used to represent sub lethal physiological markers of biological stress caused by petroleum and other contaminants, previous assessments of edema were based on qualitative or semi-qualitative assessments. The degree of variation associated with qualitative assessments can be significant and has led to conflicting conclusions among investigators. Efforts to develop quantitative biological metrics for evaluation of early life history studies should be continued (48-56). It is evident that the use of quantitative measurements following procedures outlined in this paper led to statistically significant differences among treatments, whereas application of our best professional judgment assessment of semi-qualitative scoring procedures identified similar trends but did not identify significant differences in any metric.

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