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PRINCE WILLIAM SOUND HERRING EMBRYO STUDY: SUBLETHAL EFFECTS IN SITU AND IN VITRO 1991-1992

R.M. KOCAN

PROGRESS REPORT
TO
ALASKA DEPARTMENT OF FISH AND GAME

Project Director: Evelyn D. Biggs

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Director

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The authors gratefully acknowledge the technical assistance of the following people: L. Bolles, J. Wilcock, J. Gilman, J. Allen, M. Greene, N. McKrell and J.E. Hose.

KEY WORDS

genetic damage, herring embryos, hydrocarbons, monitoring, mussel uptake, oil toxicity, Prince William Sound, reproductive impairment

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SECTION I. IN VITRO AND IN SITU HERRING STUDIES

R.M. KOCAN

INTRODUCTION

The oil spill that resulted from the grounding of the tanker *Exxon Valdez* on March 23, 1989 coincided with the return of adult herring (*Clupea harengus pallasi*) to Prince William Sound (PWS) in preparation for the spring spawning season. These fish were potentially exposed to petroleum hydrocarbons, as were their eggs and larvae. Histopathologic examination of adult herring collected after the spill demonstrated lesions consistent with oil exposure (Marty et al. 1992). Similarly, naturally spawned herring eggs collected from oiled sites in 1989 produced significantly more physically deformed and genetically damaged larvae than did eggs collected from unoiled sites (Hose et al. submitted). Such sublethal effects of environmental pollution are believed to lead to reduced survival potential at some later period in the organism's life cycle (Rosenthal and Alderdice 1976).

Because the embryos and larvae present at the time of the spill were potentially exposed to petroleum hydrocarbons and would be entering the breeding population in 1993 for the first time as 4-year-olds, these studies attempted to determine if there was indeed any significant damage that could affect subsequent spawning success. To do this, we experimentally exposed artificially spawned Prince William Sound herring embryos to a range of Prudhoe Bay crude oil concentrations for varying periods during their incubation and then evaluated embryo survival, hatching, normal larval development, biomass, and sublethal genetic damage. The objective of the study was to evaluate the toxicity of Prudhoe Bay crude oil in seawater for developing herring embryos and larvae.

MATERIALS AND METHODS

PREPARATION OF WATER SOLUBLE FRACTION

Crude oil was supplied by the National Marine Fisheries Service (NMFS) at Auke Bay, Alaska. For toxicity experiments, a water soluble fraction (WSF) was prepared by shaking 25 ml of crude oil with 1 L (1:40) of 29 ppt synthetic seawater in a 2–L separatory funnel at 8°C for 5 min, then

allowing the mixture to separate in the funnel for 18 hr at 8°C. The funnel was tapped lightly several times to release oil droplets adhering to the glass. The aqueous layer was then drained into a glass-stoppered bottle, designated 100% WSF, and diluted with clean seawater over a range from 100 to 0.1%. A new stock solution and dilutions were prepared every 48 hr until the embryos began hatching.

CHEMICAL ANALYSIS OF WSF

Chemical analyses of WSF were based on the total gas chromatographic peaks observed for low molecular weight (LMW; C6-C12) gasoline range hydrocarbons, and high molecular weight (HMW; C12-C28) diesel range hydrocarbons. LMW samples were analyzed by GC/FID (Purge and Trap) and the HMW samples by GC/FID (both by modified EPA method 8015). Analyses were carried out by Analytical Resources Inc. (Seattle, Washington) on samples prepared at the University of Washington. Analyses were performed on two separate seawater extracts of crude oil, which were split into two equal aliquots. One aliquot was sealed immediately after extraction in a glass-stoppered, acid-washed flask. The second was allowed to stand uncovered with gentle aeration at 8°C for 48 hr, simulating the conditions under which the herring embryos were exposed. The final analyses were then performed on two 0-hr (t_o) and two 48-hr (t_{48}) samples. These values were used to convert "% WSF" to ppm (mg L^{-1}) of dissolved petroleum hydrocarbons. Because LMW hydrocarbons volatilize quickly, the more stable HMW values were used for dilution calculations.

WSF EFFECT ON PHYSICAL CHARACTERISTICS OF SEAWATER

To determine whether WSF had an effect on O_2, pH, or salinity of the incubation seawater independent from the presence of herring embryos, two groups of glass vessels containing 300 ml of WSF in various dilutions were monitored for 48 hr, one with aeration (+) and one without (-). This was done to ensure that any observed embryotoxic effects were not the result of changes in physical or chemical characteristics of the seawater created by interaction with WSF.

SPAWNING

Ripe spawning herring from PWS were used as a source of eggs and sperm. In 1991 spawners were obtained from commercial pounds and consisted primarily of 6 and 7-year-olds. In 1992 spawners were gillnetted during spawning events and consisted primarily 4-year-olds. Eggs were artificially spawned onto glass slides in natural PWS seawater by placing clean glass microscope slides on the bottom of a 4-quart tupperware container, covering them with 6 in of 6°C seawater and broadcasting the eggs over the slides by hand. To minimize inter-female variability, eggs from 6-8 females were randomly distributed to all slides at the desired egg density per slide. Sperm from 3-4 males was then pooled and used to fertilize the eggs. After 1 hr, several slides were examined microscopically to verify that a desired fertilization rate of at least 90% was attained.

EMBRYO TRANSPORT

Slides containing fertile eggs from PWS were placed into an inert plexiglass carrier to prevent slide-to-slide contact during transit, submerged in seawater in a tupperware container, gassed with O_2 and placed in a cooler containing wet ice for transport back to the University of Washington in Seattle. The total elapsed time from fertilization to arrival at the University was 8 hr, and the water temperature on arrival was 6.5°C. Once at the University, the embryos were placed into 29 ppt seawater in an environmental chamber, and the water temperature was allowed to rise slowly to 8°C. This temperature was maintained ± 0.5°C for the remainder of the study.

EXPERIMENTAL VARIABILITY

In order to control for possible transport effects, ripe Puget Sound herring (PS) were transported to the University of Washington and spawned in the environmental chamber described above. These were then incubated simultaneously with the PWS embryos, exposed similarly to WSF, and compared for differences that could be attributed to transport under the described conditions.

EMBRYO EXPOSURE

Continuous Exposure (1991)

Upon arrival at the University of Washington, embryos were placed into 400-ml glass vessels containing 300 ml of WSF ranging from 100% to 0.1%. Each concentration consisted of two vessels containing five slides each. Three control vessels with five slides each received clean seawater. The exposure and control water was changed every 48 hr for 18 days post-fertilization. Larvae were allowed to hatch into clean seawater so that only embryotoxic effects would be measured.

Intermittent Exposure (1992)

To evaluate the sensitivity of various embryonic stages to WSF, we subjected herring embryos to intermittent exposures of WSF. Beginning 24 hr after fertilization (e.g., early blastodisc), and every 24 hr thereafter, a different group of embryos-on-slides was exposed to WSF for 36 hr. This resulted in four groups of embryos being exposed to WSF at 24, 48, 72, and 96 hr post-fertilization. On the basis of previous continuous exposures, it was determined that concentrations of WSF above 10% would be required to elicit a measurable toxic effect over such a short exposure period. The concentrations selected were 2.4, 4.8, and 9.7 ppm, (e.g., 25, 50, and 100% WSF). At the end of each exposure period, the embryos were washed and incubated to hatch in flowing seawater with a complete water exchange every 6 hr.

Continuous and intermittent exposures were carried out in 300 ml of seawater (temperature 8°C and salinity of 29–30 ppt). The embryos were gently aerated for the entire exposure period with an aquarium air pump which delivered approximately 60 bubbles min^{-1} to each exposure vessel

through a fine-tipped pipette. The combination of a high-humidity/low-temperature environmental room and a low rate of aeration resulted in minimal evaporative loss over a 48-hr period. Prior to hatching, the slides were placed in a flow-through system with natural filtered seawater, which was introduced at the surface by means of a pipette. When hatching began, larvae were collected twice daily, fixed in 5% formalin, labeled, and archived for later evaluation.

Scoring Gross Abnormalities

Once eye pigmentation was evident in the embryos (7-10 days), two slides from each treatment group were microscopically examined and the number of infertile, dead and live eggs recorded. After all of the exposure groups had hatched and the larvae were preserved, counts were made of the number of live-hatched larvae (=total hatch). Then the preserved larvae were evaluated for gross physical defects, which included (1) craneo-facial defects, (2) eye defects, (3) spinal defects, (4) fin defects, and (5) pericardial/yolk sac defects. These were recorded separately, but the values were pooled to calculate the total percent normal and abnormal live larvae.

Scoring Genotoxic Damage

The germinal layer between the muscle cells and developing ray structure of the pectoral fin of the newly hatched herring larvae proved to be a good source of mitotic cells (Hose et al. submitted). Twenty-five larvae from each treatment group and controls were randomly selected from a pool of 100+ formalin-preserved larvae for cytogenetic analyses. Pectoral fins were dissected from the larvae and placed on clean microscope slides in a drop of 45% acetic acid for post-fixation. Acetic acid was then removed, replaced with 19 parts saturated orcein in 45% acetic acid plus 1 part proprionic acid, and allowed to stain for 30 min. A cover slip was placed on the stained fins, and they were examined under 1000 X magnification for the presence of mitotic cells and abnormal anaphase/telophase mitotic configurations using the criteria described by Kocan et al. (1982).

Larval Weights

To evaluate changes in larval biomass, we collected larvae from each continuous exposure concentration within 24 hr of hatching; the larvae were fixed in 5% formalin, blotted dry on filter paper, and dehydrated in a desiccator for 5 days. Larvae were then weighed daily until the weights stabilized. Twenty normal and 20 abnormal larvae were weighed from each exposure groups and the results compared with weights obtained from three groups of control larvae. Because many of the larvae were so severely deformed, it was not possible to obtain accurate lengths.

FIELD EXPOSURES IN PWS

Alaska Department of Fish & Game (ADF&G) personnel were shown how to spawn herring onto glass slides and how to deploy them in the field for in situ exposures. They were then allowed to practice the procedures before attempting to carry them out for the final exposures. Field deployment occurred between April 20 and April 25, 1991, at oiled and unoiled sites in Prince William Sound (Fig. I.1). The cassettes containing the field exposed embryos were retrieved from the field

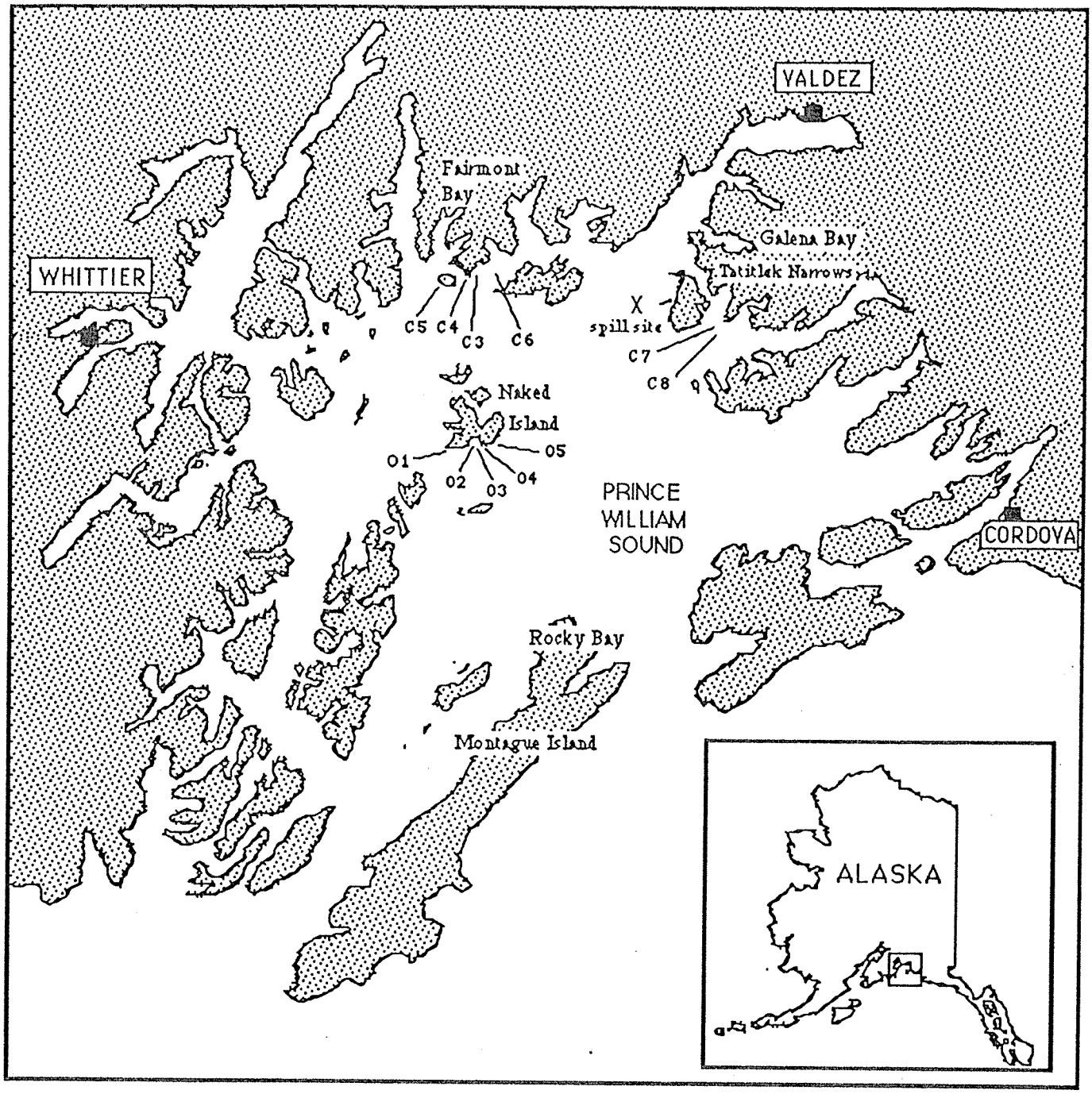

Figure I.1. Map of Prince William Sound showing the oiled sites (O) and unoiled sites (C) where embryos were exposed in situ, and Tatitlak Narrows and Rocky Bay, the sites where 4-year-old herring were collected for reproductive impairment evaluation.

on May 3, 1991. In the laboratory, all slides from each exposure group were placed in a 400-ml glass vessel containing 300 ml of 27 ppt seawater. Each vessel was aerated continuously; the water was changed every 48 hr until hatching.

Each exposure site received 250-350 eggs which were exposed 10-12 days. Embryos were exposed at 10 sites within PWS (designated "C#" and "O#") with deployments at -5 ft and -15 ft below mean low water mark (Fig. I.2).

EXPOSURE SCHEDULE FOR INTERMITTENT EMBRYO EXPOSURES

Hours post fertilization						
0	24	48	72	96	//	hatch
->expose>>>>>>>>>>	---------- clean seawater ----------					->
---------->expose>>>>>>>>>>	---------- clean seawater ----------					->
----------->expose >>>>>>>>>>	--------- clean seawater ----------					->
----------->expose >>>>>>>>>>	-- clean seawater -->					
controls ---------- clean seawater ----------						->

ADULT REPRODUCTIVE FAILURE

This project was designed to evaluate the reproductive potential of individual female herring from four sites within Prince William Sound. Two sites were known to be previously oiled and the other two were unoiled. The target fish were 4-year-olds that were exposed to Prudhoe Bay crude oil as 1-year-olds at the time of the *Exxon Valdez* oil spill in 1989 and were returning as spawners in 1992.

Running ripe females were collected by gillnet from one site in Boulder Bay (Tatitlek Narrows) (April 11 1992) and two sites in Rocky Bay on Montague Island (April 21–22). The Boulder Bay fish were designated "Group A," while the first and second collections at Rocky Bay were designated "Group B" and "Group C," respectively. Because of severe weather and transportation requirements, we were not able to collect two groups from the unoiled site (Boulder Bay).

The females were packed in a cooler to maintain their ambient temperature and returned to Cordova by air where they were given ID numbers and artificially spawned onto glass slides. Approximately 250-500 eggs were obtained from each of 25 females and fertilized with pooled sperm from 5 males. Following spawning, the fish were examined for length, weight, and age, and tissues were collected and preserved for histopathology. The entire process from field collection of fish to completion of spawning occurred within 6 hr. The slides were then placed into a transport

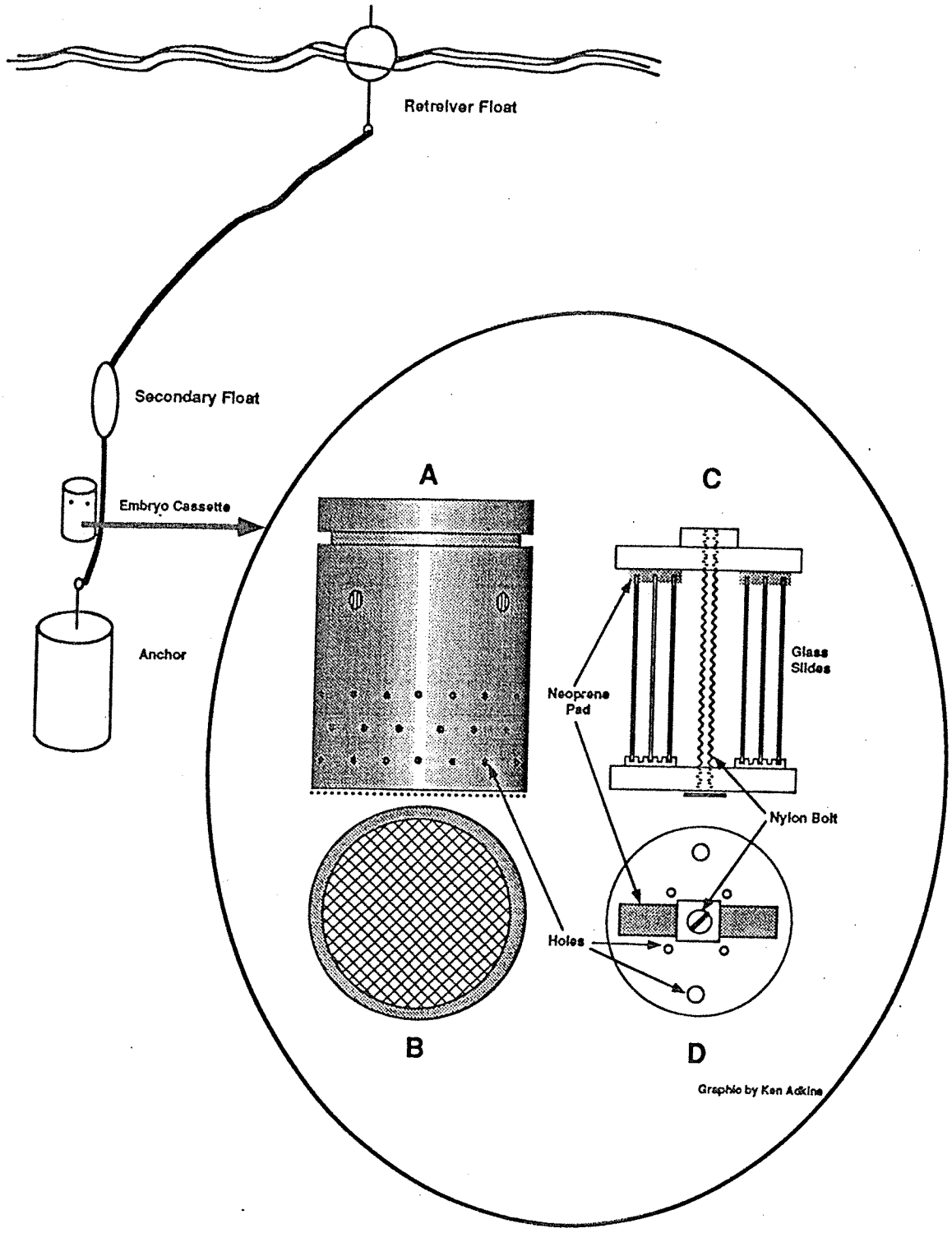

Figure I.2. Diagram of deployment system used to expose developing herring embryos in situ. A. 3" PVC pipe with netting on each end and 1/8" holes in side, used to hold cassette. B. End view of A. C. Plexiglass cassette with six slides. D. End view of C.

SAMPLING SCHEME FOR EVALUATING REPRODUCTIVE FAILURE

Oiled Site I (Rocky Bay) 25 females	**Oiled Site II** (Rocky Bay) 25 females
Unoiled Site I (Boulder Bay) 25 females	**Unoiled Site II** (not collected)

chamber, gassed with pure O_2 and transported by air to the University of Washington Friday Harbor Laboratories for incubation. When embryos arrived at Friday Harbor, they were 24 hr old and in the early blastodisc stage of development. Water temperature was maintained at 5–6°C from time of collection until their arrival at Friday Harbor, where it was allowed to slowly rise to local ambient seawater temperature of 9°C.

Developing eggs from each female were placed in flowing seawater chambers for incubation and were examined for fertility, embryo survival (hatching) and, following hatching, physical defects that would affect their viability. These included (1) spinal deformities, (2) craneo-facial deformities, (3) eye abnormalities, and (4) pericardial abnormalities. Total abnormalities were evaluated in relation to female-to-female variability and oiled vs. unoiled sites.

We anticipated that this study would clarify whether site differences or oiling differences caused abnormal larvae, as well as give some insight into the potential natural variability from individual females of a specific year class.

RESULTS (IN VITRO EXPOSURE)

CONCENTRATION OF WSF IN SEAWATER

The analytical data for WSF of crude oil is presented in Table I.1. The mean concentration of LMW components (C6-C12) at t_o was 64 (62-66) mg L^{-1} and at t_{48} was 9.7 (8.4-11) mg L^{-1}, indicating a loss of 54.3 mg/L into the atmosphere. The mean concentration for the HMW components (C12-C28) was 9.2 (9.0-9.4) mg L^{-1} at t_o and 10.2 (7.3-13) at t_{48}, indicating no net loss. The mean for all values (t_o to t_{48}) of HMW components was 9.67 mg L^{-1}, the value used to calculate the EC_{50} for the embryo toxicity titration.

Table I.1. Concentration of WSF components of Prudhoe Bay crude oil in seawater.

		t_o	t_{48}
C6 - C12	A	62	8.4
(gasoline)	B	66	11.0
C12-C28	A	9.4	13.0
(diesel)	B	9.0	7.3

PHYSICAL CHARACTERISTICS OF SEAWATER

Table I.2 summarizes the results of physical measurements taken from both aerated and unaerated WSF-treated seawater without herring embryos. Previous experience has indicated that dissolved oxygen is a critical factor for successful hatching of herring larvae. Under laboratory conditions it appears that even slight reductions in O_2 will result in large numbers of larvae dying during the hatching process. This experiment was performed to determine whether WSF had an effect on oxygen concentration, independent of the presence of hatching embryos.

WSF TITRATION IN VITRO

Table I.3 summarizes the slide-to-slide variability data. No significant differences were apparent in infertile eggs, spontaneous mortality of embryos, and live hatched larvae when exposed and unexposed embryos were compared on a slide-by-slide basis.

Hatching Dynamics

Observations on the day-to-hatch and mean hatching day post-fertilization revealed that exposure to the higher concentrations (>0.24 mg L^{-1}) of WSF resulted in embryos hatching 4 to 5 days earlier (mean = 15 days) than the controls and lower WSF concentration exposures (mean = 19–20 days). This data is summarized in Figure I.3. The concentration of WSF required to produce this effect is similar to that which produces significant increases in physically deformed larvae (Fig. I.4).

Table I.4 summarizes the data obtained from continuous exposure of PWS herring embryos to varying concentrations of Prudhoe Bay crude oil WSF. In general, WSF of crude oil had no effect on survival or live hatch of herring embryos, but it did have a dramatic effect on the percent of physically defective larvae that successfully hatched. Physical deformities included spinal deformity (scoliosis/lordosis), optic malformations, mandibular malformations, and an enlarged pericardial region. These defects appeared not to be pathognomonic, so they were all considered together for the purpose of this study (e.g., total abnormal larvae). Figure I.4 summarizes the EC_{50} titration for physical deformities using real data from larvae exposed to WSF as embryos. Figure I.5 shows the relationship between pericardial edema and all

Table I.2. Physical characteristics of seawater with WSF.

% WSF	± Air	Temp. (°C)	DO (mg/L)	pH	ppt
time 0					
0	-	9.6	9.40	8.34	27
1	-	9.6	9.10	8.34	27
10	-	9.5	9.20	8.33	28
50	-	9.6	9.00	8.21	29
100	-	9.6	9.00	8.06	29
mean		**9.58**	**9.14**	**8.26**	**28.O**
stdev		0.04	0.17	0.12	1.00
0	+	9.7	9.40	8.19	29
1	+	9.5	9.20	8.15	29
10	+	9.5	9.20	8.17	30
50	+	9.5	9.30	8.1	30
100	+	9.4	9.10	8.09	30
mean		**9.52**	**9.24**	**8.14**	**29.6**
stdev		0.11	0.11	0.04	0.55
time 24					
0	-	8.6	10.3	8.36	29
1	-	8.6	10.2	8.36	29
10	-	8.7	10.1	8.36	29
50	-	8.7	10.2	8.34	29
100	-	8.7	10.2	8.33	30
mean		**8.66**	**10.2**	**8.35**	**29.2**
stdev		0.05	0.07	0.01	0.45
0	+	8.6	10.2	8.43	30
1	+	8.5	10.1	8.42	30
10	+	8.5	10.2	8.43	30
50	+	8.5	10.2	8.41	30
100	+	8.6	10.1	8.35	31
mean		**8.54**	**10.16**	**8.41**	**30.2**
stdev		0.05	0.05	0.03	0.45
time 48					
0	-	8.1	11.8	8.60	29
1	-	8.1	11.7	8.55	29
10	-	8.1	11.6	8.56	29
50	-	8.1	11.6	8.56	30
100	-	8.1	11.7	8.58	31
mean		**8.10**	**11.68**	**8.57**	**29.6**
stdev		0.00	0.08	0.02	0.89
0	+	8.1	11.7	8.47	30
1	+	7.9	11.5	8.48	31
10	+	7.9	11.6	8.48	31
50	+	8.1	11.6	8.47	31
100	+	8.1	11.7	8.50	32
mean		**8.02**	**11.62**	**8.48**	**31**
stdev		0.11	0.08	0.01	0.71

Table I.3. Interslide variability.

Slide #	Total eggs per slide	Infertile eggs	Dead embryos	Live hatch
1	213	7	31	175
2	198	4	28	166
3	242	8	31	203
4	258	3	18	237
5	230	4	28	198
6	224	17	27	180
7	174	7	31	136
8	231	11	20	200
9	186	11	38	137
10	201	9	28	164
Total	**2157**	**81**	**280**	**1796**
%	**---**	**3.76**	**12.98**	**86.51**
Mean/slide	**216**	**8**	**28**	**180**
Stdev	**26.13**	**4.20**	**5.70**	**31.20**
25%1	181	8	37	136
2	242	10	32	200
3	182	7	38	137
4	198	7	27	164
5	191	6	28	157
50%1	328	4	26	298
2	370	2	15	353
3	249	12	57	180
4	358	13	60	285
5	238	5	35	198
Total	**2537**	**74**	**355**	**2108**
%	**---**	**2.92**	**13.99**	**85.59**
Mean/slide	**254**	**7**	**36**	**211**
St. dev.	**72.87**	**3.47**	**13.83**	**75.04**

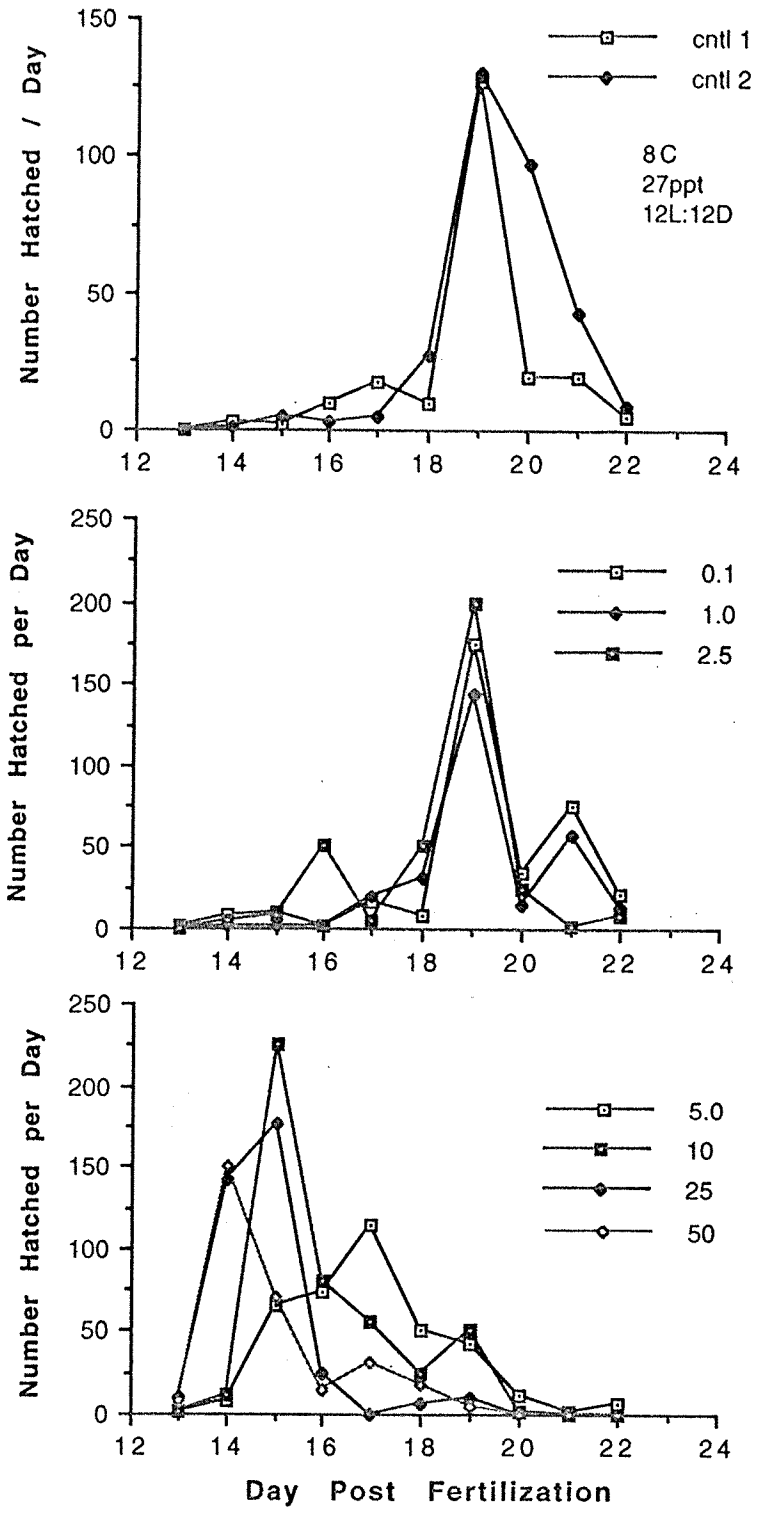

Figure I.3. Exposure of herring embryos to WSF of Prudhoe Bay crude oil throughout incubation resulted in a precocious hatch for those exposed to 0.48 ppm or greater. Mean hatch day for control embryos was 19–20 days post fertilization. Hatching dynamics for embryos exposed to WSF concentrations <0.48 ppm were the same as for controls.

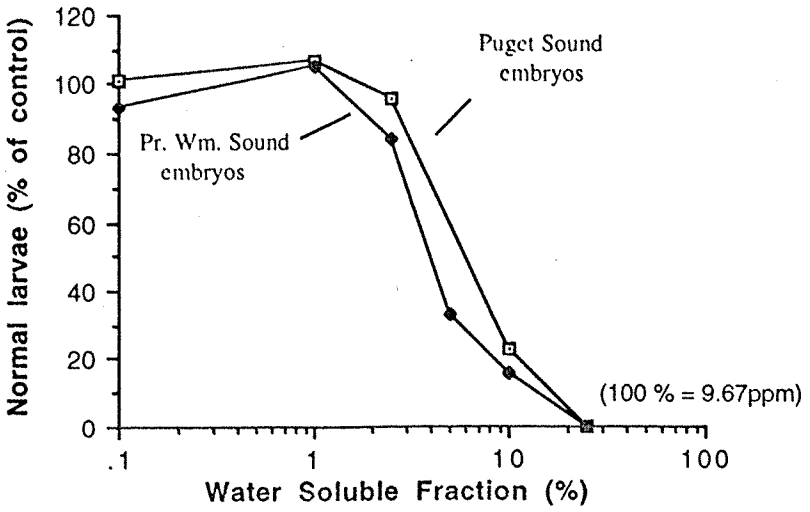

Figure I.4. Dose-response for physical deformities in herring embryos from Prince William Sound, Alaska, and Puget Sound, Washington. The EC_{50} for gross larval abnormalities is approximately 4.5% of the stock WSF. No significant difference occurred in response between Prince William Sound and Puget Sound herring embryos.

Table I.4. Results of continuous exposure of herring embryos to varying concentrations of WSF.

%	ppm[a] (mg L^{-1})	Live hatch	% hatch	Normal larvae	% normal
Controls					
0[b]	0	175	61.40	103	59
0	0	166	75.80	84	51
0	0	203	83.20	110	54
Mean	0	181	73.47	99	55
stdev	-	19.3	11.1	13.5	4.0
Treated					
0.1	0.01	237	72.92	122	51
1.0	0.10	198	79.20	114	58
2.5	0.24	180	84.91	82	46
5.0	0.48	136	86.08	25	18
10	0.97	200	92.17	17	9
25	2.41	137	74.86	0	0
50	4.83	164	72.89	0	0
100	9.67	157	70.40	0	0
Mean	---	176	79.18	45	---
stdev	---	34.69	7.78	---	---

[a]ppm dilutions calculated from HMW value (100% WSF = 9.67 mg/L).

[b]Three replicates of untreated controls.

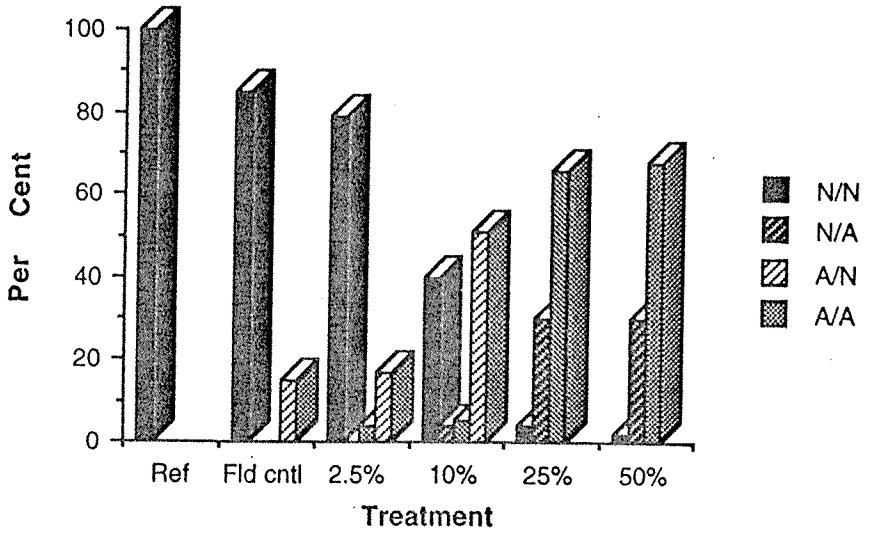

Figure I.5. Relationship between pericardial edema and all other physical defects. Ref = normal larvae; Fld cntl = larvae from clean seawater; % = % WSF of crude oil. N/N = normal body/normal pericardium; A/A = abnormal body/abnormal pericardium, etc. (i.e., body/pericardium).

other physical defects. Some investigators consider this pericardial edema to be diagnostic of oil exposure, but since the data presented here show that it is not expressed independently of other defects, it was not reported separately.

Using EPA's Probit Analysis Program for data from acute and short-term toxicity tests with aquatic organisms (EPA Biological Methods Branch, Cincinnati, Ohio), we analyzed the data from Table I.4 and generated an EC_1 to EC_{99} curve (Fig. I.6). From this analysis, it is apparent that the EC_{50} is 4.46% or 0.43 mg L^{-1} of the HMW components of Prudhoe Bay WSF. The EC_1 is 0.8% (0.08 mg L^{-1}) and the EC_{99} is 24.7% (2.39 mg L^{-1}). By comparing these values to those measured in the field, we may predict the number of larvae that would be affected following exposure to WSF in situ.

To evaluate any significant change in larval biomass which may have resulted from exposure to WSF, we collected 20 larvae from each exposure concentration within 24 hr of hatching; the larvae were fixed in 5% formalin and dehydrated in a desiccator for 5 days. The entire group of 20 was weighed together and the results compared with those obtained from control larvae (Fig. I.7). The normal untreated larvae were 71% heavier (2.4 mg) than the untreated abnormal larvae (1.4 mg). Weights of normal larvae decreased as the WSF concentration increased, but weights of abnormal larvae remained constant.

Genotoxicity

Table I.5 summarizes the results obtained from cytogenetic analysis of newly hatched herring larvae. A significant reduction in the number of mitotic cells per fin occurred at levels ≥0.24 ppm ($P < 0.05$; one-way ANOVA). Also, anaphase/telophase aberrations increased significantly at

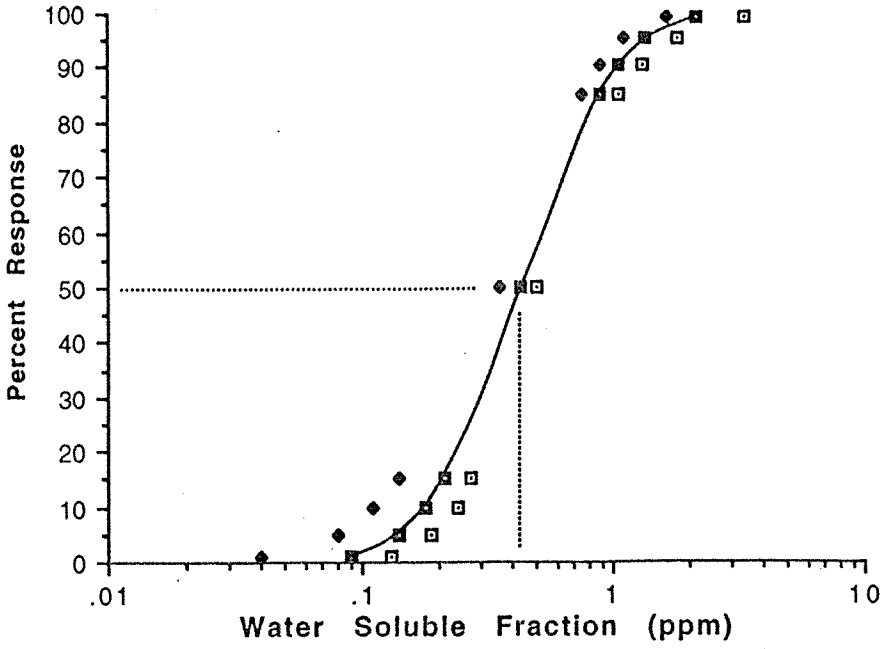

Figure I.6. Probit analysis of physical defect data showing EC_1 to EC_{99} values and 95% confidence limits for herring larvae exposed continuously to WSF from early blastodisc to just pre-hatch. $EC_5 = 0.12$ mg L^{-1}, $EC_{50} = 0.43$ mg L^{-1}, $EC_{95} = 1.12$ mg L^{-1}.

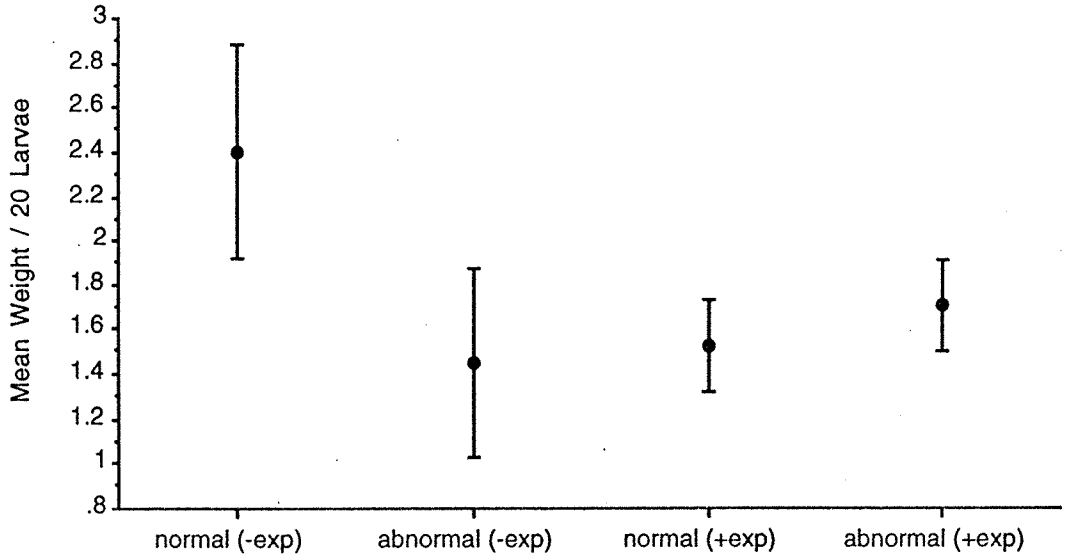

Figure I.7. Mean weight (mg ±SD) for herring larvae exposed continuously to WSF as embryos. Unexposed (-exp) and exposed (+exp) groups both produced physically normal and abnormal larvae, but larvae from the normal-unexposed group (e.g., normal controls) were significantly larger than all other groups ($P < 0.05$; Fisher PLSD). Petroleum hydrocarbons appear to affect the weight of normal larvae but not abnormal larvae. Data represent mean of three replicates with 20 larvae each (N=60).

Table I.5. Mitotic activity and genotoxic damage in larvae continuously exposed as embryos to WSF of Prudhoe Bay crude oil.

Dose (ppm)	Mitoses per fin	% anaphase aberrations
0.00	7.6	8.3
0.01	8.1	16.1
0.10	7.6	18.0 [b]
0.24	5.5 [a]	25.4 [b]
0.48	4.4 [a]	24.0 [b]
0.97	6.8 [a]	26.6 [b]
2.42	3.9 [a]	29.6 [b]
4.84	5.2 [a]	39.6 [b]
9.67	1.1 [a]	57.1 [b]

[a]Significantly different from control ($P < 0.05$).
[b]Significantly different from control ($P < 0.01$).

0.1 ppm ($P < 0.05$; G stat.). The number of aberrant anaphase/telophase cells from the 0.01ppm exposure was double the control value, but not statistically significant.

EMBRYO STAGE SENSITIVITY

Table I.6 summarizes the data obtained from exposure of herring embryos in various stages of development to WSF of Prudhoe Bay crude oil. Exposures began at 24, 48, 72, and 96 hr post fertilization and lasted for 36 hr.

The higher WSF concentrations and earliest exposure periods had the greatest effect on hatching success of herring embryos (Fig. I.8). The 24– and 48–hr developmental stages were the most severely affected, with as much as 20–45% reduction in hatching success relative to the controls. By 96 hr post-fertilization, hatching success returned to 80–100% of the control levels.

Increasing concentrations of WSF produced a decrease in the percent of normal (viable) larvae (Fig. I.9), while the embryonic stage exposed to WSF had no detectable influence on normal larval development (Fig. I.10).

RESULTS (IN SITU EXPOSURE)

Table I.7 summarizes the results obtained from herring embryos incubated in the field (in situ) at oiled and unoiled sites and then hatched in clean seawater in the laboratory. The mean number of embryos hatching from the unoiled group (C) was significantly lower than in the oiled group (O) ($p < 0.01$; one-tail t-test). Since no differences in hatching success were observed in the in vitro experimental exposures, it is not possible to interpret this data at this time.

Table I.6. Summary of the data from 36 hr exposure of herring embryos to increasing concentrations of WSF of Prudhoe Bay crude oil at four different stages of development.

Treatment conc/time	N	% infertile	Mean total live hatch	% live hatch (a)	% (b) abnormal	% viable hatch (c)
cntl A	177	4				
cntl B	270	1				
mean	224	3	297	66	22	58
24 hr						
25% A	181	1				
25% B	156	4				
mean	169	2	203	60	16	51
50% A	200	3				
50% B	160	6				
mean	180	4	162	45	20	36
100% A	140	6				
100% B	160	4				
mean	150	5	146	49	43	28
48 hr						
25% A	161	5				
25% B	138	2				
mean	150	4	126	42	12	37
50% A	137	5				
50% B	141	4				
mean	139	5	130	47	20	37
100% A	120	0				
100% B	185	1				
mean	153	0	111	36	44	20

Table I.6—cont.

Treatment conc/time	N	% infertile	Mean total live hatch	% live hatch (a)	% (b) abnormal	% viable hatch (c)
72 hr						
25% A	132	2				
25% B	105	3				
mean	119	2	136	59	14	49
50% A	155	5				
50% B	171	5				
mean	163	5	126	40	29	27
100% A	178	4				
100% B	133	7				
mean	156	6	116	39	28	27
96 hr						
25% A	183	2				
25% B	167	1				
mean	175	1	186	53	40	32
50% A	64	0				
50% B	220	2				
mean	142	1	139	49	47	26
100% A	167	4				
100% B	174	5				
Mean	171	4	189	57	48	29

[a]% of fertile eggs producing living larvae.
[b]% of live larvae which were abnormal.
[c]% of fertile eggs producing normal live larvae (e.g., viable larvae).

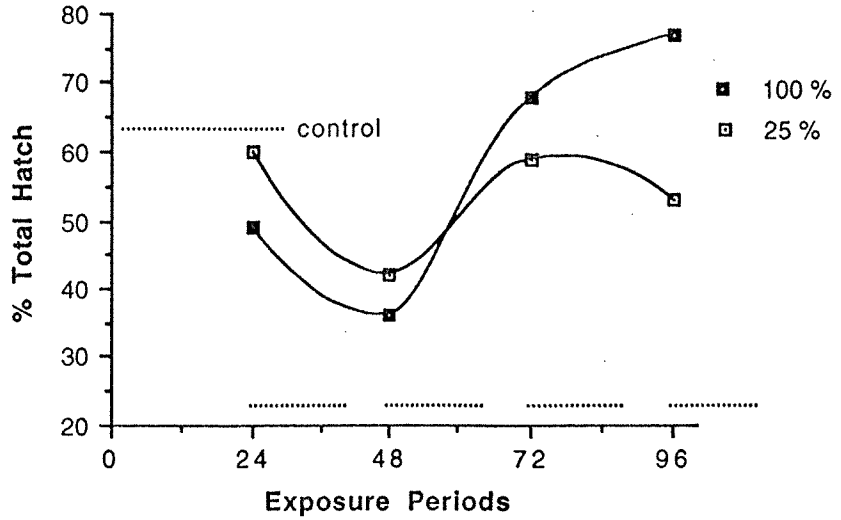

Figure I.8. Effect of WSF on herring hatch relative to developmental stage exposed.

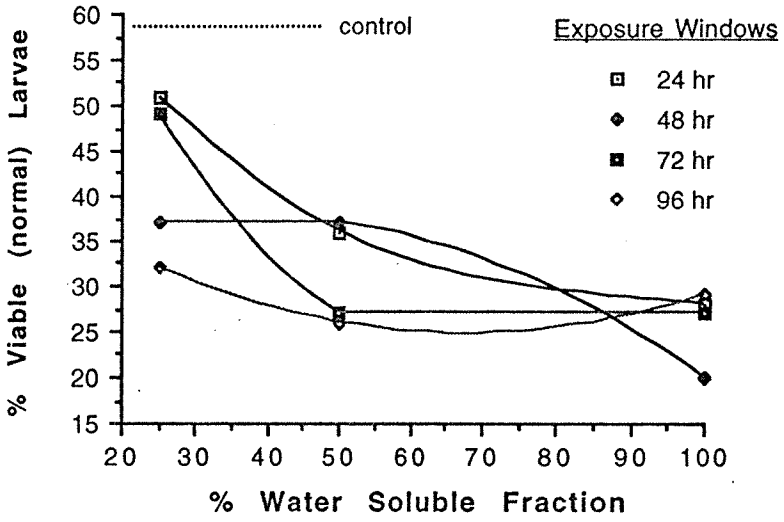

Figure I.9. Percent viable larvae over the range of WSF at 24-, 48-, 72-, and 96-hr. Increasing WSF concentrations produced a reduction in normal (viable) larvae at all exposure stages tested.

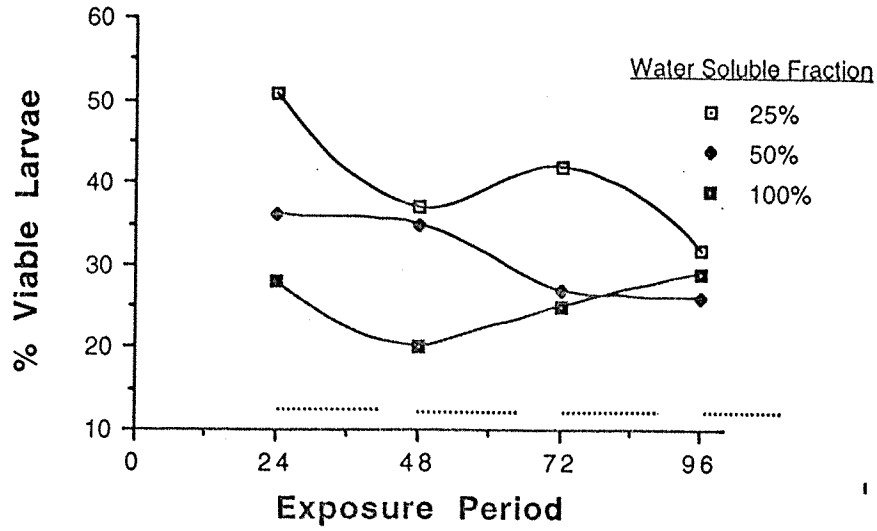

Figure I.10. Percent viable larvae relative to WSF concentration and embryonic stage exposed.

Table I.7. Percent hatch, normal and viable larvae from in situ exposures of herring embryos in Prince William Sound 3 years after the *Exxon Valdez* oil spill.

	Unoiled sites (C)					Oiled sites (O)			
	% hatch		% normal			% hatch		% normal	
Depth	(-5)	(-15)	(-5)	(-15)		(-5)	(-15)	(-5)	(-15)
Site					Site				
3	64.8	71.8	66.5	78.2	1	64.4	–	55.2	–
4	56.4	–	67.1	–	2	66.3	70.5	53.3	45.7
6	58.9	59.1	33.7	72.6	3	68.8	–	29.2	–
7	61.8	54.2	50.5	70.2	4	68.5	68.7	65.3	47.9
8	54.9	47.8	67.9	63.1	5	70.5	69.4	54.7	59.1
Mean	59.3	58.2	57.1	70.9		67.7	69.5	51.5	50.9
Stdev	4.0	10.1	14.9	6.3		2.4	0.9	13.4	7.2

Total		mean	SD	Total		mean	SD
% hatch	=	58.8	6.9	% hatch	=	68.4	2.1
% normal	=	63.3	12.6	% normal	=	51.3	10.1
% viable	=	37.2	10.0	% viable	=	35.1	10.8

Note: % hatch and % viable based on total fertile eggs
% normal based on live hatch
– no exposure at this depth

Abnormal Larvae

A significantly greater number of normal larvae hatched from "C," the unoiled group (63.3%), than from "O," the oiled group (51.3%) ($p < 0.002$; t-test). This response is similar to that observed in vitro when embryos were exposed to the WSF of crude oil. Depth did not appear to affect the production of abnormal larvae in either oiled or unoiled sites (Table I.7).

Larval Weights

A dry weight analysis of larvae showed that the mean dry weight of both normal and abnormal larvae from the "0" group was significantly lower than the weights of the "C" group ($p < 0.01$; t-test). Table I.8 summarizes the weight data ($\mu g\ larva^{-1}$) from each sample group as normal, abnormal, and combined mean dry weights.

ADULT REPRODUCTIVE FAILURE

The percent total hatch observed in the two spawning groups (B, C) collected in Rocky Bay, a previously oiled site, was significantly lower than that observed in spawners collected in Boulder Bay, a non-oiled site. Figure I.11 summarizes the mean ± SD data for the three spawning groups. Percent hatch was calculated by the following equation:

Table I.8. Mean dry weights of herring larvae exposed in situ at unoiled (C) and oiled (O) sites 3 years after the *Exxon Valdez* oil spill in Prince William Sound (values = $\mu g\ larvae^{-1}$; mean of 20 individuals).

	Unoiled sites (C)					Oiled sites (O)			
Depth	(-5) norm	(-15) abnorm	(-5) norm	(-15) abnorm		(-5) norm	(-15) abnorm	(-5) norm	(-15) abnorm
Site					Site				
3	52	71	66	112	1	66	81	-	-
4	83	138	-	-	2	63	101	97	56
6	78	106	104	98	3	93	82	-	-
7	119	143	135	83	4	48	71	51	86
8	103	96	106	61	5	94	86	89	84
Mean	87	111	102	88		72	84	79	75
Stdev	26	30	28	22	20	11	24	17	

"C"		Mean	SD	"O"		Mean	SD
All normal	=	94	25	All normal	=	75	19
All abnormal	=	101	26	all abnormal	=	81	12
Total	=	97	26	Total	=	78	17

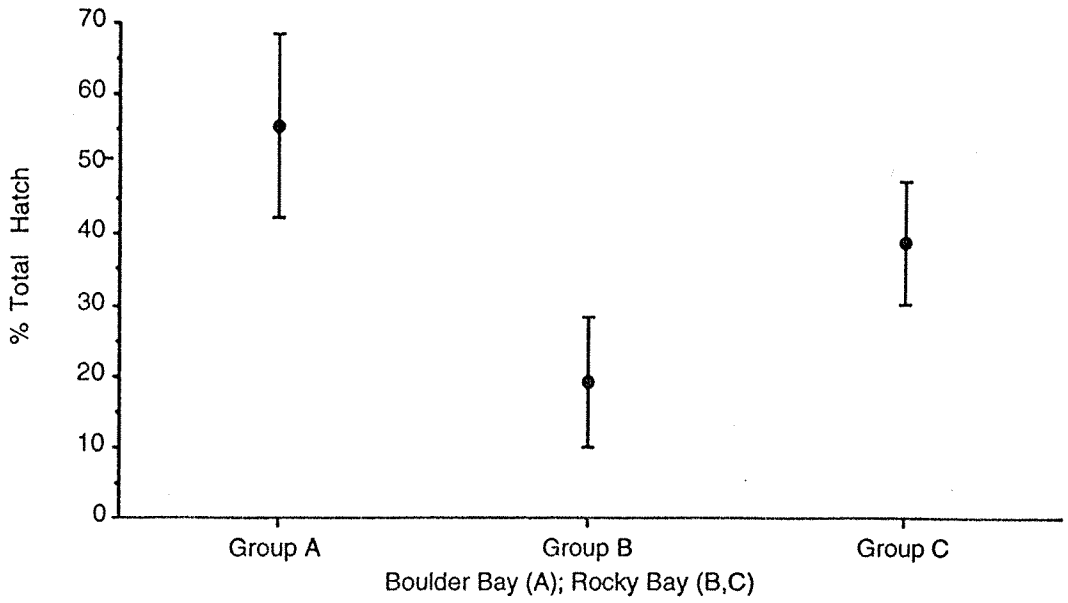

Figure I.11. Percent total hatch of eggs derived from females collected at oiled and unoiled sites in Prince William Sound.

$$\text{Mean \% hatch} = \frac{\text{\# live larvae}}{\text{\# fertile eggs}} \times 100. \qquad \text{(I.1)}$$

Figure I.12 summarizes the fish-by-fish embryo hatching success for the same three groups of spawners, displayed in ranked order. The number of normal (e.g., viable) larvae resulting from the total live hatch was calculated as follows:

$$\text{\% viable larvae} = \frac{\text{(\# live larvae) - (\# abnormal larvae)}}{\text{\# fertile eggs}} \times 100. \qquad \text{(I.2)}$$

Figure I.13 summarizes the mean ± SD of viable larvae produced by the three groups of spawners. The percent of abnormal (e.g., non-viable) larvae was significantly lower in groups B ($P < 0.001$) and C ($P < 0.003$; one-tail t-test). This same relationship held true when the data are presented for each female in rank order (Fig. I.14).

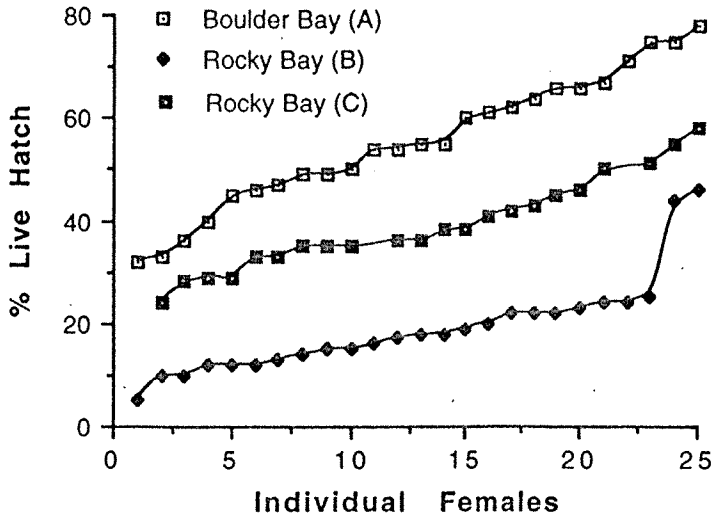

Figure I.12. Hatching success of individual spawners from Boulder Bay and Rocky Bay displayed in ranked order. Significantly fewer larvae successfully hatched from the two groups collected at the previously oiled Rocky Bay.

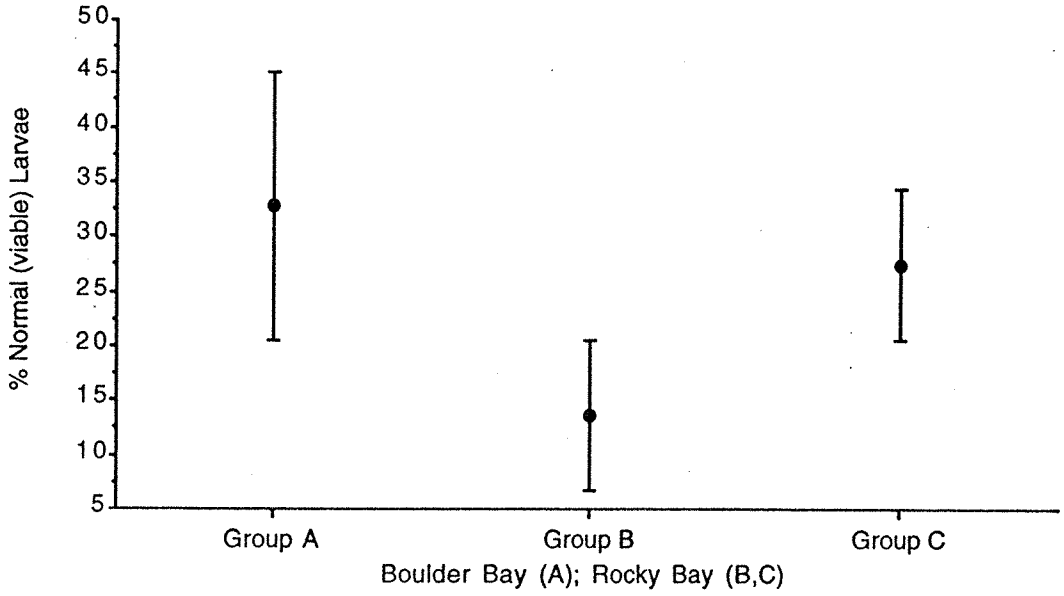

Figure I.13. Viable (normal) larvae produced by female herring collected from oiled and unoiled sites in Prince William Sound.

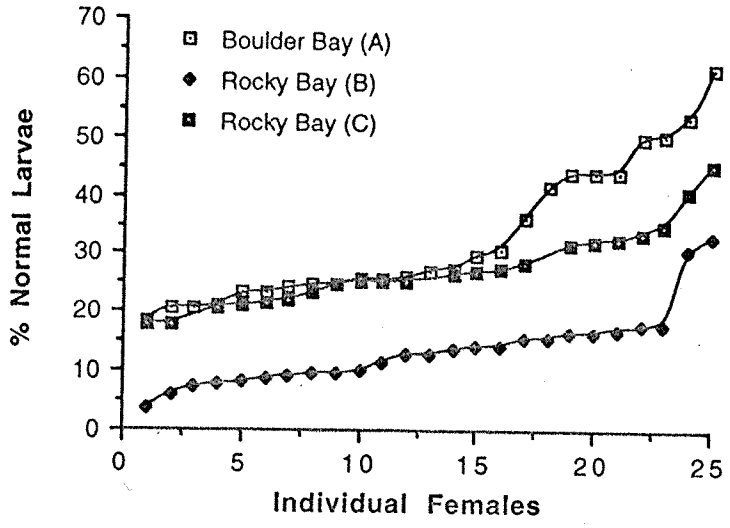

Figure I.14. The percentage of viable (normal) larvae produced by two groups of spawners. Normal spawner production at Rocky Bay (the oiled site) was significantly lower than that of Boulder Bay (a non-oiled site) ($P < 0.001$ and 0.003; one-tail t-test).

DISCUSSION

EMBRYO SENSITIVITY

Water Soluble Fraction

The concentration of LMW petroleum hydrocarbons (C6–C12) decreased from 64 (±2) ppm (mg L^{-1}) to 9.7 (±1.3) ppm between 0 and 48 hr. HMW hydrocarbons (C12–C28) remained constant for the entire 48–hr period at 9.67 (±2) ppm. Linden (1978) reported concentrations of 4.6–14.1 ppm WSF for two different crude oils, and these concentrations declined by 25 to 40% after 48 hr. Consequently, it was concluded that embryos in this study were exposed intermittently to high concentrations of LMW petroleum hydrocarbons and to constant concentrations of HMW hydrocarbons throughout their incubation.

Embryo Survival (Live Hatch)

With the exception of concentrations >2.0 ppm, embryo survival was not significantly affected by continuous exposure to WSF. This is in contrast to other studies that demonstrate high embryo mortality following exposure to WSF. Smith and Cameron (1979) report no decrease in hatching success of embryos exposed to 0.68 ppm WSF for up to 48 hr, but that 100% mortality occurred when exposures lasted for 6 days. Rice et al. (1987) reported mortality rates up to 100% in embryos exposed for 12 days (LC_{50} = 1.5 ppm), but reported no mortality in embryos exposed for

2 days (LC_{50} >5.3 ppm). Linden (1978) observed no effect on hatching when embryos were exposed to 1 ppm or less, but demonstrated over 25% embryo mortality at 10 ppm for two different crude oils. Pearson et al. (1985) reported results similar to ours, except that their exposures lasted only 24 hr.

Other investigators have also carried exposed herring embryos to petroleum hydrocarbons with and without aeration and, in most cases, it appears that oxygen concentration at the time of hatching is extremely critical and should be optimized to avoid obscuring true WSF toxicity. Holliday et al. (1964) demonstrated a constant increase in QO_2 for developing herring embryos and a 100-150% increase in O_2 consumption during hatching. McQuinn et al. (1983) demonstrated high field mortality of herring embryos associated with rapid loss in oxygen when photosynthesis ceased at night. The experiment reported here (Table I.1) demonstrates that the WSF does not directly affect pH, O_2 or salinity in the absence of living embryos. Consequently, increased activity and respiratory rate of the embryos at the time of hatching reduced dissolved oxygen and is the most likely cause of the elevated mortality observed by some investigators.

Hatching Dynamics

Embryos exposed continuously to 0.48 ppm WSF hatched 4-5 days earlier than untreated controls and embryos exposed to <0.48 ppm. Perturbation of mean hatching day has been reported by several authors, but the effect is not consistent from study to study. Kocan et al. (1987) reported precocious hatching in Baltic herring embryos exposed to petroleum hydrocarbons in the sea surface microlayer, while other authors reported no difference in hatching time (Smith and Cameron 1979, Pearson et al. 1985), and others report both precocious and delayed hatching depending on the source of the crude oil and exposure concentration (Linden 1978).

Physical Defects

The most obvious effect of continuous WSF exposure on developing herring embryos in 1991 was increased physical defects in live-hatched larvae. The EC_{50} for total abnormalities was 0.48 ppm with no normal larvae appearing at or above 0.97 ppm. Significant post-hatching larval abnormalities were reported by Linden (1978), who saw significant increases in malformed herring larvae after exposure to light fuel oil (3.1 ppm), Venezuelan crude oil (5.4 ppm), and Tuimazan crude oil (2.6 ppm). Herring embryos exposed to 0.68-ppm WSF for 48 hr exhibited a significant increase in gross abnormalities (Smith and Cameron 1979), while Pearson et al. (1985) concluded that the concentration of both total saturated hydrocarbons and total hydrocarbons was the best indicator of larval abnormalities.

Larval Weights

Water-soluble petroleum hydrocarbons appear to affect only the weight of normal larvae. The mean weight of normal-unexposed larvae was 120 µg (2.40 mg 20 larvae^{-1}), similar to 114 µg larva^{-1} reported by Schnack (1981). The weight of physically malformed larvae appears to be consistently lower than that of normal-unexposed larvae. When embryos are exposed to WSF of

Prudhoe Bay crude oil, only the weight of physically normal WSF-exposed larvae is significantly reduced.

Genotoxicity

Mitotic activity and anaphase-telophase aberrations were the most sensitive biomarkers indicative of exposure to WSF. Mitotic activity was significantly reduced in newly hatched larvae exposed to 0.24 ppm WSF, one-half the EC_{50} for larval deformities. The lowest concentration of WSF tested (0.01 ppm) produced a doubling of anaphase-telophase aberrations in mitotically active larval cells, while higher concentrations of WSF produced significantly elevated A/T aberrations ($P < 0.05$). This observation is particularly significant because damage was evident in post-hatch larvae while exposure occurred during embryogenesis.

Inhibition of, or damage to, mitotic cells are possible mechanisms to explain for the reduced weight observed in normal WSF-treated larvae. A reduction in mitotic rate as well as cell death would result in fewer cells being produced in a given amount of time, resulting in slower growth and smaller larvae.

The long-term consequence of genetic damage to any population is twofold. First, genetic damage to somatic cells is believed to be the mechanism for neoplasia (cancer) induction and some physical defects. Second, genetic damage to germ cells (sperm and ova) can result in mutations capable of being passed on to subsequent generations. The presence of recessive mutations in a population presents an insidious threat that might not manifest itself until several generations have passed and the cause of the genetic damage has been forgotten in ensuing events. Reduced fertility, embryo death, physical and biochemical defects and behavioral modification are some of the possible consequences of mutant gene amplification in a population (Brusick 1980).

Intermittent Exposure

Unlike the embryos exposed continuously to WSF in 1991, embryos exposed intermittently to WSF in 1992 showed significantly increased mortality rather than increased larval abnormalities. This may be due to the higher concentrations of WSF used for intermittent exposures. However, a difference in the gene pool from which the embryos were derived is also possible. The 1992 embryos were produced almost exclusively from 4-year-olds, while the 1991 embryos were from a mixed population of primarily 6- and 7-year-old spawners. It is not clear whether this difference results from exposure kinetics or if it is biologically based on a genetic difference in year classes or previous adult experiences affecting the survival potential of their offspring.

On the basis of intermittent exposure data, it appears that the embryonic stage most sensitive to WSF is the blastodisc, occurring approximately 48 hr after fertilization. The total hatch was affected most dramatically when 48-hr-old embryos were exposed to relatively high concentrations of WSF for 36 hr. The mechanism responsible for differential sensitivity of embryonic stages is not presently evident, but differential sensitivity suggests that the age of the embryo at the time of exposure could influence the degree to which subsequent life stages might be affected, a phenomenon originally predicted by Rosenthal and Alderdice (1976).

The differences in embryo mortality, hatching success and physical malformations reported here and by various other investigators are probably a reflection of the different techniques used for incubation, exposure, oil source, and other variables such as parental stock, and possibly the presence of other competing marine organisms in the incubation or exposure water. Only further investigations specifically designed to detect differential spawning success between year classes and sub-populations of spawners will answer these questions.

Experimental data such as this, which relates pathologic response to dose and time, can be used to predict the extent of damage done in the field. By comparing experimental laboratory data with data obtained from field samples following events such as the *Exxon Valdez* oil spill, researchers should be able to predict the number of larvae that would be affected, provided that field data on WSF concentration and exposure time are collected at spawning sites.

IN SITU EXPOSURE

Significantly fewer larvae hatched from eggs exposed in situ at unoiled sites when compared with eggs exposed at oiled sites. Oiled sites however, produced significantly more abnormal larvae than did unoiled sites. When the percentage of viable larvae (Equation I.1) was calculated, no difference was apparent between the oiled and unoiled sites.

When larval biomass was measured as dry weight, the larvae exposed at oiled sites weighed significantly less than those exposed at unoiled sites, regardless of condition. There did not appear to be a depth dependent difference. This result is similar to that found when herring embryos were exposed to water soluble fraction (WSF) of Prudhoe Bay crude oil under controlled laboratory conditions.

This reduction in hatch weight has been addressed by Crowder et al. (1992), who showed that even small increases in body size (e.g., weight) at hatching confer large benefits in terms of surviving to first feeding and subsequently gaining an advantage over smaller cohorts. If this is true, then Equation (I.1) is an overestimate of the percent viable larvae and should be modified to include a reduced survival potential for smaller larvae.

Other studies that examined the effects of oil on herring larval size report similar results, although most data are presented as length (mm) rather than dry weight. Linden (1978) and Smith and Cameron (1979) reported that embryos exposed to oil produced significantly shorter larvae than controls, and embryos exposed to Prudhoe Bay WSF by Pearson et al. (1985) also produced shorter larvae than their controls.

Interpretation of field data and correlation with laboratory generated data is at best difficult. By definition, experiments (e.g., lab data) are controlled, with ideally only one variable, while field data are collected under constantly changing and infinitely varying conditions. Our attempt to evaluate previously oiled sites and unoiled sites in Prince William Sound has demonstrated the importance of having extensive baseline data on all aspects of a species life history before one can begin to interpret the effects of catastrophic events such as the *Exxon Valdez* oil spill. The major difficulty with interpreting the extensive data collected since March 1989 is the lack of background

data with which it can be compared, and our inability to distinguish "effects" from natural biological variation.

REPRODUCTIVE IMPAIRMENT

Total hatch and percent viable larvae were significantly reduced in the two groups of females collected at oiled sites. Both of these groups of spawners were collected in Rocky Bay on Montague Island, but from different locations within the bay during separate spawning events and on different days. They may represent different waves of cohort of spawners. The one sample obtained from an unoiled site was collected at Boulder Bay in Tatitlek Narrows. Our original objective was to target 4-year-old first time spawners. Because age evaluations had to be made in the field at the time of spawning, and ageing took place by scale evaluation several days later, some fish from older year classes were included in the sample. When data from these older fish were removed, the overall results remained the same, so they were included in the final analyses to maintain a constant of 25 fish per site.

All three populations were similar in age composition, length and weight. None of these measurements could be correlated with the observed reduction in hatching success or larval viability.

The fish from oiled site C had a significantly lower fertility rate than the other two sites. However, infertility at all three sites ranged from 3.2–5.8%, below that reported for normal herring eggs (Linden 1978, Rosenthal and Hourston 1982, Munk and Rosenthal 1983). Because the infertility rate was so low, the difference between A, B and C is likely an artifact and not related to the spawning population or the site.

Spawners from the unoiled site at Tatitlek Narrows produced a mean of 56% (32-78%) total live larvae, significantly higher than either of the two oiled groups (19 and 38%), with a variability within that expected from reports of other investigators. Rosenthal and Hourston (1982) reported a mean hatch of 42% (6.5–91%) from a total of 30 individual herring, while Smith and Cameron (1979) reported a mean hatch of 53% (39–83%) from three groups of approximately 200 eggs. Rice et al. (1987) reported a mean hatching success of 78–85% for control spawners and 66% mean hatch for spawners previously exposed to WSF of Cook Inlet crude oil. No range was given for these hatching data, but a standard error of ±8 was indicated.

Significantly more viable (e.g., normal live) larvae were produced by herring collected at the unoiled site (Group A) than by those collected from oiled sites. Examination of the data indicates that the opposite is true when only the total live larvae produced by each group is considered. The discrepancy between percent normal-live larvae and percent viable larvae is due to the significantly greater number of live larvae produced by group A spawners. This is probably the result of many defective embryos from the oiled sites dying during development, resulting in a high proportion of normal larvae, but fewer total live larvae. Consequently, when "viable" larvae was calculated (Equation I.2), the oiled sites produced significantly fewer normal-live larvae than did the unoiled site (Fig. I.13). The loss of abnormal larvae is reflected in the significantly lower variability observed in the two oiled groups.

It is possible that with only three sites sampled, the differences found reflect natural variability, and the relationship to oil is a coincidence. However, an additional study year with sample stratification over time, area and age of spawners would resolve the issue of natural variability over oil, assuming that spawning fidelity does not change significantly between years with respect to cohort.

CONCLUSIONS

Every life stage of the herring examined in the field and in the laboratory has shown significant oil-related damage, and this damage has the potential of affecting the survival and long-term productivity of the 1989 cohort. Embryos and larvae (the most sensitive life stages) of the herring present in Prince William Sound in 1989 were exposed directly and indirectly to oil following the *Exxon Valdez* oil spill. These fish will return as 4-year-old spawners for the first time in 1993, and are the most important link in determining the long-term effect of that early exposure to crude oil. Consequently, it is critical that data be collected from the 1993 4-year-old spawners in order to accurately assess whether they were significantly damaged and to determine what extent this damage will be passed on to their offspring and persist into the future. Without this data, we will lose the opportunity to conclusively determine whether the *Exxon Valdez* oil spill of 1989 had a lasting and significant effect on the herring population(s) of Prince William Sound.

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