Exxon Valdez Oil Spill
Restoration Project Annual Report

The impact of exposure of adult pre-spawn herring
(*Clupea harengus pallasi*) on subsequent progeny

Restoration Project 94166
Annual Report

This annual report has been prepared for peer review as part
of the *Exxon Valdez* Oil Spill Trustee Council restoration
program for the purpose of assessing project progress. Peer
review comments have not been addressed in this annual
report.

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Exxon Valdez Oil Spill
Restoration Project Final Report

Restoration Project 94166
Annual Report

Study history: This project was initiated in 1994 as Restoration Project 94166 with NOAA as a cooperating agency and ADF&G as the lead agency. This report represents only the NOAA portion of the project.

Abstract: The *Exxon Valdez* oil spill in Prince William Sound may have impaired reproduction and caused disease in herring stock. The primary objective of this laboratory study was to determine if exposure of parent fish would cause genetic damage in progeny. Reproductively ripe adult herring were exposed to oil in water to determine the presence or absence of direct toxic effects, damage to gonads, reduced resistance to disease, and genetic damage in larvae. Because it was not practical to measure germ line damage directly in the laboratory, efforts were focused on detection of chromosomal damage in the actively dividing somatic cells in the pectoral fins of larvae. Hydrocarbons accumulated in tissues of exposed herring, and mixed function oxidase activity was induced in liver tissue. Prevalence of viral hemorrhagic septicemia virus in adult herring increased as a function of oil concentration. Parental exposure to oil did not affect progeny. Parameters not affected included egg fertility, hatching success, hatch timing, embryo death, larval health (95 to 99% not moribund or dead within 24 h of hatch), larval swimming, larval abnormalities, stage of larval development at hatch, anaphase-telophase abnormalities, number of mitotic figures, number of pycnotic cells, and number of multinucleated or karyorrhectic cells.

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EXECUTIVE SUMMARY

A significant proportion of the pre-spawning adult herring population in Prince William Sound was likely exposed to oil spilled by the *Exxon Valdez*. The herring population crash in the Sound in 1993, poor recruitment of juvenile fish to the spawning population, and a high incidence of disease have raised concern that the oil spill directly or indirectly caused these delayed problems. Several studies were initiated to determine if oil toxicity could cause chromosome aberrations in larvae, disease problems in adult fish and reproductive impairment. Two years of controlled laboratory studies concerning potential reproductive impairment of herring by exposure to oil have been largely completed. The life stage exposed to oil was varied each year, but the endpoint measurement (analysis of chromosomal condition in actively dividing cells of larvae) remained the same. In this annual report, results of the first year (1994) are detailed.

In 1994, gravid adult herring were exposed to oil in water for a period of 8 or 16 d to determine the presence or absence of direct toxic effects, damage to gonads, reduced resistance to disease, and heritable genetic damage in the progeny. Because it was not practical to measure germ line damage directly in the laboratory, efforts were focused on detection of chromosomal damage in the actively dividing somatic cells in the pectoral fins of larvae. Prediction of impacts on meiosis, therefore, was based on the premise that genetic damage in somatic cells would be correlated with germ line damage. In a follow-up experiment, a group of post-spawn herring were similarly exposed to oil.

Adult herring were impacted by exposure to oil. Hydrocarbons accumulated in tissues of exposed herring, and mixed function oxidase enzyme activity was induced in liver tissue. Induction of mixed function oxidases in pre-spawn fish was about half that in post-spawn fish. In pre-spawn fish exposed 16 d, prevalence of viral hemorrhagic septicemia virus increased as a function of oil concentration ($r^2 = 0.59$, $P = 0.001$).

Effects of exposure of adult herring to oil were not discernable in the progeny. Parameters not affected included egg fertility, hatching success, hatch timing, embryo death, larval health (95 to 99% not moribund or dead within 24 h of hatch), larval swimming, larval abnormalities, stage of larval development at hatch, anaphase-telophase abnormalities, number of mitotic figures, number of pycnotic cells, and number of multinucleated or karyorrhectic cells.

INTRODUCTION[1]

In Chapter 1, the linkage between exposure of adult herring to oil, viral hemorrhagic septicemia virus, and other disease conditions is explored. Exposure conditions, mortality, and accumulation of hydrocarbons are also quantified. Because gamete production requires considerable energy, and because pre-spawning herring do not feed (Iles 1965; Carlson 1980), we considered that reproduction was a significant secondary stress factor, thus disease response in pre-spawn fish was compared to that in post-spawn fish.

In Chapter 2, genetic response of progeny to exposure of parents to oil in water is presented; fertilization success, hatch timing, hatch success, egg mortality, larval size at hatch, and morphological abnormalities were also tested for treatment effects. Hydrocarbon concentrations and depuration from eggs was also quantified.

In Chapter 3, induction of cytochrome P-450 dependent mixed function oxidase enzymes (MFO) by exposure of pre- and post-spawn herring is explored. MFO enzymes can be induced by polycyclic aromatic hydrocarbons (PAH) and other xenobiotics, and are involved in the metabolism, thus clearance, of PAH and other xenobiotics (Jimenez and Stegeman 1990). Based on previous research (Forlin et al., 1984; Walton et al., 1983; Collier et al., 1986) it was likely that induction of MFO would be different in pre- and post-spawn herring. Depression of induction levels in pre-spawn fish probably has something to do with the energy cost of reproduction and the hormonal constituency of the reproductively ripe animals.

OBJECTIVES

Objectives as proposed in the 1994 detailed study plan

The goal of this component is to determine if exposure of herring to oil can cause genetic damage, and if this damage is transmissible to subsequent generations. The premise is that somatic genetic damage will predict germ line damage. It is not practical to measure germ line damage directly in the laboratory because it is not possible to rear herring larvae from eggs to maturity; the specific objectives of this study are to determine if genetic damage to early live stages of herring could be caused by exposure of pre-spawning adult, egg, and larval life stages to oil. The primary objective is to measure genetic aberrations in eggs and larvae after 1) exposure of pre-spawning adults, and 2) exposure directly to eggs and larvae. Genetic aberrations caused by exposure of adults will be compared to egg and larval survival, abnormalities, and growth. Secondary objectives are to 1) measure exposure concentrations and hydrocarbon accumulation in adult, egg, and larval tissues, 2) measure MFO function (or lack of it) in pre-spawning adults. In research year 1, only the adult exposure portion of the experiment will be studied, and larvae will be maintained only through yolk resorption. Detailed objectives are as follows:

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[1]Citations made in the Introduction can be found in Chapter 1 Literature Cited.

1. Determine if exposure of pre-spawning adult herring to oil can cause genetic damage in progeny. After exposure, measure or quantify in eggs or larvae:
   a. genetic aberrations
   b. morphological abnormalities
   c. histological changes
   d. fertilization success
   e. hatch timing and success
   f. growth changes (length and weight)
   g. mortality

2. Determine if herring can protect gametes from petroleum hydrocarbons by metabolic processes:
   a. Measure MFO induction in pre- and post-spawning adult tissue (liver).
   b. Measure hydrocarbon loads in pre- and post-spawning adult tissues after exposure, including muscle and ovarian tissue.

3. Compare genetic effects in eggs and larvae caused by exposure of adults to oil to genetic effects caused by direct exposure of eggs and larvae to oil.
   a. Expose eggs and larvae to water contaminated via contact with oiled substrate.
   b. Measure the same parameters listed for Objective 1.

4. Predict whether genetic damage is transmissible to subsequent generations, based on the premise that somatic genetic damage predicts germ line damage.

5. Quantify and characterize:
   a. Oil concentration in water
   b. Oil concentration in ovarian tissue
   c. Oil concentration in bile

Changes

1f. Larval size was quantified with length only; weight was not measured due to erratic crystalline growth (artifact) in the preserved collection.

3. Comparison of exposure methods will be completed after the 1995 data analysis is complete.

5c. Original objective should have read "metabolite concentration in bile". This objective was secondary to other MFO objectives and will likely not be completed.

Chapter 1: Disease, mortality, and bioaccumulation of hydrocarbons in pre-spawn herring (*Clupea harengus pallasi*)

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INTRODUCTION

On March 24, 1989 the *Exxon Valdez* oil tanker grounded in PWS; the resultant oil spill was the largest in U.S. history. In the short-term aftermath of the spill, damage caused by oil was measured in several species of fish, including herring, salmonids, and rockfish (Brown et al., In press; Bue et al., In press ; Carls et al., In press; Geiger et al., In press; Hepler, In press; Meacham and Sullivan 1993; Moles et al. 1993; Norcross and Frandsen, In press; Willette, In press; Wertheimer and Celewycz, In press). Herring were just beginning to spawn when the oil spill occurred, thus most or all of the life stages may have been exposed to the oil (Personal communication, Evelyn Brown, ADF&G, 1994). Over 40% of the spawning areas were oiled, and biologically available oil was widespread in PWS for several months after the spill (Short and Rounds 1995a). The biomass of spawning herring peaked in 1988 and 1989, but declined between 1991 and 1994. In 1993, the year the 1989 year class fully recruited into the spawning population, a VHSV epizootic occurred; up to half of every school had ulcerations typical of VHSV. The herring population crash in PWS in 1993, poor recruitment of juvenile fish to the spawning population, and a high incidence of disease (VHSV and *Ichthyophonus*) have raised concern that the oil spill directly or indirectly caused these delayed problems. Significant histopathological damage was observed in adults collected in oiled areas in PWS in 1989 (Moles et al. 1993). Although petroleum hydrocarbons were not detected in adult herring tissue in 1989, bile was contaminated (Haynes et al. 1995). Correlations of gut parasitism and histopathological damage with damage caused by laboratory exposure is perhaps the best evidence that adult herring were exposed to oil in PWS, despite the fact that petroleum hydrocarbons were not detected in herring in PWS (Moles et al. 1993).

Although herring may be reservoirs of VHS infection, the disease requires a stressor to initiate an epizootic capable of producing mass mortality (Meyers et al. 1994). Known initiating events include spawning, commercial fishing, nutritional deficiency, and concurrent infections (Meyers et al. 1994). Exposure to hydrocarbons may initiate VHSV lesions in herring (Traxler and Kieser 1994). The epizootic in 1993 coincided with entry of possibly the most oil-impacted year class (1989) into the spawning population (Brown et al., In press), suggesting a possible linkage of disease with the 1989 oil spill.

The objective of this experiment was to determine if herring exposed to oil in water would be more susceptible to VHSV and other opportunistic diseases. Fish were not inoculated with VHSV or other disease pathogens; any disease was present in the originally collected specimens or in water. In addition to disease observations, hydrocarbon concentrations were measured in treatment water, muscle, and ovaries. Polycyclic aromatic hydrocarbon (PAH) concentrations in

water (0.03 to 50.76 ppb) were chosen to include maximum mean PAH concentrations observed in water in PWS following the *Exxon Valdez* oil spill (EVOS) (6.24 ppb; Short and Rounds 1995b). Fish mortality was also quantified.

The type of oil tested in this study (Alaska North Slope Crude Oil), and the methods used to contaminate seawater were designed to mimic conditions in PWS following the EVOS. Because earlier methods to prepare water-soluble fractions (WSF) of oil generally resulted in an enrichment of mono-aromatic hydrocarbons and compounds heavier than naphthalenes were generally not present (Moles et al. 1985; Rice et al. 1987), water in this experiment was contaminated via contact with rock coated with weathered Alaska North Slope crude oil. The PAH composition produced by the latter method closely mimicked the PAH composition observed in PWS with naphthalenes through chrysenes represented.

METHODS

Pre-spawn herring were collected near Shelter Island, Southeast Alaska (58.4°N lat., 134.8°W long.) by purse seine on March 20, 1994. To minimize scale loss, fish were transferred in water and not netted. Approximately 1,500 fish were maintained in a 24,000 liter holding tank at the Auke Bay Laboratory. Seawater flow was 60 to 130 L/min at 3.5 to 5.2°C and 31 ppt. Fish were not fed during holding or experimental periods. Fish ripened during holding, and were ripe during experimental treatment.

Disease became apparent in some fish by April 1, 1994. Mortalities were noted together with observations of white raised areas along the lateral surfaces of diseased fish near the dorsal fin. There was scale loss in this area, and *Flexibacter*, a gram negative filamentous rod bacterium was present, but no viral infections were detected. Beginning April 5, all fish in the holding tank received three successive one-hour static formalin baths (150 ppm) at 2 d intervals to treat external *Flexibacter* infections. Disease treatment was successful; mean holding mortality was low (approximately 0.1 to 0.2% per day).

Water was oiled by contact with oiled rock; seawater flowed into a plenum at the bottom of 30 cm diameter x 122 cm tall polyvinyl chloride plastic cylinders and upwelled through gravel (Fig. 1.1). Water flowed from these oil generators to the bottom of individual treatment tanks; a trap inside the generator prevented slick overflow. Control generators were charged with clean gravel. Before the gravel was oiled, Alaska North Slope Crude oil was artificially weathered by heating to 70°C overnight (12 h) in a beaker with continuous stirring. Pea gravel (approximately 2 to 33 mm diameter) was washed on 3 mm screen and thoroughly air dried. A cement mixer was cleaned with soap and water and thoroughly air dried. Weathered crude oil, heated to 40°C, was applied to 45 kg batches of tumbling gravel with a paint sprayer (trace, low, and mid treatments) or with a Teflon squirt bottle (high treatment). Batches of gravel were mixed by treatment; each generator was charged with 45 kg of freshly oiled gravel (or non-oiled control rock), except high treatment generators were charged with 90 kg gravel.
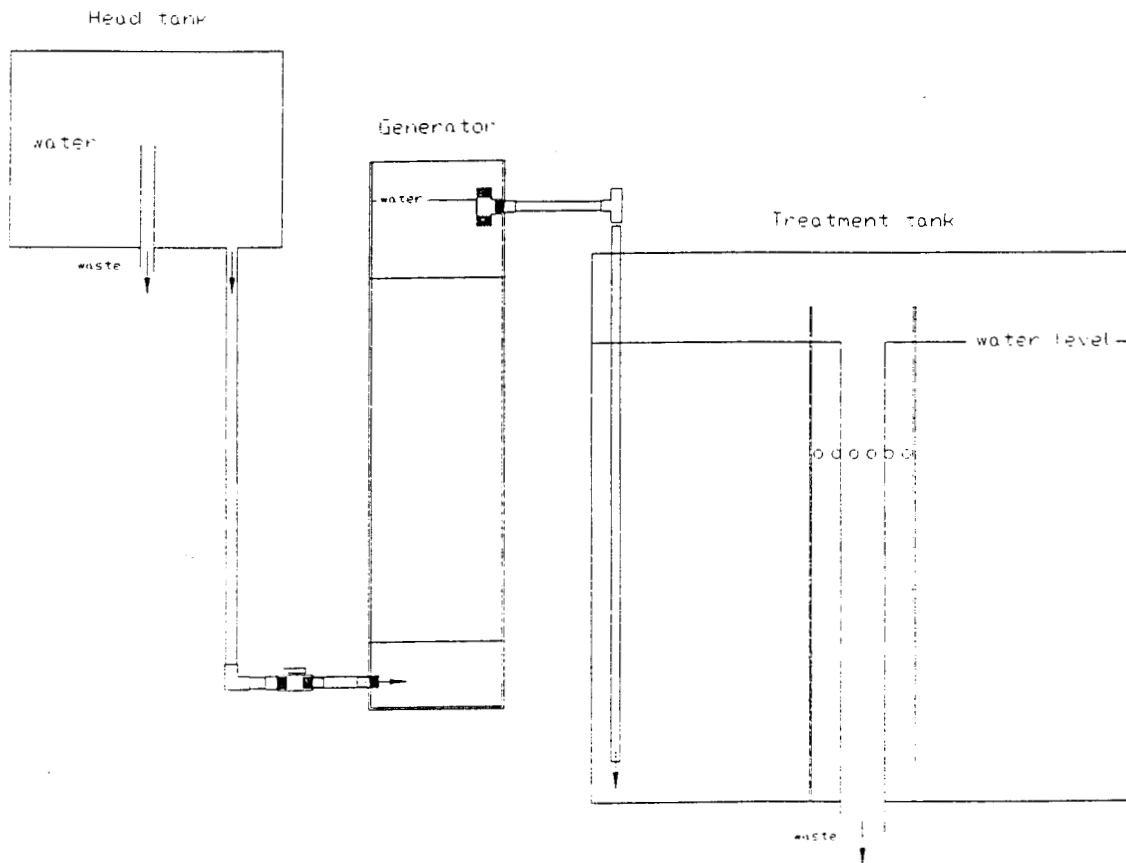
Figure 1.1.--Seawater for each replicate was delivered at a constant rate to a plenum at the bottom of a cylindrical tube partially filled with rock or oiled rock. Water overflowed from the "generator" tube to the bottom of the treatment tank as shown.

There were five treatments, including control, with three replicates per treatment. Water for each replicate tank flowed through an independent generator at 6.9 L/minute and 4.2°C. Healthy herring were randomly distributed among fifteen 700 L treatment tanks (50 fish per tank). For logistic reasons, exposures began over a three day period. Exposures lasted 8 or 16 d, with an average of 11 fish per tank sampled on day 8. To characterize the oil and quantify treatment concentrations, composite samples (1.27 L from each of 3 replicate tanks) were collected on days 0, 8, and 16 for analysis by gas chromatography (Short et al., In press). Mortality and dissolved oxygen were monitored once per day in each tank. Tanks were located outside, but were protected from weather by a translucent shed roof. Fish ranged in size from 20.3 to 29.9 cm fork length and 96 to 261 g wet weight; age ranged from 3 to 10 years.

After exposure, fish were killed by a blow to the head, measured (fork length to the nearest millimeter), weighed (wet weight to the nearest 0.01 g), and bled by clipping a gill arch. A 10 g (minimum) gonad subsample from each fish was frozen for hydrocarbon analysis. For age analysis, six scales were removed from the skin near the posterior margin of the dorsal fin, placed succulus down on a glass slide, and covered with a second slide. From each fish, 10 g (minimum) of muscle tissue (right side, tail to gill arch) was frozen for hydrocarbon analysis. To prevent contamination, one knife was used to fillet the fish, and a second knife was used to remove the muscle from the skin. All tools and glass vials used to collect samples for hydrocarbon analysis were hydrocarbon free. Glassware was hydrocarbon free as received from the manufacturer; other tools were washed with soapy water, rinsed, dried, and rinsed with methylene chloride. For each of 7 to 14 fish per replicate tank sampled for VHS and histopathological observation, gill, liver, spleen, and posterior kidney samples were pickled in 10% neutral buffered formalin (Table 1.1). In addition, half of the spleen and a portion of the anterior kidney were frozen for virus analysis, and blood smears were prepared.

Disease observations were recorded during the processing previously described and for all specimens that died. Data recorded included condition of skin, fins, eyes, jaw, gill, liver, kidney, ovaries, spleen, and peritoneum. Bacterial infections were assessed from skin scrapings.

Data processing and statistics

The 8 and 16 d exposure groups were generally treated as independent experiments. The statistical design was essentially balanced in the 8 d exposure group, but due to mortality there were insufficient fish available in the 16 d exposure group for a balanced design. The two exposure groups were not completely independent because fish were sampled from the same population pool in each tank on two occasions.

Before analysis of variance (ANOVA), percentage data were arc-sin transformed, and corrected for small n where necessary (Snedecor and Cochran 1980).

Table 1.1.--Number of fish sampled for histopathological and viral analysis.

| Treatment | Number | | |
	Males	Females	Total
Control	21	9	31[1]
Trace	17	13	30
Low	17	15	32
Mid	20	10	30
High	22	8	30

[1]Gender of one fish not recorded.

Prevalence of VHSV was tested with single factor ANOVA. Treatments were compared to controls with *a priori* multiple comparisons. All data were analyzed with general linear model procedures (SAS).

RESULTS

Hydrocarbon exposure and accumulation

Mean hydrocarbon concentrations in water, estimated for 8 and 16 d exposures, ranged from 1 to 232 ppb total hydrocarbons; mean PAH concentrations ranged from 0.03 to 39.79 ppb (Table 1.2). Hydrocarbon concentrations in treatment water declined during the 16 d exposure period (Fig. 1.2). Because concentration declines were similar in all treatments, treatment conditions remained clearly separable.

PAHs detected in treatment water ranged from naphthalene through C-4 chrysene (Fig 1.3). The PAH fraction in water was comprised of 59.4% naphthalenes, 22.9% phenanthrenes, 6.1% fluorenes, 5.6% dibenzothiophenes, 0.8% chrysenes, and 5.2% other PAH. Naphthalene concentrations were relatively high earlier in the experiment but declined significantly over time (P = 0.012); relative dibenzothiophene and phenanthrene concentrations increased significantly over time (P = 0.005 and P = 0.011, respectively) (Fig. 1.3).

PAH accumulated in muscle and ovaries of fish exposed to oil (Fig. 1.4). Concentrations of PAH in tissues were correlated with mean water concentrations ($0.86 \le r \le 0.98$, $P < 0.001$; x^2 transformation). In the highest treatment, concentrations of PAH in tissues exceeded those in water by more than two orders of magnitude. PAH concentrations in muscle tissue exceeded those in ovarian tissue; these differences were significant in the high oil treatment ($P \le 0.001$).

Composition of PAH hydrocarbons in muscle and ovarian tissue of fish exposed to oil was similar to PAH composition in water (Fig. 1.5). Relative concentrations of naphthalenes tended to decline with depuration time ($0.092 \le P \le 0.157$), and relative concentrations of all heavier compounds tended to increase with time ($0.001 < P \le 0.093$). Chrysenes were detected in muscle but not in ovaries.

Disease response

Disease related mortality occurred during exposure to oil. After 16 d, the cumulative percentage of moribund and dead fish removed from tanks due to disease averaged 20%, but was highly variable (2 to 60%). Disease related mortality was lower (4%) during the first 8 d, and exceeded 10% in only one high treatment tank (11%) (Fig. 1.6).

Death due to disease was generally correlated with hydrocarbon concentration in water and tissue ($0.37 \le r \le 0.55$, $0.034 \le P \le 0.178$) (Fig. 1.7). Relatively high mortality in two mid oil replicates (48 and 60%) appeared as outliers.

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Table 1.2.--Mean hydrocarbon concentrations in water (ppb), measured by gas chromatograph. Reproductively ripe adults were exposed for either 8 or 16 d.

	Treatment	n	Mean	Standard Error	Low Range	High Range
8 d mean:						
PAH	0	2	0.03	0.00	0.03	0.04
	1	2	1.69	1.48	0.21	3.16
	2	2	5.60	4.69	0.91	10.29
	3	2	17.10	9.90	7.21	27.00
	4	2	39.79	18.53	21.26	58.31
Alkanes	0	2	1.29	0.01	1.28	1.29
	1	2	2.38	1.02	1.37	3.40
	2	2	3.53	1.80	1.74	5.33
	3	2	11.20	2.19	9.01	13.39
	4	2	78.62	37.26	41.36	115.88
UCM	0	2	0.00	0.00	0.00	0.00
	1	2	3.96	1.72	2.24	5.68
	2	2	5.69	3.51	2.18	9.20
	3	2	26.80	6.96	19.84	33.77
	4	2	113.90	59.89	54.01	173.79
16 d mean:						
PAH	0	3	0.03	0.00	0.03	0.04
	1	3	1.17	1.00	0.13	3.16
	2	3	3.94	3.18	0.61	10.29
	3	3	13.07	7.00	4.99	27.00
	4	3	30.56	14.13	12.11	58.31
Alkanes	0	3	1.20	0.09	1.01	1.29
	1	3	1.98	0.71	1.18	3.40
	2	3	2.85	1.24	1.47	5.33
	3	3	8.86	2.66	4.17	13.39
	4	3	57.57	30.10	15.47	115.88
UCM	0	3	0.56	0.56	0.00	1.69
	1	3	7.44	3.62	2.24	14.40
	2	3	4.49	2.35	2.10	9.20
	3	3	20.67	7.34	8.39	33.77
	4	3	82.54	46.68	19.82	173.79

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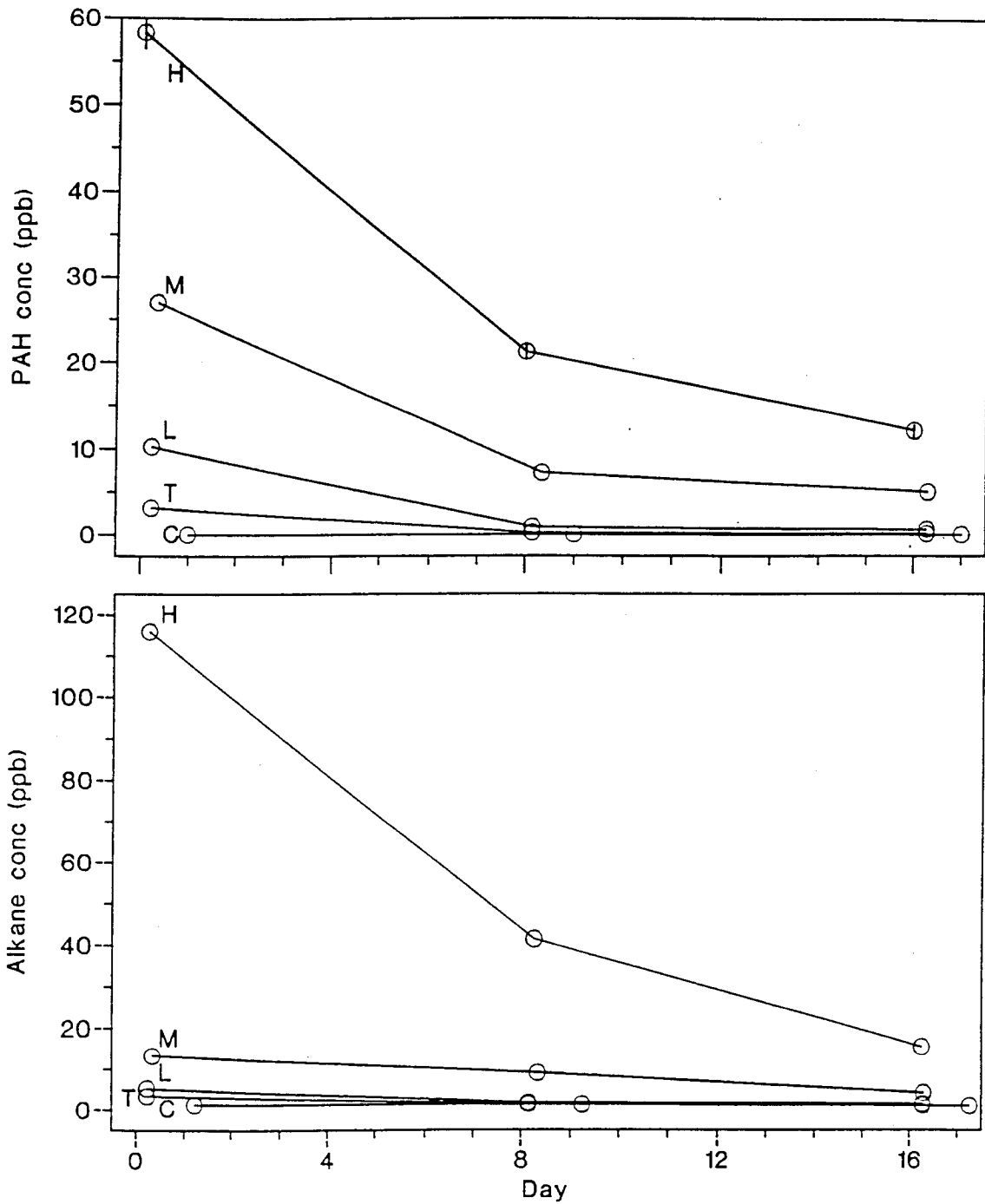
Figure 1.2.--Hydrocarbon concentrations in treatment water as a function of time. Data plotted are single composite values for replicate tanks at each time; standard error was estimated for the high oil treatment from replicate fluorescence analyses.
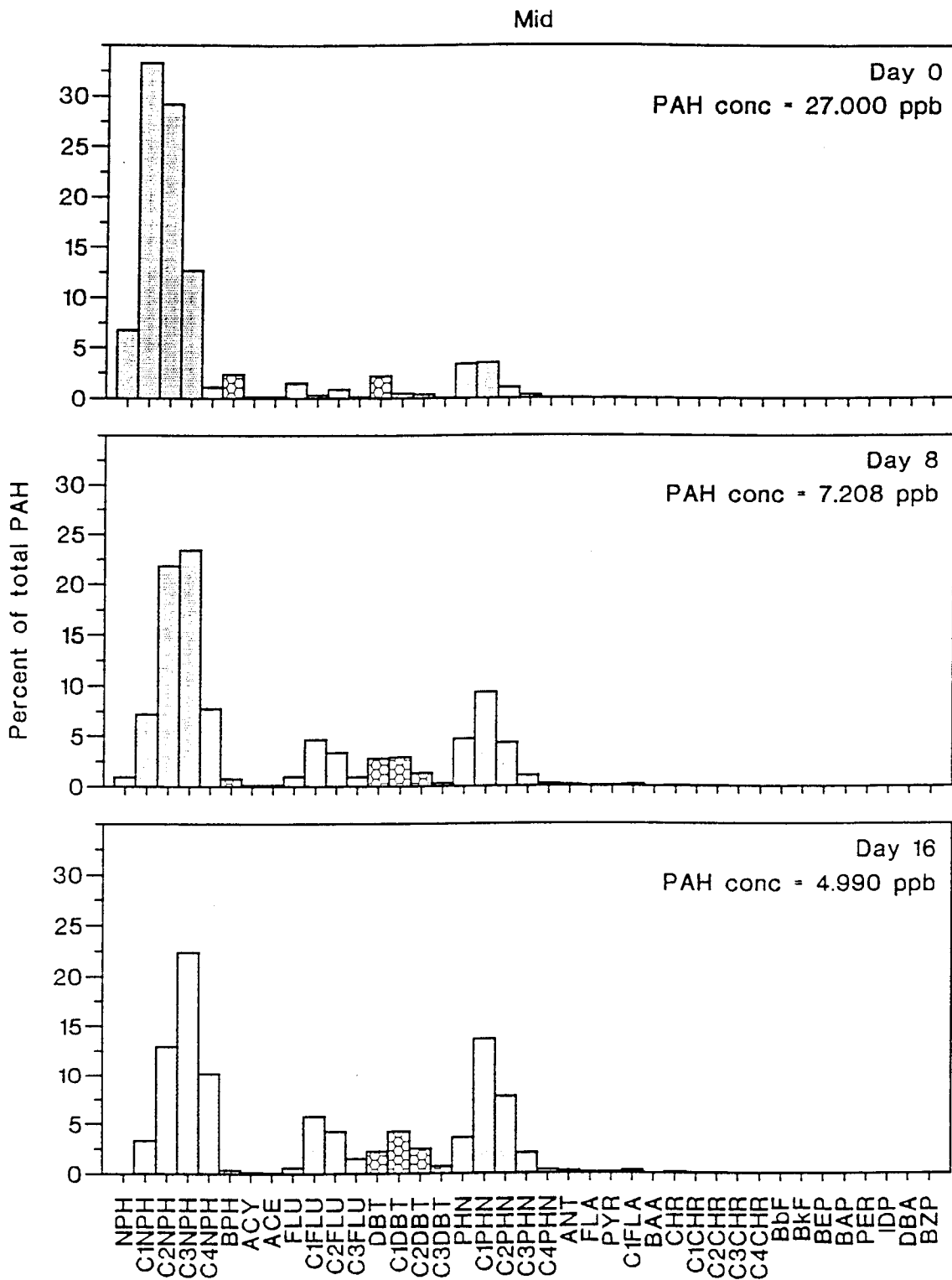
Figure 1.3.--Example PAH composition in water (mid oil treatment). As time progressed, absolute concentrations declined, but concentrations of heavier compounds, particularly phenanthrenes, increased relative to those of naphthalenes.
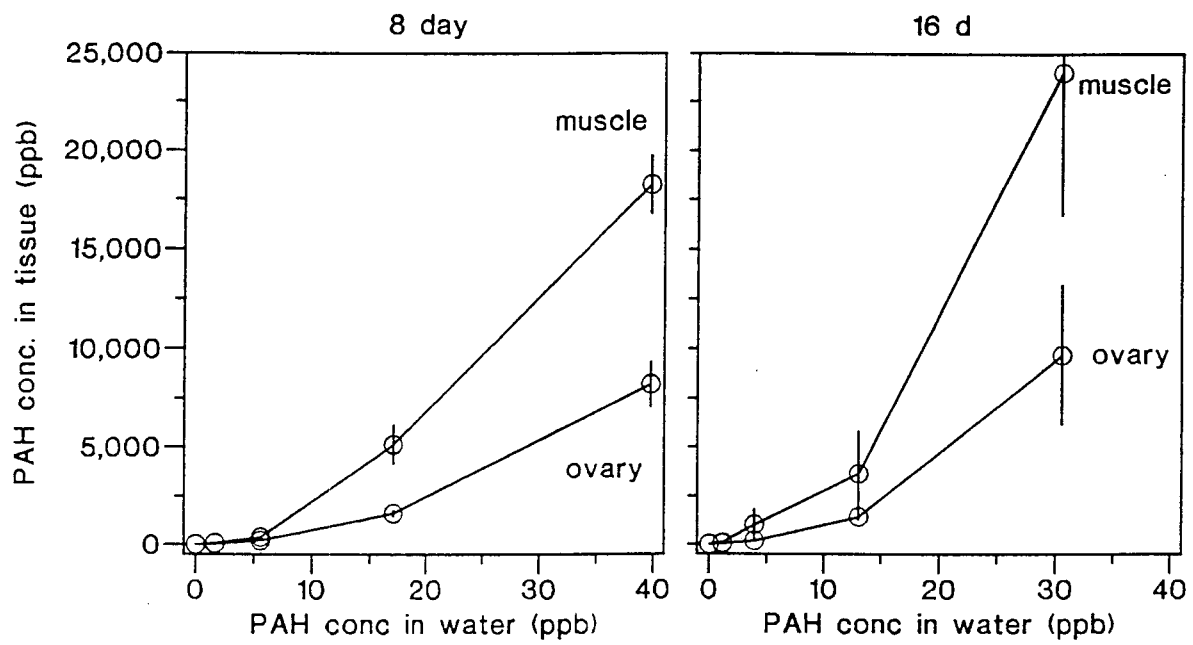
Figure 1.4.--Relationship between PAH concentration in treatment water and that in muscle and ovary tissue. Reported water concentrations are 8 or 16 d means, as appropriate. Data displayed are means ± standard error.
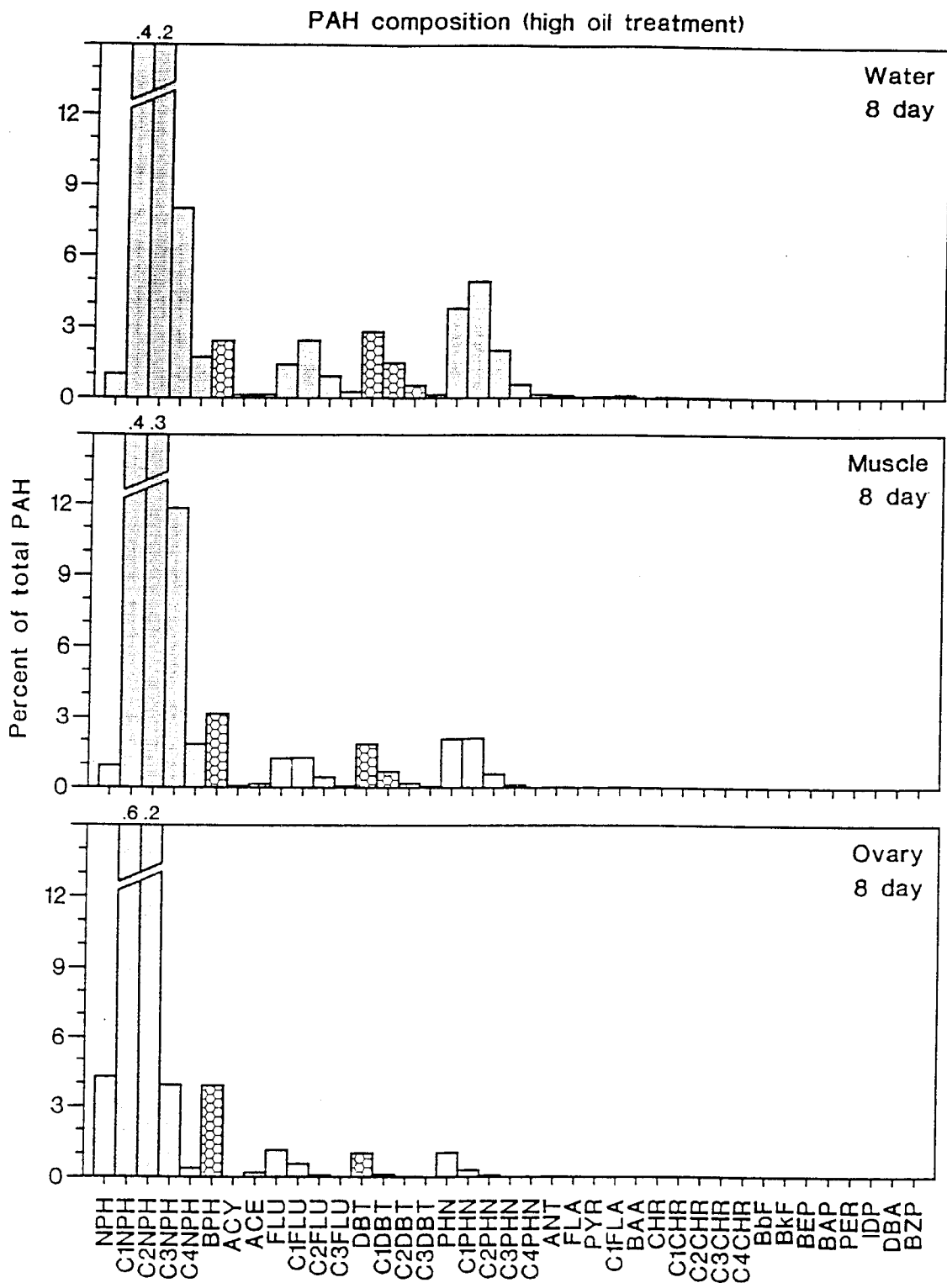
Figure 1.5.--Composition of PAH in muscle and ovary tissue was similar to that in treatment water. This example is from the high oil treatment after 8 d exposure.
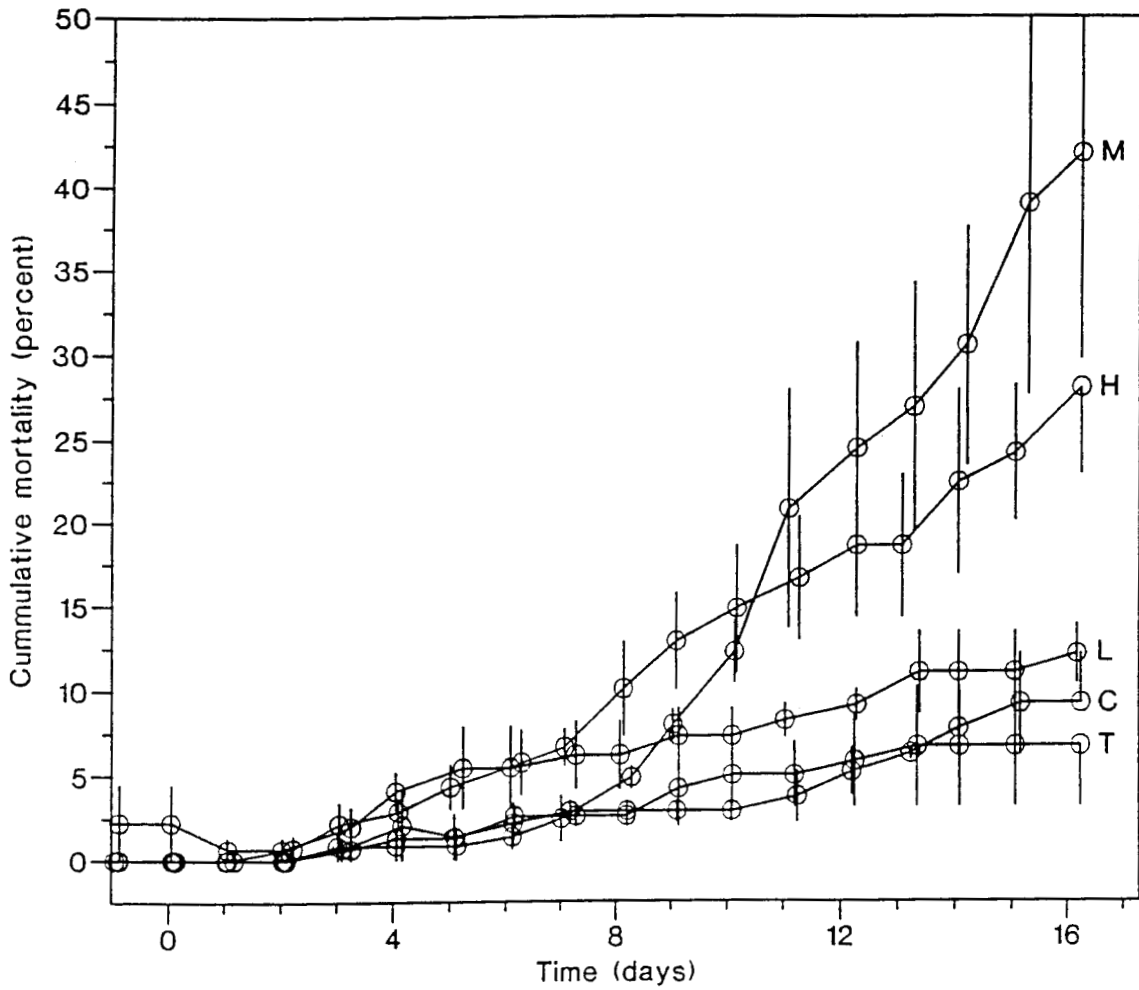
Figure 1.6.--Cumulative mortality increased with time in all treatments, but was greatest in mid and high treatments. Data displayed are means ± standard error.
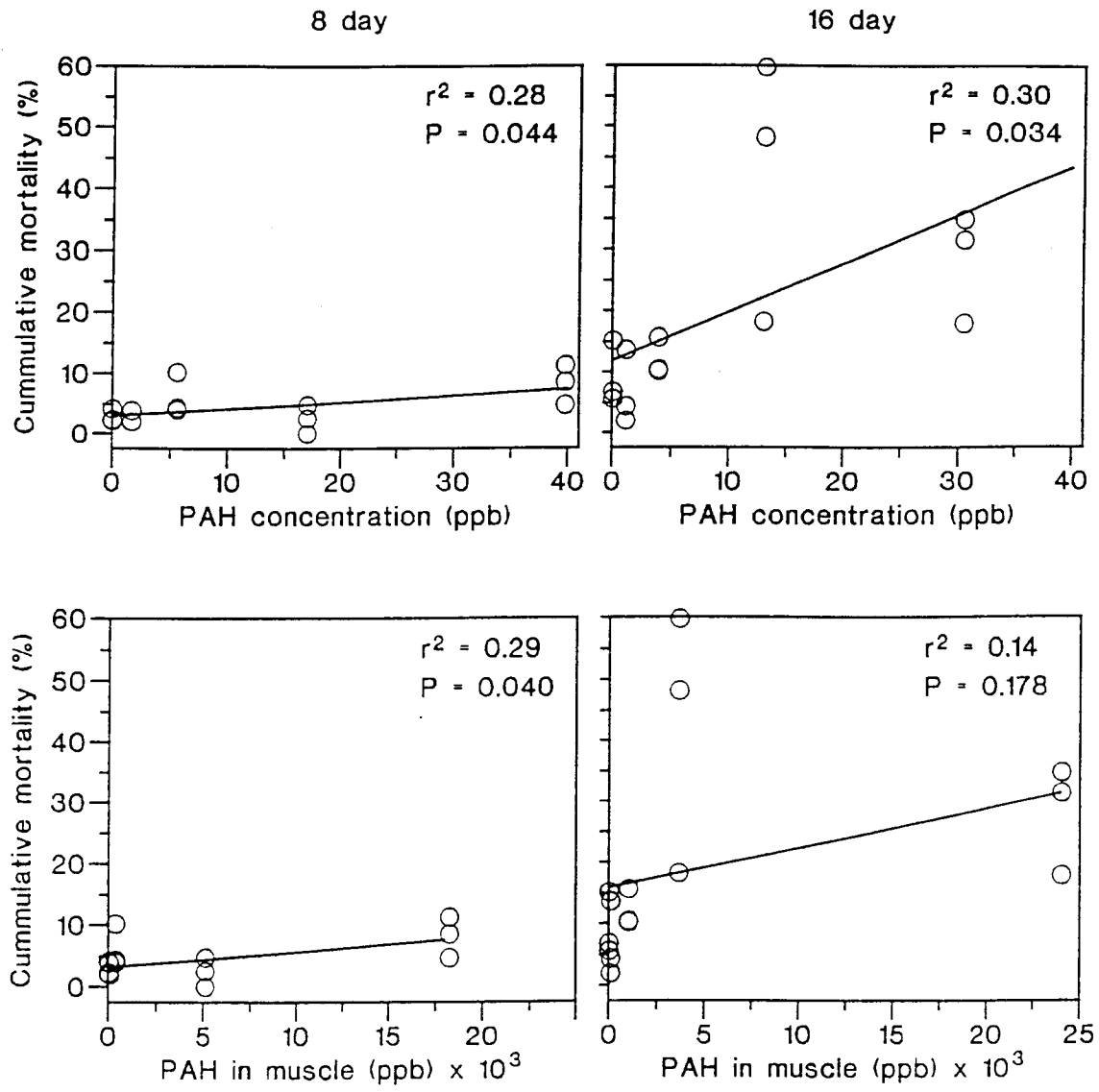
Figure 1.7.--Cumulative mortality was generally correlated with PAH concentration in treatment water and in muscle tissue. Cumulative mortality was unexpectedly high in two mid oil replicates by day 16.

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Disease in adult herring was significantly correlated with treatment in some cases, and exhibited dose-related trends in others (Fig. 1.8). Disease problems encountered included fin erosion, hemorrhaging in skin, eye, ovaries, kidney, and peritoneum, skin lesions, fin fray, hemorrhaging and erosion of the jaw, spleen enlargement, changes in liver color, and gill anemia; the number, type, and severity of these problems varied from fish to fish. *Flexibacter* infections (gram negative, filamentous rod bacteria) were identified from skin scrapings. Diseased jaws and changes in liver color varied significantly with oil treatment (P = 0.001) (Fig. 1.8). Consistent dose related trends were evident in several other disease categories (integument, fin fray, and peritoneum). In contrast, enlargement of the spleen did not vary as a function of oil treatment.

Viral hemorrhagic septicemia (VHS) virus was detected in live fish collected at the end of the 16 d exposure period, but viral erythrocytic necrosis was not found. The prevalence of VHS was correlated with treatment (r^2 = 0.59, P = 0.001), and prevalence was significantly greater in high treatment fish than in controls (Fig. 1.9). Corresponding histopathological analyses have not been completed.

DISCUSSION

This discussion is incomplete and is considered only a starting point. Further data (histopathological observations) are expected before this project is complete, and input from other authors will undoubtedly be of great value: the report in its current form was drafted by a single author (Carls). Reference to existing literature awaits completion.

In this experiment, we explored the linkage between oil exposure, VHSV, and other diseases. Fish were not inoculated with VHSV or other disease pathogens. Disease was either originally present in the population or in water; herring may be reservoirs of VHSV infection (Meyers et al. 1994). Because gamete production requires considerable energy, and because pre-spawning herring do not feed (Iles 1965; Carlson 1980), we considered that reproduction was a significant secondary stress factor.

Pre-spawn adult herring were impacted by exposure to oil in water. Hydrocarbons accumulated in tissues of exposed herring; the prevalence VHSV, jaw hemorrhaging and erosion, and changes in liver function were significantly correlated with oil concentration, and dose related mortality occurred. Others have found exposure to hydrocarbons may initiate VHSV lesions in herring (Traxler and Kieser 1994). In contrast, the post-spawn herring analogously tested in a separate study (Chapter 3) did not develop disease symptoms, and dose related mortality did not occur. This suggests that mortality in pre-spawn fish was related to disease, not direct toxic effects, and also that reproductive condition was a significant factor. Nearly all pre-spawn fish that died had evidence of disease (97%).

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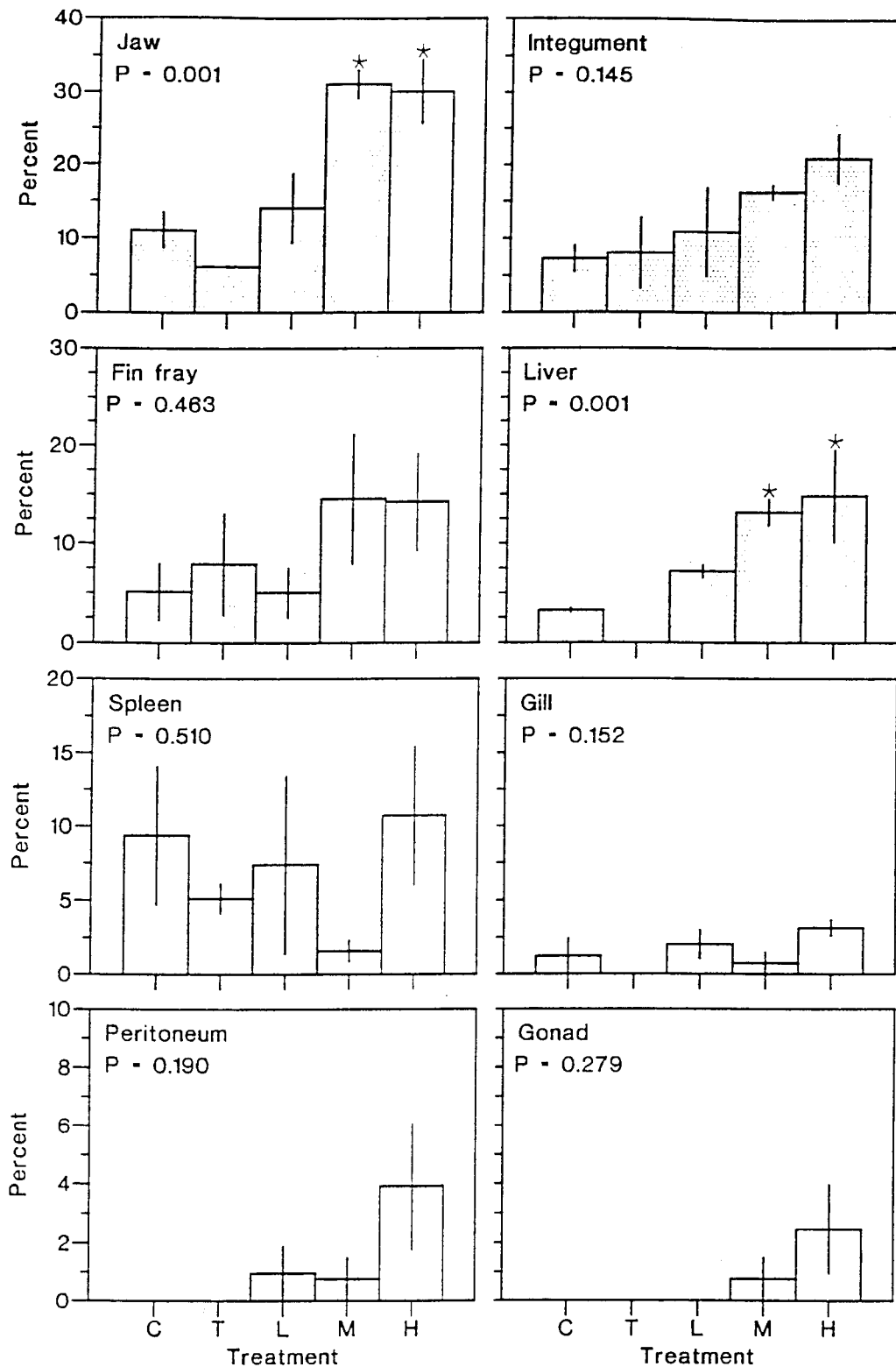
Figure 1.8.--Percentages of fish with jaw and liver abnormalities as a function of treatment. Treatments were control (C), trace (T), low (L), mid (M) and high (H) oil; P indicates probability of treatment effect, and asterisks indicate significant differences from controls. Data displayed are means ± standard error.

16 day exposures

P = 0.023
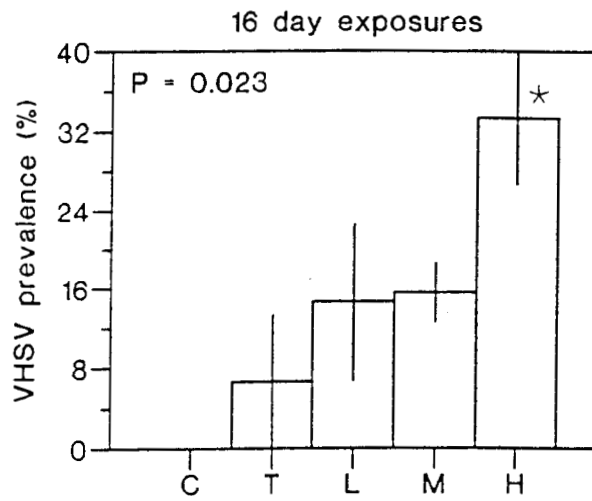
Figure 1.9.--Prevalence of VHSV as a function of treatment. Treatments were control (C), trace (T), low (L), mid (M) and high (H) oil; P indicates probability of treatment effect; the asterisk indicates a significant difference from controls. Data displayed are means ± standard error.

It is plausible that disease resistance in herring populations in PWS was significantly reduced by exposure to oil, and that epizootics observed after exposure were an indirect manifestation of the spill. An alternative explanation may be that the herring population, which was at an all time historical high at the time of the spill, was biologically susceptible to epizootics, mediated through density-dependent relationships.

CONCLUSIONS

A. Herring accumulated hydrocarbons from oil-contaminated water.

B. Exposure to oil in the range observed in Prince William Sound did not cause acute toxicity.

C. Disease resistance declined as oil concentration increased; significant dose-related mortality occurred.

D. Prevalence of VHSV increased with hydrocarbon concentration.

ACKNOWLEDGMENTS

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Chapter 2: The impact of exposure of adult pre-spawn herring (*Clupea harengus pallasi*) on subsequent progeny.

M.G. Carls, D. Fremgen, J.E. Hose, D. Love, and R.E. Thomas

INTRODUCTION

The *Exxon Valdez* oil spill (EVOS) in Prince William Sound (PWS) occurred at a particularly inopportune time for the herring population. Herring typically migrate from offshore feeding grounds to nearshore areas prior to spawning; the spill occurred just prior to this migration (Personal communication, Evelyn Brown, ADF&G, 1994). Thus, most or all of the life stages of herring in PWS may have been exposed to oil after the 1989 spill. An estimated 43% of the spawning areas were oiled (Personal communication, Evelyn Brown, ADF&G, 1994), and elevated levels of abnormalities and gene breakage were observed in newly hatched larvae (Hose et al., In press). Biologically available hydrocarbons from the EVOS were generally pervasive in the upper water column along the spill trajectory inside PWS during the summer of 1989 (Short and Rounds 1995). Thus, the potential for exposure of herring eggs and larvae persisted for several months after the spill (Brown et al., In press), and there was evidence of adverse effects in the planktonic larvae (Norcross et al., In press). The failure of the 1989 year class to recruit to the herring fishery in 1992 and 1993 suggests the early life stages of herring may have been impacted either from exposure of pre-spawning adults or by direct exposure of eggs and larvae.

Contamination by petroleum hydrocarbons may have an adverse impact on herring populations. Year-class strength may be determined by survival of herring larvae (Stevenson 1962; Taylor 1964; Outram and Humphreys 1974). Contamination of eggs and larvae by oil that leads to significant mortality or genetic damage, therefore, may have a negative impact on the population. Direct exposure of eggs to sufficiently elevated petroleum hydrocarbon concentrations frequently results in abnormal larvae with poor survival potential (Linden 1976; Rosenthal and Alderdice 1976; Pearson et al. 1985; Kocan et al., In press). These abnormalities may be caused, in part, by somatic genetic damage. For example, an elevated frequency of anaphase aberrations was detected in the pectoral fins of exposed herring embryos (Hose et al., In press). It is not clear, however, if the effect of hydrocarbons on adult herring can cause genetic damage in the progeny, and if so, whether this damage can occur in the germ line and thus affect subsequent generations. In a study by Rice et al. (1987), egg survival was not reduced by exposure of adults to the water-soluble fraction of Cook Inlet crude oil unless the adults were killed, but this study did not look for evidence of morphological or genetic abnormalities.

The purpose of this study was to determine if exposure of pre-spawning adult herring to petroleum hydrocarbons can cause genetic damage in larvae, and to determine if this damage is transmissible to subsequent generations. Genetic damage was assessed by observation of anaphase-telophase aberration rates in the pectoral fins of newly hatched larvae. Observation of

genetic damage was confined to disruption of somatic cell division because it is not practical to rear herring from eggs to maturity in the laboratory. We assume that disruption of normal somatic mitotic processes may signal the possibility of future meiotic disruption, or reduce the potential of larvae to survive to reproductive age. Fertilization success, hatch timing, hatch success, egg mortality, larval size at hatch, and morphological abnormalities were also tested for treatment effects. Exposure concentrations and hydrocarbon accumulation in adults and eggs were quantified.

METHODS

Adult herring were captured and maintained as described in Chapter 1. Fish were gravid but not ripe when captured. Ripeness was judged from percent gonad to total body weight [gonosomatic index (GSI)] and from gamete color and texture. Males began to show evidence of ripening (softening of testes and milt extrusion) by April 11. Females showed evidence of ripening by April 14 (loosening of eggs and yellowing of ovaries). Excluding individuals that spawned, GSI increased significantly from the time of capture through May 12, 1994 (P < 0.001). Fish were artificially spawned on May 2 to May 4 and again on May 10 to May 12; these spawnings occurred at about the same time natural spawning events in the area were observed (April 28 to May 6 in Berner's Bay (Personal communication, Andy McGregor, ADF&G, 1994). Volitional spawning was observed in captive fish as early as April 11.

Adult herring were exposed to oil as described in Chapter 1. There were five treatments, including controls, each replicated three times. Exposures lasted 8 or 16 d, with an average of 11 of 50 fish per tank sampled after 8 d. To characterize the oil and quantify treatment concentrations, composite samples (1.27 L from each of 3 replicate tanks) were collected on days 0, 8, and 16 for analysis by gas chromatography (Short et al., In press).

After 8 and 16 d exposure, fish were artificially spawned. On day eight, 21 females were spawned per treatment. On day 16, 9 to 18 females were spawned per treatment; the number of fish available for spawning was limited by reproductive condition and by disease mortality. Each fish was assigned a unique identification number. Fish were killed by a blow to the head, measured (fork length to the nearest millimeter), weighed (wet weight to the nearest 0.01 g), and bled by clipping a gill arch. For age analysis, six scales were removed from the skin near the posterior margin of the dorsal fin, placed succulus down on a glass slide, and covered with a second slide. Testes from up to three ripe males per replicate tank (three was the norm) were sealed in plastic bags and maintained in chilled seawater until use. Ovarian membranes were cut longitudinally and eggs were removed with a hydrocarbon-free stainless steel spatula. From each female, approximately 150 eggs were deposited with a gentle swirling on each of ten 25 x 75 mm glass slides placed at the bottom of a shallow glass dish in ambient seawater. Eggs from each female were placed in a staining rack and suspended in separate beakers of seawater. Milt was prepared from collected testes by cutting sections from each contributing male into small segments; segments plus a small amount of seawater were mixed with a spatula. A few milliliters of milt were added to the beakers containing eggs; chunks of tissue were avoided. Eggs and milt

remained in contact 5 min with gentle stirring; milt was then poured off and the eggs were gently rinsed in seawater. Eggs were fertilized within 34 min of deposition. Gonad and muscle samples (10 g minimum) were frozen for hydrocarbon analysis. All tools and glass vials used to collect samples for hydrocarbon analysis were hydrocarbon free. Glassware was hydrocarbon free as received from the manufacturer; other tools were washed with soapy water, rinsed, dried, and rinsed with methylene chloride.

For the purpose of measuring hydrocarbon depuration, additional eggs from multiple contributing females in each replicate high oil treatment tank were deposited directly into shallow glass pans and fertilized with the previously described milt. These eggs were immersed in a 220 L seawater bath. Eggs were collected 0, 0.5, 1, 2, 4, 8, 16, and 24 d after fertilization and frozen for hydrocarbon analysis.

Eggs, grouped by replicate treatment tank, were incubated in 40 to 50 L tanks for 15 to 18 d. Seawater input was approximately 1 L/min at 5.3 to 5.4°C. Staining racks containing eggs were suspended from monofilament line attached to a pivoting overhead framework driven by an offset cam to cause slow movement (1 rpm) through the water. Lighting was natural, supplemented by overhead fluorescent light during daylight hours.

Eggs were examined for fertilization success and development 1 to 10 d after fertilization. Excess eggs were removed from all slides by scraping, i.e., those along slide margins susceptible to mechanical damage, and clumps of eggs where not all eggs were exposed to water. This processing was accomplished in water with a minimum of emersion. Two of ten slides were randomly selected to quantify fertilization success and development; all eggs were counted on these slides.

Before hatch one randomly chosen slide of eggs per female was isolated by female in 1 L glass jars. Temperature was controlled by placing jars in a flowing seawater bath. To avoid oxygen depletion in water immediately surrounding the eggs and other potential problems, slides containing eggs were attached to mobile racks designed to cause slow movement through the water. Lighting was natural, supplemented by overhead fluorescent light during daylight hours. Temperature and salinity were monitored.

Hatch timing, hatching success, larval viability, and larval abnormalities were observed daily for each fish (except every other day before hatch began). Hatched larvae were assessed for swimming ability and gross morphological deformities, anesthetized with tricaine methanesulfonate, and preserved in 10% phosphate buffered formalin. Live larvae were preserved independently from dead larvae. After hatch was complete, remaining eggs were inspected; the number dead, number infertile, and number of dead embryos were enumerated.

Preserved larvae were measured with an optical micrometer. Total length, standard length (snout to tip of spine), yolk length, and yolk height were measured. Preserved larvae were transferred to physiological saline (2 parts distilled water, 1 part seawater) for measurement; only

straight larvae were measured. Measurements were restricted to a 7 to 8 d period (8 and 16 d exposures, respectively) that encompassed the majority of the hatch. Roughly 50 larvae were measured per fish.

For genetic analysis, a maximum of 15 females were randomly selected from control and high treatment, and 10 stage 1b larvae (Humphrey et al., In press) were randomly selected from each female; 300 larvae were analyzed. Larvae were transferred to 5% phosphate buffered formalin during this procedure. In the pectoral fins, the total number of cells undergoing mitosis were counted, and the number of anaphase-telophase aberrations recorded, including translocation bridges, attached fragments, acentric fragments, stray and lagging chromosomes, and sidearm bridges. The number of micronucleated cells in the pectoral fins was recorded, and interphase cells were categorized as normal or pathologic (swollen, vacuolated, or containing marginated chromatin). Degenerating cells (pycnotic and multinucleated or karyorrhectic) were also recorded. Larvae were also assessed for skeletal, craniofacial, and finfold deformities.

Data processing and statistics

The 8 and 16 d exposure groups were generally treated as independent experiments. The statistical design was essentially balanced in the 8 d exposure group, but due to volitional spawning activity and disease, there were insufficient eggs available in the 16 d exposure group for a balanced design. The two exposure groups were not completely independent because fish were sampled from the same population pool in each tank on two occasions. Reported means were based on least squares estimates unless otherwise stated.

The denominator used to calculate percentages varied according by response type. Percentages of eggs fertile, infertile, and initially dead were based on the total number of eggs counted near the beginning of the experiment. Percentages of eggs that hatched or died were based on the total number of hatched larvae plus the number of dead eggs determined at the endpoint. The number of hatched larvae was subdivided into number alive, number moribund, and number dead. Accordingly, percent alive was the number of living larvae (excluding moribund larvae) divided by the total number hatched. Similarly, number hatched was used as the denominator to calculate percent moribund and percent dead larvae. Swimming ability of alive larvae was categorized as good, fair to poor, or incapable. Swimming ability of moribund and dead larvae was, by definition, nonexistent, thus alive was used as the denominator for swimming categories. All larvae categorized as incapable of swimming had spinal defects. Spinal aberrations were assessed only in alive and moribund larvae. Because dead larvae quickly become distorted after death, they were not assessed for spinal condition. Percent spinal aberrations, therefore, was number of aberrations divided by number alive plus moribund.

To estimate hatch timing, the peak hatch day was determined for each fish. In cases where two peaks of equal magnitude occurred, the first peak was reported. Median hatch day was calculated by two-point linear interpolation of cumulative percent hatch (y) and day (x), from data points that spanned 50% hatch, based on the number of eggs that hatched.

Percentage data and hatch timing were analyzed with analysis of variance (ANOVA), with treatment, replicate, and fish as class variables, replicate nested in treatment, and fish nested in replicate and treatment. Before analysis with ANOVA, percentage data were arc-sine transformed, and corrected for small n where necessary (Snedecor and Cochran 1980). [The same general conclusions were reached with untransformed data].

Larval length and yolk volume were analyzed with analysis of covariance, with time as a covariate, and considered treatment, replicate, and fish as class variables with replicate nested in treatment and fish nested in replicate and treatment. Yolk sac volume was estimated as follows: $V = 4\pi/3 * (L_y/2)^2 * (H/2)$, where V = volume (mm^3), L_y = length (mm) of yolk sac, and H = height (mm) of yolk sac (Hourston et al. 1984).

Morphological scores assigned to larvae assessed for genetic aberrations were analyzed with the Kruskal-Wallis nonparametric test.

RESULTS

Reproductive condition of parental stock

The adult herring sampled after 8 and 16 d exposure to oil were reproductively ripe, partially spawned, or completely spawned out (Table 2.1). No immature gonads were observed. There were significantly more partially spawned males than females (P = 0.040); gender differences were not significant in the "ripe" and "spawned out" categories (P = 0.181 and P = 0.746, respectively). Significantly more herring in the 16 d group had released some or all gametes than in the 8 d group (P = 0.036). Ripeness did not vary significantly among oil treatments (0.245 ≤ P ≤ 0.494).

Hydrocarbon uptake and depuration

Results of exposure of parent fish to hydrocarbons for 8 or 16 d is detailed in Chapter 1. Concentrations of PAH hydrocarbons increased in ovarian tissue during exposures to a maximum of 9688 ppb in the high oil treatment (Fig. 1.5).

Shortly after fertilization, hydrocarbon concentrations in eggs were very similar to concentrations observed in ovaries (see Chapter 1), but declined linearly during incubation in clean seawater (r^2 = 0.97 and r^2 = 0.96 for 8 and 16 d exposures, respectively) (Fig. 2.1). After 24 d, PAH concentrations in high treatment eggs (2,359 ± 138 ppb) remained significantly elevated above baseline concentrations (11 ± 1 ppb, as measured in control ovaries) (P < 0.001).

Composition of PAH hydrocarbons in eggs was similar to PAH composition in water and ovaries (Fig. 2.2). Relative concentrations of naphthalenes tended to decline with depuration time (0.092 ≤ P ≤ 0.157), and relative concentrations of all heavier compounds tended to increase with time (0.001 < P ≤ 0.093). Chrysenes were not detected in eggs.

Table 2.1.--Reproductive condition of adult herring at time of artificial spawning. Data reported are least squares adjusted means with standard error (SE). No immature gonads were observed.

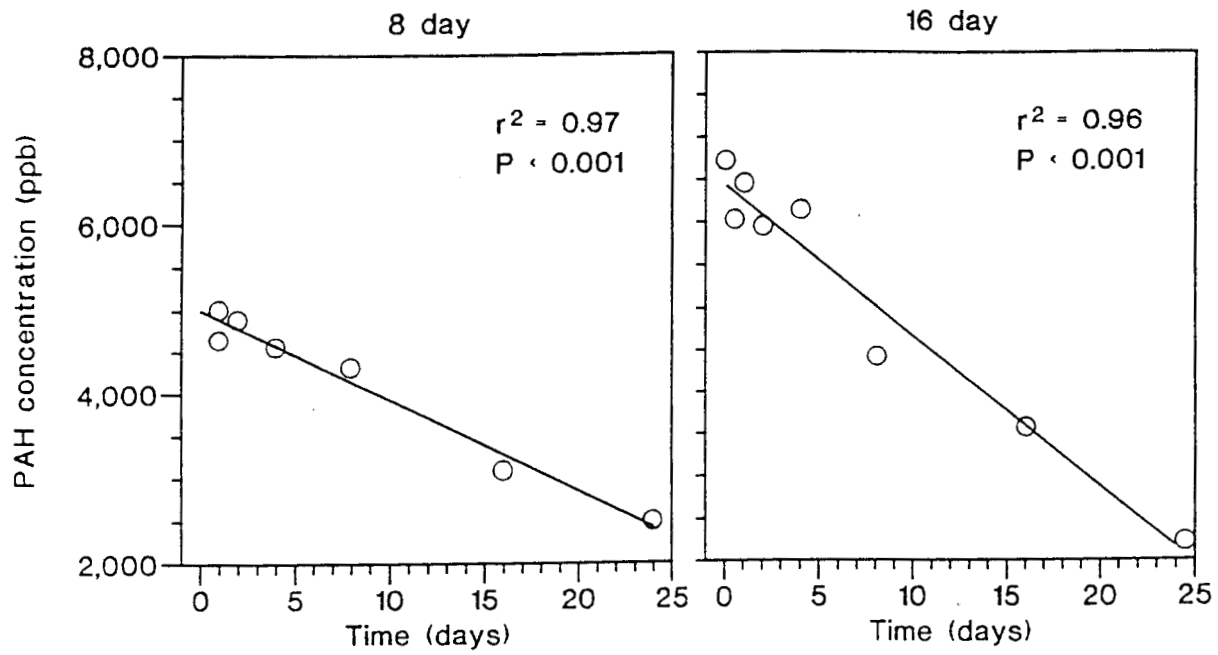|	Gender	8d	16d	SE
Percent ripe:				
	Female	98.33	89.72	5.08
	Male	92.22	77.78	
Percent partially spawned:				
	Female	0.00	1.11	3.80
	Male	1.67	15.56	
Percent spawned out:				
	Female	1.67	9.17	3.70
	Male	6.11	6.67	

Figure 2.1.--PAH concentrations in high treatment eggs as a function of time. Data displayed are means ± standard error.
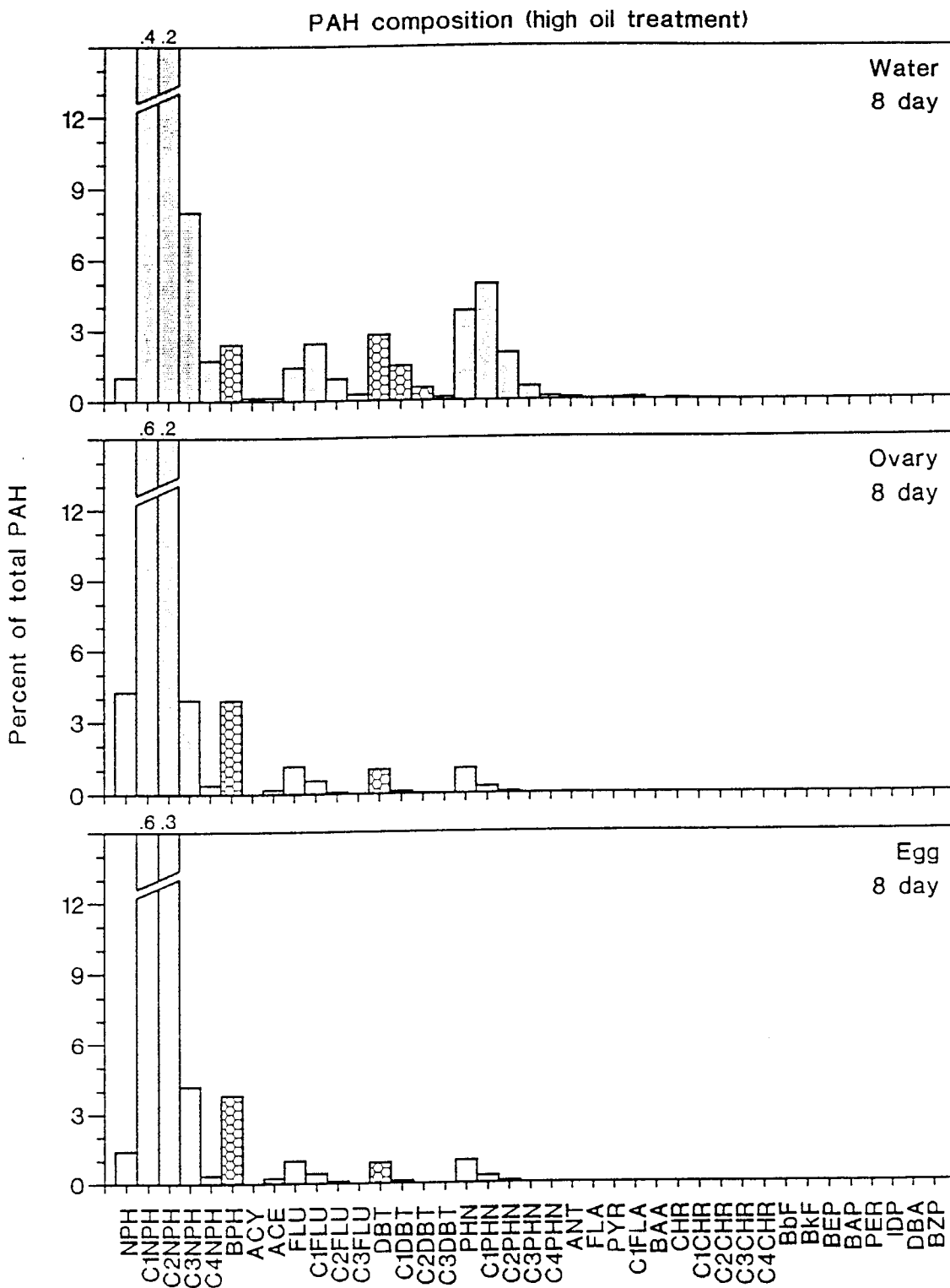
Figure 2.2.--Composition of PAH in eggs was similar to composition in ovary tissue and in treatment water. This example is from the high oil treatment after 8 d exposure.

Eggs and larvae

Egg fertility was not affected by exposure of adult herring to oil. Mean egg fertility exceeded 91% in all treatments (Fig. 2.3). Exposure of adults to oil did not affect egg fertility in the 8 d exposures (P = 0.331). In the 16 d exposures, fertility varied significantly among treatments (P = 0.001), but differences were unrelated to exposure because only the trace oil treatment differed significantly from controls (Fig. 2.3). Mean fertility was greater in 16 d exposures than in 8 d exposures, but variability was greater in the 16 d exposures.

The percentage of eggs that hatched was not affected by exposure of adult herring to oil. The mean percentage of eggs that hatched exceeded 87% in 8 d exposures and 78% in 16 d exposures (Fig. 2.3). In 8 d exposures, percent hatch varied significantly among treatments (P = 0.025), but differences were unrelated to exposure because only the trace oil treatment differed significantly from controls (Fig. 2.3). Percent hatch did not vary significantly among treatments in 16 d exposures (P = 0.122). A significantly smaller percentage of eggs hatched in 16 d exposures than in 8 d exposures; variability was greater in the 16 d exposures.

Incubation time was not affected by exposure of adult herring to oil. The percentage of eggs that hatched each day was highly similar among treatments (Fig. 2.4). In 8 d exposures, mean peak hatch and median hatch were 31 d, and were not affected by treatment (P = 0.946 and P = 0.791, respectively) (Fig. 2.5). In 16 d exposures, mean peak and median hatch times ranged from 28 to 30 d; peak hatch varied significantly among treatments (P < 0.001), but differences were probably not related to oil exposure because results conflict (peak hatch was significantly later in the mid oil treatment than in controls, but was significantly earlier in the high oil treatment) (Fig. 2.5). Similarly, differences in median hatch time in the 16 d group were probably not related to treatment, and response in the high oil treatment did not differ significantly from control (P = 0.972) (Fig. 2.5).

The percentage of eggs that died was not affected by exposure of adult herring to oil. Mean number of dead eggs did not exceed 11% and 21% in 8 and 16 d exposures, respectively (Fig. 2.3). Differences among treatments were not significant (P = 0.062 and P = 0.164 for 8 and 16 d exposures, respectively). Significantly more eggs died in 16 d exposures than in 8 d exposures (P = 0.002); variability was greater in 16 d exposures.

The percentage of embryos that developed at least to the eyed stage but died before hatch was not affected by exposure of adult herring to oil. (This category was a subset of all eggs that died.) The mean number of dead embryos did not exceed 8% and 17% in 8 and 16 d exposures, respectively (Fig. 2.3). Differences among treatments were not significant (P = 0.075 and P = 0.074 for 8 and 16 d exposures, respectively). Significantly more embryos died in 16 d exposures than in 8 d exposures (P < 0.001); variability was greater in 16 d exposures.

Larval health was not affected by exposure of adult herring to oil. The mean percentage of larvae classified as moribund did not exceed 0.17% and 0.25% in 8 and 16 d exposures,
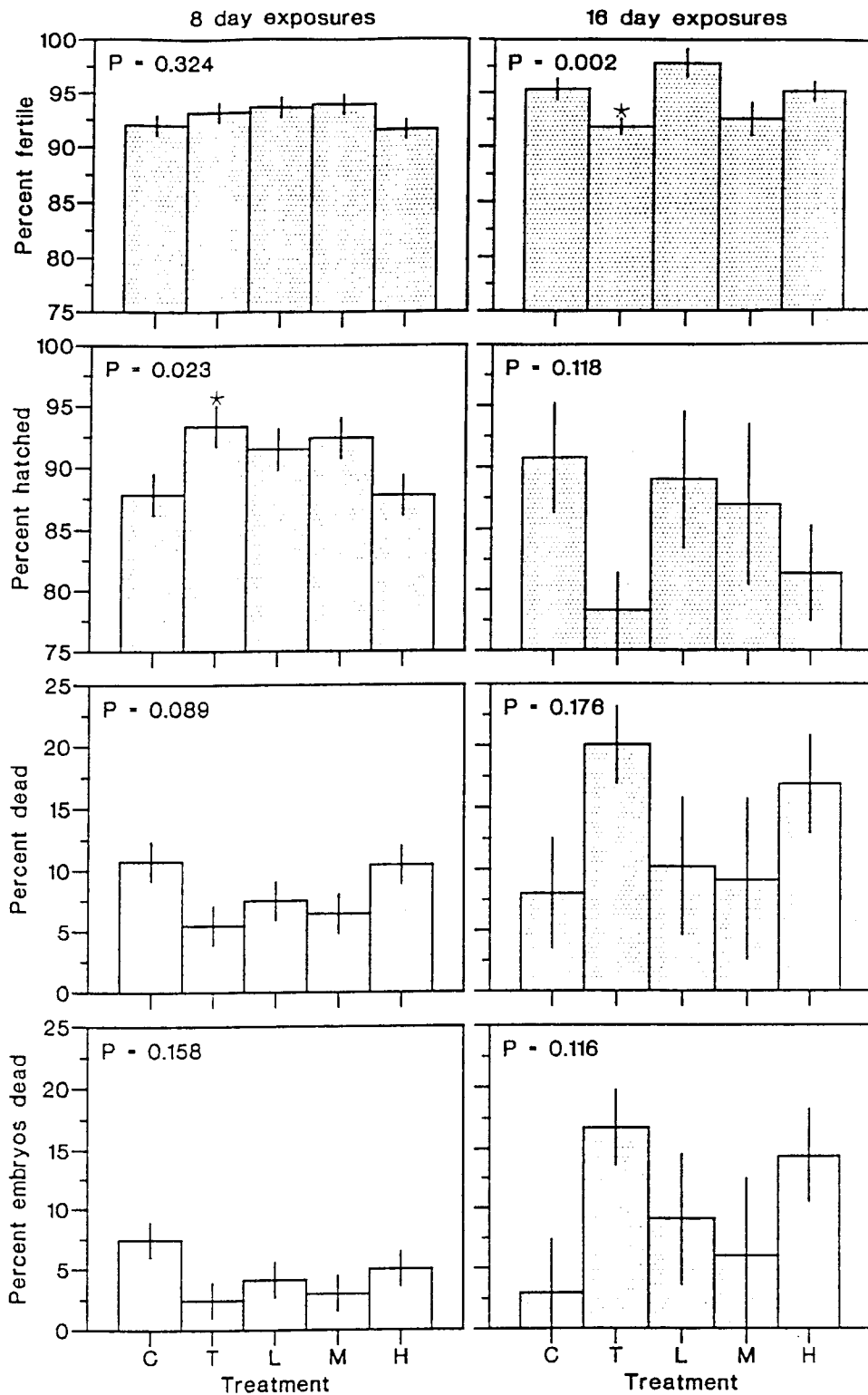
Figure 2.3.--Percent fertility, hatching, and death of eggs spawned from adult herring exposed to oil 8 or 16 d. Treatments were unexposed controls (C), and trace (T), low (L), and mid (M), and high (H) oil; P indicates probability of treatment effect, and asterisks indicate significant differences from controls. Data displayed are least squares adjusted means ± standard error
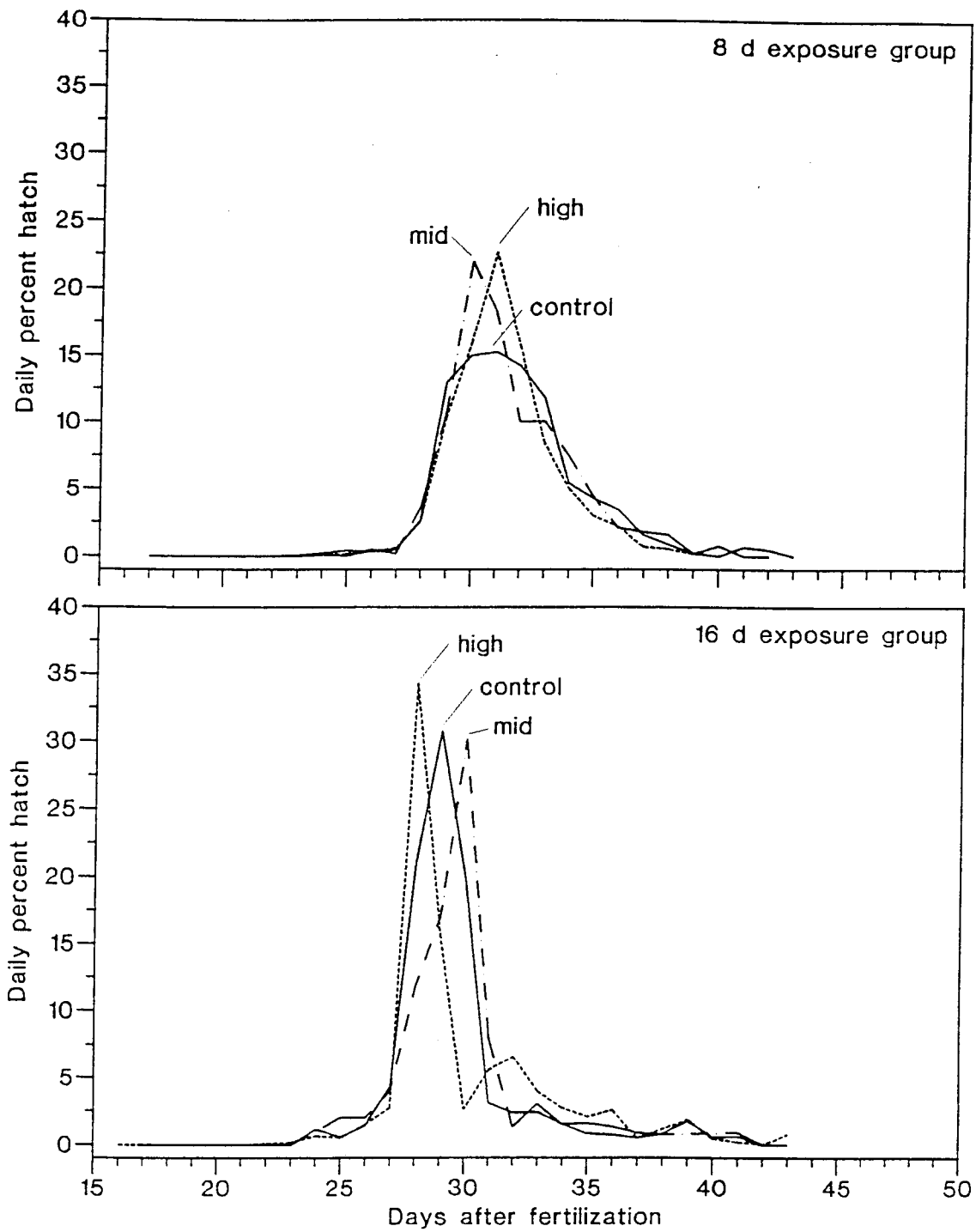
Figure 2.4.--Daily percent hatch as a function of time after fertilization of eggs spawned from adult herring exposed to oil in water for 8 or 16 d. For clarity, trace and low oil treatments were not displayed.

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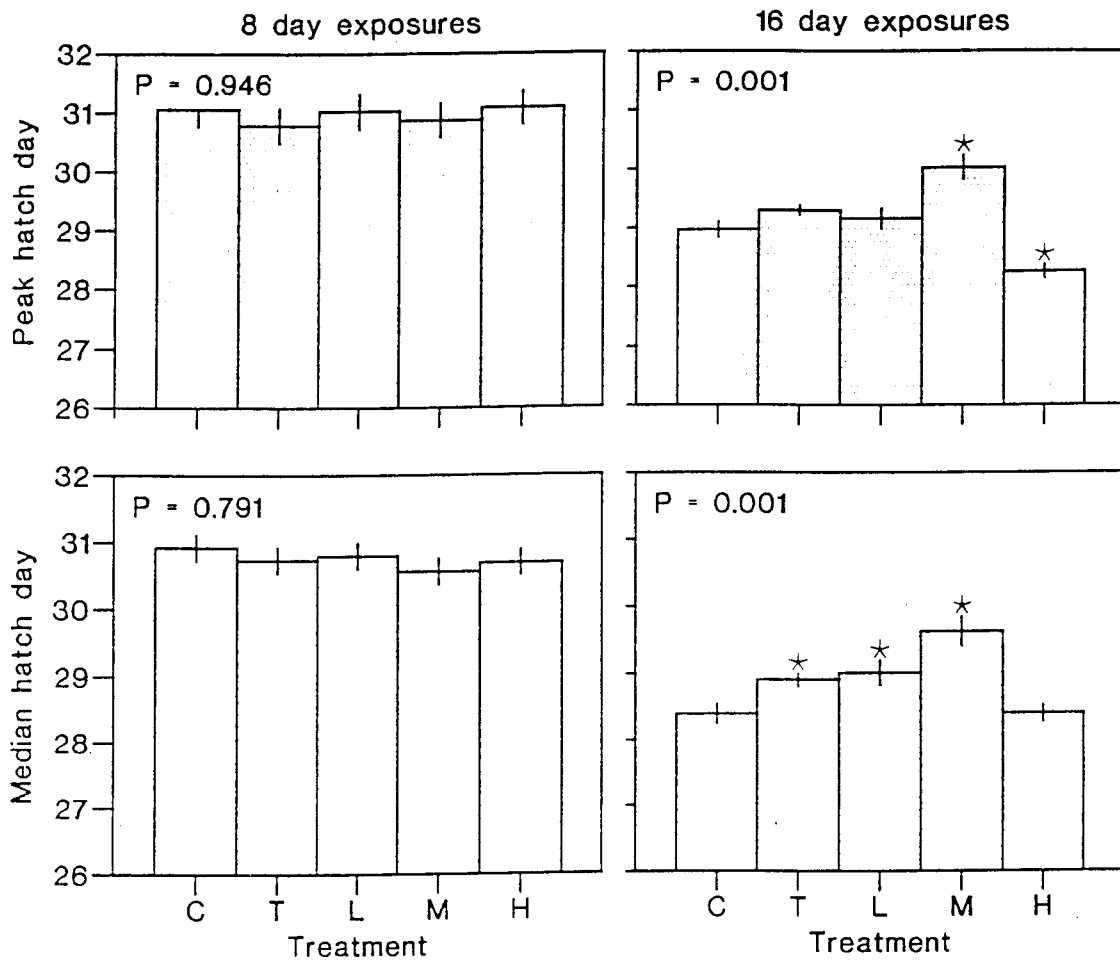
Figure 2.5.--Summary of peak and median incubation times by treatment; parent herring were exposed to oil 8 or 16 d. Treatments were unexposed controls (C), and trace (T), low (L), mid (M) and high (H) oil; P indicates probability of treatment effect, and asterisks indicate significant differences from controls. Data displayed are least squares adjusted means ± standard error.

respectively (Fig. 2.6). Differences in morbidity among treatments were not significant (P = 0.995 and P = 0.780, respectively). The mean percentage of larvae that died within the 24 h sampling periods did not exceed 2% and 5% in 8 and 16 d exposures, respectively. Differences in larval death among treatments were not significant (P = 0.867 and P = 0.458, respectively). More larvae died in the 16 d exposures than in 8 d exposures; variability was greater in 16 d exposures.

Larval swimming was not affected by exposure of adult herring to oil. The mean percentage of larvae classified as good swimmers exceeded 97% and 90% in 8 and 16 d exposures, respectively (Fig. 2.7). Differences in good swimmers among treatments were not significant (P = 0.097 and P = 0.789, respectively). The mean percentage of fair to poor swimmers did not exceed 2% and 10% in 8 and 16 d exposures, respectively; treatment differences were not significant (P = 0.384 and P = 0.637, respectively). The percentage of larvae incapable of swimming did not exceed 1.4% and 1.1% in 8 and 16 d exposures, respectively (Fig. 2.7). Differences in larvae incapable of swimming were significant among treatments in 8 d exposures (P = 0.024), but no treatment differed significantly from the control. In 16 d exposures, differences in larvae incapable of swimming were not significant (P = 0.787).

Exposure of adult herring to oil did not cause larval abnormalities. The mean percentage of larvae with spinal defects did not exceed 1.3% and 1.1% in 8 and 16 d exposures, respectively (Fig. 2.6). Differences in spinal defects among treatments were not significant (P = 0.152 and P = 0.932, respectively).

Length of newly hatched larvae varied by female, time, and treatment, but there were no uniform trends. In this analysis, only control and high treatment larvae were measured, and observation was restricted to the period of peak hatch (29 to 35 d and 27 to 34 d for 8 and 16 d exposure groups, respectively). Larval lengths varied significantly with time, but changes were not predicable or consistent (Fig. 2.8). The time effect was weak, and from the majority of females (42 of 56) length at hatch did not vary significantly as a function of time. In the 8 d exposure group, length at hatch tended to decline over time in control larvae, but remain constant in high treatment larvae, thus high treatment larvae were significantly longer than control larvae (9.5 and 9.4 mm, respectively). However, in the 16 d exposure group high treatment larvae were significantly shorter than control larvae (9.0 and 8.9 mm, respectively). When both exposure groups were analyzed simultaneously, the conclusion reached was that the length of larvae at hatch was not significantly affected by parental exposure to oil (P = 0.449).

Yolk volumes in newly hatched larvae also varied by female, time, and treatment, but there were no uniform trends. In the 8 d exposure group, yolk volume in the control (n = 622) increased over the observation period (29 to 35 d), but volume in the high treatment (n = 572) remained constant; measured with ANOVA slopes were not parallel (P < 0.001, Fig. 2.8). In the 16 d exposure group yolk volumes did not exhibit clear trends over time for either treatment, but were significantly greater (P < 0.001) in the high treatment group (n = 838) than in controls (n = 497).

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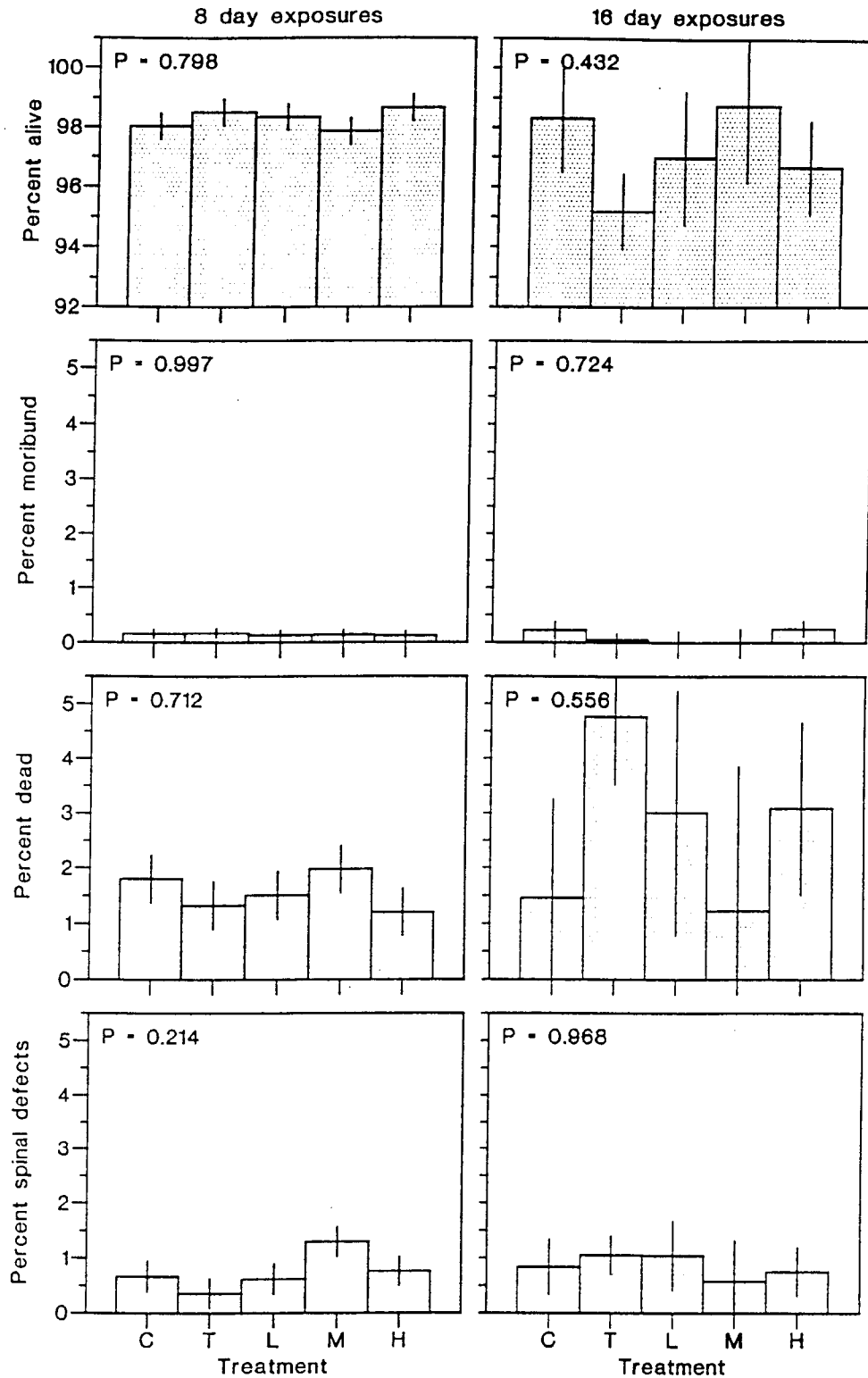
Figure 2.6.--Health and survival of larvae collected within 1 d of hatch. Spinal defects were also enumerated. Treatments were unexposed controls (C), and trace (T), low (L), mid (M) and high (H) oil; P indicates probability of treatment effect, and asterisks indicate significant differences from controls. Data displayed are least squares adjusted means ± standard error.

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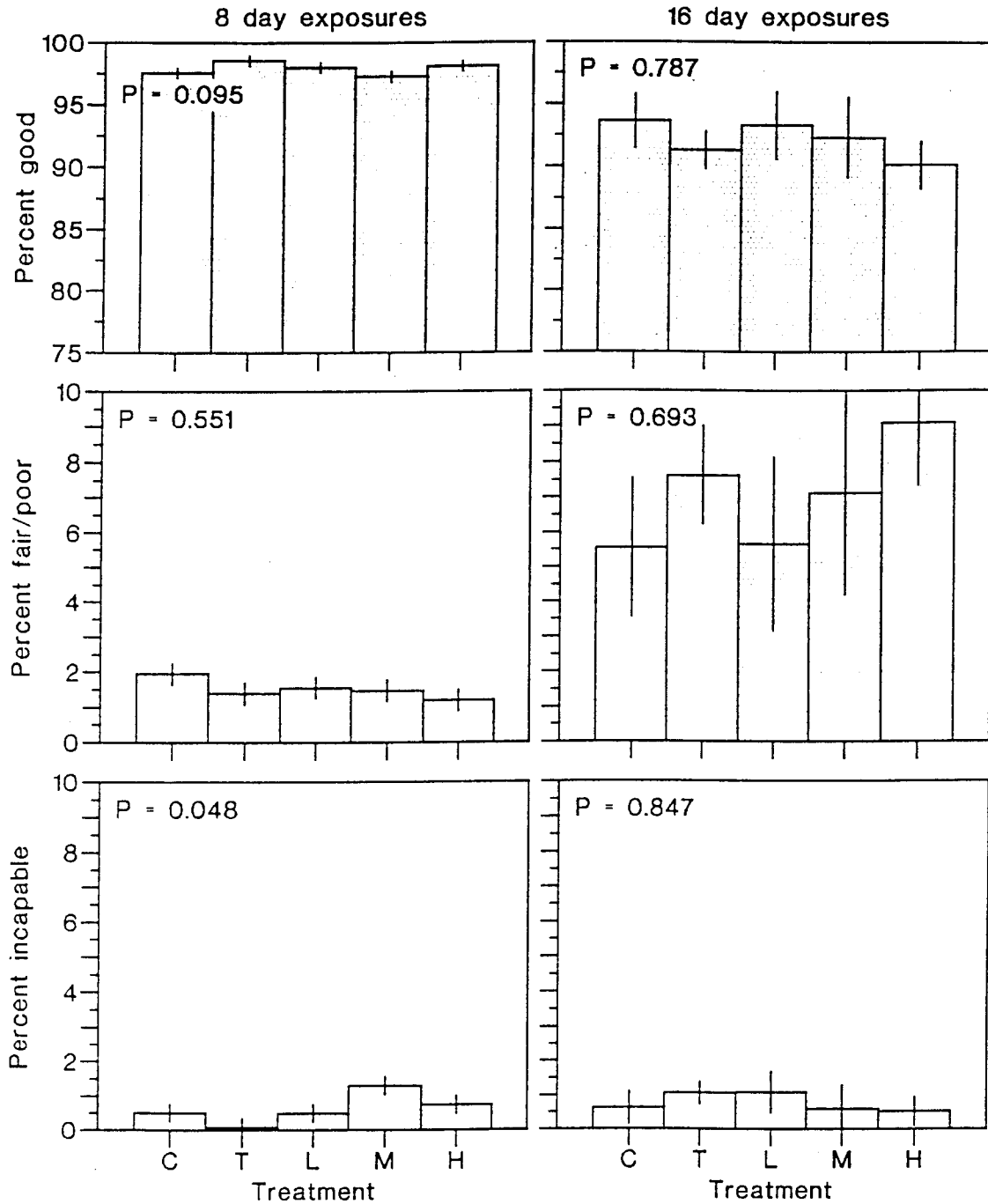
Figure 2.7.--Larval swimming ability as a function of exposure of parent fish to oil in water. Treatments were unexposed controls (C), and trace (T), low (L), mid (M) and high (H) oil; P indicates probability of treatment effect. Data displayed are least squares adjusted means ± standard error.
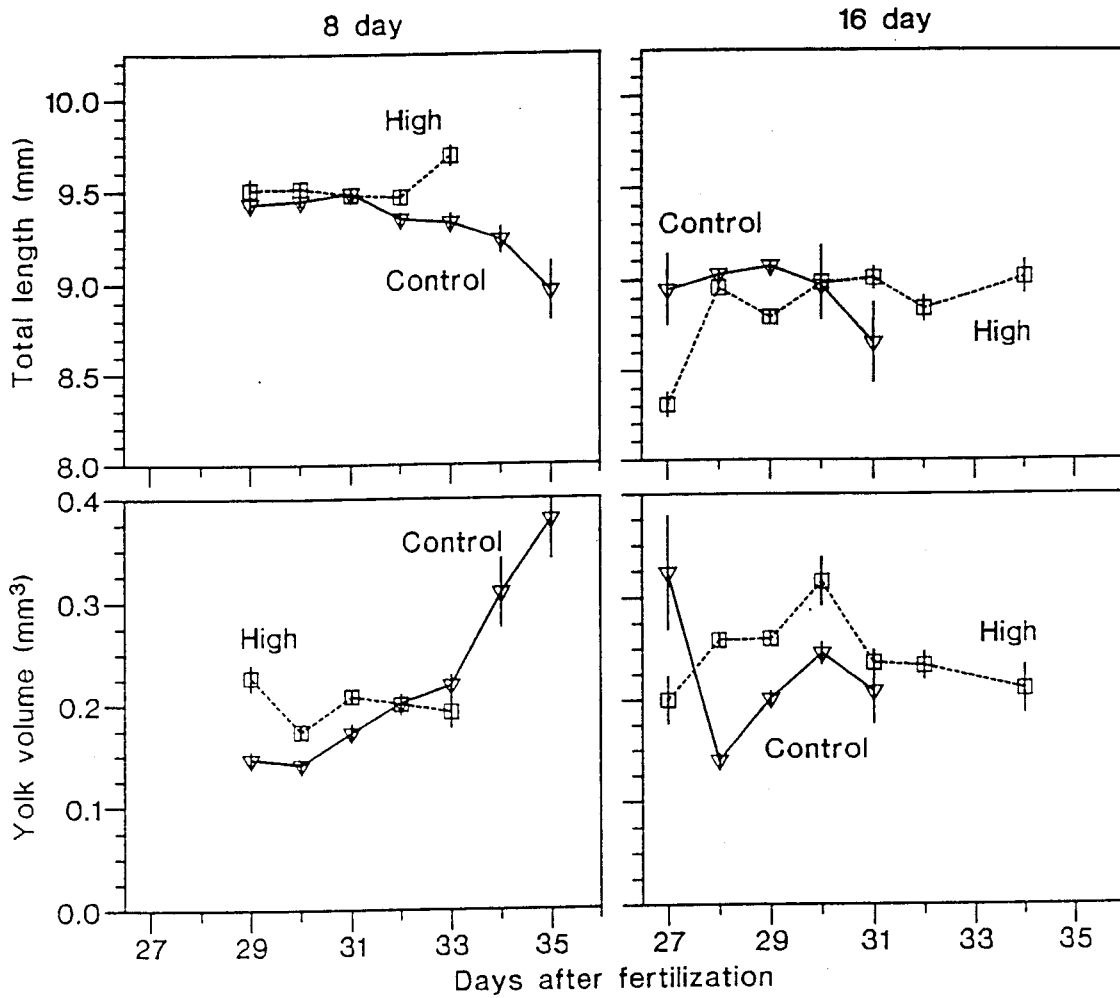
Figure 2.8.--Comparison of larval size in control and high treatments as a function of time after fertilization. Parent herring were exposed to oil for 8 or 16 d. Data displayed are least squares adjusted means ± standard error.

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Embryonic maturation was not influenced by exposure of adult herring to oil (P ≥ 0.260). The majority (60%) of newly hatched larvae were classified as stage 1b; only 3% were stage 1a, and the remainder were stage 1c.

The rate of cell division in newly hatched larvae was not affected by exposure of adult herring to oil (Fig. 2.9). The number of cells undergoing mitosis in pectoral fins did not differ significantly between control (18.5 ± 0.4) and high treatment (19.6 ± 0.4) larvae (P = 0.090).

Exposure of adult herring to oil for 8 d did not cause a significant elevation of genetic aberrations in the pectoral fins of newly hatched larvae (Fig. 2.9). Percent anaphase-telophase aberration was significantly greater in controls (5% ± 1) than in the high treatment (4% ± 1) (P = 0.032, n = 134 larvae per treatment) (Fig. 2.9). Analysis was restricted to control and high oil treatment larvae. Based on the 8 d results, and lack of response in other 16 d parameters, larvae from 16 d exposures were not analyzed for chromosomal aberrations.

Other cell problems were rare and generally not associated with treatment. Although statistically significant (P = 0.036), micronucleation was observed in only 2% of the larvae. All interphase cells were normal. Pycnotic cells were observed in 10% of the larvae; treatment was not significant (P = 0.722). Multinucleated or karyorrhectic cells were detected in 7% of the larvae; treatment was not significant (P = 0.215). Exposure of adult herring to oil did not cause morphological deformities ($P_{Kruskal-Wallis}$ = 0.516).

DISCUSSION

This discussion is incomplete and is considered only a starting point. In its current form the report was drafted by a single author (Carls); input from other authors will be of value. Reference to existing literature has not been completed.

Effects of exposure of reproductively ripe parental herring to oil in water were not discernable in the progeny. Parameters not affected included egg fertility, hatching success, hatch timing, embryo death, larval health (95 to 99% not moribund or dead within 24 h of hatch), larval swimming, larval abnormalities, stage of larval development at hatch, anaphase-telophase abnormalities, number of mitotic figures, number of pycnotic cells, and number of multinucleated or karyorrhectic cells. Exposure of adult herring to oil did cause accumulation of PAH in gametes, and although they declined, concentrations remained significantly elevated in high treatment eggs through incubation.

The experiment was, in essence, repeated twice (8 and 16 d), although mean PAH concentrations were greater in the shorter exposure than in the longer exposure. Results of the two experiments were consistent, the primary difference being more variability and poorer overall performance of eggs and larvae in the longer exposure. Differences between experiments may be attributable to increasing water temperatures or bacterial loads.
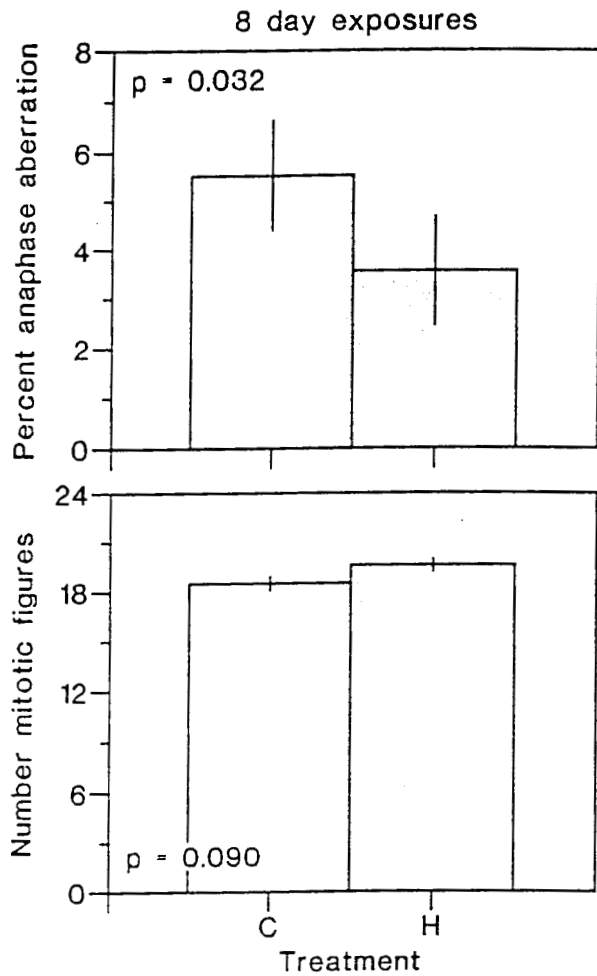
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Figure 2.9.--Anaphase aberration and number of mitotic figures in pectoral fins of newly hatched larvae as a function of treatment; parent herring were exposed to oil in water for 8 d. Treatments were control (C) and high (H); P indicates probability of treatment effect.

To determine the efficacy of the experiment, power analysis would be prudent, and awaits completion. Power analysis is not clearly defined in statistical literature for the nested ANOVA models employed in this study, so approximation with simpler models or Monte Carlo techniques may be necessary. However, the total number of eggs observed was high in both treatment series (>12,000 and >7,500, respectively) so statistical power should have been reasonably good.

Direct exposure of herring eggs to oil during incubation is more detrimental than indirect exposure shortly before spawning. Direct exposure of herring eggs to sufficiently elevated petroleum hydrocarbon concentrations frequently results in abnormal larvae with poor survival potential (Linden 1976; Pearson et al. 1985; Kocan et al., In press). In a study similar to ours, egg survival was not reduced by exposure of adults to the water-soluble fraction of crude oil unless the adults were killed (Rice et al. 1987). These findings suggest that direct exposure of spawn to *Exxon Valdez* oil in PWS in 1989 was much more likely to cause problems observed in larvae than exposure of adult herring.

Exposure of reproductively ripe parental herring did not measurably damage gametes, as judged by egg fertility and subsequent development. Herring in this experiment were reproductively similar to those in PWS at the time of the EVOS. Arguably, gametes might have been damaged if exposure had occurred during gamete formation (meiosis), but this was beyond the context of the present experiment. There was no indication that exposure of adult herring to oil caused chromosomal damage in somatic cells in progeny, thus we infer that heritable genetic damage was unlikely.

CONCLUSIONS

A. Exposure of reproductively ripe parental herring did not measurably damage gametes, as judged by egg fertility and subsequent development.

B. Gametes accumulated hydrocarbons as a result of exposure of adult herring to oil; these hydrocarbons were partially depurated during incubation in clean seawater.

C. Effects of exposure of reproductively ripe herring to oil in water were not discernable in progeny. Genetic damage was not detected in larvae.

D. It is unlikely that exposure of adult herring to oil caused genetic damage that was transmissible to future generations.

ACKNOWLEDGMENTS

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Chapter 3: Induction of mixed function oxidases by petroleum hydrocarbons in pre- and post-spawn herring (*Clupea harengus pallasi*)

R. E. Thomas, M. G. Carls, and S. D. Rice

INTRODUCTION

Several laboratory studies have demonstrated a potential for organic contaminants such as polycyclic aromatic hydrocarbons (PAH) to interfere with the reproductive process in several fish species (reviewed by Weis and Weis, 1989). In fish, as in other vertebrates, PAHs can be activated to mutagenic, teratogenic, or carcinogenic compounds by the cytochrome P-450 dependent mixed function oxidase (MFO) system: MFO enzymes are involved in the metabolism, and thus clearance, of PAH and other xenobiotics (Jimenez and Stegeman 1990). Activation of carcinogenic PAH (e.g. benzo[a]pyrene is converted to an active carcinogen by aryl hydrocarbon hydroxylase, AHH) results in ovarian toxicity and oocyte destruction in mammals (Mattison et al., 1983). A negative correlation has been shown to exist between reproductive success and hepatic AHH activity in species of flatfish (Spies et al. 1984; Johnson et al. 1988). That exposure of adult fish to contaminants can also impact the offspring is suggested by elevated AHH activity in the eggs of lake trout after exposure of adults to environmental contaminants (Binder and Lech 1984). It is possible, therefore, that even if MFO induction is suppressed in reproductively ripe herring, sufficient activation of PAH might occur to cause damage to offspring.

In previous tests, exposure of pre-spawning (reproductively ripe male and female) adult herring to water soluble fraction (WSF) of crude oil in the laboratory resulted in high concentrations of hydrocarbons in ovarian tissue (Rice et al. 1987). Accumulation of high levels of hydrocarbons in the eggs might indicate adult herring have little capacity to metabolize and excrete hydrocarbons, or that their ability to do so is impaired by their reproductive condition. Induction of MFOs has been shown to be depressed in other fish species in their spawning condition (Forlin et al. 1984; Walton et al. 1983; Collier et al. 1986; Lindstrom-Seppa 1985). Suppression of MFO induction could result in retention and increased bioaccumulation of PAH in fish tissues.

The objective of this study was to compare MFO induction, in pre- and post-spawn herring.

METHODS

MFO induction was determined in pre-spawn and post-spawn herring. Herring were collected, maintained and exposed to oil in water as previously described. In order to determine MFO induction time, pre-spawn herring were exposed to oil in water for 0, 0.5, 1, 2, 4, 8, and 16 d. There were two treatments (control and high concentration) with 25 fish per treatment.

In addition to determining MFO induction time, the effect of concentration on induction was also investigated. Twenty-five fish were exposed for 8 and 16 d to trace, low, or mid oil concentrations. Effect of the high concentration was noted from the 8 and 16 d samples taken from the induction time experiment. All 8 and 16 d exposed animals were sampled at the time they were artificially spawned for the genetics study.

To compare MFO induction between pre-spawn and post-spawn groups, post-spawn herring were exposed to oil in water in the same manner as the pre-spawn group. There were two treatment groups (control and high concentration) with 35 animals per treatment. The oil generators were recharged with freshly oiled rock between pre- and post-spawn exposures, but water temperatures were higher in the post-spawn exposure, thus PAH concentration in water declined more rapidly than in pre-spawn exposures (Fig. 3.1). As a result, post-spawn herring were exposed to much lower mean concentration of PAH than the pre-spawn herring. Fish exposed to the high concentration were sampled at 4, 8 and 16 d. Control fish were sampled at 4 and 16 d.

At the time of sampling, fish were killed by a blow to the head, length and weight determined, and fish exsanguinated by clipping a gill arch. Livers were removed, washed in cold 0.15M KCl, weighed and stored in pre-frozen 20 ml glass vials at -80 C. At least 10 g of muscle tissue was taken from each fish for hydrocarbon analysis (Short et al., In press). In the fish which were spawned, male and female gonads were also removed and subsampled for hydrocarbon analysis. All tissue samples were stored at -14°C until analyzed.

Livers were homogenized in a Potter-Elvehjem homogenizer in 4 vol of 0.15M KCl in 0.1M potassium phosphate buffer, pH 7.4. Microsomal fraction was isolated using a modification of the technique previously described (Thomas et al. 1989) and stored at -80 C until assayed. Prior to freezing, the microsomes were suspended in 0.1M potassium phosphate buffer, pH 7.4; prepared to 0.1M KCl, 1mM EDTA, 1mM dithiothreitol, and 0.1mM phenanthroline. Microsomal protein concentration was determined by the method of Lowery et al. (1951) using bovine albumin standards.

AHH activity was assayed according to the method of Nebert and Gelboin (1968) and was determined for each fish in the study. In addition to AHH activity, ethoxyresorufin 0-deethylase (EROD) activity and cytochrome P-450 concentration were determined for all animals exposed to the high oil concentration and the corresponding controls. Cytochrome P-450 concentration was determined according to the method of Omura and Sato (1964) and EROD activity according to the method of Burke and Mayer (1974). AHH and EROD assays were run in triplicate and cytochrome P-450 assays were conducted in duplicate. Differences between treatments were analyzed by analysis of variance.
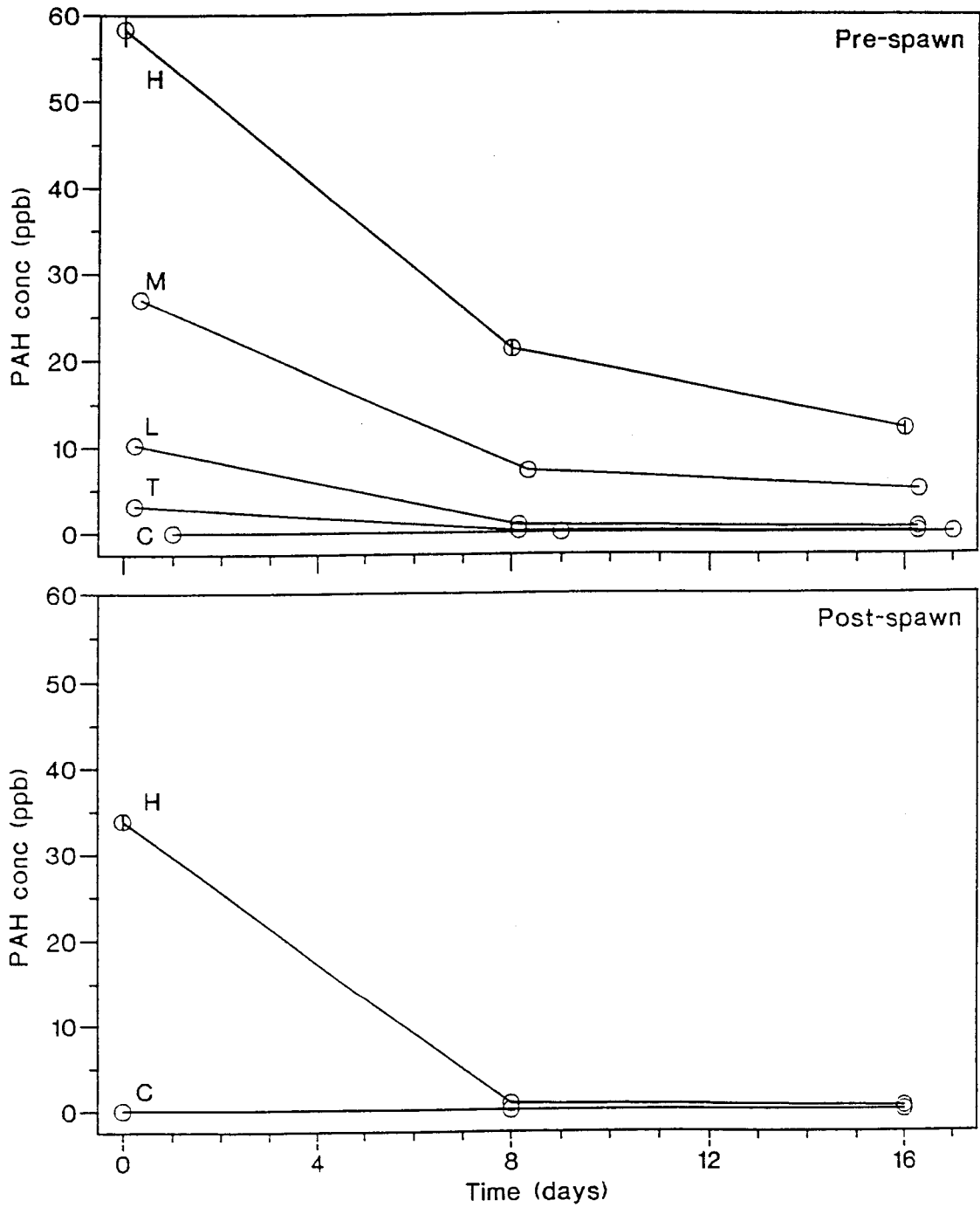
Figure 3.1.--PAH concentrations in treatment water. Treatments were control (C), trace (T), low (L), mid (M), and high (H) oil. Data plotted are single composite values for replicate tanks at each time; standard error was estimated for the high oil treatment from replicate fluorescence analyses.

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RESULTS

PAH in the exposure tanks are shown in Figure 3.1. These concentrations bracketed the maximum mean concentrations observed in water of PWS following the EVOS (6.24 ppb; Short and Rounds 1995). Hydrocarbon concentrations in both the pre- and post-spawn exposure tanks declined with time. Probably because of higher water temperatures in post-spawn exposures, the PAH concentration was much lower at 8 and 16 d than in the pre-spawn exposure tanks; concentrations were elevated above control levels at the beginning of the exposure of the post-spawn tests but then dropped to control levels (Fig. 3.1).

Exposure of pre-spawn herring to the high concentration (decreasing from approximately 55 ppb on day 1 to 12 ppb on day 16) was sufficient to cause a significant increase in AHH activity after only 48 hours. Exposure at this same concentration for 12 and 24 hours did not result in a significant increase in AHH activity (Fig. 3.2). Even the lowest concentration, the trace dose (1.69 ppb), resulted in a significant increase in AHH activity after 8 d of exposure. All exposure concentrations caused a significant increase in AHH activity after 8 or 16 d of exposure, except AHH was not significantly elevated following the 16 d exposure to the trace dose (Fig. 3.3).

Exposure of post-spawn herring to the high concentration for either 4, 8 or 16 d (approximately 18, 0.792 and 0.476 ppb) resulted in a nearly two-fold greater induction in AHH activity than was measured in pre-spawn herring exposed under the same conditions (Fig. 3.2). AHH activity was greater in post-spawn herring even though they were exposed at significantly lower concentrations than the pre-spawn herring. Although exposure concentration was less at 16 d than at 8 d, and not significantly elevated above control levels in the post spawn exposure, AHH activity was greater with the longer exposure in both the pre-spawn and post-spawn groups (Fig. 3.2).

PAH concentration in muscle tissue reached a maximum after 4 d in the pre-spawn group exposed to the high concentration, and remained elevated for the duration of the 16 d exposure (Fig. 3.4). After only one day of exposure to the high concentration, the level of PAH in the muscle was significantly greater than in the controls. During this same period, exposure concentration was decreasing and AHH activity was increasing. Muscle PAH in the post-spawn herring decreased with exposure time and was significantly less than in the pre-spawn group on days 4 and 16 but not on day 8 (Fig. 3.4). Muscle PAH was significantly greater in the exposed than in the control post-spawn herring on days 4 and 8 but not on day 16. This decrease in tissue concentration was occurring at the same time water PAH concentration was declining and AHH activity was increasing.

EROD activity and cytochrome P-450 concentration were assayed in the pre-spawn herring exposed for 16 d to the high concentration. EROD activity, although increased, was not significantly greater than the controls (Fig. 3.5). Cytochrome P-450 concentration, on the other hand, was significantly greater in these same animals (Fig. 3.5).
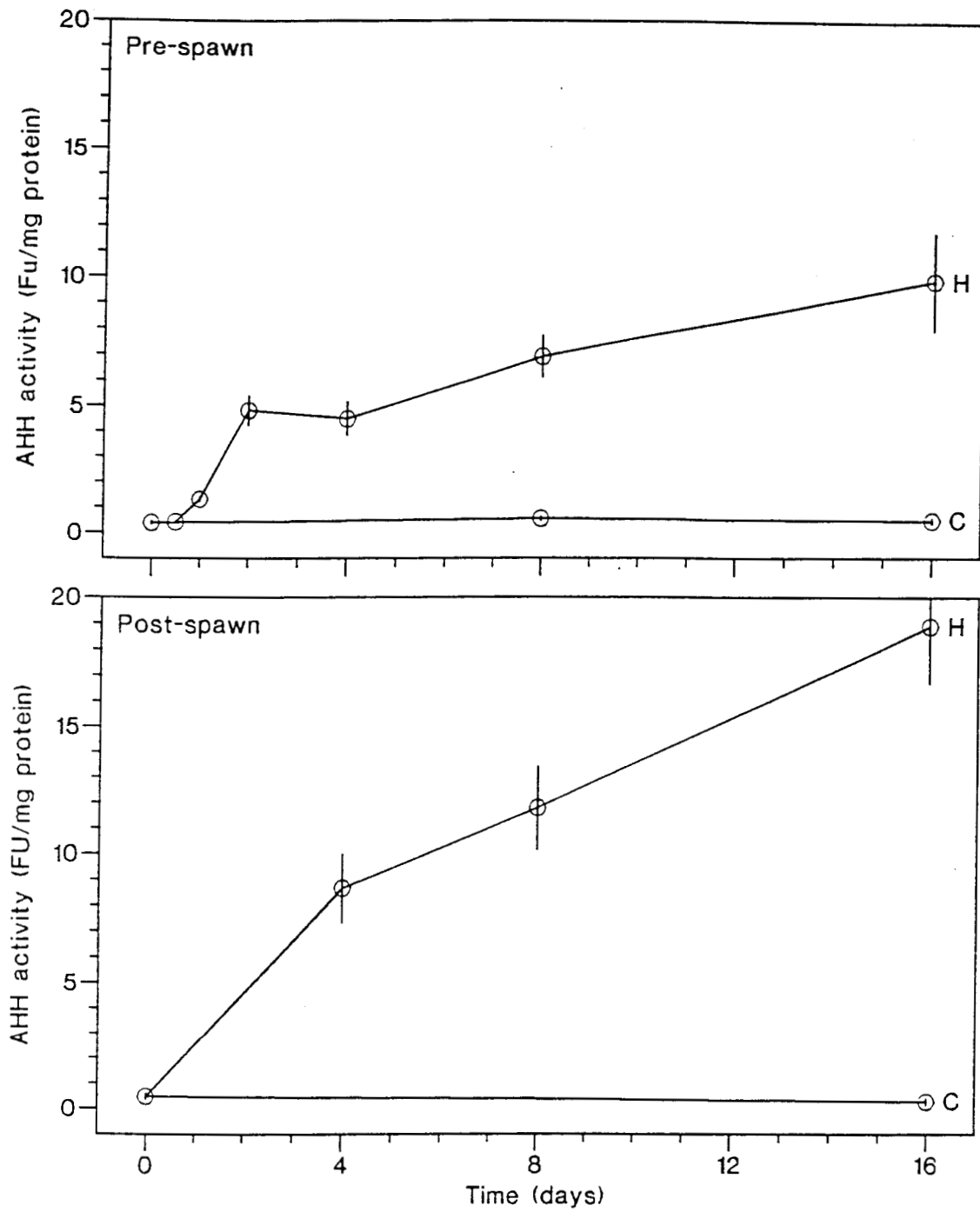
Figure 3.2.--AHH activity in livers of pre- and post-spawn herring as a function of time. Treatments were control (C) and high (H) oil. Data plotted are means ± standard error.
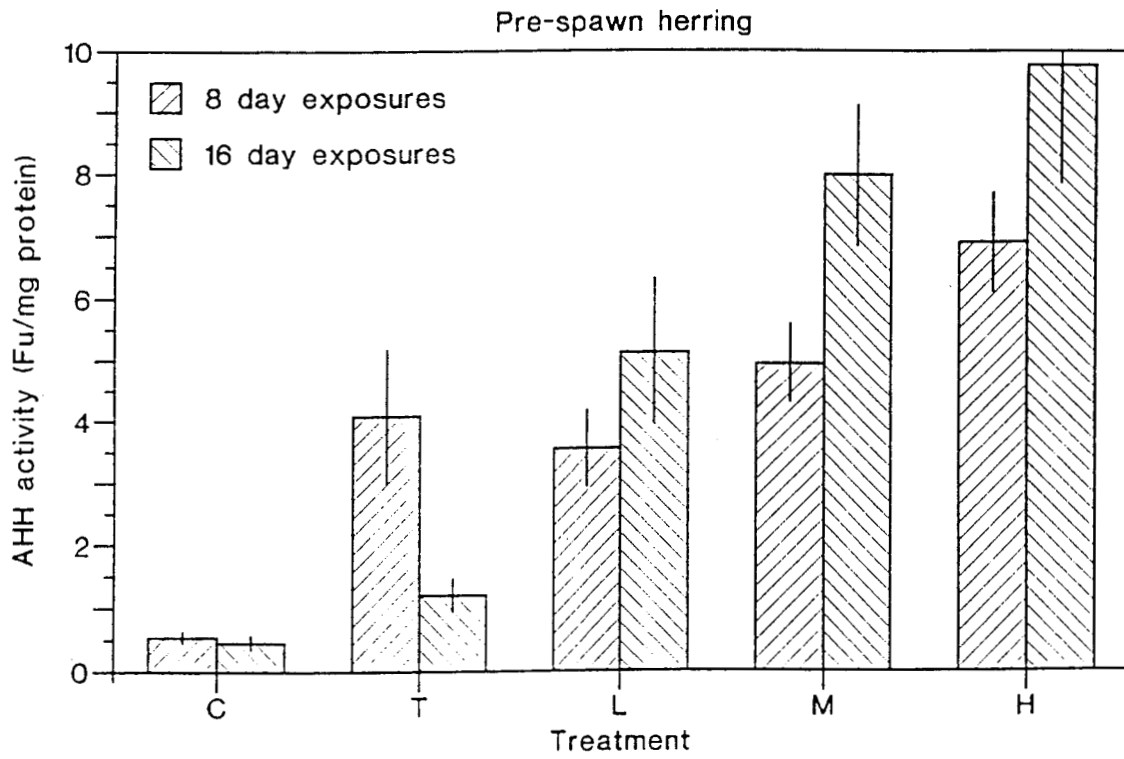
Figure 3.3.--AHH activity in livers of pre-spawn herring as a function of oil treatment. Fish were exposed to oil for 8 or 16 d. Data plotted are means ± standard error.
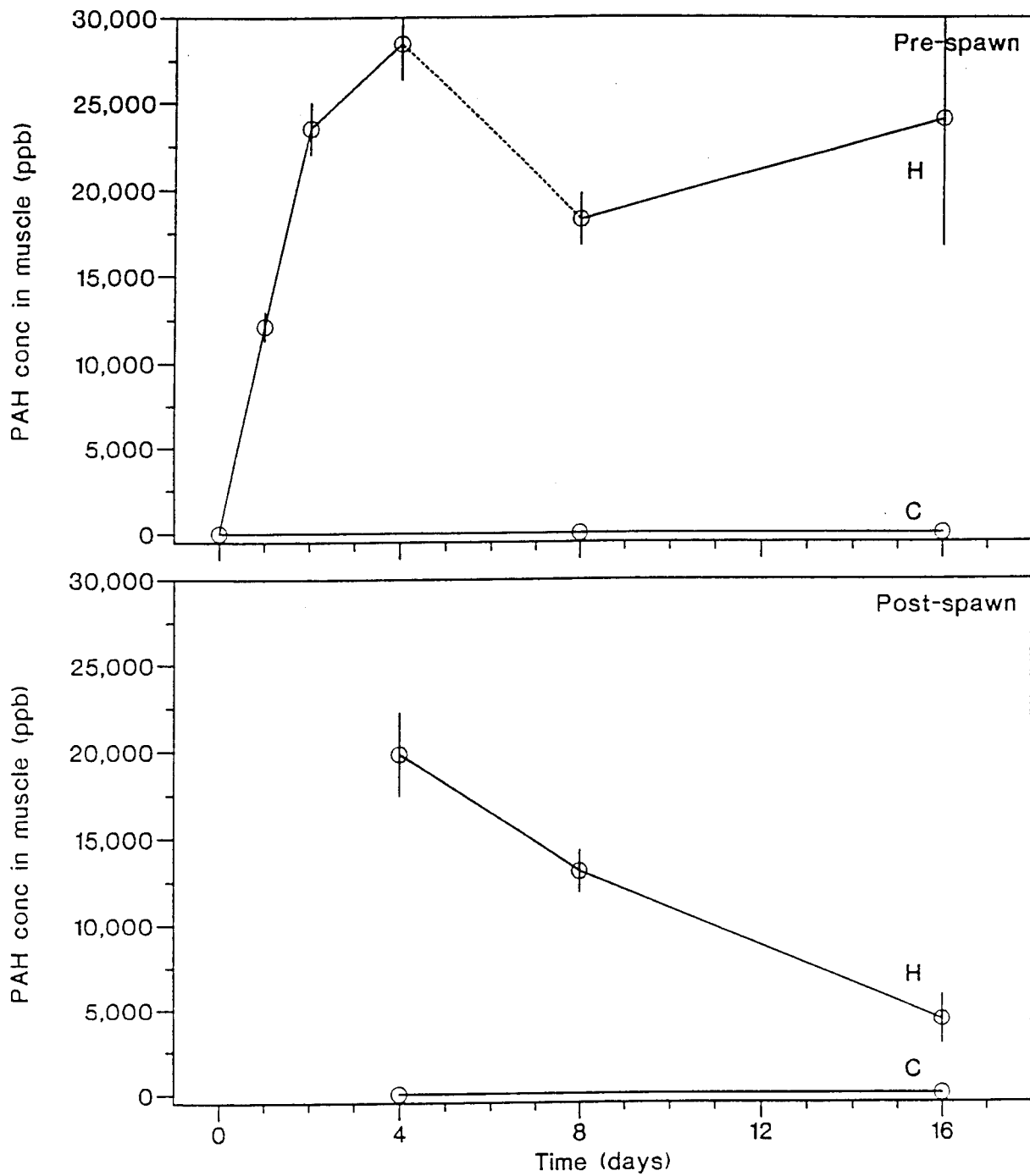
Figure 3.4.--PAH concentrations in muscle of pre- and post-spawn herring exposed to oil in
water. Treatments were control (C) and high (H) oil. Data for pre-spawn fish were collected in
two separate exposure series; the dashed line indicates where the series were joined. Data plotted
are means ± standard error.

Figure 3.5.--EROD activity and cytochrome P-450 concentration in pre- and post-spawn herring as a function of time. Treatments were control (C) and high (H) oil. Data plotted are means ± standard error.
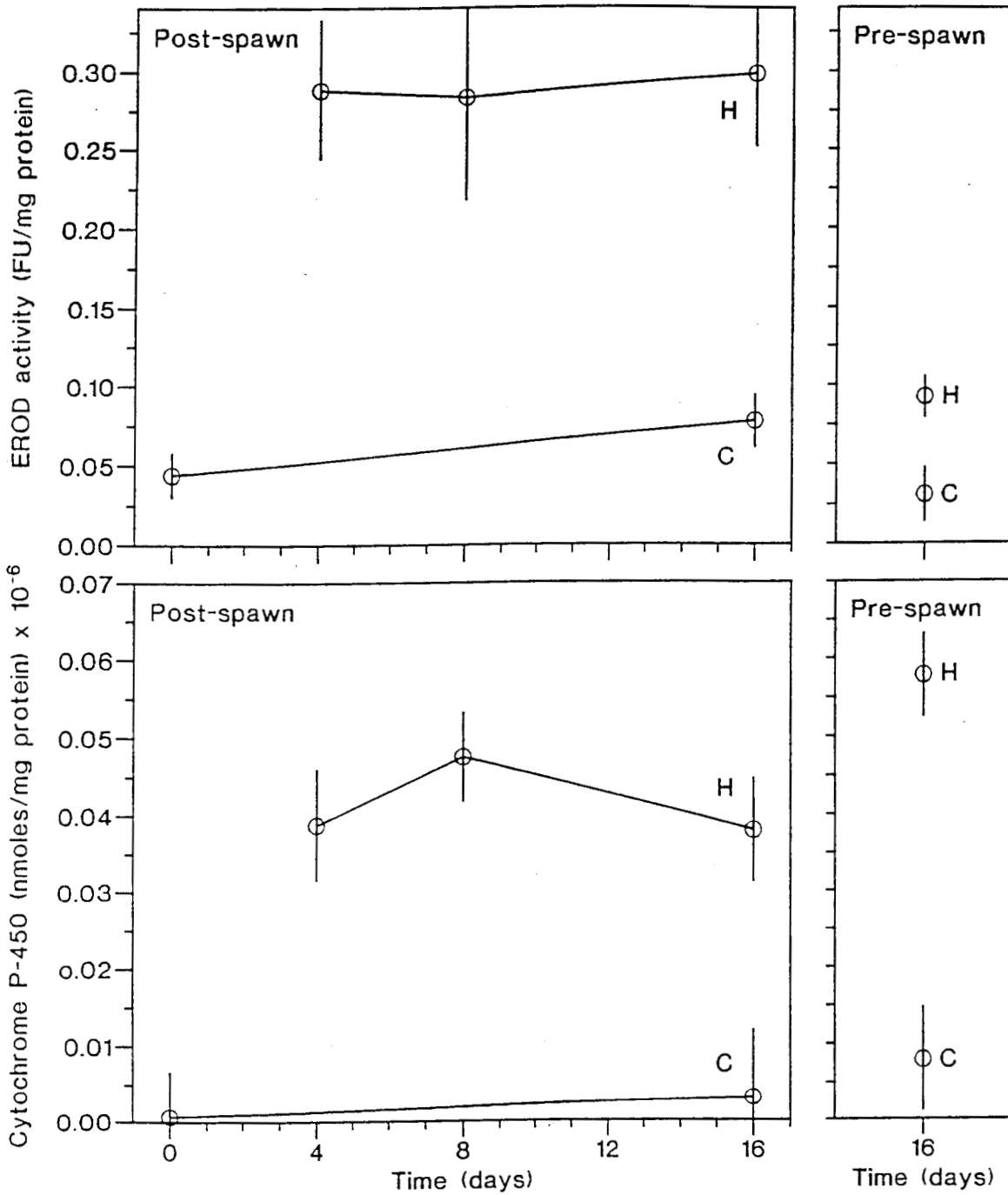
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Exposure of the post-spawn herring to the high concentration resulted in a significant increase in EROD activity after only 4 d and persisted at about the same level following 8 and 16 d exposures. Induction of EROD activity in post-spawn herring was approximately 3 times greater than in the pre-spawn animals (Fig. 3.5). Cytochrome P-450 concentration increased to about the same level in the exposed pre- and post-spawn herring. P-450 was significantly elevated after only 4 d of exposure in the post-spawn group and remained elevated following the 8 and 16 d exposures (Fig. 3.5). EROD and P-450 assays were not determined for 4 and 8 d exposures of the pre-spawn herring.

DISCUSSION

Bioaccumulation of PAH in muscle tissue of exposed pre- and post-spawn herring was significant. All exposure concentrations were sufficient to induce the MFO system in both groups of fish after at least 8 d of exposure. Induction of this enzyme system could have both beneficial and harmful effects. While cytochrome P-450 dependent MFOs can result in increased toxicity of PAHs (Nebert and Gelboin 1968; Wood et al. 1976) and activation of carcinogenic aromatic hydrocarbons (Glover and Sims 1968; Gelboin 1972; Sims et al. 1974, Ahokas 1979), they also make hydrocarbons more polar and thus more excretable (Jimenez and Stegemen 1990).

Activation of carcinogenic PAH compounds could well be cause of the negative correlation between reproductive success and AHH activity noted in flatfish (Spies et al. 1984; Johnson et al. 1988). On the other hand failure to induce MFOs, or induction at lower levels in reproductively ripe animals, could explain the high concentrations of hydrocarbons noted by Rice et al. (1987) in pre-spawn herring eggs.

Exposure of pre-spawn herring to petroleum hydrocarbons resulted in induction of MFOs but at a lower level than in post-spawn herring. Reduced levels of induction are noted although pre-spawn herring had greater PAH levels in muscle tissue and were exposed to higher concentrations. Failure of fish in the reproductive condition to express AHH induction at levels comparable to non-reproductive fish has been noted in other species as well (Forlin et al. 1984; Walton et al. 1983; Collier et al. 1986). Although lower levels of induction in reproductively ripe fish has not been explained, depression of induction levels probably have something to do with the energy cost of reproduction and the hormonal constituency of the reproductively ripe animals. Treatment of trout with estradiol reduces cytochrome P450 content and some MFO activities, and content and activity of P4501A is positively correlated with estradiol in winter flounder (Hansson and Gustaffson 1981; Forlin and Hansson 1982; Vodicnik and Lech 1983; Elskus 1992).

The post-spawn herring were exposed to much lower PAH concentrations than were the pre-spawn herring. Post-spawn herring also had significantly lower muscle PAH concentrations and these concentrations decreased with time. The importance of metabolism via the MFO system and subsequent clearance of the metabolites from the animal cannot be assessed in this study due to the differing exposure concentrations. It is very possible that the significantly higher

level of MFO induction in the post-spawn herring contributed to a lowering of tissue PAH levels.

The determination of induction of AHH activity in pre-spawn herring is important. Although induction was less than in post-spawn animals, induction of AHH would permit the conversion of PAHs to reactive compounds that could interact with gonadal macromolecules. Interaction of activated compounds has been shown in spawning English sole (Varanasi et al. 1982) and is the method by which PAHs are thought to destroy oocytes and cause ovarian toxicity in mammals (Mattison and Nightingale 1980). Egg hatch and short-term larval survival were not influenced after 8 and 16 d of exposure in this study, but interaction of activated compounds at the macromolecular level and subsequent effects cannot be ruled out.

CONCLUSIONS

A. PAH accumulated in muscle tissue of pre- and post-spawn herring.

B. MFO enzymes were induced by exposure to hydrocarbons; induction was less in pre- than in post-spawn fish.

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