Exxon Valdez Oil Spill
Restoration Project Final Report

Health and Reproductive Implications of Exposure of Pacific Herring (*Clupea pallasi*) Adults and Eggs to Weathered Crude Oil, and Reproductive Condition of Herring Stock in Prince William Sound Six Years After the *Exxon Valdez* Oil Spill

Restoration Project 95074
Final Report

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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. It was continued in 1995 as Restoration Project 95074 with NOAA as the sole agency. This report represents only the NOAA portion of the project.

Abstract: Herring spawned in Prince William Sound a few weeks after the *Exxon Valdez* oil spill; all life stages were potentially exposed. In 1993, the herring population in Prince William Sound collapsed, suggesting possible reproductive impairment. Reproductive condition of herring in Prince William Sound was assessed in 1995; adult herring and eggs were also experimentally exposed to oil. In laboratory tests, pre-spawn herring were negatively impacted by exposure to oil, principally by suppression of the immune system and increased expression of disease. Induction of aryl hydrocarbon hydroxylase, suppression of leukocytes, increased prevalence of viral hemorrhagic septicemia virus, and mortality were correlated with polynuclear aromatic hydrocarbon concentration. However, exposure of adult herring caused negligible damage in progeny at high concentrations (58 ppb aqueous polynuclear aromatic hydrocarbon). In contrast, exposure of incubating eggs to comparably weathered oil caused significant morphological defects at 9 ppb and effects of more weathered oil were significant at concentrations as low as 0.2 ppb polynuclear aromatic hydrocarbons; chromosomal aberrations were observed at 0.7 ppb. Most larvae with genetic defects would likely die due to concomitant morphological abnormalities. There was no evidence of oil-related reproductive impairment in Prince William Sound herring six years after the spill.

Key words: aryl hydrocarbon hydroxylase, *Exxon Valdez*, genetic, herring, immunosuppression, morphological abnormality, petroleum hydrocarbon, reproductive impairment, viral hemorrhagic septicemia, weathering

Project data: *Description of data* - Data sets were developed for hydrocarbon concentrations, adult herring, progeny of adult herring, herring eggs and larvae, and a field survey of reproductive success. There is a wide range of data, as described in this report and appendices. Data are archived primarily as ASCII files, LOTUS spreadsheets, and RBASE databases. LOTUS spreadsheets contain detailed data descriptions, and include descriptions of data extracted from databases. Graphics files are in AUTOCAD, PHOTOSHOP, POWERPOINT, and TIFF formats. Text files are in WordPerfect 6.1 format. *Custodian* - Contact Mark G. Carls, NOAA/NMFS, Auke Bay Laboratory, 11305 Glacier Highway, Juneau, AK 99801 (work phone:

(907) 789-6019; fax: (907) 789-6094, or E-mail mark.carls@noaa.gov. *Availability* - Copies of all data and related text files are available on CDROM for the cost of duplication.

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EXECUTIVE SUMMARY

A significant fraction of the Pacific herring population in Prince William Sound was likely exposed to oil spilled by the *Exxon Valdez*. The spill occurred just prior to the spring migration of herring from offshore feeding grounds to nearshore spawning areas in Prince William Sound. Most or all life stages may have been exposed to oil; studies in 1989 found evidence of adult, egg, and larval exposure. Post-spill studies did not detect large scale population changes, but isolated population collapses of herring and pink salmon in Prince William Sound in 1993 stimulated laboratory and field studies by several government agencies to determine causes of collapse, status of current stocks, and if the oil spill was directly or indirectly responsible. Poor recruitment of juvenile fish to the spawning population, and a high incidence of disease raised concern that the oil spill directly or indirectly caused these delayed problems. Proposed mechanisms included reduced reproductive capacity and resistance to disease. This study reports laboratory and field measurements to determine if exposure of herring to oil could cause reproductive impairment, including genetic damage, and suppression of immune function or other disease problems. Two years of controlled laboratory studies involving experimental exposure to oil were completed, plus a survey of Prince William Sound herring stock to assess reproductive condition six years post-spill. In the laboratory tests, the life stage exposed to oil was varied (adult and egg), but endpoint measurements (including analysis of chromosomal condition in actively dividing cells of larvae) remained the same. In the field survey, the current reproductive quality of females from four sites in Prince William Sound and three control sites in southeast Alaska were assessed by rearing their spawn and determining larvae viability.

Our intent in the experimental portions of this study, was to expose adult herring and eggs to oil concentrations in the range encountered in Prince William Sound following the *Exxon Valdez* spill, and to ensure that the composition of this oil was similar to that of the spill. Thus the oil chosen for study (Alaska North Slope crude) and the toxicant delivery method were designed to mimic conditions observed in Prince William Sound. Seawater was contaminated as it passed through columns of oil-coated rock. Test concentrations were chosen to bracket concentrations observed in open water in Prince William Sound after the *Exxon Valdez* oil spill.

Hydrocarbon concentrations declined during test exposures, and composition changed. As the oil weathered, composition typically shifted from naphthalene dominated to phenanthrene dominated, and alkyl-substituted homologs were retained longer than their unsubstituted counterparts. Because initial polynuclear aromatic hydrocarbon concentration in water was used as the measure of toxicant exposure, we may have underestimated toxicity. Nonetheless, this is the first report of biologically significant effects of polynuclear aromatic hydrocarbons consistently occurring in the low parts per billion range.

Experimental exposure of adult Pacific herring to oil (1994)

Pre-spawn adult herring were exposed to oil in water for a period of 8 or 16 d to determine toxic effects, resistance to disease, damage to gametes, and genetic damage in

progeny. Although adults were exposed, major emphasis was placed on the survival and viability of resultant larvae. Actively dividing somatic cells in the pectoral fins of larvae were examined for chromosomal aberrations. In a follow-up experiment, post-spawn herring were similarly exposed to oil to determine if differences in physiological and reproductive condition would affect hydrocarbon accumulation and biological response.

Adult herring were negatively impacted by exposure to oil. Hydrocarbons accumulated in tissues of exposed fish, including muscle and ovary. Aryl hydrocarbon hydroxylase activity was induced in liver tissue. The immune systems of adult fish were suppressed. Evidence for immunosuppression in the liver included significant declines in eosinophilic granular leukocytes and focal or multifocal parenchymal leukocytes in liver. Prevalence of viral hemorrhagic septicemia virus, subclinical or latent in our wild herring stock, was positively correlated with oil exposure, and negatively correlated with leukocyte abundance, suggesting a causal link between immunosuppression and disease. Death and disease were positively correlated, and 97% of the fish that died had lesions characteristic of viral hemorrhagic septicemia virus, suggesting a causal link between disease and mortality. Although expression of latent virus in response to toxicant exposure has not previously been reported, evidence strongly indicates that activation of viral hemorrhagic septicemia virus in Pacific herring might be encountered after an oil spill.

Oil impacts in post-spawn herring were smaller than in pre-spawn herring, but induction of mixed function oxidases was two to three times that in pre-spawn fish. In contrast to pre-spawn fish, there was no external evidence of disease in post-spawn fish, and there was no evidence of dose-dependent mortality. Depression of induction levels in pre-spawn fish was probably related to the energetic cost of reproduction and the hormonal constituency of the reproductively ripe animals.

Exposure of pre-spawn adult herring to oil caused no damage to gametes and little or none in progeny, even though initial polynuclear aromatic hydrocarbon exposure concentrations extended up to 58 ppb. There was no evidence that gamete viability was affected; mean fertility ranged from 93 to 95%. Fertile eggs produced normal larvae; there were no differences in larval survival (within 1 d of hatch), spinal defects, or swimming behavior among treatments. There was an apparent increase in yolk volume in larvae from adults exposed to the high-oil treatment, but this change was not reflected in other parameters that should have also responded if it were a true treatment effect. The change in yolk volume was about one-third that of an increase known to be related to oil exposure in 1995 tests. Larval size and maturity at hatch were not affected by exposure of adults, and there was no indication of an elevation of somatic chromosomal aberrations during mitosis. The rate of cell division was also unaffected.

Experimental exposure of Pacific herring eggs to oil during incubation (1995)

In 1995, herring eggs were directly exposed to oil-contaminated water for 16 d during incubation to determine morphological and genetic damage, and to relate this damage to survival potential. As in the 1994 adult exposures, the emphasis was on the survival and viability of the resulting larvae. We also observed mortality, behavior, and hatch timing. The test was repeated

twice to allow comparison of the biological impacts of weathered oil and more highly weathered oil. Additional tests were conducted to determine effective exposure duration.

Exposure of eggs to oil during incubation induced early hatch, reduced hatching success, larval swimming, survival, and size, and caused edema, skeletal, craniofacial, and finfold abnormalities. The frequency of chromosomal aberrations was significantly elevated in the pectoral fins of exposed larvae. Sensitivity of a wide variety of larval herring responses to oil were similar, and indicated significance at 0.7 ppb aqueous polynuclear aromatic hydrocarbons of the more highly weathered oil. Yolk-sac edema and fin ray formation were the most sensitive measures of response, and indicated significance at 0.2 ppb of the more highly weathered oil.

Larval survival potential was reduced by sublethal effects. For example, larval swimming, necessary for feeding and predator avoidance, was adversely affected by exposure of eggs to oil. Although spinal deformation appeared to be the dominant factor, edema, reduced finfold surface area, and retarded pectoral fin development undoubtedly contributed to decreased larval swimming ability. Premature hatch may also have contributed to reduced survival potential because swimming ability improves with maturity.

Larvae that hatched from oil-exposed eggs were genetically damaged. The genotoxicity endpoint used here, anaphase-telophase aberration rate, measured microscopically visible chromosome or chromatid breaks and bridges during the later stages of mitosis. Consequences of the genetic damage observed in this study cannot be predicted with certainty, but might include reductions in successful cell division and growth. However, it is likely that most affected individuals died due to concomitant morphological abnormalities. The question of whether observed chromosomal damage could be heritable has not been definitively answered.

Toxicity of the more highly weathered oil was greater than could be predicted by total polynuclear aromatic hydrocarbon concentration in the less weathered oil, suggesting that toxicity of the more persistent, heavier aromatic hydrocarbons and more substituted homologs was greater than that of lighter, less substituted aromatic hydrocarbons. Minimum effective concentrations were about 9 ppb polynuclear aromatic hydrocarbons for weathered oil, but fell to 0.2 ppb for more highly weathered oil.

Exposures as short as 2 d caused significant biological response and most responses were significant within 8 d exposure. These results imply that relatively brief exposure to low concentrations of oil in Prince William Sound could have adversely affected herring eggs spawned in oiled areas after the spill and may explain morphological and genetic abnormalities observed in larvae in 1989.

Comparison of pre-spawn adult exposures and direct egg exposures.

Differences in toxicant composition and concentration within herring eggs probably explains why viability of larvae was affected when eggs were exposed directly to oil, but not

when adults were exposed just prior to spawning. Composition of hydrocarbons in eggs from adult exposures was weighted toward lighter polynuclear aromatics (naphthalenes accounted for more than 90% of the aromatics on day 16), and, of the remaining heavier aromatic hydrocarbons, unsubstituted homologs dominated. In contrast, eggs exposed directly to hydrocarbons during incubation contained greater percentages of large ring compounds. Furthermore, due to oil weathering, percentages of large ring compounds in directly exposed eggs increased during incubation, as did percentages of alkylated compounds. Other studies have shown that toxicity increases both with ring size and alkylation, including our direct egg exposures. Although direct exposure of adult herring to oil can increase their vulnerability to disease, adults have significant tissue mass and some hydrocarbon metabolizing capabilities, which result in apparent protection of gametes from toxicants until they are spawned into the environment. Abnormal larvae in Prince William Sound in 1989 were not caused by exposure of adult herring to oil.

Survey of reproductive condition of Prince William Sound herring stock (1995)

Pacific herring stock in Prince William Sound were surveyed six years after the *Exxon Valdez* oil spill to determine reproductive condition of the population. There were two major foci: 1) a comparison of reproductive success between regions (Prince William Sound and southeast Alaska), and 2) a comparison of reproductive success between year classes within sites, particularly the 1989 year class (most likely impacted by the oil spill in Prince William Sound). Mature herring from four sites in Prince William Sound and three sites in southeast Alaska were collected in 1995 and artificially spawned. Reproductive success of female herring was defined as the production of morphologically and functionally normal larvae. Responses known to be sensitive to oil exposure were analyzed. Larvae were not inspected for genetic damage because morphological responses in egg exposure studies met or exceeded genetic sensitivity.

Six years after the *Exxon Valdez* oil spill, reproductive impairment was not detected in herring in Prince William Sound. Egg fertility, hatching success, larval viability and swimming, spinal abnormalities, and yolk-sac edema did not differ significantly between regions, including response of the 1989 year-class. Discrimination of responses between regions was not possible because the best and worst responses were usually found in southeast Alaska. Similarly, there were no consistent trends indicating reproductive impairment of the 1989 year class at specific sites. Further, adult fish from all sites appeared to be healthy; weight, length, and condition factor did not vary significantly among regions. Whether or not herring in Prince William Sound were ever reproductively impaired by the *Exxon Valdez* oil spill is unknown, but the time lapse between the spill and our study probably precluded any detection of reproductive impairment. Measurable effects likely declined, probably most rapidly during the first year as the most adversely affected individuals died. Regardless of the life stage and likelihood of possible oil exposure, herring we sampled in Prince William Sound in 1995 appeared to be reproductively fit and similar to herring in southeast Alaska. Although herring stocks are still depressed in Prince William Sound, factors other than reproductive impairment are probably limiting recovery.

INTRODUCTION

In Chapter 1, the linkage between exposure of adult herring to oil, accumulation of hydrocarbons in tissue, induction of mixed function oxidase enzymes, immune response, opportunistic disease, and mortality is explored. Pre-spawn herring were negatively impacted by exposure to oil. Induction of aryl hydrocarbon hydroxylase, suppression of leukocytes, increase in viral hemorrhagic septicemia virus, and cumulative mortality all correlated with polynuclear aromatic hydrocarbon concentration. In contrast to pre-spawn fish, there was no external evidence of disease in post-spawn fish, and there was no evidence of dose-dependent mortality.

In Chapter 2, induction of cytochrome P-450 dependent mixed function oxidase enzymes by exposure of pre- and post-spawn herring is explored. Activity of aryl hydrocarbon hydroxylase, a mixed-function oxidase enzyme, was induced in liver tissue; induction in post-spawn fish was two to three times that in pre-spawn fish. Depression of induction levels in pre-spawn fish was probably related to the energetic cost of reproduction and the hormonal constituency of the reproductively ripe animals.

In Chapter 3, the effects of pre-spawn adult exposure in gametes, eggs, and larvae are compared to those where herring eggs were exposed directly to oil during incubation. Exposure of pre-spawn adult herring to oil caused no damage to gametes and little or none in progeny even though initial aqueous polynuclear aromatic hydrocarbon exposure concentrations extended up to 58 ppb. In sharp contrast, eggs exposed to comparably weathered oil during incubation were significantly and adversely affected by exposure as brief as 2 d, and at polynuclear aromatic hydrocarbon concentrations as low as 9 ppb. Differences in toxicant composition in egg tissue may, in part, explain the radically different responses between types of exposure.

In Chapter 4, the effects of incubating herring eggs in oil-contaminated water are detailed, including comparison to contamination with a more highly weathered oil. Additional tests were conducted to determine effective exposure duration. Exposure adversely affected eggs and larvae. For example, survival and size declined, and morphological and genetic defects increased. Toxicity of the more highly weathered oil was greater than could be predicted by total polynuclear aromatic hydrocarbon concentration in the less weathered oil, suggesting that toxicity of the more persistent, heavier aromatic hydrocarbons and more substituted homologs was greater than that of lighter, less substituted aromatic hydrocarbons. Significant sublethal response was observed at concentrations of 0.2 ppb aqueous polynuclear aromatic hydrocarbons. Exposures as short as 2 d caused significant biological response. Although the consequences of the genetic damage observed in this study cannot be predicted with certainty, it is likely that most affected individuals died due to concomitant morphological abnormalities.

In Chapter 5, results of field surveys designed to compare the reproductive condition of Prince William Sound herring stock in 1995 with that in southeast Alaska herring are presented. Reproductive success between year classes within sites was also compared, particularly the 1989 year class (most likely impacted by the oil spill in Prince William Sound). Reproductive success

of female herring was defined as the production of morphologically and functionally normal larvae. Regardless of the life stage and likelihood of possible oil exposure in 1989 and 1990, herring we sampled in Prince William Sound in 1995 appeared to be reproductively fit and similar to herring in southeast Alaska. Response of the 1989 year-class generally did not differ significantly from any other year class within each site.

OBJECTIVES

Objectives as proposed in the 1995 detailed study plan

1. The objective of the laboratory portion of the study is to determine if genetic damage to early life stages of herring can be caused by exposure of pre-spawning adult, egg, and larval stages to oil and relate this damage to larval survival potential. Impacts will primarily be measured in larvae. Both short term and long term impacts may be caused by exposure. Short term impacts include effects on hatching success, morphological abnormalities, and larval size. Long term effects may include disruption of cell division, determined by observation of anaphase-telophase aberration rates of somatic cells. From estimates of anaphase-telophase aberration, we will infer the possibility that exposure of herring to oil can cause genetic damage that is transmissible to subsequent generations. It is not practical to measure germ line damage directly in the laboratory because it is not practical to rear herring from eggs to maturity. In 1994, pre-spawning adults were exposed and artificially spawned; the pectoral fins of newly hatched larvae were inspected for genetic aberrations. In 1995, herring eggs will be exposed to oil during incubation, and genetic aberrations will be measured. Aberration rates will be compared across exposure doses and life stage exposed.

> Primary test hypothesis: Anaphase-telophase aberration in mitotic cells of the pectoral fins of herring larvae are caused by exposure of herring adults (1994) or eggs (1995) to oil in water. (Proposed for 1996 is direct exposure of herring larvae to oil.) Other measurements include egg fertility, percent hatch, larval viability, morphological abnormality, and amount of exposure time required to cause damage.
>
> Assumption: Disruption of normal somatic mitotic processes may signal future meiotic disruption, or reduce the potential of larvae to survive to reproductive age.[1]

2. Survey herring in Prince William Sound for reproductive impairment by measuring larval viability by location and age class. Herring reproduction may have been impaired as a result of past oil exposures at one or more life stages. In 1995, we will measure herring reproduction success from several age classes collected from several sites in Prince William Sound. Some of

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[1]The assumption of linkage between somatic and germline genetic response was abandoned as unsupportable. In higher vertebrates, available data suggests that the susceptibilities of these two tissue types is not equivalent: there are no data for fish.

the age classes were exposed to oil, but post-1990 year classes were not. Spawn will be returned to the lab and reared until hatch to determine larval viability and abnormality rates.

Primary test hypothesis: Fertility, percent hatch, larval viability and morphological abnormalities were caused by the *Exxon Valdez* oil spill.

Assumptions: 1) The year classes prior to 1989 were potentially exposed to oil in 1989, 2) the 1989 year class was potentially exposed to oil in pre-spawning adults, eggs, and larvae, and 3) year classes after 1989 were not exposed to oil, except that the 1990 year class may have been exposed to residual oil in intertidal areas.

Controls will be post-spill year classes and two or three sites in southeastern Alaska, including Sitka.

Chapter 1: Immunosuppression, expression of viral hemorrhagic septicemia virus, and mortality in pre-spawn Pacific herring (*Clupea pallasi*) exposed to weathered crude oil in the laboratory.

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

ABSTRACT

Expression of subclinical viral hemorrhagic septicemia virus (VHSV) in response to toxicant exposure has not previously been reported, but evidence presented herein strongly indicates that activation of VHSV is very likely in Pacific herring exposed to crude oil encountered after an oil spill. Decreased inflammation as a function of polynuclear aromatic hydrocarbon (PAH) concentration suggested generalized immunosuppression in exposed fish, and decreased immune surveillance as a possible mechanism by which subclinical VHSV could be reactivated. Correlations suggest possible causal links between oil exposure and immunosuppression, immunosuppression and expression of VHSV, and between VHSV and mortality. Adult herring of unknown status regarding infection by VHSV were captured from the wild and exposed to weathered crude oil for 16 to 18 d. Concentration of PAH in tissue, VHSV prevalence, and mortality were correlated with dose; 97% of the fish that died had lesions characteristic of VHSV. Histopathologic lesions were significantly correlated with PAH concentration and prevalence of VHSV, but not gender or length. Significant lesions included increased hepatocellular necrosis, splenic thrombosis, and decreased inflammation in the liver.

INTRODUCTION

On March 24, 1989, the *Exxon Valdez* oil tanker grounded in Prince William Sound (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 adults of several species of fish, including herring, rockfish, and salmonids (Moles et al. 1993; Meacham and Sullivan 1993; Hepler et al. 1996). Pacific herring (*Clupea pallasi*) 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 (Brown et al. 1996). Although petroleum hydrocarbons were not detected in adult herring tissue in 1989, bile was contaminated (Haynes et al. 1995), and significant tissue alterations were observed in adults collected in two oiled areas of PWS as compared to three unoiled sites (Moles et al. 1993).

Many fish are asymptomatic carriers of pathogens that under normal conditions are held in check by the immune system. When that system is impaired by stress, the disease-causing agent may multiply and injure or kill its host (Anderson 1990).

More problematic, however, is establishing causative links in wild fish among infectious disease, immune suppression, and environmental stress (Sindermann 1993). Oil spills occur

periodically in marine systems throughout the world, and histopathology of the effects of hydrocarbon exposure on fish has been documented in laboratory and field studies (Haensly 1982; Malins 1982; Solangi and Overstreet 1982). For example, prevalence and intensity of infections with gastrointestinal parasites were lower in fish treated with crude oil in the laboratory (Khan and Kiceniuk 1983). By comparison, other fish had increased numbers of branchial *Trichodina* after chronic exposure to oiled sediments in the laboratory. In Pacific herring (*Clupea pallasi*) numbers of larval *Anisakis simplex* in the peritoneal cavity were lower in fish collected 14 d after the *Exxon Valdez* oil spill, and these results were confirmed in Pacific herring exposed to crude oil in the laboratory (Moles et al. 1993). Changes in expression of bacterial and viral pathogens after oil exposure have not been documented.

Pacific herring sampled from oiled sites immediately after the spill had hepatic necrosis that was attributed to oil exposure (Moles et al. 1993). However, Pacific herring sampled annually from the same sites from 1990 through 1992 did not have hepatic necrosis and there was no longer any evidence of hydrocarbon exposure (G. Marty, unpublished data; Kocan 1996). In PWS, in 1993, an abundant 1988 year-class was fully recruiting into the spawning population of Pacific herring, but the population collapsed. Morbidity of herring was primarily caused by *Ichthyophonus*, but VHSV was considered a significant secondary cause (Marty et al. 1995). Of the remaining herring, 15 to 43% exhibited external ulceration or hemorrhages associated with VHSV (Meyers et al. 1994), and fish again had hepatic necrosis. In 1994, VHSV isolated from individual fish was statistically associated with hepatic necrosis (Marty et al. 1995). Meyers et al. (1994) and Marty et al. (1995) hypothesized that a combination of environmental stressors (e.g., spawning, inadequate food supply, or increased predation) resulted in activation of subclinical VHSV which resulted in several lesions, including hepatic necrosis.

Our objective was to search for immunosuppressive effects of crude oil in Pacific herring as related to exposure, opportunistic disease infection, and mortality. Wild-caught Pacific herring in spawning condition were randomly separated into groups and exposed to petroleum hydrocarbons for 16 to 18 d in the laboratory. Hydrocarbon concentrations were measured in treatment water, muscle, and ovaries. To document biochemical response, induction of mixed function oxidase (MFO) enzymes was observed in livers (Thomas et al. 1997). Initial PAH concentrations in water (0.03 (controls) to 58 ppb) were chosen to include maximum mean PAH concentrations observed in open water in PWS following the spill (6.24 ppb; Short and Harris 1996). Fish were not exposed to exogenous VHSV during the experiment.

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-L holding tank; 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 matured sexually during holding, and were reproductively mature during experimental treatment.

Some herring developed signs of disease by April 1, 1994. Fish mortality was associated with scale loss and raised white cutaneous foci near the dorsal fin. A gram-negative filamentous rod resembling *Flexibacter* sp. was present, but no viral infections were detected in pooled liver and kidney tissues cultured from five fish. Fish were treated for bacterial infection beginning April 5; all fish received three successive 1-h static formalin baths (150 mg/L) at 2 d intervals. Mean holding mortality after treatment was 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 polyvinyl chloride plastic cylinders and upwelled through the rock (Appendix 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 loaded with clean rock. Before the gravel was oiled, Alaska North Slope Crude oil was artificially weathered by heating to 70°C overnight (circa 12 h) in a beaker with continuous stirring. Gravel (2 - 33 mm diameter (Mdϕ = 6.8 mm, Q_1-Q_3 = 4.7 to 9.8 mm)) 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 with a paint sprayer to 45 kg batches of gravel tumbling in the cement mixer (trace-, low-, and mid-oil treatments) or with a Teflon squirt bottle (high-oil treatment). Batches of gravel were mixed by treatment; each generator was loaded with 45 kg of freshly oiled gravel (or non-oiled control rock), except high treatment generators were loaded with 90 kg gravel.

There were five treatments, including control, with three replicates per treatment. Apparently healthy Pacific herring were randomly distributed among 15 700-L treatment tanks (50 fish per tank). Exposures lasted 16 to 18 d and began over a 3-d period for logistic reasons (April 24 - 26, 1994); fish were also sampled on day 8 for hydrocarbon measurement, and a reproductive study (Carls et al. Chapter 3.). Water for each replicate tank flowed through an independent generator at 6.9 L/min and 4.2°C. To characterize the oil and quantify treatment concentrations, composite samples (1.27 L from each of 3 replicate tanks) were collected and extracted on days 0, 8, and 16 for analysis by gas chromatography using methods of Short et al. (1996). Fish 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 or by anesthetization (tricaine methane sulfonate), measured (fork length to the nearest millimeter), weighed (wet weight to the nearest 0.01 g), and bled by clipping a gill arch. Livers were removed, frozen at -80°C, and later processed for MFO induction by Thomas et al. (1997); aryl hydrocarbon hydroxylase (AHH) activity was assayed according to the method of Nebert and Gelboin (1968). Gonad and muscle samples (10 g minimum) were collected with hydrocarbon-free dissection tools and frozen in hydrocarbon-free vials for analysis. Hydrocarbon samples were analyzed according to the

methods of Short et al. (1996). Scales were collected near the posterior margin of the dorsal fin for age analysis. An additional 7 to 14 fish per replicate tank were sampled for viral erythrocytic necrosis (gill arches were clipped, and blood smears were prepared), isolation of VHSV (anterior kidney and half of the spleen were frozen) and histopathology (gill, liver, and the other half of the spleen were preserved in 10% neutral buffered formalin; Table 1.1). Virus isolation, in the EPC cell line, media formulation, and tissue preparation for cell line inoculation were performed as described by Meyers et al. (1994). VHSV was identified by DNA probe as described by Meyers et al. (1994). Additional necropsy data were collected from all fish, including those that died before the test ended. These data included condition of skin, fins, eyes, jaw, gill, kidney, ovaries, spleen, and peritoneum. Percentages of fish with abnormal liver colors were determined; normal liver color was red. External bacterial infections were assessed from skin scrapings.

Spleen, gill, and liver from 153 Pacific herring were assigned a code number known only to Alaska-based coauthors, and samples were shipped to the University of California Davis, for processing and analysis. Tissues from each fish were re-coded with a histopathology number, processed routinely into paraffin, sectioned at 5 µm, and stained with hematoxylin and eosin. Sections from each organ were read in ascending numerical order using the random histopathology number. Lesions were scored using a four-point scale as none (0), mild (1), moderate (2), or severe (3). For quality control, autolysis and artifact in each organ were scored on the same four-point scale. Ranking of lesions was often based on the number of inflammatory cells or organisms (e.g., resting spores of *Ichthyophonus hoferi*) per 100x or 400x field on a binocular light microscope. For example, hepatic perivascular and pericholangial eosinophilic granular leukocytes (EGL) were scored as follows:

score = 0: ≤2 EGL per perivascular or pericholangial 400x field;
score = 1: >2 but ≤25 EGL per perivascular or pericholangial 400x field;
score = 2: >25 EGL in at least 1 perivascular or pericholangial 400x field, and EGL extended to the margins of the surrounding parenchyma;
score = 3: >25 EGL in at least 1 perivascular or pericholangial 400x field, and EGL extended into the surrounding parenchyma

A sum-thrombosis score and a sum-*Ichthyophonus* score were calculated by summing the thrombosis and *Ichthyophonus* scores from each organ. After all organs were examined and lesions scored, data were rearranged by necropsy number, the codes were revealed, and results were subjected to statistical analysis.

Data processing and statistics

Hydrocarbon data were processed according to the methods of Short et al. (1996) before further analysis. Hydrocarbon concentrations were accepted only if corresponding recovery of deuterated internal standards ranged from 30 to 150%; records with unacceptable recoveries were deleted (3% of the data). In addition, concentrations below minimum detection limits were set to zero (Short et al. 1996).

Table 1.1. Length, weight, VHSV and Anisakidae prevalence, and mean lesion scores in spawning Pacific herring after exposure to various concentrations of crude oil for 16 to 18 d in the laboratory. Lesions were scored as none (0), mild (1), moderate (2), or severe (3); sum scores for all organs have a maximum score of 9. Males dominated in these samples because females were preferentially sampled for a reproductive study (Carls et al. Chapter 3).

	Treatment				
Organ/Variable	Control	Trace	Low	Mid	High
Gross necropsy and virology	31	30	32	30	30
Number of males	22	17	20	20	22
Mean fork length (mm)	228	232	229	227	232
± SE	±3.9	±3.3	±2.9	±3.3	±3.4
Mean bodyweight (g)	119.6	124.8	113.8	108.8	128.0
± SE	±0.20	±0.19	±0.16	±0.15	±0.14
VHSV prevalence (%)	0.0	6.7	14.6	15.6	33.3
Anisakidae prevalence (%)	13	20	25	6.9	13
All organs					
sum-Thrombosis mean scores	0.13	0.10	0.19	0.53	0.67
sum-Thrombosis prevalence (%)	13	10	13	33	37
sum-*Ichthyophonus* mean scores	0.23	0.73	0.25	0.47	0.17
sum-*Ichthyophonus* prevalence (%)	6.5	23	6.3	10	6.7
	Mean score				
Liver Lesions					
Artifact	1.39	1.43	1.31	1.30	1.57
Autolysis	0.42	0.27	0.59	0.57	0.47
Cholangitis or biliary hyperplasia	0.13	0.07	0.06	0.03	0.03
Eosinophilic granular leukocytes (perivascular or pericholangial)	0.71	0.77	0.50	0.37	0.17
Focal necrosis	0.03	0.00	0.06	0.07	0.10
Focal or multifocal parenchymal leukocytes	0.26	0.27	0.19	0.17	0.03
Glycogen depletion	3.00	3.00	3.00	2.97	3.00
Goussia clupearum	1.35	1.17	1.16	1.00	1.07
Granulomatous inflammation	0.26	0.53	0.25	0.17	0.23
Ichthyophonus hoferi	0.06	0.37	0.09	0.23	0.03
Lipidosis	0.13	0.10	0.16	0.13	0.23
Macrophage aggregates	1.19	1.13	1.03	1.13	1.03
Pericholangial leukocytes	0.13	0.30	0.13	0.17	0.10
Single cell necrosis	0.13	0.17	0.13	0.27	0.23
Thrombosis	0.00	0.03	0.09	0.17	0.03

Organ/Variable	Treatment				
	Control	Trace	Low	Mid	High
	Mean score				
Gill Lesions					
Artifact	1.65	1.50	1.50	1.60	1.60
Autolysis	0.13	0.40	0.16	0.20	0.30
Ciliated protozoa (e.g., *Trichodina*)	0.00	0.00	0.00	0.00	0.00
Epitheliocystis	0.10	0.03	0.09	0.07	0.17
Foreign body granuloma	0.06	0.13	0.09	0.07	0.00
Gill arch inflammation or hematopoiesis	1.10	1.00	1.03	1.07	0.93
Gill lamellar telangiectasis	0.16	0.67	0.44	0.67	0.60
Ichthyophonus hoferi	0.06	0.13	0.03	0.03	0.07
Lamellar epithelial lifting (artifact)	0.87	0.77	0.94	1.03	1.03
Lamellar hyperplasia	0.00	0.00	0.00	0.00	0.07
Monogenetic trematode	0.00	0.00	0.00	0.03	0.00
Thrombosis	0.03	0.00	0.03	0.20	0.13
Spleen Lesions	31	30	31[a]	27[a]	30
Artifact	0.94	0.87	1.03	1.00	0.93
Autolysis	0.03	0.07	0.03	0.04	0.03
Congestion	1.61	1.47	1.16	1.41	1.50
Ellipsoid hyalinization	1.16	1.23	1.16	1.30	1.27
Focal intimal hyperplasia in arterioles[a]	0.13	0.17	0.10	0.19	0.03
Granulomatous inflammation	0.06	0.17	0.10	0.07	0.00
Ichthyophonus hoferi	0.10	0.23	0.13	0.22	0.07
Macrophage aggregates	1.29	1.60	1.26	1.56	1.37
Thrombosis	0.10	0.07	0.06	0.19	0.50

[a]Principal components analysis eliminates all data from a fish if a single value is missing. Because spleens from four fish were not examined microscopically, those fish were not used in statistical analysis, but scores for liver and gill from these four fish are included above.

Histopathological data were analyzed in two ways. First, overall significance of histopathology scores was determined using a multivariate analysis of variance (MANOVA). Histopathology scores were summarized through use of a principal components analysis. For a given set of scores, the first eight principal components based on the covariance matrix were used as a summary for most of the variability in the raw data. The principal components were then tested for systematic effects of oil exposure, VHSV status, length (as an estimate of age), and sex using MANOVA. Second, differences in VHSV and lesion prevalence were tested as functions of initial PAH concentration with linear regression. Histopathological lesions categorized as artifact were not significantly related to oil treatment or VHSV in preliminary tests and were, therefore, not analyzed further. Five other lesions were removed from consideration because of insufficient response (ciliated protozoa, monogenetic or digenetic trematodes, and gill lamellar hyperplasia ($n_{nonzero} < 3$)), total response (glycogen depletion ($n_{nonzero} = 153$)), or near total response (macrophage aggregates in liver ($n_{nonzero} = 151$)). Gall bladder myxosporeans were not analyzed due to incomplete data. Thus, only 25 of the original 38 lesion scores were analyzed. Stepwise regressions were used to compare the relative importance of PAH and VHSV predictors. When regressions were significant, treatments were compared to controls with *a priori* multiple comparisons in single factor ANOVA; percentage data were arcsin transformed and corrected for small n as necessary (Snedecor and Cochran 1980). Fish size and gender were eliminated as factors because they were never significant in tissues responsive to oil or VHSV in preliminary, non-parametric (Kruskal-Wallis) tests (SAS Institute, Inc. 1989).

RESULTS

Hydrocarbon exposure and accumulation

Initial PAH concentrations in water ranged from 0.03 (control) to 58.3 ppb total PAH (Figure 1.1). Concentrations in treatment water declined during the 16 d exposure period, but concentration declines were similar in all treatments, thus, treatment conditions remained clearly separable (Figure 1.1). Similarly, initial alkane concentrations in water ranged from 1.28 (control) to 116 ppb. Declines in alkane concentration over time were similar to those of PAH (Figure 1.1).

PAHs accumulated in muscle and ovaries of fish exposed to oil (Figure 1.2). PAH concentrations in muscle tissue exceeded those in ovarian tissue; these differences were significant in the high-oil treatment ($P \leq 0.001$). Concentrations of PAH in tissues after 16 d exposure were correlated with initial PAH concentrations ($0.92 \leq r \leq 0.99$, $P < 0.001$, where y = (PAH in tissue)$^{1/2}$). Bioconcentration was difficult to estimate because of declining treatment concentrations. In the highest oil treatment, estimated bioconcentration (PAH_t / PAH_w, where PAH_t was concentration in muscle, and PAH_w was concentration in water) was 787 times (range 412 to 1,986 times), estimated by varying PAH_w (mean, initial, and 16 d concentrations, respectively). Similarly, bioconcentration in ovarian tissue was 317 times (166 to 800 times).

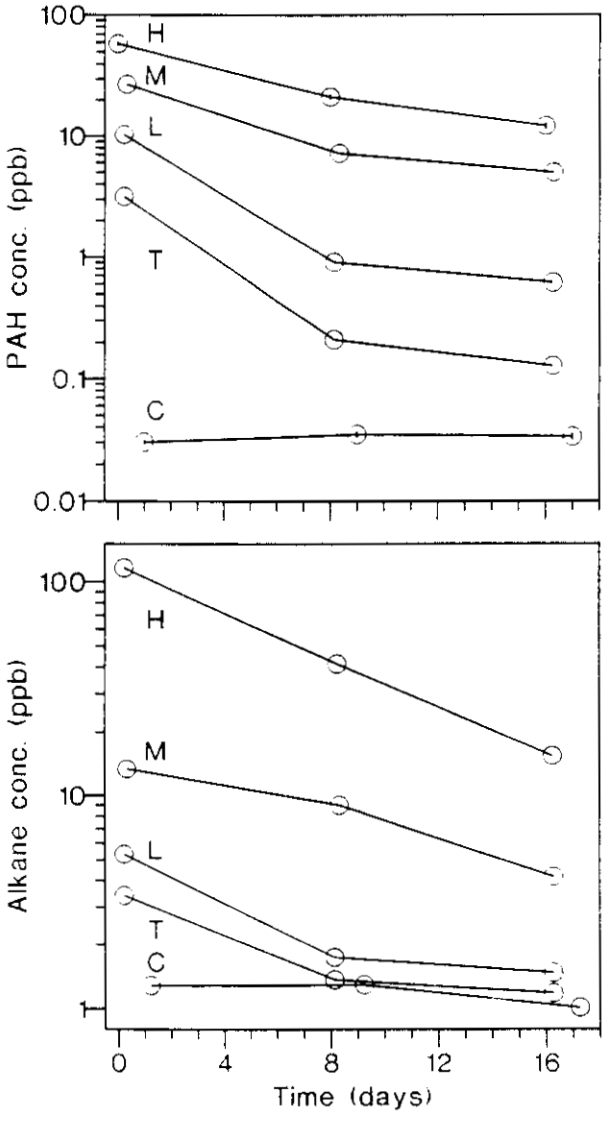

Figure 1.1. PAH and alkane concentrations in treatment water as a function of time. Data plotted are single composite values for replicate tanks at each time; standard error was estimated for the high oil treatment from replicate fluorescence analyses (n = 3).

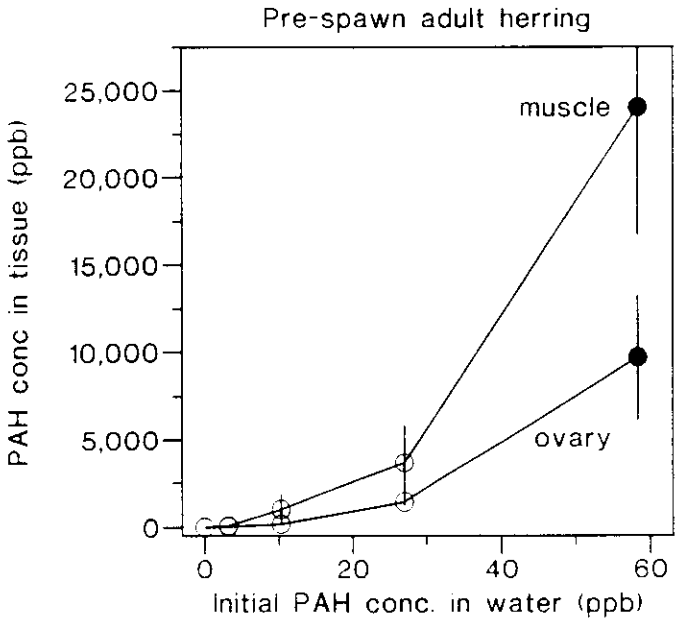

Figure 1.2. Relationship between initial PAH concentration in treatment water and that in muscle and ovary tissue (mean ± SE). Solid symbols indicate significant differences from controls.

Bioconcentration varied as a function of treatment, and was substantially lower in the trace-oil treatment (78 times for muscle and 40 times for ovary).

Biological response

Mortality exceeded 10% in only one high treatment tank during the first 8 d, but increased in the latter half of the experiment (Figure 1.3). Daily mortality was significantly related to time ($P = 0.011$) and treatment ($P < 0.001$), and cumulative mortality was significantly correlated with initial PAH concentration ($r = 0.62$, $P = 0.013$; Table 1.2). Daily mortality was significantly greater in the mid-oil treatment than in controls, but because mortality rates were unusually high in two of the mid-oil treatment tanks, the significance of elevated mortality in the high-oil treatment was masked in the first round ANOVA. However, when all mid-oil responses were removed from the test to achieve homogeneous variance, the significance of daily mortality in the high-oil treatment was also apparent. Tank-specific differences in mortality, estimated by nesting tank in treatment, were not significant ($P = 0.672$). Of the fish that died, 97% had lesions characteristic of VHSV, including external and internal hemorrhaging, fin erosion, changes in liver color, and gill pallor.

Gross lesions and other changes in adult herring were frequently correlated with initial PAH exposure concentration. Jaw hemorrhaging and erosion, changes in liver color, and hemorrhaging of integument, peritoneum, and gonad were significantly and positively correlated with initial PAH concentration ($0.64 \le r \le 0.80$, $0.001 \le P_{reg} \le 0.010$; Figure 1.4). Trends in fin erosion and gill pallor were similar, but not significant ($P_{reg} = 0.088$ and 0.068, respectively). Prevalence of jaw lesions and non-normal liver color was significantly elevated in mid- and high-oil treatments ($P_{ANOVA} < 0.001$; Figure 1.4). In contrast, enlargement of the spleen, and hemorrhaging of eye and kidney did not vary as a function of oil treatment ($0.375 \le P_{reg} \le 0.672$). Correlation of gross lesions, or lack thereof, to prevalence of VHSV was similar to that of PAH.

Table 1.2. Correlation matrix showing relationships of major biological responses AHH activity, EGL, VHSV, and cumulative mortality (Mort) and PAH. Tabled values are correlation coefficients (r): n = 15 for all correlations not involving AHH, n = 116 for correlations involving AHH. Square root (x) transformations were applied unless otherwise noted: [1]no transformation, [2]$\log_e(x)$. Asterisks indicate significance: *** $P \le 0.001$, ** $P \le 0.01$, * $P \le 0.05$.

	AHH	EGL	VHSV	Mort
PAH	0.52***	-0.84***[1]	0.79**	0.62*
AHH	-	-0.50***	0.52***	0.46***
EGL		-	-0.54*[1]	-0.70**[2]
VHSV			-	0.52*[2]

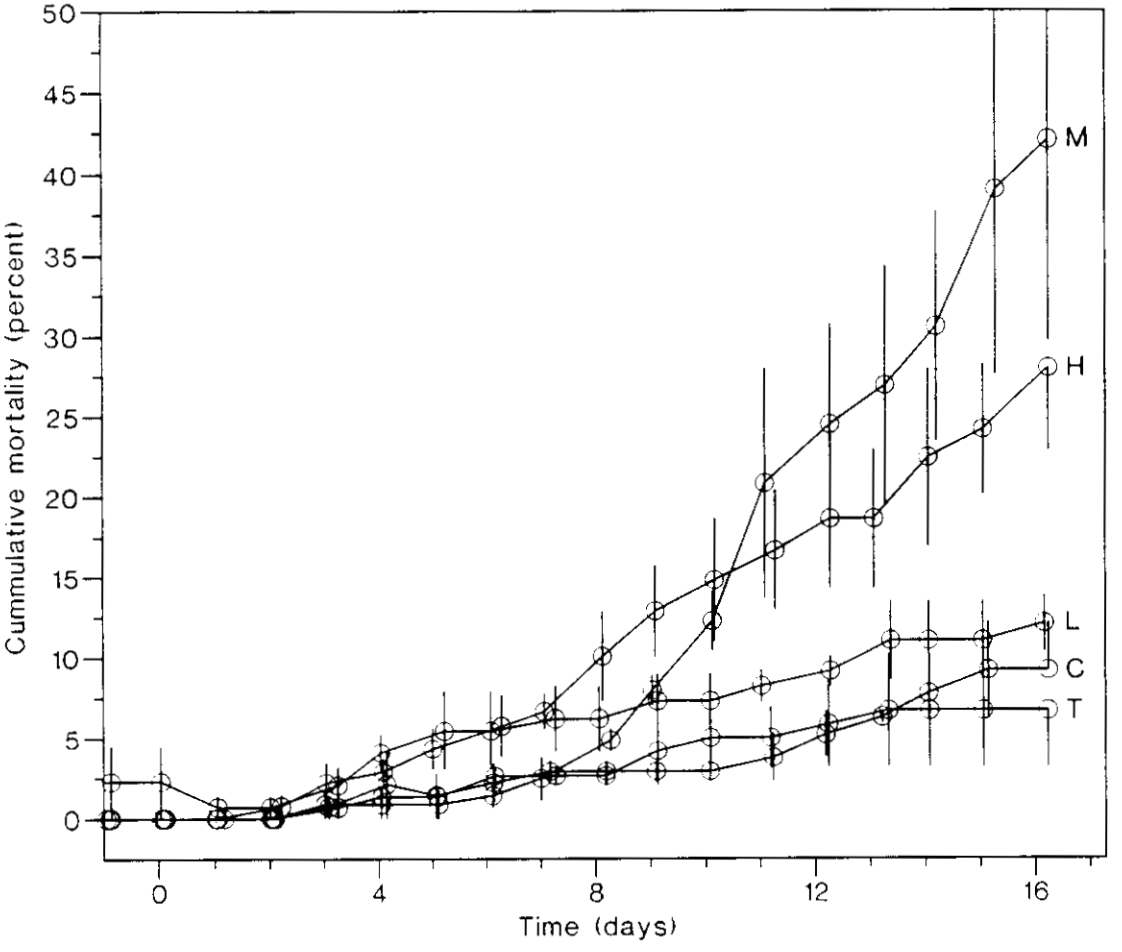

Figure 1.3. Cumulative mortality (mean ± SE) in Pacific herring exposed to weathered crude oil. Treatments were control (C), trace (T), low (L), mid (M), and high (H).

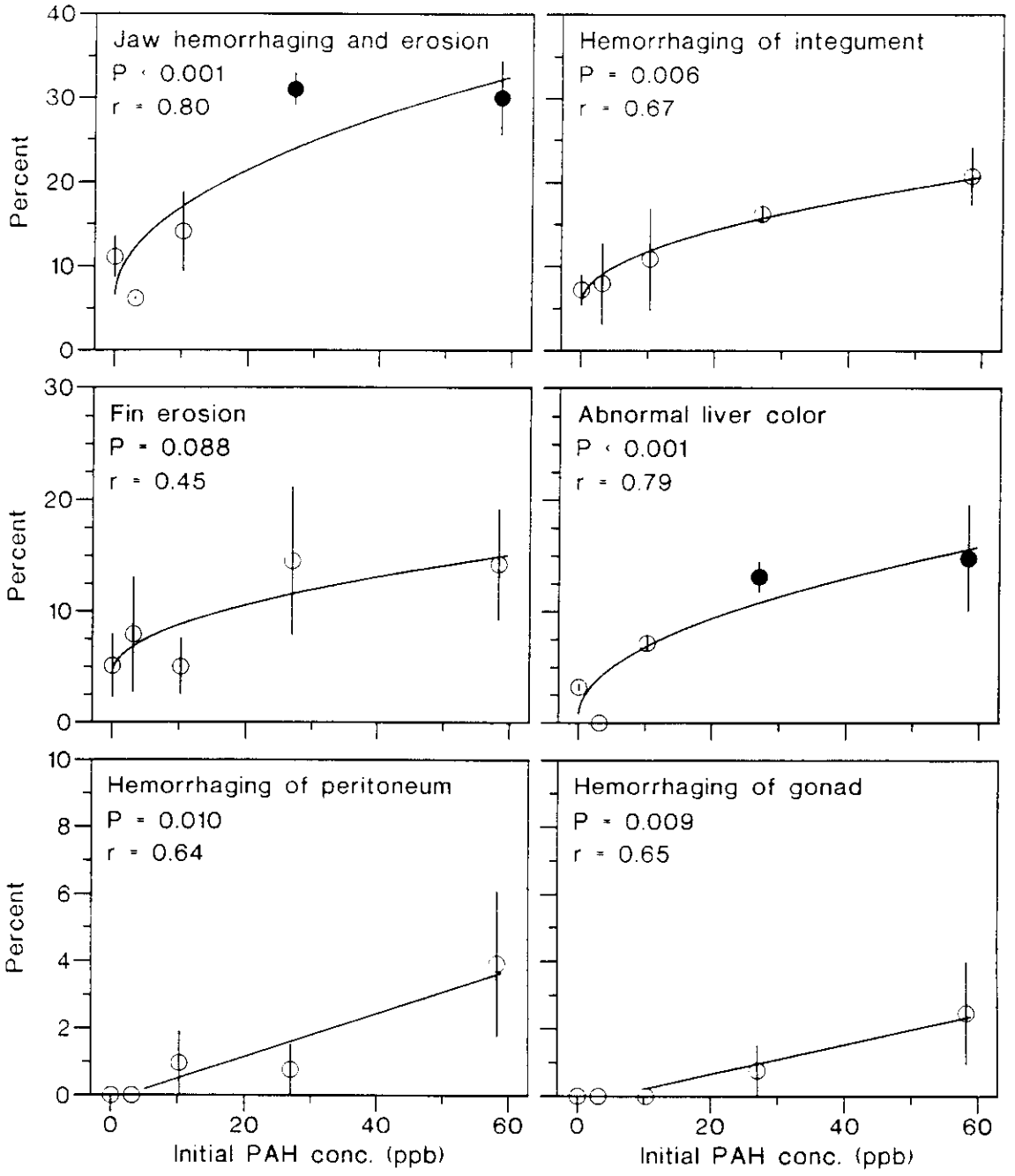

Figure 1.4. Gross lesions (mean ± SE) in Pacific herring that significantly correlated with initial PAH concentration. Percentages were regressed against PAH or $\sqrt{PAH}$; P is probability of significant slope, and r is correlation coefficient. Solid symbols indicate significant differences from controls determined by ANOVA.

except gonadal hemorrhaging did not correlate with VHSV ($P = 0.290$). Tested with stepwise regressions, PAH was a better predictor of response than VHSV, except hemorrhaging of peritoneum correlated more strongly with VHSV.

VHSV was detected in fish collected at the end of the 16 d exposure, while only one fish (of 140 total) had erythrocyte inclusion bodies typical of viral erythrocytic necrosis, another pathogen commonly found in herring. Prevalence of VHSV (Table 1.1) was correlated with initial PAH concentration ($r = 0.79$, $P = 0.001$; Table 1.2), and was significantly greater in high-oil treatment fish than in controls (Figure 1.5).

Differences in lesion scores (Table 1.1) among treatment groups were highly significant with respect to hydrocarbon concentration and VHSV (MANOVA, Wilks' Lambda, $P < 0.001$). Prevalences of several histopathological lesions were correlated with PAH concentration or VHSV prevalence. Significant lesions, listed from most to least significant P value, included EGL, splenic thrombosis, gill arch inflammation or hematopoiesis, focal parenchymal leukocytes in the liver, branchial *Epitheliocystis*, single cell hepatocellular necrosis or apoptosis, hepatic focal necrosis, foreign body granuloma, and splenic arteriolar focal intimal hyperplasia (Table 1.3 and Figures 1.6-1.7). Conservatively, the overall error rate for multiple, independent analyses could be controlled by dividing α (0.05) by the number of tests (25); only EGL and splenic thrombosis passed this criterion. Gill arch inflammation or hematopoiesis declined as PAH concentration increased ($r = -0.68$, $P = 0.005$) and with prevalence of VHSV ($r = -0.73$, $P = 0.002$). Differences in length and gender were not significant for any comparisons (MANOVA).

Focal hepatic necrosis and disseminated vascular thrombosis were among the most severe lesions associated with oil level and VHSV. Hepatic necrosis usually affected multiple random foci throughout the parenchyma. Affected hepatocytes had hypereosinophilic cytoplasm with pyknotic or karyorrhectic nuclei (Figure 1.8). Of the six affected fish, one was a control but the other five were from the three highest dose groups. No fish had severe hepatic necrosis, but the two moderate cases were in the two highest exposure groups. Four of the six fish with hepatic necrosis were positive for VHSV; among the two moderate cases, one was negative for VHSV.

Vascular thrombosis was more common, occurred in all 3 organs, and affected 29 of 153 fish (19%). Only one affected fish was a control. Thrombus composition varied from cellular to fibrinous. Cellular thrombi, usually < 100 µm in diameter, were composed of clusters of thrombocytes and scattered macrophages, with comparatively little extracellular material; cellular thrombi were never occlusive. Fibrinous thrombi were usually larger, sometimes > 1 mm in diameter and occlusive, but thrombocytes were rare (Figure 1.8). Prevalence of thrombi increased with oil treatment in spleens and possibly gills. Splenic thrombosis increased as PAH concentration increased ($r = 0.79$, $P = 0.001$) and with prevalence of VHSV ($r = 0.75$, $P = 0.001$); thrombi were significantly elevated in mid- and high-oil treatments (Table 1.3 and Figure 1.6). Gill thrombosis was rare (eight nonzero responses), and correlation with PAH was not significant ($r = 0.40$, $P = 0.141$), but trends were similar to those in spleens (Table 1.3). Thrombosis in livers did not correlate with PAH ($r = 0.13$, $P = 0.654$).

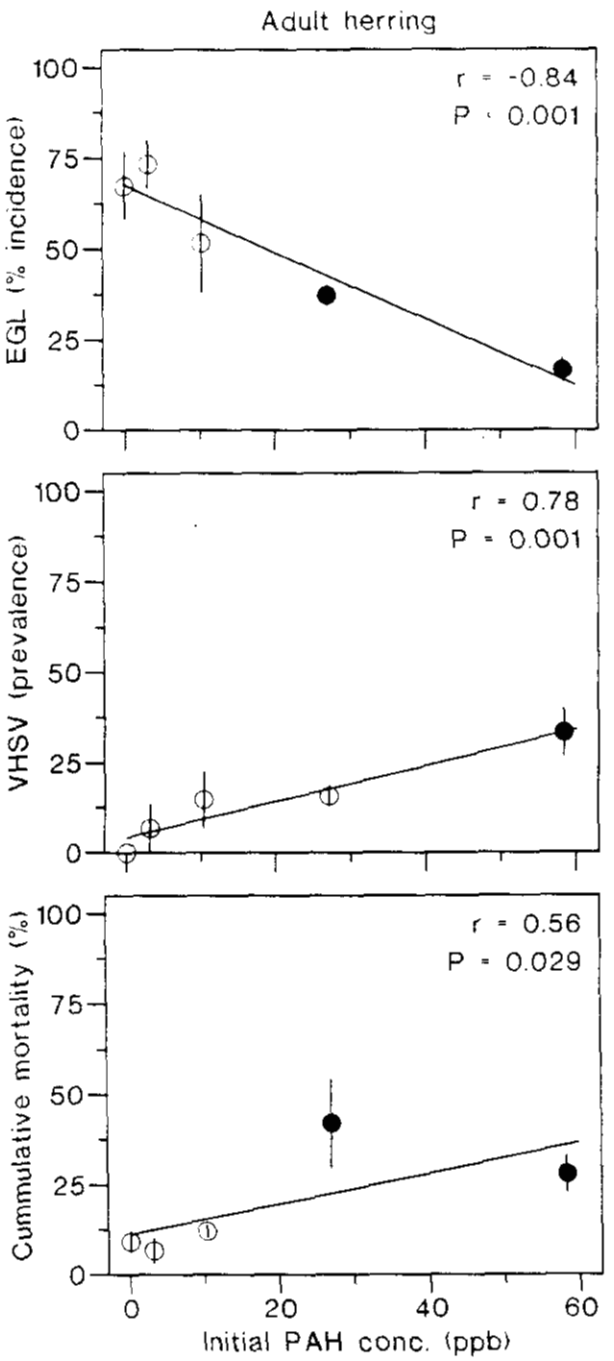

Figure 1.5. Relationship of EGL, VHSV, and cumulative mortality to initial PAH concentration. Percentages were regressed against PAH or $\sqrt{PAH}$; P is probability of significant slope, and r is correlation coefficient. Plotted data are means ± SE; solid symbols indicate significant differences from controls.

Table 1.3. Relationship of histological lesions to oil and VHSV. r = correlation coefficient, P = probability, and n is the total number of nonzero lesion scores of 153 possible. Lesions were grouped into three categories, based on response or lack of response to oil treatment or VHSV. The responsive category included lesions where P_{oil} or $P_{VHSV} \leq 0.05$. The 'possibly responsive' category included lesions where $P \leq 0.15$. Lesions where $P > 0.15$ were considered unrelated to oil treatment. Bold type indicates statistical significance ($P \leq 0.05$); [1]indicates which factor, if any, entered stepwise regression first, [2]indicates which factor, if any, entered second. In the one instance where both PAH and VHSV entered the regression (FN), final model correlations and probabilities are reported; in all other cases reported values indicate separate regressions against PAH or VHSV.

	Oil regression		VHSV regression		
Lesion	*r*	*P*	*r*	*P*	n
1. Lesions responsive to oil or VHSV					
Thrombosis, spleen	0.79	0.001[1]	0.75	0.001	16
Leukocytes, eosinophilic granular	-0.84	0.001[1]	-0.54	0.038	75
Gill arch inflammation	-0.68	0.005	-0.73	0.002[1]	149
Leukocytes, focal or multi-focal	-0.55	0.034[1]	-0.22	0.422	28
Epitheliocystis	0.53	0.040[1]	0.51	0.051	13
Necrosis, single cell	0.47	0.077	0.65	0.008[1]	22
Necrosis, focal	0.34	0.146[2]	0.56	0.028[1]	6
Foreign body granuloma	-0.45	0.096	-0.54	0.036[1]	11
Focal intimal hyperplasia	-0.45	0.090	-0.53	0.041[1]	19
2. Lesions possibly responsive to oil or VHSV ($P \leq 0.15$)					
Granulomatous inflammation	-0.51	0.052[1]	-0.20	0.482	10
Thrombosis, gill	0.40	0.141[1]	0.15	0.597	8
3. Lesions not related to oil or VHSV ($P > 0.15$)					
Macrophage aggregates	0.37	0.175	0.35	0.196	138
Lipidosis	0.37	0.175	0.04	0.877	16
Gill lamellar telangiectasis	0.03	0.270	0.13	0.656	62
Leukocytes, pericholangial	-0.30	0.271	-0.39	0.153	25
Ichthyophonus, liver	-0.26	0.355	-0.17	0.534	12
Goussia clupearum	0.25	0.360	0.01	0.981	124
Cholangitis or biliary hyperplasia	-0.25	0.375	-0.15	0.592	8
Granulomatous inflammation	-0.23	0.400	-0.32	0.240	35
Ellipsoid hyalinization	-0.21	0.461	0.03	0.906	142
Anisakis	-0.21	0.468	-0.03	0.903	24
Ichthyophonus, spleen	-0.16	0.570	-0.22	0.441	12
Thrombosis, liver	0.13	0.654	0.01	0.967	7
Ichthyophonus, gill	-0.07	0.797	-0.21	0.454	9
Congestion, spleen	0.05	0.871	0.16	0.565	139

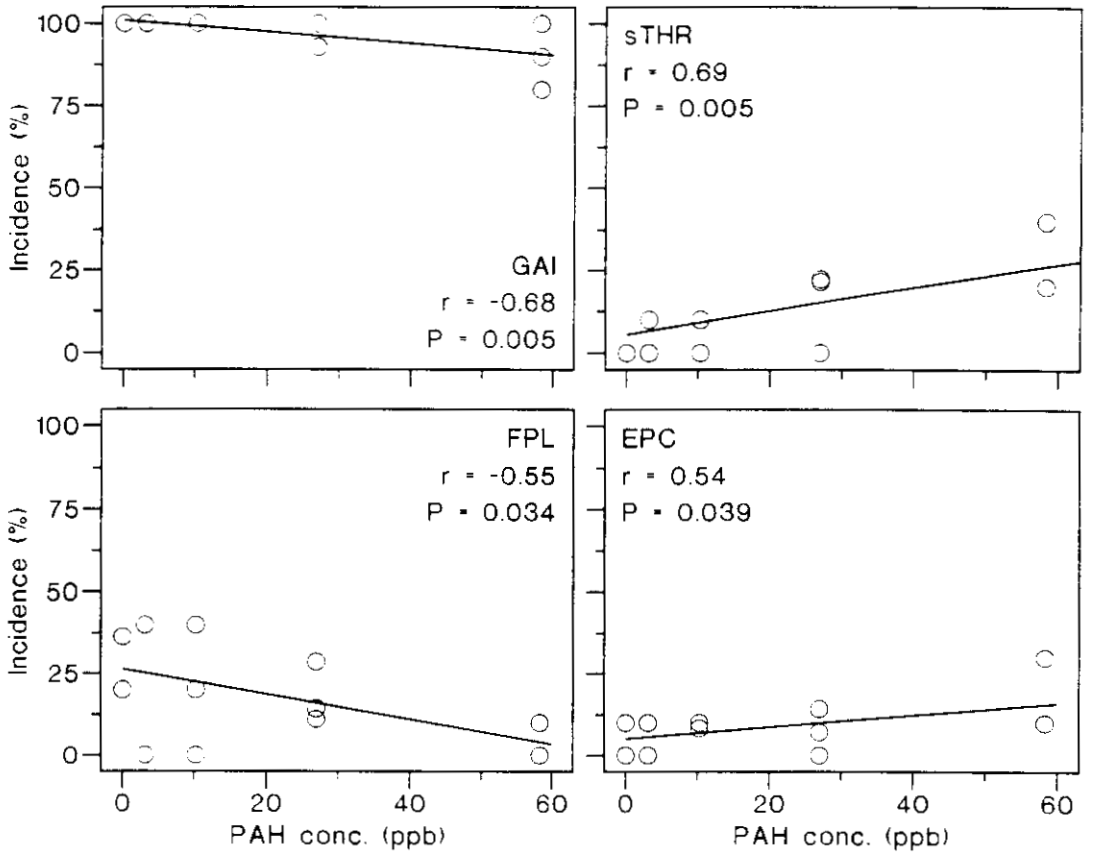

Figure 1.6. Histopathological responses that correlated significantly with initial PAH concentration. Also reported are probability of significant slope (P), and correlation coefficient (r).

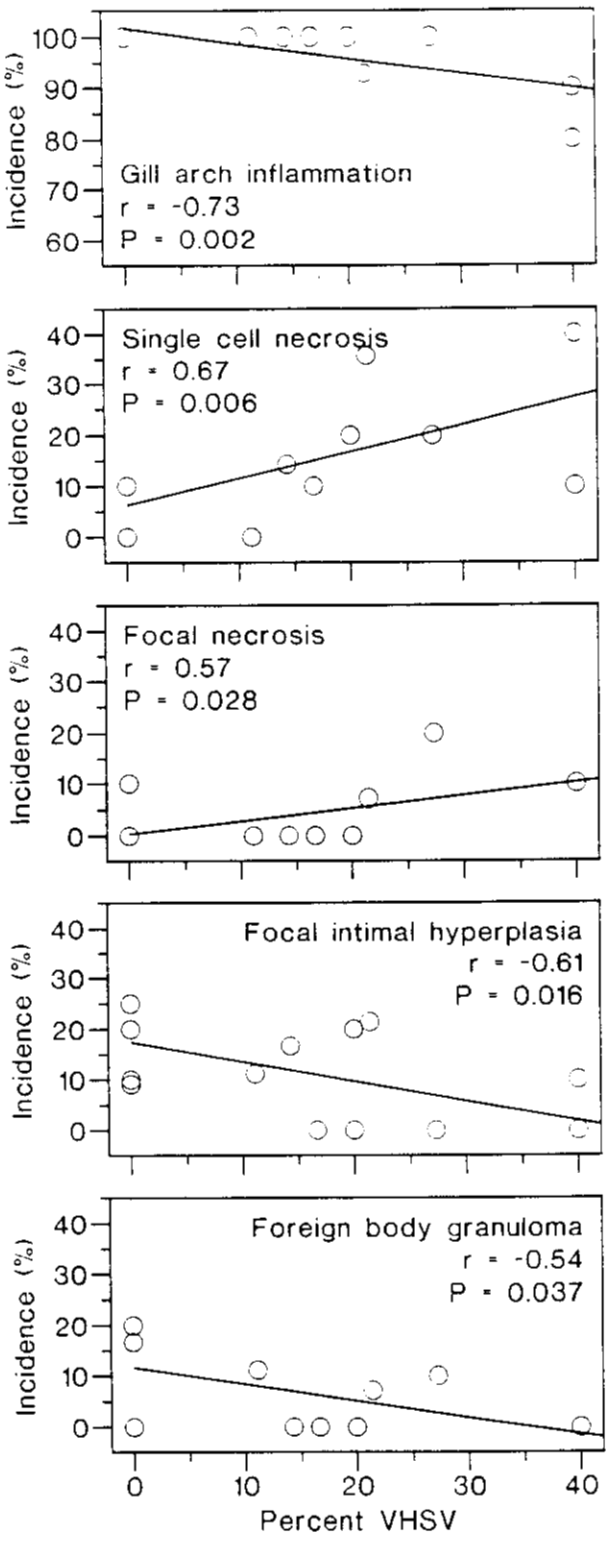

Figure 1.7. Histological lesions that correlated significantly with prevalence of VHSV. Also reported are probability of significant slope (P), and correlation coefficient (r).

Figure 1.8. Microscopic lesions in adult Pacific herring exposed to weathered crude oil in the laboratory. Top - moderate focal hepatocellular necrosis (arrows); scale bar = 40 µm. Bottom - fibrinous thrombi (arrows) in a congested spleen; scale bar = 100 µm.

Abundance of tissue leukocytes was depressed as oil treatment increased. The number of EGL declined as PAH concentration increased (r = -0.84, $P < 0.001$) and with increased VHSV prevalence (r = -0.54, P = 0.038); EGL were significantly depressed in mid- and high-oil treatments (Figure 1.5). Prevalence of EGL was also negatively correlated with AHH activity (r = -0.50, $P < 0.001$) reported in a companion study (Thomas et al. 1997). Focal or multifocal parenchymal leukocytes were also inversely correlated with PAH concentration (r = -0.55, P = 0.034), but not with VHSV (r = -0.22, P = 0.422) (Table 1.3). In contrast, pericholangial leukocytes did not correlate with either PAH (r = -0.30, P = 0.271) or VHSV (r = -0.39, P = 0.153), although trends were similar to those of other leukocytes (Table 1.3).

The paucity of inflammatory cells in Pacific herring exposed to the highest dose of oil was particularly obvious. Among the five treatment groups, organs from fish exposed to the highest dose of oil had significantly lower mean scores for several inflammatory lesions: 1) hepatic perivascular or pericholangial EGL; 2) gill arch inflammation or hematopoiesis; 3) focal or multifocal hepatic parenchymal leukocytes; and 4) granulomatous inflammation in the spleen (Table 1.1). For example, 21 of 31 livers from control fish had pericholangial or perivascular EGL, whereas only 5 of 30 livers from the high-dose group had EGL. EGL were nearly always scored as none or mild; the two moderate cases were in the control and lowest-dose groups. Mean scores for other inflammatory lesions were lower in the high-dose fish, but trends were not significant: 1) *Ichthyophonus* in the spleen and liver; 2) cholangitis or biliary hyperplasia; and 3) intrahepatic pericholangial leukocytes.

Lesions caused by parasites were not related to oil exposure or VHSV. Among 153 fish examined in this study, overall prevalences of various parasites were as follows: *Ichthyophonus hoferi* (11%), branchial *Epitheliocystis* (8.6%), hepatic *Goussia clupearum* (81%), and hepatic Anisakidae (16%).

DISCUSSION

We have shown that exposure of wild Pacific herring to environmentally relevant concentrations of crude oil resulted in depression of immune function and expression of VHSV. Histological lesions in these fish could have resulted either directly from the presence of hydrocarbons in tissues, or secondarily to VHSV. Concentrations of axenic hydrocarbons were elevated in tissues of fish exposed to oil, and MFO enzymes were induced (Thomas et al. 1997). Thus, it is possible that organ, tissue, or cellular damage was caused directly by hydrocarbons (concentrations of PAH in tissues exceeded those in water by more than two orders of magnitude). More likely, however, certain histological lesions were caused by expression of VHSV, which proliferated when immune function was suppressed by oil-induced stress. Alternatively, the lesions may have been caused by a combination of oil exposure and VHSV expression.

Concentrations of oil to which herring were exposed were environmentally relevant. Conservatively, we analyzed biological responses of herring against initial PAH exposure concentrations, which bracketed, but extended well beyond those reported in open water of PWS after the spill (up to 6.24 ppb, Short and Harris 1996). However, because test concentrations fell rapidly with time, effective exposure concentrations were lower. For example, mean PAH concentrations in low- and mid-oil treatments (4 and 13 ppb) bracketed the 6 ppb value, and significant biological response was observed in this range. In PWS, PAH concentrations were also variable, and generally declined with time. It is possible, dependent on the volume of stranded oil and other factors, that concentrations were higher in intertidal areas and that significant concentrations persisted longer intertidally than in open water. For these reasons it is plausible that reproductively ripe herring could have encountered biologically significant quantities of oil during intertidal spawning activity, or as they migrated through the slick trajectory. In a companion paper (Carls et al. Chapter 3), we show that PAH composition in this study, and weathering patterns, were very similar to composition and weathering of *Exxon Valdez* crude oil in PWS. Additionally, Short and Heintz (In press) compared weathering in similar apparatus to that in PWS, and concluded underlying processes were the same.

Because fish used in this study were captured from the wild, the original status of VHSV infection in them was unknown. Fish are frequently asymptomatic carriers of disease pathogens controlled by their immune system (Anderson 1990), and Pacific herring are reservoirs of VHSV (Meyers et al. 1994). Preliminary estimates are that 10 to 20% of wild Pacific herring carry VHSV below detectable levels (G. D. Marty and R. M. Kocan, unpublished data). This explanation was likely true for our fish prior to the experiment. That virus was not isolated from control fish indicated that exposure to VHSV through the common water supply was not likely in our experiments.

We hypothesize that decreased immune surveillance is a mechanism by which subclinical VHSV could be reactivated. Decreased inflammation in livers as oil concentration increased suggested generalized immunosuppression in exposed herring. However, because a variety of toxicants have been shown to compromise the immune system (Anderson 1990), decreased inflammation was more likely a nonspecific response to stress than a specific response to VHSV or oil exposure. Examination of Pacific herring tissues after exposure to other stressors such as crowding or altered water temperature is needed to confirm the role of stress in intrahepatic inflammation.

Decreased intrahepatic inflammation in response to oil exposure has not previously been described. This lack of published information is probably not due to lack of occurrence; rather, diagnosis of decreased inflammation may have been missed. In our experience, low-grade inflammation is normal in the liver of adult Pacific herring (Kocan et al. 1996; Marty et al. 1995). The absence of subtle lesions, however, is difficult to diagnose. To increase chances of detecting potentially significant lesions, we recommend the use of randomization, semiquantitative scoring, and blind study.

Increased expression of VHSV was probably directly related to suppression of immune function. Stressed animals are more susceptible to disease pathogens. For example, the titer of infectious pancreatic necrosis virus was elevated in brook trout (*Salvelinus fontinalis*) treated with immunosuppressant and stressed with warm water (McAllister et al. 1994). Susceptibility of rainbow trout to infectious hematopoietic necrosis virus increased when they were exposed to copper (Hetrick et al. 1979). Brook trout known to carry *Aeromonas salmonicida* developed disease symptoms after injection of a synthetic corticosteroid (Bullock and Stuckey 1975). Reproductive condition in our study may have also been a contributing stress factor because gamete production requires considerable energy (gonad mass exceeded 20% of total body weight), and because pre-spawning Pacific herring do not feed (Iles 1965; Carlson 1980); only 6% of the pre-spawn fish we captured had food in their gastro-intestinal tract. Meyers et al. (1994) suggested nutritional deficiency can lead to stress and expression of VHSV.

Results from published studies support the hypothesis that hepatic necrosis was more a result of VHSV than oil exposure. Lesions commonly associated with oil include decreased hepatocellular glycogen, and variable alterations in hepatocellular lipid (Hawkes 1977; McCain et al. 1978; Eurell and Haensly 1981; Haensly et al. 1982; Solangi and Overstreet 1982; Woodward et al. 1983). Although hepatocellular necrosis has been associated with environmental oil exposure (Haensly et al. 1982), this finding has not been confirmed in the laboratory. By comparison, hepatic necrosis is common in salmonids with the European strain of VHSV (Amlacher 1980; Wolf 1988).

Thrombosis has not been described in association with either oil exposure or VHSV. Deficiency of thrombocytes and leukocytes, and anemia have been associated with the European strain of VHSV in salmonids (Wolf 1988). In contrast, a relationship between PAH concentration and gill-vessel aneurysms that could lead to thrombosis has been observed in sanddab (*Citharichthys sordidus*) (R. Spies, Personal communication).

In our study, accumulation of hydrocarbons in tissues of exposed herring was sufficient to strongly induce AHH activity; measurement of AHH induction was made on fish sampled from the same tanks and reported separately (Thomas et al. 1997). AHH activity may have contributed to suppressed immune function in our herring, but we hesitate to suggest causality because other biochemical responses may have been responsible. For example, when fish are stressed, a general response is elevation of plasma cortisol; it is likely that corticosteroids in our herring were elevated in a dose-dependent manner. Elevation of cortisol concentration may lead to immunosuppression (Anderson 1990). Although, it has been reported that activation of the aromatic hydrocarbon receptor, signaled by induction of AHH activity, may suppress immunological function in mice (Silkworth et al. 1984), this has not been demonstrated in fish.

Expression of VHSV caused mortality in the laboratory, and may also cause mortality in wild populations. Moribund herring with VHSV were observed in PWS in 1993; although it was not possible to quantify mortality, only one-third of the expected spawning biomass returned (Meyers et al. 1994). In contrast, post-spawn herring analogously tested in a companion study

(Thomas et al. 1997) did not develop clinical signs of disease, and mortality related to oil dose did not occur. This suggests that mortality in pre-spawn fish was related to disease, and possibly reproductive or nutritional condition, not direct toxic effects. Research to determine if the toxic effects of environmentally relevant concentrations of crude oil on Pacific herring are entirely a result of VHSV expression are underway (J.R. Winton and R.M. Kocan, Personal communication).

Although effects due to oil and VHSV effects were not definitively separable in our study, we suggest that gross and histological lesions previously attributed solely to oil exposure in Pacific herring captured from PWS in 1989 (Moles et al. 1993) may have been caused by expression of VHSV, induced by oil exposure. This suggestion is further supported by occurrance of similar lesions in VHSV-positive Pacific herring sampled from PWS in 1993 and 1994, when hydrocarbon exposure was no longer detectable (Meyers et al. 1994; Marty et al. 1995).

In summary, correlations between oil exposure, induction of AHH, suppression of leukocytes, prevalence of VHSV, and cumulative mortality were strong, suggesting possible causal links between oil exposure and immunosuppression, immunosuppression and expression of VHSV, and between VHSV and mortality in Pacific herring exposed to oil. Results of this study do not prove causality, but they do show that stress caused by hydrocarbon exposure, and resultant immune response, disease, and mortality are closely related.

CONCLUSIONS

Herring accumulated hydrocarbons from oil-contaminated water; muscle tissue accumulated more PAH than ovarian tissue. Accurate estimation of biomagnification was difficult because of declines in treatment concentrations, but biomagnification in upper oil treatments apparently exceeded two orders of magnitude.

Decreased inflammation as PAH concentration increased suggested generalized immunosuppression in exposed fish, and decreased immune surveillance as a possible mechanism by which subclinical VHSV could be reactivated.

Gross pathological lesions correlated with PAH concentration and prevalence of VHSV.

Prevalence of VHSV increased with PAH concentration, and was negatively correlated with EGL.

Histopathological lesions correlated with PAH concentration and VHSV, but not with gender or length. Significant microscopic lesions included increased hepatocellular necrosis, disseminated vascular thrombosis, and decreased inflammation as determined by EGL.

Significant mortality related to oil dose occurred; 97% of fish that died had clinical signs of disease. Mortality was correlated with PAH concentration and VHSV prevalence.

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Chapter 2: Mixed function oxygenase induction in pre- and post-spawn herring (*Clupea pallasi*) by petroleum hydrocarbons

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

ABSTRACT

We compared the uptake of water-accommodated fractions of Alaska North Slope crude oil in pre- and post-spawn herring (*Clupea pallasi*) and the resulting induction of mixed function oxygenase (MFO) activity. Both groups of herring accumulated significant quantities of polynuclear aromatic hydrocarbons (PAHs) in muscle tissue. Pre-spawn herring accumulated higher concentrations of hydrocarbons and retained them longer. Induction of MFO activity in pre-spawn fish was one-third to one-half that in post-spawn fish. Inability of pre-spawn herring to induce MFOs at as high a level as post-spawn fish may explain higher tissue concentrations and retention of hydrocarbons in the pre-spawn animals.

INTRODUCTION

Herring spawned in Prince William Sound (PWS) a few weeks after the 1989 *Exxon Valdez* oil spill. Contamination in the sound was so severe that all stages in the life cycle of herring were potentially exposed to oil. Direct negative effects on eggs and larvae, such as increased abnormalities were summarized by Brown et al. (1996). Even those larvae not hatched in oiled areas were likely advected by currents to oiled parts of the sound (Norcross et al. 1996). Although the impacts of oil on reproductive processes were not studied in the few years following the spill, concern increased as the adult population declined rapidly between 1989 and 1994, particularly in 1993 when the spawning population dropped 25% to 50% below expectation (Brown et al. 1996). Disease appeared to be the primary cause (Brown et al. 1996), but indirect links to oil from the spill are suspected.

Although mature herring are harvested commercially in PWS, stock structure, winter residence, and seasonal movements are poorly understood, consequently there was little damage assessment effort invested in the adult life stage following the spill. The vulnerability of the herring adults and the reproductive process to oil was the subject of speculation by Rice et al. (1987) following a laboratory study where high concentrations of oil were found in ovary and other tissues of fish exposed to water soluble fractions (WSF) of crude oil.

Several laboratory studies have demonstrated a potential for organic contaminants, such as PAHs, to interfere with the reproductive process in several fish species (Weis and Weis 1989). 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 cytochrome P-450 dependent MFOs, a group of enzymes

involved in the metabolism and thus clearance of PAH and other xenobiotics (Stegeman et al. 1979; Britvic' et al. 1993), have been shown to be depressed in other fish species in their spawning condition (Walton et al. 1983; Förlin et al. 1984; Lindstrom-Seppa 1985; Collier et al. 1986; Porter et al. 1989).

In fish, as in other vertebrates, PAHs are detoxified or activated by the cytochrome P-450 dependent MFO system. Two of the most studied and sensitive of these enzymes in fish are aryl hydrocarbon hydroxylase (AHH) and ethoxyresorufin 0-deethylase (EROD). Induction of AHH and EROD in fish has been suggested as a useful and sensitive biomonitor of marine pollution (Payne et al. 1987; Holdway et al. 1994; Kocan et al. 1996).

Activation of carcinogenic PAH (e.g. benzo[a]pyrene is converted to an active carcinogen by AHH) results in ovarian toxicity and oocyte destruction in mammals (Mattison and Nightingale 1980). A negative correlation has been shown to exist between reproductive success and hepatic AHH activity in species of flatfish (Spies and Rice 1988; 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).

The primary goal of this study was to examine the physiological vulnerability of adult pre-spawn herring to oil exposure. Specifically, we measured hydrocarbon uptake in pre- and post-spawn herring adults from similar exposures, and MFO induction in both sets of fish. MFO components measured were AHH, EROD, and cytochrome P-450. All oil exposures were flow-through, for up to 16 d to low ppb PAH concentrations of North Slope crude oil. In companion studies, to be reported, we noted the impacts of oil exposure to adults on disease vulnerability and on spawning success.

METHODS

Pre-spawn herring were collected by purse seine near south Shelter Island, Alaska (58.4°N lat., 134.8°W long.) on 20 March 1994. Post-spawn herring were collected by jigging in the same general area between 6 and 13 July 1994 (approximately 40 d after natural spawning was observed in the area). Pre-spawn fish were gravid but not ripe when captured. Ripeness was judged from percent gonad to total body weight, gonad color, and looseness of gametes. Approximately 1,500 pre-spawn herring were placed in a 24,000 L holding tank with running seawater at 60 to 130 L/min at 3.5° to 5.2° C and 31 ppt. Some of these fish were artificially spawned beginning 2 May 1994. This spawning occurred at about the same time natural spawning events in the area were observed (A. McGregor, Personal communication). Post-spawn herring were placed in 700 L tanks with running seawater at approximately 7 L/min, 6.5°C, and 32 ppt. Morphometrically the two groups were closely matched in age, weight, and length, indicating that both the pre-spawn and post-spawn groups were probably obtained from the same population.

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. Water flowed from the top of these oiled-water generators to the bottom of individual 700 L treatment tanks. Control generators were charged with clean gravel. Before 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. This process removed most of the low molecular weight monoaromatic hydrocarbons and produced a fraction more similar to that which occurs following an oil spill in the natural environment. Pea gravel (2 to 33 mm diameter) was washed on 3 mm screen and throughly 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 in the mixer with a paint sprayer (trace, low, and mid treatments) or with a Teflon squirt bottle (high treatment). Batches of gravel were mixed by treatment to provide four concentrations of oiled water; trace, low, mid, and high-oil. Concentrations were chosen to bracket actual PAH concentrations measured in PWS during the spill (Short and Harris 1996). Each generator was charged with 45 kg of freshly oiled gravel (or non-oiled control gravel), except high treatment generators were charged with 90 kg gravel. Hydrocarbon concentrations in the treatment tanks were routinely monitored by fast-screen ultra-violet fluorescence (Krahn et al. 1993) and treatments were characterized and quantified by gas chromatography (Short et al. 1996). The PAH composition of the water accomodated fraction reflected the PAH concentrations in the oil, which was similar to the PAH composition in PWS measured by Short and Harris (1996).

To determine MFO induction time, pre-spawn herring were exposed to a single oil treatment (high) and compared to untreated controls. At 0, 0.5, 1, 2, 4, 8, and 16 d, 25 fish were sampled from each treatment. To determine the effect of oil dose, 25 fish from each of three additional, concurrent treatments (trace, low, mid) were sampled at 8 and 16 d. Ripeness of pre-spawn fish was confirmed at the end of the 16 d exposures by artificial spawning. The female:male ratio was 1.4:1. In pre-spawn tests, water flow was 6.9 L/min at 3.9° to 4.4°C (mean = 4.2°C) and 31 ppt.

To compare MFO induction between the pre-spawn and post-spawn groups, post-spawn herring were exposed to oil in water in the same manner as the pre-spawn group except there were only two treatment groups, control and high oil concentration. Fish were exposed for 4, 8, and 16 d. At each observation, 35 fish were sampled from each treatment, except controls were not sampled on day 8. The female:male ratio was 1:1. In post-spawn tests, water flow was 6.4 L/min at 6.4° to 7.6°C (mean = 6.8°C) and 32 ppt.

At the time of sampling, fish were killed by a blow to the head, length and weight determined, and 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 muscle tissue was taken from each fish for hydrocarbon analysis. In fish which were spawned, male and female gonads were also subsampled for hydrocarbon analysis; PAHs were analyzed by gas chromatography/mass spectrometry according to the method of Short et al. (1996).

Microsomal fractions for MFO induction measurements were prepared as follows. Livers were homogenized in a Potter-Elvehjem homogenizer in 4 volumes of 0.15M KCl in 0.1M potassium phosphate buffer, pH 7.4. The 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 Lowry 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, EROD activity and cytochrome P-450 concentration were determined for all animals exposed to the high oil concentration and the corresponding controls. EROD activity was determined according to the method of Burke and Mayer (1974) and cytochrome P-450 concentration according to the method of Omura and Sato (1964). AHH and EROD assays were run in triplicate and cytochrome P-450 assays were conducted in duplicate.

Observations were analyzed with analysis of variance (ANOVA), with treatment, time, and spawning condition as factors. When the overall test was significant, factors were compared to controls and each other with *a priori* multiple comparisons; probabilities less than or equal to 0.05 were considered significant.

RESULTS

Initial hydrocarbon concentrations were low; measured PAH concentrations in the pre-spawn tests were 0.03, 3.2, 10.3, 27.0, and 58.3 ppb for control, trace-, low-, mid-, and high-oil treatments, respectively. In post-spawn tests, initial PAH concentrations were 0.05 and 33.8 ppb for control and high-oil treatments. Composition of PAH in treatment water was similar to that in North Slope crude oil, except monoaromatic hydrocarbons were removed by artificial weathering. This PAH composition was similar to that found in PWS shortly after the spill (Short and Harris 1996).

Hydrocarbon concentrations declined with time; PAH concentrations generally remained above baseline throughout the pre-spawn test, but dropped to near control levels within circa 8 d in the post-spawn test (Figure 2.1). Test concentrations bracketed the maximum mean concentrations observed in the water of PWS following the spill (6.2 ppb PAH) (Short and Harris 1996). Slightly warmer temperatures in post-spawn tests than in pre-spawn tests (3°C difference) probably contributed to the more rapid decline in PAH concentrations in post-spawn tests. Composition of the doses also changed with time; naphthalenes dominated the initial concentrations, accounting for 65-87%, but declined to 31-66% in 16 d. Conversely, the relative percentages of large compounds increased with time.

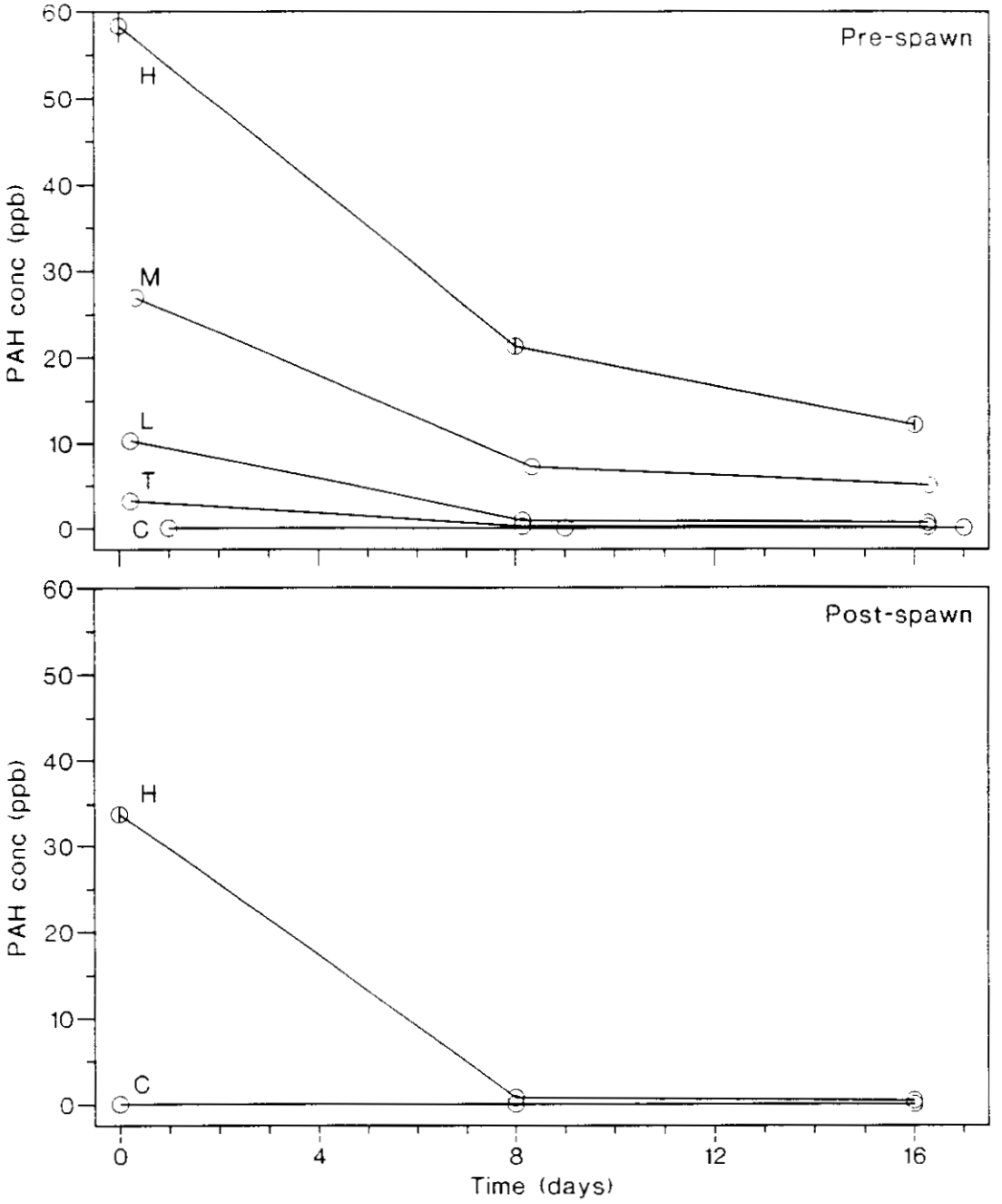

Figure 2.1. PAH concentrations in treatment water. Treatments were control (C), trace (T), low (L), mid (M), and high (H) oil. Data plotted are single composite values for replicate tanks at each time: standard error was estimated for the high oil treatment from replicate fluorescence analyses.

The PAH concentration in muscle and ovary tissue rose to a steady state in 4 d in the pre-spawn group and remained elevated for the duration of the 16 d exposure (Figure 2.2). Although ovaries did not accumulate as many hydrocarbons as muscle, accumulations were significant in both tissues. In contrast, PAH concentrations in post-spawn fish reached a maximum in 4 d, but declined significantly over the next 12 d and were not significantly different from those in controls on day 16 (Figure 2.2). Ovaries were depleted in post-spawn fish and were not measured for PAH accumulation.

Induction of AHH in pre-spawn herring, measured on days 8 and 16, increased with increasing dose (Figure 2.3). Even the lowest concentration, the trace dose (initial PAH, 3.2 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 that AHH was not significantly elevated following the 16 d exposure to the trace dose (Figure 2.4). Induction of AHH in pre-spawn males was generally not significantly different than in pre-spawn females ($0.179 \leq P \leq 0.909$), except on day 16, induction was significantly greater in males ($P = 0.001$). Induction of AHH in pre-spawn fish was rapid, requiring 48 h of exposure to the high dose to cause a significant increase over controls (Figure 2.4).

Induction of AHH in post-spawn herring was about two-fold greater than induction in pre-spawn fish (Figure 2.4), even though the exposure concentration was significantly lower in the post-spawn test (Figure 2.1). Although exposure concentration was less at 16 d than at 8 d, AHH activity was greater with the longer exposure in both the pre-spawn and post-spawn groups (Figure 2.4). Gender differences were not significant in time series tests ($0.463 \leq P \leq 0.929$), but AHH induction in pre-spawn females was consistently depressed with respect to post-spawn females ($0.001 < P \leq 0.023$), while induction differences between pre- and post-spawn males were smaller and not significant ($0.243 \leq P \leq 0.793$).

Induction of EROD was similar to AHH induction: EROD increased in both pre- and post-spawn fish, but induction in post-spawn fish was about triple that in pre-spawn fish (Figure 2.4). In pre-spawn fish, EROD activity doubled by 16 d, but exposed fish were not statistically different from controls. In contrast, EROD activity in exposed post-spawn fish increased about six times over the control values, were statistically significant, and were substantially greater than the pre-spawn fish. In contrast to AHH induction, the high levels of EROD induction in post-spawn fish changed little between days 4 and 16.

Cytochrome P-450 was induced in both pre-spawn and post-spawn fish after exposures to oil; there was no obvious difference between the two fish groups (Figure 2.4). After 16 d, P-450 induction in pre-spawn fish was slightly greater than in post-spawn fish, but differences were not significant. As with EROD, induction of P-450 between days 4 and 16 was relatively stable. Neither EROD or P-450 were assayed in 4 and 8 d exposures of pre-spawn herring.

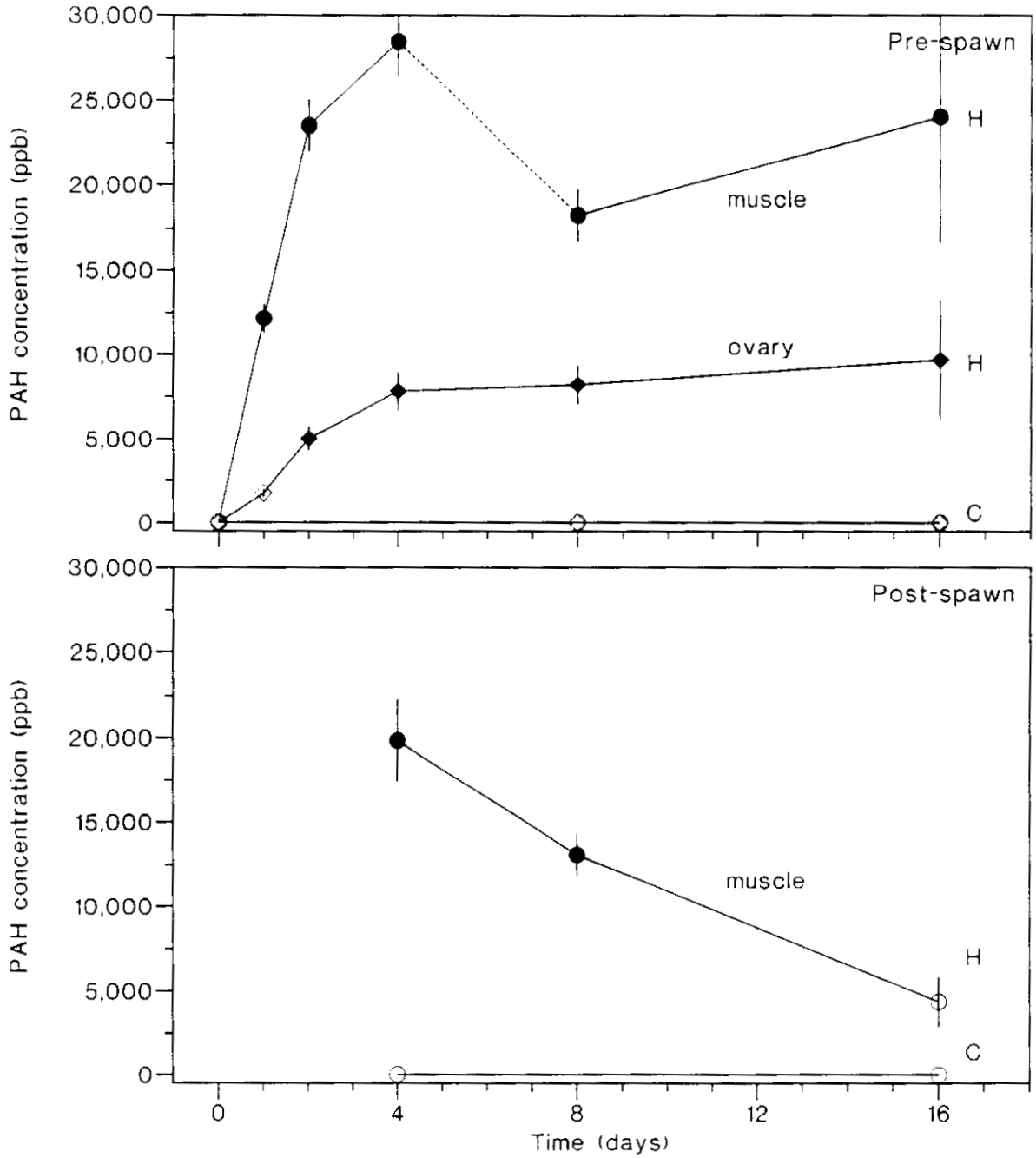

Figure 2.2. PAH concentrations in muscle and ovary of pre-spawn and muscle of post-spawn herring exposed to oil in water. Treatments were control (C) and high (H) oil. Data plotted are means ± SE. Filled symbols indicates significant difference from controls, $P \leq 0.05$.

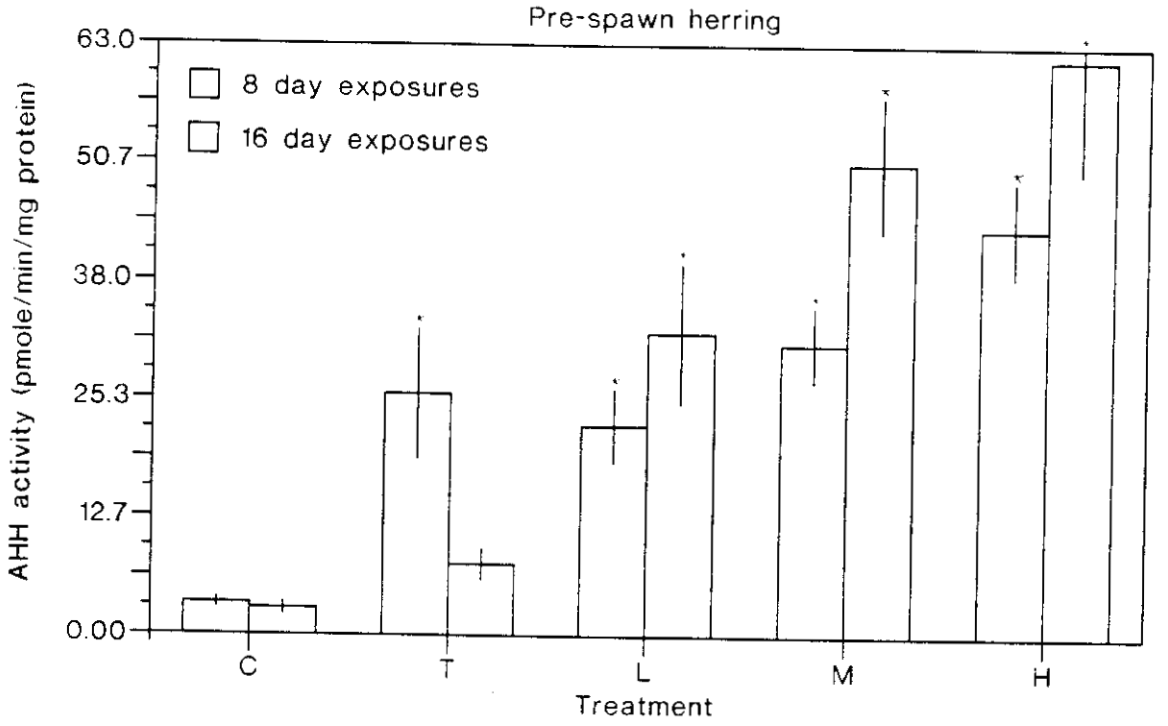

Figure 2.3. AHH activity in livers of pre-spawn herring as a function of oil treatment. Fish were exposed to oil for 8 or 16 d. Treatments were control (C), trace-oil (T), low-oil (L), mid-oil (M), and high-oil (H). Data plotted are means ± SE. * Indicates significant difference from controls. $P \leq 0.05$.

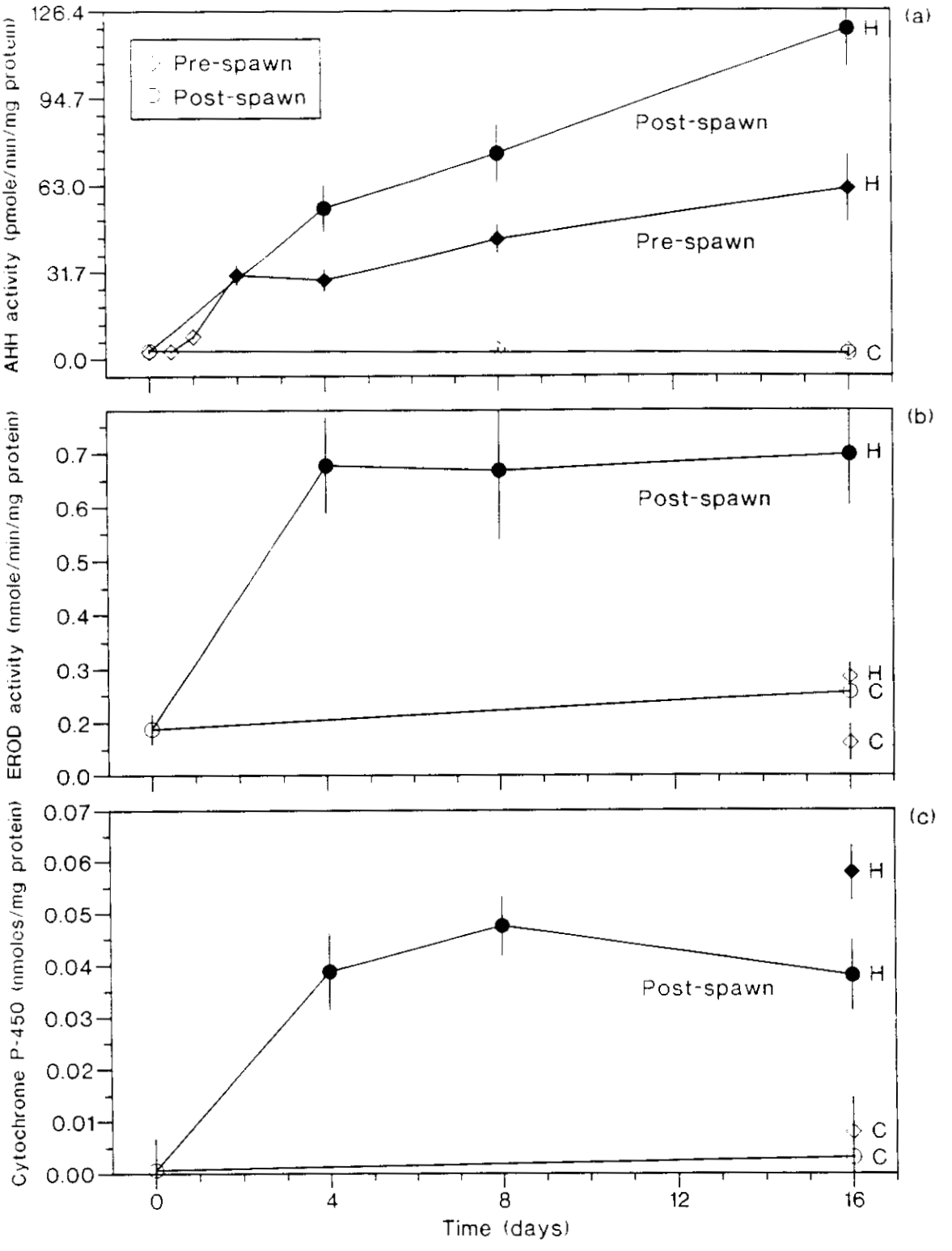

Figure 2.4. Induction of MFO in pre- and post-spawn adult herring. a) AHH activity in livers as a function of time; b) EROD activity and c) cytochrome P-450 concentration in pre- and post-spawn herring as a function of time. Treatments were control (C) and high (H) oil. Data plotted are means ± SE. Filled symbols indicates significance difference from controls, $P < 0.05$.

DISCUSSION

Induction of AHH and EROD activity in pre-spawn herring was one-third to one-half of that in post-spawn fish despite reduced exposure of post-spawn fish to hydrocarbons. Thus, pre-spawn fish are subject to greater tissue accumulation of hydrocarbons during oil exposure than post-spawn fish, because diminished MFO capacity can result in increased bioaccumulation. The greater retention of PAH, as measured in pre-spawn fish, strongly supports diminished MFO capacity. Excretion of hydrocarbons, as monitored by bile fluorescence, has been positively correlated to level of MFO induction (Collier and Varanasi 1991; Britvic' et al. 1993). 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 (Förlin and Hansson 1982; 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 has something to do with the energy cost of reproduction and the hormonal constituency and control of the reproductively ripe animals. For example, treatment of trout with estradiol reduces cytochrome P-450 content and some MFO activities, and content and activity of P4501A is positively correlated with estradiol in winter flounder (Hansson and Gustafson 1981; Förlin and Hansson 1982; Vodicnik and Lech 1983; Elskus 1992).

The diminished capability of pre-spawn herring to induce MFO enzymes, compared to post-spawn fish was undoubtedly caused by their different physiological states. Estrogens, which generally reach higher circulating levels in pre-spawning fish, directly suppress P4501A (Hansson and Gustafson 1981; Förlin and Hansson 1982; Vodicnik and Lech 1983; Elskus 1992). Also, pre-spawn fish were generally not feeding and the primary metabolic demand was gonad maturation. In contrast, post-spawn fish were actively feeding and converting much of their absorbed energy into somatic growth and fat reserves. In the post-spawn fish, enzyme induction was not limited by the energy demands of reproduction. Additionally, there was a normal seasonal temperature difference between the two tests of about 3°C, which likely also influenced metabolic rates, but this contribution was probably minor compared to the impact of different physiological states.

Although we generally did not find a significant difference in AHH induction between males and females, there were apparent differences. This was most evident when pre- and post-spawn results were compared by gender; females showed significant pre-reproductive depression of AHH activity, but males did not. These differences were likely due to gender-specific differences in hormonal constituency, and possibly lipid distribution. However, we did not measure hormone and lipid content, and do not know specifically how they relate to the reproductive cycle. Possible gender-specific differences in hydrocarbon accumulation might also be a factor, but only female tissues were analyzed in pre-spawn tests. Although the relationship might be different in post-spawn fish, differences in hydrocarbon accumulation in were not apparent between sexes.

Oil exposures in our tests were low, a few parts per billion, but were sufficient to cause bioaccumulation of PAHs and induce MFO enzymes. Test concentrations bracketed concentrations measured after the spill in PWS (Short and Harris 1996), and the PAH composition was similar to that observed in PWS. Over the 16 d test period, composition of the water accommodated fraction shifted from naphthalene dominated toward phenanthrene dominated, mimicking weathering patterns observed in PWS (Short and Heintz In press).

Bioaccumulation and retention of PAH was greater in the pre-spawn fish with diminished MFO capacity as compared to post-spawn fish. Bioaccumulation was significant, and reached 500 times the water concentration (up to 30,000 ppb in muscle tissue). Bioaccumulation and retention were significant in ovary tissue as well, although not as high as in muscle tissue. Composition of PAH hydrocarbons in tissues was similar to that in water. Accumulation of hydrocarbons in earlier studies with pre-spawn herring demonstrated similar patterns of uptake (Rice et al. 1987), although the accumulation of the unweathered oil (Cook Inlet crude) was significantly greater. In the previous study (Rice et al. 1987), herring were exposed mostly to mono- and di-aromatic hydrocarbons; in our tests with weathered oil, mono-aromatics were mostly absent and water-accommodated PAHs ranged from di-aromatics upwards through chrysenes.

All exposure concentrations were sufficient to induce the MFO system in both groups of fish after at least 8 d of exposure, including the lowest PAH concentration of about 3 ppb (initial aqueous concentration). Induction of this enzyme system could have both beneficial and harmful effects. Cytochrome P-450 dependent MFOs can make hydrocarbons more polar and thus more excretable (Jimenez and Stegeman 1990; Collier and Varanasi 1991; Britvic' et al. 1993), resulting in less exposure to toxic parent hydrocarbons. However, metabolism may also result temporarily in increased toxicity of PAH metabolites (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). The MFO system may therefore compromise the health of fishes in contaminated environments while concurrently preventing tissue hydrocarbon accumulation. Activation of carcinogenic PAH compounds could well explain the negative correlation between reproductive success and AHH activity noted in flatfish (Spies and Rice 1988; Johnson et al. 1988). 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 et al. 1983). In a companion to this study however, no notable toxic effects on eggs of oil exposed pre-spawn herring were noted (Carls et al. Chapter 3).

The effect of exposure of pre-spawn herring adults to weathered crude oil was probably more significant to mature fish than to their progeny. Eggs and larvae are vulnerable to direct exposures to oil, as evidenced by observations in the laboratory (Linden 1978; Pearson et al. 1985; Carls 1987; Kocan et al. 1987; Rice et al. 1987; Kocan et al. 1996) and field (Omura and Sato 1964; Brown et al. 1996; Kocan et al. 1996), but there is neither field nor laboratory evidence that eggs or larvae are harmed when herring adults are exposed to oil. In a companion

to the current study, measurement of egg and larval survival, larval abnormalities, and several other parameters for 40 d after exposure of adult fish indicated that the progeny were not impacted by exposure (Carls et al. Chapter 3). However, interaction of activated compounds at the macromolecular level and subsequent effects showing up at much later time intervals cannot be ruled out. In contrast to gamete exposure as a result of exposure of pre-spawn fish to oil, adult herring are apparently vulnerable to oil exposure. Exposure concentrations were below lethal levels, but oil exposed fish were more vulnerable to disease (Carls et al. Chapter 1). Disease lesions were prevalent in the higher exposures, even though exposure concentrations were in the ppb range. These results will be reported in a subsequent article. In PWS , the herring population collapsed, and the collapse is credited to disease (Brown et al. 1996). While the linkage of the oil spill to disease is very speculative, particularly considering the time between events, the cause and effect relationship is worrisome. The relationship between oil, MFO induction, immune system depression, and disease is unknown. However, it is clear that pre-spawning herring have diminished capacity to induce MFOs, and a greater potential for hydrocarbon accumulation than post-spawn fish.

CONCLUSIONS

PAHs accumulated in muscle tissue of pre- and post-spawn herring. Pre-spawn herring accumulated higher concentrations of hydrocarbons and retained them longer.

Mixed function enzymes were induced by exposure to hydrocarbons. Induction in pre-spawn fish was one third to one-half that in post-spawn fish.

Inability of pre-spawn herring to induce MFOs at as high a level as post-spawn fish may explain higher tissue concentrations and retention of hydrocarbons in the pre-spawn animals.

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Chapter 3: The impact of exposure of adult pre-spawn herring (*Clupea pallasi*) to oil on progeny compared to that of direct exposure of eggs to oil during incubation

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ABSTRACT

Reproductively ripe Pacific herring were exposed to water contaminated with weathered crude oil to determine if exposure could cause morphological and genetic effects in progeny. Herring were exposed to oil concentrations in the range encountered in open water of Prince William Sound (PWS) following the *Exxon Valdez* oil spill (6 ppb) and beyond (initial polynuclear aromatic hydrocarbon (PAH) concentrations ranged up to 58 ppb). Hydrocarbons accumulated in ovarian tissue, and similar concentrations were observed in newly spawned eggs. There were, with one possible exception, no discernible effects in progeny. The exception, an increase in yolk volume, was not reflected in other parameters that should have also responded if there were a true treatment effect, and the change in volume was about one-third of an increase known to be related to oil exposure. In sharp contrast to the lack of response observed in offspring as a result of adult exposure, direct exposure of herring eggs to oil during incubation was detrimental to developing embryos at low concentrations (9 ppb). Many of the parameters observed in this study were sensitive measures of response in direct exposure tests, including egg death, hatch timing, larval death, spinal defects, yolk-sac edema, larval swimming, larval length, stage of larval development at hatch, and chromosomal abnormalities. Differences in toxicant composition in egg tissue may, in part, explain the radically different responses between types of exposure. Hydrocarbon accumulation was strongly biased toward naphthalenes in eggs from exposed adults, but greater percentages of larger ring compounds and alkyl-substituted homologs were present in directly exposed eggs. Mechanisms leading to the abnormal herring larvae in PWS in 1989; therefore, were probably due to exposure of eggs during incubation (or possibly exposure of larvae to oil), and not a consequence of adult herring encountering oil. Our results do not exclude the possibility of longer-term damage to progeny of contaminated adults, but hypothetical mechanisms for delayed effects are difficult to devise. Genetic damage was not detected in somatic tissue of progeny, hence we predict it was unlikely in reproductive tissues.

INTRODUCTION

Pacific herring typically migrate from offshore feeding grounds to nearshore areas prior to spawning; the *Exxon Valdez* oil spill in PWS occurred just prior to this migration (Brown et al. 1996). Thus, most or all of the life stages of herring in PWS may have been exposed to oil after the 1989 spill. About half the spawned biomass was exposed to oil (Brown et al. 1996), and elevated levels of abnormalities and chromosome breakage were observed in newly hatched larvae (Hose et al. 1996). Biologically available hydrocarbons from the *Exxon Valdez* oil spill were generally pervasive in the upper water column along the spill trajectory inside PWS during

the summer of 1989 (Short and Harris 1996). Thus, the potential for exposure of herring eggs and larvae persisted for several months after the spill (Brown et al. 1996), and there was evidence of adverse effects in the planktonic larvae (Norcross et al. 1996). The low recruitment of the 1989 year class to the fishery in 1992 and 1993 (Brown et al. 1996) suggested, but did not prove, a possible population effect.

Herring populations may be adversely impacted by exposure to petroleum hydrocarbons. Because 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 causes significant mortality or genetic damage may have a negative impact on populations. 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. 1996). 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. 1996). It is not clear, however, if exposure of adult herring to hydrocarbons leads to genetically damaged progeny, and if genetic damage occurs in the germ line where it can affect subsequent generations.

The purpose of this study was to determine if exposure of pre-spawning adult herring to petroleum hydrocarbons could cause genetic and morphological damage in larvae. Genetic damage was assessed by observation of anaphase-telophase aberration rates in the pectoral fins of newly hatched larvae. We anticipated that morphological damage might co-occur with genetic damage, and that size at hatch and maturation could be influenced by treatment. Accordingly, morphological data included yolk-sac and pericardial edema, and spinal abnormalities; size data included total larval length and yolk volume; developmental data included jaw maturation and stage at hatch. Effects of exposure on gametes were judged by fertilization success, egg mortality, and hatch timing, and hatching success. We recognize that egg mortality, hatch timing, and hatch success effects could also be due to exposure of embryonic tissue to previously sequestered hydrocarbons as lipid reserves are metabolized.

A similar study was conducted in 1985 (Rice et al. 1987) to search for effects in progeny of oil-exposed pre-spawn herring. There were two major differences in our study and the preceding study. First, we exposed fish to water containing PAH ranging from naphthalenes through chrysenes; the earlier study exposed fish to mono- and di-aromatic hydrocarbons. Thus we anticipated that our exposures, which mimicked spill conditions in PWS, might be potentially more toxic to progeny. Second, a wider range of biological responses was examined, including morphological and genetic assessment.

Hydrocarbon concentrations and composition were determined in treatment water, ovaries, and in subsequently spawned eggs. Aliphatic and aromatic hydrocarbon data were collected, but because it is likely that the aromatic fraction accounts for most toxic effects, we present only the PAH data in detail. Water in this experiment was contaminated via contact with rock coated with weathered Alaska North Slope crude oil. The composition of PAH in water

produced by our method closely mimicked that observed in PWS with naphthalenes through chrysenes represented.

We report the effect of exposure of reproductively ripe, pre-spawn herring to oil on gametes and progeny, and compare responses to those where eggs were directly exposed to oil-contaminated water during incubation. In companion studies we report the effects of oil exposure on adult herring (Carls, et al. Chapter 1; Thomas et al. 1997) and the effects of direct exposure to oil of incubating herring eggs (Carls et al. Chapter 4), thus we detail only the pre-spawn experiment and compare pre-spawn and direct exposure results in the discussion. In the discussion we demonstrate that PAH concentrations and composition in treatment water to which fish or eggs were exposed were very similar. For the purpose of interpreting and comparing pre-spawn and direct exposure results, direct exposure results have been included in several graphs.

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 L holding tank; 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 were gravid but not ripe when captured. Ripeness was judged from percent gonad to total body weight to total body weight (gonosomatic index) 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, the gonosomatic index increased significantly from the time of capture through May 12, 1994 ($P < 0.001$). Fish were artificially spawned on May 2 to 4, and again on May 10 to 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 (A. McGregor, Personal communication)). Volitional spawning was observed in captive fish as early as April 11.

There were five treatments, including the control, with three replicates per treatment. Water for each replicate tank flowed at 6.9 L/min and 4.2°C through independent cylinders containing rock or oiled rock (Appendix 1.1). Healthy pre-spawn herring were randomly distributed among fifteen 700 L treatment tanks (50 fish per tank). To characterize the oil and quantify treatment concentrations, composite water samples (1.27 L from each of three replicate tanks) were collected on days 0, 8, and 16 for analysis by gas chromatography according to the methods of Short et al. (1996). Mortality and dissolved oxygen were monitored once per day in each tank. 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 8 and 16 d exposure, fish were artificially spawned. On day 8, 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 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. Additional eggs from multiple contributing females in each replicate high-oil treatment tank were deposited in 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. Tissue hydrocarbons were analyzed according to the methods of Short et al. (1996).

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. Indirect natural light entered through windows along one side; direct sunlight could enter only briefly at very low sun angles. At each location eggs were incubated, light levels were measured with a GE type 213 meter approximately 1.5 h after local noon on July 5, 1994; the sky was overcast. The ultraviolet contribution was estimated by placing a photographic UV filter (Tristar number 8730) over the light meter and recording the difference in light intensity. Light intensity ranged from 4 to 68 footcandles (mean 18 ± 2.0 footcandles). The estimated ultraviolet energy was 13% of the total light energy.

Eggs were examined for fertilization success and development 1 to 10 d after fertilization. Eggs susceptible to mechanical damage (those along slide margins), clumped eggs, and excess

eggs were removed from all slides by scraping. 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. After processing, the average number of eggs per slide was 126.

Before hatch, a randomly selected slide of eggs from each female was isolated in a 1 L glass jar. 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 was monitored daily, salinity weekly.

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. Dead larvae were discarded. 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, 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 (for 8 and 16 d exposures, respectively) that encompassed the majority of the hatch. Roughly 50 larvae were measured per fish.

Edema and jaw size were scored from digital images. Yolk-sac edema was indicated if the anterior margin of the yolk membrane was bounded by an area of clear fluid. Pericardial edema was noted when the pericardial area was convex ventrally or had an unusually large area. Jaws were classified as small if absent or posterior to the anterior margin of the eye.

Genetic and additional morphologic responses were evaluated using blind review. A maximum of 15 females were randomly selected from control and high-oil treatments, and 10 stage 1b larvae (Humphrey et al. 1995) 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. The severity of skeletal, craniofacial, and finfold defects was scored using the graduated severity index (GSI) method described in Hose et al. (1996), where 0 = no effect, 1 = slight defect in

structure or size, 2 = moderate defect in structure or size, and 3 = severe defect in structure or size.

Data processing and statistics

Hydrocarbon data were processed according to the methods of Short et al. (1996) before further analysis. Hydrocarbon concentrations were accepted only if corresponding recovery of deuterated internal standards ranged from 30 to 150%; records with unacceptable recoveries were deleted (3% of the data). In addition, concentrations below minimum detection limits were set to zero (Short et al. 1996).

Composition of individual PAH and homologous chemical groups (naphthalenes, fluorenes, dibenzothiophenes, phenanthrenes, and chrysenes) was expressed as percent of total PAH. Composition of PAH as functions of initial PAH concentration and time was modeled with linear regression techniques; independent variables (initial PAH concentration or time) were transformed along ladder of power series to determine best fit (Velleman and Hoaglin 1981). Biological responses were regressed against $\log_e$ (initial PAH concentration).

To compare PAH composition in water, ovaries, and eggs, concentrations were first divided by total PAH to yield percentage vector **X**. A new vector, $\mathbf{y} = (y_1, y_2, \ldots y_{d-1})$, where d = *number of compounds*, was defined as:

$$y_i = \log\left(\frac{x_i}{x_d}\right) \qquad i = 1,2,\ldots d-1$$

Vector **y** was analyzed as though it were a multivariate normal vector (Aitchison 1986); multivariate analysis of variance (MANOVA) was used to test for differences in PAH composition among treatments, tissue, and time. Because MANOVA required the presence of all compounds in the vector (i.e., no $x_i = 0$ allowed), the number of PAH was reduced from 39 to 22 for this test. (Compounds that remained in multivariate tests are identified with an asterisk in Table 3.1). Although data reduction eliminated many zero values, some remained, so the remaining data were shifted upward 0.001 units before calculation of the y-vector; this translation was approximately an order of magnitude smaller than the smallest non-zero values present in the data set. Biphenyl was present in every sample, and was chosen as the denominator (x_d). *Distributions of transformed compounds did not, in general, appear to be normal*, so significance of the MANOVA result was evaluated by randomization. To randomize, tissue type was randomly reassigned to each sample, and the MANOVA statistic (Wilks' Lambda) was recomputed 100 times. The observed statistic was compared with its empirical null distribution to assess its probability under the null hypothesis.

Table 3.1. PAH identified by chemical analysis, and the abbreviations used in figures. Asterisks indicate which compounds were accepted for multivariate analysis (see text). No compounds heavier than C4-chrysene were detected in water or tissue samples.

Abbreviation	Name
NPH*	naphthalene
C1NPH*	C-1 naphthalenes
C2NPH*	C-2 naphthalenes
C3NPH*	C-3 naphthalenes
C4NPH*	C-4 naphthalenes
BPH*	biphenyl
ACY	acenaphthylene
ACE*	acenaphthene
FLU*	fluorene
C1FLU*	C-1 fluorenes
C2FLU*	C-2 fluorenes
C3FLU*	C-3 fluorenes
DBT*	dibenzothiophene
C1DBT*	C-1 dibenzothiophenes
C2DBT*	C-2 dibenzothiophenes
C3DBT*	C-3 dibenzothiophenes
PHN*	phenanthrene
C1PHN*	C-1 phenanthrenes/anthracenes
C2PHN*	C-2 phenanthrenes/anthracenes
C3PHN*	C-3 phenanthrenes/anthracenes
C4PHN*	C-4 phenanthrenes/anthracenes
ANT*	anthracene
FLA	fluoranthene
PYR	pyrene
C1FLA	C-1 fluoranthenes/pyrenes
BAA	benz-a-anthracene
CHR*	chrysene
C1CHR	C-1 chrysenes
C2CHR	C-2 chrysenes
C3CHR	C-3 chrysenes
C4CHR	C-4 chrysenes
BbF	benzo-b-fluoranthene
BkF	benzo-k-fluoranthene
BEP	benzo-e-pyrene
BAP	benzo-a-pyrene
PER	perylene
IDP	indeno-123-cd-pyrene
DBA	dibenzo-a,h-anthracene
BZP	benzo-g,h,i-perylene

To augment the multivariate analysis of PAH composition, unsubstituted fluorenes, dibenzothiophenes, and phenanthrenes in tissue were independently compared. Each compound within its respective homologous chemical series was normalized to the sum of that particular homolog (e.g., fluorene/sum fluorenes) and analyzed with two-way analysis of variance (ANOVA) (tissue and time). Variation in composition among treatments was small enough to ignore in this test.

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.

The denominator used to calculate percentages varied 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 effective, ineffective, or incapable. Swimming ability of moribund and dead larvae was, by definition, nonexistent, thus number 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 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 arcsin transformed, and corrected for small n where necessary (Snedecor and Cochran 1980). [The same conclusions were reached with untransformed data]. Scored abnormality data (yolk-sac edema and jaw size) were recoded to yield incidence of abnormality (e.g., percent larvae with small jaw) then analyzed with ANOVA.

Because correlation of size (larval length and yolk volume) and time was generally negligible or inconsistent, time was not included as a factor in final size analyses. 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). Larval length and yolk volume were analyzed with ANOVA with replicate nested in treatment and fish nested in replicate and treatment; size measurements were considered to be random variables.

Graduated severity index 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 3.2). 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 ($0.245 \leq P \leq 0.494$).

Table 3.2. Reproductive condition of adult herring at time of artificial spawning. Data reported are least squares adjusted means and 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	

Hydrocarbon exposure, accumulation, and composition

Initial PAH concentrations in water ranged from 0.03 (control) to 58.3 ppb (Figure 3.1). Concentrations in treatment water declined during the 16 d exposure period, but concentration declines were similar in all treatments, thus treatment conditions remained clearly separable (Figure 3.1).

Composition of PAH at the outset of the experiment varied as a function of initial PAH concentration in water. For example, on day 0 naphthalenes comprised 65% of the PAH in water in the trace-oil treatment, but comprised 87% in the high-oil treatment (Figure 3.2 and Appendix 3.1). Initial percentages of naphthalene increased significantly as a function of initial PAH concentration ($r^2_{day\,0} > 0.99$, $P = 0.001$) (Figure 3.2). Conversely, percentages of fluorenes, dibenzothiophenes, phenanthrenes, and chrysenes declined significantly as a function of initial PAH concentration ($r^2_{day\,0} \geq 0.99$, $P \leq 0.006$) (Figure 3.2).

The composition of PAH in treatment water also changed as a function of time. Percent naphthalenes declined significantly with time ($P \leq 0.043$); percentages of fluorenes, dibenzothiophenes, phenanthrenes, and chrysenes increased with time ($0.014 \leq P \leq 0.055$) (Figure 3.3). Rates of change over time differed among treatments, and best-fit models varied, consistently suggesting initial rate of change was more rapid in the low-oil treatment, but becoming more asymptotic than in the high-oil treatment (Figure 3.3). Composition changes in the trace-oil treatment were not analyzed because of marginal analyte detectability.

Ovaries of fish exposed to oil accumulated PAH to a maximum mean of 9,688 ppb in the high-oil treatment (Figure 3.1). Concentrations of PAH in tissues were correlated with water concentrations ($0.86 \leq r \leq 0.97$, $P < 0.001$; $x = PAH^2$). Bioconcentration was difficult to estimate because of declining treatment concentrations. In the highest oil treatment, estimated bioconcentration (PAH_t / PAH_w, where PAH_t was concentration in ovary, and PAH_w was concentration in water) was 317 times (range 166 to 800 times), estimated by varying PAH_w (mean, initial, and 16 d concentrations, respectively). Bioconcentration varied as a function of treatment, and was substantially lower in the trace-oil treatment (40 times).

Composition of PAH hydrocarbons in ovaries of fish exposed to oil differed significantly from that in water ($P_{MANOVA} < 0.01$) (Figure 3.4; see also Appendices 3.1 and 3.2). Naphthalenes preferentially accumulated in ovaries; C1-naphthalenes were usually dominant, and C2-naphthalenes were usually next in abundance (Appendix 3.3). Relatively few compounds heavier than biphenyl were detected in ovaries (2-13%); chrysenes and heavier PAH were never detected. Of the heavy compounds present, unsubstituted fluorene, dibenzothiophene, and phenanthrene homologs were dominant in ovarian tissue, and highly substituted homologs were under-represented or not present. In contrast to the time-dependent composition shifts observed in water, percentages of naphthalenes in ovary tended to be the same or slightly higher on day 16 than on day 8 (Appendix 3.3). Not only were percentages of naphthalenes in ovaries greater than

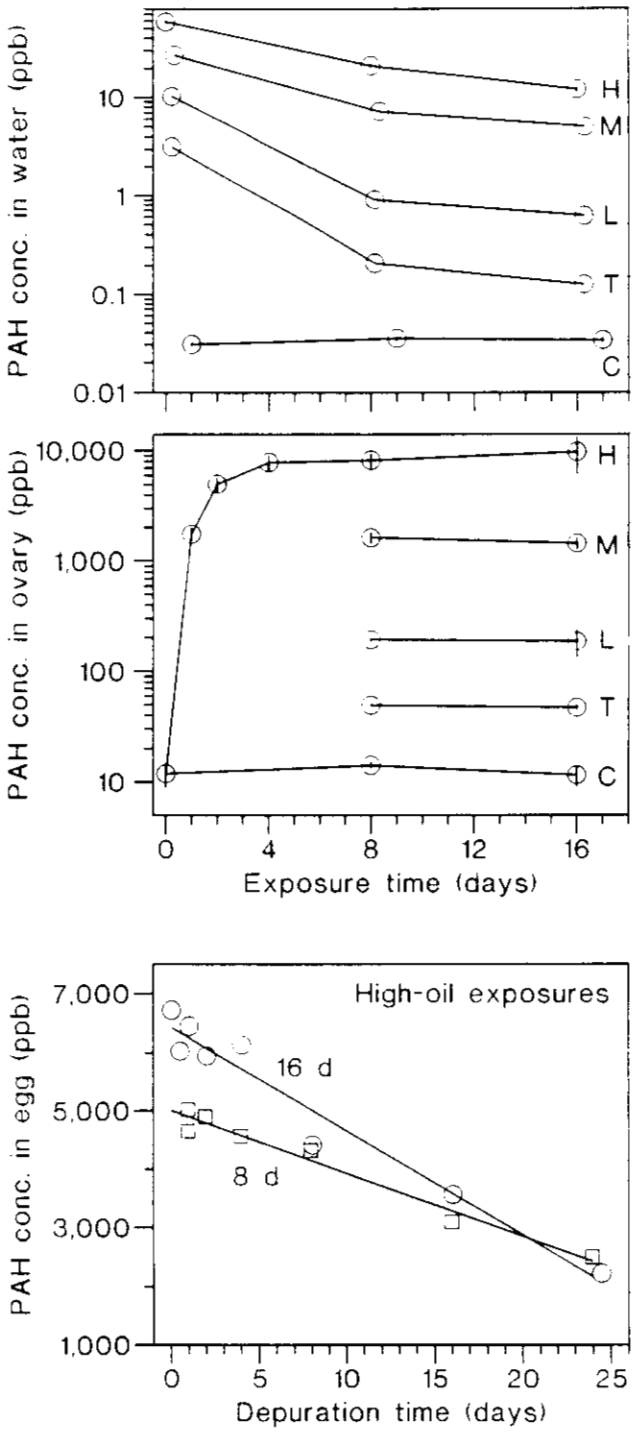

Figure 3.1. Mean PAH concentrations in water, ovaries, and in high-oil treatment eggs as functions of time (± SE).

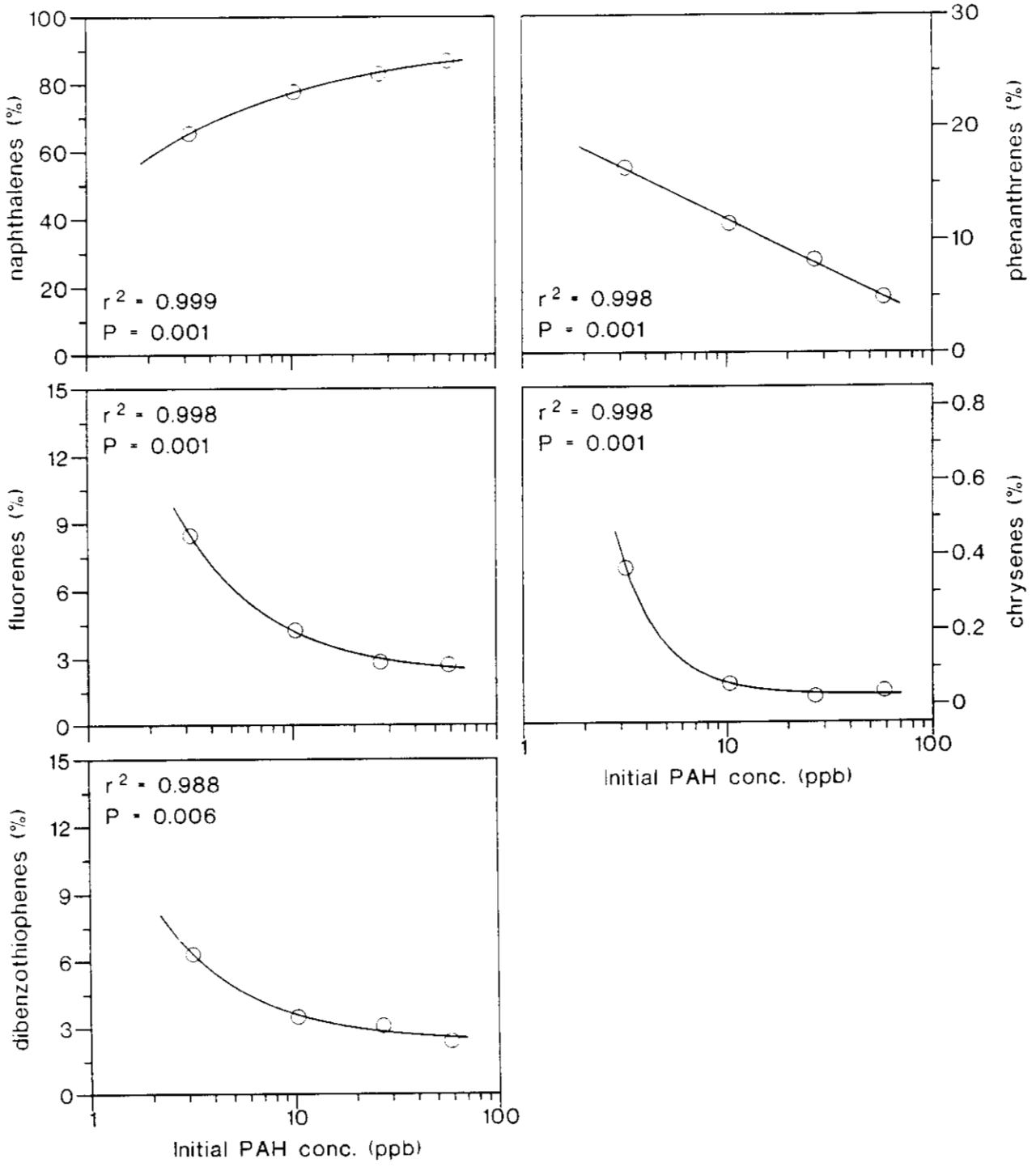

Figure 3.2. PAH composition in water varied as a function of initial PAH concentration in water. There were relatively fewer naphthalenes and more heavy compounds in the lower treatments. Plotted lines are model fits; x-axis transforms were $-1/\sqrt{PAH}$, $-1/PAH$, $-1/PAH$, $\ln(PAH)$, and $-1/PAH^2$, for naphthalenes, fluorenes, dibenzothiophenes, phenanthrenes, and chrysenes, respectively.

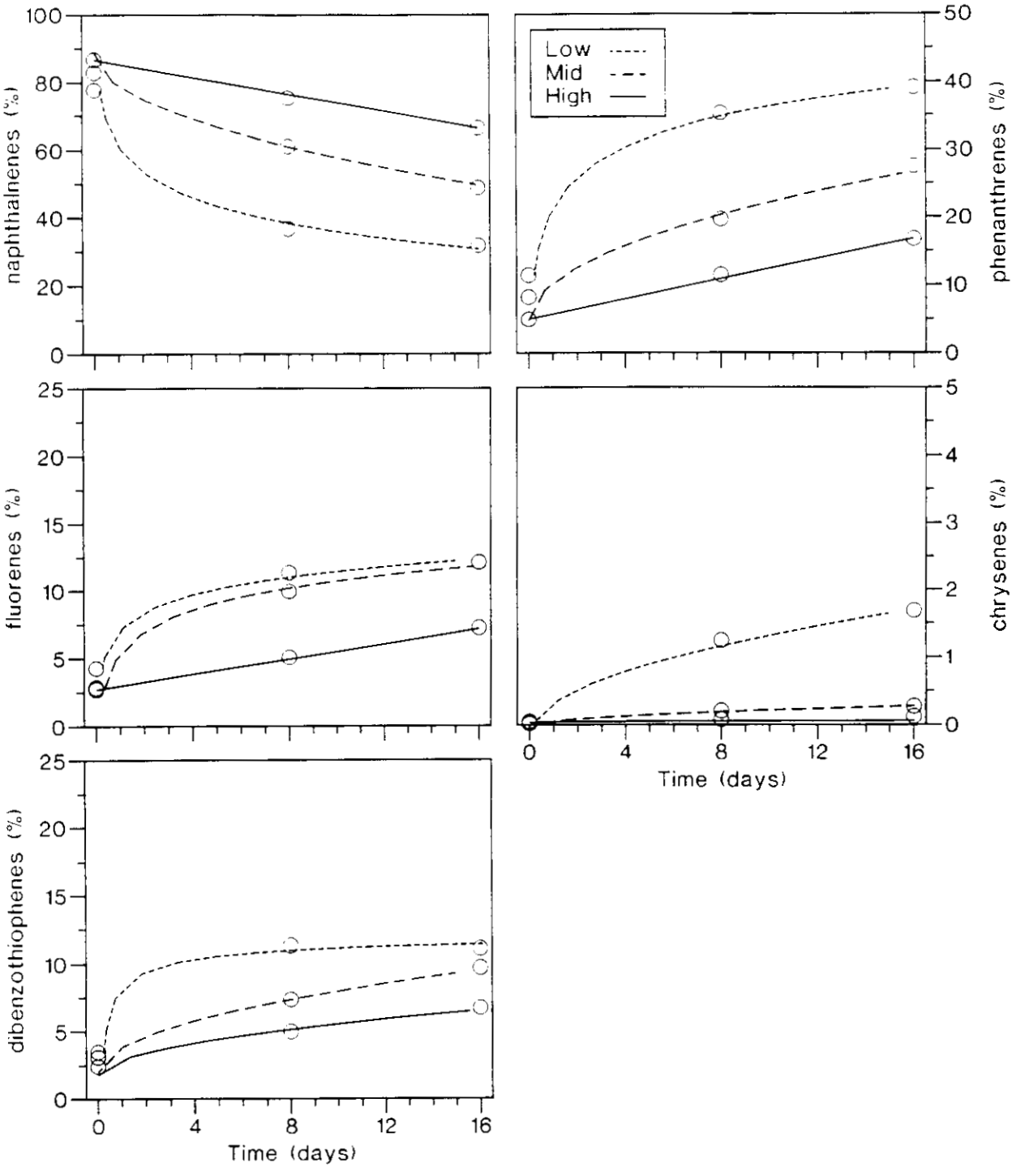

Figure 3.3. Composition of PAHs varied as a function of time. Percentages of naphthalenes declined significantly; percentages of heavier compounds increased. To avoid minimum detection limit artifacts, trace-oil concentrations were not included.

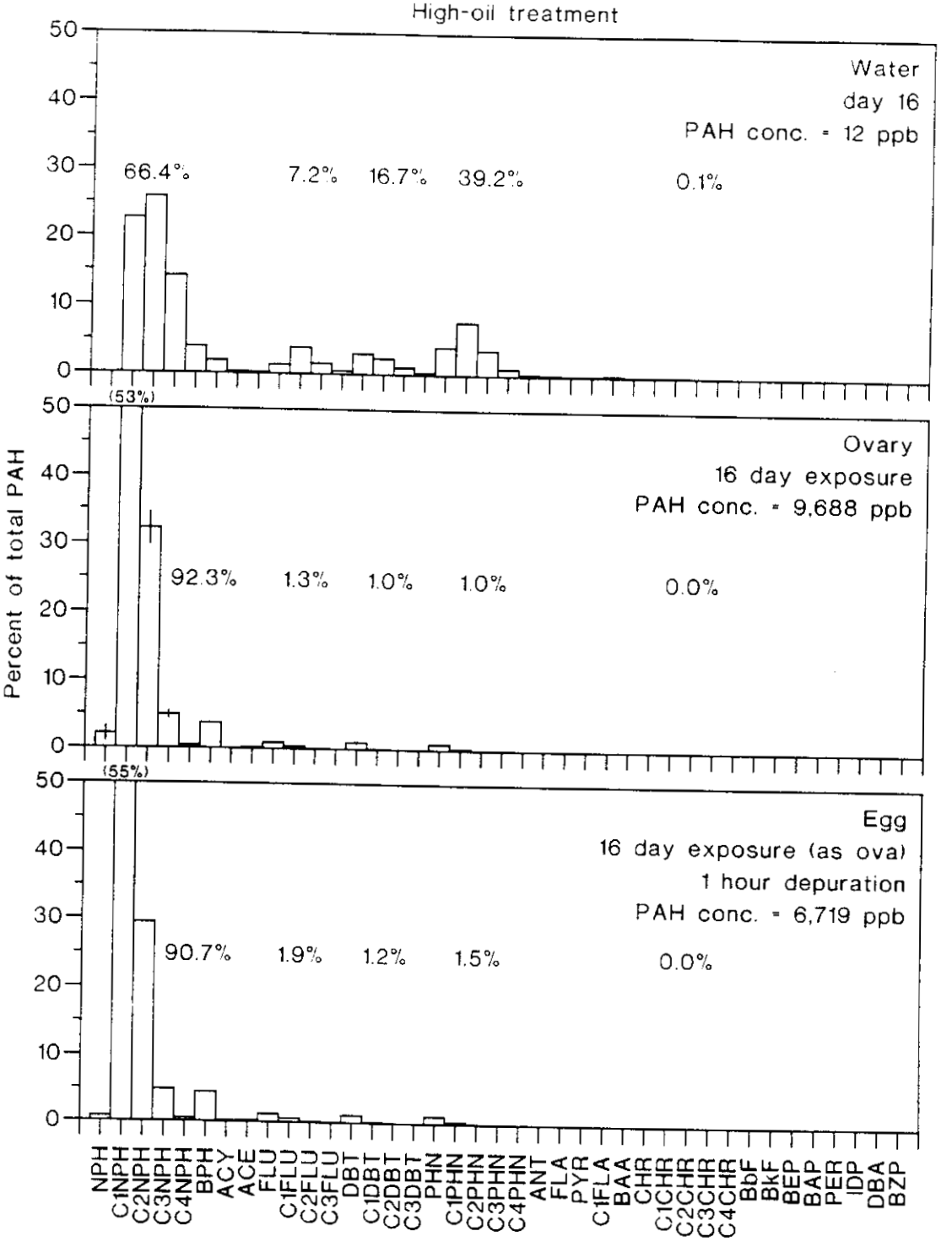

Figure 3.4. Composition of PAHs in ovaries and eggs compared to that in treatment water. There were relatively more naphthalenes in tissue than in water. This example represents the high-oil treatment after 16 d exposure. Percentages printed inside the graphs indicate total percentages of the following homologous chemical groups (left to right): naphthalenes, fluorenes, dibenzothiophenes, phenanthrenes, and chrysenes.

those in water sampled at the same time, but also consistently exceeded percentages of naphthalenes in initial water samples (see Appendices 3.1 to 3.3).

Shortly after fertilization, hydrocarbon concentrations in eggs were very similar to concentrations observed in ovaries, but declined linearly during incubation in clean seawater (r^2 = 0.97 and r^2 = 0.96 for 8 and 16 d exposures, respectively) (Figure 3.1). After 24 d, PAH concentrations in high-oil treatment eggs (2,359 ± 138 ppb) remained significantly elevated above baseline concentrations (11 ± 1 ppb, measured in control ovaries) ($P < 0.001$).

Composition of PAH hydrocarbons in eggs of fish exposed to oil did not differ significantly from composition in ovaries (P_{MANOVA} = 0.388) (Figure 3.4). Composition in eggs appeared to be fairly constant; evidence of time-dependent change was equivocal ($0.36 \leq r^2 \leq 0.99$, $0.001 < P \leq 0.155$). Rates of composition change in tissue were significantly less than corresponding rates of change in water ($P < 0.001$). For example, after 16 d in the high-oil treatment, percentages of naphthalenes changed negligibly in eggs, but dropped 20% in water. As in ovaries, chrysenes were not detected in eggs.

Eggs and larvae

Egg fertility was not correlated with PAH concentration to which adult herring were exposed ($0.00 \leq r \leq 0.06$, $P_{regression} \geq 0.544$) (Figure 3.5). Fertility did not differ significantly among treatments in 8 d exposures (P_{ANOVA} = 0.331). In 16 d exposures, fertility varied significantly among treatments (P_{ANOVA} = 0.001), but not in a dose-dependent manner; mid- and high-oil treatments did not differ significantly from controls. Mean fertility was less in 8 d exposures than in 16 d exposures (93% and 95%, respectively), but variability was greater in the 16 d exposures.

The percentage of eggs that died was not correlated with PAH concentration to which adult herring were exposed ($-0.08 \leq r \leq 0.05$, $P_{regression} \geq 0.410$) (Figure 3.5). Egg death did not differ significantly among treatments ($P_{ANOVA} \geq 0.062$). Mean percentages of eggs that died were less than 11% in 8 d exposures and 20% in 16 d exposures. Significantly more eggs died in 16 d adult 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 ($-0.12 \leq r \leq 0.17$, $P_{regression} \geq 0.171$) (Figure 3.6). In 8 d exposures, mean peak hatch and median hatch were 31 d, and were not affected by treatment ($P_{ANOVA} \geq 0.791$). In 16 d exposures, mean peak and median hatch times ranged from 28 to 30 d and varied significantly among treatments ($P < 0.001$), but significant differences did not appear to be dose-related (Figure 3.6). For example, median hatch time was significantly late in trace-, low-, and mid-oil treatments, but not in the high-oil treatment.

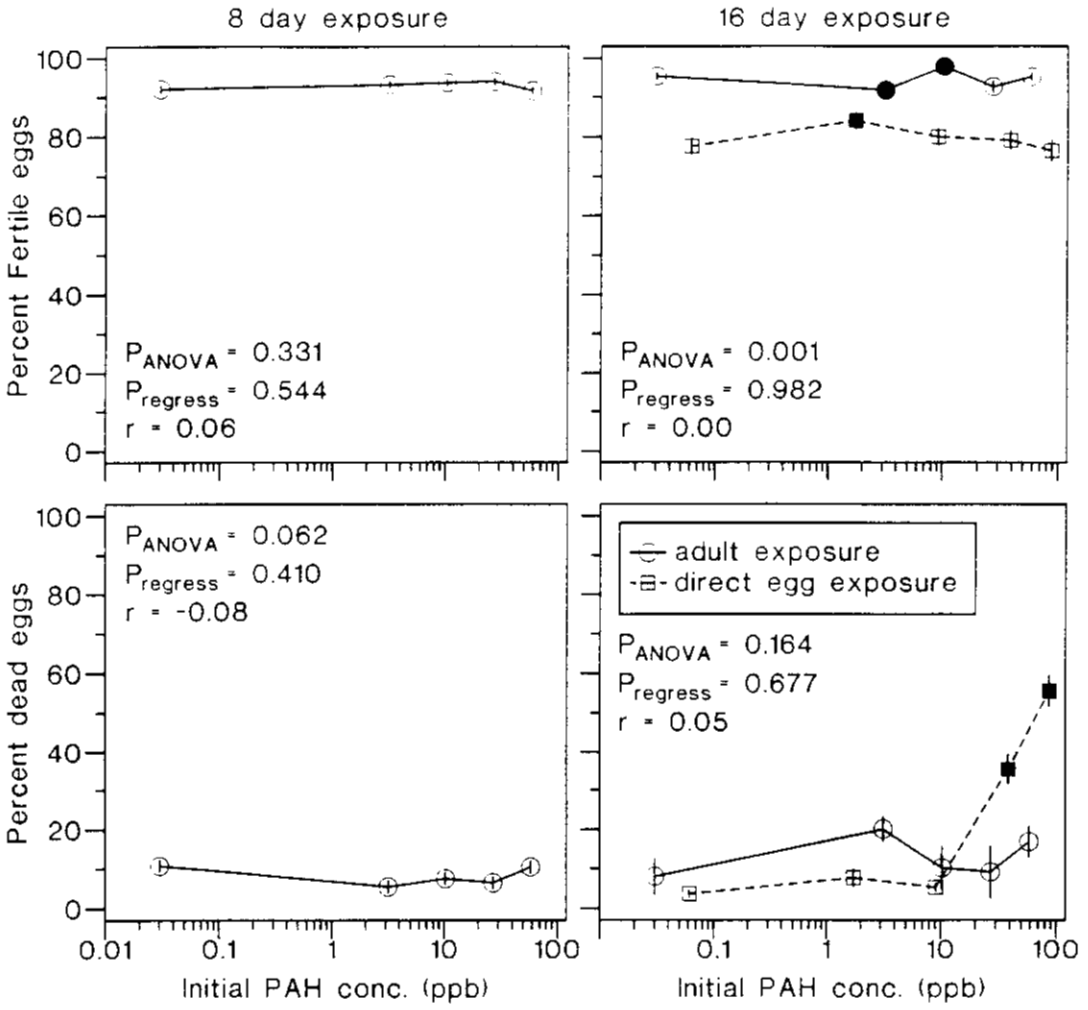

Figure 3.5. Fertility and death of eggs spawned from adult herring exposed to oil 8 or 16 d as functions of initial PAH. Included for comparison are responses of eggs incubated in similarly contaminated water for 16 d. P_{ANOVA} is probability of overall treatment effect estimated by ANOVA; solid symbols indicate significant differences from controls. $P_{regress}$ reports probability of significant slope, estimated by regression (x = $\log_e$(concentration)), and r = correlation coefficient. Data displayed are least squares adjusted means ± SE.

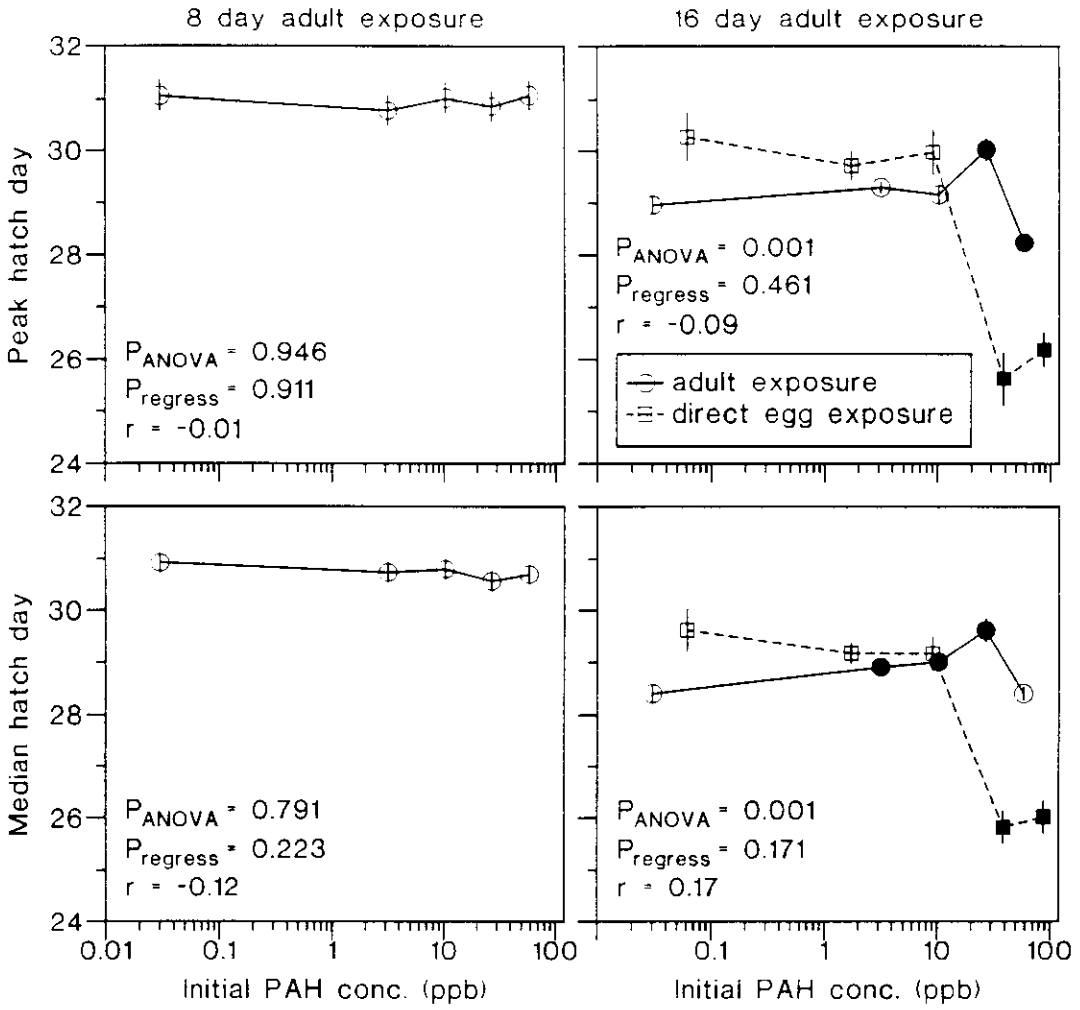

Figure 3.6. Peak and median hatch day of eggs spawned from adult herring exposed to oil 8 or 16 d as functions of initial PAH concentration. Included for comparison are responses of eggs incubated in similarly contaminated water for 16 d. P_{ANOVA} is probability of overall treatment effect estimated by ANOVA; solid symbols indicate significant differences from controls. $P_{regress}$ reports probability of significant slope, estimated by regression (x = $\log_e$(concentration)), and r = correlation coefficient. Data displayed are least squares adjusted means ± SE.

The percentage of larvae that were moribund or died within 1 d of hatch was not correlated with PAH concentration to which adult herring were exposed ($-0.05 \leq r \leq 0.01$, $P_{regression} \geq 0.596$) (Figure 3.7). Differences among treatments in percentages of moribund plus dead larvae were not significant ($P \geq 0.485$). Fewer larvae were moribund or dead in the 8 d exposure than in 16 d exposures ($\leq 2.1\%$ and $\leq 4.8\%$, respectively).

Exposure of adult herring to oil did not cause larval abnormalities. The percentage of larvae with spinal defects was not correlated with PAH concentration ($0.03 \leq r \leq 0.09$, $P_{regression} \geq 0.359$) (Figure 3.7). Differences in spinal defects among treatments were not significant ($P \geq 0.152$). The mean percentage of larvae with spinal defects did not exceed 1.3% and 1.1% in 8 and 16 d exposures, respectively (Figure 3.7). Incidence of larvae with yolk-sac edema (mean $\leq$ 2.9%) was not elevated in high-oil treatments ($0.109 \leq P \leq 0.608$) (Figure 3.8). Incidence of larvae with undeveloped lower jaws (mean $\leq$ 2.8%, $0.164 \leq P \leq 0.251$) was not elevated in high-oil treatments. Similarly, GSI scores did not indicate significant morphological response ($P_{Kruskal-Wallis} = 0.516$).

Larval swimming ability was not correlated with PAH concentration to which adult herring were exposed ($-0.04 \leq r \leq 0.03$, $P_{regression} \geq 0.742$) (Figure 3.7). Differences in swimming ability among treatments were not significant ($P \geq 0.097$). Swimming ability was better in 8 d exposures than in 16 d exposures: percentages of larvae classified as effective swimmers exceeded 97% and 90%, respectively.

Exposure of adult herring to oil did not affect progeny size, with one possible exception. Larval lengths in high-oil treatments did not differ significantly from those in controls ($P \geq 0.351$) (Figure 3.9). Yolk volumes in high-oil treatments did not differ significantly from those in controls in 8 d adult exposures ($P = 0.852$), but were significantly larger in 16 d exposures ($P = 0.034$).

Embryonic maturation was not influenced by exposure of adult herring to oil ($P \geq 0.229$) (Figure 3.9). The majority (62%) of newly hatched larvae were classified as stage 1b (Humphrey et al. 1995); 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 (Figure 3.10). The number of cells undergoing mitosis in pectoral fins did not differ significantly between control (18.5 ± 0.4) and high-oil 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 (Figure 3.10). Percent anaphase-telophase aberration was significantly greater in controls ($5\% \pm 1$) than in the high-oil treatment ($4\% \pm 1$) ($P = 0.032$, n = 134 larvae per treatment). Analysis was restricted to control and high-oil treatment larvae. Based on the 8 d results, and lack of response in other 16 d measurements, larvae from 16 d exposures were not analyzed for chromosomal aberrations.

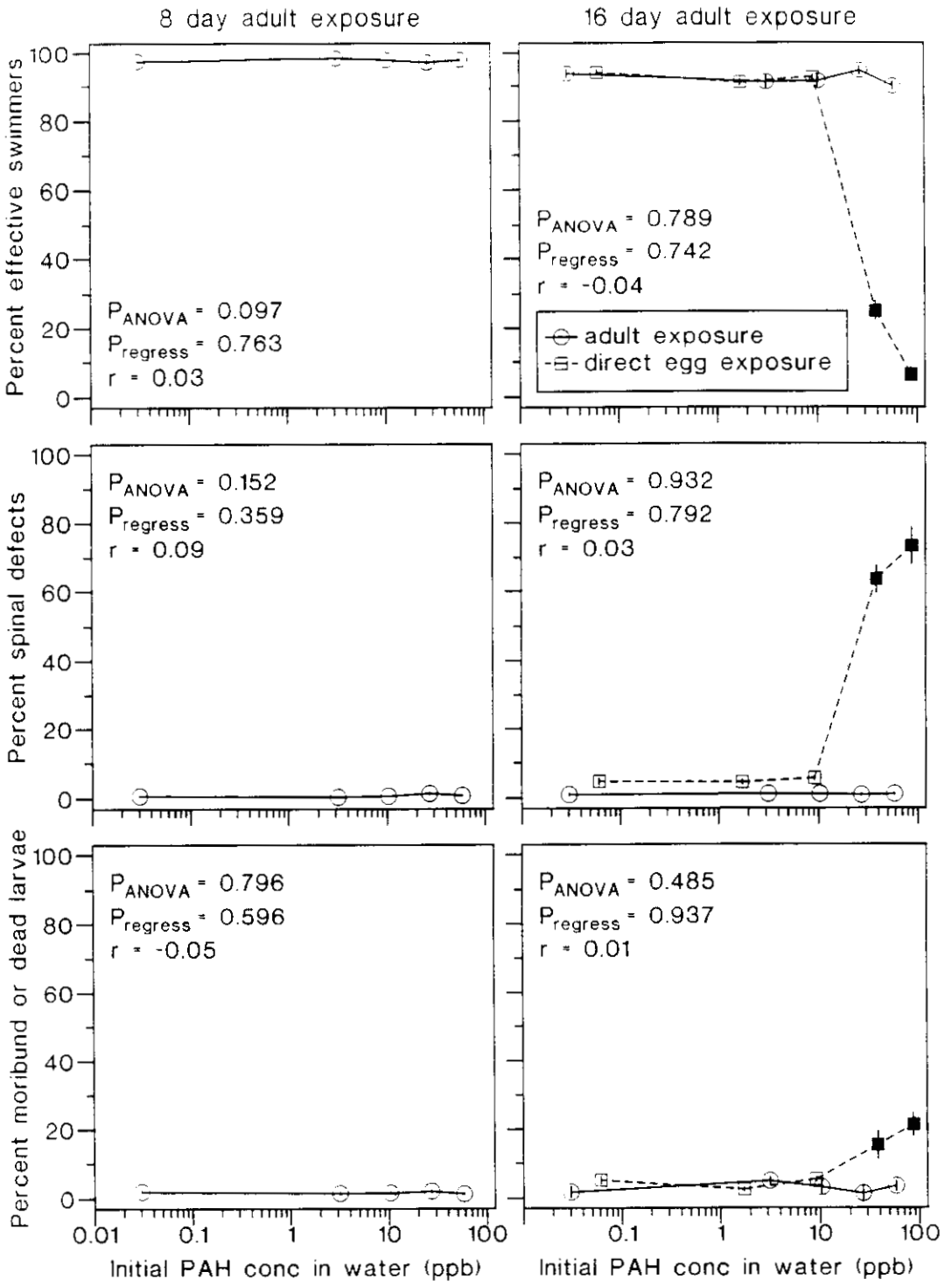

Figure 3.7. Swimming ability, spinal defects, and death of larvae from adult herring exposed to oil 8 or 16 d as functions of initial PAH concentration. Included for comparison are responses of larvae hatched from eggs incubated in similarly contaminated water for 16 d. P_{ANOVA} is probability of overall treatment effect estimated by Analysis of Variance; solid symbols indicate significant differences from controls. $P_{regress}$ reports probability of significant slope, estimated by regression ($x = \log_e(\text{concentration})$), and r = correlation coefficient. Data displayed are least squares adjusted means ± SE.

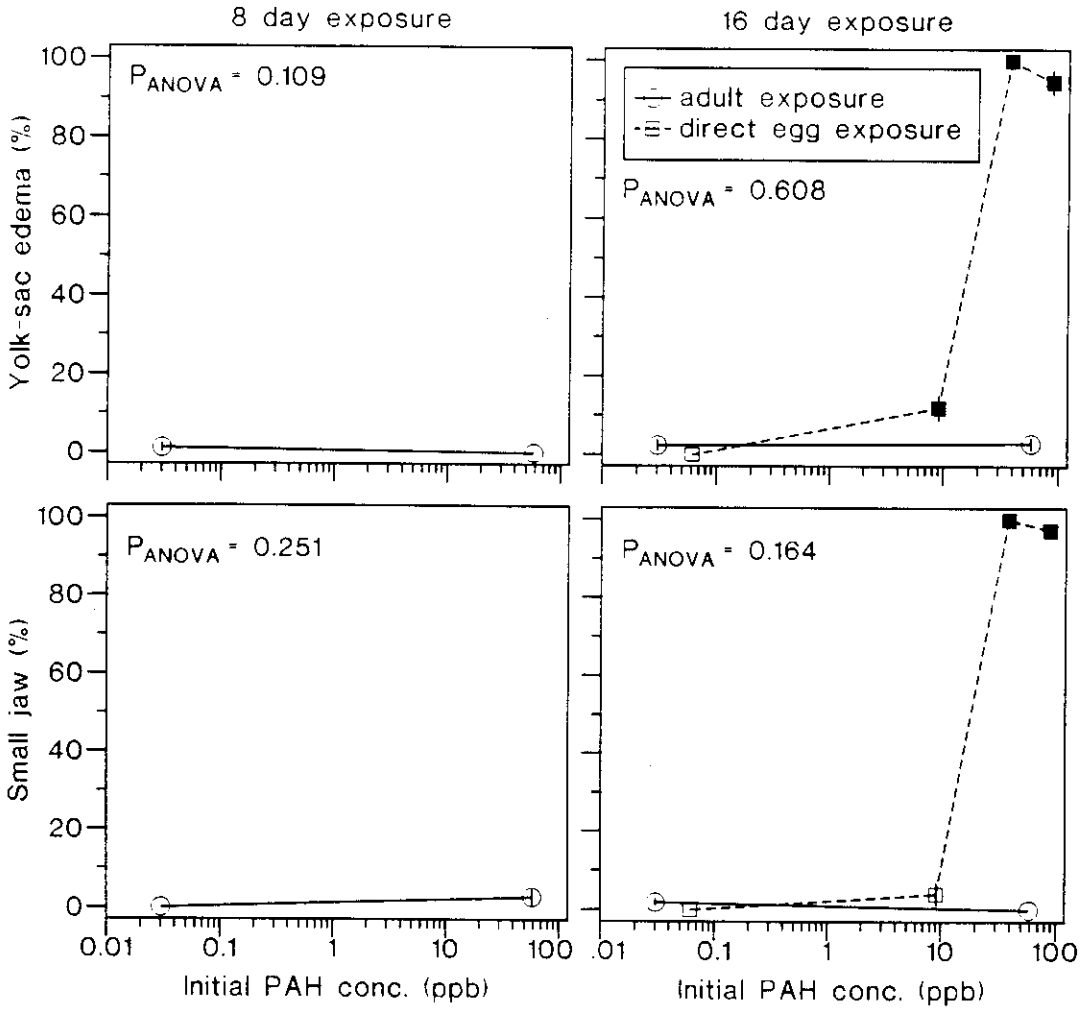

Figure 3.8. Incidence of larvae with yolk-sac edema and underdeveloped lower jaws from adult herring exposed to oil 8 or 16 d as functions of initial PAH concentration. Included for comparison are responses of larvae hatched from eggs incubated in similarly contaminated water for 16 d. P_{ANOVA} is probability of overall treatment effect estimated by ANOVA; solid symbols indicate significant differences from controls. Data displayed are least squares adjusted means ± SE.

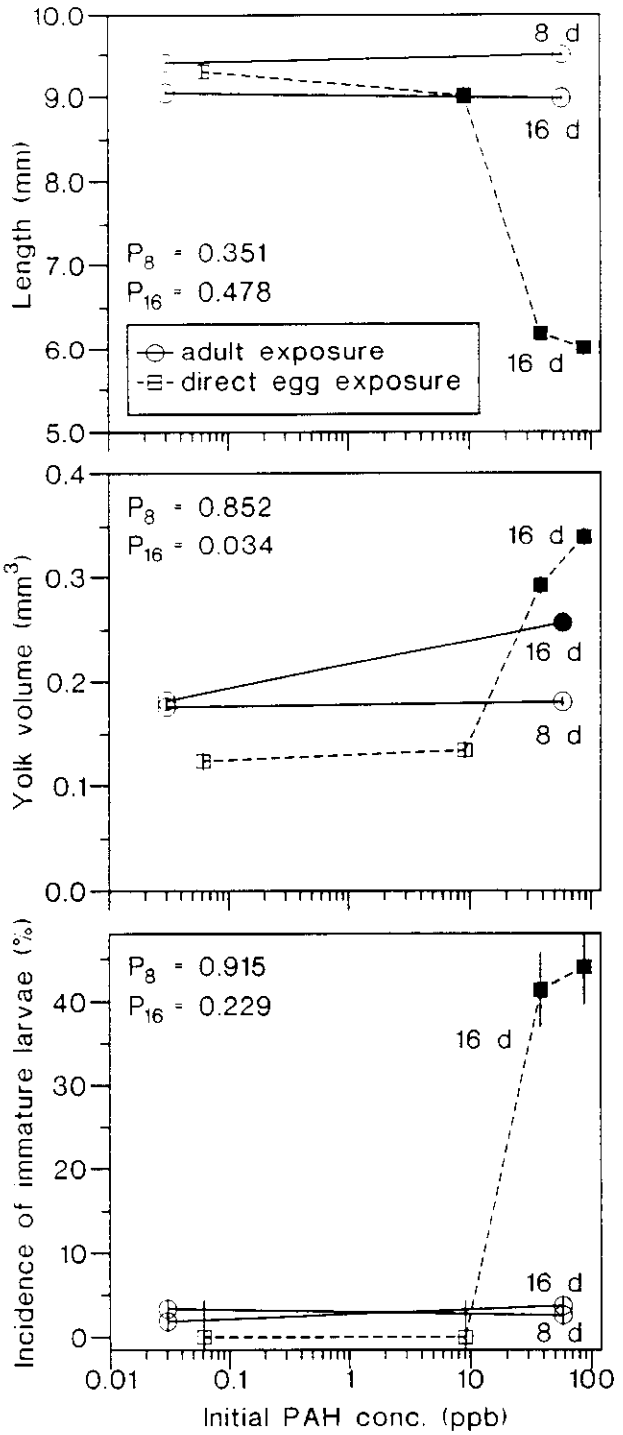

Figure 3.9. Length, yolk volume, and maturity of larvae hatched from adult herring exposed to oil 8 or 16 d as functions of initial PAH concentration. Included for comparison are responses of larvae hatched from eggs incubated in similarly contaminated water for 16 d. P_{ANOVA} is probability of overall treatment effect estimated by ANOVA; solid symbols indicate significant differences from controls. $P_{regress}$ reports probability of significant slope, estimated by regression ($x = \log_e(\text{concentration})$), and r = correlation coefficient. Data displayed are least squares adjusted means ± SE.

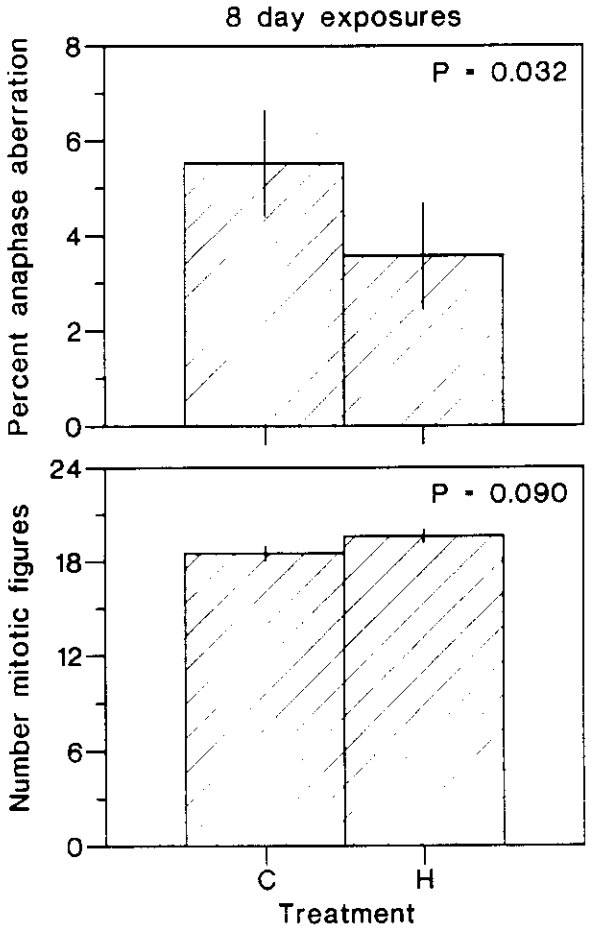

Figure 3.10. Anaphase aberration and number of mitotic figures in pectoral fins of larvae from adult herring exposed to oil 8 d. Treatments were control (C) and high-oil (H); *P* indicates probability of treatment effect.

Other cell problems were rare and generally not associated with treatment. Although statistically significant ($P = 0.036$), micronucleation was observed in only 5 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 ($P = 0.215$).

DISCUSSION

In this study, we exposed reproductively ripe Pacific herring to water contaminated with weathered crude oil and looked for effects in artificially spawned progeny. Adults were exposed to oil concentrations in the range encountered in open water in PWS following the spill (up to 6.24 ppb PAH (Short and Harris 1996)) and beyond (initial PAH concentrations ranged up to 58 ppb). PAH hydrocarbons accumulated in herring tissue, including muscle and ovary (Carls et al. Chapter 1). Although hydrocarbon concentrations were allowed to fall as the oil weathered, adult herring remained in contaminated water up to 16 d. Hydrocarbons that accumulated in ovaries were detectable at similar concentrations shortly after fertilization, but declined during incubation. Despite adult exposure, and presence of hydrocarbons in eggs during incubation, there were, with one possible exception, no discernable effects in progeny. We will argue that the exception, an elevation in yolk volume in 16 d exposures, was more likely due to random chance than treatment related. Response of the adult fish to oil exposure, including suppression of immune function, increased incidence of disease, and disease-related mortality, is covered in a separate paper (Carls et al. Chapter 1).

As in PWS, oil weathered as time progressed, resulting in reduced concentrations in water and changes in PAH composition. Composition in water shifted from naphthalene dominated to phenanthrene dominated, particularly at lower doses. Also due to weathering, PAH composition varied in a predictable manner across treatments. Rates of naphthalene loss were apparently dependent on the volume of oil in our delivery system; thickness of the oil film on the rock substrate may have been a crucial factor in controlling loss rates. (Similar loss rate patterns were observed in another experiment employing the same apparatus, but with different rock (Carls et al. Chapter 4). Thus, although weathering was different in each treatment at the beginning of the study, all treatments could be back-extrapolated to the same starting point. The same physical and chemical processes that caused weathering in our experimental apparatus were at play in PWS (Short and Heintz, In press), although the situation in PWS was infinitely more complex.

The least toxic PAH preferentially accumulated in ovaries, and were transferred to eggs. Accumulation was strongly biased toward naphthalenes, and, of the few large ring PAH detected, there was a tendency to accumulate unsubstituted homologs. Other studies have shown that toxicity increases both with ring size (Rice et al. 1977; Carls et al. Chapter 4) and alkylation (Rice 1985; Rice et al. 1977; Carls et al. Chapter 4), thus uptake patterns by ovarian tissue may have favored reduced toxicity to ova. We do not, however, fully understand the chemical

partitioning that caused these compositional differences. Composition of PAH in tissues may have been partially modified by metabolic action: mixed function oxidase (MFO) enzymes were induced in these pre-spawn herring (Thomas et al. 1997). Hydrocarbons accumulated by ova transferred to subsequently spawned eggs; the PAH composition matched that in ovarian tissue, and concentrations in eggs immediately after spawning were similar to those in ovaries.

Effects of exposure of reproductively ripe herring to oil in water were not discernable in the progeny. Parameters not affected included egg fertility, egg death, hatch timing, larval death, spinal defects, yolk-sac edema, larval swimming, larval length, stage of larval development at hatch, jaw development, rate of cell mitosis, anaphase-telophase chromosome abnormalities, number of pycnotic cells, and number of multinucleated or karyorrhectic cells. In several cases significant departure from control responses were observed, but not in a dose-dependent manner For example, in the 16 d exposure group, fertility was significantly low in the trace-oil treatment, and significantly high in the low-oil treatment, but not in mid- and high-oil treatments (Figure 3.5). These significant differences were invariably confined to the 16 d exposure group, where variability of response was consistently greater than in the 8 d group. In most cases, interpretation of randomly significant responses as not dose dependent was clearly put in context by comparison to responses to direct exposure of eggs during incubation (Figures 3.5 through 3.9).

There was one significant result, an elevation in yolk volume, that may have been dose related, but we hesitate to draw this conclusion. Although yolk volume was significantly elevated in the high-oil treatment of the 16 d exposure group ($P = 0.034$), it is unlikely that it represents a significant response. First a technicality; the acceptance criterion ($\alpha = 0.05$) should be divided by two tests (8 and 16 d), thus marginalizing the statistical significance. Second, the 16 d exposure group tended to exhibit a pattern of random significance that was not dose related, and we suspect the increase in yolk volume belongs in this category. Third, there was no indication of similar response in the 8 d exposure group. Fourth, if the response were real, other parameters, including larval length, developmental stage, jaw development, and yolk-sac edema should have also shown significant response or trends, but did not. Jaw development and yolk-sac edema were more sensitive measures of response to oil than yolk volume in direct egg exposure tests (Carls et al. Chapter 4). Comparison of increased yolk volume to that observed in eggs directly exposed to oil during incubation does not exclude the possibility that the response was real; a 0.22 mm^3 increase was significant in direct egg exposures, but an increase of 0.01 mm^3 was not. The yolk volume increase observed in this study was intermediate (0.08 mm^3), but substantially smaller than the significant 0.22 mm^3 increase.

Lack of response was not due to insufficient data or poor test design. The total number of eggs observed was high in 8 and 16 d tests (>12,000 and >7,500, respectively). Second, in a highly similar experiment where eggs were exposed during incubation (Carls et al. Chapter 4), many of the same parameters measured in this study were sensitive measures of response, including egg death, hatch timing, larval death, spinal defects, yolk-sac edema, larval swimming, larval length, apparent stage of larval development at hatch, jaw size, and anaphase-telophase

chromosomal abnormalities. Finally, in corroboration with our results, a previous study also found herring egg survival was not reduced by exposure of adults to the water-soluble fraction (WSF) of Cook Inlet crude oil unless the adults were killed (Rice et al. 1987).

We conclude that exposure of reproductively ripe parental herring did not measurably damage gametes, as judged by egg fertility and subsequent normal egg development. Herring in this experiment were reproductively similar to those in PWS at the time of the spill. Arguably, gametes might have been damaged if exposure had occurred during gamete formation (meiosis), but this was beyond the context of the present experiment.

Comparison of indirect and direct exposure of eggs to hydrocarbons

To understand why progeny were not affected when pre-spawn adult herring were exposed to oil, we compare results of this experiment (1994) to a similar experiment where eggs were directly exposed to oil of similar concentration and composition during incubation (1995) (Carls et al. Chapter 4), and also draw on other published studies. To validate this comparison, we will first establish that treatment concentrations were similar, and that the PAH composition (in water) to which adult fish or eggs were exposed was highly similar. Comparisons were restricted to 16 d exposures, and a second 1995 experiment involving more highly weathered oil was not included in direct comparisons.

Total PAH concentrations in treatment water were similar for each paired 1994 and 1995 treatment (Figure 3.11). Although treatment concentrations were not identical, there were no consistent upward or downward shifts in concentration, and data for each treatment pair closely overlapped. Tested with analysis of covariance (x = time, y = $\log_e$(PAH concentration)), all treatment pairs were coincident ($P \geq 0.277$), except the rate of concentration decline in the mid-oil treatment in 1995 was a bit faster than in 1994 (-0.19 ± 0.02 and -0.11 ± 0.03, respectively).

The PAH composition in treatment water was similar in 1994 and 1995 tests. Extreme differences in percentages ranged from -11.8 to 16.5, but most differences were much smaller (mean = 0.03, sd = 2.8, n = 337 for non-zero comparisons) (Appendix 3.4). In lower treatments in 1994 there was a tendency for more naphthalenes and fewer large ring compounds than in 1995, but in the upper treatments the situation reversed, particularly by the end of exposure (Appendix 3.4). Differences in analyte percentages between 1994 and 1995 fluctuated in time, hence there were also minor differences in weathering rates between years. However, treatment water contained large ring compounds in both years (including fluorenes, dibenzothiophenes, phenanthrenes, and chrysenes), and the composition of PAH to which pre-spawn adult herring and eggs were exposed was very similar.

Although pre-spawn adult herring (1994) and eggs (1995) were exposed to PAH of similar concentration and composition, PAH composition in resultant eggs was not similar. Naphthalenes were enriched in eggs spawned from exposed adults, and of the remaining heavier PAH, unsubstituted homologs dominated (Figure 3.4 and Appendix 3.3). In contrast,

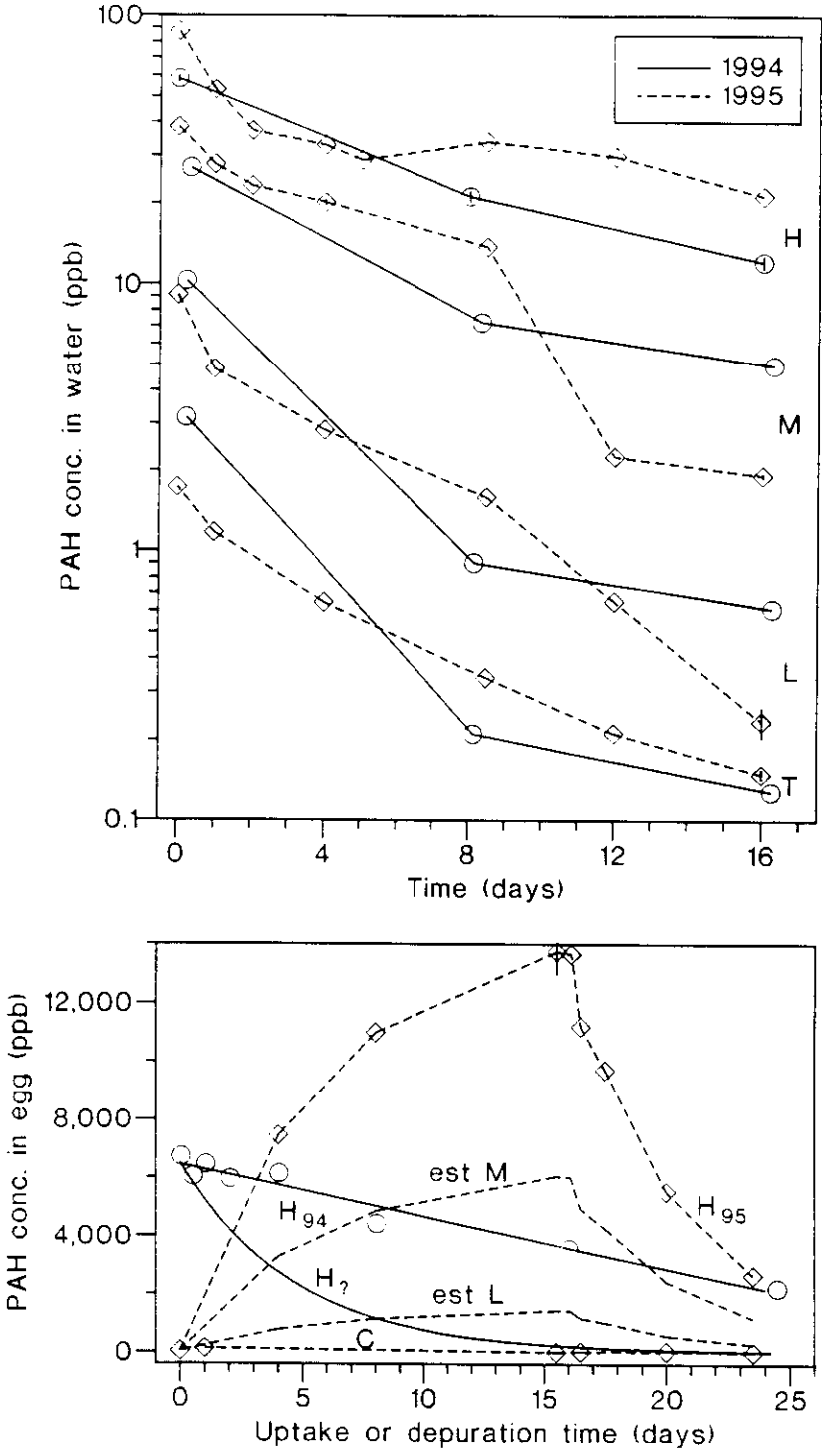

Figure 3.11. Comparison of PAH concentration in treatment water and egg tissue in experiments where pre-spawn adult herring were exposed to oil-contaminated water (1994) or where herring eggs were exposed directly to oil-contaminated water during incubation (1995). In the upper graph, H = high-oil treatment, M = mid-oil treatment, L = low-oil treatment, and T = trace-oil treatment. In the lower graph, H_{94} = observed concentrations in 1994 egg tissue (high-oil treatment) and H_{95} = concentrations in 1995 egg tissue (also high-oil treatment). Tissue concentrations in mid- and low-oil treatments of the 1995 study were estimated from uptake in the high-oil treatment (est M and est L, respectively). The relatively slow, linear decline of PAH in 1994 eggs may have been an experimental artifact (see text), thus a hypothesized 1994 decline ($H_?$) has also been included.

composition of PAH in directly exposed eggs tended to be similar to that in water, and although there was a tendency for naphthalene enrichment, this enrichment was not as pronounced as in 1994. Consequently, eggs exposed directly to hydrocarbons during incubation contained higher percentages of large ring compounds than did indirectly exposed eggs. Furthermore, percentages of large ring compounds in directly exposed eggs increased during incubation, and percentages of alkyl-substituted compounds were greater in 1995 than in 1994 (compare Appendices 3.3 and 4.4.1).

Another difference between 1994 and 1995 experiments was PAH concentration in eggs. (Data collection was restricted to the high-oil treatment in both years.) In 1994, concentrations were maximum at the beginning of the incubation, and declined throughout (Figure 3.11). In 1995, concentrations increased throughout incubation, then declined when eggs were transferred to clean water on day 16 (Figure 3.11). Apparent concentrations were equal on day 3 and approximately equal on day 25. Peak concentrations in 1995 were twice those in 1994. The relatively slow, linear, concentration decline in 1994 may have been an artifact of incubation conditions; the rate of decline in eggs on glass slides was probably much more rapid. In both years, spawn density of eggs collected for hydrocarbon analysis was much higher than spawn density of eggs followed for biological observation, particularly in 1994. Additionally, the 1994 uptake eggs were spawned in shallow pans, thus water circulation was likely significantly reduced compared to the 1995 uptake eggs, which were spawned on plankton net and suspended from mobile overhead racks much as other eggs were. We therefore suspect the true loss of hydrocarbons from eggs studied for biological effects in 1994 was exponential, much as in 1995, and may have approached baseline within roughly 15 d. Irrespective of loss rate adjustment, average concentrations in high-oil treatment eggs in 1995 were greater than those in 1994, and were greater than 1994 concentrations for the majority of incubation.

Determination of the relative importance of PAH composition and concentration as causes of differences in biological response between years was difficult because of uncertainties in true oil loss rates (1994) and because hydrocarbon collections were limited to the high-oil treatment in 1995. To understand the importance of PAH concentration, it appeared reasonable to compare the highest 1994 treatment against lower oil treatments in 1995. Thus, PAH concentrations in 1995 low- and mid-oil treatment eggs were roughly approximated from the high-oil uptake data by applying calculated biomagnification at each observation time to the other treatments (Figure 3.11). Assuming, for the moment, that 1994 loss rates were accurate, we find that mean concentrations in the 1994 high-oil eggs (5,185 ppb) were somewhat greater than in the mid-oil eggs in 1995 (3,668 ppb), but that peak concentrations were highly similar (6,719 and 6,049 ppb, respectively). This scenario suggests that PAH concentration in eggs in the 1994 high-oil treatment was roughly equivalent to that in the 1995 mid-oil treatment, and implied that differences in PAH composition were primarily responsible for differences in biological response. (Recall that some biological responses were significant in the low-oil treatment in 1995, and all responses were significant in the mid-oil treatment.) If, on the other hand, we dispute the 1994 hydrocarbon loss rate data, and assume loss rates were similar to those in 1995, the recalculated 1994 mean concentration becomes 1,335 ppb, intermediate to

those in 1995 low- and mid-oil treatments; peak 1994 concentration exceeded the estimated low-oil peak concentration (1,435 ppb) by a substantial margin. Thus we estimate that concentrations in low- and mid-oil treatment eggs in 1995 bracketed those in high-oil treatment eggs in 1994, and conclude that differences in PAH composition were likely a more important factor than concentration in causing differing biological responses. There was some evidence (Carls et al. Chapter 4) that biomagnification increased as a function of treatment, thus PAH concentrations in low- and mid-oil treatments in 1995 may have been overestimated. The smaller the 1995 concentrations were relative to 1994 concentrations, the more likely differences in biological response were due to differences in composition rather than in concentration.

Comparison of herring eggs indirectly and directly exposed to hydrocarbons supports the hypothesis that toxicity varies as a function of ring size and alkylation. In this study, PAH concentrations up to 58 ppb PAH (in water) did not evoke biological response in progeny, but when eggs were exposed directly to the same compounds during incubation, responses were detectable at PAH concentrations as low as 9 ppb (Carls et al. Chapter 4). When incubating eggs were exposed to more highly weathered oil, where the proportion of large-ring and alkyl-substituted compounds was even greater, significant biological responses were detected at 0.2 ppb (Carls et al. Chapter 4). (Our minimum effective PAH concentrations may be high; they represent initial concentrations, and do not account for generally rapid declines in exposure concentration.) Previous studies have shown that much larger concentrations of mono- and di-aromatic hydrocarbons are required to cause toxicity. For example, in tests where herring eggs were incubated in the WSF of Cook Inlet crude oil (82% mono- and 18% di-aromatics), significant reduction in hatch was observed at 1,400 ppb but not at 1,000 ppb (Rice et al. 1987; data were reanalyzed with authors' permission). In contrast, we observed significant reduction in hatch (inverse of increase in dead eggs) at concentrations as low as 9 ppb for highly weathered oil (Carls et al. Chapter 4). Similarly, part-per-million concentrations of WSF were necessary to cause toxic effects in adult and larval herring (Carls 1987), well above the part-per-billion concentrations effective in these studies.

Concluding remarks

The reproductive implications of shifts towards naphthalene and unsubstituted PAH homologs in gametes of exposed adult herring are that gametes are protected from the more toxic PAH. This protection may be a consequence of differing affinities of hydrocarbons for lipids or other tissue components, perhaps mediated by gradients in blood plasma. However, metabolic action may have also been a factor: MFO enzyme activity was induced in adult fish (Thomas et al. 1997).

We have shown that short-term reproductive effects as a result of exposure of pre-spawn adult herring to water contaminated with oil, as occurred in PWS after the spill are unlikely. Progeny of adult herring that were exposed to oil during migration to spawning grounds, or as they spawned intertidally, were probably not affected by this exposure. Our results do not exclude the possibility of longer-term damage to progeny of contaminated adults, but

hypothetical mechanisms for delayed effects are difficult to devise. Genetic damage was not detected in somatic tissue, hence we suspect that it was unlikely in reproductive tissues. However, available data suggests that the susceptibilities of these two tissue types in vertebrates is not equivalent, thus lack of genetic effect in somatic cells does not guarantee germline response. Hydrocarbons depurated from eggs during incubation, thus prolonged retention of biologically significant hydrocarbon concentrations beyond hatch was also unlikely.

We have also shown that direct exposure of herring eggs to oil during incubation is detrimental to developing embryos, in stark contrast to the lack of response observed as a result of gamete exposure. Mechanisms leading to the abnormal herring larvae in PWS following the spill (Norcross et al. 1996) were, therefore, probably due to exposure of eggs during incubation, or possibly exposure of larvae to oil (but direct larval exposure to similarly weathered oil has not been studied). It is also possible that exposed gametes were spawned in oiled areas, thus adding to total toxicant exposure.

CONCLUSIONS

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

The least toxic PAHs preferentially accumulated in ovaries, and were transferred to eggs. Accumulation was strongly biased toward naphthalenes, and, of the few large ring PAHs detected, there was a tendency to accumulate unsubstituted homologs.

In sharp contrast to lack of biological response in progeny to exposure of adult herring to oil, direct exposure of herring eggs to oil during incubation is detrimental to developing embryos.

Effects of exposure of reproductively ripe herring to oil in water were not discernable in progeny. Somatic genetic damage was not detected in larvae, hence we conclude that developing reproductive tissue will not be genetically damaged.

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Chapter 4: Effects of incubating herring (*Clupea pallasi*) eggs in water contaminated with weathered crude oil.

M. G. Carls, D.M. Fremgen, J. E. Hose, S. W. Johnson, and S. D. Rice

ABSTRACT

Herring spawned in Prince William Sound (PWS) a few weeks after the 1989 *Exxon Valdez* oil spill occurred, thus most or all life stages may have been exposed. Genetic and histopathological effects consistent with oil exposure were detected in larval herring collected from oiled areas in 1989. In the years following the spill, the herring population in PWS collapsed, leading to speculation that the spill was directly or indirectly responsible, possibly through reproductive impairment by oil. In this laboratory study, designed to assess the potential impacts of the spill in PWS, herring eggs were exposed to varying concentrations of weathered Alaska North Slope Crude oil (plus unexposed controls) for 16 d. Additional groups of eggs were exposed for variable amounts of time (0 to 8 d). The entire experiment was repeated twice, first with higher polynuclear aromatic hydrocarbon (PAH) concentrations biased toward naphthalenes, and second, with lower concentrations biased toward heavier aromatics (e.g., phenanthrenes). In both experiments, exposure to oil induced early hatch, reduced hatching success, larval swimming, survival, and size, and caused edema, skeletal, craniofacial, and finfold abnormalities. The frequency of chromosomal aberrations was significantly elevated in the pectoral fins of exposed larvae. Eggs exposed to weathered oil during incubation were significantly affected by concentrations as low as 9 ppb, but effects of more highly weathered oil were significant at 0.2 ppb, suggesting the toxicity of the more persistent, heavier and more substituted PAHs was greater than that of lighter, less substituted PAHs. Exposures as short as 2 d elicited significant biological response. These results imply that relatively brief exposure to low concentrations of oil in PWS may have adversely affected herring eggs spawned in oiled areas shortly after the spill. The implications of chromosomal aberrations in mitotic cells is not clear. It is likely most affected individuals died due to concomitant morphological abnormalities, but the question of whether observed chromosomal damage could be heritable remains unanswered.

INTRODUCTION

On March 24, 1989, the T/V *Exxon Valdez* grounded in PWS, Alaska; 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. 1996; Carls et al. 1996; Wertheimer and Celewycz 1996; Willette 1996; Meacham and Sullivan 1993). Herring were just beginning to spawn when the spill occurred, thus most or all of the life stages may have been exposed to oil (Brown et al. 1996). Herring stock in PWS may have been reproductively impaired by the oil, and it is feared that continuing long-lasting effects

could hamper restoration of the stocks that have collapsed since the spill. The herring population collapse in PWS 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. Significant tissue alterations were observed in adult herring collected in oiled areas of PWS in 1989 (Moles et al. 1993), and about half of the spawned biomass was oiled (Brown et al. 1996). Exposure of herring eggs to petroleum hydrocarbons frequently results in abnormal larvae with poor survival potential (Linden 1976; Rosenthal and Alderdice 1976; Pearson et al. 1985; Kocan et al. 1996). Herring larvae collected throughout PWS (May through July, 1989), exhibited conditions associated with oil exposure, including morphologic malformations, genetic damage, and small size (Norcross et al. 1996). Anaphase aberrations were elevated in the pectoral fins of herring embryos exposed to oil (Hose et al. 1996), giving some credence to the hypothesis that long term genetic damage was possible to the germ line. Because year-class strength is heavily influenced by survival of herring larvae (Stevenson 1949; Taylor 1964; Outram and Humphreys 1974), contamination of pre-spawn adults, eggs, or larvae by petroleum hydrocarbons may have an adverse impact on herring populations. At present, recruitment failures in herring in PWS are apparently continuing, and the standing biomass has decreased; the toxicological influence of oil on either is unknown.

Our goal was to examine the possibility of long term impacts of the spill in PWS herring; this paper represents only part of the effort. The purpose of this particular study was to determine if genetic damage to early life stages of herring could be caused by exposure of eggs to oil similar in composition and concentration to that observed in PWS, and relate this damage to larval survival potential. Genetic damage was assessed by observation of anaphase-telophase aberrations in the pectoral fins of newly hatched larvae. Observation of genetic damage was confined to disruption of somatic cell division. We assumed that disruption of normal somatic mitotic processes might reduce the potential of larvae to survive to reproductive age. Other related studies investigated effects of oil exposure in pre-spawn adults (Carls et al. Chapter 1; Thomas et al. 1997), gametes and progeny (Carls et al. Chapter 3), and surveyed reproductive condition of herring in PWS herring six years after the spill (Johnson et al. In press).

Specific experimental objectives were as follows: 1) to determine if exposure of incubating herring eggs to oil would cause genetic defects in somatic cells; 2) to determine if exposure of incubating eggs to oil would cause mortality, morphological effects, behavioral effects, and changes in hatch timing; 3) to determine how much oil was required to cause significant effects, and how much exposure time was required before significant response; 4) to quantify and characterize accumulation of hydrocarbons by eggs; and 5) to compare the toxicity of lighter PAHs with heavier PAHs.

METHODS

There were two consecutive experiments, each with a dose-series component (16 d exposures) and a time series component (0, 1, 2, 4, 8, and 16 d) at a single dose. Fertile herring

eggs, attached to glass slides, were incubated in clean or contaminated seawater. Water was contaminated by passage through oiled rock; the rock used in the first experiment was reused in the second experiment to yield a lower concentration series and a shift in PAH composition toward heavier compounds.

Pre-spawn herring were collected from two locations in southeast Alaska. Herring for experiment one (E1) were collected near Cat Island (55.0°N lat., 131.2°W long.) on April 11, 1995, maintained on ice, transported to Ketchikan, and artificially spawned. Fish for experiment two (E2) were collected in Seymour Canal (57.5°N lat., 133.8°W long.) on May 13, 1995, maintained on ice, transported to Juneau, and artificially spawned. Ovarian membranes were cut longitudinally and eggs were removed with a stainless steel spatula. From each female, eggs were deposited in a single layer on nine 25 x 75 mm glass slides placed at the bottom of a shallow glass dish in ambient seawater. Deposition was accomplished with a gentle swirling motion; approximately 100 to 150 eggs were deposited per slide. Eggs from each female were placed in a staining rack and suspended in separate beakers of seawater. Milt was prepared from testes by cutting sections from each of three contributing males into small segments; segments plus a small amount of seawater were mixed with a spatula. A few milliliters of milt were added to each beaker. Eggs and milt remained in contact 5 min; eggs were removed and gently rinsed in seawater. Eggs were spawned within 30 min of deposition. In both experiments, eggs from 21 females were fertilized in three separate spawning events; a total of 9 males contributed sperm in each experiment. Fertilized eggs were incubated 1 d before experimental treatment.

Eggs were examined for fertilization success within 5 d of spawning. Excess eggs were removed by scraping; eggs along slide margins and clumps of eggs where not all eggs were exposed to water were removed. Processing was accomplished in water with a minimum of emersion. Remaining fertile, infertile, and dead eggs were counted.

There were some fertilization problems associated with the eggs used in E1; mean fertilization rates were generally above 65%, but were poor for six females (17% to 55%). We suspect that this poor fertility was caused by excessive chilling before spawning. Because previous experience indicated that bacteria would overrun all eggs when a high percentage of eggs were infertile, we removed the eggs from these six females from study. All ensuing results for E1 refer only to the remaining 15 females (the average fertility rate was 80%). Fertility rates in E2 averaged 93%.

Water was oiled by contact with oiled rock; seawater flowed into a plenum at the bottom of a 30-cm diameter x 122-cm polyvinyl chloride plastic cylinders and upwelled through rock (Appendix 1.1). Water flowed from these oil generators to the bottom of 40 to 50 L individual treatment tanks; a trap inside the generator prevented slick overflow. Seawater flow rates were 6 L/min in E1 and 5 L/min in E2. Water was delivered via manifold to treatment tanks; one generator was used per dose. Before the rock was oiled, Alaska North Slope Crude oil was artificially weathered by heating to 70°C overnight (12 h) in a beaker with continuous stirring. Rock, approximately 1 - 11 mm (Mdϕ = 5.2 mm, Q1-Q3 = 4.2 - 6.6 mm), 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 rock with a paint sprayer (trace-, low-, and mid-oil treatments) or with a Teflon squirt bottle (high-oil treatment). Each generator was loaded with 45 kg of oiled rock (or non-oiled control rock), except the high-oil treatment generator was loaded with 90 kg rock.

PAH concentrations in water (0.04 ppb (control) to 87 ppb) were chosen to bracket maximum mean concentrations observed in open water of PWS following the spill (6.24 ppb; Short and Harris 1996a). At the beginning of treatment, and every fourth day thereafter, 1 to 3 replicate 3.8 L water samples were extracted from each dose for analysis by gas chromatography (Short et al. 1996) to characterize the oil and quantify treatment concentrations.

Eggs from each female were represented in each of 9 treatments (control, trace-, low-, mid-, and high-oil, plus additional 1, 2, 4, and 8 d exposures in the mid-oil treatment). Eggs were first exposed to oil 1 d after fertilization. Exposure continued for a maximum of 16 d; time series groups were transferred to clean seawater at appropriate intervals. After 16 d, all remaining eggs were transferred to clean seawater. Eggs were isolated by female and treatment 1 d later; each slide was individually suspended in a 1 L jar filled with seawater. Temperature was controlled by placing jars in a flowing seawater bath. To avoid oxygen depletion in water immediately surrounding the eggs, slides containing eggs were attached to mobile racks designed to cause slow movement through the water. Mean temperature was 5.1°C (4.0 to 6.7°C) in E1, and 6.2°C (5.6 to 7.1°C) in E2; estimated temperature increase over the entire study period was $0.05°C \cdot d^{-1}$. Mean salinity was 32 ± 0.3 ppt.

Lighting was natural, supplemented by overhead fluorescent light during daylight hours. Indirect natural light entered through windows along one side; direct sunlight could enter only briefly at very low sun angles. To minimize natural light, all experimental tanks were located away from the windows. Light levels were estimated with a GE type 213 meter on May 14, 1997; the sky was clear. The ultraviolet (UV) contribution was estimated by placing a photographic UV filter (Tristar number 8730) over the light meter and recording the difference in light intensity.

To more accurately estimate UV emission, an intensity spectrum for a fluorescent light was measured with a Perkin-Elmer model 650-10S fluorescence spectrophotometer. The spectrophotometer was operated at the lowest sensitivity setting with the light source off; emission slit width was 2 nm. The standard sample cell was replaced with a small (13.3 cm) fluorescence tube operated with weak batteries to minimize intensity. Area under the intensity curve was measured for UV light (≤ 400 nm) and for visible light (400 to 750 nm); the UV contribution to total light energy was expressed as a percentage.

Dosing generators were covered with black plastic tops to exclude light. Light could enter the glass distribution manifolds, but they were protected from direct light, and water flow rates were high (5 to 6 L/min), further reducing the likelihood of significant molecular excitation.

The incubation tanks were black, so any light entering them was probably quickly absorbed. When isolated for hatch, eggs were placed in brighter surroundings (smooth, green tanks), but lights were located at least 1.5 m from the water surface.

Light intensity ranged from 6 to 70 footcandles (mean 31 footcandles). (By comparison, at the time of these measurements, outside light was 4,367 footcandles, but contributed only 7% of light in the laboratory.) The estimated UV energy was 16 ± 1% of the total energy, as measured with the hand-held meter. Spectral output of the representative fluorescent tube was 14.4% UV (Appendix 1.7). There were two prominent UV peaks, centered at 315 and 365 nm. The shorter wavelength UV peak was at the border (310 to 330 nm) of UV-A and UV-B wavelengths, and represented 1.7% of the spectral energy.

During peak hatch, hatch timing and success, larval viability, and larval abnormalities were observed daily. During periods of low hatch, observation frequency was reduced to two or three day intervals, and larvae were collected only if five or more were present per jar. Living larvae were assessed for swimming ability and gross morphological deformities, anesthetized with tricaine methanesulfonate, and preserved in 5% buffered formalin. Dead larvae were discarded. After hatch was complete, the remaining eggs and embryos were inspected and enumerated.

The swimming ability of live larvae was categorized as effective, ineffective, or incapable. Effective swimmers were active, frequented the water column, and avoided capture. Ineffective swimmers were generally more lethargic than effective swimmers, and were more likely to be found on jar bottoms. Incapable larvae were unable to swim in a straight line, and were often only capable of spasmodic twitching.

Hydrocarbon uptake and depuration by eggs was measured by gas chromatography. Eggs from several females were spawned on nylon plankton netting; egg density was high (often multiple layers) and fertilized with milt from several males. In E1, eggs were incubated in the high-oil treatment or clean seawater (controls) for 1 to 16 d. Approximately 10 g samples were collected for hydrocarbon analysis after 1, 2, 4, 8, and 16 d exposure. After 16 d exposure, remaining eggs were transferred to clean water and sampled periodically (0.5, 1, 2, 4, and 8 d) until hatch. Control eggs were collected at similar frequencies. In E2, uptake was measured for all treatments after 4, 8, and 16 d; in addition, eggs were sampled from the mid-oil treatment after 1 and 2 d exposure. As they were collected, eggs were inspected visually for coating by oil; no evidence of coating was detected in any treatment (this observation was also recorded photographically).

For morphological measurement (total body length, yolk volume, and spinal curvature), five preserved larvae were randomly subsampled from each of 15 or 21 females in each treatment group. Lateral views were digitized with a video camera and a frame grabber. To minimize variance, specimens were rotated to align eyes before image capture. Initial magnification varied from 6 or 12x for total length measurement to 50x for yolk measurement. Total length was

measured from snout to tip of notochord, using as many line segments as necessary to approximate larval posture. Spinal curvature was estimated from the same line segments as the sum of (180° - segment angle). Yolks were generally elliptical: the major axis, defined as yolk length, was measured parallel to the notochord, and the minor axis, defined as yolk height, was perpendicular to the body axis. Yolk volume was estimated from length and height measures according to Hourston et al. (1984). Muscle width, measured for staging purposes, was immediately dorsal to the yolk height measurement.

Edema and jaw size were scored from digital images. Yolk-sac edema was indicated if the anterior margin of the yolk membrane was bounded by an area of clear fluid. Pericardial edema was noted when the pericardial area was convex ventrally or had an unusually large area. Jaws were classified as small if absent or posterior to the anterior margin of the eye.

Genetic and additional morphologic responses were evaluated using blind review. The number of treatments thus analyzed was limited due to cost; 10 larvae were randomly subsampled from 15 randomly selected females. Each larvae was examined at 30X magnification. The severity of skeletal, craniofacial, and finfold defects was scored using the graduated severity index (GSI) method described in Hose et al. (1996) where 0 = no effect; 1 = slight defect in structure or size; 2 = moderate defect in structure or size; and 3 = severe defect in structure or size. Types of skeletal defects evaluated were kyphosis or lordosis of the notochord and stunting. Craniofacial abnormalities consisted of reduction, malformation or absence of one or both jaws, ocular and otic capsule defects, and microcephaly. Reductions of the dorsal and ventral finfold were observed. The development of both pectoral fins was categorized as a bud (no fin rays visible) or containing fin rays.

Both pectoral fins were removed, placed onto a glass microscope slide, and stained with aceto-orcein. Cytogenetic analysis was performed at 1,000X magnification using the method of Hose et al. (1996). All mitotic figures in a fin were enumerated, and anaphase-telophase configurations (AT) were visually assessed for evidence of chromosome/chromatid breakage (bridges and attached fragments), multipolar spindles, and aneuploidy (unequal masses of chromatin in daughter cells). The mitotic index was the number of mitoses per fin. Thus, two mitotic indices were calculated per larva and were generally in good agreement. An anaphase aberration rate was calculated for each dose treatment by dividing the total number of aberrant AT by the total number of AT present.

Data processing and statistics

The denominator used to calculate percentages varied according to response type. Percentages of eggs fertile, infertile, and initially dead were based on the total number of eggs counted near the beginning of each 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, moribund, and 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 effective, ineffective, or incapable; swimming ability of moribund and dead larvae was, by definition, nonexistant. Thus, alive was used as the denominator for swimming categories. Spinal aberrations were assessed 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.

The primary experimental design was a randomized block : 15 or 21 females were represented in each of 9 treatments. All treatments (dose series and time series) were analyzed simultaneously with analysis of variance (ANOVA); where the overall test was significant, treatments were compared to controls with *a priori* multiple comparisons. Before analysis with ANOVA, percentage data were arcsin transformed, and corrected for small n where necessary (Snedecor and Cochran, 1980). [The same general conclusions were reached with untransformed data]. In cases where multiple larval observations occurred for each female (e.g., larval lengths), larvae were nested in female. In addition, time series data were analyzed with linear regression techniques.

Scored abnormality data were either analyzed with the Kruskal-Wallis nonparametric test, or recoded to yield incidence of abnormality (e.g., percent larvae with small jaw). If the overall *P* in Kruskal-Wallis tests (SAS Institute, Inc. 1989) was significant, treatment groups were compared to controls using the Dunn multiple comparison procedure (SigmaStat 1994). Incidence of abnormality was calculated on a per female basis, arcsin transformed, and analyzed with single factor ANOVA; dose series and time series data were analyzed simultaneously. [Conclusions reached with either technique were similar, but incidence data tended to detect smaller differences than scored data].

Median effective concentrations (EC50) were estimated from logit fits by female (x = ↓ initial PAH concentration); independently estimated EC50s were averaged. Analyses were restricted to cases where response in controls and highest treatments spanned 50%.

Stepwise regression was used to test the influence of morphological abnormalities on larval swimming ability. In this assessment, dose-series and time-series data from both experiments were combined. Predictive factors tested included incidence of spinal defects, yolk-sac edema, pericardial edema, and lower jaw size. Although jaw size probably did not affect swimming directly, it served as an estimator of maturation; immature larvae might not swim as well as their more mature counterparts.

Hydrocarbon data were processed according to the methods of Short et al. (1996) before further analysis. Hydrocarbon concentrations were accepted only if corresponding recovery of deuterated internal standards ranged from 28 to 150%; records with unacceptable recoveries were deleted (6% of the data). In addition, concentrations below minimum detection limits were set to zero (Short et al. 1996).

RESULTS

Hydrocarbons

Exposure

Concentrations of hydrocarbons in water declined over time, thus treatment concentrations in the second of two consecutive experiments were smaller than in the first experiment. Initial PAH concentrations ranged from 0.06 (control) to 87 ppb in E1 and from 0.04 (control) to 7.6 ppb in E2 (Figure 4.1). The rate of decline of PAHs from water was greater in E1 treatments than in corresponding E2 treatments (Figure 4.1). The PAHs detected in treatment water ranged from naphthalene through C-1 chrysene, with occasional occurrence of heavier compounds (through benzo[b]fluoranthene). Initial PAH concentrations were linearly related to concentration on sediment in dosing apparatus (Appendix 4.5).

Alkane concentrations in treatment water were similar to PAH concentrations, but tended to be more variable (Appendix 4.1.1). Alkane concentrations in the trace-oil treatment were not distinguishable from controls in either experiment, and there was little difference between low-oil alkane concentrations and control concentrations in the second experiment. The ratios of alkanes/PAHs and phytane/PAHs, measured in E1, were smaller in water than in the oiled rock substrate (Table 4.1).

Table 4.1. Alkane/PAHs and phytane/PAHs ratios were lower in water than in oiled rock, indicating dissolution of the more soluble PAHs. Observations were made in E1 on day 2, except day 1 for low-oil treatment water. Ratios were not reported for the trace-oil treatment because alkanes were below detection limits. None of these observations were replicated, hence estimation of variance is not possible.

	Alkane/PAHs ratio		Phytane/PAHs ratio	
Treatment	water	rock	water	rock
Low	0.59	6.65	0.004	0.133
Mid	0.64	6.54	0.001	0.118
High	2.75	6.03	0.001	0.106

Composition and weathering

Oil was more weathered in lower treatments than in higher treatments at the start of E1, despite uniform oil preparation, rock drying, and pre-experimental water flow. For example, on day 0, naphthalenes comprised 47% of the PAHs in water in the trace-oil treatment, but comprised >80% of the PAHs in the upper treatments (Appendix 4.2). Percentages of naphthalene increased significantly as a function of log initial PAH concentration ($r^2_{\text{day 0}}$ >0.99, P

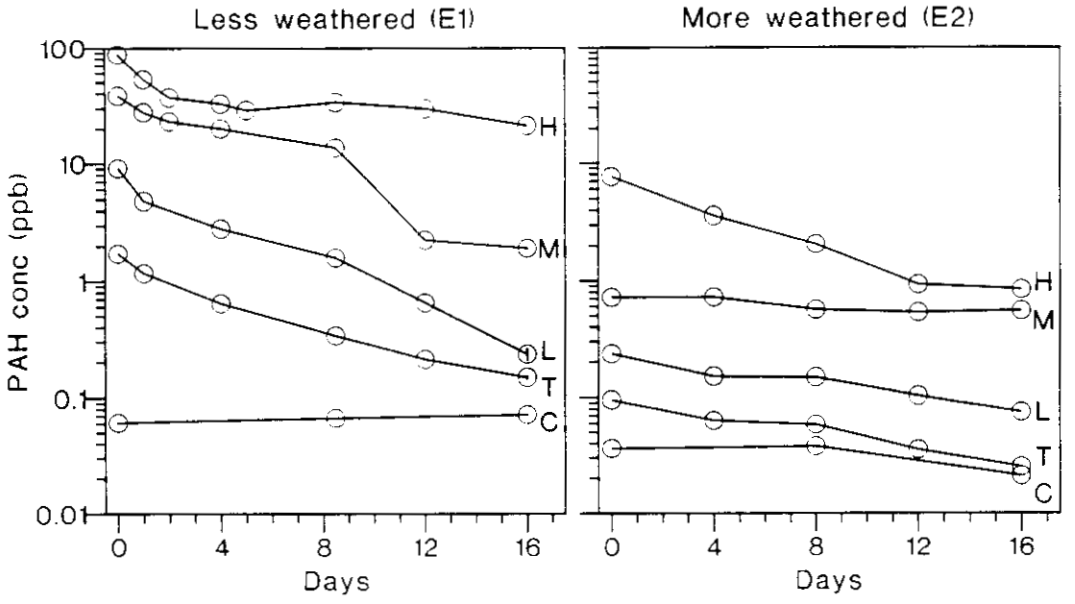

Figure 4.1. Total PAH concentrations in water as a functions of time, by experiment and treatment. Most points represent single observations: where present vertical bars indicate SE.

= 0.042) (Figure 4.2). Conversely, percentages of phenanthrenes declined significantly as a function of log initial PAH concentration ($r^2_{\text{day 0}} > 0.99$, $P = 0.033$) (Figure 4.2). Percentages of chrysenes also declined as a function of initial PAH concentration ($r^2_{\text{day 0}} > 0.99$, $P = 0.013$) (Figure 4.2). These composition - treatment relationships persisted over the first 12-16 d of treatment. Relationships between fluorenes and inital concentration, and dibenzothiophenes and initial concentration, were not as clear (Figure 4.2), and varied with time.

In contrast to the composition relationships observed in water in E1, the relationship of most homologous PAH families and initial PAH concentration in E2 was not clear; relative quantities of naphthalenes, fluorenes, dibenzothiophenes, and phenanthrenes either peaked or were depressed in intermediate treatments (Figure 4.3). Only chrysenes exhibited a relationship with treatment; percentage declined as a function of -1/PAH ($r^2_{\text{day 0}} = 0.99$, $P = 0.05$) (Figure 4.3). Chrysenes remained related to initial PAH concentration in similar fashion through the 16 d exposure.

Relationships between PAH composition and concentration in the rock substrate used to prepare treatment water were similar to those observed in E1 treatment water (Appendice 4.4.1 and 4.4.2). The percentage of naphthalenes increased as concentration increased; percentages of fluorenes, dibenzothiophenes, phenanthrenes, and chrysenes declined with concentration increases. However, few measurements were taken (five), and these were completed on a single day.

Up to this point, PAH composition has been presented on a relative basis. In terms of absolute concentration, total naphthalenes, fluorenes, dibenzothiophenes, and phenanthrenes concentrations in E1 consistently exceeded those in E2, but for chrysenes, the situation was generally reversed (Appendix 4.3). However, concentrations of the more substituted compounds within homologous families in the high-oil treatment were generally greater in E2 than E1; these included C3-fluorenes (days 0-8), C3-dibenzothiophenes (days 0-8), C3- and C4-phenanthrenes (days 0-8) (Appendix 4.3). Concentrations of all chrysenes in the high-oil treatment of E2 were consistently greater than in E1, except no C4-chrysenes were detected. Additionally, C3 and C4-phenanthrene, and C2-chrysene concentrations in the mid-oil treatment of E2 frequently exceeded those in E1.

The oil weathered with time in both experiments; trends observed in E1 generally continued through E2. Described linearly, percent naphthalenes declined with time ($P < 0.001$); percentages of fluorenes, dibenzothiophenes, phenanthrenes, and chrysenes increased with time ($P < 0.001$) (Figure 4.4). Data from both experiments were analyzed together, with a 1 d offset between experiments. Rates of change over time frequently differed among treatments, and fitted lines were generally not coincident. Correlation of percentage naphthalenes, phenanthrenes, and chrysenes with time ($0.61 \leq r^2 \leq 0.90$) was generally better than that of fluorenes and dibenzothiophenes with time ($0.12 \leq r^2 \leq 0.87$). Weathering in the trace-oil treatment was not analyzed because of marginal analyte detectability.

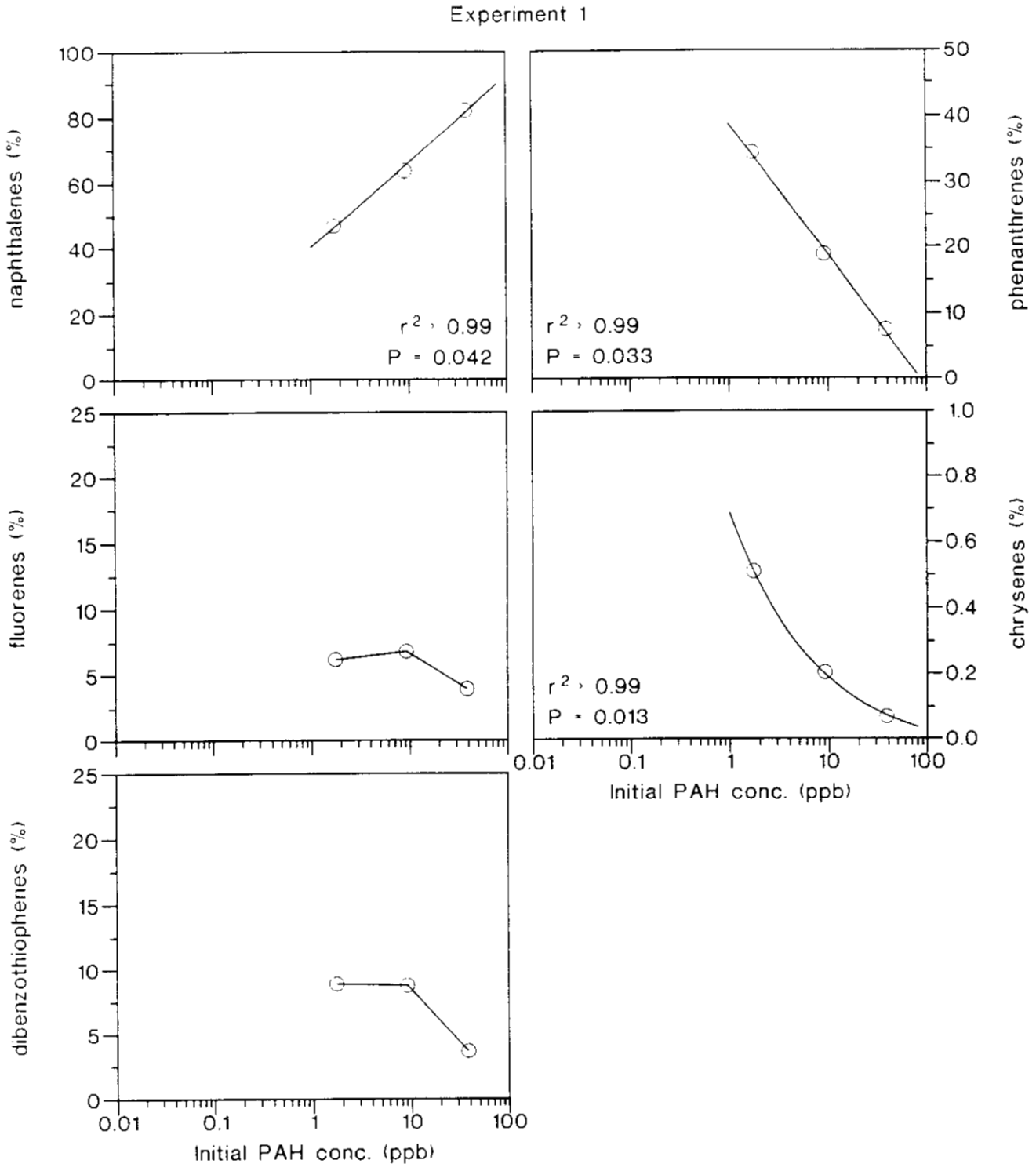

Figure 4.2. PAH composition on day 0 in E1 as a function of initial PAH concentration. Naphthalenes and phenanthrenes were related to $\log_e$(PAH); chrysenes were related to -1/√PAH; r = correlation coefficient, P = probability. Each data point represents a single observation.

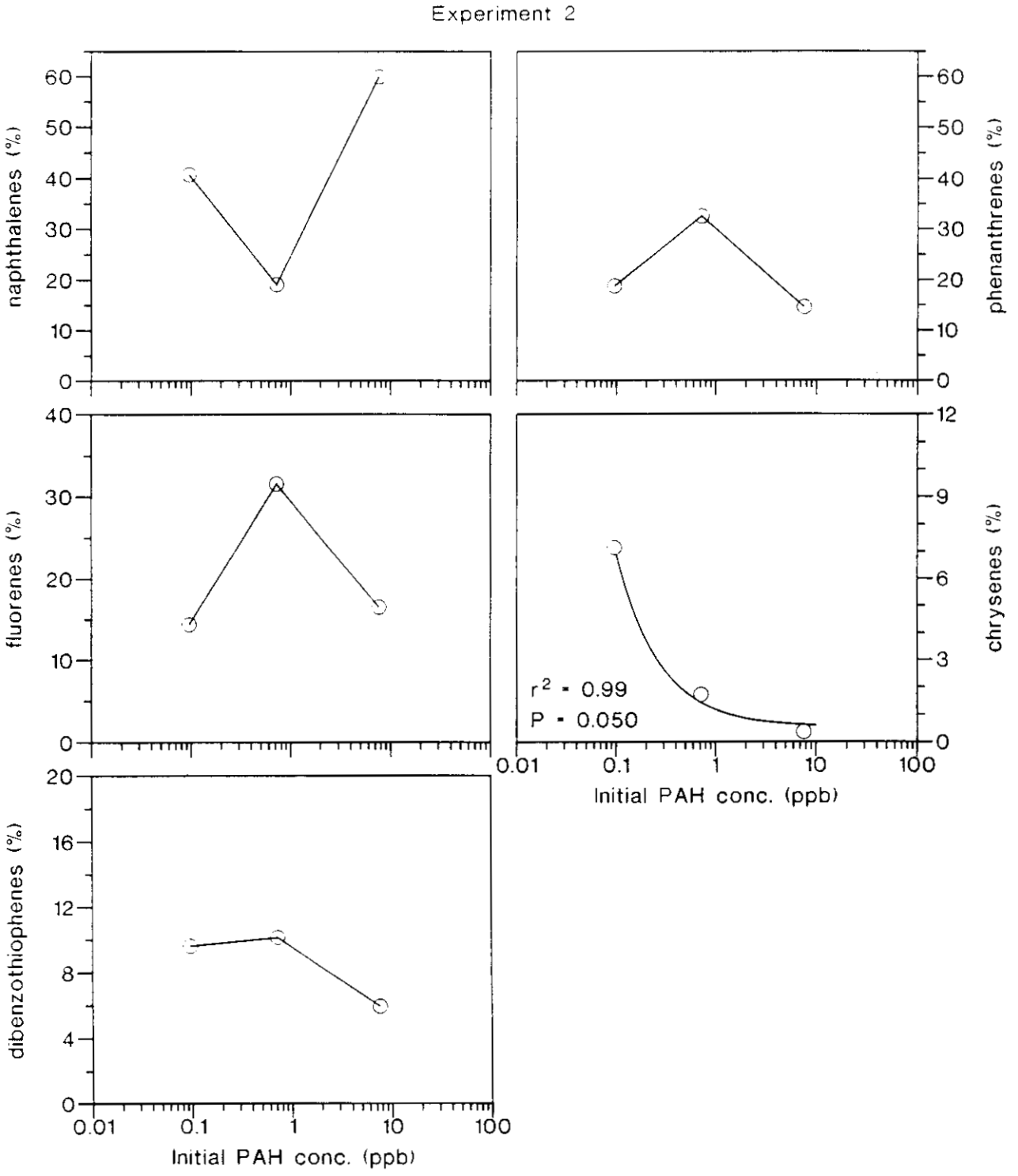

Figure 4.3. PAH composition on day 0 in E2 as a function of initial PAH concentration. Except for chrysenes, the relationship of most homologous families and initial concentration was not clear; relative quantities either peaked or were depressed in intermediate treatments. Chrysenes were related to -1/PAH; r = correlation coefficient, P = probability.

Most PAH concentrations were negatively correlated with time, except that concentrations of C3-fluorenes, and C1-chrysenes were not correlated with time, and concentrations of C3-dibenzothiophenes, C3- and C4-phenanthrenes, chrysene, and C2-chrysene were positively correlated with time in the high-oil treatment (Appendix 4.3). Correlation of PAH concentrations and time in the low-oil treatment were consistently negative; correlations in the mid-oil treatment were intermediate between low- and high-oil treatments (Appendix 4.3). Both experiments were included in these observations, with a 1 d offset between day 16 of E1 and day 0 of E2.

Accumulation in egg tissue

Hydrocarbons accumulated in exposed eggs in both experiments, but maximum concentrations were smaller in E2, and there were differences in accumulation patterns. In E1, PAH concentrations in egg tissue increased throughout exposure and peaked at 13,746 ppb (high-oil treatment) on day 16. Hydrocarbons depurated exponentially from egg tissue when contaminated eggs were transferred to clean water (Figure 4.5). In E2, PAH concentrations in eggs accumulated during the first 4 d of exposure, but declined thereafter (Figure 4.5).

Bioconcentration was difficult to estimate because of declining PAH concentrations in water and variable concentrations in tissue. Mean estimated bioaccumulation in E1 was 406 times (range 157 to 643), determined by varying the estimate of water concentration (mean, initial, and 16 d concentrations, respectively). Estimated bioconcentration in E2 was 157 (19 to 335) times the aqueous PAH concentration (summarized across time and treatment).

Composition of PAHs in egg tissue tended to change in a manner similar to that in water in E1, but at a slower rate; there was no evidence of consistent composition change in E2 eggs. In E1 eggs, naphthalene percentages tended to decline linearly ($P = 0.104$), while slopes of heavier compounds increased ($0.001 \leq P \leq 0.236$ for other homologs). Rates of change were at least twice as fast in water as in tissue ($2.3 \leq \beta_w / \beta_t \leq 86.4$, where β_w = rate of change in water, and β_t = rate of change in egg tissue, estimated linearly for each homologous chemical family). In E2 eggs, variation in composition was unrelated to time ($0.214 \leq P \leq 0.961$ except $P = 0.083$ for chrysenes in the high-oil treatment).

Compared on a per treatment basis, composition of PAHs in eggs differed significantly between the two experiments. (Because of limited sampling, the only possible inter-experiment comparison was the high-oil treatment.) Data from days 4, 8, and 16 were grouped (by experiment) and analyzed by ANOVA. This combination of times was reasonable because the rate of change in PAH composition in eggs was small in E1 ($0.01 < |\beta_t| \leq 0.14$ %d^{-1}) or unrelated to time (E2). The percentage of naphthalenes was significantly greater in the first experiment (85%) than in the second (49%) ($P < 0.001$). Thus, percentages of heavier compounds (fluorenes, dibenzothiophenes, phenanthrenes, and chrysenes) were consistently higher in E2 than in E1 ($P \leq 0.014$) (Appendix 4.4.1 and 4.4.4).

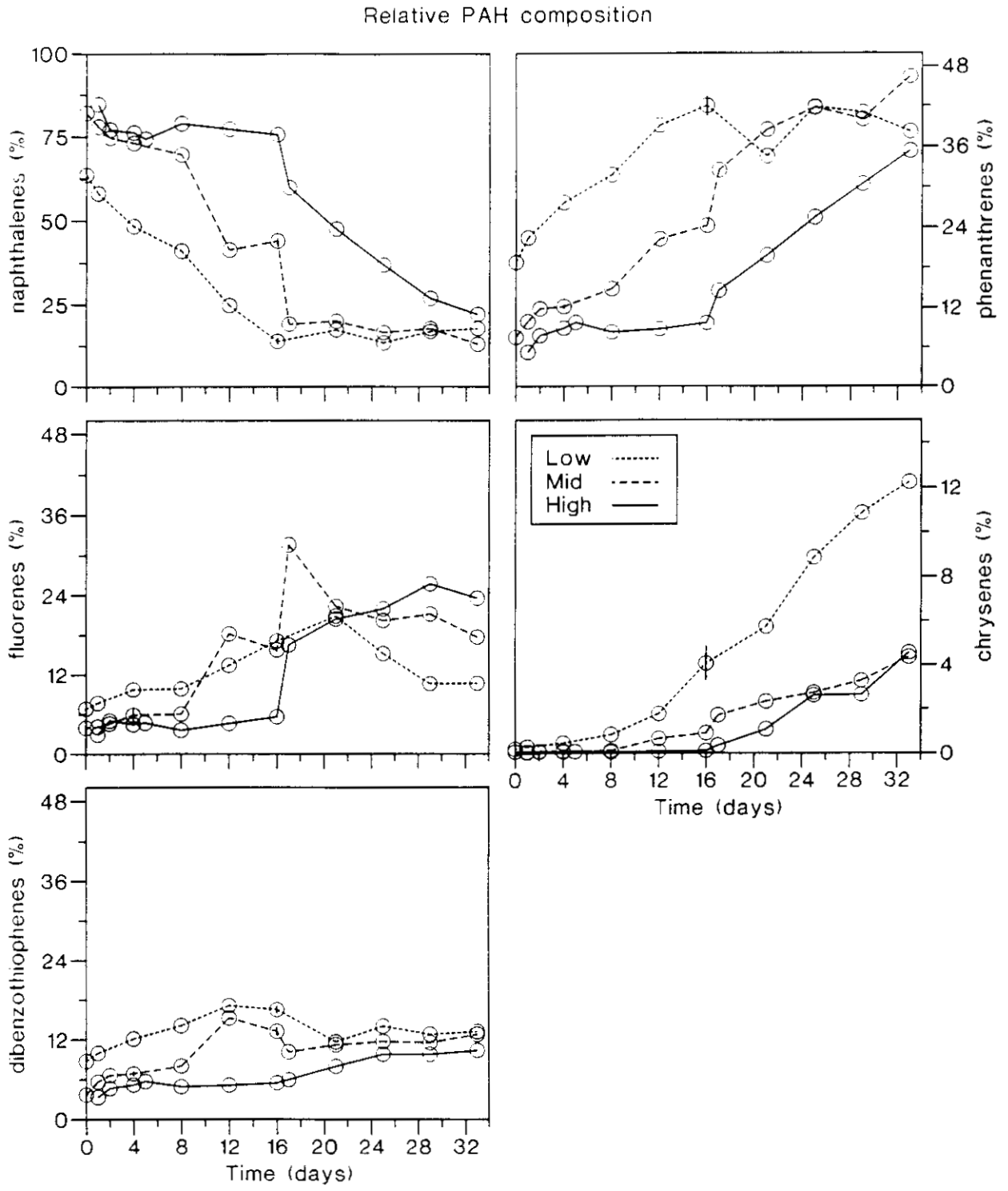

Figure 4.4. Changes in PAH composition as a function of time. Percentages of naphthalenes declined significantly over the course of the two experiments ($P < 0.001$). Percentages of fluorenes, dibenzothiophenes, phenanthrenes, and chrysenes increased significantly ($P < 0.001$). Trace-oil observations were not included because of marginal analyte detectability. Observations ≥ 16 d represent E1; all others represent E2. The majority of symbols represent single observations; where present, error bars indicate means ± SE.

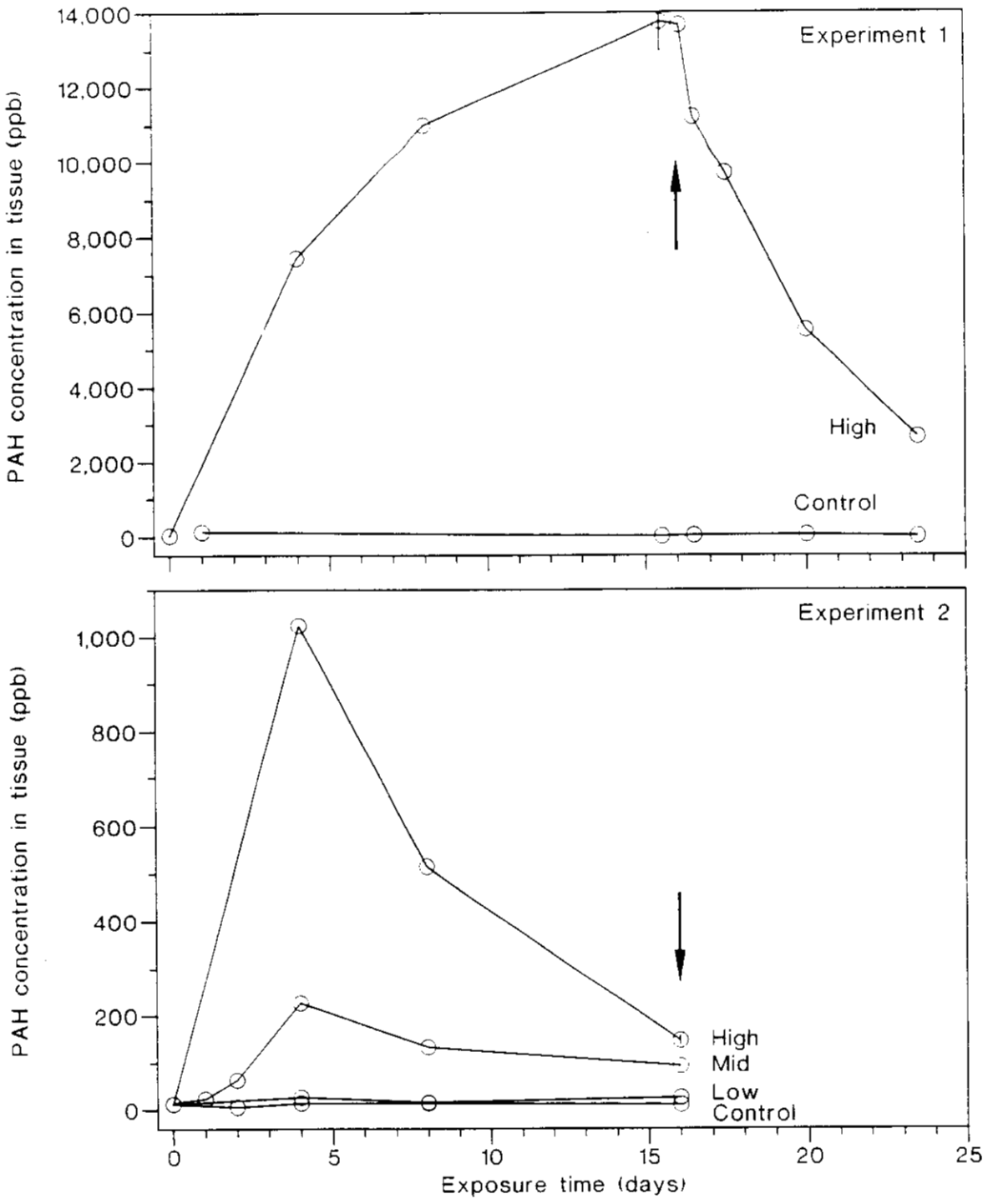

Figure 4.5. Concentrations of PAHs increased in egg tissue over the entire 16 d exposure in E1, but peaked after circa 4 d in E2. Arrows indicate when eggs were transferred to clean water. Only control and high-oil treatments were observed in E1. Depuration in clean water was not observed in E2. For clarity, the trace-oil concentration was not displayed in E2. Variance was estimated in high-oil treatments (only) on day 16.

Composition of PAHs in eggs was generally similar to that in water (compare Appendices 4.2 and 4.4). Some differences in composition were evident, but these differences were not consistent across experiments or treatments. In E1, naphthalenes through C1-naphthalenes were enriched in eggs, but C3-C4 naphthalenes were depressed; C2-naphthalene was enriched in some cases, but depressed in others. In E2, C2-C4 naphthalenes tended to be enriched relative to composition in water, but not in every treatment. Also in E2, composition of C2-C4 phenanthrenes was generally depressed in eggs relative to that in water. Composition of substituted chrysenes was also generally depressed in eggs relative to that in water. All differences in PAH composition between tissue and water phases were less than 17%, and were usually less than 10%.

Dose series

Experiment 1

Exposure of eggs to oil during incubation reduced incubation time. Peak and median hatch time were significantly shorter in the upper two treatments than in controls ($P < 0.001$) (Figure 4.6). Mean incubation time was reduced by 4 d in the high-oil treatment.

Egg survival, larval survival, and swimming ability were significantly reduced by exposure of eggs to oil during incubation ($P < 0.001$); in each case, responses in the upper two treatments differed significantly from those in controls (Figures 4.7 and 4.8). Absolute differences between the control and high-oil treatment response were 52, 16, and 88 for percent dead eggs, percent moribund plus dead larvae, and percent effective swimmers, respectively.

Exposure of eggs to oil during incubation caused morphological abnormalities in larvae. The percentage of larvae with spinal defects was significantly higher in the upper two treatments than in controls ($P < 0.001$) (Figure 4.8). Percentages of larvae with yolk-sac edema were significantly elevated in the upper three treatments ($P \leq 0.019$) (Figure 4.9). Percentages of larvae with pericardial edema were significantly elevated in the mid- and high-oil treatments (Figure 4.9). Percentages of larvae with abnormally small lower jaws significantly increased in the upper two treatments ($P < 0.001$). Differences between the control and high-oil treatment response were 69, 95, 27, and 97 for percent spinal defects, yolk-sac edema, pericardial edema, and small lower jaws.

Larval length was reduced by exposure of eggs to oil during incubation, and spinal curvature and yolk-sac volume increased. Length was significantly reduced in the upper three oil treatments ($P \leq 0.029$) (Figure 4.10). Curvature was significantly elevated in the upper two treatments, as was yolk-sac volume ($P < 0.001$) (Figure 4.10). Compared to controls, larval length in the high-oil treatment decreased by 3 mm, spinal curvature increased by 166°, and yolk-sac volume increased by 0.2 mm^3.

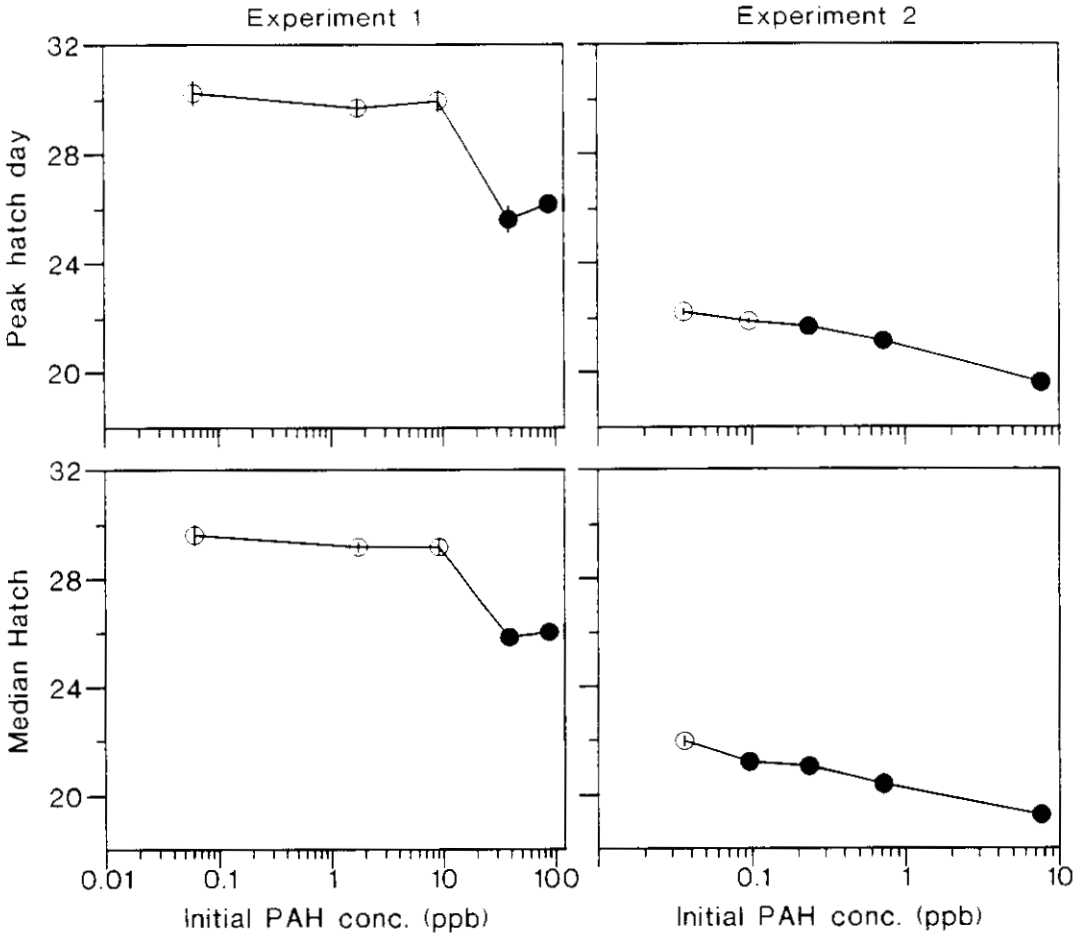

Figure 4.6. Exposure of herring eggs to oil during incubation stimulated early hatch. Hatch timing was measured by peak and median hatch day, as functions of PAH concentration; results were similar for both measures. Data displayed are means ± SE. Solid symbols indicate significant differences from controls.

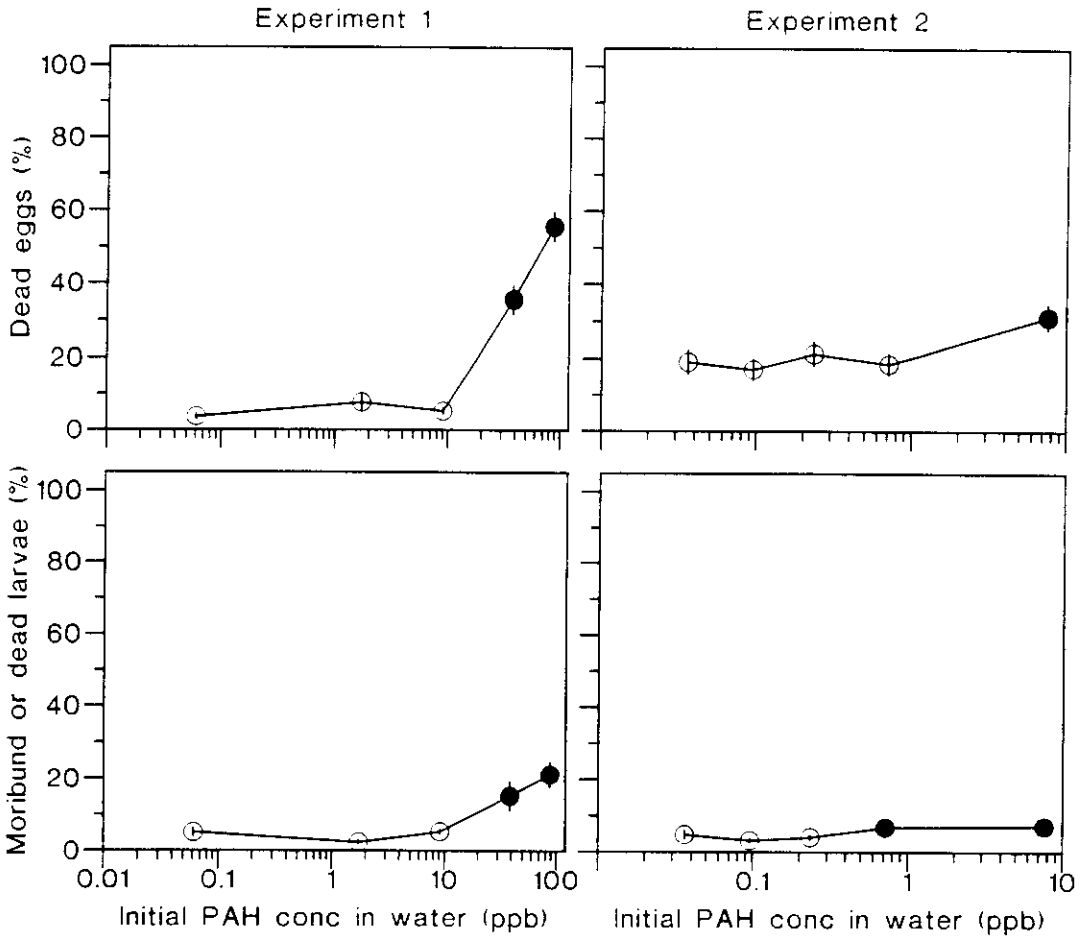

Figure 4.7. Exposure of eggs to oil during incubation caused egg and larval mortality. Larval mortality observations were generally completed within 24 h of hatch. Data displayed are means ± SE, as functions of PAH concentration. Solid symbols indicate significant differences from controls.

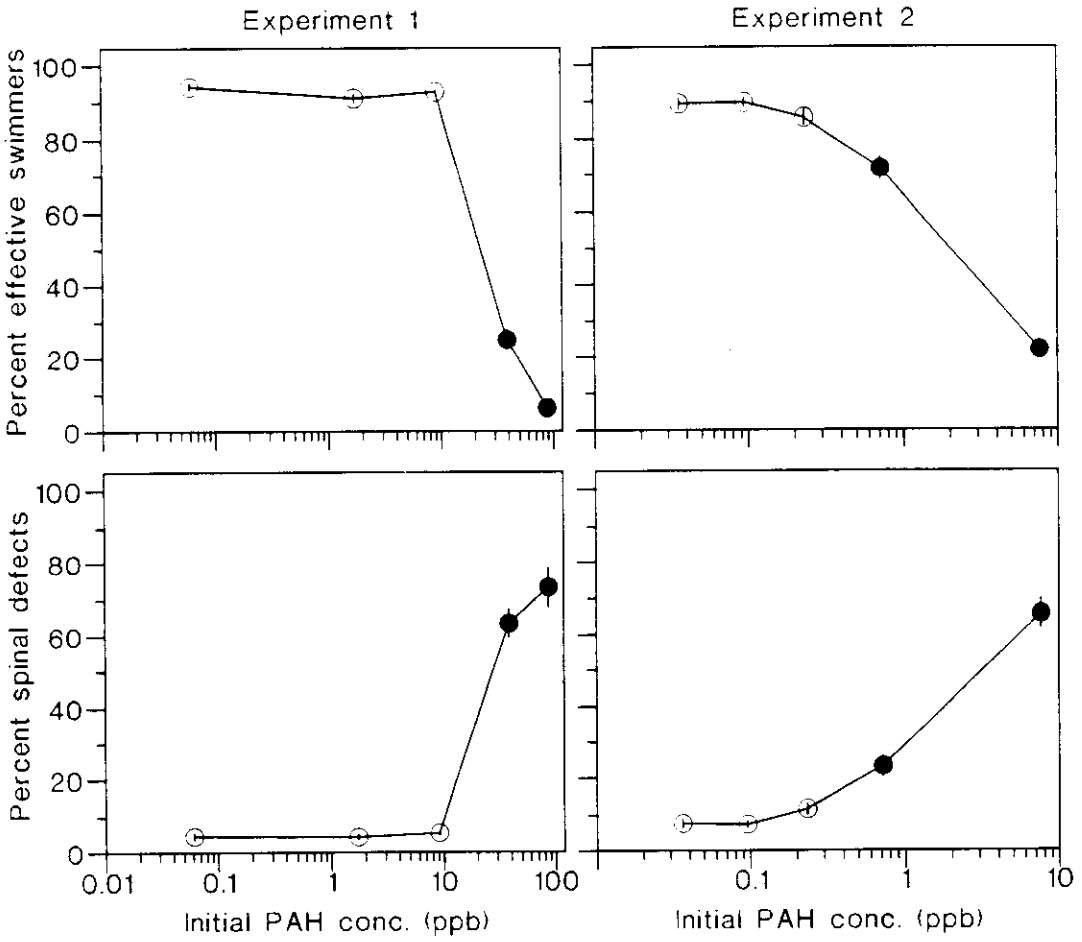

Figure 4.8. Larval swimming ability was reduced by exposure of incubating eggs to oil, and the incidence of spinal abnormalities increased. Data displayed are means ± SE, as functions of PAH concentration. Solid symbols indicate significant differences from controls.

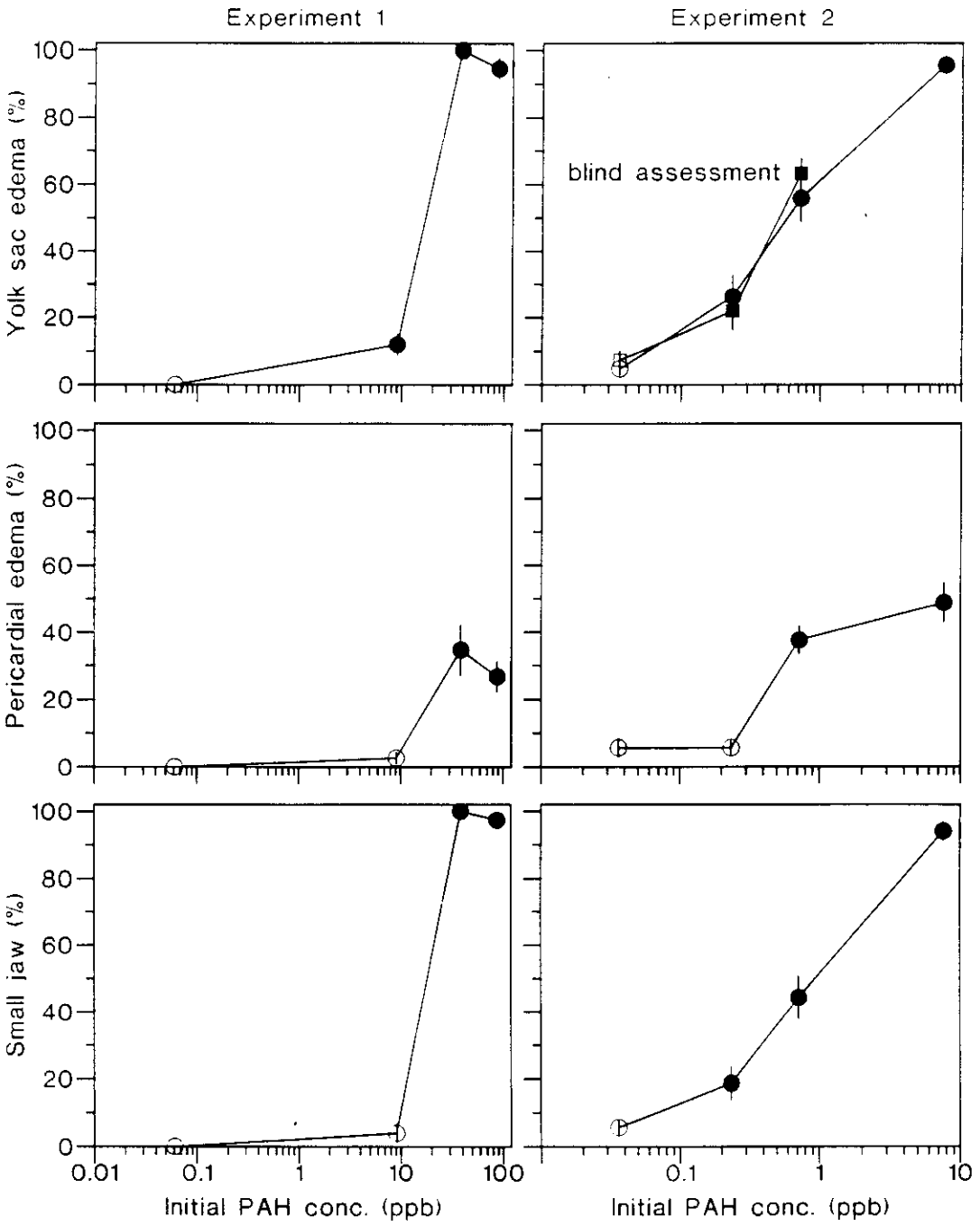

Figure 4.9. Edema was observed in larvae hatched from oil-exposed eggs, and the incidence of larvae with small lower jaws increased. Incidence of yolk-sac edema as verified by blind assessment in E2 (square symbols). Data displayed are means ± SE, as functions of PAH concentration. Solid symbols indicate significant differences from controls.

The ratio of yolk height to muscle width was significantly elevated in mid- and high-oil treatments ($P < 0.001$). For non-polluted situations, this ratio has been proposed as a way of distinguishing stage 1a, 1b, and 1c Pacific herring larvae (Humphrey et al. 1995). Incidence of immature larvae (stage 1a), categorized from yolk height to muscle width ratios, was significantly elevated in mid- and high-oil treatments ($P < 0.001$).

Comparison of E1 and E2

The magnitude of biological response was generally less in E2 than in E1, except that pericardial edema in E2 was about the same or slightly greater than that in E1 (Figures 4.6 to 4.10). However, pericardial edema in E1 tended to be masked by prominent downward rotation of the head. Responses in E2 were usually significant in the upper two treatments, except for percent dead eggs (Figures 4.6 to 4.10). Observed only in E2, results of a blind analysis of yolk-sac edema were highly similar to those obtained in-house (Figure 4.9).

Additional larval abnormalities, assessed only in E2, included a broader evaluation of skeletal and craniofacial defects, finfold defects, and failure to develop pectoral fin rays. Incidence of defects was significantly elevated at 0.7 ppb PAHs in every case ($P < 0.001$); the high-oil treatment (7.6 ppb) was not tested (Figure 4.11). The most frequently observed skeletal defects were kyphosis or lordosis of the notochord followed by stunting. Craniofacial abnormalities consisted of the reduction or absence of the lower jaw and, less frequently, the upper jaw. Occasionally smaller brain size (microcephaly) or reduced retinal pigmentation were also present in severely affected larvae. Most individuals with yolk-sac edema also had reductions in the width of the dorsal and ventral finfolds. Incidence of larvae without fin rays was significantly elevated in the low-oil treatment ($P = 0.034$) (Figure 4.11).

Anaphase aberrations were caused by exposure of eggs to oil during incubation. (Control, low-, and mid-oil treatments were examined in E2 only). Anaphase aberration was significantly elevated to 10% at 0.7 ppb PAHs ($P = 0.008$); aberration frequency was 6% in controls (Figure 4.11). The number of mitoses per pectoral fin averaged 11, and were not influenced by treatment.

Median effective concentrations

Median effective concentrations were calculable for some, but not all biological responses. Estimated EC50 values in E1 were substantially higher than those in E2, but were based on initial PAH concentrations and did not account for other variables, such as differential weathering. In E1, EC50s generally ranged from 19 to 35 ppb , except 61 ppb for egg death (Table 4.2). In E2, EC50s ranged from 0.3 to 3.4 ppb, with spinal defects producing the highest estimate (Table 4.2).

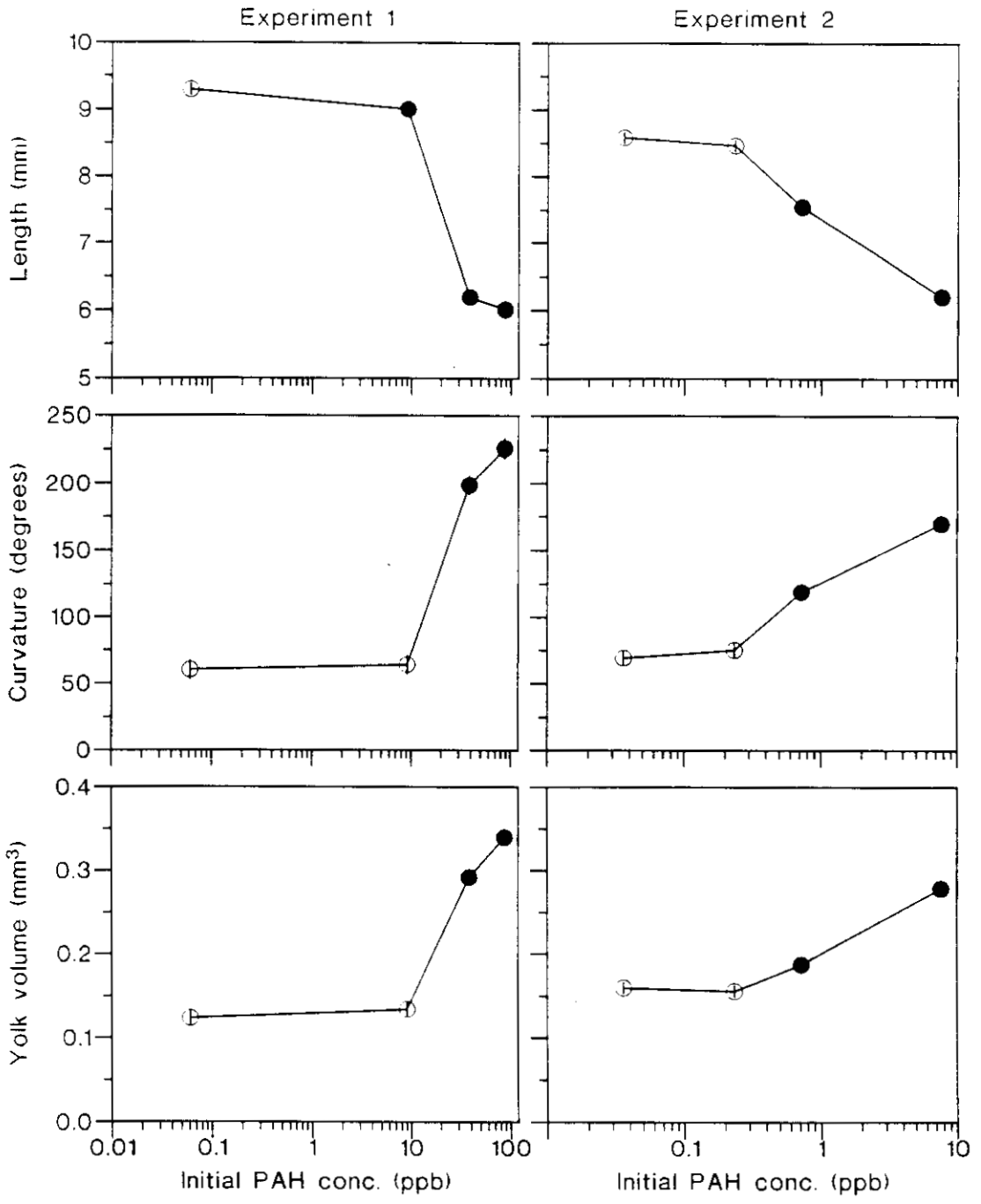

Figure 4.10. Larval length decreased as a result of oil exposure; body curvature and yolk volume increased. Data displayed are means ± SE, as functions of PAH concentration. Solid symbols indicate significant differences from controls.

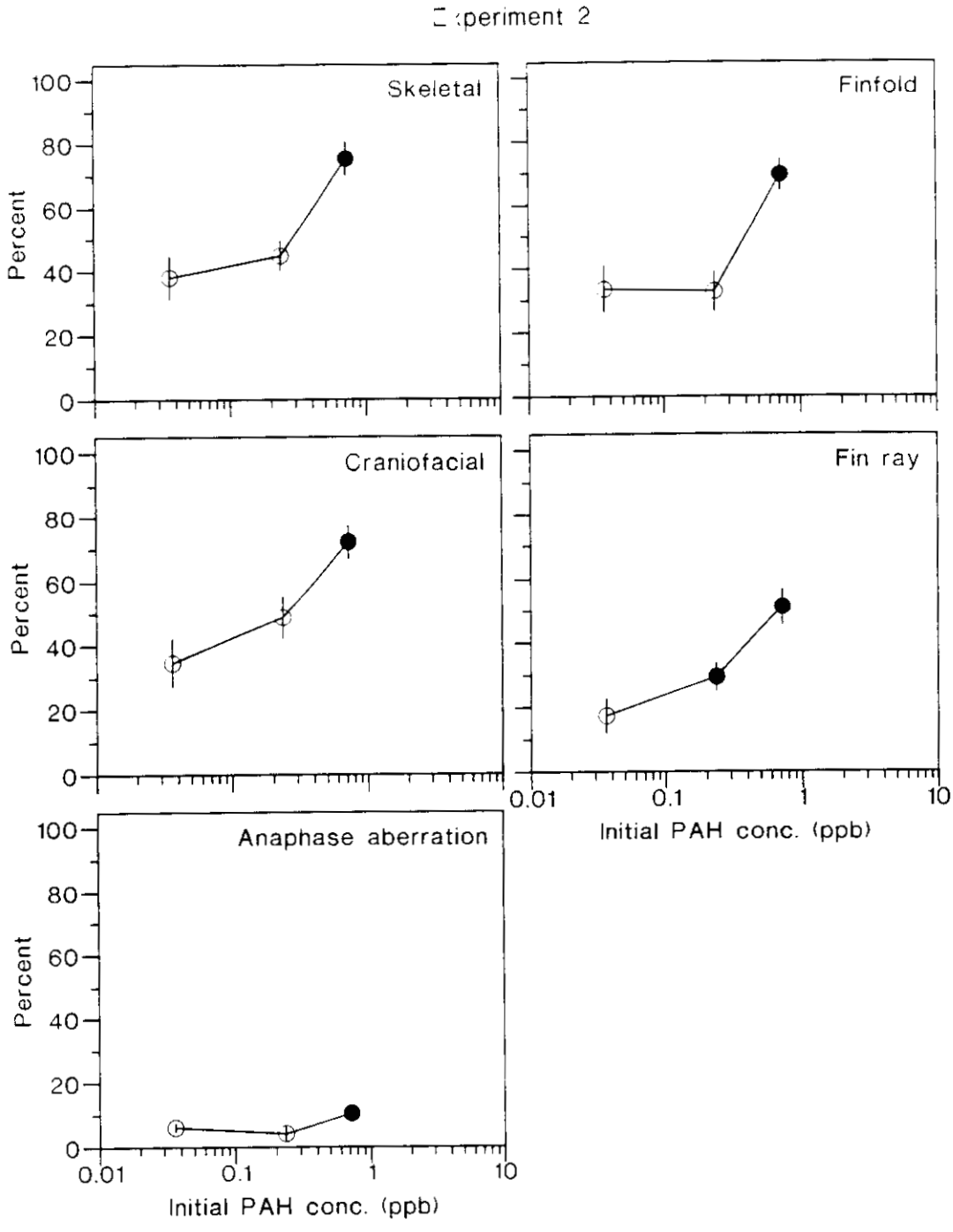

Figure 4.11. In E2, a random subsample of larvae were assessed blind for morphological deformities and genetic aberration. Skeletal, craniofacial, and finfold deformities increased as a result of oil exposure, and fin ray formation was delayed. The incidence of anaphase-telophase aberration also increased as a result of oil exposure. Data displayed are means ± SE, as functions of PAH concentration. Solid symbols indicate significant differences from controls.

Table 4.2. Median effective PAH concentrations (EC50s). Initial PAH concentrations in water were used for estimations; SE is standard error, n is number of calculable observations, nc is not calculable, - is not tested.

Response	Experiment 1 EC50	SE	n	Experiment 2 EC50	SE	n
Death						
egg	61	6.4	10	nc	nc	0
larvae	nc	nc	0	nc	nc	0
Abnormalities						
skeletal	-	-	-	0.3	0.1	8
spinal	35	3.2	13	3.4	0.5	17
craniofacial	-	-	-	0.3	0.0	8
finfold	-	-	-	0.4	0.1	10
yolk-sac edema	21	1.7	3	0.7	0.1	13
pericardial edema	nc	nc	nc	3.0	0.5	10
Developmental rate						
small jaw	21	0.7	4	0.9	0.2	14
absence of fin rays	-	-	-	0.5	0.1	7
Genetic						
anaphase aberration	-	-	-	nc	nc	0
Behavioral						
effective swimmers	19	1.2	15	2.3	0.2	21

Time series, E1 (mid-oil treatment)

In this section we show how much exposure time was required to elicit a biological response. For each biological parameter, the relationship of response and time was modeled with linear regression. Regressions were usually significant, although scatter, measured by r^2, tended to be large. Times at which responses became significant were determined with ANOVA.

Hatch time decreased as the time eggs were incubated in crude oil increased. Peak and median hatch times declined with increasing exposure time ($P < 0.001$); significant reductions in hatch time were detected after 8 d exposure (Figure 4.12). Estimated reductions in hatch time were -0.34 to -0.27 d per exposure day.

Percent hatching, larval survival, and swimming ability declined significantly as exposure time increased ($P < 0.001$). Percent egg hatch was significantly reduced after 2 d exposure, but not after 4 d; reductions were significant for ≥8 d exposures (Figure 4.12). Percentages of living larvae were significantly reduced after an 8 d exposure (Figure 4.13). The percentage of larvae that swam normally were significantly reduced by 4 d exposures (Figure 4.14). Hatch declined by 1.84%·d^{-1}, living larvae declined by 0.75%·d^{-1}, and effective swimmers declined by 4.86%·d^{-1}.

Incidence of morphological abnormalities increased significantly with increasing exposure time ($P < 0.001$). Incidence of spinal abnormalities and yolk-sac edema became significant after 4 d exposure (Figures 4.14 and 4.15). Incidence of pericardial edema was significant after 8 d exposure (Figure 4.15). Incidence of larvae with small or missing lower jaws became significant after 2 d exposure (Figure 4.15). Incidence of abnormalities increased by 6.65%·d^{-1}, 2.53%·d^{-1}, and 6.27%·d^{-1} for yolk-sac edema, pericardial edema, and larvae with small jaws, respectively.

Larval length decreased significantly as exposure time increased, and spinal curvature and yolk-sac volume increased significantly ($P < 0.001$); 2 d exposures caused significant changes in each case (Figure 4.16). Length decreased by 0.20 mm·d^{-1}, spinal curvature increased by 9.13°·d^{-1}, and yolk-sac volume increased by 0.010 mm^3·d^{-1}.

Comparison of E1 and E2

As observed in the dose series, the magnitude of biological response in E2 time series tests was less than in E1. Magnitudes of corresponding slopes were smaller in E2 than E1, and in two cases, percent eggs hatched and percent larvae alive, regression slopes were not significant. In E2, significant biological responses were observed after 4 to 16 d exposure, except no significant reduction in percent hatch occurred (Figures 4.12 to 4.16). In some cases, the exposure time required to elicit a significant response was the same in E2 as in E1, (e.g., percent effective swimmers and spinal defects) while in other cases, longer exposures were required (e.g., larval length and yolk volume). Observed only in E2, results of a blind analysis of yolk-sac edema were similar to those obtained in-house; slopes were parallel, but the blind assessment indicated a slightly greater frequency of edema ($P_{\text{analysis of covariance}} = 0.001$) (Figure 4.15).

Other larval abnormalities, assessed only in E2, included general skeletal and craniofacial defects, finfold defects, and failure to develop pectoral fin rays. Incidence of defects increased significantly with exposure time in every case ($P < 0.001$); except for skeletal defects, these responses were significant after 8 d exposure (Figure 4.17). The magnitude of these responses were similar, ranging from 2.14 to 2.58%·d^{-1}.

Swimming and larval condition

The ability of newly hatched larvae to swim was likely a function of physical condition; defective larvae were less able to swim normally. Spinal condition was the most important

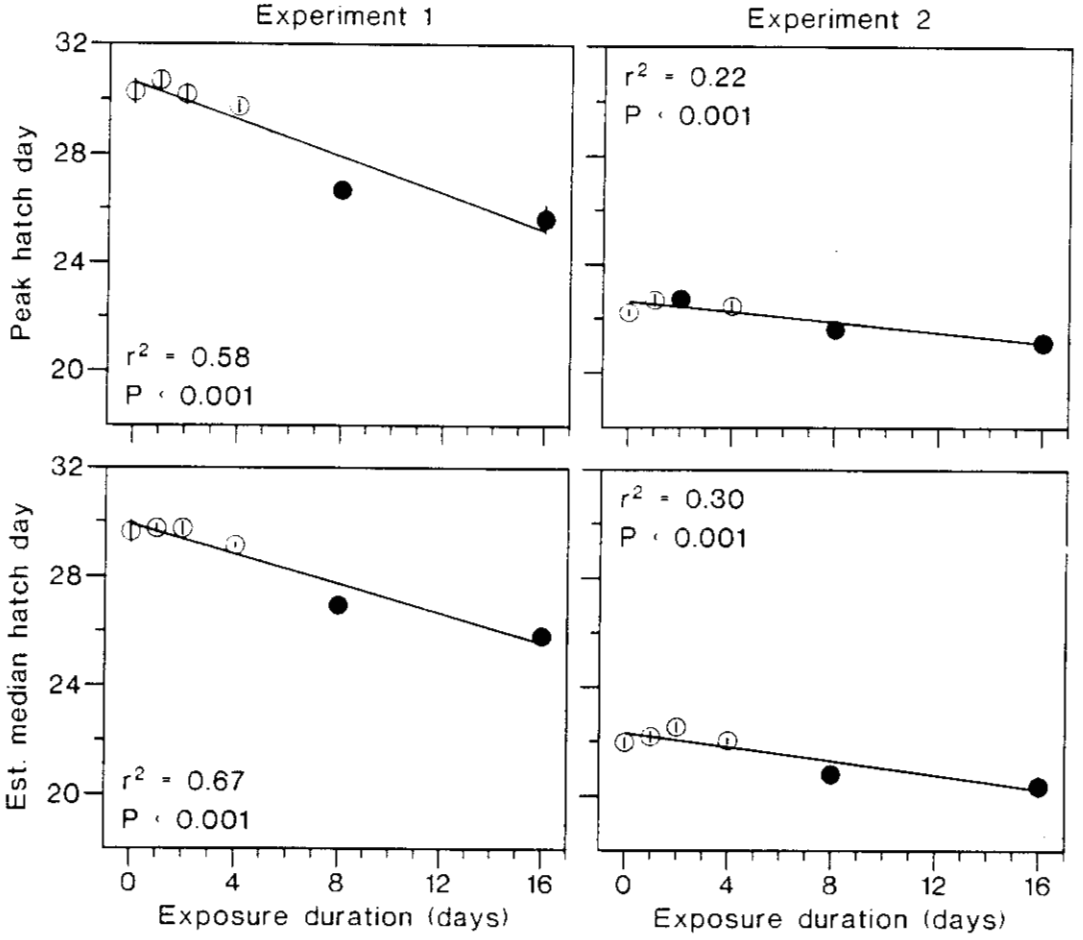

Figure 4.12. Hatch timing, measured by peak hatch day and by median hatch day, declined as exposure time increased. Initial PAH concentrations were 38 ppb in E1 and 0.7 ppb in E2. Data displayed are means ± SE and fitted regression lines. Solid symbols indicate significant differences from controls.

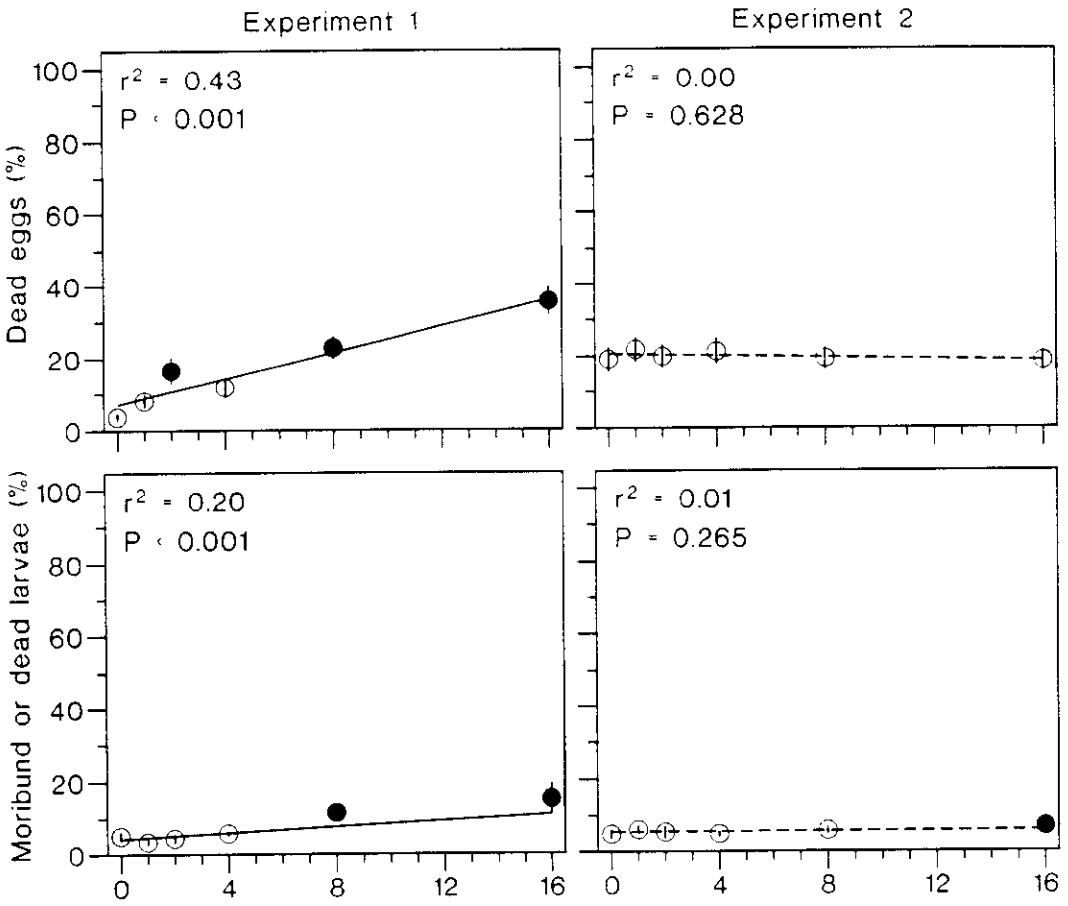

Figure 4.13. In E1, egg and larval mortality increased as exposure time increased, but regressions were not significant in E2. Initial PAH concentrations were 38 ppb in E1 and 0.7 ppb in E2. Data displayed are means ± SE and fitted regression lines. Solid symbols indicate significant differences from controls. Non-significant regression lines are dashed.

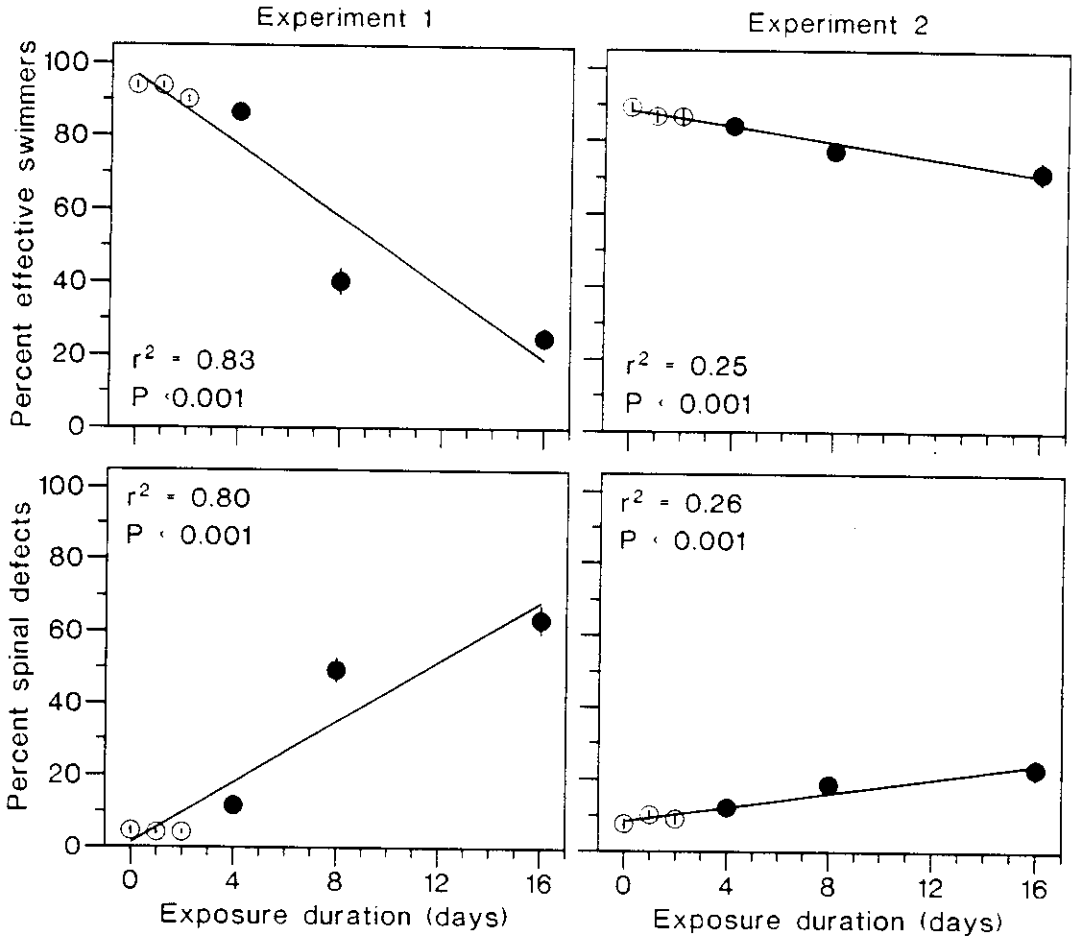

Figure 4.14. Larval swimming ability declined as exposure time increased, and the incidence of spinal abnormalities increased. Initial PAH concentrations were 38 ppb in E1 and 0.7 ppb in E2. Data displayed are means ± SE and fitted regression lines. Solid symbols indicate significant differences from controls.

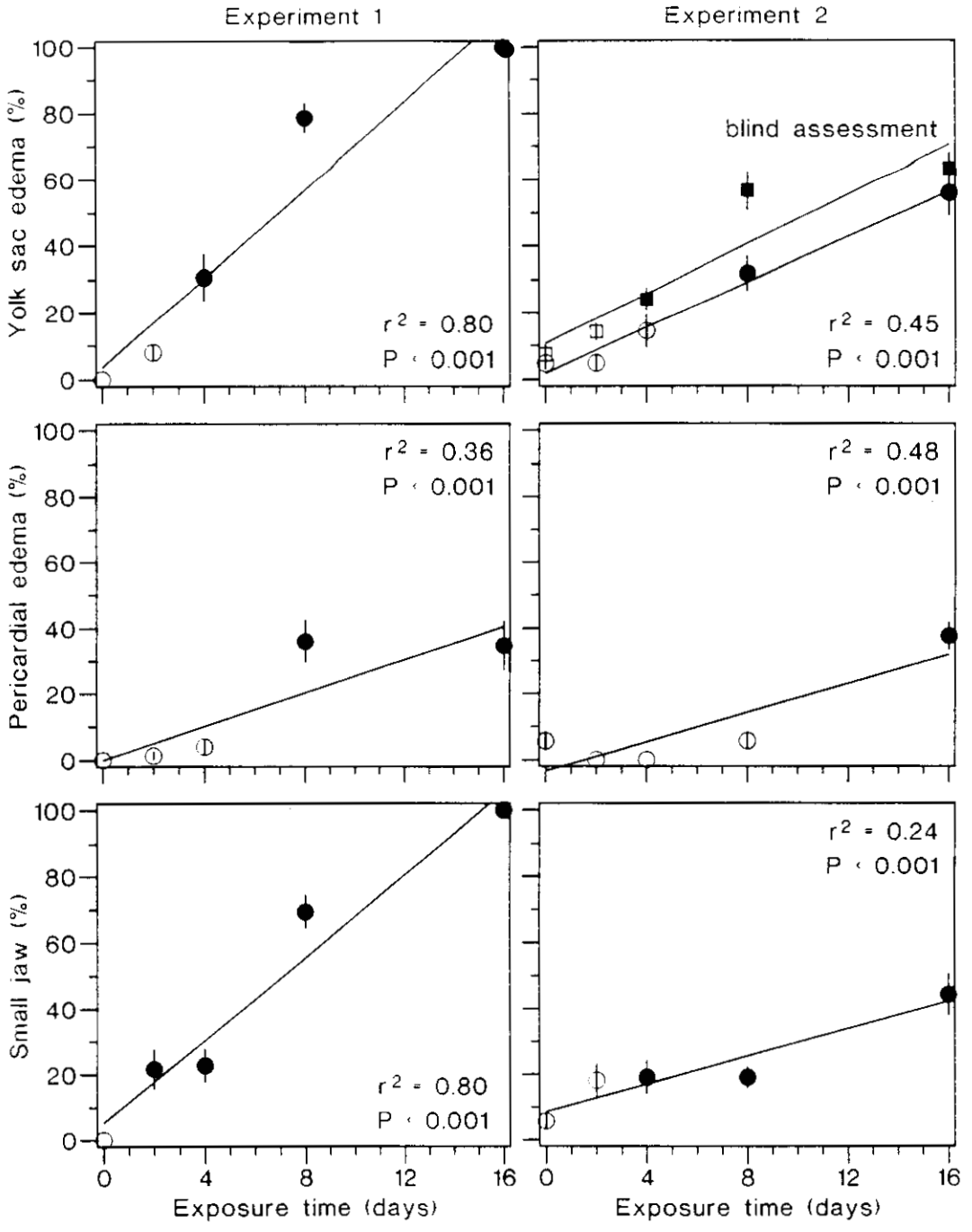

Figure 4.15. Edema increased as exposure time increased, and the incidence of larvae with small lower jaws increased. Initial PAH concentrations were 38 ppb in E1 and 0.7 ppb in E2. Incidence of yolk-sac edema as verified by blind assessment in E2 (square symbols). Data displayed are means ± SE and fitted regression lines. Solid symbols indicate significant differences from controls.

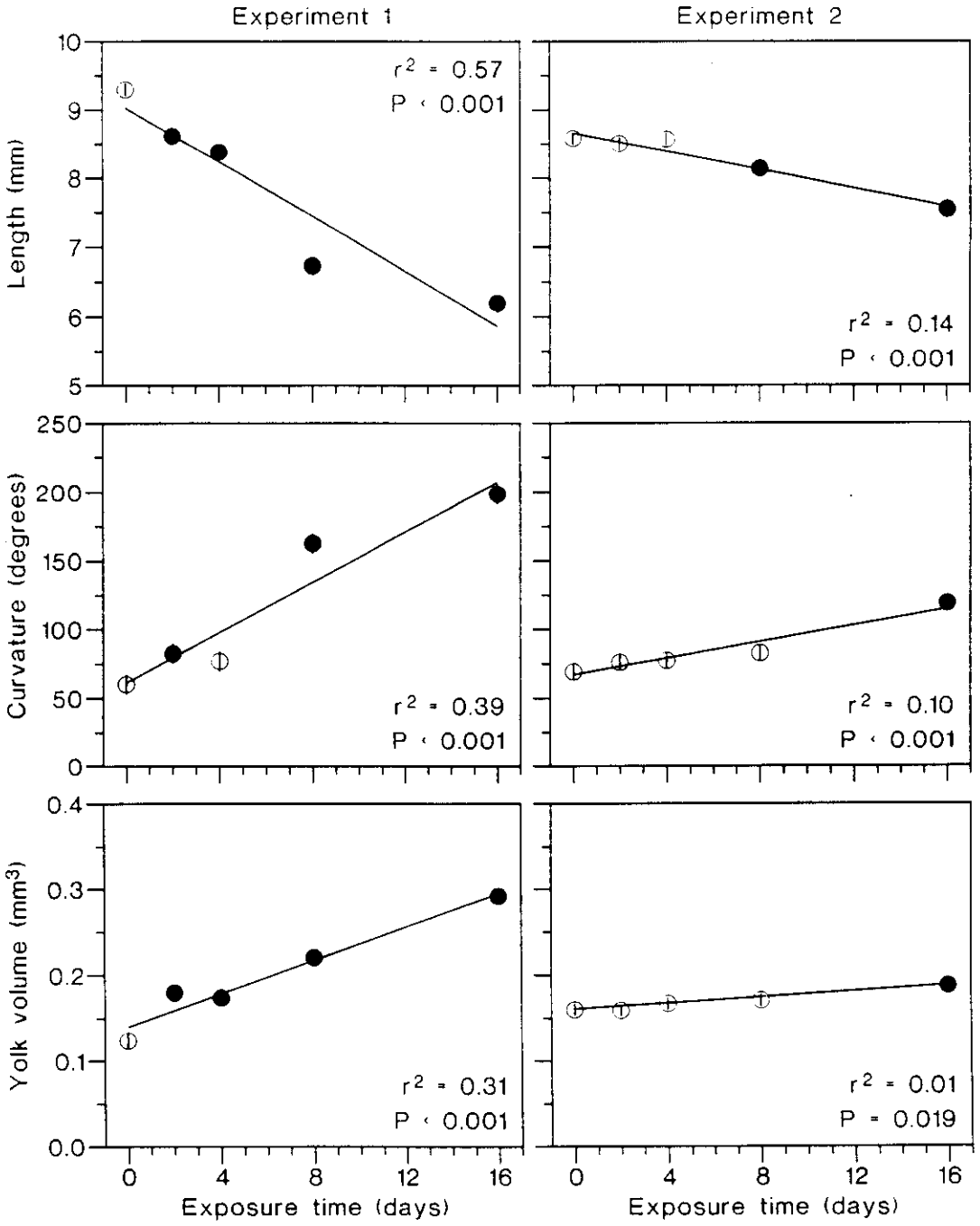

Figure 4.16. Larval length decreased as exposure time increased; body curvature and yolk volume increased. Initial PAH concentrations were 38 ppb in E1 and 0.7 ppb in E2. Data displayed are means ± SE and fitted regression lines, as functions of PAH concentration. Solid symbols indicate significant differences from controls.

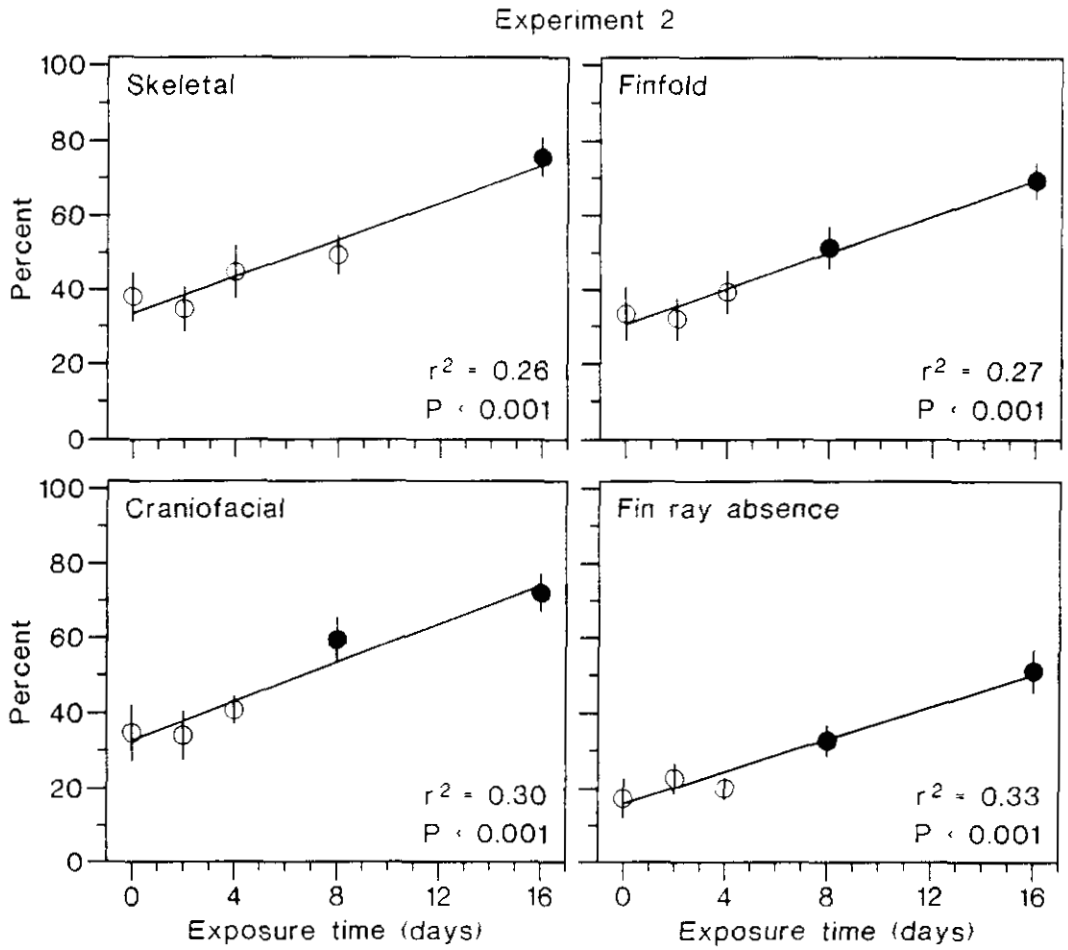

Figure 4.17. In E2, a random subsample of larvae were assessed blind for morphological deformities. Initial PAH concentration was 0.7 ppb. Deformities increased as exposure time increased, and fin ray formation was delayed. Data displayed are means ± SE and fitted regression lines. Solid symbols indicate significant differences from controls.

predictor of swimming ability (partial $r^2 = 0.906$, $P < 0.001$) (see also Figure 4.8). Tested with stepwise regression, lower jaw size was the second predictor (partial $r^2 = 0.021$, $P < 0.001$), and yolk-sac edema was third (partial $r^2 = 0.002$, $P = 0.018$); pericardial edema did not enter the equation. Although all dose and time series data from both experiments were included in this analysis, logical subgroupings produced comparable results.

DISCUSSION

Exposure of herring eggs to oil during incubation caused mortality as well as a number of sublethal effects, including morphological abnormalities, reduced swimming ability, and genetic damage. Incubation time declined with increasing PAH concentration; exposed larvae were physiologically more immature and smaller than unexposed larvae. Most of these adverse responses to oil have been reported elsewhere (e.g., Linden 1976; Rosenthal and Alderdice 1976; Pearson et al. 1985; Kocan et al. 1996); however, this is the first study to link chemistry with effects thresholds to compare the sensitivity of the observed responses. This is also the first report of mortality and biologically significant sublethal effects consistently occurring after exposure to PAHs in the low parts-per-billion range.

The intent of this study was to expose herring eggs to oil concentrations in the range encountered in PWS following the spill, and to ensure that the composition of this oil was similar to that spilled. Thus the oil chosen for study, pre-weathering, and the toxicant delivery method were designed to mimic conditions observed in PWS. After the spill, PAH concentrations in open seawater ranged up to 6.24 ppb, and concentrations up to 1.59 ppb were observed five weeks after the spill (Short and Harris 1996a). It is likely that concentrations were even higher in intertidal spawn areas where oil was stranded on beach substrate and reworked by each tide. In our E2, initial PAH concentrations only exceeded values reported by Short and Harris (1996a) in the highest treatment (7.6 ppb PAH). In an oil-weathering experiment employing similar toxicant delivery apparatus, Short and Heintz (In press) concluded first-order rate loss kinetics accounted for PAH weathering processes in the laboratory and for the dominant weathering processes PWS. Thus the biological responses observed in these experiments should accurately reflect conditions that occurred in PWS in 1989.

Biological response to oil

Despite the low exposure concentrations tested in these experiments, significant egg and larval mortality was observed. This may explain field observations in 1989 in PWS that egg-to-larval mortality was three to six times higher at oiled sites than at unoiled sites (McGurk and Brown 1996); although deposition of eggs was approximately equal, estimated larval production was 200 times higher at unoiled areas (Brown et al. 1996). Since oiled sites tended to be more subject to turbulence than unoiled sites; however, oil exposure may not have been the only significant factor that differentially influenced instantaneous mortality rates in PWS.

Of the observed abnormalities, edema was a highly sensitive measure of oil exposure, and likely reduced survival potential. Edema was induced in larvae exposed to initial PAH concentrations as low as 0.2 ppb and was apparent after only a 4 d exposure to 0.7 ppb PAH. In mild cases, slight edema was visible only in the yolk sac; more severe cases were accompanied by increased fluid within the pericardial cavity and the ventricular spaces of the nervous system. The incidence and severity of edema were directly related to exposure duration and dose. Most individuals with yolk-sac edema also had reductions in the width of the dorsal and ventral finfolds, which are the respiratory surfaces in pelagic fish larvae. Thus, the increased energetic demands of swimming with edema would be exacerbated by lowered respiratory potential.

Two important consequences of the observed sublethal effects would be impairment of swimming and feeding in oil-exposed larvae. Although spinal deformation appeared to be the dominant factor, observation of live larvae suggested that edema also adversely affected swimming. Larvae with enlarged yolk sacs had greater difficulty orienting in the water column than normal larvae, and appeared to swim more slowly, even with apparent vigorous exertion. Physical alterations due to edema, concomitant physiological changes (decreased blood flow to tissues, interference with nervous system function and increased energy expenditures), reduced finfold surface area, and retarded pectoral fin development undoubtedly contributed to decreased larval swimming ability. As spinal curvature became more pronounced, larvae were less able to swim normally; at the extreme, larvae were only capable of swimming in a circle, or spasmodic twitching - movement that resulted in no directed motion. The development of yolk-sac edema, spinal deformities, and swimming problems were correlated temporally and in terms of dose, implying all these abnormalities had a causal role in the loss of swimming ability. Decreased swimming ability likely reduced the ability of larvae to capture prey and to avoid predators. Premature hatching of larvae with immature or absent jaws would also be expected to severely limit prey capture (von Westernhagen 1988). Marty et al. (In press (a)) found that larvae from oiled areas in PWS which had higher incidences of edema also grew less and had significantly less food within their gastrointestinal tract than did larvae from unoiled areas.

Although oil-exposed eggs hatched early, embryonic growth was retarded, as indicated by shorter body length, larger yolks, smaller lower jaws, and immature pectoral fins. Because swimming ability improves with maturity, immature larvae are also probably less capable of prey capture and predator avoidance than mature counterparts. Although categorization of larvae by developmental stage indicated treated larvae were less mature than controls, estimates of physiological development based on the egg to muscle width relationship described by Humphrey et al. (1995) may be misleading because of yolk-sac edema.

Genetic damage was also induced in oil-exposed larvae. Although not as sensitive a response as edema, pectoral fin development, and finfold reduction, genotoxicity was significantly elevated following exposure to an initial PAH concentration of 0.7 ppb. Our genotoxicity endpoint, anaphase aberration rate, measured microscopically visible chromosome/chromatid breaks and bridges during the later stages of mitosis. In a previous study using an oil-water dispersion of Prudhoe Bay crude oil, the most sensitive endpoints were the

anaphase aberration rate, craniofacial defects, and finfold abnormalities. This method has been previously used to demonstrate genotoxicity in field studies of the *Exxon Valdez* oil spill (Hose et al. 1996; Brown et al. 1996), the *Argo Merchant* oil spill (Longwell 1977), the New York Bight (Longwell and Hughes 1980), and in sediment from San Francisco Bay (Long et al. 1990). In each case, mitotic aberrations were quantitatively or spatially related to oil. The consequences of the low level of genetic damage observed in this study cannot be predicted but might include reductions in successful cell division and growth. It may be possible to develop more sensitive endpoints using methods to assess breakage of individual chromosomes.

In sharp contrast to the distinct biological responses caused by exposure of incubating herring eggs to oil, exposure of adult herring to similar quantities of oil shortly before spawning did not produce any discernable effects in progeny (Carls et al. Chapter 3). The clear implication is that contamination of intertidal habitat where herring eggs incubated in PWS likely caused adverse developmental effects, but exposure of reproductively ripe herring as they migrated to spawning grounds was reproductively inconsequential. However, neither study addressed whether the presence of oil in water affected migratory behavior, site selection, or spawn timing.

Toxicity of weathered oil

Based on concentration alone, a stronger biological response to oil was expected in E1 than in E2 because oil concentrations were much higher in E1. Nonetheless, biological responses in the upper two treatments of both experiments were usually significant. Had the study ended after the first experiment, the conclusion would have been that initial PAH concentrations in water of about 9 ppb could cause significant problems in developing herring eggs, and that smaller concentrations would not cause significant biological response. When the second experiment was completed with all initial PAH concentrations below the originally predicted effective concentration; however, significant biological responses occurred, and the predicted initial effective dose fell to 0.2 ppb.

Based on biological response, it is apparent that weathered oil is relatively more toxic than unweathered oil. Similar results were obtained when pink salmon eggs were incubated in oiled rock; eggs incubated in weathered rock were affected more strongly than was predicted by concentration alone (Heintz and Short, In prep.). The implication is that oil persistent in intertidal spawning areas may have adversely affected egg development a year or more after the spill. Such a circumstance was documented for pink salmon; egg mortality in oiled streams was higher than in unoiled streams for three years after the spill (Bue et al. 1996).

Two factors, time and initial oil concentration, were important in the oil-weathering process. As time progressed, concentrations of lighter aromatics declined relative to those of heavier aromatic compounds. Within homologous groups, concentrations of parent compounds declined more rapidly over time relative to substituted homologs. Oil weathered more rapidly on lightly oiled rock than heavily oiled rock; with less oil available and thinner layers of oil, water flow (equal in all treatments) depleted the lighter aromatic compound reservoir more rapidly in

lightly oiled rock. Some weathering, due to volatilization, may also have taken place when the rock was allowed to stand in air.

Due to time-dependent weathering, differences in PAH composition were obvious within the same treatments between experiments. In these comparisons, concentrations of heavier aromatics were greater than those of lighter aromatics in the second experiment. This leads to the general inference that the toxicity of heavier PAHs (e.g., phenanthrenes and chrysenes) is greater than toxicity of lighter PAHs (e.g., naphthalenes). Increases in toxicity of PAHs with increasing molecular size has been documented in other studies and summarized by Rice et al. (1977).

Up to this point, discussion of PAH composition in the less and more weathered experiments has been presented on a relative basis. Comparison of PAH concentrations on an absolute basis supports the idea that larger PAH molecules are more toxic, including increased alkylation within homologous familes; except in naphthalenes, concentrations of the more substituted compounds and all chrysenes in E2 generally equaled or exceeded those in E1. However, this raises an interesting chemical problem - what was the underlying mechanism responsible for these relationships?.

We expected oil would be lost from rock in the oil generators as time progressed; more specifically, we expected the slope of each component PAH concentration in water would be negatively correlated with time. Under this hypothesis, time-dependent changes in PAH composition would be entirely dependent on differential loss rates. In practice, most PAH concentrations were negatively correlated with time, except that concentrations of the more refractory (multi-ring, and more substituted components) did not correlate with time (C3-fluorenes, and C1-chrysenes) or were positively correlated with time (C3-dibenzothiophenes, C3- and C4-phenanthrenes, chrysene, and C2-chrysene), particularly in the uppermost oil treatment (most C3- and C4-chrysene concentrations were below minimum detection limits). These relationships were not as clear in an earlier experiment, but concentrations of C3-fluorenes, C3-dibenzothiophenes, C4-phenanthrenes, chrysene, and C1-chrysene were constant over time; C2-chrysenes were positively correlated with time (Carls et al, Chapter 3). The presence of constant or increasing concentrations of components suspected of disproportionately high toxicity explains the high toxicity of weathered oil, but brings the mechanism whereby this might happen into question. We suggest that the oil-coated rock acted as a chromatographic column: component PAH molecules may have migrated through the column at different rates, depending on their tendency to re-enter the organic phase. The fact that the rock column was twice as long in the high-oil treatment as in other treatments, and that more oil was present, thereby increasing the probability of resorption, is in keeping with observation. More study is required to confirm or refute the chromatographic column hypothesis.

The issue of relative PAH toxicity may be more complex than indicated by simple comparison of composition within treatments. Two factors lead to awareness of increased complexity in interpretation of PAH toxicity. First, differential weathering rates as a function of initial hydrocarbon concentrations yielded treatments with different composition at the outset of

experimentation. Second, total PAH concentrations in the low-oil treatment of E1 were very similar those in the high-oil treatment of E2 (Figure 4.1). Additionally, biological responses in the low-oil treatment of E1 were generally not significant, but responses in the high-oil treatment of E2 were usually significant. Because total PAH concentrations were very similar in the two experiments, we suggest differences in composition caused the distinctly different biological responses. In the $E2_{high}$ treatment, there were consistently more C4 naphthalenes, fluorenes, and compounds heavier than C2 phenanthrenes than in the $E1_{low}$ treatment (Figure 4.18). Phenanthrene concentrations were initially greater in $E1_{low}$ than in $E2_{high}$, but were greater in $E2_{high}$ by day 8. Comparison of these two specific treatments supports the idea higher molecular weight PAHs are more toxic, but also suggests that the more substituted homologs may be more toxic than less substituted homologs. The same conclusions were reached when PAH percentages were compared (Appendix 4.4.2 and 4.2.8). It has been previously reported that alkylation of aromatic rings leads to increased toxicity (Rice 1985; Rice et al. 1977) These two factors (ring size and substitution) are confounding, thus the specific compounds responsible for increased toxicity of weathered oil in our tests were not readily identifiable.

Because the composition of PAHs that accumulated in egg tissues was generally similar to that in water (usually within 10%), the previously presented comparison of PAH composition in water appears to be a reasonable first approximation for describing chemical toxicity. Composition of PAHs in tissue of pink salmon eggs and alevins was also similar to that in treatment water in a study by Marty et al. (In press (b)). Some minor differences in PAH composition did occur between tissue and water phases (generally less than 10%). These differences, however, were not consistent across experiments, thus interpretation was not clear. For example, in E1, naphthalene through C1-naphthalene composition was enriched in tissue relative to that in water, but composition of C3- to C4-naphthalenes was depressed; these enrichments and depressions declined over time. In contrast, C2- to C4-naphthalenes in E2 were generally, but not always, more enriched in tissue. Others have observed more rapid uptake rates of lighter PAHs by teleosts, but after sufficient time, accumulation of heavier PAHs may exceed that of lighter PAHs (Kuhnhold and Busch 1978; Sharp et al. 1979; Solbakken et al. 1984).

Improved determination of which compounds contributed to toxicity might have been possible had tissues from all treatments been analyzed, but the fact that percentages of heavier compounds were consistently greater in egg tissues in E2 than in E1 also suggests toxicity increases with molecular weight.

Bioaccumulation, depuration, and chemical toxicity

Observed biomagnification in E1 (406 times) was similar to that reported in ova (317 times) of similarly exposed adult herring (Carls et al. Chapter 1). Biomagnification of mono- and di-aromatics in ova of herring exposed to the WSF of crude oil was somewhat lower (170 - 240 times) (Rice et al. 1987), but others have observed accumulation increases with ring size and methylation (Lee et al. 1972; Korn et al. 1977; Roubal et al. 1977).

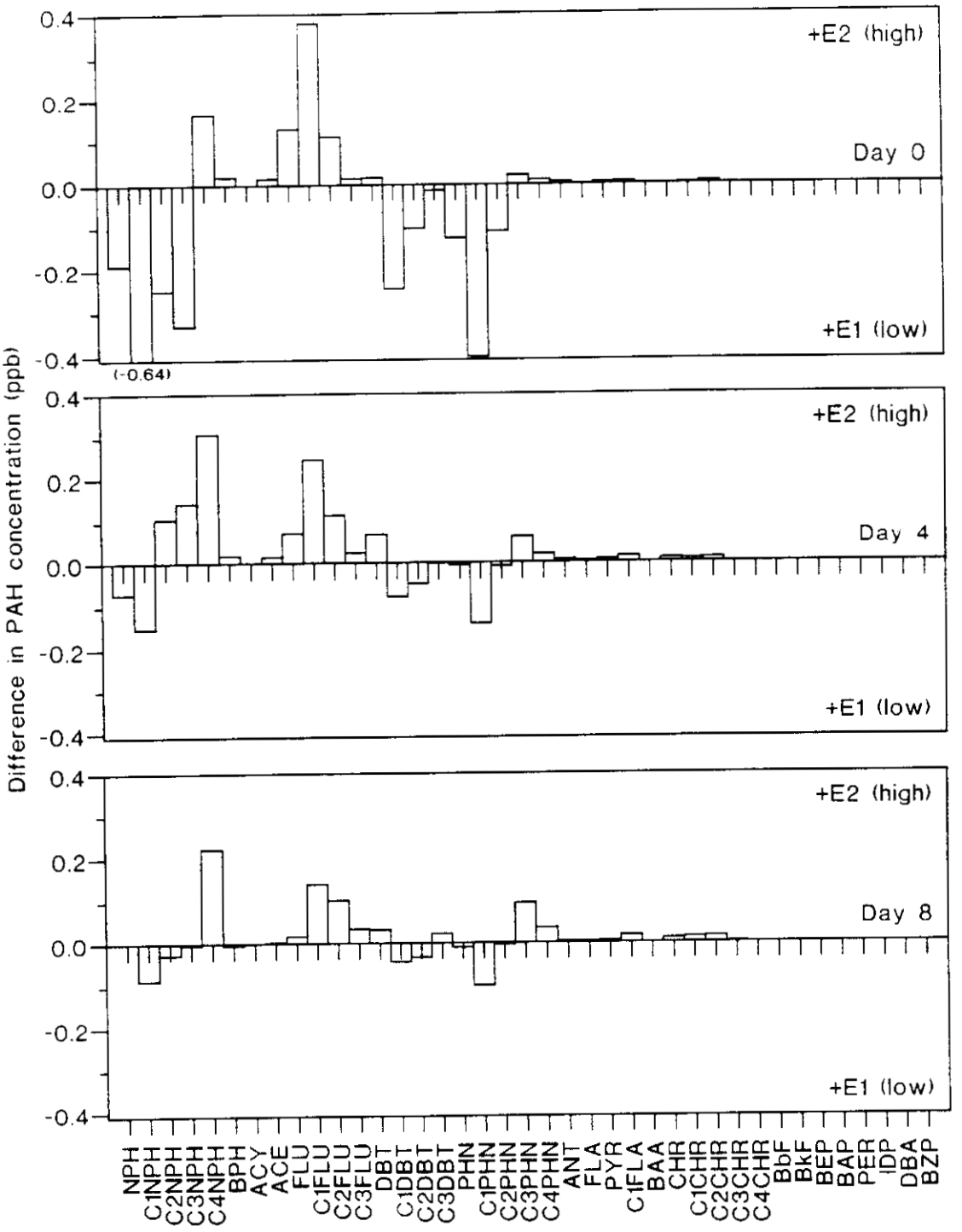

Figure 4.18. Comparison of PAH composition in the low-oil treatment of E1 (initial PAH concentration was 9 ppb) and the high-oil treatment of E2 (initial PAH concentration was 8 ppb). Total PAH concentrations in these two treatments were highly similar throughout the exposure period. Values greater than zero indicate higher relative composition in E2 than in E1.

The pattern of bioaccumulation observed in the more weathered exposures was not anticipated; PAH concentrations in eggs peaked within circa 4 d, then declined. Declines may have simply been caused by the entropic relationship between PAH accumulation in tissue and loss in water. Another possibility was active metabolism and elimination of hydrocarbons by eggs. In juvenile and adult fish with inducible mixed function oxidase (MFO) enzymes, including herring, similar patterns of PAHs decline from tissue have been observed, even when PAH concentrations in water or food remained elevated (Schwartz 1985; Thomas et al. 1997). Cod eggs may metabolize aromatic hydrocarbons (Solbakken et al. 1984), thus occurrence of MFO activity in herring eggs is plausible. We recommend that additional egg exposures should be conducted to determine MFO activity according to the immunohistochemical procedures of Smolowitz et al. (1991).

The biological response of herring eggs to oil and weathered oil suggests that observed effects were due to chemical toxicity, not other factors such as surface coating as has been suggested by some researchers (Pearson et al. 1985). This conclusion is based on several factors, including the dissolved state of PAHs, toxicity at very low concentrations, absence of visible coating, absence of visible slicks in incubation tanks, and demonstration of PAHs in internal tissues of similarly exposed adult herring. Had surface coating been the mechanism causing biological effects, toxicity should have been dramatically reduced at lower concentrations because significantly less coating would have been possible. Instead, toxicity in E2 remained high even though absolute PAH concentration dropped more than an order of magnitude, and effective concentrations were less than 1 ppb. Eggs were carefully inspected visually for evidence of oil coating, but no coating was observed. Furthermore, the toxicant delivery system was designed to exclude surface slicks, and slick formation was not apparent in treatment tanks. During incubation, the eggs remained below the water surface, further reducing the likelihood of coating.

Our data indicate that dissolved PAHs entered eggs and interacted directly with embryonic tissue. Enrichment of PAHs to alkanes in water relative to that in parent oil indicated solublization because PAHs are more soluble than aliphatic hydrocarbons (Marty et al. In press (b)). Dissolved, lipophilic PAHs can pass through egg chorions and bind to lipid rich material inside (Kuhnhold and Busch 1978; Solbakken et al. 1984). In a study involving cod (*Gadus morhua*), most of the radiolabeled hydrocarbons (including naphthalenes, phenanthrenes, and benzo[a]pyrene) accumulated by eggs were associated with the yolk, not the chorion (Solbakken et al. 1984). Similarly, 98% of radiolabeled naphthalene and 90% of labeled benzopyrene penetrated the chorion in Atlantic salmon (*Salmo salar*) within 3 d (Kuhnhold and Busch 1978). The amount of aromatic hydrocarbon accumulated by herring eggs correlated with yolk size (Eldridge et al. 1978; Struhsaker 1977), indicating that the lipid rich yolk may store significant amounts of hydrocarbon. As yolk reserves are metabolized, hydrocarbons sequestered in yolk lipid likely interact with embryonic tissues. In a companion study, PAH concentrations increased in interior tissues of similarly exposed adult herring, including ova (Carls et al. Chapter 3), clearly demonstrating that these hydrocarbons passed through biological membranes. Other

investigators have also observed that hydrocarbons pass through biological membranes in adult fish (Korn et al. 1976; Struhsaker 1977; Rice et al. 1987).

The intensity of UV light in our laboratory during the test procedures was probably not sufficient to activate a significant fraction of the PAH molecules in our tests. However, elevated toxicity of PAHs as a result of photoactivation by UV light has been well documented (e.g., Landrum et al. 1987). Although we did not measure the intensity of our representative fluorescent light source below 300 nm, our data suggested rapidly decreasing spectral energy at shorter wavelengths. Other investigators have found UV-B (265 to 330 nm) was not detectable from fluorescent light sources, and have used fluorescent lights as controls in tests designed to study photoactivation by UV light (Ankley et al. 1994). In addition, dosing apparatus was covered, distribution manifolds were protected from direct light, and estimated light intensity was ≤ 70 footcandles, of which 14-16% was UV, and less than 2% was below 330 nm.

Although we cannot prove that PAH toxicity was not elevated as a result of UV radiation in the laboratory, photoactivation of PAHs in PWS after the spill was more likely because of direct exposure to sunlight at greater intensities. Thus, even if photoactivation did occur in the laboratory, it likely had a greater role in the natural environment, and thus one could argue that the surprisingly low effective PAH concentrations we measured (0.2 ppb) were conservative. We concede that light conditions in the natural environment were more variable in the laboratory, e.g., varying water depth and clarity, but contend that the potential for photoactivation was greater in PWS than in the laboratory.

Water quality standards

In light of the variable toxicity of petroleum products due to weathering, and the high toxicity of weathered oil, the water quality standards of the State of Alaska, 10 ppb aromatics, need refinement. In these experiments, significant biological responses were observed as low as 0.2 ppb PAH, well below the published standard.

The relatively high toxicity exhibited by weathered oil in these experiments also suggests the conclusions reached by some investigators (Neff and Stubblefield 1995) that PAHs in PWS in 1989 did not adversely effect biota is likely erroneous. Short and Harris (1996a) observed PAH concentrations in the water column up to 6.2 ppb, declining to ≤ 1.6 ppb five weeks after the spill. Although mean PAH concentrations declined to 0.01 to 0.10 ppb by midsummer, concentrations in excess of 2 ppb were apparently observed in open waters of PWS five months post-spill (Neff and Stubblefield 1995).

Concentrations of PAHs in water may have been even higher in intertidal areas of PWS than suggested by the preceding observations. Oiled substrate can serve as a reservoir of oil capable of contaminating water that comes in contact with it. This was clearly demonstrated in our experiments where this mechanism was exploited to contaminate treatment water. In an oil spill situation, such as in PWS, we expect that tidal interaction with oiled substrate would lead to

similar water contamination patterns, and that the concentrations in and very near to the oiled substrate would be higher than in open water. Indeed, the weathering patterns observed in these experiments were very similar to weathering patterns observed in oiled substrate in PWS (Babcock and Short 1996), and the underlying weathering mechanism was the same (Short and Heintz, In press). Loss rates of PAHs in this type of laboratory study and in PWS both fit first-order loss-rate kinetics, and demonstrated that laboratory results predict weathering behavior of petroleum introduced into subarctic marine environments (Short and Heintz, In press).

The aromatic compounds observed in water in this experiment, ranging from naphthalenes to chrysenes, were similar to those observed in PWS (e.g., Babcock and Short 1996; Carls et al. 1996), but are fundamentally different from those observed in many earlier studies (e.g., Rice et al. 1979). Prior to 1989, oil-contamination tests routinely conducted at the Auke Bay Laboratory involved principally mono- and di-aromatic hydrocarbons, and the presence of compounds with more than two rings was negligible. Because the toxicity of aromatics increases with ring size, the toxicity observed in our experiments is correspondingly greater than the toxicity published in earlier work. For example, compare the 12 d LC50 for herring eggs published by Rice et al. (1987) of 1,500 ppb to the 61 ppb value observed in this study. Again we emphasize that it is not only how much oil is important, but also that the composition of the oil should be considered when formulating water quality standards.

Recommendations for future observation

Sensitivity of a wide variety of larval herring responses to oil were similar, and indicated significance at 0.7 ppb PAH in E2. Yolk-sac edema and fin ray formation were the most sensitive measures of response, and indicated significance at 0.2 ppb (in E2). Lower jaw size was also significantly affected at 0.2 ppb (in E2), but was a less sensitive measure of response in the first experiment.

Our experience indicated that observation of yolk-sac edema yields maximum experimental sensitivity at minimum cost: preserved larvae could be assessed rapidly for edema. For little more cost, these same individuals could be simultaneously assessed for other morphological defects, fin ray formation, and lower jaw size. However, larval age must be considered when determining measurement parameters. For example, edema is obvious in yolked larvae but may not be obvious in older larvae. Other abnormalities, such as spinal deformation may be more important in older larvae.

Genetic assessment may be the best assessment tool in field situations because capture may cause tissue damage, thus obscuring true morphological condition. Because it is labor intensive and not as sensitive as morphological assessment, the genetic assessment technique used in this study would be of less importance in similar experimental research, but was necessary in these tests to establish the relationship between morphological and genetic response. Development of more sensitive genetic techniques would be useful.

To reduce labor-intensive costs, observation of live larvae may not be necessary in similar studies. Nonetheless, observation of larval swimming in these experiments allowed assessment of the relationship between morphological response and ability to swim, important for inferring survival potential. Similarly, although preservation of larvae does cause some distortion, it appears that assessment of spinal condition in preserved larvae gave similar results to live assessment, thus obviating the need for live assessment. Even if live observations are not routine, moribund and dead larvae should be preserved (or discarded) independently of living larvae.

Interpretation of 1989 *Exxon Valdez* oil spill results

Sublethal effects identical to those described here at low ppb doses of PAHs were also observed in herring larvae from oiled areas within PWS during spring 1989 (Brown et al. 1996) and in other laboratory exposures using oil-water dispersions of Prudhoe Bay crude oil (Pearson et al. 1985; Kocan et al. 1996). These include skeletal and craniofacial abnormalities, finfold reductions, edema or ascites (the histological equivalent of edema), premature hatching, genetic damage, and small size. Although the morphological defects reported in these studies can also be caused by natural stressors such as extreme salinity, temperature, or desiccation (Dushkina 1973; Purcell et al. 1990), environmental conditions during 1989 were not unusual and were well within optimal ranges for herring development. Incidences of all the types of morphological and genetic abnormalities reported here were significantly elevated at oiled areas relative to unoiled sites and decreased throughout the spring of 1989 until no measurable differences remained in 1990.

It is likely that the morphological defects in herring larvae observed in PWS in 1989 lead to death. Death of deformed larvae was predicted by our experiment where larval mortality was significantly elevated within 24 h of hatch. In a study where similarly deformed pollock larvae were observed for a longer period of time, Carls and Rice (1990) observed that abnormalities became more pronounced with time, and that the majority of abnormal larvae died early. Therefore, we expect that the abnormal herring larvae observed in PWS in 1989 did not recover, but rather died prematurely. Field observation in PWS also suggested early death, because incidence and severity of malformations decreased in larval populations sampled over time. Newly hatched larvae more frequently displayed skeletal bends, absent jaws, microcephaly and ocular defects than did pelagic larvae (Hose et al. 1996; Marty et al. In press (a)). Jaws were absent only in the smallest, least mature larvae, and it is assumed that these larvae died after their yolk reserves were utilized. It is also assumed that larvae with severe skeletal bends and ocular defects would succumb to predators. Manifestations of retarded development (immature pectoral fins and jaws) were generally the only types of morphological defects present in older larvae (Norcross et al. 1996; McGurk et al. 1990). Larvae with reduced jaws might later recover their ability to feed since jaws continue to grow throughout the larval period (Alderdice and Velsen 1971). Tracking of individual larvae in the field is virtually impossible, and it is thought that the reduction in malformation rates in older larvae results from death of severely affected

individuals, mixture with unaffected larvae from other areas, as well as some recovery of jaw development (Hose et al. 1996).

Although the existence of toxic effects in PWS herring eggs and larvae has been disputed (Pearson et al. 1995), PAH concentrations in eggs measured at many locations in 1989 appeared to be sufficient to elicit toxicity, based on the minimum effective concentrations estimated here (22 to 108 ppb in egg tissue). Herring eggs collected from some oiled sites in PWS in 1989 had PAH concentrations in excess of 100 ppb (Pearson et al. 1995). Although Pearson et al. (1995) claim the *Exxon Valdez* crude oil fingerprint was not strong, sites exhibiting elevated concentrations were located within slick trajectories, suggesting that *Exxon Valdez* crude oil was the *de facto* source of contamination. Pearson et al. (1995) found correlation between egg mortality and total PAHs, due principally to response at the most contaminated site observed (about 350 ppb PAH in egg tissue), but was unable to find a significant correlation between abnormalities and PAHs. In contrast, other studies (Brown et al. 1996; Hose et al. 1996) observed morphologic and genetic responses identical to ours among herring larvae of differing ages that decreased with distance and time from the spill.

The mechanisms whereby herring eggs in PWS may have been exposed to oil likely varied. Some exposure may have been due to dissolved PAHs, as was tested in our study. Other exposure mechanisms may have involved mechanically dispersed oil (Short and Harris 1996b), adherent oil droplets (Pearson et al. 1985), exposure of intertidally spawned eggs to a PAH-rich surface layer (von Westernhagen et al. 1987), and direct coating by surface film or slick. Pearson et al. (1985) demonstrated that short exposure (1 d) of herring eggs to low concentrations (4 ppb) of mechanically dispersed oil could cause a significant increase in larval abnormalities when oil droplets adhered to the eggs. Direct coating by visible slicks is an extension of this situation; the amount of adherent oil was apparently the major factor in determining toxicity in tests by Pearson et al. (1985). Even in the absence of a visible slick, petroleum hydrocarbons may be significantly concentrated in the upper 50 μm of the water column, leading to elevated toxicity when intertidally spawned eggs come in contact with the microlayer during low tides (von Westernhagen et al. 1987; Kocan et al. 1987).

Although it is impossible to directly relate *Exxon Valdez* oil-PAH concentrations in ambient seawater, mussel tissue, and herring eggs, it is likely that biologically effective oil concentrations were exceeded at many oiled sites. To overcome problems with detection of low level PAHs, Hose et al. (1996) relied on PAH concentrations in intertidal mussels adjacent to herring egg beds since mussels bioaccumulate, but do not metabolize PAHs. All areas within the oil trajectory sustained some level of oil exposure in 1989, and over 85% of pelagic larvae were collected within the oil trajectory. *Exxon Valdez* PAH fingerprints were present at all oiled sites, although oiling was highly patchy. Identical types of sublethal damage were found in newly hatched and pelagic (offshore) larvae from oiled areas, consisting of premature hatching, growth depression, morphological defects, and genetic damage (Brown et al. 1996). In general, morphologic and genetic effects in herring larvae were significantly correlated to the *Exxon*

Valdez oil-PAH concentration in adjacent mussels (Hose et al. 1996) in a dose-dependent fashion.

CONCLUSIONS

The frequency of chromosomal aberrations was significantly elevated in the pectoral fins of larvae hatched from eggs exposed to oil during incubation.

Exposure to oil induced early hatch, caused egg and larval mortality, affected larval swimming and size, and caused skeletal, craniofacial, finfold, and pericardial abnormalities.

Minimum effective concentrations of the less weathered oil were 9 ppb (initial PAH). In the more weathered oil, minimum effective concentrations were 0.2 ppb PAH.

In the less weathered oil, 2 d exposures to 38 ppb (initial PAH concentration) occasionally elicited significant responses; responses were frequently significant after 4 d exposure. In the more weathered oil, significant biological responses occurred after 4 d exposure to 0.7 ppb PAH.

Responses of herring larvae to the more weathered oil were greater than could be predicted by total PAH concentrations in less weathered tests, suggesting that toxicity of heavier, more substituted PAHs was greater than that of lighter, less substituted PAHs.

The implications of chromosomal aberrations in mitotic cells is not clear. However, it is likely most affected individuals would die due to concomitant morphological abnormalities.

Based on the biologically effective PAH concentrations determined in this study, and concentrations observed in PWS, it is likely that concentrations of *Exxon Valdez* crude oil caused damage in incubating Pacific herring eggs in PWS. This conclusion is consistent with the results of Brown et al. (1996) and Hose et al. (1996), but not Pearson et al. (1995).

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Chapter 5: Reproductive success of Pacific herring (*Clupea pallasi*) in Prince William Sound, Alaska, six years after the *Exxon Valdez* Oil Spill

S.W. Johnson, M.G. Carls, R.P. Stone, C.C. Brodersen, and S.D. Rice

ABSTRACT

The *Exxon Valdez* oil spill occurred just prior to the spring migration of Pacific herring (*Clupea pallasi*) from offshore feeding grounds to nearshore spawning areas in Prince William Sound (PWS), Alaska. Most or all of the life stages of herring in PWS may have been exposed to oil after the March 1989 spill. Delayed impacts from the spill were suspected as one possible cause in the unprecedented crash of the adult herring population in 1993 and stimulated studies to assess reproductive success. In spring 1995, mature herring were collected from four sites in PWS and from three unoiled sites in southeast Alaska (SEAK) to determine if reproductive impairment was evident in PWS herring six years after the spill. Herring were artificially spawned and resultant eggs were reared in a laboratory until hatch. Observed response parameters included fertilization success, hatch timing, hatch success, and larval viability, swimming ability, and spinal abnormalities. Responses of all year-classes combined or restricted to the same year-class did not differ significantly between regions ($P > 0.50$); the best and worst responses generally occurred in SEAK. Within each site, response of the 1989 year-class (most likely impacted by the oil spill in PWS), generally did not differ significantly from any other year class. To verify macroscopic observations, a subset of larvae from the 1989 year class was also inspected microscopically for yolk and pericardial abnormalities and yolk volume was measured; no significant regional differences were observed for any morphological category. Based on the parameters examined in this study, evidence of reproductive impairment of Pacific herring in PWS by the spill was not detected in 1995, and the chances of detecting any oil related effects against the natural background variation appear negligible.

INTRODUCTION

The oil spill in PWS occurred just a few weeks prior to the Pacific herring (*Clupea pallasi*) spawning season. Most or all of the life stages of herring in PWS may have been exposed to oil after the March 1989 spill. Biologically available hydrocarbons were present in the upper water column of PWS for several weeks following the spill (Short and Harris 1996a) and residual oil may have persisted in some areas into 1990 (Short and Harris 1996b). An estimated 40-50% of the egg biomass in PWS was deposited within the oil trajectory in 1989 (Brown et al. 1996a). The failure of the 1989 year class to recruit to the fishery and subsequent population crash in 1993 (Meyers et al. 1994) suggested the early life stages of herring were impacted either from exposure of pre-spawning adults or by direct exposure of eggs and larvae. Thus, as fish exposed to oil were recruiting into the fishery (20% by age 3, 80% by age 4, 100% by age 5 (Funk 1994)), the herring population crashed, and recovery was minimal through the

1996 season (J. Wilcock, Personal communication). Genetic damage, morphological deformities, and small size were reported for newly hatched larvae following the spill (Brown et al. 1996a; Hose et al. 1996; Norcross et al. 1996; Marty et al. In press), but long-term effects remain unknown. In a preliminary study in 1992, Kocan et al. (1996b) observed decreased reproductive success of herring from an oiled area in PWS compared to an unoiled area; results were inconclusive, however, because only two sites were compared. Delayed impacts from the spill were suspected as one possible cause in the population decline and stimulated the need for more definitive studies to assess reproductive success of herring.

The purpose of this study was to determine if reproductive impairment potentially caused by the spill was evident in PWS herring six years after the spill. There were two major focuses in the study: 1) a comparison of reproductive success between regions (PWS and SEAK) and 2) a comparison of reproductive success between year classes within sites, particularly the 1989 year class (most likely impacted by the oil spill) to other year classes in PWS.

Sites sampled within PWS included all areas where spawning occurred in 1995; spawning was absent in areas that were heavily oiled in 1989. For example, Naked Island which was in the middle of the spill trajectory, had 22 km of spawn in 1989 (Brown et al.1996a) but none in 1995. Although some have speculated that herring home to the same general spawning area each year (Zijlstra 1963; Hourston 1982), site fidelity is poorly understood and we can only assume that herring sampled in PWS in 1995 may or may not have been exposed to oil at some earlier time in their life history (adult, eggs, or larvae).

METHODS

Herring were collected at four sites in PWS and at three sites in SEAK (Figure. 5.1); all sites had spawn in previous years. Two of the sites in PWS (St. Mathews Bay and Fish Bay) were not directly oiled from the spill, whereas the other two sites (Port Chalmers and Rocky Bay) were at least lightly oiled. Shortly after the spill, elevated hydrocarbon levels were detected in mussels at Rocky Bay (Brown et al. 1996b) and in seawater at Port Chalmers (Carls 1996). Additionally in Port Chalmers, concentrations of oil metabolites in bile of adult herring sampled in spring 1990, were similar to metabolite concentrations observed in 1989, suggesting continued contamination (Brown et al. 1996b). Herring were collected in St. Mathews Bay on 7 April, Fish Bay on 14 April, Port Chalmers on 30 April, and Rocky Bay on 1 May 1995. In SEAK, herring were collected in Sitka on 29-30 March, Ketchikan on 11 April, and Seymour Canal on 13 May 1995.

Mature herring were captured during or just prior to spawning at all sites, sorted by size, and artificially spawned. Capture methods included gill net, cast net, and purse seine. Fish were chilled immediately after capture and transported within 2 h to a field laboratory, except Seymour Canal fish that were transported directly to the Auke Bay Laboratory (ABL). To approximate the

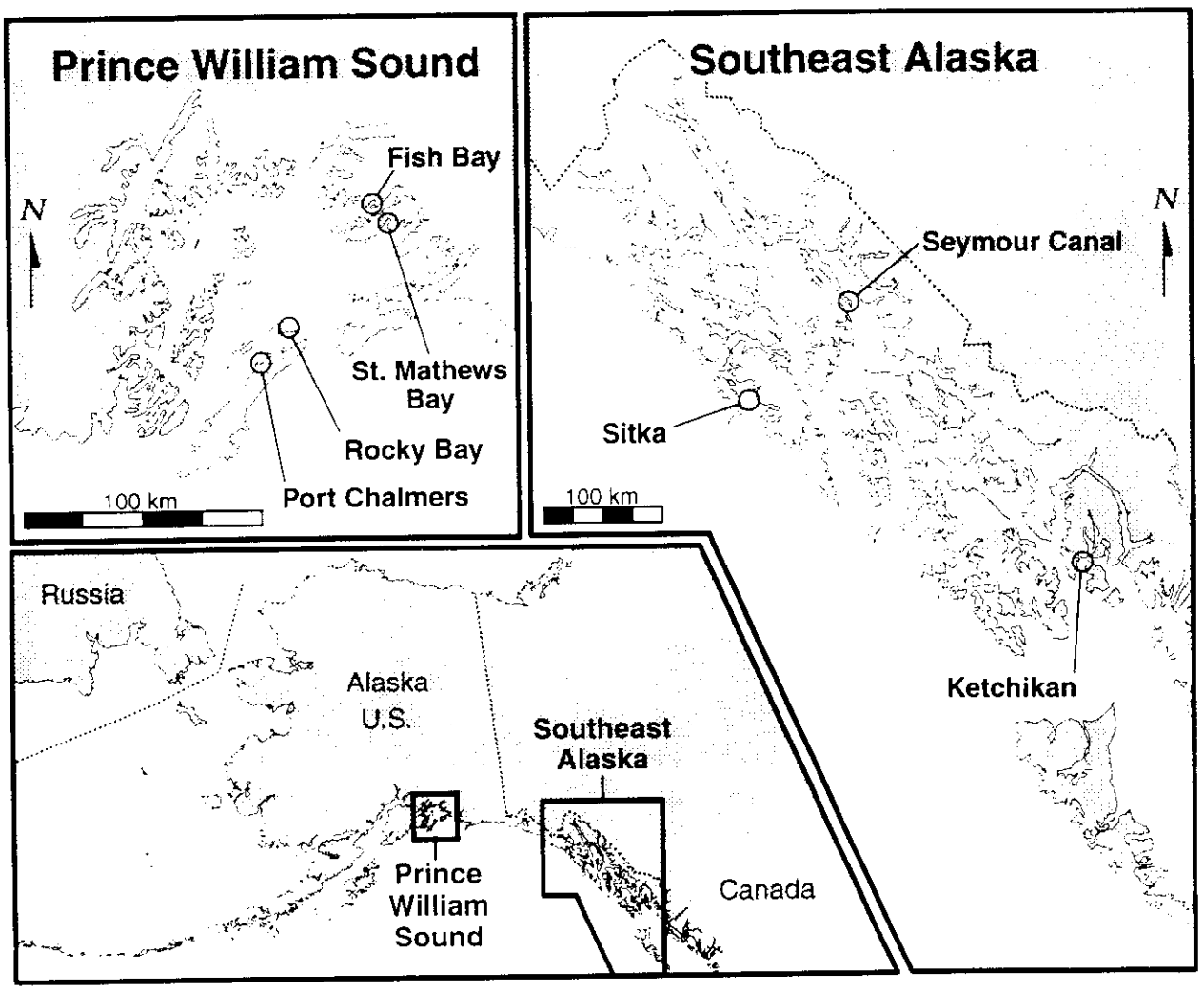

Figure 5.1. Collection sites of mature Pacific herring in PWS and SEAK Alaska in spring 1995.

different age-classes present, fish were sorted by sex and size (usually in 10 mm increments; e.g., 220-230 mm fork length). Six or more size classes were usually identified at each site. From each size class, 25 females were artificially spawned with males of the same size; generally 3 males contributed sperm for all 25 crosses. Size classes were processed at random. Each fish was assigned a unique identification number, measured to the nearest mm (fork length), and weighed to the nearest 0.1 g (wet weight). To determine age, three scales were removed from the left side of each spawned fish near the posterior margin of the dorsal fin, placed succulus side down on a glass slide, and covered with a second slide.

For spawning, testes were removed and sealed in a plastic bag and maintained in chilled seawater until use; ovarian membranes were cut longitudinally and eggs were removed with a hydrocarbon-free stainless steel spatula similar to methods used by E.D. Brown (Personal communication). From each female, approximately 150 eggs were deposited with gentle swirling onto a 25 x 75 mm glass slide placed on the bottom of a shallow plastic dish filled with seawater. Individual slides from each female were placed in a staining rack and suspended in separate 1 L 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 ml of milt were added to each beaker containing eggs. Eggs and milt remained in contact for 5 min; milt was then poured off and the eggs were gently rinsed in seawater. Slides were kept in staining racks and maintained in ambient seawater with constant aeration until transport to ABL by air. To transport the eggs, staining racks were placed in plastic containers filled with seawater and placed in coolers with blue ice.

Slides with eggs from each site were randomly distributed among twelve, 600 L living streams with flow-through seawater. Slides were suspended from monofilament line attached to a pivoting overhead framework designed to cause slow movement (1 rpm) through the water. During the first 16-18 d of incubation, all slides were maintained directly in the seawater bath. A few days before hatch, each slide was isolated in a 1 L glass jar containing seawater; jars were surrounded by flowing seawater. Lighting was natural, supplemented by overhead fluorescent light during daylight hours. Seawater flow was approximately 1 L/min at 3.9 °C, warming to 7.1 °C due to normal seasonal change. Salinity was 32 ± 1 ppt.

Reproductive success of female herring was defined as the production of morphologically and functionally normal larvae. Key reproductive parameters included hatching success and larval viability, swimming ability, and spinal abnormalities. These four parameters were sensitive to oil in laboratory studies (Carls et al. Chapter 4). Other parameters examined included fertility and hatch timing. Fertility was not considered a key parameter because it may have been negatively influenced by unavoidable handling conditions at the different sites and by variable gamete storage time prior to spawning. Hatch timing was not considered a key parameter because it was strongly influenced by seasonal increases in water temperature.

Fertilization success and stage of development were determined 1 to 10 d after spawning. Eggs along slide margins that were susceptible to mechanical damage, and clumps of eggs not

directly exposed to water were removed from all slides by scraping. This process was accomplished in water with minimal exposure to air.

Isolated eggs were inspected every 2 d to determine onset of hatch. Once hatch began, larvae were enumerated and assessed daily for swimming ability and gross morphological deformities. Without exposing eggs to air, seawater was changed in each jar every 2 d prior to hatching and daily after hatch began. All hatched larvae were collected, anesthetized with tricaine methanesulfonate, and preserved in 10% phosphate buffered formalin. Approximately the first and last 10% of larvae hatched were preserved in separate bottles (by female). Live larvae were preserved independently from dead larvae. After hatch was complete, remaining eggs were inspected; infertile eggs and dead embryos were enumerated.

A subset of preserved larvae was scored for yolk-sac edema, pericardial edema, and yolk volume. Ten females from the 1989 year class were randomly selected from each site, and ten larvae per female were randomly selected from the central portion of hatch for analysis. At Fish Bay, only five females from the 1989 year class were present, so the number of larvae analyzed per female was doubled. Sitka and St. Mathews Bay were excluded because of an insufficient number of females from the 1989 year class. Lateral views of larvae were digitized with specimens rotated to align eyes to minimize variance. Yolk shapes were generally elliptical; major and minor axes were measured perpendicular to the body axis. Yolk volume was estimated from these linear measures according to the method of Hourston et al. (1984). Yolk-sac edema was indicated if the anterior margin of the yolk membrane was bounded by an area of clear fluid. Pericardial edema was scored if the pericardium was unusually large or convex ventrally.

Data processing and statistics

To assess the general health of parent fish, condition factor (K) was calculated for each female:

$$K = \frac{100(W)}{FL^{b}}$$

(Bagenal and Tesch 1978) where W = somatic wet weight in g, FL = fork length in cm, and b was determined by site from length-weight regressions. Gonad weight was subtracted from body weight to avoid variation in spawning condition.

Hatch timing among sites, which was temperature dependent, was compared using peak hatch time as the estimator. Peak hatch day was defined as the day the most larvae hatched from eggs of a given female; where two hatch peaks of equal magnitude occurred, the first peak was reported. Mean incubation temperature for eggs from each female was calculated by weighing

mean water bath temperatures by the number of eggs hatched daily. This avoided possible under or over estimates of mean incubation temperature caused by early or late hatching eggs as seasonal temperature increased.

Most observations were expressed as percentages. The denominator used to calculate percentages varied by response parameter (Table 5.1). Percentages of eggs fertile and initially dead were based on the total number of eggs counted near the beginning of the experiment. Percentages of eggs that hatched 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 live, moribund, and dead. Hearts of moribund larvae were beating, but these larvae were incapable of movement. Accordingly, percent live was the number of living larvae (excluding moribund larvae) divided by the total number hatched. Swimming ability of live larvae was categorized as effective, ineffective, or incapable. Effective swimmers were active, frequented the water column, and avoided capture. Ineffective swimmers were generally more lethargic than effective swimmers, and were more likely to be found on jar bottoms. Incapable larvae were unable to swim in a straight line and were often only capable of spasmodic twitching. Swimming ability of moribund and dead larvae was, by definition, nonexistent, thus the number of live larvae was used as the denominator for swimming ability categories. Because larvae quickly became distorted after death, spinal aberrations were assessed only in live and moribund larvae. Percent spinal abnormalities, therefore, was the number of larvae with spinal aberrations divided by the sum of live and moribund larvae.

Table 5.1. Description of key response parameters used to evaluate reproductive impairment in Pacific herring collected from a PWS and SEAK Alaska. Herring were collected in 1995, artificially spawned and reared in a laboratory until hatch. Moribund larvae were alive (heart beating) but incapable of swimming.

Parameter (%)	Description
Hatch	100 · (total number of eggs that hatched) /(total number of eggs that hatched + total number of dead eggs)
Live (viable)	100 · (total number of live larvae excluding moribund larvae) / (total number of eggs that hatched)
Effective swimmers	100 · (total number of effective swimmers) / (total number of live larvae excluding moribund larvae)
Spinal abnormalities	100 · (number of live + moribund larvae with spinal defects) / (total number of live + moribund larvae)

One-way analysis of variance (ANOVA) was used to examine for differences among sites, among age classes, and between regions. Each reproductive parameter was separately tested by individual age class and for all age classes combined; percentage data were arcsin transformed, and corrected for small n as necessary (Snedecor and Cochran 1980). To account for variance among sites, the F test comparison between regions was:

$$F = \frac{MS_{between\ regions}}{MS_{among\ sites}}$$

where MS = mean square. Somatic weight, FL, and K were similarly analyzed. Age class responses within site were compared because in PWS different age classes were potentially exposed to varying levels of oil (Table 5.2). When the overall ANOVA was significant ($P \leq 0.05$), *a priori* multiple comparisons were used to identify which ages differed:

$$F = \frac{MS_{between\ age\ classes}}{MS_{error}}$$

Maternal age was used as the standard in all age comparisons because ages frequently differed in the male and female crosses. Age 3 and 4 herring were not exposed to oil at any life stage in PWS so they were combined (age 4-) as site-specific controls. Few older age fish were captured, thus, ages ≥ age 9 were combined and reported as age 9+.

Table 5.2. Age, year-class, and possible oil exposure of Pacific herring collected in PWS, Alaska in 1995. The *Exxon Valdez* oil spill occurred in March 1989.

Age	Year-class	Possible oil exposure
3	1992	no direct oil exposure of any life stage
4	1991	no direct oil exposure of any life stage
5	1990	all life stages possibly exposed to residual oil
6	**1989**	**all life stages likely exposed to oil**
7	1988	juveniles at time of spill
8	1987	juveniles/immature at time of spill
9+	1986	mature; reproductive at time of spill

Because cold storage of adult fish (mean time of fish capture to mean spawning time) varied among sites (0.7-12.9 h), we also examined the possible effect of storage time on all reproductive parameters using storage time as a covariate in the ANOVA. Storage times up to 7 h did not significantly affect any of the key reproductive parameters ($P \geq 0.376$, except $P = 0.084$ for % live larvae). We repeated the ANOVA for regional differences with storage times in the model as a covariate, and restricted the analysis to include only those fish sampled within the same time period ($\leq$ 7 h).

Scored yolk-sac edema was analyzed with the Kruskal-Wallis nonparametric test (SAS Institute, Inc. 1989). Yolk-sac edema was also re-expressed as a percentage by female, arcsin transformed, and analyzed by ANOVA. Yolk volume was analyzed by ANOVA.

RESULTS

Regional comparison

Herring sampled from all sites appeared healthy and showed no obvious external signs of disease. For fish of the same age, there were no significant differences in FL ($P \geq 0.09$), weight ($P \geq 0.09$), or condition factor ($P \geq 0.41$) between regions. For herring in PWS, mean FL ranged from 196 to 260 mm and mean weight from 60.7 to 151.7 g, whereas in SEAK, mean FL ranged from 198 to 253 mm and mean weight from 65.1 to 140.4 g (Table 5.3).

For all age-classes combined, reproductive success of herring did not differ significantly between regions ($P > 0.50$); the best and worst responses generally occurred in SEAK, whereas PWS sites were intermediate (Figure 5.2). Statistical power of these tests was high (≥ 0.99) and remained high for most analyses. Restricting the analysis to fish stored $\leq$ 7 h did not alter the overall results; no significant ($P > 0.39$) regional differences existed for any reproductive parameter. In SEAK, mean responses ranged from 63 to 91% for hatch success, 95 to 98% for live larvae, 90 to 96% for effective swimmers, and 1 to 7% for spinal abnormalities. In PWS, mean responses ranged from 78 to 86% for hatch success, 95 to 96% for live larvae, 92 to 93% for effective swimmers, and 4 to 6% for spinal abnormalities. Among all sites, reproductive success was consistently best at Sitka (e.g., highest hatch success, 91%, and fewest spinal abnormalities, 1%) and worst at Seymour Canal or Ketchikan (e.g., lowest hatch success, 63%, and most spinal abnormalities, 7%) (Figure 5.2). Of the sites in PWS, reproductive success was usually best at St. Mathews Bay or Port Chalmers (e.g., highest hatch success, 86%, and fewest spinal abnormalities, 4%) and worst at Fish Bay (e.g., lowest hatch success, 78%, and most spinal abnormalities, 6%) (Figure 5.2). Similarly, when reproductive success was estimated for each age class individually, regional differences were not significant ($P > 0.50$). This was true for all age comparisons (age 3 to 9+). For example, age 6 (1989 year-class) herring in PWS did not differ significantly from those in SEAK (Figure 5.3). Among all sites where more than four age 6 fish were collected (excludes St. Mathews Bay and Sitka), hatch success ranged from 66% (Seymour Canal) to 91% (Port Chalmers), live larvae from 95% (Fish Bay) to 98% (Port

Chalmers), effective swimmers from 93% (Rocky Bay) to 96% (Port Chalmers), and spinal abnormalities from 2% (Port Chalmers) to 6% (Fish Bay).

No significant regional differences were observed in progeny of the 1989 year class scored for morphological condition. Only one larva of 500 had pericardial edema. Analyzed with the Kruskal-Wallis test, the site with the most yolk-sac edema (Port Chalmers) was significantly different from that with the least (Ketchikan), but there was no regional trend. Percentages of larvae with yolk-sac edema were low (≤ 16%), and differences among sites and between regions were not significant ($P > 0.348$) (Figure 5.4).

Table 5.3. Fork length (mm) and somatic weight (g) of mature female Pacific herring captured in SEAK and PWS, Alaska in spring 1995. Values are mean ($\bar{x}$) and ± SE; sample size = n.

		Age								
		3	4	5	6	7	8	9	10	11
Fork length										
SEAK	$\bar{x}$	198	211	217	221	236	234	241	236	253
	±	1.9	2.9	2.0	1.2	1.3	3.5	8.4	3.5	7.7
	n	86	21	49	94	95	15	3	2	3
PWS	$\bar{x}$	196	219	225	236	242	260	259	259	260
	±	1.1	2.3	1.1	1.7	1.0	3.2	1.3	2.2	2.9
	n	81	18	65	25	149	10	16	7	13
Weight										
SEAK	$\bar{x}$	65.1	79.1	87.2	91.7	112.9	112.7	117.5	108.3	140.4
	±	1.8	3.2	2.1	1.7	1.9	5.1	4.6	6.4	14.7
	n	81	21	49	93	94	15	3	2	3
PWS	$\bar{x}$	60.7	90.3	95.0	107.4	121.0	149.0	147.8	148.9	151.7
	±	1.2	2.2	1.3	2.7	1.5	8.3	3.3	6.8	5.1
	n	80	18	65	25	149	10	16	7	13

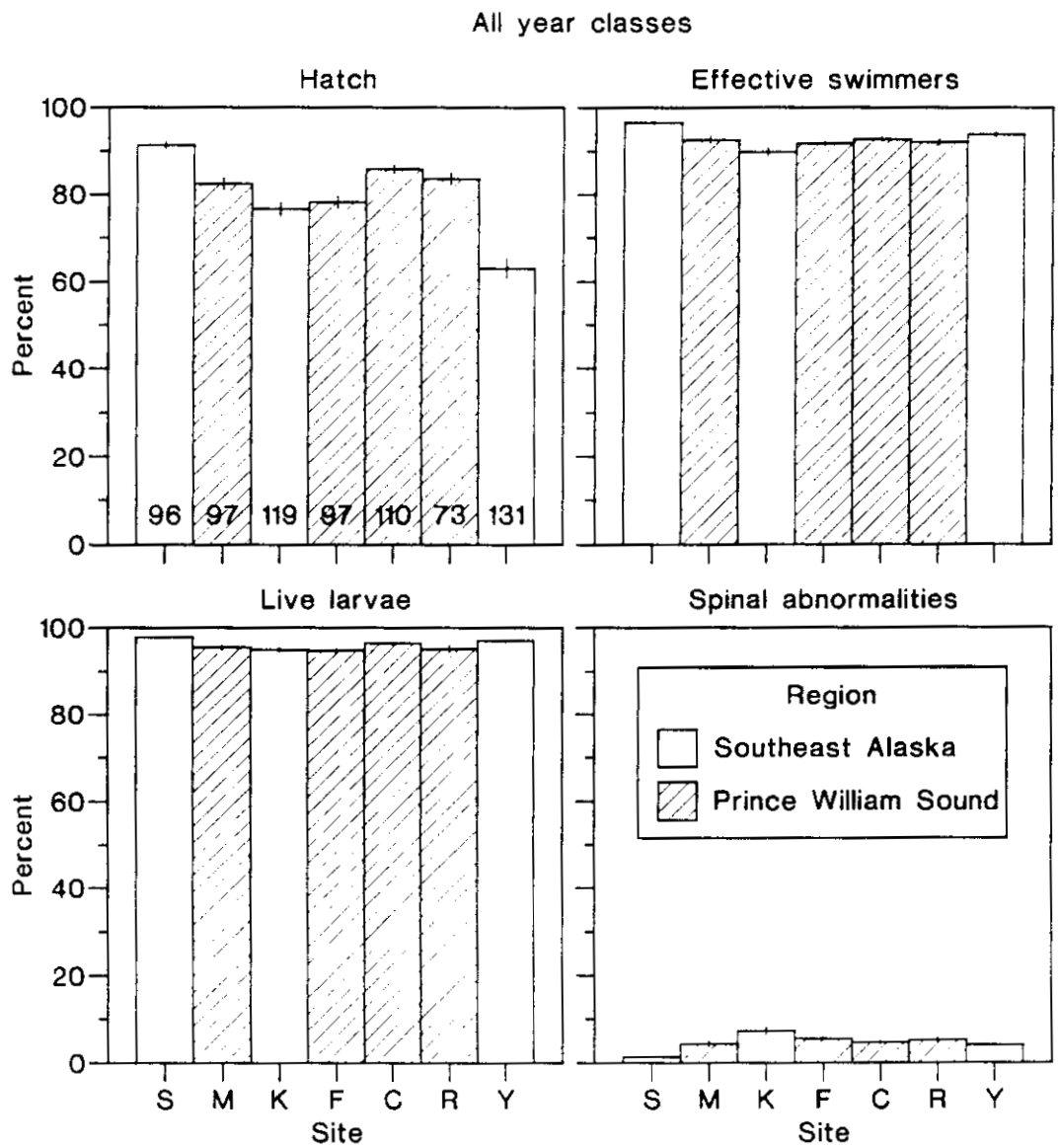

Figure 5.2. Mean (± SE) percent hatch, live, effective swimmers, and spinal abnormalities of larval Pacific herring by site and region in Alaska, 1995. (S = Sitka, M = St. Mathews Bay, K = Ketchikan, F = Fish Bay, C = Port Chalmers, R = Rocky Bay, Y = Seymour Canal; sites in chronological order of spawning date). Sample size is shown in hatch graph. No significant differences existed between regions for any reproductive parameter ($P > 0.50$).

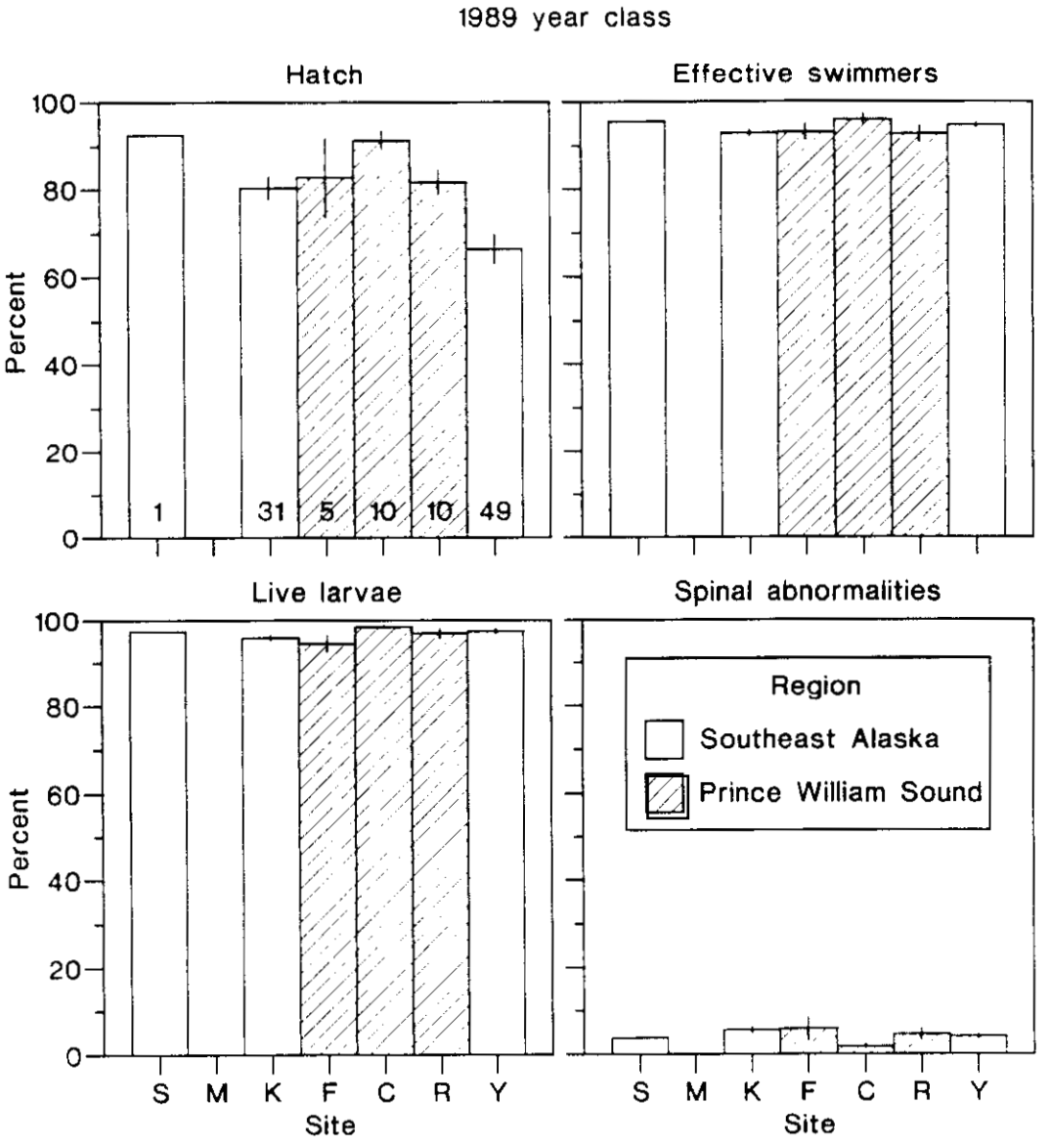

Figure 5.3. For the 1989 year-class, mean (± SE) percent hatch, live, effective swimmers, and spinal abnormalities of larval Pacific herring by site and region in Alaska, 1995. The 1989 year-class, sampled in PWS in 1995, was more likely exposed to oil as eggs or larvae than other year-classes. (S = Sitka, M = St. Mathews Bay, K = Ketchikan, F = Fish Bay, C = Port Chalmers, R = Rocky Bay, Y = Seymour Canal). Sample size is shown in hatch graph. Progeny of the 1989 year-class did not differ significantly between regions for any reproductive parameter ($P > 0.50$).

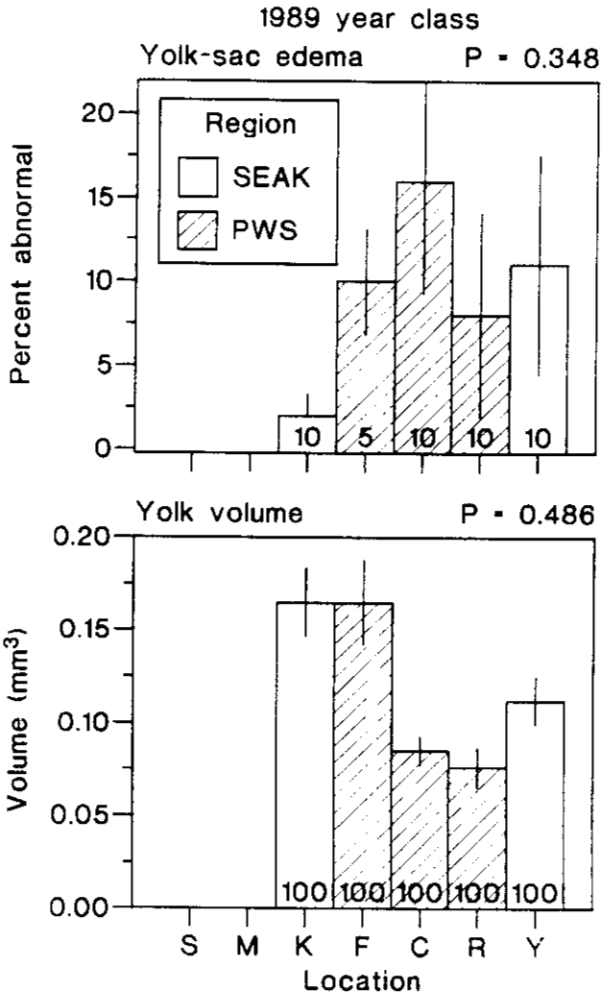

Figure 5.4. For the 1989 year-class, mean (± SE) percent yolk-sac abnormalities (edema) and yolk volume for larval Pacific herring by site and region in Alaska, 1995. (S = Sitka, M = St. Mathews Bay, K = Ketchikan, F = Fish Bay, C = Port Chalmers, R = Rocky Bay, Y = Seymour Canal). No significant differences existed between regions for either parameter ($P \geq 0.348$).

Yolk volume in larvae from the 1989 year class did not differ significantly ($P = 0.486$) between regions, but may have been related to incubation temperature. The largest and smallest mean yolk volumes were observed in PWS, but closely overlapped those in SEAK (Figure 5.4). Although scatter was high ($r^2 = 0.13$), yolk volumes declined significantly ($P < 0.001$) as temperature increased. It is possible, however, that site differences and incubation temperature were confounding factors.

Comparison among age-classes within sites

Reproductive success differed significantly among some age-classes at Sitka, Ketchikan, Port Chalmers, and Rocky Bay but not among age classes at St. Mathews Bay, Fish Bay, and Seymour Canal (Figures 5.5 to 5.8). The few significant differences we observed were highly variable, inconsistent among sites, and no pattern existed for the 1989 year class. For example, at Rocky Bay, age 4- fish had a significantly lower percentage of live larvae than age 5, 6, and 7 fish (Figure 5.6), whereas at Sitka, age 4- fish had a significantly higher percentage of effective swimmers and a significantly lower percentage of spinal abnormalities than age 7 fish (Figures 5.7 and 5.8).

Other parameters

Hatch timing decreased steadily with increasing incubation temperature (Figure 5.9). For Sitka, the first site sampled, peak hatch occurred about 33 d after start of incubation at a mean temperature of about 4.5°C, whereas in Seymour Canal, the last site sampled, peak hatch occurred about 26 d after start of incubation at a mean temperature of about 6.0°C.

Fertility did not differ significantly ($P > 0.50$) between regions for all ages combined or when the comparison was restricted to fish of the same age. For all ages combined, fertility in SEAK ranged from 80% at Seymour Canal to 96% at Sitka; in PWS, fertility ranged from 88% at Fish Bay to 94% at St. Mathews Bay.

DISCUSSION

Six years after the spill, reproductive impairment was not detected in PWS herring. This conclusion was reached by comparing reproductive success of fish collected in PWS and SEAK and among age classes within specific sites. Specifically, hatch success, larval viability, and fertility did not differ significantly between PWS and SEAK, including response of the 1989 year-class. In fact, discrimination of responses between regions was not possible because the best and worst responses were usually found in one region (SEAK). Therefore, the chances of detecting any oil related effects against the natural background variation were negligible when herring were compared between regions. Although responses among some age-classes within Port Chalmers and Rocky Bay were occasionally significant, these differences were highly

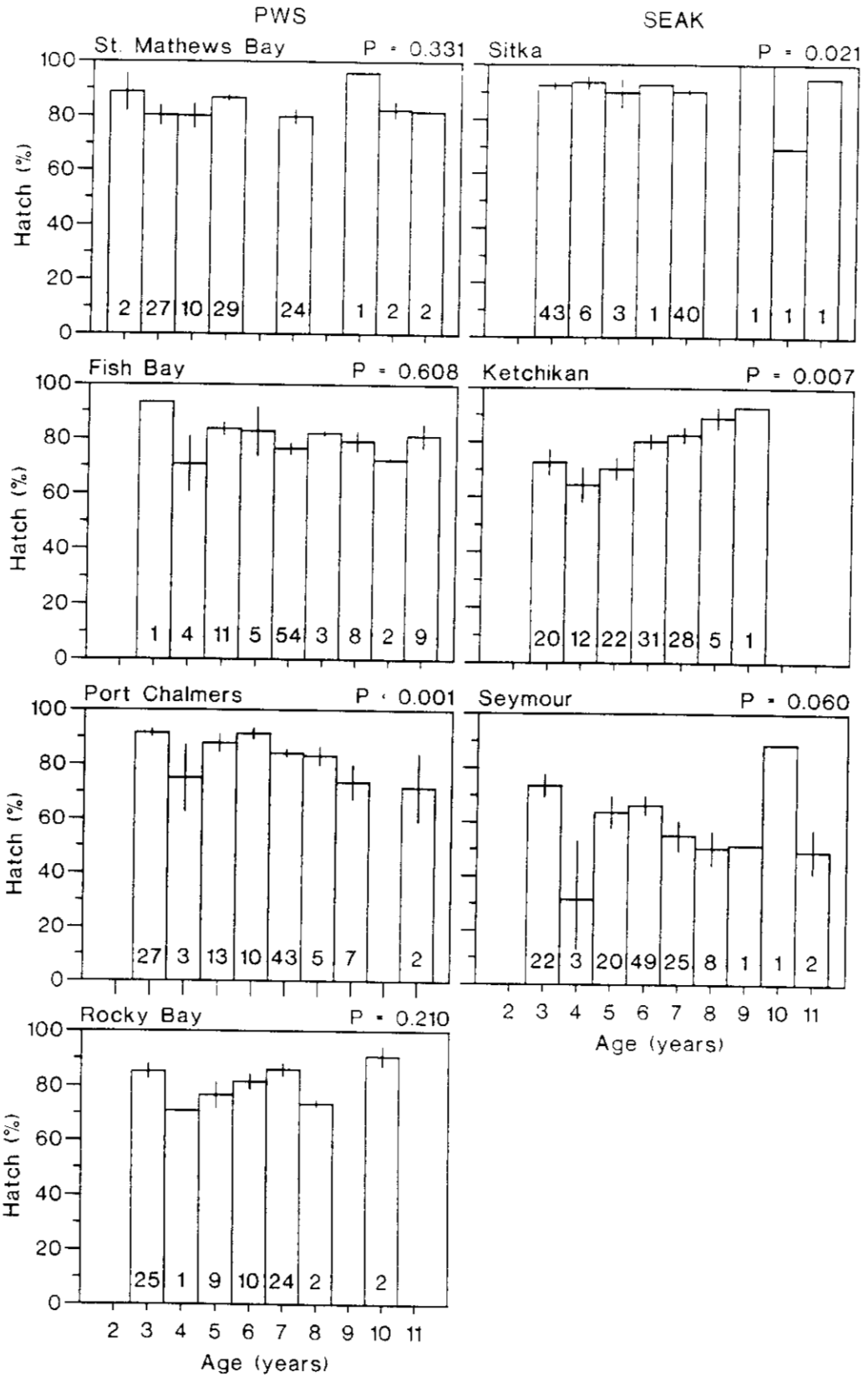

Figure 5.5. Mean (± SE) percent hatch of larval Pacific herring by female parent age, site, and region in Alaska, 1995. Sample size is shown in each bar. Overall P value from ANOVA is listed above each graph. Significant differences were: Ketchikan, age 4- < age 6, 7, and 8 ($P \leq 0.015$); Port Chalmers, age 4- > age 9+ ($P = 0.050$).

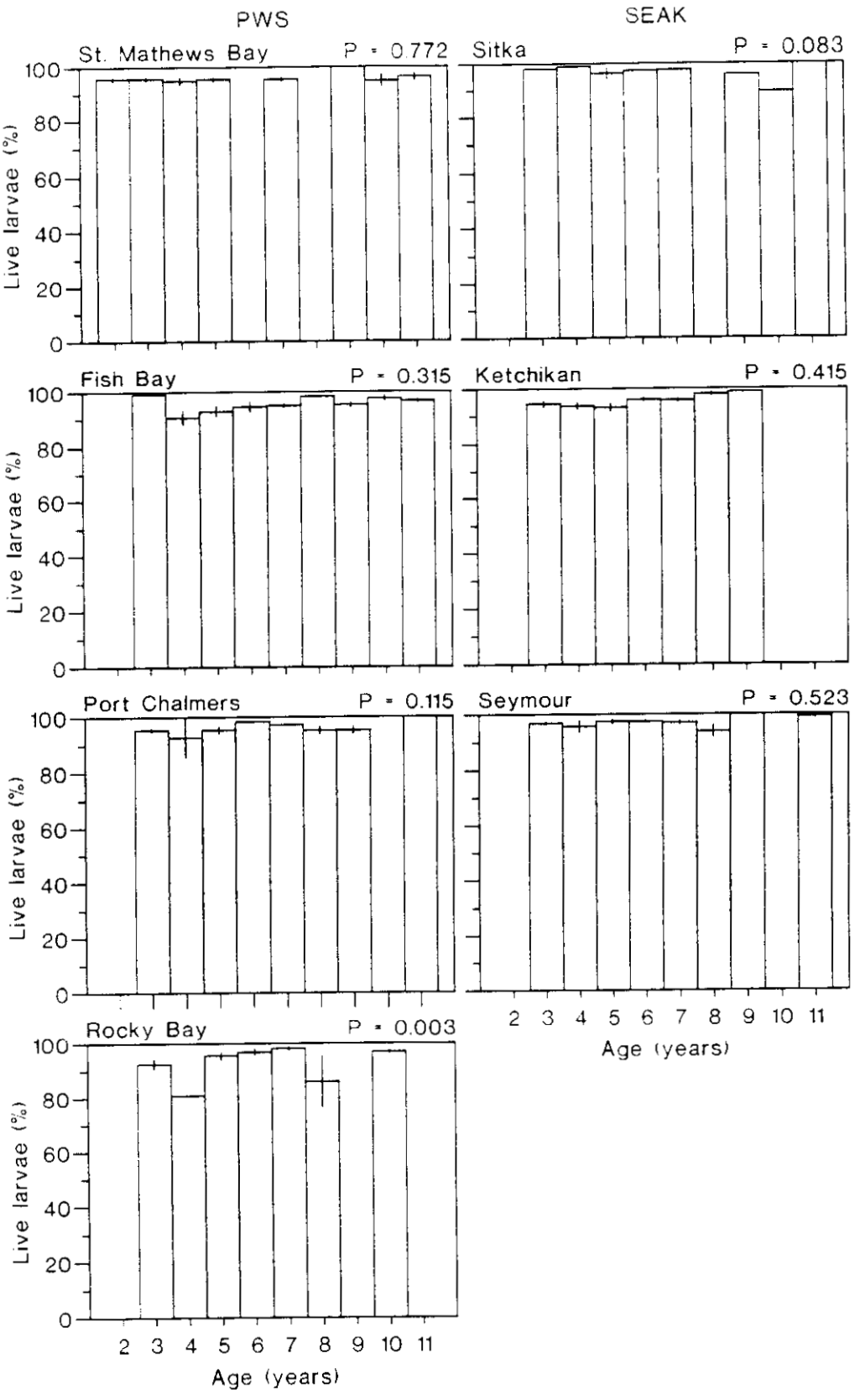

Figure 5.6. Mean (± SE) percent live larvae of Pacific herring by female parent age, site, and region in Alaska, 1995. Sample sizes are indicated in Figure 5.5. Overall P value from ANOVA is listed above each graph. Significant differences were: Rocky Bay, age 4- < age 5, 6, and 7 (P = 0.047).

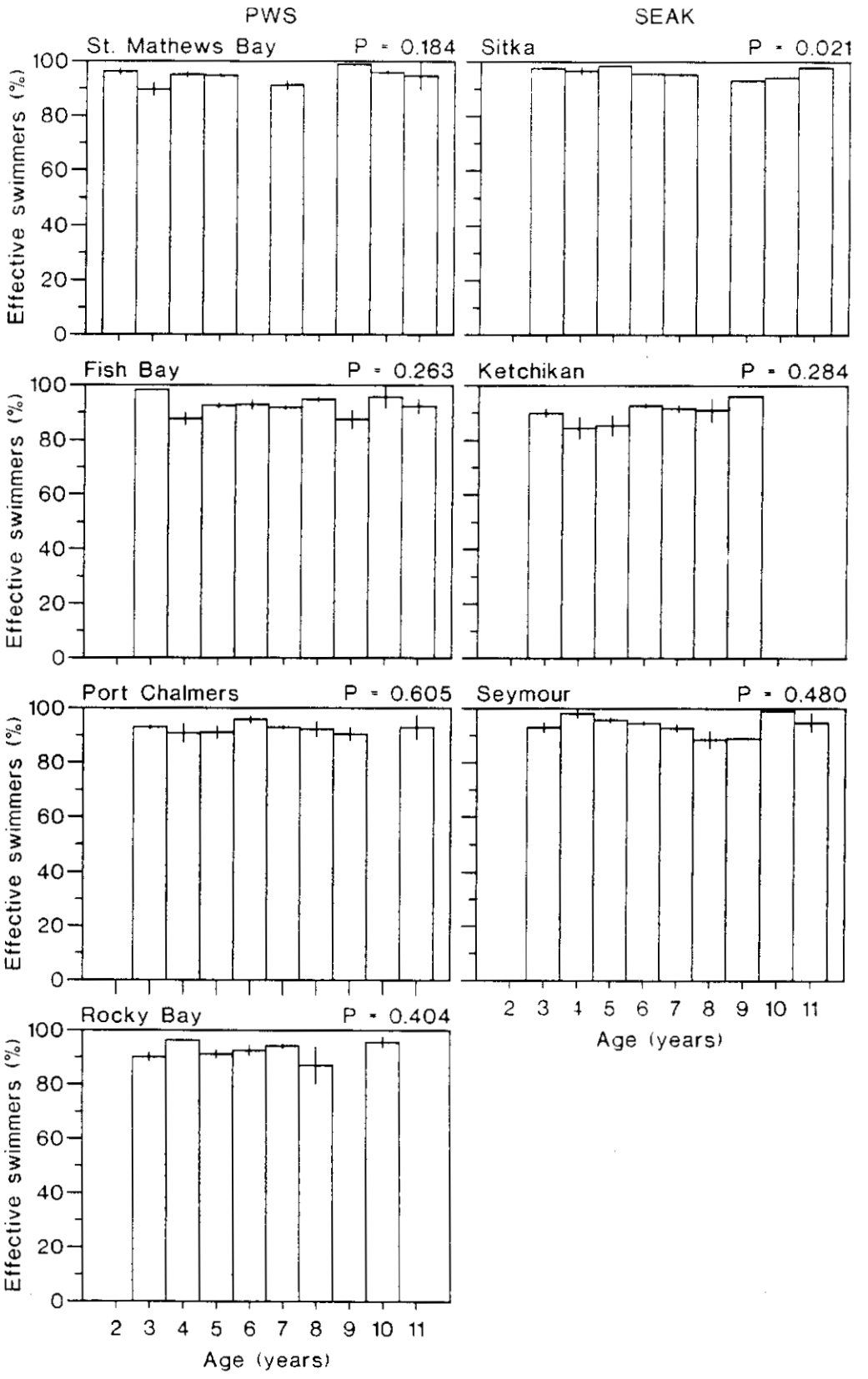

Figure 5.7. Mean (± SE) percent effective swimmers of larval Pacific herring by female parent age, site, and region in Alaska, 1995. Sample sizes are indicated in Figure 5.5. Overall *P* value from ANOVA is listed above each graph. Significant differences were: Sitka, age 4- > age 7 (*P* = 0.011).

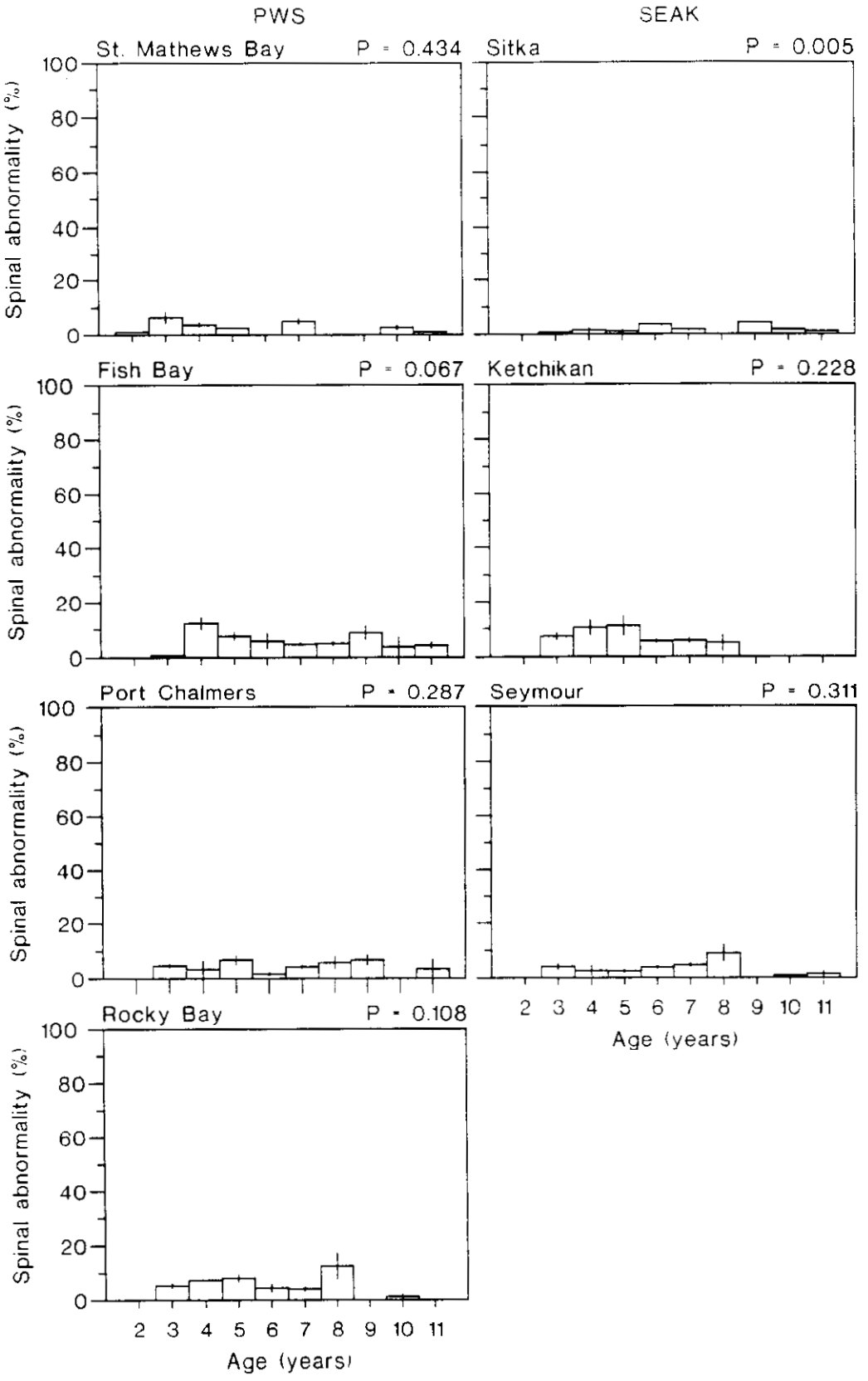

Figure 5.8. Mean (± SE) percent spinal abnormalities of larval Pacific herring by female parent age, site, and region in Alaska, 1995. Sample sizes are indicated in Figure 5.5. Overall P value from ANOVA is listed above each graph. Significant differences were: Sitka, age 4- < age 7 (P = 0.024).

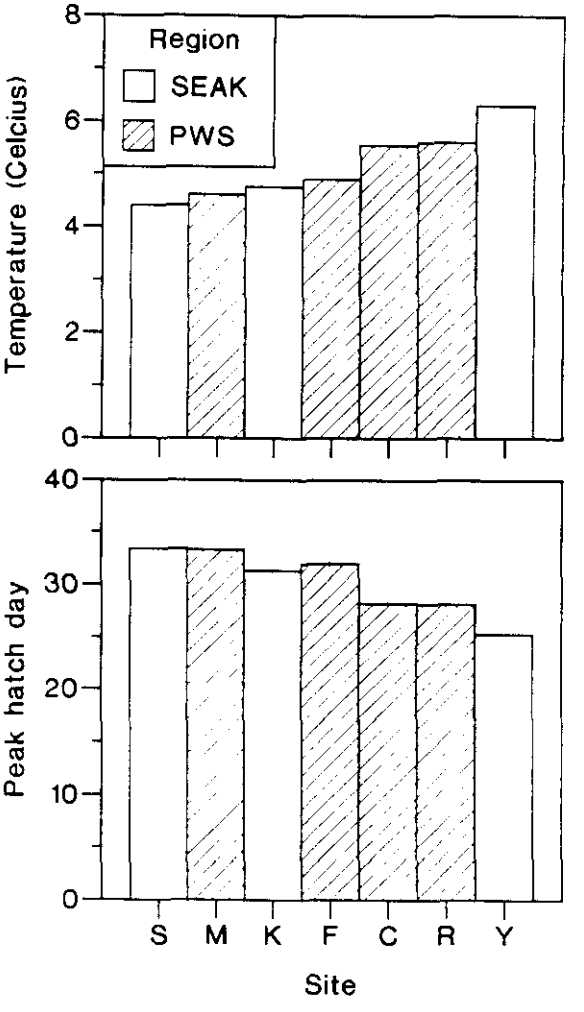

Figure 5.9. Peak hatch day and mean (± SE) incubation temperature (°C) for eggs spawned from mature Pacific herring by site and region in Alaska, 1995. (S = Sitka, M = St. Mathews Bay, K = Ketchikan, F = Fish Bay, C = Port Chalmers, R = Rocky Bay, Y = Seymour Canal; sites in chronological order of spawning date).

variable, did not indicate reproductive impairment of the 1989 year-class, and were inconsistent between sites.

Of the four key reproductive parameters we examined, spinal defects were particularly important because exposure of herring eggs to oil frequently causes spinal defects (Linden 1978; Pearson et al. 1985; Kocan et al. 1987; Rice et al. 1987) that could result in reduced swimming ability and long-term survival. Spinal defects, however, can also occur naturally as a result of other environmental factors. In our study, herring from an unoiled site, Ketchikan, had the highest percentage of spinal defects (7%). Ketchikan samples were collected at least 40 km from any urban area and it is unlikely that these fish were exposed to industrial or other urban pollutants. Whether the incidence of spinal defects at Ketchikan was just random noise or a response to some underlying environmental factor is impossible to determine, but is evidence that similar results could occur in PWS without implicating oil as a cause. Environmental variation, such as extremes in temperature, salinity, dissolved oxygen and ultraviolet radiation can also induce abnormalities in larval fish (Alderdice and Velsen 1971; Hunter et al. 1979). A 10% incidence of gross abnormalities was observed in PWS herring 23 years prior to the spill (Smith and Cameron 1979).

Reproductive success of herring in PWS was consistently better in 1995 than reported in earlier studies. For example, we observed a mean hatch success of 78-86% compared to 53% in 1976 (Smith and Cameron 1979), 62%[2] in 1989 (McGurk et al. 1990), 85% in 1990 (McGurk et al. 1991), 59-79% in 1991 (Kocan et al. 1996a), and 19-56% in 1992 (Kocan et al. 1996b). The viable hatch[3] we observed in PWS (79%) also exceeded previously reported percentages: 53%[4] in 1989 (McGurk et al. 1990), 57% in 1990 (McGurk et al. 1991), 35-37%[5] in 1991 (Kocan et al. 1996a), and 13-33% in 1992 (Kocan et al. 1996b). Incidence of spinal abnormalities in PWS was about 5% in our study compared to 7% in 1989 (McGurk et al. 1990). Although

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[2]To avoid desiccation effects, and because egg survival was significantly less in the +1.5 m collections in the McGurk et al. (1990) data set, these data were not included in this comparison. Estimated egg survival was 59% when the +1.5 m data was included.

[3]To conform with McGurk et al. (1990, 1991), % viable hatch was defined as 100·[(n live larvae - n abnormal larvae)/(n hatched)] · (n eggs hatched/n eggs total). The value defined by Kocan et al. (1996a,b) as % viable larvae is nearly synonymous with % viable hatch. Our % live larvae (Table 5.1) included abnormal larvae, but McGurk et al. (1990, 1991) excluded abnormal larvae in their definition of % viable larvae (% viable = 100·(n live larvae - n abnormal larvae)/n hatched)).

[4]As previously, +1.5 m data were not included; estimated % viable hatch was 50% when these data were included.

[5] Percent viable larvae values reported by Kocan et al. (1996a,b) should be increased by 2% to approximate percent viable hatch.

procedural differences between earlier studies and ours may partially account for differences in assessment of reproductive success, responses we observed in 1995 were consistently the best.

To interpret the effects of the spill on herring in PWS requires an understanding of the life stage exposed and the magnitude and duration of exposure. Which life stages were impacted, and to what extent, however, is largely a matter of conjecture. Adult fish may have encountered oil before, during, or after spawning, but determining what percentage of the population was significantly impacted is impossible. Metabolites of aromatic hydrocarbons were detected in adult herring (Haynes et al. 1995), but sample sizes were very low. Nematode prevalence in adult body cavities differed significantly between oiled and non-oiled areas (Moles et. al. 1993), also indicating adult exposure. The duration and magnitude of oil exposure of herring eggs and larvae is also unknown. After hatch, herring larvae from both oiled and unoiled sites may have been exposed to oil as they passively traversed the spill trajectory. For example, some of the largest concentrations of larvae in June were found in the southwest portion of PWS, well within the oil trajectory (Norcross et al. 1996). By inference, juvenile herring occupying the same nearshore habitat utilized by juvenile salmonids may have also been exposed to oil; such exposure was documented in juvenile pink and chum salmon (Carls et al. 1996).

Response of wild herring to an oil spill can be partially inferred from laboratory studies. For example, laboratory exposure of mature herring to hydrocarbons did not cause discernable damage in progeny, including fertility, viability, and larval swimming, morphological, and genetic abnormalities (Rice et al. 1987; Carls et al. Chapter 3). In contrast, the early life stages of herring are more susceptible to the effects of oil based on laboratory (Linden 1978; Pearson et al. 1985; Carls 1987; Kocan et al. 1987; Rice et al. 1987) and field studies (Brown et al. 1996a; Norcross et al. 1996). Abnormal larvae have poor survival potential (Kocan et al. 1996a) and thus, the exposure of eggs and larvae to oil in PWS may have resulted in increased mortality. Furthermore, the same oil concentrations that caused significant genetic damage also caused significant morphological damage in developing embryos (Carls et al. Chapter 4), suggesting that early death would likely preclude recruitment of genetically damaged individuals to spawning populations.

Although genetic damage was detected in larvae collected in oiled areas of PWS in 1989 (Hose et al. 1996; Brown et al. 1996a), we did not inspect larvae for genetic damage. Concomitant laboratory measurements of artificially oiled larvae suggested genetic response was not a more sensitive measure of oil exposure than the parameters we examined (Carls et al. Chapter 4). In addition, artificial exposure of pre-spawn adults to relatively high oil concentrations (58 ppb, initial PAH) did not cause genetic defects in artificially spawned progeny (Carls et al. Chapter 3). Other defects observed in larvae from PWS in 1989 included morphological damage, assessed by scored indices (Hose et al. 1996). Carls et al. (Chapter 4) observed that two of these indices, pericardial edema and finfold condition, were more sensitive to oil than the genetic response. Failure to detect significant pericardial abnormalities in larvae from PWS six years after the spill suggests that the genetic condition of these larvae has not been adversely affected.

The failure of the 1989 year-class of herring in PWS to recruit to the spawning population may have been partly attributable to the spill, but it is impossible to separate oil effects from other natural factors. At the sites we sampled in PWS, the 1989 year-class usually comprised <4.0% of the spawning population (J. Wilcock, Personal communication). Larval survival in PWS was reduced an estimated 52% in 1989 as a result of the spill (Brown et al. 1996a); such loss supports inferences of poor survival based on laboratory observation. Natural environmental conditions, however, can also cause a high degree of variability in herring recruitment (Stevenson 1962; Anthony and Fogarty 1985). For example, the 1989 year-class at Sitka also comprised a small proportion of the spawning population in 1995 (<2%; J. Wilcock, Personal communication), indicating that factors other than oil are important determinants of cohort size.

Whether or not herring in PWS were ever reproductively impaired by the spill is unknown, but the time lapse between the spill and our study probably precluded any detection of reproductive impairment. Measurable effects likely declined, probably most rapidly during the first year as the most adversely affected individuals died. Although oil-related abnormalities were observed in larvae immediately following the spill, both developmental and genetic damage progressively decreased with time (Brown et al. 1996a) and were undetectable in 1990 and 1991 (Hose et al. 1996). The extent of spawning site fidelity in herring is poorly understood but unaffected individuals from other geographic areas have probably joined remaining, less affected spawners, diluting possible residual effects. The disease epidemic observed in PWS in 1993 (Meyers et al. 1994) may have removed additional marginal spill survivors. Thus, it is not particularly surprising that reproductive impairment was not detected in 1995.

Understanding the long-term implications of exposure of Pacific herring to oil in PWS was the principal objective of this research. Regardless of the life stage and likelihood of possible oil exposure, herring we sampled in PWS in 1995 appeared to be reproductively fit and similar to herring in SEAK. Although herring stocks are still depressed in PWS, factors other than reproductive impairment are probably limiting recovery.

CONCLUSIONS

Reproductive impairment of PWS herring by the *Exxon Valdez* oil spill was not detected in 1995.

At this time, discrimination of any oil-related reproductive effects in herring from natural background variation appears negligible.

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APPENDICES

Appendix Contents

Appendix 1

General Information

Information common to the complete report is contained in this appendix, including schematic representation of the dosing apparatus, lists of analytes reported in chemical analyses (including abbreviations used in figures), and structural formulae of the five major homologous polynuclear aromatic hydrocarbon (PAH) groups discussed in this study (including examples of alkyl substitution within a group)

Appendix 1.1 Method of contamination of seawater.

Appendix 1.2 Polynuclear aromatic hydrocarbons names and abbreviations.

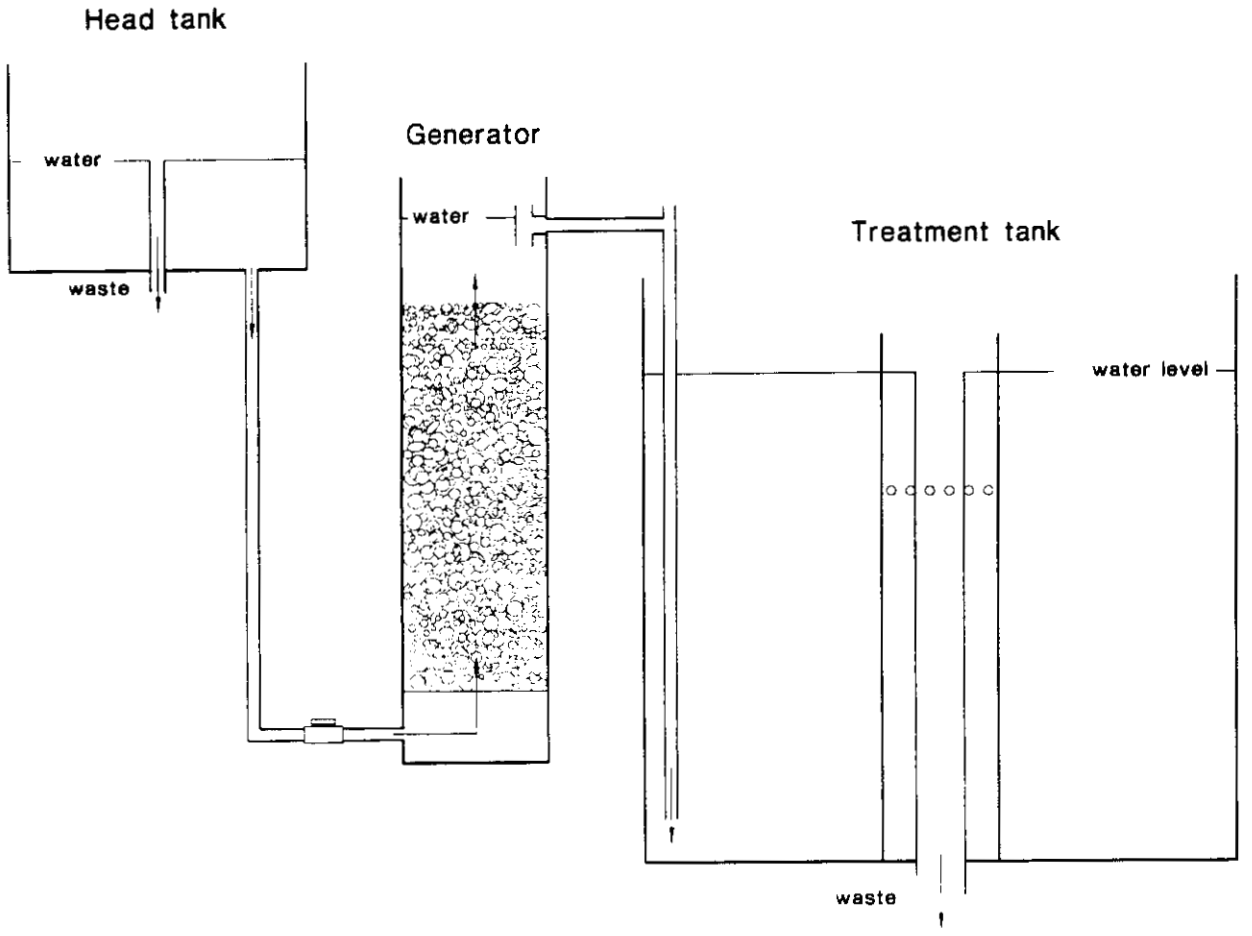
Appendix 1.3 Alkane hydrocarbons and their abbreviations.

Appendix 1.4 Structural formulas of naphthalenes, fluorenes, dibenzothiophenes, phenanthrenes, and chrysenes.

Appendix 1.5 Structural formulas demonstrating substitution in naphthalenes.

Appendix 1.6 Polynuclear aromatic hydrocarbon composition of *Exxon Valdez* crude oil compared to the Alaska North Slope crude oil used in these studies.

Appendix 1.7 Representative spectral intensity output of a fluorescent light source, including ultraviolet (UV) and visible wavelengths.



Appendix 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. Tank sizes were smaller in egg exposure experiments (1995), but other components remained the same.

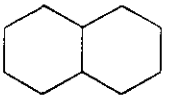
Appendix 1.2. Polynuclear aromatic hydrocarbons identified by gas chromatography, confirmed by mass spectrometry, and their abbreviations.

NPH	naphthalene
C1NPH	C-1 naphthalenes
C2NPH	C-2 naphthalenes
C3NPH	C-3 naphthalenes
C4NPH	C-4 naphthalenes
BPH	biphenyl
ACY	acenaphthylene
ACE	acenaphthene
FLU	fluorene
C1FLU	C-1 fluorenes
C2FLU	C-2 fluorenes
C3FLU	C-3 fluorenes
DBT	dibenzothiophene
C1DBT	C-1 dibenzothiophenes
C2DBT	C-2 dibenzothiophenes
C3DBT	C-3 dibenzothiophenes
PHN	phenanthrene
C1PHN	C-1 phenanthrenes/anthracenes
C2PHN	C-2 phenanthrenes/anthracenes
C3PHN	C-3 phenanthrenes/anthracenes
C4PHN	C-4 phenanthrenes/anthracenes
ANT	anthracene
FLA	fluoranthene
PYR	pyrene
C1FLA	C-1 fluoranthenes/pyrenes
BAA	benz-a-anthracene
CHR	chrysene
C1CHR	C-1 chrysenes
C2CHR	C-2 chrysenes
C3CHR	C-3 chrysenes
C4CHR	C-4 chrysenes
BbF	benzo-b-fluoranthene
BkF	benzo-k-fluoranthene
BEP	benzo-e-pyrene
BAP	benzo-a-pyrene
PER	perylene
IDP	indeno-123-cd-pyrene
DBA	dibenzo-a,h-anthracene
BZP	benzo-g,h,i-perylene

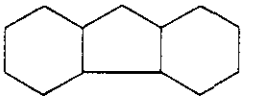
Appendix 1.3. Alkane hydrocarbons identified by gas chromatography and their abbreviations.

C10alk	c-10 alkanes
C11alk	c-11 alkanes
C12alk	c-12 alkanes
C13alk	c-13 alkanes
C14alk	c-14 alkanes
C15alk	c-15 alkanes
C16alk	c-16 alkanes
C17alk	c-17 alkanes
Pristane	pristane
C18alk	c-18 alkanes
Phytane	phytane
C19alk	c-19 alkanes
C20alk	c-20 alkanes
C21alk	c-21 alkanes
C22alk	c-22 alkanes
C23alk	c-23 alkanes
C24alk	c-24 alkanes
C25alk	c-25 alkanes
C26alk	c-26 alkanes
C27alk	c-27 alkanes
C28alk	c-28 alkanes
C29alk	c-29 alkanes
C30alk	c-30 alkanes
C31alk	c-31 alkanes
C32alk	c-32 alkanes
C33alk	c-33 alkanes
C34alk	c-34 alkanes
ALKANES	sum alkanes, including unidentified peaks, but excluding UCM.
UCM	unresolved complex mixture

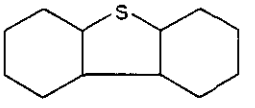
Naphthalene



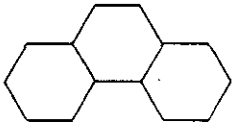
Fluorene



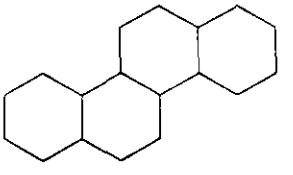
Dibenzothiophene



Phenanthrene

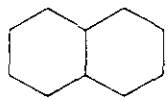


Chrysene

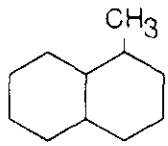


Appendix 1.4 Structural formulas of naphthalenes, fluorenes, dibenzothiophenes, phenanthrenes, and chrysenes.

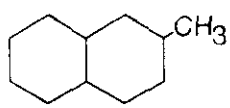
Naphthalene



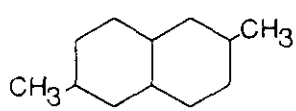
1-methylnaphthalene



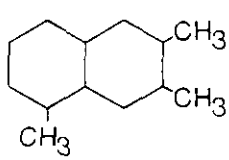
2-methylnaphthalene



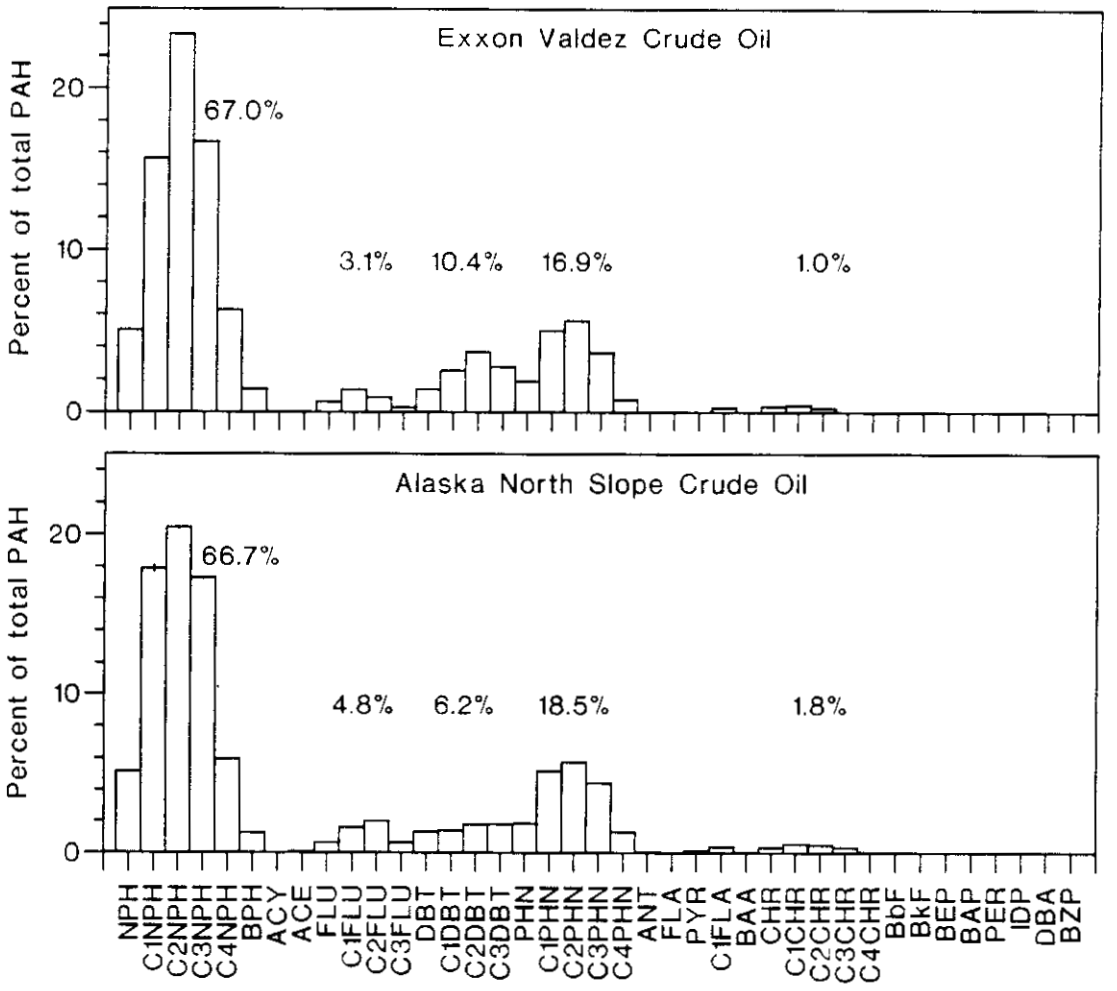
2,6-dimethylnaphthalene



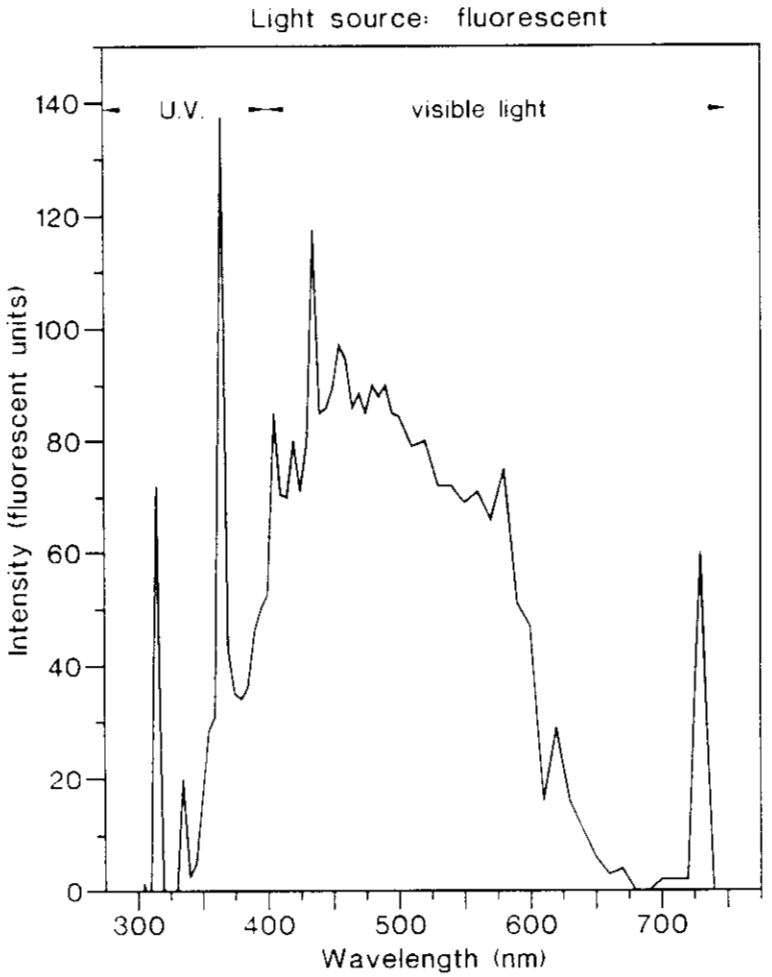
2,3,5-trimethylnaphthalene



Appendix 1.5. Structural formulas demonstrating substitution in naphthalenes. Unless otherwise indicated, hydrogen occupy ring corners. 1- and 2-methylnaphthalene are c1-naphthalenes, 2,6-dimethylnaphthalene is a c2-naphthalene, and 2,3,5-trimethylnaphthalene is a c3-naphthalene.



Appendix 1.6. Polynuclear aromatic hydrocarbon composition of *Exxon Valdez* crude oil compared to the Alaska North Slope crude oil used in these studies.



Appendix 1.7. Representative spectral intensity output of a fluorescent light source, including ultraviolet (UV) and visible wavelengths.

Appendix 2

Histopathological scores: preliminary, non-parametric analyses

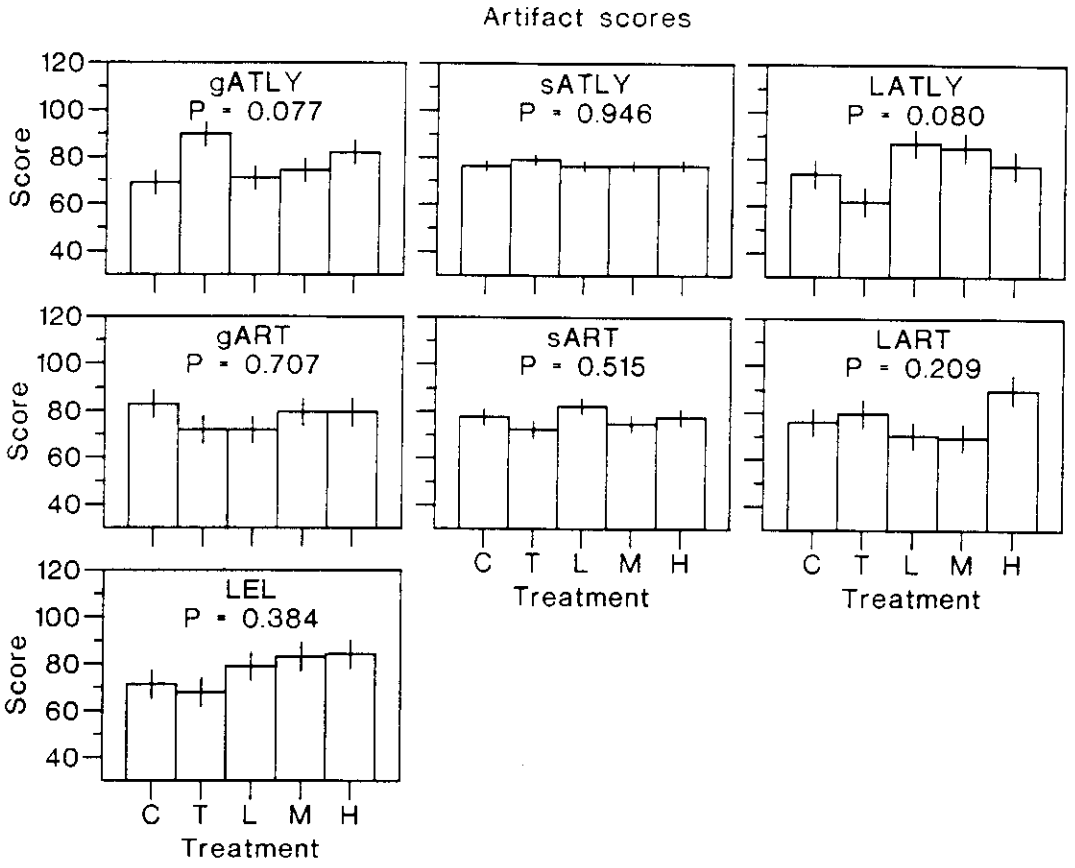
Individual histopathological scores were analyzed with the Kruskal-Wallis non-parametric test. Results were pooled by treatment in these tests.

Appendix 2.1. Lesion scores classified as artifact or autolysis as functions of treatment.

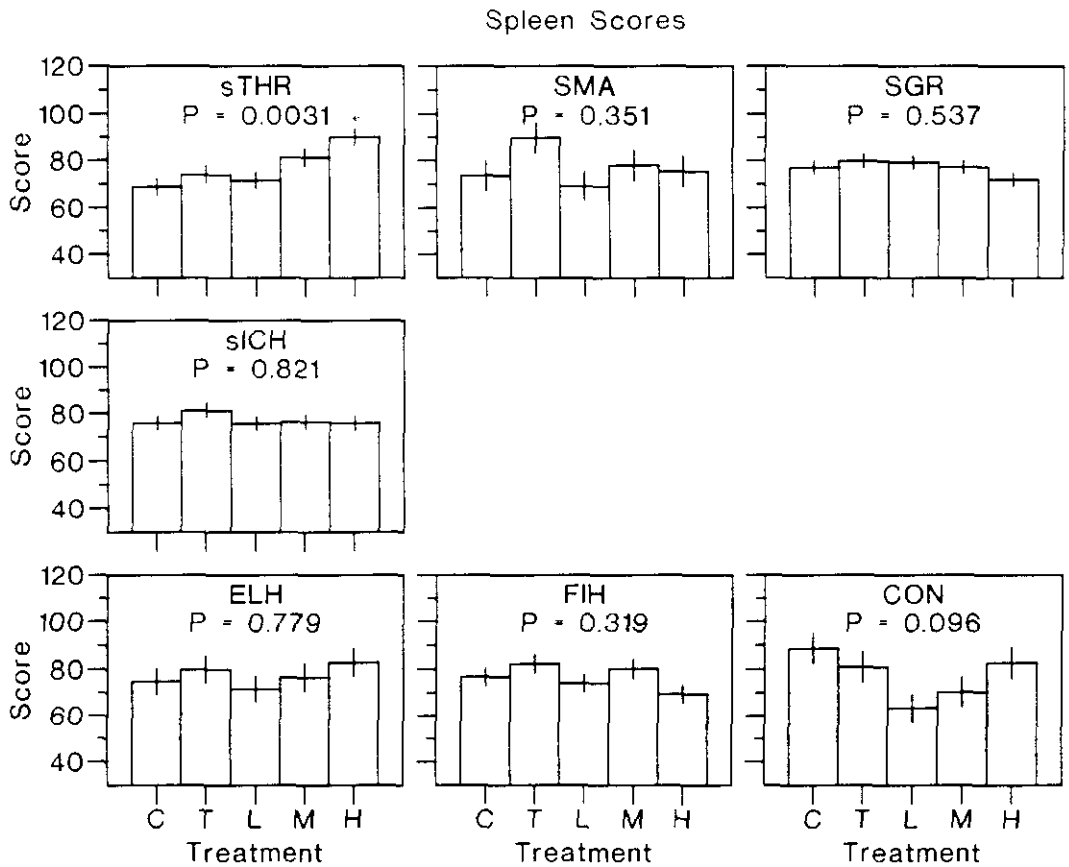
Appendix 2.2. Lesion scores in spleen as functions of treatment.

Appendix 2.3. Lesion scores in liver as functions of treatment.

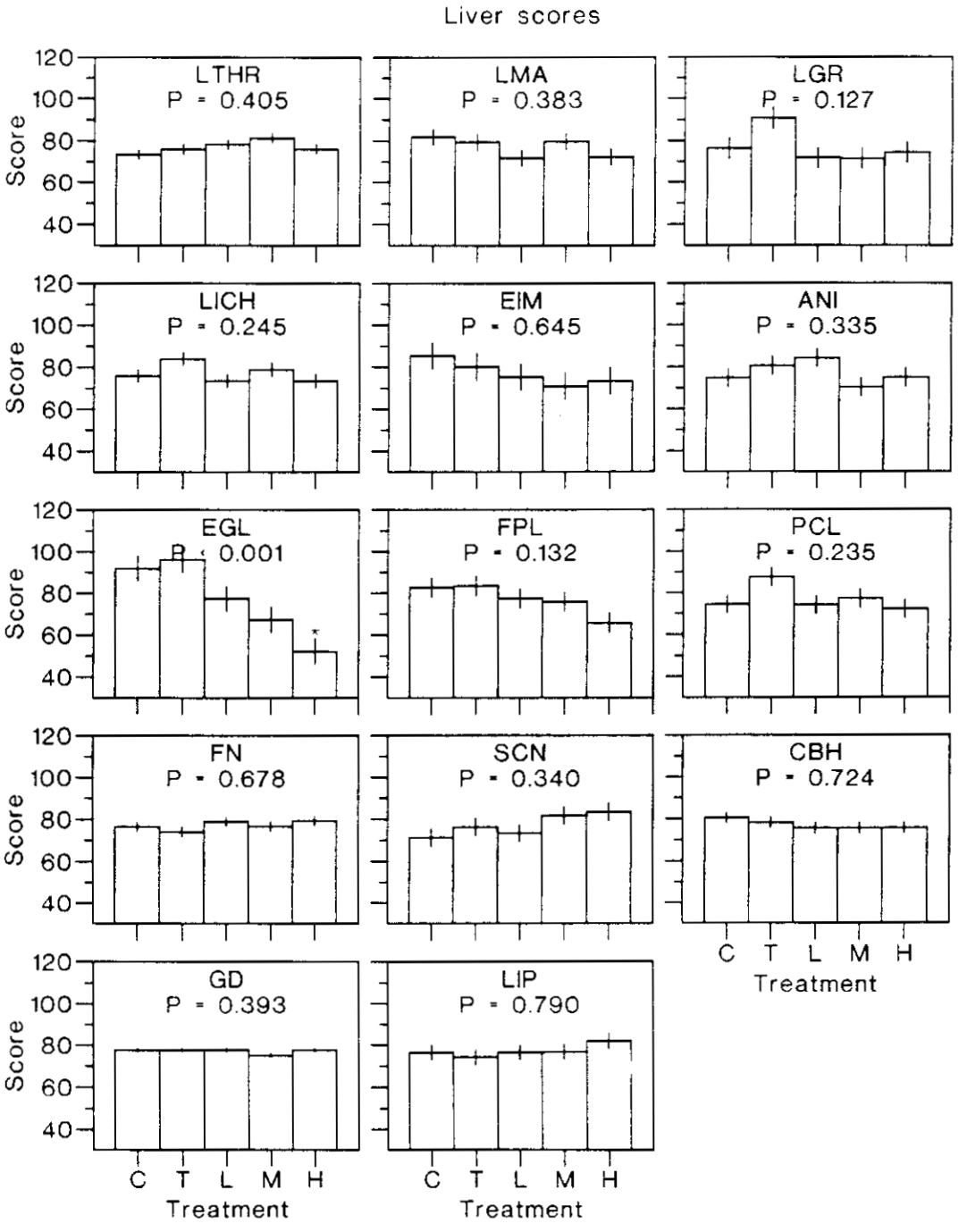
Appendix 2.4. Lesion scores in gill as functions of treatment.



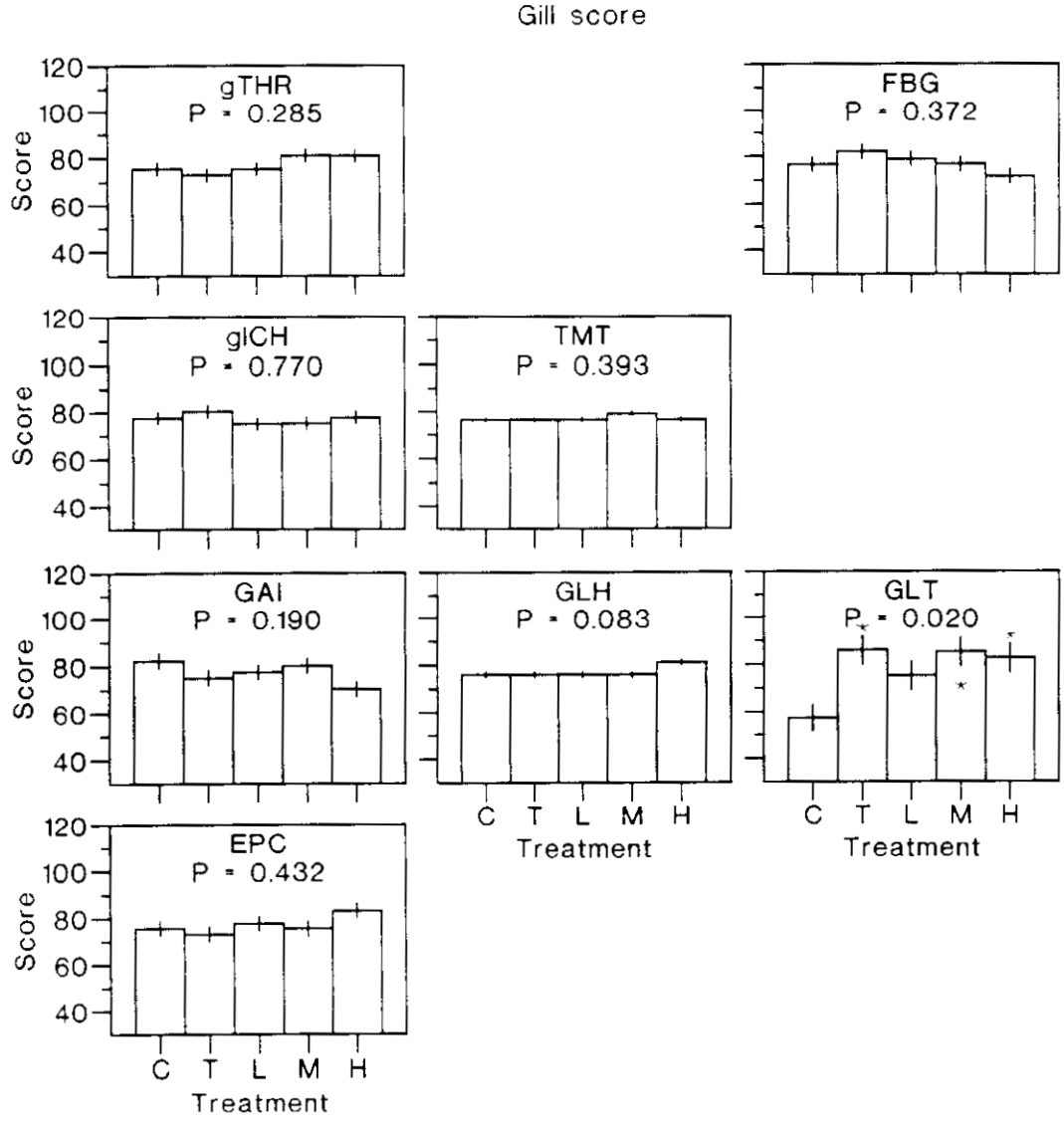
Appendix 2.1. Lesion scores classified as artifact or autolysis as functions of treatment: gill autolysis (gATLY), gill artifact (gART), lamellar epithelial lifting (LEL), spleen autolysis (sATLY), spleen artifact (sART), liver autolysis (LATLY), and liver artifact (LART). None of these lesions varied significantly as a function of oil treatment. Data displayed are Kruskal-Wallis scores ± standard deviation.



Appendix 2.2. Lesion scores in spleen as functions of treatment: thrombosis (sTHR), *Ichthyophonus hoferi* (sICH), ellipsoid hyalinization (ELH), macrophage aggregates (SMA), focal intimal hyperplasia in blood vessels (FIH), granulomatous inflammation (SGR), and congestion (CON). Asterisk indicates significant difference from control. Data displayed are Kruskal-Wallis scores ± standard deviation.



Appendix 2.3. Lesion scores in liver as functions of treatment: thrombosis (LTHR), *Ichthyophonus hoferi* (LICH), eosinophilic granular leukocytes (EGL), focal necrosis (FN), glycogen depletion (GD), macrophage aggregates (LMA), *Goussia clupearum* (EIM), focal or multifocal parenchymal leukocytes (FPL), single cell necrosis (SCN), lipidosis (LIP), granulomas (LGR), *Anisakis spp* (ANI), pericholangial leukocytes (PCL), and cholangitis or biliary hyperplasia (CBH). Asterisk indicates significant difference from control. Data displayed are Kruskal-Wallis scores ± standard deviation.



Appendix 2.4. Lesion scores in gill as functions of treatment: thrombosis (gTHR), *Ichthyophonus hoferi*, (gICH), gill arch inflammation (GAI), Epitheliocystis (EPC), monogenetic or digenetic trematode (TMT), gill lamellar hyperplasia (GLH), foreign body granuloma (FBG), and gill lamellar telangiectasis (GLT). Asterisks indicate significant differences from control. Data displayed are Kruskal-Wallis scores ± standard deviation.

Appendix 3.1

Composition of polynuclear aromatic hydrocarbons in 1994 treatment water

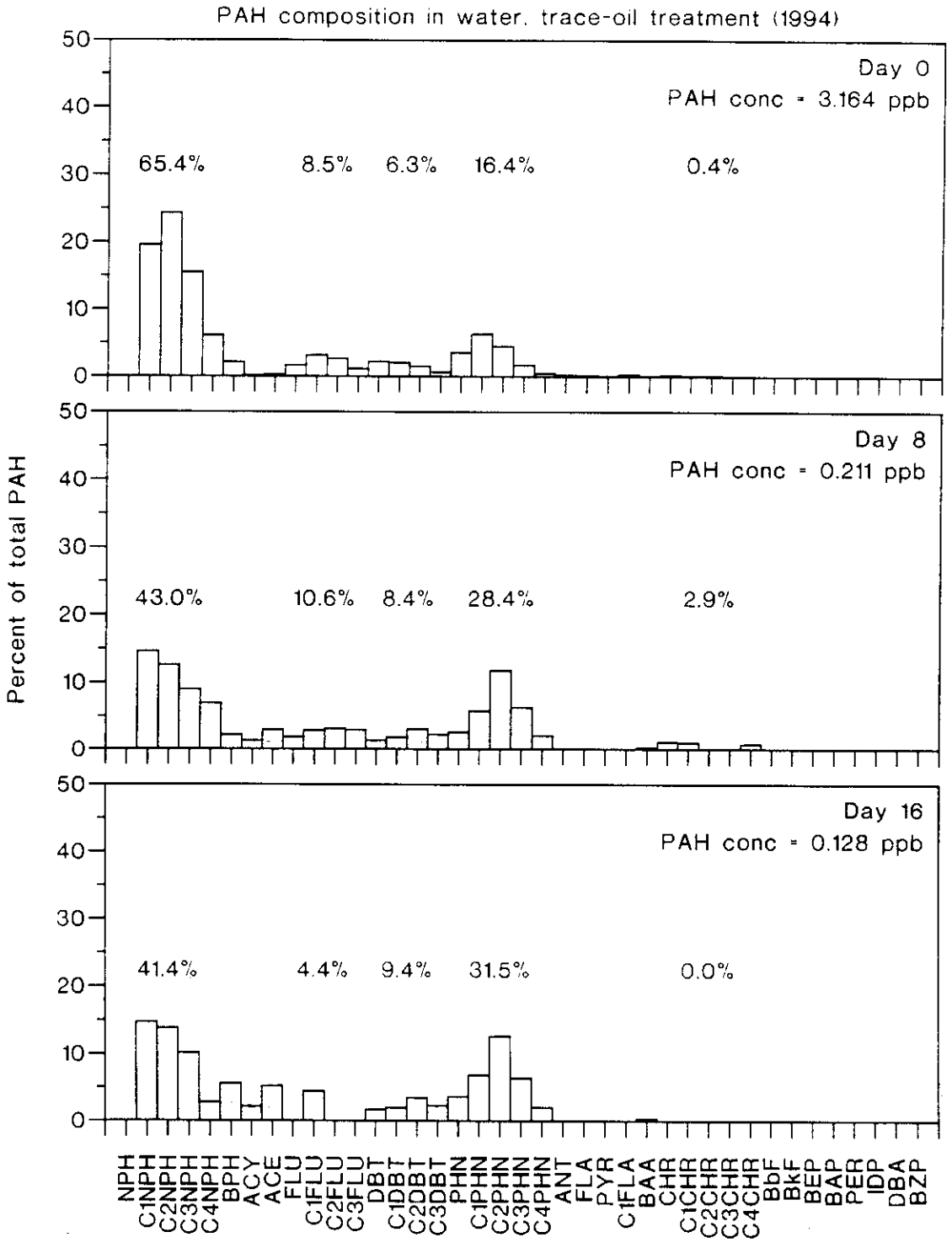
Composition of polynuclear aromatic hydrocarbons (PAH) in water in 1994. Characteristic time-dependent weathering patterns were evident: percentages of lighter compounds, particularly naphthalenes, decreased, while percentages of heavier compounds, particularly phenanthrenes, increased. Differential weathering across treatments was also evident; for example compare total naphthalenes on day 0 for each treatment. Abbreviations of chemical compounds are found in Appendix 0.2.

Appendix 3.1.1. Composition of PAH in trace-oil treatment water in 1994 at the beginning, midpoint, and end of the experiment.

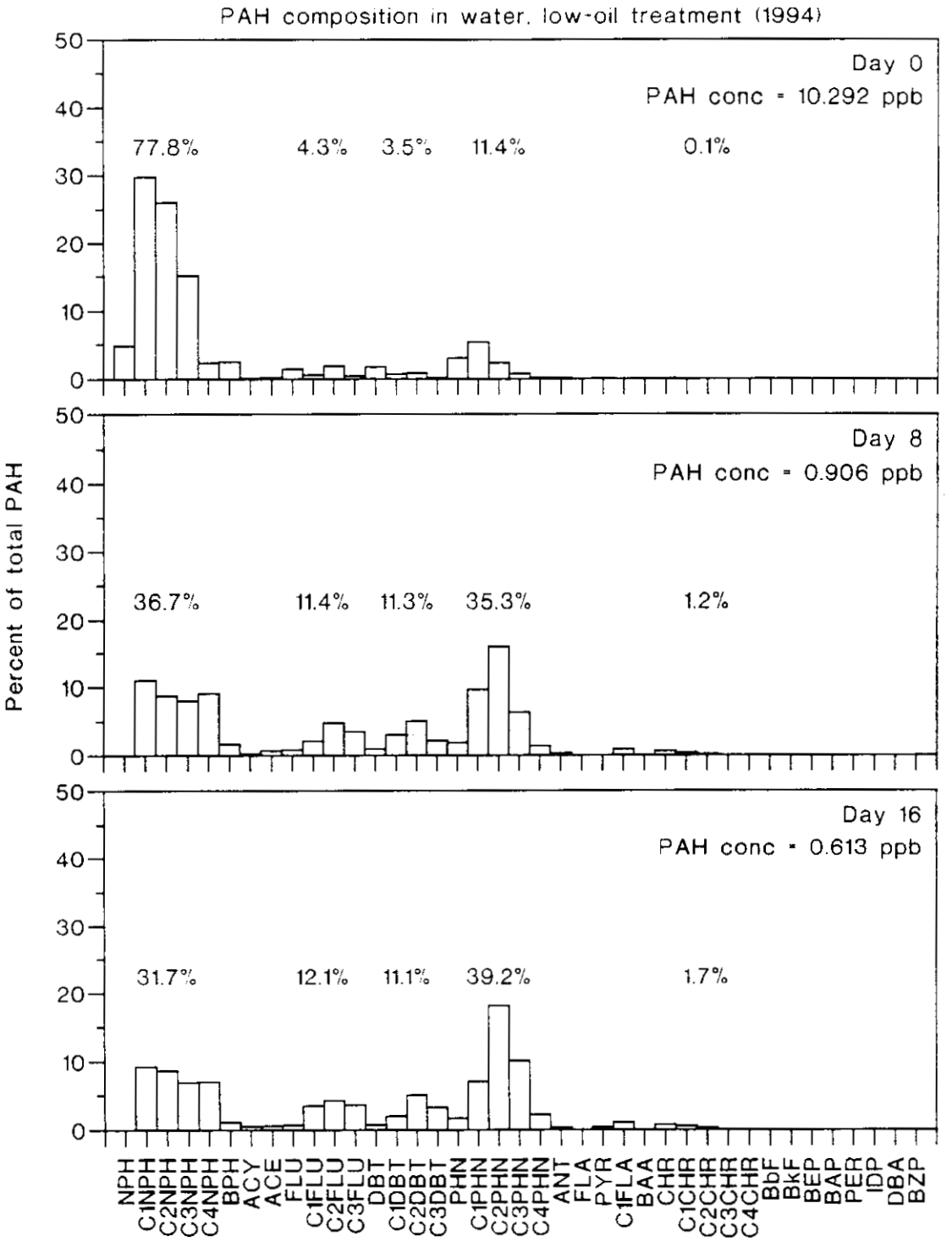
Appendix 3.1.2. Composition of PAH in low-oil treatment water in 1994 at the beginning, midpoint, and end of the experiment.

Appendix 3.1.3. Composition of PAH in mid-oil treatment water in 1994 at the beginning, midpoint, and end of the experiment.

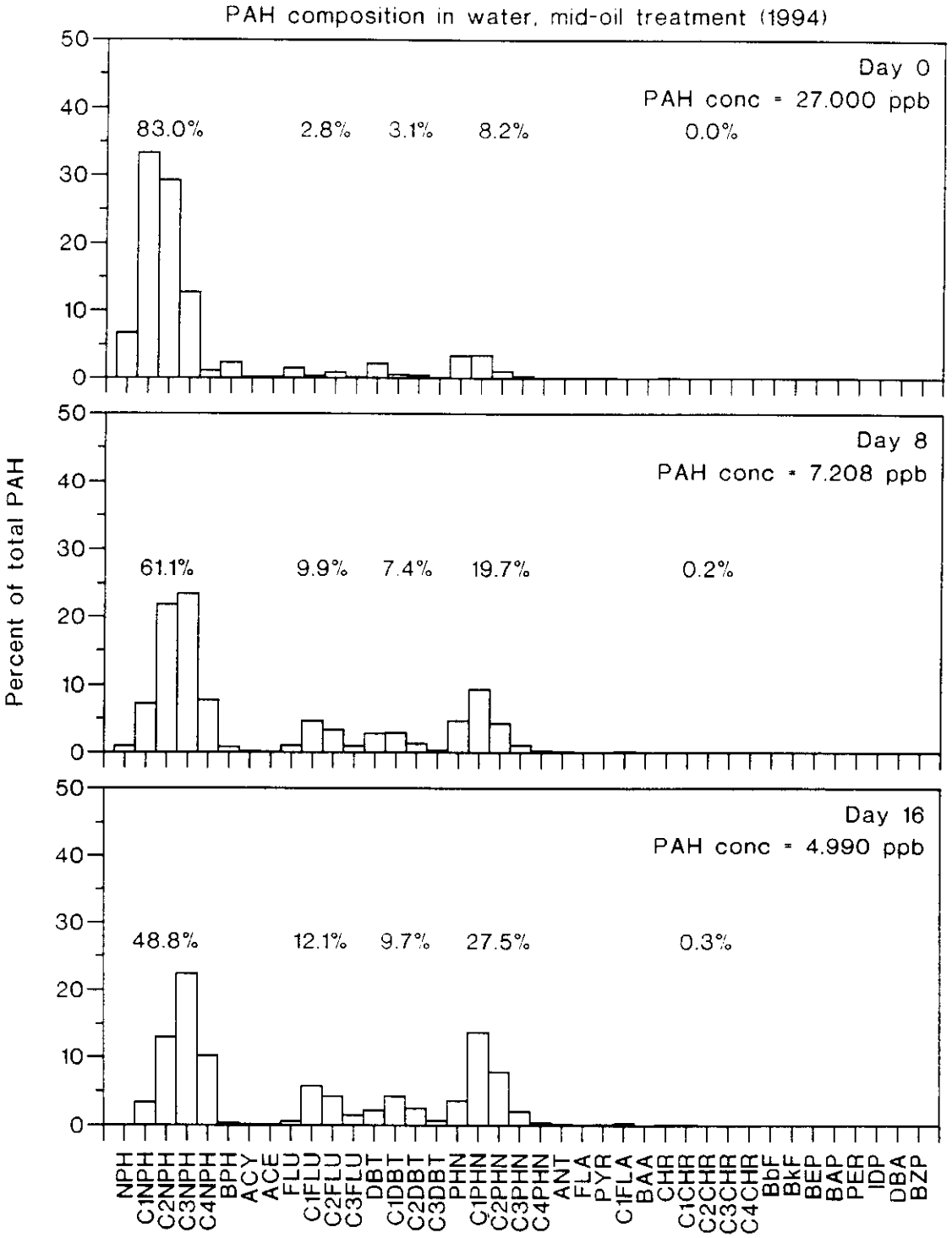
Appendix 3.1.4. Composition of PAH in high-oil treatment water in 1994 at the beginning, midpoint, and end of the experiment.



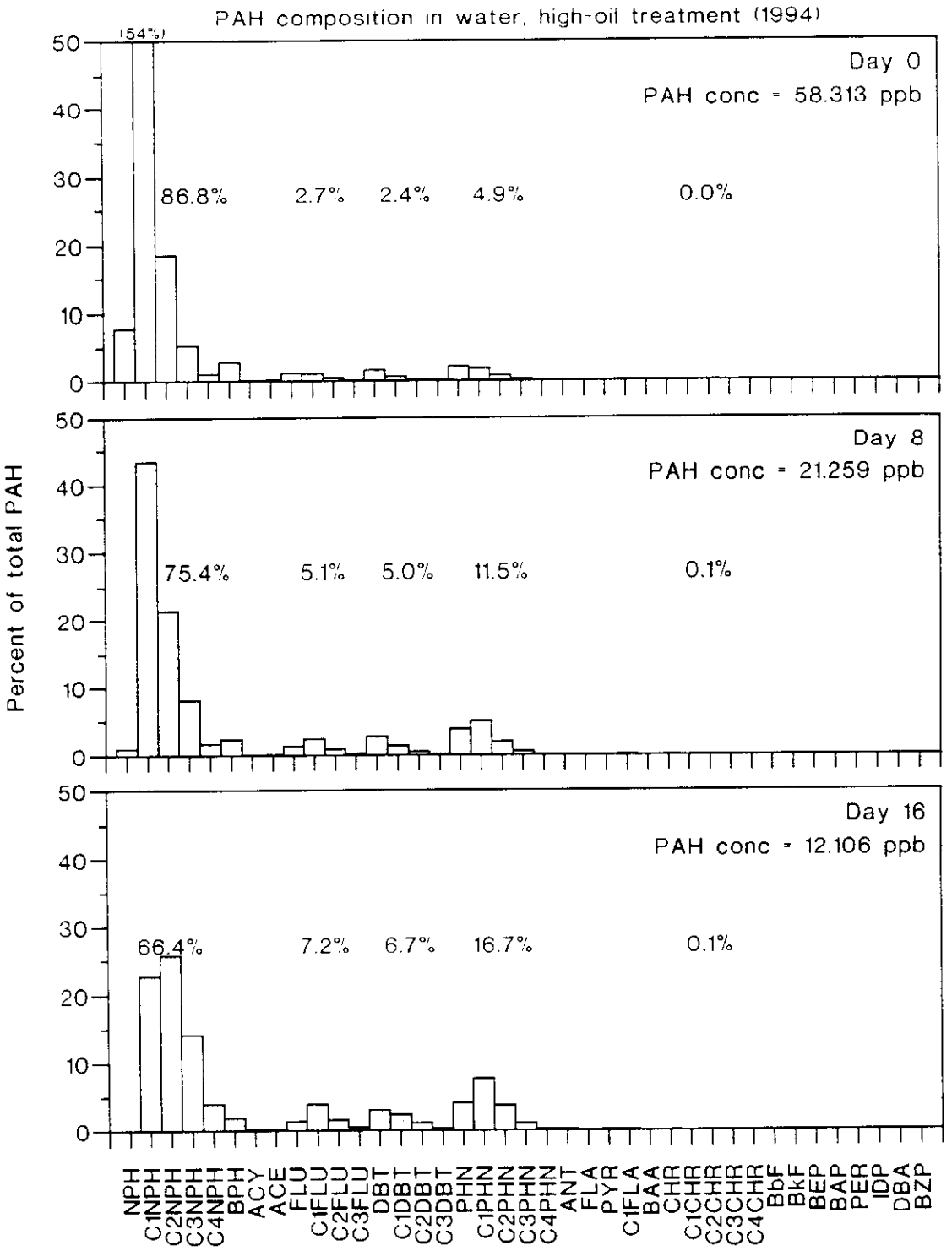
Appendix 3.1.1. Composition of PAH in trace-oil treatment water in 1994 at the beginning, midpoint, and end of the experiment. Percentages printed inside the graphs indicate total percentages of the following homologous chemical groups (left to right): naphthalenes, fluorenes, dibenzothiophenes, phenanthrenes, and chrysenes.



Appendix 3.1.2. Composition of PAH in low-oil treatment water in 1994 at the beginning, midpoint, and end of the experiment. Percentages printed inside the graphs indicate total percentages of the following homologous chemical groups (left to right): naphthalenes, fluorenes, dibenzothiophenes, phenanthrenes, and chrysenes.



Appendix 3.1.3. Composition of PAH in mid-oil treatment water in 1994 at the beginning, midpoint, and end of the experiment. Percentages printed inside the graphs indicate total percentages of the following homologous chemical groups (left to right): naphthalenes, fluorenes, dibenzothiophenes, phenanthrenes, and chrysenes.



Appendix 3.1.4. Composition of PAH in high-oil treatment water in 1994 at the beginning, midpoint, and end of the experiment. Percentages printed inside the graphs indicate total percentages of the following homologous chemical groups (left to right): naphthalenes, fluorenes, dibenzothiophenes, phenanthrenes, and chrysenes.

Appendix 3.2

Composition of polynuclear aromatic hydrocarbons in muscle and ovary (1994)

Composition of polynuclear aromatic hydrocarbon (PAH) in muscle and ovaries compared to that in water. Composition in ovaries differed significantly from that in water as did composition in muscle tissue ($P_{MANOVA} < 0.01$). Composition in tissue was strongly biased toward naphthalenes; in compounds heavier than biphenyl, there was also a shift toward unsubstituted homologs. Shifts in composition were more pronounced in ovaries than in muscle, thus differences in PAH composition were also significant between ovary and muscle ($P_{MANOVA} < 0.01$). Percentages of unsubstituted fluorenes, dibenzothiophenes, and phenanthrenes were greater in ovarian tissue than in muscle tissue ($P_{ANOVA} < 0.001$). Abbreviations of chemical compounds are found in Appendix 0.2.

Appendix 3.2.1. Composition of PAH in muscle and ovarian tissue in the trace-oil treatment, 8 d exposure (1994), compared to that in water at the midpoint of the experiment.

Appendix 3.2.2. Composition of PAH in muscle and ovarian tissue in the low-oil treatment, 8 d exposure (1994), compared to that in water at the midpoint of the experiment.

Appendix 3.2.3. Composition of PAH in muscle and ovarian tissue in the mid-oil treatment, 8 d exposure (1994), compared to that in water at the midpoint of the experiment.

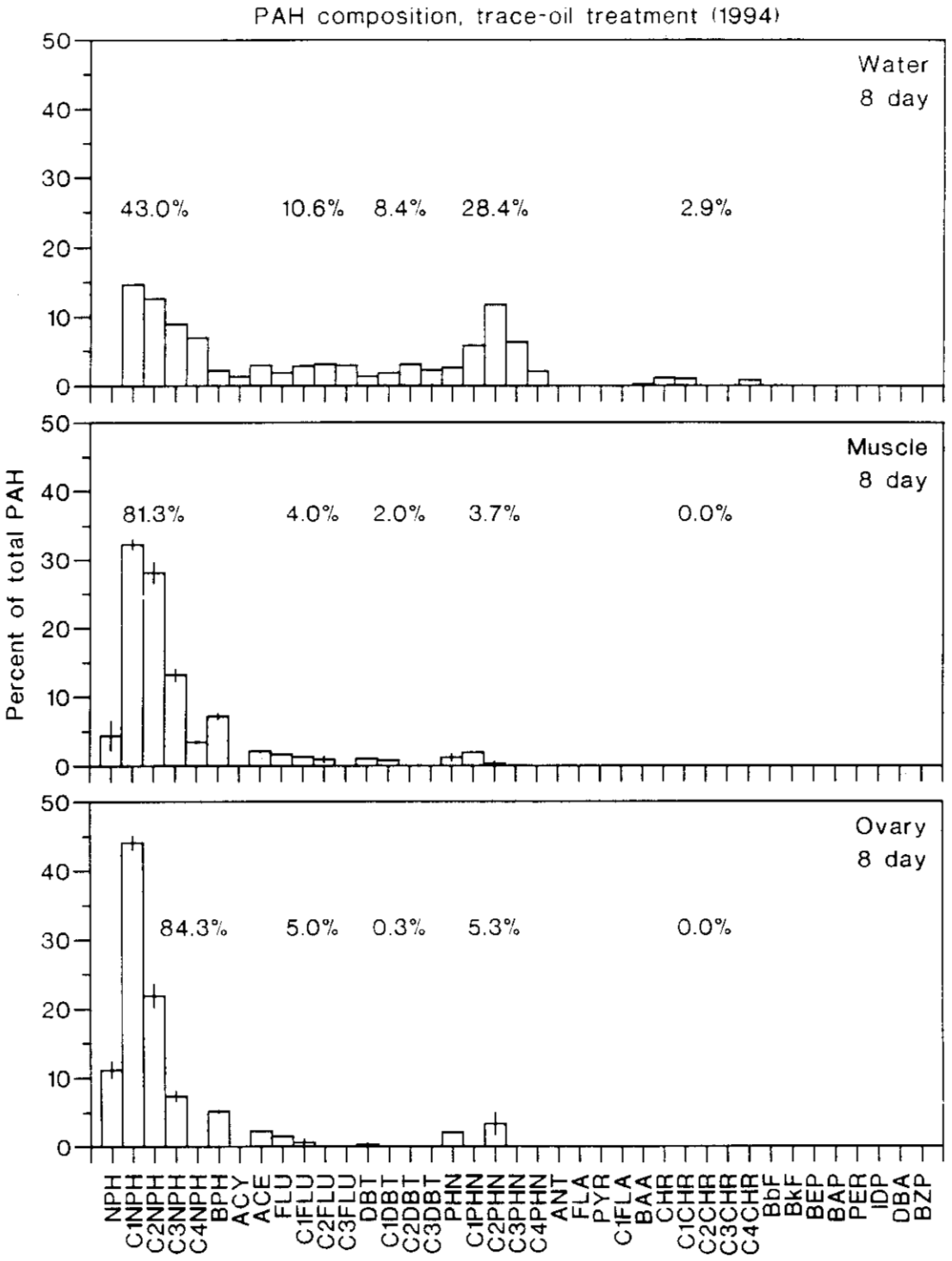
Appendix 3.2.4. Composition of PAH in muscle and ovarian tissue in the high-oil treatment 8 d exposure (1994), compared to that in water at the midpoint of the experiment.

Appendix 3.2.5. Composition of PAH in muscle and ovarian tissue in the trace-oil treatment, 16 d exposure (1994), compared to that in water at the endpoint of the experiment.

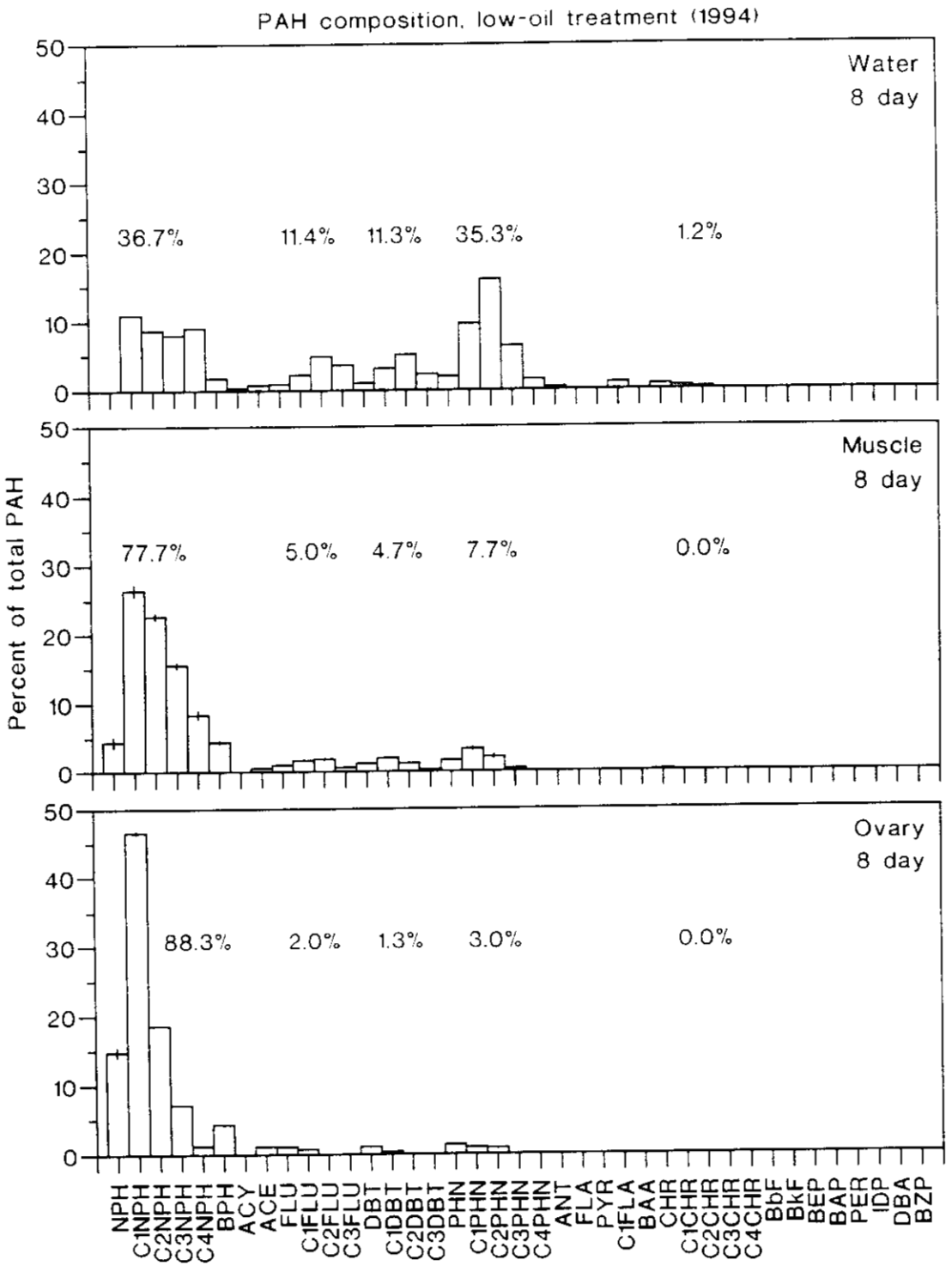
Appendix 3.2.6. Composition of PAH in muscle and ovarian tissue in the low-oil treatment, 16 d exposure (1994), compared to that in water at the endpoint of the experiment.

Appendix 3.2.7. Composition of PAH in muscle and ovarian tissue in the mid-oil treatment, 16 d exposure (1994), compared to that in water at the endpoint of the experiment.

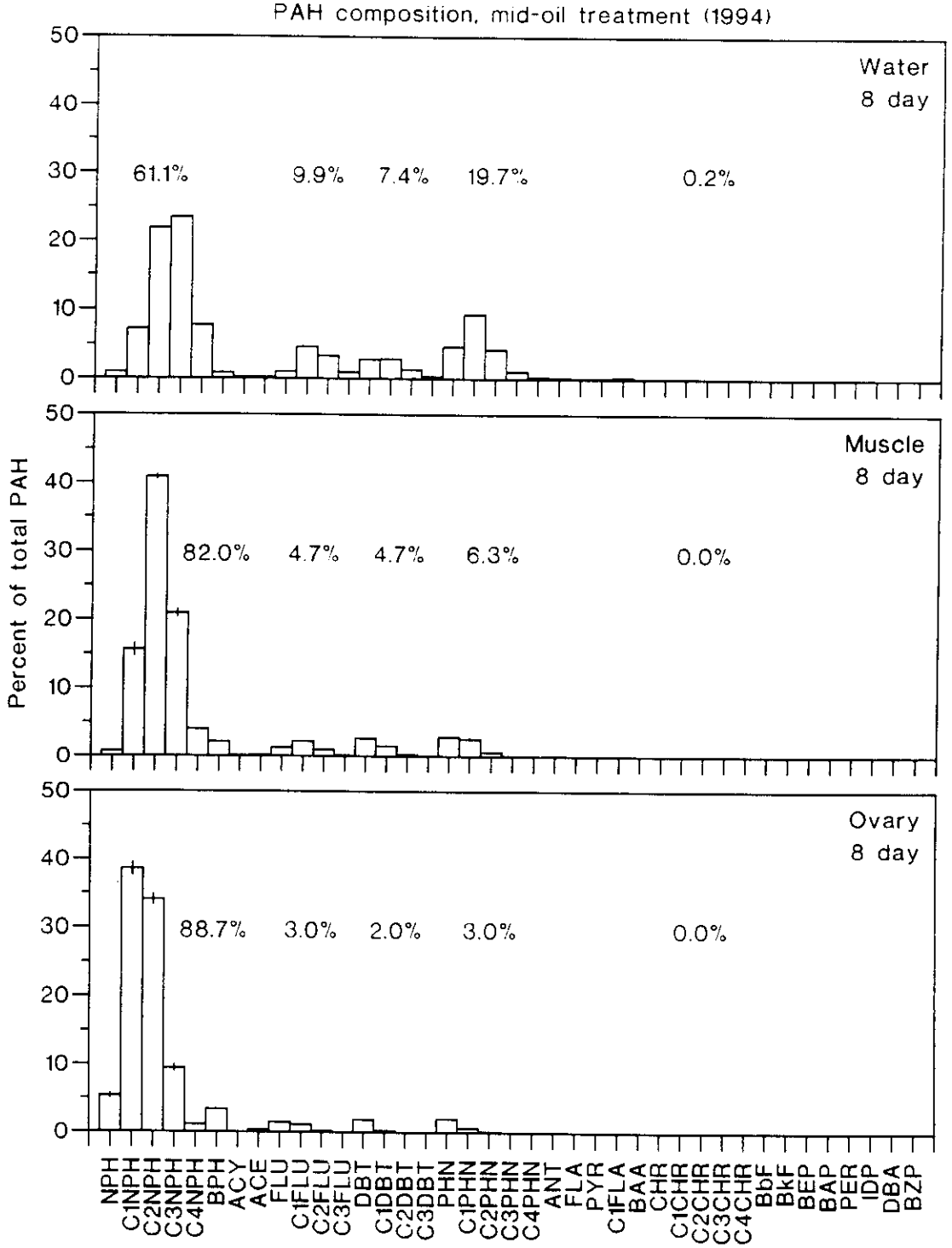
Appendix 3.2.8. Composition of PAH in muscle and ovarian tissue in the high-oil treatment, 16 d exposure (1994), compared to that in water at the endpoint of the experiment.



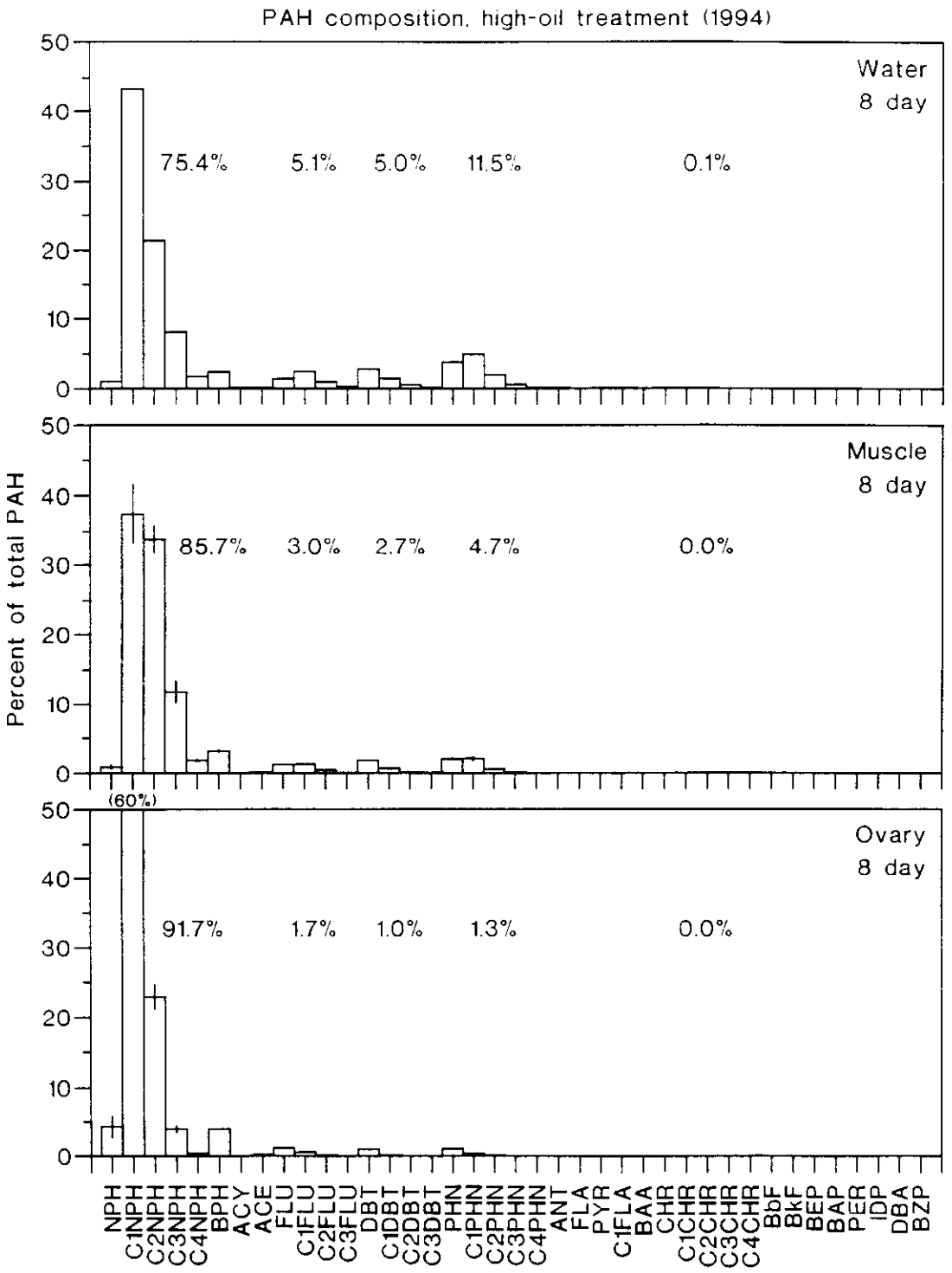
Appendix 3.2.1. Composition of PAH in muscle and ovarian tissue in the trace-oil treatment, 8 d exposure (1994), compared to that in water at the midpoint of the experiment. Percentages printed inside the graphs indicate total percentages of the following homologous chemical groups (left to right): naphthalenes, fluorenes, dibenzothiophenes, phenanthrenes, and chrysenes.



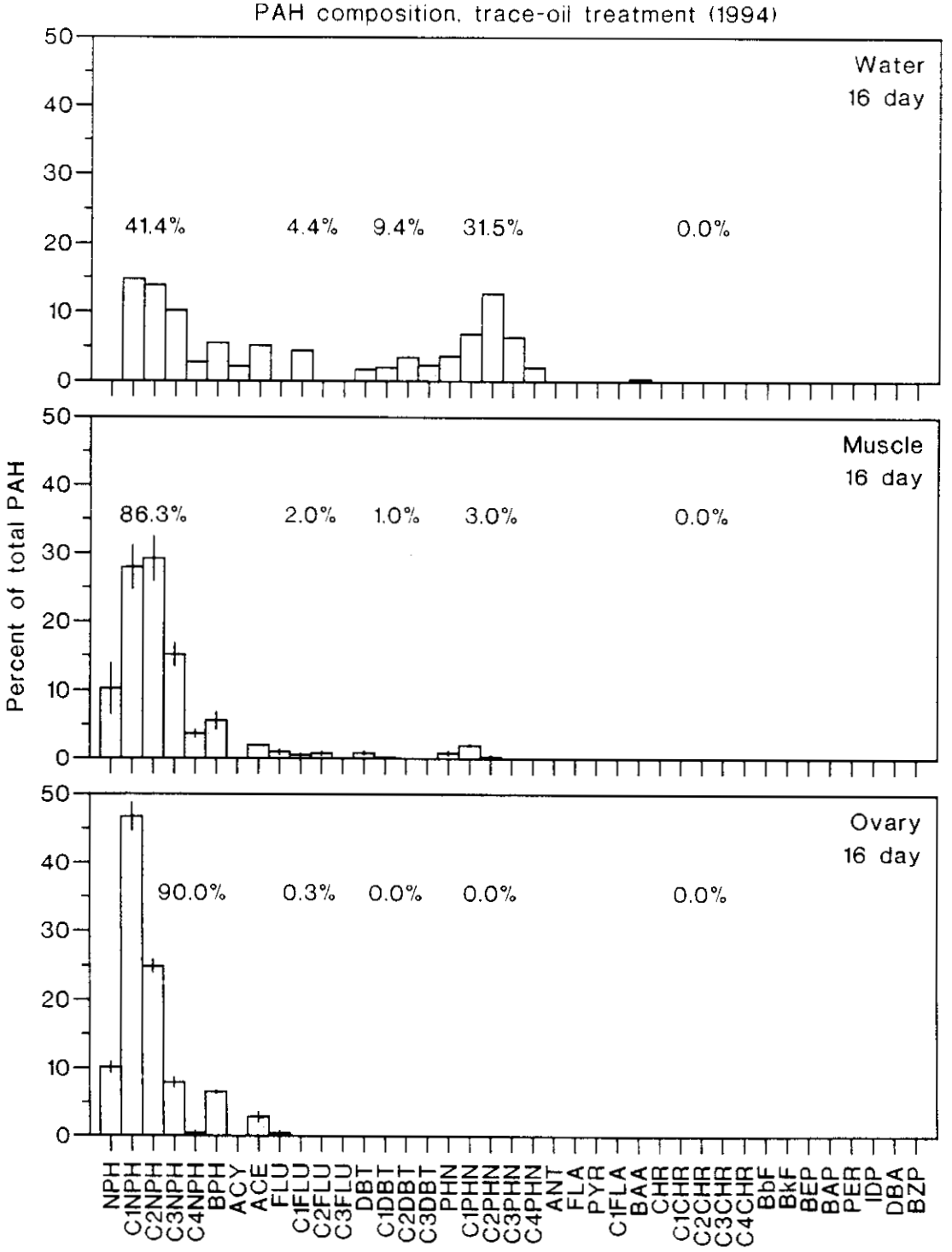
Appendix 3.2.2. Composition of PAH in muscle and ovarian tissue in the low-oil treatment, 8 d exposure (1994), compared to that in water at the midpoint of the experiment. Percentages printed inside the graphs indicate total percentages of the following homologous chemical groups (left to right): naphthalenes, fluorenes, dibenzothiophenes, phenanthrenes, and chrysenes.



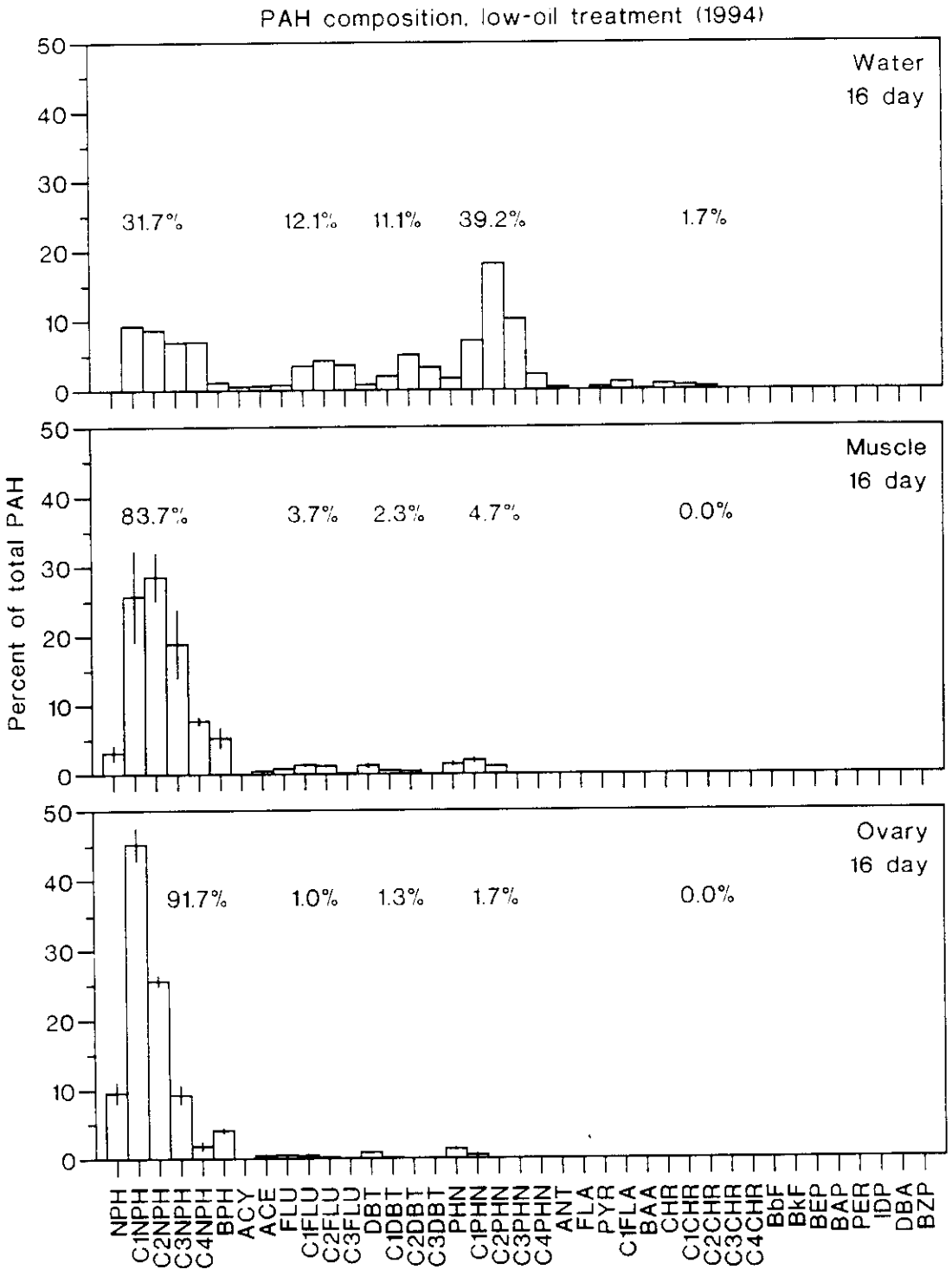
Appendix 3.2.3. Composition of PAH in muscle and ovarian tissue in the mid-oil treatment, 8 d exposure (1994), compared to that in water at the midpoint of the experiment. Percentages printed inside the graphs indicate total percentages of the following homologous chemical groups (left to right): naphthalenes, fluorenes, dibenzothiophenes, phenanthrenes, and chrysenes.



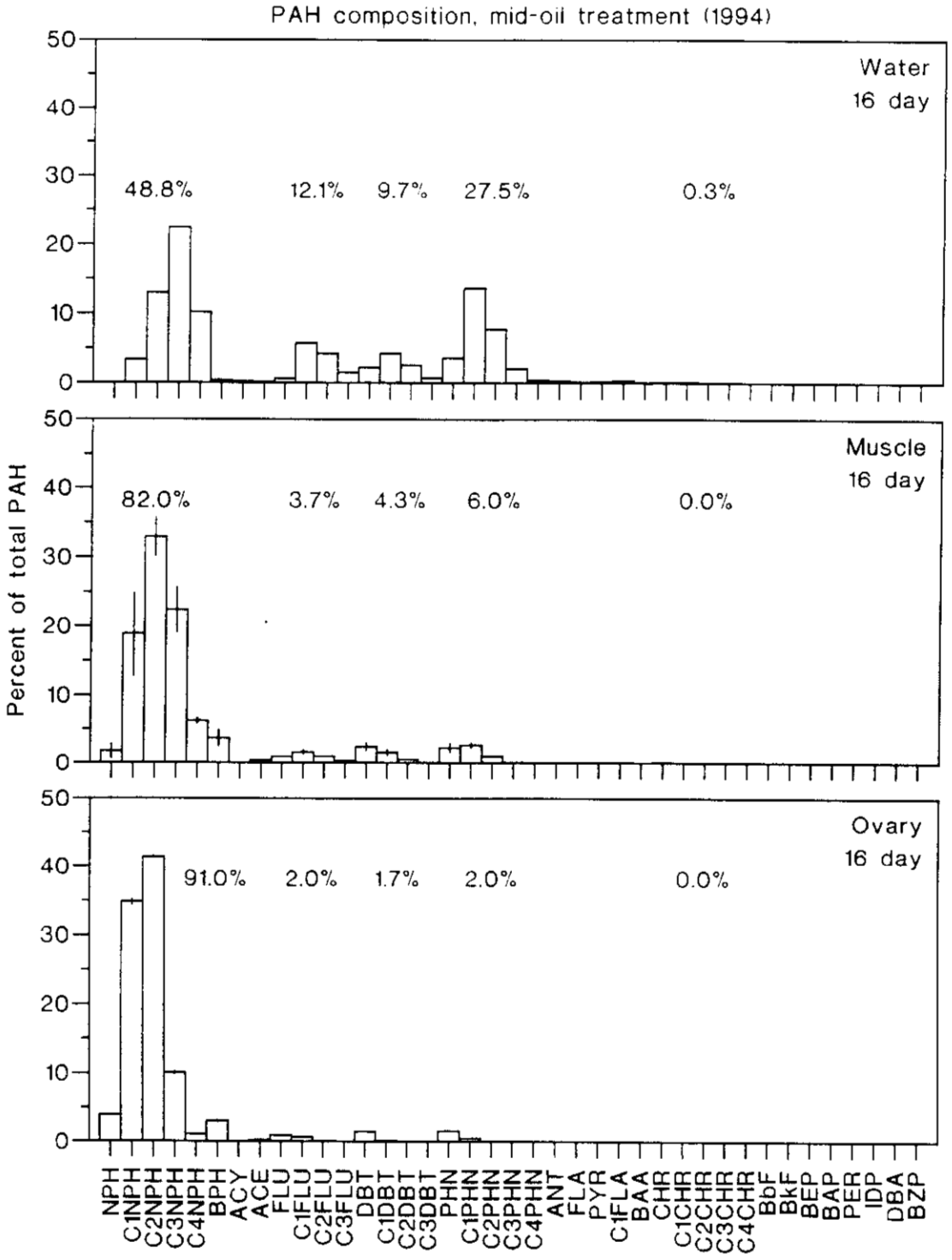
Appendix 3.2.4. Composition of PAH in muscle and ovarian tissue in the high-oil treatment 8 d exposure (1994), compared to that in water at the midpoint of the experiment. Percentages printed inside the graphs indicate total percentages of the following homologous chemical groups (left to right): naphthalenes, fluorenes, dibenzothiophenes, phenanthrenes, and chrysenes.



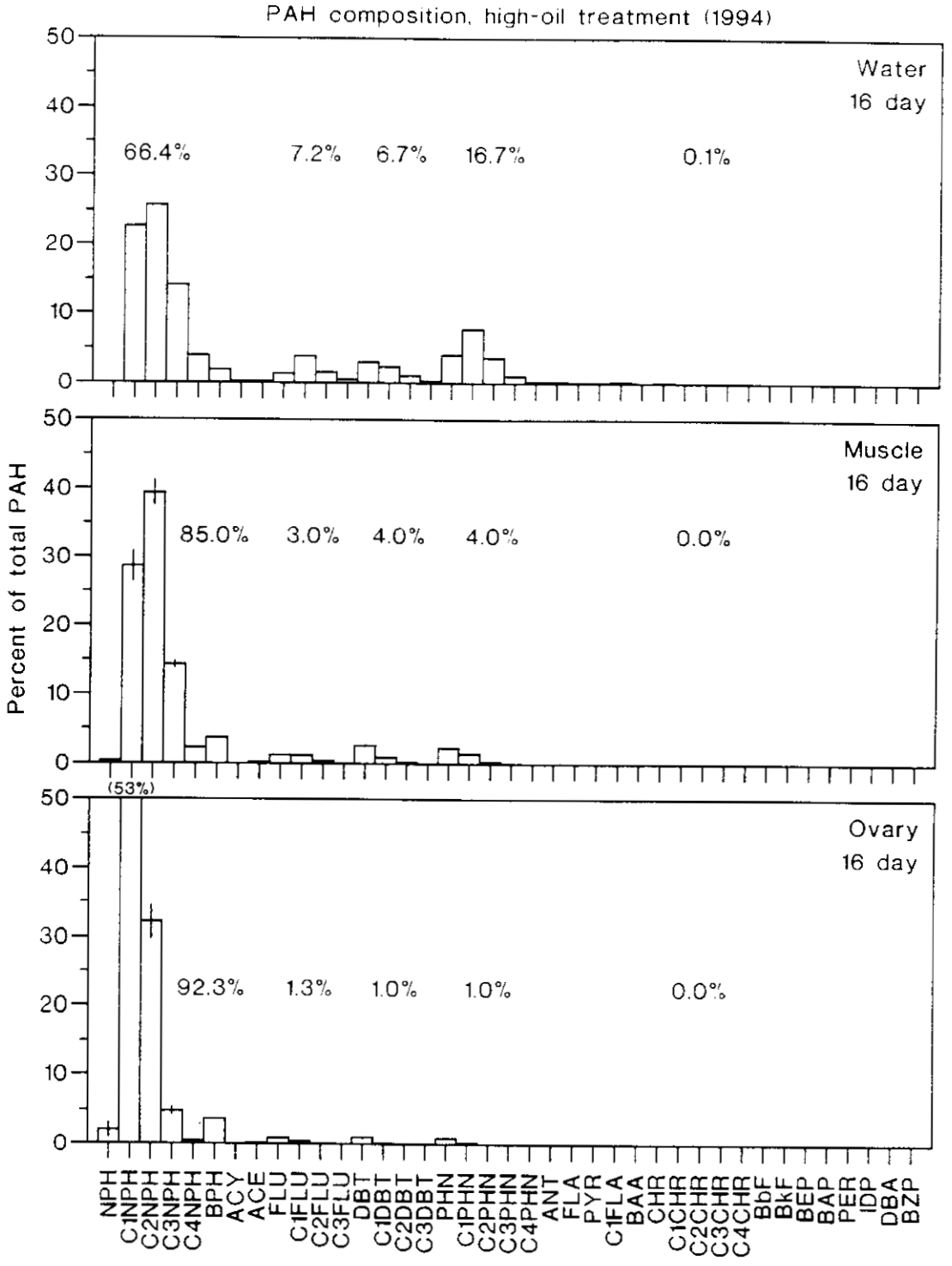
Appendix 3.2.5. Composition of PAH in muscle and ovarian tissue in the trace-oil treatment, 16 d exposure (1994), compared to that in water at the endpoint of the experiment. Percentages printed inside the graphs indicate total percentages of the following homologous chemical groups (left to right): naphthalenes, fluorenes, dibenzothiophenes, phenanthrenes, and chrysenes.



Appendix 3.2.6. Composition of PAH in muscle and ovarian tissue in the low-oil treatment, 16 d exposure (1994), compared to that in water at the endpoint of the experiment. Percentages printed inside the graphs indicate total percentages of the following homologous chemical groups (left to right): naphthalenes, fluorenes, dibenzothiophenes, phenanthrenes, and chrysenes.



Appendix 3.2.7. Composition of PAH in muscle and ovarian tissue in the mid-oil treatment, 16 d exposure (1994), compared to that in water at the endpoint of the experiment. Percentages printed inside the graphs indicate total percentages of the following homologous chemical groups (left to right): naphthalenes, fluorenes, dibenzothiophenes, phenanthrenes, and chrysenes.



Appendix 3.2.8. Composition of PAH in muscle and ovarian tissue in the high-oil treatment, 16 d exposure (1994), compared to that in water at the endpoint of the experiment. Percentages printed inside the graphs indicate total percentages of the following homologous chemical groups (left to right): naphthalenes, fluorenes, dibenzothiophenes, phenanthrenes, and chrysenes.

Appendix 3.3

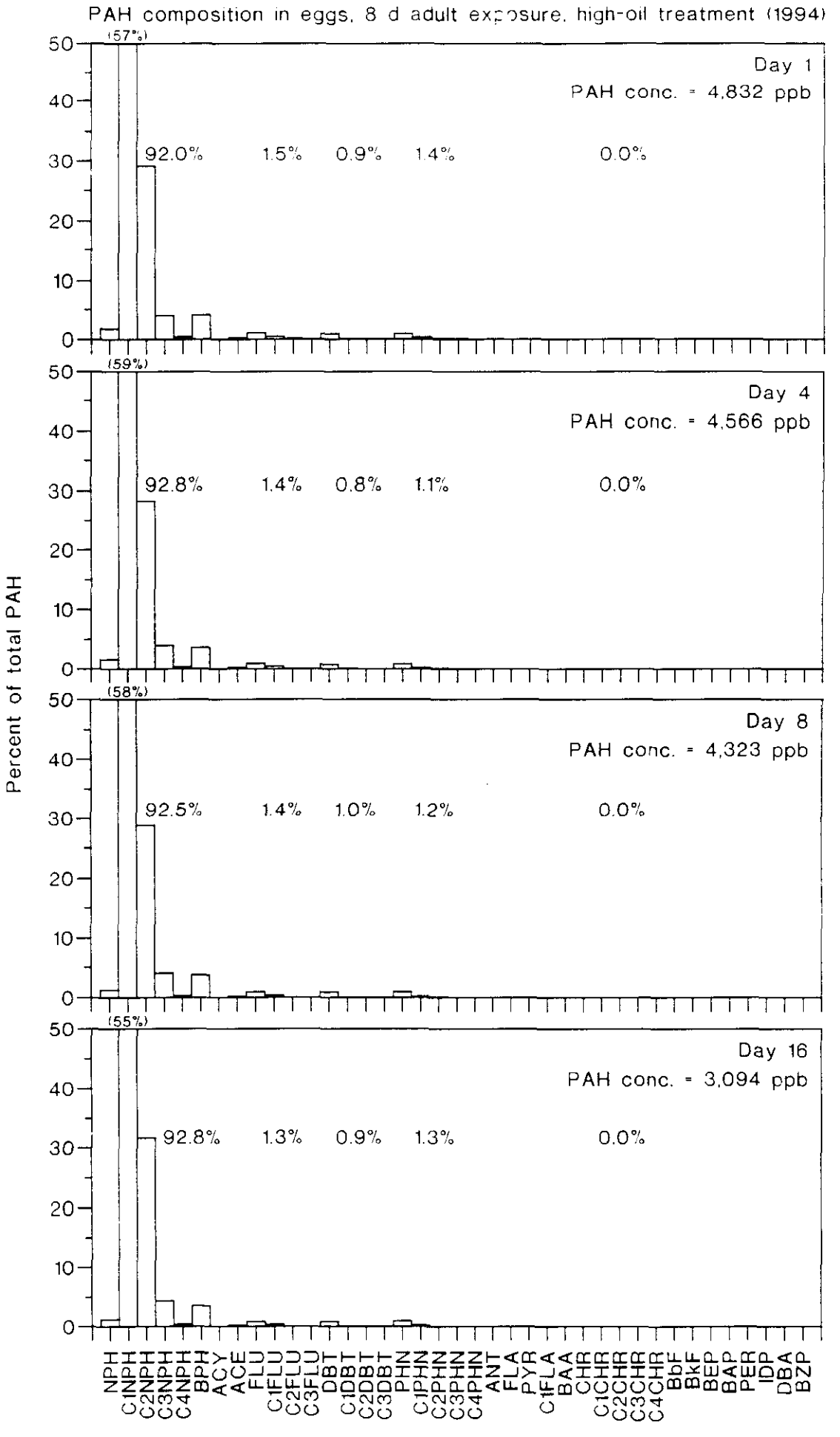
Composition of polynuclear aromatic hydrocarbons in eggs (1994)

Composition of polynuclear aromatic hydrocarbons (PAH) in eggs resulting from exposure of pre-spawn adult herring to oil-contaminated water. Composition in eggs appeared to be fairly constant over the 16 d observation period, but relative concentrations of naphthalenes did tend to decline with depuration time ($0.092 \leq P \leq 0.157$), and relative concentrations of all heavier compounds tended to increase with time ($0.001 < P \leq 0.093$). These changes in composition in eggs during incubation were slight. Abbreviations of chemical compounds are found in Appendix 0.2.

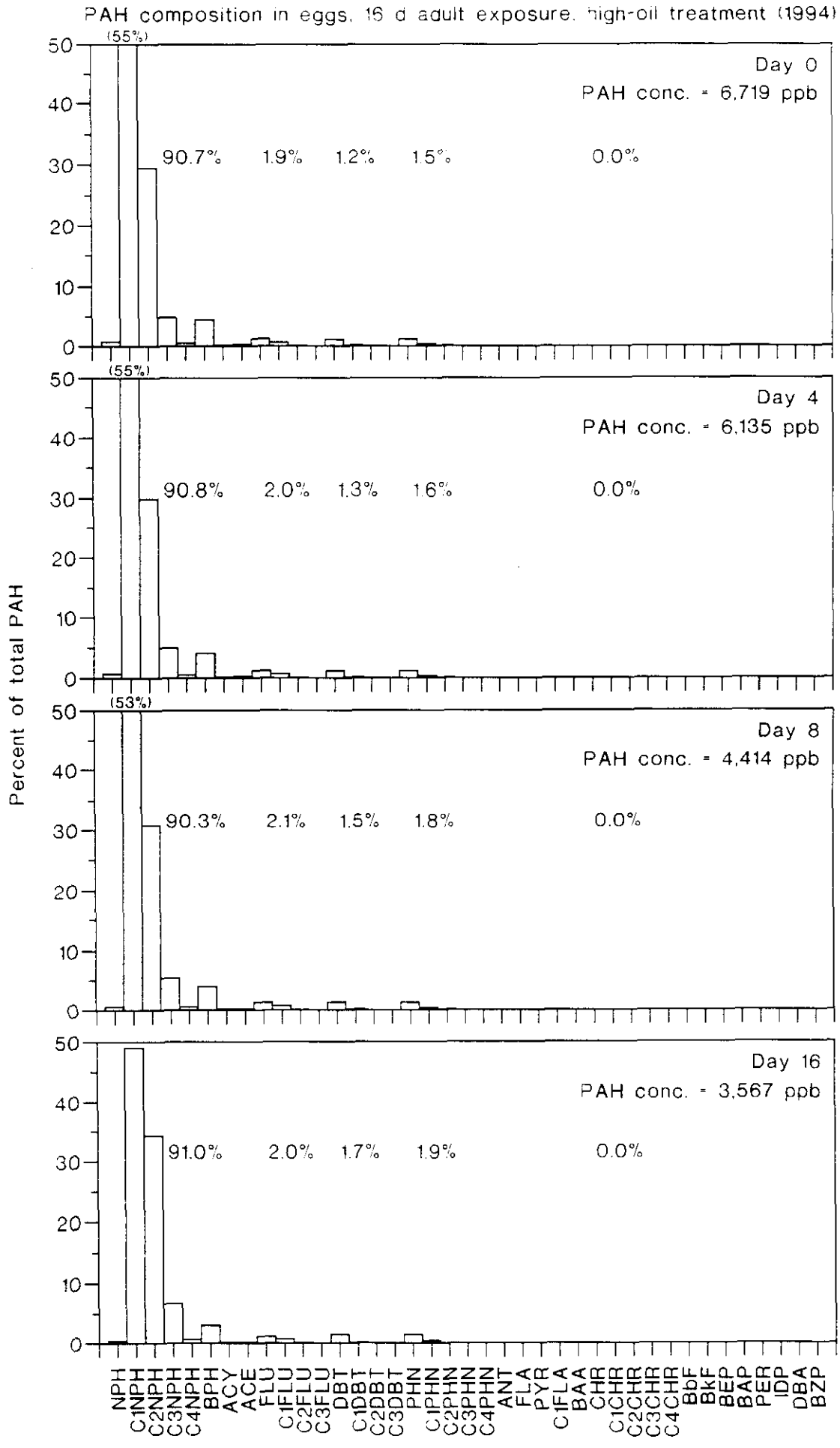
Appendix 3.3.1. Composition of PAH in egg tissue, high-oil treatment, 8 d adult exposure. Indicated times are depuration times.

Appendix 3.3.2. Composition of PAH in egg tissue, high-oil treatment, 16 d adult exposure. Indicated times are depuration times.

Appendix 3.3.1 *(at right).* Composition of PAH in egg tissue, high-oil treatment, 8 d adult exposure. Indicated times are depuration times. Percentages printed inside the graphs indicate total percentages of the following homologous chemical groups (left to right): naphthalenes, fluorenes, dibenzothiophenes, phenanthrenes, and chrysenes.


PAH composition in eggs, 8 d adult exposure, high-oil treatment (1994)
Percent of total PAH
Day 1
PAH conc. = 4,832 ppb
(57%)
92.0%
1.5%
0.9%
1.4%
0.0%
Day 4
PAH conc. = 4,566 ppb
(59%)
92.8%
1.4%
0.8%
1.1%
0.0%
Day 8
PAH conc. = 4,323 ppb
(58%)
92.5%
1.4%
1.0%
1.2%
0.0%
Day 16
PAH conc. = 3,094 ppb
(55%)
92.8%
1.3%
0.9%
1.3%
0.0%
0
10
20
30
40
50
NPH
C1NPH
C2NPH
C3NPH
C4NPH
BPH
ACY
ACE
FLU
C1FLU
C2FLU
C3FLU
DBT
C1DBT
C2DBT
C3DBT
PHN
C1PHN
C2PHN
C3PHN
C4PHN
ANT
FLA
PYR
C1FLA
BAA
CHR
C1CHR
C2CHR
C3CHR
C4CHR
BbF
BkF
BEP
BAP
PER
IDP
DBA
BZP

Appendix 3.3.2 *(at right).* Composition of PAH in egg tissue, high-oil treatment, 16 d adult exposure. Indicated times are depuration times. Percentages printed inside the graphs indicate total percentages of the following homologous chemical groups (left to right): naphthalenes, fluorenes, dibenzothiophenes, phenanthrenes, and chrysenes.


PAH composition in eggs, 16 d adult exposure, high-oil treatment (1994)
Percent of total PAH
Day 0
PAH conc. = 6,719 ppb
(55%)
90.7%
1.9%
1.2%
1.5%
0.0%
Day 4
PAH conc. = 6,135 ppb
(55%)
90.8%
2.0%
1.3%
1.6%
0.0%
Day 8
PAH conc. = 4,414 ppb
(53%)
90.3%
2.1%
1.5%
1.8%
0.0%
Day 16
PAH conc. = 3,567 ppb
91.0%
2.0%
1.7%
1.9%
0.0%
0
10
20
30
40
50
NPH
C1NPH
C2NPH
C3NPH
C4NPH
BPH
ACY
ACE
FLU
C1FLU
C2FLU
C3FLU
DBT
C1DBT
C2DBT
C3DBT
PHN
C1PHN
C2PHN
C3PHN
C4PHN
ANT
FLA
PYR
C1FLA
BAA
CHR
C1CHR
C2CHR
C3CHR
C4CHR
BbF
BkF
BEP
BAP
PER
IDP
DBA
BZP

Appendix 3.4

Comparison of polynuclear aromatic hydrocarbon composition in water between 1994 and 1995

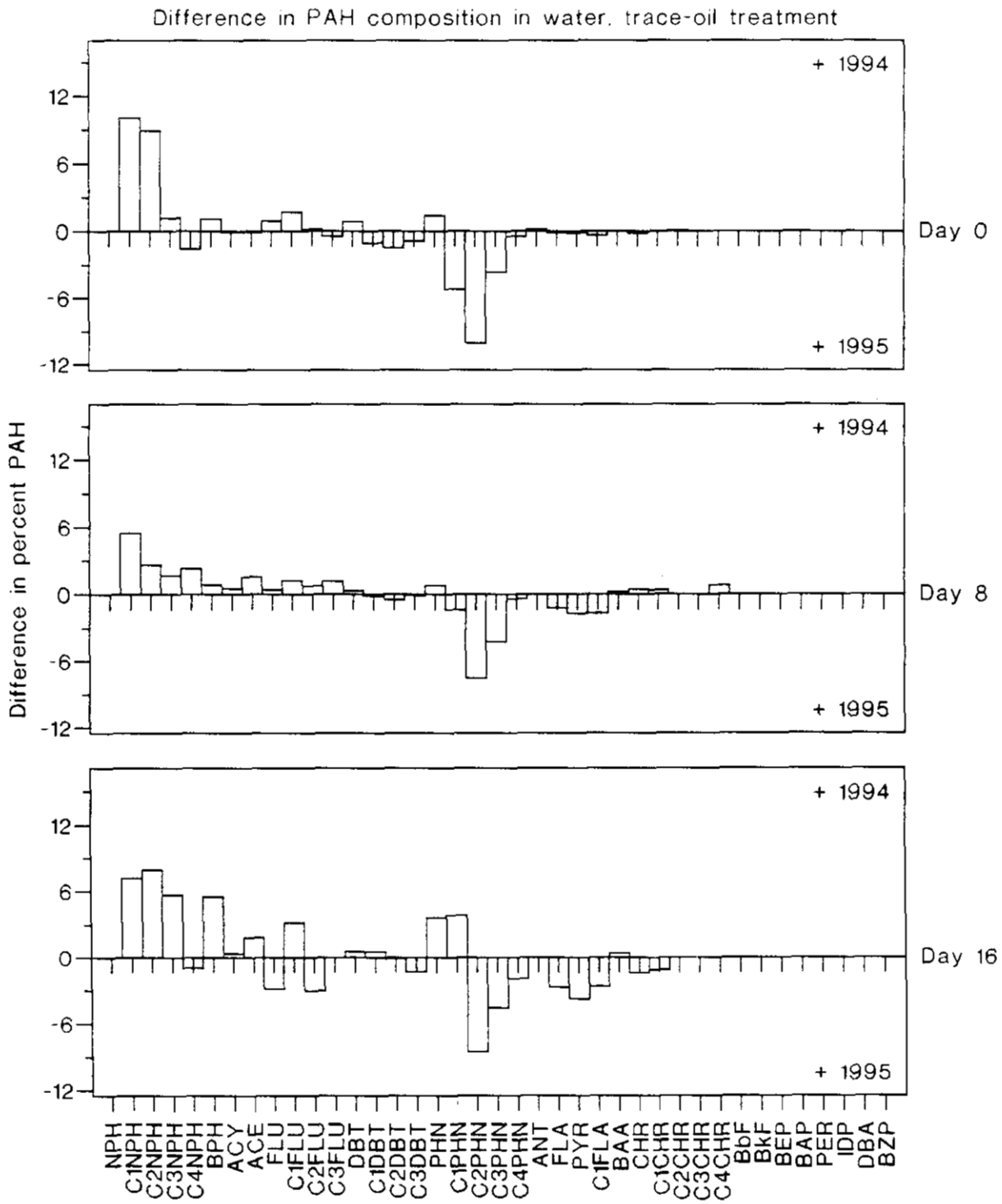
Polynuclear aromatic hydrocarbon (PAH) composition in treatment water was compared between years 1994 and 1995 visually by subtracting 1995 values from 1994 values. Beginning, mid-, and end-point exposure times were compared (days 0, 8, and 16, respectively). There were insufficient data to allow a detailed multivariate comparison between years. An alternative way to compare composition between years is to match appropriate graphs in Appendices 2.1 and 4.2. Abbreviations of chemical compounds are found in Appendix 0.2.

Appendix 3.4.1. Comparison of PAH composition in trace-oil treatment water, years 1994 and 1995.

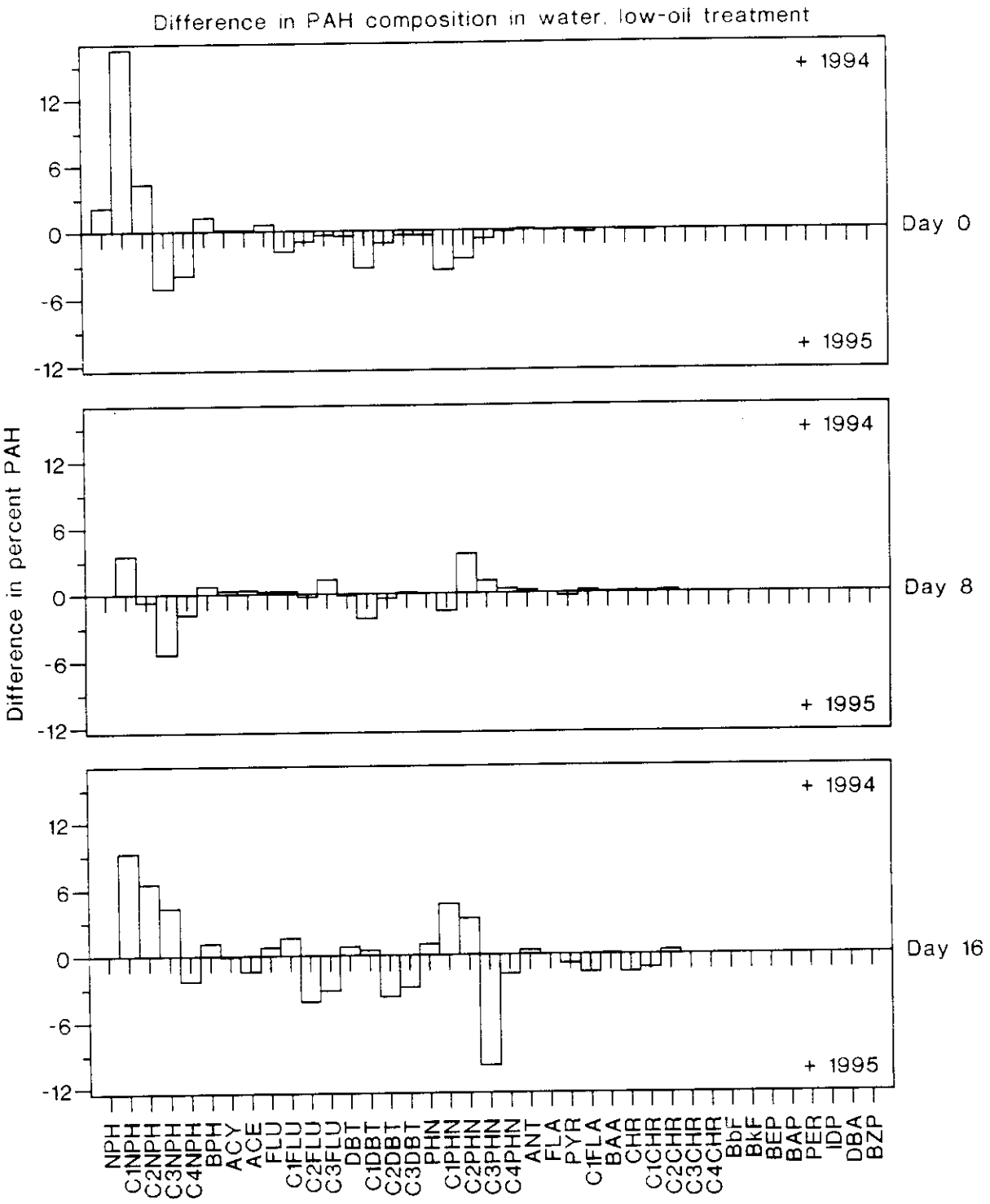
Appendix 3.4.2. Comparison of PAH composition in low-oil treatment water, years 1994 and 1995.

Appendix 3.4.3. Comparison of PAH composition in mid-oil treatment water, years 1994 and 1995.

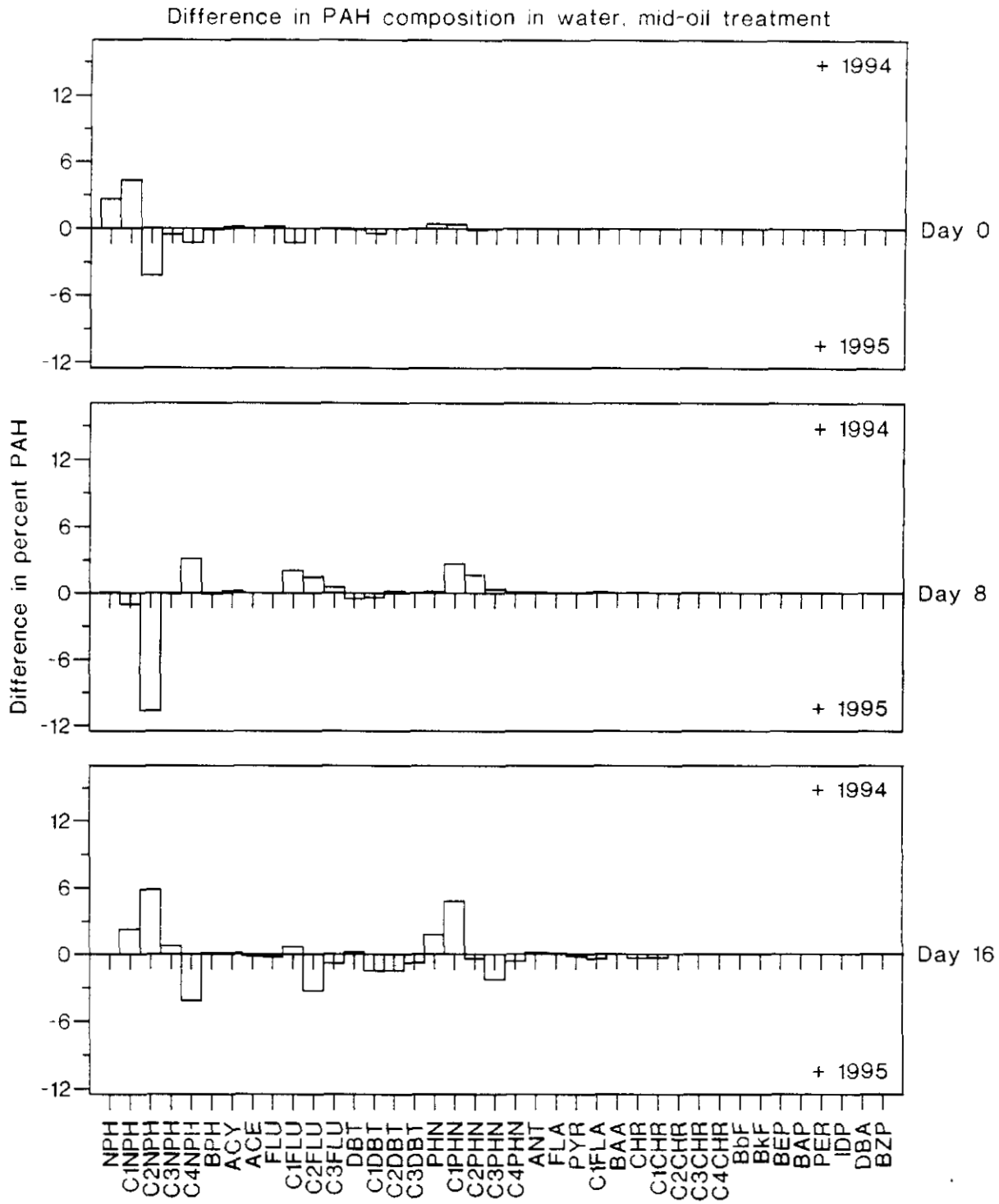
Appendix 3.4.4. Comparison of PAH composition in high-oil treatment water, years 1994 and 1995.



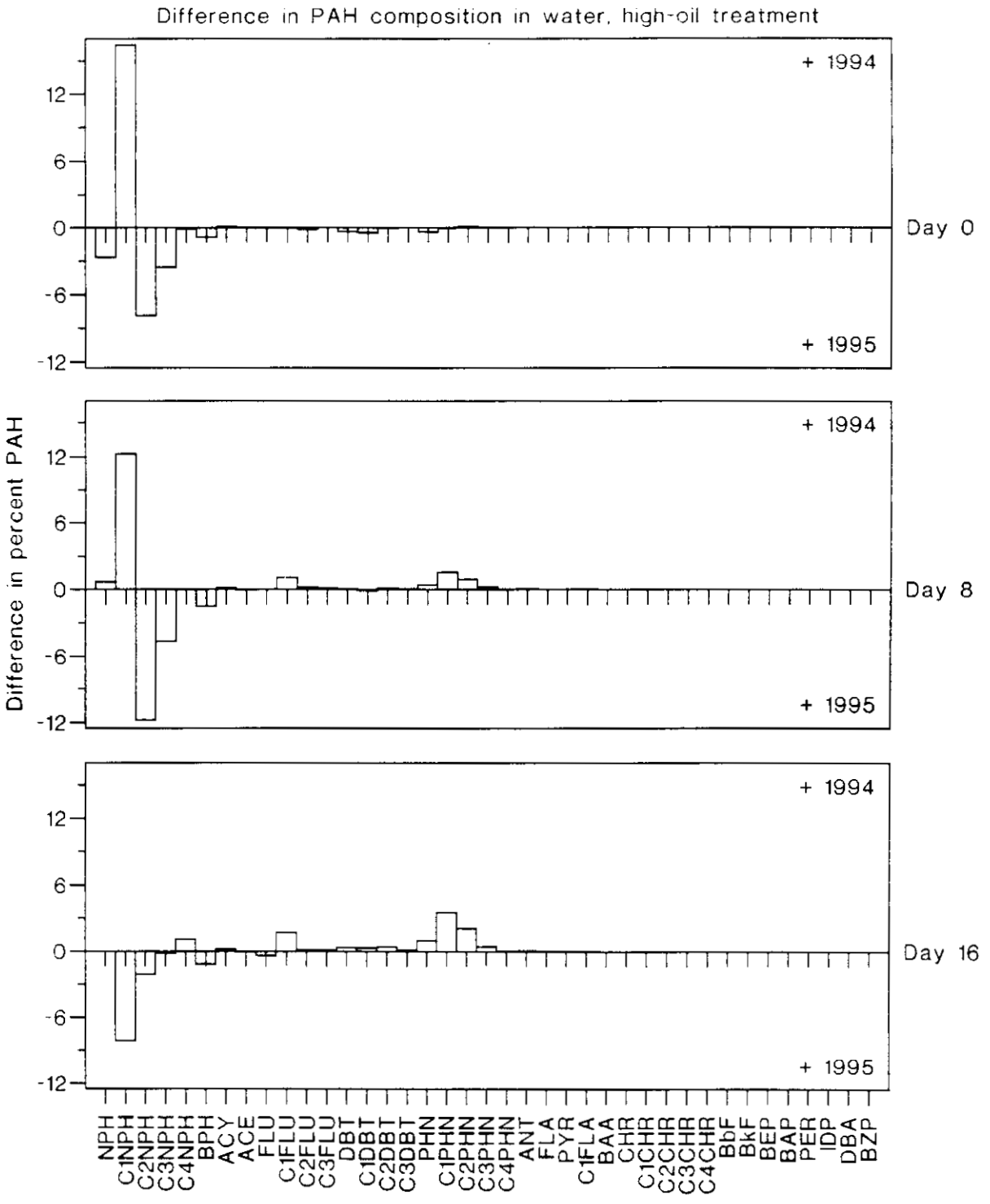
Appendix 3.4.1. Comparison of PAH composition in trace-oil treatment water, years 1994 and 1995. Positive values indicate more compound was present in 1994, negative values indicate more in 1995. Beginning, mid-, and end-point exposure times are compared (day 0, 8, and 16, respectively).



Appendix 3.4.2. Comparison of PAH composition in low-oil treatment water, years 1994 and 1995. Positive values indicate more compound was present in 1994, negative values indicate more in 1995. Beginning, mid-, and end-point exposure times are compared (day 0, 8, and 16, respectively).



Appendix 3.4.3. Comparison of PAH composition in mid-oil treatment water, years 1994 and 1995. Positive values indicate more compound was present in 1994, negative values indicate more in 1995. Beginning, mid-, and end-point exposure times are compared (day 0, 8, and 16, respectively).



Appendix 3.4.4. Comparison of PAH composition in high-oil treatment water, years 1994 and 1995. Positive values indicate more compound was present in 1994, negative values indicate more in 1995. Beginning, mid-, and end-point exposure times are compared (day 0, 8, and 16, respectively).

Appendix 3.5

Example polynuclear aromatic hydrocarbon concentration in water, 1994

Polynuclear aromatic hydrocarbon (PAH) concentration in treatment water of three consecutive studies, each utilizing the same oil generator. Study 2 (S2) refers to the MFO research reported in chapter 2 of this report. Study 3 (S3) refers to the adult and gamete exposures reported in chapters 1 and 3. Study 5 (S5) refers to an otherwise unreported ancillary test. Although the same contaminated substrate was used in these consecutive studies, there were some unexplained concentration discontinuities, particularly between S2 and S3 in c1-naphthalenes, c1-fluorenes, and c1-dibenzothiophenes. Despite discontinuities, grouping across these studies allowed sufficient data for credible regression analysis.

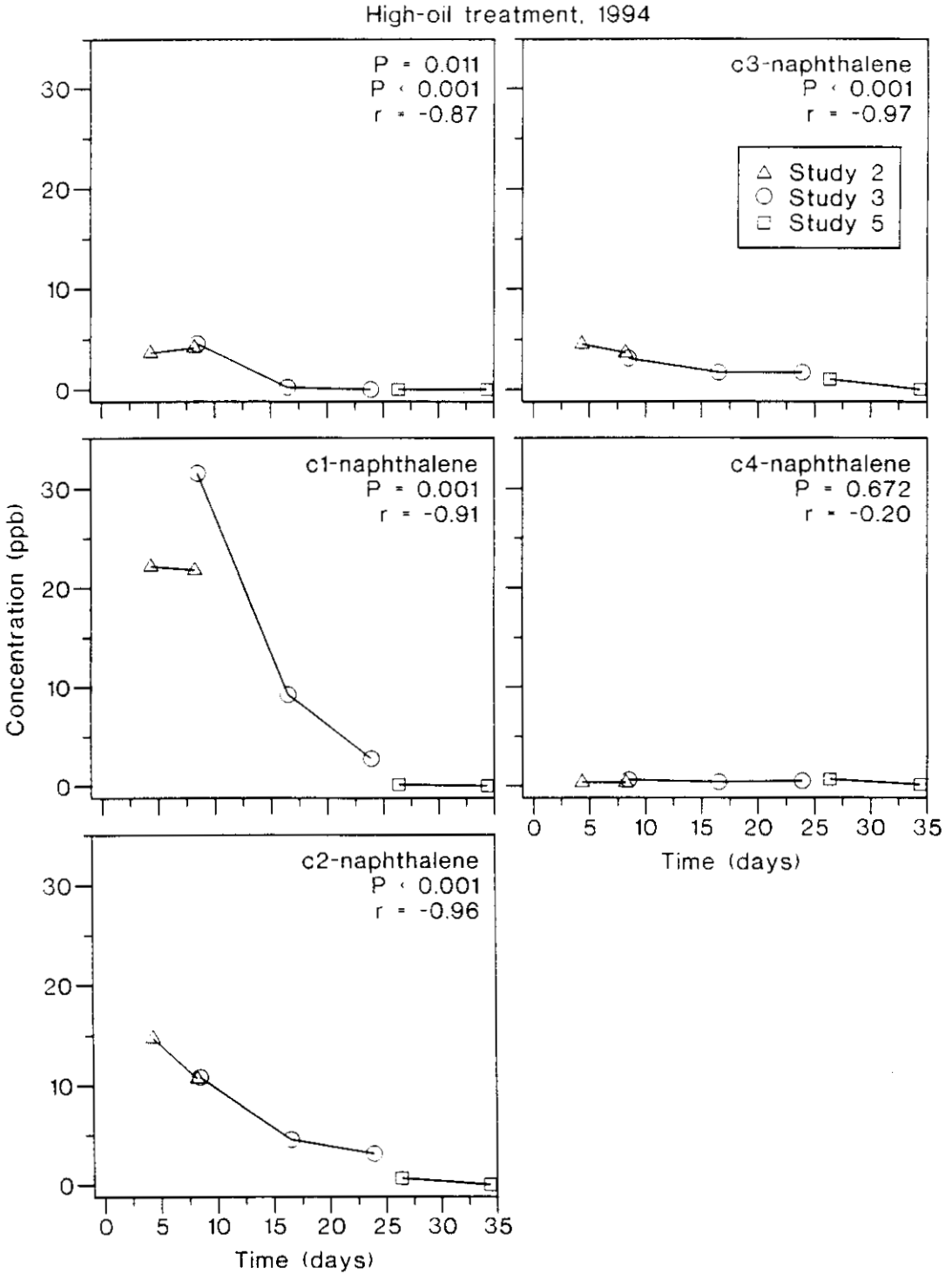
Appendix 3.5.1. Naphthalene concentrations in the high-oil treatment of three consecutive 1994 studies.

Appendix 3.5.1. Fluorene concentrations in the high-oil treatment of three consecutive 1994 studies.

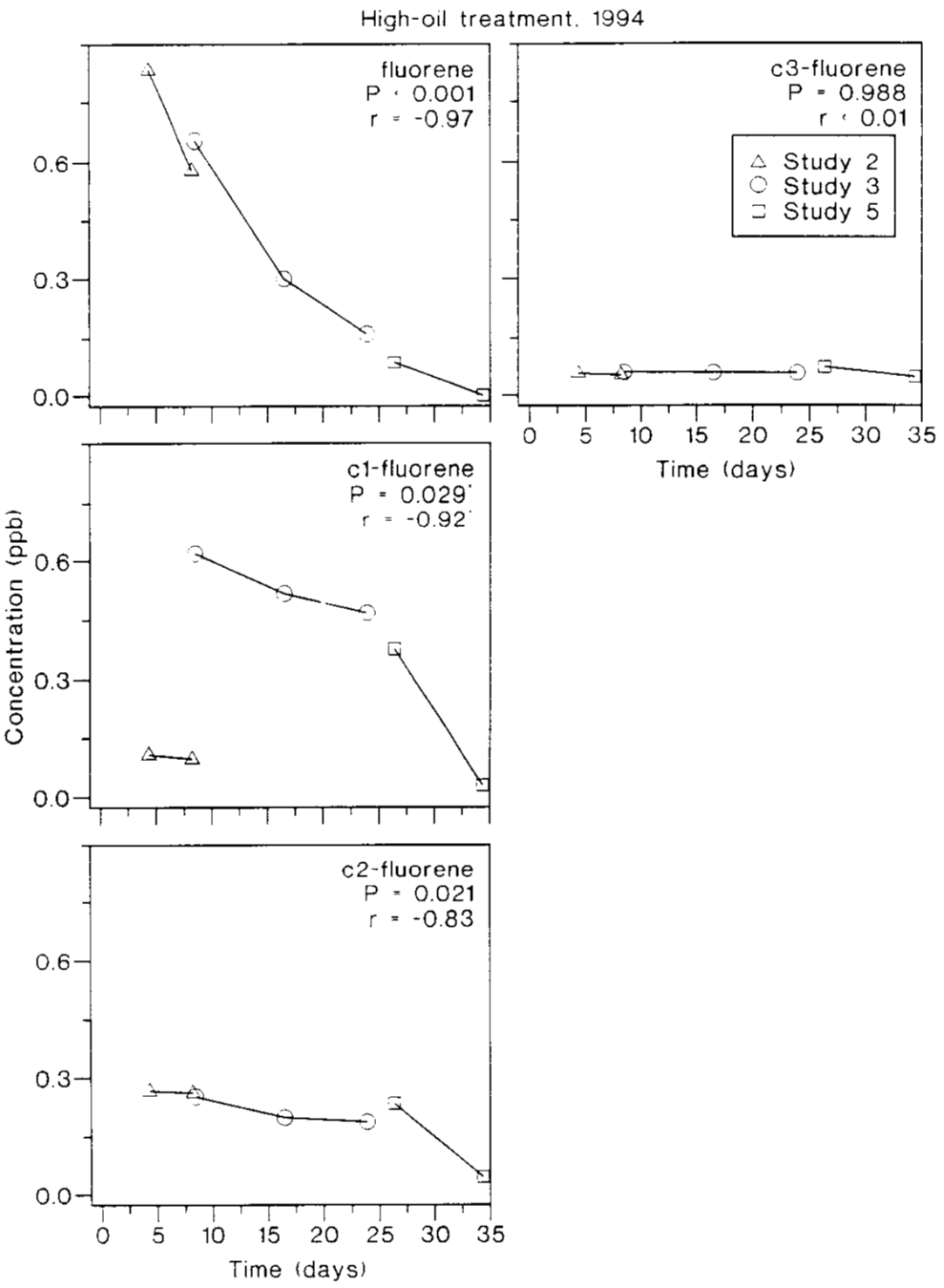
Appendix 3.5.1. Dibenzothiophene concentrations in the high-oil treatment of three consecutive 1994 studies.

Appendix 3.5.1. Phenanthrene concentrations in the high-oil treatment of three consecutive 1994 studies.

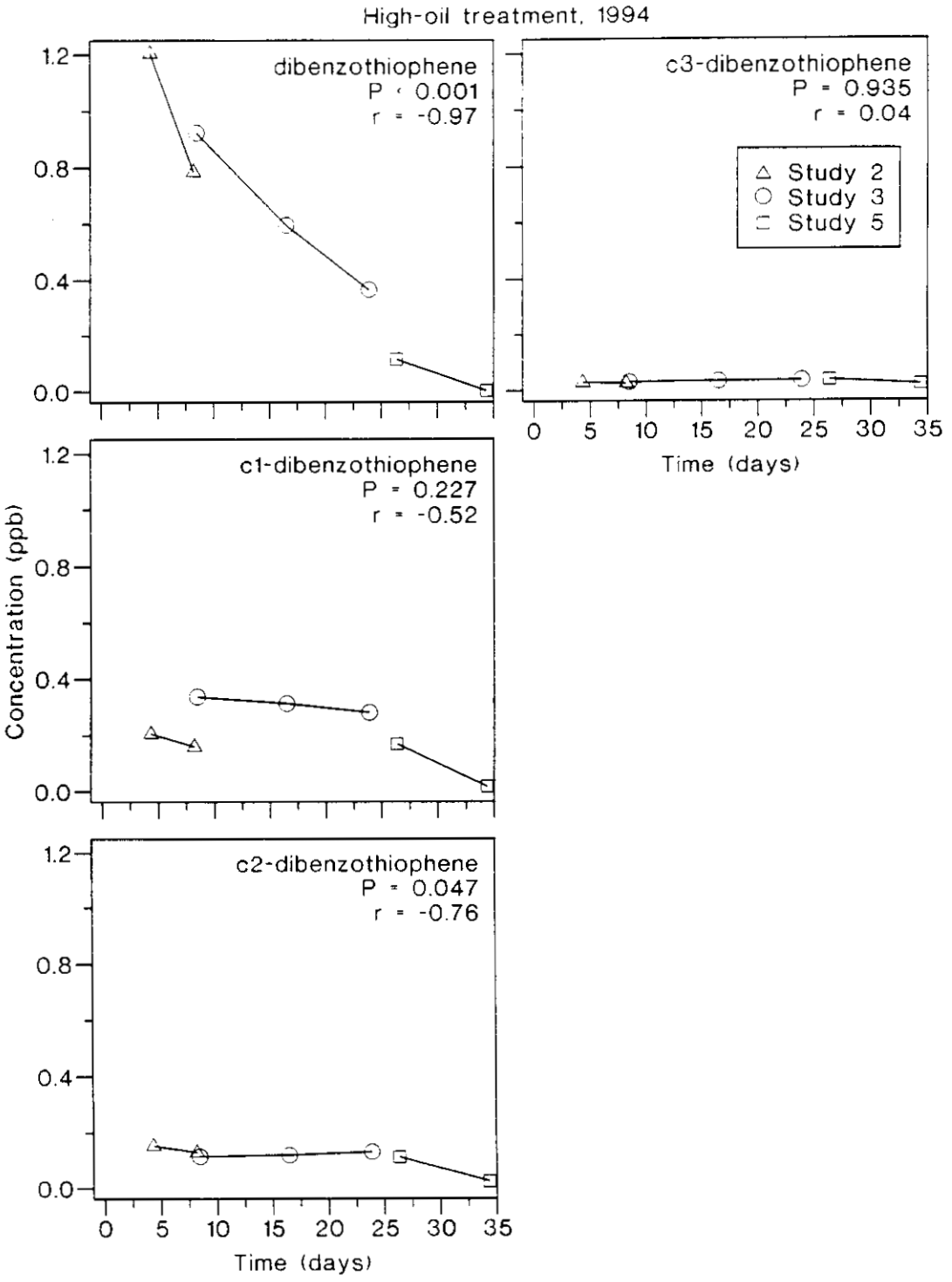
Appendix 3.5.1. Chrysene concentrations in the high-oil treatment of three consecutive 1994 studies.



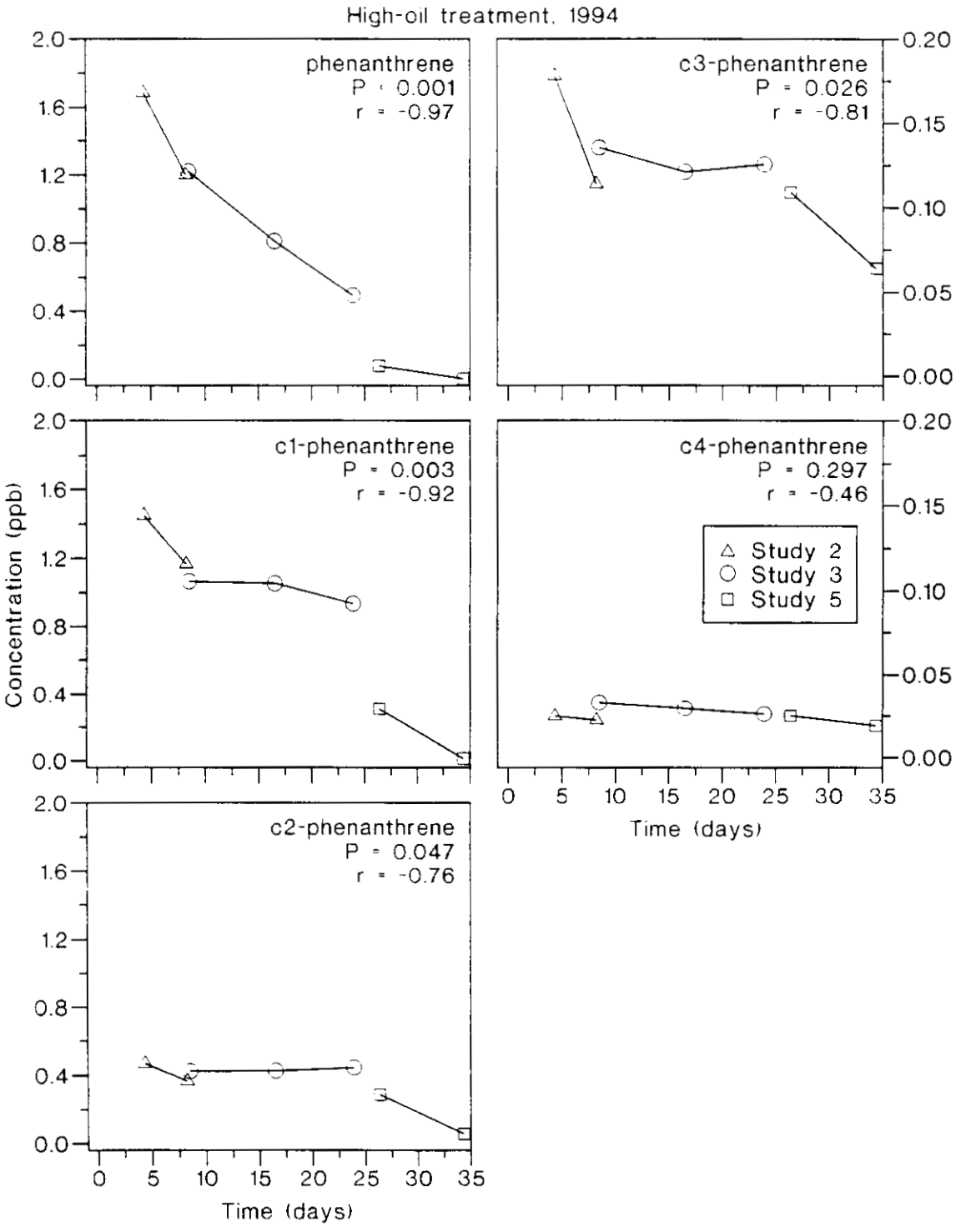
Appendix 3.5.1. Naphthalene concentrations in the high-oil treatment of three consecutive 1994 studies. Studies were S2 (Chapter 2), S3 (Chapters 1 and 3), and S5 (an unreported ancillary test): r = correlation coefficient, P = probability of significant slope.



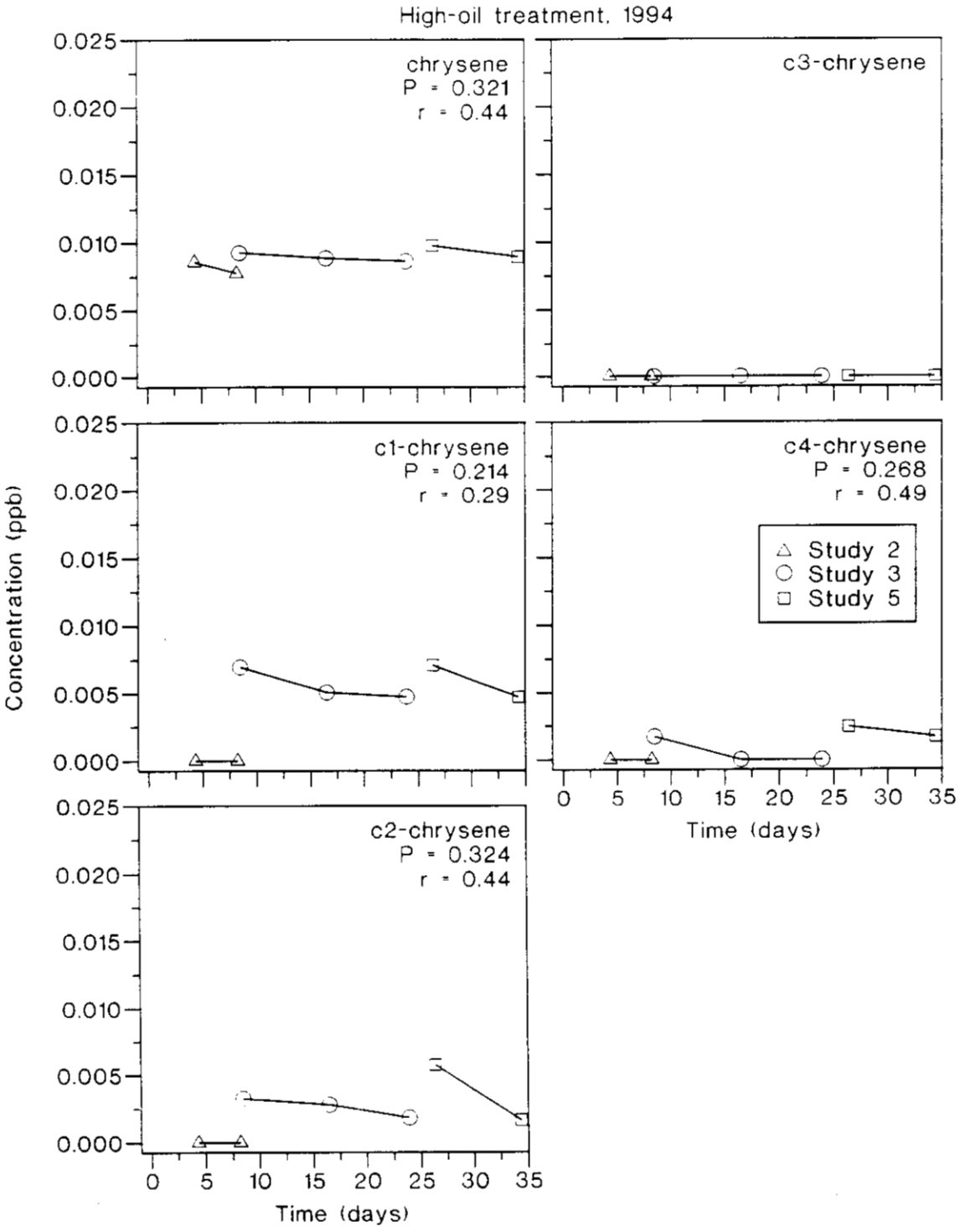
Appendix 3.5.2. Fluorene concentrations in the high-oil treatment of three consecutive 1994 studies. Studies were S2 (Chapter 2), S3 (Chapters 1 and 3), and S5 (an unreported ancillary test): r = correlation coefficient, P = probability of significant slope. *The P and r values reported for c1-fluorene were calculated without S2 data; when S2 data were included, P = 0.837, and r = -0.10.



Appendix 3.5.3. Dibenzothiophene concentrations in the high-oil treatment of three consecutive 1994 studies. Studies were S2 (Chapter 2), S3 (Chapters 1 and 3), and S5 (an unreported ancillary test): r = correlation coefficient, P = probability of significant slope.



Appendix 3.5.4. Phenanthrene concentrations in the high-oil treatment of three consecutive 1994 studies. Studies were S2 (Chapter 2), S3 (Chapters 1 and 3), and S5 (an unreported ancillary test); r = correlation coefficient, P = probability of significant slope.



Appendix 3.5.5. Chrysene concentrations in the high-oil treatment of three consecutive 1994 studies. Studies were S2 (Chapter 2), S3 (Chapters 1 and 3), and S5 (an unreported ancillary test): r = correlation coefficient, P = probability of significant slope.

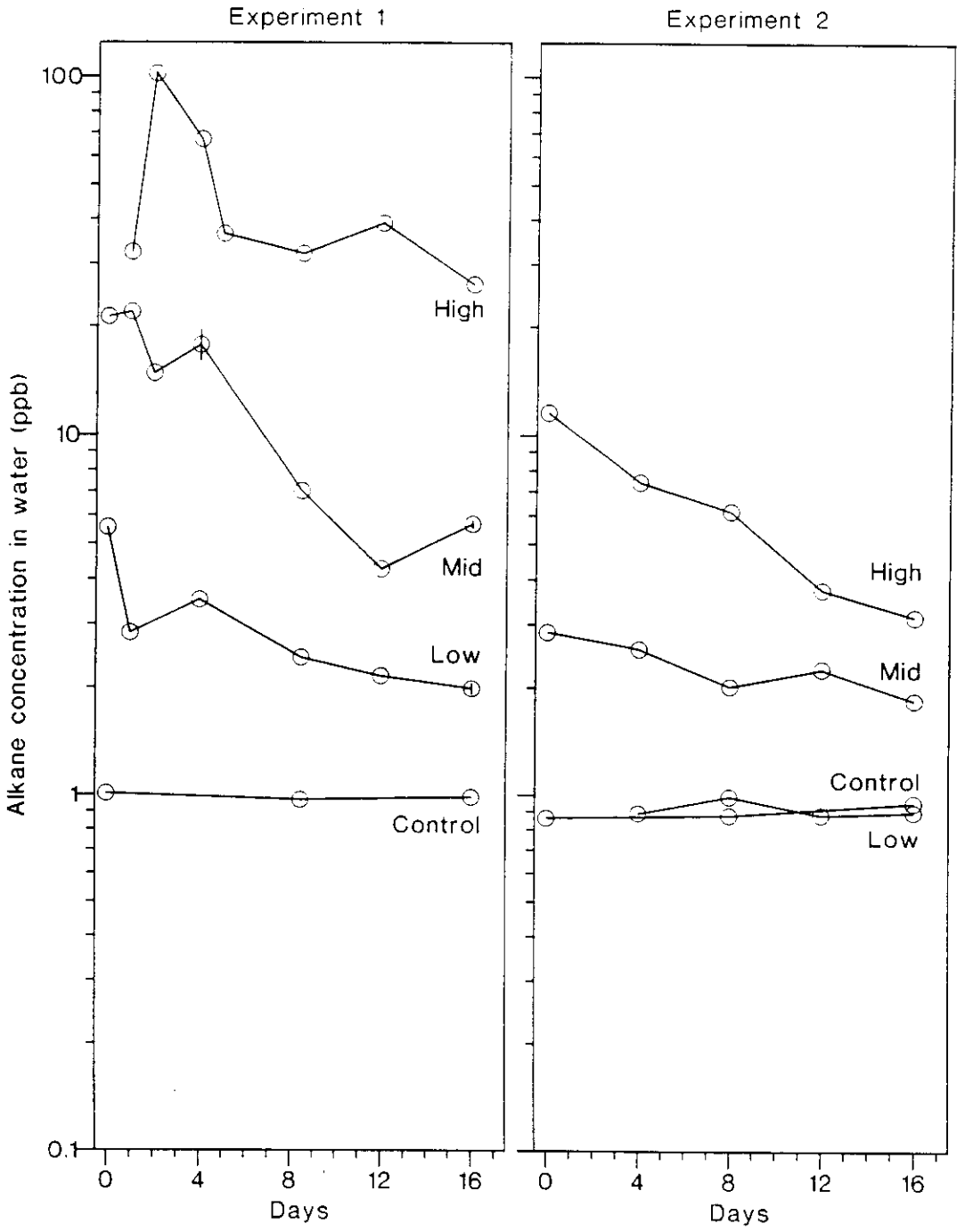
Appendix 4.1

Alkane concentrations in water (1995)

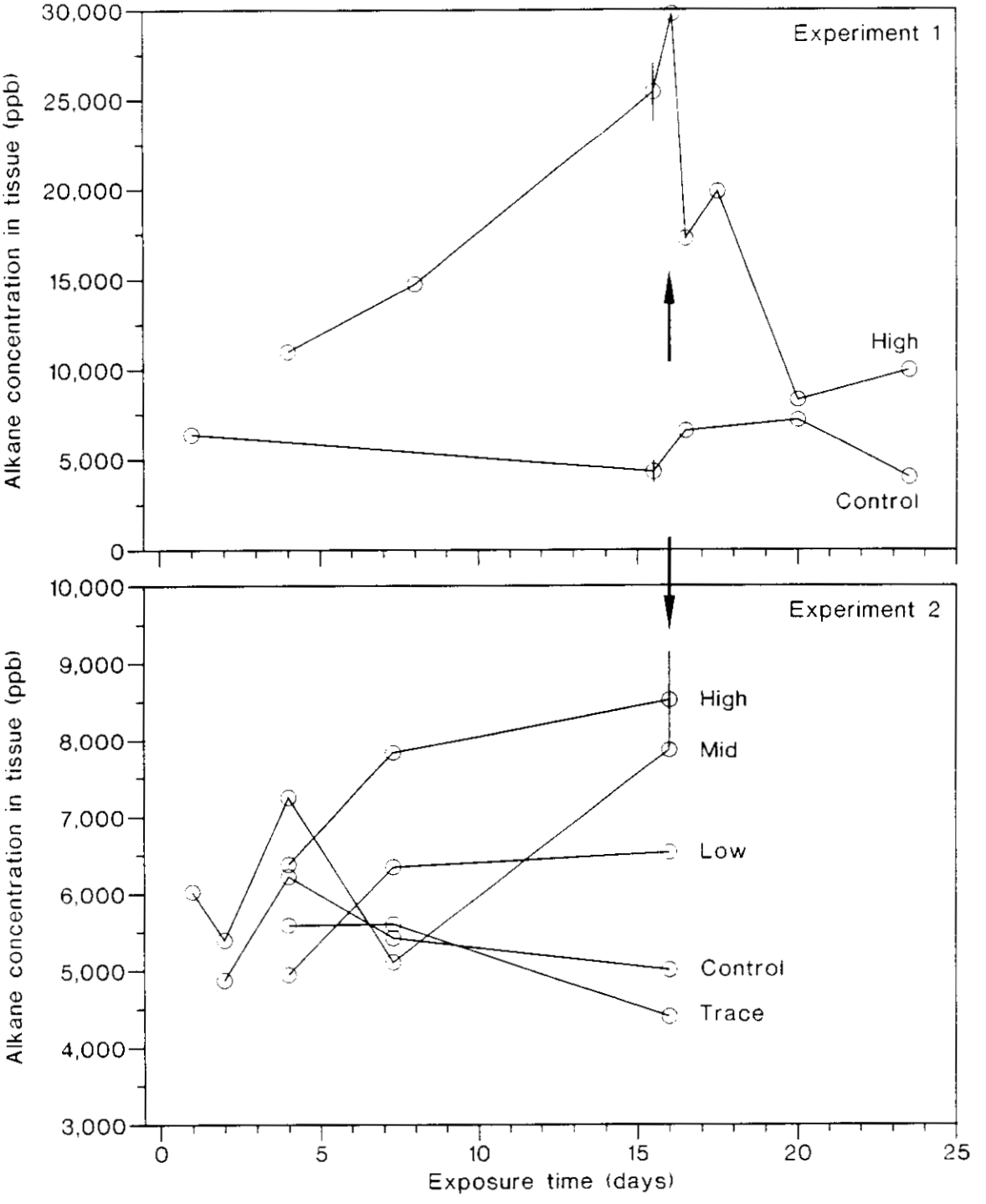
More alkanes were detected in treatment water and tissues than polynuclear aromatic hydrocarbons (PAH), but were not emphasized in this report because PAH are more toxic. Variability in the alkane data was proportionately much greater than in PAH data.

Appendix 4.1.1. Total alkane concentrations in water, by experiment and treatment.

Appendix 4.1.2. Concentrations of total alkanes in experiments 1 and 2.



Appendix 4.1.1. Total alkane concentrations in water, by experiment and treatment. Most points represent single observations; where present vertical bars are ± standard error.



Appendix 4.1.2. Concentrations of total alkanes increased in egg tissue over the entire 16 d exposure in experiment 1. In the upper three treatments of experiment 2, alkane concentrations tended to rise throughout the exposure period, but apparent variance was high, particularly in the mid-oil treatment. Concentrations in the trace-oil treatment of experiment 2 closely followed control values. Arrows indicate when eggs were transferred to clean water. Variance was estimated only in high-oil treatments on day 16; vertical bars are ± standard error.

Appendix 4.2

Composition of polynuclear aromatic hydrocarbons in 1995 treatment water.

Polynuclear aromatic hydrocarbon (PAH) composition data in water, by treatment and experiment. Characteristic time-dependent weathering patterns were evident in most treatments: percentages of lighter compounds, particularly naphthalenes, decreased while percentages of heavier compounds, particularly phenanthrenes, increased. Differential weathering across treatments was also evident; in particular, compare day 0 observations among treatments in experiment 1. Abbreviations of chemical compounds are found in Appendix 0.2.

Appendix 4.2.1. Composition of PAH in trace-oil treatment water, experiment 1 (1995).

Appendix 4.2.2. Composition of PAH in low-oil treatment water, experiment 1 (1995).

Appendix 4.2.3. Composition of PAH in mid-oil treatment water, experiment 1 (1995).

Appendix 4.2.4. Composition of PAH in high-oil treatment water, experiment 1 (1995).

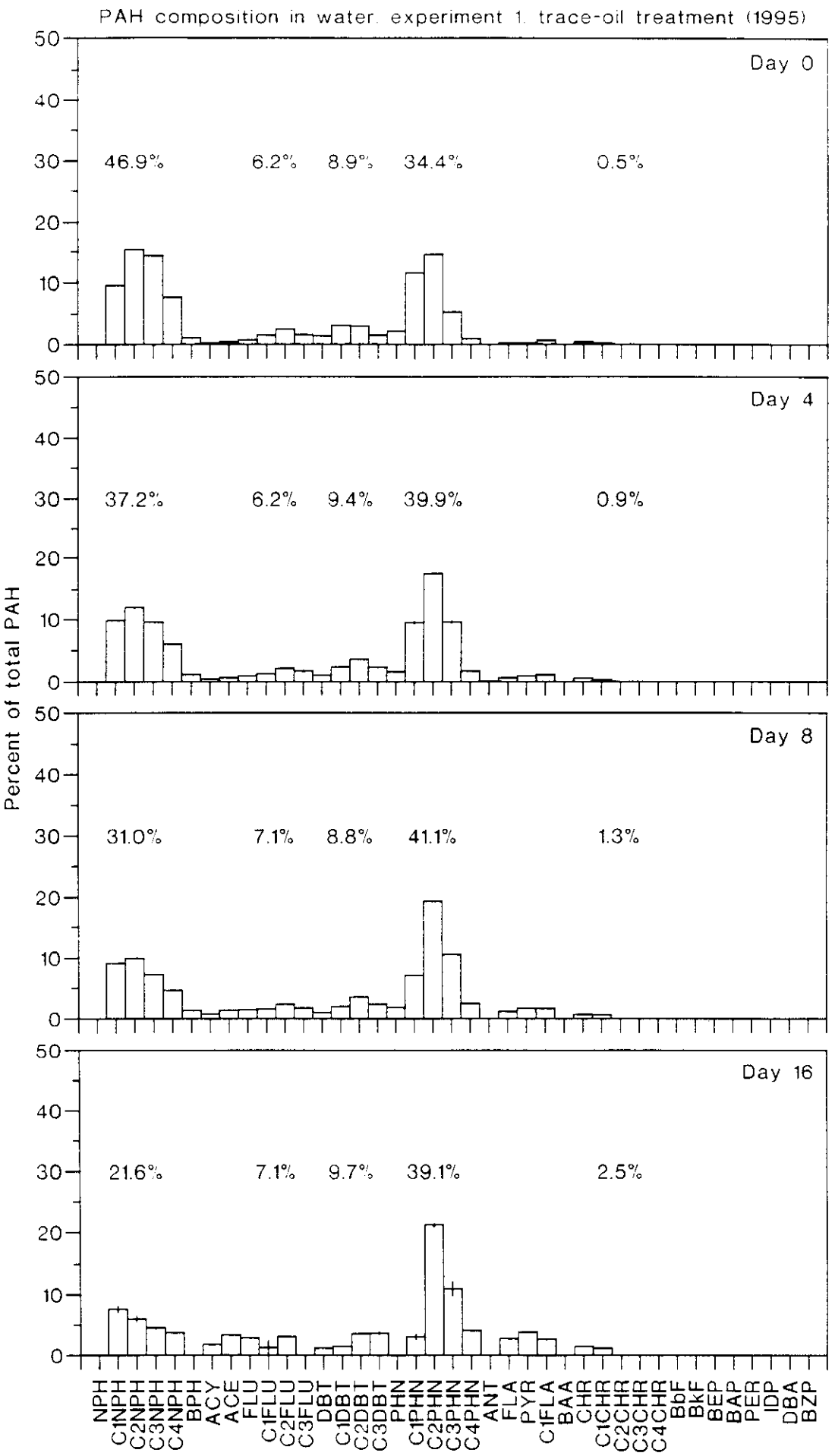
Appendix 4.2.5. Composition of PAH in trace-oil treatment water, experiment 2 (1995).

Appendix 4.2.6. Composition of PAH in low-oil treatment water, experiment 2 (1995).

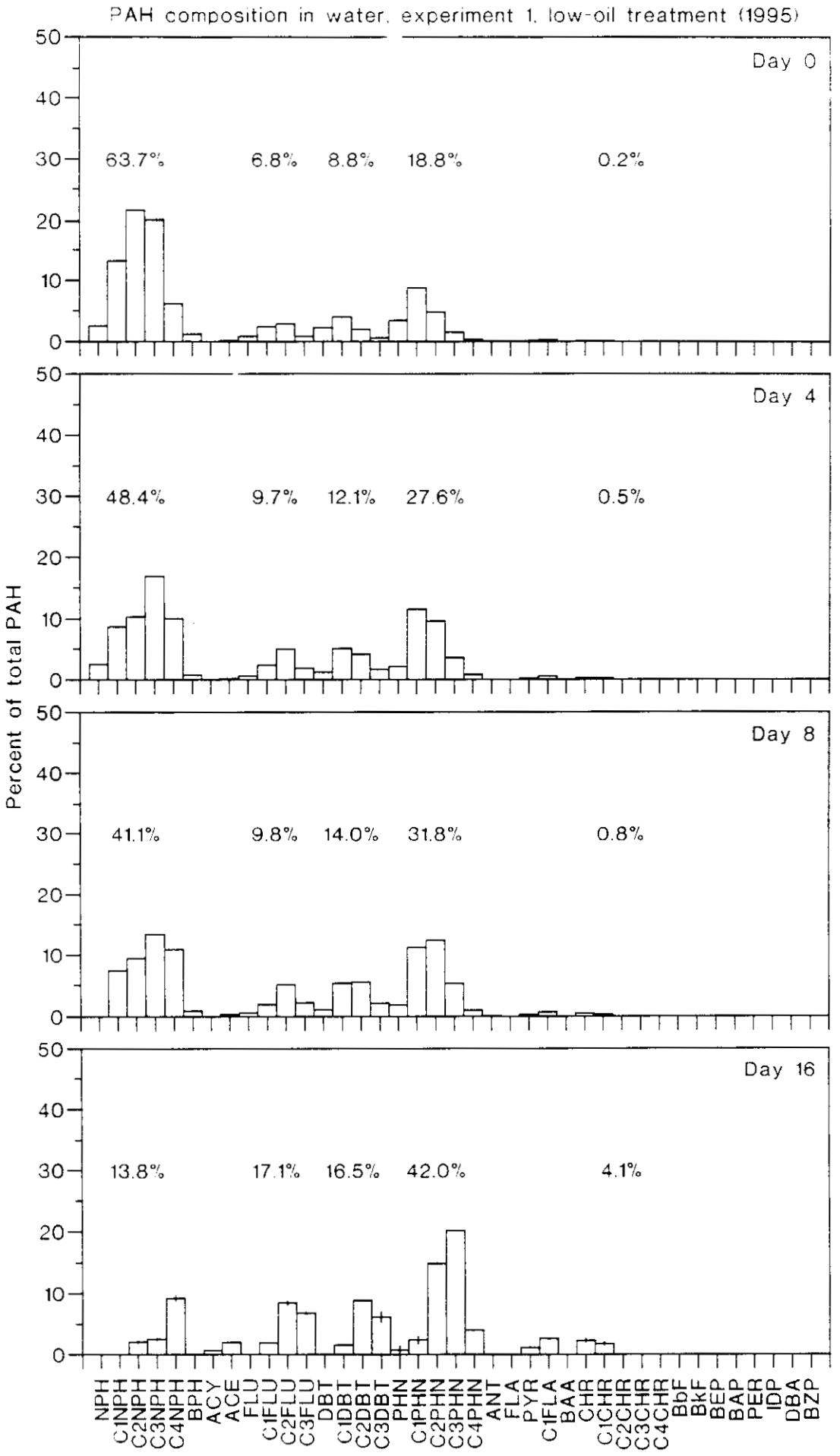
Appendix 4.2.7. Composition of PAH in mid-oil treatment water, experiment 2 (1995).

Appendix 4.2.8. Composition of PAH in high-oil treatment water, experiment 2 (1995).

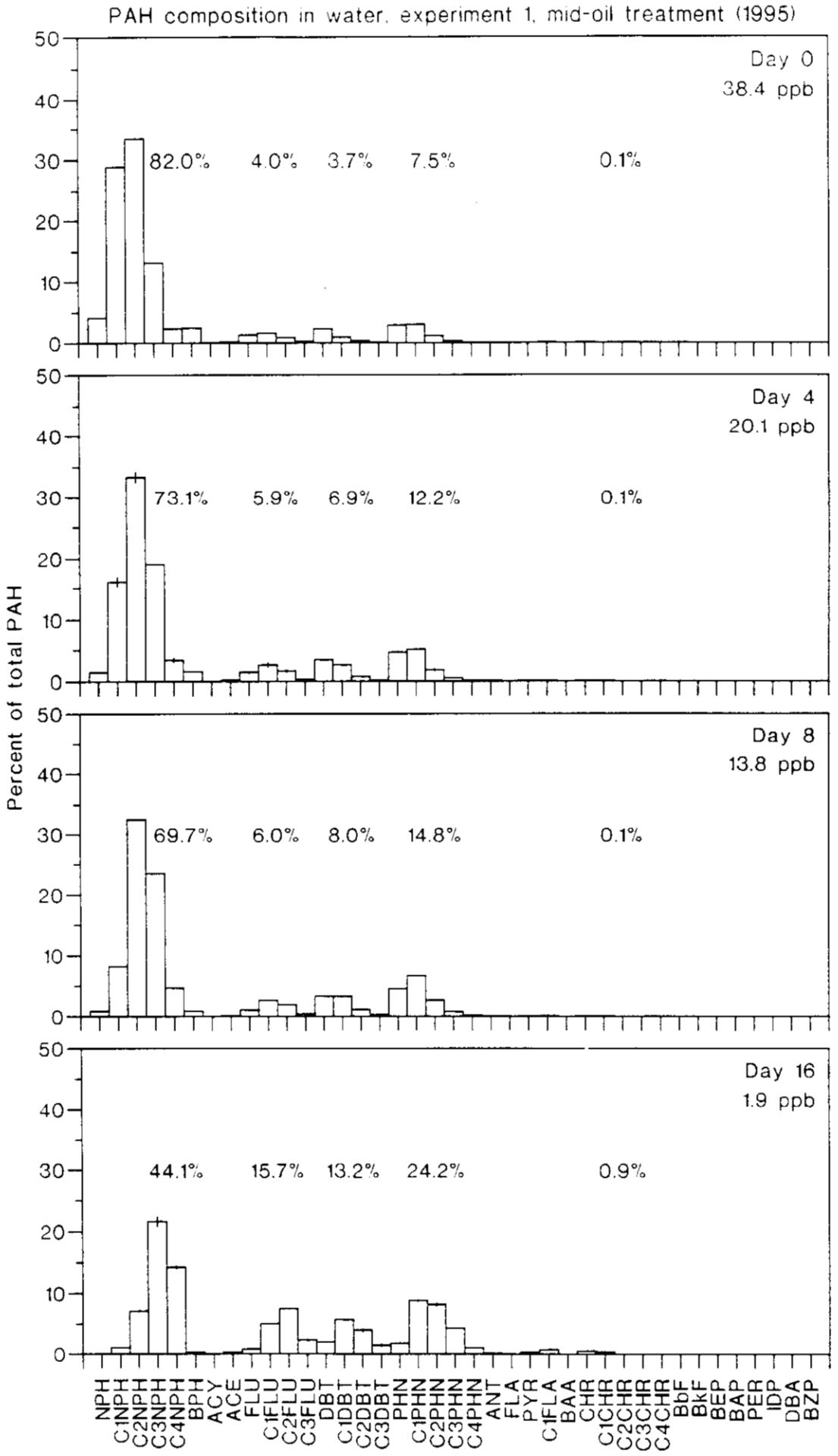
Appendix 4.2.1 *(at right).* Composition of PAH in trace-oil treatment water, experiment 1 (1995). Data for days 4 and 16 are means of paired observations ± standard error. Percentages printed inside the graphs indicate total percentages of the following homologous chemical groups (left to right): naphthalenes, fluorenes, dibenzothiophenes, phenanthrenes, and chrysenes.


PAH composition in water, experiment 1, trace-oil treatment (1995)
Day 0
46.9%
6.2%
8.9%
34.4%
0.5%
Day 4
37.2%
6.2%
9.4%
39.9%
0.9%
Day 8
31.0%
7.1%
8.8%
41.1%
1.3%
Day 16
21.6%
7.1%
9.7%
39.1%
2.5%
Percent of total PAH
0
10
20
30
40
50
NPH
C1NPH
C2NPH
C3NPH
C4NPH
BPH
ACY
ACE
FLU
C1FLU
C2FLU
C3FLU
DBT
C1DBT
C2DBT
C3DBT
PHN
C1PHN
C2PHN
C3PHN
C4PHN
ANT
FLA
PYR
C1FLA
BAA
CHR
C1CHR
C2CHR
C3CHR
C4CHR
BbF
BkF
BEP
BAP
PER
IDP
DBA
BZP

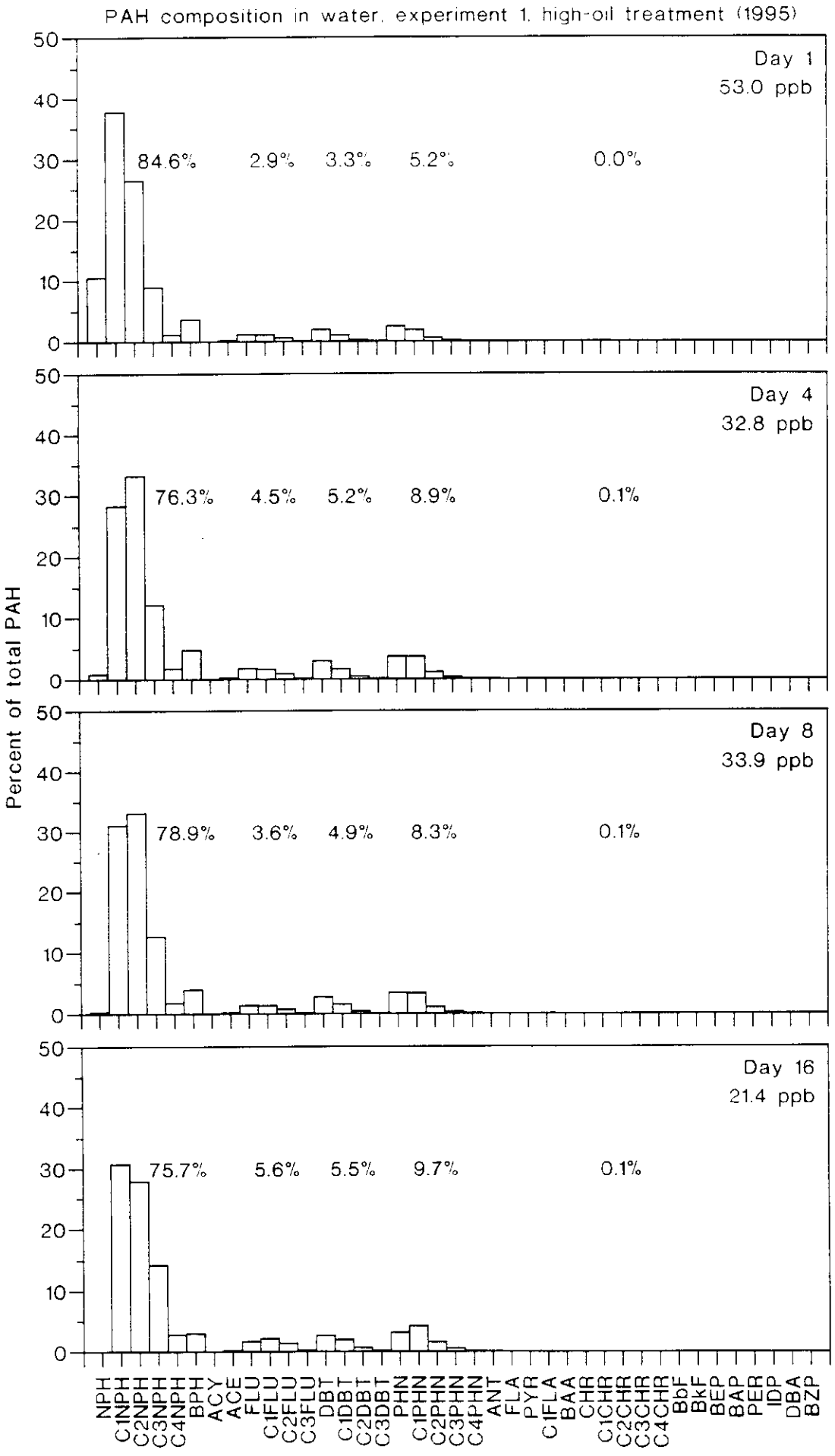
Appendix 4.2.2 *(at right)*. Composition of PAH in low-oil treatment water, experiment 1 (1995). Data for days 4 and 16 are means of paired observations ± standard error. Percentages printed inside the graphs indicate total percentages of the following homologous chemical groups (left to right): naphthalenes, fluorenes, dibenzothiophenes, phenanthrenes, and chrysenes.


PAH composition in water, experiment 1, low-oil treatment (1995)
Day 0
63.7%
6.8%
8.8%
18.8%
0.2%
Day 4
48.4%
9.7%
12.1%
27.6%
0.5%
Day 8
41.1%
9.8%
14.0%
31.8%
0.8%
Day 16
13.8%
17.1%
16.5%
42.0%
4.1%
Percent of total PAH
0
10
20
30
40
50
NPH
C1NPH
C2NPH
C3NPH
C4NPH
BPH
ACY
ACE
FLU
C1FLU
C2FLU
C3FLU
DBT
C1DBT
C2DBT
C3DBT
PHN
C1PHN
C2PHN
C3PHN
C4PHN
ANT
FLA
PYR
C1FLA
BAA
CHR
C1CHR
C2CHR
C3CHR
C4CHR
BbF
BkF
BeP
BAP
PER
IDP
DBA
BZP

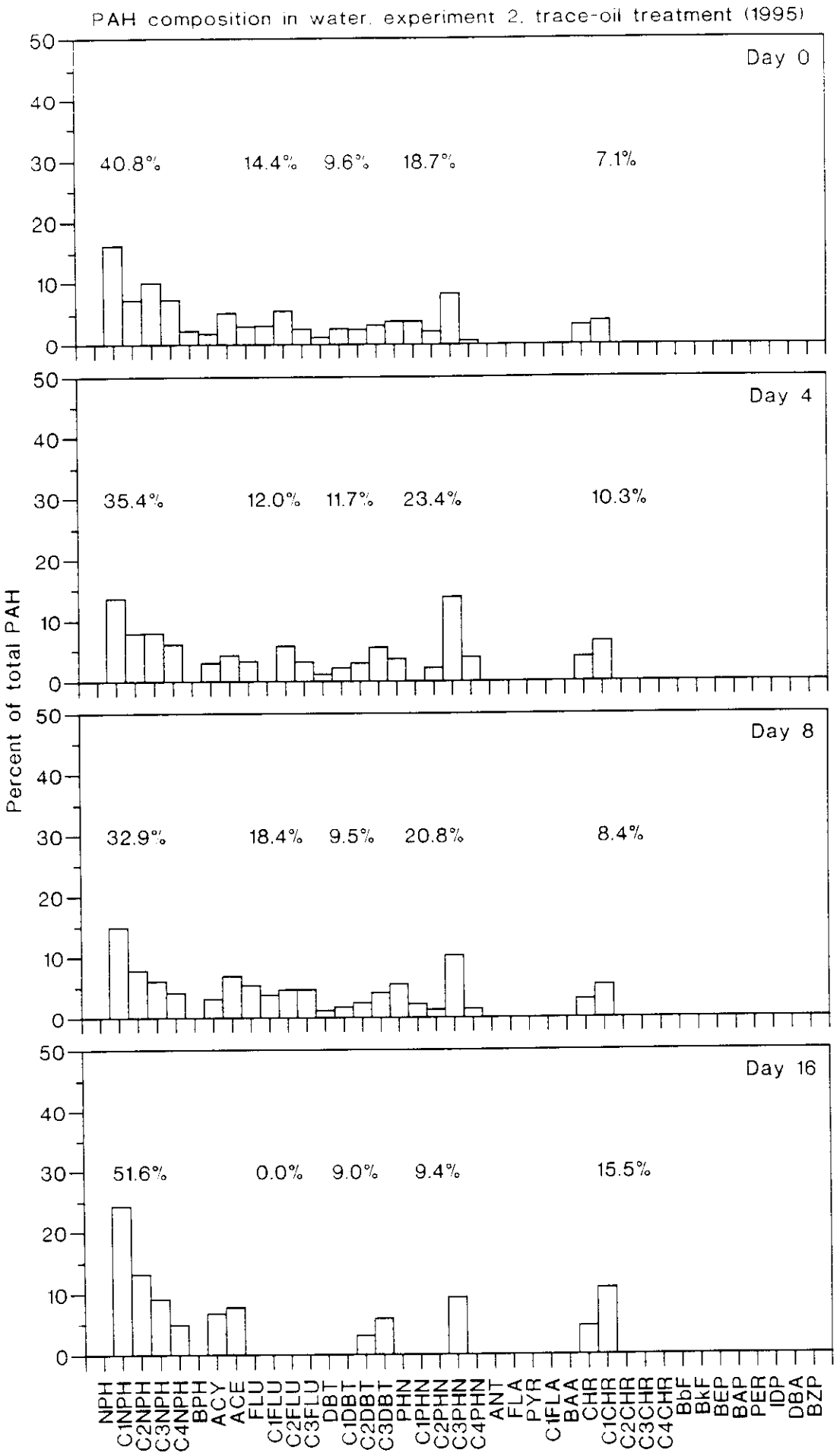
Appendix 4.2.3 *(at right).* Composition of PAH in mid-oil treatment water, experiment 1 (1995). Data for day 16 are means of paired observations ± standard error. Percentages printed inside the graphs indicate total percentages of the following homologous chemical groups (left to right): naphthalenes, fluorenes, dibenzothiophenes, phenanthrenes, and chrysenes.


PAH composition in water, experiment 1, mid-oil treatment (1995)
Percent of total PAH
Day 0
38.4 ppb
82.0%
4.0%
3.7%
7.5%
0.1%
Day 4
20.1 ppb
73.1%
5.9%
6.9%
12.2%
0.1%
Day 8
13.8 ppb
69.7%
6.0%
8.0%
14.8%
0.1%
Day 16
1.9 ppb
44.1%
15.7%
13.2%
24.2%
0.9%
0
10
20
30
40
50
NPH
C1NPH
C2NPH
C3NPH
C4NPH
BPH
ACY
ACE
FLU
C1FLU
C2FLU
C3FLU
DBT
C1DBT
C2DBT
C3DBT
PHN
C1PHN
C2PHN
C3PHN
C4PHN
ANT
FLA
PYR
C1FLA
BAA
CHR
C1CHR
C2CHR
C3CHR
C4CHR
BbF
BkF
BEP
BAP
PER
IDP
DBA
BZP

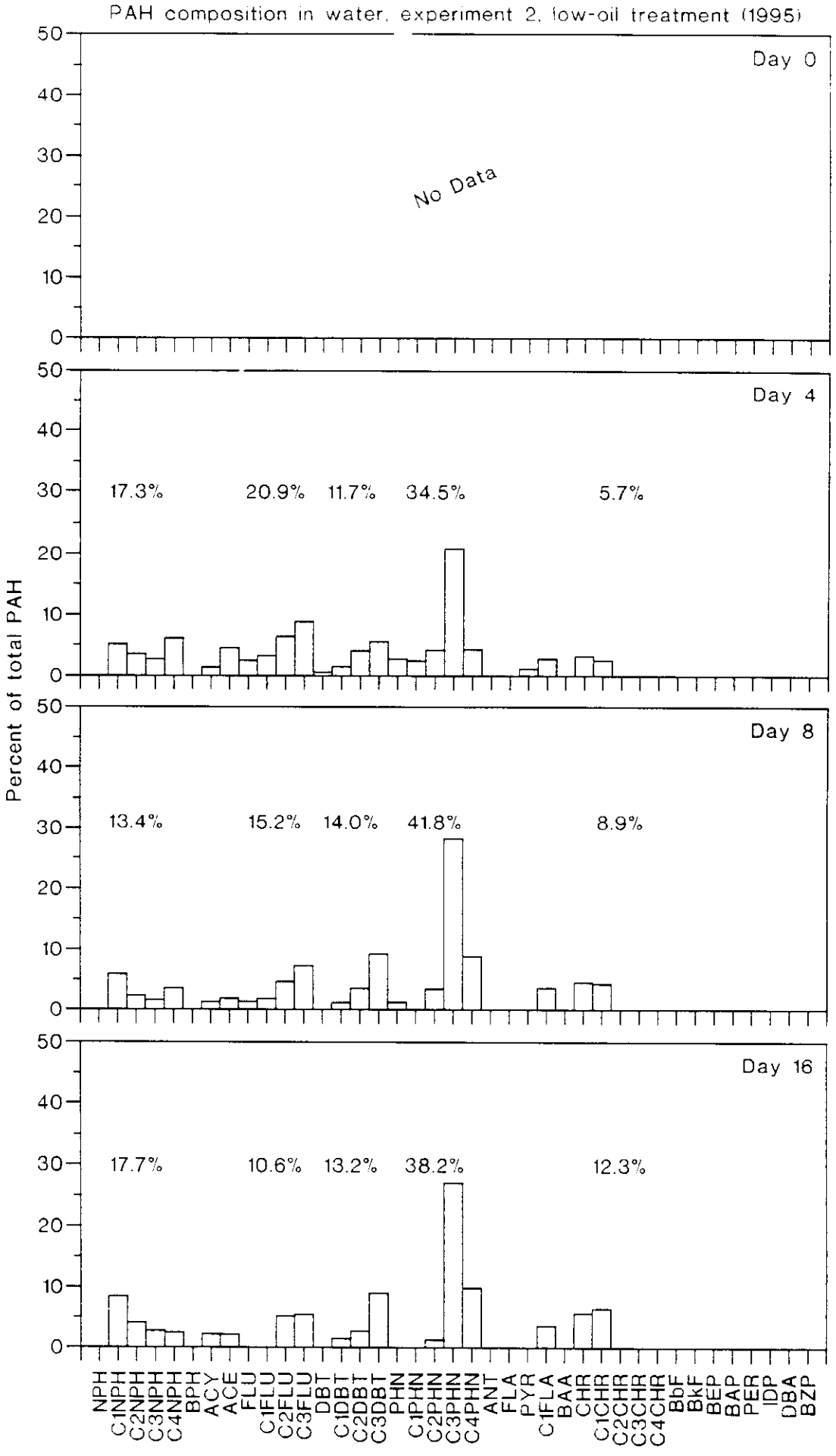
Appendix 4.2.4 *(at right)*. Composition of PAH in high-oil treatment water, experiment 1 (1995). Percentages printed inside the graphs indicate total percentages of the following homologous chemical groups (left to right): naphthalenes, fluorenes, dibenzothiophenes, phenanthrenes, and chrysenes.


PAH composition in water, experiment 1, high-oil treatment (1995)
Day 1
53.0 ppb
84.6%
2.9%
3.3%
5.2%
0.0%
Day 4
32.8 ppb
76.3%
4.5%
5.2%
8.9%
0.1%
Day 8
33.9 ppb
78.9%
3.6%
4.9%
8.3%
0.1%
Day 16
21.4 ppb
75.7%
5.6%
5.5%
9.7%
0.1%
Percent of total PAH
0
10
20
30
40
50
NPH
C1NPH
C2NPH
C3NPH
C4NPH
BPH
ACY
ACE
FLU
C1FLU
C2FLU
C3FLU
DBT
C1DBT
C2DBT
C3DBT
PHN
C1PHN
C2PHN
C3PHN
C4PHN
ANT
FLA
PYR
C1FLA
BAA
CHR
C1CHR
C2CHR
C3CHR
C4CHR
BbF
BkF
BEP
BAP
PER
IDP
DBA
BZP

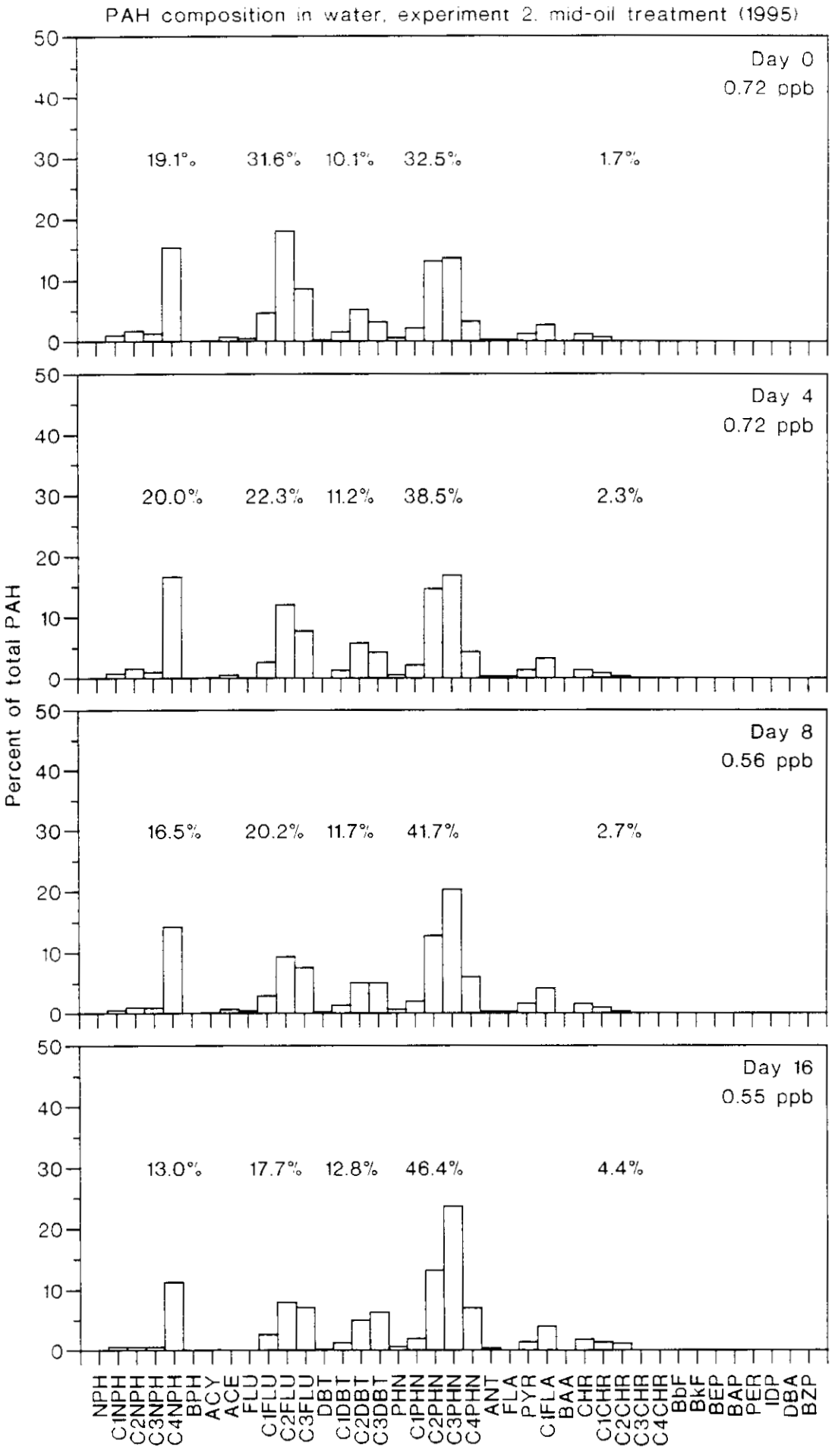
Appendix 4.2.5 *(at right).* Composition of PAH in trace-oil treatment water, experiment 2 (1995). Percentages printed inside the graphs indicate total percentages of the following homologous chemical groups (left to right): naphthalenes, fluorenes, dibenzothiophenes, phenanthrenes, and chrysenes.


PAH composition in water, experiment 2, trace-oil treatment (1995)
Day 0
40.8%
14.4%
9.6%
18.7%
7.1%
Day 4
35.4%
12.0%
11.7%
23.4%
10.3%
Day 8
32.9%
18.4%
9.5%
20.8%
8.4%
Day 16
51.6%
0.0%
9.0%
9.4%
15.5%
Percent of total PAH
0
10
20
30
40
50
NPH
C1NPH
C2NPH
C3NPH
C4NPH
BPH
ACY
ACE
FLU
C1FLU
C2FLU
C3FLU
DBT
C1DBT
C2DBT
C3DBT
PHN
C1PHN
C2PHN
C3PHN
C4PHN
ANT
FLA
PYR
C1FLA
BAA
CHR
C1CHR
C2CHR
C3CHR
C4CHR
BbF
BkF
BEP
BAP
PER
IDP
DBA
BZP

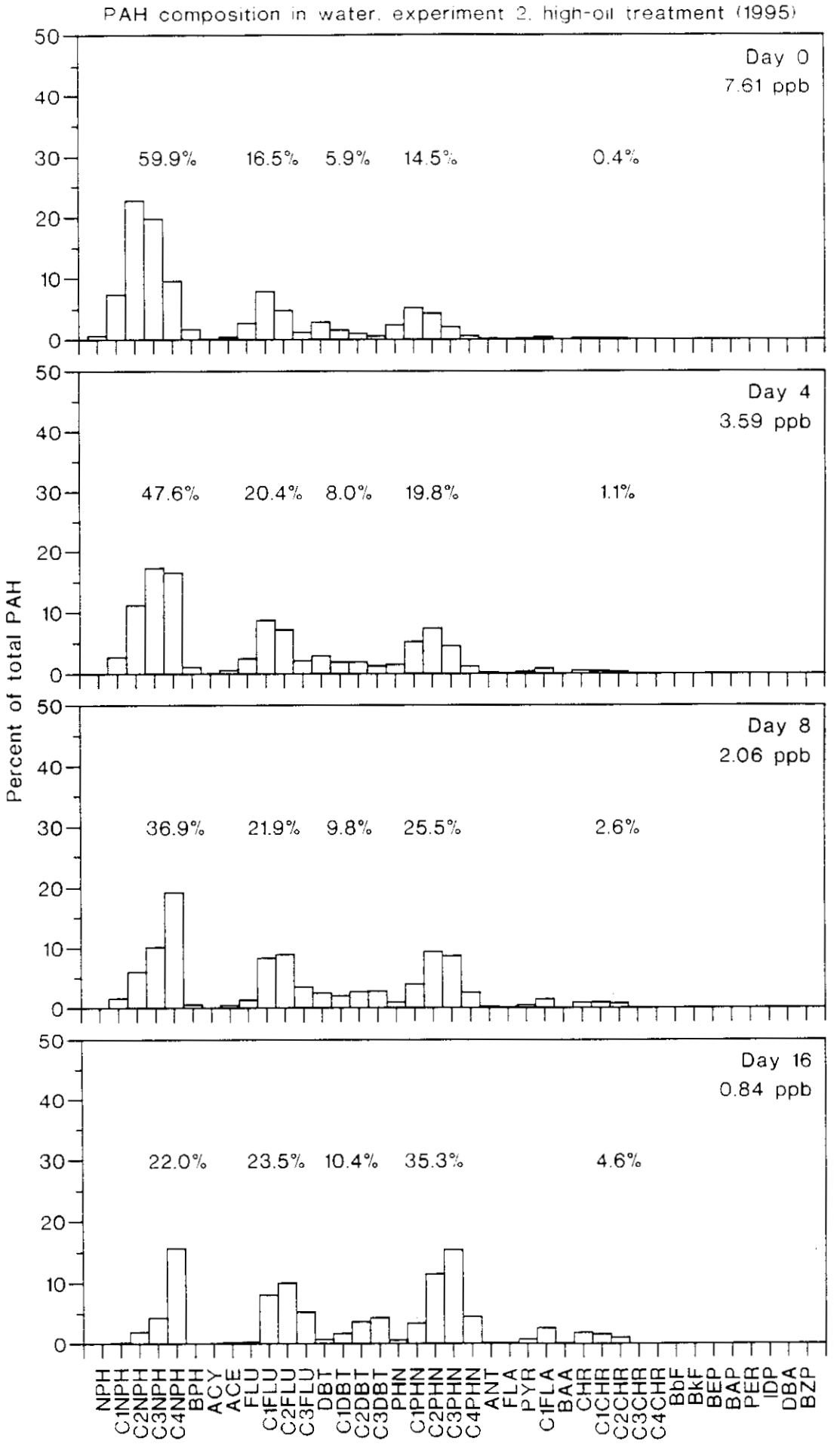
Appendix 4.2.6 *(at right).* Composition of PAH in low-oil treatment water, experiment 2 (1995). Percentages printed inside the graphs indicate total percentages of the following homologous chemical groups (left to right): naphthalenes, fluorenes, dibenzothiophenes, phenanthrenes, and chrysenes.


PAH composition in water, experiment 2, low-oil treatment (1995)
Day 0
No Data
Day 4
17.3%
20.9%
11.7%
34.5%
5.7%
Day 8
13.4%
15.2%
14.0%
41.8%
8.9%
Day 16
17.7%
10.6%
13.2%
38.2%
12.3%
Percent of total PAH
0
10
20
30
40
50
NPH
C1NPH
C2NPH
C3NPH
C4NPH
BPH
ACY
ACE
FLU
C1FLU
C2FLU
C3FLU
DBT
C1DBT
C2DBT
C3DBT
PHN
C1PHN
C2PHN
C3PHN
C4PHN
ANT
FLA
PYR
C1FLA
BAA
CHR
C1CHR
C2CHR
C3CHR
C4CHR
BbF
BkF
BEP
BAP
PER
IDP
DBA
BZP

Appendix 4.2.7 *(at right).* Composition of PAH in mid-oil treatment water, experiment 2 (1995). Percentages printed inside the graphs indicate total percentages of the following homologous chemical groups (left to right): naphthalenes, fluorenes, dibenzothiophenes, phenanthrenes, and chrysenes.


PAH composition in water, experiment 2, mid-oil treatment (1995)
Percent of total PAH
Day 0
0.72 ppb
19.1%
31.6%
10.1%
32.5%
1.7%
Day 4
0.72 ppb
20.0%
22.3%
11.2%
38.5%
2.3%
Day 8
0.56 ppb
16.5%
20.2%
11.7%
41.7%
2.7%
Day 16
0.55 ppb
13.0%
17.7%
12.8%
46.4%
4.4%
0
10
20
30
40
50
NPH
C1NPH
C2NPH
C3NPH
C4NPH
BPH
ACY
ACE
FLU
C1FLU
C2FLU
C3FLU
DBT
C1DBT
C2DBT
C3DBT
PHN
C1PHN
C2PHN
C3PHN
C4PHN
ANT
FLA
PYR
C1FLA
BAA
CHR
C1CHR
C2CHR
C3CHR
C4CHR
BbF
BkF
BeP
BaP
PER
IDP
DBA
BZP

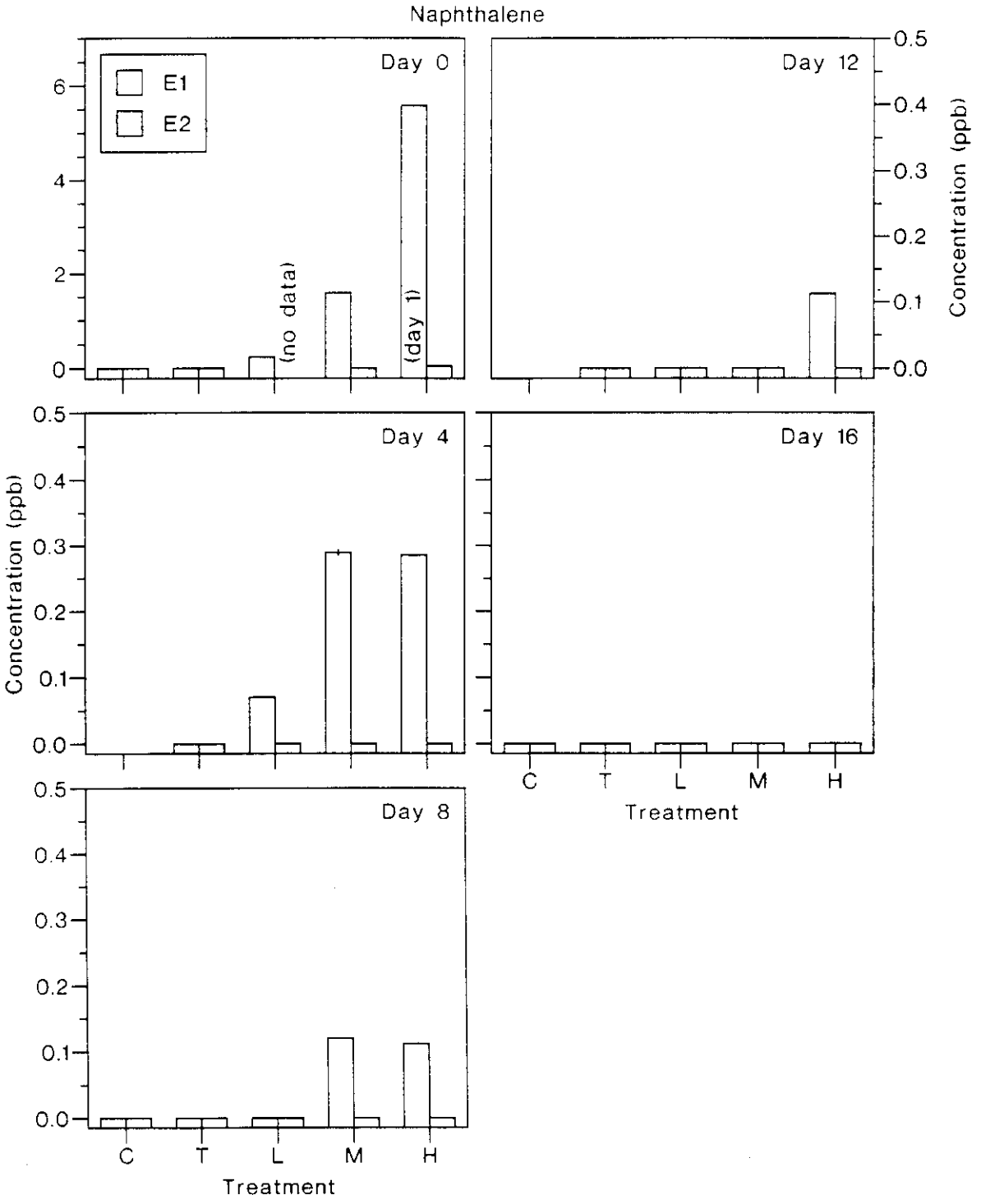
Appendix 4.2.8 *(at right).* Composition of PAH in high-oil treatment water, experiment 2 (1995). Percentages printed inside the graphs indicate total percentages of the following homologous chemical groups (left to right): naphthalenes, fluorenes, dibenzothiophenes, phenanthrenes, and chrysenes.


PAH composition in water, experiment 2, high-oil treatment (1995)
Day 0
7.61 ppb
59.9%
16.5%
5.9%
14.5%
0.4%
Day 4
3.59 ppb
47.6%
20.4%
8.0%
19.8%
1.1%
Day 8
2.06 ppb
36.9%
21.9%
9.8%
25.5%
2.6%
Day 16
0.84 ppb
22.0%
23.5%
10.4%
35.3%
4.6%
Percent of total PAH
0
10
20
30
40
50
NPH
C1NPH
C2NPH
C3NPH
C4NPH
BPH
ACY
ACE
FLU
C1FLU
C2FLU
C3FLU
DBT
C1DBT
C2DBT
C3DBT
PHN
C1PHN
C2PHN
C3PHN
C4PHN
ANT
FLA
PYR
C1FLA
BAA
CHR
C1CHR
C2CHR
C3CHR
C4CHR
BbF
BkF
BEP
BAP
PER
IDP
DBA
BZP

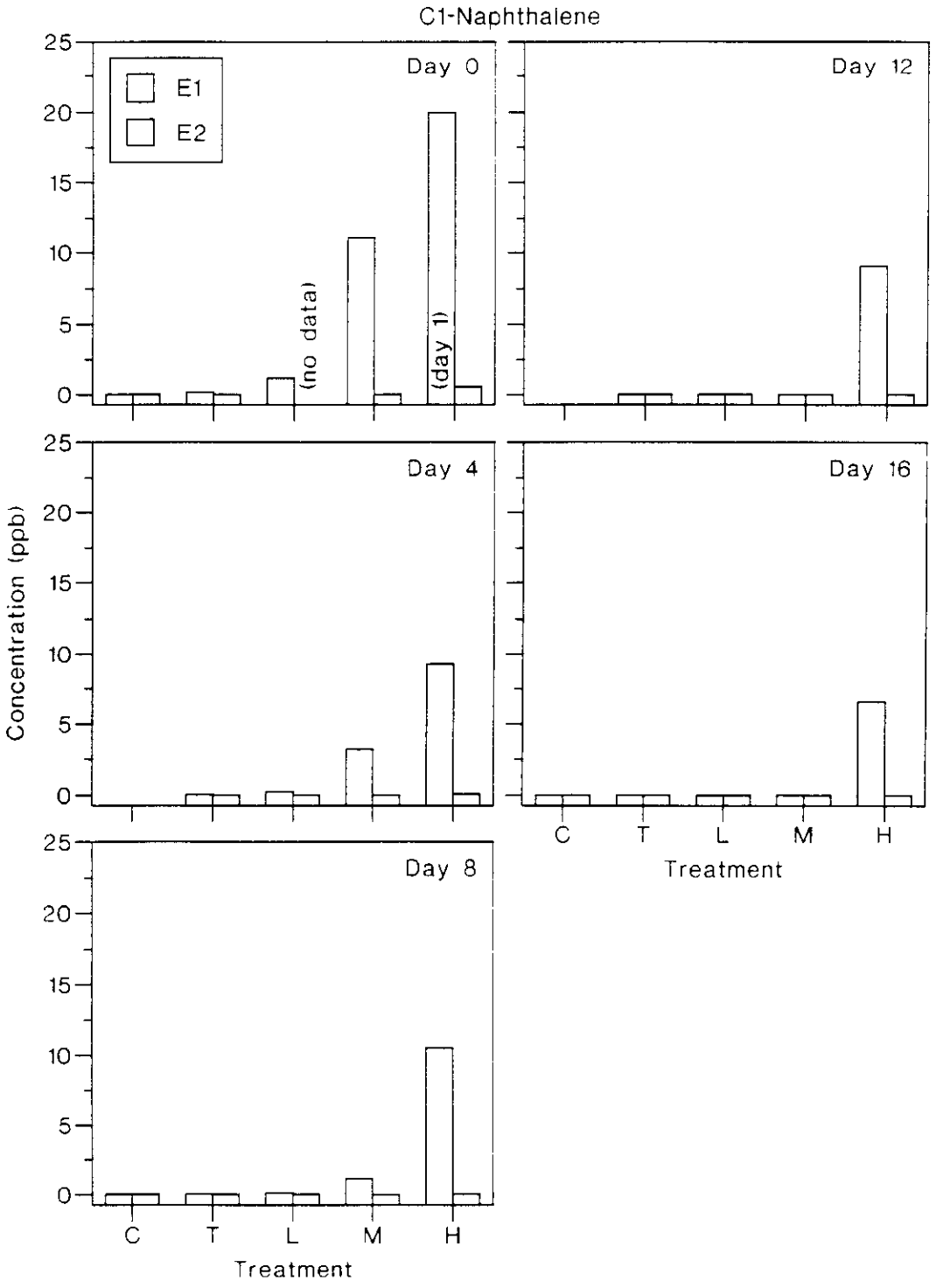
Appendix 4.3

Comparison of polynuclear aromatic hydrocarbons concentrations in water of less and more weathered oil experiments (1995)

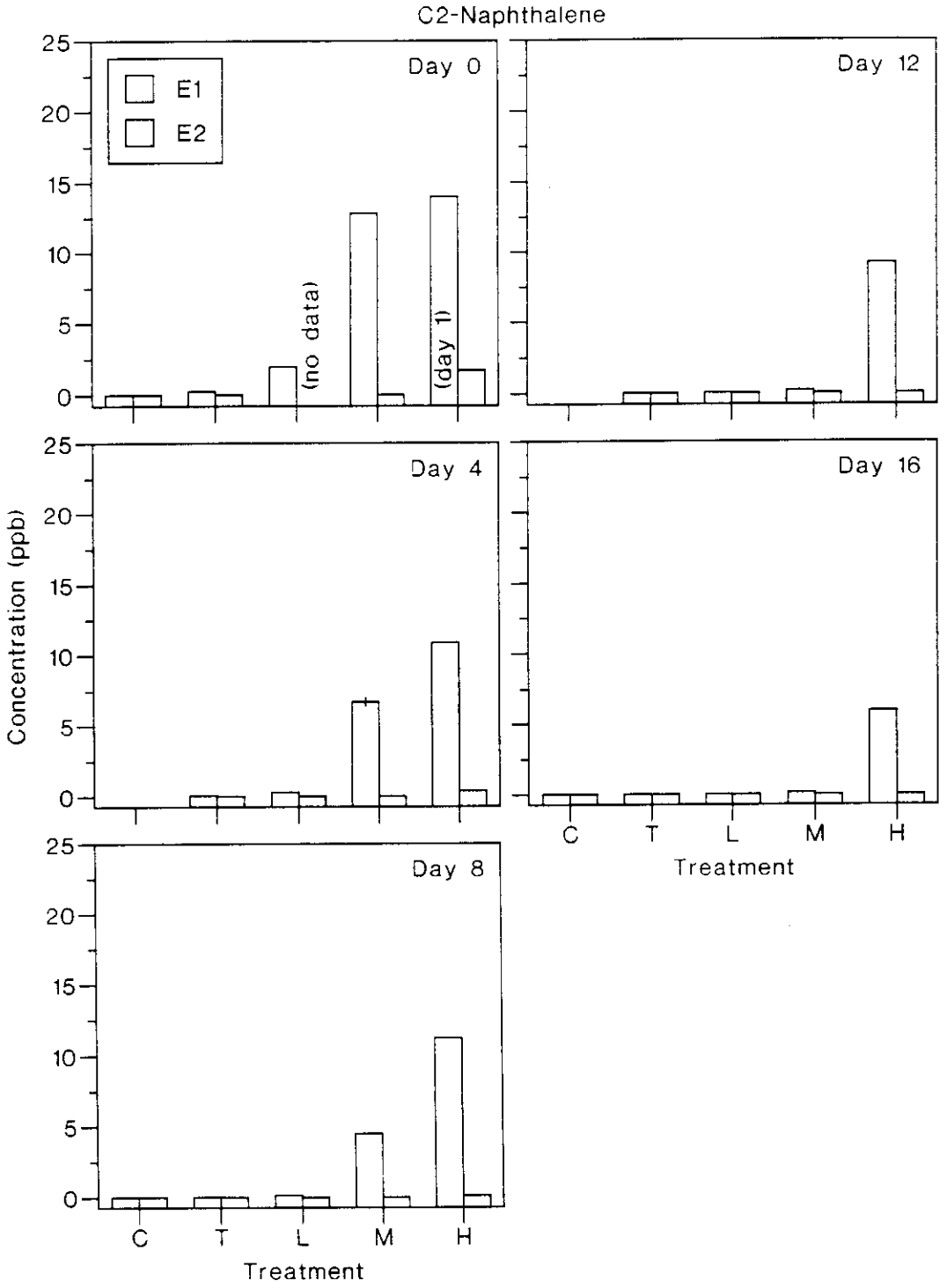
Polynuclear aromatic hydrocarbon (PAH) concentrations in water by family (napthalenes, fluorenes, dibenzothiophenes, phenanthrenes, and chrysenes) and by individual compound to compare concentrations between the less weathered (E1) and more weathered (E2) experiments. Concentrations of compounds with the most rings (chrysenes) were consistently greater in E1 than in E2, despite the lower total PAH concentration. In addition, concentrations of the more substituted compounds within homologous families were generally greater in the more weathered experiment. No C4-chrysenes were detected.



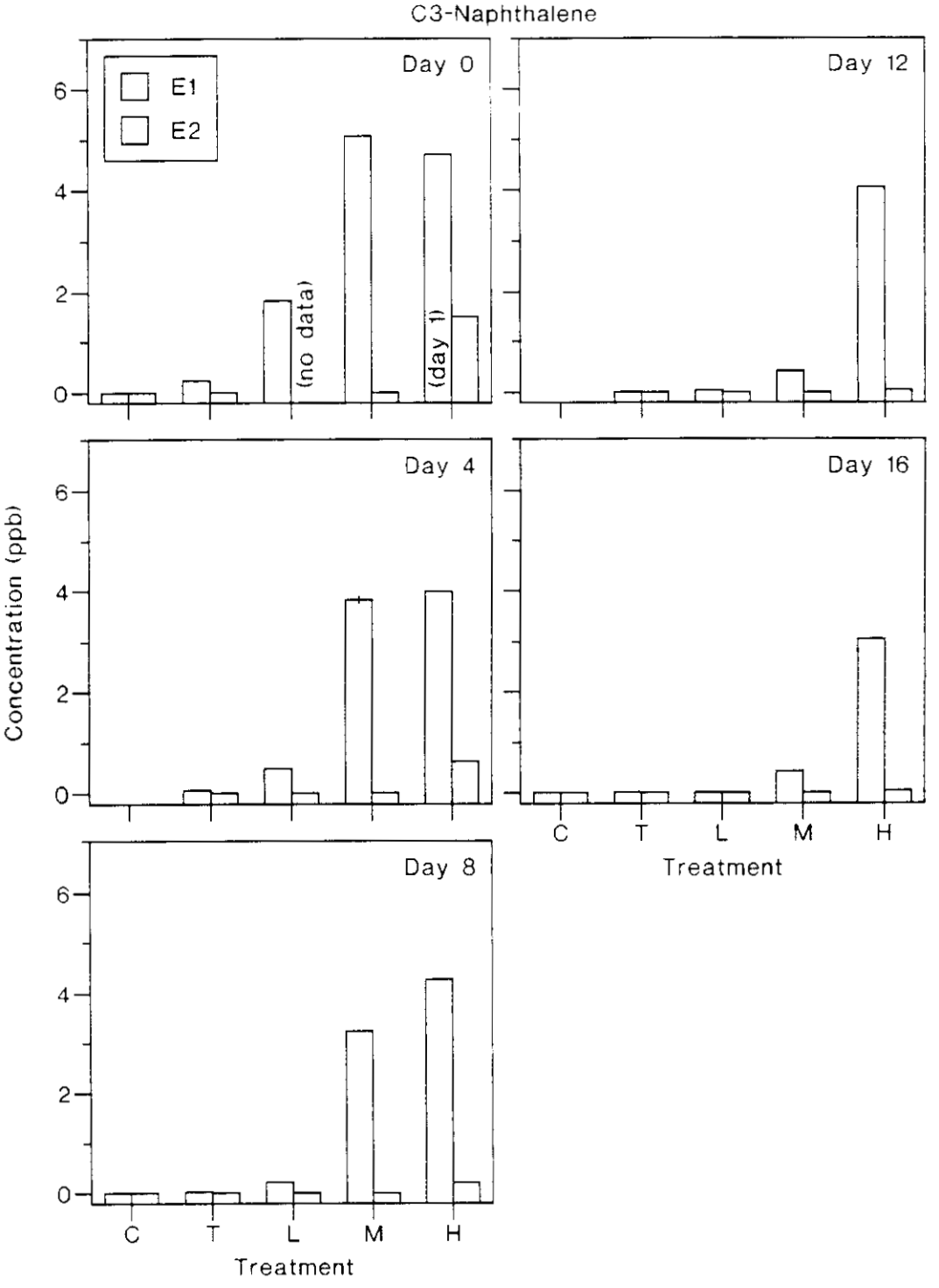
Appendix 4.3.1. Comparison of naphthalene concentrations in experiments one (E1) and two (E2). Observation times were as indicated on graphs, except day 1 data were substituted for day 0 in the high-oil treatment of E1. The majority of symbols represent single observations; where present, error bars indicate means ± SE.



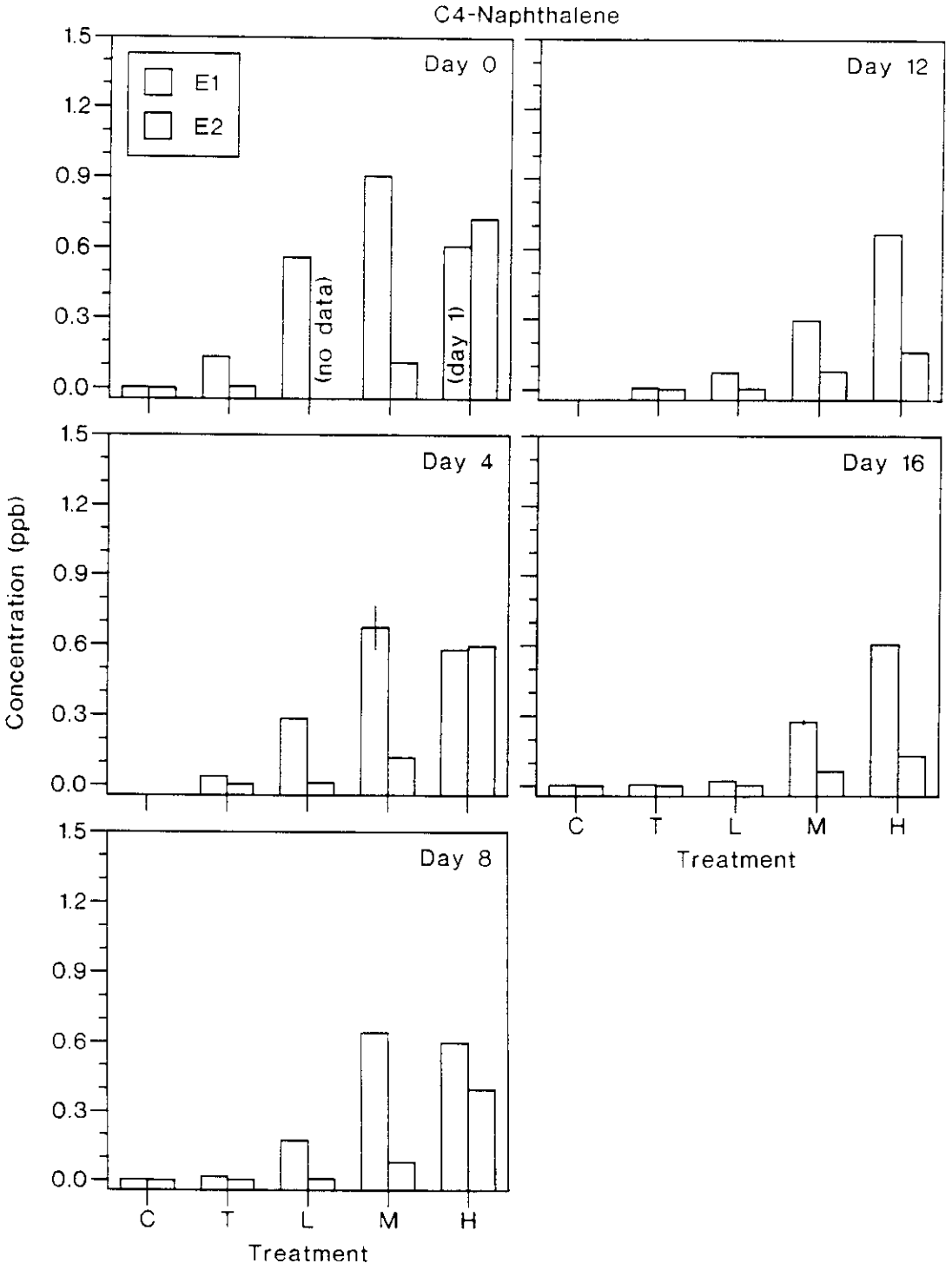
Appendix 4.3.2. Comparison of C1-naphthalene concentrations in experiments one (E1) and two (E2). Observation times were as indicated on graphs, except day 1 data were substituted for day 0 in the high-oil treatment of E1. The majority of symbols represent single observations; where present, error bars indicate means ± SE.



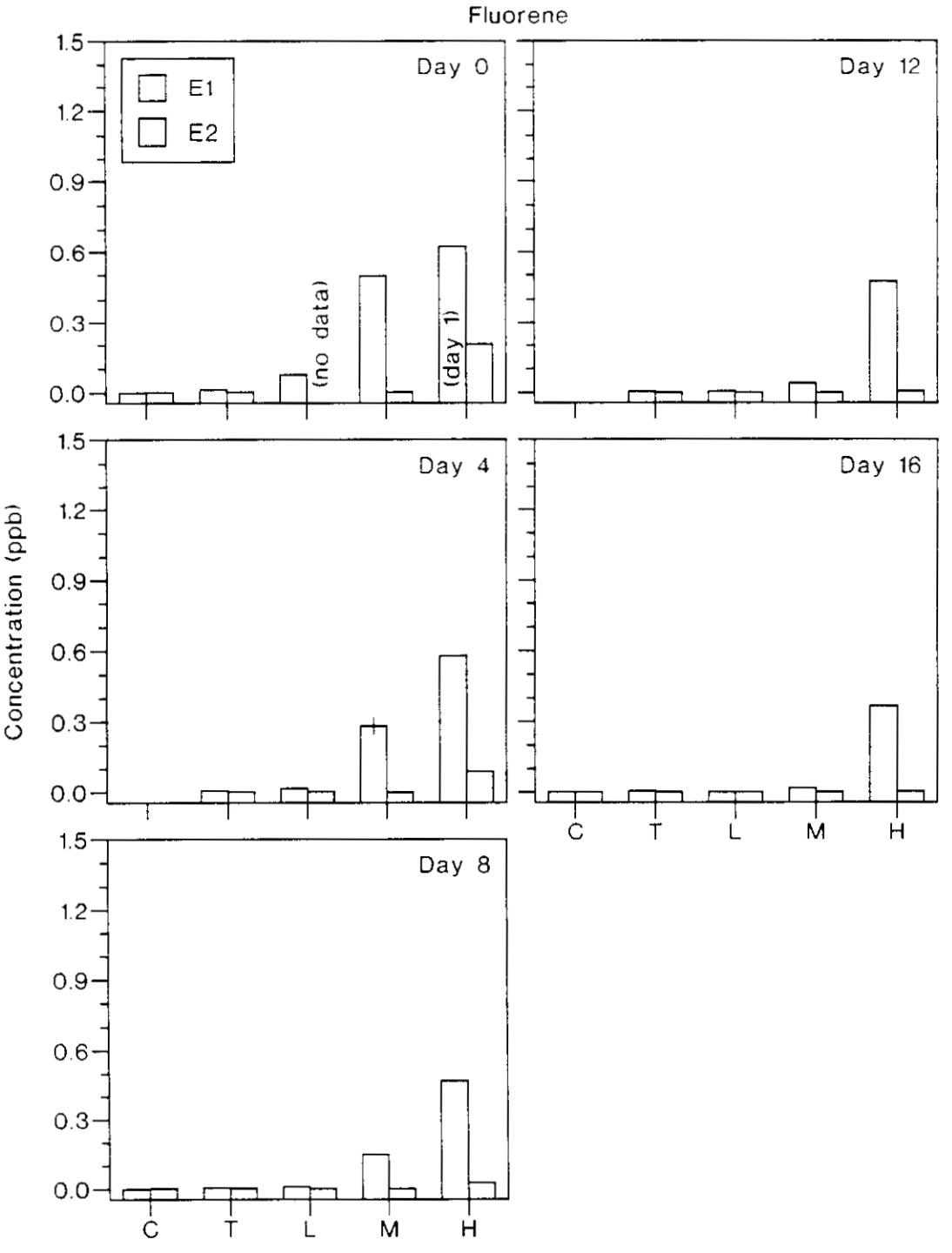
Appendix 4.3.3. Comparison of C2-naphthalene concentrations in experiments one (E1) and two (E2). Observation times were as indicated on graphs, except day 1 data were substituted for day 0 in the high-oil treatment of E1. The majority of symbols represent single observations; where present, error bars indicate means ± SE.



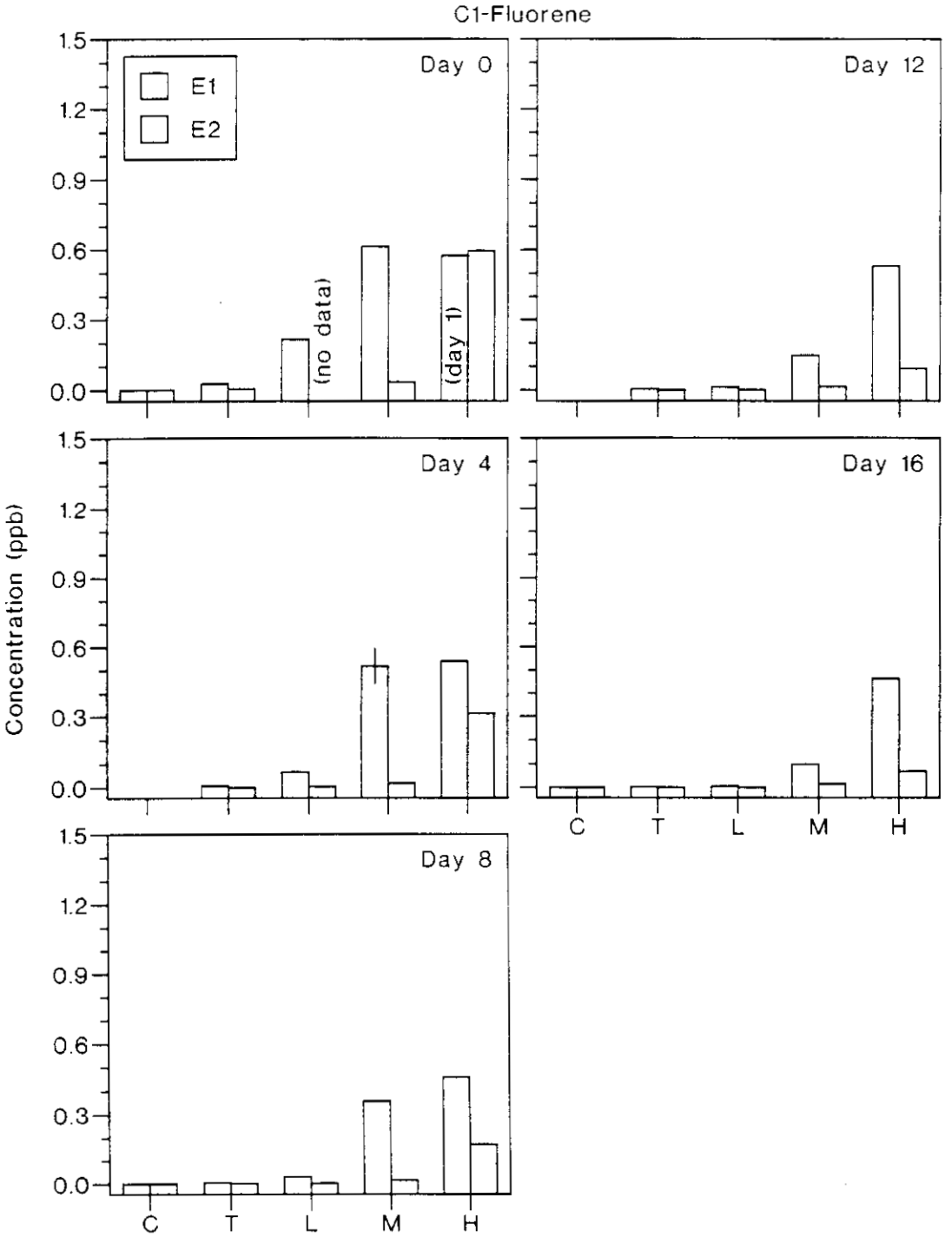
Appendix 4.3.4. Comparison of C3-naphthalene concentrations in experiments one (E1) and two (E2). Observation times were as indicated on graphs, except day 1 data were substituted for day 0 in the high-oil treatment of E1. The majority of symbols represent single observations; where present, error bars indicate means ± SE.



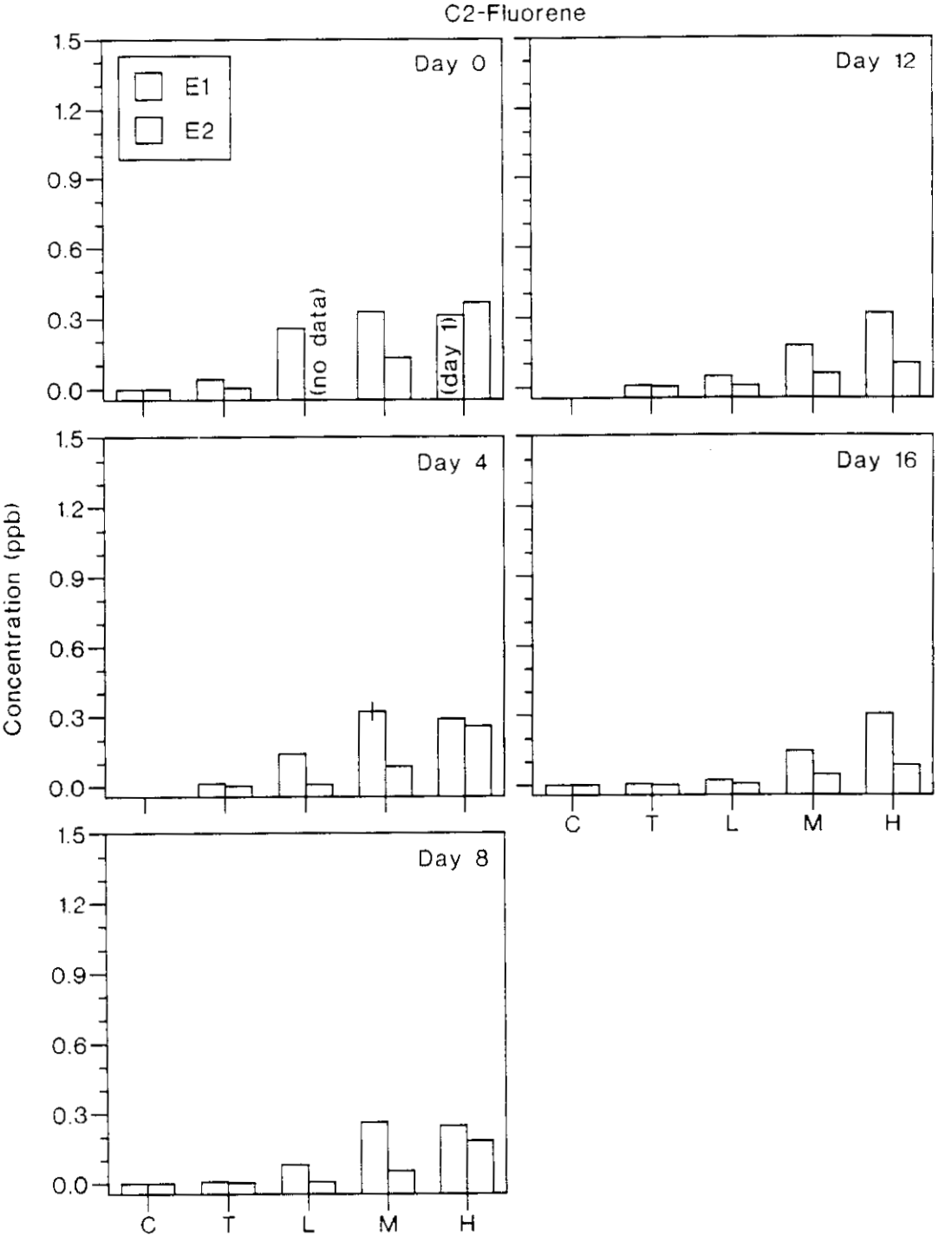
Appendix 4.3.5. Comparison of C4-naphthalene concentrations in experiments one (E1) and two (E2). Observation times were as indicated on graphs, except day 1 data were substituted for day 0 in the high-oil treatment of E1. The majority of symbols represent single observations; where present, error bars indicate means ± SE.



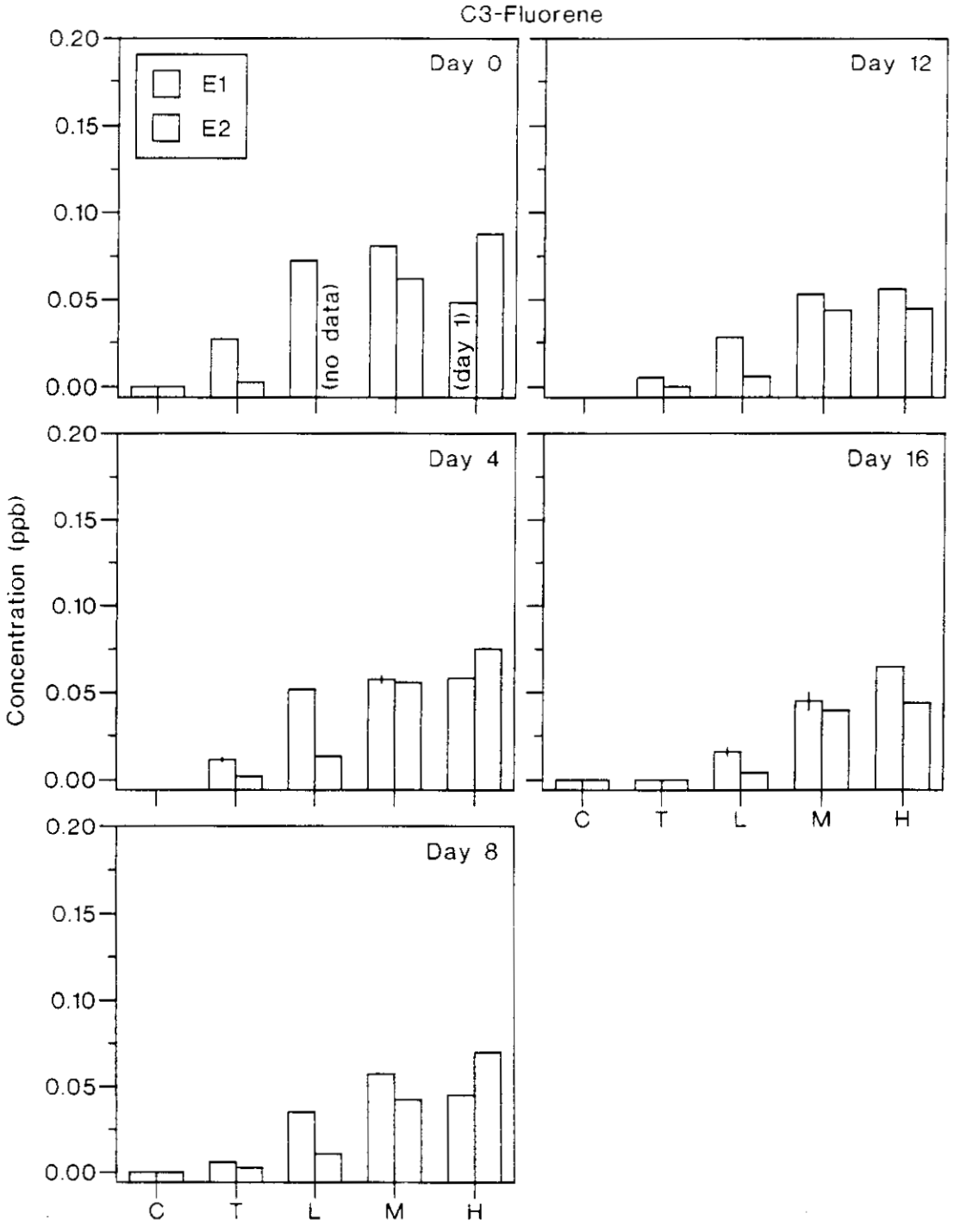
Appendix 4.3.6. Comparison of fluorene concentrations in experiments one (E1) and two (E2). Observation times were as indicated on graphs, except day 1 data were substituted for day 0 in the high-oil treatment of E1. The majority of symbols represent single observations; where present, error bars indicate means ± SE.



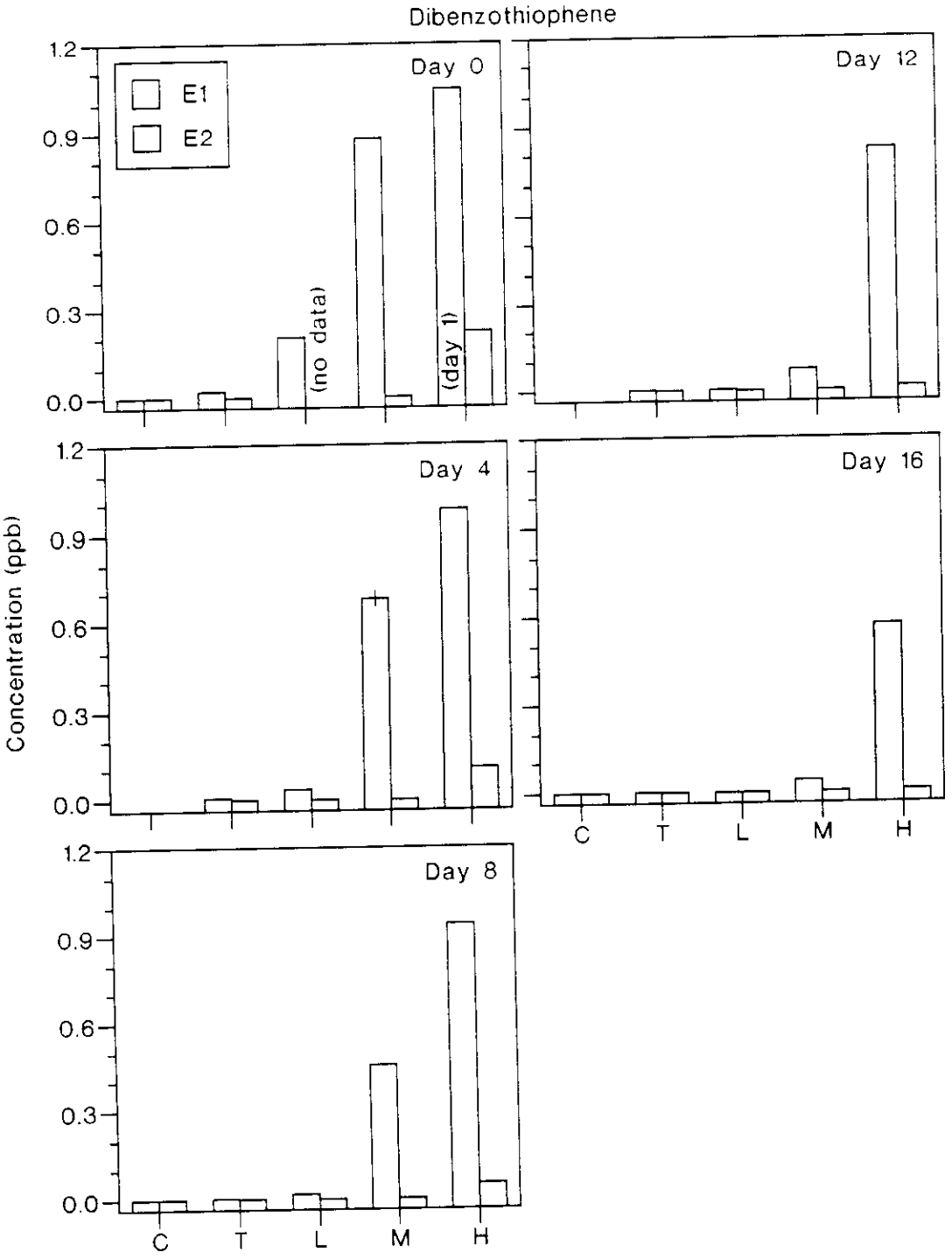
Appendix 4.3.7. Comparison of C1-fluorene concentrations in experiments one (E1) and two (E2). Observation times were as indicated on graphs, except day 1 data were substituted for day 0 in the high-oil treatment of E1. The majority of symbols represent single observations; where present, error bars indicate means ± SE.



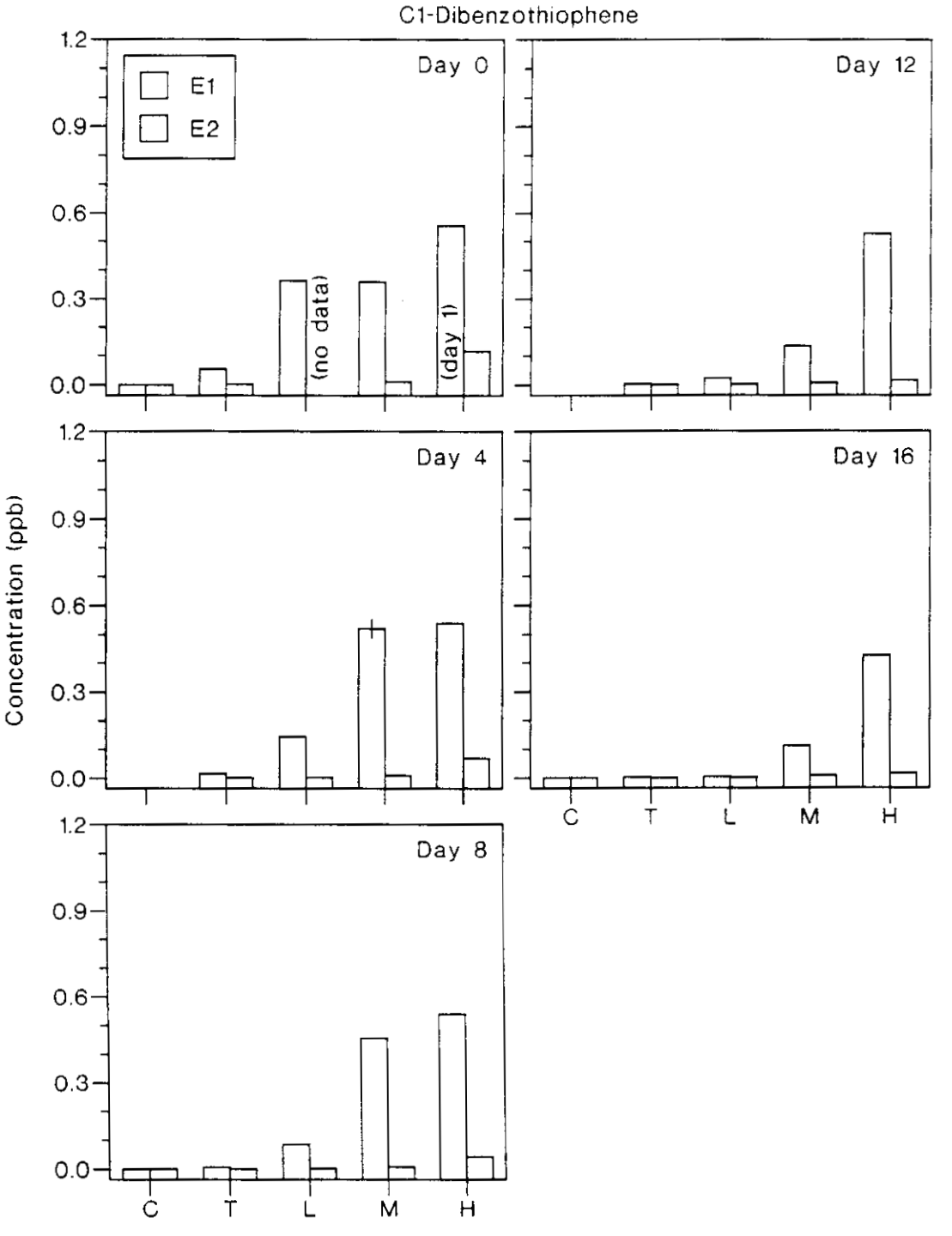
Appendix 4.3.8. Comparison of C2-fluorene concentrations in experiments one (E1) and two (E2). Observation times were as indicated on graphs, except day 1 data were substituted for day 0 in the high-oil treatment of E1. The majority of symbols represent single observations; where present, error bars indicate means ± SE.



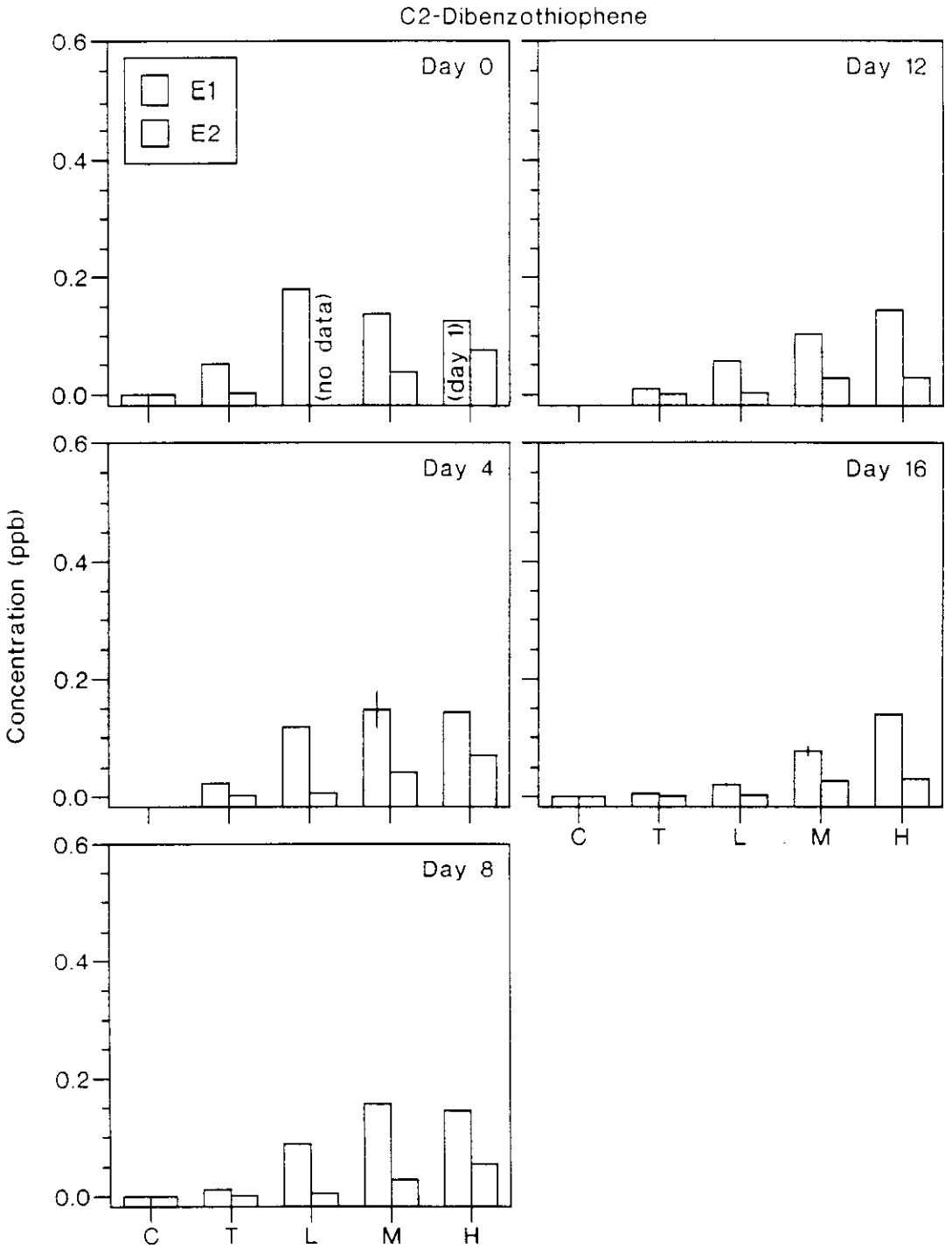
Appendix 4.3.9. Comparison of C3-fluorene concentrations in experiments one (E1) and two (E2). Observation times were as indicated on graphs, except day 1 data were substituted for day 0 in the high-oil treatment of E1. The majority of symbols represent single observations; where present, error bars indicate means ± SE.



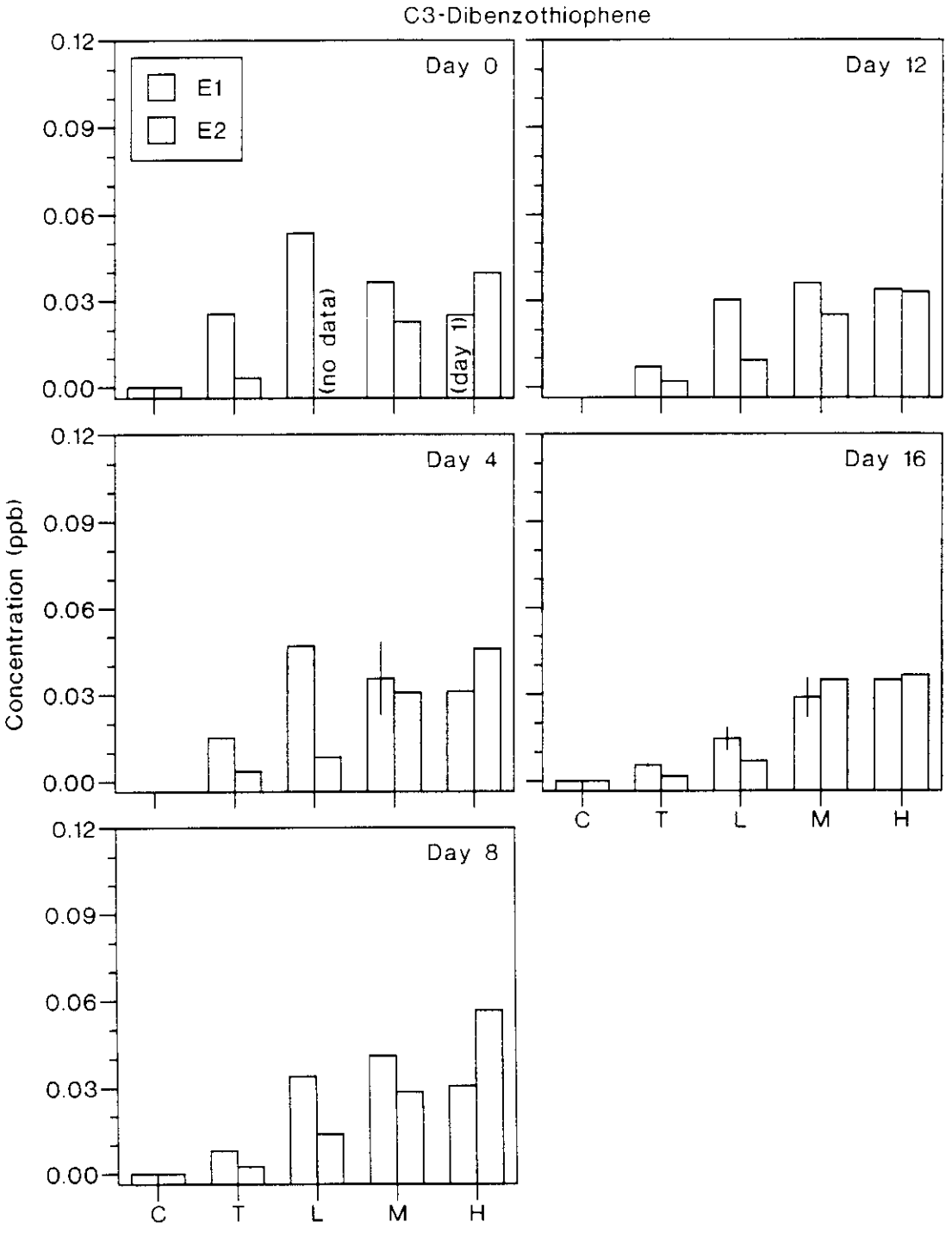
Appendix 4.3.10. Comparison of dibenzothiophene concentrations in experiments one (E1) and two (E2). Observation times were as indicated on graphs, except day 1 data were substituted for day 0 in the high-oil treatment of E1. The majority of symbols represent single observations; where present, error bars indicate means ± SE.



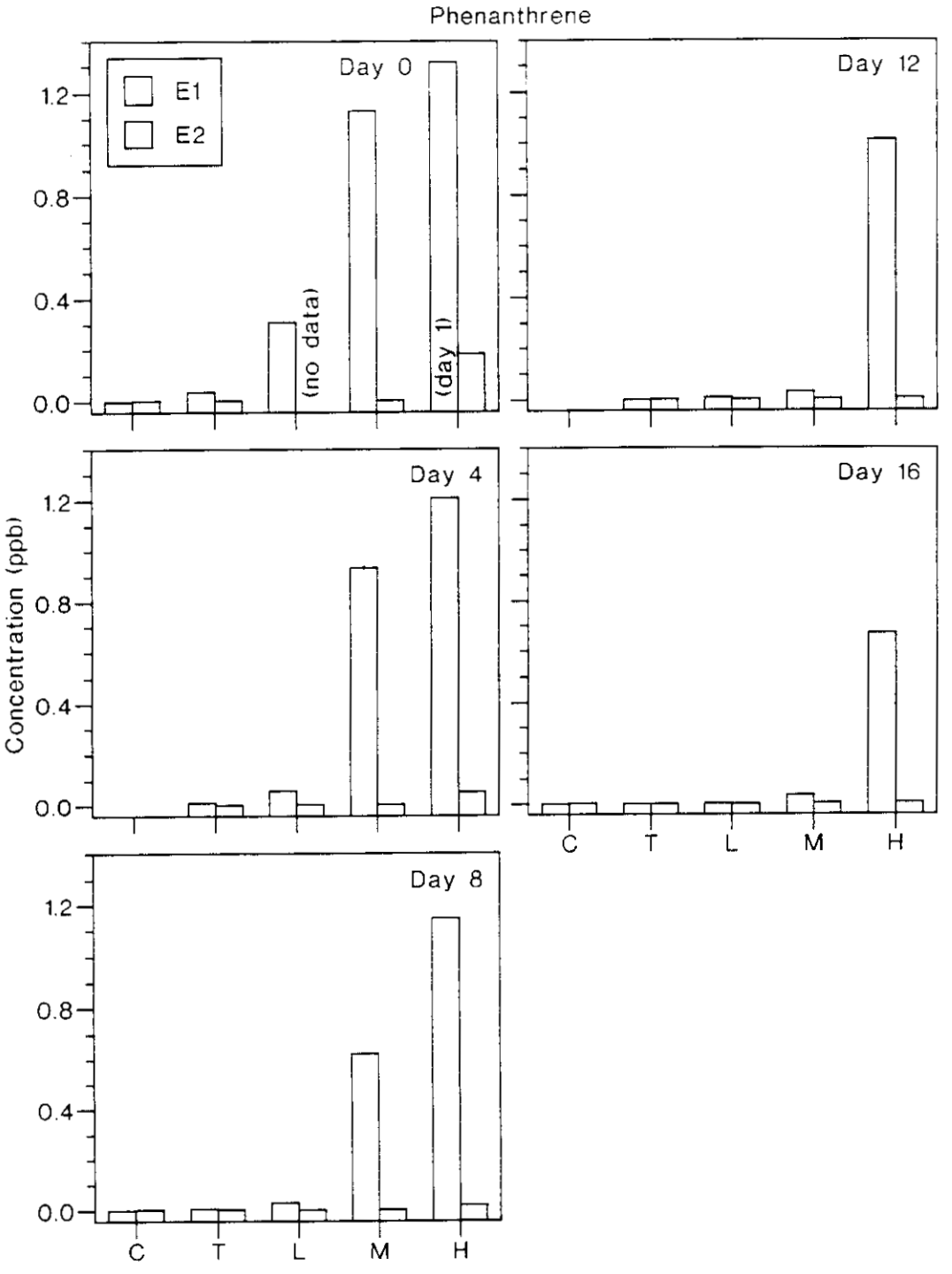
Appendix 4.3.11. Comparison of C1-dibenzothiophene concentrations in experiments one (E1) and two (E2). Observation times were as indicated on graphs, except day 1 data were substituted for day 0 in the high-oil treatment of E1. The majority of symbols represent single observations; where present, error bars indicate means ± SE.



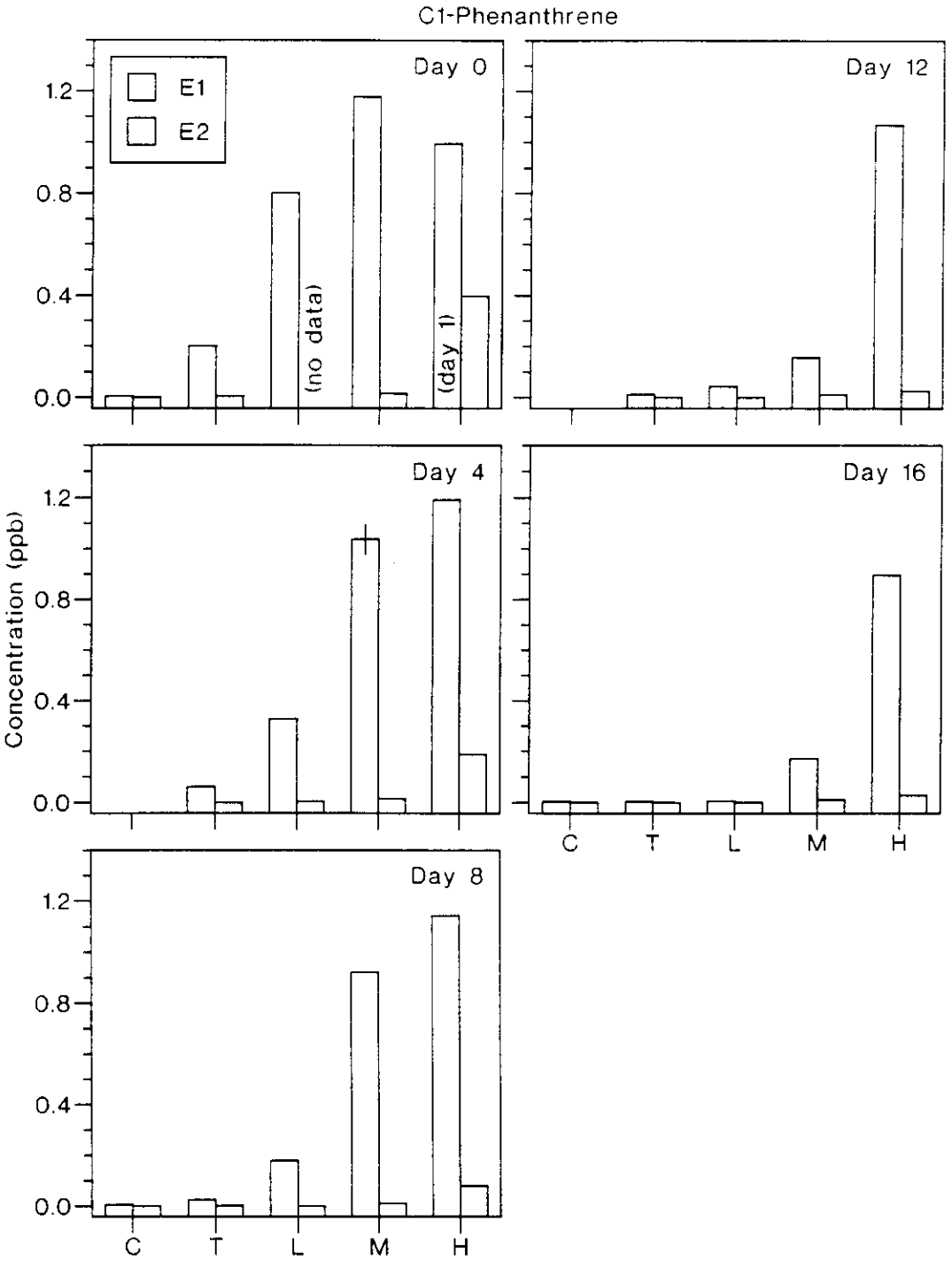
Appendix 4.3.12. Comparison of C2-dibenzothiophene concentrations in experiments one (E1) and two (E2). Observation times were as indicated on graphs, except day 1 data were substituted for day 0 in the high-oil treatment of E1. The majority of symbols represent single observations; where present, error bars indicate means ± SE.



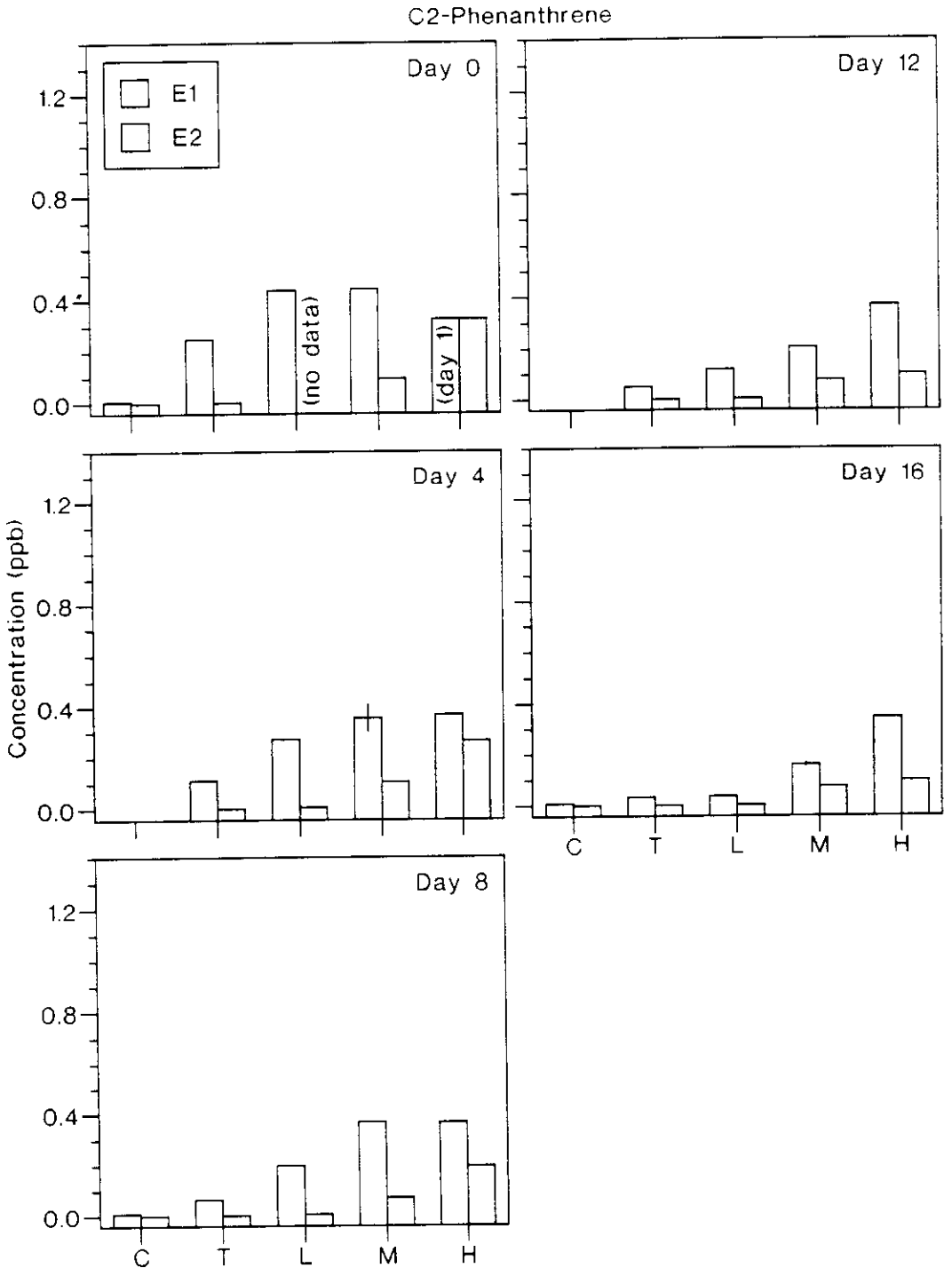
Appendix 4.3.13. Comparison of C3-dibenzothiophene concentrations in experiments one (E1) and two (E2). Observation times were as indicated on graphs, except day 1 data were substituted for day 0 in the high-oil treatment of E1. The majority of symbols represent single observations; where present, error bars indicate means ± SE.



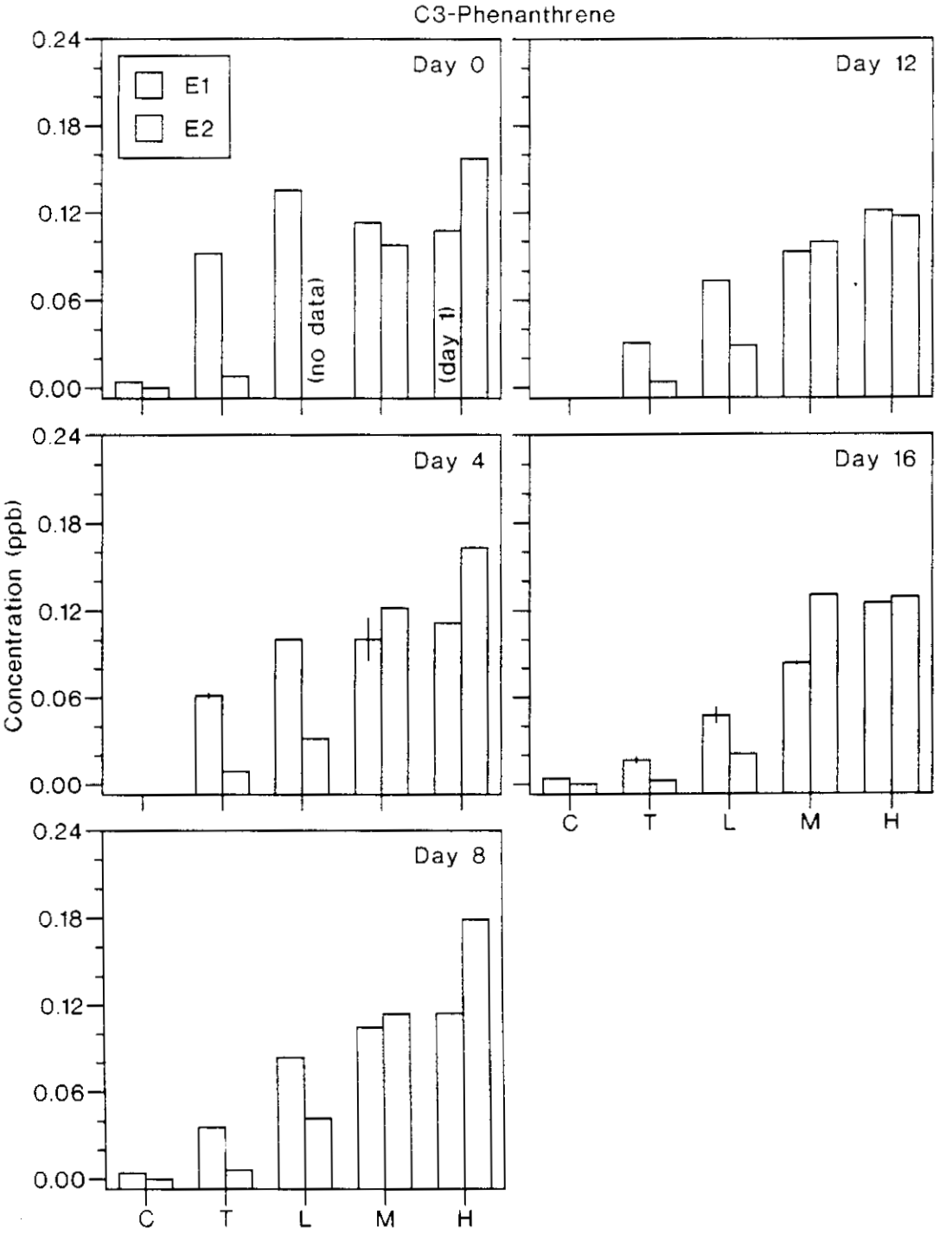
Appendix 4.3.14. Comparison of phenanthrene concentrations in experiments one (E1) and two (E2). Observation times were as indicated on graphs, except day 1 data were substituted for day 0 in the high-oil treatment of E1. The majority of symbols represent single observations; where present, error bars indicate means ± SE.



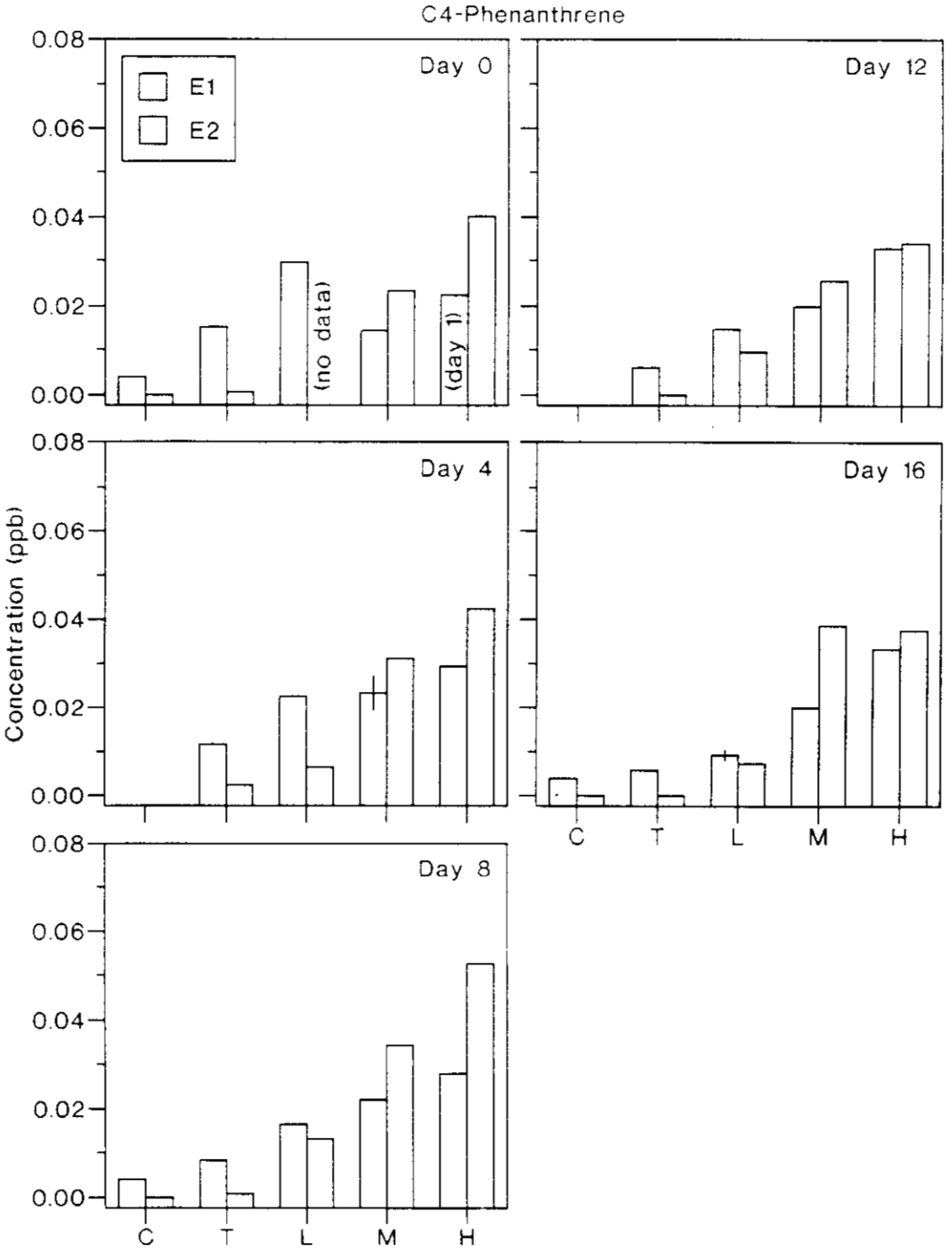
Appendix 4.3.15. Comparison of C1-phenanthrene concentrations in experiments one (E1) and two (E2). Observation times were as indicated on graphs, except day 1 data were substituted for day 0 in the high-oil treatment of E1. The majority of symbols represent single observations; where present, error bars indicate means ± SE.



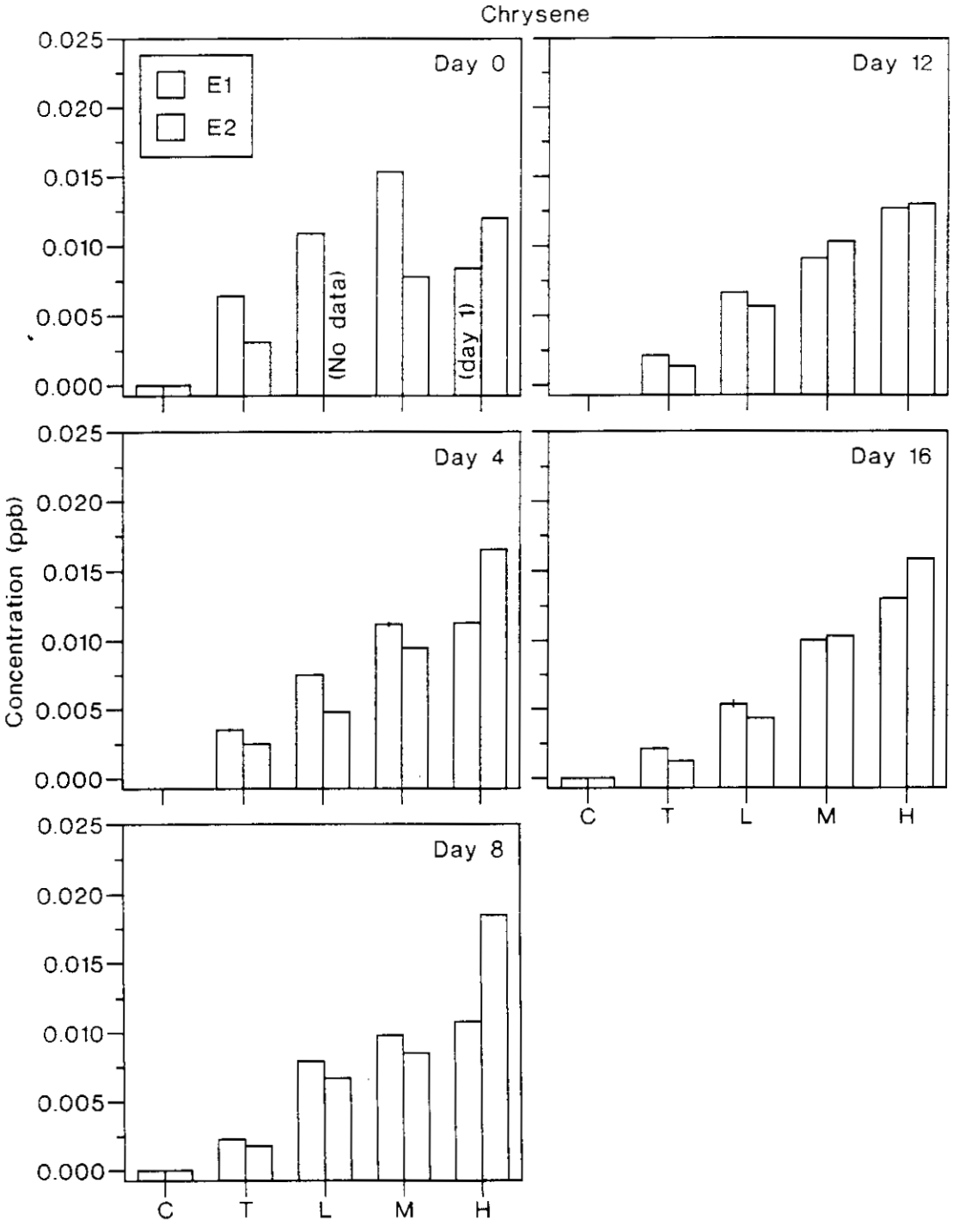
Appendix 4.3.16. Comparison of C2-phenanthrene concentrations in experiments one (E1) and two (E2). Observation times were as indicated on graphs, except day 1 data were substituted for day 0 in the high-oil treatment of E1. The majority of symbols represent single observations; where present, error bars indicate means ± SE.



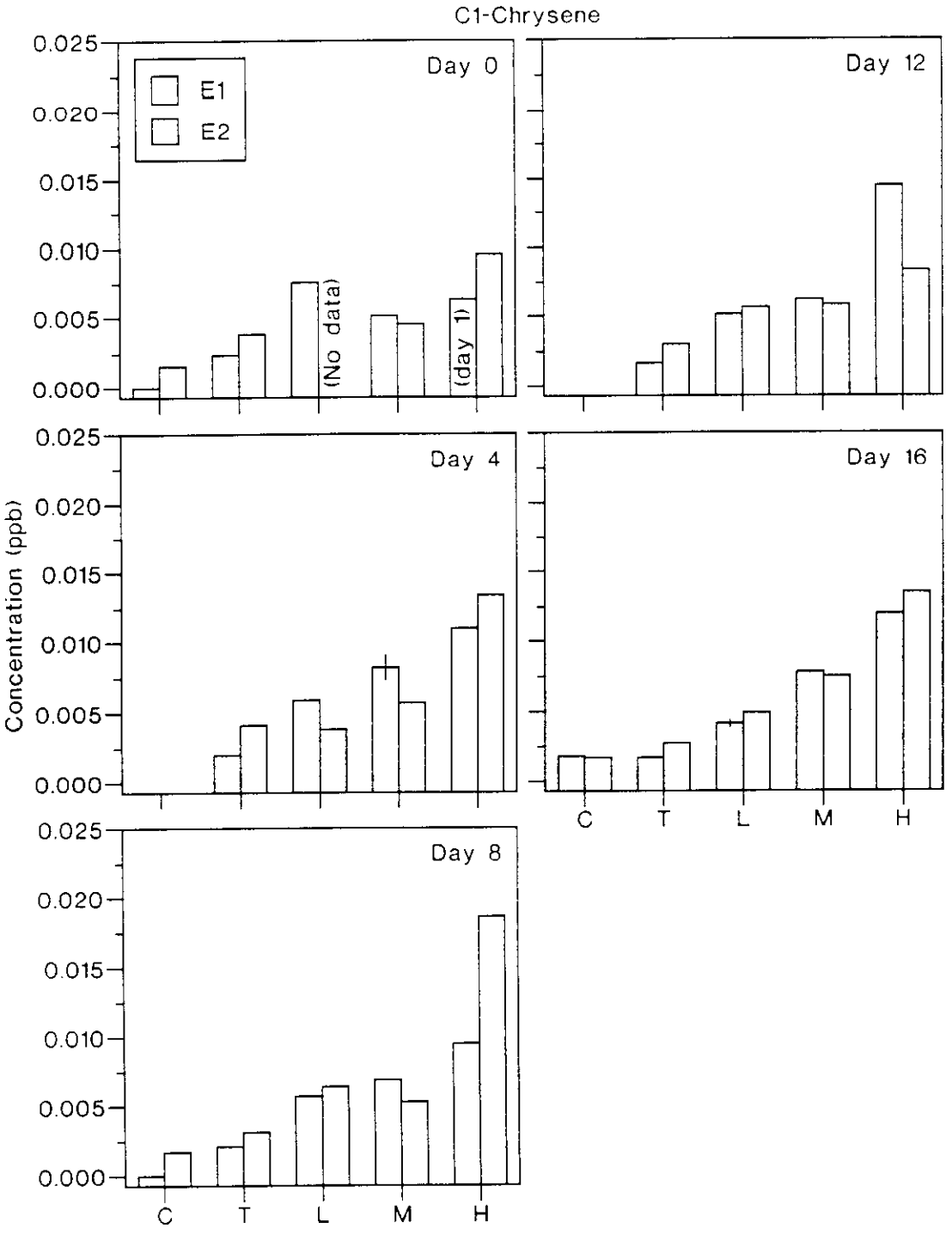
Appendix 4.3.17. Comparison of C3-phenanthrene concentrations in experiments one (E1) and two (E2). Observation times were as indicated on graphs, except day 1 data were substituted for day 0 in the high-oil treatment of E1. The majority of symbols represent single observations; where present, error bars indicate means ± SE.



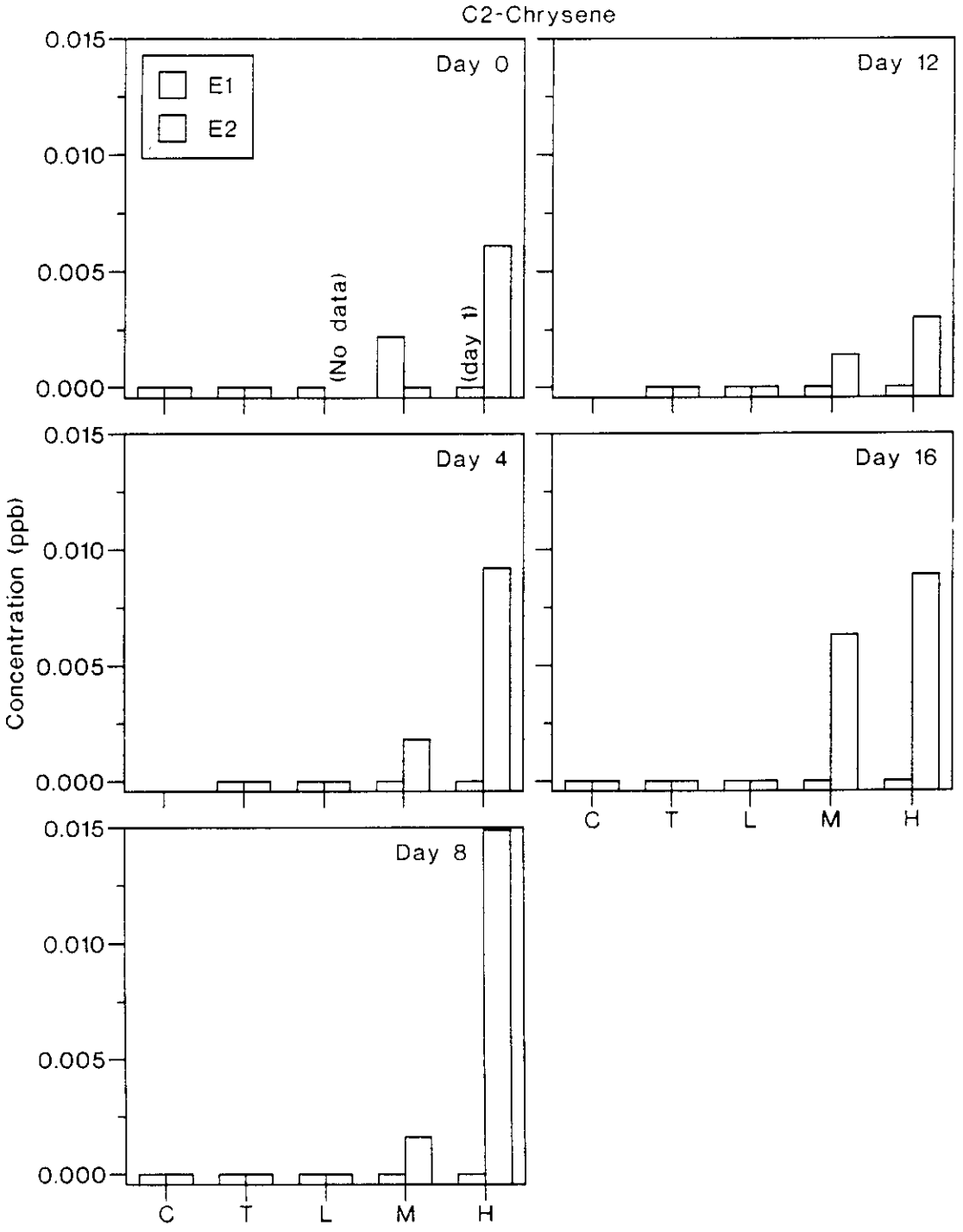
Appendix 4.3.18. Comparison of C4-phenanthrene concentrations in experiments one (E1) and two (E2). Observation times were as indicated on graphs, except day 1 data were substituted for day 0 in the high-oil treatment of E1. The majority of symbols represent single observations; where present, error bars indicate means ± SE.



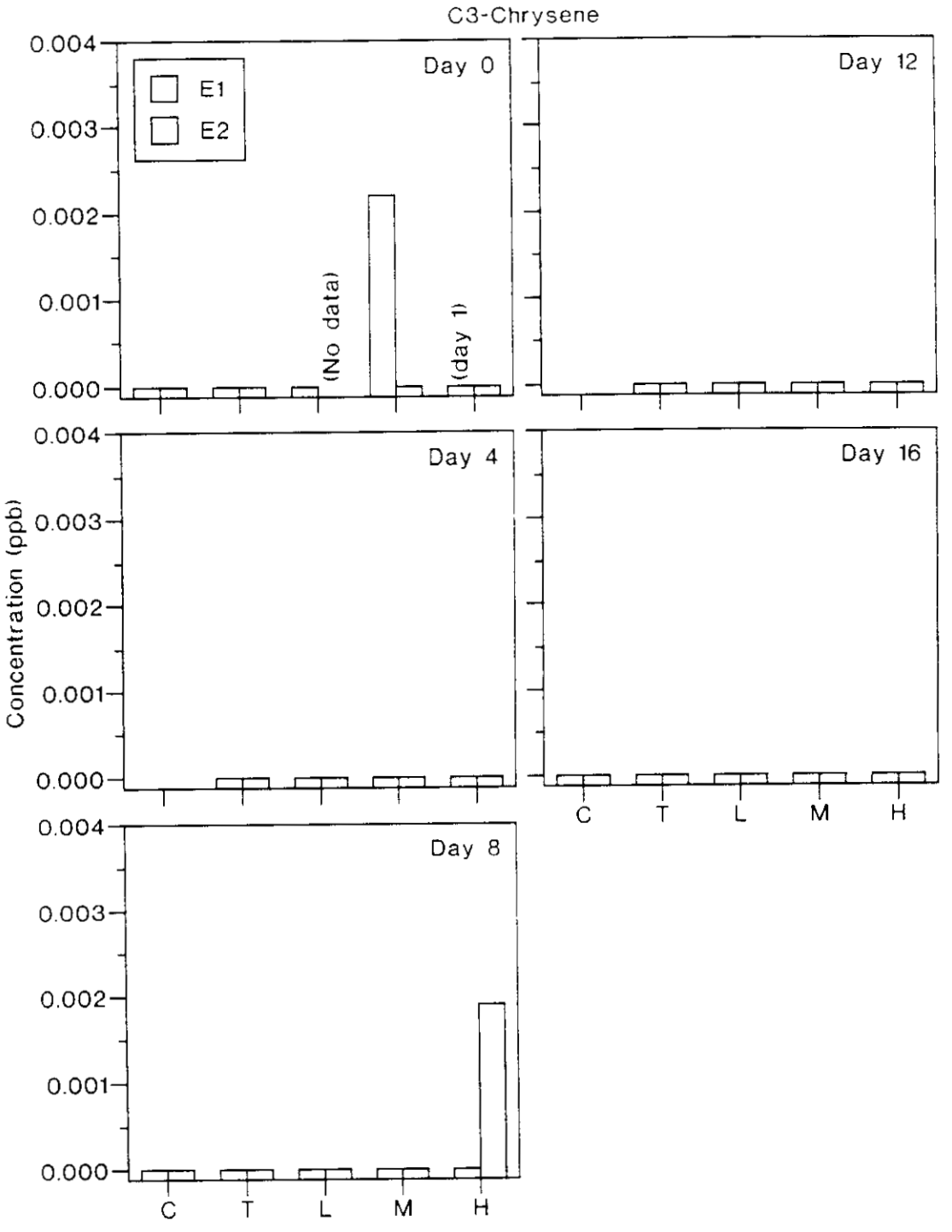
Appendix 4.3.19. Comparison of chrysene concentrations in experiments one (E1) and two (E2). Observation times were as indicated on graphs, except day 1 data were substituted for day 0 in the high-oil treatment of E1. The majority of symbols represent single observations; where present, error bars indicate means ± SE.



Appendix 4.3.20. Comparison of c1-chrysene concentrations in experiments one (E1) and two (E2). Observation times were as indicated on graphs, except day 1 data were substituted for day 0 in the high-oil treatment of E1. The majority of symbols represent single observations; where present, error bars indicate means ± SE.



Appendix 4.3.21. Comparison of C2-chrysene concentrations in experiments one (E1) and two (E2). Observation times were as indicated on graphs, except day 1 data were substituted for day 0 in the high-oil treatment of E1. The majority of symbols represent single observations; where present, error bars indicate means ± SE.



Appendix 4.3.22. Comparison of C3-chrysene concentrations in experiments one (E1) and two (E2). Observation times were as indicated on graphs, except day 1 data were substituted for day 0 in the high-oil treatment of E1. The majority of symbols represent single observations; where present, error bars indicate means ± SE.

Appendix 4.4

Composition of polynuclear aromatic hydrocarbons in egg tissue (1995)

Composition of polynuclear aromatic hydrocarbons (PAH) in eggs resulting from exposure of eggs during incubation to oil-contaminated water. Contaminant oil in experiment two (E2) was more weathered than that in experiment one (E1). In E1, there was weak evidence that PAH composition in egg tissue changed over time ($0.001 \leq P_{regression} \leq 0.513$), much as in water, but at a far slower rate. In E2 there was no evidence of a time-dependent change in PAH composition in egg tissue. Abbreviations of chemical compounds are found in Appendix 0.2.

Appendix 4.4.1. Composition of PAH in eggs exposed during incubation to the high-oil treatment, experiment 1 (1995).

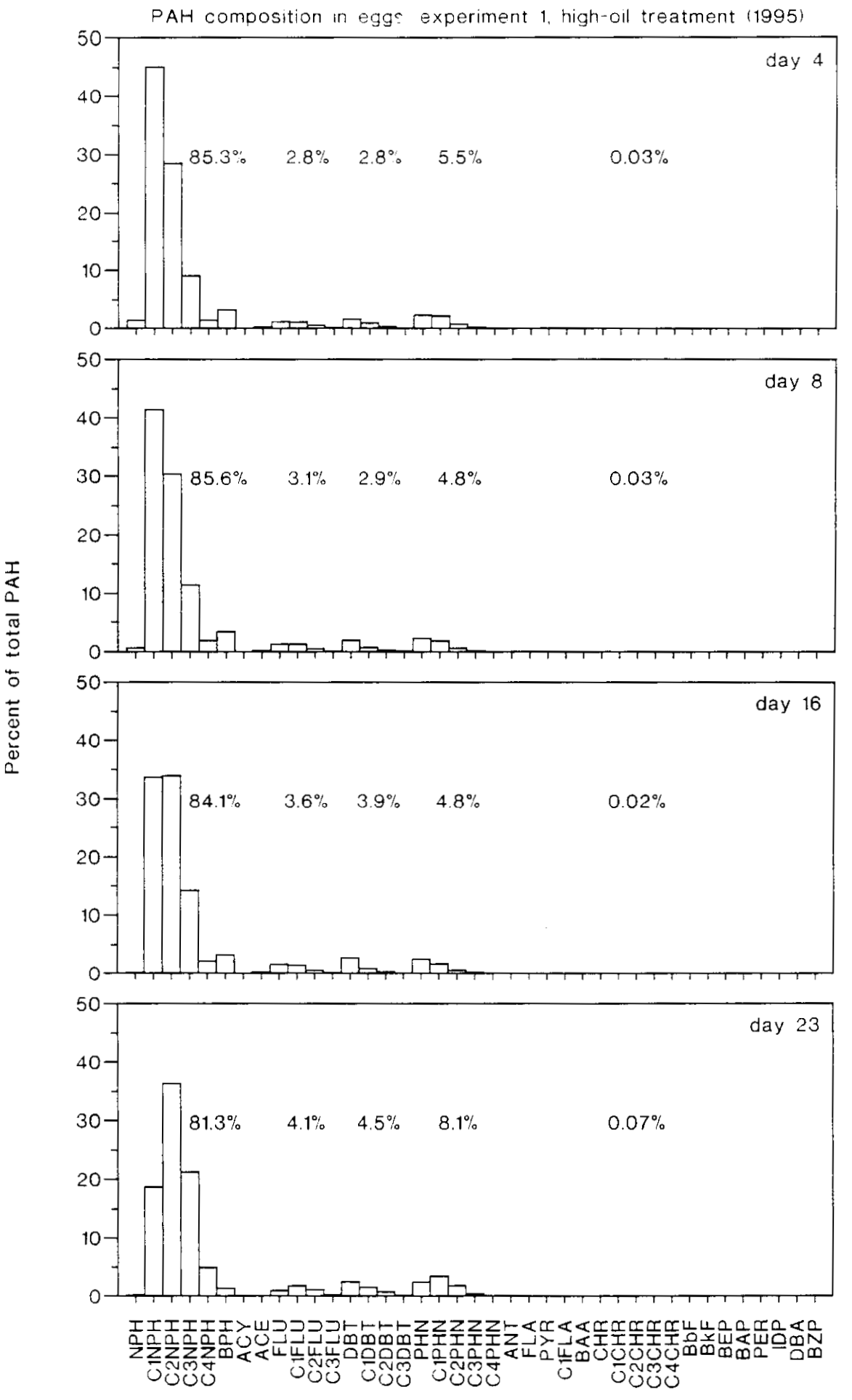
Appendix 4.4.2[1]. Composition of PAH in eggs exposed during incubation to the low-oil treatment, experiment 2 (1995).

Appendix 4.4.3. Composition of PAH in eggs exposed during incubation to the mid-oil treatment, experiment 2 (1995).

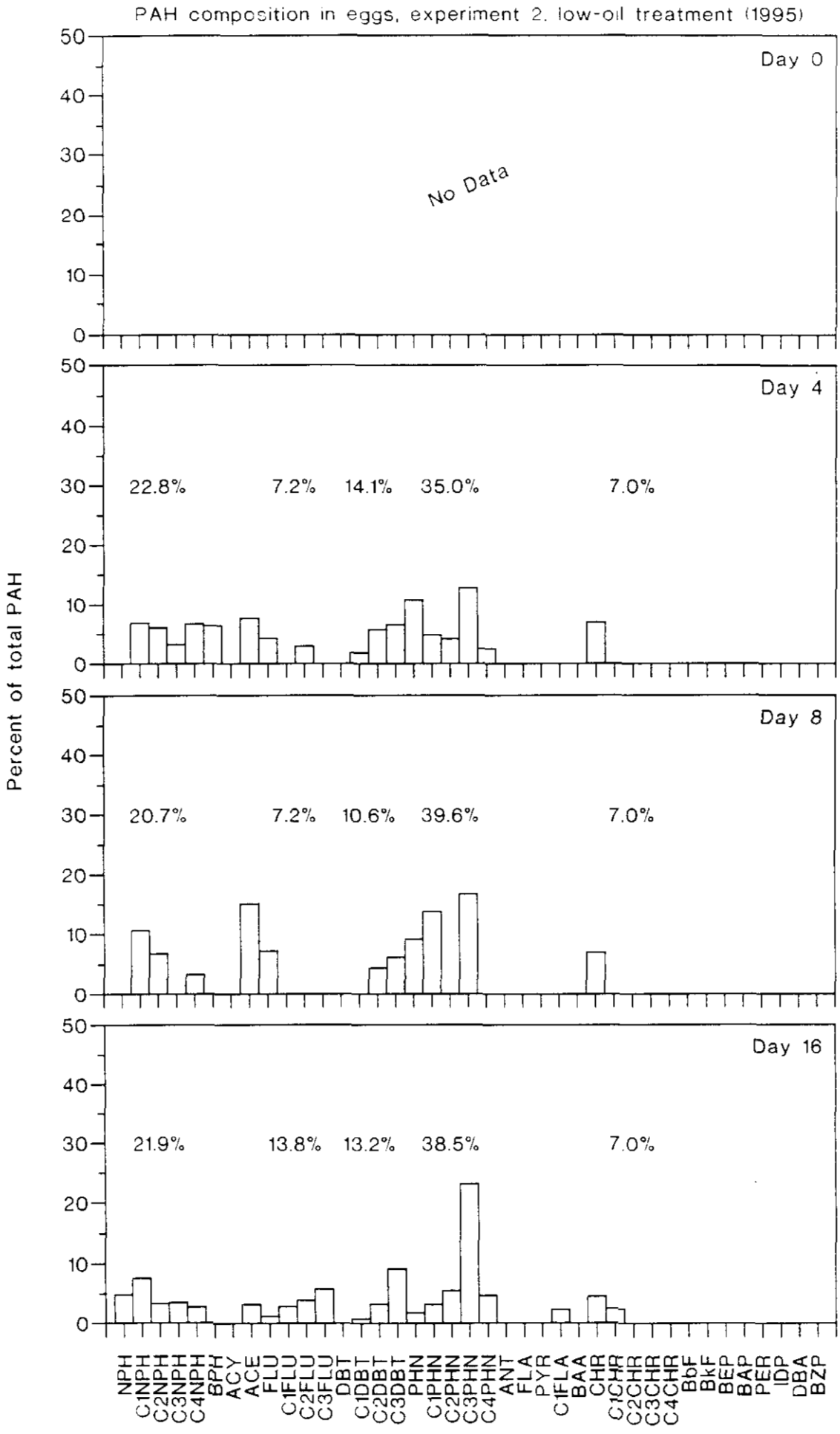
Appendix 4.4.4. Composition of PAH in eggs exposed during incubation to the high-oil treatment, experiment 2 (1995).

[1]Trace-oil composition was not reported because PAH concentrations were at or below detection limits.

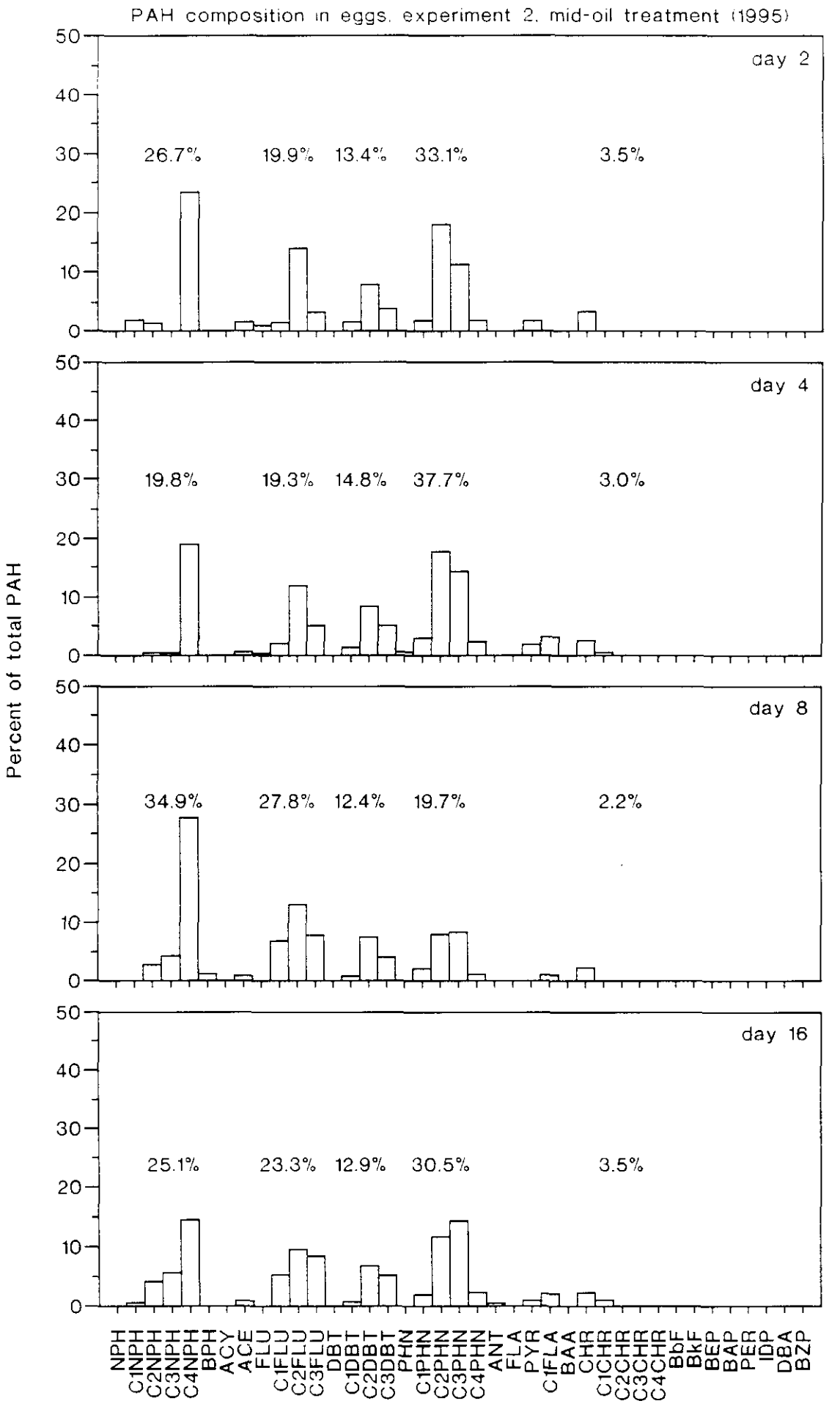
Appendix 4.4.1 *(at right).* Composition of PAH in eggs exposed during incubation to the high-oil treatment, experiment 1 (1995). Exposure continued for 16 d, then eggs were transferred to clean water. Percentages printed inside the graphs indicate total percentages of the following homologous chemical groups (left to right): naphthalenes, fluorenes, dibenzothiophenes, phenanthrenes, and chrysenes.


PAH composition in eggs experiment 1, high-oil treatment (1995)
Percent of total PAH
day 4
85.3%
2.8%
2.8%
5.5%
0.03%
day 8
85.6%
3.1%
2.9%
4.8%
0.03%
day 16
84.1%
3.6%
3.9%
4.8%
0.02%
day 23
81.3%
4.1%
4.5%
8.1%
0.07%
0
10
20
30
40
50
NPH
C1NPH
C2NPH
C3NPH
C4NPH
BPH
ACY
ACE
FLU
C1FLU
C2FLU
C3FLU
DBT
C1DBT
C2DBT
C3DBT
PHN
C1PHN
C2PHN
C3PHN
C4PHN
ANT
FLA
PYR
C1FLA
BAA
CHR
C1CHR
C2CHR
C3CHR
C4CHR
BbF
BkF
BEP
BAP
PER
IDP
DBA
BZP

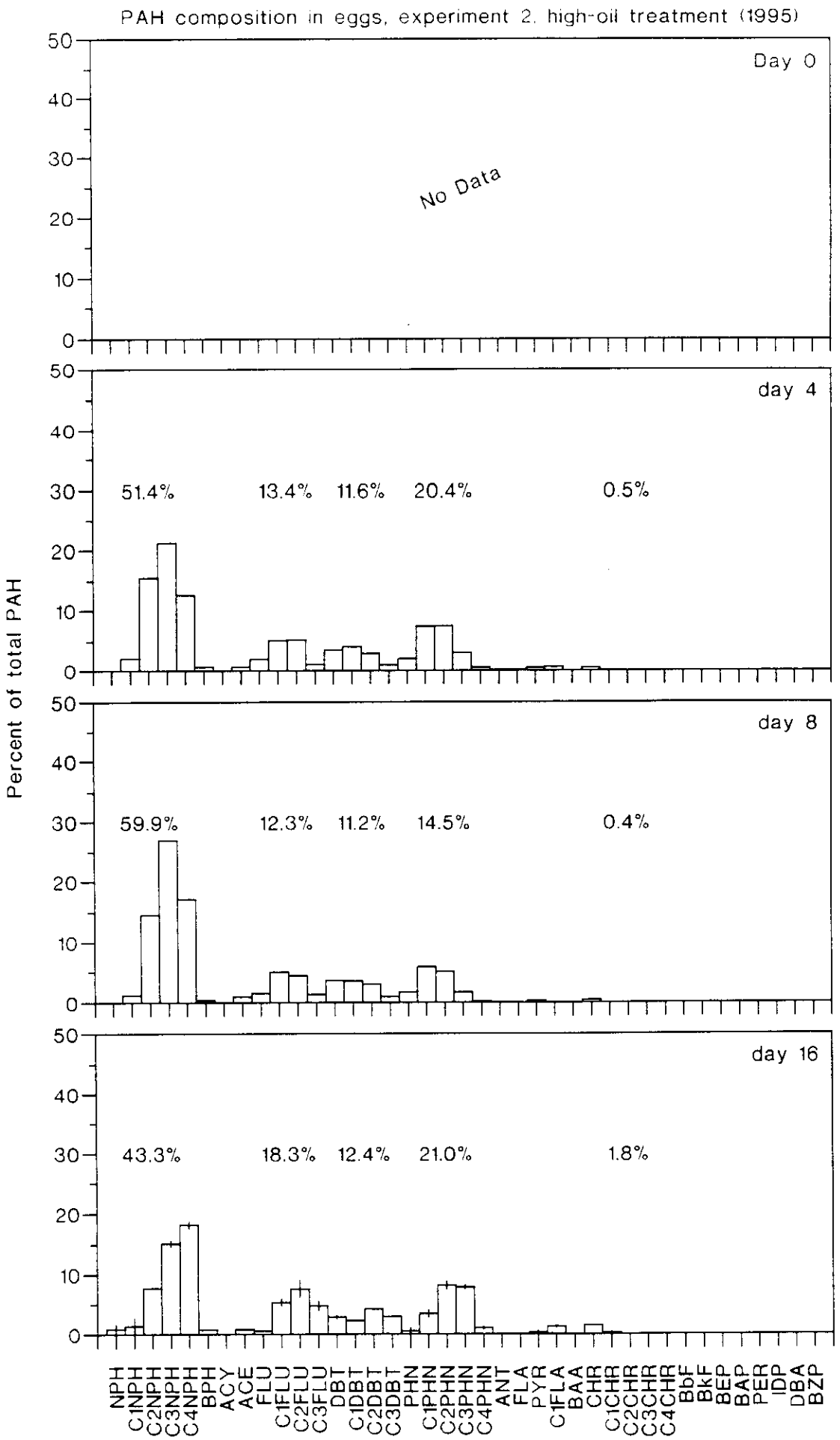
Appendix 4.4.2 *(at right).* Composition of PAH in eggs exposed during incubation to the low-oil treatment, experiment 2 (1995). Exposure continued for 16 d, then eggs were transferred to clean water. Percentages printed inside the graphs indicate total percentages of the following homologous chemical groups (left to right): naphthalenes, fluorenes, dibenzothiophenes, phenanthrenes, and chrysenes.


PAH composition in eggs, experiment 2, low-oil treatment (1995)
Percent of total PAH
Day 0
No Data
Day 4
22.8%
7.2%
14.1%
35.0%
7.0%
Day 8
20.7%
7.2%
10.6%
39.6%
7.0%
Day 16
21.9%
13.8%
13.2%
38.5%
7.0%
0
10
20
30
40
50
NPH
C1NPH
C2NPH
C3NPH
C4NPH
BPH
ACY
ACE
FLU
C1FLU
C2FLU
C3FLU
DBT
C1DBT
C2DBT
C3DBT
PHN
C1PHN
C2PHN
C3PHN
C4PHN
ANT
FLA
PYR
C1FLA
BAA
CHR
C1CHR
C2CHR
C3CHR
C4CHR
BbF
BkF
BeP
BAP
PER
IDP
DBA
BZP

Appendix 4.4.3 *(at right)*. Composition of PAH in eggs exposed during incubation to the mid-oil treatment, experiment 2 (1995). Exposure continued for 16 d, then eggs were transferred to clean water. Percentages printed inside the graphs indicate total percentages of the following homologous chemical groups (left to right): naphthalenes, fluorenes, dibenzothiophenes, phenanthrenes, and chrysenes.


PAH composition in eggs, experiment 2, mid-oil treatment (1995)
Percent of total PAH
day 2
26.7%
19.9%
13.4%
33.1%
3.5%
day 4
19.8%
19.3%
14.8%
37.7%
3.0%
day 8
34.9%
27.8%
12.4%
19.7%
2.2%
day 16
25.1%
23.3%
12.9%
30.5%
3.5%
0
10
20
30
40
50
NPH
C1NPH
C2NPH
C3NPH
C4NPH
BPH
ACY
ACE
FLU
C1FLU
C2FLU
C3FLU
DBT
C1DBT
C2DBT
C3DBT
PHN
C1PHN
C2PHN
C3PHN
C4PHN
ANT
FLA
PYR
C1FLA
BAA
CHR
C1CHR
C2CHR
C3CHR
C4CHR
BbF
BkF
BEP
BAP
PER
IDP
DBA
BZP

Appendix 4.4.4 *(at right).* Composition of PAH in eggs exposed during incubation to the high-oil treatment, experiment 2 (1995). Exposure continued for 16 d, then eggs were transferred to clean water. Data for day 16 are means of paired observations ± standard error. Percentages printed inside the graphs indicate total percentages of the following homologous chemical groups (left to right): naphthalenes, fluorenes, dibenzothiophenes, phenanthrenes, and chrysenes.


PAH composition in eggs, experiment 2, high-oil treatment (1995)
Day 0
No Data
day 4
51.4%
13.4%
11.6%
20.4%
0.5%
day 8
59.9%
12.3%
11.2%
14.5%
0.4%
day 16
43.3%
18.3%
12.4%
21.0%
1.8%
Percent of total PAH
0
10
20
30
40
50
NPH
C1NPH
C2NPH
C3NPH
C4NPH
BPH
ACY
ACE
FLU
C1FLU
C2FLU
C3FLU
DBT
C1DBT
C2DBT
C3DBT
PHN
C1PHN
C2PHN
C3PHN
C4PHN
ANT
FLA
PYR
C1FLA
BAA
CHR
C1CHR
C2CHR
C3CHR
C4CHR
BbF
BkF
BEP
BAP
PER
IDP
DBA
BZP

Appendix 4.5

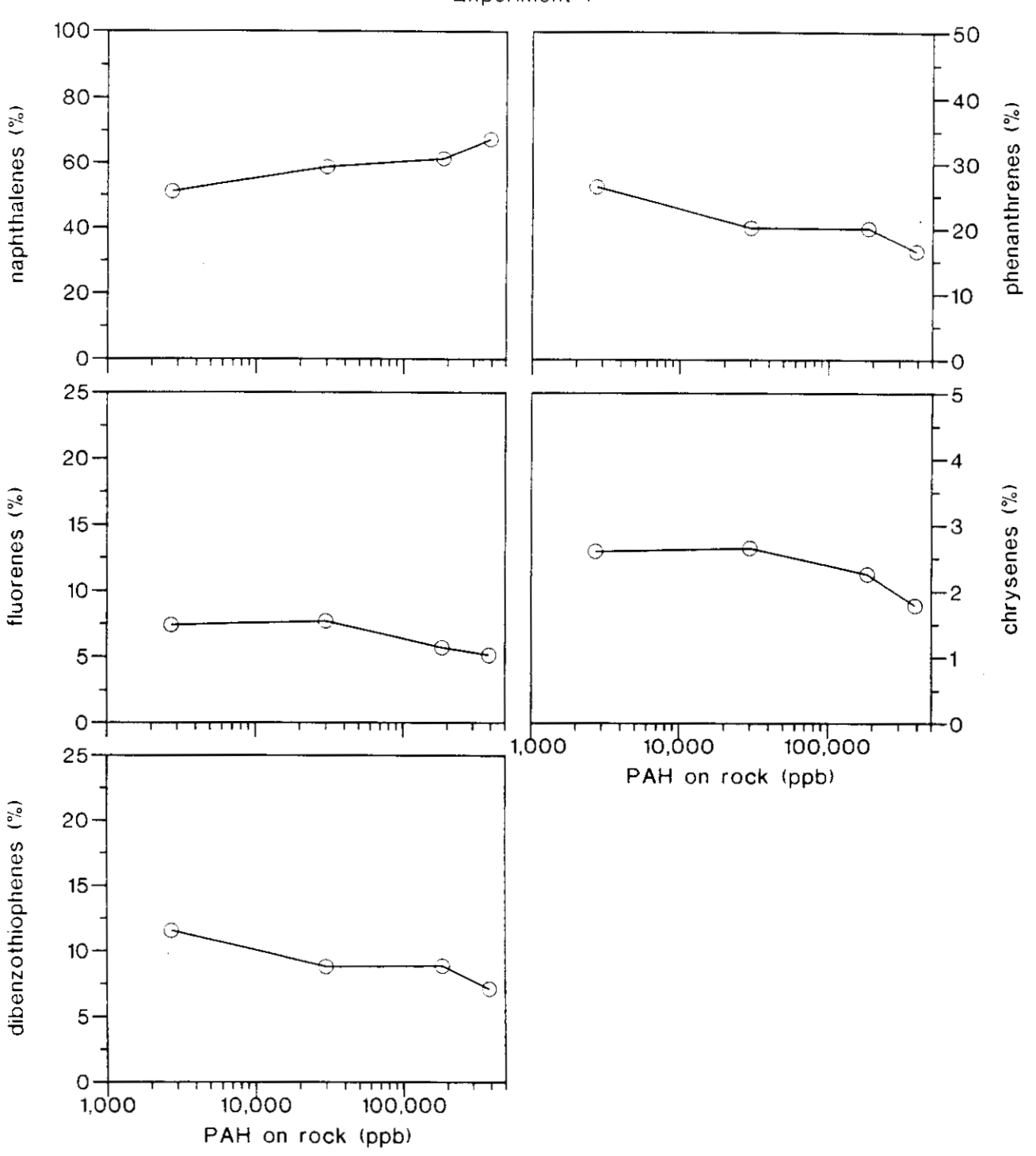
Polynuclear aromatic hydrocarbons in rock substrate of oil delivery apparatus (1995)

Weathering of polynuclear aromatic hydrocarbons (PAH) in the rock substrate of the oil delivery apparatus, and the relationship between PAH concentration and composition in rock substrate and those in water.

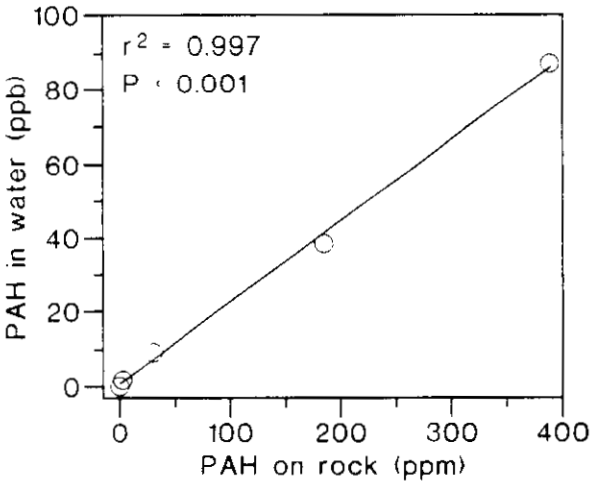
Appendix 4.5.1. Composition of PAH in rock as a function of PAH concentration in rock on day 2, experiment 1.

Appendix 4.5.2. Initial PAH concentration in treatment water and that measured in rock on day 2 (experiment 1) were linearly related ($r^2 = 0.997$, $P < 0.001$).

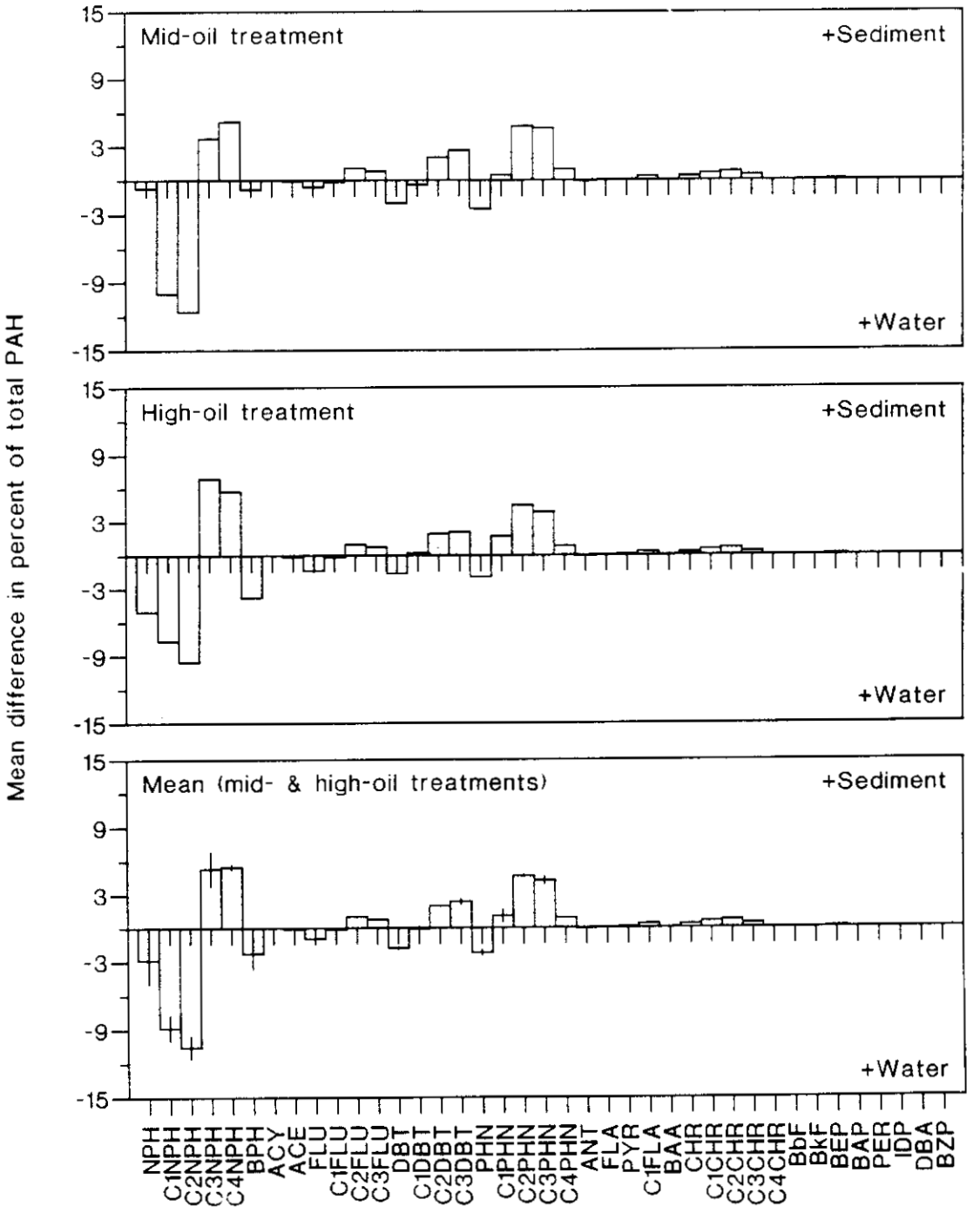
Appendix 4.5.3. Polynuclear aromatic hydrocarbon (PAH) composition in rock substrate and treatment water.



Appendix 4.5.1. Composition of PAH in rock as a function of PAH concentration in rock on day 2, experiment 1. As in water, percent naphthalenes increased with PAH concentration, and percentages of phenanthrenes and chrysenes declined. Compositional shifts were less pronounced in rock than in water (compare this figure to figure 2.2).



Appendix 4.5.2. Initial PAH concentration in treatment water and that measured in rock on day 2 (experiment 1) were linearly related ($r^2 = 0.997$, $P < 0.001$).



Appendix 4.5.3. Polynuclear aromatic hydrocarbon (PAH) composition in rock substrate and treatment water was compared in 1995 visually by subtracting percentages in water from those in rock. Individual comparisons were for mid- and high-oil treatments; a combined mid- and high-treatment graph was also prepared. Abbreviations of chemical compounds are found in Appendix 0.2.