EFFECTS OF BENZENE (A TOXIC COMPONENT OF PETROLEUM) ON SPAWNING PACIFIC HERRING, *CLUPEA HARENGUS PALLASI*

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ABSTRACT

When female Pacific herring were exposed to low (parts per billion) levels of benzene for 48 h just prior to their spawning, a significant reduction occurred in survival of ovarian eggs and resultant embryos and larvae through yolk absorption. The reduction in survival of ovarian eggs was approximately 10-25%, for embryos from fertilization to hatching, 26%, and for embryos and larvae through yolk absorption, 43%. Exposure to benzene also induced premature spawning and resulted in aberrant swimming behavior and disequilibrium in adults of both sexes.

The maximum accumulation of ^{14}C-labeled benzene and/or metabolites in ovarian eggs (14 times initial concentration in water in 24-48 h; 1.4 μl/g from 0.1 μl/liter) was greater than in later egg and larval stages as measured in other experiments.

Conservatively estimating the total reduction in survival in these experiments to be approximately 50% through yolk absorption, I surmise that the effect of exposing spawning herring to only one toxic component of petroleum could have a significant effect on the population. The fish in these experiments were exposed to relatively high parts per billion levels, but they were exposed for a relatively short period (48 h); it is probable that in the estuary, if chronically exposed over a longer period of time to low parts per billion levels of aromatic components, the populations could be seriously affected.

When the spawning female herring is compared with other life history stages, we find that the spawning stage is clearly the most sensitive of those tested. If fishes prove generally to be most sensitive to petroleum components during their spawning seasons, fishery management decisions should take this factor into consideration in protecting the resources.

In studies of pollutant effects on marine organisms, emphasis should be placed on critical or sensitive life history stages. With this in view, research on petroleum effects on fish has been directed more recently toward egg, embryo, and larval stages (Kühnhold 1969, 1972; Evans and Rice 1974; Struhsaker et al. 1974). Results in many studies revealed that fish egg and larval stages were surprisingly resistant to crude oil and water-soluble and aromatic fractions of crude oil. Some of this resistance in fish is probably attributable to the presence of enzymes for metabolic detoxification of components with ensuing rapid depuration and physiological homeostasis (Lee et al. 1972; Neff 1975; Korn, Hirsch, and Struhsaker 1976, footnote 2).

I have observed, as expected, that the effects of exposure of monoaromatics such as benzene are more severe at all life history stages if fishes are otherwise stressed by environmental extremes or are in poor "condition" from inadequate nutrition. On this basis it is suggested that the female at time of spawning may be the most sensitive stage to toxic oil components. In herring, for example, the fish often feed poorly for some time prior to spawning and have low fat and energy reserves associated with the production of eggs (Blaxter and Holliday 1963). Anadromous fishes or fishes such as herring which migrate into estuaries for spawning may also be exposed to environmental extremes, particularly to changes in salinity, which produce additional stress. Further, since aromatics are highly lipid-soluble, it might be expected that benzene would accumulate to high levels in ovarian eggs. These factors could lead to significant reductions in fecundity and serious consequences for populations over long chronic exposures.

The purpose of this experiment was to examine the effect of benzene on female Pacific herring, *Clupea harengus pallasi* Valenciennes, just prior to spawning. We have also studied benzene effects on other life history stages of the herring (Struhsaker et al. 1974; Korn et al. see footnote 2;

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[2]Korn, S., N. Hirsch, and J. W. Struhsaker. 1976. The uptake, distribution, and depuration of ^{14}C-benzene and ^{14}C-toluene in Pacific herring (*Clupea pallasi*). Unpubl. manuscr.

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Eldridge et al.[3]). So far as we know, there is no similar study, exposing fish just prior to spawning, for any oil component.

Benzene was selected for most of our studies on herring because of its relatively high proportion in the water-soluble fraction of crude oil and refined products (Anderson et al. 1974), high solubility in water and relative toxicity (Benville and Korn 1974, footnote 4; Korn, Struhsaker, and Benville 1976). Monoaromatics were tested individually rather than exposing fishes to the total oil or total water-soluble fraction in order to more specifically delineate physiological responses to a known toxic component.

Initial research on Pacific herring adults, eggs, and larvae was conducted with high (ppm level) concentrations of benzene (Struhsaker et al. 1974; Korn, Struhsaker, and Benville 1976). Because of the high volatility of benzene, such concentrations would probably occur only briefly after catastrophic incidents, such as tanker accidents and well blowouts. Subsequently, we tested levels in the low ppb (parts per billion) range as being more representative of chronic exposures and potentially more damaging over a long period to marine populations.

In this study, ripe male and female herring were exposed just prior to spawning to 100 nl/liter (ppb) and 800 nl/liter (ppb) benzene for 48 h. The ^{14}C-labeled benzene and its metabolites were measured in the ovaries to determine uptake, accumulation, and depuration. Exposure effects on behavior, the mortality of eggs in the gonads of females, and rate of delayed mortality in embryos at hatching and larvae through yolk absorption were also recorded.

METHODS

Pacific herring were captured 4 December 1974 during the spawning season in San Francisco Bay by a local bait dealer. The fish were captured with a lampara net and wet-brailed from the net into the vessel bait wells. The fish were transported immediately in the bait vessel to the Tiburon Laboratory dock and then transferred to 1,900-liter tanks in the laboratory. Fish were "running ripe" when captured. Because the purpose of these experiments was to expose fish prior to spawning, an acclimation period of only 24 h was allowed. Previous experience with ripe herring has shown that they usually spawn shortly after capture.

Fish were initially placed in circular tanks with double sand-filtered, open flow seawater at ambient conditions in the bay at the time. Initial handling mortality was negligible. During the experiment, conditions were as follows: salinity, 23.0-24.0‰; temperature, 10.0°-11.5°C; oxygen, 6.0-10.5 ppm. An ambient benzene concentration was undetectable at the ppb level. Since herring generally feed poorly when spawning, neither exposed nor control fish were fed during the experiment. The exposure treatments were as follows:

Control: 0 nl/liter (ppb) benzene; open flow system, no benzene exposure; approximately 100 fish (50 males, 50 females).

Exposed: 800 nl/liter (ppb) benzene, open flow system, constant exposure for 48 h; approximately 100 fish (50 males, 50 females).

Exposed: 100 nl/liter (ppb) ^{14}C-labeled benzene; static system, declining exposure, 48 h; 25 females only; linear decrease in benzene concentration to approximately 10% of initial concentration remaining at end of 48 h.

All benzene exposures were terminated and open flow reestablished in the 100 ppb static exposure tank at the end of 48 h. The static exposure of ^{14}C-labeled benzene was to determine the uptake, accumulation, and depuration of benzene in the gonads of females. The open flow constant exposure and control were primarily to establish morphological and mortality effects on the ovarian eggs and delayed effects on subsequent larval development and mortality.

The behavior of fish was observed before sampling. Subsamples of females were taken daily for 6 days—2 days during exposure and 4 days after. Fish were removed randomly until 10 females were obtained from the control and 800 ppb exposure conditions. Five females were removed daily from the static 100 ppb exposure. Concentrations of benzene in the water of all tanks were also measured daily.

Each female sampled was measured (standard length), weighed (wet weight), and the ovaries dissected out. The ovaries were also measured (total length) and weighed (wet weight); the left ovaries were examined microscopically, the right

[3]Eldridge, M. B., T. Echeverria, and J. W. Struhsaker. Manuscr. in prep. The effect of benzene on the energetics of Pacific herring (*Clupea harengus pallasi*) embryos and larvae.

[4]Benville, P., Jr., and S. Korn. Manuscr. in prep. The acute toxicity of six mono-cyclic aromatics to striped bass (*Morone saxatilis*) and bay shrimp (*Crago* sp.).

ovaries prepared for radiometric or gas chromatograph analyses. Methods of preparation for radiometric and chromatograph measurements are described elsewhere (Benville and Korn 1974; Korn, Hirsch, and Struhsaker 1976, see footnote 2). It should be emphasized that the radiometric technique measures total radioactivity and concentrations calculated may include metabolites of benzene as well as benzene itself.

Ovaries were examined under a dissecting microscope for developmental stage [Hjort's stage (Bowers and Holliday 1961)] and the presence of opaque dead or dying eggs, and the gross appearance (color and degree of deliquescence) was ranked. The maximum diameters of 10 eggs from the ovary of each female were measured and the eggs examined for abnormal development.

On day 3, after cessation of exposure, pieces of clean plastic screening were placed around the standpipe in the center of the 800 ppb and 100 ppb exposure and control tanks to provide substrate for spawned eggs. Males were placed with females in the 100 ppb tank. After spawning occurred, the screens were removed and eggs examined for developmental stage and mortality. Pieces of screen with 75 eggs on each (most in 4-cell stage) were cut apart. Pieces of screen were selected with a single layer of relatively separated eggs because previous experience showed reduced survival in dense egg clusters. Two pieces of screen with 75 eggs each were placed in each 8-liter rearing container (total of 150 eggs). There were five replicate containers for each treatment and control (total of 15 containers). Temperature during development was 11.0°-12.0°C, and salinity, 22.0‰. Other rearing conditions were as previously described (Struhsaker et al. 1974). Hatching occurred 10 days after fertilization, and percent survival at hatching was determined from three replicate counts of swimming larvae in each container and by counting the number of dead and abnormal embryos left on the screen. The screens were removed and surviving larvae fed the rotifer, *Brachionus plicatilis,* through the remainder of the experiment (past yolk absorption to larval day 7). Surviving larvae were counted and the percent survival through yolk absorption determined from the original egg number.

Data were analyzed, depending upon variables, with the methods of analysis of variance and covariance using University of California Biomedical programs, BMD 01V, 02V, and 03V (Dixon 1970).

RESULTS

No adult mortalities occurred during the 6 days of the experiment. Stress behavior was noted in exposed fish, particularly at the constant 800 ppb exposure. Definite distress was observed by the end of the first day, although oxygen levels were above saturation. Milling was disrupted, fish were gaping at the surface, and many exhibited disequilibrium. Even after cessation of exposure, stress behavior continued for the duration of the experiment. Control fish may also have been stressed by the capture conditions and the short acclimation period, but they exhibited none of the stress symptoms of exposed fish and milled normally.

Although behavior was abnormal in exposed fish, spawning occurred in the tanks. In fact, the stress from benzene exposure appeared to prematurely induce spawning. This is illustrated in Table 1 by the percentage of exposed fish which were spent (Stage VII) compared with control fish. At the end of the 6-day experimental period, 73% (100 ppb) and 70% (800 ppb) of the exposed fish were spent, compared with only 25% of the controls. The higher percentage of spent females in the 100 ppb static treatment than in the 800 ppb open flow treatment during the first 4 days may be a result of additional stress imposed by static conditions. At all treatments, most unspent ovaries were ripe (Stage VI); only 7-10% were immature (Stages III-V) (Table 1).

No changes in growth (as indicated by wet weight and length) were expected in females over the short experimental period. However, these measurements were taken to determine the similarity of fish between the treatments and to adjust effect of size on the differences in weights of ovaries between the treatments. Ovary length and weight and egg diameters were measured to determine if benzene uptake affected the growth or resorption of ovaries or eggs and to determine the ripeness or proximity to spawning. Data are summarized in Table 2. Egg diameter did not correlate with any other measurement variable. Analysis of variance revealed no significant difference ($P>0.25$) in egg diameter between 0 and 800 ppb benzene treatments. Since the size range of females varied somewhat between the two treatments (Table 2), analysis of covariance was used to compare the weights of females and ovaries between concentrations and days after adjustment for the effect of lengths (Table 3). No

TABLE 1.—Effects of benzene exposure on ovaries and eggs of Pacific herring.

Hours (Days)	Benzene concentration (nl/l; ppb)	No. of ovaries examined	Percent of eggs in stage[1] III-V Immature	VI Ripe	VII Spent	No. of ripe ovaries examined	Stages III-VI dead eggs No.	%
24	0	10	10	80	10	9	0	0
(1)	100	5	0	40	60	2	0	0
	800	9	40	49	11	8	0	0
48	0	10	0	90	10	9	0	0
(2)	100	5	0	20	80	1	1	100
	800	10	10	60	30	7	1	14
72	0	10	20	70	10	9	0	0
(3)	100	5	20	40	40	2	2	100
	800	9	0	56	44	6	6	100
96	0	10	0	70	30	7	1	14
(4)	100	5	20	20	60	1	1	100
	800	10	10	57	33	6	6	100
120	0	10	10	40	50	5	0	0
(5)	100	5	0	0	100	0-All spent	—	—
	800	9	0	0	100	0-All spent	—	—
148	0	10	0	60	40	6	0	0
(6)	100	5	0	0	100	0-All spent	—	—
	800	10	0	0	100	0-All spent	—	—
Totals	0	60	7	68	25	36	1	3
(6 days)	100	30	7	20	73	6	4	67
	800	57	10	20	70	24	13	54

[1]Hjort's stage; Bowers and Holliday (1961).

TABLE 2.—Mean and range of female standard length, wet weight; ovary length and wet weight; and maximum egg diameter for Pacific herring. Linear equation describes the regression of wet weights on lengths for both whole female fish and left ovaries. Sample size = 59 females; 59 ovaries (spent females excluded).

Benzene concentration (ppb)	Female: Standard length (X) Range (cm)	Mean (cm)	Female: Wet weight (Y) Range (g)	Mean (g)	Ovary (Stages III-VI): Total length (X) Range (cm)	Mean (cm)	Wet weight (Y) Range (g)	Mean (g)	Max egg diameter Range (mm)	Mean (mm)
0	16.8-22.4	19.3	76.8-239.6	136.8	7.7-11.5	10.4	6.7-30.8	18.2	1.20-1.50	1.30
800	16.4-21.5	18.7	75.3-189.6	120.3	7.5-14.3	9.3	6.3-26.5	13.6	1.20-1.56	1.30
Total	16.4-22.4	19.0	75.3-239.6	126.2	7.5-14.3	9.9	6.3-30.8	15.9	1.20-1.56	1.30
	Regressions[1]									
0	$\hat{Y} = -339.96+24.98X$				$\hat{Y} = -19.26+3.56X$					
800	$\hat{Y} = -267.50+20.89X$				$\hat{Y} = -12.84+2.90X$					

[1]Tests of significance between slopes (*b*) and elevations (*a*) of regressions showed no significant difference ($0.100<P<0.250$) between concentrations (Snedecor and Cochran 1967:432-436).

TABLE 3.—Analysis of covariance of wet weight on standard length of female, wet weight of ovary on wet weight of female, and wet weight on total length of ovary for Pacific herring. Analysis of ripe ovaries (Stage VI) only. Treatments: Concentrations (0 vs. 800 ppb); Days (1 to 4); 2×4=8 treatment combinations × 5 observations per treatment combination = 40.

Analysis of dependent variable (wet wt female) after adjustment for covariate (standard length female)						
Source of variation	df	SS	MS	*F* ratio[1]	Probability	
Between concentrations (C) (0 vs. 800 ppb)	1	5.2508	5.2508	0.24	$P>0.250$	NS[2]
Between days (D)	3	675.2348	225.0783	1.04	$P>0.250$	NS
Interaction (CD)	3	485.9035	161.9678	0.75	$P>0.250$	NS
Within cells	31	6,721.2742	216.8153			
Analysis of dependent variable (wet wt ovary) after adjustment for covariate (wet wt female)						
Source of variation	df	SS	MS	*F* ratio	Probability	
Between concentrations (C) (0 vs. 800 ppb)	1	0.6940	0.6940	0.13	$P>0.250$	NS
Between days (D)	3	2.5351	0.8450	0.16	$P>0.250$	NS
Interaction (CD)	3	19.4057	6.4686	1.21	$P>0.250$	NS
Within cells	31	165.5181	5.3393			
Analysis of dependent variable (wet wt ovary) after adjustment for covariate (total length ovary)						
Source of variation	df	SS	MS	*F* ratio	Probability	
Between concentrations (C) (0 vs. 800 ppb)	1	0.4585	0.4585	0.04	$P>0.250$	NS
Between days (D)	3	27.2532	9.0844	0.71	$P>0.250$	NS
Interaction (CD)	3	8.0860	2.6953	0.21	$P>0.250$	NS
Within cells	31	398.2616	12.8471			

[1]F 0.05=4.16, df=1,31; F 0.05=2.91, df=3,31.
[2]NS = not significant.

significant difference ($P>0.25$) between concentrations or days or interaction was found. Tests between slopes (b) and elevations (a) of the regression lines of weights on lengths of females and weights on lengths of ovaries (Snedecor and Cochran 1967:432-436) showed no significant differences ($P>0.10$) between 0 and 800 ppb concentrations (Table 2).

Microscopic examination of the ovaries, however, revealed the presence of dead eggs in ovaries of exposed fish by the second day of exposure (Table 1). No dead eggs were found in control fish until day 4, and then only a few (15-20 eggs) in one female, the rest of the ovary appearing normal. Ovaries of exposed fish contained significantly larger numbers of opaque dead eggs (more than 10%) and were generally paler yellow and deliquescent. By the end of 6 days, 67% (100 ppb) and 54% (800 ppb) of exposed females were found with ovaries containing dead or dying eggs.

The uptake and depuration of benzene in ovaries of females exposed to a static initial concentration of 100 nl/liter (ppb) ^{14}C-labeled benzene is shown in Figure 1, together with data determined from other larval studies for later stages (Eldridge, Struhsaker, and Echeverria[5]). Uptake was rapid, so that a maximum accumulation (1.4 μl/g; ppm) was reached in 24 h. This level was maintained through the 48-h exposure period. After open flow was reestablished and exposure ended, benzene and/or metabolites were depurated until they reached an undetectable level in 96 h. The figure shows that levels accumulated in ovarian eggs were higher and sustained longer than in later egg and larval stages from other experiments with comparable exposure conditions.

Results of rearing experiments with eggs from females exposed to 0 and 800 ppb unlabeled benzene are summarized in Tables 4 and 5. Survival was also reduced in eggs and larvae from females exposed to an initial concentration of 100 ppb labeled benzene. However, results were obscured by an additional variable. Eggs taken from the static exposure tank were covered by filamentous bacterial growth early in development and many eggs died as a result. In the other treatment with open flow and in controls, eggs did not undergo this mortality due to epifloral growth.

[5]Eldridge, M. B., J. W. Struhsaker, and T. Echeverria. Manuscr. in prep. The uptake, accumulation and depuration of ^{14}C-labeled benzene in embryos and larvae of Pacific herring (*Clupea harengus pallasi*).

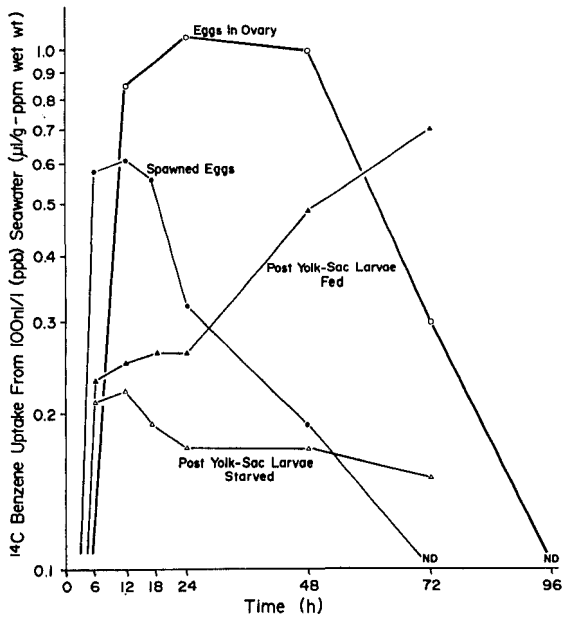

FIGURE 1.—Accumulation of ^{14}C-labeled benzene in different early Pacific herring developmental stages exposed to an initial concentration of 100 nl/liter (ppb) in a static system. Concentrations shown on y-axis were calculated from total radioactivity and may include metabolites derived from benzene as well as benzene. Spawned eggs were in a stage just prior to blastopore closure; post yolk-sac larvae were fed the rotifer, *Brachionus plicatilis*, containing high accumulated levels of labeled benzene. ND = not detectable.

The 100 ppb treatment, therefore, was not included in the analysis. Analysis of variance showed survival at hatching and survival of larvae through yolk absorption were significantly less in exposed eggs (800 ppb) than in control eggs ($P<0.1$; Table 5). Exposure to ppb benzene levels for only 48 h reduced survival by about 43% through yolk absorption to larval day 7 (Table 4).

DISCUSSION

When female herring were briefly exposed to low levels of benzene for 48 h just prior to spawning, a significant reduction occurred in survival of eggs and resultant larvae from the ovary through yolk absorption. It is probable that further mortality would have occurred in later larval stages if the experiments were continued. When this result is compared with that from exposing other life history stages after spawning (Struhsaker et al. 1974; Eldridge et al. see footnote 5) where survival is not affected except at ppm levels, it appears that the spawning female and ovarian eggs are the most sensitive stages.

TABLE 4.—Mean percent survival through hatching and yolk absorption of Pacific herring larvae from eggs of benzene-exposed and control females.

Stage	Benzene concentration (nl/l; ppb)	Total no. of eggs	Mean survival (%)	95% confidence interval (%)	Mean reduction survival[1] (%)
Embryos to hatching	0	750	92.9	91.5-94.3	
	800	750	66.6	64.1-69.1	−26.3
Hatched larvae through yolk absorption	0	750	76.7	74.5-78.9	
	800	750	34.4	32.0-36.8	−43.3

[1]See Table 5 for test of significance.

TABLE 5.—One-way analysis of variance in survival of Pacific herring embryos to hatching and larvae through yolk absorption (larval day 7). Ripe females exposed prior to spawning. Five replicate containers per treatment; 150 eggs/container. (Arcsin transformation applied to percent survival data.)

Percent survival to hatching					
Source of variation	df	SS	MS	*F* ratio	Probability
Between concentrations 0 vs. 800 ppb	2	1.3442	0.6721	95.6098**	$P<0.01$
Within groups	12	0.0843	0.0070		
Total	14	1.4285			
Percent survival through yolk absorption					
Source of variation	df	SS	MS	*F* ratio	Probability
Between concentrations 0 vs. 800 ppb	2	0.8053	0.4026	30.2147*	$P<0.05$
Within groups	12	0.1599	0.0133		
Total	14	0.9652			

Although male herring were not studied in detail here, their behavior was severely disrupted, as in the females. Testes of mature, spawning herring have been found to contain higher levels of cholesterol (a lipid) during spawning than at other times in their adult life (Blaxter and Holliday 1963), and it is possible the lipid-soluble benzene may accumulate to high levels in testes of ripe males. Effects on males and their spermatozoa, as well as effects on females, may have contributed to reduction in survival of fertilized eggs through yolk absorption in these experiments.

Reference to Figure 1 shows that the maximum accumulation of labeled benzene in ovarian eggs was greater than in later egg and larval stages as measured in other experiments. Accumulation in ovarian eggs of exposed females was approximately twice that in eggs exposed just after spawning and prior to blastopore closure and about six times that in embryos exposed just after yolk-sac absorption. Accumulation for the first 48 h of water column exposure in these stages appears to correlate with the yolk volume of the eggs and larvae, decreasing as yolk is utilized, as would be expected with lipid-soluble benzene. However, the decreased accumulation may also relate to the development of enzymes enabling later stages to metabolize benzene and subsequently depurate more rapidly. After being fed *Brachionus plicatilis*, which accumulate high levels of benzene (Echeverria[6]), the fish larvae rapidly accumulated benzene from their food (Figure 1). Other studies of accumulation in tissues of adult herring (Korn et al. see footnote 2) show that only one site, the gall bladder with bile, accumulates higher concentrations than ovarian eggs (30 times and 14 times initial concentration, respectively).

I have noted previously (Struhsaker et al. 1974) that the percentage survival of eggs through hatching is significantly less (approximately 25% less; $P<0.01$) in Pacific herring eggs collected from San Francisco Bay than in those from Tomales Bay. Although other environmental differences may be involved, this reduction in hatching success may well relate to the effects of accumulated pollutants in the gonads of spawning fish in the relatively more polluted San Francisco Bay waters and warrants further study.

Estimating that the reduction in survival of eggs through yolk absorption of spawning exposed females is at least 43%, the effect on Pacific herring populations exposed to only one toxic component of petroleum could be significant. Considering that the total water-soluble fraction contains many other toxic aromatics, it is possible

[6]Echeverria, T. Manuscr. in prep. Uptake and depuration of ^{14}C benzene in the rotifer, *Brachionus plicatilus*.

that long-term chronic exposures to low levels may be decreasing population survival in polluted areas. In addition, chlorinated hydrocarbons in pesticides may also be accumulating in the gonadal lipids and interacting with petroleum hydrocarbons producing even more deleterious effects. More studies of the effects of these components on spawning fish are clearly needed. If fishes prove generally to be the most sensitive to accumulated oil components during their spawning season, fisheries management decisions should take into consideration their protection from damaging levels, particularly at spawning time.

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