DETERMINATION OF ASSIMILATIVE CAPACITY: IMPACT OF CONTAMINANTS ON REPRODUCTION AND MICRONUCLEUS FORMATION OF MARINE FISHES

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FINAL REPORT ON GRANT NO. NA85ABD00003

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Southern California Coastal Water Research Project Authority, A Public Agency
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May 16, 1988

Dr. Alan J. Mearns
Ocean Assessment Division N/OMA 32X2
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7600 Sand Point Way NE
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Dear Alan:

Enclosed are two copies of the final report for NOAA Grant No. NA85ABD00003.

Sincerely,

Jeffrey N. Cross, Ph.D.
Senior Environmental
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PROJECT SUMMARY

The objectives of this project were: 1) to determine the reproductive success of important commercial and sport fish living in contaminated coastal waters off Los Angeles, and 2) to evaluate the incidence of micronuclei in peripheral erythrocytes as an indicator of exposure to environmental contamination and a predictor of reproductive dysfunction.

We have shown: 1) reduced reproductive success (fecundity, fertilization success, early oocyte growth) of white croaker (Sciaenidae: Genyonemus lineatus) with ovary DDT concentrations above 4 mg/kg (wet weight); 2) a marked effect of the reproductive cycle on tissue contaminant concentrations and liver histology; and 3) the frequency of micronuclei in peripheral erythrocytes was inversely correlated with fertilization success.

The final report contains two published papers, one manuscript in review, and one technical report on the data collected during the NOAA-funded study. The first paper was presented at the Fourth International Symposium on Responses of Marine Organisms to Pollutants at Woods Hole, Massachusetts in April 1987, and is in press in Marine Environmental Research. The second paper is a manuscript submitted to Environmental Pollution and is in review. The research covered in these publications was also presented at SETAC Annual Meeting in Alexandria, Virginia in November 1986. The third paper appeared in Marine Environmental Research (Vol. 22, 1987) and was presented at the California Water Pollution Control Association Annual Conference in San Deigo in April 1987, and also at the Aquatic Habitat Institute Seminar Series in San Francisco in November 1987. The fourth paper appeared in the SCCWRP 1986 Annual Report; an expanded version of this research is being prepared for publication.

The results of this study were very encouraging. With NOAA sponsorship (Grant No. NA87ABD00003), we are continuing our research on reproductive success of coastal fishes, and the utility of micronuclei as an indicator of chronic exposure to environmental contamination.

Marine Environmental Research, In Press

Evidence for Impaired Reproduction in White Croaker (Genyonemus lineatus) from Contaminated Areas off Southern California

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ABSTRACT

Thousands of tons of contaminants were dumped into the coastal waters off Los Angeles in the last 40 years. Contaminant exposure has been implicated in the declines in catches of several sport and commercial fishes. Laboratory spawning studies demonstrated that white croaker (Sciaenidae: Genyonemus lineatus) inhabiting contaminated areas near Los Angeles had higher chlorinated hydrocarbon body burdens, greater early oocyte destruction and preovulatory atresia, lower batch fecundities, and lower fertilization rates than fish from a reference area 80 km away.

The coastal waters off Los Angeles receive a large portion of the domestic and industrial wastes generated by 8 million people. Contaminant exposure has been implicated in the declines in catches of several sport and commercial fishes. The objective of this study was to determine if reproduction was impaired in white croaker (Sciaenidae: Genyonemus lineatus), an important sport and commercial species inhabiting contaminated areas near Los Angeles.

White croaker were collected from San Pedro Bay (SPB), a contaminated site near Los Angeles, and Dana Point (DP), a reference site 80 km to the southeast. Females were induced to spawned in the laboratory with human chorionic gonadotropin. Eggs from each female were fertilized with sperm pooled from at least three males from the same site.

Forty-one percent of SPB females and 54% of DP females were induced to spawn; the difference was not significant (Chi-square, $p>0.25$). Ovaries of non-spawning females were catheterized and oocytes were staged microscopically. All of the non-spawning DP fish (n=13) had hydrated oocytes indicating that the oocytes were maturing and spawning was imminent. Twenty-seven percent of the non-spawning SPB fish (n=30) had hydrated oocytes; the remaining fish had only yolky oocytes and were unresponsive to the gonadotropin injections.

Chlorinated hydrocarbon concentrations were higher in livers and gonads of white croaker from SPB (Table 1).

Females from SPB produced fewer eggs per spawn and had lower fertilization rates (Table 1).

To control for potential differences in the timing of oocyte maturation between sites, numbers of early oocytes were compared among females at the beginning of the reproductive season (October). Fish from SPB had fewer early oocytes and a higher proportion of early oocytes undergoing atresia (Table 1).

Destruction of early oocytes should be evident in the population as early cessation of reproduction or decreased fecundity among older individuals.[1] Based on regressions of batch fecundity against female size, the predicted number of eggs for a large female (200 g) was 70,900 (SE=64,400) from SPB and 122,200 (SE=28,100) from DP.

Ovarian DDT concentrations of spawning SPB croakers (X=2.1 ppm, SD=0.9, n=19) were less than ovarian concentrations of fish from the general population in the bay (X=4.3 ppm, SD=1.5, n=16) (U test, p=0.025). None of the spawning fish had ovarian levels greater than 3.8 ppm while 38% of the fish from the general population had higher levels. This suggests that fish with levels above 4 ppm total DDT did not spawn. A gonadal threshold of 3 ppm was determined in hatchery studies on salmonids.[2,3]

Ovarian PCB concentrations were not different between spawning SPB fish (X=1.7 ppm, SD=1.0) and fish from the general population in the bay (X=1.5, SD=0.8) (U test, p>0.05; Fig. 4). With the exception of one study,[4] PCB body

burdens in excess of those measured in this study appear necessary to cause impaired reproduction.

Although reductions in reproductive success are correlated with body burdens of total DDT, it is probably not solely responsible for the observed effects since other contaminants (polycyclic aromatic hydrocarbons and metals) occur at high concentrations in SPB sediments and fishes.[5]

The mechanisms of reproductive toxicity are not completely understood but may include modulation of hormone levels essential for oocyte maturation and ovulation, toxicity to developing gametes or nutritive cells, and generalized stress responses.[6,7,8,9] Effects similar to those observed in this study (primordial oocyte destruction, preovulatory atresia, and decreased fecundity and fertility) were reported in mammals after laboratory exposures to polycyclic aromatic hydrocarbons and in fishes after laboratory exposures to certain chlorinated hydrocarbons.[1,10,11]

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TABLE 1

Contaminant body burdens (mg/wet kg) and reproductive success of female white croakers collected during December and January (1985-86) and spawned between January and March (1986). Data are mean + standard deviation (sample size). All site comparisons with Mann-Whitney U test.

	San Pedro Bay	Dana Point	Significance
DDT - Liver	1.52 + 0.77 (19)	0.17 + 0.07 (8)	$p<0.001$
DDT - Ovary	2.10 + 0.85 (19)	0.31 + 0.18 (8)	$p<0.001$
PCB - Liver	1.35 + 1.34 (19)	0.03 + 0.06 (8)	$p<0.001$
PCB - Ovary	1.67 + 1.02 (19)	0.16 + 0.08 (8)	$p<0.001$
Number eggs spawned (x1000) per female	67.4 + 62.8 (21)	104.5 + 32.0 (9)	$p<0.01$
Percent fertilization	80 + 16 (21)	93 + 3 (6)	$p<0.05$
Early oocytes/field	1.5 + 0.6 (6)	2.7 + 0.8 (6)	$p<0.01$
Percent atretic	15.0 + 8.8 (6)	2.1 + 2.4 (6)	$p<0.01$

Environmental Pollution, In Review

REPRODUCTIVE IMPAIRMENT IN A FISH INHABITING A CONTAMINATED COASTAL ENVIRONMENT OFF SOUTHERN CALIFORNIA

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ABSTRACT

White croaker (Genyonemus lineatus), collected from a highly contaminated site in San Pedro Bay and from a reference site 80 km away (Dana Point), were induced to spawn in the laboratory. Forty-one percent of San Pedro Bay females and 54% of Dana Point females spawned. Examination of the ovaries of non-spawning females revealed that spawning was imminent in the remainder of Dana Point fish but only in 16% of the San Pedro Bay fish. The remainder of the San Pedro Bay fish (43%) contained only immature, yolky oocytes. No croakers containing more than 3.8 ppm ovarian total DDT could be induced to spawn whereas 36% of a contemporaneous San Pedro Bay sample had ovarian total DDT residues in excess of 4 ppm. This suggests that the inability to induce spawning in white croaker may be associated with an ovarian total DDT threshold of about 4 ppm. These data, coupled with observed decreases in fecundity (32%), fertility (14%), and early oocyte loss (30%) relative to reference fish, could partially explain the population declines observed for many southern California fishes since the 1940's.

INTRODUCTION

Several of the major sport and commercial fish stocks in the coastal waters off southern California declined substantially over the last 40 years (Bell, 1971; Oliphant, 1979). Coastal waters near Los Angeles have received significant inputs of chlorinated organic hydrocarbon (CHC) contaminants, notably DDT and PCBs. An estimated 2400 metric tons (mt) of DDT wastes were discharged or dumped into San Pedro Bay between 1947 and the early 1970's; approximately 40 mt of DDT and 50 mt of PCB were discharged from 1971 to 1983 (Schafer, 1984; Chartrand et al., 1985; Brown et al., 1986). Because the decline in fishery stocks was temporally related to CHC discharges, it has been suggested that exposure to these contaminants contributed to reduced fecundity and/or larval survival of sportfishes such as white seabass (Atractoscion nobilis) and kelp bass (Paralabrax clathratus) (Young, 1963; Vojkovich & Reed, 1983).

Large numbers of white croaker (Sciaenidae:Genyonemus lineatus), a species related to the white sea bass, live close to the sediment-water interface in degraded habitats near Los Angeles sewage discharges (Love et al., 1984). White croaker was the principal sportfish landed by fisherman on piers in the Los Angeles area in the early 1980's (Puffer et al., 1982). Over 700,000 pounds were landed commercially at Los Angeles area ports in 1984 (NMFS, 1985). Human consumption warnings were posted by California Department of Health Services in early 1985 at several popular sportfishing locations near Los Angeles because of high CHC levels in white croaker muscle (Gossett et al., 1982;

California Dept. Fish & Game, 1987). Consequently, commercial landings dropped to 84,000 pounds in 1986 (NMFS, 1987). The effect on sport landings of white croaker is not known.

Female white croaker are batch spawners, spawning 18-24 times during the season (November to April). Peak spawning occurs in February and March (Love et al., 1984). During autumn, hepatic DDT and PCB stores are mobilized into the maturing gonads. Peak gonadal CHC concentrations occur in October with mean total DDT and PCB concentrations of 4.9 ppm and 1.1 ppm wet weight, respectively (Cross & Hose, 1986). Because high frequencies of preovulatory atresia were observed in small benthic fishes from San Pedro Bay which also contain high CHC residues (Cross et al., 1988), the relationship between reproductive impairment in white croaker and environmental exposure to chlorinated hydrocarbons was investigated.

MATERIALS AND METHODS

Fish were collected by hook-and-line from San Pedro Bay and the reference area, Dana Point. Only females with total lengths > 19 cm (the length at which 100% of the population is sexually mature [Love et al., 1984]) were returned to the laboratory. Following a one week acclimation period, 79 females (28 from Dana Point and 51 from San Pedro Bay) were injected with 1 IU/g body weight human chorionic gonadotrophin (HCG). Forty hours later, eggs were manually stripped and fertilized with pooled milt (n > 3) from the respective sites. Variability in fertilization success in marine fish is not influenced by sperm characteristics, provided they are motile (Spies & Rice, 1988). Fertilization success was estimated 12 hours later as the percentage of eggs exhibiting a visible perivitelline space. Six Dana Point fish which released only immature hydrated oocytes (identified by the presence of multiple oil globules) were not used for fertilization success determinations. Average weights of San Pedro Bay croaker were not significantly different from those of Dana Point fish. Ovaries of females that did not spawn were catheterized and 200 oocytes were staged microscopically (Wallace & Selman, 1981; Hunter & Macewicz, 1985).

To ensure that site-specific differences in fecundity were not due to variations in the timing of oocyte maturation, numbers of early oocytes (which do not undergo cyclic atresia) were compared. Histological sections of ovaries were prepared from six fish from each site collected in October, the onset of the reproductive season (Love et al., 1984). This time point was

chosen to reduce the substantial variability in ovarian CHC concentrations after spawning commenced (Cross & Hose, 1986). Numbers of viable and atretic early oocytes (oogonia, primordial oocytes plus primary oocytes not yet entering primary growth) (Gulyas & Mattison, 1979; Mattison, 1980) were counted in five high power (400X magnification) fields. Because of individual differences in reproductive state, numbers of early oocytes were standardized to 25 total oocytes per field. Standardized early oocyte counts were compared using a Mann-Whitney test.

Total DDT and PCB concentrations were measured individually in the livers and ovaries of females spawning between November 1985 and January 1986 (Gossett et al., 1982). The ovaries consisted primarily of stromal tissue containing unspawned immature oocytes and occasionally, unspawned maturing oocytes. Samples (1 to 5 g) were homogenized in a 20 ml aliquot of pesticide-quality acetone, filtered and re-extracted into n-hexane. The hexane fraction was prepared by cleaning on activated Florisil (750^{o}C for 4 hrs). Analysis for CHC was performed using a Tracor MT220 gas chromatograph equipped with an electron capture detector and a 1.8 m x 2 mm ID glass column packed with 1.5% OV17 + 1.95% QF1 on 80 to 100 mesh Gaschrom Q. Column temperature was 200^{o}C with a nitrogen flow of 20 ml/min. Total DDT measurements were comprised of approximately 85% DDE isomers, a lesser percentage of DDT isomers and trace amounts of DDD isomers. Total PCB measurements usually represented Aroclor 1254 equivalents; rarely trace amounts of Aroclor 1242 equivalents were detected.

RESULTS

Forty-one percent of San Pedro Bay females (21 individuals) and 54% of Dana Point females (15 individuals) were induced to spawn; the difference is not significant (Chi-square, $p > 0.25$). Females from San Pedro Bay produced a mean of 67,400 eggs, significantly less than the mean of 104,500 for Dana Point fish (Mann-Whitney U-test, $p < 0.01$) (Fig. 1). Based on regressions of the number of eggs spawned against body weight, the predicted number of eggs spawned by an average size female (182 g) from San Pedro Bay was 75,000 + 14,900 ($\hat{Y}$ + SE) compared to 111,900 + 9,100 for the same size female from Dana Point. Batch fecundity estimates are the only useful fecundity measurement for batch spawners such as white croaker (Hunter et al., 1985).

Fertilization success was also significantly lower among San Pedro Bay fish (X = 80%) compared to fish from Dana Point (X = 93%) (Mann-Whitney U-test, $p < 0.05$). All of the non-spawning Dana Point fish (n = 13) contained hydrated oocytes, indicating that oocytes were maturing and spawning was imminent (Hunter et al., 1985). Of the non-spawning San Pedro Bay fish, eight (16%) contained hydrated oocytes and 22 (43%) had only yolky oocytes which were unresponsive to HCG induction.

Livers of San Pedro Bay croakers averaged 1.5 ppm total DDT wet weight and 1.4 ppm total PCB wet weight (Table 1) with hepatic CHC concentrations at least an order of magnitude lower in Dana Point fish. Mean total DDT and PCB concentrations in the ovaries of San Pedro Bay croakers were 2.1 ppm and 1.7 ppm, respectively. Ovarian DDT concentrations were not correlated

with liver measurements of San Pedro Bay fish, although ovarian and hepatic PCB concentrations were significantly correlated ($r = 0.88$, $p < 0.05$). CHC measurements of San Pedro Bay fish spawning during January-March were compared to those measured in the general population of fishes caught at the same time. Ovaries of spawning fishes contained an average of only 1.9 ppm total DDT (SD = 0.7, n = 15), significantly less than the mean of 4.4 ppm (SD = 3.5, n = 14) measured in the ovaries of the general population (Mann-Whitney U-test, $p < 0.05$) (Fig. 2). Thirty-five percent of the fish from the general population had ovarian total DDT concentrations greater than 3.3 ppm (the upper 95% confidence limit of DDT concentrations in spawners); 43% had levels greater than 3.0 ppm. The latter figure is identical to the percentage of fish which were unresponsive to hormone induction. None of the fish spawned in this study (n=19, tissues of two fish were unavailable for CHC analysis) had ovarian DDT concentrations greater than 3.8 ppm. Distributions of ovarian total PCB were similar between the spawning group (X = 1.5 ppm, SD = 1.0, n = 15) and the general population (X = 1.8 ppm, SD = 1.1, n = 14).

Ovarian sections of San Pedro Bay fish contained fewer early oocytes than did Dana Point samples (Mann-Whitney U-test, $p < 0.01$). Atresia was rarely observed in early oocytes of reference fishes (2.1% of all those examined) while 15.0% of the early oocytes from San Pedro Bay fish were atretic (Chi-square, $p < 0.01$). Mean numbers of viable early oocytes were 2.6 (SD=0.5) and 1.3 (SD=0.8) for Dana Point and San Pedro Bay, respectively. Because the finite nature of early oocytes has not been established for fish as it has for mammals (Hunter et al., 1985),

the consequences of the observed atresia are unclear. Nevertheless, manifestations of early oocyte destruction might include premature cessation of reproduction or decreased fecundity in older individuals (Mattison et al., 1980). The data in Fig. 1 suggest a size-related decrease in the fecundity of croakers from San Pedro Bay. Three-quarters of fish weighing 200 g or more spawned under 70,000 eggs, approximately 50% of the average number spawned by similarly-sized reference fish.

DISCUSSION

Our results indicate that the reproductive success of an important sport and commercial fish is impaired in a highly contaminated area off southern California. Contaminant-related reproductive impairment in a benthic flatfish, the starry flounder (Platichtys stellatus), was recently reported from San Francisco Bay (Spies & Rice, 1988). Although the authors of that study mentioned that some of the flounder failed to spawn following their hormone induction regimen, chemical analyses of those fish were not conducted. Data from white croaker suggest that ovarian total DDT concentrations above a threshold of 4 ppm prevents spawning in white croakers, a figure similar to the previously reported LC50 for salmon eggs, 2.9 ppm (Burdick et al., 1964; 1972). Other researchers have observed DDT-dependent larval mortality in rainbow trout at ovarian DDT concentrations of 7.1 ppm (Hopkins et al., 1969), winter flounder (Pseudopleuronectes americanus) between 2.0 and 4.6 ppm DDT in eggs (Smith & Cole, 1973), and in spotted sea trout (Cynoscion

nebulosus) at 8.0 ppm DDT in eggs (Butler et al., 1972). In contrast, a recent study by Hansen et al. (1985) found that viable hatch of Baltic herring (Clupea harengus) was reduced at ovary DDE concentrations >18 ppb. With the exception of a single study by the same group (von Westernhagen et al., 1981), ovarian PCB burdens in excess of those measured in croakers from the contaminated site appear necessary to elicit reproductive impairment or larval mortality (Hogan & Brauhn, 1975; DeFoe et al., 1978; Sivarajah et al., 1978; Monod, 1985).

It is likely that DDT is not solely responsible for observed reproductive effects since many other contaminants (such as polycyclic aromatic hydrocarbons and metals) found at high concentrations in San Pedro Bay water and fishes (Brown et al., 1986; Malins et al., 1986) are generally acknowledged to produce reproductive toxicity (Mattison et al., 1980; Horning & Neiheisel, 1979; Hose et al., 1981). Contaminant interactions through induction of bioactivating enzyme systems, synergism or differential localization within subcellular compartments probably modulate the toxicity of CHC (Freeman & Idler, 1975; Brown et al., 1982). The mechanisms involved in reproductive toxicity are not completely understood but are thought to include modulation of hormone levels essential for oocyte maturation and ovulation, toxicity to developing gametes or nutritive cells, and generalized stress responses (Saxena & Garg, 1979; Truscott et al., 1983; Spies & Rice, 1988;). Effects similar to those described here (destruction of early oocytes, preovulatory atresia, decreased fertility and fecundity) were induced following laboratory exposure to polycyclic aromatic

hydrocarbons in mammals (Mattison et al., 1980) and to certain CHC in fishes (Buckler et al., 1981; Nagler et al.., 1986).

Survival and normal development of fish embryos and larvae are dependent not only on gametic body burdens of contaminants but on ambient water contamination as well. Like many commercially important fishes, white croaker release positively-buoyant, pelagic eggs which develop in upper surface waters. Surface waters from San Pedro Bay are highly toxic to pelagic fish eggs and larvae and the toxicity is related to total CHC (DDT plus PCB) concentrations (Cross et al., 1988).

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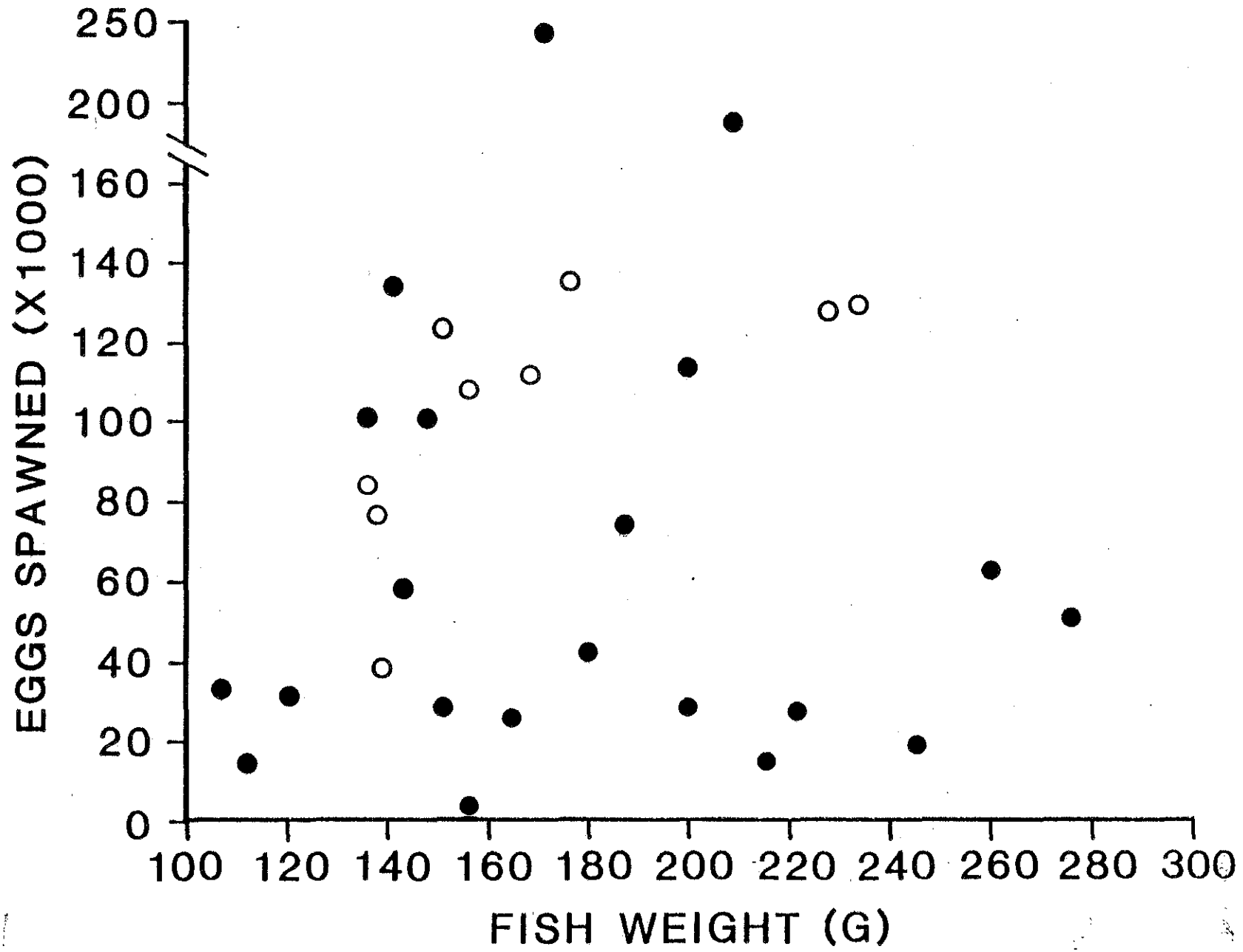
FIGURE LEGENDS

Figure 1. Relationship between fecundity and fish weight in white croaker induced to spawn with HCG. ● = San Pedro Bay, ○ = Dana Point.

Figure 2. Distributions of ovarian total DDT (Fig. 2A) and PCB (Fig. 2B) in San Pedro Bay white croaker spawned in the laboratory ▨ compared to the general population of females trawled ▧ in January through March.


EGGS SPAWNED (X1000)
250
200
160
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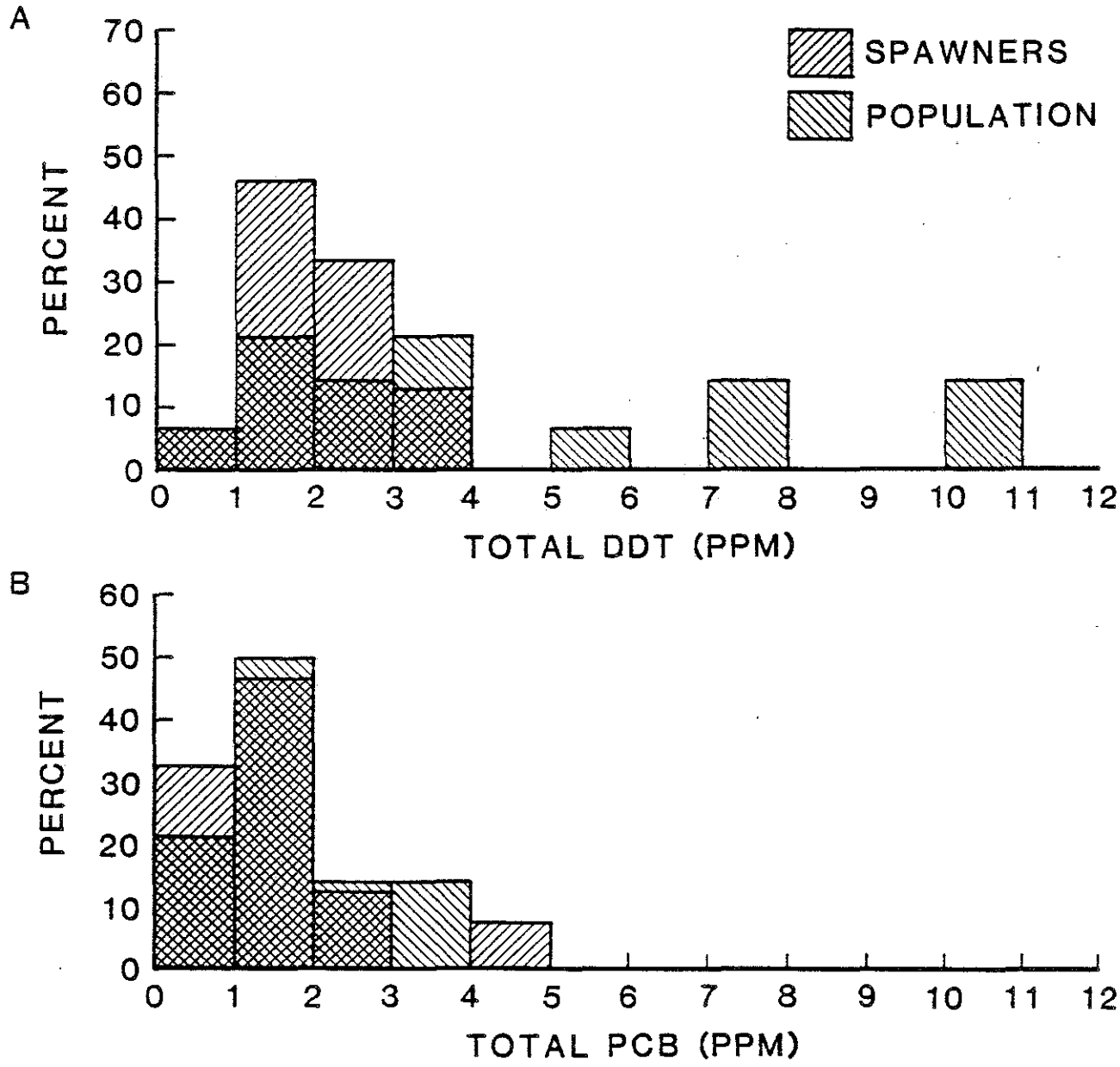
100 120 140 160 180 200 220 240 260 280 300
FISH WEIGHT (G)


A
70
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SPAWNERS
POPULATION
0 1 2 3 4 5 6 7 8 9 10 11 12
TOTAL DDT (PPM)
B
60
50
40
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PERCENT
0 1 2 3 4 5 6 7 8 9 10 11 12
TOTAL PCB (PPM)

TABLE 1

Hepatic and Ovarian Contaminant Concentrations in White Croaker from San Pedro Bay and the Less Contaminated Reference Site, Dana Point. (Values are in ppm wet weight.)

	San Pedro Bay		Dana Point	
Organ	Total DDT X + SD (n)	Total PCB X + SD (n)	Total DDT X + SD (n)	Total PCB X + SD (n)
Liver	1.52+0.77(19)	1.35+1.34(19)	0.17+0.07(8)	0.03+0.06(8)
Ovary	2.10+0.85(19)	1.67+1.02(19)	0.31+0.18(8)	0.16+0.08(8)

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Elevated Circulating Erythrocyte Micronuclei in Fishes from Contaminated Sites off Southern California

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ABSTRACT

*Frequencies of circulating erythrocyte micronuclei in two marine fish species from contaminated areas off southern California were elevated relative to fishes from less contaminated sites. Micronuclei frequencies from contaminated sites were four times higher in white croaker (*Genyonemus lineatus*) and eleven times higher in kelp bass (*Paralabrax clathratus*). The increased micronuclei frequency was related to previously determined environmental concentrations of chlorinated hydrocarbons (DDTs and PCBs) and polycyclic aromatic hydrocarbon metabolites. However, micronuclei frequency was only weakly correlated to individual body burdens of chlorinated hydrocarbons in white croaker as determined in this study. Applications and limitations of piscine micronucleus measurements are discussed.*

INTRODUCTION

One primary goal of aquatic toxicology is the development of animal screening techniques that measure environmental contamination. Among the most commonly used techniques are chemical contaminant, biochemical, cytogenetic and histopathological analyses (Dixon, 1985). One of the most promising, inexpensive and rapid screening techniques suitable for

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evaluating exposure to contaminants for marine and freshwater fishes is the micronucleus test (Landolt & Kocan, 1983). Micronuclei are smaller, secondary nuclei formed following chromosomal breakage (Schmid, 1976). Although micronuclei may arise spontaneously, induction of micronuclei is commonly used to detect genotoxic damage resulting from mutagen exposure (Heddle *et al.*, 1983). Originally developed as a test using rodent polychromatic erythrocytes in bone marrow and later extended to circulating erythrocytes (MacGregor *et al.*, 1980), the micronucleus test has also been applied to nucleated piscine erythrocytes (Hooftman & de Raat, 1982; Hose *et al.*, 1984).

Although all the causes of micronucleus formation in fish are not known, micronuclei have been induced following continuous exposure to ethyl methanesulfonate (Hooftman & de Raat, 1982) and benzo(a)pyrene (Hose *et al.*, 1984). In both cases the incidence of micronucleus formation was dose-dependent. Hooftman & de Raat (1982) observed the presence of additional anomalies such as the loss of the usual elliptical shape of the nucleus and the appearance of irregular Feulgen-positive structures in the cytoplasm. Results from these two studies indicate that the piscine micronucleus test may be a simple and rapid alternative to the more routinely used cytogenetic tests such as the occurrence of chromosomal aberrations and sister chromatid exchanges. Since most marine fishes have numerous small chromosomes, cytogenetic methods that require visualization of individual chromosomes are difficult and time-consuming (Landolt & Kocan, 1983). The micronucleus test is applied independent of karyotypic characteristics and is equally suitable for any fish species.

The objective of this study was to determine if the incidence of micronuclei in circulating erythrocytes was elevated in fishes from a highly contaminated environment. White croaker (Sciaenidae: *Genyonemus lineatus*) and kelp bass (Serranidae: *Paralabrax clathratus*) were chosen for this study because they are important local sportfishes (Fitch & Lavenberg, 1971; Puffer *et al.*, 1982) and they respectively occupy increasing trophic levels in the marine food web (Mearns & Young, 1980). White croaker reside near the sediment–water interface and consume a variety of benthic and epibenthic organisms. Because white croaker from portions of San Pedro and Santa Monica bays have high concentrations of DDTs and PCBs (Brown *et al.*, 1982, 1986), human consumption warnings have been posted in several areas. Kelp bass live in kelp beds and rocky reefs and feed on small fish, including young croaker, and invertebrates; frequently they contain high concentrations of chlorinated hydrocarbons (Gossett *et al.*, 1982).

In this study, frequencies of erythrocyte micronuclei were compared between fishes collected from the highly contaminated San Pedro Bay and from less contaminated sites away from metropolitan Los Angeles. The San

Pedro Bay–White Point area received enormous amounts of DDT from municipal wastewater discharge until the early 1970s (Schafer, 1984). Current levels of DDT and PCB in sediment exceed those measured in Commencement Bay, Washington (Brown *et al.*, 1986), a federal Superfund site (NOAA, 1986). San Pedro Bay also receives significant inputs of polycyclic aromatic hydrocarbons from natural oil seeps, shipping activity and the Los Angeles River (Gossett *et al.*, 1983).

METHODS

Fishes were collected by hook-and-line from contaminated sites near metropolitan Los Angeles and from reference sites 40 to 80 km away (Fig. 1). White croaker were obtained from outer Los Angeles Harbor in San Pedro Bay and off Dana Point. Kelp bass were collected from White Point on the Palos Verdes Peninsula and off the west end of Santa Catalina Island.

Fishes were taken to the laboratory and acclimatized for two to three weeks prior to sampling. They were anaesthetized with methyl tricaine sulfonate. Using cardiac puncture, approximately 1 cc of blood was

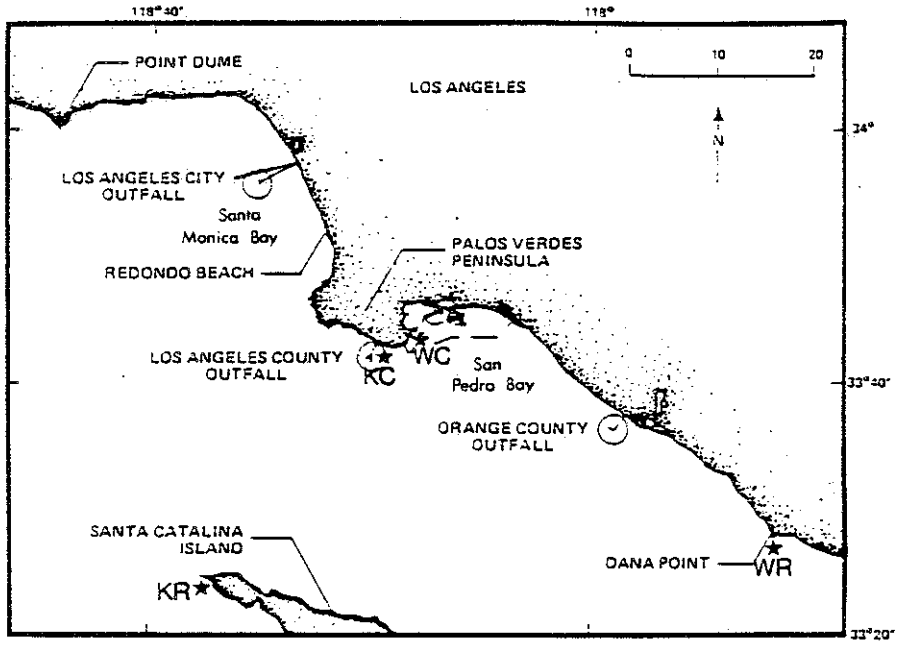

Fig. 1. Map of fish collection sites in southern California. WC = contaminated site (outer Los Angeles Harbor) for white croaker; WR = reference site (Dana Point) for white croaker; KC = contaminated site (White Point) for kelp bass; KR = reference site (Catalina Island) for kelp bass. Scale bar is calibrated in miles.

withdrawn into a heparinized 1-cc tuberculin syringe fitted with a 21-G needle. Blood smears were immediately prepared and fixed in absolute methanol for 15 min. Smears were then stained with May-Grunwald Giemsa.

Blood smears were examined microscopically under high (1000×) power. Numbers of micronucleated erythrocytes (MN) per 1000 erythrocytes were determined on coded slides and scored using blind review by a single observer. The number of micronucleated erythrocytes was the average of two determinations and was expressed as MN‰.

Selected fishes were analyzed for total DDT (DDE + DDD + DDT) and total PCB (Aroclor 1242 + Aroclor 1254). Methods for chlorinated hydrocarbon contaminant analysis follow Gossett *et al.* (1983). Liver samples (1 to 5 g) were homogenized in a 20-ml aliquot of pesticide-quality acetone, filtered and re-extracted into *n*-hexane. The hexane fraction was prepared by cleaning on activated Florisil (750°C for 4 h). Analysis for chlorinated hydrocarbons was performed using a Tracor MT220 gas chromatograph (GC) equipped with an electron-capture detector and a 1·8 m × 2 mm i.d. glass column packed with 1·5% OV17 + 1·95% QF1 on 80 to 100 mesh Gaschrom Q. Column temperature was 200°C with a nitrogen flow of 20 ml/min.

RESULTS

Frequencies of MN were elevated fourfold in white croaker from San Pedro Bay and elevenfold in kelp bass from White Point compared to less contaminated sites (Table 1). The raw data were transformed to $\log_{10}(x+1)$ (where $x = \text{MN‰}$) because the data fit a negative binomial distribution (variance greater than the mean) and the variances of the contaminated fishes were significantly greater than those of the reference fishes. *T*-tests of the log-transformed data showed that the incidences of MN were significantly higher in white croaker and kelp bass from contaminated sites than those of the reference sites (white croaker: $t = 6{\cdot}384$, df = 54, $p < 0{\cdot}001$; kelp bass: $t = 7{\cdot}511$, df = 28, $p < 0{\cdot}001$). Micronuclei counts were significantly higher in kelp bass than in white croaker at the contaminated sites ($t = 2{\cdot}743$, df = 41, $p < 0{\cdot}01$) but not at the reference sites ($t = 0{\cdot}155$, df = 41, $p > 0{\cdot}50$). There was no difference between MN frequencies in male and female white croaker at either site.

Piscine erythrocytes are elliptical with central elliptical nuclei (Fig. 2). Micronuclei observed in this study usually ranged in diameter from 1/20 to 1/10 of that of the erythrocyte nucleus; rarely MN approaching 1/3 the size of the parent nucleus were found. Staining characteristics of nuclei and MN

TABLE 1

Frequency of Micronucleated Erythrocytes in Peripheral Circulation of Fishes from Contaminated and Reference Sites in Southern California (values are the mean number of micronucleated erythrocytes per 1000 cells (MN‰); SD = standard deviation; n = sample size)

Species	*Contaminated site*[a] *(MN‰ + SD (n))*	*Reference site*[b] *(MN‰ + SD (n))*
White croaker	3·4 ± 2·7 (28)	0·8 ± 1·1 (28)
$\log_{10}$-transformed	0·59 ± 0·24 (28)	0·19 ± 0·22 (28)
Kelp bass	6·8 ± 5·1 (15)	0·6 ± 0·6 (15)
$\log_{10}$-transformed	0·81 ± 0·27 (15)	0·18 ± 0·16 (15)

[a] Contaminated site for white croaker = San Pedro Bay; contaminated site for kelp bass = Palos Verdes Peninsula.

[b] Reference site for white croaker = Dana Point; reference site for kelp bass = Catalina Island.

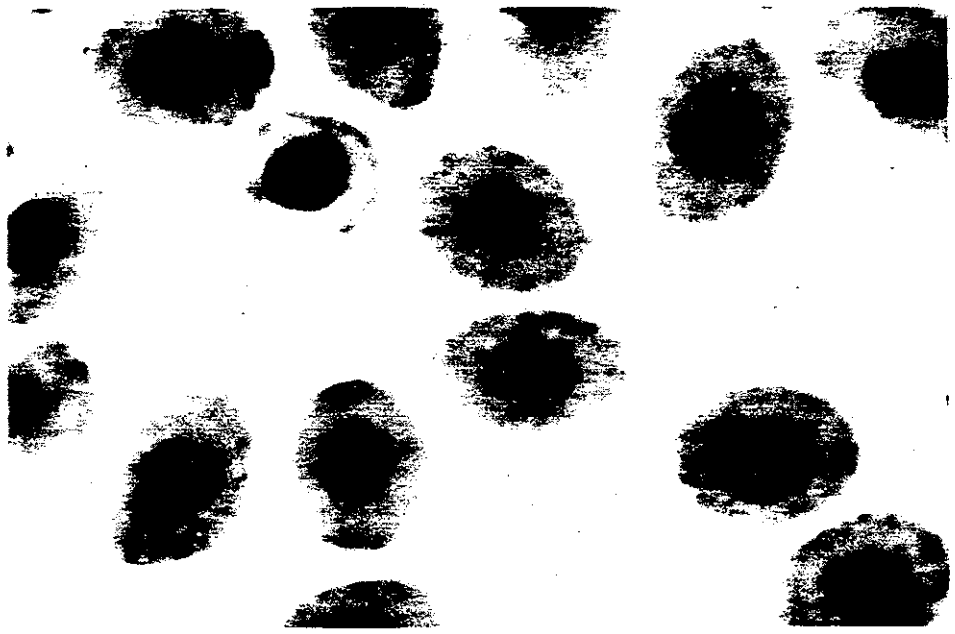

Fig. 2. . Erythrocytes from white croaker (*Genyonemus lineatus*) caught at reference site off Dana Point, California. Nuclei are elliptical and rarely contain micronuclei. May-Grunwald Giemsa. 3000 × .

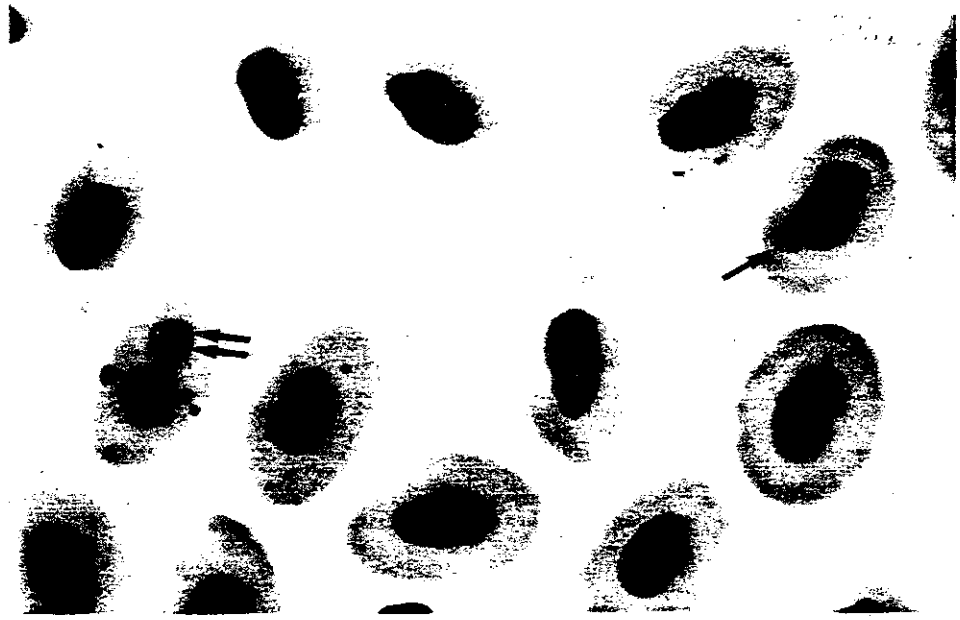

Fig. 3. Micronucleated erythrocytes (arrows) of white croaker from contaminated San Pedro Bay site. Micronuclei are visible as attached, knoblike structures (single arrow) or large detached secondary nuclei (double arrow). Note extreme nuclear pleomorphism in this fish. Small dark dots in cytoplasm are bacteria. They are easily distinguished from isolated micronuclei because of their refractile nature and their location on the cell surface. Bacteria were identified using a Gram stain. May-Grunwald Giemsa. 3000 ×.

are similar. Two types of MN were observed: attached, knoblike nuclear segments (Fig. 3) resembling those described by Hooftman & de Raat (1982) and isolated nuclear fragments identical to MN in mammalian cells. Although the attached nuclear fragments do not conform to the classical definition of a micronucleus in a mammalian cell (Schmid, 1976), they are quantifiable manifestations of genotoxicity in lower vertebrates (Hooftman & de Raat, 1982) and hence have been included in the MN counts conducted in this study. MN frequencies in reference fishes, averaging from 0·6 to 0·8‰, are lower than the spontaneous MN frequency of 1 to 3‰ reported for mice polychromatic erythrocytes (Heddle *et al.*, 1983). Nuclear pleomorphism, also mentioned by Hooftman & de Raat (1982), was found in this study in smears with high MN frequencies.

Chlorinated hydrocarbon contaminants were at least ten times higher in fishes from contaminated sites when compared to those from the reference sites (Table 2). Correlations between log-transformed MN frequencies and individual log-transformed total DDT ($r = 0{\cdot}156$, df = 34, $0{\cdot}20 < p < 0{\cdot}50$) and total PCB ($r = 0{\cdot}186$, df = 34, $0{\cdot}20 < p < 0{\cdot}50$) in white croaker were not significant.

TABLE 2
Hepatic Contaminant Concentrations in Fishes from Contaminated and Reference Sites (values are in ppm wet weight; X = mean; SD = standard deviation; n = sample size)

Species	*Contaminated site*[a]		*Reference site*[b]	
	Total DDT ($X+SD(n)$)	*Total PCB* ($X+SD(n)$)	*Total DDT* ($X+SD(n)$)	*Total PCB* ($X+SD(n)$)
White croaker	3·13 + 5·44 (28)	1·36 + 0·98 (28)	0·17 + 0·07 (8)	0·03 + 0·05 (8)
Kelp bass	8·28 + 7·37 (5)	2·57 + 3·18 (5)	0·85 + 0·31 (5)	0·42 + 0·18 (5)

[a] Contaminated site for white croaker = San Pedro Bay; contaminated site for kelp bass = Palos Verdes Peninsula.
[b] Reference site for white croaker = Dana Point; reference site for kelp bass = Catalina Island.

DISCUSSION

Genotoxic damage was greater in fishes from highly contaminated San Pedro Bay compared to fishes collected at Dana Point and Catalina Island. Kelp bass, which generally had higher body burdens of chlorinated hydrocarbons than white croaker, also had higher frequencies of MN. Although factors which modify MN induction are poorly understood and may include factors such as blood cell kinetics, temperature, life history stage and possibly sex differences (Heddle *et al.*, 1983), results obtained in this study do support the overall relationship between contaminant exposure and *in vivo* genotoxicity. Induced spawning experiments have shown that maternal MN frequencies in white croaker were predictive of reproductive success. Eggs from fish with elevated MN counts had lower fertilization rates (Cross & Hose, 1986).

Micronucleus counts in white croaker were only weakly correlated with body burdens of total DDTs and PCBs; these results are consistent with the non-clastogenic properties of these substances (Heddle *et al.*, 1983). San Pedro Bay white croaker body burdens of oxygenated metabolites of DDT and PCB (primarily hydroxylated and conjugated derivatives) are up to 60 times the concentrations of the parent compounds (Brown *et al.*, 1982). The mutagenic potential of the chlorinated hydrocarbon metabolites has not been adequately defined. Other potential clastogens, including chlorinated benzenes, have been found in San Pedro Bay fishes (Young *et al.*, 1980; Malins *et al.*, 1986). Although polycyclic aromatic hydrocarbons (many of which are well-known mutagens) were not measured in this study, previous work has shown that San Pedro Bay sediments contain high (up to 18 ppm dry weight) concentrations of benzo(a)pyrene (Gossett *et al.*, 1983). Biliary

polycyclic aromatic hydrocarbon metabolites were highest in white croakers from outer Los Angeles Harbor (3·70 ± 3·10 ppm wet weight, $X \pm SD$, $n = 7$), less off White Point (0·96 ± 1·60 ppm, $n = 12$) and lowest off Dana Point (0·072 ± 0·074 ppm, $n = 9$) (Malins *et al.*, 1986).

The piscine MN test may be a more sensitive indicator of chronic, rather than acute, exposure to contaminants. After a three-week exposure, Hooftman & de Raat (1982) did not observe MN induction in *Umbra pygmaea* except at 200 mg/liter ethyl methanesulphonate. Micronucleus formation was evident at their lowest dosage of 8 mg/liter after six weeks. The time-dependent response could limit the use of the piscine MN test to monitor chronic pollution rather than as an early warning system.

Mammalian studies suggest that the *in vivo* MN test is less sensitive than the Ames test due to the lack of activated metabolites reaching target bone marrow cells (Natarajan & Obe, 1986). The piscine MN test does not have this limitation since head kidney (the major site of hematopoiesis in adult fish) contains high mixed function oxygenase activity (James *et al.*, 1979*a,b*). Formation of MN can also be measured in larval fishes by preparing squash–smear preparations of liver (Hose *et al.*, 1984), which is the hematopoietic organ during larval stages. This technique can be applied to archived, formalin-fixed specimens.

Results of this study support the use of the piscine micronucleus test as a rapid monitoring tool to detect the presence of genotoxic agents in the environment. A similar system using circulating erythrocytes has been proposed for use in humans (MacGregor *et al.*, 1980). Recently, two promising MN models, the newt (*Pleurodeles waltl*) (Grinfield *et al.*, 1986) and the tadpole (*Rana catesbeiana*) (Krauter *et al.*, in press), have been described for assessing contaminants in freshwater. The simplicity, rapidity and biological relevance of the piscine MN test suggest that further laboratory validation studies are warranted. Background information on fish erythrocyte kinetics, persistence of micronucleated erythrocytes, and interspecific and environmental variables that affect MN formation are necessary for the development of an *in vivo* monitoring system based on MN frequencies.

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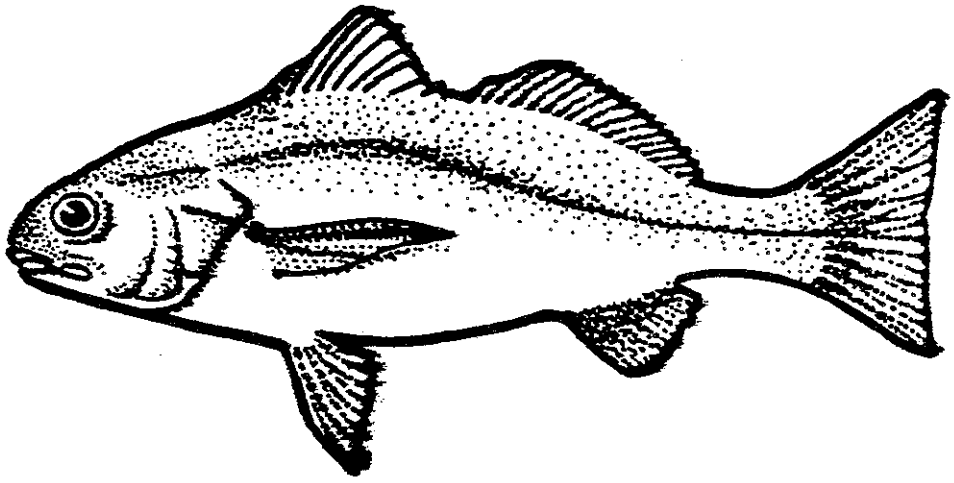

CHANGES IN DDT AND PCB CONCENTRATION IN WHITE CROAKER ARE RELATED TO THE REPRODUCTIVE CYCLE

Most marine fishes and invertebrates living in temperate waters spawn during one or at most two seasons of the year. As part of this seasonal spawning cycle, the gonad weight, particulary of females, varies seasonally, both on an absolute basis and as a percentage of body weight. The yearly reproductive cycle also affects body chemistry: for example, the concentration of lipids in the liver drops as lipid is diverted to the gonad during egg development.

As a result, contaminants from the environment such as DDT and PCBs that are lipid soluble are affected by lipid dynamics in the organism. In this study of the reproductive cycle of whit croaker (Sciaenidae: *Genyonemus lineatus*), J. N. Cross and his colleagues* examines seasonal changes in gonad weight, lipid content of the liver, and contaminant content of the liver. His study confirms that DDT and PCB concentrations in the liver vary over time, reflecting the seasonal reproductive cycle, even when the effects of changes in lipid content are removed.

Cross recommends that researchers report fish reproductive status when comparing contaminant body burdens of fish collected at different times of year. A report could include the gonadosomatic index (GSI) or alternatively histological condition of the gonad. At the very least, lipid content should be reported.

White croaker are the mainstay of the pier and small boat sport fish catches in southern California (Wine, 1979; Puffer et al, 1982). They are abundant in coastal areas with soft substrates from the surf zone down to over 100 m (Miller and Lea, 1972). They consume a wide variety of epibenthic organisms and live up to 15 years.

In his study, Cross determined the timing of the croaker reproductive cycle by examining the ratio of gonad weight to body weight. Expressed as a percent, this ratio is known as the gonadosomatic index (GSI). Among female white croakers collected in outer Los Angeles Harbor, GSI was lowest in the summer, rose to a peak in the winter, and declined through spring (Figure 1). Most spawning occurred between November and April; peak spawning occurred in February and March. Females are batch spawn-

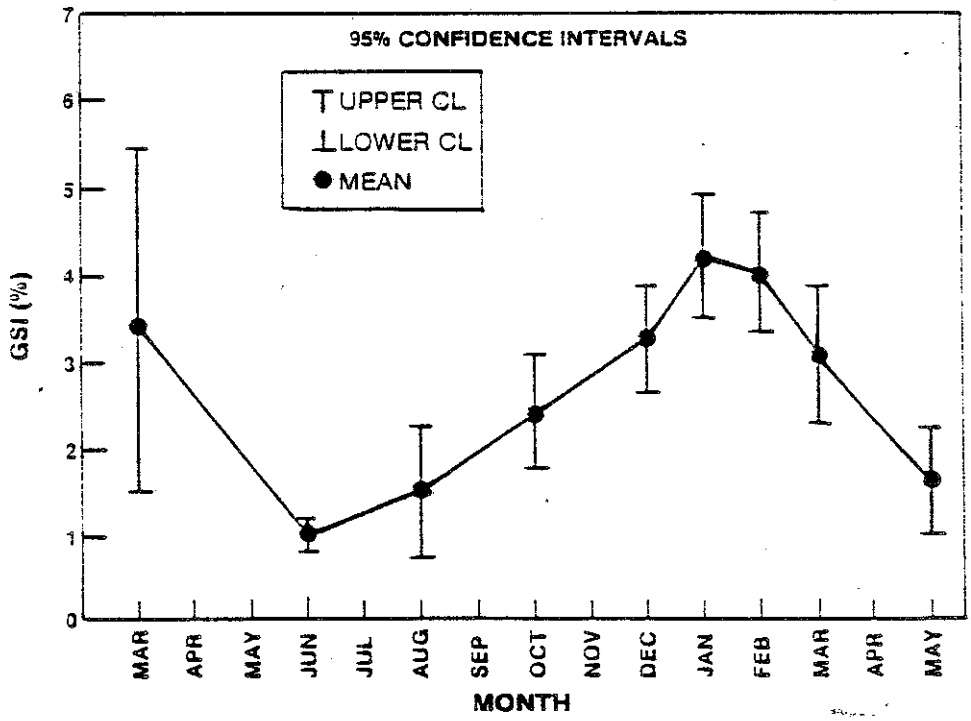

Figure 1. Los Angeles Harbor Female GSI

rs producing 18 to 24 batches per ear with 800 to 37,000 eggs per atch. Approximately 50 percent of he population is mature after one ear and 100 percent are mature by our years (Love et al., 1984).

The concentration of lipids in the livers of the fish changed seasonally with the reproductive cycle (Figure 2). After spawning, liver lipid content was at the lowest level of the year. Lipid content increased rapidly during the summer when the fish were feeding and accumulating energy reserves for the following spawning season. The decrease in liver lipids during the fall and winter was due to the provisioning of eggs with yolk (Figure 3). The liver produces a lipid-rich substance called vitellogenin that is released into the blood and taken up by the developing egg cells (Wallace and Selman, 1981).

The liver concentrations of total DDT (DDT + DDD + DDE) and total PCB (Aroclor 1242 + 1254) among female white croakers varied seasonally with the reproductive cycle (Figure 4). Both organic contaminants were accumulated during the summer when the fish were feeding and accumulating fat. Concentrations in the liver peaked in the fall and declined during the spawning season. The decline in liver contaminant concentration paralleled the decline in lipid.

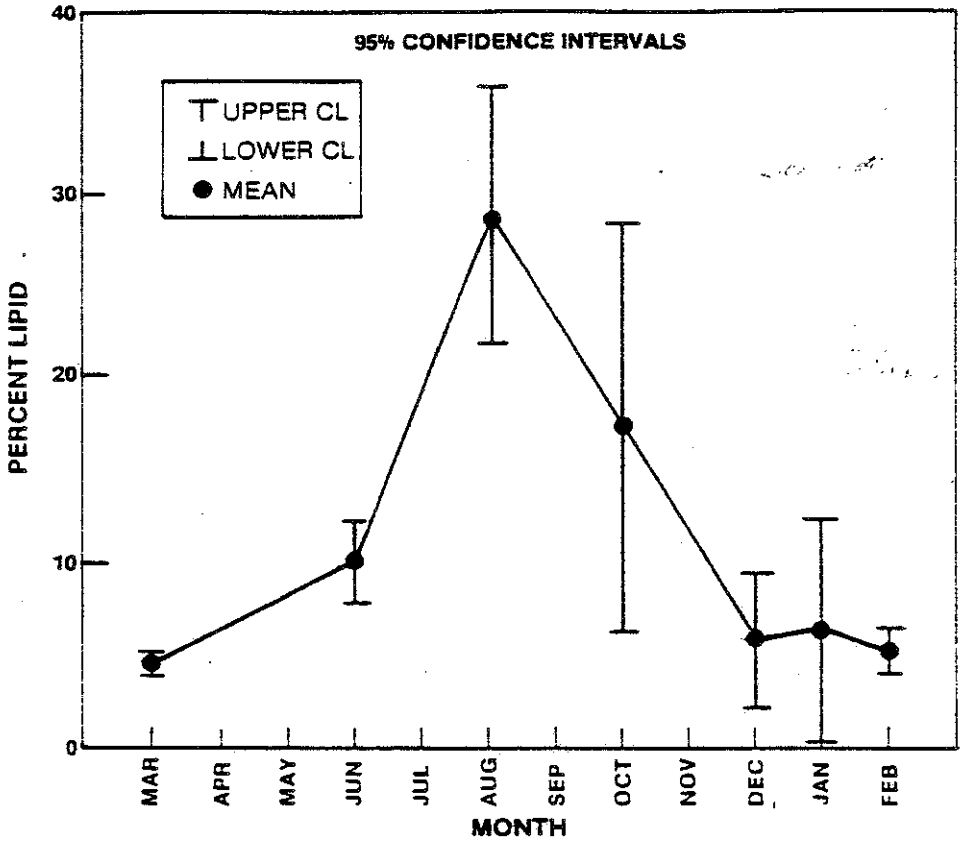

Figure 2. LA Harbor Female Livers — Mean % Lipid

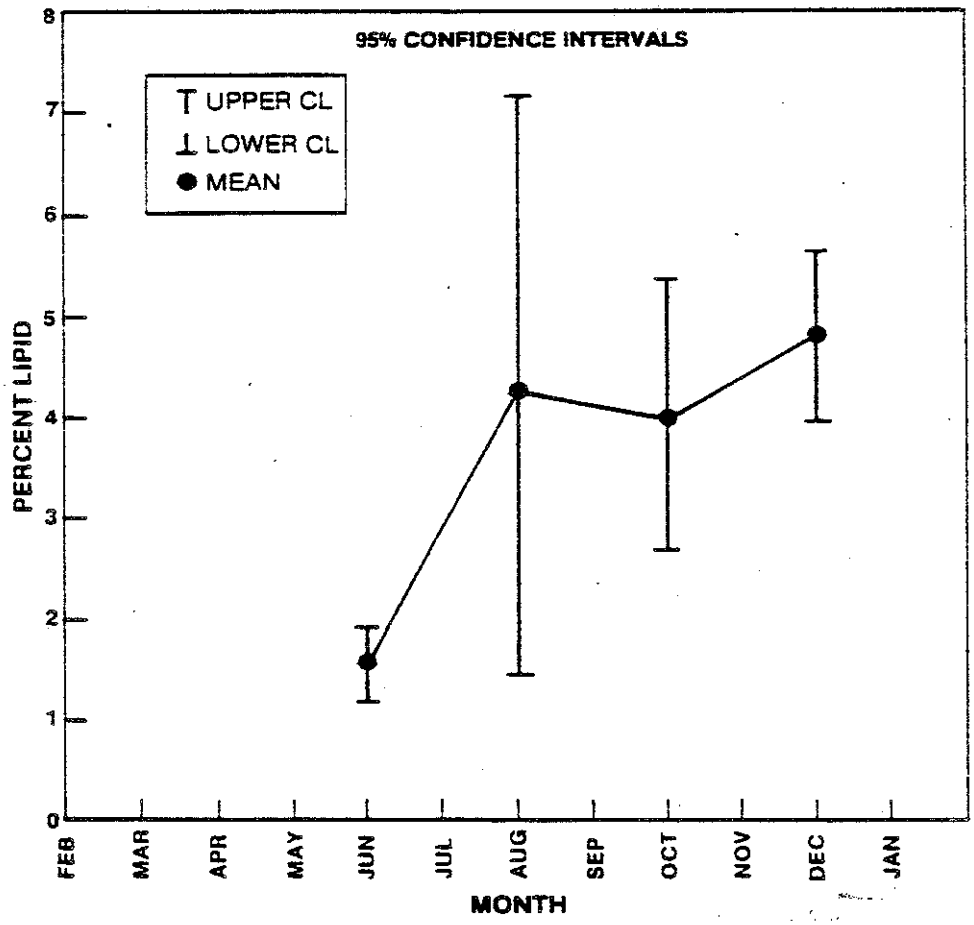

Figure 3. Palos Verdes Female Gonads — Mean % Lipid

content. These lipid soluble contaminants are deposited in the ovaries with vitellogenin (Figure 5). Normalizing the liver concentrations to lipid content does not remove the seasonality (Figure 6).

Cross points out that the variation in lipid and contaminant concentrations was greatest prior to spawning. This is related to the age of the fish and the onset of spawning. Older, larger females spawn earlier and more frequently than younger, smaller females. Consequently, some females are still accumulating lipids and lipid soluble contaminants in their livers while other females are depositing them in their ovaries.

The relationship between contaminant body burdens and the reproductive cycle is only one part of a larger study funded by NOAA. Cross is also studying the reproductive cycle and contaminant levels of the kelp bass, *Paralabrax clathratus*. He plans to add a third species to the study, probably one of southern California's flatfishes, such as Pacific sanddab or fantail sole.

Work with additional fishes will help to confirm the pattern seen in the white croaker. Also, there has been extensive research with flatfish in relation to water quality in other urban coastal areas, including Puget Sound, Boston Harbor and San Francisco Bay. Thus, Cross will be able to compare the contaminant-reproductive cycle relationship in several fish species and to compare his flatfish data with results of flatfish studies from other coastal areas.

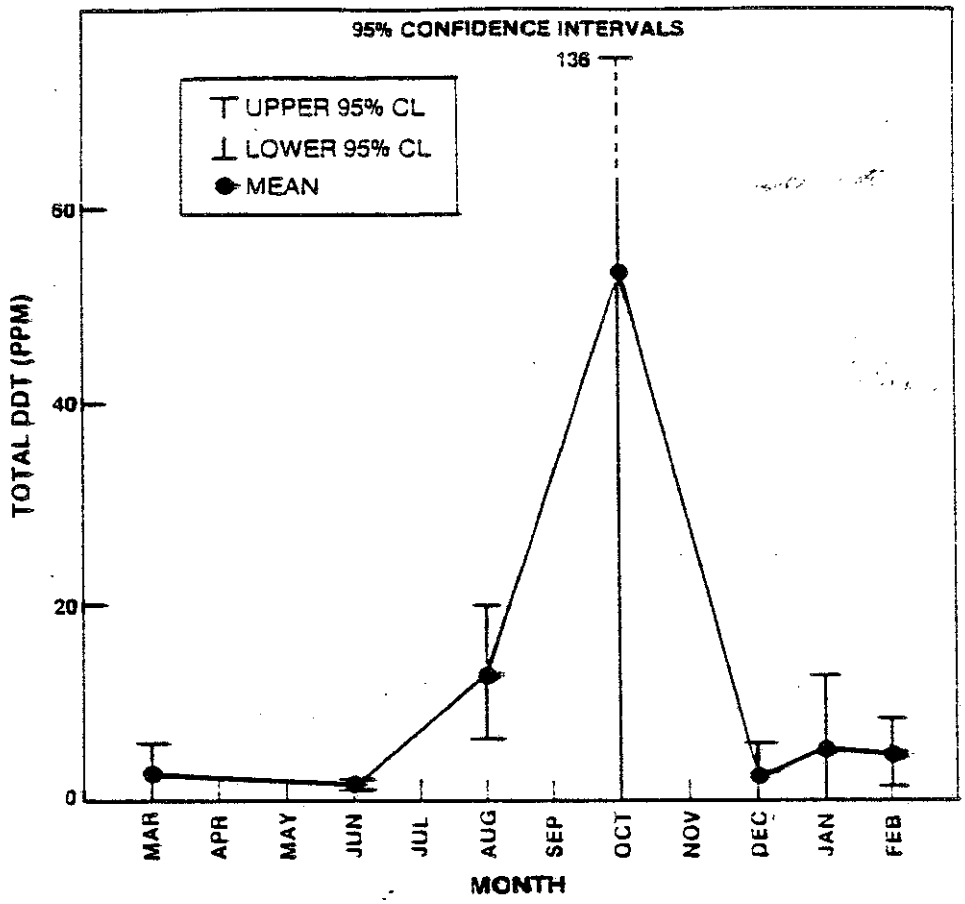

Figure 4a. L.A. Harbor Female Livers - DDT Conc.

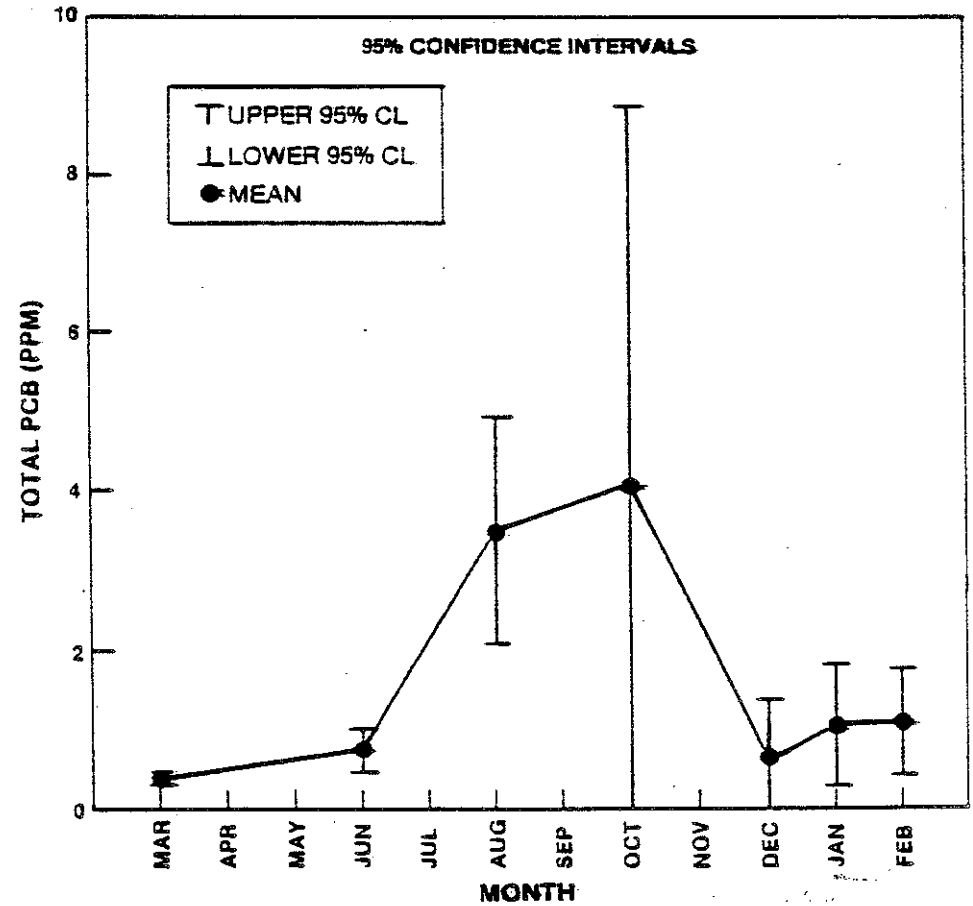

Figure 4b. L.A. Harbor Female Livers - PCB Conc.

Figure 5a. San Pedro Bay Female Gonads — DDT Conc.

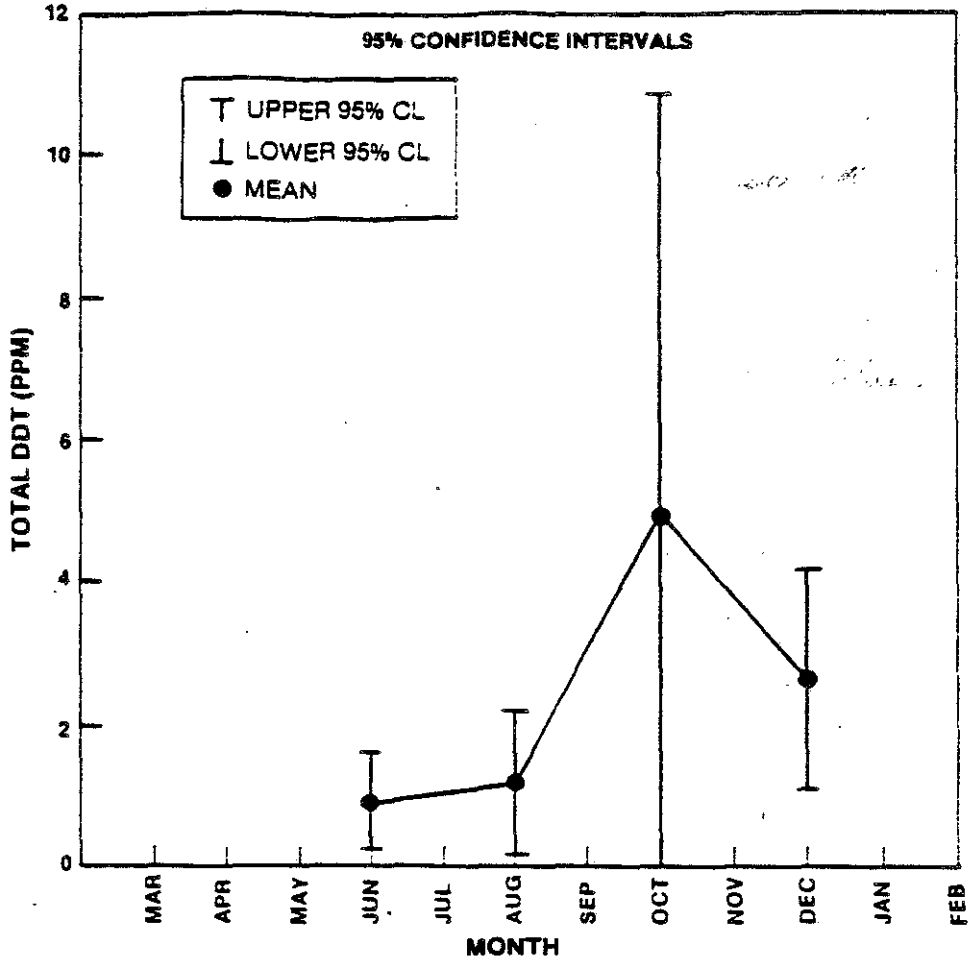

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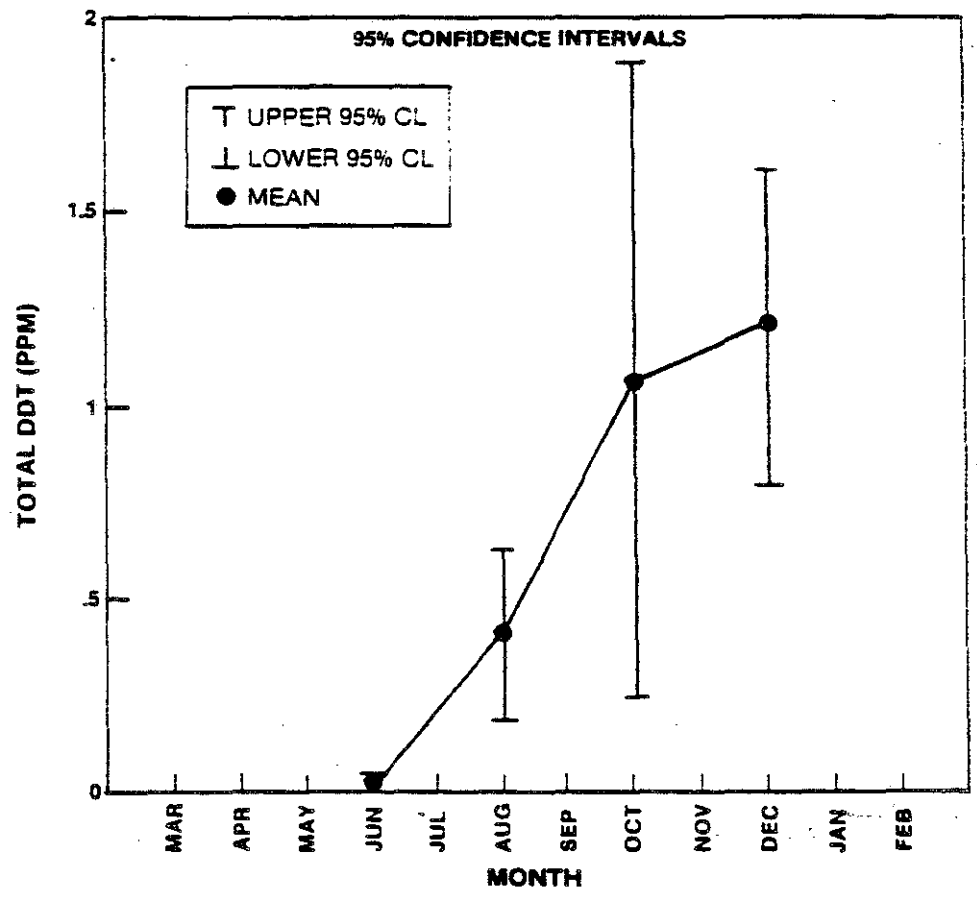
Figure 5b. San Pedro Bay Female Gonads — PCB Conc.



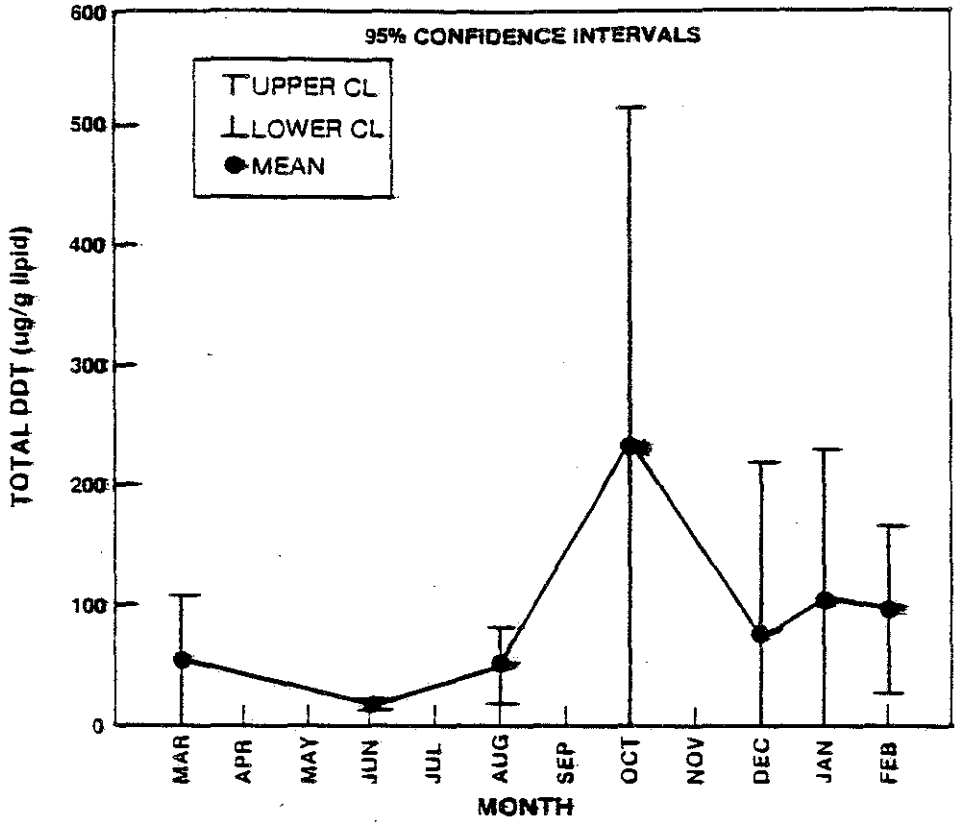


Figure 6a. L.A. Harbor Female Liver Lipid Normalized DDT Conc.

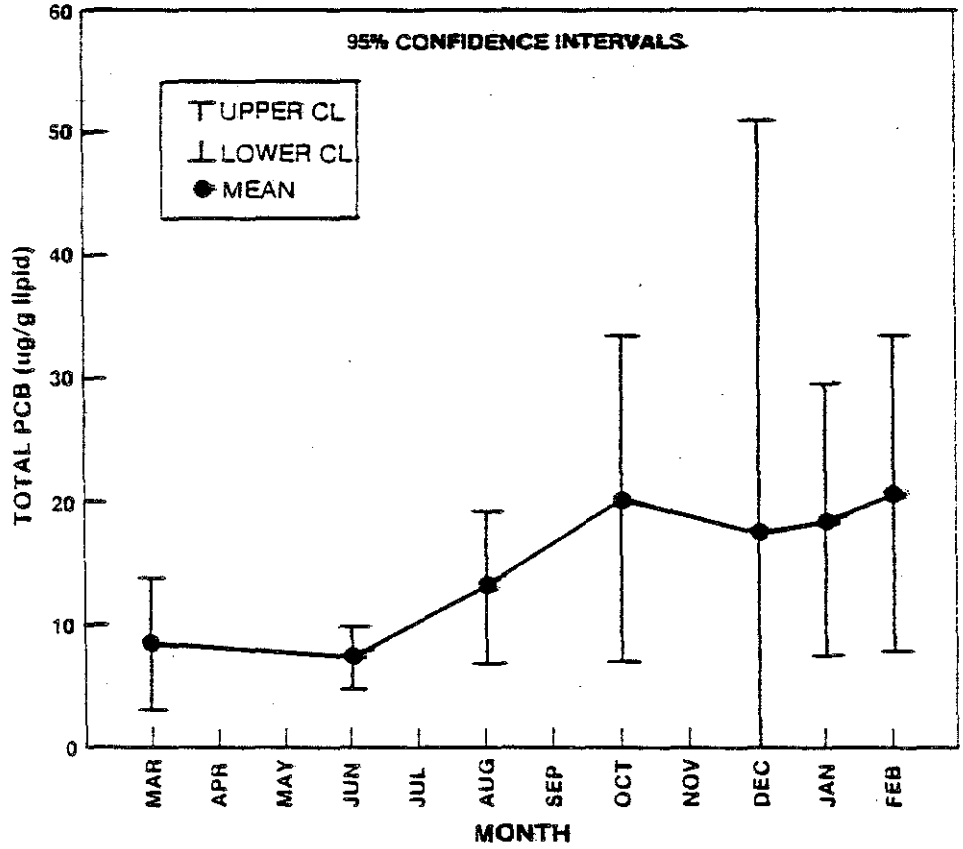


Figure 6b. L.A. Harbor Female Liver Lipid Normalized PCB Conc.