EFFECTS OF HEPTACHLOR AND TOXAPHENE ON LABORATORY-REARED EMBRYOS AND FRY OF THE SHEEPSHEAD MINNOW[1]

by
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ABSTRACT

Flow-through seawater bioassays of 28-days duration were conducted with the organochlorine pesticides heptachlor and toxaphene to determine their toxicity to and bioconcentration by embryos and fry of the sheepshead minnow (Cyprinodon variegatus). *At technical heptachlor measured concentrations of 4.3, 3.5, 2.2, 2.0, and 1.2 μg/l (ppb), test animal survival was 1, 5, 61, 79, and 88%, respectively. At toxaphene measured concentrations of 2.5, 1.1, 0.6, 0.3, and 0.2 μg/l, test animal survival was 10, 85, 79, 88, and 80% respectively. Average standard length of fry continuously exposed from fertilization to heptachlor concentrations of 4.3 and 3.5 μg/l was significantly reduced ($\alpha = 0.01$). Concentration factors (concentration in fish/measured concentration in water) for heptachlor averaged 3,600 and for trans-chlordane averaged 8,600. Heptachlor epoxide and cis-chlordane were also present in the fish. Concentration factors for toxaphene in fry averaged 9,800.*

Various histopathological characteristics not seen in control fish were observed in the liver, kidney, pancreas, and intestine of the few fish that survived 4.3 and 3.5 μg/l of heptachlor.

INTRODUCTION

The persistent organochlorine insecticides heptachlor and toxaphene have been used extensively throughout the United States. Estimated U.S. production in 1971 was 2.7 million kilograms (6 million lbs.) of heptachlor (active ingredient) (Midwest Research Institute, 1972), and in 1975, 36-50 million kilograms (80-110 million lbs.) of toxaphene (Midwest Research Institute, 1976). In 1975, toxaphene production exceeded that of all other insecticides, and its use may become even more widespread if new restrictions are imposed on other organochlorine pesticides.

Heptachlor and toxaphene have been found in the tissues of estuarine animals. Heptachlor concentrations as high as 1.5 μg/g (ppm) have been reported from muscle of winter flounder (*Pseudopleuronectes americanus*) from the Weweantic River estuary, Massachusetts (Smith and Cole, 1970). Heptachlor has also been found in oysters from Galveston Bay (Casper, 1967) and in water and oysters from estuarine areas of Louisiana (Hammerstrom et al., 1967). Toxaphene concentrations as high as 1 μg/g have been found in eastern oysters (*Crassotrea virginica*) from South Carolina (Bugg et al., 1967). Toxaphene concentrations greater than 200 μg/g were found in mummichogs (*Fundulus heteroclitus*) during dredging of a Georgia coastal creek that receives effluent from a toxaphene manufacturing plant (Reimold and Durant, 1974).

Both heptachlor and toxaphene are acutely toxic to estuarine animals. The 96-hr LC50 values (concentration in water estimated to kill 50% of test animals) based on measured concentrations for sheepshead minnows (*Cyprinodon variegatus*) and spot (*Leiostomus xanthurus*) exposed to technical grade heptachlor under flow-through conditions were 3.68 μg/ℓ (ppb) and 0.85 μg/ℓ, respectively (Schimmel et al., 1976). The 96-hour LC50 values (based on measured concentrations) for sheepshead minnows and pinfish (*Lagodon rhomboides*) exposed to toxaphene were 1.1 μg/ℓ and 0.5 μg/ℓ, respectively (Schimmel et al., in press). The 96-hour LC50's (measured concentrations) for pink shrimp (*Penaeus duorarum*) exposed to technical grade heptachlor and toxaphens were 0.11 μg/ℓ (Schimmel et al., 1976) and 1.4 μg/ℓ (Schimmel et al., in press), respectively.

To determine the toxicity of these compounds to the young of the estuarine fish *Cyprinodon variegatus*, we conducted 28-day embryo/fry bioassays.

[1] Contribution No. 297, Gulf Breeze Environmental Research Laboratory

We wish to acknowledge the technical assistance of Walter Burgess in these bioassays and of Johnnie Knight in the chemical analyses of water.

MATERIALS AND METHODS

Bioassay Methods

The embryo/fry bioassays were conducted with the apparatus described by Schimmel *et al.* (1974), which is a modification of the diluter designed by Mount and Brungs (1967). Seawater from Santa Rosa Sound, Florida, was pumped through a sand-filled swimming pool filter and a 20μ polypropylene filter into a head box where it was heated to 30±2°C and aerated before intermittent delivery with the diluter. To each of four 960-ml experimental aquaria in each treatment, our diluter delivered 87.5 ml of this seawater per cycle for heptachlor exposures and 125 ml per experimental aquaria for toxaphene exposures. Number of daily cycles averaged 183 for heptachlor and 207 for toxaphene.

Each bioassay consisted of two controls, one with and one without carrier, and five toxicant concentrations with four replicates per concentration for a total of 28 aquaria per experiment. Nominal heptachlor concentrations were 6.25, 9.37, 12.5, 18.75, and 25 $\mu g/\ell$. Nominal toxaphene concentrations were 0.25, 0.50, 1.0, 2.0, and 4.0 $\mu g/\ell$. Hereafter, all concentrations will be reported as μg of the insecticide measured in the test water. Toxicants[1] were dissolved in triethylene glycol and this carrier was delivered at 7.8 mg/ℓ of water to each carrier control and toxicant aquarium.

Adult sheepshead minnows were collected near the Environmental Research Laboratory at Gulf Breeze and acclimated to 30°C water at ambient salinity for at least 2 days prior to hormonal induction of egg production by the procedure of Hansen *et al.* (1973). Ten days prior to the first hormonal injection, adults for the heptachlor, but not the toxaphene bioassay, were treated with 1:4,000 formalin in ambient seawater for 30 minutes to remove trematodes. Seventeen female and 7 male fish were used to obtain gametes in the heptachlor experiment; 21 females and 8 males were used in the toxaphene experiment. Fertilization was obtained by combining eggs with macerated testes in approximately 60 ml of control water for one hour. Chemical analysis of a sample of eggs from each experiment revealed no detectable amounts of commonly occurring organochlorine pesticides (n.d. = <0.01 mg/kg heptachlor experiment, <0.03 mg/kg toxaphene experiment) or PCB's (n.d. = <0.08 mg/kg heptachlor experiment, <0.24 mg/kg toxaphene experiment).

Twenty embryos, confirmed microscopically, were placed in each of four replicate egg cups per concentration one hour after eggs and milt were combined. Egg cups consisted of a 9-cm I.D. petri dish bottom to which a 10 cm high, 450μ nylon mesh collar was attached. To assure good water exchange, egg cups were changed before the mesh became clogged. Exposure aquaria were located in a recirculating freshwater bath that maintained the exposure temperature at 30 ± 2°C. Embryos, fry, or juvenile fish were checked daily for mortality or behavioral aberrations. A feeder (Schimmel and Hansen, 1975) automatically delivered equal portions of live brine shrimp, (*Artemia salina*), nauplii six times daily to the fry. Dissolved oxygen was measured weekly using the modified Winkler method of Strickland and Parsons (1968). Each of the seven samples measured was a composite of water from the four replicates in that treatment. On the last day of the exposure, day 28, fish in each replicate were photographed for subsequent standard length measurement. Fish to be chemically analyzed were sacrificed and rinsed thoroughly in warm freshwater, towel-drained, weighed, and frozen until analyzed.

Statistical Analyses

Analyses of variance and the Newman-Keuls range test were used to determine treatment differences in embryo hatching and fry survival. Fry growth data were analyzed by analysis of covariance (number of surviving fry per egg cup on last day of experiment was the covariate). The Newman-Keuls range test was then applied to the adjusted means.

Chemical Analysis

Heptachlor.—Samples of juvenile fish were weighed into a 200 mm x 25 mm (O.D.) screw-top test tube and extracted with 10 ml acetonitrile for 30 minutes at 65°C in a sonic

[1]Technical grade heptachlor was supplied by Velsicol Chemical Corporation, Chicago, Ill., and the technical toxaphene was supplied by Hercules, Inc., Wilmington, Del. Mention of commercial products does not constitute endorsement by the Environmental Protection Agency.

cleaner. The test tube was centrifuged and the acetonitrile extract transferred to another test tube. The sample was then extracted three times for 15 minutes with 5 ml of acetonitrile, centrifuged, and the acetonitrile extracts added to the second test tube.

Egg samples were weighed into a 150 mm x 25 mm (O.D.) screw top test tube and were extracted twice with 5 ml volumes of acetonitrile for 30 seconds with a model PT 10-ST Willems Polytron (Brinkman Instruments, Westbury, New York). After centrifugation, the acetonitrile extract was transferred to a clean, 200 mm x 25 mm test tube. Following a second identical extraction, the tissue was rinsed with 5 ml of acetonitrile by agitation on a Vortex mixer for 30 seconds, centrifuged, and the acetonitrile added to the above extracts. This rinse was repeated a second time.

Following extraction, 25 ml of 2% aqueous sodium sulfate and 5 ml hexane were added to the combined extracts for both tissue and egg samples. The test tube was sealed with a teflon-lined cap and was shaken for one minute. After the solvent phases separated, the upper hexane layer was transferred with a dropping pipette to a 25 ml Kuderna-Danish concentrator tube. This extraction was repeated three times with 5 ml of hexane. The combined extracts, concentrated to about 0.5 ml on a steam table using a Micro Snider Column, were transferred to a 200 mm x 9 mm (I.D.) chromatographic column containing 3.0 g Florisil topped with 2.0 g of anhydrous sodium sulfate. Heptachlor, heptachlor epoxide, cis-chlordane, and trans-chlordane were eluted from the column with 20 ml of 5.0% ethyl ether in hexane. Extracts were concentrated or diluted to an appropriate volume for analysis by electron capture gas chromatography. Egg samples were eluted a second time with 20 ml of 20% ethyl ether in hexane to check for more polar pesticides.

Composite water samples from each concentration were analyzed at least weekly by extracting one liter with two 100 ml portions of petroleum ether. The extracts were cleaned up with the chromatographic column described above and were adjusted to an appropriate volume for analysis.

The operating parameters of the Model 5710 Hewlett-Packard Gas Chromatographs were: 183 cm x 2 mm (I.D.) glass columns packed with 2.0% OV-101 on 100/120 mesh Gas Chrom Q and 0.75% OV-17: 0.97% OV 210 on 100/120 mesh Gas Chrom Q; oven temperature, 200 C; detector (^{63}Ni) temperature, 300 C; injector temperature, 200 C; carrier gas, 90/10 Argon/Methane with a flow rate of 25 ml/minute.

All samples were fortified with an internal standard (2,3,4,5,6,2′5′ heptachlorobiphenyl) prior to analysis to evaluate the integrity of the results. Extracts of tissue fortified with heptachlor, heptachlor epoxide, cis-chlordane, and trans-chlordane gave recoveries greater than 90%. Pesticide concentrations were calculated on a wet-weight basis without a correction factor for percentage recovery. Technical heptachlor used in these experiments contained 65% heptachlor, 22% trans-chlordane, 2% cis-chlordane, and less than 2% nanochlor when analyzed by gas chromatography. Identities of these compounds were confirmed by mass spectrometry.

Toxaphene.—Tissue and egg samples were extracted by the same method used for heptachlor egg samples, except that tissue samples were not eluted a second time. Toxaphene was eluted from the column with 20 ml of 5% ethyl ether in hexane. Extracts were concentrated or diluted to an appropriate volume for analysis by electron capture gas chromatography. Water samples were analyzed by extracting one liter with two 100-ml portions of petroleum ether. The extract was cleaned up on a Florisil column.

Toxaphene was analyzed on a Hewlett-Packard model 5710 gas chromatograph as described in heptachlor methods, except that only a 2% OV-101 column was used.

All samples were fortified with an internal standard (o,p′—DDE) prior to analysis in order to evaluate the integrity of the results.

Extracts of tissue fortified with toxaphene gave recoveries greater than 90%. Residue concentrations were calculated on a wet-weight basis without a correction factor for percentage recovery.

Toxaphene is a multiple peak compound and concentrations were quantitated by comparing the total area under the major peaks of the sample with the total area under the major peaks of a standard of known concentration. The lower limit of detectability of toxaphene was 0.2 $\mu g/l$ (0.2 parts per billion, ppb) in water and 0.2 mg/kg (0.2 parts per million, ppm) in tissue samples.

Pathological Methods

Fish from aquaria containing 4.3, 3.5, and 2.0 μg/ℓ of heptachlor and control aquaria containing the carrier were fixed in Davidson's fixative for 24 hours, transferred to 70% ethyl alcohol, and processed for sagittal and parasagittal sections (7 μm) for later pathological examination. Sections were stained with Harris hematoxylin and eosin and examined with a Zeiss Photomicroscope III. Tissues and organs studied histologically were: tegument, gills, digestive system, (buccopharyngeal region, stomach, intestine), liver, pancreas, kidney, spinal cord, brain, and thymus. Samples of fish were identified by number rather than by history of experimental treatment (exposed vs. control) so the pathologist had no *a priori* knowledge of the history of study samples. Therefore, histopathological findings (damage) was the sole basis for classifying samples of fish as *effect* or *no-effect* groups. Only after the hispathological classification of samples were the groups identified according to their history of exposure.

Fish from the toxaphene bioassay were not given pathological examinations.

RESULTS AND DISCUSSION

Neither heptachlor nor toxaphene significantly reduced embryo survival to hatching at concentrations tested, but each insecticide was lethal to fry that developed from the exposed embryos. Mortality in measured heptachlor concentrations of 4.3, 3.5, and 2.2 μg/ℓ (99%, 95%, and 39%, respectively) was significantly greater than that of both controls (Table 1). Schimmel et al. (1976) reported a heptachlor 96-hour LC50 of 3.68 μg/ℓ for previously unexposed juvenile sheepshead minnows. Our data, therefore, demonstrates that mortality of juvenile sheepshead minnows continuously exposed to 3.5 μg/ℓ heptachlor from embryos onward was nearly twice as great as mortality of juveniles exposed for 96 hours to 3.68 μg/ℓ during the juvenile stage only.

Table 1. Survival of embryos and fry of *Cyprinodon variegatus* exposed for 28 days to technical heptachlor and bioconcentration of this insecticide and its metabolites[a]. Concentration factors (concentration in tissues divided by concentration measured in water) are in parentheses.

Exposure Concentration μg/ℓ			*Combined Embryo/Fry Survival %*	*Average Standard Length (mm)*	*Covariate Adjusted Standard Length (mm)*	*Concentration in Fry, μg/g Wet Wt.*					
Heptachlor (Nominal)	*Heptachlor (Measured)*	*Trans-chlordane Measured*				*Heptachlor*		*Heptachlor Epoxide*	*Trans-Chlordane*		*Cis-Chlordane*
Control	0.021	0.015	81	9.2	10.0	0.011	(500)	0.035	0.030	(2,000)	0.019
Control-C[b]	0.022	0.021	96	8.0	10.2	0.038	(1,700)	0.056	0.104	(5,000)	0.024
6.25	1.22	0.44	88	8.6	10.0	4.5	(3,700)	3.6	3.2	(7,300)	0.65
9.375	2.04	0.67	79	6.6	7.2	4.8	(2,400)	4.2	4.6	(6,900)	1.0
12.5	2.24	0.78	61[c]	9.3	9.6	10.4	(4,600)	8.0	9.1	(11,700)	2.6
18.75	3.5	1.2	5[c]	11.0	6.3[c]						
25.0	4.3	1.5	1[c]	12.0	6.2[c]						

[a] Salinity averaged 25.2 ‰ (range, 19-29) and dissolved oxygen averaged 3.8 mg/ℓ (range, 2.1-5.5).
[b] Contained 7.8 mg/ℓ of the solvent triethylene glycol.
[c] Significantly different from both controls ($\alpha = 0.05$).
[d] Significantly different from both controls ($\alpha = 0.01$).

Behavioral effects observed in the heptachlor exposure ranged from decreased swimming in 1.2 μg/ℓ to decreased swimming accompanied, when disturbed, by abnormal hyperkinetic activity in higher concentrations. Hyperkinetic activity was often followed by temporary paralysis persisting from a few seconds to over a minute before return to a semi-normal state. Effect increased with concentration and preceded death in severely affected individuals. The most pronounced behavioral effects occurred during weeks 2 and 3, subsiding slightly during week 4.

A concentration of 2.5 μg toxaphene/ℓ of water was lethal to 90% of the fry. Mortality of fry in other concentrations did not differ from controls (Table 2). In contrast, the 96-hr LC50 of toxaphene to adult sheepshead minnows was 1.1 μg/ℓ (Schimmel et al., in press). Behavioral effects observed in 1.1 and 2.5 μg/ℓ of toxaphene were similar to those noted previously for fish exposed to heptachlor, the effect increasing with concentration and decreasing with time.

The length of fish was not affected by the toxaphene concentrations tested. This is in contrast to the study by Mayer et al. (1975) in which significant mortality and reduced fry

Table 2. Survival of embryos and fry of *Cyprinodon variegatus* exposed for 28 days to toxaphene and bioconcentration of this insecticide[a]. Concentration factors (concentration in tissue divided by concentration measured in water) are in parentheses.

Exposure Concentration, μg/ℓ		*Combined Embryo/Fry Survival*	*Average Standard Length*	*Concentration In*
Nominal	*Measured*	*%*	*(mm)*	*Fry, μg/g*
Control	ND[b]	80	11.2	ND
Control-C[c]	ND	85	10.3	ND
0.25	0.20	80	10.0	2.3 (11,500)
0.5	0.29	88	10.9	2.3 (7,900)
1.0	0.62	79	11.1	3.8 (6,100)
2.0	1.1	85	11.3	10. (9,100)
4.0	2.5	10[d]	13.3	36. (14,400)

[a] Salinity averaged 12.9 °/₀₀ (range, 7-23.5) and dissolved oxygen averaged 4.9 mg/ℓ (range, 2.9-6.5).
[b] ND = not detectable. < 0.2 μg/ℓ.
[c] Contained 7.8 mg/ℓ of the solvent triethylene glycol.
[d] Significantly different from both controls ($\alpha = 0.01$).

length occurred in brook trout exposed to 39, 68, and 139 ηg/ℓ (parts per trillion) toxaphene continuously from eyed eggs through 90-day fry. However, length was significantly affected by heptachlor concentrations of 3.5 and 4.3 μg/ℓ. The few fish surviving in these concentrations were longer than fish in other concentrations. However, when the number of fish in each egg cup on the last day of exposure was considered a covariate, adjusted length of fish in 3.5 and 4.3 μg/ℓ was less than the length of control fish.

Three of the ingredients of technical heptachlor (heptachlor, trans-chlordane, and cis-chlordane) as well as the metabolite heptachlor epoxide concentrated within the fry (Table 1). Disregarding controls, the average whole-body concentration factor (concentration in fish divided by measured concentration in water) for heptachlor was 3,600; for trans-chlordane, 8,600.

Toxaphene was also bioconcentrated by the fry at all exposure concentrations; the average whole-body concentration factor being 9,800 (range, 6,100-14,400) (Table 2). These concentration factors are similar to those reported by Schimmel et al. (in press) in a 96-hour bioassay with adult sheepshead minnows.

Mehrle and Mayer (1975) reported a "broken-back" syndrome in fathead minnows that had been exposed to 55-1230 ηg/ℓ of toxaphene for 150 days. This effect was not observed in our bioassay, but our external observations were gross and our exposure was for considerably shorter duration.

PATHOLOGY RESULTS—HEPTACHLOR EXPOSED FISH

The one fish that survived exposure to 4.3 μg/ℓ and the four fish that survived 3.5 μg/ℓ heptachlor showed consistent histological alterations not evident in 10 fish from control aquaria containing carrier or in 9 fish from 2.0 μg/ℓ aquaria. Liver alterations in fish exposed to 4.3 and 3.5 μg/ℓ ranged from moderate to severe fatty change (accumulation), as indicated by vacuolation in paraffin sections of liver and loss of normal muralia orientation (Figs. 1-3). Kidney damage consisted of degenerative changes in tubular epithelium (Figs. 4,5). Pancreatic alterations were characterized by extreme fatty accumulation in pancreatic exocrine tissue (Figs. 6,7). Intestinal changes consisted of lytic, frothy mucosal epithelium and possible nuclear proliferation or epithelial cell hyperplasia in foci (Figs. 8-10). The most striking histopathological changes were those in the liver, pancreas, and intestinal epithelium. Gill alterations in fish exposed to 4.3 μg/ℓ consisted of edematous separation of lamellar respiratory epithelium and lamella capillary

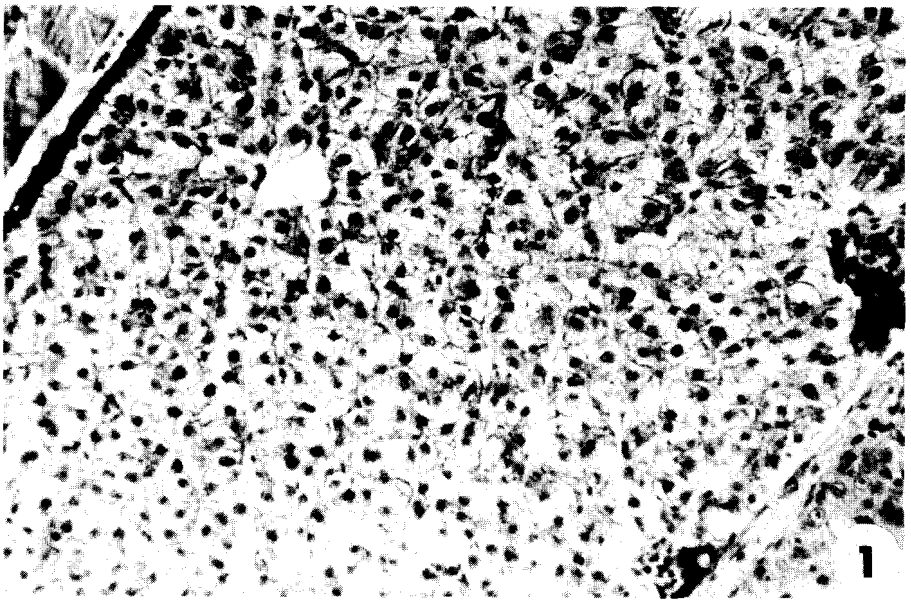

Figure 1. Sections from normal liver of fish from control aquaria; light areas within hepatocytes probably resulted from glycogen extraction during processing.

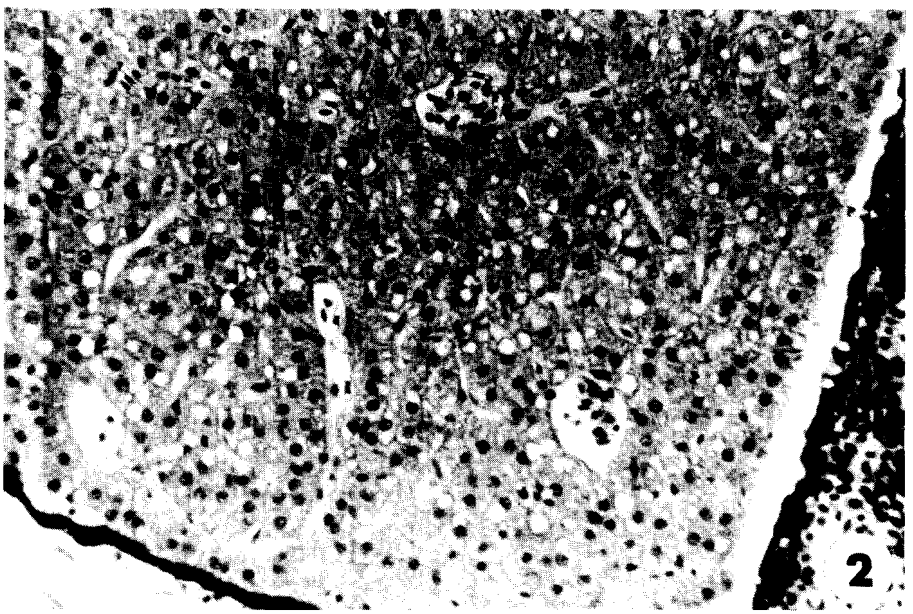

Figure 2. Section from liver of fish exposed to 4.3 $\mu g/l$ heptachlor and 1.5 $\mu g/l$ trans-chlordane; note extensive vacuolation of liver parenchyma probably due to abnormal accumulation of lipid.

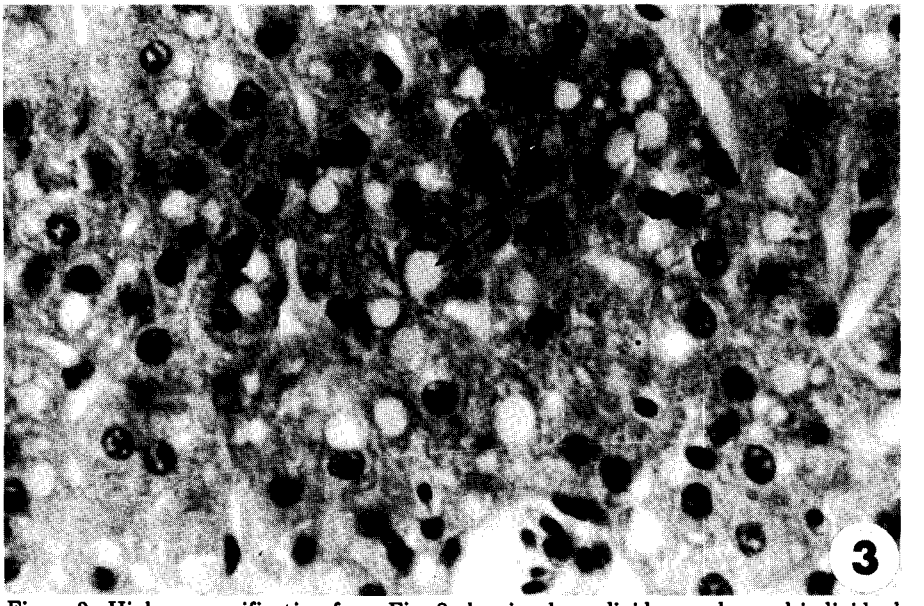

Figure 3. Higher magnification from Fig. 2 showing large lipid vacuolas and individual hepatocytes (arrow).

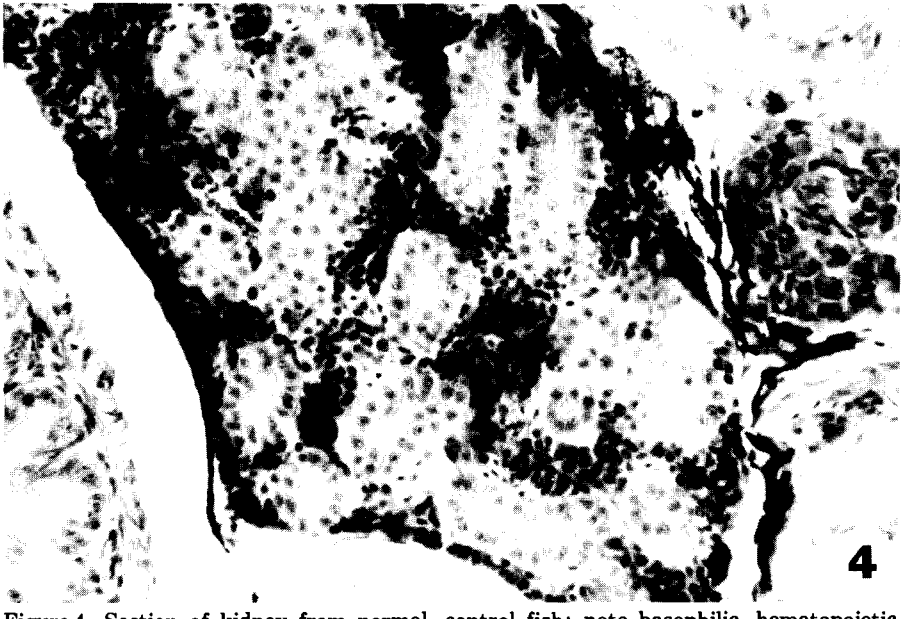

Figure 4. Section of kidney from normal, control fish; note basophilic, hematopoietic tissue between kidney tubules.

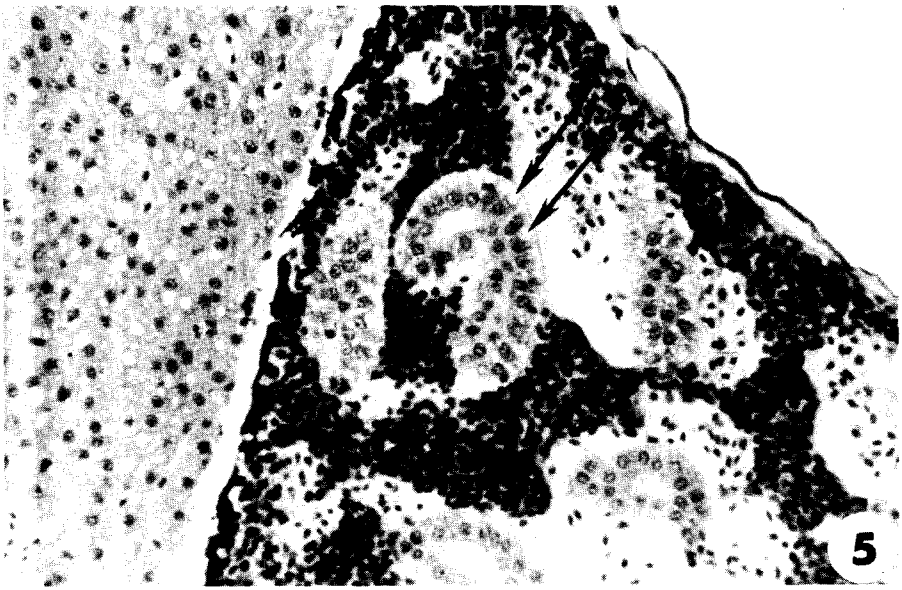

Figure 5. Sections of kidney and liver from fish exposed to 4.3 $\mu g/\ell$ heptachlor and 1.5 $\mu g/\ell$ trans-chlordane; arrows point to vacuoles in basal regions of kidney tubular epithelium cells.

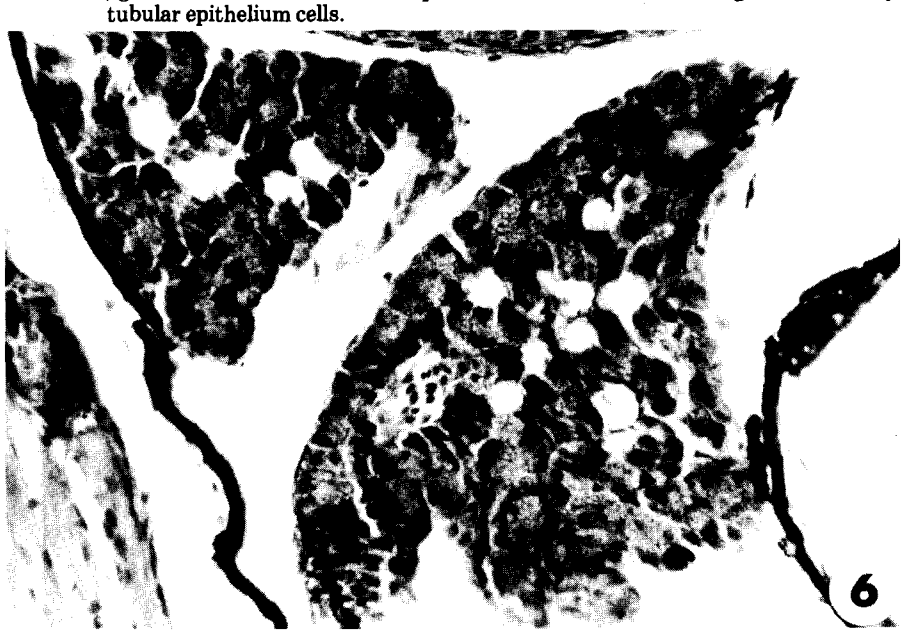

Figure 6. Normal pancreatic exocrine tissue from control fish; note few large lipid vacuoles and abundant granular material in exocrine cells.

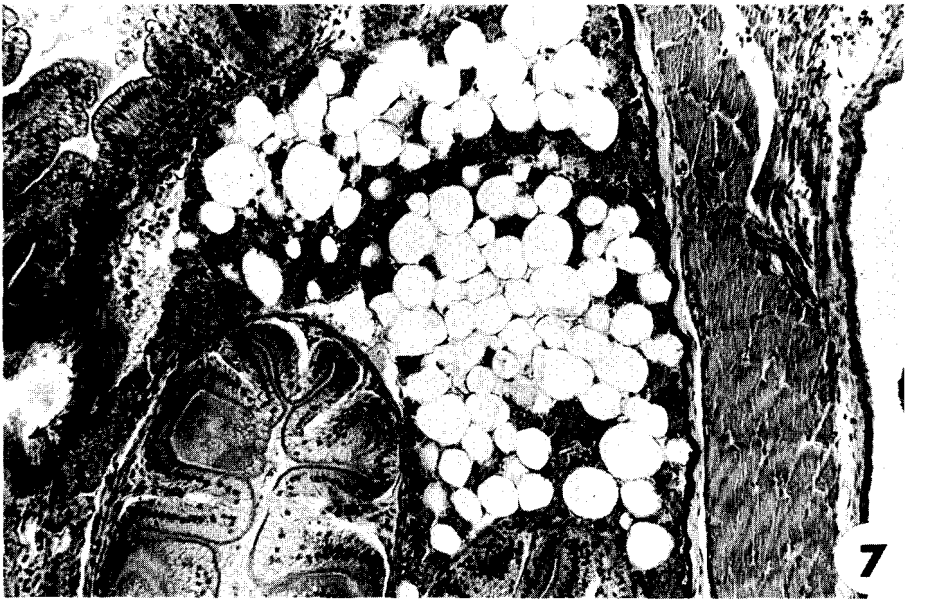

Figure 7. Apparent excessive lipid accumulation in pancreatic exocrine tissue from fish exposed to 4.3 $\mu g/\ell$ heptachlor and 1.5 $\mu g/\ell$ trans-chlordane.

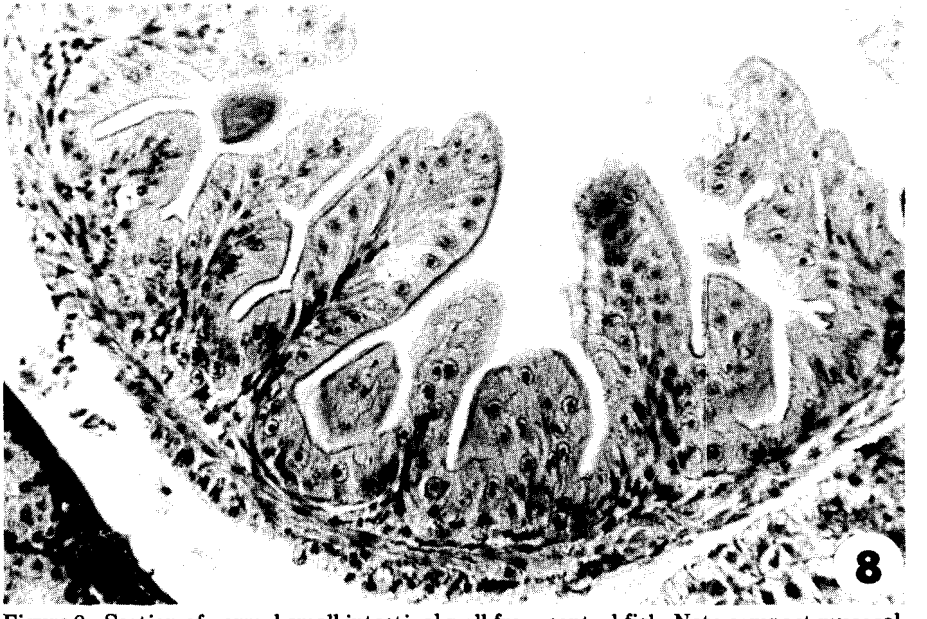

Figure 8. Section of normal small intestinal wall from control fish. Note compact mucosal epithelium.

Figure 9. Section of small intestine from fish exposed to 4.3 $\mu g/l$ heptachlor and 1.5 $\mu g/l$ trans-chlordane; note frothy vacuolated mucosal epithelium and loss of brush border.

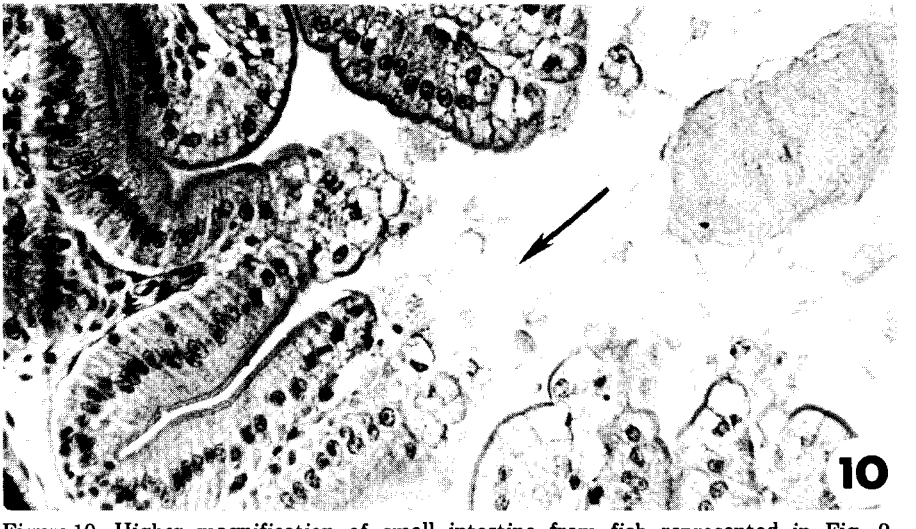

Figure 10. Higher magnification of small intestine from fish represented in Fig. 9. Extensive vacuolation and degeneration of tips of villi of intestinal epithelium with sloughing of some villi apparent (arrow). These degenerative changes are similar to those resulting from necrosis of inadequately fixed intestinal tissue; however, these changes were not found in control fish nor in fish exposed to lower concentrations that were fixed and processed identically to the fish represented in this figure.

tissue. Certain specimens showed evidence of possible leukocytic invasion of basal lamellar epithelium.

Final conclusions on the histopathological effects of heptachlor in the sheepshead minnow cannot be drawn from the relatively small number of fish examined in this study. However, initial results strongly suggest that this fish demonstrates histopathological characteristics not observed in control fish at the higher levels of heptachlor exposure (3.5 $\mu g/\ell$ and 4.3 $\mu g/\ell$). Further tests utilizing larger numbers of fish would be desirable to confirm or refute these observations.

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