

**A Comparative Evaluation
of Biomarker Methods
Using Fish Captured from
the Los Angeles Harbor Area**
(Goby Biomarker Study)

Final Report

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by

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Executive Summary

The Goby Biomarker Study was conducted as part of the Bay Protection and Toxic Cleanup Program (mandated by the California State Legislature in 1989). The project was designed to assess the impact of sediment contaminants on fish, with special emphasis on evaluation of bay gobies (*Lepidogobius lepidus*) as a potential indicator species for the California coast. The study area included nine sites in the Los Angeles Harbor area. Sediments were analyzed by the California Department of Fish and Game for metals, pesticides, polyaromatic hydrocarbons, and polychlorinated biphenyls. In addition, pore water was tested for metals and sediments assayed for toxicity to amphipods. Analysis revealed that six sites were moderately to severely contaminated and designated as impacted. The remaining three were substantially less contaminated and were designated as reference sites.

A total of 127 fish were collected in October 1993. None of the sampled fish were bay gobies. Instead, four different teleost species (yellowfin gobies, white croakers, tonguefish, and basketweave cusk-eels) and one elasmobranch (round stingrays) were caught. Contaminant exposure was assessed: 1) grossly via determination of hepatosomatic index (HSI), gonadosomatic index (GSI), and condition index (CI); 2) biochemically via evaluation of cytochrome P4501A (CYP1A) induction with the ethoxyresorufin O-deethylase (EROD) assay; and 3) histologically via examination of liver and spleen. In addition, to augment the EROD assay, P450 immunohistochemistry was used to localize CYP1A induction in tissue sections. All organ and tissue samples were assayed blind and site codes revealed only when analyses were finished.

The most striking lesions were splenic lymphoid and myeloid necrosis. Lesions were observed in all five species and average scores were consistently higher in fish from contaminated sites. Since both cell lines are major components of the immune system, their loss probably results in significant immunosuppression and increased susceptibility to infections. Hepatic lesions were not as severe, but three (eosinophilic cytoplasmic inclusions, megalocytes, and hyalinization of vessel walls) were consistently associated with impact sites. Both splenic and hepatic lesions were taken as direct evidence of deleterious effect.

Of the two methods used to evaluate CYP1A activity, P450 immunohistochemistry proved more valuable and revealed clear differences between impact and reference sites. CYP1A was induced in all (gill, spleen, gonad, liver, kidney, and intestine) organs examined and scores were consistently and sometimes significantly higher in fish from impact sites. In contrast, EROD activity was often erratic and only when activity was evaluated based on the predominant species collected did differences emerge between reference and impact sites.

Of the three indices examined, only HSI distinguished between impact and reference sites. HSI was consistently higher in croakers from impact sites. GSI is a valuable biomarker, but was not useful in this study because fish were often so small that obtaining accurate gonad weights was difficult or impossible.

Overall, the biomarker approach was effective in separating reference from impact sites and enabled us to assess both contaminant exposure (CYP1A induction) and effect (splenic and hepatic lesions). Although we were unable to identify a single "indicator" species which could be used throughout California coastal waters, all five species examined did have lesions consistent with contaminant exposure. Recommendations for future studies include: 1) development of species priority lists based on habitat, availability, and responsiveness; 2) increasing sample size to allow for valid comparisons when multiple species are used; 3) aging fish to exclude age-related lesions; and 4) expanding organ sampling to thoroughly assess damage to the immune system.

Goby Biomarker Study

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Introduction

The Bay Protection and Toxic Cleanup Program (BPTCP) was mandated by the California State Legislature in 1989. The BPTCP is administered by the California State Water Resources Control Board, in cooperation with the state's Regional Water Quality Control Boards, California Department of Fish and Game, and Office of Environmental Health and Hazard Assessment. This study ("Goby Biomarker Study") was one of several special studies conducted as part of the BPTCP, but was also planned and conducted in cooperation and partial funding from two National Oceanic and Atmospheric Administration's (NOAA's) programs: National Status and Trends Program; and the Coastal Ocean Program.

Hundreds of samples (primarily sediment) from California's coastal bays and estuaries have been collected and analyzed since the BPTCP began its field sampling effort in 1992, with most of the assessment effort focused on toxic "hot spot" screening. Results have identified numerous coastal locations, throughout the state, which were termed impacted, as demonstrated by sediment contaminants, benthic community data, and laboratory toxicity bioassays.

Included in the field studies were about 100 sampling stations, nine of which were selected for this study with fish. All nine sites were in the Los Angeles Harbor area (see Map 1). Sediments were analyzed for metals (appendix 1), pesticides (appendix 3), polyaromatic hydrocarbons (appendix 4), and polychlorinated biphenyls (appendix 5). In addition, pore water was tested for metals (appendix 2) and sediments assayed for toxicity to amphipods (appendix 6). Analysis of the data revealed that six sites were moderately to severely contaminated and designated as impacted (appendices 6-10). The remaining three were substantially less contaminated and were designated as reference sites.

In order to assess the impact of sediment contaminants on fish, it was proposed that bay gobies (*Lepidogobius lepidus*) be collected, at all nine Los Angeles sites, and evaluated by a variety of biomarker assays. Although several species of marine fish were considered, bay gobies were selected as the target species of choice because their habitat (burrows in the mud), habits (territorial), distribution (throughout the California coast), relative abundance, and ease of capture (by bottom trawls). Contaminant exposure was assessed grossly via evaluation of condition and gonado-somatic indices, biochemically via analysis of cytochrome P4501A induction, and histologically via examination of liver and spleen. The ultimate goal of the study was to evaluate bay gobies as a potential "indicator species" for contaminated marine ecosystems along the California coast.

Objectives

Objective 1: To determine the type and frequency of histopathological disorders, enzyme levels, and contaminant levels at reference and study sites.

- Approach:
- 1) To determine type and frequency of histopathological disorders; fish were necropsied, samples of liver and spleen fixed and paraffin processed, sections histologically evaluated for the presence of lesions, and lesions scored semi-quantitatively.
 - 2) To determine enzyme levels; liver samples were homogenized and an EROD assay run to determine level of P450 induction. In addition, step-sections of paraffin blocks were cut and immunohistochemistry run using a monoclonal antibody specific for P450.
 - 3) To determine contaminant exposure; fish carcasses, following organ removal, were frozen and assayed.

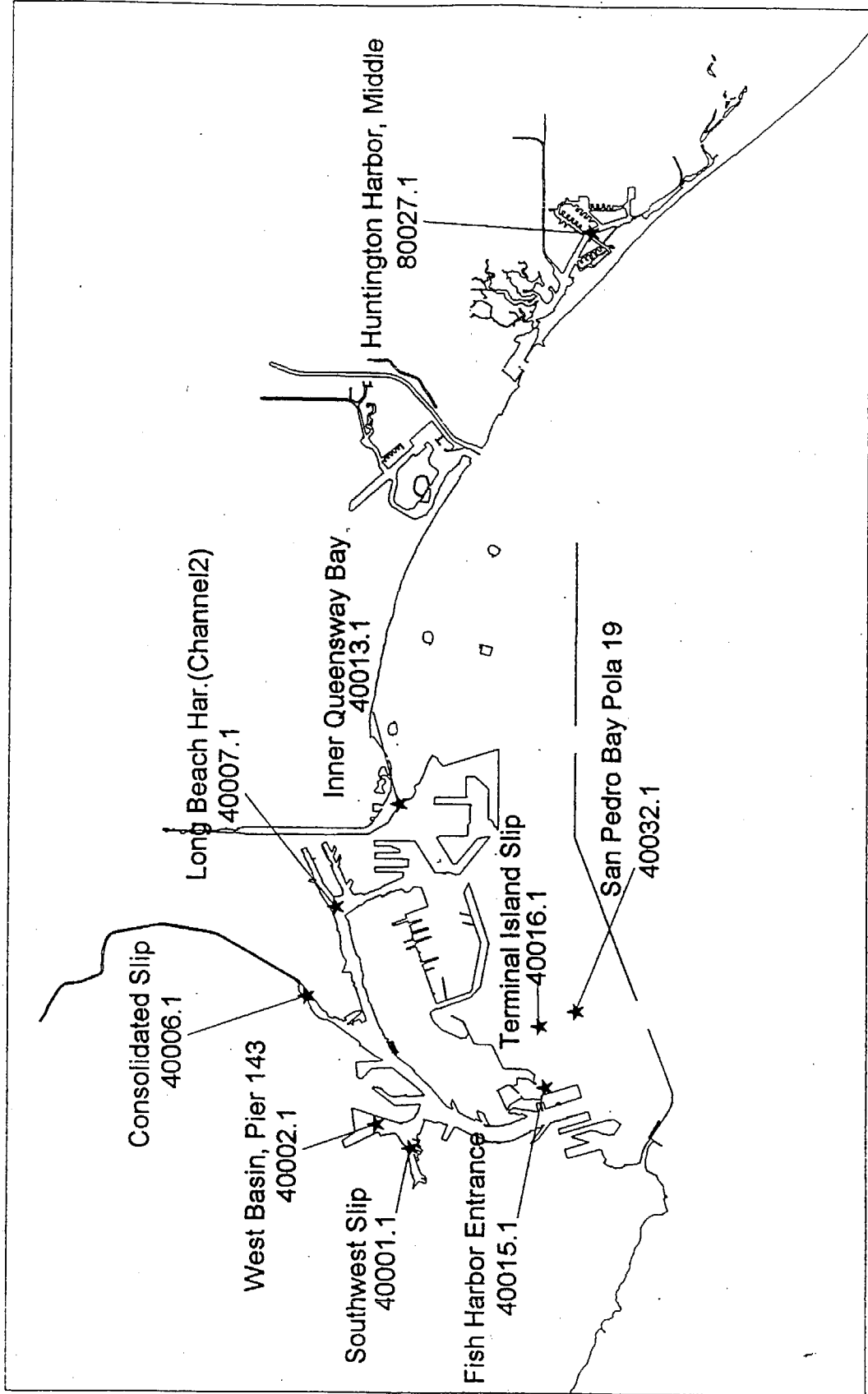
Objective 2: To determine the appropriateness of goby biomarkers for use as indicators of bay and estuarine pollution.

Approach: Evaluate the various gross (HSI, GSI, and CI), biochemical (EROD), histologic (liver and spleen histopathology), and immunohistochemical (P450) assays used in this study to determine if they can differentiate impact from reference sites, and if those differences are relevant (ie. consistent with contaminant exposure) and statistically significant.

Specific Data Evaluation Includes Determination of:

1. the relative bioaccumulation of sediment-associated toxicants in the tissues of sampled fish
2. the relative performance of each of the biomarkers
3. the presence/absence of statistically significant results
4. the relative degree of severity of effects observed at each site
5. the relationships between the sediment, chemistry, and biological data

MAP 1
Fish Sampling Locations



Materials and Methods

Fish Collection: Fish collection was done by California Department of Fish and Game from a 19 ft Boston Whaler and sampling was conducted over a period of five days in October 1993. Initial collections were made using a fine mesh net (eye size approximately 1 cm) and beam trawls. A larger mesh net (approximately 2 cm eye) was also tried with both beam and otter trawls. Following capture, fish were held on the boat and maintained with frequent water changes. When sampling was complete, fish were transferred to the Southern California Coastal Waters Research Project (SCCWRP) laboratory where they were held in flow-through fiberglass tanks.

Necropsy and Tissue Sampling: Although provided with a NOAA protocol regarding a "clean technique" for necropsies, some equipment and supplies (ie. teflon cutting boards and 10% HCl) were not available and some procedures proved impractical. As such, NOAA protocols were modified and streamlined. A large plastic cutting board was used to perform the initial dissection. A second smaller polyethylene board was used to separate individual organs for formalin fixation. Between fish, cutting boards were wiped clean with paper towels. Occasionally, the smaller board was briefly scrubbed and rinsed in tap water. Between sites, the smaller cutting board was wiped clean, rinsed in tap water, and dried with a paper towel. The larger board was scrubbed clean, then rinsed with tap water, distilled (DI) water, 10% nitric acid, methanol, and milli-Q (MQ) water between sites. Two sets of instruments were used, one for the initial dissection (opening the abdomen), and a second set for the final dissection (separating organs). Between fish, the first set was wiped clean and rinsed in tap, DI, and MQ water. The second set was wiped clean between fish. Between sites, both sets were wiped clean, rinsed with tap and DI water, rinsed with 10% nitric acid and methanol, and then rinsed with MQ water.

Fish were killed with an overdose of ticaine methanesulfonate (MS222), rinsed in tap, DI, and MQ water, then transferred to a clean paper towel covering the larger cutting board. Dissections were made using two sets of gloves, an inner latex set and an outer polyethylene set. With each fish, a new pair of polyethylene gloves was used. Following the initial dissection, polyethylene gloves were discarded and the final dissection made with the latex set. Latex gloves were used until torn or extremely soiled.

Euthanized fish were weighed ("total weight"), measured for standard length (SL), and placed in right lateral recumbency on the larger board. The left abdominal wall was opened and left operculum removed to expose the gills. Internal organs were briefly examined for gross lesions. Gastrointestinal (GI) tract, liver, and spleen were removed together by severing rectum and esophagus, and then transferred to the smaller cutting board. The heart and gills were then excised. Finally, gonads were excised, weighed, and transferred to the second cutting board. Although initially targeted for histologic analysis, kidneys were not routinely sampled. That decision was based on the small size of many fish and concern that renal excision would result in excessive loss of tissue from the carcass (slated for chemical residue analysis). A few kidney samples were taken if fish were large enough and if kidneys were readily accessible. Following organ removal, the carcass was weighed ("chemistry weight"), placed in a glass jar, and frozen at -20°C.

The final dissection was made on the smaller cutting board. In teleost fish, gall bladders were dissected free of the liver, placed in amber vials, and frozen on dry ice. Bile samples from stingrays were taken by aspirating with needle and syringe. Following bile

sampling, the liver was weighed ("total liver weight") and a piece cut free with razor blade. The smaller section of liver was then weighed ("P450 liver weight") and homogenized (using a hand tissue grinder) with a volume of phosphate buffered saline (PBS) approximately three times the weight of the liver sample. The homogenate was centrifuged for 10 minutes at 11,000 x g and 2°C. The supernatant was decanted into a cryotube and frozen on dry ice.

After the first day, it was apparent that many fish were too small for both liver EROD and histologic analyses. To augment biochemical EROD analysis, it was decided to take samples for P450 immunohistochemistry. The two organs selected for P450 immunohistochemistry were liver and gill. Liver histopathology samples were fixed in formalin, along with spleen, heart, and gill in one 20 ml glass scintillation vial (vial "A"). Gonad, GI tract, and any skin or kidney samples were fixed in a second vial ("B").

Histopathology: Histology samples were fixed in 10% formalin for 14 to 17 days. Prior to cassetting, a list of random numbers was generated and one assigned to each fish (Appendix 11). Each cassette was labelled with a processing number (93H63), a random number (from 1-127), and a letter ("A" = liver, spleen, and gonad; "B" = gill; and "C" = skin). All tissues, except skin, were routinely paraffin processed. Skin samples were decalcified in dilute hydrochloric acid for 24 hours, rinsed in tap water for 24 hours, and then paraffin processed. Paraffin blocks were sectioned at 4 µm and stained with hematoxylin and eosin (HE). All slides were read blind.

An initial screen of 50 slides was used to identify the range of lesions present and to determine which were included in the final score sheet. The initial screen was also used to identify "type lesions" in both liver and spleen. "Type lesions" were specific examples of lesions with emphasis on separating lesion types based on severity. Severity scores were semiquantitative and based on a scale of 0 to 3 (0 = not present, 1 = mild, 2 = moderate, and 3 = severe). Once "type lesions" were identified, specific criteria (size, number, composition) for severity scores were generated and the slides read. Two organs, liver and spleen, were scored for lesions. Four other other organs (skin, kidney, gill, and gonad) were screened for lesions, but were not scored.

P450 Immunohistochemistry: Sections for P450 immunohistochemistry were taken from the same paraffin blocks used for routine histopathology. There were two blocks for each fish and both blocks were cut at 4 µm and mounted on a single "Superfrost" (Fisher Scientific) electrically charged glass slide. Slides were air dried and shipped to Dr. John Stegeman in Woods Hole, Massachusetts. Immunohistochemistry was performed with a monoclonal antibody (MAb 1-12-3p5) developed against scup (*Stenotomus versicolor*) cytochrome P4501A. All slides were stained using a standard ABC (avidin-biotin complex) technique. Slides were read and scored blind. P450 score was reported as a staining index by multiplying the "occurrence" by the "intensity" (O x I) of the stain.

EROD: Frozen liver samples (homogenate supernatant) were shipped to Dr. John Stegeman in Woods Hole, Massachusetts on dry ice. 7-ethoxyresorufin O-deethylase (EROD) activity was evaluated either spectrophotometrically, according to the method of Klotz *et al.* (1984), or flurometrically, by modifications of the method of Eggens and Galgani (1992) using Millipore Cytofluor fluorescent plate reader. Hepatic EROD activity was reported as pmol/min-mg.

Indices: Gross measurements included standard length (SL in millimeters), body weight (BW in grams), liver weight (LW), and gonad weight (GW). Gross measurements were used to

determine three indices; hepatosomatic index (HSI), gonadosomatic index (GSI), and condition index (CI). HSI was determined by taking LW/BW and multiplying by 100. GSI was determined by taking GW/BW and multiplying by 100. CI was determined by taking BW/SL³ and multiplying by 100,000.

Statistical Analysis: Dr. Neil Willits (Senior Statistician, UCD) was the statistical consultant for this study. Two types of data were generated, non-continuous and continuous. Non-continuous data included semi-quantitative scores used with both the histopathology lesions in spleen and liver (range = 0-3), and similar semi-quantitative P450 immunohistochemistry scores (range = 0-15). Continuous data included EROD activity, gross measurements, and indices.

Non-continuous data was analyzed using Principal Components Analysis (PCA), followed by analysis of variance (ANOVA) or multivariate analysis of variance (MANOVA) of the scale values derived from PCA. This type of analysis has several advantages including: 1) accounting for the presence and severity of lesions; 2) identifying the source of variability; 3) identifying the most significant lesions; and 4) determining the significance of "impact" versus reference site differences with a single P value. PCA was run separately on the lesion (spleen and liver) data and on the P450 immunohistochemistry data. Four principal components were used with the lesion data and only one for the P450 analysis (only one component accounted for appreciable variability). Two types of post hoc comparisons among sites were used following PCA and MANOVA; Tukey's and Least Square Means. The disadvantage to the Tukey comparisons are they do not adjust for possible impact of gender and species. The Least Square Means comparisons do adjust for gender and species, and were considered the more relevant of the two.

Continuous data was analyzed using MANOVA, and site to site comparisons made using Least Square Means.

Results

Fish Collection: Initial collection attempts were made at night on 10-4-93 at sites 40015 and site 40006. Trawling speed and time were varied. Through trial and error, a 15 minute trawl at a speed just above idle provided the best results. Faster speeds tended to "fly" the sled above the bottom, and longer trawls resulted in excessive mud in the "caught end" of the net. Longer trawls also resulted in higher morbidity and mortality.

10 hours of sampling yielded no Bay gobies and only 13 yellowfin gobies. Following a meeting between UCD, AMS, and CADFG personnel, and a subsequent conference call on 10-5-93 with NOAA, the decision was made to go with multiple species. The four species selected were: 1) yellowfin goby (*Acanthogobus flavimanus*); 2) basketweave cusk-eel (*Ophidion scrippsae*); 3) California tonguefish (*Symphurus atricauda*); and 4) white croaker (*Genyonemus lineatus*). Selection was based on; relative abundance and availability at the initial reference site (site 40015), habits and habitat, size, and distribution. In addition, a fifth species - round stingrays (*Urolopus halleri*) - was added because it was the predominant species at site 80027 and no croakers, cusk-eels, or tonguefish were caught. Although the stingrays were relatively large (they could not be weighed on the balance used), rays did fit some selection criteria being bottom dwellers and relatively abundant.

The final collection total of 127 fish included; 31 yellowfin gobies, 49 white croakers, 7 basketweave cusk-eels, 30 tonguefish, and 10 round stingrays (Table 1). Fish were collected from nine different sites over a period of four days, from 10-4-93 to 10-7-93. Sorting the data by site (Appendix 12) revealed that the distribution of species was highly variable. Two examples are site 40013, where only white croakers were caught, and site 80027 where 10 of 12 fish were stingrays.

Necropsy and Tissue Sampling: Histopathology samples included; 127 liver, 127 spleen, 127 heart, 127 gill, 127 gonad, 127 GI tract, 14 skin, and a small (<20) number of kidney samples (Appendix 13). 85 liver samples were collected for EROD analysis and 212 (106 liver and 106 gill) samples taken for P450 immunohistochemistry. The total number of liver and gill samples for P450 immunohistochemistry was less than the histopathology totals (127) because 21 fish died prior to necropsy and tissues were mildly to severely autolyzed. 99 gall bladder/bile samples were taken, frozen, and archived at -80°C.

Table 1. Goby Biomarker Study. Sample Number and Species of Teleost Fish Collected from Nine Sites in the Los Angeles Harbor area.

#	Site #	Site Name	Fish Species (N)							total
			Goby	Croaker	Cusk-eel	Tonguefish	Stingray			
1	40001	Southwest Slip	3	2	0	0	0	0	5	
2	40002	West Basin Pier 143	4	5	0	6	0	0	15	
3	40006	Consolidated Slip	7	11	0	0	0	0	18	
4	40007	Long Beach Harbor, channel 2	2	5	5	3	0	0	15	
5	40013	Inner Queensway Bay	0	15	0	0	0	0	15	
6	40015	Entrance to Fish Harbor	12	3	0	0	0	0	15	
7	40016	Term Island Stop	1	3	1	17	0	0	22	
8	40032	Pola 19	0	5	1	4	0	0	10	
9	80027	Huntington Harbor, middle	2	0	0	0	10	0	12	
		total	31	49	7	30	10	0	127	

Histopathology - Results:

Definitions

Basophilic: Blue to purple color when sections are stained with hematoxylin and eosin (HE).

Congestion: Stasis of blood in a blood vessel (usually a vein).

Diffuse: Spread out.

Eosinophilic: Red to orange color when sections are stained with HE.

Focus: A small area.

Granuloma / granulomatous: Granuloma refers to a mass lesion centered around either a foreign body (ie. a parasite) or cluster of bacteria (ie. tuberculosis tubercles). The wall of a granuloma is composed of a mixture of macrophages, giant cells (fused macrophages), and a peripheral rim of connective tissue. Granulomatous refers to a more diffuse inflammatory reaction composed of primarily macrophages, but mixed with some giant cells and lymphocytes.

Hyalinization: Hyalinization refers to thickening or infiltration of a tissue or organ by an acellular, hyaline (glassy) material which can be composed of a variety of materials (ie. immune complexes or amyloid).

Hyperplasia: Hyperplasia is an increase in the number of cells of a particular organ or tissue. Hyperplasia is differentiated from neoplasia (cancer) in that it is usually a reversible condition.

Inflammation: Inflammation refers to the influx, into an organ or tissue, of inflammatory cells. Inflammatory cells can be broadly classified as mononuclear (macrophages, lymphocytes) and polymorphonuclear (PMN) cells (neutrophils, eosinophils, basophils), which have "segmented" nuclei (pinched in multiple places).

Karyomegaly: Enlarged nucleus.

Karyorrhexis: Karyorrhexis is the fragmentation of a pyknotic nucleus in a dead cell.

Lymphocyte: Lymphocytes are mononuclear inflammatory cells which specifically target foreign antigens. They can be divided into B-cells which produce antibody and T-cells which do not produce antibody, but are responsible for cell-mediated immunity.

Macrophage: Macrophages are mononuclear inflammatory cells which have the capability of phagocytizing foreign material or micro-organisms.

Necrosis: Cell death.

Definitions continued:

Nematode: Round worm.

Neoplasia / neoplasms: Neoplasms are tumors and can be divided into benign and malignant. Cancer usually refers to malignant neoplasms.

Megalocyte: Megalocytes are excessively large cells which often have large (karyomegalic) nuclei.

Phagocytosis: Phagocytosis is the process whereby by a cell (usually an inflammatory cell) surrounds, engulfs, and digests a small fragment of foreign material or a micro-organism (ie. bacteria).

Pyknosis: Pyknosis is one of the initial changes the nucleus of a dying cell undergoes. Pyknosis involves nuclear shrinkage with condensation of chromatin and hyperpigmentation (usually dark blue-black with HE stain).

Tinctorially Altered Foci: Tinctorially Altered Foci (TAF) are small focal preneoplastic lesions in the liver which are primarily distinguished by color (ie. basophilic foci = blue; eosinophilic foci = red).

Histopathology - Results:

I. Splenic Lesions:

A. Splenic Necrosis

1. **Lymphoid Necrosis (LN):** Lymphoid necrosis was a surprisingly common lesions. The necrosis involved individual lymphocytes in the white pulp and was characterized by cellular shrinkage, nuclear pyknosis, and karyorrhexis (Figures 1 & 2). Necrotic lymphocytes were often phagocytized by macrophages and macrophage aggregates were often centered within the white pulp.

Type lesions for LN:

- a. Score = 0; no necrosis (type specimens = 57*)
- b. Score = 1; 1-4 necrotic lymphocytes per 150 micron diameter field in a lymphoid follicle (type specimen = 76)
- c. Score = 2; 5-10 necrotic lymphocytes per 150 micron diameter field (type specimen = 1)
- d. Score = 3; >10 necrotic lymphocytes per 150 follicle diameter field (type specimen = 69)

* "Type specimen" numbers refer to UCD random ID numbers; ie. 57 = 93H63-57.

2. **Red Pulp Necrosis (RPN):** The red pulp of the spleen is composed of hematopoietic cells, including both red and white blood cells in various stages of maturation from stem cells through well-differentiated blood cells. Careful examination revealed that many fish had necrosis of individual cells in the red pulp, similar to that observed in the lymphoid follicles. Necrotic hematopoietic cells were characterized by nuclear pyknosis and karyorrhexis, and phagocytosis by individual macrophages (Figures 3 & 4).

Type lesions for RPN:

- a. Score = 0; no necrosis (type specimen = 18)
- b. Score = 1; 1-4 necrotic cells per 150 micron diameter field of hematopoietic tissue (type specimen = 23)
- c. Score = 2; 5-10 necrotic cells per 150 micron diameter field (type specimen = 9)
- d. Score = 3; >10 necrotic cells per 150 micron diameter field (type specimen = 73)

B. Splenic Hyperplasia:

1. **Periarteriolar macrophage sheath hyperplasia (PSH):** The periarteriolar macrophages are generally considered antigen presenting cells, in mammalian spleens, and are believed to process antigen and present it to T-cells. The lesions which occurs in some fish appears to be an increase in the thickness of the sheath surrounding small arterioles in the spleen (Figure 5). The increased sheath thickness is the result of increased numbers of macrophages forming multiple concentric layers around the arteriole.

Type lesions for PSH:

- a. Score = 0; 0-2 layers of APC per arteriole (type specimen = 18)
 - b. Score = 1; 3-4 layers of APC per arteriole (type specimen = 4)
 - c. Score = 2; 5-6 layers of APC per arteriole (type specimen = 6)
 - d. Score = 3; >6 layers of APC per arteriole (type specimen = 3)
2. **Lymphoid hyperplasia (LH):** Lymphoid hyperplasia can be characterized by increase in the number and size of the lymphoid aggregates in the spleen. Although lymphoid hyperplasia was left on the final score sheet, none of the spleens examined had any significant hyperplasia and there are no "type specimens."

C. Vascular Lesions

1. **Splenic Congestion (SC):** Splenic congestion was a relatively common finding and characterized by dilation of splenic blood vessels, and stasis and pooling of blood in the red pulp (Figures 6 and 7). The white pulp was often obscured with severe congestion, making it difficult to evaluate.

Type lesions for SC:

- a. Score = 0; no congestion (type specimen = 18)
 - b. Score = 1; mild red pulp congestion, but no expansion or compression of white pulp (type specimen = 10)
 - c. Score = 2; moderate congestion resulting in partial to complete obscuring of interstitial connective tissue and white pulp (type specimen = 44)
 - d. Score = 3; severe expansion of the red pulp with complete loss of interstitium and white pulp, often with bulging capsule (type specimen = 90)
2. **Splenic hemorrhage and necrosis (SHN):** One fish (109) had a large focus of hemorrhage and necrosis in the spleen. The lesion was characterized by an irregular necrotic central mass of pale eosinophilic proteinaceous material and fibrin, surrounded by extravasated red blood cells (figures 8 & 9). This lesion was not included in the final score sheet and there are no "type specimens."

- D. **Splenic macrophage aggregates (SMA):** Macrophage aggregates were a common finding in the spleen and were characterized by accumulations of large macrophages packed with granular green-brown pigment (Figures 10 & 11). Macrophage aggregates were often located within the white pulp, amongst lymphocytes.

Type lesions for SMA:

1. Score = 0; none present (type specimen = 5)
2. Score = 1; 1-5 macrophage aggregates per 50X field, macrophage aggregates are \geq 50 microns diameter (type specimen = 18)
3. Score = 2; 5-10 aggregates per 50X field (type specimen = 27)
4. Score = 3; >10 aggregates per 50X field (type specimen = 4)

- E. **Lymphoid depletion (LD):** Lymphoid depletion was another relatively common lesion characterized by decreased number and size of lymphoid follicles in the spleen (Figures 12 and 13).

Type lesions for LD:

- a. Score = 0; \geq 5 follicles per 50X field with a follicle defined as a cluster of lymphocytes 150 microns in diameter (type specimen = 18)
- b. Score = 1; 3-4 follicles per 50X field (type specimen = 23)
- c. Score = 2; 1-2 follicles per 50X field (type specimen = 8)
- d. Score = 3; \leq 1 follicle per 50X field (type specimen = 10)

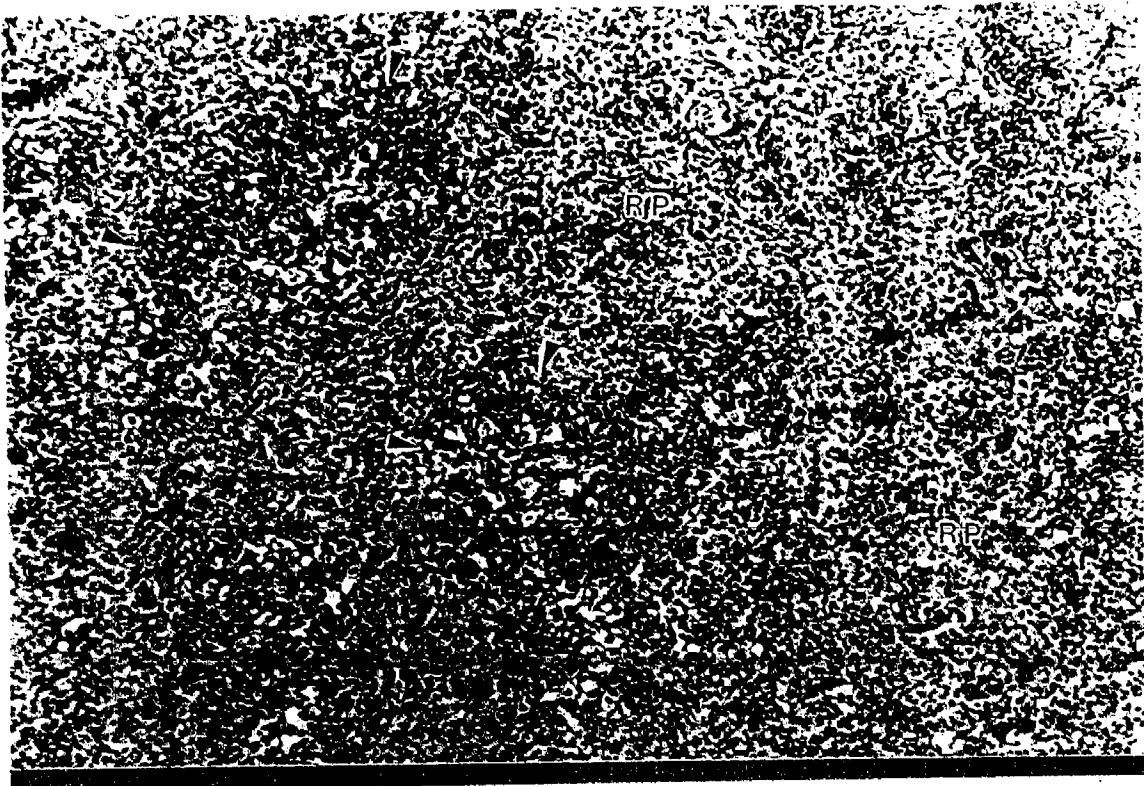


Figure 1. Spleen of fish 93H63-69 (white croaker from site 40013) with severe lymphoid necrosis (arrowheads). The necrosis is confined to the lymphoid follicles (white pulp) and does not involve the hematopoietic tissue of the red pulp (RP). HE 50X.

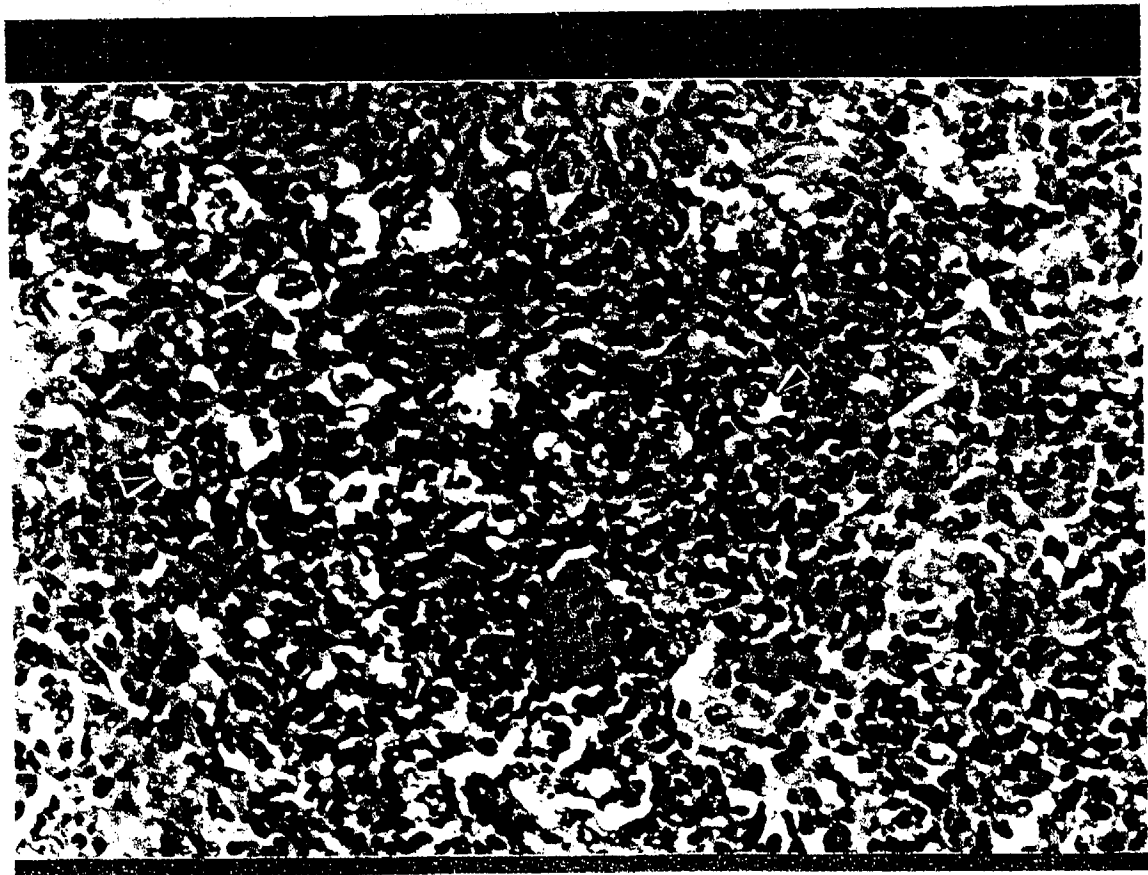


Figure 2. Higher magnification of figure 1. Note the large numbers of necrotic lymphocytes (arrowheads) characterized by cytoplasmic swelling, nuclear pyknosis, and karyorrhexis. HE 100X.

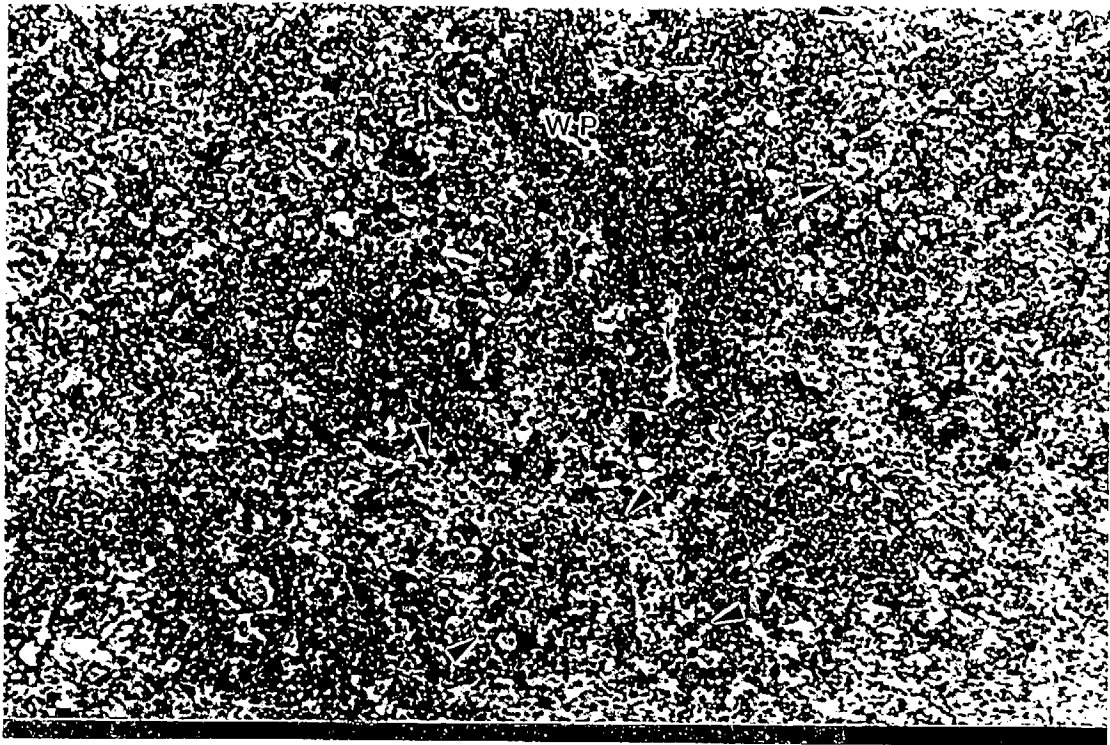


Figure 3. Spleen of fish 93H63-83 (white croaker from site 40002) with severe red pulp necrosis (arrowheads). Note that the lymphoid follicles (white pulp; WP) is only mildly affected with scattered individually necrotic lymphocytes (arrow). HE 50X.

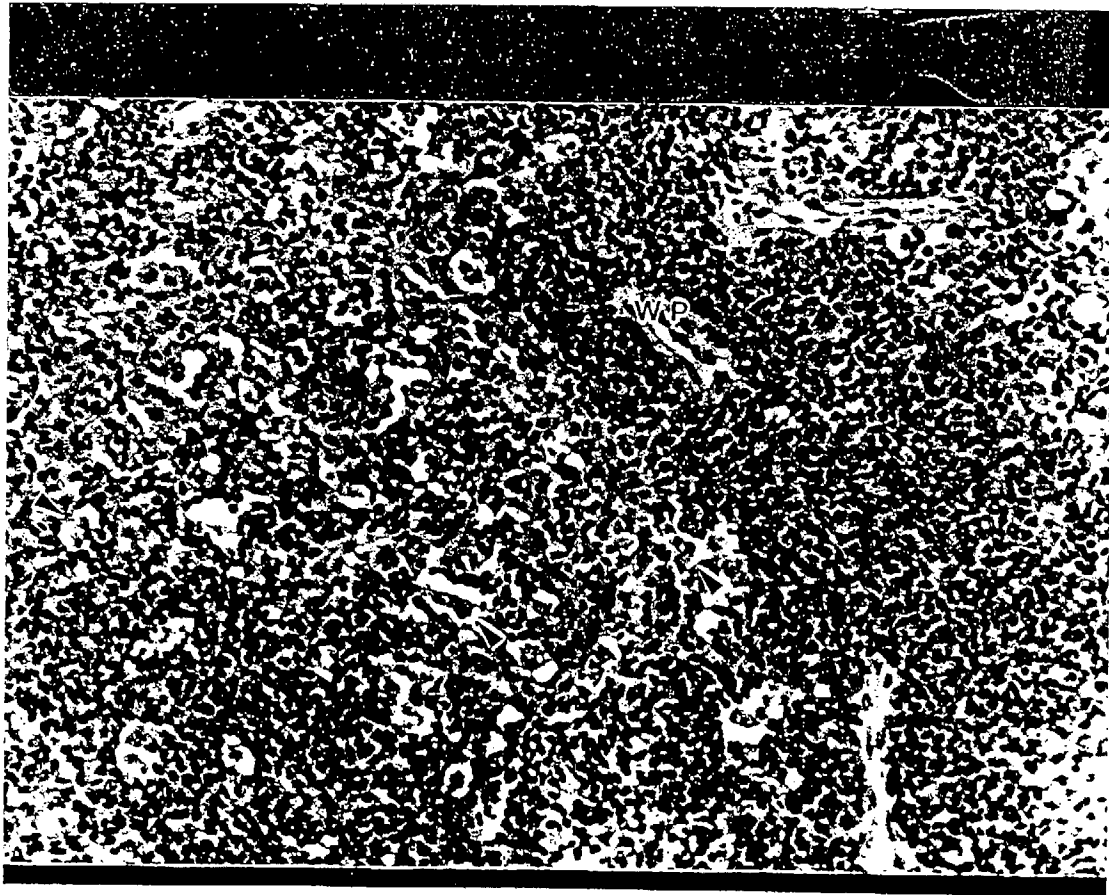


Figure 4. Higher magnification of figure 3. Note the large number of individually necrotic cells (arrowheads) in the red pulp. The lymphocytes in the white pulp (WP) are relatively unaffected. HE 100X.

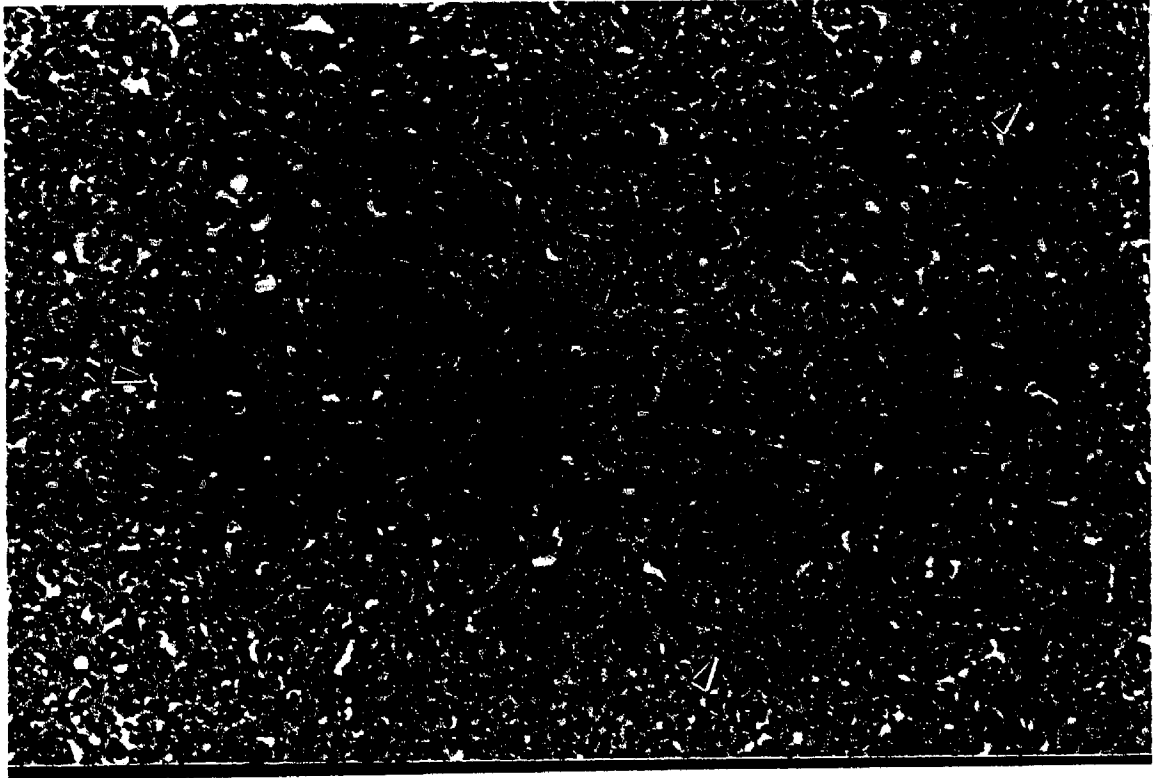


Figure 5. Spleen of fish 93H63-88 (yellowfin goby from site 40015) with moderate periaarteriolar sheath hyperplasia. The arteriole in the center (arrow) has only two layers, but several others (arrowheads) have three to six layers of antigen-presenting cells. HE 100X.

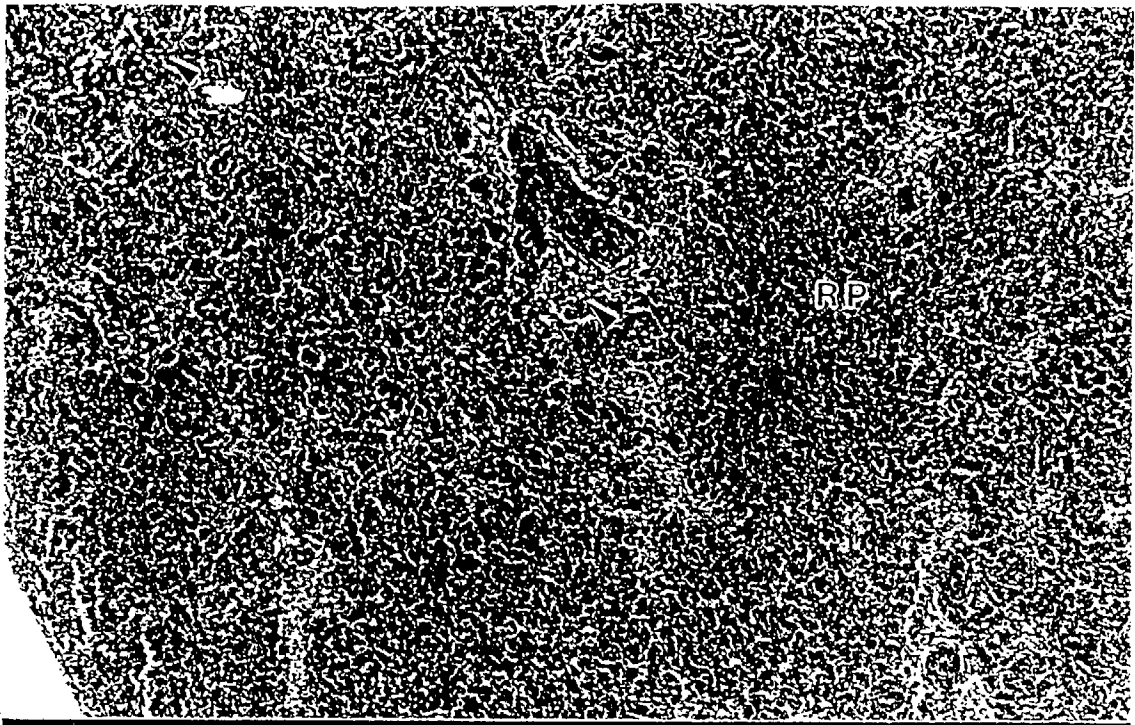


Figure 6. Spleen of fish 93H63-44 (yellowfin goby from site 40015) with severe congestion. The vasculature of the red pulp (RP) is markedly expanded and congested with blood. Lymphoid follicles (arrowheads) are small and often partially obscured by the dilated, congested blood vessels. HE 25X.

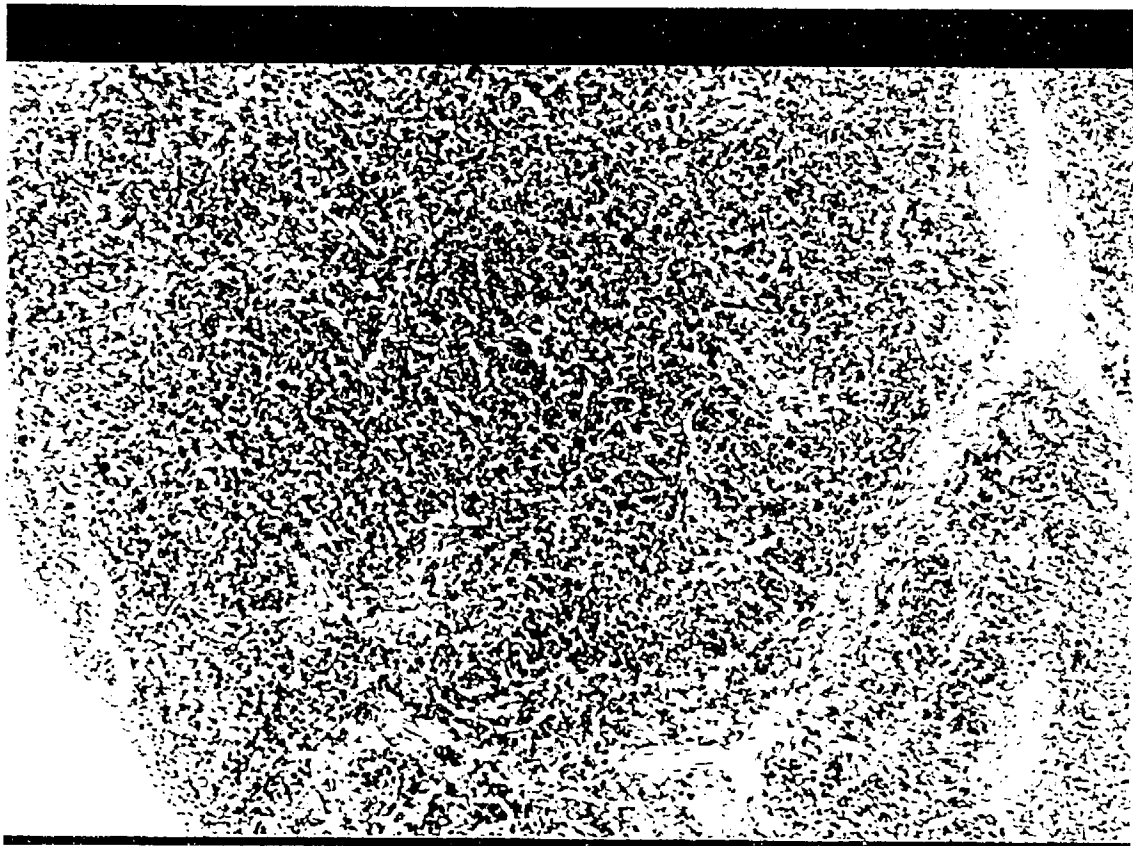


Figure 7. Higher magnification of figure 6. The hematopoietic cells of the red pulp are very difficult to identify because of the severe congestion. HE 50X.



Figure 8. Spleen of fish 93H63-109 (yellowfin goby from site 40002) with two large foci of hemorrhage and coagulation necrosis (arrows). HE 25X.

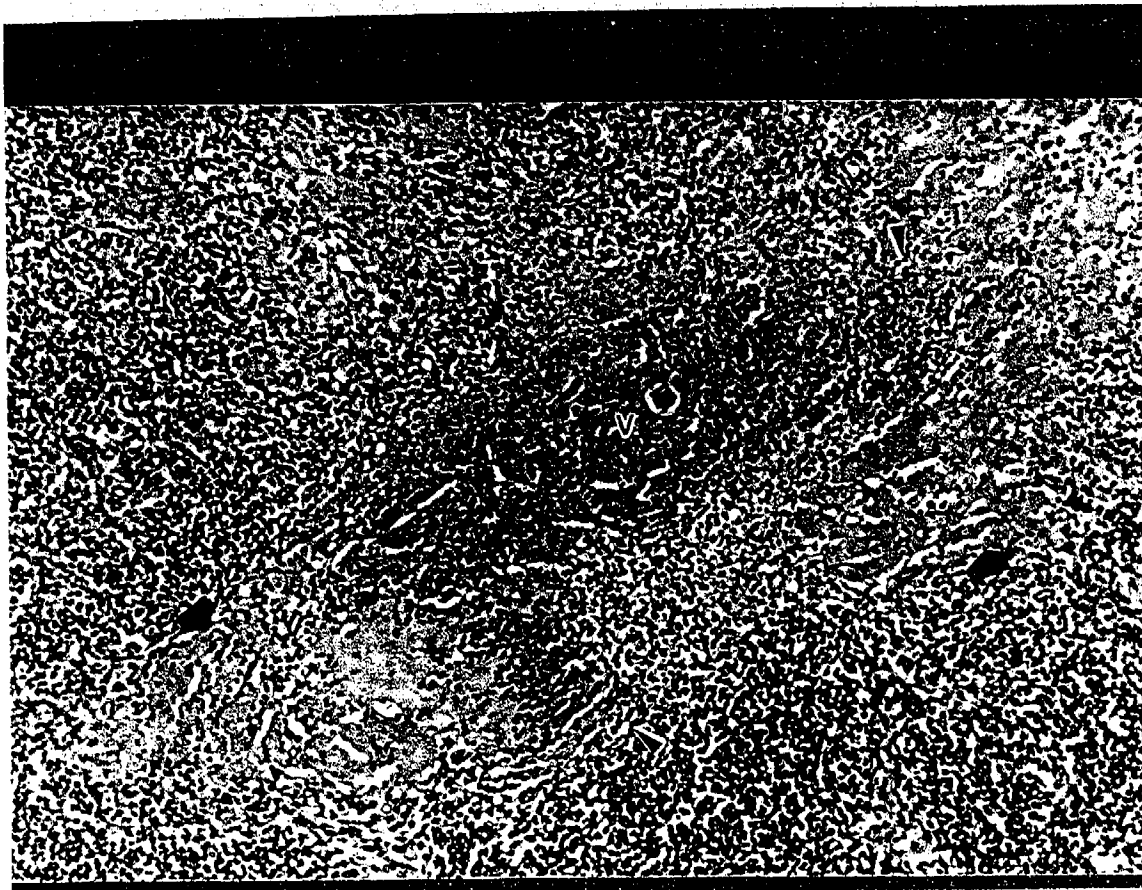


Figure 9. Higher magnification of figure 9. The splenic vein (V) is hemorrhaging (arrowheads), into the parenchyma and there are multiple foci of pale eosinophilic acellular material (arrows) which may represent either serum or necrotic material. HE 50X.



Figure 10. Spleen of fish 93H63-88 (yellowfin goby from site 40015) with large numbers of macrophage aggregates (arrowheads). HE 50X.

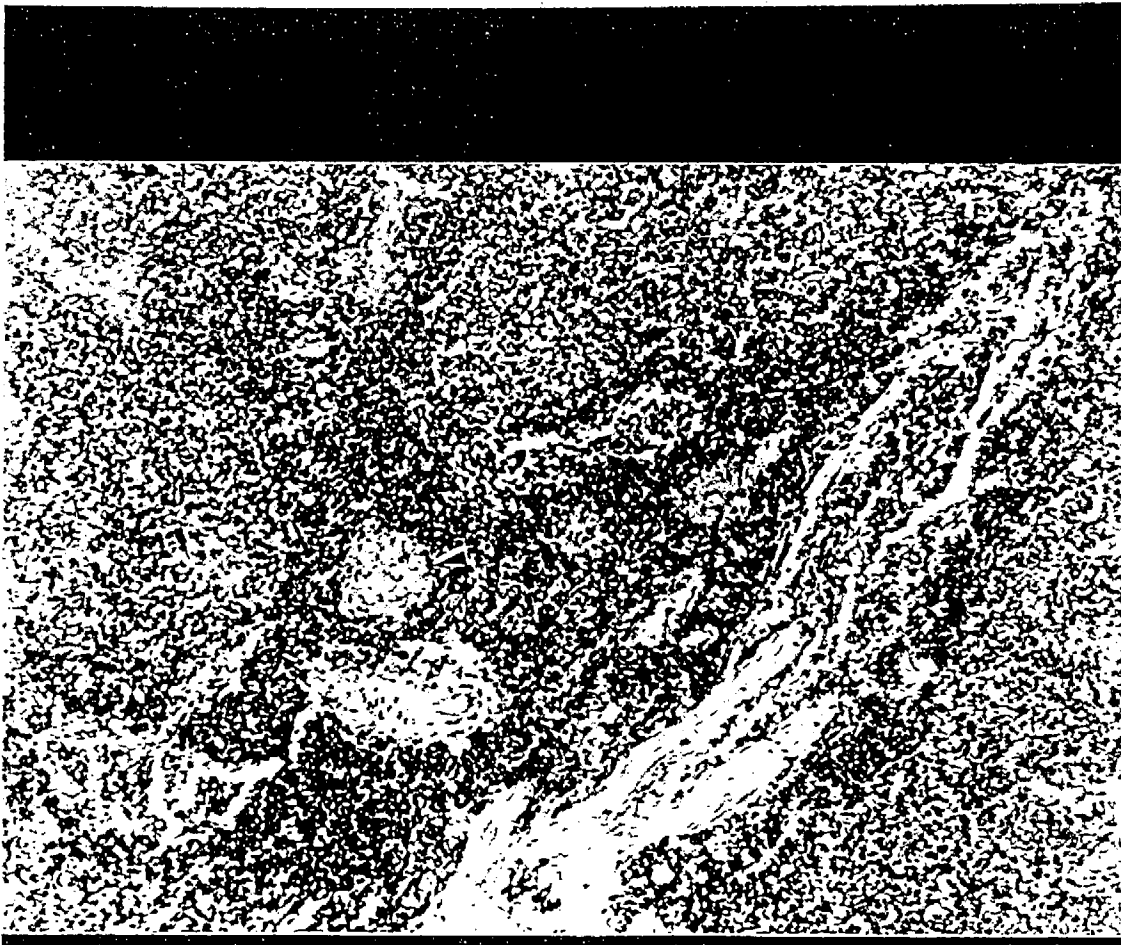


Figure 11. Spleen of fish 93H63-66 (yellowfin goby from site 40006) with a macrophage aggregate (arrowhead) centered within a lymphoid follicle. HE 50X.

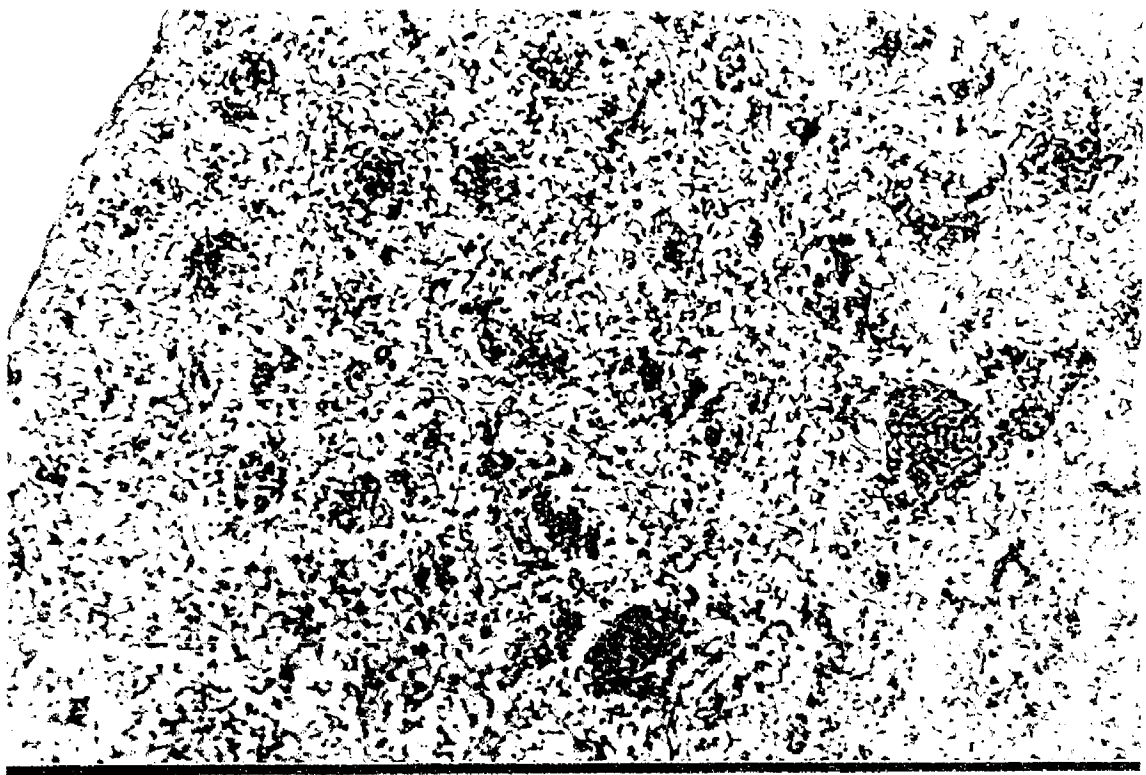


Figure 12. Spleen of fish 93H63-70 (basketweave cusk-eel from site 40007) with severe lymphoid depletion. Note that there are no lymphoid follicles present. HE 50X.

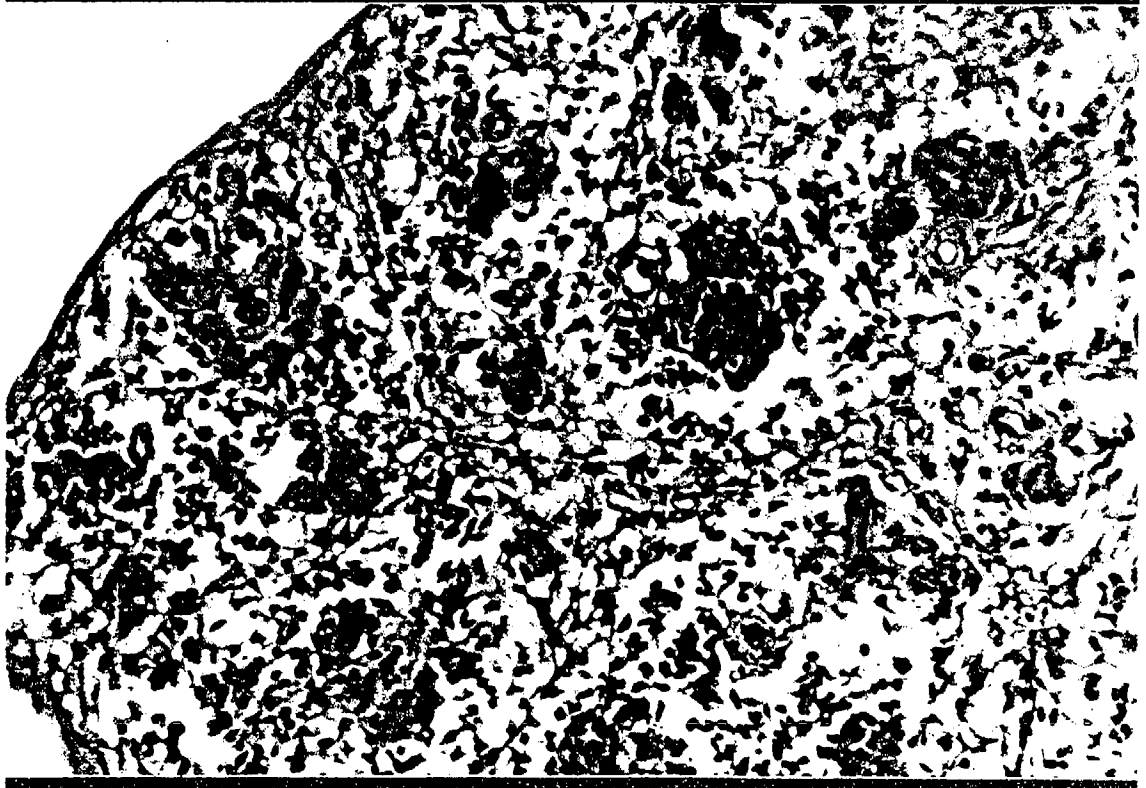


Figure 13. Higher magnification of figure 12. Organized lymphoid follicles are absent and even the red pulp appears depleted of hematopoietic cells. HE 100X.

Histopathology - Results:

Splenic Histopathology Summary: Splenic lesion scores are given in appendix 14. Appendices 14a-14c include scores for all fish sorted on the basis of site. Scores in appendices 14d-14h are sorted on the basis of both species and site.

Average lesion scores are depicted in graphs 1-3. Graph 1a shows average lesion scores for lymphoid necrosis (LN) and red pulp necrosis (RPN) in all fish from all sites. Average scores for both LN and RPN are in general markedly lower in the three reference sites (40015, 40016, and 40032) when compared to the six "impact" sites. The only exception was impact site 40007 which had a lower average LN score than two reference sites (40015 and 40032). Of the three reference sites, site 40015 had the highest LN and RPN scores.

When splenic lesion scores are examined on the basis of both site and species, the same general trend holds, with the majority of impact sites having higher average LN and RPN scores than the three reference sites. Average LN and RPN scores for gobies (graph 1b) at the two impact sites, with sample sizes greater than three (40002 and 40006), were higher than those in the only reference site (40015) with an "N" over three. In croakers (graph 1c), average LN scores were only markedly higher at impact site 1 when compared to the three reference sites, but RPN scores were consistently higher in four of five impact sites where croakers were collected. Tonguefish (graph 1d) were collected from four sites and average LN and RPN scores at the two impact sites (40002 and 40007) were elevated above scores from two reference sites (40016 and 40032).

Graph 2a depicts average lesion scores for splenic macrophage aggregates (SMA) and splenic lymphoid depletion (LD). There were no consistent trends with either lesion. Average LD scores were high at two reference sites (40016 and 40032) and one impact site (40007). SMA scores were highest at reference site 40032. Impact site 80027 (primarily composed of stingrays) had the lowest SMA and LD scores.

Additional analysis of SMA and LD scores, with respect to species, uncovered trends in croakers (graph 2c) and tonguefish (graph 2d). In croakers, there were no consistent findings with regard to SMA, but croakers from the impact sites tended to have more LD than those from the reference sites. LD scores in croakers from four impact sites were slightly higher than scores from reference site 40016, and the other two reference sites had no LD. Average SMA scores in Tonguefish were highest in fish from reference site 40032, second highest in reference site 40016, and lowest at the two impact sites. Interestingly, the same pattern with respect to size (both standard length and body weight) also occurs in Tonguefish, with the largest (and presumably oldest) fish coming from reference site 40032, second largest from reference site 40016, and smallest from the two impact sites (Graph 17c). LD scores were slightly lower in tonguefish from the two impact sites.

Graph 3a shows average lesion scores for periarteriolar sheath hyperplasia (PSH) and splenic congestion (SC). There were no consistent trends with respect to these two lesions. Sorting of the data, based on species, did not reveal any trends in gobies (graph 3b). Analysis of average scores in croakers (graph 3c) and tonguefish (graph 3d) reveals that fish from reference sites tend to have higher SC scores than fish from impact sites. The only exception was impact site 40013 which had the highest average SC score in croakers.

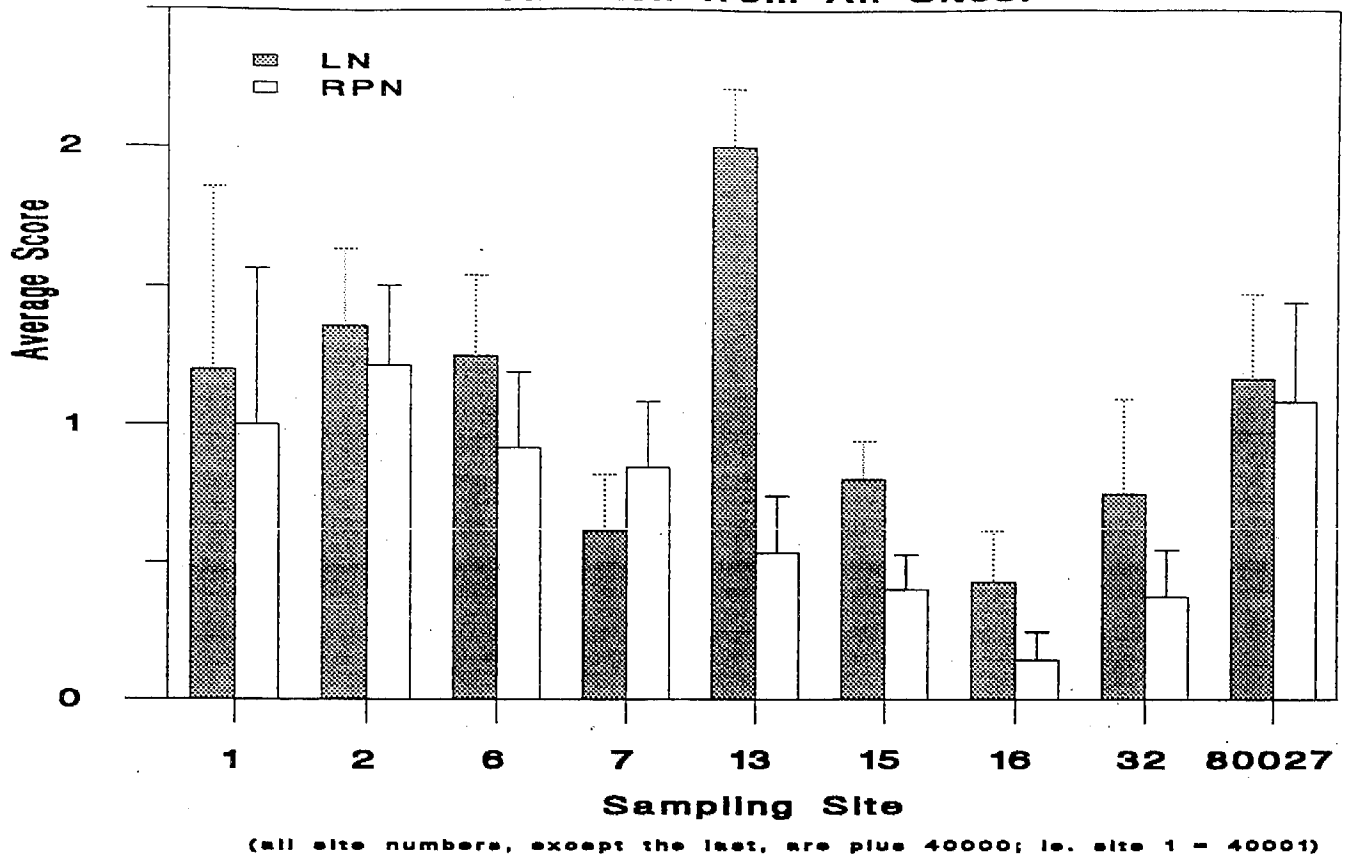
Histopathology - Results:

Splenic Histopathology - Statistics: The SAS statistical program was used to analyze for differences in individual scale values with MANOVA, nested for site effect and blocked for species. Due to missing values only 115 of 127 fish were used in the analysis. With principal components analysis (PCA), a correlation matrix, eigenvalues of the correlation matrix, and eigenvectors were calculated. From the proportion part of the "eigenvalues of the correlation matrix," the first principal component accounted for 11.8% of the variability; the second principal component, 10%; the third principal component, 8.9%; and the fourth, 7.7%.

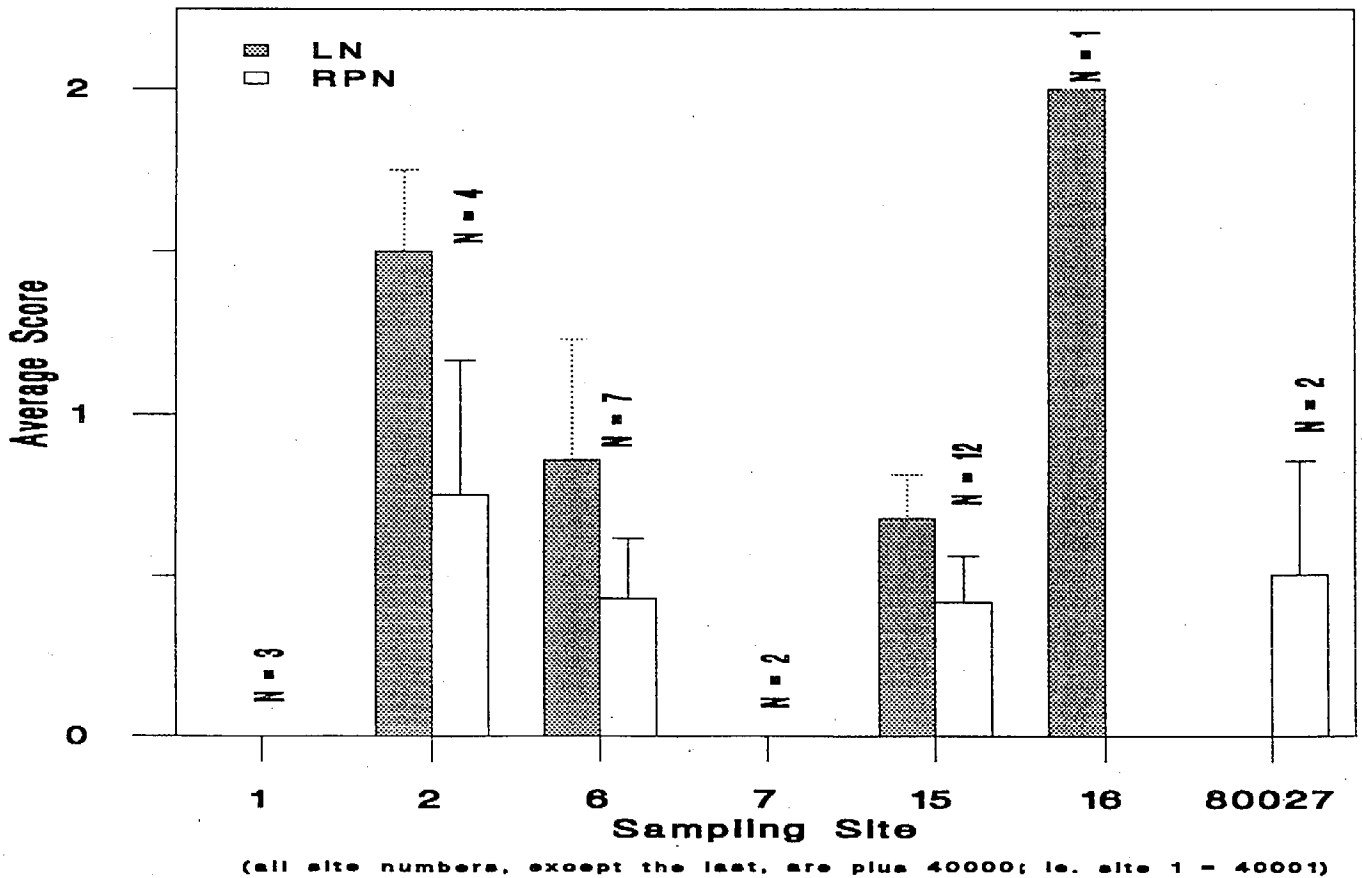
From individual scale values for the first principal component, splenic lymphoid necrosis (LN) and red pulp necrosis (RPN) were most important (eigenvectors with the greatest absolute values contribute most to variability). With the second principal component, hepatic glycogen depletion (GD) and lipidosis (LIP) were most important. With the third principal component, hepatic melanomacrophage aggregates (MM) and splenic periarteriolar sheath hyperplasia (PSH) were most important. With the fourth principal component, hepatic macrophage aggregates (HMA) and splenic macrophage aggregates (SMA) contributed most to variability.

MANOVA, using all four principal components, showed that there were significant ($P < 0.05$) differences among the nine (six impact and three reference) sites, but not any overall species effect. Comparisons among sites, with respect to the first principal component, using Least Square Means revealed that impact site 40002 was highly significantly ($P \leq 0.01$) different from all three reference sites. The fact that the two lesions contributing most to variability in the first principal component were LN and RPN indicates that these two splenic lesions are important markers differentiating impact from reference sites.

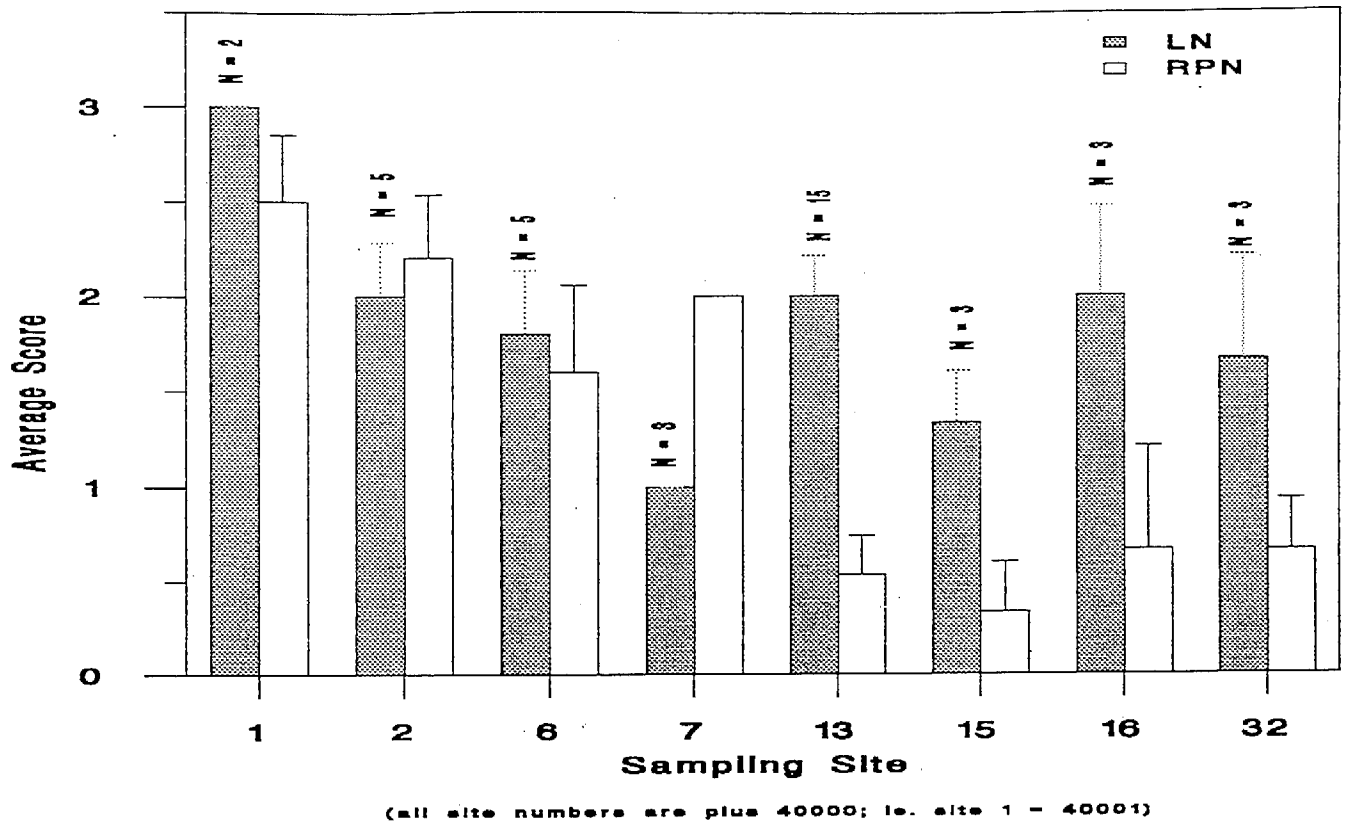
Graph 1a. Average Scores for Lymphoid Necrosis (LN) and Red Pulp Necrosis (RPN) for All Fish from All Sites.



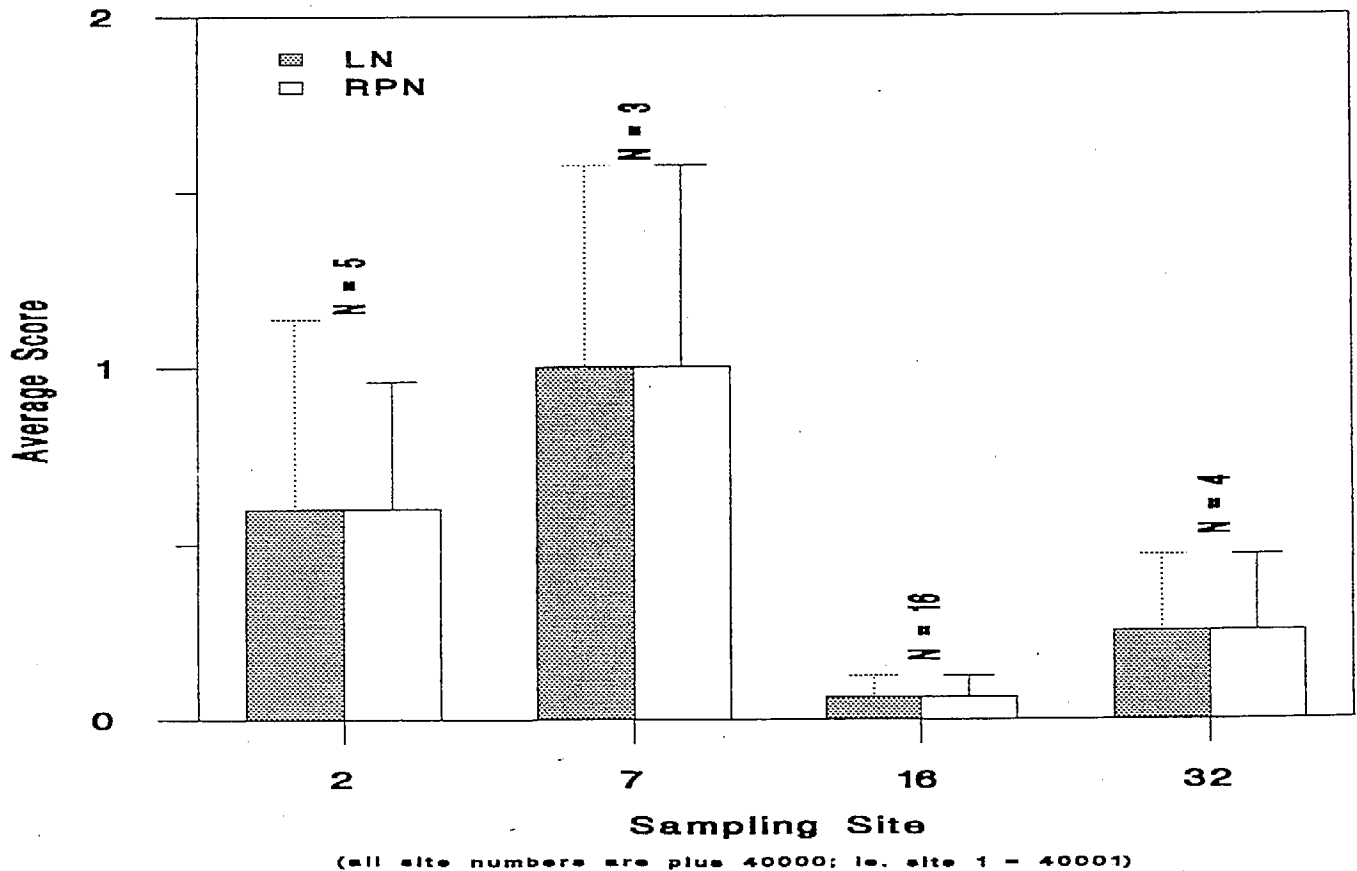
Graph 1b. Average Scores for Splenic Lymphoid Necrosis (LN) and Red Pulp Necrosis (RPN) in Yellowfin Gobies.



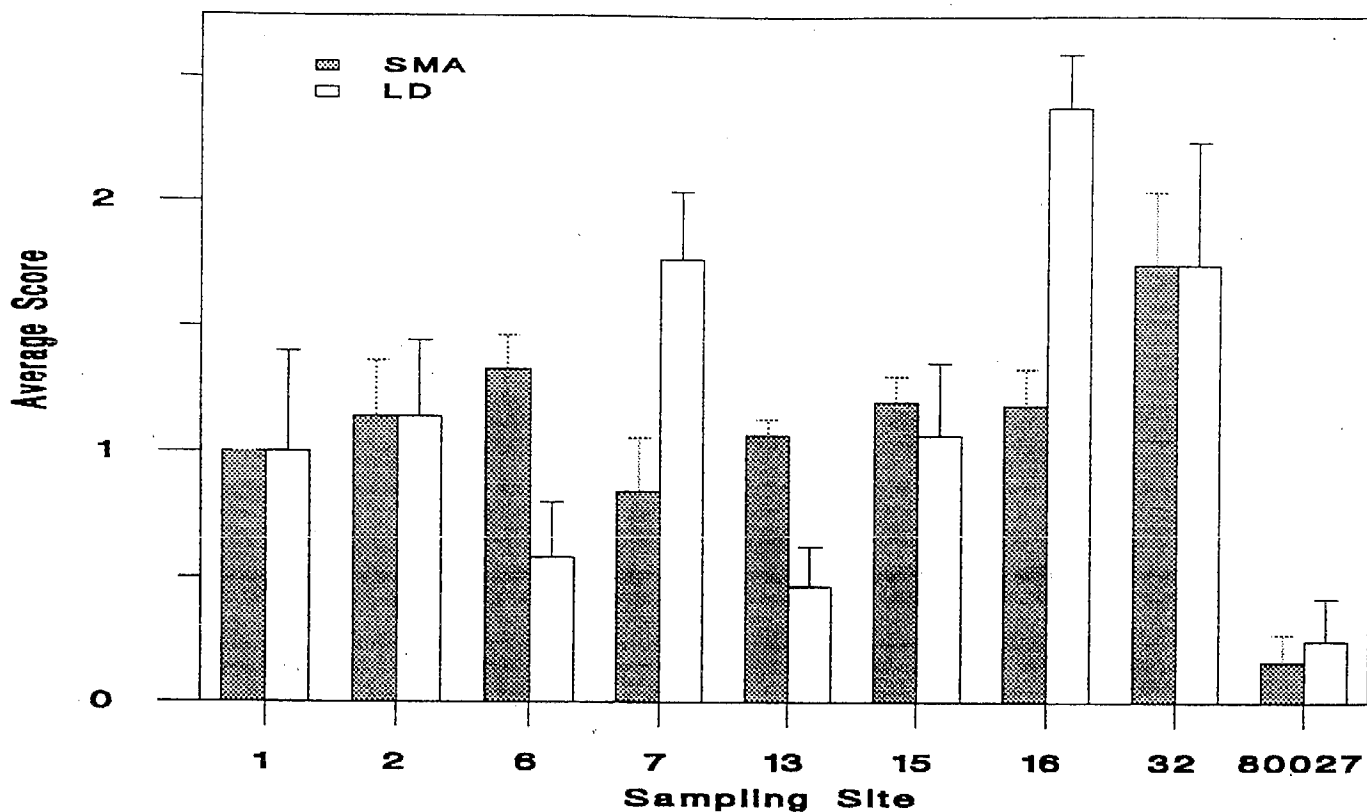
Graph 1c. Average Scores for Splenic Lymphoid Necrosis (LN) and Red Pulp Necrosis (RPN) in White Croakers.



Graph 1d. Average Scores for Splenic Lymphoid Necrosis (LN) and Red Pulp Necrosis (RPN) in Tonguefish.

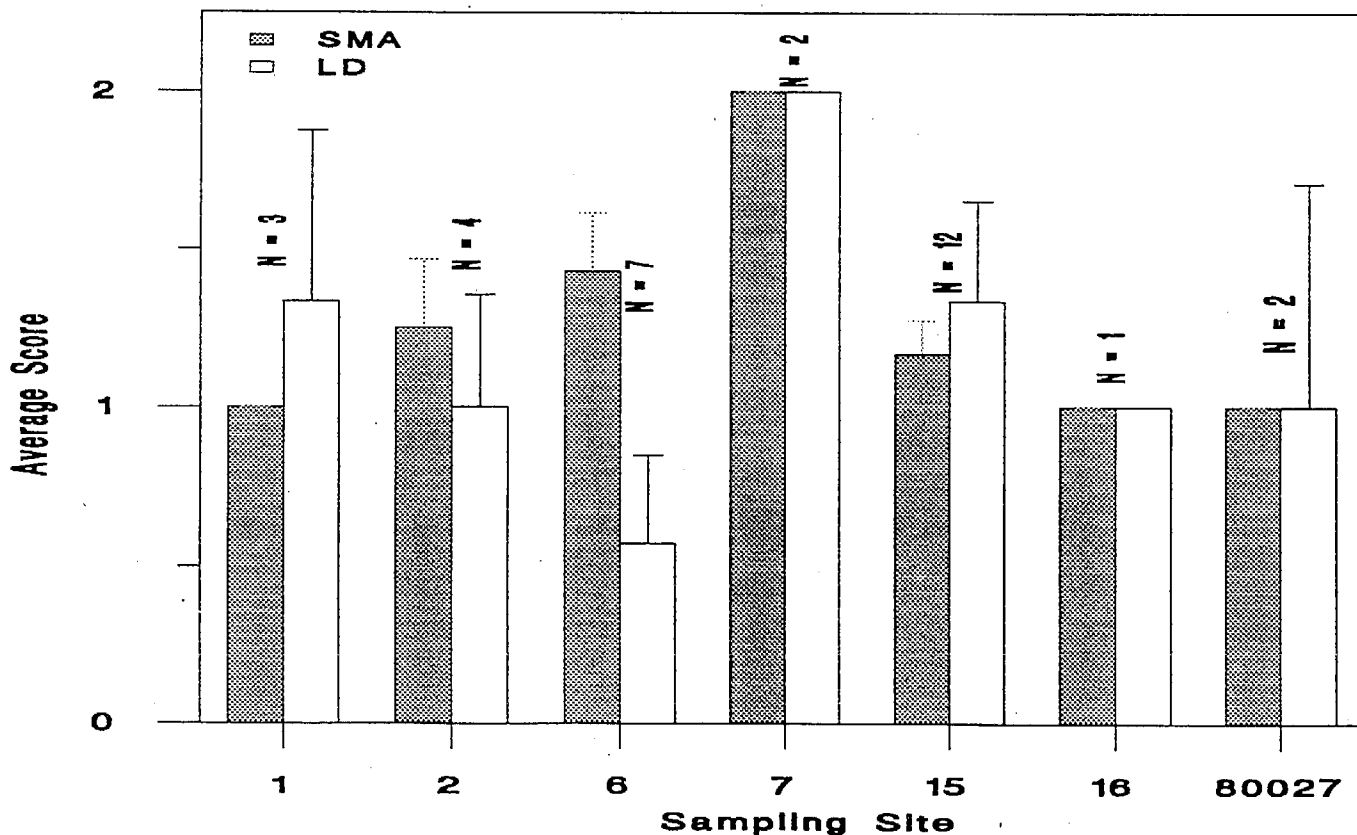


Graph 2a. Average Scores for Splenic Macrophage Aggregates (SMA) and Lymphoid Depletion (LD) in All Fish from All Sites.



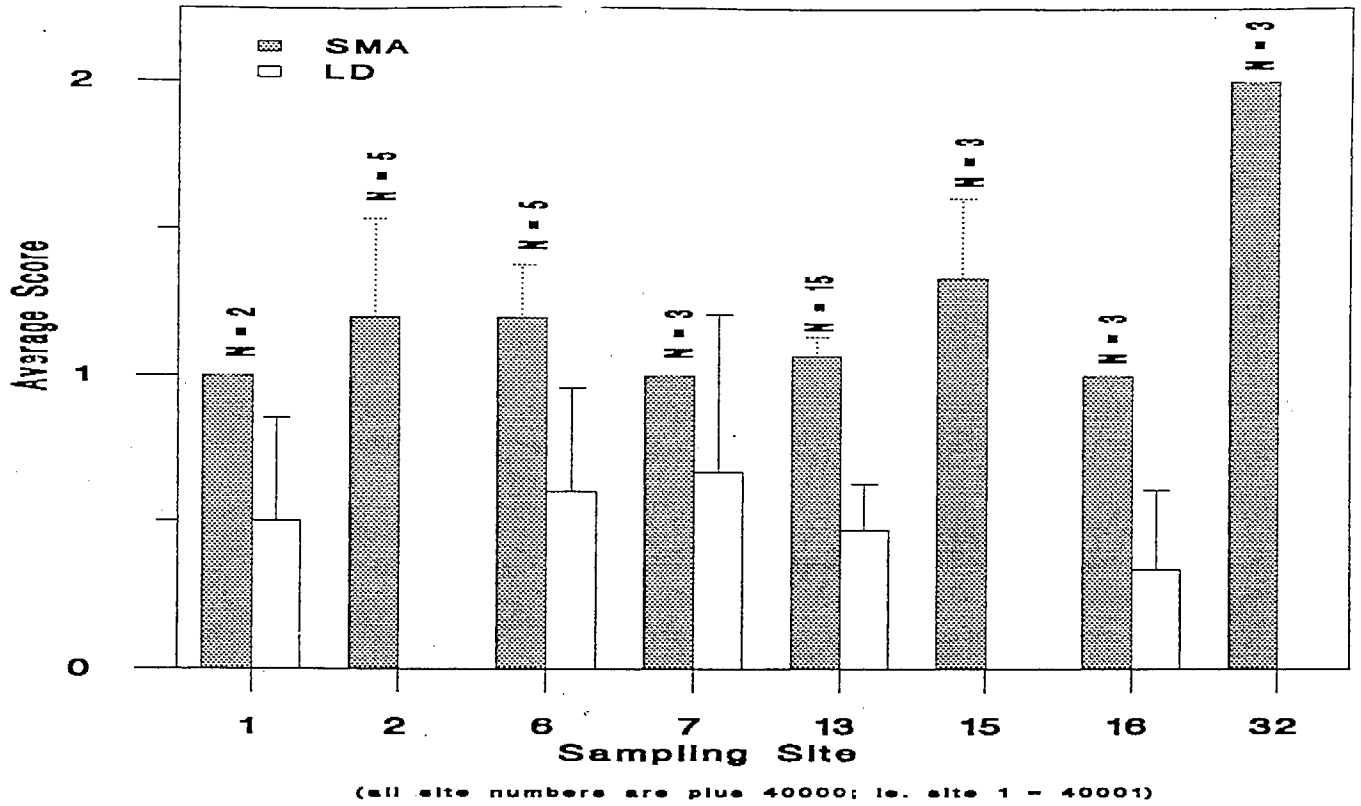
(all site numbers, except the last, are plus 40000; i.e. site 1 = 40001)

Graph 2b. Average Score for Splenic Macrophage Aggregates (SMA) and Lymphoid Depletion (LD) in Yellowfin Gobies.

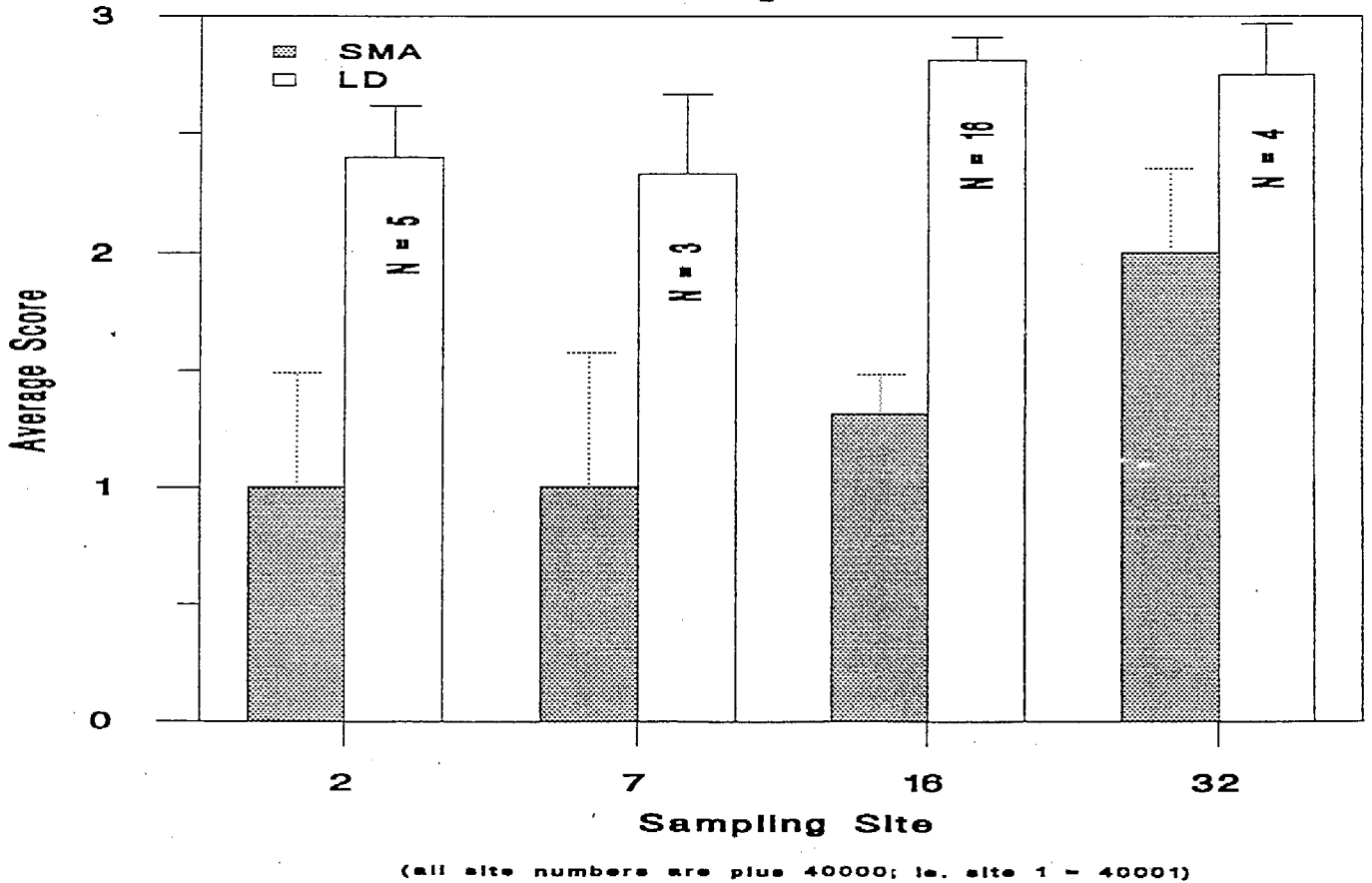


(all site numbers, except the last, are plus 40000; i.e. site 1 = 40001)

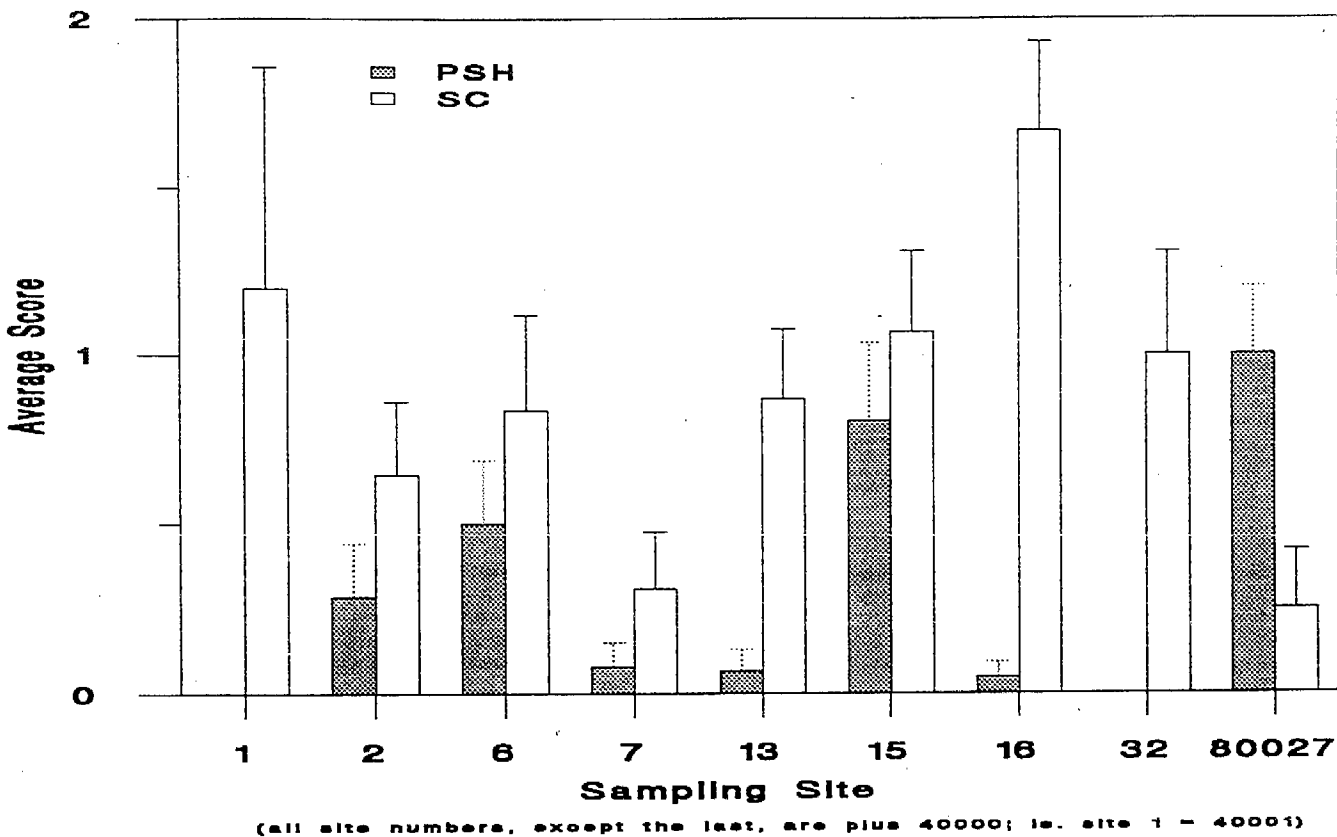
Graph 2c. Average Scores for Splenic Macrophage Aggregates (SMA) and Lymphoid Depletion (LD) in White Croakers.



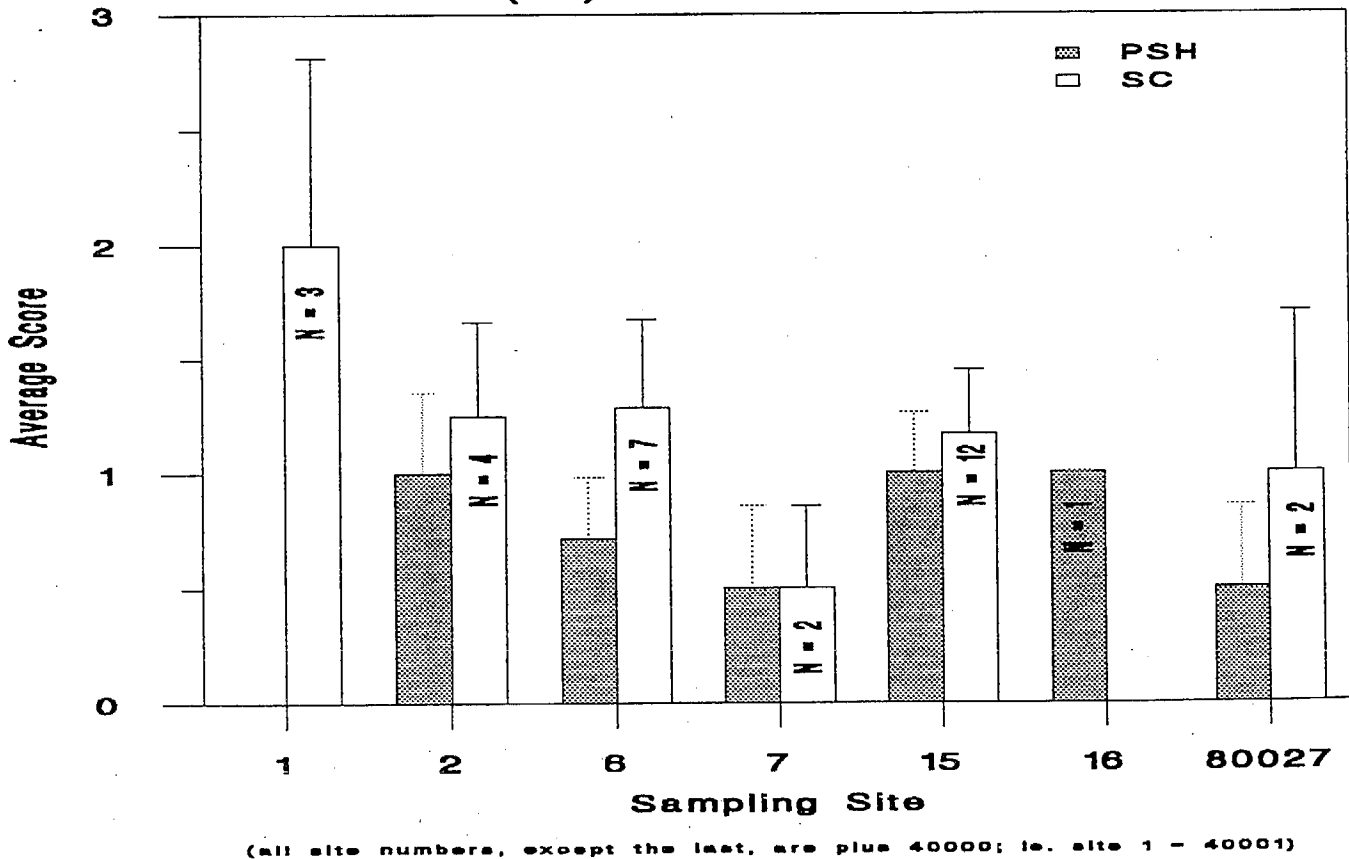
Graph 2d. Average Scores for Splenic Macrophage Aggregates (SMA) and Lymphoid Depletion (LD) in Tonguefish.



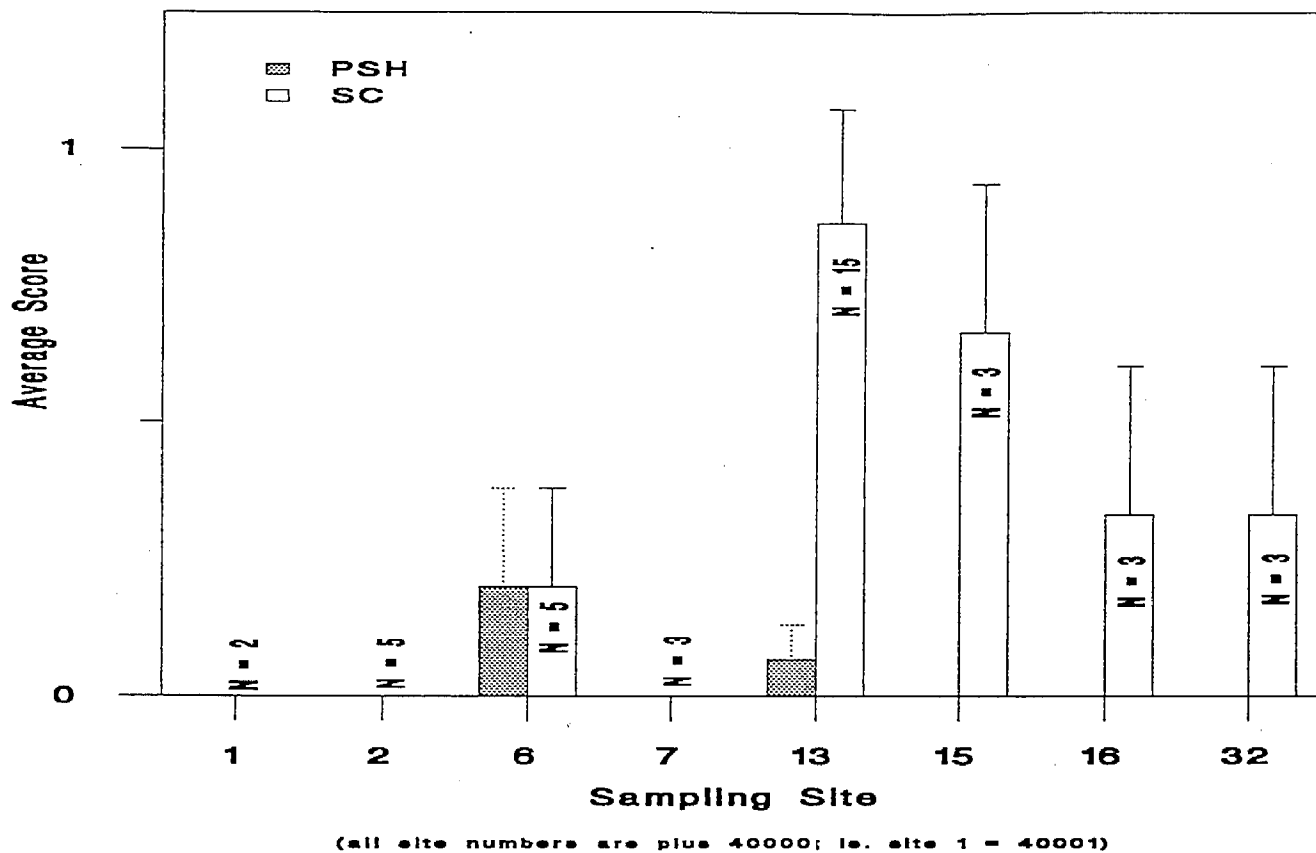
Graph 3a. Average Scores for Periarteriolar Sheath Hyperplasia (PSH) and Splenic Congestion (SC) in All Fish from All Sites.



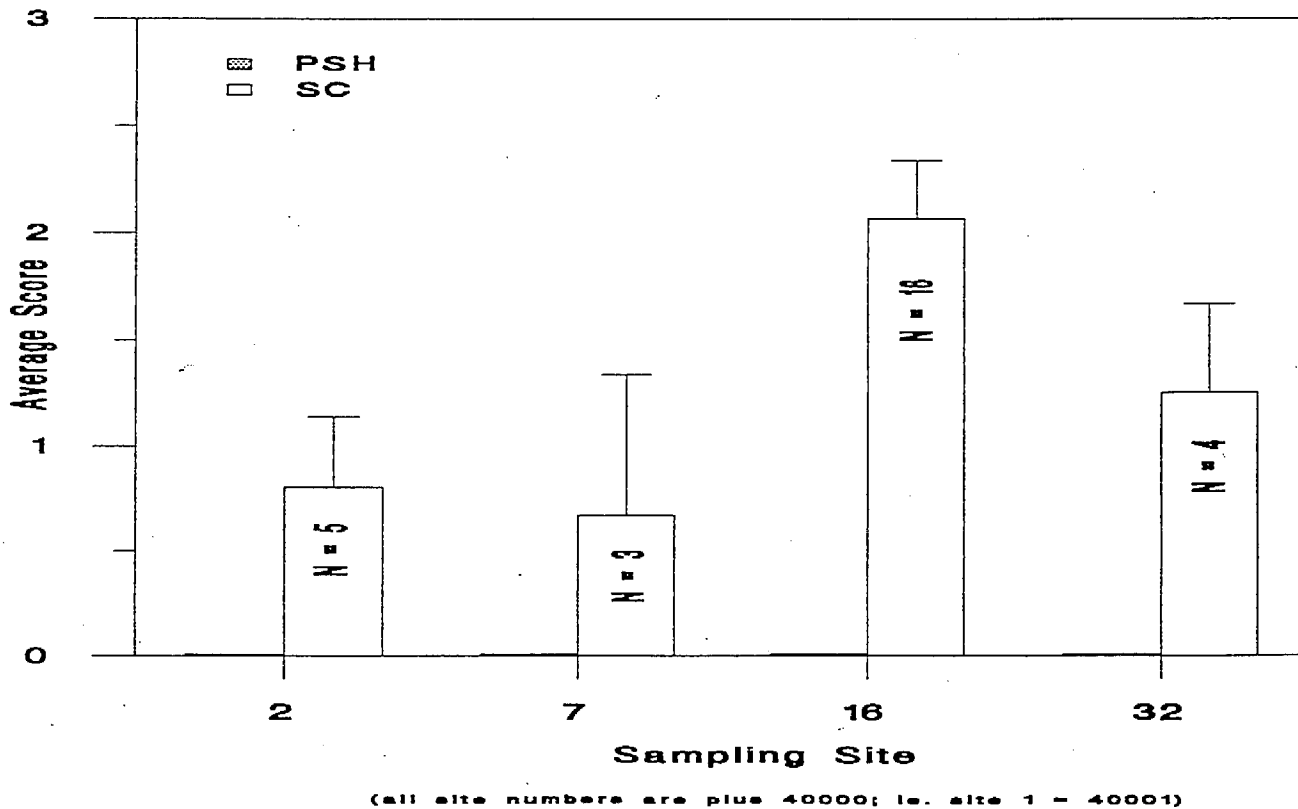
Graph 3b. Average Scores for Periarteriolar Sheath Hyperplasia (PSH) and Splenic Congestion (SC) in Yellowfin Gobies.



Graph 3c. Average Scores for Perilarteriolar Sheath Hyperplasia (PSH) and Splenic Congestion (SC) In White Croakers.



Graph 3d. Average Scores for Perilarteriolar Sheath Hyperplasia (PSH) and Splenic Congestion (SC) In Tonguefish.



Histopathology - Results:

II. Liver Lesions:

A. Hepatocyte Storage Defects:

1. **Glycogen depletion (GD):** Hepatic glycogen depletion was a common finding. In contrast to "normal" liver with abundant glycogen stores (Figures 14 & 15), glycogen depleted livers were characterized by; decreased size of individual hepatocytes, loss of the "lacy" cytoplasmic vacuolation typical of glycogen, and increased cytoplasmic basophilia (Figure 16).

Type lesions for GD:

- a. Score = 0; no glycogen depletion (type specimen = 8)
 - b. Score = 1; mild, glycogen vacuoles are present, vacuoles are smaller than normal, but larger than hepatocyte nuclei, hepatocytes are slightly smaller in size due to loss of glycogen (type specimen = 34)
 - c. Score = 2; moderate, glycogen vacuoles are present and the aggregate of vacuoles are smaller than hepatocyte nuclei, hepatocytes are moderately smaller than normal; or there is patchy loss of glycogen with some areas of mild glycogen depletion and other foci of complete glycogen depletion (type specimen = 16)
 - d. Score = 3; severe, glycogen vacuoles are absent from the majority of hepatocytes, hepatocytes may be significantly smaller than normal and are often markedly basophilic, glycogen vacuoles may be replaced by lipid vacuoles (type specimen = 21)
2. **Lipidosis (LIP):** Hepatic lipidosis was another common finding and was characterized by the presence of discrete, round, clear, cytoplasmic vacuoles. Hepatocytes were often enlarged and nuclei displaced to the periphery (Figure 17).

Type lesions for LIP:

- a. Score = 0; no lipidosis (type specimen = 8)
- b. Score = 1; mild, diffuse lipidosis with hepatocytes with small lipid vacuoles (vacuoles smaller than hepatocyte nuclei) or patchy lipidosis with scattered small foci of hepatocytes with large vacuoles (vacuoles larger than hepatocyte nuclei)(type specimen = 56)
- c. Score = 2; moderate, diffuse lipidosis with 50-80% of hepatocytes with large vacuoles (type specimen = 11)
- d. Score = 3; severe, diffuse lipidosis with 80-100% of hepatocytes with large lipid vacuoles, hepatocyte nuclei displaced to the periphery of the cell (type specimen = 15)

3. **Eosinophilic cytoplasmic inclusions (ECI):** Hepatocytes in a few livers had distinct, round, refractile, eosinophilic cytoplasmic inclusions (Figure 18).

Type lesions for ECI:

- a. Score = 0; no inclusions (type specimen = 8)
- b. Score = 1; <50% of hepatocytes have inclusions (type specimen = 56)
- c. Score = 2; 50-80% of hepatocytes have inclusions (type specimen = 105)
- d. Score = 3; 80-100% of hepatocytes have inclusions (type specimen = 67)

B. **Hepatic Inflammation (Hepatitis):**

1. **Macrophages:**

- a. **Macrophage Aggregates (HMA):** The majority of macrophage aggregates were clusters of mature macrophages packed with coarsely granular yellow-brown pigment (Figure 19). Occasionally, an aggregate would be composed of activated macrophages with little or no pigment.

Type lesions for HMA:

- 1) Score = 0; no MA (type specimen = 8)
- 2) Score = 1; <1 MA per 50X field (type specimen = 16)
- 3) Score = 2; 1-3 per 50X field (type specimen = 15)
- 4) Score = 3; >3 MA per 50X field (type specimen = 88)

- b. **Melanomacrophages (MM):** Melanomacrophages were individual macrophages packed with dark brown-black melanin pigment and were characteristic of stingray livers (Figure 20).

Type lesions for MM:

- 1) Score = 0; none present (type specimen = 5)
- 2) Score = 1; <10 per 100X field (type specimen = 33)
- 3) Score = 2; 10-20 per 100X field (type specimen = 40)
- 4) Score = 3; >20 per 100X field (type specimen = 72)

- c. **Granulomatous inflammation (HGI):** Granulomatous inflammation was a rare finding and not scored for. The lesion was characterized by infiltration of the hepatic parenchyma with a mixed population of mononuclear inflammatory cells; macrophages, lymphocytes, and multinucleated giant cells (Figure 21).

- d. **Foreign body granulomas (FBG):** Foreign body granulomas were focal accumulations of macrophages, lymphocytes, and occasionally multinucleated giant cells, clustered around a foreign body (Figures 21 & 22). The foreign body could often be identified as a nematode larva.

Type lesions for FBG:

- 1) Score = 0; none present (type specimen = 8)
- 2) Score = 1; mild, <1 per 25X field (type specimen = 13)
- 3) Score = 2; moderate, 1-3 per 25X field (type specimen = none)
- 4) Score = 3; severe, >3 per 25X field or fewer large granulomas (type specimen = none)

2. **Lymphocytic inflammation (LYM):** Lymphocytic inflammation in the liver was an uncommon finding, except in stingrays. Lymphocytes were usually perivascular in location, but could occasionally be found within the parenchyma (Figure 23).

Type lesions for LYM:

- a. Score = 0; no inflammation (type specimen = 8)
- b. Score = 1; <1 per 50X field (type specimen = 32)
- c. Score = 2; 1-3 per 50X field (type specimen = 33)
- d. Score = 3; >3 per 50X field (type specimen = none)

C. **Hepatic Necrosis:**

1. **Focal necrosis (FN):** Focal necrosis was not observed.
2. **Individual hepatocyte necrosis (IHN):** Individual hepatocyte necrosis was occasionally seen and characterized by shrinkage, rounding up, cytoplasmic eosinophilia, and nuclear pyknosis (Figures 24 & 25). Necrotic hepatocytes were often phagocytized by macrophages.

Type lesions for IHN:

- a. Score = 0; no necrosis (type specimen = 8)
- b. Score = 1; <1 per 100X field (type specimen = 56)
- c. Score = 2; 1-3 per 100X field (type specimen = 110)
- d. Score = 3; >3 per 100X field (type specimen = 88)

D. Hepatic Preneoplasia and Neoplasia:

1. **Tinctorially Altered Foci (TAF):** TAF were a rare occurrence and characterized by small clusters of hepatocytes distinguished from the adjacent parenchyma by altered staining. Types of TAF include basophilic, eosinophilic, amphophilic, and clear cell. TAF were not scored on the basis of severity, but were simply counted and described. There were only four fish with TAF; two with basophilic foci (1 & 42), one with an amphophilic focus (95), and one with a clear cell focus (118).
2. **Liver neoplasms:** Neoplasms were not observed.

E. Other liver lesions:

1. **Megalocytosis (MEG):** Hepatocyte megalocytosis was characterized primarily by nuclear enlargement or karyomegaly (Figures 26 & 27). Hepatocytes were not considered megalocytes unless nuclei were at least twice the average size in the section. There was usually little or no cellular enlargement associated with karyomegalic nuclei.

Type lesions for MEG:

- a. Score = 0; none present (type specimen = 8)
- b. Score = 1; <1 per 100X field (type specimen = 109)
- c. Score = 2; 1-3 per 100X field (type specimen = none)
- d. Score = 3; >3 per 100X field (type specimen = none)

2. **Fibrin whorls (FW):** Fibrin whorls were an unusual lesion and were seen only in stingray livers. Fibrin whorls were characterized by nodular accumulation of pale eosinophilic, acellular material which was fibrinous and laminated in irregular, concentric, laminated whorls (Figures 28 & 29). Small numbers of spindle cells mixed in with the fibrinous material.

Type lesions for FW:

- a. Score = 0; none present (type specimen = 8)
- b. Score = 1; <1 per 25X field (type specimen = 5)
- c. Score = 2; 1-3 per 25X field (type specimen = none)
- d. Score = 3; >3 per 25X field (type specimen = none)

3. **Exocrine Pancreas (EP):** Exocrine pancreas was not a lesion, but was assessed on the basis of presence (1) or absence (0).

4. **Nematodes (NEM):** A few fish had small numbers of nematode larvae either in the capsule of the liver or hepatic parenchyma (Figure 22). The nematodes were usually associated with small granulomas.

Type lesions for NEM:

- a. Score = 0; none present (type specimen = 8)
- b. Score = 1; <1 per 25X field (type specimen = 13)
- c. Score = 2; 1-3 per 25X field (type specimen = none)
- d. Score = 3; >3 per 25X field (type specimen = none)

5. **Hyalinization of Vessel Walls (HVW):** A few fish had mild to moderate intimal and medial thickening of hepatic blood vessels (both arteries and veins). The thickening was often irregular and composed of pale eosinophilic to eosinophilic, acellular, amorphous, acellular material. In some vessels, this hyalin thickening was associated with mixed mononuclear and EGL inflammation.

Type lesions for HVW:

- a. Score = 0; none present (type specimen = 8)
- b. Score = 1; <3 vessels per 25X field (type specimen = 1)
- c. Score = 2; 3-5 vessels per 25X field (type specimen = none)
- d. Score = 3; >5 vessels per 25X field (type specimen = none)



Figure 14. Liver of fish 93H63-8 (white croaker from site 40013) with no lesions. Hepatocytes are laden with glycogen and exocrine pancreatic cells (arrow) have abundant zymogen granules. HE 25X.

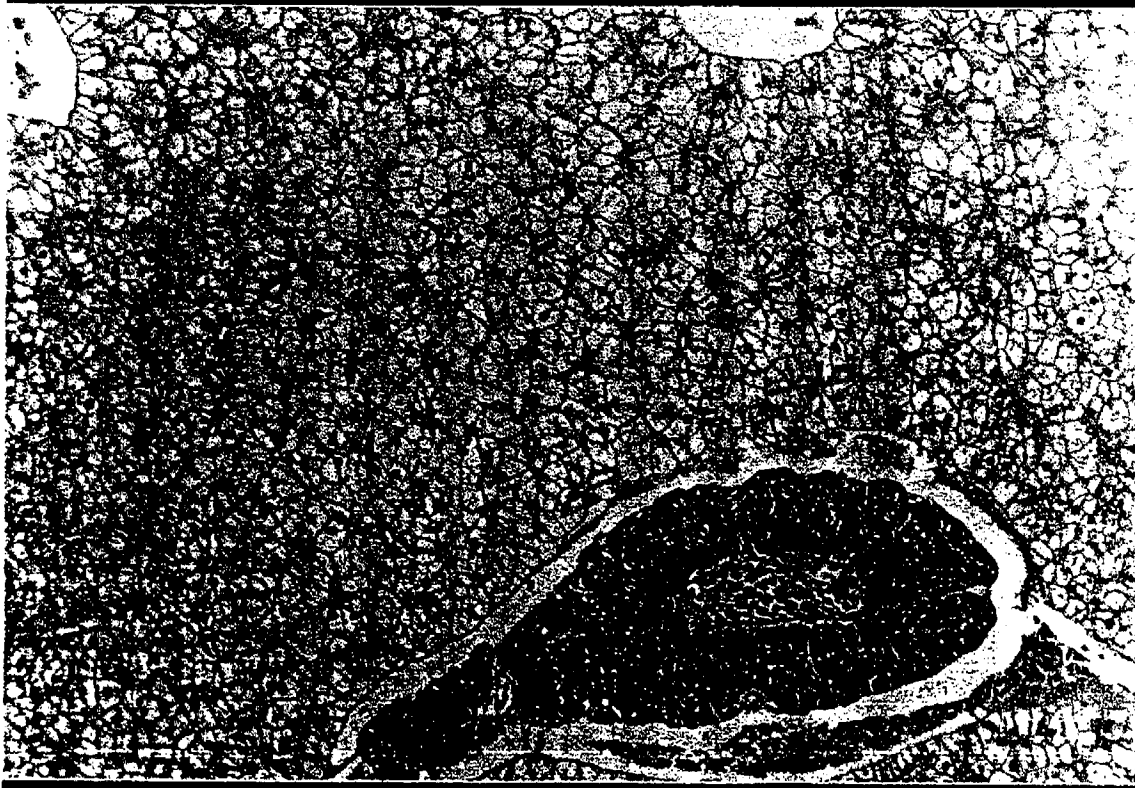


Figure 15. Higher magnification of figure 14. Note the normal glycogen laden hepatocytes and exocrine pancreatic cells with prominent eosinophilic zymogen granules. HE 50X.

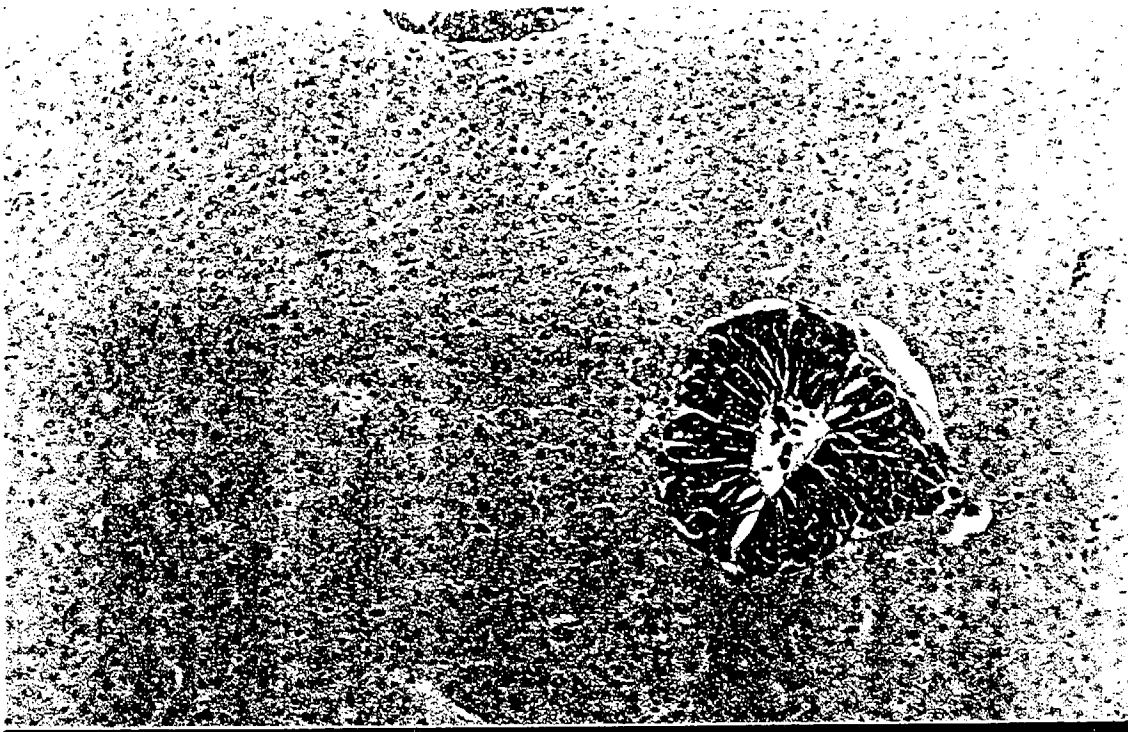


Figure 16. Liver of fish 93H63-21 (white croaker from site 40032) with severe glycogen depletion. Note hepatocytes are devoid of cytoplasmic vacuoles indicative of glycogen. HE 50X.

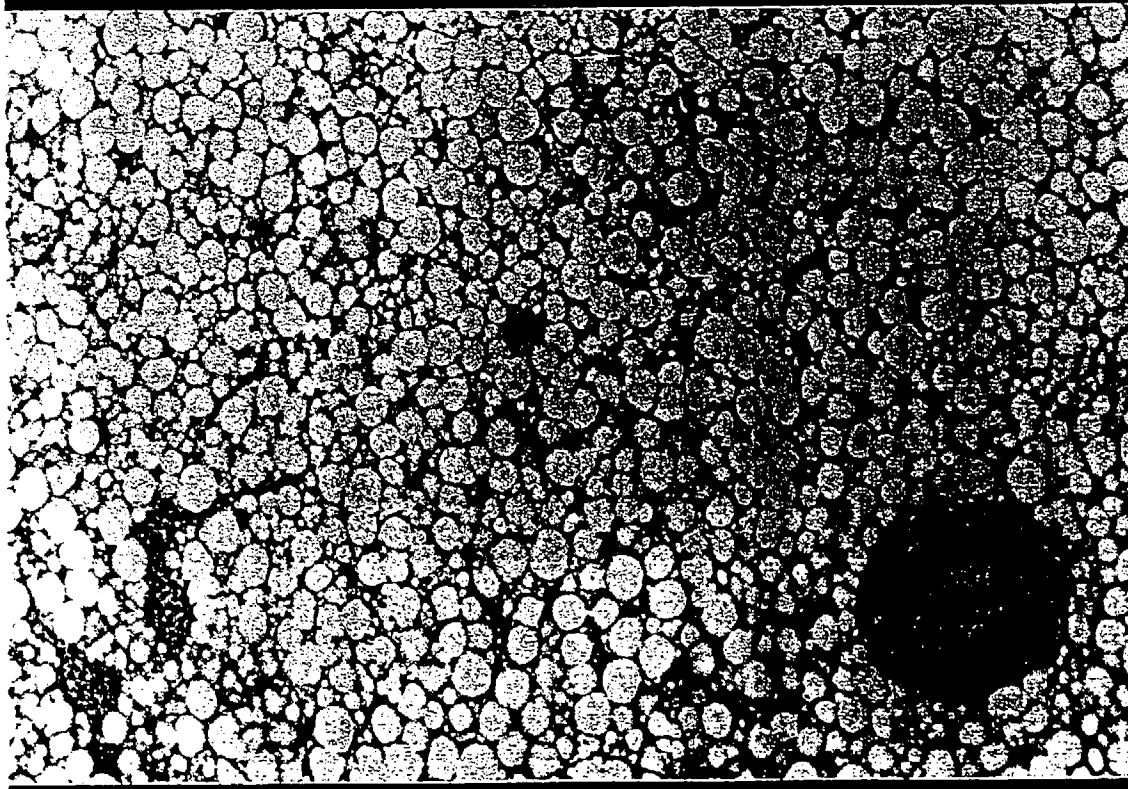


Figure 17. Liver of fish 93H63-15 (yellowfin goby from site 40006) with severe lipidoses. Lipid is characterized by large, round, discrete, cytoplasmic vacuoles. HE 50X.

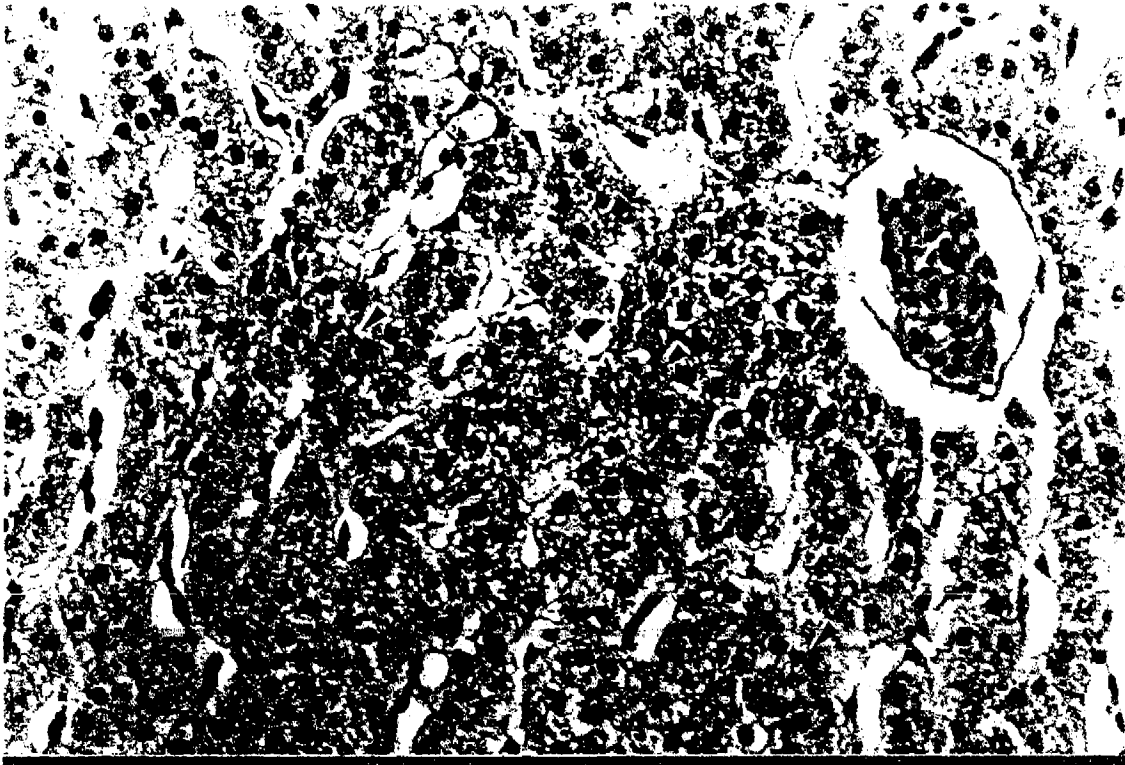


Figure 18. Liver of fish 93H63-67 (tonguefish from site 40002) with large numbers of eosinophilic cytoplasmic inclusions (arrowheads). HE 132X.

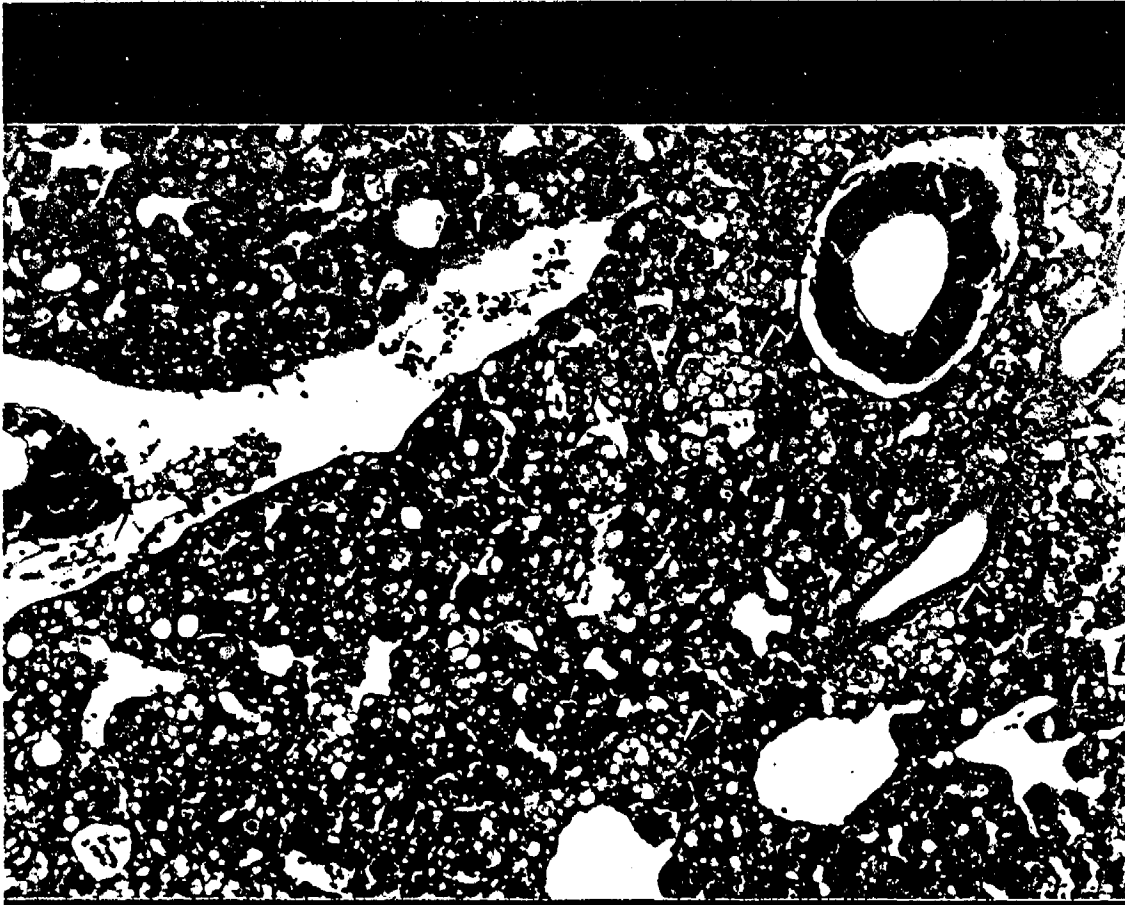


Figure 19. Liver of fish 93H63-88 (yellowfin goby from site 40015) with severe individual hepatocyte necrosis and numerous macrophage aggregates (arrowheads). HE 50X.

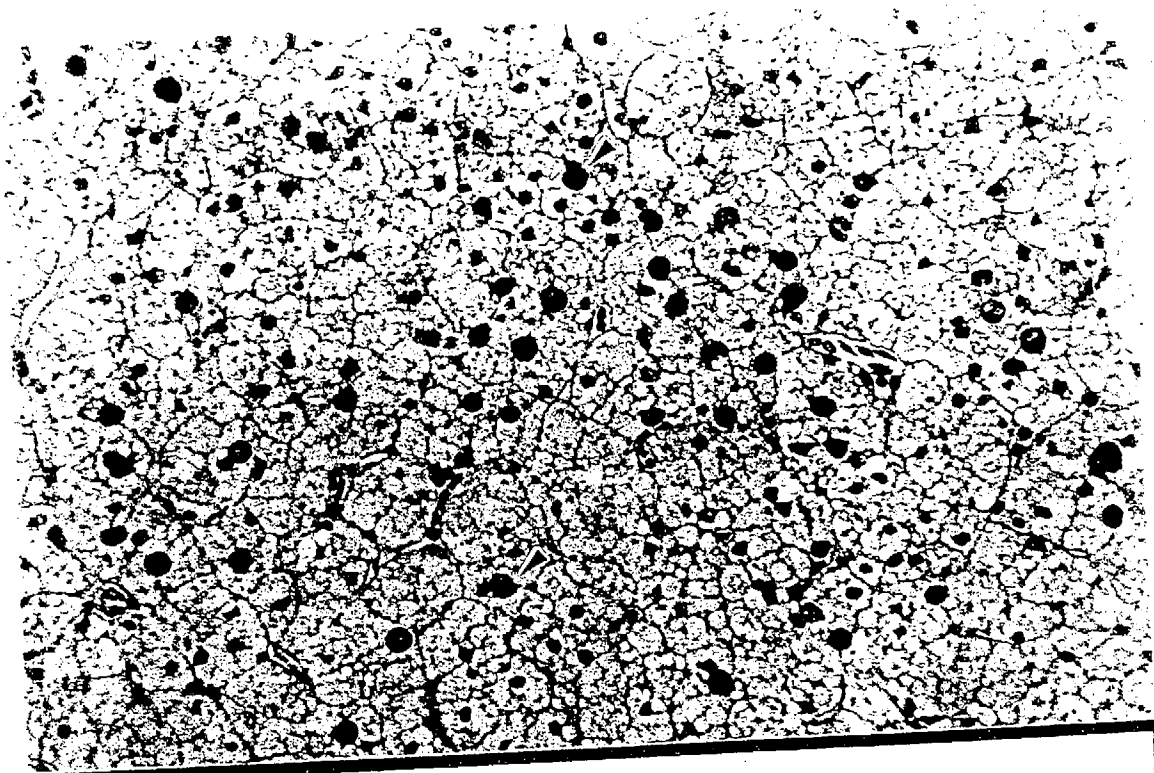


Figure 20. Liver of fish 93H63-14 (stingray from site 80027) with large numbers of melanomacrophages (arrowheads). Hepatocytes are packed with lipid. HE 50X.

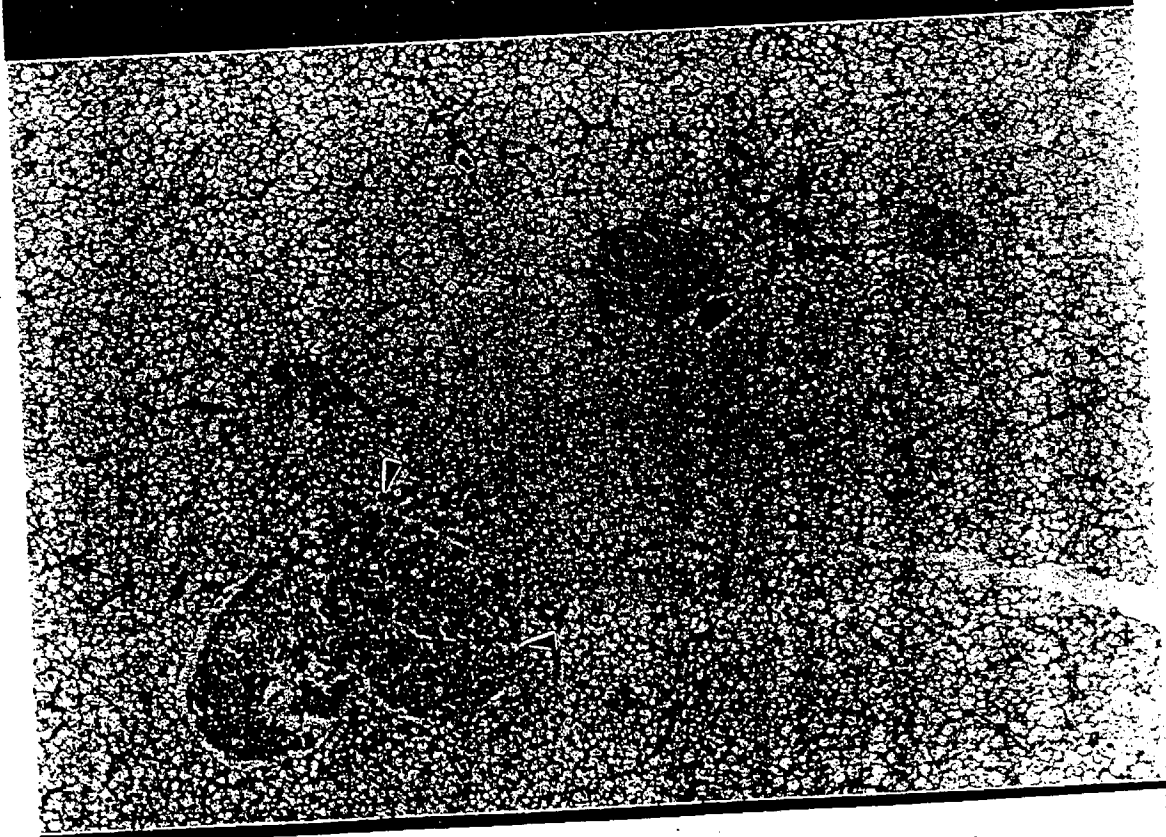


Figure 21. Liver of fish 93H63-13 (basketweave cusk-eel from site 40016) with moderate lipidosis, a large focus of granulomatous inflammation (arrowheads), and a foreign body granuloma (arrow). HE 25X.



Figure 22. Liver of fish 93H63-13 (basketweave cusk-eel from site 40016) with severe lipidosis. There are several granulomas on the hepatic capsule and many are centered around nematode larvae (arrowheads). HE 50X.

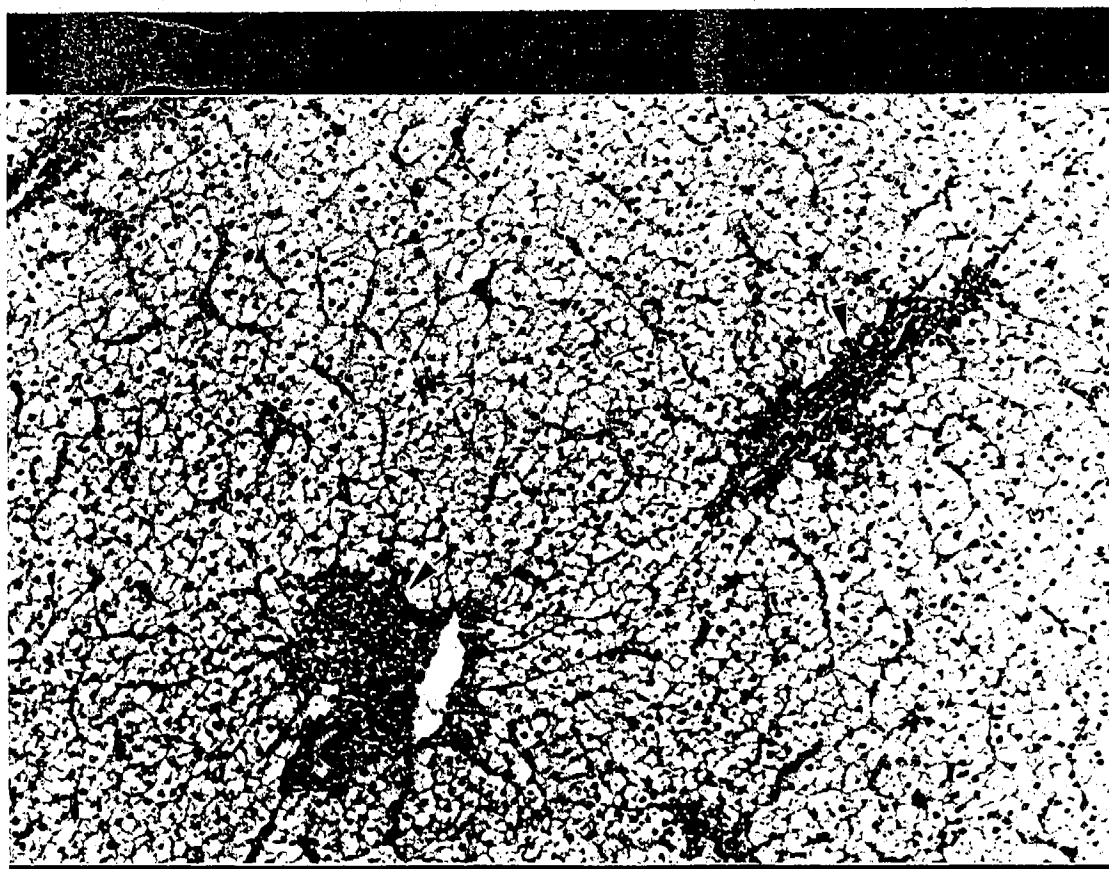


Figure 23. Liver of fish 93H63-33 (stingray from site 80027) with multiple, perivascular aggregates of lymphocytes (arrowheads). HE 25X.

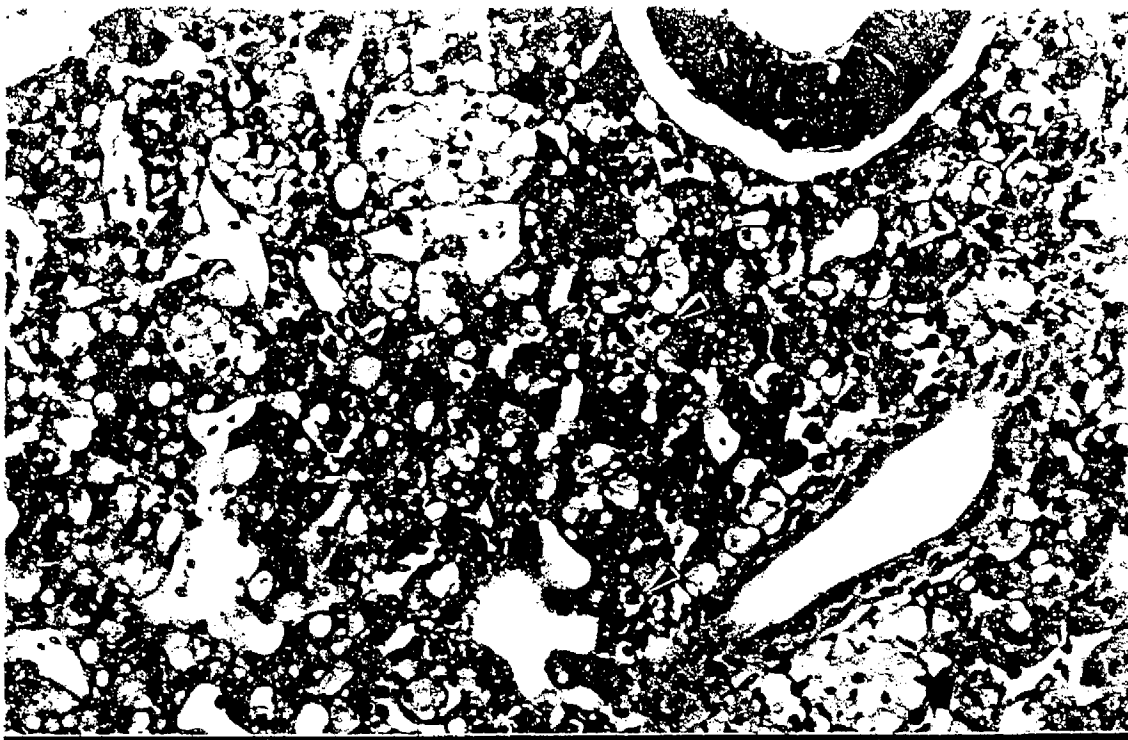


Figure 24. Liver of fish 93H63-88 (yellowfin goby from site 40015) with severe necrosis of individual hepatocytes (arrowheads) and numerous macrophage aggregates (arrows). HE 100X.

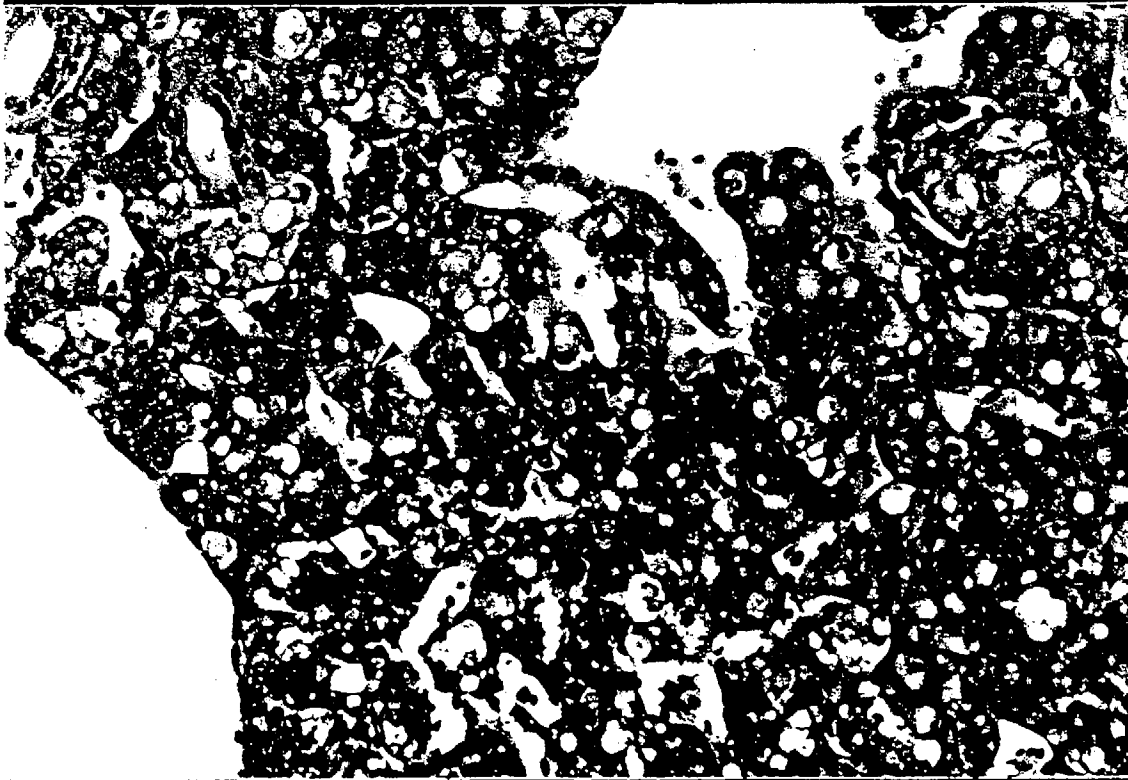


Figure 25. Liver of fish 93H63-88 (yellowfin goby from site 40015). Necrotic hepatocytes (arrowhead) are characterized by cytoplasmic hypereosinophilia, nuclear pyknosis and karyorrhexis. Many necrotic hepatocytes have been phagocytized by individual macrophages (arrows). HE 100X.

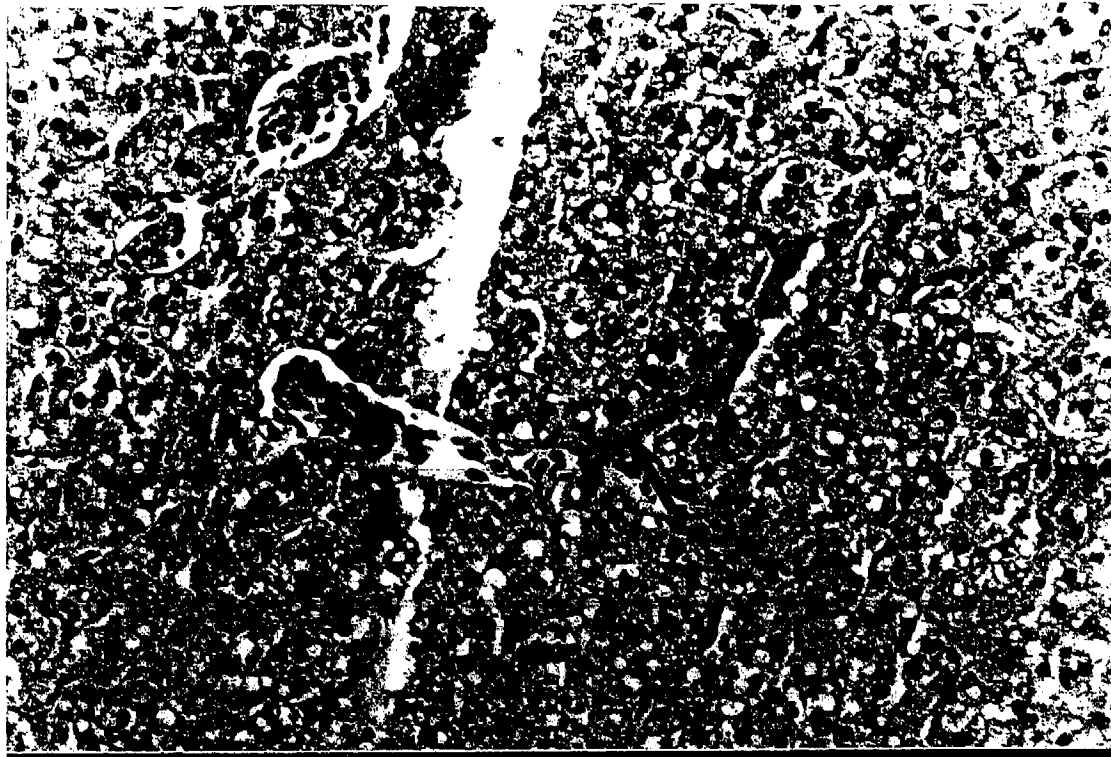


Figure 26. Liver of fish 93H63-109 (yellowfin goby from site 40002). The liver has severe glycogen depletion and mild lipidosis. There are scattered megalocytes (arrow) present. HE 100X.

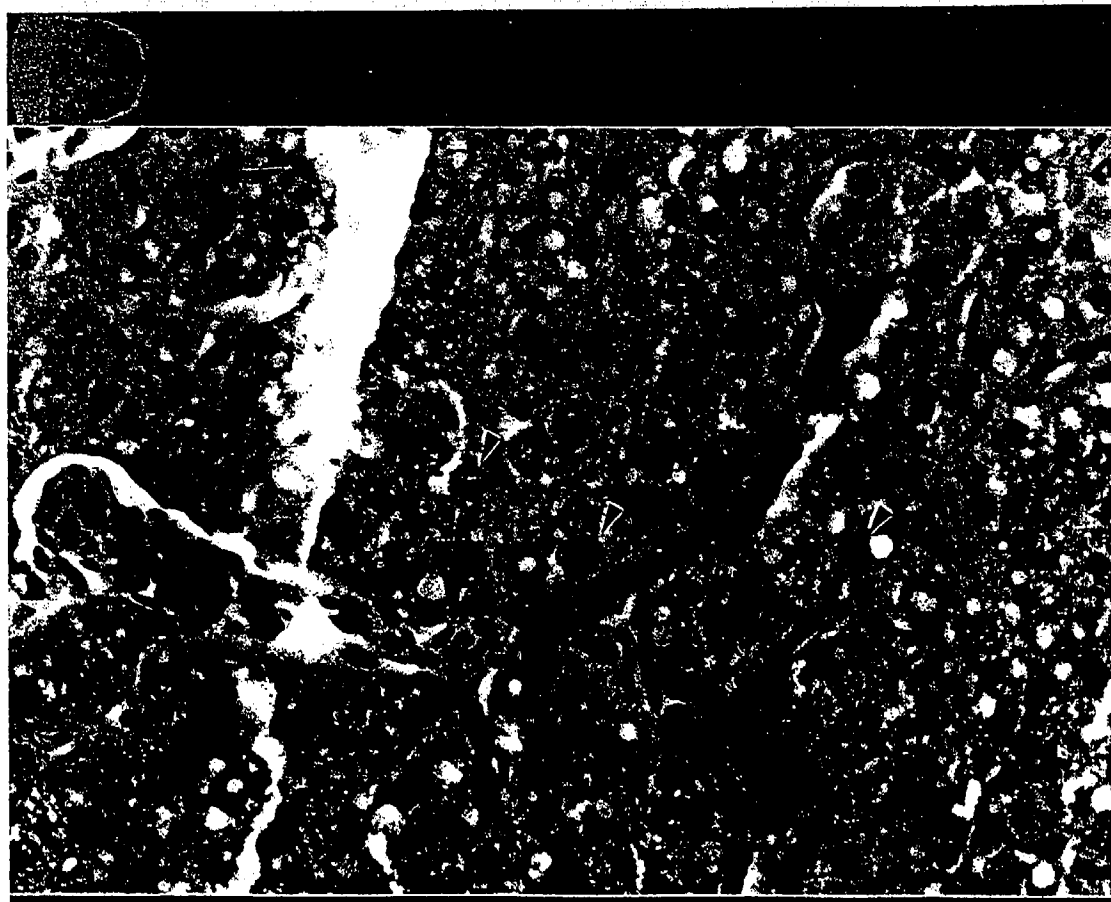


Figure 27. Higher magnification of figure 26. Megalocytes (arrowheads) are characterized by varying degrees of karyomegaly. HE 160X.

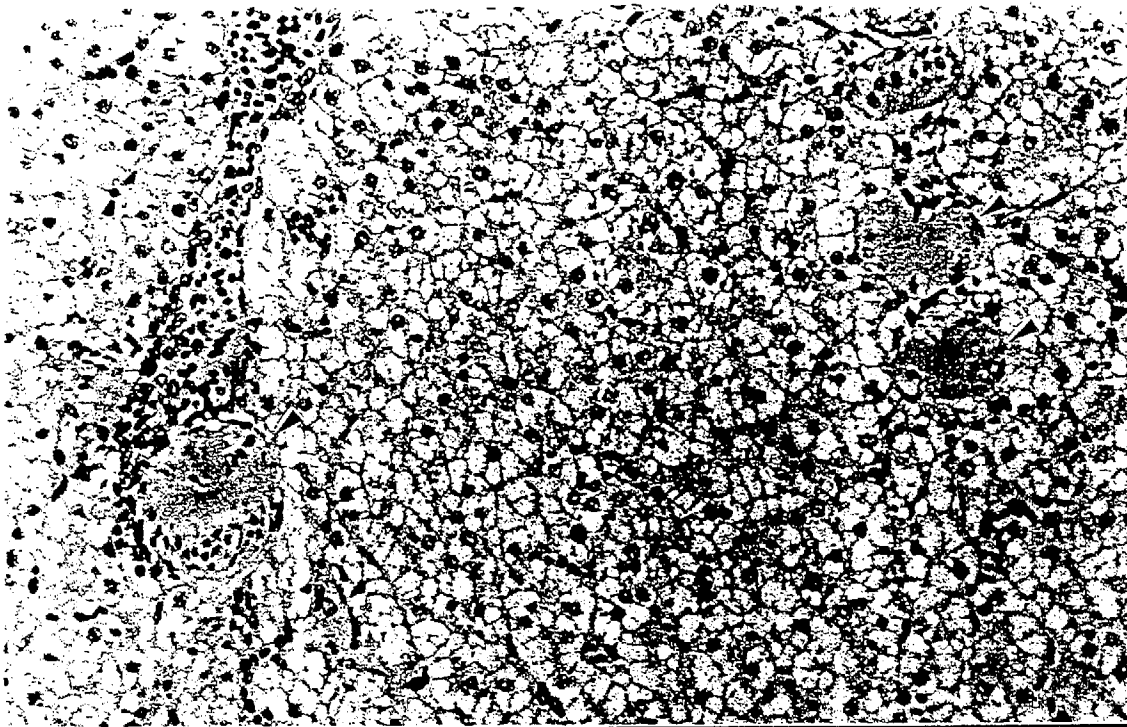


Figure 28. Liver of fish 93H63-5 (stingray from site 80027) with numerous fibrin whorls (arrowheads). HE 50X.

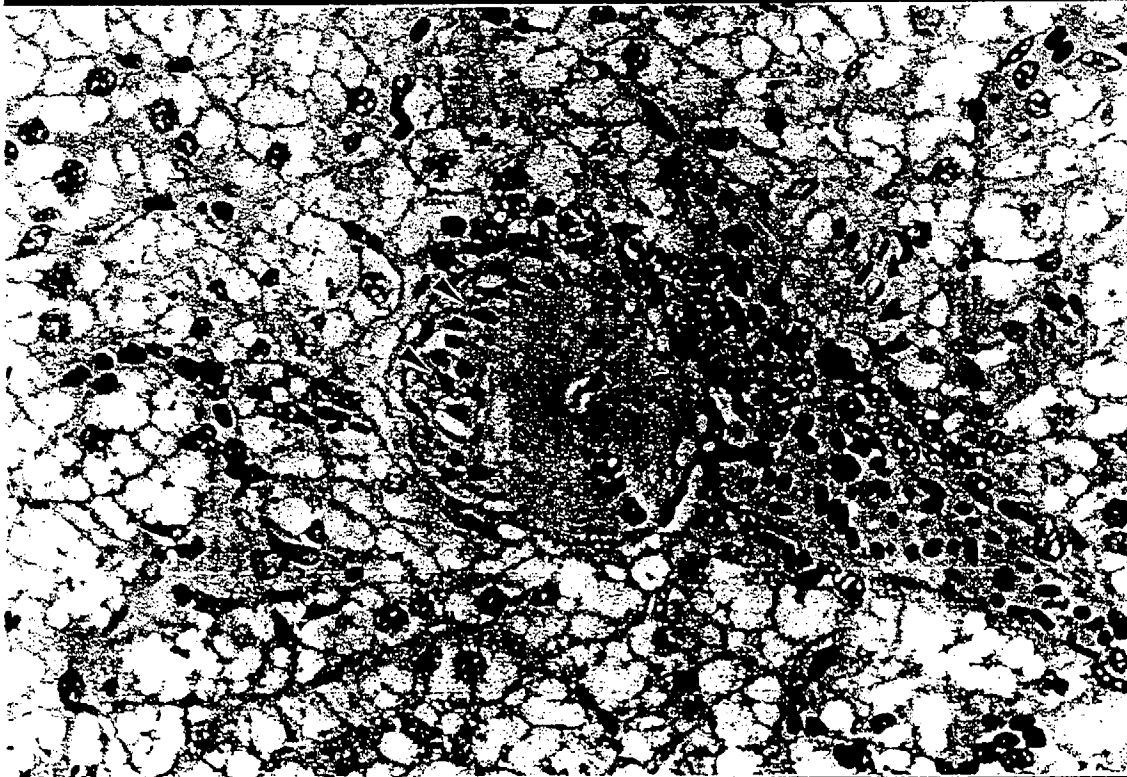


Figure 29. Higher magnification of figure 28. Note the laminated whorls of acellular eosinophilic material and the palisade of hyperchromatic small nuclei (arrowheads). HE 100X.

Histopathology - Results:

Hepatic Histopathology Summary: Hepatic lesion scores are given in appendix 15. Appendices 15a-15c include scores for all fish from all sites sorted on the basis of site. Average scores and standard error were computed for each lesion. Appendices 15d-15h lesion scores sorted on the basis of both site and species.

Average lesion scores for three species (gobies, croakers, and tonguefish), which were found at more than one site, are depicted in graphs 4-6. Graph 4a shows average lesion scores for three hepatic storage defects; glycogen depletion (GD), lipidosis (LIP), and eosinophilic cytoplasmic inclusions (ECI). Average scores for GD, LIP, and ECI were consistently lower at two reference sites (40016 and 40032) when compared to five of the six impact sites. The primary exceptions to this trend were impact site 40013 which had lower average scores than all three reference sites and reference site 40015 which had the highest average scores for both GD and LIP. Site 40015 also had some ECI, whereas the other two reference sites had no ECI.

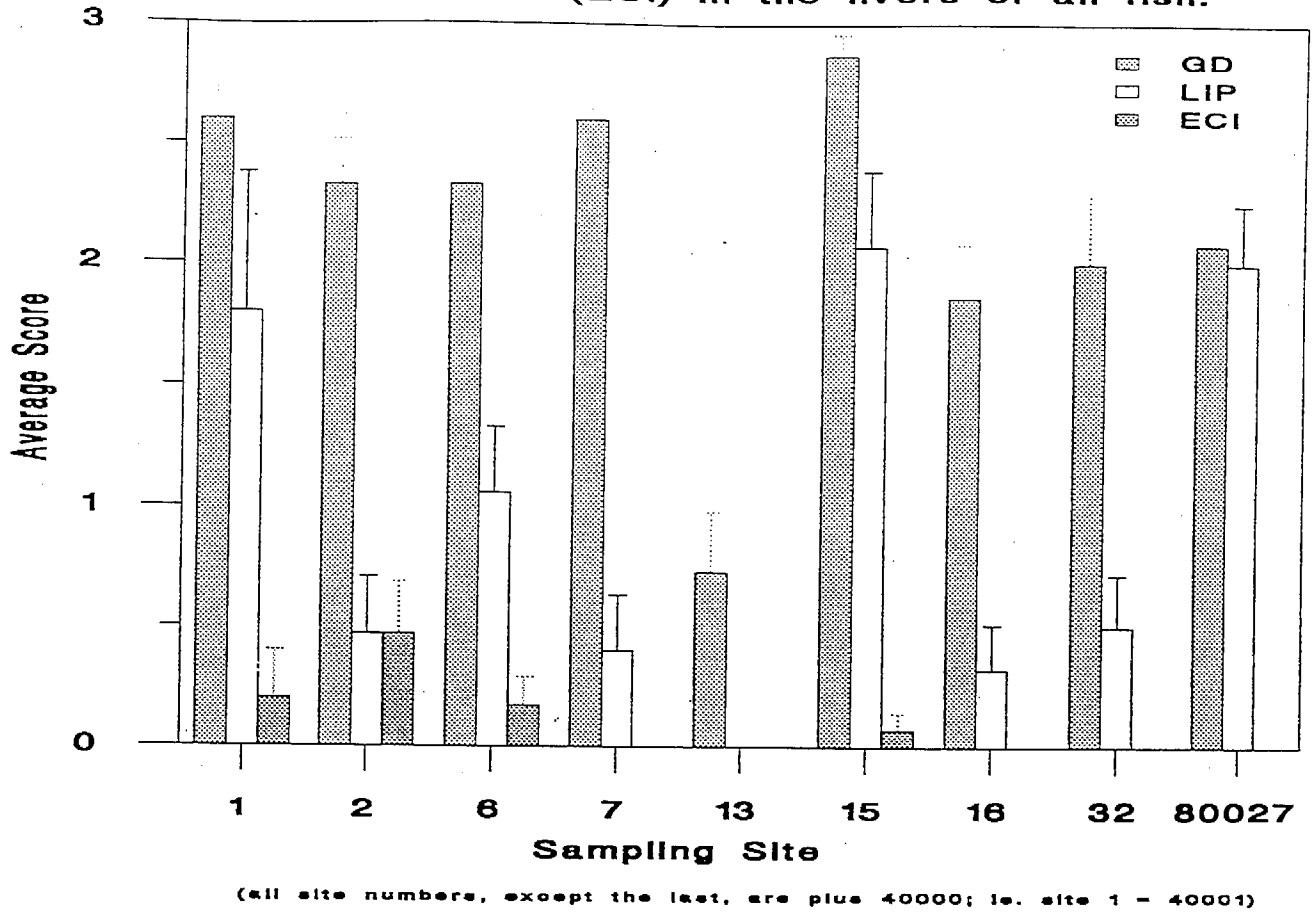
Further separation of average lesion scores on the basis of species revealed that there were some significant differences between species. With gobies (graph 4b), ECI correlated well with three of five impact sites, but there were no striking differences with respect to GD, and LIP tended to be lower at the two reference sites. With croakers, low levels of LIP were consistently found at the impact sites (versus none at the two reference sites), but there were no obvious trends with either GD or ECI. In tonguefish, GD scores were higher at the two impact sites when compared to two reference sites, and the only site with any ECI was at one of the impact sites.

Graph 5a depicts average lesion scores for hepatic macrophage aggregates (HMA), individual hepatocyte necrosis (IHN), and megalocytes (MEG). There were no obvious trends with respect to any of the three lesions. Sorting of the data on the basis of species revealed that average HMA scores for gobies (graph 5b) were highest at impact site 40001 and that gobies from impact site 40002 had an average MEG score which was markedly higher than that in the two reference sites. With croakers, there were no consistent trends with respect to HMA and IHN, but two impact sites had low levels of MEG while the three reference sites had none. In tonguefish, the only consistent finding was higher average HMA score at the two reference sites.

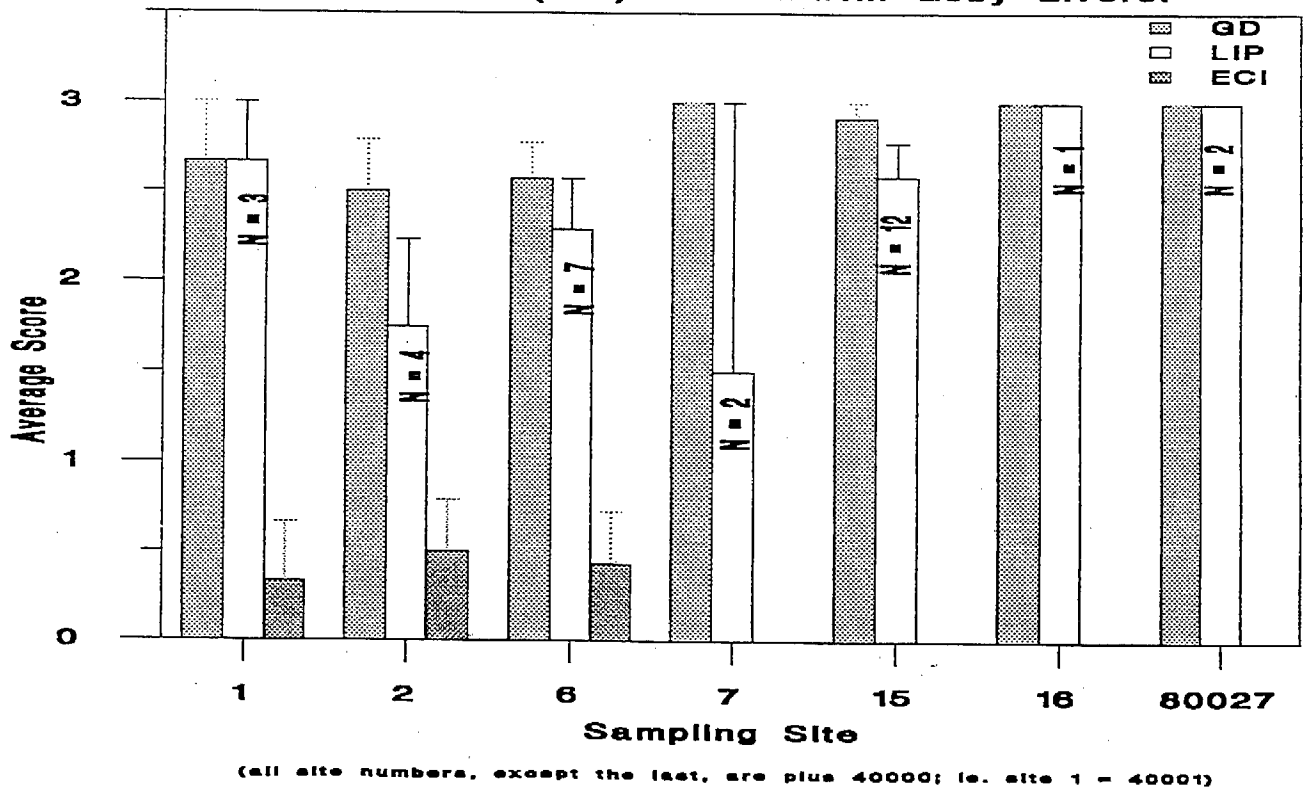
Graph 6a shows average lesion scores for foci of cellular alteration (FCA) and hyalinization of vessel walls (HVW) for all fish from all sites. There were no consistent trends. Additional sorting based on species revealed higher average HVW score in both gobies and croakers from impact sites when compared to the same species from reference sites. With tonguefish, the opposite trend was in effect, with the two reference sites having higher HVW scores than the two impact sites.

Hepatic Histopathology - Statistics: Hepatic lesion scores were statistically analyzed together with splenic lesion scores. Principal components Analysis (PCA) was covered in the splenic histopathology statistics section and in the material and methods. None of the hepatic lesions were significantly higher in the six impact sites when compared to the three reference sites. All comparisons were made between individual impact and reference sites.

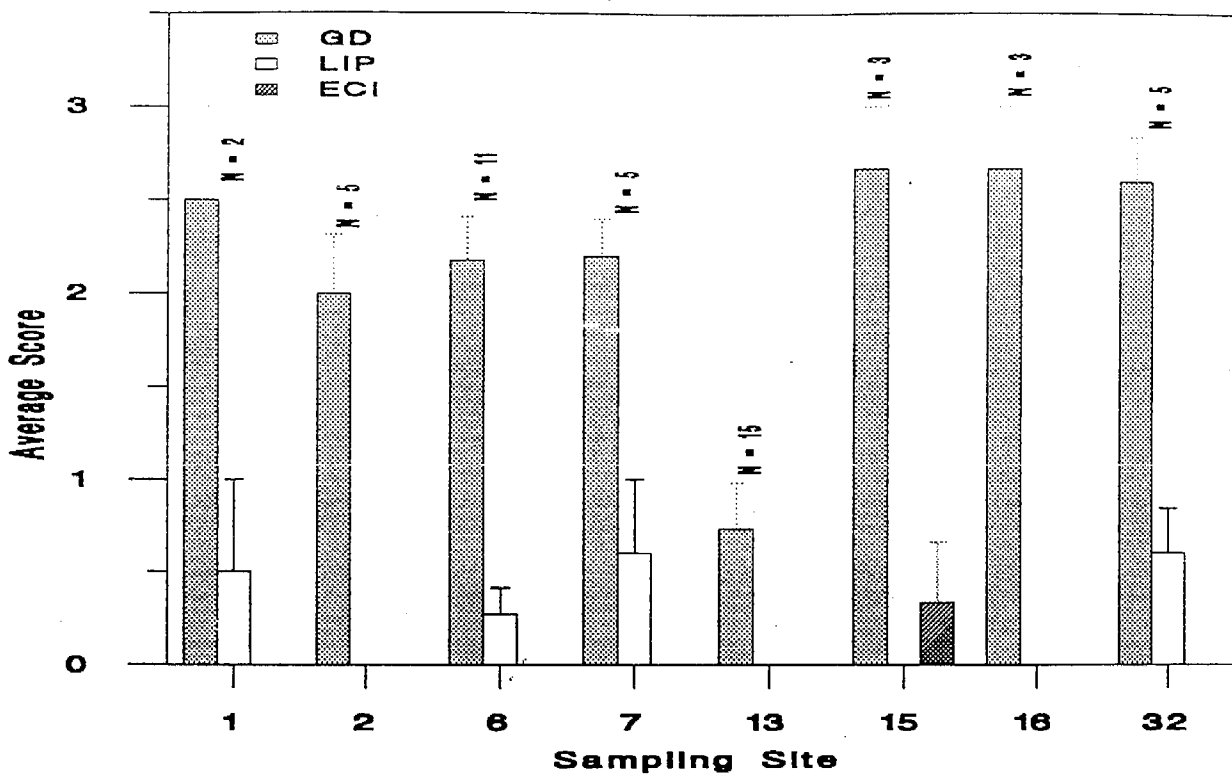
Graph 4a. Average Scores for Glycogen Depletion (GD), Lipidosis (LIP), and Eosinophilic Cytoplasmic Inclusions (ECI) in the livers of all fish.



Graph 4b. Average Scores for Glycogen Depletion (GD), Lipidosis (LIP), and Eosinophilic Cytoplasmic Inclusions (ECI) in Yellowfin Goby Livers.

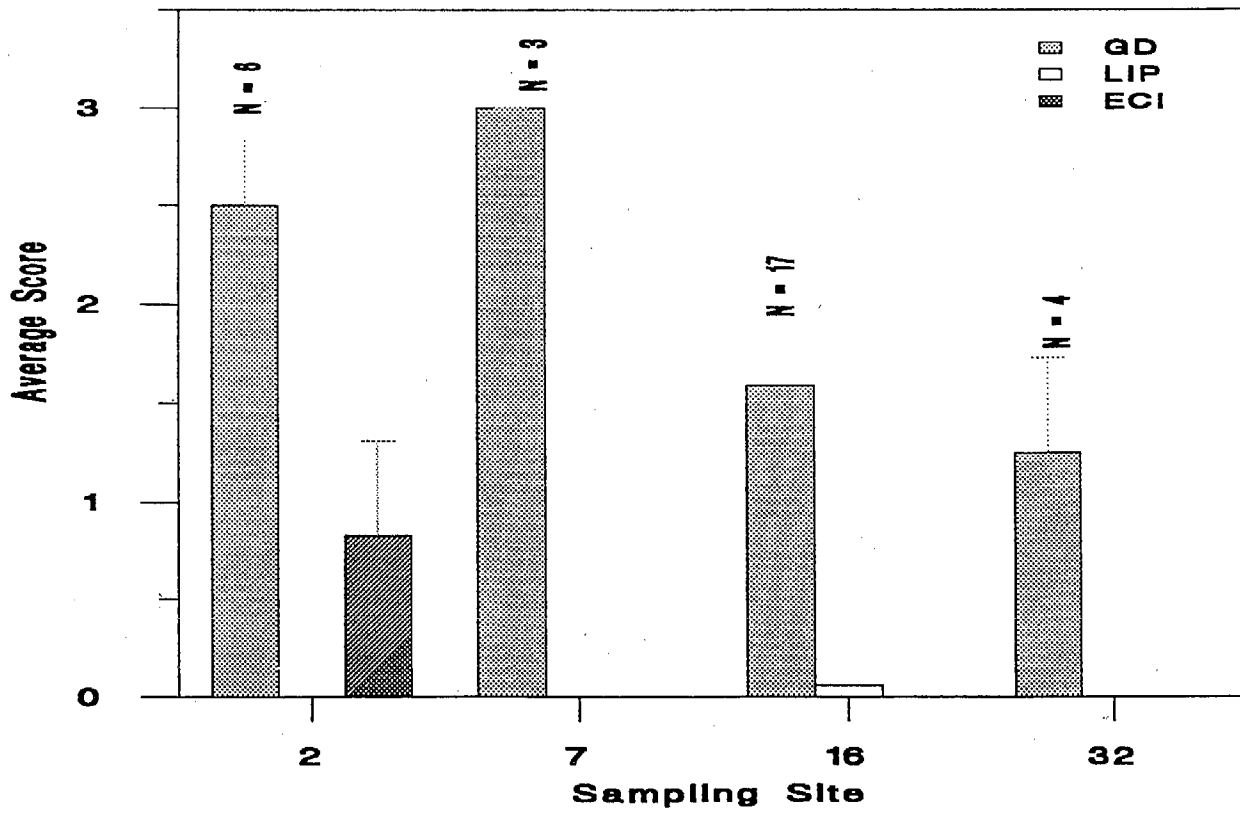


Graph 4c. Average Scores for Glycogen Depletion (GD), Lipidosis (LIP), and Eosinophilic Cytoplasmic Inclusions (ECI) in White Croaker Livers.



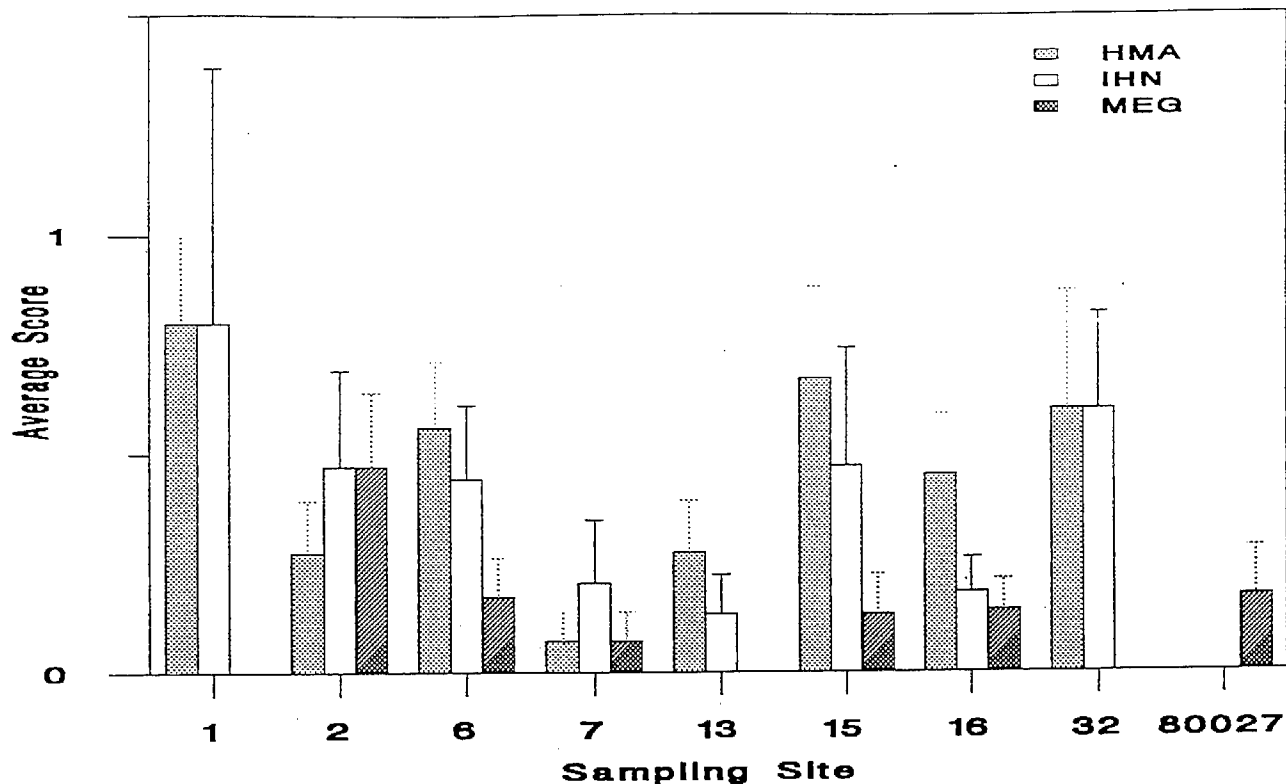
(all site numbers are plus 40000; i.e. site 1 = 40001)

Graph 4d. Average Scores for Glycogen Depletion (GD), Lipidosis (LIP), and Eosinophilic Cytoplasmic Inclusions (ECI) in Tonguefish Livers.



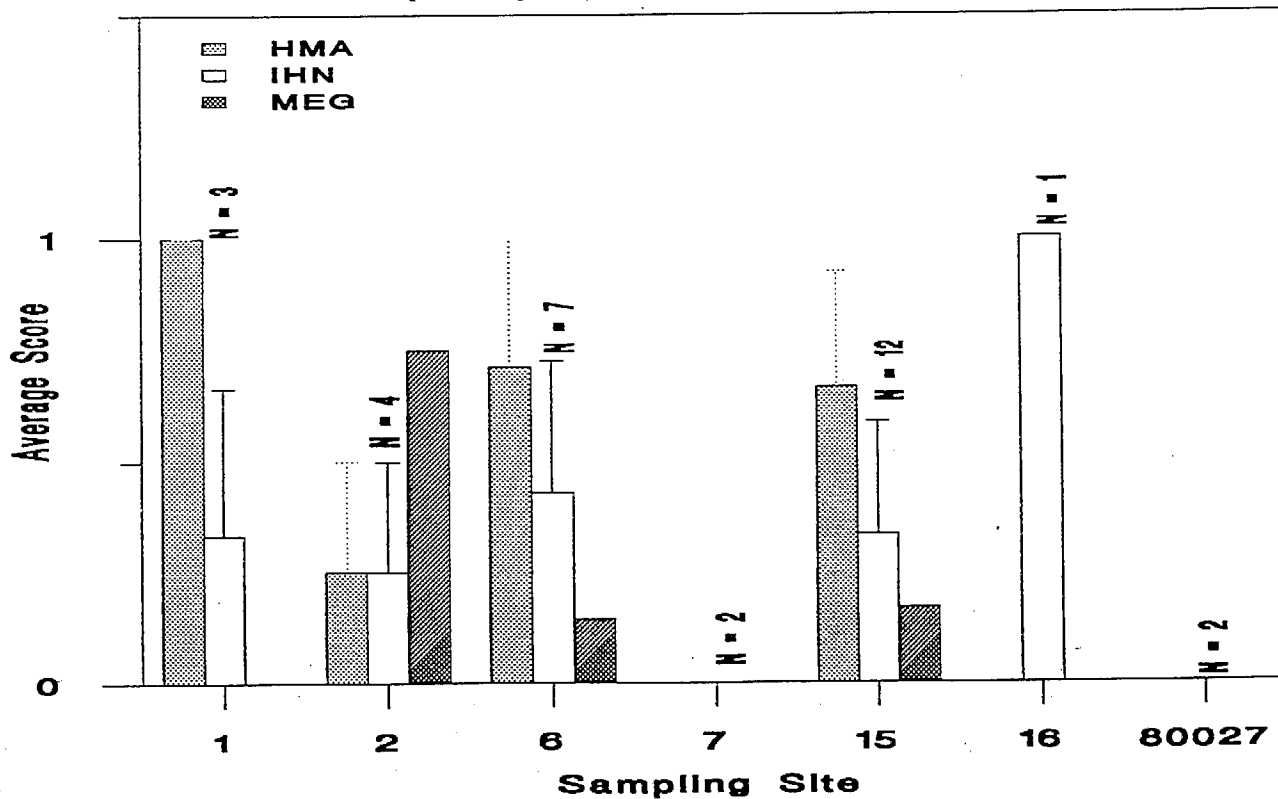
(all site numbers are plus 40000; i.e. site 1 = 40001)

Graph 5a. Average Scores for Hepatocyte Macrophage Aggregates (HMA), Individual Hepatocyte Necrosis (IHN), and Megalocytosis in all fish.



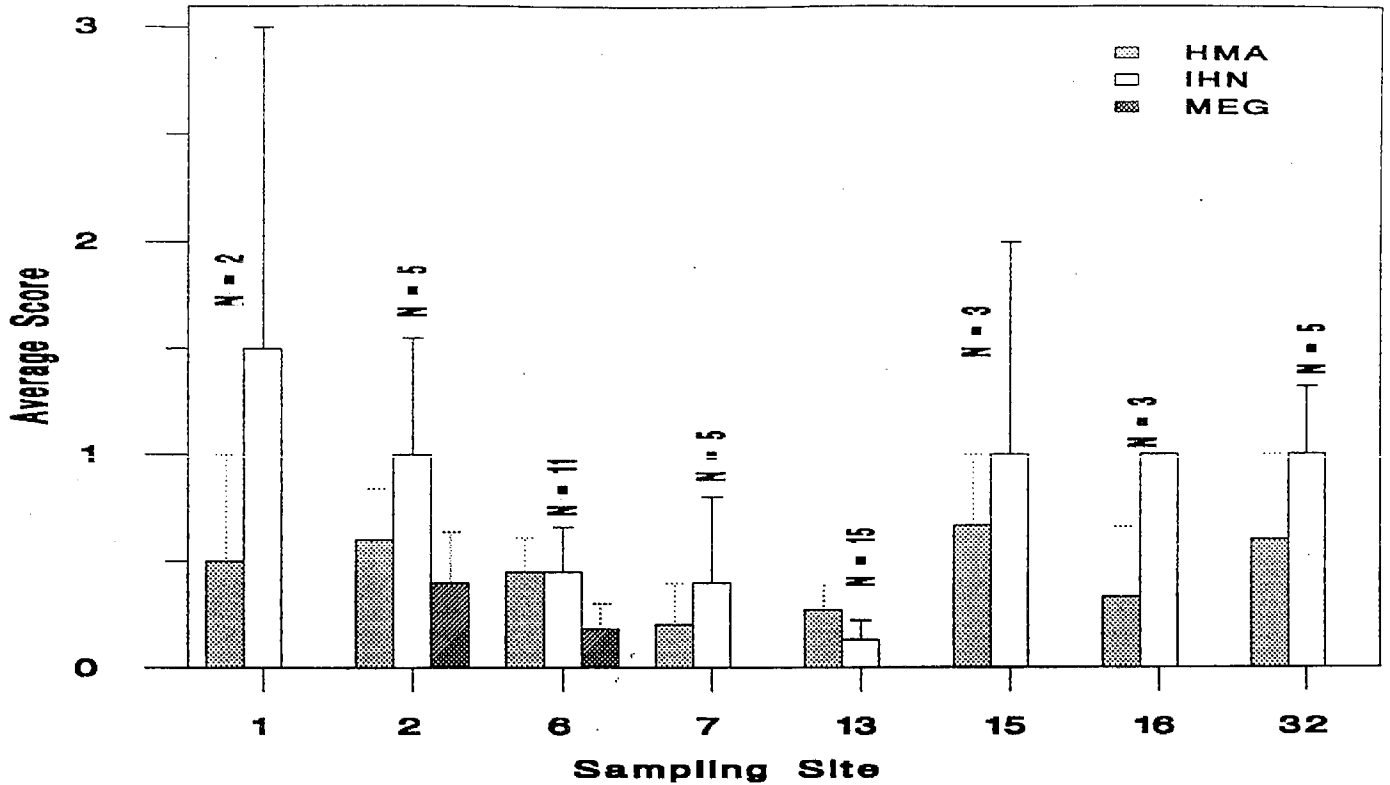
(all site numbers, except the last, are plus 40000; i.e. site 1 - 40001)

Graph 5b. Average Score for Hepatocyte Macrophage Aggregates (HMA), Individual Hepatocyte Necrosis (IHN), and Megalocytosis (MEG) in Yellowfin Gobies.



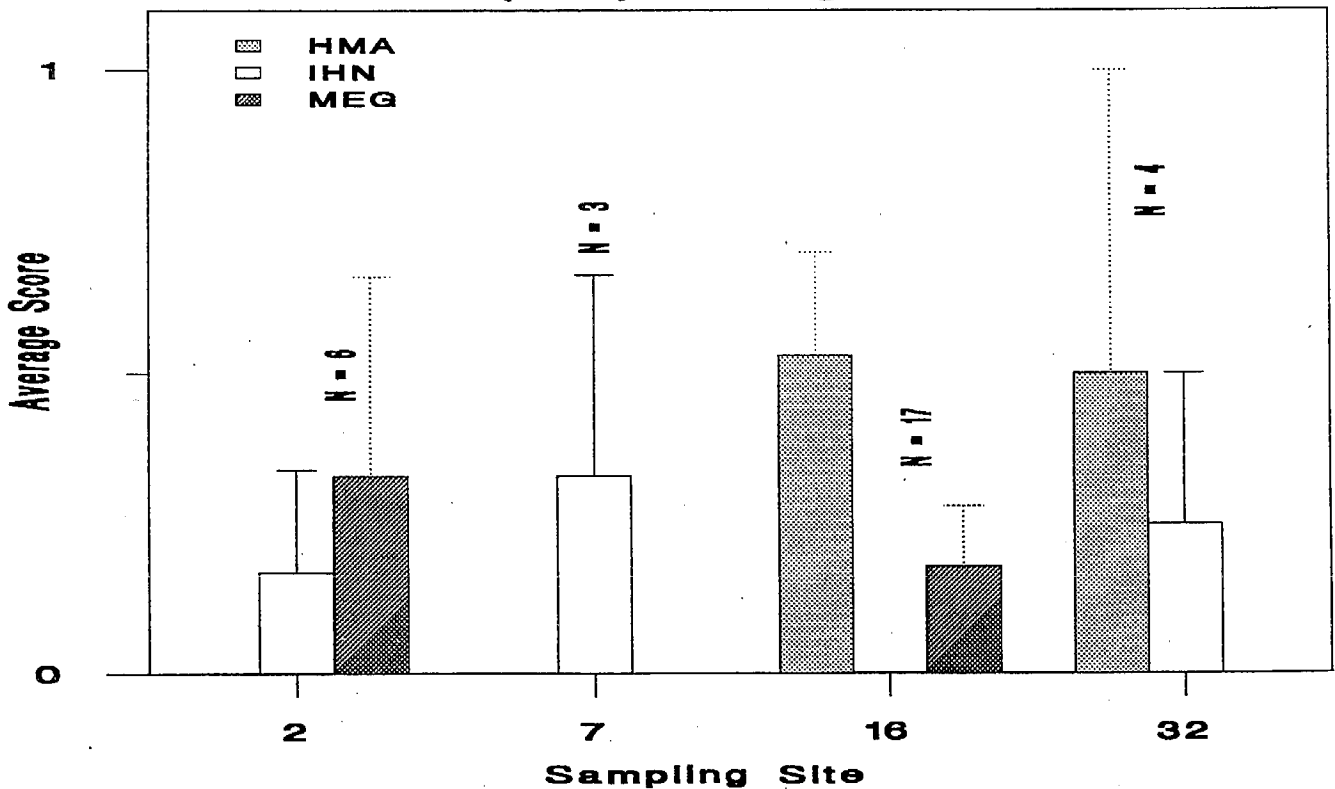
(all site numbers, except the last, are plus 40000; i.e. site 1 - 40001)

Graph 5c. Average Scores for Hepatocyte Macrophage Aggregates (HMA), Individual Hepatocyte Necrosis (IHN), and Megalocytosis (MEG) in White Croakers.



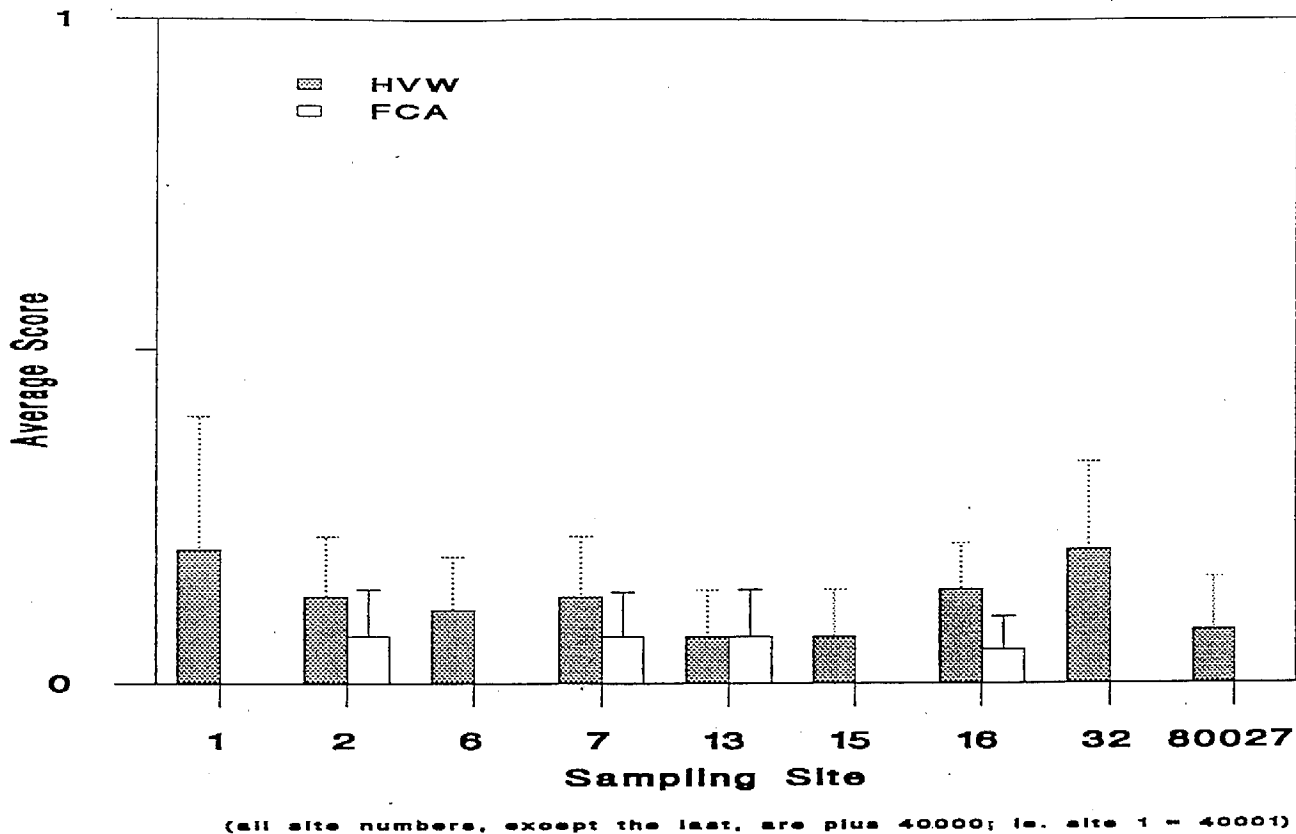
(all site numbers are plus 40000; i.e. site 1 = 40001)

Graph 5d. Average Scores for Hepatocyte Macrophage Aggregates (HMA), Individual Hepatocyte Necrosis (IHN), and Megalocytosis (MEG) in Tonguefish.

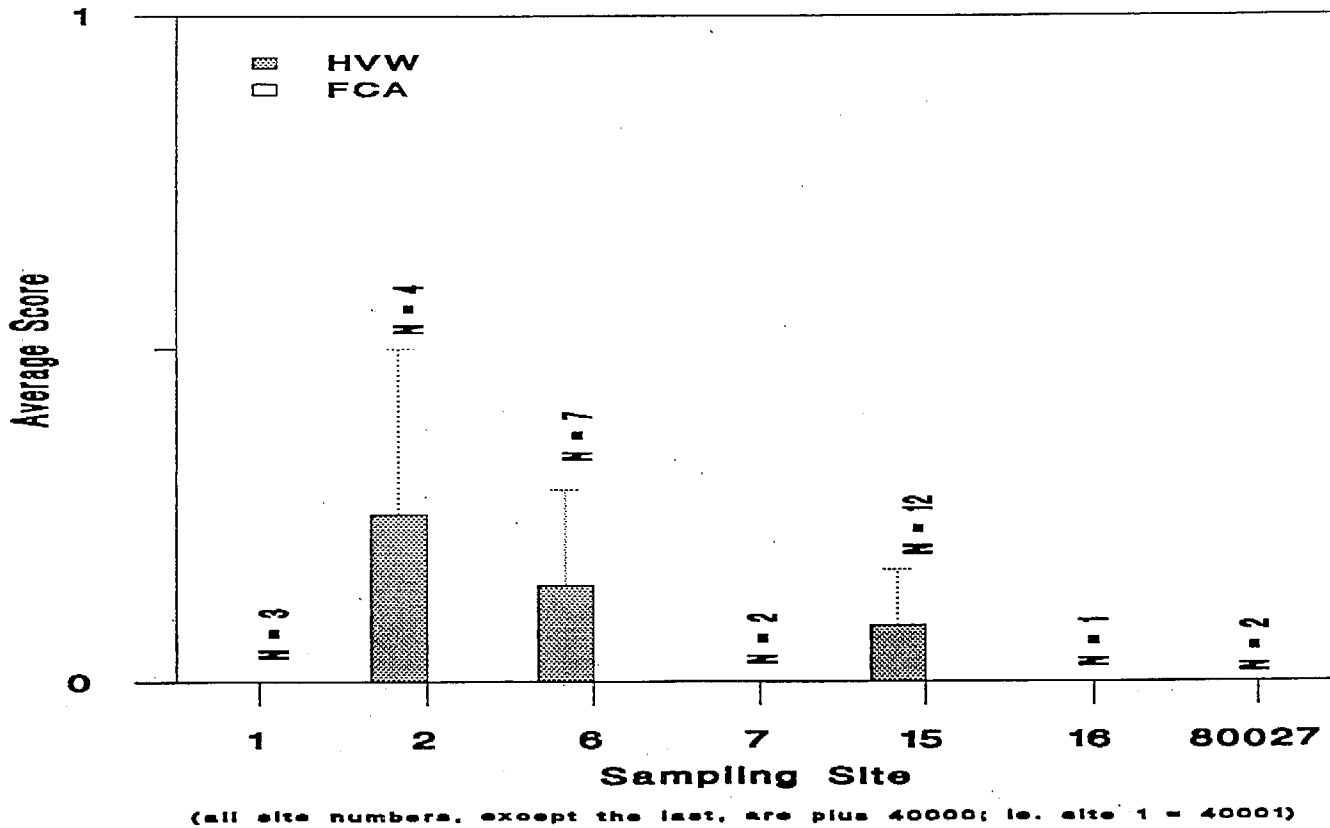


(all site numbers are plus 40000; i.e. site 2 = 40002)

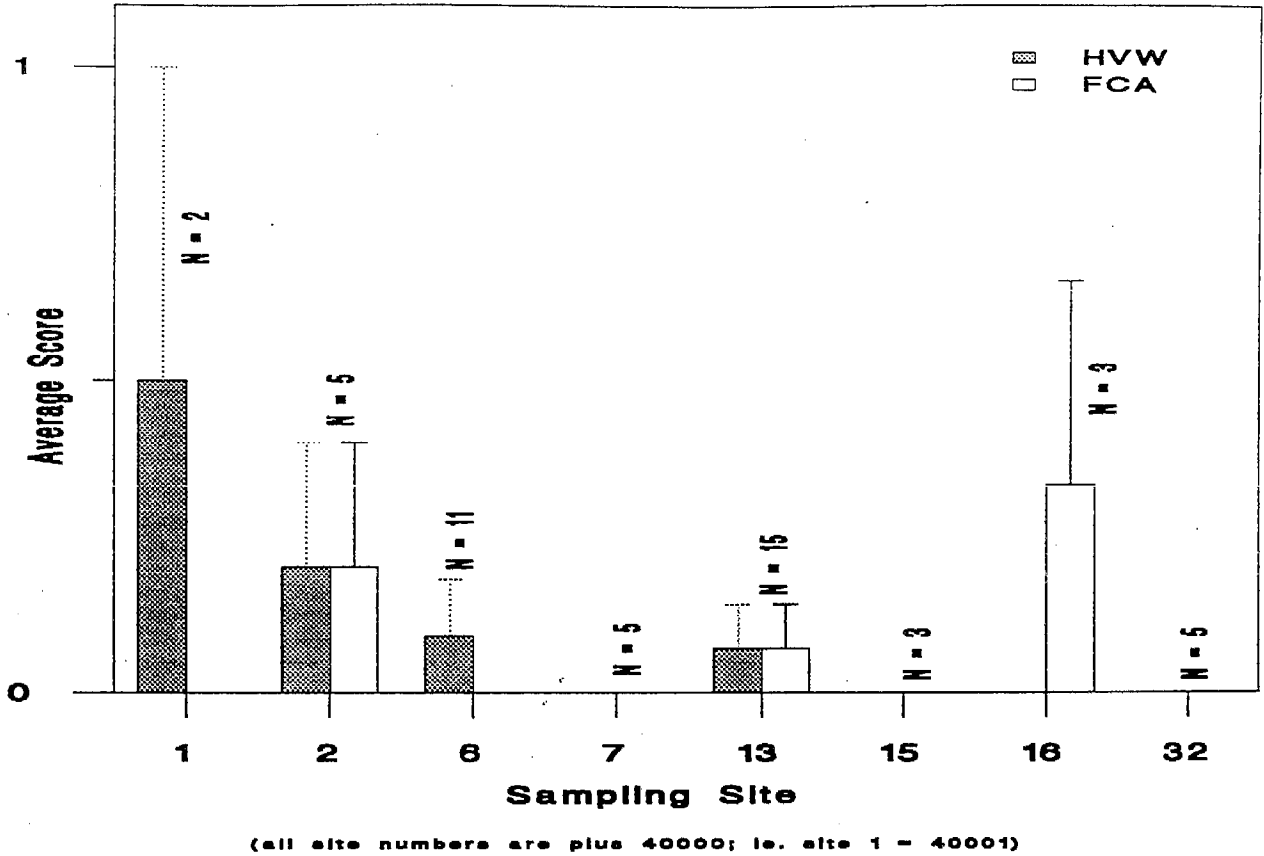
Graph 6a. Average Scores for Foci of Cellular Alteration (FCA) and Hyallnization of Vessel Walls (HVW) in all fish.



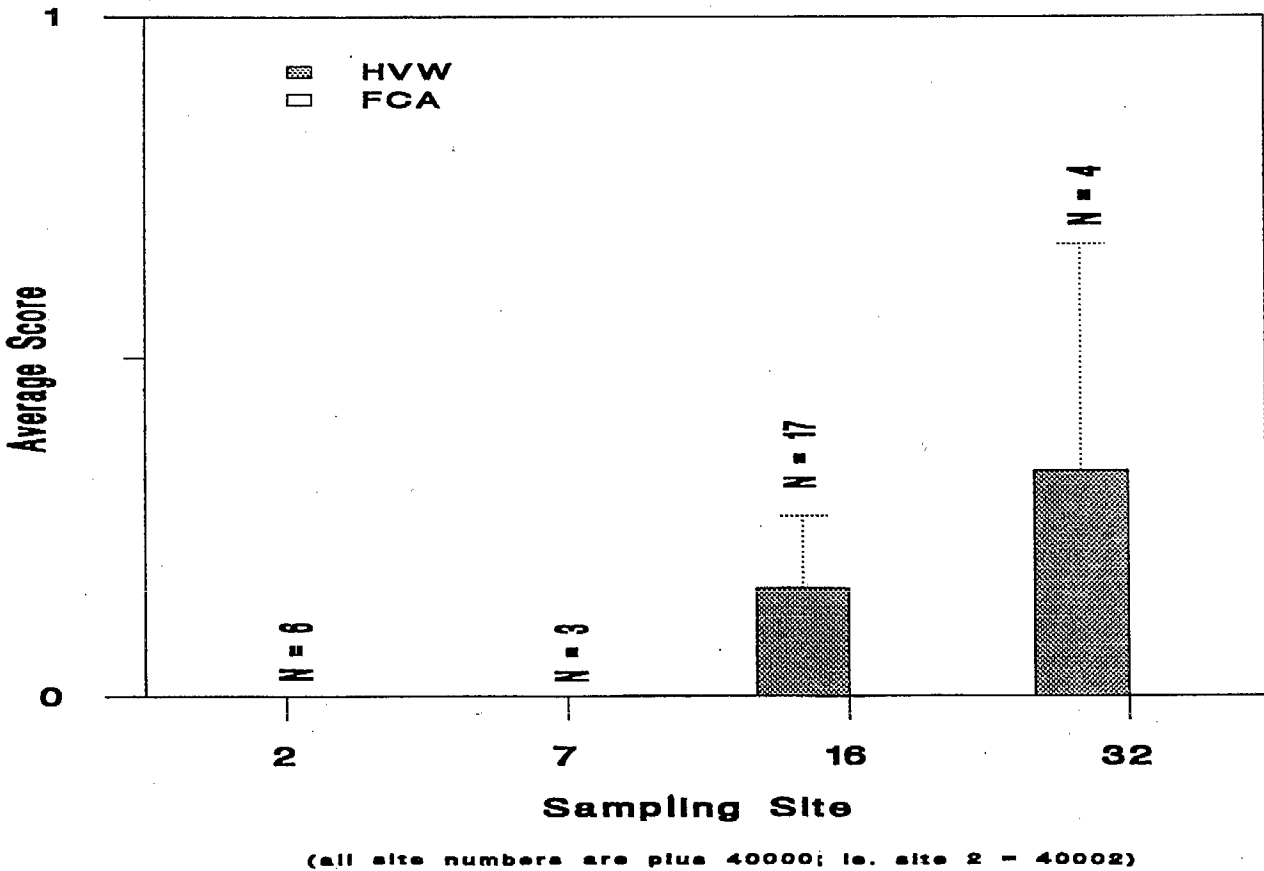
Graph 6b. Average Scores for Foci of Cellular Alteration (FCA) and Hyallnization of Vessel Walls (HVW) In Yellowfin Goby Livers.



Graph 6c. Average Scores for Foci of Cellular Alteration (FCA) and Hyalinization of Vessel Walls (HVW) in White Croakers.



Graph 6d. Average Scores for Foci of Cellular Alteration (FCA) and Hyalinization of Vessel Walls (HVW) in Tonguefish.



Histopathology - Results:

III. Histopathology of Other Organs

A. Skin:

1. **Stingrays:** Ten stingrays were caught at site 80027. Five rays had foci of melanosis over their dorsal surface and one had a black focus on the ventral skin. Foci varied from 0.1 to 8 mm in diameter and were non-raised and grey to black. Histologically, these foci of melanosis were characterized by mild hyperplasia of melanophores in both the dermis and epidermis. In contrast to normal skin (Figures 30 & 31) with only widely scattered small melanophores in the superficial dermis, melanophores in hyperplastic foci were prominent with dark brown melanin pigment. Hyperplastic melanophores were spindle to stellate with finely tapered cell processes (Figures 32-35).

In addition, some rays also had acanthosis (hyperplasia of squamous epithelial cells), mucous cell hyperplasia, and scattered single cell necrosis in the epidermis (Figures 34 & 35).

2. **Tonguefish:** Many tonguefish had mild to marked melanosis involving the caudal aspect of the dorsal and ventral fins, and the tail. Histologically, the melanosis was characterized by mild to moderate hyperplasia of melanophores in the superficial dermis (Figures 36 & 37). With mild melanophore hyperplasia, the melanophore layer was still intermittent. With moderate hyperplasia, the melanophore layer was continuous and in some areas, in multiple layers.

- #### B. Kidney:
- A few stingray kidneys were examined histologically and some had marked membranous glomerulonephritis, along with a few tubular casts (Figures 38 & 39).

- #### C. Gills:
- The gills were briefly screened, in some fish, and observed lesions included: 1) mucus cell hyperplasia (Figures 40 & 41); 2) lamellar epithelial hyperplasia; 3) interstitial fibrosis (Figures 40 & 41); and 4) inflammation (primarily with EGLs).

Histopathology - Results:

III. Histopathology of Other Organs

- D. **Gonads:** Ovaries had varying degrees of oocyte atresia and mixed inflammation involving both lymphocytes and eosinophilic granular leukocytes (EGLs). Testes in some white croakers were very small (<1mm diameter) with little or no sperm production. These testes were either atrophic (testes of sexually mature males which had undergone atrophy) or were immature (testes of young sexually immature male fish). A few croakers with atrophic/immature testes also had small numbers of developing oocytes and these fish were classified as "intersex."

[Processing note: Gonads were fixed and processed together with the liver, spleen, and gonad. In retrospect, this was a mistake. Because gonads, especially testes, were often very small many were lost during routine paraffin processing. Tissues could have been lost either via passing through the slots in the cassettes or may have been inadvertently "sectioned through" when the histotech was sectioning the blocks. In the future, liver, spleen, and gonad should be fixed in separate vials and processed in separate cassettes. The addition of small sponges in the cassettes would prevent loss during processing. Having individual blocks for each tissue would also prevent accidental "sectioning through" when looking for other tissue.]

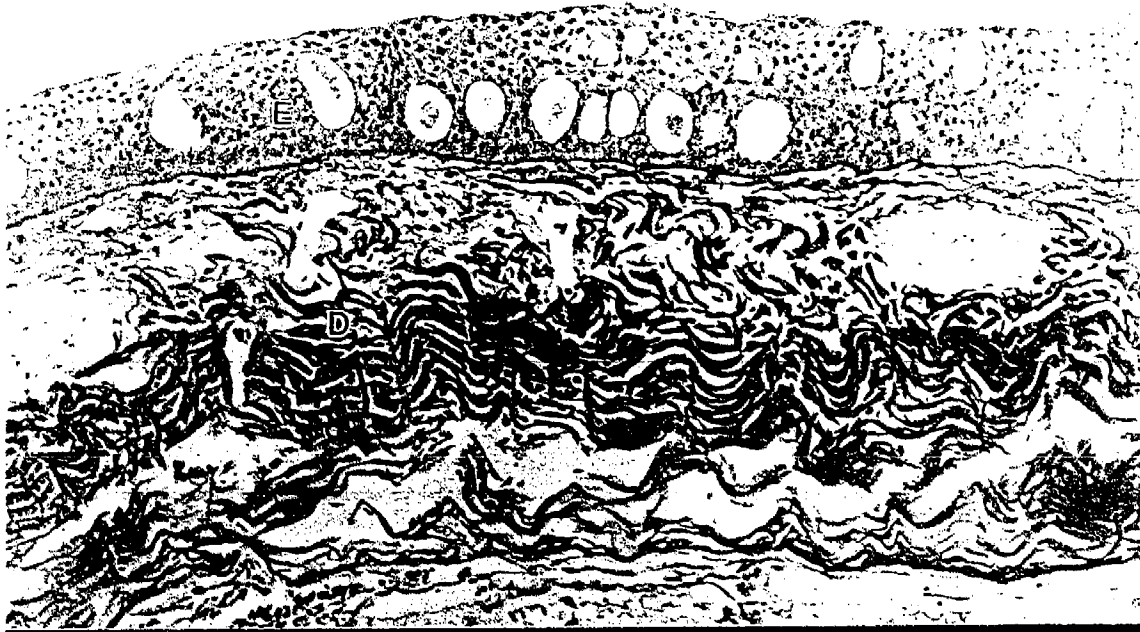


Figure 30. Normal dorsal skin from fish 93H63-40 (stingray from site 80027). Note the absence of pigmented melanophores in both the epidermis (E) and dermis (D). HE 25X.

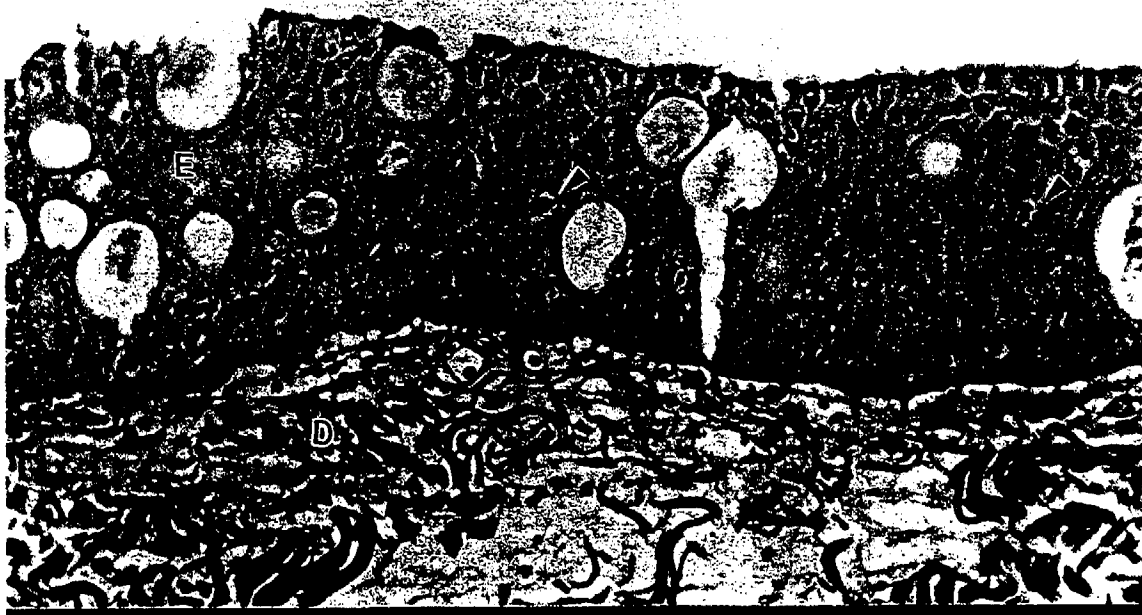


Figure 31. Normal dorsal skin from fish 93H63-40 (stingray from site 80027). Note the absence of melanophores in the epidermis (E) and dermis (D). There are a few dead keratinocytes in the epidermis (arrowheads). HE 50X.

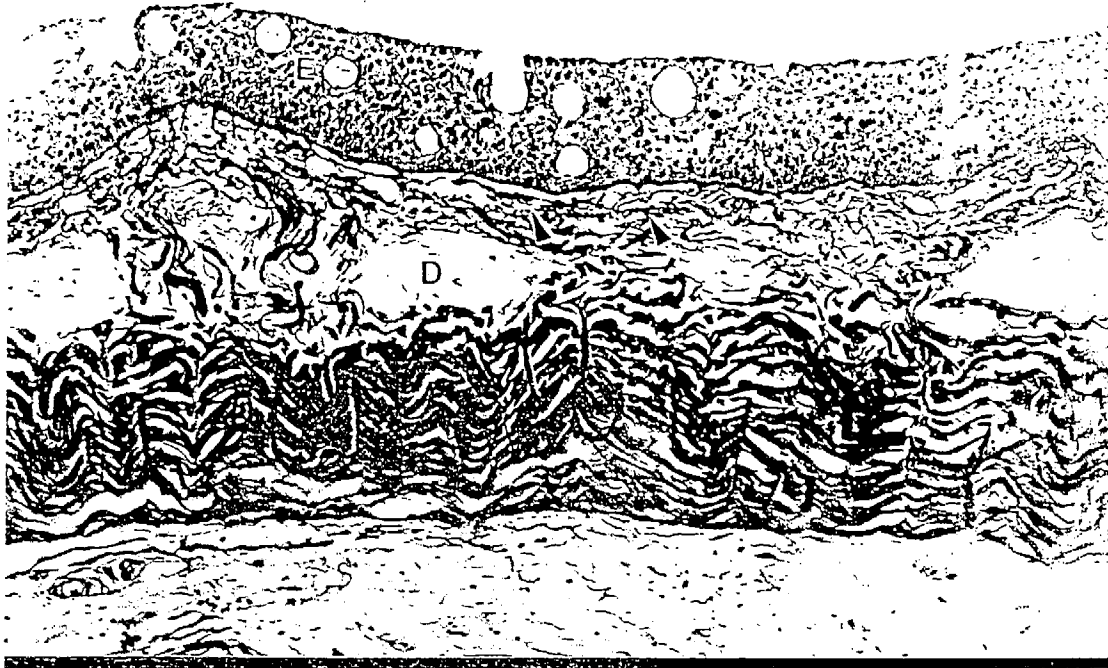


Figure 32. Dorsal skin from fish 93H63-40 (stingray from site 80027). There is mild melanophore hyperplasia in both the epidermis (E) and dermis (D). Melanophores are concentrated (arrowheads) in the dermis near the junction with the epidermis. HE 25X.

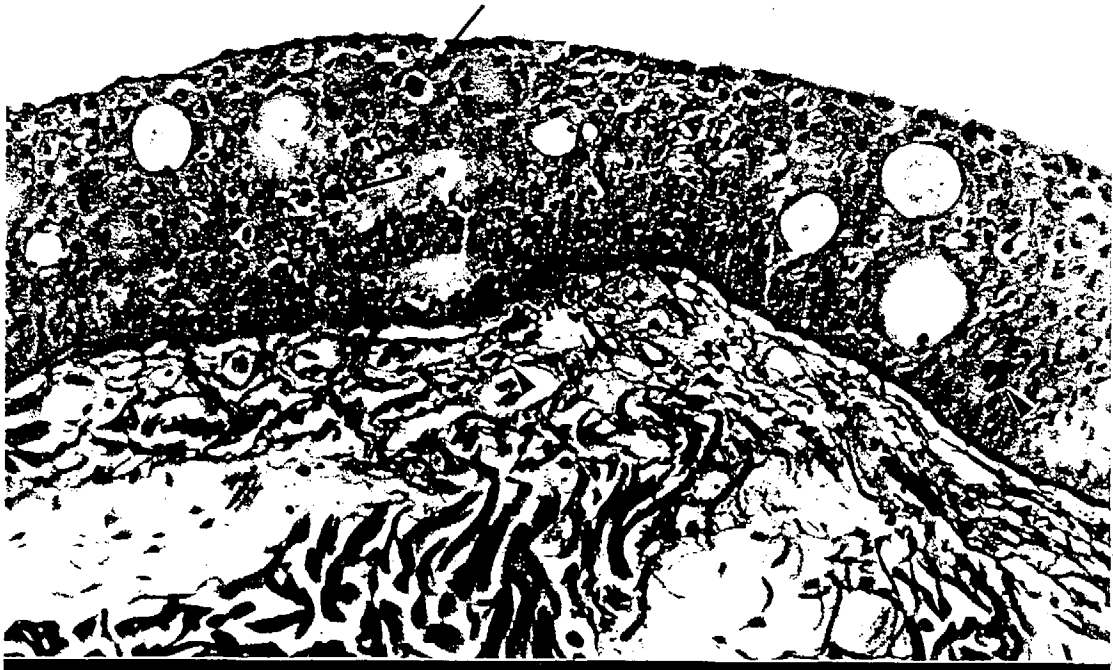
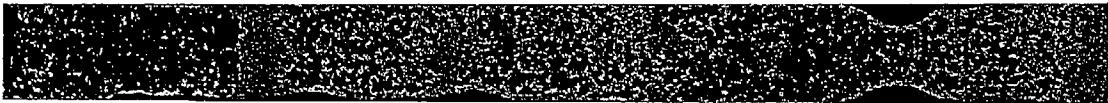


Figure 33. Dorsal skin from fish 93H63-40 (stingray from site 80027). Melanophores (arrowheads) are present both within the epidermis and dermis. Note the hyper eosinophilic dead keratinocytes (arrows). HE 50X.

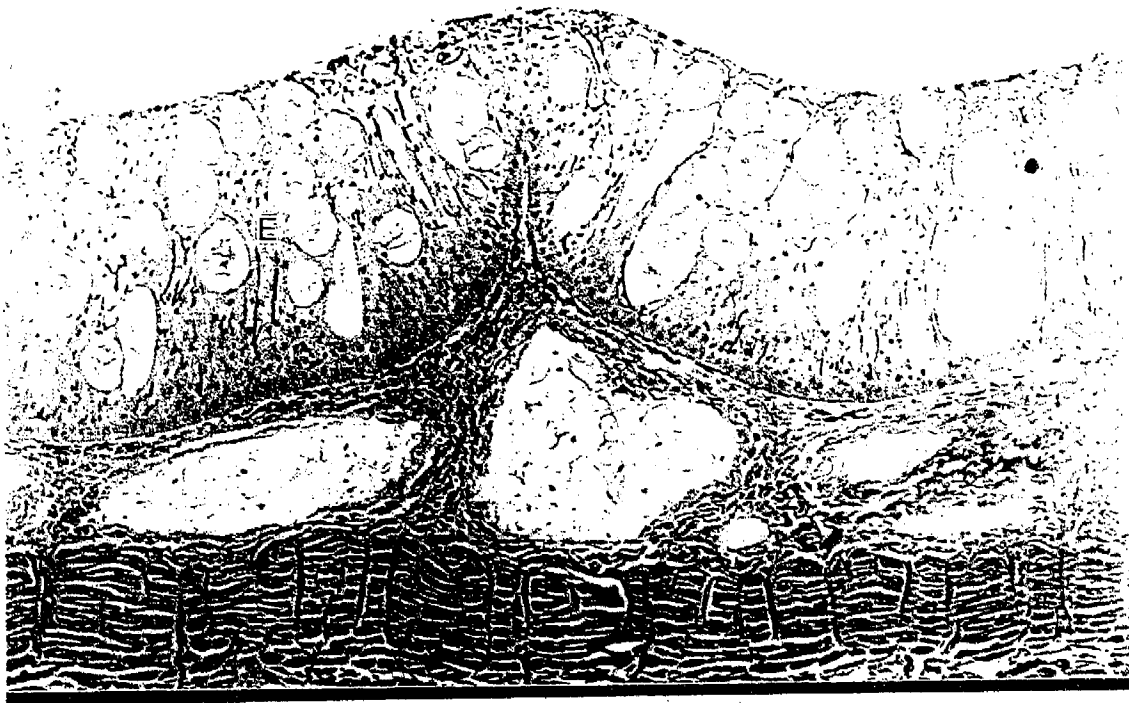


Figure 34. Dorsal skin from fish 93H63-14 (stingray from site 80027). There is marked acanthosis of the epidermis (E) and mild melanophore hyperplasia in both the epidermis and dermis. HE 25X.

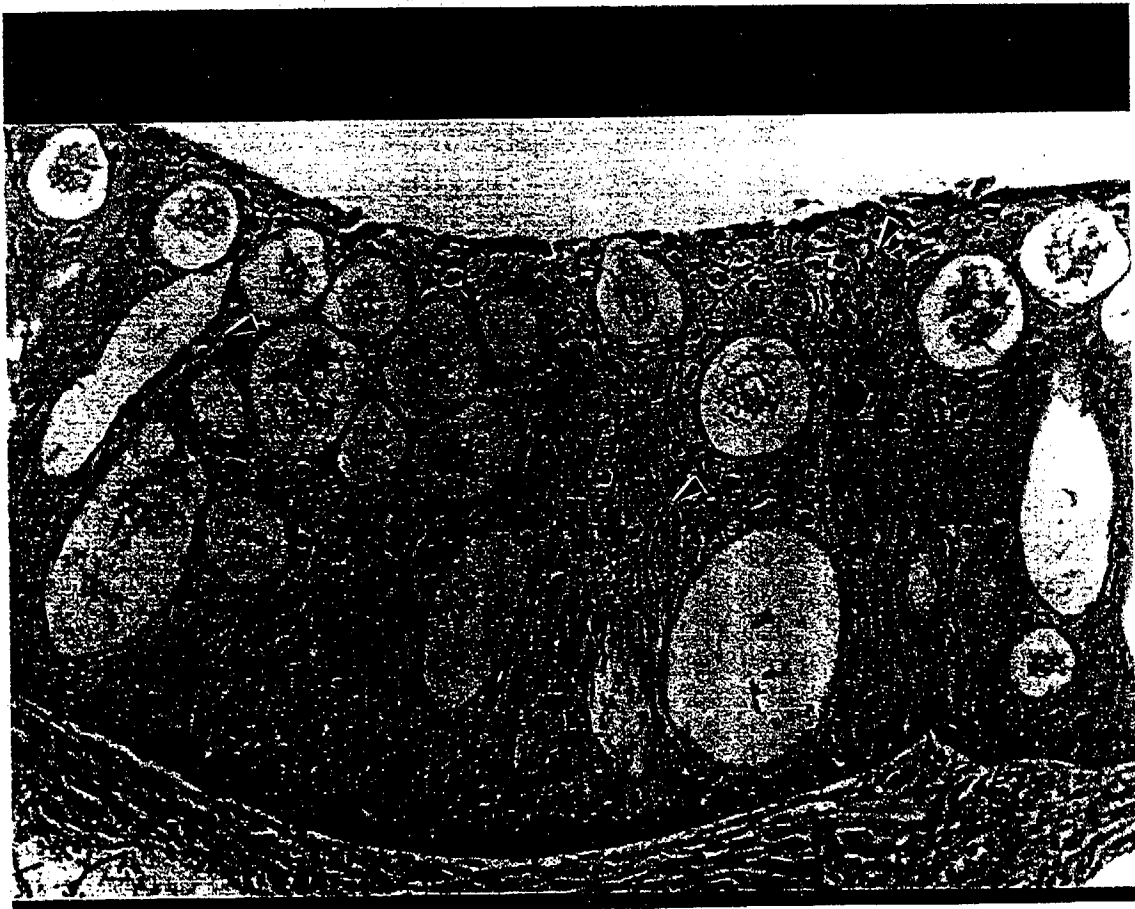


Figure 35. Dorsal skin from fish 93H63-14 (stingray from site 80027). Note how thick the epidermis is and the numerous melanophores (arrowheads). HE 50X.

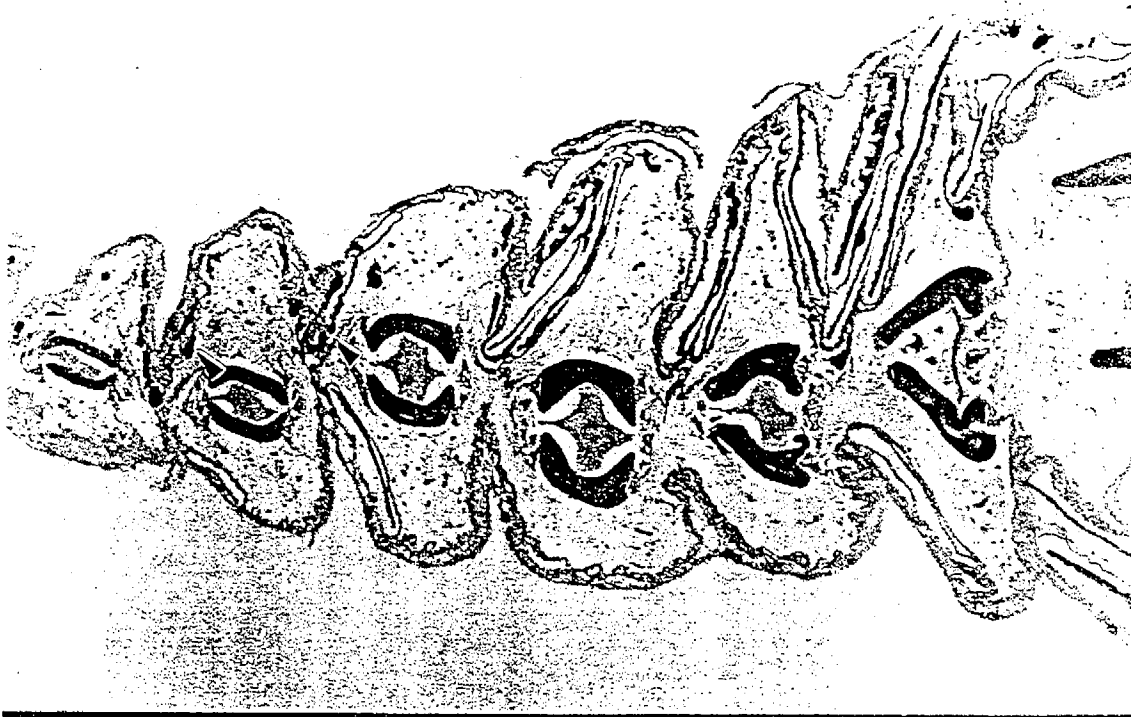


Figure 36. Dorsal and ventral skin from fish 93H63-16 (tonguefish from site 40016). There is mild melanophore hyperplasia (arrowheads) in the dermis of the dorsal skin. Note that there are still gaps in the melanophore layer where no pigment cells are visible. HE 25X.

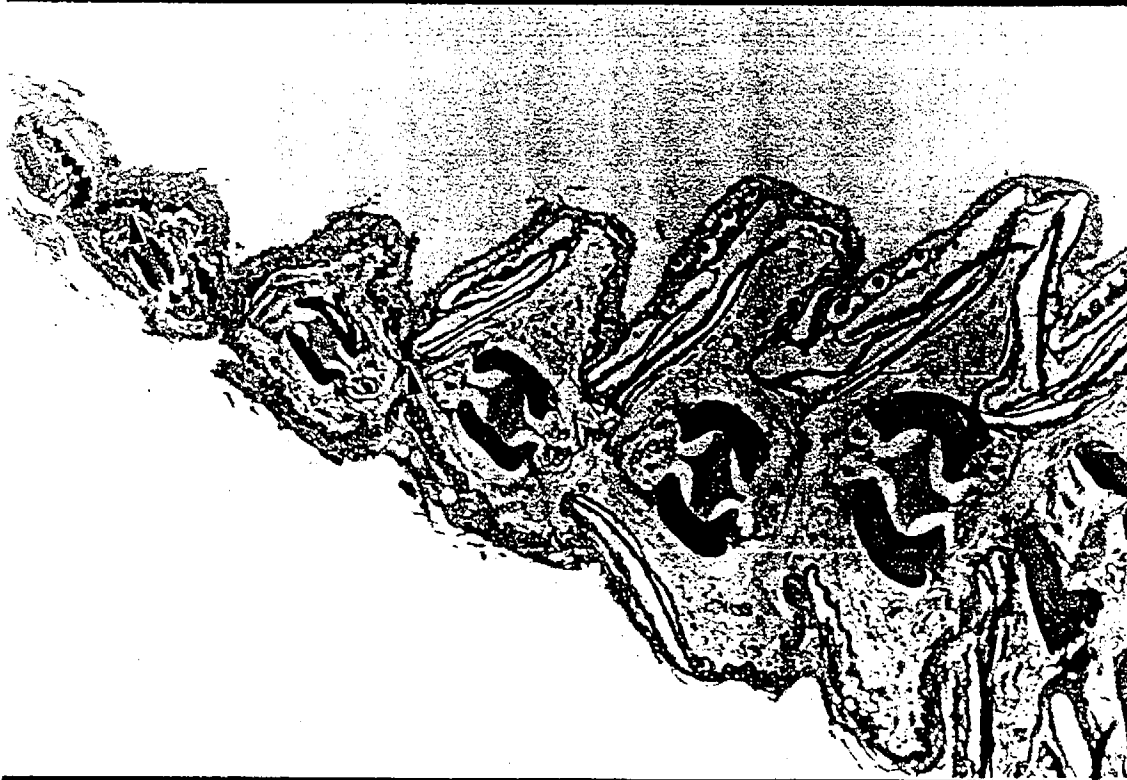


Figure 37. Dorsal and ventral skin from fish 93H63-90 (tonguefish from site 40016). There is mild to moderate melanophore hyperplasia (arrowheads) in the dermis of the dorsal skin. Note that the melanophore layer is continuous and multilayered in places. HE 25X.

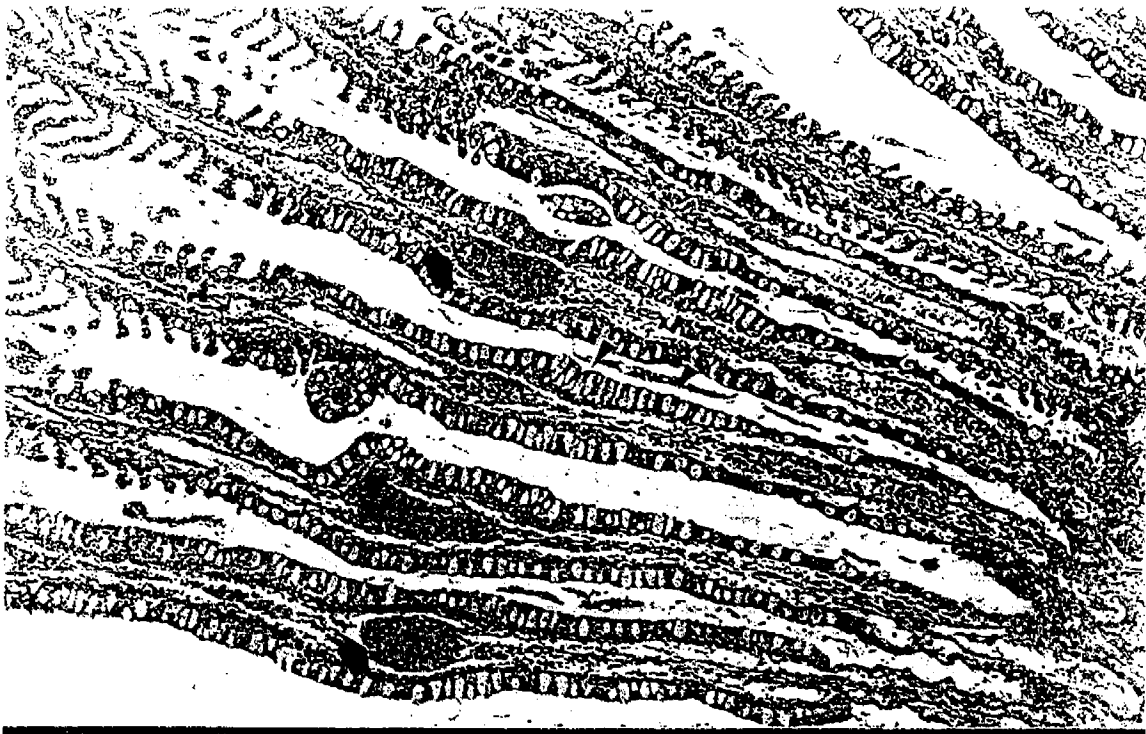


Figure 40. Gill of fish 93H63-41 (stingray from site 80027). Note the mucous cell hyperplasia (arrowheads) along the filaments and the numerous interstitial foci of fibrosis (arrows). HE 10X.



Figure 41. Higher magnification of figure 40. Note the hyperplastic mucous cells (arrowheads) in the filament epithelium and the focus of interstitial fibrosis (F). HE 50X.

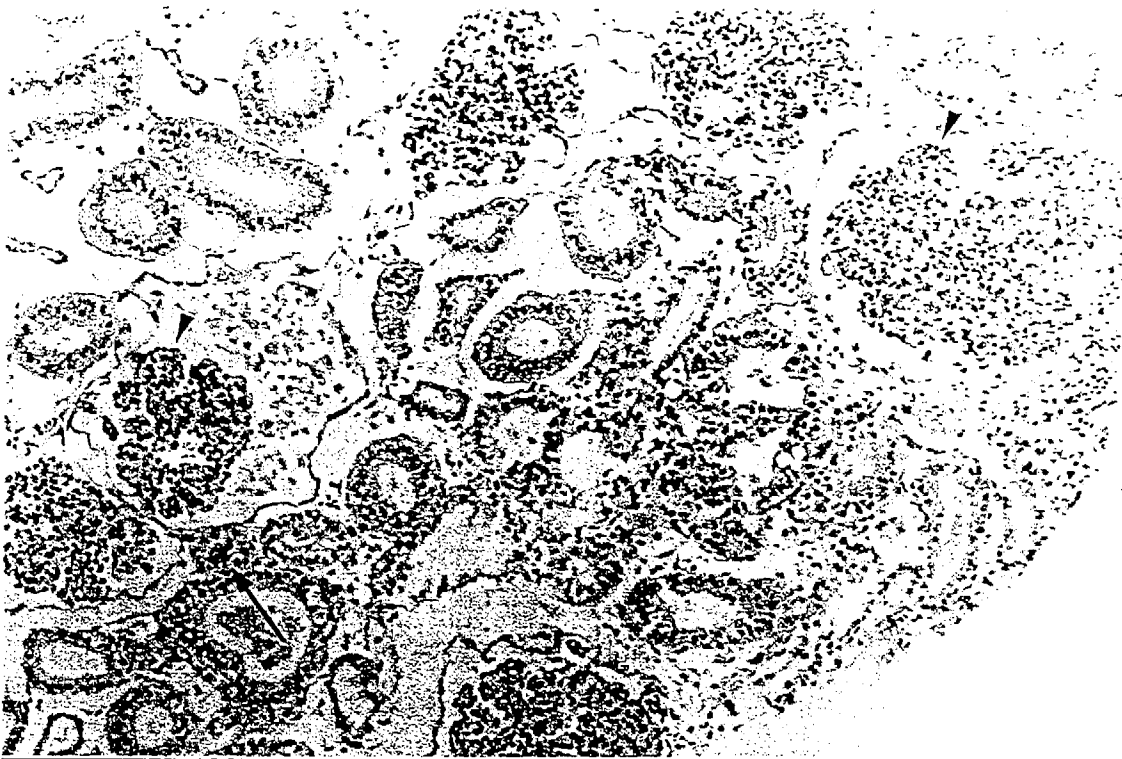


Figure 38. Kidney from fish 93H63-5 (stingray from site 80027) severe membranous glomerulonephritis. Note the thickened glomerular tufts (arrowheads) which are occasionally surrounded by a dilated Bowman's space. A few tubules have casts (arrow). HE 25X.

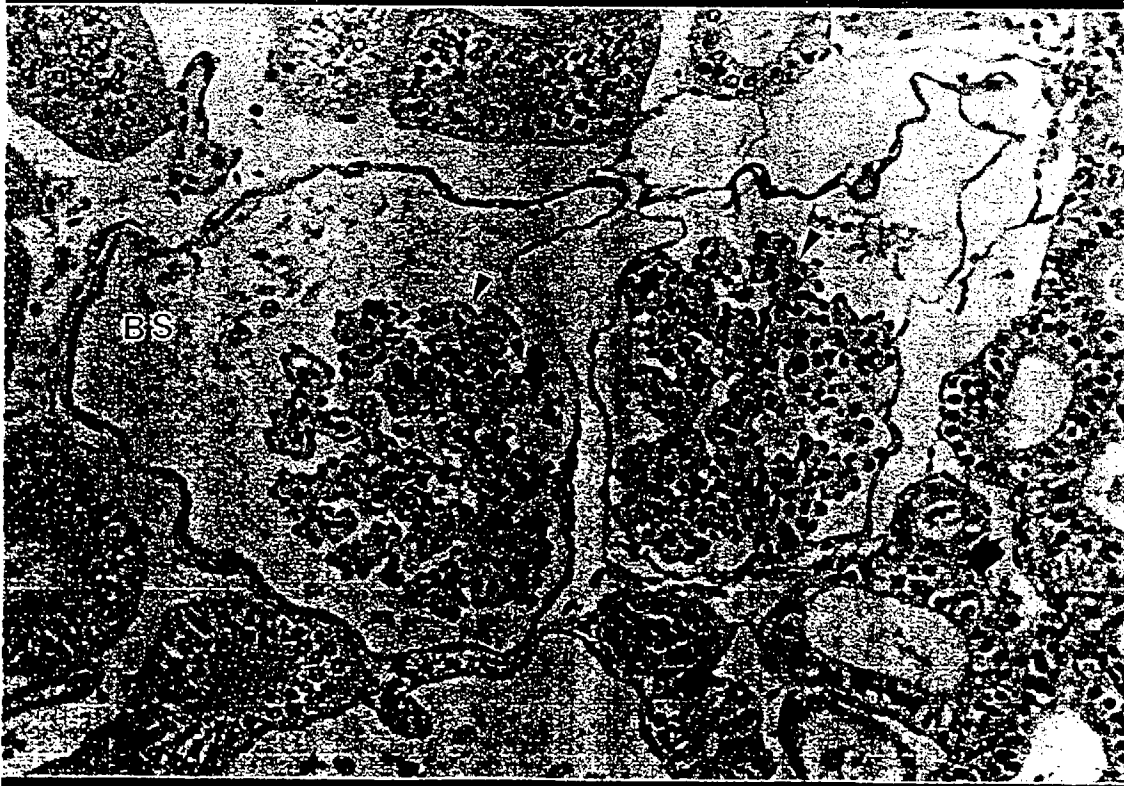


Figure 39. Kidney of fish 93H63-5 (stingray from site 80027). Note the markedly thickened glomerular basement membranes (arrowheads), dilated Bowman's space (BS), and tubular casts (arrows). HE 50X.

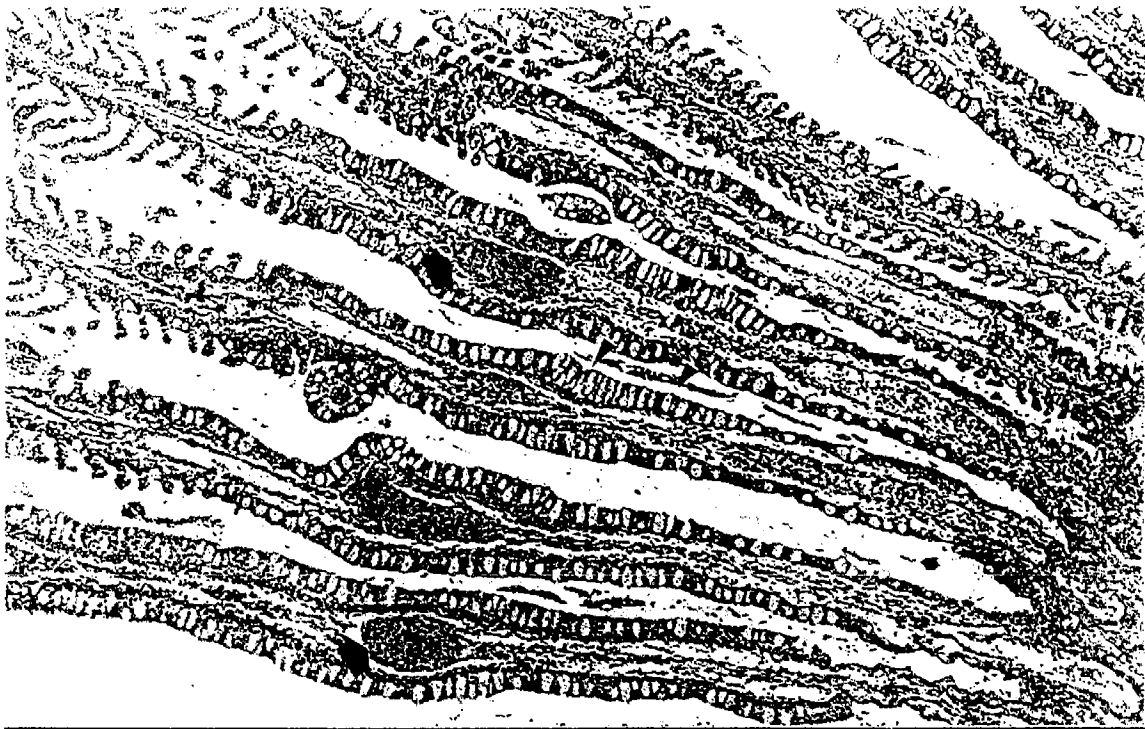


Figure 40. Gill of fish 93H63-41 (stingray from site 80027). Note the mucous cell hyperplasia (arrowheads) along the filaments and the numerous interstitial foci of fibrosis (arrows). HE 10X.

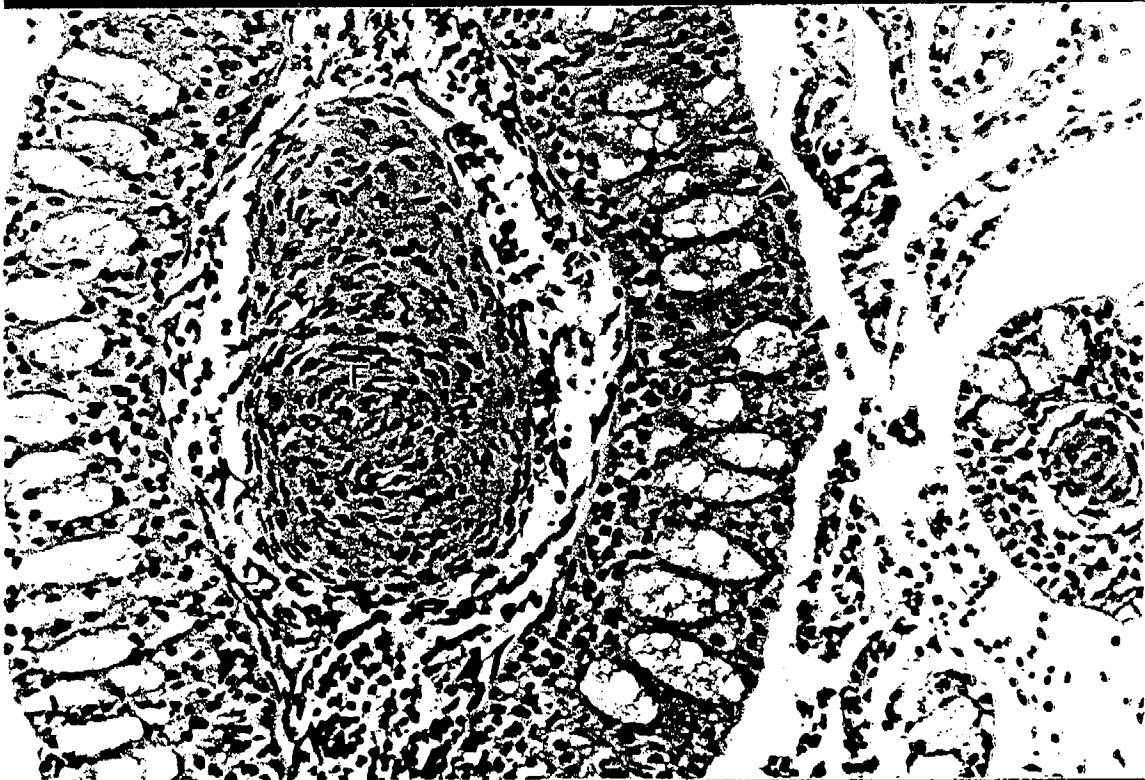


Figure 41. Higher magnification of figure 40. Note the hyperplastic mucous cells (arrowheads) in the filament epithelium and the focus of interstitial fibrosis (F). HE 50X.

P450 Immunohistochemistry - Results: P450 activity was detected in all organs examined. In the gills, P450 was concentrated in lamellar epithelium, pillar cells, and endothelium of large blood vessels in the arch (Figures 42 & 43). In the gonad and spleen, P450 was detected in the endothelium of major blood vessels (Figures 44 & 45). In liver, P450 was induced in hepatocytes and in the endothelium of major blood vessels (Figure 46). In kidney, P450 was detected in tubular epithelium and blood vessels. In intestine, P450 was found in the lining epithelium and blood vessels.

Immunohistochemical P450 scores for gill, gonad, spleen, liver, kidney, and intestine are given in appendix 16. Appendices 16a-16c include scores for all fish from all sites sorted on the basis of site. Average P450 score and standard error were determined for each organ at each site. Appendices 16d-16g are P450 scores sorted on the basis of both site and species.

Average P450 scores of selected organs are depicted in graphs 7-9. Graph 7a shows that the three reference sites (40015, 40016, and 40032) had markedly lower P450 scores in gill epithelial cells (GEC) and endothelium of gill arch (E-GA) when compared to the five impact sites where teleost fish were caught. Impact site 80027 (where 10 of 12 fish were stingrays) had low P450 gill scores which were comparable to those in two reference sites. Of the three reference sites, site 40032 appeared to be the "cleanest" with respect to induction of gill P450. When gill P450 scores were sorted on the basis of species, the same general trend (higher scores at impact sites) was also observed in gobies (graph 7b), croakers (7c), and tonguefish (graph 7d).

Graph 8a shows average P450 scores for endothelium in gonadal blood vessels (GO-VE) and splenic blood vessels (SVE). Again, the three reference sites have markedly lower P450 scores when compared to impact sites where teleost fish were caught. In addition, impact site 80027, where stingrays were collected, also had higher vascular P450 scores. Sorting the data based on species revealed similar patterns among gobies (graph 8b), croakers (8c), and tonguefish (graph 8d) with lower P450 scores at reference sites.

Graph 9a depicts average P450 scores for hepatocytes (HEP) and major liver blood vessels (LVE). The trends were similar to P450 scores in other organs, with the three reference sites having markedly lower average P450 scores. The only exception was with the LVE P450 score at site 80027 which was the lowest amongst all nine sites. Sorting the data based on species revealed that although gobies from impact sites still had higher average liver P450 scores, when compared to reference sites, goby scores (graph 9b) were lower than those in tonguefish (graph 9d) from impact sites and markedly lower when compared to croakers from impact sites (graph 9c). Hepatic (both HEP and LVE) P450 goby scores from impact sites were even lower than scores from croakers collected from reference sites. Average HEP P450 scores in croakers from two reference sites (40015 and 16) were similar or higher than three of five impact sites where croakers were collected. LVE in contrast was consistently higher in croakers from impact sites when compared to the reference sites. Both HEP and LVE P450 scores were markedly higher in tonguefish from two impact sites when compared with those from two reference sites.

P450 Immunohistochemistry - Statistics: Due to missing values, only 82 fish were used with this analysis. Only one principal component was used as only one accounted for appreciable variability. From individual scale values, three vascular P450 categories [liver vascular endothelium (LVE), splenic blood vessels (SVE), and endothelium of gill arch blood vessels (E-GA)] were most important (eigenvectors with the greatest absolute values). Three other categories [gill pillar cells (GPC), gill epithelial cells (GEC), and hepatocytes (HEP)] also had high eigenvector values and there was strong positive correlation between almost all tissues where P450 activity was observed.

MANOVA revealed that there were significant ($P = 0.01$) differences among the nine sites, but no overall species effect. Comparisons among sites using both Tukey's and Least Square Means revealed significant differences between impact and reference sites. With Tukey's, P450 scores from impact site 40001 were significantly ($P < 0.05$) higher than scores at reference sites 40016 and 40032. P450 scores were also significantly ($P < 0.05$) higher at impact site 40002 when compared to reference site 40032.

Using Least Squares Means, three impact sites (40001, 40002, and 40006) had P450 scores which were significantly ($P < 0.01$) elevated over two reference sites (40016 and 40032). In addition, P450 scores from two impact sites (40001 and 40002) were also significantly ($P \leq 0.01$) higher than scores from the third reference site, 40015.

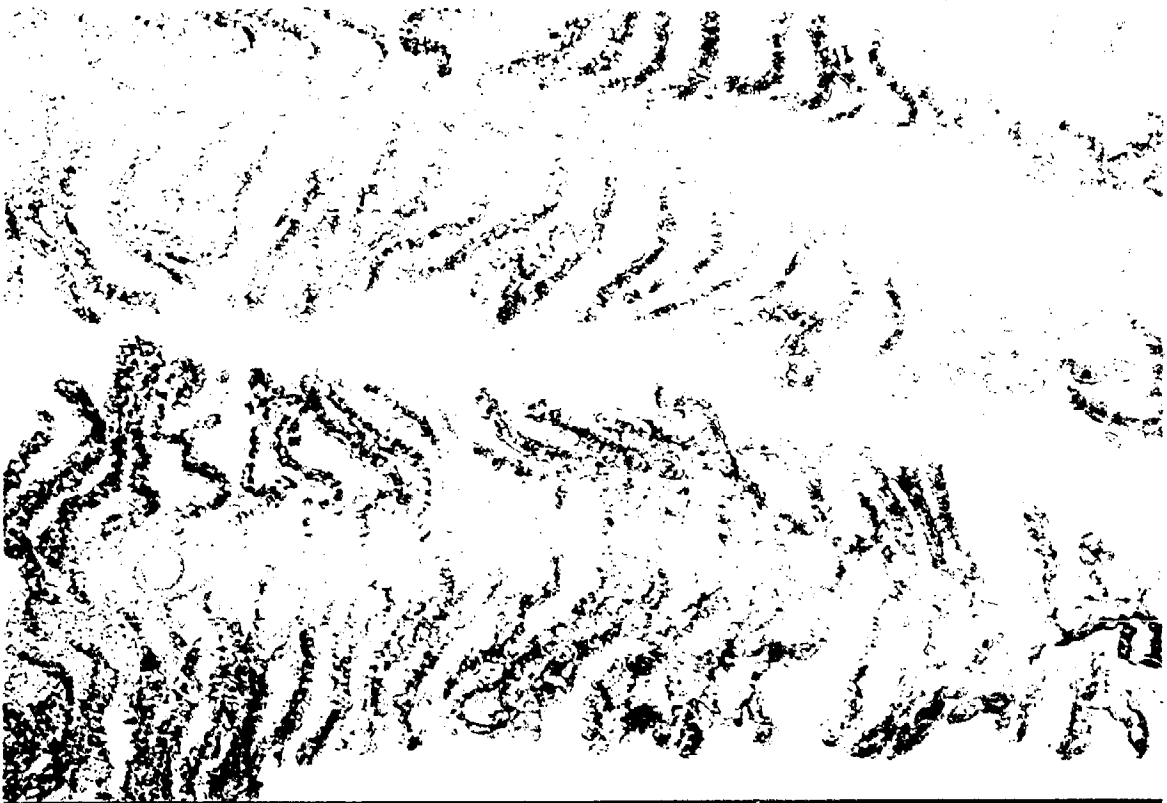


Figure 42. Gill of fish 93H63-98 (white croaker from site 40006). There is marked induction of P450 activity in both lamellar epithelium and pilar cells of gill filaments. Immunohistochemical stain with hematoxylin counterstain.



Figure 43. Gill of fish 93H63-98 (white croaker from site 40006). There is marked induction of P450 activity in both lamellae (arrowheads) and endothelium of gill arch blood vessels (arrows). Immunohistochemical stain with hematoxylin counterstain.

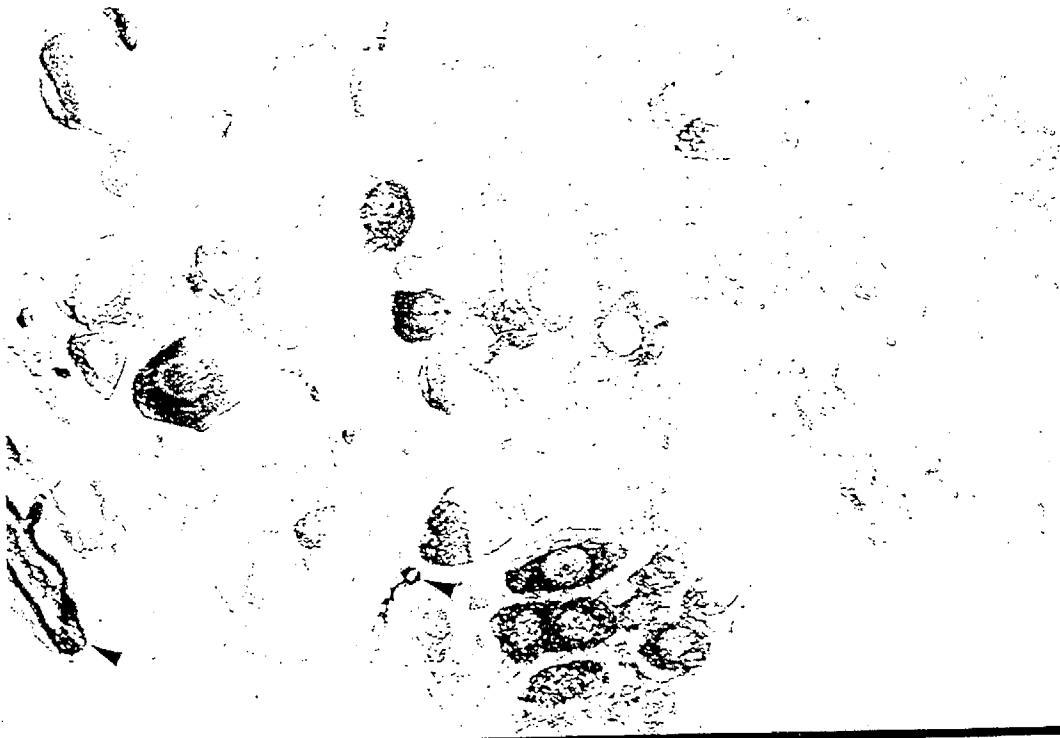


Figure 44. Ovary of fish 93H63-98 (white croaker from site 40006). There is marked induction of P450 activity in the endothelium of interstitial blood vessels (arrowheads). Immunohistochemical stain with hematoxylin counterstain.



Figure 45. Spleen of fish 93H63-91 (white croaker from site 40002). The endothelium of major splenic blood vessels is positive for P450 (arrowheads). Immunohistochemical stain with hematoxylin counterstain.

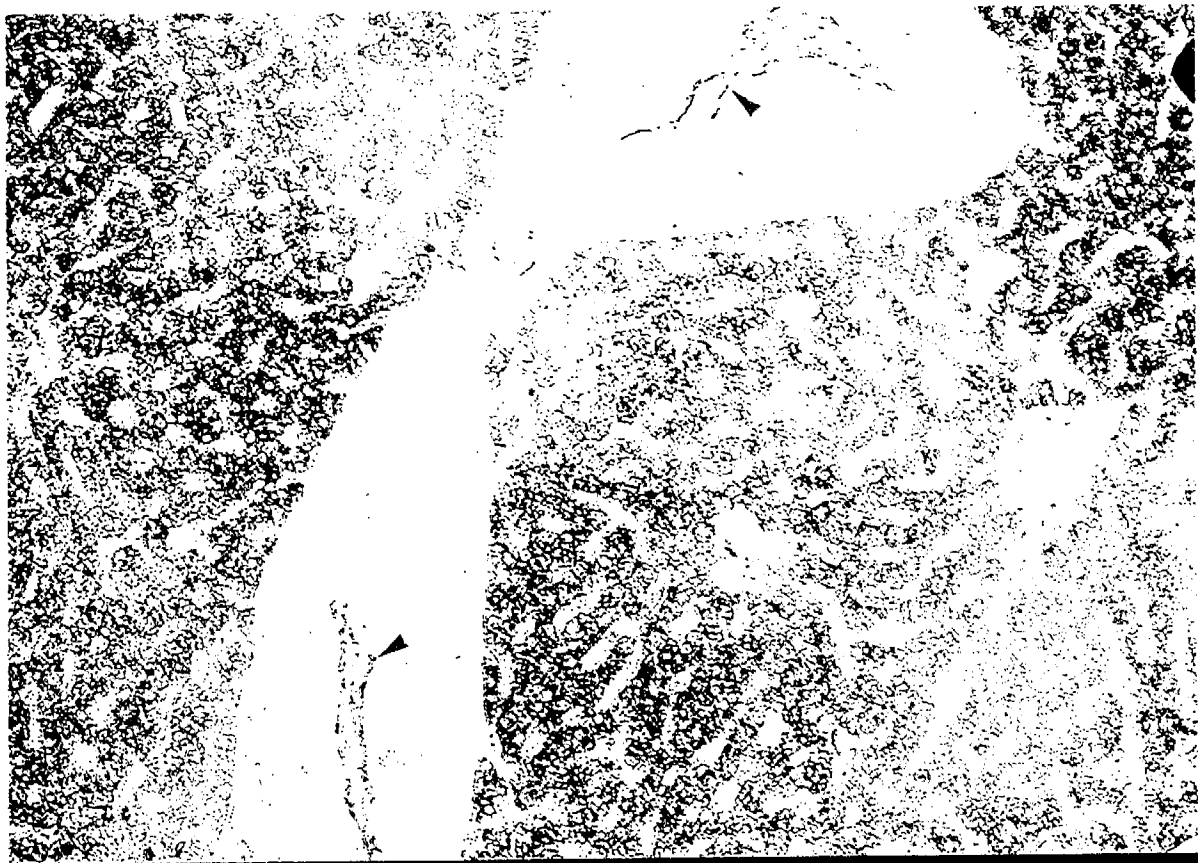
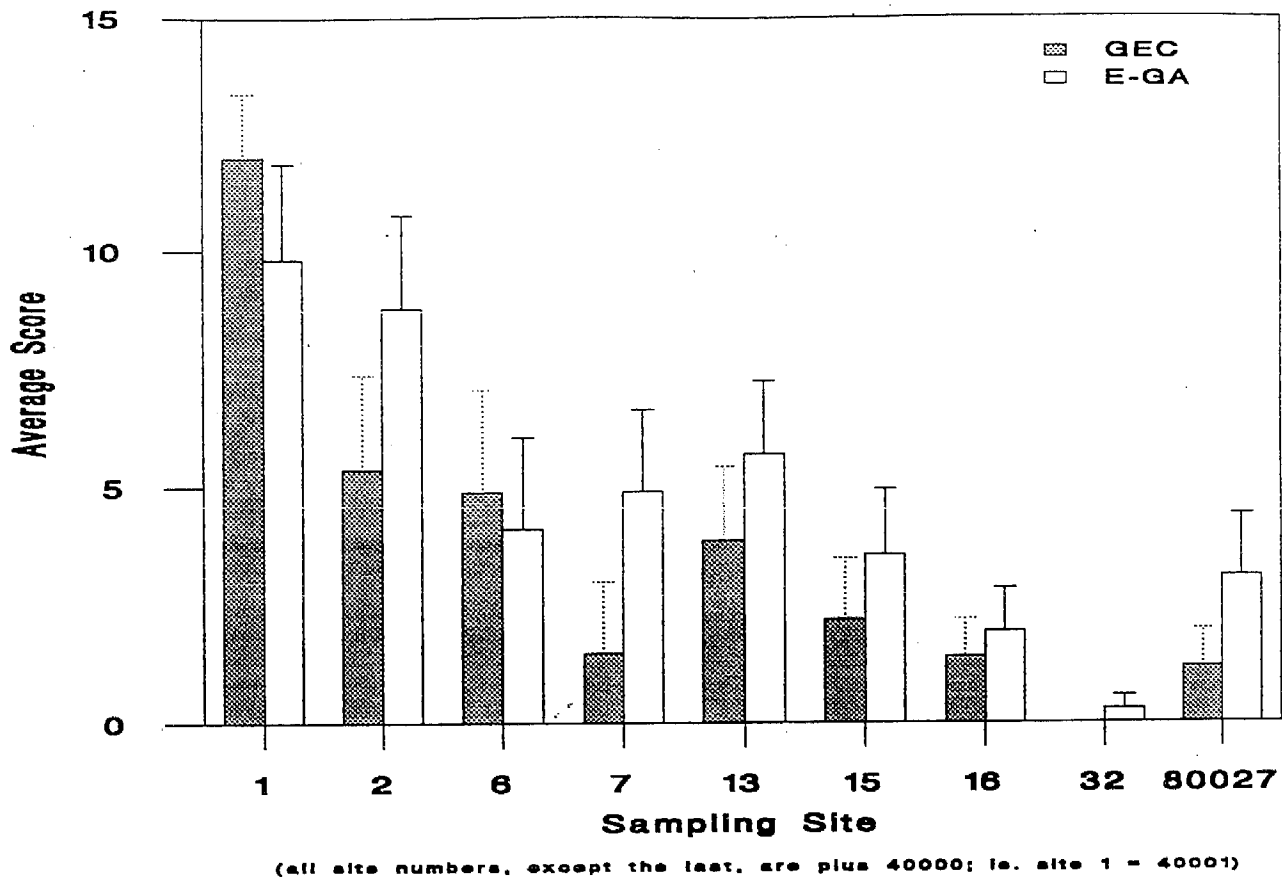
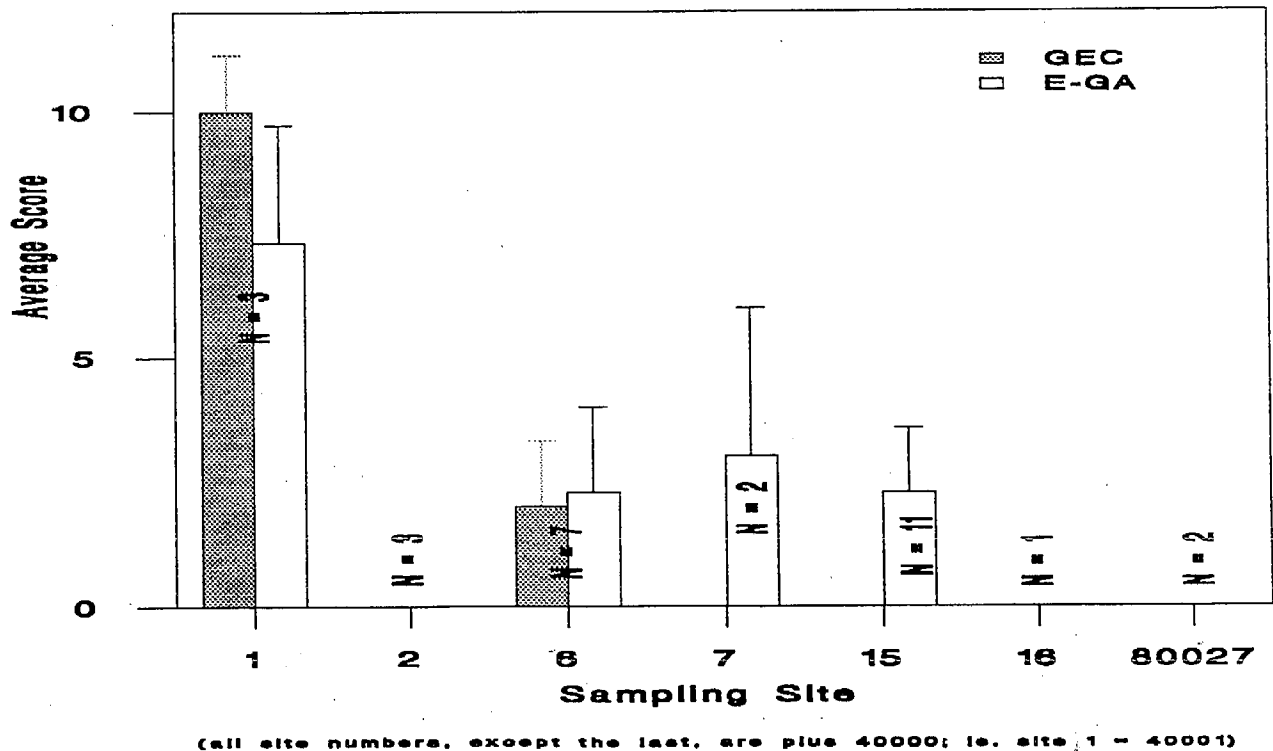


Figure 46. Liver of fish 93H63-98 (white croaker from site 40006). There is marked induction of P450 in hepatocytes throughout the liver. Blood vessels (arrowheads) centered within foci of exocrine pancreas are also positive. Immunohistochemical stain with hematoxylin counterstain.

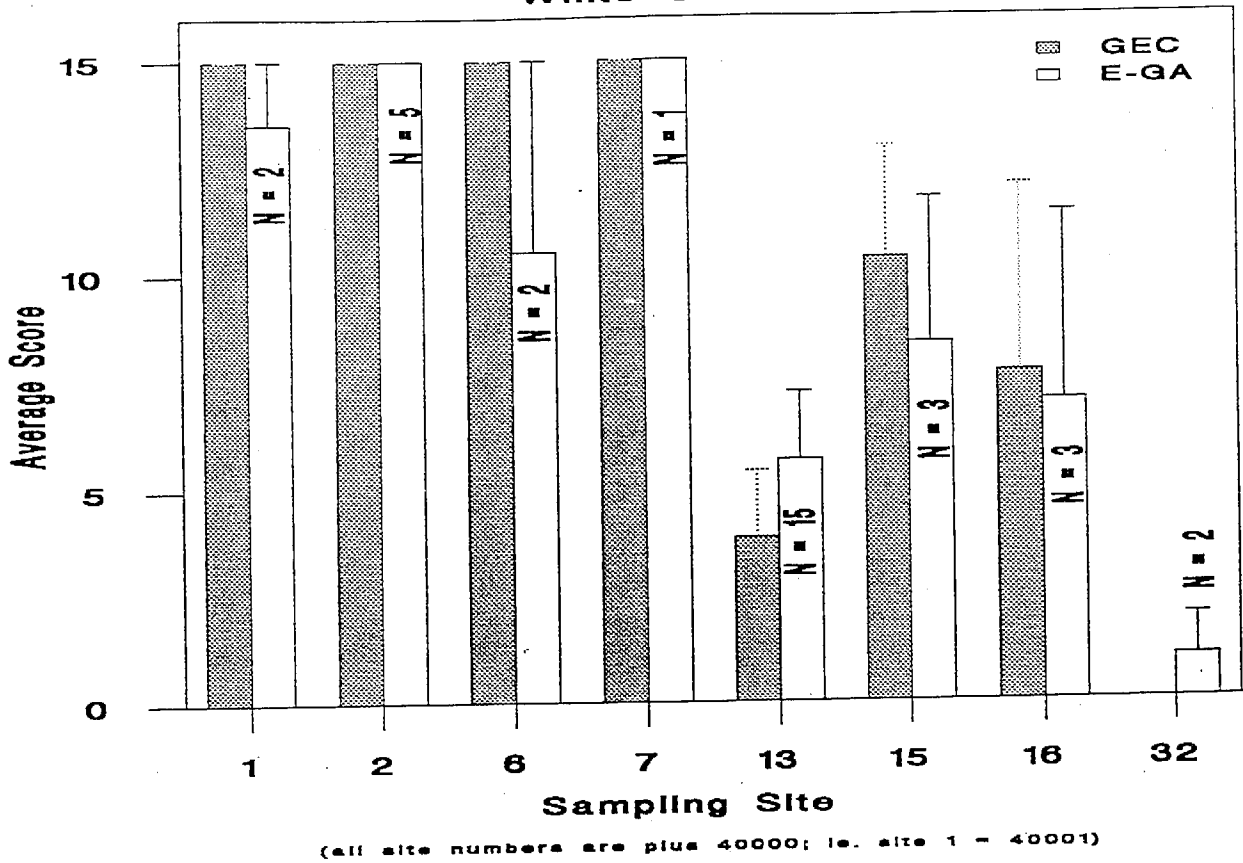
Graph 7a. Average Immunohistochemical P450 Scores for Gill Epithelial Cells (GEC) and Endothelium of Gill Arch Vessels (E-GA) for all fish from all sites.



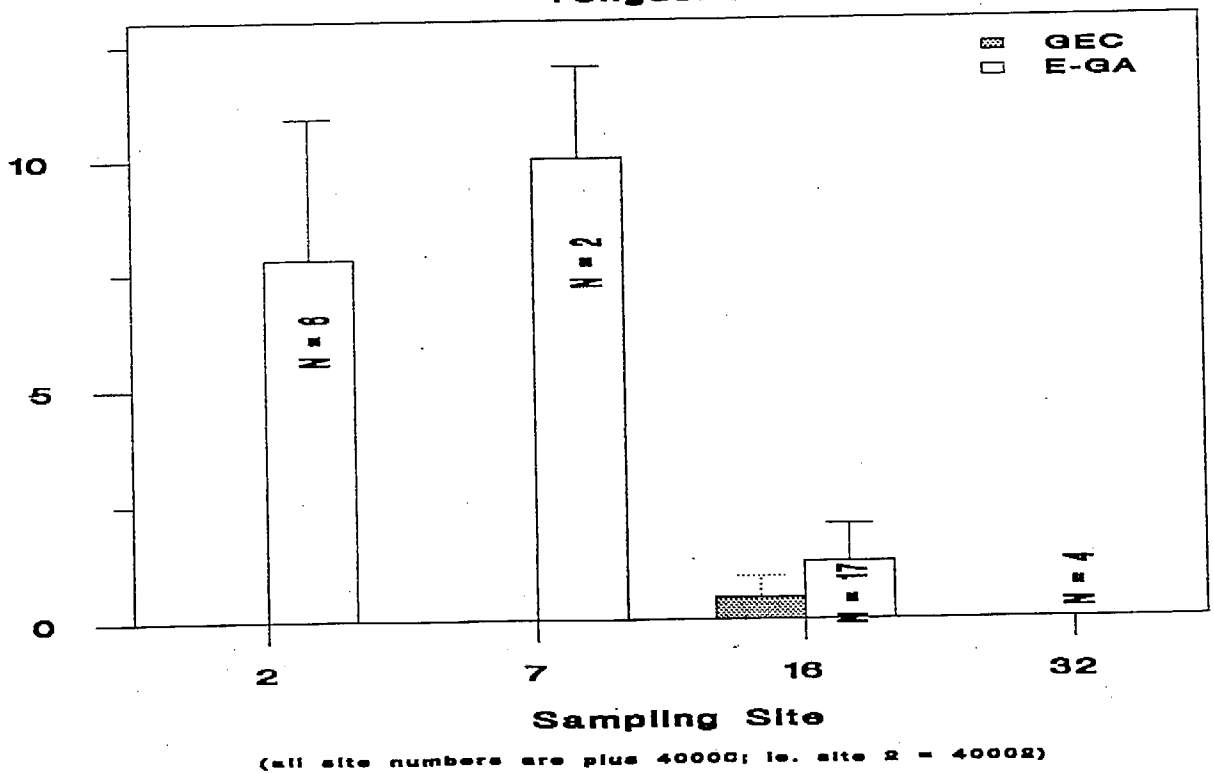
Graph 7b. Average Immunohistochemical P450 Scores for Gill Epithelial Cells (GEC) and Endothelium of Gill Arch Vessels (E-GA) in Yellowfin Gobies.



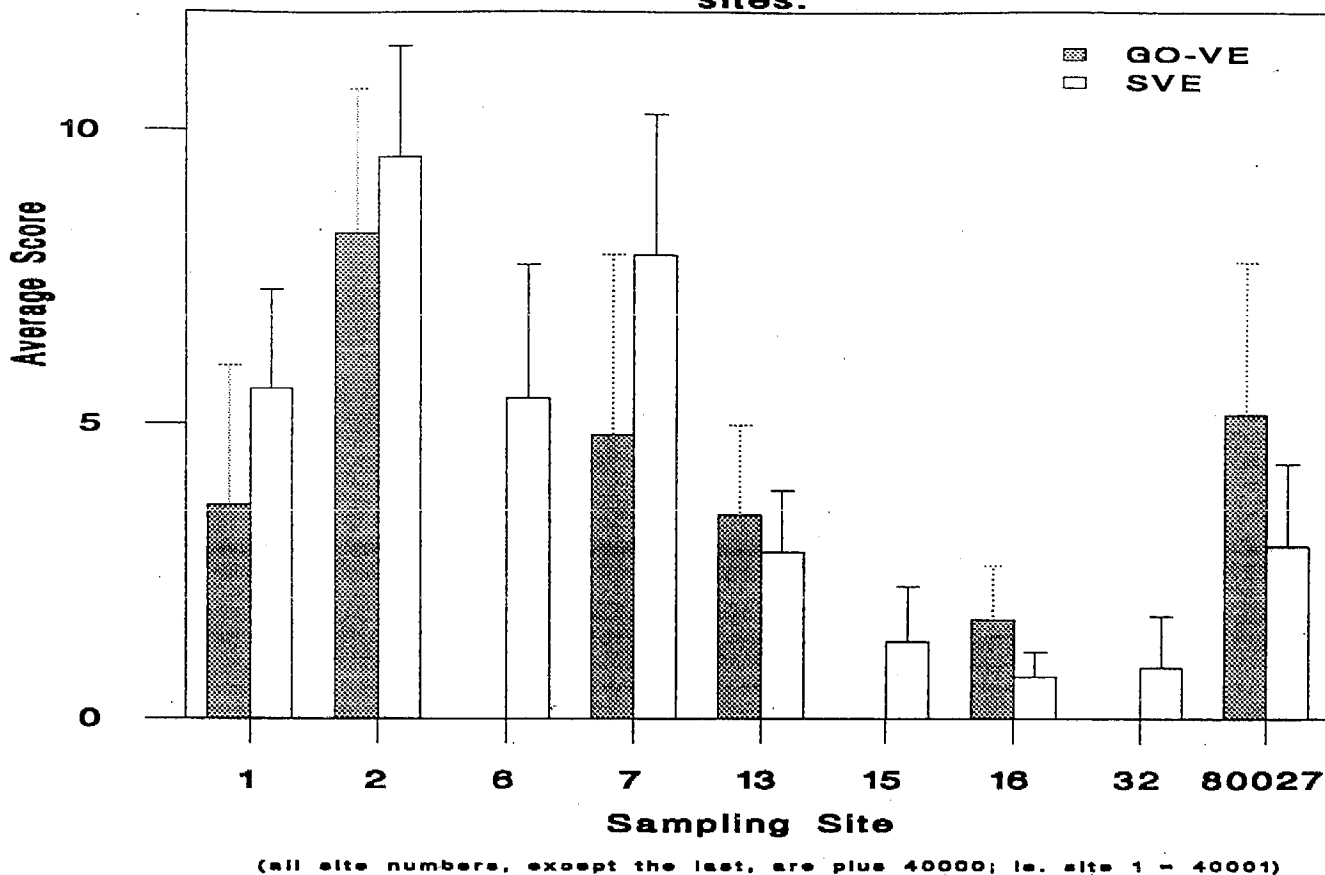
Graph 7c. Average Immunohistochemical P450 Scores for Gill Epithelial Cells (GEC) and Endothellum of Gill Arch Vessels (E-GA) In White Croakers.



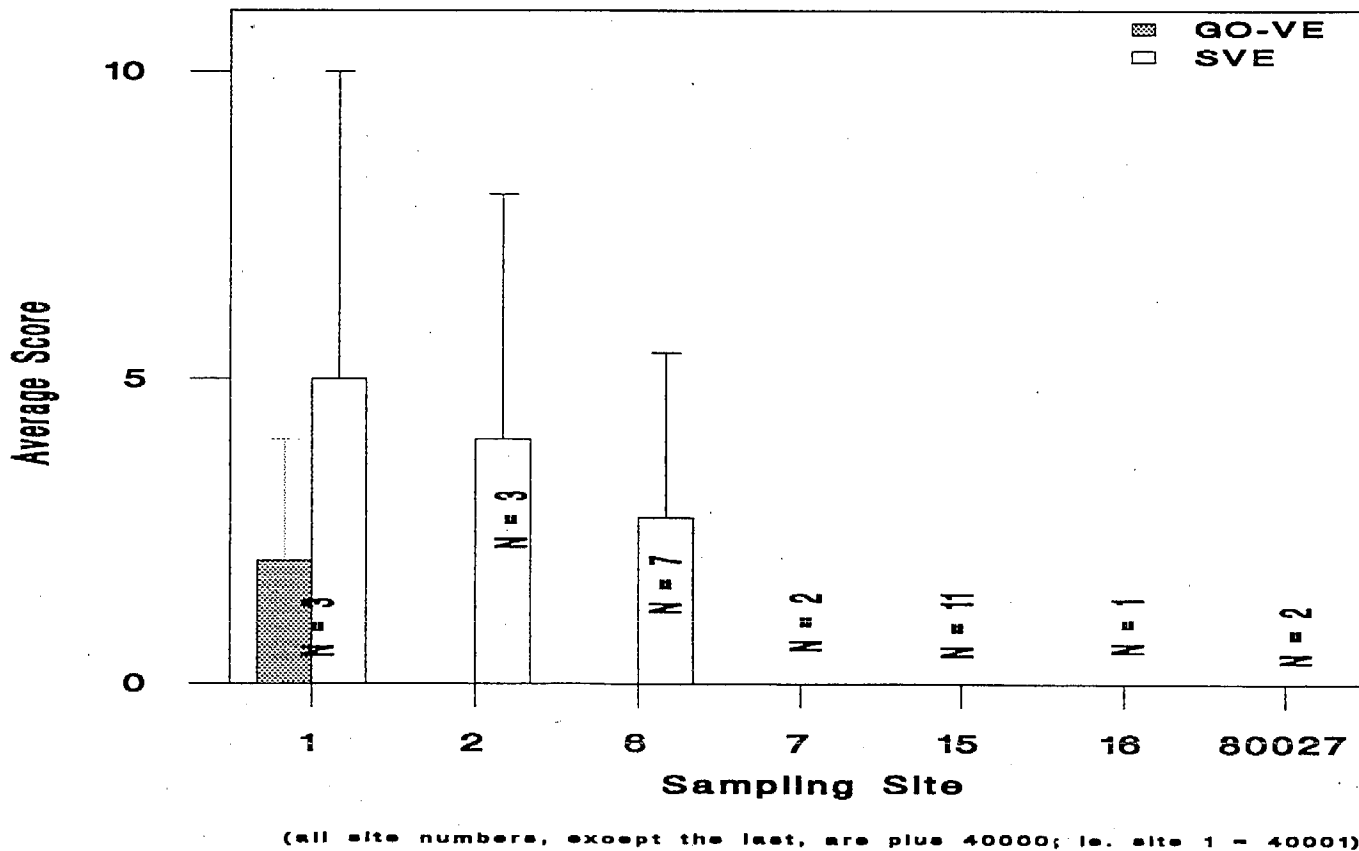
Graph 7d. Average Immunohistochemical P450 Scores in Gill Epithelial Cells (GEC) and Endothellum of Gill Arch Vessels (E-GA) In Tonguefish.



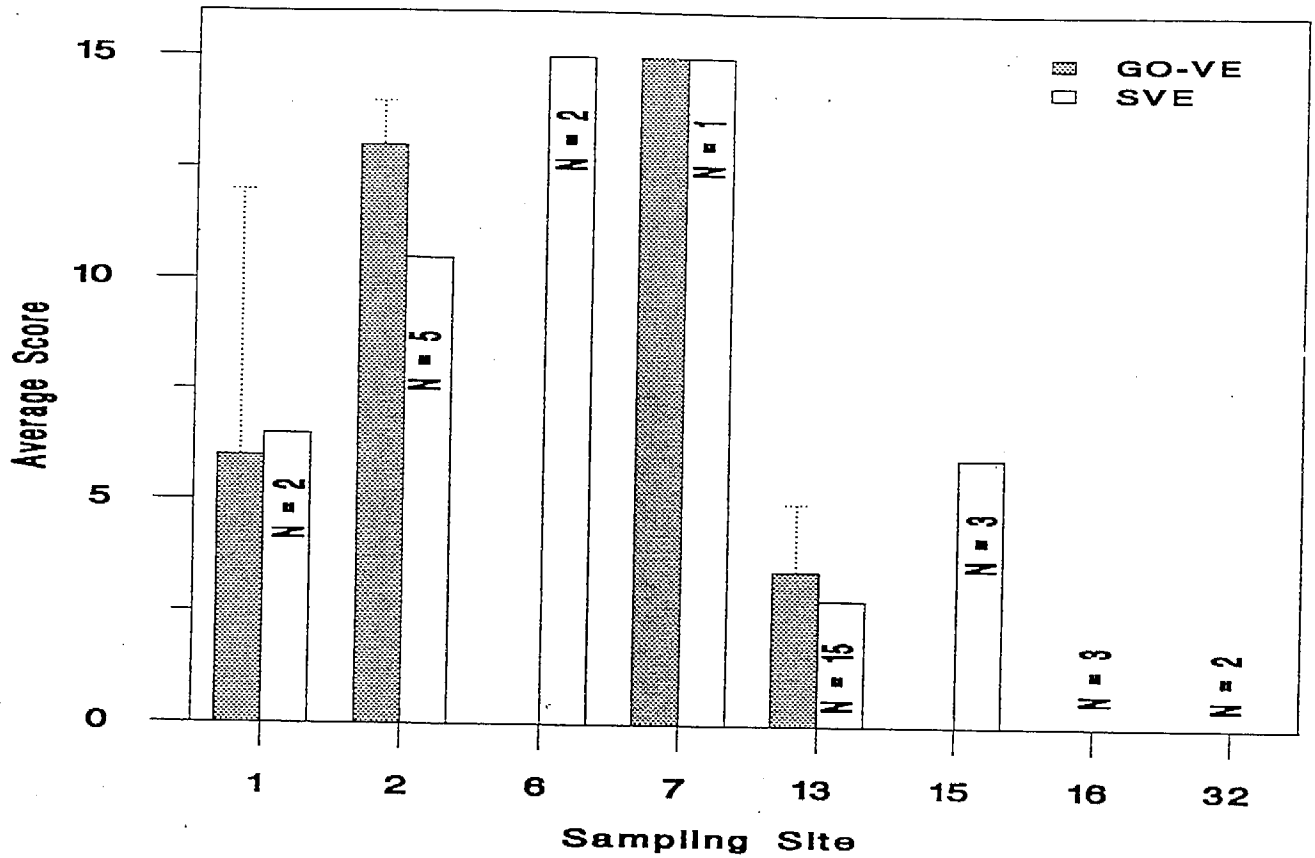
Graph 8a. Average Immunohistochemical P450 Scores for Gonadal Blood Vessels (GO-VE) and Splenic Blood Vessels (SVE) for all fish from all sites.



Graph 8b. Average Immunohistochemical P450 Scores for Gonadal Blood Vessels (GO-VE) and Splenic Blood Vessels (SVE) in Yellowfin Gobies.

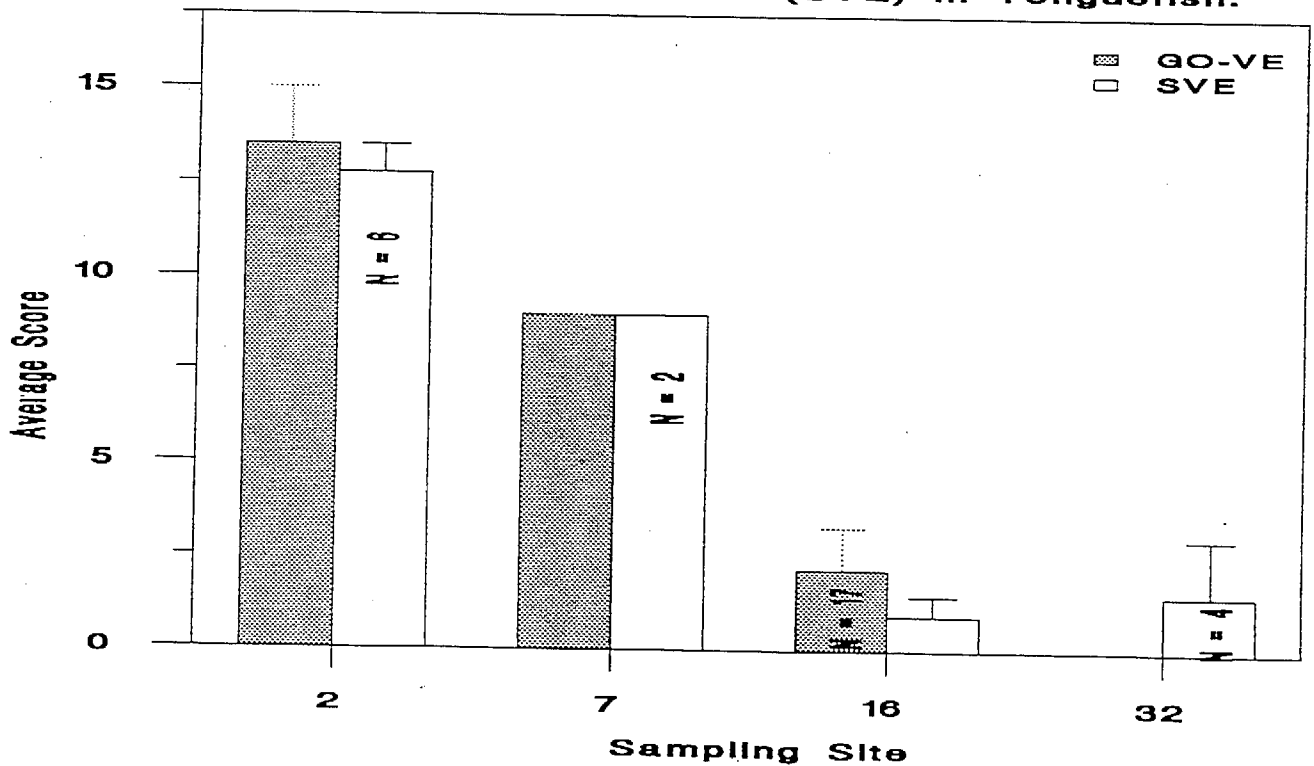


Graph 8c. Average Immunohistochemical P450 Scores for Gonadal Blood Vessels (GO-VE) and Splenic Blood Vessels (SVE) in White Croakers.



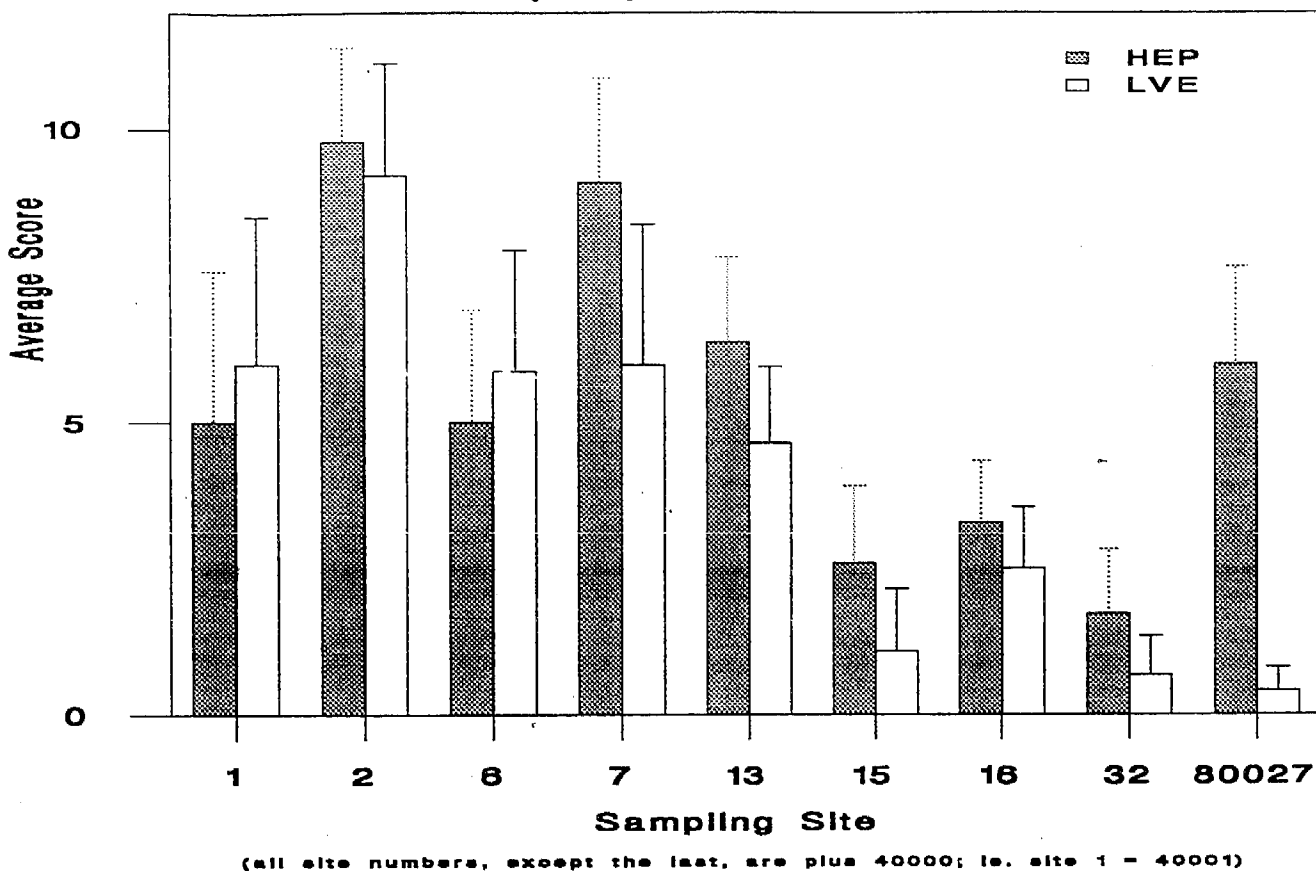
(all site numbers are plus 40000; i.e. site 1 = 40001)

Graph 8d. Average Immunohistochemical P450 Scores for Gonadal Blood Vessels (GO-VE) and Splenic Blood Vessels (SVE) in Tonguefish.

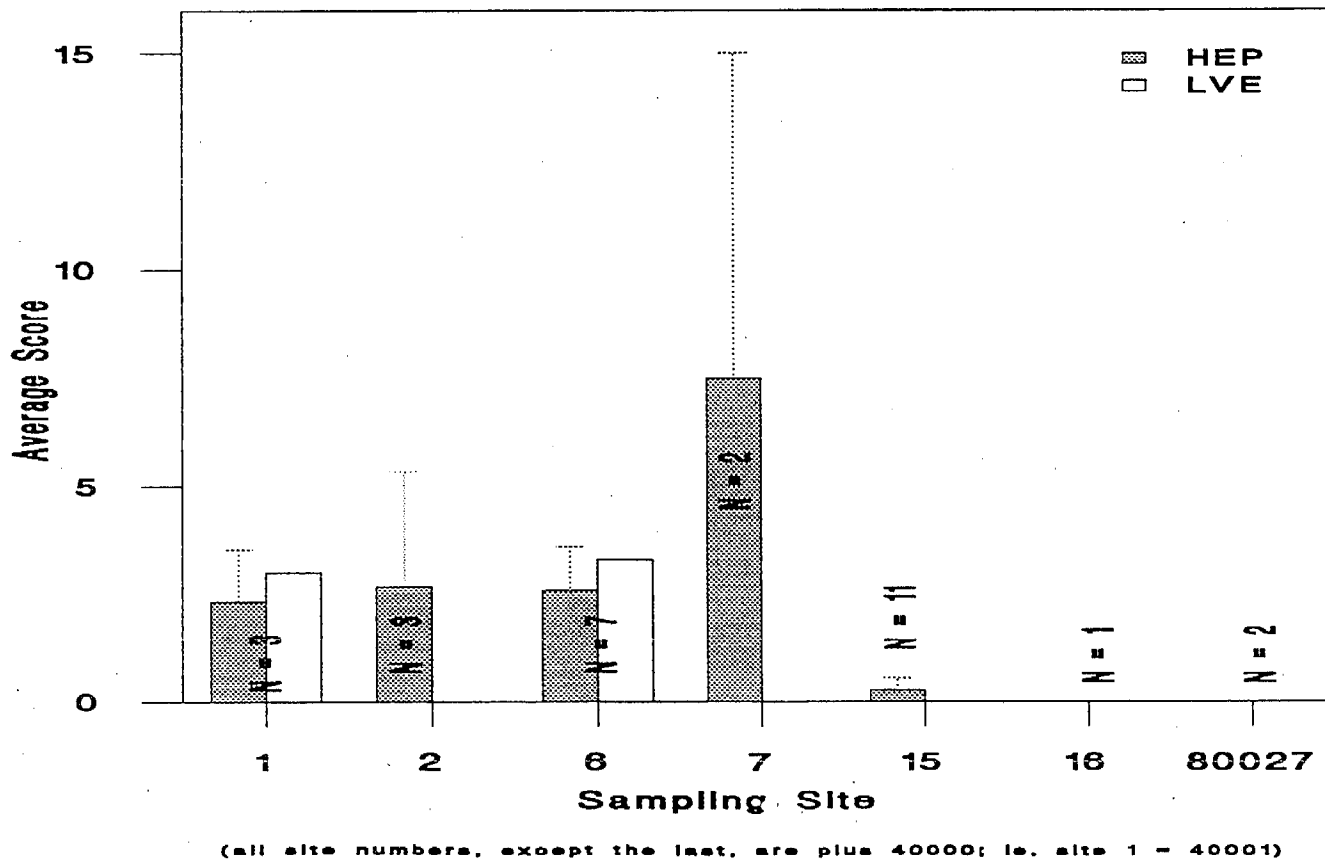


(all sampling sites are plus 40000; i.e. site 2 = 40002)

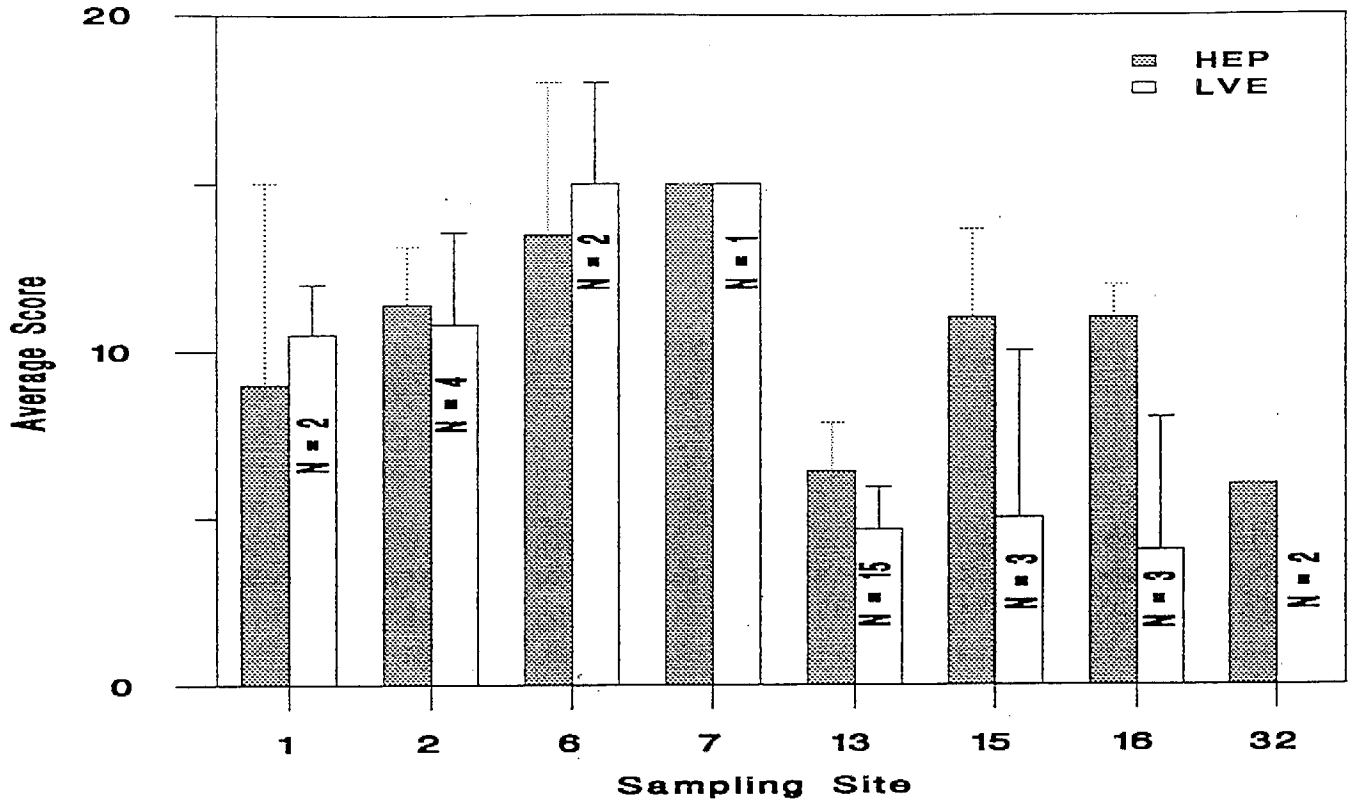
Graph 9a. Average Immunohistochemical P450 Scores for Hepatocytes (HEP) and Liver Vascular Endothelium (LVE) for all fish from all sites.



Graph 9b. Average Immunohistochemical P450 Scores for Hepatocytes (HEP) and Liver Vascular Endothelium (LVE) in Yellowfin Gobies.

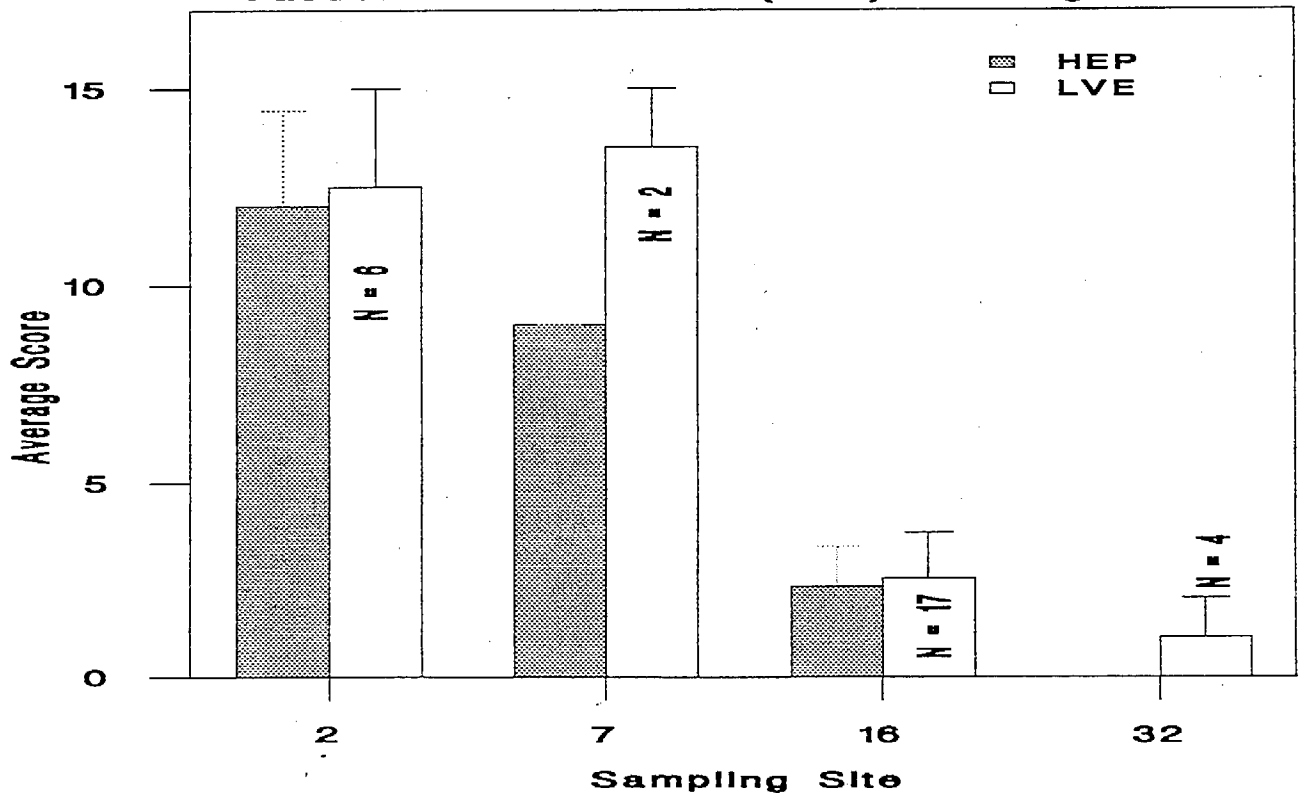


Graph 9c. Average Immunohistochemical P450 Scores for Hepatocytes (HEP) and Liver Vascular Endothelium (LVE) in White Croakers.



(all site numbers are plus 40000; i.e. site 1 = 40001)

Graph 9d. Average Immunohistochemical P450 Scores for Hepatocytes (HEP) and Liver Vascular Endothelium (LVE) in Tonguefish.



(all site numbers are plus 40000; i.e. site 1 = 40001)

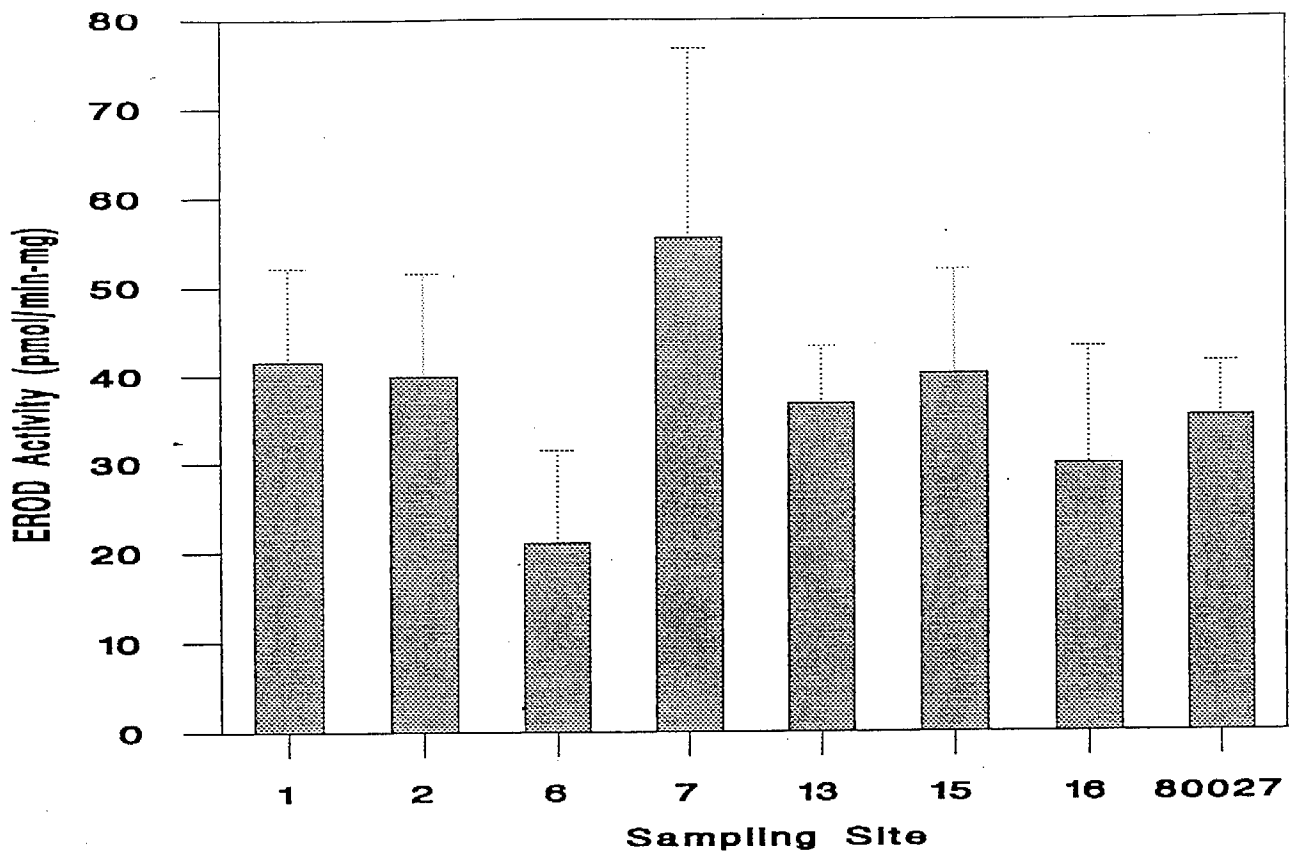
EROD - Results: Hepatic EROD activity as expressed in pmol/min-mg is given in Appendix 17. The data was sorted on the basis of site and species. Average EROD activity and standard error was determined for each site.

EROD activity for all fish from all sites is depicted on graph 10a. There are no sharp trends, but one reference site (40016) does have lower average EROD activity than five of six impact sites. The second reference site (40015) had higher EROD activity than three impact sites, but lower activity when compared to the other three impact sites. Sorting the data, with respect to species (graphs 10b-10d), did not clarify matters.

Graph 11 shows average hepatic EROD activity for the dominant species taken from each site. Although it is somewhat unconventional way of examining the data, the differences between sites does become more distinct. Reference site 40016 now has the lowest average EROD activity and the second reference site (40015) has EROD activity which is lower than five of six impact sites.

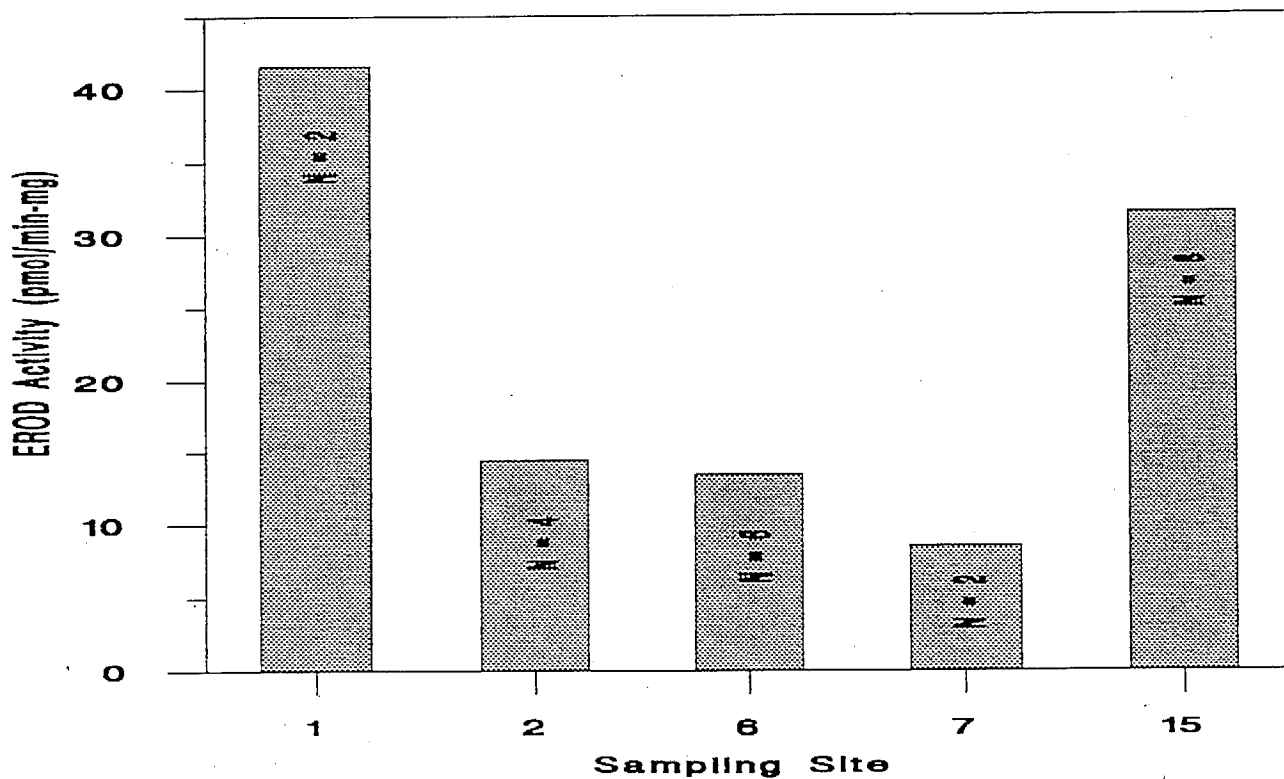
EROD - Statistics: MANOVA revealed that there were significant ($P < 0.01$) differences with respect to site and highly significant differences ($P = 0.0001$) with respect to species. Comparison between sites, using Least Squares Means, revealed that impact site 40007 (composed of primarily cusk-eels) had significantly ($P < 0.01$) higher EROD activity when compared to reference sites 40015 (gobies) and 40016 (tonguefish). Impact site 40006 (gobies) had significantly ($P < 0.05$) lower EROD activity than fish from reference site 40015 (gobies).

Graph 10a. Hepatic EROD Activity for All Fish from ALL Sites.



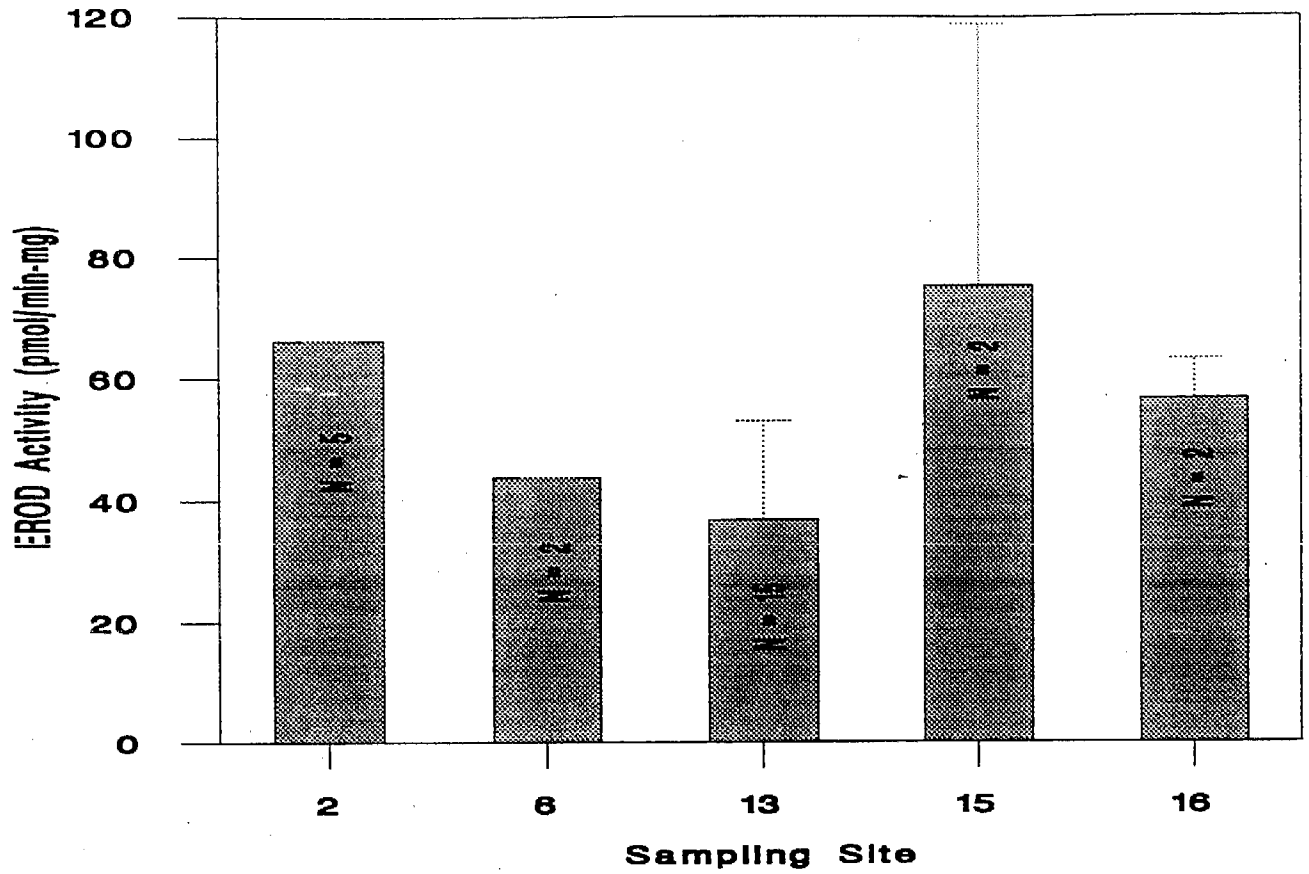
(all site numbers, except the last, are plus 40000; i.e. site 1 = 40001)

Graph 10b. Hepatic EROD Activity In Yellowfin Gobles.



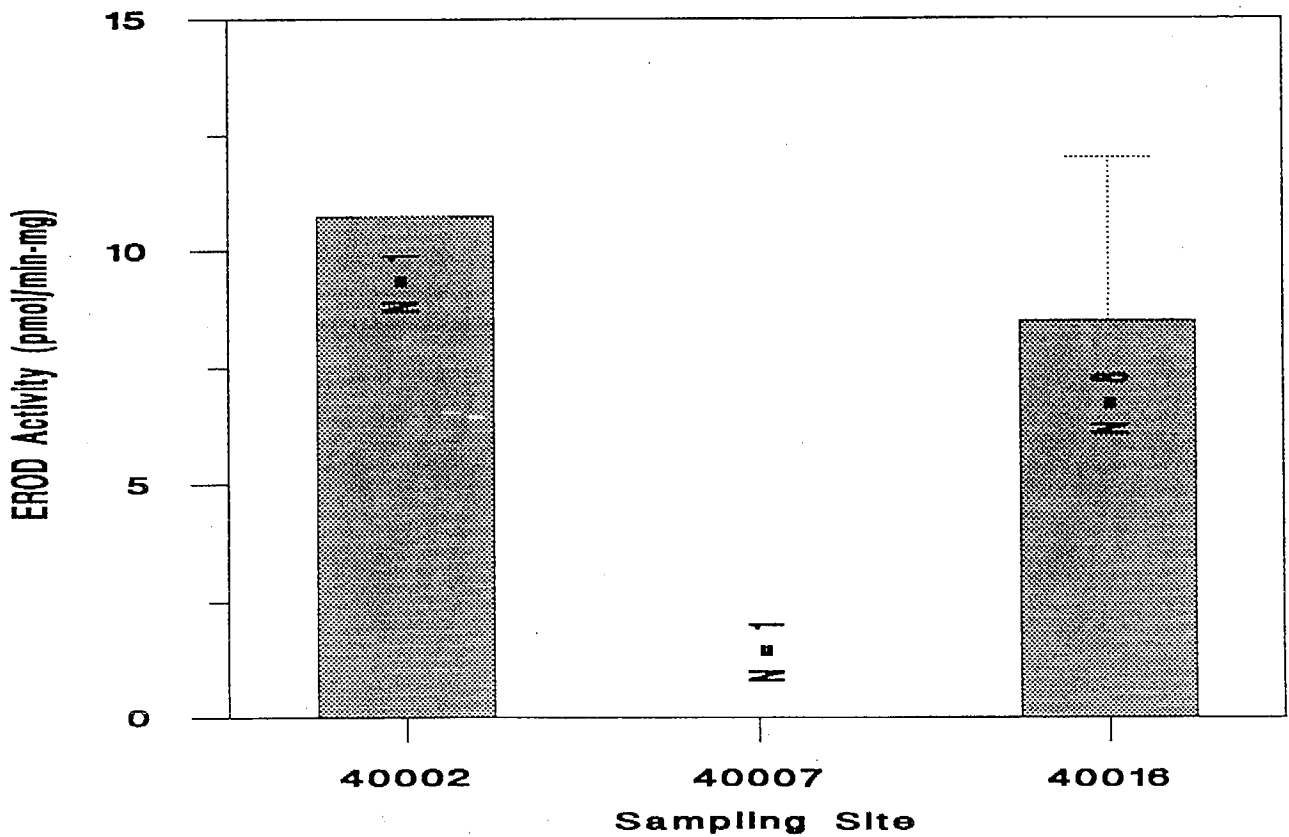
(all site numbers are plus 40000; i.e. site 1 = 40001)

Graph 10c. Hepatic EROD Activity in White Croakers.

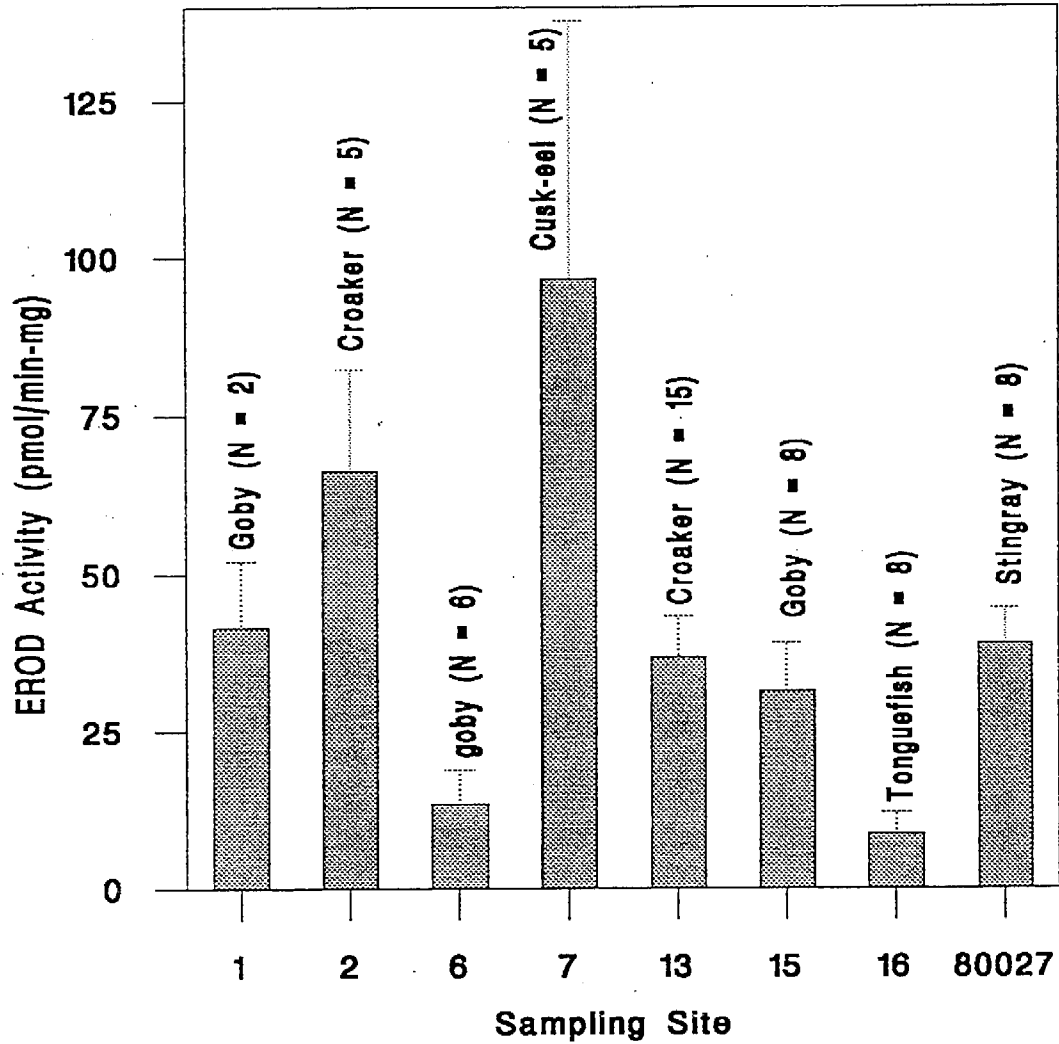


(all site numbers are plus 40000; i.e. site 2 = 40002)

Graph 10d. Hepatic EROD Activity in Tonguefish.



Graph 11. Hepatic EROD Activity of Fish Species which Comprised the Majority of Fish Sampled from Each Site.



(all site numbers, except the last, are plus 40000; i.e. site 1 = 40001)

Indices - Results:

Hepatosomatic Index: Hepatosomatic indices (HSI), sorted on the basis of site, are given in appendices 18a-18c and depicted in graph 12a. HSI from two reference sites (40016 and 40032) are moderately to markedly lower when compared to the six impact sites. Site 80027, where 10 of 12 fish were stingrays, had the highest HSI.

HSI, sorted on the basis of site and species, are given in appendices 18d-18h and depicted in graphs 12b-12d. Average HSI in gobies (graph 12b) and tonguefish (graph 12d), from impact and reference sites, were similar. Average HSI in croakers from five impact sites was higher than HSI in croakers in all three reference sites. The highest HSI was in croakers from impact site 40013.

MANOVA revealed that there were significant ($P = 0.05$) differences in HSI with respect to site, and highly significant ($P = 0.0001$) differences with respect to species. Comparisons among sites using Least Squares Means revealed that HSI in fish from impact site 40013 was significantly ($P < 0.05$) higher than HSI in fish from all three reference sites. Average HSI was also higher in fish from site 40013 when compared to two other impact sites (40006 and 40007).

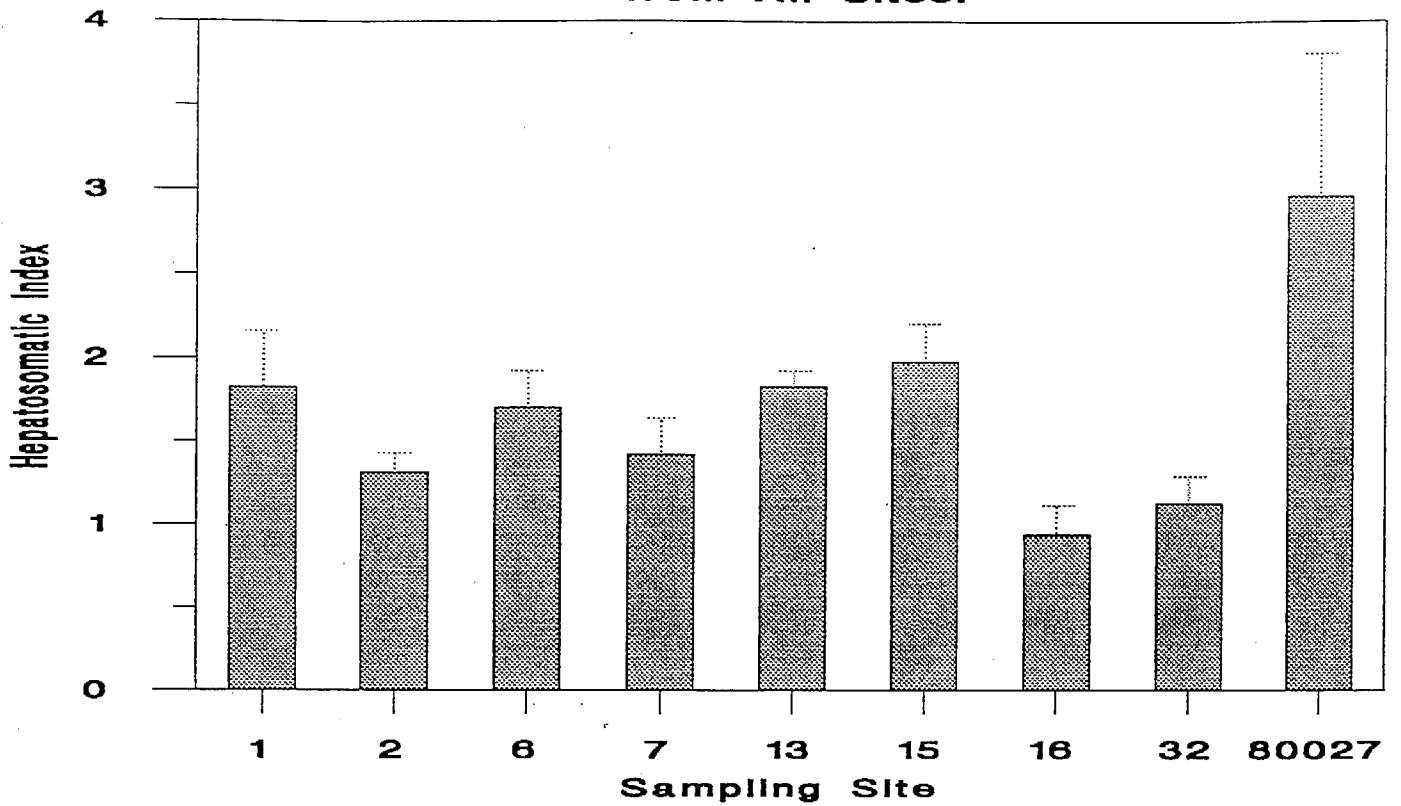
Comparisons between species, using Least Squares Means, showed that there were numerous differences with respect to HSI. HSI in cusk-eels was significantly ($P < 0.001$) different from tonguefish and croakers. HSI in stingrays was significantly ($P < 0.01$) different from tonguefish and croakers. HSI in tonguefish was significantly ($P = 0.0001$) different from croakers and gobies. HSI in croakers was significantly ($P = 0.0001$) different from gobies.

Gonadosomatic Index: Gonadosomatic indices (GSI), sorted on the basis of site, are given in appendices 18a-18c. GSI, sorted on the basis of site and species, are given in appendices 18d-18h. Although there appears to be a slight trend towards higher GSI at the three reference sites (graph 13a), separation of GSI scores on the basis of species (graph 13b-13d) did not reveal any consistent patterns. MANOVA and comparisons among sites, using Least Squares Means analysis, did not reveal any significant differences in GSI between sites or species. GSI differences between sexes were highly significant ($P < 0.001$), but this was expected.

Condition Index: Condition indices (CI), sorted on the basis of site, are given in appendices 18a-18c. CI, sorted on the basis of site and species, are given in appendices 18d-18h. Average CI for all fish from all sites is depicted in graph 14a. The highest average CI were in two impact sites (40013 and 40006), but there were no consistent trends. Additional sorting of the data based on species also did not reveal any consistent trends. Average CI, with very few exceptions was very similar in both impact and reference sites for gobies, croakers, and tonguefish. Comparisons between sites could not be made for cusk-eels and stingrays, as they were only collected in significant numbers from a single site.

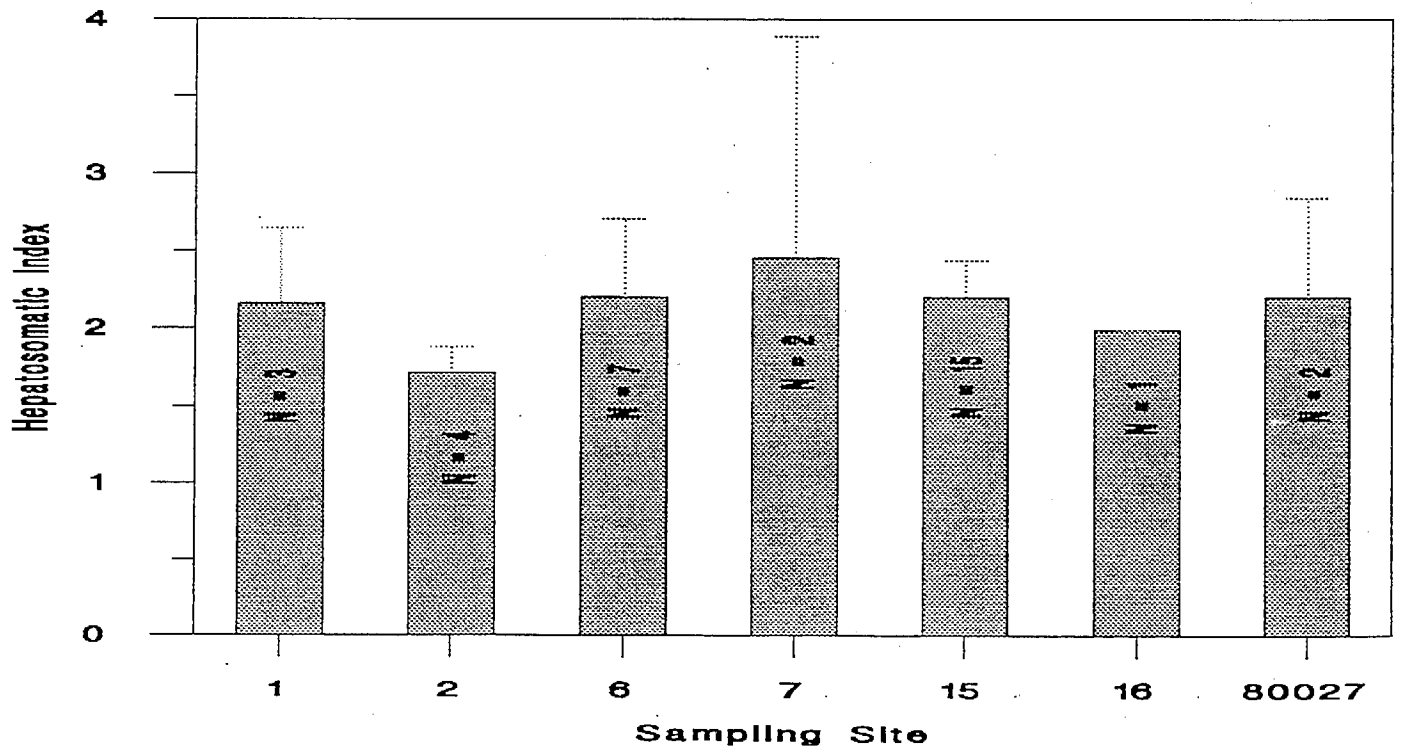
MANOVA revealed that there were significant differences in CI with respect to both site ($P < 0.01$) and species ($P = 0.0001$). Comparisons between sites, using Least Squares Means, revealed that average CI in fish from impact site 40007 was significantly ($P < 0.01$) lower than CI in all three reference sites. Average CI from impact site 40007 was also significantly ($P < 0.01$) lower than CI in fish from two other impact sites (40002 and 40006) and cusk-eels comprised 33.3% of the sample taken from site 40007. Comparisons between species, using Least Squares Means, revealed that CI in all five species were significantly ($P < 0.01$) different from one another.

Graph 12a. Hepatosomatic Indices for All Fish from All Sites.



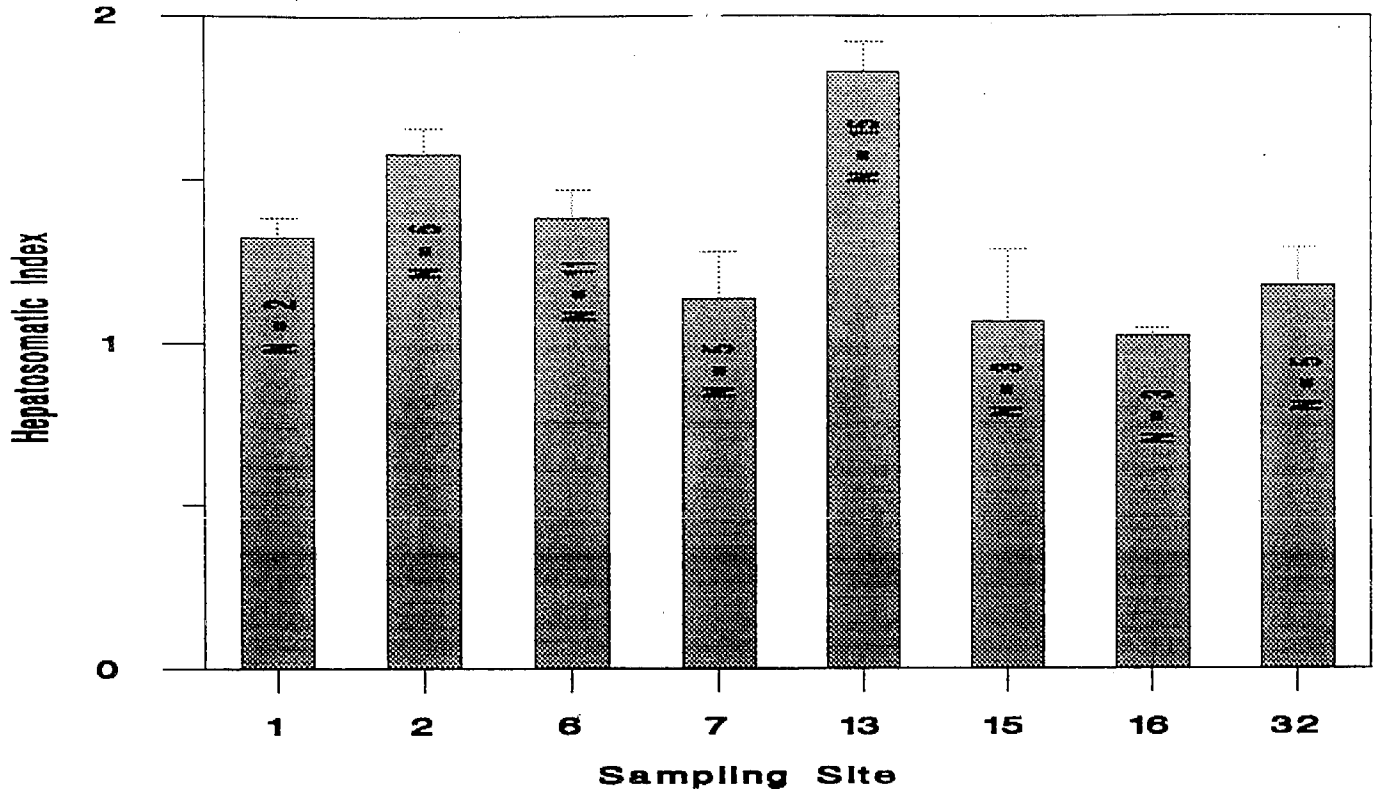
(all site numbers, except the last, are plus 40000; i.e. site 1 = 40001)

Graph 12b. Hepatosomatic Indices in Yellowfin Gobles.



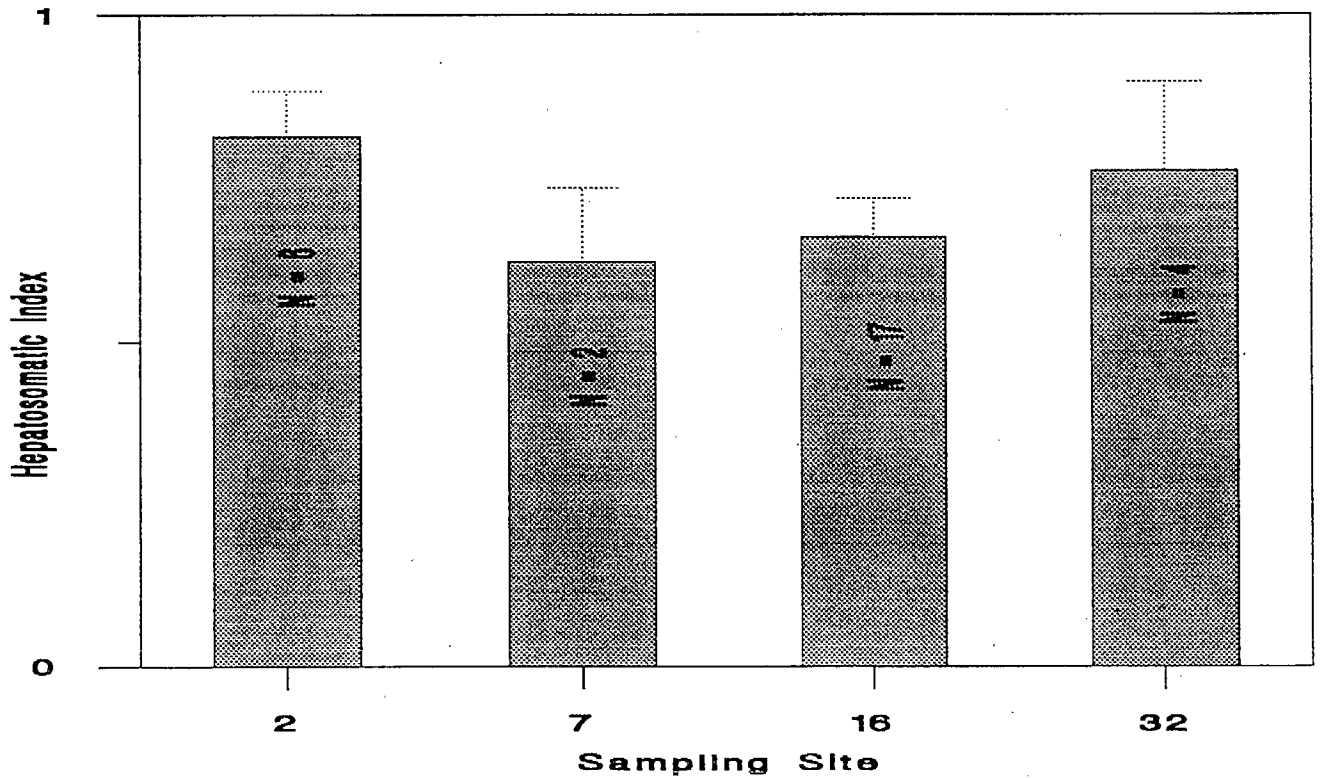
(all site numbers, except the last, are plus 40000; i.e. site 1 = 40001)

Graph 12c. Hepatosomatic Indices In White Croakers.



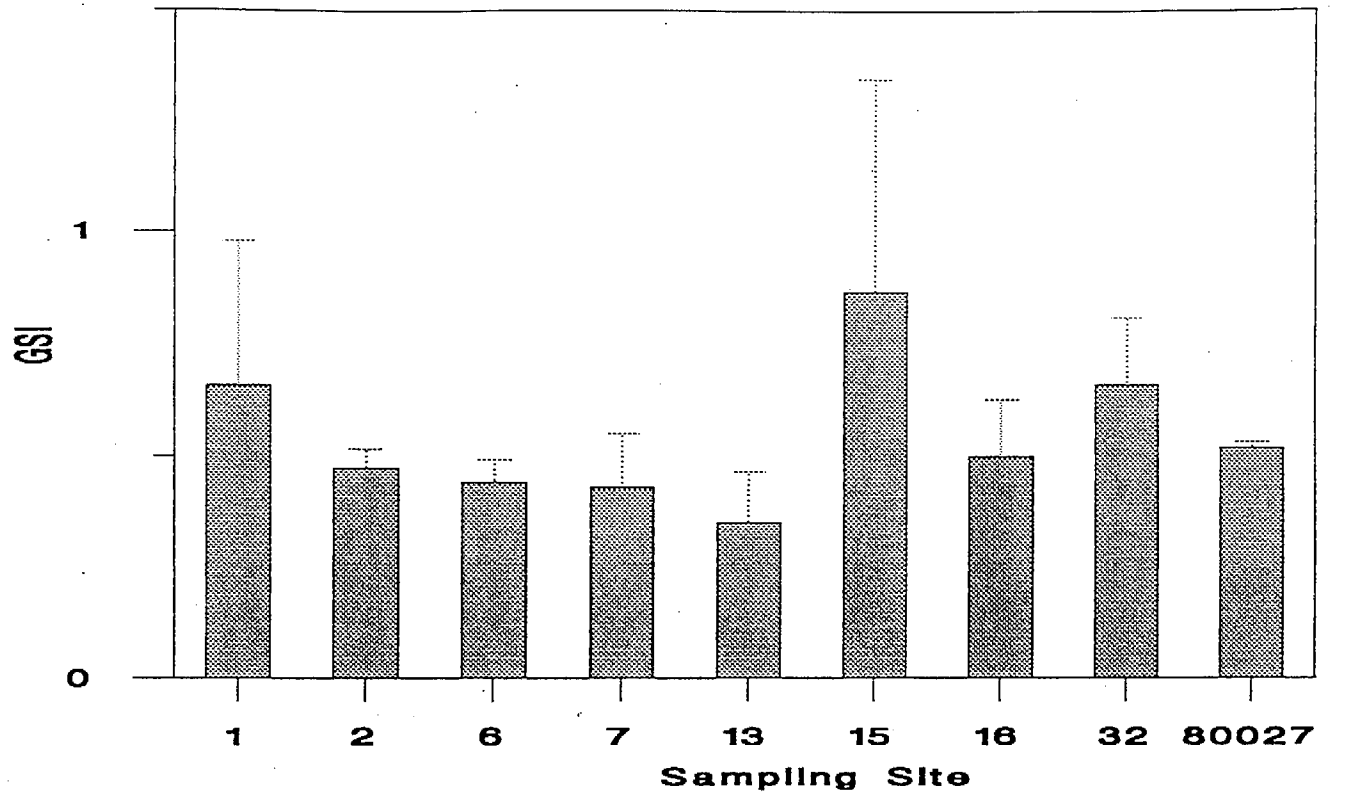
(all site numbers are plus 40000; i.e. site 1 - 40001)

Graph 12d. Hepatosomatic Indices In Tonguefish.



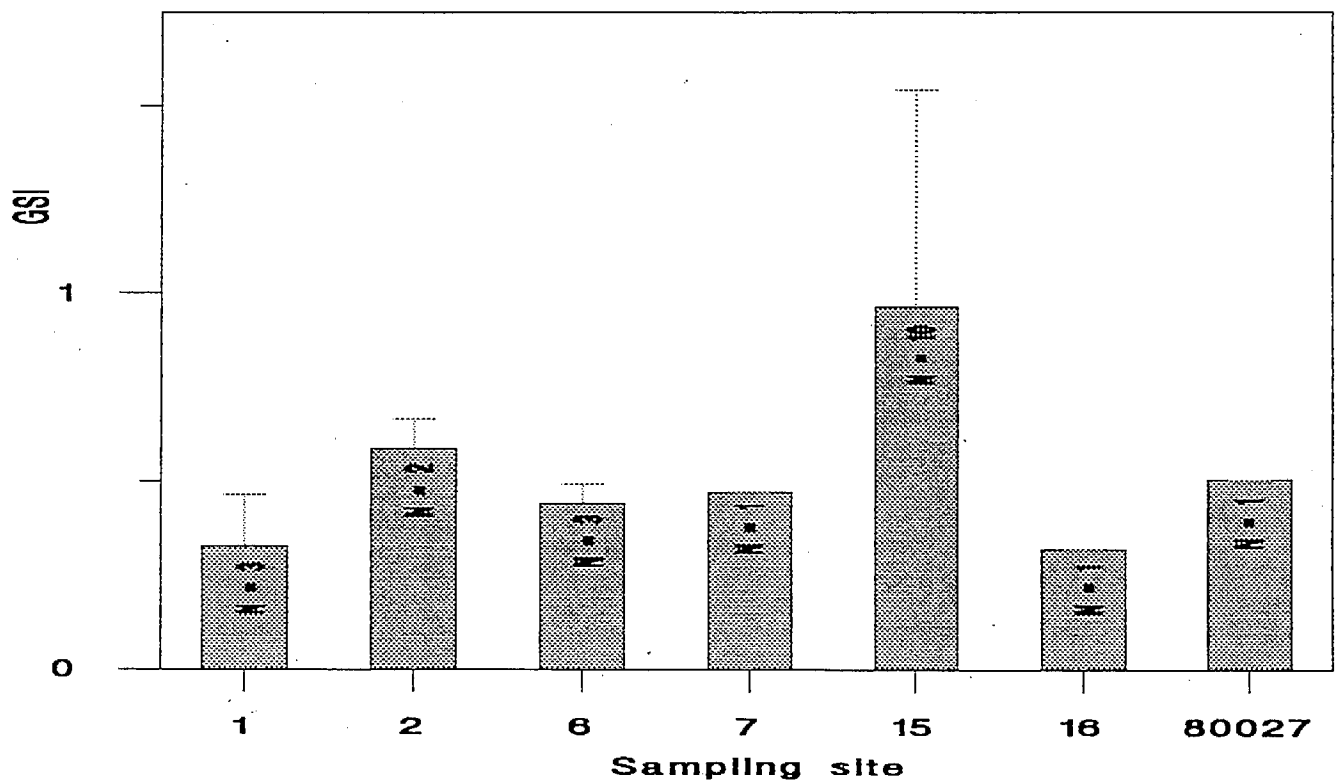
(all site number are plus 40000; i.e. site 2 - 40002)

Graph 13a. Gonadosomatic Indices (GSI = gonad weight/body weight X 100) for all fish from all sites.



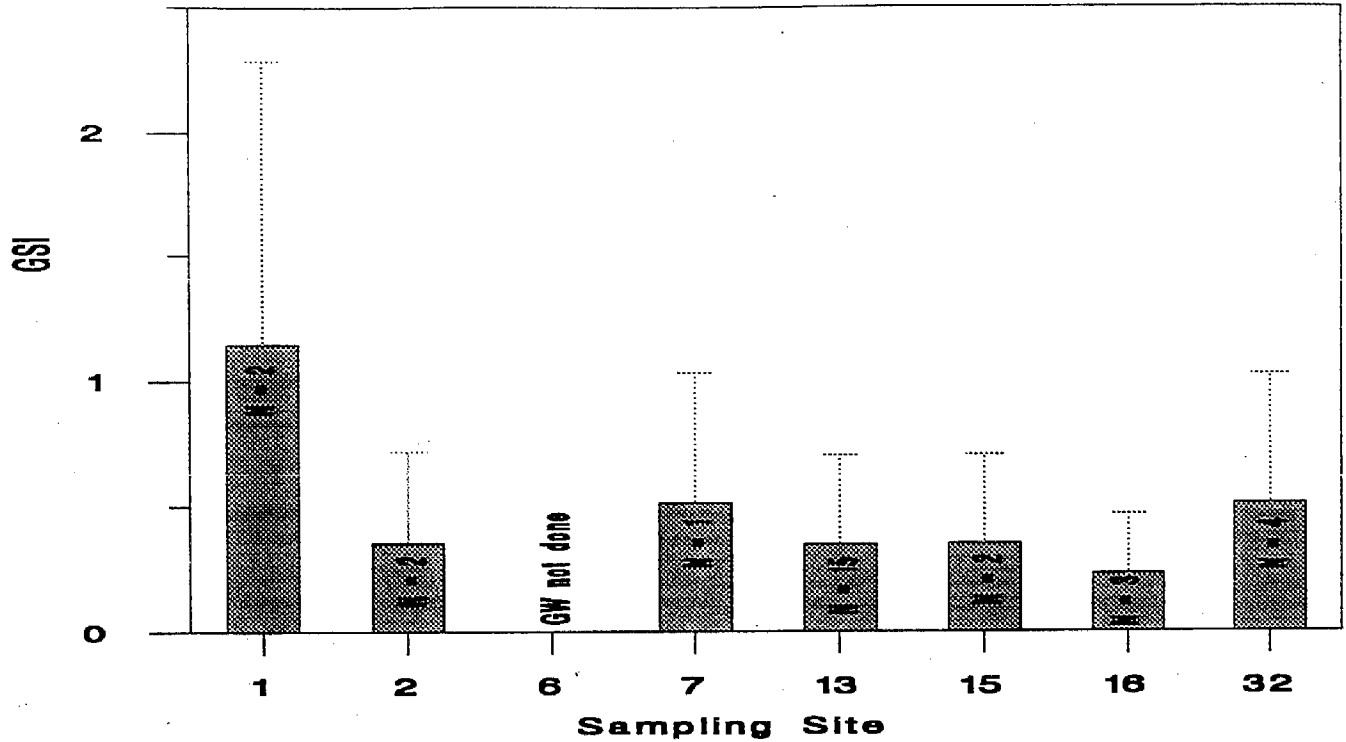
(all site numbers, except the last, are plus 40000; i.e. site 1 = 40001)

Graph 13b. Gonadosomatic Indices (GSI = gonad weight/body weight X 100) for Yellowfin Gobles.



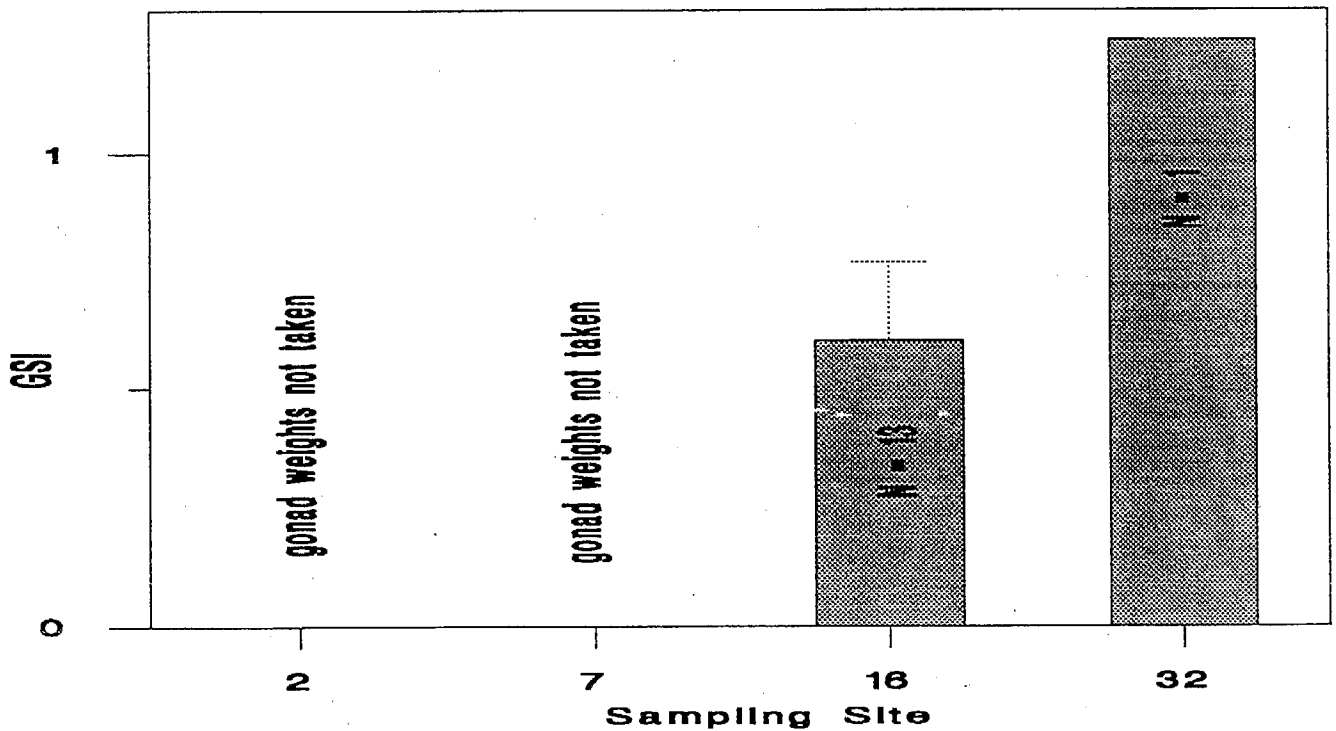
(all site numbers, except the last, are plus 40000; i.e. site 1 = 40001)

Graph 13c. Gonadosomatic Indices (GSI = gonad weight/body weight X 100) for White Croakers.



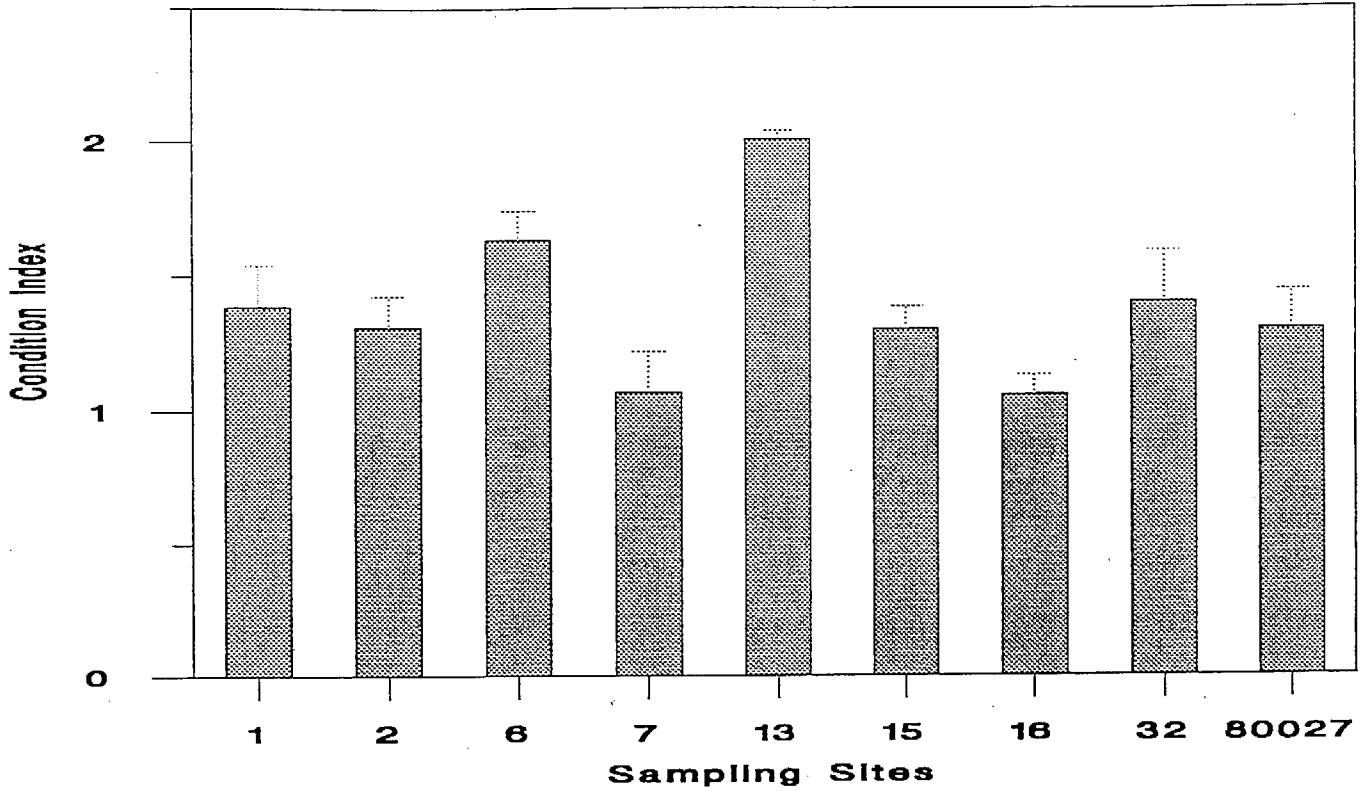
(all site numbers are plus 40000; ie. site 1 = 40001)

Graph 13d. Average Gonadosomatic Index (GSI = gonad weight/body weight X 100) for Tonguefish.



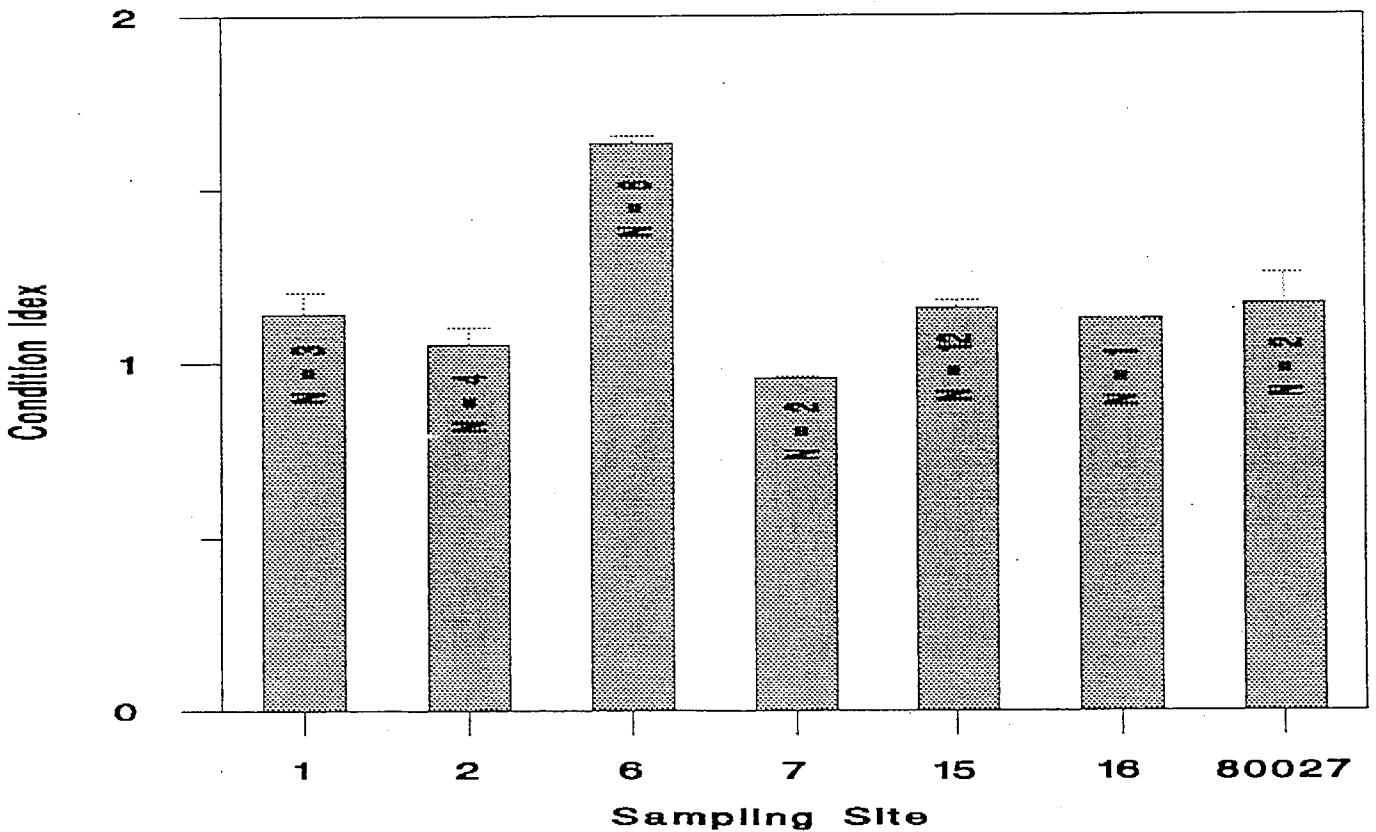
(all site numbers are plus 40000; ie. site 1 = 40001)

Graph 14a. Condition Index (CI = body weight/standard length³ X 100,000) of All fish from All sites.



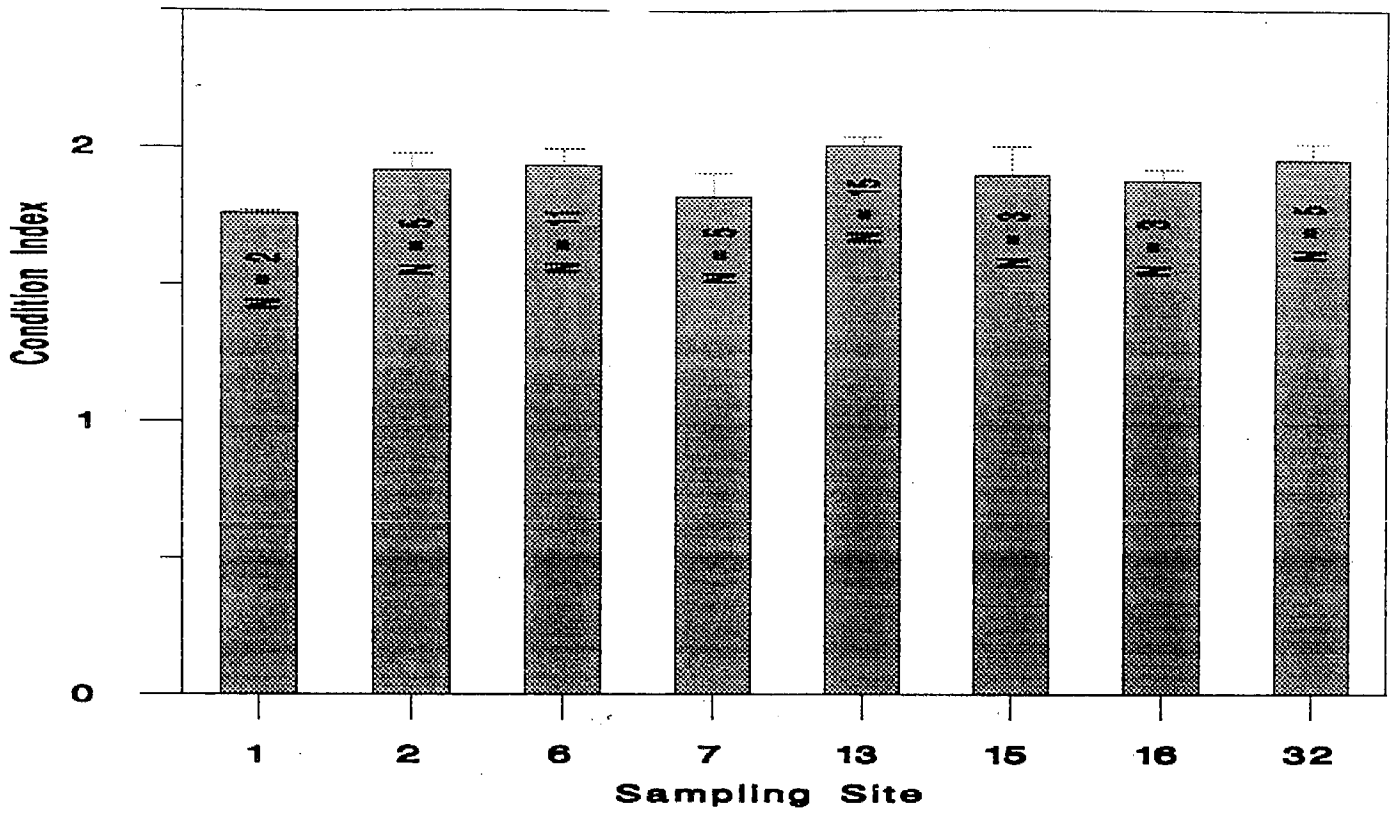
(all site numbers, except the last, are plus 40000; i.e. site 1 = 40001)

Graph 14b. Condition Indices (CI = body weight/standard length³ X 100,000) for Yellowfin Gobles.



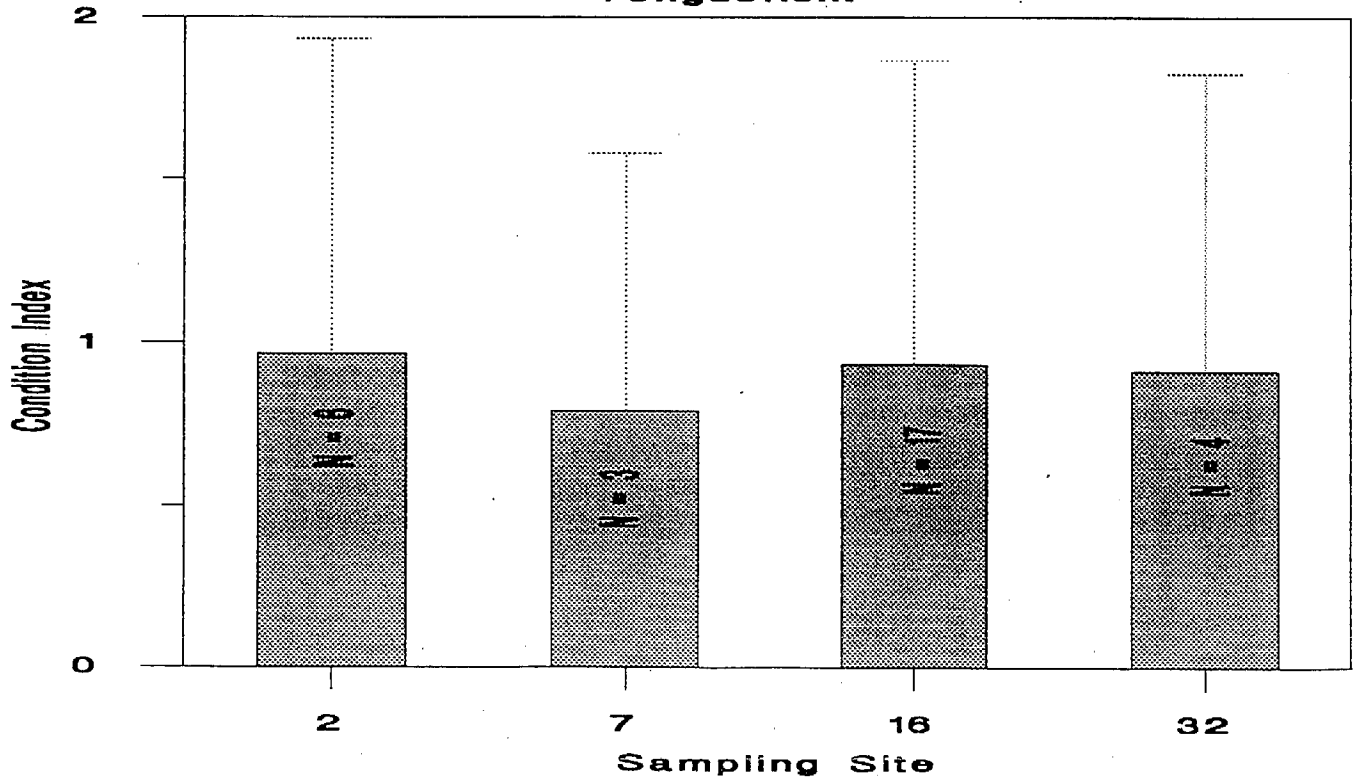
(all site numbers, except the last, are plus 40000; i.e. site 1 = 40001)

Graph 14c. Condition Indices (body weight/standard length³ X 100,000) for White Croakers.



(all site numbers are plus 40000; i.e. site 1 = 40001)

Graph 14d. Average Condition Indices (CI = body weight/standard length³ X 100,000) for Tonguefish.



(all site numbers are plus 40000; i.e. site 1 = 40001)

Sex Determination - Results: Determination of sex was made via gross and histologic examination of the gonads. In the majority of fish, histology was needed to determine sex. Unfortunately, 17 gonad samples were lost either during processing or sectioning. The data, sorted on the basis of site, is given in appendices 18a-18c. Additional sorting on the basis of species is shown in appendices 18d-18h and depicted in graphs 15a-15d. Graph 15a shows the distribution of mature males (testes with sperm) and females (ovaries with developing oocytes) in all fish for all sites. There were no obvious differences in between impact and reference sites.

When gobies were examined separately (graph 15b), there appear to be sharp site to site difference with respect to the sex. Two sites, impact site 40007 and reference site 40016, had samples with 100% female gobies. Sample sizes, however, were small with an "N" of two at site 40007 and only one at site 40016. Sample sizes were also small in the two other species (croakers and tonguefish) found at multiple sites, and no trends were uncovered with respect to prevalence of mature male or female fish.

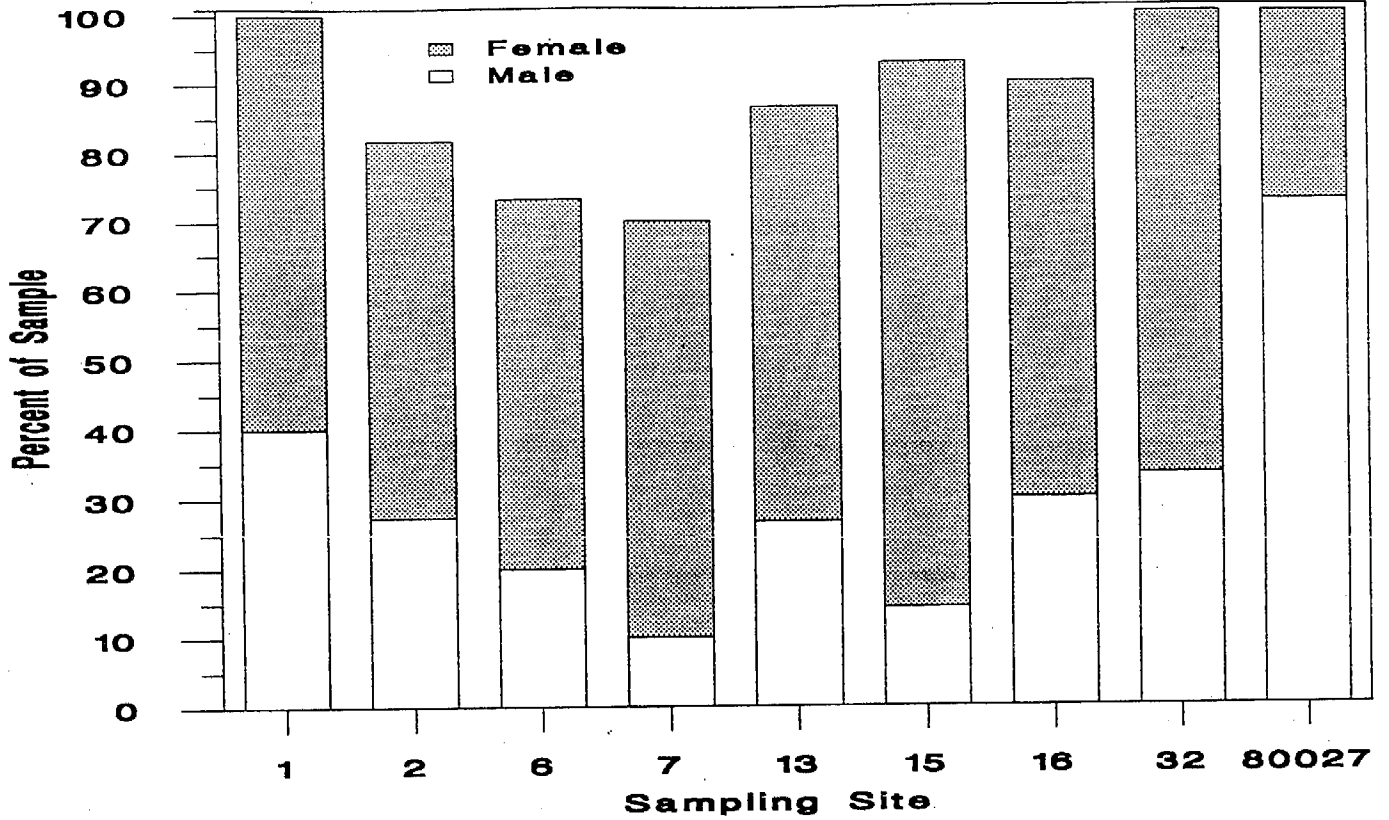
Some male fish with very small (< 1 mm diameter) testes were observed when gonads were surveyed to determine sex. These testes had minimal to no sperm production and were classified as immature and/or atrophic (Mi/a). In addition, a few male fish had immature/atrophic testes which had small numbers of immature oocytes. These fish were classified as intersex. Prevalence of intersex and Mi/a male fish for all sites is shown in graph 16a. All three intersex fish were from impact sites. Two impact sites (40006 and 40007) had markedly higher prevalences of Mi/a males when compared to the three reference sites. Reference site 40032 had no intersex fish nor any Mi/a males.

Sorting the data on the basis of sex revealed that almost all the intersex and Mi/a fish were croakers (graph 16b). Prevalence of Mi/a males among croakers was not consistent with the pattern when all five species were evaluated together. Prevalence of Mi/a croakers at reference site 40016 was higher than prevalences in all of the impact sites. In addition, the Mi/a males at reference site 40015 was higher than four of five impact sites where croakers were collected. Sample sizes at both reference and impact sites were often small (N = 2-5 at six of eight sites where croakers were collected). Average size of Mi/a croakers (mean SL = 77.9 mm) was markedly smaller than croakers with mature testes (mean SL = 118.3 mm).

No intersex or Mi/a males were observed in gobies, tonguefish, or stingrays. One intersex fish and one Mi/a male cusk-eel were found at impact site 40007.

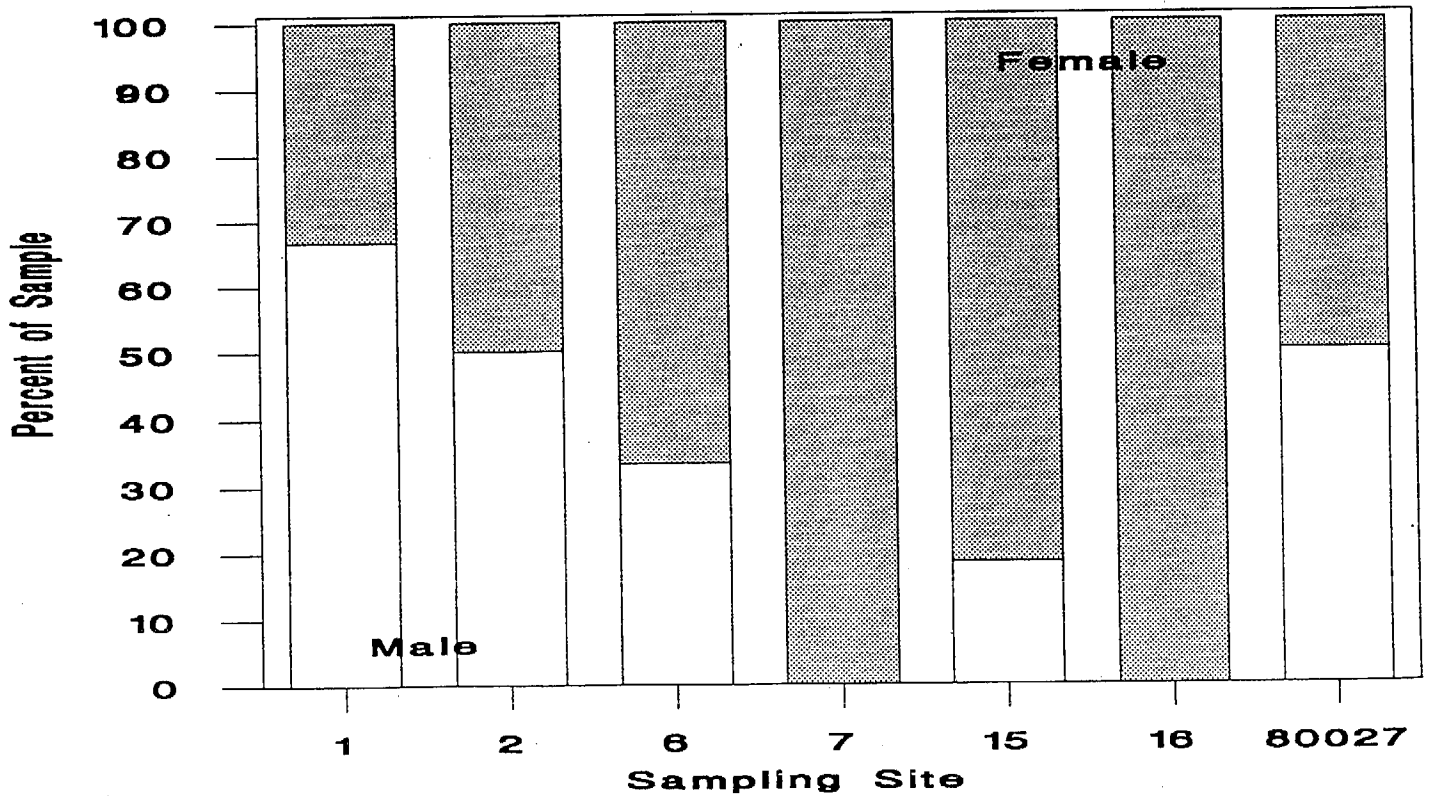
Size - Results: Although fish were not aged, both standard length (mm) and body weight (g) were determined for the majority of fish sampled (appendices 18a-18h). SL and BW data were sorted on the basis of both site and species for the three species which were collected at more than one site. Average SL and BW were similar in gobies from both impact and reference sites (graph 17a). In croakers (graph 17b), fish from four of five impact sites were consistently smaller (shorter and lighter) than croakers from the three reference sites. A similar trend was observed with tonguefish, where fish from the two impact sites (40002 and 40007) were markedly smaller than fish from two reference sites (40016 and 40032).

Graph 15a. Percent of Sample Composed of Mature Male and Female Fish.



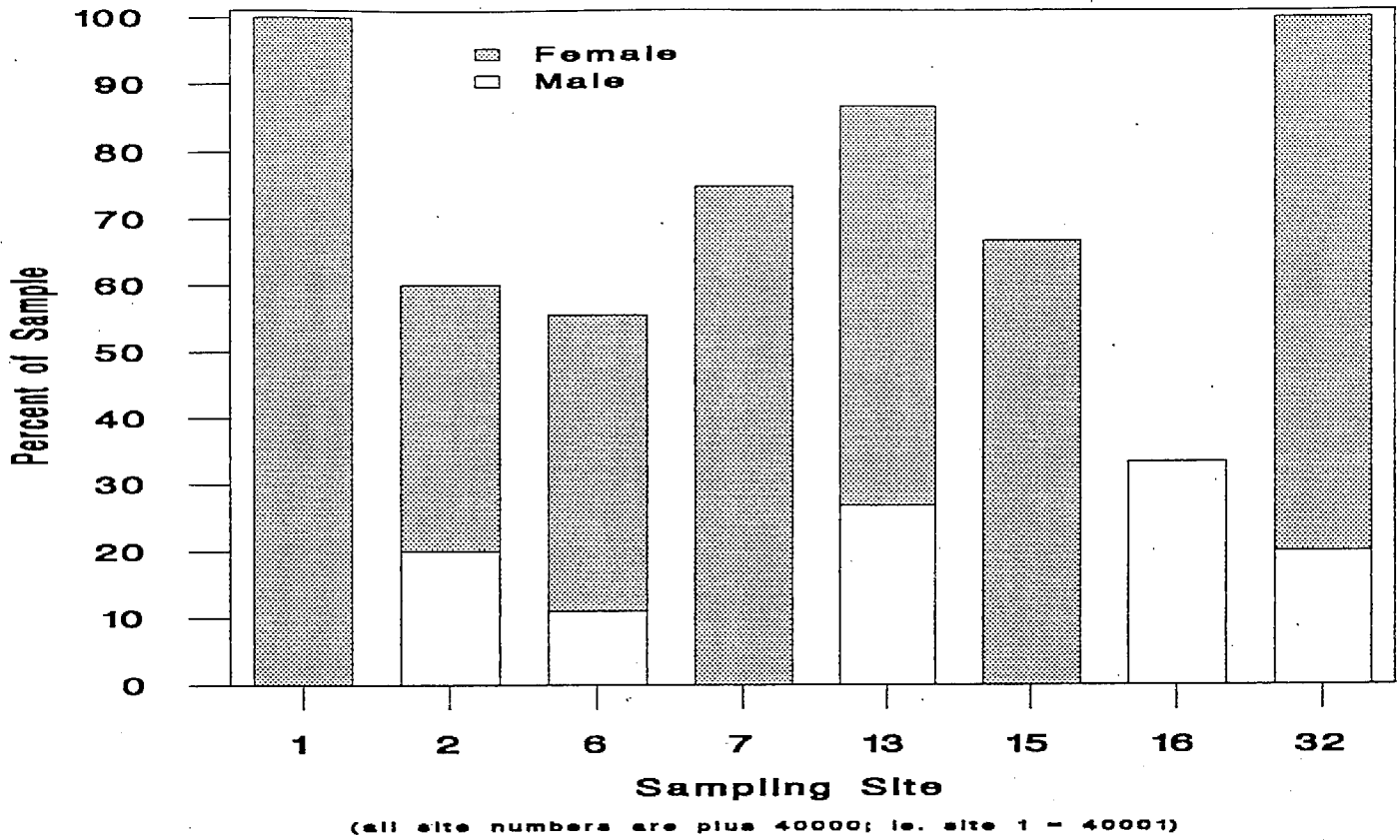
(all site numbers, except the last, are plus 40000; i.e. site 1 - 40001)

Graph 15b. Percent of Yellowfin Gobles which were Mature Males or Females.

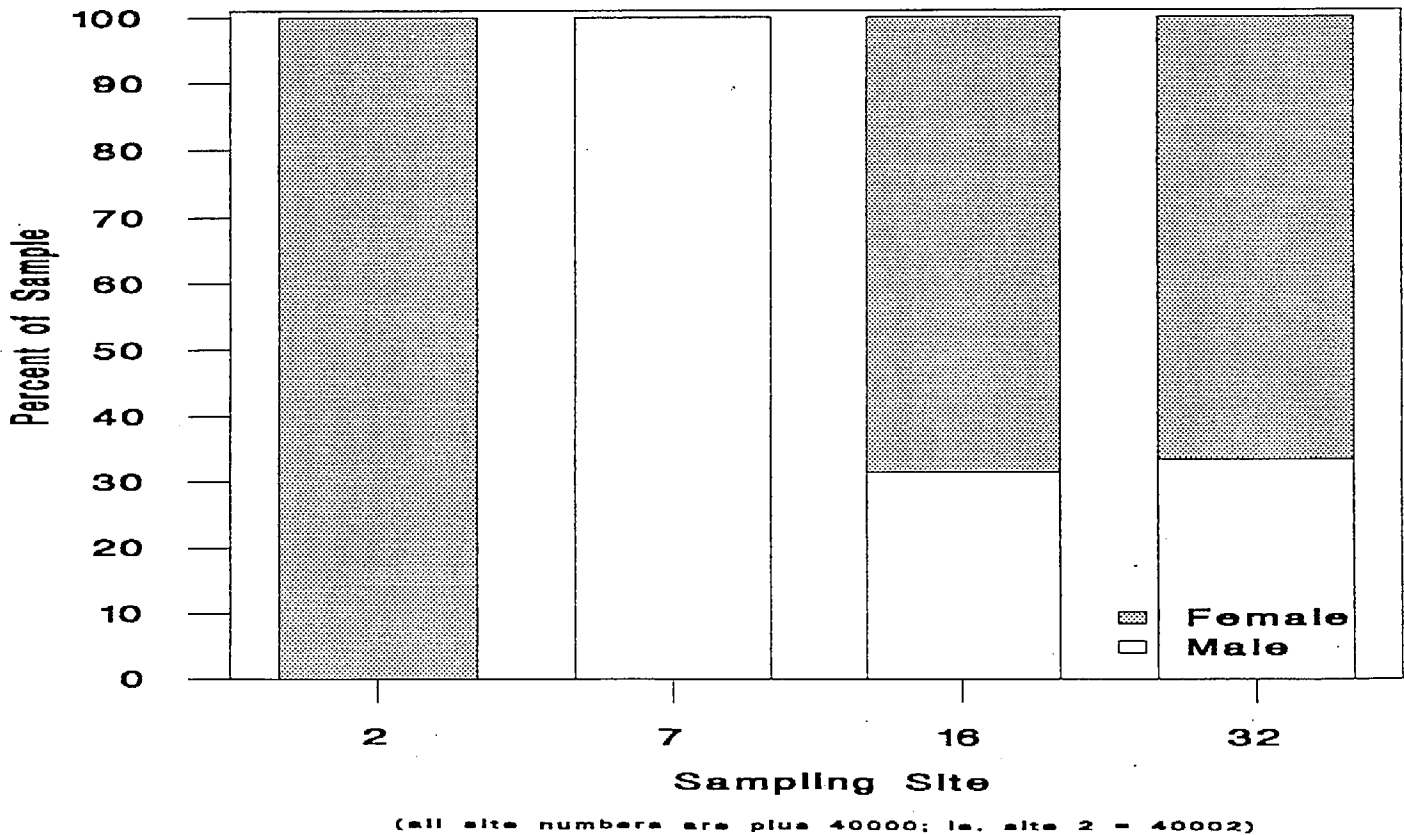


(all site numbers, except the last, are plus 40000; i.e. site 1 - 40001)

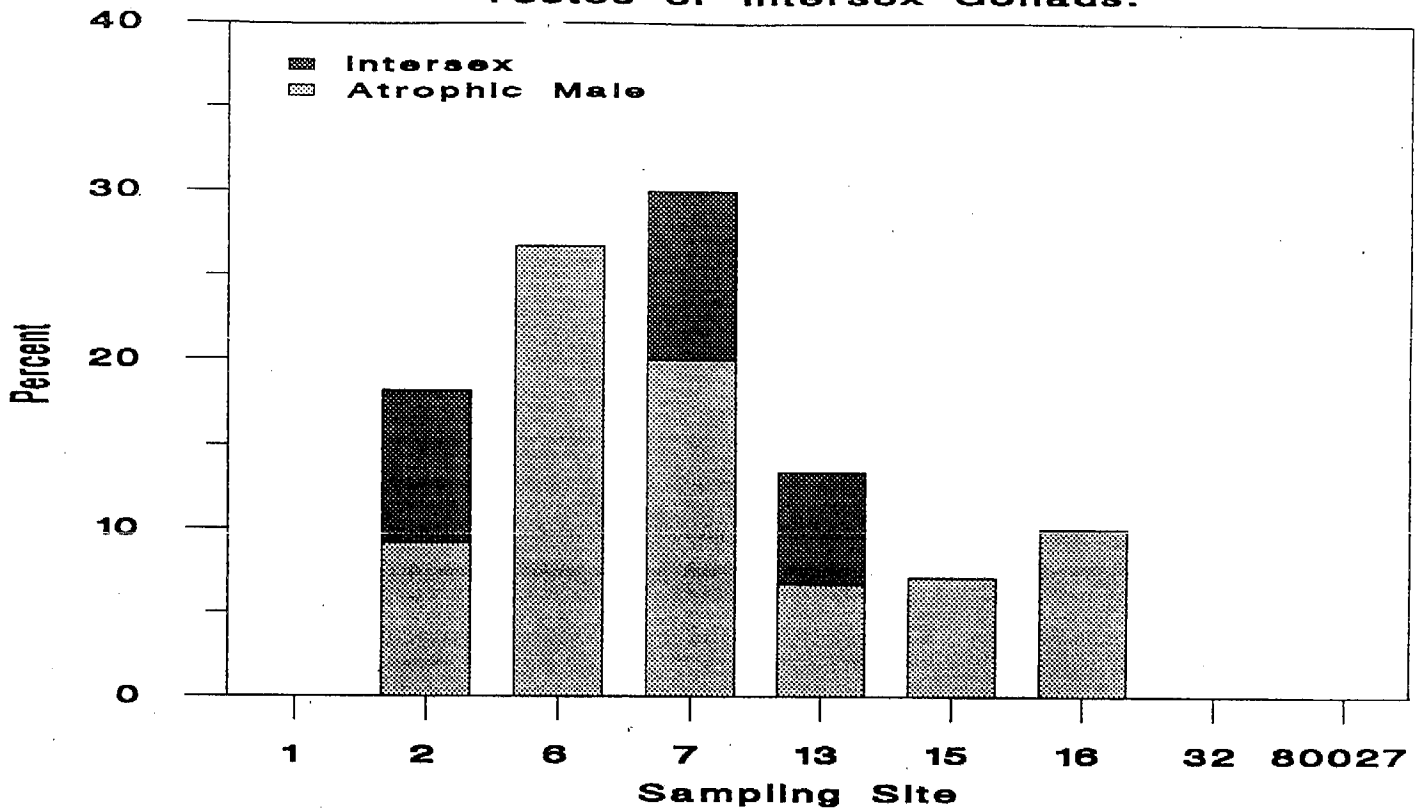
Graph 15c. Percent of White Croakers which were Mature Males or Females.



Graph 15d. Percent of Tonguefish which were Mature Males or Females.

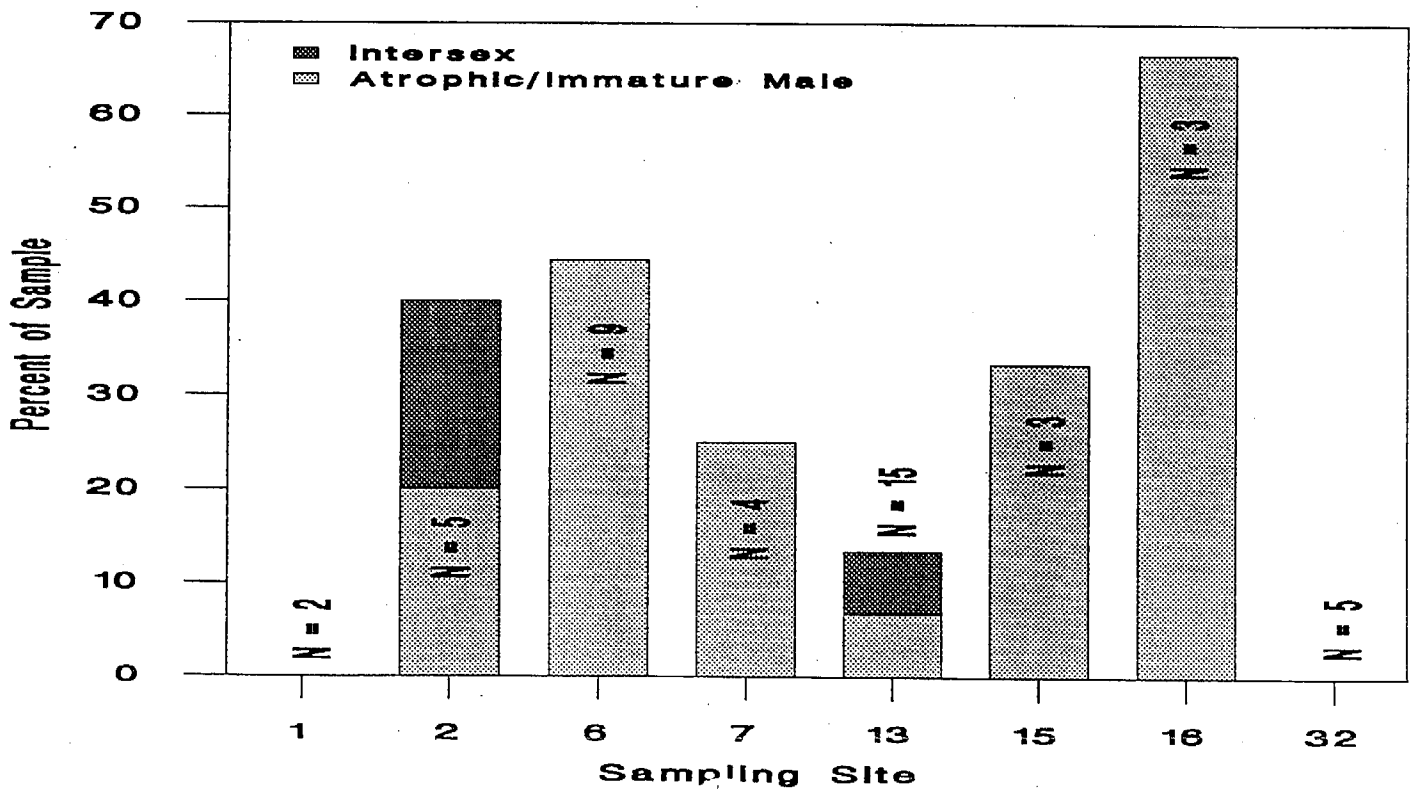


Graph 16a. Percent of All Samples from All Sites Composed of Fish with Atrophic/Immature Testes or Intersex Gonads.



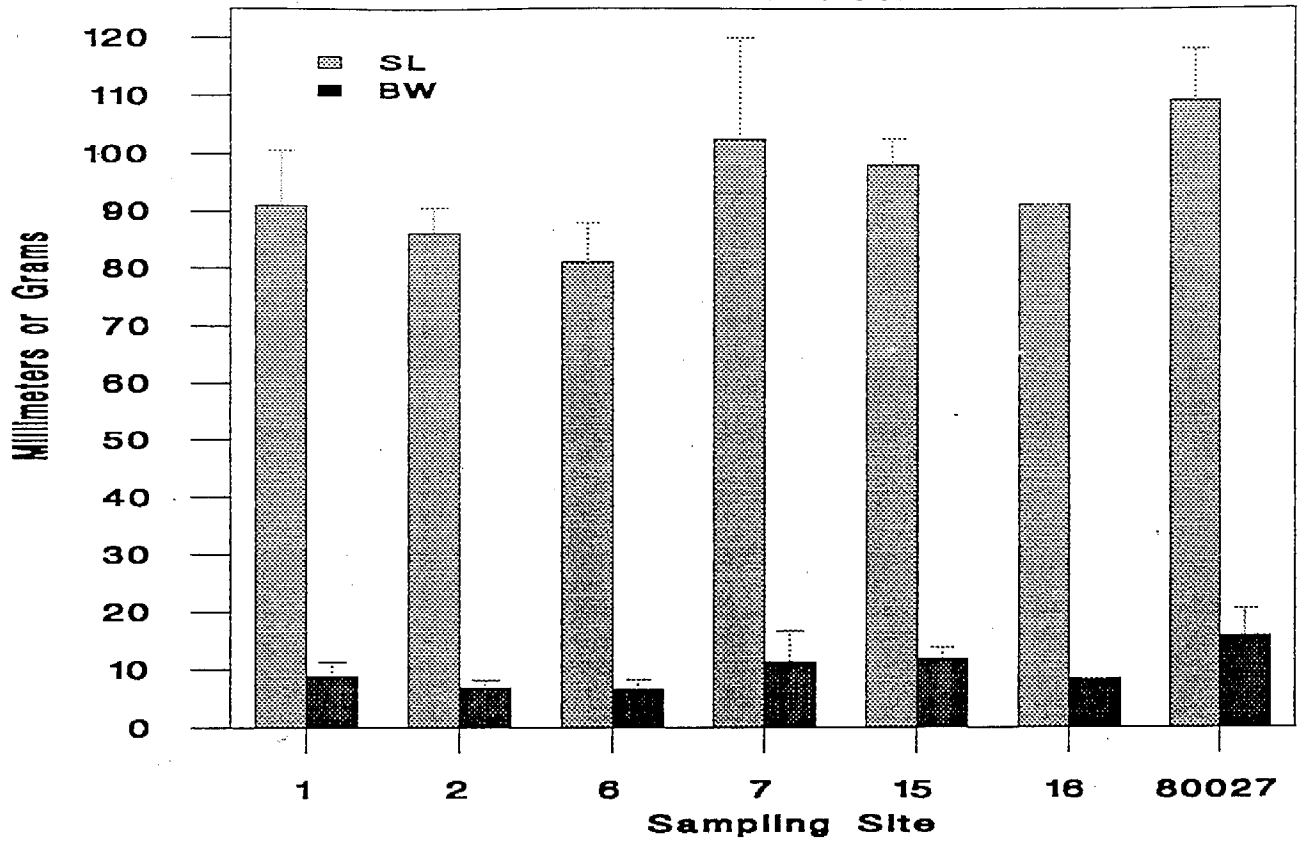
(all site numbers, except the last, are plus 40000; i.e. site 1 = 40001)

Graph 16b. Percent of White Croakers which had Atrophic/Immature Testes or Intersex Gonads.



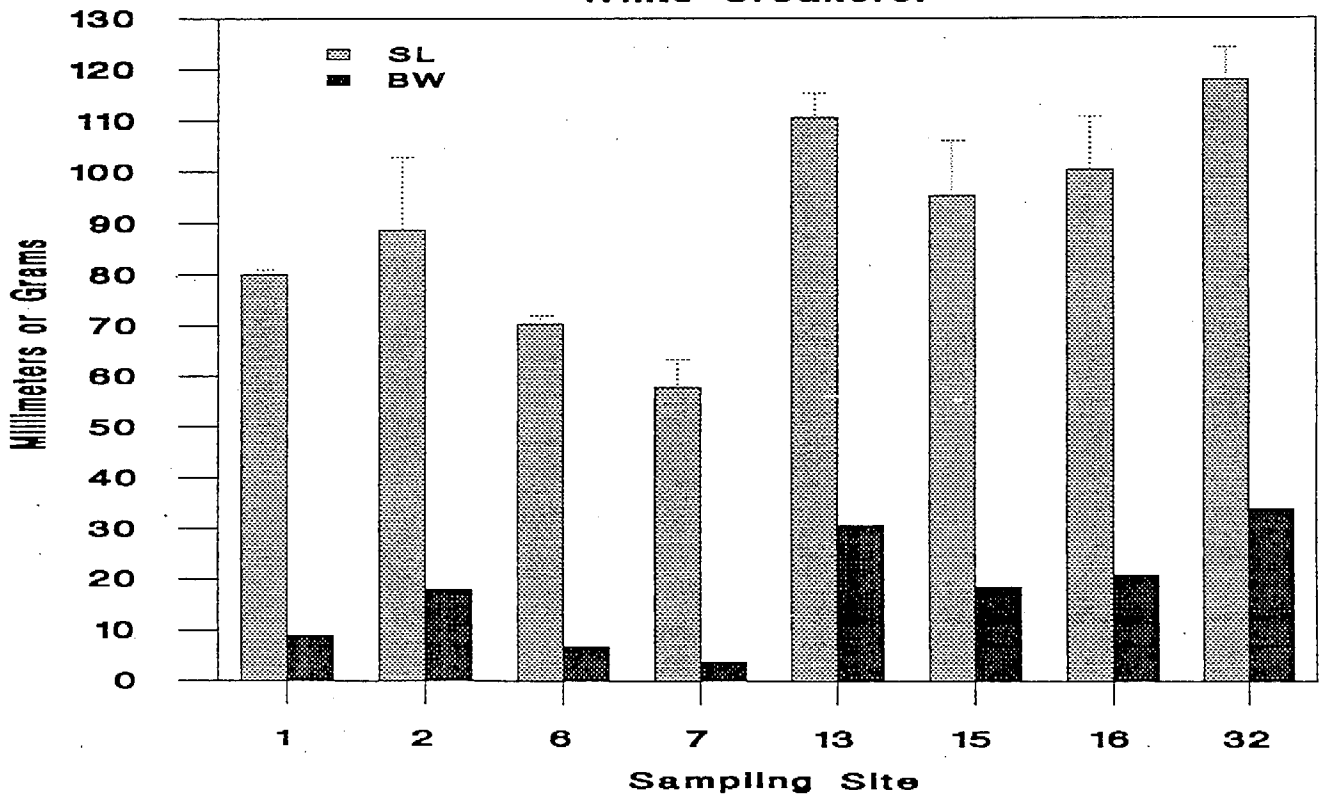
(all site numbers are plus 40000; i.e. site 1 = 40001)

Graph 17a. Average Standard length (SL in millimeters) and Body Weight (BW in grams) of Yellowfin Gobles.



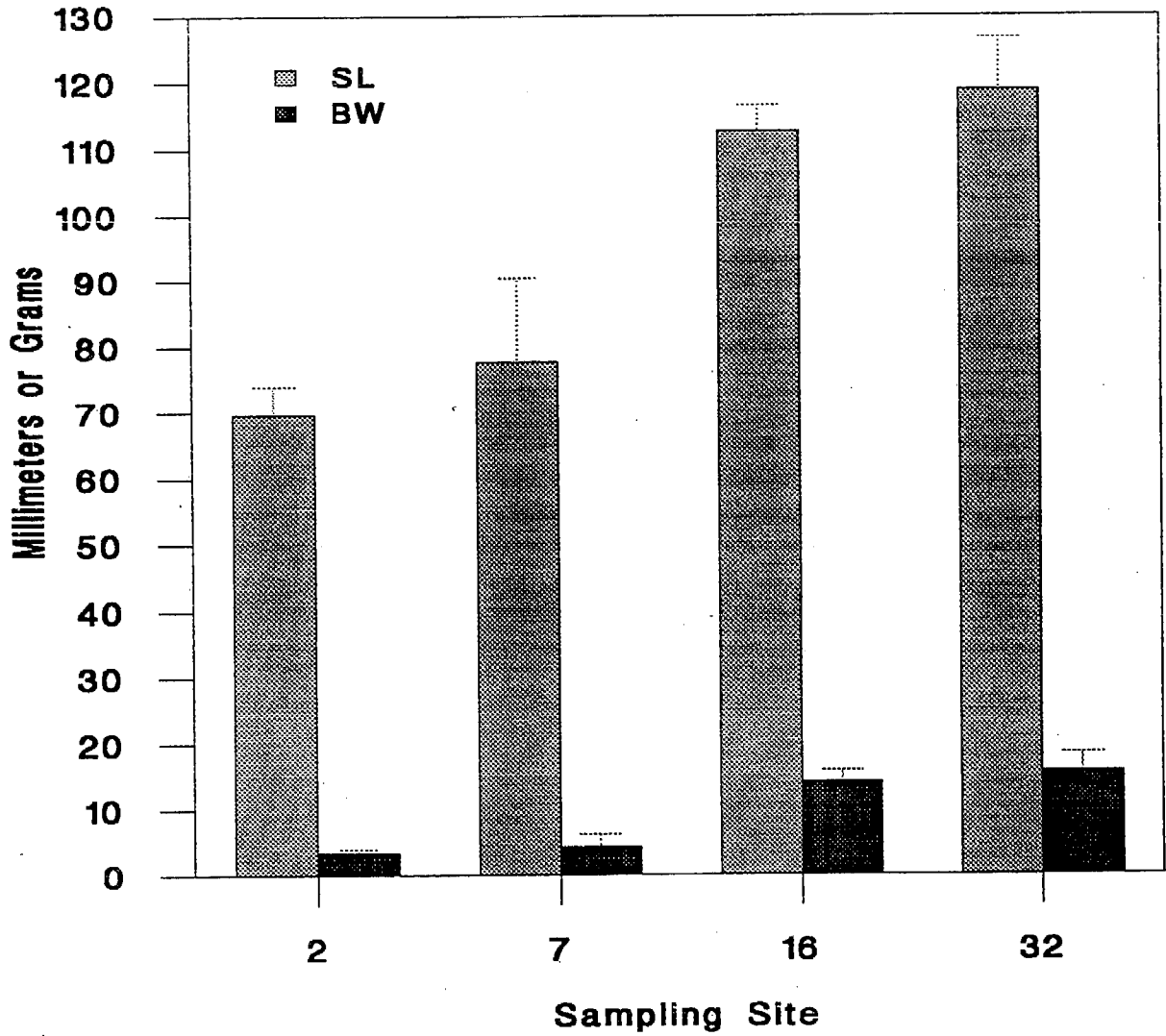
(all site numbers, except the last, are plus 40000; i.e. site 1 = 40001)

Graph 17b. Average Standard Length (SL in millimeters) and Body Weight (BW in grams) of White Croakers.



(all site numbers are plus 40000; i.e. site 1 = 40001)

Graph 17c. Average Standard Length (SL in millimeters) and Body Weight (BW in grams) of Tonguefish.



(all site numbers are plus 40000; i.e. site 2 = 40002)