INFLUENCE OF REPRODUCTIVE STATUS, SEX, AND PETROLEUM HYDROCARBON EXPOSURE ON HEPATIC AND EXTRAHEPATIC BIOTRANSFORMATION ENZYMES OF THE CUNNER, TAUTOGOLABRUS ADSPERSUS

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by

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ABSTRACT

Laboratory and field trials have validated liver mixed function oxygenase (MFO) induction as a sensitive biomonitor for petroleum hydrocarbon exposure. However, basal enzyme levels are known to vary seasonally and the usage of extrahepatic tissues for biological monitoring has received little attention. Laboratory experiments were conducted in the summer of 1985 to determine the induction potential of ethoxyresorufin O-deethylase (EROD) in liver, kidney, gill, and heart tissues of cunners (Tautogolabrus adspersus) exposed to no. 2 fuel oil (diesel oil) during the reproductive season. Although basal levels varied, induction was readily resolved in hepatic and extrahepatic tissues of both male and female cunners during prespawning, early spawning and late spawning. The induction potential of heart (7-18 fold) was much greater than liver (4-6 fold), kidney (4-7 fold) and gill (2-5 fold). Male cunners displayed a much higher induction potential compared to females in heart EROD during prespawning (18 versus 13 fold) and early spawning (12 versus 7 fold) but no significant sex differences were observed during late spawning. Sex differences were also evident in liver and kidney tissues throughout the reproductive season, males exhibiting higher enzyme activities than females in each case.

The potential for induction of a conjugating enzyme, glutathione S-transferase (GST), was also studied. Unlike EROD, GST was refractory to induction in all tissues of male and female cunners throughout gonad maturation/spawning. Depression of GST was observed in the heart tissue of diesel exposed males during early spawning and male liver and kidney during late spawning.

These experiments show the feasibility of using extrahepatic as well as hepatic MFO enzymes for monitoring studies even during the reproductive season.

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Finally, to my parents and sister, thank you for your life-long patience and support in all my endeavours.

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List of Abbreviations and Acronymns

AHH	Aryl hydrocarbon hydroxylase
ANOVA	Analysis of Variance
BHA	Butyl hydroxy anisole
BNF	β-Naphthoflavone
BOD	Biological Oxygen Demand
BSA	Bovine Serum Albumin
CDNB	Chlorodinitrobenzene
DBA	Dibenzanthracer
ER	Ethoxyresorufin
EROD	Ethoxyresorufin O-deethylase
GSH	γ-glutamylcysteinylglycine
GST	Glutathione S-transferase
HPLC	High performance liquid chromatography
MAB	Monomethylaminoazobenzene
MC	Methylcholanthrene
MFO	Mixed function oxidase
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
PAH	Polycyclic aromatic hydrocarbon
PB	Phenobarbital
PCB	Polychlorinated biphenyl
PTFE	Polytetrafluoroethylene
RER	Rough endoplasmic reticulum
SER	Smooth endoplasmic reticulum

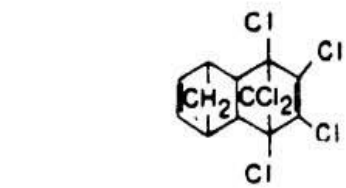
1. INTRODUCTION

1.1 Mixed Function Oxygenase in Mammals

Mixed function oxygenases (MFO's), also referred to as cytochrome P-450 monooxygenases, are responsible for the oxidative metabolism of a myriad of lipophilic organic substrates. These lipophilic substrates include a diversity of chemical structures, and include drugs, carcinogens, and environmental pollutants such as polychlorinated biphenyls (PCB's), pesticides, and petroleum hydrocarbons (Conney, 1967; Snyder and Remmer, 1979; Gelboin, 1980). Besides the oxidation of xenobiotic compounds, MFO enzymes are involved in the metabolism of endobiotics like steroids, fatty acids, vitamins, hormones, and bile acids (Ahmad, 1979). Some common MFO reactions are illustrated in Figure 1.1.

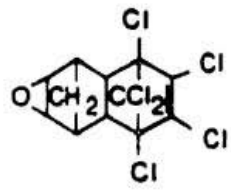
Mixed function oxygenases are found in virtually all organisms including vertebrates, invertebrates, and many bacteria, with the exception of anaerobic bacteria. Components of the mammalian P-450 monooxygenase system, isolated from the microsomal fraction (smooth endoplasmic reticulum vesicles) of the liver include the heme-containing cyctochrome P-450, the flavoprotein NADPH-cyctochrome P-450 reductase, and phospholipid. The highest concentrations of cyctochrome P-450-dependent monooxygenases are localized in the smooth endoplasmic reticulum (SER) of the liver cell (Hodgson, 1979). Intracellular locations besides

Figure 1.1: Common mixed function oxygenase reactions (Adapted from Lee, 1981.)

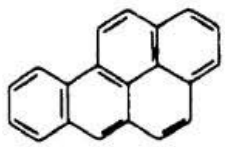


ALDRIN

Epoxidation

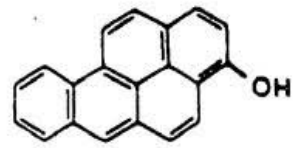


DIELDRIN

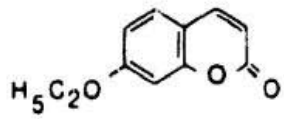


BENZO (a) PYRENE

Hydroxylation

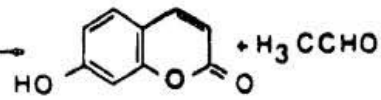


3-HYDROXYBENZO (a) PYRENE

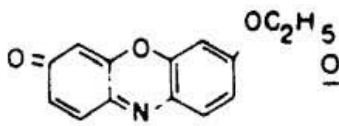


7 - ETHOXYCOUMARIN

O-Deethylation

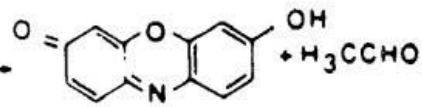


UMBELLIFERONE

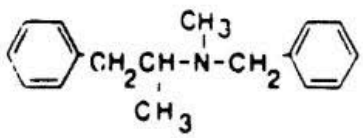


ETHOXYRESORUFIN

O-Deethylation

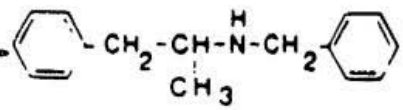


RESORUFIN



BENZPHETAMINE

N-Demethylation



+HCHO

the SER that usually contain lower MFO activity include the rough endoplasmic reticulum (RER) and the mitochondrial membrane (Hodgson, 1979) as well as the nuclear envelope of the liver cell (Viviani et al., 1978). The mitochondrial electron transport system (inner membrane matrix) is quite different from the microsomal system and appears to be specialized for steroid metabolism. Sato et al. (1977) noticed that partially purified cyctochrome P-450 from rat liver mitochondria was incapable of catalyzing the NADPH-dependent benzphetamine N-demethylation, unlike a cyctochrome P-450 isolated from rat liver microsomes. Both cyctochrome P-450's, however, exhibited 26-hydroxylase activity, which is involved in cholesterol metabolism. Extra-hepatic tissues including kidney, small intestine, lung, placenta, and skin appear to be active to some degree in the biotransformation of lipophilic compounds (Brattsten, 1979).

Monooxygenases function primarily as a detoxification system (so-called phase 1 enzymes), but many compounds became more toxic or carcinogenic upon conversion to chemically reactive metabolites by mixed function oxygenases (Conney, 1982). Primary oxidation products arising from phase 1 reactions are excreted or further transformed into more hydrophilic metabolites by a series of conjugating "phase 2" enzymes e.g. glutathione transferases, glucuronyl transferases and sulphotransferases (Gelboin, 1980).

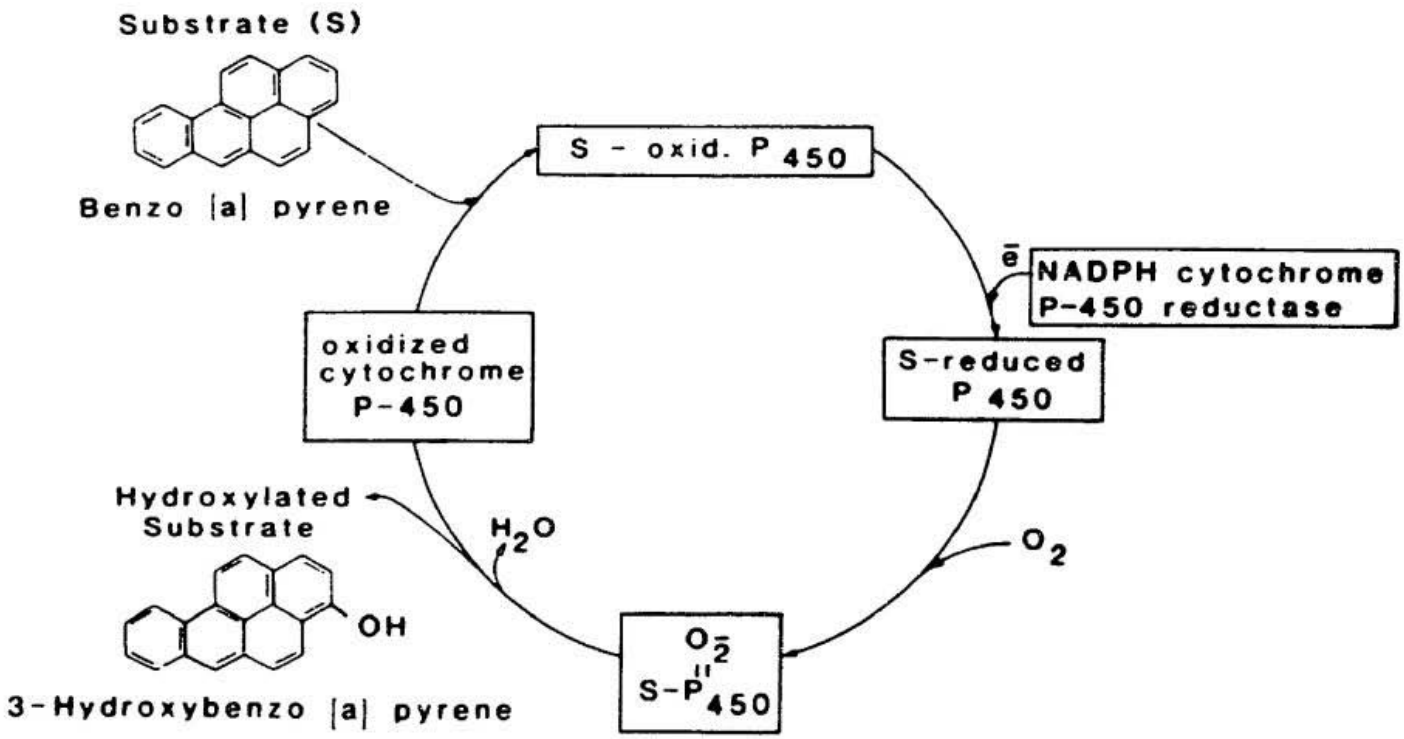
Manifestation of cellular toxicity in an organism is governed by various factors including species, type of xenobiotic, dose, levels of conjugating enzymes, and type of MFO enzymes involved (Gelboin, 1980).

It is quite clear that monooxygenases "activates" molecular oxygen with one atom being incorporated into the substrate and the other atom reduced in the form of water (Mason, 1957). Detailed catalytic events mediated by MFO are still ambiguous. There appears to be four common phases involved in catalysis: (1) substrate binding, (2) reduction of enzyme-substrate complexes by NADPH cytochrome P-450 reductase, (3) oxygen activation and (4) oxygen atom transfer (Ahmad, 1979). The substrate molecule combines with the oxidized ferric form of cyctochrome (Fe+3) and the complex undergoes reduction to the ferrous form (Fe+2), which interacts with oxygen in such a way that the hydroxylated substrate and a molecule of water leave the now re-oxidized cyctochrome P-450 (Estabrook et al., 1971). The sequential events of cytochrome P-450 mediated metabolism of benzo[a]pyrene are schematically depicted in Figure 1.2. The complex nature of MFO enzyme reactions is obvious when one considers that, depending on the substrate, all the reaction steps except possibly the association of substrate and enzyme, could be rate-limiting (Bjorkhem, 1977). However, in many hydroxylation reactions either

Figure 1.2: Oxidative metabolism of benzo[a]pyrene.

(Adapted from Lee, 1981.)

Membrane of Endoplasmic Reticulum



Membrane of Endoplasmic Reticulum

of the electron transfer steps appear to regulate the rate of reaction (Bjorkhem, 1977).

A number of different cyctochrome P-450 hemoproteins have been detected in rat tissues (Wolf et al., 1986), and this may account for the broad substrate specificities of the MFO enzyme system. The enzyme system is unique in that increased levels as well as variant forms are commonly found in the tissues of animals exposed to chemicals referred to as "inducing" compounds (Snyder and Remmer, 1979). Early studies on hepatic enzyme induction by Brown et al. (1954) revealed that the rate at which rat or mouse liver preparations de-methylated the hepatocarcinogen, 3-methyl-4-monomethylaminoazobenzene (3-methyl MAB), was dependent upon the diet fed to the animals. Brown et al. (1954) eventually attributed the increased rate of catalysis to polycyclic aromatic hydrocarbons (PAH's) like 3-methylcholanthrene (3-MC) present in the animal feed. At about the same time, Conney et al. (1956) presented evidence indicating that the increased rate of de-methylation was a result of de novo synthesis of new enzyme rather than the activation of nascent enzyme. The level of response depends upon the type of inducers, species, age, sex, and physiological state of the animal including reproductive and nutritional status (Vessel, 1982).

In depth study of two forms of purified cytochrome P-450 hemoproteins (Lu and West, 1980) demonstrated that they possess different spectral properties along with differing affinities for certain types of inducers. Phenobarbital administered to rats resulted in the proliferation of hepatic cytochrome P-450 monooxygenases possessing a Soret band at 450 nm (Orrenius and Ernster, 1964). Omura and Sato (1962) first coined the term "cytochrome P-450" upon observing this unique spectral band in microsomes bubbled with carbon monoxide and further reduced with sodium dithionite. Aromatic hydrocarbons like 3-MC and β-napthoflavone (BNF) induce cytochromes with a Soret band at 448 nm (Orrenius and Ernster, 1964). Recently another form of cytochrome P-448 from the liver of 3-MC pretreated rats has been purified and characterized (Seidel and Shires, 1986), further demonstrating the multiplicity of the cytochrome P-450 monooxygenases. Even though the cytochrome P-450 system possesses biological ubiquity and displays the capacity to metabolize a wide range of chemicals, selectivity towards some substrates may be enhanced.

1.2 Mixed Function Oxygenase in Fish

Metabolism of PAH's and barbiturates to oxidised derivatives has been studied in mammals since the 1940's. Subsequent characterization of the enzymes responsible

for oxidation led to the discovery of the cytochrome P-450dependent mixed function oxygenase system. Until the late 1960's research on drug metabolism in aquatic organisms was limited, probably in part to the premise that "ocean dwelling" species could simply remove toxic materials via passive diffusion processes. Contrary to such a belief, Adamson (1967) reported that many lipophilic compounds are relatively impermeable to fish gills compared to other membranes. Evidence was subsequently presented demonstrating that marine and freshwater fish species are quite efficient in transforming xenobiotics into water soluble derivatives for easy excretion via renal or biliary routes (Dewaide and Henderson, 1968). The microsomal fraction of trout liver was found to contain cytochrome P-450 (Chan et al., 1967). In fact, the xenobiotic metabolizing system of aquatic organisms was observed to have qualities similar to the complex mammalian MFO system, although the rates of oxidative metabolism were reported to be slower in aquatic species (James et al., 1977). This early work involved in vitro systems, but it is realized now that fish are capable of many phase 1 and phase 2 reactions in vivo (Table 1.1). Since many xenobiotics to which fish are exposed are readily taken up and sequestered in tissues like liver, blood, muscle, and brain (Melancon and Lech, 1978) the necessity to metabolize toxicants for easy excretion is apparent.

Table 1.1 Biotransformation reactions demonstrated in vivo by several fish species.

Biotransformation reaction		Species	Chemical
Phase I	Phase II		
O-Dealkylation		Fathead minnow	p-Nitrophenylethers
		Rainbow trout	Pentachloroanisole
			Fenithrothion
N-Dealkylation		Carp	Dinitramine
Oxidation		Mudsucker, sculpin	Naphthalene, benzo[a]pyrene
		Coho salmon	Naphthalene
		Rainbow trout	Methylnaphthalene
		Carp	Rotenone
		Mosquito fish	Aldrin, dieldrin
Hydrolysis		Catfish, bluegill	2,4-Dichlorophenoxyacetic acid ester
		Rainbow trout	Diethylhexylphthalate
		Pinfish	Malathion
		Mosquito fish	Parathion
	Acetylation	Dogfish shark	Ethyl-m-aminobenzoate
		Rainbow trout	Ethyl-m-aminobenzoate
	Glutathione conjugation	Carp	Molinate
	Taurine "	Flounder	2,4-Dichlorophenoxyacetic acid
	Sulfate "	Goldfish	Pentacholorophenol
	Glucuronide "	Rainbow trout	Pentachlorophenol
		Rainbow trout	3-Trifluoromethyl-4-nitrophenol
		Goldfish	Pentachlorophenol
	Glycine "	Flounder	Aminobenzoic acid

Taken from Lech and Vodicnik, 1984.

Mammalian MFO and fish MFO have several characteristics in common. Both systems are dependent upon molecular oxygen and NADPH, and the cytochrome P-450's exhibit most of the same spectral patterns. The monooxygenase activity of trout liver microsomes has been shown to be sensitive to inhibition by carbon monoxide and responsive to mammalian MFO modulators like α-naphthoflavone and metyrapone (Ahokas et al., 1977). As in mammals, MFO enzyme activities are highest in fish liver, and other extrahepatic tissues including kidney, gill, and heart have been shown to possess xenobiotic metabolizing ability (Payne and May, 1979; Porter et al., 1986). The head kidney of teleost fish (attached to the trunk kidney which is responsible for urine production) seems analogous to the mammalian adrenal cortex (Butler, 1973) which is known to be instrumental in steroid and xenobiotic metabolism (Burke and Orrenius, 1979). The fish monooxygenase system also responds to a diversity of substrates that may undergo biotransformation. Different fish species display a wide variation in the rates of xenobiotic metabolism (James and Bend, 1980) as do different mammalian species. Mixed function oxygenases in fish are also influenced by physiological and environmental factors including age, sex, diet, gonadal maturity, ambient temperature, seasonal changes, developmental stage, and exposure to inducers/inhibitors (Walton et al., 1978; Stegeman and Chevion, 1980; Addison and Willis, 1982; Walton

et al., 1983; Binder and Stegeman, 1984; Andersson and Koivusaari, 1985).

Most hepatic monooxygenase activities are higher in male rats than in female rats (Shapiro, 1986). Fish species also appear to exhibit sexual dimorphism in MFO metabolizing ability, generally with higher activities in males versus females. Sex differences have been reported in the specific content of cytochrome P-450 in hepatic and renal microsomes from adult rainbow trout (Salmo gairdneri) and brook trout (Salvelinus fontinalis) (Stegeman and Chevion, 1980). Kidney microsomes from mature male trout showed a 20-fold higher cytochrome c reductase activity, as well as faster hydroxylation rates of various substrates including progesterone and aflatoxin B1 compared to females (Williams et al., 1986). The same study, however, showed no significant sex differences in benzo[a]pyrene hydroxylase or benzphetamine N-demethylase activities (Williams et al., 1986), and sex differences in cytochrome P-450 content and associated MFO activities were less pronounced with liver microsomes compared to kidney microsomes. Other investigators have shown that sex-related changes in monooxygenase activities during certain stages of gonad maturation/spawning of fish are also dependent on the substrates used to assay the activity. Hepatic MFO activities measured in rainbow trout using benzo[a]pyrene,

7-ethoxycoumarin, aminopyrene, and ethylmorphine as substrates were significantly higher in males than in females during the pre-spawning period, yet 7-ethoxyresorufin deethylase activity displayed no such sex difference (Koivusaari et al., 1984). In the rat, sex differences have been shown to be regulated through the hypothalamo-pituitary axis (Gustafsson et al., 1983) and growth hormone (somatotrophin) secretory patterns are important for the sexual dimorphism seen in this species. Androgen "imprinting" during neonatal life is postulated to play an important role in producing MFO activities observed during the adult stage of the rat (Gustafsson et al., 1983). Neonatal gonadectomy and androgen exposure studies reveal the existence of sex-specific cytochrome P-450s in the rat (Dannan et al., 1986), however, we know little of the underlying mechanisms. Gonadal steroids (estradiols and testosterones) may play an important role in regulating observed sex differences in MFO activity of fish (Stegeman and Chevion, 1981).

Habitat temperature appears to influence the time course and intensity of the induction process in fish (Andersson and Koivusaari, 1985). Constitutive levels of MFO activity in isolated liver cells of rainbow trout are affected by temperature changes (Andersson and Koivusaari, 1986). The enzymes ethoxycoumarin O-deethylase

and benzo[a]pyrene hydroxylase were higher in liver cells from cold acclimated trout than those from warm acclimated fish at all assay temperatures used (Andersson and Koivusaari, 1986). Hepatic MFO of bluegill (Lepomis macrochirus r.) displayed temperature compensation (Ankley et al., 1985), since fish acclimated to cool water (12°c) hydroxylate benzo[a]pyrene more rapidly than fish kept at a warmer temperature (32°c). This compensatory response is important for poikilotherms which live in an environment with large temperature fluctuations.

1.3 Mixed Function Oxygenase Induction: A Useful Biological Monitor for Organic Pollution in the Aquatic Environment

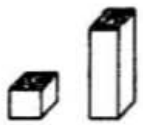
During the early 1970's more intensive studies on xenobiotic metabolism in fish and other aquatic species began to appear. Investigations were also more related to environmental health interests than in previous years (reviewed by Payne, 1984). Mixed function oxygenase induction was proposed (Payne and Penrose, 1975) as a sensitive biological monitor of environmental pollutants, particularly oil contamination. Elevated MFO enzyme levels in fish have been shown in a number of field trials over the past decade to be related to hydrocarbon pollution in the aquatic environment (Figure 1.3). Field studies carried out in the early 1970's in Newfoundland demonstrated that brown trout (Salmo trutta) captured from a small urban

Figure 1.3: Mixed function oxygenase trials in fish in association with petroleum hydrocarbon pollution.

(Units are relative enzyme activities for liver tissues in all cases. Low tower = control site; high tower = experimental site.)

(Adapted from Payne et al., 1987.)

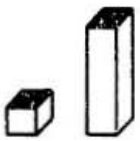
LOCALE : Newfoundland
SITE : Urban Lake
SPECIES : Brown Trout *(Salmo gairdneri)*
ASSAY : AHH
REF. : Payne and Penrose, 1975



LOCALE : Newfoundland
SITE : Oil Refinery
SPECIES : Cunner *(Tautogolabrus adspersus)*
ASSAY : AHH
REF. : Payne, 1976

LOCALE : Adriatic Sea
SITE : Oil Spill
SPECIES : Blenny *(Blennius pavo)*
ASSAY : AHH
REF. : Kurelec et al, 1977

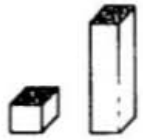
LOCALE : New England
SITE : Old Oil Spill
SPECIES : Mummichog *(Fundulus heteroclitus)*
ASSAY : Aldrin Epoxidation
REF. : Burns, 1976



LOCALE : Adriatic Sea
SITE : Petroleum Industry
SPECIES : Blenny *(Blennius pavo)*
ASSAY : AHH
REF. : Rijavec et al, 1981

LOCALE : California
SITE : Natural Oil Seep
SPECIES : Pacific Sanddab *(Citharichthys sordidus)*
ASSAY : AHH
REF. : Spies et al, 1982

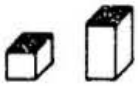
LOCALE : California
SITE : Oil Seep
SPECIES : Speckled Sanddab *(Citharichthys stigmaeus)*
ASSAY : AHH
REF. : Spies et al, 1982



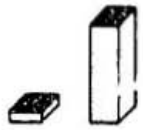
LOCALE : California
SITE : Oil Seep
SPECIES : White Sea-perch *(Phanerodon furcatus)*
ASSAY : AHH
REF : Spies et al, 1980

LOCALE : North Sea
SITE : Oil Rigs
SPECIES : Atlantic Cod *(Gadus morhua)*
ASSAY : AHH
REF. : Davies et al, 1984

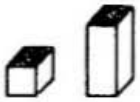
LOCALE : North Sea
SITE : Oil Rigs
SPECIES : Haddock *(Melanogrammus aeglefinus)*
ASSAY : AHH
REF : Davies et al, 1984



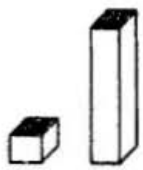
LOCALE : North Sea
SITE : Oil Rigs
SPECIES : Whiting *(Merlangius merlangus)*
ASSAY : AHH
REF : Davies et al, 1984



LOCALE : Newfoundland
SITE : Baie Verte Oil Spill
SPECIES : Winter Flounder *(Pseudopleuronectes americanus)*
ASSAY : AHH
REF : Payne et al, 1984



LOCALE : Vasso Archipelago, Finland
SITE : Oil Spill
SPECIES : Perch *(Perca fluviatilis)*
ASSAY : AHH
REF. : Lindstrom-Seppa, 1985



LOCALE : Newfoundland
SITE : Elevated Level Of Sediment Hydrocarbons
SPECIES : Winter Flounder *(Pseudopleuronectes americanus)*
ASSAY : EROD
REF. : PAYNE et al, unpublished

lake in St. John's with a history of hydrocarbon pollution exhibited elevated benzo[a]pyrene hydroxylase levels in liver tissues (Payne and Penrose, 1975). Other field studies were also successful in demonstrating the association between petroleum hydrocarbon exposure and increased MFO enzyme levels in fish species. Cunners (Tautogolabrus adspersus) collected in the vicinity of a large oil refinery in Placentia Bay, Newfoundland, had elevated MFO enzyme levels in both liver and gill tissues compared to fish taken from control sites (Payne, 1976). Likewise, a small boat marina putatively contaminated with petroleum hydrocarbons harbored cunners with induced MFO enzyme levels (Payne, 1976). Blennies (Blennius parvo) collected from a diesel oil spill site in the Adriatic sea exhibited marked benzo[a]pyrene hydroxylase induction in liver tissues and the induction was maintained for three weeks (Kurelec et al., 1977). Another field trial in the Adriatic revealed blennies taken from the site of a refinery outfall had highly induced liver MFO enzyme levels (Britvic et al., 1983) relative to control sites. Two species of sanddabs (Citharicthys sardidus and C. stigmeus) and white perch (Phanerodon furcatus) collected near a natural petroleum seep in the Santa Barbara channel displayed increased benzo[a]pyrene hydroxylase levels (Spies et al., 1980; Spies et al., 1982). Mummichogs (Fundulus heteroclitis) collected near the site of an oil spill off the coast of Massachusetts were reported to have elevated aldrin epoxidase (Burns, 1976) and

benzo[a]pyrene hydroxylase (Stegeman, 1978) enzyme activities. Since one would expect extremely low levels of hydrocarbons or other chemicals in the open ocean, particularly interesting are the reports of elevated MFO levels in various fish species including codfish (Gadus morhua) collected near oil-rigs in the North Sea (Davies et al., 1984). A recent biomonitoring study was carried out in Finland near the site of an oil spill in the Vassa Archipelago (Lindstrom-Seppa et al., 1985). Perch (Perca fluviatilis) at this site were found to exhibit slightly elevated benzo[a]pyrene hydroxylase enzyme activities and significantly induced glutathione S-transferase activities compared to control sites. An important revelation from this study was that glutatione S-transferase enzyme levels were a more powerful indicator of hydrocarbon exposure than the MFO enzyme levels. Most of the earlier field studies have focused on elevated liver MFO, but Payne et al. (1984) reported elevated MFO enzyme levels in kidney tissues of flounder (Pseudopleuronectes americanus) collected at the site of a no. 2 fuel oil spill in Baie Verte, Newfoundland.

Some workers have proposed that exposure to levels of pollutants sufficient to disturb serum chemistry may be applicable to monitoring programs (Lockhart and Metner, 1984). However, a study in Puget Sound (Casillas et al., 1985) revealed that changes in serum chemistry (e.g. glucose

levels, adrenaline) and gross pathology were comparable in diagnosing presumptive pollution mediated diseases of English Sole (Parophrys vetulus). Contaminant levels sufficient to produce gross pathological damage (primary effect) make measurement of changes in biochemical parameters like serum ions, sugars, cortisol, or adrenaline (secondary effects) redundant in the sense of providing an early warning system. Mixed function oxygenase enzyme induction is, in essence, a primary detoxification response and, in the case of exposure to potent inducers such as petroleum hydrocarbons, enzyme change can be expected to occur before the onset of more serious pathological change. A recent field study carried out in Finland (Nikunen, 1985) cataloging the differences in 25 biochemical variables of rainbow trout held in cages near a reference site compared to a water waste discharge site from a petrochemical complex, showed significant differences between only two of the parameters: increases in activity of the detoxification enzymes, MFO and glucuronyltransferase of fish near the chemically polluted site compared to the control site.

An attempt to interpret biochemical responses in terms of whole-organism or population effects might be optimistic at present, but it is not unreasonable to want to do so. However, from a regulatory or environmental perspective, one of the primary values of such a sensitive response

as MFO induction is its value as an indicator in defining boundary limits for point sources of pollution e.g. around oil-rigs or major spill sites. Also in relation to mixed organic pollution, studies in the Great Lakes and Europe (Figure 1.4), have demonstrated the potential to discriminate water quality over broad geographical regions by using the MFO induction response. Although it is quite reasonable to state that chemical analysis is a more efficient means of quantitatively and qualitatively defining water quality, it lacks biological significance and is time consuming and expensive. Kurelec et al. (1982) stated "the use of MFO as a monitoring tool would help fill the gap which usually exists between the estimated concentration of xenobiotics in water and corresponding biological effects."

1.4 Phase 2 Detoxification: Glutathione S-Transferase

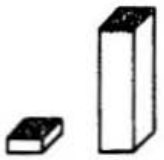
Xenobiotics catalyzed by MFO enzymes produce electrophilic products that are often conjugated with endogenous substances such as sugars, amino acids, sulphate, phosphate or a tripeptide referred to as glutathione (Brattsten, 1979). Glutathione (GSH), gamma-glutamylcysteinylglycine, possesses a nucleophilic thiol moiety, the cysteinyl residue, which combines with highly reactive electrophiles and other oxidative products of MFO enzymatic reactions (Ketterer et al., 1983). The negative charge and high hydrophilicity of GSH greatly increases the aqueous solubility of lipophilic

Figure 1.4: Mixed function oxygenase trials in fish in association with mixed organic pollution.

(Units are relative enzyme activities for liver tissues in all cases. Low tower = control site; high tower = experimental site.)

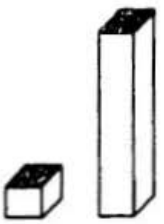
(Adapted from Payne et al., 1987.)

LOCALE : New England
SITE : Pesticide Contamination
SPECIES : Mummichog *(Fundulus heteroclitus)*
ASSAY : Aldrin Epoxidation
REF. : Burns, 1976

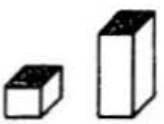


LOCALE : California
SITE : La Sewage Outfall
SPECIES : Pacific Sanddab *(Citharichthys sordidus)*
ASSAY : AHH
REF. : Spies et al, 1982

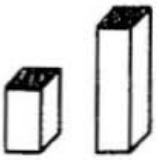
LOCALE : Yugoslavia
SITE : River Krka
SPECIES : Chub *(Leuciscus cephalus)*
ASSAY : AHH
REF. : Kezic et al, 1963



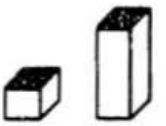
LOCALE : Great Lakes
SITE : Lake Michigan
SPECIES : Rainbow Trout *(Salmo gairdneri)*
ASSAY : AHH
REF. : Binder and Lech, 1984



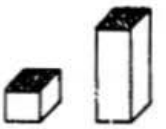
LOCALE : Gulf of Mexico
SITE : Incineration Vessel
SPECIES : Mummichog *(Fundulus grandis)*
ASSAY : Cytochrome
REF. : Kamlet, 1981



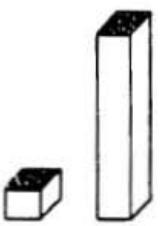
LOCALE : New York Bight
SITE : Marine Sewage Dump
SPECIES : Mummichog *(Fundulus grandis)*
ASSAY : Cytochrome P_{450}
REF. : Pequegnat and Wastler, 1980



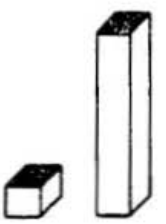
LOCALE : Yugoslavia
SITE : River Krka
SPECIES : Barbel *(Barbus barbus)*
ASSAY : AHH
REF. : Kezic et al, 1983



LOCALE : Yugoslavia
SITE : River Krka
SPECIES : Nase *(Chondrostoma nasus)*
ASSAY : AHH
REF. : Kezic et al, 1983



LOCALE : Great Lakes
SITE : Green Bay
SPECIES : Rainbow Trout *(Salmo gairdneri)*
ASSAY : AHH
REF. : Binder and Lech, 1984



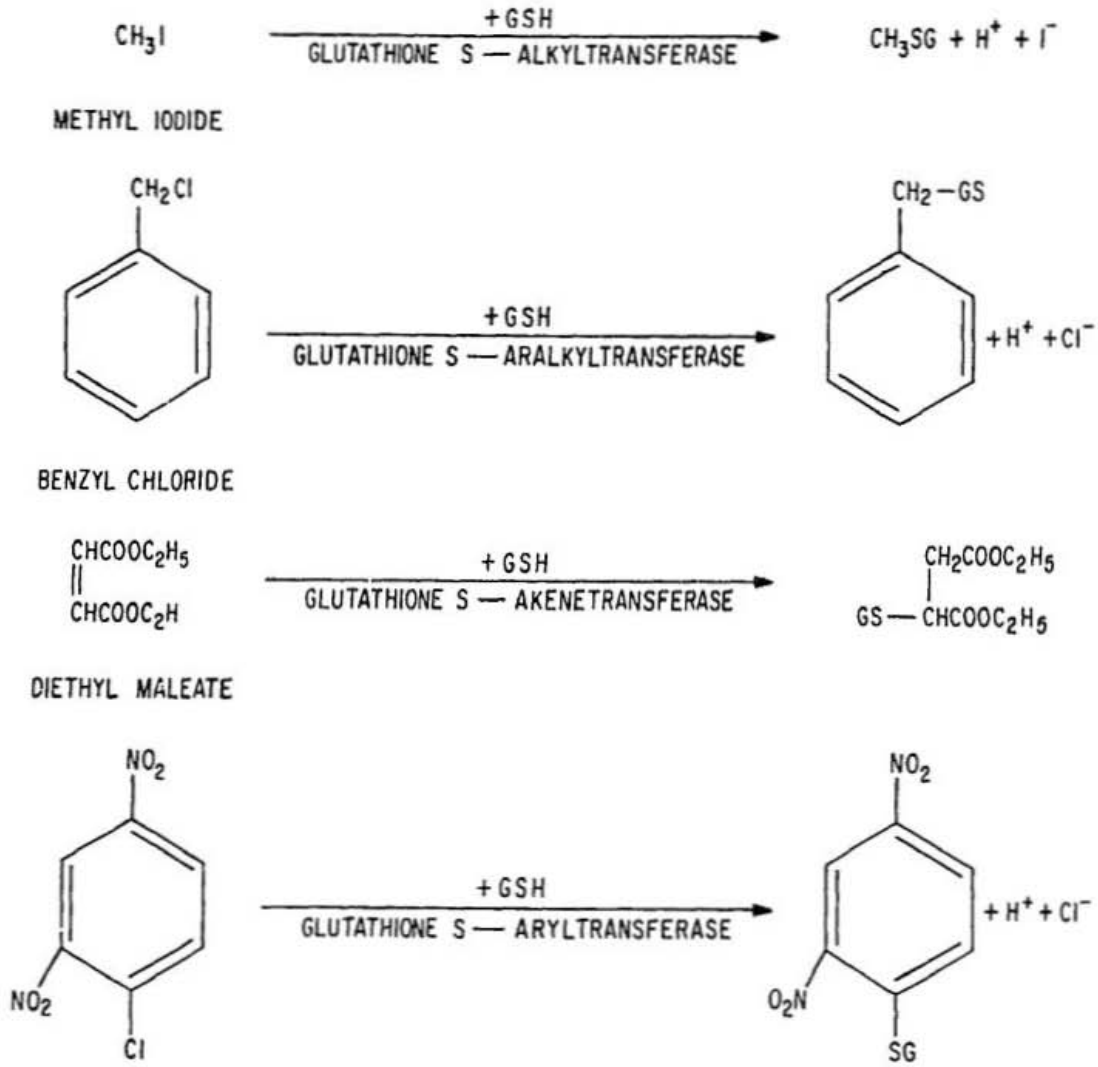
LOCALE : Swedish Coast
SITE : 8 km from Pulp Mill
SPECIES : Perch *(Perca fluviatilis)*
ASSAY : EROD
REF : Forlin et al, 1985

compounds that conjugate with GSH, enhancing biliary excretion (Ketterer et al., 1983) or the GSH conjugates are further metabolized to form mercapturic acids which are excreted in the urine (Fukami, 1984). Enzymatic catalysis is involved in many GSH conjugation reactions and executed by a group of enzymes referred to as the glutathione S-transferases (GST) which are primarily cytosolic (Fukami, 1984). A microsomal glutathione S-transferase has also been characterized (Boyer et al., 1986). Figure 1.5 show the conjugation of some substrates catalysed by glutathione S-transferases.

Since conjugation is preceded by oxidation reactions mediated by the MFO enzyme system, the former is sometimes considered a secondary detoxification process while the latter is a primary detoxification process (Fukami, 1984). This does not lessen the importance of the GSH S-transferases. Substrates like some insecticides are detoxified primarily by the GSH conjugation pathway rather than by the MFO enzyme system (Fukami, 1984). Furthermore, the MFO enzyme system may produce both toxic and nontoxic metabolites whereas the GSH S-transferases are primarily involved in detoxification. The enhancement of GSH S-transferase activity in the forestomach of mice by compounds including benzyl isothiocyanate, p-methoxynitrophenol, coumarin and 2-tert-butyl-4-hydroxyanisole (2-BHA)

Figure 1.5: Conjugation reactions catalyzed by glutathione S-transferase

(Adapted from Fukami, 1984.)


CH3I
+GSH
GLUTATHIONE S — ALKYLTRANSFERASE
CH3SG + H+ + I−
METHYL IODIDE
CH2Cl
+GSH
GLUTATHIONE S — ARALKYLTRANSFERASE
CH2—GS
+H+ +Cl−
BENZYL CHLORIDE
CHCOOC2H5
CHCOOC2H
+GSH
GLUTATHIONE S — AKENETRANSFERASE
CH2COOC2H5
GS—CHCOOC2H5
DIETHYL MALEATE
NO2
NO2
Cl
+GSH
GLUTATHIONE S — ARYLTRANSFERASE
NO2
+H+ +Cl−
O2N
SG

significantly reduced benzo[a]pyrene induced neoplasia in that organ (Sparnins et al., 1982). However, in some instances the GSH conjugation process has been reported to activate xenobiotics like alkylnitrosoguanidines, dihalomethanes, and the antineoplastic agent, bleomycin, to mutagenic and/or carcinogenic derivatives (Igwe, 1986).

Glutathione conjugation has been demonstrated to occur in a variety of species including mammals, fish, birds, amphibians, insects and other invertebrates (Boyland and Chasseaud, 1969). Five GSH S-transferases have been isolated from rat liver and they display a wide range of catalytic activity - it is hypothesized that a general mechanism of catalysis exists for the isoenzymes which involves a nucleophilic attack of enzyme-bound GSH on the electrophilic center of the xenobiotic (Keen et al., 1976). Similar to the MFO system, GSH S-transferases show a broad overlapping substrate specificity (Habig and Jakoby, 1981).

The majority of research on the GSH S-transferases have been performed using rat liver cytosol, but extrahepatic tissues as well as other species have also been employed (Habig et al., 1974). Five cytosolic GSH S-transferases have been isolated from the liver of the male little skate, Raja erinacea (Foureman and Bend, 1984). Glutathione

S-transferase activity has also been demonstrated in black sea bass (Centropristis striata) liver, kidney, brain, muscle and red blood cells (Braddon et al., 1985).

Glutathione S-transferases, like MFO enzymes, exhibit the highest activities in mammalian and fish liver tissues relative to other organs (Jakoby, 1978; Braddon et al., 1985). Intracellular GSH concentrations in the rat liver are as high as 5-10 mM (Kosower and Kosower, 1978) with GSH S-transferase concentrations as high as 0.2 mM (Ketterer et al., 1983). Liver biotransformation enzyme activities are generally higher in mammals than aquatic species. However, whole Odonata larva were found to exhibit GSH S-transferase activities 6 times that of rat liver (Dierickx and De Brabander, 1984).

Many factors that influence MFO enzyme activities in fish may also affect GSH S-transferases. Xenobiotics such as PCB's, BNF, and PAH's have been shown to induce liver GSH S-transferases in fish (Ankley et al., 1986; Andersson et al., 1985b; Lindstrom-Seppa et al., 1985); although others claim that various MFO-type inducers have no enhancing affect on GSH S-transferase activities (Bend and James, 1978; Fair, 1986). The effects of other factors like environmental temperature and starvation on various MFO related activities and UDP glucoronyltransferase

activities (conjugating enzyme) have been thoroughly characterized (Andersson et al., 1985a; Andersson and Koivusaari, 1986), however, little information is available about these effects on the GSH S-transferases. This study investigated the potential for GST induction in various tissues of cunners exposed to petroleum hydrocarbons during the critical stages of gonad maturation/spawning.

2. MATERIALS AND METHODS

2.1 Chemicals

All reagents used in these studies were of standard chemical grade obtained from various suppliers. Other chemicals are listed below:

ethoxyresorufin: Pierce Chemical Co., Rockford, Illinois.

resorufin: Eastman-Kodak Ltd., Rochester, New York.

diesel oil: Texaco (local).

glutathione (reduced form): Sigma Chemical CO., St. Louis, Missouri.

1-choloro-2, 4-dinitrobenzene: Sigma Chemical Co., St. Louis, Missouri

naphthalene, fluorene, phenanthrene, pyrene, benzo[a]pyrene: Supelco Ltd., Oakville, Ontario.

hexane (HPLC grade): Fisher Scientific, Montreal, Quebec.

methyl-tert-butyl-ether (HPLC grade): BDH, Dartmouth, Nova Scotia.

2.2 Fish Collection

Male and female cunners (Tautogolabrus adspersus) were caught using a hoop net and/or gill net from a relatively pristine area of Portugal Cove during the summer of 1985. The fish ranged in weight from 100-300 g and were collected during three periods of gonad maturation/spawning based on gonad indices and according to Pottle and Green (1978):

(1) prespawning (June 25th, 26th, 27th)

(2) early spawning (July 16th, 17th, 19th)

(3) late spawning (August 4th).

2.3 Exposure System

After the fish were transported to the laboratory in 52 L containers, they were acclimated for 1-2 weeks in 2000 L holding tanks. This was followed by exposure to no. 2 fuel oil (diesel oil) in a flow-through sea water tank maintained at ambient temperature (Prespawn temperature = 8°C; early spawn temperature = 12°C; late spawn temperature = 13°C). The exposure consisted of introducing 200 ml of oil into a head tank (25 L) and mixing it with a constant stream of seawater sprayed onto the surface of the head tank (Kiceniuk et. al., 1982). Seawater containing the oil was then drawn from the bottom of the tank into the experimental tank (200 L, flow rate = 2 L/min). This type of exposure set-up allows for a pulse delivery of diesel oil. Refer to Figure A-1 of the Appendix for experimental design.

A control tank was set up next to the experimental tank and received the same manipulations except for chemical exposure. Due to the difficulty in sexing cunners, each tank contained 35-40 fish to provide sufficient numbers of each sex for appropriate statistical analysis. The experimental fish received two equivalent exposure doses,

200 ml of diesel, 45 h apart. Hourly water samples were taken from each tank throughout the duration of the experiment, in such a way as not to disturb the oil sheen on the surface water of the experimental tank.

2.4 Oil Analysis in Water

Each water sample was subjected to a modified version of the fluorimetric method of Keizer and Gordon (1973) for determination of total oil concentration. The control and experimental tanks each had a glass tube (1 cm diameter; 1 m length) immersed approximately 20 cm below the water surface for withdrawing samples into 300 ml glass BOD sampling bottles. Each seawater sample was extracted with 100 ml of spectroanalyzed hexane in a 1 L separatory funnel. The hexane extract of both control and experimental tanks was analyzed by fluorescence spectroscopy (308 nm excitation/344 nm emission) for the presence of mono- and polyaromatic hydrocarbons. A standard curve was constructed by spiking hexane with diesel oil (Figure A.2).

2.5 Preparation of S9 Fractions from Liver, Kidney, Gill and Heart

Fish were killed (90 hr following first diesel exposure) with a blow to the head and the hepatic and extra-hepatic organs were removed immediately. Necropsy data (organ weights, body weight, length, sex) were recorded. The bile duct was clamped with a haemostat and the liver and

other tissues of the fish were excised, weighed, and placed on ice. All organs were minced with scissors and approximately 1 g of each was homogenized in 4 volumes of ice-cold 50 mM Tris-HCL, (pH 7.5) using ten passes of a glass Ten Broek hand homogenizer. The S9 supernatant was obtained by removing cell debris by centrifugation at 4°C for 10 min at 9000 x g. The S9 fraction of each sample was stored in triplicate in polyproplylene Eppendorf micro test-tubes at -80°C.

2.6 Protein Determination

Protein was determined by the procedure of Lowry et al. (1951), using a Perkin-Elmer UV-Visible scanning spectrophotometer. Lowry reagent consisted of 20 g Na_2CO_3 (anhydrous) and 4 g NaOH dissolved in 950 ml of distilled water and diluted to 1 L. Five ml of a solution consisting of 1 ml of 1% CuSO4, 1 ml of 2% Na-K tartate and 100 ml of Lowry reagent was added to 500 µl of S9 suspension (10-20 µl of S9 to 480-490 µl of distilled water). Various concentrations of bovine serum albumin (BSA) ranging from 50 µg/ml to 400 µg/ml were used as standards. Distilled water (500 µl) served as a blank. After a 10 min incubation period at room temperature, 500 µl of 1 N Folin-Ciocalteu reagent was added to the mixture. The mixture was immediately vortexed and incubated for 30 min at room temperature. The absorbance was read at 620 nm. A linear

standard curve for protein concentration versus absorbance was produced from the BSA standards and used to calculate S9 protein concentrations in mg/ml and mg/g organ.

2.7 Total Lipid Determination

Total lipids were measured gravimetrically after extraction by the method of Bligh and Dyer (1959). Liver S9 fractions were removed from the -80°C freezer and thawed. Approximately 2 ml of the S9 fraction was transferred to a conical volumetric test tube and the volume recorded. The S9 fraction was transferred to an Omni mixer and blended for 60 sec. with 10 ml of methanol:water (1:1). The mixture was blended for another 2 min after the addition of 20 ml of chloroform and then filtered. Filtration through a Whatman #1 filter paper was followed by blending of the filter paper with 10 ml of methanol:water (1:1) and 20 ml of chloroform. The blender was rinsed with 10 ml of methanol:water (1:1) and 20 ml of chloroform followed by filtration through a second filter paper. The total filtrate was transferred to a 200 ml Erlenmeyer flask and 20 ml of 0.88% potassium chloride was added. The mixture was allowed to settle for 10 min before the upper phase was discarded. A 30 ml solution of methanol:water (1:1) was added to 115 ml of the filtrate, mixed thoroughly and the solution was allowed to settle before the upper phase was discarded. This step was repeated twice. Two grams of

anhydrous sodium sulphate were added to the flask which was swirled and allowed to stand for at least 30 min. The solution was then filtered through glass wool into a pre-weighed 250 ml roundbottom flask. The glass funnel was washed three times with chloroform and allowed to drain into the flask. The filtrate was evaporated on a Brinkmann rotary evaporator (42°C, -700 Torr) and the flask placed in a dessiccator overnight.

The flask was weighed after a 24 h period and the lipid weight determined. The extract was taken up in 2 ml of HPLC grade hexane and filtered through a Millex SR 0.5 µm PTFE filter. The filtrate was stored in an amber vial with a Teflon lid at 4°C in preparation for HPLC analysis.

2.8 Polycyclic Aromatic Hydrocarbon (PAH) Analysis

Chromatographic hardware included a Beckman Model 110 liquid chromatograph and the following Perkin-Elmer instrumentation:ISS-100 Autoinjector and a Model 3600 Data Station.

Chromatography was carried out on a Nucleosil NH_2 column (5 µm, 5mm id, 25 cm) with an injection volume of 145 µl for samples and 25 µl for standards. The mobile phase consisted of 100% hexane (Fisher HPLC grade) for

14 min. followed by a column backflush of 90% hexane and 10% methyl-t-butyl-ether (Fisher HPLC grade). The solvent flow rate was 2 ml/min in the forward flow mode and 3 ml/min on backflush.

Benzene plus 5 groups of polycyclic aromatic hydrocarbons (naphthalene, fluorene, phenanthrene, pyrene and benzo[a]pyrene) were used as external standards and peaks were identified on the basis of retention times and quantitated by peak height of the absorbance (254 nm) of the extracts with those of the standards.

2.9 Ethoxyresorufin O-deethylase Determination

The mixed function oxygenase system deethylates 7-ethoxyresorufin (7-ER) to produce resorufin. This substrate is relatively specific for mammalian cytochrome P-448 monoxygenases. Burke and Mayer (1974) developed a fluorimetric assay which measures the increase in resorufin formation as a linear increase in fluorescence.

Ethoxyresorufin O-deethylase (EROD) activity was assayed fluorimetrically as described by Pohl and Fouts (1980) using a Perkin-Elmer LS-5 fluorescence spectrophotometer. The reaction mixture, final volume 1.25 ml, consisted of 53 nmol Tris- Sucrose buffer (50 mM, pH 7.5), 50 µl S9 liver (100 µl for the extra-hepatic organs and the buffer

volume adjusted accordingly), 2.25 nmol 7-ER (150 μM) and the reaction mixture was started by addition of 0.16 mg NADPH (1.25 mg/ml). After a 15 min incubation at 25°C in a temperature controlled water bath, the reaction was terminated by the addition of 2.5 ml of ice-cold spectro-analyzed methanol. A methanol blank contained the same components as the sample tubes except the addition of methanol to denature the protein occurred before the addition of NADPH. Assay tubes were vortexed and the protein precipitate removed by centrification at 3600 x g for 2 min. The fluorescence of resorufin formed in the supernatants was measured in a matched set of fluorimetric cuvettes (1 cm path length) at 585 nm using an excitation wavelength of 550 nm (slit width of 0.5 mm). Enzyme activity was linear with time and protein concentration. The rate of enzyme activity in nmol/min/mg protein was obtained from the regression of fluorescence against standard concentrations of resorufin. The level of sensitivity of this assay was calculated to be 3 pmol product formed/min/mg protein.

2.10 Glutathione S-transferase Determination

Glutathione transferase activity was assayed based on Habig et al. (1974) by measuring the conjugation of 1-chloro-2, 4-dinitrobenzene (CDNB) with glutathione (GSH) as a change in absorbance.

The reaction mixture was prepared in a spectrophotometer cuvette with a total volume of 3 ml. The reaction mixture consisted of 2.875 ml of 0.1 mM potassium phosphate (pH 6.5), 60 µl of 50 mM CDNB and 50 µl of a 1/10 dilution of liver S9 (100 µl of a 1/10 dilution of kidney S9 and gill S9; 200 µl of a 1/2 dilution of heart S9 and buffer was adjusted accordingly). The incubation was carried out at 25°C. The reaction was initiated by the addition of 25 µl of 1 mM GSH and the sample cuvette was read against the reference cuvette (minus GSH and adjusted with buffer) at a wavelength of 340 nm in a dual-beam Perkin-Elmer UV Visible scanning spectrophotometer (model 571). The change in absorbance was monitored on a chart recorder for a period of 3 min. The specific activity was expressed as µg CDNB-GSH conjugate formed/min/mg protein based on the extinction coefficient of 9.6 mM/cm. All enzyme assays were linear with time and protein concentration.

2.11 Statistical Methods

Analysis of variance (ANOVA) was applied to determine significant relationships between sex, treatment, and sample period and their interactions on enzyme activities. Duncan's multiple range test, which compares all possible pairs of means, was used to determine which means were different. Means of data sets were considered to differ significantly from each other if $p<0.05$ for the F-value. All data points

in the tables and figures represent means +/- standard error (S.E.Mn.). Univariate analysis was applied to determine distribution patterns. Correlations were computed using the Spearman ranked correlation method. All statistical analysis were carried out using SAS Statistical programs.

3. RESULTS

Univariate analysis of the hepatic and extrahepatic ethoxyresorufin O-deethylase (EROD) and glutathione S-transferase (GST) specific activities revealed the absence of a normal distribution. Therefore, the data were ranked for the purpose of meaningful statistical analysis. Many investigators carrying out biochemical studies do not perform univariate analysis on their data, i.e. they assume normality. However, this assumption in most cases will be invalid. To illustrate the pitfalls that may be encountered when interpreting toxicological data (or any experimental data set), an example is presented in the Appendix. Table A.1 shows the differences in p-values generated from three-way ANOVA of non-normal data (liver EROD) that have and have not been ranked.

3.1 Ethoxyresorufin O-deethylase (EROD) Induction

A laboratory study was conducted to measure the effect of petroleum hydrocarbon exposure on EROD in cunner (Tautogolabrus adspersus) during the critical period of gonad maturation/spawning. The exposure system (Figure A.1 of the Appendix) delivered a pulse of diesel oil with a mean concentration of 50 µg/ml (50 ppm), calculated from the standard curve depicted in Figure A.2 of the Appendix. A representative concentration curve of diesel oil in the

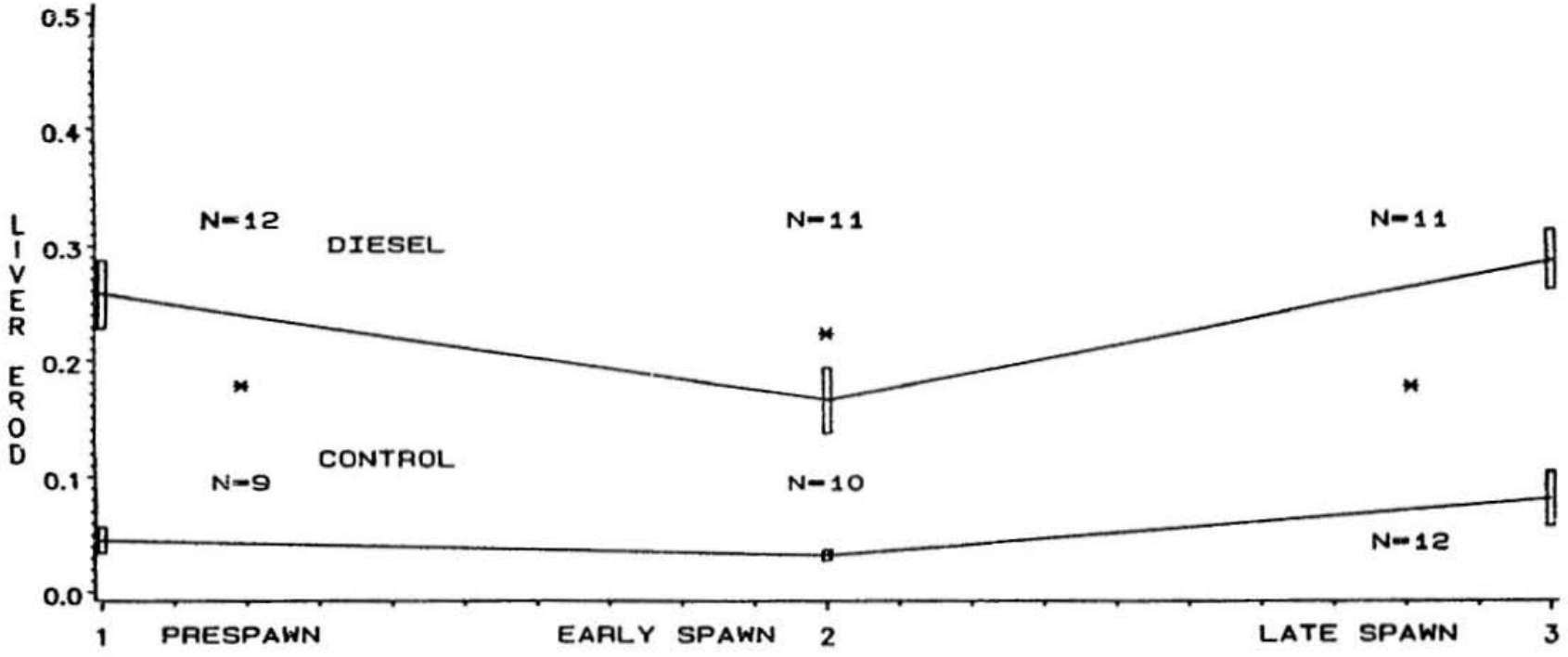
tank water during the experimental trials is presented in Figure A.3. A relatively high concentration of diesel oil was used in order to decrease the variability of oil concentrations in water (which is difficult to attain with low concentrations of oil) during the various exposure periods.

Figures 3.1-3.8 show EROD specific activities in liver, kidney, gill and heart of cunners throughout the reproductive season. Results indicate EROD in hepatic and extrahepatic tissues of diesel exposed cunners were significantly higher relative to the control cunners during prespawning, early spawning and late spawning. Ethoxyresorufin O-deethylase specific activities of diesel exposed cunners were 4-6 fold higher in the liver, 4-7 fold higher in the kidney, 2-5 fold higher in the gill, and 7-18 fold higher in the heart than enzyme activities of control cunners. The EROD induction potential of each organ is tabulated in Table 3.1.

3.2 Sex Differences in Ethoxyresorufin O-deethylase (EROD)

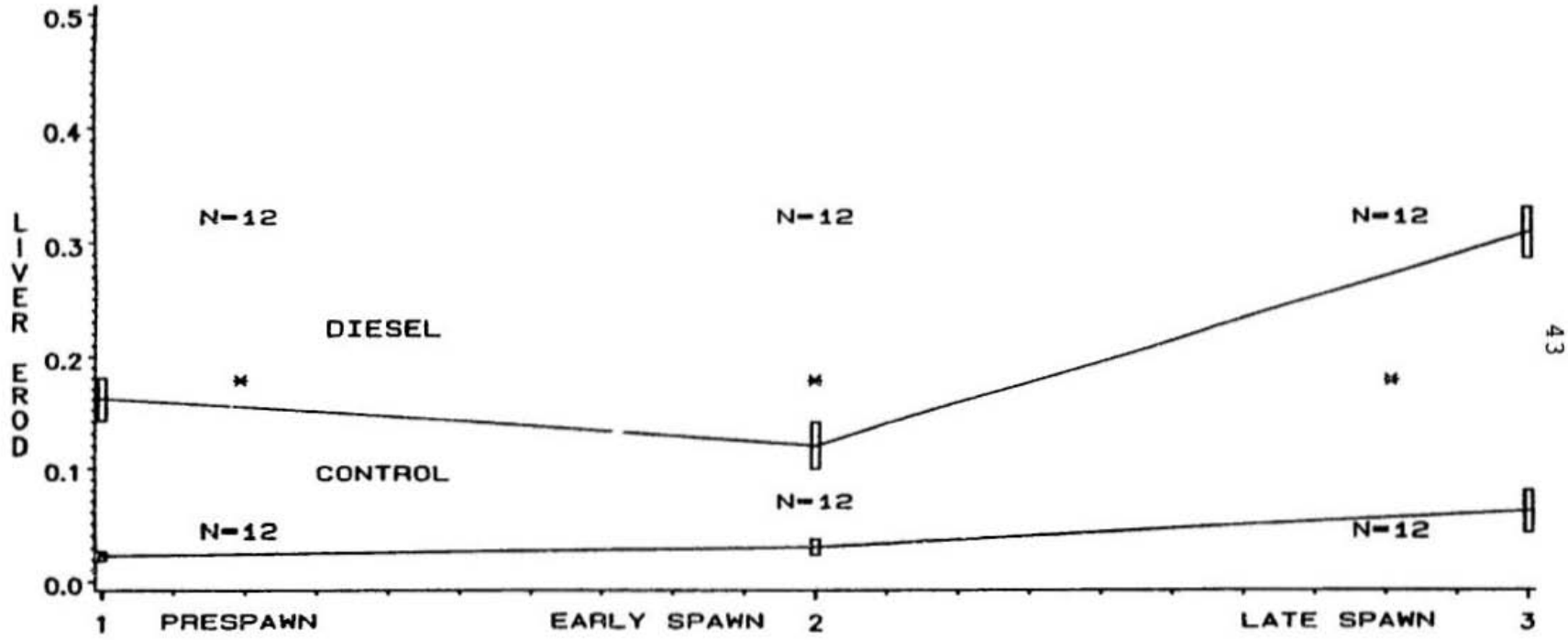
The generation of p-values by one-way ANOVA for the determination of sex differences in the ability of cunners to deethylate 7-ethoxyresorufin are shown in Table 3.2. Sex differences in gill and heart EROD were not significant throughout the reproductive season. However, induction

Figure 3.1 : Male liver EROD specific activity (nmol/min/mg protein) of control and diesel exposed cunners.



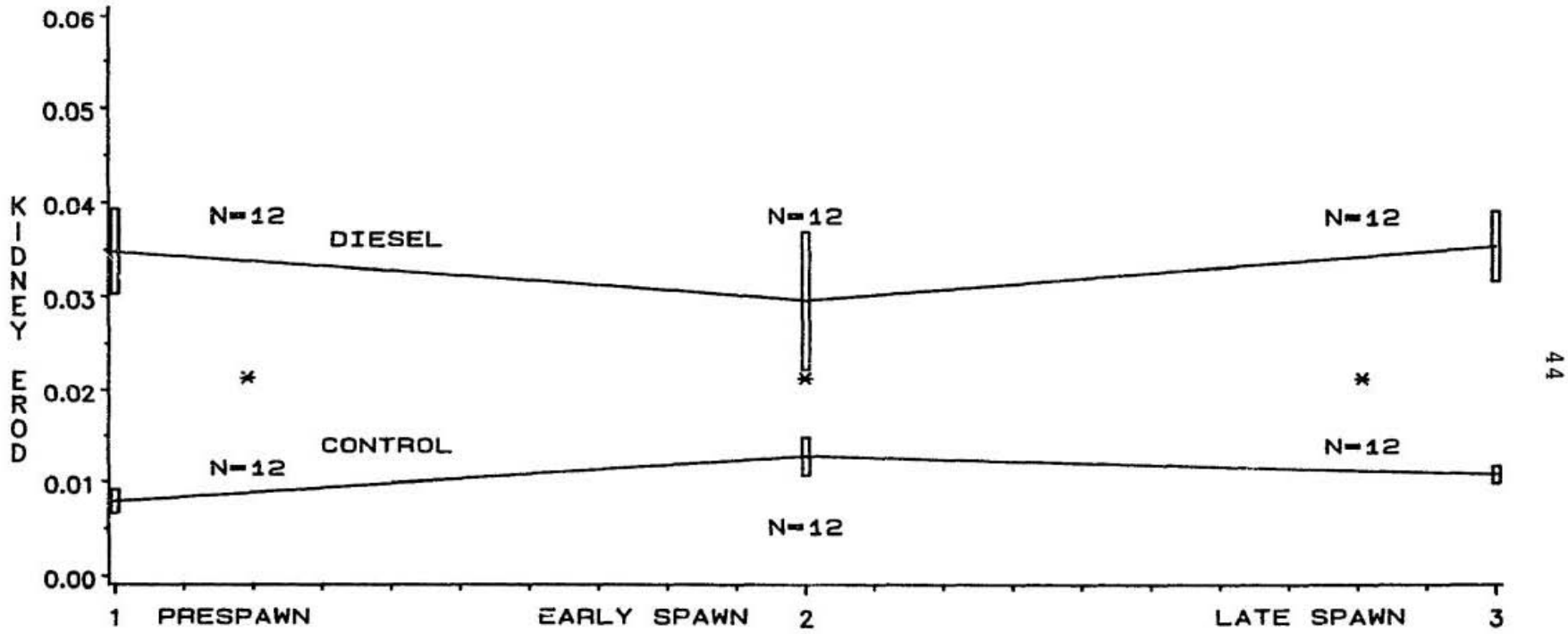
* : statistically significant (p<0.05).

Figure 3.2 :Female liver EROD specific activity (nmol/min/mg protein) of control and diesel exposed cunners.



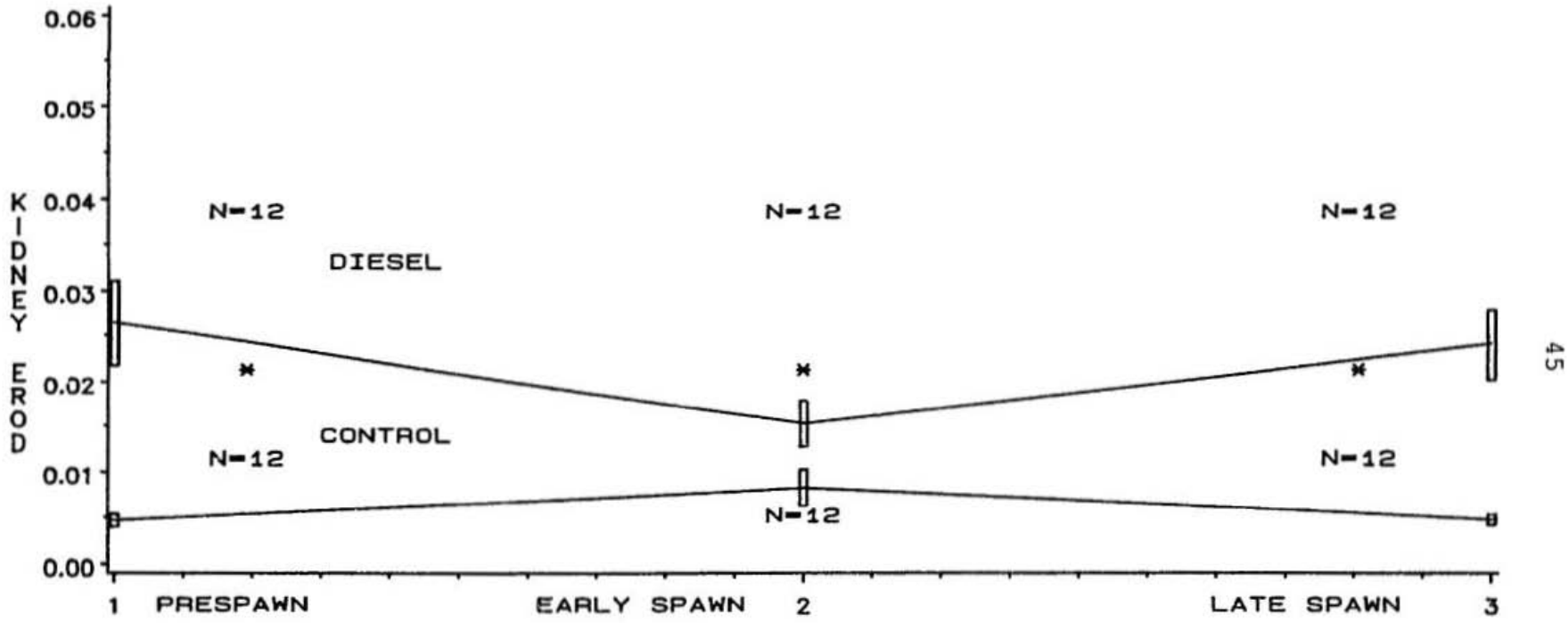
* : statistically significant (p<0.05) .

Figure 3.3 : Male kidney EROD specific activity (nmol/min/mg protein) of control and diesel exposed cunners.



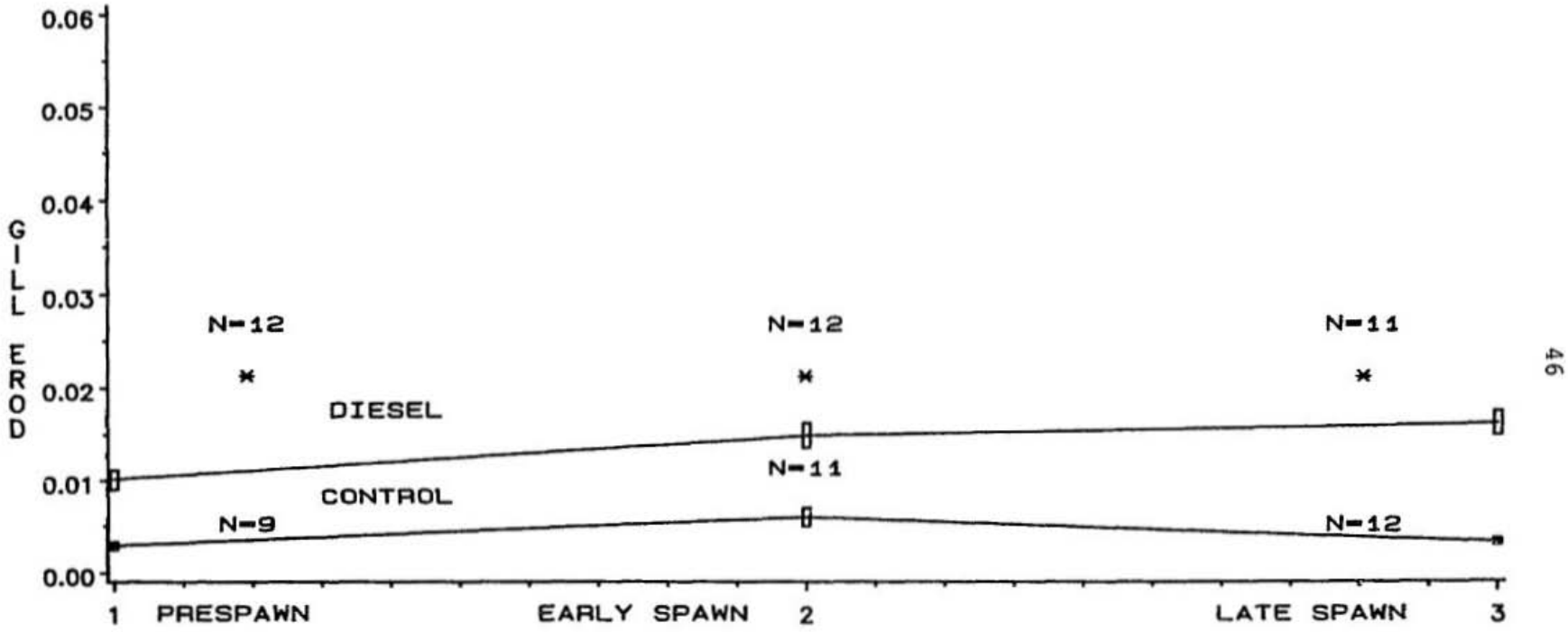
* : statistically significant (p<0.05).

Figure 3.4 : Female kidney EROD specific activity (nmol/min/mg protein) of control and diesel exposed cunners.



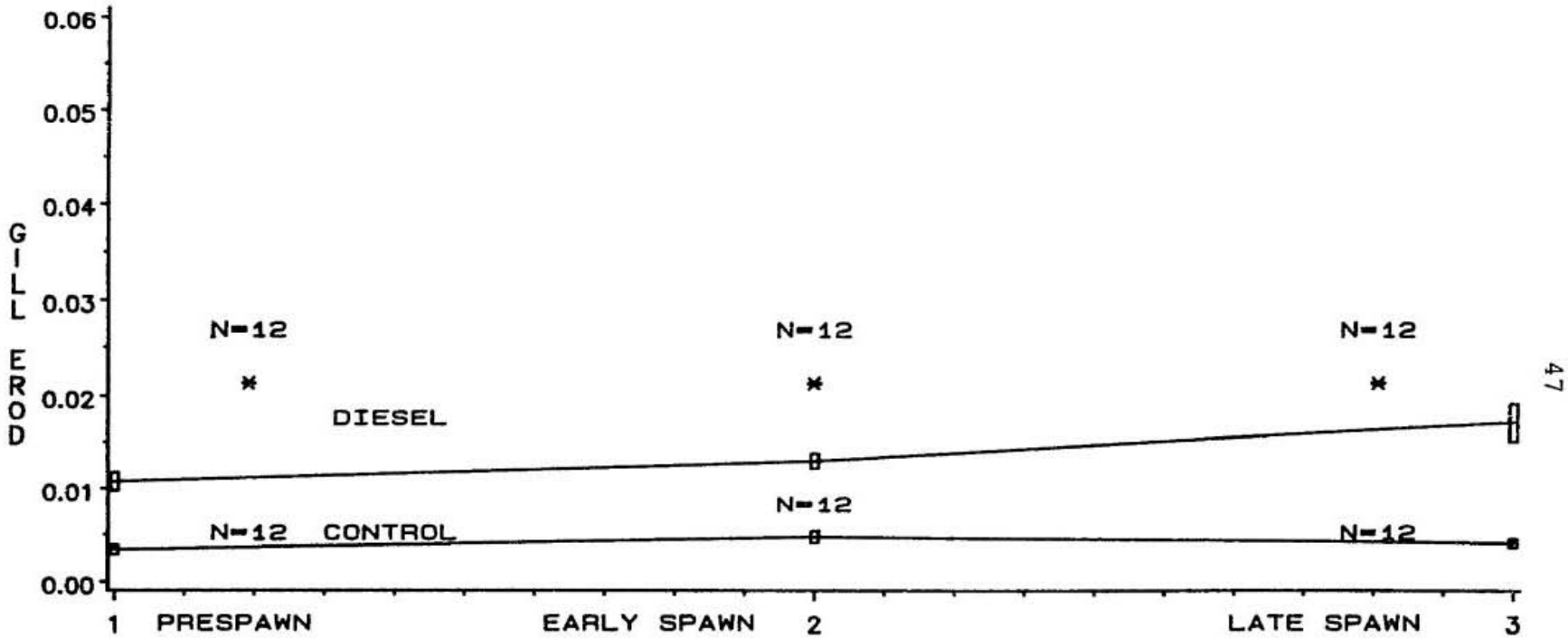
* : statistically significant (p<0.05) .

Figure 3.5 : Male gill EROD specific activity (nmol/min/mg protein) of control and diesel exposed cunners.



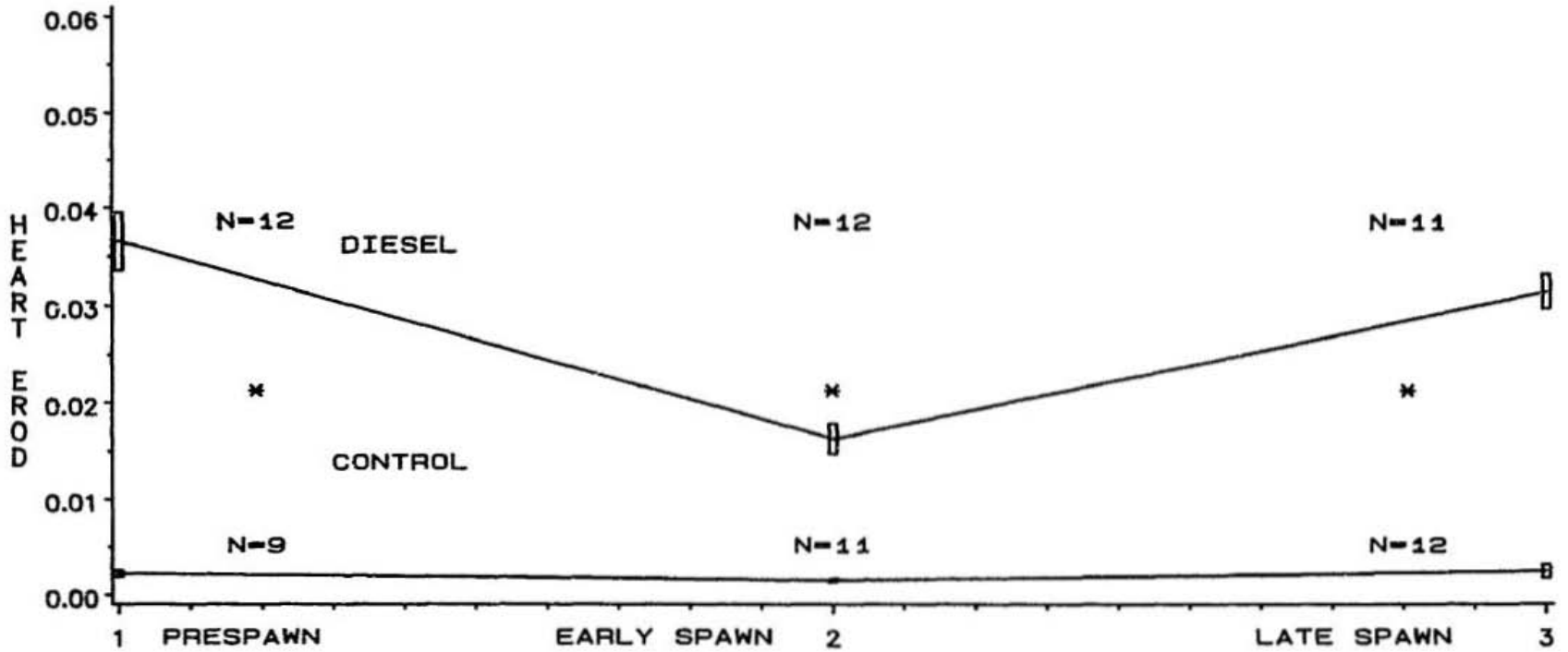
* : statistically significant (p<0.05).

Figure 3.6 : Female gill EROD specific activity (nmol/min/mg protein) of control and diesel exposed cunners.



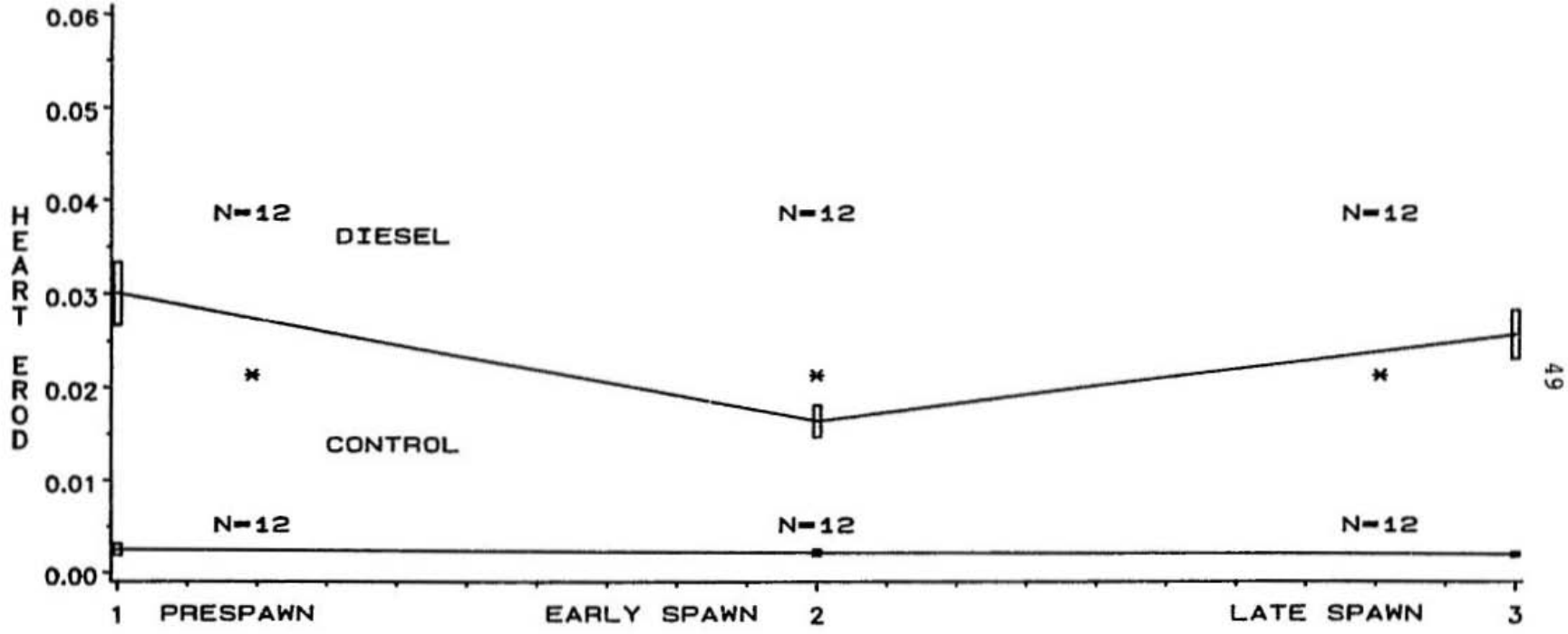
* : statistically significant (p<0.05).

Figure 3.7 : Male heart EROD specific activity (nmol/min/mg protein) of control and diesel exposed cunners.



* : statistically significant (p<0.05) .

Figure 3.8 : Female heart EROD specific activity (nmol/min/mg protein) of control and diesel exposed cunners.



* : statistically significant (p<0.05).

Table 3.1: Ethoxyresorufin O-deethylase (EROD) induction factors of cunners exposed to diesel oil.

	Male Cunners				Female Cunners			
	Liver	Kidney	Gill	Heart	Liver	Kidney	Gill	Heart
Prespawn	6	7	3	18	6	5	3	13
Early spawn	5	5	2	12	4	5	2	7
Late spawn	4	5	5	15	5	4	4	16

$$\text{Induction factor} = \frac{\text{mean EROD specific activities of oil exposed cunners}}{\text{mean EROD specific activities of control cunners}}$$

Table 3.2: Level of significance of sex differences in EROD specific activities of cunners.

Sample	Treatment	Organ			
		Liver	Kidney	Gill	Heart
Prespawn	control	p<0.1023	p<0.0191	p<0.6952	p<0.9454
	diesel	p<0.0046	p<0.0121	P<0.4919	p<0.2568
Early spawn	control	p<0.4843	p<0.1297	p<0.3118	p<0.1403
	diesel	p<0.2899	p<0.0367	p<0.3718	p<0.7372
Late spawn	control	p<0.4073	p<0.0001	p<0.1064	p<0.7367
	diesel	p<0.2473	p<0.0287	p<0.7889	p<0.0584

potential in heart (Table 3.1) was higher in males versus females during prespawning (18 versus 13 fold) and late spawning (12 versus 7 fold). There were significant sex differences in liver EROD of cunners exposed to diesel during early spawning. Significant sex differences in kidney EROD were observed within both control and treated cunners throughout the reproductive season with the exception of early spawning control cunners. In each case where significant sex differences were evident, males always displayed higher EROD specific activities than females.

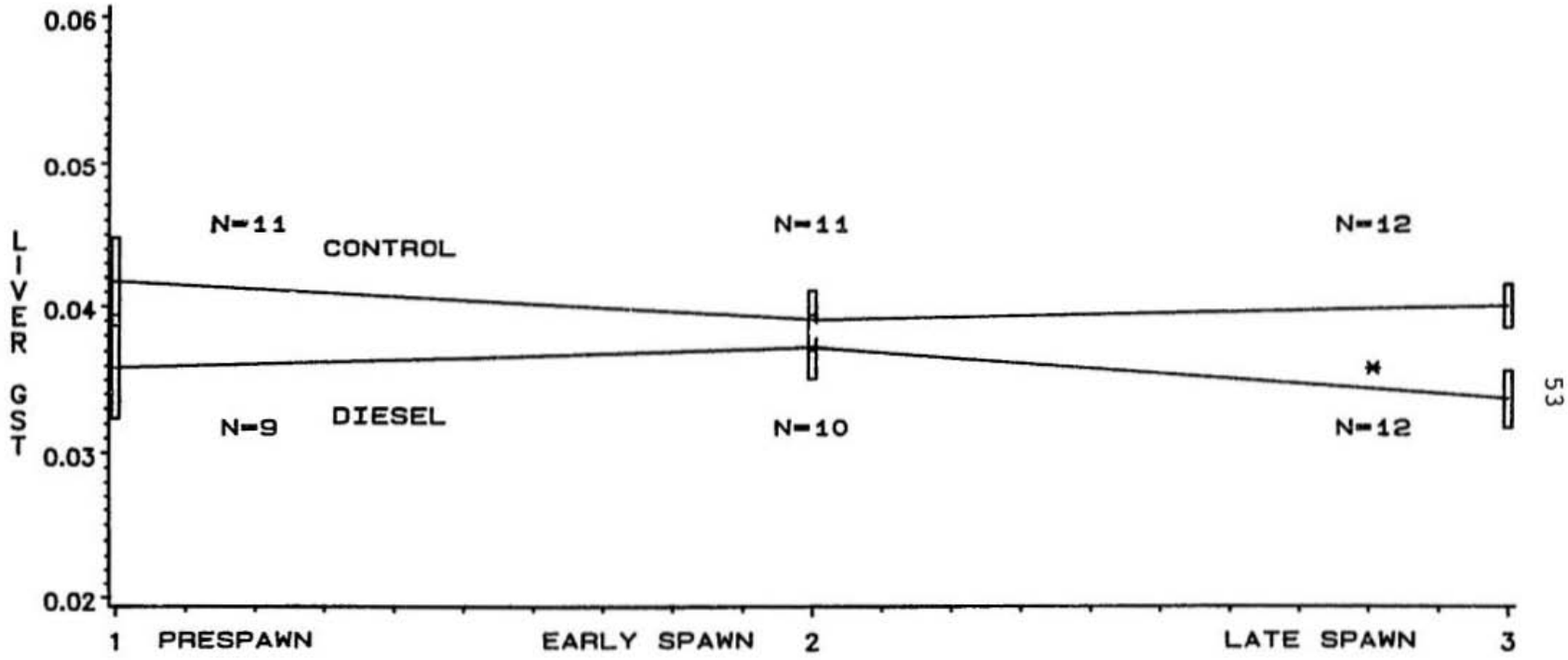
3.3 Seasonal Variability in Ethoxyresorufin O-deethylase (EROD)

Control cunners showed no significant variability in liver, kidney, gill or heart EROD ($p<0.05$) throughout the reproductive season. Also, there was no significant seasonal variability in hepatic or extrahepatic EROD specific activities of diesel treated males. Induced females also displayed negligable differences in EROD of kidney, gill and heart, whereas liver EROD was significantly higher during late spawning compared to prespawning and early spawning.

3.4 Glutathione S-transferase (GST) Inducibility.

Figures 3.9-3.16 depict the hepatic and extrahepatic GST specific activities of control and diesel exposed cunners

Figure 3.9 : Male liver GST specific activity (umol/min/mg protein) of control and diesel exposed cunners.



* : statistically significant (p<0.05).

Figure 3.10 : Female liver GST specific activity (umol/min/mg protein) of control and diesel exposed cunners.

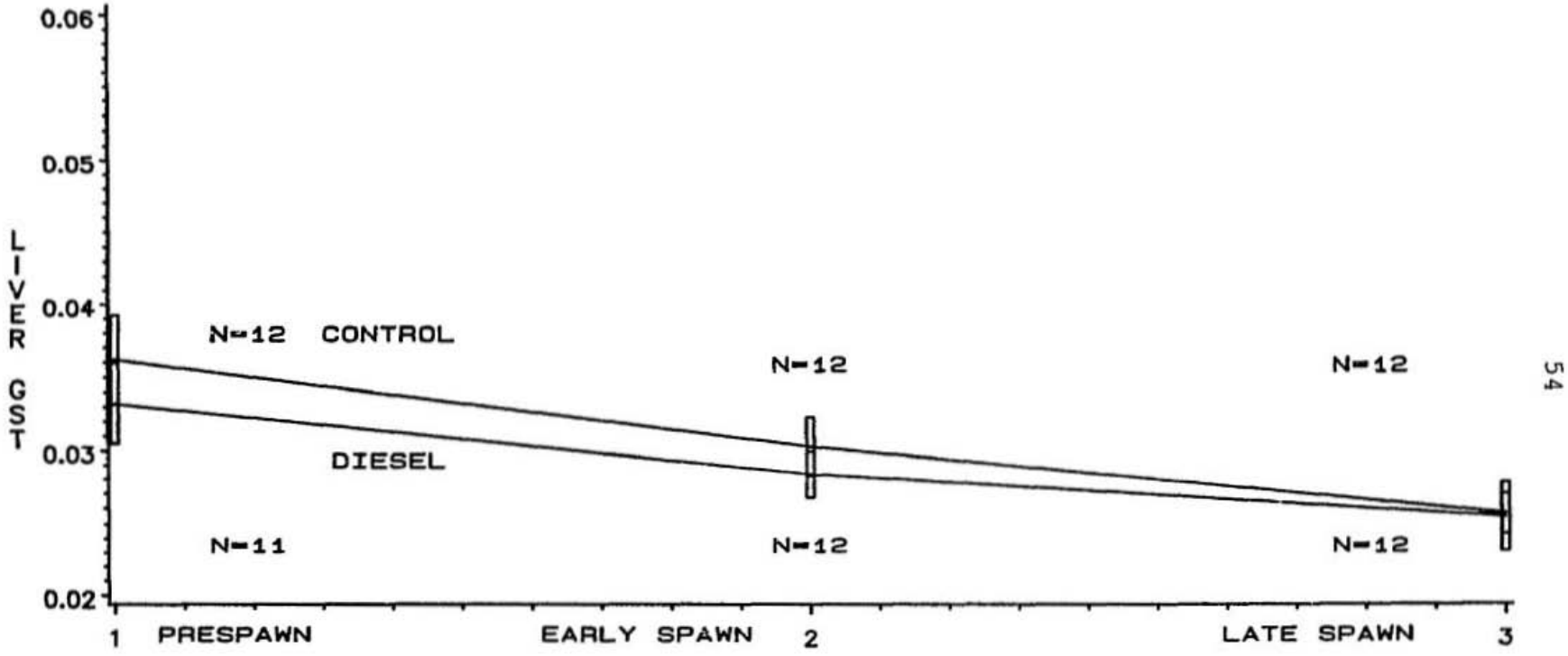
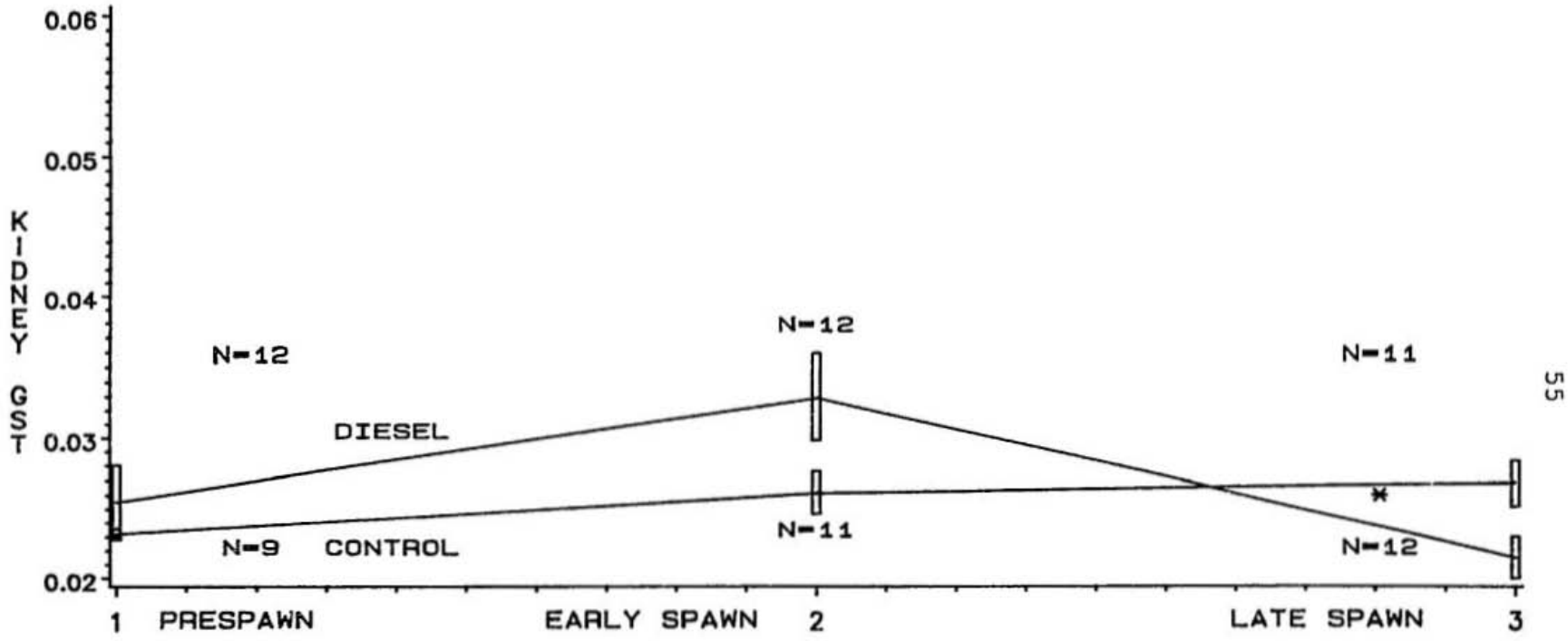


Figure 3.11 : Male kidney GST specific activity (umol/min/mg protein) of control and diesel exposed cunners.



* : statistically significant (p<0.05) .

Figure 3.12 : Female kidney GST specific activity (umol/min/mg protein) of control and diesel exposed cunners.

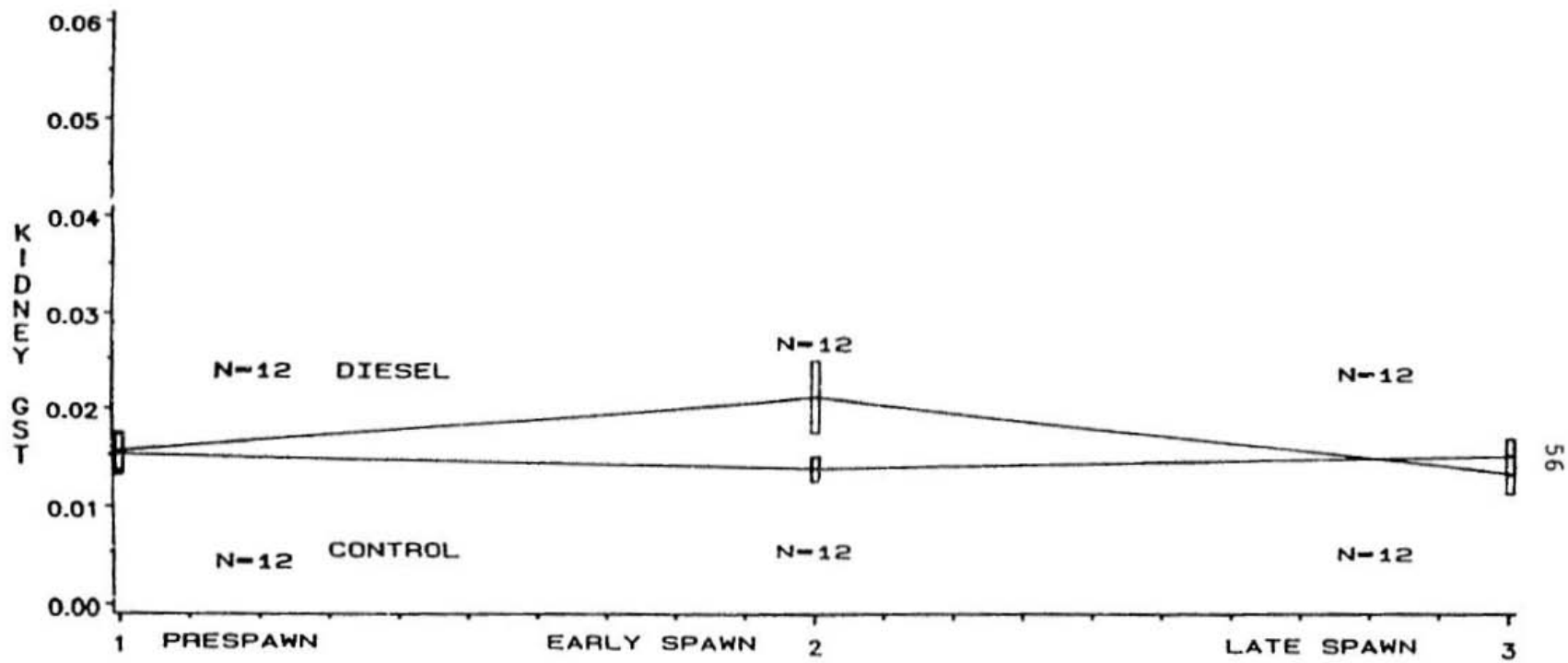


Figure 3.13 : Male gill GST specific activity (umol/min/mg protein) of control and diesel exposed cunners.

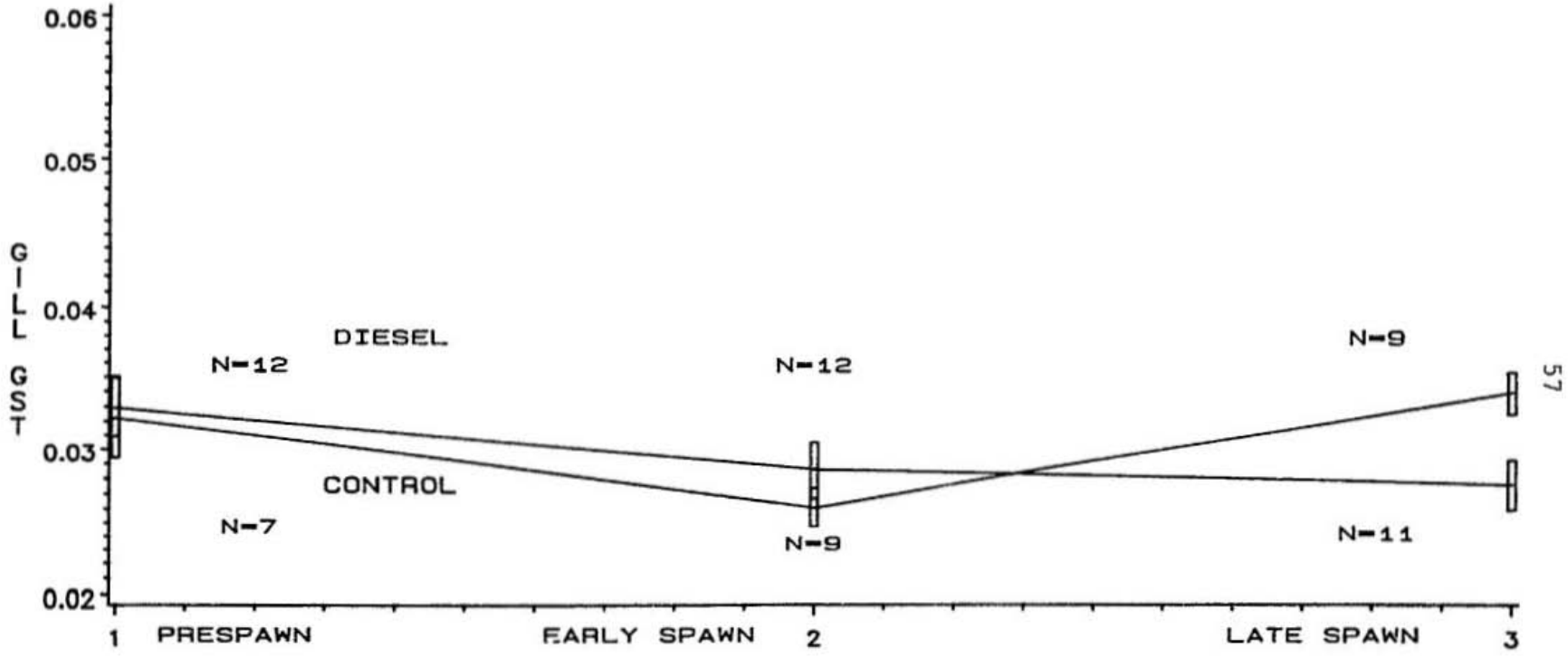
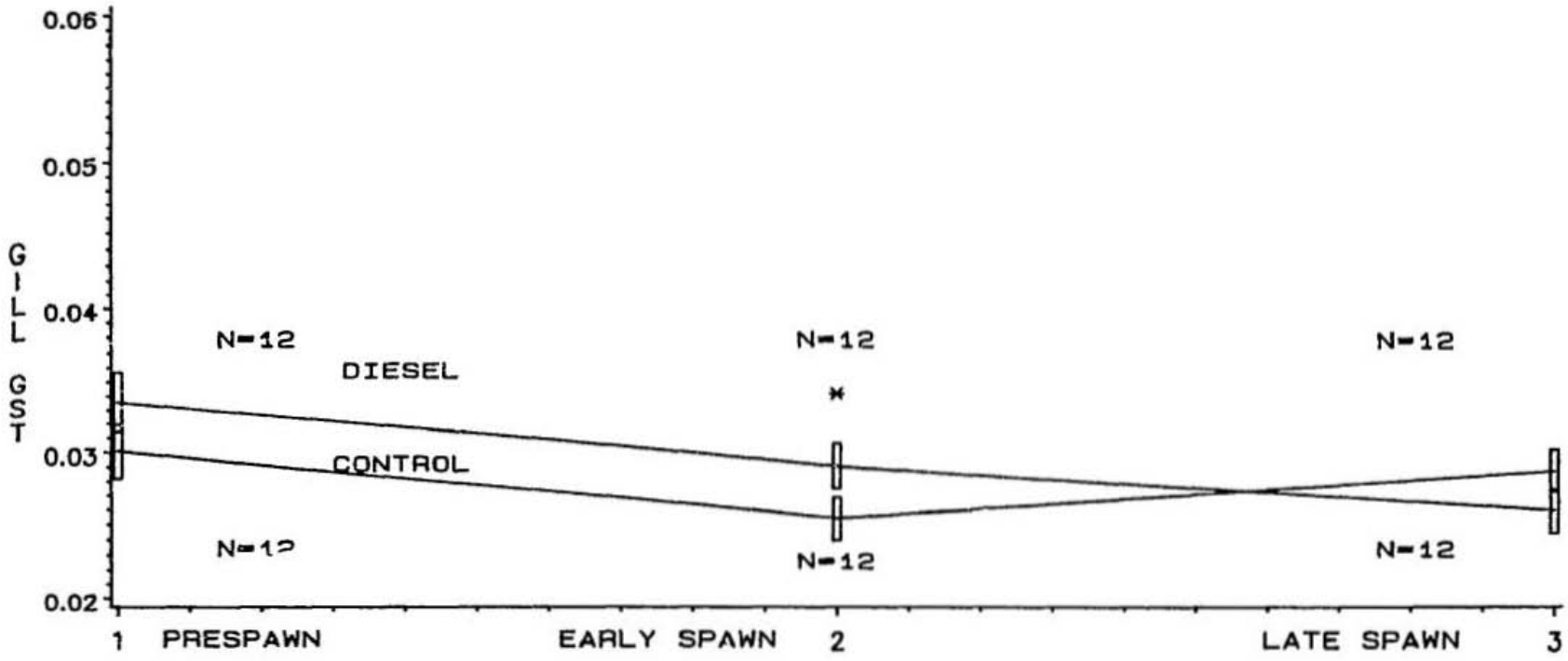
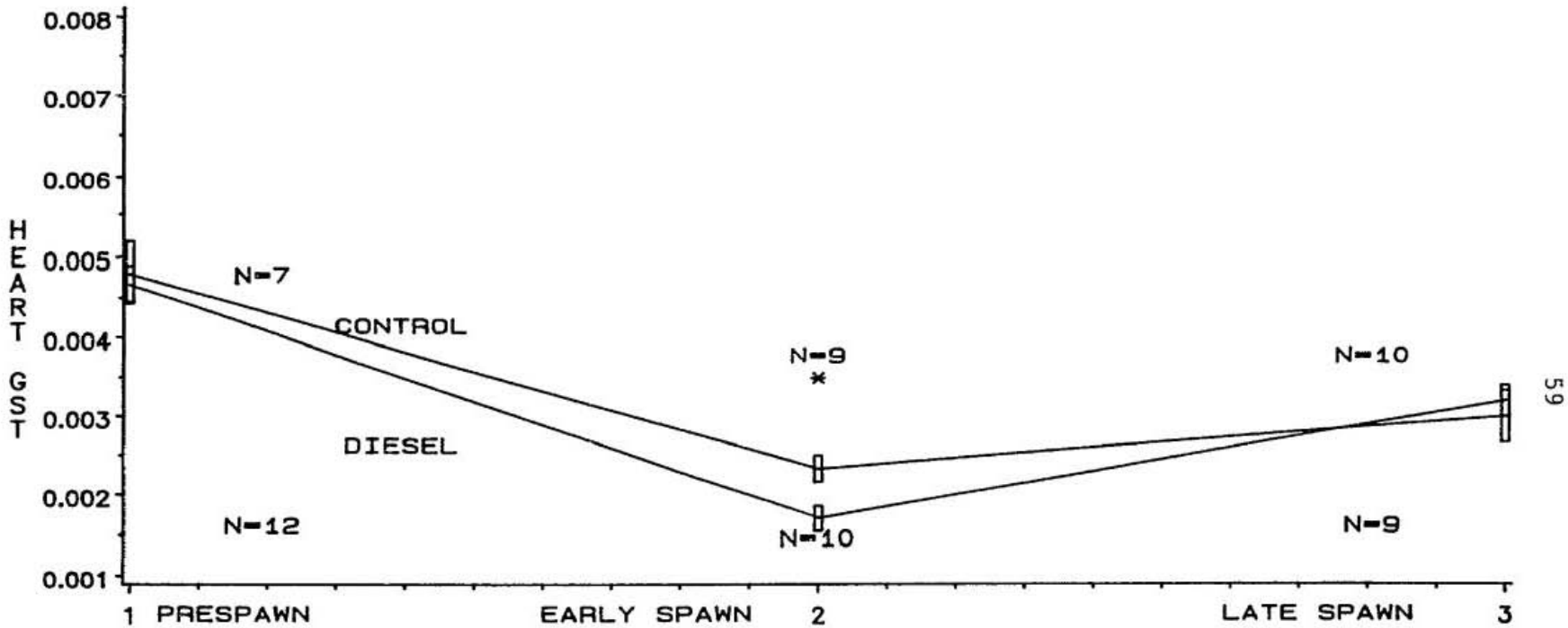


Figure 3.14 : Female gill GST specific activity (umol/min/mg protein) of control and diesel exposed cunners.



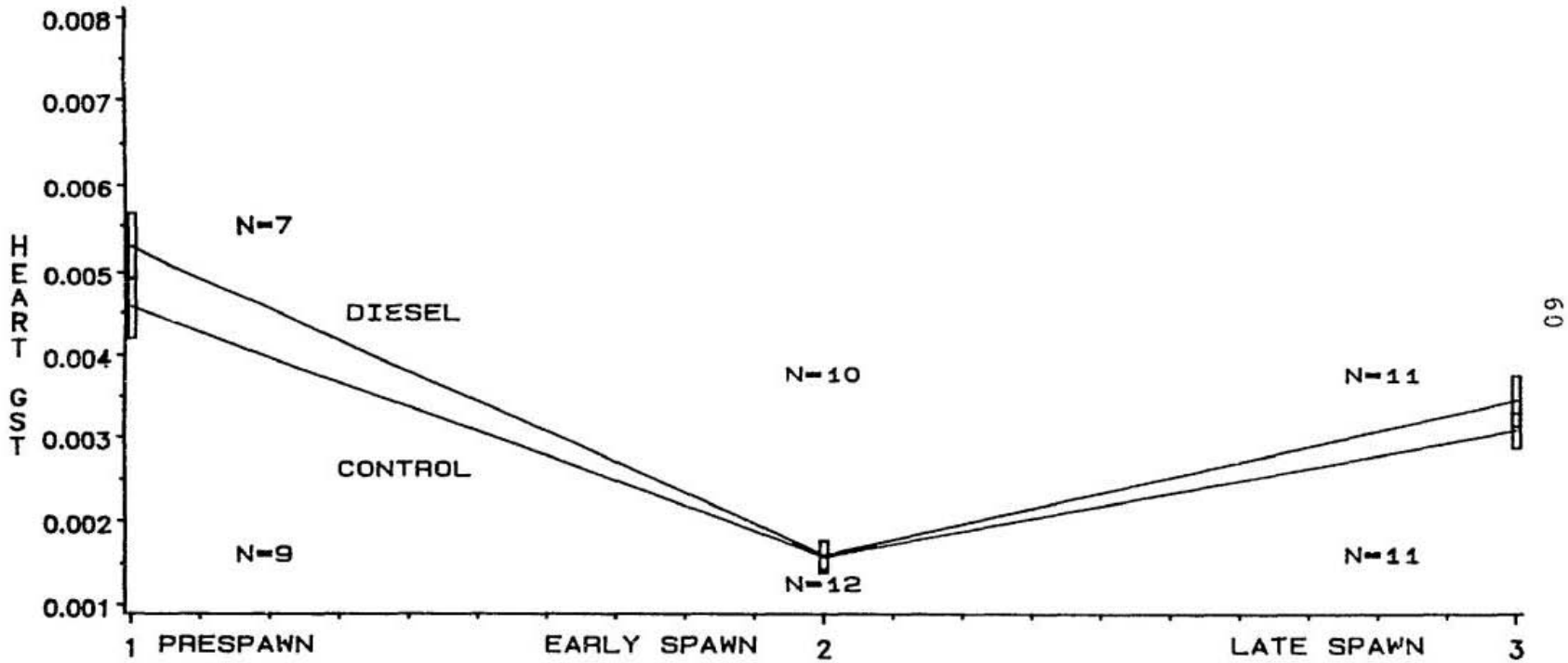
* : statistically significant (p<0.05).

Figure 3.15 : Male heart GST specific activity (umol/min/mg protein) of control and diesel exposed cunners.



* : statistically significant (p<0.05) .

Figure 3.16 : Female heart GST specific activity (umol/min/mg protein) of control and diesel exposed cunners.



during the three experimental trials. Asterisks indicate significant treatment differences in the enzyme activity. Unlike EROD, GST was not induced in the fish exposed to no. 2 fuel oil. There were some significant, but marginal, differences between control and diesel exposed cunners. However, no special trend was apparent enabling distinction between GST specific activities of control and experimental fish throughout the reproductive season.

3.5 Sex Differences in Glutathione S-transferase (GST)

Table 3.3 indicates the level of significance for sex differences in GST specific activity. Although significant differences were seen, these differences were small and did not constitute a clear trend in any tissue. The sex with the higher GST specific activities is shown below the p-values generated by one-way ANOVA.

3.6 Seasonal Variability in Glutathione S-transferase (GST).

Similar to EROD, seasonal variability in gill and heart GST specific activities was not significant. The only significant change was in kidney GST which was lower in control males during early spawning relative to pre-spawning and late spawning. Also, liver GST in control and experimental females varied significantly throughout the reproductive season with enzyme activities lower during

Table3.3: Level of significance of sex differences in GST specific activities of cunners.

Sample	Treatment	Organ			
		Liver	Kidney	Gill	Heart
Prespawn	control	p<0.0723	p<0.0150 (female)	p<0.3234	p<0.7455
	diesel	p<0.1649	p<0.1595	p<0.5748	p<0.1558
Early spawn	control	p<0.0088 (male)	p<0.0116 (male)	p<0.4891	p<0.0087 (male)
	diesel	p<0.0003 (male)	p<0.0528 (male)	p<0.7044	p<0.6601
Late spawn	control	p<0.0001 (male)	p<0.0011 (female)	p<0.1728	p<0.6771
	diesel	p<0.0003 (male)	p<0.0783	p<0.5191	p<0.6398

late spawning relative to the two previous periods. Even though differences in GST were statistically significant in some cases, the absolute differences were quite small.

3.7 Polycyclic Aromatic Hydrocarbon (PAH) Levels in Cunner Liver

The levels of unsubstituted PAH in liver tissues of cunners during the early spawning period are listed in Table 3.4. Naphthalene equivalents were higher than the other PAH in both control and diesel exposed fish. There were no significant differences in PAH levels between male control and experimental cunners. Experimental females had significantly higher levels of naphthalene and fluorene equivalents relative to control females. No other significant differences in PAH levels between treatments were evident.

There were no significant sex differences in PAH levels between experimental males and females. Only one PAH, fluorene, was found to be significantly higher in control males relative to control females. Table 3.5 list p-values for sex differences within control and diesel exposed cunners.

Table 3.4: Levels of PAH in cunner liver tissues during early spawning.

		[b]PAH units/mg lipid				
Sex	Treatment	Naphthalene	Fluorene	Phenanthrene	Pyrene	Benzo[a]pyrene
male	control	[a]45.4±7.76 p<0.5501	4.0±0.81 p<0.9395	4.2±0.83 p<0.8519	2.1±1.76 p<0.6524	1.5±0.50 p<0.6232
	diesel	63.5±14.68	3.8±0.75	3.9±0.84	2.9±1.87	1.4±0.73
female	control	33.2±5.48 p<0.0022	2.01±0.20 p<0.0012	5.5±1.33 p<0.6384	0.3±0.34 p<0.1308	1.6±0.83 p<0.4227
	diesel	57.7±5.13	5.5±1.96	6.3±1.59	3.3±1.6	0.4±0.19

[a] means ± S.E.

[b] 1 PAH unit = 1 µg/ml = 1 ppm

Table 3.5: Level of significance of sex differences in PAH within control and diesel exposed cunners during early spawning.

	PAH Equivalents				
Treatment	Naphthalene	Fluorene	Phenanthrene	Pyrene	Benzo[a]pyrene
Control	P<0.2442	p<0.0480	p<0.8785	P<0.0961	p<0.4813
Diesel	p<0.4836	p<0.4750	p<0.3959	p<0.7447	p<0.2930

3.8 Correlations Between Enzyme Specific Activities and Body Characteristics

Correlation coefficients (R-values) of enzyme-enzyme interaction and enzyme-body characteristic interaction for cunners during the reproductive season are presented in Tables A.2-A.13 of the Appendix. Although a number of significant correlations are seen within each cunner group, no obvious trend was observed throughout gonad maturation/spawning.

4. DISCUSSION

4.1 Mixed Function Oxygenase (MFO) in the Cunner: Induction, Sex Differences and Seasonal Variability During Gonad Maturation/Spawning.

Fish are known to display a multiplicity of cytochrome P-450's (Elmamlouk et al., 1977). Hepatic and extrahepatic organs of fish are known to exhibit MFO enzyme activities (Stegeman, 1980). Induction in fish, however, appear to be more limited to P-448 type inducers. Inducers include important environmental contaminants such as PAH's, PCB's, and complex petroleum products (Addison et al., 1981; Payne and Penrose, 1975; Walton et al., 1978). Field trials have demonstrated the usefulness of MFO induction as an indicator of early biological effects due to these pollutants (reviewed by Payne, 1987). However, the liver may be refractory to induction during the reproductive period and studies were carried out to investigate the induction potential of extrahepatic as well as hepatic tissues in cunners during the summer reproductive period.

The cunner was a suitable test species for this experiment because of its contracted gonad maturation/spawning period relative to such species as Atlantic cod (Templeman, 1976) or winter flounder

(Pleuronectes americanus) (Fletcher and King, 1978). This work successfully demonstrated the ability to resolve ethoxyresorufin O-deethylase (EROD) induction in liver, kidney, gill, and heart tissues of cunners in all experimental trials when exposed to diesel oil. Also, constitutive levels of EROD did not vary significantly throughout the reproductive season in hepatic or extrahepatic tissues. Sex differences in EROD were observed in liver and kidney tissues during certain periods of gonad maturation/spawning. In each case, males had higher EROD specific activities than females.

Most investigations on the inducibility of MFO enzymes in aquatic biota have focussed on fish liver (Addison, 1984). This may be a more practical organ for monitoring since it has been established as the major detoxification tissue of animals. Mammalian liver generally contains the highest concentrations of enzymes involved in xenobiotic metabolism (Bend and Singh, 1984), and preparation of liver fractions are easier to work with relative to extrahepatic organs. However, besides elucidating the importance of chemical metabolism in extrahepatic organs from a toxicological perspective, it is essential to study the potential use of biotransformation enzymes in other organs as indices of environmental pollution. The results of this thesis show EROD inducibility of kidney and gill tissues

is comparable to liver. Furthermore, heart EROD induction was even greater than liver throughout the reproductive season. Previous investigations have provided evidence that fish kidney has the capacity to execute cytochrome P-450 mediated reactions (Lindstrom-Seppa et al., 1981; Stegeman et al., 1984); other studies have revealed comparable MFO activities in liver and kidney tissues (Pesonen et al., 1985). Furthermore, field studies have shown the importance of measuring MFO in extrahepatic organs as well as liver. Flounder (with ripe gonads) collected at the site of an oil spill in Newfoundland in June exhibited no induction potential in liver tissues but marked induction in kidney tissues compared to reference sites (Payne et al., 1984). The potential for induction of MFO in gill tissue was also demonstrated in fish collected near a refinery outfall (Payne, 1976). The low constitutive EROD levels observed in heart tissues of the cunners in this study may account for the excellent induction seen in this tissue. Information on the xenobiotic metabolizing capacity of fish heart is lacking, especially with respect to the influence of reproductive status. Stegeman et al. (1982) demonstrated that the cytochrome P-450 in the heart of scup (Stenotomus chrysops) was similar to a 3-methylcholanthrene (P-448) inducible form found in rat heart. Overall, the results of this study demonstrate

a role for extrahepatic as well as hepatic MFO enzymes in biological monitoring including throughout the reproductive period.

No significant variability in EROD specific activities was noted during gonad maturation/spawning. Walton et al. (1983) found large differences in constitutive levels and induction of liver MFO (aryl hydrocarbon hydroxylase) in female and male cunners throughout the gonad maturation/spawning season. This earlier study, which was restricted to analysis of liver tissues, indicated that aryl hydrocarbon hydroxylase (AHH) may not be a sensitive indicator of exposure to petroleum hydrocarbons during intense reproductive activity. It should be noted that different MFO enzyme activities (EROD vs AHH) were evaluated in this study. The 'noise' generated by the reproductive cycle of cunners in the study conducted by Walton et al. (1983) does suggest the use of AHH, unlike the EROD assay which was specially developed for inducers like PAH's, should be scrutinized when used as a biological monitor (especially it seems during reproduction).

No significant sex differences between control and diesel exposed cunners were observed for gill or heart EROD. However, the heart EROD induction potential of male cunners was higher than female cunners during prespawning

and early spawning. No differences in heart EROD induction potential were seen during late spawning, whereas sex differences were observed in liver and kidney tissues. Within diesel exposed cunners, early spawning males had higher liver EROD specific activities compared to early spawning females. Also, kidney EROD of both male control and male diesel exposed cunners were higher than females throughout the reproductive season. The only exception in kidney EROD sex differences was in early spawning (control) cunners, where no significant sex differences were noted. An investigation concerned with natural variation in benzo-[a]pyrene hydroxylation in flounder (Platichthys flesus) revealed higher specific activities in liver tissues of males versus females throughout the year, especially during gonad maturation when MFO activity appeared to be inhibited in the females (Tarlebo et al., 1985). In another study, liver MFO (benzo[a]pyrene, 7-ethoxycoumarin, and 7-ethoxyresorufin as substrates) of the freshwater vendace (Coaregonus albula) varied during the seasons with lowest levels being detected prior to and during spawning, but these levels were consistantly higher in males relative to females (Lindstrom-Seppa, 1985). Stegeman and Chevion (1980) found a higher cytochrome P-450 content in male brook trout and rainbow trout relative to females during spawning, yet benzo[a]pyrene hydroxylase activity was higher in females.

Different ratios of androgens have been found in the plasma of both sexes of rainbow trout (Campbell et al., 1980) and during prespawning, females have higher concentrations of plasma estradiol-17-β levels (Whitehead et al., 1978). Growing evidence supports the idea that sex steroids may play a major role in regulating MFO activities in fish species (Forlin et. al., 1984; Hansson and Gustafsson, 1981). Furthermore, sex-specific cytochrome P-450's have been isolated from rat liver (Dannan et. al., 1986). Circulating steroids and sex specific cytochromes P-450 may be postulated to help explain seasonal and sex-linked differences in MFO enzymes.

Analytical procedures have traditionally been an integral aspect of environmental monitoring programs. Besides analytical determination of chemical concentrations in the aquatic environment, it is also important to measure tissue concentrations of xenobiotics in target organisms. Biological effects of chemicals are a manifestation of biological concentrations and not environmental concentrations (Tan and Singh, 1987). The importance of measuring a sublethal response such as MFO induction, in addition to chemical analysis, is clearly seen in this thesis when the sensitivities of both approaches are compared. Determination of total PAH levels in cunner liver does not easily discriminate between control fish

and diesel exposed fish. When EROD induction is used as a sole indicator of petroleum exposure, control fish are readily distinguished from diesel exposed fish. Thus, incorporation of a biomonitor such as MFO induction in environmental monitoring programs is an essential complement to analytical methods.

In summary, EROD induction was readily resolved in male and female cunners in both hepatic and extrahepatic tissues throughout the reproductive season. Some sex-linked differences were apparent in EROD of liver, kidney and heart. These studies support the versatility of MFO enzyme induction as a sensitive biological monitoring tool.

4.2. Glutathione S-transferase (GST) in the Cunner: Induction, Sex Differences, and Seasonal Variability During Gonad Maturation/Spawning.

One major role of reduced glutathione (GSH) in mammals is the direct conjugation of xenobiotics. Conjugation may be spontaneous or achieved by a group of cytosolic/ microsomal enzymes known as glutathione S-transferases (GST) (Igwe, 1986) found in mammalian and non-mammalian species (Morgenstern et al., 1984). In this study, the potential for GSH S-transferase induction in cunners was also investigated during the gonad maturation/spawning period.

The results of this study indicate that male and female cunners are refractory to GST induction when exposed to No. 2 fuel oil during the reproductive season. It is difficult to relate these observations to other investigations due to species differences, physiological differences and differing methodologies. Induction of hepatic GSH S-transferase specific activity has been shown to occur in rats exposed to PAH-type compounds. Intraperitoneal injection of 3-methylcholanthrene or benzo[a]pyrene significantly induced GST activities in female and male rats (Kaplowitz et al., 1975) with higher enzyme activity being observed in males. Conflicting reports in the literature on the inducibility of GST in fish species appear to be due to species differences and variations of experimental protocol. Winter flounder administered 1, 2, 3, 4-dibenzanthracene (DBA) or 5, 6-benzoflavone (BNF) intraperitoneally displayed no significant induction in hepatic GST activity over controls (Foureman et al., 1983). In another study, BNF treated rainbow trout exhibited significantly higher hepatic GST activity relative to control fish (Andersson et al., 1985a).

No significant sex differences in GST were observed in gill tissues. Heart GST of control male cunners were significantly higher than females during early spawning. There were some variability and sexual differences in GST

specific activity of cunner liver and kidney tissues. Where sex differences were observed in liver and kidney tissues, males generally had higher GST enzyme activities than females. A number of investigations have been conducted studying MFO sexual dimorphism in fish (Stegeman and Chevion, 1980; Koivusaari et al., 1984; Williams et al., 1986), yet information on sex differences in enzyme-catalyzed conjugation is lacking. There are some reports on sex differences in mammalian GST. Examination of five strains of mice indicated a ten-fold higher hepatic GST content in males compared to females (Hatayama et al., 1986). Kaplowitz et al. (1975) demonstrated higher hepatic GST specific activities in male rats than female rats. Testosterone has been shown to influence the levels of GST in mouse liver (Hatayama et al., 1986) and this steroid is postulated to be an important developmental regulator of GST in mice. The postulated control of MFO in mammals by androgen "imprinting" (Gustafsson et al., 1983) may also be important in the expression of sex-specific GST in mammals and fish. Sex differences seen in kidney and liver GST of cunners may be related to sex-specific enzymes and circulatory steroids.

The relationship between the biological oxidation of drugs/chemicals and subsequent conjugation in mammals implies that the higher the detoxifying/toxifying enzyme

ratio the more protection afforded against xenobiotic toxicity (Conney, 1985). Several studies with fish have also supported this premise. Starry flounder appear to have a lower incidence of hepatocarcinoma than English sole in areas of Puget Sound (Collier and Varanasi, 1986) which are known to be heavily polluted with aromatic hydrocarbons. Field studies have revealed that English sole exhibit substantially higher levels of AHH (activation enzyme) and lower levels of GST (deactivating enzyme) activities than do the nonsusceptible starry flounder (Collier and Varanasi, 1986). Another investigation with the same two species showed higher increases in hepatic DNA adduct formation in English sole compared to starry flounder when exposed to equal doses of benzo[a]pyrene (Varanasi et al., 1987). Even though the cunners were refractory to GST induction, the high MFO induction potential implies that this species may be very susceptible to aquatic pollutants, especially during the sensitive reproductive stages. However, the results of this study suggests that GST has little potential as a biomonitor for hydrocarbon pollution.

CONCLUSIONS

1. Mixed function oxygenase induction, measured as ethoxyresorufin O-deethylase (EROD) was readily resolved in hepatic and extrahepatic tissues of cunners throughout the reproductive season when exposed to diesel oil.
2. Male EROD was generally higher than female EROD in hepatic and extrahepatic tissues throughout gonad maturation/spawning.
3. Heart EROD had a higher induction potential than liver, kidney and gill EROD in cunners throughout the reproductive season.
4. GST was not inducible in hepatic or extrahepatic tissues of cunners throughout gonad maturation/spawning.
5. Unlike EROD induction, which was observed in all tissues at all sampling periods in fish exposed to petroleum, GST was demonstrated to have little potential as a biomonitor for hydrocarbon pollution.
6. Except for a few incidental correlations among EROD, GST and the body characteristics, no meaningful relationships were apparent.

REFERENCES

Adamson, R.H. (1967): Drug metabolism in marine vertebrates. Fed. Proc. 26: 1047-1055.

Addison, R.F. (1984): Hepatic mixed function oxidase (MFO) induction in fish as a possible biological monitoring system. pp. 51-59 in Contaminant Effects on Fisheries, eds., V.W. Cairns, P.V. Hodson, J.O. Nriagu; J. Wiley & Sons Inc.

Addison, R.F., D.E. Willis (1982): Variation of hepatic ethoxycoumarin O-deethylase activity with body weight and other factors in brook trout (Salvelinus fontinalis). Can. J. Fish. Aquat. Sci. 39: 924-926.

Addison, R.F., M.E. Zinck, D.E. Willis (1981): Time- and dose-dependence of hepatic mixed function oxidase activity in brook trout Salvelinus fontinalis on polychlorinated biphenyl residues: Implications for 'biological effects monitoring'. Environ. Pollution. 25A: 211-218.

Ahmad, S. (1979): The functional roles of cytochrome P-450 mediated systems: present knowledge and future areas of investigations. Drug Metab. Rev. 10: 1-14.

Ahokas, J.T., O. Pelkonen, N.T. Kärki (1977): Characterization of benzo[a]pyrene hydroxylase of trout liver. Cancer Res. 37: 3737-3743.

Andersson, T., U. Koivusaari (1985): Influence of environmental temperature on the induction of xenobiotic metabolism by β-naphthoflavone in rainbow trout, Salmo gairdneri. Toxicol. Appl. Pharmacol. 80: 43-50.

Andersson, T., U. Koivusaari (1986): Oxidative and conjugative metabolism of xenobiotics in isolated liver cells from thermally acclimated rainbow trout. Aquat. Toxicol. 8: 85-92.

Andersson, T., U. Koivusaari, L. Förlin (1985a): Xenobiotic biotransformation in the rainbow trout liver and kidney during starvation. Comp. Biochem. Physiol. 82C: 221-225.

Andersson T., M. Pesonen, C. Johansson (1985b): Differential induction of cytochrome p-450-dependent monooxygenase, epoxide hydrolase, glutathione transferase and UDP glucuronosyl transferase activities in the liver of the rainbow trout by β-naphthoflavone or Clophen A50. Biochem. Pharm. 34: 3309-3314.

Ankley, G.T., R.E. Reinert, A.E. Wade, R.A. White (1985): Temperature compensation in the hepatic mixed-function oxidase system of bluegill. Comp. Biochem. Physiol. 81C: 125-129.

Ankley, G.T., V.S. Blazer, R.E. Reinert, M. Agosin (1986): Effects of Aroclor 1254 on cytochrome P-450-dependent monooxygenase, glutathione S-transferase, and UDP-glucuronosyl-transferase activities in channel catfish liver. Aquat. Toxicol. 9: 91-103.

Balk, L., S. Månér, A. Bergstrand, W. Birberg, Å. Pilotti, J.W. DeJierre (1985): Preparation and characterization of subcellular fractions from the head kidney of the northern pike (Esox lucius), with particular emphasis on xenobiotic-metabolizing enzymes. Biochem. Pharm. 34: 789-802.

Bend, J.R., M.O. James (1978): Xenobiotic metabolism in marine and freshwater species. pp. 125-188 in Biochemical and Biophysical Perspectives in Marine Biology. eds. D.C. Malins, J.R. Sargent. Academic Press, New York.

Bend, J.R., C.J. Serabjit-Singh (1984): Xenobiotic metabolism by extrahepatic tissues: relationship to target organ and cell toxicity. pp. 99-136 in Drug Metabolism and Drug Toxicity. eds. J.R. Mitchell, M.G. Horning. Raven Press, New York.

Binder, R.L., J.J. Stegeman (1984): Microsomal electron transport and xenobiotic monooxygenase activities during the embryonic period of development in the killifish, Fundulus heteroclitus. Toxicol. Appl. Pharmacol. 73: 432-443.

Björkhem, I. (1977): Rate limiting step in microsomal cytochrome P-450 catalyzed hydroxylations. Pharmacol. Ther. A.1: 327-348.

Bligh, E.G., W.J. Dyer (1959): A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37: 911-917.

Boyer, T.D., D.A. Vessey, E. Kempner (1986): Radiation inactivation of microsomal glutathione S-transferase. J. Biol. Chem. 261: 16963-16968.

Boyland, E., L.F. Chasseaud (1969): The role of glutathione and glutathione S-transferases in mercapturic acid biosynthesis. Advances in Enzymology 32: 173-219.

Braddon, S.A., C.M. McIlvaine, J.E. Balthrop (1985): Distribution of GSH and GSH cycle enzymes in black sea bass (Centropristis striata). Comp. Biochem. Physiol. 80B: 213-216.

Brattsten, L.B. (1979): Ecological significance of mixed-function oxidations. Drug Metab. Rev. 10: 35-58.

Britvić, S., M. Protić, M. Rijavec, R.K. Zahn, B. Kurelec (1983): Detection of xenobiotics in the northern Adriatic waters by estimating their biochemical effects in fish. Rapp. Comm. int. Mer Mèdit. 28: 7.

Brown, R.R., J.A. Miller, E.C. Miller (1954): The metabolism of methylated aminoazodyes. IV. Dietary factors enhancing demethylation in vitro. J. Biol. Chem. 209: 211-222.

Burns, K.A. (1976): Microsomal mixed function oxidases in an estaurine fish, Fundulus heteroclitus, and their induction as a result of environmental contamination. Comp. Biochem. Physiol. 53B: 443-446.

Burke, M.D., R.T. Mayer (1974): Ethoxyresorufin: direct fluorimetric assay of a microsomal O-dealkylation which is preferentially inducible by 3-methylcholanthrene. Orug. Metab. Disp. 2: 583-588.

Burke, M.D., S. Orrenius (1979): Mixed function oxidase activities from mammalian extrahepatic tissues. Pharm. Ther. 7: 549-599.

Butler, D.G. (1973): Structure and function of the adrenal gland of fishes. Amer. Zool. 13: 839-879.

Campbell, C.M., A. Fostier, B. Jalabert, B. Truscott (1980): Identification and quantification of steroids in the serum of rainbow trout during spermiation and oocyte maturation. J. Endocrinol. 85: 371-378.

Casillas, E., M.S. Myers, L.D. Rhodes, B.B. McCain (1985): Serum chemistry of diseased English sole, Parophys vetulus Girard from polluted areas of Puget Sound, Washington. J. Fish. Diseases 8: 437-449.

Chan, T.M., J.W. Gillett, L.C. Terriere (1967): Interaction between microsomal electron transport systems of trout and male rat in cyclodiene epoxidation. Comp. Biochem. Physiol. 20: 731-742.

Collier, T.K., U. Varanasi (1988): Xenobiotic metabolizing enzymes in two species of benthic fish, showing different prevalences of hepatic neoplasms in Puget Sound, Washington. Aquat. Toxicol. 11: 400-401.

Conney, A.H. (1967): Pharmacological implications of microsomal enzyme induction. Pharm. Rev. 19: 317-366.

Conney, A.H. (1982): Induction of microsomal enzymes by foreign chemicals and carcinogenesis by polycyclic aromatic hydrocarbons: G.H.A. Clowes Memorial Lecture. Cancer Res. 42: 4875-4917.

Conney, A.H., E.C. Miller, J.A. Miller (1956): The metabolism of methylated aminoazodyes. V. Evidence for induction of enzyme synthesis in the rat by 3-methylcholanthrene. Cancer Res. 16: 450-459.

Dannan, G.A., F.P. Guengerich, D.J. Waxman (1986): Hormonal regulation of rat liver microsomal enzymes. Role of gonadal steroids in programming, maintenance and suppression of Δ^4-steroid 5α-reductase, flavin-containing monooxygenase, and sex-specific cytochromes P-450. J. Biol. Chem. 261: 10728-10735.

Davies, J.M., J.S. Bell, C. Houghton (1984): A comparison of the levels of hepatic aryl hydrocarbon hydroxylase in fish caught close to and distant from North Sea Oil Fields. Mar. Envir. Res. 14: 23-45.

Dewaide, J.H., P.Th. Henderson (1968): Hepatic N-demethylation of aminopyrene in rat and trout. Biochem. Pharm. 17: 1901-1907.

Dierickx, P.J., K. De Brabander (1984): Glutathione S-transferase in aquatic macro-invertebrates as a bio-degradation source of organic micropollutants. Arch. Int. Physiol. Biochem. 92: 82-83.

Elmamlouk, T.H., R.M. Philpot, J.R. Bend (1977): Separation of two forms of cytochrome P-450 from hepatic microsomes of 1, 2, 3, 4- dibenzanthracene (DBA) pretreated little skates (Raja erinacea). Pharmacologist 19: 160.

Estabrook, R.W., A.G. Hildebrandt, J. Baron, K.J. Netter, K. Leibman (1971): A new spectral intermediate associated with cytochrome P-450 function liver microsomes. Biochem. Biophys. Res. Commun. 42: 132-139.

Fair, P.H. (1986): Interaction of benzo[a]pyrene hydroxylase in the black sea bass Centropristis striata. Arch. Environ. Contam. Toxicol. 15: 257-263.

Fletcher, G.L., M.J. King (1978): Seasonal dynamics of Cu^{+2}, Zn^{+2}, Ca^{+2}, and Mg^{+2} in gonads and liver of winter flounder (Pseudopleuronectus americanus): evidence for summer storage of Zn^{+2} for winter gonad development in females. Can. J. Zool. 56: 284-290.

Förlin, L., T. Andersson, U. Koivusaari, T. Hansson (1984): Influence of biological and environmental factors on hepatic steroid and xenobiotic metabolism in fish: interaction with PCB and β-naphthoflavone. Mar. Environ. Res. 14: 47-58.

Foureman, C.L., N.B. White Jr., J.R. Bend (1983): Biochemical evidence that winter flounder (Pseudopleuronectes americanus) have induced hepatic cytochrome P-450-dependent monooxygenase activities. Can. J. Fish. Aquat. Sci. 40: 854-865.

Foureman, G.L., J.R. Bend (1984): The hepatic glutathione transferases of the male little skate, Raja erinacea. Chem. Biol. Interact. 49: 89-103.

Fukami, J. (1984): Metabolism of several insecticides by glutathione S-transferase. pp. 223-264 in Differential Toxicities of Insecticides and Halogenated Aromatics. ed., F. Matsumura.

Gelboin, H.V. (1980): Benzo[a]pyrene metabolism, activation, and carcinogenesis: role and regulation of mixed function oxidases and related enzymes. Physiol. Rev. 60: 1107-1165.

Gustafsson, J.-Å., A. Mode, G. Norstedt, P. Skett (1983): Sex steroid induced changes in hepatic enzymes. Ann. Rev. Physiol. 45: 51-60.

Habig, W.H., W.B. Jakoby (1981): Assays for differentiation of glutathione S-transferases. Meth. Enzymol. 77: 398.

Habig, W.H., M.J. Pabst, W.B. Jakoby (1974): Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249: 7130-7139.

Hansson, T., J.-Å. Gustafsson (1981): In vitro metabolism of 4-androstene-3, 17-dione by hepatic microsomes from the rainbow trout (Salmo gairdneri): effects of hypophysectomy and oestradiol-17β. J. Endocrinol. 90: 103-112.

Hatayama, I., K. Satoh, K. Sato (1986): Developmental and hormonal regulation of the major form of hepatic glutathione S-transferase in male mice. Biochem. Biophys. Res. Comm. 140: 581-588.

Hazal, J.R. (1979): Influence of thermal acclimation on membrane lipid composition of rainbow trout liver. Am. J. Physiol. 236: 91-101.

Hodgson, E. (1979): Comparative aspects of the distribution of cytochrome P-450 dependent mono-oxygenase systems: an overview. Drug Metab. Rev. 10: 15-33.

Hollebone, B.R. (1986): Categorization of lipophilic xenobiotics by the enthalpic structure-function response of hepatic mixed-function oxidase. Drug Metab. Rev. 17: 93-143.

Igwe, O.J. (1986): Biologically active intermediates generated by the reduced glutathione conjugation pathway. Toxicological implications. Biochem. Pharm. 35: 2987-2994.

James, M.O., J.R. Fouts, J.R. Bend (1977): Xenobiotic metabolizing enzymes in marine fish. pp. 171-189 in Pesticides in Aquatic Environments. ed., M.A.Q. Khan, Plenum Press.

James, M.O., J.R. Bend (1980): Polycyclic aromatic hydrocarbon induction of cytochrome P-450-dependent mixed-function oxidases in marine fish. Toxicol. Appl. Pharmacol. 54: 117-133.

Jakoby, W.B. (1978): The glutathione-S-transferases: a group of multi-functional detoxification proteins. Adv. Enzym. 46: 383-414.

Kaplowitz, N., J. Kuhlenkamp, G. Clifton (1975): Drug induction of hepatic glutathione S-transferases in male and female rats. Biochem. J. 146: 351-356.

Keen, J.H., W.H. Habig, W.B. Jakoby (1976): Mechanism for the several activities of the glutathione S-transferases. J. Biol. Chem. 251: 6183-6188.

Keizer, P.D., D.C. Gordon Jr. (1973): Detection of trace amounts of oil and seawater by fluorescence spectroscopy. J. Fish. Res. Bd. Can. 30: 1039-1046.

Ketterer, B. B. Coles, D.J. Meyer (1983): The role of glutathione in detoxication. Environ. Health Persp. 49: 59-69.

Kiceniuk, J.W., R.A. Khan, M. Dawe, U. Williams (1982): Examination of interaction of Trypanosome infection and crude oil exposure on hematology of the longhorn sculpin (Myoxocephalus octodecemspinosus). Bull. Environm. Contam. Toxicol. 28: 435-438.

Koivusaari, U., M. Pesonen, O. Hänninen (1984): Polysubstrate monooxygenase activity and sex hormones in pre- and postspawning rainbow trout (Salmo gairdneri). Aquat. Toxicol. 5: 67-76.

Kosower, N.S., E.M. Kosower (1978): The glutathione status of cells. Int. Rev. Cytol. 54: 109-160.

Kurelec, B., S. Britvić, M. Rijavec, W.E.G. Müller, B.K. Zahn (1977): Benzo[a]pyrene monooxygenase induction in marine fish molecular response to oil pollution. Mar. Biol. 44: 211-216.

Kurelec, B., M. Protić, M. Rijavec, S. Britivić, W.E.G. Müller, R.K. Zahn (1982): Induction of benzo[a]pyrene monooxygenase in fish after i.p. application of water hexane extract. A Prescreen tool for detection of xenobiotics. United States Environmental Protection Agency. EPA-600/9-82-013, July, 1982.

Lech, J.J., M.J. Vodicnik, C.R. Elcombe (1982): Induction of monooxygenase activity in fish. pp. 107-148 in Aquatic Toxicology, ed., L.J. Weber, Raven Press, New York.

Lech, J.J., M.J. Vodicnik (1984): Biotransformation of chemicals by fish: an overview. Natl. Cancer Inst. Monogr. 65: 355-358.

Lee, R.F. (1981): Mixed Function Oxygenases (MFO) in Marine Invertebrates. Marine Biology Letters, 2: 87-105.

Lewis, D.F.V., C. Ioannides, D.V. Parke (1986): Molecular dimensions of the substrate binding site of cytochrome P-448. Biochem. Pharm. 35: 2179-2185.

Lindström-Seppä, P. (1985): Seasonal variation of the xenobiotic metabolizing enzyme activities in the liver of male and female vendace (Coregonus albula l.). Aquat. Toxicol. 6: 323-331.

Lindström-Seppä, P., O. Hänninen, R. Hudd (1985): Biomonitoring of the petroleum spill in Vaasa Archipelago, Finland by biotransformation activities in fish. pp. 357-360 in Cytochrome P-450: Biochemistry, Biophysics and Induction, eds., L. Vereczkey, K. Magyar, Akademiai, Kiado, Budapest.

Lindström-Seppä, P., U. Koivusaari, O. Hänninen (1981): Extrahepatic xenobiotic metabolism in North-European freshwater fish. Comp. Biochem. Physiol. 69C: 259-263.

Lockhart, W.L., D.A. Metner (1984): Fish serum chemistry as a pathology tool. pp. 73-85 in Contaminants Effects on Fisheries, eds., V.W. Cairns, P.V. Hodson, J.O. Nriagu, J. Wiley & Sons Inc.

Lowry, O.H., N.J. Rosebrough, A.L. Farr, R.J. Randall (1951): Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265-275.

Lu, A.Y.H., S.B. West (1980): Multiplicity of mammalian microsomal cytochromes P-450. Pharm. Rev. 31: 277-295.

Mason, H.S. (1957): Mechanisms of oxygen metabolism. pp. 79-233 in Advances in Enzymology, Vol. 19. ed., F.F. Nord, Academic Press, New York.

Melancon, M.J., J.J. Lech (1978): Distribution and elimination of naphthalene and 2-methylnaphthalene in rainbow trout during shortand long-term exposures. Arch. Environm. Contam. Toxicol. 7: 207-220.

Morgenstern, R., G. Lundqvist, G. Andersson, L. Balk, J.W. DePierre (1984): The distribution of microsomal glutathione transferase among different organelles, different organs, and different organisms. Biochem. Pharm. 33: 3609-3614.

Nikunen, E. (1985): Toxic impact of effluents from petrochemical industry. Ecoto. Envir. Safety, 9: 84-91.

Omura, T., R. Sato (1962): A new cytochrome in liver microsomes. J. Biol. Chem. 237: 1375-1376.

Orrenius, S., L. Ernster (1964): Phenobarbital-induced synthesis of the oxidative demethylating enzymes of rat liver microsomes. Biochem. Biophys. Res. Commun. 16: 60-65.

Payne, J.F. (1976): Field evaluation of benzopyrene hydroxylase induction as a monitor for marine pollution. Science (Wash. D.C.) 191: 945-946.

Payne, J.F. (1984): Mixed-function oxygenases in biological monitoring programs: review of potential usage in different phyla of aquatic animals. pp. 625-655 in Ectoxicological Testing for the Marine Environment,

eds., G. Personne, E. Jaspers, C. Claus, Vol. 1, State Univ. Ghent and Inst. Mar. Scient. Res., Bredene, Belgium.

Payne, J.F., C. Bauld, A.C. Dey, J.W. Kiceniuk, U. Williams (1984): Selectivity of mixed-function oxygenase enzyme induction in flounder (Pseudopleuronectes americanus) collected at the site of the Baie Verte, Newfoundland oil spill. Comp. Biochem. Physiol. 79C: 15-19.

Payne, J.F., L.L. Fancey, A.D. Rahimtula, E.L. Porter (1987): Review and perspective on the use of mixed-function oxygenase enzymes in biological monitoring. Comp. Biochem. Physiol. 86C: 233-245.

Payne, J.F., N. May (1979): Further studies on the effects of petroleum hydrocarbons on mixed function oxidases in marine organisms. pp. 339-347 in Pesticide and Xenobiotic Metabolism in Aquatic Organisms. eds., M.A.Q. Khan, J.J. Lech, J.J. Menn, American Chemical Society, Washington, D.C.

Payne, J.F., W.R. Penrose (1975): Induction of aryl hydrocarbon (Benzo[a]pyrene) hydroxylase in fish by petroleum. Bull. Environ. Contam. Toxicol. 14: 112-116.

Pesonen, M., T. Andersson, L. Förlin (1985): Characterization and induction of cytochrome P-450 in the rainbow trout kidney. Mar. Environ. Res. 17: 106-108.

Plack, P.A., N.W. Fraser (1971): Incorporation of L-[^{14}C] into egg proteins by liver slices from cod. Biochem. J. 121: 857-862.

Pohl, R.J., J.R. Fouts (1980): A rapid method for assaying the metabolism of 7-ethoxyresorufin microsomal subcellular fractions. Anal. Biochem. 107: 150-155.

Porter, E., L. Fancey, A. Rahimtula, J.F. Payne (1986): Induction of hepatic and extrahepatic mixed-function oxygenases in cunners exposed to petroleum hydrocarbons during various stages of gonad maturation. Can. Fed. Biol. Soc. 29: 141.

Pottle, R.A., J.M. Green (1978): Field observations on the reproductive behavior of the cunner, Tautogolabrus adspersus. (Walbaum), in Newfoundland. Can. J. Zool. 57: 247-256.

Sato, R., Y. Atsuta, Y. Imai, S. Taniguchi, K. Okuda (1977): Hepatic mitochondrial cytochrome P-450: isolation and functional characterization. Proc. Natl. Acad. Sci. USA 74: 5477-5481.

Seidel, S.L., T.R. Shires (1986): Purification and characterization of a previously unreported form of cytochrome P-448 from the liver of 3-methylcholanthrene -pretreated rats. Biochem. J. 235: 859-868.

Shapiro, B.H. (1986): Sexually dimporphic response of rat hepatic monoxygenases to low-dose phenobarbital. Biochem. Pharm. 35: 1766-1768.

Smith, G.J., V.S. Ohl, G. Litwack (1977): Ligandin, the glutathione S-transferases, and chemically induced hepatocarcinogenesis: a review. Cancer Res. 37: 8-14.

Snyder, R., H. Remmer (1979): Classes of hepatic microsomal mixed function oxidase inducers. Pharmacol. Ther. 7: 203-244.

Spies, R.B., P.H. Davis and D.H. Stuermer (1980): Ecology of a submarine petroleum seep off the California coast. pp. 229-263 in Marine Environmental Pollution, I. Hydrocarbons, ed., R.A. Geyer, Elsevier, Amsterdam.

Spies, R.B., J.S. Felton, L. Dillard (1982): Hepatic mixed-function oxidases in California flatfishes are increased in contaminated environments and by oil and PCB ingestion. Mar. Biol. 70: 117-127.

Sparnins, V.L., J. Chuan, L.W. Wattenberg, (1982): Enhancement of glutathione S-transferase activity of the esophagus by phenols, lactones, and benzyl isothiocyanate. Cancer Res. 42: 1205-1207.

Stegeman, J.J. (1978): Influence of environmental contamination of cytochrome P-450 mixed function oxygenases in fish: implications for recovery in the Wild Harbour Marsh. J. Fish. Res. Board Can. 35: 668-674.

Stegeman, J.J. (1980): Mixed-function oxygenase studies in monitoring for effects of organic pollution. Rapp. P.-v. Réun. Cons. int. Explor. Mer., 179: 33-38.

Stegeman, J.J., M. Chevion (1980): Sex differences in cytochrome P-450 and mixed-function oxygenase activity in gonadally mature trout. Biochem. Pharm. 29: 553-558.

Stegeman, J.J., B.R. Woodin, R.L. Binder (1984): Patterns of benzo[a]pyrene metabolism by varied species, organs, and developmental stages of fish. Natl. Cancer Inst. Monogr. 65: 371-377.

Stegeman, J.J., B.R. Woodin, A.V. Klotz, R.E. Wolke, N.R. Orme-Johnson (1982): Cytochrome P-450 and monoxygenase activity in cardiac microsomes from the fish Stenotomus chrysops. Mol. Pharmacol. 21: 517-526.

Tan, W.Y., K.P. Singh (1987): Assessing the effects of metabolism of environmental agents on cancer tumor development by a two-stage model of carcinogenesis. Environ. Health Persp. 74: 203-210.

Tarlebo, J., J.E. Solbakken, K.H. Palmork (1985): Variation in hepatic aryl hydrocarbon hydroxylase activity in flounder, Platichthys flesus: a baseline study. Helgoländer Meeresunters. 39: 187-199.

Templeman W. (1976): Biological and oceanographic background of flemish Cap as an area for research on the reasons for year-class success and failure in cod and redfish. ICNAF Res. Bull. 12: 91-117.

Varanasi, U., J.E. Stein, M. Nishimoto, W.L. Reichert, T.K. Collier (1987): Chemical carcinogenesis in feral fish: uptake, activation and detoxification of organic xenobiotics. Environ. Health Persp. 71: 155-170.

Vessell, E.S. (1982): On the significance of host factors that affect drug disposition. Clin. Pharm. Ther. 31: 1-7.

Viviani, A., W.K. Lutz, C. Schlatter (1978): Time course of induction of aryl hydrocarbon hydroxylase in rat liver nuclei and microsomes by phenobarbital, 3-methyl-cholanthrene, 2, 3, 7, 8-tetrachloro-dibenzo-p-dioxin, dieldrin and other inducers. Biochem. Pharm. 27: 2103-2108.

Walton, D.G., W.R. Penrose, J.M. Green (1978): The petroleum-inducible mixed-function oxidase of cunner (Tautogolabrus adspersus, Walbaum 1792): some characteristics relevant to hydrocarbon monitoring. J. Fish. Res. Board Can. 35: 1547-1552.

Walton, D.G., L.L. Fancey, J.M. Green, J.W. Kiceniuk, W.R. Penrose (1983): Seasonal changes in aryl hydrocarbon hydroxylase activity of a marine fish Tautogolabrus adspersus (Walbaum) with and without petroleum exposure. Comp. Biochem. Physiol. 76C: 247-253.

Whitehead, C., N.R. Bromage, J.R.M. Forster (1978): Seasonal changes in reproductive function of the rainbow trout (Salmo gairdneri). J. Fish Biol. 12: 601-608.

Williams, D.E., B.S.S. Masters, J.J. Lech, D.R. Buhler (1986): Sex differences in cytochrome P-450 isozyme composition and activity in kidney microsomes of mature rainbow trout. Biochem. Pharm. 35: 2017-2023.

Wolf, C.R., S. Seilman, F. Oesch, R.T. Mayer, M.D. Burke (1986): Multiple forms of cytochrome P-450 related to forms induced marginally by phenobarbital. Differences in structure and in the metabolism of alkoxyresorufins. Biochem. J. 240: 27-33.

APPENDIX

Figure A.1: Pulse delivery exposure system of diesel oil to cunners.

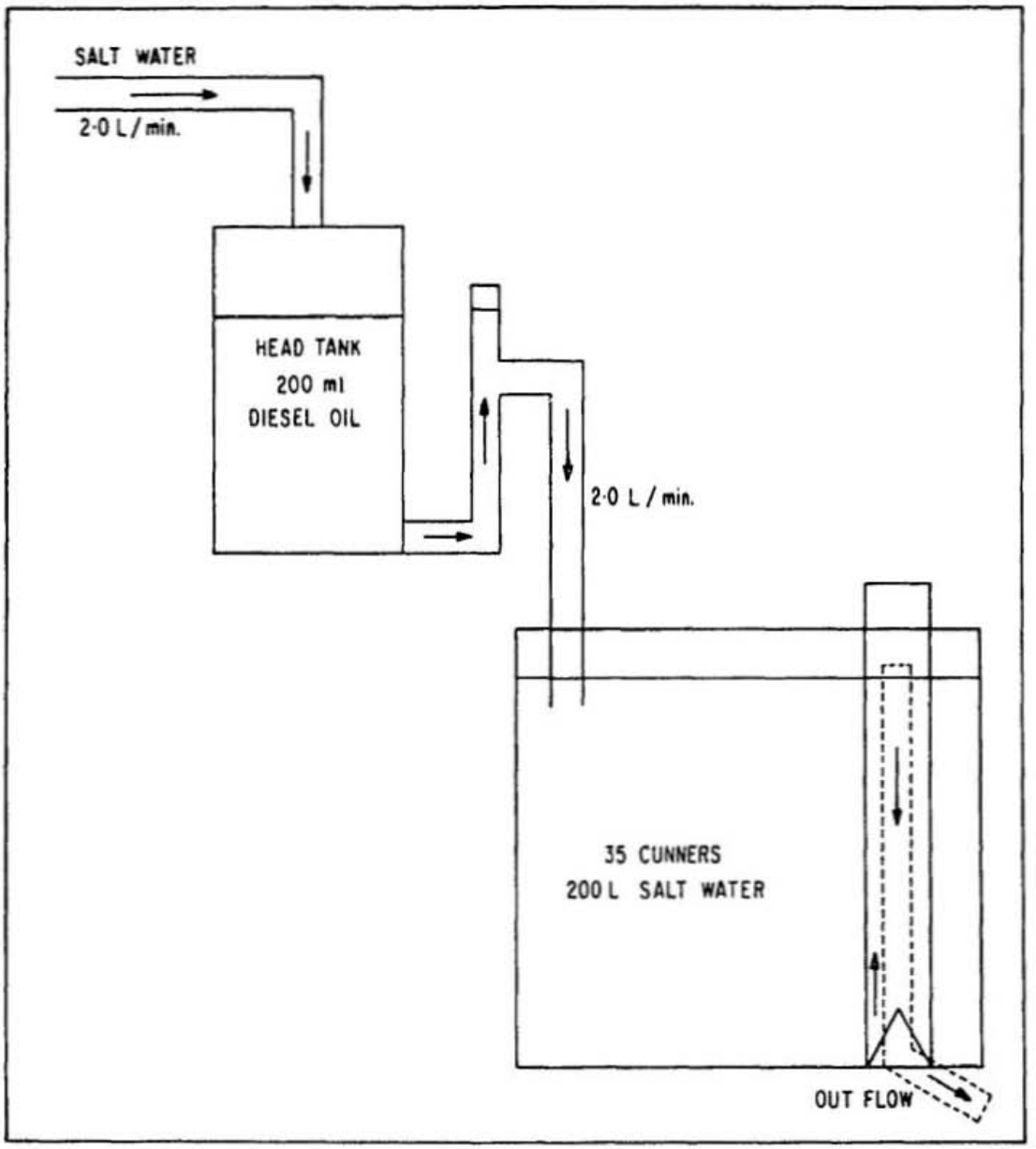

SALT WATER
2·0 L / min.
HEAD TANK
200 ml
DIESEL OIL
2·0 L / min.
35 CUNNERS
200 L SALT WATER
OUT FLOW

Figure A.2: Standard curve of fluorescence (excitation 308 nm, emission 344 nm) versus diesel oil concentration.

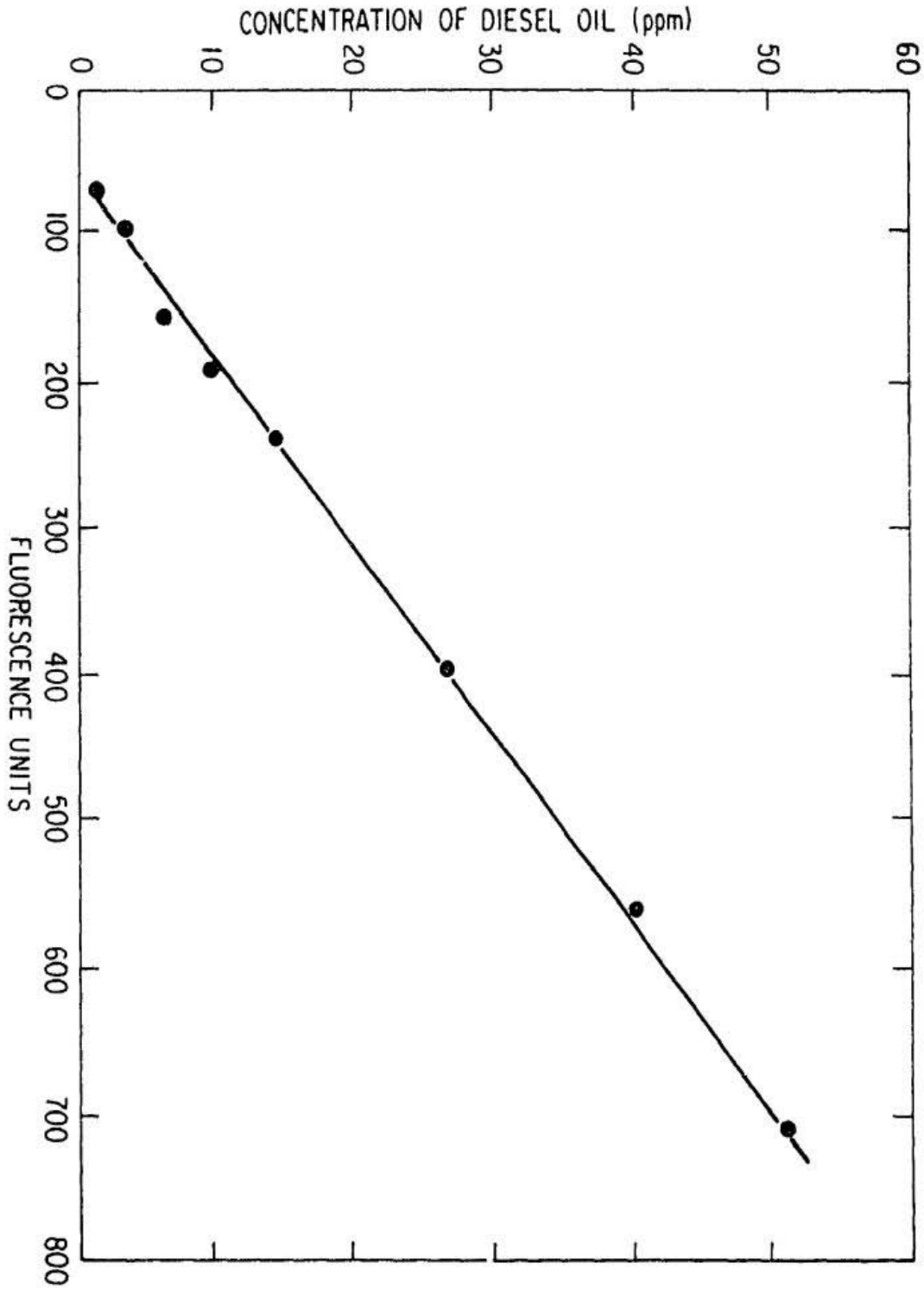

CONCENTRATION OF DIESEL OIL (ppm)
0
10
20
30
40
50
60
FLUORESCENCE UNITS
0
100
200
300
400
500
600
700
800

Figure A.3: Diesel concentration in exposure tank during early spawning trial.

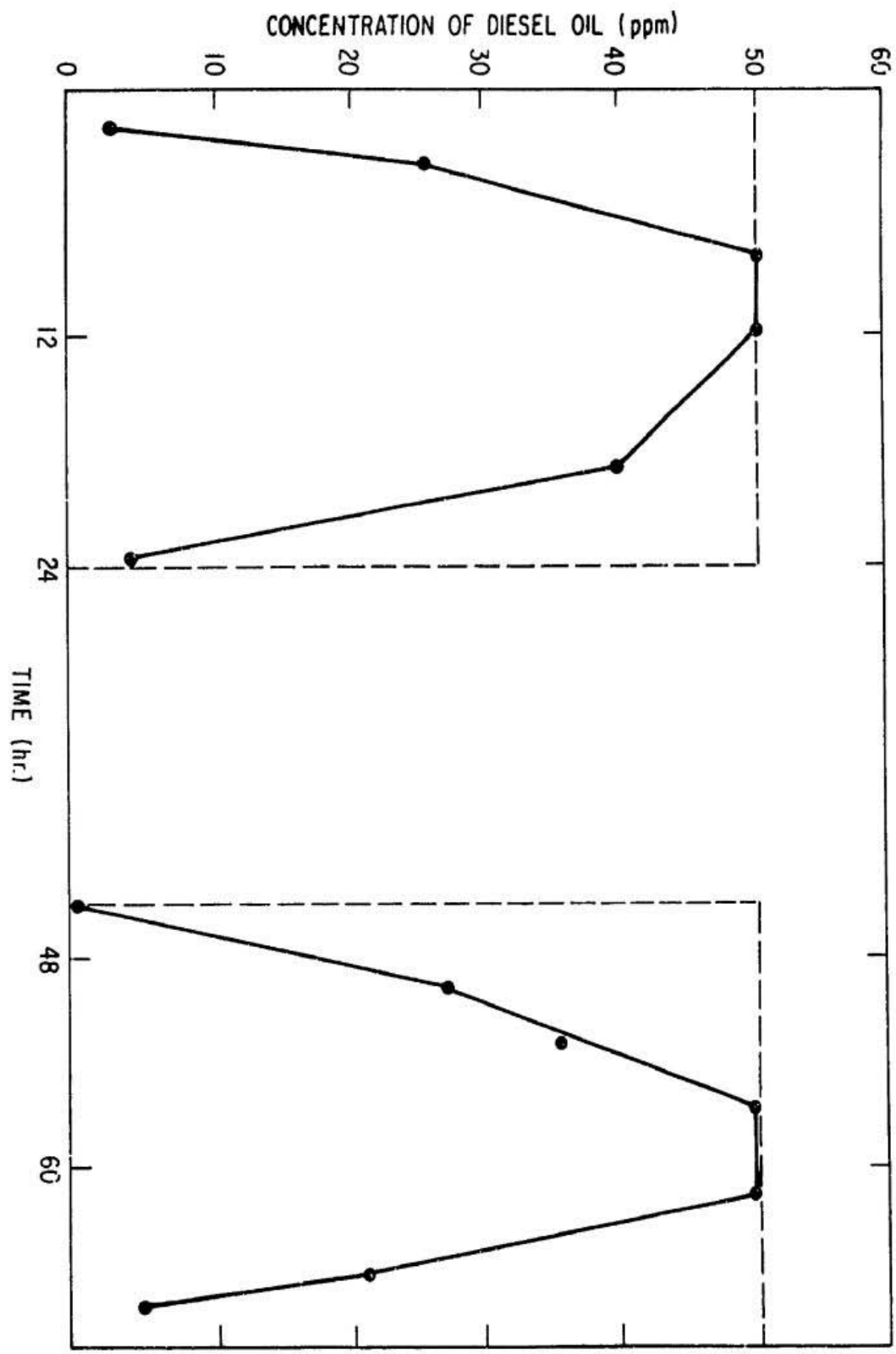

CONCENTRATION OF DIESEL OIL (ppm)
0
10
20
30
40
50
60
TIME (hr.)
12
24
48
60

TABLE A.1: **Comparison of p-values of non-normal data that have been ranked and non-ranked.**

Source		EROD specific activities			
		Liver	Kidney	Gill	Heart
Sample	U	0.0001*	0.0001	0.0001	0.0001
	R	0.5313	0.5741	0.3746	0.3401
Treatment	U	0.0001	0.0001	0.0001	0.0001
	R	0.0001	0.0001	0.0001	0.0
Sex	U	0.0471	0.0043	0.8565	0.7898
	R	0.0200	0.0001	0.9212	0.5035
Sample x Treatment	U	0.0001	0.0001	0.0001	0.0001
	R	0.8026	0.2381	0.8975	0.9035
Sample x Sex	U	0.0028	0.1730	0.4053	0.8189
	R	0.1105	0.6278	0.1656	0.3464
Treatment x Sex	U	0.3696	0.0283	0.6909	0.4949
	R	0.8068	0.8332	0.6085	0.0371
Sample x Treatment x Sex	U	0.0024	0.2323	0.8572	0.8692
	R	0.2589	0.7877	0.4860	0.8534

* p-value

U unranked

R ranked

Table A.2: Correlation coefficients among enzyme activities and body characteristics of control male cunners during prespawning.

	EK	EG	EH	GL	GK	GG	GH	CGO	CCO	CLSI	LTH	WT
EL	0.04	0.53	0.18	0.61	0.37	0.04	0.11	-0.07	0.20	0.38	0.15	0.27
EK		-0.10	0.13	0.29	0.77*	0.16	0.77*	0.45	-0.55	-0.58	-0.45	-0.44
EG			0.51	0.12	0.20	0.60	0.16	0.31	0.06	0.48	-0.13	-0.03
EH				0.40	0.41	0.46	0.66	0.29	0.12	0.22	-0.60	-0.33
GL					0.56	0.07	0.54	-0.12	-0.12	-0.17	-0.21	-0.10
GK						0.56	0.79*	0.17	-0.30	-0.39	-0.52	-0.43
GC							0.33	0.19	-0.22	-0.16	-0.54	-0.51
GH								0.27	-0.71	-0.54	-0.38	-0.41
CGO									-0.33	-0.17	-0.68*	-0.68*
CCO										0.81*	0.26	0.47
CLSI											0.42	0.62
LTH												0.95*

* $p<0.05$

EL= Liver Ethoxyresorufin O-Deethylase
EK= Kidney Ethoxyresorufin O-Deethylase
EG= Gill Ethoxyresorufin O-Deethylase
EH= Heart Ethoxyresorufin O-Deethylase
GL= Liver Glutathione S-Transferase
GK= Kidney Glutathione S-Transferase
GL= Gill Glutathione S-Transferase
GL= Heart Glutathione S-Transferase

CGO= Gonad Index = [(Gonad/Weight-Tl)] x 100
CCO= Condition Index = [(Weight-Tl)/(lengthx3)] x 100
CLSI= Liver Somatic Index = [(Liver/Weight-Tl)] x 100
LTH= Length
WT= Weight
Tl= Gonad + Liver

Table A.3: Correlation coefficients among enzyme activities and body characteristics of control male cunners during early spawning.

	EK	EG	EH	GL	GK	GG	GH	CGO	CCO	CLSI	LTH	WT
EL	-0.09	-0.10	0.15	0.74*	0.18	-0.27	-0.73*	-0.03	0.03	0.08	0.11	0.22
EK		0.08	0.04	-0.32	0.30	-0.18	-0.09	0.47	0.09	0.43	-0.07	-0.07
EG			0.45	-0.19	0.10	0.59	0.23	-0.26	-0.16	0.18	0.49	0.50
EH				0.17	0.002	0.33	-0.28	0.49	0.20	0.40	0.08	0.40
GL					0.03	-0.27	-0.28	-0.21	0.34	-0.05	-0.37	-0.22
GK						0.02	-0.09	-0.02	0.14	-0.50	-0.20	-0.18
GG							-0.10	-0.16	-0.13	0.29	-0.27	0.24
GH								-0.36	0.18	-0.27	-0.23	-0.27
CGO									0.42	0.54	-0.10	0.19
CCO										0.11	-0.47	-0.14
CLSI											0.40	0.55
LTH												0.90*

* p<0.05

EL= Liver Ethoxyresorufin O-Deethylase
EK= Kidney Ethoxyresorufin O-Deethylase
EG= Gill Ethoxyresorufin O-Deethylase
EH= Heart Ethoxyresorufin O-Deethylase
GL= Liver Glutathione S-Transferase
GK= Kidney Glutathione S-Transferase
GL= Gill Glutathione S-Transferase
GL= Heart Glutathione S-Transferase

CGO= Gonad Index = [(Gonad/Weight-Tl)] x 100
CCO= Condition Index = [(Weight-Tl)/(Lengthx3)] x 100
CLSI= Liver Somatic Index = [(Liver/Weight-Tl)] x 100
LTH= Length
WT= Weight
Tl= Gonad + Liver

Table A.4: Correlation coefficients among enzyme activities and body characteristics of control male cunners during late spawning.

	EK	EG	EH	GL	GK	GG	GH	CGO	CCO	CLSI	LTH	WT
EL	0.15	0.19	-0.22	0.35	-0.42	0.49	0.06	-0.22	0.01	-0.42	-0.20	-0.22
EK		0.71*	-0.09	-0.07	0.07	-0.08	-0.29	0.12	0.38	-0.15	0.49	0.54
EG			-0.09	0.05	0.05	0.02	-0.27	0.22	0.11	0.02	0.43	0.34
EH				-0.45	-0.07	-0.43	0.40	0.15	0.06	0.22	0.25	0.22
GL					0.12	0.50	0.05	-0.39	-0.56	-0.72*	-0.26	-0.54
GK						0.27	0.21	-0.07	0.24	0.07	-0.22	-0.17
GG							0.14	-0.49	-0.18	-0.22	-0.70*	-0.80*
GH								0.27	0.28	0.50	-0.70*	-0.44
CGO									0.17	0.53	0.19	0.35
CCO										0.33	0.05	0.48
CLSI											-0.16	0.12
LTH												0.85*

* $p<0.05$

EL= Liver Ethoxyresorufin O-Deethylase
EK= Kidney Ethoxyresorufin O-Deethylase
EG= Gill Ethoxyresorufin O-Deethylase
EH= Heart Ethoxyresorufin O-Deethylase
GL= Liver Glutathione S-Transferase
GK= Kidney Glutathione S-Transferase
GL= Gill Glutathione S-Transferase
GL= Heart Glutathione S-Transferase

CGO= Gonad Index = [(Gonad/Weight-Tl)] x 100
CCO= Condition Index = [(Weight-Tl)/(Lengthx3)] x 100
CLSI= Liver Somatic Index = [(Liver/Weight-Tl)] x 100
LTH= Length
WT= Weight
Tl= Gonad + Liver

Table A.5: Correlation coefficients among enzyme activities and body characteristics of control female cunners during prespawning.

	EK	EG	EH	GL	GK	GG	GH	CGO	CCO	CLSI	LTH	WT
EL	0.20	-0.04	0.46	-0.12	0.07	-0.25	0.14	0.25	-0.31	0.02	-0.03	0.06
EK		-0.28	-0.54	0.16	0.52	-0.63*	-0.85*	-0.34	0.56	-0.22	0.10	0.21
EG			0.41	0.10	-0.11	0.17	0.61	-0.15	0.06	0.01	-0.11	-0.22
EH				0.02	-0.45	0.23	0.68*	0.29	-0.19	0.03	-0.02	0.09
GL					0.14	-0.67*	-0.40	-0.06	0.34	0.20	0.08	0.27
GK						-0.39	-0.28	-0.04	0.29	-0.27	-0.28	-0.23
GG							0.71*	-0.02	-0.23	-0.10	-0.24	-0.45
GH								0.44	-0.44	0.51	-0.01	-0.11
CGO									-0.42	0.74*	-0.12	0.11
CCO										-0.04	-0.18	0.01
CLSI											0.13	0.40
LTH												0.89*

* p<0.05

EL= Liver Ethoxyresorufin O-Deethylase
EK= Kidney Ethoxyresorufin O-Deethylase
EG= Gill Ethoxyresorufin O-Deethylase
EH= Heart Ethoxyresorufin O-Deethylase
GL= Liver Glutathione S-Transferase
GK= Kidney Glutathione S-Transferase
GL= Gill Glutathione S-Transferase
GL= Heart Glutathione S-Transferase

CGO= Gonad Index = [(Gonad/Weight-Tl)] x 100
CCO= Condition Index = [(Weight-Tl)/(Lengthx3)] x 100
CLSI= Liver Somatic Index = [(Liver/Weight-Tl)] x 100
LTH= Length
WT= Weight
Tl= Gonad + Liver

Table A.6: Correlation coefficients among enzyme activities and body characteristics of control female cunners during early spawning.

	EK	EG	EH	GL	GK	GG	GH	CGO	CCO	CLSI	LTH	WT
EL	0.33	-0.23	0.16	0.07	-0.25	-0.07	0.31	-0.28	-0.04	0.10	0.55	0.41
EK		0.47	0.81*	-0.02	0.28	0.67*	0.14	0.47	-0.20	0.34	0.46	0.40
EG			0.30	-0.21	0.04	0.39	-0.19	-0.05	-0.26	0.11	-0.16	-0.13
EH				0.28	0.63*	0.80*	0.28	0.58*	0.05	0.48	0.68+	0.68+
GL					0.78*	0.06	0.10	-0.07	-0.32	0.09	0.11	0.07
GK						0.43	0.22	0.35	-0.25	0.16	0.20	0.21
GG							-0.18	0.75*	0.17	0.70*	0.52	0.62*
GH								-0.16	0.04	-0.30	0.31	0.19
CGO									0.29	0.53	0.31	0.40
CCO										0.58*	0.26	0.38
CLSI											0.33	0.46
LTH												0.97*

* $p<0.05$

EL= Liver Ethoxyresorufin O-Deethylase
EK= Kidney Ethoxyresorufin O-Deethylase
EG= Gill Ethoxyresorufin O-Deethylase
EH= Heart Ethoxyresorufin O-Deethylase
GL= Liver Glutathione S-Transferase
GK= Kidney Glutathione S-Transferase
GL= Gill Glutathione S-Transferase
GL= Hearth Glutathione S-Transferase

CGO= Gonad Index = [(Gonad/Weight-Tl)] x 100
CCO= Condition Index = [(Weight-Tl)/(Lengthx3)] x 100
CLSI= Liver Somatic Index = [(Liver/Weight-Tl)] x 100
LTH= Length
WT= Weight
Tl= Gonad + Liver

Table A.7: Correlation coefficients among enzyme activities and body characteristics of control female cunners during late spawning.

	EK	EG	EH	GL	GK	GG	GH	CGO	CCO	CLSI	LTH	WT
EL	-0.003	0.03	-0.57*	-0.19	-0.06	0.41	-0.04	-0.47	-0.27	-0.75*	-0.76*	-0.82*
EK		0.30	-0.29	0.17	0.37	-0.27	-0.01	0.23	-0.16	0.43	-0.14	-0.19
EG			-0.47	-0.18	-0.28	-0.62*	0.07	0.03	-0.48	0.27	-0.14	-0.18
EK				0.25	0.25	0.24	0.06	0.09	0.52	0.06	0.59*	0.59*
GL					0.39	0.05	0.22	-0.34	0.45	0.15	-0.005	0.03
GK						-0.12	0.16	-0.29	-0.12	-0.19	-0.06	-0.19
GG							0.20	0.08	0.29	-0.43	-0.28	-0.24
GH								0.14	-0.23	0.01	-0.17	-0.21
CGO									-0.14	0.67*	0.46	0.50
CCO										0.14	0.14	0.31
CLSI											0.49	0.59*
LTH												0.96*

* p<0.05

EL= Liver Ethoxyresorufin O-Deethylase
EK= Kidney Ethoxyresorufin O-Deethylase
EG= Gill Ethoxyresorufin O-Deethylase
EH= Heart Ethoxyresorufin O-Deethylase
GL= Liver Glutathione S-Transferase
GK= Kidney Glutathione S-Transferase
GL= Gill Glutathione S-Transferase
GL= Heart Glutathione S-Transferase

CGO= Gonad Index = [(Gonad/Weight-Tl)] x 100
CCO= Condition Index = [(Weight-Tl)/(Lengthx3)] x 100
CLSI= Liver Somatic Index [(Liver/Weight-Tl)] x 100
LTH= Length
WT= Weight
Tl= Gonad + Liver

Table A.8: Correlation coefficients among enzyme activities and body characteristics of diesel exposed male cunners during prespawning.

	EK	EG	EH	GL	GK	GG	GH	CGO	CCO	CLSI	LTH	WT
EL	0.13	0.31	0.34	0.69*	-0.13	-0.28	-0.11	-0.52	-0.13	-0.44	0.21	0.18
EK		0.39	0.24	0.27	0.72*	-0.01	0.14	-0.43	-0.22	-0.53	-0.19	-0.25
EG			-0.32	0.39	0.49	-0.32	0.15	-0.01	-0.17	-0.29	-0.39	-0.37
EH				0.34	0.005	0.12	-0.04	-0.27	-0.07	-0.05	0.46	0.45
GL					-0.02	-0.21	0.11	-0.05	0.01	0.01	-0.26	-0.06
GK						0.32	0.50	-0.08	0.004	-0.15	-0.10	-0.11
GG							0.18	0.17	0.49	0.24	0.03	0.16
GH								0.43	0.37	0.37	-0.24	-0.14
CGO									0.20	-0.38	-0.52	-0.50
CCO										0.29	-0.26	0.29
CLSI											-0.07	0.16
LTH												0.77*

* $p<0.05$

EL= Liver Ethoxyresorufin O-Deethylase
EK= Kidney Ethoxyresorufin O-Deethylase
EG= Gill Ethoxyresorufin O-Deethylase
EH= Heart Ethoxyresorufin O-Deethylase
GL= Liver Glutathione S-Transferase
GK= Kidney Glutathione S-Transferase
GL= Gill Glutathione S-Transferase
GL= Heart Glutathione S-Transferase

CGO= Gonad Index = [(Gonad/Weight-T1)] x 100
CCO= Condition Index = [(Weight-T1)/(Lengthx3)] x 100
CLSI= Liver Somatic Index = [(Liver/Weight-T1)] x 100
LTH= Length
WT= Weight
T1= Gonad + Liver

Table A.9: Correlation coefficients among enzyme activities and body characteristics of diesel exposed male cunners during early spawning.

	EK	EG	EH	GL	GK	GG	GH	CGO	CCO	CLSI	LTH	WT
EL	0.84*	-0.14	-0.22	-0.13	0.47	0.71*	0	-0.34	-0.37	-0.74*	0.24	0.26
EK		-0.10	-0.02	-0.22	0.45	0.40	0.34	-0.42	-0.47	-0.85*	0.38	0.32
EG			0.21	0.41	0.55	0.07	-0.27	0.40	-0.09	0.42	0.27	-0.08
EH				0.11	-0.28	0.003	0.26	-0.11	-0.46	0.007	-0.06	-0.47
GL					-0.06	0.14	-0.21	0.18	-0.04	0.13	-0.22	-0.23
GK						0.35	-0.15	0.14	0.14	-0.12	0.25	0.32
GG							-0.27	-0.33	-0.45	-0.39	0.41	0.38
GH								-0.42	-0.04	-0.42	0.31	0.34
CGO									0.16	0.74*	-0.35	-0.40
CCO										0.38	-0.51	-0.08
CLSI											-0.27	0.78*
LTH												0.78*

* p<0.05

EL= Liver Ethoxyresorufin O-Deethylase
EK= Kidney Ethoxyresorufin O-Deethylase
EG= Gill Ethoxyresorufin O-Deethylase
EH= Heart Ethoxyresorufin O-Deethylase
GL= Liver Glutathione S-Transferase
GK= Kidney Glutathione S-Transferase
GL= Gill Glutathione S-Transferase
GL= Heart Glutathione S-Trasferase

CGO= Gonad Index = [(Gonad/Weight-Tl)] x 100
CCO= Condition Index = [(Weight-Tl)/(Lengthx3)] x 100
CLSI= Liver Somatic Index = [(Liver/Weight-Tl)] x 100
LTH= Length
WT= Weight
Tl= Gonad + Liver

Table A.10: Correlation coefficients among enzyme activities and body characteristics of diesel exposed male cunners during late spawning.

	EK	EG	EH	GL	GK	GG	GH	CGO	CCO	CLSI	LTH	WT
EL	-0.11	0.67*	-0.14	0.11	-0.20	-0.33	0.25	0.16	0.35	0.55	-0.41	-0.34
EK		-0.05	-0.13	0.48	0.62*	-0.40	-0.15	-0.44	-0.61*	-0.43	0.29	-0.22
EG			-0.41	0.08	-0.25	-0.34	-0.10	0	0.24	0.22	-0.10	-0.03
EH				0.07	-0.02	-0.01	0.76*	0	-0.19	-0.03	0.19	0.13
GL					0.69*	0.21	-0.09	-0.57	-0.42	-0.38	0.30	-0.17
GK						0.34	0.11	-0.79*	-0.60*	-0.74*	0.02	-0.50
GG							-0.18	-0.37	0.14	-0.34	-0.30	-0.24
GH								-0.12	0.02	-0.05	-0.25	-0.14
CGO									0.32	0.63*	0.18	0.60*
CCO										0.68*	-0.64*	-0.09
CLSI											-0.30	0.04
LTH												0.77*

* $p<0.05$

EL= Liver Ethoxyresorufin O-Deethylase
EK= Kidney Ethoxyresorufin O-Deethylase
EG= Gill Ethoxyresorufin O-Deethylase
EH= Heart Ethoxyresorufin O-Deethylase
GL= Liver Glutathione S-Transferase
GK= Kidney Glutathione S-Transferase
GL= Gill Glutathione S-Transferase
GL= Heart Glutathione S-Transferase

CGO= Gonad Index = [(Gonad/Weight-Tl)] x 100
CCO= Condition Index = [(Weight-Tl)/(Lengthx3)] x 100
CLSI= Liver Somatic Index = [(Liver/Weight-Tl)] x 100
LTH= Length
WT= Weight
Tl= Gonad + Liver

Table A.11: Correlation coefficients among enzyme activities and body characteristics of diesel exposed females during prespawning.

	EK	EG	EH	GL	GK	GG	GH	CGO	CCO	CLSI	LTH	WT
EL	0.45	0.47	-0.37	0.61*	0.66*	0.04	-0.29	0.13	-0.22	-0.52	-0.61*	-0.82*
EK		0.44	0.15	0.09	0.23	-0.59*	-0.81*	-0.31	-0.11	-0.68*	-0.34	-0.34
EG			-0.37	0.39	0.36	-0.52	-0.81*	0.27	0.02	-0.21	-0.53	-0.59*
EH				0.002	-0.38	0.02	0.51	-0.47	-0.04	0.11	0.71*	0.77*
GL					0.36	0.10	-0.26	0.30	-0.44	-0.06	0.02	-0.30
GK						0.30	-0.37	0.17	-0.55	-0.29	-0.47	-0.66*
GG							0.70	0.25	-0.14	0.46	0.15	0.11
GH								-0.37	-0.02	0.57	0.67	0.71
CGO									0.24	0.32	-0.45	-0.33
CCO										0.31	-0.09	0.16
CLSI											0.44	0.50
LTH												0.90*

* $p<0.05$

EL= Liver Ethoxyresorufin O-Deethylase
EK= Kidney Ethoxyresorufin O-Deethylase
EG= Gill Ethoxyresorufin O-Deethylase
EH= Heart Ethoxyresorufin O-Deethylase
GL= Liver Glutathione S-Transferase
GK= Kidney Glutathione S-Transferase
GL= Gill Glutathione S-Transferase
GL= Heart Glutathione S-Transferase

CGO= Gonad Index = [(Gonad/Weight-Tl)] x 100
CCO= Condition Index = [(Weight-Tl)/(Lengthx3)] x 100
CLSI= Liver Somatic Index = [(Liver/Weight-Tl)] x 100
LTH= Length
WT= Weight
Tl= Gonad + Liver

Table A.12: Correlation coefficients among enzyme activities and body characteristics of diesel exposed female cunners during early spawning.

	EK	EG	EH	GL	GK	GG	GH	CGO	CCO	CLSI	LTH	WT
EL	0.37	-0.34	-0.27	0.54	-0.22	-0.34	-0.35	-0.78*	0.32	-0.74*	-0.38	-0.36
EK		-0.31	0.07	0.59*	0.18	-0.30	0.28	-0.42	-0.01	-0.24	-0.10	-0.20
EG			0.44	-0.26	-0.07	0.32	0.43	0.35	0.20	0.06	-0.17	-0.20
EH				-0.09	-0.46	-0.47	0.64*	0.34	0.25	0.50	-0.23	-0.20
GL					0.13	-0.30	-0.43	-0.65*	0.56	-0.55	0.17	0.17
GK						0.37	0.04	-0.15	-0.35	-0.23	0.38	0.28
GG							-0.04	0.14	-0.43	-0.13	0.27	0.17
GH								0.21	-0.43	0.43	-0.57	-0.64*
CGO									-0.26	0.94*	0.35	0.34
CCO										-0.39	-0.04	0.08
CLSI											0.20	0.20
LTH												0.98*

* p<0.05

EL= Liver Ethoxyresorufin O-Deethylase
EK= Kidney Ethoxyresorufin O-Deethylase
EG= Gill Ethoxyresorufin O-Deethylase
EH= Heart Ethoxyresorufin O-Deethylase
GL= Liver Glutathione S-Transferase
GK= Kidney Glutathione S-Transferase
GL= Gill Glutathione S-Transferase
GL= Heart Glutathione S-Transferase

CGO= Gonad Index = [(Gonad/Weight-Tl)] x 100
CCO= Condition Index = [(Weight-Tl)/(Lengthx3)] x 100
CLSI= Liver Somatic Index = [(Liver/Weight-Tl)] x 100
LTH= Length
WT= Weight
Tl= Gonad + Liver

Table A.13: Correlation coefficients among enzyme activities and body characteristics of diesel exposed female cunners during late spawning.

	EK	EG	EH	GL	GK	GG	GH	CGO	CCO	CLSI	LTH	WT
EL	0.83*	0.03	0	-0.47	-0.20	0.15	-0.29	0.38	0.35	0.34	0.02	0.32
EK		0.11	0.29	-0.31	-0.41	-0.06	-0.32	0.38	0.19	0.49	0.23	0.47
EG			0.29	0.03	-0.29	0.18	0.31	-0.16	0.20	0.03	-0.18	-0.10
EH				-0.32	-0.49	-0.55	-0.49	-0.06	-0.19	-0.13	0.31	0.30
GL					0.06	-0.07	0.55	-0.37	0.07	-0.20	-0.28	-0.35
GK						-0.12	0.17	0.09	-0.01	0.07	-0.19	-0.38
GG							0.49	0.25	0.21	0.33	-0.49	-0.38
GH								-0.17	0.24	-0.23	-0.62*	-0.68*
CGO									0.51	0.73*	-0.13	0.12
CCO										0.16	-0.47	-0.07
CLSI											0.11	0.21
LTH												0.87*

* $p<0.05$

EL= Liver Ethoxyresorufin O-Deethylase
EK= Kidney Ethoxyresorufin O-Deethylase
EG= Gill Ethoxyresorufin O-Deethylase
EH= Heart Ethoxyresorufin O-Deethylase
GL= Liver Glutathione S-Transferase
GK= Kidney Glutathione S-Transferase
GL= Gill Glutathione S-Transferase
GL= Heart Glutathione S-Transferase

CGO= Gonad Index = [(Gonad/Weight-Tl)] x 100
CCO= Condition Index = [(Weight-Tl)/(Lengthx3)] x 100
CLSI= Liver Somatic Index = [(Liver/Weight-Tl)] x 100
LTH= Length
WT= Weight
Tl= Gonad + Liver

Table A.14: Gonad Indices of Cunners Throughout the Reproductive Season

		Gonad Index (CGO)		
Sex	Treatment	Prespawn	Early Spawn	Late Spawn
Male	Control	5.20±0.71	6.34±0.44	2.79±0.22
	Diesel	6.18±0.28	3.13±0.35	3.16±0.16
Female	Control	6.06±0.23	9.05±0.33	5.08±0.32
	Diesel	5.25±0.34	9.95±0.15	4.63±0.48

Gonad Index (CGO) = (Gonad/Weight - Tl) x 100
Tl = Gonad + Liver