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VITELLOGENESIS IN WINTER FLOUNDER
(*Pleuronectes americanus*)
UNDER HYDROCARBON CONTAMINATED CONDITIONS

by

Jean-Marc Nicolas

Submitted in partial fulfillment of the requirements
for the degree of Doctorate of Philosophy

at

Dalhousie University
Halifax, Nova Scotia
September 1999

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by Jean-Marc Nicolas

in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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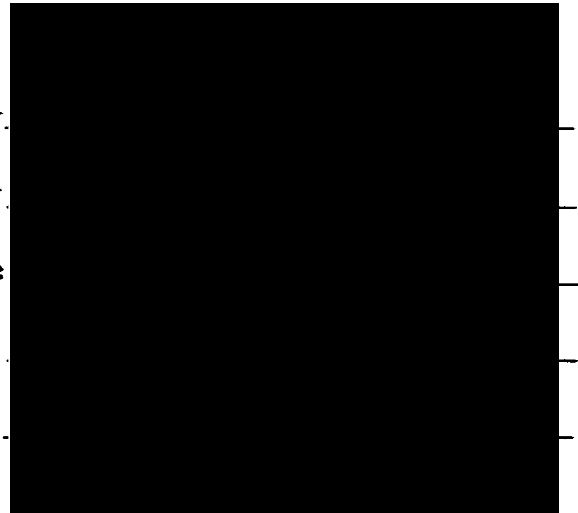

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ABSTRACT

This project was two-part, consisting of both extensive field and laboratory studies. This work has focused on the reproductive cycle of winter flounder and particularly on related endocrine disruption due to hydrocarbon contaminant exposure. The goal of the field study was to assess the effects of sediment contaminants on the main components of the vitellogenic cycle in winter flounder, and determine seasonal and inter-annual variations. The objectives of the laboratory experiment were to monitor the complete reproductive cycle of winter flounder in captivity, confirm that contaminated sediments alone could cause adverse effects to winter flounder reproduction and validate non-destructive sampling of fish for the purpose of biomonitoring.

The field work aspect of this research took place in Sydney Harbour (Nova Scotia, Canada), which is one of the most polycyclic aromatic hydrocarbon contaminated estuaries (PAH) in North America. Concentrations of PAHs above 500 ppm (wet weight) have been measured in the sediments. Five stations were selected representing a gradient of bottom sediment PAH concentrations ranging from 200 ppm to less than detectable. Feral winter flounder were sampled at three different stages of the reproductive cycle for two consecutive years (30 females and 10 males at each station). Ten variables were used in a principal component analysis, which clearly demonstrated the existence of an effect of the site of capture. When analyzing the effect of the sampling site on individual variables (ANOVA), the response was not as clear cut (in part because of the high variance associated with some of the variables). However, serum estradiol concentrations were found to be significantly reduced in fish captured at stations with sediment PAH concentrations at or above 10 ppm. In the same way, egg maturation was significantly retarded for part of the reproductive cycle in fish from the same stations. Furthermore, between 25% and 30% of the fish from the two most contaminated sites (50 ppm and 200 ppm) did not spawn. Female serum vitellogenin concentrations were inconclusive, but a significant increase of serum vitellogenin in males was measured. This phenomenon correlated positively (and highly significantly) with sediment PAH concentrations, suggesting the possibility of an estrogenic effect of the contamination.

In the laboratory, an attempt was made at reproducing the conditions found in the Sydney Estuary. Three treatment tanks for female fish were set up, having PAH sediment concentrations of 168, 41 and 20 ppm respectively, a control (female fish on clean sediment) and a negative control (male fish on clean sediment). Each tank was randomly assigned 30 individually tattooed fish captured at an uncontaminated site. Fish were sampled each month for two complete reproductive cycles. The results were more difficult to interpret than in the field. Heavy PAH metabolites significantly accumulated (up to 100 times) in the bile. Growth was reduced, and mortality and fin erosion were increased in the contaminated tanks. Estradiol concentrations were slightly lower in the treated fish. Contrary to the field results, vitellogenin concentrations tended to be more sensitive than estradiol in detecting significant differences between treatments and suggested the existence of an estrogenic effect at the intermediate PAH concentration (41 ppm). Maybe the most interesting and significant result of the laboratory work, was that the number of fish failing to spawn correlated positively with the level of contamination in the tank, ranging from less than 10% to more than 30%, similar to field results.

This study has demonstrated that coal-tar contaminated sediments significantly adversely affected some aspects of the reproduction of winter flounder in Sydney Harbour. It is estimated overall, that under the present conditions an average of 10% of the female winter flounder from the Sydney estuary fail to mature and spawn.

LIST OF ABBREVIATIONS

%	percent
μg.g	microgram per gram
μl	microlitre
μm	micrometer
μM	micromolar
$\mu g.l^{-1}$	micrograms per litre
1-NAP	1-naphthol
1-OH-P	1-hydroxypyrene
2-NAP	2-naphthol
4-PHE	4-phenylphenol
9-ANT	9-anthraldehyde
9-OH-FL	9-hydroxyfluorene
9-PHE	9-phenanthrol
Ah	aromatic hydrocarbon
ALGH-I	aldehyde dehydrogenase ezyme
ANOVA	analysis of variance
ANT	anthraldehyde isomers
Anthr	anthracene
ANTQ	anthraquinone
Athe	acenaphthalene
Athy	acenaphthylene
BaA	benzo(a)anthracene
BaP	benzo(a)pyrene
BASF	Biota Sediment Accumulation Factor
BbFlu	benzo(b)fluoranthene
BghiPeryl	benzo(ghi)perylene
BkFlu	benzo(k)fluoranthene
BNF	β-naphthoflavone
Bzaa	benzo(a)anthracene
Bzap	benzo(a)pyrene

Bzbk	benzo(b+k)fluoranthene
Bzep	benzo(e)pyrene
Bzghi	benzo(ghi)perylene
Chrys	chrysene
cm	centimeter
CYP1A1	cytochrome P4501A1 gene
CYP1A2	cytochrome P4501A2 gene
DDD	dichlorodiphenyldichloroethane
DDE	dichlorodiphenylethane
DDT	dichlorodiphenyltrichloroethane
Di-b-A	dibenzo(ah)anthracene
Dibz	dibenzo(ah)anthracene
DNA	deoxyribonucleic acid
EH	epoxide hydrolase
EPS	Environmental Protection Agency
ER	estrogen receptor
ERE	estrogen response element
EROD	7-ethoxyresorufin-O-deethylase
FCI	fish condition index
Fe	iron
Flu	flurene
Fluor	fluoranthene
g	gravity
GB	George's Bay
GC/MS	gas chromatography/mass spectrometry
GnRH	gonadotropin-releasing hormone
GSI	gonadosomatic index
GST	glutathione S-transferase
Gt-I	glutathione transferase enzyme
H^+	proton
H_2O	water
H_2O_2	peroxide
HSI	hepatosomatic index

Indo	indopyrene
$kg.m^{-3}$	kilograms per cubic meter
L	Length
$L.min.^{-1}$	litres per minute
m	meter
m^3	cubic meter
Met-2-Nap	methyl-2-naphthalene methanol
MFO	mixed function oxygenase
$mg.kg^{-1}$	milligrams per kilogram
MH	Main Harbour
min.	minute
MP	melting point
mRNA	messenger ribonucleic acid
MS 222	tricaine methanesulfonate; 3-aminobenzoic acid ethyl ester ethanesulfonate
MW	molecular weight
N-V	non-vitellogenic
n.d.	not detectable
NA	Northwest Arm
NaCl	sodium chloride
NaOH	sodium hydroxide
Naph	naphthalene
Naph	naphthalene
$ng.g^{-1}$	nanograms per gram
$ng.ml^{-1}$	nanogram per milligram
$ng.ml^{-1}$	nanograms per millilitre
Nmo-I	NAD(P)H:menadione oxidoreductase enzyme
°C	degrees Celsius
OH	hydroxide
OH-FL	hydroxyflourene
P4501A	cytochrome P4501A1 gene
PAHs	polycyclic aromatic hydrocarbons
PCBs	polychlorinated biphenyls

pers. obs.	personal observation
Peryl	perylene
PHE	phenanthrol
PHE-9	phenanthrene-9-carboxaldehyde
Phen	phenanthrene
ppm	parts per million
ppt	parts per thousand
Pyr	pyrene
RIA	radioimmunoassay
RNA	ribonucleic acid
SB	South Bar
SD	standard deviation
ST	sulfotransferase
SYS	Sysco
Tris-HCl	Tris-hydrochloric acid
UDPGT	UDP-glucuronosyl-transferase
Ugt-I	UDP-glucuronosyltransferase enzyme
VTG	vitellogenin
W	weight
W_e	eviscerate weight
W_g	gonad weight
W_l	liver weight
WSF	water soluble fraction

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CHAPTER 1

Vitellogenesis in fish and the effects of polycyclic aromatic hydrocarbon contaminants

INTRODUCTION

In the last two decades, public concern about environmental preservation has steadily increased, largely because of scientific and media attention to major pollution accidents worldwide. Thus, the monitoring of the effects of environmental contamination on wildlife and human populations has become a political and, in some cases, a marketing issue. This has been the driving force behind the development of recent monitoring techniques involving biological organisms (i.e. biomonitoring). These techniques have been particularly useful in the survey of aquatic environments.

Polycyclic aromatic hydrocarbons (PAHs) constitute one of several classes of organic molecules released into the environment largely as the result of human activities (GESAMP, 1993; 1991; Neff, 1985; Vandermeulen, 1984), resulting in the ubiquity of these toxic chemicals in the marine environment. Unlike other anthropogenic sources of contamination, emissions of PAHs are very difficult to control. The main reason for this is that the primary source of PAHs is the combustion of carbon based materials (fossil fuels, wood, plastics, petroleum derivatives, etc.). As a consequence of increasing human population and economic development of many countries, the demand for energy is increasing (Cohen, 1995) and so is the global amount of PAHs released to the environment. Even if PAHs do not seem to be an immediate threat to the marine environment at the global scale (GESAMP, 1993), there are reasons for concern. This is true at the local level in coastal areas and on a broader scale, because of the probable exponential increase of emissions linked to economic development. Furthermore, PAHs have been demonstrated to be mutagenic and carcinogenic precursors (Maccubin, 1994; McElroy et al., 1991; Gelboin and Ts'o, 1981; Lutz, 1979), as well as impair some aspects of fish reproduction (as well as other organisms). In female fish, these impairments can occur at different stages of vitellogenesis.

Vitellogenesis is the process through which maturing oocytes in the ovary accumulate yolk. In most species of fish, this maturation process is seasonal and spawning only occurs once in the yearly reproductive cycle. Consequently, any factor causing an impairment of the vitellogenic cycle can dramatically reduce the reproductive success

(number of eggs, hatching rate and viability of embryos) of an individual. This, in turn, has the potential to affect the entire population of the species.

The objective of this chapter is to review our understanding of the effects of PAHs on vitellogenesis in fish. After providing relevant background information on PAHs and the biological pathways of toxic effects associated with these chemicals, this review discusses several aspects of the impairment of the vitellogenic cycle in fish exposed to PAHs and other organic compounds, both in field studies and in laboratory experiments. This is followed by a discussion of the use of bioindicators to monitor environmental contamination (biomonitoring), and by recommendations for future environmental surveys.

The research project presented herein was two-part, consisting of both extensive field and laboratory studies. This work has focused on the reproductive cycle of winter flounder and particularly on related endocrine disruption due to hydrocarbon contaminant exposure. The goal of the field study was to assess the effects of sediment contaminants on the main components of the vitellogenic cycle in winter flounder, and determine seasonal and inter-annual variations. The objectives of the laboratory experiment were to monitor the complete reproductive cycle of winter flounder in captivity, confirm that contaminated sediments alone could cause adverse effects to winter flounder reproduction and validate non-destructive sampling of fish for the purpose of biomonitoring.

BACKGROUND INFORMATION

Contamination of the marine environment by anthropogenic substances is virtually global. At the scale of the open ocean there seem to be few detectable adverse effects of human activities. On the other hand, because most sources of contamination (e.g. ocean dumping, atmospheric emissions, industrial and urban effluents) are land based, there is a strong impact and a wide variety of adverse effects of these contaminants at the local level. Coastal zones in particular show the highest levels of pollution, and this should be of concern for several reasons:

- the coastal zone is the natural habitat of a large number of species, many of them commercially important;

- there is a risk of direct impact of contaminated coastal areas on human health, as more than 50% of the human population reside in these areas;
- coastal zones often are the location of nursery areas for many species of fish, and some of the open ocean species come into coastal waters for a part of their life cycle.

1. Marine pollution

The GESAMP (Joint Group of Experts on the Scientific Aspects of Marine Pollution; 1993) report commissioned by the United Nations, defines marine pollution (p iii) as "*the introduction by man, directly or indirectly, of substances or energy into the marine environment (including estuaries) resulting in such deleterious effects as harm to living resources, hazards to human health, hindrance to marine activities including fishing, impairment of quality for use of seawater and reduction of amenities.*"

Traditionally, humans have considered the world an infinite body, capable of supporting an unlimited number of individuals and assimilating their waste. While this might have been a reasonable assumption as long as the human population remained small, the exponential increases of both population and industry (and with it energy demand) have made this presumption obsolete. Unfortunately, the perception of the world has not evolved as rapidly. As a result many ecosystems are threatened, and waste management has become a critical issue.

Human health is at risk directly by exposure to hazardous chemicals and indirectly by the loss of critically important ecosystems that can affect global ecology.

1.1 Contaminants of prime concern

The perception of what are the most hazardous substances released into the environment varies. In a 1991 report, the GESAMP provided a summary table of the substances that give rise to the greatest concern (Table 1). The order of entry in this table qualitatively ranks the severity of adverse effects associated with the release of these substances into the marine environment. This ranking does not reflect the general perception that synthetic organic chemicals are of the highest concern. Interestingly, persistence of contaminants in the marine environment was not accounted for in this table and is an important factor affecting their potential long-term toxic effects.

As mentioned in the introduction, this review will focus on organic contaminants and PAHs specifically. These chemicals have been demonstrated to be the cause of numerous biological disorders at concentrations that are commonly found in urbanized areas (Vandermeulen and Mossman, 1996; Johnson et al., 1988; Payne et al., 1988). Because of their low water solubility, PAHs have a tendency to adsorb to particulate matter and sink to the bottom sediments (Neff, 1985).

In fact, the GESAMP (1993; p 77) stated "*that sediment levels of PAH in the range 3 to 5 ppm are of importance in producing adverse biological effects in fish, namely flatfish, which exist in intimate contact with the sediment. [...] It is of interest that values of 3 to 5 ppm generally represent a concentration approximately 10 times the background, or more, for PAHs from combustion sources.*"

The following sections will review the structure, sources and bioavailability of PAHs in the marine environment.

2. Polycyclic aromatic hydrocarbons

PAHs constitute one of several classes of organic molecules released into the environment largely as the result of human activities (GESAMP, 1993; 1991; Neff, 1985; Vandermeulen, 1984).

2.1 Chemical structure

PAHs are large organic molecules composed of two or more aromatic rings (Fig. 1). The low molecular weight (MW) PAHs ranging from naphthalene (MW=128.16) to coronene (MW=300.36) are of primary environmental concern (Neff, 1985) because of their higher solubility and bioavailability. Larger molecules have such low solubility and volatility that they are virtually immobile.

Electrophilic substitution, oxidation and reduction are the three types of chemical reactions that affect PAHs (Buhler and Williams, 1989; Neff, 1985). The latter two involve the breakdown of the aromatic structure of the molecule. All three types of reactions can take place inside an organism where they are catalyzed by enzymatic activities (see Section 3).

Table 1: Priority contaminants from land-based sources (from GESAMP; 1991):

Substance	Status of Science and Management	Known/Suspected Targets/Effects
Sewage	Science Adequate Inadequate Management	Human Health Pathogens Eutrophication
Nutrients	Science Limited Conservative Management Possible	Eutrophication Harmful Algal Blooms
Synthetic Organics	Science Limited Conservative Management Possible	Human Health Animal Health
Sediment	Science Limited Conservative Management Possible	Destruction of Amenities (Habitat/Organisms) Decreased Productivity
Litter	Science Adequate Management Deficient	Animal Life Destruction of Amenities
Metals	Science Adequate Management Deficient	Human Health Animal Health
Radionuclides	Science Adequate Management Adequate	Human Health Animal Health
Oil/Hydrocarbons	Science Generally Adequate Management Deficient	Animal Health Destruction of Amenities Decreased Productivity
PAHs	Science Limited Management Deficient	Human Health Animal Health Foodstuff Taint

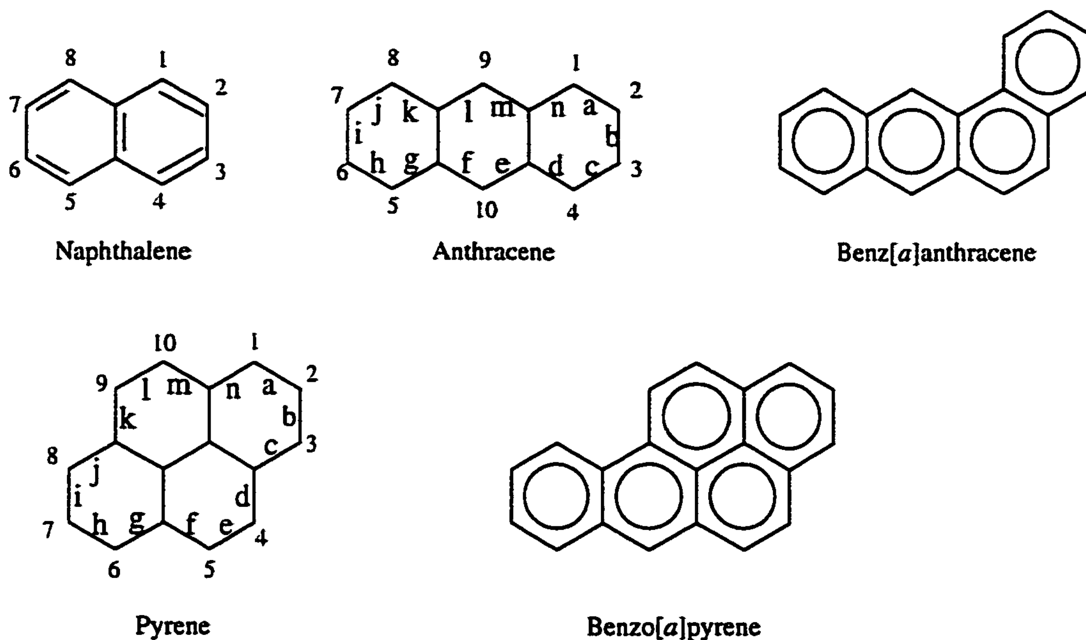


Figure 1: Chemical structure of five specific PAHs (Naphthalene, Anthracene, Benz[*a*]anthracene, Pyrene, Benzo[*a*]pyrene). Carbon atoms are numbered in a clockwise direction starting with the carbon atom that is not part of another ring and is in the most counterclockwise position of the uppermost ring farthest to the right; carbon atoms common to two or more rings are not numbered. Ring faces (except for those common to two rings) are lettered in alphabetical order starting with the side between carbon atoms 1 and 2. Alphabetical order is continued clockwise around the molecule.

2.2 Sources, distribution and bioavailability of PAHs in the marine environment

2.2.1 Sources

PAHs have been reported to be formed through three types of processes (Neff, 1985; Stegeman, 1981): low to moderate temperature diagenesis of sedimentary organic materials to form fossil fuel, direct biosynthesis by micro-organisms and plants, and high temperature combustion of organic materials (pyrolysis).

Total yearly input of PAHs into the aquatic environment is estimated to be approximately 230,000 tonnes (GESAMP, 1993; Neff, 1979), of which 4-5% (10,000 tonnes) originates directly from crude oil discharges. The remaining is the result of high temperature combustion of carbon containing materials, surface runoffs, and atmospheric fallout.

2.2.2 Distribution

Except for oil spills and seepage, the main sources of PAHs are land based, entering the aquatic environment via rivers and estuaries. Not suprisingly, this is reflected by the marine environment as the highest concentrations of PAHs are found in coastal zones. However, on a global scale, PAHs associated with airborne particles are the main source of marine contamination (Neff, 1985).

Generally, PAHs are not distributed evenly; concentrations tend to decrease with distance from the source of contamination. Because of their low solubility and hydrophobic chartacter, PAHs tend to adsorb to particulate matter and sediment out (Neff, 1985; Stegeman, 1981). They have also been shown to adsorb to organic colloidal material (Witjayaratne and Means, 1980). As a result, bottom sediments become the ultimate sink for PAHs and can contain concentrations 1000 times higher than the overlying water column (Neff, 1985).

Domestic sewage typically contains up to 1 $\mu g.l^{-1}$ of total PAHs, industrial sewage 5-15 $\mu g.l^{-1}$ and sewage sludge 1-30 $mg.kg^{-1}$ (Neff, 1985). Water bodies in the vicinity of heavily industrialized areas typically display PAH levels of 1-5 $\mu g.l^{-1}$ (Neff,1979).

Finally, extremely contaminated sediments have been reported to contain up to 500 mg.kg^{-1} (Vandermeulen, 1989).

2.2.3 Bioavailability

Bioavailability of PAHs is a function of several abiotic and biotic factors. Solubility is the main factor affecting abiotic PAH bioavailability. The available evidence indicates that sediment-adsorbed PAHs have a limited bioavailability (Neff, 1985). Neff (1985; p 435) also points out that "*accumulation of PAH from sediment, when it occurs at all, may be attributed in large part to uptake of PAH desorbed from sediment particles into the interstitial water*". However, bioturbation can reportedly increase the bioavailability of PAHs by resuspending sediment bound molecules and/or producing PAH metabolites (Aller, 1978; Wood, 1975). Bioavailability of sediment PAHs has been shown to also depend on the origin (i.e. the structure) of the molecules: PAHs originating from oil are more bioavailable than PAHs with a pyrogenic origin (Varanasi et al., 1985; Farrington et al., 1983). This observation is true even if sediment concentrations of pyrogenic PAHs are higher than those of oil PAHs (Farrington et al., 1983) and is consistent with the physical-chemical speciation of PAHs in an estuarine ecosystem (Rheadman et al., 1984).

Conversely, the water-soluble fraction of PAHs has been demonstrated to be far more bioavailable than sediment-bound PAHs (Lee et al., 1972). Availability of water-soluble PAHs decreases with molecular weight and octanol-water partition coefficient (Neff, 1985; Lee et al., 1972).

Temperature and salinity can also affect PAH bioavailability by modulating the solubility of the chemicals. Lower temperatures and higher salinities have been shown to decrease PAH solubility (Whitehouse, 1984).

The two principal biotic factors affecting PAH bioavailability are behaviour and feeding strategy. Depending on the ecological niche of an animal, it will be exposed to different sources and different concentrations of PAHs. Since sediments are a sink for PAHs, bottom dwelling organisms such as echinoderms, molluscs, polychetes and flatfish will tend to be exposed to higher levels of PAHs. Yet, as was discussed earlier, the water

soluble fraction of PAHs is more toxic than the larger molecular weight sediment-bound fraction.

PAHs and their metabolites have been shown to bioaccumulate in invertebrates, making them available to predators for contamination through the food chain (Gardner et al., 1991; Kleinow et al., 1989; McElroy and Sisson, 1989; Foster et al., 1987; Mix, M.C., 1986; Vandermeulen and Penrose, 1978). However, there seems to be no evidence of biomagnification of PAHs through the food chain (D'Adamo et al., 1997; Dunn and Fee, 1979), as some invertebrates and all vertebrate species studied to date, rapidly metabolize PAHs into more soluble molecules (see Section 3.3).

3. Biological pathways of toxic effects of PAHs

3.1 Toxicity

3.1.1 Acute toxicity

Generally, acute toxicity of PAHs decreases as a function of molecular weight (i.e. naphthalene would be the most toxic PAH) (Neff, 1985; Stegeman, 1981). However this is not an absolute rule, and PAHs of similar molecular weight can have different toxic effects. It also seems that molecules containing four rings or more have low acute toxicities (Neff, 1985). This is apparently because their solubility falls below the aqueous concentration required to cause a response (Gehrs, 1978). Hence the most toxic molecules are also the most mobile, which means that they are the most bioavailable, and have the shortest residence time (Malins et al., 1980). PAH acute toxicity is thought to be mainly the result of interference with cellular membrane functions (Neff, 1985). Aromatic hydrocarbons, in general, tend to bind preferentially to the surface of plasma membranes (Roubal and Collier, 1975; Roubal, 1974), causing perturbations of surface organization (i.e. modifying permeability) and affecting the activity of essential membrane-associated enzymes (Neff, 1985).

3.1.2 Chronic sublethal toxicity

Chronic exposure to low concentrations of PAHs in water, sediment or food can cause sublethal effects in aquatic organisms which may affect the long-term survival of the organism (Neff, 1985; McCain et al., 1978). Moreover, chronic sublethal exposure to PAHs has been shown to induce mutations and cancer (see section 3.3). Sublethal effects of PAHs in fish include hyperventilation, reduced feeding rate (Malins et al., 1980), depression of the immune system resulting in secondary infections (Payne and Fancey, 1989; Khan, 1986), reduced lymphocyte proliferation (Faisal and Huggett, 1993), increased stress (estimated by elevated cortisol concentrations) (Fletcher et al., 1981), histologic and skeletal abnormalities (Hose et al., 1984), necrosis of neurosensory cells of tissues in contact with PAHs, induction of detoxification enzymes (Neff, 1985; Malins et al., 1980) and reduced fecundity (Ghosh and Thomas, 1995; Black et al., 1988). Early life stages are the most susceptible to chronic sublethal exposure (Hose et al., 1984). Some research has also demonstrated that effects are greater at low salinities (Neff, 1985).

In many aquatic organisms PAHs are rapidly metabolized by detoxifying enzymes of the monooxygenase family. Thus most of the toxic effects associated with PAH contamination may be the result of highly reactive metabolites (such as 7,8-diols). This aspect of PAH toxicity will be reviewed in more detail in Section 3.3.

3.2 Bioaccumulation

Fish readily accumulate PAHs from environmental sources, including from the water column, sediments and food (Hellou and Warren, 1997; Kleinow et al., 1989; McElroy and Sisson, 1989; McElroy et al., 1989; McCain et al., 1978). However, most studies of PAH bioaccumulation show a decrease of parent compounds in the organisms which coincides with the appearance of metabolites (Vandermeulen et al., unpubl. data; Varanasi and Stein, 1991; Goddard et al., 1987; Krahn et al., 1987; Statham et al., 1978). Using radio-labeled molecules, laboratory studies have demonstrated that only 10-15% of force-fed PAHs were retained by fish (Vandermeulen et al., unpubl. data; Lech and Vodicnik, 1985; Malins et al., 1980). Most of this fraction was rapidly metabolized. Such

studies suggest that biomagnification of PAHs along the food chain may be negligible in many fish species.

The distribution of PAHs and the loadings per tissue type seem to be influenced, in part, by the fat content of the tissue. Tissue function plays an important role as does relative position to the point of entry of the contaminant (i.e. from the gut if the PAHs were force fed) (Vandermeulen et al., unpubl. data; Hellou and Warren, 1997; Al-Yakoob et al., 1993; Goddard et al., 1987). Furthermore, accumulation is affected by the type of fat present in the tissue, as triglycerides have the highest potential for bioaccumulation of PAHs.

Reported results seem to indicate that the tissue accumulations of metabolites parallel those of the parent compound (Vandermeulen et al., unpubl. data; Krahn et al., 1987). However, generalizations should certainly not be made based on the information available, as rates of metabolism of PAHs are very specific and can even differ substantially from individual to individual.

As a general rule, estimates of body burdens of PAHs in organisms should include accumulation of parent compounds *and* their metabolites (Van Der Oost et al., 1991). This would provide a better estimate of the ability of the organisms to metabolize the chemicals considered.

3.3 Biotransformation

Many xenobiotics, including PAHs, are so lipophilic that they would remain in the body of an organism if they were not metabolically rendered more water-soluble (Pelkonen and Nebert, 1982). The enzymatic biotransformation reactions associated with this process are usually grouped into two categories; Phase I and Phase II reactions. Phase I reactions introduce one or more polar groups into the target molecule (e.g. PAH, PCB [Poly Chlorinated Biphenyls], steroids: Stegeman and Hahn, 1994; Andersson and Förlin, 1992; Buhler and Williams, 1989; Pelkonen and Nebert, 1982). Phase II reactions conjugate the xenobiotic molecules or their Phase I metabolites with endogenous molecules (Andersson and Förlin, 1992; Buhler and Williams, 1989; Pelkonen and Nebert, 1982). At this point the products are usually sufficiently water soluble to be excreted (via bile, gill or kidney).

3.3.1 The Aromatic hydrocarbon-response (Ah) receptor

The response of animals to aromatic hydrocarbon contamination can result from a receptor-mediated induction of Phase I and Phase II metabolizing enzymes (Nebert et al., 1990). The Aromatic hydrocarbon-response (Ah) receptor is one such receptor which has been extensively studied. A wide variety of aromatic compounds are known to bind to the Ah receptor, including among many others PAHs, PCBs, dioxins and sex steroids. By binding to the receptor, the ligand induces a transcription of each of the Ah-dependent genes known as the Ah gene battery. In the mouse for example (Nebert et al., 1990), the Ah-receptor dependent induction by dioxins involves two Phase I genes, cytochrome P4501A1 (or CYP1A1) and cytochrome P4501A2 (or CYP1A2), and four Phase II genes, NAD(P)H:menadione oxidoreductase (*Nmo-1*), aldehyde dehydrogenase (*Aldh-1*), UDP-glucuronosyltransferase (*Ugt-1*), and glutathione transferase (*Gt-1*).

3.3.2 Cytochrome P450-dependent mixed function oxygenase (MFO) Phase I reaction enzymes

Xenobiotic metabolizing enzymes have been the focus of many studies in the last 20 years (for reviews see Stegeman and Hahn, 1994; Andersson and Förlin, 1992; Buhler and Williams, 1989; Foureman, 1989; Payne et al., 1987; Pelkonen and Nebert, 1982; Stegeman, 1981). The primary role of these enzymes is to rid the body of potentially toxic lipophillic organic molecules.

The cytochrome P450 dependent MFO are the main enzymes involved in phase I reactions. MFO are a family of membrane bound enzymes that are principally located in the endoplasmic reticulum (Goksøyr and Förlin, 1992; Larsen et al., 1992; Smolowitz et al., 1989). Although present in most organs of the body, the liver is the site of the highest activity (Smolowitz et al., 1989; Payne and May, 1979). These enzymes catalyze the introduction of a polar group into an organic molecule through an oxidative process (Fig. 2) (Lech and Vodicnick, 1985; Pelkonen and Nebert, 1982; Stegeman, 1981). This results in increased water solubility of the molecule and provides a substrate for the phase II enzymatic reactions.

The synthesis of the P450 enzymes has been shown to be induced by many chemicals, including PAHs, in a dose dependent manner (Goksøyr and Förlin, 1992; Stegeman and Lech, 1991; Elskus and Stegeman, 1989; Ahokas and Pelkonen, 1984). This feature has been of prime interest for the use of MFO as bioindicator of environmental contamination. PAH induction of MFO is rapid (12-18 hrs) and can persist over several weeks (Vandermeulen et al., unpub. data; Kloepper-Sams and Stegeman, 1989). Pre-exposure and/or multiple exposure of fish to certain PAHs has been shown to increase the rate of metabolism of the chemical (Carr and Neff, 1988). The mechanism of the induction is primarily the promotion of the transcription of the P450 gene, specifically P4501A. This results in the production of new cytochrome P450 proteins which, after activation, yields the catalytically active enzymes (for review see Stegeman and Hahn, 1994; Goksøyr and Förlin, 1992). Table 2 provides a few examples of xenobiotic inducers of the P4501A gene family and the main substrates of the induced enzymes.

P450-dependent enzymes may have two biological functions according to the type of substrate of the reaction: synthesis and degradation of endogenous substrates, and metabolism of foreign compounds (Stegeman and Hahn, 1994; Foureman, 1989; Pelkonen and Nebert, 1982; Parke, 1981; Stegeman, 1981). As shown in Table 2, endogenous substrates include sex steroids, and xenobiotic substrates include organic chemicals (PAH, PCB), pesticides (DDT), drugs, and plant derived compounds (e.g. caffeine).

The P450 activity is regulated by steroids. In fish, cortisol (kidney) and estradiol (ovary) seem to be the main hormones involved in this process (Koivussaari et al., 1984; Vodicnik and Lech, 1983; Hansson et al., 1982; Hansson and Gustafsson, 1981). Consequently, PAH metabolism is intimately linked to physiological and hormonal cycles and displays strong sexual-seasonal and individual variability (Linström-Seppä and Stegeman, 1995; Larsen et al., 1992; George et al., 1990; Stegeman and Chevion, 1980). In this way, PAHs can have an indirect effect on the reproductive cycle of fish, and measurments of hormone levels may be a useful biomarker, providing another method of detecting contaminant effects.

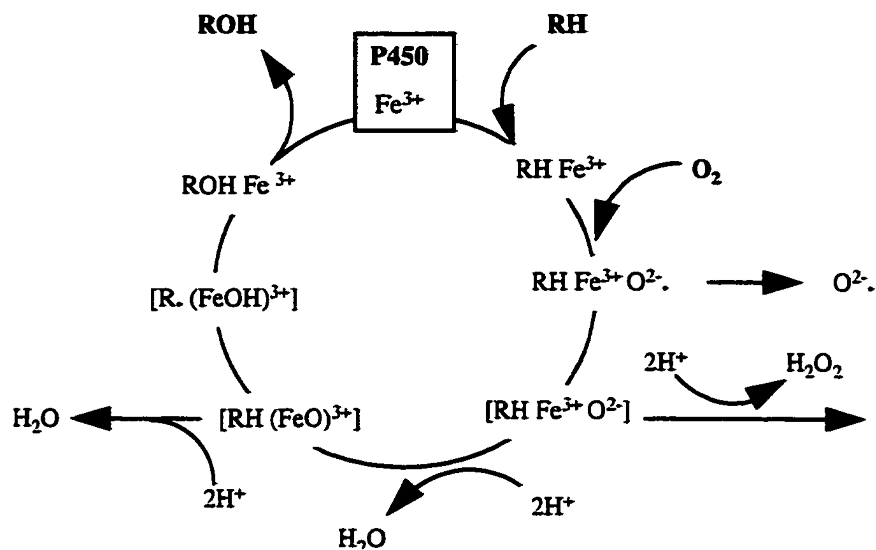


Figure 2: P450 catalytic cycle (from Stegeman and Hahn, 1994).

Table 2. Induction in selected P450 gene subfamilies (Phase I).

Gene family and subfamily	Selected protein members	Prominent substrates	Common inducers
P4501A (or CYP1A)	1A1	PAH, planar PCB, 7-ethoxyresorufin	PAH, planar PCB, BNF, chlorinated dioxins and furans
	1A2	Estradiol, caffeine	PAH, planar PCB, BNF, chlorinated dioxins and furans

From Stegeman and Hahn, 1994. Abbreviations used: PCB, polychlorinated biphenyls; BNF, β-naphthoflavone.

3.3.3 Conjugating enzymes: Phase II reaction enzymes

Following the oxidative processes catalysed by the cytochrome P450-dependent MFO, some PAH metabolites may undergo further enzymatic metabolism. The enzymes involved in this secondary metabolism are called conjugating or phase II enzymes. They include glutathione S-transferase (GST), UDP-glucuronosyl-transferase (UDPGT) and sulfotransferase (ST) (George, 1994; Foureman, 1989; Lech and Vodicnik, 1985). One of the effects of these enzymes is to increase the polarity of the xenobiotic metabolites thus increasing their solubility and subsequent excretion (James, 1986; Gelboin and Ts'o, 1981). This result is acheived by conjugating endogenous molecules with the metabolites of the foreign compounds (Fig. 3).

Contrary to MFO, conjugating enzymes activities do not seem to be significantly induced by the presence of the pollutants. Mammalian UDPGT and GST for example, are only increased by two to five fold at the most in the presence of PAHs, whereas P4501A levels can be induced by one to two orders of magnitude (George, 1994; Scott et al., 1992). Results for phase II enzyme activities in fish are equivocal and species specific (Table 3).

Of the three enzymes listed previously, UDPGT and GST have received the most attention in fish as they are the prominent pathways of conjugation (Leaver et al., 1992; Baumann, 1989; Lech and Vodicnik, 1985; Varanasi et al., 1984; Varanasi and Gmur, 1981). UDPGT catalyzes the conjugation of glucuronic acid with an acceptor substrate (George, 1994; Foureman, 1989). Glucuronide conjugates are then excreted in the bile or the urine (Clarke et al., 1991; Fouremen, 1989). GST conjugates the nucleophilic thiol group of the glutathione molecule with the electrophilic metabolites generated by the monooxygenases (Foureman, 1989; Stenersen et al., 1987). The glutathione conjugates are then further metabolized and ultimately excreted in the urine as mercapturic acids (Foureman, 1989).

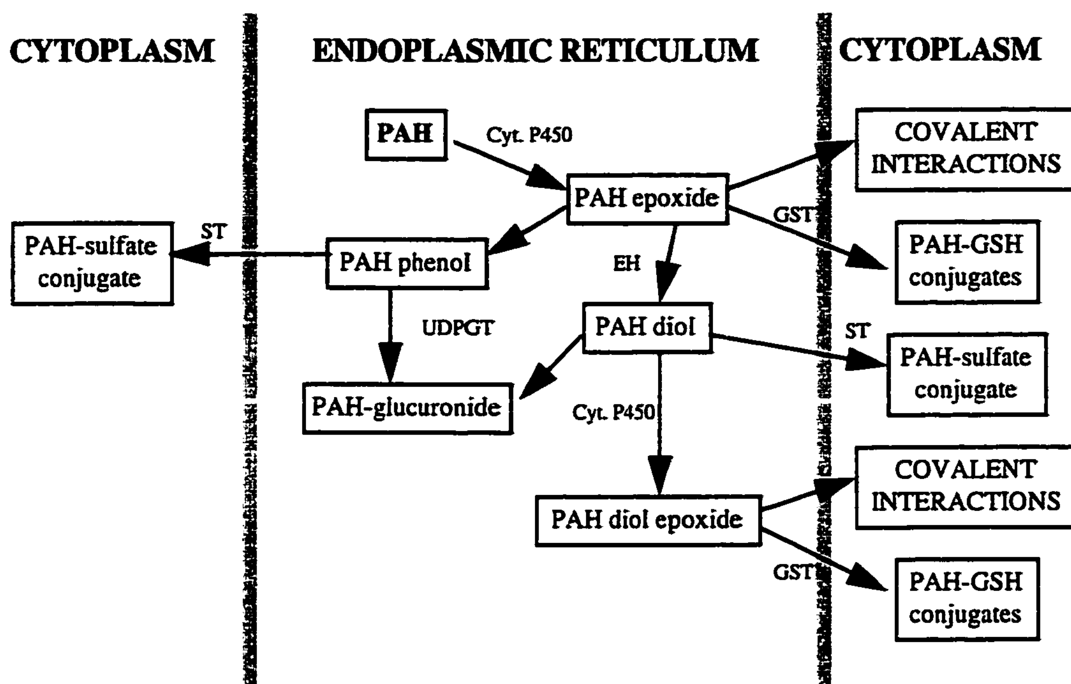


Figure 3: Representation of the possible metabolic fates of PAHs and their metabolites in the cell. Phase I reactions involve Cyt. P450 and EH, phase II reactions involve GST, ST and UDPGT. Abbreviations used: EH: epoxide hydrolase (From Foureman, 1989).

Table 3: Effect of inducer treatments on hepatic GST activity in different fish (from George 1994).

Species	Inducer	Effect
Cod	β-Naphthoflavone	No effect
Flathead minnow	3-Methylcholanthrene	No effect
Flounder	3-Methylcholanthrene	Repressed
	Arochlor 1254	No effect
	trans-Stilbene oxide	Induced
Killifish	3-Methylcholanthrene	No effect
Plaice	3-Methylcholanthrene	Induced
	Arochlor 1254	Induced
	trans-Stilbene oxide	Induced
	Butylated hydroxyanisole	Induced
Sheepshead minnow	3-Methylcholanthrene	No effect
Trout	β-Naphthoflavone	No effect
	β-Naphthoflavone	Slightly induced
	Clophen A50	Slightly induced
Anabas	Phenol	Induced

3.3.4 Metabolic activation

Metabolic activation of pollutants by the MFO enzymes produces highly reactive intermediates which have been demonstrated to bind with cellular macromolecules such as DNA, RNA and protein (Peters et al., 1997; Varanasi et al., 1989; Lech and Vodicnik, 1985; Ahokas and Pelkonen, 1984; Lutz, 1979). Under normal physiological conditions, these metabolites are conjugated and transformed into non-reactive water soluble molecules by Phase II enzymes and excreted (see Section 3.3). However, if the P450 dependent system is induced by the presence of xenobiotics for example, the increased production of reactive metabolites may exceed the capacity of the conjugating (deactivating) Phase II enzymes (Ahokas and Pelkonen, 1984; Pelkonen and Nebert, 1982). The reactive intermediates would then have the opportunity to bind to the macromolecules and generate cellular disorders such as pre- and post-transcriptional disruptions, mutations and carcinogenesis (Maccubbin, 1994; Stegeman and Hahn, 1994; Baumann, 1989; Varanasi et al. 1989; Smolarek et al., 1987; Lech and Vodicnik, 1985). In fact, reactive intermediates are responsible for most of the toxicity of higher molecular weight PAHs. They have been demonstrated to be carcinogenic, mutagenic and cytotoxic (Moore et al., 1989; Gelboin and Ts'o, 1981; Stegeman, 1981; Lutz, 1979).

The present state of knowledge suggests that PAH-induced cancers are initiated by covalent interactions between the reactive metabolite of the toxicant and cellular DNA (Maccubbin, 1994; Varanasi et al., 1989; 1982; Lutz, 1979). Mutations can occur in the DNA if the repair system is not fast or accurate enough to remove adducts before the replication of the genetic material (Baan et al., 1994). In fish the most prominant carcinogens and mutagens seem to be the diol epoxides (for review see Buhler and Williams, 1989). These highly reactive molecules have been demonstrated to bind to DNA both in feral fish collected in contaminated sites and in laboratory-exposed animals (Peters et al., 1997; Maccubbin, 1994; McElroy et al., 1991; Lutz, 1979). DNA adducts have been used as indices of carcinogenic potential. However, the extent of binding of carcinogens *in vitro* has not always been found to be directly related to the development of cancer *in vivo* (Varanasi et al., 1989). Furthermore, the rate of DNA damage repair

seems to be relatively low in fish suggesting that these organisms may be at greater risk when exposed to environmental carcinogens such as PAHs (Walton et al., 1984; 1983).

4. Vitellogenesis in fish

The primary event during oogenesis is the synthesis by the liver and uptake by the oocytes of the yolk precursor protein vitellogenin (Johnson et al., 1991; LeMenn et al., 1988; DeVlaming et al., 1984; Wiegand, 1982). Oogenesis is triggered by environmental cues and controlled by a series of regulating hormones (Lam, 1983; Billard et al., 1978; DeVlaming, 1974) as illustrated by Figure 4. Under the influence of temperature and/or photoperiod for example (Bye, 1990; DeVlaming, 1972), the brain stimulates the pituitary gland to secrete gonadotropins (peptide hormones).

These are the gonadal function-regulating hormones in vertebrates (Campbell and Idler, 1976; Campbell et al., 1976) and they also promote meiotic maturation and ovulation (Harmin and Crim, 1992). The secretory functions of the pituitary are, in turn, regulated by brain neurohormones, such as gonadotropin-releasing hormone (GnRH), which is released by the hypothalamus (Browder et al., 1991). Once oogenesis is triggered, the gonadotropins are released into the bloodstream and carried to the ovaries where they induce oocyte growth, and ultimately, ovulation. They also stimulate the follicle cells to synthesize estrogens, primarily estradiol (Nagler and Idler, 1992; Singh and Singh, 1991; Vanderkraak et al., 1990). Estradiol is then released into the serum where it is bound by steroid-binding proteins or albumin (Lazier and MacKay, 1993; Pottinger, 1986; Lazier et al., 1985).

One of the functions of estradiol is to stimulate the liver to synthesize vitellogenin (MacKay and Lazier, 1993; Wallace, 1985; LeMenn et al., 1980; Emmersen et al., 1979; Emmersen and Petersen, 1976). Vitellogenin is a female-specific serum protein which contains phosphorus, lipids, carbohydrates, calcium and iron and has been identified as the egg-yolk precursor in most oviparous vertebrates (Nagler and Idler, 1990; Wiley et al., 1979; Craik 1978a, 1978b). Estradiol enters the hepatocytes by diffusion and is retained in target cells by high affinity binding to a specific receptor protein (ER: estrogen receptor) (Smith and Thomas, 1990; McPherson et al., 1988; Pottinger, 1986; Lazier et al., 1987,

1985; Sloop et al., 1984; Turner et al., 1981; Lazier and Haggarty, 1979). This results in activation of transcription of the vitellogenin *loci* (Pakdel et al., 1991; Tata et al., 1987; Lazier et al., 1987; Wiskocil et al., 1980). Binding affinity of ER for estradiol has been reported to increase in the presence of higher doses of estradiol (Anderson et al., 1996b; Donohoe and Curtis, 1996). Estradiol stimulation of hepatic cells also induces proliferation of rough endoplasmic reticulum and Golgi apparatus where modifications of the vitellogenin precursor take place (Wallace, 1985).

Vitellogenin is subsequently released into the bloodstream and is transported to the ovaries. It reaches the follicle via a capillary network located within the thecal layer of the follicle. After exiting the capillaries, vitellogenin passes through channels between the follicle cells to reach the oocyte surface. Under gonadotropin stimulation, vitellogenin is then incorporated by receptor-mediated endocytosis into yolk platelets (Tyler et al., 1991; Stifani et al., 1990; Tyler et al., 1990; Nunez Rodriguez and LeMenn, 1988; LeMenn et al., 1988; Wallace and Selman, 1981) where it is rapidly proteolytically cleaved to form the yolk proteins lipovitellin and phosvitin (Tyler et al., 1988; Wallace, 1978).

The presence of yolk-precursor has been demonstrated in the plasma of vitellogenic females of numerous teleosts (Ando and Matsuzaki, 1996; MacKay and Lazier, 1993; Vaisius et al., 1991; Tyler et al., 1991, 1990; Riazi and Fremont, 1988; DeVlaming et al., 1980; Emmersen and Petersen, 1976). Several studies using radiolabelled ^{32}P and ^{3}H-leucine have demonstrated that vitellogenin is the most prevalent phosphorus-containing protein in the blood of oviparous animals (Tyler et al., 1988; Whitehead et al., 1983; Emmersen and Petersen, 1976).

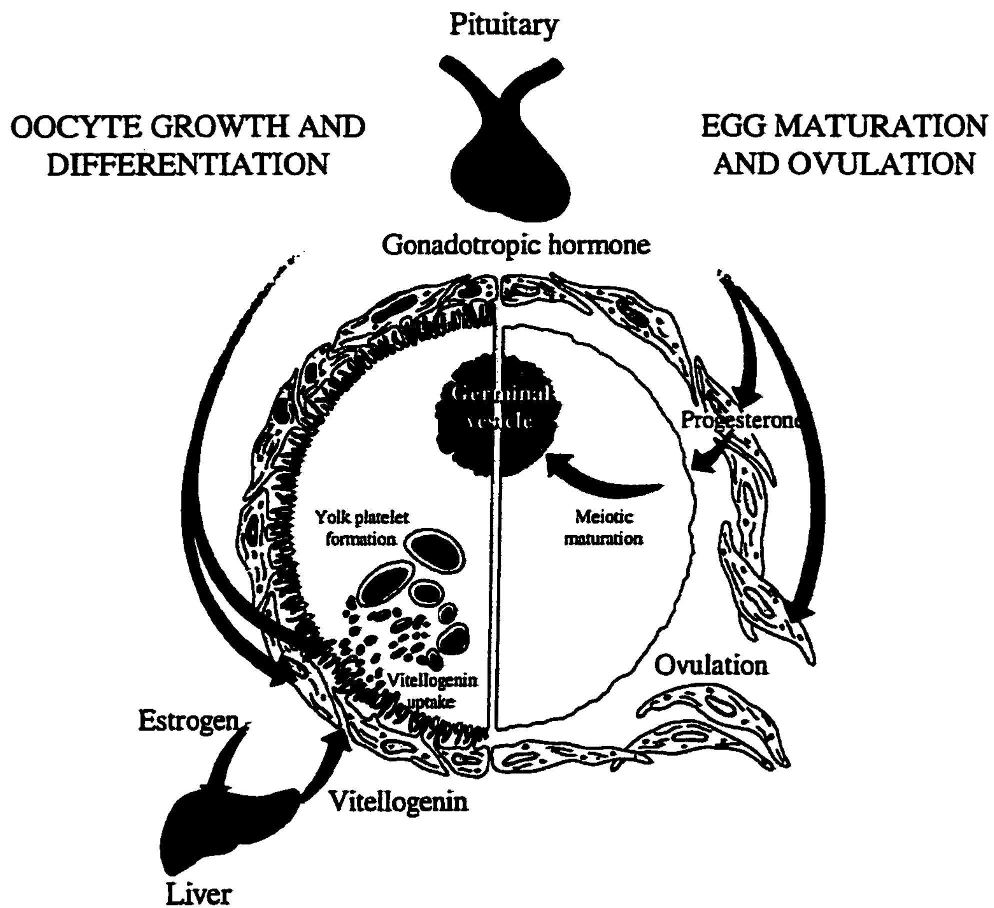


Figure 4: Example of hormonal regulation of oocyte growth and differentiation (left) and egg maturation and ovulation (right). From Browder et al., 1991.

EFFECTS OF PAHS AND OTHER ORGANIC XENOBIOTICS ON FISH VITELLOGENESIS

Fish reproduction can be sensitive to chemical and physical stressors (Johnson et al., 1992; Spies et al., 1990; Thomas, 1990a; Gerking, 1982). Vitellogenesis, in particular, is a complex series of hormonally regulated biochemical reactions (Peter and Crim, 1979). The sublethal effect of a contaminant on any of the steps described previously has the potential to affect the whole reproductive cycle of an organism. This, in turn, could result in a lower reproductive success of the species leading to a decline in the population. Stressor-induced changes of some of the reproductive parameters within an organism that could be correlated with reproductive impairment could, therefore, be valuable as early warning indicators of the potential long-term hazard of environmental deterioration at the population level (Thomas, 1990a).

5. Xenobiotic effects

Although the focus of this review is on the effects of PAHs on fish vitellogenesis, some results investigating the effects of other organic xenobiotics have been included. These other organic chemicals such as PCBs, DDT congeners and certain pesticides have been shown to interact similarly to PAHs with some biological systems. This does not imply however, that the effects generated by these substances would necessarily be the same as those generated by PAHs.

Field studies of contaminant effects on the reproductive cycle of fish regularly provide contradicting results. Because the sampling designs and the species studied are usually different it is difficult to draw general conclusions from field work alone. Moreover, wild fish are usually exposed to a mixture of contaminants, and observed effects are difficult to link to one specific type of chemical. One of the problems with results obtained in field studies, in the case of non-detection of an effect of the contaminant(s), is that a power test is rarely performed to ensure that the negative result is statistically reliable. Nevertheless some extensive studies have given very complete and valuable information on the effects

of anthropogenic pollution on the reproductive cycle of fish. Laboratory research has, in many cases, confirmed these results.

5.1 Pituitary gland

Very little work has been published on the effects of organic toxicants on levels and functions of pituitary secretion of gonadotropin in fish. A few studies in the eighties showed conflicting results. Singh and Singh (1982) found reduced plasma levels of gonadotropin in catfish (*Heteropneustes fossilis*) from a pesticide-contaminated area. Also, Thomas (1989) showed that Atlantic croaker (*Micropogonias undulatus*) fed a PCB-contaminated diet for 17 days displayed a decrease in gonadotropin secretion by the pituitary, whereas Hansson et al. (1982), in a laboratory experiment, were unable to detect any impairment of the pituitary function of rainbow trout (*Oncorhynchus mykissi*) exposed to PAHs.

Finally, in 1995, the work of Thomas and Budiantara with Atlantic croaker (*Micropogonias undulatus*) suggested that naphthalene and fuel oil were similarly responsible for a decrease in pituitary tissue responsiveness to hormonal stimulation *in vitro* (LHRHa, 10 $ng.ml^{-1}$) as the result of interference with hormone membrane receptors (although the data were not presented).

5.2 Estradiol

The numerous studies that include plasma estradiol concentrations as a variable have demonstrated with few exceptions the reduction of circulating estradiol in the presence of organic contaminants both in the field (Johnson et al., 1993, 1988; Casillas et al., 1991), and in laboratory exposures (Thomas and Budiantara, 1995; Pajor et al., 1990; Thomas, 1990b, 1988; Singh, 1989; Snowberger and Stegeman, 1987). Recently however, Janssen et al. (1997) demonstrated in a large scale mesocosm experiment, that female flounder (*Platichthys flesus*) displayed increased serum estradiol and vitellogenin concentrations if exposed to harbour sediment contaminated mostly with PCBs (5 $\mu g.g$ organic C^{-1}) and PAHs (62 $\mu g.g$ organic C^{-1}) when compared with reference sediment. The understanding of the processes by which organic contaminants affect estradiol levels is not clear. It has been established that the drop in plasma estradiol in the presence of PAHs, when it

occurs, is associated with an increase of the excretion of estradiol metabolites in the bile (Johnson et al., 1993; Stein et al., 1991, 1988; Förlin and Haux, 1985; Hansson and Rafter, 1983). Janssen et al. (1997) explained the increased circulating estradiol by the reverse process (reduced catabolism). The mechanism of the enhanced excretion of estradiol metabolites remains under debate. Förlin and Haux (1985), Hansson and Rafter (1983) and Sivarajah et al. (1978) claim that the induction of Phase I (oxidative) and/or II (conjugative) detoxifying enzyme activities associated with PAH exposure increases the catabolism and excretion of estradiol into the bile (as steroids are a substrate for some forms of P450 enzymatic activities; Table 1). This result was challenged by the studies of Johnson et al. (1993) and Stein et al. (1991) who found no sign of induced steroid-metabolizing activity associated with enhanced excretion of estradiol metabolites. Stein et al. (1991) suggest that the increased excretion of estradiol metabolites might be the result of alterations in physiological factors such as increased bile flow. Furthermore, the reduction of estradiol concentration does not seem to be the consequence of reduced steroid synthesis by the follicle cells according to *in vitro* studies by Janssen et al. (1997) and Thomas (1988). This is contrary to the hypothesis of Anderson et al. (1996a) who suggested a disruption of the pituitary-gonadal axis, including reduction of serum gonadotropin levels and decreased steroid biosynthesis in the ovary, as the cause for reduced steroid metabolism. Interestingly, Thomas and Budiantara (1995) reported a decline in plasma concentrations of estradiol and testosterone after the exposure of Atlantic croaker (*Micropogonias undulatus*) to naphthalene and diesel oil, and related this result with decreased responsiveness of ovarian tissue to hormonal stimulation, suggesting that some PAHs could interfere with hormone membrane receptors in target tissues. However, as in the case of pituitary tissue, no actual results were presented.

Numerous forms of P450 in fish have been characterized of which only a few are responsible for the metabolism of PAHs. Stegeman and Hahn (1994) have described in some detail the variety of functions undertaken by these enzymes. Cytochrome P450 enzyme systems are regulated in part by steroids. In fish, cortisol (kidney) and estradiol (ovary) appear to be the main hormones involved in this process (Snowberger Gray et al., 1991; Koivussaari et al., 1984; Vodicnik and Lech, 1983; Hansson et al., 1982; Hansson

and Gustafsson, 1981). Consequently, PAH metabolism is intimately linked to physiological and hormonal cycles and displays strong sexual-seasonal and individual variability (Vandermeulen and Mossman, 1996; Vignier et al., 1994: Larsen et al., 1992; Stegeman and Chevion, 1980). In laboratory experiments, it seems that the elevated plasma concentration of estradiol in maturing female fish systematically reduces the detoxifying enzyme response to the presence of PAHs in a dose-dependent way (Anderson et al., 1996a; Larsen et al., 1992; Snowberger Gray et al., 1991; Klotz et al., 1986; Lindström-Seppä, 1985; Förlin et al., 1984; Waxman et al., 1983; Wood et al., 1983; Förlin and Hansson, 1982; Ryan et al., 1982; Stegeman et al., 1982), and field studies have provided results confirming this outcome (Van Den Heuvel et al., 1995; Spies et al., 1990, 1988; Johnson et al., 1988; Koivussaari et al., 1984; Stegeman and Woodin, 1984; Vodicnik and Lech, 1983; Hansson et al., 1982). Elskus et al. (1992) demonstrated that suppression of P450 by estradiol occurs at the pretranslational level (as P4501A mRNA content was reduced), and that this endogenous regulation could "override" the exogenous regulation by high concentrations of inducers.

5.3 Vitellogenin in females

Plasma vitellogenin concentrations are not a routine measurement in environmental surveys. This is partly the result of the difficulty to obtain a sensitive and accurate estimate of vitellogenin concentrations. Vitellogenin is a species specific protein. Its structure varies significantly among types of fish, and characterizing vitellogenin in one species is costly and labor intensive. Short of creating an assay for each species, there are few methods available to measure vitellogenin concentrations. Recently, Heppell et al. (1995) seemed confident that the development of a universal assay for the measurement of vitellogenin was achievable. This was based on a polyclonal antiserum raised against a synthetic peptide representing a sequence of vitellogenin conserved across several species. In addition, some chemical measurements (such as protein-bound phosphorus concentrations) provide an indirect estimate of vitellogenin concentrations but they lack accuracy. Those studies that have measured vitellogenin report conflicting results.

In field studies, Pereira et al. (1992), Casillas et al. (1991) and Spies et al. (1990) found reduced levels of plasma vitellogenin in flatfish (*Pleuronectes americanus, Parophrys vetulus, Platichthys stellatus* respectively) from heavily industrialized harbors (i.e. San Francisco, Puget Sound and Boston) as compared with uncontaminated reference sites. Contradicting those findings were the results of Spies et al. (1990) and Johnson et al. (1988) which did not detect any difference between the vitellogenin levels in starry flounder (*Platichthys stellatus*) and English sole (*Parophrys vetulus*) from clean and contaminated sites of capture. Interestingly, Pereira et al. (1992) reported that under low contaminated conditions, vitellogenin concentrations increased as compared to a reference site, but were significantly reduced when contaminant concentrations were high. This outcome was supported by the results of Anderson et al. (1996b), showing that under low circulating estradiol concentration, exposure to a low concentration of β-naphthoflavone (12.5 $mg.kg^{-1}$ fish) stimulated vitellogenin synthesis in rainbow trout (*Oncorhynchus mykiss*) whereas higher concentrations (25 and 50 $mg.kg^{-1}$ fish) inhibited vitellogenin production. Additionally, under high circulating estradiol concentration, all β-naphthoflavone concentrations promoted vitellogenin production. This suggests that early vitellogenic stages could be more susceptible to PAH contamination. Several processes could be causing the effects observed: as mentioned previously, PAHs have the potential to reduce estradiol circulating levels, but could also directly induce the release of cortisol (Anderson et al., 1996b) which has been shown to down-regulate hepatic ER in brown trout (*Salmo trutta*; Pottinger and Pickering; 1990). Furthermore, vitellogenin levels could be reduced through an Ah receptor-mediated decrease in the response to estradiol (Safe et al., 1991). Several mechanisms for this phenomenon have been suggested: (1) induction of the Ah gene battery can cause an increase of the catabolism of estradiol (see Section 5.2), (2) down-regulation of the ER caused by blocking of ER gene transcription (White and Gasiewicz, 1993) and/or of estrogen responsive gene transcription (Zacharewski et al., 1994; 1991), and (3) decrease the binding of estradiol to the ER and/or ER binding to the estrogen response element (ERE) (Anderson et al., 1996b; Wang et al., 1993). Estrogenic (and antiestrogenic) effects are discussed further in the following section.

The results of other laboratory exposures show a decrease of the level of vitellogenin in the blood of fish (*Oncorhynchus mykiss, Monopterus albus*) exposed to organic contaminants (PAH and PCB) (Anderson et al., 1996b; Thomas, 1990b; Singh, 1989; Chen et al., 1986). This observation has been mostly attributed to decreased levels of circulating estradiol, however, no information is available on the rate of transcription of the vitellogenin gene in exposed fish to confirm this hypothesis.

5.4 Estrogenic/antiestrogenic effect of organic contaminants

A large and increasing number of organic xenobiotics has been demonstrated to display endocrine disrupting effects in wildlife populations. These compounds are suspected of causing significant impairments in the human reproductive system (for review see Colborn et al., 1993). This has renewed interest in the vitellogenic cycle of fish which appear to be a valuable surrogate organism to model the effects of endocrine disrupting chemicals. Moreover, a large number of the chemicals of concern ultimately enter aquatic environments (sewage, fallout, spills, etc.) causing fish to be prime targets. In addition, the shorter generation time allows for the observation and anticipation of long-term trans-generation effects which are ecologically more relevant.

Estrogenic (or antiestrogenic) effects are a class of endocrine disrupting effects which generally refer to a disruption of the hormonal regulation of the reproductive cycle resulting in an estrogen-like response. Strictly speaking however, estrogenic effects should only be comprised of direct interactions of a substance with estrogen receptors. By comparison, reduced levels of circulating estradiol in the presence of a toxicant resulting in a decreased vitellogenic response (as described in Section 3.1.2), or xenobiotic-related reduced numbers of estrogen receptor sites (as a result of DNA damage or repression of transcription for example) would not be considered true antiestrogenic effects.

Little has been published on the estrogenic effects of PAHs specifically. These chemicals are thought to be less estrogenic and therefore less of a concern than organochlorines and dioxins. However, recent results suggest that PAHs can have a significant antiestrogenic effect through an Ah-receptor mediated mechanism (Anderson

et al., 1996b). Several mechanisms are currently proposed to describe the toxicity of environmental estrogens:

(1) They could act as an ER agonist or antagonist (Garcia et al., 1997; Nimrod and Benson, 1996; Stancel et al., 1995; Thomas and Smith, 1993; Thomas, 1990b; Hatakeyama, 1989). The binding of environmental agonists to the hepatic ER artificially induces or enhances the transcription of the vitellogenin gene. Thus, in male fish, an estrogenic contaminant would reduce reproductive potential and could even induce vitellogenesis (Kelce et al., 1995; Korach and McLachlan, 1995; Sumpter and Jobling, 1995). In females it would promote the synthesis of vitellogenin. This could initiate or artificially stimulate vitellogenesis if the fish is exposed to the contaminant at an early stage of the vitellogenic cycle, or inhibit ovulation and spawning if the fish is exposed at a late stage of the cycle (Colborn et al., 1993; Thomas and Smith, 1993; Thomas, 1990b; Wester et al., 1985). Conversely, the binding of an antagonist to the ER would reduce the rate of transcription of vitellogenin. This could delay vitellogenesis (Anderson et al., 1996a,b).

(2) Some environmental estrogens could produce an imbalanced estrogenic response in a target tissue. This more subtle type of toxicity is discussed in more detail by Stancel et al. (1995). These authors suggest that the hormonal imbalance could lead to deleterious effects during critical periods of cell development.

(3) The xenobiotics could cause Ah receptor-mediated estrogenicity by affecting estrogen binding to ER and/or ER binding to the estrogen response element (ERE) (Anderson et al., 1996b; Zacharewski et al., 1991). This mode of action would result in a reduction of the transcription of vitellogenin and could potentially retard vitellogenesis.

It appears that a variety of organic molecules (e.g. PAH, PCB, pesticides, dioxins, alkyl substituted phenols) have a mild estrogenic effect. Generally, the affinity of environmental estrogens for the ER is several hundred to often thousand times lower than that of estradiol (Knudsen and Pottinger, 1999; Garcia et al., 1997; Donohoe and Curtis, 1996; Nimrod and Benson, 1996). Synergistic effects leading to increased estrogenic responses have been "demonstrated" (Arnold et al., 1996; Simons Jr., 1996). However, this result was challenged in a subsequent study which found the effects of the same

pesticides to be additive (Ramamoorthy et al., 1997). The article by Arnold et al. (1996) was eventually removed from the journal. Knudsen and Pottinger (1999) also reported a lack of synergistic effect of mixtures of estrogenic contaminants. While certain chemicals such as PCB (Garcia et al., 1997; Thomas and Smith, 1993; Wester and Canton, 1986) and herbicides (Thomas and Smith, 1993; Hatakeyama, 1989) compete directly with estradiol for binding with liver ER, other chemicals, like certain PAHs, require metabolic transformation before they can produce an estrogenic response in an organism (Anderson et al., 1996a; Thomas and Smith, 1993; Thomas, 1990b; Bulger et al., 1985). Benzo(a)pyrene (Thomas and Smith, 1993) and β-naphthoflavone (Anderson et al., 1996a) for example, were found to have no direct estrogenic effect. However, microsomal metabolites of benzo(a)pyrene did produce an estrogenic response when tested *in vitro* (Bulger et al., 1985).

5.5 Gonadal maturation and reproductive success

Further investigations of the effects of PAHs on gonadal maturation and reproductive success were performed on wild fish. Most studies report a reduction in gonadal and egg maturation (Ghosh and Thomas, 1995; Thomas and Budiantara, 1995; Kirby, 1994; Collier et al., 1993; Casillas et al., 1991; Johnson et al., 1988; Thomas, 1988; Cross and Hose, 1986; Spies et al., 1985; Hose et al., 1981) and a lower reproductive success (lower percent hatch and survival. Kocan et al., 1996; Casillas et al., 1991; Nelson et al., 1991; Tilghman Hall and Oris, 1991; Black et al., 1988; Spies and Rice, 1988; Spies et al., 1985; Hose et al., 1981) of fish exposed to PAH concentrations commonly found in contaminated harbours. Furthermore, Tilghman Hall and Oris (1991) have reported the maternal transfer of PAH (anthracene) to offspring in fathead minnows. Other studies have reported similar results with other types of organic chemicals (PCB, DDT) (Ghosh and Thomas, 1995; Monosson et al., 1994; Munkittrick et al., 1992; Thomas, 1989; Binder and Lech, 1984; Cross et al., 1984). Binder and Lech (1984) also demonstrated that the inherited xenobiotics caused a biological effect. In cases of unusually high levels of sediment PAH (above 100 ppm), Collier et al. (1993) described cases of English sole (*Parophrys vetulus*) in which spawning had been totally inhibited. This concurs with

results in the field and in laboratory exposures obtained by Nicolas and Vandermeulen (1999, unpublished data) with winter flounder (*Pleuronectes americanus*) and Cross and Hose (1988) with white croaker (*Genyonemus lineatus*), and by Thomas and Budiantara (1995) with Atlantic croaker (*Micropogonias undulatus*) in the laboratory. However, inhibition of egg maturation by PAH and PCB was demonstrated to be reversible when Atlantic croaker (*Micropogonias undulatus*) were transferred to an uncontaminated environment (Ghosh and Thomas, 1995). Impairment of gonadal maturation and reproductive success can occur at lower doses of PCB than would impairment of circulating levels of estradiol or vitellogenin (Monosson et al., 1994). Generally, the response to the presence of contaminants in the sediment or the water column is non-linear and suggests the existence of a threshold level of contaminant. Depending on the study, this threshold varies from 5 to 10 ppm of PAH in the sediment.

Studies by Casillas et al. (1991) and Johnson et al. (1988), did not detect any impairment of ovarian recrudescence in the presence of liver lesions in English sole (*Parophrys vetulus*). Finally, Spies et al. (1990) found no significant alteration of gonadal maturation in starry flounders (*Platichthys stellatus*) exposed to sediments contaminated primarily with PAHs as compared with starry flounders exposed to uncontaminated sediments.

BIOMONITORING OF MARINE POLLUTION

Practical experience seems to indicate that biomonitoring is not independent of chemical or physical monitoring of the study environment. To be useful as a diagnostic tool it must be part of a multidisciplinary survey that includes chemical, physical and biological characterization of the environment.

Stegeman and Hahn (1994) proposed that aquatic toxicology has four main objectives:

1) to model systems for investigating fundamental biochemical and cellular processes involved in the action of xenobiotics,
2) to evaluate new chemicals as environmental or human health hazard(s),
3) to evaluate and monitor effluents and/or other waste streams,
4) to delineate and evaluate the biological and ecological significance of local and global contamination.

6. Bioindicators of Marine Pollution

6.1 Definitions

Bioindicators are organisms that best fit a selection criteria (see below) in which biomarkers of environmental contamination can be quantified.

Biomarkers are tests which can be used to measure exposure to, or sublethal effects of, a contaminant on living organisms.

6.2 Rationale and limitations

What is the rationale behind using biomonitoring as opposed to simply measuring, for example, chemical concentrations or toxic effects ? There are several justifications for the use of bioindicators of aquatic contamination:

According to Phillips and Rainbow (1993), the main drive to use biomarkers to measure organic contaminants in aquatic environments was the initial difficulty and unreliability of chemical measurements. Low pollutant concentrations in the water and contamination of the samples can considerably reduce the accuracy of results. The authors also acknowledge the problem of temporal variability associated with contaminant

concentrations in aquatic environments. McCarthy and Shugart (1990) in their overview of biological markers of environmental contamination agreed with this concern. In their view, chemical and/or physical measurements only provide a "*snapshot*" image of the system under investigation, whereas biomarkers offer a "*temporally and spatially integrated measure of bioavailable pollutants*". This may be the most important benefit of using biomarkers, as changes in concentration resulting from random events such as storms, accidental or intermittent releases for example, could be missed by conventional sampling. Furthermore, contamination is often geographically patchy, depending on distance from the source and physical factors (e.g. hydrography, sediment type). The responses of organisms integrate the concentration and bioavailability of a contaminant over time and a specific spatial range, thus providing a measure that is more relevant to evaluating ecological and health risks.

McCarthy and Shugart (1990) suggest three additional reasons for monitoring biomarkers in organisms:

1) they may provide a cause and effect relationship between exposure to pollutants and observed deleterious effects;
2) they may indicate the main route of exposure (e.g. depending on the type of markers selected, it might be possible to determine whether an organism was exposed through contact with sediment or through the food chain);
3) they offer more information than short-term bioassays.

The first two aspects can also provide valuable insight on the threat of exposure to human health.

Even if biomarkers are very appealing, it is necessary to be aware of the limitations associated with their use. Biomonitoring programs usually require large amounts of money, equipment, personnel and time. They are often multidisciplinary and require a high level of expertise in each field of competence.

Interpretation of the results is often difficult because of high variability which can be associated with some biomarkers. In some cases, cause and effect relationships are not easy to establish.

6.3 Selection of test organisms

When selecting a test organism, several criteria should be considered. Usually the selection will differ from ecosystem to ecosystem, and could even be site-specific. An ideal test species would fit the six basic criteria defined by Rand and Petrocelli (1985):

1) Representative of a broad range of sensitivities (since sensitivities vary among species)
2) Abundant and readily available
3) Indigenous and representative of the impacted system
4) Ecologically important (and recreationally or commercially)
5) Amenable to routine maintenance and techniques for possible laboratory culture and testing
6) Adequate background information available (life cycle, physiology and behaviour)

6.4 Selection of biomarkers

The ultimate goal of biomonitoring is to answer two critical questions:

1) Are organisms exposed to levels of toxicants that exceed the capacity of normal detoxification and repair systems ?
2) If there is evidence of exposure, is the contaminant stress impacting the integrity of the communities ?

Therefore the choice of biomarkers should be dictated by the type of environment and the kind of contaminant present, in accordance to the selective criteria presented in a previous section, to best provide information towards answering those questions. In addition, it is necessary to understand biotic and abiotic factors influencing a biological variable before that variable can be used as an indicator of environmental deterioration (Thomas, 1990a). Stress and temperature are prime examples of biotic and abiotic factors, respectively. They have been found to influence many biomarkers (Carragher et al., 1989; Vignier et al., 1994).

Three types of biomarkers can be distinguished: biochemical, physiological and ecological. These can be subsequently separated into two groups: specific indicators which can provide direct information on the exposure, uptake and effects of a toxicant

(e.g. detoxification enzyme systems); and non-specific indicators which would measure an effect that could only be attributed indirectly to the toxicant (e.g. plasma steroid levels).

7. Integrative approach

The list of publications demonstrating the high variability associated with the use of biomarkers to monitor the environment is too long to include here. In many cases the variance associated with the data is such that it precludes any convincing interpretations. Some authors found that the use of an increased number of biomarkers associated with multivariate statistical analysis reduced the variability in the results significantly, and provided with a much better diagnostic and predictive tool (Van Der Oost et al., 1997; Adams et al., 1996; Wirgin et al., 1994; Casillas et al., 1991). It seems that an integrative approach to monitoring the environment is necessary to obtain reliable results. As was recommended by Thomas (1990a), the choice of biological markers will depend on the nature of the contamination and the results expected. In an ideal situation, environmental monitoring programs should include a suite of markers providing information on the type of stressor, its mechanism of action, the extent of the physiological impairment and the potential long-term population consequences of the exposure.

CONCLUSION

Based on the review of the literature available, PAHs have a deleterious effect on the vitellogenesis of fish. How the presence of elevated levels of PAHs affects the vitellogenic cycle of the fish can vary with species, population and can even differ between individuals. As a result, drawing general conclusions regarding the effects of PAHs on vitellogenesis in fish based on the study of a few individuals from one population and one species is uncertain at best.

This review illustrates a lack of consensus between studies and that we are far from grasping all the mechanisms at play in the effects of PAHs on the various phases of

vitellogenesis in fish. It also underlines the necessity for further research in several areas of this field:

(1) Further work is required to assess effects on the pituitary-ovary axis and estrogen regulation, since no clear mechanistic explanation for the reduction in estradiol in the presence of PAHs has yet been put forth.

(2) In recent years, several symposia, conferences and workshops have stressed the necessity for increased scientific efforts in the field of endocrine disruption. Government agencies have also made this topic a priority agenda. Establishing and validating screening assays for the identification of estrogen disrupters should be a prime objective.

(3) A broader range of organisms has to be investigated in order to gain a better understanding of the processes involved in the disruption of the vitellogenic cycle. One possible way of achieving this would be to routinely include reproductive bioindicators in contaminant effects monitoring protocols. This would be especially valuable if a standardized universal assay for vitellogenin becomes available (with emphasis on males as indicators of estrogen disruption).

It seems apparent that one of the difficulties associated with environmental research is the high variability associated with the data (i.e. effects of season/sexual maturation can be 10x higher than effects of contamination) which often precludes any detection of statistically significant effects. This problem might be overcome by the use of multivariate statistical models to interpret the data.

In general, the goal of environmental research is to assess the risk to ecosystems in order to provide the framework to protect and maintain. Thus the determination of what is irreparable and what is reversible damage seems to be critical to establish proper regulatory guidelines (especially with respect to the impact on human populations). This can only be achieved by understanding long-term trans-generational effects and gaining better knowledge of the effects of mixtures of xenobiotics to which wild populations of organisms are exposed.

RESEARCH OUTLINE

The research included in this dissertation was associated with a larger program (Department of Fisheries and Oceans, Canada) investigating the long-term effects of coal-tar derived contamination on fish in Sydney Harbour (Nova Scotia, Canada). The Sydney Estuary is one of the worst known cases of PAH contamination of a coastal environment. As such, it has attracted much public attention over the past 15 years.

The research that will be presented here has focused on the effects of coal-tar derived contaminants on the reproduction of both male and female winter flounder in an attempt to identify potential risks at the ecologically more relevant population level. This has included (1) two consecutive years of field work in Sydney Harbour (Chapter 2), allowing for the characterization of seasonal and inter-annual variations and (2) a laboratory experiment (Chapter 3) in which captive winter flounder were exposed to a range of sediment contamination for two full reproductive cycles under a monthly sampling regime.

CHAPTER 2

Effects of long-term exposure to coal-tar derived contamination on the reproductive cycle of a feral population of Winter Flounder

1. Introduction

This part of the work was focused on assessing the state of the reproductive cycle of feral fish exposed to long-term coal-tar derived contamination. This contamination, of which PAHs are the major component, was hypothesized to have deleterious effect on vitellogenesis. Because estradiol, and sex steroids in general are substrates for some MFO enzymes, it was expected that a high level of organic contaminants which would induce a higher MFO activity, would provoke a depletion of the circulating levels of estradiol. This would result in a weaker vitellogenic response and slower oocyte maturation.

For this purpose, an estuary chronically contaminated with hydrocarbons and PAHs for decades was selected as the study site, and winter flounder was chosen as the test species.

1.1 Sydney Harbour

The Sydney Estuary, in northern Nova Scotia, is one of the worst cases of contamination of a coastal environment in Canada. The sediments of the estuary are the most contaminated of Nova Scotia and the most PAH-contaminated in Canada. This is mainly the result of almost 100 years of activity of a large coking facility located on the premises of the Sydney Steel Corporation (Sysco) plant (Matheson et al., 1983). The Sysco facilities are adjacent to the South Arm of the estuary (Figs. 5, 6). The coking ovens were in activity until 1988 (Mossman and Vandermeulen, 1995). Effluents from the operation together with surface and groundwater from the immediate area, including coal storage, were allowed to flow via Coke Oven Brook into Muggah Creek (Figs. 5, 6) (Vandermeulen, 1989; Matheson et al., 1983). Over time the outflow area in Muggah Creek expanded to eventually form the "tar pond". Depths of coal-tar sludge of up to 4.35 m have been reported (Vandermeulen, 1989). The total amount of contaminated sediment in the tar pond is estimated to be $5x10^8$ kg dry weight (ca. 729,000 m^3) and to contain approximately $3.5x10^6$ kg of PAHs (Vandermeulen, 1989). Muggah Creek is tidal and over time, substantial amounts of contaminants have been flushed into the harbour. Levels of up to 2.8 ppt of total sediment PAH (dry weight) have been reported at the mouth of Muggah Creek (Matheson et al., 1983). Based on the analysis of bottom

Figure 5: Aerial photography of the Sydney Steel Corporation plant. Muggah Creek, the Tar Pond and the settling pond are clearly visible. The Tar Pond opens to the South Arm of the estuary at the top of the picture.

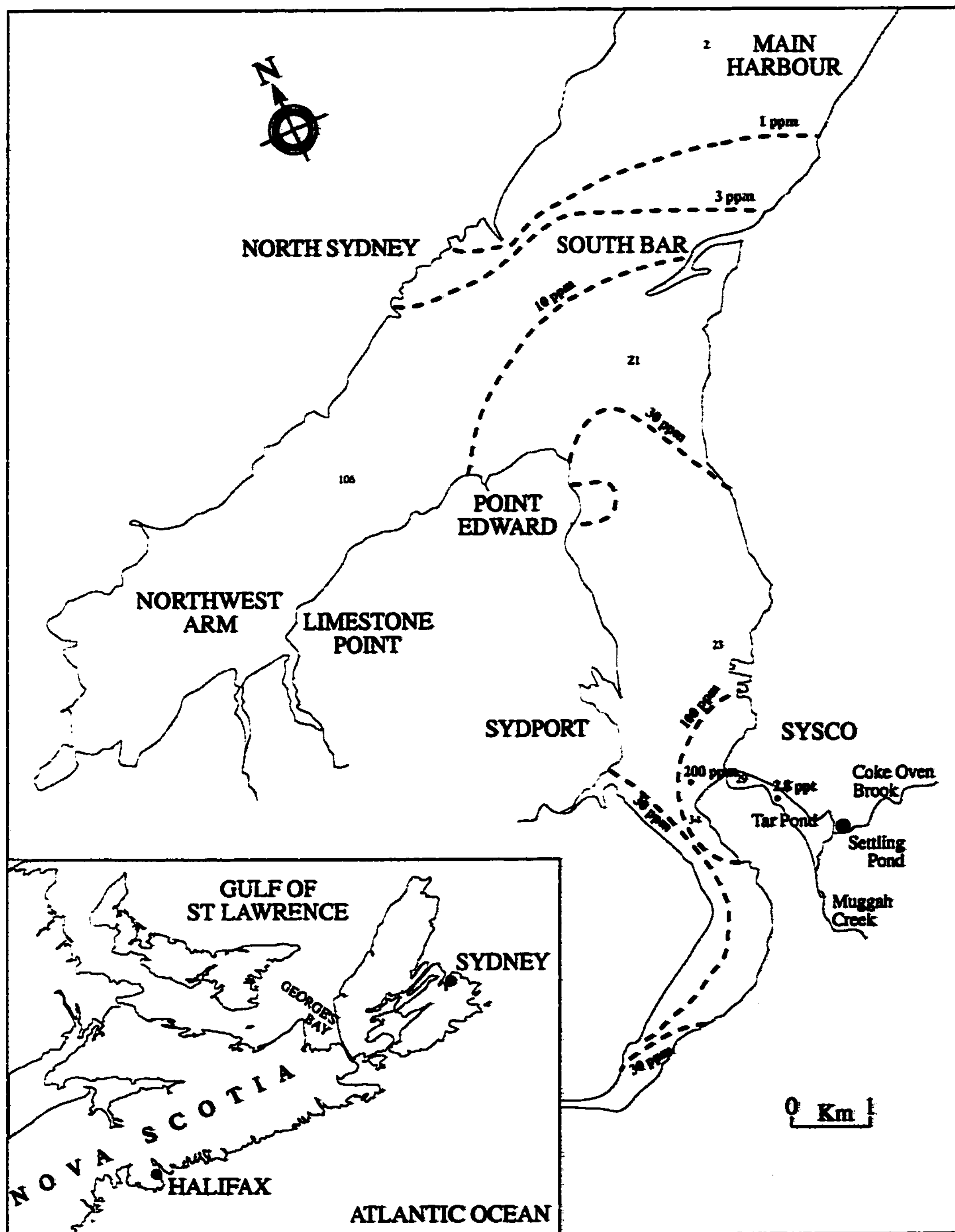


Figure 6: Contour map of Sydney Harbour sediment PAH concentrations. Adapted from Vandermeulen (1989) and Matheson et al. (1983). 34, 29, 23, 106, Z1 and 2 indicate core positions for PAH profile.

sediment samples and tracer dye studies by Matheson et al. (1983), Vandermeulen (1989) extrapolated a map of PAH concentrations in the sediment from Sydney Harbour (Fig. 6).The chronic hydrocarbon contamination of Sydney Harbour has been an environmental concern for many years. Several studies have investigated the effects of this contamination on the major species of invertebrates (King et al., 1993; Uthe and Musial, 1986; Sirota et al., 1984; Matheson et al., 1983) and fish (Vandermeulen and Mossman, 1996; Addison et al., 1994; Vignier et al., 1994) present in the estuary. However, the effects on the reproductive biology of organisms exposed to the contaminants and the implications at the population level of organization had yet to be studied. The present study was specifically aimed at investigating the effects of long-term exposure to PAH-contaminated sediments on vitellogenesis in feral female fish. In the course of this work, the response of male fish to potentially estrogenic chemicals was also investigated.

1.2 Winter flounder as a bio-indicator for environmental contamination

Winter flounder (*Pleuronectes americanus*, Walbaum) are flatfish from the Pleuronectidae family. This predominantly inshore species is common along the Atlantic coast of Canada and displays a limited migratory behaviour (Klein-MacPhee, 1978; Howe and Coates, 1975). Along the coast of Nova Scotia, the spawning season extends from late April to June, depending on environmental conditions (Nicolas, pers. obs.; Harmin and Crim, 1992; Klein-Macphee, 1978). Estuaries and near-shore areas are important spawning and nursery grounds for winter flounder. There is evidence of territorial behaviour, with a tendency to return to the same spawning locations in consecutive years and restricted movement patterns suggesting relatively discrete groups of individuals (Phelan, 1992; Perry et al., 1991; Black et al., 1988). Furthermore, Saucerman and Deegan (1991) demonstrated in two consecutive years that 98 and 90% of winter flounder released in in-shore and coastal waters where recaptured (after 1 and 3 weeks) within a 100 m of the release site and that only 1% of the fish had moved more than 200 m.

After spawning, seasonal gonadal recrudescence begins with the summer feeding period (Harmin and Crim, 1992). The development of the oocytes in the ovary is synchronous.

Although there is always more than one year-class of oocytes in the ovary, only some undergo vitellogenesis, and the recruited oocytes mature at the same rate throughout the ovary (Wallace and Selman, 1981; Rosenthal and Alderice, 1976; Dunn and Tyler, 1969). In fish from coastal Nova Scotia, the developing oocytes mature from a previtellogenic stage at the beginning of the summer to an advanced stage of vitellogenesis in early winter. Over the winter oocyte development stops. This phenomenon is correlated with low water temperatures and reduced feeding. As in most other poikilotherm species, the rate of maturation of the oocytes is temperature-dependent (Klein-MacPhee, 1978). When flounder move closer inshore into warmer waters, the oocyte maturation resumes shortly prior to spawning.

Winter flounder is one of the most abundant species of fish in the Sydney estuary. In addition, being a bottom dwelling species, these fish are exposed to contaminants originating from both the sediment and the water column. Furthermore, as these fish are opportunistic sight feeders (Pearcy, 1962), and prey on benthic organisms, they can also be exposed to contaminants through the food chain. Thus, winter flounder appeared well suited as organisms in which to look for signs of adverse effects.

2. Materials and methods

2.1 Sampling stations

Based on previous environmental studies in the Sydney Estuary (Vandermeulen, 1989; Matheson et al., 1983), four stations were selected in the harbour. The stations were chosen to reflect a gradient of bottom sediment PAH contamination ranging from less than 1 ppm at the mouth of the harbour to approximately 200 ppm (Note: all sediment concentrations of contaminants are expressed as dry weight) at the station closest to the Sysco operation (Fig. 7). This station was the closest to the point source of the contamination that the research vessel could safely reach. Sediment PAH profiles for several characteristic areas of the harbour were available (Fig. 8) from earlier work by Vandermeulen and associates (unpublished data). The stations at which the cores were sampled are indicated on Figure 6. Sediment PCB concentrations at the two most

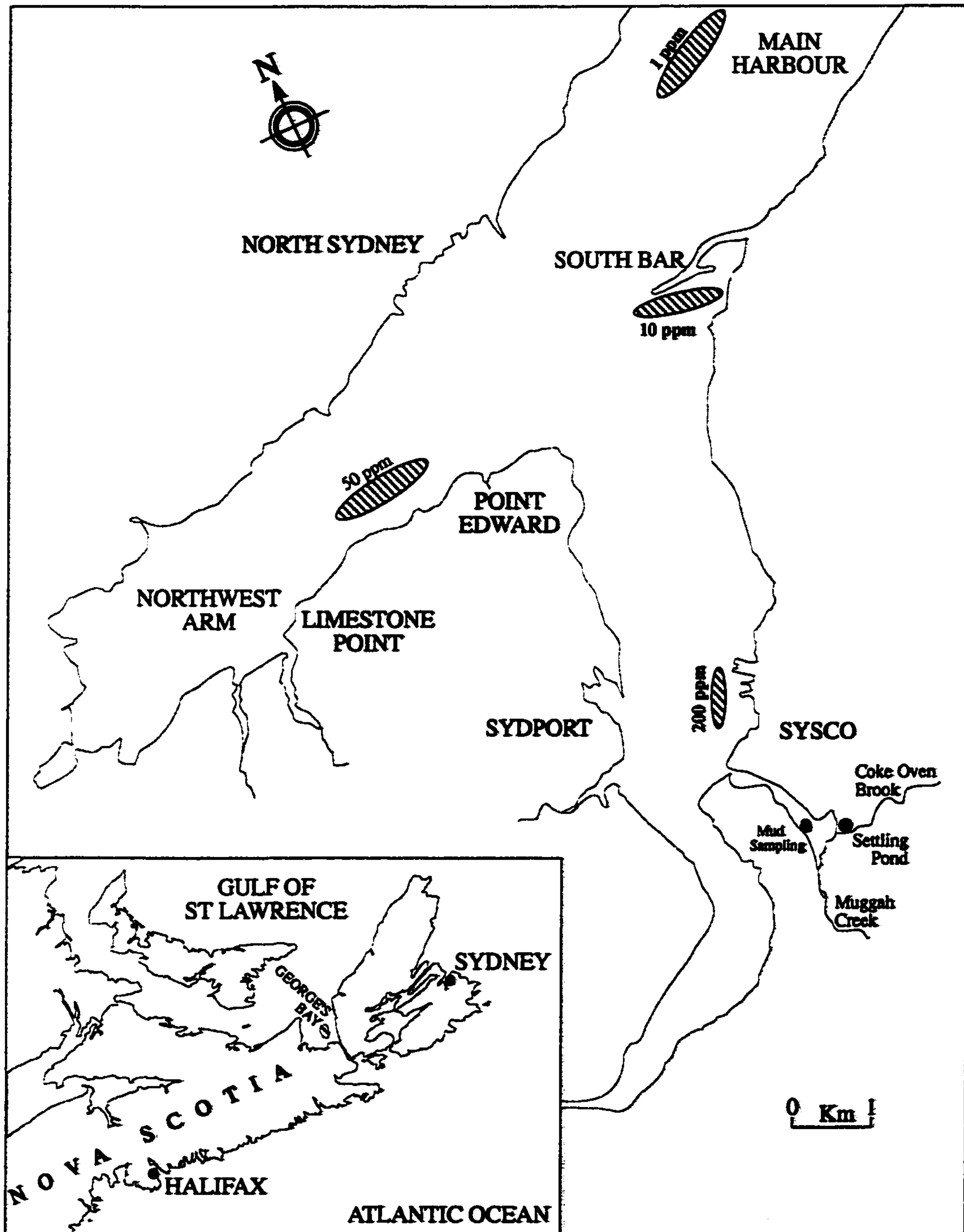


Figure 7: Location of trawling stations in Sydney Harbour and George's Bay. Sediment PAH concentrations measured from grab samples at each station of the Sydney Estuary are also indicated.

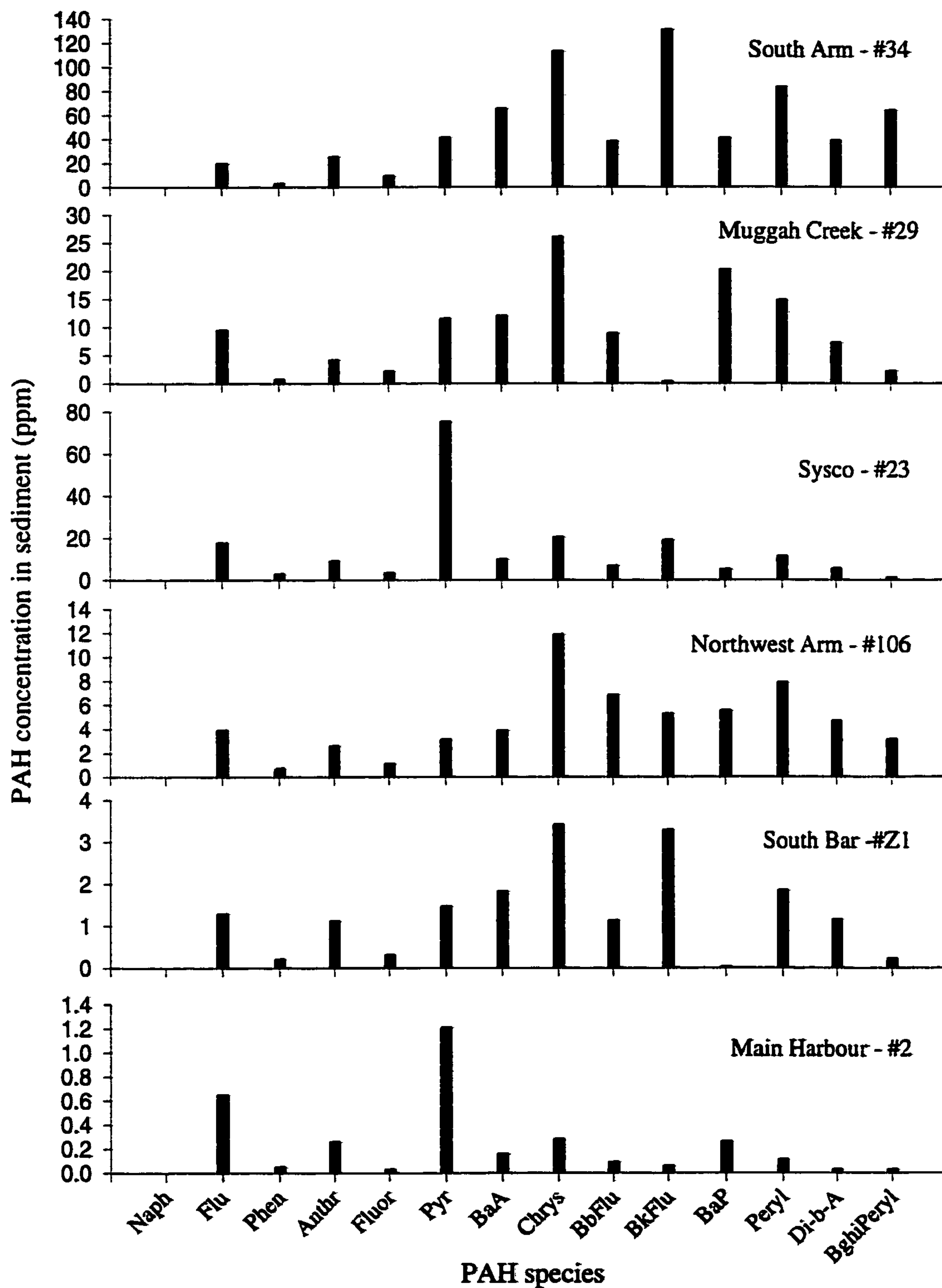


Figure 8: PAH concentrations in sediment core samples from Sydney Harbour. Core numbers are indicated by the sampling station (see Fig. 6). Abbreviations used: Naph: Naphthalene; Flu: Fluorene; Phen: Phenanthrene; Anthr: Anthracene; Fluor: Fluoranthene; Pyr: Pyrene; BaA: Benzo(a)anthracene; Chrys: Chrysene; BbFlu: Benzo(b)fluoranthene; BkFlu: Benzo(k)fluoranthene; BaP: Benzo(a)pyrene; Peryl: Perylene; Di-b-A: Dibenzo(ah)anthracene; BghiPeryl: Benzo(ghi)perylene.

contaminated stations (Sysco and Northwest Arm) were 2.8 ppm and 0.15 ppm respectively (Vandermeulen and Mossman, 1996). A reference station was selected in George's Bay (Fig. 7) with sediment PAH concentrations below detection limit (see section 2.8). All stations were sampled within a few days of each other at three time intervals in 1992 (June, September, October) and 1993 (June, September, November). No sampling could be done from December through April as the Sydney estuary is ice-bound.

2.2 Sample collection

Based on preliminary work, a minimum sample size of 26 fish was required to allow the detection of a 20% difference in the mean of the primary variables. Sample size was therefore set at 30 fish per station. An additional 10 male flounders were captured at each station, to be used as negative controls for serum reproductive parameters. Fish were captured by otter trawl at all stations except Sysco where a beam trawl was used due to very soft bottom sediment conditions. In order to ensure that all animals were sexually mature, only fish longer than 27 cm (Beacham, 1982; Kennedy and Steele, 1971) were kept and placed in an aerated tank containing sea water pumped from the trawling site. The time interval between capture and dissection of the fish varied from 1 to 4 hours. Bottom water temperature was recorded once at each station using a Seatemp Digital® thermometer.

For each fish, morphometric measurements were recorded and a blood sample was collected from the caudal vein using heparinized Vacutainers® (Fisher Scientific). This sample was immediately split and one half (approx. 2 ml) received 50 µl of trypsin inhibitor, Aprotinin (Sigma). The blood was left to clot overnight at 4°C and centrifuged for 7 minutes at 2500 x g. The serum was then frozen and held in liquid nitrogen until transfer into a cryofreezer where it was maintained at -70°C until assayed. Livers were excised, rinsed in ice-cold saline and frozen in liquid nitrogen until transfer into a cryofreezer maintained at -70°C. Once weighed, a section of the posterior region of the left ("ventral") ovary were preserved in 10 volumes of 10% buffered formaldehyde. The remainder of the ovaries was treated in the same way as the livers. Gall bladders were

excised, pooled in groups of ten, frozen and maintained at -20°C until assayed for PAH metabolites. Otoliths were removed, rinsed in ice-cold distilled water, dried and stored in capped vials (1992) or glassine envelopes (1993) until age determination was performed.

2.3 Morphometric measurements

Length (L), weight (W), eviscerate weight (W_e), liver weight (W_l) and gonad weight (W_g) were measured for each fish. Subsequently, fish condition index (FCI), gonadosomatic index (GSI) and hepatosomatic index (HSI) were calculated from the following equations:

$$FCI = \frac{W_e}{L^3} x100 \qquad GSI = \frac{W_g}{W_e} x100 \qquad HSI = \frac{W_l}{W_e} x100$$

2.4 Estradiol

Estradiol serum concentrations were measured by radioimmunoassay (RIA) modified from a Sigma Chemicals kit. All assays were run in duplicate. A third assay was performed if the variation between duplicates exceeded 5%. Human 17β-estradiol (Sigma Chemicals) was used as standard. A volume of 100 µl of serum was extracted twice with 1 ml of ethyl acetate:cyclohexane (1:1). This method was found to be more consistent than extraction with ether. Extracts were pooled, evaporated under nitrogen and resuspended in 100 µl of assay buffer (Tris-HCl 0.05 M, pH 8.0, containing 0.1 M NaCl, 0.1% gelatin and 0.1% sodium azide). Samples and standards were pre-incubated for 30 minutes at room temperature with the antibody (1:10,000 working dillution) and further incubated for 70 minutes at 35°C in the presence of the radioactive tracer ([^{3}H]estradiol; Amersham). Excess radioactive tracer was removed by addition of activated charcoal and stirring for 10 minutes at 0°C, followed by 15 minutes centrifugation at 2500 x g. Counts in the supernatant were determined on a Beckman LSC2000 liquid scintillation counter. The efficiency of the steroid extraction ranged from 97% to 104% and the detection limit of the assay was 50 pg.ml^{-1}. Interassay variability was 2 $^+$/-2%.

2.5 Vitellogenin

Serum vitellogenin levels were estimated by serum protein-bound phosphorus concentrations (DeVlaming et al., 1984). All assays were run in duplicate. Protein was precipitated out of 100 µl of serum (containing Aprotinin) by addition 1 ml of ice-cold TCA (12%). Samples were then centrifuged for 10 minutes at 2500 x g. The precipitated proteins were rinsed twice; once with 1 ml of ethanol:ether (3:1) and once with 1 ml of ether. The pellet was then resuspended in NaOH (1N), hydrolyzed for 20 minutes at 100°C and the solution was neutralized with glacial acetic acid. The proteins were precipitated with 1 ml of ice-cold TCA (12%) and 100 µl of the supernatant was then diluted with 5 ml of distilled water and assayed for phosphate according to the method of Strain and Clement (1996) on a Technicon AutoAnalyzer II segmented flow analyzer.

Throughout this study and the laboratory experiments (Chapter 3), serum protein-bound phosphorus concentrations will be considered directly proportional to serum vitellogenin concentrations. This assumption is based on work by Nagler et al. (1987), DeVlaming et al. (1984) and Whitehead et al. (1983) and to date their findings have not been disputed. Several variations of this method have been used as recently as 1998 (Kramer et al., 1998). The technique developed here offers the convenience and rapidity of automation.

2.6 Serum protein

Total serum protein was measured according to Lowry et al. (1951), using bovine serum albumin as a standard.

2.7 Histology

The formaldehyde preserved ovary tissues underwent dehydration according to Preece (1956) in increasing concentrations of 2-propanol (70 to 90%) for 30 minutes in each bath, and 60 minutes in two consecutive baths of 100% 2-propanol. This was followed by two consecutive baths of clearing agent (Histoclear®). Samples were then embedded in paraffin wax (MP 56°C). Paraffin blocks were sliced at 5-15 µm depending on the yolk

content of the sample, and sections were stained with Mayer's Haematoxylin and conterstained with Eosin (standard staining procedure).

Sections were subsequently assessed for oocyte maturity. The system of ranking of the development stage of the samples was modified from a number of different sources (Burton and Idler, 1984; Tokarz, 1978; Dunn, 1970; Dunn and Tyler, 1969; Yamamoto, 1956). The developmental cycle of the oocyte was divided in five stages (see Appendix for photographic illustrations of oocyte maturation stages)

Stage 1: resting or newly recruited oocytes. Nucleus occupies most of the cell. Few prominent nucleoli and granular ooplasm (size approx. 30 μm).

Stage 2: recently recruited oocytes. Increased size with nucleoli dispersed around the periphery of the nucleus. Beginning of eosinophilic yolk droplets appearing around the periphery of the oocyte. Presence of a thin zona pellucida (size approx. 100 μm).

Stage 3: maturing oocytes. Increased cell size and number of yolk inclusions (occupying 1/2 to 2/3 of the cell volume). Ooplasm appears to occupy less of the cellular volume. Thickening of the eosinophilic zona pellucida (size approx. 200 μm).

Stage 4: maturing oocytes. Very large cell (15-20 x Stage 1). Well developed zona pellucida. Oocyte almost completely filled with yolk inclusions. Compressed nucleus (size approx. 500 μm).

Stage 5: mature oocyte. Slight increase in cell size. Coalescent yolk inclusions (homogenous yolk). Absence of nucleus. Thick zona pellucida (size approx. 600 μm).

2.8 Chemical measurements

Measurements of PAH concentrations in the sediment and ovaries as well as bile metabolites of PAH were performed by RPC Chemistry Laboratory (Fredericton, N.B., Canada). Briefly, for sediment PAH, samples were fortified with d-PAH surrogates (perdeuterated naphthalene, fluorene and fluoranthene) and Soxhlet extracted with

hexane/acetone (1:1) for 20 hours. Following the removal of residual acetone by washing with saline, the hexane solution was treated with concentrated sulfuric acid and fractionated on a silica gel column. Concentrations of PAHs were determined by GC/MS. The detection limit was less than 0.02 ppm. Due to budget limitations only 114 ovary samples and 3 samples of pooled bile per station and month were tested for PAHs and PAH metabolites respectively (samples collected in 1992 only). For gonadal PAH, a 5 g portion of ovarian tissue was mixed with 50 ml of ethanol, 3 g of potassium hydroxide and 0.1 μg of each d-PAH surrogate in 1 ml of methanol and refluxed for 2 hours (Musial and Uthe, 1986). The ethanol solution was transferred to a 250 ml separatory funnel. The flask and condenser were rinsed with 50 ml of distilled water, followed by 25 ml of pentane. The content of the funnel was shaken for 3 minutes and the pentane layer was collected. Extraction was repeated with 25 ml of pentane. Extracts were combined and reduced to 1 ml. Extracts were then cleaned through an activated acidic alumina packet column. PAHs were eluted with 5 ml of hexane, followed by 25 ml of dichloromethane. Concentrations of PAHs were determined by GC/MS. The detection limit of the method was approximately 0.005 ppm (wet weight) in the final extract. For PAH bile metabolites, 0.5 to 1.0 g of sample was analyzed. The sample was fortified with a d-PAH surrogate solution (at 0.1 ppm each of perdeuterated naphthalene, fluorene and fluoranthene), acidified and extracted 3 times with 2 ml of benzene. The extracts were combined and reduced to 1 ml. The extracts were fractionated on a micro-silica gel column. PAH metabolites were eluted with 5 ml of benzene/acetone (1:1). Concentrations were determined by GC/MS. The detection limit was less than 0.02 ppm per sample.

2.9 Statistics

Statistical analyses were performed using SigmaStat® 1.0 (Jandel) and Statgraphics® 5.0 (STSC). For all analyses, probability of type I error (α) was less than or equal to 0.05 and power was at or above 0.8 unless otherwise noted.

3. Results

3.1 Sample size

In 1992, the predetermined optimal number of fish per station (30) was not always obtained. The catch was particularly low at the Sysco (SYS) station: 4 females and no males captured in June, 12 females and 5 males in September and 16 females and 4 males in October. This was the result of gear inefficiency in difficult trawling conditions (very soft muddy sediments). At all other stations, catches ranged from 23 to 30 for females and from 8 to 14 for males. The gear was modified for the 1993 field work, and a complete sample set was caught.

3.2 Multivariate analysis

A total of 821 female winter flounder were captured over the two year period of the study. For each fish, ten variables were taken into consideration: station of capture (S), month of capture (M), serum estradiol concentration (E2), serum vitellogenin concentration (V), egg maturation stage (ES), gonadosomatic index (G), hepatosomatic index (H), length (L), weight (W) and age (A). Several variables were excluded from the multivariate model because of the low number of data (serum protein, bile metabolites and PAH concentration in the ovary). The resulting data set was submitted to a factor analysis in an attempt to isolate and identify the different sources of variability affecting the data.

The first three factors accounted for 71.8% of the variability in the data (36.3%, 24.6% and 10.9% respectively; Table 4). Variables with a strong seasonal component (M, E2, ES, V and to a lesser degree H and G) loaded heavily on factor 1 and the weights of the remainder of the variables was comparatively low (Table 5, Fig. 9).This lead to the

Table 4: Results of a Factor Analysis on Sydney Harbour winter flounder samples. Ten variables considered: station of capture, month of capture, serum estradiol concentration, serum vitellogenin concentration, egg maturation stage, GSI, HSI, length, weight and age. % variance is the proportion of the variance in the data accounted for by the associated factor.

Factor	Eigenvalue	% variance	Cum. % Var.
1	**3.632**	**36.3**	**36.3**
2	2.464	**24.6**	**61.0**
3	**1.088**	**10.9**	**71.8**
4	0.805	8.0	79.9
5	0.624	6.2	86.1
6	0.424	4.2	90.4
7	0.408	4.1	94.4
8	0.246	2.5	96.9
9	0.179	1.8	98.7
10	0.131	1.3	100.0

Table 5: Results of a Factor Analysis on Sydney Harbour winter flounder samples. Weights of variables on each factor.

Variable	Factor 1	Factor 2	Factor 3	Factor 4
Station	0.08513	-0.00213	**0.98915**	0.01464
Month	**0.78684**	-0.11886	0.02573	-0.12102
Estradiol	**0.87321**	-0.01565	-0.10844	-0.36631
Vitellogenin	**0.78763**	0.00306	-0.03293	0.36879
Egg Stage	**0.86860**	-0.00618	0.05227	0.00397
GSI	0.48271	0.12396	-0.06516	0.29878
HSI	0.63288	0.11050	0.19738	0.61580
Length	0.06836	**0.93467**	0.00601	-0.13096
Weight	0.20870	**0.91246**	-0.02006	-0.04434
Age	-0.17996	**0.84114**	-0.03194	0.24496

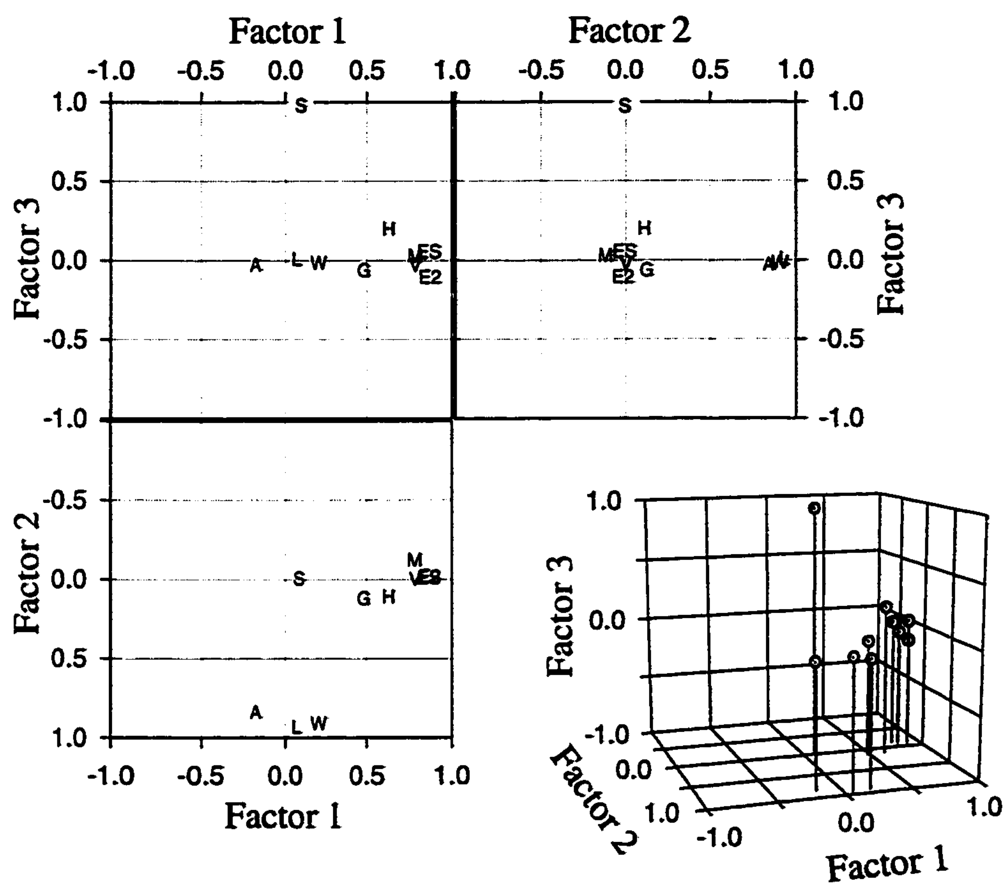


Figure 9: Results of a Factor Analysis on Sydney Harbour winter flounder samples. Loadings of variables on the first 3 factors.
Abbreviations used: S: station of capture, M:month of capture, E2: serum estradiol, V: serum protein-bound phosphorus, ES: egg maturation stage, G: GSI, H: HSI, L: length, W: weight, A: age.

conclusion that season dominated factor 1 and was the main source of variability in the data set. Using the same approach, a comparable clear-cut pattern was found for factors 2 and 3. Variables associated with the size of the fish (A, L, W) scored very high (>0.84) on factor 2 whereas all other variables scored low (<0.13). The second highest source of variability was therefore associated with the morphological differences between fish. Factor 3 appeared to be almost exclusively associated with the station of capture of the fish (weight >0.989). All other variables scored below 0.2 (Table 5, Fig. 9). Interestingly, HSI was the only variable to score more than 0.1 on factor 3 (Table 5) and was also significantly correlated with the station of capture of the fish (Table 6).

Hence, three distinct sources of variability (associated with three groups of variables) could be identified: seasonal effect, morphological effect and station effect. Yet, all the variables were strongly associated with the first three factors as communalities within the factor analysis model and were all above 80% (Table 7).

Within each set of variables associated with each of the factors, the correlation between variables was highly significant (Table 6). Also, length and weight were significantly positively correlated with GSI and HSI (Table 6), indicating that larger fish tended to have comparatively larger gonads and livers.

Following this, a discriminant analysis was performed on the same data set in an attempt to discriminate between sampling stations using the 9 remaining variables. Four discriminant functions were derived from the data (number of stations -1 or numbers of degrees of freedom). Table 8 contains the coefficients that relate each of the original variables to each of the canonical variables. Only the first 2 functions were significant in discriminating between stations and accounted for 48.8% and 31.9% of the variance respectively (Table 9). For both functions, HSI and egg maturation stage had a strong influence. As well, the month of capture strongly affected the first function. Results also show that using the discriminant functions derived from the data, 45% to 67% of the fish from Sydney Harbour could be correctly attributed to their station of capture, while only 25% of the fish from George's Bay (GB) could be correctly predicted to have been

Table 6: Spearman rank correlations between variables. Correlation coefficient and significance level. Results printed in bold are statistically significant (N=821).

	Month	Estradiol	VTG	Egg Stage	GSI	HSI	Length	Weight	Age
Station	0.07	0.04	***0.11***	0.12	-0.03	**0.26**	0.07	0.04	-0.01
	0.05	0.34	***0.01***	0.05	0.51	**$<10^{-4}$**	0.07	0.26	0.79
Month		**0.66**	**0.57**	**0.56**	**0.55**	**0.54**	-0.004	***0.14***	**-0.37**
		$<10^{-4}$	**$<10^{-4}$**	**$<10^{-4}$**	**$<10^{-4}$**	**$<10^{-4}$**	0.91	***0.0003***	**$<10^{-4}$**
Estradiol			**0.56**	**0.69**	**0.52**	**0.43**	0.08	***0.22***	***-0.13***
			$<10^{-4}$	**$<10^{-4}$**	**$<10^{-4}$**	**$<10^{-4}$**	0.06	***$<10^{-4}$***	***0.02***
VTG				**0.65**	**0.52**	**0.55**	0.02	***0.16***	-0.06
				$<10^{-4}$	**$<10^{-4}$**	**$<10^{-4}$**	0.62	***0.0001***	0.36
Egg Stage					**0.76**	**0.63**	0.09	***0.19***	-0.11
					$<10^{-4}$	**$<10^{-4}$**	0.13	***0.002***	0.06
GSI						**0.67**	***0.29***	***0.41***	0.14
						$<10^{-4}$	***$<10^{-4}$***	***$<10^{-4}$***	0.05
HSI							***0.22***	***0.33***	0.14
							$<10^{-4}$	***$<10^{-4}$***	0.06
Length								**0.92**	**0.68**
								$<10^{-4}$	**$<10^{-4}$**
Weight									**0.63**
									$<10^{-4}$

	Station	Month	Estradiol	VTG	Egg Stage	GSI	HSI	Length	Weight	Age
EROD	0.23	-0.42	-0.61	-0.50	-0.61	-0.70	-0.58	-0.17	-0.27	-0.02
N=389	0.004	$<10^{-4}$	$<10^{-4}$	$<10^{-4}$	$<10^{-4}$	$<10^{-4}$	$<10^{-4}$	0.008	$<10^{-4}$	0.72

Abbreviations used: VTG: vitellogenin; GSI: Gonado-Somatic Index; HSI: Hepato-Somatic Index.

Table 7: Communalities estimating the proportion of the variability of each variable that is based on common factors (in this case the first 3 factors). Results of a Factor Analysis on Sydney Harbour winter flounder samples.

Variable	Estimated Communality
Station	0.986
Month	0.928
Estradiol	0.919
Vitellogenin	0.822
Egg Stage	0.911
GSI	0.967
HSI	0.839
Length	0.897
Weight	0.904
Age	0.861

Table 8: Function coefficients for the discrimination between the stations of capture.

Variables	Function 1	Function 2	Function 3	Function 4
Month	**-0.954**	-0.16	-0.241	0.769
Estradiol	-0.003	0.002	-0.0003	-0.007
VTG	-0.0003	-0.0009	0.0004	0.0004
ES	**0.539**	**0.83**	-0.066	0.274
GSI	-0.172	-0.018	0.018	0.044
HSI	**1.05**	**-0.67**	1.5	-0.022
Length	-0.01	0.014	0.018	-0.027
Weight	0.006	-0.0006	-0.005	0.01
Age	-0.103	0.077	-0.163	0.262
Constant	2.01	-4.65	-5.56	1.51

Abbreviations used: VTG: vitellogenin, ES: egg maturation stage, GSI: gonadosomatic index, HSI: hepatosomatic index.

captured at that station (Table 10). Furthermore, close to 30% of the GB fish were predicted by the model to have been caught at the Northwest Arm station (NA).

Some of the enzymatic activities associated with the MFO system were available from Vandermeulen and Mossman (1996) for the winter flounder captured in 1992. These authors reported that the enzymatic activities measured were significantly correlated to one another. Therefore only one of them was taken into consideration here. As one of the more commonly used P450 dependent enzymes, 7-ethoxyresorufin-O-deethylase (EROD) activities for 1992 females were added to the data (N=389) and submitted to factor and discriminant analyses as described previously. Overall, the results remained very similar to the original tests performed. The factor analysis confirmed the presence and the hierarchy of the three main sources of variability in the data (i.e. season>fish morphology>station of capture; Table 11). EROD displayed a strong seasonality, and was also the variable which had the strongest association with the station of capture (Table 11). The multivariate model demonstrated that EROD was negatively correlated with all the other variables excluding the station of capture (Table 6). Also, HSI was found to be better correlated with estradiol (R^2=0.61), vitellogenin (R^2=0.59), egg maturation stage (R^2=0.60) than with EROD (R^2=-0.51). The addition of EROD as a discriminating variable increased the fraction of the variance explained by the discriminant analysis (Table 12) as well as the accuracy of the prediction of the station of capture (Table 13). It showed that EROD became the most influential variable in discriminating between sampling stations (Table 14). Interestingly, the model remained unable to discriminate between GB and NA.

Because some of the variables were not normally distributed (even after extensive transformation), and in order to be consistent, all the statistical tests performed in the following section are non-parametric (unless otherwise noted).

Table 9: Discriminant analysis for the station of capture.

Discriminant Function	Eigenvalue	Percentage Variance	Canonical Correlation	Significance Level
1	0.259	**48.81**	0.45	**0.004**
2	0.169	**31.91**	0.38	**0.05**
3	0.056	10.48	0.23	0.54
4	0.047	8.80	0.21	0.43

Table 10: Classification of captured fish by discriminant analysis.

	Predicted station of capture (%)				
Actual station of capture	GB	MH	SB	NA	SYS
GB	**25.93**	11.11	14.81	**29.63**	18.52
MH	2.78	**44.97**	21.69	19.44	11.11
SB	9.76	12.20	**46.59**	9.51	21.95
NA	9.52	4.76	9.52	**66.67**	9.52
SYS	8.33	0.00	25.00	0.00	**66.67**

Abbreviations used: GB: George's Bay, MH: Main Harbour, SB: South Bar, NA: Nortwest Arm, SYS: Sysco.

Table 11: Results of a Factor Analysis on Sydney Harbour winter flounder samples. Weights of variables on each factor.

Variable	Factor 1	Factor 2	Factor 3	Factor 4
Station	0.03214	-0.13093	**0.93076**	-0.07095
Month	**0.69781**	-0.37253	0.03610	-0.41333
Estradiol	**0.77613**	-0.23665	-0.15551	-0.38802
Vitellogenin	**0.75369**	-0.18843	0.09230	0.07386
Egg Stage	**0.85025**	-0.19961	0.05097	0.05048
GSI	0.64861	0.02785	0.01984	0.33557
HSI	**0.71320**	-0.02441	0.28545	0.44911
Length	0.27073	**0.86861**	0.07528	-0.24951
Weight	0.40529	**0.80199**	0.10193	-0.25707
Age	0.03778	**0.87784**	0.14189	0.16238
EROD	-0.62723	**-0.26393**	0.52742	-0.19278

Table 12: Discriminant analysis for the station of capture (including EROD as a variable).

Discriminant Function	Eigenvalue	Percentage Variance	Canonical Correlation	Significance Level
1	0.605	**58.50**	0.61	**$<10^{-5}$**
2	0.257	**24.87**	0.45	**0.004**
3	0.125	12.04	0.33	0.18
4	0.047	4.59	0.21	0.55

Table 13: Classification of captured fish by discriminant analysis (including EROD as a variable).

Actual station of capture	Predicted station of capture (%) GB	MH	SB	NA	SYS
GB	**38.46**	7.69	15.38	**38.46**	0.00
MH	8.33	**52.78**	11.11	22.22	5.56
SB	7.32	14.63	**56.10**	19.51	2.44
NA	9.52	0.00	9.52	**71.43**	9.52
SYS	0.00	8.33	16.67	8.33	**66.67**

Abbreviations used: GB: George's Bay, MH: Main Harbour, SB: South Bar, NA: Nortwest Arm, SYS: Sysco.

Table 14: Function coefficients for the discrimination between the stations of capture.

Variables	Function 1	Function 2	Function 3	Function 4
Month	-0.133	**-0.807**	0.171	0.485
Estradiol	0.162	-0.410	-0.515	-1.023
VTG	0.368	-0.239	0.149	0.188
ES	-0.163	**0.847**	-0.813	0.185
GSI	0.002	**-1.053**	-0.006	0.327
HSI	**0.605**	**0.497**	0.007	0.502
Length	0.136	-0.374	-0.893	-0.623
Weight	-0.064	**0.790**	0.579	0.846
Age	-0.122	-0.129	0.030	0.253
EROD	**1.181**	-0.131	-0.303	-0.043
Constant	-4.11	2.38	8.29	-0.49

Abbreviations used: VTG: vitellogenin, ES: egg maturation stage, GSI: gonadosomatic index, HSI: hepatosomatic index.

3.3 Seasonal variation

The data show a strong seasonality of all the reproductive parameters monitored (E2,ES,G,V) both in 1992 and in 1993.

Generally, serum estradiol concentrations increased at each station from June (0.15-1.09 $ng.ml^{-1}$) to September (0.94-2.51 $ng.ml^{-1}$) and from September to October/November (1.53-3.53 $ng.ml^{-1}$) for both years (Fig. 10). Most of these increases were highly significant (Table 15). For the majority of fish, the concentrations measured in June were close to the minimum levels reached in the reproductive cycle (0.1-0.5 $ng.ml^{-1}$). However, a certain number of fish displayed high concentrations of estradiol (up to 4.6 $ng.ml^{-1}$). These were individuals captured at the George's Bay (GB), Main Harbour (MH) and South Bar (SB) stations in 1992 and at the GB and MH stations in 1993. These fish account for the higher variability in the results from those stations.

The pattern for serum protein-bound phosphorus concentrations was slightly different although an increase over time was obvious. Concentrations were at their lowest level in June of both years (400-800 μM) (Fig. 11). However in 1992 there appeared to be very little increase between June and September, whereas in 1993 the increase was highly significant for the same period (Table 16). With the exception of GB in 1993, serum protein-bound phosphorus was significantly higher in October/November than in September (Table 16) and reached 1500-2500 μM (Fig. 11).

In June, the ovaries of the majority of the fish from the five stations contained primordial oocytes (Stage 1). However, a small number of fish from GB, MH and SB in 1992 and GB and MH in 1993, had ovaries containing Stage 5 oocytes. These fish were the same which displayed high estradiol concentrations. Also, more than 25% of the samples at NA displayed atretic oocytes. Similarly, more than 20% of the fish from NA and 33% of the fish from SYS were resorbing their eggs. In September, depending on the station of origin, oocytes ranking ranged from Stage 1 to Stage 4, although globally the median value increased from Stage 1 to Stage 2. In October/November, the majority of samples were ranked at Stage 4, with a few individuals only at Stage 3.

GSI and HSI displayed identical trends (Tables 17-18). There appeared to be no significant increase in the first interval, and a very significant increase in the second for both sampling years (Table 19). FCI remained unaffected by the month of capture (Table 19).

Serum protein levels in fish did not increase significantly between June and September (1.7-4.1 $mg.ml^{-1}$). Individuals with high serum estradiol, protein-bound phosphorus and mature oocytes also had higher serum protein concentrations (up to 7.0 $mg.ml^{-1}$). By October/November serum protein levels had significantly increased to 3.2-5.1 $mg.ml^{-1}$ ($p<0.001$).

Concentrations of PAHs in the ovaries showed no seasonal variations. The number of samples analyzed for PAH bile metabolites was too low to run any meaningful statistics, however no differences between sampling seasons were apparent.

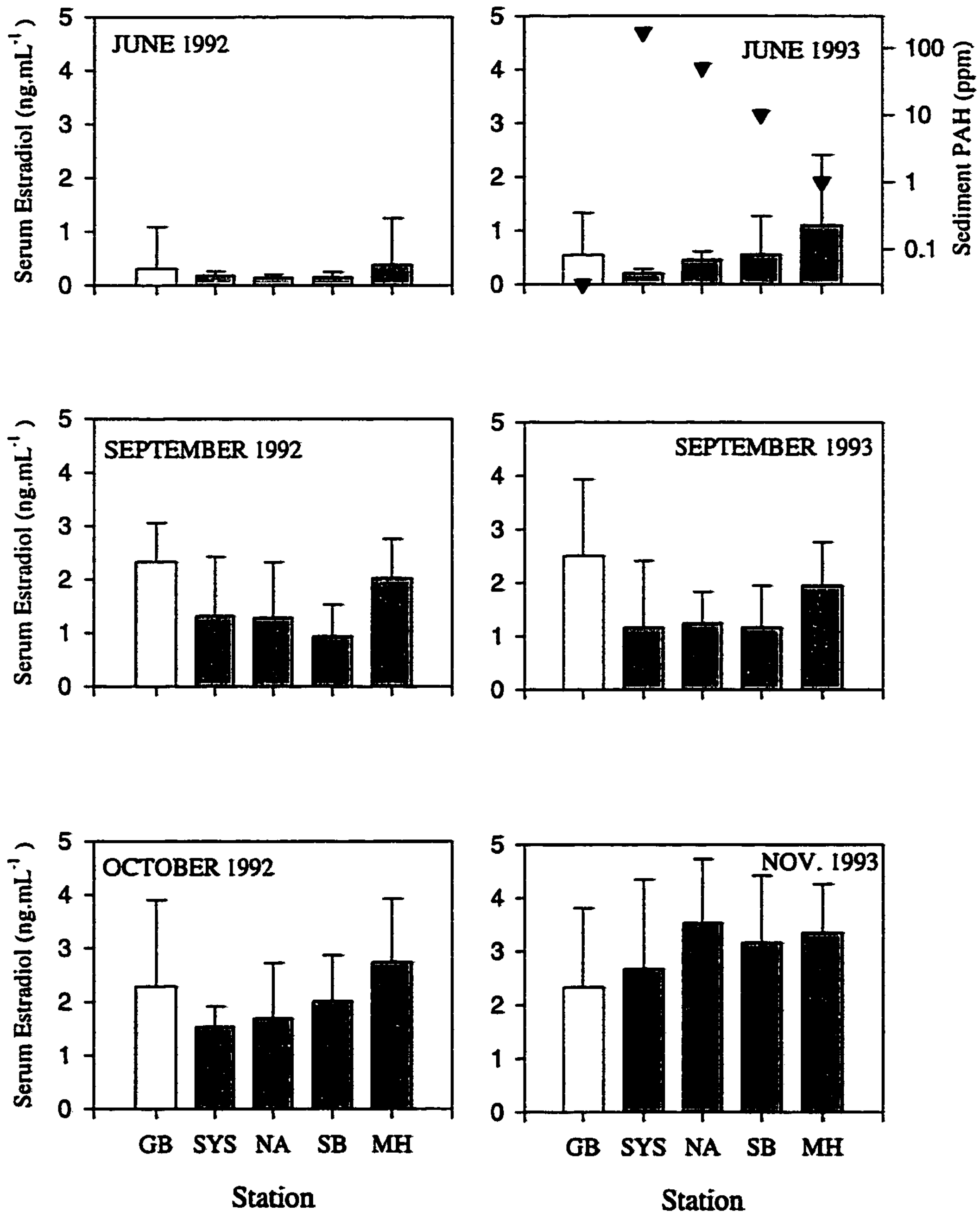


Figure 10: Serum estradiol concentrations in female winter flounder from Sydney Harbour and George's Bay (mean +/- SD).
Abbreviations used: GB: George's Bay, SYS: Sysco, NA: Northwest Arm SB: South Bar, MH: Main Harbour.
▼ indicate sediment PAH concentrations at each station.

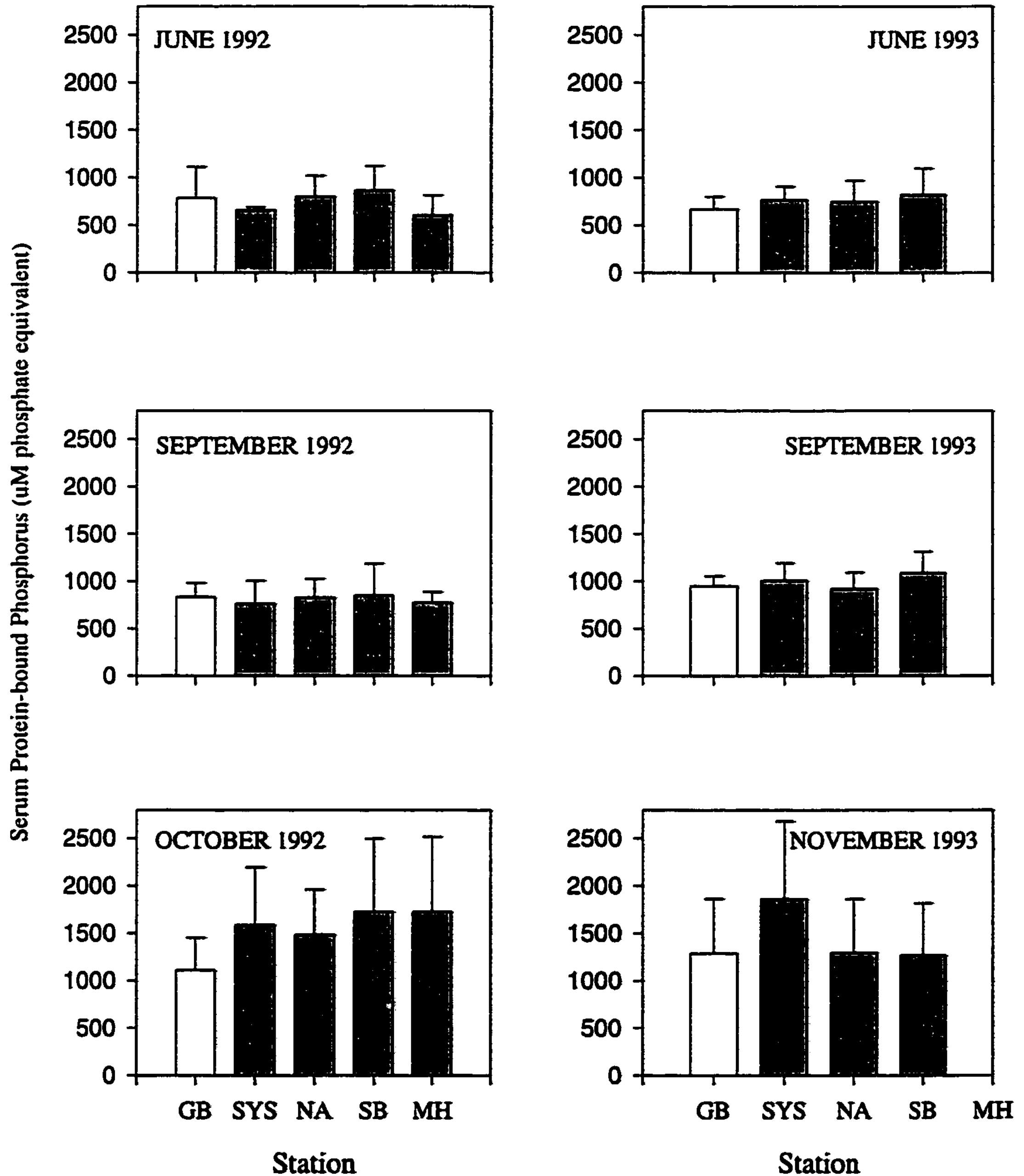


Figure 11: Serum protein-bound phosphorus concentrations in female winter flounder from Sydney Harbour and George's Bay (mean +/-SD).
Abbreviations used: GB: George's Bay, SYS: Sysco, NA: Northwest Arm SB: South Bar, MH: Main Harbour.

Table 15: Comparison of mean serum estradiol concentrations (in female winter flounder) at each station between two consecutive sampling windows. Results of Mann-Whitney rank sum test (P values).

Year	Interval	GB	SYS	NA	SB	MH
1992	June-September	$<10^{-4}$	NS	$<10^{-4}$	$<10^{-4}$	0.017
	September - October	NS	$<10^{-4}$	$<10^{-4}$	$<10^{-4}$	$<10^{-4}$
1993	June-September	$<10^{-4}$	$<10^{-4}$	$<10^{-4}$	$<10^{-4}$	$<10^{-4}$
	September-November	0.039	NS *power=0.05*	NS	0.0013	0.0029

Table 16: Comparison of mean serum protein-bound phosphorus concentrations (in female winter flounder) at each station between two consecutive sampling windows. Results of Mann-Whitney rank sum test (P values).

Year	Interval	GB	SYS	NA	SB	MH
1992	June-September	NS	NS *power=0.18*	NS	NS	0.017
	September - October	0.016	0.043	$<10^{-4}$	0.0011	0.0013
1993	June-September	$<10^{-4}$	$<10^{-4}$	0.029	0.0002	$<10^{-4}$
	September-November	NS	$<10^{-4}$	$<10^{-4}$	$<10^{-4}$	$<10^{-4}$

Table 17: Comparison of mean GSI values (in female winter flounder) at each station between two consecutive sampling windows. Results of Mann-Whitney rank sum test (P values).

Year	Interval	GB	SYS	NA	SB	MH
1992	June-September	NS	NS *power=0.54*	NS	NS	NS
	September - October	0.014	0.028	$<10^{-4}$	$<10^{-4}$	$<10^{-4}$
1993	June-September	NS	NS	NS	NS	NS
	September-November	0.032	0.001	0.011	$<10^{-4}$	$<10^{-4}$

Table 18: Comparison of mean HSI values (in female winter flounder) at each station between two consecutive sampling windows. Results of Mann-Whitney rank sum test (P values).

Year	Interval	GB	SYS	NA	SB	MH
1992	June-September	NS	NS *power=0.05*	NS	NS *power=0.34*	NS
	September - October	$<10^{-4}$	0.01	0.01	$<10^{-4}$	$<10^{-4}$
1993	June-September	NS	NS	NS	NS	NS
	September-November	0.021	0.001	0.009	$<10^{-4}$	$<10^{-4}$

Table 19: Mean FCI, GSI and HSI (± s.d.) at each station for the three sampling months.

1992	June	GB	SYS	NA	SB	MH
	FCI	1.2±0.2	1.1±0.2	1.1±0.2	1.2±0.2	1.1±0.1
	GSI	6.9±7.6	2.9±0.4	19.0±18.7	3.6±2.9	6.3±6.2
	HSI	1.4±0.3	2.1±0.5	1.7±0.3	1.9±0.5	1.8±0.3
	September					
	FCI	1.3±0.2	1.2±0.1	1.2±0.1	1.2±0.1	1.2±0.1
	GSI	3.6±1.3	2.0±1.0	2.8±0.6	3.4±0.7	2.8±0.8
	HSI	1.7±0.5	1.6±0.5	1.8±0.4	1.7±0.3	1.5±0.4
	October					
	FCI	1.3±0.2	1.3±0.1	1.2±0.1	1.3±0.1	1.3±0.1
	GSI	7.0±4.2	6.1±3.7	6.5±2.6	7.9±2.8	7.1±2.9
	HSI	2.3±0.7	2.5±0.8	2.3±0.6	2.6±0.7	2.4±0.7
1993	June	GB	SYS	NA	SB	MH
	FCI	1.1±0.2	1.1±0.2	1.2±0.2	1.2±0.2	1.1±0.1
	GSI	5.8±4.9	21.4±19.8	18.3±17.7	3.1±2.9	6.1±5.7
	HSI	1.7±0.3	2.2±0.6	1.8±0.3	1.9±0.6	1.8±0.2
	September					
	FCI	1.1±0.2	1.3±0.1	1.2±0.1	1.1±0.1	1.2±0.1
	GSI	3.8±1.4	2.2±0.9	3.0±0.9	3.2±0.8	3.1±0.8
	HSI	1.5±0.5	1.9±0.4	1.9±0.4	1.6±0.3	1.4±0.3
	November					
	FCI	1.2±0.1	1.3±0.2	1.1±0.1	1.3±0.3	1.2±0.2
	GSI	7.4±3.9	6.2±4.0	6.3±2.5	7.7±3.0	7.3±2.7
	HSI	2.5±0.9	2.7±0.6	2.4±0.5	2.4±0.8	2.2±0.5

3.4 Influence of the sampling site

Fewer variables were significantly influenced by the site of capture than by the month of capture. Site of capture had a significant effect on serum estradiol concentrations for every sampling month (Table 20). The pairwise comparisons between stations generally showed that fish from GB and MH were not different (with the exception of October 1992), and had significantly higher levels of serum estradiol than fish from the three most contaminated sites in the harbour (SYS, NA, SB; Fig. 10). Furthermore, no differences between the latter stations were significant. In June 1992 however, no significant differences between stations could be measured. This was the result of the presence of several fish with unusually high levels of estradiol in the serum at stations GB, MH and SB, which increased the variance in the data considerably. If these individuals were to be removed from the data, GB and MH would then become significantly different from SYS, NA and SB. In November 1993 surprisingly, the fish captured at the reference site appeared to have, on average, lower serum estradiol concentrations than the fish captured in Sydney Harbour (Fig. 10).

Although for some sampling months ANOVA detected a significant effect of the station of capture on serum protein-bound phosphorus concentrations (Table 21), no identifiable trends emerged from the data (Fig. 11). Furthermore, as shown on Figure 12, the correlations between serum estradiol and protein-bound phosphorus concentrations were unexpectedly low ($25\%<R^2<40\%$).

The results of the analysis of the oocytes maturity stages were remarkably similar for both sampling years. In June 1992, more than 25% of the fish captured at NA displayed a majority of atretic oocytes. In June 1993, more than 20% of the fish from NA and 33% of the fish from SYS had mostly atretic oocytes. For both years however, the differences in oocyte maturity between all stations were not statistically significant. In September of both years, the oocytes of the fish from the stations with the highest sediment PAH concentrations (SYS and NA) were at a significantly less advanced stage of maturity than the fish from the remaining stations (Table 22).

Table 20: Effect of sampling station on serum estradiol concentrations in female winter flounder. Results of Kruskal-Wallis ANOVA on ranks followed by all pairwise multiple comparison procedures (Dunn's Method) :

Year	Month	P value	Significant differences between stations
1992	June	0.0027	no significant differences between stations within the model
	September	0.0005	GB and MH different from NA, SB, SYS
	October	0.0014	MH≠SYS; MH≠NA; MH≠SB GB≠SYS; GB≠NA
1993	June	0.0020	MH≠SYS; MH≠NA; MH≠SB GB≠SYS; GB≠NA
	September	$<10^{-4}$	GB and MH different from SB, NA, SYS
	November	0.0006	NA≠GB; NA≠SYS

Table 21: Effect of sampling station on serum protein-bound phosphorus concentrations in female winter flounder. Results of Kruskal-Wallis ANOVA on ranks followed by all pairwise multiple comparison procedures (Dunn's Method) :

Year	Month	P value	Significant differences between stations
1992	June	0.0116	MH≠SB
	September	NS	-
	October	NS *power=0.079*	-
1993	June	0.0343	MH≠SB
	September	0.0104	SB≠NA; MH≠NA
	November	NS	-

Analysis of variance was unable to detect any effect of the sampling station on the mean values of FCI, GSI, HSI and serum protein at any sampling period.

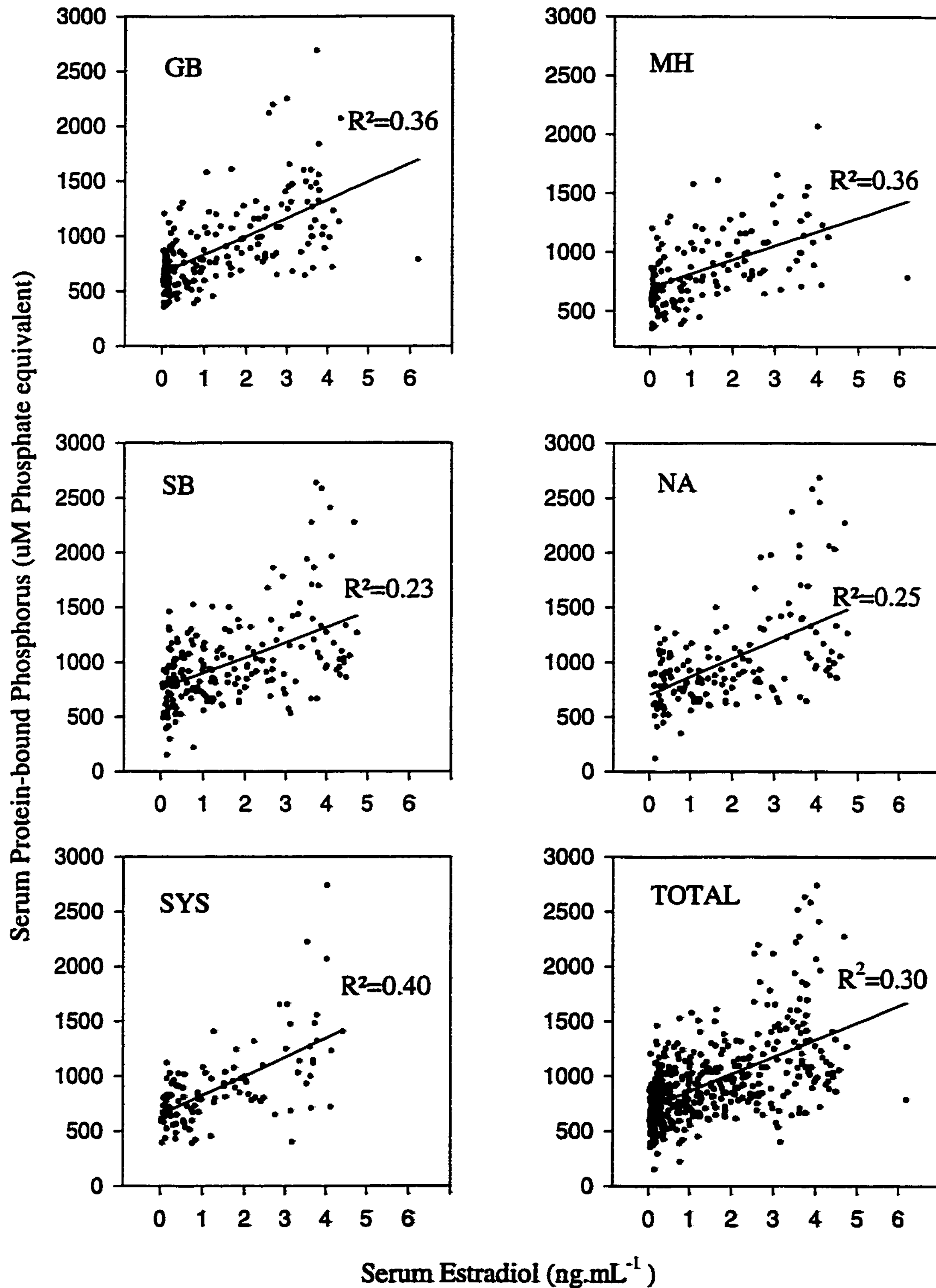


Figure 12: Linear regressions between serum protein-bound phosphorus and serum estradiol concentrations in female winter flounder from Sydney Harbour and George's Bay.

Oocytes from the SYS and NA fish had on average reached Stage 2, while fish from SB, MH and GB displayed mainly Stage 3 oocytes. Interestingly, of the latter stations, SB had the most fish with Stage 2 oocytes in 1992. In October 1992 and November 1993, no differences between stations were statistically detectable. The oocytes of the majority of the fish were ranked at Stage 4.

There appeared to be little or no accumulation of PAHs in the ovaries. Of the 16 parental PAHs investigated (recommended priority pollutants), only 5 were found to be present above the detection limit (Table 23). Acenaphthene was present in 69% and naphthalene was detected in 15% of the samples. These were the only two PAHs present in any significant amounts. Levels of the PAHs present were generally low (0.01-0.10 ppm). Two fish had ovarian concentrations of acenaphthene above 1 ppm because of probable interference (RPC, pers. comm.) and were removed from the data set. Also, there appeared to be no correlation between ovarian PAH concentration and fat content.

Three to six PAH metabolites were detected in the bile of the fish from all stations (Fig. 13). However the concentrations were generally higher in the fish from Sydney Harbour and highest in fish captured at SYS. Concentrations of higher molecular weight compounds such as 1-hydroxypyrene and hydroxyfluoranthene were generally higher (up to 10 times higher at SYS) than the lower MW metabolites 4-phenylphenol and 9-hydroxyfluorene.

Table 22: Effect of sampling station on oocyte maturation in female winter flounder. Results of Kruskal-Wallis ANOVA on ranks followed by all pairwise multiple comparison procedures (Dunn's Method) :

Year	Month	P value	Oocyte maturation stages
1992	June	NS	All stations at Stage 1
	September	0.009	GB,MH,SB at Stage 3
			SYS,NA at Stage 2
	October	NS	All stations at Stage 4
1993	June	NS	All stations at Stage 1
	September	0.0104	GB,MH,SB at Stage 3
			SYS,NA at Stage 2
	November	NS	All stations at Stage 4

Table 23: Mean ovarian PAH concentrations of (n) winter flounder from Sydney Harbour and George's Bay (units are ng.g^{-1}): Detection limit of the method is 10 ng.g^{-1}.

Station	GB			MH			SB			NA			SYS		
PAH (by increasing MW)	June N=5	Sept. N=5	Oct. N=5	June N=5	Sept. N=10	Oct. N=10	June N=5	Sept. N=13	Oct. N=15	June N=5	Sept. N=9	Oct. N=11	June N=4	Sept. N=6	Oct. N=6
Naphthalene		11.92(1)			11.38(2)	11.21(3)		12.94(3)			15.22(1)			12.23(2)	
Acenaphthylene															
Acenaphthene	21.19(4)	67.20(3)	46.89(4)	16.37(4)	31.90(2)	36.45(8)	18.52(5)	27.72(5)	45.12(12)	27.52(5)	57.37(9)	30.42(11)	27.11(4)	15.74(6)	41.30(3)
Fluorene											13.80(1)				
Phenanthrene						31.10(1)					11.19(1)				
Anthracene															
Fluoranthene															
Pyrene											12.21(1)				
Bz(a)anthracene															
Chrysene/ Triphenylene															
Bz(b+k)fluoranthene															
Bz(e)pyrene															
Bz(a)pyrene															
Indopyrene															
Bz(ghi)perylene															
Dibz(ah)anthracene															

Abbreviations used: GB: George's Bay, MH: Main Harbour, SB: South Bar, NA: Northwest Arm, SYS: Sysco, N: number of samples.
Numbers in () indicate the number of samples in which the PAH was detected.

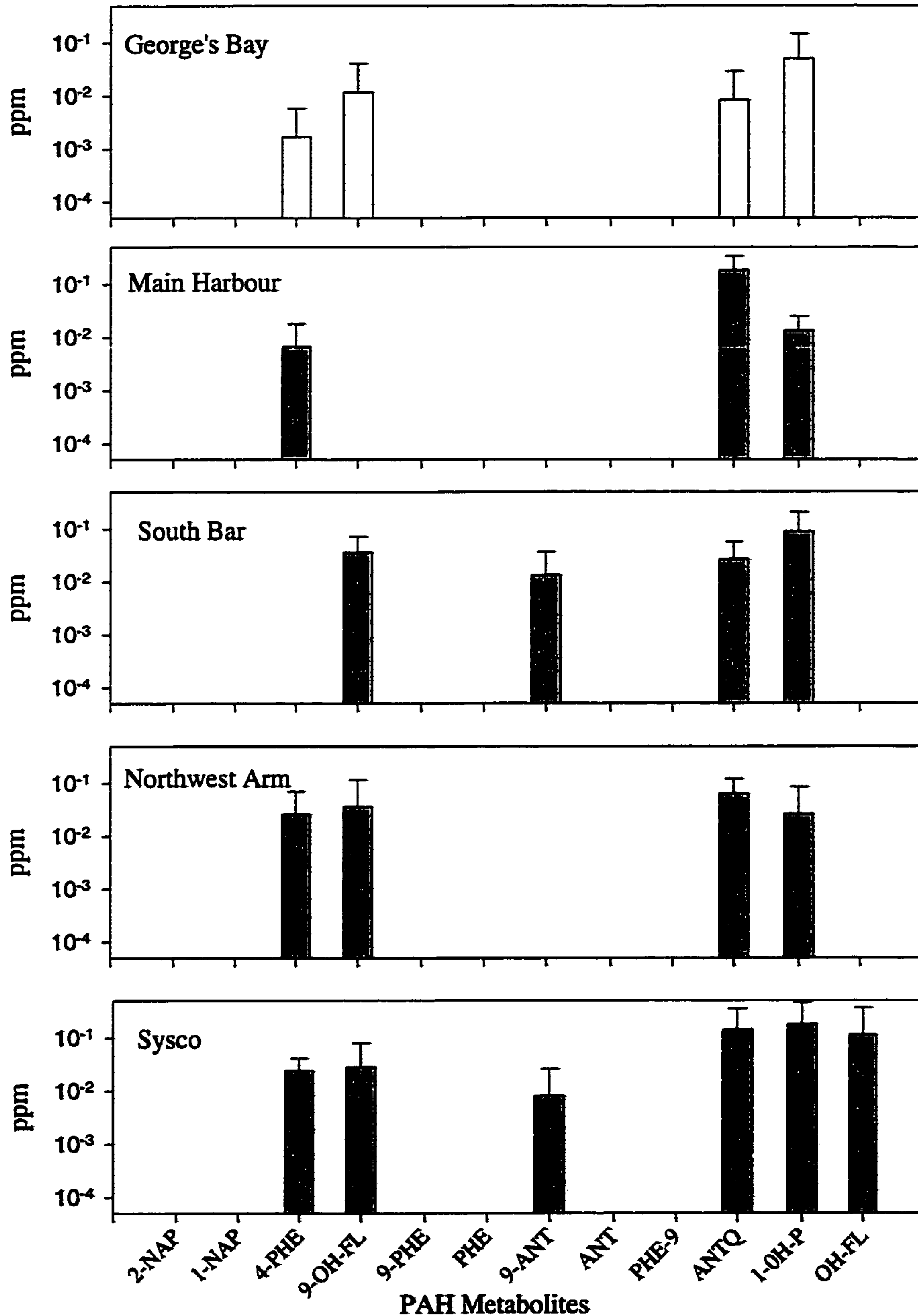


Figure 13: Concentrations of PAH metabolites (by increasing MW) in the bile of female winter flounder from Sydney Harbour and George's Bay (mean =/- SD).
Abbreviations used: 2-NAP: 2-naphthol, 1-NAP: 1-naphtol, 4-PHE: 4-phenylphenol, 9-OH-FL: 9-hydroxyfluorene, 9-PHE: 9-phenanthrol, PHE: phenanthrol, 9-ANT: 9-anthraldehyde, ANT: anthraldehyde isomers, PHE-9: phenanthrene-9-carboxaldehyde, ANTQ: anthraquinone, 1-OH-P: 1-hydroxypyrene, OH-FL: hydroxyfluoranthene.

3.5 Endocrine disrupting effect in male winter flounder

There appeared to be no detectable effect of the season on male serum estradiol (Table 24) and protein-bound phosphorus concentrations (Table 25), although in many cases the power of the Mann-Whitney rank test was very low (5-31%), due to a small sample size and a high variance.

Analysis of variance on ranks revealed that in some cases the station of capture of the fish affected the serum estradiol concentrations and that significant differences could be detected between stations (Table 26). However, no pattern was evident, and the levels remained extremely low throughout the reproductive cycle (Fig. 14). In the case of serum protein-bound phosphorus concentrations, except for the June 93 results, ANOVA on ranks showed a significant effect of the sampling site (Table 27). Differences between stations (Dunn's pairwise comparison) demonstrated that fish from the reference site (GB) had consistently significantly lower concentrations of protein-bound phosphorus in the serum than most fish from Sydney Harbour (Fig. 15). Furthermore, it was apparent that protein-bound phosphorus concentrations in the fish from Sydney Harbour decreased with distance from the tar ponds. In fact, association of serum protein-bound phosphorus with sediment PAH concentrations established that higher PAH concentrations where significantly correlated with higher protein-bound phosphorus concentrations (Fig. 16). Moreover, the slope of the relationship increased from June to September and from September to October/November. This result was consistent over the two consecutive years of the study.

4. Discussion

This study has monitored half of the reproductive cycle of the winter flounder for two consecutive years in fish collected from stations with pollution profiles ranging from high to very low. In both females and males, an effect of the station of capture was detected, suggesting that coal-tar derived sediment contaminants adversely influenced the reproductive cycle of the fish.

Table 24: Comparison of mean serum estradiol concentrations (in male winter flounder) at each station between two consecutive sampling windows. Results of Mann-Whitney rank sum test (P values).

Year	Interval	GB	SYS	NA	SB	MH
1992	June-September	NS	no data	NS *power=0.17*	NS *power=0.05*	0.0044
	September - October	0.0014	NS	NS	NS	NS
1993	June-September	NS	NS *power=0.05*	NS	NS *power=0.31*	$<10^{-4}$
	September-November	NS	NS	NS	NS	NS

Table 25: Comparison of mean serum protein-bound phosphorus concentrations (in male winter flounder) at each station between two consecutive sampling windows. Results of Mann-Whitney rank sum test (P values).

Year	Interval	GB	SYS	NA	SB	MH
1992	June-September	NS *power =0.05*	no data	NS *power =0.05*	NS *power=0.05*	NS *power=0.05*
	September - October	NS *power=0.09*	NS	NS *power=0.05*	NS	0.0308
1993	June-September	NS	NS	NS	NS	NS *power=0.06*
	September-November	NS	0.0303	NS	NS	NS

Table 26: Effect of sampling station on serum estradiol concentrations in male winter flounder. Results of Kruskal-Wallis ANOVA on ranks followed by all pairwise multiple comparison procedures (Dunn's Method).

Year	Month	P value	Significant differences between stations
1992	June	0.0030	MH different from SB, GB, NA
	September	0.0134	SB≠MH
	October	0.0276	no significant differences between stations within the model
1993	June	NS	-
	September	10^{-4}	GB different from SB, NA, SYS
	November	10^{-4}	GB≠SYS

Table 27: Effect of sampling station on serum protein-bound phosphorus concentrations in male winter flounder. Results of Kruskal-Wallis ANOVA on ranks followed by all pairwise multiple comparison procedures (Dunn's Method).

Year	Month	P value	Significant differences between stations
1992	June	0.0009	no significant differences between stations within the model
	September	0.0002	GB different from SYS, NA, SB SYS≠MH
	October	0.0156	GB different from SYS, NA, SB
1993	June	NS	-
	September	10^{-4}	GB different from SYS, NA, SB MH≠SB;
	November	10^{-4}	GB different from SYS, NA SYS≠MH

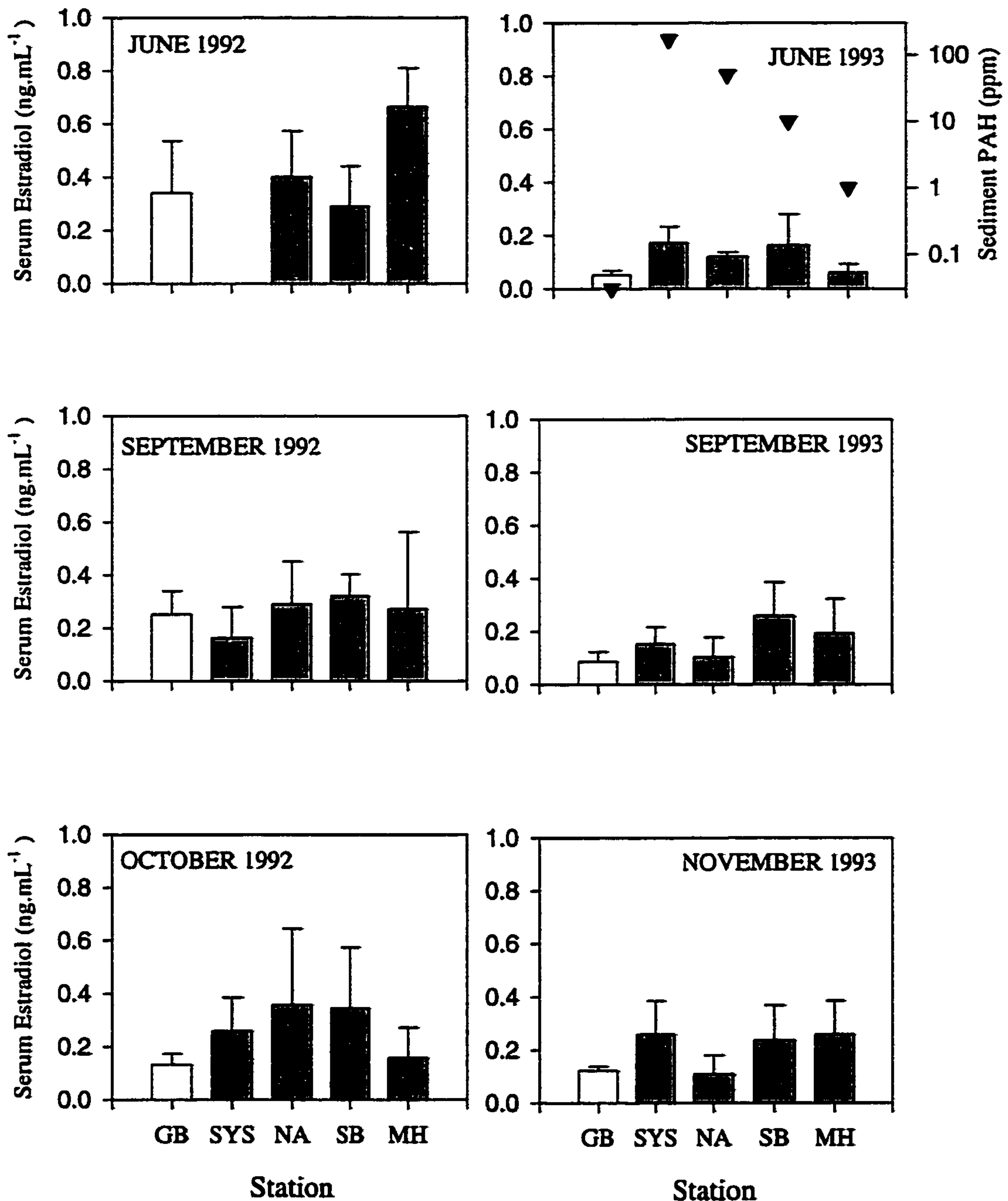


Figure 14: Serum estradiol concentrations in male winter flounder from Sydney Harbour and George's Bay (mean +/- SD).
Abbreviations used: GB: George's Bay, SYS: Sysco, NA: Northwest Arm SB: South Bar, MH: Main Harbour.
▼ indicate sediment PAH concentrations at each station.

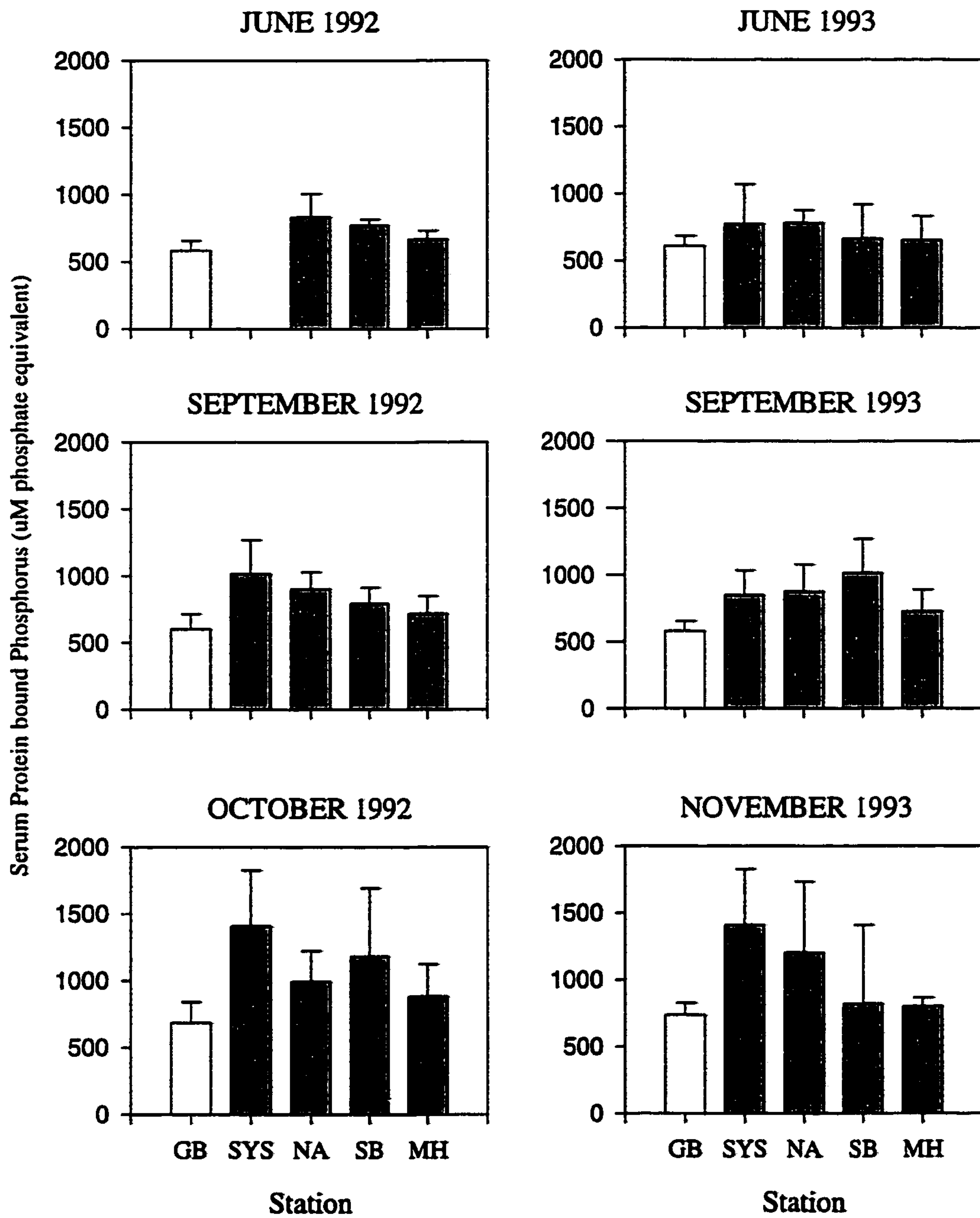


Figure 15: Serum protein-bound phosphorus concentrations in male winter flounder from Sydney Harbour and George's Bay (mean +/- SD).
Abbreviations used: GB: George's Bay, SYS: Sysco, NA: Northwest Arm SB: South Bar, MH: Main Harbour.

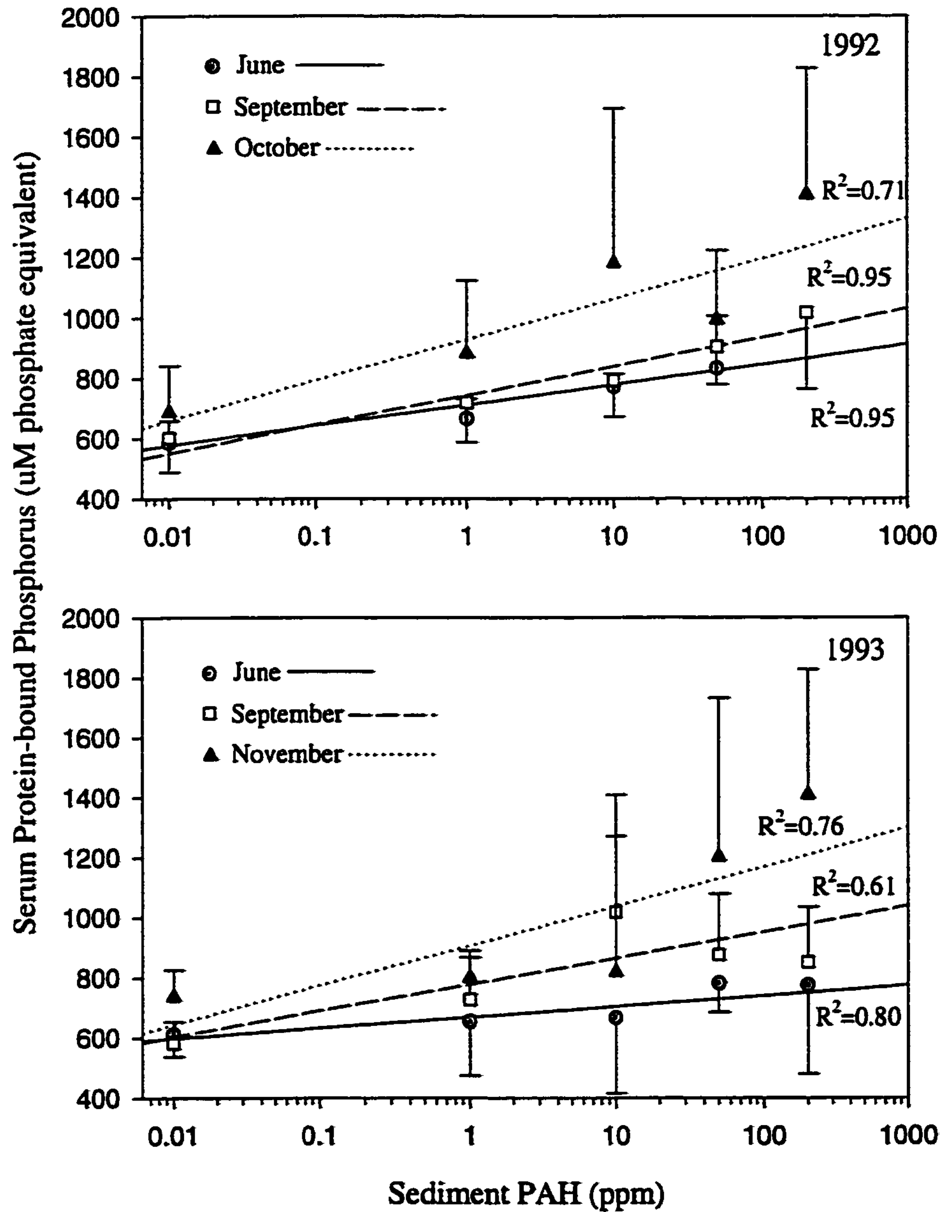


Figure 16: Linear correlations between serum protein-bound phosphorus concentrations and log(sediment PAH concentration) in male winter flounder from Sydney Harbour and George's Bay (mean +/- SD).
The sediment PAH concentration for George's Bay was arbitrarily set at 0.01 ppm (half the detection limit).

PAHs are more probably responsible than PCBs and heavy metals (or other pollutants) for the effects detected in this study. First the concentrations of PAHs are 50 to 100 times higher than PCB concentrations and second PAHs have been reported to be more closely associated to ovarian development inhibition and depressed plasma estradiol than PCBs (Johnson et al., 1988). Therefore, for the purpose of this discussion, PAHs will be assumed to be the contaminants causing the observed effects, and will be used as a surrogate for all the contaminants present in the sediments. However, it should be kept in mind that the fish were exposed to a mixture of contaminants. The effects observed here could be the result of some other contamination which was not taken into account.

4.1 Multivariate analysis

The almost inherent high variability associated with environmental studies has often been cause for concern (Vandermeulen and Mossman, 1996). However, few authors have addressed this problem, which, in many cases, has made interpretation of results very difficult. In such cases, the sample size would have to be unrealistically increased in order for "conventional" statistical tests (i.e. univariate) to effectively detect an environmental impact.

In this study, univariate statistics were unable to discriminate between stations (for all variables) and in some cases, were unable to detect any effect at all. This was the result of the variance associated with most of the variables being higher than anticipated. Calculation of the original sample size (30) was based on preliminary analysis of a few samples and published results from other studies. Based on the results presented here, and depending on the variable considered, the sample size necessary to detect a 20% difference between the mean values of 5 stations in a one-way ANOVA model ranged from 55 to 96. These are clearly not realistic numbers of samples for this kind of study. Therefore, only extreme effects can be detected by this kind of analysis.

Multivariate statistics, on the other hand, were able to clearly detect the effect of the station of capture in the data set (factor analysis), and even accurately discriminated between Sydney Harbour stations in 45 to 67% of cases (discriminant analysis). This,

along with other studies (Van Der Oost, et al., 1997; Wirgin et al., 1994; Casillas et al., 1991), illustrates the value of multivariate statistics to analyze data sets with high variability. The results of the discriminant analysis (even after addition of EROD as a variable) also indicate that GB is very "similar" to the Sydney Harbour stations in terms of effects on the variables. This was surprising, and suggests the existence at this station of conditions inducing the same kind of response in fish as those present in Sydney Harbour.

4.2 Seasonal variation

Overall, female winter flounder from Sydney Harbour conformed with the description of the vitellogenic cycle in Pleuronectidae. Most fish (90% overall) successfully complete the vitellogenic cycle. Serum estradiol concentrations were similar in June and September but as much as 10 times lower in October/November than those reported by Harmin et al. (1995) for the same periods. Vitellogenin and protein concentrations (Kramer et al., 1998; Janssen et al., 1997; Johnson et al., 1991; 1988), as well as GSI and HSI (Harmin et al., 1995; DeVlaming et al. 1982; Htun-Han, 1978), were found to be in the same range as those of other fish species. As in *Pleuronectes flesus* (Janssen et al., 1995), oogonia were present in the ovary throughout the sampling season (and most probably throughout the whole reproductive cycle). By June, very few late spawning fish, displaying Stage 5 oocytes as well as high serum estradiol and vitellogenin, were observed. These late spawning fish were only found at the outer stations of the estuary (MH and SB) and at the reference site, suggesting that they might be late in their in-land migration. Also, the presence of post-ovulatory follicles indicated that spawning had occurred recently in some individuals. These observations strongly suggest that spawning occurs in May in this population of winter flounder. Oocyte size (30-700 µm) over the range of the whole reproductive cycle corroborated the results of Harmin et al. (1995) and Burton and Idler (1987) and was comparable with that of other flatfish (Janssen et al., 1995; Yamamoto, 1956). There was no evidence of a starvation-induced 3-year maturation cycle of the female winter flounder from Sydney Harbour as described by Burton (1994, 1991) and Burton and Idler (1984). This would tend to indicate that food is not a limiting factor in

the Sydney Estuary. Indeed, most of the stomachs of the fish dissected contained fairly large amounts of food.

The absence of seasonal variation of bile metabolite concentrations suggests that the depuration rate of PAHs in the fish sampled adjusts to the levels of PAHs present in the sediment, as the bioavailability of PAH is strongly affected by temperature (Neff, 1985; Whitehouse, 1984). This is supported by numerous studies demonstrating the dose-dependant relationship between detoxifying enzymes activities and PAH exposure (Goksøyr and Förlin, 1992; Stegeman and Lech, 1991; Elskus and Stegeman, 1989; Ahokas and Pelkonen, 1984). Furthermore, no significant accumulation of PAHs was detected in the ovaries sampled (all concentrations measured were at or just above detection limit). This could indicate that the depuration rate was sufficient to prevent any PAHs from reaching the ovaries (see Chapter 1, Section 3.2). However, it should be noted that the method to measure ovarian PAHs used here was 100 times less sensitive than that, for example, of Hellou and Warren (1997). As a result, ovarian concentrations of PAHs could have been significantly underestimated. In studies which have demonstrated a significant accumulation of PAHs and metabolites in the ovaries (Varanasi et al., 1982; Hose et al., 1981), the contaminants were generally injected peritoneally or force-fed in high doses. These modes of exposure, although useful in the investigation of mechanisms of action, do not realistically simulate the natural routes of uptake of hydrocarbons and could explain the discrepancy with the results presented here.

4.3 Influence of the sampling site

Regrettably, since the question of the mechanism(s) of action of the contaminants was not addressed by this study, one can only speculate as to how the PAHs affect the different aspects of the reproductive cycle considered here.

Nonetheless, the present study confirms that high sediment concentrations of PAHs reduce circulating levels of estradiol in fish. In this particular case however, one way ANOVA was unable to discriminate between stations with sediment PAH concentrations below 10 ppm (GB, MH) or between stations with sediment PAH above this

concentrations (SB, NA, SYS). Moreover, serum estradiol concentrations correlated poorly with sediment PAHs (data not shown), illustrating the non-linear nature of the response. This suggests the existence of a threshold level of sediment PAHs (around 10 ppm) for estradiol impairment. The decrease in estradiol is consistent with increase catabolism associated with the induction of MFO activity in the liver of the fish captured in 1992 (Vandermeulen and Mossman, 1996). However, no information on the rate of excretion of estradiol metabolites is available to confirm this mechanism. The weak but highly significant correlation between HSI and the station of capture (Table 6) could be the result of increased protein synthesis by the liver. This could be linked to the induction of detoxifying enzymes (Janssen, 1996; Truscott et al., 1992) as well as the production of vitellogenin (Lye et al., 1997; Sumpter and Jobling, 1995). Vitellogenin being significantly correlated with the station of capture (Table 6), while estradiol was not, could be indicative of a weak estrogenic effect (see below). This could also account for the correlation of HSI and station of capture. Since HSI was better correlated with estradiol, vitellogenin and egg maturation stage than with EROD, it would seem that the increase in relative liver size is due more to vitellogenin production than to MFO induction. This is further supported by Vandermeulen and Mossman's (1996) results which show no increase in liver microsomal protein content for the fish captured in 1992.

It is not known why the fish from GB had low levels of estradiol in the serum in November 1993. These fish were caught within 4 days from the others and bottom temperatures at GB (9.7°C) were not different from those in Sydney Harbour (9.4-9.9°C). One possible explanation is the presence of unusually high numbers of dogfish (*Squalus acantatus*). Spiny dogfish were by far the most abundant species caught at that station (several hundred) and winter flounder are known to be one of their many prey species in this area (Scott and Scott, 1988). The increased predation by the sharks could have caused stress related elevation of circulating levels of cortisol. This has been demonstrated to reduce estradiol and vitellogenin concentrations in trout *Salmo trutta* and *S. gairdneri* (Carragher and Sumpter, 1990; Carragher et al, 1989). However, in the present case, vitellogenin concentrations did not appear to be similarly reduced.

No pattern emerged from the vitellogenin concentrations. Although significant, correlations between serum estradiol and protein-bound phosphorus concentrations remained low even within the multivariate model (Table 6, Fig. 11). This is surprising as estradiol has been demonstrated to induce a dose-dependant synthesis of vitellogenin (Johnson et al., 1991; DeVlaming et al., 1980; Emmersen and Petersen, 1976). The results could not show that sediment PAHs significantly affected vitellogenin concentrations, or that PAHs influenced the association between estradiol and vitellogenin. However, as can be seen on Figure 11, vitellogenin concentrations tended to increase more rapidly than estradiol concentrations. This result is consistent with the findings of Anderson et al. (1996b), which showed that under high circulating concentrations of estradiol, serum vitellogenin concentrations were significantly increased by β-naphthoflavone. Furthermore, as in the present study, this increase was not dose-dependant in the presence of 2 and 4-fold increases in PAH concentrations. This mild estrogenic effect could account for the significant weak correlation between vitellogenin concentrations and station of capture (Table 6) and would be consistent with the slight increase in HSI discussed previously.

It is significant that the development of the oocytes of the fish captured in September at SYS, NA and SB was slower, having only reached Stage 2 which is pre-vitellogenic. By comparison, the fish captured at GB and MH had normal oocyte development reaching Stage 3, which is vitellogenic. The slower development of ovarian follicles was also documented by Thomas and Budiantara (1995) in a laboratory experiment (Atlantic croaker exposed to 5% WSF of oil and 1 ppm naphthalene) and by Stott et al. (1983, 1981) in fish from environments heavily contaminated with oil. Thomas and Budiantara (1995) also reported that 30-56% of the oil- and naphthalene-exposed fish failed to undergo sexual maturation. Furthermore, there was great individual variability, as "*oocyte development appeared to be arrested in some fish, whereas it was merely delayed in others since some individuals eventually developed fully grown oocytes capable of final maturation and fertilization*" (p. 149). This also concurs with the results presented here, since the oocytes of most fish sampled in October and November had reached an advanced stage of vitellogenic development (Stage 4). In the case of heavy oil

contamination, Stott et al. (1983) observed that no mature oocytes were observed at any season in the plaice (*Pleuronectes platessa*) sampled. The significantly slower maturation of egg in the fish from the two most contaminated stations (SYS, NA) in September of both years could be the result of a combination of effects: reduced circulating estradiol (Casillas et al., 1991), disruption of membrane organization and permeability (Neff, 1985), binding of PAH metabolites to vitellogenin (Varanasi et al., 1982) which could result in the impairment of endocytosis. The latter two mechanisms are more likely responsible for the effect since estradiol did not correlate with vitellogeninin. PAH metabolites have a high affinity for plasmatic macromolecules with high lipid content (Neff, 1985; Varanasi et al., 1982). The presence of adducts could interfere with the endocytosis of vitellogenin by the oocytes and with normal lipid metabolism. Dey et al. (1982) showed than long-term exposure of winter flounder to crude oil significantly affected lipid metabolism in cod and winter flounder. Free fatty acids and total phospholipids were elevated and triglycerides depleted significantly in both males and females experimentally exposed to crude oil.

Also, the presence of atretic oocytes in the ovaries at these stations (20-25% and 33% respectively) indicates that spawning was inhibited in a large proportion of fish. This phenomenon has been documented in other estuarine environments with high levels of urban contaminants (Johnson et al., 1993; Spies et al., 1990; Johnson et al., 1988). It could be the result of a disruption of an endocrine regulation of the hypothalamus-pituitary-ovary axis. The small body of literature available to date on this subject does not shed much light on the possible modes of action of endocrine disrupting chemicals. As was demonstrated by Harmin and Crim (1992), ovulation and spawning in winter flounder are under GnRH control. Singh and Singh (1982) and Thomas (1989) reported reduced gonadotropins in catfish and Atlantic croaker exposed to pesticides and PCBs respectively (see Chapter 1, Section 5.1). No such evidence of the effects of PAHs was found, however a similar scenario seemed conceivable. However, in a recently published article, Khan and Thomas (1998) reported that force-fed o,p'-DDT (0.02 and 0.1 $\mu g.g^{-1}$ body weight.day^{-1}) exerted an estrogen-like action by significantly stimulating

gonadotropin release in Atlantic croaker (*Micropogonias undulatus*) during the gonadal recrudescence.

As mentioned previously, no meaningful statistical analysis of PAH bile metabolites could be performed. In hindsight, pooling gall bladder samples was a bad decision which reduced the information available. However, due to budget considerations, extensive bile analyses would probably not have been performed even if individual samples had been available. Nevertheless, as can be seen on Figure 12, the bile of the fish from SYS generally appeared to contain more metabolites and in higher concentrations (up to 10 times) than that of the fish from other stations. However, these concentrations were low when compared to those of winter flounder exposed to tar-pond sediments in the laboratory for 3 weeks (Mossman and Vandermeulen, 1995; Fig. 17). The "signature" of the metabolite profile also confirmed the pyrogenic origin of the PAHs, as the higher molecular weight compounds dominated the samples.

The lack of accumulation of PAHs in the ovaries, even at the most contaminated site, was surprising and contradicted results found for English sole (*Parophrys vetulus*; Varanasi et al., 1982; Hose et al., 1981) and flathead sole (*Hippoglossoides elassodon*; Hose et al., 1981). Whether administered by force-feeding or by intra-peritoneal injection, PAHs were found to significantly accumulate in the ovaries of both species (15-37% of total administered PAH as parent compound and 6-35% as metabolites). The cause of the low ovarian PAH concentrations is unclear. It was originally thought that a high rate of hepatic metabolism and excretion could have been responsible. However this was difficult to confirm, as the MFO enzymatic activities measured in the fish captured in 1992 were inconsistent and the bile samples were pooled in groups of 10. Similarly, in the study of a particularly contaminated bay of Boston Harbour (up to 113 ppm of sediment PAHs), winter flounder were found to display numerous signs of adverse effects, including liver neoplasia, inflammation of the digestive tract and respiratory organs, and necrosis of gill tissue, and yet did not accumulate PAHs (U.S. Environmental Protection Agency, 1988). The authors concluded that although the effects were induced by PAHs, these chemicals did not accumulate because of metabolism.

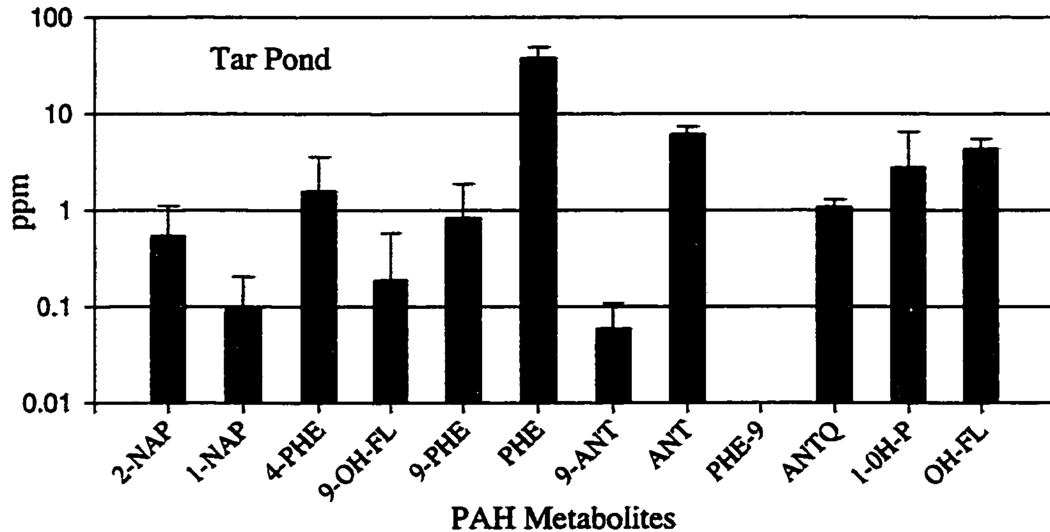


Figure 17: Concentrations of PAH metabolites in the bile of male winter flounder exposed to Tar Pond sediments for 3 weeks (mean +/- SD). From Mossman and Vandermeulen (1995).
Abbreviations used: 2-NAP: 2-naphthol, 1-NAP: 1-naphtol, 4-PHE: 4-phenylphenol, 9-OH-FL: 9-hydroxyfluorene, 9-PHE: 9-phenanthrol, PHE: phenanthrol, 9-ANT: 9-anthraldehyde, ANT: anthraldehyde isomers, PHE-9: phenanthrene-9-carboxaldehyde, ANTQ: anthraquinone, 1-OH-P: 1-hydroxypyrene, OH-FL: hydroxyfluoranthene.

4.4 Endocrine disrupting effect in male winter flounder

Increased vitellogenin levels in the blood of males is one of the most documented estrogenic effects in fish (Christiansen et al., 1998; Folmar et al., 1996; Sumpter and Jobling, 1995). This is usually accompanied by testicular inhibition (Matthiessen et al., 1998; Panter et al., 1998). Under normal conditions, vitellogenin concentrations in the serum of male fish are very low or undetectable (Copeland et al., 1986; Emmersen et al., 1979). Although the vitellogenin gene is present in males, it remains silent if circulating levels of estrogens are too low to trigger its expression. Originally, male winter flounder were included in the sampling design as negative controls, in an attempt to estimate background levels of serum estradiol and vitellogenin. However, as the data was analyzed, it was obvious that concentrations of serum protein-bound phosphorus were unusually high. This phenomenon also appeared to vary with the station at which the fish were captured. There was, therefore, an opportunity for assessing the estrogenic potential of coal-tar derived contamination. Regrettably, as this investigation was not included in the design of the study, no gonadal tissue was sampled in males. Hence it was impossible to further assess the potential damage to the reproductive tract of the fish.

For the fish from the reference site (GB), protein-bound phosphorus concentrations remained around 600-700 μM throughout the sampling season, both in 1992 and in 1993 (Fig. 14). These values are close to the minimum concentration of serum protein-bound phosphorus recorded in females in June (Fig. 10), at an early stage of the vitellogenic cycle (Pereira et al., 1993; Nagler et al., 1987). Moreover, these relatively elevated concentrations further support the existence at GB of conditions inducing similar effects as the presence of organic contaminants. Contrary to the results with females, a dose-dependant relationship between serum protein-bound phosphorus and sediments PAH was demonstrated. The significant increase of protein-bound phosphorus in the fish from Sydney Harbour could not be accounted for by the circulating levels of estradiol which remained extremely low (Fig. 13). Furthermore, the high correlations between serum protein-bound phosphorus and sediment PAH and the consistency of this association over time strongly suggest the existence of an estrogenic effect of the estuary's contaminants.

This differs from the results of Anderson et al. (1996b), which showed that under low circulating concentrations of estradiol, β-naphthoflavone only increased vitellogenin synthesis at low doses and that 2 and 4-fold increases of β-naphthoflavone concentrations significantly reduced serum vitellogenin in juvenile rainbow trout (*Oncorhynchus mykiss*) of both sexes.

The increase in the slope of the regression between serum protein-bound phosphorus and sediment PAH over time, is suspected to be a factor of temperature and duration of exposure to the contaminants. Bottom temperatures varied considerably over time (-0.2° to 17.6°C). As mentioned previously, this would strongly affect the bioavailability of PAHs and the rate of uptake of these compounds.

Vandermeulen and Mossman (1996) have reported that in June, GSI was 2.5 times higher in male winter flounder from Sydney Harbour than in males from George's Bay. One possible explanation for this important difference could be that the testes of the fish from Sydney Harbour were accumulating vitellogenin. The results of Matthiessen et al. (1998) tend to support this observation although in their case no statistically significant difference was detected. This phenomenon, called intersex condition and feminization, has been reported in flounder (*Platichthys flesus* L.; Matthiessen et al., 1998), in carp (*Cyprinus carpio*; Gimeno et al., 1996) and Japanese medaka (*Oryzias latipes*; Gray and Mertcalfe, 1997) exposed to environmental contaminants and would be consistent with the results presented here. However, with no histological information, any discussion of the potential damage to the reproductive organs of the fish or of its long-term effects is speculative. The study by Vandermeulen and Mossman (1996) also shows a slight increase in HSI and no evidence of a significant increase in liver microsomal protein content in the fish from Sydney Harbour. This suggests that the increase in liver size could be the result of vitellogenin synthesis by the liver. This observation has been documented by other authors in the presence of different natural and synthetic estrogens (Von Der Decken et al., 1992; Copeland et al., 1986) as well as potentially estrogenic contaminants (Matthiessen et al., 1998; Lye et al., 1997; Sumpter and Jobling, 1995; Truscott et al., 1992).

5. Conclusion

The results presented here show that even under extreme contaminated conditions (sediment PAHs above 200 ppm), 90% of female winter flounder were able to complete their vitellogenic cycle and spawn. However, serum estradiol and egg maturation were significantly adversely affected by the presence of sediment contaminants. The observed effects were non-linear, and suggest the existence of a threshold level of sediment PAHs around 10 ppm. Above this concentration, serum estradiol concentrations were reduced, egg maturation was retarded during a portion of the reproductive cycle, and up to 33% of females were unable to spawn. Not surprisingly, season was clearly identified as the most influential source of variability in the data, accounting for over 36% of the variance. By comparison, station of capture was only the third source of variability, accounting for 10.9% of the overall variance. Discrimination between sampling stations could only be achieved using multivariate statistics. Furthermore, there is evidence that the choice of George's Bay as reference site was poor. The addition of detoxifying enzyme activities to the 1992 data did not affect the overall result of the statistical analysis and discrimination between stations in the harbour was improved by less than 10%. However, EROD activity was the only variable to be meaningfully associated with the station of capture.

In addition, this study strongly suggests that coal-tar derived sediment contamination has a significant estrogenic effect on the population of male winter flounder from Sydney Harbour. Such documentation of the estrogenic effect of PAHs in feral fish has been found in few studies published to date.

Although the reproductive success of the fish was not investigated, these results suggest the existence of a potential threat to the flounder population as part of both male and female fish reproduction was significantly affected by the presence of contaminants.

CHAPTER 3

Effects of long-term exposure to coal-tar derived contamination on the reproductive cycle of captive female Winter Flounder

1. Introduction

The purpose of the second part of this study was to attempt to reproduce the environmental conditions encountered in Sydney Harbour in a laboratory experiment. It was hypothesized that the laboratory study would validate non-destructive sampling which would give results similar to those obtained from the field study and could be used as a tool for predicting contaminant effects in the field from laboratory results. The experiment had multiple goals: (1) to provide data for the period of the reproductive cycle not sampled during the field work (December-May), (2) to confirm that the Tar Pond sediments are the source of the effects detected in the flounder from Sydney Harbour, and (3) to assess any temporal variations of these effects over two consecutive reproductive cycles. In addition, exposing fish to a range of contamination in captivity would provide the ability to control most of the parameters of the experimental environment. This could allow access to more information on the life history of the animals and ensure that all animals are treated identically, possibly reducing the variability associated with the variables measured. Finally, the comparison of the results with those of the Sydney Harbour study using identical methods could also help validate laboratory to field extrapolations.

Hence, fish acclimated in captivity were exposed to a range of sediment contamination, simulating the environmental conditions present at the Sydney Harbour sampling sites described in the previous chapter. The additional purpose of such an experimental design was to evaluate and validate a non-destructive sampling of individual fish. Most biological sampling usually requires the sacrifice of the individuals sampled. If it can be demonstrated to be a sensitive and efficient tool to detect adverse effects of contaminants on fish reproduction, non-destructive sampling could become a useful technique to survey small populations while affecting them as little as possible.

2. Materials and methods

2.1 Fish collection

Winter flounder were captured by otter trawl in April 1993 and by SCUBA diving in June 1993 from uncontaminated coastal sites south of Halifax Harbour (Nova Scotia, Canada). All fish were longer than 27 cm to ensure that they were sexually mature (Beacham, 1982; Kennedy and Steele, 1971). Fish were transported back to the Bedford Institute of Oceanography (Dartmouth, Nova Scotia) where they were transferred to holding tanks. Fish were acclimated for 6-13 weeks during which they were fed a diet of chopped squid to satiation every other day. Several fish died in the first few days following their transfer to holding tanks. This stress induced mortality stopped after four days. Subsequently, only six fish (less than 3%) died during the acclimation period.

2.2 Experimental design

The experimental design consisted of three tanks containing contaminated sediments and two control tanks containing uncontaminated sediments. Experimental tanks measuring 2.5 m in diameter received flow-through filtered sea water from the Bedford Basin at a rate of 10 $L.min.^{-1}$ (the time for the replacement of the total volume of the tanks was approximately 8.5 hours) and were aerated with compressed air. The sea water was allowed to reach a depth of approximately 1 m (total volume of approximately 5000 L). The bottom of the tanks were covered with a layer of approximately 5 cm of sediment. The sediment in the contaminated tanks consisted of a dilution of coal-tar contaminated mud collected in Sydney Tar Pond (Fig. 7, Chapter 2). This mud, containing a total PAH concentration of approximately 500 ppm wet weight, was mixed with clean beach sand in a proportion of 1:4 (Tank 3), 1:8 (Tank 4) and 1:16 (Tank 5) respectively. This was done in an attempt to simulate the environmental conditions found at the sampling stations selected in the Sydney Estuary (Chapter 2). The control tank holding females (Tank 1) received only clean sand. A negative control tank holding only male winter flounder (N=30) on clean sand was also included in the design (Tank 2). Prior to being introduced into the experimental tanks, all sediments were frozen (-20°C)

and thawed three times to ensure destruction of most organisms (McCain et al., 1978). Each tank received 30 female winter flounder. An opaque enclosure was erected around the tanks, within which artificial light was adjusted weekly to simulate natural photoperiod. Tanks were screened from direct light with landscape fabric in order to provide a light intensity approximately equivalent to that present at a depth of 10 m (average depth of the field stations in Sydney Harbour).

The fish were sexed by feeling the scales on the caudal peduncle on the blind (left) side (Smigielski, 1975). They were subsequently individually identified by cold branding on the blind side and randomly assigned to an experimental tank. The technique used was modified from Herbinger et al. (1990) and consisted of the jet injection of Alcian Blue pigment in the dermis at the base of the dorsal, ventral and/or caudal fin. Only a few individuals developed an infection around the tattoo area and were replaced. Overall, the load of fish in each tank was similar: 12418±857 g. Fish were fed to satiation a diet of chopped squid every other day (approximately 4% of tank load; Vercaemer, 1991). The amount of food offered was adjusted to the total weight in each tank (checked monthly). Food not eaten within 24 hours was removed from the tanks, drained and weighed.

2.3 Sampling regime

Each month, fish were removed from their tanks and anaesthetized by immersion in sea water containing 150 $mg.L^{-1}$ of MS 222 (tricaine methanesulfonate; 3-aminobenzoic acid ethyl ester methanesulfonate; Syndel®) for 2-3 minutes depending on their size. This procedure has been shown to be the least stressful to fish and to have few side effects (Kleinow et al., 1986; Strange and Schreck, 1978). Length, weight and general condition were recorded, and a 1 ml blood sample was collected from the caudal vein using heparinized Vacutainers (Fisher Scientific). Fish were then placed in a recovery tank until actively swimming (2-3 min.) and subsequently returned to their experimental tank. All fish were sacrificed and dissected at the end of the experiment in the same way as the fish collected in Sydney Harbour (see Chapter 2). Fish that died during the experiment were also dissected identically. At each sampling date the water temperature was recorded. As

well, the sediments in the tanks were mixed and raked while the fish were out of the tank, to try and maintain a uniform layer.

2.4 Chemical and biochemical analyses

All sample handling as well as chemical and biochemical analyses, were performed as described in Chapter 2. Because of budget considerations, only one sediment sample, taken at the end of the experiment from each tank was analyzed for PAH concentrations and only 4-6 bile samples per tank were analyzed for PAH metabolite concentrations. Results were compared using one-way ANOVA followed by Bonferroni *t* tests adjusted for multiple comparisons. Statistical tests were run with the same constraints as in the previous chapter ($\alpha \leq 0.05$ and Power$\geq$0.8 unless otherwise specified).

3. Results

3.1 Sediment and water chemistry

Sediment concentrations of PAHs in the experimental tanks were 167.53 ppm, 40.95 ppm and 19.88 ppm in Tank 3, Tank 4 and Tank 5 respectively. No PAHs were detected in the sediment from the control tanks. Sediment PAH profiles in the contaminated tanks (Fig. 18) at the end of the 24 month exposure were very similar to those found in the core samples from Sydney Harbour (Fig. 8).

Sea water at the Bedford Institute of Oceanography was monitored routinely by Environment Canada for contaminants of major concern (metals, pesticides and PAHs). All contaminants were found to be within acceptable limits for EPS bioassay work (K. Doe, pers. comm.; Tables 24-25). The concentration of PAHs in the seawater in particular was 140 to 1200 times lower than the sediment PAH concentrations.

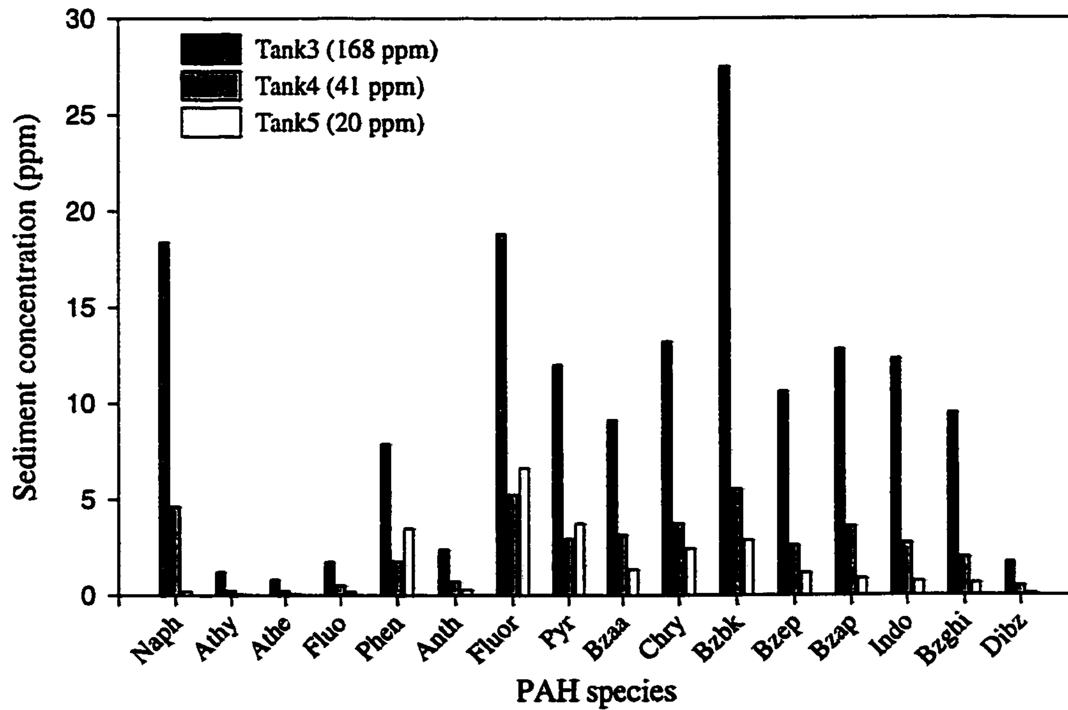


Figure 18: Concentrations of PAHs in the sediments of the three treatment tanks at the end of the 25 month exposure. Total sediment PAH concentrations are indicated in brackets.

Abbreviations used: Naph: naphthalene, Athy: acenaphthylene, Athe: acenaphthene, Fluo: fluorene, Phen: phenanthrene, Anth: anthracene, Fluor: fluoranthene, Pyr: pyrene, Bzaa: benzo(a)anthracene, Chry: chrysene, Bzbk: benzo(b+k)fluoranthene, Bzep: benzo(e)pyrene, Bzap: benzo(a)pyrene, Indo: indopyrene, Bzghi: benzo(ghi)perylene, Dibz: dibenzo(ah)anthracene.

Table 28: Salinity, inorganic elements and total PAHs in seawater at the Bedford Institute of Oceanography facility. From K. Doe (pers. comm.).

Parameters	Concentrations ($\mu g.L^{-1}$)
Salinity ($^{0}/_{00}$)	30.5
Cadmium	1
Copper	1
Iron	40
Mercury	0.7
Lead	1
Zinc	4
Phosphate phosphorus (total)	20
Total PAH (pyrene equivalent)	140

Table 29: Pesticides in seawater at the Bedford Institute of Oceanography facility. From K. Doe (pers. comm.).

Organophosphorus pesticides	(μg.L⁻¹)	Organochloride pesticides	(μg.L⁻¹)	Carbamate pesticides	(μg.L⁻¹)
Amino-fenitrothion	0.01	Arochlors (total PCBs)	0.005	Aminocarb	0.05
Diaziron	0.01	p,p'-DDT	0.001	Carbaryl	0.08
Fenitrooxon	0.05	o,p'-DDT	0.001	Carbofaran	0.80
Fenitrothion	0.01	p,p'-DDD	0.001	Methiocarb	3.80
Malathion	0.01	p,p'-DDE	0.001	Propoxur	2.60
Methyl-parathion	0.01	p,p'-methoxychlor	0.010	Zectran	0.40
Parathion	0.01	Heptachlor	0.001		
Thimet	0.005	Heptachlor epoxide	0.001		
		α-endosulfan	0.010		
		β-endosulfan	0.010		
		α-chlordane	0.005		
		γ-chlordane	0.005		
		α-BHC	0.005		
		γ-BHC (Lindane)	0.001		
		Aldrin	0.001		
		Endrin	0.010		
		Dieldrin	0.001		
		Dibutyl phtalate	1.2		
		Diethylhexyl phtalate	42.0		
		Mirex	0.001		

3.2 External factors

Beginning in July 1994, areas of the "wet lab" facility at the Bedford Institute of Oceanography located in the proximity of the experimental tanks were undergoing extensive renovations. This resulted in a variety of construction debris contaminating the tanks (Gyprock fragments, plaster dust, paint, fiberglass, polyester gelcoat, etc.). Some preventative measures were taken rapidly (plastic covers; cautioning of workers). This prevented any further contamination of Tanks 1 to 4. However, in Tank 5 which was closest to the working area, numerous debris had to be removed on a daily basis for the following 3 months. Following these incidents, the mortality sharply increased in Tank 5 (Fig. 19). As a result, the number of fish in this tank dropped from 26 in July 1994 to 11 in October 1994. Furthermore, the fish appeared to be severely stressed: lack of feeding, fin erosion (Fig. 20), hyperventilation, unusual behaviour. Therefore, starting in July 1994, the results obtained from Tank 5 fish had to be analyzed with extreme caution.

3.3 Adverse effects

3.3.1 Fin erosion, growth and mortality

There was no occurrence of fin erosion during the first 4 months in any of the experimental tanks. Fin erosions were first observed in November/December 1993 in Tanks 1, 2, 4 and 5 (Fig. 20). The numbers of fish with fin erosion remained low in all tanks until the late stages of vitellogenesis, and subsequently increased sharply in April and May 1994 in the contaminated tanks, coinciding with the onset of spawning. During this period, 9 fish developed fin erosions in Tank 3, 4 in Tank 4 and 2 in Tank 5. During the second reproductive cycle, the number of fish with fin erosion remained comparatively stable. The lesions appeared mostly in the caudal fin, but there were also some occurrences of dorsal and ventral fin erosion especially in Tank 3.

Overall the number of fish with fin erosion remained low in the control tanks (10 and 15% respectively). By comparison, 50% of the fish in Tank 3, 29% in Tank 4 and 13% in Tank 5 (before July 1994) displayed eroded fins over the span of the experiment.

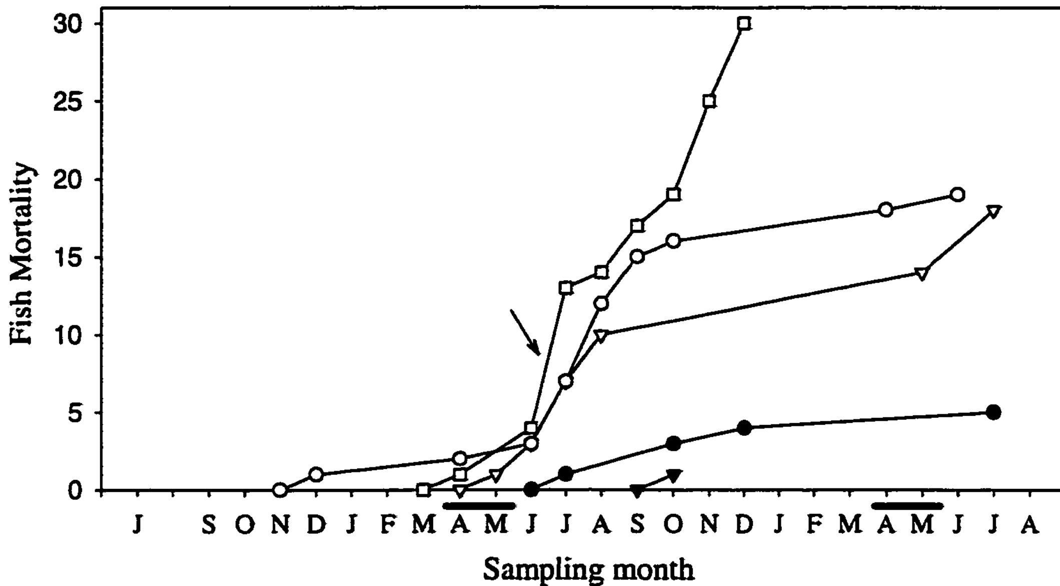


Figure 19: Cumulative mortalities of captive winter flounder exposed to PAH contaminated sediments.

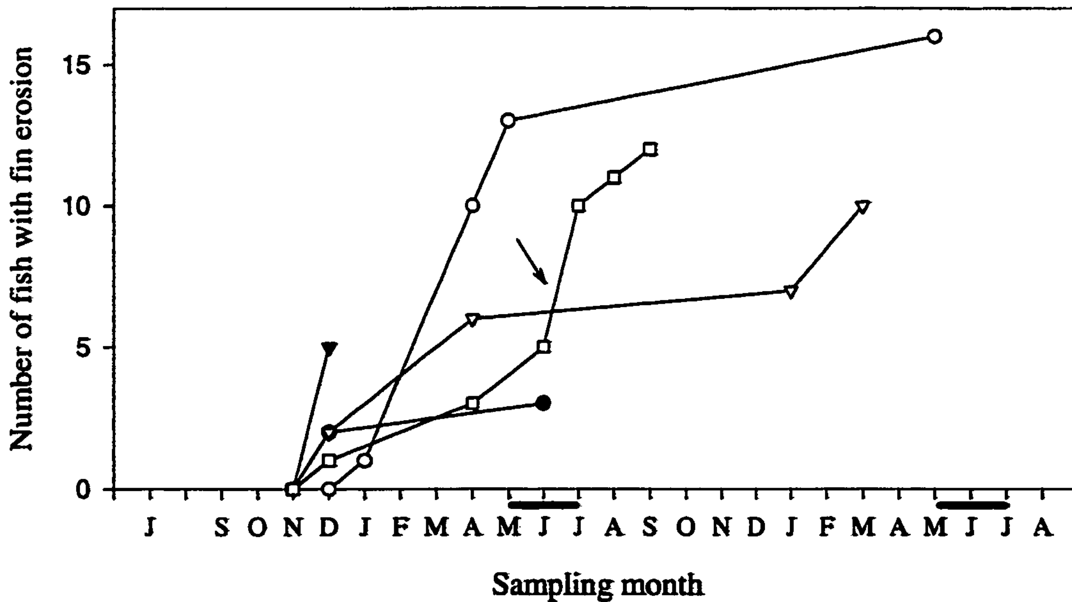


Figure 20: Cumulative occurences of fin erosion in captive winter flounder exposed to PAH contaminated sediments.

Arrow indicates first occurence of debris in Tank 5.
● Tank 1 (females control) ▼Tank 2 (males control)
○ Tank 3 (168 ppm of PAHs) ▽ Tank 4 (41 ppm of PAHs)
□ Tank 5 (20 ppm of PAHs) ▬ indicate spawning periods.

The occurrence of fin erosion increased dramatically in Tank 5 after the building renovations began.

Growth, as expressed by weight increase normalized to the number of fish, was negatively affected by the treatment. The average increase in weight over the two year study period was 35% in the control tank and was -10%, -11% and 4% in Tanks 3, 4 and 5 respectively (in the case of Tank 5, the growth rate was calculated between July 1993 and July 1994). Furthermore, the fish in Tanks 3 and 4 were slower to recover after the first spawning period (Fig. 21). This was especially clear in Tank 3, as the growth rate remained negative from April to October 1994. By comparison, growth in the control tanks had become positive as early as August 1994. The low or negative growth in the contaminated tanks was the result of a larger number of individuals which did not undergo vitellogenesis or did not recover after the first reproductive cycle.

Winter flounder food intake decreased as water temperature decreased. Beginning in January-February 1994, as the temperature dropped below 3°C (Fig. 23), the fish did not eat all the food offered. In Tanks 1 and 5, the amount of uneaten food reached 8% and 7%, respectively by March 1994 and returned to 0% by May 1994, when water temperature rose above 3°C. The pattern was repeated for Tank 1 in 1995 (Fig. 22). In Tanks 3 and 4, the reduction of feeding occurred one month earlier than in the control tank and remained between 4% and 25% for the rest of the experiment. The amount of uneaten food was generally greater in Tank 3 than in Tank 4 (Fig. 22). At the peak of the building renovations, in July and August, the feeding of the fish in Tank 5 was markedly reduced. The amount of food uneaten decreased slightly thereafter.

Mortality appeared to be associated with sediment contamination, if Tank 5 was not taken into consideration past July 1994. Mortality was higher in Tank 4 than in the controls and highest in the most contaminated tank (Fig. 19). Final mortality rates were 16%, 3%, 63%, 60% and 100% respectively, in Tanks 1, 2, 3, 4 and 5. Mortality increased during the first spawning period (April-June 1994) in all tanks containing females. Furthermore, as mentioned previously, 10 fish in Tank 3, and 7 fish in Tank 4 did not recover from spawning.

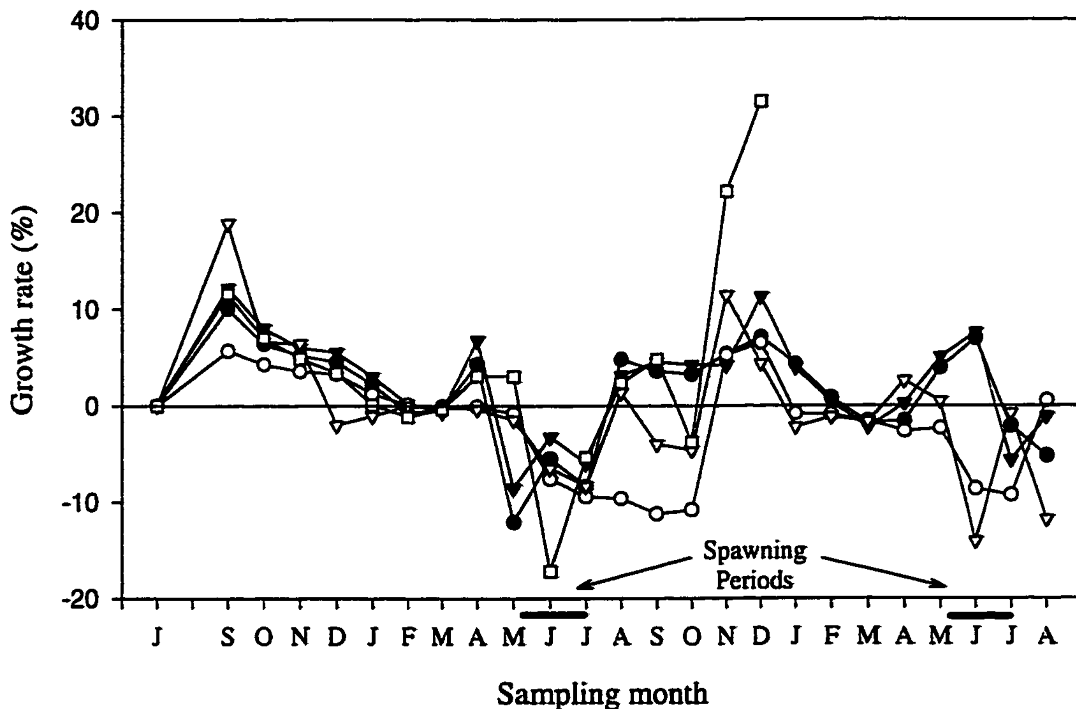


Figure 21: Growth rate of captive winter flounder estimated by the variation of the average fish weight per tank between sampling months.

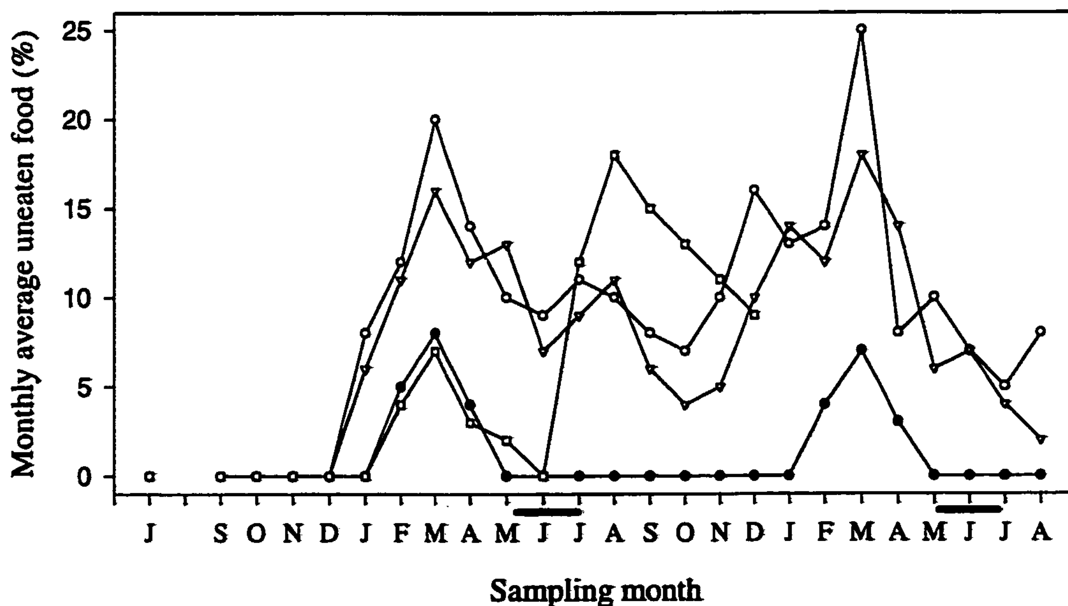


Figure 22: Monthly average uneaten food as % of food ration.

● Tank 1 (females control) ▼ Tank 2 (males control)
○ Tank 3 (168 ppm of PAHs) ▽ Tank 4 (41 ppm of PAHs)
□ Tank 5 (20 ppm of PAHs)

These individuals remained very thin and some were anemic (3 fish in Tank 3 and 2 in Tank 4). Several died over the course of the summer. A similar increase in mortality was not apparent during the second spawning season.

Without any reliable data for Tank 5 past July 1994, it is difficult to assess the effect of the treatment on the mortality of the fish in this tank. Mortality before July 1994 was close to that in the control tanks (11%) and rose sharply after the beginning of the constructions (Fig. 19). All fish in this tank were dead by December 1994.

3.3.2 Reproductive variables

Spawning and vitellogenesis inhibition

Most fish (70-100% per tank) successfully completed two reproductive cycles in captivity (Table 26). The majority of the fish spawned spontaneously, however, some were stripped manually if eggs were running out freely during sampling. In 1994, the spawning period of the fish from the 3 contaminated tanks appeared to be slightly shifted when compared to the control tank. Fewer fish spawned in May and more fish spawned in July (Table 26). This did not hold true for the following reproductive season. Spawning occurred in June of both years in 30 to 77% of fish from all tanks with the exception of Tank 4 where 27% of the fish spawned in May 1995. In 1995, spawning seemed to spread out more evenly over the three months (May-July) in all tanks.

A small number of the fish that underwent vitellogenesis in Tanks 3 and 4 were unable to spawn (even stripping of these fish was unsuccessful). The ovaries of these flounders were very rubbery, and the mostly atretic oocytes within the ovary were agglomerated into a mass. Animals displaying this condition died shortly after the spawning period.

Complete inhibition of vitellogenesis was apparent in several fish from all three contaminated tanks (Table 26). The oocytes in these fish did not undergo vitellogenesis and remained at Stages 1 or 2 of maturation (pre-vitellogenic; see Chapter 2). Consequently, ovarian growth was absent and spawning did not occur. The frequency of this "condition" ranged from 10% in Tank 5 up to 32% in Tank 3 and appeared to be closely associated with sediment PAH concentrations.

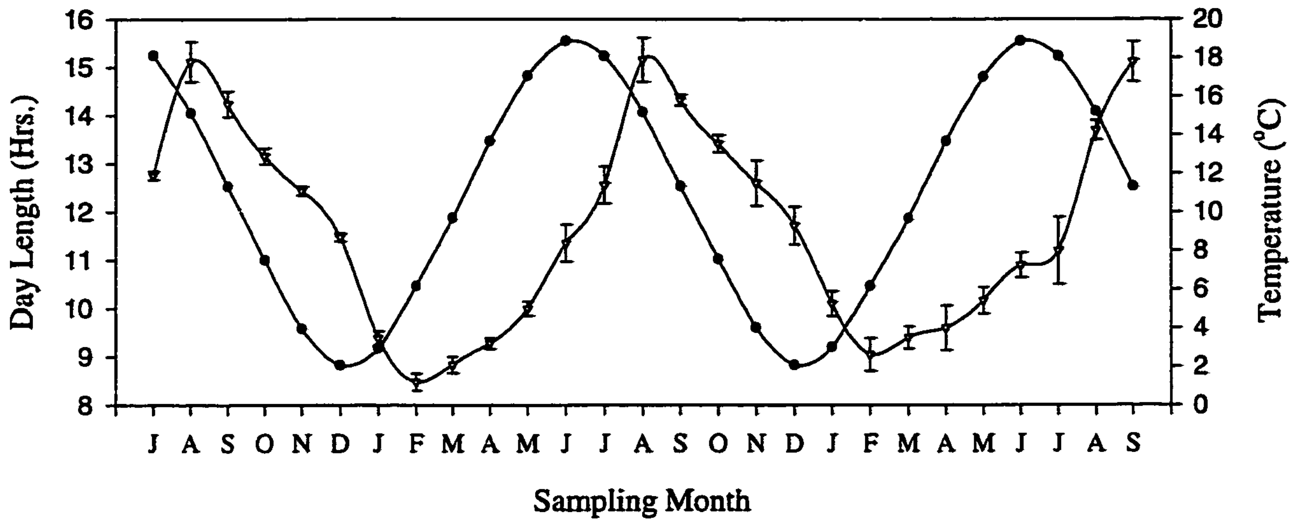


Figure 23: Photoperiod and average water temperature (mean +/- SD) over the duration of the experiment.

- Day length
- Average water temperature in 5 experimental tanks

Table 30: Number of spawning female winter flounder in each experimental tank. Numbers in brackets indicate the corresponding percentage of total number of fish present in each tank at the time of sampling.

	PAH (ppm)	1994				1995			
		May	June	July	N-V	May	June	July	N-V
Tank 1	n.d.	4(13)	23(77)	3(10)	0	9(35)	11(42)	5(20)	0
Tank 3	168	3(11)	8(30)	5(22)	9(32)	2(17)	4(33)	2(18)	3(25)
Tank 4	41	2(7)	11(41)	8(35)	5(20)	7(27)	4(15)	2(8)	3(12)
Tank 5	20	1(3)	13(50)	5(29)	2(10)	-	-	-	-

Abbreviations used: N-V: non-vitellogenic fish; n.d.: not detectable.

Although only performed on the data from two consecutive reproductive cycles, the correlation coefficient of the regression of the percentage of vitellogenesis inhibition on the sediment PAH concentrations was 0.93 (R^2=0.86; $P<10^{-4}$). The equation of the regression was: %Non-vitellogenic fish = 20.01 x [PAH] - 16.21.

Two of the non-vitellogenic fish in Tank 3 and one in Tank 4 started undergoing ovarian maturation during the second cycle. All the other non-vitellogenic fish died during the summer of 1994. Therefore, the non-vitellogenic fish in 1995 were individuals which did undergo vitellogenesis in 1994.

Estradiol

Serum estradiol concentrations in female winter flounder ranged from 0.07 $ng.ml^{-1}$ to 4.16 $ng.ml^{-1}$ during the first cycle and from 0.08 $ng.ml^{-1}$ to 4.75 $ng.ml^{-1}$ in the second cycle (Fig. 24). The peak values were measured in February 1994 and in April 1995. In males the concentration remained close to the detection limit of the assay for the duration of the experiment (0.05 $ng.ml^{-1}$). The variability of circulating estradiol levels between female winter flounder was high in all experimental tanks (SD~20% to 63% of the mean), and was the highest in Tanks 3 and 4. This was primarily the result of some individuals not undergoing vitellogenesis and their serum estradiol levels remaining very low. In an attempt to reduce this variability, non-vitellogenic females were removed from the Tank 4 and Tank 5 data and a second set of statistical analyses run (dotted line on Fig. 24). Although this constitutes an *a posteriori* bias, it can be justified by the fact that the non-vitellogenic females clearly represented a distinct group.

The two yearly cycles of serum estradiol in females were fairly different in the control tank and similar to each other in the contaminated tanks. In 1994, levels remained low in the fish from all tanks through the fall and early winter (< 0.5 $ng.ml^{-1}$), then increased sharply in January, reaching a maximum (3-3.5 $ng.ml^{-1}$) and plateaued until spawning (April-May). During the second captive reproductive cycle the increase of serum estradiol concentrations was more gradual in the control tank (Fig. 24).

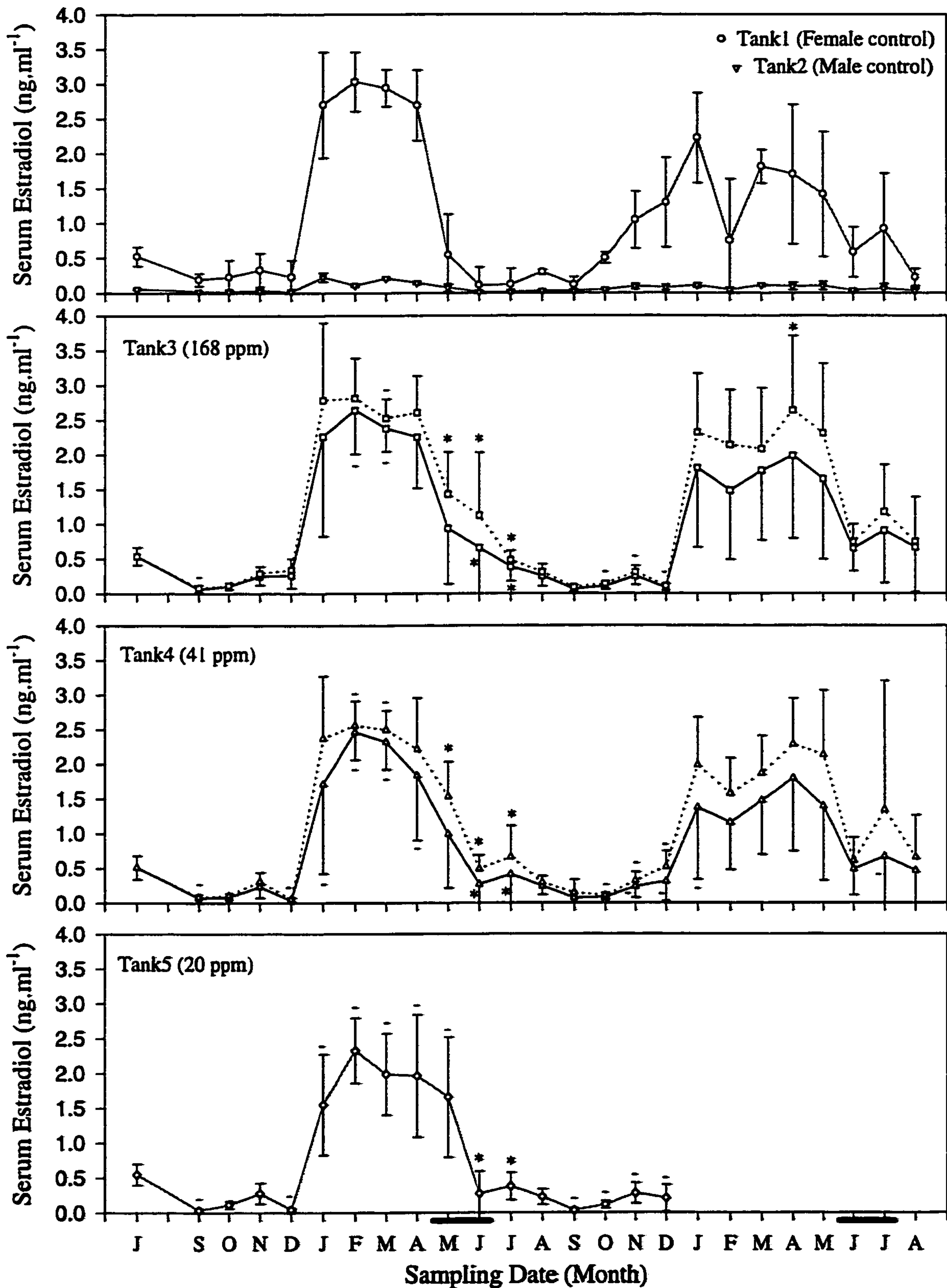


Figure 24: Serum estradiol concentrations (mean +/- SD) in winter flounder held for 25 months on a range of PAH contaminated sediments. Dotted lines: non-vitellogenic females removed.
* indicate a concentration significantly higher than the control.
\- indicate a concentration significantly lower than the control.
▬ indicate the spawning periods.

In contrast, the trend in the contaminated tanks was similar to the previous year, and concentrations remained significantly lower than the control until January 1995. From January to May, the serum estradiol concentrations fluctuated considerably in all tanks from month to month (within the same individuals). For most fish, estradiol did not reach the levels of the previous year.

Even with the removal of the non-vitellogenic females from the data, variability of the results remained high which made the power of the tests low and the detection of statistically significant differences between treatments difficult. Generally, fish in all three contaminated tanks had lower estradiol concentrations than the controls, except during the first spawning period when this trend was reversed (Fig. 24). Concentrations seemed to be the lowest in Tank 5, although no significant difference between Tanks 3, 4 and 5 could be detected. During the second cycle, serum estradiol levels dropped sharply in February 1995 in the control tank and more slightly in Tanks 3 and 4.

Vitellogenin

Serum protein-bound phosphorus concentrations in female winter flounder ranged from 214 µM to 2945 µM during the first cycle and from 256 µM to 2264 µM in the second cycle (Fig. 25). The highest values were measured in May of both years, however, the maximum mean value for the control tank in 1995 was reached in March. In males the concentration fluctuated between 200 µM and 400 µM throughout the experiment. Paralleling estradiol measurements during the first year, vitellogenin levels remained lower than would be expected until December 1993 then increased until reaching the maximum at the onset of the spawning period. During 1994-95, protein-bound phosphorus levels increased gradually during the vitellogenic cycle and peaked in May before dropping during the spawning season (Fig. 25).

Contrary to estradiol results, removing the non-vitellogenic females from the data substantially reduced the variability of the protein-bound phosphorus measurements. Also, the difference between the two sets of data appeared greater than in the case of estradiol. This allowed for the detection of significant differences and trends which would otherwise have been missed (Fig. 25).

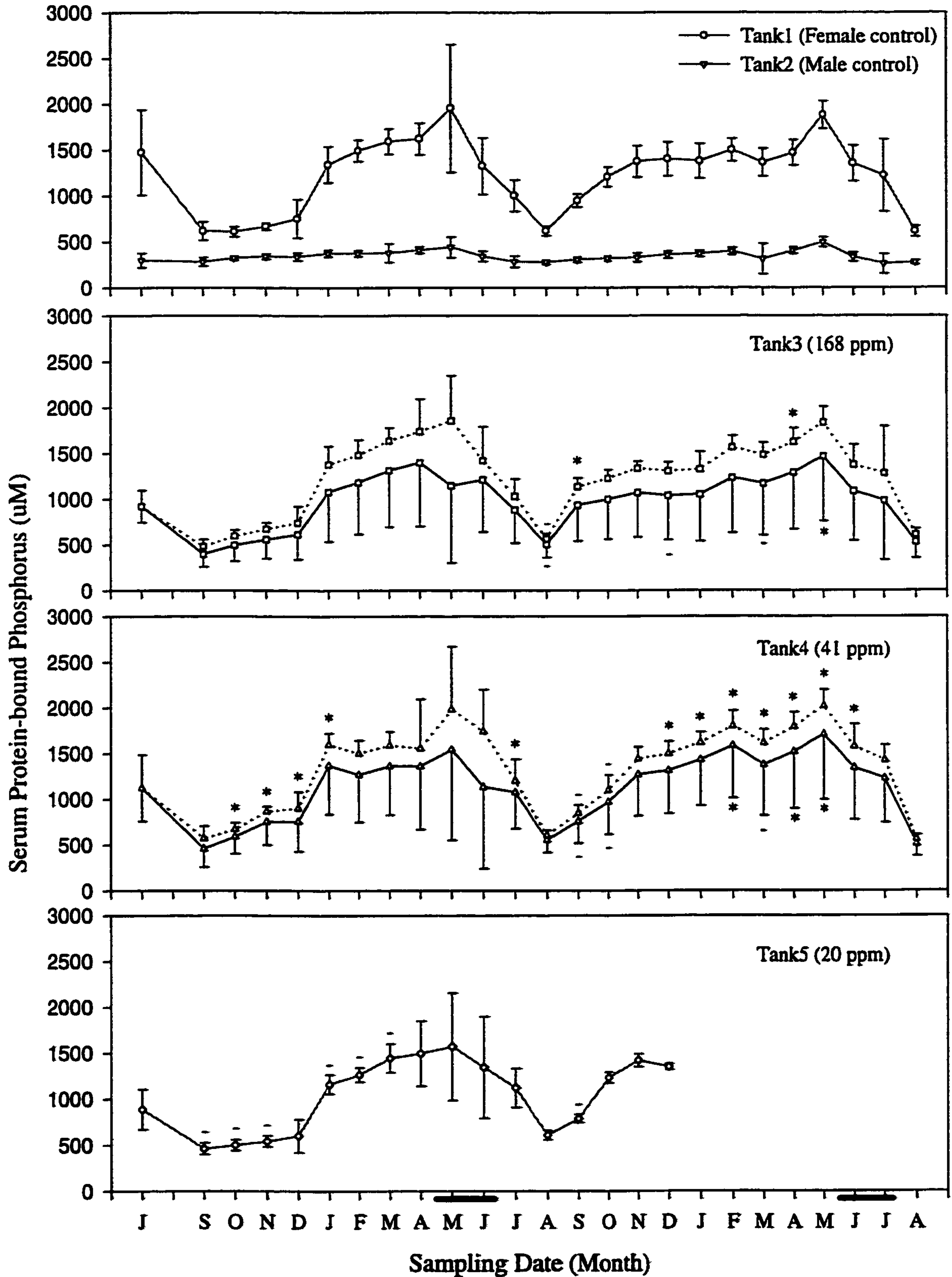


Figure 25: Serum protein-bound phosphorus concentrations (mean +/- SD) in winter flounder held for 25 months on a range of PAH contaminated sediments. Dotted lines: non-vitellogenic females removed.
* indicate a concentration significantly higher than the control.
- indicate a concentration significantly lower than the control.
▬ indicate the spawning periods.

Under these conditions, vitellogenin was found to be more sensitive than estradiol in detecting significant differences between tanks, as the variability between fish was less. Protein-bound phosphorus levels were the most similar in the fish from Tanks 1 and 3. When a significant difference was detected between those tanks, the mean concentration was higher in Tank 5 in 3 of the 4 sampling months (Fig. 25). Generally, protein-bound phosphorus concentrations measured in the fish from Tank 5 were lower than in the control. However significant differences could only be detected in 7 of the 17 sampling months. Tank 4 had the most significant protein-bound phosphorus differences relative to the control (14 of the 25 sampling months). In all cases but 2, concentrations were higher in the flounder from Tank 4 than from the control tank.

3.3.3 Bile metabolites

Analysis of the bile revealed that PAH metabolites were present in higher concentrations in the gall bladders of fish from Tanks 3 (P=0.009) and 4 (P=0.015) when compared with fish from the control Tank (Fig. 26). Concentrations of higher molecular weight metabolites especially (anthraldehydes, 1-hydroxypyrene and hydroxyfluoranthene) were 10 to 100 times higher in those fish than in the controls. These molecules also dominated the samples, representing 7%, 6.5% and 55% respectively of the total PAH metabolite content of the bile of the fish from Tank 3 and 11%, 16% and 56% respectively, from Tank 4.

4. Discussion

A number of studies have investigated the effects of chronic exposure of captive fish to contaminants (see Chapter 1). However, few have extended the exposure period beyond a few months and analyzed the results on an individual basis. This project has allowed the monitoring of individual female winter flounder through two complete yearly reproductive cycles under clean and hydrocarbon-contaminated conditions. Although the results led to some speculative conclusions, the effects detected clearly demonstrate the toxicity of the contaminated sediments (which impaired vitellogenesis and spawning). All the variables considered in this experiment were affected to some extent by the treatment.

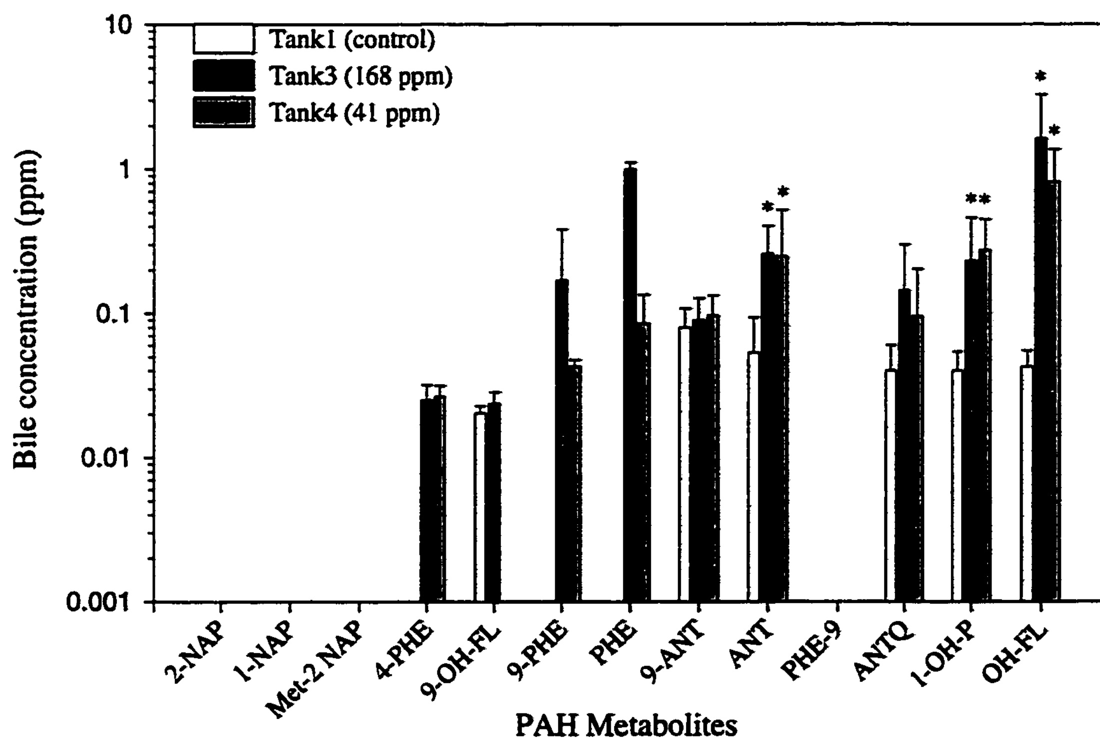


Figure 26: Concentrations of PAH metabolites (by increasing MW) in the bile of female winter flounder exposed to contaminated sediments for 25 months (mean +/- SD).
* indicate statistically significant differences from the control.

Abbreviations used: 2-NAP: 2-naphthol, 1-NAP: 1-naphtol, Met-2 NAP: methyl-2 naphthalene methanol, 4-PHE: 4-phenylphenol, 9-OH-FL: 9-hydroxyfluorene, 9-PHE: 9-phenanthrol, PHE: phenanthrol, 9-ANT: 9-anthraldehyde, ANT: anthraldehyde isomers, PHE-9: phenanthrene-9-carboxaldehyde, ANTQ: anthraquinone, 1-OH-P: 1-hydroxypyrene, OH-FL: hydroxyfluoranthene.

Although sediment conditions were close to those found at the stations sampled in Sydney Harbour, the results of this experiment were unexpectedly different from those of the field study. The laboratory experiment failed to reproduce the effects observed in Sydney Harbour and it is therefore implicit that validating laboratory to field extrapolations is impossible. The possible causes for these differences will be discussed in the following chapter (Chapter 4).

4.1 Sediment and water chemistry

There appeared to be very little if any weathering of the PAHs in the experimental sediments. Even lighter, more volatile molecules such as naphthalene were still present in significant amounts in Tanks 3 and 4 at the end of the 25 month exposure (Fig. 18). The abundance of heavier compounds (penta- and hexa-aromatics; molecules heavier than chrysene) confirmed the pyrogenic origin of the PAH contamination. The lack of weathering of the PAHs could be explained by the fact that the Tar Pond sediments are well weathered to begin with since coke ovens activities ceased in 1988 (Mossman and Vandermeulen, 1995). Therefore, at the time the mud for the experiment was collected in 1992, no substantial amount of coal-tar had been released to Muggah Creek for 4 years. Furthermore, although the Tar Ponds are tidal, the creek is very sheltered and the energy of the system is low which would favour a slow weathering of the hydrocarbons (Neff, 1979).

The concentration of total PAHs in the seawater was found to be negligible in comparison to sediment concentrations in the contaminated tanks (several orders of magnitude difference). Likewise, the very low concentrations of organic contaminants and metals in the seawater were assumed to have no effect on the variables considered in this experiment.

4.2 Fish acclimation

Although 4 to 6 weeks appeared to be adequate for the acclimation of winter flounder to captivity when measuring hepatic MFO activities (Vandermeulen and Mossman, 1996),

an even longer period in this study (6-13 weeks) did not appear sufficient to acclimate female flounder to captivity and allow them to undergo a typical reproductive cycle. Serum concentrations of both estradiol and protein-bound phosphorus remained significantly lower than would be expected during the first 5 months of the experiment. This result held true regardless of which tank the fish were held in and therefore appears not to be related to the experimental treatment. These low levels could have been caused by stress-induced elevated levels of cortisol (fish still not completely acclimated to captivity), which have been demonstrated to reduce circulating steroid levels (Carragher and Sumpter, 1990; Carragher et al, 1989). This is supported by the fact that fin erosion in the control was more prevalent during the first cycle than during the second, indicating a higher stress level (Sinderman, 1994; Ziskowski and Murchelano, 1975). Similarly, captivity was demonstrated to induce chronic stress (elevated cortisol; Clearwater and Pankhurst, 1997; Jardine et al., 1996), reduce estradiol and increase the proportion of atretic follicle in vitellogenic female red gurnard (*Chelidonichthys kumu*; Clearwater and Pankhurst, 1997). Stocking density of the experimental tanks does not seem to be the cause of the stress as it was relatively low (2.4 $kg.m^{-3}$) and was not different from that used in other studies where spawning occurred normally (Devauchelle et al., 1987). However, during the acclimation period, winter flounder were held in stocking tanks at a higher density (not measured) than during the experiment. It would have been interesting to measure serum cortisol concentrations in the experimental fish to confirm this hypothesis, however, because of time and budget constraints, these analyses were not performed.

4.3 Bile metabolites

As was described by Moles et al. (1994) in juveniles, the winter flounder in the present experiment did not avoid the contaminated sediments. In fact, the animals spent most of the time buried in them. Not surprisingly, PAH metabolites significantly accumulated in the gall bladders of the female winter flounder from treatment Tanks 3 and 4. This demonstrates that the parent PAHs where bioavailable and that winter flounder

accumulated these molecules. It also demonstrates that the fish were actively metabolizing and excreting the PAHs.

4.4 Adverse effects

After the initial low, the yearly cycles of serum estradiol and protein-bound phosphorus were fairly typical in the control tank. The sharp increase in serum estradiol and protein-bound phosphorus concentrations of the first cycle appeared to be associated with a return to increasing day length (Fig. 23). This could be a significant trigger for vitellogenesis in winter flounder. However this did not hold true for the following year. After the January 1994 increase, the fish which did undergo vitellogenesis had reached a concentration of serum estradiol and protein-bound phosphorus within the normal range in all tanks. However, as for the winter flounder sampled in Sydney Harbour, estradiol concentrations were approximately 10 times lower than those measured by Harmin et al. (1995) for the same period.

Serum estradiol concentrations tended to be lower in the fish from the contaminated tanks than in the control animals. However, statistically significant differences were few because of high individual variability (Fig. 24). Nonetheless, two important results emerged from the data: (1) during the first spawning period, estradiol concentrations were significantly higher than the control in fish held on contaminated sediments, and (2) subsequently remained significantly lower than the control from September to December 1994. The first result was the consequence of the greater number of slower maturing/late spawning winter flounder with high estradiol levels in the contaminated tanks (Table 26). These fish significantly increased the monthly mean estradiol values. The low estradiol concentrations during early vitellogenesis could result from a combination of effects, including a contaminant-induced decrease in circulating estradiol (see Chapter 1), stress related increase in cortisol levels reducing circulating estradiol (Carragher and Sumpter, 1990; Carragher et al, 1989), and reduction in circulating estradiol induced by a lower ration (Cerdá et al., 1994). The latter effect will be discussed further subsequently. With the data available, it is impossible to determine which of these mechanisms was the most influential.

None of the variables considered in this study could account for the drop in serum estradiol measured in all tanks during February 1995 and the associated drop in protein-bound phosphorus in March 1995.

Outside of the spawning periods, serum protein-bound phosphorus appeared to be more sensitive to the experimental treatment than estradiol concentrations. The variability of the protein-bound phosphorus concentrations was significantly lower than that of estradiol concentrations in all tanks, whether non-vitellogenic females were ignored or not.

The effect of the treatment on serum protein-bound phosphorus concentrations in female winter flounder varied with the sediment concentration of contaminants (Fig. 25). In Tank 5 (20 ppm), the serum vitellogenin of the fish was significantly reduced by the treatment, possibly as a result of low circulating estradiol levels. In Tanks 3 (168 ppm) and 4 (41 ppm), the female serum vitellogenin concentration was higher than in Tank 5 and close to (Tank 3) or higher than (Tank 4) in the control. These results could not be accounted for by serum estradiol concentrations which were lower in those fish than in the control females. The elevated protein-bound phosphorus concentrations could have two non-exclusive origins: (1) the existence of a mild estrogenic effect and/or (2) a lower rate of uptake of vitellogenin by the oocytes, thus increasing circulating concentrations.

Although the fish in Tank 3 had a lower growth rate and feeding, higher incidence of fin erosion and higher mortality (all indicative of stress), serum protein-bound phosphorus concentrations were very close to those of the control females and significantly higher than those of the females from Tank 5. This could reflect a lower rate of uptake of vitellogenin by the oocytes. It seems unlikely that fish feeding poorly and under severe stress would undergo intensified vitellogenin synthesis. Furthermore, Tank 3 fish had by far the highest concentration of metabolites in the bile, indicating high detoxifying activity in the liver.

Conversely, females in Tank 4 did not display signs of stress as severe, and had the highest vitellogenin concentrations. In this case, the elevated concentrations of protein-bound phosphorus could result from an estrogenic effect similar that described by

Anderson et al. (1996b). One of the consequences of estrogenic effects in females can be the premature onset of vitellogenesis and spawning (Janssen et al., 1997). The results reported here show no evidence of this. Although vitellogenin concentrations in females from Tank 4 were higher than in the control females, spawning occurred during the same 3 month period as the other tanks.

Spawning of captive winter flounder, whether under clean or contaminated conditions, was spread over a 3 month period (May-July). This differs from other studies which have reported spawning occurring over a one month period (Burton and Idler, 1984; Klein-MacPhee, 1978).

Possibly the most important result of this study remains that non-vitellogenic females were present in all three contaminated tanks (Table 26). This condition correlated significantly with sediment PAH concentrations, suggesting that concentrations as low as 20 ppm can inhibit vitellogenesis in winter flounder. These results are similar to those of Thomas and Budiantara (1995) who also reported that 30-56% of the oil- and naphthalene-exposed fish (Atlantic croaker exposed to 5% WSF of oil and 1 ppm naphthalene) failed to undergo sexual maturation. In feral English sole (*Parophrys vetulus*), Collier et al. (1993) described similar cases of total inhibition of spawning only at sediment PAH concentrations above 100 ppm, and Stott et al. (1983) could not find any mature oocytes in plaice (*Pleuronectes platessa*) sampled in estuaries which had been heavily oiled 3 years earlier. The slower development of ovarian follicles was also documented by Thomas and Budiantara (1995) in the same experiment and by Stott et al. (1983, 1981) in fish from environments heavily contaminated with oil.

Growth, ration, fin erosion and mortality all tended to indicate that the fish were more severely affected with increasing sediment contamination. The difference between the fish from the control tanks and those from the contaminated tanks was greatest after the first spawning period and during the second reproductive cycle. However, because of the nature of these data, no statistical comparisons could be made.

The reduction of growth and feeding in the contaminated tanks are of significant interest. Indeed, food intake has been demonstrated to be positively correlated with

ovarian growth in captive winter flounder (Tyler and Dunn, 1976). In cases of very low ration or starvation, a non-vitellogenic state can be induced (Burton, 1994, 1991; Burton and Idler, 1987) and winter flounder have been shown to stop feeding when exposed to oiled sediments for 4-5 months in the laboratory (Fletcher et al., 1981). The summer and early fall appear to be feeding periods critical to the onset of vitellogenesis in winter flounder (Burton, 1994). As well, Cerdá et al. (1994) reported that a reduction of approximately 50% of the daily ration delayed spawning by a month in sea bass (*Dicentrarchus labrax*) and extended the spawning period by a similar period. These authors also reported that oocyte maturation was delayed. Furthermore, ration reduction significantly reduced circulating estradiol concentration, fecundity and somatic growth. Interestingly, circulating vitellogenin concentrations were not affected by the treatment. The similarity between these adverse effects and the results described here strongly suggests that at least a portion of the effects detected could be caused by the lack of feeding of the fish in Tanks 3 and 4. However, it is clear from the bile metabolite concentrations, that the fish in Tanks 3 and 4 were exposed to very high levels of PAHs. As a result, direct effects of the PAHs on vitellogenesis are probable and should not be minimized.

Reduced feeding is not only of consequence because of direct effects on fitness and ovarian maturation, but because there is the potential for a trans-generational effect, since reduced female size has been linked with reduced hatching and larval survival (Cerdá et al. 1994; Buckley et al., 1991a,b).

Captivity prohibited the natural migration behaviour of the fish which could be critical to the reproductive cycle of the winter flounder. However, all females in the control tank underwent vitellogenesis and were able to spawn.

Most of the adverse effects described in this chapter have been reported for fish exposed to hydrocarbon contamination as well for fish on a low ration feeding. It is unclear given the present data, whether the results described here were the consequence of a direct toxic effect of the PAHs, of an indirect feeding-induced effect, or more likely, of a combination of both effects.

5. Conclusion

The results of this laboratory experiment were more difficult to interpret than those of the Sydney Harbour study. With the exception of bile metabolite concentrations, the discussion of the cause of the effects documented in this chapter was mostly speculative. It remains unclear whether the observed effects were the result of a direct toxic effect or whether they were the result of a combination of toxic effects and reduction in ration induced by the contaminants and confinement.

Nonetheless, some important conclusions could be drawn. This study confirms that the Tar Pond sediments are toxic to winter flounder and adversely affect vitellogenesis. Heavy PAH metabolites significantly accumulated in the bile (up to 100 times) of the fish from Tanks 4 and 5. Growth and feeding were reduced and fin erosion and mortality increased with increasing sediment contamination. These adverse effects were enhanced during the spawning season. Between 10% and 30% of the female winter flounder exposed to Sydney Harbour Tar Pond sediments for up to 25 months did not undergo vitellogenesis. The frequency of fish failing to mature was positively correlated with sediment PAH concentration.

It is suggested here that the elevated concentration of vitellogenin in the female winter flounder exposed to a 1:8 dilution of Tar Pond mud (41 ppm total PAHs) results from an estrogenic effect. The timing of vitellogenesis and of spawning did not appear to be affected by the estrogenic effect.

Finally, the present experiment demonstrated that estradiol and vitellogenin alone are not sensitive enough variables to detect adverse effects of hydrocarbon contamination on vitellogenesis. This shows that using the techniques described here, non-destructive sampling methods are insufficient to detect adverse effects on vitellogenesis of even extreme hydrocarbon contamination because of high variability.

CHAPTER 4

Summary and Conclusions

1. Field study

The results reported from the field study demonstrate that the contaminants present in the sediments of the Sydney estuary have a significant adverse effect on the reproduction of winter flounder.

The effects detected on variables measured in females were non-linear and suggest the existence of a threshold concentration of PAHs in the sediment. The sediment PAH concentration of 10 ppm is proposed as threshold value, since no difference could be detected between stations with sediment PAH above or below this concentration. This is in agreement with the GESAMP report (1993) which specified that PAH sediment concentrations in the range of 3 to 5 ppm produced adverse biological effects in flatfish. It is interesting to note however, that in several of the cases where no difference could be detected between stations, the power of the univariate statistical analyses was low. This indicates that the probability of wrongly concluding that the stations are similar is high.

When compared to fish from stations with sediment PAH levels below the threshold, female winter flounder captured at the three most contaminated stations, Sysco (sediment PAH>200 ppm), Northwest Arm (50 ppm) and South Bar (10 ppm) displayed a 30% to 50% reduction in serum estradiol concentration. Also, the oocyte maturation of these fish was retarded, and by September had only reached a pre-vitellogenic Stage 2. By comparison, most flounder at the reference station displayed vitellogenic Stage 3 oocytes. However, all the fish sampled in late fall (October/November) had on average reached an advanced stage of oocyte maturity (Stage 4). Finally and maybe most significantly, 20% to 33% of the fish from the more contaminated stations failed to spawn.

The fact that no differences between stations were detected above or below the threshold, even when the sediment PAH concentrations differed by an order of magnitude or more is compelling and could possibly be the result of other sources of variability masking the effect of the contaminants. A factor analysis of the data indicated that 36.3% of the variance was associated with seasonal variations, 24.6% with the morphological differences between fish and only 10.9% with the station of capture. Not surprisingly, only extreme differences caused by the contaminants (more than 50% difference in the

mean) could be detected by univariate statistical tests. Discrimination between stations could only be achieved by using multivariate statistics (discriminant analysis). This test was able to predict the station of capture of Sydney Harbour fish with a 45% to 67% accuracy. Also based on the variables measured in this study, this analysis was unable to discriminate between the females captured at the reference site (George's Bay) and those captured in the Northwest Arm of the harbour. This strongly suggests that a factor unaccounted for in this study affects the variables measured in the female winter flounder from George' Bay in the same way as the contamination of the Sydney estuary.

Male winter flounder, which were originally only included in the study as negative controls, were also significantly affected by the contaminated sediments. Male serum protein-bound phosphorus (vitellogenin) was significantly positively correlated with the log of the sediment PAH concentration. The elevated serum vitellogenin could not be accounted for by the concentrations of estradiol measured in the serum of these fish. Therefore it is likely the result of a sediment contaminant-induced estrogenic effect. Such documentation of the estrogenic effect of PAHs in feral fish has been found in few studies published to date.

All these results were consistent over three sampling seasons and the same trend was evident again the second sampling year.

2. Laboratory exposure

The laboratory study confirmed that the Tar Pond sediments from Sydney Harbour are toxic to winter flounder and adversely affected the female reproductive cycle. The three dilutions of Tar Pond mud, 1:4 (168 ppm total PAH), 1:8 (41 ppm) and 1:16 (20 ppm) all induced adverse effects in winter flounder.

The PAHs in the sediment were bioavailable and accumulated in the fish, as demonstrated by the significantly higher concentrations of metabolites in the bile. Heavier compounds accumulated up to 100 times more than lighter molecules. This confirmed the pyrogenic origin of the PAHs.

Somatic indexes also appeared to be altered by the contaminants, although no statistics could be run on these data. Growth and feeding were reduced and fin erosion and

mortality were increased with increasing concentration of PAHs in the sediment. Reduced growth, increased occurrence of fin erosion and higher mortality seemed to coincide with the onset of the spawning season, possibly associated with increased stress and water temperatures. Spawning during this experiment happened over a period of three months (May-July). In 1994, most females (30% to 70%) spawned in June, whereas in 1995 spawning was more evenly distributed over the three month period. During the 1995 season, more fish from the contaminated tanks spawned later than the fish from the control tank, suggesting a slightly slower maturation.

In all three contaminated tanks, the sediments induced a non-vitellogenic state in the female winter flounder. The frequency of fish affected ranged from 10% in the tank with the least contaminated sediments (20 ppm) to 30% in the tank with the most contaminated sediments (168 ppm). This condition was observed during both reproductive cycles and correlated significantly with PAH concentrations in the sediment.

With the data available in this study, it was not possible to determine whether the effects described here were the result of a direct toxic effect alone or whether the reduction of feeding and/or stress also played an important role. These three mechanisms have been reported to induce effects consistent with the results presented (Thomas and Budiantara, 1995; Cerdá et al., 1994; Collier et al., 1993; Carragher and Sumpter, 1990; Carragher et al, 1989; Stott et al., 1983, 1981).

Serum estradiol results were highly variable in all tanks. Concentrations tended to be lower in the contaminated tanks. However, few of the differences noted were statistically significant. Serum estradiol concentrations appeared to be the most affected through the summer and fall, at the onset of vitellogenesis. During this critical period (August-December), concentrations of estradiol were significantly lower in fish held on PAH contaminated sediments. Serum concentrations of protein-bound phosphorus, by comparison, were less variable and therefore a more sensitive indicator of effect. Vitellogenin concentrations were found to be lower in the females from Tank 5 relative to the control fish, higher in the females from Tank 4, and no different from the control in Tank 3. The results from Tank 4 and 5 could not be accounted for by the circulating estradiol levels. It was speculated, given other variables measured in the fish, that the

high vitellogenin concentrations in the fish from Tank 4 were the consequence of an estrogenic effect. In Tank 5, a similar mechanism seemed unlikely, and the vitellogenin levels measured were attributed to a reduced rate of endocytosis by the oocytes, as corresponding serum estradiol concentrations were low.

3. Discussion

3.1 Similarities

One of the most important results reported in this manuscript is that whether in the wild or under controlled laboratory conditions, a large number of winter flounder exposed to Sydney sediments failed to mature and spawn. The frequency of inhibition was very similar in the laboratory and the field results: 33-35% when exposed to sediments containing approximately 167-200 ppm of PAH, 20% when exposed to approximately 50 ppm and 10% when exposed to 20 ppm. One possible mechanism to explain this condition could be that PAHs reduce the rate of endocytosis of vitellogenin by the oocytes, thereby slowing down or in extreme cases completely inhibiting vitellogenesis.

3.2 Differences

It was hoped that by having the same source of contamination in the laboratory and in the field, the results would help corroborate predictions of impact of toxic chemicals in the field from laboratory studies (McDowell Capuzzo et al., 1988). It was also hoped that this work would assist in the validation of non-destructive collection of data to assess toxic effects on vitellogenesis. Neither of these objectives could be achieved because of important differences between field and laboratory results.

With few exceptions, serum estradiol concentrations in feral fish were significantly different from those measured in captive fish. In general, concentrations were higher in the flounder from Sydney Harbour (Fig. 27), although the maxima reached in the laboratory animals were not significantly different from the highest values detected in the Sydney flounder.

In the case of serum protein-bound phosphorus concentrations, the results were slightly different.

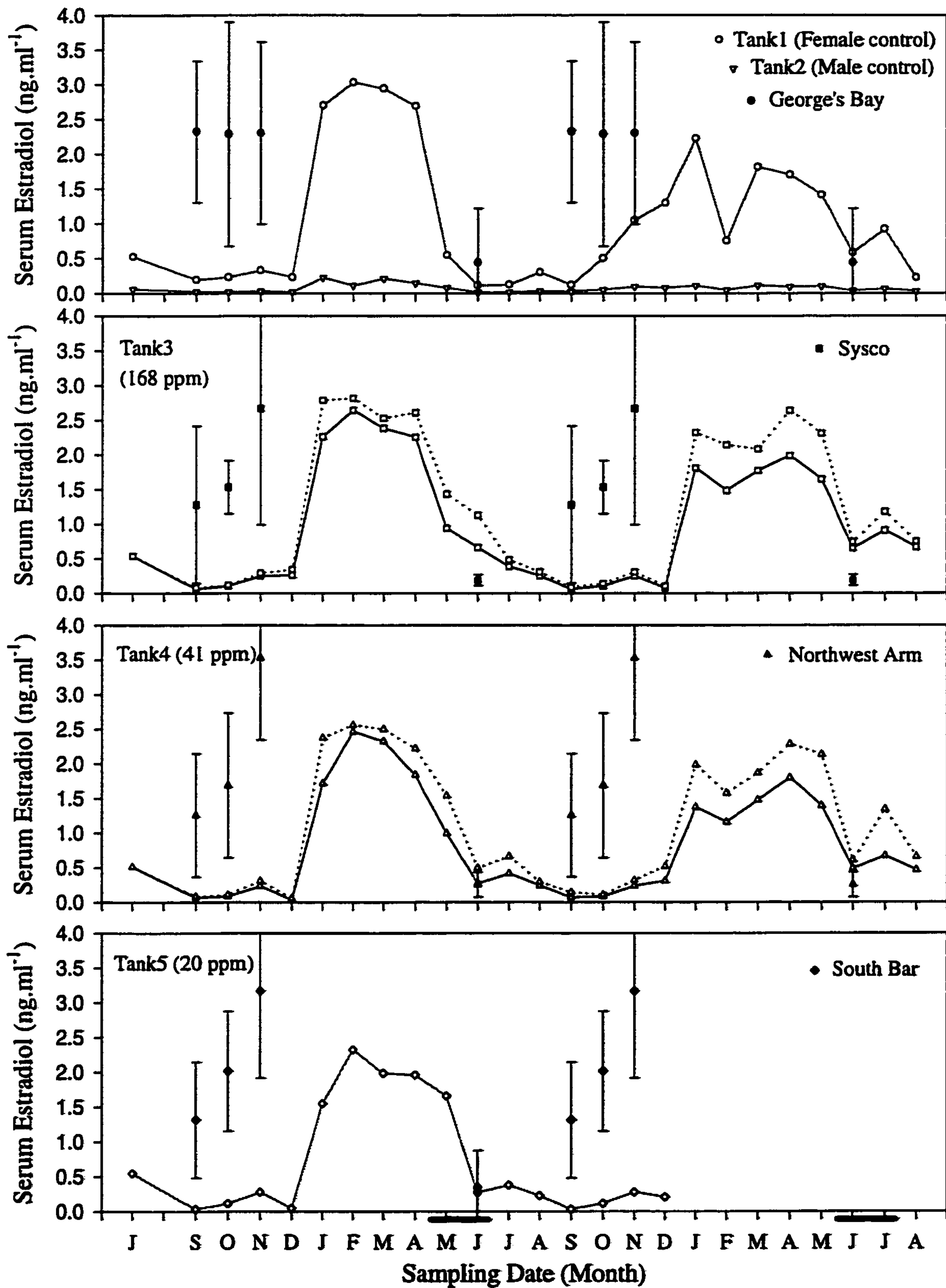


Figure 27: Comparison of mean (+/- SD) serum estradiol concentrations in winter flounder from field and laboratory studies.
▬ indicate the spawning periods.

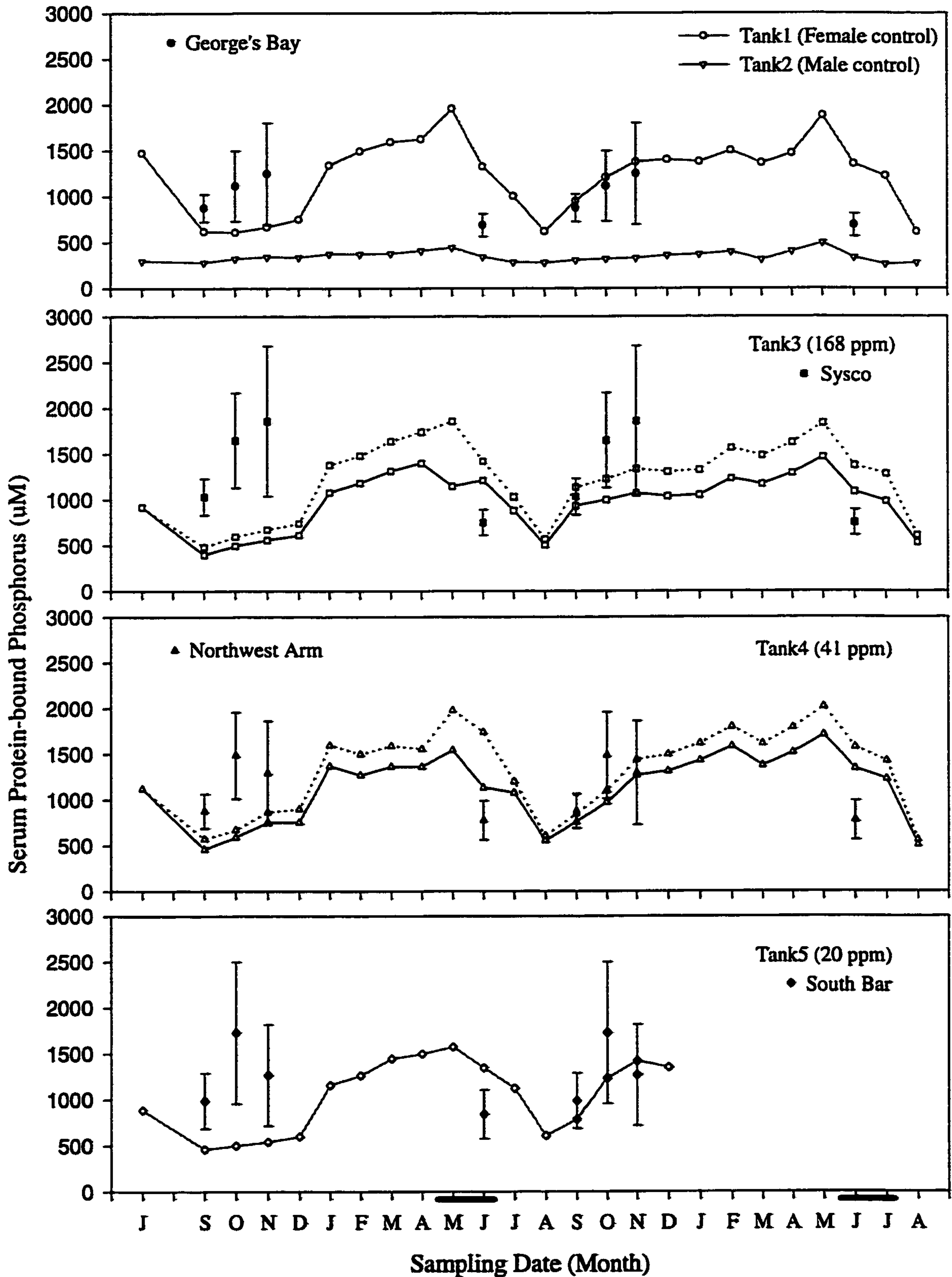


Figure 28: Comparison of mean (+/- SD) serum protein-bound phosphorus concentrations in winter flounder from field and laboratory studies.
▬ indicate spawning periods.

First, results for the month of June remained significantly lower in the fish from the field (Fig. 28). During the first reproductive cycle in captivity, serum vitellogenin concentrations were significantly lower in the laboratory held flounder than in the wild ones. During the second captive cycle, serum protein-bound phosphorus in feral and laboratory fish were not different. These results held true whether non-vitellogenic females were removed from the laboratory data or not.

Although sediment PAH concentrations were similar in both studies, the laboratory experiment failed to reproduce the effects observed in Sydney Harbour. The difference between the field and the laboratory results could be associated with two important factors:

(1) The route of exposure might have been different. Fish in Sydney Harbour probably feed on contaminated prey, as well as being exposed to contaminants originating from the water and the sediment. In the laboratory experiment, the food offered was uncontaminated, and the levels of contaminants in the water supply was several orders of magnitude lower than in the sediments. Therefore, the only source of contamination in the laboratory was the sediment, whether ingested with the food or through desorption of PAHs from sediment particles into the interstitial water (Neff, 1985).

Based on results published to date, diet appears to be a significant route of uptake of PAHs in winter flounder. In a somewhat older study, Zdanowics et al. (1986) noted that levels of PAHs and PCBs in stomach contents of winter flounder from contaminated estuaries were higher than in bottom sediments (biomagnification). Several more recent laboratory studies have demonstrated that PAHs and their metabolites accumulate in winter flounder feeding on contaminated infauna (James and Kleinow, 1994; McElroy et al., 1991; Kleinow et al., 1989; McElroy and Sisson, 1989). Finally, a recent study surveyed mussels (*Mytilus edulis* and *Mytilus galloprovincialis*) in coastal areas including the vicinity of an aluminum smeltering plant using coke ovens (Baumard et al.; 1999). The results show that where particulate-bound pyrogenic PAHs were the primary source of contaminants, mussels had a Biota Sediment Accumulation Factor (BASF) ranging from 1 to 34% with an average of 15%. This suggests that, depending on the area of the

Sydney Harbour they forage in, winter flounder feeding on contaminated molluscs could be ingesting 0.15 ppm to 30 ppm of PAHs through their diet alone.

(2) In Sydney Harbour, winter flounder migrate out of the estuary from December to April into deeper waters and a less contaminated environment. Feeding is very reduced during this period, which would decrease further the intake of contaminants via the diet and allow the fish to depurate. In captivity, the winter flounder were denied any migratory behaviour. The fish were permanently in contact with the contaminated sediments (with no escape). Furthermore, water temperatures remained above 2°C (compared to -0.5°C for other years; Vandermeulen and Mossman, 1995), and the fish kept on feeding through the winter (however, much less than in the summer).

Although sediment concentrations of PAHs were similar in both studies, it is unclear why concentrations of bile metabolites were 10 to 100 times higher in captive winter flounder than in Sydney Harbour animals. The higher levels of metabolites in the experimental fish could be the result of lower food intake, resulting in a decrease of bile secretion and excretion of PAHs. The lower concentrations of metabolites in the winter flounder from Sydney Harbour might also be the result of an acclimation to the presence of the contaminants. The fact that winter flounder return to the same contaminated estuary year after year would create a strong selective pressure in favour of animals with a better capacity for PAH metabolism and depuration.

4. Conclusion

In conclusion, the Tar Pond sediments significantly adversely affected the reproduction of winter flounder. The consequences for the flounder population of the Sydney estuary are difficult to assess. It seems that even under the most severe PAH contamination most female flounder were able to reach maturity and spawn. Although estradiol concentrations were decreased and egg maturation was retarded in contaminant exposed fish, 70% to 100% (per station or experimental tank) of the females reached maturity and were able to spawn. Conversely, this result means that 10% to 32% of female winter flounder exposed to coal-tar contaminated sediments were unable to

achieve maturity and spawn. This is a somewhat encouraging result, as environmental conditions in the Sydney estuary should improve over time.

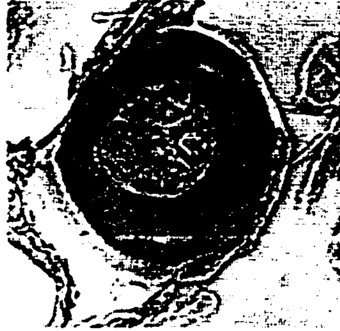
Since the closure of the coke ovens (1988), increased sedimentation of clay/silt has been observed in benthic sediments (Mossman, Nicolas and Willis, personal observations). Core samples taken from the harbour in 1995 showed a thin depositional layer (ca. 5 mm) on top of the tarry black mud. However, until the contaminated layers are sufficiently buried by new sediments, local bioturbation by worms and other invertebrates will keep the upper layers of the benthic sediments relatively mixed. And as long as PAH concentrations in the upper layer of the sediment remain at or above 10 ppm, some aspects of the reproduction of both female and male winter flounder from Sydney Harbour could be impaired to some degree.

Another interesting point is that the comparison of the results from the field and the laboratory suggest that the winter flounder from Sydney Harbour could be acclimated to high levels of sediments contaminants.

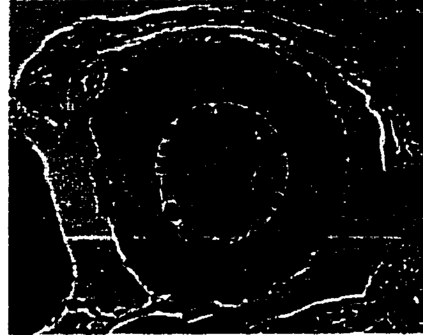
Unfortunately, the quantification of blood variables (serum estradiol, protein-bound phosphorus, protein) obtained from non-destructive sampling were incapable of assessing alone the effects of the contaminants on the reproductive cycle of female flounder. In males however, the quantification of serum estradiol and protein-bound phosphorus allowed the detection of a contaminant-induced estrogenic effect. They do not however, provide any information on the potential damage caused to the reproductive tract by this condition.

Finally, in this study, as in many others, bile metabolites were found to be the best biomarker of exposure of benthic fish to sediment PAHs.

APPENDIX

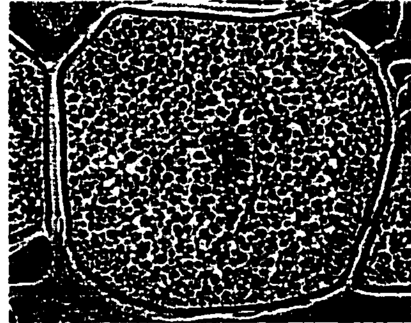


Stage 1: resting or newly recruited oocyte.



Stage 2: recently recruited oocyte.

Stage 3: vitellogenic oocyte.



Stage 4: advanced vitellogenic oocyte.

Stage 5: mature oocyte.

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