# Development and Application of Non-destructive Biomarkers for Biomonitoring Endocrine Disruption In Pinnipeds

Ourania Liori

PhD

KINGSTON UNIVERSITY LIBRARY
Acc. No. 0622637 X. PREF
Class No. THESES. PHD/L.
R0429822KP

2006

**Abstract**

Over the past years there has been an increased concern regarding widespread pollution of the environment with persistent organic pollutants (POPs) that biomagnify and bioaccumulate along the food chain. Marine mammals, especially seals being top predators of the marine food chain, are significantly exposed to biomagnified levels of POPs that bioaccumulate in the blubber which acts as a reservoir for these lipophilic compounds. POPs have been associated with a number of pathological changes in the seals, thus early detection of the adverse health effects resulting from pollutant exposure is vital, since exposure may cause irreversible damage to entire populations or ecosystems in the long term. Biomonitoring studies to investigate changes in fecundity of marine mammal populations due to exposure to POPs are needed. Biological indicators (biomarkers) that reflect the exposure levels or toxic effects of the contaminant load in wildlife populations can be used as early warning system for the reproduction and survival of the animals with non-destructive or minimal invasion. The aim of this study was to research and develop new and existing exposure and effect biomarkers for biomonitoring endocrine disruption in Ringed (*Phoca hispida*), Grey (*Halichoerus grypus*) and Harbour (*Phoca* vitulina) seals from different geographical areas using non-destructive/non-invasive sampling. Sex steroids, lactoferrin and blood chemistry parameters were investigated in seal faecal and serum samples as potential biomarkers. Exposure level (PCBs, DDT, PBDEs) was determined using chromatographic and immunoassay techniques. Estrogens in the exposed populations showed a negative correlation with contaminant exposure and higher LTF, glucose and lipoprotein levels showed a positive correlation with contaminant exposure and negative correlation with sex steroids, thus they are proposed as biomarkers of endocrine disruption. This is the first study to quantify and measure LTF as well as sex steroids in seal faecal samples and to investigate their correlation with environmental exposure and the promising results justify further research in this area. Since the biomarkers in this study are influenced by endogenous hormones it is recommended that they should be used in conjunction with sex steroids.

**Acknowledgements**

I would like to express my deepest gratitude to Kingston University for supporting and funding this project as well as to all the collaborators that provided me with the seal samples (Dr. M. Nyman, Finish Game and Fisheries Research Institute, Helsinki; Dr. P. Thompson, Lighthouse Research Station, University of Aberdeen; Dr. A. Hall, Sea Mammal Research Unit, St. Andrews University; Prof. Reijnders and Durham Univeristy). A special thanks to RSPCA Norfolk Wildlife Hospital, Lincolnshire Wildlife Trust and Ministry of Defence for their support and assistance in collecting the samples.

I am most grateful to my first supervisor Dr. Gera Troisi who introduced me to the world of environmental toxicology, encouraged and supported me and who enthusiastically took me in to her home to allow me to complete this work (May and Sotiri thanks for putting up with me all those days).

Dr. Steve Barton thank you so much for all the long nights you stayed behind and kept me company in the lab and the continuous support throughout this project. You have been a great supervisor (2nd does not apply to you) and a valuable friend!!!

Dr. Ralph Manly I owe you my deepest gratitude for giving me so much support and help at my most difficult times and believing in me to complete this project. The 8-ball did make it this time!!!

I would also like to thank Prof. Russell who always had her door open and the time to listen to me when I needed someone to speak to and Dr. D. Mackintosh for his support and encouragement.

A wish to thank Dr. Julien Swinden for giving me excellent technical assistance and being so patient with all of my questions considering GC analysis and Mrs. Di Watts, a very special friend, for always being there for me. Mrs Barbara and Mrs Kay thank you for all of your help and your patience when I was asking all of those questions.

Delphine Aigreau has been invaluable to me during various phases of this project. Besides being a great friend she developed the contaminant analysis as part of her MSc and has been of great help in many ways.

This work would have never been accomplished without the unconditional support and love from my family and friends. I owe my deepest thanks to my family and Ilia that even though they were so far away there wasn't one single moment that I felt alone. I thank them for showing so much love, patience and support especially during the year of write up and I thank them deeply for giving me so much love and being my inspiration. A special thanks goes to all of my friends back home (Natasha, Eirini, Bruce…) and of course to all the people at the General Hospital of Patission who supported me and helped me so much at the final stages of my PhD.

I would also like to express my gratitude and love to two very special people, Jill and Lino, who took me into their home and had me as their daughter. I will always love you and be there for you. Thank you so much!

There are no words to describe my gratitude to my two most valuable friends Humma and Simon who have been there with me from day one. Their optimism, unconditional love and support helped me to overcome my most difficult times all through these years and gave me the strength to carry on when there was no light ahead. I thank you so much and I would be honoured to stand next to you the following January.

**Contents**


1.0 General introduction 1
1.1 Polychlorinated biphenyls (PCBs) 3
1.2 DDT 6
1.3 Polybrominated diphenyl ethers (PBDEs) 8
1.4 Toxic effects of POPs on marine mammals 10
1.5 Biological Monitoring 14
1.5.1 Analytical procedures used in biological monitoring 15
1.5.2 Pollutant biomonitoring in Grey, Harbour and Ringed seals 16
1.5.3 Biomarkers 20
1.5.4 Biomarkers in this study 22
1.5.4.1 Blood chemistry parameters 22
1.5.4.2 Lactoferrin 26
1.5.4.3 Sex steroids and reproduction in seals 31
1.6 Biological information on study species 39
1.6.1 Geographical distribution 39
1.6.2. Taxonomy, diet and risks 41
1.7 Study groups 44
1.7.1 Experimental group 44
1.7.2 Control group 45
1.7.3 Exposed group(s) 45
1.7.3.1 Tees Estuary (UK) 46
1.7.3.2 The Wash: Blakeney Point and Donna Nook (UK) 48
1.7.3.3 Moray Firth (UK) 49
1.7.3.4 Firth of Tay – Abertay Sands, UK 50
1.7.3.5 Baltic Sea 50
1.8 Project aim and objectives 54
2.0 Characterisation of persistent organic pollutant (POP) exposure in study populations 56
2.1 Introduction 56
2.2 POPs investigated in this study 56
2.2.1 Polychlorinated biphenyls (PCBs) 56
2.2.2 DDT 60
2.2.3 Polybrominated diphenyl ethers (PBDEs) 60
2.2.4 Toxic effects of POPs on seals 62
2.3 Biological monitoring 63
2.4 Chromatographic analysis of organohalogen pollutants 64
2.4.1 Extraction 64
2.4.2 Clean-up 65
2.4.3 Detection 65
2.5 Pollutant immunoassays 65
2.6 Materials and methods 67
2.6.1 Sampling 67
2.6.1.2 Faecal samples 67
2.6.1.3 Blood (serum) samples 68
2.6.2 Chemicals 71
2.6.2.1. Chemicals used for faecal analysis 71
2.6.2.2. Chemicals used for blood (serum) analysis 71
2.6.3 Preparation of faecal samples 72

2.6.4 Faecal sample clean up 73
2.6.5 Chromatographic analysis 74
2.6.6 QA/QC 77
2.6.7 PCB analysis of blood (serum) samples (ELISA) 78
2.7 Results 84
2.7.1 Contaminant concentrations in seal samples 84
2.7.2 Age-dependent variation of PCB concentrations in serum samples 92
2.7.3 Sex-dependent variation of PCB concentrations in serum samples 92
2.7.4 Exposure level comparison in all study populations 94
2.8 Discussion 98
2.8.1 Immunoassay development 99
2.8.2 Intra-species differences in contaminant concentrations 100
2.8.3 Geographical and inter-species variations in contaminant concentrations 101
2.8.4 Exposure levels of study populations 105
3.0 Sex steroids and their application as biomarkers of xenoestrogenic exposure in pinnipeds 108
3.1 Introduction 108
3.2 Materials and methods 113
3.2.1 Sampling and study populations 113
3.2.1.1 Blood samples 113
3.2.1.2 Faecal samples 113
3.2.2 Sample preparation 114
3.2.3 Reproductive biology information of study populations 114
3.2.4 Reagents 117
3.2.5 Quantification of sex steroids (ELISA) 117
3.2.5.1 Serum samples 117
3.2.5.2 Assay protocol for serum samples 117
3.2.5.3 Faecal samples 118
3.2.5.4 Assay protocol for faecal samples 118
3.2.6 Quantification of serum ΣPCB concentrations 119
3.2.7 Cross reactivity of ELISA kits 119
3.3 Results 121
3.3.1 Sex steroid concentrations in Ringed, Grey and Harbour seals 121
3.3.2 Sex and age dependence differences within species 121
3.3.3 Population and species differences 127
3.3.4 Sex steroid concentrations in faecal samples 131
3.3.5 High-exposed vs. low exposed populations 133
3.3.5.1 Serum samples 133
3.4 Discussion 144
3.4.1 Relationship between faecal and serum steroid concentrations 146
3.4.2 Intra-species differences in steroid concentrations 148
3.4.3 Inter-species variation in steroid concentrations 149
3.4.4 Validation of steroids as biomarkers of endocrine disruption 151
3.4.4.1 Normal values for steroids 151
3.4.4.2 Dose-dependence 152
3.4.5 Biomonitoring ED with steroid biomarkers in study populations 153
4.0 Characterisation of phocine lactoferrin and its application as a biomarker of xenoestrogenic exposure in pinnipeds 155
4.1 Introduction 155

4.2 Materials and methods 160
4.2.1 Sampling and study populations 160
4.2.2 Reproductive biology information of study populations 160
4.2.3 Sample preparation 161
4.2.4 Reagents 163
4.2.5 Lactoferrin characterisation 163
4.2.6 Quantification of LTF protein (ELISA) 164
4.2.6.1 Assay development 164
4.2.6.2 Assay protocol 165
4.2.7 Quantification of serum ΣPCB, 17β-estradiol and progesterone concentrations 166
4.3 Results 168
4.3.1 Characterisation of LTF in seal serum and uterine epithelium tissue 168
4.3.2 LTF concentrations in Ringed, Grey and Harbour seals 170
4.3.2.1 Sex and age dependent differences within species 171
4.3.2.2 Population and species differences 174
4.3.2.3 Tissue dependent-differences in LTF concentrations 176
4.3.2.4 High-exposed vs. low-exposed populations 177
4.3.2.5 Hormonal-dependence variations 182
4.4 Discussion 187
4.4.1 Characterisation of pinniped LTF 187
4.4.2 LTF concentrations in pinnipeds 188
4.4.2.1 LTF concentration in seal uterine flushings and uterine epithelium tissue 188
4.4.2.2 LTF concentration in seal serum 190
4.4.3 Validation of LTF as biomarker of endocrine disruption 191
5.0 Blood chemistry parameters as biomarkers of PCB exposure and effects in pinnipeds 195
5.1 Introduction 195
5.2 Materials and methods 200
5.2.1 Sampling 200
5.2.2 Materials 200
5.2.3 Blood chemistry analysis 201
5.2.4 Quantification of serum ΣPCB and 17β-estradiol concentrations 204
5.3 Results 206
5.3.1 Sex and age-dependent variations 206
5.3.2 Inter-species differences 215
5.3.3 High-exposed vs. low-exposed populations 226
5.4 Discussion 244
5.4.1 Age, sex and geographically-dependent differences in blood chemistry parameters within species 244
5.4.2 Validation of blood chemistry parameters as biomarkers of endocrine disruption 246
5.4.3 Biomonitoring ED with blood chemistry biomarkers in study populations 247
6.0 General Discussion 253
6.1 Contaminant analysis 254
6.2 Sex steroids 256
6.3 Lactoferrin (LTF) 259
6.4 Blood chemistry parameters 261

6.5 Limitations and future work 264
7.0 References 267
8.0 Appendix 291
8.1 Appendix I – Contaminant Analysis 291
8.1.1 Soxhlet extraction 291
8.1.2 Soxhlet extraction and HPLC – recovery 291
8.1.3 PCB analysis (ELISA) 296
8.2 Appendix II – Sex steroid Analysis 297
8.2.1 Cross reactivity of ELISA kits 297
8.3 Appendix III – Lactoferrin 301
8.3.1 Homogeniser buffer 301
8.3.2 SDS-PAGE Gel electrophoresis 301
8.3.3 Molecular weight Marker 304

## 1.0 General Introduction

Over the past decades there has been increased concern regarding widespread pollution of the environment with pesticides and industrial chemicals, resulting from industrial, agricultural and other anthropogenic activity (traffic exhaust fumes, municipal waste, fires, etc). Many of these chemicals are known to possess a wide range of toxic effects, including reproductive failure, immunosuppression and endocrine disruption, in a range of vertebrate and invertebrate species including fish, gastropods, turtles, aquatic mammals, birds, alligators, panthers and humans (Safe, 1984; Ruus *et al.*, 1999; MacDonald *et al.*, 2000; Smeds and Saukko, 2001; UNEP, 2001; AMAP, 2002). Chemicals shown to cause effects on the reproductive, immune and endocrine system of marine mammals include industrial chemicals and pesticides, which reach the aquatic environment via industrial discharges, sewage effluent, atmospheric deposition, agricultural run-off and accidental spillage. The more lipophilic and persistent of these compounds bioaccumulate in the lipid of organisms and biomagnify, reaching higher concentrations in lipid-rich tissues of top predators (primarily blubber in the case of seals). Exposed animals remain contaminated for long periods of time since these chemicals are not easily metabolised and/or excreted (McFarland and Clarke, 1989; Aguilar *et al.*, 2002; Breivik *et al.*, 2004). Chemicals that fall in to this category are known collectively as Persistent Organic Pollutants (POPs) and the main categories are listed in Table 1.

POPs pose a risk to human health and wildlife due to their known toxicity. Combined with evidence of their potential for long-range transport leading to widespread contamination even of pristine regions (e.g. Arctic), POPs are clearly a threat to global environmental health. As a result international regulations and agreements have been put in place to reduce and eliminate releases of these chemicals. For example in 1997, the United Nations Environment Programme (UNEP, 2001) Governing Council decided that immediate international action should be initiated to protect the environment through measures which will reduce and/or eliminate the emissions and discharges of a majority of these POPs. All Polychlorinated Biphenyls (PCBs), polychlorinated dibenzo–*p*–furans (PCDFs) and nine organochlorine pesticides (OCPs: DDT, aldrin, dieldrin, endrin, heptachlor, mirex, toxaphene, chlordane, and hexachlorobenzene) have been banned internationally, but are still widely used in undeveloped countries, such as Africa and India, for public health and agricultural purposes (Karlsson *et al.*, 2000).

**Table 1: Summary of categories of POPs, their sources, uses and modes of action/effect**

| Category | Examples | Uses | Modes of Action / Effects |
|---|---|---|---|
| Polychlorinated organic compounds | PCBs | No longer manufactured but some equipment (mainly electrical) containing PCBs remain in use | Anti-estrogenic (ED), Uterine stenosis & occlusions; Reproduction; Immunosuppression; Carcinogenicity; ED |
| | Dioxins (PCDDs) | By-products from incineration & industrial chemical processes | |
| | Furans (PCDFs) | By-products from combustion & incineration | Reproduction; Immunotoxicity |
| Organochlorine pesticides | DDT; dieldrin; lindane | Insecticides (some now banned or being phased out) | Reproduction &/or Developmental Effects; Immune System; Carcinogenic |
| Polybrominated organic compounds | PBDEs | Flame retardants | Carcinogenic, Mutagenic, Teratogenic |
| Organotins | Tributyltin (TBT); | Used in anti-fouling paint | Reproductive; ED; Nervous System |
| Alkyl phenols | Nonyl/octyl phenol | In production of nonyl-phenol ethoxylates & polymers | Estrogenic (ED) |
| Phthalates | Dibutyl phthalate (DBP) | Plasticisers | Estrogenic |
| Hydrocarbons | PAHs | Combustion of organic material | Carcinogenic |

ED; endocrine disruption

Ref: McFarland and Clarke, 1989; Colborn *et al.*, 1993; Koistinen *et al.*, 1997; Tyler *et al.*, 1998; Hoyer, 2001; Aguilar *et al.*, 2002; de Wit, 2002; Birnbaum *et al.*, 2003; Chen *et al.*, 2003; Hamm *et al.*, 2003; Hagger *et al.*, 2005; Heiden *et al.*, 2005; Routti *et al.*, 2005; Van den Berg *et al.*, 2006.

In 2005, the most stringent EU regulations have now been put in to force to restrict the use and subsequent release of hazardous substances (which include a range of POP chemicals, including PCBs and dioxins) to the environment from industry and other sectors (Restriction of Hazardous Substances Directive, RoHS; Waste Electrical Equipment Directive, WEE; EU, 2005).

## 1.1 Polychlorinated Biphenyls (PCBs)

Of the great number of POPs, polychlorinated biphenyls (PCBs) are of the most studied chemicals in terms of environmental contamination and toxicology. They have been used industrially since 1929 and by 1970 hundreds of millions of kilograms had been produced. Total worldwide production of PCBs is estimated at 1.68 million tonnes (AMAP, 2000). PCBs are a group of synthetic halogenated aromatic hydrocarbons with the formula $C_{12}H_{10-n}Cl_n$, where n = 1-10 (Safe, 1994). They have a general chemical structure of one biphenyl ring into which varying numbers of chlorine atoms are incorporated (Fig.1). There are 10 possible sites for chlorination of the biphenyl molecule (mono- to decachlorobiphenyl) and any particular polychlorinated biphenyl is named in accordance with the number of chlorine atoms in the molecule and their positions of substitution (de Voogt et *al.*, 1990).

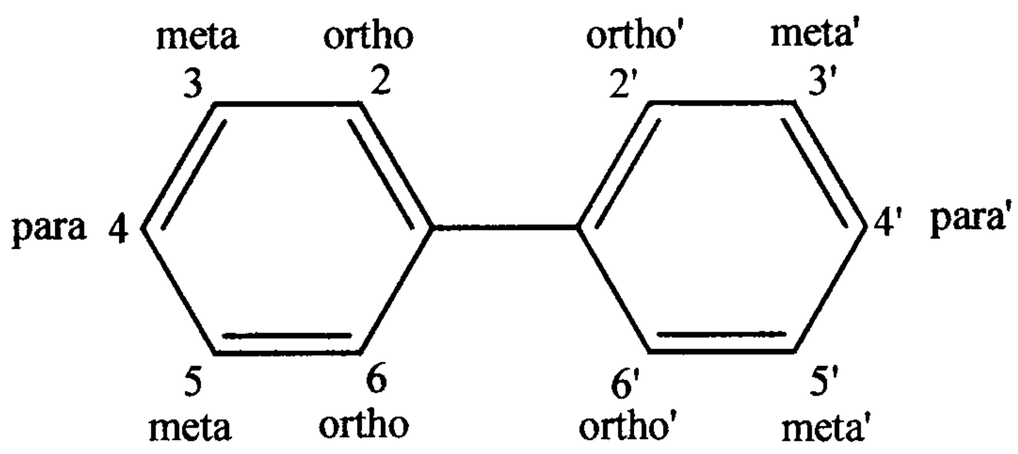



**Figure 1:** General structure of PCBs

There are 209 theoretically possible individual isomers or congeners, ranging from monochlorinated through to one fully chlorinated decachlorobiphenyl congener, created by different substitutions and numbers of chlorine atoms in the *ortho*, *meta* and/or *para* positions (Fig. 1; Table 2). Each possible configuration is named according to the IUPAC numbering system (congeners 1 to 209) according to Ballschmitter and Zell (1980). Generally, the water solubility and vapour pressure decrease as the degree of substitution increases, and the lipid solubility increases with increasing chlorine substitution. Some of

the known trade names for PCB mixtures are; Aroclor, Pyranol, Pyroclor, Clophen, Phenochlor and Kanechlor (UNEP, 2000).

**Table 2:** Description of the 209 possible chlorinated biphenyl isomers

| *Name (-chlorobiphenyl)* | *Structural Formula* | *Mol. Wt*.* | *IUPAC Systemic Numbering* | *Isomer Number* | *% Chlorination* |
|---|---|---|---|---|---|
| Mono- | $C_{12}H_9Cl$ | 188.65 | 1-3 | 3 | 18.79 |
| Di- | $C_{12}H_8Cl_2$ | 233.10 | 4-15 | 12 | 31.77 |
| Tri- | $C_{12}H_7Cl_3$ | 257.54 | 16-39 | 24 | 41.30 |
| Tetra- | $C_{12}H_6Cl_4$ | 291.99 | 40-81 | 42 | 48.65 |
| Penta- | $C_{12}H_5Cl_5$ | 326.43 | 82-127 | 46 | 54.30 |
| Hexa- | $C_{12}H_4Cl_6$ | 360.88 | 128-169 | 42 | 58.93 |
| Hepta- | $C_{12}H_3Cl_7$ | 395.32 | 170-193 | 24 | 62.77 |
| Octa- | $C_{12}H_2Cl_8$ | 429.77 | 194-205 | 12 | 65.98 |
| Nona- | $C_{12}HCl_9$ | 464.21 | 206-208 | 3 | 68.73 |
| Deca- | $C_{12}Cl_{10}$ | 498.66 | 209 | 1 | 71.10 |

** Biphenyl = 154.22, Cl = 35.45; Mol. Wt.= Molecular weight*

Jensen (1969) was the first to discover the environmental occurrence of PCBs and since then a large number of reviews have been published. PCBs are chemically inert and inflammable. They are resistant to oxidation, reduction and highly apolar (non water soluble; Hutzinger *et al.*, 1974). They are resistant to heat, hydrolysis and photo-degradation reactions, they have high boiling point and low electrical conductivity (Erickson, 1986). The chemical and physical properties of PCBs are the reason for their extensive use in a wide variety of open, nominally open and mainly closed electrical systems and industrial processes (Breivik *et al.*, 2004). PCBs have been used as organic diluents, plasticisers, wax extenders, heat transfer fluids, dielectric fluids for transformers and capacitors, hydraulic lubricants, sealants, adhesives, pesticide extenders, cutting oils, dust-reducing agents, flame-retardants and in carbonless copy paper (Safe, 1994). These same properties have also contributed to their environmental problems. They enter into the environment and reach the aquatic ecosystems mainly from discharge points of industrial and urban wastes through runoff, leakage, dumping and atmospheric transport.

Once introduced into the environment, PCBs degrade very slowly and due to their lipophilicity, cross biological membranes from the surrounding medium and bioaccumulate in plant and animal lipids. Their environmental persistence, resistance to biodegradation and lipophilicity, causes them to biomagnify in food chains reaching highest concentrations in top predators (Jensen, 1969; McFarland and Clarke, 1989; Safe,

1994; UNEP, 2000). In 1977 and 1979, a ban on PCB manufacture was imposed in the United States, and it was specified that PCBs could only continue to be used in totally enclosed systems. Although most countries have instituted bans on the production and use of PCBs, significant quantities of PCBs are still used in old transformers and capacitors as dielectric fluids and other electrical equipment and leakage into the environment still occurs (Safe, 1984).

The bioaccumulation, biotransformation and toxicity of PCBs differ between congeners. The toxicity of PCBs is affected by the number and position of the chlorine atoms, as substitution in the *ortho* position hinders the rotation of the rings (Safe, 1994; UNEP, 2000). The non–*ortho* (without chlorine atoms in the *ortho* position) also called planar PCB congeners (PCB 77, 81, 126 and 169) are the most toxic. They adopt a planar configuration similar to planar and highly toxic polychlorinated dibenzo-*p*-dioxins (PCDDs), commonly known as dioxins (Fig.2), and are the energetically most favourable structure for hepatic biotransformation (McKinney, 1996). Another group of toxic PCBs are congeners with one chlorine atom in the *ortho* position called mono-*ortho* CBs (105, 114, 118, 123, 156, 157, 167 and 189). These are structural analogues of non-*ortho* PCBs but with less planar configuration and are classified as coplanars (Safe, 1990). As with dioxins, planar and coplanar PCBs bind the cytosolic aryl hydrocarbon (A*h*) receptor with strong affinity. Upon binding this receptor, the A*h*R-bound complex translocates to the cell nucleus to initiate transcription of CYP4501A mRNA to stimulate the synthesis of cytochrome P450A1 (CYP450) by the microsomes. This is a family of aryl hydrocarbon–metabolising enzymes which generate bioactivated oxidative metabolites which are cytotoxic. These reactive contaminant intermediates also act as DNA adducts and are classed as potent carcinogens (Colborn and Smolen, 1996; Schlezinger *et al*., 1999; Slim *et al*., 1999).

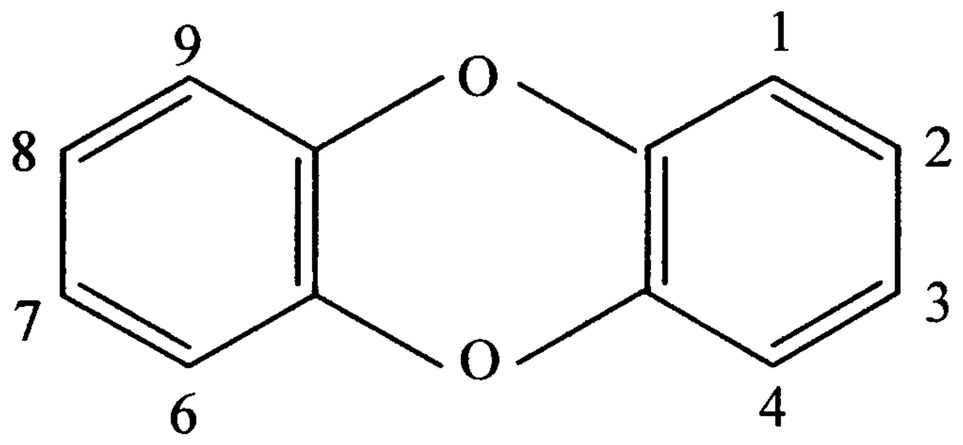



**Figure 2:** General structure of PCDDs

The di-*ortho* PCBs (128, 137, 138, 153, 158, 166, 168, 170, 180, 190, 191, 194 and 205) have two *ortho*-substituted chlorines and are mixed-type inducers but less potent than the

non- and mono-*ortho* coplanar congeners. CBs 138 and 153 are major components of technical PCB formulations and appear to have the greatest potency among the di-*ortho* coplanar congeners both as inducers and as potential toxicants (McFarland and Clarke, 1989).

## 1.2 DDT

In the past decades the use of organochlorine pesticides (OCPs) such as dichlorodiphenyl-trichloroethane (DDT), dieldrin, lindane, chlordecone and others was extensive but it has decreased in recent years. OCPs are generally formed from carbon, hydrogen and chlorine and consist of the three main families: aliphatic diphenyls (e.g. DDT), benzene derivatives (e.g. lindane) and cyclodienes (e.g. aldrin) (Tomlin, 1993).

DDT is one of the most used and more persistent compounds of the organochlorine pesticides. It was widely used during the Second World War to prevent the spread of malaria, typhus and other insect vector-borne diseases. After the war, DDT continued to be used for the control of disease vectors and it was also widely used on a variety of agricultural crops (Weatherley *et al.*, 1997; UNEP, 2000). In general, DDT in insects affects the normal transmission of nerve impulses and poisons the nervous system in humans if the dose is sufficiently high. Therefore, dose is an important factor in determining the severity of the response. The principal neurophysiological mechanism of action of DDT is to slow the closing of the voltage-dependent sodium channel once it has been opened by the action potential, with the result that the hyperexcitable phase of the action potential is prolonged. This is exhibited at the organism level in behavioural hyperexcitability. The dose of DDT required in mammals to provoke signs and effects such as ataxia, tremor and avoidance responding is more than 100 times the dose used for insect exposure (Carson, 1962; AMAP, 2002).

DDT is semi-volatile, highly stable and insoluble in water (apolar). Its presence in the environment is ubiquitous as a result of atmospheric deposition resulting in contamination of remote pristine areas such as the Arctic (Macdonald, *et al.*, 2000) and its bioaccumulation was first reported in the 1950s (Carson, 1962). Technical grade DDT which is used as an insecticide, comprises several isomers (up to fourteen chemical compounds), the most prevalent and active ingredient being the *p,p'*-DDT form, which makes up about 65-80% of the total. Other components included is the nearly inactive *o,p'*-DDT which comprises 15-21% of the mixture and *p,p'*-DDD (up to 4%) (Metcalf,

1995). The chemical structure of *p,p'*-DDT is similar to that of the bisphenols and the isomeric *o,p'*-DDT (Fig.3) which is present as an impurity in commercial *p,p'*-DDT, is known to exhibit estrogenic activity (EPA, 2002). The main metabolite of DDT is *p,p'*-DDE (dichlorodiphenyl-chloroethane). This metabolite is the most persistent, binds the androgen receptor (AR) and exhibits a broad spectrum of anti-androgenic activities as demonstrated in both *in vivo* and *in vitro* bioassays (Kelce *et al.*, 1995). *p,p'*-DDD (dichlorodiphenyl-dichloroethane) (Fig.4) is a less persistent metabolite of DDT known to target the adrenal glands in humans and wildlife (EPA, 2002).

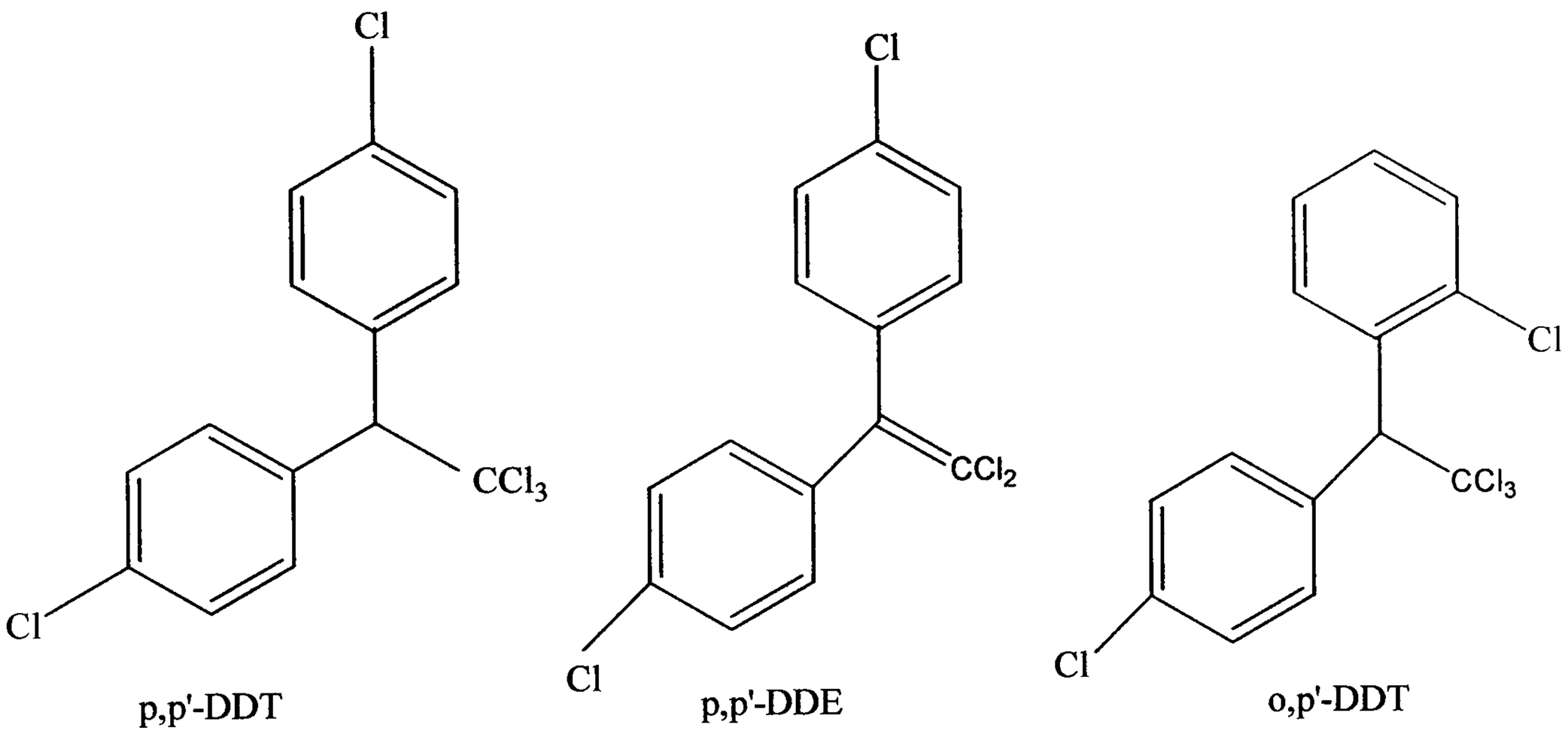



**Figure 3:** Chemical structures of DDT and its metabolites

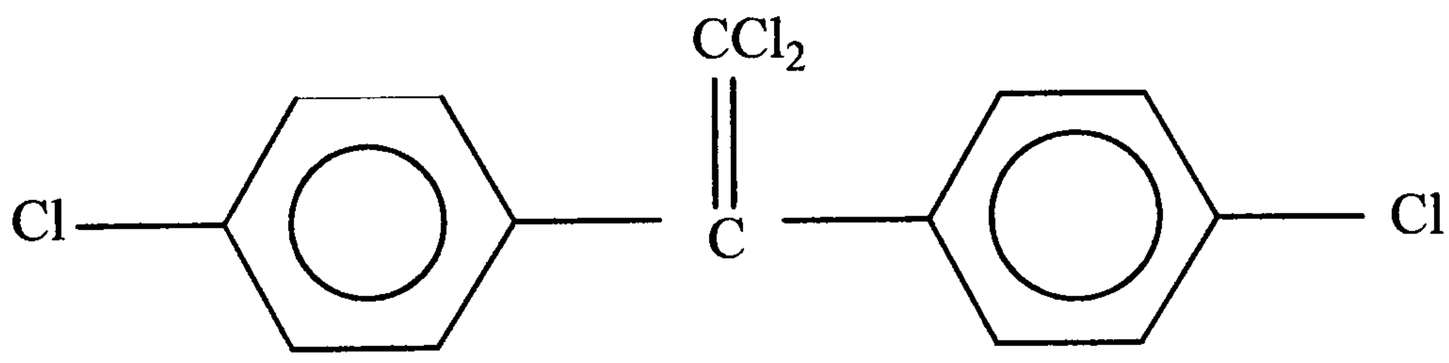



**Figure 4:** Chemical structure of *p,p'*-DDD

A notable characteristic of most of these compounds is their ability to concentrate in biological systems. Bioaccumulation of pesticides was first reported in the 1950s, when residues of the then widely used insecticide, 2,2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane or dichlorodiphenyl-trichloroethane (*p,p'*-DDT) (Fig.3), were discovered in birds following the spraying of crops (Carson, 1962). The production and use of many

organochlorine pesticides such as DDT has been banned or severely restricted in most industrialized and developing countries since the 1970s due to their bioaccumulative, persistent and toxic properties (Schafer *et al.*, 2001). As a result environmental concentrations of these chemicals have been declining over the last two decades (Thomas *et al.*, 2005). However, DDT is still used in the developing countries such as Africa and India, to control mosquitoes, flies and lice infestations and to avoid the spread of malaria, cholera and typhus (Loganathan and Kannan, 1994; Karlsson *et al.*, 2000). As a result DDT still poses great threat to the environment.

## 1.3 Polybrominated Diphenyl Ethers (PBDEs)

In the last decade, PBDEs have aroused concern because of their detection at significant levels in air, water, fish, marine and terrestrial mammals as well as in humans. They are aromatic hydrocarbons with a general formula $C_{12}H_{10-n}Br_nO$, where n = 1-10 (Fig.5). They are structurally similar to PCBs and therefore have similar properties and a large number of congeners depending on the number and positions of the bromine atoms on the two phenyl rings. There are 10 possible sites for bromination of the molecule (mono- to decabromodiphenyl) and any particular polybrominated diphenyl is named in accordance with the number of bromine atoms on the two phenyl rings and their positions of substitution (WHO/ICPS, 1994b). The individual PBDE congeners are numbered according to the IUPAC system used for numbering PCBs based on the position of the halogen atoms on the rings (Pijnenburg *et al.*, 1995; de Wit, 2002).

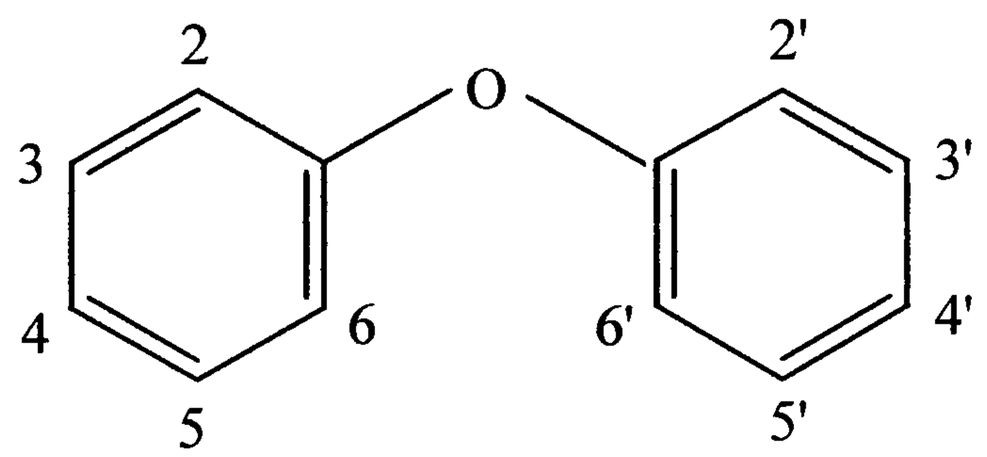



**Figure 5:** General structure of PBDEs

PBDEs are used as flame retardants and their use has been able to significantly reduce fire hazards over the past years. Since the application of stricter fire regulations in many countries, the PBDE demand is increasing rapidly, they are extensively used in controlling the risks of fire and therefore their production has been highly increased. PBDEs are industrially produced from diphenyl ether and bromine under catalytic conditions, which results in mixtures of PBDE congeners. Most industrially manufactured

PBDEs contain mixtures of brominated diphenyl ethers, their isomers and homologues (Elvers *et al.*, 1992). The commercial PBDEs are predominantly penta-(PeBDE), octa-(OBDE) and deca-(DeBDEs) but contain other PBDEs as well. They are very stable lipophilic compounds with high boiling points (310 – 425°C), are resistant towards acids, bases and to reducing or oxidising compounds (Pijnenburg *et al.*, 1995; Allchin *et al.*, 1999; Rahman *et al.*, 2001). According to the UK Department of Trade and Industry (DTI, 1999), the market for flame retardants in Europe is approximately 200,000 tonnes per year in which the demands for organobromines are 64,000 tonnes. According to the International Programme for Chemical Safety (IPCS), there are eight world major PBDE manufacturers in Europe, Japan and the USA and the annual global PBDE consumption has been estimated to be 40,000 tonnes (30,000 tonnes DeBDE, 6,000 tonnes OBDE and 4,000 tonnes PeBDEs; WHO, 1994).

The major uses of PBDE flame retardants are in resins and polymers such as high impact polystyrene (electrical housing, TV cabinets and back covers), flexible polyurethane foam (cushioning/packaging materials), textile coatings (carpets, automotive seating, furniture, tents, military safety clothing), wire and cable insulation, electrical and electronic connectors and other interior parts (DTI, 1999).

Due to their properties (highly lipophilic, readily bioaccumulative and resistant to degradation) PBDEs are of environmental concern. PBDE release to the environment occurs from waste domestic disposals, landfills and incineration processes and since some PBDEs are used as additive chemicals mixed into polymers they are not chemically bound to the plastic or textiles and therefore may separate or leach out from the surface of their product applications into the environment (de Wit, 2002). Moreover, during PBDE incineration toxic dioxins are produced (Rahman *et al.*, 2001).

Trace quantities of congeners of different PBDEs have already been detected in biological and sediment samples from remote areas suggesting that they are being distributed world-wide due to long-range atmospheric transport (Jansson *et al.*, 1987; Sellström, 1996; De Boer *et al.*, 1998). Concentrations of PBDEs in Sperm whale blubber have been reported to be 100ng/g wet weight indicating that PBDEs have reached deep oceanic waters since these marine mammals feed at great depths (De Boer *et al.*, 1998). Several species of seal from different sites have also been analysed for PBDE concentrations. For example, female Baltic Grey seals (*Halichoerus grypus*) contained 730ng/g lipid weight in their blubber (sum of BDE-47, -99, and -100), male Baltic Grey seals had 280ng/g and male

Ringed seals (*Pusa hispida*) were found with 320ng/g lipid weight (Andersson and Wartanian, 1992; Jansson *et al.*, 1993; Sellström *et al.*, 1993a; Sellström, 1996). The main PBDEs reported from environmental samples are PBDE 47, 99, 100, 153, 154 and 209 (De Boer *et al.*, 2001)

## 1.4 Toxic Effects of POPs on Marine Mammals

Marine mammals are apex predators of the marine food chain and therefore significantly exposed to biomagnified levels of persistent, lipophilic organic pollutants, particularly PCBs, PBDEs and DDT (Aguilar *et al.*, 2002; Law *et al.*, 2002). Aquatic mammals in general, have a poor capacity for detoxifying and excreting organic pollutants compared to terrestrial mammals due to the low activity or inactivity of hepatic microsomal cytochrome P450 enzymes of xenobiotic metabolism (Troisi and Mason, 1997). For example, seals have functional CYP450 1A1 and 1A2 iso-enzymes that enable them to metabolise planar compounds but have weak CYP450 2B function and therefore low capacity for metabolising non-planar PCBs (AMAP, 2000). PCB and DDT tissue concentrations and severity of resultant toxic effects, vary according to exposure level and physiological factors, such as age, species, sex, nutritional and health status (Addison, 1989). There is substantial evidence that marine mammal populations, especially those inhabiting polluted waters are more susceptible to the toxic effects of POPs (Thomas, *et al.*, 2005). The main toxic effects of POPs in marine mammals are summarised in Table 3.

**Table 3:** Summary of POP Effects on Marine Mammals

| Types of Effects | PCBs | DDT | References |
|---|---|---|---|
| Endocrine Disruption | + | + | Hook & Johnels, 1972; DeLong et *al.*,1973; Freeman & Sangalang, 1976 & 1979; Helle & Olsson,1976a,b; Reijnders, 1980 & 1986; Bergman & Olsson, 1985; Subramanian *et al.*, 1987; Baker, 1989; Béland *et al.*, 1993; De Swart *et al.*, 1996; O'Shea, 1999; Troisi & Mason, 2000 |
| Hepatotoxicity | + | + | O'Shea, 1999; Dean *et al.*, 2002 |
| CYP 450 Induction | + | + | Boon *et al.*, 1997; Nyman *et al.*, 2003 |
| Immunosuppression | + | + | deSwart *et al.*, 1996; Brouwer *et al.*, 1991; Simmonds *et al.*, 1993; Aguilar & Borrell, 1994: Oberdörster & Cheek, 2000 |
| Retinol depletion | + | + | Brouwer *et al.*, 1989; Zile, 1992; Simms *et al.*, 2000; Debier *et al.*, 2004 |
| Carcinogenicity | + | + | Martineau *et al.*, 1994; Dean *et al.*, 2002 |
| Neurotoxicity | | + | O'Shea, 1999 |

Evidence of reproductive failure in seals (e.g. Harbour; Reijnders, 1986) and cetaceans (e.g. beluga whales; Béland *et al.*, 1993) has been recorded, as well as cancer (Martineau *et al.*, 1994) and lesions in numerous organs (Olsson *et al.*, 1994). Moreover, there are few feeding studies providing evidence linking PCB and DDT exposure to reproductive disorders, immunotoxicity and hepatotoxicity (Brouwer & van den Berg, 1986; Reijnders, 1986; Brouwer *et al.*, 1989; O'Shea, 1999). PCBs are metabolised by cytochrome P450 dependent mixed function oxidases and their induction is associated with toxic effects in the liver (Safe, 1990), including altered liver function enzymes and carcinogenesis (Dean *et al.*, 2002). One such study is a controlled feeding study of adult female captive Harbour seals, where one group was fed "contaminated" Wadden Sea fish (containing ~1.5mg PCB and 0.22mg *pp*'-DDE/day) while the other group was fed "cleaner" Atlantic fish (containing ~0.22mg PCB and 0.13mg *pp*'-DDE/day) over a two year period. This study demonstrated organochlorine-induced reproductive failure. The exposed group produced fewer live pups and daily monitoring showed a difference in plasma sex hormone levels between the groups (Reijnders, 1986). Daily plasma monitoring also showed that thyroid hormone (thyroxin) and vitamin A levels were significantly depressed in the exposed seals and there was lower resistance to secondary bacterial infections compared with the control seals (Brouwer *et al.*, 1989). This was believed to be due to the displacement of thyroxin hormone from its plasma receptor transthyretin by circulating hydroxy metabolites of PCBs, which reduced thyroxin transport to target epithelial cells necessary for functional immune response indicating that endocrine disruption was involved (Brouwer & van den Berg, 1986).

Endocrine disruption has been observed in aquatic wildlife and particularly in marine mammals with evidence available since the 1960s, before the term 'endocrine disruptor' was even conceived. One commonly accepted definition of an endocrine disrupting chemical (EDC) is an "exogenous substance which causes adverse health effects in an intact organism, or its progeny, consequent to changes in endocrine function" (EU, 1996). The endocrine system includes the pituitary, thyroid, adrenal glands and the female/male reproductive system. It is one of the most important homeostatic systems in animals and functions to transfer information from one cell or organ to another, through controlled release of chemical messengers. The chemical messengers are known as hormones and are potent, natural, secretory products of the endocrine glands which travel in the blood in very small concentrations. Reproductive hormones are composed of sex steroid and trophic peptide hormones (Griffin and Ojeda, 1996). EDCs can mimic natural estrogens due to similarities in their molecular structure to 17-β estradiol (phenol group). More

specifically, Fielden *et al.*, (1997) defined 'estrogenic' chemicals as substances whose effects are mediated through the estrogen receptor (ER), by initiating a cascade of cellular and/or tissue effects similar to those initiated by 17-β estradiol (E2). Furthermore, chemicals whose effects resemble those of estrogen but are not mediated through the estrogen receptor are referred to as 'estrogen-like'. For example as previously mentioned, PCBs induce certain iso-enzymes (e.g. 1A1, 1A2, 1B1) of cytochrome P450 in mammalian liver via the A*h*R. Some of these enzymes are involved in E2 metabolism and might disrupt the endogenous hormone levels (Boon *et al.*, 1992). Consequently, any compound found in the environment that exhibits potential 'estrogenicity' is known as 'xenoestrogen'. Steroidogenic tissues are another target of EDCs, where altered steroid biosynthesis leads to changes in circulating hormone levels and hormone-dependent events (e.g., gametogenesis, sexual development and fertility) (AMAP, 2002).

There have been numerous wildlife endocrine disruptive effects reported. Perhaps the clearest evidence of endocrine disruption resulting from a chemical pollutant comes from marine gastropods. Widespread development of a male sexual organ in female marine snails, such as the dog whelk has been observed. Tributyltin (TBT) a biocide in antifouling paints and wood preservatives has been one of the early examples of an EDC that causes impairments in growth, development, reproduction and survival of many marine species. This condition called the 'imposex' phenomenon which is characterised by the development of male sex organs (penis and sperm duct) by female gastropods, results in female sterilization and leads to reproductive failure and has resulted in population declines in many harbours and coastal waters. TBT inhibits the steroidogenic enzyme, aromatase that transforms androgens into estrogens in gastropods and is now known to be responsible for these effects (Hagger *et al.*, 2005). Other examples of endocrine disruption include hatchment failure in Florida alligators while males that do hatch have abnormally small penises (Selcer *et al.*, 2006). Enlargement of the thyroid glands in Great Lake salmons, decreased fertility in birds, feminisation of male fish and birds, and masculinisation of female fish are also common examples caused by EDCs (Milnes, *et al.*, 2006). Male fish living downstream of sewage treatment plants in Southern England have been found to have high concentrations of the female egg-yolk protein, vitellogenin, not normally present in males. Such an effect has been shown to result from synthetic estrogens which are released into the aquatic environment via the sewage treatment plant effluent (van Aerle *et al.*, 2001).

A number of endocrine disruptive effects have also been reported in marine mammals attributable to PCBs, PBDEs, DDT and their metabolites. For example, Subramanian *et al.*, (1987) reported the effects of anti-androgenic activities in adult male Dall's porpoises (*Phocoenoides dalli*) in the North-western Pacific. PCBs and DDE blubber levels were found to be inversely correlated with circulating androgen levels which may have adverse consequences for normal sexual development and function. Significant population declines and poor population recovery have been observed in Baltic and Wadden Sea seal populations over the last fifty years that have been attributed to their high tissue organochlorine burdens. For example, the Harbour seal (*Phoca vitulina*) population inhabiting the Wadden Sea, collapsed from 3000 to less than 500 animals between 1950 and 1975, as a result of PCB-induced reproductive failure (Reijnders, 1980 and 1986). In the 1970s, Hook and Johnels (1972) showed a significant relationship between blubber PCB concentration greater than 70μg/g (lipid weight) and infertility due to uterine stenosis/occlusions in sexually mature females of Grey (*Halichoerus grypus*) and Ringed (*Phoca hispida baltica*) Baltic seal populations. Other studies have also reported similar findings in Baltic Grey and Ringed seals such as uterine stenosis and occlusions, adrenocortical hyperplasia and hormonal osteoporosis (Bergman and Olsson, 1985).

Population-level evidence of PCB-associated immunosuppression in seals (mediated by an endocrine disruption toxic mechanism) also exists. This has been seen as an increased incidence in epizootic episodes in Northern European seal populations in the last two decades (De Swart *et al.*, 1995a; Thomas, 2005). There is now considerable evidence available that PCBs were responsible for causing significantly greater mortality of seals originating from more heavily polluted waters compared with those from cleaner waters during the 1988 Phocine Distemper Virus (PDV) epizootic (Barret *et al.*, 1992; Hall *et al.*, 1992; Osterhaus *et al.*, 1992; De Swart *et al.*, 1995a) The immunotoxicity of PCBs was shown to be mediated by an endocrine disrupting toxic mechanism (thyroid hormone antagonism) causing immunosuppression and increased susceptibility to PDV (deSwart *et al.*, 1995a).

There is also population-level evidence of the reproductive effects of organochlorines caused by endocrine disrupting mechanisms. Reduced reproductive success (Reijnders, 1986), still births (DeLong *et al.*, 1973), uterine pathological deformities (e.g. stenosis and occlusions) (Helle and Olson, 1976a,b; Reijnders, 1980; Baker, 1989), altered steroid hormone metabolism, altered steroid biosynthesis (Freeman and Sangalang, 1979; Troisi and Mason, 2000) and progesterone antagonism (Gillner *et al.*, 1988; Troisi and Mason,

2000), are some of the organochlorine-induced reproductive effects reported in seals, mediated by an endocrine disrupting mechanism.

Tissue concentrations of organic pollutants tend to increase with age, in reproducing females however, the levels fluctuate due to the loss of organic chemicals to their offspring during gestation and lactation (Addison & Brodie, 1987). A female Grey seal for example, can lose up to 30% of her total body burden of DDT and up to 15% of her PCB burden by feeding her pup with lipid rich milk (Pomeroy *et al.*, 1996; Debier *et al.*, 2003). Exposure of seal pups to PCBs is of particular concern since developing organisms suffer more from pollution than adults. For example, PCB-related toxicity on vitamin A which is essential for growth, vision, cell differentiation as well as immune system development has been observed in laboratory animals and in marine mammals (Debier, *et al.*, 2004). Therefore, seal pups are under significant risk from PCB-induced Vitamin A deficiency (Brouwer *et al.*, 1989 & 1991; Zile, 1992; Simms *et al.*, 2000).

## 1.5 Biological Monitoring

*Biological monitoring* or *biomonitoring* is a direct method to determine pollutant exposure in biological samples collected from a population, including magnitude of exposure and temporal fluctuation in exposure. This is achieved by the collection of biological samples from individuals of a population (e.g. tissues, biological fluids, etc) and analysis of pollutant concentrations and/or biological indicators of pollutant exposure or effects. Biological indicators are also referred to as *biomarkers* and their level of expression is proportional to pollutant exposure (dose-dependent). These are either biological responses to pollutant exposure (exposure biomarkers) or adverse health effects/toxicity resulting from pollutant exposure (effect biomarkers) (Kamrin, 2004). An important strength of biomonitoring is that it provides a direct measure of exposure in target populations which is a measure of total integrated exposure from multiple sources. It also provides an understanding of exposure burden in individuals which reaches the circulation and target organs, following pollutant biotransformation and partitioning. Most importantly, biomonitoring involving the application of effect biomarkers provides a quantitative indication of the severity of specific adverse health effects in individuals of an exposed population (Marsili *et al.*, 1998; Fossi *et al.*, 1999; Nyman *et al.*, 2003; Troisi and Borjesson, 2005).

### 1.5.1 Analytical Procedures Used in Biological Monitoring

The process to identify and quantify POPs, in an environmental or biological sample matrix, involves a stepwise analytical procedure. The first step is the extraction of the lipids in which lipophilic pollutants are retained, into an organic (apolar) solvent (e.g. hexane) or mixture of solvents (Smeds & Saulko, 2001), usually by soxhlet extraction or liquid-liquid extraction (De Boer, 1999). After extraction, a clean-up method follows to remove co-extractives/impurities and non-target analytes. Clean-up techniques for separation of PCBs and organochlorine pesticides from the lipid matrix include open column chromatography using silica or other adsorbents (Hall *et al.*, 1999), gel permeation chromatography (Tindle & Stalling, 1992; Cameron *et al.*, 1997) and/or High Performance Liquid Chromatography (HPLC) (van der Hoff *et al.*, 1996). The last step of this procedure is the detection of target analytes by Gas Chromatography (GC), usually by highly sensitive Electron Capture Detection (GC/ECD) (Errickson, 1997) and/or Mass Spectrometry (GC/MS) in Electron Impact (EI) or Negative Chemical Ionisation (NCI) using Selective Ion Monitoring (SIM) (De Boer, 1999).

Chromatographic methods for the analysis of organochlorines are the most accurate available for detecting such chemicals in a variety of samples. However the analytical procedure described above is complex and expensive, especially if large numbers of samples need to be processed to examine how contaminant levels vary with different factors (e.g. location, diet, sex, age and health status). Furthermore, chromatographic analysis is time-consuming, requires large sample volumes and must be undertaken by an analytical chemist in a dedicated analytical laboratory using specialised equipment (Kamrin, 2004). Therefore, alternative more high-throughput, cost-effective and simple methods for the determination of organochlorines are very useful for population-level biomonitoring. Pollutant immunoassays, such as Enzyme Linked Immunosorbent Assays (ELISA), provide an efficient screening tool that can satisfy the above requirements. Furthermore, ELISAs require only small volumes of sample such as plasma, which facilitates ethical non-destructive sampling. ELISA analyses can be completed in less than 12 hours for up to 40 samples in duplicates (or 80 in singles) with appropriate standards and controls per 96 well microtiter plate. Non-destructive sampling enables repeat sampling to monitor exposure over time. Usually costs associated with repeat chromatographic analysis are inhibitive with chromatographic methods however this is not the case with pollutant ELISAs, which encourages temporal monitoring (Zajicek *et al.*, 2000; Johnson *et al.*, 2001; Deng *et al.*, 2002; Troisi & Borjesson, 2005). Unfortunately,

pollutant immunoassays have the limitation of providing only group-specific data, for example total PCB concentration as opposed to congener-specific data provided by chromatographic methods. However, pollutant immunoassays are still well-suited for population exposure monitoring.

### 1.5.2 Pollutant Biomonitoring in Grey, Harbour and Ringed Seals

PCBs and organochlorine pesticides have been widely studied in seal populations from a wide range of different locations, from industrialised areas to remote pristine areas such as the Arctic. Consequently, there is considerable information on contaminant levels in seals from different parts of the world. An overview of the levels of PCBs and OC pesticides in blubber samples of Grey, Harbour and Ringed seals from different geographic areas is shown in Table 4.

**Table 4 (A):** Mean PCB and OC pesticide concentrations in blubber of Grey seals from different geographic areas

| (A) Mean Concentrations (µg/g lipid weight) | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Species** | **Location** | **ΣDDT** | **DDE** | **Dieldrin** | **ΣHCH** | **ΣPCB** | **Ref** |
| Grey Seal | Farne Islands | 5.0 (1.2-7.5) | 3.1 | 0.16-0.19 | 0.02 | 22 (6.9-34) | 1,2 |
| | Swedish Coast | - | - | - | - | 6.1 | 3 |
| | Cardigan Bay | 1.4 | 1.1 | 0.26 | 0.02 | 17 | 4* |
| | Dee Estuary | - | 1.20 | 0.334 | - | 46.79 | 5 |
| | Blackpool | - | 4.8 | - | - | 55.7 | 6 |
| | Baltic | M 41 (11-100) | - | - | - | M 110 (66-180) | 7 |
| | | F 110 (13-180) | | | | F 305 (57-770) | |
| | | F 224 (87-460)** | | | | F 723 (300-1900)** | |
| | | 420 | | | | 140 | 10 |
| | Bothnian Bay, Baltic | M 51 (10-101) | - | - | - | M 84 (17-152) | 8, 31 |
| | | F 20 (10-53) | - | - | - | F 44(35-56) | |
| | | F 39 (15-57)** | | | | F 64 (24-93)** | |
| | | 7.6 (3.3-14) | | | | 28 (10-74) | 9 |
| | | 210 | | | | 100 | 10 |
| | Gulf of Finland | - | - | - | - | 52.6 (14-90) | 11 |
| | | 51 (7.9-110) | 29 | - | - | 66 (31-140) | 12 |
| | St. Lawrence, Canada | 3.55 (1.2-5.2) | - | - | 0.07 (0.02-0.12) | 6.58 (1.55-11.2) | 13 |
| | | M 2.23 ± 1.68 | 3.9 (1.3-11.6) | - | M 0.06 | M 5.59 ± 5.23 | 28 |
| | | F 1.7 ± 0.8 | | - | 0.05 ± 0.2 | F 6.56 ± 5.87 | |
| | Amet Island, Canada | F 2.55 (1.5-4.6) | F 2.15 (1.26-4.2) | - | - | F 6.8 (3.7-10.2) | 29 |
| | Norway | 2.8 (0.6-8.2) | - | - | - | 6.6 (1.9-15) | 1, 14 |
| | Northern Norway, Jarfjord | 1.98 (0.8-6) | - | - | 0.03 (0.01-0.097) | 5.6 (2.9-10.1) | 15 |
| | Sable Island, Canada | 1.8 (0.3-4.5) | - | - | - | 8.2 (0.3-58) | 9, 16 |
| | North Sea | 1 (0.23-21.8) | 0.86 (0.16-3.8) | - | 0.02 (0.004-0.21) | 4.6 (0.1-93) | 17 |

**Table 4 (B):** Mean PCB and OC pesticide concentrations in blubber of Harbour seals from different geographic areas

**(B) Mean Concentrations** (μg/g lipid weight)

| Species | Location | ΣDDT | DDE | Dieldrin | ΣHCH | ΣPCB | Ref |
|---|---|---|---|---|---|---|---|
| Harbour Seal | W. Scotland | 5.3 | - | - | - | - | 18 |
| | | M 3.04 | - | - | - | M 28.5 | 19 |
| | E. Scotland | 1.97 | 1.34 | - | - | 7.62 | 19 |
| | | - | 1.37 | - | - | - | 5 |
| | N. Shetland | - | - | - | - | 4 | 18 |
| | Summer Island, W. Scotland | - | - | - | - | 12 | 18 |
| | E. England | 6.8 (3.3-14) | 4.4 | 0.33 | 0.039 | 23 | 1 |
| | | 3.64 | - | - | - | 25.5 | 19 |
| | Norfolk Wash | - | 2.07 | 0.031 | 0.131 | 7.17 | 5 |
| | Gulf of St. Lawrence, Canada | 5.09 (3.8-8.3) | - | - | 0.05 (0.03-0.08) | 33.3 (12.1-81.6) | 13 |
| | | M 7.06 ± 4.19 | - | - | M 0.07 ± 0.02 | M 53.9 ± 25.3 | 28 |
| | | F 3.48 ± 1 | - | - | F 0.07 ± 0.02 | 14.4 ± 3.93 | |
| | Denmark, Wadden Sea | 17 (4.0-4.7) | 6.1 (3.55-8.83) | - | 0.8 | 110 (23-340) | 20, 26 |
| | Germany, Wadden Sea | 9.3 (2.5-31) | 2.87 | 0.22 | 0.039 | 180 (31-640) | 27, 21 |
| | Dutch, Wadden Sea | 30 (3.3-130) | 16 | 0.5 | - | 130 (5.0-680) | 26 |
| | Norway | 2.1 (1.2-4.6) | - | - | - | 16 (13-23) | 14 |
| | Northern Norway, Jarfjord | 2.06 (0.6-0.7) | - | - | 0.08 (0.04-0.18) | 4.87 (2.25-11.32) | 15 |
| | Iceland (Hofn) | 1.55 | - | - | 0.017 | 5.22 | 21 |
| | Svalbard, Arctic | - | M 1.32 | - | - | M 2.2 | 22 |
| | | - | F 0.19 | - | - | F 0.46 | |

**Table 4 (C):** Mean PCB and OC pesticide concentrations in blubber of Ringed seals from different geographic areas

**(C) Mean Concentrations** (µg/g lipid weight)

| Species | Location | ΣDDT | DDE | Dieldrin | ΣHCH | ΣPCB | Ref |
|---|---|---|---|---|---|---|---|
| Ringed Seal | Bothnia Bay, Baltic | 38 (10-101) | - | - | - | 66 (17-152) | 9 |
| | | M 289 (150-820) | - | - | - | M 266 (120-770) | 7 |
| | Svalbard, Arctic | - | M 3.6 (1.4-6.1) F 1.1 (0.78-1.5) | - | - | M 4.16 (1.16-7.47); F 1.7 (1.12-2.9) | 23 |
| | | - | - | - | - | 1.1 (0.3-8.8) | |
| | | 0.4 (0.2-1.7) | | - | - | | 9 |
| | Barent Sea, East of Svalbard | - | 1.4 ± 0.378 | - | - | 0.07 | 24 |
| | Greenland | M 298; F 301 | - | - | M 69.5; F 59.9 | M 237; F 235 | 30 |
| | Gulf of Finland | 96 (11-200) | 64 | - | - | 96 (14-340) | 12 |
| | NW Russia, White Sea, Arctic | M 0.6 (0.3 - 1.4) | M 0.4 (0.2-1.13) | - | M 0.02 (0.01-0.04) | M 0.96 (0.51-2.12) | 25 |
| | | F 0.52 (0.4-0.7) | F 0.36 (0.3-0.5) | - | F 0.02 (0.01-0.03) | F 1 (0.65-1.54) | |

*: (µg/g wet weight); F**: Females with uterine stenosis

References: 1, Law *et al.*, 1989; 2, Holden, 1978b; 3, Jensen *et al.*, 1969; 4, Morris *et al.*, 1989; 5, Simmonds *et al.*, 1993; 6, Law, 1994; 7, Blomkvist *et al.*, 1992; 8, Nyman *et al.*, 2002; 9, Nyman *et al.*, 2003; 10, Helle *et al.*, 1976a; 11, Koistinen *et al.*, 1997; 12, Perttila *et al.*, 1986; 13, Hobbs *et al.*, 2002; 14, Ofstad and Martinsen, 1983; 15, Ruus *et al.*, 1999; 16, Addison and Stobo, 1993; 17, Kalantzi *et al.*, 2005; 18, Holden, 1972; 19, Hall *et al.*, 1992; 20, Storr-Hansen and Spliid, 1993a; 21, Luckas *et al.*, 1990; 22, Wolkers *et al.*, 2004; 23, Severinsen *et al.*, 2000; 24, Hop *et al.*, 2002; 25, Muir *et al.*, 2003; 26, Reijnders, 1980; 27, Drescher *et al.*, 1977; 28, Bernt *et al.*, 1999; 29, Sørmo *et al.*, 2003; 30, Riget *et al.*, 2004; 31, Routti *et al.*, 2005.

### 1.5.3 Biomarkers

A biological indicator or biomarker is generally defined as 'a biological response to a chemical or chemicals that gives a measure of exposure and/or toxic effect', and can be used as early warning system for the reproduction and survival of the animal (Depledge, 1994). This definition encompasses responses that range from biochemical changes (e.g. receptor binding) to whole ecosystems (e.g. community structure). Biomarker expression should be dose-dependent, i.e. the degree of biomarker expression is proportional to the degree of contaminant exposure and sensitive enough to monitor low-level exposure. A biomarker can be specific to certain groups of contaminants, or non-specific, where it provides only a general indicator of exposure to pollutants. Moreover, it can be quantified in samples taken non-destructively or with minimal invasion (e.g. blood, milk, urine, faeces, and blubber biopsy) and can be used to facilitate the investigation of POP exposure and effects in live animals. The quantification can be accomplished using cost-effective and straightforward biological assays, facilitating larger sample sizes and reproducible results. Expression of baseline/control biomarkers can also be determined in less exposed animals/populations and this expression can be validated with data from chemical analyses (quantification of pollutant burdens in tissue samples) or by at least one other biomarker that is validated by chemical analyses (Addison *et al.*, 1986; Hall *et al.*, 1997; Fossi *et al.*, 1999; Nyman *et al.*, 2003).

Early detection of the adverse health effects resulting from pollutant exposure is vital, since exposure may cause irreversible damage to entire populations or ecosystems in the long term. More biomonitoring studies to investigate changes in fecundity of marine mammal populations due to exposure to POPs are needed. However, there are several logistical difficulties which need to be taken in to consideration to facilitate an ethical and scientifically-robust biomonitoring programme. Firstly, there is the difficulty in obtaining suitable samples from representative animals of a study population. Samples from dead animals (e.g. net entanglement/hunting victims, or diseased stranded animals) are rarely suitable due to poor sample availability in the short term, small sample size and poor sample quality, which limit the number and type of analyses which can be undertaken. Secondly, no unexposed control populations are available to provide baseline information to establish normal biological responses which may be used as biomarkers. Thirdly, there are as to be expected, ethical considerations and government licensing requirements which need to be addressed. Only non-destructive/minimally-invasive sampling is acceptable to avoid death/injury to study animals. Seals are far easier to sample than

cetaceans (hence there has been biomonitoring of pinnipeds) but require trained personnel to access, anaesthetise and sample individuals. In the UK, home office licences are required before any sampling of wild animal populations which include a demonstration of qualified sampling personnel and justification for study. Fourthly, the cost of analysing large numbers of samples for statistically-relevant population data is extremely prohibitive, particularly for chromatographic determination of pollutant levels in biological sample (Schumacher *et al.*, 1995; Hall, 1998; Fossi *et al.*, 1999; Hall *et al.*, 1999).

Naturally, most research to date has been limited to measuring pollutant levels, histo-pathological changes and *in vitro* biochemical changes using tissue samples collected from small numbers of dead animals to investigate pollutant-mediated effects. However, application of biomarkers to biomonitor exposure and effects of POPs in wild populations, overcomes many of the issues. For example, POP exposure biomarkers provide information of the total biological response to POP mixtures and infer whether resultant biological responses are within physiological limits. Biomarker expression can be measured in samples taken non-destructively using cost-effective, high-throughput immunoassays, thus enabling processing of larger sample numbers to achieve more biologically-relevant data on exposure and effects. Furthermore, re-sampling individuals for temporal exposure studies is also possible for surveillance monitoring.

Biomarkers provide vital information on the way in which varying degrees of contaminant exposure affect biological variables such as reproductive or endocrine in male and female, adult and juvenile marine mammals (de Swart *et al.*, 1995; Hall, 1998). Furthermore, biomarker data from a representative proportion of marine mammals in a population can provide vital information on effects of POPs on the population level, which can be borne out of differences in biomarker expression between POP exposed and control (less-exposed) populations (Reijnders, 1986; Nyman *et al.*, 2003). There are few reliable biomarkers (Table 5) of POP exposure and effect that have been used successfully in marine mammal studies, identifying an important area for research, particularly pertaining to existing biomarkers used in other mammalian species, developed for laboratory animals and humans. The biomarkers most commonly used in marine mammal biomonitoring require the sampling of organs such as liver or kidneys and therefore in most cases, imply sacrifice of the animals. However, blood, faeces and skin biopsy can all be sampled non-destructively and are easy to obtain with minimal stress to individuals.

**Table 5:** Biomarkers of exposure to and effects of POPs employed for marine mammal biomonitoring

| Biomarkers | Exposure | Effect | References |
|---|---|---|---|
| **MFO Induction** | + | | Addison *et al.*,1986; Watanabe *et al.*,1989; White *et al.*, 1994; Marsili *et al.*, 1998 |
| **CYP450** | + | | Boon *et al.*, 1997, Troisi & Mason, 1997; Nyman *et al.*, 2003 |
| **A*h* Receptor Binding** | + | | Nyman *et al.*, 2003 |
| **DNA Integrity** | | + | Colborn *et al.*, 1993 |
| **DNA Adducts** | + | | Peakall, 1992 |
| **Thyroid Hormones** | | + | Hall *et al.*, 1998 |
| **Sex Steroid Changes** | | + | Reijnders, 1986; Subramanian *et al.*, 1987 |
| **Vitamin A & E Changes** | | + | Brouwer *et al.*, 1989 |
| **Serum Enzymes** | | + | Nyman *et al.*, 2003 |
| **Immune Responses** | | + | Duignan *et al.*,1993,1995; Shaw *et al.*, 2005 |

### 1.5.4 Biomarkers in This Study

#### *1.5.4.1 Blood Chemistry Parameters*

The health and nutritional status of animals and humans are generally assessed from blood chemistry parameters and a large variety of readily available diagnostic tests are used in order to generate results for common diseases (Bossart *et al.*, 2001). Although marine mammal clinical laboratory medicine has advanced in recent years, very few marine mammal biomonitoring studies have included blood chemistry parameters as biomarkers of contaminant exposure and effects, since blood sampling presents obvious logistical problems (Hall *et al.*, 1997; Hall, 1998; Nyman *et al.*, 2003). A range of blood chemistry parameters are elevated/depressed as a direct response to contaminant exposure/effects. Although confounding factors such as diet, level of physical activity, reproductive status and trauma alter blood chemistry parameters, these remain useful tools for population non-destructive marine mammal biomonitoring, provided more information on baseline levels can be made available.

In rodents and humans, PCB exposure most commonly results in increased total serum lipids, cholesterol and triglycerides (Allen *et al.*, 1976; Kreiss *et al.*, 1981; Borlakoglu and Welch, 1992; Yamamoto *et al.*, 1994). Increased total serum lipids, lipoproteins (such as very-low density lipoprotein; VLDL) cholesterol and triglycerides indicate an accumulation of lipid in the liver, resulting in a fatty liver (Vandenberghe, 1996). This may lead to hepatomegaly, degeneration and necrosis. Hepatic lipid accumulation has been proposed as a protective response against oxidative damage from oxidative metabolites of PCB biotransformation (Borlakoglu and Welsch, 1992).

Plasma aspartate aminostransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALKP), and lactate dehydrogenase (LDH) have all been shown to be affected *in vitro* by a range of environmental pollutants, but corresponding effects *in vivo* are difficult to demonstrate (Christensen *et al*., 1982). Elevations in ALKP, one of the most widely distributed enzymes in the body is associated with pregnancy, liver disease, intestinal inflammation and growth in young animals (Bossart and Dierauf, 1990; Nyman 2000). ALT and AST are associated with hepatocellular necrosis, hepatic cirrhosis and neoplasia, whereas measurement of gamma glutamyl transferase (γGT) levels is a sensitive test reflecting hepatocellular injury (Bossart *et al*, 2001). Plasma creatinine kinase (CK) and creatinine have also been found to be induced by exposure to diethylstilbestrol (DES). In general, liver function enzymes, such as AST, plasma ALKP and γGT are used in clinical screening for liver failure since they become elevated well before the onset of clinical symptoms of liver disease making them very valuable diagnostic tools for biomonitoring hepatotoxicity of organic pollutants such as organochlorines, in marine mammals (Burtis and Ashwood, 1999).

Increased serum levels of total bilirubin (TBIL) are associated with hepatic or post-hepatic problems (Medway and Geraci, 1986; Sweeney, 1974; Sweeney and Ridgway, 1975; Bossart and Dierauf, 1990). In dogs, plasma uric acid levels have been used as indicators of liver disease, however very limited information exists to determine if it is valuable for assessing liver insufficiency in marine mammals (Bossart and Dierauf, 1990). Blood urea nitrogen (BUN) in marine mammals is higher than terrestrial due to high dietary protein and fat and in liver represents the principal product of protein catabolism. The measurement of BUN levels can provide information on changes in metabolic activity of marine mammals, as well as give indications of liver cirrhosis (Nyman, 2000). The urea cycle operates to eliminate excess nitrogen. On high-protein diets the carbon skeletons of the amino acids are oxidised for energy or stored as fat and glycogen but the amino nitrogen must be excreted. Under stress conditions, such as starvation, enzyme levels increase as proteins are degraded and amino acid carbon skeletons are used to provide energy, therefore, increasing the quantity of nitrogen that must be excreted. Inhibition of enzymes in the urea cycle can cause severe conditions such as hepatomegaly. Hyperammonemia is also a urea cycle defect where increased ammonia levels lead to ammonia intoxication. Increased BUN levels have also been found to associate with high corticosteroid levels (Bossart *et al*., 2001). Increase in plasma albumin (ALB), when associated with increased globulin (GLOB) levels, is also indicative of chronic liver disease and stimulation of the immune system (Bossart and Dierauf, 1990).

Moreover, measurement of ALB levels in marine mammals is very important since it is found to be associated with stress and the transport of PCBs after their release into the blood stream (Peakall *et al.*, 1972, 1992; Busbee *et al.*, 1985).

POPs are believed to adversely alter the physiology of seals by significant alterations in their blood chemistry (Brouwer *et al.*, 1989; Schumacher *et al.*, 1995; Nyman, 2000). Some limited reference values (Table 6) have been suggested for Grey, Harbour and Ringed seals from studies of stranded and captive animals (Engelhardt, 1979; Hall, 1998; McConnell *et al.*, 1983; Schumacher *et al.*, 1995; Nielsen, 1995; Roletto, 1993; Dierauf and Gulland, 2001). These baseline values should be treated as general guidelines because they were derived from measurements of a small number of individuals living under unnatural conditions. Moreover, there is wide variation in published values from different sources resulting from inter-laboratory variation and differences in environmental/husbandry conditions of study animals.

As mentioned earlier, blood chemistry parameters have been used in very few studies as biomarkers of contaminant exposure in marine mammals and therefore more research is needed in order to establish a clear idea about their validity as biomarkers. However, increased total serum lipids, cholesterol and triglycerides are the most commonly found changes in rodent and human blood chemistry parameters after deliberate and accidental PCB exposure respectively (Allen *et al.*, 1976; Kreiss *et al.*, 1981; Borlakoglu and Welch, 1992; Yamamoto *et al.*, 1994). Moreover, elevated ALB levels during stress and starvation have been associated with an increased demand for contaminant (e.g. PCBs) blood transport (Peakall *et al.*, 1972; Busbee *et al.*, 1985) and elevated AST, ALT and γGT are used in clinical screening for liver failure (Nyman *et al.*, 2003). A positive relationship between blubber PBDEs and blood cholesterol and albumin levels in juveniles and post-weaned Grey seals (Hall *et al.*, 2003), elevated albumin levels in Baltic Grey and Ringed seals with high contaminant exposure (PCBs and OCPs), as well as increased ALT, AST, ALKP and γGT levels in Harbour seals fed on contaminated fish (PCBs and DDT; Reijnders, 1988) are examples that it is quite possible that these relationships are due to uptake of contaminants and therefore further studies are required to determine the significance of blood chemistry parameters used as biomarkers.

**Table 6:** Ranges of blood chemistry parameters in Harbour, Grey and Ringed seals

| | Harbour Seals | | Grey Seals | Ringed Seals |
|---|---|---|---|---|
| **Parameter** | **Juveniles (M & F)** | **Adults (M & F)** | | |
| **Serum Proteins (g/L)** | 65-90[a]<br>67-84[e] | 61-88[a]<br>27-119[b]<br>57-89[c] | 76 (69-87)[f]<br>79.1[a] | 73 (53-88)[f] |
| **Albumin (g/L)** | 27-37[a] | 25-33[a] | 27 (22-30)[f]<br>27.6[a] | 29 (23-33)[f] |
| **Globulin (g/L)** | 27-64[a] | 32-60[a] | 55.3[a] | n.a. |
| **Creatinine (umol/L)** | 27-97[d]<br>75-111[e] | 35.36-123.76[ab] | 93 (73-112)[f] | 77 (54-114)[f] |
| **Uric Acid (umol/L)** | n.a | 53.53-101.12[f] | 61 (43-121)[f] | 98 (43-206)[f] |
| **BUN (mmol/L)** | 12.13-21-42[a]<br>2.9-12.7[d]<br>8.0-14.8[e] | 10-34.6[af] | 14 (8.9-21.3)[f]<br>11.42-38.56[a] | 13 (5.7-25)[f] |
| **T.BILIR. (umol/L)** | 0.1-6.8[d]<br>2-6.6[e] | 1.71-8.55[ab] | 3.6 (2-6)[f] | |
| **Total CHOL (mmol/L)** | 4.3-10.3[ae] | 4.3-9.1[ac]<br>1.65-7.16[b] | 6.2 (4.7-9.1)[f] | 7.7 (4.9-12.1)[f] |
| **HDLC (mmol/L)** | n.a. | 3.9-6.0[g] | 4.9 (3.9-6.0)[f] | 2.8 (3.5-8.4)[f] |
| **LDL (mmol/L)** | n.a. | n.a. | 0.9 (0.1-2.6)[f] | |
| **VLDL (mmol/L)** | n.a. | *Estimated*<br>0.12-1.82[a] | *Estimated*<br>0.5 (0.3-1.34)[f] | *Estimated*<br>0.63 (0.3-0.7)[f] |
| **TRIG (mmol/L)** | 0-2.9[d]<br>0.2-1[e] | 0.25-4.1[a]<br>0.23-2.95[b] | 1.1 (0.6-2.9)[f] | 1.4 (0.6-3.7)[f] |
| **Glucose (mmol/L)** | 4.8-10.32[df]<br>6.9-9.8[e] | 2.28-9.5[af] | 4.7 (2.5-6.1[f]<br>3.88-5.22[a] | 5.7 (2.4-8.9)[f] |
| **Liver Function Enzymes** | | | | |
| **ALT (U/L)** | 36-95[ad]<br>6-90[e] | 8-75[a] | 34 (10-69)[f]<br>11-30[a] | 72 (24-236)[f] |
| **AST (U/L)** | 5-91[e]<br>50-159[a] | 29-168[a] | 49 (32-79)[f]<br>24-59[a] | 83 (35-174)[f] |
| **γGT (U/L)** | 4.9-23[de] | 2-15[ab] | 11 (7-26)[f] | 17 (6-54)[f] |
| **ALKP (U/L)** | 23-259[ad]<br>20-84[e] | 11-176[a] | 89 (17-634)[f] | 54 (0-148)[f] |
| **CK (U/L)** | n.a. | 34-4572[a] | 629 (250-1495)[f] | 762 (104-3502)[f] |
| **LDH (U/L)** | 292-1330[df]<br>285-1071[e] | 167-1000[a] | 996 (849-1254)[f] | 1591 (692-2790)[f] |

BUN, Blood Urea Nitrogen; CHOL, Cholesterol; HDLC, High Density Lipid Cholesterol; T.BILIR, Total Bilirubin; TRIG, Triglycerides; LDH, lactate dehydrogenase; VLDL, Very Low Density Lipoprotein; ALKP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CK, creatinine kinase; γGT, gamma glutynil transferase; n.a., no data available. References: [a] Dierauf & Gulland, 2001; [b] Schumacher, 1995; [c] Bossart and Dierauf, 1990; [d] Roletto, 1993; [e] de Swart *et al.*, 1995, [f] Nyman, 2002.

### *1.5.4.2 Lactoferrin*

Lactoferrin (LTF) is an iron-binding glycoprotein (molecular weight ~70kDa) of the transferrin family, originally discovered in bovine whey in 1939 (Farnaud & Evans, 2003) and later in human breast milk (Schäfer, 1951). It is the major estrogen-inducible glycoprotein in the mouse uterus (Pentecost and Teng, 1987; Ranhotra & Teng, 2005) and the most abundant protein in human milk (Masson & Heremans, 1971; Hennart *et al.*, 1991). LTF is naturally present in most exocrine secretions including milk, nasal secretions, saliva, tears, semen, plasma, uterine fluid and intestinal secretions (Masson *et al.*, 1966; Teng *et al.*, 1989 & 2004; Inoue *et al.*, 2004). LTF is produced mainly in the bone marrow by immature neutrophilic leukocytes (McMaster *et al.*, 1991; Rebelo *et al.*, 1995), mammary glands and certain secretory epithelia such as uterine epithelium (Pacora *et al.*, 2000). LTF is also expressed and secreted by the specific granules of polymorphonuclear leukocytes (PMN) (Masson *et al.*, 1969).

LTF is a multifunctional protein to which several physiological functions have been ascribed (Fig.6; Brock, 2002). Some of its functions include anti-oxidant protection from iron-induced lipid peroxidation, cell growth regulation, stimulation of DNA synthesis and DNA binding (Nichols *et al.*, 1987), bacteriostatic and bacteriocidal activities against gram-negative and gram-positive bacteria (e.g. inhibits growth of *Eserichia coli, Streptococcus* spp, and *Clostridium* spp) (Orsi, 2004), activity against fungi, viral DNA and RNA, modulation of immune responses (Conneely, 2001) and inhibition of tumour growth (Teng *et al.*, 1992; Bezault *et al.*, 1994; Levay and Viljoen, 1995; Kanyshkova *et al.*, 2003; Valenti *et* al., 2004; Larkins, 2005).

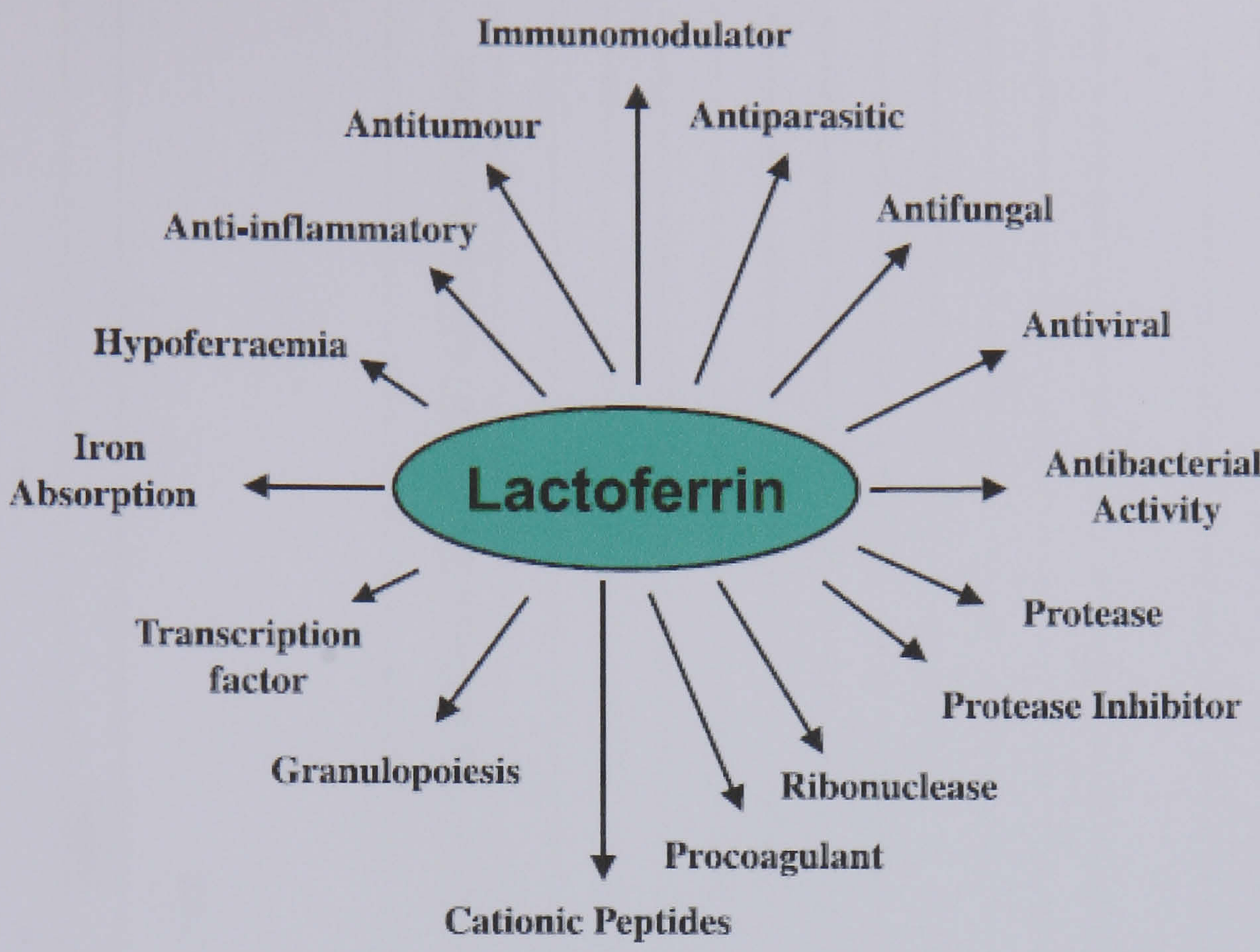



**Figure 6:** Possible functions of LTF (*source*: Brock, 2002)

LTF levels have been reported for humans, rodents and domestic animals, but not marine mammals. Human colostrum – the first breast milk produced post-partum, has higher levels of LTF (up to 7g/l) than breast milk produced later (Masson and Heremans, 1971). Human milk is by far the most abundant source of LTF and has the highest concentration of LTF compared with milk from other species (Teng *et al*., 2002). Human plasma LTF concentrations are low - approximately 0.2µg/ml (Baynes *et al*., 1986; van der Strate *et al*., 2001), whereas LTF levels in tears, nasal secretions and seminal fluids have levels of over 100µg/ml (Lakritz *et al*., 2000). Substantial amounts of LTF are also found in sow, cow and goat milk (Yang *et al*., 2000; Teng *et al*., 2002). Table 7 provides a summary of LTF concentrations in biological fluids of different species.

**Table 7:** LTF concentrations in biological fluids of different species

| Species | Sample | LTF µg/ml | Author |
|---|---|---|---|
| **Humans** | | | |
| M & F | Plasma | 0.13 - 1.62 | Bennet & Mohla, 1976; Hansen *et al.*, 1976; Boxer *et al.*, 1982 |
| Pregnant | Plasma | 2.6 – 3.9 | Rebelo *et al.*, 1995 |
| Puerperium* | Plasma | 5.72 ± 0.99 | Rebelo *et al.*, 1995 |
| Preeclamptic | Plasma | 4.63 ± 1.08 | Rebelo *et al.*, 1996 |
| | Serum | 0.31 – 0.39 | Oloffson *et al.*, 1977; Hetherington *et al.*, 1983 |
| | Milk | 1000 - 7000 | Nagasawa *et al.*, 1972; Lönnerdal *et al.*, 1976a & b; Bezwoda *et al.*, 1985; Bezwoda & Mansoor, 1989; van der Strate *et al.*, 2001 |
| | Tears | 100 - 2000 | Farnaud & Evans, 2003 |
| | Nasal Secretions | >100 | Lakritz *et al.*, 2000 |
| | Umbilical Cord Plasma | 0.3(L) - 0.2(BL) | Pacora *et al.*, 2000 |
| | Seminal Plasma | 100 - 1000 | Lakritz *et al.*, 2000; Inagaki *et al.*, 2002 |
| **Boar** | Seminal Plasma | 100 - 1000 | Inagaki *et al.*, 2002 |
| **Horse** | Seminal Plasma | 157 ± 118 | Inagaki *et al.*, 2002 |
| **Guinea Pig** | Milk | 100 - 1000 | Mason & Heremans, 1971 |
| **Mouse** | Milk | 100 – 1000 | Mason & Heremans, 1971 |
| **Mare** | Milk | 100 – 1000 | Mason & Heremans, 1971 |
| **Cow** | Milk | 10 - 100 | Mason & Heremans, 1971 |
| **Goat** | Milk | 10 - 100 | Mason & Heremans, 1971 |
| **Sow** | Milk | 10 – 100 | Mason & Heremans, 1971; Elliot *et al.*, 1984 |
| | Serum | 19 - 42 | Yang *et al*, 2000 |
| **Rat** | Milk | <50 | Mason & Heremans, 1971 |
| **Rabbit** | Milk | <50 | Mason & Heremans, 1971 |
| **Dog** | Milk | <50 | Mason & Heremans, 1971 |

* 24-48h after childbirth; L = Labour; BL = Before Labour

Levels of LTF in mammals increase during inflammation, in blood, milk and faeces (Thomas *et al.*, 2002; Kane *et* al., 2003) and also during pregnancy, thus it is very important to monitor the changes of LTF levels in biological fluids to detect any physiological changes or infection. For this reason and the properties that were mentioned above, LTF has been identified as a useful marker of stress during pregnancy, for the studies of the role of specific granules in PMN function (Hetherington, 1983), for determining the infection status of udder halves in dairy goats (Chen *et al.*, 2004) and cows (Hagiwara *et al.*, 2003), inflammation (Rebelo *et al.*, 1995) and as a pathological marker for distinguishing between adenocarcinomas of the cervix and the uterus in humans (Farley *et al.*, 1997), to mention only a few.

LTF is a major secretory and estrogen-inducible protein in mammals (Newbold *et al.*, 1992; Walmer *et al.*, 1992; Teng *et al.*, 2002; Ranhotra & Teng, 2005). Uterine LTF synthesis is mainly under the control of estrogen in both the female and male mammalian reproductive tract LTF secretion (Teng *et al.*, 1992 and 1995). During the mouse estrus cycle serum LTF levels positively correlate with circulating estrogen and therefore vary remarkably over the estrus cycle (Fig.7; Walmer *et al.*, 1992).

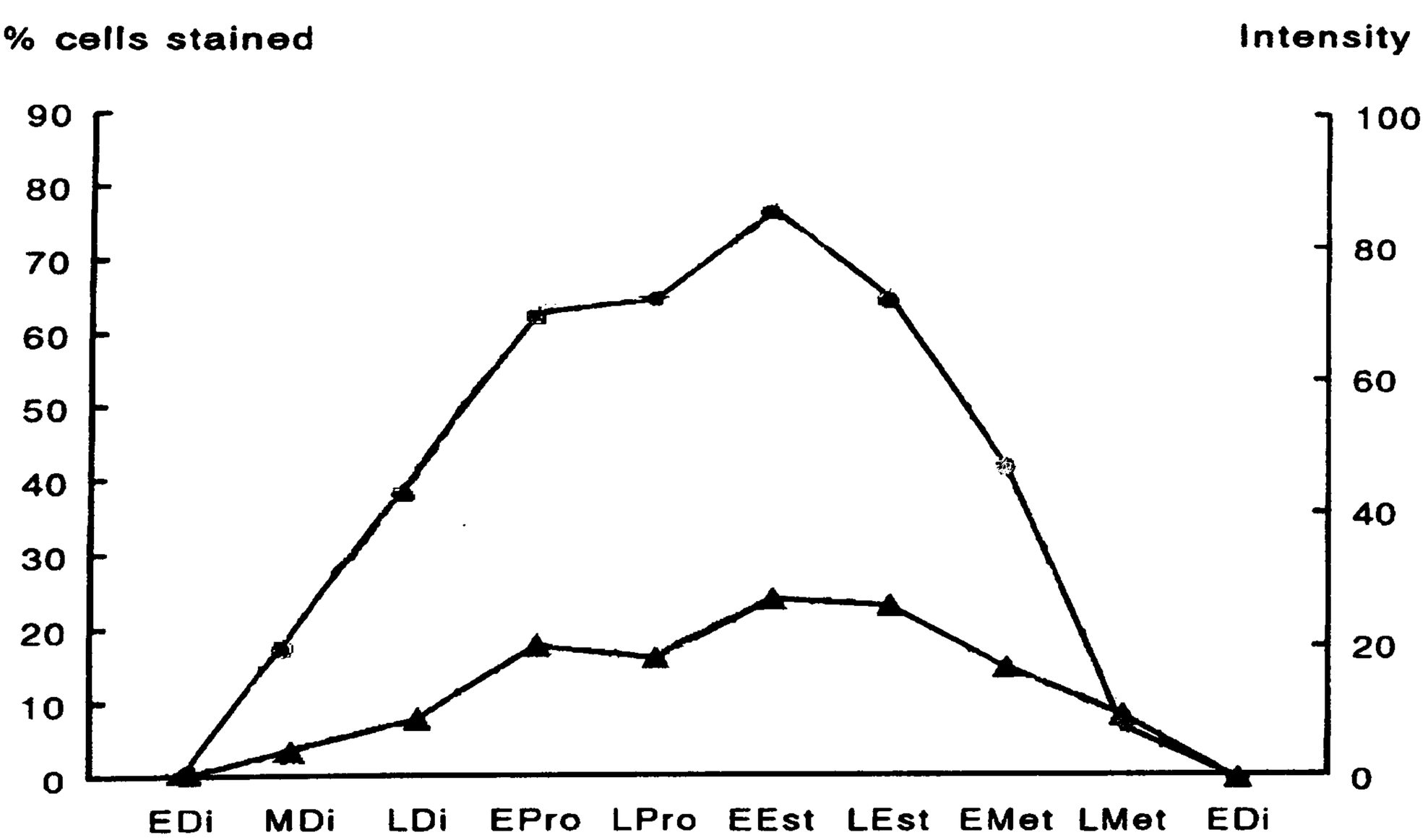



**Figure 7:** LTF levels indicated by the intensity of stained cells obtained from quantitative immunohistochemistry in the endometrial luminal epithelium throughout the CD-1 mouse estrus cycle. Two staining patterns were observed: (●) cytoplasm and (▲) nucleus.
E=Early; M=Mid; L=Late; Di=Diestrus; Pro=Proestrus; Est=Estrus; Met=Estrus
(*source*: Walmer *et al.*, 1992)

Progesterone and estradiol are the two major ovarian steroids that co-ordinate the LTF protein expression (Kurita *et al.*, 2000). As serum estrogen rises during proestrus and progesterone levels are low, LTF protein expression increases reaching highest levels at early estrus in concert with maximal estrogen concentrations and as progesterone levels rise during metestrus, LTF levels begin to decline becoming undetectable during diestrus (Newbold *et al.*, 1992; Walmer *et al.*, 1992; Jefferson *et al.*, 2000; Kurita *et al.*, 2000). LTF protein expression has been shown to increase during the proliferative phase and diminish during the luteal phase in human endometrium and vaginal epithelium (Teng *et al.*, 2002; Walmer *et al.*, 1995). The tight hormonal control of LTF suggests that this protein plays an important role in the uterus during early pregnancy (Ward *et al.*, 1999; Teng *et al.*, 2002).

Since the discovery that LTF is an estrogen-responsive protein in the mouse uterus, LTF has been a biomarker for the study of environmental estrogenic chemicals and thus far, many natural estrogens, estrogen metabolites, synthetic estrogens and xenoestrogens have been examined for their effects on LTF expression either in mouse uterus or in cultured human endometrial cells (Teng *et al.*, 2002; Zhang and Teng, 2002; Newbold *et al.*, 2004; Stokes *et al.*, 2004; Ranhotra and Teng; 2005). At present no studies have looked at whether in marine mammals LTF in the blood is influenced by EDCs (such as PCBs, DDT and PBDEs) however it has been demonstrated that the level of serum LTF correlates positively with circulating estrogen levels in humans and mice and can vary remarkably in response to the levels of endogenous estrogen (Newbold *et al.*, 1992; Walmer *et al.*, 1992). Therefore the use of LTF as a biomarker of endocrine disruption in marine mammals requires investigation and for this reason was chosen for the present study as a potential biomarker of xenoestrogenic exposure/effects in seal populations.

### *1.5.4.3. Sex Steroids and Reproduction in Seals*

The endocrine system is one of the body's main systems for controlling, communicating and coordinating bodily functions achieved via a network of endocrine glands which secrete hormones and target organs which receive/store hormones via hormone receptors. The major glands of the endocrine system are the hypothalamus, pituitary, thyroid, parathyroids, adrenals, pineal body and the reproductive glands (ovaries and testes). Although these glands are the body's main hormone producers, other organs, such as the liver, thymus, pancreas, placenta etc., also produce hormones. Hormones are the chemical messengers that transfer information from one cell/organ to another and regulate a number of critical functions such as growth, development, reproduction and metabolic homeostasis. The male and female reproductive glands are the main sources of sex hormones called steroids (androgens, estrogens and progestins) to regulate biochemical and physical changes associated with sexual development (e.g. differentiation of genitalia), pregnancy, regulation of the menstrual cycle and control of sexual behaviour. Steroids are lipophilic, low molecular weight organic compounds derived from cholesterol (Fig.8). Besides the ovaries and the testis, steroids are also synthesized by the adrenals and the feto-placental unit during gestation (Griffin and Ojeda, 1996). The first biosynthetic reaction product is pregnenolone and a series of microsomal CYP-catalysed dehydrogenation and hydroxylation reactions give rise to the androgens dehydroepiandrosterone (DHEA) and androstenedione (Fig.8). The final reaction steps of sex steroid biosynthesis take place in the gonads and lead to the production of testosterone from androstenedione precursor and the female sex hormones estradiol and estrone. Hormone levels can be influenced by several factors such as infection, stress and changes in the balance of fluid and minerals in blood as well as EDCs (Fielden *et al.*, 1997; Portier, 2002).

In female mammals, decreased levels of circulating progesterone and the onset of parturition trigger increased secretion of gonadotropin-releasing hormone (GnRH), luteinizing hormone (LH) and follicle-stimulating hormone (FSH) for the initiation of follicular recruitment and development. Follicular maturation subsequently leads to a rise in estrogen production and ovulation following the LH surge (Atkinson, 1997).

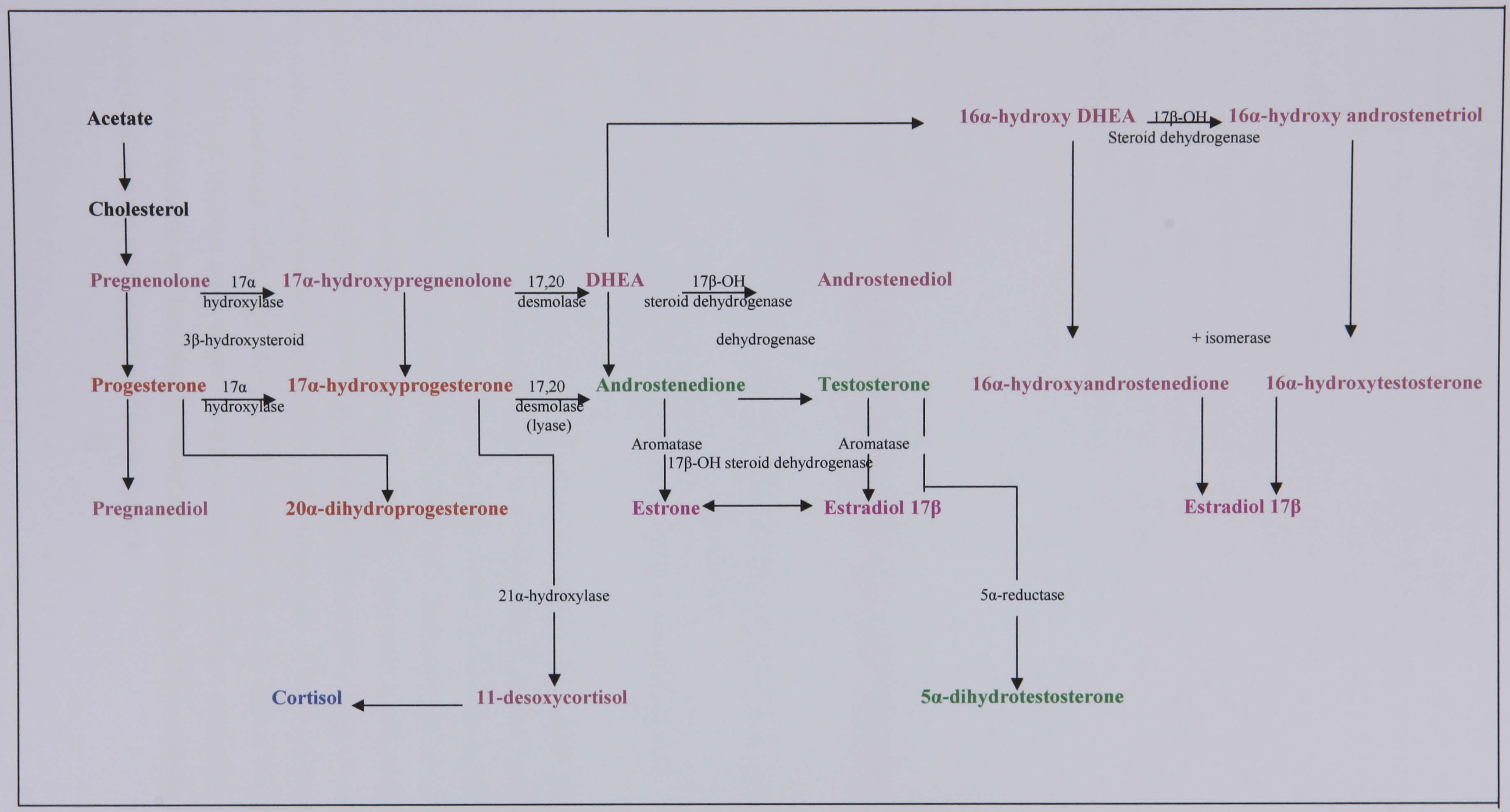



**Figure 8:** Pathway of interconversion of the 4 major classes of sex steroids
Progestagens: Red; Androgens: Green; Estrogens: Pink; and Corticosteroids: Blue
(*Source*: Johnson & Everitt, 2000)

Although the reproductive cycles of species can vary, the function and activity of sex steroids involved in the control of reproduction tend to be similar among all vertebrates. The reproductive cycle is defined as 'the normal minimum time period for all stages of reproduction in the female from ovulation through conception, embryonic diapause (fertilised embryo held in stasis), implantation (embryo attachment) and pregnancy to birth, lactation and moulting followed by a short rest period' (Boyd *et al.*, 1999). There are three distinctive phases of the reproductive cycle of female pinnipeds: estrus (ovulation from alternate ovaries), delayed implantation/embryonic diapause, and foetal growth and development. In the case of male pinnipeds, there is a profound annual cycle of testicular regression and recrudescence (Boyd, 1982). In Harbour, Grey and Ringed seals, estrus begins toward the end of lactation or soon after weaning (Fig.9) (Atkinson, 1997).

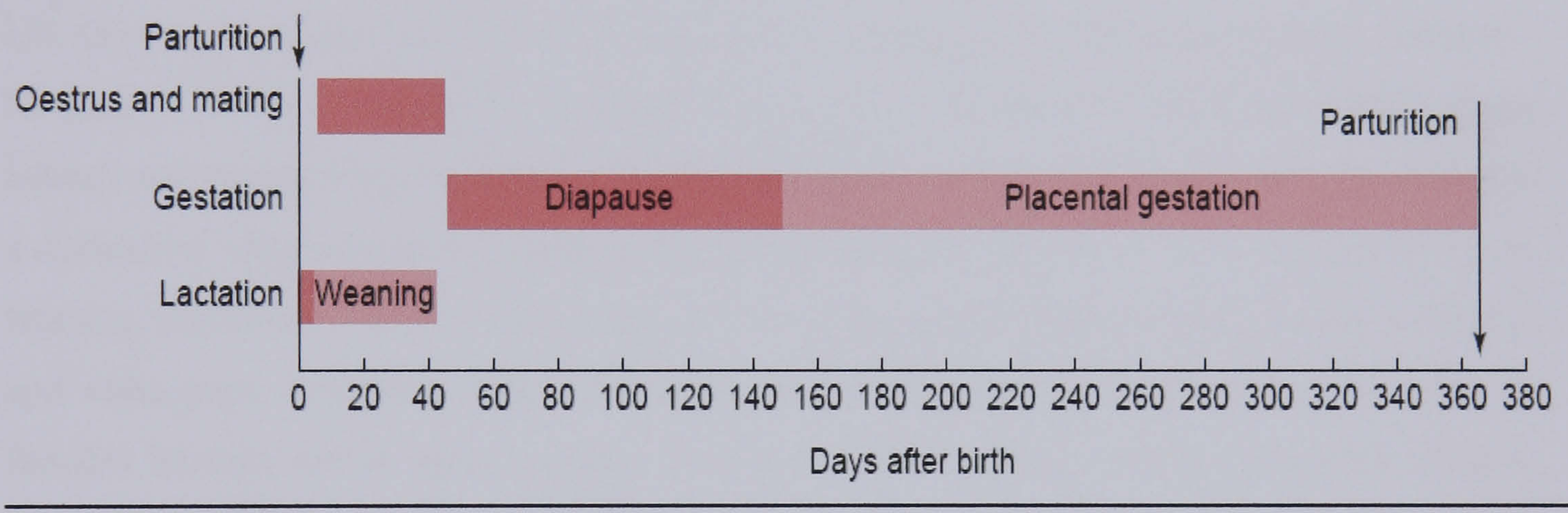



**Figure 9:** Annual reproductive events of Grey, Harbour and Ringed seals (*source*: Atkinson, 1997)

Harbour and Grey seals exhibit typical pinniped reproductive behaviour. Sexually mature seals are polygynous and females give birth to a single offspring. Both species undergo a complete moult every year at the end of the breeding season, which lasts approximately three to four weeks (Corbet and Harris, 1991). Adult male seals compete for good breeding territories on the beach and access to estrus females. They also aggressively defend these territories during the pupping and mating seasons with the expense of significant body weight loss as less time is devoted to foraging during this season (rate of loss for Grey seals, female: 3.8kg/day and male: 2.2kg/day) (Boyd, 1982). Similarly the short intense nursing periods of both Grey and Harbour seals, cause mothers to lose a significant proportion of their body weight due to lactation and less time for foraging. The timing of the pupping and breeding seasons for all species studied are presented in Table 8.

Grey seals give birth on floe-ice and have the shortest lactation periods (16-21 days) compared to species such as Harbour seals, which give birth on land (23-42 days) (Boness, 1979; Kovacs, 1986; Renouf, 1991). This occurs because harsh environment and high latitudes favour the short lactation period. Moreover, Grey seals rarely feed during lactation, therefore depending on body reserves, whereas Harbour seals have the ability to feed during lactation and therefore have relative longer lactation duration. Baltic Ringed seals moult in April and May in small groups or alone on the ice and during this period they greatly reduce their feeding activity and can loose up to half of their weight (Nyman, 2000).

Reproductive activity in Grey seal females begins at 3-5 years and 6-10 years in males. Male Harbour seals reach sexual maturity at 5-6 years whereas females at 3-4 years (Corbet and Harris, 1991). Baltic Ringed female seals become sexually mature at 3 years and males a few years later (Nyman, 2000). There is a clockwise cline in the timing of the pupping season in UK Grey seal populations (Table 8); September – October in south-west England, October – November in West and North Scotland, November – December in the Isle of May, Farne Islands and Donna Nook (Corbet and Harris, 1991). Grey seal females give birth to pups with a distinctive white coat that is replaced by another after the first moult (2-4 weeks after birth). Nursing (lactation) lasts for an average of 16-21 days with female pups gaining 1.6kg/day and male pups 1.9kg/day. Once the pups are weaned mating ensues soon afterwards as females become fertile again (estrus). During the non-breeding season Grey seals disperse widely, but reassemble to moult, forming large mixed aggregations with Harbour seals making identification sometimes difficult at high-density sites. In UK waters females moult from December to March and males from March to May. Outside of these seasons, Grey seals are usually solitary or in small dispersed groups.

## Table 8: Reproductive cycle for Harbour, Grey and Ringed seals

| Species | Study Population | Pupping / Birth | Nursing / Lactation | Estrus / Mating | Embryonic Diapause | Moult |
|---|---|---|---|---|---|---|
| Harbour Seals | Moray Firth | Late June – mid July | Late June – mid August | Early August-mid September | 2-3 months from conception | Mid August |
| | Tees Estuary | " | " | " | " | " |
| | Abertay Sands | " | " | " | " | " |
| | Norfolk Wash | " | " | " | " | " |
| | Blakeney Point, Norfolk | " | " | " | " | " |
| Grey Seals | Donna Nook, Linconshire | Mid October-early December | Mid October-Late December | Mid November-mid December | 4 months from conception | Mid December-mid January |
| | Baltic | February-March | For 18 days | February-April | 3 months from conception | April, May and June |
| | Sable Island, West Atlantic | January-February | For 16 days | January-February-March | " | March-April-May |
| Ringed Seals | Baltic | February-March | For 5-7 weeks | March-April | " | April-May |
| | Svalbard, European Arctic | Late February-March | " | Late March-April-May | " | April-June |

(Corbet and Harris, 1991; Nyman, 2000)

Mating for Harbour seals takes place in water any time from February to October soon after females come into estrus once their pups have been weaned between April and July (peaks mid-June). The timing of each stage of the life cycle varies between populations from different areas of the UK coast. Pups are born on inter-tidal areas and possibly in the water and suckled for over 3-6 weeks. During weaning pups increase their birth weight of 9-11kg to 24kg (Reijnders *et al.*, 1997).

In Northern fur seals there is an annual peak in plasma progesterone levels to 20-30ng/ml around July, indicative of ovulation (Robeck *et al.*, 2001). During embryonic diapause between August and October, progesterone concentrations drop to 5-10ng/ml, increasing again in November to 25-35ng/ml (Robeck *et al.*, 2001). In non-pregnant Harbour seals, however, blood progesterone levels decline rapidly after implantation (Reijnders, 1990; Atkinson, 1997). During the breeding season, in Grey and Harbour seals, testosterone levels are 10-20 times higher than the non-breeding season (Sangalang and Freeman, 1976; Atkinson, 1997). In female Harbour and Grey seals, plasma progesterone concentrations are decreased through lactation (Boyd, 1983; Reijnders, 1990), increase after mating and remain stable during the three months when delayed implantation takes place (Boyd, 1982; Reijnders, 1990). After implantation, progesterone concentrations gradually increase throughout gestation and peak in the final days before parturition (Raeside and Roland, 1981; Boyd, 1984; Reijnders, 1990). Reijnders (1990) has suggested that estradiol levels are elevated at implantation. In pregnant seals estradiol concentrations increase after implantation (Raeside and Roland, 1981; Boyd, 1982; Reijnders, 1990; Gardiner *et al.*, 1999) and peak at estrus (Boyd, 1983; 1991a).

Due to the need for non-destructive assessment of endocrine status of captive and wild mammals, new hormone monitoring methods have been adopted in recent years, thanks to the availability of sensitive immunoassay detection methods. These endocrine assessment methods include blood, urine, sputum and faecal hormonal measurement. Of particular interest for monitoring seals are faecal hormone assessments, due to the ease of sampling faeces without the need to handle study animals. Immunoassays are able to cope with the complex nature of the faecal matrix, making faecal steroids analysis both practicable, high-throughput and cost-effective (Schwarzenberger *et al.*, 1996). A number of studies have employed faecal monitoring to successfully monitor gonadal and adrenal steroids concentrations in a wide variety of mammalian species (including dogs, buffalo, primates,

elephants, wolves, caribou, wild horses, cows, wild baboons and otters (Wasser *et al.*, 1991; Schwarzenberger *et al.*, 1996; Matsumuro *et al.*, 1999; Linklater, *et* al, 2000; Turner *et al.*, 2001, Lynch *et al.*, 2003). All the available data confirm that faecal steroids accurately reflect plasma/serum profiles, confirming the value of this non-invasive approach to hormone monitoring which also has the added benefit of inferring reproductive status (Schwarzenberger *et al.*, 1996; Moreira *et al.*, 2001). Faecal steroid analysis is now the most commonly used diagnostic tool to study fundamental reproductive endocrinology in wild, farm and zoo animals. This approach offers clear advantages over repeated blood sampling for monitoring reproductive cycles/status of seals, where faecal deposition occurs mainly on the shore or in caves (depending on species).

Organochlorines such as PCBs and DDT are known to cause a wide range of effects in experimental animals, wildlife and humans and there is extensive data on endocrine-related effects (Safe, 1994). PCBs for example are known to affect a variety of systems including the estrogen and androgen system, the thyroid hormone system, retinoid system and several other endocrine pathways (Golden *et al.*, 1998; Brouwer *et al.*, 1998). Possible effects of endocrine disruptors in wildlife animals are difficult to assess because of the generally low background levels of exposure. However, seals being top predators in the marine food chain are highly exposed to contaminants such as PCBs, PBDEs and DDT and the endocrine effects of these chemicals are important when taking into account that there are studies were contaminants such as PCBs for example have been found to be responsible for significant reduction in reproduction. Harbour seals for example that were fed fish originating from the highly polluted Baltic Sea showed reduced reproduction as well as reduced total and free thyroxine and triiodothyronine levels in the plasma (Reijnders, 1986; Brouwer *et al.*, 1989). Aroclor 1254 (a mixture of PCBs) has been found to cause a decrease in plasma progesterone in female adult minks as well as Harbour seals (Reijnders, 1986; Aulerich *et al.*, 1985).

Very little information is available concerning altered plasma sex steroid levels in seals due to EDC exposure; however there are data from cetaceans. Subramanian *et al.*, (1987) demonstrated that in Dall's Porpoises (*Phocoenoides dalli*) increasing organochlorine levels such as PCBs and DDT where associated with a significant decrease of testosterone levels. The probability of organochlorine compounds affecting the reproduction of seals is also supported by Reijnders (1986).



KINGSTON UNIVERSITY LIBRARY

Measuring steroid concentrations in blood and faecal samples can provide accurate information on endocrine/reproductive status/cycle of wild seals in a non-destructive manner. However, hormone changes must be shown to be directly related to pollutant exposure by a known toxic mechanism in a dose-dependent manner to be considered reliable effect biomarkers. Clearly, hormone levels warrant further study to validate their use as non-destructive biomarkers of endocrine disruption in seal populations.

## 1.6 Biological Information on Study Species

### 1.6.1 Geographical Distribution

Harbour seals (*Phoca vitulina vitulina*) are among the most widespread species of pinnipeds, comprising four subspecies. They are found in the Northern hemisphere (North Atlantic and North Pacific) ranging from temperate to Polar Regions and in Europe are found in UK (Fig.10) and in Icelandic, Norwegian, Danish, German and Dutch waters. Harbour seals are non-migratory and found mainly in coastal waters off the continental shelf and slope. They haul out and breed on beaches, low-lying rocks, terraces and ice. In the UK Harbour seals are widespread along the West Coast of Scotland and throughout the Hebrides and Northern Isles. On the East Coast they are more restricted occurring in estuaries such as the Moray Firth, the Tay and the Wash, with smaller groups on the sandbanks of East Anglia and Lincolnshire (Corbet and Harris, 1991).

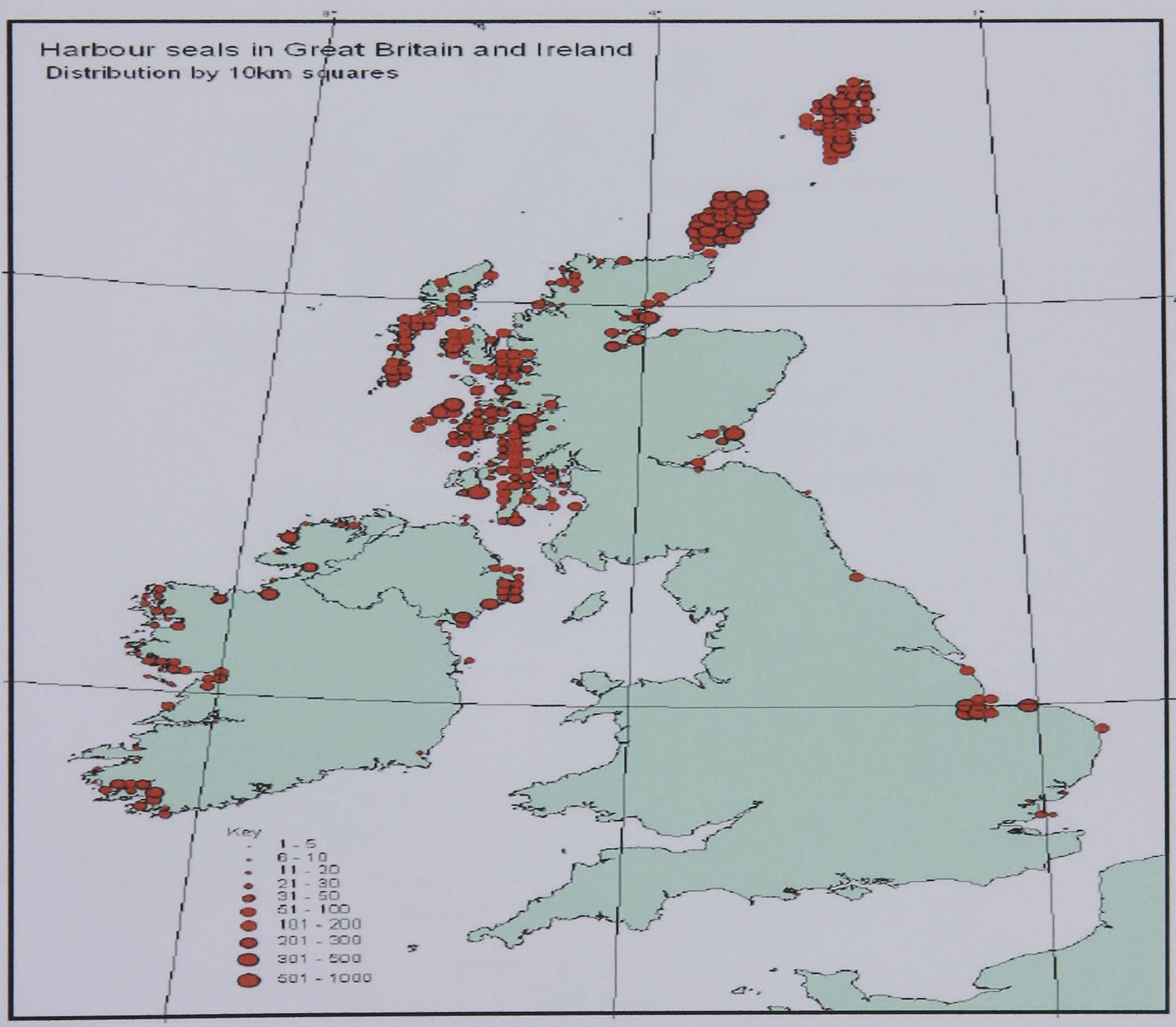



**Figure 10:** Distribution of Harbour seals in Great Britain and Ireland Surveys between 1996-2003.
(*source*: UK Special Committee on Seals, 2004)

Grey seals (*Halichoerus grypus*) are distributed across the North Atlantic Ocean (Canada, UK; Fig.11) where the total population is comprised of three isolated stocks. The first, in the West Atlantic centred on the North-Eastern coast of North America. The second, is in the East Atlantic distributed across a wide geographic range along Iceland, the Faeroe Islands, Ireland and Norway, although the majority (about 80%) breeds around the British Isles with largest colonies occurring off the North-west coast of Scotland. The third stock is found in the Baltic Sea (Corbet and Harris, 1991). Accounts vary, but UK population size range between 130,000 – 200,000 (www.greenchannel.com and www.holidaymull.org). The UK coastal waters support around 40% of the European Harbour and Grey seal population of which 90% is found in Scottish Waters (SMRU, 2004).

**Figure 11:** Distribution of Grey seals in Britain
(*source*: UK Special Committee on Seals, 2004)

Ringed seals (*Phoca hispida*) inhabit the Northern and Central parts of the Baltic Sea. The largest populations are found in areas such as the Gulfs of Bothnia, Finland and Riga where the most permanent and thickest ice cover is found (Helle *et al*., 1990). In 1998, the haul-out population of Baltic Ringed seals was estimated to be approximately 5500 individuals (Härkönen *et al*., 1998).

### 1.6.2 Taxonomy, Diet and Risks

The Harbour (Fig.12), Grey (Fig.13) and Ringed (Fig.14) seal belong to the same subfamily *Phocidae* (Table 9) or 'true' seals. Harbour and Grey seals are very similar in their physical appearance and size and share overlapping habitats.

**Figure 12**: Harbour seal, Norfolk

**Figure 13**: Grey seal, Donna Nook

Both Harbour and Grey seals are generalist predators and their diet varies considerably in time and space. They feed on a variety of fish, cephalopods and crustaceans of surface, mid-water and benthic habitats (Jefferson, 1993; Corbet and Harris, 1991).

**Table 9**: Taxonomy of Harbour, Grey and Ringed seals

| Species | Common Name | Class | Order | Family | Subfamily |
|---|---|---|---|---|---|
| ***Phoca vitulina vitulina*** | Harbour seal<br>Common seal | Mammalia | Carnivora | Phocidae | Phocinae<br>(Northern Phocids) |
| ***Halichoerus grypus*** | Grey seal | Mammalia | Carnivora | Phocidae | Phocinae<br>(Northern Phocids) |
| ***Phoca hispida hispida*** | Baltic Ringed seal | Mammalia | Carnivora | Phocidae | Phocinae<br>(Northern Phocids) |

Ringed seals are opportunistic feeders and prey on a variety of food sources, ranging from fish to crustaceans and amphipods (Lowry *et al*., 1980; Smith, 1987).

**Figure 14**: Ringed seal, Canada (*source*: Canadian Arctic Profile)

A variety of human and ecological factors influence seal population health and size, making it extremely difficult to establish clear links between pollutant exposure and impacts of endocrine disruption at the population-level, such as reduced reproductive success. Small-scale subsistence hunting poaching and incidental catch in fishing gear (particularly gillnets) account for an unknown level of annual mortality in seal populations (Jefferson, 1993). Subsequent to the impact of direct hunting and incidental

mortality, pollution has had the greatest impact on population size. The close proximity of seal habitats to coastal pollution point sources, such as agricultural runoff, industrial discharging and municipal sewage discharges results in high pollutant exposure and subsequent adverse health effects (Reijnders, 1986; Brouwer *et al.*, 1989; De Swart *et al.*, 1994). In the case of Tees estuary, UK, pollutant exposure in Harbour seals is more important than for Grey seals, as the Harbour seals are the main breeding residents (Industry Nature Conservation Association, INCA, *personal communication*). The Baltic is a relative young and shallow water basin and is one of the most polluted water bodies in the world. Three decades ago, extremely high levels of organochlorines were found in marine sediment and biota, with pathological changes and reproductive impairment observed in several species of Baltic aquatic wildlife, including seals (Helle *et al.*, 1976a, b; Bergman and Olsson, 1985; Bengston *et al.*, 1999).

## 1.7 Study Groups

The selection of study groups and seal populations was based on the requirements of this study- to obtain non-destructive samples for biomarker validation studies and to undertake a biomonitoring study of POP exposure and endocrine disrupting effects in seal populations. To achieve this ambitious goal, archived samples from other projects were provided from collaborators outside of this study and extensive field sampling undertaken for the collection of further samples within this study, to represent seal species and populations covering Harbour, Grey and Ringed seals from several locations across Europe, comprising a diverse range of pollutant exposure levels.

### 1.7.1. Experimental Group

To study the effects of contaminated food on reproduction in seals and the kinetics of PCBs, an experiment was conducted on captive Harbour seals at the Research Institute for Nature Management at Texel by Prof. Reijnders (Reijnders, 1984). During the experimental period (1981-1984), two groups of 12 females were fed with fish originating from the Dutch Wadden Sea (exposed group) and the Atlantic Ocean (reference / control group). This equated to an average daily intake of 1.5mg of PCBs and 0.4mg of *pp'*-DDE for the exposed group and 0.22mg and 0.13mg for the control group (Reijnders, 1986). This group of harbour seals were sampled 3-4 weeks before end of pregnancy and the average total PCB concentration in the exposed group (n=11) was 25.7µg/g lipid weight and in the control group (n=12) was 5.3 µg/g lipid weight (Reijnders, *pers. comm*).

Archived samples collected from the seals in the Reijnders study were donated to this project from the controlled dosing experiments of Reijnders and are unique (Table 11). They were included in this study for the specific purpose of biomarker validation studies. They were invaluable to this project because the samples were collected from identically treated seals under controlled conditions, whereby the experimental treatment was either high or low dosing with organochlorines via the diet. Thus any differences in biomarker expression measured in samples collected from each group would differ solely due to exposure, allowing dose-response relationships to be established for candidate biomarkers required as part of their validation. Unfortunately faecal samples were not collected from the same individuals at the time of plasma sampling which would have been ideal for the validation of faecal biomarkers in this study. The limitations of these samples were that they were sampled in 1988.

### 1.7.2. Control Group

Due to the lack of baseline knowledge on normal expression of biological parameters being considered in this project as candidate endocrine disruption biomarkers, it was necessary to obtain samples from populations widely-accepted to be relatively un-exposed to pollutants. There are no pristine areas in the UK, so it was necessary to find samples to compare with our findings from seals originating from highly-polluted areas. Samples were made available by collaborators (Table 11), collected from seals from relatively un-polluted areas to provide us with a "control group". These were from Sable Island Grey seals (Canada) and Svalbard Ringed seals (European Arctic). The average sum concentration of PCBs and DDT levels in seals from these areas is only 0.4-8mg/kg lipid, considered as low background contamination (Nyman, 2002).

Samples from Svalbard and Sable Island were provided by Dr. M. Nyman, *Finnish Game and Fisheries Research Institute* (Helsinki, Finland) and were collected with permission of the Department of Fisheries and Oceans, Canada. These samples were used as reference materials in terms of pollution load in a study in which several biochemical parameters were investigated as potential biomarkers for exposure to and the effects of the contaminant load in Baltic Ringed and Grey seals, two populations that have been suffering from pathological impairments, including reproductive disturbances, which have resulted in a depressed reproductive capacity (Nyman, 2003).

### 1.7. 3. Exposed Group(s)

This group of seals comprised several seal populations from around the UK and the Baltic known to be exposed to pollutants, to provide information on biomarker expression in exposed populations. These samples were required to achieve a gradient of pollutant exposures for validating dose-dependency of candidate biomarker as part of their validation, and to investigate population-level differences in biomarkers expression to identify populations at risk from endocrine disruption/reproductive effects. Specific seal populations were selected on the basis of evidence of (or likelihood of) POP exposure and/or evidence of endocrine disruptive/reproductive effects (e.g. low reproductive success, disease). Of particular focus were the seal populations inhabiting waters close to reasonable levels of agricultural and industrial activity and populations where various studies have indicated/observed elevated pollutant exposure.

In UK, Harbour and Grey seals are found at rocky-shore haul-out sites such as Shetland, Orkney and the Outer Hebrides and inter-tidal sandbanks in the east coast estuaries and coastline (e.g. The Wash, Tees Estuary, Moray Firth; Thompson *et al.*, 1996 and 1997). Estuaries and coastal areas have long been affected by agricultural run-off in farming areas and the disposal of industrial and municipal effluences containing a range of pollutants, such as PCBs and pesticides. These chemicals can also enter estuaries by wet and dry atmospheric deposition, via agricultural run-off and groundwater leaching. Therefore, marine mammals and in particular coastal species such as seals using coastal areas as haul-out sites (e.g. Tees estuary, Donna Nook) are more likely to be exposed to POPs. Most studies reporting concentrations of organochlorines in pinnipeds have found blubber PCB concentrations in Ringed, Grey and Harbour seals to be of the highest recorded. These pollutants are also believed to have contributed to low fertility in Wadden Sea Harbour seal and Baltic Sea Grey and Ringed seal populations (Reijnders, 1986; Hall *et al.*, 1992; Hutchinson and Simmonds, 1994; Nyman *et al.*, 2002 and 2003).

#### *1.7.3.1 Tees Estuary (UK)*

The Tees Estuary, in the north-east of England, has been subject to considerable change due to land reclamation and dredging and is a well known pollution 'hot spot' in the UK, as a result of a long history of intense industrial activity (since the nineteenth century) in the surrounding areas. The River Tees has been used for large volumes of domestic and industrial waste disposal for over 150 years where it received $137x10^4m^3$ of chemical pollutants and $11x10^4m^3$ of sewage per day (Shillabeer and Tapp, 1989; Hardy *et al.*, 1993). However, since 1973 numerous changes such as the introduction of primary sewage treatment and the closure of many industrial plants, have led to a significant improvement in the water quality and biology of the estuary (Tapp *et al.*, 1993; Jones and Turki, 1997).

The Tees seal colony had disappeared in the early nineteenth century due to the intensive reclamation and industrial development. There is a small colony of approximately 60-70 seals that has re-established composed of resident breeding Harbour seals and a smaller proportion of Grey seals (which join the population from neighbouring areas; Isle of May and Farne Islands) which inhabit the Tees Estuary area (Tees Valley Wildlife Trust, 2005). Over the last two decades the seal population has increased from a handful of 6-10 animals due to the survival of all pups born (Table 10) but it is unlikely that this increase is due to major improvement in reproductive success. It is thought that the high pup

mortality rate (100% mortality for several years pre-1994) and high contaminant burdens in these animals due to localised industrial activity (organochlorines and heavy metals) are causing reproductive problems in the group (Industry Nature Conservation Agency-INCA-*personal communication*). It is possible that Harbour seals from southern neighbouring populations such as Donna Nook may be joining the group contributing to the increase in population number. Although there is no published data available on tissue pollutant burdens, particularly POPs in this population, two samples have been analysed in a dead aborted foetus and one pup and the levels were found to be appreciably high, warranting further study of these animals (INCA-*personal communication*).

Despite the lack of toxicological data, the Tees Estuary Harbour seal population is an ideal population to study since data on population dynamics for the last two decades are available. Due to clear evidence of poor reproductive success in adult females, poor pup survival in this population and likely high exposure to organochlorines (and other pollutants) in their environment, it is logical to investigate the impact of the effects of POPs on this population. Samples were available from this population in collaboration with Durham University, UK and further field sampling was undertaken as part of this study between August 2000 and January 2002 with permission of the local warden (Table 11).

**Table 10:** Population size & composition of Harbour seals in Tees Estuary, 1989-2000

| Year | Population Size and Composition** |
|---|---|
| **1989 – 1998** | 24 – 50 Harbour seals |
| | 18 – 27 Grey seals |
| **1989 – 1993** | 1 pup born in alternate years |
| **1994 – 1996** | 2 pups born each year 1995 and 1996, 1 pup rescued and rehabilitated |
| **1997** | 4 pups born (1 stillborn, 2 of the 3 – rescue and rehabilitation) |
| **1998** | 4 pups born (3 weaned successfully) |
| **1999** | 56 Harbour seals; 5 pups born (4 healthy pups survived to weaning), |
| | 5$^{th}$ pup either stillborn / or died soon after birth |
| **2000** | 69 Harbour seals; 4 pups born – all successful births, no deaths |
| **2001** | 5 pups born – all successful births, no deaths |

**Monitoring was undertaken from Huntsman Tioxide hide, approx. 250m from most regular haul-out site using a high-power, 60X magnification telescope

*Source*: INCA, UK

### *1.7.3.2 The Wash: Blakeney Point and Donna Nook (UK)*

The Norfolk Wash (East Anglia) is encircled by areas of high industrial and agricultural activity, but also by less industrialised tourist areas and nature reserves. Just North-east of the Wash is Blakeney Point where there is a resident population of Harbour seals that haul out on sand banks exposed at low tide in the middle of the bay. The RSPCA Norfolk Wildlife Hospital has been monitoring this population for over two decades. The Lincolnshire and Norfolk coast holds >95% of the English Harbour seal population (UK Special Committee on Seals, 2004).

There are several studies of the organochlorine contaminant burdens found in individuals from this population, particularly in the years preceding the 1988 Phocine Distemper Epizootic (Table 6). There is evidence of variable levels of PCB and DDT exposure in this population. There is little knowledge on daily movements of individual seals which complicates our understanding of their exposure history. There is no evidence to date of any gross pathological deformities in this population; however no previous studies have actually studied the reproductive tract for presence of abnormalities. This population therefore, warrants further investigation since contaminant burdens may reach levels of concern. In the case of individuals at the lower end of the exposure gradient which have very low/undetectable contaminant burdens are useful to obtain baseline data on biological variables for Tees Estuary Harbour seals.

Donna Nook (Fig. 11) is one of the most accessible sites in UK for observation of seals during the breeding season (autumn and early winter) when Grey and common seals haul out on the sand flats. The Ministry of Defence (MoD) maintains part of the area as a bombing range close to the haul-out areas. Though noisy, this has significantly reduced disturbance from other human activities since public access by land (tourists, walkers, etc) and sea (boat traffic) is restricted and strictly controlled. This has allowed the colony to establish itself since the early 1970s resulting in good statistics for pup survival (only 15 - 25% pup mortality per year; Lincolnshire Wildlife Trust).

The numbers of Harbour seals in The Wash declined in 1988 by approximately 50% due to the PDV epidemic and increased again at almost 6% per year. However, the population was affected by the recurrence of the PDV epidemic in August 2002 (pre-epidemic counts in 2002: 3037, post-epidemic counts in 2003: 2496). Counts at both Blakeney Point and

Donna Nook decreased between 2002 and 2003 and this decline is believed to have occurred due to human disturbance (UK Special Committee on Seals, 2004). Faecal samples were collected from this population as part of this study (Table 11).

#### *1.7.3.3 Moray Firth (UK)*

The Moray Firth is a large triangular embayment in North-east Scotland of approximately 5230km$^2$. It is the largest firth of its kind on the East coast of Scotland (www.crru.org.uk) and contains within it four smaller firths. Onto the inter-tidal sandbanks, Harbour seals come ashore in the three inner firths (Thompson and Miller, 1992). Systematic counts of the number of Harbour seals began in 1988 by Dr. P. Thompson (University of Aberdeen) and prior to this, occasional counts over parts of the Moray Firth were conducted by Sea Mammal Research Unit (SMRU; National Environment Research Council NERC Research Centre) and other organisations.

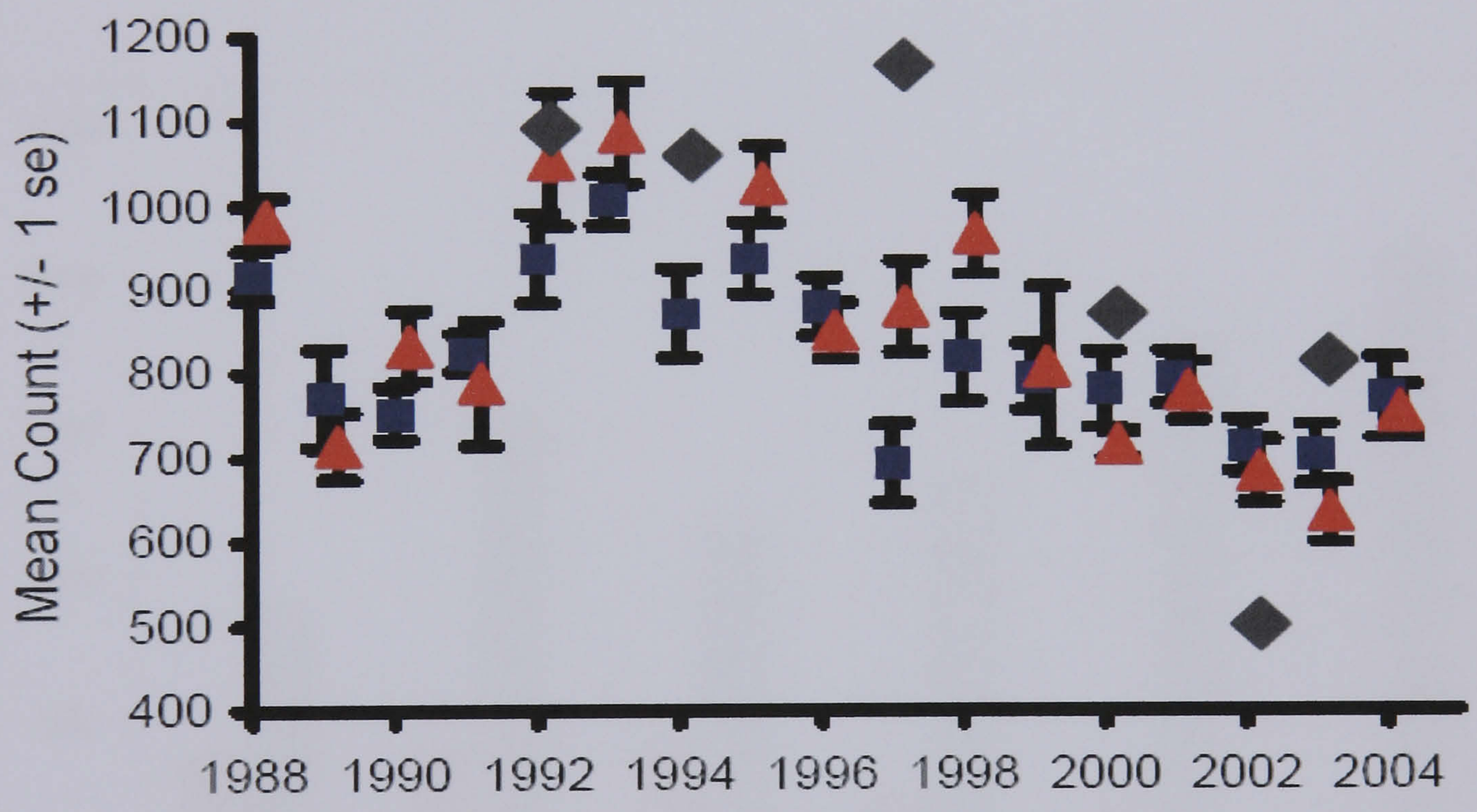



**Figure 15:** Counts of Harbour seals hauled out at Moray Firth.
■: *Pupping season survey (Aberdeen University);* ▲: *Moult survey (AU);*
♦: *Moult survey (source*: UK Special Committee on Seals, Advice, 2004)

In 1993, a population estimate of 1653 (1471-1863) Harbour seals was published by Thompson *et al*., (1997b) from telemetry data. These estimates were made mid-summer when maximum haul-out occurs providing the best estimate of population numbers. Temporal data on Harbour seal abundance from 1988-2004 are shown in figure 15 (UK Special Committee on Seals, Advice 2004). The Moray Firth has a reasonable level of

industrial and agricultural activity, and studies of contaminant levels in this population indicate variable degrees of exposure to organochlorine pollutants (Table 4) making this an ideal study population. Samples from this population (Table 11) were provided by Dr. Paul Thompson (Lighthouse Research Station, University of Aberdeen).

#### *1.7.3.4 Firth of Tay - Abertay Sands (UK)*

Abertay Sands stretches eastwards into the North Sea for nearly 8 miles from the mouth of river Tay at Tayport in Fife (www.geo.ed.ac.uk). Large numbers of both Grey and Harbour seals use the area throughout the year. Sometimes as many as 2000 Grey seals haul-out on Abertay Sands and on the sandbank near the Southern sea fence. Up to several hundred Harbour seals use all the sandbanks and the southern foreshore. Temporal population counts for Harbour and Grey seals at Abertay Sands between 1990 and 2000 are presented in figure 16 (Scottish Natural Heritage, 2002). Samples for the Harbour seal population inhabiting Abertay Sands (Table 11) were provided by Dr. Ailsa Hall (Sea Mammal Research Unit, St. Andrews University).

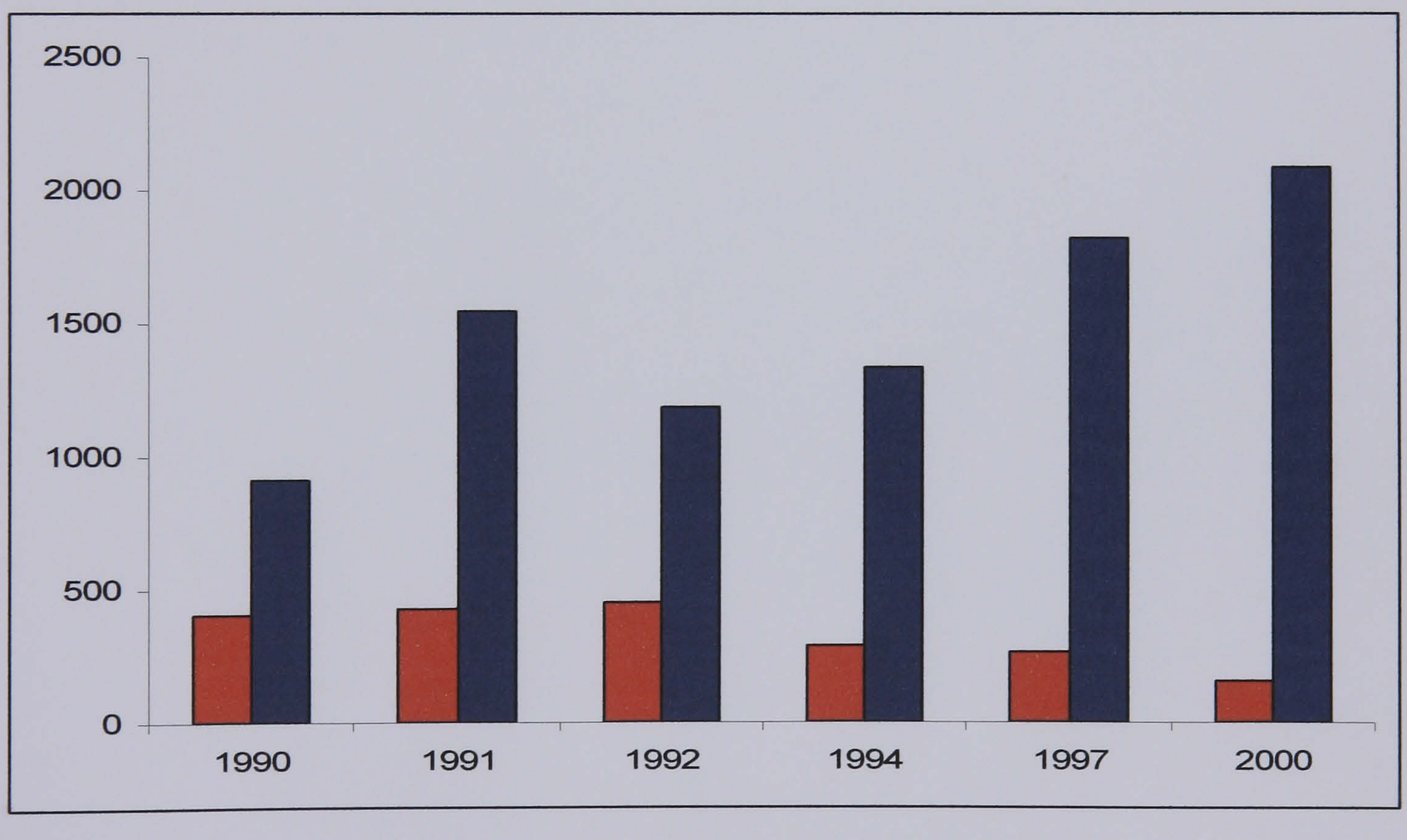



**Figure 16:** Population counts of **Harbour** and **Grey** seals at Abertay Sands

#### *1.7.3.5. Baltic Sea*

As already mentioned, the Baltic Sea is one of the most polluted water bodies in the world. It is extremely vulnerable to pollution since it has slow water exchange and high

levels of industrial activity around it. In general, since 1960s, high organochlorine levels (PCBs, DDT and/or their metabolites) have been associated with low fertility due to a high incidence of sterility in the population caused by uterine deformities and other reproductive tract deformities/abnormalities in Ringed and Grey seals (Helle *et al.*, 1976a and b; Bergman and Olsson, 1985). It is estimated that there were around 200,000 Baltic Ringed seals at the beginning of the 20$^{th}$ century, but intensive hunting and pollution have reduced their numbers considerably. Even though PCBs and DDT have been banned in the countries around the Baltic Sea, pollutant levels are still high enough (Table 4) to cause a threat to the aquatic system (Nyman *et al.*, 2003). Blood (serum, Table 11) from Baltic Grey and Ringed seals were provided by Dr. M. Nyman of the *Finnish Game & Fisheries Research Institute* (Helsinki, Finland). These samples were archived material taken to monitor the health status of the two populations as part of a Finnish government Baltic Sea research expedition.

**Table 11:** Summary of study groups / populations

| Groups | Study Site / Population | Species | Sex | Age | Sample Type | Number of Samples |
|---|---|---|---|---|---|---|
| **Experimental** | | | | | | **23** |
| | Control | *Phoca vitulina* | FC | A | Serum | 12 |
| | Exposed | | FE | A | Serum | 11 |
| **Control** | | | | | | **20** |
| | **Sable Island,** Canada | *Halichoerus grypus* | F | A | Serum | 10 |
| | | | M | A | Serum | 10 |
| | | | | | | **21** |
| | **Svalbard,** European Arctic | *Phoca hispida* | F | A | Serum | 11 |
| | | | M | A | Serum | 10 |
| **Exposed** | | | | | | |
| | **Tees Estuary,** North-east England | *Phoca vitulina* | na | A | Faeces | **47** |
| | **Blakeney Point,** The Wash, UK | *Phoca vitulina* | na | A | Faeces | **10** |
| | | | | | | **13** |
| | **Donna Nook** [a], The Wash, UK | *Phoca vitulina* | na | A | Faeces | na |
| | | *Halichoerus grypus* | na | A | Faeces | na |
| | **Moray Firth,** North-east Scotland | *Phoca vitulina* | | | | **67** |
| | | | F | A | Serum | 17 |
| | | | F | SA | Serum | 4 |
| | | | F | J | Serum | 14 |
| | | | M | A | Serum | 21 |
| | | | M | SA | Serum | 2 |
| | | | M | J | Serum | 9 |
| | **Abertay Sands,** Firth of Tay, UK | | | | | **56** |
| | Firth of Tay, UK | *Phoca vitulina* | F | A | Serum | 20 |
| | | | M | A | Serum | 36 |

**Table 11:** Summary of Study Groups / Populations (*continued*)

| Groups | Study Site / Population | Species | Sex | Age | Sample Type | Number of Samples |
|---|---|---|---|---|---|---|
| | | | | | | **41** |
| | **Baltic Sea** | *Halichoerus grypus* | F | A | Serum | 20 |
| | | | | | Uterine Tissue | 2 |
| | | | | | Uterine Flushing | 5 |
| | | | M | A | Serum | 14 |
| | | | | | | **26** |
| | | *Phoca hispida baltica* | F | A | Serum | 10 |
| | | | | | Uterine Tissue | 2 |
| | | | | | Uterine Flushing | 2 |
| | | | M | A | Serum | 12 |

Abbreviations: F: Female; M: Male; A: Adult; SA: Sub-adult; J: Juvenile; FC: females fed North-eastern Atlantic fish; FE: females fed with Wadden Sea fish; na; not available [a]: could not distinguish species of faecal sample collected

## 1.8 Project Aim and Objectives

The overall aim of this project was to research and develop new and existing exposure and effect biomarkers for biomonitoring endocrine disruption in pinniped populations. The research aim was addressed by the following specific objectives;

I. To quantify exposure level (PCBs, DDT and PBDEs) of study seal populations using chromatographic and immunoassay techniques;

   i) Use of a congener-specific method employing GC-ECD for the quantification of PCB, DDT and PBDE concentrations in seal faecal samples

   ii) Develop an immunoassay (ELISA) method for the quantification of total PCB concentrations in seal serum samples.

   iii) Determine exposure level in Harbour, Ringed and Grey seals in order to determine whether they are exposed or control populations

II. To investigate the reliability of sex steroids as biomarkers of endocrine disruption effects of PCBs and DDT in seals;

   i) Quantification of selected sex steroids in all seal serum and faecal samples by immunoassay and confirmation of exposure (PCB, PBDE and DDT)-dependent expression of steroid biomarkers.

   ii) Use BM and population data to identify populations at risk from endocrine disruption/reproductive effects.

III. To investigate the reliability of LTF as a biomarker of estrogenic PCB exposure in seals;

   i) Undertake the first characterisation of LTF protein in seal species

   ii) Establish male and female baseline LTF values over the reproductive cycle by LTF immunoassay of serum samples from control/reference seals.

   iii) Quantification of LTF biomarker concentration by immunoassay in all seal serum samples and confirmation of exposure (PCB) dependent LTF biomarker expression.

   iv) Use LTF expression to identify populations at risk from ED

IV. To investigate the reliability of blood chemistry parameters as biomarkers of PCB exposure and effects in seals;

i) Investigation of a range of blood chemistry parameters in seal serum to establish baseline values for the three study species

ii) Identify blood chemistry parameters with exposure (PCB)-dependent expression and quantify these biomarkers in all serum samples from study populations.

iii) Use BM expression to identify populations at risk from POP-associated adverse health effects

# Characterisation of Persistent Organic Pollutant (POP) Exposure in Study Populations

## 2.1 Introduction

Over the years many different compounds not normally found in nature have been synthesised in order to improve living standards and quality of life. In the past, such compounds were not adequately tested before registration and they were spread in industry and the environment. Over the past decades, there is increased concern regarding this widespread pollution of the environment with pesticides and industrial chemicals, resulting from industrial, agricultural and other anthropogenic activities. The more lipophilic and persistent of these compounds bioaccumulate to high concentrations in lipid-rich tissues of top predators (primarily blubber in the case of seals). Exposed animals remain contaminated for long periods of time since these chemicals are not easily metabolised and/or excreted.

Chemicals that fall in to this category are known collectively as Persistent Organic Pollutants (POPs) and include industrial chemicals and pesticides, which reach the aquatic environment via industrial discharges, sewage effluent, atmospheric deposition, agricultural run-off and accidental spillage. Many POPs such as PCBs and DDT are known to possess a wide range of toxic effects, including reproductive failure, immunosuppression and endocrine disruption, in a range of vertebrate and invertebrate species including fish, gastropods, aquatic mammals, birds, alligators, panthers and humans (Jones & de Voogt, 1999; Schafer *et al.*, 2001). Since POPs pose a risk to human health and wildlife due to their known toxicity international regulations and agreements have been put in place to reduce and eliminate releases of these chemicals.

## 2.2 POPs Investigated in this Study

### 2.2.1 Polychlorinated Biphenyls (PCBs)

Of the great number of POPs, PCBs are of the most studied chemicals in terms of environmental contamination and toxicology and have been used industrially since 1929 (AMAP, 2000). They are a group of synthetic halogenated aromatic hydrocarbons with the formula $C_{12}H_{10-n}Cl_n$, where n = 1-10 (Safe, 1994) and have a general chemical structure of one biphenyl ring into which varying numbers of chlorine atoms are incorporated (Fig.1, Chapter 1). There are 10 possible sites for chlorination of the

biphenyl molecule (mono- to decachlorobiphenyl) with 209 theoretically possible individual isomers or congeners created by different substitutions and numbers of chlorine atoms in the *ortho*, *meta* and/or *para* positions (Fig. 1 & Table 2, Chapter 1). Any particular polychlorinated biphenyl is named in accordance with the number of chlorine atoms in the molecule and their positions of substitution (de Voogt *et al.*, 1990) and each possible configuration is named according to the IUPAC numbering system (congeners 1 to 209) according to Ballschmitter and Zell (1980). Generally, the water solubility and vapour pressure decrease as the degree of substitution increases, and the lipid solubility increases with increasing chlorine substitution. Some of the known trade names for PCB mixtures are; Aroclor, Pyranol, Pyroclor, Clophen, Phenochlor and Kanechlor (UNEP, 2000).

The chemical and physical properties of PCBs are the reason for their extensive use in a wide variety of open, nominally open and closed mainly electrical systems and industrial processes (Breivik *et al.*, 2004, Table 2.1). These same properties have also contributed to their environmental problems.

**Table 2.1:** Uses, physical and chemical properties of PCBs

| **Uses** | **Physico-chemical Properties** |
|---|---|
| Organic diluents | High boiling point |
| Plasticisers | Highly apolar (low water solubility) |
| Wax Extenders | High dielectric constant |
| Heat transfer fluids | Excellent thermal stability |
| Dielectric fluid for transformers and capacitors | Highly resistant to acidic and basic conditions, hydrolysis and photo-degradation reactions |
| Hydraulic lubricants | Good thermal conductors |
| Sealants | Good lubricating characteristics |
| Adhesives | |
| Pesticide extenders | |
| Cutting oils | |
| Dust-reducing agents | |
| Flame-retardants | |
| In carbonless copy paper | |

Sources: Hutzinger et *al.*, 1974; Erickson, 1986 and 1997; Safe, 1994.

The bioaccumulation, biotransformation and toxicity of PCBs differ between congeners. The toxicity of PCBs is affected by the number and position of the chlorine atoms, as substitution in the *ortho* position hinders the rotation of the rings (Safe, 1994; UNEP, 2000). The non–*ortho* (without chlorine atoms in the *ortho* position) or "coplanar" PCB congeners (PCB 77, 126 and 169) are the most toxic and are the energetically most favourable structure for hepatic biotransformation (McKinney, 1996). Coplanar PCBs

bind the cytosolic aryl hydrocarbon (A*h*) receptor with strong affinity, act as DNA adducts and are classed as potent carcinogens (Colborn and Smolen, 1996, Schlezinger *et al.*, 1999, Slim *et al.*, 1999). Another group of toxic PCBs are congeners with one chlorine atom in the *ortho* position (mono-*ortho* CBs) such as congeners 105, 118, 156, 167 and 189. These are structural analogues of non-*ortho* PCBs but with less planar configuration and also classified as coplanars (Safe, 1990). Of the 209 possible congeners of PCB, around a 150 have been reported at significant concentrations in commercial PCB mixtures or in the environment (Tanabe, 1988) and only 36 (Table 2.2) have been highlighted as most environmentally-threatening due to their toxicity and their high occurrence in environmental samples and animal tissues (McFarland and Clarke, 1989).

**Table 2.2:** The 36 most environmentally threatening PCB congeners

| PCBs | Reported toxicity and occurrence |
|---|---|
| 77, 126, 169 | Most potent MFO inducing congeners |
| 105, 118, 128, 138, 156, 170 | Less potent but at high concentrations in the environment |
| 87, 99, 101, 153, 180, 183, 194 | Highly abundant but relative weak MFO inducing congeners |
| 18, 44, 49, 52, 70, 74, 151, 177, 187, 199 | Weak or non-MFO inducers but frequently present in the environment |
| 37, 81, 114, 119, 123, 157, 158, 167, 168, 169 | MFO inducing PCBs present at low concentrations in tissues |

In the present study blood samples from Baltic and Svalbard (Arctic) Ringed seals, Baltic and Sable Island (Canada) Grey seals, and UK Harbour seals as well as faecal samples from UK Harbour and Grey seals were collected. For the congener-specific analysis of faecal samples a total of 32 congeners were investigated including most of the 36 PCBs mentioned earlier with some additional congeners (Table 2.3; (*) indicates the additional PCB congeners selected for this study).

**Table 2.3:** PCB congeners included in the study

| IUPAC No. | Homolog Group | Isomer |
|---|---|---|
| 28 | tri- | 2,4,4'- |
| 44 | tetra- | 2,2',3,5'- |
| 52 | | 2,2',5,5'- |
| 70 | | 2,3'4',5- |
| 77 | | 3,3',4,4'- |
| 101 | penta- | 2,2',4,5,5'- |
| 105 | | 2,3,3',4,4'- |
| 110* | | 2,3,3',4',6- |
| 114 | | 2,3,4,4',5- |
| 118 | | 2,3',4,4',5- |
| 123 | | 2',3,4,4',5- |
| 126 | | 3,3',4,4',5- |
| 128 | hexa- | 2,2',3,3',4,4'- |
| 132* | | 2,2',3,3',4,6'- |
| 138 | | 2,2;,3,4,4',5'- |
| 149* | | 2,2',3,4',5',6- |
| 151 | | 2,2',3,5,5',6- |
| 153 | | 2,2',4,4',5,5'- |
| 156 | | 2,3,3',4,4',5- |
| 157 | | 2,3,3',4,4',5'- |
| 163* | | 2,3,3',4',5,6- |
| 167 | | 2,3',4,4',5,5'- |
| 169 | | 3,3',4,4',5,5'- |
| 170 | hepta- | 2,2',3,3',4,4',5- |
| 180 | | 2,2',3,4,4',5,5'- |
| 183 | | 2,2',3,4,4',5',6- |
| 187 | | 2,2',3,4',5,5'6- |
| 190* | | 2,2',3',4,4',5,6- |
| 194 | octa- | 2,2',3,3',4,4',5,5'- |
| 199 | | 2,2',3,3',4,5,5'6'- |
| 206* | nona- | 2,2',3,3',4,4',5,5',6- |
| 209* | deca- | 2,2',3,3',4,4',5,5',6,6'- |

### 2.2.2 DDT

Organochlorine pesticides (OCPs) such as DDT, dieldrin, lindane and others had been extensively used the past decades but their use has decreased in the recent years. DDT [2,2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane or dichlorodiphenyl-trichloroethane (*p,p'*-DDT)] is one of the most used and more persistent compounds of the organochlorine pesticides (Fig.3, Chapter 1). It was widely used during the Second World War to prevent the spread of malaria, typhus and other insect vector-borne diseases. After the war, DDT continued to be used for the control of disease vectors and it was also widely used on a variety of agricultural crops (Weatherley et *al.*, 1997; UNEP, 2000). DDT is semi-volatile, highly stable and insoluble in water (apolar). Its presence in the environment is ubiquitous as a result of atmospheric deposition resulting in contamination of remote pristine areas such as the Arctic (Macdonald, *et al.*, 2000) and its bioaccumulation was first reported in the 1950s (Carson, 1962).

Technical grade DDT which is used as an insecticide, comprises several isomers, the most prevalent and active ingredient being the *p,p'*-DDT form. Other components included is the *p,p'*-DDD (Fig.4, Chapter 1) and nearly inactive *o,p'*-DDT (Metcalf, 1995). The main and most persistent DDT metabolite is *p,p'*-DDE which binds the androgen receptor (AR) and exhibits a broad spectrum of anti-androgenic activities as demonstrated in both *in vivo* and *in vitro* bioassays (Kelce *et al.*, 1995). *p,p'*-DDD, is also known to be an endocrine disruptor but is less persistent.

### 2.2.3 Polybrominated Diphenyl Ethers (PBDEs)

PBDEs are aromatic hydrocarbons with a general formula $C_{12}H_{10-n}Br_nO$, where n = 1-10 (Fig. 5, Chapter 1). They are ubiquitous chemicals with large and global industrial use and many of them are still produced in large volumes. There are 10 possible sites for bromination of the molecule (mono- to decabromodiphenyl) and any particular polybrominated diphenyl is named in accordance with the number of bromine atoms on the two phenyl rings and their positions of substitution (WHO / ICPS, 1994b). PBDE congeners are identified by the IUPAC nomenclature originally invented for denomination of PCB congeners (Ballschmiter and Zell, 1980; Pijnenburg *et al.*, 1995; de Wit, 2002).

Decabromodiphenyl ether (De-BDE) makes up over 80% of the total production of PBDEs whereas PeBDE and OcBDE products constitute about 12% and 6% respectively of the total PBDE production (de Wit, 2002; Darnerud, 2003). They are very stable, resistant, lipophilic compounds with high boiling points that are now finding their way into the terrestrial and marine environment where they bioaccumulate and bioconcentrate through the food chain (de Boer *et al.*, 1998; Rahman *et al.*, 2001; Hale *et al.*, 2003; Hall *et al.*, 2003).

Trace quantities of congeners of different PBDEs have already been detected in biological and sediment samples from remote areas suggesting that they are being distributed world-wide due to long-range atmospheric transport (Jansson *et al.*, 1987; Sellström, 1996; de Boer *et al.*, 1998). Significant PBDE levels have also been reported in marine mammals and especially seals (Table 2.4). The main congeners reported in environmental samples are PBDE 47, 99, 100, 153, 154 and 209 (de Boer *et al.*, 2001).

Laboratory studies have indicated that some PBDE congeners may have endocrine disrupting effects (Byrne *et al.*, 1987) and that they may be hepatotoxic and immunotoxic (Darnerud *et al.*, 2001). PBDEs have been found to affect thyroid hormone transport and metabolism and since thyroid hormones are key components of the endocrine system any effects will have serious consequences for a range of physiological processes (Hallgren *et al.*, 2001; Zhou *et al.*, 2001 and 2002). At the present study five PBDE congeners were investigated and are presented in Table 2.5.

**Table 2.4:** PBDE levels reported in seals in ng/g lipid weight (unless otherwise stated)

| Species | Location | PBDE 47 | PBDE 99 | PBDE 100 | PBDE 153 | ΣPBDE |
|---|---|---|---|---|---|---|
| **Harbour seals** | | | | | | |
| ***Phoca vitulina*** | Eastern England[a] | 560-822 | 115-329 | 51-65 | 36-68 | 990-1070 |
| | NE England[a] | 1780 | 101 | 93 | 18 | 2020 |
| | Baltic Sea[f] | - | - | - | - | 90* |
| | Skagerrak[g] | - | - | - | - | 230* |
| | North Sea[h] | 390-4900 | 42-660 | 25-450 | - | 600-6000* |
| | San Francisco, CA[i] | 1304 | 112 | 87.1 | 107 | 1730 |
| **Grey seals** | | | | | | |
| ***Halichoerus grypus*** | NE England[a] | 366-488 | 26-27 | 10-12 | nd | 415-525 |
| | SW England[a] | 422 | 71 | 16 | 8.7 | 527 |
| | Wales[a] | 126-524 | nd-169 | 7.9-94 | 6.9-92 | 176-1080 |
| | Farne Islands, UK[b] | - | - | - | - | 222.6^ |
| | | | | | | 514.7^^ |
| | Baltic Sea[c] | 308-650 | 40-54 | 38-57 | - | 280-730* |
| **Ringed seals** | | | | | | |
| ***Phoca hispida*** | Baltic Sea[d,g] | 256 | 33 | 61 | - | 320-350* |
| | Svalbard[c] | 47 | 1.7 | 2.3 | - | 51* |
| | Canadian Arctic[e] | - | - | - | - | 25.8-50* |
| **Caspian seals** | | | | | | |
| ***Phoca caspica*** | Azerbaijan[a] | nd-15 | nd | nd | nd | nd-15 |

^, post-weaned pups; ^^, juveniles; * Sum of PBDEs (47, 99, 100); nd, not detected; a, Law *et al.*, 2003 (CEFAS unpublished data, in µg/kg wet weight); b, Hall *et al.*, 2003; c, Sellström *et al.*, 1993a and 1996; d, Haglund *et al.*, 1997; e, Alaee *et al.*, 1999; f, Jansson *et al.*, 1987; g, Andersson and Wartanian, 1992; h, de Boer *et al.*, 1998b; i, She *et al.*, 2002.

**Table 2.5:** PBDE congeners included in the study

| IUPAC No. | Homolog Group | Isomer |
|---|---|---|
| 28 | tri- | 2,4,4'- |
| 47 | tetra- | 2,2',4,4'- |
| 99 | penta- | 2,2',4,4',5- |
| 153 | hexa- | 2,2',4,4',5,5'- |
| 154 | hexa- | 2,2',4,4',5,6'- |

### 2.2.4 Toxic Effects of POPs on Seals

PCBs and organochlorine pesticides have been widely studied in seal populations from a wide range of different locations, from industrialised areas to remote pristine areas. Seals, being apex predators of the marine food chain are of special interest as monitors of trends

and biological effects of contaminants since they significantly exposed to biomagnified levels of persistent, lipophilic organic pollutants such as PCBs, PBDEs and DDT (Aguilar *et al.*, 2002; Law *et al.*, 2002). Aquatic mammals in general, have a poor capacity for detoxifying and excreting organic pollutants due to the low activity or inactivity of hepatic microsomal cytochrome P450 enzymes of xenobiotic metabolism (Troisi and Mason, 1997).

POP tissue concentrations (Table 4, Chapter 1) and severity of resultant toxic effects, vary according to exposure level and physiological factors, such as age, species, sex and nutritional and health status (Addison, 1989). There is substantial evidence that marine mammal populations, especially those inhabiting polluted waters are more susceptible to the toxic effects of POPs (Thomas, *et al.*, 2005). Significant population declines and poor population recovery have been observed in Baltic and Wadden Sea Ringed (*Phoca hispida baltica*) and Grey seal populations over the last fifty years that have been attributed to their high tissue organochlorine burdens (Bergman and Olsson, 1985). Evidence of reproductive failure and disorders (Reijnders, 1980 and 1986; Béland *et al.*, 1993) has been recorded, as well as still births (DeLong *et al.*, 1973), uterine pathological deformities (e.g. stenosis and occlusions) (Helle and Olson, 1976a,b; Reijnders, 1980; Baker, 1989), cancer (Martineau *et al.*, 1994), lesions in numerous organs (Olsson *et al.*, 1994), neuro-toxicity, immunosuppression (increased incidence in epizootic episodes in Northern European seal populations) and hepatotoxicity (Brouwer & van den Berg, 1986; Reijnders, 1986; Brouwer *et al.*, 1989; Safe, 1990; Barret *et al.*, 1992; Hall *et al.*, 1992; Osterhaus *et al.*, 1992; de Swart *et al.*, 1995a; O'Shea, 1999; Dean *et al.*, 2002; Thomas, 2005). Endocrine disruption has also been observed in marine mammals and PCBs and DDT are known EDCs which are able to alter the natural balance of endogenous hormones in tissues and circulating plasma by functioning as hormone agonists or antagonists (Fielden *et al.*, 1997; Portier, 2002). Altered steroid hormone metabolism, altered steroid biosynthesis (Freeman and Sangalang, 1979; Troisi and Mason, 2000) and progesterone antagonism (Gillner et *al.*, 1988; Troisi and Mason, 2000), are some of the organochlorine-induced reproductive effects reported in seals, mediated by an endocrine disrupting mechanism.

## 2.3 Biological Monitoring

*Biological monitoring* is a direct method to determine pollutant exposure and effects in a population, including magnitude of exposure and temporal fluctuation in exposure in

order to identify populations at risk from toxic effects. Successful biomonitoring requires the collection of biological samples from large numbers of individuals of a population that would be representative for population level significance and analysis of pollutant levels and/or biological indicators, *biomarkers*, of pollutant exposure or effects. It provides a direct measure of exposure in target populations and an understanding of exposure burden in individuals which reaches the circulation and target organs, following pollutant biotransformation and partitioning and most importantly provides a quantitative indication of the severity of specific adverse health effects in individuals of an exposed population (Kamrin, 2004). There are two methods used for quantification of organohalogens in biological samples, chromatographic analysis and immunoassays.

## 2.4 Chromatographic analysis of organohalogen pollutants

### 2.4.1 Extraction

The process to determine chemical residues in an environmental or biological sample matrix involves a stepwise analytical procedure. The first step is the extraction of the lipids in which organochlorines are retained, into an organic (apolar) solvent (e.g. hexane) or mixture of solvents (Smeds and Saukko, 2001), usually by soxhlet extraction or liquid-liquid extraction (De Boer, 1999).

As already mentioned earlier, DDT, PCBs and PBDEs are highly lipid soluble and therefore blubber is the most commonly used sample for contaminant analyses. However, blubber samples are difficult to obtain since they are usually only available from animals that have died from natural causes or net entanglements. Collecting samples from live animals is time consuming, expensive and requires home office licence. Faeces and blood samples however, even though they have a much lower lipid content when compared to blubber they still provide a good sample for contaminant analysis. Faecal samples are easy to collect, with minimal disturbance to the seal population and are widely available; therefore they were used at the present study.

Soxhlet extraction, an intense and rigorous form of extraction, is the most widely used method (Van der Hoff and van Zoonen, 1999). It consists of the use of hot solvent and prolonged contact time of the sample with the extraction fluid in a closed system. Numerous solvent systems have been widely used for lipid extraction such as hexane, dichloromethane, or mixtures of solvents (Keefe *et al.*, 1997; de Boer, 2001; Smeds and

Saukko, 2001). At the present study a mixture of hexane and diethyl ether was used for the extraction of lipids from the faecal samples.

### 2.4.2 Clean-up

After extraction, a clean-up method follows to remove co-extractives/impurities and non-target analytes that can be found in the extract together with the contaminants of interest. Many clean-up techniques for separation of PCBs and organochlorine pesticides from the lipid matrix exist and many methods have been developed (Tindle & Stalling, 1972; van der Hoff *et al*., 1996; Cameron *et al*., 1997; Hall *et al*., 1999). At the present study for the POP separation and the lipid removal, normal phase liquid chromatography (HPLC) with a silica column was used.

### 2.4.3 Detection

The last step of the analytical procedure is the detection of target analytes by Gas Chromatography (GC), usually by highly sensitive Electron Capture Detection (GC/ECD) (Errickson, 1997) and/or Mass Spectrometry (GC/MS) in Electron Impact (EI) or Negative Chemical Ionisation (NCI) using Selective Ion Monitoring (SIM) (De Boer, 1999). Chromatographic methods for the analysis of organochlorines are the most accurate available for detecting chemicals such as PCBs, PBDEs and DDT in a variety of samples. ECD is the most widely used detector for GC analysis of PCBs (Erickson, 1997) since it is highly sensitive and selective towards halogenated compounds and therefore it was used at the present study to analyse contaminant levels in seal faecal samples.

## 2.5 Pollutant immunoassays

Even though contaminant analysis by GC is the most accurate, due to the complexity of the biological samples collected from internal tissues and body fluids, it requires special extraction and separation techniques in order to concentrate and isolate analytes for analysis. Moreover, these conventional methods are expensive and time consuming and require specialised equipments (Okuyama, *et al*., 2002). A rapid, non-destructive, high-throughput, more simple and cost-effective method would facilitate analytical measurements and therefore could be useful for immediate determination of exposure. Immunochemical techniques (immunoassays), first developed for clinical chemistry and endocrinology in the 1960s, require small sample volume, satisfy the above criteria and

the past decades have been commonly used for analytical methods in laboratory practice as an efficient screening tool based on poly- or monoclonal antibodies.

Immunoassays, such as Enzyme Linked Immunosorbent Assays (ELISA) are based on highly specific binding of antibodies against foreign substances (antigens) and the final detection can be performed with the reaction of an enzyme linked to antibody or antigen (ELISA or EIA) (Knopp, 1995; Fránek *et al.*, 2001; Troisi & Borjesson, 2005). ELISA techniques are usually based on the analysis of PCBs as Aroclors and not as congeners as with chromatography and therefore are more sensitive to those PCBs with greater similarity to the hapten used to produce the antibody. When analysing biological samples with ELISA the results obtained represent total concentration of PCBs in the sample and therefore it is best to use as standard the main Aroclor expected in the sample (Díaz-Ferrero *et al.*, 1997).

Blood sampling is ethical and non-destructive and because serum levels represent the contaminant load circulating to target organs causing toxic effects it is ideal for exposure assessment. Moreover, enables repeated sampling to monitor exposure over time. At the present study the seal blood samples provided were analysed by ELISA using Aroclor 1254 as standard.

The aim of the present study was to quantify exposure level (PCBs, PBDEs and DDT) of study seal populations using chromatographic and immunoassay techniques;

i) Use of a congener-specific method employing GC-ECD for the quantification of PCBs, PBDEs and ΣDDT concentrations in seal faecal samples
ii) Develop an immunoassay (ELISA) method for the quantification of total PCB concentrations in seal serum samples.
iii) Analyse faecal and blood samples from the three seal species (Harbour, Grey and Ringed).
iv) Determine exposure level in seals under study in order determine whether they are exposed or control populations

## 2.6 Materials and Methods

### 2.6.1 Sampling

Due to strict UK Home office licensing requirements, collection of blood samples from seals in the UK is heavily restricted. Therefore all sampling undertaken within this study was of faecal samples only, for which permission was first obtained from the Nature Reserve Wardens at Blakeney Point (Norfolk Wash), Donna Nook and Tees Estuary. Archival samples provided by collaborators were all collected under UK Home Office project licences that were assigned to each institute.

#### *2.6.1.2. Faecal Samples*

UK seal populations were selected for study on the basis of elevated pollutant exposure due to localised industrial activity and accessibility for sampling. Seal faecal samples were collected from The Norfolk Wash, East Anglia at Blakeney Point (Harbour seal samples) and Donna Nook (Harbour & Grey seal samples), in December 2001 with the permission of the local wardens. There is a resident population of Harbour seals that haul out on the sand banks at these locations. Sampling was undertaken with the support and assistance of staff at the RSPCA Norfolk Wildlife Hospital (East Winch, King's Lynn). Blakeney Point and Donna Nook were selected as suitable sampling points in The Wash, as they are the most accessible sites in the area, particularly during the breeding season (autumn and early winter) when large numbers of Grey and Harbour seals haul out on the sand flats. Collection of samples from Donna Nook was accomplished with the help from Lincolnshire Wildlife Trust and the permission and help of The Ministry of Defence (MoD) since it maintains part of the area as a bombing range close to the haul-out areas. Harbour Seal faecal samples were also collected from the Tees Estuary (north-east England), between August 2000 and January 2002 with permission of the local warden. The Tees seal colony is composed of resident breeding Harbour seals and a smaller proportion of Grey seals that join the population from neighbouring areas from time to time. It was therefore assumed that samples collected would mostly originate from Harbour seals. The faecal samples from the Tees Estuary were collected in collaboration with Durham University, UK.

The species, age and sex of the seals sampled could not be determined because it was not possible to identify the originating seal for each faecal sample; however, it is believed that the samples were originating from adult seals since from personal observation on the field

only adult seals (indicated by body size) were in close proximity of the samples collected. It is also assumed that the volume of the faecal sample originating from an adult seal would be sufficiently higher than from a juvenile or a pup. Moreover, faecal samples originating from pups are expected to be watery in consistency since their diet doesn't consist of fish and therefore no fish bones would be found present in the samples.

After collection, faecal samples were wrapped in hexane-washed aluminium foil and transported in liquid nitrogen to the lab and stored at –70°C until analysis to maintain sample quality. It was not possible to take blood samples from these populations as UK Home office licence approval was not available to the project. Sample information is summarised in Table 2.6.

**Table 2.6:** Summary of faecal sample information for study groups /populations

| | **Study Site / Population** | **Species** | **Sample number (n)** |
|---|---|---|---|
| **Experiment Group** | **n/a** | | 0 |
| **Control Group** | **n/a** | | 0 |
| **Exposed Groups** | | | Sum = 70 |
| *Tees Estuary* | (North-east England) | *Harbour seals* | 47 |
| *Blakeney Point* | (The Norfolk Wash, UK) | *Harbour seals* | 10 |
| *Donna Nook* [a] | (The Norfolk Wash, UK) | *Harbour & Grey Seals* | 13 |

[a]: could not distinguish species of faecal sample collected ; n/a – not available for this study

### *2.6.1.3. Blood (Serum) Samples*

Seal populations were selected for this study on the basis of known/predicted elevated pollutant exposure and availability of archival blood samples, as summarised in Table 2.7. All of the blood samples from the three study species (Ringed, Grey and Harbour seals) were provided by collaborators and were collected from six different seal populations; Baltic and Svalbard (Arctic) Ringed seals, Baltic and Sable Island (Canada) Grey seals and UK Harbour seals from Abertay Sands (Dundee, Scotland) and Moray Firth (Northeast Scotland). Samples from Harbour seals from a control feeding study in which seals were fed fish from the highly contaminated Wadden Sea were also provided.

All blood samples were collected from seals at approximately the same phase of their respective annual reproductive cycle and were available as serum/plasma prepared in the same manner and ranging in volume from 500µl to 1.5ml. Briefly, whole blood collected from venu-puncture in EDTA-treated vacutainers, was centrifuged in the field for 5

minutes at 3000 rpm to obtain serum / plasma, transported on dry ice or liquid nitrogen to the lab and stored at –70°C until analysis to maintain sample quality.

*Experimental Group:* Prof. P. Reijnders provided serum samples from female Harbour seals that were fed fish taken from Wadden Sea and Northeast Atlantic. This group of Harbour seals were sampled at late pregnancy (3-4 weeks before pregnancy termination) and the average of total PCB concentration in the exposed group (n=11) was 25.6µg/g lipid weight and in the control group (n=12) was 5.3µg/g lipid weight (Reijnders, *pers. Comm.*).

*Control Group*: Blood samples provided from Svalbard Ringed Seals (Arctic) and Sable Island Grey seals (Canada) were collected by Dr. M. Nyman, *Finnish Game and Fisheries Research Institute* (Helsinki, Finland). Reference samples from Svalbard (Arctic) were collected during the hunting season according to the local hunting law whereas Grey seals from Sable Island (Canada) were collected with permission of the Department of Fisheries and Oceans, Canada (Nyman, 2003).

*Exposed Group*: Blood samples from UK and Baltic Sea populations were selected for study on the basis of availability of blood samples and elevated pollutant exposure. The blood samples from Ringed, Grey and Harbour seals were all provided by collaborators and originated from six different seal populations (Table 2.7.). Moray Firth (Northeast Scotland) Harbour Seal samples were provided by Dr. Paul Thompson (Lighthouse Research Station, University of Aberdeen), Abertay Sands (Dundee, Scotland) Harbour Seal samples were provided by Dr. Ailsa Hall (Sea Mammal Research Unit, St. Andrews University) and samples from Baltic Ringed and Grey seals were provided by Dr. M. Nyman, and were taken primarily to monitor the health status of the two populations, as part of a government sanctioned research expedition in the Baltic Sea. Both Baltic species were sampled on the ice in the Bothnian Bay, northern Baltic Sea.

**Table 2.7:** Blood sample information for each study population.

| **Population Category** | **Exposed** | **Control** | **Exposed** | **Control** | **Exposed** | **Exposed** | **Experimental Group** | |
|---|---|---|---|---|---|---|---|---|
| **Study Site** | **North Baltic Sea** | **Svalbard** | **North Baltic Sea** | **Sable Island** | **Moray Firth** | **Abertay Sands** | **Exposed** | **Control** |
| | | **European Arctic** | | **Canada** | **North-east Scotland** | **Firth of Tay, Scotland** | | |
| **Species** | Ringed Seals | | Grey Seals | | | Harbour Seals | | |
| | *Phoca hispida baltica* | *Phoca hispida* | *Halichoerus grypus* | | | *Phoca vitulina* | | |
| **Number of Samples** | **23** | **19** | **19** | **19** | **47** | **48** | **11** | **12** |
| **Sex & Age** | | | | | | | | |
| **Females** | **11** | **11** | **10** | **10** | **25** | **17** | **11** | **12** |
| Adult | All | All | All | All | 7 | All | All | All |
| Sub-Adult | - | - | - | - | 4 | - | - | - |
| Juvenile | - | - | - | - | 14 | - | - | - |
| **Males** | **12** | **8** | **9** | **9** | **22** | **31** | **-** | **-** |
| Adult | All | All | All | All | 12 | All | - | - |
| Sub-Adult | - | - | - | - | 2 | - | - | - |
| Juvenile | - | - | - | - | 8 | - | - | - |
| **Year of Sampling** | April to June | April to June | April to June | April to June | 1992-1995 | 1999-2000 | 1988 | 1988 |
| | 1997-1998 | 1996 | 1997-1998 | 1998 | | | | |
| | 2000 | 1998 | 2000 | | | | | |
| | 2002 | | | | | | | |
| **Stage of Reproduction** | | Moulting, Delayed Implantations | | | Estrus, Moulting | All Stages | Late Pregnancy | |

### 2.6.2 Chemicals

#### *2.6.2.1 Chemicals Used for Faecal Analysis*

All solvents used were high-purity glass distilled grade (GDG) purchased from *Rathburn Chemicals Ltd.* (Scotland, UK). Anhydrous sodium sulphate was purchased from *Fluka-Sigma* which prior to use was activated at 500oC for 12h. Individual analytical standards for the PCB congeners under study were purchased from *ChemService* (2ml at 100µg/ml in hexane) and mixed to produce a PCB congener mixture at 1 µg/ml (each PCB congener) in hexane (Table 2.5). Five PBDEs, 2,4,4'- triBDE (28), 2,2',4,4'- tetraBDE (47); 2,2',4,4',5- pentaBDE, 2,2',4,4',5,5'- hexaBDE (153) and 2,2',4,4',5,6'- hexaBDE (154) were purchased as individual standards from *Cambridge Isotope Laboratories* (1.2ml at 50µg/ml in nonane). Dichlorodiphenyl-trichloroethane (4.4'-DDT), dichlorodiphenyl-chloroethane (4,4'-DDE) and dichlorodiphenyl-dichloroethane (4,4'-DDD) standards were purchased from *Fluka-Sigma.* All compounds under study were combined and multi-point calibration mixtures of 1ml were prepared with a total of five levels ranging from 0.1 to 0.005µg/ml (in hexane). 2,4,5,6-Tetrachloro-m-xylene (*Fluka-Sigma*) was added to these mixtures in the concentration of 0.009µg/ml and was used as internal standard (ISTD) for GC analysis. All glassware was hexane washed before use.

#### *2.6.2.2 Chemicals Used for Blood (Serum) Analysis*

Goat anti-rabbit IgG (whole molecule) affinity purified antibody, bovine serum albumin (BSA), Tris-HCl buffered saline tablets, carbonate-bicarbonate buffer capsules and Aroclor 1254 were purchased from Sigma-Aldrich (St. Louis, USA). Nunc C bottom Immunoplate 96 well modules and frames were purchased from *BETHYL Laboratories, Inc.*, (USA). 3,3',5,5'-tetramethyl benzidine (TMB) Peroxidase substrate and Peroxidase Solution B were purchased from *Kirkegaard and Perry* (Gaithersburg, MD). Pentachloro-phenol (PCP)-Horseradish Peroxidase (HRP) and polyclonal rabbit anti-PCB antibody (raised against Aroclor 1254) were purchased from *Abraxis Kits Inc.* (USA).

### 2.6.3 Preparation of Faecal Samples

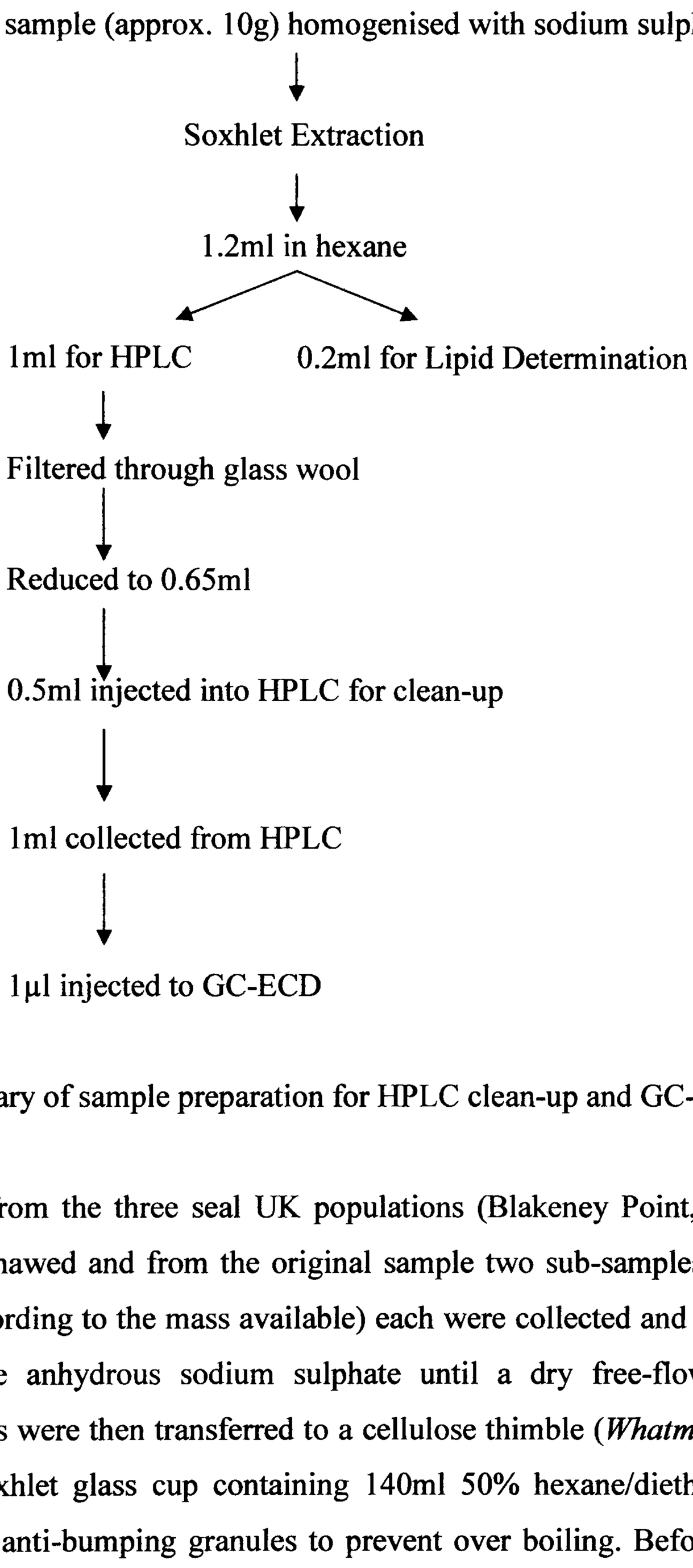



**Figure 2.1** Summary of sample preparation for HPLC clean-up and GC-ECD analysis.

The faecal samples from the three seal UK populations (Blakeney Point, Donna Nook and Tees Estuary) were thawed and from the original sample two sub-samples of approximately 10g (wet weight, according to the mass available) each were collected and were homogenised with analytical grade anhydrous sodium sulphate until a dry free-flowing mixture was obtained. The samples were then transferred to a cellulose thimble (*Whatman)* and inserted in to a pre-weighed Soxhlet glass cup containing 140ml 50% hexane/diethyl ether (v/v) and hexane-washed glass anti-bumping granules to prevent over boiling. Before extraction, each sub-sample was spiked with 100µl of PCB 151 and 100µl of PCB 209 at 1µg/ml and extracted using an automatic Soxhlet extraction system (Soxtherm; *Gerhardt Gmbh*) for ~ 2.5 hrs at

180°C (*details of the soxtherm programme is presented in Appendix*). One blank sample was included with each batch of 12 faecal samples.

After extraction each sample was left to cool to room temperature and excess solvent evaporated under a stream of nitrogen until a final volume of 1.2ml extract was obtained. This extract was divided into two sub-extracts. One sub-sample (200µl) was used to determine the lipid content of the sample gravimetrically and the second sub-sample (1ml) was filtered through a Pasteur pipette containing glass wool. At the end of filtration the glass wool was hexane washed and the filtrate was collected in a vial and a final volume of 0.65ml was recorded. The extracts were then ready for injection into HPLC system for clean-up. A schematic representation of the method is described in figure 2.1.

### 2.6.4 Faecal Sample Clean up

Clean-up methodology for separating target analytes (PCBs, OCPs and PBDEs) was according to the method of Aigreau, 2002 with minor modification. Briefly, extracts were cleaned up using normal phase liquid chromatography (HPLC). The HPLC system consisted of an *Econosphere* preparative column (*Alltech Inc.*, L = 250mm x I.D. = 10mm) packed with 10µm silica which was connected to two HPLC pumps (2150 *LKB Bromma*). One pump was used to deliver the mobile phase (100% hexane) at a flow rate of 4ml/min and the second pump delivered 100% acetone for back-flushing the column at same flow rate. A 0.5ml injection loop with 7125 *Rheodyne* valve was used to make sample injections of 500µl and a second *Rheodyne* valve was used to reverse flow for column-backflushing. Upon elution, a 40ml fraction was collected for each sample and evaporated to near dryness under vacuum using a *Syncore ® Polyvap System* (*Büchi*) set to 40°C and rotary speed 220rpm. After this step, m-xylene (ISTD) was added and the samples were made up to 1ml with hexane. M-xylene final concentration in the samples was 0.009µg/ml. After each run the HPLC column was cleaned (back-flushed) with acetone in order to remove any impurities introduced into the column and it was reconditioned for 10min (flow rate: 2ml/min) with hexane in order to remove acetone residues.

### 2.6.5 Chromatographic Analysis

Methodology for GC analysis of PCBs, PBDEs, DDT and its metabolites was according to the method of Aigreau, (2002). Analyses were carried out using a *Hewlett-Packard* (HP) 5890 Series II Gas Chromatograph (GC) with Electron Capture Detection (ECD), fitted with an HP 7673 autosampler. The fused silica capillary column was a BPX-5 (*SGE*, 5% Phenyl equiv. Polysilphenylene-siloxane; 50m x 0.22mm i.d., 0.25µm film thickness). Hydrogen was used as carrier gas (Head Pressure: 30psi) and nitrogen as make up gas (flow rate: 30ml/min). The injection volume was 1µl. Injector and detector temperatures were held constant at 250°C and 300°C respectively, with the injector set for splitless injection 1min after injection. The oven temperature programme with minor modifications was as follows: 85°C for 1 min, ramped to 210°C at 30°C/min, ramped to 300°C at 3°C/min and held for 15min. Data was analysed using the Varian Star Chromatography Workstation (Version 5.5) software.

To establish the linear working range of the GC-ECD detector, 5 concentrations of standards were prepared to generate calibration curves for each contaminant group (PCBs, PBDE, DDT and its metabolites), down to low (ppb) concentrations. For every 10 samples analysed, one blank sample was included to monitor background contamination of the column. The limit of detection (LOD - the lowest concentration of target compound from the linear calibration curve; Kealey and Haines, 2002), elution order, retention times (RTs), relative (to PCB 209) retention times (RRTs) and calibration curve results for each contaminant group, are presented in Table 2.8. A sample chromatogram showing the elution order and quality of analyte separation is presented in Figure 2.2.

**Table 2.8:** Elution order, RTs, RRTs, LoD and calibration curves for each target analyte

| Elution order | Analyte | RT (min) | RRT | LoD (ng/ml) | Linear Regression Equation | Correlation Coefficient ($R^2$) |
|---|---|---|---|---|---|---|
| 2 | PCB28 | 14.837 | 0.40 | 0.085 | y = 0.5906x + 0.3868 | 0.9947 |
| 3 | PCB 52 | 15.889 | 0.43 | 0.093 | y = 0.4773x + 0.3376 | 0.9922 |
| 4 | PCB 44 | 16.698 | 0.45 | 0.068 | y = 0.6065x + 0.4190 | 0.9935 |
| 5 | PCB 70 | 18.305 | 0.49 | 0.077 | y = 0.6863x + 0.5103 | 0.9945 |
| 6 | PCB 101 | 19.257 | 0.52 | 0.071 | y = 0.6642x + 0.5475 | 0.9909 |
| 7 | 4,4'-DDE | 20.454 | 0.55 | 0.049 | y = 0.072x – 0.7701 | 0.9957 |
| 8 | PCB 110 | 21.014 | 0.57 | 0.060 | y = 0.7001x + 0.4534 | 0.9934 |
| 9 | PCB 77 | 21.38 | 0.57 | 0.163 | y = 1.254x + 1.0536 | 0.9913 |
| 10 | PCB 151 | 21.385 | 0.58 | 0.063 | y = 1.254x + 1.0536 | 0.9913 |
| 11 | PCB 149 | 21.992 | 0.59 | 0.077 | y = 0.6432x + 0.5481 | 0.9900 |
| 12 | PCB 123 | 22.094 | 0.59 | 0.068 | y = 0.7427x + 0.4881 | 0.9951 |
| 13 | PCB 118 | 22.287 | 0.60 | 0.076 | y = 0.7614x + 0.6831 | 0.9918 |
| 14 | 4,4'-DDD | 22.724 | 0.61 | 0.127 | y = 0.5403x – 5.0557 | 0.9910 |
| 15 | PBDE 28 | 22.868 | 0.61 | 0.104 | y = 0.512x + 0.2145 | 0.9954 |
| 16 | PCB 153 | 23.161 | 0.62 | 0.070 | y = 0.8585x + 0.6931 | 0.9917 |
| 17 | PCB 132 | 23.529 | 0.63 | 0.060 | y = 0.731x + 0.5664 | 0.9930 |
| 18 | PCB 105 | 23.725 | 0.64 | 0.067 | y = 0.815x + 0.5562 | 0.9932 |
| 19 | PCB 163 | 24.593 | 0.66 | 0.053 | y = 1.0659x + 0.6805 | 0.9947 |
| 20 | 4,4'-DDT | 24.598 | 0.66 | 39.3 | y = 0.2892x – 2.2184 | 0.9936 |
| 21 | PCB 138 | 24.733 | 0.67 | 0.055 | y = 1.8312x + 1.0976 | 0.9946 |
| 22 | PCB 160 | 25.281 | 0.68 | 0.051 | y = 0.8222x + 0.6264 | 0.9937 |
| 23 | PCB 187 | 25.481 | 0.69 | 0.064 | y = 1.5563x + 0.8899 | 0.9951 |
| 24 | PCB 126 | 25.58 | 0.69 | 0.106 | y = 0.8745x + 0.7190 | 0.9924 |
| 25 | PCB 166 | 26.18 | 0.70 | 0.059* | y = 0.8699x + 0.5882 | 0.9938 |
| 26 | PCB 183 | 26.186 | 0.70 | 0.059* | y = 0.8699x + 0.5882 | 0.9938 |
| 27 | PCB 167 | 26.28 | 0.71 | 0.033** | y = 0.9135x + 0.5864 | 0.9941 |
| 28 | PCB 128 | 26.283 | 0.71 | 0.033** | y = 0.9135x + 0.5864 | 0.9941 |
| 29 | PCB 156 | 27.542 | 0.74 | 0.069 | y = 0.8965x + 0.6723 | 0.9949 |
| 30 | PCB 157 | 27.829 | 0.75 | 0.068 | y = 0.9069x + 0.5436 | 0.9950 |
| 31 | PCB 180 | 228.193 | 6.14 | 0.060 | y = 0.9442x + 0.6341 | 0.9949 |
| 32 | PBDE 47 | 29.072 | 0.78 | 0.139 | y = 0.4463x + 0.1194 | 0.9959 |
| 33 | PCB 169 | 29.684 | 0.80 | 0.113 | y = 0.8355x + 0.4637 | 0.9956 |
| 34 | PCB 170 | 29.953 | 0.81 | 0.029*** | y = 2.2022x + 1.2131 | 0.9948 |
| 35 | PCB 190 | 29.961 | 0.81 | 0.029*** | y = 2.2022x + 1.2131 | 0.9948 |
| 36 | PCB 199 | 30.103 | 0.81 | 0.066 | y = 0.9788x + 0.6851 | 0.9936 |
| 37 | PCB 194 | 33.435 | 0.90 | 0.070 | y = 1.0717x + 0.6306 | 0.9939 |
| 38 | PCB 206 | 35.418 | 0.95 | 0.069 | y = 1.9108x + 1.0575 | 0.9954 |
| 39 | PBDE 99 | 35.623 | 0.96 | 0.206 | y = 0.3386x – 0.0272 | 0.9954 |
| 40 | PCB 209 | 37.186 | 1.00 | 0.077 | y = 1.1201x + 0.7153 | 0.9942 |
| 41 | PBDE 154 | 40.046 | 1.08 | 0.249 | y = 0.2277x – 0.1060 | 0.9900 |
| 42 | PBDE 153 | 43.022 | 1.16 | 0.364 | y = 0.1313x – 0.0828 | 0.9965 |

*, **, *** assessed together due to co-elution

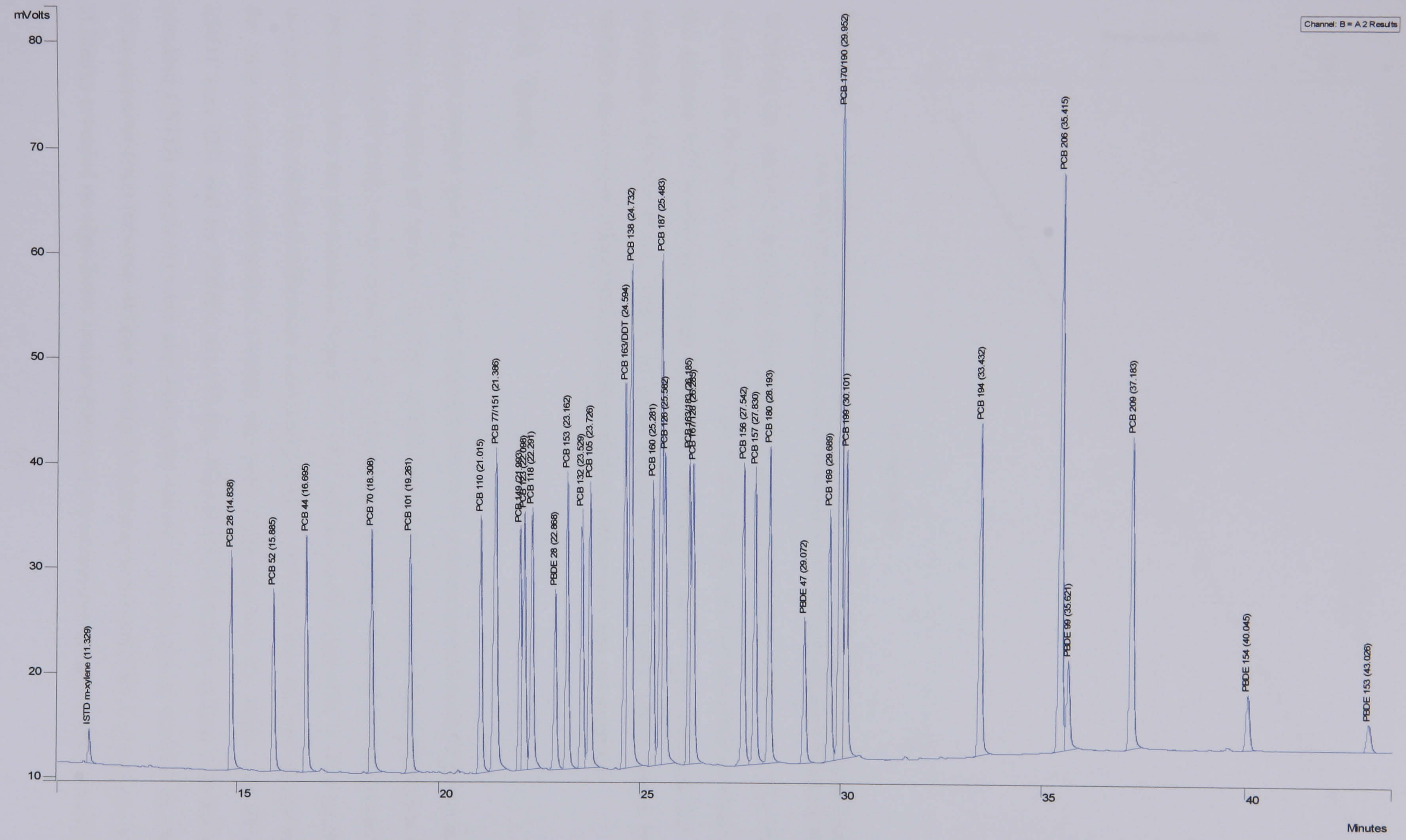



**Figure 2.2** Contaminant mixture at 0.1µg/ml in hexane

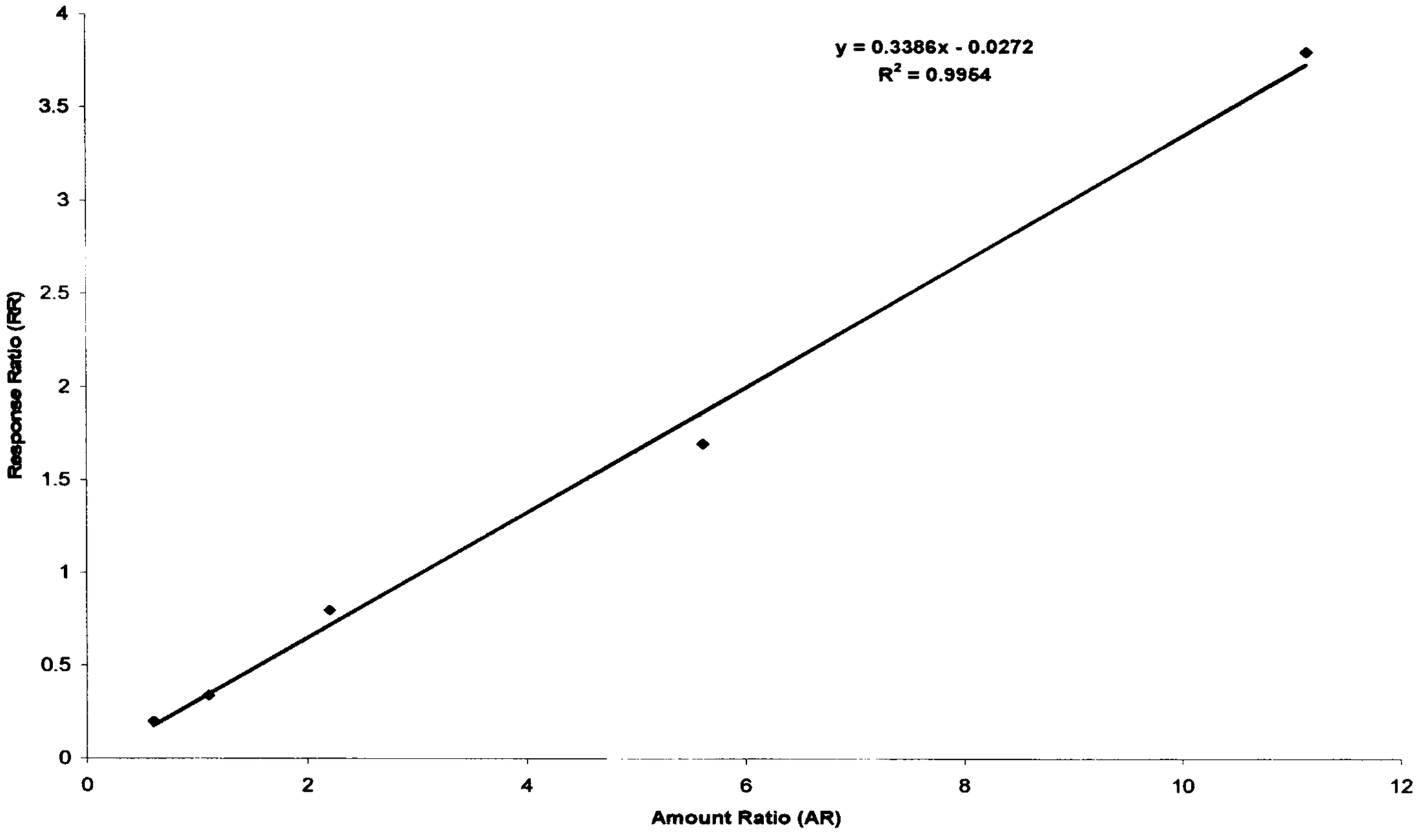



**Figure 2.3** Example of typical calibration plot (PBDE 99) and linear regression equation.
(AR, PBDE 99 concentration / m-xylene concentration; RR, PBDE 99 peak area / m-xylene peak area)

With the GC column employed, five groups of analytes co-eluted; PCBs 77 & 151; PCB 163 & DDT; PCBs 166 & 183; PCBs 167 & 128; PCBs 170 & 190. Despite these co-eluting pairs, the column still performed better than other comparable SE-54 type columns for analyte separation (Schultz *et al.*, 1989). In cases where co-eluting peaks where found in faecal samples results were interpreted for both co-eluting compounds and not separate.

### 2.6.6 QA/QC

To assess analyte-specific recovery through the whole preparation and analysis process, spiked blanks, consisting of sodium sulphate spiked with 1 ml standard mixture (0.01ppm of each analyte under study) were extracted and analysed by GC along with real samples (examples of chromatograms are presented in Figures 8.1, 8.2 and 8.3 in the Appendix I). The results were compared with standard calibration curves analysed on the same day and percentage recovery for each compound determined. Overall, the percentage recovery for PCBs was 97.2%, for ΣDDT was 92% and for PBDEs, was 96.8%. Blanks (0.5ml hexane spiked with the internal standard (ISTD) m-xylene) were also run under identical conditions as samples to check for cross-contamination between samples during Soxhlet extraction and HPLC clean-up. Analyses of blanks revealed no significant contamination of co-extractives using the above method.

Since the lipid weight of each faecal sample was known before being injected in the HPLC column, acetone back-flush efficiency of the HPLC column was also assessed in order to investigate if there was any retention of lipid and other impurities in the column. A volume of 40ml of acetone was found by experimentation, to be sufficient to remove all the lipid and co-extractives. The determination of back-flush efficiency was repeated for every ten seal faecal samples injected into the HPLC system for clean-up. The colouration of back-flush eluate and decrease in HPLC pump pressure following lipid elution was used as visual confirmation that all lipids and co-extractives were completely removed from the HPLC column.

### 2.6.7 PCB Analysis of Blood (Serum) Samples (ELISA)

*Method Development*

To achieve a reliable method for quantification of total PCB concentrations in seal serum the immunoassay method needed to be optimised (possible matrix effects, incubation times, competitor and capture antibody dilutions) although some incubation times and antibody dilutions were already determined by the manufacturer who provided reagents from a soil testing kit for adaptation in this study for serum analyses.

*Development of the Immunoassay*

A competitive enzyme-linked immunosorbent assay (ELISA) was developed in this study to quantify ΣPCB concentrations in serum samples (Fig 2.4). In the assay, the PCP-HRP conjugate competed with unlabeled PCB in the sample/standard for antibody binding sites. Consequently, due to the presence of a colour label on the competing antigen, colour development was inversely proportional to PCB concentration.

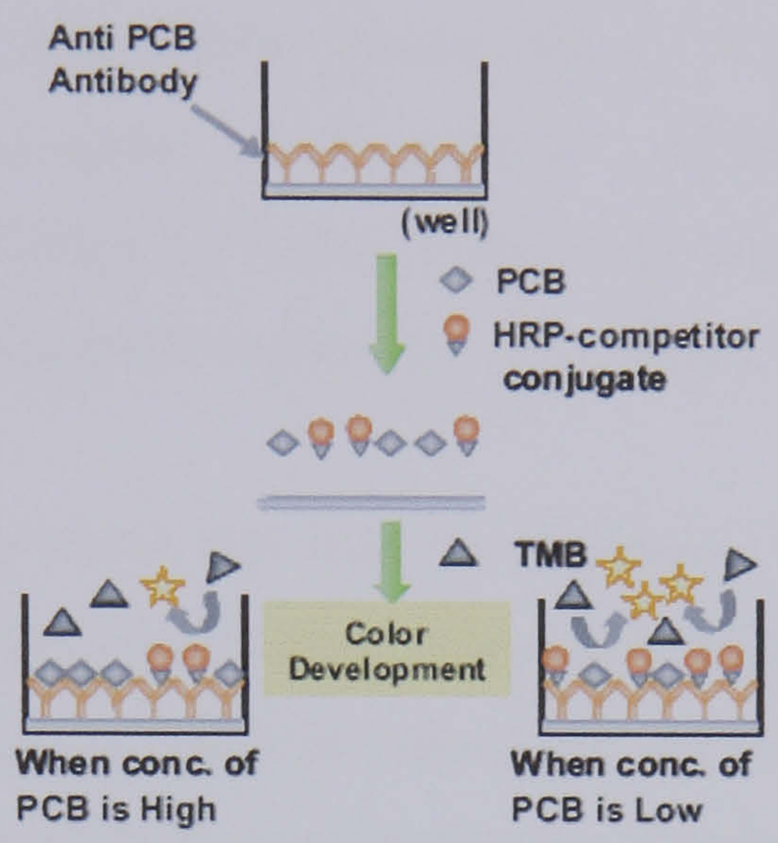



**Fig. 2.4** Format of the competitive PCB ELISA
(*Source*: Amersham Biosciences)

The ELISA method was developed by experimentation with competitor and capture antibody dilutions with guidance from the manufacturer and the final optimal method is described below.

Firstly, each of the 96 wells of the microplate were coated with 200µl goat anti-rabbit IgG (affinity-purified) antibody at a concentration of 10µg/ml in 0.05M sodium carbonate, pH 9.6 and left overnight at room temperature. Following this, the plate was washed three times with 250µl washing buffer (50mM Tris buffered saline with 0.05% Tween 20, pH 8.0). A range of dilutions of PCB standard Aroclor 1254 was prepared from the stock solution at 200µg/ml (in methanol) covering a concentration range of 0.06 – 20 µg/ml. Dilutions were made using 50% methanol/Tris-HCl buffered saline (v/v), 150mM NaCl, pH 7.6 containing 7% BSA, which is added to reduce non-specific adsorption of the analyte to the wells of the microtiter plate (Deng *et al*., 2002). Buffer was added to Aroclor 1254 in order to match the serum matrix. The working dilution of PCB antibody was 1/5,000 diluted with 50mM Tris-HCl buffered saline (pH 7.4), containing 0.1% BSA and PCP-HRP conjugate 1/2,500 diluted with 50mM Tris-HCl buffered saline (pH 7.4) containing 0.1% BSA. These dilutions were optimal due to the resultant absorbance range/colour reaction which was necessary to increase sensitivity of method for detection of low PCB concentrations. Each well was loaded with 50µl of sample or

standard, 50µl PCP-HRP and 50µl of PCB antibody, and the plate was incubated for 1h at room temperature. Following this the plate was washed three times with 250µl washing buffer (50mM Tris buffered saline with 0.05% Tween 20, pH 8.0) and then 150µl of enzyme substrate (TMB in $H_2O_{2,\ v/v}$) added to all wells for colour development. Reaction was terminated by the addition of 100µl 2N $H_2SO_4$ to each well after 20mins. The absorbance was measured at 450nm using a microplate reader (*Bio-Rad*, Model 550).

The Aroclor 1254 calibration curve was used to extrapolate ΣPCB concentrations in each sample using the equation of the linear portion of the calibration curve. The limit of detection of this ELISA was 0.03µg/ml. To minimise inter-plate variation, samples were analysed in triplicate on the same plate and ΣPCB concentrations extrapolated from the calibration curve derived on the same plate. A blank (duplicate) was also analysed with each sample set in order to control any background absorbance attributable to non-specific binding and to avoid over-presentation of the data. Sample ΣPCB concentrations were expressed as mean of triplicate values for each sample.

The standard calibration curve of Aroclor 1254 is presented in Fig. 2.5 ($R^2$ = 0.99, standard deviations ranging from 0.01 to 0.04). The % Coefficient Variance (CV) ranged from 1.19 to 8.93 (<10%). The detection limit (defined as the lowest detectable concentration from the linear curve) was 0.03µg/ml which was sufficient for the quantification of total PCB concentrations in serum.

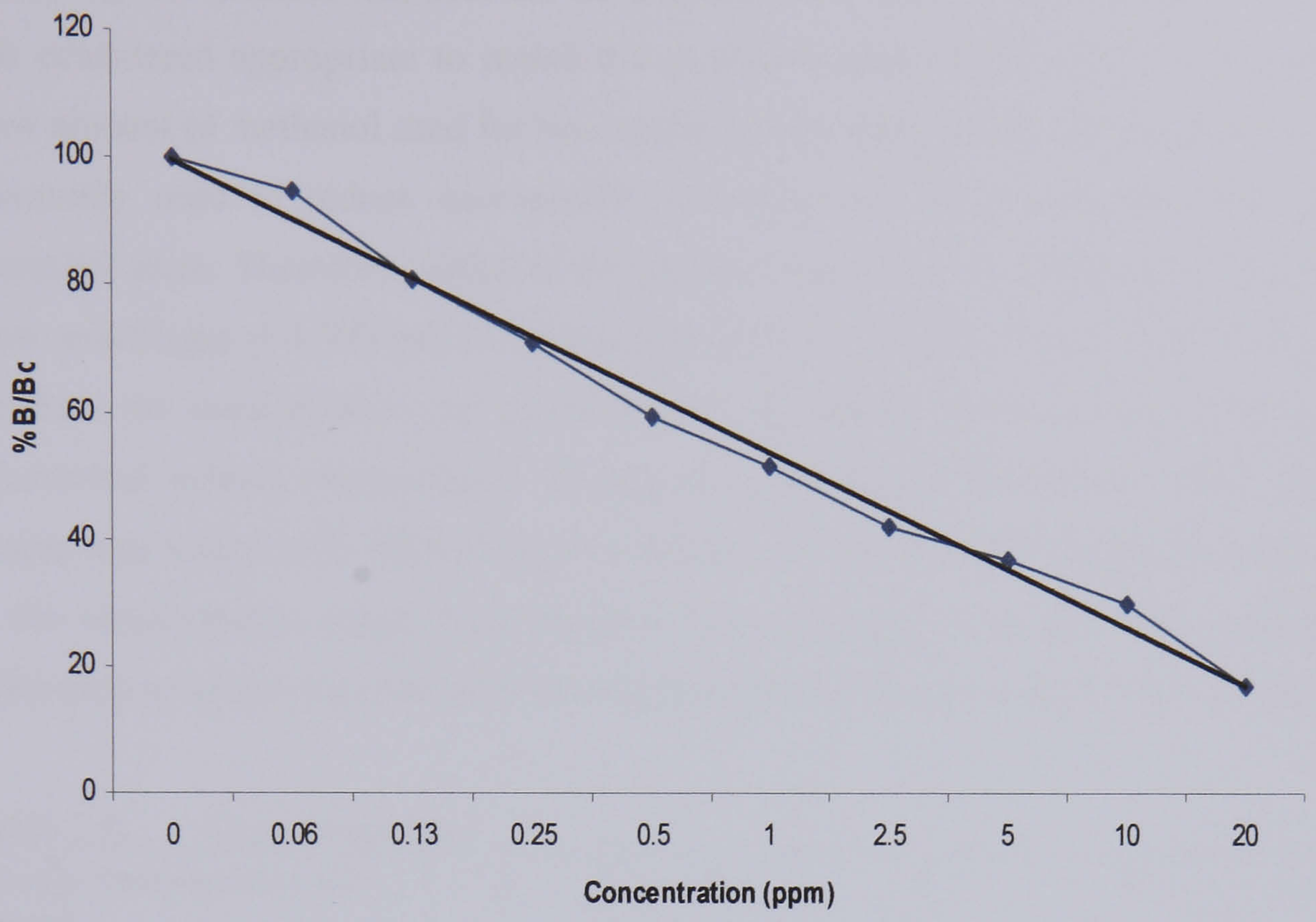



**Figure 2.5:** Standard curve for Aroclor 1254

*Matrix Effects*

To test for matrix effects (interference of other components in the matrix that might influence the absorbance reading by resulting in falsely elevated PCB concentrations in serum samples) two experiments were conducted.

Experiment 1

For the extraction of lipophilic environmental pollutants in matrices such as PCB, non-polar or of low polarity organic solvents are usually required. Since ELISA is usually performed in aqueous phase then an organic solvent that is water-miscible (e.g. methanol) can be directly applied to ELISA analysis. On the other hand, if the solvent is not (e.g. hexane), then before the analysis samples need to be reconstituted in another suitable assay-compatible solvent by evaporation of the water-immiscible solvent (Setford, 2000). In the present study the assay solution used for PCB analysis from serum samples was a mixture of 50% methanol: Tris buffered Saline (7%BSA v/v).This mixture solution was chosen since methanol is a water miscible solvent that has been used in other studies for extraction of PCBs in environmental samples (Deng *et al*., 2002).

Moreover, this mixture was used for the preparation of Aroclor 1254 standard solutions and it was considered appropriate to match the methanol used for the sample extraction with the same amount of methanol used for the standard preparation. BSA was used since it is a protein commonly used to reduce non-specific adsorption of the analyte onto the wells of the microtiter plate. Therefore, 100µl of the solution was added to 100µl seal serum and samples were centrifuged at 4,000 rpm at room temperature for 10mins. This was performed in order to facilitate the precipitation and sedimentation of sample proteins, and to produce a clear protein-free extract (supernatant) for analysis (Troisi and Borjesson, 2005). 50µl of each sample was spiked with 50µl of 5µg/ml Aroclor 1254 and added to 100µl solution, standards in the concentration range 10-0.06µg/ml were prepared with the assay solution and the calibration solutions together with the samples were subjected to the ELISA procedure.

**Table 2.9:** Experiment No.1

| **Sample Concentration (C) (µg/ml)** | **1** | **2** | **3** | **4** | **5** |
|---|---|---|---|---|---|
| **Conc Without Extraction** | 2.11 ± 0.01 | 1.12 ± 0.02 | 4.39 ± 0.04 | 1.32 ± 0.02 | 6.37 ± 0.02 |
| **Conc After Extraction** | 0.37 ± 0.01 | 0.14 ± 0.01 | 0.51 ± 0.01 | 0.23 ± 0.04 | 0.75 ± 0.04 |
| **(C) + 1.25 (1 in 4 dilution)** | 0.40 ± 0.20 | 0.24 ± 0.10 | 0.32 ± 0.2 | 0.53 ± 0.1 | 0.64 ± 0.1 |
| **Expected (C)** | 1.62 | 1.39 | 1.76 | 1.48 | 2 |
| **Recovery (%)** | 24.7 | 17.3 | 18.2 | 35.8 | 32 |

From the % recovery of the samples (Table 2.9) it was apparent that this method of extraction was not working according to the desired results.

Experiment 2

At the same time with experiment No.1, 100µl of the same serum samples used were spiked with 100µl of 5µg/ml (final concentration: 2.5µg/ml) and analysed with the same PCB method (Table 2.10). The % recovery obtained from the second experiment indicated that the interference in the assay by the matrix was negligible and therefore the serum samples were analysed for PCBs without extraction.

**Table 2.10:** Experiment No.2

| **Sample Concentration (C) (µg/ml)** | **1** | **2** | **3** | **4** | **5** |
|---|---|---|---|---|---|
| ***Conc Without Extraction** | 2.11 ± 0.01 | 1.12 ± 0.02 | 4.39 ± 0.04 | 1.32 ± 0.02 | 6.37 ± 0.02 |
| **Conc + 2.5µg/ml (1in 2 dilution)** | 4.52 ± 0.11 | 3.74 ± 0.08 | 6.95 ± 0.09 | 3.78 ± 0.09 | 8.62 ± 0.09 |
| **Expected Concentration** | 4.61 | 3.62 | 6.89 | 3.82 | 8.87 |
| **Recovery (%)** | 98.0 | 103.3 | 100.9 | 99.0 | 97.2 |

*Conc, Concentration

Dilution of the serum samples was not necessary due to the resultant absorbance range/colour reaction; however, when a sample was found to have higher ΣPCB level compared to the highest point of the calibration curve, it was diluted and re-analysed.

The cross-reactivity of the assay antibody against a range of Aroclor standards was determined by the manufacturer (*Abraxis Kits Inc.* (USA) and is presented in Table 2.11. Also other possible cross-reacting compounds were tested by the manufacturer (on the basis of their similarity of molecular structure with PCBs) namely biphenyl, 2,5-dichlorophenol, 2,3,5-trichlorophenol, di-*n*-octyl-phthalate at concentrations up to 10ppm. No detectable cross-reactivity was observed confirming the specificity of the antibody to PCBs.

**Table 2.11:** Cross-reactivity of goat-anti rabbit PCB antibody with a range of Aroclor PCB Mixtures

| Aroclor | LDD (ppb) | 50%B/Bo (ppb) |
|---|---|---|
| 1254 | 0.11 | 9.0 |
| 1260 | 0.35 | 4.4 |
| 1248 | 0.40 | 18 |
| 1242 | 1.3 | 38 |
| 1262 | 0.25 | 4.0 |
| 1232 | 0.60 | 46 |
| 1268 | 0.36 | 20 |
| 1016 | 0.46 | 38 |
| 1221 | 1.6 | 42 |

LDD, least detectable dose which is estimated at 90% B/Bo and at the concentration required to displace 50% (50% B/Bo)
*Source*: *Abraxis Kits Inc.* (USA)

## 2.7 Results

### 2.7.1 Contaminant Concentrations in Seal Samples

For the statistical analysis, the data were divided according to species, geographical location, sex and age. The differences were tested by student t-test and significance levels were set at $p<0.05$ and $p<0.005$. The $p<0.05$ and $p<0.005$ were selected because they are typically used in similar studies and are the norm for biological investigations. They correspond to the 95% and 99.5% confidence levels respectively. Results are presented as mean ± SE and ranges.

All blood samples were analysed for PCB concentrations by ELISA since antibodies for the other groups of contaminants (PBDEs and DDT) were not available. Faecal samples were analysed by GC-ECD and a representative sample chromatogram showing the prominent peaks of the analytes is presented in figure 2.6.

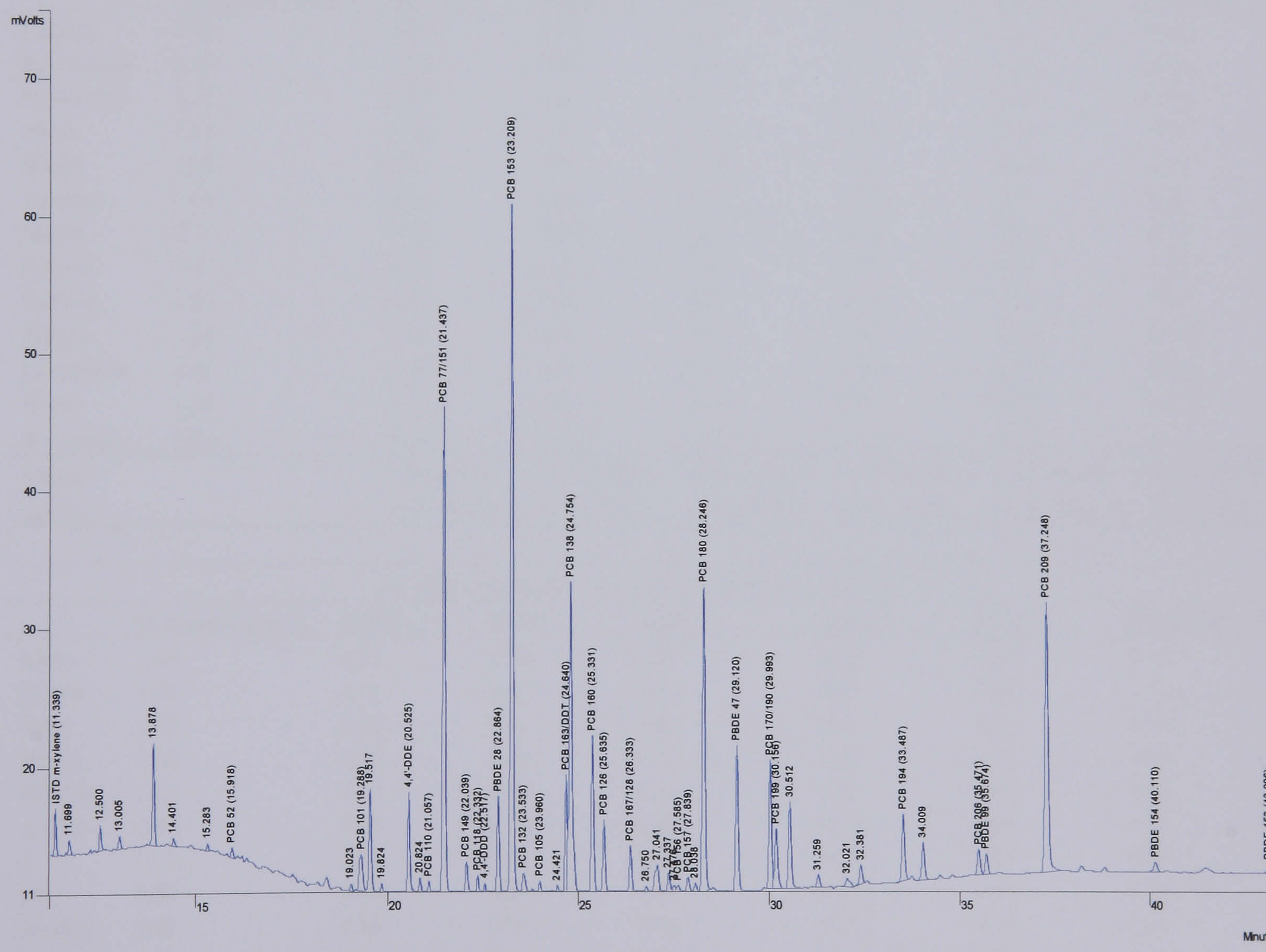



**Figure 2.6:** PCB, ΣDDT and PBDE patterns in a seal faecal sample from the Tees Estuary (UK)

Concentrations of contaminant groups in each seal population are presented in Table 2.12 (faecal samples: a, b, c; blood samples: d, e, f) and the % contribution representing the ΣPCB, ΣDDT and ΣPBDE pattern in faecal samples is presented in figures 2.7, 2.8 and 2.9 respectively. Of the analysed contaminant groups, ΣPCBs ($p<0.005$), DDE ($p<0.05$) and DDT ($p<0.005$) were found to be statistically significant higher in the faecal samples collected from Tees Estuary compared to Blakeney Point. ΣPBDEs were found to be statistically significant lower in Blakeney Point and Donna Nook compared to faecal samples from the Tees Estuary ($p<0.005$). No statistically significant differences between faecal samples collected from Blakeney Point and Donna Nook were observed.

**Table 2.12:** Mean Concentrations of contaminants (µg/g L.wt) in faecal and blood samples for each study population (Results are presented as means ±standard errors (ranges). The significance of geographical variation is presented as * $p<0.05$ or ** $p<0.005$)

**(a) Donna Nook - Harbour (*Phoca vitulina*) and Grey (*Halichoerus grypus*) Seals**

| | % Lipid Content | ΣPCBs | DDE | DDD | DDT | ΣDDT | ΣPBDEs |
|---|---|---|---|---|---|---|---|
| **Charlie** | 0.78 | 0.57 | 0.54 | n.d. | 1.33 | 1.87 | 0.12 |
| **Red Baron** | 6.18 | 0.98 | 0.66 | n.d. | 0.36 | 1.02 | 0.16 |
| **Enola Gay** | 1.22 | 0.45 | n.d. | n.d. | 0.61 | 0.61 | 0.21 |
| **Flash** | 1.82 | 0.19 | n.d. | n.d. | 0.40 | 0.40 | 0.04 |
| **Bikini** | 1.08 | 8.89 | 7.92 | 0.91 | 1.78 | 10.61 | 2.10 |
| **Tommy** | 1.02 | 0.32 | n.d. | n.d. | 0.72 | 0.72 | 0.07 |
| **Mario** | 4.75 | 1.68 | 1.39 | 0.21 | 0.33 | 1.93 | 0.77 |
| **Patriot** | 0.33 | 1.98 | n.d. | n.d. | 4.96 | 4.96 | 0.22 |
| **Spitfire** | 2.43 | 0.05 | n.d. | n.d. | 0.31 | 0.31 | 0.01 |
| **Jaguar** | 1.44 | 0.27 | n.d. | n.d. | 1.05 | 1.05 | 0.002 |
| **Hurricane** | 1.52 | 0.06 | n.d. | n.d. | 0.48 | 0.48 | 0.01 |
| **Rover** | 1.05 | 0.36 | n.d. | n.d. | 0.66 | 0.66 | 0.05 |
| **Jeronimo** | 0.90 | 0.40 | n.d. | n.d. | 1.67 | 1.67 | n.d. |
| **Mean** | | **1.25 ± 0.66** | **2.63 ± 0.98** | **0.56 ± 0.14** | **1.13 ± 0.35** | **2.02 ± 0.79** | **0.31 ± 0.17**** |
| **(n=13)** | | (0.05-8.89) | (n.d.-7.92) | (n.d.-0.91) | (0.31-4.96) | (0.31-10.61) | (n.d.-0.17) |

**(b) Blakeney Point - Harbour Seals (*Phoca vitulina*)**

| | % Lipid Content | ΣPCBs ** | DDE* | DDD | DDT** | ΣDDT | ΣPBDEs** |
|---|---|---|---|---|---|---|---|
| **Larry** | 3.94 | 0.23 | 0.08 | n.d. | 0.39 | 0.47 | 0.14 |
| **Nereis** | 1.58 | 0.11 | n.d. | n.d. | 0.49 | 0.49 | 0.11 |
| **Tray** | 2.48 | 0.20 | n.d. | n.d. | n.d. | n.d. | 0.24 |
| **Wane** | 1.40 | 0.14 | n.d. | n.d. | 0.27 | 0.27 | 0.28 |
| **WWW** | 1.81 | 0.12 | 0.05 | n.d. | n.d. | 0.05 | 0.21 |
| **A (25)** | 0.97 | 0.05 | n.d. | n.d. | 0.44 | 0.44 | 0.12 |
| **J** | 1.17 | 0.50 | n.d. | 0.88 | 0.76 | 1.64 | 0.46 |
| **EE** | 0.92 | 0.44 | n.d. | n.d. | 0.85 | 0.85 | 0.98 |
| **Freddy** | 2.72 | 0.18 | 0.10 | 0.42 | 0.31 | 0.83 | 0.01 |
| **Iris** | 1.43 | 0.14 | n.d. | n.d. | 0.49 | 0.49 | 0.11 |
| **Mean** | | **0.21 ± 0.05** | **0.08 ± 0.01** | **0.65 ± 0.10** | **0.50 ± 0.07** | **0.62 ± 0.15** | **0.27 ± 0.09** |
| **n=10** | | (0.05-0.5) | (n.d.-0.10) | (n.d.-0.88) | (n.d.-0.85) | (n.d.-1.64) | (0.01-0.98) |

**(c) Tees Estuary - Harbour Seals (*Phoca vitulina*)**

| | % Lipid Content | ΣPCBs | DDE | DDD | DDT | ΣDDT | ΣPBDEs |
|---|---|---|---|---|---|---|---|
| **Daizy** | 3.88 | 0.18 | n.d. | 0.60 | n.d. | 0.60 | 0.17 |
| **Eleanor** | 5.96 | 0.27 | 0.10 | 0.18 | 0.25 | 0.54 | 0.06 |
| **George** | 5.01 | 0.28 | 0.08 | 0.24 | 0.18 | 0.49 | 0.10 |
| **Hana** | 2.63 | 0.75 | 0.47 | 0.20 | 0.15 | 0.82 | 0.21 |
| **Jeremy** | 5.90 | 0.40 | 0.47 | 0.20 | 0.13 | 0.80 | 0.20 |
| **Katie** | 2.86 | 1.05 | 0.65 | 0.39 | 0.28 | 1.32 | 0.17 |
| **Mary** | 1.47 | 0.41 | 0.35 | n.d. | 0.58 | 0.92 | 0.39 |
| **Oasis** | 1.40 | 0.38 | n.d. | 1.51 | 1.09 | 2.60 | 0.17 |
| **Queen** | 5.16 | 0.37 | 0.03 | n.d. | 0.34 | 0.37 | 0.40 |
| **Ringo** | 0.74 | 0.12 | n.d. | n.d. | 0.49 | 0.49 | 0.07 |
| **Star** | 0.59 | 1.63 | 1.77 | n.d. | 1.53 | 3.29 | 1.70 |
| **Uranus** | 0.47 | 0.51 | n.d. | n.d. | 1.59 | 1.59 | 3.50 |
| **Venus** | 2.30 | 0.51 | 0.34 | 0.48 | 0.35 | 1.16 | 0.33 |
| **Becky** | 8.05 | 0.06 | 0.07 | 0.15 | 0.09 | 0.31 | 0.25 |
| **15/09 (1)** | 0.58 | 8.90 | 3.79 | 0.96 | 2.97 | 7.73 | 3.45 |
| **15/09 (3)** | 0.59 | 0.89 | n.d. | 0.95 | 1.32 | 2.28 | 0.41 |
| **A** | 0.97 | 0.20 | 0.38 | n.d. | n.d. | 0.38 | 0.20 |
| **B** | 0.55 | 2.19 | 0.13 | n.d. | 1.03 | 1.16 | 0.30 |
| **C** | 0.09 | 1.88 | n.d. | n.d. | 7.25 | 7.25 | 11.85 |
| **D** | 0.31 | 1.25 | n.d. | n.d. | 2.47 | 2.47 | 1.26 |
| **E** | 0.42 | 0.59 | n.d. | n.d. | 1.88 | 1.88 | 2.73 |
| **F** | 3.83 | 0.44 | 0.06 | 0.28 | 0.19 | 0.54 | 0.09 |
| **G** | 0.12 | 1.42 | n.d. | 13.28 | n.d. | 13.28 | 14.42 |
| **H** | 0.30 | 0.34 | n.d. | n.d. | 2.41 | 2.41 | 2.00 |
| **K** | 0.49 | 2.35 | n.d. | 2.28 | 1.64 | 3.92 | 3.83 |
| **L** | 0.52 | 0.04 | n.d. | n.d. | 1.35 | 1.35 | 0.59 |
| **M** | 1.40 | 1.59 | 2.61 | 0.38 | 0.83 | 3.82 | 0.34 |
| **N** | 0.44 | 0.72 | n.d. | n.d. | 1.89 | 1.89 | 2.31 |
| **O** | 0.54 | 0.47 | n.d. | n.d. | 1.29 | 1.29 | 0.83 |
| **P** | 0.27 | 3.49 | 0.48 | n.d. | 2.45 | 2.93 | 1.31 |
| **Q** | 0.66 | 0.52 | n.d. | n.d. | 1.17 | 1.17 | 0.11 |
| **R** | 2.12 | 0.63 | 0.65 | n.d. | 0.47 | 1.12 | 0.18 |
| **S** | 0.24 | 2.60 | n.d. | n.d. | 3.54 | 3.54 | 2.07 |
| **T** | 0.59 | 8.90 | 3.37 | 1.26 | 2.76 | 7.38 | 2.18 |
| **U** | 1.04 | 1.35 | 0.47 | n.d. | 0.77 | 1.24 | 0.61 |
| **W** | 0.07 | 1.30 | n.d. | n.d. | 10.35 | 10.35 | 9.06 |
| **Y** | 0.15 | 1.55 | n.d. | n.d. | n.d. | n.d. | 0.81 |
| **Z** | 0.30 | 1.27 | n.d. | 1.59 | 2.74 | 4.32 | 0.78 |
| **AA** | 0.25 | 1.65 | n.d. | 11.53 | 7.57 | 19.10 | n.d. |
| **BB** | 0.23 | 3.00 | 7.93 | n.d. | 3.83 | 11.76 | 5.45 |
| **DD** | 0.60 | 0.43 | n.d. | n.d. | n.d. | n.d. | 1.43 |
| **FF** | 0.35 | 2.28 | n.d. | 2.58 | 2.22 | 4.80 | 5.14 |
| **GG** | 0.13 | 3.94 | n.d. | n.d. | 0.35 | 0.35 | 5.57 |
| **HH** | 1.25 | 0.16 | n.d. | 0.49 | 0.41 | 0.89 | 0.17 |
| **II** | 0.67 | 0.30 | 0.45 | n.d. | 1.15 | 1.61 | 0.35 |
| **JJ** | 0.52 | 0.55 | n.d. | n.d. | 0.73 | 0.73 | 0.32 |
| **KK** | 0.15 | 6.85 | 1.31 | n.d. | 1.91 | 3.22 | 0.85 |
| **Mean (n=47)** | | **1.51 ± 0.29** (0.04-8.90) | **1.18 ± 0.27** (n.d.-7.93) | **1.98 ± 0.53** (n.d.-13.28) | **1.81 ± 0.31** (n.d.-10.35) | **3.14 ± 0.57** (n.d.-19.10) | **1.31 ± 0.45** (n.d.-14.42) |

(d) **Harbour Seals** (*Phoca vitulina*)

| **Abertay Sands** | | | | **Moray Firth** | | | | | | | | **Exptal (C)** | | **Exptal (E)++** | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Adult (M)* | | *Adult (F)* | | *Adult (M)* | | *Juvenile (M)* | | *Adult (F)++* | | *Juveniles (F)* | | *Adult (F)++* | | *Adult (F)* | |
| **20** | 8.15 | **17** | 2.35 | **14** | 12.16 | **40** | 8.63 | **10** | 21.29 | **49** | 20.33 | **2C** | 6.31 | **1E** | 11.80 |
| **25** | 2.92 | **26** | 1.29 | **16** | 6.93 | **41** | 4.10 | **17** | 8.78 | **50** | 7.36 | **6C** | 6.09 | **3E** | 14.37 |
| **51** | 8.08 | **56** | 18.22 | **26** | 12.09 | **24** | 3.15 | **4** | 15.40 | **47** | 21.93 | **8C** | 3.97 | **4E** | 4.82 |
| **48** | 5.96 | **39** | 14.30 | **28** | 9.98 | **31** | 7.60 | **3** | 14.13 | **48** | 3.60 | **10C** | 4.29 | **5E** | 4.74 |
| **45** | 7.84 | **29** | 2.48 | **2** | 11.87 | **33** | 3.72 | **27** | 5.11 | **22** | 36.54 | **11C** | 5.55 | **7E** | 17.82 |
| **38** | 18.22 | **34** | 3.93 | **5** | 22.22 | **38** | 25.23 | **6** | 5.43 | **34** | 14.97 | **14C** | 4.32 | **9E** | 23.81 |
| **54** | 19.80 | **15** | 2.24 | **11** | 23.70 | **36** | 5.76 | **21** | 6.86 | **35** | 26.23 | **15C** | 2.29 | **12E** | 7.71 |
| **55** | 17.37 | **42** | 12.52 | **15** | 21.37 | **46** | 2.43 | **1** | 9.26 | **29** | 4.17 | **16C** | 4.09 | **13E** | 6.38 |
| **37** | 5.78 | **21** | 2.25 | **19** | 9.88 | | | **9** | 15.24 | **45** | 1.97 | **18C** | 2.62 | **17E** | 4.16 |
| **7** | 3.72 | **8** | 2.18 | **23** | 7.73 | | | **20** | 11.30 | **37** | 2.85 | **19C** | 9.51 | **21E** | 15.52 |
| **33** | 2.46 | **24** | 11.82 | **25** | 6.56 | | | **7** | 3.69 | **42** | 6.48 | **20C** | 4.40 | **22E** | 29.22 |
| **6** | 2.45 | **28** | 3.20 | **32** | 6.45 | | | | | **30** | 31.51 | **23C** | 5.60 | | |
| **9** | 16.66 | **49** | 14.29 | **18** | 20.65 | | | | | **39** | 9.42 | | | | |
| **11** | 17.58 | **31** | 27.49 | **13** | 21.48 | | | | | **43** | 14.29 | | | | |
| **46** | 24.54 | **22** | 9.67 | | | | | | | | | | | | |
| **47** | 19.69 | **4** | 2.46 | | | | | | | | | | | | |
| **16** | 2.11 | **10** | 12.08 | | | | | | | | | | | | |
| **52** | 16.97 | | | | | | | | | | | | | | |
| **40** | 10.87 | | | | | | | | | | | | | | |
| **1** | 4.31 | | | | | | | | | | | | | | |
| **43** | 4.22 | | | | | | | | | | | | | | |
| **44** | 7.33 | | | | | | | | | | | | | | |
| **27** | 8.84 | | | | | | | | | | | | | | |
| **35** | 3.97 | | | | | | | | | | | | | | |
| **53** | 4.90 | | | | | | | | | | | | | | |
| **5** | 0.68 | | | | | | | | | | | | | | |
| **50** | 17.00 | | | | | | | | | | | | | | |
| **12** | 4.42 | | | | | | | | | | | | | | |
| **13** | 7.06 | | | | | | | | | | | | | | |
| **41** | 20.12 | | | | | | | | | | | | | | |
| **18** | 5.71 | | | | | | | | | | | | | | |

**Table 2.12 (d)** *(continued)*

| (d) | Harbour Seals (Phoca vitulina) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Abertay Sands | | Moray Firth | | | | Exptal (C) | Exptal (E)++ |
| | *Adult (M)* | *Adult (F)* | *Adult (M)* | *Juvenile (M)* | *Adult (F)++* | *Juveniles (F)* | *Adult (F)++* | *Adult (F)* |
| **n** | **31** | **17** | **14** | **8** | **11** | **14** | **12** | **11** |
| **Mean (µg/ml)** | **9.67 ± 1.23** | **8.40 ± 1.81** | **13.79 ± 1.76** | **7.58 ± 2.64** | **10.59 ± 1.64** | **14.40 ± 3.02** | **4.92 ± 0.55** | **12.76 ± 2.52** |
| **Range** | **(0.68-24.54)** | **(1.29-27.49)** | **(6.45-23.70)** | **(2.43-25.23)** | **(3.69-21.29** | **(1.97-36.54)** | **(2.29-9.51)** | **(4.16-29.22)** |

| (e) | Grey Seals (*Halichoerus grypus*) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **Baltic Sea** | | | | **Sable Island, Canada** | | | |
| | *Adult (M)* +* | | *Adult (F)++* | | *Adult (M)+* | | *Adult (F)* | |
| | **H4-97** | 54.68 | **H1-98** | 14.97 | **C1-98** | 10.09 | **C4-98** | 2.28 |
| | **H6-97** | 8.11 | **H2-98** | 6.71 | **C2-98** | 6.21 | **C8-98** | 3.44 |
| | **H11-97** | 21.07 | **H3-98** | 8.59 | **C3-98** | 24.05 | **C9-98** | 0.36 |
| | **H13-97** | 53.56 | **H4-98** | 39.73 | **C5-98** | 0.3 | **C10-98** | 0.8 |
| | **H5-98** | 87.06 | **H1-00** | 6.04 | **C6-98** | 0.67 | **C11-98** | 0.44 |
| | **H6-98** | 38.63 | **H3-00** | 9.73 | **C7-98** | 6.87 | **C12-98** | 4.89 |
| | **H7-98** | 82.44 | **H5-00** | 2.35 | **C14-98** | 0.72 | **C13-98** | 0.51 |
| | **H2-00** | 7.1 | **H6-00** | 4.58 | **C15-98** | 10.34 | **C16-98** | 0.75 |
| | **H4-00** | 8.94 | **H7-00** | 3.95 | **C19-98** | 49.99 | **C17-98** | 3.34 |
| | | | **H8-00** | 37.96 | | | **C18-98** | 1.07 |
| **n** | **9** | | **10** | | **9** | | **10** | |
| **Mean (µg/ml)** | 40.18 ± 10.43 | | 13.46 ± 4.38 | | 12.14 ± 5.33 | | 1.79 ± 0.51 | |
| **Range** | (7.10-87.06) | | (2.35-39.73) | | (0.30-16.00) | | (0.36-4.89) | |

| (f) | Ringed Seals (*Phoca hispida*) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **Baltic Sea** | | | | **Svalbard, European Arctic** | | | |
| | *Adult (M)* | | *Adult (F)++* | | *Adult (M)++* | | *Adult (F)++* | |
| | **N8-97** | 17.72 | **N3-99** | 23.75 | **S6-96** | 2.38 | **S10-96** | 0.509 |
| | **N9-97** | 45.76 | **N2-00** | 12.1 | **S12-96** | 6.95 | **S11-96** | 1.513 |
| | **N10-97** | 25.34 | **N6-00** | 8.01 | **S13-96** | 1.91 | **S15-96** | 6.484 |
| | **N6-98** | 13.98 | **N7-00** | 10.39 | **S17-96** | 3.84 | **S16-96** | 0.862 |
| | **N7-98** | 17.84 | **N1-02** | 14.17 | **S3-98** | 2.04 | **S19-96** | 3.064 |
| | **N10-98** | 14.4 | **N2-02** | 10.86 | **S5-98** | 0.88 | **S1-98** | 1.334 |
| | **N4-00** | 13.78 | **N3-02** | 25.49 | **S6-98** | 1.09 | **S2-98** | 0.468 |
| | **N5-00** | 8.08 | **N4-02** | 18.32 | **S7-98** | 1.36 | **S4-98** | 1.307 |
| | **N8-00** | 13.22 | **N5-02** | 10.5 | | | **S8-98** | 2.186 |
| | **N9-00** | 13.82 | **N8-02** | 10.95 | | | **S9-98** | 1.443 |
| | **N6-02** | 7.32 | **N9-02** | 9.8 | | | **S10-98** | 4.615 |
| | **N7-02** | 6.05 | | | | | | |
| **n** | **12** | | **11** | | **8** | | **11** | |
| **Mean (µg/ml)** | 16.44 ± 3.06 | | 14.03 ± 1.78 | | 2.56 ± 0.71 | | 2.16 ± 0.57 | |
| **Range** | (6.05-45.76) | | (8.01-25.49) | | (0.88-6.95) | | (0.47-6.48) | |

n, number of samples; n.d., non detectable; F, females; M, males; Exptal, experimental group; E, exposed; C, control
Results are presented as means ±standard errors (ranges). The significance of sex variation within each species is presented as * $p<0.05$ and the significance of geographical variation within each species is presented as + $p<0.05$ or ++ $p<0.005$.

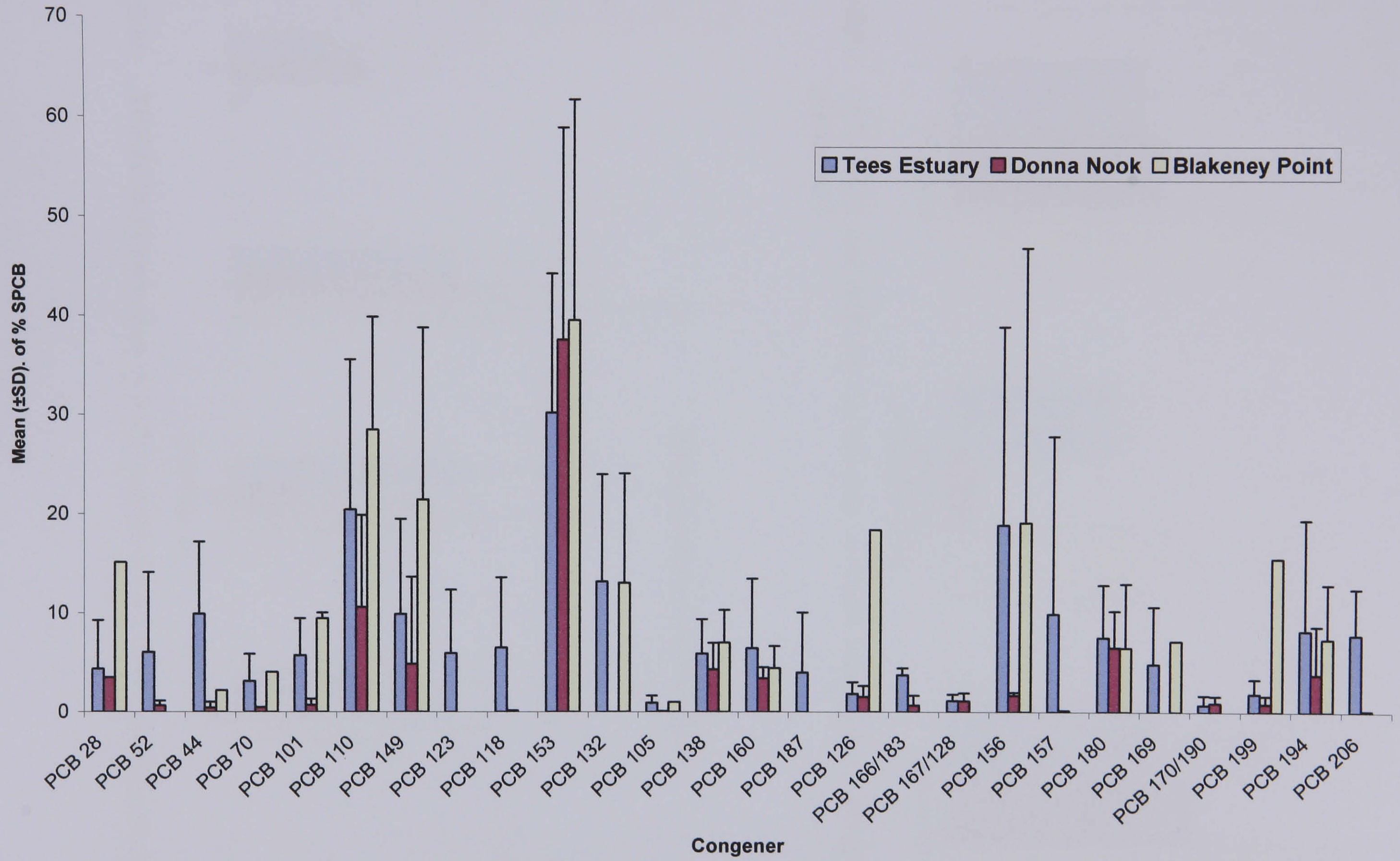



**Figure 2.7:** ΣPCB % Contribution in faecal samples from all three study populations.

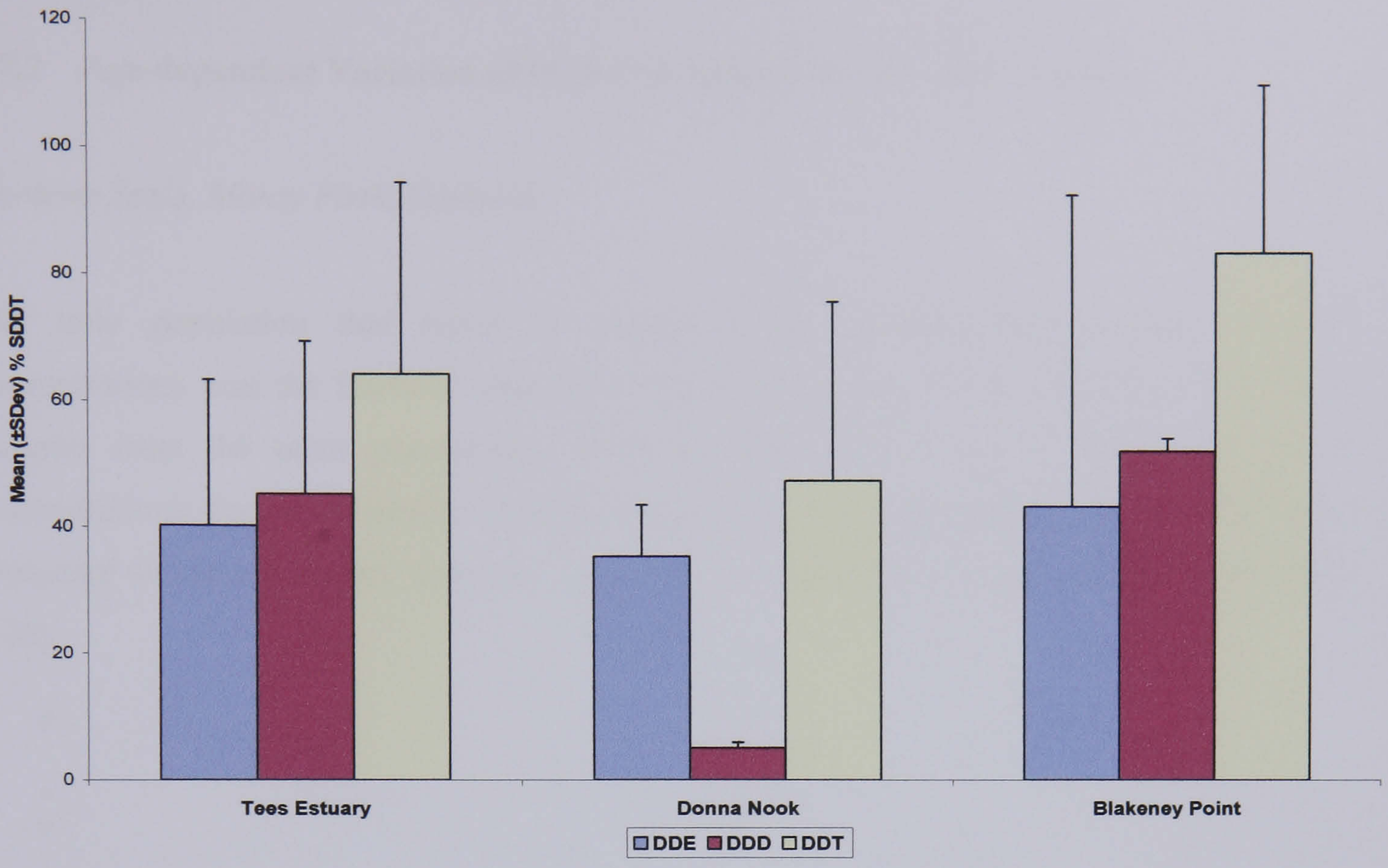



**Figure 2.8:** DDT, DDE and DDD % Contribution in faecal samples from all three study populations.

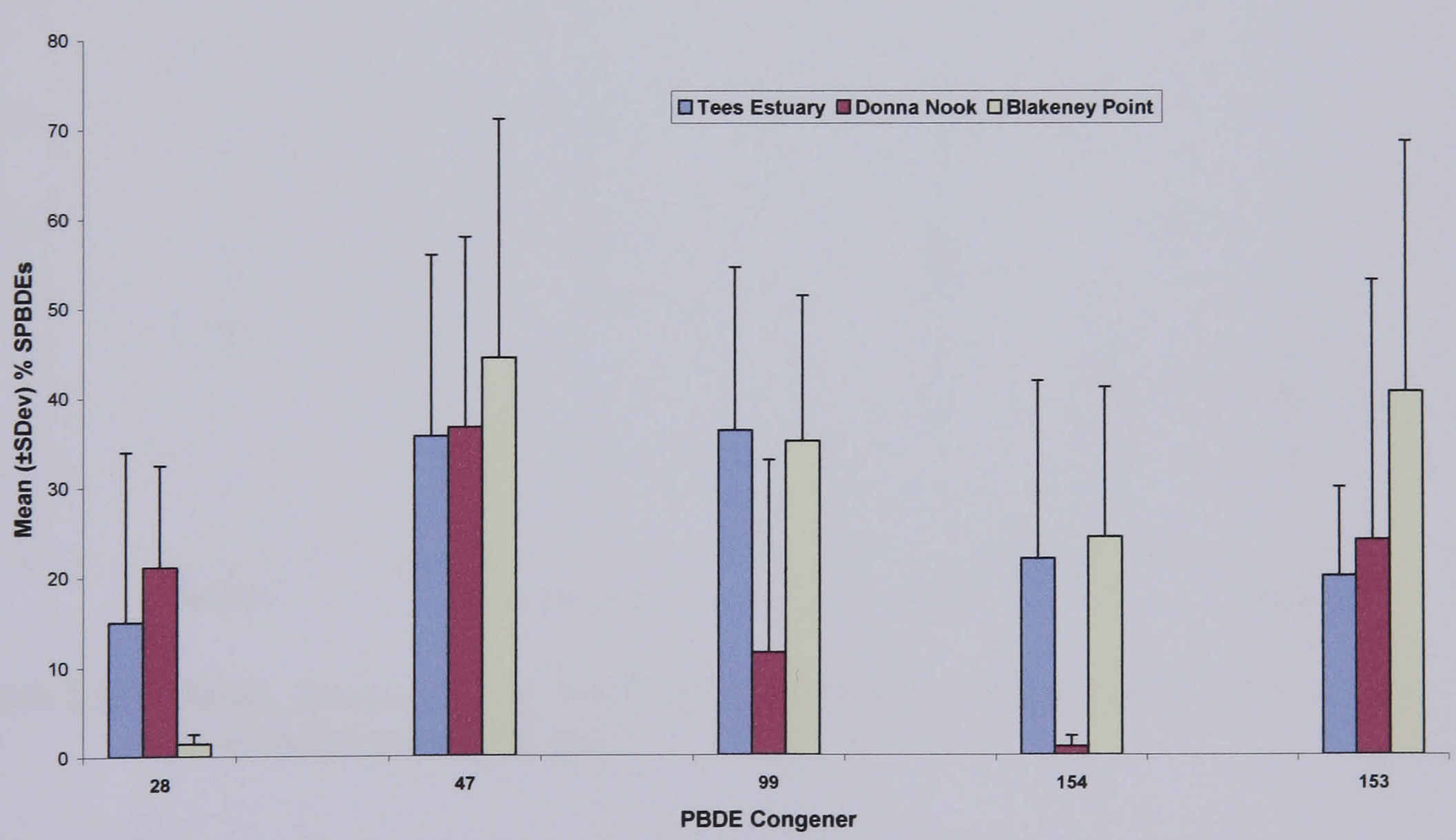



**Figure 2.9:** ΣPBDE % Contribution in faecal samples from all three study populations.

### 2.7.2 Age-dependent Variation of PCB Concentrations in Serum Samples

*Harbour Seals, Moray Firth, Scotland.*

The only population that could be examined for age-dependent variation of PCB concentrations was the Harbour seals from Moray Firth, due to the lack of availability of samples from the other populations. Male juveniles were found to have lower ΣPCB concentrations than male adults, whereas female juveniles had higher concentrations when compared to female adults; however, there was no statistically significant difference (Fig. 2.10).

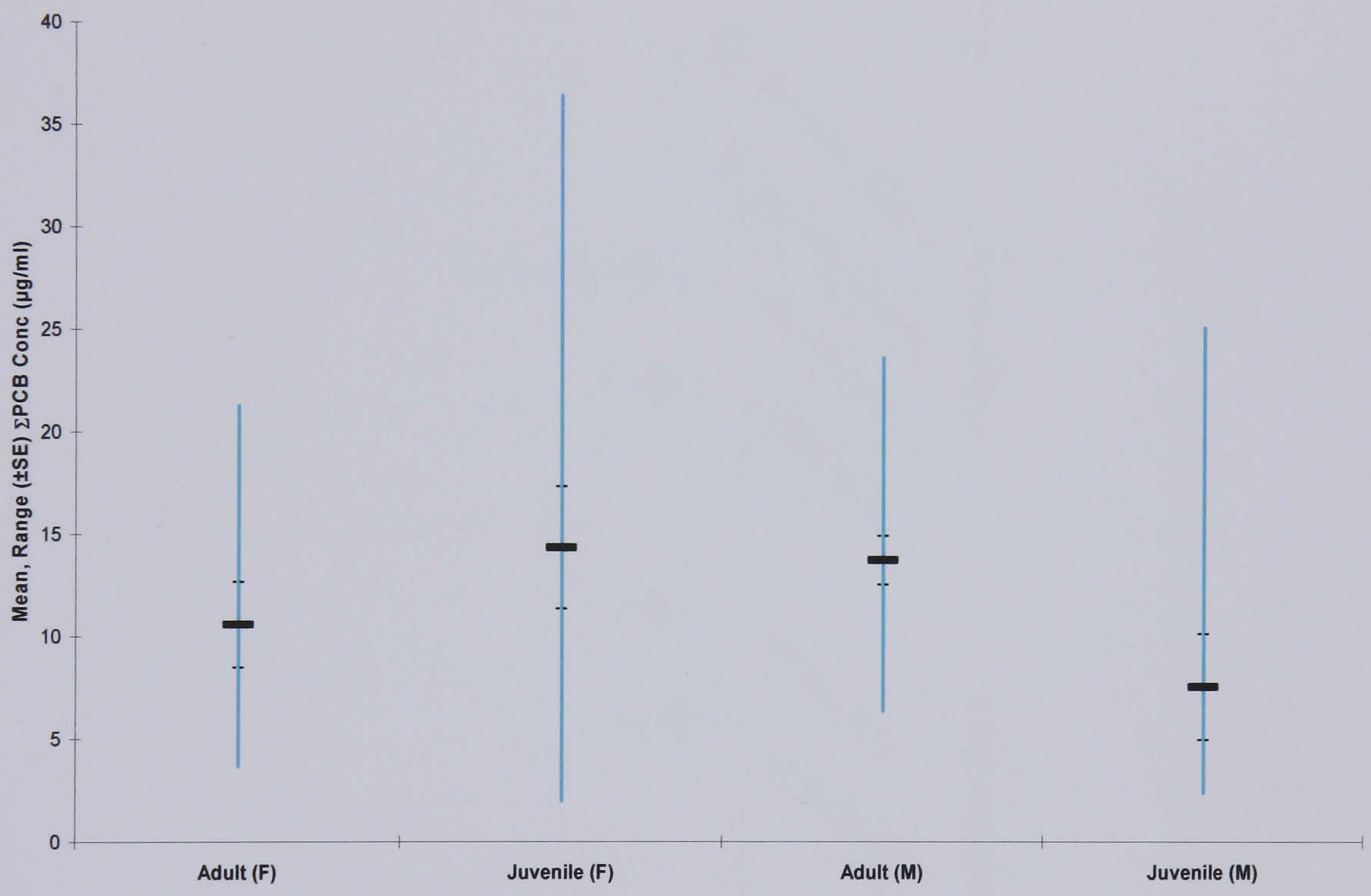



**Figure 2.10:** Mean, Range ±SE of ΣPCB concentrations in Harbour seals (Moray Firth, Scotland) F, Females; M; Males

### 2.7.3 Sex-dependent Variation of PCB Concentrations in Serum Samples

Sex-dependent differences were only looked at for blood samples since the identification of the origin of faecal samples was not feasible at the time of collection.

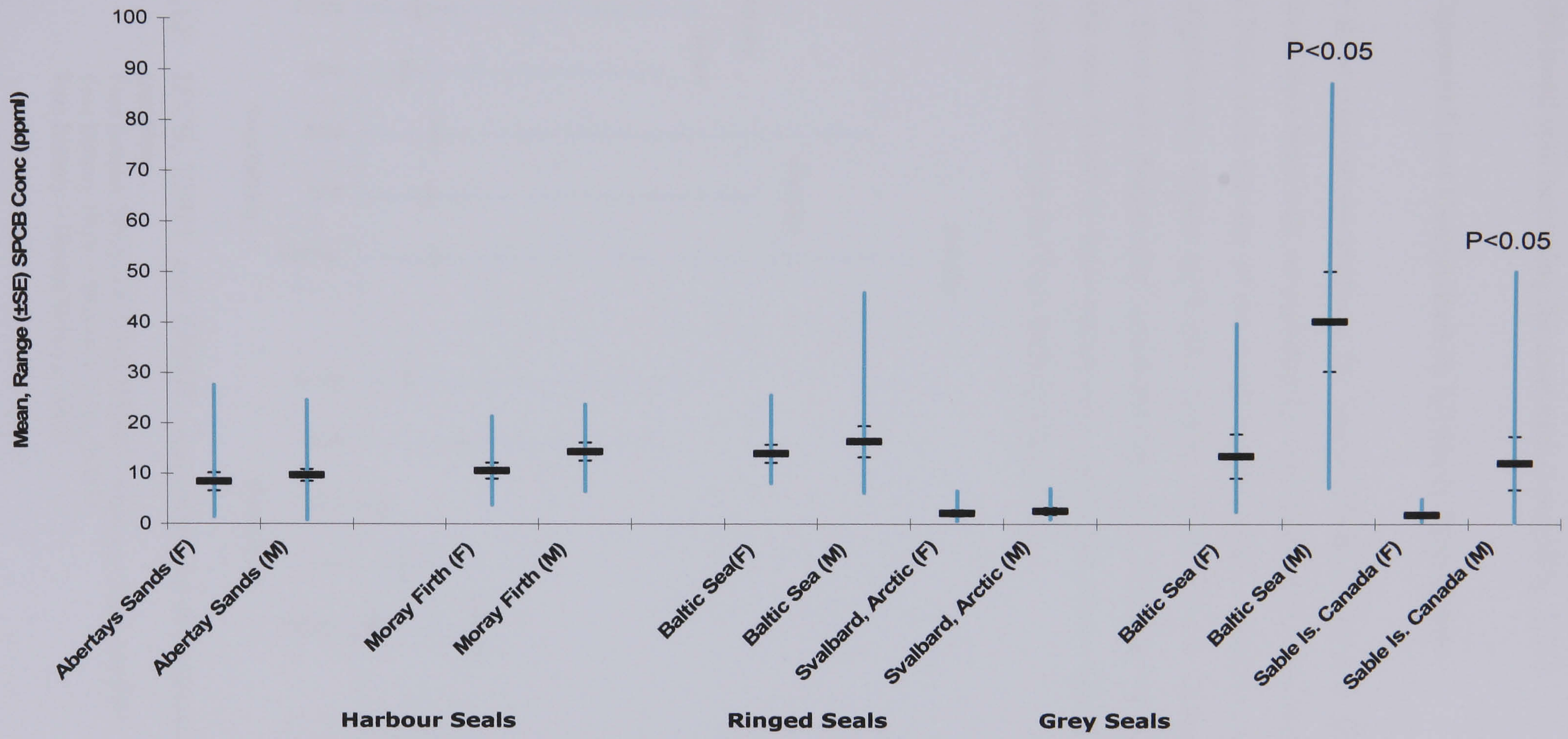



**Figure 2.11:** Sex-dependent variation in ΣPCB concentrations in study populations
F, Females; M; Males
Grey Baltic M > Grey Baltic; $p<0.05$
Grey Sable Island M > Grey Sable Island F; $p<0.05$

Statistically significant difference between genders was only observed among the Grey seals from both Baltic Sea and Sable Island, where males showed higher ΣPCB levels ($p<0.05$) although the trend was increasing for most seal groups (Fig. 2.11).

### 2.7.4 Exposure Level Comparison in All Study Populations

From the three populations analysed for faecal ΣPCB, ΣDDT and ΣPBDE concentrations it was observed that there was no statistically significant difference between Donna Nook and Blakeney Point seals for any of the contaminant groups. However, seals from Tees Estuary showed significantly higher ($p<0.005$) ΣPCB, DDT, ΣPBDE concentrations compared to Blakeney Point and statistically significant increased DDE ($p<0.05$) concentrations. Donna Nook seals were found to have statistically significant lower ΣPBDE concentrations when compared with seals from the Tees Estuary (Fig. 2.12).

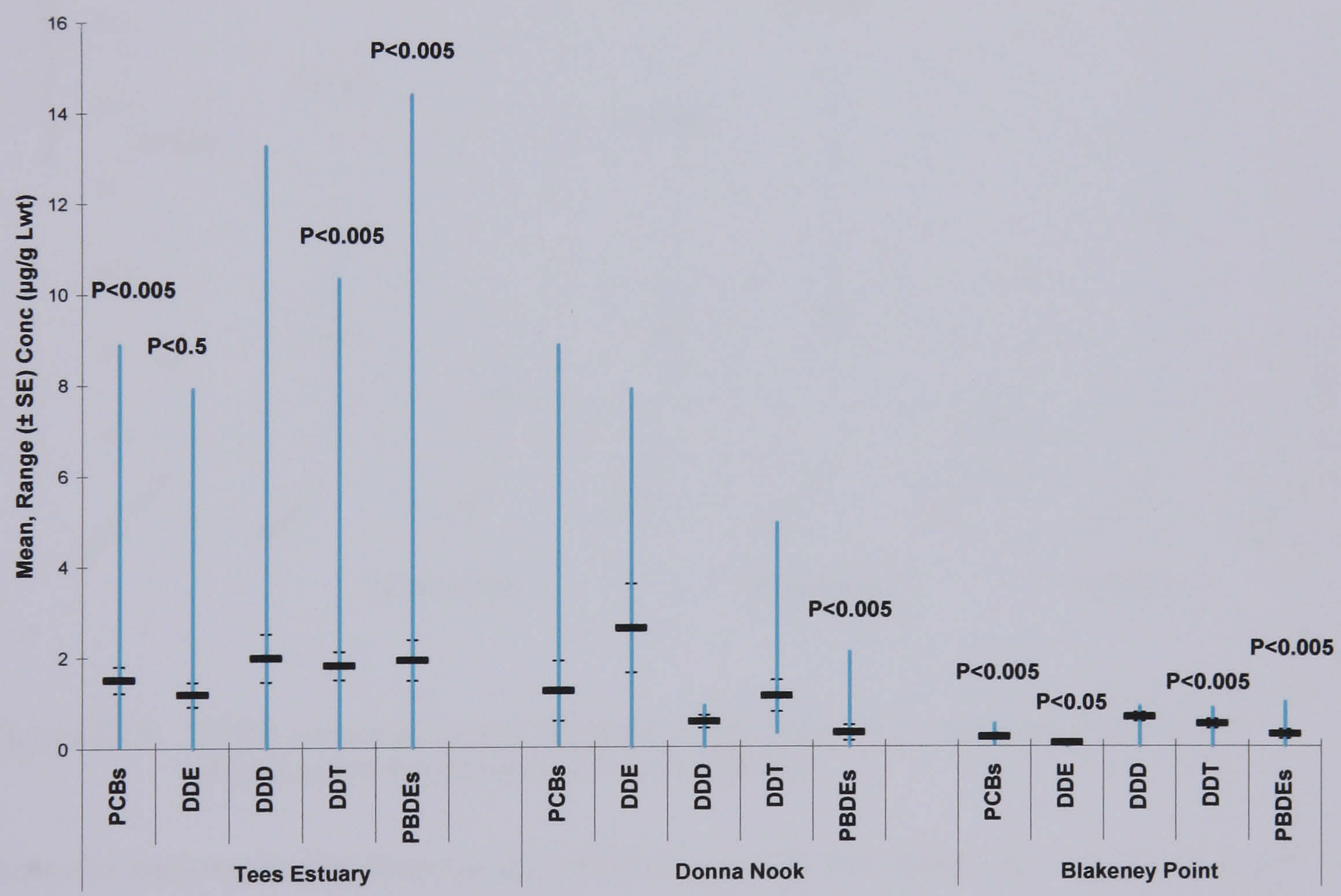



**Figure 2.12:** ΣPCB, ΣDDT and ΣPBDE concentrations in Harbour and Grey seal faecal samples
Tees Estuary ΣPCB, DDT and ΣPBDE > Blakeney Point; $p<0.005$
Tees Estuary DDE > Blakeney Point; $p<0.05$
Tees Estuary > Donna Nook; $p<0.005$

For the purposes of contaminant exposure comparison within seals from different geographical areas, the results for ΣPCB concentrations in the blood samples from females and males were pooled together and are presented in figure 2.13. Figure 2.14 shows the ΣPCB concentrations in blood and faeces for all populations under study.

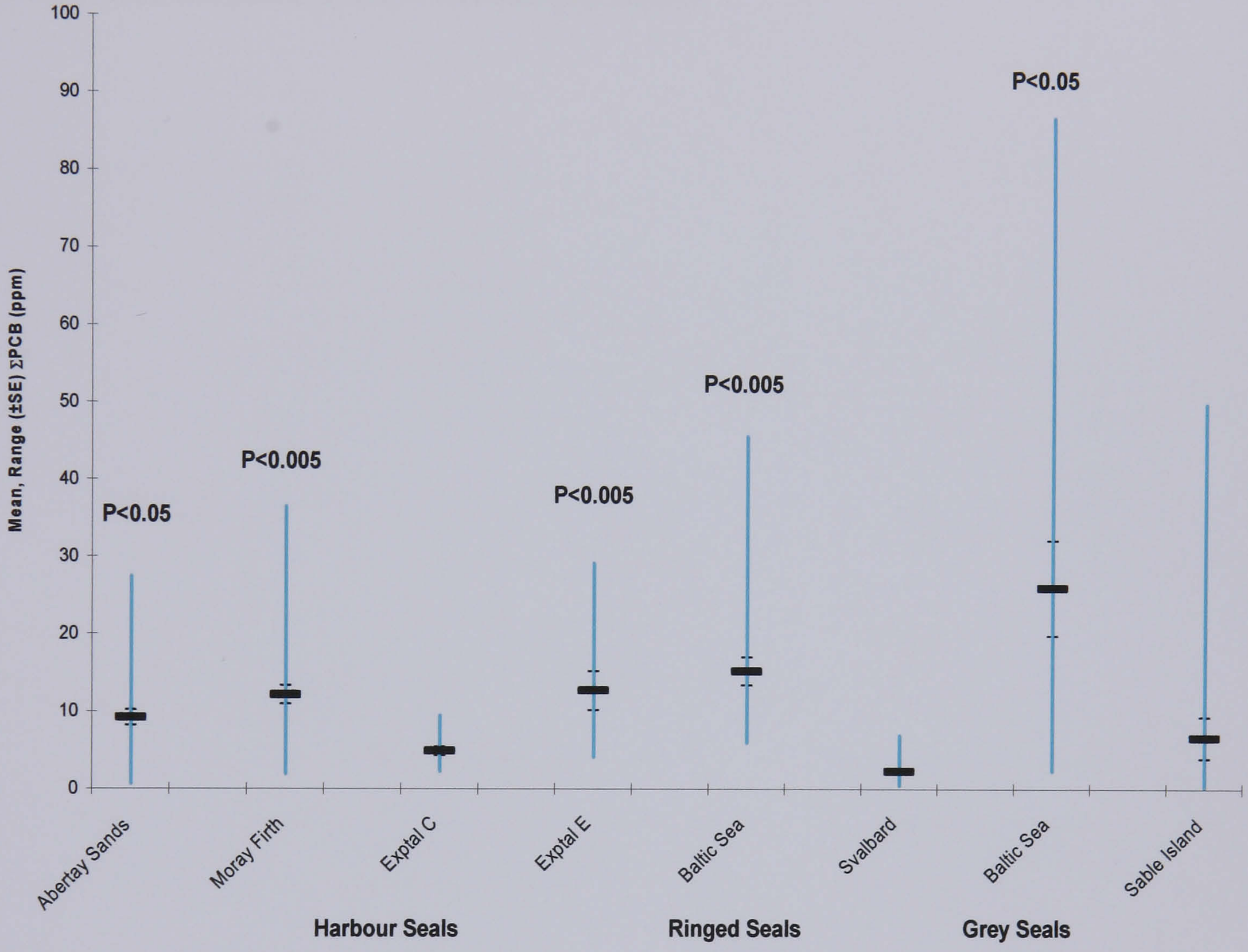



**Figure 2.13:** ΣPCB concentrations in Harbour, Ringed and Grey serum samples (ELISA) (Exptal, experimental group; C, control; E, exposed).

From the analyses it was observed that all Harbour seals had statistically significant higher blood ΣPCB concentrations compared to the control experimental group whereas they showed no difference when compared to the exposed experimental group. ΣPCB levels were higher in the Baltic Ringed and Grey populations compared to the Svalbard ($p<0.005$) and Sable Island ($p<0.05$) seals. Baltic Grey seals showed significantly higher ΣPCB concentrations when compared to Svalbard Ringed ($p<0.005$), Abertay Sands ($p<0.005$), Moray Firth ($p<0.005$)

and the experimental control ($p<0.05$) Harbour seal populations whereas Baltic Ringed seals showed statistically significant higher levels only when compared to Sable Island Grey ($p<0.05$), Abertay Sands and the experimental control Harbour seals ($p<0.005$). There was no statistically significant difference between the two Baltic Sea seal populations as well as between the seal populations of the two reference areas (Svalbard and Sable Island). On the other hand, the Ringed Svalbard seals showed statistically significant lower ΣPCB levels ($p<0.005$) when compared with all other seal populations.

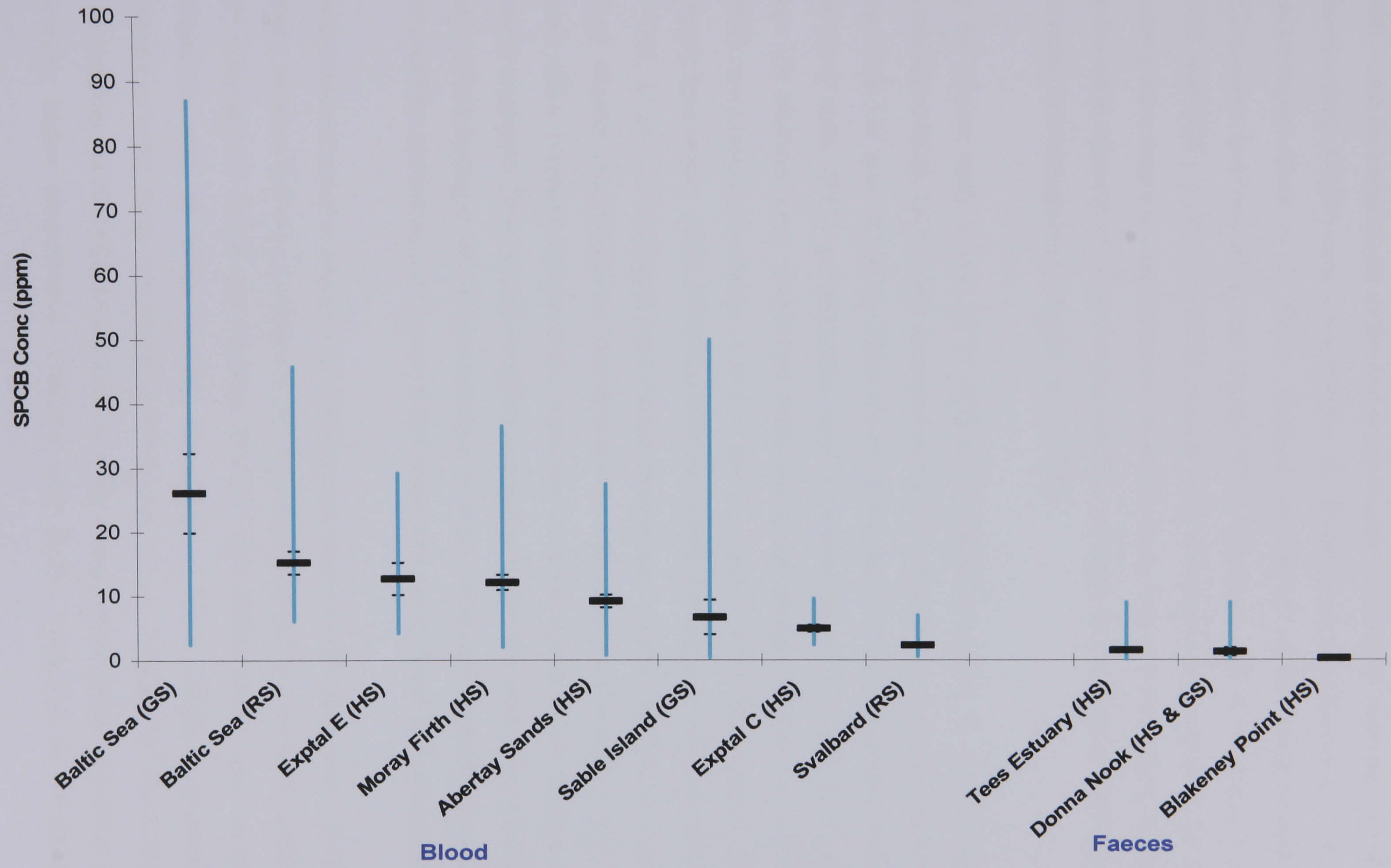



**Figure 2.14:** Mean ± SE and Range of ΣPCB concentrations in all study populations (faeces and serum)
GS, Grey seals; RS, Ringed seals; HS, Harbour seals

## 2.8 Discussion

Marine mammals and especially seals being top predators in the marine food chain have been widely used for contaminant evaluation in the marine environment since they accumulate high concentrations of POPs over a long lifetime and therefore are good indicators of the ecosystem contamination (Shaw *et al.*, 2005). Concern has focused on the polyhalogenated aromatic hydrocarbons including PCBs and organochlorine pesticides such as DDT and its metabolites (DDD and DDE). In the late 1970s, another class of contaminants emerged, the PBDEs that recently have received great attention since they have been shown to have increased in the environment whereas PCB and organochlorine pesticide concentrations such as DDT have generally been dropping (Meironyté, *et al.*, 1999).

At the present study a total of 29 PCB and 5 PBDE congeners as well as DDT and its metabolites (DDD, DDE) were measured using GC analysis in seal faecal samples collected from three UK sites (Blakeney Point, Donna Nook and Tees Estuary) inhabited by Harbour and Grey seals. ΣPCB concentrations were also analysed using ELISA in serum samples from UK Harbour, Grey (Baltic and Sable Island) and Ringed (Baltic and Svalbard) seals. ΣPCB concentrations in faeces in all UK seal populations under study ranged from 0.04 to 8.9μg/g lipid weight. DDE, DDD and DDT concentrations ranged from being non-detectable to 7.93, 13.28 and 10.35μg/g lipid weight respectively whereas ΣPBDE concentrations in faecal samples ranged between non-detection to 14.42μg/g lipid weight. Due to strict UK Home office licensing requirements, collection of blood samples from seals in the UK is heavily restricted. Therefore all sampling undertaken within this study was of faecal samples only without having matching blood samples in order to compare contaminant concentrations and evaluate relationships of their level and composition in different body compartments.

Archival blood samples provided by collaborators were all collected under UK Home Office project licences that were assigned to each institute. However, due to small volume they were only analysed for ΣPCB and therefore ΣPBDEs and ΣDDT levels were not available for comparison with the faecal samples. The range of blood ΣPCBs concentration for all populations under study was found to be between 0.36 to 87.06μg/ml. These measurements indicated that faecal ΣPCB levels were an order of magnitude lower than blood levels. This relatively higher concentration measured with ELISA compared to GC-ECD could be

expected because of the limited number of PCB congeners measured by the GC-ECD method. The antibody used in the assay most probably has cross-reacted with other PCBs present in the blood samples besides the 29 congeners investigated with the GC. Moreover, the antibody has probably cross-reacted with PCB metabolites (hydroxy-PCBs) present in the samples that are not measured by the GC-ECD and contribute to the ELISA-derived concentrations. Hydroxy-PCBs show highly selective retention in blood due to their affinity for transthyretin (TTR) (Bergman *et al.*, 1994; Hovander, *et al.*, 2002; Chuang *et al.*, 2003). Boon *et al.*, (1994) found seal blood PCB concentrations higher up to a factor of five compared to blubber and liver due to adsorption of significant portions of PCBs to non-lipid constituents such as the non-polar parts of albumin molecules.

### 2.8.1 Immunoassay development

Cost and time limitation of chromatographic techniques as well as sampling requirements for the analysis of POPs make detailed studies of contaminated sites and biomonitoring of seal populations difficult. On the other hand, immunoassays currently provide an alternative means for estimation of total POPs such as PCB, or organochlorine pesticide contamination in a variety of samples including soil, water and to a lesser extend biological matrices such as fish tissue (Harrison and DeBaise, 1994; Lawruk *et al.*, 1996; Chuang, *et al.*, 1998; Zajicek *et al.*, 2000; Johnson *et al.*, 2001). At present there are no commercially available immunoassays to measure total PCB concentration in blood samples from seal populations therefore a logical extension on the ELISA method is to adapt it for determination of PCB concentrations in serum samples. Others have developed sensitive and selective immunoassays for hydrophobic contaminants such as PAHs in plasma samples from oiled birds (Troisi and Borjesson, 2005), polychlorinated dibenzo-*p*-dioxins (Sherry *et al.*, 1989) and PCBs in fish samples (Zajicek *et al.*, 2000) but in most cases the success of the method was dependent on extensive sample preparation prior to the ELISA measurements. This is the first study to quantify ΣPCBs in serum samples in Ringed, Grey and Harbour seals using a competitive enzyme-linked immunosorbent assay (ELISA).

Although GC methods are the most accurate since they provide congener-specific PCB analysis in biological samples they are expensive, time-consuming and require large sample volume and dedicated analytical laboratories. Immunoassay techniques however, provide a

non-destructive, high-throughput, cost effective, simple and rapid analytical technique for semi-quantitative determination of ΣPCBs concentrations in biological matrices. Analysis by immunoassays is a fast developing field with numerous possibilities for further improvement. For example, besides measuring the ΣPCBs, a direct competitive immunoassay for analysis of coplanar PCBs also exists that can provide specific information on the most toxic congeners at very low levels in environmental matrices. Such immunoassays could be used for future screening analysis of biological samples such as blood and faeces to provide a quick estimate of environmental contamination in order to identify contaminant exposure in seals speedily and economically. Moreover, PCB screening in environmental samples could provide valuable information on metabolism and bioaccumulation of these compounds in the environmental and food chain matrices (Fránek *et al.*, 2001).

### 2.8.2 Intra-species differences in Contaminant Concentrations

Age, species and sex related differences in results obtained from faecal samples were not possible to be determined since identification of the originating seal for each faecal sample did not occur. However, it is believed that the samples were originating from adult seals since from personal observation on the field only adult seals were in close proximity of the samples collected. Therefore, only comparisons among the seals that blood samples were analysed could be made in this section.

The only population that could be examined for age-dependent variation of ΣPCB concentrations was the Harbour seals from Moray Firth due to the lack of the availability of samples from the other populations. Even though male juveniles were found to have lower ΣPCB concentrations than male adults and female juveniles higher levels when compared with the female adults, there was no statistical difference observed. Difference between genders was only observed among the Baltic and Sable Island Grey seals where males showed statistically significant ($p<0.05$) higher levels compared to females. However, even if there were not any statistical differences between sexes at the other populations, the trend was increasing for all the seal groups. Organochlorine levels vary with gender, reproductive status, age and condition of animal in marine mammals. Differences between the sexes do generally occur after the animals have become mature (Nyman *et al.*, 2003). The contamination levels tend to increase with age in males but not in adult females since females

can transfer a major part of their contaminant burden through lactation to their offspring whereas male seals with time accumulate higher concentrations of PCBs (Boon *et al.*, 1992). These differences also appear to depend on the degree of the contamination of the area from where the animals originate. For example, no differences between adult females and males have been observed in seals from Wadden Sea, a relative highly polluted area (Reijnders, 1980) whereas seals originating from areas with medium PCB levels compared to the Wadden Sea, blubber PCB levels are found to increase with age in mature males but not females and in other marine mammals from areas with low PCB levels, such as the Arctic, blubber PCB levels increase with age in mature males but remain constant or slightly decrease in females (Subramanian *et al.*, 1988; Boon *et al.*, 1992). In the present study for example, the Svalbard Ringed seals represent an unpolluted area in which no statistically significant sex differences were observed whereas the Sable Island Grey seals show increased levels of ΣPCBs in males indicating a contaminant exposure at a level where female Grey seals are able to keep their contaminant burden low by transferring a proportion to their pups. Nyman *et al.*, (2003) also observed an absence of sex difference in contaminant levels with age-dependent PCB accumulation in both sexes, suggesting that level of exposure exceeds the capacity of the seals to keep a stable contaminant level with increasing age in the whole population.

### 2.8.3 Geographical and Inter-species variation in Contaminant Concentrations

*Blood (Serum) Samples*

A geographic comparison of the PCB data obtained from this study with previous reports on the same species was difficult due to the difference in the sampling material (blood samples instead of blubber, age, reproductive and health status) and the analytical method used (ELISA instead of GC analysis). However, few specific comparisons are discussed together with the general trends.

The data obtained from this study show that PCB levels in Baltic and especially Grey seals are higher compared with the Svalbard Ringed and UK Harbour seals (Abertay Sands and Moray Firth). Even though male and female Baltic Grey seals had higher and lower PCB levels and when compared to male and female Baltic Ringed seals respectively, there was no statistically

significant difference between the two seal populations. No statistically significant different PCB levels were also observed between the seal populations of the two reference areas (Sable Island and Svalbard). However, Ringed Svalbard seals showed statistically significant lower ΣPCB levels ($p<0.005$) when compared with all other seal populations. The current species difference of PCB levels in the Baltic seals is difficult to explain. At a similar study it was observed that even though there was no statistically significant difference among the two Baltic seal populations, male Ringed seals were found with higher PCB levels than Grey seals (Nyman *et al.*, 2002). This difference in the results could be attributed to three reasons. Firstly the PCB level data from the other study were derived from blubber analysis and secondly they were analysed by GC in which particular congeners where studied. At the present study, however an enzyme immunoassay was used by which blood samples were analysed for total PCB levels. Therefore differences should be expected in the total PCB concentrations between the two different methods and matrices used for analysis. An explanation for this species difference could also be attributed to the condition of the animals reflected in the proportion of the blubber in the body layer. A decrease in the blubber layer during the fasting period releases the contaminants from the blubber to other organs, including blood. Moreover, the present study and the one described above used samples from Baltic seals that were sampled in different years so this fact could have attributed to the slight different results obtained in this study.

Comparison between the UK Harbour seals revealed that both females and males from the Moray Firth had higher PCB levels compared with Abertay Sands seals although this was not statistically significant. An explanation could be attributed to the fact that these seals were sampled at different stages of the reproductive cycle which plays a significant role on the PCB burdens (Boon *et al.*, 1992). Moray Firth Harbour seals were sampled in summer whereas Abertay Sands Harbour seals were sampled throughout the year making it impossible to estimate the contaminant equilibrium load. Significant differences between different stages of reproductive cycle (winter and summer sampling) have been observed in Grey seals in the North Sea attributed to blubber loss during the fasting period of lactation and moulting (Kalantzi *et al.*, 2005).

*Faecal Samples*

Of the analysed contaminant groups, ΣPCBs ($p<0.005$), DDE ($p<0.05$) and DDT ($p<0.005$) levels were found to be higher in the Tees Estuary compared to Blakeney point seals. Tees Estuary seals also had significantly higher ΣPBDE levels when compared to the other two populations. There were no statistically significant differences between contaminant levels in faecal samples collected from Blakeney Point and Donna Nook. Moreover, when comparing the ΣDDT there was no statistically significant difference among the three populations under study. The pattern of the different groups of contaminants analysed at the present study for each population was the same for Tees Estuary and Donna Nook seals (ΣDDT > PCBs > ΣPBDEs) whereas Blakeney Point Harbour seals showed higher ΣPBDEs compared to ΣPCBs (ΣDDT > ΣPBDEs > ΣPCBs) highlighting the emergence of PBDEs as a significant concern in the marine environment.

Congener profiles in faeces were not well correlated with blubber or blood congener profiles in literature although some congeners that were detected in other matrices were also detected in faeces (Krahn *et al.*, 2001). Higher-chlorinated, coplanar and metabolically stable PCB congeners such as 153, 156, 194, 180 and 118 were found to predominate in faeces from all three study populations in comparison with more readily metabolised congeners such as 52, 123, 169 and 126. In seal blood higher concentrations for the most persistent congeners such as 183, 153 and 180 are usually seen whereas concentrations of the lower chlorinated congeners are relatively low (Boon *et al.*, 1992). Vrecl *et al.*, (2005) also found similar high chlorinated congener pattern in ovine milk and faeces. The blood serum PCB congener profile supports the hypothesis that the lower chlorinated congeners show higher net absorption efficiencies because they are metabolised fairly efficiently while the higher chlorinated congeners are generally influenced by the body burden. The lack of correlation between faecal PCB congener profiles and blubber or blood reported in other studies was expected since faecal OC levels not only reflect excretion of PCB congeners not metabolized or retained in the body but also recent dietary intake (Krahn *et al.*, 2001).

The PCB content is the result of a complex interaction of varying exposures, metabolism and clearance over the period of life. There are important differences between congeners in their susceptibility to metabolism. In general, lower chlorinated congeners are metabolized most readily. The lower chlorinated congeners are apparently absorbed very efficiently (Juan *et al.*, 2002). The fact that seals from Blakeney Point and Donna Nook were sampled right after

lactation (beginning of breeding) could have attributed to the increased non-metabolised PCB concentrations in faeces. Body weight loss during sampling period causes the release of PCBs from blubber to blood and subsequently into faeces. Therefore, the amount of body fat in an individual will have a major impact on their capacity to store PCBs and whether they are losing (fasting-negative energy balance) or gaining fat (feeding) will also have an influence. The level of PCBs in faeces is not only influenced by the recent dietary intake, but also the body burden – which itself is influenced by (and reflects) lifetime exposure, explaining therefore the higher concentrations of PCBs found in seals from the Tees Estuary which is a well known pollution 'hot spot' in the UK as a result of a long history of intense industrial activity in the surrounding areas (Shillabeer and Tapp, 1989; Hardy *et al.*, 1993; Tapp *et al.*, 1993).

In all three seal populations reported at the present study ΣDDT levels were lower than ΣPCB levels. This could be explained by a more rapid decrease of DDT in the environment since it was banned in 1970s whereas PCBs are still continuously released (Bignert *et al.*, 1998). The predominant OCP in all three populations under study was DDT followed by DDD and DDE in seals from Tees Estuary and Blakeney Point, whereas DDE was found to be in higher concentrations compared to DDD in seals from Donna Nook. Harbour and Grey seals were found in Donna Nook whereas in the other two populations only Harbour seals were observed. Therefore a difference in the diet is likely to have an impact on the POP input. The results obtained at the present study come in contrast with previously published levels of ΣDDT in seal blubber and blood where DDT concentrations are found to be lower than DDE levels (Reijnders, 1980; Kalantzi, *et al.*, 2005; Shaw *et al.*, 2005). The fact that from Donna Nook and Blakeney Point there were only few seals to sample could have attributed to underestimation of POP data. However, it has been observed that DDE in rat faeces is excreted as polar metabolites with only 3-4% of a single dose of DDE excreted as DDE (Muhlebach *et al.*, 1991). Therefore, DDE excretion in seals could be limited by the slow formation of polar metabolites of DDE.

From the five PBDEs under investigation, congener 47 was found at the highest concentration followed by 99 in Tees Estuary seals and 153 in Donna Nook and Blakeney Point. PBDEs 47 and 99 are two of the main components of the PentaBDE technical product Bromkal 70-5DE which has been heavily used in Europe (Kalantzi *et al.*, 2005). Ikonomou *et al.*, (2002)

described an exponential increase of PBDEs in the ringed seals since 1981, which correlated with the industrial production of the penta-mix PBDE formulation. The dominance of PBDE 47 in the current PBDE congener profile has also been observed in marine and terrestrial mammals including human blood plasma, grey seal blubber, whales and human milk in the highest concentrations of all PBDEs measured (Haglund *et al.*, 1997; Meironyté *et al.*, 1999; Lindström *et al.*, 1999). The concentrations measured at the present study were lower compared to other studies (Kalantzi *et al.*, 2005; Sellström *et al.*, 1993 and 1997). This is related to two factors: firstly the above studies did not measure ΣPBDEs in faeces. Secondly, the Baltic is generally considered to be more contaminated with PBDEs than the UK (de Wit, 2002). However, ΣPBDE levels of the present study were higher compared with ringed seals from Svalbard, Arctic (Wolkers *et al.*, 2004). In general, European samples appear to follow the concentration trend Baltic>UK/North Sea> North Atlantic (Kalantzi *et al.*, 2005).

### 2.8.4 Exposure Levels of Study Populations

A statistically significant difference ($p<0.005$) was found between the exposed and control ΣPCB levels in Harbour seals from the experimental group. This was anticipated since these seals belonged to a feeding study in which the exposed group was fed contaminated fish from the Wadden Sea whereas the control group was fed with cleaner Atlantic fish.

Of the field sampled groups, Baltic Grey and Ringed seals were found to have slightly higher ΣPCB levels compared to the experimental-exposed group (although not statistically significant) and they were classified as exposed populations. Svalbard Ringed seals were found to have lower ΣPCB levels when compared to the control experimental group. For the purposes of the present study these two groups (experimental control and Svalbard Ringed seals) were considered control seal populations (fig. 2.14). UK Harbour seals were found to be at the same level of exposure with the exposed experimental group although with slight less ΣPCB concentrations and they were therefore classified as exposed seal populations. The order of exposure of the seal populations under study was as follows:

Grey Baltic seals>Ringed Baltic seals>**Exptal (E)**>Harbour Moray Firth seals>Harbour Abertay Sands seals>Grey Sable Island seals>**Exptal (C)**>Ringed Svalbard seals. It has to be taken into consideration that the above order is derived from pooling female and male data.

When considering the sexes separately, Grey seals from Sable Island also account as control population.

The serum ΣPCB concentrations observed at the present study were in agreement with published data. This observation shows that the immunoassay method selected for PCB quantification in seal serum was efficient. According to the manufacturer of the immunoassay antibody used in the study (*Abraxis Kits Inc*, LLC, U.S.A; Fig. 8.5, Appendix I) a strong positive correlation ($R^2 = 0.91$) was obtained between data from the ELISA soil test kit and data from GC-MS for same soil samples, confirming the accuracy and reliability of immunoassay antibody when used as part of the Abraxis soil test kit which is based on the same principals as the plasma ELISA in this project.

The levels of organic contaminants are still high in the Baltic seal populations, although ΣDDT has decreased during the last decades (Nyman *et al.*, 2002), whereas OC levels in the European Arctic and Canada have been reported to be low compared with seal populations from the Baltic and the North Sea (Muir *et al.*, 2000c; Braune *et al.*, 2005). Serum ΣPCB levels in Harbour seals were found to be lower when compared with studies in West Scotland and East England (Law *et al.*, 1989; Hall *et al.*, 1992) and higher when compared to Harbour seals from East Scotland, North Shetland and Norfolk Wash (Holden *et al.*, 1972; Hall *et al.*, 1992; Ruus *et al.*, 1999). It has to be noted that the current study investigated ΣPCB in serum samples whereas the above studies used blubber and therefore significant differences in the concentrations between the results are expected. The relationship between OC levels in blubber and those circulating in the blood is not well understood. However, it is reported that the representation of individual CB congeners in seal milk is different from that in blubber (Addison and Brodie, 1987) and therefore the same is presumed to be true of blood and blubber. It has to be taken into account that the stage of reproduction that the seals are sampled plays a significant role since OCs in blubber are metabolized when a seal is in negative energy balance such as lactation when seals are in fasting, mating and moulting seasons and when an animal is sick (Hall *et al.*, 1992). Therefore it could be proposed that seals with high blubber OC levels would also have high circulating levels at this time. Lipid mobilization would also influence faecal OC concentrations along with dietary intake since faecal OC levels reflect excretion of PCB congeners not metabolized or retained in the body in addition to recent dietary intake. Therefore, use of faecal OCs has value as an indicator of recent OC exposure,

particularly in the area where the sample was collected and as an index to compare relative spatial and temporal exposure. It is not necessary a reflection of the individual congener concentrations in body depot stores. This matrix, as a contaminant exposure-monitoring tool, has great promise because it can be collected without physical capture of animals which is very expensive and potentially dangerous to both humans and seals. Unfortunately, this matrix is not useful in predicting the body burden of a particular individual animal (Krahn *et al.*, 2001).

The major limitation of the present study was that no matching faecal and blood samples could be obtained in order to provide a direct correlation of contaminant levels between these two matrices and to validate extrapolation of faecal to blood levels. Thomas *et al.*, (2005) developed a model for predicting contaminant burdens in seals from diet, whereas matching faecal and blood samples would be ideal in order to develop a model for predicting seal population burdens directly from faeces thus providing a valuable tool for non-destructive sampling and seal biomonitoring. For future work, sampling should take place during all the stages of reproduction in order for changes in the physiology of individual seals to be accounted (e.g. lactation, moulting etc.) Moreover, larger sample sizes are needed in order to have a better picture of the contaminant exposure of the population under study. In the present study, the identity of the seals from which the faecal samples originated was not known. Faecal DNA testing would have been ideal to identify the sex of animals sampled and provide more information on sex-dependent differences in contaminant exposure. Another limitation of the present study was that ΣPBDE and ΣDDT levels were not quantified for blood samples, because the sample volume was too small for GC analysis and antibodies or dedicated immunoassay kits have yet to be developed for PBDE quantification. On the other hand, immunoassays for OCPs already exist, but due to resource and funding limitations could not be used in this study. Future development and validation of a PBDE immunoassay is strongly recommended as an ideal tool for PBDE exposure biomonitoring in seal populations.

# Sex Steroids and their Application as Biomarkers of Xenoestrogenic Exposure in Pinnipeds

## 3.1 Introduction

Being top predators in the marine food chain, seals are significantly exposed to biomagnified levels of persistent, lipophilic organic pollutants such as PCBs, PBDEs and organochlorine pesticides whose tissue concentrations and severity of resultant toxic effects, vary according to exposure level and physiological factors, such as age, species, sex and nutritional and health status (Addison, 1989; Aguilar *et al.*, 2002; Law *et al.*, 2002). Endocrine disruption has been observed in marine mammals with evidence available since the 1960s. The endocrine system is one of the most important homeostatic systems in animals and EDCs are capable to disrupt the endocrine system in several ways. They can either mimic the endogenous hormones by binding to receptors and initiate a new cellular response (e.g. mimicking 17-β estradiol (E2) and bind to estrogen receptor (ER)) or bind and block the receptor, making these regulatory switches unavailable to signals from the body's naturally produced hormone messengers, thus disrupting the body's normal functions (Griffin and Ojeda, 1996). Moreover, disruption can occur through altering normal hormone levels by interfering with the breakdown of the hormones by the liver's enzyme system thus affecting the functions that these hormones control (Portier, 2002). Less direct interferences involve interference with the ways hormones travel through the body and alterations in numbers of receptors in developing tissues thereby predisposing these tissues to abnormal response later in life (Fielden *et al.*, 1997; Thomas, *et al.*, 2005).

Some examples of chemicals that are known endocrine disruptors include dioxins, PCBs and organochlorine pesticides such as DDT that have been reported to cause a number of different estrogenic and anti-androgenic effects in marine mammals (Reijnders, 1986; Hall *et al.*, 1992; Koistinen *et al.*, 1997; AMAP, 2002; Nyman *et al.*, 2002). Over the last fifty years significant declines and poor population recovery have been observed in Baltic and Wadden Sea seal populations that have been attributed to high tissue organochlorine burdens (Reijnders, 1980 and 1986). In the 1970s, Hook and Johnels (1972) showed a significant relationship between blubber PCB concentration greater than 70μg/g (lipid weight) and infertility due to uterine stenosis/occlusions in sexually mature females of Grey and Ringed Baltic seal populations and since then the estimated threshold level of total PCBs was set between 25-70μg/g L.wt for reproductive effects in marine mammals. Other studies have also

reported similar findings in Baltic Grey and Ringed seals such as uterine stenosis and occlusions, adrenocortical hyperplasia and hormonal osteoporosis (Bergman and Olsson, 1985). There is population-level evidence of the reproductive effects of POPs caused by endocrine disrupting mechanisms. Reduced reproductive success (Reijnders, 1986), still births (DeLong *et al.*, 1973), uterine pathological deformities (Helle and Olson, 1976a,b; Reijnders, 1980; Baker, 1989), altered steroid hormone metabolism, altered steroid biosynthesis (Freeman and Sangalang, 1977; Troisi and Mason, 2000) and progesterone antagonism (Gillner *et al.*, 1988; Troisi and Mason, 2000) are some of the organochlorine-induced reproductive effects reported in seals, mediated by an endocrine disrupting mechanism. Aroclor 1254 has also been found to cause a decrease in plasma progesterone in female adult minks as well as Harbour seals (Reijnders, 1986; Auerlich *et al.*, 1985).

Early detection of the adverse health effects resulting from pollutant exposure is vital, since exposure may cause irreversible damage to entire populations or ecosystems in the long term. A direct method to determine pollutant exposure and effects in a population includes the collection of biological samples from large numbers of individuals of a population and analysis of pollutant concentrations and/or biomarkers of pollutant exposure or effects. (Depledge, 1994) which will provide a direct measure of exposure of the entire population, an understanding of exposure burden and a quantitative indication of the severity of specific adverse health effects in individuals of an exposed population (Kamrin, 2004). Biomarkers can be dose-dependent, sensitive enough to monitor low-level exposure and they can provide vital information on the way in which varying degrees of contaminant exposure affect biological variables such as reproductive or endocrine in male and female, adult and juvenile seals. Reproducible results can be facilitated using large sample sizes obtained non-destructively (e.g. faeces) or with minimal invasion (e.g. blood) and the use of cost-effective and straightforward biological assays (immunoassays). Baseline/control biomarkers can be determined in less exposed animals or populations and can be validated with data from chemical analysis or at least one other biomarker that has already been validated by chemical analysis. Furthermore, re-sampling individuals for temporal exposure studies is also possible for surveillance monitoring (Addison *et al.*, 1986; Schumacher *et al.*, 1995; Hall *et al.*, 1997; Hall, 1998; Fossi *et al.*, 1999; Hall *et al.*, 1999; Nyman *et al.*, 2003)

Sex steroids are likely targets for contaminant-induced impairment of reproduction since EDCs are able to change their balanced levels (Portier, 2002). EDCs also target the steroidogenic tissues and organs, such as the adrenals and gonads to alter hormone biosynthesis leading to changes in circulating steroid levels and hormone-dependent events (e.g., gametogenesis, sexual development and fertility) (Fielden *et al.*, 1997). In the present study, estrogens were expected to be decreased with increasing POP concentrations.

Due to the need to assess non-destructively the endocrine status of captive and wild animals, new hormone monitoring methods have been adopted in recent years, thanks to the availability of sensitive immunoassay detection methods. These endocrine assessment methods include blood, urine, sputum and faecal hormonal measurement. Of particular interest for monitoring seals are faecal hormone assessments, due to the ease of sampling faeces without the need to handle study animals. Immunoassays are able to cope with the complex nature of the faecal matrix, making faecal steroids analysis both practicable, high-throughput and cost-effective (Schwarzenberger *et al.*, 1996). A number of studies have employed faecal monitoring to successfully monitor gonadal and adrenal steroids concentrations in a wide variety of mammalian species (including dogs, buffalo, primates, elephants, wolves, caribou, wild horses, cows, wild baboons, otters and others (Wasser *et al.*, 1991; Schwarzenberger *et al.*, 1996; Matsumuro *et al.*, 1999; Linklater, *et* al, 2000; Turner *et al.*, 2001, Larson *et al.*, 2003; Lynch *et al.*, 2003). All the available data confirm that faecal steroids accurately reflect plasma/serum profiles, confirming the value of this non-invasive approach to hormone monitoring which also has the added benefit of inferring reproductive status (Schwarzenberger *et al.*, 1996; Moreira *et al.*, 2001). Faecal steroid analysis is now the most commonly used diagnostic tool to study fundamental reproductive endocrinology in wild, farm and zoo animals. This approach offers clear advantages over repeated blood sampling for monitoring reproductive cycles/status of seals, where faecal deposition occurs mainly on the shore or in caves (depending on species).

Measuring steroid levels together with contaminants in blood and faecal samples not only can provide accurate measures of the reproductive cycle of seals but also additional information about the changes in the reproductive status of these animals. Sex steroids have been successfully used as biomarkers in human studies in order to assess reproductive health by measuring the absolute level of hormones and the pattern of hormone fluctuation throughout

the menstrual cycle and during pregnancy (Lasley and Overstreet, 1998). Serum PCB levels and Great Lake fish consumption has been associated with significantly lower levels of serum thyroxine (T4) in women and men whereas in males there was no significant association with serum free testosterone (T), estrone sulfate and dehydroepiandosterone sulfate (DHEA-S) (Persky *et al.*, 2001). So far there are very few studies that have used serum steroid levels as biomarkers for endocrine disruption in seals. Reijnders (1986) was the first to demonstrate that a causal relationship between naturally occurring levels of pollutants and reproductive failure in Harbour seals from the Dutch Wadden Sea exists. However, sex steroid levels have been measured in seals in order to obtain baseline levels of steroids through the reproductive cycle to provide better understanding of the reproductive biology of the animals under study (Sangalang and Freeman, 1976; Reijnders, 1990; Boyd, 1991; Noonan *et al.*, 1991; Atkinson, 1997; Gales *et al.*, 1991; Gardiner *et al.*, 1999; Renfree and Shaw, 2000; Greig, 2002). Using sex steroids as biomarkers of endocrine disruption in seals would be a valuable tool since they are measurable in non-destructive blood samples and non-invasive faecal samples.

Even though measuring serum levels of hormones has already been used, as already mentioned earlier faecal steroid measurement so far has only been applied to reproductive/endocrine assessments in mammals. There is clear potential of using faecal samples for biomonitoring altered function and EDC exposure in seals because sampling faeces is a completely non-invasive method. However, in wild populations it will be difficult to identify the originating animal.

There is lack of baseline information on sex steroid levels in seals and clearly there is the need of knowledge of the stage of reproductive cycle and exposure of the population under study in order to examine hormone level changes due to EDCs. Therefore, it is important to firstly establish normal values in seals as part of biomarker validation. Moreover, studies in control/less exposed seal populations are a necessary step in the biomarker validation process in order to compare hormone concentrations in blood and the corresponding concentrations in faecal samples (Lasley and Overstreet, 1998). Lastly, it is important to establish that hormone changes are directly related to pollutant exposure by a known mechanism in a dose-dependent manner in order to be considered reliable effect biomarkers. Clearly, hormone levels warrant further study to validate their use as non-destructive biomarkers of endocrine disruption in seal populations.

The aim of this study was to investigate the reliability of sex steroids as biomarkers of estrogenic POP exposure and effects in seals. This was accomplished by the following objectives:

i. To quantify sex steroid concentrations in seal serum and faeces samples in low exposure/control seal groups to establish normal baseline values for steroids in Harbour, Grey and Ringed seals, as part of their validation as ED exposure and effect biomarkers
ii. Identify sex steroids whose expression is dose-dependent with POP exposure, again, as part of their validation as biomarkers using samples from the experimental seal group
iii. Quantify validated sex steroid BMs in serum and faecal samples of highly polluted Baltic Ringed and Grey seal populations (serum), exposed UK Harbour seals (serum and faeces) and less polluted (control) Grey (Sable Island, Western Atlantic Ocean) and Ringed seals (Svalbard, European Arctic) to represent changes in BM expression over a range of POP exposure.
iv. Identify which populations are at risk from ED effects

## 3.2 Materials and methods

### 3.2.1 Sampling and Study Populations

#### *3.2.1.1 Blood Samples*

Samples were obtained from three species, Grey (*Halichoerus grypus*), Ringed (*Phoca hispida*) and Harbour seals (*Phoca vitulina*). All of the serum samples used in the present study were provided by collaborators and were collected from six different seal populations over a 7 year period (1999 – 2002); Baltic and Svalbard (Arctic) Ringed seals, Baltic Grey and Sable Island (Canada) seals and UK Harbour seals from Abertay Sands (Dundee, Scotland) and Moray Firth (Northeast Scotland). Baltic Ringed and Grey seals, Svalbard Ringed seals and Grey seals from Sable Island, Canada were provided from Dr. M. Nyman, *Finnish Game and Fisheries Research Institute* (Helsinki, Finland). Harbour seals from Abertay Sands (Dundee, Scotland) were provided by Dr. Ailsa Hall (Sea Mammal Research Unit, St. Andrews University) and Moray Firth (Northeast Scotland) Harbour seals were provided by Dr. Paul Thompson (Lighthouse Research Station, University of Aberdeen). Harbour seals were also provided from a controlled dosing study, in which the seals were fed fish from the highly contaminated Wadden Sea or less contaminated Atlantic Sea (Prof. P. Reijnders).

#### *3.2.1.2 Faecal Samples*

UK seal populations were selected for study on the basis of elevated pollutant exposure due to localised industrial activity and accessibility for sampling. Seal faecal samples were collected from the Norfolk Wash, East Anglia at Blakeney Point (Harbour seal samples) and Donna Nook (Harbour & Grey seal samples), in December 2001 with the support and assistance of staff at the RSPCA Norfolk Wildlife Hospital (East Winch, King's Lynn), the Lincolnshire Wildlife Trust and the permission and help of The Ministry of Defence (MoD). Harbour Seal faecal samples were also collected from the Tees Estuary (north-east England), between August 2000 and January 2002 in collaboration with Durham University, UK and the permission of the local warden (*For more information refer to Chapter 2-Contaminant Analysis*).

Sample information is summarised in Table 3.1.

**Table 3.1:** Summary of faecal sample information for study groups/populations

| | Study Site / Population | Species | Sample number (n) |
|---|---|---|---|
| **Experiment Group** | **n/a** | | 0 |
| **Control Group** | **n/a** | | 0 |
| **Exposed Groups** | | | Sum = 70 |
| *Tees Estuary* | (North-east England) | *Harbour seals* | 47 |
| *Blakeney Point* | (The Norfolk Wash, UK) | *Harbour seals* | 10 |
| *Donna Nook* [a] | (The Norfolk Wash, UK) | *Harbour & Grey Seals* | 13 |

[a]: could not distinguish species of faecal sample collected ; n/a – not available for this study

### 3.2.2 Sample preparation

After collection, faecal samples were wrapped in hexane-washed aluminium foil and transported in liquid nitrogen to the lab and stored at –70°C until analysis to maintain sample quality. It was not possible to take blood samples from these populations as UK Home office licence approval was not available to the project.

All blood samples were available as serum prepared in the same manner and ranging in volume from 500µl to 1.5ml. Briefly, whole blood collected, was centrifuged in the field for 5 minutes at 3000 rpm to obtain serum, transported on dry ice or liquid nitrogen to the lab and stored at –70°C until analysis to maintain sample quality.

### 3.2.3 Reproductive Biology Information of Study Populations

There are three distinctive phases of the annual reproductive cycle of sexually mature female pinnipeds: estrus, delayed implantation and gestation. In the case of sexually mature male pinnipeds, there is a profound annual cycle of testicular regression and recrudescence (Boyd, 1999). In the Baltic Sea, Grey seals breed between February and April and moult in April, May and June. Pups are born in February and March and are nursed for about 18 days (Bergman & Olson, 1985). Female Baltic Ringed seals give birth in February and March and nurse their pups for five to seven weeks. Mating occurs around the time of weaning and the implantation of the foetus is delayed for three months. Post implantation period lasts approximately for nine months. Baltic Ringed seals moult in April and May in small groups or alone on the ice (Bergman & Olson, 1985; Helle *et al.*, 1990). In UK Harbour seals, mating takes place any time from February to October soon

after females come into estrus once their pups have been weaned between April and July (peaks mid-June). Embryonic diapause lasts 2-3 months and is followed by a 10-11 month gestation period (Corbet & Harris, 1991). Pups are nursed for approximately 3-6 weeks.

The reproductive cycle of Ringed, Harbour and Grey seals was divided into four stages (I-IV) that represent different reproductive states during the annual cycle (Gardiner *et al.*, 1999) and changes in hormone concentrations during the reproductive cycle were analysed with respect to these changes. The four different stages of the reproductive cycle of the three species under study are presented in Table 3.2 and a summary of sample information is provided in Table 3.3 (*for further sampling and hormone information refer to Chapter 2 and 1 respectively*).

**Table 3.2:** Stages in the reproductive cycle of Harbour, Ringed and Grey seals.

| Stage | Species | Months | Female | Male |
|---|---|---|---|---|
| **I** | *Harbour seals* | June – August | Postpartum Period | Copulation |
| | *Ringed seals* | February – April | | |
| | *Grey seals* | February – April | | |
| **II** | *Harbour seals* | Sep – November | Delayed Implantation | Post-copulation |
| | *Ringed seals* | May – July | | |
| | *Grey seals* | May – July | | |
| **III** | *Harbour seals* | December – February | Post-implantation Gestation | Mid-cycle |
| | *Ringed seals* | August – October | | |
| | *Grey seals* | August – October | | |
| **IV** | *Harbour seals* | March – May | Late Gestation or Non-Pregnant | Pre-copulation |
| | *Ringed seals* | November – January | | |
| | *Grey seals* | November – January | | |

**Table 3.3:** Sample information for each study population

| **Population Category** | **Exposed** | **Control** | **Exposed** | **Control** | **Exposed** | **Exposed** | **Experimental** | |
|---|---|---|---|---|---|---|---|---|
| | **North Baltic Sea** | **Svalbard** | **North Baltic Sea** | **Sable Island** | **Moray Firth** | **Abertay Sands** | **Experimental Group** | |
| | | **European Arctic** | | **Canada** | **NE Scotland** | **Firth of Tay, Scotland** | **Exposed** | **Control** |
| **Species** | **Ringed Seals** | | **Grey Seals** | | **Harbour Seals** | | | |
| | *Phoca hispida baltica* | *Phoca hispida* | *Halichoerus grypus* | | *Phoca vitulina* | | | |
| **Number of Samples** | **26** | **18** | **41** | **19** | **68** | **56** | **11** | **12** |
| *Serum* | 23 | 18 | 27 | 19 | 68 | 56 | 11 | 12 |
| **No. analysed for ΣPCBs** | **23** | **18** | **19** | **19** | **47** | **48** | **11** | **12** |
| **Sex & Age** | | | | | | | | |
| **Females** | **11** | **10** | **16** | **10** | **36** | **20** | **11** | **12** |
| Adult | All | All | All | All | 18 | All | All | All |
| Sub-Adult | - | - | - | - | 4 | - | - | - |
| Juvenile | - | - | - | - | 14 | - | - | - |
| **Males** | **12** | **8** | **11** | **9** | **32** | **36** | - | - |
| Adult | All | All | All | All | 21 | All | - | - |
| Sub-Adult | - | - | - | - | 2 | - | - | - |
| Juvenile | - | - | - | - | 9 | - | - | - |
| **Year of Sampling** | April to June | April to June | April to June | April to June | 1992-1995 | 1999-2000 | 1988 | 1988 |
| | 1997-1998 | 1996 | 1997-1998 | 1998 | | | | |
| | 2000 | 1998 | 2000 | | | | | |
| | 2002 | | | | | | | |
| **Stage of Reproduction** | II | II | II | II | II and IV | I, II, III and IV | IV | IV |

### 3.2.4 Reagents

Dehydroepiandrosterone sulphate (DHEA-S), 17β-estradiol (E2), free estriol (E3), progesterone (P), 17-α-hydroxy progesterone (17-α-OHP), testosterone (T) and androstenedione (A) ELISA quantitation kits were purchased from *DRG Diagnostics* (Germany). Estrone (E1) ELISA quantitation kit was purchased from *Takeda Chemical Industries*, Ltd. Methanol was glass distilled grade and was purchased from *Rathburn* (Scotland). For information on faecal and blood contaminant analysis reagents refer to Chapter 2.

### 3.2.5 Quantification of sex steroids (ELISA)

#### *3.2.5.1 Serum Samples*

Commercially-available ELISA kits were selected in order to quantify sex steroid levels in seal serum samples. Prior to analyses the samples were grouped according to the stage of reproductive cycle. Dilution of the serum samples was not necessary due to the resultant absorbance range/colour reaction. However, when samples were above the high standard of the calibration curve they were diluted with sample diluent provided by the manufacturer. Seal sex steroid concentrations were extrapolated using the equation of the linear portion of the four-parameter logistic calibration curve that was derived using the computer software: Microplate Manager, Ver.5.2.1; Bio-Rad Lab. To minimise inter-plate variation, samples were analysed in triplicate on the same plate and sex steroid concentrations extrapolated from the calibration curve derived on the same plate. A blank (in duplicate) was also analysed with each sample set in order to control any background absorbance attributable to non-specific binding and to avoid over-presentation of the data. Sample sex steroid concentrations were expressed as mean of triplicate values for each sample. Absorbance at 450nm was measured using an ELISA microplate reader (*Bio-Rad*, Model 550).

#### *3.2.5.2 Assay Protocol for Serum Samples*

No special pre-treatment of the seal serum samples was necessary. The sex steroid ELISA kits (P, 17-α-OHP, E2, E3, T, A, DHEA-S) were based on the competition principle and the microplate separation. An unknown amount of sex steroid in the sample and a fixed amount of

sex steroid conjugate with horse-radish peroxidase competed for the binding sites of a polyclonal steroid antiserum coated onto the wells. After one hour incubation the microtiterplate was washed to stop the competition reaction. Having added the substrate solution the concentration of the sex steroid in the sample was inversely proportional to the optical density measured at 450nm. The lowest detectable level (limit of detection) defined as the sex steroid concentration given by the mean absorbance of the zero standard minus two standard deviations was assessed for each sex steroid under study and it was the following: P: 0.05ng/ml; 17-α-OHP: 0.05ng/ml; E2: 4.6pg/ml; E3: 0.02ng/ml; T: 0.069ng/ml; A: 0.043ng/ml and DHEA-S: 0.02µg/ml.

#### *3.2.5.3 Faecal Samples*

Faecal samples were extracted with methanol as described by Schwarzenberger *et al.*, (1996b). Briefly, approximately 0.5g of wet faeces were homogenised with anhydrous sodium sulphate until a dry-free flowing mixture was obtained. Before extraction samples were spiked with 100ng/ml progesterone as internal standard since unmetabolised progesterone is barely present, if at all, in faecal samples (Schwarzenberger *et al.*, 1996). Then the sample was mixed with 0.5ml water and 4ml of methanol. Samples were then vortexed for 30min and then defatted by vortexing for 10s in 3ml diethyl ether. The methanol fraction was then transferred into a new vial, diluted in the assay buffer and analysed using ELISA.

#### *3.2.5.4 Assay Protocol for Faecal Samples*

Commercially-available ELISA kits were selected in order to quantify E1, E2, E3 levels in seal faecal samples. Prior to analyses the samples were grouped according to the stage of reproductive cycle. E2 and E3 analysis was performed the same way as described earlier for serum samples. E1 analysis was based on the competition principle and the microplate separation. Briefly, the test was based on the recognition of E1 by specific monoclonal antibody. E1 present in the sample and an E1-enzyme conjugate were premixed and added into each well of the microplate and allowed to compete for limited number of binding sites of the antibody immobilised on the surface of the wells. Unbound E1 and excess E1-enzyme conjugate were washed out and a chromogenic substrate was added to develop colour in conjunction with the enzyme conjugate. The concentration of E1 in the sample was inversely

proportional to the optical density measured at 450nm. The lowest detectable level (limit of detection) defined as the E1concentration given by the mean absorbance of the zero standard minus two standard deviations was 0.05ng/ml.

### 3.2.6 Quantification of serum ΣPCB concentrations

Serum and faecal samples were analysed for contaminants in order to determine the level of exposure in study animals. Full details of analysis are provided in Chapter 2. Mean faecal ΣPCBs, ΣDDTs and ΣPBDEs are presented in Table 3.4 whereas ΣPCBs concentrations in serum samples are presented in Table 3.5.

**Table 3.4** Mean Concentrations of ΣPCBs, ΣDDTs and ΣPBDEs in faecal samples for each study population (Results are presented as means ±standard errors and ranges)

| **Population** | **Tees Estuary** | **Donna Nook** | **Blakeney Point** |
|---|---|---|---|
| **Study Sites** | North-east England | The Norfolk Wash, UK | The Norfolk Wash, UK |
| **Species** | Harbour Seals | Harbour & Grey Seals | Harbour Seals |
| **Number of Samples** | **47** | **13** | **10** |
| **POPs (µg/g l.wt)** | | | |
| **ΣPCB** | **1.51 ± 0.29** (0.04 – 8.90) | **1.25 ± 0.66** (0.05 – 8.89) | **0.21 ± 0.05** (0.05 – 0.5) |
| **ΣDDT** | **3.14 ± 0.57** (nd – 19.10) | **2.02 ± 0.79** (0.31 – 10.61) | **0.62 ± 0.15** (nd – 1.64) |
| **ΣPBDE** | **1.31 ± 0.45** (nd – 14.42) | **0.31 ± 0.17** (nd – 0.17) | **0.27 ± 0.09** (0.01 – 0.98) |

*(Table 3.4 was reproduced from Chapter 2 – Contaminant Analysis)*

### 3.2.7 Cross reactivity of ELISA kits

Cross reactivity information for each sex steroid analysed at the present study was provided by the manufacturer and is presented in Appendix II.

**Table 3.5** Mean Concentrations of ΣPCBs in blood samples for each study population (Results are presented as means ±standard errors; ranges)

| **Population** | **Exposed** | **Control** | **Exposed** | **Control** | **Exposed** | **Exposed** | **Experimental** | |
|---|---|---|---|---|---|---|---|---|
| | **North Baltic Sea** | **Svalbard** | **North Baltic Sea** | **Sable Island** | **Moray Firth** | **Abertay Sands** | **Experimental Group** | |
| | | **European Arctic** | | **Canada** | **NE Scotland** | **Firth of Tay, Scotland** | **Exposed** | **Control** |
| **Species** | **Ringed Seals** | | **Grey Seals** | | **Harbour Seals** | | | |
| | *Phoca hispida baltica* | *Phoca hispida* | *Halichoerus grypus* | | *Phoca vitulina* | | | |
| **No of Serum Samples** | **23** | **18** | **19** | **19** | **47** | **48** | **11** | **12** |
| **Female Adults** | **11** | **11** | **10** | **10** | **11** | **31** | **11** | **12** |
| **Mean (µg/ml)** | 14.03 ± 1.78 | 2.16 ± 0.57 | 13.46 ± 4.38 | 1.79 ± 0.51 | 10.59 ± 1.64 | 8.40 ± 1.81 | 12.76 ± 2.52 | 4.92 ± 0.55 |
| **Range** | (8.01-25.49) | (0.47-6.48) | (2.35-39.73) | (0.36-4.89) | (3.69 – 21.29) | (1.29-27.49) | (4.16 – 29.22) | (2.29 – 9.51) |
| **Female Juveniles** | **na** | **na** | **na** | **na** | **14** | **na** | **na** | **na** |
| **Mean (µg/ml)** | | | | | 14.40 ± 3.02 | | | |
| **Range** | | | | | (1.97 – 36.54) | | | |
| **Male Adults** | **12** | **8** | **9** | **9** | **14** | **17** | **na** | **na** |
| **Mean (µg/ml)** | 16.44 ± 3.06 | 2.56 ± 0.71 | 40.18 ± 10.43 | 12.14 ± 5.33 | 13.79 ± 1.76 | 9.67 ± 1.23 | | |
| **Range** | (6.05-45.76) | (0.88-6.95) | (7.10-87.06) | (0.30-16.00) | (6.45 – 23.70) | (0.68 – 24.54) | | |
| **Male Juveniles** | **na** | **na** | **na** | **na** | **8** | **na** | **na** | **na** |
| **Mean (µg/ml)** | | | | | 7.58 ± 2.64 | | | |
| **Range** | | | | | (2.43 – 25.23) | | | |

na, not available

*(Table 3.4 was reproduced from Chapter 2 – Contaminant Analysis)*

## 3.3 Results

### 3.3.1 Sex steroid concentrations in Ringed, Grey and Harbour seals

For statistical analysis, seal data were grouped according to species, reproductive stage, sex and age. The differences between groups were tested by analysis of variance (ANOVA). Significance levels were set at $p<0.05$ and $p<0.005$. Results are presented as means ± standard error (s.e.) and ranges (min- max).

Sex steroid concentrations detected in seal serum are presented in Table 3.6 and in faecal samples in Table 3.7.

### 3.3.2 Sex and age-dependent differences within species

*Harbour Seals, Moray Firth, Scotland*

All female and male Harbour seals from Moray Firth were sampled at Stage I of reproductive cycle (estrus / breeding), whereas only one from each sex was sampled during pregnancy in Stage II. Moray Firth, Harbour seals were the only group that could be tested for age dependent differences since all the rest of the populations consisted of adult seals. There was no statistically significant difference between juvenile female and male Harbour seals. When comparing female adult with juvenile sex steroid concentrations, it was observed that female adults had significantly lower 17-α-OHP levels ($p<0.05$) than juveniles (Fig.3.1) whereas there was no statistically significant difference observed for the other sex steroids. There was no statistically significant difference between male adult and juveniles. Sex steroid levels between female and male adults also showed no statistically significant difference.

*Harbour Seals, Abertay Sands, Scotland*

Female and male Harbour seals from Abertay Sands were compared at all the stages of the reproductive cycle except Stage II, since there was only one female sample available. In Stage I of the reproductive cycle females were found to have statistically significant higher P and 17α-OHP mean levels ($p<0.05$) compared to males. In Stage III females had statistically significant higher P ($p<0.005$), 17α-OHP ($p<0.05$) and A ($p<0.05$) mean levels compared to males, whereas in Stage IV only P levels were found to be statistically significant higher ($p<0.05$) in females compared to males (Fig.3.2).

**Table 3.6(a,b,c):** Sex steroid concentrations in Harbour Grey and Ringed seals

**a.) Harbour seals, Moray Firth, Scotland – Exposed population**

| **Sex Steroids**<br>**Units** | **P**<br>**(ng/ml)** | **17-α-OHP**<br>**(ng/ml)** | **E2**<br>**(ng/ml)** | **E3**<br>**(ng/ml)** | **T**<br>**(ng/ml)** | **A**<br>**(ng/ml)** | **DHEA-S**<br>**(µg/ml)** |
|---|---|---|---|---|---|---|---|
| **Age, Sex & Reproductive Stage** | | | | | | | |
| **Female Adults** | | | | | | | |
| **Stage I** | **24.00 ± 9.79**<br>(3.73 – 89.46) | **0.91 ± 0.14**<br>(0.30 – 1.94) | **0.12 ± 0.03**<br>(0.02 – 0.26) | **0.41 ± 0.16**<br>(0.003 – 1.73) | **1.02 ± 0.31**<br>(0.04 – 3.04) | **0.63 ± 0.13**<br>(0.19 – 1.45) | **0.07 ± 0.02**<br>(0.005 – 0.23) |
| **Stage IV*** | **70.00** | **7.92** | **0.84** | **1.81** | **4.73** | **2.41** | **0.81** |
| **Female Juveniles** | **14.73 ± 5.26**<br>(2.94 – 68.37) | **1.83 ± 0.32**<br>(0.63 – 5.04) | **0.17 ± 0.04**<br>(0.05 – 0.49) | **1.05 ± 0.35**<br>(0.06 – 4.04) | **2.11 ± 0.68**<br>(0.18 – 9.56) | **0.82 ± 0.17**<br>(0.26 – 2.17) | **0.12 ± 0.04**<br>(0.02 – 0.63) |
| **Male Adults** | | | | | | | |
| **Stage I** | **7.81 ± 3.94**<br>(0.53 – 40.00) | **1.12 ± 0.47**<br>(0.18 – 6.49) | **0.16 ± 0.05**<br>(0.02 – 0.68) | **1.55 ± 0.98**<br>(0.01 – 12.70) | **3.46 ± 1.64**<br>(0.15 – 19.45) | **0.86 ± 0.26**<br>(0.22 – 3.42) | **0.28 ± 0.26**<br>(0.01 – 1.63) |
| **Stage IV*** | **1.49** | **0.52** | **0.07** | **0.06** | **2.77** | **0.39** | **0.09** |
| **Male Juveniles** | **15.28 ± 1.48**<br>(2.16 – 59.82) | **2.79 ± 0.47**<br>(0.46 – 11.17) | **0.17 ± 0.02**<br>(0.02 – 0.98) | **2.29 ± 0.10**<br>(0.01 – 18.63) | **3.43 ± 0.3**<br>(0.29 – 21.38) | **1.43 ± 0.16**<br>(0.20 – 9.20) | **0.32 ± 0.03**<br>(bdl – 1.63) |

Stage IV*=only one sample available for this stage

**b.) Harbour seals, Abertay Sands, Scotland – Exposed population and Experimental Group (Exposed & Control)**

| Sex Steroids<br>Units | P<br>(ng/ml) | 17-α-OHP<br>(ng/ml) | E2<br>(ng/ml) | E3<br>(ng/ml) | T<br>(ng/ml) | A<br>(ng/ml) | DHEA-S<br>(µg/ml) |
|---|---|---|---|---|---|---|---|
| **Sex & Reproductive Stage** | | | | | | | |
| **Female Adults** | | | | | | | |
| **Stage I** | **40.18 ± 0.60**<br>(1.12 – 70.00) | **2.65 ± 0.45**<br>(0.67 – 5.36) | **0.16 ± 0.05**<br>(0.02 – 0.47) | **0.51 ± 0.20**<br>(0.09 – 2.02) | **1.18 ± 0.35**<br>(0.20 – 3.62) | **1.39 ± 0.35**<br>(0.49 – 3.70) | **0.21 ± 0.09**<br>(0.05 – 0.39) |
| **Stage II*** | **70.00** | **4.90** | **0.42** | **5.88** | **24.12** | **15.36** | **n.a.** |
| **Stage III** | **52.71 ± 9.74**<br>(11.34 – 72.00) | **2.42 ± 0.22**<br>(1.87 – 3.58) | **0.12 ± 0.02**<br>(0.05 – 0.17) | **0.95 ± 0.23**<br>(0.12 – 1.61) | **1.44 ± 0.36**<br>(0.21 – 2.59) | **2.58 ± 0.81**<br>(0.46 – 6.02) | **0.19 ± 0.13**<br>(0.06 – 0.13) |
| **Stage IV** | **22.53 ± 9.39**<br>(11.54 – 41.22) | **1.95 ± 0.31**<br>(1.33 – 2.32) | **0.21 ± 0.06**<br>(0.10 – 0.32) | **2.60 ± 2.27**<br>(0.23 – 7.15) | **2.62 ± 1.99**<br>(0.63 – 4.60) | **1.00 ± 0.72**<br>(0.28 – 1.72) | **0.17 ± 0.04**<br>(0.13 – 0.21) |
| **Male Adults** | | | | | | | |
| **Stage I** | **1.81 ± 0.40**<br>(0.98 – 3.34) | **0.75 ± 0.19**<br>(0.30 – 1.24) | **0.07 ± 0.02**<br>(0.04 – 0.12) | **0.59 ± 0.25**<br>(0.25 – 1.57) | **6.75 ± 4.35**<br>(0.32 – 22.59) | **0.82 ± 0.15**<br>(0.53 – 1.35) | **0.07 ± 0.01**<br>(0.05 – 0.10) |
| **Stage II** | **4.59 ± 0.92**<br>(1.21 – 7.80) | **0.87 ± 0.14**<br>(0.36 – 1.28) | **0.09 ± 0.02**<br>(0.04 – 0.14) | **0.52 ± 0.13**<br>(0.18 – 0.93) | **2.53 ± 1.01**<br>(0.38 – 6.93) | **0.60 ± 0.12**<br>(0.20 – 0.92) | **0.09 ± 0.02**<br>(0.01 – 0.14) |
| **Stage III** | **9.15 ± 3.30**<br>(0.74 – 45.00) | **1.42 ± 0.26**<br>(0.19 – 3.41) | **0.10 ± 0.01**<br>(0.04 – 0.21) | **0.86 ± 0.23**<br>(0.15 – 3.19) | **2.59 ± 0.67**<br>(0.47 – 9.46) | **0.89 ± 0.11**<br>(0.27 – 1.50) | **0.22 ± 0.07**<br>(0.20 – 9.20) |
| **Stage IV** | **4.95 ± 1.10**<br>(1.76 – 7.84) | **1.33 ± 0.14**<br>(0.54 – 1.93) | **0.13 ± 0.03**<br>(0.03 – 0.24) | **1.47 ± 0.39**<br>(0.13 – 3.27) | **8.89 ± 2.67**<br>(0.42 – 24.82) | **1.30 ± 0.27**<br>(0.36 – 2.71) | **0.45 ± 0.11**<br>(0.03 – 0.88) |
| **Harbour Seals (Experimental Group)** | | | | | | | |
| **Exposed** | | | | | | | |
| **Stage IV** | **11.35 ± 5.67**<br>(0.10 – 58.9) | **1.19 ± 0.45**<br>(0.07 – 3.85) | **0.11 ± 0.05**<br>(0.47 – 0.01) | **0.08 ± 0.04**<br>(b.d.l. – 0.41) | **n.a.** | **n.a.** | **n.a.** |
| **Control** | | | | | | | |
| **Stage IV** | **27.01 ± 5.92**<br>(0.09 – 70.00) | **2.43 ± 0.77**<br>(0.01 – 10.40) | **0.22 ± 0.05**<br>(0.03 – 0.66) | **0.53 ± 0.23**<br>(0.01 – 2.61) | **n.a.** | **n.a.** | **n.a.** |

Stage II* = only one sample available; n.a. = not available; b.d.l. = below detection limit

**c.) Ringed seals, Baltic Sea (Exposed) and Svalbard (Control) and Grey seals, Baltic Sea (Exposed) and Sable Island (Control)**

| Sex Steroids<br>Units | | P<br>(ng/ml) | 17-α-OHP<br>(ng/ml) | E2<br>(ng/ml) | E3<br>(ng/ml) | T<br>(ng/ml) | A<br>(ng/ml) | DHEA-S<br>(µg/ml) |
|---|---|---|---|---|---|---|---|---|
| **Ringed Seals (Baltic Sea – Exposed)** | | | | | | | | |
| **Female Adults** | | | | | | | | |
| | **Stage II** | **6.51 ± 0.90**<br>(2.73 – 9.88) | **0.50 ± 0.08**<br>(0.15 – 0.76) | **0.08 ± 0.02**<br>(0.02 – 0.19) | **0.08 ± 0.03**<br>(b.d.l. – 0.24) | **0.27 ± 0.17**<br>(b.d.l. – 1.07) | **0.33 ± 0.18**<br>(0.02 – 1.01) | **0.18 ± 0.05**<br>(0.04 – 0.44) |
| **Male Adults** | | | | | | | | |
| | **Stage II** | **0.11 ± 0.04**<br>(b.d.l. – 0.25) | **0.11 ± 0.04**<br>(b.d.l. – 0.54) | **0.04 ± 0.01**<br>(0.02 – 0.06) | **0.19 ± 0.10**<br>(b.d.l. – 1.22) | **2.74 ± 1.54**<br>(0.03 – 18.95) | **0.21 ± 0.03**<br>(0.05 – 0.40) | **0.13 ± 0.03**<br>(0.01 – 0.32) |
| **Ringed Seals (Svalbard – Control)** | | | | | | | | |
| **Female Adults** | | | | | | | | |
| | **Stage II** | **18.33 ± 7.77**<br>(0.84 – 55.00) | **3.10 ± 1.38**<br>(0.85 – 9.90) | **0.28 ± 0.09**<br>(0.05 – 0.77) | **1.66 ± 0.76**<br>(0.10 – 5.69) | **1.05 ± 0.69**<br>(0.35 – 1.74) | **0.36 ± 0.18**<br>(0.18 – 0.53) | **0.18 ± 0.08**<br>(0.05 – 0.72) |
| **Male Adults** | | | | | | | | |
| | **Stage II** | **2.12 ± 1.48**<br>(b.d.l. – 7.54) | **0.54 ± 0.25**<br>(b.d.l. – 1.89) | **0.10 ± 0.06**<br>(0.02 – 0.44) | **0.80 ± 0.39**<br>(0.16 – 3.09) | **3.33 ± 1.30**<br>(0.23 – 8.57) | **0.31 ± 0.11**<br>(0.07 – 0.83) | **0.20 ± 0.06**<br>(0.05 – 0.49) |
| **Grey Seals (Baltic Sea – Exposed)** | | | | | | | | |
| **Female Adults** | | | | | | | | |
| | **Stage II** | **27.36 ± 5.92**<br>(0.82 – 82.00) | **2.68 ± 0.54**<br>(0.28 – 6.67) | **0.13 ± 0.03**<br>(0.03 – 0.48) | **1.02 ± 0.48**<br>(b.d.l. – 7.44) | **1.48 ± 0.74**<br>(0.08 – 5.06) | **0.48 ± 0.16**<br>(0.11 – 1.55) | **0.29 ± 0.09**<br>(0.0003 – 1.22) |
| **Male Adults** | | | | | | | | |
| | **Stage II** | **0.88 ± 0.30**<br>(0.21 – 2.22) | **0.66 ± 0.22**<br>(0.05 – 2.38) | **n.a.** | **2.85 ± 0.99**<br>(0.31 – 10.38) | **2.96 ± 1.02**<br>(0.56 – 9.15) | **0.70 ± 0.23**<br>(0.16 – 2.42) | **0.22 ± 0.04**<br>(0.07 – 0.53) |
| **Grey Seals (Sable Island - Control)** | | | | | | | | |
| **Female Adults** | | | | | | | | |
| | **Stage II** | **32.29 ± 7.25**<br>(6.04 – 85.25) | **8.72 ± 2.95**<br>(0.91 – 33.36) | **0.46 ± 0.19**<br>(0.07 – 2.10) | **13.38 ± 7.71**<br>(0.90 – 80.00) | **6.33 ± 3.49**<br>(0.09 – 12.14) | **3.65 ± 2.67**<br>(0.20 – 8.90) | **0.61 ± 0.49**<br>(0.07 – 4.98) |
| **Male Adults** | | | | | | | | |
| | **Stage II** | **14.34 ± 10.09**<br>(0.57 – 54.25) | **3.99 ± 1.82**<br>(1.34 – 18.42) | **0.56 ± 0.16**<br>(0.06 – 1.30) | **28.77 ± 10.12**<br>(2.05 – 70.00) | **33.59 ± 9.74**<br>(2.85 – 85.82) | **2.85 ± 0.97**<br>(0.56 – 10.16) | **0.26 ± 0.04**<br>(0.08 – 0.41) |

n.a. = not available; b.d.l. = below detection level

**Table 3.7** Sex steroid concentrations in faecal samples from UK Harbour and Grey seals

| Sex Steroids (ng/ml) | | E1 | E2 | E3 |
|---|---|---|---|---|
| **Tees Estuary Harbour seal** | | | | |
| | **Stage I** | **337.26 ± 99.89** (32.00 – 597.12) | **4.23 ± 1.27** (2.10 – 9.08) | **6.71 ± 2.05** (2.02 – 12.74) |
| | **Stage II** | **361.96 ± 50.54** (130.34 – 1023.04) | **3.78 ± 0.49** (0.80 – 10.03) | **9.67 ± 1.15** (1.60 – 18.50) |
| | **Stage III** | **324.89 ± 88.77** (36.58 – 752.00) | **2.66 ± 0.16** (1.95 – 3.38) | **8.28 ± 1.11** (4.77 – 13.50) |
| | **Stage IV** | **243.20 ± 50.95** (75.55 – 605.12) | **5.00 ± 0.97** (2.02 – 12.92) | **17.00 ± 8.13** (2.30 – 97.47) |
| **Donna Nook (Harbour & Grey seals)** | | | | |
| | **Stage III** | **334.04 ± 57.43** (100.29 – 664.96) | **6.08 ± 1.02** (2.95 – 11.97) | **19.23 ± 8.28** (1.73 – 105.50) |
| **Blakeney Point (Harbour seals)** | | | | |
| | **Stage III** | **210.85 ± 65.71** (42.59 – 458.88) | **2.98 ± 0.51** (0.78 – 5.75) | **7.40 ± 1.51** (0.61 – 15.26) |

n.a. = not available; b.d.l. = below detection level

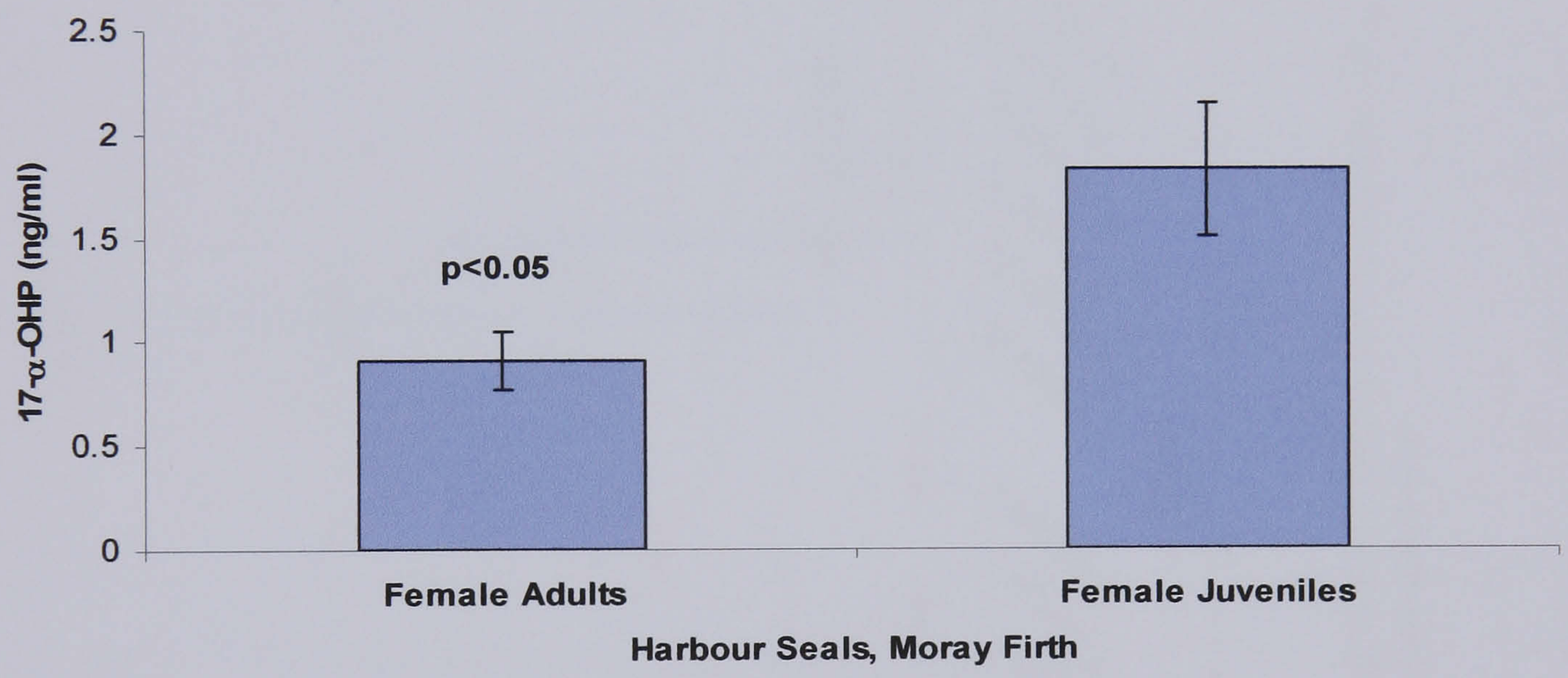



**Figure 3.1:** Age-dependent difference in Harbour seals, Moray Firth
(Female Adults, n:18; Female Juveniles, n:14 )

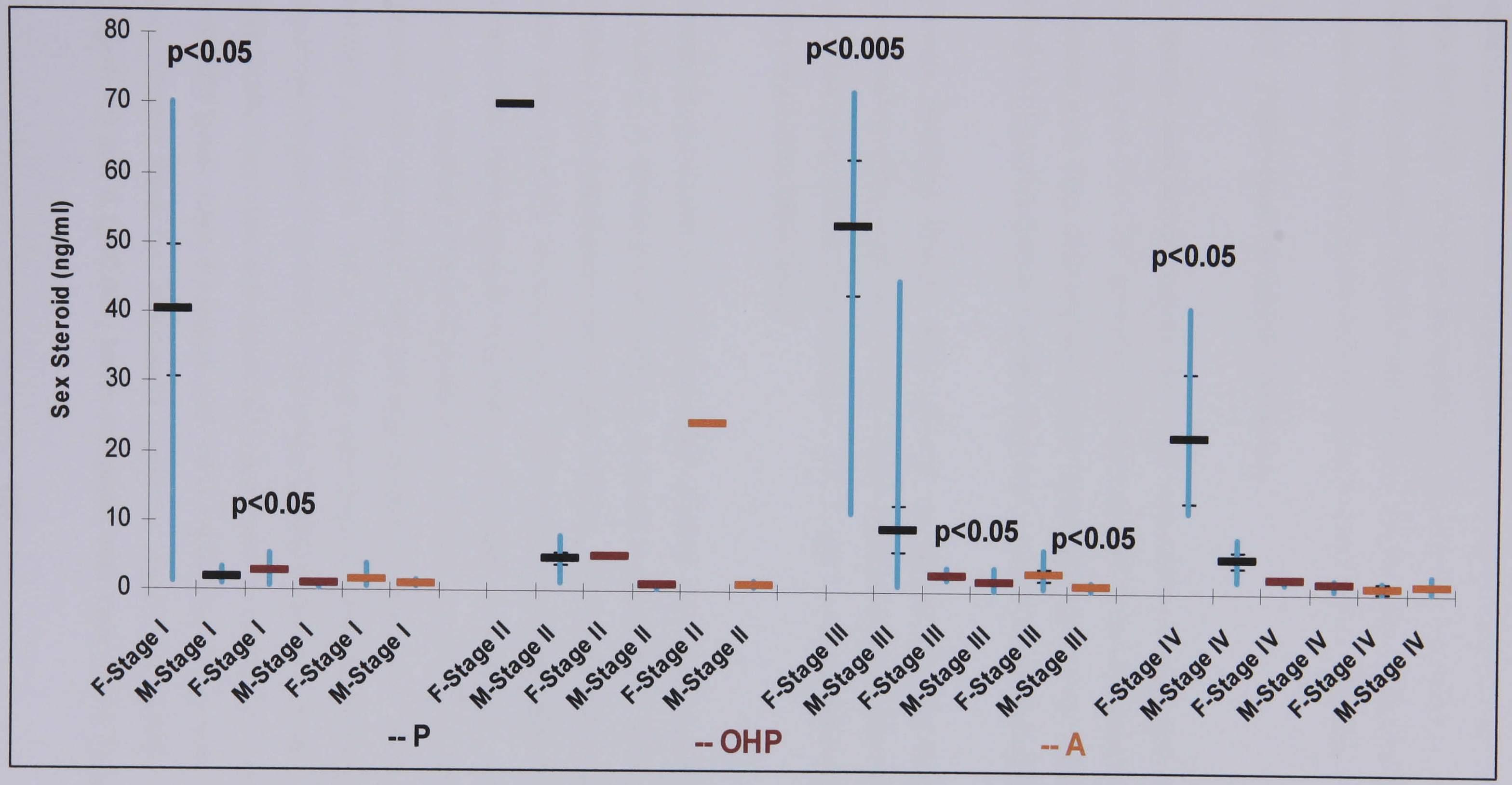



**Figure 3.2:** Sex-dependent differences in Harbour seals from Abertay Sands through the reproductive cycle
P, Progesterone; OHP, 17α-OHProgesterone; A, Androstenedione

*Ringed and Grey Seals*

The serum sex steroid concentrations for Ringed and Greys seals are presented as means ±SE and ranges in Table 3.6c.

P and 17α-OHP mean levels were statistically significant higher ($p<0.005$) in female compared to male Baltic Ringed seals as well as in female compared to male Baltic Grey seals ($p<0.005$), whereas the remaining sex steroids under study showed no statistically significant difference (Fig.3.3 and Fig.3.4). Female and male Svalbard Ringed and Sable Island Grey seal serum sex steroids showed no statistically significant difference.

### 3.3.3 Population and Species Differences

A species comparison between the Baltic seals showed statistically significantly higher P ($p<0.05$) and 17α-OHP ($p<0.005$) levels in female Grey compared to female Ringed seals whereas male Grey seals had statistically significant higher P ($p<0.05$), 17α-OHP ($p<0.05$), E3 ($p<0.05$) and A ($p<0.05$) levels compared to male Ringed seals (Fig.3.3 & Fig.3.4).

Female Svalbard Ringed seals showed no statistically significant difference when compared to Grey seals from Sable Island whereas male Svalbard Ringed seals showed statistically significant lower mean E2, E3, T and A concentrations ($p<0.05$) compared to Grey seals from Sable Island.

Female Harbour seals from Abertay Sands showed statistically significant higher 17α-OHP ($p<0.005$), A ($p=0.05$) and DHEA-S ($p<0.05$) concentrations when compared to female Harbour seals from Moray Firth (Table 3.6a, b), whereas male Harbour seals from the two study sites showed no sex steroid statistically significant differences. Comparison of Moray Firth Harbour seals with Grey and Ringed seals was not possible since the later were only sampled in Stage II (Table 3.6c). Moreover, comparison between Abertay Sands females with Grey and Ringed seal was not possible since there was only one seal that was sampled in Stage II. Male Harbour seals from Abertay Sands however, had statistically significant higher P ($p<0.005$) and lower DHEA-S ($p=0.05$) levels when compared with male Baltic Grey seals and statistically significant lower E2, E3, T ($p<0.05$) and DHEA-S ($p<0.005$) levels when compared with male Sable Island Grey seals. Male Harbour seals from Abertay Sands had statistically significant higher P ($p<0.005$), 17α-OHP ($p<0.005$), E2 ($p<0.05$) and A ($p<0.005$) levels compared with male Baltic Ringed seals whereas no

statistically significant difference was observed for any of the sex steroids when compared to Svalbard male Ringed seals.

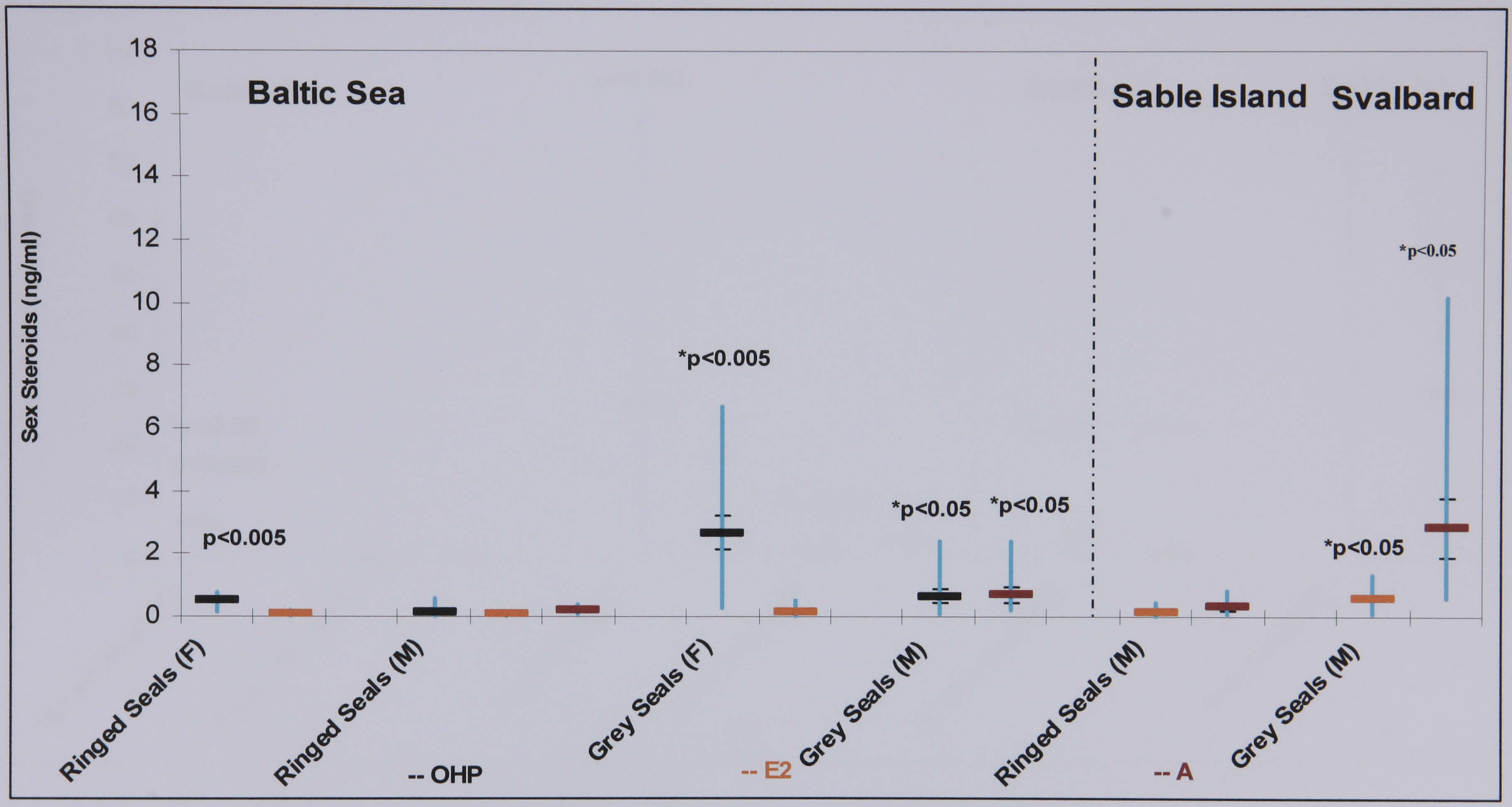



**Figure 3.3:** Sex and geographical variation (*) of 17α-OH Progesterone (OH), 17β-Estradiol (E2) and Androstenedione (A) in Grey and Ringed Seals

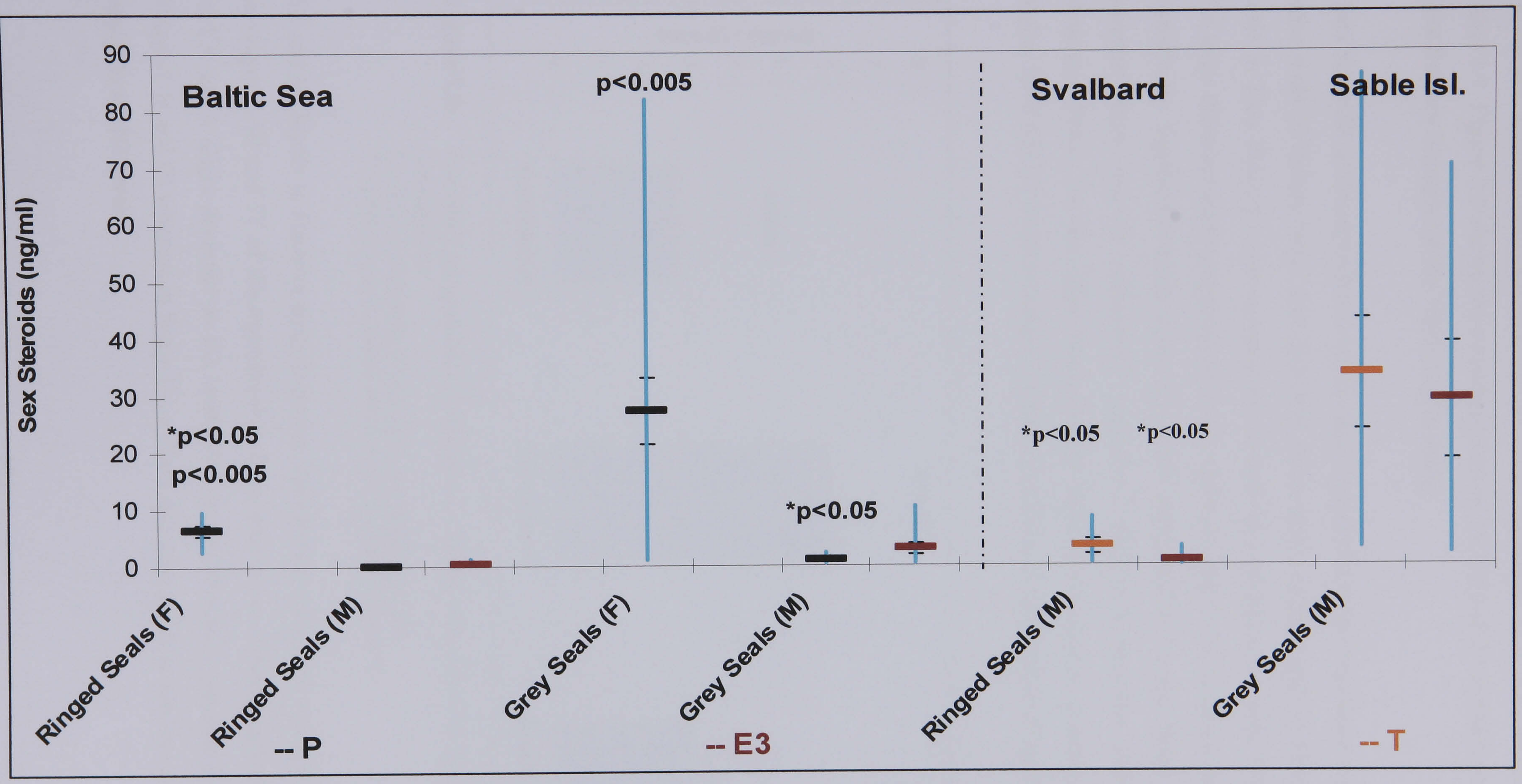



**Figure 3.4:** Sex and geographical variation (*) of Progesterone (P), Estriol (E3) and Testosterone (T) in Grey and Ringed Seals

### 3.3.4 Sex steroid concentration in faecal samples

Faecal sex steroid concentrations are presented as means ±s.e. and ranges (min-max) in Table 3.7. Figure 3.5 shows the concentrations of E1, E2 and E3 in Harbour seals from the Tees Estuary through out the reproductive cycle.

Only Stage III of the reproductive cycle was compared between the three study populations since seals in Donna Nook and Blakeney Point were sampled only in December whereas seals in Tees Estuary were sampled all through the reproductive cycle. When comparing the three different study populations it was observed that Tees Estuary Harbour seals had statistically significant mean lower E2 levels compared to Donna Nook ($p<0.05$) seals whereas there was no statistically significant difference between Tees Estuary and Blakeney Point. On the other hand, Donna Nook seals showed statistically significant higher ($p<0.05$) E2 levels compared to Blakeney Point Harbour seals (Fig. 3.8).

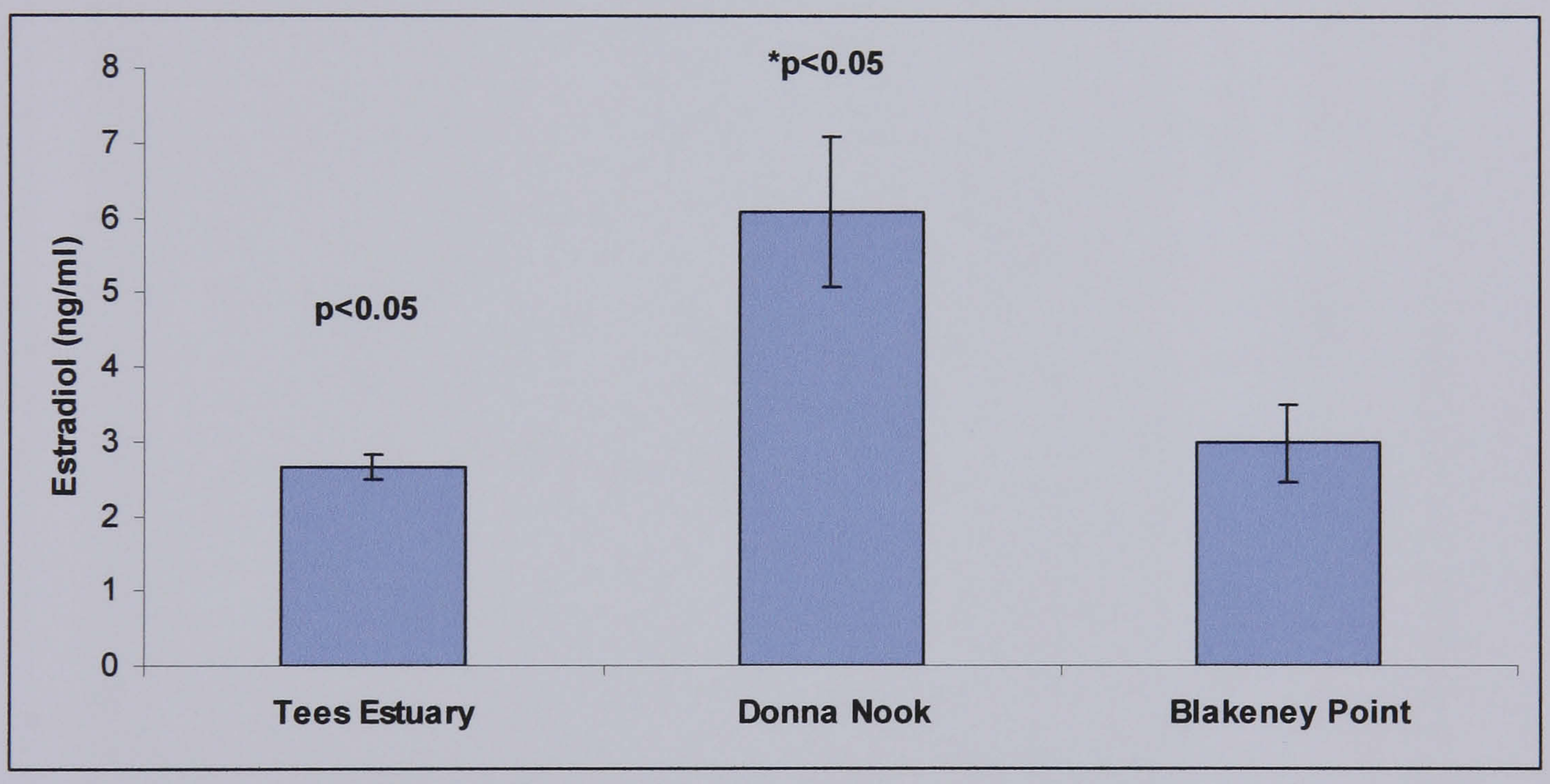



**Figure 3.8:** Estradiol geographical variations in faecal samples from the three UK study populations
(*, difference between Donna Nook and Blakeney Point)
Tees Estuary, n:47; Donna Nook, n:13; Blakeney Point, n:10

E2 and E3 levels in Harbour seals from the Tees Estuary (Fig.3.9) showed a parallel trend in Stages I, III and IV of the reproductive cycle whereas in Stage II there was an increase in E3 and a slight decrease in E2 levels. E1 levels showed a parallel trend with E3 in Stages I, II and III whereas in Stage IV, a sharp decrease in E1 levels was observed and the opposite for E3 levels.

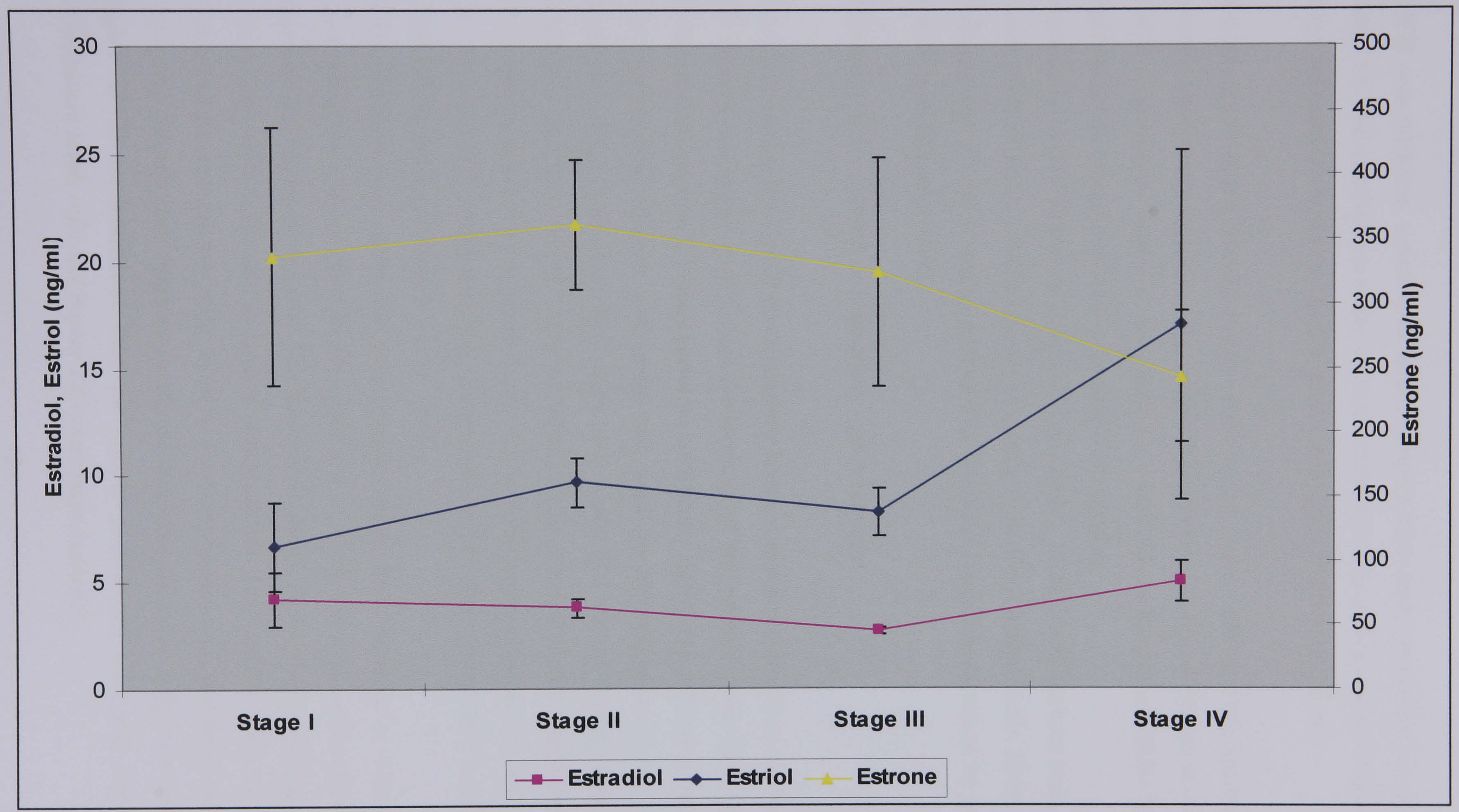



**Figure 3.9:** Estradiol, Estriol and Estrone faecal concentrations in Harbour seals (Tees Estuary), for all stages of the reproductive cycle

### 3.3.5 High-exposed vs. low-exposed populations

#### *3.3.5.1 Serum Samples*

ΣPCB concentrations detected in serum samples from each population are presented in Table 3.5. Full justification of the exposure classifications is provided in Chapter 2. These data confirmed the exposure classification for the present study as follows:

**Female seals:** Baltic Ringed seals > Baltic Grey seals > Moray Firth Harbour seals > Abertay Sands Harbour seals > Sable Island Grey seals > Svalbard Ringed seals. From the ΣPCB concentration results it was concluded that Svalbard Ringed and Sable Island Grey seals would be treated as control populations for females.

**Male seals:** Baltic Grey seals > Baltic Ringed seals > Moray Firth Harbour seals > Abertay Sands Harbour seals > Sable Island Grey seals > Svalbard Ringed seals. From the ΣPCB concentration results it was concluded that Svalbard Ringed seals would be treated as a control population for males.

*Validation of sex steroids as biomarkers – dose response*

When comparing sex steroid concentrations between exposed and control Harbour seals of the experimental group even though the levels of all sex steroids under study were higher in the exposed compared to the control no statistically significant difference was observed. The experimental Harbour seal group was used for the validation of sex steroids as biomarkers (Fig. 3.10) since these seals were a part of a control feeding study with fish taken from Wadden Sea and Northeast Atlantic. This group of Harbour seals were sampled at late pregnancy and the average ± s.d. of total PCB concentration in the exposed group (n=11) was 25642 ± 9699 ng/g lipid weight and in the control group (n=12) was 5336ng/g ± 1632 lipid weight (Reijnders, *pers. Comm.*).

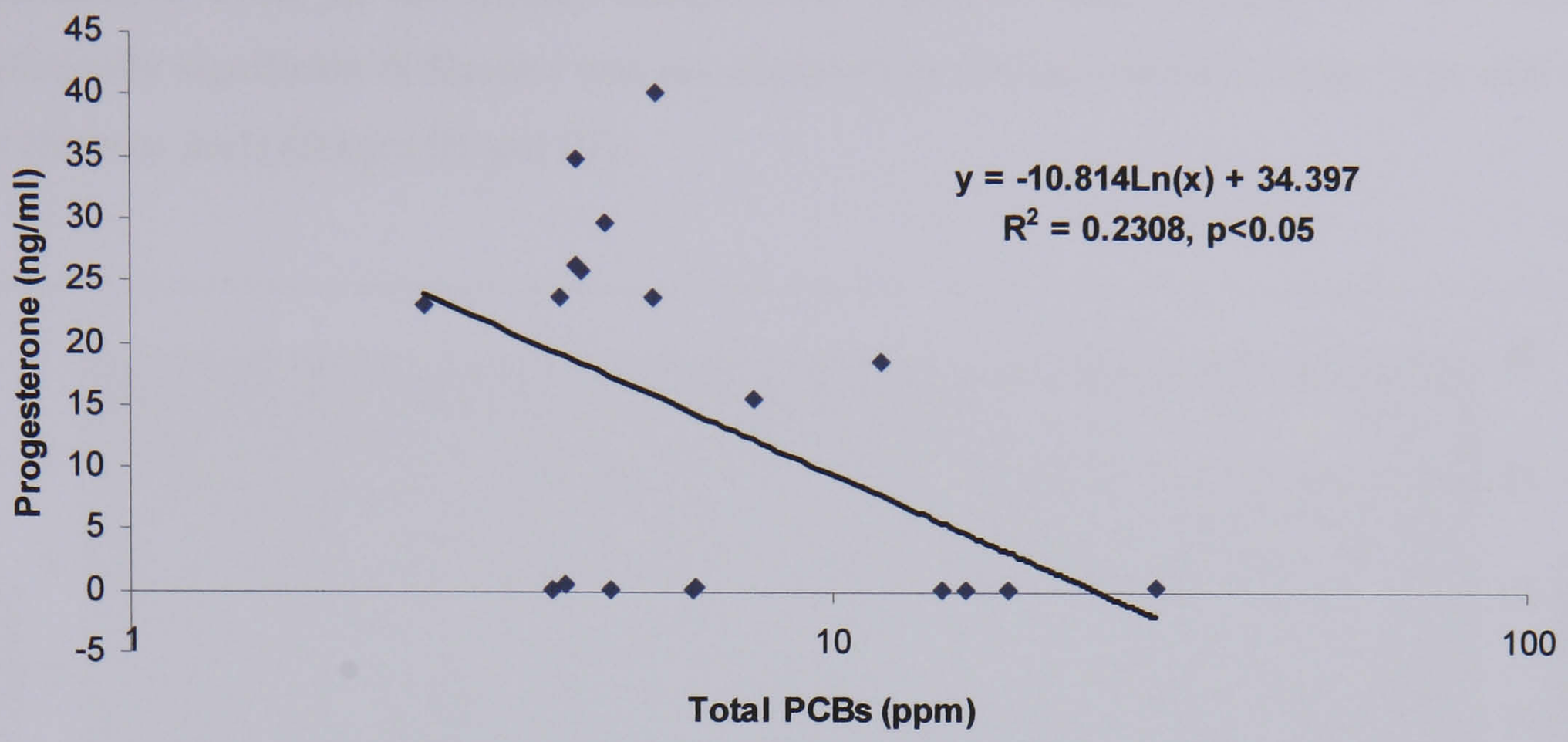
Since Harbour seals from the experimental group were sampled at late pregnancy, control/baseline data of sex steroids could not be compared with seals sampled at other stages of the reproductive cycle. For this reason, levels from Svalbard Ringed and Sable Island Grey seals were used as control concentrations in this study for seals sampled at Stage II of the reproductive stage. Baseline levels for juveniles are not presented since only one group (Moray Firth, Harbour seals) was available and these seals were found to have higher ΣPCB concentrations than the control populations (*For full justification of contaminant levels refer to Chapter 2-Contaminant Analysis*).

**Table 3.8:** 'Control / Baseline' steroid levels for Stage II and IV of reproductive cycle for Harbour, Ringed and Grey Seals

| Sex Steroids<br>Units | | P<br>(ng/ml) | 17-α-OHP<br>(ng/ml) | E2<br>(ng/ml) | E3<br>(ng/ml) | T<br>(ng/ml) | A<br>(ng/ml) | DHEA-S<br>(µg/ml) |
|---|---|---|---|---|---|---|---|---|
| **Ringed Seals (Svalbard – Control)** | | | | | | | | |
| **Female Adults** | | | | | | | | |
| | **Stage II** | **18.33 ± 7.77**<br>(0.84 – 55.00) | **3.10 ± 1.38**<br>(0.85 – 9.90) | **0.28 ± 0.09**<br>(0.05 – 0.77) | **1.66 ± 0.76**<br>(0.10 – 5.69) | **1.05 ± 0.69**<br>(0.35 – 1.74) | **0.36 ± 0.18**<br>(0.18 – 0.53) | **0.18 ± 0.08**<br>(0.05 – 0.72) |
| **Male Adults** | | | | | | | | |
| | **Stage II** | **2.12 ± 1.48**<br>(b.d.l. – 7.54) | **0.54 ± 0.25**<br>(b.d.l. – 1.89) | **0.10 ± 0.06**<br>(0.02 – 0.44) | **0.80 ± 0.39**<br>(0.16 – 3.09) | **3.33 ± 1.30**<br>(0.23 – 8.57) | **0.31 ± 0.11**<br>(0.07 – 0.83) | **0.20 ± 0.06**<br>(0.05 – 0.49) |
| **Grey Seals (Sable Island – Control)** | | | | | | | | |
| **Female Adults** | | | | | | | | |
| | **Stage II** | **32.29 ± 7.25**<br>(6.04 – 85.25) | **8.72 ± 2.95**<br>(0.91 – 33.36) | **0.46 ± 0.19**<br>(0.07 – 2.10) | **13.38 ± 7.71**<br>(0.90 – 80.00) | **6.33 ± 3.49**<br>(0.09 – 12.14) | **3.65 ± 2.67**<br>(0.20 – 8.90) | **0.61 ± 0.49**<br>(0.07 – 4.98) |
| **Male Adults** | | | | | | | | |
| | **Stage II** | **14.34 ± 10.09**<br>(0.57 – 54.25) | **3.99 ± 1.82**<br>(1.34 – 18.42) | **0.56 ± 0.16**<br>(0.06 – 1.30) | **28.77 ± 10.12**<br>(2.05 – 70.00) | **33.59 ± 9.74**<br>(2.85 – 85.82) | **2.85 ± 0.97**<br>(0.56 – 10.16) | **0.26 ± 0.04**<br>(0.08 – 0.41) |
| **Harbour Seals (Experimental Group)** | | | | | | | | |
| **Control** | | | | | | | | |
| | **Stage IV** | **27.01 ± 5.92**<br>(0.09 – 70.00) | **2.43 ± 0.77**<br>(0.01 – 10.40) | **0.22 ± 0.05**<br>(0.03 – 0.66) | **0.53 ± 0.23**<br>(0.01 – 2.61) | **n.a.** | **n.a.** | **n.a.** |

n.a. = not available; b.d.l. = below detection level

a.)




b.)

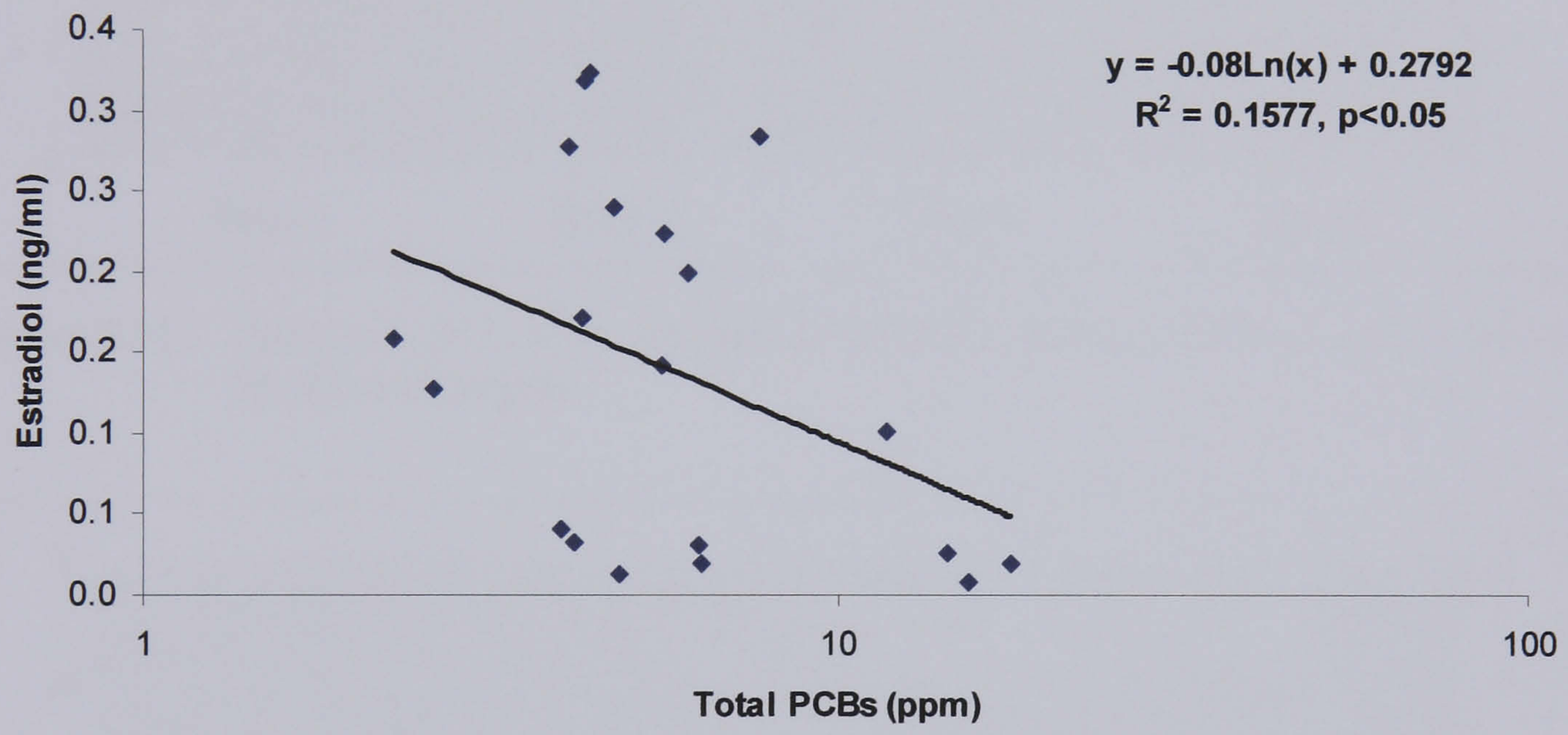



**Figure 3.10:** Stage IV: ΣPCBs Progesterone (a) and Estradiol (b) concentrations in female Harbour Seals – Experimental Group

From figure 3.10a,b it can be seen that Harbour seal (Experimental group) serum P and E2 concentrations were negatively correlated with ΣPCBs in dose-dependent manner and this relationship was statistically significant ($p<0.05$). There was no statistically significant correlation between ΣPCBs, 17α-OHP and E3. It was not possible to test any relationship between T, A and DHEA-S with ΣPCBs in Harbour seals from the experimental group since analysis of these steroids was not possible at the time of the experiment.

ΣPCBs and sex steroid concentrations according to species and stage of the reproductive cycle for all females are presented in figures 3.10, 11 and 12 and for all males in figures 3.13.

In females, statistically significance difference was observed for all the stages of the reproductive cycle in all species except from Harbour seals at Stage III. In males, statistically significant difference was not observed in for Grey seals in Stage II as well as for Harbour seals (Stages III and IV).

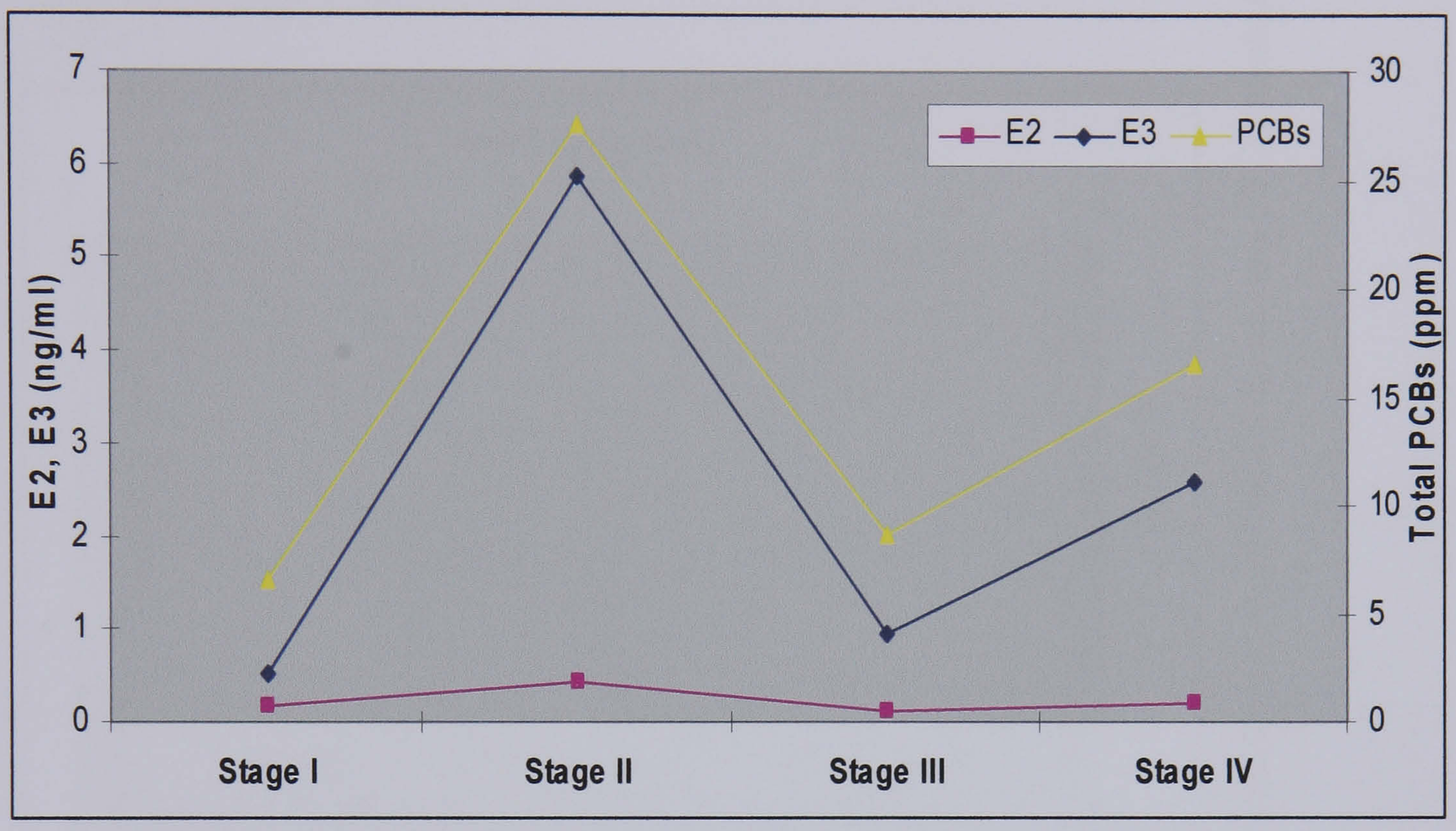



**Figure 3.11:** Estrogens (E2, E3) and ΣPCB levels in female Harbour seals (Abertay Sands) (All stages)

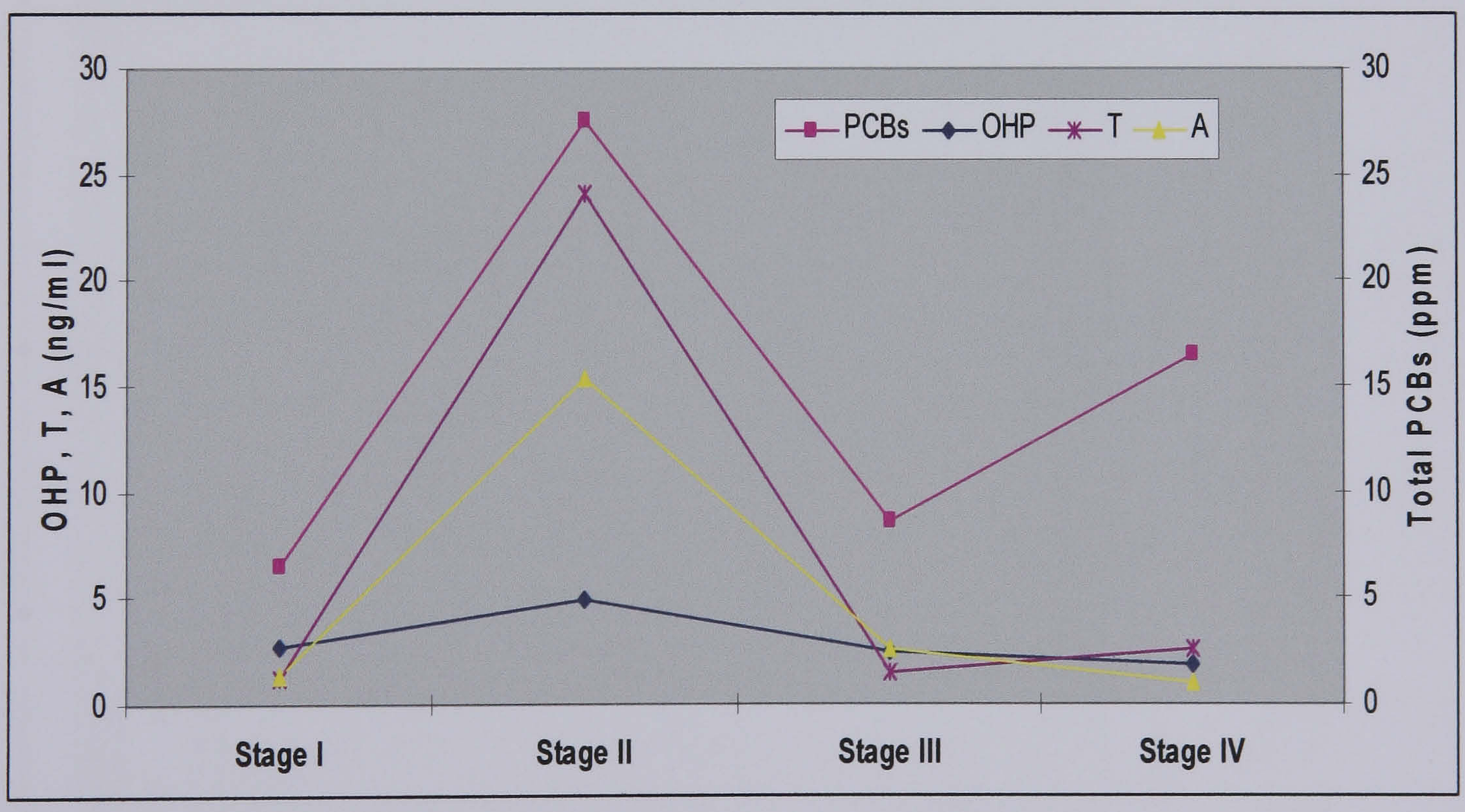



**Figure 3.12:** 17α-OH-Progesterone (OHP), Testosterone (T), Androstenedione (A) and ΣPCB levels in female Harbour seals (Abertay Sands) (All stages)

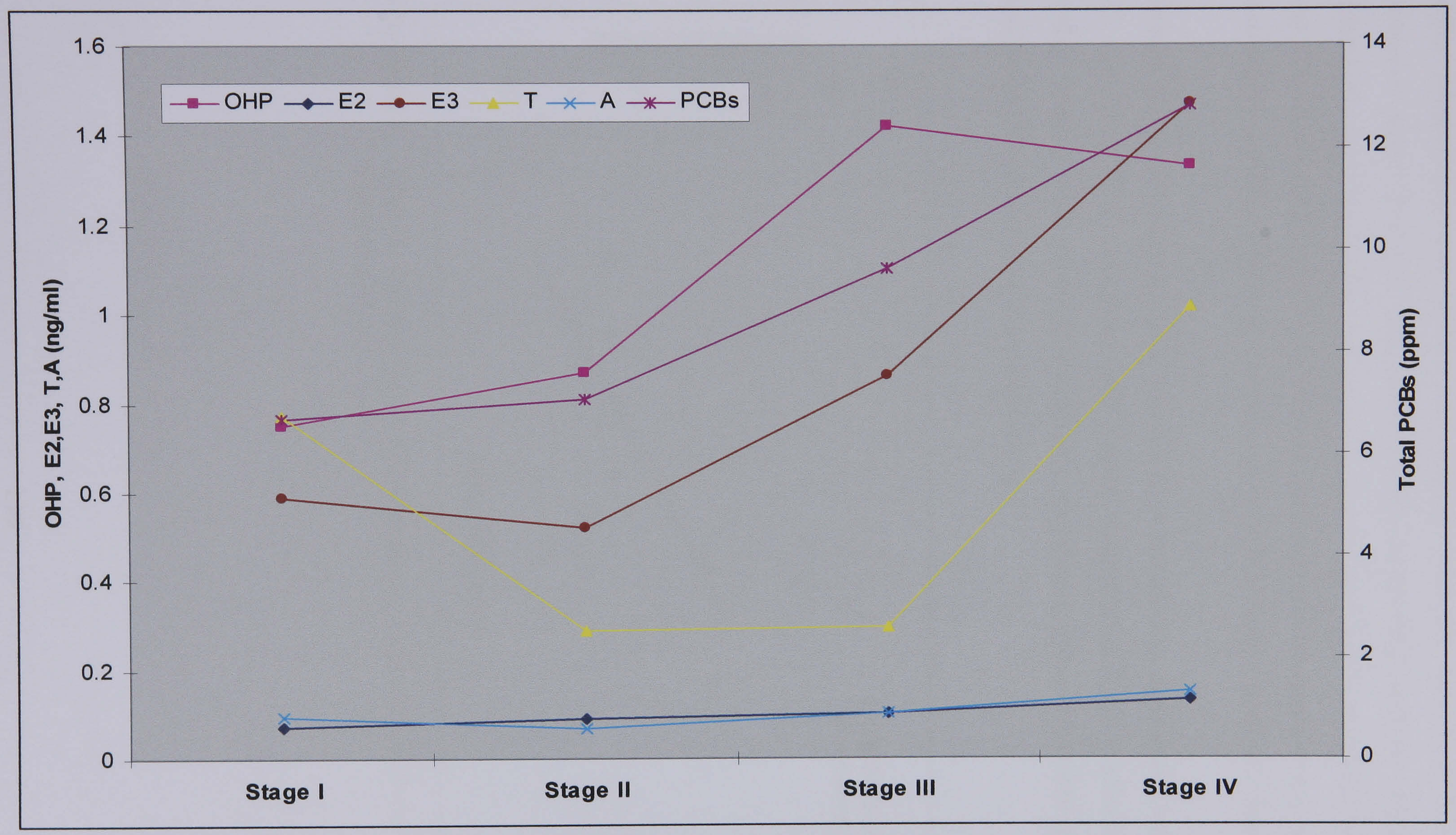



**Figure 3.13:** 17α-OH-Progesterone (OHP), 17β-Estradiol (E2), Estriol (E3), Testosterone (T), Androstenedione (A) and ΣPCB levels in male Harbour seals (Abertay Sands) (All stages)

The biomarker (BM) expression over a gradient of contaminant exposure was investigated using linear regressions (Fig. 3.14 to 3.28). To identify specific populations affected by ED (being at risk), BM expression in exposed groups was compared with control populations and differences were tested using student t-test. Where BM expression was statistically significant, populations were classified at risk from ED.

Baltic Ringed and Grey seals were compared with the control populations from the reference areas (Svalbard and Sable Island respectively) and the results indicated a statistically significant difference between female Baltic and Sable Island Grey seals for E2 and 17α-OHP ($p<0.05$). When the male seals from the above populations were compared a statistically significant difference in E3 and A ($p<0.05$) and T ($p<0.005$) was observed.

Female Baltic Ringed seals were found to have statistically significant lower P, 17α-OHP, E2 and E3 ($p<0.05$) compared with female Ringed seals from Svalbard, whereas male Baltic Ringed seals showed statistically significant lower 17α-OHP ($p<0.05$) levels compared with males from the control group.

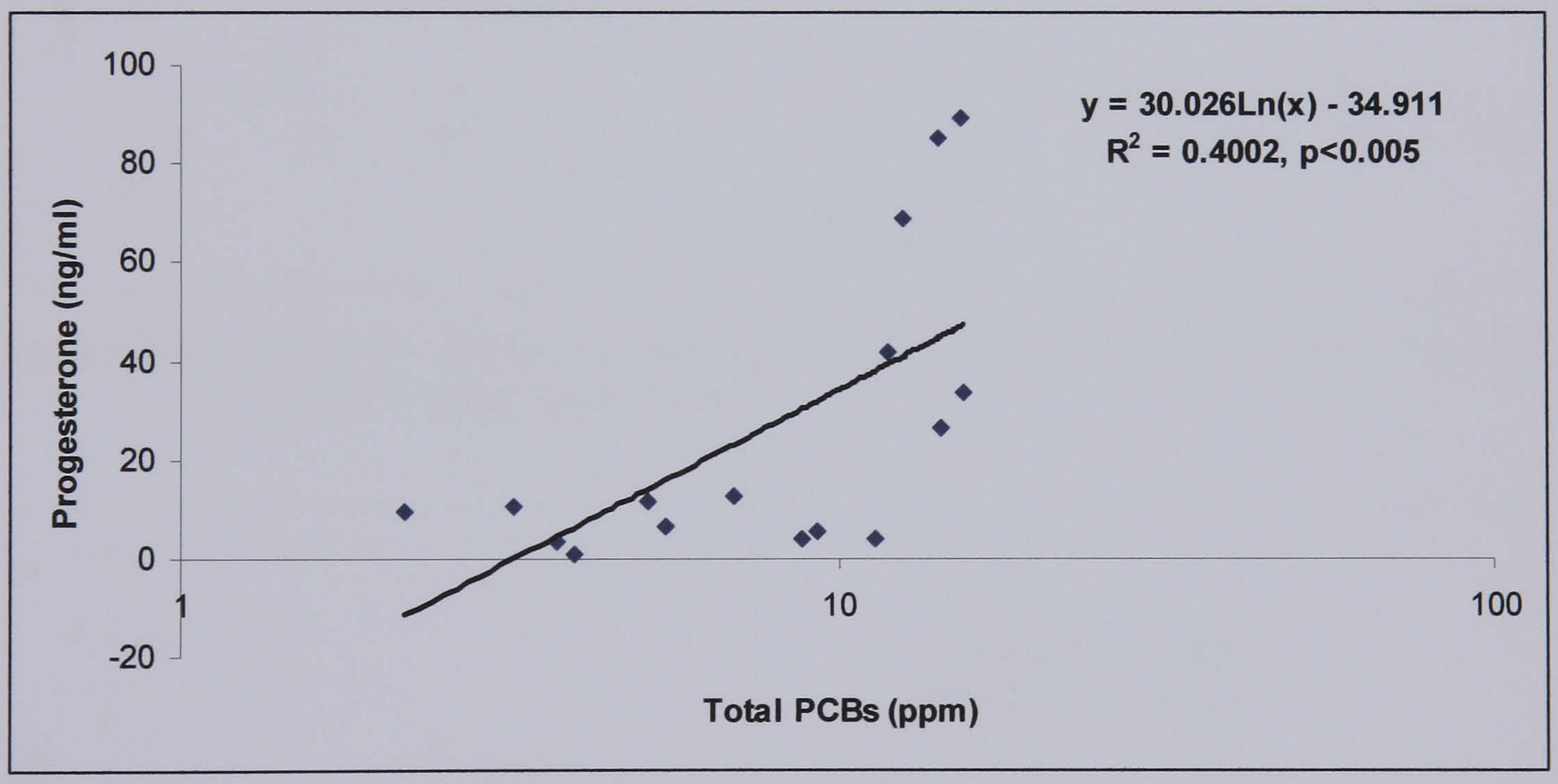



**Figure 3.14:** Stage I: ΣPCBs and P concentrations in female Harbour Seals (Abertay Sands and Moray Firth)

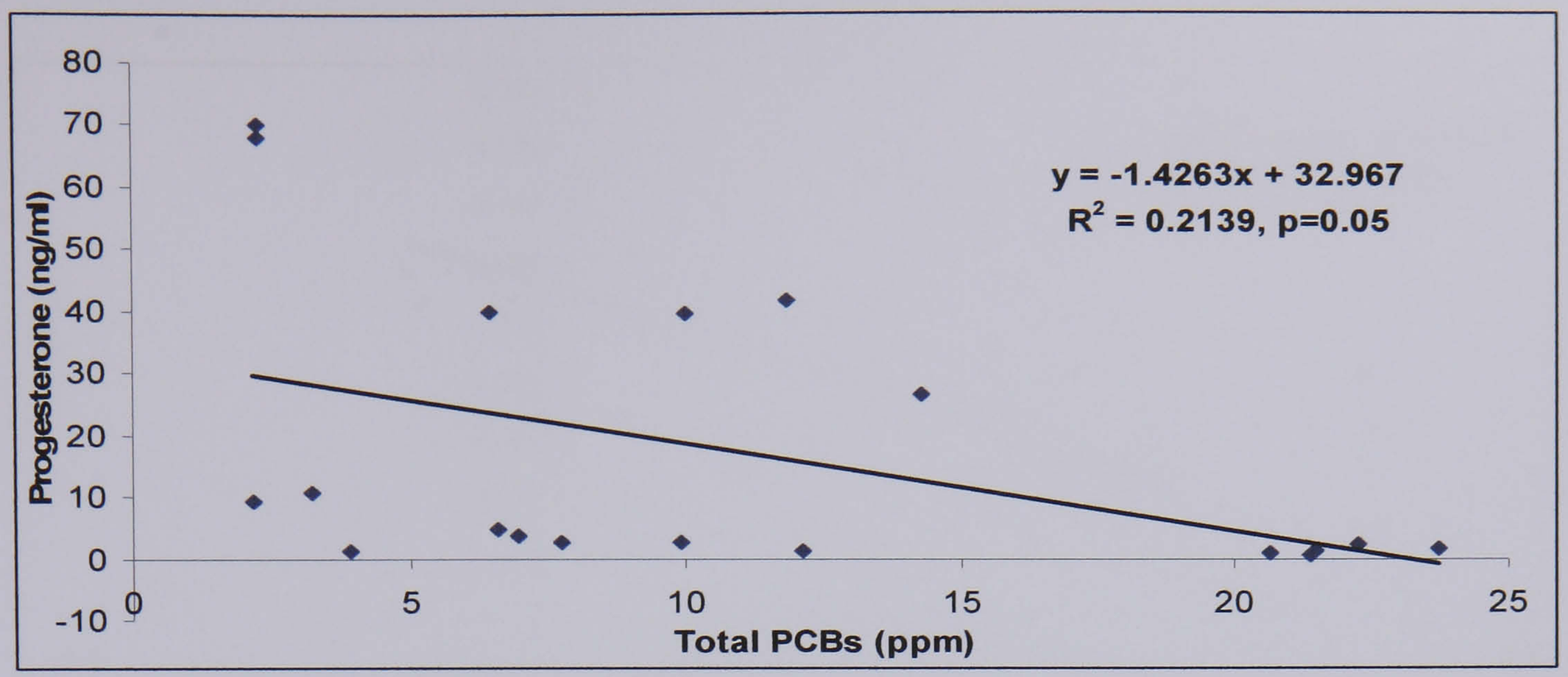



**Figure 3.15:** Stage I: ΣPCBs and P concentrations in male Harbour Seals (Abertay Sands and Moray Firth)

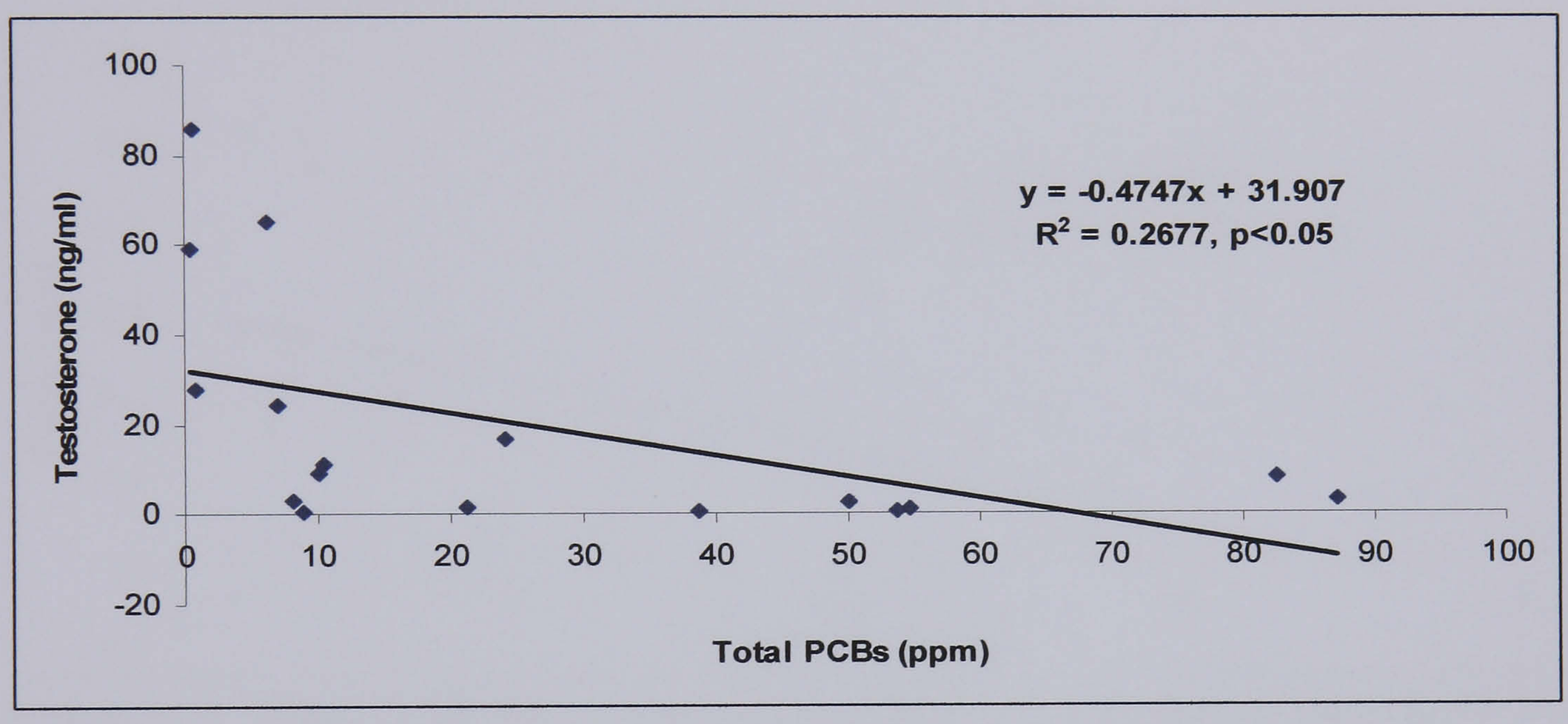



**Figure 3.16:** Stage II: ΣPCBs and testosterone (T) concentrations in male Grey seals (Baltic Sea and Sable Island)

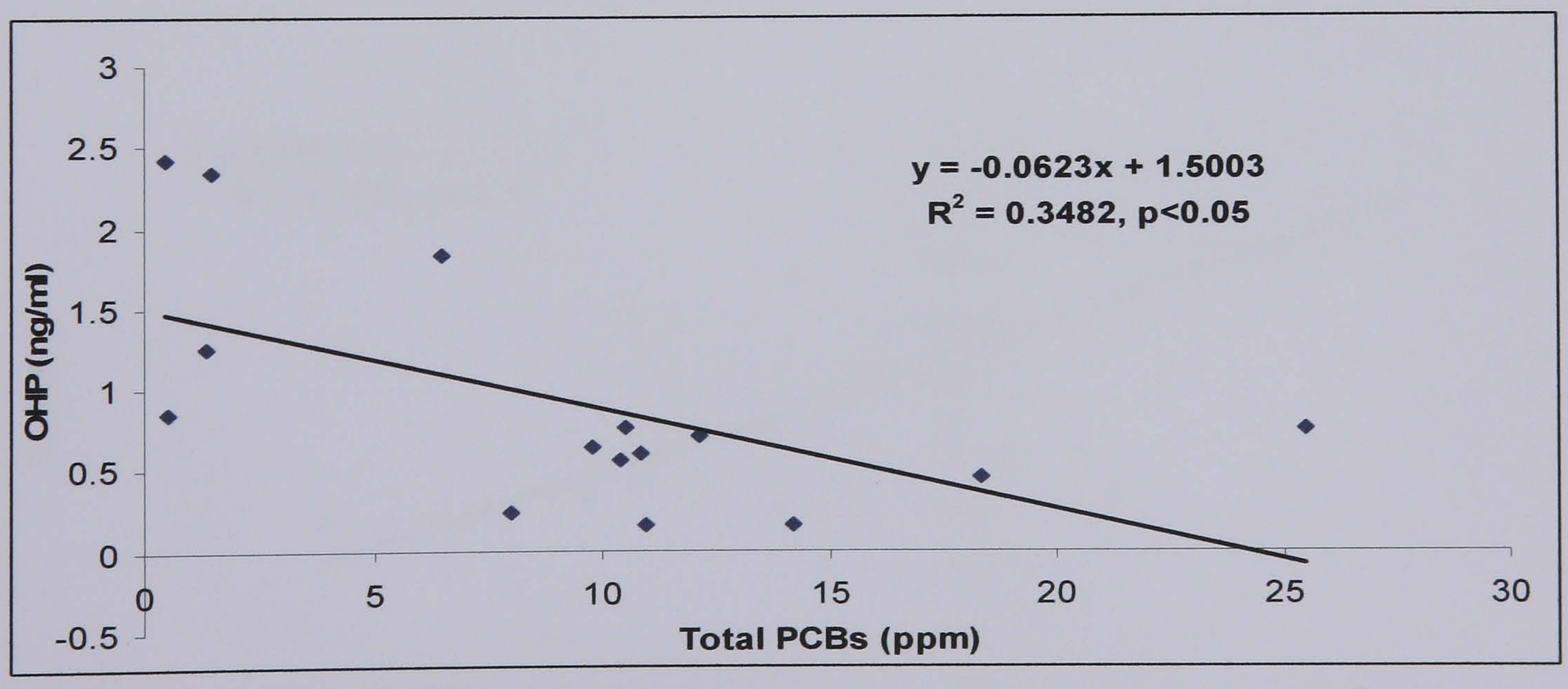



**Figure 3.17:** Stage II: ΣPCBs and 17α-OH-progesterone (OHP) concentrations in female Ringed seals (Baltic Sea and Svalbard)

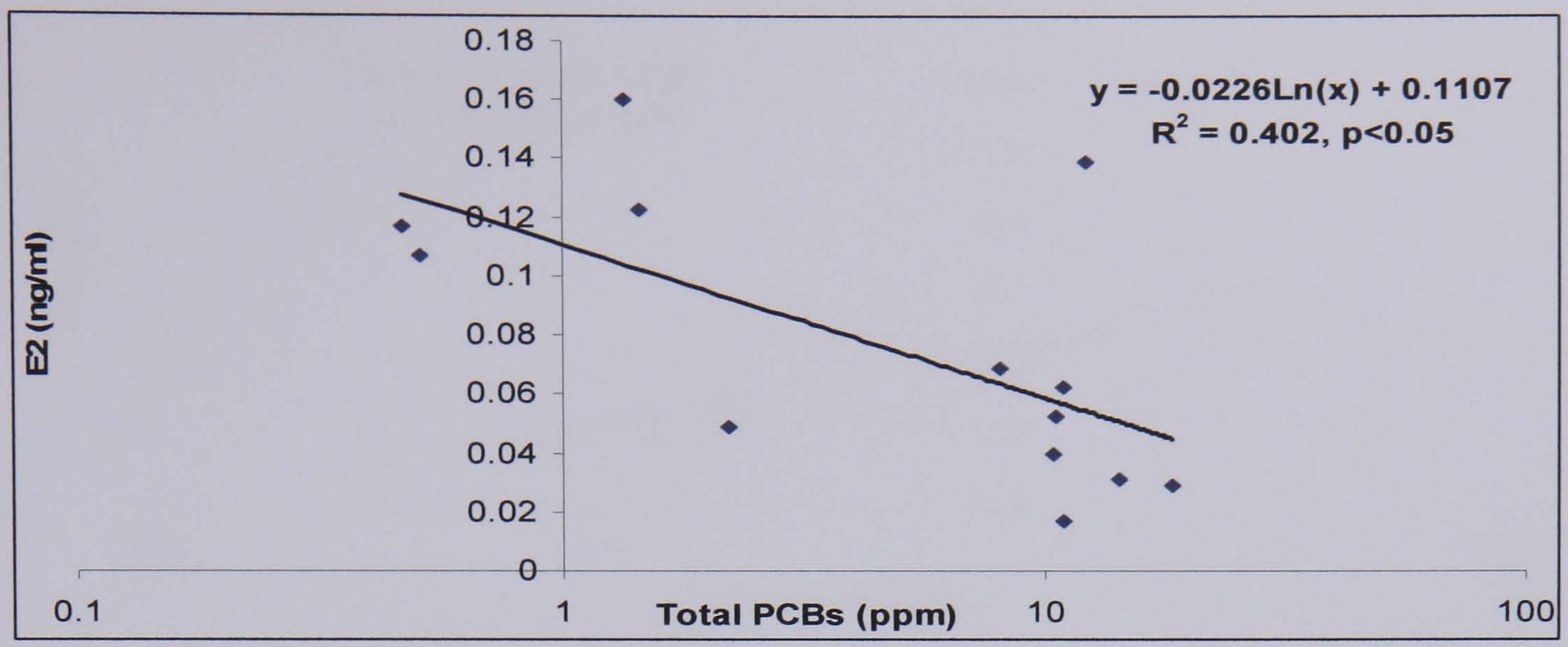



**Figure 3.18:** Stage II: ΣPCBs and 17β-estradiol (E2) concentrations in female Ringed seals (Baltic Sea and Svalbard)

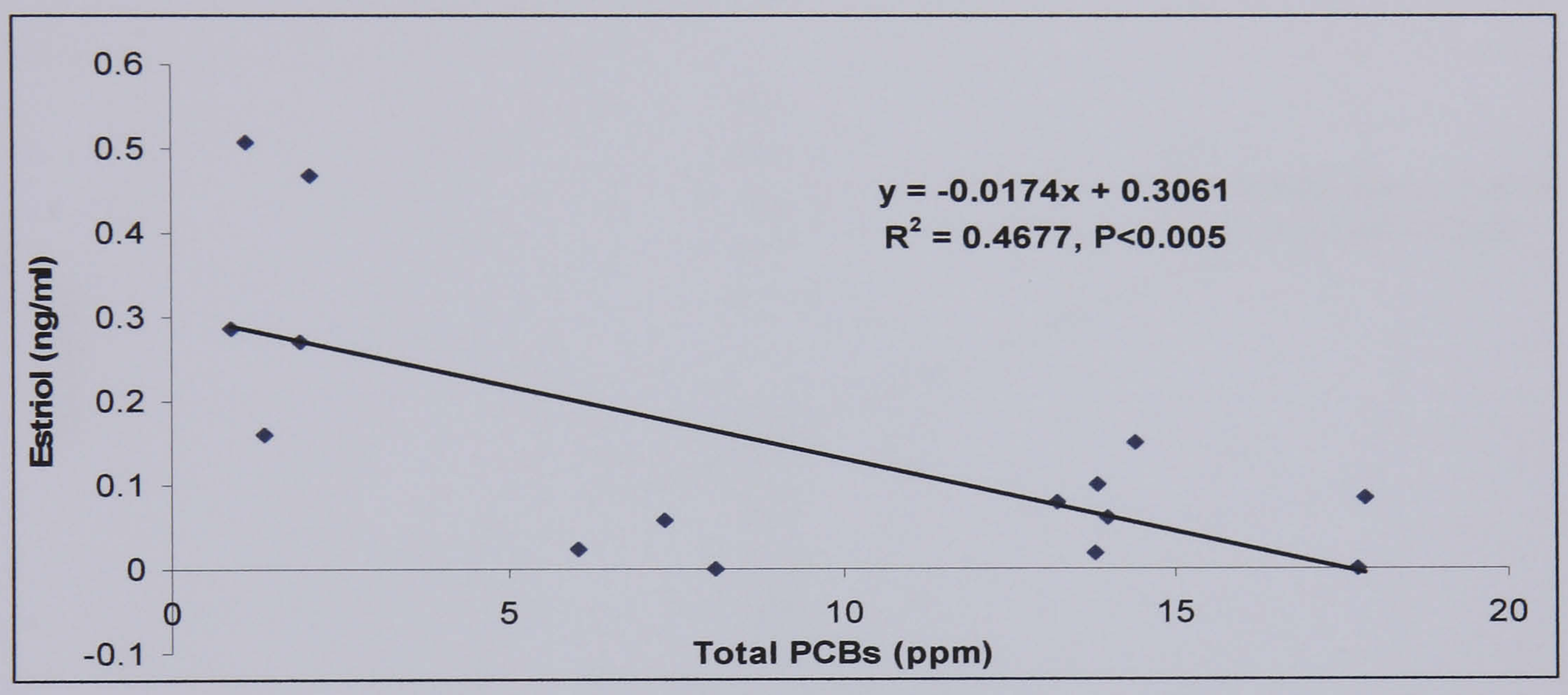



**Figure 3.19:** Stage II: ΣPCBs and Estriol concentrations in male Ringed seals (Baltic Sea and Svalbard)

### *3.3.4.2 Faecal samples*

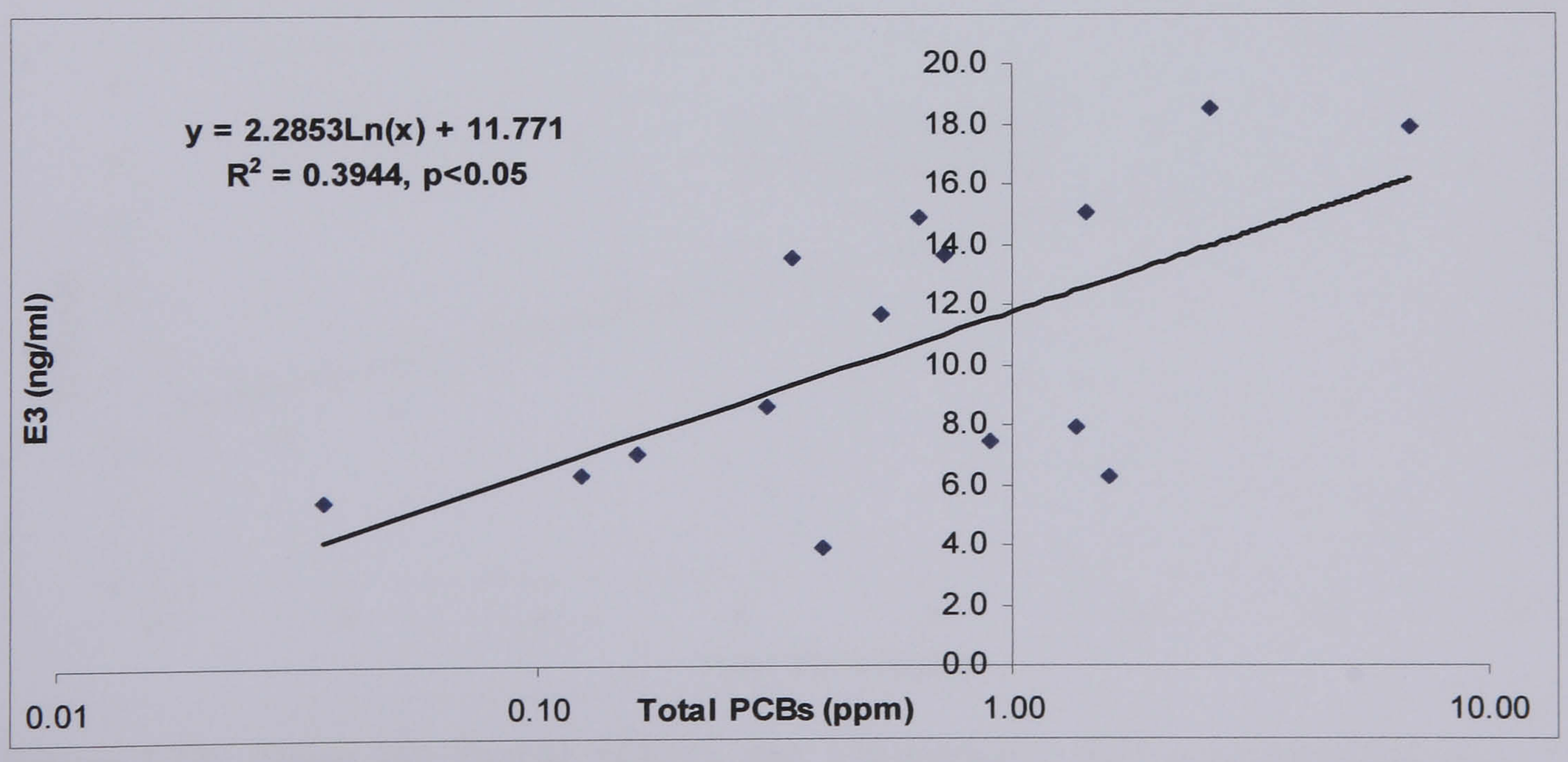



**Figure 3.20**: Stage II: Faecal ΣPCBs and free estriol (E3) concentrations in male Harbour seals from Tees Estuary

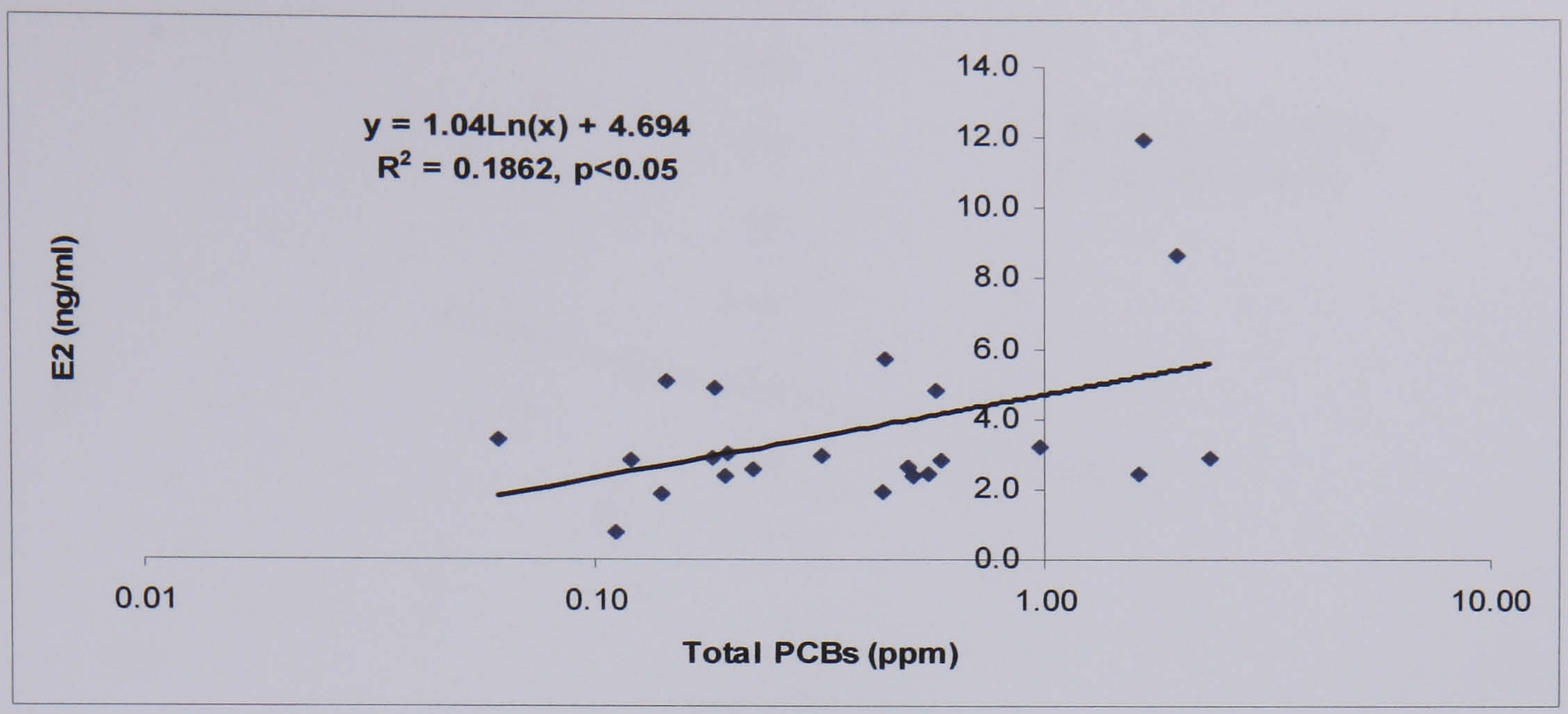



**Figure 3.21**: Stage III: Faecal ΣPCBs and 17β-estradiol (E2) concentrations in seals from all three study populations (Tees Estuary, Donna Nook and Blakeney Point)

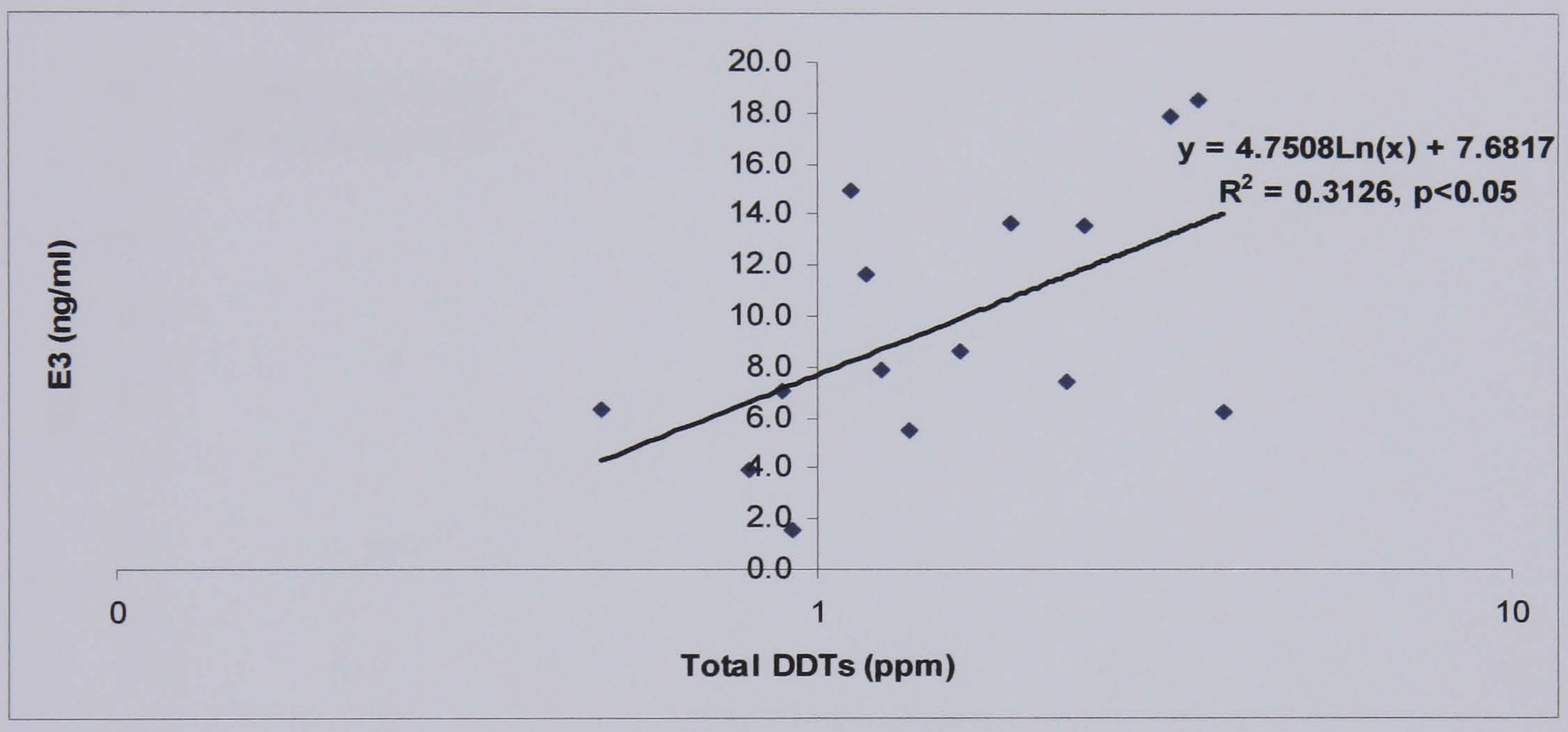



**Figure 3.22**: Stage II: Faecal ΣDDTs and free estriol (E3) concentrations in Harbour seals from Tees Estuary

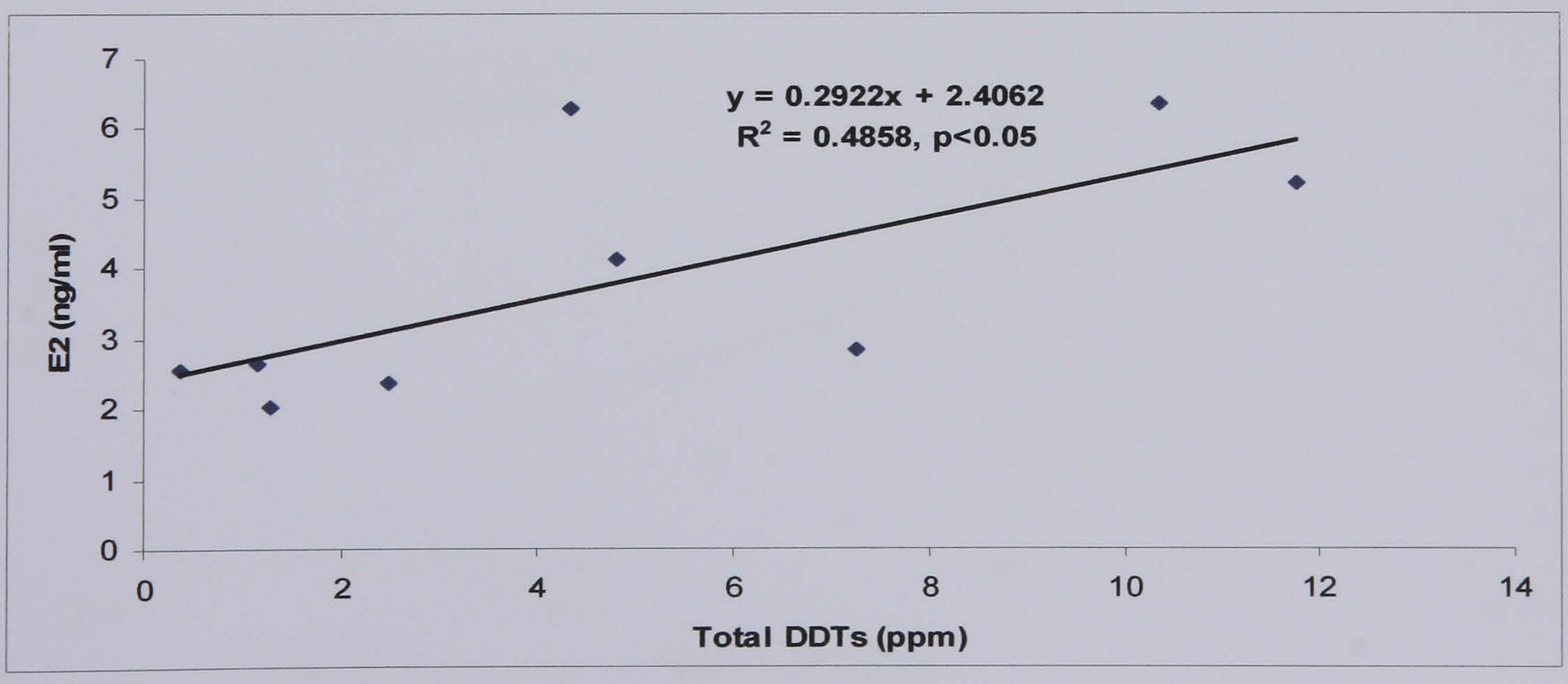



**Figure 3.23**: Stage IV: Faecal ΣDDTs and 17β-estradiol (E2) concentrations in seals from Harbour seals from Tees Estuary

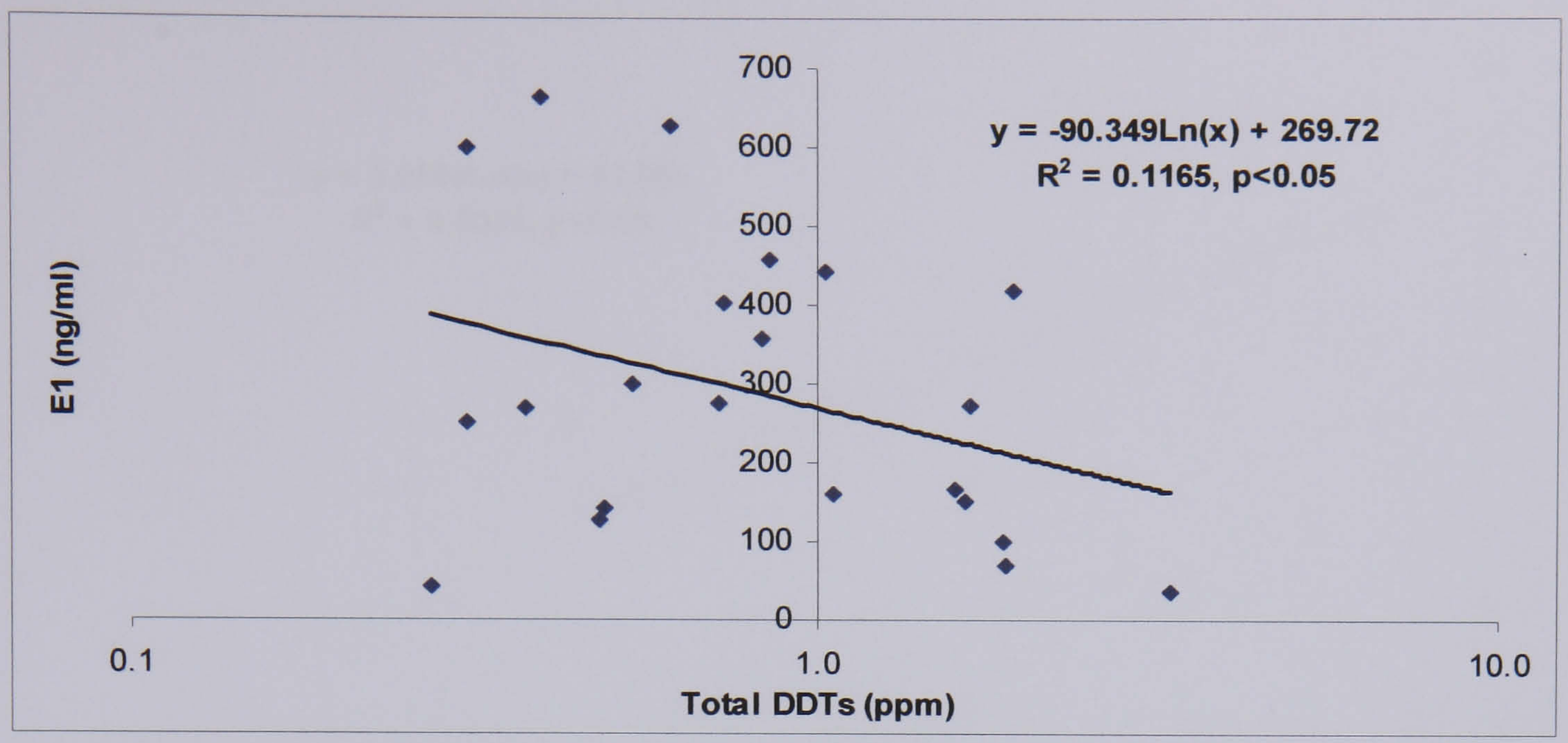



**Figure 3.24**: Stage III: Faecal ΣDDTs and estrone (E1) concentrations in seals from all three study populations (Tees Estuary, Donna Nook and Blakeney Point)

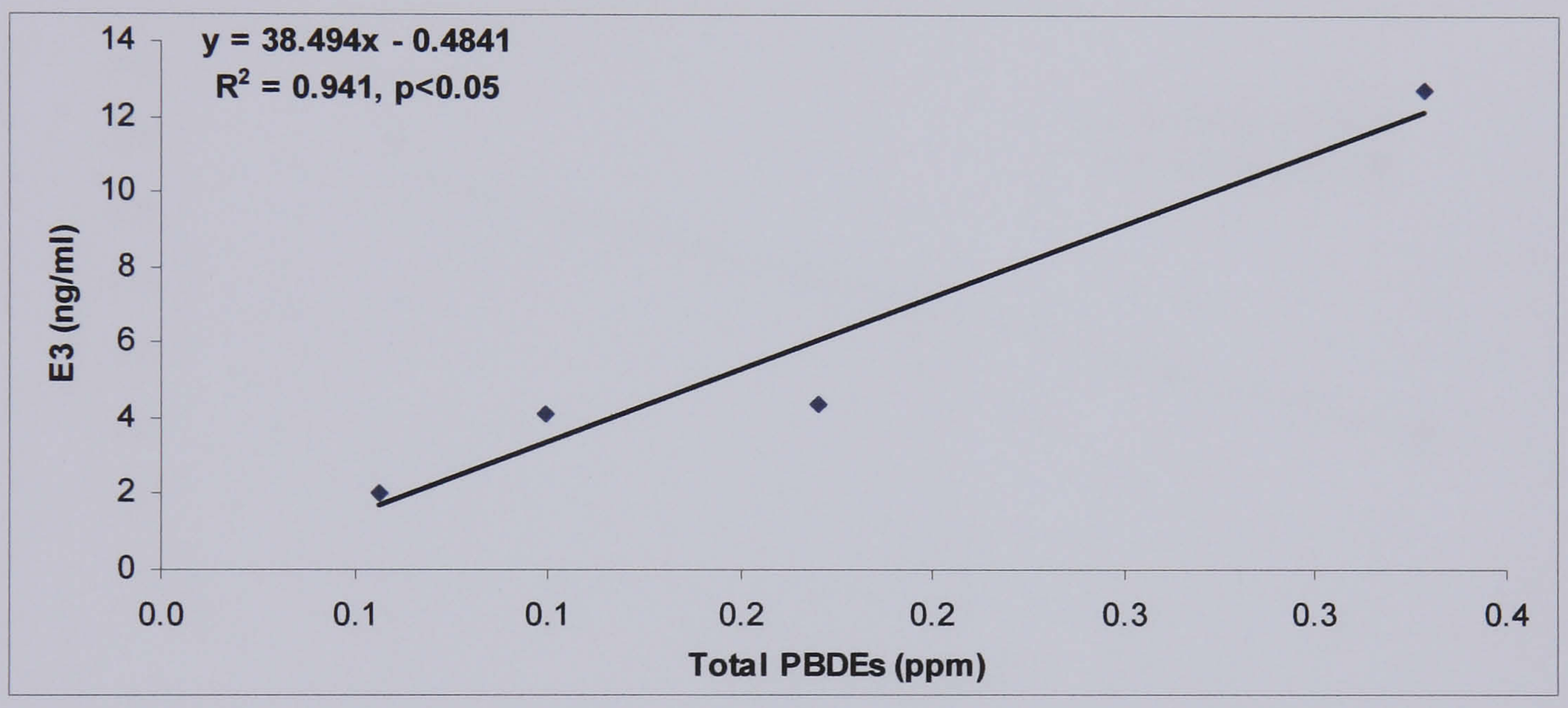



**Figure 3.25**: Stage I: Faecal ΣPBDEs and free estriol (E3) concentrations in seals from Tees Estuary

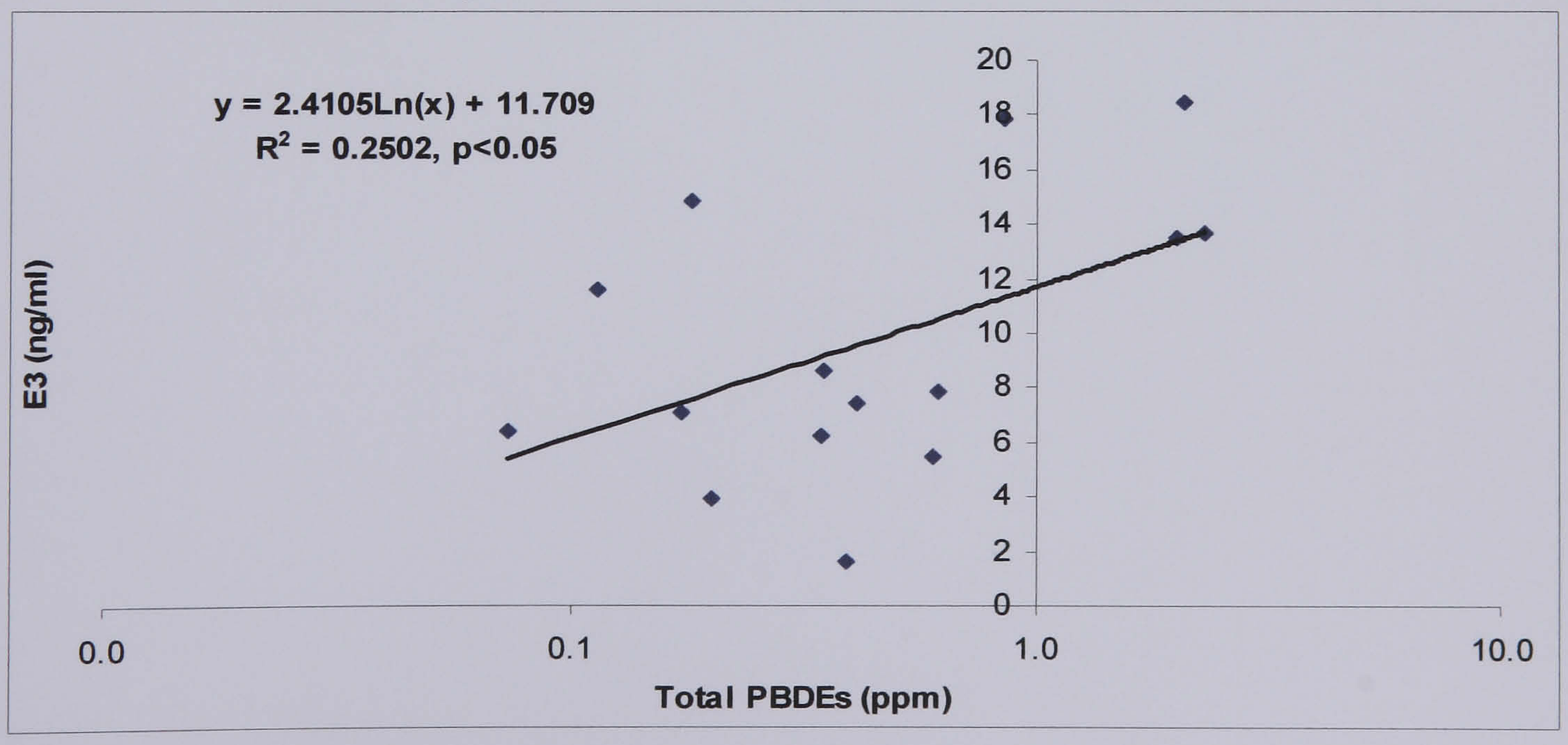



**Figure 3.26**: Stage II: Faecal ΣPBDEs and free estriol (E3) concentrations in seals from Tees Estuary

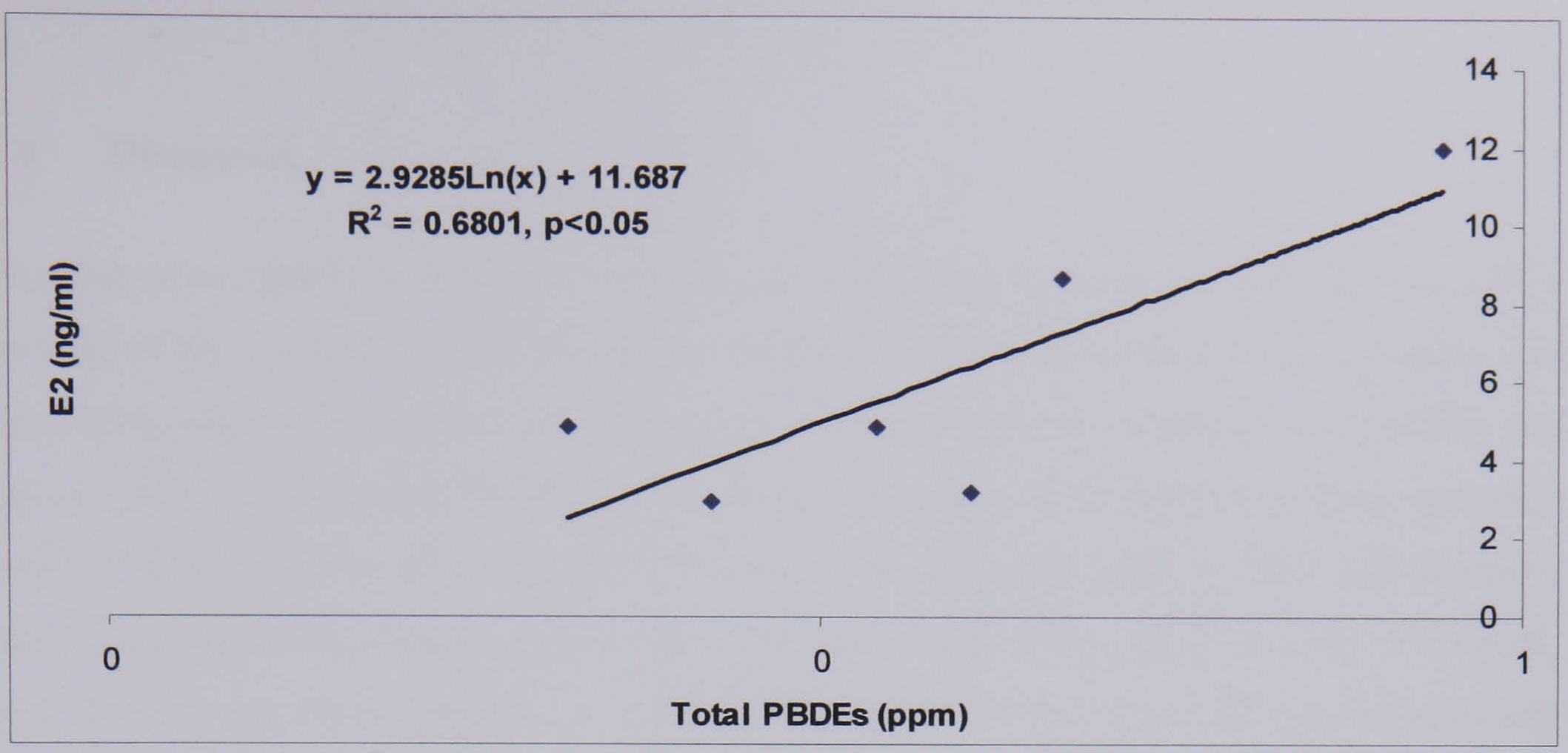



**Figure 3.27**: Stage III: Faecal ΣPBDEs and 17β-estradiol (E2) concentrations in seals from Harbour seals from Tees Estuary

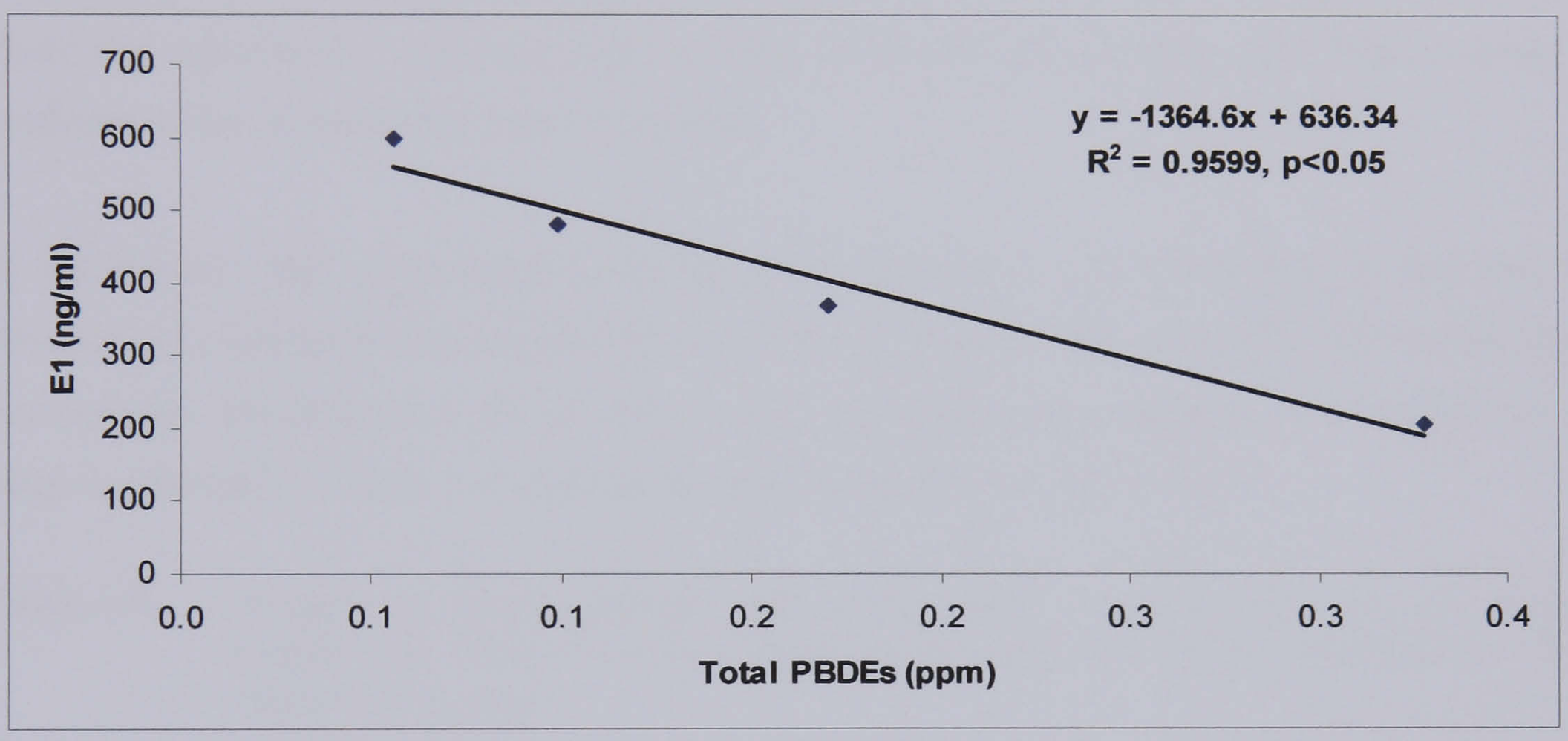



**Figure 3.28**: Stage I: Faecal ΣPBDEs and estrone (E1) concentrations in seals from Tees Estuary

## 3.4 Discussion

The endocrine system is one of the most important homeostatic systems in animals and EDCs such as PCBs are able to alter the natural balance of endogenous hormones in tissues and circulating plasma or serum by functioning as hormone agonists or antagonists (Griffin and Ojeda, 1996; Portier, 2002; Thomas, *et al.*, 2005). A number of different estrogenic and anti-androgenic effects in marine mammals have been reported attributable to POPs and therefore early detection of the adverse health effects resulting from contaminant exposure is crucial. Sex steroids are likely targets for contaminant-induced impairment of reproduction and therefore due to the increasing need to assess the endocrine status of marine mammals a non-destructive monitoring diagnostic tool is required that can provide fast and reliable assessment. Serum and recently faecal steroid analysis have been used as diagnostic tools to study the reproductive endocrinology in wild, farm and zoo animals providing accurate measures of the reproductive cycle of animals.

In the present study progestagens, estrogens and androgens were quantified in seal serum whereas only estrogens were quantified in seal faecal samples. The range of faecal steroid and contaminant concentrations for all three study populations according to each reproductive stage is presented in Table 3.9, whereas for serum samples in Table 3.10.

**Table 3.9:** Ranges of faecal steroid and contaminant concentrations for all study populations (Tees Estuary, Donna Nook, Blakeney Point) according to the reproductive stage

| **Sex Steroid (ng/ml)** | **Stage of Reproduction** | | | |
|---|---|---|---|---|
| | **Stage I** | **Stage II** | **Stage III** | **Stage IV** |
| **E1** | 32.00 – 597.12 | 130.34 – 1023 | 36.58 – 752.00 | 75.55 – 605.12 |
| **E2** | 2.10 – 9.08 | 0.80 – 10.03 | 0.78 – 11.97 | 2.02 – 12.92 |
| **E3** | 2.02 – 12.74 | 1.60 – 18.50 | 0.61 – 105.50 | 2.30 – 97.47 |
| **Contaminant (µg/gL.wt)** | | | | |
| **ΣPCBs** | 0.27 – 1.05 | 0.04 – 8.90 | 0.05 – 8.89 | 0.43 – 3.94 |
| **ΣDDTs** | 0.49 – 1.32 | 0.49 – 13.28 | 0.05 – 10.61 | 0.35 – 11.76 |
| **ΣPBDEs** | 0.06 – 0.33 | 0.07 – 14.42 | 0.002 – 3.83 | 0.30 – 11.85 |

Seal serum PCB concentrations were analysed using an ELISA method since the volume of the samples was not sufficient to carry out GC analysis. Unfortunately, only PCBs could be

quantified in serum samples in contrast to faecal samples where ΣPBDEs and ΣDDTs were also quantified using GC. The reason for analyzing only PCBs in the serum samples was due to the lack of antibodies for PBDEs as well as the lack of funding for the commercially available DDTs.

**Table 3.10:** Ranges of serum steroid and ΣPCB concentrations for all study populations (Harbour, Ringed and Grey seals) according to the reproductive stage

| **Sex Steroid** | **Stage of Reproduction** | | | |
|---|---|---|---|---|
| | **Stage I** | **Stage II** | **Stage III** | **Stage IV** |
| **P (ng/ml)** | | | | |
| *Females* | 1.12 – 89.46 | 0.82 – 70.00 | 11.34 – 72.00 | 11.54 – 70.00 |
| *Males* | 0.53 – 40.00 | b.d.l. – 54.25 | 0.74 – 45.00 | 1.49 – 7.84 |
| **17α-OHP (ng/ml)** | | | | |
| *Females* | 0.30 – 5.36 | 0.15 – 33.36 | 1.87 – 3.58 | 1.33 – 7.92 |
| *Males* | 0.18 – 6.49 | b.d.l. – 18.42 | 0.19 – 3.41 | 0.52 – 1.93 |
| **E2 (ng/ml)** | | | | |
| *Females* | 0.02 – 0.47 | 0.02 – 0.77 | 0.05 – 0.17 | 0.10 – 0.84 |
| *Males* | 0.02 – 0.68 | 0.02 – 1.30 | 0.04 – 0.21 | 0.03 – 0.24 |
| **E3 (ng/ml)** | | | | |
| *Females* | 0.003 – 2.02 | b.d.l. – 80.00 | 0.12 – 1.61 | 0.23 – 7.15 |
| *Males* | 0.01 – 12.70 | b.d.l. – 70.00 | 0.15 – 3.19 | 0.06 – 3.27 |
| **T (ng/ml)** | | | | |
| *Females* | 0.04 – 3.62 | b.d.l. – 24.12 | 0.21 – 2.59 | 0.63 – 4.73 |
| *Males* | 0.15 – 22.59 | 0.03 – 85.82 | 0.47 – 9.46 | 0.39 – 24.82 |
| **A (ng/ml)** | | | | |
| *Females* | 0.19 – 3.70 | 0.02 – 8.90 | 0.46 – 6.02 | 0.28 – 2.41 |
| *Males* | 0.22 – 3.42 | 0.05 – 0.92 | 0.27 – 1.50 | 0.36 – 2.71 |
| **DHEA-S (µg/ml)** | | | | |
| *Females* | 0.005 – 0.39 | 0.0003 – 4.98 | 0.06 – 0.13 | 0.13 – 0.81 |
| *Males* | 0.01 – 1.63 | 0.01 – 0.88 | 0.20 – 9.20 | 0.03 – 0.88 |
| **ΣPCBs (µg/ml)** | | | | |
| *Females* | 2.18 – 15.40 | 0.36 – 39.73 | 1.29 – 18.22 | 2.48 – 30.57 |
| *Males* | 4.22 – 23.70 | 0.30 – 87.06 | 2.92 – 20.12 | 2.11 – 24.54 |

b.d.l., below detection limit

### 3.4.1 Relationship between faecal and serum steroid concentrations

Due to strict UK home office licensing requirements collection of blood from seals in the UK is heavily prohibited, therefore no matching faecal and seal samples were available to compare sex steroid concentrations and evaluate relationships of their level and composition in different body compartments. This is the first study to measure sex steroid levels in faeces of seals and since no comparison could be made with similar published data it is not possible to make accurate conclusions on faecal – serum relationship. However, inferences on the trend of faecal steroids over the whole reproductive cycle for Harbour seals could be made by combining data from Harbour seals from Abertay Sands (all four stages of reproductive cycle). This could not be done for Grey and Ringed seals since this seals were sampled at Stage II of the reproductive cycle and also faecal samples were not available for this species. When comparing serum for female Harbour seals (Abertay Sands) and Harbour seal faecal (Tees Estuary) estrogen levels it was observed that in general faecal samples had higher concentrations of E2 and E3 in all reproductive stages compared with serum samples.

As it can be seen from Fig. 3.29 by combining faecal and serum steroid data from Harbour seals of different populations it was possible to investigate the change in faecal steroids throughout the reproductive cycle of Harbour seals with E2 and E3 in both matrices to follow the same trend in the different stages of reproductive cycle even though the samples originated from two different seal populations. However, it has to be noted that an inevitable introduction of error occurs from using different individuals since there was no identification of the origin of the faecal samples (sex, species and age) and no DNA testing of the faeces to confirm sex and source as well as using small number of samples representing each stage of reproductive cycle. The trend between faecal and serum estrogens observed in the present study has also been observed in female monkeys, with faeces having higher concentrations compared to serum samples. Moreover, in female monkeys faecal E2 lagged behind serum E2 due to steroid processing in liver and reabsorption in the gut (Matsumuro *et al.*, 1999).

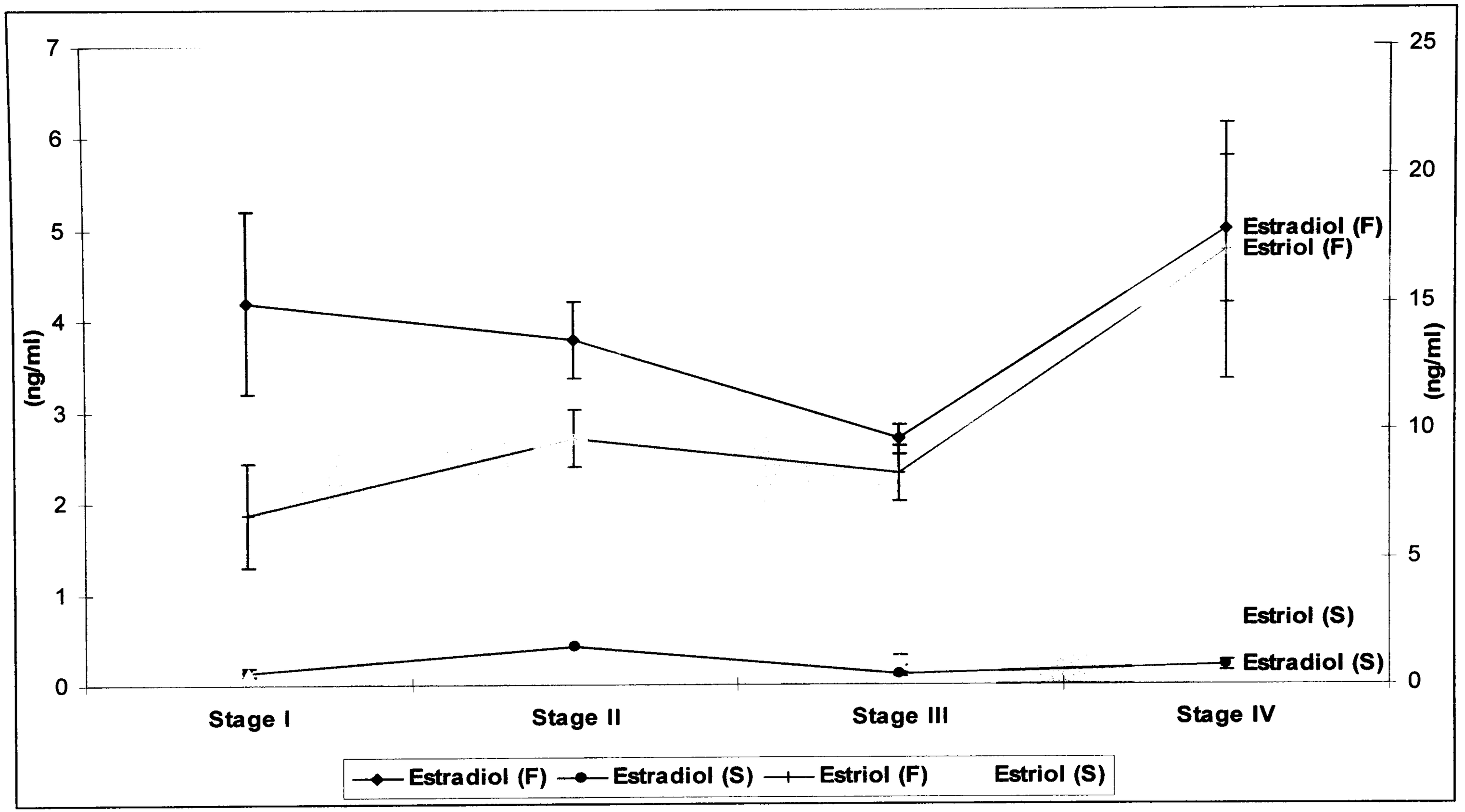



**Figure 3.29:** Comparison of faecal (F) and serum (S) estrogen levels (E2, E3) in Harbour seals from Tees Estuary and Abertay Sands.

Estrone levels in the two matrices could not be compared since this analysis was not available for serum samples. The levels observed in the faeces however are higher than reported levels in pinniped serum samples (Atkinson, 1997) and statistically significant higher from E2 and E3. A possible explanation could be the metabolism that estradiol undergoes. Urinary and faecal estrone concentrations in primates have been found to be 100 times higher than estradiol concentrations due to the metabolism of nearly 50% of circulating estradiol into estrone before excretion into urine and faeces. Therefore, circulating estradiol is contributing to the already abundant excretion of estrone (Ziegler *et al.*, 1989; Ziegler *et al.*, 1993).

### 3.4.2 Intra-species differences in Steroid Concentrations

*Harbour seals*

Harbour seals from Moray Firth, were the only population where age-dependent differences could be investigated since all other populations consisted only of adult seals. The only hormone that was observed to be statistically significant different between female adult and juvenile Harbour seals was 17α-OHP (Fig.3.1) which was found to be lower in adults compared to juveniles ($p<0.05$). There was no statistical difference observed between male adult and juvenile Harbour seals. This finding comes in contradiction with previously reported levels of prepubertal seals that are characterized by low or undetectable concentrations of sex steroids, including progesterone (< 0.5ng/ml), testosterone (< 1ng/ml) and various forms of estrogen (Atkinson, 1997) that show only slight fluctuations. A possible explanation that could be given for this difference is that the juvenile seals could have entered puberty earlier than expected or that the Harbour seal population in Moray Firth is exposed to endocrine disruption.

Sex steroid levels between female and male adult Harbour seals from Moray Firth showed no statistically significant difference. Female and male Harbour seals from Abertay Sands were compared at all the stages of the reproductive cycle except Stage II, since there was only one female sample available for that stage. As it can bee seen from Fig.3.2 female Harbour seals in all stages of reproductive cycle (I, III, IV) had statistically significant higher P levels ($p<0.05$) compared with male Harbour seals. In Stages I and III, females also were found to have statistically significant higher 17α-OHP ($p<0.05$) compared to males whereas in Stage III, A mean levels were also statistically significant higher in males compared to females.

*Ringed and Grey Seals*

The same statistically significant difference between females and males was observed in Baltic Ringed and Grey seals that were sampled in Stage II of the reproductive cycle, with P and 17α-OHP to be significantly higher ($p<0.005$) in female compared to male seals. Female and male Svalbard Ringed and Sable Island Grey seals showed no statistically significant difference for any of the sex steroids under study. There are problems associated with characterising individual reproductive status based on sex steroid levels in blood (single blood sample) since results can be misleading because they may not accurately reflect the physiological activity of these hormones in the body (Greig, 2002). Using a single hormone sample from an individual to characterize reproductive status or possible endocrine disruption has the potential of error since several sex steroids, such as progesterone, are released into the body in pulses and pulse length and amplitude vary among species. Moreover, hormone activity can be affected by the rate of hormone secretion, binding capacity, the rate of conversion to metabolites or the density of cellular receptors (Aubin, 2001). However, since there was a significant number of samples obtained for this study it was assumed that a range of possible values would cover the highs and lows in circulating sex steroid concentrations. Moreover, hormone concentrations are more likely to depend on the reproductive stage that the seal is than on a particular date and therefore, choosing to sample the seals at each stage of the reproductive cycle would have reduced the error from using a single hormone sample.

### 3.4.3 Inter-species variation in Steroid Concentrations

A species comparison between the Baltic seals showed statistically significant higher P ($p<0.05$) and 17α-OHP ($p<0.005$) levels in female Grey seals compared to Ringed seals, whereas male Grey seals had statistically significant higher ($p<0.05$) P, 17α-OHP, E3 and A levels compared to male Ringed seals (Fig.3.3 and Fig.3.4).

Female Harbour seals from Abertay Sands showed statistically significant higher 17α-OHP ($p<0.005$), A ($p=0.05$) and DHEA-S ($p<0.05$) concentrations when compared with Moray Firth female Harbour seals, whereas males from the two populations showed no significant differences. Comparison of Harbour seals from Moray Firth with Grey and Ringed seals was not possible since the different seal species were sampled in different stages of the

reproductive cycle. No comparison could be made between female Harbour seals from Abertay Sands and Ringed and Grey seals since there was only one sample available for Stage II of the reproductive cycle representing Harbour seal sex steroid levels. However, male Harbour seals from Abertay Sands were found to have statistically significant higher P ($p<0.005$) and lower DHEA-S ($p=0.05$) levels compared to male Baltic Grey seals and statistically significant lower E2, E3, T ($p<0.05$), 17α-OHP ($p<0.005$), E2 when compared with male Sable Island, Grey seals. P, 17α-OHP , A ($p<0.005$) and E2 levels were found to be statistically significant higher in male Harbour seals from Abertay Sands compared to male Baltic Ringed seals whereas no statistically significant difference was observed for any of the sex steroids when compared to Svalbard male Ringed seals.

The above comparisons were made between seal species sampled at the same stage of the reproductive cycle. Progesterone levels differences have been previously reported among species as a result of the variation of the functional life of the corpora lutea between species (Atkinson, 1997). In Harbour seals circulating progesterone levels remain high for 4-5 month (prolonged luteal phase – known as pseudopregancy) whereas in Hawaiian Monk seals circulating progesterone levels decrease abruptly 15-18 days after ovulation (Reijnder, 1990; Atkinson, 1997). In Northern fur seals there is an annual peak in plasma progesterone levels to 20-30ng/ml around July, indicative of ovulation (Robeck *et al.*, 2001). During embryonic diapause in Harbour seals between August and October, progesterone concentrations drop to 5-10ng/ml, increasing again in November to 25-35ng/ml (Robeck *et al.*, 2001). In non-pregnant Harbour seals, however, blood progesterone levels decline rapidly after implantation (Reijnders, 1990; Atkinson, 1997). During the breeding season, in Grey and Harbour seals, testosterone levels are 10-20 times higher than the non-breeding season (Sangalang and Freeman, 1976; Atkinson, 1997). In female Harbour and Grey seals, plasma progesterone concentrations are decreased through lactation (Boyd, 1983; Reijnders, 1990), increase after mating and remain stable during the three months when delayed implantation takes place (Boyd 1982; Reijnders, 1990). After implantation, progesterone concentrations gradually increase throughout gestation and peak in the final days before parturition (Raeside and Roland, 1981; Boyd, 1984; Reijnders, 1990).

### 3.4.4 Validation of steroids as biomarkers of endocrine disruption

The use of blood and faecal samples for steroid analyses is a useful biomonitoring tool for assessing the reproductive status in seals as well as to investigate if these mammals suffer from endocrine disruption, i.e adverse health effects in an intact organism such as alteration in the levels of the endogenous hormones caused by an exogenous substance (e.g. POPs). The use of faeces for steroid analysis enables data to be collected in situations in which blood collection may prove either detrimental to the animals or impossible to obtain (Brillitti *et al.*, 1998). The use of sex steroids in serum and faeces as valuable biomarkers have been proposed by several studies including marine mammal monitoring (Reijnders, 1990; Wasser *et al.*, 1991; Schwarzenberger *et al.*, 1996; Lasley and Overstreet, 1998; Matsumuro *et al.*, 1999; Greig, 2002; Linklater, *et* al, 2000; Persky *et al.*, 2001; Turner *et al.*, 2001, Larson *et al.*, 2003; Lynch *et al.*, 2003). These two matrices are ideal for population monitoring of protected species such as seals since reproducible results can be facilitated with large sample sizes obtained non-destructively (faeces) or with minimal invasion (blood). Moreover, re-sampling individuals for temporal exposure studies is also possible for surveillance monitoring (Fossi *et al.*, 1999).

Little information is available for serum sex steroid concentrations and none for faecal concentrations, thus it is important to establish normal baseline levels of sex steroids over the reproductive cycle for study species. Moreover, very little information is available concerning altered sex steroid levels in seals due to EDC exposure, thus it s crucial to establish dose-response relationship of sex steroids with exposure as well as it is important to establish the degree of deviation from normal levels due to endocrine disruption (toxic threshold). All of the above would aid the validation of other biomarkers whose normal expression may be regulated by steroids such as lactoferrin (LTF) and Very Low Density Lipoprotein (VLDL). This is the first study reported so far to investigate the concentrations of sex steroids in faecal samples obtained from seals in correlation with contaminant exposure.

#### *3.4.4.1 Normal values for Steroids*

As already mentioned earlier, there is little information available on baseline blood sex steroid levels in seals whereas none is available for faecal sex steroids. Most of the studies have

concentrated on progesterone and estrogen levels through the reproductive cycle and some of the species investigated include Harbour, Grey, Antarctic Fur and Hawaiian Monk seals, (Reijnders, 1990; Boyd, 1991; Atkinson, 1997; Gales *et al.*, 1997; Boyd *et al.*, 1999; Renfree and Shaw, 2002). This is the first study to investigate faecal estrogens (E1, E2 and E3) and their correlation with PCB exposure in Harbour, Grey and Ringed seals. This study therefore constitutes a major contribution to providing vital information needed as part of the validation of steroids as biomarkers of endocrine disruption.

The experimental control group levels agreed with progesterone published levels for Harbour seals (Reijnders; 1990) whereas E2 levels were found to be slightly higher. However, no statistically significant differences were found in the P and E2 profiles of pregnant seals from exposed and control groups. Moreover, the results demonstrated that the mean level of E2 in the exposed group was lower than the control group. The results obtained in this study for the experimental group were in agreement with Reijnders (1986 and 1989). Since this group of seals was sampled at Stage IV of the reproductive stage, sex steroid levels could not be compared with the other samples at a different reproductive stage. For this reason, baseline levels for other stages of the reproductive cycle were ascertained from the other control groups in this study, i.e. the Svalbard Ringed seals and Sable Island Grey seals that had lower contaminant levels than concentrations found in the experimental group (*For full justification of exposure level refer to Chapter 2-Contaminant Analysis*). Control Grey and Ringed seal hormone profiles were in close agreement with published information available for seals (Atkinson, 1997). On this basis Grey and Ringed seal hormone levels from the reference areas were used as baseline levels for Stage II of the reproductive cycle whereas hormone levels derived from the control (experimental group) were used for Stage IV of the reproductive cycle (Table 3.9). These values were used for identifying deviations from normal due to endocrine disruption in the seal samples of this study.

#### *3.4.4.2 Dose-dependence*

Only P, 17α-OHP, E2 and E3 were analysed in serum samples of Harbour seals from the experimental group. Serum P and E2 concentrations were negatively correlated with ΣPCBs in dose-dependent manner and this relationship was statistically significant ($p<0.05$, Fig.3.10a, b). Since there was no control population of sex steroid levels in faecal samples, in order to

investigate whether sex-steroid expression was dose-dependent with exposure as observed in the serum samples of the experimental group, contaminant data and sex steroid results were investigated using linear regressions. For ΣPCBs (Fig.3.20 and 3.21) there was a positive correlation with E3 in Stage II (Tees Estuary) and with E2 in Stage III (all three study populations) in a dose-dependent manner and this relationship was statistically significant ($p<0.05$). For ΣDDTs (Fig.3.22, 23 and 24) there was a positive correlation with E3 in Stage II (Tees Estuary) and with E3 in Stage IV (Tees Estuary) and a negative correlation with E1 in stage III (all three study populations) in a dose-dependent manner and this relationship was statistically significant ($p<0.05$). Finally, for ΣPBDEs (Fig. 3.25 to 28) there was a statistically significant dose-dependent positive correlation with E3 ($p<0.05$) in Stages I and II, with E3 ($p<0.05$) in Stage III and a dose-dependent negative correlation with estrone ($p<0.05$) in Stage I.

Even though the sex steroids analysed in the study showed a statistically significant correlation with exposure, a low correlation coefficient was observed in the linear regressions indicating that this relationship was not as strong as was anticipated. However, this does not exclude the use of sex steroids in biological monitoring. There is a clear trend observed in the linear regression that could have been amplified if more samples from all the stages of the reproductive cycle were available for the study. In terms of minimizing disturbance, faecal steroid biomonitoring is ideal for studying seal populations as long as sex and originating individual of the faecal sample is known by DNA testing or identification at the field. These samples can be collected all through the reproductive cycle providing a large sample size that would be optimal for biomonitoring study design.

### 3.4.5 Biomonitoring ED with steroid biomarkers in study populations

Biomarker (BM) expression over a gradient of contaminant exposures was investigated in the seal populations under study using linear regressions (Fig.3.14 to 3.20). To identify specific populations affected by ED (being at risk), BM expression in exposed groups was compared with control populations and differences were tested using student t-test. Where BM expression was statistically significant, populations were classified at risk from ED. From the sex steroid results obtained in this study and the contaminant data obtained in Chapter 2 it can be concluded that Baltic Ringed and Grey seals are considered populations at risk since they

significantly deviated from the normal/control group data. This study shows that especially the Baltic Ringed seals are still suffering from a very high contaminant burden that poses a threat to their health status. Therefore, these populations could be at risk from toxic effects to endocrine-dependent processes such as growth, development and reproduction (Portier, 2002). There is population-level evidence of the reproductive effects of POPs caused by endocrine disrupting mechanisms (DeLong *et al.*, 1973; Helle and Olson, 1976a, b; Freeman and Sangalang, 1979; Reijnders, 1980; Auerlich *et al.*, 1985; Reijnders, 1986; Gillner et *al.*, 1988; Baker, 1989; Troisi and Mason, 2000; *for detailed information please refer to Chapter 1-General Introduction*).

This is the first study to measure sex steroid concentrations in faecal samples from UK Harbour and Grey seals and to investigate the correlation of these steroids in blood and in faecal samples in a dose-dependent manner with contaminant exposure. The major limitation of the present study was that no matching faecal and blood samples were available in order to provide a direct correlation of sex steroid levels between the two matrices. This could have provided a valuable tool for development of a model for predicting seal circulating hormone levels using only faecal samples and therefore offering minimal disturbance to the animals. Using blood and faecal sex steroids as biomarkers of endocrine disruption in seals is a promising and valuable tool since both matrices can be sampled non-destructively and non-invasively (faeces and serum). Faecal steroid measurement so far has only been applied to reproductive/endocrine assessments in mammals. There is clear potential of using faecal samples for biomonitoring altered function and EDC exposure in seals since sex steroid measurement in combination with contaminant data from faecal samples is an ideal tool for non-destructive sampling and seal biomonitoring. More samples could be collected during all the stages of the reproductive cycle and individual seals could be monitored, tagged and re-sampled therefore providing a data base of information on sex steroids baseline levels and possible endocrine disruption through out the seal reproductive cycle.

# Characterisation of Phocine Lactoferrin and its application as a Biomarker of Xenoestrogenic Exposure in Pinnipeds

## 4.1 Introduction

Lactoferrin (LTF) is a major estrogen-inducible glycoprotein in the mammalian uterus (Pentecost and Teng, 1987). It was originally identified in bovine milk in 1939 (Sorensen and Sorensen, 1939) and in 1960 was isolated by Johansson from human milk (Johansson, 1960). It is a single-subunit glycoprotein (molecular weight: 65-80kDa) with an isoelectric point of 8.7 and consists of two metal-binding sites, each of which can bind a ferric ($Fe^{3+}$) cation and a bicarbonate anion (Metz-Boutigue *et al.*, 1984). It belongs to the serum transferrin gene family and is synthesised by neutrophils and glandular epithelial cells. Besides its presence in high concentrations in milk, LTF in mammals is also present in the mammary glands, saliva, tears, semen, plasma, uterine fluid, vaginal fluid, wet surface mucosa and in the specific granules of polymorphonuclear leukocytes (PMN) (Masson *et al.*, 1966; Teng *et al.*, 1989).

LTF appears to be a multifunctional protein; it is involved in iron metabolism by controlling iron availability and is known to play a role in the host defence mechanism. This includes a broad spectrum of antimicrobial activity with bacteriostatic activity against *Escherichia coli, Staphylococus aureus* and *Candida albicans* (Arnold *et al.*, 1977 and 1980; Lönnerdal and Iyer, 1995; Bhimani *et al.*, 1999, Valenti *et al.*, 2004; Larkins, 2005). Its high affinity for iron is the factor that contributes to the inhibition of the bacterial growth since iron is a necessary element for bacterium survival. LTF also has immuno-modulatory actions (Levay and Viljoen, 1995; Teng *et al.*, 1992) and it is known to have anti-tumour and anti-metastatic activity (Bezault *et al.*, 1994).

LTF is produced mainly in the bone marrow, mammary glands and the uterus and in each of these organs is regulated differently. For example, it has been shown that prolactin (PRL) stimulates LTF secretion in explants of mammary tissue from mid-pregnancy mice (Teng *et al.*, 1989). LTF expression in maturing neutrophils is part of the differentiation process (Panella *et al.*, 1991), whereas mouse uterine LTF synthesis is under the control of estrogen. In the female and male reproductive tract (uterus, seminal vesicles), LTF secretion is under the influence of sex steroids, primarily 17β-estradiol (Teng *et al.*, 1992 and 1995; Ranhotra and Teng, 2005) which explains the significantly higher concentrations of the protein in female reproductive tract compared with that of males.

LTF has been reported in rodents, domestic animals (e.g. cow, goat, dog etc), humans but not marine mammals (Mason and Heremans, 1971; Rebelo *et al.*, 1995 and 1996; Farnaud and Evans, 2003). Human milk is by far the most abundant source of LTF and has the highest concentration of LTF (up to 7g/L in human colostrum) compared with milk from other species (Mason and Heremans, 1971; Teng, 2002). Human plasma LTF levels are low (0.2µg/ml) whereas seminal fluid, tears and nasal secretions contain up to 100µg/ml (Baynes *et* al., 1986; Lakritz *et al.*, 2000; van der Strate *et* al., 2001). LTF levels in blood, faeces and milk increase during inflammation and pregnancy (Thomas *et al.*, 2002; Kane *et al.*, 2003).

As already mentioned previously, LTF is a major secretory and estrogen-inducible protein (Newbold *et al.*, 1992; Walmer *et al.*, 1992; Teng *et al.*, 2002; Ranhotra and Teng, 2005). In the adult mouse estrus cycle it has been demonstrated that LTF protein expression fluctuates in parallel with the rise and fall of estrogens (Newbold *et al.*, 1992; Walmer *et al.*, 1992), the circulating LTF level correlates positively with circulating estrogen levels and can vary remarkably in response to the levels of endogenous estrogen (Walmer *et al.*, 1992). LTF in the uterine epithelial cells has been found to be in much higher concentrations in the cytoplasm rather than the nucleus. However, over the estrus cycle the fluctuations in LTF levels in the nucleus parallel those in the cytoplasm. Two major ovarian steroids, progesterone and estradiol are key regulatory components of reproduction in females. Uterine function is co-ordinately regulated by progesterone and estrogen and in many cases progesterone antagonises the effect of estradiol (Kurita *et al.*, 2000). LTF protein expression increases in association with the rising serum estradiol concentrations in proestrus, a time where progesterone concentrations are very low. From early to mid-diestrus, LTF levels are comparatively low, rise during late diestrus/early proestrus and peak at early estrus due to maximum estrogen concentrations. During metestrus, there is small elevation of 17β-estradiol, which is associated with elevated progesterone. At this stage, LTF levels begin to decline reaching lowest levels again in late metestrus/early diestrus (Newbold *et al.*, 1992; Walmer, 1992; Jefferson *et al.*, 2000). This tight hormonal control of LTF suggests that this protein may play an important physiological role in the uterus during early pregnancy (Ward *et al.*, 1999). In humans, LTF is also found in female reproductive organs as well as in seminal vesicles and the prostate. From a study carried out by Yu and Chen, (1993) it was found that LTF mRNA in the pre-pubertal period increases with growth of epididymis and that the levels could be stimulated by 17β-estradiol, but not influenced by testosterone. It has also been reported that LTF interacts with the surface of sperm and becomes one of its major coating antigens (Autiero *et al.*,

1991; Teng *et al*., 1992). Therefore, LTF might have additional functions in fertilisation in the reproductive tract.

Over the past years, there has been an increased concern on the impact of environmental chemicals on the health of humans and wildlife. Amongst these chemicals, synthetic and environmental estrogens (e.g. PCBs) in particular have drawn a lot of attention due to their potential harmful effect on the endocrine system by acting as endocrine disrupting chemicals (EDCs). One of the primary targets of EDCs is the hormone nuclear receptors which bind steroid hormones and modulate activities in target genes (Ranhorta and Teng, 2005). In mammals, EDCs by having similar structure to endogenous estrogens are able to bind to estrogen receptors and by mimicking or antagonising estrogens elicit estrogenic responses and therefore altering the effects of endogenous estrogens (McLachlan, 1993). For example, elevated estrogenic exposure during critical periods of development leads to teratogenic effects and development of carcinogenic lesions in the reproductive tract (Nelson *et al*., 1994).

Many natural estrogens, estrogen metabolites, synthetic estrogens and xenoestrogens have been examined for their effects on LTF expression in mouse uterus or cultured human endometrial cells. Pentecost *et al*. (1988) showed uterine LTF mRNA expression increased approximately 300-fold after three daily subcutaneous injections of 17β-estradiol. Neonatal exposure of mice to synthetic estrogen (diethylstilbestrol; DES) resulted in persistent ovarian-independent induction of LTF mRNA and LTF protein expression in the uterus and vagina (Gray *et al*., 1994). Curtis *et al*. (1997) also examined the effects of synthetic estrogens DES and indenestrol A (IA) on the stimulation of LTF promoter activity demonstrating induction of LTF expression *in vivo* and in *vitro*. Ovariectomised mice exposed to the insecticides methoxychlor, chlordecone (Kepone), DDT metabolites *p,p'*-DDD and *o,p'*-DDT and the catecholestrogen 4-hydroxy-estradiol, demonstrated significant increases in uterine levels of LTF mRNA (Das *et al*., 1998; Ghosh *et al*., 1999). Markey *et al*. (2001) also demonstrated that bisphenol A (BPA) induced a >300% increase in LTF in luminal and glandular epithelium of the murine uterus. In the same study, BPA was also capable of inducing changes in the vaginal opening, uterine wet weight and epithelial cell height. Moreover, animal studies on mouse epididymis, showed LTF mRNA was stimulated by 17β-estradiol (Yu and Chen, 1993). In men, serum and testicular estradiol levels were found to be associated with oligospermia (Krause, 1988; Levalle *et al*., 1994). It is thought that in men, increased circulation of seminal estradiol may increase seminal LTF secretion (Buckett, 1997).

These studies show that exposure to certain xenoestrogens as with natural estrogens, stimulates LTF expression in both the male and female reproductive tract. Environmental pollutants such as certain PCB congeners and DDT are known endocrine disruptors and aspects of their toxicity and effects in the marine environment have been well documented (Aguilar and Borrell, 1996; *for more information please refer to Chapter 1-General Introduction*). It is plausible therefore, that LTF homeostasis may be disturbed in animals exposed to elevated levels of these and other EDCs, indicative of endocrine disrupting effects. LTF therefore has the potential to be used as a marker of estrogen exposure in animals exposed to endocrine disruptors (LeBlanc and Bain, 1997).

Being top predators of the marine food chain, pinnipeds are significantly exposed to biomagnified concentrations of persistent, lipophilic organic pollutants, particularly organohalogens (PCBs, DDT, dioxins and PBDEs), polyaromatic hydrocarbons (PAHs) and organometals (e.g., tributyl tin; TBT and methyl mercury) from their diet (Simmonds *et al.*, 1993; DeSwart *et al.*, 1994; Tanabe, 1999; She *et al.*, 2000). Baltic and Wadden Sea seal populations in particular, have been shown to be exposed to significantly elevated concentrations of PCBs and DDT since the 1960s, with organochlorine concentrations reaching highest levels in the late 1970s (Jensen *et al.*, 1969; Helle *et al.*, 1976a,b, Nyman *et al.*, 2002). Olsson *et al.*, (1974) reported that samples of Ringed seals from the Gulf of Bothnia contained DDT concentrations in the range of 200μg $g^{-1}$ and PCBs in the range of 110μg $g^{-1}$ (lipid weight). Similarly, Reijnders (1980) reported that German and Wadden Sea Harbour seal samples contained PCBs at concentrations of up to 340μg $g^{-1}$ and DDT at up to 47μg $g^{-1}$(lipid weight) These seals suffered various pathological impairments leading to infertility (Helle *et al.*, 1976a, b) and suffered significantly greater mortality during the 1988 seal epizootic due also to the immunosuppressive toxic effects of organochlorines (Reijnders, 1986; DeSwart *et al.*, 1994). As a result these seal populations suffered severe crashes during the 20$^{th}$ century with a slow recovery (Addison, 1989).

Many of these chemicals are believed to mediate their effects in marine mammals via an endocrine disrupting mechanism, for example by altering gonadal steroid biosynthesis, hepatic steroid metabolism, steroid receptor binding and causing general disturbance of endocrine homeostasis (Freeman and Sangalang, 1977; Colborn and Smolen, 1996; Troisi and Mason, 2000). Possibly, LTF could provide a sensitive, estrogen-specific biomarker of exposure in pinnipeds and other marine mammal populations exposed to environmental pollutants.

For LTF to be a successful biomarker for population biomonitoring, it needs to be measured in non-destructive samples, such as serum. However, whether the regulation of LTF expression is under estrogen control outside the reproductive tract (e.g. in serum) has yet to be established; therefore research is needed to determine this in the first instance. Baseline normal levels of LTF in tissues and biological fluids of seals are not known. Clearly, it is necessary for validation of LTF as an estrogen-specific biomarker in pinnipeds, that information on normal levels of LTF according to sex, age, species and reproductive cycle be determined. Thirdly, the relationship between LTF expression and xenoestrogenic exposure (dose-dependence) independent of confounding factors (e.g. estrus cycle), must also be demonstrated in the validation of LTF as a reliable biomarker.

The aim of this study was to investigate the reliability of LTF as a biomarker of PCB estrogenic exposure and effects in seals by the development of methods for characterisation and quantification of LTF in pinniped reproductive tissues and serum, and subsequently employing these methods to establish baseline LTF expression over the reproductive cycle. The dose-response relationship between PCB exposure and serum LTF concentrations in exposed and relatively unexposed seal populations was investigated as well as changes in LTF concentrations in association with reproductive steroid hormones at certain stages of the reproductive cycle.

## 4.2 Materials and methods

### 4.2.1 Sampling and Study Populations

Samples were obtained from three species, Grey (*Halichoerus grypus*), Ringed (*Phoca hispida*) and Harbour seals (*Phoca vitulina*). All of the serum and uterine samples used in the present study were provided by collaborators and were collected from six different seal populations over a 7 year period (1999 – 2002); Baltic and Svalbard (Arctic) Ringed seals, Baltic Grey and Sable Island (Canada) seals and UK Harbour seals from Abertay Sands (Dundee, Scotland) and Moray Firth (Northeast Scotland). Baltic Ringed and Grey seals, Svalbard Ringed seals and Grey seals from Sable Island, Canada were provided from Dr. M. Nyman, *Finnish Game and Fisheries Research Institute* (Helsinki, Finland). Harbour seals from Abertay Sands (Dundee, Scotland) were provided by Dr. Ailsa Hall (Sea Mammal Research Unit, St. Andrews University) and Moray Firth (Northeast Scotland) Harbour seals were provided by Dr. Paul Thompson (Lighthouse Research Station, University of Aberdeen). Harbour seals were also provided from a controlled dosing study, in which the seals were fed fish from the highly contaminated Wadden Sea or less contaminated Atlantic Sea (Prof. P. Reijnders).

### 4.2.2 Reproductive Biology Information of Study Populations

Information on the reproductive cycle of pinnipeds is critical to understanding the natural endogenous regulation of LTF expression in these species. The reproductive cycle of Ringed, Harbour and Grey seals was divided into four stages (I-IV) that represent different reproductive states during the annual cycle (Gardiner *et al.*, 1999) and changes in hormone concentrations during the reproductive cycle were analysed with respect to these changes (*for further information refer to Chapter 3-Sex Steroid Analysis*). The four different stages of the reproductive cycle of the three species under study are presented in Table 4.1 and a summary of sample information is provided in Table 4.2 (*for further information refer to Chapter 2-Contaminant Analysis*).

**Table 4.1:** Stages in the reproductive cycle of Harbour, Ringed and Grey seals.

| Stage | Species | Months | Female | Male |
|---|---|---|---|---|
| I | *Harbour seals* | June – August | Postpartum Period | Copulation |
| | *Ringed seals* | February – April | | |
| | *Grey seals* | February – April | | |
| II | *Harbour seals* | Sep – November | Delayed Implantation | Postcopulation |
| | *Ringed seals* | May – July | | |
| | *Grey seals* | May – July | | |
| III | *Harbour seals* | December – February | Postimplantation Gestation | Midcycle |
| | *Ringed seals* | August – October | | |
| | *Grey seals* | August – October | | |
| IV | *Harbour seals* | March – May | Late Gestation or Non-Pregnant | Precopulation |
| | *Ringed seals* | November – January | | |
| | *Grey seals* | November – January | | |

*(Table 4.1 was reproduced from Chapter 3-Sex Steroid Analysis)*

### 4.2.3 Sample preparation

Uterine samples and uterine flushings (Baltic Ringed and Grey seals) were taken immediately after death and frozen in liquid nitrogen during transfer to the laboratory. Uterine flushings were taken by flushing the uterus with sterile 0.1M phosphate buffer (pH 7.4) using a sterile plastic syringe. Flushings where centrifuged at 2000 x g for 10 min at 4°C. The supernatant was further centrifuged at 3,000 x g for 10 min at 4°C and then frozen. Whole blood samples were centrifuged for 5 minutes at 3000rpm to obtain the serum.

| Population Category | Exposed | Control | Exposed | Control | Exposed | Exposed | Experimental | |
|---|---|---|---|---|---|---|---|---|
| | North Baltic Sea | Svalbard | North Baltic Sea | Sable Island | Moray Firth | Abertay Sands | Experimental Group | |
| | | European Arctic | | Canada | NE Scotland | Firth of Tay, Scotland | Exposed | Control |
| **Species** | **Ringed Seals** | | **Grey Seals** | | | **Harbour Seals** | | |
| | *Phoca hispida baltica* | *Phoca hispida* | *Halichoerus grypus* | | | *Phoca vitulina* | | |
| **Number of Samples** | **26** | **18** | **41** | **19** | **68** | **56** | **11** | **12** |
| *Serum* | 23 | 18 | 27 | 19 | 68 | 56 | 11 | 12 |
| **No. analysed for ΣPCBs** | **23** | **18** | **19** | **19** | **47** | **48** | **11** | **12** |
| *Uterine Epithelium* | 3 | - | 7 | - | - | - | - | - |
| *Uterine Flushings* | - | - | 7 | - | - | - | - | - |
| **Sex & Age** | | | | | | | | |
| **Females** | **11** | **10** | **16** | **10** | **36** | **20** | **11** | **12** |
| Adult | All | All | All | All | 18 | All | All | All |
| Sub-Adult | - | - | - | - | 4 | - | - | - |
| Juvenile | - | - | - | - | 14 | - | - | - |
| **Males** | **12** | **8** | **11** | **9** | **32** | **36** | - | - |
| Adult | All | All | All | All | 21 | All | - | - |
| Sub-Adult | - | - | - | - | 2 | - | - | - |
| Juvenile | - | - | - | - | 9 | - | - | - |
| **Year of Sampling** | April to June 1997-1998 2000 2002 | April to June 1996 1998 | April to June 1997-1998 2000 | April to June 1998 | 1992-1995 | 1999-2000 | 1988 | 1988 |
| **Stage of Reproduction** | II | II | II | II | II and IV | I, II, III and IV | IV | IV |

*(Table 4.2 was reproduced from Chapter 2 – Contaminant Analysis)*

### 4.2.4 Reagents

All chemicals were purchased from Sigma (UK) unless stated otherwise. Bromophenol blue, goat anti-bovine LTF – Horseradish Peroxidase (HRP) and purified bovine LTF protein, were purchased from *ICN Biochemicals Inc* (UK). Tris (hydroxymethyl) methylamine, Glycine, β-mercaptoethanol, Glycerol, Coomassie Brilliant Blue, and Sodium Dodecyl Sulphate (SDS) were all purchased from *BDH* (UK). A Bovine Lactoferrin ELISA Quantitation kit was purchased from *BETHYL Laboratories, Inc.*, (USA) including, affinity purified Goat anti-Bovine Lactoferrin, Bovine Milk Protein Calibrator, Goat x-Bovine Lactoferrin – Horseradish Peroxidase and Nunc C bottom Immunoplate 96 well Modules and Frames. 3,3',5,5'-tetramethyl benzidine (TMB) Peroxidase substrate and Peroxidase Solution B were purchased from *Kirkegaard and Perry* (Gaithersburg, MD).

### 4.2.5 Lactoferrin Characterisation

Uterine epithelial tissue was macerated into small pieces using a sterile scalpel. Two volumes of homogenising buffer were used per gram of tissue. The macerated tissue with buffer was placed in a tube held on ice to prevent degradation and homogenised with two volumes of homogenising buffer (50mM Tris-HCl, pH 7.5) per gram of tissue, for 10sec intervals with an ultra-turax homogeniser to obtain a homogeneous mixture. The mixture was further homogenised in a teflon-glass Potter homogeniser packed on ice. Homogenates were then centrifuged at 105,000 x g for 60 min at 4°C using an ultracentrifuge (MSC Eureopa 65). The soluble fraction (supernatant) was collected ready for analysis.

LTF protein was separated from interfering proteins using discontinuous Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis SDS-PAGE mini gel system according to the method of Laemmli (1970) with minor modifications. An upper - 10% acrylamide separating gel and a lower - 4.5% stacking gel, comprised the discontinuous mini gel system. A colour molecular weight marker (SIGMA; 6.5-205kDa) was loaded into the first lane and 10µl of uterine tissue homogenates, uterine flushings and serum were loaded into remaining lanes. The diluent buffer (Tris-HCl, pH 7.5) was used as the negative control and purified bovine LTF as positive control (10mg/ml). Duplicate gels (one to be kept and the second to be transferred into a membrane) were run for 40min at 200 volts. To verify loading efficiency, each time one

of the gels was stained with Coomassie Brilliant Blue. For LTF immunodetection the second gel was then transferred overnight at 30volts to nitrocellulose paper by electrophoretic Western blotting technique using the method of Towbin *et al.*, (1979) with minor modifications. Briefly, the nitrocellulose was washed with 10ml Tris-saline and then incubated for 1h at room temperature with 10ml Tris-saline containing 3% Tween20 (blocking agent). Goat x-bovine Lactoferrin – HRP in Tris-saline and 0.1%Tween20 (1/1000 dilution) was added to the nitrocellulose. After a 1h incubation at room temperature, the nitrocellulose was washed three times in Tris-saline and the blot developed for 1-5min using 3,3'-diaminobenzidine tetrahydrochloride (DAB) peroxidase substrate. The reaction was stopped with Tris-saline (Details on SDS-Page and western blotting techniques are presented in the Appendix-II).

### 4.2.6 Quantification of LTF protein (ELISA)

#### *4.2.6.1 Assay Development*

There are no commercially available antibodies to pinniped LTF and at the present study there were no facilities available to generate our own in the laboratory. As a result a commercially-available ELISA was selected with polyclonal capture antibody to increase likelihood of cross reactivity with pinniped LTF. LTF enzyme-linked immunosorbent assay (ELISA; *Bethyl Inc.*) was firstly optimised by trying different dilutions of the goat anti-bovine LTF-HRP conjugate in order to derive the best LTF detection possible in the seal samples. Goat anti-bovine LTF was selected as a suitable capture antibody since it was also used for LTF characterisation and provided positive results. The working dilution of the goat anti-bovine LTF antibody was 1 in a 100 (10µg/ml; diluted in 0.05M sodium carbonate, pH 9.6) and the HRP conjugate antibody was 1 in 80,000 diluted with 50mM Tris-HCl buffered saline (pH 8.0), containing 0.1% BSA and 10% Tween 20 (1/200,000 suggested by manufacturer). These dilutions were optimal due to the resultant absorbance range/colour reaction which was necessary to increase sensitivity of method for detection of low LTF concentrations in seal samples. Moreover, this working dilution of the goat anti-bovine LTF antibody was selected to increase the cross reactivity with the seal LTF since it was expected to be low due to difference in the species. The assay was then calibrated with known concentrations of purified bovine LTF protein (positive

control) due to unavailability of pinniped LTF. A sample of seal colostrum was sent for purification but until this stage the purification has not been completed.

Bovine milk protein calibrator (stock solution concentration: 1mg/ml; supplied by manufacturer) was used for the calibration curve. Seal LTF concentrations were extrapolated using the equation of the linear portion of the calibration curve. To minimise inter-plate variation, samples were analysed in triplicate on the same plate and LTF concentrations extrapolated from the calibration curve derived on the same plate. A blank (in duplicate) was also analysed with each sample set in order to control any background absorbance attributable to non-specific binding and to avoid over-presentation of the data. Sample LTF concentrations were expressed as mean of triplicate values for each sample.

*4.2.6.2 Assay Protocol*

Prior to analyses the samples were grouped according to the stage of reproductive cycle. Dilution of the blood samples was not necessary due to the resultant absorbance range/colour reaction. Briefly, the plate was coated with 100µl capture antibody (affinity purified Goat anti-Bovine LTF) at 10µg/ml for 1h. Following this it was postcoated with 1% Bovine Serum Albumin (BSA) in 50mM Tris (pH 8.0, 0.15M NaCl (TBS)). After a 30min-incubation period, Bovine Milk Protein Calibrator was diluted (initial lactoferrin concentration 1mg/ml) in 1%BSA in TBS with 0.05% Tween20 for the calibration curve. Subsequent LTF concentrations ranging from 500 to 0.02ng/ml were prepared. Each well was loaded with 100µl of either sample or LTF standard. The plate was incubated for 1h and then 100µl of Goat anti-Bovine LTF – HRP (diluted in 1% BSA in TBS with 0.05% Tween20) added to each well and incubated for 1h. Finally, 100µl of enzyme substrate (TMB/$H_2O_2$) were added to each well for colour development and the reaction was terminated with 100µl/well of 2 M $H_2SO_4$ after 20mins. Absorbance at 450nm was measured using an ELISA microplate reader (*Bio-Rad*, Model 550). From the LTF calibration curve the concentration of LTF protein in samples (uterine epithelium tissue, uterine flushings and serum) was extrapolated from the equation of the linear portion of the calibration curve. The limit of detection of this ELISA was 0.02ng/ml.

### 4.2.7 Quantification of serum ΣPCB, 17β-estradiol and progesterone concentrations

Serum samples were analysed (ELISA) for the sex hormones 17β-estradiol (E2) and progesterone (P) as well as for ΣPCBs in order to determine the level of exposure in study animals. Full details of analysis are provided in Chapter 3 and 2 respectively. Mean hormone and ΣPCBs concentrations are presented in Tables 4.3 and 4.4.

**Table 4.3:** Mean Concentrations of progesterone and 17β-estradiol in serum samples for each study population (Results are presented as means ±standard errors; ranges)

| Species<br>Study Population | Sample No. | Sex Steroid Hormone | | Reproduction Stage |
|---|---|---|---|---|
| **Harbour Seals (*Phoca vitulina*)** | **(E – P)** | **17β-estradiol (E) (ng/ml)** | **Progesterone (P) (ng/ml)** | |
| **Abertay Sands, Firth of Tay, Scotland** | | | | |
| *Male Adults* | (5) – (5) | 0.07 ± 0.02 (0.04 – 0.12) | 1.81 ± 0.40 (0.98 – 3.34) | **I** |
| | (6) – (6) | 0.09 ± 0.02 (0.04 – 0.14) | 4.59 ± 0.92 (1.21 – 7.80) | **II** |
| | (14) – (13) | 0.10 ± 0.01 (0.04 – 0.21) | 9.15 ± 3.30 (0.74 – 45.00) | **III** |
| | (10) – (5) | 0.13 ± 0.03 (0.03 – 0.24) | 4.95 ± 1.10 (1.76 – 7.84) | **IV** |
| *Female Adults* | (9) – (9) | 0.16 ± 0.05 (0.02 – 0.47) | 41.07 ± 9.91 (1.12 – 70.00) | **I** |
| | (1) – (1) | 0.42 | 70 | **II** |
| | (7) – (7) | 0.12 ± 0.02 (0.05 – 0.17) | 52.71 ± 9.74 (11.34 – 72.00) | **III** |
| | (3) – (3) | 0.21 ± 0.06 (0.10 – 0.32) | 22.53 ± 9.39 (11.54 – 41.22) | **IV** |
| **Moray Firth, NE Scotland** | | | | |
| *Male Adults* | (14) – (14) | 0.15 ± 0.05 (0.02 – 0.68) | 7.38 ± 3.68 (0.53 – 40.00) | **I** |
| | (1) – (1) | 0.07 | 1.49 | **IV** |
| *Male Juveniles* | (9) – (9) | 0.17 ± 0.10 (0.02 – 0.98) | 15.28 ± 7.57 (2.16 – 59.82) | **I** |
| *Female Adults* | (11) – (11) | 0.12 ± 0.03 (0.02 – 0.26) | 24.04 ± 9.79 (3.73 – 89.46) | **I** |
| | | 0.84 | 78 | **IV** |
| *Female Juveniles* | (14) – (14) | 0.17 ± 0.04 (0.05 – 0.49) | 14.73 ± 5.26 (2.94 – 68.37) | **I** |
| **Experimental Group** | | | | |
| *Females Control* | (12) – (12) | 0.22 ± 0.05 (0.03 – 0.66) | 27.01 ± 5.92 (0.09 – 70.00) | **IV** |
| *Females Exposed* | (11) – (11) | 0.11 ± 0.04 (0.01 – 0.47) | 11.35 ± 5.67 (0.10 – 58.90) | **IV** |
| **Ringed Seals (*Phoca hispida*)** | | | | |
| **North Baltic Sea** | | | | |
| *Male Adults* | (6) – (9) | 0.04 ± 0.01 (0.02 – 0.06) | 0.11 ± 0.04 (b.d.l. – 0.11) | **II** |
| *Female Adults* | (10 – (9) | 0.08 ± 0.02 (0.02 – 0.19) | 6.51 ± 0.90 (2.73 – 9.88) | **II** |
| **Svalbard, European Arctic** | | | | |
| *Male Adults* | (7) – (5) | 0.10 ± 0.06 (0.02 – 0.44) | 2.12 ± 1.48 (b.d.l. – 7.54) | **II** |
| *Female Adults* | (8) – (7) | 0.31 ± 0.09 (0.05 – 0.77) | 24.28 ± 8.86 (0.84 – 60.00) | **II** |
| **Grey Seals (*Halichoerus grypus*)** | | | | |
| **North Baltic Sea** | | | | |
| *Male Adults* | (0) – (6) | n.a. | 0.88 ± 0.30 (0.21 – 2.22) | **II** |
| *Female Adults* | (15) – (15) | 0.13 ± 0.03 (0.0.3 – 0.45) | 27.36 ± 5.92 (0.82 – 82.00) | **II** |
| **Sable Island, Canada** | | | | |
| *Male Adults* | (9) – (5) | 0.56 ± 0.16 (0.06 – 1.30) | 14.34 ± 10.09 (0.57 – 54.25) | **II** |
| *Female Adults* | (10) – (10) | 0.46 ± 0.19 (0.07 – 2.10) | 32.29 ± 7.25 (6.04 – 85.25) | **II** |

b.d.l, below detection limit; n.a, not available *(Table 4.3 was reproduced from Chapter 3 – Sex steroid analysis)*

| Population | Exposed | Control | Exposed | Control | Exposed | Exposed | Experimental | |
|---|---|---|---|---|---|---|---|---|
| | North Baltic Sea | Svalbard | North Baltic Sea | Sable Island | Moray Firth | Abertay Sands | Experimental Group | |
| | | European Arctic | | Canada | NE Scotland | Firth of Tay, Scotland | Exposed | Control |
| **Species** | **Ringed Seals** | | **Grey Seals** | | | **Harbour Seals** | | |
| | *Phoca hispida baltica* | *Phoca hispida* | *Halichoerus grypus* | | | *Phoca vitulina* | | |
| **No of Serum Samples** | **23** | **18** | **19** | **19** | **47** | **48** | **11** | **12** |
| **Female Adults** | **11** | **11** | **10** | **10** | **11** | **31** | **11** | **12** |
| **Mean (µg/ml)** | 14.03 ± 1.78 | 2.16 ± 0.57 | 13.46 ± 4.38 | 1.79 ± 0.51 | 10.59 ± 1.64 | 8.40 ± 1.81 | 12.76 ± 2.52 | 4.92 ± 0.55 |
| **Range** | (8.01-25.49) | (0.47-6.48) | (2.35-39.73) | (0.36-4.89) | (3.69 – 21.29) | (1.29-27.49) | (4.16 – 29.22) | (2.29 – 9.51) |
| **Female Juveniles** | **n.a.** | **n.a.** | **n.a.** | **n.a.** | **14** | **n.a.** | **n.a.** | **n.a.** |
| **Mean (µg/ml)** | | | | | 14.40 ± 3.02 | | | |
| **Range** | | | | | (1.97 – 36.54) | | | |
| **Male Adults** | **12** | **8** | **9** | **9** | **14** | **17** | **n.a.** | **n.a.** |
| **Mean (µg/ml)** | 16.44 ± 3.06 | 2.56 ± 0.71 | 40.18 ± 10.43 | 12.14 ± 5.33 | 13.79 ± 1.76 | 9.67 ± 1.23 | | |
| **Range** | (6.05-45.76) | (0.88-6.95) | (7.10-87.06) | (0.30-16.00) | (6.45 – 23.70) | (0.68 – 24.54) | | |
| **Male Juveniles** | **n.a.** | **n.a.** | **n.a.** | **n.a.** | **8** | **n.a.** | **n.a.** | **n.a.** |
| **Mean (µg/ml)** | | | | | 7.58 ± 2.64 | | | |
| **Range** | | | | | (2.43 – 25.23) | | | |

n.a., not available

*(Table 4.4 was reproduced from Chapter 2 – Contaminant Analysis)*

## 4.3 Results

### 4.3.1 Characterisation of LTF in seal serum and uterine epithelium tissue

Representative western blots of Ringed and Grey seal serum samples can be seen in Figures 4.1 (A, B and C) and Figure 4.2. The gel (Fig. 4.1(A)) revealed clear bands showing immunodetection of bovine LTF standard (positive control) at ~ 65 - 70kDa (lanes 3 and 4). Corresponding bands were also detected in the serum samples (lanes 5-8), indicating that an LTF-like protein was also present in the Ringed, Grey and Harbour seals serum samples. The western blots (Fig. 4.1(B) and 4.1(C); Fig.4.2) showed staining (lanes 4-8) at approximately 70kDa. The band in lane 7 (Fig.4.2) was darker than the other lanes, indicating a higher LTF-like protein concentration in the serum sample from the male Baltic Grey seal (H5-00) compared with serum samples tested from other seals: two Grey female Baltic seals (H8-00 and H2-98), one male Baltic Grey seal (H5-00) and two Harbour seals from Moray Firth (female: No.27 and male No. 73).

The protein components of Ringed and Grey seal uterine epithelium tissue can be seen in Figure 4.3. From this blot it can be seen that a protein band was again present at ~ 65-70kDa, indicating that the antibody cross-reacted with a seal LTF-like protein confirming the presence of this protein in both Baltic Ringed and Grey seal uterine epithelium. There were other proteins present in the uterine epithelial tissue extracts of all the seal samples tested, seen as weaker stained bands above and below the LTF band. The weaker staining could have been indicative of poorer cross-reactivity of the detection antibody with these proteins or that there was less LTF-like protein to bind to (low concentration).

These results concluded that *Bethyl Inc.* goat anti-bovine LTF antibody was found to be capable of cross-reacting with an LTF-like protein present in uterine epithelium and serum from Ringed, Grey and Harbour seals (males and females), as evidenced by a distinct staining band at ~65-70kDa. There was also successful immunodetection of bovine LTF (positive control) and no detection in blanks (negative control western blots–no sample/standard applied (Figure 4.1, lane 2). Although the intensity of staining was weaker for seal LTF-like protein than that for bovine LTF positive control, the binding affinity of the detection antibody was adequate for immunodetection and this justified the selection of a polyclonal goat anti-bovine LTF antibody based ELISA for subsequent LTF quantification.

Examination of the western blots in all figures shows that the bovine LTF protein standard (positive control) used was not completely pure. Other proteins of a lower molecular weight than LTF were present, as shown in lane 3 (Fig 4.1,2 and 3). Information on the purity of bovine LTF was not available from *Sigma Co., Ltd.* The goat anti-bovine LTF antibody cross-reacted with these impurities, but is unlikely to have had any major effect on the binding to the LTF-like protein in seal samples, and therefore does not affect the accuracy of the results. These impurities could be LTF degradation products and were not considered a problem since LTF quantitation using ELISA includes them.

**a.)**

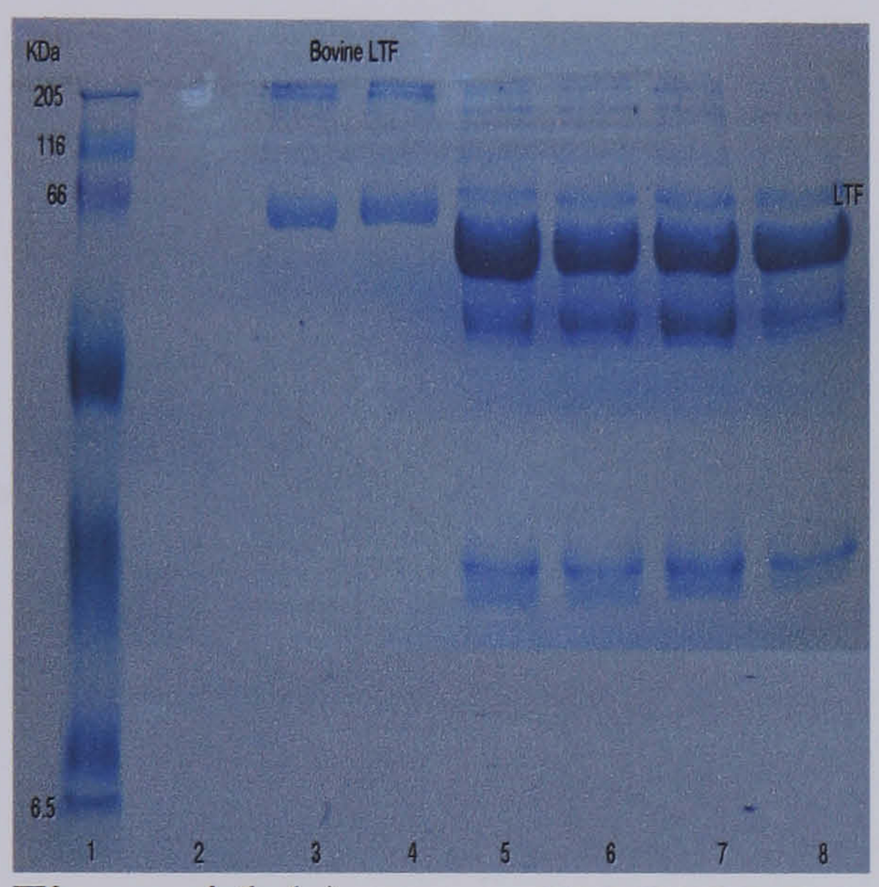
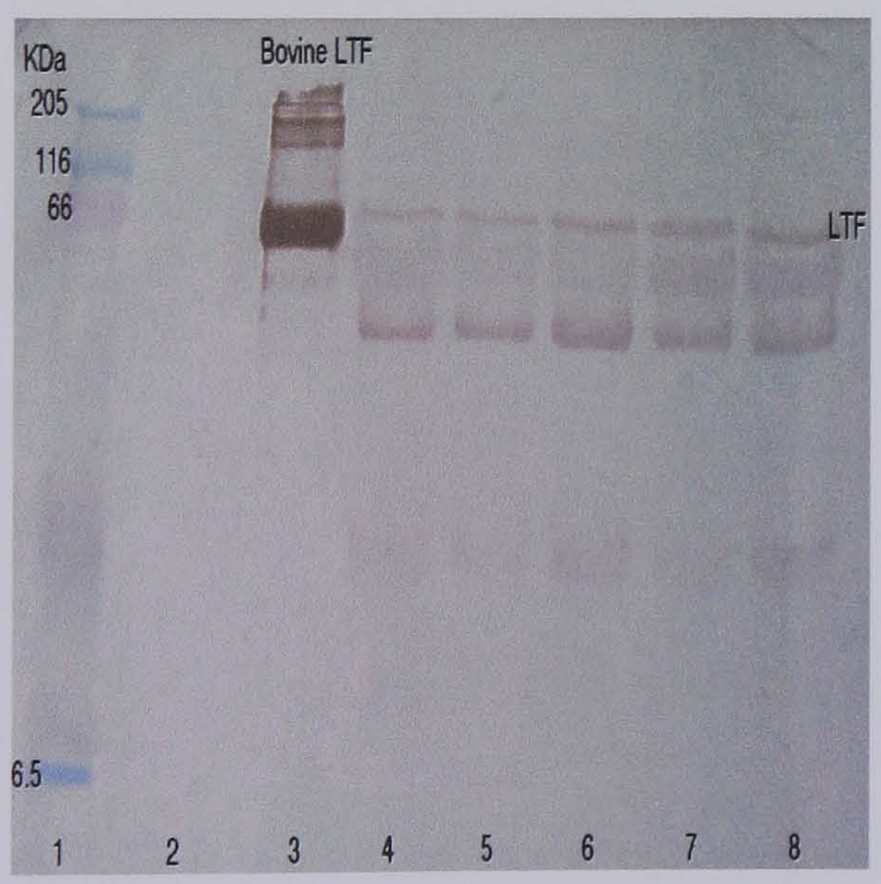
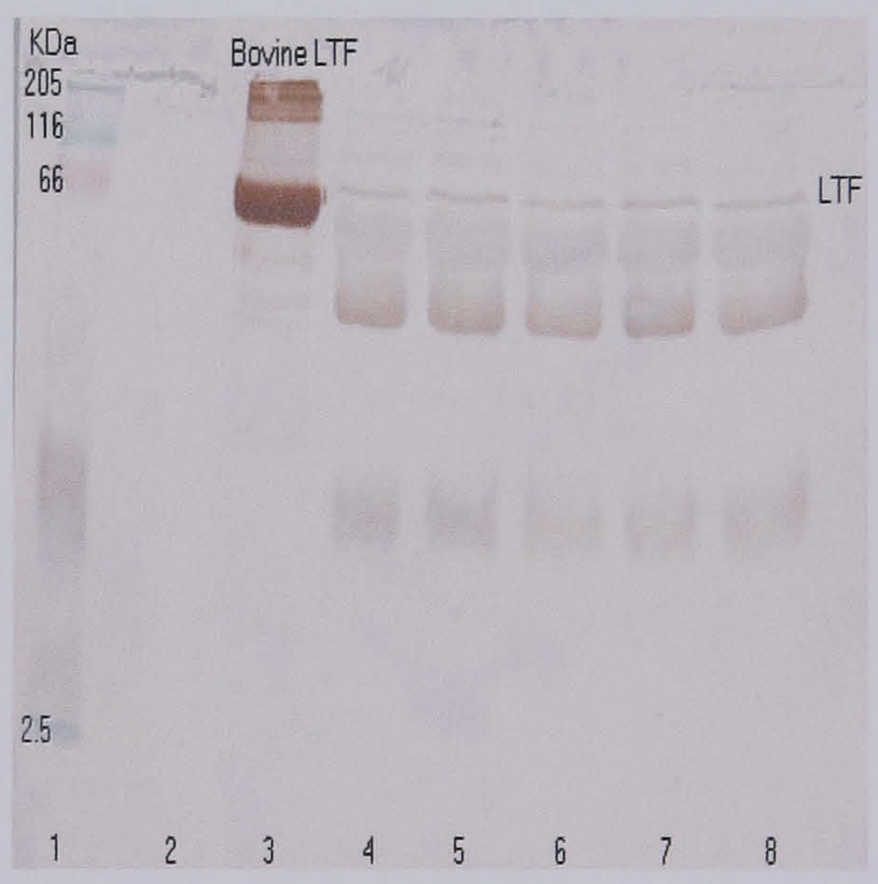



**Figure 4.1 (a):** SDS-PAGE Gel of Ringed Seal Serum.
Lane 1, Coloured MW marker; lane 2, negative control (sample loading buffer, Tris-HCl, pH 7.6); lanes 3 and 4, bovine LTF (2µg protein); lane 5, female seal-N2; lane 6, male seal-N4; lane 7, male seal-N5; lane 8, female seal-N6 (for all samples 10µl serum was diluted in 790µl Tris-HCl, pH 7.6).

**b.)**




**c.)**




b.) Western Blot of Ringed Seal Serum:
Lane 1, Coloured MW marker; lane 2, negative control (sample loading buffer, Tris-HCL, pH 7.6); lane 3, bovine LTF (2µg protein); lane 4, female seal-N2; lane 5, male seal-N4; lane 6, male seal-N5; lane 7, female seal-N6; lane 8, female seal-N7 (for all samples 10µl serum was diluted in 790µl Tris-HCl, pH 7.6)
c.) Western Blot of Grey Seal Serum:
Lane 1, Coloured MW marker; lane 2, negative control (sample loading buffer, Tris-HCL, pH 7.6); lane 3, bovine LTF (2µg protein); lane 4, male seal-H4; lane 5, female seal-H5; lane 6, female seal-H6; lane 7, female seal-H7; lane 8, female seal-H8 (for all samples 10µl serum was diluted in 790µl Tris-HCl, pH 7.6)

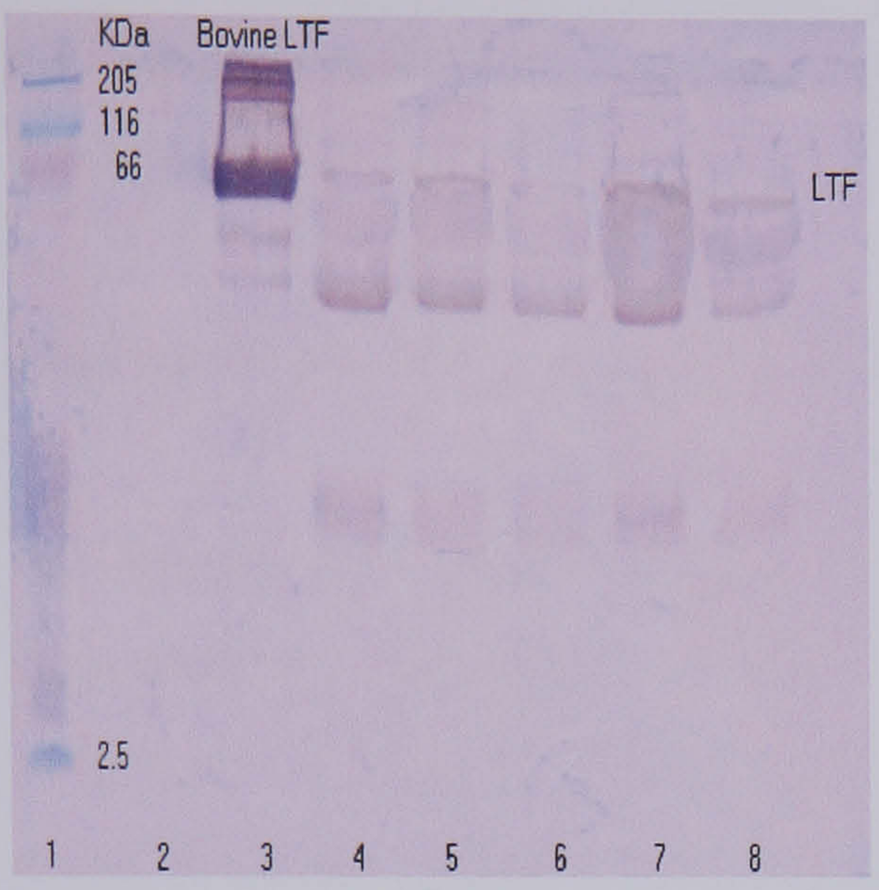



**Figure 4.2.** Western Blot of Ringed, Harbour and Grey Seal Sera.
Lane 1, Coloured MW marker; lane 2, negative control (sample loading buffer, Tris-HCL, pH 7.6); lane 3, bovine LTF (2µg protein); lane 4, female Grey seal-H8; lane 5, female Grey seal-H2; lane 6, female Harbour seal-Moray Firth (No.27); lane 7, male Grey seal-H5; lane 8, male Harbour seal (No. 73)-Moray Firth (for all samples 10µl serum was diluted in 790µl Tris-HCl, pH 7.6).

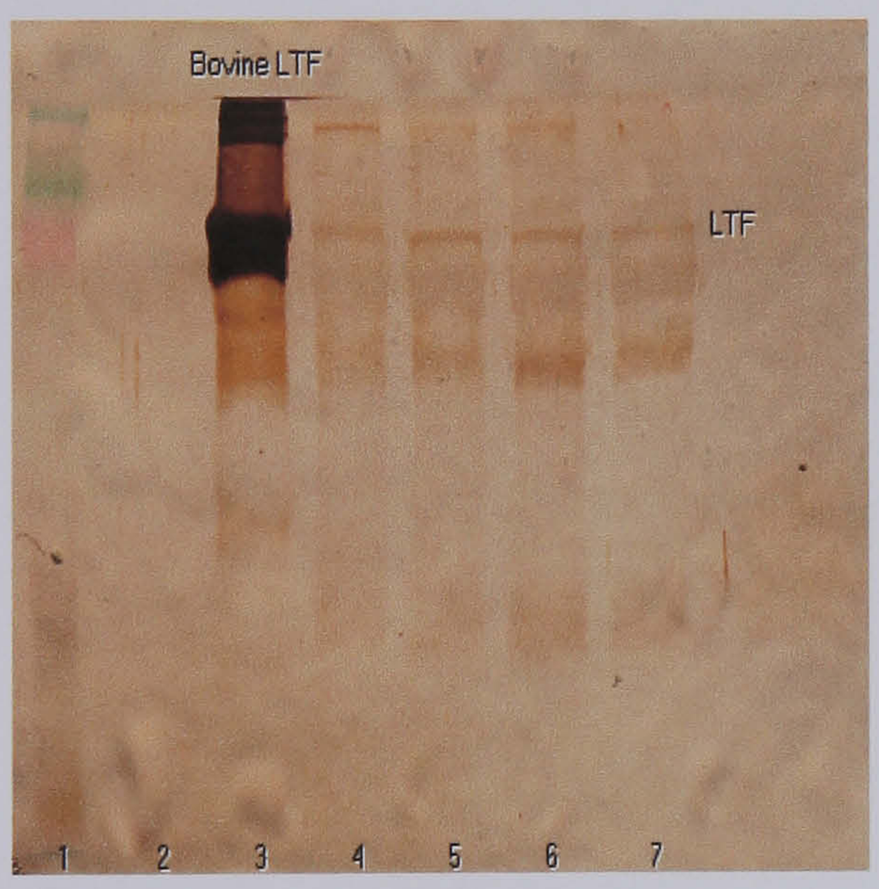



**Figure 4.3.** Western Blot of Ringed and Grey Seal Uterine Epithelium
Lane 1, Coloured MW marker; lane 2, negative control (sample loading buffer, Tris-HCL, pH 7.6); lane 3, bovine LTF (2µg protein); lane 4, Ringed seal-N2; lane 5, Ringed seal-N3; lane 6, Grey seal-H2; lane 7, Grey seal-H6 (for all samples 10µl of uterine epithelial tissue was diluted in 90µl Tris-HCl, pH 7.6).

### 4.3.2. LTF concentrations in Ringed, Grey and Harbour seals

For statistical analysis, seal data were grouped according to species, reproductive stage, sex and age. The differences between groups were tested by two-tailed student t-test. Significance levels were set at $p<0.05$ and $p<0.005$. Results are presented as means ± standard error (s.e.) and ranges (min- max).

#### 4.3.2.1 Sex and age-dependent differences within species

LTF-like concentrations detected in seal serum from all study populations are presented in Table 4.5.

*Harbour Seals, Moray Firth, Scotland*

All female and male Harbour seals from Moray Firth were sampled at Stage I of reproduction cycle (estrus / breeding), whereas only one from each sex was sampled during pregnancy in Stage II. Therefore no comparison between different stages of reproduction could be made. Moray Firth, Harbour seals were the only group that could be tested for age dependent differences since all the rest of the populations consisted of adult seals. There was no statistically significant difference between juvenile female and male Harbour seals. However, when comparing female adult with juvenile LTF concentrations, it was observed that female adults had significantly higher LTF levels ($p<0.05$) than juveniles (Fig.4.4). Female adult LTF levels were also statistically significant higher than male adults ($p<0.005$; Fig. 4.4). Male and female juvenile Harbour seals showed no statistically significant difference.

*Harbour Seals, Abertay Sands, Scotland*

Female and male Harbour seals from Abertay Sands were compared at all the stages of the reproductive cycle and as it can be seen from Figure 4.4, female adult LTF concentrations were significantly higher than male adults ($p<0.05$) in all stages. Even though seals were sampled during different reproductive stages when comparing LTF concentrations of the same sex but different reproductive stage it was observed that only males from Stage I had statistically significant lower LTF levels with males from Stage III ($p<0.05$). No statistically significant differences were observed between the females of different reproductive stages.

**Table 4.5:** Serum LTF concentrations (ng/ml) for Harbour, Ringed and Grey seals

| **Population Category** | **Exposed** | **Control** | **Exposed** | **Control** | **Exposed** | **Exposed** | **Experimental Group** | |
|---|---|---|---|---|---|---|---|---|
| **Study Site** | North Baltic Sea | Svalbard, European Arctic | North Baltic Sea | Sable Island Canada | Moray Firth North-east Scotland | Abertay Sands Firth of Tay, Scotland | Control | Exposed |
| **Species** | Ringed Seals *Phoca hispida* | | Grey Seals *Halichoerus grypus* | | Harbour Seals *Phoca vitulina* | | | |
| **Age, Sex & Repr.Stage** | | | | | | | | |
| *Female - Adults* | | | | | | | | |
| Stage I | na | na | na | na | 2.54 ± 0.06** (2.15 - 3.15) | 1.81 ± 0.09* (1.43 - 2.25) | na | na |
| Stage II | 2.51 ± 0.03* (2.38 - 2.63) | 0.14 ± 0.04 (0.02 - 0.44) | 2.97 ± 0.06 (2.60 - 3.43) | 0.27 ± 0.07** (0.03 - 0.57) | na | 2.26 | na | na |
| Stage III | na | na | na | na | na | 1.75 ± 0.07* (1.52 - 2.13) | na | na |
| Stage IV | na | na | na | na | 2.43 | 1.82 ± 0.07* (1.68 - 1.93) | 1.59 ± 0.04 (1.36 - 1.85) | 3.00 ± 0.16 (2.35 - 3.83) |
| *Female - Juveniles* | na | na | na | na | 2.34 ± 0.07^ (2.03 - 2.75) | na | na | na |
| *Male - Adults* | | | | | | | | |
| Stage I | na | na | na | na | 1.95 ± 0.14 (1.05 - 3.04) | 1.42 ± 0.09 (1.16 - 1.68) | na | na |
| Stage II | 2.40 ± 0.05 (2.12 - 2.64) | 0.25 ± 0.06 (0.07 - 0.53) | 2.90 ± 0.19 (1.52 - 3.95) | 1.31 ± 0.28 (0.22 - 2.23) | na | 1.70 ± 0.09 (1.35 - 1.94) | na | na |
| Stage III | na | na | | | na | 1.59 ± 0.03 (1.36 - 1.85) | na | na |
| Stage IV | na | na | na | na | 1.06 | 1.49 ± 0.07 (1.24 - 1.87) | na | na |
| *Male - Juveniles* | na | na | na | na | 2.29 ± 0.09 (2.10 - 3.02) | na | na | na |

Results are presented as means ±standard errors (ranges). The significance of sex variation is presented as * $p<0.05$ or ** $<0.005$ and the significance of age variation is shown as ^ $p<0.05$.
na, not available

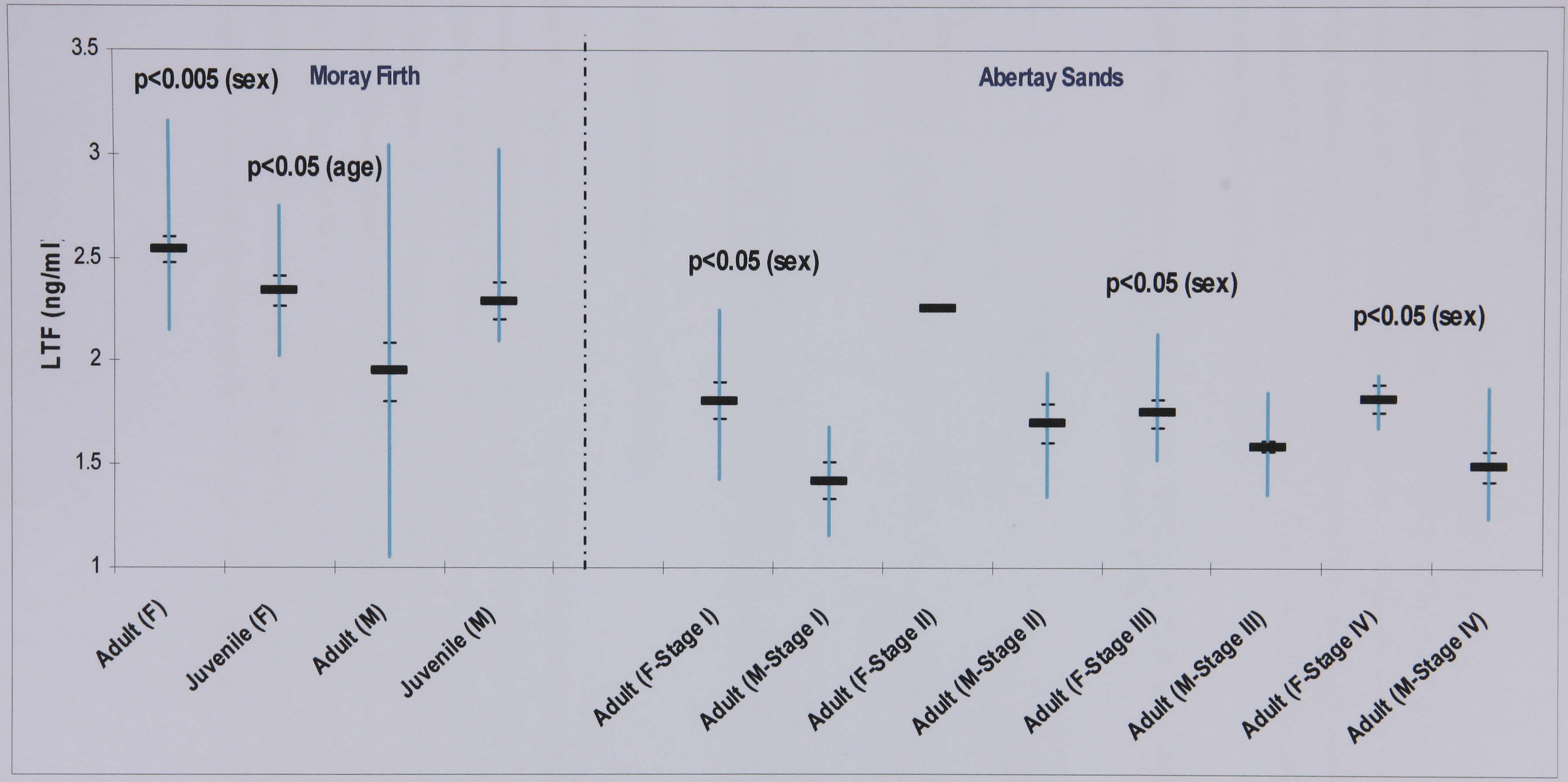



**Figure 4.4:** Sex and age-dependent difference in Harbour seals (Moray Firth and Abertay Sands).

*Ringed and Grey Seals*

The serum, uterine epithelium and uterine flushing LTF-like concentrations for Ringed and Greys seals, are presented as means ±SE and ranges in Table 4.6.

LTF levels of Baltic and Svalbard female Ringed seals (Fig.4.6) were higher than males with only females from the Baltic area having statistically significant higher values compared to the males ($p<0.005$). Baltic male and female Grey seals had similar LTF values whereas male Grey seals from Sable Island had statistically significant higher LTF levels ($p<0.005$) when compared to females. All Grey and Ringed seals were sampled at the same stage of reproductive cycle (Table 4.2).

**Table 4.6:** Summary of serum, uterine epithelium tissue and uterine flushing LTF values (ng/ml) for Ringed and Grey seals

| **Species** | **Ringed Seals** | | **Grey Seals** | |
|---|---|---|---|---|
| | *Phoca hispida* | | *Halichoerus grypus* | |
| | **Baltic Sea** | **Svalbard** | **Baltic Sea** | **Sable Island** |
| **Females** | | | | |
| *Sample No.(Serum)* | **n = 11** | **n = 10** | **n = 16** | **n = 10** |
| *Serum* | **2.5 ± 0.03** (2.4-2.6)$^{00}$ | **0.14 ± 0.04** (0.02-0.44) | **3.0 ± 0.06** (2.6-3.4)* | **0.3 ± 0.1** (0.03-0.6)^^ |
| *Uterine Epithelium* | **2.3 ± 0.3** (1.8-2.7) **(n=3)** | - | **3.9 ± 0.5** (2.2-6.1)* **(n=7)** | - |
| *Uterine Flushing* | - | - | **2.5 ± 0.4** (1.6-4.5)* **(n=7)** | - |
| | | | | |
| **Males** | | | | |
| *Sample No.* | **n = 12** | **n = 8** | **n = 11** | **n = 9** |
| *Serum* | **2.4 ± 0.05** (2.1-2.6)$^{00}$ | **0.3 ± 0.1** (0.1-0.5) | **2.9 ± 0.2** (1.5-3.9) | **1.3 ± 0.3** (0.2-2.2)^^ |

Results are presented as means ±standard errors (ranges). The significance of sample dependence difference is presented as * $p<0.05$, the significance of geographical variation between Grey seals is shown as ^^ $p<0.005$ and between Ringed seals as $^{00}$ $p<0.005$.

## 4.3.2.2 Population and Species Differences

Female Harbour seals from Moray Firth showed statistically significant higher LTF concentrations ($p<0.005$) when compared to Harbour seals from Abertay Sands (Table 4.5; Fig.4.4). Comparison of Moray Firth Harbour seals with Grey and Ringed seals was not possible since the later were only sampled in Stage II (Table 4.2). Moreover, comparison between Abertay Sands females with Grey and Ringed seal was not possible since there was only one seal that was sampled in Stage II. Male Abertay Sands however, had statistically significant lower LTF levels ($p<0.005$) when compared with Baltic Grey and Ringed seals and higher compared with Svalbard male Ringed seals ($p<0.005$).

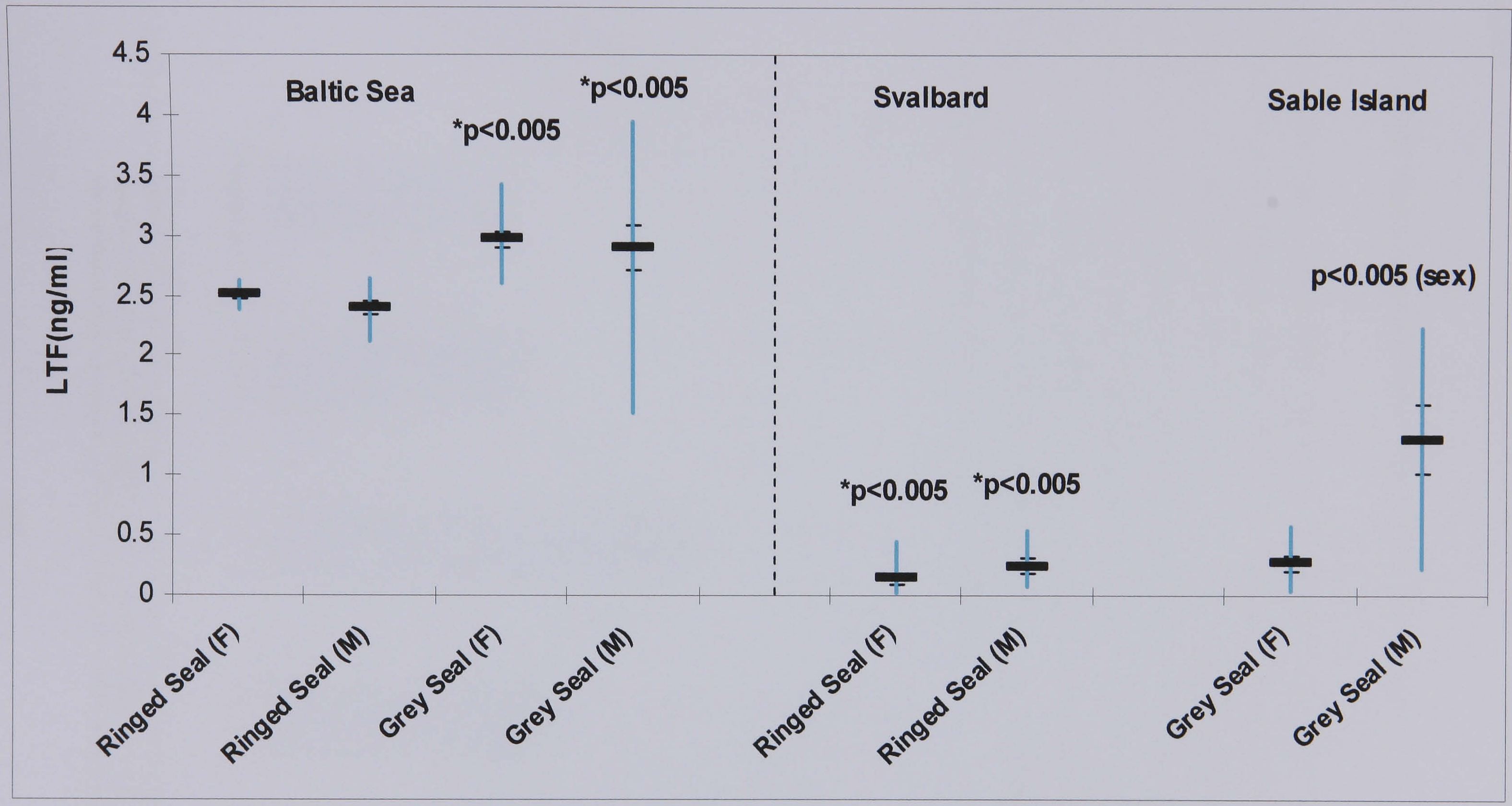



**Figure 4.5** Sex and geographical variation (*) in Grey and Ringed Seals

A species comparison between the Baltic seals showed statistically significantly higher LTF levels in female ($p<0.005$) and male ($p<0.005$) Grey than in Ringed seals. Female Svalbard Ringed seals showed no statistically significant difference when compared to Grey seals from Sable Island whereas male Svalbard Ringed seals showed statistically significant lower mean LTF concentrations ($p<0.005$) compared to Grey seals from Sable Island.

#### 4.3.2.3 Tissue dependent-differences in LTF concentrations

In the present study, uterine flushings were only available from seven Grey seals. The LTF concentrations in female Ringed and Grey seal serum, uterine epithelium tissue and uterine flushings are presented in Table 4.6, Fig.4.6. In Baltic Grey seals, uterine epithelium LTF concentrations were statistically significantly higher when compared with serum ($p<0.05$) and uterine flushings ($p<0.05$). Uterine epithelium LTF tissue levels from Baltic Ringed seals showed no significant differences when compared with serum LTF concentrations. However, sample number for uterine epithelium tissue was very small ($n=3$) and there were no uterine flushing samples available for comparison.

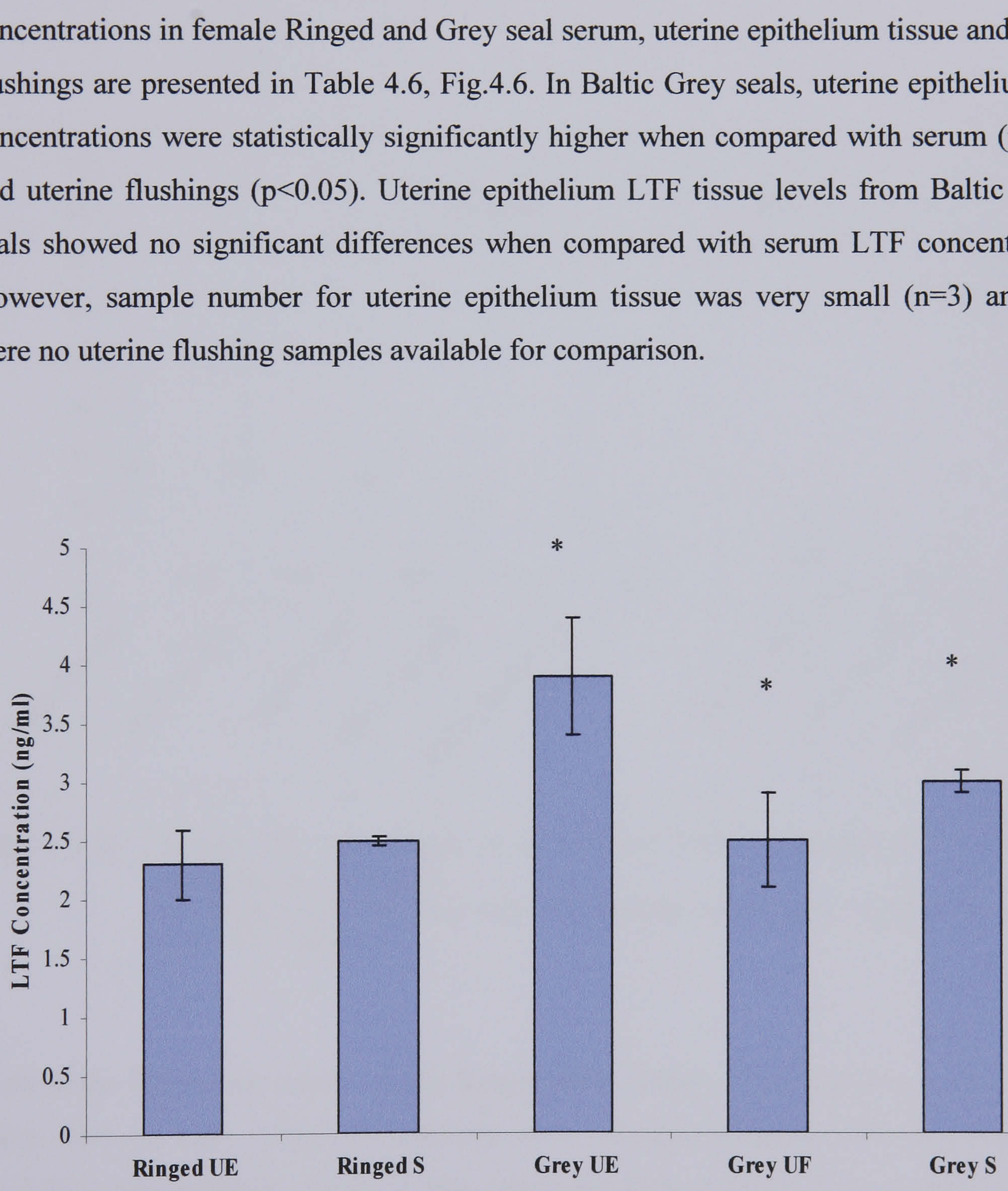



**Figure 4.6:** Mean ±SE of lactoferrin concentrations in serum (S), uterine epithelium tissue (UE) and uterine flushings (UF) from Ringed and Grey seals
The significance of sample dependence difference in Baltic Grey seals is presented as * $p<0.05$

### 4.3.2.4. High-exposed vs. low-exposed populations

Serum ΣPCB concentrations detected in serum samples from each population are presented in Table 4.4. Full justification of the exposure classifications is provided in Chapter 2. These data confirmed the exposure classification for the present study as follows:

**Female seals:** Baltic Ringed seals > Baltic Grey seals > Moray Firth Harbour seals > Abertay Sands Harbour seals > Sable Island Grey seals > Svalbard Ringed seals (Fig. 4.7). From the ΣPCB concentration results it was concluded that Svalbard Ringed and Sable Island Grey seals would be treated as control populations for females.

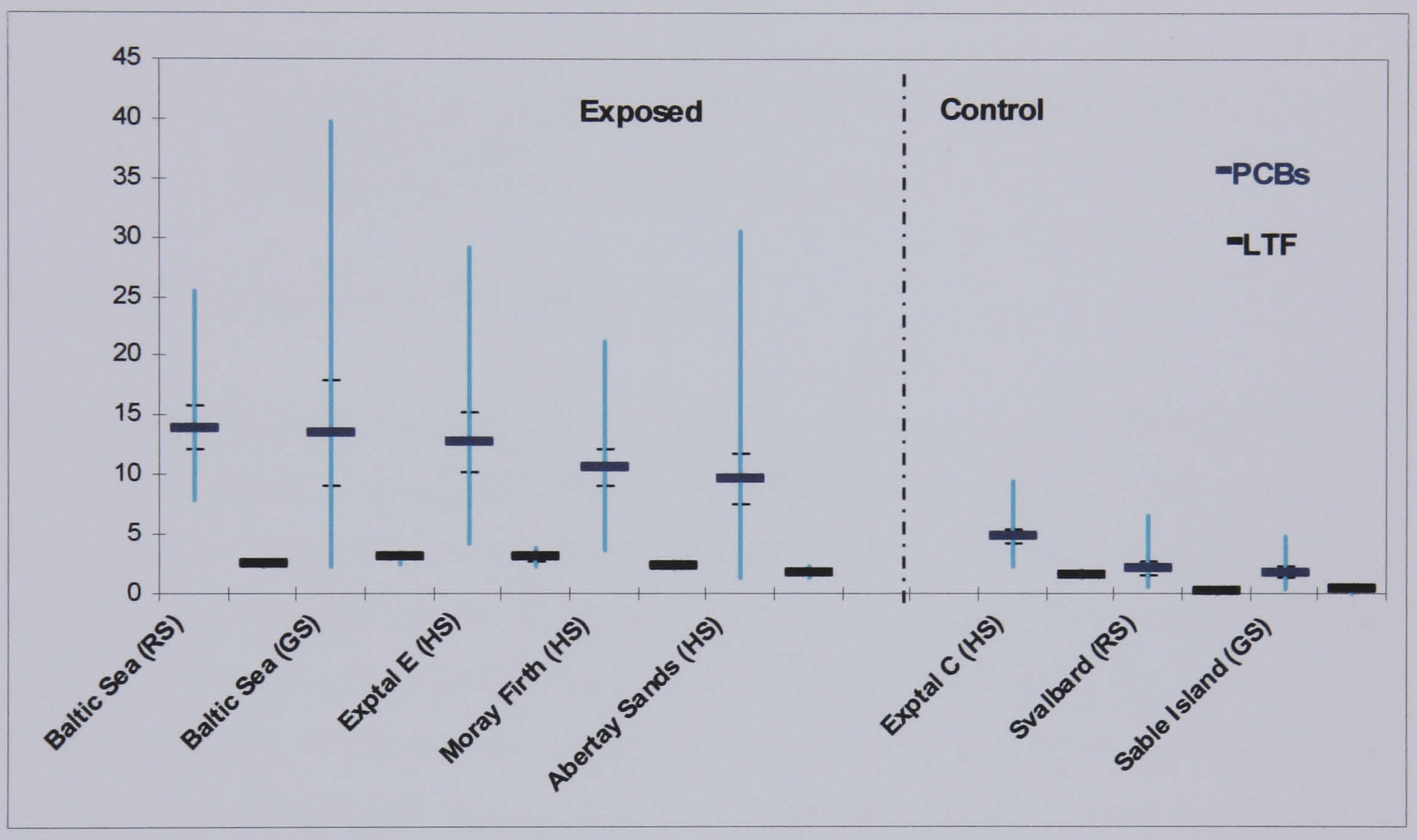



**Figure 4.7:** Mean± SE and range of ΣPCB and LTF concentrations in all study populations - Females
RS, Ringed seals; GS, Grey seals; HS, Harbour seals; Exptal, Experimental Group; C, Control; E, Exposed

**Male seals:** Baltic Grey seals > Baltic Ringed seals > Moray Firth Harbour seals > Abertay Sands Harbour seals > Sable Island Grey seals > Svalbard Ringed seals (Fig. 4.8). From the ΣPCB concentration results it was concluded that Svalbard Ringed seals would be treated as a control population for males.

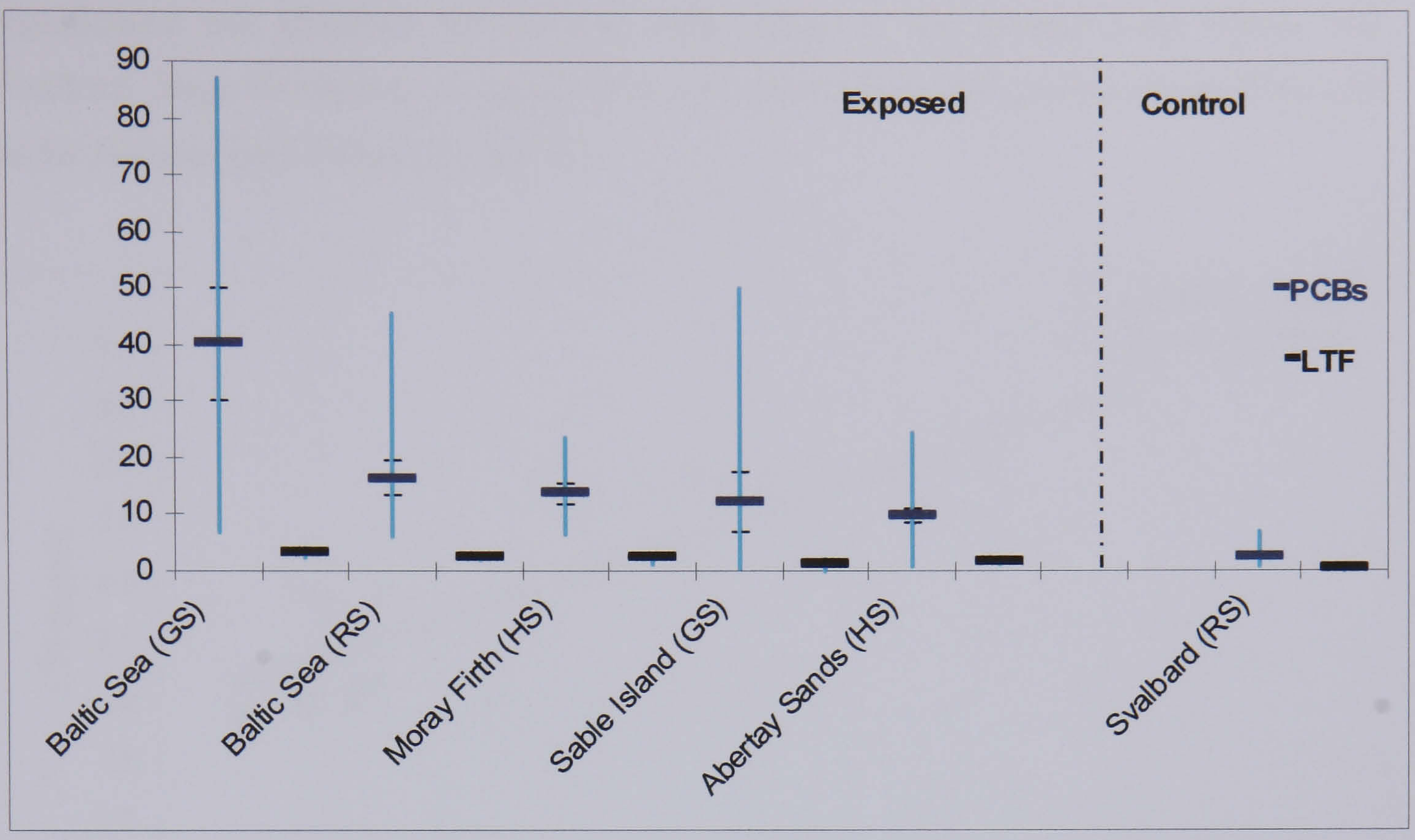



**Figure 4.8:** Mean± SE and range of ΣPCB and LTF concentrations in all study populations - Males
RS, Ringed seals; GS, Grey seals; HS, Harbour seals

*Validation of LTF as biomarker – dose response*

When comparing LTF concentrations between exposed and control Harbour seals of the experimental group a statistically significant difference was observed with mean LTF concentrations being higher ($p<0.005$) in exposed seals (Table 4.5). The experimental Harbour seal group was used for the validation of LTF as biomarker (Fig.4.9) since these seals were a part of a control feeding study with fish taken from Wadden Sea and Northeast Atlantic. This group of Harbour seals were sampled at late pregnancy and the average ± s.d. of total PCB concentration in the exposed group (n=11) was 25642 ± 9699 ng/g lipid weight and in the control group (n=12) was 5336ng/g ± 1632 lipid weight (Reijnders, *pers. Comm.*). From figure 4.9 a statistically significant positive correlation ($p<0.001$) between ΣPCBs and LTF concentrations can be observed for Harbour seals from the experimental group.

ΣPCBs and LTF concentrations according to species and stage of the reproductive cycle for all females are presented in figures 4.10, 11 and 12 and for all males in figures 4.13 and 4.14.

In females, statistically significant correlation was observed for all the stages of the reproductive cycle in all species except from Harbour seals at Stage III. In males, statistical

significance was observed for Harbour seals (Stage I) and Ringed seals (Baltic and Svalbard, Stage II) whereas no significance was observed for Grey seals in Stage II as well as for Harbour seals (Stages III and IV).

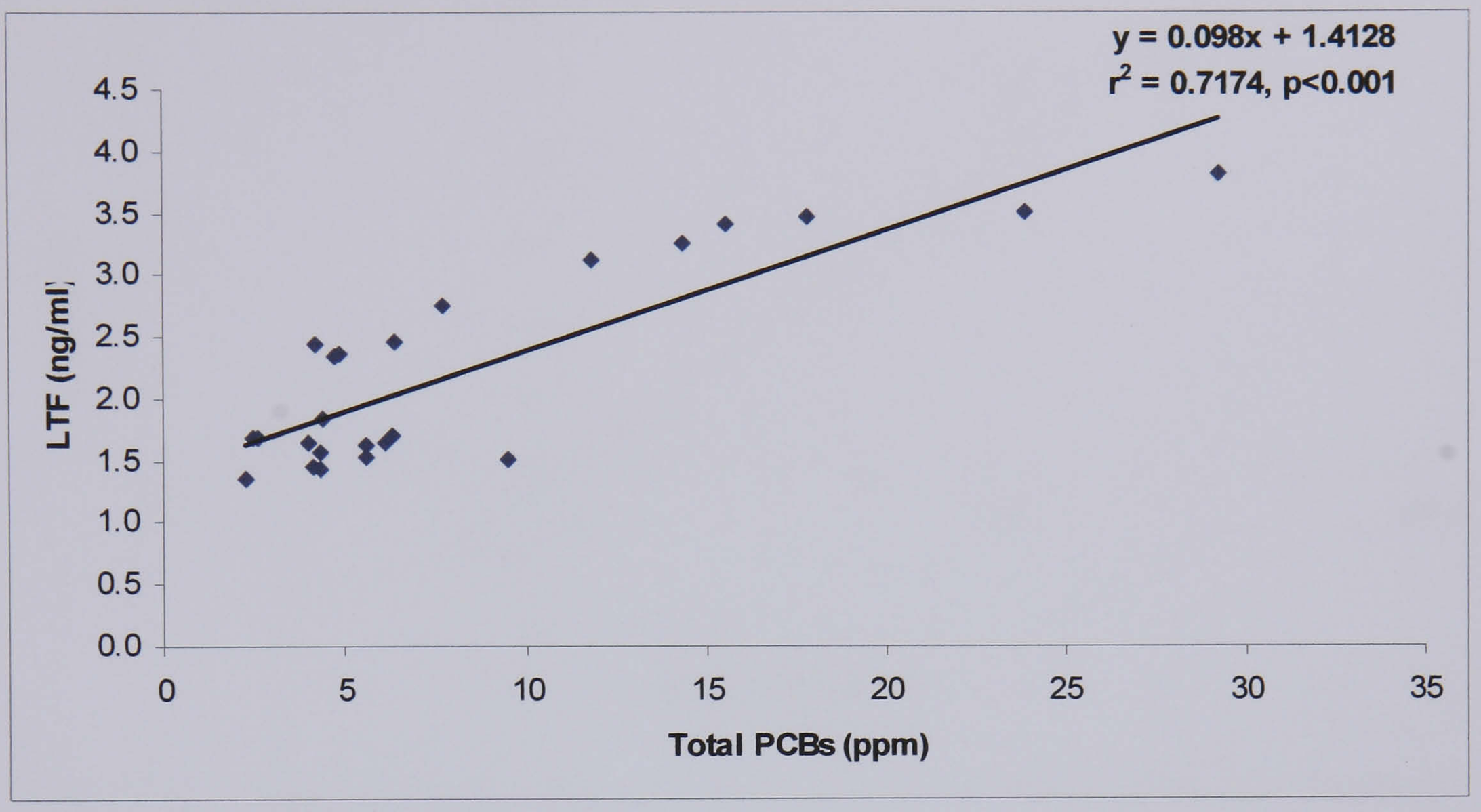



**Figure 4.9** Stage IV: ΣPCBs and LTF concentrations in female Harbour Seals – Experimental Group

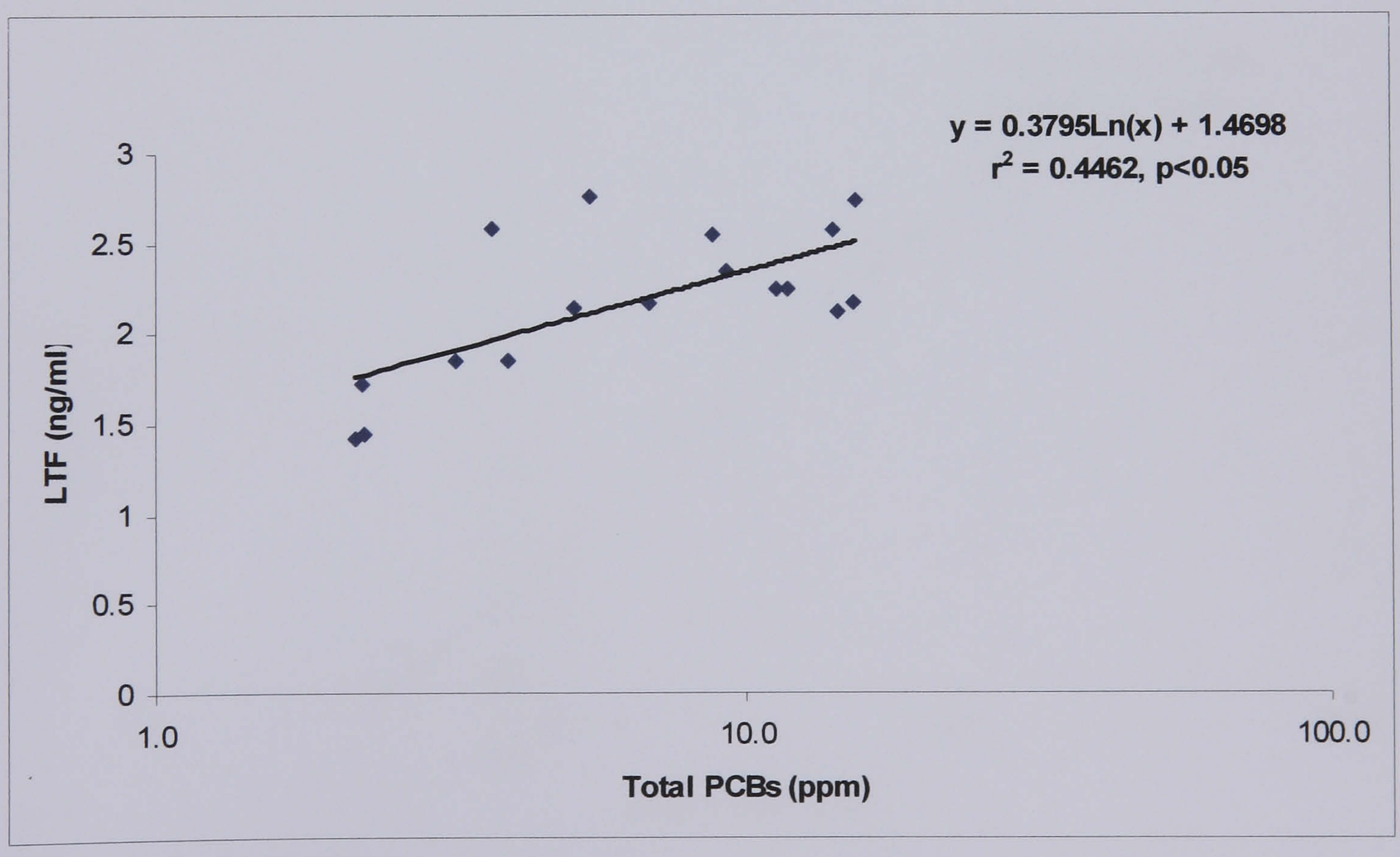



**Figure 4.10** Stage I: ΣPCBs and LTF concentrations in female Harbour Seals

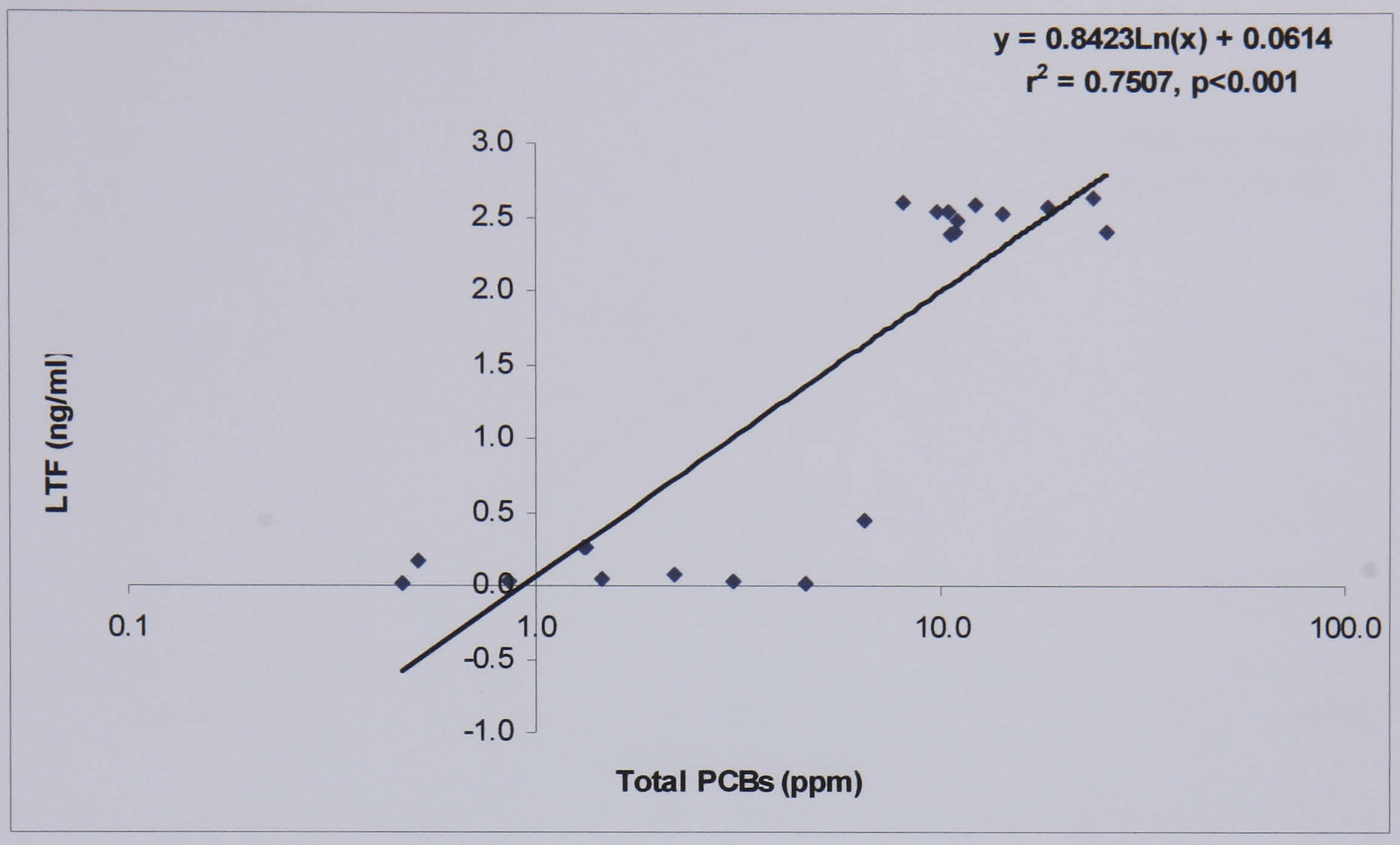



**Figure 4.11** Stage II: ΣPCBs and LTF concentrations in female Baltic and Svalbard Ringed Seals

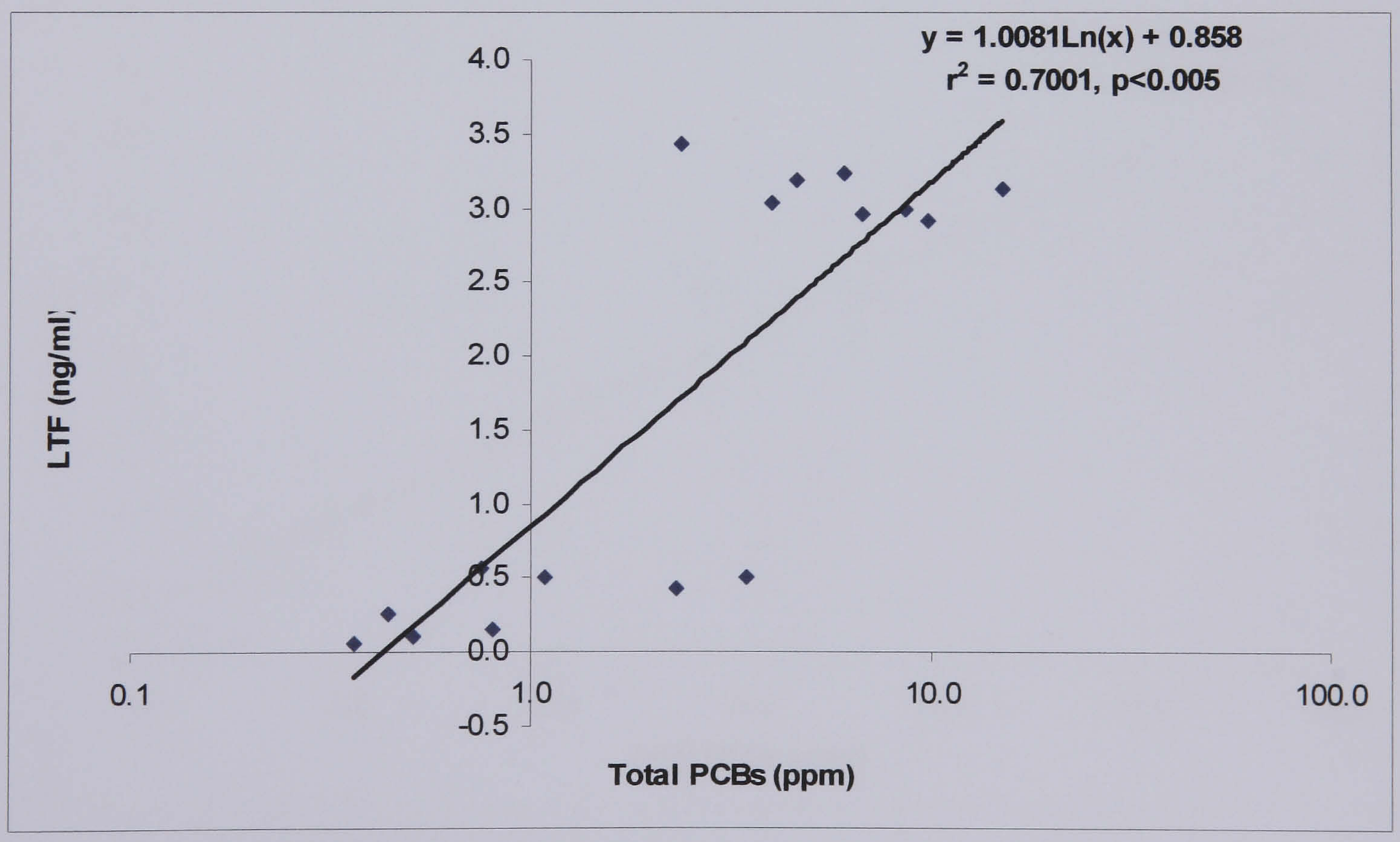



**Figure 4.12** Stage II: ΣPCBs and LTF concentrations in female Baltic and Sable Island Grey Seals

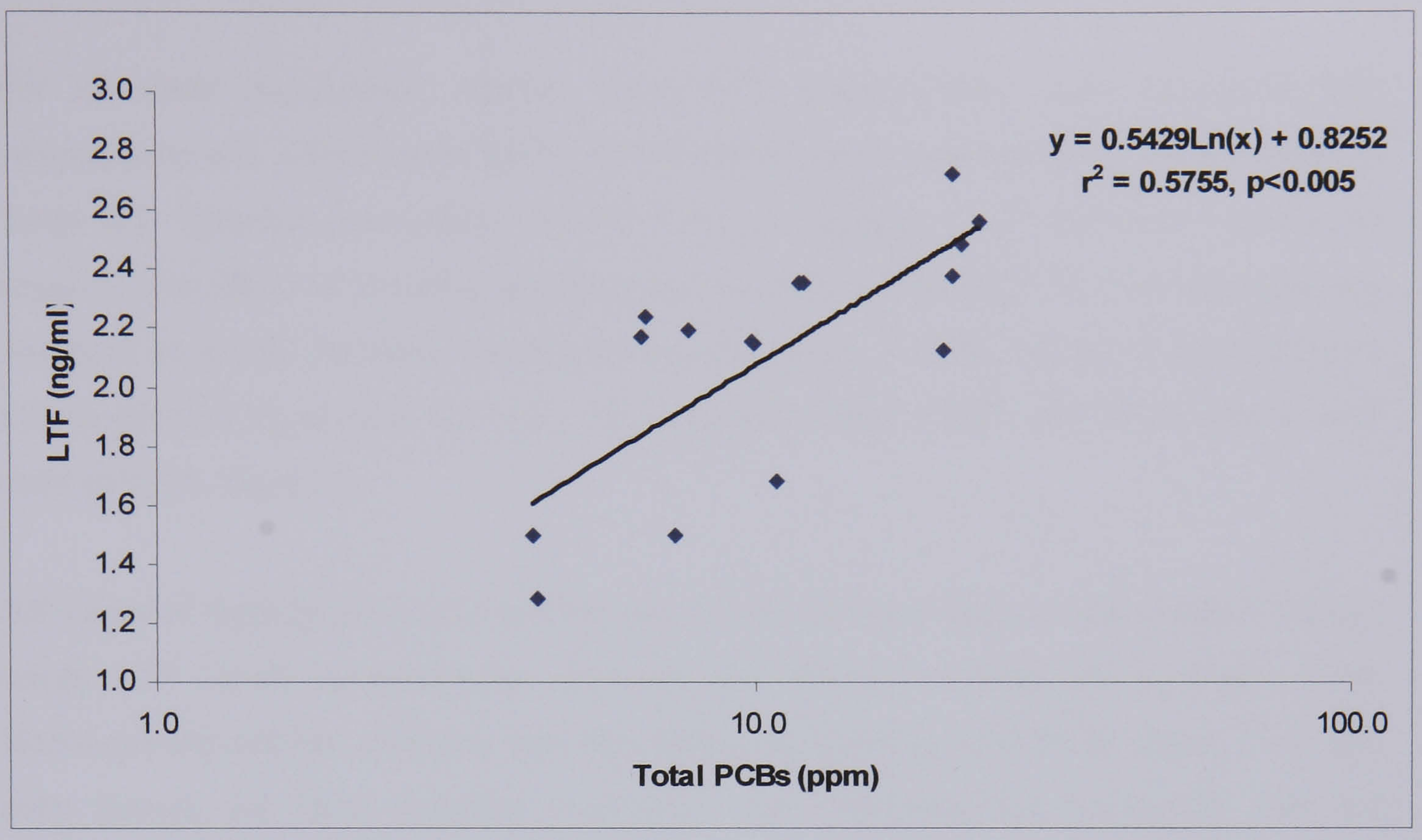



**Figure 4.13** Stage I: ΣPCBs and LTF concentrations in male Harbour seals

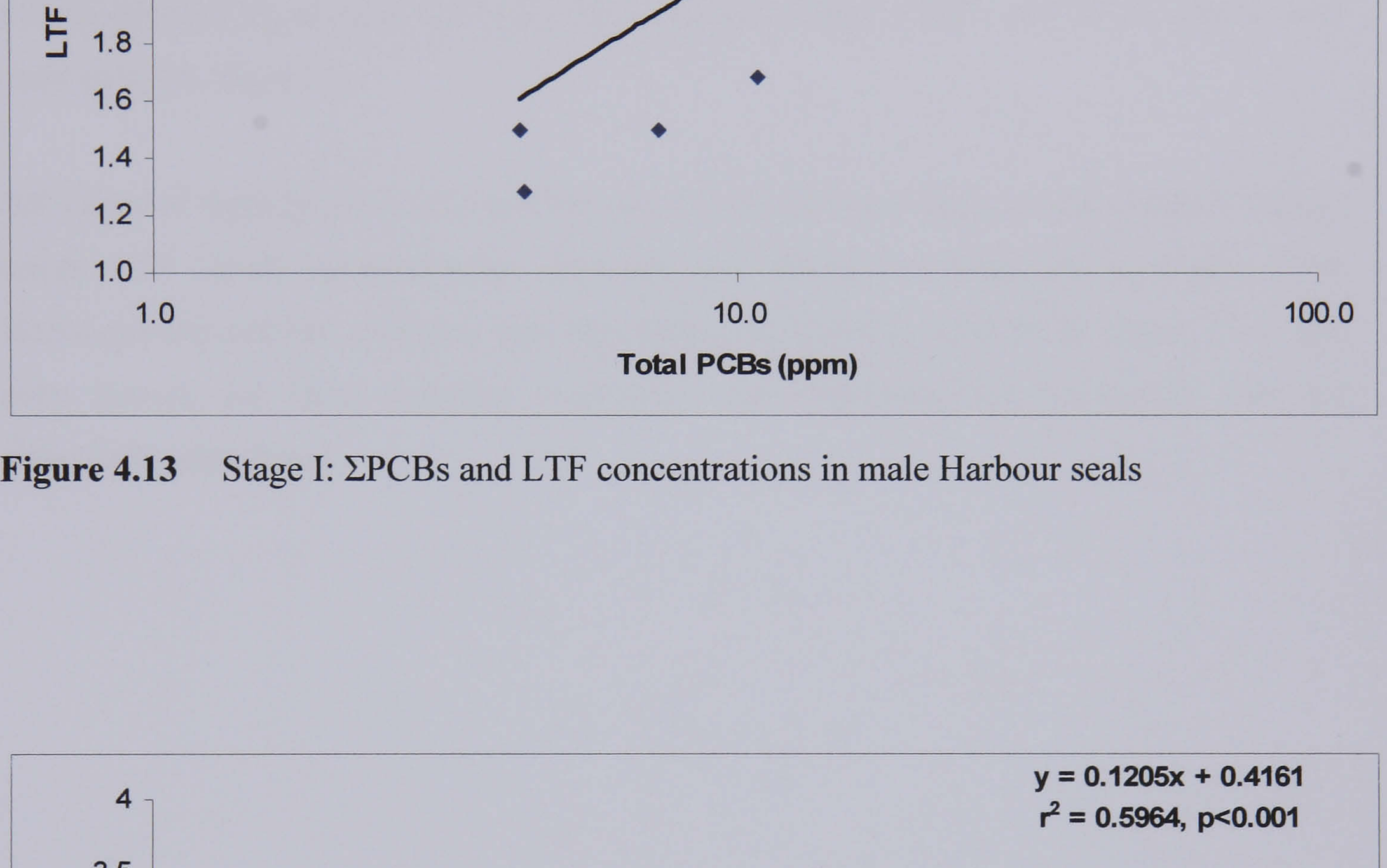


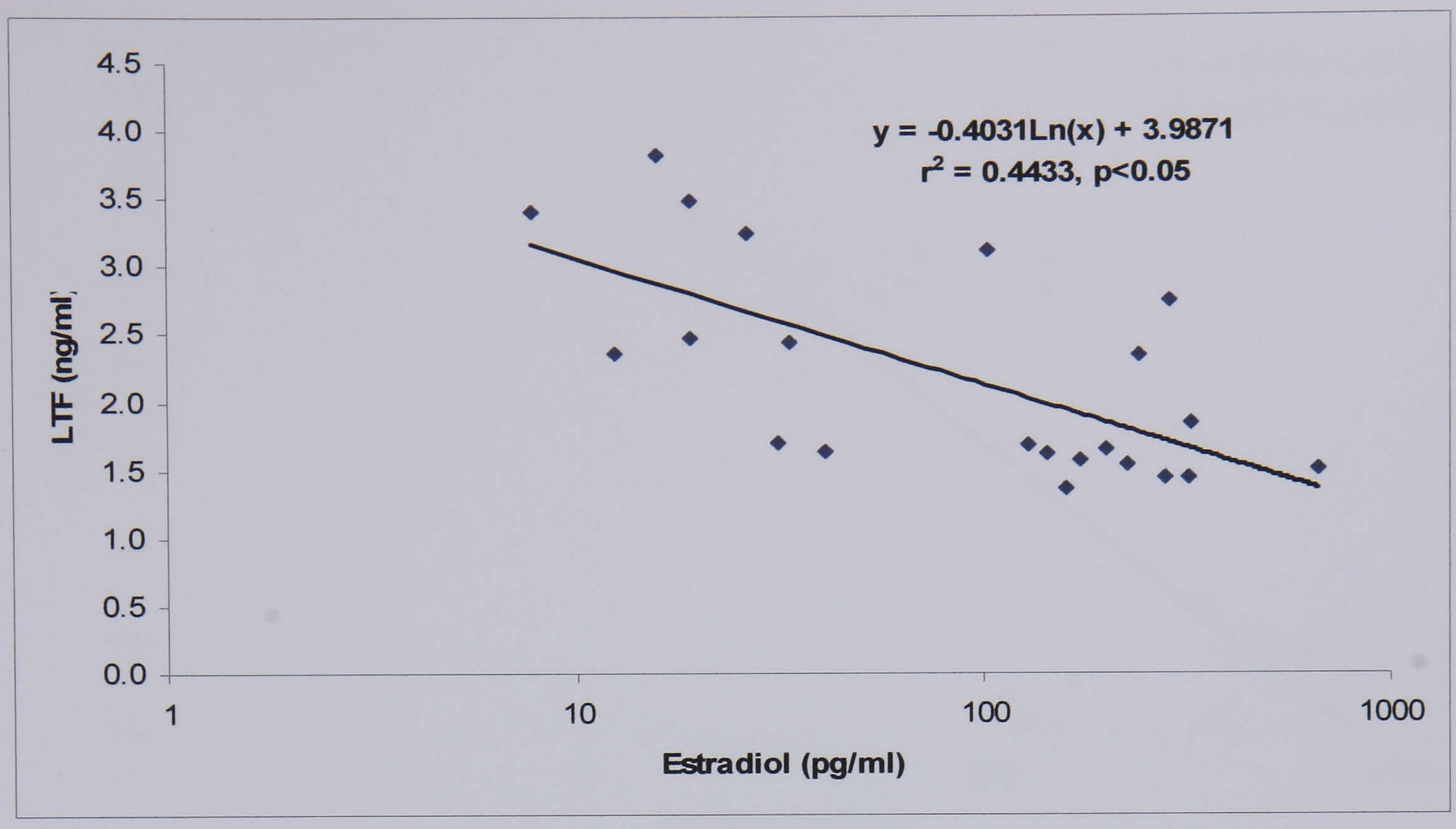

**Figure 4.14** Stage II: ΣPCBs and LTF concentrations in male Baltic and Svalbard Ringed seals

### 4.3.2.5. Hormonal-dependence Variations

For all study populations, female serum LTF concentrations were compared with progesterone and 17β-estradiol levels. Results of hormone concentrations are presented in Table 4.3. Detailed information can be found in Chapter 3. A statistically significant negative correlation of estradiol and progesterone with seal serum LTF concentrations was observed in female Harbour seals (experimental group, $p<0.05$, Fig.4.15), female Baltic and Svalbard Ringed seals ($p<0.05$, Fig.4.16) and female Baltic and Sable Island Grey seals ($p<0.05$, Fig.4.17).

All stages of reproductive cycle and the association between Harbour seal (Abertay Sands) serum LTF levels, progesterone, estradiol and ΣPCBs is presented in Figure 4.18. Hormonal-dependence variation was also tested for Harbour seals from Moray Firth and even though the same negative correlation was observed, this correlation was not statistically significant.

a.)




b.)

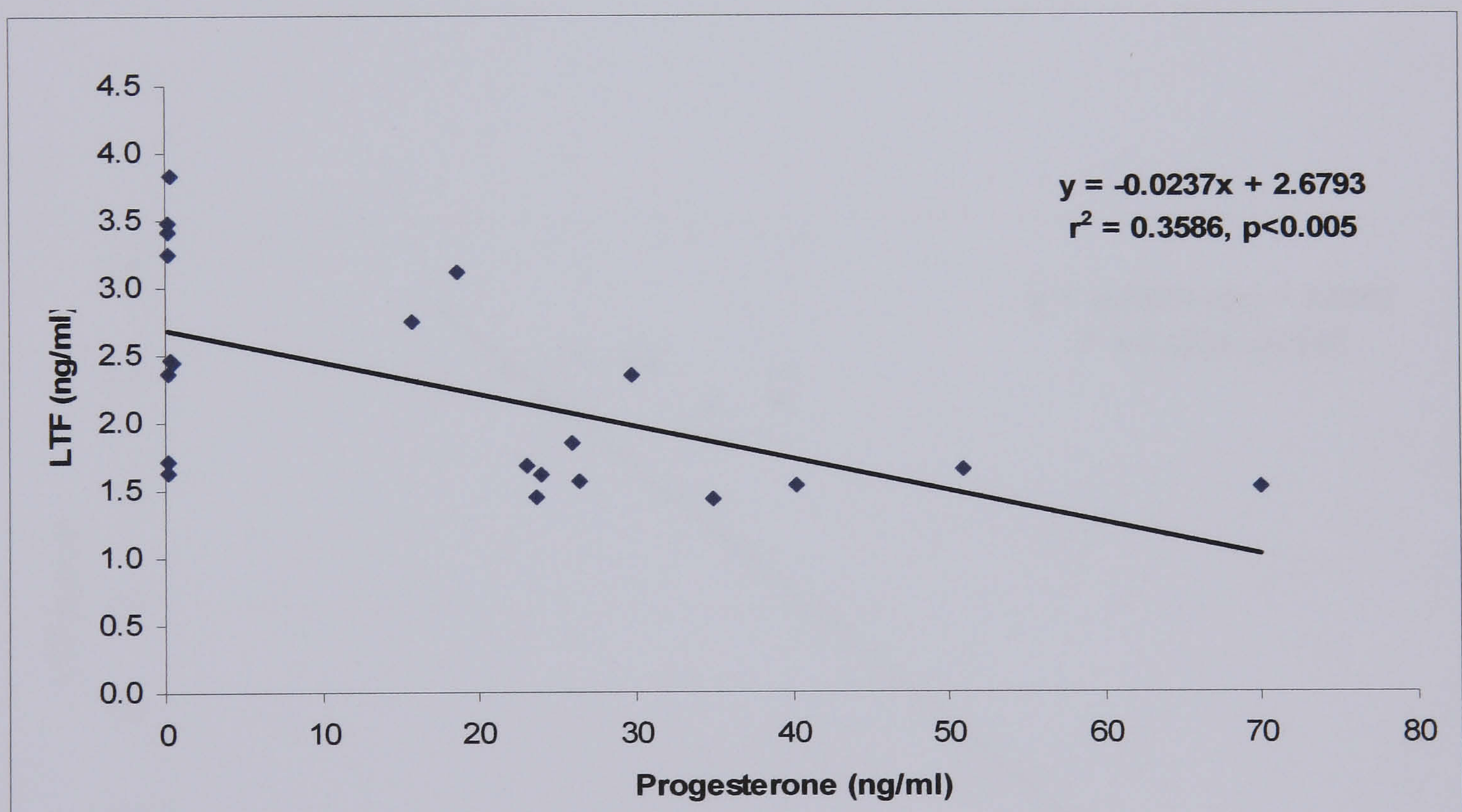
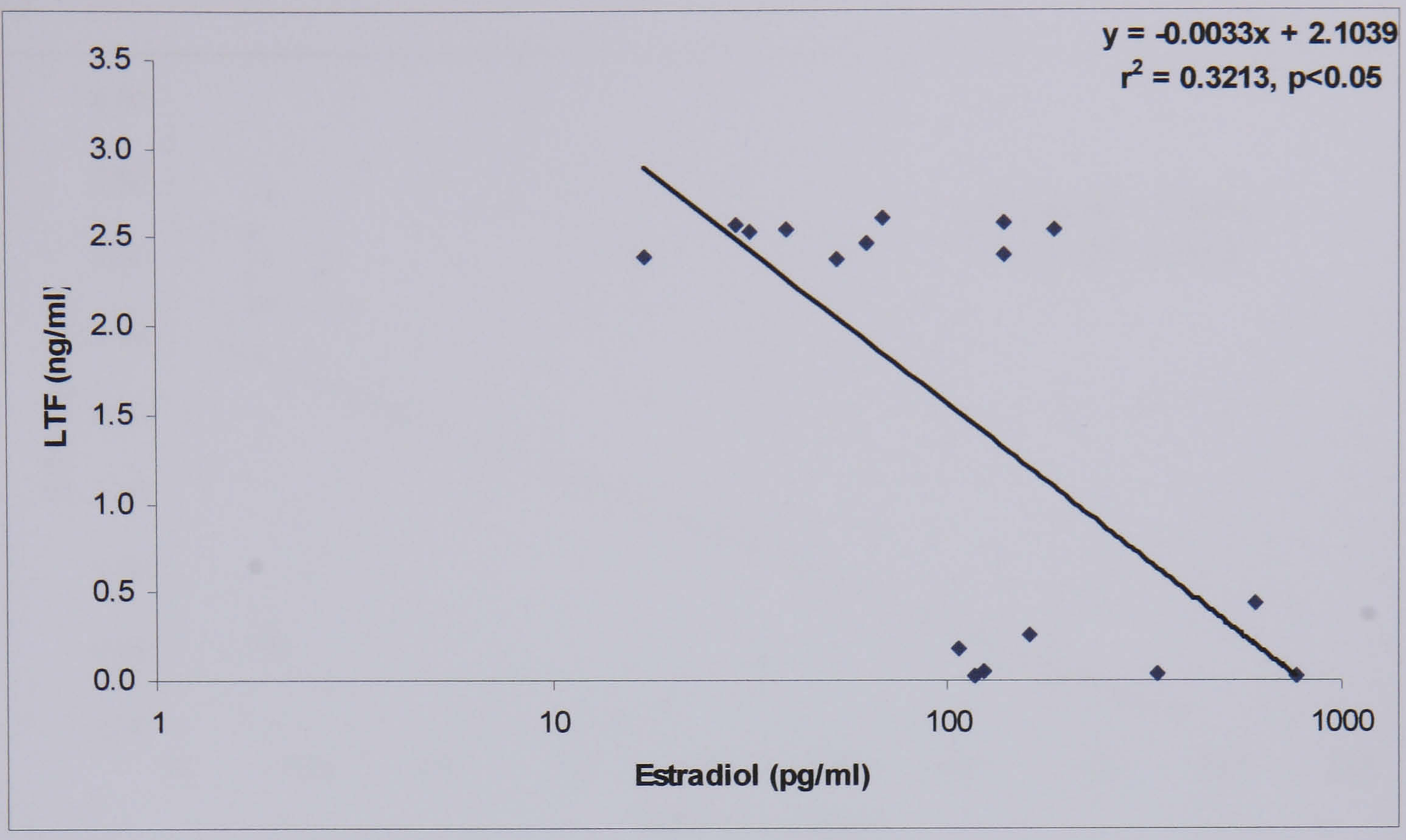



**Figure 4.15**: 17β-estradiol (a), progesterone (b) and LTF levels in female Harbour seals (Experimental Group – Stage IV)

a.)




b.)

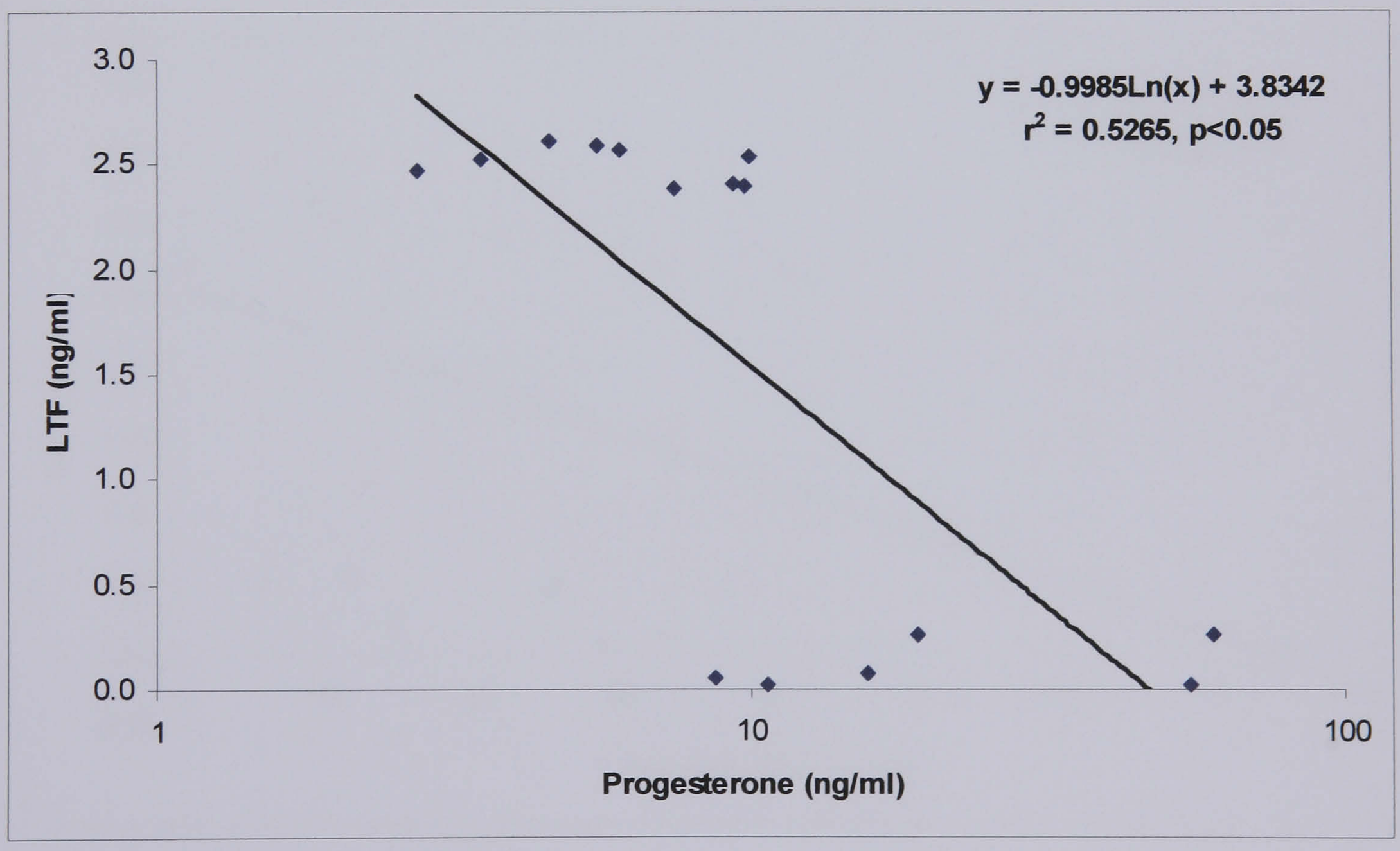
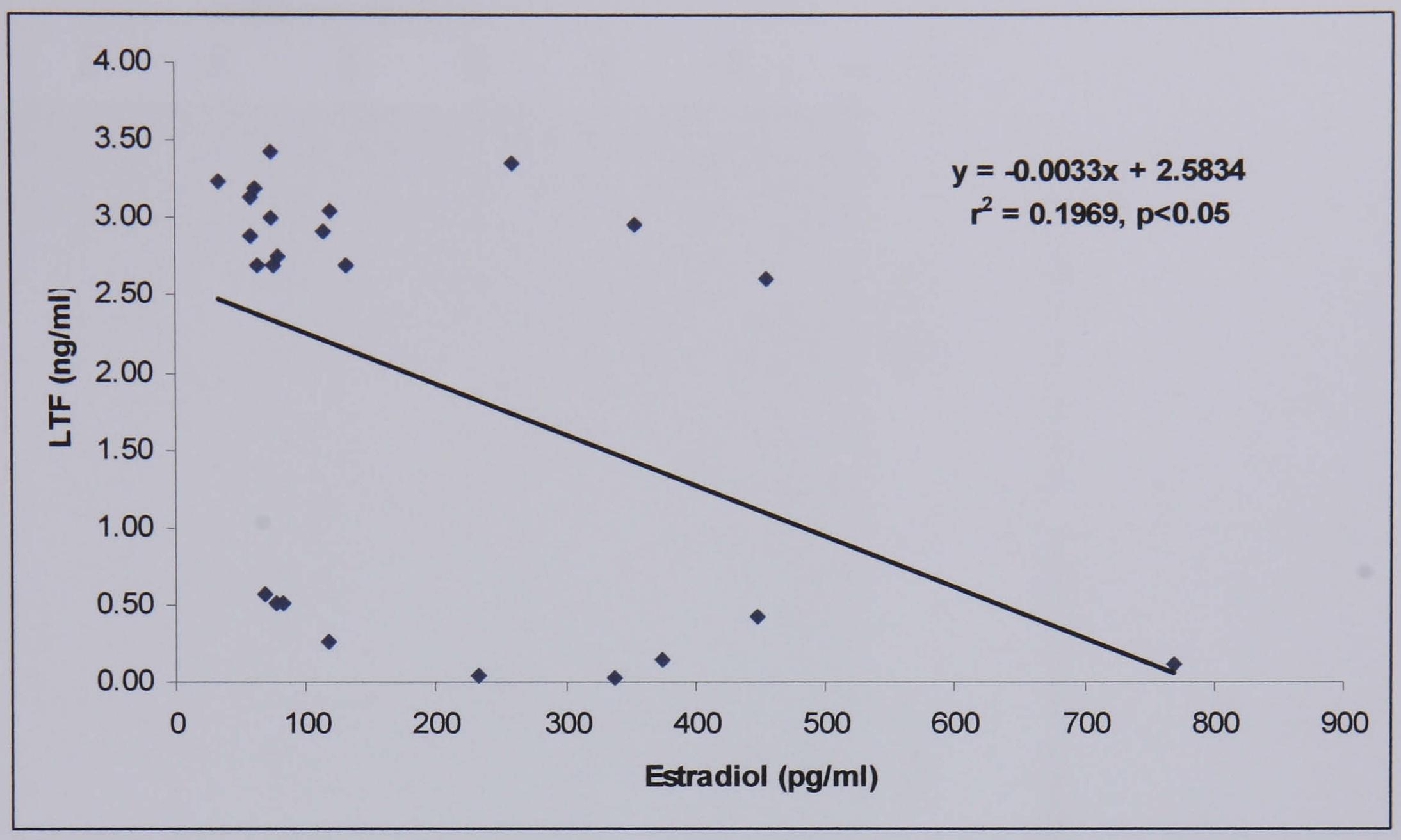



**Figure 4.16**: 17β-estradiol (a), progesterone (b) and LTF levels in female Baltic and Svalbard Ringed seals (Stage II)

a.)




b.)

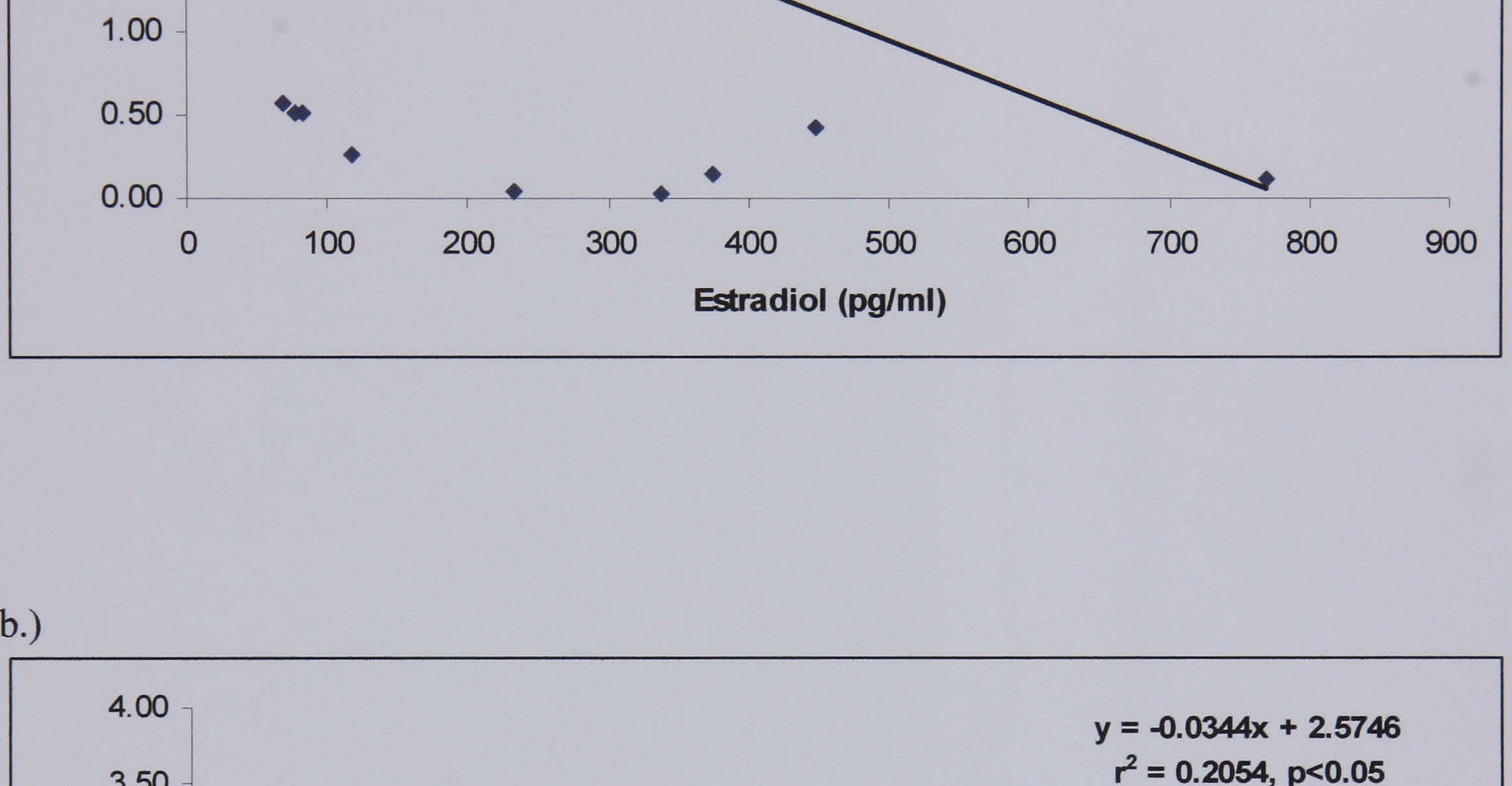



**Figure 4.17**: 17β-estradiol (a), progesterone (b) and LTF levels in female Baltic and Sable Island Grey seals (Stage II)

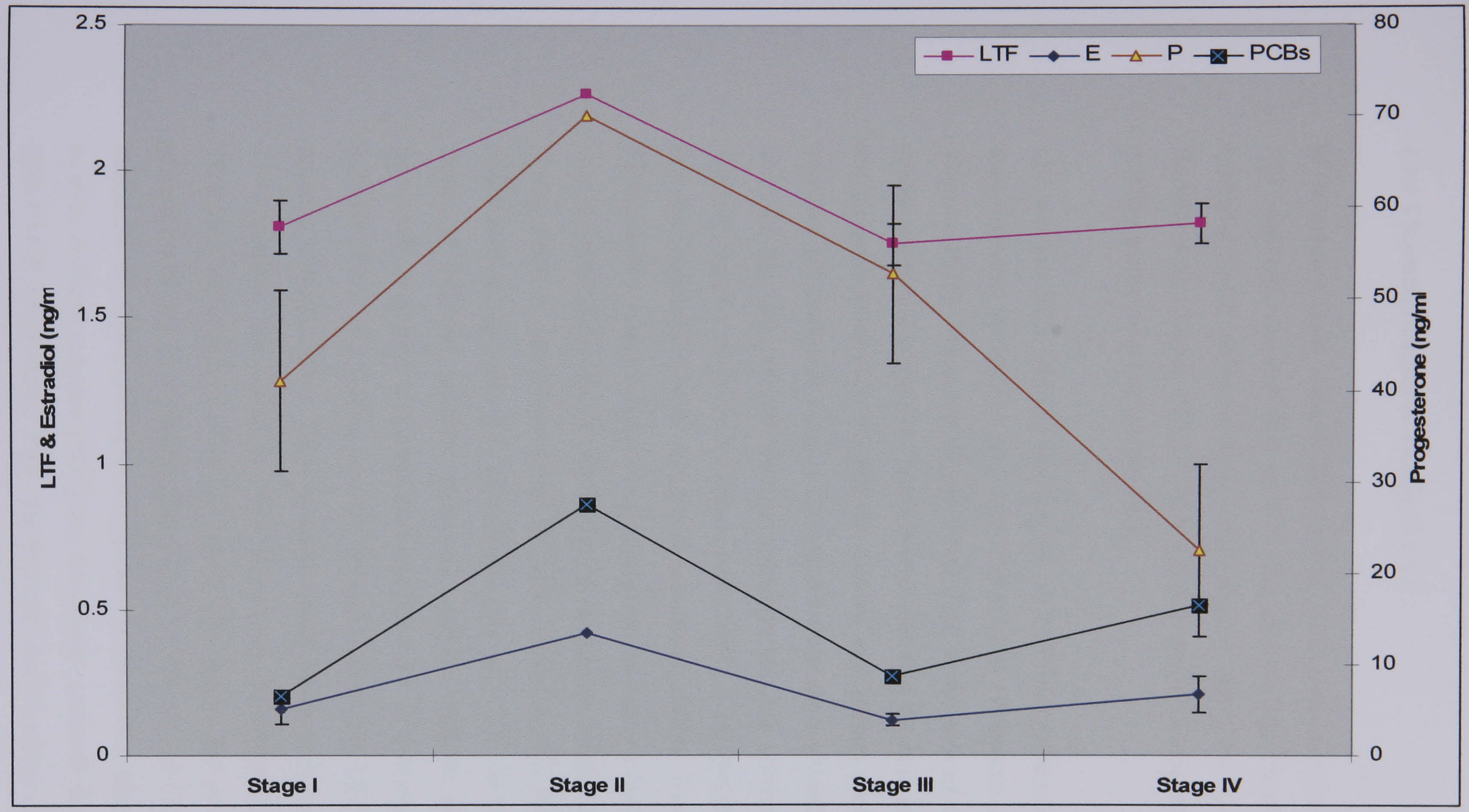



**Figure 4.18**: 17β-estradiol, progesterone, ΣPCBs and LTF levels in female Harbour seals (Abertay Sands) (All stages)

## 4.4 Discussion

### 4.4.1 Characterisation of Pinniped LTF

In the present study, western blot analysis using a commercially-available goat anti-bovine LTF antibody showed that an LTF-like protein was detectable in serum and uterine epithelial tissue obtained from Baltic Ringed and Grey seals and in serum from UK Harbour seals (Moray Firth). There was successful immunodetection of both bovine LTF positive control and the seal samples.

The western blots showed staining for an LTF-like protein at a lower molecular weight than that seen in humans and mice (70-80kDa) (Pentecost and Teng, 1987; Levay and Viljoen, 1995). There are no published data on the size of LTF for comparison for any marine mammalian species. There is evidence that the state of glycosylation accounts for the apparent molecular weights for bovine and human proteins. Bovine LTF has five potential glycosylation sites as determined from the nucleotide sequence whereas human LTF has two glycosylated sites per molecule but four potential sites exist (Hurley *et al.*, 1993). Also, in a study by Jefferson *et al.* (2000), it was found using western blot analysis that the molecular weight of LTF in the mouse uterine homogenate was approximately 70kDa with a minor band at 60kDa, whereas the sole immunoreactive band in rat tissues was approximately 60kDa. The different molecular weights may represent LTF peptides glycosylated at variable number of sites. Therefore the presence of specific types or combinations of glycans may affect the migration of LTF on SDS-PAGE and result in LTF bands of different apparent molecular weight. Multiple isoforms of LTF have been previously described and may relate to function. In humans, three isoforms have been isolated. Two with RNase activity termed LTF-β and LTF-γ, and one without RNase activity, termed LTF-α. These isoforms share the same physical, chemical and antigenic characteristics, but differ in their functional properties (Levay and Viljoen, 1995). Therefore, the existence of an LTF multi-gene family that produces the different mobility protein could not be excluded (Teng *et al.*, 1989; Li and Chen, 1999). Another possible reason for the lower molecular weight seen in the western blot analysis could be due to the sodium dodecyl sulphate (SDS) not adequately removed from the sample wells during SDS-PAGE preparation. Post translational modifications such as phosphorylation or acetylation might also contribute to different electromobilities (Teng *et al.*, 1989). Last but not least, someone cannot exclude the possibility that the lower band is not a form of LTF even though it interacts with the antibody.

### 4.4.2 LTF Concentrations in Pinnipeds

After characterisation of an LTF-like protein in Baltic Ringed and Grey seal serum and uterine epithelium tissue and UK Harbour seal serum by SDS-PAGE and western blotting, concentrations of the LTF-like protein were quantified with a commercially-available ELISA based on the same detection antibody (goat anti-bovine LTF) used for LTF characterisation. In the absence of a purified pinniped LTF protein standard, bovine LTF protein standard was employed as the positive control and assay calibrant.

#### *4.4.2.1 LTF concentration in seal uterine flushings and uterine epithelium tissue*

LTF concentrations were found to be statistically significantly higher ($p<0.05$) in Baltic Grey uterine epithelium tissue than serum and uterine flushings, with no difference observed between serum and uterine epithelium tissue in Baltic Ringed seals. A possible reason for low LTF levels in uterine flushings, may possibly be high sample dilution during sample collection and/or the fact that these seals were sampled during Stage II of the reproductive cycle - the early stages of pregnancy (3-4 months of embryonic diapause) when there is a down-regulation of LTF expression due to lower levels of circulating estrogen (Walmer, 1992). In a study by McMaster *et al.*, (1991) it was shown that P blocked estrogen-stimulated LTF expression in the mouse uterus and confirmed that P at least in this species has little effect on uterine LTF mRNA expression. LTF might also be estrogen-inducible in the pinniped uterus, as shown in other mammalian species. However, the role of progesterone and other factors during the estrus cycle can not be excluded and warrant further investigation.

In humans and mice, LTF levels are generally higher in uterine epithelium tissue than in serum and uterine flushings during the estrous cycle in response to the rise and fall of estrogens and it has been established as the major uterine secretory protein under the control of estrogen (McMaster *et al.*, 1991; Li and Chen, 1999; Teng *et al.*, 2002). In our study, LTF-like concentrations were also higher in uterine epithelial tissue compared to serum and uterine flushings for both Baltic Ringed and Grey seals ($p<0.05$, Table 4.3). However, when compared with other mammals (Table 7, Chapter 1), seal LTF concentration in uterine epithelial tissue and flushings are lower than expected, therefore, more samples are required before conclusions can be drawn.

LTF-like protein concentrations were found to be relatively low compared with other mammals such as mice and humans (Bennet and Mohla, 1976; Hansen *et al.*, 1976; Oloffson *et al.*, 1977; Boxer *et al.*, 1982; Hetherington *et al.*, 1983; Rebelo *et al.*, 1995; Rebelo *et al.*, 1996; Yang *et al.*, 2000). Much higher LTF concentrations have been reported in serum of female mice at estrus, due to the high circulating levels of endogenous estrogen hormones at this stage of the reproductive cycle (Walmer, 1992). Baltic seal samples were collected at the end of the moulting period following the breeding season and therefore many if not all of the female seals were pregnant at the time of sampling at the onset of embryonic diapause (Nyman, *person. comm.*). At this time, there are low levels of estrogen and elevated progesterone, when it would be expected that estrogen-induced LTF expression would also be down-regulated (Gardiner, 1996). As can be seen here, the stage of reproductive cycle of female seals plays a critical role in governing LTF expression as has been seen in mice (McMaster *et al.*, 1991; Newbold *et al.,* 1992). However, possible protein degradation could have occurred during sample storage and transit (despite storage at -70$^{o}$C) and due to the amount of time that elapsed between sampling and analysis, further depressing sample LTF-like levels. Another factor which may have under-presented the data, is the reduced cross-reactivity of the detection antibody used. An antibody raised against bovine LTF is not 100% specific for pinniped LTF. However, a high sequence homology at certain regions of the LTF gene and its protein product has been reported among different species (Teng *et al.*, 2002). For example, human and bovine LTF share 69% sequence homology and structurally are very similar (Farnaud and Evans, 2003). A 40ml milk sample from a Grey seal was obtained and pinniped LTF was planed to be purified from this sample under the guidance of a dedicated LTF research group based at King's College (Dr. R. Evans, University of London). However, due to limited time and resources, purification and raising antibodies to the protein was not possible. Without the availability of fresh purified pinniped LTF protein standard and dedicated anti-pinniped LTF antibodies it is impossible to confirm whether seal LTF are indeed generally lower than in studies of other mammals to date, or are the cause of experimental problems.

Aside from hormonal influences on LTF expression, binding affinity of the detection antibody used for LTF quantification in seal samples, may be a significant factor resulting in the under-representation of LTF-like protein expression in seal samples. Despite this, the relative differences in LTF expression between uterine epithelial tissue, flushings and serum was as expected according to observations in other mammals, indicating that antibody LTF binding level is consistent across in all sample matrices, permitting valid comparison of data.

Uterine epithelium tissue levels in Baltic Grey seals (3.9±0.5ng/ml), although not statistically different were higher than in Ringed seals (2.3±0.3ng/ml). The amount of LTF protein secreted into the uterine lumen varies with the stage of the estrus cycle in the mouse, ranging from low (800 ± 400μg/ml) to high levels at estrus (3.2 ± 0.6 mg/ml) (Newbold *et al.*, 1992). Even though both Baltic seal species were sampled at Stage II of the reproductive cycle (Nyman, *person. comm.*), the Grey seals were likely approaching the end of embryonic diapause when estrogen levels in seals increase for preparation of blastocyst implantation (Gardiner *et al.*, 1996), which would have lead to the induction of LTF expression. Baltic Ringed seals might have not reached this stage yet and therefore having lower LTF levels. Estrogen is also considered to trigger the expression of a unique set of genes in the pre-implantation endometrium of the uterus that in turn controls implantation (Teng *et al.*, 2002). Therefore, it is likely that rising estrogen levels in Baltic Grey seals caused uterine LTF levels to be higher than Baltic Ringed seals because they were closer to the blastocyst implantation stage.

#### *4.4.2.2 LTF concentration in seal serum*

##### *Age, Sex and Geographical-dependent Differences within Species*

This is the first study to report levels of an LTF-like protein in seals since LTF levels have been reported for humans, rodents and domestic animals, but not marine mammals (Birgens, 1985; Baynes *et al.*, 1986; Kähler *et al.*, 1988; Caccavo *et al.*, 1999; van der Strate *et al.*, 2001; Teng *et al.*, 2002). Serum LTF concentrations were higher in adult versus juvenile Harbour seal females from Moray Firth ($p<0.05$) but no difference was apparent between male age groups. Although there is little information on sex-dependent differences in LTF expression in mammals in general, higher mean plasma LTF levels in healthy adult males than females have been reported in humans (Bennett and Mohla, 1976). However, there is also evidence of this not being a general rule since in humans similar LTF levels have been recorded in healthy males and females (Bezwoda *et al.*, 1985). LTF levels in male Ringed seals from Svalbard and Grey seals from Sable Island were found to be higher than in the female seals. However, only in the case of Grey seals were these differences statistically significant ($p<0.05$). These two seal populations were used as a reference (low exposure) group for comparison with highly exposed seals from the Baltic. In the case of exposed Baltic seals, there were no sex-dependent differences in LTF serum levels. In contrast with females, the presence of the LTF protein in males is independent of reproductive status, suggesting that this difference could be the fact that normal levels of

the LTF-like protein in male seals could actually be in this range seen in Grey seals from Sable Island. When comparing UK male Harbour seals with male Grey seals from Sable Island there was also no statistically significant difference in LTF levels between the two different species. Moreover, female Ringed and Grey seals from the two reference areas were sampled during the delayed implantation period where progesterone levels are high and estrogen levels low thus the very low LTF serum levels.

When comparing LTF-like serum levels among the Harbour seals it was observed that UK Harbour seals had significantly higher mean LTF concentrations ($p<0.005$) compared to control Harbour seals from the experimental group. As already mentioned earlier, seals from the experimental group were sampled at the last stage of pregnancy when progesterone levels are elevated which could have caused the difference between the seals from the different geographical areas.

### 4.4.3 Validation of LTF as biomarker of endocrine disruption

Many natural estrogens, estrogen metabolites, synthetic estrogens and xenoestrogens have been examined for their effects on LTF expression in mouse uterus or cultured human endometrial cells (Pentecost *et al.*, 1988; Yu and Chen, 1993; Nelson *et al.*, 1994; Curtis *et al.*, 1997; Das *et al.*, 1998; Ghosh *et al.*, 1999; Markey *et al.*, 2001). These studies show that exposure to xenoestrogens as with natural estrogens, stimulates LTF expression in the reproductive tract. The toxicity of environmental pollutants such as PCBs in the marine environment has been documented (Aguilar and Borrell, 1996), it is plausible therefore, that LTF homeostasis may be disturbed in animals exposed to elevated levels of these and other xenoestrogens, indicative of endocrine disrupting effects. LTF therefore has the potential to be used as a marker of estrogen exposure in animals exposed to environmental estrogens (LeBlanc and Bain, 1997). This is the first study to measure LTF levels in pinnipeds, to investigate the relationship with progesterone and 17β-estradiol through the reproductive cycle and to investigate the dose-response relationship with PCB exposure. This study therefore constitutes a major contribution to providing vital information needed as part of the validation of LTF as biomarker of endocrine disruption.

It was not possible to make any comparison of seal LTF levels with other studies since baseline levels are not available. However, as already mentioned earlier the difference in the levels between serum, uterine epithelium tissue and uterine flushing were in accordance with published information. Serum LTF levels in the experimental control

group (Harbour seals) were statistically significant lower than the exposed group. Since Harbour seals from the experimental group were sampled during Stage IV of the reproductive cycle no comparison of the data could be made with seals sampled at other stages. Baseline levels for Stage II could be ascertained from the other two control groups (Grey, Sable Island and Ringed, Svalbard seals), since the PCB levels were less than the concentrations found in the experimental group (*For contaminant justifications refer to Chapter 2-Contaminant Analysis*).

*Dose-dependence*

Statistically significant elevated serum LTF levels were observed in the exposed female Harbour seals (experimental group) when compared with UK Harbour seals (Abertay Sands and Moray Firth, $p<0.005$). Baltic Ringed and Grey seal LTF levels were also significantly higher from seals inhabiting the reference areas (Svalbard and Sable Island, $p<0.005$).

Serum LTF concentrations according to species and stage of the reproductive cycle for all females and males of the three seal species under study were compared with ΣPCB concentrations previously measured (Chapter 2). This could not be applied to uterine epithelium and uterine flushing LTF levels since no contaminants could be analysed. Serum LTF concentrations of female Harbour seals (experimental group, Stage IV) were positively correlated with exposure in dose-dependent manner and this relationship was statistically significant ($p<0.001$). In female seals statistical significance was observed for all stages of the reproductive cycle in all species except for Harbour seals at Stage III (Fig. 4.10, 11 and 12) whereas male Harbour seals showed a positive correlation with ΣPCBs only in Stage I (Fig.4.13). Male Grey seals showed positive correlation with ΣPCB, however no statistically significant, whereas LTF concentrations in male Ringed seals (Baltic and Svalbard) were found to be positively correlated with ΣPCBs (Fig.4.14).

In order to validate the above results hormonal-dependence variation (estrogen and progesterone) was also tested for the female serum samples from all the three species and it was concluded that there was a negative correlation between LTF levels and estradiol and progesterone (Fig.4.15-4.17).

Since Baltic Ringed and Grey seal were sampled in Stage II (delayed implantation) of the reproductive cycle, endogenous estrogen levels were expected to be low and progesterone

levels high. Due to progesterone inhibition of LTF protein in the uterus (Walmer *et al.*, 1992; Teng *et al.*, 2002), seal LTF protein could be expected to be absent or in very low concentrations at this stage. The detection of high levels of LTF protein in these samples as well as with the results obtained from the linear regressions may signify the action of environmental estrogens mimicking the action of the endogenous estrogen hormones, which may have potential implications on implantation. Since LTF levels in all female exposed seals were significantly higher that the reference seals and because there was a statistically significant positive correlation with ΣPCBs it could be proposed that these seals could be expressing high LTF levels due to the action of EDCs mimicking the action of endogenous hormones since there was a negative correlation established with estradiol. Moreover the presence of the LTF-like protein in males is independent of the reproductive status in contrast with females. In the present study male seals from Baltic Grey and Ringed seals had statistically significant higher LTF levels when compared with seals from reference areas as well as with male seals from UK Harbour seals. Moreover, male Harbour seals in Stage I of the reproductive cycle as well as Ringed seals (Stage II) were positively correlated with ΣPCBs indicating a dose-dependent effect on LTF levels. It has been demonstrated that abnormally elevated levels of LTF in serum from males occur as a result of xenoestrogen exposure (e.g., rodents and primates prenatally dosed with DES; Pentecost, *et al.*, 1988).

In conclusion, this study demonstrates the first characterisation of an LTF-like protein using goat anti-bovine LTF antibodies in a range of seal tissue and serum samples. This information facilitated selection of an appropriate ELISA to quantify LTF in a variety of pinniped samples. Tissue-dependent differences in LTF were observed; uterine epithelium > uterine flushings > serum. The weak staining of LTF for pinniped samples on western blots in general and the relatively low levels of LTF concentrations determined by ELISA may be attributed to a weak binding affinity of the goat anti-bovine LTF antibody for pinniped LTF. This indicates the need to obtain purified pinniped LTF protein and production of monoclonal anti-seal LTF antibodies for more accurate immunodetection and quantification of pinniped LTF protein in future studies. If purified pinniped LTF had been used as positive control then a more accurate immunodetection and quantification of LTF would have been expected. Moreover, some degradation of LTF protein could also have occurred during sample storage and transit despite storage at -70°C and furthermore due to the amount of time elapsed between sampling and analysis. As mentioned earlier, LTF can be found in very high concentrations in milk. Obtaining milk sample from a pregnant seal or during lactation can be very difficult since the animals are very hostile at

this period. On the other hand, this difference in LTF concentrations could be attributed to the difference in species studied or that seals might in general have lower LTF levels compared with other mammals.

LTF serum values obtained in the study are very valuable since baseline values could provide an important tool for monitoring the health status of free-ranging seals especially during stress conditions (e.g. pregnancy and inflammation) since LTF levels in blood have been previously demonstrated to fluctuate during stress conditions (Rebelo *et al.*, 1995 & 1996; Thomas *et al.*, 2002, Kane *et al.*, 2003).

LTF is also found in seminal vesicles and it has been reported to interact with the surface of sperm and becomes one of its major coating antigens (Goodman and Young, 1981; Lakritz *et al.*, 2000; Inagaki *et al.*, 2002). Moreover, it has been demonstrated in men, that serum and testicular estradiol levels are associated with oligospermia (Krause, 1988; Levalle *et al.*, 1994) and that increased circulation of seminal estradiol may increase seminal LTF secretion (Buckett, 1997). Therefore, baseline LTF levels obtained from this study could be a valuable tool in order to assess the male reproductive status.

Since LTF levels were found to fluctuate in different contaminant exposure levels as previously observed in the mouse after contaminant administration suggests that LTF is a sensitive marker for endocrine disruption and its expression could be a useful tool when studying effects of EDC exposure in seals. This preliminary study is the first to characterise an LTF-like protein and quantify its levels in pinnipeds as well as to establish a positive correlation with ΣPCBs in seal serum and a negative correlation with estradiol and progesterone levels. Even though, the sample number used in this study was not big it was sufficient in order to derive conclusions on the LTF baseline levels in seals and the dose-dependent effect of EDCs. The results of the present study and evidence from laboratory animals strongly support the use of LTF as a biomarker of endocrine disruption and since it can be measured in serum samples taken non-destructively, LTF remains a highly suitable biomarker for wildlife population biomonitoring and should continue to be studied on the basis of the preliminary findings of this study.

# Blood Chemistry Parameters as Biomarkers of PCB Exposure and Effects in Pinnipeds

## 5.1 Introduction

Blood chemistry parameters are often used to assess physical condition and nutritional status in humans and animals. Collection of blood samples from wild seal populations presents obvious logistical problems, as the animals need to be captured and restrained at haul-out sites or at sea. Despite this, limited reference values have been determined for Grey (*Halichoerus grypus*), Harbour (*Phoca vitulina*) and Ringed seals (*Phoca hispida*) from studies of stranded and captive seals (Engelhardt, 1979; Hall, 1997; McConnell *et al.*, 1983; Schumacher, 1995; Nielsen, 1995; Roletto, 1993; Bossart *et al.*, 2001). These values should be considered a general guideline, because they have been determined in only small numbers of individuals under unnatural conditions. Furthermore, there is variation in the data ranges, from different published sources resulting from inter-laboratory variation, differences in environmental and husbandry (captive seals) conditions.

The effects of exposure to organochlorines and especially PCBs have been studied on a large scale in experimental animals and humans. In general, pathological changes have been more obvious than variations in physiological parameters such as blood chemistry values. Pathological disturbances have been found mainly in the liver, thymus and skin, during development, and in the reproductive, immune, nervous and endocrine systems. The liver, being the major organ for xenobiotic metabolism, often shows pathological changes such as generalised hypertrophy and focal hepatocellular degeneration (Allen *et al.*, 1976). Furthermore, the liver function enzymes become elevated well before the onset of clinical symptoms of liver disease making them very valuable diagnostic tools for biomonitoring hepatotoxicity of organic pollutants in marine mammals (Burtis and Ashwood, 1999).

Alkaline phosphatase (ALKP) is one of the most widely distributed enzymes in the body located on the cell membranes of a variety of tissues including liver, kidney, bone, heart and skeletal muscle. ALKP induced activity is the most specific parameter available for bile stasis. In general, ALKP shows minimal activity in normal hepatic tissue, but it is found to be highly elevated in serum as a result of increased enzyme production due to impaired bile flow (Bossart *et al.*, 2001). Increased ALKP levels are also associated with liver disease and intestinal inflammation and with growth in young animals (Bossart and Dierauf, 1990; Nyman 2000 and 2003). In addition, an increase of 2-3 times above normal

can be observed in pregnant women due to the additional circulating ALKP of placental origin (Burtis and Ashwood, 1999). In marine mammals, ALKP activity has also been found to be liver specific, with increased levels indicating liver damage (Medway and Geraci, 1986).

Alanine aminotransferase (ALT or SGPT), aspartate aminotransferase (AST or SGOT), and gamma glutamyl transferase (γGT) are enzymes widely used in veterinary clinical pathology to diagnose liver failure or dysfunction. Like ALKP, AST is also quite widely distributed and is particularly found in muscle, liver and erythrocytes (Kerr, 1989), ALT is found primarily in the liver, whereas γGT is located on the cellular membranes of many organs, such as heart, liver, kidneys, skeletal muscle and pancreas. Increased ALT levels are associated with hepatocellular necrosis as well as with neoplasia, parasitism, and hepatic and muscular trauma. Rises in AST also indicate liver disease and neoplasia as well as skeletal muscle damage and heart disease. Moreover, decreased AST levels are associated with hepatic cirrhosis (Bossart *et al.*, 2001). Measurement of γGT levels is a sensitive test reflecting hepatocellular injury-primary acute and chronic obstructive liver diseases are associated with increased levels of γGT; but it discriminates poorly among various liver diseases. Moreover, increased serum γGT activity is also seen in nursing animals (Meyer and Harvey, 1998).

Serum creatine kinase (CK) and creatinine are not only elevated at certain stages during the course of diseases leading to muscular dystrophy, but they can also be elevated by exposure to certain drugs and phytoestrogens, such as isoflavones (Zhao *et al.*, 2005). Diethylstilbestrol (DES), a well-known endocrine disrupting chemical (estrogen mimic) can significantly increase serum CK activities (Burtis & Ashwood, 1999). Although CK is an indicator of muscular dystrophy, its activity in humans is also inversely influenced by thyroid activity. For example, in hyperthyroidism, serum CK activity is found to be highly decreased (Burtis and Ashwood, 1999).

Low serum albumin (ALB) levels when associated with elevated globulin levels are also indicating chronic liver disease and stimulation of the immune system (Bossart and Dierauf, 1990). Liver diseases can lead to decreased serum albumin levels by altering ALB synthesis (decrease), by increasing degradation or by promoting extra-vascular loss. ALB is also an important blood chemistry parameter for marine mammals. It is believed to transport PCBs that during stress and starvation are released into the blood stream from the blubber where they were accumulated (Peakall *et al.*, 1972; Busbee *et al.*, 1985). Total

bilirubin (TBIL) in humans is a sensitive indicator of hepatic disease and increased levels are associated with liver disease, bile duct obstruction, hepatic or post-hepatic problems, cirrhosis and neonatal physiologic jaundice (Medway *et al.*, 1966; Sweeney and Ridgway, 1975; Bossart and Dierauf, 1990). In humans, levels of TBIL have been reported to decrease by the use of oral contraceptives (Cowan *et al.*, 1982) and slightly increase in DES and methoxychlor treatment (Golub *et al.*, 2004). In dogs, uric acid (UA) levels have been used as indicators of liver disease (Bossart and Dierauf, 1990). In humans, it has been observed that serum UA levels increase after menopause indicating that this effect maybe mediated by the decrease in the levels of estrogens (Simon *et al.*, 2005). In marine mammals however, very limited information exists to determine that its measurement is valuable for assessing liver insufficiency. Therefore, collection of data is necessary to determine if blood chemistry parameters are useful to show exposure and effect of organochlorine compounds.

Decreases in blood urea nitrogen (BUN) level is an indicator of changes in the metabolic activity (e.g. reduction of food intake due to fasting) and liver failure (cirrhosis) and can be used as a biological indicator together with other parameters, such the increase in albumin and changes in CK levels (Nyman, 2000). Decreased glucose levels are indicators of malnutrition and starvation whereas increased levels have been found in marine mammals as a response to stress (Bossart and Dierauf, 1990). Increased lactate dehydrogenase (LDH) values show excess muscular activity but LDH has been minimally investigated in marine mammals (Bossart *et al.*, 2001). Normal ranges need to be established in order for this enzyme to be useful in marine mammal medicine.

The liver plays a key role in lipoprotein metabolism and hepatic disorders are associated with alterations in lipoprotein profiles. It is well established that elevated serum lipids indicate an accumulation of lipid in the liver, resulting in lipidosis (Vandenberghe, 1996). In humans and rodents, it is well established that increases in total serum lipids, lipoproteins (such as very-low density lipoprotein; VLDL, intermediate density lipoprotein; IDL and low density lipoprotein; LDL), cholesterol and triglycerides result from PCB exposure (Allen *et al.*, 1976; Kreiss *et al.*, 1981). This is a pathological condition, commonly associated with liver degeneration and necrosis. However, hepatic lipid accumulation has conversely been proposed as a protective response against the oxidative stress caused by enhanced metabolism of PCBs (Borlakoglu and Welsch, 1992). In Harbour seals, it has been reported that cholesterol and triglyceride levels rise during pregnancy (Kuiken, 1985). Moreover, serum lipids also can be raised by hypothyroidism

and dosing with drugs, such as corticosteroids and estrogen analogues (Bossart *et al.*, 2001).

Although there are few studies of blood chemistry parameters as indicators of contaminant-induced toxicity in marine mammals, elevated exposure to organochlorine pollutants has been found to adversely alter seal physiology as evidenced by significant alterations in their blood chemistry (Brouwer *et al.*, 1989; Schumacher *et al.*, 1995; Nyman, 2000). Since these chemistry parameters are measured in blood, they are an ideal tool for non-destructive biomonitoring. Although blood chemistry parameters have proven to be very useful tools in veterinary medicine, the dose-response relationships between contaminant exposure and changes in blood chemistry parameters must first be established as part of the validation of these parameters as reliable biomarkers of pollutant exposure and/or effects in marine mammal populations. Furthermore, controlling the influence of other factors altering blood chemistry parameters (e.g. diet, physical activity, infection, trauma, and reproductive status) must also be achieved.

Baltic Ringed (*Phoca hispida baltica*) and Grey (*Halichoerus grypus*) seals have been heavily exposed to organochlorine contaminants since the 1960s and recovery of both populations have been slow due to reproductive pathological deformities reducing reproductive success, such as uterine occlusions (Helle *et al.*, 1976a, b; Bergman and Olsson, 1985, Nyman 2003). Elevated pollutant exposure has also been reported in Harbour and Grey seals from the UK and Wadden Sea (Reijnders, 1980 and 1986; Osterhaus and Vedder, 1988).

The aim of the present study was to investigate the reliability of blood chemistry parameters as biomarkers of PCB exposure and effects in seals. This was accomplished by the following objectives:

i. Determining a range of blood chemistry parameters in seal serum samples in low exposure/control seal groups in order to establish baseline values for Harbour, Grey and Ringed seals to contribute to the limited knowledge of normal ranges for these species.
ii. Identifying blood chemistry parameters whose expression is dose-dependent with PCB exposure as part of their validation as biomarkers using samples from the experimental seal group

iii. Quantify validated blood chemistry parameters in serum samples of highly polluted Baltic Ringed and Grey seal populations and less polluted (control) Grey (Sable Island, Western Atlantic Ocean) and Ringed seals (Svalbard, European Arctic) to represent changes in BM expression over a range of PCB exposure

iv. Investigate changes in VLDL, HDLC and LDL concentrations in association with estradiol

v. Identify which populations are at risk from ED effects

## 5.2 Materials and Methods

### 5.2.1 Sampling

All of the blood samples from the three study species (Ringed, Grey and Harbour seals) were provided by collaborators. The samples were collected from six different seal populations over a 7 year period (1999 - 2002); Baltic Ringed and Grey seals, Svalbard Ringed seals and Grey seals from Sable Island, Canada were provided from Dr. M. Nyman, *Finnish Game and Fisheries Research Institute* (Helsinki, Finland). Harbour seals from Abertay Sands (Dundee, Scotland) were provided by Dr. Ailsa Hall (Sea Mammal Research Unit, St. Andrews University) and Moray Firth (Northeast Scotland) Harbour seals were provided by Dr. Paul Thompson (Lighthouse Research Station, University of Aberdeen). Harbour seals were also provided from a controlled dosing study, in which the seals were fed fish from the highly contaminated Wadden Sea or less contaminated Atlantic Sea (Prof. P. Reijnders). Details of samples are summarised in Table 5.1 (*Full details are provided in Chapter 2-Contaminant Analysis*).

### 5.2.2 Materials

| | |
|---|---|
| *Instrumentation*: | Vitros ® DT60 II chemistry analyser, Vitros ® DTSC II module and rechargeable pipette. |
| *Consumables/Controls*: | DT calibrator plus, DT Control I and II purchased from *Axis Shield*, (Dundee, Scotland) and stored at -20°C until use. |

Blood chemistry parameters were analysed in serum using the automatic analysers, Ektachem DT60 II and DTSC II module. The Ektachem DT slides used to perform the clinical tests were colour-coded to indicate required storage temperatures (-20°C or <8°C).

| | |
|---|---|
| *DT 60 Chemistry Slides*: | Cholesterol (CHOL), High Density Lipid Cholesterol (HDLC), Glucose (GLU), Total Bilirubin (TBIL), Total Protein (TP), Triglycerides (TRIG), Blood Urea Nitrogen (BUN/UREA) and Uric Acid (URIC). |

*DTSC Chemistry Slides*: Alanine Aminotransferase (ALT), Albumin (ALB), Alkaline Phosphatase (ALKP), Aspartate Aminotransferase (AST), Creatinine Kinase (CK), Creatinine (CRSC-single slide), Gamma glutamyl transferase (γGT) and Lactate Dehydrogenase (LDH).

### 5.2.3 Blood Chemistry Analysis

All samples were analysed in singles. However, at the start and the end for every day of seal serum analysis calibration controls for each blood chemistry parameter were analysed in order to check the efficiency and reproducibility of the automatic analyser.

All reactions needed for a single quantitative measurement took place within the multi-layered analytical element of the slide. Different dedicated slides were used to measure each parameter and were for single use. All processes performed by the analyser were controlled by the self-contained microcomputer in the analysers. Periodic calibration of the analyser was carried out to ensure the accuracy of test results with a daily quality control test carried out once a day before testing. The procedure analysis was carried out as follows. The Ektachem DT slide was placed into the loading station of the analyser and moved into the spotting position (as the slide entered the spotting position, a bar code reader read the laser bar code on the slide to identify the test and the slide generation number for the microcomputer). When reading was done, 10μl of serum was dispensed using an Ektachem DT pipette onto the slide. After spotting and verification of spotting by the optical drop detector the slide was incubated for five minutes at 37°C within the analyser.

Following incubation, the slide was read by the DT 60 II analyser to obtain a measure of the intensity of colour formation. Measurements are read when the light transmitted to the slide by the optical fibres is reflected off the slide and conducted to a photo-detector, the signal is translated by the microcomputer against readings from reference slides and calibration slides calculate the concentration of the parameter in the sample. In the case of the DTSC II analyser, measurements are made from the light reflected from the slide that is transported to the reflectometer and then through an interference filter of the appropriate set wavelength (according to parameter being measured). The light is detected by a silicon photo-detector and

the signal intensity translated by the microcomputer into a quantitative measure in the same manner as the DT 60 II analyser (*Johnson & Johnson* Clinical Diagnostics, Inc.).

**Table 5.1:** Background information for each study population

| **Population Category** | **Exposed** | **Control** | **Exposed** | **Control** | **Exposed** | **Exposed** | **Experimental** | |
|---|---|---|---|---|---|---|---|---|
| **Study Site** | **North Baltic Sea** | **Svalbard** | **North Baltic Sea** | **Sable Island** | **Moray Firth** | **Abertay Sands** | **Experimental Group** | |
| | | **European Arctic** | | **Canada** | **NE Scotland** | **Firth of Tay, Scotland** | | |
| **Species** | Ringed Seals | Ringed Seals | Grey Seals | Grey Seals | Harbour Seals | Harbour Seals | Harbour Seals (E) | Harbour Seals (C) |
| | *Phoca hispida baltica* | *Phoca hispida* | *Halichoerus grypus* | *Halichoerus grypus* | *Phoca vitulina* | *Phoca vitulina* | *Phoca vitulina* | *Phoca vitulina* |
| **No. of Serum Samples** | **23** | **18** | **27** | **19** | **68** | **56** | **11** | **12** |
| **No. analysed for ΣPCBs** | **23** | **18** | **19** | **19** | **47** | **48** | **11** | **12** |
| **Sex & Age** | | | | | | | | |
| **Females** | **11** | **10** | **16** | **10** | **36** | **20** | **11** | **12** |
| Adult | All | All | All | All | 18 | All | All | All |
| Sub-Adult | - | - | - | - | 4 | - | - | - |
| Juvenile | - | - | - | - | 14 | - | - | - |
| **Males** | **12** | **8** | **11** | **9** | **32** | **36** | **-** | **-** |
| Adult | All | All | All | All | 21 | All | - | - |
| Sub-Adult | - | - | - | - | 2 | - | - | - |
| Juvenile | - | - | - | - | 9 | - | - | - |
| **Year of Sampling** | April to June<br>1997-1998<br>2000<br>2002 | April to June<br>1996<br>1998 | April to June<br>1997-1998<br>2000 | April to June<br>1998 | 1992-1995 | 1999-2000 | 1988 | 1988 |
| **Stage of Reproduction** | II | II | II | II | I and IV | I, II, III and IV | IV | IV |

### 5.2.4 Quantification of serum ΣPCB and 17β-estradiol concentrations

Serum samples were analysed (ELISA) for the sex hormone 17β-estradiol (E2) as well as for ΣPCBs in order to determine the level of exposure in study animals. Full details of analysis are provided in Chapter 3 and 2 respectively. Mean hormone and ΣPCBs concentrations are presented in Tables 5.2 and 5.3.

**Table 5.2:** Mean Concentrations of 17β-estradiol (E2) in serum samples for each study population (Results are presented as means ±standard errors; ranges)

| Species / Study Populations | Sample No. | Sex Steroid | Reproduction Stage |
|---|---|---|---|
| **Harbour Seals (*Phoca vitulina*)** | | **17β-estradiol (E2) (ng/ml)** | |
| **Abertay Sands, Firth of Tay, Scotland** | | | |
| *Male Adults* | 5 | 0.07 ± 0.02 (0.04 – 0.12) | **I** |
| | 6 | 0.09 ± 0.02 (0.04 – 0.14) | **II** |
| | 14 | 0.10 ± 0.01 (0.04 – 0.21) | **III** |
| | 10 | 0.13 ± 0.03 (0.03 – 0.24) | **IV** |
| *Female Adults* | 9 | 0.16 ± 0.05 (0.02 – 0.47) | **I** |
| | 1 | 0.42 | **II** |
| | 7 | 0.12 ± 0.02 (0.05 – 0.17) | **III** |
| | 3 | 0.21 ± 0.06 (0.10 – 0.32) | **IV** |
| **Moray Firth, NE Scotland** | | | |
| *Male Adults* | 14 | 0.15 ± 0.05 (0.02 – 0.68) | **I** |
| | 1 | 0.07 | **IV** |
| *Male Juveniles* | 9 | 0.17 ± 0.10 (0.02 – 0.98) | **I** |
| *Female Adults* | 11 | 0.12 ± 0.03 (0.02 – 0.26) | **I** |
| | 1 | 0.84 | **IV** |
| *Female Juveniles* | 14 | 0.17 ± 0.04 (0.05 – 0.49) | **I** |
| **Experimental Group** | | | |
| *Females Control* | 12 | 0.22 ± 0.05 (0.03 – 0.66) | **IV** |
| *Females Exposed* | 11 | 0.11 ± 0.04 (0.01 – 0.47) | **IV** |
| **Ringed Seals (*Phoca hispida*)** | | | |
| **North Baltic Sea** | | | |
| *Male Adults* | 6 | 0.04 ± 0.01 (0.02 – 0.06) | **II** |
| *Female Adults* | 10 | 0.08 ± 0.02 (0.02 – 0.19) | **II** |
| **Svalbard, European Arctic** | | | |
| *Male Adults* | 7 | 0.10 ± 0.06 (0.02 – 0.44) | **II** |
| *Female Adults* | 8 | 0.31 ± 0.09 (0.05 – 0.77) | **II** |
| **Grey Seals (*Halichoerus grypus*)** | | | |
| **North Baltic Sea** | | | |
| *Male Adults* | na | Na | **II** |
| *Female Adults* | 15 | 0.13 ± 0.03 (0.0.3 – 0.45) | **II** |
| **Sable Island, Canada** | | | |
| *Male Adults* | 9 | 0.56 ± 0.16 (0.06 – 1.30) | **II** |
| *Female Adults* | 10 | 0.46 ± 0.19 (0.07 – 2.10) | **II** |

*(Table 5.2 was reproduced from Chapter 3 –Sex Steroid Analysis)*

**Table 5.3** Mean Concentrations of ΣPCBs in blood samples for each study population (Results are presented as means ±standard errors; ranges)

| **Population** | **Exposed** | **Control** | **Exposed** | **Control** | **Exposed** | **Exposed** | **Experimental** | |
|---|---|---|---|---|---|---|---|---|
| | **North Baltic Sea** | **Svalbard** | **North Baltic Sea** | **Sable Island** | **Moray Firth** | **Abertay Sands** | **Experimental Group** | |
| | | **European Arctic** | | **Canada** | **NE Scotland** | **Firth of Tay, Scotland** | **Exposed** | **Control** |
| **Species** | **Ringed Seals** | | **Grey Seals** | | | **Harbour Seals** | | |
| | *Phoca hispida baltica* | *Phoca hispida* | *Halichoerus grypus* | | | *Phoca vitulina* | | |
| **No of Serum Samples** | **23** | **18** | **19** | **19** | **47** | **48** | **11** | **12** |
| **Female Adults** | **11** | **11** | **10** | **10** | **11** | **31** | **11** | **12** |
| **Mean (µg/ml)** | 14.03 ± 1.78 | 2.16 ± 0.57 | 13.46 ± 4.38 | 1.79 ± 0.51 | 10.59 ± 1.64 | 8.40 ± 1.81 | 12.76 ± 2.52 | 4.92 ± 0.55 |
| **Range** | (8.01-25.49) | (0.47-6.48) | (2.35-39.73) | (0.36-4.89) | (3.69 – 21.29) | (1.29-27.49) | (4.16 – 29.22) | (2.29 – 9.51) |
| **Female Juveniles** | **na** | **na** | **na** | **na** | **14** | **na** | **na** | **na** |
| **Mean (µg/ml)** | | | | | 14.40 ± 3.02 | | | |
| **Range** | | | | | (1.97 – 36.54) | | | |
| **Male Adults** | **12** | **8** | **9** | **9** | **14** | **17** | **na** | **na** |
| **Mean (µg/ml)** | 16.44 ± 3.06 | 2.56 ± 0.71 | 40.18 ± 10.43 | 12.14 ± 5.33 | 13.79 ± 1.76 | 9.67 ± 1.23 | | |
| **Range** | (6.05-45.76) | (0.88-6.95) | (7.10-87.06) | (0.30-16.00) | (6.45 – 23.70) | (0.68 – 24.54) | | |
| **Male Juveniles** | **na** | **na** | **na** | **na** | **8** | **na** | **na** | **na** |
| **Mean (µg/ml)** | | | | | 7.58 ± 2.64 | | | |
| **Range** | | | | | (2.43 – 25.23) | | | |

na, not available

*(Table 5.3 was reproduced from Chapter 2 – Contaminant Analysis)*

## 5.3 Results

Seal samples were grouped according to species, geographical location, sex and age. Differences between groups were tested by a two-tailed student t-test. Significance levels were set at $p<0.05$ and $p<0.005$. Results are presented as mean ± SE and ranges.

### 5.3.1 Sex and age-dependent variations

*Harbour Seals, Moray Firth, Scotland*

The blood chemistry values for the analysed Harbour seal population from Moray Firth and the reference population are presented in Table 5.4. Of the analysed parameters female adults showed reduced globulin, cholesterol, triglycerides and HDLC concentrations ($p<0.05$) and increased ALKP concentrations ($p<0.005$) compared to male adults (Fig.5.1). Female adults also showed increased uric acid and AST/ALT compared to female juveniles ($p<0.05$). Male adults also showed increased AST/ALT compared to juveniles ($p<0.005$). Male and female juveniles showed increased ALT, AST, γGT and ALKP compared to male and female adults, respectively ($p<0.005$). When comparing female juveniles with female adults, cholesterol levels where significantly higher in juveniles ($p<0.005$).

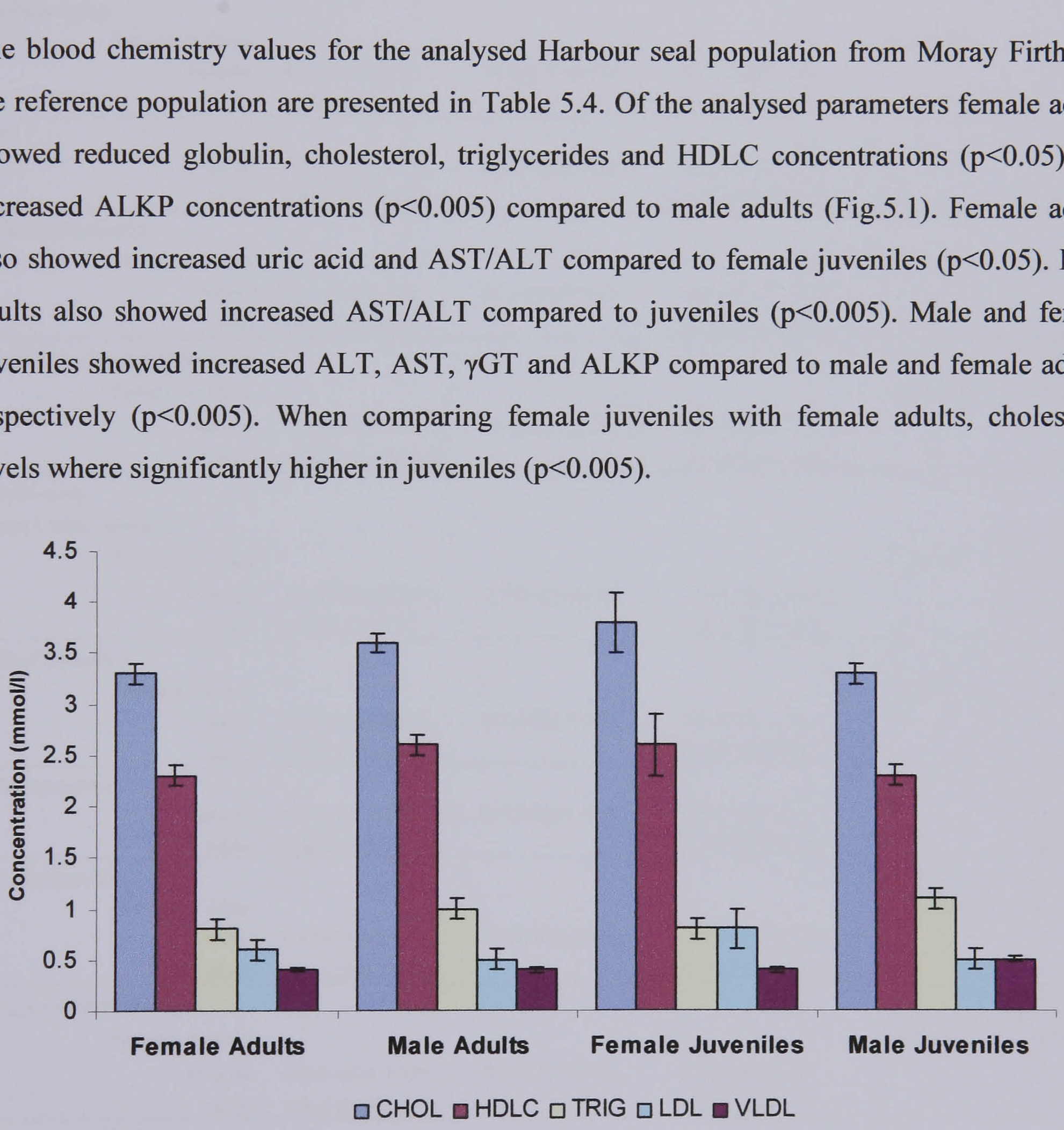



**Figure 5.1:** Mean ±SE Cholesterol, HDLC, Triglycerides, LDL and VLDL concentrations in Harbour seals (Moray Firth, Scotland).

**Table 5.4:** Blood chemistry values for Harbour seals from Moray Firth, Scotland.

| | This Study | | | Reference | |
|---|---|---|---|---|---|
| **Blood chemistry Parameter** | **Adults** | **Sub-Adults** | **Juveniles** | **Adults** | **Juveniles** |
| **Total Protein (g/L)** | | | | | |
| *Females & Males* | | | | 27.0-119.0[abc] | 65.0-90.0[ae] |
| *Females* | 81.1±1.2 (70-89) | 82.3±2.0 (78-87) | 83.5±1.4(77-91) | | |
| *Males* | 83.9±1.3(73-93) | | 78.6±3.6(55-90) | | |
| **Albumin (g/L)** | | | | | |
| *Females & Males* | | | | 25.0-33.0[a] | 27.0-37.0[a] |
| *Females* | 31.9±0.6 (25-35) | 33.5±0.6(32-35) | 33.3±0.9(28-38) | | |
| *Males* | 31.5±0.4 (28-35) | | 33.0±1.6(23-39) | | |
| **Globulin (g/L)** | | | | | |
| *Females & Males* | | | | 32.0-60.0[a] | 27.0-64.0[a] |
| *Females* | 49.2±0.9 (41-55) | 48.8±2.4(45-55) | 50.2±1.5(41-57) | | |
| *Males* | 52.3±1.3 (40-64) | | 45.6±2.8(32-58) | | |
| **A/G** | | | | | |
| *Females* | 0.7±0.01(0.5-0.7) | 0.7±0.04(0.6-0.8) | 0.7±0.03(0.5-0.9) | n.a. | n.a. |
| *Males* | 0.6±0.02(0.4-0.9) | | 0.7±0.05(0.6-0.9) | n.a. | n.a. |
| **Uric Acid (µmol/L)** | | | | | |
| *Females & Males* | | | | 53.53-101.12[f] | n.a. |
| *Females* | 64.4±3.1(41-82) | 64.5±2.1(61-69) | 48.6±8.3(25-100)^^ | | |
| *Males* | 64.0±4.0(18-93) | | 49.2±9.4(19-88) | | |
| **Blood Urea Nitrogen (mmol/L)** | | | | | |
| *Females & Males* | | | | 10.0-34.6[af] | 2.9-21.42[ade] |
| *Females* | 16.8±0.3(13.3-19.1) | 16.0±0.5(15.2-17.2) | 16.4±0.9(12.6-19.8) | | |
| *Males* | 16.9±0.3(14.9-20.8) | | 16.1±0.9(11.2-20.9) | | |
| **Cholesterol** | | | | | |
| **Total CHOL (mmol/L)** | | | | | |
| *Females & Males* | | | | 1.65-9.1[abc] | 4.3-10.3[ae] |
| *Females* | 3.2±0.1(2.3-3.9)* | 3.7±0.1(3.6-3.8) | 3.8±0.3(2.5-5.5)^^ | | |
| *Males* | 3.6±0.1(2.7-4.2) | | 3.3±0.1(2.9-3.8) | | |
| **HDLC (mmol/L)** | | | | | |
| *Females & Males* | | | | 3.9-6.0[g] | n.a. |
| *Females* | 2.2±0.1(1.3-2.8)* | 2.7±0.4(1.4-3.3) | 2.6±0.3(1-3.9) | | |
| *Males* | 2.6±0.1(1.8-3.4) | | 2.3±0.1(1.8-2.8) | | |
| **LDL (mmol/L)** | | | | | |
| *Females* | 0.6±0.1(0.1-1.5) | 0.7±0.4(0.1-1.9) | 0.8±0.2(0.2-2.4) | n.a. | n.a. |
| *Males* | 0.5±0.1(0.1-1) | | 0.5±0.1(0.2-0.9) | n.a. | n.a. |
| **VLDL (mmol/L)** | | | | | |
| *Females & Males* | | | | 0.12-1.82[a] (E) | n.a. |
| *Females* | 0.4±0.03(0.2-0.6) | 0.3±0.03(0.3-0.4) | 0.4±0.03(0.2-0.5) | | |
| *Males* | 0.4±0.03(0.2-0.8) | | 0.5±0.03(0.4-0.6) | | |
| **Triglycerides (mmol/L)** | | | | | |
| *Females & Males* | | | | 0.23-4.1[ab] | 0-2.9[de] |
| *Females* | 0.8±0.1(0.4-1.3)* | 0.7±0.1(0.6-0.8) | 0.8±0.1(0.5-1.2) | | |
| *Males* | 1.0±0.1(0.3-1.8) | | 1.1±0.1(0.8-1.4) | | |
| **CHOL/HDLC** | | | | | |
| *Females* | 1.5±0.1(1.1-2.3) | 1.5±0.4(1.1-2.6) | 1.6±0.3(1.2-3.9) | n.a. | n.a. |
| *Males* | 1.4±0.04(1.1-1.8) | | 1.4±0.04(1.3-1.6) | n.a. | n.a. |

**Table 5.4** (*continued*)

| Blood chemistry Parameter | Adults | This Study Sub-Adults | Juveniles | Reference Adults | Juveniles |
|---|---|---|---|---|---|
| **Glucose (mmol/L)** | | | | | |
| *Females & Males* | | | | 2.28-9.5[af] | 4.8-10.32[def] |
| *Females* | 6.2±0.5(3.3-10.2) | 5.8±0.4(5.1-6.7) | 6.6±0.5(4.6-9.6) | | |
| *Males* | 6.0±0.3(2.1-8.8) | | 6.7±0.2(5.6-7.5) | | |
| **Enzymes** | | | | | |
| **ALT (U/L)** | | | | | |
| *Females & Males* | | | | 8.0-75[a] | 6.0-95.0[ade] |
| *Females* | 8.7±0.4(5-11) | 9.5±0.6(8-11) | 36.9±2.0(28-48)^^ | | |
| *Males* | 8.9±0.4(5-12) | | 37.3±1.7(29-44)^^ | | |
| **AST (U/L)** | | | | | |
| *Females & Males* | | | | 29.0-168.0[a] | 5.0-159.0[ae] |
| *Females* | 32.4±1.7(20-51) | 32.0±3.0(26-39) | 59.0±4.8(37-79)^^ | | |
| *Males* | 30.7±1.8(21-57) | | 60.3±3.9(48-85)^^ | | |
| **AST/ALT** | | | | | |
| *Females & Males* | | | | n.a. | n.a. |
| *Females* | 4.0±0.3(1.8-7.6) | 3.4±0.3(2.5-3.9) | 1.6±0.2(1-2.4)^^ | | |
| *Males* | 3.7±0.3(2.2-8.1) | | 1.6±0.1(1.2-2.4)^^ | | |
| **γGT (U/L)** | | | | | |
| *Females & Males* | | | | 2.0-15.0[ab] | 4.9-23.0[de] |
| *Females* | 17.6±1.4(4-25) | 22.3±4.0(14-33) | 22.6±2.9(16-47) | | |
| *Males* | 16.2±0.4(12-19) | | 21.3±1.6(13-28)^^ | | |
| **ALKP (U/L)** | | | | | |
| *Females & Males* | | | | 11.0-176.0[a] | 20-259[ade] |
| *Females* | 23.5±1.6(11-36)** | 28.0±4.3(17-38) | 54.8±4.8(39-86)^^ | | |
| *Males* | 14.9±0.7(8-20) | | 56.8±1.5(50-63)^^ | | |

CHOL, Cholesterol; HDLC, High Density Lipid Cholesterol; VLDL, Very Low Density Lipoprotein; ALKP, Alkaline Phosphatase; ALT, Alanine Aminotransferase; AST, Aspartate Aminotransferase; γGT, Gamma Glutynil Transferase; n.a., no data available; E, estimated.

Results are presented as means ±standard errors (ranges). The significance of sex variation is presented as * P<0.05 or ** P<0.005 and the significance of age variation is shown as ^ P<0.05 or ^^ P<0.005.

References: [a] Dierauf and Gulland, 2001; [b] Schumacher, 1995; [c] Bossart and Dierauf, 1990; [d] Roletto, 1993; [e] de Swart, 1995; [f] Nyman, 2003.

## *Harbour Seals, Abertay Sands, Scotland*

The blood chemistry values of the analysed samples are presented in Table 5.6. Reference values for this population, are presented in Table 5.4. Of the analysed parameters AST and ALKP were found to be significantly higher in females compared to male Harbour seals ($p<0.005$, Fig.5.2)

**Table 5.6:** Blood chemistry values for Harbour seals from Abertay Sands, Scotland and the experimental group fed with Wadden Sea fish.

| | | *Exposed* | *Experimental Group* | |
|---|---|---|---|---|
| **Blood chemistry Parameter** | | **Abertay Sands** | **Exposed** | **Control** |
| **Total Protein (g/L)** | | | | |
| | *Females* | 72.8±1.4(64-84) | 81.8±2.1(72-99) | 84.3±1.9(75-98) |
| | *Males* | 74.0±1.7(49-92) | | |
| **Albumin (g/L)** | | | | |
| | *Females* | 28.0±0.3(25-31) | 34.6±1.0(31-43)^ | 31.6±0.9(28-40) |
| | *Males* | 28.2±0.3(24-31) | | |
| **Globulin (g/L)** | | | | |
| | *Females* | 44.8±1.3(36-58) | 47.2±2.2(37-63) | 52.7±2.3(37-69) |
| | *Males* | 45.8±1.7(21-65) | | |
| **A/G** | | | | |
| | *Females* | 0.6±0.02(0.4-0.8) | 0.8±0.1(0.6-1.1) | 0.6±0.05(0.4-1.1) |
| | *Males* | 0.7±0.03(0.4-1.3) | | |
| **Uric Acid (µmol/L)** | | | | |
| | *Females* | 64.5±4.2(22-94) | 68.8±2.2(54-78)^ | 63.4±1.3(57-71) |
| | *Males* | 64.0±3.4(30-99) | | |
| **Blood Urea Nitrogen (mmol/L)** | | | | |
| | *Females* | 17.1±0.7(12.5-24.4) | 16.5±0.3(15.1-18.3) | 17.0±0.4(15.4-20) |
| | *Males* | 16.9±0.7(12.2-28.7) | | |
| **Cholesterol** | | | | |
| **Total CHOL (mmol/L)** | | | | |
| | *Females* | 2.9±0.2(1.9-4.6) | 4.7±0.2(4.1-5.8) | 4.5±0.1(3.7-5.8) |
| | *Males* | 3.0±0.1(1.3-4.2) | | |
| **HDLC (mmol/L)** | | | | |
| | *Females* | 1.9±0.2(0.8-3.5) | 2.4±0.2(1.3-3.3)^ | 3.1±0.1(2.7-4.1) |
| | *Males* | 2.0±0.1(0.5-3.3) | | |
| **LDL (mmol/L)** | | | | |
| | *Females* | 0.7±0.1(0.2-1.6) | 1.3±0.2(0.4-2.4)^^ | 0.6±0.1(0.02-0.9) |
| | *Males* | 0.8±0.1(0.1-1.4) | | |
| **VLDL (mmol/L)** | | | | |
| | *Females* | 0.3±0.03(0.1-0.5) | 1.0±0.1(0.8-1.3)^ | 0.8±0.05(0.5-1.1) |
| | *Males* | 0.3±0.02(0.1-0.6) | | |
| **Triglycerides (mmol/L)** | | | | |
| | *Females* | 0.7±0.1(0.3-1.2) | 2.2±0.1(1.8-2.8)^ | 1.7±0.1(1.1-2.4) |
| | *Males* | 0.6±0.04(0.3-1.4) | | |
| **CHOL/HDLC** | | | | |
| | *Females* | 1.6±0.1(1.3-2.4) | 2.1±0.2(1.4-3.1)^ | 1.5±0.03(1.2-1.7) |
| | *Males* | 1.6±0.1(1.1-3.1) | | |
| **Glucose (mmol/L)** | | | | |
| | *Females* | 2.5±0.4(1.1-6.6) | 8.0±0.2(6.8-8.8)^^ | 6.4±0.3(5.2-7.7) |
| | *Males* | 2.9±0.4(1.1-10.4) | | |

**Table 5.6** (*continued*)

| Blood chemistry Parameter | | Exposed | *Experimental Group* | |
|---|---|---|---|---|
| | | Abertay Sands | Exposed | Control |
| **Enzymes** | | | | |
| **ALT (U/L)** | | | | |
| | *Females* | 11.8±0.8(3-20) | 14.4±0.5(12-17)^^ | 9.1±1.2(1-18) |
| | *Males* | 11.6±1.1(3-25) | | |
| **AST (U/L)** | | | | |
| | *Females* | 41.7±2.6(23-67)** | 52.8±6.6(21-97)^ | 36.5±4.0(10-53) |
| | *Males* | 32.3±1.2(18-46) | | |
| **AST/ALT** | | | | |
| | *Females* | 3.8±0.3(2.2-7.7) | 3.6±0.4(1.8-6.5) | 8.0±4.1(1-53) |
| | *Males* | 4.4±0.6(1.2-13) | | |
| **γGT (U/L)** | | | | |
| | *Females* | 17.0±1.0(5-24) | 24.3±2.6(15-46)^ | 17.2±1.5(12-32) |
| | *Males* | 17.1±0.9(11-37) | | |
| **ALKP (U/L)** | | | | |
| | *Females* | 36.0±2.2(16-54)** | 47.0±5.4(34-91)^ | 34.1±0.9(30-42) |
| | *Males* | 23.4±2.1(10-59) | | |
| **CK (U/L)** | | | | |
| | *Females* | 700.8±48.4(264-1186) | n.a. | n.a. |
| | *Males* | 767.1±54.1(206-1508) | | |

CHOL, Cholesterol; HDLC, High Density Lipid Cholesterol; VLDL, Very Low Density Lipoprotein; ALKP, Alkaline Phosphatase; ALT, Alanine Aminotransferase; AST, Aspartate Aminotransferase; γGT, Gamma Glutynil Transferase; CK, Creatinine Kinase.

Results are presented as means ±standard errors (ranges). The significance of sex variation is presented as * P<0.05 or ** P<0.005 and the significance of contaminant variation for the experimental group is shown as ^ P<0.05 or ^^ P<0.005.

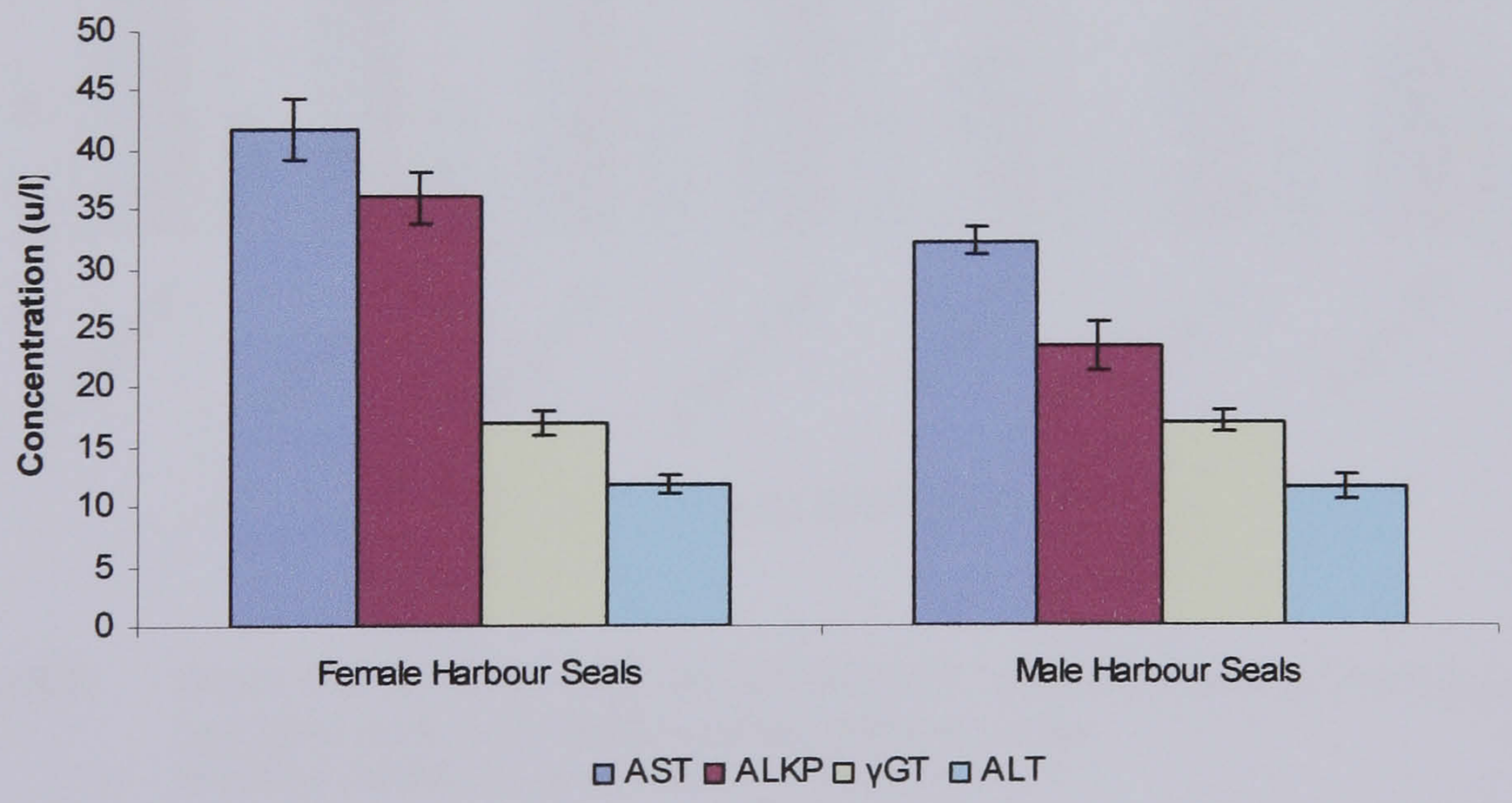



**Figure 5.2:** Mean ±SE AST, ALKP, γGT and ALT concentrations in Harbour seals (Abertay Sands, Scotland).

*Ringed and Grey Seals, Baltic*

The blood chemistry values in Ringed and Grey seals are presented in Table 5.7 as mean±SE and ranges. References for these populations are also included. Of the analysed parameters, female Baltic and Svalbard Ringed seals showed reduced ALT ($p<0.05$ and $p<0.005$ respectively) concentrations compared to males and increased ALKP concentrations ($p<0.005$) (Fig.5.3). Female Svalbard Ringed seals also showed reduced AST levels ($p<0.05$) compared to males. Female Baltic Grey seals showed reduced ALT and increased ALKP concentrations compared to adult Grey seals ($p<0.05$). Female Grey seals from Sable Island also showed increased ALKP concentrations compared to males ($p<0.05$) (Fig.5.3).

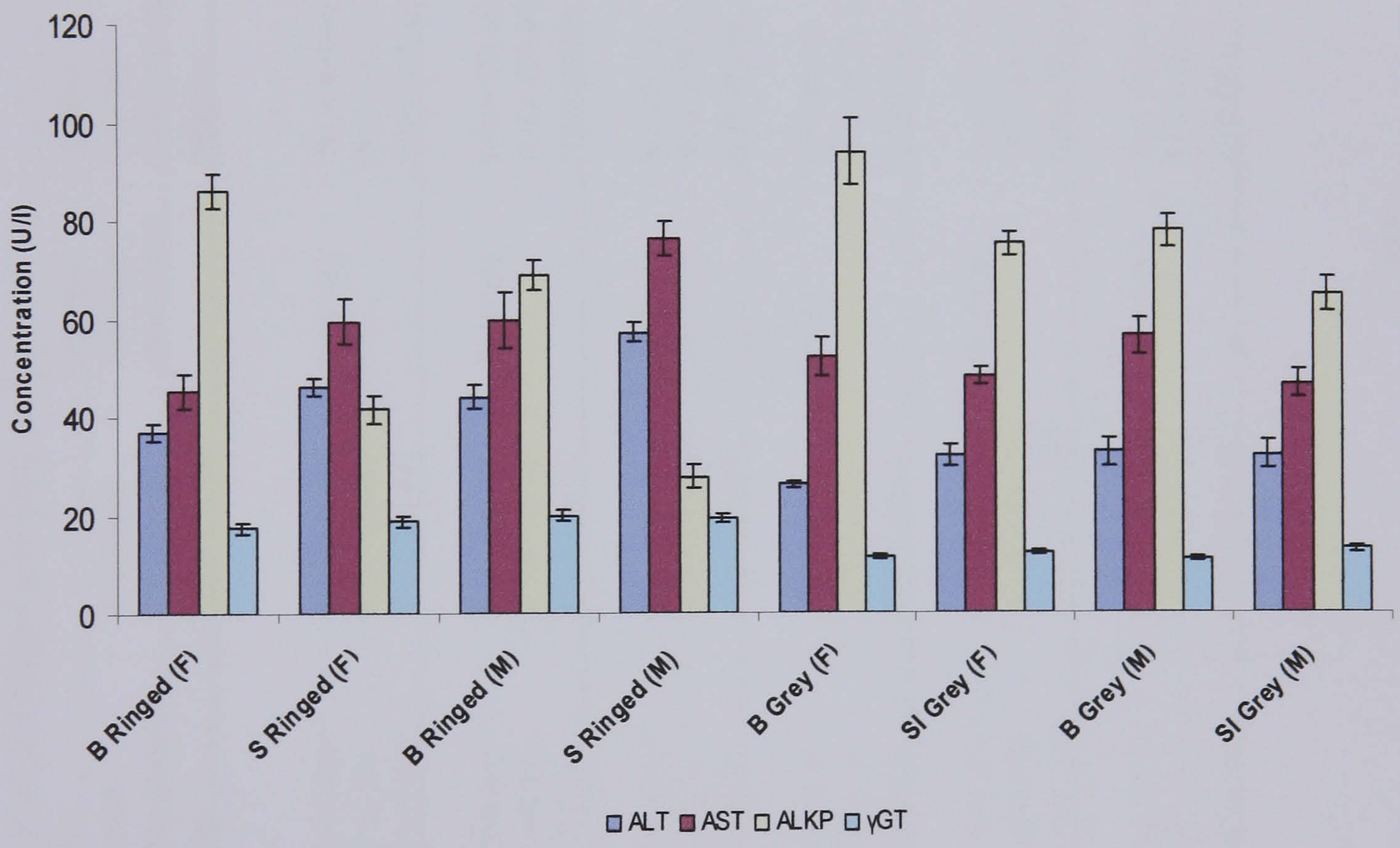



**Figure 5.3:** Mean ±SE of ALT, AST, ALKP and γGT concentrations in female and male Ringed and Grey seals from Baltic and the reference areas
B, Baltic; S, Svalbard; SI, Sable Island; F, Females; M, Males

**Table 5.7:** Blood chemistry values for Ringed and Grey seals

| | This Study | | Reference[0] | This Study | | Reference |
|---|---|---|---|---|---|---|
| | **Ringed Seals** | **Ringed Seals** | **Ringed Seals** | **Grey Seals** | **Grey Seals** | **Grey Seals** |
| | ***Phoca hispida baltica*** | ***Phoca hispida*** | ***Phoca hispida*** | ***Halichoerus grypus*** | ***Halichoerus grypus*** | ***Halichoerus grypus*** |
| | **Baltic Sea** | **Svalbard, Arctic** | | **Baltic Sea** | **Sable Island, Canada** | |
| **Blood chemistry Parameter** | | | | | | |
| **Total Protein (g/L)** | | | | | | |
| *Females & Males* | 69.4±2.3(57-102)^^ | 78.1±1.4(61-87) | 73 (53-88) | 72.1±2.0(45-82) | 76.6±2.1(50-97) | 76 (69-87)[a0] |
| *Females* | 70.2±3.1(61-89) | 79.5±1.3(75-87) | | 69.1±1.5(62-75) | 76.5±1.5(71-86) | |
| *Males* | 68.8±3.4(57-102) | 76.4±2.6(61-84) | | 75.0±3.5(45-82) | 76.7±4.2(50-97) | |
| **Albumin (g/L)** | | | | | | |
| *Females & Males* | 34.1±0.8(30-46)^^ | 29.6±1.1(22-38) | 29 (23-33) | 33.4±0.5(29-36)^ | 27.5±0.7(20-33) | 27 (22-30)[a0] |
| *Females* | 34.3±1.1(30-39) | 29.7±1.7(22-38) | | 33.6±0.7(29-36) | 27.4±1.3(20-33) | |
| *Males* | 34.0±1.3(30-46) | 29.5±1.3(25-37) | | 33.1±0.7(29-36) | 27.6±0.6(25-30) | |
| **Globulin (g/L)** | | | | | | |
| *Females & Males* | 35.3±2.2(19-68)^^ | 48.5±1.7(34-59) | 44 (E) | 38.7±2.3(9-52) | 49.1±2.3(24-72) | 55.3[a] |
| *Females* | 35.9±2.8(26-54) | 49.8±2.3(37-59) | | 35.5±2.0(27-44) | 49.1±2.1(42-66) | |
| *Males* | 34.8±3.4(19-68) | 46.9±2.3(34-54) | | 41.9±3.9(9-52) | 49.1±4.4(24-72) | |
| **A/G** | | | | | | |
| *Females & Males* | 1.0±0.1(0.5-2.4) | 0.6±0.04(0.4-1) | 0.7 (E) | 1.0±0.2(0.6-4) | 0.6±0.02(0.3-1.1) | 0.5 (E)[a0] |
| *Females* | 1.0±0.1(0.6-1.5) | 0.6±0.1(0.4-1) | | 1.0±0.1(0.7-1.3) | 0.6±0.02(0.3-0.7) | |
| *Males* | 1.1±0.1(0.5-2.4) | 0.6±0.02(0.5-0.9) | | 1.1±0.3(0.6-4) | 0.6±0.1(0.3-1.1) | |
| **Uric Acid (µmol/L)** | | | | | | |
| *Females & Males* | 92.9±1.6(78-112)^^ | 106.8±3.4(86-139) | 98 (43-206) | 74.6±2.7(56-104)^^ | 61.5±1.5(49-72) | 61 (43-121)[0] |
| *Females* | 92.8±3.1(78-112) | 106.8±5.1(86-139) | | 74.4±4.0(56-104) | 62.1±2.2(51-72) | |
| *Males* | 93.0±1.6(83-104) | 106.9±4.7(87-124) | | 74.8±3.7(56-89) | 60.9±2.2(49-72) | |
| **Blood Urea Nitrogen (µmol/L)** | | | | | | |
| *Females & Males* | 8.6±0.8(5.6-21.3)^^ | 13.8±1.1(7.8-24.5) | 13 (5.7-25) | 9.1±0.4(6.4-14.6)^^ | 14.4±0.7(9.1-19.9) | 11.4-38.6[a0] |
| *Females* | 8.3±1.0(6.2-14.9) | 13.9±1.2(8.1-21.5) | | 8.8±0.3(7.2-10.4) | 15.1±0.8(10.9-19.9) | |
| *Males* | 8.9±1.2(5.6-21.3) | 13.8±2.1(7.8-24.5) | | 9.5±0.7(6.4-14.6) | 13.6±1.1(9.1-19.6) | |

**Table 5.7** (*continued*)

| | This Study | | Reference[0] | This Study | | Reference |
|---|---|---|---|---|---|---|
| | **Ringed Seals** | **Ringed Seals** | **Ringed Seals** | **Grey Seals** | **Grey Seals** | **Grey Seals** |
| | ***Phoca hispida baltica*** | ***Phoca hispida*** | ***Phoca hispida*** | ***Halichoerus grypus*** | ***Halichoerus grypus*** | ***Halichoerus grypus*** |
| | **Baltic Sea** | **Svalbard, Arctic** | | **Baltic Sea** | **Sable Island, Canada** | |
| **Total CHOL (mmol/L)** | | | | | | |
| *Females & Males* | 7.2±0.2(4.5-9.9) | 7.1±0.1(6.3-8.3) | 7.7 (4.9-12.1) | 6.3±0.2(5.2-7.8) | 6.1±0.2(4.7-8.5) | 6.2 (4.7-9.1)[0] |
| *Females* | 7.0±0.2(6.2-7.6) | 7.2±0.2(6.3-8.3) | | 6.4±0.3(5.2-7.6) | 6.3±0.2(5.6-7.7) | |
| *Males* | 7.5±0.4(4.6-9.9) | 6.9±0.2(6.5-7.7) | | 6.2±0.3(5.3-7.8) | 5.9±0.4(4.7-8.5) | |
| **HDLC (mmol/L)** | | | | | | |
| *Females & Males* | 3.5±0.2(1.9-5.5)^^ | 4.9±0.1(3.8-5.7) | 5.8 (3.5-8.4) | 3.9±0.2(2.8-5.4)^^ | 4.8±0.1(4.1-6.2) | 4.9 (3.9-6.0)[0] |
| *Females* | 3.7±0.3(2.5-5.5) | 4.8±0.2(3.8-5.6) | | 4.2±0.3(3.1-5.4) | 4.9±0.2(4.1-6.2) | |
| *Males* | 3.3±0.2(1.9-4.6) | 5.1±0.2(4.3-5.7) | | 3.6±0.2(2.8-5) | 4.6±0.2(4.2-5.7) | |
| **LDL (mmol/L)** | | | | | | |
| *Females & Males* | 3.1±0.2(1.1-5.4)^^ | 1.7±0.2(0.02-4.2) | 1.3 (0.3-2.5) | 1.6±0.2(0.5-4)^ | 0.9±0.1(0.1-2) | 0.9 (0.1-2.6)[0] |
| *Females* | 2.6±0.3(1.2-3.8) | 2.0±0.3(0.9-4.2) | | 1.3±0.2(0.5-2.2) | 1.0±0.2(0.1-2) | |
| *Males* | 3.5±0.3(1.1-5.4) | 1.3±0.2(0.02-2) | | 1.9±0.4(0.5-4) | 0.8±0.2(0.1-1.8) | |
| **VLDL (mmol/L)** | | | | | | |
| *Females & Males* | 0.7±0.1(0.3-1.2)^ | 0.5±0.04(0.2-0.8) | 0.6 (E) | 0.8±0.05(0.5-1.3)^^ | 0.5±0.05(0.3-0.9) | 0.5 [0](E) |
| *Females* | 0.7±0.1(0.4-1) | 0.5±0.1(0.3-0.8) | | 0.9±0.1(0.7-0.8) | 0.4±0.1(0.3-0.8) | |
| *Males* | 0.7±0.1(0.3-1.2) | 0.5±0.1(0.2-0.8) | | 0.7±0.1(0.5-1.2) | 0.5±0.1(0.3-0.9) | |
| **Triglycerides (mmol/L)** | | | | | | |
| *Females & Males* | 1.4±0.1(0.8-2.6)^ | 1.1±0.1(0.5-1.7) | 1.4 (0.6-3.7) | 1.8±0.1(1.2-2.8)^^ | 1.0±0.1(0.6-2.1) | 1.1 (0.3-2.9)[0] |
| *Females* | 1.5±0.2(0.8-2.2) | 1.0±0.1(0.6-1.7) | | 1.9±0.1(1.5-2.8) | 1.0±0.1(0.6-1.7) | |
| *Males* | 1.4±0.2(0.8-2.6) | 1.1±0.1(0.5-1.7) | | 1.6±0.1(0.5-1.2) | 1.1±0.2(0.6-2.1) | |
| **CHOL/HDLC** | | | | | | |
| *Females & Males* | 2.2±0.1(1.3-4.3)^^ | 1.5±0.1(1.1-2.2) | 1.3 (E) | 1.7±0.1(1.3-2.7)^^ | 1.3±0.03(1.1-1.5) | 1.3 [0](E) |
| *Females* | 2.0±0.2(1.3-2.9) | 1.5±0.1(1.3-2.2) | | 1.6±0.1(1.4-2) | 1.3±0.04(1.1-1.5) | |
| *Males* | 2.3±0.2(1.6-4.3) | 1.4±0.05(1.1-1.5) | | 1.8±0.2(1.3-2.7) | 1.3±0.04(1.2-1.5) | |
| **Glucose (mmol/L)** | | | | | | |
| *Females & Males* | 6.5±0.2(5.2-9.3)^ | 5.8±0.3(3.3-7.5) | 5.7 (2.4-8.9) | 5.9±0.2(4.5-8.2)^^ | 4.5±0.2(2.2-6.3) | 3.9-6.1[a0] |
| *Females* | 6.2±0.5(3.3-10.2) | 5.6±0.4(3.3-7.5) | | 5.9±0.4(4.5-8.2) | 4.6±0.3(3.2-6.3) | |
| *Males* | 6.0±0.3(2.1-8.8) | 5.9±0.5(3.6-7.5) | | 6.0±0.3(4.5-7.5) | 4.4±0.4(2.2-6.2) | |

**Table 5.7** (*continued*)

| | This Study | | Reference[0] | This Study | | Reference |
|---|---|---|---|---|---|---|
| | **Ringed Seals** | **Ringed Seals** | **Ringed Seals** | **Grey Seals** | **Grey Seals** | **Grey Seals** |
| | ***Phoca hispida baltica*** | ***Phoca hispida*** | ***Phoca hispida*** | ***Halichoerus grypus*** | ***Halichoerus grypus*** | ***Halichoerus grypus*** |
| | **Baltic Sea** | **Svalbard, Arctic** | | **Baltic Sea** | **Sable Island, Canada** | |
| **Enzymes** | | | | | | |
| **ALT (U/L)** | | | | | | |
| *Females & Males* | | | 72 (24-236) | | | 34 (10-69)[a0] |
| *Females* | 36.9±1.7(28-46)*^^ | 46.2±1.7(38-53)** | | 26.3±0.7(24-30)* | 31.9±2.2(17-39) | |
| *Males* | 44.1±2.4(32-58)^^ | 57.3±1.9(49-64) | | 32.9±2.8(21-43) | 32.3±2.8(25-49) | |
| **AST (U/L)** | | | | | | |
| *Females & Males* | | | 83 (35-174) | | | |
| *Females* | 45.4±3.6(28-65)^ | 59.4±4.6(43-97)* | | 52.3±4.0(36-72)^ | 48.3±1.8(37-56) | 49 (24-79)[a0] |
| *Males* | 59.8±5.9(37-97)^ | 76.5±3.6(64-90) | | 56.6±3.8(40-75) | 46.8±3.0(34-58) | |
| **AST/ALT** | | | | | | |
| *Females & Males* | 1.3±0.1(0.8-2.1) | 1.3±0.1(0.9-1.9) | 1.2 (E) | 1.9±0.1(1.2-3.3)^ | 1.6±0.1(0.9-2.6) | 1.4 [0](E) |
| *Females* | 1.3±0.1(0.8-2.1) | 1.3±0.1(0.9-1.9) | | 2.0±0.1(1.4-2.3) | 1.6±0.2(0.9-2.6) | |
| *Males* | 1.3±0.1(0.9-1.9) | 1.3±0.1(1.1-1.6) | | 1.8±0.2(1.2-3.3) | 1.5±0.1(1.1-2.2) | |
| **γGT (U/L)** | | | | | | |
| *Females & Males* | 18.7±0.8(13-26) | 19.0±0.6(15-23) | 17 (6-54) | 11.3±0.3(9-13)^ | 12.7±0.4(10-16) | 11 (7-26)[0] |
| *Females* | 17.4±1.0(13-22) | 18.7±0.9(15-23) | | 11.5±0.4(9-13) | 12.4±0.5(10-15) | |
| *Males* | 19.8±1.1(15-26) | 19.4±0.8(15-23) | | 11.1±0.5(9-13) | 13.1±0.6(10-16) | |
| **ALKP (U/L)** | | | | | | |
| *Females & Males* | | | 54 (0-148) | | | 89 (17-634)[0] |
| *Females* | 86.0±3.5(69-103)**^ | 41.7±2.9(30-55)** | | 94.1±6.8(68-129)*^^ | 75.4±2.4(63-86)* | |
| *Males* | 69.0±2.9(54-83)^ | 27.9±2.4(18-36) | | 78.1±3.2(62-96) | 65.2±3.5(46-78) | |
| **CK (U/L)** | | | | | | |
| *Females & Males* | 229.7±12.1(144-385)^ | 286.3±18.5(215-573) | 762 (104-3502) | 677.0±26.8(513-1043)^^ | 551.5±25.7(320-762) | 996 (849-1254)[0] |
| *Females* | 230.4±24.7(144-385) | 287.7±33.3(215-573) | | 703.2±49.7(513-1043) | 520.3±37.3(320-729) | |
| *Males* | 229.1±12.9(171-339) | 284.6±9.4(253-336) | | 650.7±20.4(552-750) | 586.2±33.4(439-762) | |

CHOL, Cholesterol; HDLC, High Density Lipid Cholesterol; VLDL, Very Low Density Lipoprotein; ALKP, Alkaline Phosphatase; ALT, Alanine Aminotransferase; AST, Aspartate Aminotransferase; γGT, Gamma Glutynil Transferase; E, estimated.
Results are presented as means ±standard errors (ranges). The significance of sex variation is presented as * P<0.05 or ** P<0.005, the significance of geographical variation within each species is shown as ^ P<0.05 or ^^ P<0.005 and the significant species differences within the Baltic Sea or the two reference areas is shown as + P<0.05 or ++ P<0.005.
References: [a], Dierauf and Gulland, 2001; [0], Nyman *et al.*, 2003.

### 5.3.2 Inter-species differences

The blood chemistry values for all the adult seal populations are presented in Table 5.8. Where biochemical parameters showed no sex-dependent differences, female and male samples were pooled.

*Harbour Seals*

Of the analysed parameters, mean total protein and albumin levels were significantly lower in seals from Abertay Sands ($p<0.005$), whereas only male Harbour seals from Abertay Sands showed significantly lower globulin concentration compared to seals from Moray Firth ($p<0.005$). Of the analysed enzymes seals from Abertay Sands showed significantly reduced ALT, AST and ALKP levels ($p<0.005$) (Fig.5.4). In male seals from Moray Firth, mean cholesterol, triglycerides, VLDL and HDLC values (Fig.5.5) were significantly higher from male seals from Abertay Sands ($p<0.005$). In seals from Moray Firth, LDL levels were significantly reduced ($p<0.05$) whereas glucose levels were significantly higher compared with seals from Abertay Sands ($p<0.005$).

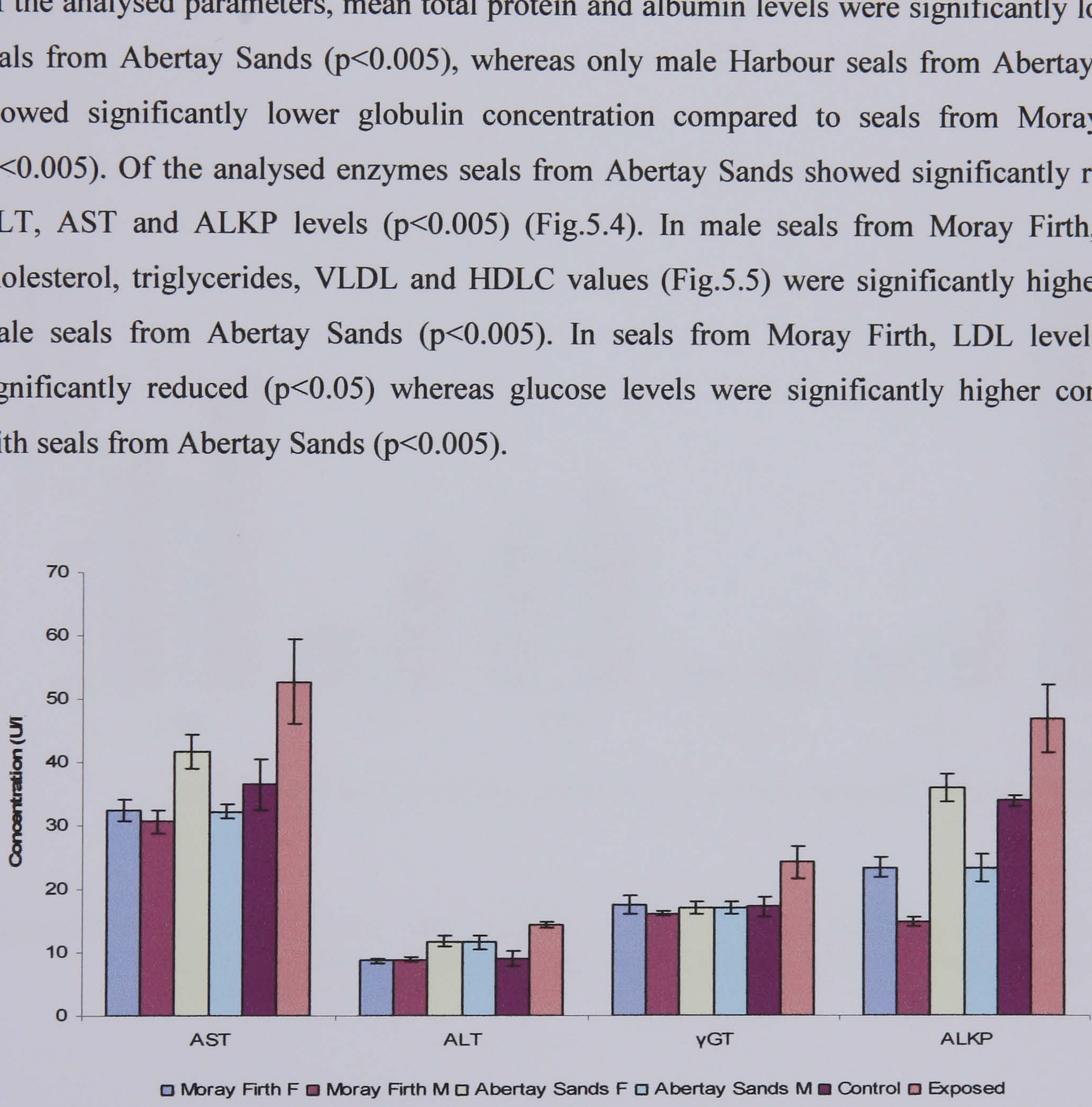



**Figure 5.4:** Mean ±SE of AST, ALT, γGT and ALKP concentrations in female and male Harbour seals from Moray Firth, Abertay Sands and the experimental group
F, Females; M, Males

The experimental group was comprised only by female Harbour seals therefore only females from Moray Firth were compared. Of the analysed parameters Harbour seals from Moray Firth showed statistically significant reduced ALKP (Fig.5.4), cholesterol, triglycerides, VLDL and HDLC (Fig.5.5) levels when compared to the control Harbour seals ($p<0.005$).

When comparing female Harbour seals from Abertay Sands with the control female experimental group, it was seen that females from Abertay Sands had significantly reduced globulin ($p<0.05$), cholesterol, triglycerides, VLDL, HDLC and glucose levels ($p<0.005$; Fig.5.5).

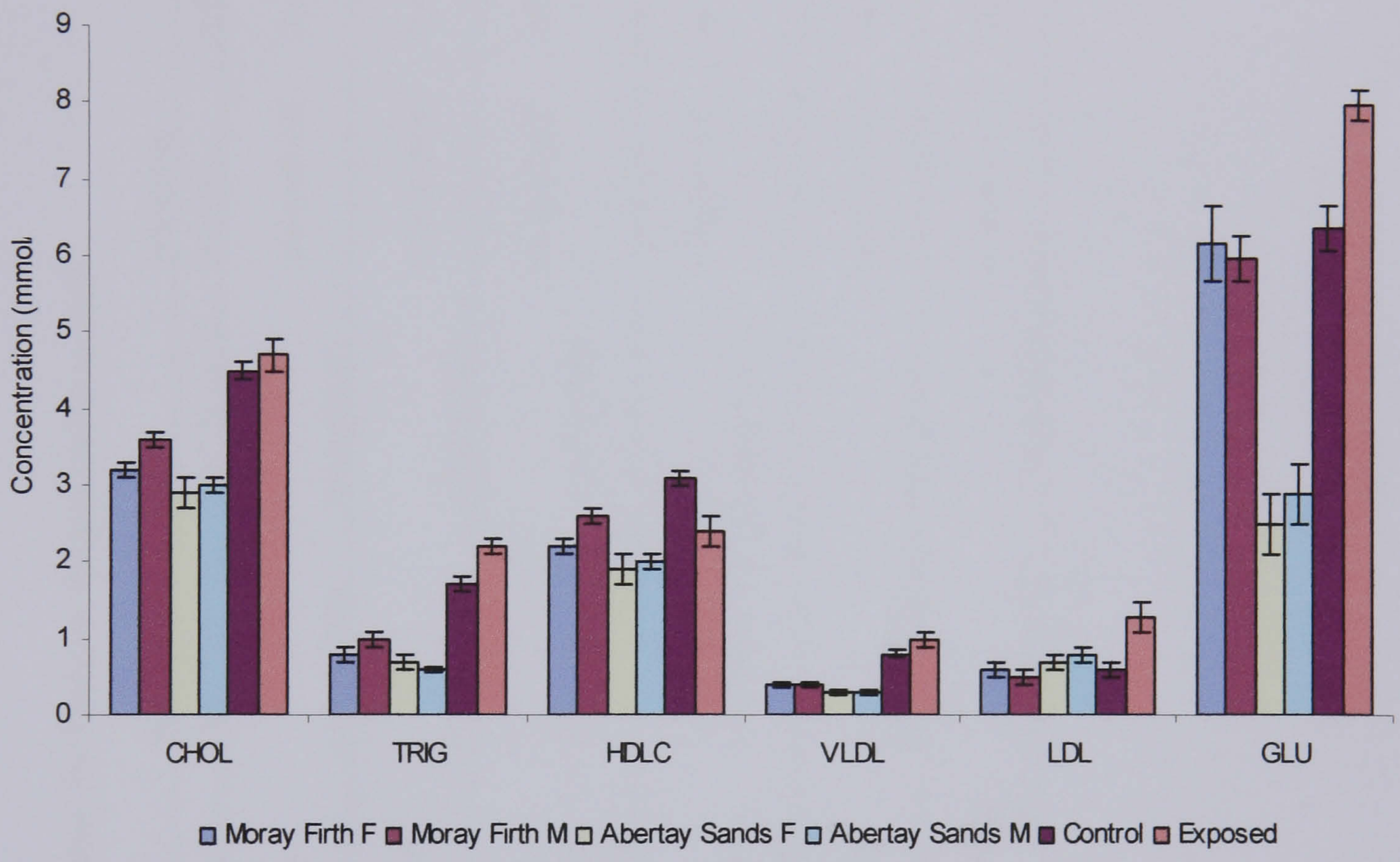



**Figure 5.5:** Mean ±SE CHOL, TRIG, HDLC, VLDL, LDL and glucose concentrations in female and male Harbour seals from Moray Firth, Abertay Sands and the experimental group
F, Females; M, Males

**Table 5.8:** Summary of blood chemistry values for Harbour, Ringed and Grey seals

| Species | Harbour Seals | Harbour Seals | Harbour Seals | | Ringed Seals | | Grey Seals | |
|---|---|---|---|---|---|---|---|---|
| **Clinical Chem. Param.** | **Abertay Sands** | **Moray Firth** | **Exper. Group (E)** | **Exper. Group (C)** | **Baltic Sea** | **Svalbard** | **Baltic Sea** | **Sable Island** |
| **Total Protein (g/L)** | | | | | | | | |
| *Females & Males* | 73.6±1.2(49-92) | 82.6±0.8(70-93) | | | 69.4±2.3(57-102) | 78.1±1.4(61-87) | 72.1±2.0(45-82) | 76.6±2.1(50-97) |
| *Females* | | | 81.8±2.1(72-99) | 84.3±1.9(75-98) | | | | |
| **Albumin (g/L)** | | | | | | | | |
| *Females & Males* | 28.1±0.2(24-31) | 31.9±0.3(25-35) | | | 34.1±0.8(30-46) | 29.6±1.1(22-38) | 33.4±0.5(29-36) | 27.5±0.7(20-33) |
| *Females* | | | 34.6±1.0(31-43) | 31.6±0.9(28-40) | | | | |
| **Globulin (g/L)** | | | | | | | | |
| *Females & Males* | 45.4±1.2(21-65) | | | | 35.3±2.2(19-68) | 48.5±1.7(34-59) | 38.7±2.3(9-52) | 49.1±2.3(24-72) |
| *Females* | | 49.2±0.9(41-55) | 47.2±2.2(37-63) | 52.7±2.3(37-69) | | | | |
| *Males* | | 52.3±1.3(40-64) | | | | | | |
| **A/G** | | | | | | | | |
| *Females & Males* | 0.6±0.02(0.4-1.3) | | | | 1.0±0.1(0.5-2.4) | 0.6±0.04(0.4-1) | 1.0±0.2(0.6-4) | 0.6±0.02(0.3-1.1) |
| *Females* | | 0.7±0.01(0.5-0.7) | 0.8±0.1(0.6-1.1) | 0.6±0.05(0.4-1.1) | | | | |
| *Males* | | 0.6±0.02(0.4-0.9) | | | | | | |
| **Uric Acid (µmol/L)** | | | | | | | | |
| *Females & Males* | 64.2±2.7(22-99) | 64.2±2.4(18-93) | | | 92.9±1.6(78-112) | 106.8±3.4(86-139) | 74.6±2.7(56-104) | 61.5±1.5(49-72) |
| *Females* | | | 68.8±2.2(54-78) | 63.4±1.3(57-71) | | | | |
| **Urea (mmol/L)** | | | | | | | | |
| *Females & Males* | 17.0±0.5(12.2-28.7) | 16.8±0.2(13.3-20.8) | | | 8.6±0.8(5.6-21.3) | 13.8±1.1(7.8-24.5) | 9.1±0.4(6.4-14.6) | 14.4±0.7(9.1-19.9) |
| *Females* | | | 16.5±0.3(15.1-18.3) | 17.0±0.4(15.4-20) | | | | |

**Table 5.8** (*continued*)

| Species | Harbour Seals | Harbour Seals | Harbour Seals | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Clinical Chem. Param.** | **Abertay Sands** | **Moray Firth** | **Exper. Group (E)** | **Exper. Group (C)** | **Ringed Seals (B)** | **Ringed Seals (S)** | **Grey Seals (B)** | **Grey Seals (SI)** |
| **Cholesterol** | | | | | | | | |
| **Total CHOL (mmol/L)** | | | | | | | | |
| *Females & Males* | 3.0±0.1(1.3-4.6) | | | | 7.2±0.2(4.5-9.9) | 7.1±0.1(6.3-8.3) | 6.3±0.2(5.2-7.8) | 6.1±0.2(4.7-8.5) |
| *Females* | | 3.2±0.1(2.3-3.9) | 4.7±0.2(4.1-5.8) | 4.5±0.1(3.7-5.8) | | | | |
| *Males* | | 3.6±0.1(2.7-4.2) | | | | | | |
| **HDLC (mmol/L)** | | | | | | | | |
| *Females & Males* | 2.0±0.1(0.5-3.5) | | | | 3.5±0.2(1.9-5.5) | 4.9±0.1(3.8-5.7) | 3.9±0.2(2.8-5.4) | 4.8±0.1(4.1-6.2) |
| *Females* | | 2.2±0.1(1.3-2.8) | 2.4±0.2(1.3-3.3) | 3.1±0.1(2.7-4.1) | | | | |
| *Males* | | 2.6±0.1(1.8-3.4) | | | | | | |
| **LDL (mmol/L)** | | | | | | | | |
| *Females & Males* | 0.7±0.05(0.1-1.6) | 0.5±0.1(0.1-1.9) | | | 3.1±0.2(1.1-5.4) | 1.7±0.2(0.02-4.2) | 1.6±0.2(0.5-4) | 0.9±0.1(0.1-2) |
| *Females* | | | 1.3±0.2(0.4-2.4) | 0.6±0.1(0.02-0.9) | | | | |
| **VLDL (mmol/L)** | | | | | | | | |
| *Females & Males* | 0.3±0.01(0.1-0.6) | | | | 0.7±0.1(0.3-1.2) | 0.5±0.04(0.2-0.8) | 0.8±0.05(0.5-1.3) | 0.5±0.05(0.3-0.9) |
| *Females* | | 0.4±0.03(0.2-0.6) | 1.0±0.1(0.8-1.3) | 0.8±0.05(0.5-1.1) | | | | |
| *Males* | | 0.4±0.03(0.2-0.8) | | | | | | |
| **Triglycerides (mmol/L)** | | | | | | | | |
| *Females & Males* | 0.6±0.03(0.3-1.4) | | | | 1.4±0.1(0.8-2.6) | 1.1±0.1(0.5-1.7) | 1.8±0.1(1.2-2.8) | 1.0±0.1(0.6-2.1) |
| *Females* | | 0.8±0.1(0.4-1.3) | 2.2±0.1(1.8-2.8) | 1.7±0.1(1.1-2.4) | | | | |
| *Males* | | 1.0±0.1(0.3-1.8) | | | | | | |
| **CHOL/HDLC** | | | | | | | | |
| *Females & Males* | 1.6±0.05(1.1-3.1) | 1.4±0.05(1.1-2.6) | | | 2.2±0.1(1.3-4.3) | 1.5±0.1(1.1-2.2) | 1.7±0.1(1.3-2.7) | 1.3±0.03(1.1-1.5) |
| *Females* | | | 2.1±0.2(1.4-3.1) | 1.5±0.03(1.2-1.7) | | | | |
| **Glucose (mmol/L)** | | | | | | | | |
| *Females & Males* | 2.7±0.3(1.1-10.4) | 6.0±0.3(2.1-10.2) | | | 6.5±0.2(5.2-9.3) | 5.8±0.3(3.3-7.5) | 5.9±0.2(4.5-8.2) | 4.5±0.2(2.2-6.3) |
| *Females* | | | 8.0±0.2(6.8-8.8) | 6.4±0.3(5.2-7.7) | | | | |

**Table 5.8:** (*continued*)

| Species | Harbour Seals | Harbour Seals | Harbour Seals | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Clinical Chem. Param.** | **Abertay Sands** | **Moray Firth** | **Exper. Group (E)** | **Exper. Group (C)** | **Ringed Seals (B)** | **Ringed Seals (S)** | **Grey Seals (B)** | **Grey Seals (SI)** |
| **Enzymes** | | | | | | | | |
| **ALT (U/L)** | | | | | | | | |
| *Females & Males* | 11.7±0.8(3-25) | 8.9±0.3(5-12) | | | | | | |
| *Females* | | | 14.4±0.5(12-17) | 9.1±1.2(1-18) | 36.9±1.7(28-46) | 46.2±1.7(38-53) | 26.3±0.7(24-30) | 31.9±2.2(17-39) |
| *Males* | | | | | 44.1±2.4(32-58) | 57.3±1.9(49-64) | 32.9±2.8(21-43) | 32.3±2.8(25-49) |
| **AST (U/L)** | | | | | | | | |
| *Females & Males* | | 31.5±1.1(20-57) | | | | | | |
| *Females* | 41.7±2.6(23-67) | | 52.8±6.6(21-97) | 36.5±4.0(10-53) | 45.4±3.6(28-65) | 59.4±4.6(43-97) | 52.3±4.0(36-72) | 48.3±1.8(37-56) |
| *Males* | 32.3±1.2(18-46) | | | | 59.8±5.9(37-97) | 76.5±3.6(64-90) | 56.6±3.8(40-75) | 46.8±3.0(34-58) |
| **AST/ALT** | | | | | | | | |
| *Females & Males* | 4.2±0.4(1.2-13) | 3.8±0.2(1.8-8.1) | | | 1.3±0.1(0.8-2.1) | 1.3±0.1(0.9-1.9) | 1.9±0.1(1.2-3.3) | 1.6±0.1(0.9-2.6) |
| *Females* | | | 3.6±0.4(1.8-6.5) | 8.0±4.1(1-53) | | | | |
| **γGT (U/L)** | | | | | | | | |
| *Females & Males* | 17.0±0.7(5-37) | 17.3±0.7(4-33) | | | 18.7±0.8(13-26) | 19.0±0.6(15-23) | 11.3±0.3(9-13) | 12.7±0.4(10-16) |
| *Females* | | | 24.3±2.6(15-46) | 17.2±1.5(12-32) | | | | |
| **ALKP (U/L)** | | | | | | | | |
| *Females & Males* | | | | | | | | |
| *Females* | 36.0±2.2(16-54) | 23.5±1.6(11-36) | 47.0±5.4(34-91) | 34.1±0.9(30-42) | 86.0±3.5(69-103) | 41.7±2.9(30-55) | 94.1±6.8(68-129) | 75.4±2.4(63-86) |
| *Males* | 23.4±2.1(10-59) | 14.9±0.7(8-20) | | | 69.0±2.9(54-83) | 27.9±2.4(18-36) | 78.1±3.2(62-96) | 65.2±3.5(46-78) |
| **CK (U/L)** | | | | | | | | |
| *Females & Males* | 743.4±38.8(206-1508) | n.a. | n.a. | n.a. | 229.7±12.1(144-385) | 286.3±18.5(215-573) | 677.0±26.8(513-1043) | 551.5±25.7(320-762) |

CHOL, Cholesterol; HDLC, High Density Lipid Cholesterol; VLDL, Very Low Density Lipoprotein; ALKP, Alkaline Phosphatase; ALT, Alanine Aminotransferase; AST, Aspartate Aminotransferase; γGT, Gamma Glutynil Transferase; Exper., Experimental; E, Exposed; C, Control; B, Baltic Sea; S, Svalbard, Arctic; SI, Sable Island, Canada.

Results are presented as means ±standard errors (ranges).

A species comparison between the Baltic Grey seal population and the Harbour seal populations (Abertay Sands, Moray Firth and the experimental group) showed significantly higher mean γGT ($p<0.005$) and blood urea nitrogen ($p<0.005$) levels in Harbour than the Grey seals. Albumin (Fig.5.8) levels were significantly lower in Harbour seals from Moray Firth ($p<0.05$) and Abertay Sands ($p<0.005$) compared to Baltic Grey seals. ALKP, ALT (Fig.5.6&9), cholesterol and HDLC levels were significantly higher ($p<0.005$) in Baltic Grey seals than Harbour seals from all geographical areas. When compared to Baltic Grey seals AST levels were significantly reduced ($p<0.005$) in all Harbour seals. Compared to Baltic Grey seals, triglycerides, VLDL and LDL (Fig.5.7) levels were significantly reduced ($p<0.005$) in Harbour seals from Moray Firth and Abertay Sands and glucose levels ($p<0.005$) were significantly lower in Harbour seals from Abertay Sands. Glucose levels were higher ($p<0.005$) in the exposed Harbour seals compared to Baltic Grey seals. Exposed Harbour seals also showed increased triglycerides ($p<0.05$) and VLDL ($p<0.005$; Fig.5.7) levels compared with Baltic Grey seals.

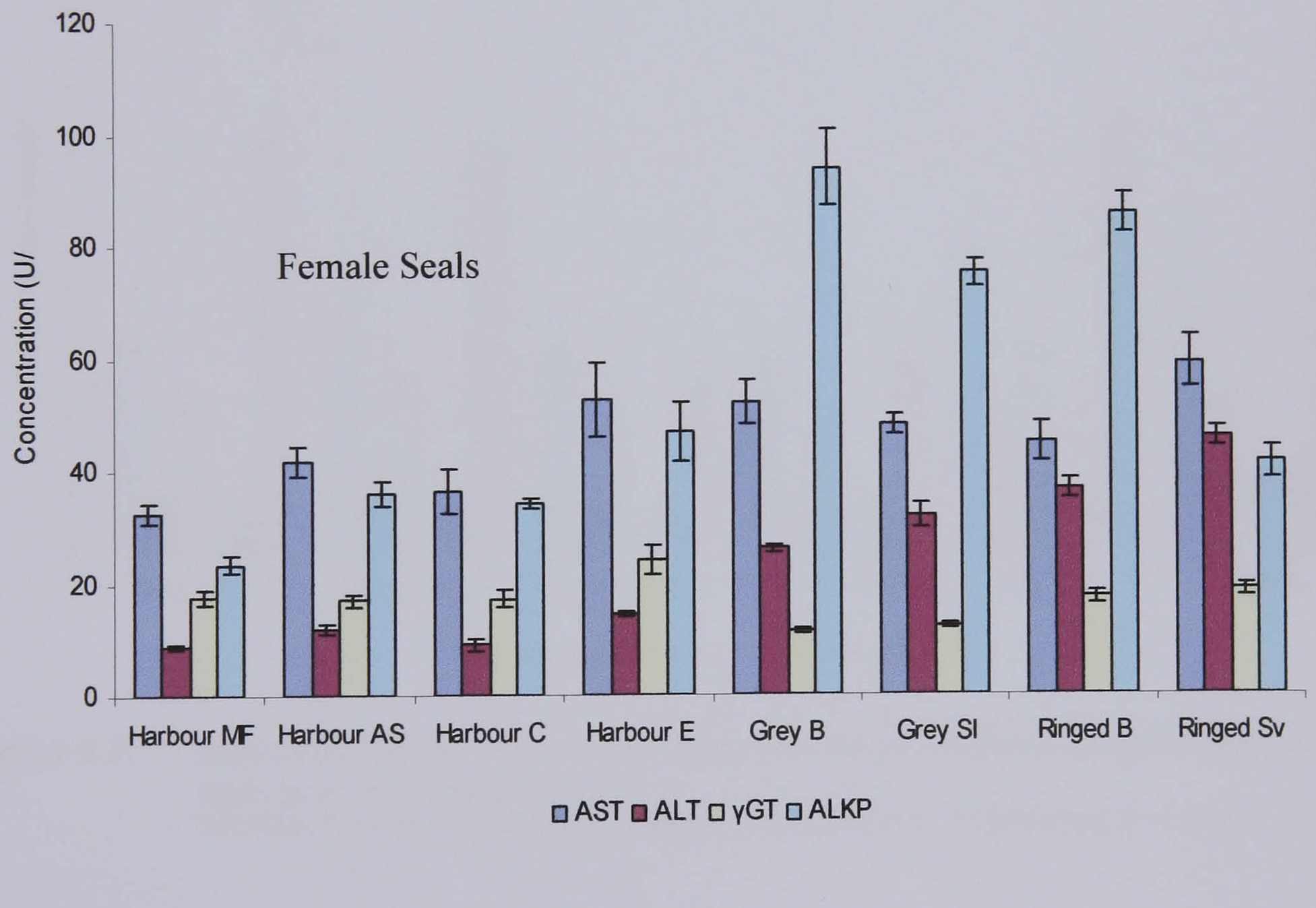



**Figure 5.6:** Mean ±SE AST, ALT, γGT and ALKP concentrations in female seals from all study populations
MF, Moray Firth; AS, Abertay Sands; C, Control; E, Exposed; B, Baltic; SI, Sable Island; Sv, Svalbard

Harbour seals from all geographical areas showed significantly reduced ALT, ALKP (Fig.5.6&9), cholesterol and HDLC levels ($p<0.005$; Fig.5.7) and increased γGT and glucose ($p<0.005$) concentrations compared to Grey seals from Sable Island. Except the control seals from the experimental group all Harbour seals showed significantly reduced AST levels compared to Sable Island Grey seals ($p<0.005$). Harbour seals from Moray Firth showed significantly increased total protein, albumin and urea levels ($p<0.005$) and decreased LDL levels ($p<0.05$) compared to Grey seals from Sable Island. Of the control Harbour seals, total protein ($p<0.005$), albumin ($p<0.005$; Fig.5.8), urea ($p<0.05$), triglycerides ($p<0.005$) and VLDL ($p<0.005$; Fig.5.7&10) were significantly higher compared to Sable Island Grey seals. Exposed Harbour seals showed increased mean uric acid levels ($p<0.05$) compared to Grey seals from Sable Island. Harbour seals from Abertay Sands showed significantly reduced triglycerides ($p<0.005$), VLDL ($p<0.005$; Fig.5.7&10) and urea levels ($p<0.05$) and higher CK levels ($p<0.05$) compared to Grey seals from Sable Island.

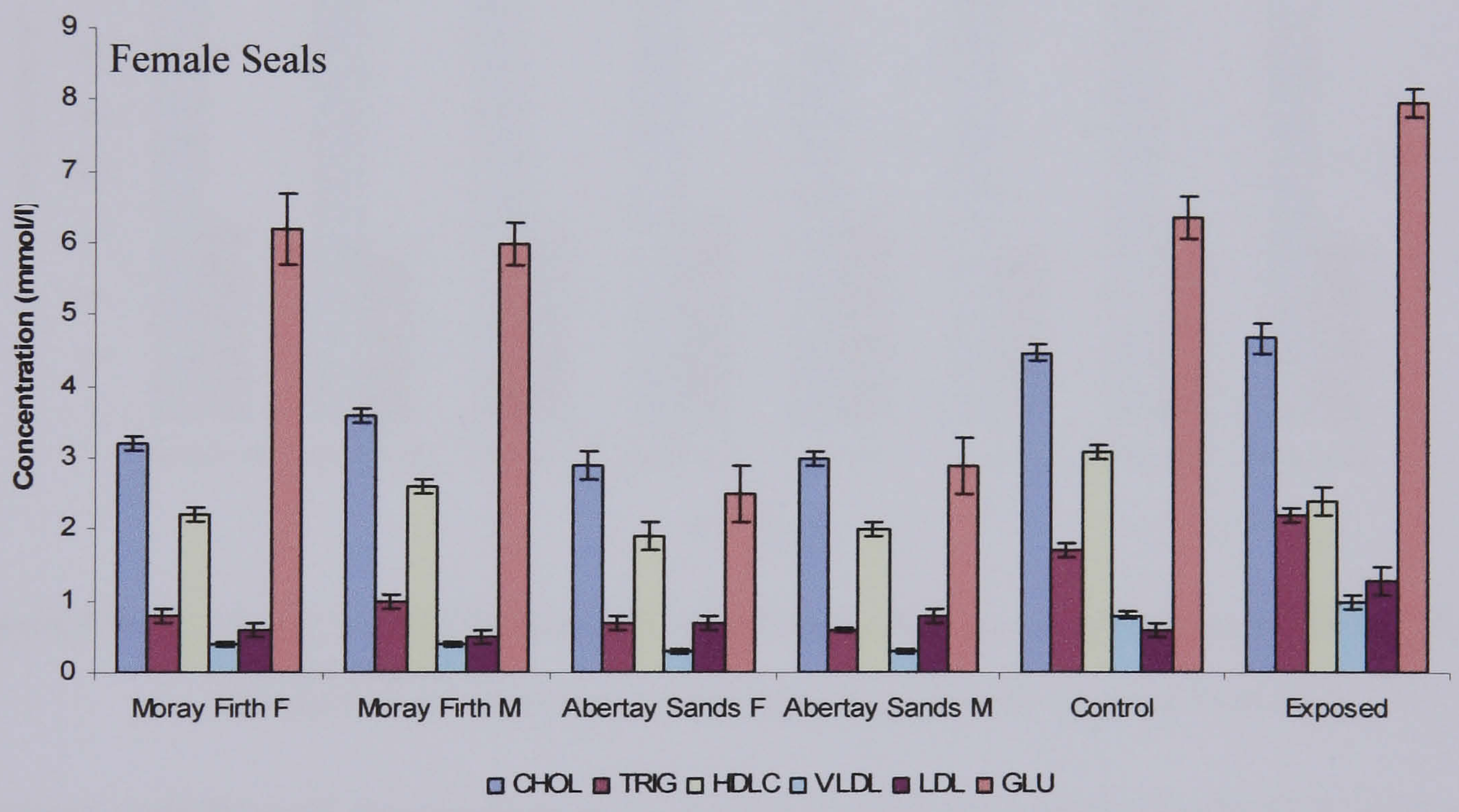



**Figure 5.7:** Mean ±SE CHOL, TRIG, HDLC, VLDL, LDL and Glucose concentrations in female seals from all study populations
MF, Moray Firth; AS, Abertay Sands; C, Control; E, Exposed; B, Baltic; SI, Sable Island; Sv, Svalbard

A species comparison between the Baltic Ringed seal population and the Harbour seal populations (Abertay Sands, Moray Firth and experimental group) showed significantly higher mean urea levels ($p<0.005$) and lower uric acid, cholesterol, LDL, ALT (Fig.5.6&9) and ALKP levels ($p<0.005$) in Harbour than Baltic Ringed seals. Mean levels of albumin (Fig.5.8),

AST (Fig.5.6&9), triglycerides, VLDL and HDLC were significantly lower ($p<0.005$) whereas total protein concentrations were significantly higher ($p<0.005$) in Harbour seals from Moray Firth than Baltic Ringed seals. Harbour seals from Abertay Sands showed significantly higher CK levels ($p<0.005$) lower triglyceride, VLDL, HDLC, AST (only males) and glucose levels compared to Baltic Ringed seals. In the experimental group the control Harbour seals showed significantly reduced albumin (Fig.5.8&11) and AST levels ($p<0.005$; Fig.5.6&9) whereas total protein was higher compared to Baltic Ringed seals. Mean levels of total protein, triglycerides, VLDL, glucose ($p<0.005$) and γGT ($p<0.05$; Fig.5.6&9) were also higher in the exposed Harbour seals compared to Baltic Ringed seals, whereas HDLC were significantly lower ($p<0.005$).

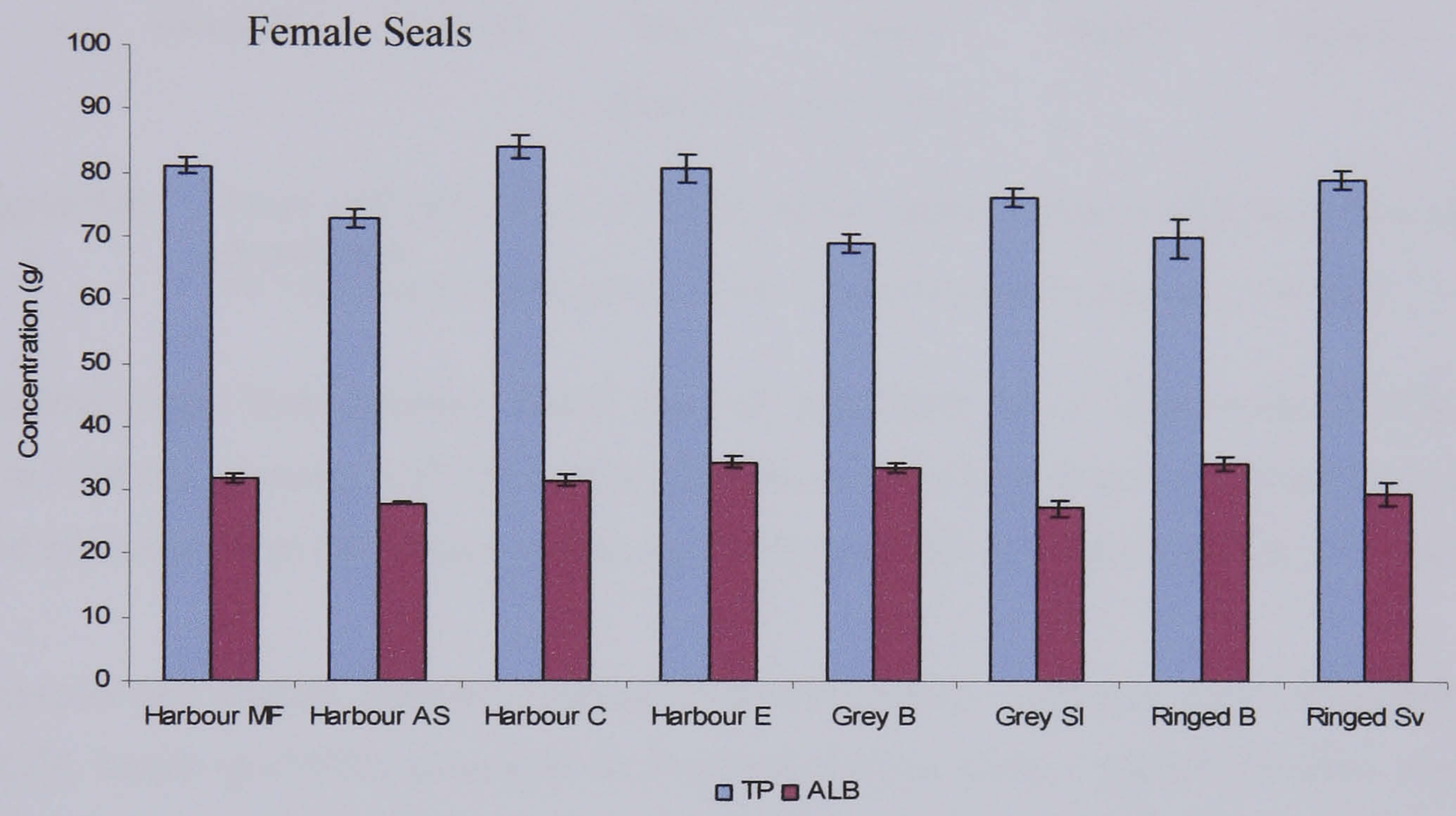



**Figure 5.8:** Mean ±SE Total Protein (TP) and Albumin (ALB) concentrations in female seals from all study populations
MF, Moray Firth; AS, Abertay Sands; C, Control; E, Exposed; B, Baltic; SI, Sable Island; Sv, Svalbard

Harbour seals from all geographical areas showed significantly reduced mean ALT, uric acid, cholesterol and HDLC levels ($p<0.005$) compared to Ringed seals from Svalbard. Statistically significant reduced AST, ALKP (Fig.5.6&9), LDL ($p<0.005$), triglycerides and VLDL levels ($p<0.05$) and increased mean total protein and albumin ($p<0.05$) was seen in Harbour seals from Moray Firth compared to Ringed seals from Svalbard. Control Harbour seals showed significantly increased mean triglyceride, VLDL levels ($p<0.005$), total protein and urea ($p<0.05$) and decreased AST ($p<0.005$; Fig.5.6&8), ALKP ($p<0.05$) and LDL ($p<0.005$) levels compared to Svalbard Ringed seals.

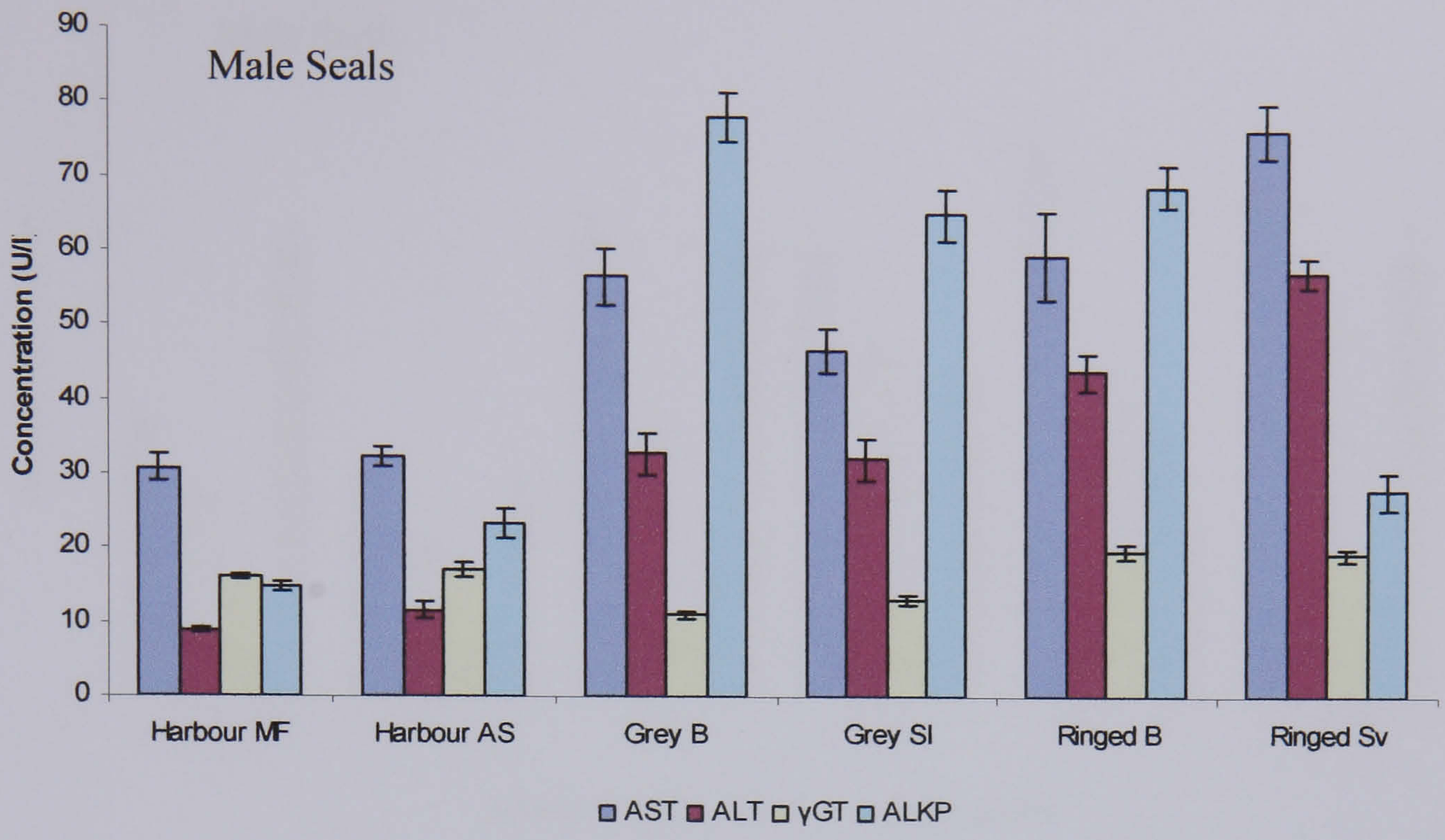



**Figure 5.9:** Mean ±SE AST, ALT, γGT and ALKP concentrations in male seals from all study populations

MF, Moray Firth; AS, Abertay Sands; C, Control; E, Exposed; B, Baltic; SI, Sable Island; Sv, Svalbard

Harbour seals from Abertay Sands showed significant lower triglyceride, VLDL, LDL (Fig.5.7&10), glucose, AST ($p<0.005$), albumin and total protein ($p<0.05$; Fig.5.8&11) levels and increased mean CK concentrations ($p<0.05$) compared to Ringed seals from Svalbard.

Exposed and control Harbour seals showed significantly increased mean triglyceride and VLDL levels ($p<0.005$) compared to Svalbard Ringed seals. Exposed, Harbour seals also showed increased albumin, γGT (Fig.5.6&9) and glucose levels ($p<0.005$) whereas control Harbour seals showed increased total protein and urea ($p<0.05$), and decreased AST ($p<0.005$; Fig.5.6&9), ALKP ($p<0.05$) and LDL ($p<0.005$) levels compared to Svalbard Ringed seals.

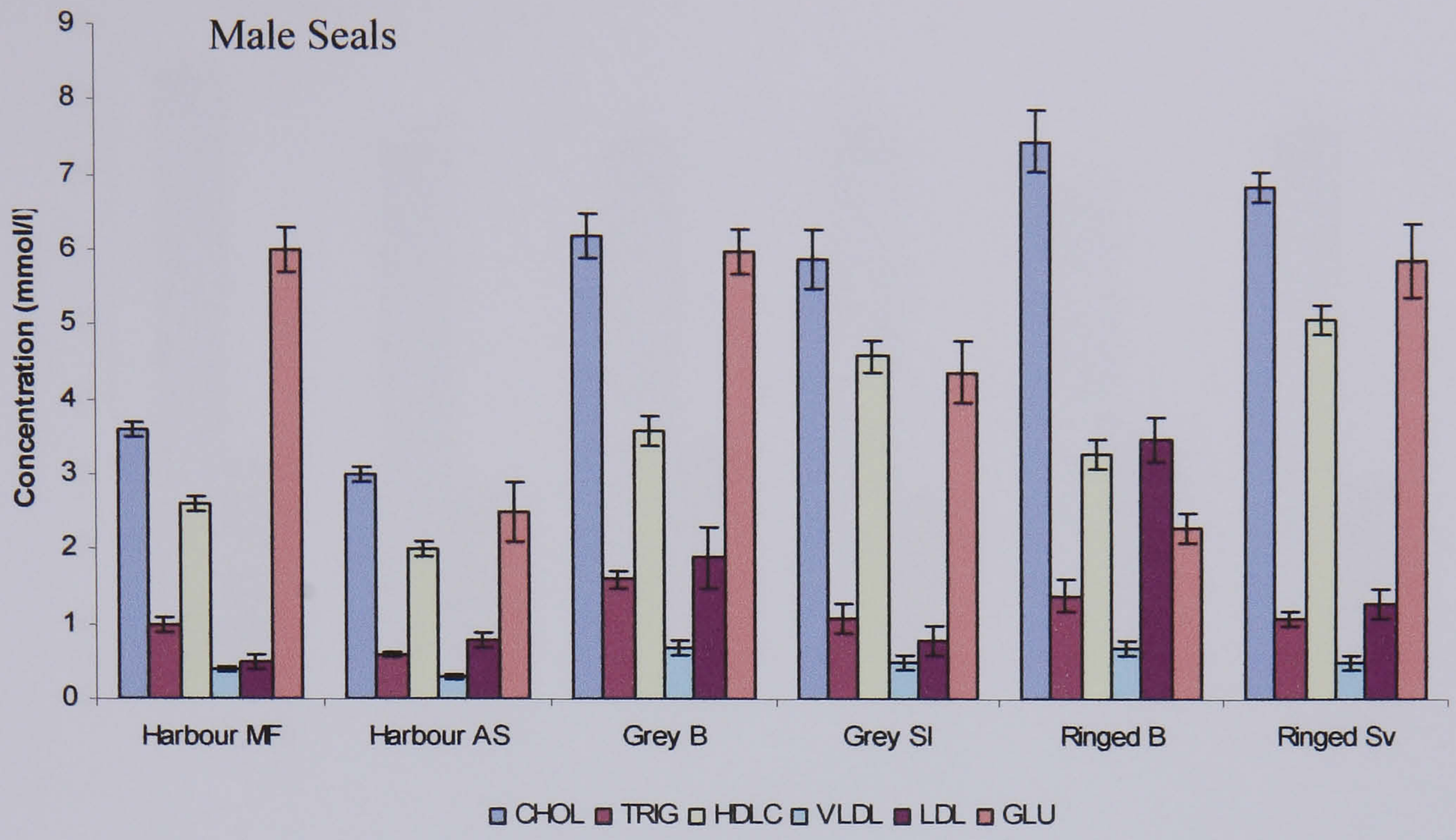



**Figure 5.10:** Mean ±SE CHOL, TRIG, HDLC, VLDL, LDL and Glucose concentrations in male seals from all study populations
MF, Moray Firth; AS, Abertay Sands; C, Control; E, Exposed; B, Baltic; SI, Sable Island; Sv, Svalbard

*Grey vs. Ringed Seals*

A species comparison between the Baltic seals showed significantly higher levels of triglyceride ($p<0.05$), VLDL ($p<0.05$) and CK ($p<0.005$) and reduced ALT, γGT, uric acid, cholesterol and LDL levels ($p<0.005$) in Grey than in Ringed seals. Svalbard Ringed seals showed significantly higher ALT, AST, γGT, uric acid, cholesterol, HDLC, LDL and glucose levels ($p<0.005$) and reduced mean ALKP and CK concentrations ($p<0.005$) compared to Grey seals from Sable Island. Grey seals from Baltic also showed significantly increased albumin, ALKP, triglyceride, VLDL and CK levels ($p<0.005$) compared to Ringed seals from Svalbard. Mean total protein ($p<0.05$), ALT, AST, γGT, ALKP (Fig.5.6&9), blood urea nitrogen, uric acid, cholesterol and HDLC levels were significantly lower ($p<0.005$) in Baltic Grey seals compared to Ringed seals from Svalbard.

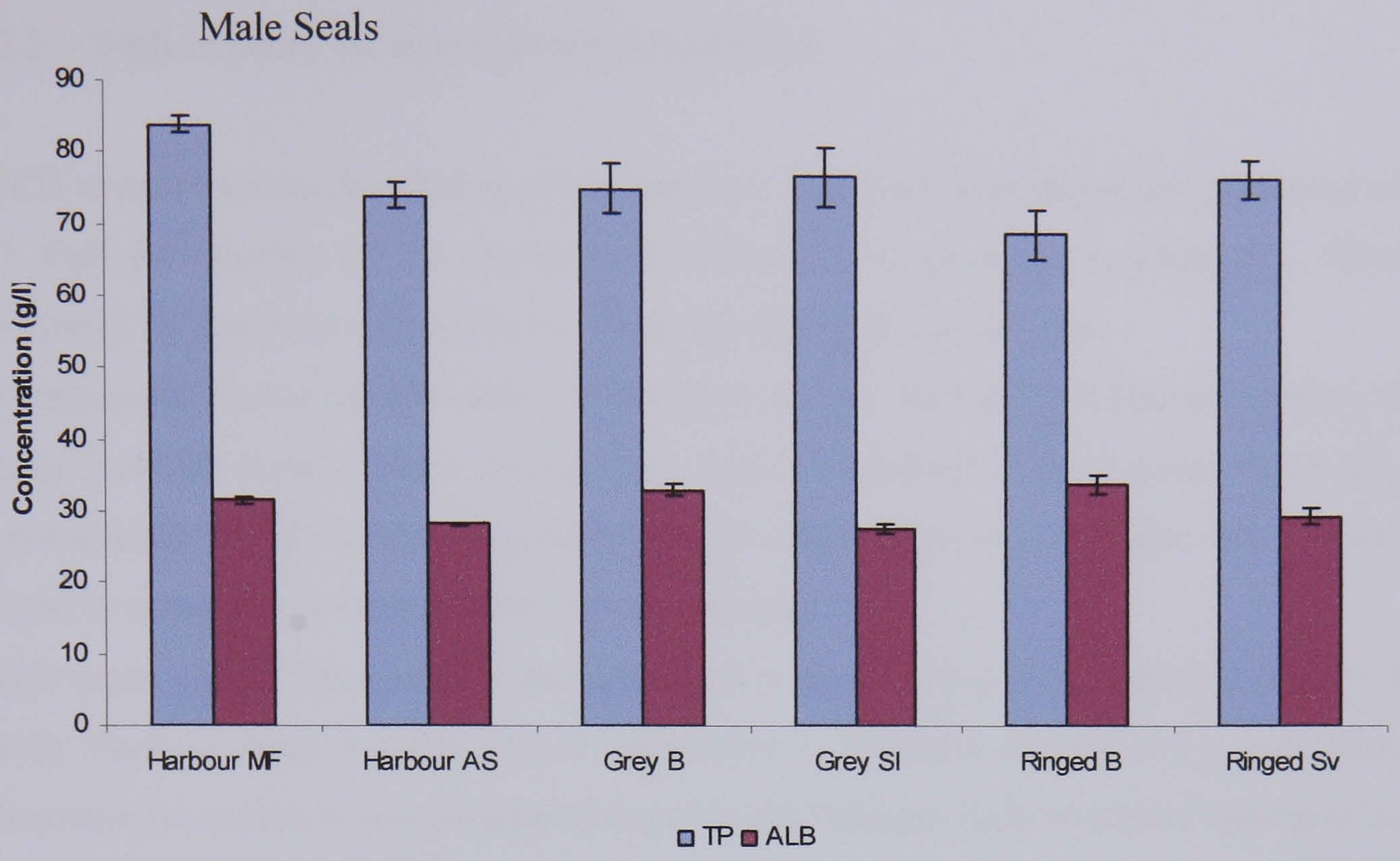



**Figure 5.11:** Mean ±SE Total Protein (TP) and Albumin (ALB) concentrations in male seals from all study populations

MF, Moray Firth; AS, Abertay Sands; C, Control; E, Exposed; B, Baltic; SI, Sable Island; Sv, Svalbard

Baltic Ringed seals showed reduced total protein ($p<0.05$), urea, HDLC and CK ($p<0.005$) concentrations and significantly increased ALKP ($p<0.05$), albumin, ALT, γGT, uric acid, cholesterol, triglyceride, VLDL, LDL and glucose levels ($p<0.005$) compared to Grey seals from Sable Island.

### 5.3.3 High-exposed vs. low-exposed populations

ΣPCB concentrations detected in serum samples from each population are presented in Table 5.2. Full justification of the exposure classifications is provided in Chapter 2. These data confirmed the exposure classification for the present study as follows:

**Female seals:** Baltic Ringed seals > Baltic Grey seals > Moray Firth Harbour seals > Abertay Sands Harbour seals > Sable Island Grey seals > Svalbard Ringed seals. From the ΣPCB concentration results it was concluded that Svalbard Ringed and Sable Island Grey seals would be treated as control populations for females.

**Male seals:** Baltic Grey seals > Baltic Ringed seals > Moray Firth Harbour seals > Abertay Sands Harbour seals > Sable Island Grey seals > Svalbard Ringed seals. From the ΣPCB concentration results it was concluded that Svalbard Ringed seals would be treated as a control population for males.

*Validation of blood chemistry parameters as biomarkers – dose response*

The control Harbour seals (Fig.5.12) were found to have significantly higher HDLC ($p<0.005$) and significantly lower triglycerides ($p<0.05$), LDL ($p<0.005$) and VLDL ($p<0.05$). Of the analysed parameters the liver function enzymes (ALT, AST and γGT, Fig.5.13) were found to be significantly different, being higher in the exposed group ($p<0.005$, $p<0.05$ and $p<0.05$ respectively). Albumin ($p<0.05$), ALKP ($p<0.05$, Fig.5.13), uric acid ($p<0.05$) and glucose ($p<0.005$) were also seen to be higher in the exposed compared to the control group.

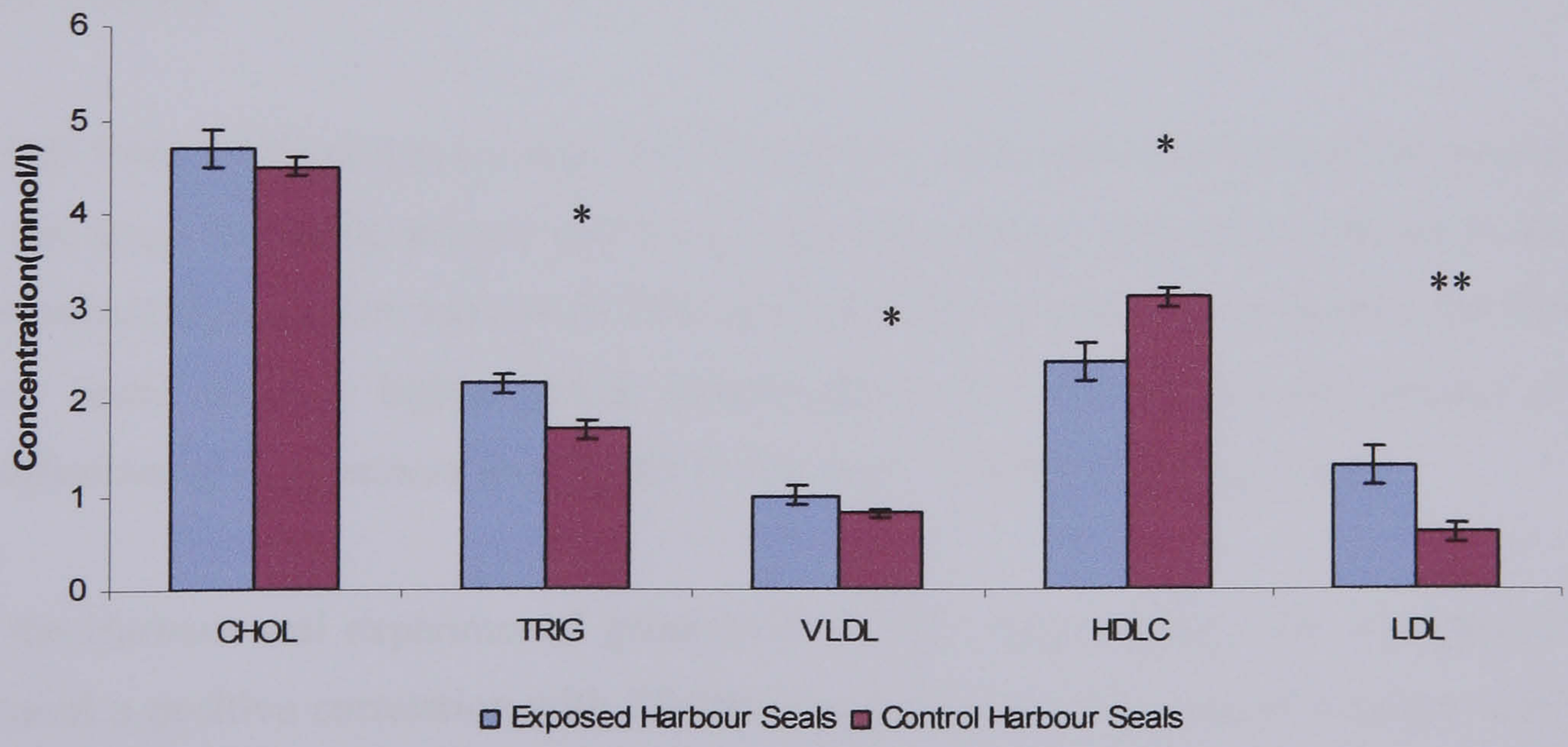



**Figure 5.12:** Mean ±SE CHOL, TRIG, VLDL, HDLC and LDL concentrations in Harbour seals (experimental group).

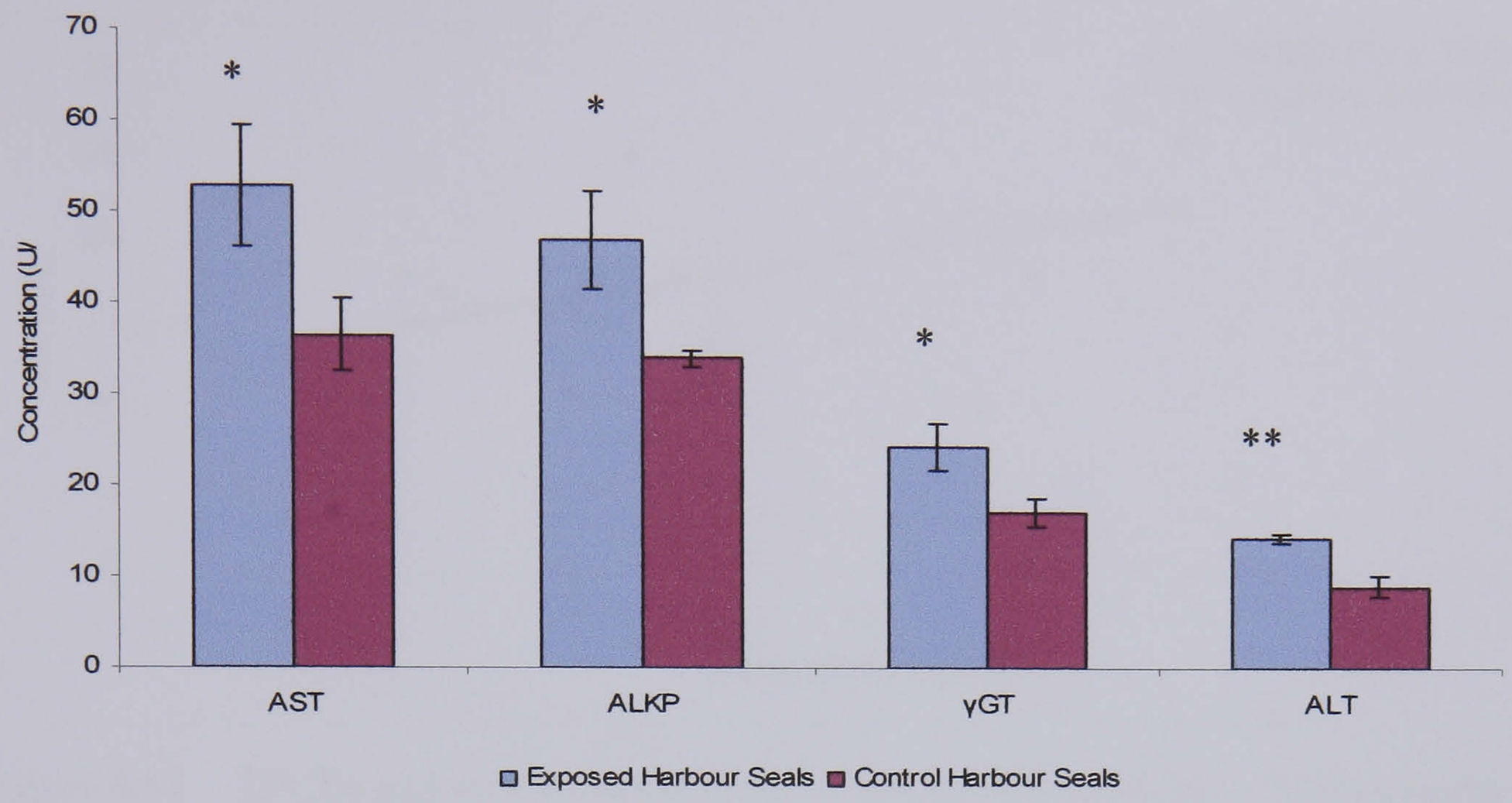



**Figure 5.13:** Mean ±SE AST, ALKP, γGT and ALT concentrations in Harbour seals (experimental group).
The significance of exposure variation is presented as * p<0.05 or ** p<0.005

The experimental Harbour seal group was used for the validation of sex steroids as biomarkers since these seals were a part of a control feeding study with fish taken from Wadden Sea and Northeast Atlantic. This group of Harbour seals were sampled at late pregnancy and the average ± s.d. of total PCB concentration in the exposed group (n=11) was 25642 ± 9699 ng/g lipid weight and in the control group (n=12) was 5336ng/g ± 1632 lipid weight (Reijnders, *pers. Comm.*).

Levels from Svalbard Ringed and Sable Island Grey seals were used as control concentrations in this study for Baltic Ringed and Grey seals respectively. Baseline levels for juveniles are not presented since only one group (Moray Firth, Harbour seals) was available and these seals were found to have higher ΣPCB concentrations than the control populations (*For full justification of contaminant levels refer to Chapter 2-Contaminant Analysis*).

In the Harbour seal experimental group γGT, VLDL, triglycerides, LDL and glucose levels showed a positive correlation with ΣPCBs in a dose-dependent manner and this relationship was statistically significant, whereas HDLC levels showed a statistically significant negative

correlation with contaminants. VLDL levels were also compared with serum estradiol and from Fig. 5.17 a statistically significant negative correlation can be observed.

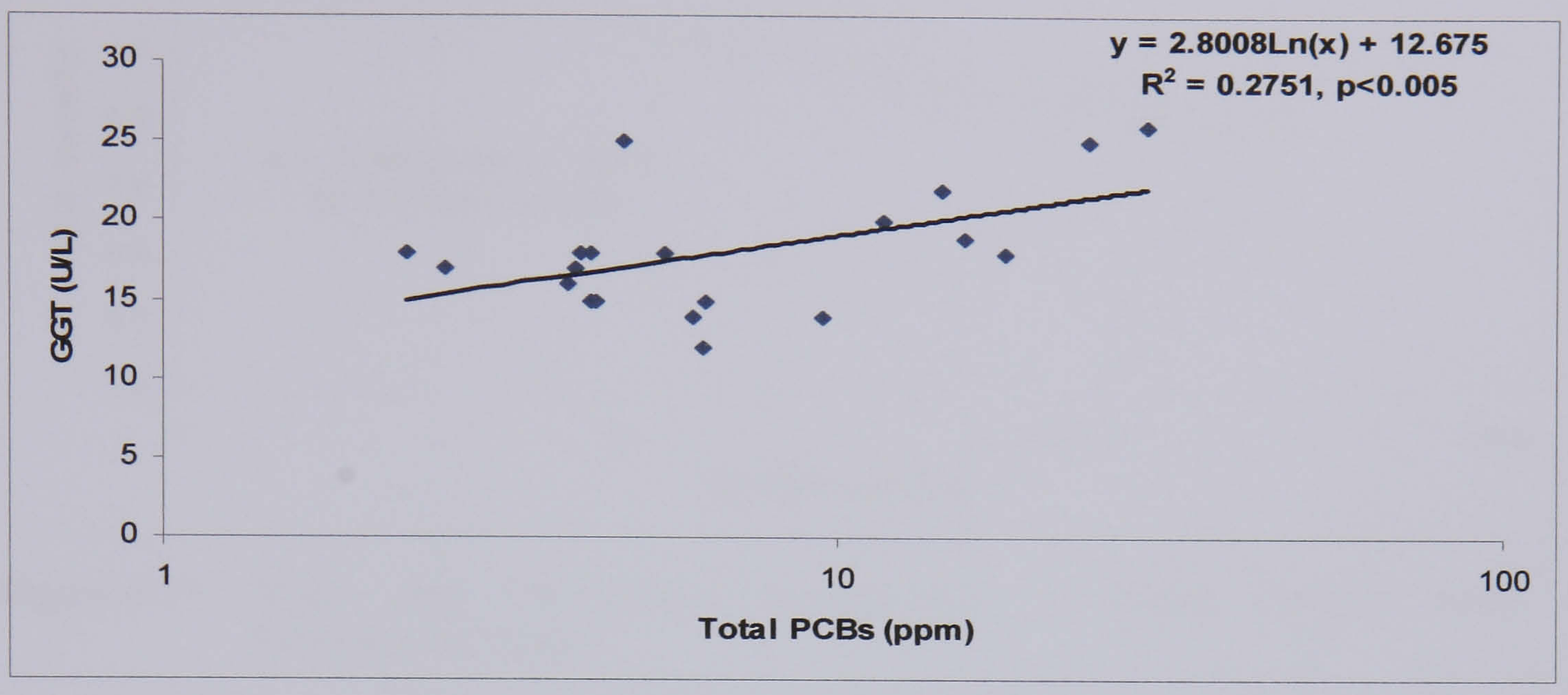



**Figure 5.14** ΣPCBs and γGT concentrations in female Harbour Seals – Experimental Group

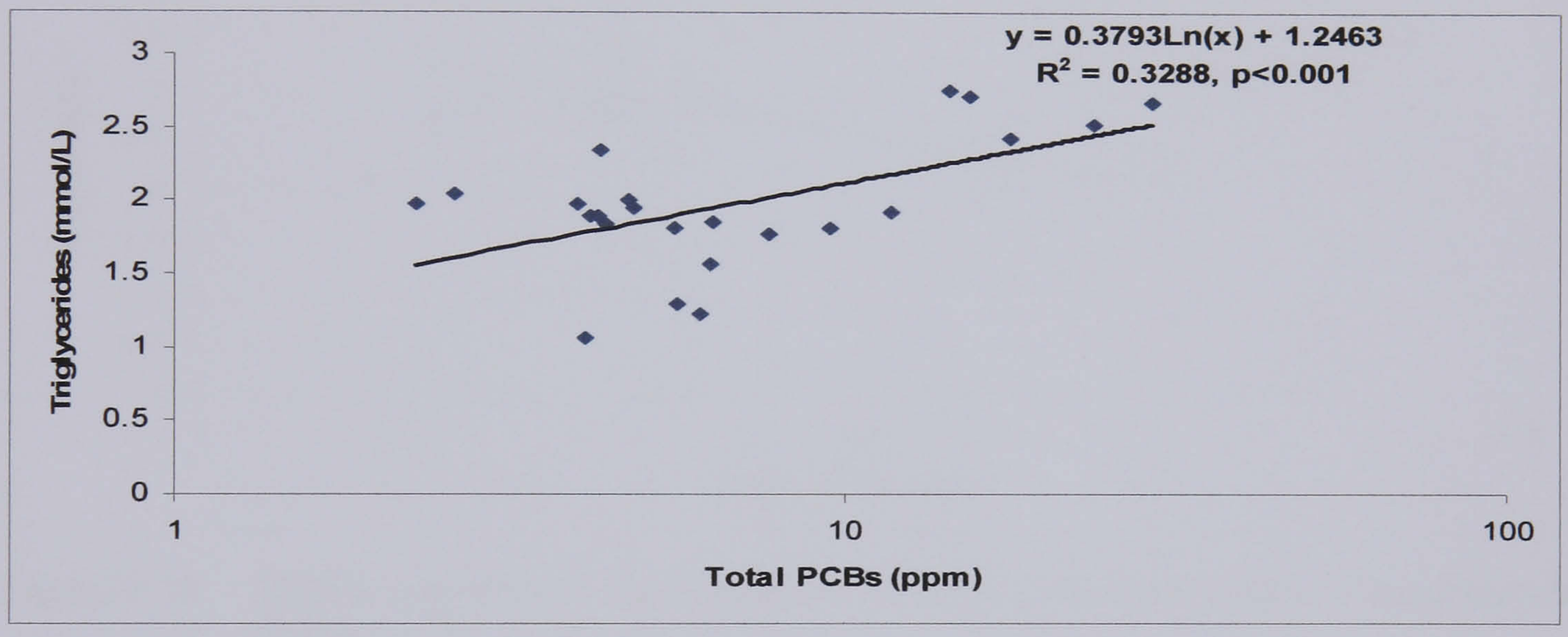



**Figure 5.15** ΣPCBs and triglyceride concentrations in female Harbour Seals – Experimental Group

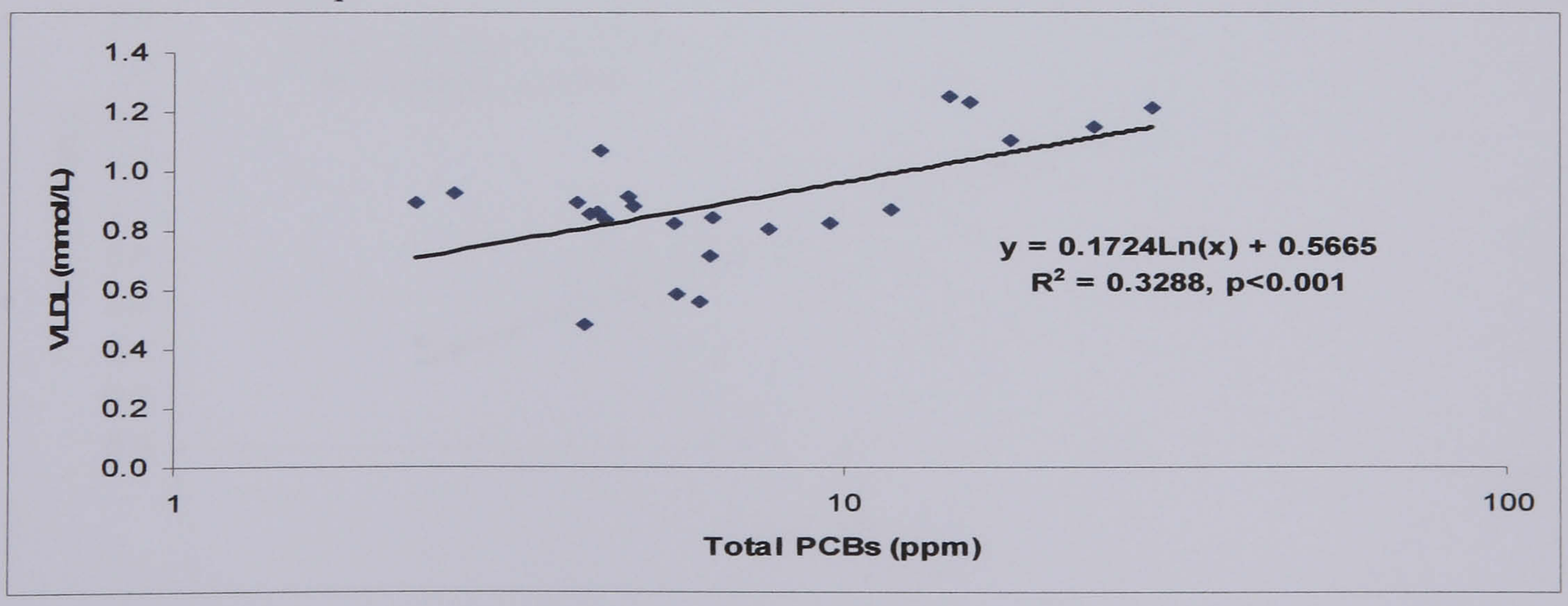



**Figure 5.16** ΣPCBs and VLDL concentrations in female Harbour Seals – Experimental Group

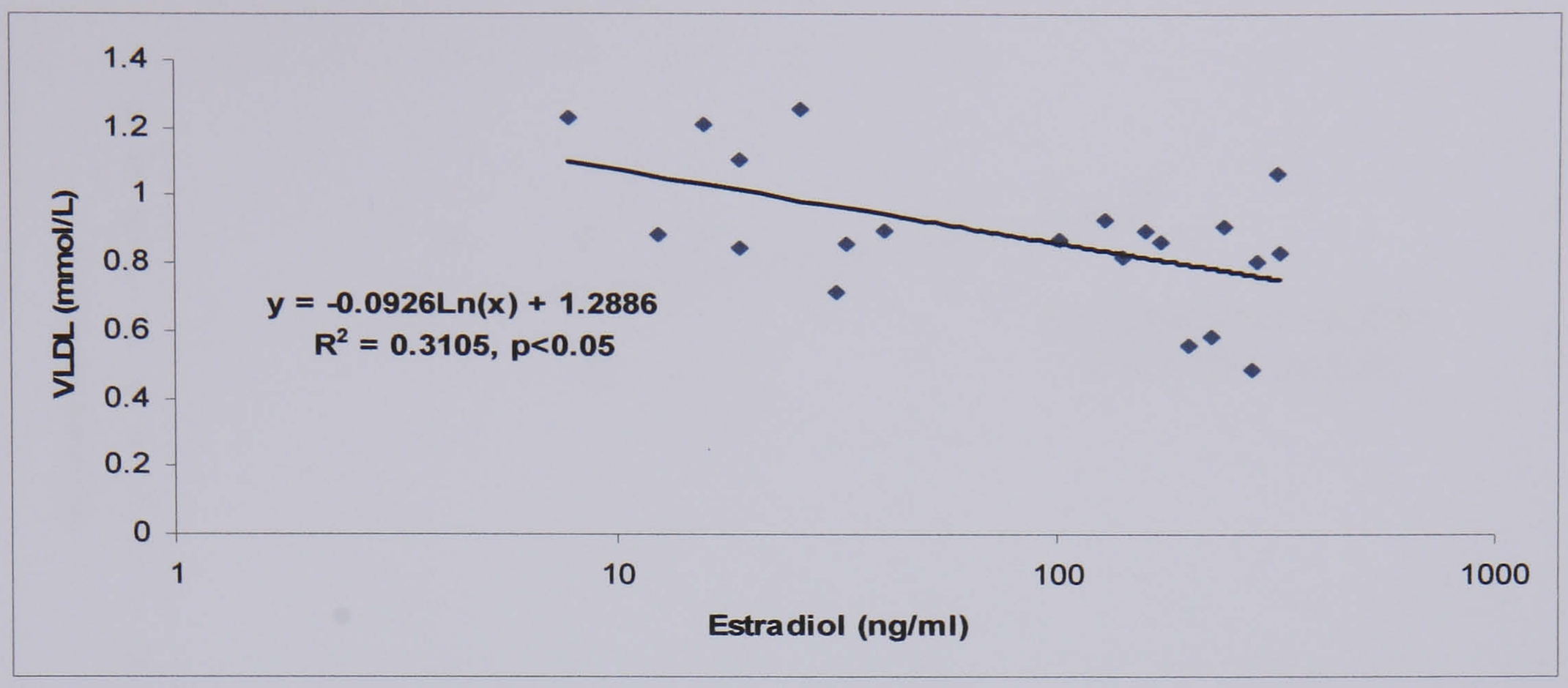



**Figure 5.17** VLDL and 17β-estradiol concentrations in female Harbour Seals – Experimental Group

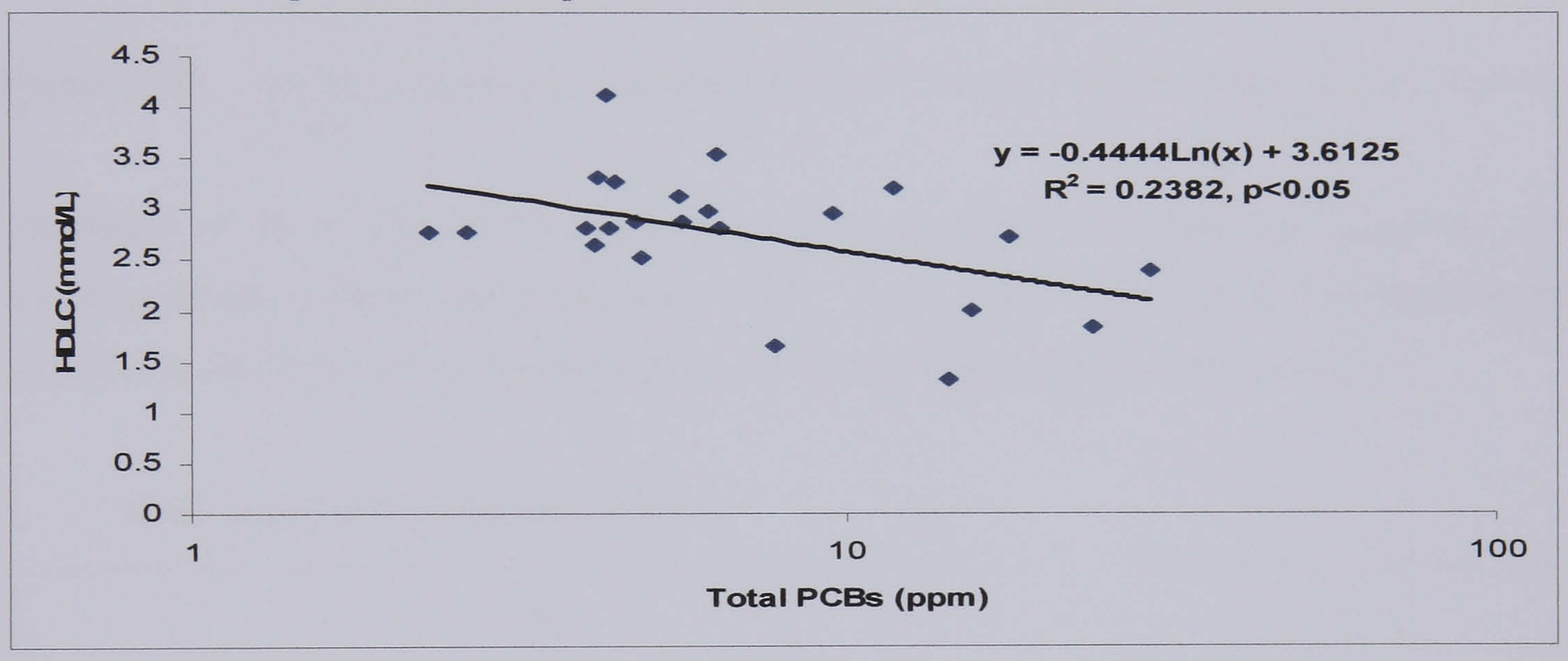



**Figure 5.18** ΣPCBs and HDLC concentrations in female Harbour Seals – Experimental Group

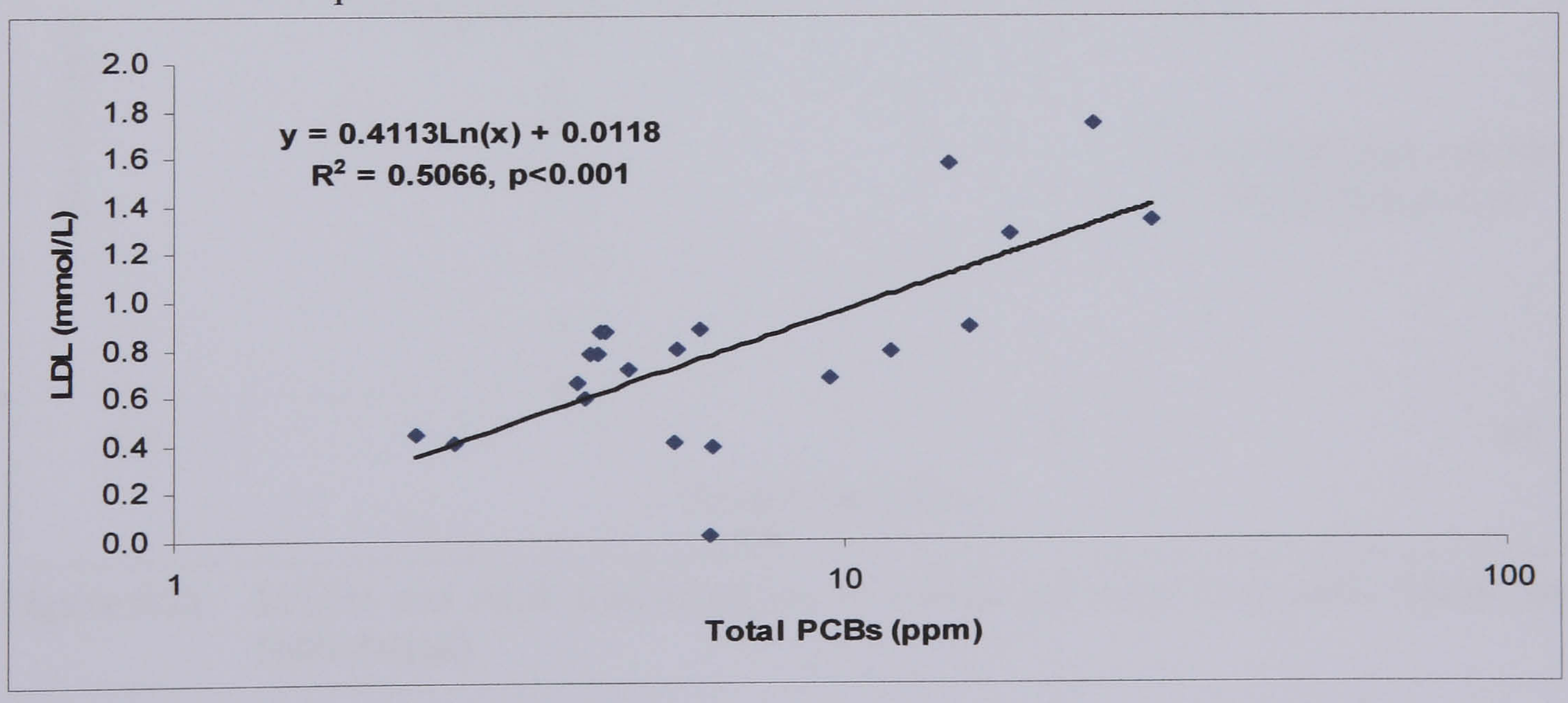



**Figure 5.19** ΣPCBs and LDL concentrations in female Harbour Seals – Experimental Group

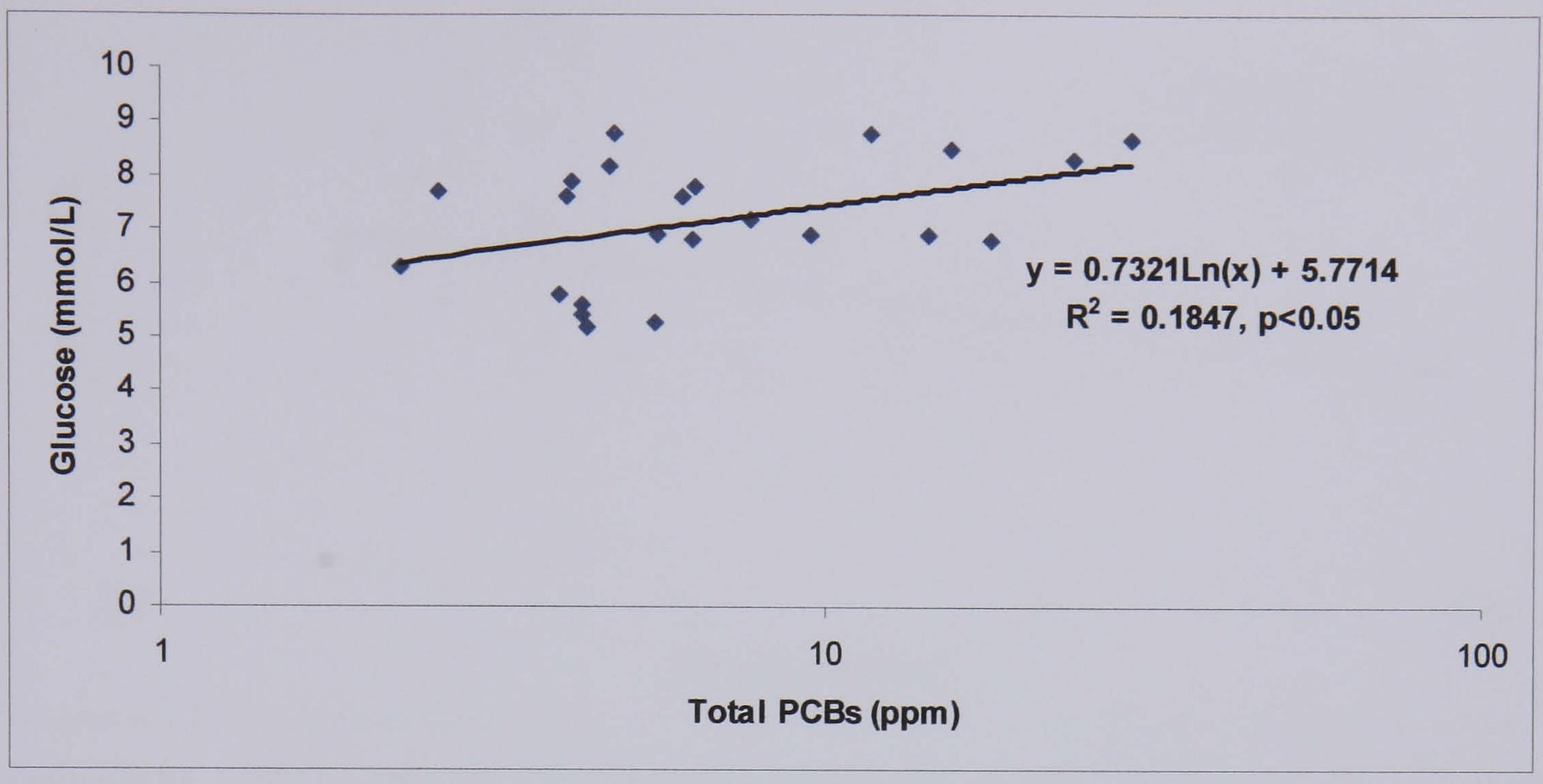



**Figure 5.20** ΣPCBs and glucose concentrations in female Harbour Seals – Experimental Group

Validation of Biomarker (BM) expression over a gradient of contaminant exposure was investigated using linear regressions (Fig. 5.21 – 5.39). When there was no sex-dependence variation in the blood chemistry parameters, female and male samples were pooled.

*Grey seals (Baltic and Sable Island)*

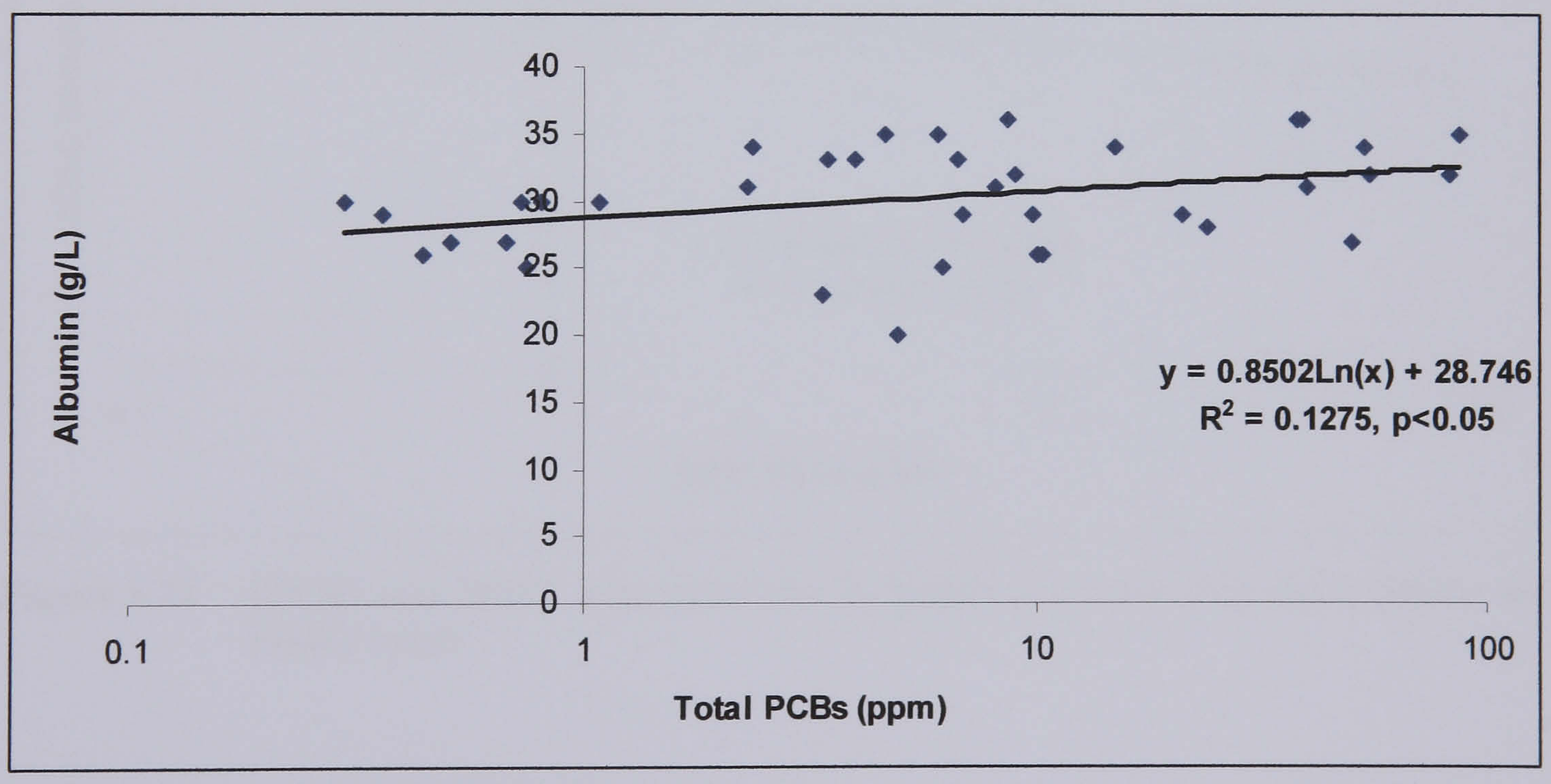



**Figure 5.21** ΣPCBs and ALB concentrations in female and male Grey seals (Baltic and Sable Island)

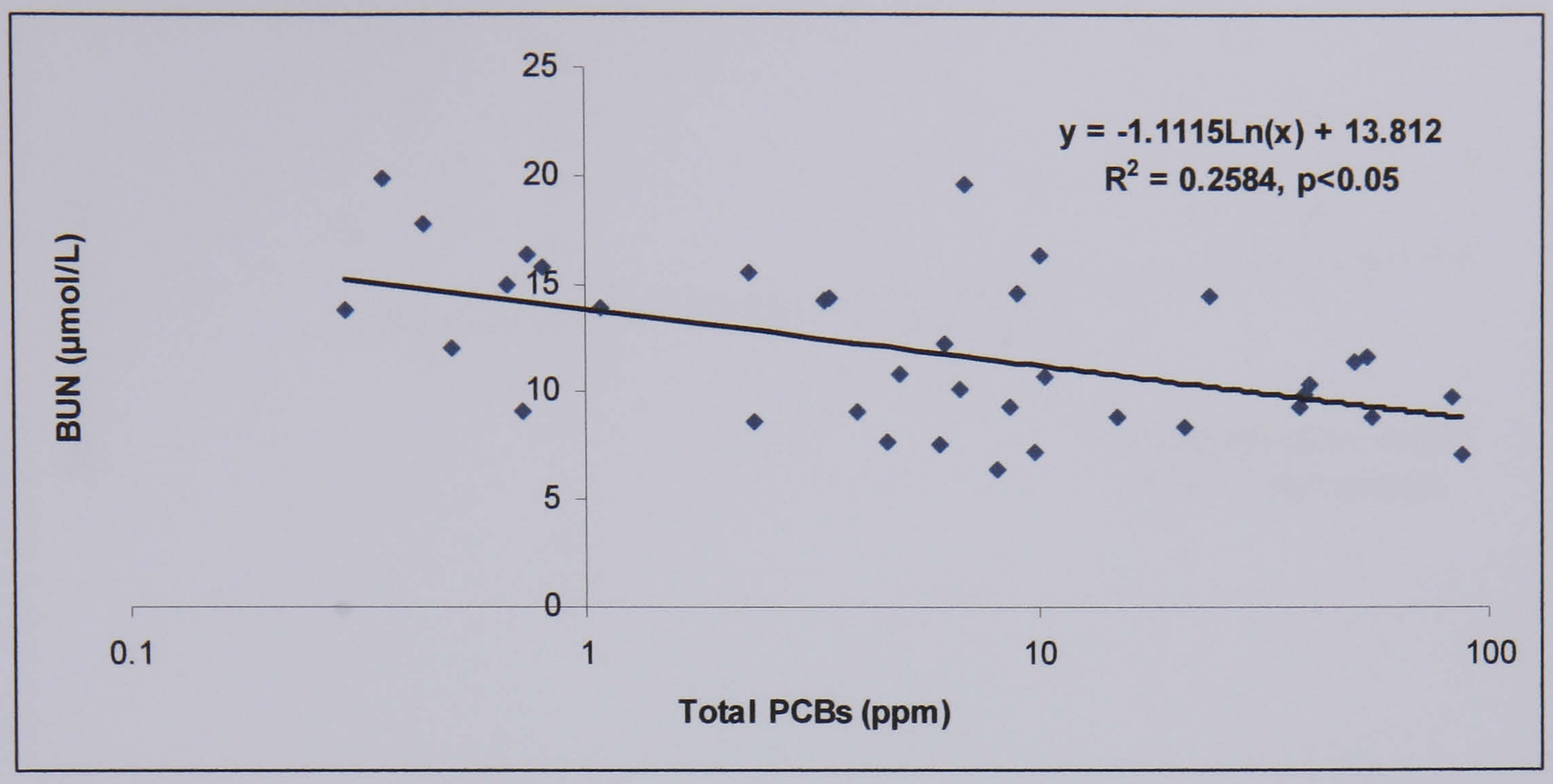



**Figure 5.22** ΣPCBs and Blood Urea Nitrogen (BUN) concentrations in female and male Grey seals (Baltic and Sable Island)

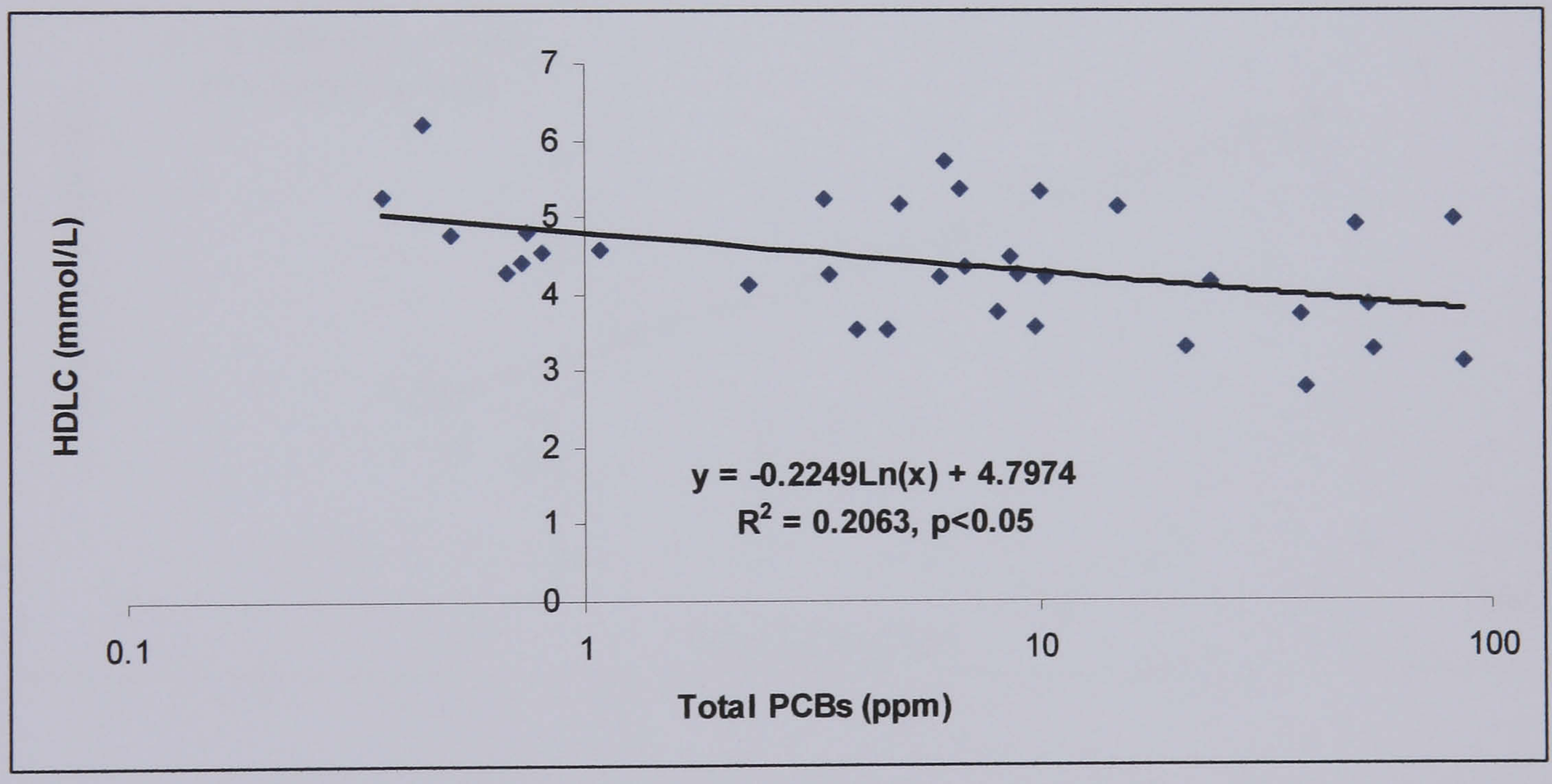



**Figure 5.23** ΣPCBs and HDLC concentrations in female and male Grey seals (Baltic and Sable Island)

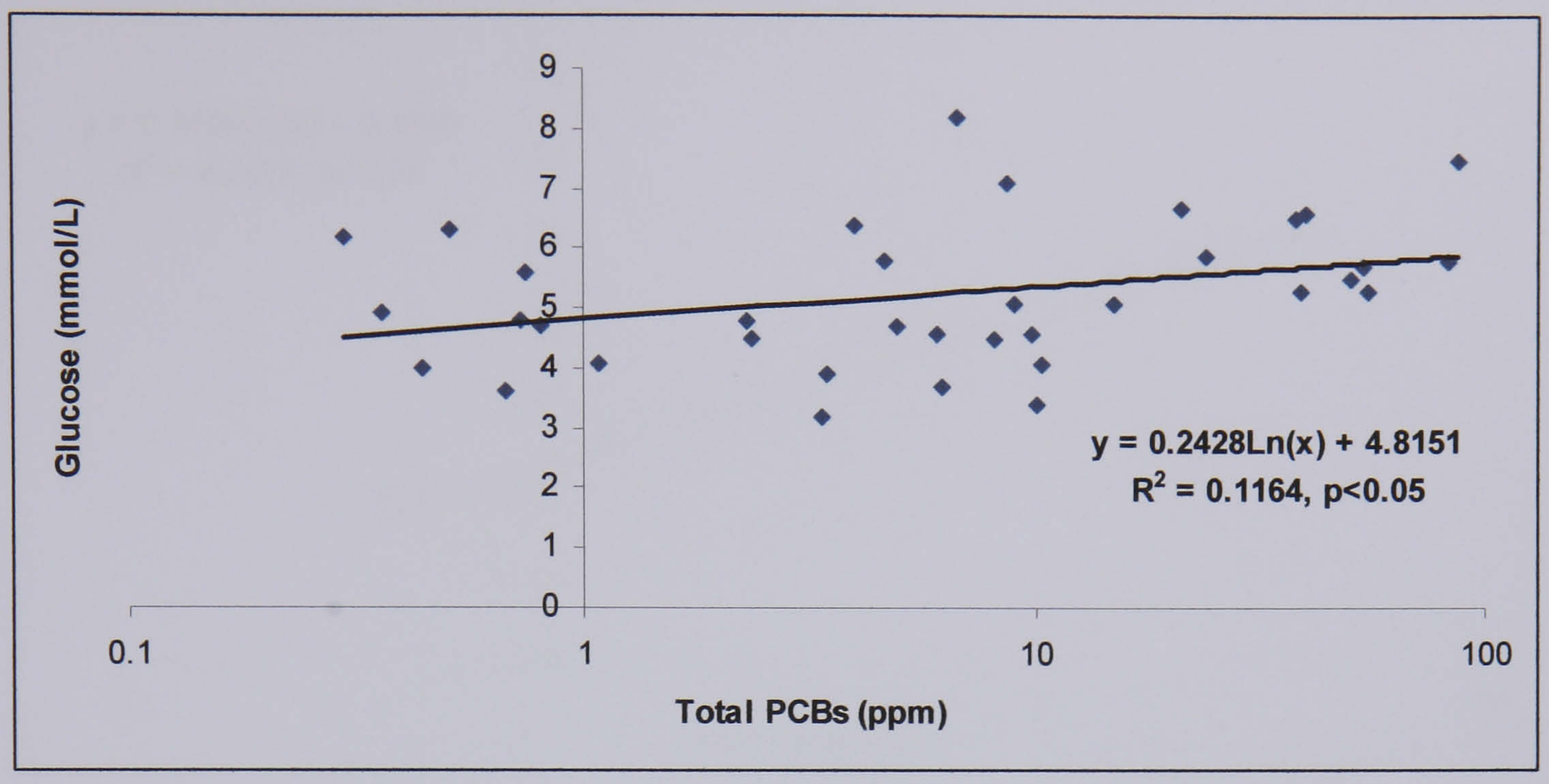



**Figure 5.24** ΣPCBs and glucose concentrations in female and male Grey seals (Baltic and Sable Island)

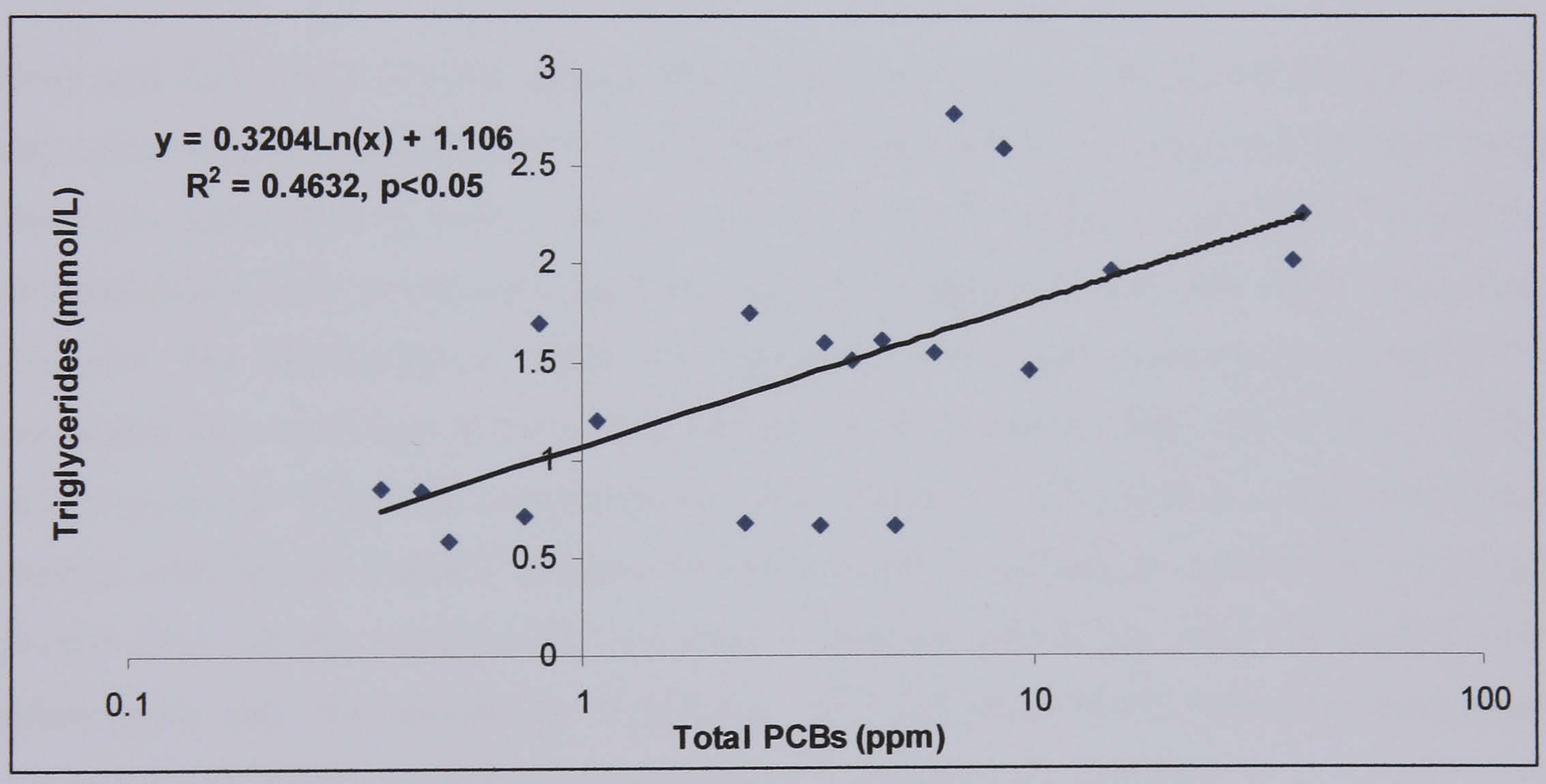



**Figure 5.25** ΣPCBs and triglyceride concentrations in female Grey seals (Baltic and Sable Island)

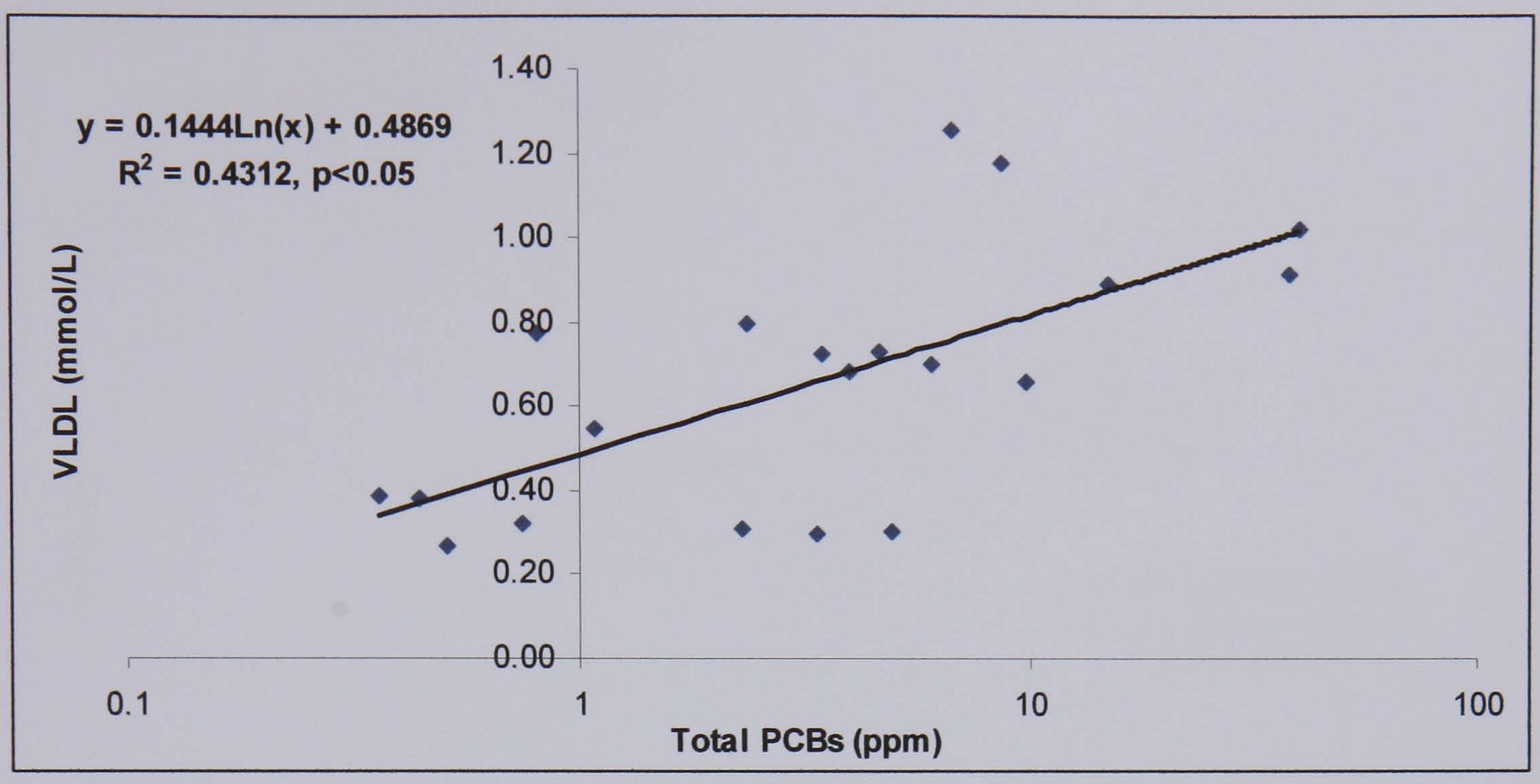



**Figure 5.26** ΣPCBs and VLDL concentrations in female Grey seals (Baltic and Sable Island)

Grey seal ALB (Fig.5.21) and glucose (Fig.5.24) levels were positively correlated in a dose-dependent manner with ΣPCBs. This relationship was statistically significant for both blood chemistry parameters ($p<0.05$). On the other hand, BUN (Fig.5.22) and HDLC (Fig.5.23) concentrations were statistically significant ($p<0.05$) negatively correlated with contaminant exposure. The relationship of VLDL and triglyceride levels with contaminant exposure was not statistically significant in the pooled data set, however, female Grey seal VLDL (Fig.5.26) and triglyceride (Fig.5.25) concentrations were positively correlated in a dose-dependent manner with ΣPCBs and this relationship was statistically significant ($p<0.05$). Even though female Grey VLDL concentrations showed a negative correlation with 17β-estradiol this relationship was not statistically significant. The remaining blood chemistry parameters showed no statistically significant correlation with contaminant exposure.

*Ringed seals (Baltic and Svalbard)*

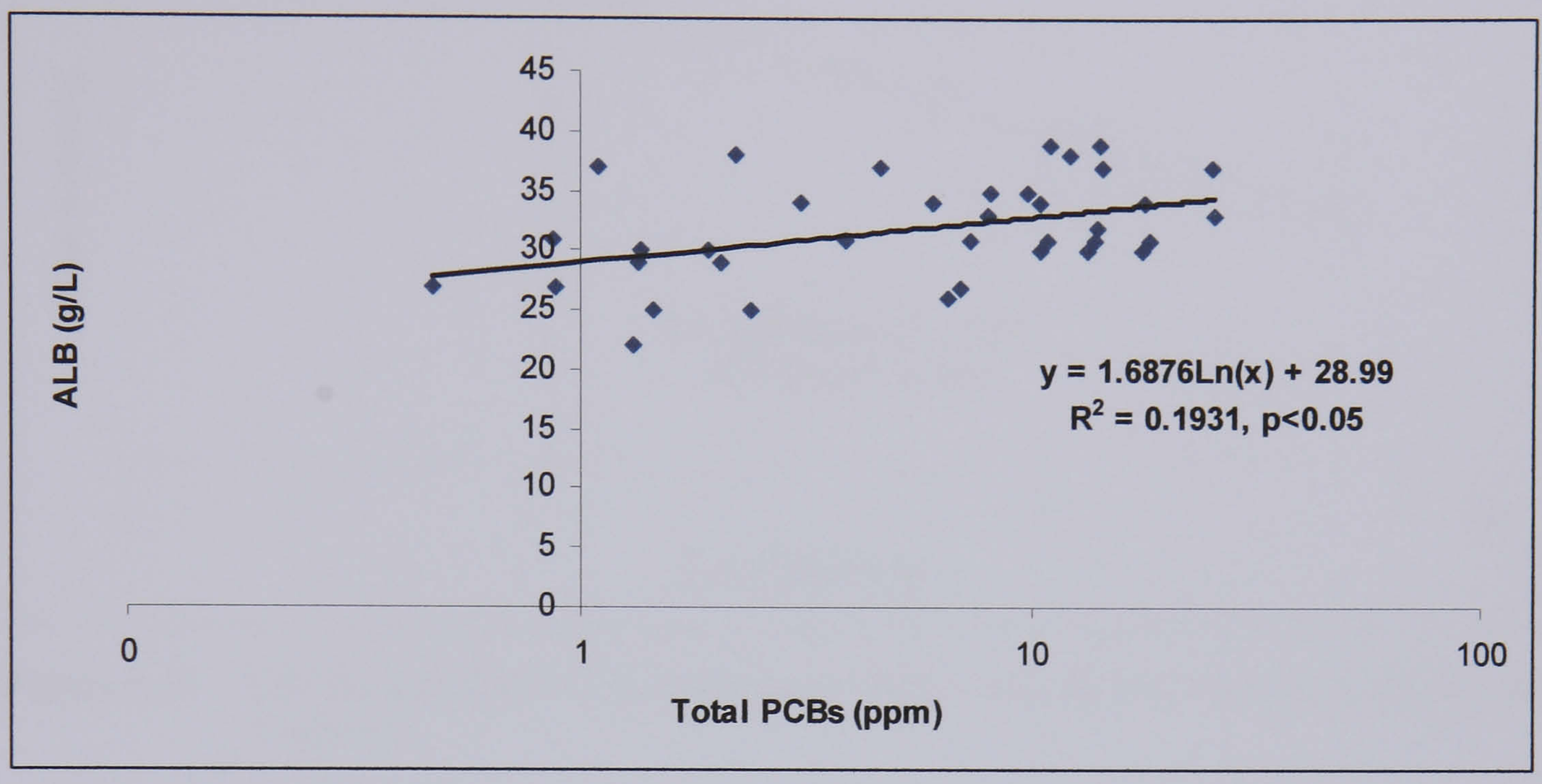



**Figure 5.27** ΣPCBs and ALB concentrations in female and male Ringed seals (Baltic and Svalbard)

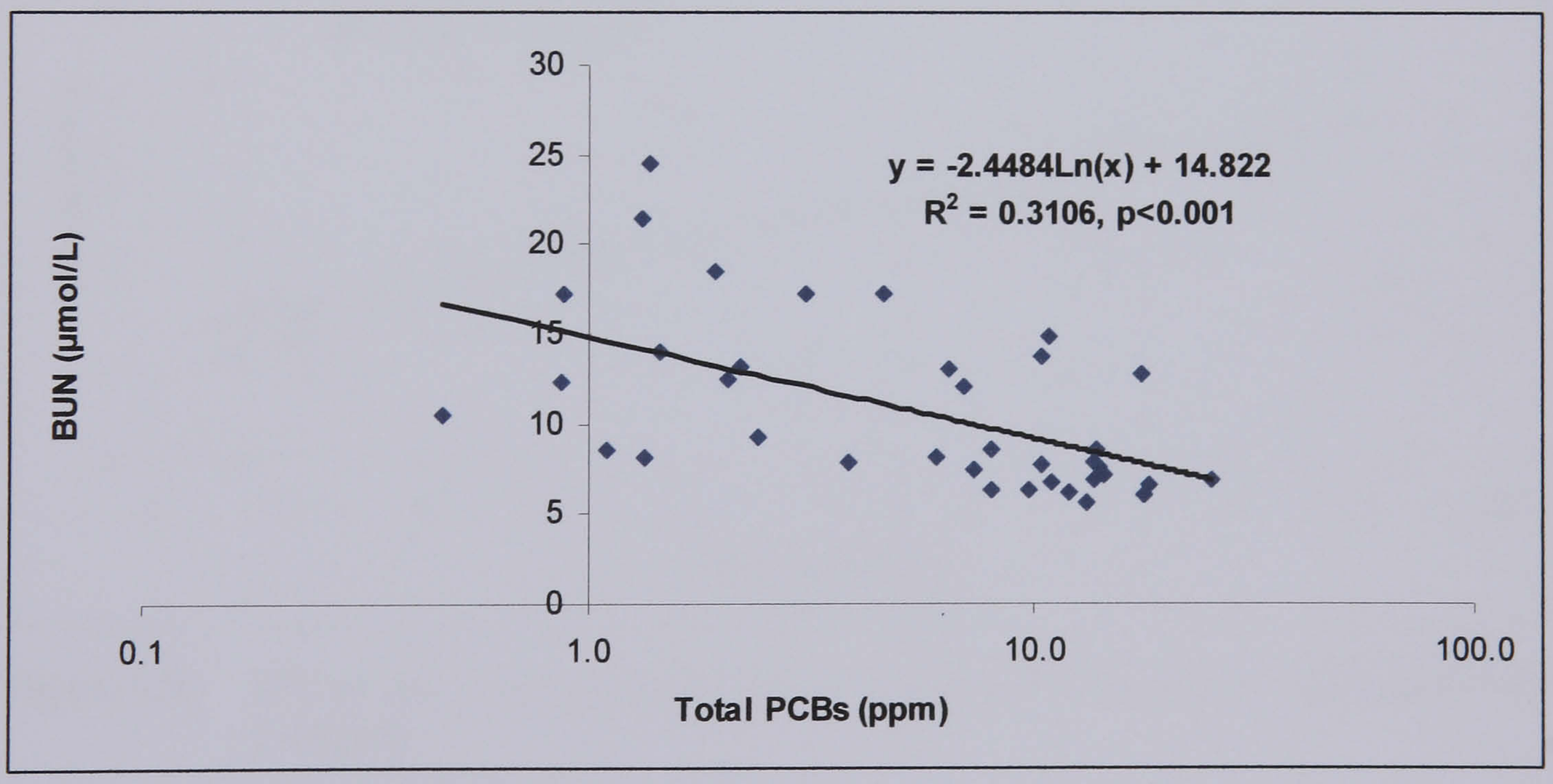



**Figure 5.28** ΣPCBs and blood urea nitrogen (BUN) concentrations in female and male Ringed seals (Baltic and Svalbard)

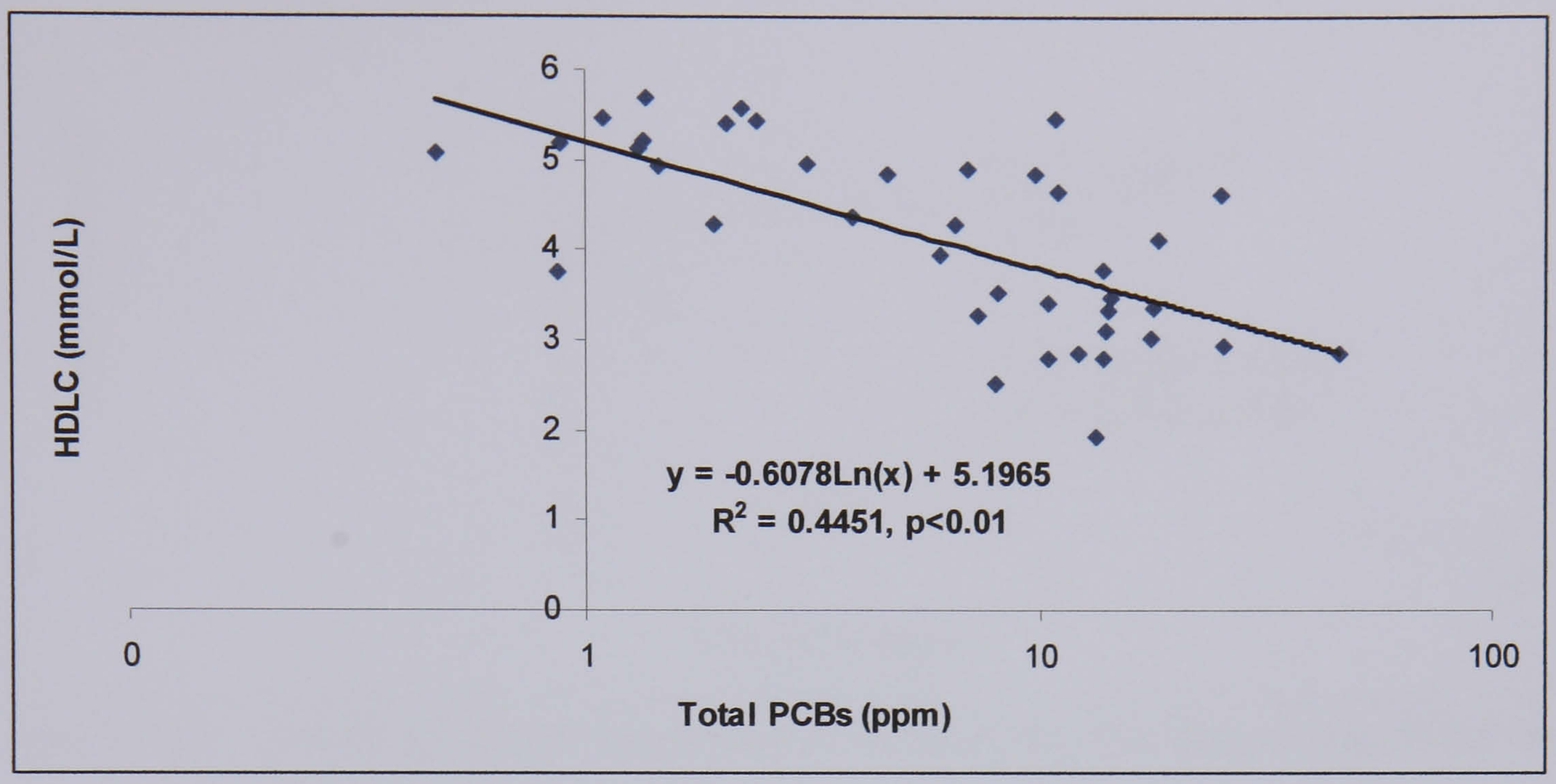



**Figure 5.29** ΣPCBs and HDLC concentrations in female and male Ringed seals (Baltic and Svalbard)

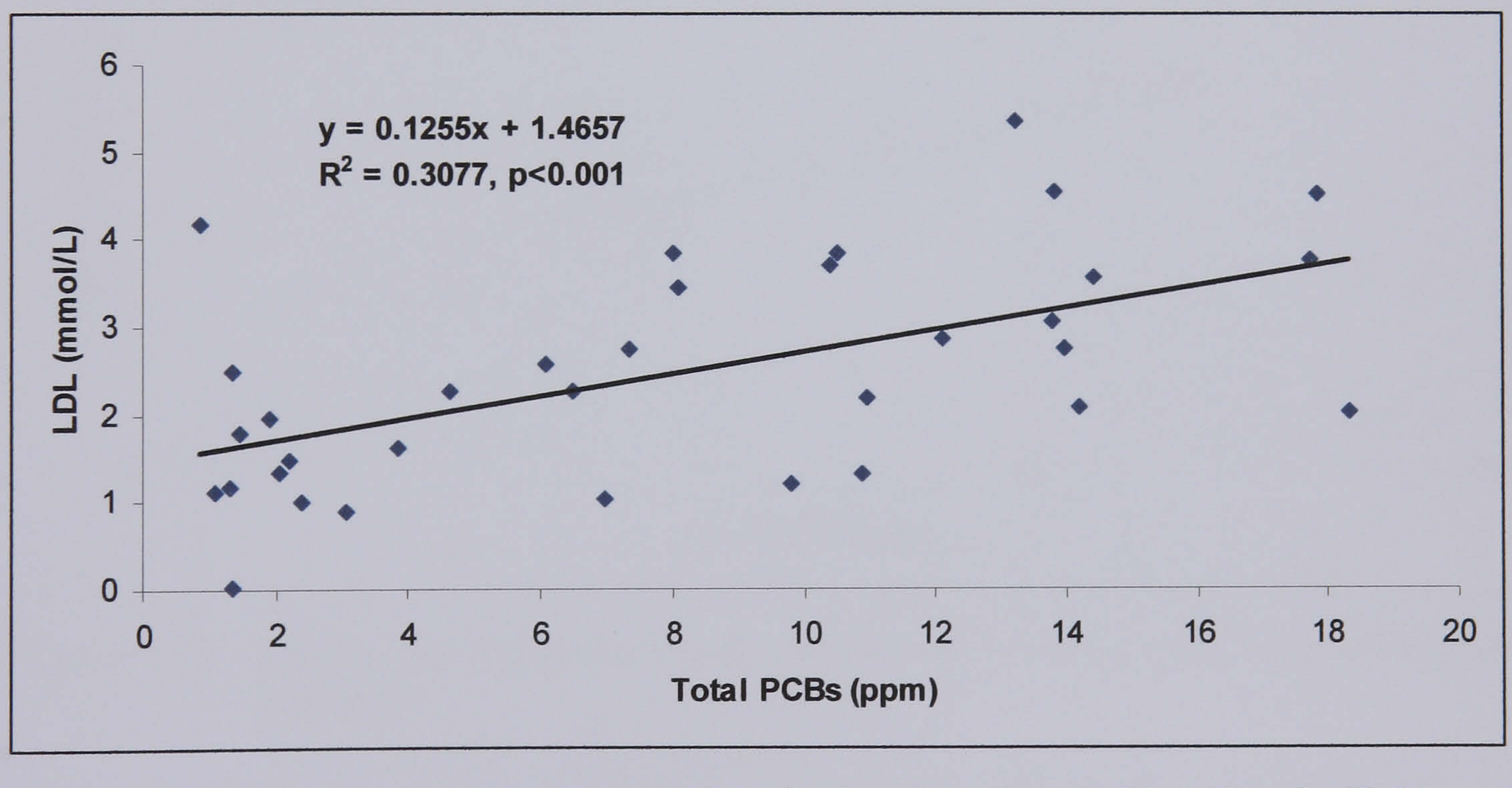



**Figure 5.30** ΣPCBs and LDL concentrations in female and male Ringed seals (Baltic and Svalbard)

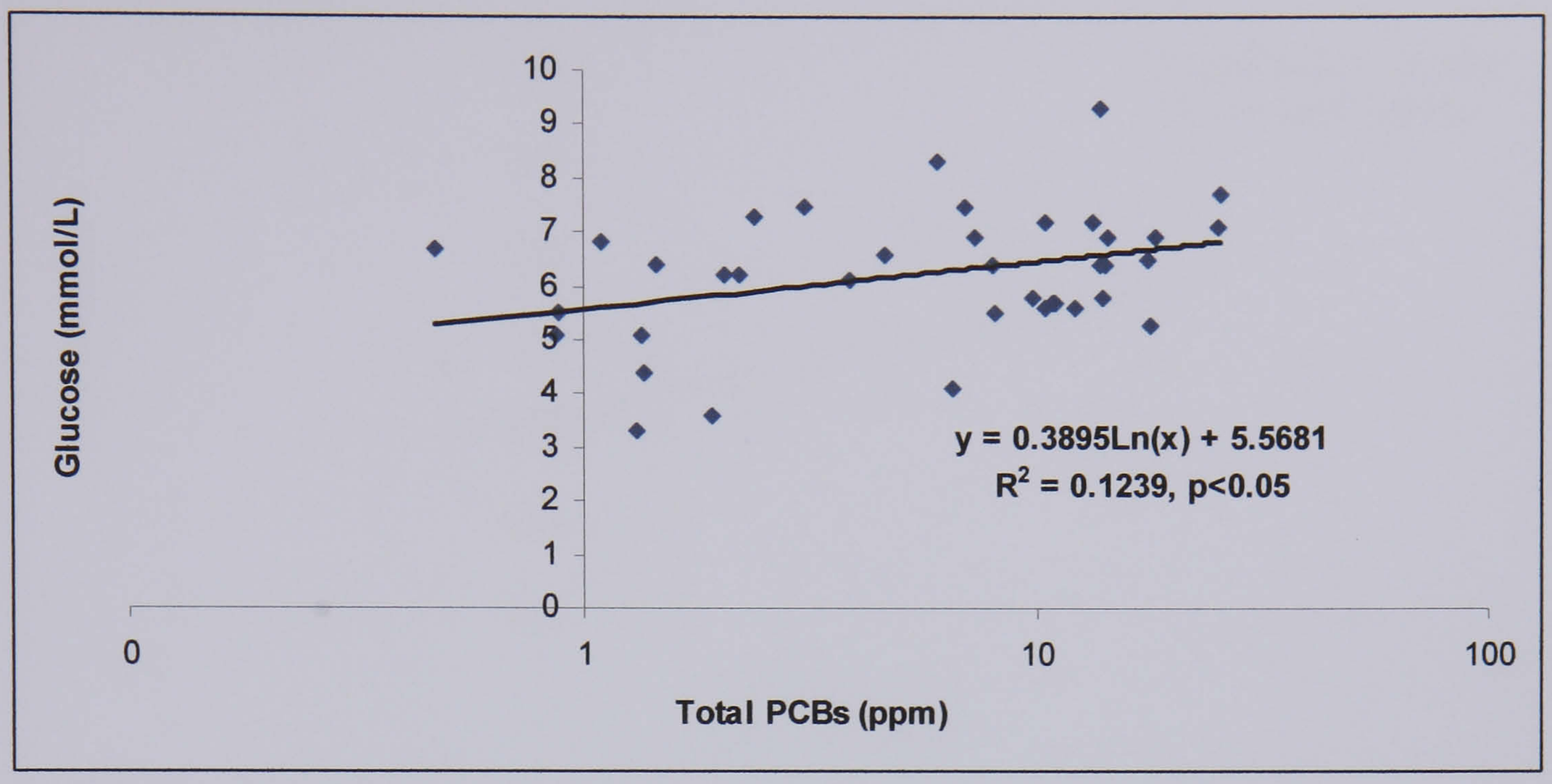



**Figure 5.31** ΣPCBs and glucose concentrations in female and male Ringed seals (Baltic and Svalbard)

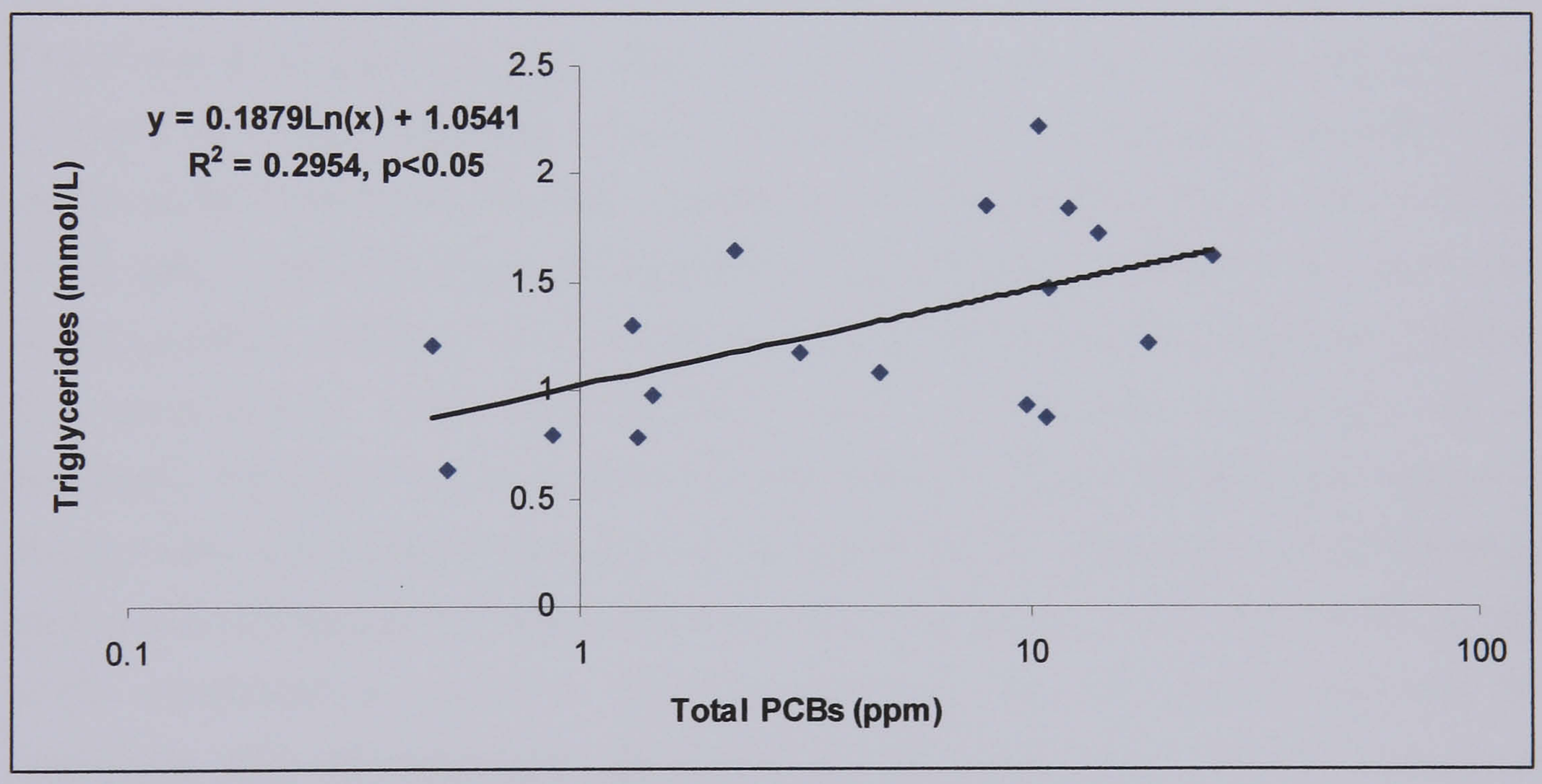



**Figure 5.32** ΣPCBs and triglyceride concentrations in female Ringed seals (Baltic and Svalbard)

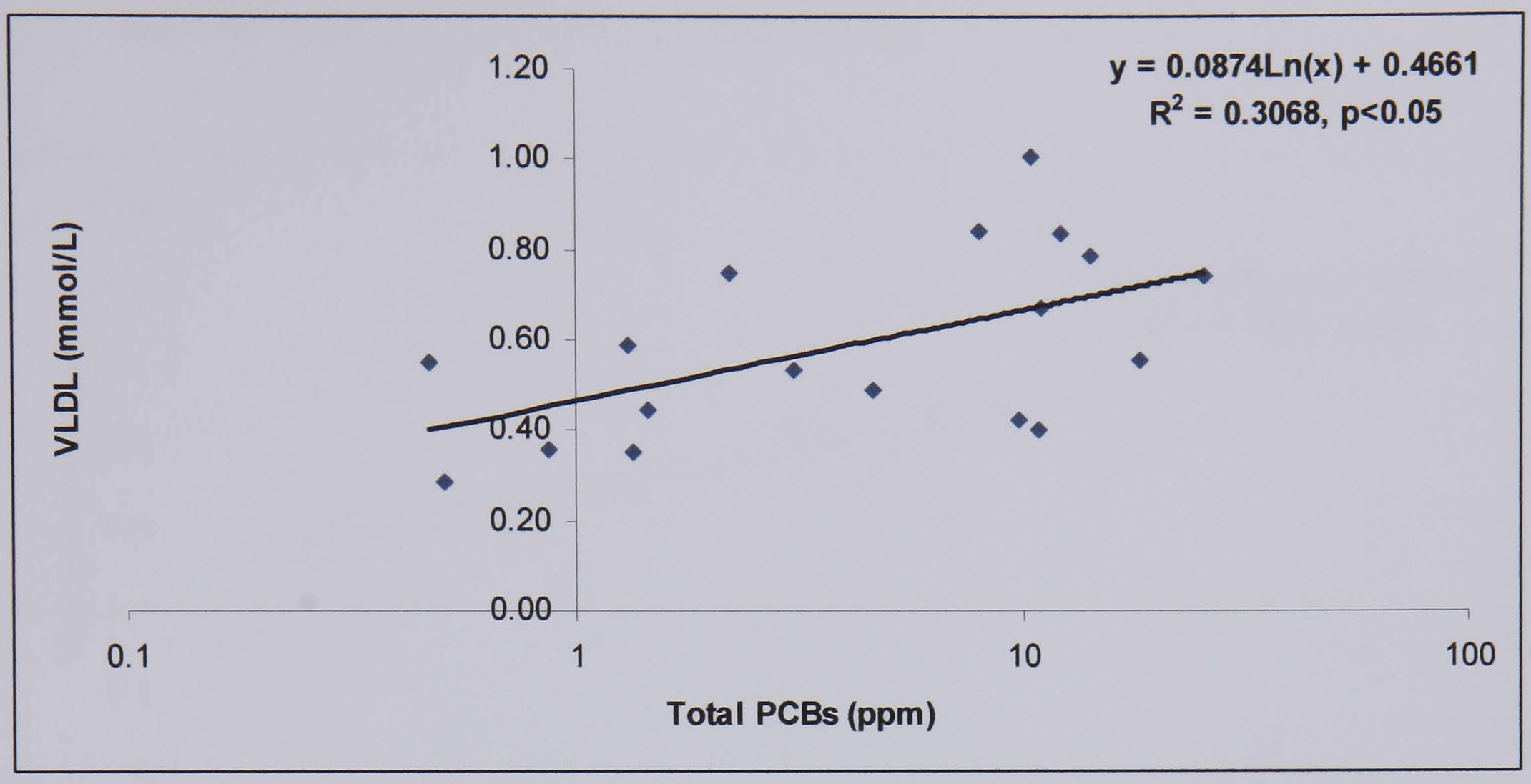



**Figure 5.33** ΣPCBs and VLDL concentrations in female Ringed seals (Baltic and Svalbard)

Ringed seal ALB (Fig.5.27), LDL (Fig.5.30) and glucose (Fig.5.31) levels were positively correlated in a dose-dependent manner with ΣPCBs. This relationship was statistically significant for all the blood chemistry parameters (ALB and glucose, $p<0.05$; LDL, $p<0.001$). On the other hand, BUN (Fig.5.28) and HDLC (Fig.5.29) concentrations showed statistically significant (BUN, $p<0.001$; HDLC, $p<0.01$) negatively correlation with contaminant exposure. The relationship of VLDL and triglyceride levels with contaminant exposure was not statistically significant in the pooled data set, however, female Ringed seal triglyceride (Fig.5.32) and VLDL (Fig.5.33) concentrations were positively correlated in a dose-dependent manner with ΣPCBs and this relationship was statistically significant ($p<0.05$). Female Ringed VLDL concentrations showed a negative correlation with 17β-estradiol however, this relationship was not statistically significant. The remaining blood chemistry parameters showed no statistically significant correlation with contaminant exposure.

*Harbour seals (Abertay Sands and Moray Firth)*

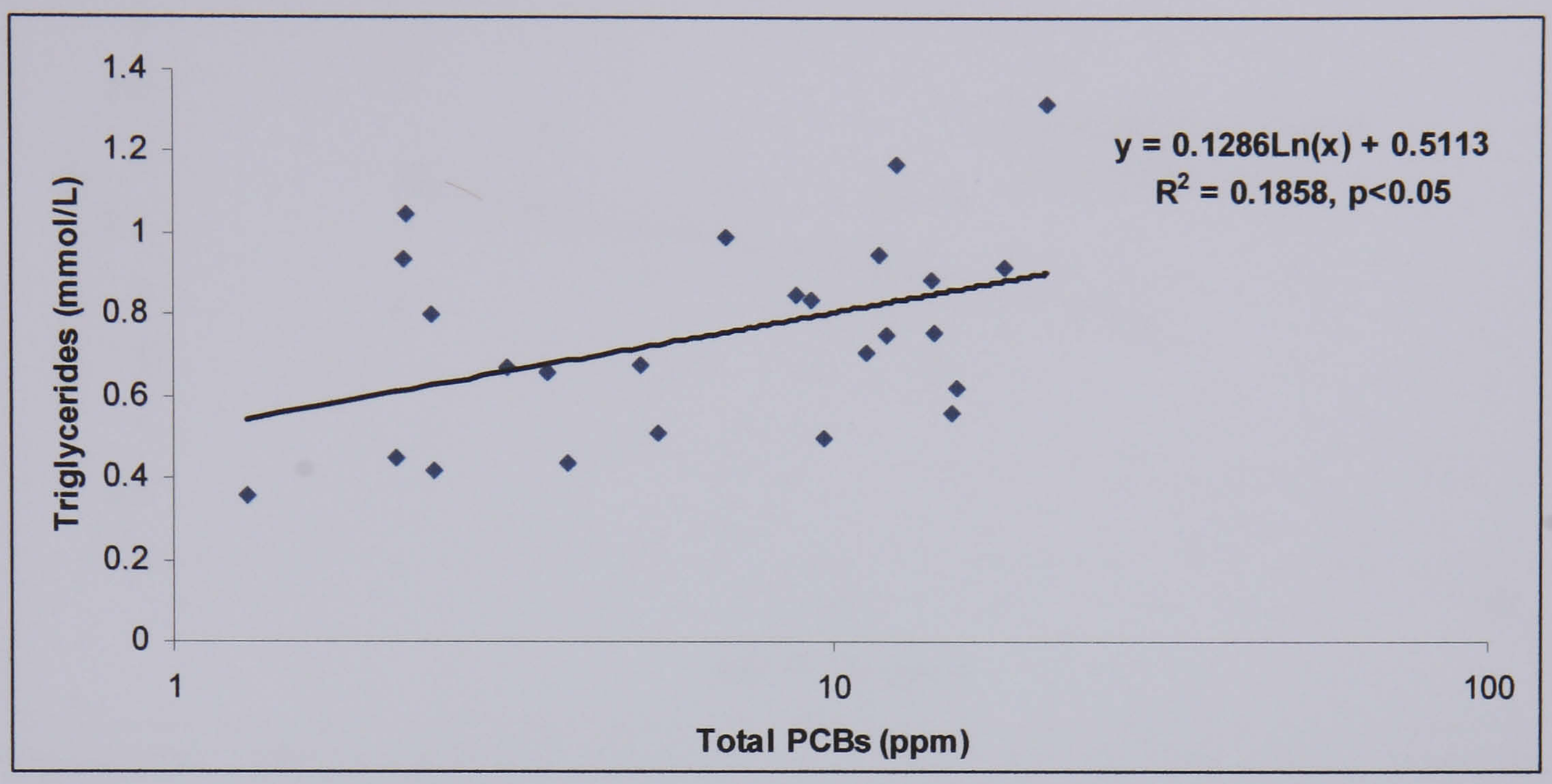



**Figure 5.34** ΣPCBs and triglyceride concentrations in female Harbour seals (Abertay Sands and Moray Firth)

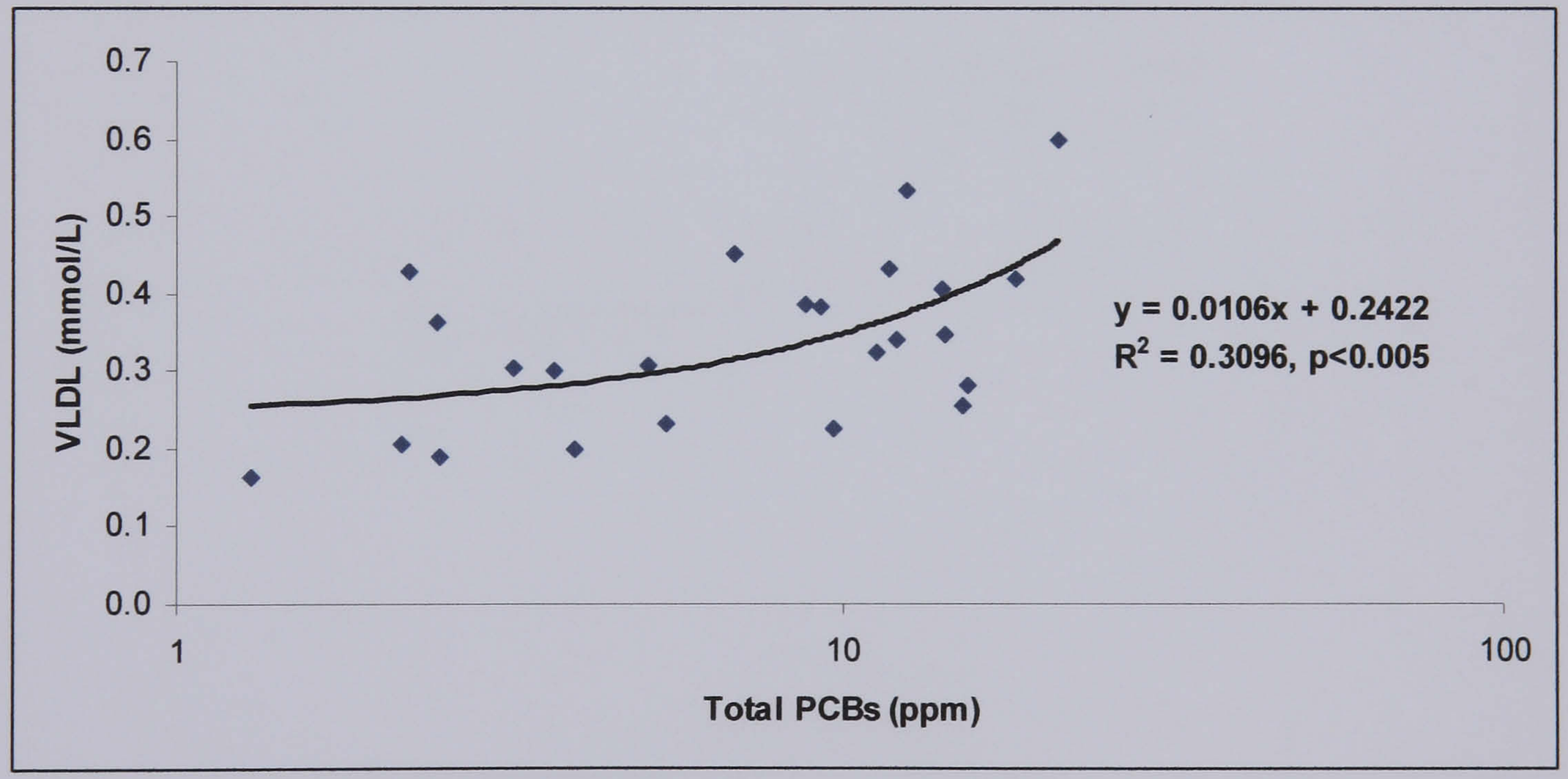



**Figure 5.35** ΣPCBs and VLDL concentrations in female Harbour seals (Abertay Sands and Moray Firth)

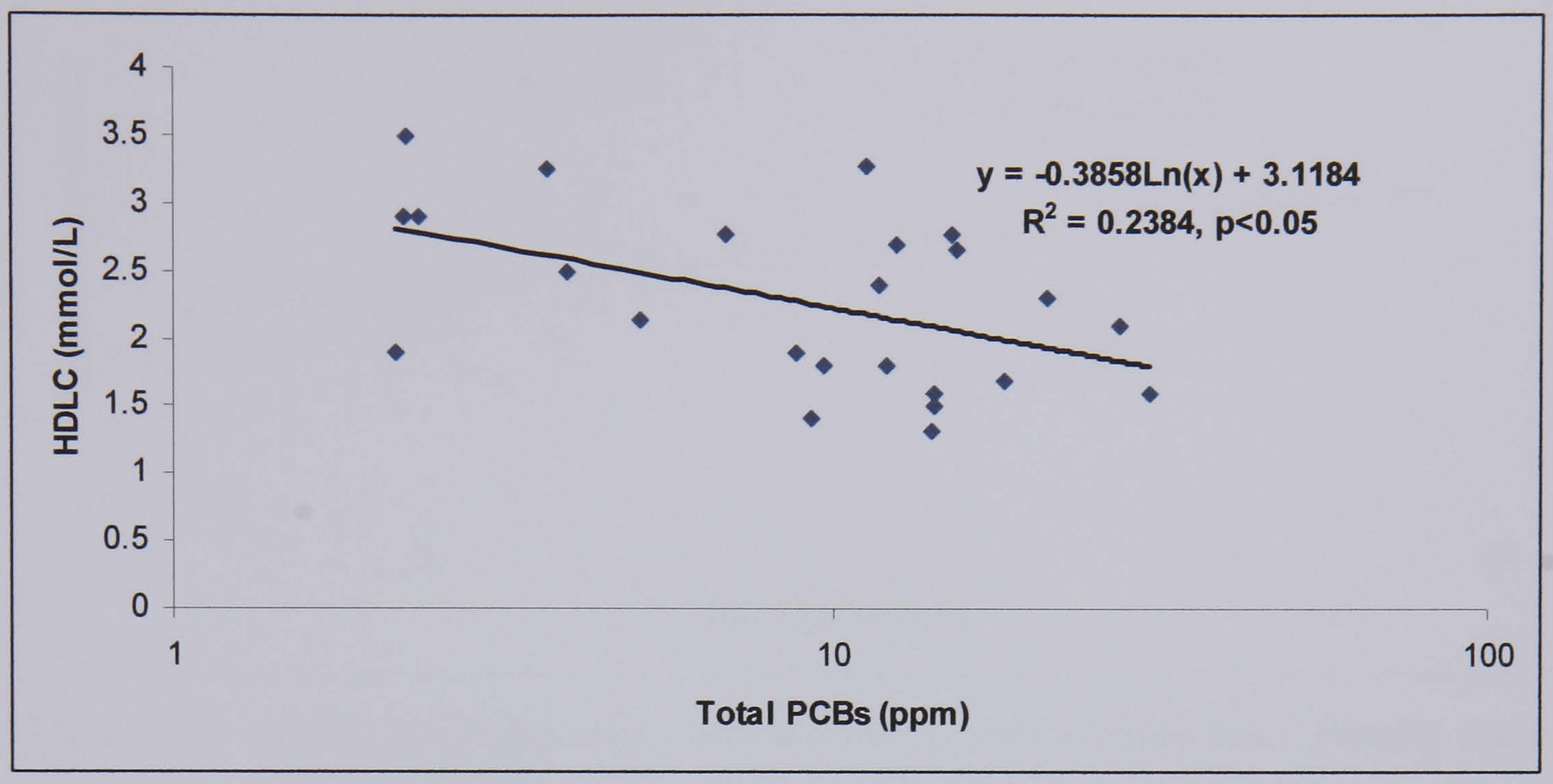



**Figure 5.36** ΣPCBs and HDLC concentrations in female Harbour seals (Abertay Sands and Moray Firth)

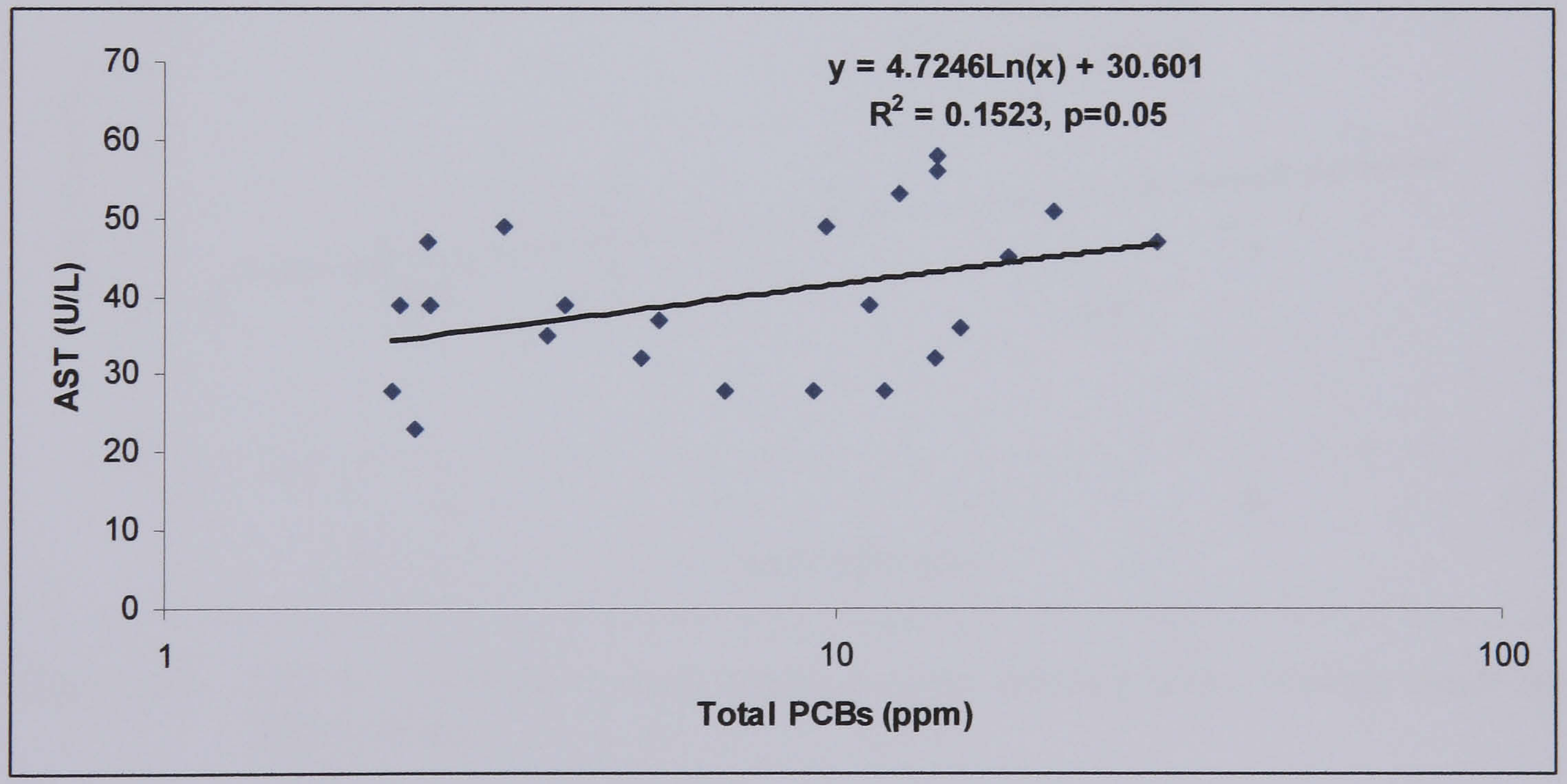



**Figure 5.37** ΣPCBs and AST concentrations in female Harbour seals (Abertay Sands and Moray Firth)

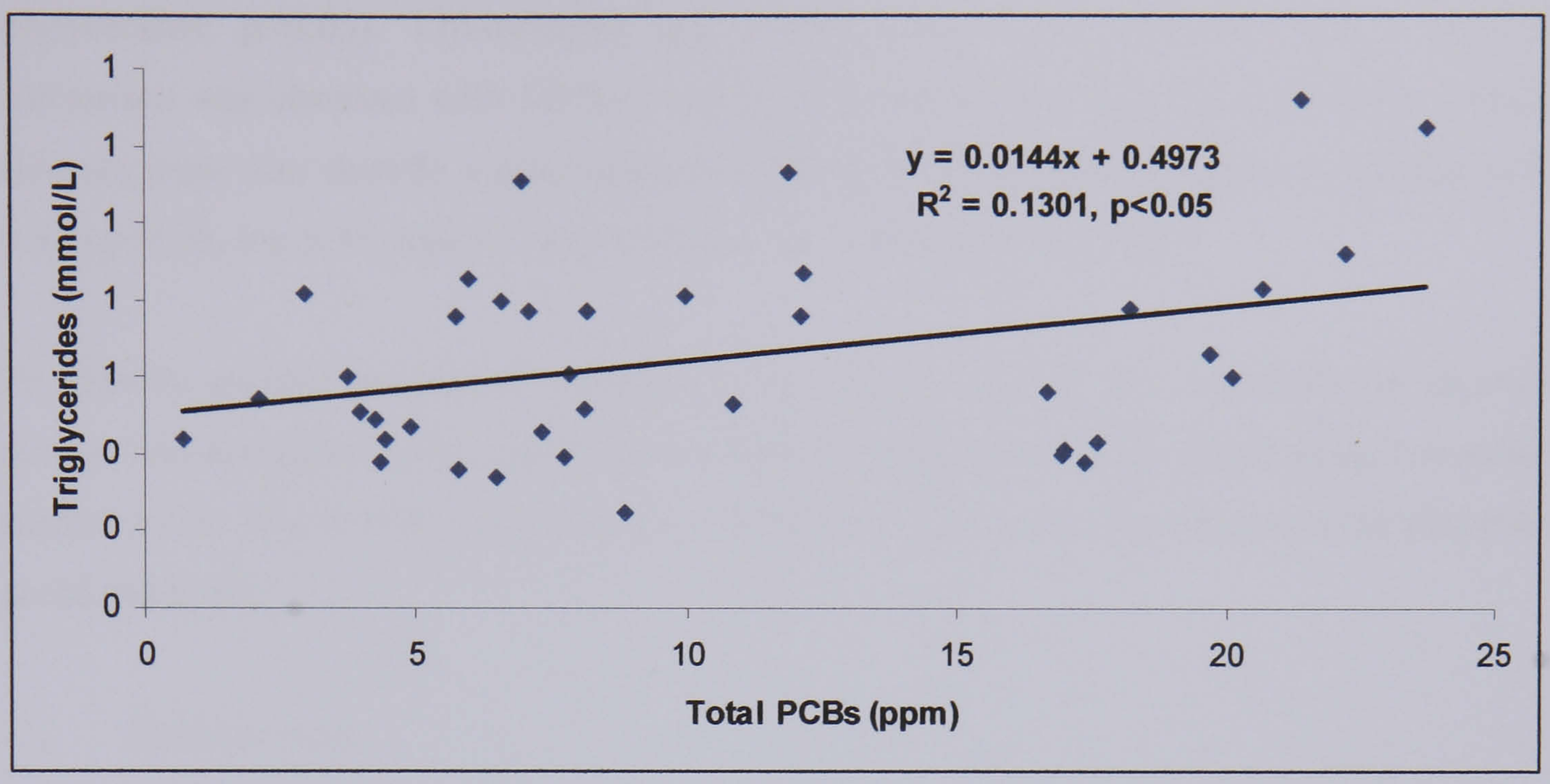



**Figure 5.38** ΣPCBs and triglyceride concentrations in male Harbour seals (Abertay Sands and Moray Firth)

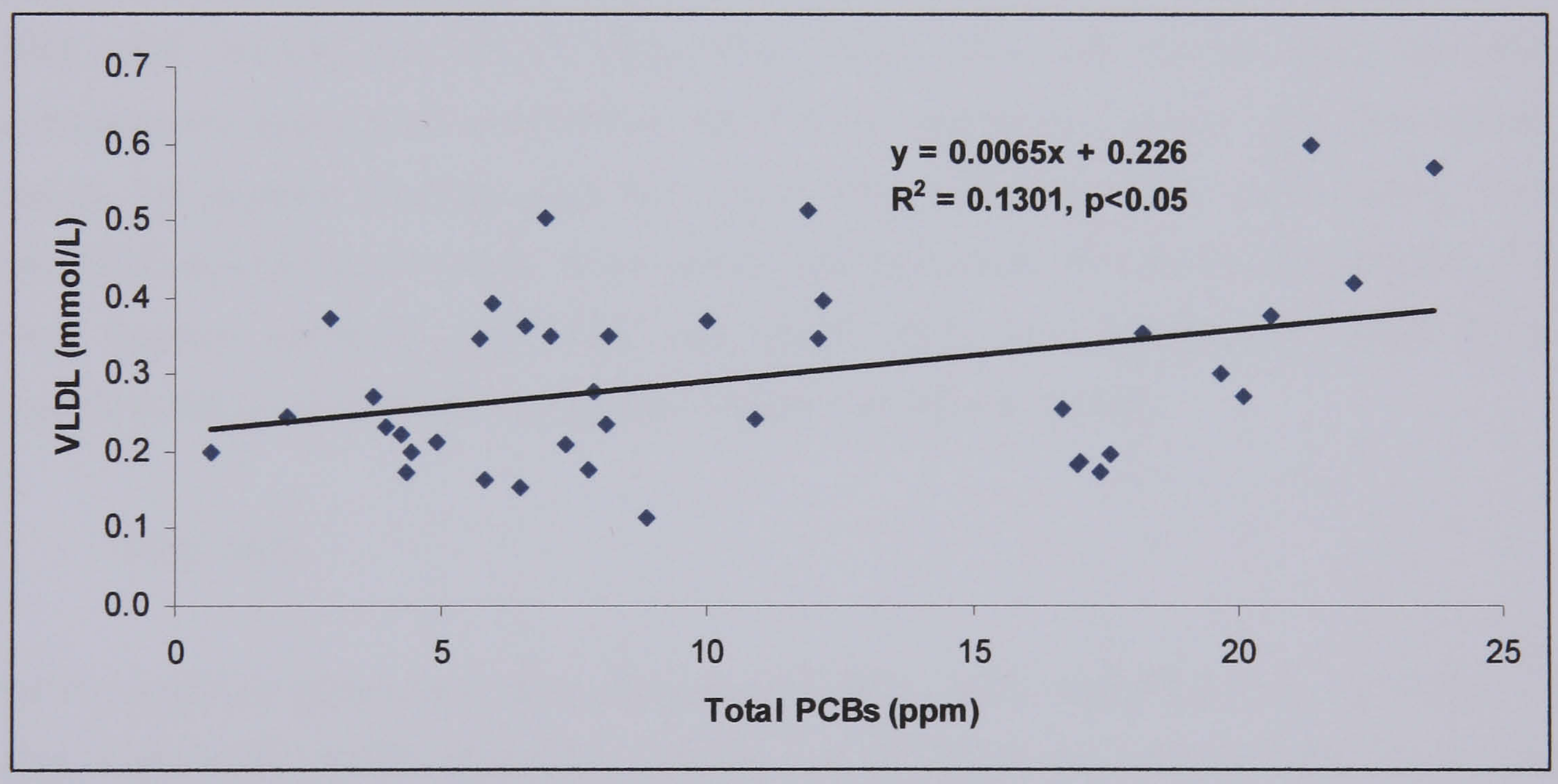



**Figure 5.39** ΣPCBs and VLDL concentrations in male Harbour seals (Abertay Sands and Moray Firth)

Harbour seal blood chemistry parameters were analysed according to sex-dependence variations since there were statistically significant differences between females and males. From all the blood chemistry parameters studied, in both male and female Harbour seals VLDL and triglycerides showed a dose-dependent positive correlation with ΣPCBs that was statistically significant (females: VLDL: $p<0.005$; triglycerides: $p<0.05$; males; VLDL,

triglycerides: $p<0.05$). 17β-estradiol and VLDL levels were compared and a positive correlation was observed with ΣPCBs, however not statistically significant ($p=0.08$). Female Harbour seals also showed a dose-dependent statistically significant positive correlation with AST ($p<0.05$, Fig.5.37) and a negative association with HDLC ($p<0.05$).

To identify specific populations affected by ED (being at risk), BM expression in exposed groups was compared with control populations and differences were tested using two-tailed student t-test. Where BM expression was statistically significant, populations were classified at risk from ED.

*Harbour seals*

Although there were statistically significant differences between the two Scottish Harbour seal populations, all of the chemical blood parameters were within the 'normal' ranges except for HDLC and γGT. The exposed Harbour seals showed significantly increased albumin, ALT, AST, γGT, ALKP, cholesterol, triglycerides, VLDL, LDL and glucose levels ($p<0.005$) compared to Harbour seals from Moray Firth. When compared to Harbour seals from Abertay Sands, the exposed Harbour seals had higher total protein ($p<0.05$) and albumin levels ($p<0.005$) as well as cholesterol, triglycerides, VLDL, HDLC and LDL levels ($p<0.005$). The liver function enzymes, AST, ALT and ALKP where also significantly higher in the experimental group ($p<0.005$) compared to seals from Abertay Sands.

*Grey Seals*

Of the analysed parameters, mean albumin ($p<0.005$), AST, ALKP ($p<0.05$, Fig.5.14), uric acid, triglyceride, VLDL (Fig.5.15), glucose, CK ($p<0.005$) and LDL ($p<0.05$) levels were significantly higher in Grey seals from the Baltic Sea than from Sable Island. ALT ($p<0.05$), γGT ($p<0.05$, Fig.5.14), urea and HDLC (Fig.5.15) concentrations ($p<0.005$) were found to be significantly reduced in Baltic Grey seals compared to the low exposed/control seals population (Sable Island).

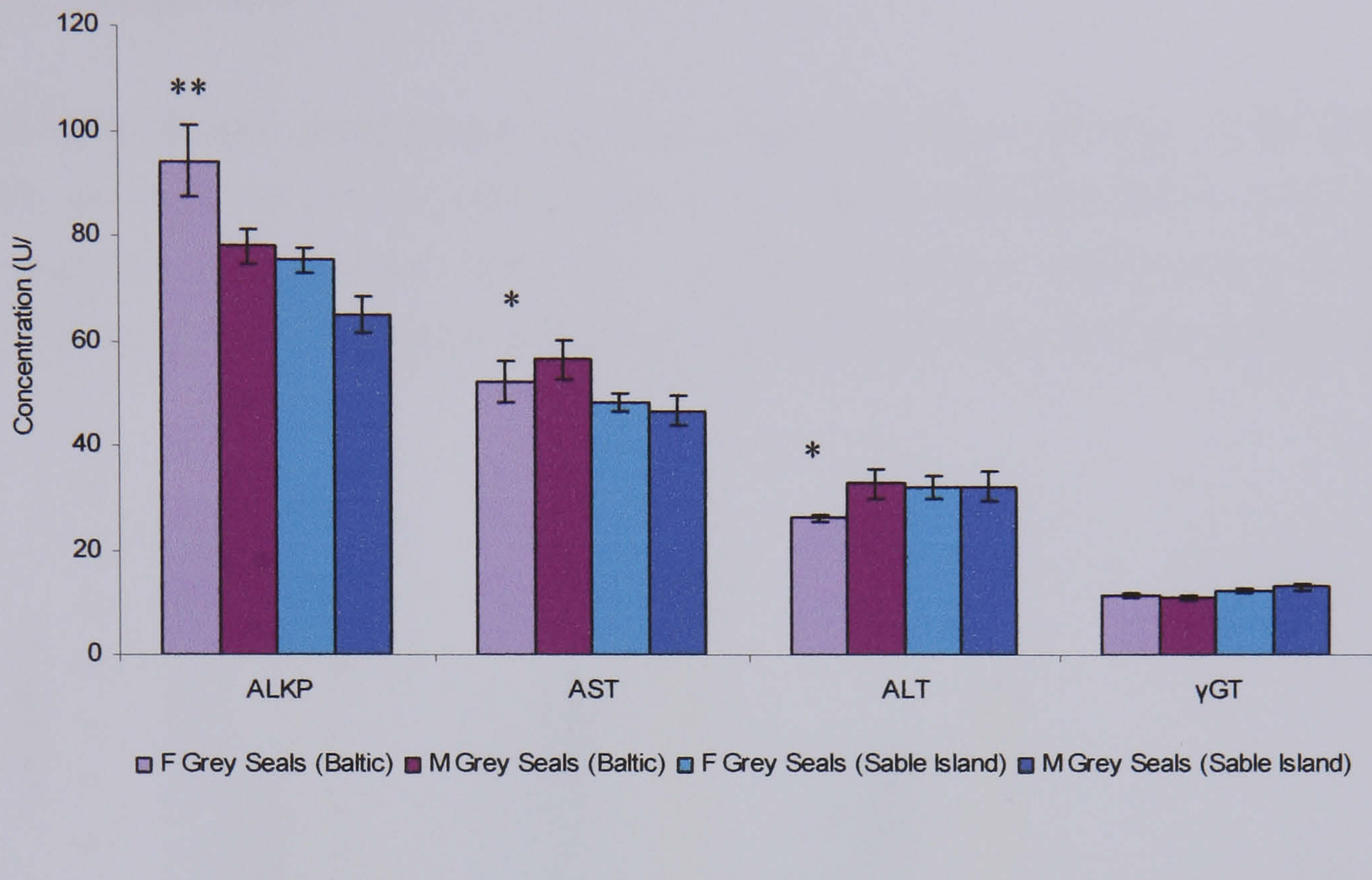



**Figure 5.14:** Mean ±SE AST, ALKP, γGT and ALT concentrations in Grey seals
The significance of exposure variation is presented as * $p<0.05$ or ** $p<0.005$
F, Females; M, Males

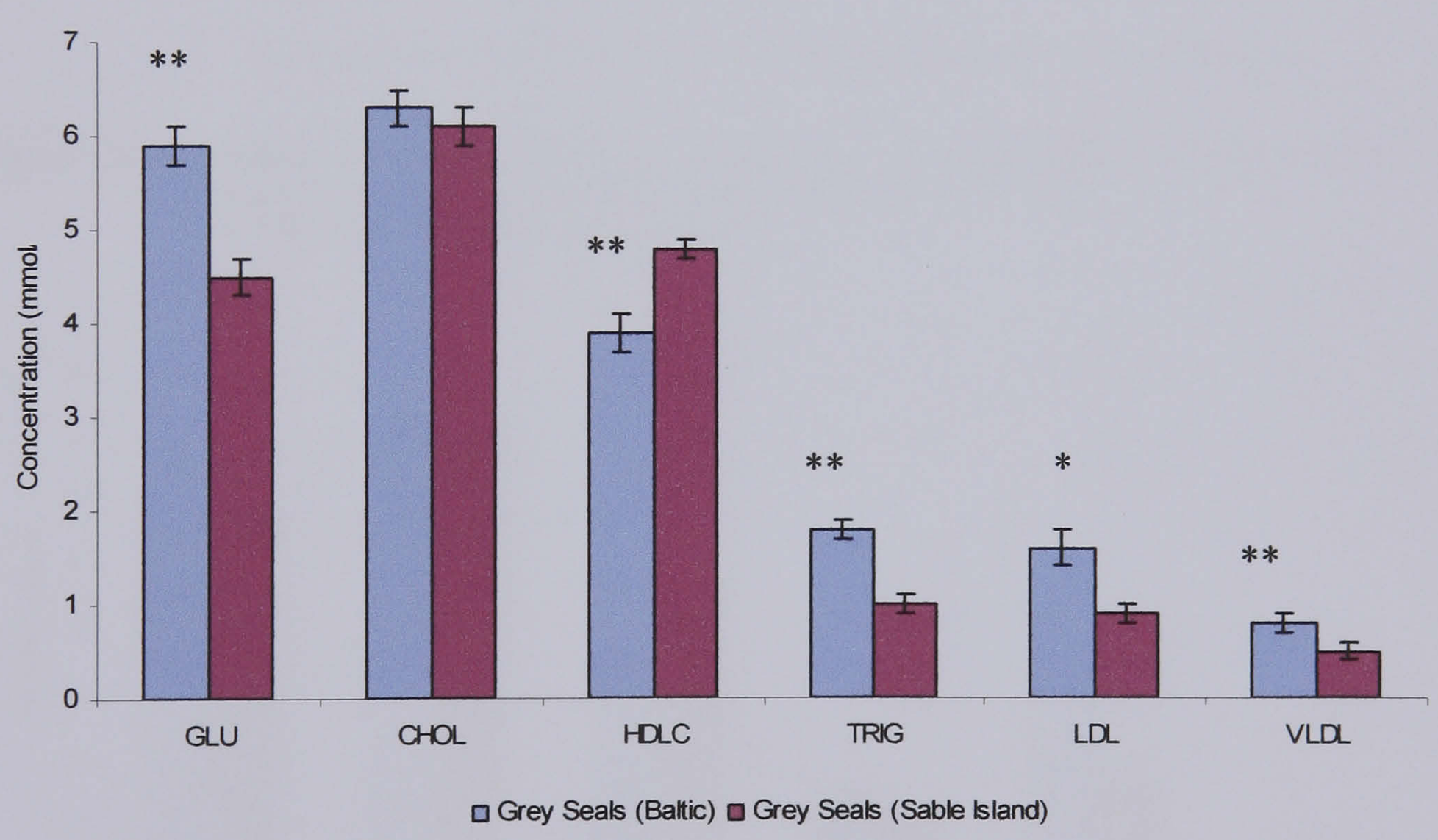



**Figure 5.15:** Mean ±SE glucose, CHOL, HDLC, TRIG, LDL and concentrations in Grey seals
The significance of exposure variation is presented as * $p<0.05$ or ** $p<0.005$

*Ringed Seals*

The Baltic Ringed seals showed statistically significant higher albumin, ALKP (Fig.5.16), LDL ($p<0.005$), CK, VLDL, triglyceride (Fig.5.17) and glucose ($p<0.05$) levels compared to Ringed seals from Svalbard. Mean ALT, urea, uric acid, HDLC, total protein ($p<0.005$) and AST ($p<0.05$) levels were found to be statistically significant reduced in Baltic Ringed seals.

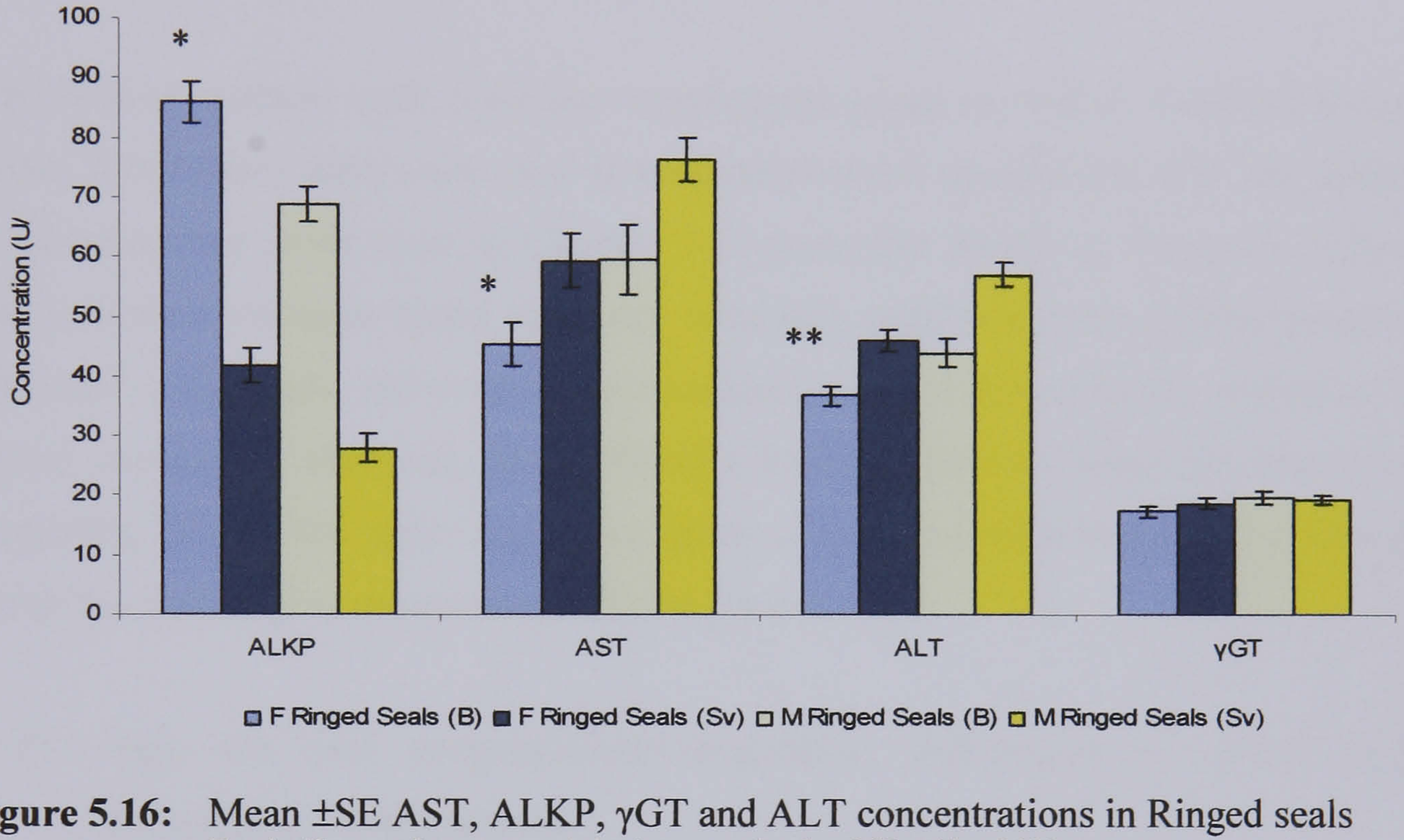



**Figure 5.16:** Mean ±SE AST, ALKP, γGT and ALT concentrations in Ringed seals
The significance of exposure variation is presented as * $p<0.05$ or ** $p<0.005$
F, Females; M, Males; B, Baltic; Sv, Svalbard

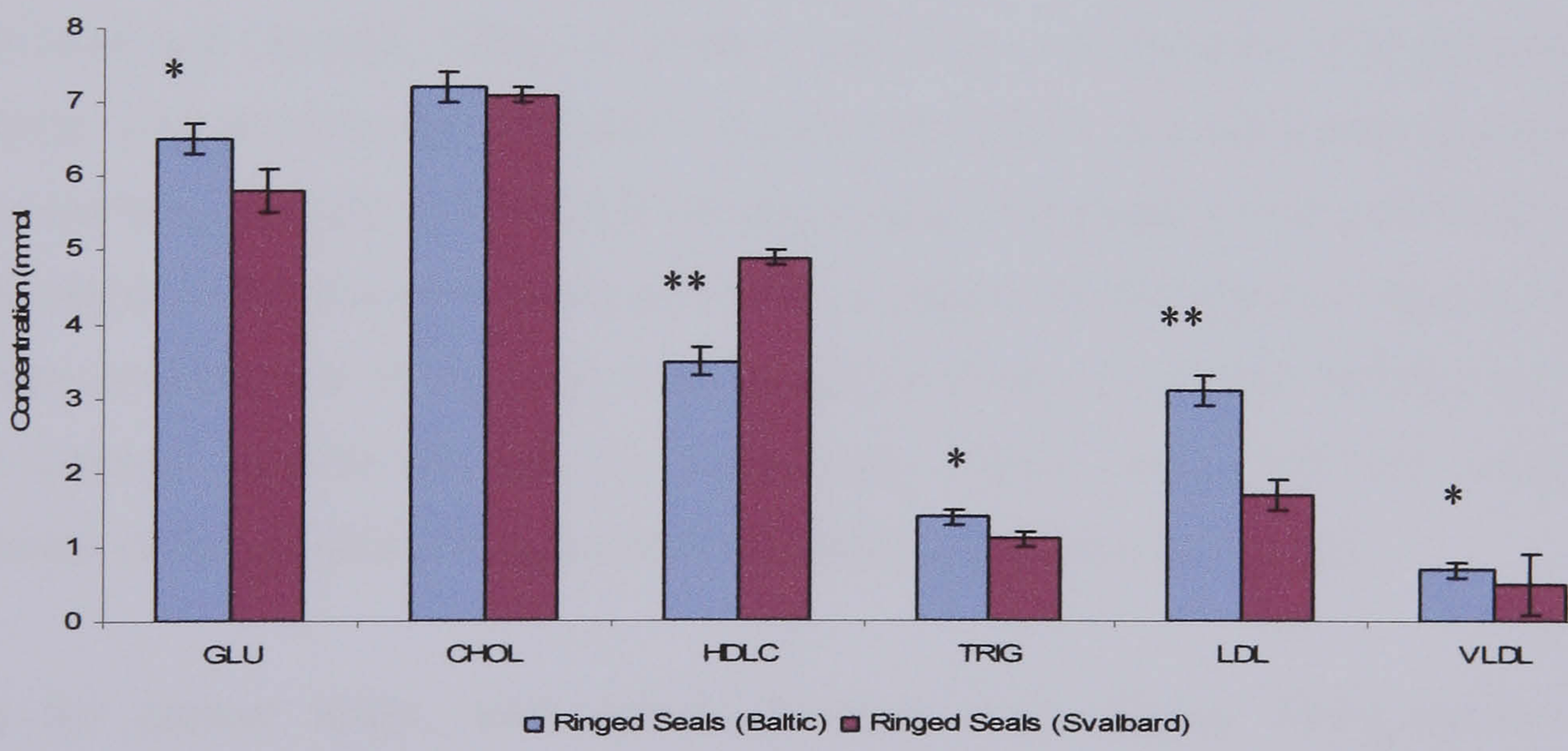



**Figure 5.17:** Mean ±SE glucose, CHOL, HDLC, TRIG, LDL and VLDL concentrations in Ringed seals
The significance of exposure variation is presented as * $p<0.05$ or ** $p<0.005$

## 5.4 Discussion

Published data on blood chemistry values for seals are rare and (McConnell and Vaughan, 1983; Bossart and Dieurauf, 1990; Schumacher *et al.*, 1995) and there can be considerable variation between published sources of normal ranges. Therefore, new baseline data for seals is clearly critical for improving existing knowledge and forming the basis for investigating the suitability of these parameters as biomarkers for monitoring pollutant exposure and effects in free-ranging seal populations.

The control Harbour seals from the experimental group as well as Svalbard Ringed and Sable Island Grey seals were used as reference/control populations (*For full justification on contaminant levels refer to Chapter 2-Contaminant Analysis*). However, values from the published reference blood chemistry were also used to support reference/control data obtained in this study. All samples were analysed using analysers for the analysis of human blood parameters. However, they offered a broad calibration range for each parameter extending beyond the upper and lower limits of human normal ranges ensuring adequate scope for quantitative measurement for the present study.

### 5.4.1 Age, sex and geographically-dependent differences in blood chemistry parameters within species

ALKP levels are associated with bile stasis, liver disease and intestinal inflammation (Bossart and Dierauf, 1990). In humans, age does not influence liver function enzyme levels, with the exception of ALKP. As this enzyme is associated with growth and bone remodelling it is found in higher levels in children (Schumacher *et al.*, 1995). In this study, age-dependent differences could only be investigated for UK Harbour seals (Moray Firth) since samples from all the other study populations were from adult animals only. As seen in humans, juvenile females had significantly higher levels of ALKP compared with female adults and this difference was statistically significant ($p < 0.005$)

In the present study, adult female Harbour seals (Moray Firth) showed reduced triglycerides, HDLC, VLDL and ALKP concentrations compared to adult males. Such differences in lipid levels could be attributed to sex-dependent differences in metabolism and feeding habits (Ellfolk *et al.*, 1997). In humans, lipid variations between male and females has also been attributed to an estrogen-dependence influence on lipids in which

estrogens increase the VLDL pool size and plasma triglyceride concentrations (Wolfe and Huff, 1995; Wolfe *et al.*, 2000; *Zhang et al.*, 2001).

AST activity predominates in hepatocytes and is used in screening for liver failure (Ellfolk *et al.*, 1997). AST and ALKP were found to be significantly higher in female adult compared to male adult Harbour seals in this study (Abertay Sands). However when compared with published reference/baseline AST values were found to be within the same range.

When the Scottish Harbour seals where compared, mean total protein, albumin, ALT, glucose, ALKP and the liver function enzymes ALT and AST were significantly lower ($p<0.005$) in seals from Abertay Sands whereas LDL ($p<0.05$) was significantly higher in Harbour seals from Abertay Sands compared to seals from Moray Firth. All levels however, were within the range of reported baseline levels for Harbour seals except for γGT levels that were found reduced in both populations. These differences could be attributed to different metabolism and feeding habits (e.g., albumin, total protein, glucose; Nyman *et al.*, 2003).

Of the analysed blood chemistry parameters, ALT was found to be higher in male Baltic Grey seals compared to females ($p<0.05$) whereas ALKP was seen to be reduced ($p<0.05$). ALKP was also found to be reduced in male Grey seals from Sable Island compared to females. As Grey seals were sampled during Stage II (delayed implantation) possibly some of the female seals could have been pregnant and therefore attributing to the higher levels of ALKP concentrations. However, when comparing ALKP levels with reference values it was found that Grey seals were within the range of the published reference values for Grey seals.

Female Baltic and Svalbard Ringed seals also showed reduced ALT ($p<0.05$ and $p<0.005$ respectively) concentrations compared to males and increased ALKP concentrations ($p<0.005$). Moreover it was found that female Ringed seals from Svalbard showed slightly reduced AST levels ($p<0.05$) compared to males.

### 5.4.2 Validation of blood chemistry parameters as biomarkers of endocrine disruption

The physical condition and nutritional status of humans, domestic and captive animals is generally assessed using blood chemistry parameters. However, some blood chemistry parameters have also been associated with contaminant exposure. For example in humans and rodents, VLDL, LDL and triglycerides have been demonstrated to increase from PCB exposure (Allen *et al.*, 1976; Kreiss *et al.*, 1981). Moreover, hepatic lipid accumulation has been proposed as a protective response against the oxidative stress caused by enhanced metabolism of PCBs (Borlakoglu and Welsch, 1992). Since blood chemistry parameters are measured in blood, they are an ideal tool for non-destructive biomonitoring and therefore their investigation as potential markers of PCB exposure in animals is necessary.

*Dose-dependence*

The control Harbour seals from the experimental group were found to have statistically significant higher HDLC ($p<0.005$) and significantly lower triglyceride ($p<0.05$), LDL ($p<0.005$) and VLDL ($p<0.05$) (Fig.5.12) levels compared to the exposed group. Moreover, the liver function enzymes ALT ($p<0.005$), AST ($p<0.05$) and γGT ($p<0.05$) as well as albumin ($p<0.05$), ALKP ($p<0.05$), uric acid ($p<0.05$) and glucose ($p<0.005$) levels were found to be statistically significant higher in the exposed group compared to the control (Fig.5.13).

Female Harbour seals from Abertay Sands were found to have significantly reduced cholesterol ($p<0.05$), triglycerides, VLDL, HDLC and glucose levels ($p<0.005$; Fig.5.5) compared with the control Harbour seals from the experimental group. ALKP, cholesterol, triglycerides, VLDL and HDLC levels were also statistically significant reduced in Harbour seals from Moray Firth compared to the experimental control Harbour seals ($p<0.005$).

Albumin, glucose, triglyceride, VLDL and LDL levels were found to be statistically significant higher in both Baltic Ringed and Grey seals when compared to Svalbard Ringed and Sable Island seals whereas HDLC, blood urea nitrogen and ALT levels were found significant reduced in the Baltic seals compared to Ringed and Grey seals from reference areas. AST levels were found reduced in Baltic compared to Svalbard Ringed seals and significantly increased in Baltic Grey seals compared to seals from Sable Island.

Blood chemistry parameters according to species for all females and males of the three seal species under study were compared with ΣPCB concentrations previously measured (Chapter 2). In the Harbour seal experimental group there was statistically significant positive correlation of γGT ($p<0.005$), triglyceride ($p<0.001$), VLDL ($p<0.001$), LDL ($p<0.001$) and glucose ($p<0.05$) levels with ΣPCBs in a dose-dependent manner. HDLC levels showed a statistically significant negative correlation ($p<0.05$) with contaminant exposure (Fig.5.14 to 5.19). In order to validate the VLDL results, hormone-dependence variation (estrogen) was tested for the female Harbour seals and a statistically significant negative correlation ($p<0.05$) was observed. This was expected since Harbour seals were sampled at pregnancy and therefore estradiol levels are expected to be low at this stage of reproductive cycle (Atkinson, 1997). High lipid levels together with this significant observation may signify the action of environmental estrogens mimicking the action of endogenous estrogen hormones which may have potential implications on lipid metabolism having as a result lipid accumulation in the liver. The fact that a low correlation coefficient was observed in the linear regressions indicating that the above relationships were not as strong as desired does not exclude the use of these blood chemistry parameters as biomarkers of endocrine disruption. There is a clear trend observed in the linear regressions that could have been amplified if more samples from all the stages of the reproductive cycle were available for the study.

### 5.4.3 Biomonitoring ED with blood chemistry biomarkers in study populations

Biomarker (BM) expression over a gradient of contaminant exposures was investigated in all three seal species using linear regressions (Fig.5.21-5.39). When there was no sex-dependent difference, female and male samples were pooled. Moreover, in order to identify specific populations affected by ED (being at risk), BM expression in exposed groups was compared with control populations and differences were tested using student t-test. Where BM expression was statistically significant, populations were classified at risk from ED. From all the blood chemistry parameters studied it was observed that VLDL and triglyceride levels had a positive correlation with ΣPCBs in a dose-dependent manner for all three species (Harbour, Grey and Ringed seals) and this relationship was statistically significant (Fig.5.25, 26, 30, 32, 33,34, 35, 38 and 39). Albumin (Fig.5.21 and 5.27) and glucose (Fig.5.24 and 5.31) levels were also found to be positively correlated with contaminant exposure but this relationship was only observed in Ringed and Grey seals. HDLC levels were found to be statistically significant negatively correlated with PCB exposure in female and male Ringed (Fig.5.29, $p<0.01$) and Grey (Fig.5.23, $p<0.05$) seals

but only in female Harbour (Fig.5.36, $p<0.05$) seals (Abertay Sands and Moray Firth). Female Harbour seal AST levels were found to be positively correlated with contaminant exposure in a dose-dependent manner but the statistical significance was not as strong ($p=0.05$). This relationship was not observed in male Harbour seals. Finally, there was a negative correlation observed between blood urea nitrogen levels and contaminant exposure in both Ringed ($p<0.001$) and Grey seals ($p<0.05$) and a positive correlation between LDL levels ($p<0.001$) and PCBs in Ringed seals.

In general, the blood chemistry parameters in the Ringed, Grey and Harbour seal populations studied were within the ranges previously reported for wild and captive pinnipeds (Greenwood, 1971, Engelhardt, 1979; Geraci *et al.*, 1979; McConnell and Vaughan, 1983; Bossart and Dierauf, 1990; De Swart *et al.*, 1995; Nyman *et al.*, 2003).

Elevated albumin was observed in both Baltic Ringed and Grey seals when compared with control Svalbard and Sable Island seal values. The Ringed seals were sampled in May, the season when they moult. During this period, seals gather in small groups or alone on the ice and reduce their food intake drastically. Therefore, during moulting season and especially during the first month of the fast, seals can loose up to half of their weight (Nyman *et al.*, 2003). Organochlorines, being lipophilic and persistent, are bioaccumulated in fat reserves and in the case of the seals, in blubber. During this extensive seasonal change in the fat reserves, organochlorines and especially PCBs that are accumulated in the blubber are released into the blood stream, in which they are transported by albumin (Peakall *et al.*, 1972; Busbee *et al.*, 1985). Therefore, this elevation in albumin in association with the statistically significant positive correlation with contaminant exposure could be a result of an increased demand for PCB blood transport during fasting. When PCBs enter circulation they are metabolised or released from chylomicrons during hepatic chylomicron clearance (Boon *et al.*, 1994). Therefore, these resulting PCBs or PCB derivatives circulate in association with albumins. Moreover, when comparing the albumin values with other species, the levels slightly diverged from the reference seal populations.

Blood urea nitrogen levels in Baltic Ringed and Grey seals were negatively correlated with contaminant exposure in a dose-dependent manner and were also found statistically significant reduced compared to Ringed and Grey seals from reference areas. BUN levels are found to decrease during liver failure and starvation (Bossart *et al.*, 2001). As stated earlier, moulting season is a period of stress and starvation for the seals. Grey and Ringed seals were sampled during this period. Due to changes in metabolic activity, blood urea

levels reduce significantly during the first two weeks of fasting. At the same time of this negative energy balance PCBs are released from the blubber into the bloodstream (Nyman, 2003). Reduced urea levels in the Baltic seal populations could indicate that these seal populations had proceeded further in the fasting than in the reference areas (Greenwood, 1971; Geraci *et al.*, 1979; Nyman *et al.*, 2003).

Glucose, triglyceride, VLDL and LDL levels were found to be statistically significant higher in Baltic Ringed and Grey seals compared to reference areas whereas HDLC levels were found to be reduced. The availability of baseline values for many blood chemistry parameters is limited. Harbour, Ringed and Grey seals belong to the same family, Phocidae and the same sub-family, Phocinae (Northern Phocids) and therefore comparison between the species is used extensively in literature (Schumacher *et* al., 1993 and 1995; Nyman, 2003). In the present study, comparison of biochemistry data for the Baltic Ringed and Grey seals with data from Harbour seals (Abertay Sands and Moray Firth), showed significantly higher levels of cholesterol, LDL and VLDL in Ringed and Grey than Harbour seals. LDL ($p<0.005$), VLDL ($p<0.05$) and triglycerides ($p<0.05$) were also found significantly increased in the exposed Harbour seals compared to control from the experimental group. It has to be noted that HDLC levels in all exposed populations (Harbour, Grey and Ringed seals) where found to be statistically decreased compared to the reference population of each species. Moreover, HDLC levels in male and female Grey and Ringed seals as well as in female Harbour seals were negatively correlated in a dose-dependent manner with PCB exposure. In studies on humans and rodents, increased total serum lipids, cholesterol and triglycerides are the most commonly found changes in blood chemistry parameters after PCB exposure (Allen *et al.*, 1976; Kreiss *et al.*, 1981; Yamamoto *et al.*, 1994; Nyman *et al.*, 2003). Elevated serum lipids indicate an accumulation of lipid in the liver, which results in a fatty liver. This is a pathological state commonly associated with degeneration and necrosis. In mammals, liver or peripheral lipid accumulation may result from the concentration of plasma triacylglycerols (most concentrated form of energy available) transported by VLDL and therefore plasma VLDL concentrations are a good indicator of hepatic lipogenesis (Davail *et al.*, 2000; Gibbons *et al.*, 2000). Moreover, non-metabolised VLDL and catabolised forms such as LDL return to the liver where the triacylglycerols can be stored and contribute to hepatic steatosis (relative imbalance between lipid synthesis and VLDL assembly and secretion; Hermier *et* al., 1996; Davail *et al.*, 2000, Scheen and Luyckx, 2002). Hepatic lipid accumulation has also been proposed as a protective response against the oxidative stress caused by enhanced metabolism of PCBs (Borlakoglu and Welsch, 1992). The observed differences

in cholesterol levels could also be related to possible differences in fatty acid composition in the diet between the different populations. Moreover, in the case of Harbour seals (experimental group) pregnancy can attribute to the high levels of lipoproteins observed since these seals were sampled approximately four weeks before termination of pregnancy. During pregnancy plasma triglycerides increase in parallel with VLDL (Sattar *et al.*, 1997, Duggan *et al.*, 2001). However, pre-existing hypertriglyceridaemia or hyperlipidaemia can be exacerbated in pregnancy (Halpern, 1995).

TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin), the most potent organochlorine carcinogen, due to its coplanar molecular structure causes a diversity of toxic effects in animals, including effects on metabolism and reproduction. Like PCBs, TCDD due to its lipophilic properties partition into fatty tissues such as adipose (blubber) and the liver. In mammals TCDD has been found to decrease the lipoprotein lipase (LpL) activity, a key enzyme responsible for the catabolism of VLDL, with a loss of triglycerides and a parallel increase of cholesterol in high density lipoprotein (HDL; Sirtori, 1995). Therefore inhibition of LpL activity in adipose tissue results in increased levels of circulating lipids, promotion of TCDD partitioning into peripheral circulation and increase of the potential for systemic TCDD adverse effects (Stanton *et al.*, 2001). Since certain PCB congeners (e.g.118) have similar molecular structure to TCDD (Boon, 1992) it could be proposed that they may function in a similar toxic mechanism on lipid metabolism. For example, VLDL not hydrolysed by LpL may return to liver and contribute to hepatic steatosis. Therefore, the significantly elevated levels found in all studied exposed populations at the present study and since the use of triglycerides and lipoproteins such as VLDL are well established by published data in laboratory animals that increase during PCB exposure, indicates that they could be used as possible biomarkers of endocrine disruption in marine mammals.

As already mentioned, exposed Harbour, Ringed and Grey seals showed statistically significant increased glucose levels compared to reference populations. Hypertriglyceridaemia associated with increased insulin resistance is a product of increased VLDL secretion since increase influx of free fatty acids to liver associated with increased insulin resistance will further contribute to increased VLDL synthesis (Vega-López *et al.*, 2002). In humans, patients with liver steatosis express significant increased fasting plasma glucose, insulin, triglycerides and ALT levels (Scheen and Luyckx, 2002). Insulin resistance is associated with various metabolic abnormalities such as increased triglycerides, high levels of small dense LDL and decreased HDLC. Moreover, decreased LpL activity in combination with increased VLDL synthesis results in elevated plasma

concentrations of triglyceride rich lipoproteins leading to triglyceride enrichment of LDL and HDL associated with decreased HDLC. The combination of increased cortisol and insulin concentrations coupled with decreased sex steroid levels is thought to exert powerful effects leading to increased serum triglyceride concentrations (Weltman *et al.*, 2003). Hepatic steatosis is acquired from metabolic disorders and associated with insulin resistance i.e. increased in plasma glucose levels (Hermier *et al.*, 2003). Insulin stimulates the activity of enzymes that catalyse the hydrolysis of triglycerides contained in particles such as VLDL (i.e. LpL) and therefore contaminants such as TCDD and possibly PCBs and DDT that decrease LpL activity can contribute to increase glucose and lipoprotein levels in plasma resulting in hepatic steatosis (Davail *et al.*, 2003).

AST and ALT are mainly active in the hepatocytes where their activity predominates, and therefore are used in clinical screening for liver failure (Ellfolk, *et al.*, 1997). ALT levels were significantly reduced in Baltic Ringed and Grey seals when compared with the reference populations whereas AST levels were found reduced in Baltic compared to Svalbard Ringed seals and significantly increased in Baltic Grey seals compared to seals from Sable Island. Moreover, AST levels were found to be positively associated with PCB exposure in female Harbour seals (Abertay Sands and Moray Firth). In a study by Schumacher *et al.*, (1995) free-living seals from highly contaminated waters, showed higher urea and cholesterol levels while ALT, AST and γGT levels were lower than in seals from less polluted waters. In the present study Baltic Ringed seal γGT levels showed no statistically significant difference from the reference area (Svalbard) whereas Baltic Grey seals showed a slight increase compared to the reference population from Sable Island. The observed values (Schumacher *et al.*, 1995) differ from those observed previously by Reijnders (1988). In a captive feeding study in which two groups of Harbour seals were fed fish containing different levels of contaminants he found elevated ALT, AST, ALKP and γGT levels in the seals fed on contaminated fish. In the present study these seals were used for biomarker validation and were re-analysed in order to get values for other blood chemistry parameters that were not previously studied. The results obtained had the same associations as previously described (Reijnders, 1988). Moreover, γGT levels were found to be positively correlated with PCB exposure ($p<0.005$). The reason of the varying changes in the blood chemistry parameters between the Ringed and Harbour seals could be attributed to the fact that they are different species. However, association with toxic exposure should not be excluded for the observed concentration changes in the liver function enzymes, however due to scarcity of information on baseline values more samples should be analysed (Reijnders, 1988; De Swart *et al.*, 1995). Last but not list, the

difference in the levels of liver function enzymes between this study and others could be attributed to different types of damage in the liver. For example, ALT and AST levels increase in hepatocellular necrosis, neoplasia or parasitism whereas they are found to decrease with hepatic cirrhosis (Bossart *et al.*, 2001).

In conclusion, the blood chemistry levels measured in the three seal species were within the range of published references. Liver function enzymes such as ALT and AST did not show indication that changes could be attributed to disease or toxicity. However, the strong correlation of triglycerides and lipoproteins (VLDL, LDL, HDLC) with PCB exposure observed in the exposed populations suggests the use of these parameters as biomarkers of endocrine disruption. Nevertheless a larger number of samples is necessary for a more scientifically robust evaluation. Therefore, more research is necessary in order to establish a link between environmental pollution and changes in the blood chemistry parameters. Moreover, the levels of other blood chemistry parameters such as insulin and the activity of enzymes such as LpL could also be studied in future in order to establish any correlation with contaminant exposure. Last but not least, the blood chemistry values are valuable as baseline values for monitoring the health of free-ranging seals and especially during stress conditions such as fasting as it was observed for Baltic Ringed and Grey seals.

## 6.0 General Discussion

Over the past years there has been increased concern regarding widespread pollution of the environment with lipophilic, persistent organic pollutants (POPs) such as PCBs, DDT (DDD and DDE) and PBDEs resulting from industrial, agricultural and other anthropogenic activity. Many of these chemicals are known to possess a wide range of toxic effects, including reproductive failure, immunosuppression and endocrine disruption in humans and wildlife (Addison, 1989; Thomas, et *al*., 2005). Marine mammals and especially seals being top predators of the marine food chain are significantly exposed to biomagnified levels of POPs (Aguilar et *al*., 2002; Law et *al*., 2002) and have been widely used for contaminant evaluation in the marine environment since they are good indicators of the ecosystem contamination (Shaw *et al*., 2005).

In marine mammals evidence of reproductive failure (DeLong *et al*., 1973; Helle and Olson, 1976a, b; Freeman and Sangalang, 1977; Reijnders, 1980; Gillner et *al*., 1988; Baker, 1989; Troisi and Mason, 2000), cancer (Martineau et *al*., 1994) neurotoxicity, hepatotoxicity (O'Shea, 1999) and endocrine disruption (Fielden *et al*., 1997) has been observed. A number of different estrogenic and anti-androgenic effects, immunosuppression (De Swart et *al*., 1995a; Thomas *et al*., 2005) significant population declines and poor recovery in marine mammals have been reported attributable to POPs (Hook and Johnels, 1972; Reijnders, 1980; Bergman and Olsson, 1985; Reijnders, 1986; Subramanian et *al*., 1987; Barret et *al*., 1992; Hall et *al*., 1992; Osterhaus et *al*., 1992; De Swart et *al*., 1995a). Therefore, early detection of the adverse health effects resulting from pollutant exposure is vital and biomonitoring studies of marine mammal populations are needed. A biomarker that can be used as an early warning system for the reproduction and survival of the animal is an ideal tool for population biomonitoring (Depledge, 1994).

The overall aim of this project was to research and develop new and existing exposure and effect biomarkers for biomonitoring endocrine disruption in pinniped populations. The research aim was firstly addressed by quantifying the exposure level (PCBs, DDT and PBDEs) in non-destructive/non-invasive (faeces and serum) samples of the study seal populations using chromatographic and immunoassay techniques. Then sex steroids, lactoferrin (LTF) and blood chemistry parameters were investigated for establishing baseline

values and for their reliability to be used as biomarkers of PCBs, DDT and PBDE exposure and effects in seals. Finally, the biomarker expression was used in order to identify populations at risk from POP-associated adverse health effects.

## 6.1 Contaminant Analysis

In the present study a total of 29 PCB, 5 PBDE congeners as well as DDT and its metabolites (DDD, DDE) were measured using GC analysis in seal faecal samples collected from three UK sites (Blakeney Point, Donna Nook and Tees Estuary) inhabited by Harbour and Grey seals. This is the first study to analyse ΣPCB concentrations using an immunoassay (ELISA) technique in serum samples from UK Harbour, Grey (Baltic and Sable Island) and Ringed (Baltic and Svalbard) seals. At the present there are no commercially available immunoassays to measure total PCB in blood samples from seal populations. Even though GC methods are the most accurate since they provide congener-specific analysis in biological samples the ELISA method was chosen in order to provide a non-destructive, cost-effective, simple and rapid method for the analysis of ΣPCB in serum samples.

ΣPCB concentrations in faeces in all UK seal populations under study ranged from 0.04 to 8.9μg/g lipid weight. ΣDDT concentrations ranged from being non-detectable to 19.10μg/g lipid weight whereas ΣPBDE concentrations in faecal samples ranged between non-detection to 14.42μg/g lipid weight. The range of blood ΣPCB concentration for all populations was found to be between 0.36 to 87.06μg/ml, indicating that faecal ΣPCB levels were an order of magnitude lower than blood levels.

The data obtained from the serum analysis showed that PCB levels in Baltic and especially Grey seals are higher compared with the Svalbard Ringed and UK Harbour seals (Abertay Sands and Moray Firth). Ringed Svalbard seals showed statistically significant lower ΣPCB levels ($p<0.005$) when compared with all other seal populations. The pattern of the different groups of contaminants analysed in seal faecal samples was the same for Tees Estuary and Donna Nook seals (ΣDDT > PCBs > ΣPBDEs) whereas Blakeney Point Harbour seals showed higher ΣPBDEs compared to ΣPCBs (ΣDDT > ΣPBDEs > ΣPCBs) highlighting the emergence of PBDEs as a significant concern in the marine environment. The congener profile in faeces from all three populations was characterized with the dominance of higher-chlorinated, coplanar and metabolically stable PCB congeners such as 153, 156, 194, 180 and 118. This

pattern was expected since faecal OC levels are the result of a complex interaction of varying exposures, metabolism and clearance over the period of life and they represent the contaminant load not metabolized or retained in the body as well as recent dietary intake (Krahn *et al.*, 2001). In all three seal populations reported in the present study ΣDDT levels were lower than ΣPCB levels and the predominant OCP was DDT followed by DDD and DDE in seals from Tees Estuary and Blakeney Point, whereas DDE was found to be in higher concentrations compared to DDD in seals from Donna Nook. From the five PBDEs under investigation, congener 47 was found at the highest concentration followed by 99 in Tees Estuary seals and 153 in Donna Nook and Blakeney Point. The dominance of PBDE 47 in the current PBDE congener profile has also been observed in marine and terrestrial mammals including human blood plasma, grey seal blubber, whales and human milk in the highest concentrations of all PBDEs measured (Haglund *et al.*, 1997; Meironyté *et al.*, 1999; Lindström *et al.*, 1999).

The order of exposure of the seal populations under study was as follows:
Grey Baltic seals>Ringed Baltic seals>**Exptal (E)**>Harbour Moray Firth seals>Harbour Abertay Sands seals>Grey Sable Island seals>**Exptal (C)**>Ringed Svalbard seals. It has to be taken into consideration that the above order is derived from pooling female and male data. When considering the sex-dependent variations in biomarker expression, Grey seals from Sable Island were also accounted as a control population since the contaminant levels were found to be statistically significant lower from the exposed.

The serum ΣPCB concentrations observed in the present study were in agreement with published data. The levels of organic contaminants are still high in the Baltic seal populations, although ΣDDT has decreased during the last decades (Nyman *et al.*, 2002), whereas OC levels in the European Arctic and Canada have been reported to be low compared with seal populations from the Baltic and the North Sea (Muir *et al.*, 2000c; Braune *et al.*, 2005). Serum ΣPCB levels in Harbour seals were found to be lower when compared with studies in West Scotland and East England (Law *et al.*, 1989; Hall *et al.*, 1992) and higher when compared to Harbour seals from East Scotland, North Shetland and Norfolk Wash (Holden *et al.*, 1972; Hall *et al.*, 1992; Ruus *et al.*, 1999). It has to be noted that the current study investigated ΣPCB in serum samples whereas the above studies used blubber and therefore significant differences between the results are expected. The stage of reproduction that the seals are

sampled plays a significant role since OCs in blubber are metabolized when a seal is in negative energy balance such as lactation and when seals are fasting, mating and moulting seasons and when an animal is sick (Hall *et al.*, 1992). Therefore it could be proposed that seals with high blubber OC levels would also have high circulating levels at this time. Lipid mobilization would also influence faecal OC concentrations along with dietary intake since faecal OC levels reflect excretion of PCB congeners not metabolized or retained in the body in addition to recent dietary intake. Therefore, use of faecal OCs has value as an indicator of recent OC exposure, particularly in the area where the sample was collected and as an index to compare relative spatial and temporal exposure, however it is not necessary a reflection of the individual congener concentrations in body depot stores. This matrix, as a contaminant exposure-monitoring tool, has great promise because it can be collected without physical capture of animals which is very expensive and potentially dangerous to both humans and seals. Unfortunately, this matrix is not useful in predicting the body burden of a particular individual animal (Krahn *et al.*, 2001).

## 6.2 Sex Steroids

Sex steroids are likely targets for contaminant-induced impairment of reproduction and therefore due to the increasing need to assess the endocrine status of marine mammals, a non-destructive monitoring diagnostic tool is required that can provide fast and reliable assessment (Griffin and Ojeda, 1996; Portier, 2002; Thomas, *et al.*, 2005). The use of sex steroids in serum and faeces as valuable biomarkers has been proposed by several studies including marine mammal monitoring (Reijnders, 1990; Wasser *et al.*, 1991; Schwarzenberger *et al.*, 1996; Lasley and Overstreet, 1998; Matsumuro *et al.*, 1999; Greig, 2002; Linklater, *et* al, 2000; Persky *et al.*, 2001; Turner *et al.*, 2001, Larson *et al.*, 2003; Lynch *et al.*, 2003). However, little information is available for seal serum sex steroid concentrations and none for faecal concentrations, thus it is important to establish normal baseline levels of sex steroids over the reproductive cycle. Moreover, very little information is available concerning altered sex steroid levels in seals due to EDC exposure thus investigation of the relationship of sex steroids with contaminant exposure is needed.

This is the first study to measure sex steroid levels in faeces of seals and to investigate their correlation with PCB exposure. This study therefore constitutes a major contribution to

providing vital information needed as part of the validation of steroids as biomarkers of endocrine disruption. Progestagens, estrogens and androgens were quantified in seal serum and estrogens were quantified in seal faecal samples. Detailed data on levels and ranges of sex steroids measured in faecal and serum samples are presented in Chapter 3 as well as with sex and age-dependent differences. Matrix-dependence and statistically significant inter-species variations in steroid concentrations are presented in short.

Since no comparison could be made with similar published data it was not possible to make accurate conclusions on faecal-serum relationship. However, when comparing serum for female Harbour seals (Abertay Sands) and Harbour seal faecal (Tees Estuary) estrogen levels it was observed that in general faecal samples had higher concentrations of E2 and E3 in all reproductive stages compared with serum samples. Moreover, it was observed that E2 and E3 in both matrices followed the same trend in the different stages of reproductive cycle. The results of the present study were supported in other mammalian studies in which faecal estrogen levels were found in higher concentrations compared to serum levels (Matsumuro *et al.*, 1999).

P ($p<0.05$) and 17α-OHP ($p<0.005$) levels in Baltic female Grey seals were higher compared to Ringed seals, whereas male Grey seals had higher ($p<0.05$) P, 17α-OHP, E3 and A levels compared to male Ringed seals. Female Harbour seals from Abertay Sands showed higher 17α-OHP ($p<0.005$), A ($p=0.05$) and DHEA-S ($p<0.05$) concentrations when compared with Moray Firth female Harbour seals. Harbour male seals from Abertay Sands were found to have statistically significant higher P ($p<0.005$) and lower DHEA-S ($p=0.05$) levels compared to male Baltic Grey seals and statistically significant lower E2, E3, T ($p<0.05$), 17α-OHP ($p<0.005$), E2 when compared with male Sable Island, Grey seals. P, 17α-OHP , A ($p<0.005$) and E2 levels were found to be statistically significant higher in male Harbour seals from Abertay Sands compared to male Baltic Ringed seals.

Serum P and E2 concentrations were negatively correlated with ΣPCBs in dose-dependent manner and this relationship was statistically significant ($p<0.05$). In faecal samples ΣPCBs were in a positive correlation with E3 in Stage II and with E2 in Stage III ($p<0.05$). For ΣDDTs a positive correlation was observed with E3 in Stage II and with E2 in Stage IV and a negative correlation with E1 in stage III of the reproductive cycle ($p<0.05$). Finally, for

ΣPBDEs there was a statistically significant dose-dependent positive correlation with E3 ($p<0.05$) in Stages I and II, with E2 ($p<0.05$) in Stage III and a dose-dependent negative correlation with estrone ($p<0.05$) in Stage I of the reproductive cycle.

Serum and faecal sex steroids analysed in the study showed a statistically significant correlation with exposure, however a low correlation coefficient was observed in the linear regressions indicating that this relationship was not as strong as it was expected. However, this observation should not be a limiting factor in the use of sex steroids in biomonitoring of endocrine disruption since EDCs are able to alter the natural balance of endogenous hormones in tissues and circulating plasma (Griffin and Ojeda, 1996; Portier, 2002; Thomas, *et al.*, 2005). There is a clear trend observed in the linear regression that could have been amplified if more samples from all the stages of the reproductive cycle were available for the study.

In general, the use of blood and faecal samples for steroid analyses is a useful biomonitoring tool for assessing the reproductive status in seals as well as to investigate if these mammals suffer from endocrine disruption. The use of faeces for steroid analysis enables data to be collected in situations in which blood collection may prove either detrimental to the animals or impossible to obtain (Billitti *et al.*, 1998). These two matrices are ideal for population monitoring of protected species since large sample sizes can be obtained non-destructively (faeces) or with minimal invasion (blood) providing reproducible results. Re-sampling individuals for temporal exposure studies is also possible for surveillance monitoring (Fossi *et al.*, 1999). Blood and faecal sex steroids can be analysed by immunoassays that are able to cope with the complex nature of faecal matrix, making blood and faecal steroid analysis practicable, cost-effective and high-throughput (Schwarzenberger *et al.*, 1996). In terms of minimizing disturbance, faecal steroid biomonitoring is proposed as more ideal for studying seal populations since these samples can be collected at all stages of the reproductive seal cycle providing large sample size that is optimal for biomonitoring studies. However, the sex and originating individual of the faecal sample should be known for a better understanding of the normal fluctuations of sex steroids due to ED.

From this study it can be concluded that Baltic Ringed and Grey seals are considered populations at risk since they significantly deviated from the normal/control group data. This study shows that especially the Baltic Ringed seals are still suffering from a very high contaminant burden that poses a threat to their health status. Therefore, these populations are

at risk from toxic effects to endocrine-dependent processes such as growth, development and reproduction (Portier, 2002).

## 6.3 Lactoferrin (LTF)

Many natural and synthetic estrogens, estrogen metabolites and xenoestrogens have been examined for their effects on LTF expression in mouse uterus or cultured human endometrial cells (Pentecost *et al.*, 1988; Yu and Chen, 1993; Gray *et al.*, 1994; Curtis *et al.*, 1997; Das *et al.*, 1998; Ghosh *et al.*, 1999; Markey *et al.*, 2001). These studies show that exposure to xenoestrogens stimulates LTF expression in the reproductive tract. The toxicity of environmental pollutants such as PCBs in the marine environment has been documented (Aguilar and Borrell, 1996), it is plausible therefore, that LTF homeostasis may be disturbed in animals exposed to elevated levels of these and other xenoestrogens, indicative of endocrine disrupting effects. LTF therefore has the potential to be used as a marker of estrogen exposure in animals exposed to environmental estrogens (LeBlanc and Bain, 1997). This is the first study to quantify and measure LTF levels in pinniped serum, uterine epithelium tissue and uterine flushings, to investigate the relationship with progesterone and 17β-estradiol through the reproductive cycle and to investigate the dose-response relationship with PCB exposure. This study therefore constitutes a major contribution to providing vital information needed as part of the validation of LTF as a biomarker of endocrine disruption.

The western blots showed staining for LTF protein at a lower molecular weight than that seen in humans and mice (70-80kDa) (Pentecost and Teng, 1987; Levay and Viljoen, 1995), however there are no published data on the size of LTF for comparison for any marine mammalian species. LTF levels, age and sex-dependent differences are presented in Chapter 4. Tissue-dependence and statistically significant inter-species variations in LTF concentrations are presented in short.

Tissue-dependent differences in LTF were as follows: uterine epithelium > uterine flushings > serum. The relative differences in LTF expression between uterine epithelial tissue, flushings and serum was as expected according to observations in other mammals, indicating that

antibody LTF binding level is consistent across in all sample matrices, permitting valid comparison of data.

When comparing LTF serum levels among the Harbour seals it was observed that UK Harbour seals had significantly higher mean LTF concentrations ($p<0.005$) compared to control Harbour seals from the experimental group. Statistically significant elevated serum LTF levels were observed in the exposed female Harbour seals (experimental group) when compared with UK Harbour seals (Abertay Sands and Moray Firth, $p<0.005$). Baltic Ringed and Grey seal LTF levels were also significantly higher from seals inhabiting the reference areas (Svalbard and Sable Island, $p<0.005$).

Serum LTF concentrations according to species and stage of the reproductive cycle for all females and males of the three seal species under study were compared with ΣPCB concentrations. Serum LTF concentrations of female Harbour seals (experimental group, Stage IV) were positively correlated with contaminant exposure ($p<0.001$). In female seals, statistical significance was observed for all stages of the reproductive cycle in all species except for Harbour seals at Stage III whereas male Harbour seals showed a positive correlation with ΣPCBs only in Stage I. LTF concentrations in male Ringed seals (Baltic and Svalbard) were found to be positively correlated with ΣPCBs. In order to validate the above results hormonal-dependence variation (estrogen and progesterone) was also tested for the female serum samples from all the three species and it was concluded that there was a negative correlation between LTF levels and estradiol and progesterone.

From the detection of high levels of LTF protein in the serum samples, the positive correlation with contaminant exposure and the negative correlation with estradiol and progesterone it could be proposed that these seals could be expressing high LTF levels due to the action of environmental estrogens mimicking the action of endogenous which may have potential implications on implantation. Moreover the presence of LTF protein in males is independent of the reproductive status in contrast with females and it has been demonstrated that abnormally elevated levels of LTF in serum from males occur as a result of xenoestrogen exposure (e.g., rodents and primates prenatally dosed with DES; Pentecost, *et al.*, 1988).

LTF serum values obtained in the study are very valuable since baseline values could provide an important tool for monitoring the health status of free-ranging seals especially during stress conditions (e.g. pregnancy and inflammation) since LTF levels in blood have been previously demonstrated to fluctuate during stress conditions (Rebelo *et al.*, 1995 & 1996; Thomas *et al.*, 2002, Kane *et al.*, 2003). LTF is also found in seminal vesicles and it has been reported to interact with the surface of sperm and becomes one of its major coating antigens (Goodman and Young, 1981; Lakritz *et al.*, 2000; Inagaki *et al.*, 2002). Moreover, it has been demonstrated in men, that serum and testicular estradiol levels are associated with oligospermia (Krause, 1988; Levalle *et al.*, 1994) and that increased circulation of seminal estradiol may increase seminal LTF secretion (Buckett, 1997). Therefore, baseline LTF levels obtained from this study could be a valuable tool in order to assess the male reproductive status as well endocrine disruptive effects from contaminant exposure.

Being hormonally regulated, as in the mouse, suggests that LTF is a sensitive marker for estrogen in the uterus and its expression could be a useful tool when studying effects of environmental estrogen exposure in seals. This preliminary study is the first to establish a positive correlation with ΣPCBs in seal serum and a negative correlation with estradiol and progesterone levels. Even though, the sample number used in this study was not big it was sufficient in order to derive conclusions on the LTF baseline levels in seals and the dose-dependent effect of xenoestrogens. The results of the present study and evidence from laboratory animals strongly support the use of LTF as a biomarker of endocrine disruption and since it can be measured in serum samples taken non-destructively, LTF remains a highly suitable biomarker for wildlife population biomonitoring and should continue to be studied on the basis of the preliminary findings of this study.

## 6.4 Blood chemistry parameters

Published data on blood chemistry values for seals are rare and are mainly derived from captive animals (McConnell and Vaughan, 1983; Bossart and Dieurauf, 1990; Schumacher *et al.*, 1995). Furthermore, there can be considerable variation between, published sources of normal ranges due to inter-laboratory variation and differences in animal husbandry. New data on blood chemistry parameters for seals is clearly critical for improving existing knowledge and forming the basis for investigating the suitability of these parameters as biomarkers for

monitoring pollutant exposure and effects in free-ranging seal populations. In the present study a range of blood chemistry parameters was analysed in seal serum from Harbour, Ringed and Grey seals and the correlation with contaminant exposure was investigated. Detailed data of mean and ranges of the parameters are presented in Chapter 5 as well as sex and age-dependent differences. Geographical dependent differences that were statistically significant are presented in short.

In general, the blood chemistry parameters in the Harbour, Ringed and Grey seal populations studied were within the ranges previously reported for wild and captive pinnipeds (Greenwood, 1971, Engelhardt, 1979; Geraci *et al.*, 1979; McConnell and Vaughan, 1983; Bossart and Dierauf, 1990; De Swart *et al.*, 1995; Nyman, 2003). When the Scottish Harbour seals where compared, mean total protein, albumin, ALT, glucose, ALKP and the liver function enzymes ALT and AST were significantly lower ($p<0.005$) in seals from Abertay Sands whereas LDL ($p<0.05$) was significantly higher in Harbour seals from Abertay Sands compared to seals from Moray Firth. All levels however, were within the range of reported baseline levels for Harbour seals except for γGT levels that were found reduced in both populations.

The control Harbour seals from the experimental group were found to have statistically significant higher HDLC ($p<0.005$) and significantly lower triglyceride ($p<0.05$), LDL ($p<0.005$) and VLDL ($p<0.05$) levels compared to the exposed group. Moreover, the liver function enzymes ALT ($p<0.005$), AST ($p<0.05$) and γGT ($p<0.05$) as well as albumin ($p<0.05$), ALKP ($p<0.05$), uric acid ($p<0.05$) and glucose ($p<0.005$) levels were found to be statistically significant higher in the exposed group compared to the control.

Female Harbour seals from Abertay Sands were found to have significantly reduced cholesterol ($p<0.05$), triglycerides, VLDL, HDLC and glucose levels ($p<0.005$; Fig.5.5) compared with the control Harbour seals from the experimental group. ALKP, cholesterol, triglycerides, VLDL and HDLC levels were also statistically significant reduced in Harbour seals from Moray Firth compared to the experimental control Harbour seals ($p<0.005$).

Albumin, glucose, triglyceride, VLDL and LDL levels were found to be statistically significant higher in both Baltic Ringed and Grey seals when compared to Svalbard Ringed

and Sable Island seals whereas HDLC, blood urea nitrogen and ALT levels were found significant reduced in the Baltic seals compared to Ringed and Grey seals from reference areas. AST levels were found reduced in Baltic compared to Svalbard Ringed seals and significantly increased in Baltic Grey seals compared to seals from Sable Island.

From all the blood chemistry parameters studied it was observed that VLDL and triglyceride levels had a positive correlation with ΣPCBs in a dose-dependent manner for all three species and this relationship was statistically significant. In studies on humans and rodents, increased total serum lipids, cholesterol and triglycerides are the most commonly found changes in blood chemistry parameters after PCB exposure (Allen *et al.*, 1976; Kreiss *et al.*, 1981; Yamamoto *et al.*, 1994; Nyman, 2003). In mammals TCDD has been found to decrease the lipoprotein lipase (LpL) activity, a key enzyme responsible for the catabolism of VLDL, with a loss of triglycerides and a parallel increase of cholesterol in high density lipoprotein (HDL; Sirtori, 1995). The planar PCBs have similar molecular structure and toxic effects with TCDD (Boon, 1992) thus it could be proposed that they may function in a similar toxic mechanism on lipid metabolism. Therefore, the elevated levels found in all studied exposed populations at the present study and since the use of triglycerides, lipoproteins, such as VLDL, and cholesterol are well established by published data in laboratory animals that increase during PCB exposure, indicates that they could be used as possible biomarkers of endocrine disruption in marine mammals.

Albumin and glucose levels were also found to be positively correlated with contaminant exposure but this relationship was only observed in Ringed and Grey seals. Organochlorines, being lipophilic and persistent, are bioaccumulated in seal blubber. During negative energy balance (fasting) PCBs are released into the blood stream, in which they are transported by albumin (Peakall *et al.*, 1972; Busbee *et al.*, 1985). Therefore, this elevation in albumin in association with the statistically significant positive correlation with contaminant exposure could be a result of an increased demand for PCB blood transport during fasting. Insulin resistance is associated with various metabolic abnormalities such as increased triglycerides, high levels of small dense LDL and decreased HDLC (Weltman *et al.*, 2003). Insulin stimulates the activity of enzymes that catalyse the hydrolysis of triglycerides contained in particles such as VLDL and therefore contaminants such as TCDD and possibly PCBs and

DDT that decrease lipoprotein lipase (LpL) activity can contribute to increase glucose and lipoprotein levels in plasma resulting in hepatic steatosis (Davail *et al.*, 2003).

HDLC levels were found to be statistically significant negatively correlated with PCB exposure in female and male Ringed ($p<0.01$) and Grey ($p<0.05$) seals but only in female Harbour ($p<0.05$) seals (Abertay Sands and Moray Firth). Finally, there was a negative correlation observed between blood urea nitrogen levels and contaminant exposure in both Ringed ($p<0.001$) and Grey seals ($p<0.05$) and a positive correlation between LDL levels ($p<0.001$) and PCBs in Ringed seals.

In conclusion, the blood chemistry levels measured in the three seal species were within the range of published references. The levels obtained in the presented study are valuable as baseline values for monitoring the health of free-ranging seals and especially during stress conditions such as fasting as it was observed for Baltic Ringed and Grey seals. The strong correlation of triglycerides and lipoproteins (VLDL, LDL, HDLC) with PCB exposure observed in the exposed populations suggests the use of these parameters as biomarkers of endocrine disruption. However, a larger number of samples is necessary for a more scientifically robust evaluation. Therefore, more research is necessary in order to establish a link between environmental pollution and changes in the blood chemistry parameters. Moreover, the levels of other blood chemistry parameters such as insulin and the activity of enzymes such as LpL could also be studied in future research in order to establish any correlation with contaminant exposure.

## 6.5 Limitations and future work

The major limitation of the contaminant analysis was that no matching faecal and blood samples could be obtained in order to provide a direct correlation of contaminant levels between these two matrices and to validate extrapolation of faecal to blood levels. The same limitation stands for sex steroid analysis because a direct correlation of sex steroid levels between the two matrices originating from the same individual was not able to be accounted. This could have provided a valuable tool for development of a model for predicting seal circulating hormone levels using only faecal samples and therefore offering minimal disturbance to the animals. Moreover, matching samples would be ideal in order to develop a

model for predicting seal population burdens directly from faeces thus providing a valuable tool for non-destructive sampling and seal biomonitoring. Another drawback in the study was that there was no information of the origin of the faecal samples (age, sex) collected. Therefore, faecal DNA testing would be ideal in order to identify the sex of the animals sampled and to provide more information on the sex-dependent differences of contaminant exposure. Another limitation of the contaminant analysis was that ΣPBDEs and ΣDDTs levels were not quantified for blood samples since sample volume was too small for GC analysis and there are no immunoassays at present developed for detection of PBDEs in environmental samples such as blood or faeces. On the other hand, immunoassays for OCPs already exist but due to resource limitations could not be used. Future development and validation of a PBDE immunoassay would be an ideal tool for non-destructive biomonitoring of seal populations. In future studies sampling should take place during all the stages of reproduction in order for changes in the physiology of individual seals to be accounted (e.g. lactation, moulting etc.) Moreover, larger sample sizes should be used in order to have a better picture of the contaminant exposure of the seal population under study. Analysis by immunoassays is a fast developing field with numerous possibilities for further improvement. They could be used for future screening analysis of biological samples such as blood and faeces to provide a quick estimate of environmental contamination in order to identify contaminant exposure in seals speedily and economically. Moreover, PCB screening in environmental samples could provide valuable information on metabolism and bioaccumulation of these compounds in the environmental and food chain matrices (Fránek *et al.*, 2001).

Using blood and faecal sex steroids as biomarkers of endocrine disruption in seals is a promising and valuable tool since both matrices can be sampled non-destructively (blood) and non-invasively (faeces). Faecal steroid measurement so far has only been applied to reproductive/endocrine assessments in mammals. There is clear potential of using faecal samples for biomonitoring altered function and EDC exposure in seals since sex steroid measurement in combination with contaminant data from faecal samples is an ideal tool for non-destructive sampling and seal biomonitoring. More samples could be collected from each individual and during all the stages of the reproductive cycle since using a single hormone sample from an individual in order to characterise reproductive status or possible endocrine disruption introduces error into the study. This occurs because certain hormones are released into the body in pulses (Aubin, 2001). However, if more samples are obtained individual seals could be monitored, tagged and re-sampled providing a data base of information on sex

steroids baseline levels and possible endocrine disruption through out the seal reproductive cycle.

Due to limited time and resources, purification and raising antibodies to the LTF protein was not possible and therefore without the availability of a purified pinniped LTF protein and production of monoclonal anti-seal LTF antibodies it is impossible to confirm whether seal LTF levels are indeed generally lower than in studies of other mammals to date, or are the cause of experimental problems. However, from the results obtained from this study and since LTF is proven to be a major estrogen-inducible protein, the use of sex steroid biomarkers in association with LTF is proposed as a valuable tool for seal biomonitoring since these two parameters complement each other.

Most toxicological studies in wild animals are focused on the chemical profile of specific compounds without taking consideration that animals in the wild are exposed to a mixture of compounds with varying toxicity. Naturally, most research to date has been limited to measuring pollutant levels, histo-pathological changes and *in vitro* biochemical changes using tissue samples collected from small numbers of dead animals to investigate pollutant-mediated effects. However, application of biomarkers to biomonitor exposure and effects of POPs in wild populations, overcomes many of the issues. POP exposure biomarkers provide information of the total biological response to POP mixtures and infer whether resultant biological responses are within physiological limits. Biomarker expression can be measured in samples taken non-destructively using cost-effective, high-throughput immunoassays, thus enabling processing of larger sample numbers to achieve more biologically-relevant data on exposure and effects. Furthermore, re-sampling individuals for temporal exposure studies is also possible for surveillance monitoring. Of the potential biomarkers assessed in the present study glucose as well as lipoproteins such as VLDL, LDL and HDLC could be proposed as potential biomarkers of exposure and effects in seals. These parameters should be used as biomarkers in conjunction with sex steroids such as estradiol. LTF in combination with sex steroids such as estradiol and progesterone are proposed as potential exposure biomarkers for PCBs in Harbour, Ringed and Grey seals.

## 7.0 References


**Addison, R.F.,** Brodie, P.F., Edwards, A. and M.C. Sadler (1986). Mixed Function Oxidase Activity in the Harbour Seal (*Phoca vitulina*) from Sable Island, N.S. *Comparative Biochemistry and Physiology*, **85C** (1)**:** 121-124.

**Addison, R.F.** and P.F. Brodie (1987). Transfer of Organochlorine Residues from Blubber through the Circulatory System in Milk in the Lactating Grey Seal, *Halichoerus grypus*. *Canadian Journal of Fisheries and Aquatic Sciences*, **44**: 782-786.

**Addison, R.F.** (1989). Organochlorines and Marine Mammal Reproduction. *Canadian Journal of Fisheries and Aquatic Sciences*, **46:** 360-368.

**Addison, R.F.** and W.T. Stobo (1993). Organochlorine Residue Concentration and Burdens in Grey Seal (*Halichoerus grypus*) Blubber during the First Year of Life. *Journal of Zoology*, **230**: 443-450.

**Aguilar, A.** and A. Borrell (1994). Abnormally High PCB Levels in Striped Dolphin (*Stenella coeruleoalba*) Affected by the 1990-1992 Mediterranean Epizootic. *The Science of the Total Environment*, **154**: 237-247.

**Aguilar, A.** and A. Borrell (1996). Marine Mammals and Pollutants, an Annotated Bibliography. Fundació pel Desenvolupament Sostenible, Barcelona.

**Aguilar, A.,** Borrell, A., and P.J.H. Reijnders (2002). Geographical and Temporal Variation in Levels of Organochlorine Contaminants in Marine Mammals. *Marine Environmental Research*, **53**: 425-452.

**Aigreau, D** (2002). Method Development for Multiresidue Analysis of Persistent Organic Pollutants in Seal Faecal Samples. MSc, Kingston University, UK.

**Alaee, J.M.M.J.G.,** Luross, J., Sergeant, D.B., Muir, D.C.G., Whittle, D.M. and K. Solomon (1999). Distribution of Polybrominated Diphenyl Ethers in the Canadian Environment. *Organohalogen Compounds*, **40**: 347-350.

**Allchin, C.R.,** Law, R.J. and S. Morris (1999). Polybrominated Diphenylethers in Sediments and Biota Downstream of Potential Sources in the UK. *Environmental Pollution*, **105**: 197-207.

**Allen, J.R.,** Carstens, L.A., and L.J. Abrahamson (1976). Responses of Rats Exposed to Polychlorinated Biphenyls for Fifty – Two Weeks I. Comparison of Tissue Levels of PCB and Biological Changes. *Archives of Environmental Contamination and Toxicology*, **4**: 404-419.

**AMAP,** (2000). AMAP Assessment Report: Arctic Pollution Issues. Chapter 6: Persistent Organic Pollutants. Arctic Monitoring and Assessment Programme (AMAP), Oslo, Norway.

**Andersson, Ö.** and A. Wartanian (1992). Levels of Polychlorinated Camphenes (Toxaphene), Chlordane Compounds and Polybrominated Diphenyl Ethers in Seals from Swedish Waters. *Ambio*, **21**: 550-552.

**Arnold, R.R.,** Cole, M.F. and J.R. McGhee (1977). A Bactericidal Effect for Human Lactoferrin. Science, **197**: 263-265.

**Arnold, R.R.,** Brewer, M. and J.J. Gauthier (1980). Bactericidal Activity of Human Lactoferrin: Sensitivity of a Variety of Micro-organisms. *Infection and Immunology*, **28**: 893-898.

**Atkinson, S.** (1997). Reproductive Biology of Seals. *Reviews of Reproduction*, **2**: 175-194.

**Aubin, D.J.** (2001). Endocrinology. *In*: CRC handbook of Marine Mammal Medicine. 2nd Edition, Dierauf, L.A., Gulland, F.M.D (Eds). Boca Raton, FL, CRC Press, pp: 165-192.

**Aulerich R.J.,** Bursian, S.J., Breslin, W.J., Olson, B.A. and R.K. Ringer (1985). Toxicological Manifestations of 2,4,5,2',4',5'-, 2,3,6,2',3',6'-, and 3,4,5,3',4',5'-Hexachlorobiphenyl and Aroclor 1254 in Mink. *Journal of Toxicology and Environmental Health*, **15**: 63-79

**Autiero, M.,** Sansone, G. and P. Abrescia (1991). Relative Ratios of Lactoferrin, Albumin, and Acid Phosphatase Seminal Levels as Sperm Quality Markers in Fertile and Infertile Men. *Journal of Andrology*, **12**(3): 191-200.

**Baker, J.R.** (1989) Pollution – Associated Uterine Lesions in Grey Seals from the Liverpool Bay Area of the Irish Sea. *The Veterinary Record*, **125**: 303.

**Ballschmiter, K.** and M. Zell (1980). Analysis of PCBs by Glass Capillary Chromatography. *Arbeit. Anal. Chem.* **302**: 20-31.

**Barret, T.,** Crowther, J., Osterhaus, A.D.M.E., Subbaro, S.M., Groen, J., Hass, L., Mamaev, L.V., Titenko, A.M., Visser, J.K.G. and C.J. Bostock (1992). Molecular Serological Studies on the Recent Seal Virus Epizootics in Europe and Siberia. *The Science of the Total Environment*, **115**: 117-132.

**Baynes, R.D.,** Bezwoda, W.R., Khan, Q. and N. Mansoor (1986). Plasma Lactoferrin Content: Differential Effect of Steroid Administration and Infective Illnesses: Lack of Effect of Ambient Temperature at Which Specimens are Collected. *Scandinavian Journal of Haematology*, **37**: 353-359.

**Béland, P.**, DeGuise, S., Girard, C., Lagacé, A., Martineau, D., Michaud, R., Mukir, D.C.G., Nortström, R.J., Pelletier, É., Ray, S., and L.R. Shugart (1993). Toxic Compounds and Health and Reproductive Effects in St. Lawrence Beluga Whales. *Journal of Great Lakes Research*, **19**(4): 766-775.

**Bengston, B-E.,** Hill, C., Bergman, Å., Brandt, I., Johansson, N., Magnhage, C., Södergren, A. and A. Thulin (1999). Reproductive Disturbances in Baltic Fish: A Synopsis of the FiRe Project. *Ambio*, **28**: 2-8.

**Bennet, R.M.** and C. Mohla (1976). A Solid-Phase Radioimmunoassay for the Measurement of Lactoferrin in Human Plasma: Variations with Age, Sex and Disease. *The Journal of Laboratory and Clinical Medicine*, **88**(1): 156-166.

**Bergman, A.** and M. Olsson (1985). Pathology of Baltic Grey Seal and Ringed Seal Females with Special Reference to Adrenocortical Hyperplasia: Is Environmental Pollution the Cause of a Widely Distributed Disease Syndrome? *Finnish Game Research*, **44**: 47-62.

**Bergman, Å.,** Klasson-Wehler, E. and H. Kuroki (1994). Selective Retention of Hydroxylated PCB Metabolites in Blood. *Environmental Health Perspectives*, **102**(5): 464-469.

**Bernt, K.E.,** Hammill, M.O., Lebeuf, M. and K.M. Kovacs (1999). Levels and Patterns of PCBs and OC Pesticides in Harbour and Grey Seals from the St. Lawrence Estuary, Canada. *The Science of the Total Environment*, **243/244**: 243-262.

**Bezault, T.,** Bhimani, T., Wiprovnick, J. and P. Furmanski (1994). Human Lactoferrin Inhibits Growth of Solid Tumors and Development of Experimental Metastases in Mice. *Cancer Research*, **54**: 2310-2312.

**Bezwoda, W.R.,** Baynes, R.D., Khan, Q. and N. Mansoon (1985). Enzyme Linked Immunosorbent Assay for Lactoferrin, Plasma and Tissue Measurements. *Clinical Chimica Acta*, **151**: 61-69.

**Bezwoda, W.R.** and N. Mansoor (1989). Lactoferrin from Human Breast Milk and from Neutrophil Granulocytes. Comparative Studies of Isolation, Quantitation, Characterization and Iron Binding Properties. *Biomedical Chromatography*, **3**: 121-126.

**Bhimani, R.S.,** Vendrov, Y. and P. Furmanski (1999). Influence of Lactoferrin Feeding and Injection Against Systemic Staphylococcal Infections in Mice. *Journal of Applied Microbiology*, **86**: 135-144.

**Bignert, A.,** Olsson, M., Persson, W., Jensen, S., Zakrisson, S., Litzén, K., Eriksson, U., Häggberg, L. and T. Alsberg (1998). Temporal Trends of Organochlorines in Northern Europe, 1967-1995. Relation to Global Fractionation, Leakage from Sediments and International Measures. *Environmental Pollution*, **99**: 177-198.

**Billitti, J.E.,** Lasley, B.L. and B.W. Wilson (1998). Development and Validation of a Fecal Testosterone Biomarker in *Mus musculus* and *Peromyscus maniculatus*. *Biology of Reproduction*, **59**: 1023-1028.

**Birgens, H.S.** (1985). Lactoferrin in Plasma Measured by an ELISA Technique: Evidence That Plasma Lactoferrin is an Indicator of Neutrophil Turnover and Bone Marrow Activity in Acute Leukaemia. *Scandinavian Journal of Haematology*, **34:** 326-331.

**Birnbaum, L.S.,** Staskal, D.F. and J.J. Diliberto (2003). Health Effects of Polybrominated Dibenzo-*p*-Dioxins (PBDDs) and Dibenzofurans (PBDFs). *Environment International*, **29**: 855-860.

**Blomkvist, G.,** Roos, A., Jensen, S., Bignert, A. and M. Olsson (1992). Concentrations of SDDT and PCB in Seals from Swedish and Scottish Waters. *Ambio*, **21**: 539-545.

**Boness, D.J.** (1979). The Social System of the Grey Seal, *Halichoerus grypus* (Fab) on Sable Island, Nova Scotia. PhD Thesis, Dallhousie University, Halifax, N.S.

**Boness, J.P.** and H. James (1979). Reproductive Behaviour of the Grey Seal, *Halichoerus grypus* (Fab), on Sable Island, Nova Scotia. *London Journal of Zoology*, **188**: 477-500.

**Boon, J.P.,** van Arnheim, E., Jansen, S., Kannan, N., Petrick, G., Schulz, D., Duinker, J.C., Reijnders, P.J.H. and A. Goksoyr (1992). The toxicokinetics of PCBs in Marine Mammals with Special Reference to Possible Interactions of Individual Congeners with the Cytochrome P450-Dependent Monooxygenase System. *In*: Persistent Pollutants in Marine Ecosystems. Walker C., Livingston,D.R. (Eds.), Pergamon Press, New York, pp. 119-159.

**Boon, J.P.,** Oostingh, J., van der Meer, J. and M.T.J. Hillebraud (1994). A Model for the Bioaccumulation of Chlorobiphenyl Congeners in Marine Mammals. *European Journal of Environmental Toxicology and Pharmacology*, **270**(2): 237-251.

**Boon, J.P.,** van der Meer, J., Allchin, C.R., Law, R.J., Klungsoyr, J., Leonards, P.E., Spliid, H., Storr-Hansen, E., McKenzie, C. and D.E. Wells (1997). Concentration – Dependent Changes of PCB Patterns in Fish – Eating Mammals: Structural Evidence for Induction of Cytochrome P450. *Archives of Environmental Contamination and Toxicology*, **33**(3): 298-311.

**Borlakoglu, J.T.** and V.A. Welch (1992). Xenobiotic-Induced Aberrations of Lipid Metabolism. *In*: Fatty Acids in Foods and their Health Implementations C.K. Chow, (Ed.), Marcel Dekker Inc., New York, pp. 613-630..

**Bossart, G.D.** and L.A. Dierauf (1990). Marine Mammal Clinical Laboratory Medicine *In*: Handbook of Marine Mammal Medicine: Health, Disease and Rehabilitation (L.A. Diearauf, Ed.), pp. 1-52. CRC Press, Boca Raton.

**Bossart, D.,** Reidarson, T.H., Dierauf, L. and D.A. Duffield (2001). Chapter 19: Clinical Pathology. *In*: CRC Handbook of Marine Mammal Medicine, 2nd Edition. L.A. Dierauf and F.M.D. Gulland (Eds.), CRC Press, Boca Raton, pp. 383-430.

**Boxer, L.A.,** Coates, T.D., Hack, R.A., Wolach, J.B., Hoffstein, S. and R.C. Baehner (1982). Lactoferrin Deficiency Associated with Altered Granulocyte Function. *The New England Journal of Medicine*, **307**: 404-410

**Boyd, I.L.** (1982). Reproduction of Grey Seal with Reference to 9 Factors Influencing Fertility. PhD Thesis, Cambridge University, Cambridge, UK, pp. 167.

**Boyd, I.L.** (1983). Luteal Regression, Follicle Growth and the Concentration of Some Plasma Steroids During Lactation in Grey Seals (*Halichoerus grypus*). *Journal of Reproduction and Fertility*, **69**: 157-164.

**Boyd, I.L.** (1984). Development and Regression of the Corpus Luteum in Grey Seal (*Halichoerus grypus*) Ovaries and its Use in Determining Fertility Rates. *Canadian Journal of Zoology*, **62**: 1095–1100.

**Boyd, I.L.** (1991). Changes in Plasma Progesterone and Prolactin Concentrations During the Annual Cycle and the Role of Prolactin in the Maintenance of Lactation and Luteal Development of the Antarctic Fur Seal (*Arctocephalus gazella*). *Journal of Reproduction and Fertility*, **91**: 637-647.

**Boyd, I.L.** (1991*a*). Environmental and Physiological Factors Controlling the Reproductive Cycles of Pinnipeds. *Canadian Journal of Zoology*, **69**: 1135–1148.

**Boyd, I.L.,** Lockyer, C. and H. Marsh (1999). Reproduction in Marine Mammals. *In* Biology of Marine Mammals, Reynolds, J.E., III, and Rommel, S.A. (Eds.), Smithsonian Institution Press, Washington, D.C., pp. 218-286.

**Braune, B.M.,** Outridge, P.M., Fisk, A.T., Muir, D.C.G., Helm, P.A., Hobbs, K., Hoekstra, P.F., Kuzyk, Z.A., Kwan, M., Letcher, R.J., Lockhart, W.L., Norstrom, R.J., Stern, G.A. and I. Stirling (2005). Persistent Organic Pollutants and Mercury in Marine Biota of the Canadian Arctic: An Overview of Spatial and Temporal Trends. *Science of the Total Environment*, **351-352**: 4-56.

**Breivik, K.,** Alcock, R., Li, Y-F., Bailey, R.E., Fiedler, H. and J.M. Pacyna (2004). Primary Sources of Selected POPs: Regional and Global Scale Emission Inventories. *Environmental Pollution*, **128**: 3-16.

**Brock, J.H.** (2002). The Physiology of Lactoferrin. *Biochemistry and Cell Biology*, **80**: 1-6.

**Brouwer, A.** and K.J. Van den Berg (1986). Binding of a Metabolite of 3,4,3',4' – Tetrachlorobiphenyl to Transthyretin Reduces Serum Vitamin A Transport by Inhibiting the Formation of the Protein Complex Carrying Both Retinol and Thyroxin. *Toxicology and Applied Pharmacology*, **85**: 301-312.

**Brouwer, A.,** Reijnders, P.J.H., and J.H. Koeman (1989). Polychlorinated Biphenyl (PCB)-Contaminated Fish Induces Vitamin A and Thyroid Hormone Deficiency in the Common Seal (*Phoca vitulina*). *Aquatic Toxicology* **15**: 99-106.

**Brouwer, A.** (1991). Role of Biotransformation in PCB-Induced Alterations in Vitamin A and Thyroid Hormone Metabolism in Laboratory and Wildlife Species. *Biochem. Soc. Trans.*, **19**: 731-737.

**Brouwer, A.,** Morse, D.C., Lans, M.C., Schuur, A.G., Murk, A.J., Klasson-Wehler, E., Bergman, A. and T.J. Visser (1998). Interactions of Persistent Environmental Organohalogens with the Thyroid Hormone System: Mechanisms and Possible Consequences for Animal and Human Health. *Toxicology and Industrial Health*, **14**(1-2): 59-84.

**Buckett, W.,** Luckas, M.J.M., Gazvani, M.R., Aird, I.A. and I. Lewis-Jones (1997). Seminal Plasma Lactoferrin Concentrations in Normal and Abnormal Semen Samples. *Journal of Andrology*, **18**(3): 302-304.

**Burtis, C.A. and E.R. Ashwood** (1999). *In:* Tietz Textbook of Clinical Chemistry, 3rd Edition, W.B. Saunders Company, Philadelphia.

**Busbee, D.L.,** Yoo, J.S., Norman, J.O. and C.O. Joe (1985). Polychlorinated Biphenyl Uptake and Transport by Lymph and Plasma Components. *Proceedings of the Society for Experimental Biology and Medicine*, **179**: 116-122.

**Byrne, J.J.,** Carbone, J.P. and E.A. Hanson (1987). Hypothyroidism and Abnormalities in the Kinetics of Thyroid Hormone Metabolism in Rats Treated Chronically with Polychlorinated Biphenyls and Polybrominated Biphenyls. *Endocrinology*, **121**: 520-527.

**Caccavo, D.,** Sebastiani, G.D., Di Monaco, C., Guido, F., Galeazzi, M., Ferri, G.M., Bonomo, L. and A. Afeltra (1999). Increased Levels of Lactoferrin in Synovial Fluid but not in Serum from Patients with Rheumatoid Arthritis. *International Journal of Clinical and Laboratory Research*, **29**: 30-35.

**Cameron, M.E.,** Metcalfe, T.L., Metcalfe, C.D. and C.R. Macdonald (1997). Persistent Organochlorine Compounds in the Blubber of Ringed Seals (*Phoca hispida*) from the Belcher Islands, Northwest Territories, Canada. *Marine Environmental Research*, **43**(1/2): 99-116.

**Canadian Arctic Profile**, Species: http://collections.ic.gc.ca/arctic/species.rsphoto.htm

**Carson, R.** (1962). Silent Spring. Houghton Mifflin; Cambridge, Mass. Riverside Press, Boston.

**Cetacean Research and Rescue Unit:** www.crru.org.uk

**Chen, G.** and N.J. Bunce (2003). Polybrominated Diphenyl Ethers as Ah Receptor Agonists and Antagonists. *Toxicological Sciences*, **76**: 310-320.

**Chen, P.W.,** Chen, W.C. and F.C. Mao (2004). Increase of Lactoferrin in Mastitic Goat Milk. *Journal of Veterinary Medicine*, **66**(4): 345-350.

**Christensen, G.M.,** Olson, D. and B. Riedel (1982). Chemical Effects on the Activity of Enzymes: a Review and a Discussion Relevant to Environmental Monitoring. *Environmental Research*, **29**(2): 247-255.

**Chuang, J.C.,** Miller, L.S., Davis, D.B., Peven, C.S., Johnson, J.C. and J.M. Van Emon (1998). Analysis of Soil and Dust Samples for Polychlorinated Biphenyls by Enzyme-Linked Immunosorbent Assay (ELISA). *Analytica Chimica Acta*, **376**: 67-75.

**Chuang, J.C.,** Van Emon, J.M., Chou, Y-L., Junod,N., Finegold, J.K. and N.K. Wilson (2003). Comparison of Immunoassay and Gas Chromatography – Mass Spectrometry for Measurement of Polycyclic Aromatic Hydrocarbons in Contaminated Soil. *Analytica Chimica Acta*, **486**: 31-39.

**Colborn, T.,** Vom Saal, F. and A.M. Soto (1993). Developmental Effects of Endocrine – Disrupting Chemicals in Wildlife and Humans. *Environmental Health Perspectives*, **101**(5): 378-384.

**Colborn, T.,** and M.J. Smolen (1996). Epidemiologic Analysis of Persistent Organochlorine Contaminants on Cetaceans. *Reviews of Environmental Contamination and Toxicology*, **146**: 91-172.

**Common Wadden Sea Secretariat,** (2004). Counting Harbour Seals in the Wadden Sea in 2004 – Expected and Unexpected Results.

**Conneely, O.M.** (2001). Review: Antiinflammatory Activities of Lactoferrin. *Journal of the American College of Nutrition*, **20**(5): 389S-395S.

**Corbet, G.B.,** and S. Harris (1991). The Handbook of British Mammals, 3rd edition. Published for the Mammal Society, Blackwell Scientific Publications, pp. 462-471.

**Cowan, L.D.,** Wallace, R.B., Barrett-Connor, E., Hunninghake, D., Pomreh, P., Wahl, P. and G. Heiss (1982). Variations in Clinical Chemistry Measures Associated with Oral Contraceptive and Estrogen Use. *Journal of Reproductive Medicine*, **27**: 275-282.

**Curtis, S.W.,** Shi, H., Teng, C. and K.S. Korach (1997). Promoter and Species Specific Differential Estrogen-Mediated Gene Transcription in the Uterus and Cultured Cells Using Structurally Altered Agonists. *Journal of Molecular Endocrinology*, **18**: 203-211.

**Darnerud, P.O.,** Ericksen, G.S., Jóhannesson, T., Larsen, P.B. and M. Viluksela (2001). Polybrominated Diphenyl Ethers: Occurrence, Dietary Exposure and Toxicology. *Environmental Health Perspectives*, **109**(Suppl.1): 49-68.

**Darnerud, P.O.** (2003). Toxic Effects of Brominated Flame Retardants in Man and in Wildlife. *Environment International*, **29**: 841-853.

**Das, S.K.,** Tan, J., Johnson, D.C. and S.K. Dey (1998). Differential Spatiotemporal Regulation of Lactoferrin and Progesterone Receptor Genes in the Mouse Uterus by Primary Estrogen, Catechol Estrogen, and Xenoestrogen. *Endocrinology*, **139**(6): 2905-2915.

**Davail, S.,** Guy, G., André, J-M., Hermier and R. Hoo-Paris (2000). Metabolism in Two Breeds of Geese with Moderate or Large Overfeeding Induced Liver-Steatosis. *Comparative Biochemistry and Physiology Part A*, **126**: 91-99.

**Davail, S.,** Rideau, N., Guy, G., Andre, J-M., Hermier, D. and R. Hoo-Paris (2003). Hormonal and Metabolic Responses to Overfeeding in Three Genotypes of Ducks. *Comparative Biochemistry and Physiology, Part A*, **134**: 707-715.

**De Boer, J.,** Wester, P., Klamer, H.J.C., Lewis, W.E. and J.P. Boon (1998). Do Flame Retardants Threaten Ocean Life? *Nature*, **394**: 28-29.

**De Boer, J.,** Wester, P.G., Pastor i Rodriquez, D., Lewis, W.E. and J.P. Boon (1998b). Polybrominated Biphenyls and Diphenylethers in Sperm Whales and Other Marine Mammals – a New Threat to Ocean Life? *Organohalogen Compounds*, **35**: 383-386.

**De Boer, J.** (1999). Capillary Gas Chromatography for the Determination of Halogenated Micro – Contaminants. *Journal of Chromatography A*, **843**(1/2): 179-198.

**De Boer, J.,** Allchin, C., Law, R., Zegers, B. and J.P. Boon (2001). Method for the Analysis of Polybrominated Diphenyl Ethers in Sediments and Biota. *Trends in Analytical Chemistry*, **20**(10): 591-599.

**De Swart, R.L.,** Ross, P.S., Vedder, L.J., Timmerman, H.H., Heisterkamp, S., Van Loveren, H., Vos, J.G., Reijnders, P.J.H. and A.D.M.E. Osterhaus (1994). Impairment of Immune Function in Harbor Seals (*Phoca vitulina*) Feeding on Fish from Polluted Waters. *Ambio*, **23**(2): 155-159.

**De Swart, R.L.,** Ross, P.S., Vedder, L.J., Boink, F.B., Reijnders, P.J., Mulder, P.G. and A.D. Osterhaus (1995). Haematology and Clinical Chemistry Values of Harbor Seals (*Phoca vitulina*) Fed Environmentally Contaminated Herring Remain Within Normal. Ranges *Canadian Journal of Zoology*, **73**: 2035-2043.

**De Swart, R.L.**, Harder, T.C., Ross, P.S., Vos, H.W. and A.D. Osterhaus (1995a). Morbilliviruses and Morbillivirus Diseases of Marine Mammals *Infectious Agents and Diseases*, **4**(3): 125-130.

**De Swart, R.L.,** Ross, P.S., Timmerman, H.H., Hijman, W.C., de Ruiter, E.M., Liem, A.K.D., Brouwer, A., van Loveren, H., Reijnders, P.J.H., Vos, J.G. and A.D.M.E. Osterhaus (1995b). Short – Term Fasting Does Not Aggravate Immunosuppression in Harbour Seals (*Phoca vitulina*) with High Body Burdens of Organochlorines. *Chemosphere*, **31**(10): 4289-4306.

**De Swart, R.L.,** Ross, P.S., Timmerman, H.H., Vos, H.W., Reijnders, P.J., Vos, J.G. and A.D. Osterhaus (1995c). Impaired Cellular Immune Response in Harbour Seals (*Phoca vitulina*) Feeding on Environmentally Contaminated Herring *Clinical and Experimental Immunology*, **101**(3): 480-486.

**De Swart, R.L.,** Ross, P.S., Vos, J.G. and A.D.M.E. Osterhaus (1996). Impaired Immunity in Harbour Seals (*Phoca vitulina*) Exposed to Bioaccumulated Environmental Contaminants: Review of a Long – Term Feeding Study. *Environmental Health Perspectives*, **104**(4): 823-828.

**De Voogt, P.,** Wells, D.E., Reutergårdh, L., and U.A.Th. Brinkman (1990). Biological Activity, Determination and Occurrence of Planar, Mono- and Di–Ortho PCBs. *International Journal of Environmental Analytical Chemistry*, **40**: 1-46.

**De Wit, C.A.**, (2002). An Overview of Brominated Flame Retardants in the Environment. *Chemosphere*, **46**: 583-624.

**Dean, C.E.Jr.,** Benjamin, S.A., Chubb, L.S., Tessari, J.D. and T.J. Keefe (2002). Nonadditive Hepatic Tumor Promoting Effects by a Mixture of Two Structurally Different Polychlorinated Biphenyls in Female Rat Livers. *Toxicological Sciences*, **66**: 54-61

**Debier, C.,** Pomeroy, P.P., Dupont, C., Joiris, C., Comblin, V., Le Boulengé, E., Larondelle, Y. and J.-P. Thomé (2003). Quantitative Dynamics of PCB Transfer from Mother to Pup During Lactation in UK Grey Seals *Halichoerus grypus*. *Marine Ecology Progress Series*, **247**: 237-248.

**Debier, C.,** Pomeroy, P.P., Thomé, J.-P., Mignolet, E., de Tillesse, T. and Y. Larondelle (2004). An Unexpected Parallelism Between Vitamin A and PCBs in Seal Milk. *Aquatic Toxicology*, **68**: 179-183.

**DeLong, RL.,** Gilmartin, W.G. and J.G. Simpson (1973). Premature Births in California Sea Lions: Association with High Organochlorine Pollutant Residue Levels. *Science*, **181**: 1168-1169.

**Deng, A.-P.,** Kolář, V., Ulrich, R. and M. Fránek (2002). Direct Competitive ELISA for the Determination of Polychlorinated Biphenyls in Soil Samples. *Analytical and Bioanalytical Chemistry*, **373**: 685-690.

**Depledge, M.H.** (1994). Genotypic Toxicity: Implications for Individuals and Populations. *Environmental Health Perspectives*, **102** (12): 101-104.

**Díaz-Ferrero, J.,** Rodríguez-Larena, M.C., Comellas, L. and B. Jiménez (1997). Bioanalytical Methods Applied to Endocrine Disrupting Polychlorinated Biphenyls,

Polychlorinated Dibenzo-*p*-Dioxins and Polychlorinated Dibenzofurans. A Review. *Trends in Analytical Chemistry*, **16**(10): 563-573.

**Dierauf, L.A.** (1990). *CRC Handbook of Marine Mammal Medicine: Health, Disease and Rehabilitation*. CRC Press, Boca Raton.

**Drescher, H. E.,** Harms, U. and E. Huschenbeth (1977). Organochlorines and Heavy Metals in the Harbour Seal *(Phoca vitulina)* from the German North Sea Coast. *Marine Biology*, 41: 99-106.

**DTI**, UK Trade and Industry Risk and Benefits in the Use of Flame Retardants in Consumer Products. DTI ref: URN 98/1026.

**Duck, C.D.** (2004). Grey Seal Pup Production in Britain in 2003. *In*: UK Special Committee on Seals, NERC Sea Mammal Research Unit, SMRU, pp. 28-37.

**Duck, C.D.** and D. Thompson (2004). The Status of British Common Seal Populations. *In*: UK Special Committee on Seals, NERC Sea Mammal Research Unit, SMRU, pp. 51-60.

**Duggan, A.,** Paolucci, M., Tercyak, A., Gigliotti, M., Small, D. and I. Callard (2001). Seasonal Variation in Plasma Lipids, Lipoproteins, Apolipoprotein A-I and Vitellogenin in the Freshwater Turtle, *Chrysemys picta*. *Comparative Biochemistry and Physiology Part A*, **130**: 253-269.

**Duignan, P.J.,** Sadove, S., Saliki, J.T. and J.R. Geraci (1993). Phocine Distemper in Harbor Seals (*Phoca vitulina*) from Long Island, New York. *Journal of Wildlife Diseases*, **29**(3): 465-469.

**Duignan, P.J.,** Saliki, J.T., Aubin, St.D.J., Early, G., Sadove, S., House, J.A., Kovacs, K. and J.R. Geraci (1995). Epizootiology of Morbillivirus Infection in North American Harbor Seals (*Phoca vitulina*) and Gray Seals (*Halichoerus grypus*). *Journal of Wildlife Diseases*, **31**(4): 491-501.

**Ellfolk, M.,** Heikkilä, R., Huotari, K., Koskela, J., and R. Pelkonen (1997). Laboratorio Käsikirja '89-99. Otava, Keuruu. p. 477.

**Elliot, J.I.,** Senft, B., Erhardt, G. and D. Fraser (1984). Isolation of Lactoferrin in Sows' Colostrum and Milk During a 21-Day Lactation. *Journal of Animals Science*, **59**: 1080-1084.

**Elvers, B.,** Hawkins, S. and G. Shulz (1992). Ullmans Encyclopaedia of Industrial Chemistry, 4th ed., VCH, Weinheim.

**Engelhardt, F.R.** (1979). Haematology and Plasma Chemistry of Captive Pinnipeds and Cetaceans. *Aquatic Mammals*, **7**: 11-24.

**EPA,** (2002). Toxicological Profile for DDT, DDE and DDD. U.S. Department of Health and Human Services, Public Health Service, Agency for Toxic Substances and Disease Registry.U.S. Environmental Protection Agency (EPA).

**Erickson, M.D.** (1986). Analytical Chemistry of PCBs, Boston, M.A: Butterworth Publishers, Stoneham, MA.

**Errickson, M.D.** (1997). Analytical Chemistry of PCBs, 2nd edition, CRC Press, Boca Raton, FL.

**European** Workshop on the Impact of Endocrine Disruptors on Human Health and Wildlife, Report of Proceedings, 2-4 December 1996, Weybridge, UK.

**Farley, J.,** Loup, D., Nelson, M., Mitchell, A., Esplund, G., Macri, C., Harrison, C. and K. Gray (1997). Neoplastic Transformation of the Endocervix Associated with Downregulation of Lactoferrin Expression. *Molecular Carcinogenesis*, **20**: 240-250.

**Farnaud, F.** and R.W. Evans (2003). Lactoferrin – A Multifunctional Protein with Antimicrobial Properties. *Molecular Immunology*, **40**: 395-405.

**Fielden, M.R.,** Chen, I., Chittim, B., Safe, S.H. and T.R. Zacharewski (1997). Examination of the Estrogenicity of 2,4,6,2′,6′ - Pentachlorobiphenyl (PCB 104), Its Hydroxylated Metabolite 2,4,6,2′,6′ - Pentachloro – 4 – Biphenylol (HO – PCB 104), and a Further Chlorinated Derivative, 2,4,6,2′,4′,6′ - Hexachlorobiphenyl (PCB 155). *Environmental Health Perspectives*, **105**(11): 1238-1248.

**Fossi, M.C.,** Casini, S., and L. Marsili (1999). Nondestructive Biomarkers of Exposure to Endocrine Disrupting Chemicals in Endangered Species of Wildlife. *Chemosphere*, **39**(8): 1273-1285.

**Fránek, M.,** Deng, A., Kolář V. and J. Socha (2001). Direct Competitive Immunoassays for the Coplanar Polychlorinated Biphenyls. *Analytica Chimica Acta*, **444**: 131-142.

**Freeman, H.C.** and G.B. Sangalang (1976). Steroids in the Plasma of the Gray Seal, *Halichoerus grypus General and Comparative Endocrinology*, **29**: 419-422.

**Freeman, H.C.** and G.B. Sangalang (1977) A Study of the Effects of Methyl Mercury, Cadmium, Arsenic, Selenium, and a CB (Aroclor 1254) on Adrenal and Testicular Steroidogenesis *in vitro* by the Grey Seal (*Halichoerus grypus*) *Archives of Environmental Contamination and Toxicology*, **5**: 369-383.

**Gales, N.J.,** Williamson, P., Higgins, L.V., Blackberry, M.A. and I. James (1997). Evidence for a Prolonged Postimplantation Period in the Australian Sea Lion (*Neophoca cinerea*). *Journal of Reproduction and Fertility*, **111**(2): 159-163.

**Gardiner, KJ.** (1996). Plasma Progesterone Concentrations Measured Using an Enzyme-linked Immunosorbent Assay Useful for Diagnosing Pregnancy in Harbor Seals (*Phoca vitulina*). *Marine Mammal Science*, **12**(2): 265-273.

**Gardiner, K.J.,** Boyd, I.L., Follett, B.K., Racey, P.A. and P.J.H. Reijnders (1999). Changes in Pituitary, Ovarian, and Testicular Activity in Harbour Seals (*Phoca vitulina*) in Relation to Season and Sexual Maturity. *Canadian Journal of Zoology*, **77**: 211-221.

**Geography at Edinburgh:** www.geo.ed.ac.uk

**Geraci, JR.,** Aubin, D.J. and T.G. Smith (1979). Influence of Age, Condition, Sampling Time, and Method on Plasma Chemical Constituents in Free-ranging Ringed Seals, *Phoca hispida. Journal of the Fisheries Research Board of Canada*, **36**: 1278-1282.

**Ghosh, D.,** Taylor, J.A., Green, J.A. and D.B. Lubahn (1999). Methoxychlor Stimulates Estrogen-Responsive Messenger Ribonucleic Acids in Mouse Uterus Through a Non-Estrogen Receptor (Non-ER)α and Non-ERβ Mechanism. *Endocrinology*, **140**(8): 3526-3533.

**Gibbons, F.,** Islam, K. and R.J. Pease (2000). Mobilisation of Triacylglycerol Stores. A Review. *Biochimica et Biophysica Acta*, **1483**: 37-57.

**Gillner, M.,** Lund, J., Cambillau, C., Alexandersson, M., Hurtig, U., Bergman, Å., Klasson-Wehler, E. and J. Gustafsson (1988). The Binding of Methylsulfonyl-polychlorobiphenyls to Uteroglobin. *The Journal of Steroid Biochemistry and Molecular Biology,* **31**: 27-33.

**Golden, R.J.,** Noller, K.L., Titus-Ernstoff, I., Kaufman, R.H., Mittendorf, R., Stillman, R. and E.A. Reese (1998). Environmental Endocrine Modulators and Human Health: An Assessment of the Biological Evidence. *Critical Reviews in Toxicology*, **28**(2): 109-227.

**Golub, M.S.,** Hogrefe, C.E., Germann, S.L. and C.P. Jerome (2004). Endocrine Disruption in Adolescence: Immunologic, Hematologic and Bone Effects in Monkeys. *Toxicological Sciences*, **82**: 598-607.

**Goodman, A.S.** and L.G. Young (1981). Immunological Identification of Lactoferrin as a Shared Antigen on Radioiodinated Human Sperm Surface and in Radioiodinated Human Seminal Plasma. *Journal of Reproductive Immunology*, **3:** 99-108.

**Gray, N.K.,** Sakai, Y., Eitzman, B., Steed, T. and J. McLachlan (1994). Exposure to Diethylstilbestrol During a Critical Developmental Period of the Mouse Reproductive Tract Leads to Persistent Induction of Two Estrogen-regulated Genes. *Cell Growth and Differentiation*, **5**: 595-606.

**Greenwood, A** (1971). Blood Values in Young Gray Seals. *Journal of the American Veterinary Medical Association*, **159**: 571-574.

**Greig, D.J.** (2002). Pregnancy and Parturition Rates of Harbor Seals in Monterey Bay, California. Thesis (MSc) presented to the Faculty of the Department of Biological Sciences, San Jose University and Moss Landing Marine Labs.

**Griffin, J.E.** and Ojeda, S.R. (1996). Textbook of Endocrine Physiology, Third Edition. Oxford University Press, New York.

**Hagger, J.A.,** Depledge, M.H. and T.S. Galloway (2005). Toxicity of Tributyltin in the Marine Mollusc *Mytilus edulis*. *Marine Pollution Bulletin*, **51**: 811-816.

**Hagiwara, S.,** Kawai, K., Anri, A. and H. Nagahata (2003). Lactoferrin Concentrations in Milk from Normal and Subclinical Mastitic Cows. *Journal of Veterinary Medicine*, **65**(3): 319-323.

**Haglund, P.,** Zook, D.R., Buser, H.-R. and J. Hu (1997). Identification and Quantification of Polybrominated Diphenyl Ethers and Methoxy-Polybrominated Diphenylethers in Baltic Biota. *Environmental Science and Technology*, **31**: 3281-3287.

**Hale, R.C.,** Alaee, M., Manchester-Neesvig, J.B., Stapleton, H.M. and M.G. Ikonomou (2003). Brominated Diphenylethers in Fishes, Sediments and Sewage Sludges in the U.S.A. and Canada. *Environment International*, **29**(6): 771-779.

**Hall, A.J.,** Law, R.J., Harwodd, J., Ross, H.M., Kennedy, S., Allchin, C.R., Campbell, L.A. and P.P. Pomeroy (1992). Organochlorine Levels in Common Seals (*Phoca vitulina*) which Were Victims and Survivors of the 1988 Phocine Distemper Epizootic. *The Science of the Total Environment*, **115**: 145-162.

**Hall, A.J.,** Pomeroy, PP., Green, N., Jones, K., and J. Harwood (1997). Infection, Haematology and Biochemistry in Grey Seal Pups Exposed to Chlorinated Biphenyls. *Marine Environmental Research* **43**(1-2): 81-98.

**Hall, A.J.** (1998). Blood Chemistry and Hematology of Gray Seal (*Halichoerus grypus*) Pups from Birth to Postweaning. *Journal of Zoo and Wildlife Medicine*, **29**(4): 401-407.

**Hall, A.J.,** Green, N.J.L., Jones, K.C., Pomeroy, P.P. and J. Harwood (1998). Thyroid Hormones as Biomarkers in Grey Seals. *Marine Pollution Bulletin*, **36**(6): 424-428.

**Hall, A.J.,** Duck, C., Law, R.J., Allchin, C.R., Wilson, S. and T. Evbator (1999). Organochlorine Contaminants in Caspian and Harbour Seal Blubber. *Environmental Pollution*, **106**(2): 203-212.

**Hall, A.J.,** Kalantzi, O.I. and G.O. Thomas (2003). Polybrominated Diphenyl Ethers (PBDEs) in Grey Seals During their First Year of Life – are they Thyroid Hormone Endocrine Disrupters? *Environmental Pollution*, **126**: 29-37.

**Hallgren, S.,** Sinjari, T., Håkansson, H. and P.O. Darnerud (2001). Effects of Polybrominated Diphenyl Ethers and Polychlorinated Biphenyls (PCBs) on Thyroid Hormone and Vitamin A Levels in Rats and Mice. *Archives of Toxicology*, **75**(4-5): 200-208.

**Halpern, M.J.** (1995). Lipids and Atherosclerosis. *Molecular Aspects of Medicine*, **16**: 509-710.

**Hamm, J.T.,** Chen, C.Y. and L.S. Birnbaum (2003). A Mixture of Dioxins, Furans, and Non-Ortho PCBs Based Upon Consensus Toxic Equivalency Factors Produces Dioxin-Like Reproductive Effects. *Toxicological Sciences*, **74**: 182-191.

**Hansen, N.E.,** Karle, H., Andersen, V., Malmquist, J. and G.E. Hoff (1976). Neutrophilic Granulocytes in Acute Bacterial Infection, Sequential Studies on Lysozyme, Myeloperoxidase and Lactoferrin. *Clinical and Experimental Immunology*, **26**(3): 463-468.

**Hardy, F.G.,** Evans, S.M. and M.A. Tremayne (1993). Long-Term Changes in the Marine Macroalgae of Three Polluted Estuaries in North-East England. *Journal of Experimental Marine Biology and Ecology*, **172**: 81-92.

**Härkönen, T.,** Stenman, O., Jussi, M., Jussi, I., Sagitov, R. and M. Verevkin (1998). Population Size and Distribution of the Baltic Ringed Seal (*Phoca hispida baltica*). *In*: Ringed Seals in the North Atlantic. M. Heide-Jorgessen and C. Lydersen (Eds.), NAMMCO Scientific Publications **1**, pp. 167-180.

**Harrison, R.O.** and J. DeBaise (1994). On-Site Analysis of PCBs in Water by Enzyme Immunoassay. Presentation at the ACS National Meeting, San Diego, CA.

**Heiden, T.K.,** Hutz, R.J. and M.J. Carvan, III (2005). Accumulation, Tissue Distribution, and Maternal Transfer of Dietary 2,3,7,8,-Tetrachlorodibenzo-*p*-Dioxin: Impacts on Reproductive Success of Zebrafish. *Toxicological Science*, **87**(2): 497-507.

**Helle, E.,** Olsson, M. and S. Jenssen (1976a). DDT and PCB Levels and Reproduction in Ringed Seal from the Bothnian Bay. *Ambio*, **5**: 188-189.

**Helle, E.,** Olsson, M. and S. Jensen (1976b). PCB Levels Correlated with Pathological Changes in Seal Uteri. *Ambio*, **5**: 261-263.

**Helle, E.** (1986). Reproduction, Size and Structure of the Baltic Ringed Seal Population of the Bothnian Bay. *Acta Univ. Ouluensis* (A) **106**: 1-47.

**Helle, E.,** Stenman, O., Olsson, M., Helander, B. and T. Harkonen (1990). Baltic Seals, Population Size, Reproduction and Organochlorines. *Ambio*, **7**: 16

**Hennart, P.E.,** Brasseur, D.J., Delogne-Desnoeck, J.B., Dramaix, M.M. and C.E. Robyn (1991). Lysozyme, Lactoferrin, and Secretory Immunoglobulin A Content in Breast Milk: Influence of Duration of Lactation, Nutrition Status, Prolactin Status, and Parity of Mother. *The American Journal of Clinical Nutrition*, **53**: 32-39.

**Hermier, D.,** Catheline, D. and P. Legrand (1996). Relationship Between Hepatic Fatty Acid Desaturation and Lipid Secretion in the Estrogenized Chicken. *Comparative and Biochemistry Physiology*, **115A**(3): 259-264.

**Hermier, D.,** Guy, G., Guillaumin, S., Davail, S., Andre, J-M. and R. Hoo-Paris (2003). Differential Channelling of Liver Lipids in Relation to Susceptibility to Hepatic Steatosis in Two Species of Ducks. *Comparative Biochemistry and Physiology, Part B*, **135**: 663-675.

**Hetherington, S.V.** Spitznagel, J.K. and P.G. Quie (1983). An Enzyme-Linked Immunoassay (ELISA) for Measurement of Lactoferrin. *Journal of Immunological Methods*, **65**: 190-183.

**Hobbs, K.E.,** Lebeuf, M. and M.O. Hammill (2002). PCBs and OCPs in Male Harbour, Grey, Harp and Hooded Seals from the Estuary and Gulf of St. Lawrence, Canada. *The Science of the Total Environment*, **296**: 1-18.

**Holden, A.V.** (1972). Monitoring Organochlorine Contamination of the Marine Environment by the Analysis of Residues in Seals. In: Ruivo M (ed) Marine Pollution and Sea Life. Fishing New Books Ltd., London, pp.266-272.

**Holden, A.V.** (1978b). Organochlorine Residues in Blubber of Grey Seals (*Halichoerus grypus*) from the Farne Islands *International Council for the Exploration of the Sea CM*, **E**: 41-45.

**Hook, O.** and A.G. Johnels (1972). The Breeding and Distribution of the Grey Seal (*Halichoerus grypus* Fab.) in the Baltic Sea, With Observations on Other Seals of the Area. *Proceedings of the Royal Society of London*, **182**: 37-58.

**Hop, H.,** Borgå, K., Gabrielsen, G.W., Kleivane, L. and J.U. Skaare (2002). Food Web Magnification of Persistent Organic Pollutants in Poikilotherms and Homeotherms from the Barents Sea. *Environmental Science and Technology*, **36**: 2589-2597.

**Hoyer, P.B.** (2001). Reproductive Toxicology: Current and Future Directions. *Biochemical Pharmacology*, **62**: 1557–1564.

**Hovander, L.,** Malmberg, T., Athanasiadou, M., Athanassiadis, S., Bergman, Å. and E. Klasson-Wehler (2002). Identification of Hydroxylated PCB Metabolites and Other Phenolic Halogenated Pollutants in Human Blood Plasma. *Archives of Environmental Contamination and Toxicology*, **42**: 105-117.

**Hurley, W.L.,** Grieve, R.C.J., Magura, C.E., Hegarty, H.M. and S. Zou (1993). Electrophoretic Comparisons of Lactoferrin from Bovine Mammary Secretions, Milk Neutrophils, and Human Milk. *Journal of Dairy Science* **76**(2): 377-387.

**Hutchinson, J.D.** and M.P. Simmonds (1994). Organochlorine Contamination in Pinnipeds. *Reviews of Environmental Contamination and Toxicology*, **136**: 123-167.

**Hutzinger, O.,** Safe, S. and V. Zitko (1974). *The Chemistry of PCBs*. CRC Press, Boca Raton, Florida.

**Ikonomou, M.G.,** Rayne, S. and R.F. Addison (2002). Exponential Increases of the Brominated Diphenyl Ethers, in the Canadian Arctic from 1981-2000. *Environmental Science and Technology*, **36**: 1886-1892.

**Inagaki, M.,** Kikuchi, M., Orino, K., Ohnami, Y. and K. Watanabe (2002). Purification and Quantification of Lactoferrin in Equine Seminal Plasma. *The Journal of Veterinary Medicine*, **64**(1): 75-77.

**INCA,** Industry Nature Conservation Association, 2000

**Inoue, H.,** Sakai, M., Kaida, Y., and K. Kaibara (2004). Blood Lactoferrin Release Induced by Running Exercise in Normal Volunteers: Antibacterial Activity. *Clinica Chimica Acta*, **341**: 165-172.

**Jansson B.,** Splund, L. and M. Olsson (1987). Brominated Flame Retardants – Ubiquitous Environmental Pollutants? *Chemosphere*, **16**(1-12): 2343-2349.

**Jansson, B.,** Andersson, R., Asplund, L., Litzén, K., Nylund, K., Sellström, U., Uvemo, U.-B., Wahlberg, C., Wideqvist, U., Odsjö, T. and M. Olsson (1993). Chlorinated and Brominated Compounds in Biological Samples from the Environment. *Environmental Toxicology and Chemistry*, **12**: 1163-1174.

**Jefferson, A.** (1993). Marine Mammals of the World, United Nations Environment Programme: Food and Agriculture Organization of the United Nations. A. Jefferson, S. Leatherwood and M.A. Webber (Eds.), Rome, pp. 214-227, 258-259 and 272-273.

**Jefferson, W.N.,** Padilla-Banks, E. and R.R. Newbold (2000). Lactoferrin is an Estrogen Responsive Protein in the Uterus of Mice and Rats. *Reproductive Toxicology*, **14**: 103-110.

**Jensen, S.,** Johnels, A.G., Olsson, M. and G. Otterlind (1969). DDT and PCB in Marine Mammals from Swedish Waters. *Nature*, **224**: 247-250.

**Jenseen, B.,** Skaare, J., Woldtad, S., Nastad, A., Haugen, O. and E. Sørmo (1995). Biomarkers in Blood to Assess Effects of Polychlorinated Biphenyls in Free – Living Grey Seal Pups. *In*: Whales, Fish and Man, A.S. Blix, L. Walløc, and O. Ulltang (Eds.), Amsterdam, pp. 607-615.

**Jenssen, B.M.** (1996). An Overview of Exposure to, and Effects of, Petroleum Oil and Organochlorine Pollution in Grey Seals (*Halichoerus grypus*). *The Science of the Total Environment*, **186**: 109-118.

**Johansson, B.G.** (1960). Isolation of an Iron Containing Red Protein from Human Milk. *Acta Chemica Scandinavica*, **14**: 510-512.

**Johnson, J.C.,** Van Emon, J.M., Clarke, A.N. and B.N. Wamsley (2001). Quantitative ELISA of Polychlorinated Biphenyls in an Oily Soil Matrix Using Supercritical Fluid Extraction. *Analytica Chimica Acta*, **428**: 191-199.

**Johnson, M.H.** and B.J. Everitt (2000). Chapter 3: Reproductive Messengers. *In*: Essential Reproduction, 5th Edition, M.H. Johnson and B.J. Everitt (Eds.), Blackwell Science, UK, pp. 36.

**Jones, B.** and A. Turki (1997). Distribution and Speciation of Heavy Metals in Surficial Sediments from the Tees Estuary, North-East England. *Marine Pollution Bulletin*, **34**(10): 768-779.

**Jones, K.C.** and P. de Voogt (1999). Persistent Organic Pollutants (POPs): State of the Science. *Environmental Pollution*, **100**(1-3): 209-221.

**Juan, C-Y.,** Thomas, G.O., Sweetman, A.J. and K.C. Jones (2002). An Input-Output Balance Study for PCBs in Humans. *Environmental International*, **28**: 203-214.

**Kähler, S.,** Christophers, E. and J.M. Schröder (1988). Plasma Lactoferrin Reflects Neutrophil Activation in Psoriasis. *British Journal of Dermatology*, **119**: 289-293.

**Kalantzi, O.I.,** Hall, A.J., Thomas, G.O. and K.C. Jones (2005). Polybrominated Diphenyl Ethers and Selected Organochlorine Contaminants in Grey Seals (*Halichoerus grypus*) in the North Sea. *Chemosphere*, **58**: 345-354.

**Kamrin, M.A.** (2004). Biomonitoring Basics. A Report from biomonitoringinfo.org. *Institute for Environmental Toxicology*, Michigan State University.

**Kane, S.V.,** Sandborn, W.J., Rufo, P.A., Zholudev, A., Boone, J., Lyerly, D., Camilleri, M. and S.B. Hanauer (2003). Faecal Lactoferrin Is a Sensitive and Specific Marker in Identifying Intestinal Inflammation. *The American Journal of Gastroenterology*, **98**(6): 1309-1314.

**Kanyshkova, T.G.,** Babina S.E., Semenov, D., Isaeva, N., Vlassov, A., Neustroev, K.N., Kul'minskaya, A.A., Buneva, V.N. and G.A. Nevinsky (2003). Multiple Enzymic Activities of Human Milk Lactoferrin. *European Journal of Biochemistry*, **270**: 3353-3361.

**Karlsson, H.,** Muir, D.C.G., Teixiera, C.F., Burniston, D.A., Strachan, W.M.J., Hecky, R.E., Mwita, J., Boostma, H.A., Grift, N.P., Kidd, K.A. and B. Rosenberg (2000). Persistent Chlorinated Pesticides in Air, Water and Precipitation from the Lake Malawi Area, Southern Africa. *Environmental Science and Technology*, **34**(21): 4490-4495.

**Kealey, D.** and P.J. Haines (2002). Instant Notes, Analytical Chemistry. BIOS Scientific Publishers Limited.

**Keefe, P.W.,** Miller, J., Smith, R., Connor, S., Clayton, W. and R. Storm (1997). Separation of Extracts from Biological Tissues into Polycyclic Aromatic Hydrocarbon, Polychlorinated Biphenyl and Polychlorinated Dibenzo-*p*-Dioxin/Polychlorinated Dibenzofuran Fractions Prior to Analysis. *Journal of Chromatography A.*, **771**: 169-179.

**Kelce, W.R.,** Stone, C.R., Laws, S.C., Gray, L.E., Kemppainen, J.A., and E.M., Wilson (1995). Persistent DDT Metabolite *p,p'*-DDE is a Potent Androgen Receptor Antagonist. *Nature*, **375**: 581-585.

**Kerr, M.G.** (1989). Veterinary Laboratory Medicine Clinical Biochemistry and Haematology. Blackwell Scientific Publications, Oxford, pp. 270.

**Knopp. D** (1995). Application of Immunological Methods for the Determination of Environmental Pollutants in Human Biomonitoring. A review. *Analytical Chimica Acta*, **311**: 383-392.

**Koistinen, J.,** Stenman, O., Haahti, H., Suonperä, M. and J. Paasivirta (1997). Polychlorinated Diphenyl Ethers, Dibenzo-p-dioxins, Dibenzofurans and Biphenyls in Seals and Sediment from the Gulf of Finland. *Chemosphere*, **35**(6): 1249-1269.

**Kovacs, K.M.,** (1986). Maternal Investment and Early Behavioural Development in the Harp (*Phoca groenlandica*) and Grey Seals (*Halichoerus grypus*). PhD Thesis, Univ. Guelph, Guelph, Ontario.

**Krahn, M.M.,** Beckmen, K.B., Pitcher, K.W. and K.A. Burek (2001). Population Survey of Organochlorine Contaminants in Alaskan Steller Sea Lions. Final Programmatic Report for the NFWF Funded Project.

**Krause, W.** (1988) Concentration of Oestradiol in Serum and Testis Tissue in Patients with Fertility Disorders. *Fertility and Sterility*, **49**: 926-927.

**Kreiss, K.,** Zack, M., Kimbrough, R., Needham, L., Smrek, A. and B. Jones (1981). Association of Blood Pressure and Polychlorinated Biphenyl Levels. *The Journal of the American Medical Association*, **245**(24): 2505-2509.

**Kuiken, T.** (1985). Influences of Diet, Gestation and Age on Haematology and Plasma Chemistry of the Harbour Seal, *Phoca vitulina*. *Aquatic Mammals*, **11**: 40

**Kurita, T.,** Lee, K., Cooke, P.S., Lydon, J.P., and G.R. Cunha (2000). Paracrine Regulation of Epithelial Progesterone Receptor and Lactoferrin by Progesterone in the Mouse Uterus. *Biology of Reproduction*, **62**: 831-838.

**Laemmli, U.K.** (1970). Cleavage of Structural Proteins During the Assembly of the Head of Bacteriophage T4. *Nature*, **227**: 680-685.

**Lakritz, J.,** Tyler, J.W., Hostetler, D.E., Marsh, A.E., Weaver, D.M., Holle, J.M., Steevens, B.J. and J.L. Denbigh (2000). Effects of Pasteurization of Colostrum on Subsequent Serum Lactoferrin Concentration and Neutrophil Superoxide Production in Calves. *American Journal of Veterinary Research*, **61**(9): 1021-1025.

**Larkins, N.** (2005). Review Article: Potential Implications of Lactoferrin as a Therapeutic Agent. *American Journal of Veterinary Research*, **66**(4): 739-742.

**Larson, S.,** Casson, C.J. and S. Wasser (2003). Noninvasive Reproductive Steroid Hormone Estimated from Faecal Samples of Captive Female Sea Otters (*Enhydra lutris*). *General and Comparative Endocrinology*, **134**: 18-25.

**Lasley, B.L.** and J.W. Overstreet (1998). Biomarkers for Assessing Human Female Reproductive Health, An Interdisciplinary Approach. *Environmental Health Perspectives*, **106**(4): 955-960.

**Law, R.J.,** Allchin, C.R. and J. Harwood, (1989). Concentrations of Organochlorine Compounds in the Blubber of Seals from Eastern and North-eastern England *Marine Pollution Bulletin,* **20**(3): 110-115.

**Law, R.J.** (1994). Collaborative UK Marine Mammal Project: Summary of Data Produced 1988-1992. Fisheries Research Technical Report No. 97, Directorate of Fisheries Research, Lowestoft.

**Law, R.J.,** Allchin, C.R., Bennett, M.E., Morris, S. and E. Logan (2002). Polybrominated Diphenyl Ethers in Two Species of Marine Top Predators from England and Wales. *Chemosphere*, **46**: 673-681.

**Law, R.J.,** Alaee, M., Allchin, C.R., Boon, J.P., Lebeuf, M., Lepom, P. and G.A. Stern (2003). Levels and Trends of Polybrominated Diphenylethers and Other Brominated Flame Retardants in Wildlife. *Environment International*, **29**: 757-770.

**Lawruk, T.S.,** Lachman, C.E., Jourdan, S.W., Fleeker, J.R., Hayes, M.C., Herzog, D.P. and F.M. Rubio (1996). Quantitative Determination of PCBs in Soil and Water by a Magnetic Particle – Based Immunoassay. *Environmental Science and Technology*, **30**(2): 695-700.

**LeBlanc, G.A.** and L.J. Bain (1997). Chronic Toxicity of Environmental Contaminants: Sentinels and Biomarkers. *Environmental Health Perspectives*, **105**(1): 65-80.

**Levalle, O.A.,** Zylberstein, C., Aszpis, S., Mariana, V., Ponzio, R., Aranda, C., Guitelman, C. and H.E. Scaglia (1994). Serum Luteinizing Hormone Pulsatility and Intratesticular Testosterone and Oestradiol Concentrations in Idiopathic Infertile Men with High and Normal Follicle Stimulating Hormone Serum Concentrations. *Human Reproduction*, **9**: 781-787.

**Levay, P.F.** and M. Viljoen (1995). Lactoferrin: A General Review. *Haematologica*, **80**: 252-267.

**Li, S.H.** and Y.H. Chen (1999). Various Forms of Mouse Lactoferrins: Purification and Characterisation. *Journal of Chromatography B: Biomedical Sciences and Applications*, **726**(1-2): 45-52.

**Lincolnshire Wildlife Trust**: www.lincstrust.org.uk

**Lindström, G.,** Wingfors, H., Dam, M. and B. van Bavel (1999). Identification of 19 Polybrominated Diphenyl Ethers (PBDEs) in Long-Finned Pilot Whale (*Globicephala melas*) from the Atlantic. *Archives of Environmental Contamination and Toxicology*, **36**: 355-363.

**Linklater, W.L.,** Henderson, K.M., Cameron, E.Z., Stafford, K.J., and E.O. Minot (2000). The Robustness of Faecal Steroid Determination for Pregnancy Testing Kaimanawa Feral Mares under Field Conditions. *New Zealand Veterinary Journal*, **48**: 93-98.

**Loganathan, B.G.** and K. Kannan (1994). Global Organochlorine Contamination Trends: An Overview. *Ambio*, **23**(3): 187-191.

**Lönnerdal, B.,** Forsum, E. and L. Hambraeus (1976a). A Longitudinal Study of the Protein, Nitrogen and Lactose Contents of Human Milk from Swedish Well-Nourished Mothers. *The American Journal of Nutrition*, **29**: 1127-1133.

**Lönnerdal, B.,** Forsum, E., Gebre-Medhin, M. and L. Hambraeus (1976b). Breast Milk Composition in Ethiopian and Swedish Mothers. II. Lactose, Nitrogen and Protein Contents. *The American Journal of Nutrition*, **29**: 1134-1141.

**Lönnerdal, B.** and S. Iyer (1995). Lactoferrin: Molecular Structure and Biological Function. *Annual Review of Nutrition*, **15**: 93-110.

**Lowry, L.F.,** Frost, K.J. and J.J. Burns (1980). Variability in the Diet of Ringed Seals, *Phoca hispida*, in Alaska. *Canadian Journal of Fisheries and Aquatic Science*, **37**: 2254-2261.

**Luckas, B.,** Vetter, W., Fischer, P., Heidemann, G., and J. Plötz (1990). Characteristic Chlorinated Hydrocarbon Patterns in the Blubber of Seals from Different Marine Regions. *Chemosphere*, **21**(1-2): 13-19.

**Lynch, J.W.,** Khan, M.Z., Altmann, J., Njahira, M.N. and N. Rubenstein (2003). Concentrations of Four Fecal Steroids in Wild Baboons: Short-Term Storage Conditions and Consequences for Data Interpretation. *General and Comparative Endocrinology*, **132**: 264-271.

**Macdonald, R.W.,** Barrie, L.A., Bidleman, T.F., Diamond, M.L., Gregor, D.J., Semkin, R.G., Strachan, W.M.J., Li, Y.F., Wania, F., Alaee, M., Backus, S., Bewers, M., Gobeil, C., Halsall, C., Hoff, J., Lockhart, L., Mackay, D., Muir, D., Pudykiewicz, J., Reimer, K., Smith, J., Stern, G., Schroeder, W., Wagemann, R., and M. Yunker (2000). Contaminants in the Canadian Arctic: Five Years of Progress in Understanding Sources, Occurrence and Pathways. *Science of the Total Environment*, **254**: 93-234.

**Markey, C.M.,** Michaelson, C.L., Veson, E.C., Sonnenschein, C. and A.M. Soto (2001). The Mouse Uterotrophic Assay: A Reevaluation of Its Validity in Assessing the Estrogenicity of Bisphenol A. *Environmental Health Perspectives*, **109**: 55-60.

**Marsili, L.,** Fossi, M.C., Notarbartolo di Sciara, G., Zanardelli, M., Nani, B., Panigada, S. and S. Focardi (1998). Relationship Between Organochlorine Contaminants and Mixed Function Oxidase Activity in Skin Biopsy Specimens of Mediterranean Fin Whales (*Balaenoptera physalus*). *Chemosphere.*, **37**: 1501-1510.

**Martineau, D.,** De Guise, S., Fournier, M., Shugart, L., Girard, C., Lagacé, A. and P. Béland (1994). Pathology and Toxicology of Beluga Whales from the St. Lawrence Estuary, Québec, Canada. Past, Present and Future. *Science of the Total Environment*, **154**: 201-215.

**Masson, P.L.,** Heremans, J.F. and C. Div (1966). An Iron-binding Protein Common to Many External Secretions. *Clinica Chimica Acta*, **14**: 735-739.

**Masson, P.L.,** Heremans, J.F. and E. Schonne (1969). Lactoferrin, an Iron-Binding Protein in Neutrophil Leukocytes. *The Journal of Experimental Medicine*, **130**(3): 643- 658.

**Masson, P.L.** and J.F. Heremans (1971). Lactoferrin in Milk from Different Species. *Comparative Biochemistry and Physiology B*, **39**: 119-122.

**Matsumuro, M.,** Sankai, T., Cho, F., Yoshikawa, Y.and T. Yoshida. (1999). A Two-Step Extraction Method to Measure Fecal Steroid Hormones in Female Cynomolgus Monkeys (*Macaca fascicularis*). *American Journal of Primatology*, **48**: 291-298.

**McConnell, L.C.** and Vaughan, R.W. (1983). Some Blood Values in Captive and Free-living Common Seals (*Phoca vitulina*). *Aquatic Mammals*, **10**: 9-13.

**McFarland, V.A.** and J.U. Clarke (1989). Environmental Occurrence, Abundance and Potential Toxicity of Polychlorinated Biphenyl Congeners: Considerations for a Congener-Specific Analysis. *Environmental Health Perspectives*, **81**: 225-239.

**McKinney, J.D.** (1996). Reactivity Parameters in Structure-Activity Relationship-Based Risk Assessment of Chemicals. *Environmental Health Perspectives*, **104**(8): 810-816.

**McLachlan, J.A.** (1993). Functional Toxicology: A New Approach to Detect Biologically Active Xenobiotics. *Environmental Health Perspectives*, **101**(5): 386-387.

**McMaster, M.T.,** Teng, C., Dey, S.K. and G.K. Andrews (1991). Lactoferrin in the Mouse Uterus: Analyses of the Preimplantation Period and Regulation by Ovarian Steroids. *Molecular Endocrinology*, **6**(1): 101-111.

**Medway, W.,** Schryver, H.F., and B. Bell (1966). Clinical Jaundice in Porpoises. *Journal of the American Medical Association*, **149**: 891.

**Medway, W.,** and J.R. Geraci (1986). Clinical Pathology of Marine Mammals. *In*: Zoo and Wild Animal Medicine, 2nd Edition, M.E. Fowler (Ed.), W.B. Saunders, Philadelphia, pp. 791.

**Meironyté, D.,** Norén, K. and Å Bergman (1999). Analysis of Polybrominated Diphenyl Ethers in Swedish Human Milk: A Time-related Trend Study, 1972-1997. *Journal of Toxicology and Environmental Health*, **58**(6): 329-341.

**Metcalf, R.L.** (1995). Insect Control Technology *In*: Kroschwitz J., Howe-Grant M., eds. Kirk-Othmer Encyclopaedia of Chemical Technology. Volume 14. New York, NY: John Wiley and Sons, Inc., 524-602.

**Metz-Boutigue, M.H.,** Jolle P,J., Mazurier, J., Schoentgen, F., Legrand, D., Spik, G, Montreuil, J. and P. Jolle (1984). Human Lactotransferrin: Amino Acid Sequence and Structural Comparisons With Other Transferrins. *European Journal of Biochemistry* **145**: 659-676.

**Meyer, D.J.** and J.W. Harvey (1998). Veterinary Laboratory Medicine: Interpretation and Diagnosis, W.B. Saunders, Philadelphia, Chaps. 3 (43-82) and 7 (157-186).

**Milnes, M.R.,** Bermudez, D.S., Bryan, T.A., Edwards, T.M., Gunderson, M.P., Larkin, I.L.V., Moore, B.C. and L.J. Guillette, Jr. (2006). Contaminant-Induced Feminization and Demasculinization of Nonmammalian Vertebrate Males in Aquatic Environments. *Environmental Research*, **100**:3-17.

**Moreira, N.,** Monteiro-Filho, E.L.A., Moraes, W., Swanson, W.F., Graham, L.H., Pasquali, O.L., Gomes, M.L.F., Morais, R.N., Wildt, D.E. and J.L. Brown (2001). Reproductive Steroid Hormones and Ovarian Activity in Felids of the *Leopardus* Genus. *Zoo Biology*, **20**: 103-116.

**Morris, R.J.,** Law, R.J., Allchin, C.R. Kelly, C.A. and C.F. Fileman (1989). Metals and Organochlorines in Dolphins and Porpoises of Cardigan Bay, West Wales. *Marine Pollution Bulletin*, **20**(10): 512-523.

**Muhlebach, S.,** Moor, M.J., Wyss, P.A. and M.H. Bickel (1991). Kinetics of Distribution and Elimination of DDE in Rats. *Xenobiotica*, **21**(1): 111-120.

**Muir, D.C.G.,** Riget, F., Cleemann, M., Skaare, J., Kleivane, L. and H. Nakata (2000c). Circumpolar Trends of PCBs and Organochlorine Pesticides in the Arctic Marine Environment Inferred from Levels in Ringed Seals. *Environmental Science and Technology*, **34**: 2431– 2438.

**Muir, D.,** Savinova, T., Savinov, V., Alexeeva, L., Potelov, V. and V. Sretochev (2003). Bioaccumulation of PCBs and Chlorinated Pesticides in Seals, Fishes and Invertebrates from the White Sea, Russia. *The Science of the Total Environment*, **306**: 111-131.

**Nagasawa, T.,** Kiyosawa, I. and K. Kuwahara (1972). Amounts of Lactoferrin in Human Colostrum and Milk. *Journal of Dairy Science*, **55**(12): 1651-1659.

**Newbold, R.R.,** Teng, C.T., Beckman, W.C JR., Jefferson, W.N., Hanson, R.B., Miller, J.V. and J.A. McLachlan (1992). Fluctuations of Lactoferrin Protein and Messenger Ribonucleic Acid in the Reproductive Tract of the Mouse During the Estrous Cycle. *Biology of Reproduction*, **47**: 903-915.

**Newbold, R.R.,** Jefferson, W.N., Padilla-Banks, E. and J. Hasemanb (2004). Developmental Exposure to Diethylstilbestrol (DES) Alters Uterine Response in Prepubescent Mice: Low versus High Dose Effects. *Reproductive Toxicology*, **18**: 399-406.

**Nichols, B.L.,** McKee, K.S., Henry, J.F. and M. Putman (1987). Lactoferrin Stimulates Thymidine Incorporation into DNA of Rat Crypt Cells. *Pediatric Research*, **21**: 563-567.

**Nielsen, J.** (1995). Immunological and Haematological Parameters in Captive Harbor Seals (*Phoca vitulina*). *Marine Mammal Science*, **11**: 314-323.

**Noonan, L.M.,** Ronals, K. and J. Raeside (1991). Plasma Testosterone Concentrations of Captive Male Hooded Seals (*Cystophora cristata*). *Canadian Journal of Zoology*, **69**: 2279-2282.

**Nyman, M.** (2000). Biomarkers for Exposure and for Effects of Contamination With Polyhalogenated Aromatic Hydrocarbons in Baltic Ringed and Grey Seals, PhD Thesis, Helsinki.

**Nyman, M.,** Koistinen, J., Fant, M.L., Vartiainen, T. and E. Helle (2002). Current Levels of DDT, PCB and Trace Elements in the Baltic Ringed Seals (*Phoca hispida baltica*) and Grey Seals (*Halichoerus grypus*). *Environmental Pollution*, **119**: 399-412.

**Nyman, M.,** Bergknut, M., Fant, M.L., Raunio, H., Jestoi, M., Bengs, C., Murk, A., Koistinen, J., Bäckman, C., Pelkonen, O., Tysklind, M., Hirvi, T. and E. Helle (2003). Contaminant Exposure and Effects in Baltic Ringed and Grey Seals as Assessed by Biomarkers. *Marine Environmental Research*, **55**: 73-99.

**O'Shea, T.** (1999). Environmental Contaminants and Marine Mammals. *In*: J.E. Reynolds III and S.A. Rommel (Eds.), Biology of Marine Mammals (pp. 485-536). Washington, USA, Smithsonian Institution Press.

**Oberdörster, E.** and A.O. Cheek (2000). Gender Bender at the Beach: Endocrine Disruption in Marine and Estuarine Organisms. *Environmental Toxicology and Chemistry*, **20**: 23-36.

**Ofstad, E.B.** and K. Martinsen (1983). Persistent Organochlorine Compounds in Seals from Norwegian Coastal Waters. *Ambio*, **12**: 262-264.

**Okuyama, A.,** Takenaka, H., Nishi, K., Mizukami, H., Kozaki, S., Kirihata, M., Miyatake, K., Takigami, H., Sakai, S. and M. Morita (2002). Development of Enzyme-Linked Immunosorbent Assay for the Pre-Screening of Coplanar Polychlorinated Biphenyls. *Organohalogen Compounds*, **58**: 333-335.

**Olofsson, T.,** Olsson, I., Venge, P. and B. Elgefors (1977). Serum Myeloperoxidase and Lactoferrin in Neutropenia. *Scandinavian Journal of Haematology*, **18**(1): 73-80.

**Olsson, M.,** Johnels, A.G. and R. Vaz (1974). DDT and PCB Levels in Seals from Swedish Waters. The Occurrence of Aborted Seal Pups. *In* Proc. Symposium of the Seal in the Baltic, June 4-6 1974, Lidingl, Sweden. Pub;. SNV PM 591, Swedish Environmental Protection Board.

**Olsson, M.,** Karlsson, B. and E. Ahnland (1994). Disease and Environmental Contaminants in Seals from the Baltic and Swedish West Coast. *Science of the Total Environment*, **154**: 217-227.

**Orsi, N.** (2004). The Antimicrobial Activity of Lactoferrin: Current Status and Perspectives. *BioMetals*, **17**: 189-196.

**Osterhaus, A.D.M.E.** and E.J. Vedder (1988). Identification of Virus Causing Recent Seal Deaths. *Nature*, **335**: 20.

**Osterhaus, A.D.M.E.,** Visser, I.K.G., de Swart, R.L., van Bressem, M.F., van de Bildt, M.W.G., Örvell, C., Barrett, T. and J.A. Raga (1992). Morbillivirus Threat to Mediterranean Monk Seals? *The Veterinary Record*, **130**: 141-142.

**Pacora, P.,** Maymon, E., Gervasi, M.T., Gomez, R., Edwin, S.S., Yoon, B.H. and R. Romero (2000). Lactoferrin in Intrauterine Infection, Human Parturition, and Rupture of Fetal Membranes. *American Journal of Obstetrics and Genecology*, **183**(4): 904-910.

**Panella, TJ.,** Liu, Y.H., Huang, A.T. and C.T. Teng (1991). Polymorphism and Altered Methylation of the Lactoferrin Gene in Normal Leukocytes, Leukemic Cells, and Breast Cancer. *Cancer Research*, **51**: 3037-3043.

**Peakall, D.,** Lincer, J.L. and S.E. Bloom (1972). Embryonic Mortality and Chromosomal Alterations Caused by Aroclor 1254 in Ring Doves. E*nvironmental Health Perspectives* **1**: 103-104.

**Peakall, D.** (1992). *Animal Biomarkers as Pollution Indicators*, pp. 291. Chapman & Hall.

**Pentecost, B.T.** and C.T. Teng (1987). Lactotransferrin Is the Major Estrogen Inducible Protein of Mouse Uterine Secretions. *The Journal of Biological Chemistry*, **262**(21): 10134-10139.

**Pentecost, B.T.,** Newbold, R.R., Teng, C.T. and J.A. McLachlan (1988). Prenatal Exposure of Male Mice to Diethylstilbestrol Alters the Expression of the Lactotransferrin Gene in Seminal Vesicles. *Molecular Endocrinology*, **2**(12): 1243-1248.

**Persky, V.,** Turyk, M., Anderson, H.A., Hanrahan, L.P., Falk, C., Steenport, D.N., Chatterton, R.Jr., Freels, S. and the Great Lakes Consortium (2001). The Effects of

PCB Exposure and Fish Consumption on Endogenous Hormones. *Environmental Health Perspectives*, **109**: 1275-1283.

**Perttillä, M.,** Stenman, O., Pyysalo, H. and K. Wickström (1986). Heavy Metals and Organochlorine Compounds in Seals in the Gulf of Finland. *Marine Environmental Research*, **18**: 43-59.

**Pijnenburg A.M.C.M.**, Evert, J.W., de Boer, J and J.P., Boon (1995). Polybrominated Biphenyl and Diphenyl Ether Flame Retardants: Analysis, Toxicity and Environmental Occurrence. *Reviews of Environmental Contamination and Toxicology*, **141**: 1-26.

**Pomeroy, P.P.,** Green, N., Hall, A.J., Walton, M., Jones, K. and J. Harwood (1996). Congener – Specific Exposure of Grey Seal (*Halichoerus grypus*) Pups to Chlorinated Biphenyls During Lactation. *Canadian Journal of Fisheries and Aquatic Science*, **53**: 1526-1534.

**Portier, C.J.** (2002) Endocrine Dismodulation and Cancer. *Neuroendocrinology Letters* **23**(2): 37-41.

**Raeside, J.I.** and K. Ronald, K (1981). Plasma Concentration of Oestrone, Progesterone and Corticosteroids During Late Pregnancy and After Parturition in the Harbour Seal. *Journal of Reproduction and Fertility*, **61**: 135–139.

**Rahman, F.,** Langford, H., Scrimshaw, M.D. and J.N. Lester (2001). Polybrominated Diphenyl Ether (PBDE) Flame Retardants. *The Science of the Total Environment*, **275**: 1-17.

**Ranhotra, H.S.** and C.T. Teng (2005). Assessing the Estrogenicity of Environmental Chemicals with a Stably Transfected Lactoferrin Gene Promoter Reporter in HeLa Cells. *Environmental Toxicology and Pharmacology*, **20**: 42-47.

**Rebelo, I.,** Carvalho-Guerra, F., Pereira-Leite, L. and A. Quintanilha (1995). Lactoferrin as a Sensitive Blood Marker of Neutrophil Activation in Normal Pregnancies. *European Journal of Obstetrics and Gynecology and Reproductive Biology*, **62**: 189-194.

**Rebelo, I.,** Carvalho-Guerra, F., Pereira-Leite, L. and A. Quintanilha (1996). Comparative Study of Lactoferrin and Other Blood Markers of Inflammatory Stress Between Preeclamptic and Normal Pregnancies. *European Journal of Obstetrics and Gynecology and Reproductive Biology*, **64**: 167-173.

**Reijnders, P.J.H.** (1980). Organochlorine and Heavy Metal Residues in Harbour Seals from the Wadden Sea and Their Possible Effects on Reproduction. *Netherlands Journal of Sea Research*, **14**(1): 30-65.

**Reijnders, P.J.H.** (1984). Man Induced Environmental Factors in Relation to Fertility Changes in Pinnipeds. *Environmental Conservation*, **11**: 61-65.

**Reijnders, P.J.H.** (1986). Abstracts from the Dutch Perinatal Biology Study Group: Association of Reproductive Disorders in Mammals with Polychlorinated Biphenyls. *European Journal of Obstetrics and Gynecology and Reproductive Biology*, **21**: 117-123.

**Reijnders, P.J.H.** (1986). Reproductive Failure in Common Seals Feeding on Fish from Polluted Coastal Waters. *Nature* **324**: 456-457.

**Reijnders, P.J.H.** (1988). Ecotoxicological Perspectives in Marine Mammalogy: Research Principles and Goals for a Conservation Policy. *Marine Mammal Science*, **4**: 91-102.

**Reijnders, P.J.H.** (1990). Progesterone and Estradiol-17β Concentration Profiles Throughout the Reproductive Cycle in Harbor Seals (*Phoca vitulina*). *Journal of Reproduction and Fertility*, **90**: 403-409.

**Reijnders, P.J.H.,** Ries, E.H., Tougaard, S., Nøgaard, N., Heidemann, G., Schwarz, J., Vareschi, E. and I.M. Traut (1997). Population Development of Harbour Seals *Phoca vitulina* in the Wadden Sea After the 1988 Virus Epizootic. *Journal of Sea Research*, **38**: 161-168.

**Reijnders, P.J.H.,** Verriopoulos, G., and S.M.J.M., Brasseur (1997). Status of Pinnipeds Relevant to the European Union, DLO Institute for Forestry and Nature Research (IBN-DLO), Wageningen.

**Renfree, M.B.** and G. Shaw (2000). Diapause. *Annual Review of Physiology*, **62**: 353-375.

**Renouf, D.** (1991). The Behaviour of Pinnipeds, Chapman and Hall, London.
**Restriction of Hazardous Substances Directive**, RoHS; Waste Electrical Equipment Directive, WEE; EU, 2005
**Ries, E.H.,** Traut, I.M., Brinkman, A.G. and P.J.H. Reijnders (1999). Net Dispersal of Harbour Seals Within the Wadden Sea Before and After the 1988 Epizootic. *Journal of Sea Research*, **41**: 233-244.
**Riget, F.,** Dietz, R., Vorkamp, K., Johansen, P. and D. Muir (2004). Levels and Spatial and Temporal Trends of Contaminants in Greenland Biota: An Updated Review. *The Science of the Total Environment*, **331**: 29-52.
**Robeck, T.R.,** Atkinson, S.K.C. and F. Brook (2001). Chapter 11: Reproduction. *In*: CRC Handbook of Marine Mammal Medicine, 2nd Edition. L.A. Dierauf and F.M.D. Gulland (Eds.), CRC Press, Boca Raton, pp. 193-236.
**Roletto, J.** (1993). Haematology and Serum Chemistry Values for Clinically Healthy and Sick Pinnipeds. *Journal of Zoo and Wildlife Medicine*, **24**: 145-157.
**Routti, H.,** Nyman, M., Bäckman, C., Koistinen, J. and E. Helle (2005). Accumulation of Dietary Organochlorines and Vitamins in Baltic Seals. *The Science of the Total Environment*, **60**: 267-287.
**Ruus., A.,** Ugland, K.I., Espeland, O. and J.U. Skaare (1999). Organochlorine Contaminants in a Local Marine Food Chain from Jarfjord, Northern Norway. *Marine Environmental Research*, **48**(2): 131-146.
**Safe, S.H.** (1984). Polychlorinated Biphenyls (PCBs) and Polybrominated Biphenyls (PBBs): Biochemistry, Toxicology, and Mechanism of Action. *CRC Critical Reviews in Toxicology*, **13**(4): 319-395.
**Safe, S.H.** (1990). Polychlorinated Biphenyls (PCBs), Dibenzo-p-dioxins (PCDDs), Dibenzofurans (PCDFs) and Related Compounds: Environmental and Mechanistic Considerations Which Support the Development of Toxic Equivalency Factors (TEFs). *Critical Reviews in Toxicology.*, **21**: 51-88.
**Safe, S.H.** (1994). Polychlorinated Biphenyls (PCBs): Environmental Impact, Biochemical and Toxic Responses, and Implications for Risk Assessment. *Critical Reviews in Toxicology*, **24**(2): 87-149.
**Sangalang, G.B.,** and H.C. Freeman (1976). Notes: Steroids in the Plasma of the Gray Seal, *Halichoerus grypus. General and Comparative Endocrinology*, **29**(3): 419-422.
**Sattar, N.,** Greer, I.A., Louden, J., Lindsay, G., McConnell, M., Shepherd, J. and J. Packard (1997). Lipoprotein Subfraction Changes in Normal Pregnancy: Threshold Effect of Plasma Triglyceride on Appearance of Small, Dense Low Density Lipoprotein. *Journal of Clinical Endocrinology and Metabolism*, **82**(8): 2483-2491.
**Schafer, K.,** Kegley, S. and S. Patton. (2001). Nowhere to Hide: Persistent Toxic Chemicals in the U.S. Food Supply. Pesticide Action Network, North America, 2nd Edition.
**Schäfer,K.H.** (1951). Elektrophoretische untersuchungen zum milcheiweissproblem. *Monatssch Kinderheilk*, **99**: 69.
**Scheen, A.J.** and F.H. Luyckx (2002). Obesity and Liver Disease. *Best Practise and Research Clinical Endocrinology and Metabolism*, **16**(4): 703-716.
**Schlezinger, J.J.,** White, R.D. and J.J. Stegeman (1999). Oxidative Inactivation of Cytochrome P-450 1A (CYP1A) Stimulated by 3,3',4,4'-tetrachlorobiphenyl: Production of Reactive Oxygen by Vertebrate CYP1As. *Molecular Pharmacology*, **56**(3): 588-597.
**Schultz, D.E.,** Petrick, G. and J. Duinker (1989). Complete Characterisation of PCB Congeners in Commercial Aroclor and Clophen Mixtures by Multidimensional Gas Chromatography – Electron Capture Detection (MD-GC-ECD). *Environmental Science and Technology*, **23**: 852-859.
**Schumacher, U.,** Zahler, S., Horny, H.P., Heidemann, G., Skirnisson, G. and U. Welsch, (1993). Histological Investigations on the Thyroid Glands of Marine Mammals and the Possible Implications of Marine Pollution. *Journal of Wildlife Diseases*, **29**: 103-108.

**Schumacher, U.,** Heidemann, G., Skírnisson, K., Schumacher, W. and R.M. Pickering (1995). Impact of Activity and Contamination Level on Blood Parameters of Harbour Seals (*Phoca vitulina*). *Comparative Biochemistry and Physiology*, **112A**(3/4): 455-462.

**Schwarzenberger, F.,** Möstl, E., Palme, R. and E. Bamberg (1996). Faecal Steroid Analysis for Non-Invasive Monitoring of Reproductive Status in Farm, Wild and Zoo Animals. *Animal Reproduction Science*, **42**: 515-526.

**Schwarzenberger, F.,** Tomášová, K., Holečková, D., Matern, B. and E. Möstl (1996b). Measurement of Fecal Steroids in the Black Rhinoceros (*Diceros bicornis*) Using Group-Specific Enzyme Immunoassays for 20-Oxo-Pregnanes. *Zoo Biology*, **15**: 159-171.

**Scottish Natural Heritage**, (2002). Surveys of Harbour (Common) Seals in Shetland and Orkney, August 2001. Report No. F01 AA417.

**Selcer, K.W.,** Nespoli, L.M., Rainwater, T.R., Finger, A.G., Ray, D.A., Platt, S.G., Smith, P.N., Densmore, L.D. and S.T. McMurry (2006). Development of an Enzyme-Linked Immunosrbent Assay for Vitellogenin of Morelet's Crocodile (*Crocodylus moreletii*). *Comparative Biochemistry and Physiology, Part C*, **143**: 50-58.

**Sellström, U.,** Jansson, B., Kierkegaard, A., de Wit, C., Odsjö T. and M. Olsson (1993a). Polybrominated Diphenyl Ethers (PBDE) in Biological Samples from the Swedish Environment. *Chemosphere*, **26**: 1703-1718.

**Sellström, U.,** Kierkegaard, A., de Wit, C., Jansson, B., Asplund, L., Bergander, L., Bignert, A., Odsjö T. and M. Olsson (1996), Polybrominated Diphenyl Ethers (PBDE) in the Swedish Environment – a Summary. *Organohalogen Compounds*, **28**: 526-529.

**Sellström, U.,** Kierkegaard, A., de Wit, C. and B. Jansson (1997). Polybrominated Diphenyl Ethers and Hexabromocyclododecane in Sediment and Fish from a Swedish River. *Environmental Toxicology and Chemistry*, **17**(6): 1065-1072.

**Setford, S.J.** (2000). Immunosensing in Organic and Mixed Aqueous – Organic Phase Environments. *Trends in Analytical Chemistry*, **19**(5): 330-339.

**Severinsen, T.,** Skaare, J.U. and C. Lydersen (2000). Spatial Distribution of Persistent Organochlorines in Ringed Seals (*Phoca hispida*) Blubber. *Marine Environmental Research*, **49**: 291-302.

**Shaw, S.D.,** Brenner, D. Bourakovsky, A., Mahaffey, C.A. and C.R. Perkins (2005). Polychlorinated Biphenyls and Chlorinated Pesticides in Harbor Seals (*Phoca vitulina concolor*) from the Northwestern Atlantic Coast. *Marine Pollution Bulletin*, **50**(10): 1069-1084.

**She, J.,** Winkler, J., Visita, P., McKinney, M. and M. Petreas (2000). Analysis of PBDEs in Seal Blubber and Human Breast Adipose Tissue Samples. Dioxin 2000: 20th International Symposium on Halogenated Environmental Organic Pollutants and POPs. Monterey, California, USA. August 13-17, 2000. **47**: 53-56.

**She, J.,** Petreas, M, Winkler, J., Visita, P., McKinney, M. and D. Kopee (2002). PBDEs in the San Francisco Bay Area: Measurements in Harbor Seal Blubber and Human Breast Adipose Tissue. *Chemosphere*, **46**: 697-707.

**Sherry, J.,** ApSimon, J., Collier, L., Wilkinson, R., Albro, P. and B. Zfghan (1989). The Use of Radioimmunoassay for the Detection of Polychlorinated Dibenzo-p-Dioxins in Fish Samples. *Chemosphere*, **19**(1-6): 255-261.

**Shillabeer, N.** and J.F. Tapp (1989). Improvements in the Benthic Fauna of the Tees Estuary After a Period of Reduced Pollution Loadings. *Marine Pollution Bulletin*, **20**(3): 119-123.

**Simmonds, M.P.,** Johnston, P.A., and M.C. French (1993). Organochlorine and Mercury Contamination in United Kingdom Seals. *The Veterinary Record*, **132**(12): 291-295.

**Simms, W.,** Jeffries, S., Ikonomou, M. and P.S. Ross (2000). Contaminant-Related Disruption of Vitamin A Dynamics in Free-Ranging Harbour Seal (*Phoca vitulina*) Pups from British Columbia, Canada and Washington State, USA. *Environmental. Toxicology and Chemistry*, **19**: 2844-2849.

**Simon, J.A.,** Lin, F., Vittinghoff, E. and V. Bittner (2005). The Relation of Postmenopausal Hormone Therapy to Serum Uric Acid and the Risk of Coronary Heart Disease Events: The Heart and Estrogen-Progestin Replacement Study (HERS). *Annals of Epidemiology*, **16**(2): 138-145.

**Sirtori, C.R.** (1995). New Targets for Lipid Lowering Atherosclerosis Prevention. *Pharmacology and Therapeutics*, **67**(3): 433-447.

**Slim, R.,** Toborek, M., Robertson, L.W. and B. Hennig (1999). Antioxidant Protection Against PCB Mediated Endothelial Activation. *Toxicological Sciences*, **52**(2): 232-239.

**Smeds, A.** and P. Saukko (2001). Identification and Quantification of Polychlorinated Biphenyls and Some Endocrine Disrupting Pesticides in Human Adipose Tissue from Finland. *Chemosphere*, **44**: 1463-1471.

**Smith, T.G.** (1987). The Ringed Seal, *Phoca hispida*, of the Canadian Western Arctic. *Canadian Bulletin of Fisheries and Aquatic Science*, **216**: 1-81.

**Sorensen, M.** and S.P.L. Sorensen (1939). The Protein in Whey. *C. R. Trav. Lab. Carlsberg Seb. Cgun.* **23**: 55-99.

**Sørmo, E.G.,** Skaare, J.U., Lydersen, C., Kovacs, K.M., Hammill, M.O. and B.M. Jenssen (2003). Partitioning of Persistent Organic Pollutants in Grey Seal (*Halichoerus grypus*) Mother-Pup Pairs. *The Science of the Total Environment*, **302**: 145-155.

**Stanton, B.J.,** El-Sabeawy, F., Yang, X.F., Enan, E. and B.L. Lasley (2001). Interaction of Estrogen and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in Immature Male Chickens *Gallus domesticus*. *Comparative Biochemistry and Physiology, Part C*, **129**: 35-47.

**Stokes, K.,** Aston-Mills, B. and C. Teng (2004). Estrogen Response Element and the Promoter Context of the Human and Mouse Lactoferrin Genes Influence Estrogen Receptor α-Meditated Transactivation Activity in Mammary Gland Cells. *Journal of Molecular Endocrinology*, **33**: 315-334.

**Storr-Hansen, E.** and H. Spliid (1993a) Coplanar Biphenyl Congener Levels and Patterns and the Identification of Separate Populations of Harbor Seals (*Phoca vitulina*) in Denmark. *Archives of Environmental Contamination and Toxicology*, **24**: 44-58.

**Subramanian, A.,** Tanabe, S., Tatsukawa, R., Saito, S. and N. Miyazaki (1987). Reduction in the Testosterone Levels by PCBs and DDE in Dall's Porpoises of Northwestern North Pacific. *Marine Pollution Bulletin*, **18**(12): 643-646.

**Subramanian, A.,** Tanabe, A. and R. Tatsukawa (1988). Use of Organochlorines as Chemical Tracers in Determining Some Reproductive Parameters in Dalli-Type Dall's Porpoises *Phocoenoides dalli*. *Marine Environmental Research*, **25**: 161-174.

**Sweeney, J.C.** (1974). Common Diseases of Pinnipeds. *Journal of the American Veterinary Medical Association*, **165**: 805-810.

**Sweeney, J.C.** and S.H. Ridgway (1975). Common Diseases of Small Cetaceans. *Journal of the American Medical Association*, **167**: 533-540.

**Tanabe, S.** (1988). PCB Problems in the Future: Foresight from Current Knowledge. *Environmental Pollution*, **50**: 5-28.

**Tanabe, S.** (1999). Butyltin Contamination in Marine Mammals – A Review. *Marine Pollution Bulletin*, **39**(1-12): 62-72.

**Tapp, J.F.,** Shillabeer, N. and C.M. Ashman (1993). Continued Observations of the Benthic Fauna of the Industrialised Tees Estuary, 1979-1990. *Journal of Marine Biology and Ecology*, **172**: 67-80.

**Tees Valley Wildlife Trust**:. http://www.wildlifetrust.org.uk/teesvalley/teesvalley/home.htm

**Teng, C.T.,** Pentecost, B.T., Chen, Y.H., Newbold, R.R., Eddy, E.M. and J.S. McLachlan (1989). Lactotransferrin Gene Expression in the Mouse Uterus and Mammary Gland. *Endocrinology*, **124**(2): 992-999.

**Teng, C.T.,** Liu, Y., Yang, N., Walmer, D. and T. Panella (1992). Differential Molecular Mechanism of the Estrogen Action that Regulates Lactoferrin Gene in Human and Mouse. *Mouse Endocrinology*, **6**(11): 1969-1981.

**Teng, C.T.** (1995). Mouse Lactoferrin Gene: A Marker for Estrogen and Epidermal Growth Factor. *Environmental Health Perspectives*, **103**(7): 17-20.

**Teng, C.T.,** Beard, C. and W. Gladwell (2002). Differential Expression and Estrogen Response of Lactoferrin Gene in the Female Reproductive Tract of Mouse, Rat and Hamster. *Biology of Reproduction*, **67**: 1439-1449.

**Teng, C.T.,** Gladwell, W., Raphiou, I. and E. Liu (2004). Methylation and Expression of the Lactoferrin Gene in Human Tissues and Cancer Cells. *BioMetals*, **17**: 317-323.

**Thomas, G.O.,** Moss, S.E.W., Asplund, L., and A.J. Hall (2005). Absorption of Decabromophenyl Ether and Other Organohalogen Chemicals by Grey Seals (*Halichoerus grypus*). *Environmental Pollution* **333**: 581-586.

**Thomas, N.J.,** Carcillo, J.A., Doughty, L.A., Sasser, H. and R.P. Heine (2002). Plasma Concentrations of Defensins and Lactoferrin in Children with Severe Sepsis. *The Pediatric Infectious Disease Journal*, **21**(1): 34-38.

**Thompson, P.M.** and D. Miller (1992). Phocine Distemper Virus Outbreak in the Moray Firth Common Seal Population: An Estimate of Mortality. *The Science of the Total Environment*, **115**: 57-65.

**Thompson, P.M.,** Mcconnell, B.J., Tollit, D.J., Mackay, A., Hunter, C. and P.A. Racey (1996). Comparative Distribution, Movements and Diet of Harbour and Grey Seals from Moray Firth, N.E. Scotland. *The Journal of Applied Ecology*, **36**(6): 1572-1584.

**Thompson, P.M.,** Tollit, D.J., Wood, D., Corpe, H.M., Hammond, P.S. and A. Mackay (1997). Estimating Harbour Seal Abundance and Status in an Estuarine Habitat in North-East Scotland. *The Journal of Applied Ecology*, **34**(1): 43-52.

**Thompson, P.M.** and T.R. Barton (2004). Recent Trends in the Abundance of Harbour Seals in the Moray Firth. *In*: UK Special Committee on Seals, NERC Sea Mammal Research Unit, SMRU, pp. 97-99.

**Tindle, R.C.** and D.L. Stalling (1972). Apparatus for Automated Gel Permeation Cleanup for Pesticide Residue Analysis. Applications to Fish Lipids. *Analytical Chemistry*, **44**(11): 1768-1773.

**Tomlin, C.** (1993). *The Pesticide Manual*. The British Crop Protection Council and the Royal Society of Chemistry.

**Towbin, H.,** Staehelin, T. and J. Gordon (1979). Electrophoretic Transfer of Proteins from Polyacrylamide Gels to Nitrocellulose Sheets: Procedure and Some Applications. *Proceedings of the National Academy of Sciences of the United States of America*, **76**(9): 4350-4354.

**Troisi, G.M.** and C.F. Mason (1997). Cytochromes P450, P420 & Mixed – Function Oxidases as Biomarkers of Polychlorinated Biphenyl (PCB) Exposure in Harbour Seals (*Phoca vitulina*). *Chemosphere* **35**(9): 1933-1946.

**Troisi, G.M.** and C.F. Mason (2000). PCB-associated Alteration of Hepatic Steroid Metabolism in Harbor Seals (*Phoca vitulina*). *Journal of Toxicology and Environmental Health Part A*, **61**: 649-655.

**Troisi, G.M.,** Haraguchi, K., Kaydoo, D.S., Nyman, M., Siebert, U. and C.F. Mason (2001). Bioaccumulation of Polychlorinated Biphenyls (PCBs) and Dichlorodiphenylethane (DDE) Methyl Sulfones in Tissue of Seal and Dolphin Morbillivirus Epizootic Victims. *Journal of Toxicology and Environmental Health, Part A*, **62**: 1-8.

**Troisi, G.M.** and L. Borjesson (2005). Development of an Immunoassay for the Determination of Polyaromatic Hydrocarbons in Plasma Samples from Oiled Sea birds. *Environmental Science and Technology*, **39**: 3748-3755.

**Turner, J.W.,** Liu, I.K.M., Flanagan, D.R., Rutberg, A.T. and J.F. Kirkpatrick (2001). Immunocontraception in Feral Horses: One Inoculation Provides One Year of Infertility. *Journal of Wildlife Management*, **65**(2): 235-241.

**Tyler, C.R.,** Jobling, S. and J.P. Sumpter (1998). Endocrine Disruption in Wildlife: A Critical Review of the Evidence. *Critical Reviews in Toxicology*, **28**: 319–361.

**UNEP**, 2000. Inventory of Information Sources on Chemicals: Persistent Organic Pollutants. United Nations Environmental Programme.

**UNEP,** 2001. The Stockholm Convention on Persistent Organic Pollutants. United Nations Environmental Programme.

**Valenti, P.,** Berlutti, F., Conte, M.P., Longhi, C. and L. Seganti (2004). Lactoferrin Functions: Current Status and Perspectives. *Journal of Clinical Gastroenterology*, **38**: S127-S129.

**van Aerle, R.,** Nolan, M., Jobling, S., Christiansen, L.B., Sumpter, J.P. and C.R. Tyler (2001). Sexual Disruption in a Second Species of Wild Cyprinid Fish (The Gudgeon, *Gobio gobio*) in United Kingdom Freshwaters. *Environmental Toxicology*, **20**: 2841-2847.

**Van der Berg, M.,** Birnbaum, L.S., Denison, M., De Vito, M., Farland, W., Feeley, M., Fiedler, H., Hakansson, H., Hanberg, A., Haws, L., Rose, M., Safe, S., Schrenk, D., Tohyama, C., Tritscher, A., Tuomisto, J., Tysklind, M., Walker, N. and R.E. Peterson (2006). Review: The 2005 World Health Organization Reevaluation of Human and Mammalian Toxic Equivalency Factors for Dioxins and Dioxin-Like Compounds. *Toxicological Sciences*, **93**(2): 223-241.

**Van der Hoff, G.R.,** Van Beuzekom, A.C., Brinkman, U.A.Th., Baumann, R.A. and P. Van Zoonen (1996). Determination of Organochlorine Compounds in Fatty Matrices. Application of Rapid-Off-Line Normal-Phase Liquid Chromatographic Clean-up. *Journal of Chromatography A*, **754**: 487-496.

**Van der Hoff, G.R.** and P. van Zoonen (1999). Trace Analysis of Pesticides by Gas Chromatography. *Journal of Chromatography A.*, **843**: 301-322.

**van der Strate, B.W.A.,** Beljaars, L., Molema, G., Harmsen, M.C. and D.K.F. Meijer (2001). Antiviral Activities of Lactoferrin, Review. *Antiviral Research*, **52**: 225-239.

**van der Strate, B.W.A.,** Harmsen, M.C., The, T.H., Sprenger, H.G., De Vries, H., Eikelboom, M.C., Kuipers, M.E., Meijer, D.K.F. and P.J. Swart (2001). Plasma Lactoferrin Levels are Decreased in End-Stage AIDS-Patients. *Viral Immunology*, **12**(3): 197-203

**Vandenberghe, J.** (1996). Hepatotoxicology: Structure, Function, and Toxicological Pathology. In: *Toxicology, Principles and Application.* R.J.M. Niesink, J de Vries and M.A. Hollinger, (Eds.), CRC Press, Boca Raton, pp. 702-723.

**Vega-López, S.,** Conde-knape, K., Vidal-Quintanar, R-L., Schachter, N.S. and M.L. Fernandez (2002). Sex and Hormonal Status Influence the Effects of Psyllium on Lipoprotein Remodelling and Composition. *Metabolism*, **51**(4): 500-507.

**Walmer, D.K.,** Wrona, M.A., Hughes, C.L. and K.G. Nelson (1992). Lactoferrin Expression in the Mouse Reproductive Tract During the Natural Estrous Cycle: Correlation With Circulating Estradiol and Progesterone. *Endocrinology*, **131**: 1458-1466.

**Walmer, D.K.,** Padin, C.J., Wrona, M.A., Healy, B.E., Bentley, R.C., Tsao, M.S., Kohler, MF., McLachlan, J.A. and K.D. Gray (1995). Malignant Transformation of the Human Endometrium is Associated with Overexpression of Lactoferrin Messenger RNA and Protein. *Cancer Research*, **55**(5): 1168-1175.

**Ward, P.P.,** Mendoza-Meneses, M., Mulac-Jericevic, B., Cunningham, G.A., Saucedo-Cardenas, O., Teng, C.T. and O.M. Conneely (1999). Restricted Spatiotemporal Expression of Lactoferrin during Murine Embryonic Development. *Endocrinology*, **140** (4): 1852-1860.

**Wasser, S.K.** Monfort, S.L. and D.E. Wildt (1991). Rapid Extraction of Faecal Steroids for Measuring Reproductive Cyclicity and Early Pregnancy in Free-Ranging Yellow Baboons (*Papio cynocephalus cynocephalus*). *Journal of Reproduction and Fertility*, **92**: 415-423.

**Watanabe, S.,** Shimada, T., Nakamura, S., Nishiyama, N., Yamashita, N., Tanabe, S. and R. Tatsukawa (1989). Specific Profile of Liver Microsomal Cytochrome P-450 in Dolphin and Whales. *Marine Environmental Research*, **27**(1): 51-65.

**Weatherley, N.S.,** Davies, G.L., and S. Ellery (1997). Polychlorinated Biphenyls and Organochlorine Pesticides in Eels (*Anguilla anguilla* L.) from Welsh Rivers. *Environmental Pollution*, **95**(1): 127-134.

**Weltman, A.,** Despres, J.P., Clasey, J.L., Weltman, J.Y., Wideman, L., Kanalev, J., Patrie, J., Bergeron, J., Thorner, M.O., Bouchard, C. and M.L. Hartman (2003). Impact of Abdominal Visceral Fat, Growth Hormone, Fitness, and Insulin on Lipids and Lipoproteins in Older Adults. *Metabolism*, **52**(1): 73-80.

**White, R.D.,** Hahn, M.E., Lockhart, W.L. and J.J. Stegeman (1994). Catalytic and Immunochemical Characterisation of Hepatic Microsomal Cytochromes P 450 in Beluga Whale (*Delphinapterus leucas*). *Toxicology and Applied Pharmacology*, **126**: 45-57.

**WHO / ICPS**, 1994. Environmental Health Criteria, 162: Brominated Diphenyl Ethers, World Health Organisation, Geneva.

**Wolfe, B.M.** and M.W.Huff (1995).Effects of Continuous Low-Dosage Hormonal Replacement Therapy on Lipoprotein Metabolism in Postmenopausal Female. *Metabolism*, **44**(3): 410-417.

**Wolfe, B.M.,** Barett, P.H.R., Laurier, L and M.W.Huff (2000).Effects of Continuous Conjugated Estrogen and Micronised Progesterone Therapy Upon Lipoprotein Metabolism in Postmenopausal Female. *Journal of Lipid Research*, **41**: 368-375.

**Wolkers, H.,** Lydersen, C. and K.M. Kovacs (2004). Accumulation and Lactational Transfer of PCBs and Pesticides in Harbor Seals (*Phoca vitulina*) from Svalbard, Norway. *The Science of the Total Environment*, **319**: 137-146.

**Wolkers, H.,** van Bavel, B., Derocher, A.E., Wiig, Ø., Kovacs, K.M., Lydersen, L. and G. Linström (2004). Congener-Specific Accumulation and Food Chain Transfer of Polybrominated Diphenyl Ethers in Two Arctic Food Chains. *Environmental Science and Technology*, **38**: 1667-1674.

**Yamamoto, K.,** Fukuda, N., Shiroi, S., Shiotsuki, Y., Negata, Y., Tani, T. and T. Sakai (1994). Ameliorative Effect of Dietary Probucol on Polychlorinated Biphenyls-Induced Hypercholesterolemia and Lipid Peroxidation in the Rat. *Life Sciences*, **54**(14): 1019-1026.

**Yang, T.S.,** Wu, S.C., and S.R. Wang (2000). Serum and Milk Lactoferrin Concentrations and the Correlation with Some Blood Components in Lactating Cows. *Research in Veterinary Science*, **69**: 95-97.

**Yu, L.C.** and Y.H. Chen (1993). The Developmental Profile of Lactoferrin in Mouse Epididymis. *Journal of Biochemistry*, **296**(Pt.1): 107-111.

**Zajicek, J.L.,** Tillitt, D.E., Schwartz, T.R., Schmitt, C.J. and R.O. Harrison (2000). Comparison of an Enzyme-Linked Immunosorbent Assay (ELISA) to Gas Chromatography (GC) – Measurement of Polychlorinated Biphenyls (PCBs) in Selected US Fish Extracts. *Chemosphere*, **40**: 539-548.

**Zhang, Z.,** Zhuang, Y., Qiang, H., Liu, X., Xu, R. and Y. Wu (2001). Relationship Between Endogenous Estrogen Concentrations and Serum Cholesteryl Ester Transfer Protein Concentrations in Chinese Females. *Clinica Chimica Acta*, **314**: 77-83.

**Zhang, Z.** and C. Teng (2002). Methoxychlor Stimulates the Mouse Lactoferrin Gene Promoter Through a GC-Rich Element. *Biochemistry and Cell Biology*, **80**: 23-26.

**Zhao, J-H.,** Sun, S-J., Horiguchi, H., Arao, Y., Kanamori, N., Kikutchi, A., Oguma, E. and F. Kayama (2005). A Soy Diet Accelerates Renal Damage in Autoimmune MRL/Mp-lpr/lpr Mice. *International Immunopharmacology*, **5**: 1601-1610.

**Zhou, T.,** Ross, D.G., de Vito, M.J. and K.M. Crofton (2001). Effects of Short-Term in *vivo* Exposure to Polybrominated Diphenyl Ethers on Thyroid Hormones and Hepatic Enzyme Activities in Weanling Rats. *Toxicological Sciences*, **61**: 76-82.

**Zhou, T.,** Taylor, M.M., de Vito, M.J. and K.M. Crofton (2002). Developmental Exposure to Brominated Diphenyl Ethers Results in Thyroid Hormone Disruption. *Toxicological Sciences*, **66**: 105-116.

**Ziegler, T.E.,** Sholl, S.A., Scheffler, G., Haggerty, M.A. and B.L. Lasley (1989). Excretion of Estrone, Estradiol, and Progesterone in the Urine and Feces of the Female Cotton-Top Tamarin (*Saguinus oedipus oedipus*). *American Journal of Primatology*, **17**: 185-197.

**Ziegler, T.E.,** Wittwer, D.J. and C.T. Snowdon (1993). Circulating and Excreted Hormones During the Ovarian Cycle in the Cotton-Top Tamarin, *Saguinus oedipus*. *American Journal of Primatology*, **31**: 55-65.

**Zile, M.H.** (1992). Vitamin A Homeostasis Endangered by Environmental Pollutants: A Minireview. *Society for Experimental Biology and Medicine*, **201**: 141-152.

**Internet Sources**

http://www.chem.unep.ch/pops/pdf/invsrce/inventpopscomb.pdf)
http://www.chem.unep.ch/sc/default.htm)
www.greenchannel.com
www.holidaymull.org

# 8.0 APPENDIX

## 8.1 APPENDIX I – Contaminant Analysis.

### 8.1.1 Soxhlet Extraction

**Table 8.1:** Soxhlet extraction program

| **Program Using 50% Hexane/diethyl ether (v/v)** | **Parameters** |
|---|---|
| Temperature limit | 200°C |
| Extraction temperature | 180°C |
| Time until boiling begins | 10min |
| Boiling time | 45min |
| Solvent reduction A | 3x15ml |
| Extraction time | 80min |
| Solvent reduction B | 8min |
| Solvent reduction C | 5min |
| Solvent reduction interval | 3min |
| Solvent reduction phase | 3sec |
| **Total time** | **2 hour 23min** |

The Soxhlet system functioned to heat (hotplates) and cool (condensers) the solvent mixture. This facilitated evaporation and condensation of the solvent mixture (refluxing) through the system. The Soxtherm had two stages of operation. In the first stage the sample was boiled in the solvent. Briefly, the heated solvent penetrated the permeable thimble and dissolved the sample lipids. Further heating caused the solvent to enter vapour phase and rise to the base of the cooled condensers, causing the solvent mixture to become liquid again and drip back down into the thimble. Repeated cycles (circulation) ensured maximum lipid recovery. At the second stage the solvent mixture volume was reduced/concentrated to approximately 10mm below the thimble allowing the condensed solvent to rinse through the sample and therefore wash out the materials to be extracted. This was facilitated by recovering the solvent mixture into the unit, which was discarded via a discharge pipe from the unit.

### 8.1.2 Soxhlet Extraction and HPLC - Recovery

In order to assess the efficiency of the soxhlet extraction using the Soxtherm, blank samples of sodium sulphate spiked with 1 ml standard mixture (0.01ppm) of the compounds under study were extracted and analysed by GC. Mixture of all the analytes under study were also injected into the HPLC. Examples of chromatograms are shown in Figures 8.1, 8.2 and 8.3.

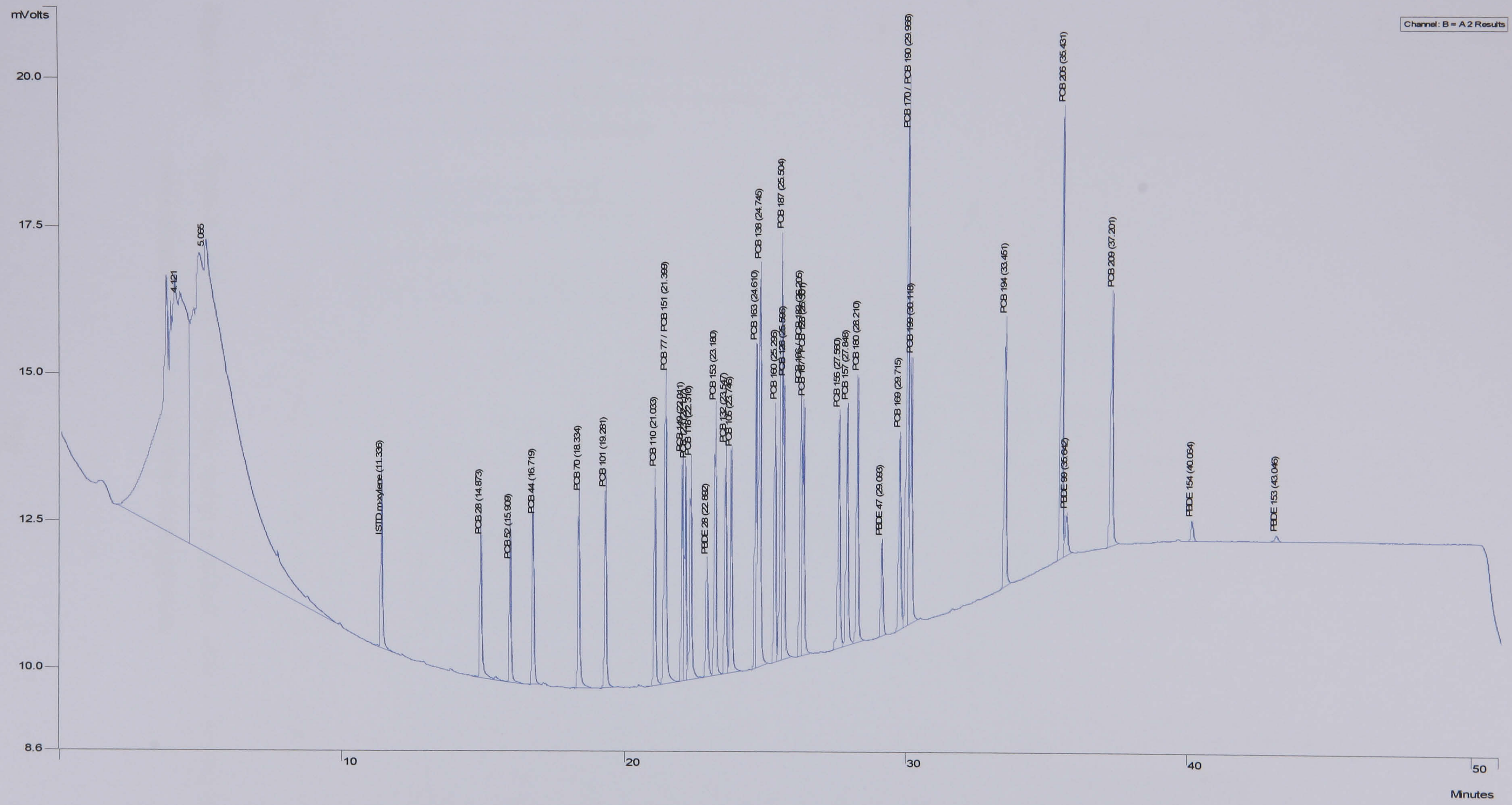



**Figure 8.1** Soxhlet extraction of blank sample spiked with a known mixture of contaminants (1 ml standard mixture of 0.01ppm)

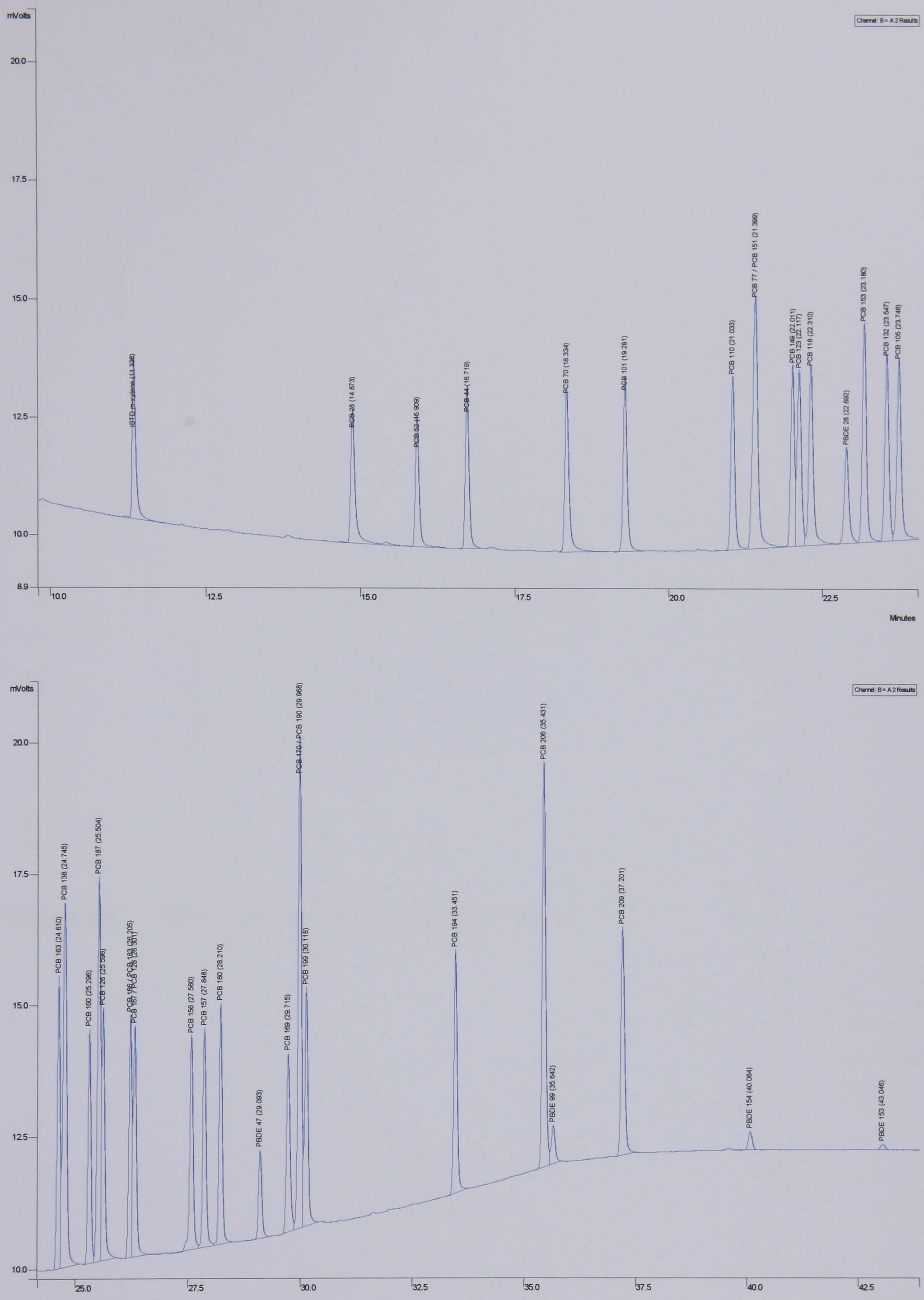



**Figure 8.2** Soxhlet extraction of blank sample spiked with a known mixture of contaminants showing co-elution of compounds

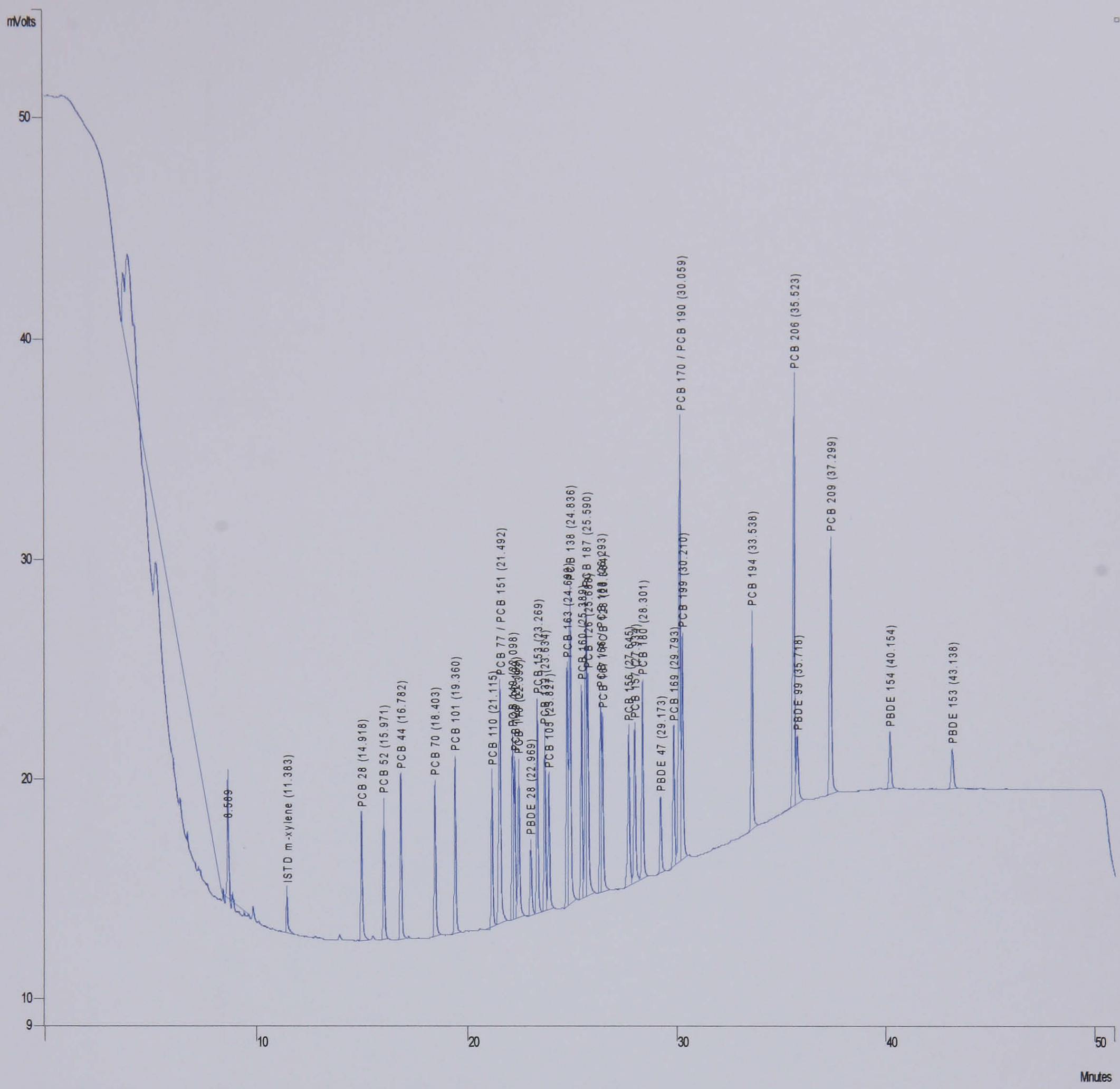



**Figure 8.3** HPLC standard mixture injected (fraction 1)

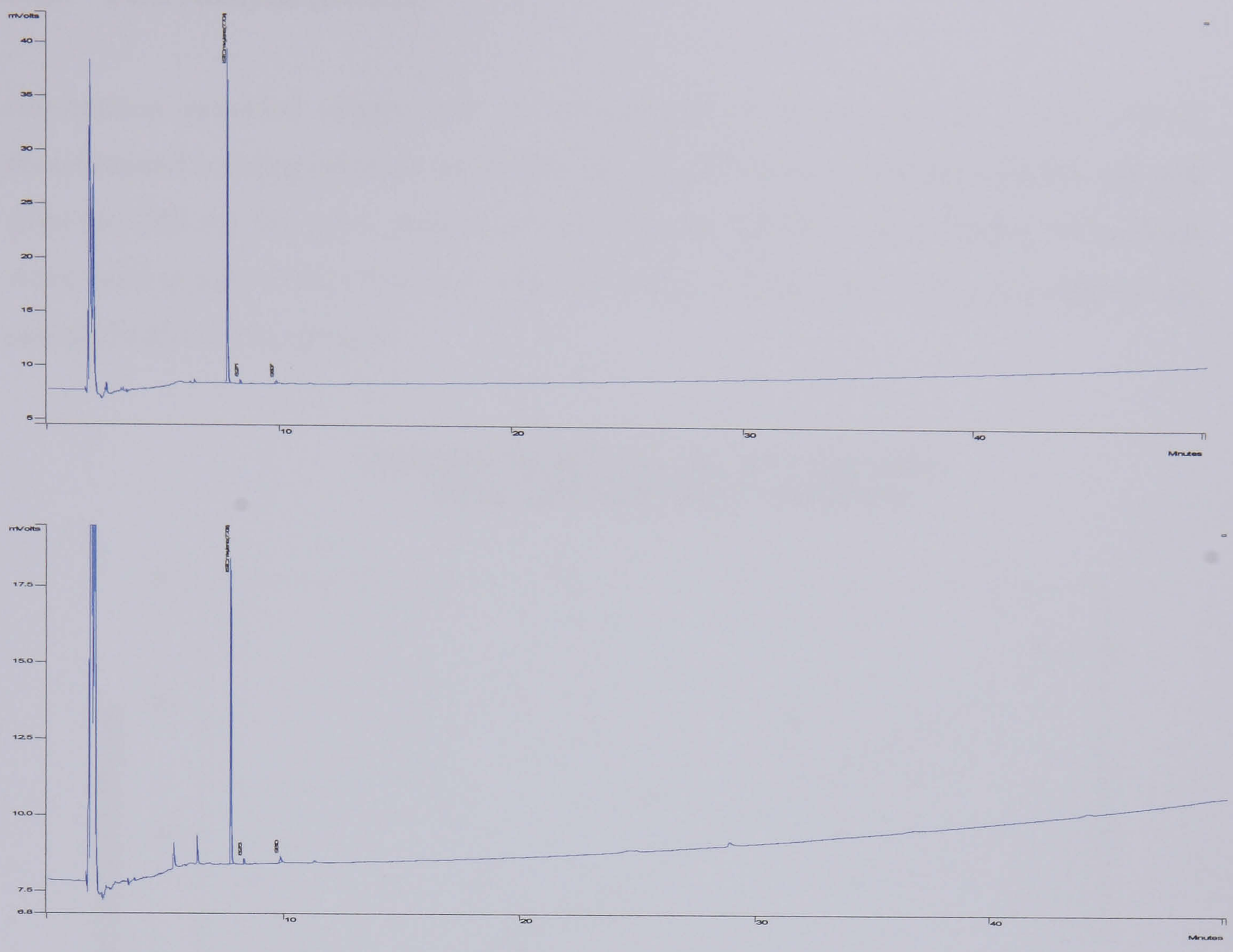



**Figure 8.4** Example of blank chromatograms (hexane)

### 8.1.3 PCB Analysis (ELISA)

Information provided (figure 8.5) by manufacturer (Abraxis Kits Inc, LLC, U.S.A.) demonstrated a strong positive correlation ($R^2 = 0.91$) between data from ELISA and data from GC-MS for the same samples, confirming the accuracy and reliability of antibody when used as part of the Abraxis soil test kit which is based on the same principals as the plasma ELISA in this project. .

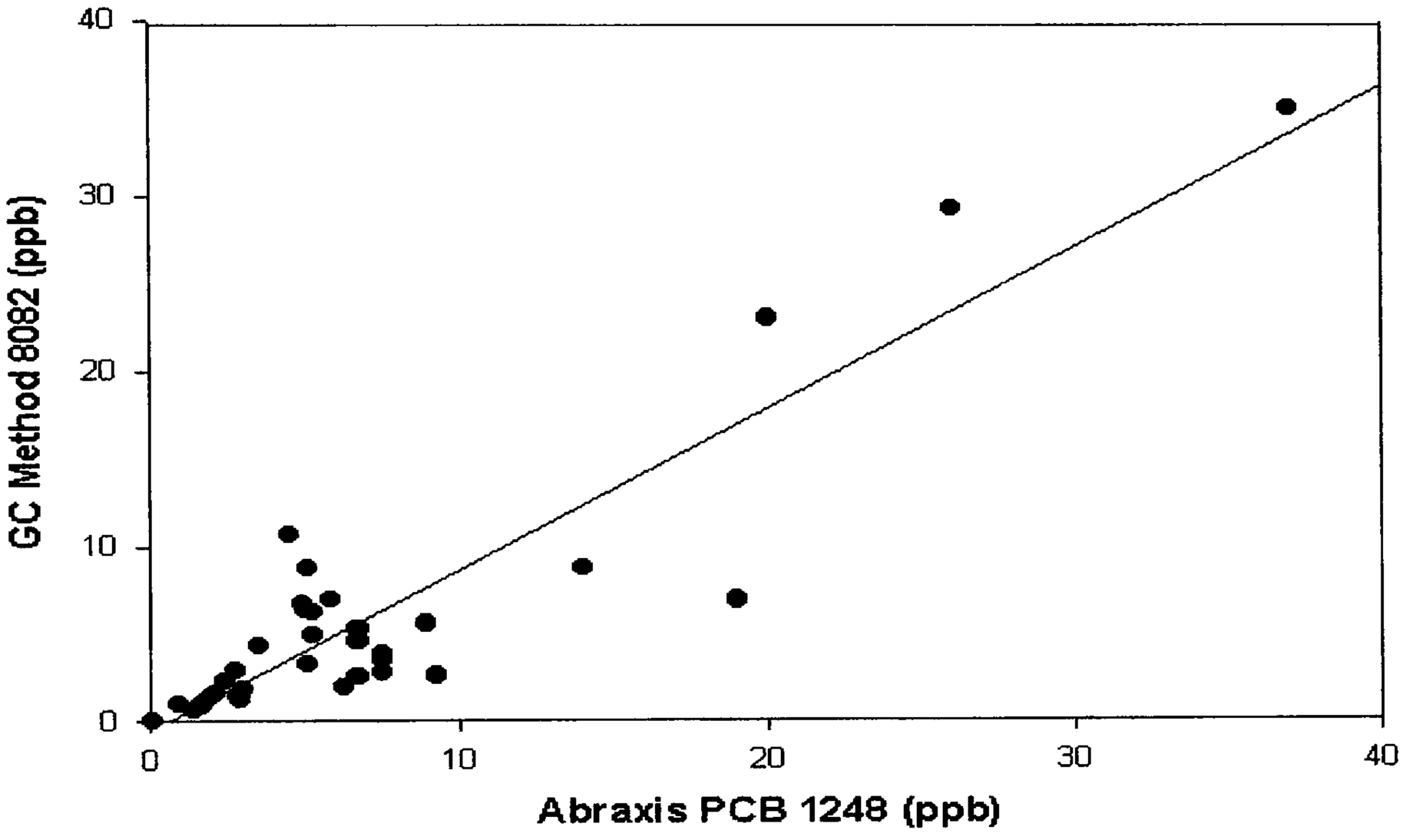



**Figure 8.5:** Correlation of ELISA and GC data for soil and sediment samples using the Abraxis Kits Soil Test kit (*Source:* Abraxis)

## 8.2 APPENDIX II – Sex Steroid Analysis

### 8.2.1 Cross reactivity of ELISA kits

Cross reactivity information for each sex steroid was provided by the manufacturer. The percentage indicates cross reactivity at 50% displacement compared to the sex steroid analysed.

**Table 8.2:** Cross Reactivity

**a.) Progesterone**

| Steroid | % Cross Reaction |
|---|---|
| Progesterone | 100 |
| 17-α-OH Progesterone | 0.3 |
| Estriol | <0.1 |
| Estradiol 17β | <0.1 |
| Testosterone | <0.1 |
| 11-Desoxycorticosterone | 1.1 |
| DHEA-S | <0.02 |
| Cortisol | <0.02 |
| Corticosterone | 0.2 |
| Pregnenolone | 0.35 |
| Cortisone | <0.1 |
| 11-Desoxycortisol | 0.1 |

**b.) 17-α-OH Progesterone**

| Steroid | % Cross Reaction |
|---|---|
| 17-α-OH Progesterone | 100 |
| Estriol | <0.01 |
| Estradiol 17β | <0.01 |
| Testosterone | <0.01 |
| Dihydrotestosterone | <0.01 |
| DOC | 0.05 |
| 11-Desoxicortisol | 1.4 |
| Progesterone | 1.2 |
| DHEA | <0.01 |
| DHEA-S | <0.001 |
| Cortisol | <0.01 |
| Corticosterone | <0.05 |
| Aldosterone | <0.01 |
| Androstenedione | <0.01 |

## c.) Estrone

| Steroid | % Cross Reaction |
|---|---|
| Estrone | 100 |
| 2-methoxy-estrone | 0.2 |
| Estradiol 17β | 0.3 |
| 16-keto-estradiol | 0.2 |
| Estradiol-17-glucuronide | <0.1 |
| Estradiol-3-glucuronide | <0.1 |
| Estradiol-3-sulfate-17-glucuronide | <0.1 |
| Estriol | <0.1 |
| 16-epi-estriol | <0.1 |
| Estriol-16-glucuronide | <0.1 |
| Ethynyl estradiol | <0.1 |

## d.) Estradiol 17β

| Steroid | % Cross Reaction |
|---|---|
| Estradiol 17β | 100 |
| Estrone | 0.2 |
| Estriol | 0..05 |
| Androstenedione | 0 |
| Androsterone | 0 |
| Corticosterone | 0 |
| Epiandrosterone | 0 |
| 16-Epiestriol | 0 |
| Estradiol-3-sulfate | 0 |
| Estradiol-3-glucuronide | 0 |
| Estradiol-17α | 0 |
| Estriol-16-glucuronide | 0 |
| Estrone-3-sulfate | 0 |
| DHEA | 0 |
| 11-Desoxycortisol | 0 |
| 11-Desoxycorticosterone | 0 |
| 21-Desoxycortisol | 0 |
| Dehydrotestosterone | 0 |
| Progesterone | 0 |
| Pregnenolone | 0 |

## e.) Free Estriol

| Steroid | % Cross Reaction |
|---|---|
| Free Estriol | 100 |
| 16-Epiestriol | 2.7 |
| 16,17-Epiestriol | 0.08 |
| Estradiol 17β | 0.038 |
| Estradiol 17α | 0.037 |
| 17-Epiestriol | 0.0128 |
| Corticosterone | 0.0003 |
| 5α-Dehydrotestosterone | 0.00004 |
| Testosterone | n.d. |
| 11-Desoxycortisol | n.d. |
| Estrone | n.d. |

n.d. = non detectable

## f.) Testosterone

| Steroid | % Cross Reaction |
|---|---|
| Testosterone | 100 |
| Dehydrotestosterone | 0.86 |
| Androstenedione | 0.89 |
| Androsterone | 1.0 |
| Estradiol 17β | 0.05 |
| Progesterone | <0.05 |
| Estrone | <0.05 |
| Epitestosterone | <0.05 |
| 17α-OH- Progesterone | <0.05 |
| Estriol | <0.05 |
| Cortisol | <0.05 |
| DHEA-S | <0.05 |

## g.) Androstenedione

| Steroid | % Cross Reaction |
|---|---|
| Androstenedione | 100 |
| Androsterone | <0.01 |
| Cortisol | <0.01 |
| Dihydrotestosterone | 0.01 |
| Dihydroepiandrosterone | <0.01 |
| Estriol | <0.01 |
| 16-Epiestriol | <0.01 |
| Estradiol | <0.01 |
| Estriol-3-glucuronide | <0.01 |
| Estriol-16-glucuronide | <0.01 |
| Estriol-16-sulfate | <0.01 |
| Estrone | <0.01 |
| 17α Pregnenolone | <0.01 |
| 17α-OH-Progesterone | <0.01 |
| Progesterone | <0.01 |
| Testosterone | 0.01 |

## h.) DHEA-S

| Steroid | % Cross Reaction |
|---|---|
| DHEA-S | 100 |
| Androstenedione | 20.9 |
| Androsterone | 8.5 |
| Androsterone-sulfate | <0.1 |
| Progesterone | 4.7 |
| Testosterone | 0.3 |
| Estrone | 0.9 |
| Estriol | <0.1 |
| Estradiol | <0.1 |
| Estradiol-sulfate | <0.1 |
| Cortisol | <0.1 |

## 8.3 APPENDIX III – Lactoferrin

### 8.3.1 Homogeniser Buffer

50mM Tris-HCL pH7.5
1mM EDTA
10% Glycerol
0.1mM PMSF

### 8.3.2 SDS-PAGE Gel Electrophoresis

### 1. List of materials and reagents

- Gel electrophoresis Kit
- Pasteur pipettes
- Conical / vacuum flasks
- 500ml and 1L reagent bottles
- eppendorf tubes
- falcon tubes
- auto-pipettes 10ul-5ml capacity
- plastic trays
- tips

- Tris
- Glycine
- SDS (Sodium Dodecyl Sulphate)
- Concentrated HCl (2M)
- TEMED
- Glycerol
- Bromophenol Blue
- 2-mercaptoethanol
- Methanol
- NaCl
- 30%acrylamide-0.8%bis solution (ready made)
- Ammonium persulphate
- Bovine LTF
- Human LTF
- Molecular Markers

## 2. Preparation of buffers

**Electrophoresis**

- **Electrode buffer (Tris-glycine SDS, pH 8.3)**

Make it 5X in 1L
:

-15g Tris (3)
-72g Glycine (14.4)
-5g SDS (1)

Make it up to 1L in distilled water and adjust pH to 8.3 with HCL 2M.
Store @ 4oC.
Every time use 200ml of buffer with 800ml of distilled water

- **Separating gel buffer (3.0M Tris-HCL pH 8.9)**

- 36.3g Tris
- 80ml distilled water – stir until dissolved

Adjust pH to 8.9 with 2M HCL
Remove stirring bar and make up to 100ml with distilled water
Add 15µl TEMED **right** before using it

- **Stacking gel buffer (0.5M Tris-HCL pH 6.7)**
  - 6.05g Tris
  - 80ml distilled water

Adjust pH to 6.7 with 2M HCL
Remove stirring bar and make up to 100ml with distilled water
Add 10µl TEMED **right** before using it

- **30% Acrylamide/Bis**
  Purchased from SIGMA

- **10% SDS**
  - 10g Sodium Dodecyl Sulphate
  - 100ml distilled water

(or 1g SDS in 10ml distilled water)

- **10% APS**
  - 1g Ammonium Persulphate
  - 10ml distilled water

- **Sample Loading Buffer**
  - 1.51g Tris
  - 20ml Glycerol
  - dissolve in 35ml distilled water

Adjust pH to 6.75 with 2M HCl
- add 4.0g SDS
- 0.002g Bromophenol Blue

Dilute to a final volume of 90ml
This solution is stable and can be stored, but requires the addition of 2-mercaptoethanol which is unstable.

***Therefore: Add*** **1ml of 2-mercaptoethanol to 9ml of buffer in a fume cupboard prior to sample preparation**

- **Stain**
    - 0.2% Coomasie Blue R
    - 40% methanol
    - 10% acetic acid
    - 50% distilled water

- **De-stain**
    - 10% methanol
    - 10% acetic acid
    - 80% distilled water

**Assemble gel plates**

- clean glass plates
- assemble gel plates and place on a levelling table
- **Mark the plates 5.5cm from bottom before pouring the gels**

**3. Preparation of separation gel (10%)**

- Pipette 6.7ml Acrylamide – Bis into a small vacuum flask and add
- 2.5ml separation buffer
- 10.5ml distilled water

Degas for about 30minutes

After degassing add:

- 200µl of 10% SDS
- 100µl of 10% APS
- 15µl of TEMED

Mix gently by swirling

*Polymerisation begins immediately so work quickly*

Pipette the solution between the glass plates until the mark.

Overlay with iso-propanol (about 100-200µl) to exclude air and make a straight line.

Leave for 20-30 minutes to polymerise

**4. Preparation of 4.5% stacking gel**

- Pipette 1.5ml of Acrylamide-Bis into a small vacuum flask and add
- 2.5ml of stacking gel buffer
- 5.8ml distilled water

Degas for about 30 minutes

After degassing add:

- 100µl of 10% SDS
- 100µl of 10% APS
- 10µl of TEMED

Mix gently by swirling

Remove the iso-propanol and clean with stacking gel buffer
Then pipette the stacking gel on top of the separating gel
Insert a well comb between the plates
Pour the remaining stacking gel
Leave to polymerise for 20-30 minutes

### 8.3.3 Molecular Weight Marker

**Table 1:** Molecular Weight Marker

| Proteins | MW (Da) | Colour of Conjugate |
|---|---|---|
| Myosin, Rabbit Muscle | 205,000 | Blue |
| β-Galactosidase, *E.coli* | 116,000 | Turquoise |
| Albumin, Bovine Serum | 66,000 | Pink |
| Ovalbumin, Chicken Egg | 45,000 | Yellow |
| Carbonic Anhydrase, Bovine Erythrocytes | 29,000 | Orange |
| Trypsin Inhibitor, Soybean | 20,000 | Green |
| α-Lactalbumin, Bovine Milk | 14,200 | Purple |
| Aprotinin, Bovine Milk | 6,500 | Blue |

SIGMA (C-3437, Lot 70K9347)