ABSTRACT

HIGH PREVALENCE OF CARCINOMA IN CALIFORNIA SEA LIONS *ZALOPHUS CALIFORNIANUS*: EVIDENCE OF A XENOBIOTIC-INDUCED CARCINOGENIC CASCADE?

By

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Reports in the literature indicate that high incidences of cancer in certain species of marine mammals may be linked to exposure to elevated levels of anthropogenic pollutants. This study aimed to determine whether stranded California sea lions with cancer exhibited higher levels of xenobiotic induced biomarkers of stress compared to animals without cancer. Pollutant concentrations in tissues were measured using GC-MS. *CYP1A* gene induction, protein content and activity were measured using RT-PCR, Western immunoblot and EROD assays, respectively. BPDE adducts were measured using ELISAs. BPDE-protein adducts were the only measured factor associated with cancer. Pollutants were positively correlated to gene induction. TEQ, gene induction, protein content and EROD activity were negatively correlated to BPDE-protein adducts in certain tissues. Based on these results involving a limited number of samples, we were unable to clearly associate cancer with xenobiotic exposure.

HIGH PREVALENCE OF CARCINOMA IN CALIFORNIA SEA LIONS *ZALOPHUS CALIFORNIANUS*: EVIDENCE OF A XENOBIOTIC-INDUCED CARCINOGENIC CASCADE?

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CHAPTER 1

INTRODUCTION

California sea lions (CA sea lions), *Zalophus californianus*, show a particularly high incidence of urogenital carcinomas (18%; Gulland et al., 1996) which have been causally linked to a multifactorial etiology that includes immune deficiency (Ross et al., 1996b; Brouwer et al., 1989), immunogenetic factors (Bowen et al., 2005), bacterial (Johnson et al., 2006) and viral (Buckles et al., 2006, 2007; King et al., 2002; Lipscomb et al., 2000) infections. Xenobiotics have also been implicated in promoting cancer in these marine mammals (Ylitalo et al., 2005) either directly or indirectly (Figure 1). Xenobiotics may have a direct effect by being metabolized into more pro-carcinogenic compounds capable of forming DNA adducts (Martineau et al., 2002). Alternatively, it has been shown that xenobiotics such as polychlorinated biphenyls (PCBs) can have an indirect effect by perturbing a marine mammal's immune system (Ross et al., 1996b; Brouwer et al., 1989), which may lead to an increased susceptibility to bacterial and viral infections with subsequent increased risk of carcinogenesis. These adducts may lead to increased carcinogenesis either through the direct activation of proto-oncogenes or indirectly by damaging genes that encode for normal immune function.

CA sea lions are found along the western coast of the United States and their habitat ranges from the tip of the Baja California peninsula to Alaska (Le Boeuf et al., 2002). Mature females tend to reside near their natal rookeries in the Southern California

Channel Islands where they pup and nurse for 6-11 months. Conversely, male and juvenile CA sea lions tend to have a larger range and migrate as far north as British Columbia to feed during the winter months (Le Boeuf et al., 2002).

CA sea lions of the Southern and Central Coast of California are exposed to high levels of xenobiotic pollutants such as polycyclic aromatic hydrocarbons (PAHs), dichlorodiphenyltrichloroethane (DDT) and PCBs (Zeng et al., 2005; Hartwell, 2008). These particular compounds are often referred to as persistent organic pollutants (POPs) because of their persistence in the environment. Most of these chemicals enter the marine environment via non-point sources through urban, industrial and agricultural run-offs, as well as through maritime activity (Hartwell, 2008), although a significant proportion of the chlorinated xenobiotics in the Southern California Bight (SCB) off Palos Verdes peninsula can be directly attributed to historical discharge of DDTs and PCBs (Zeng et al., 1999) from the 1950s to the 1970s. Although concentrations of DDT and PCBs in the sediments have been slowly declining, the levels are still significantly elevated relative to other coastal sites (Zeng et al., 1999). In a study conducted by Zeng et al. (1999), elevated levels of total DDTs (tDDTs = Dichlorodiphenyldichloroethane (DDD)+DDT+ Dichlorodiphenyldichloroethylene (DDE)) ranging from 0.5 - 9.7 ng/L in the dissolved phase and from 0.1 - 10.0 ng/L in the particulate phase suspended in the water were measured in various locations off the Palos Verdes peninsula, over 25 years after the ban on DDT dumping off the Palos Verdes peninsula. When expressed on a dry weight basis, the particulate phase concentrations of DDT were 3.0 - 360 ng/g dry weight (Zeng et al., 1999). Similarly, the PCB concentrations ranged from 0.06 - 0.79 ng/L in

the dissolved phase and from non-detected to 0.68 ng/L in the particulate phase suspended in the water (Zeng et al., 1999). This represents levels of DDTs an order of magnitude higher than levels found in the relatively unpolluted western Beaufort Sea, Alaska in 1993 (undetected to 0.78 ng/g dry weight; Valette-Silver et al., 1999).

Organochlorines (OCs) such as DDT and PCBs are persistent in organisms due to their lipophilic nature and their resistance to elimination through metabolic processes. For this reason, they bioaccumulate throughout the food chain and particularly affect apex predators that have high body lipid content, such as pinnipeds (Blasius and Goodmanlowe, 2008; Le Boeuf et al., 2002). Blasius and Goodmanlowe (2008) showed that the levels of tDDTs and total PCBs (tPCBs=sum of all detected PCB congeners) detected in the blubber samples of CA sea lions, Pacific harbor seals (*Phoca vitulina richardii)* and northern elephant seals (*Mirounga angustirostris)* that stranded near and died at the marine mammal care centers in San Pedro and Laguna Beach were among the highest found in marine mammals worldwide.

The propensity of CA sea lions to biomagnify pollutants can be estimated from a term referred to as the concentration factor. This expression can be determined by dividing the concentration of xenobiotics in the tissue by that in the water. In the SCB, CA sea lions exposed to tDDTs in the 9.7×10^{-6} µg/g range in the water column have blubber values of up to 13,271 µg/g--a biomagnification factor of 1.37×10^{9}. The tPCB concentration factors are even higher with 1.14×10^{-6} µg/g in the water column and 2,208 µg/g in the blubber--a biomagnification factor of 1.97×10^{9}. As can be seen from these data, the xenobiotic concentration factors in CA sea lions at the top of the food chain can

be considerable. As long-lived, top-level predators, CA sea lions therefore represent a convenient sentinel species that provide an integrated perspective of the potential for xenobiotic exposure and associated risks (Le Boeuf et al., 2002).

High concentrations of xenobiotic contaminants have been associated with various adverse effects in marine mammals such as depressed immune systems (experimentally exposed harbor seals with tPCBs of 17 mg/kg lipid weight; Ross et al., 1996b), reproductive impairments (experimental exposure to PCB concentrations as low as 25 μg per day; Reijnders, 1986), abnormal growth and development (Le Boeuf et al., 2002) and endocrine disruption (Brouwer et al., 1989). There is also evidence to suggest that PCB exposure may be associated with carcinogenicity in CA sea lions (Ylitalo et al., 2005). Given this proposed action of xenobiotics in promoting cancer, it is therefore surprising to note that the recorded percentage of CA sea lions with carcinomas has increased since the cessation of DDT and PCB discharge off the Palos Verdes Shelf (PVS) in the 1970s. Out of 51 diseased and beached CA sea lions from the Southern California coast necropsied in 1972 and 1973, only one (2%) adult female showed signs of carcinomas (Sweeney and Gilmartin, 1974). In a subsequent study evaluating 370 CA sea lions stranded along the Central California coast from 1979 to 1994, 66 (18%) showed signs of transitional cell carcinomas (Gulland et al., 1996). These findings imply that xenobiotics may not be a causal agent in carcinogenesis and that further research involving xenobiotics such as pharmaceuticals and chemicals of emerging concern may be of interest, although it has to be acknowledged that the diagnostic procedures for cancer detection have probably become more refined and stringent in recent years.

Some of the evidence in support of xenobiotics causing cancer in marine mammals comes from studies on beluga whales in the highly contaminated St. Lawrence Estuary. Necropsies of stranded whales from the estuary showed that 21% of the animals had malignant tumors (De Guise et al., 1994). Similar studies of 100 well-preserved carcasses of belugas stranded in the estuary between 1983 and 1999 showed cancer-associated mortality in 18% of the cases, although a total of 21% of the animals had cancers (Martineau et al., 2002). Based on various aerial and boat surveys, it was shown that these belugas lived in a restricted range of the St. Lawrence Estuary and the Gulf of St. Lawrence (Martineau et al., 1987; 2002), contaminated with PAHs from aluminum smelter plants in the region. A spatial distribution study of PAH levels in sediment in the highly industrialized Saguenay Fjord leading to the St. Lawrence Estuary showed levels of PAH to be between 500 and 4500 ppb (Martel et al., 1986). Belugas feed on benthic invertebrates, which are known to readily bioaccumulate PAHs (Ferguson and Chandler, 1998; Martineau et al., 2002). For example, the concentrations of PAHs in blue mussels from Tadoussac and Baie-Ste-Catherine at the base of the Saguenay Fjord and at a more distant site, Port-Cartier located at the mouth of the estuary, showed concentrations that were approximately 100 times higher than those from more pristine areas (Cossa et al., 1983).

Although other xenobiotics such as PCBs and DDTs have been found in the St. Lawrence Estuary (Lebeuf and Nunes, 2005), studies by Martineau et al. (2002) and De Guise et al. (1994) suggest that exposure to PAHs is probably the causal factor for the high incidence of carcinoma in this whale population since no tumors were found in the

carcasses of approximately 50 beluga whales from the Canadian Arctic, which is a relatively unpolluted environment (Martineau et al., 2002). However, direct comparisons between the St. Lawrence Estuary and the arctic populations of animals may be invalid since the arctic animals were randomly selected by hunting rather than being found stranded and dying of natural causes (De Guise et al., 1994). Although tissue residue analyses showed no PAHs in the St. Lawrence beluga whale tissues (Martineau et al., 1985), presumably because of their ability to rapidly metabolize these compounds, analysis of acid hydrolyzed DNA samples by high performance liquid chromatography (HPLC) and fluorescence detection showed the presence of benzo(a)pyrene (BaP) derived DNA adducts (Martineau et al., 2002).

Other support for xenobiotics promoting potentially carcinogenic DNA adducts comes from a study on fish from Lake Saimaa, Finland. The southern part of this lake is polluted with PAHs such as BaP generated from a pulp and paper mill. Analyses of fish taken along the pollution gradient showed a progressive correlative dose-response effect with increased EROD activity and BPDE-DNA adducts towards the point source, implicating an important role played by the cytochrome P450 (CYP450) xenobiotic metabolizing enzymes in the formation of potentially carcinogenic adducts (Kantoniemi et al., 1996). While cause and effect relationships are often difficult to determine in field studies due to confounding co-variables, a similar dose-response relationship was observed for DNA adducts and EROD activity in liver microsomes of rats exposed to soil polluted with a mixture of PAHs in a controlled environment (Fouchécourt et al., 1999). Both of these studies demonstrate a link between environmental (water and soil) PAH

exposure, EROD activity induction and PAH DNA adducts. Furthermore, PAHs have often been associated with carcinomas in humans along with experimental animals (Armstrong et al., 2004).

The pathways involved in PAH-DNA adduct formation have been well characterized and provide a mechanistic link to increased DNA damage and carcinogenesis. PAHs such as BaP bind to aryl hydrocarbon receptors (AhR) bound to the heat shock protein 90 (HSP90) in mammalian cells (Revel et al., 2003). The binding of the ligand (e.g., a xenobiotic such as BaP) allows the AhR to dissociate from the HSP90 and bind to the AhR nuclear translocator (ARNT) following which, the newly formed complex is translocated to the nucleus where it binds to the xenobiotic response elements (XRE) that regulate transcription of various downstream genes (Revel et al., 2003; Figure 2). One of the gene families that is transcriptionally upregulated is the phase 1 biotransformation enzymes, such as *CYP450*, which render certain classes of xenobiotics more polar by adding reactive and polar groups into the substrate, most often by the addition of an oxygen though a hydroxylation reaction. This process enables these anthropogenic pollutants to be directly excreted or metabolized further by phase 2 detoxification enzymes such as UDP-glucuronosyltransferases (UGT) or glutathione S-transferases (GST). These phase 2 enzymes are involved in conjugating metabolites produced during the phase 1 reactions with endogenous, water-soluble compounds such as UDP-glucuronic acid or glutathione and newly formed metabolites are then generally more easily eliminated using renal or other pathways (Kwak et al., 2001).

However, depending upon the product and its propensity to be conjugated appropriately to phase 2 enzymes, some of the compounds formed during phase 1 metabolism are rendered into more pro-carcinogenic derivatives, as is the case of certain PAHs such as BaP. BaP causes DNA damage through metabolism by CYP1A1 and epoxide hydrolase into its metabolite benzo(a)pyrene diol epoxide (BPDE). BPDEs can bind covalently on guanine residues, forming DNA adducts which can potentially lead to cancer. This metabolite is thought to be the major carcinogen involved in the etiology of lung cancer in humans (Revel et al., 2003).

The AhR xenobiotic receptor also regulates the *UGT1A* mRNA expression (Iida et al., 2010). UGTs are important phase II enzymes that help detoxify and eliminate potentially toxic carcinogens and xenobiotics through glucuronidation. The addition of a glucuronic acid to these substrates makes them more water-soluble and excretable (Bock, 2003). For example, potential carcinogens such as *N*-nitrosobutyl-(4-hydroxybutyl) amine (BBN) are conjugated by UGT to form BBN-glucuronide conjugates that are easily excreted in the urine (Bonfanti et al., 1988). However, BBN can also generate alkyl-carbonium ions, a reactive species produced when BBN is chemically cleaved through the α-hydroxylation pathway. These chemical species can bind to DNA causing DNA damage and increasing carcinogenesis in uroepithelial cells (Iida et al., 2010). Interestingly, *UGT1A* mRNA expression is down-regulated by BBN through the suppression of the AhR signaling pathway in urinary bladder carcinogenesis in experimental rats (Iida et al., 2010). The concomitant reduction in glucuronidation leads to an increase in products from the α-hydroxylation pathway, which increases the risk of

carcinogenesis through DNA adduct formation (Iida et al., 2010). Other evidence supporting the importance of phase 2 enzymes in reducing PAH-DNA adducts comes from studies on human lung tissue which showed that individuals that were null in gluthatione S-transferase, a phase 2 enzyme involved in the conjugation of gluthatione with xenobiotics, showed an increase in the presence of PAH DNA adducts (Shields et al., 1993).

A number of studies have shown CYP450 induction by xenobiotics occurring in pinnipeds. Troisi and Mason (1997) studied harbor seals and found a direct positive correlation between increases in PCBs and CYP4501A enzymatic activity. While total CYP450 is a good indicator of contaminant exposure, the CYP450 isozyme profile allows for the determination of the type of organochlorine exposure. For example, CYP1A is more actively induced by organochlorines (OCs), such as CoPlanar PCBs (cPCBs) and PAHs. On the other hand, CYP2B is primarily induced by OCs such as non-planar PCBs and chlorinated pesticides (DDT) (Troisi and Mason, 1997). Whether xenobiotic induced alterations in phase 1 or 2 metabolism lead to cancer through genotoxicity in CA sea lions remains to be established.

DNA adducts can become particularly important in carcinogenesis if they bind to and disrupt the normal activity of proto-oncogenes or tumor suppressor genes such as the *p53* tumor suppressor gene (Revel et al., 2003). p53 is a nuclear protein that plays an important role in the regulation of transcription. The p53 tumor suppressor pathway is a stress response pathway involved in the prevention of growth and survival of cells that could potentially be malignant (Ryan et al., 2001). P53 allows for the inhibition of tumor

development by either repairing damaged DNA before the next replication cycle or removing the damaged cells permanently through apoptosis (Ryan et al., 2001).

In humans, mutations in the *p53* gene can drastically affect its function. Approximately 50% of reported human tumor cases have a mutated form of the *p53* tumor suppressor. 95% of malignant *p53* mutations occur in the DNA-binding domain of the protein (Xu et al., 2011). Mutations in other functional areas of the gene can also cause aberrant functions. Xu et al. (2011) demonstrated how mutant p53 induced an oncogenic gain of function effect and dominant negative activity by aggregating both with its paralogs p63 and p73 and wild-type p53 into inactive cellular occlusions, possibly contributing to the development of tumors. Heat shock proteins are also increasingly expressed due to these aggregates, especially the heat shock protein 70 (HSP70), which is a known inhibitor of apoptosis (Xu et al., 2011).

The levels of p53 expression as well as mutations in the gene itself have been used to indicate for cellular stress. In unstressed cells, p53 is maintained at low levels by continual degradation. Conversely stressors such as DNA damage, oxidative and osmotic stress upregulate its expression.

There is evidence of *p53* gene upregulation in CA sea lions with tumors. Colegrove et al. (2009) found that elevated levels of p53 were expressed in 11 out of 12 sea lions with metastatic tumors from a carcinoma of genital origin. In contrast, p53 levels were low in the genital tract of control animals, except if intraepithelial lesions were present, in which case expression of p53 was noted in certain of these instances. Although it is not known whether the high incidences of tumorigenesis found in CA sea

lions is related to DNA adducts causing mutations on the *p53* tumor suppressor gene, the observation that CA sea lions with cancer have elevated expression of p53 protein in metastatic tumors compared to controls without tumors is indicative that there may be a causal relationship between these factors in this species.

Yamasaki et al. (1992) have reviewed epidemiological and experimental studies on animals and humans that showed that exposure to carcinogens *in utero* as well as exposure of germinal cells to chemicals prior to conception may lead to increased susceptibility to cancer development in adults upon later exposure to carcinogens. This pre-conditioning transgenerational effect of carcinogens may be particularly relevant in marine mammals where maternal offloading of xenobiotics, both in-utero and via lactation, may sensitize the offspring to increased susceptibility to cancer (Blasius and Goodmalowe, 2008; Le Boeuf et al., 2002).

Many studies have linked the exposure of marine mammals to organochlorines to reduced immune function (Ross et al., 1996a; De Guise et al., 1994, 1995; Brouwer et al., 1989) and often mention a possible association with a high prevalence of carcinomas (De Guise et al., 1994, 1995; Ylitalo et al., 2005; Martineau et al., 2002). In pinnipeds, a study by Ross et al. (1996b) showed that harbor seals fed Baltic Sea herring highly polluted with organochlorines had an impaired natural killer (NK) cell activity compared to the harbor seals fed uncontaminated herring from the Atlantic Ocean. Tumor growth is often associated with a depressed immune system and a decreased resistance to tumor development.

It is well known that T lymphocytes are able to recognize tumor specific antigens and exercise cytotoxicity towards cancer cells. NK cells also share this function. NK cells act mainly against tumor and virus-infected cells. Cancers are most frequent in immunosuppressed individuals and experimental mice, especially when these are virus-associated cancers (Catros-Quemener et al., 2003; Allison, 1977). Based on evidence in humans, it is thought that xenobiotic exposure may mediate carcinogenesis in CA sea lions through bacterial and viral infections. In a case-study on women from an indigent developing community in Durban, South Africa, Kharsany et al. (1993) demonstrated a correlation between viral infection and the pathogenesis of cervical intraepithelial neoplasia (CIN). Their study showed a significant association of bacterial vaginosis and human papilloma virus (HPV) with CIN. Pathological factors such as beta-hemolytic streptococci bacteria found in bacterial flora (Johnson et al., 2006) and Otarine Herpesvirus-1 (OtHV-1) (Buckles et al., 2006, 2007; King et al., 2002; Lipscomb et al., 2000) have been associated with the urogenital carcinomas in CA sea lions. In female CA sea lions, the beta-hemolytic streptococci were strongly associated with urogenital carcinomas but not in males. As in all correlative studies, it is difficult to ascertain cause and effect. The data could imply that promotion of carcinogenesis in female CA sea lions may be due to infection-associated inflammation. Alternatively, urogenital carcinomas in female CA sea lions may provide a favorable environment for the growth of beta-hemolytic streptococci (Johnson et al., 2006). In these cases, the papilloma virus did not appear to be associated with carcinomas but OtHV-1 was significantly associated with urogenital carcinomas in both male and female CA sea lions (Buckles et al., 2006)

suggesting that OtHV-1 might be a gammaherpesvirus that induces oncogenesis (Buckles et al., 2006). Two separate studies identified the OtHV-1 in 100% (4/4; 16/16) of stranded CA sea lions with tumors of urogenital origin (Lipscomb et al., 2000; King et al., 2002). A more recent and comprehensive study by Buckles et al. (2007) on the distribution of OtHV-1 in a free ranging CA sea lion population showed a presence of the OtHV-1 in urogenital swabs of 43.3% (26/60) of adult males, 22.2% (16/72) of adult females and in 5.2% (6/116) of juvenile, non-sexually mature individuals but only 2% of mature males, 0.1% of juvenile and none of the mature female pharyngeal swabs tested positive for the OtHV-1. This evidence suggests that the virus is sexually transmitted, as it was found more commonly in urogenital secretions of adult animals, especially males. This is likely due to the polygynous nature of this species, which normally results in males having twice as much genital contact than females over their lifespan (Buckles et al., 2007). Although it is unknown whether xenobiotics play a role in promoting bacterial and viral cofactors in urogenital carcinoma development, it is possible that viruses and bacteria play a role in the etiology of cancer development.

The major histocompatibility complex (MHC) is an immunogenetic factor that has been linked to the increased prevalence of carcinoma in CA sea lions. *MHC class II* is a family of genes that code for glycoproteins that present foreign peptides to T lymphocytes. They are important in the immune response and recognition of self versus non-self antigens. Certain MHC polymorphisms have been associated with genetic susceptibility to cervical cancer in humans (Alaez-Verson et al., 2011). A study by Bowen et al. (2005) compared the MHC genotypes of stranded CA sea lions with and

without cancer and showed that the presence of the MHC class II locus, Zaca-DRB.A was strongly associated with an increased risk of cancer in this species. Although the mechanism underlying this association is still unknown, this observed link may be accompanied by environmental contaminant exposure or pathological factors such as the herpes virus (Bowen et al., 2005).

Alterations in endocrinological and hormonal factors have also been noted with high levels of PCBs. Harbor seals fed PCB-contaminated fish from the Wadden Sea had significantly reduced levels of plasma retinol, total thyroxine (tT4), free thyroxin (FT4) and triiodothyronin (tT3) when compared to harbor seals fed fish from the relatively uncontaminated Atlantic Ocean (Brouwer et al., 1989). A reduction of retinol concentration in plasma is likely to result in vitamin A deficiency, which has been associated with an increased susceptibility to viral infection (Brouwer et al., 1989). Therefore, the possibility exists that CA sea lions exposed to xenobiotics may experience endocrine disruption and reduced protection against tumors.

As can be seen from these studies, cancer is a multifactorial disease involving many possible pathways. A comprehensive study of the relative importance of each of these pathways in promoting cancer in CA sea lions was beyond the scope of this thesis. This study therefore focuses on whether xenobiotic pollutants are associated with the induction of a carcinogenic cascade involving phase 1 enzymes and the ensuing production of carcinogenic metabolites capable of binding to DNA. Conceptually the linkages between cause and effect can be followed by the presence of a legacy biomarker signature that can be identified using a combination of molecular, biochemical and mass

spectrometric techniques (Figure 3). The purpose of this study was to establish the enzymatic biochemical pathways involved in the metabolic activation of the pro-carcinogenic compounds to their reactive electrophilic intermediates involved in DNA binding and therefore potentially proto-oncogene activation. In order to establish whether xenobiotic metabolites capable of producing DNA adducts could be a significant factor in tumorigenesis in CA sea lions, it is hypothesized that animals with cancer will exhibit higher levels of xenobiotics, as well as DNA adducts and other biomarkers of xenobiotic exposure and activation capable of eliciting a carcinogenic cascade, than animals without cancer. In order to test this hypothesis, this thesis project undertook: (i) GC-MS analyses to quantify the concentrations of POPs in blubber samples taken from stranded CA sea lions, (ii) Western immunoblots and RT-PCR analyses to quantify Cytochrome p450 content and *CYP1A* gene expression levels, (iii) EROD fluorometric assays to quantify CYP4501A enzymatic activity, (iv) ELISA assays to quantify BPDE-DNA and BPDE-protein adducts, and (v) correlative analyses to establish the presence of relationships between these factors in CA sea lions with and without cancer.

While not definitive, the identification of the various individual components will provide a continuous thread of evidence that is symptomatic of the xenobiotic induced carcinogenic cascade that has been identified in a number of model mammal species.

CHAPTER 2

MATERIALS AND METHODS: (QA/QC IN APPENDIX A)

In order to test our hypothesis, this thesis attempts to empirically ascertain whether there are correlative linkages between all of the proposed steps leading from xenobiotic exposure through P450 induction and DNA adducts to the cancerous state (Figure 3). The various procedures and methodologies required to analyze each of the various steps in the cascade, from POP tissue residue analysis through to the determination of BPDE-DNA and BPDE-protein adducts as well as the correlative analyses to establish the presence of relationships between these factors in animals with and without cancer, are outlined below.

Sample Collection

Samples of CA sea lion tissues were collected by the personnel from four different marine mammal stranding centers: the Pacific Marine Mammal center (Laguna Beach, CA), the Marine Mammal Center in Sausalito, the Marine Mammal Care Center (San Pedro, CA) and SeaWorld (San Diego, CA). Attempts were made to collect tissues within an hour of the animal's death. Harvested tissue samples included the liver, bladder, uterus, vagina or penis, urethra and sublumbar lymph nodes. Blubber, blood, serum and urine were also collected when possible. All organ and blubber samples were stored at -80°C while urine, blood and serum were stored at -20°C. For the purpose of

this study, animals diagnosed with cancer at necropsy were considered treatment animals. Conversely, necropsied animals showing no signs of cancer were considered control animals. In total, we obtained 6 samples from animals with cancer and 7 samples from animals without cancer. Pictures showing pathology were evaluated whenever possible to confirm evidence of cancer. Data from each animal were collected indicating approximate age, gender, stranding location (Table 1) and date, weight, date and cause of death, overall body condition and the time delay between death and necropsy.

Sample Preparation and Chemical Analysis

In order to compare pollutant levels between cancer and non-cancerous animals and between levels found in this study with studies previously published, pollutant levels in CA sea lions were quantified. Blubber samples were thawed and subjected to organic extractions following EPA Method 3541 (http://www.epa.gov/osw/hazard/testmethods /sw846 /pdfs/3541.pdf). An array of PCB congeners, PAHs and pesticide concentrations were quantified in the blubber of the CA sea lion samples. The toxic equivalency (TEQs) was calculated by summing the concentrations of each compound multiplied by its toxic equivalency factor (TEF) (Van den Berg et al., 2006). In brief, approximately 1 g of sample (wet weight (w/w)) was placed in a cellulose thimble with 10 g sodium sulfate and extracted with 250 mL methylene chloride using a Soxhlet apparatus system. Following extraction, the volume of the sample was reduced to approximately 1 mL using roto-evaporation, and split for organics/lipid analysis (10% for sampling/20% for lipid determination/70% was archived). The organics fraction was solvent exchanged with approximately 1 mL of hexane and the volume reduced again to approximately 0.5

mL. The reduced sample was loaded on a column packed with alumina and silica and eluted with 30 mL of 100% hexane followed by 15 mL of 30% methylene chloride in hexane and 15 ml of 100% methylene chloride.

Following sample clean up, samples were roto-evaporated, transferred to autosampler vials, and an internal standard (see QA/QC) was added to each sample prior to analysis by gas chromatography-mass spectrometry (GC-MS). The samples were injected into the GC-MS via a splitless injection onto a 5% phenyl / 95% dimethyl polysiloxane capillary column (DB-5 (Agilent), 60 m length, 0.25 mm ID and 0.25 μm film thickness. Helium carrier gas flow was applied at an average velocity of 31 cm/sec and the analytes were fractioned using a temperature gradient of the oven as follows: 45°C to 150°C at 25°C per minute then to 285°C at 2.5°C per minute, then held for 16.8 minutes. The Mass Selective Detector (MSD) was scanned from 45-500 atomic mass units (amu) at a rate of 1.64 scan/sec and was used in the Electron Ionization (EI) mode. The MSD ChemStation software by Agilent Technologies was used to quantify each target analyte based on the largest single ion with confirmation from at least two additional ions when these were present. The analyses were conducted in the Institute for Integrated Research in Material, Environment and Society (IIRMES) laboratories.

RT-PCR Analysis

DNA primers for the CA sea lion *CYP1A* gene were designed from deduced amino acid sequence within conserved regions of the mammalian *CYP1A* nucleotide sequence (Teramitsu et al., 2000; Hirakawa et al., 2007). The forward degenerate primer was determined using a conserved series of amino acids VQKKIQEE, which provided

the nucleotide sequence of 5'-GTSCAGAAGAAGATCCAGGAGGAG-3' where S stands for either G or C. A second conserved region was taken downstream and the reverse complement of 5'- SCCCTTGGGGATGTAGAASCC -3' (where S is either G or C) was derived from a highly conserved GFYIPKG amino acid sequence. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping gene sequence in CA sea lion was forward: 5'-TGAGAACGGGAAGCTTGTCA-3' and reverse: 5'-GACAATCTTGAGGGAGTTGTCA-3' (Buckles et al., 2007) and was used to normalize the loading of the samples on the PCR gels and as a control for the RT-PCR.

PCR Reactions

PCR reactions and analyses were undertaken in collaboration and under the supervision of Dr. Judith Brusslan (CSULB). Oligonucleotide primers synthesized by Operon, Inc (Eurofins), were diluted to a concentration of 10 pmol/μL of both the forward and reversed primers using RNA/DNA free water (Sigma water). One microliter of genomic DNA was used as a template and added to 0.5 mL thin-walled PCR tubes. Genomic DNA was selected from an animal with a high DNA yield (SW110866 uterus; 519 ng/μL) and one with an average yield (11-258-Zc liver; 238 ng/μL). The total volume for each PCR reaction was 20 μL comprising of 2 μL of 10x Buffer (10 mM Tris HCL, 50 mM KCl, 1.5 mM $MgCl_2$, pH 8.3), 1 μL of dNTPs (2.5 mM dNTPs), 1 μL of forward and reverse primer (10 pmol/μL), 14.9 μL of Sigma water, 0.1 μL of Taq Polymerase (5000 Units/mL, New England Biolabs, MA) and 1 μL of genomic DNA (concentrations as detailed above). The PCR reaction was run under varying annealing conditions (50°, 50.3°, 51.5°, 53.4°, 55.7°, 58.3°, 61°, 63.7°, 66.1°, 68°, 69.4°, 70°C) as

follows: 4 minutes and 30 seconds at 94°C, 30 cycles of 30 seconds at 94°C, 45 seconds at the annealing temperature, 1 minute at 72°C and 5 minutes at 72°C and then held at 20°C. The completed reactions were stored at 4°C overnight.

PCR Gels

In order to confirm the presence of the anticipated amplified sequence, DNA loading dye (3 μL) was added to each sample and 9 μL of the PCR reaction mixture was loaded onto a 2.5% Tris borate with EDTA (TBE) agarose gel. Three microliters of DNA molecular weight marker MVI (0.25 μg/μL, Roche) was added to the first well and used as a ladder to determine the size of the products generated from the PCR reaction. Gels were run at 100 V for 45 minutes and 110 V for an additional 15 minutes. The gels were removed from the apparatus and stained for 25 minutes in an aqueous solution of ethidium bromide (1% Solution, Fisher Scientific). The gels were then loaded onto a FOTO/UV 21 UV reader apparatus and a picture of the reaction products was taken.

PCR Product Purification

Samples showing the highest yield of products in the gels were combined for both individuals on the assumption that the *CYP1A* gene is highly conserved. The annealing temperatures corresponding to the highest product yield for the CYP primers were 51.5°C, 53.4°C, 55.7°C and 58.3°C and the annealing temperatures for the GAPDH primers were 55.7°C, 58.3°C, 61.0°C and 63.7°C (Figure 4). Combining all GAPDH samples and the 4 best of each of the 2 types of genomic DNA samples yielded a total of 90 μL of PCR product for both the CYP and GAPDH primers. A QIAquick PCR purification kit (QIAGEN) was used to purify the desired products from the reaction

mixtures by adding 450 μL of buffer PB to 90 μL of PCR product and applying the mixture directly onto the QIAquick column and centrifuging at 13,000 rpm for 60 seconds. The eluate was discarded and the sample washed with 0.75 mL of buffer PE, which was removed by centrifugation at 13,000 rpm for 120 seconds. The QIAquick column was placed onto a clean 1.5 mL centrifuge tube and 50 μL of Sigma water was added to the center of the QIAquick membrane. The column was centrifuged for an additional minute at 13,000 rpm and the purified PCR product concentration was read using a NanoDrop apparatus. Each sample was prepared for sequencing by adding 0.25 μL of the desired primer to 10 μL of the PCR product. Sequencing was conducted by Macrogen USA (Rockville, MD).

TOPO-TA Cloning Reaction and Transformation

The TOPO Cloning reaction (Invitrogen TOPO TA cloning kit part number (p/n) 45-0030) was prepared in a 0.5 mL thin walled PCR tube as per the manufacturer's protocol. Two microliters of PCR product, 1 μL of salt solution (1.2 mM NaCl, 0.06 M $MgCl_2$), 1 μL TOPO Vector and 2 μL of Sigma water were added to the PCR tube. The reagents were mixed well, incubated for 20 minutes at 23°C and placed on ice. TOPO cloning reaction (2 μL) was placed into a vial of one shot chemically competent E. Coli (Invitrogen p/n 44-0301) and mixed gently. The tube was incubated on ice for 5 minutes and heat shocked for 90 seconds at 42°C. The tube was transferred to ice once again and 250 μL of optimal broth with catabolite repression (SOC) medium was added. The tube was shaken horizontally for 1 hour at 37°C after which samples of the E.Coli/PCR product (50 or 200 μL) were inoculated on freshly prepared lysogeny broth (LB) agar and

carbenicillin (50 μg/mL) plates. The plates were then placed in an incubator at 37°C overnight and 20 individually selected colonies were transferred to new plates and allowed to incubate overnight at 37°C. The 16 best colonies were selected and 1 μL of each was individually transferred to a tube chilled on ice to which was added 1 μL of M13 forward (M13F) primers (5'-GTAAAACGACGGCCAG-3';(10 pmol/μL), 1 μL of M13 reverse (M13R) primers (5'-CAGGAAACAGCTATGAC-3'; 10 pmol/μL), 1 μL of dNTPs, 2μL of 10X PCR buffer, 0.1 μL of Taq Polymerase and 13.9 μL of water. The tubes were then quickly spun, placed in the PCR machine and subjected to a PCR reaction cycle of 4 minutes at 94°C, 30 cycles of 45 seconds at 94°C, 45 seconds at 58°C, 55 seconds at 72°C, then 3 minutes at 72°C after which it was held at 20°C. Once completed, 3 μL of loading dye was added to each of the samples. An aliquot of 8 μL of each sample was added to an assigned well of a 1.5% agarose TBE gel and run at 110 V for 60 minutes. The gel was stained with ethidium bromide (1% Solution, Fisher) on an orbital shaker for 15 minutes then visualized and recorded on a FOTO/UV 21 UV reader apparatus to determine the colonies that came out at the expected base pair (bp) marker (approx. 771bp) (Figure 5). The marker (MVI) was used to estimate the size of the PCR products and samples at the expected base pair size were selected and added to tubes containing 2mL of 2x Yeast extract and Tryptone (YT) media + carbenicillin and incubated with shaking overnight at 37°C.

Plasmid DNA Purification

A QIAprep spin miniprep kit (QIAGEN) was used to purify the bacterial plasmid colonies. Colonies were pelleted by centrifugation at 13,000 rpm for 90 seconds and

resuspended by lightly vortexing in 250 μL of Buffer P1. Two hundred and fifty microliters of Buffer P2 was added and within 5 minutes, 350 μL of Buffer N3 was added. After mixing by inversion, the samples were pelleted by centrifugation for 10 minutes at 13,000 rpm. The supernatant was collected and applied onto a QIAprep spin column, centrifuged for 60 seconds at 13,000 rpm and the flow-through was discarded. The QIAprep spin column was washed by adding 0.5 mL of Buffer PB and centrifuged for 60 seconds at 13,000 rpm. The column was washed again with 0.75 mL of Buffer PE. The QIAprep column was placed in a clean 1.5 mL centrifuge tube and 50 μL of Sigma water was added directly to the center of each QIAprep spin column, left to incubate for 1 minute and centrifuged for 1 minute at 13,000 rpm. The concentrations of DNA in each sample were quantified and those with sufficient yield of DNA were prepared (10 μL of the purified DNA to which was added 0.25 μL of either Primer M13F or M13R) for DNA sequencing (Macrogen USA, Rockwille, MD).

Trizol RNA Preparation

A Trizol RNA preparation was performed using a protocol developed in Dr. Judith Brusslan's lab at CSULB (http://www.csulb.edu/~bruss/LabProtocols.htm). Approximately 150 mg of liver tissue was mechanically homogenized in 2 mL of Trizol reagent and incubated for 5 minutes at room temperature (RT). Chloroform (200 μL) was added to each tube, incubated at RT for 3 minutes and then centrifuged for 15 minutes at 11,500 rpm at 4°C. The aqueous phase (top layer) was transferred into a fresh centrifuge tube and the RNA was precipitated by adding 500 μL of isopropanol. The RNA was then pelleted by centrifugation for 10 minutes at 11,500 rpm at 4°C after

incubation at RT for 10 minutes. The resulting pellets were resuspended and combined in a total of 300 μL of TE Buffer. Thirty microliters of sodium acetate, pH 5.2 and 660 μL of 100% ethanol was added, incubated at RT for 10 minutes and the solution was then centrifuged for 10 minutes at 11,500 rpm at 4°C. The resulting pellet was washed with 1 mL of 80% ethanol and then centrifuged at 9,500 rpm at 4°C for 5 minutes. The supernatant was discarded and the sample was again quickly spun and the supernatant removed using a pipette. The pellets were dried in a SpeedVac for approximately 5 minutes. The RNA pellet was dissolved in 40 μL of Sigma water by mixing, quickly spinning and incubating overnight at 4°C. The concentrations of RNA were quantified using a spectrophotometer at OD 260/280 using 3 μL of sample in 600 μL of Sigma water.

Agarose Gel Electrophoresis of RNA in Formaldehyde

A RNA formaldehyde gel was prepared using standard procedures. The RNA samples were prepared at a concentration of 1 μg RNA/μL in Sigma water. An additional 5 μL of Sample Buffer was added and the sample was heated at 65°C for 10 minutes. After cooling on ice, 2.5 μL of sample dye was added to each tube. Samples were loaded onto the gel and subjected to electrophoresis at 100 V for 50 minutes. The gel was then washed twice for 10 minutes on an orbital shaker using 100 mL of water filtered in a Milli Q water filter (Milli Q water) to remove the formaldehyde. The gel was then stained with a 1% solution of ethidium bromide for 10 minutes at RT on an orbital shaker. The gel was then destained, first for 30 minutes in 100 mL of Milli Q water on an orbital shaker, and then again in 100 mL of Milli Q water in a plastic receptacle

overnight at 4°C. The gel was then placed in the FOTO/UV 21 UV reader apparatus to assess the quality and the quantity of RNA yielded from the extraction (Figure 6).

cDNA Preparation

cDNA was prepared from the various samples using 1 μg of total RNA in a total volume of 5.3 μL Sigma water. Contaminating DNA was removed by the addition of 1 μL of DNAse I and 0.7 μL of 10X DNAse Buffer (previously prepared as Master Mix 1). After incubation at 37°C in the PCR machine for 10 minutes, 1 μL of 25 nM EDTA was added and the sample was incubated at 75°C for an additional 10 minutes. The sample tube was then centrifuged and 3 μL of random hexamers (150 ng/μL) and 1 μL of dNTPs (10 mM each) (previously prepared as Master Mix 2) were added to each tube. The tubes were then heated for 10 minutes at 70°C under the MMLV program on the PCR machine. After cooling down to 4°C with ice, 2 μL of 10X Buffer, 2 μL of 0.1 M DTT and 3 μL of Sigma water was added. The samples were briefly incubated in the PCR machine at 37°C for 2 minutes and 2 μL of Moloney Murine Leukemia Virus (MMLV) was added to each tube after which the samples were incubated at 37°C for 50 minutes. The reaction was terminated by heating the sample to 70°C for 15 minutes to inactivate the reverse transcriptase. The total volume of the reaction yielded 20 μL to which 60 μL of Sigma water was added prior to storage at -20°C.

PCR Reactions on cDNA and Genomic DNA

The PCR reaction protocol explained previously was followed for the primer sets CYP1AF + CYP1AR1, CYP1AF + CYP1AR2, GAPDHF + GAPDHR. The primers were obtained following sequencing of the plasmid DNA by Macrogen USA (Rockville,

MD). It was determined that the CA sea lion had a strong nucleotide sequence identity to the walrus *Odobenus rosmarus* sequence and therefore the exon sequence found on BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for the walrus was used to design primers for this reaction. Design tools Netprimer and Operon were used to create primers free of hairpins and dimers. A CYP1A_Reverse 1(CYP1AR1) sequence was constructed with the sequence 5'-ATG GTG AAG GGG ACG AAG GAA G-3'. CYP1A_Reverse 2 (CYP1AR2) sequence was 5'-TAT GAG GGA TGG TGA AGG GG-3' and CYP1A_Forward 1 (CYP1AF) sequence was 5'-TGG ACA CAG TGA TTG GCA GG-3'. Two reverse and two forward sequences with potential are usually designed in order to ensure the optimal sequence is utilized. In this case, one optimal forward sequence and two reverse sequences were designed. cDNA samples were used and one genomic DNA sample was used as a positive control. Samples were placed in the PCR machine using the 58°C annealing program. The samples were then placed at 4°C overnight.

PCR Gel for Primer Samples

A 2% TBE PCR gel was prepared as described previously. A single layer gel was used and run for 45 minutes at 100 V and 30 minutes at 110 V. The gel was stained using 5 μL of ethidium bromide (1% solution, Fisher Scientific) in 200 mL of Milli Q water (Figure 7).

Real Time PCR (RT-PCR) with SYBR Green

To ensure the detection of gene induction activity was not due to a false positive, non-template controls (Sigma water) were used as a negative control in the RT-PCR reactions. Pipettes used during the sample preparation were previously rinsed with

ethanol to avoid DNA contamination. Barrier tips were used as an additional precautionary measure. The prepared cDNA from 2 samples were used as templates. Three sets of primers were used: CYP1AF + CIP1AR1, CYP1AF + CYP1AR2 and GAPDHF + GAPDHR. Each sample was run in duplicate and contained 10 μL of a MasterMix containing 2 μL of 2X SYBR Green (AB Gene AB-1158A), 2 μL of Forward primer diluted to 1800 nM and 2 μL of Reverse Primer diluted to 1800 nM. 2 μL of either the cDNA sample or the non-template control was added to their respective tubes and the tubes were centrifuged. Tubes were placed in the RT-PCR instrument and underwent the following temperature control cycle: 15 minutes at 95°C, 40 cycles of 15 seconds at 95°C, 1 minute at 60°C (annealing temperature), 15 seconds at 72°C, 1 minute at 95°C, 30 seconds at 60°C and 30 seconds at 95°C.

RT-PCR with SYBR Green – Serial Dilutions

To ensure that optimal RT-PCR conditions were met, reactions were conducted using serial dilutions of one cDNA sample. The series involved cDNA diluted 1 in 4, 1 in 16, 1 in 64 and 1 in 256 (each step used 5 μL of stock diluted in 15 μL of Sigma water). The dilutions 1 in 16, 1 in 64 and 1 in 256 were used as our template. A MasterMix for each sample was prepared as described above. Primers were diluted to 540 nM instead of 1800 nM as this dilution yielded the best results. Samples were run in duplicate and non-template controls were used for each set of primers.

RT-PCR with SYBR Green –GAPDH and CYP1AF + CYP1AR1 for All Samples

RT-PCR was conducted in order to establish the induction levels of the *CYP1A* gene in each collected sample. Following determination of optimal RT-PCR conditions,

each cDNA sample (except for samples Z11-04-03-012 and Z11-04-12-014 because no liver was left) was thawed and diluted 1 in 16, 1 in 64 and 1 in 256 as described previously. The 1 in 64 and 1 in 256 dilutions were used as templates and each sample was assayed in triplicate. Six non-template controls were also assayed. Primers were diluted to 540 nM. Quantitation was done using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001).

Microsomal Preparation

Microsomes were prepared according to the methodology of Ronis et al. (1994). In brief, 1 M Na/KPi (pH = 7.4) and 1 M KPi (pH = 7.4) buffers were mixed in order to obtain a 10 mM Na/KPi, pH 7.4, 1.15% KCL, 10 mM EDTA homogenization buffer and a 50 mM KPi (pH = 7.4), 0.1 mM EDTA Assay Buffer. Approximately 2 g of liver tissue was homogenized in 6 mL of ice cold homogenization buffer. The homogenate was centrifuged at 10,000 x g (9,000 rpm) for 30 minutes and the supernatant was carefully transferred to another polycarbonate tube and centrifuged at 100,000 x g (35,000 rpm) at 4°C for 1 hour. The supernatant was decanted and 5 mL of homogenization buffer containing Halt™ Protease and Phosphatase inhibitor cocktail (Pierce Biotechnology (Thermo) p/n 78440) was added to each tube containing the resulting pellet, which was resuspended and centrifuged at 35,000 rpm for 30 minutes. The supernatant was decanted and discarded and the resulting microsomal pellet was resuspended using a glass homogenizer in approximately 1mL of assay buffer to which protease and phosphatase inhibitors were added. The samples were then aliquoted into 1.5 mL microcentrifuge tubes and 10 μL was saved for protein concentration

determination prior to storage at -80°C. The microsomal protein concentration was determined following the Thermo BCA–protein assay protocol using Bovine Serum Albumin (BSA) as a standard. Sample absorbance was determined using a microplate reader (Biorad Benchmark Plus Microplate Reader/Microplate Manager 5.2 program) on 25 μL of a 1:20 dilution (in deionized water) of sample. Standards and unknown samples were pipetted in replicate onto the microplate and 200 μL of the working reagent was added to each well. The samples on the plate were agitated on a shaker/incubator at 37°C for 30 minutes after a protective plate seal was placed on top of the plate to avoid sample cross contamination. The plate was cooled to room temperature and the absorbance of the reaction mixture was measured at 562 nm.

Cytochrome P450 Content

Western immunoblot analysis was used to determine the CYP450 content in microsome preparations. Western immunoblots were performed according to the methodology of Ronis et al. (1994). In brief, 37.5 μg of liver microsomes were loaded into each well based on the predetermined concentrations. The gels were prepared using a standard 2-step layout where the lower resolving gel was poured first into the apparatus. Two resolving gels were prepared using 37.6 mL of distilled water, 21.2 mL of 30% liquid acrylamide (19:1, acrylamide:bis-acrylamide), 20 mL of separating gel buffer and 400 μL of 10% SDS. The mixture was polymerized with 400 μL of 10% APS and 80 μL of TEMED. The solution was poured into the gel apparatus (Bio-Rad Laboratories, Hercules, CA) and a layer of 10% ethanol was added on top of the resolving gel to keep the surface straight and smooth during polymerization. Once set, a stacking gel was

prepared using a mixture of 24 mL of distilled water, 5.3 mL of 30% liquid acrylamide (19:1, acrylamide:bis-acrylamide), 10 mL stacking gel Buffer, 200 μL of 20% SDS (Sigma Aldrich Co, St. Louis, MO) which was polymerized using 200 μL of 10% ammonium persulfate and 40 μL of TEMED. The stacking gel solution was poured on top of the resolving gel after removal of the ethanol layer and a sample comb was inserted. Polymerization was complete after approximately 20 minutes following which the comb was removed and each well was cleaned out with a syringe.

The gels were run at 50 V of constant current for approximately 1 hour and the resolved proteins were then transferred to a methanol-washed PVDF membrane by sandwich blotting with absorbent paper. The sandwich apparatus was covered with transfer buffer (25mM Trizma Base (Sigma Aldrich Co, St. Louis, MO), pH 8.3, 162 mM glycine (Sigma Aldrich Co, St. Louis, MO), 0.1% SDS (Sigma Aldrich Co, St. Louis, MO), 20% methanol) and transfer was accomplished at 100 mA for about 1 hour with cooling from an ice pack. Following transfer, the PVDF membrane was placed in a 5% milk-based blocking solution for 1 hour and probed using a variety of different primary antibodies. One gel was probed with rabbit polyclonal CYP1A1 (ARP41404, Aviva Systems Biology, San Diego) 1:1,000 dilution (20 μL of AB and 20 mL 5% milk solution), a second gel was probed with rabbit polyclonal CYP1A1 (ARP41405, Aviva Systems Biology, San Diego) 1:1,000 dilution (20 μL of AB and 20 mL 5% milk solution), while a third gel was probed using Gentest CYP1A antibody (1:100,000 dilution). Gels were left on a shaker in the primary antibody solution at 4°C overnight after which excess primary antibodies were removed by washing four times for 10

minutes in a solution of Tris-buffered saline and Tween 20 (TBTS). Two different secondary antibodies were used to visualize the proteins (1 hour incubation). The first and second gels were incubated with BIORAD Anti-rabbit 1:10,000 dilution (p/n170-6515). The third gel was incubated with Bovine anti-goat HRP conjugate (Santa Cruz Biotech SC-2350). Excess secondary antibodies were removed with four 10 minutes rinses of TBTS. Once ready to be developed, the blots were placed on a clear plastic paper and covered with approximately 2mL of either ECL Prime or SuperSignal West FEMTO (Thermo Fisher, Rockford, IL) chemiluminescent substrate and incubated for 5 minutes. The blots were then developed in a dark room using an x-ray cassette and radiography film under various exposure conditions. Gels were scanned using Epson scanning software. The signals of the various gels were quantified by densitometry using Quantity One 4.6.5 software.

Cytochrome P450 Activity

Samples used to compare EROD, PROD and BROD activities were SW110866, 11-258-Zc, SW110817, Z11-04-03-012, and Z11-04-12-014. All samples were used to compare EROD activity between cancer and non-cancer animals except for Z11-04-03-012 because insufficient microsomal proteins could be prepared. EROD, PROD and BROD assays were performed according to the methodology of Ronis et al. (1994) as follows:

EROD Assay

Ethoxyresorufin-o-deethylase (EROD) assays were used to monitor mixed function oxygenase activity in thawed microsomal liver fractions. EROD activity in each

microsomal preparation was conducted by pipetting 3 mg of microsomal proteins to a total volume of 3 mL assay buffer (50 mM KPi, pH 7.4, 0.1 M EDTA) containing 1 mM NADPH and 20 μL of EROD substrate. The reaction was initiated by the addition of 30 μL of NADPH and the fluorescent yield was monitored using a Shimadzu fluorescence plate reader and analyzed using the associated RF5301PC software. The measurement parameters were set as follows: 536 nm excitation wavelength, 596 nm emission wavelength. Slit width was set at 1.5 for both the excitation and emission. The sensitivity was set to high and the reaction time was set to 900 seconds. The intensity values were noted over time and the kinetic rate of the reaction was expressed as follows:

$$\text{EROD (nmols/min/mg)}: \frac{\text{Intensity/time (mins)} \times 3\ \text{mL}/0.66}{\text{mg sample}}$$

where 0.66 F.U. is the fluorescent units in 1 nm/mL of resorufin.

BROD Assay

The Benzyloxy-resorufin-o-deethylase activity was measured in liver microsomes from five CA sea lion samples (SW110866, SW110817, 11-258 Zc, Z11-04-03-012, Z11-04-12-014) by fluorometry as described above using BROD substrate in place of EROD.

PROD Assay

The Pentoxy-resorufin-o-deethylase activity was measured in liver microsomes from five CA sea lion samples (SW110866, SW110817, 11-258 Zc, Z11-04-03-012, Z11-04-12-014) by fluorometry as described above using PROD substrate in place of EROD.

Genomic DNA Extraction for BPDE-DNA Adduct Analysis

Genomic DNA extraction was conducted using a Zymo Research Quick-gDNA Midi prep kit (Zymo research, Irvine, CA, p/n D3100). Approximately 100 mg of tissue

samples (liver and bladder for males, liver, bladder and uterus for females) were mechanically homogenized in 2.5 mL of genomic lysis buffer. The lysate was then transferred to a Zymo-Spin™ V-E Column/Zymo Midi Filter assembly and centrifuged at 1,500 x g for 5 minutes. The Zymo-Spin™ V-E Column/Zymo Midi-Filter assembly was disconnected and the Zymo Spin™ V-E column was transferred to a collection tube. The tube was centrifuged at 10,000 x g for 1 minute to remove the residue from the column. The column was then rinsed with 300 μL of DNA pre-wash buffer, which was removed by centrifugation at 10,000 x g for 1 minute. The eluate was discarded and the column was rinsed with 400 μL of gDNA wash buffer by centrifugation at 10,000 x g for 1 minute. The eluate was again discarded and the wash step was repeated. The Zymo-Spin™ V-E column was transferred onto a 1.5 mL microcentrifuge tube and 150 μL of DNA Elution Buffer was added directly onto the column matrix. The column was incubated for 1 minute at room temperature and was then centrifuged at 10,000 x g for 1 minute to elute the DNA.

A NanoDrop apparatus was used to measure the concentration and the purity of the extracted DNA. Dilute samples were concentrated by centrifugation after precipitation by the addition of 95% ethanol at -20°C. The resulting pellet was resuspended in 25 μL of Elution Buffer and the final concentration and quality was determined using the NanoDrop apparatus (See QA/QC).

BPDE-Protein Adduct Formation

BPDE-protein adducts were measured on a subset of liver, bladder and uterus cell lysate samples from female animals (SW110817, SW110866, Z11-04-03-012, Z11-04-

12-014, 11-258-Zc) using an OxiSelect™ BPDE-protein adduct ELISA kit (p/n STA-301). The recommended protocol was followed. Cell lysates were prepared from liver, bladder and uterus tissue samples by homogenizing approximately 100 mg from each tissue in 1 mL of homogenization buffer containing Halt™ Protease and Phosphatase inhibitor cocktail. Five microliters of each homogenate was collected and assayed for protein content as described previously. Hundred microliters of the reduced BPDE-BSA standards and 10 μg/mL of each CA sea lion sample were loaded individually into separate wells of the protein binding plate and incubated overnight at 4°C. Each BPDE BSA standard and unknown samples were assayed in duplicate (except sample Z11-04-012-014 bladder assayed in quadruplicate due to suspected pipetting error). The wells were washed twice with 250 μL of 1X PBS, blotted and incubated at RT in 200 μL of Assay Diluent for 2 hours on an orbital shaker. The wells were washed three times with 250 μL of 1X wash buffer, blotted and 100 μL of the diluted Anti-BPDE 1 antibody was added. After incubation for 1 hour at RT on an orbital shaker the plate was washed 3 times with buffer and 100 μL of the prediluted secondary antibody-HRP conjugate was added to all wells. The samples were incubated for 1 hour at RT with agitation and then washed 5 times prior to incubation for 2-30 minutes with 100 μL of warmed 3,3,5,5-Tetramethylbenzidine (TMB) substrate solution. The enzyme reaction was halted with 100 μL of Stop Solution and the reaction product was quantified on a microplate reader at 450 nm. The blank (or zero) concentration of the reduced BSA Standard was used as an absorbance blank.

BPDE-DNA Adduct Formation

In order to compare BPDE-DNA adduct levels between cancer and non-cancerous animals, BPDE-DNA adduct levels were measured in DNA extracted from liver (all animals), bladder (except Z11-04-12-014) and in the uterus tissue samples of all female CA sea lions (except Z11-04-12-014). For males, only the liver and bladders were used. BPDE-DNA adducts were quantified using an OxiSelect^{TM} BPDE-DNA adduct ELISA kit (p/n STA-357) using the manufacturer's recommended procedure. Extracted DNA samples and BPDE-DNA standards were diluted with phosphate buffered saline (PBS) to a concentration of 2 μg/mL and 100 μL of each sample and standard was loaded in duplicate onto a DNA High-Binding plate and incubated overnight at 4°C. The samples were washed twice with PBS and the excess fluid was removed by blotting the plate on a paper towel. Assay Diluent (200 μL) was added to each well and blocked for 1 hour at RT after which the excess fluid was removed by carefully blotting it on a paper towel. Diluted Anti-BPDE 1 antibody (100 μL) was added to each well and incubated for 1 hour at RT on an orbital shaker. The plate was then rinsed 5 times with buffer and 150 μL of prediluted 1X Blocking Reagent was added to each well prior to incubation for 1 hour at RT on an orbital shaker. Following 3 washes with a buffer, 100 μL of the diluted secondary antibody HRP conjugate was added to all the wells and incubated for 1 hour at RT on an orbital shaker. The plate was again washed 5 times and 100 μL of warmed TMB substrate solution was added to each well including the blank/empty wells. The enzyme reaction was allowed to proceed for 30 minutes at RT on an orbital shaker and stopped by adding 100 μL of Stop Solution to each well. The colorimetric reaction was

immediately read on a microplate reader at a wavelength of 450 nm. The blank (or zero) concentration of the reduced BSA Standard was used as an absorbance blank.

Data Analysis

In order to normalize the data, square root or log10 (log) transformations were applied when necessary. We used *t* tests to compare results between animals with cancer and without cancer for all assays (pollutant analysis, *CYP1A* Gene Induction, protein content, EROD activity, BPDE-DNA adduct ELISA, BPDE-protein adduct ELISA), except when the data could not be normalized in which case a Mann-Whitney U test was performed. Linear and logarithmic regression statistics were conducted to determine correlations between all measured assays. A non-parametric Spearman's rank correlation test was used to correlate BPDE-DNA adduct (bladder) data with EROD activity since the data could not be normalized. A one-way ANOVA with Tukey's post-hoc test was performed on levels of total PCBs, total Pesticides and total CoPlanar PCBs, on EROD, PROD and BROD activities obtained from a subset of samples to compare their levels and on BPDE-DNA and protein adducts to compare levels between each tissue.

CHAPTER 3

RESULTS

Comparative Analysis

Pollutant Levels

In order to compare pollutant levels between cancer and non-cancerous animals and between levels found in this study with studies previously published, pollutant levels in CA sea lions were quantified. The most prevalent pesticides were DDTs, cis- and trans-Nonachlor and Chlordane-alpha. Pesticides BHC (alpha, beta, gamma, delta), Hexachlorobenze, Heptachlor, Aldrin, Heptachlor Epoxide, Oxychlordane, Chlordane-gamma, Endosulfan I, Dieldrin, Perthane, Endrin, Endosulfan II, Endrin aldehyde, Endosulfan sulfate, Endrin ketone, Methoxychlor and Mirex were all below detection levels. Seven PCB congeners accounted for the majority (73%) of tPCBs (101, 187, 099, 118, 180, 138, 153) (Table 2). PAHs were all below detection limits. Total Pesticides were higher than tPCBs and total CoPlanar PCBs (tCoPlanar PCBs). No significant difference was found between tPCBs and tCoPlanar PCBs, $p = 0.005$; tPesticides and tPCBs, $p = 0.02$; tPesticides and tCoPlanar PCBs, $p = 0.01$; tPCBs and tCoPlanar PCBs, $p = 0.89$; F= 6.40, n=12 in ng/g, lipid weight (l/w); data not shown). No significant difference was found in the pollutant levels between cancerous animals and non-cancerous animals for any of the pollutant groups (l/w and w/w) (T=2.23; $p = 0.33$ for tCoPlanar PCBs w/w; $p = 0.76$ for tCoPlanar PCBs l/w; $p = 0.36$ for tPCBs w/w; $p = 0.93$

for tPCBs l/w; $p = 0.60$ for tPesticides w/w; $p = 0.85$ for tPesticides l/w; $n_c=n_{nc}= 6$; Figures 8-10).

TEQs

TEQs were calculated in order to compare levels in animals with cancer with non-cancerous animals and between TEQs found in this study with previously reported levels. The value for all samples was 1836 ± 4176.76 (Mean ± S.D.) ng/g in l/w. The value for samples with cancer (2889.18 ± 5902.16 (Mean ± S.D.) ng/g in l/w) was not significantly different than the value recorded in animals without cancer (783.22 ± 939.82 (Mean ± S.D.) ng/g in l/w; U=21; $n_{nc}=n_c= 6$; $p = 0.63$; Figure 11).

Gene Sequence

In order to design primers for the RT-PCR, a single amplified gene product was obtained by the TOPO-TA cloning procedure between the reverse primers designed from the conserved amino acid region GFYIPKG (single underline) and forward primer designed for the conserved region VQKKIQEE (bold underline).

3'-
GGGNNNAANNAANNNNANNTACTATANNGGCGAATTGAATTTAGCGGCCGC
GAATTCGCCCTTGCCCTTGGGGATGTAGAAGCCACTCAGACTTGTGTCTTTGG
TGGTACTGGGGAAGAAAGAAGCCCAGTCAATCTTAGGACCACAAGGACATCC
CCCTGCCTCCCATACCTGTCCCTCCCTCCGGGCCTGATCAGGTACATGGACTG
GAGGAAGGCCCACTATCAGACAGCAGTTGATCCATGGGGTCTCACCTATGAG
GGATGGTGAAGGGGACGAAGGAAGCATGTCGGAAGGTCTCCAGGATGAATG
CCTCCAGGTAGGGCAGCTGGAGCCTGTCAGAGAGCCGGGGCTGCCGGGCCCT
GCCAATCACTGTGTCTGCAGAACATAAAGGACCATGTGGGGTGCCTGGGTGG
CTCAGTGGGTTAAGCATCTACCTTCTGCTCAGGTTATGATCTCAGGGTCTTGG
GATCCAGCCCAGCCCTGCACCAGGTCCCCTGCTCAGTGGGAGTCGGCTCCTC
CCTCTCCCACCCCTCCCCCAGCTTGTGCTCTCCTCTCNCTCACTCTCTCTCAAA
TAAATAAAATCTTTAAAATAAAATAAAAAAGGACCATGTGGATGAAGGGGCT
GCCCAGAACTTGGCCAGGCCCTCTGCCTTGAGCACTTTAAAGGAAGCCACCA
CCTACCCAGCTCCTCCTGGATCTTCTTCTGCACAAGGGCAAATTCNTTTAAAC
CTGCAGGACTAGTCCCTTTGGTGNGGGTTNNTTNTGNGN-5'

Based on these results, the reverse complement of the sequence was determined with the VQKKIQEE sequence (single underline) and the GFYIPKG sequence (bold underline).

5'-
NCNCANAANNAACCCNCACCAAAGGGACTAGTCCTGCAGGTTTAAANGAATT
TGCCCTTGTGCAGAAGAAGATCCAGGAGGAGCTGGGTAGGTGGTGGCTTCCT
TTAAAGTGCTCAAGGCAGAGGGCCTGGCCAAGTTCTGGGCAGCCCCTTCATC
CACATGGTCCTTTTTTATTTTATTTTAAAGATTTTATTTATTTGAGAGAGAGTG
AGNGAGAGGAGAGCACAAGCTGGGGGAGGGGTGGGAGAGGGAGGAGCCGA
CTCCCACTGAGCAGGGGACCTGGTGCAGGGCTGGGCTGGATCCCAAGACCCT
GAGATCATAACCTGAGCAGAAGGTAGATGCTTAACCCACTGAGCCACCCAGG
CACCCCACATGGTCCTTTATGTTCTGCAGACACAGTGATTGGCAGGGCCCGG
CAGCCCCGGCTCTCTGACAGGCTCCAGCTGCCCTACCTGGAGGCATTCATCCT
GGAGACCTTCCGACATGCTTCCTTCGTCCCCTTCACCATCCCTCATAGGTGAG
ACCCCATGGATCAACTGCTGTCTGATAGTGGGCCTTCCTCCAGTCCATGTACC
TGATCAGGCCCGGAGGGAGGGACAGGTATGGGAGGCAGGGGGATGTCCTTG
TGGTCCTAAGATTGACTGGGCTTCTTTCTTCCCCAGTACCACCAAAGACACAA
GTCTGAGTGGCTTCTACATCCCCAAGGGCAAGGGCGAATTCGCGGCCGCTAA
ATTCAATTCGCCNNTATAGTANNTNNNNTTNNTTNNNCCC-3'

Note. Ns were nucleotides that were not identified during the sequencing.

The amplified fragment was 771 base pairs in length and contained both putative introns and exons based on alignments with other pinniped sequences. The CA sea lion sequence without introns was deduced from the walrus sequence found in BLAST (GenBank accession number DQ093088.1).

5'-T GTG CAG AAG AAG ATC CAG GAG GAG CTG GAC ACA GTG ATT GGC AGG GCC CGG CAG CCC CGG CTC TCT GAC AGG CTC CAG CTG CCC TAC CTG GAG GCA TTC ATC CTG GAG ACC TTC CGA CAT GCT TCC TTC GTC CCC TTC ACC ATC CCT CAT AGT ACC ACC AAA GAC ACA AGT CTG AGT GGC TTC TAC ATC CCC AAG GG-3'

The sequence was identified as a highly conserved region of the *CYP1A* gene within limits of both the alpha-helix I, containing conserved threonines, and the heme binding motif found in the Baikal seal (*Phoca sibirica*) and other mammals (Hirakawa et al., 2007).

A putative amino acid sequence was determined using In-silico software (http://in-silico.net/tools/biology/sequence_conversion) of VQKKIQEELDTVIGRARQP RLSDRLQLPYLEAFILETFRHASFVPFTIPHSTTKDTSLSGFYIPK. ClustalW software (http://www.genome.jp/tools/clustalw/), used to undertake comparative analysis of the CA sea lion bp sequence with the walrus and Baikal seal showed a 96% amino acid sequence identity to the same portion (nucleotide 1152 to 1348) of the *CYP1A* gene in Baikal seals (Hirakawa et al., 2007) and 98% identity with the walrus (Figure 12). The sequence without intron was also 98% similar to the sequence found for Stellar sea lions in GenBank (GenBank accession number AB014356.1).

Gene Induction

In order to compare gene induction levels between cancer and non-cancerous animals, *CYP1A* gene induction was measured in CA sea lion RNA samples. The mean fold-difference (relative to a calibrator) level was 911.1 delta delta CT ($\Delta\Delta C_T$) with values ranging from 1 to 2765.5 $\Delta\Delta C_T$. There was no significant difference between the *CYP1A* fold gene induction levels in animals with cancer and animals without cancer ($T=2.26$; $n_{nc}=5$, $n_c=6$; $p = 0.36$; Table 3; Figure 13).

Western Immunoblot: CYP1A Protein Content

In order to compare CYP1A protein content between cancer and non-cancerous animals and between levels found in this study with studies previously published, a Western immunoblot was conducted to determine the expression of CYP1A protein in liver microsomes. A band appeared at approximately 56 kDa for the Supersome, a rat CYP1A1 + reductase (baculovirus-insect cell expressed with cDNA-expressed rat P450

reductase; BD Biosciences catalog number 456511), which was used as a positive control, and at approximately 52 kDa for the rest of the samples. There was some cross-reactivity at approximately 50 kDa for a few samples (CSL10281, CSL10322, CSL10192) (Figure 14). CYP450 protein content from CA sea lion liver microsomes of each sample loaded onto the gel was quantified by densitometry except for animal Z11-04-03-012 and CSL120008 since insufficient liver microsomes were collected from these samples for electrophoresis. No significant difference was detected between the protein content levels of animals with cancer and animals without cancer ($U=7$; $n_{nc}=6$, $n_c=5$; $p = 0.14$; Table 4; Figure 15).

EROD, BROD and PROD

In order to determine the relative degree of induction of the various *CYP* gene families in CA sea lions, EROD (CYP1A), PROD (CYP2B) and BROD (CYP3A) fluorometric assays were run on samples SW110866, SW110817, Z11-04-03-012, Z11-04-12-014, 11-258-Zc. EROD activity was significantly higher overall than BROD and PROD ($F= 11.79$, $p = 0.002$; EROD was significantly higher than BROD, $p = 0.004$; and PROD, $p = 0.005$; no difference between BROD and PROD was found, $p = 0.98$; n=5; Figure 16).

EROD Activity

Following determination that CYP1A was the most induced enzyme in CA sea lions from this study, EROD fluorometric assays were run on all samples but Z11-04-03-012 in order to compare CYP1A enzymatic activity between cancer and non-cancerous animals and between levels found in this study with studies previously published. All

measured samples exhibited EROD activity except two: SW120008, which visually had poor liver quality (fully metastasized, light brown coloring with patchy white nodules and was therefore excluded) and sample CSL10322, which also showed no activity. No significant difference was detected between the EROD activity levels in animals with cancer and animals without cancer (U=19; n_{nc}=6, n_C=5; p = 0.27; Table 5; Figure 17).

BPDE-Protein Adducts

In order to compare BPDE-protein adduct levels between cancer and non-cancerous animals, BPDE-protein adducts were measured on a subset of liver, bladder and uterus cell lysate samples from female animals (SW110817, SW110866, Z11-04-03-012, Z11-04-12-014, 11-258-Zc). Significant variability was observed in duplicates of some samples however, for statistical reasons given the small n, all data were used. No significant difference occurred between cancer and non-cancerous animals when tissues were compared individually (T=3.18; n_{nc}=3, n_c=2; p = 0.16 in livers; T=3.18; n_{nc}=3, n_c=2; p = 0.35 in bladders; T=4.30; n_{nc}=2, n_c=2; p = 0.29 in uterus;). However, a statistical difference was observed when the data from all the tissues were pooled within each group (T=2.18; n_{nc}=8, n_c=6; p = 0.015; Table 6; Figure 18). BPDE-protein adduct levels were not significantly different between tissues (F = 0.64; df between groups= 2, df within groups = 11; p = 0.55; Figure 19).

BPDE-DNA Adducts

DNA adducts were detectable in all of the samples except 6 (3 out of 13 for livers (SW120235, CSL10305, CSL10208); 3 out of 12 for bladders (CSL10208; CSL10192, CSL10281). Adduct levels were not significantly different between cancerous and non

cancerous animals for any of the tissues or for all tissues combined (T=2.07; n_{nc}=n_c=12; p = 0.25 for all tissues combined; T=2.31; n_{nc}=6, n_c=4; p = 0.25 for livers; T=2.37; n_{nc}=4, n_c=5; p = 0.38 for bladders; T=3.18; n_{nc}=2, n_c=3; p = 0.20 for uterus; Table 7; Figure 20). Mean BPDE-DNA adduct levels were not significantly different between tissues (F = 2.61; *df* between groups= 2, *df* within groups = 21; p = 0.1; Figure 21). No significant difference was found between BPDE-protein and DNA adducts for all tissues combined (T=2.06; n_{prot}=n_{DNA}=13; p = 0.86; data not shown).

Correlation Analysis (Table 8)

Pollutant Levels and Gene Induction

No correlation was observed between the gene induction levels and CoPlanar PCBs (R=0.22, R^2=0.05, p=0.54 in w/w; R=0.54, R^2=0.29, p=0.11 l/w; data not shown), total PCBs in w/w (R=0.31, R^2=0.09, p=0.39; data not shown), tPesticides in w/w (R=0.30, R^2=0.09, p=0.39; data not shown) and total Pollutants (tPollutants) in w/w (R=0.31, R^2=0.09, p=0.39; data not shown). There was a slightly significant positive correlation between the gene induction levels and tPCBs, tPesticides and tPollutants (in l/w) (R=0.58, R^2=0.34, p=0.08 for tPCBs; R=0.62, R^2=0.39, p=0.055 for tPesticides and R=0.62, R^2=0.39, p=0.055 for tPollutants; Figures 22-24).

Pollutant Levels, Protein Content and EROD Activity

No correlation was observed between the protein content or EROD activity and the pollutant levels for CoPlanar PCBs, total PCBs, total Pesticides (tPesticides) and all pollutants combined (w/w and l/w) (protein content: R=0.09, R^2=0.01, p = 0.81 for cPCBs w/w; R=0.43, R^2=0.19, p = 0.21 for cPCBs l/w; R=0.21, R^2=0.04, p = 0.36 for

tPCBs w/w; R=0.48, R^2=0.23, p = 0.16 for tPCBs l/w; R=0.06, R^2=0.003, p = 0.87 for tPesticides w/w; R=0.40, R^2=0.16, p = 0.25 for tPesticides l/w; R=0.07, R^2=0.01, p = 0.84 for tPollutants w/w; R=0.42, R^2=0.18, p = 0.23 for tPollutants l/w; data not shown; EROD Activity: R=0.32, R^2=0.10, p = 0.36 for cPCBs w/w; R=0.49, R^2=0.24, p = 0.15 for cPCBs l/w; R=0.42, R^2=0.18, p = 0.22 for tPCBs w/w; R=0.53, R^2=0.28, p = 0.12 for tPCBs l/w; R=0.13, R^2=0.02, p = 0.71 for tPesticides w/w; R=0.40, R^2=0.16, p = 0.25 for tPesticides l/w; R=0.17, R^2=0.03, p = 0.64 for tPollutants w/w; R=0.43, R^2=0.18, p = 0.23 for tPollutants l/w; Figures 25-32).

Gene Induction, Protein Content and EROD Activity versus TEQs

There were no significant correlations between the gene induction, protein content or EROD activity and the TEQs (gene induction: R = 0.32, R^2 = 0.10, p = 0.36; data not shown; protein content: R = 0.10, R^2 = 0.01, p = 0.78; data not shown; EROD activity: R = 0.32, R^2 = 0.10, p = 0.37; Figure 33).

EROD Activity, Protein Content Levels and *CYP1A* Gene Induction

There was a positive correlation between the EROD activity and the *CYP1A* fold induction levels (R=0.86; R^2=0.74; p = 0.001; Figure 34) and between the EROD activity and the CYP1A1 protein content (R=0.60; R^2=0.35; p = 0.05; Figure 35). Similarly, there was a slightly positive correlation between the CYP1A protein content and *CYP1A* induction levels (R=0.59; R^2=0.35; p = 0.07; Figure 36).

Pollutants Levels, TEQs and BPDE-Protein Adducts

Using logarithmic regressions, slightly significant inverse correlations were observed between cPCBs (l/w) and protein adducts in livers (R = 0.99, R^2 = 0.99, p =

0.07; Table 8; Figure 37) and bladders (R = 0.99, R^2 = 0.98, p = 0.09; Table 8; Figure 38), tPCBs (l/w) and protein adducts in uterus (R = 0.99, R^2 = 0.99, p = 0.07; Table 8; Figure 39) and TEQs and protein adducts in livers (R = 0.99, R^2 = 0.98, p = 0.08; Table 8; Figure 40). There were significant inverse correlations between tPCBs (l/w) and protein adducts in livers (R = 1, R^2 = 1, p = 0.003; Table 8; Figure 41) and bladders (R = 1, R^2 = 1, p = 0.01; Table 8; Figure 42).

Pollutant Levels, TEQs and BPDE-DNA Adducts

No significant correlations were observed between pollutant levels or TEQs and BPDE-DNA adducts (Table 8).

Gene Induction, Protein Content and EROD Activity versus BPDE-Protein Adducts

Using logarithmic regressions, slightly significant inverse correlations were observed between the *CYP1A* gene induction and BPDE-protein adducts in livers (R=0.996; R^2=0.99; p = 0.058; Table 8; Figure 43) and bladders (R=0.99; R^2=0.99; p = 0.08; Table 8; Figure 44), between protein content and BPDE-protein adducts in uterus (R=0.99; R^2=0.98; p = 0.09; Table 8; Figure 45) and EROD activity and BPDE-protein adduct levels in uterus (R=0.996; R^2=0.99; p = 0.058; Table 8; Figure 46). A significant inverse correlation was found between CYP1A protein content and BPDE-protein adducts in bladders (R=0.99; R^2=0.97; p = 0.02; Table 8; Figure 47) and between EROD activity and BPDE-protein adducts in bladders (R=0.97; R^2=0.94; p = 0.03; Table 8; Figure 48).

EROD Activity versus BPDE-DNA Adducts

All data with reported values below the detection levels were removed from the data set to prevent bias in the analysis (3 out of 13 for livers (SW120235, CSL10305, CSL10208); 3 out of 12 for bladders (CSL10208; CSL10192, CSL10281,) none for the uterus) as were the data for the heavily metastasized liver from sample SW120008. No statistically significant correlation was observed between EROD activity and BPDE-DNA adduct levels for any of the tissues despite an apparent relationship between EROD activity and BPDE-DNA adducts in uterus ($R=0.25$; $R^2=0.06$; $p = 0.56$ for livers; Spearman Rank $=-0.21$; $p = 0.68$ for bladders (not shown); $R=0.98$; $R^2=0.96$; $p = 0.13$ for uterus; Table 8; Figures 49-50).

BPDE-Protein and DNA Adducts

There was a significant inverse correlation between BPDE-protein adducts and DNA adducts with levels of protein adducts increasing as DNA adducts decreased in the uterus ($R=0.98$; $R^2=0.96$; $p = 0.02$; data not shown).

CHAPTER 4

DISCUSSION

Comparative Analysis

Pollutant Levels in the Coastal California Waters

CYP450 comprises a superfamily of proteins, a number of which are known to metabolize xenobiotics and anthropogenic pollutants. Different families of the enzyme are transcriptionally induced and metabolized by different classes of pollutants. The CYP1A family metabolizes pollutants such as CoPlanar PCBs and PAHs. The CYP2B family typically metabolizes non-planar PCBs and chlorinated pesticides such as DDT (Troisi and Mason, 1997). Finally, CYP3A characteristically metabolize common drugs (Thummel and Wilkinson, 1998). Although it is difficult to directly compare levels and sensitivity of inducibility between the different CYP families, based upon the finding that there is a significantly higher level of CYP1A enzymatic activity relative to CYP2B and CYP3A activities measured using EROD, PROD and BROD fluorometric assays respectively, we tentatively conclude that CA sea lions are likely exposed to PAHs to a higher degree than to steroids, common drugs, chlorinated pesticides and ortho PCBs. POPs such as PCBs, chlorinated pesticides and PAHs originate from a variety of sources whether it is atmospheric from fossil fuel combustion (forest fires, vehicular or industrial emissions), from stormwater and urban or agricultural runoff or from maritime activity (Hartwell, 2008). Two of the more contaminated sites on the west coast are the Palos

Verdes peninsula and the San Francisco Bay area. Concentrations of tDDT measured at various sites around the Palos Verdes peninsula ranged from 0.6 ng/L to 15.8 ng/L (particulate + dissolved phase) and concentrations of tPCBs collected at the same sites ranged from 0.14 ng/L to 1.14 ng/L (Zeng et al., 1999). tPAH concentrations obtained at 4 different sites along the Southern California coast were Ballona Creek estuary: 29.8 ng/L, Los Angeles Harbor: 146±46.1 ng/L, Upper Newport Bay: 8.9 ng/mL and San Diego Bay: 97±45.9 ng/mL (particulate + dissolved phase; Sabin et al., 2010). Median total PAH concentrations found in the water column of various sites in the San Francisco Estuary contained levels of PAHs ranging from 7 to 120 ng/L (particulate +dissolved phase; Ross and Oros, 2004). PAHs are known to be a potent inducer of *CYP1A* (Kantoniemi et al., 1996) in marine mammals (Troisi and Mason, 1997) such as pinnipeds. It is therefore not surprising to see high levels of CYP1A activity in the animals collected from both Northern and Southern California. Despite the high levels of ambient PAHs found in the urban coastal waters of California, no detectable concentrations of PAHs were observed by GC-MS analysis in any of the blubber samples. It is well documented that PAHs are rapidly metabolized and eliminated by the phase 1 (CYP450 type) and phase 2 (GST-, UGT- type) enzymes such that the tissue residue levels are often below detection limits (Martineau et al., 1985). Evidence for PAH exposure in the current study can be observed, however, by the presence of BPDE-protein and -DNA adducts (Boysen and Hecht, 2003). The efficient metabolism and elimination of PAHs therefore effectively decouples CYP1A content and activity from the body burden of the compound, which may partially explain why we did not observe

any correlations between the CYP450 protein content/EROD activity and the concentrations of xenobiotics in any of the tissues that would otherwise be predicted.

There is some indication for PAH exposure being linked to carcinogenesis in free ranging belugas from the St. Lawrence Estuary (Martineau et al., 2002; De Guise et al., 1994). We see no evidence for this relationship in our study, however the sample size of our study population was inherently small and our evidence for prior PAH exposure was based upon indirect evidence and inference from the presence of BPDE-adducts. Our conclusion is therefore tentative that CYP1A activity does not correlate strongly with carcinogenesis and that the formation of cancer may be due to other extrinsic factors such as disease, immune deficiency, immunogenetic factors, as previously described or factors inherent to the individual that have not been taken into account in this study (age, gender, location, blubber thickness, etc.). Our study also showed that the tPCB levels in animals with cancer were not significantly different than those in animals without cancer. This contradicts previous results published by Ylitalo et al. (2005) demonstrating that blubber tPCB levels were associated with carcinoma formation in CA sea lions. The mechanism by which PCB causes cancer is unknown although previous studies have indicated that PCBs induce immune deficiency (Brouwer et al., 1989; Ross et al., 1996b), previously postulated as a possible cofactor in the induction of carcinogenesis.

Blubber thickness highly affects pollutant concentrations detected in this tissue and therefore is arguably one of the most confounding factors inherent to the individual. Blubber thickness and xenobiotic concentrations vary greatly with gender, age, food availability, seasonally (during lactation, pupping, moulting, migration), and with health.

The relationship between xenobiotic concentrations in the blubber and those of the environment is therefore tenuous and affected by a large number of variables, which are unique to the individual. While certain studies have attempted to take the relative contribution of each of these variables on xenobiotic loading into account (Ylitalo et al., 2005), this was beyond the scope of the present study which had very little background on the history of the sampled animals. tDDTs were not significantly different between the cancer and non-cancer group, which is consistent with previous results (Ylitalo et al., 2005).

History of Pollutant Burden in CA Sea Lion Blubber

Pollutant levels measured in the blubber of CA sea lions for this study (Table 2) were compared to levels found in the blubber of CA sea lions originating from similar locations along the California coast and reported in previous studies (Le Boeuf et al., 2002; Blasius and Goodmanlowe, 2008). Le Boeuf et al. (2002) reported mean tDDT levels similar to, and mean tPCB levels approximately 2-fold higher than the ones found in this study. Blasius and Goodmanlowe (2008) reported mean levels of tDDT and tPCB 3 fold higher than the ones found in this study, which is consistent with the overall decline in the concentrations of both classes of compounds previously reported in the literature (Le Boeuf et al., 2002). Even though tPCB concentrations are declining to levels lower than those previously reported to be associated with cancer, they are still relatively high and have not yet reached concentrations that are considered to represent minimal risk in promoting cancer (Ylitalo et al., 2005).

TEQs

CoPlanar PCB TEQs were calculated based on TEFs (van den Berg et al., 2006). The blubber TEQ value found in this study was 1836 ± 4177 (Mean ± S.D.) ng/kg (in l/w). This is approximately 6-fold higher than the lowest observed adverse effect level (LOAEL) reported to reduce vitamin A, NK cell activity, and lymphocyte proliferation in harbor seals fed herring from the Baltic Sea highly contaminated with PCBs and DDTs (Kannan et al., 2000). Deleterious effects were also noted in harbor seals fed PCB-contaminated fish from the Wadden Sea. These individuals had significantly reduced levels of plasma retinol, total thyroxine (tT4), free thyroxin (FT4) and triiodothyronin (tT3) compared to harbor seals fed fish from the relatively less contaminated Atlantic Ocean (Brouwer et al., 1989). As discussed previously, T lymphocytes are capable of recognizing tumor specific antigens and exercise cytotoxicity towards cancer cells and NK cells also share this function. NK cells act mainly against tumor and virus-infected cells. Cancers, especially virus-associated cancers, are most frequent in immunosuppressed individuals (Catros-Quemener et al., 2003; Allison, 1977). Bacterial and viral infections have also previously been associated with urogenital carcinoma in CA sea lions (Johnson et al., 2006; Buckles et al., 2006; 2007; King et al. 2002; Lipscomb et al., 2000). Immune function was not determined in the current study, but based upon the evidence in the literature, a causal relationship between xenobiotic exposure and immunodeficiency leading to tumorigenesis in CA sea lions is clearly plausible.

EROD Activity in Other Species

There have been numerous attempts to relate the difference in CYP enzymatic expression and enzymatic activity both intra- and interspecifically (Lin and Lu, 2001; Chiba et al., 2002; Snawder and Lipscomb, 2000). In humans for example, there was a 36 fold difference between the lowest and highest reported CYP1A protein content values, although it should be acknowledged that these results were obtained using liver microsomes of humans with different health assessments (Snawder and Lipscomb, 2000). These intrinsic differences complicate comparative assessments in estimating the threshold levels of EROD activity necessary to cause adverse effects (Snawder and Lipscomb, 2000; Gelboin, 1980). Nyman et al. (2000) studied the expression and inducibility of *CYP1A* in ringed seal (*Phoca hispida*) from the Baltic Sea and Svalbard and grey seals (*Halichoerus grypus*) from the Baltic Sea and Sable Island (Canada) and observed a species difference in EROD activity between the grey seals from the Sable Island reference site and the ringed seals from Svalbard, also considered a reference site. These interspecies differences may make it difficult to generate meaningful comparative analysis of EROD activity levels, TEQs, no observed adverse effect level (NOAEL) or LOAEL, certainly if the ultimate goal is to equate carcinogenesis potential.

The mean EROD activity measured in CA sea lions was 2.98 ± 4.37 nmol of metabolites formed/min/mg of microsomal protein which is approximately one order of magnitude higher than what has previously been found in Largha seals (*Phoca largha*) and Ribbon seals (*Phoca fasciata*) from Hokkaido, Japan (Chiba et al., 2002) and Baikal seals from Lake Baikal (Hirakawa et al., 2007). There have been documented cases of

early stage carcinomas in Largha seals (Honma et al., 2000) but whether the particularly high prevalence of cancer noted in stranded CA sea lions (18%; Gulland et al., 1996) is indexed to the high levels of EROD activity is uncertain. Certainly, high levels of cancer-associated death have been shown in PAH and PCB exposed (Martineau et al., 2002) beluga whales from the St. Lawrence Estuary having levels of EROD activity (87 pmol/mg/min) at least an order of magnitude lower than those found in CA sea lions from the current study (McKinney et al., 2004).

BPDE-DNA and Protein Adduct Formation

Our data indicate that the formation of BPDE-protein and -DNA adducts can occur in a variety of tissues and is not necessarily localized to those showing elevated levels of xenobiotic metabolism. We were unable to report our results in the units commonly reported in the literature due to the manufacturer's inability to provide us with calibrated and certified values for the number of BPDE modifications that occurred per unit mass of DNA in their standard. Interestingly, elevated levels of BPDE-protein adducts but not DNA adducts were associated with animals exhibiting cancer. Although protein adducts are not causally linked to carcinogenesis, they have been frequently used as a biomarker of carcinogenic exposure because of their high abundance and the fact they do not undergo enzymatic repair. They may therefore provide for a better index of chronic or intermittent exposure to carcinogens than DNA adducts (Skipper et al., 1994). The lack of correlation between BPDE-DNA adducts and carcinogenesis may also be due to the inherent lack of sensitivity of the technique. Indeed, many of the samples failed to

register BPDE-adducts above the detection limits of the assay and had to be excluded from the analysis.

Gene Induction and Pollutant Levels

Xenobiotic exposure directly induces the activation of the gene transcription through the XRE (Revel et al., 2003); thus, we anticipated a strong correlation between pollutant exposure and gene induction. There were slightly significant relationships between the *CYP1A* gene induction levels and tPCBs, tPesticides and tPollutants when reported in lipid weights. The correlations observed in this study are consistent with previous studies demonstrating this relationship in other animals. In male Atlantic cod from the North Sea, a similar significant correlation was observed between the pollutant exposure to PCBs, PBDEs, perfluorooctanesulfonate (PROs) and PAHs and gene induction levels (Bratberg et al., 2013). In the Baikal seal, the total TEQs comprising of polychlorinated dibenzodioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), mono-ortho PCBs and non-ortho PCBs was strongly positively correlated to the ratio of *CYP1B1/CYP1A1* mRNA expression levels (Hirakawa et al., 2007). We did not observe a similar correlation with CoPlanar PCBs and TEQs, which reflect the exposure to dioxin-like compounds known to be the most potent inducers of the AhR and *CYP1A.* The absence of a correlation with CoPlanar PCBs and TEQs implies that associated co-localized PAHs might be the primary inducer of *CYP1A* in CA sea lions, even though PAHs were undetected in the chemical analyses.

CYP1A Protein Content, EROD Activity and Gene Induction

Western immunoblot (WB) and RT-PCR analyses allowed the determination of protein content and mRNA expression levels respectively. In the WB, the Supersome expressed a band at a slightly larger molecular weight (MW) than the CYP1A band in our samples, which is not surprising given the species differences (rat versus CA sea lion). The anticipated MW region for CYPs can be anywhere from 47.5 to 62 kDa (Chiba et al., 2002). Some cross-reactivity with the CYP1A1 antibody was observed below the CYP1A1 band, which has previously been attributed to cross-reactivity with CYP1A2, a closely related family member with a slightly smaller MW (Chiba et al., 2002). As anticipated, there was a positive correlation between EROD activity and CYP1A protein content as has been observed in both Largha and Ribbon seals (Chiba et al., 2002). There was also a positive correlation between protein content and gene induction as has previously been observed in Baikal seals (Hirakawa et al., 2007). Taken collectively, these results confirmed that the induction of *CYP1A* mRNA is correlated with the CYP1A protein content, which in turn, is correlated with CYP1A enzyme activity.

EROD Activity, Protein Content and Pollutant Levels

Correlations between pollutants levels and EROD activity have been observed in harbor seals (Troisi and Mason, 1997) and Largha seals (Chiba et al., 2002) but not in ribbon seals (Chiba et al., 2002). Contrary to gene induction, EROD activity and CYP1A protein content in CA sea lions did not correlate significantly with the concentration of any of the pollutants measured (tCoPlanar PCBs, tPCBs, tPesticides and TEQs). Although one would anticipate that the *CYP1A* gene response level should be related to

upstream xenobiotic exposure as measured by POP residues as well as downstream protein content and EROD activity, it appears that the relationships are lost as we start to correlate more distal events on the proposed carcinogenic cascade and the cause-effect associations become more tenuous. Perhaps more significantly, as described previously, we suspect that the primary xenobiotic inducers in CA sea lions are PAHs. These are undetectable in the tissues in the chemical analyses due to their rapid metabolic elimination and therefore cannot be used in order to establish any causative correlation. Their presence as the inductive culprit is nonetheless captured in the form of metabolite adducts conjugated to proteins and DNA.

Pollutant Levels, Gene Induction, Protein Content and EROD Activity versus BPDE-DNA and Protein Adduct Formation

Given the relationship between exposure to PAHs and induction of the CYP1A enzyme, we predicted a correlation between the CYP1A activity and BPDE-protein and -DNA adduct formation. Our data show no significant correlations between the BPDE-DNA adducts and pollutants, *CYP1A* gene induction, protein content or EROD activity in any of the tissues. Evidence for a relationship between CYP1A activity and DNA adduct formation in the literature is equivocal. Aas et al. (2000) showed a positive correlation between DNA adducts and pollutant exposure in Atlantic cod exposed to crude oil but Lyons et al. (2000) found no correlation between EROD activity and DNA adducts in dab, a fish found in polluted sites of the British coastal waters. In humans, DNA adduct levels in lungs are significantly elevated in cigarette smokers compared to non-smokers (Phillips, DH, 2002). This relationship is not surprising, given the dosages of PAHs applied directly to the lung epithelia however, smoking also increases associated DNA

adducts in the cervix of women (Simons et al., 1993; 1995; Ali et al., 1994) implying that there is systemic distribution of these compounds to distant tissues. Whether the uterus has tissue specific characteristics that make it susceptible to this distal interaction is unknown but, it is probably worth noting that CA sea lions appear to be particularly prone to carcinomas of urogenital origin (Ylitalo et al., 2005) and that there is a tenuous positive relationship between all pollutant levels measured, gene induction, protein content as well as EROD activity, and BPDE-DNA adducts in the uterus of females in the current study, a relationship that is not observed for any of the other tissues taken individually.

In this study, we observed significant inverse correlations between the tPCBs (l/w) and BPDE-protein adducts in livers and bladders as well as between protein content and EROD activity and BPDE-protein adducts in bladders. We also observed slightly significant inverse correlations between cPCBs (l/w), tPCBs (l/w), TEQs, gene induction, protein content and EROD activity and BPDE-protein adducts in various tissues as shown in Table 8. To the best of our knowledge, this inverse correlation has never been observed before and is therefore a novel finding. This result was not expected and it implies that increased primary metabolism by CYP1A through xenobiotic exposure may reduce the prevalence of metabolite conjugated protein adducts in these animals. The mechanisms in these CA sea lions that could cause the selective and highly efficient elimination of proteins damaged through the activation of the xenobiotic metabolic pathway are unknown, but increased clearance of proteins has been observed in response to exposure to dithiolethiones, which act as indirect antioxidants (Kwak et al., 2003). In

this instance, the antioxidant compound appears to operate at the transcriptional level through the tandem antioxidant response element in the proximal promoter of the 20S subunit of the proteasome complex. Expression of the 20S subunit appears to be the rate-limiting step in the construction of the active 26S proteasome. Whether there is a similar protective effect caused by certain xenobiotics inducing the proteosomal degradation pathway in CA sea lions is unknown, but is suggested in our current findings.

Temporal displacement is another potentially confounding factor in explaining the negative correlations observed between BPDE-protein adducts and the other biomarkers of xenobiotic stress. There is a lack of mechanistic understanding of the turnover rates for many of the components of the carcinogenic cascade. For example, the turnover rates of *CYP1A* induction and EROD activity are known to be much higher than for DNA adducts. Ericson et al. (1999) showed that EROD activity was significantly increased 21 days after the first exposure and up to 9 days post-cessation (or 59 days after the first exposure) with a significantly marked decline 16 days post exposure in experimentally exposed northern pike (*Esox lucius*) to BaP, benzo(k)fluoranthene and 7H-dibenzo(c,g)carbazole. On the other hand, DNA adduct formation in the liver reached their highest expression levels 66 days after the first exposure (or 16 days post-cessation) and remained high long after the cessation of exposure to pollutants (up to 78 days post-cessation or 128 days after first being exposed). The delay in the appearance of protein adducts relative to the exposure event are largely unknown but it appears that the turnover rate of the protein adducts is much slower than that for DNA adducts (Skipper et al., 1994). It is therefore conceivable that the early inductive events, indicated by *CYP*

induction and activity, may be decoupled in time from the ultimate products of the metabolic pathway. Ultimately, even with exact knowledge of the varying turnover rates in each component in the cascade, a rationalization of the observed negative correlations with BPDE-protein adducts would be impossible because of the unknown exposure history of these wild stranded animals.

Limitations

The ability of this study to make definitive statements on the effects of xenobiotics on tumorigenesis in CA sea lions is limited primarily because of the sample size and the absence of any data on the exposure history of the sampled animals. These inherent limitations were understood at the initiation of the project but could not easily be rectified without unreasonably expanding the scope and duration of the present study beyond the requirements of a Master's thesis.

One of the key principles of establishing cause and effect is temporal precedence; proving that the effects seen in the study happened after and as a consequence of the cause. Another is the ability to distinguish, within a sample set, those individuals in the treatment and those in the control group. Having a known control or baseline group enables one to determine intrinsic variability and the thresholds of detection that are necessary for meaningful comparisons and conclusions to be drawn. Given that this study was done on a transient species from the wild, we could not readily account for a multitude of variables, which are likely to confound interpretation of the data. Indeed, within all the samples it was difficult to ascertain a single individual that, based upon the longitudinal profile of the studied biomarkers, could be classified as a calibrant control.

Sample CSL10322 showed low levels of tissue residue burden as well as the lowest levels of *CYP1A* gene induction and EROD activity but simultaneously, this sample also showed some of the highest levels of BPDE-DNA adducts. While this single case exemplifies the difficulties of working with field exposed animals, the concepts outlined in this thesis do provide a practical framework for more elaborate, better controlled studies involving a larger sample size.

Conclusions

Although there have been a number of published studies that tie cancer to xenobiotic exposure in pinnipeds including CA sea lions, to the best of our knowledge this is the first study performed on any pinniped species that attempts to ascertain the strength of the correlative relationships of each of the individual components in a pollutant induced carcinogenic cascade to cancer. Thus, isolated studies have measured blubber pollutant levels in various pinnipeds (Blasius and Goodmanlowe, 2008; Le Boeuf et al., 2002). Similarly, EROD activity and CYP1A protein content has been studied in various seal species (Nyman et al., 2000; Chiba et al., 2002) as well as *CYP* gene induction in Baikal seals (Hirakawa et al., 2007). A longitudinal study of the effects of chlorinated dioxins and related compounds (DRC) on inflammation and DRC-induced oxidative stress was conducted by Hirakawa et al. (2011) on Baikal seals but thus far, no single longitudinal study in pinnipeds has ever been conducted with the expressed intent of studying the xenobiotically-related factors implicated in a carcinogenic cascade in this group of organisms. Prior studies of this nature have been conducted on beluga whales from the St. Lawrence Estuary (Martineau et al., 2002) however, and based upon their

findings it was hypothesized that a similar cascade of biomarkers would be evident in CA sea lions exhibiting cancer.

Despite the prediction of correlative relationships informed by this earlier study, the current research project did not provide any strong evidence for causal linkages between xenobiotic exposure and carcinogenesis in CA sea lions. Each component of the cascade has inherent variability and we chose to measure only those responses that seemed to be the most compelling in providing a direct route between cause and effect. In the final analysis it should be acknowledged that it would have required extremely strong correlative linkages between each adjacent component in the cascade for a correlation to exist between the ends of the chains.

One of the major limitations of our experimental approach was the assumption that PAH exposure, something we could not measure by direct GC-MS analysis, could be ascertained by proxy from the signatures of more resistant chlorinated xenobiotics such as DDTs and PCBs. These compounds coexist in the urbanized waters of Southern California and San Francisco Bay but the strength and the variability of the stoichiometric relationship between these compounds in the environment is unknown. Our linkage from PAH exposure, the predicted primary effector at one end of the cascade, to BPDE-DNA adducts, the putative causal agent for cancer at the other end, was therefore tenuous.

The action of PAH exposure in causing cancer is well established. In humans, DNA adducts are much higher in lungs (Phillips, DH, 2002) and cervix (Simons et al., 1993; Simons et al., 2005; Ali et al., 1994) of smokers than non-smokers which correlates

with susceptibility to cancer. There is also suggestive evidence of PAH linked carcinomas in beluga whales (Martineau et al., 2002; De Guise et al., 1994). Based on these findings we anticipated that CA sea lions with cancer would demonstrate higher levels of CYP1A enzymatic activity, protein content and gene induction, BPDE-DNA and -protein adducts than animals without cancer, even if PAH residues could not be measured in the tissues. While the levels of BPDE-DNA adducts were generally higher in the tissues of animals with cancer than those without, the only statistically positive association with cancer was with BPDE-protein adducts when all tissues were combined. There is no known mechanistic linkage to show protein adducts induce cancer, therefore this is a novel, if unexplainable, finding. Unlike DNA adducts, protein adducts do not undergo excision and DNA repair and are thought to have a slower turnover rate. They may therefore be better biomarkers for cancer than DNA adducts.

Stowers and Anderson (1985) have suggested that the disappearance rates of BPDE-DNA adducts may vary in tissues and may be a reflection of, not only enzymatic excision repair, but cell turnover as well. In mice livers, a high rate of excision repair and a low rate of DNA synthesis may explain why BaP induced cancers are low in this tissue while the opposite occurs in lungs possibly rendering this tissue more susceptible to mutations and carcinogenesis. It would be interesting to determine the rates of DNA excision, turnover and synthesis in the uterus of CA sea lions since the majority of cancers in these animals are of suspected urogenital origin (Ylitalo et al., 2005).

Our inability to analyze for PAH-residues may also account for the lack of any strong correlations between non-ortho PCBs and EROD activity in our study since these

compounds are known to induce *CYP1A*. Nyman et al. (2000) reported significantly higher levels of EROD activity in ringed and grey seals from the Baltic Sea, exposed to high levels of *CYP1A* inducing pollutants, than in seals from less polluted Svalbard and Sable Island. Although no significant relationships were observed with EROD activity, marginally significant correlations were observed between the *CYP1A* gene induction levels and tPCBs (l/w), tPesticides (l/w) and tPollutants (l/w), again suggesting that while there are direct relationships between adjacent components on the xenobiotic-cancer cascade these correlations become increasingly tenuous as one compares components that are separated by one or more steps where intervening cellular or biochemical control mechanisms are possible.

One of the unexpected and intriguing findings of the current study were the negative correlations observed between pollutants, TEQs, gene induction, and EROD activity and BPDE-protein adducts. Why these factors should be negatively correlated, while the levels of BPDE-protein adducts are generally higher (although not statistically significant because of the high variances except when all tissues are pooled) in animals having cancer, is unknown. As mentioned previously, it is possible to speculate that this effect may be due to the interpretational complexities caused by the temporal misalignment of the various components in the system or, more provocatively, a response caused by a yet undiscovered pathway in which xenobiotics increase protein turnover by proteosomal activation. Further studies involving carefully controlled experiments will need to be conducted to verify these findings and elucidate the underlying mechanisms that may be at play.

Although we were unable to verify the hypothesis that the xenobiotic metabolism induced pathway was the primary mechanism involved in promoting carcinogenesis in stranded CA sea lions, we were able to establish a robust methodology that could be used by others in the future. Our findings were constrained by our small sample size and future studies would also benefit greatly from knowledge of the exposure history of each animal. Analytically the study was also limited by our inability to detect PAHs (and therefore deduce exposure) in the tissues and the poor sensitivity of the BPDE-DNA adduct ELISA assay. Alternative methods for DNA-adduct analysis, with high sensitivity and fidelity do exist including ^{32}P-post labeling, HPLC and fluorescence spectroscopy or mass spectrometry detection (Strickland et al., 1993; Martineau et al., 2002). Adopting one of these more sensitive alternative assays would have undoubtedly allowed us to obtain more accurate and precise data and a better grasp of any relationship between pollutant levels, these DNA adducts and carcinogenesis.

On a positive note we were able to confirm a continuing trend towards a reduced bioaccumulation of PCBs and DDTs in CA sea lions from coastal California waters although the residue levels were still high and above thresholds known to cause immunodeficiency in harbor seals. In future studies, it would be interesting to ascertain if cancerous animals positively correlated for reduced vitamin A and reduced immune function, such as NK cell activity and lymphocyte proliferation. It was also apparent that the observed levels of EROD activity in our sample population of CA sea lions were an order of magnitude higher than those observed in beluga whales from the St. Lawrence Estuary suspected to have pollutant induced cancers as well as Largha and Ribbon seals.

Correlations with cancer may have therefore been anticipated, but as described earlier, intra- and interspecific differences in enzymatic response to xenobiotics make calibration of these biomarkers of carcinogenic susceptibility across species difficult. This could be an interesting avenue to explore further.

The findings of this project were meant to provide evidence-based data for determining the effects of anthropogenic waste on the top trophic level predators in the ecosystem communities of California's marine and coastal region. While this thesis did not achieved this goal, data driven mechanistic research that ties cause with effect still provides the best and most convincing scientific arguments for promoting stewardship and sustainable use of California's coastal resources. Furthermore, definitive studies are necessary in order to convincingly advocate the adoption of better waste management practices to protect top-level predators in the ecosystem communities of California's marine and coastal region. The data derived from these future studies could help EPA and local authorities involved in managing discharges in making well-informed decisions regarding the establishment of threshold levels of lipophilic pollutants with bioamplification potential.

APPENDICES

APPENDIX A

QUALITY ASSURANCE AND QUALITY CONTROL

QUALITY ASSURANCE/QUALITY CONTROL (QA/QC)

Compounds Analyzed

All standards were purchased from a commercial supplier and traceable to NIST. The Custom PCB standard solution (Accustandard, New Haven, Connecticut) contained PCB congeners -3, -8, -31, -33, -56 (-60), -95, -97, - 132, -141, -174, -195, -209 at 100 μg/mL. The PCB custom solution contained the following PCB congeners: -18, -28, -37, -44, -49, -52, -66, -70, -74, -77, -81, - 87, - 99, -101, -105, -110, -114, -118, -119, -123, -126, - 128, -138, -149, -151, -153, - 156, -157, -158, -167, -168, - 169, -170, -177, -180, - 183, - 187, -189, -194, -199, -201, -206 at 20 μg/mL. Both Solutions were combined into a 25 mL volumetric flask and diluted to a final concentration of 800 ng/mL with hexane.

The custom Pesticide solution contained the following pesticides: BHC-alpha, Hexachlorobenzene, BHC-beta, BHC-gamma, BHC-delta, Heptachlor, Aldrin, Heptachlor Epoxide, Oxychlordane, Chlordane-gamma, 2,4'-DDE, Endosulfan I, Chlordane-alpha, trans-Nonachlor, 4,4'-DDE, Dieldrin, 2,4'-DDD, Perthane, Endrin, Endosulfan II, 4.4'-DDD, 2,4'-DDT, cis-Nonachlor, Endrin aldehyde, Endosulfan sulfate, 4,4'-DDT, Endrin ketone, Methoxychlor, Mirex at 100 μg/mL concentration diluted with hexane to a final concentration of 1,000 ng/mL in 100 mL volumetric flask.

The PAH custom solution contained the following 25 compounds: Naphthalene, 2-Methylnaphthalene, 1-Methylnaphthalene, Biphenyl, 2,6-Dimethylnaphthalene, Acenaphthylene, Acenaphthene, 2,3,5-Trimethylnaphthalene, Fluorene, Dibenzothiophene, Phenanthrene, Anthracene, 1-Methylphenanthrene, Fluoranthene,

Pyrene, Benz(a)anthracene, Chrysene, Benzo(b)fluoranthene, Benzo(k)fluoranthene, Benzo(e)pyrene, Benzo(a)pyrene, Perylene, Indeno (1,2,3-c,d)pyrene, Dibenz(a,h)anthracene, Benzo(g,h,i)perylene at 100 μg/mL diluted to a final concentration of 1,000 ng/mL using methylene chloride in 100 mL volumetric flask. Standards were added to the blank spikes and matrix spikes to test for recovery and matrix effect.

Internal Standards/Surrogates

The surrogates standard solution for the PAH were prepared from a custom solution (Accustandard, New Haven, Connecticut) and contained d8-Naphthalene, d10-Acenaphthene, d10-Phenanthrene, d12-Chrysene, and d12-Perylene at 4000 μg/mL concentration diluted to 40,000 ng/mL with methylene chloride in a 100 mL volumetric flask. The surrogates for pesticides (CHCs) were Tetrachloro-m-xylene (TCMX), PCB030, PCB112 and PCB198 at a 2,000 μg/mL concentration (Accustandard, New Haven, Connecticut) diluted to 8,000 ng/mL using hexane in a 250 mL volumetric flask. Recovery surrogates were added to each sample to test for the recovery of the analytes that could be affected by the sample preparation/extraction process. Typically, recoveries should be between 70-130% and average 100%. This goal was met with an average recovery of 109% for CHC surrogates and 108% for PAH surrogates.

The internal standard used for the PAH were deuterated d10-Anthracene (2000 μg/mL, Accustandard, New Haven, Connecticut) and d12-benzo(g,h,i) perylene (1000 μg/mL, Cambridge Isotope Lab, Andover, MA) diluted to a final concentration of 20,000 ng/mL and 10,000 ng/mL respectively using methylene chloride in a 100 mL volumetric

flask. The internal standard used for PCBs and Pesticides were 4,4-Dibromobiphenyl (DBBP) and 2,2',5,5'- Tetrabromobiphenyl (TBBP). 4000 μg of each DBBP and TBBP were diluted in 1mL of hexane and each solution was added to a 100 mL volumetric flask and diluted to a final concentration of 40,000 ng/mL with hexane. The internal standard was added to each sample at a fixed concentration to act as a calibrant to control for changes in conditions and sensitivity of the GC-MS over time.

Instrument Calibration

Calibration solutions traceable to NIST purchased from a commercial supplier were diluted at 6 different concentrations and used to generate the calibration curve.

Continuous Calibration

A continuing calibration verification (CCV) sample containing the custom standard solution of interest, the internal standard and the recovery surrogate solution was analyzed at the beginning of each batch. It was also analyzed at the end of a batch run to verify for differences in sensitivity that occurred during the analysis, possibly due to sample contamination of the ion source in the instrument.

Method Blank

A procedural blank was also extracted to demonstrate that no contaminants were introduced during the sample preparation procedure. The sample blank underwent the same manipulations as all other samples.

Sample Duplicates

Each sample was analyzed in duplicate and matrix spike/matrix spike duplicate (MS/MSD) were also analyzed to measure for the precision and recovery of the

extraction procedure as well as for possible interferences caused by the sample matrix. The relative standard deviation (RSD) was measured and was deemed to be acceptable and within EPA guidelines if 90% of the duplicates had a RSD value below 30%. Reproducibility for this study exceeded these guidelines with 92% of the samples (in l/w) having an average RSD of 11.5%.

Certified Reference Material

A Certified Reference Material (CRM1947) was also analyzed to measure the accuracy of the procedure. The CRM contained known quantities of the majority of the analytes of interest. QA/QC required that analyte recovery should be between 70-130% of the known CRM values. This goal was met at 82% for PCBs and 103% for Pesticides.

EROD Activity

A blank was assayed to demonstrate the lack of activity without the presence of microsomes. A 20 μL sample of Supersome (120 μg of protein content) containing a mixture of rat CYP1A1+P450 reductase was used as a positive control. One sample was analyzed in triplicate to estimate the variance in the procedure and also to determine the detection limits of the assay. A linear relationship between the enzyme expression and the activity was previously demonstrated (Ronis and Walker, 1985).

Western immunoblots

A BIORAD Kaleidoscope ladder (p/n 161-0305) was used to determine the approximate molecular weight of the resolved protein bands and the Supersome was used as a positive control to demonstrate the procedure could identify CYP1A proteins in the gels.

NanoDrop for determining protein concentrations

The NanoDrop instrument determines concentration and quality of DNA and RNA. The 260 nm/280 nm absorbance ratio provides an estimate of DNA or RNA purity relative to protein contamination. The sample concentration in ng/μL is given based on the absorbance read at 260 nm and the selected analysis constant, a value determined based on the type of nucleic acid being analyzed, and is calculated according to Beer's Law, used to correlate the absorbance read with a concentration. The spectrum is normalized at 340 nm, an absorbance value where the absorbance should be very near zero and all spectra are using this zero as a reference.

BPDE-DNA and Protein Adduct Formation

Standards were used to determine DNA Adduct and protein adduct concentrations and the zero concentration of the Reduced DNA Standard was used as an absorbance blank.

APPENDIX B

TABLES

TABLE 1. Sample Description

Sample	Location	Sex	Age	Cancer?
SW110817	San Diego	Female (F)	Adult	Yes
SW120008	San Diego	F	Adult	Yes
SW120235	San Diego	Male (M)	Adult	Yes
11-258-Zc	San Pedro	F	Adult	Yes
CSL10305	Sausalito	M	SubAdult	Yes
CSL10281	Sausalito	M	SubAdult	Yes
Z11-04-03-012	Laguna	F	Juvenile	No
Z11-04-12-014	Laguna	F	SubAdult	No
SW110866	San Diego	F	Adult	No
CSL10192	Sausalito	M	Adult	No
CSL10208	Sausalito	M	SubAdult	No
CSL10219	Sausalito	M	SubAdult	No
CSL10322	Sausalito	M	Juvenile	No

TABLE 2. Percent Lipid and Concentrations (Mean ± S.D.) of tCoPlanar PCBs, tPCBs and tPesticides in Blubber of CA Sea Lions

		ug/g wet weight				ug/g lipid weight		
	n	%Lipid	tCoplanar PCBs	tPCBs	tPesticides	tCoplanar PCBs	tPCBs	tPesticides
Mean±S.D.	12	46.82±20.5	0.68±0.46	7.07±4.3	47.83±32.18	2.17±2.11	25.68±31.17	173.18±217.62
Range (min-max)			(0.138-1.65)	(2.37-15.26)	(5.9-119.15)	(0.28-8.92)	(3.19-119.52)	(13.44-849.18)
Cancer	6		wet weight			lipid weight		
		%Lipid	tCoplanar PCBs	tPCBs	tPesticides	tCoplanar PCBs	tPCBs	tPesticides
Mean±S.D.		44.06±8.69	0.78±0.42	8.22±4.5	56.74±35.24	1.81±0.94	19.07±9.92	130.72±75.83
No Cancer	6		wet weight			lipid weight		
		%Lipid	tCoplanar PCBs	tPCBs	tPesticides)	tCoplanar PCBs	tPCBs	tPesticides
Mean±S.D.		49.58±34.31	0.58±0.51	5.92±4.23	27.42±7.96	2.53±2.93	32.28±43.99	228.39±341.2

TABLE 3. *CYP1A* Gene Fold Induction (in delta delta CT; $\Delta\Delta C_T$) of Animals with Cancer and without Cancer

Sample (Cancer)	Fold Induction (delta delta CT)	Sample (No Cancer)	Fold Induction (delta delta CT)
11-258-Zc	1915	SW110866	1302
CSL10281	146	CSL10322	1
SW120235	97	CSL10192	2766
CSL10305	128	CSL10291	1771
SW110817	12	CSL10208	1876
SW120008	9		

TABLE 4. Cytochrome P450 Protein Content Measured in Liver Microsomes of CA Sea Lions

Protein Content		
	N	Mean±S.D.
Cancer	5	1.32±0.88
No Cancer	6	2.33±1.27

Note. Results are expressed in arbitrary absorbance densitometry units (A.U.)

TABLE 5. EROD Activity Measured in Liver Microsomes from CA Sea Lions

EROD		
	n	Mean±S.D.
Cancer	6	1.97±3.03
No Cancer	6	3.83±5.38

Note. Results are expressed in nmol/min/mg protein.

TABLE 6. BPDA Protein Adduct Levels in Livers, Bladders and Uterus of CA Sea Lions

BPDE Protein Adducts	**All Tissues**		**Livers**		**Bladders**		**Uterus**	
	N	Mean±S.D.	n	Mean±S.D.	n	Mean±S.D.	n	Mean±S.D.
Cancer	6	0.023±0.013	2	0.0193± 0.012	2	0.022±0.021	2	0.013±0.012
No Cancer	8	0.009±0.004	3	0.007±0.003	3	0.009±0.004	2	0.017±0.039

Note. Levels are expressed in μg of protein adducts/μg of protein)

TABLE 7. BPDE-DNA Adduct Levels in Livers, Bladders and Uterus of CA Sea Lions

BPDE DNA Adducts	**All Tissues**		**Livers**		**Bladders**		**Uterus**	
	N	Mean±S.D.	n	Mean±S.D.	n	Mean±S.D.	n	Mean±S.D.
Cancer	12	0.015±0.002	4	0.016±0.002	5	0.016±0.001	3	0.014±0.0009
No Cancer	12	0.016±0.002	6	0.018±0.003	4	0.015±0.002	2	0.016±0.0002

Note. Levels are expressed in μg of DNA adducts/μg of DNA)

TABLE 8. Correlations Between Pollutant Levels, TEQs, *CYP1A* Gene Induction, Protein Content and Enzymatic Activity, BPDE-Protein and -DNA Adducts

	cPCBs w/w	cPCBs l/w	tPCBS w/w	tPCBs l/w	tPesticides w/w	tPesticides l/w	tPollutants w/w	tPollutants l/w	tTEQs l/w	Gene Induction	Protein content	EROD
cPCBs w/w	-	-	-	-	-	-	-	-	-	-	-	-
cPCBs l/w	-	-	-	-	-	-	-	-	-	-	-	-
tPCBS w/w	-	-	-	-	-	-	-	-	-	-	-	-
tPCBs l/w	-	-	-	-	-	-	-	-	-	-	-	-
tPesticides w/w	-	-	-	-	-	-	-	-	-	-	-	-
tPesticides l/w	-	-	-	-	-	-	-	-	-	-	-	-
tPollutants w/w	-	-	-	-	-	-	-	-	-	-	-	-
tPollutants l/w	-	-	-	-	-	-	-	-	-	-	-	-
tTEQs l/w	-	-	-	-	-	-	-	-	-	-	-	-
Gene Induction	0.22	0.22	0.31	0.58*	0.3	0.62*	0.31	0.62*	0.32	-	-	-
Protein content	0.09	0.43	0.21	0.48	0.06	0.4	0.07	0.42	0.1	0.59*	-	-
EROD	0.32	0.49	0.42	0.53	0.13	0.4	0.17	0.43	0.32	0.86***	0.6**	-
Protein Adduct Liver	-	L.R.(-) 0.99*	-	L.R.(-) 1***	-	L.R.(-) 0.96	-	L.R.(-) 0.97	L.R.(-) 0.99*	L.R.(-) 1*	L.R.(-) 0.51	L.R.(-) 0.42
Protein Adduct Bladder	-	L.R.(-) 0.99*	-	L.R.(-) 1***	-	L.R.(-) 0.95	-	L.R.(-) 0.96	L.R.(-) 0.99	L.R.(-) 0.99*	L.R.(-) 0.99**	L.R.(-) 0.97**
Protein Adduct Uterus	-	L.R.(-) 0.97	-	L.R.(-) 0.99*	-	L.R.(-) 0.92	-	L.R.(-) 0.93	L.R.(-) 0.97	L.R.(-) 0.98	L.R.(-) 0.99*	L.R.(-) 1*
DNA Adduct Liver	(-) 0.44	0.09	(-) 0.43	0.11	(-) 0.42	0.06	(-) 0.43	0.07	0.1	(-) 0.05	(-) 0.33	(-) 0.24
DNA Adduct Bladder	0.2	(-) 0.42	0.11	0.3	0.02	(-) 0.27	(-) 0.03	(-) 0.29	0.18	(-) 0.49	(-) 0.49	S.R. -0.21
DNA Adduct Uterus	0.59	0.87	0.72	0.96	0.43	0.62	0.46	0.67	0.86	0.79	0.92	0.98

*p<0.1; **p<0.05; ***p<0.01 S.R = Spearman Rank L.R. = Log Regression

APPENDIX C

FIGURES

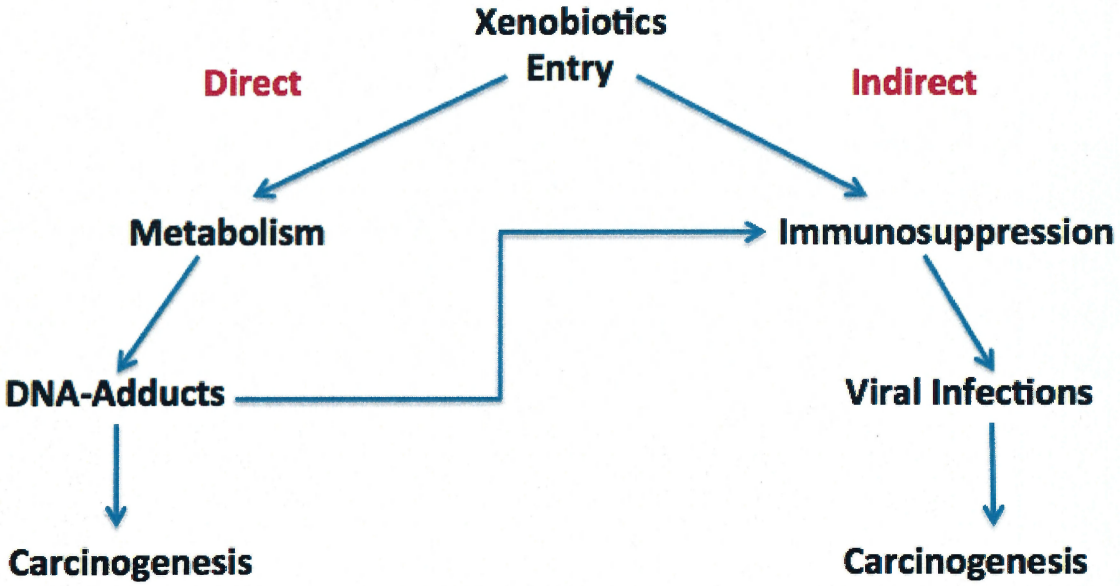

FIGURE 1. Possible indirect and direct modes of interaction of xenobiotics leading to carcinogenesis.

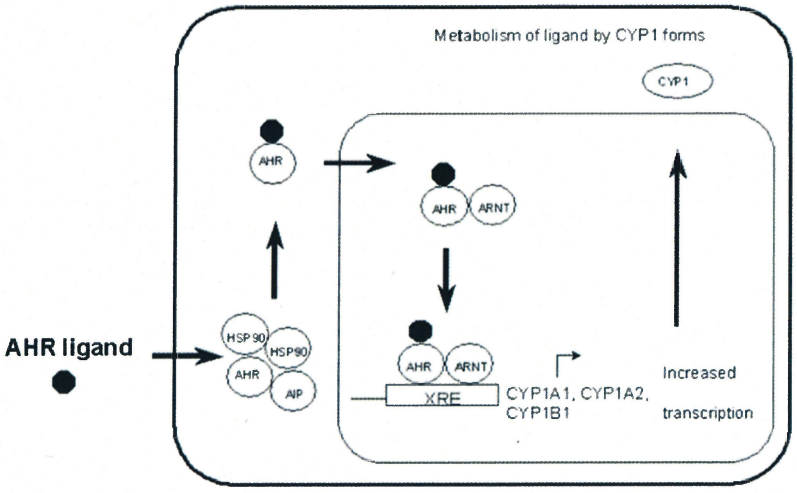

FIGURE 2. Mechanism of activation of the *CYP1A* gene in the cell. *Note*. Used with the permission of Dr. Andrew Z. Mason.

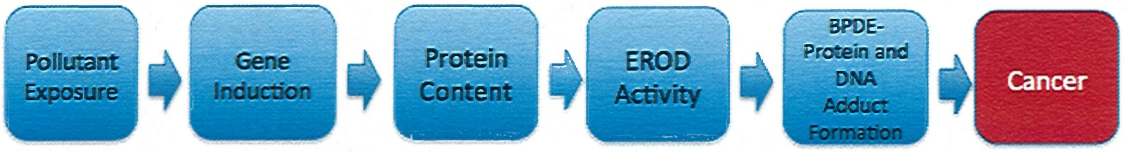

FIGURE 3. Pollutant induced carcinogenic cascade.

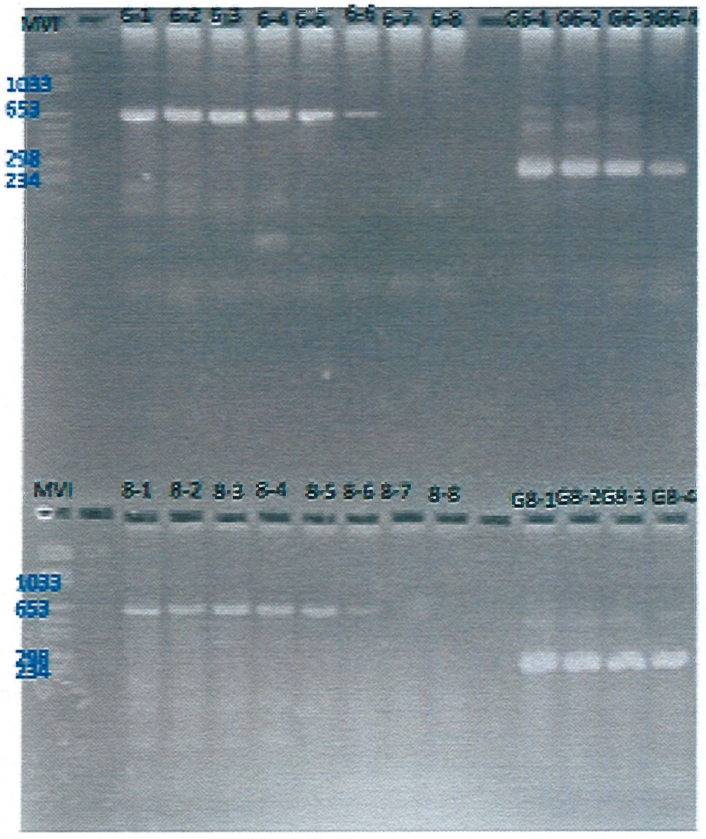

FIGURE 4. Agarose gel on gradient PCR amplification samples. Samples 6-1 to 6-8 were high (SW110866) and samples 8-1 to 8-8 were low (11-258-Zc) DNA content samples incubated with forward and reversed CYP1A primers. Samples G6-1 to G6-4 were high (SW110866) and samples G8-1 to G8-4 were low (11-258-Zc) DNA content samples incubated with forward and reversed GAPDH primers. MVI is for Marker VI (with base pair markers of interest labeled in blue).

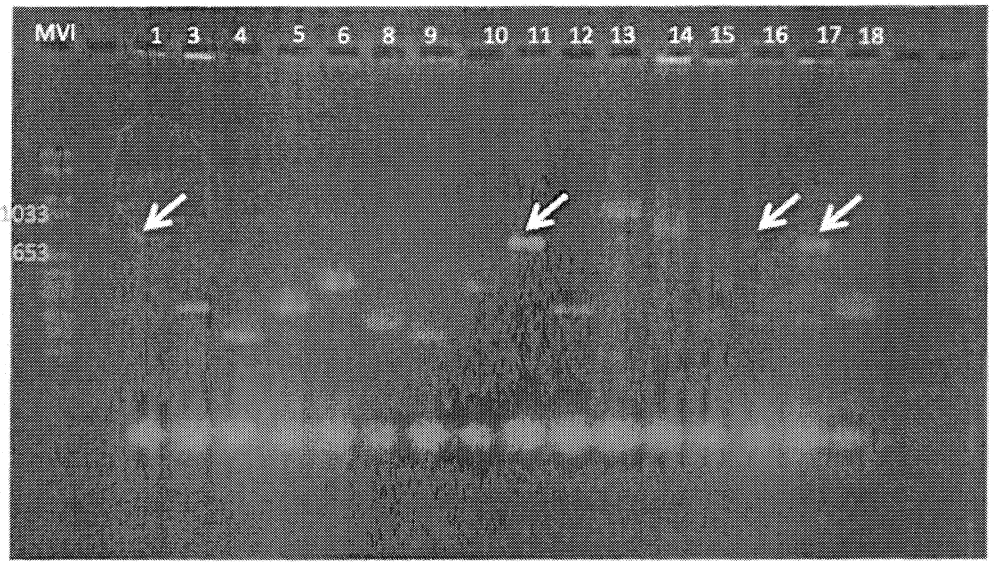

FIGURE 5. Agarose gel on the TOPO TA cloning reaction colonies. Colonies 1, 11, 16 and 17 were located at the expected bp marker (approximately 770 bp). The number in orange are the base pairs at these markers.

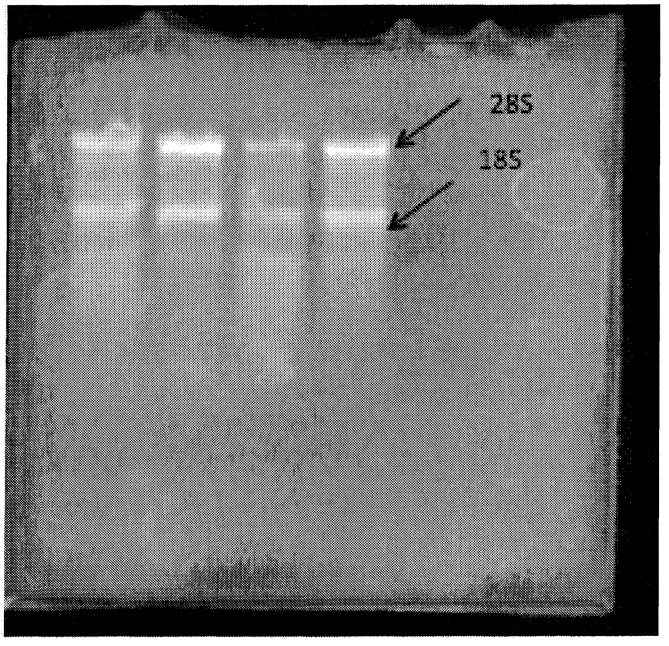

FIGURE 6. RNA gel on a set of Trizol RNA samples. The quality of extracted RNA is assessed by the visualization of the two bands for each sample (from top to bottom: large ribosomes (28 Svedberg (S) rRNA), small ribosomes (18S rRNA) indicating RNA is intact. A smeared appearance would indicate the presence of degraded RNA.

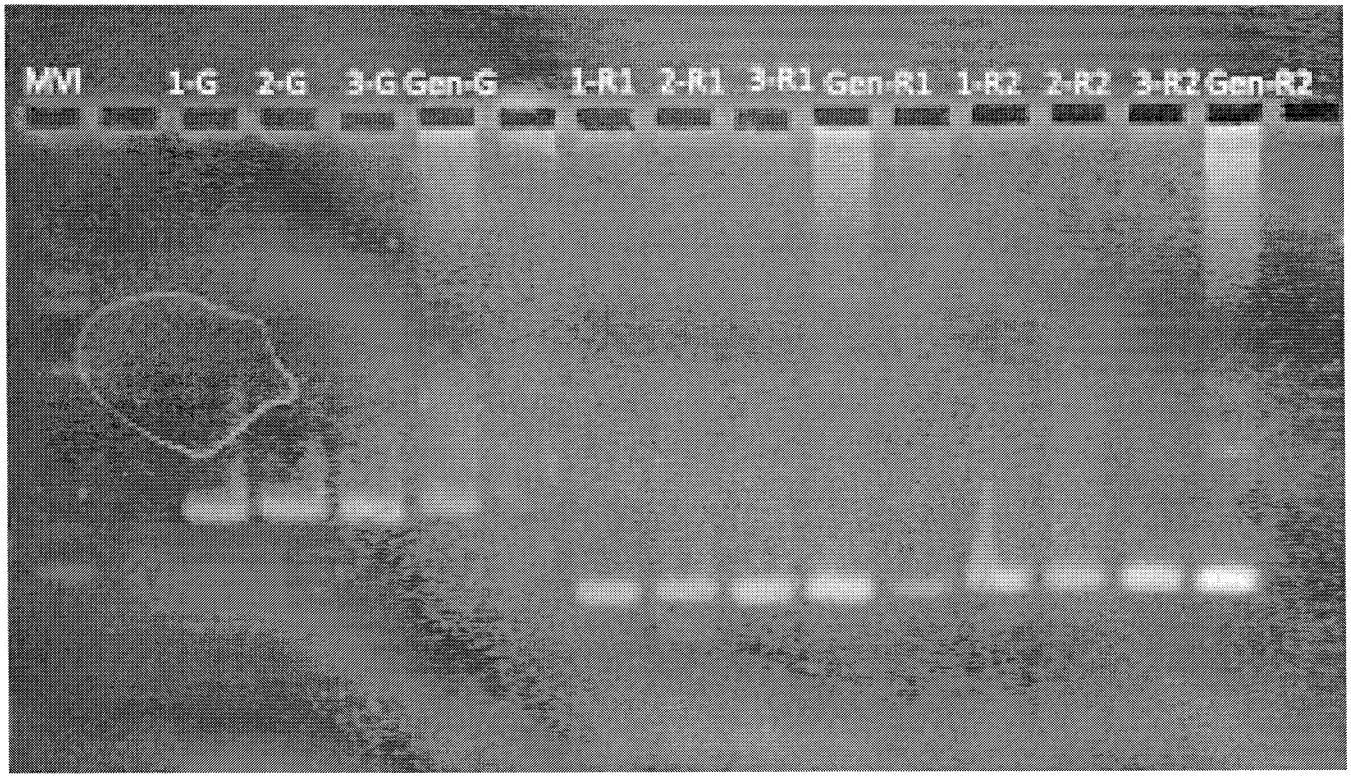

FIGURE 7. PCR gel ran on cDNA samples. Samples 1-G, 2-G, 3-G, 1-R1, 2-R1, 3-R1, 1-R2, 2-R2, 3-R2 were cDNA samples and samples Gen-G, Gen-R1 and Gen-R2 were from genomic DNA used as a control. Samples 1-G, 2-G, 3-G and Gen-G were for primer pair GAPDHF + GAPDHR. Samples 1-R1, 2-R1, 3-R1 and Gen-R1 are for primer pair CYP1AF + CYP1AR1. Samples 1-R2, 2-R2, 3-R2, Gen-R2 are for primer pair CYP1AF + CYP1AR2.

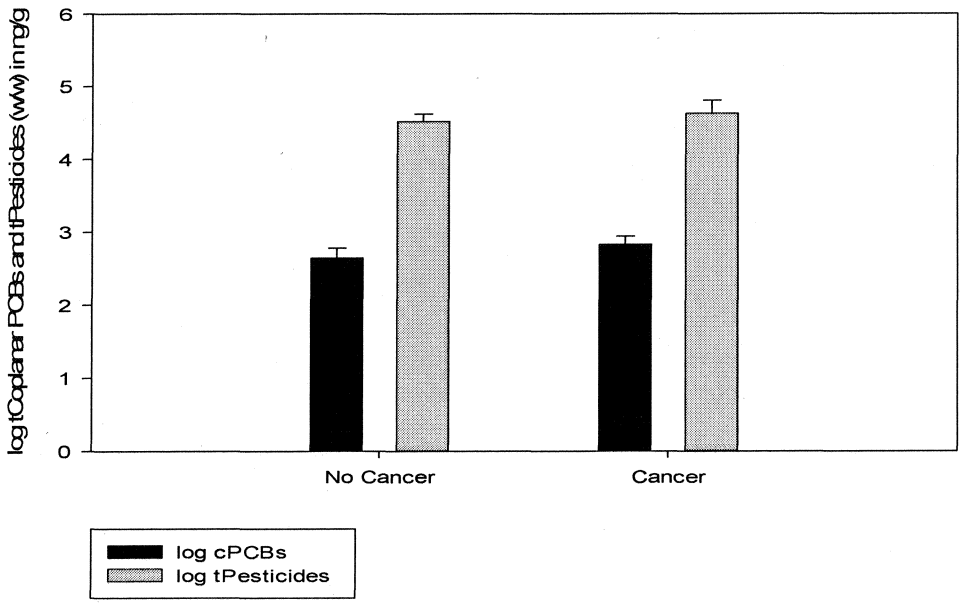

FIGURE 8. Mean ± S.E. of cPCB and tPesticide values (in w/w) in CA sea lions with and without cancer. No significant difference was found between log cPCBs ($T=2.23$, $n_1=n_2=6$, $p=0.33$) or log tPesticides ($T=2.23$, $n_1=n_2=6$, $p=0.60$) measured in blubber of CA sea lion without and with cancer.

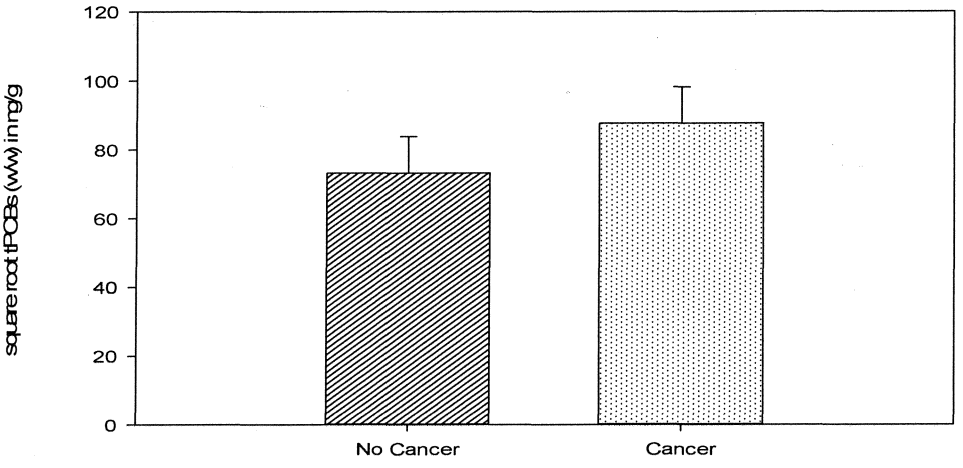

FIGURE 9. Mean ± S.E. of tPCB values (in w/w) in CA sea lions with and without cancer. No significant difference was found between the mean square root of tPCB pollutant values measured in blubber of CA sea lions without and with cancer ($T=2.23$, $n_1=n_2=6$, $p=0.36$).

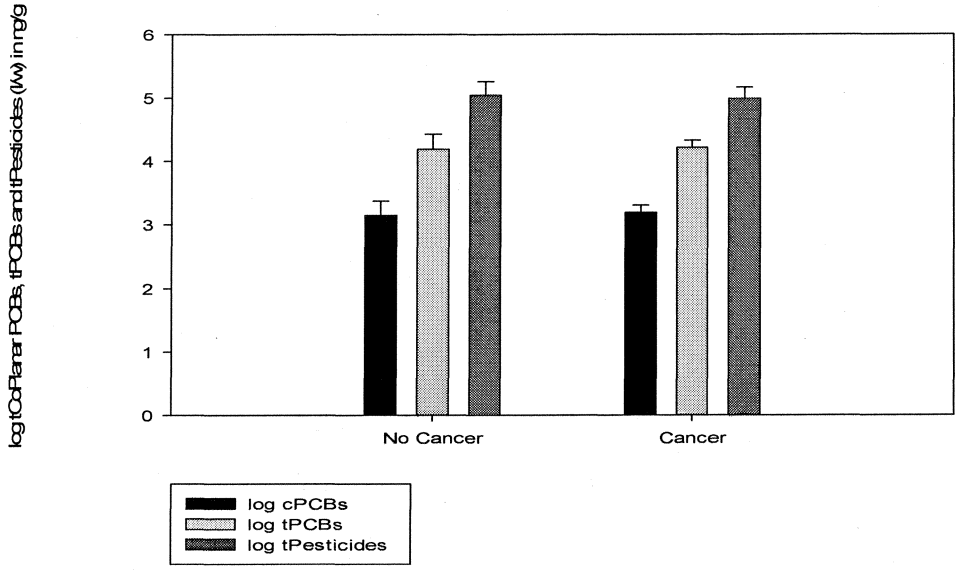

FIGURE 10. Mean ± S.E. of cPCB, tPCB and tPesticide values (in l/w) in CA sea lions with and without cancer. No significant difference was found between the mean log tCoPlanar PCB pollutant values (T=2.23, $n_1=n_2= 6$, p=0.76), tPCBs (T=2.23, $n_1=n_2= 6$, p=0.93) or tPesticides (T=2.23, $n_1=n_2= 6$, p=0.85) measured in blubber of CA sea lions without and with cancer.

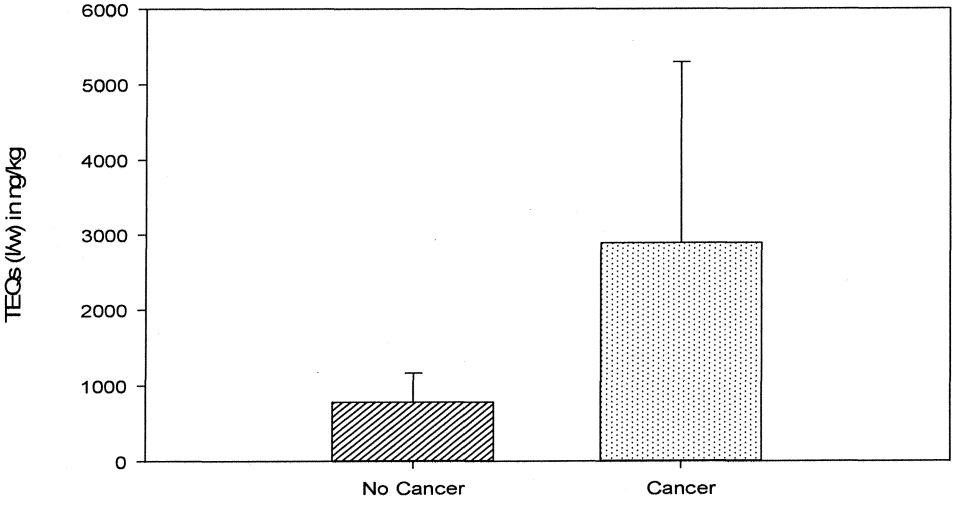

FIGURE 11. Mean ± S.E. of TEQ levels in CA sea lions with and without cancer. No significant difference was found between mean TEQ levels measured in blubber samples from CA sea lions without and with cancer (U=21; $n_{nc}=n_c= 6$; p = 0.63).

```
walrus          ------------------------------------------------------------
CSL             ------------------------------------------------------------
Baikal          CACATTCGGGATATCACAGACAGCCTGATCAAACATTGTCAGGACAAGAGGCTGGATGAG

walrus          ------------------------------------------------------------
CSL             ------------------------------------------------------------
Baikal          AATGCCAACATTCAGCTGTCTGATGAGAAGATCGTTAATGTTGTCTTGGACCTCTTTGGA

walrus          ----GATTTGACACCGTCACAACTGCCATCTCCTGGAGCCTCTTATACCTGGTGACAAGC
CSL             ------------------------------------------------------------
Baikal          GCCGGATTTGACACCGTGACAACTGCCATCTCCTGGAGCCTCCTGTACCTGGTGACAAGC

walrus          CCCAATGTACAGAAAAAGATCCAGGAGGAGCTGGACACAGTGATTGGCAGGGCCCGGCAG
CSL             -----TGTGCAGAAGAAGATCCAGGAGGAGCTGGACACAGTGATTGGCAGGGCCCGGCAG
Baikal          CCCAGTGTACAAAAAAAGATCCAGGAGGAGTTGGACACAGTGATTGGCAGGGCCCGGCAG
                     ***.**.**.*************** ******************************

walrus          CCCCGGCTCTCTGACAGGCTCCAGCTGCCCTACCTGGAGGCATTCATCCTGGAGACCTTC
CSL             CCCCGGCTCTCTGACAGGCTCCAGCTGCCCTACCTGGAGGCATTCATCCTGGAGACCTTC
Baikal          CCCCGGCTCTCTGACAGGCCCCAGTTGCCCTACCTGGAGGCATTCATCCTGGAGACCTTC
                ****************** **** **********************************

walrus          CGACATGCTTCCTTCGTCCCCTTCACCATCCCTCATAGTACCACCAAAGACACAAGTCTG
CSL             CGACATGCTTCCTTCGTCCCCTTCACCATCCCTCATAGTACCACCAAAGACACAAGTCTG
Baikal          CGACACGCTTCCTTCGTCCCCTTCACCATCCCTCATAGTACCACCAAAGACACAAGTCTG
                ***** ******************************************************

walrus          AGTGGCTTTTACATCCCCAAGGGACGTT-------------------------------
CSL             AGTGGCTTCTACATCCCCAAGGG-------------------------------------
Baikal          AGTGGCTTTTACATCCCCAAGGGACGTTGTGTCTTTGTGAACCAGTGGCAGATCAACCAT
                ******** **************

walrus          ------------------------------------------------------------
CSL             ------------------------------------------------------------
Baikal          GACCAGGAGCTATGGGGTGACCCATCTGAGTTCCGACCAGAACGATTTCTCACTCTTGAT

walrus          ------------------------------------------------------------
CSL             ------------------------------------------------------------
Baikal          GGCACCATCAACAAGGCACTGAGTGAGAAGGTGATTCTCTTTGGAATGGGCAAGCGGAAG
```

FIGURE 12. Comparative analysis of the *CYP1A* gene in Walrus, CA sea lion (CSL) and Baikal seal nucleotide sequences. An asterisk underlines similar bp. *Note*. Only a portion of the fully sequenced gene for Baikal seal is shown.

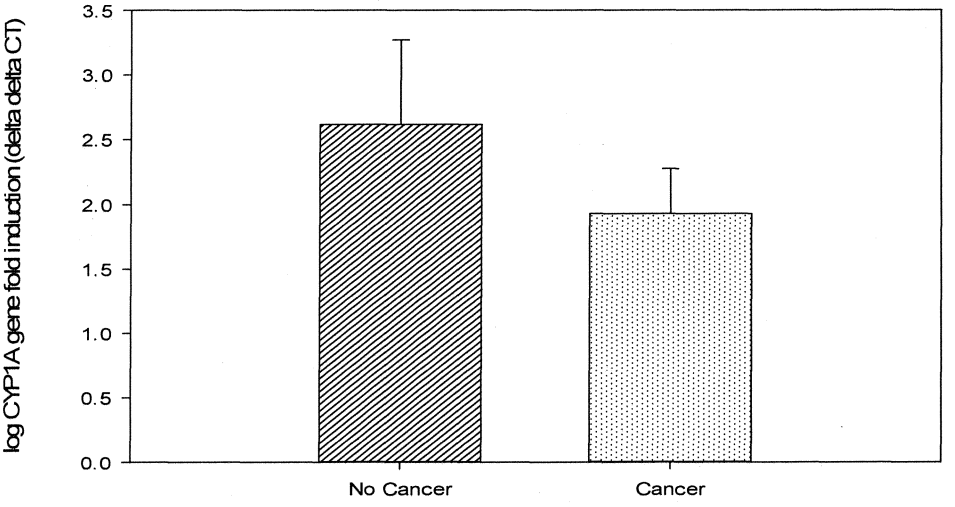

FIGURE 13. Mean ± S.E. of the *CYP1A* gene induction levels in CA sea lions with and without cancer. No significant difference was found between the mean log *CYP1A* gene induction levels (delta delta CT; $\Delta\Delta C_T$) measured in liver microsomes of CA sea lions without and with cancer ($T=2.26$; $n_{nc}=5$, $n_c=6$; $p = 0.36$). *Note.* Expression is relative to a calibrator sample set at 1.

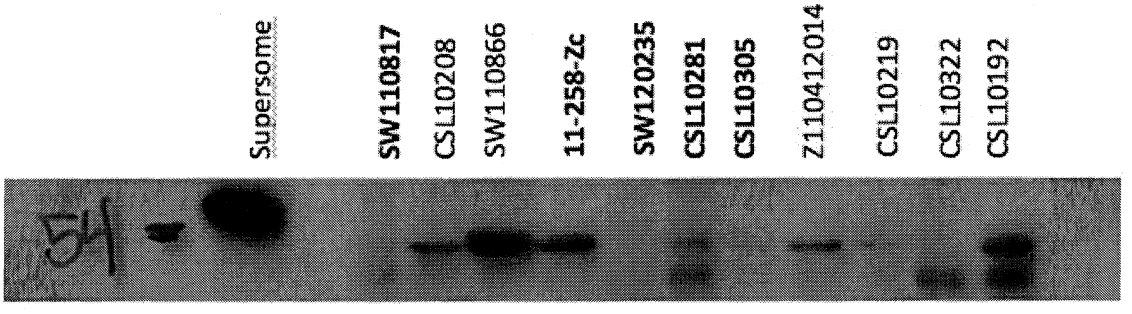

FIGURE 14. Western Immunoblot for CYP1A protein determination in CA sea lion liver microsomes. Samples are (from left to right): Supersome, (Blank Space), SW110817, CSL10208, SW110866, 11-258-Zc, SW120235, CSL10281, CSL10305, Z-11-04-12-014, CSL10219, CSL10322, CSL10192.

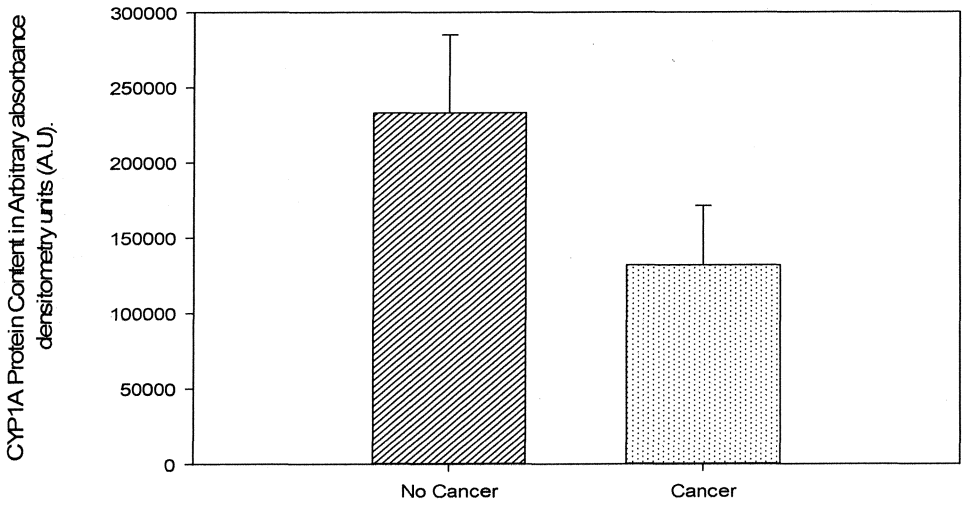

FIGURE 15. Mean ± S.E. of cytochrome p450 protein content in CA sea lions with and without cancer. No significant difference was found between the mean cytochrome p450 protein content in arbitrary absorbance densitometry units (A.U.) measured in liver microsomes from CA sea lions without and with cancer (U=7; n_{nc}=6, n_c=5; p = 0.14).

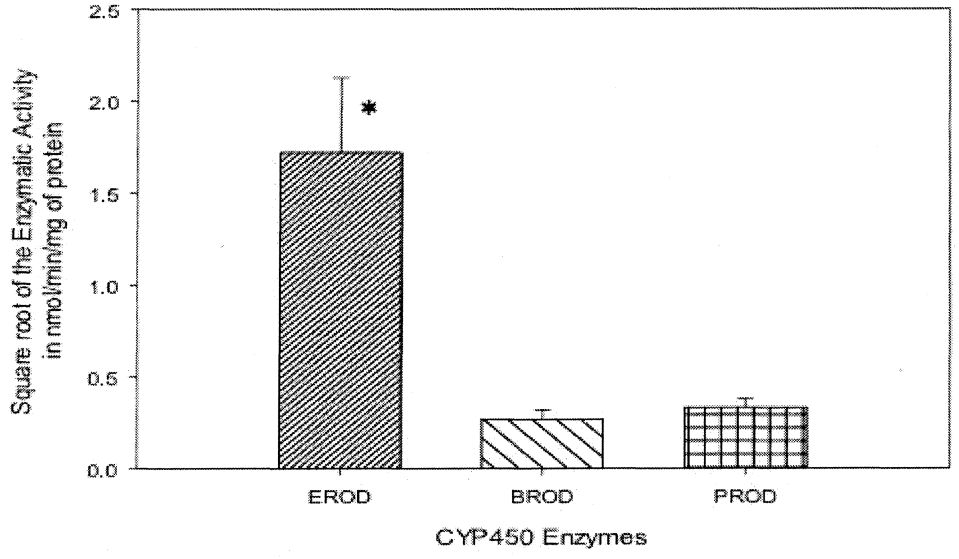

FIGURE 16. Mean ± S.E. of EROD, BROD and PROD activity in CA sea lion liver microsomes. (F= 11.79, n=5, p = 0.002; EROD was significantly higher than BROD p = 0.004(*); EROD was significantly higher than PROD p = 0.005 (*); no significant difference was found between BROD and PROD p = 0.98).

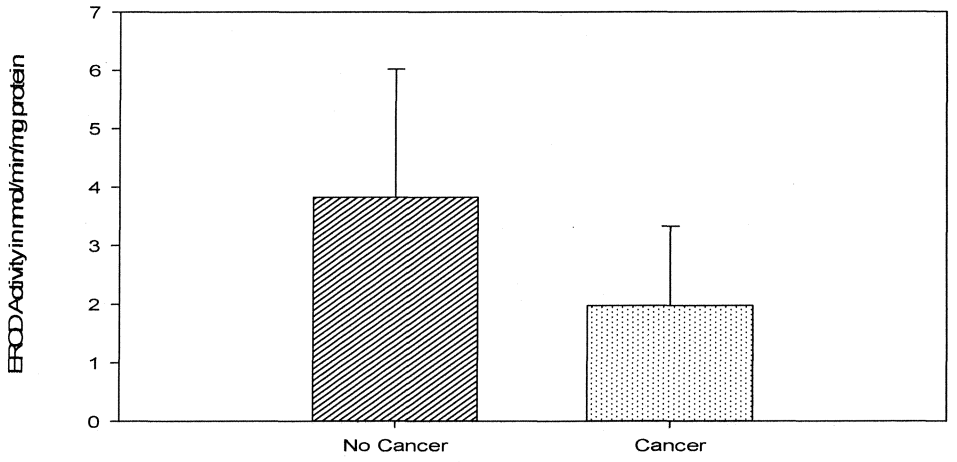

FIGURE 17. Mean ± S.E. of EROD activity in CA sea lions with and without cancer. No significant difference was found in EROD Activity measured in liver microsomes from CA sea lions without and with cancer (U=19; n_{nc}=6, n_C=5; p = 0.27).

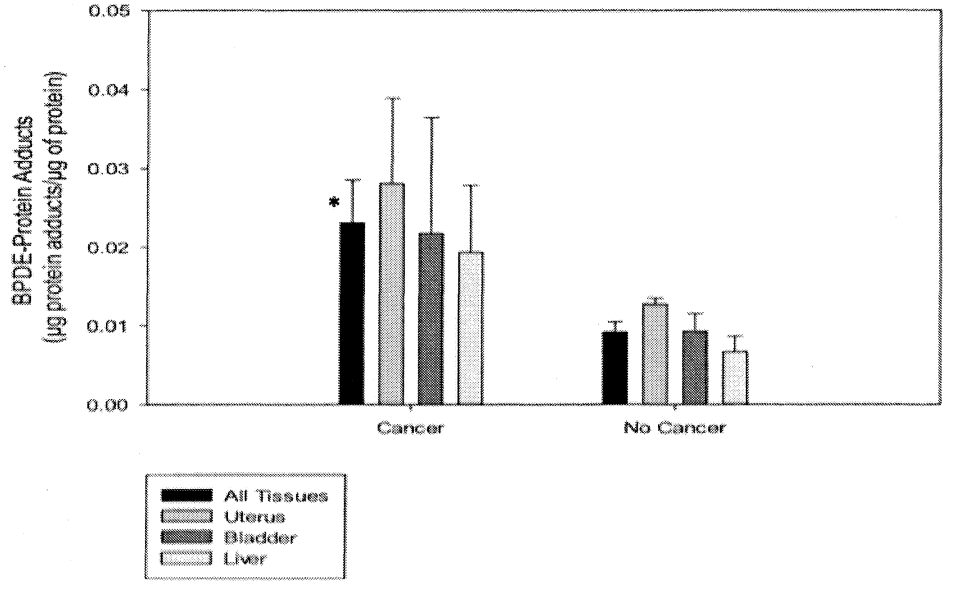

FIGURE 18. Mean ± S.E. of BPDE-protein adducts in CA sea lions with and without cancer. BPDE-protein adduct levels measured in tissues from CA sea lions with cancer and without cancer showed no significant difference for Livers: T=3.18, n_{nc}=3, n_c=2, p = 0.16; Bladders: T=3.18; n_{nc}=3, n_c=2; p = 0.35; Uterus: T=4.30; n_{nc}=2, n_c=2; p = 0.29. A significant difference was found for all tissues combined: T=2.18; n_{nc}=8, n_c=6; p = 0.015 (*)).

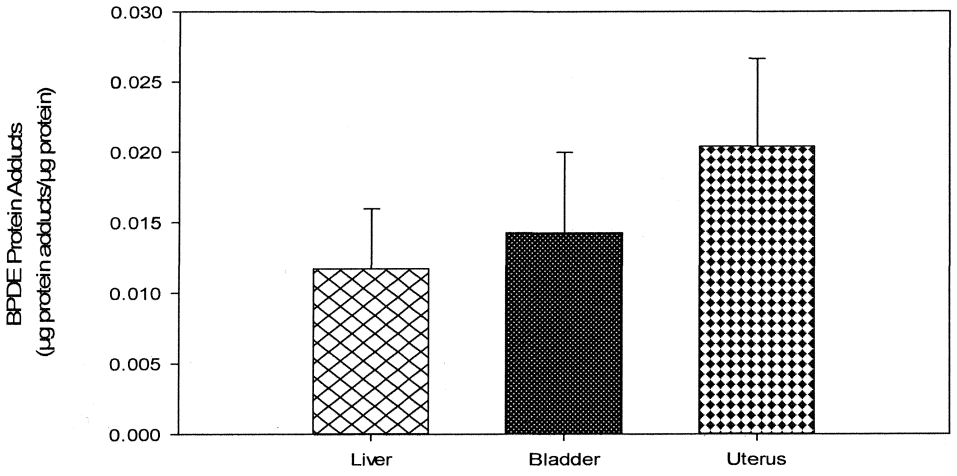

FIGURE 19. Mean ± S.E. of BPDE-protein adduct levels and S.E. measured in livers, bladders and uterus of CA sea lions. No significant difference was found between groups ($F = 0.64$; *df* between groups= 2, *df* within groups = 11; $p = 0.55$).

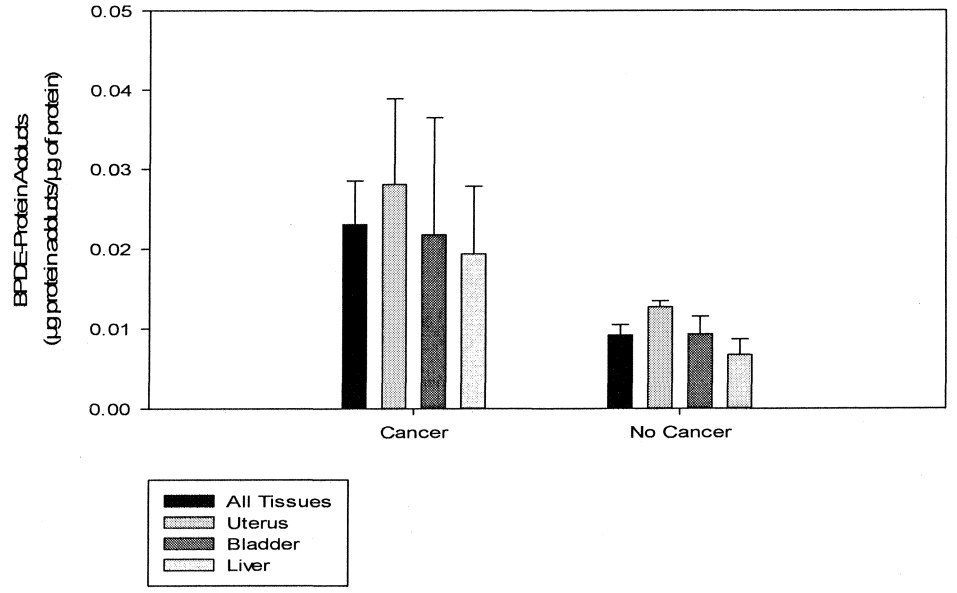

FIGURE 20. Mean ± S.E. of BPDE-DNA adduct levels measured in tissues from CA sea lions with and without cancer. No significant difference was found between levels in all tissues combined: T=2.07; $n_{nc}=n_c=12$; $p = 0.25$; Livers: T=2.31; $n_{nc}=6$, $n_c=4$; $p = 0.25$; Bladders: T=2.37; $n_{nc}=4$, $n_c=5$; $p = 0.38$; Uterus: T=3.18; $n_{nc}=2$, $n_c=3$; $p = 0.20$). *Note.* Non-detectable results were excluded.

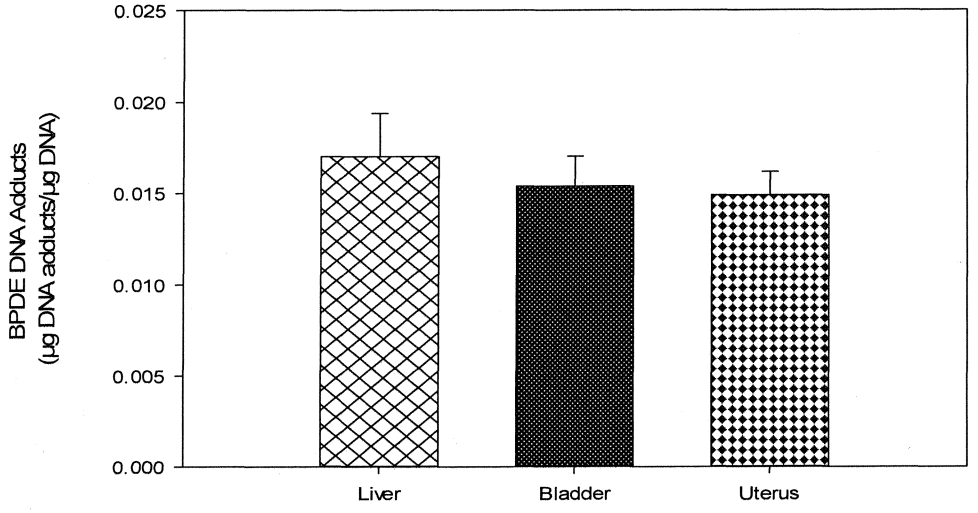

FIGURE 21. Mean ± S.E. of BPDE-DNA adducts in CA sea lion liver, bladder and uterus. No significant difference was found between tissues in CA sea lions ($F = 2.61$; *df* between groups= 2, *df* within groups = 21; $p = 0.1$).

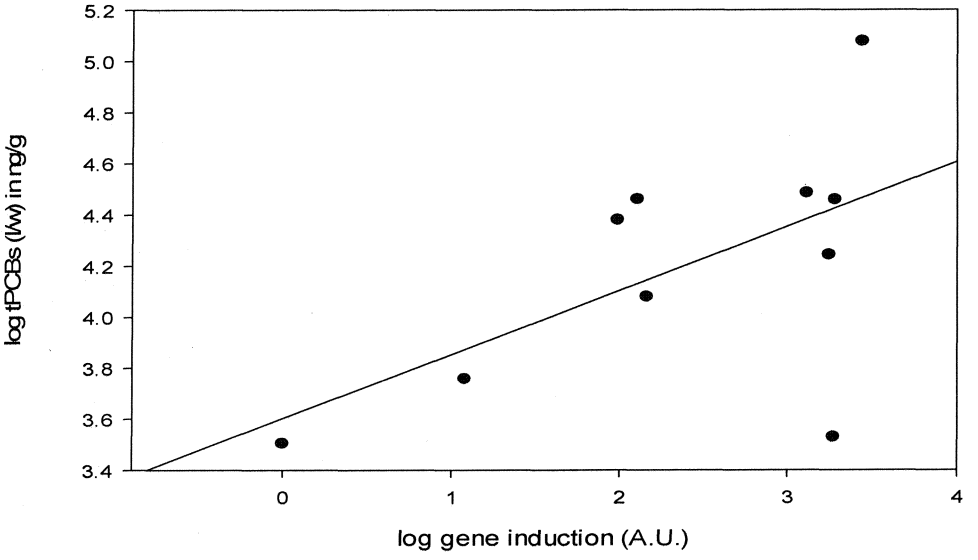

FIGURE 22. Log *CYP1A* gene induction levels versus log tPCBs (in l/w). A slightly significant positive correlation was found (R=0.58, R^2=0.34, p=0.08; y = 0.251x + 3.602).

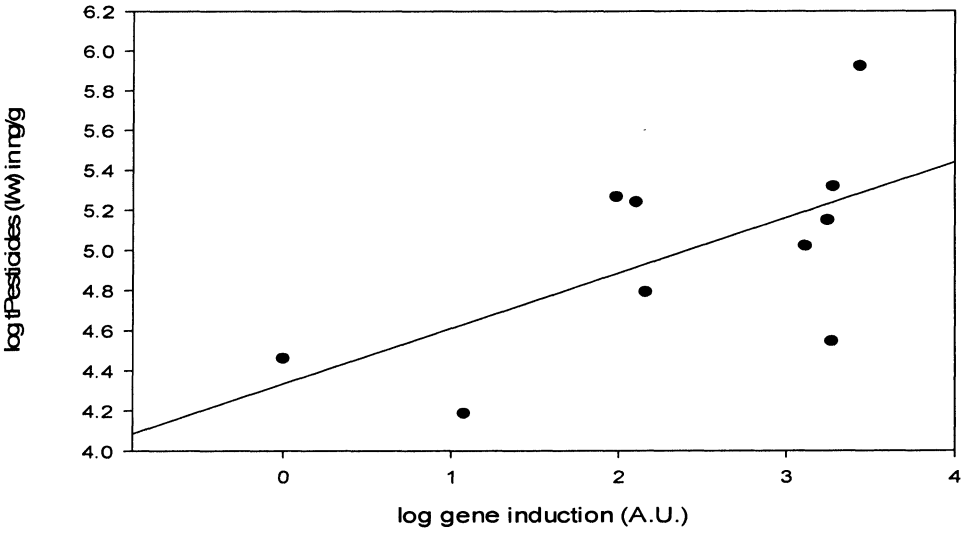

FIGURE 23. Log *CYP1A* gene induction levels versus log tPesticides (in l/w). A slightly significant positive correlation was found (R=0.62, R^2=0.39, p=0.055; y = 0.276x + 4.335).

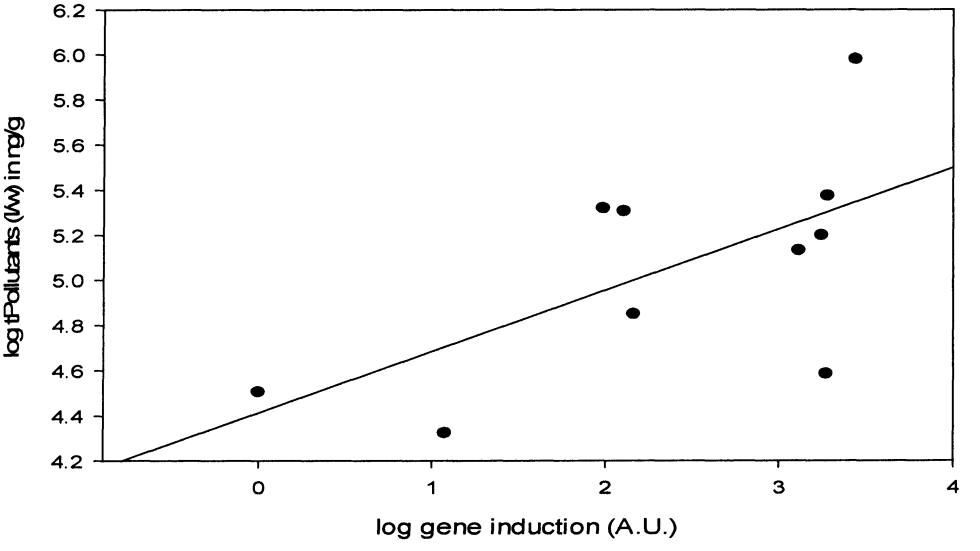

FIGURE 24. Log *CYP1A* gene induction levels versus log tPollutants (in l/w). A slightly significant positive correlation was found (R=0.62, R^2=0.39, p=0.055; y = 0.2709x + 4.4148).

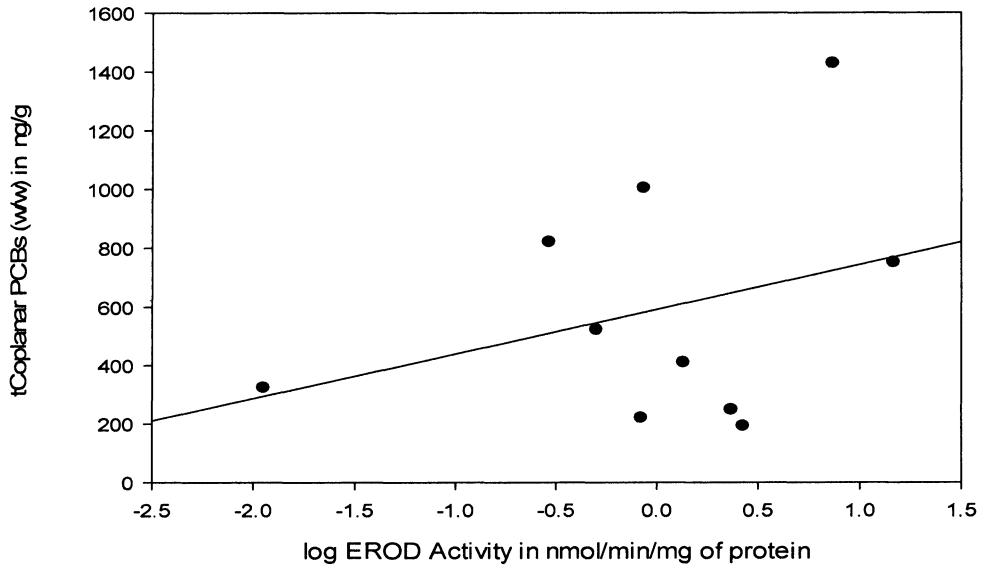

FIGURE 25. Log EROD activity levels versus tCoPlanar PCBs (in w/w). No significant correlation was found ($R=0.32$, $R^2=0.10$, $p = 0.36$; $y = 152.43x + 592.31$).

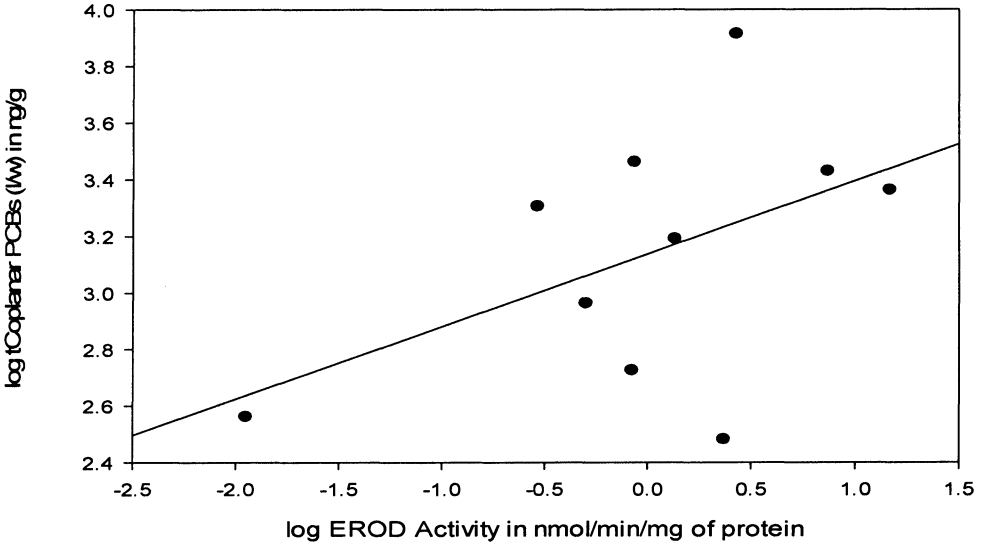

FIGURE 26. Log EROD activity levels versus log tCoPlanar PCBs (in l/w). No significant correlation was found ($R=0.49$, $R^2=0.24$, $p = 0.15$; $y = 0.2572x + 3.1389$).

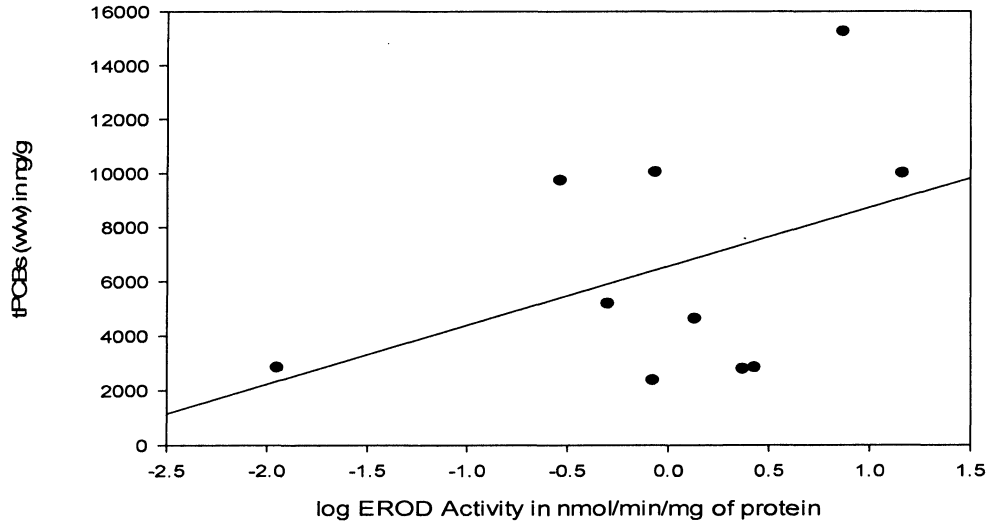

FIGURE 27. Log EROD activity levels versus total PCBs (in w/w). No significant correlation was found ($R=0.42$, $R^2=0.18$, $p = 0.22$; $y = 2162.9x + 6560.3$).

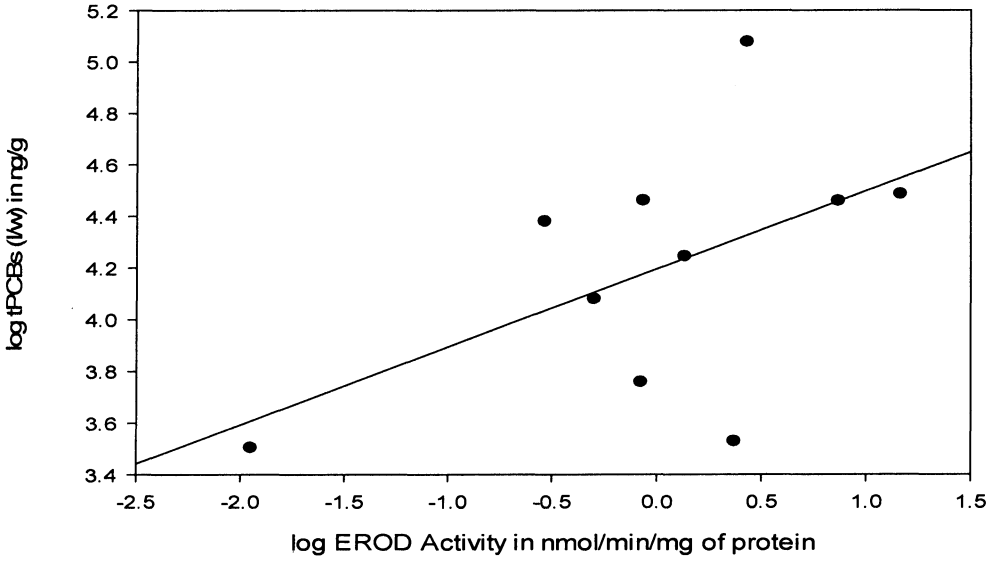

FIGURE 28. Log EROD activity levels versus log total PCBs (in l/w). No significant correlation was found ($R=0.53$, $R^2=0.28$, $p = 0.12$; $y = 0.3015x + 4.1962$).

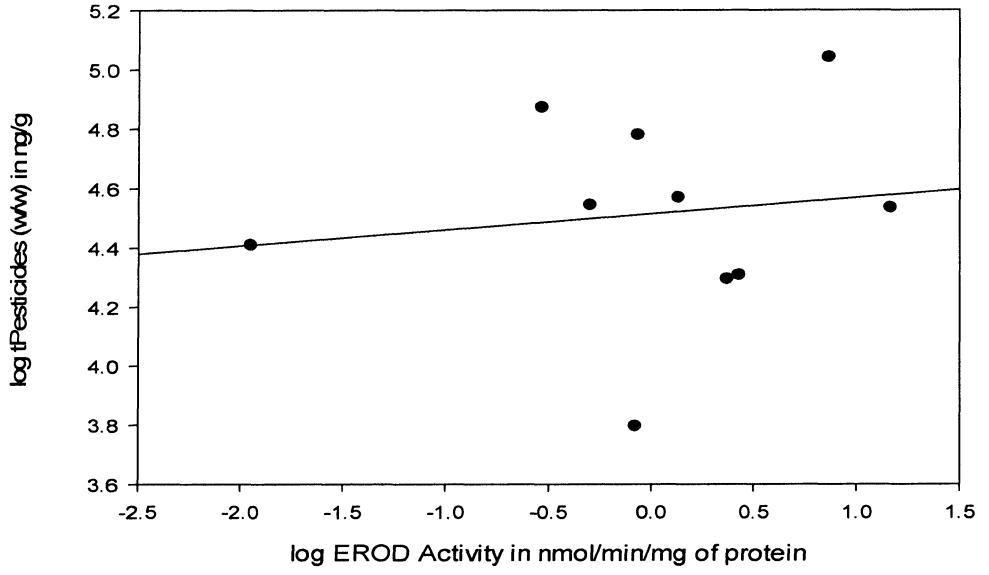

FIGURE 29. Log EROD activity levels versus log total Pesticides (in w/w). No significant correlation was found ($R=0.13$, $R^2=0.02$, $p = 0.71$; $y = 0.0542x + 4.5151$).

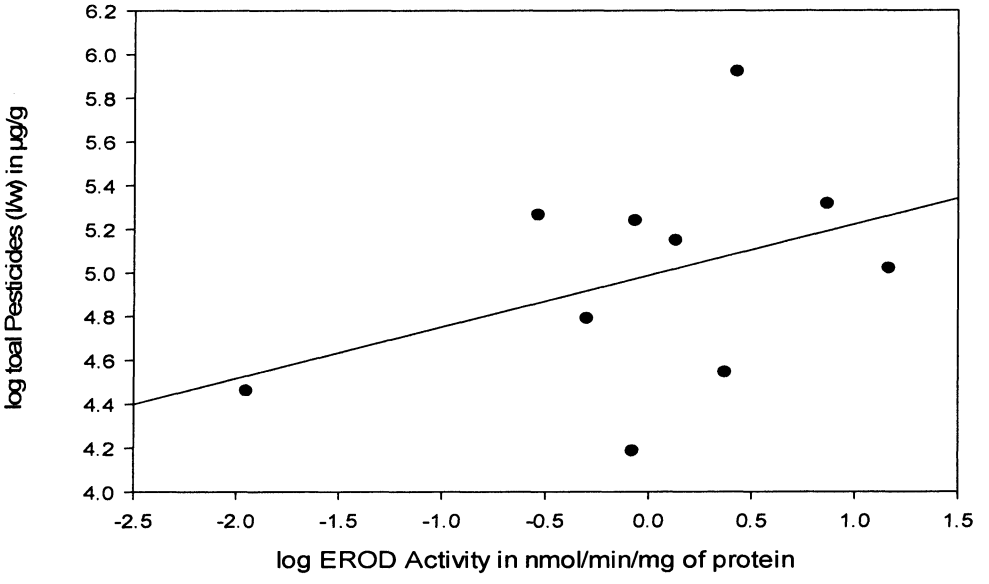

FIGURE 30. Log EROD activity levels versus log total Pesticides (in l/w). No significant correlation was found ($R=0.40$, $R^2=0.16$, $p = 0.25$; $y = 0.2355x + 4.9879$).

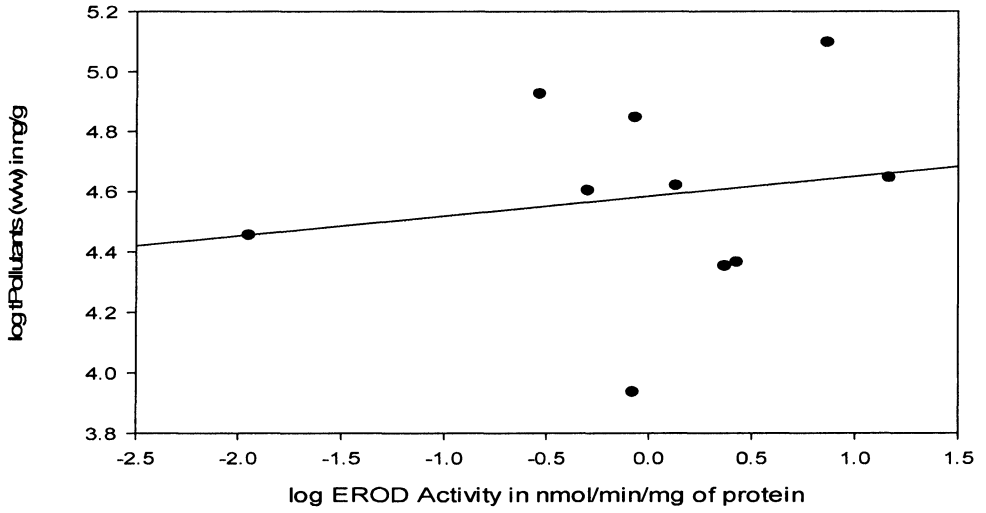

FIGURE 31. Log EROD activity levels versus log total Pollutants (in w/w). No significant correlation was found ($R=0.17$, $R^2=0.03$, $p = 0.64$; $y = 0.0659x + 4.5848$).

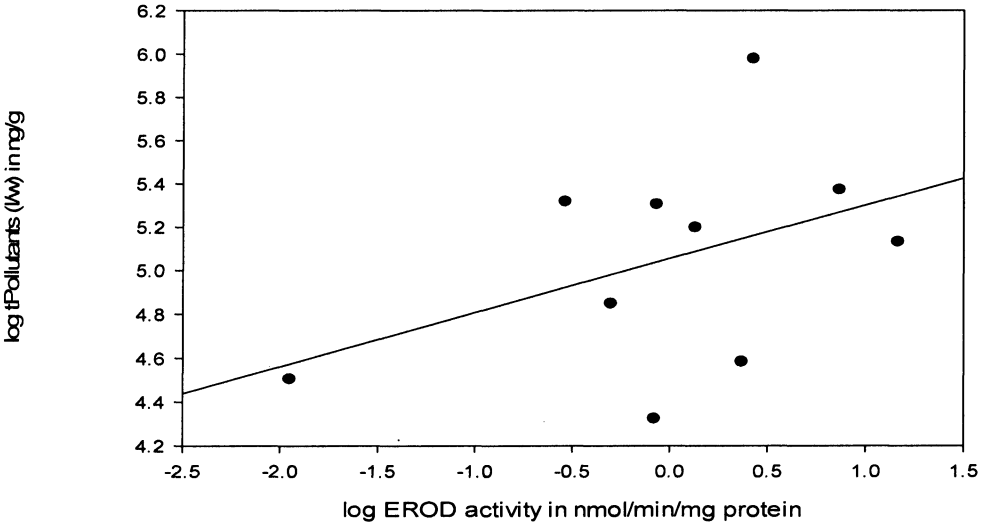

FIGURE 32. Log EROD activity levels versus log total Pollutants (in l/w). No significant correlation was found ($R=0.43$, $R^2=0.18$, $p = 0.23$; $y = 0.2464x + 5.0558$).

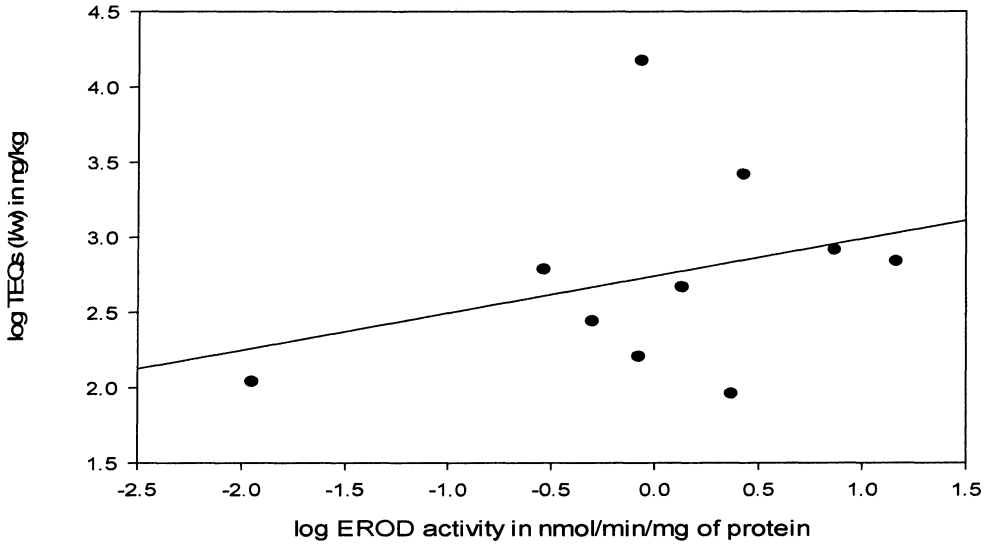

FIGURE 33. Log EROD activity levels versus log TEQs (in l/w). No significant correlation was found ($R = 0.32$, $R^2 = 0.10$, $p = 0.37$; $y = 0.247x + 2.7431$).

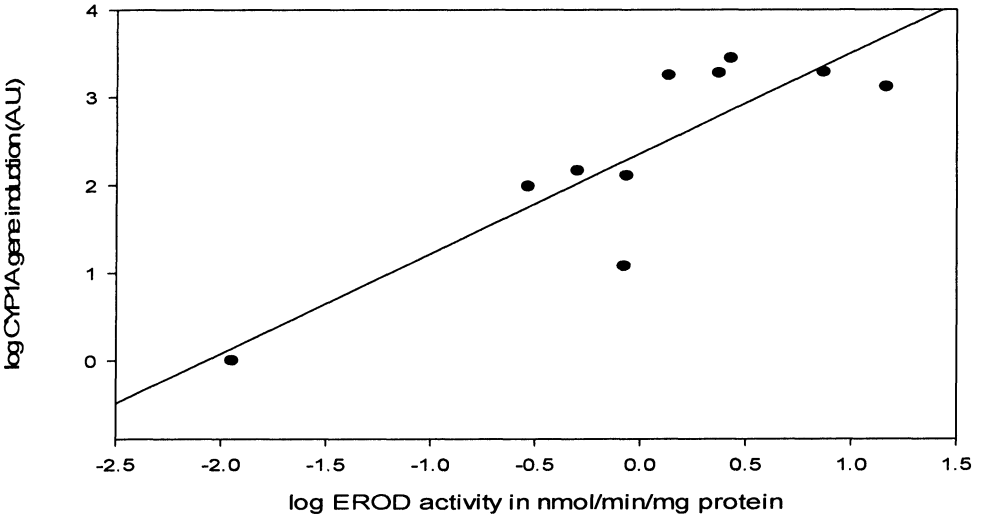

FIGURE 34. Log EROD activity levels versus the log of the fold induction of the *CYP1A* gene. A significant positive correlation was found ($R=0.86$; $R^2=0.74$; $p = 0.001$; $y = 1.1405x + 2.365$).

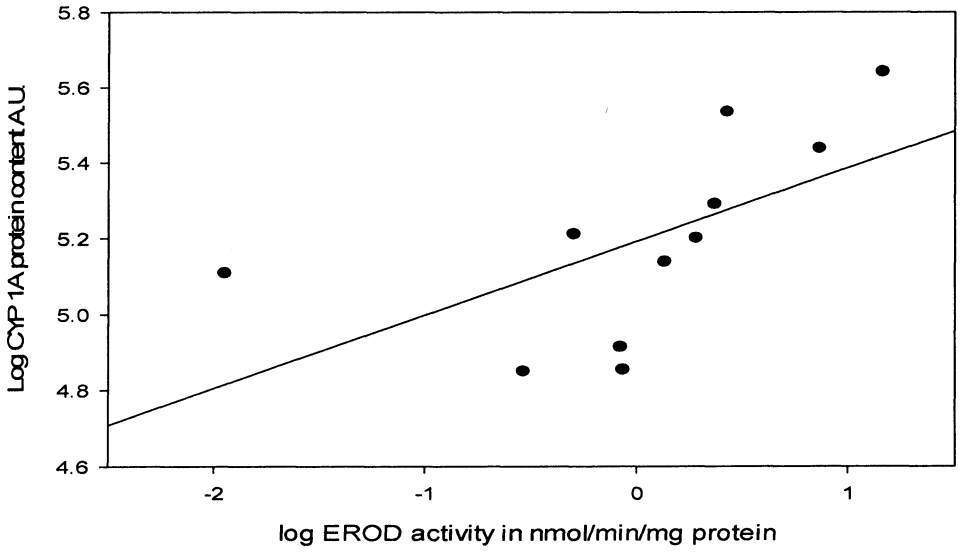

FIGURE 35. Log EROD activity versus log CYP1A protein content. A significant positive correlation was found (R=0.60; R^2=0.35; p = 0.05; y = 0.1933x + 5.1922).

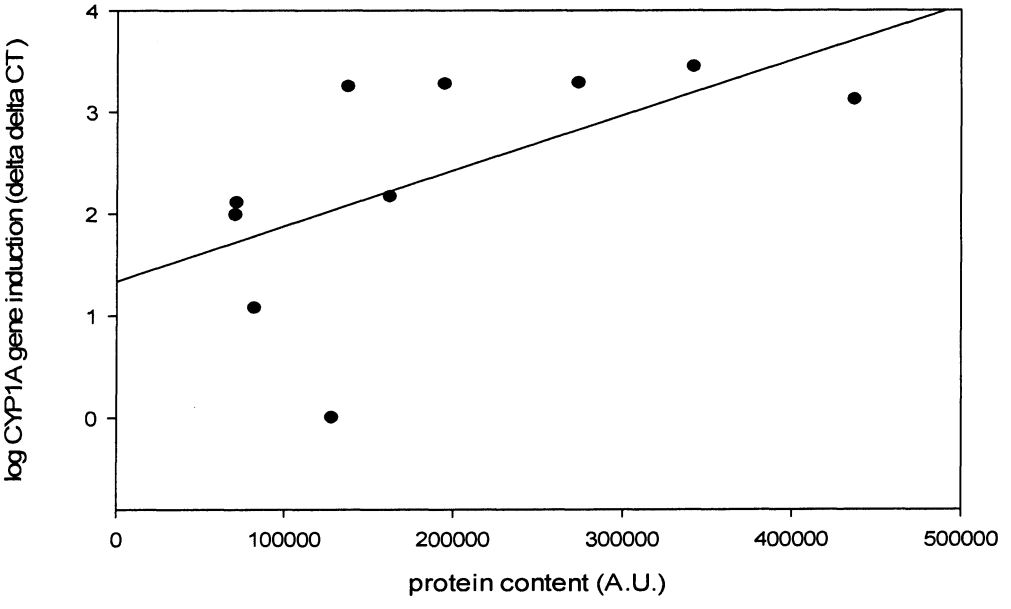

FIGURE 36. Protein content levels versus the log of *CYP1A* gene induction. A slightly positive correlation was found (R=0.59; R^2=0.35; p = 0.07; y = 5E-06x + 1.3413).

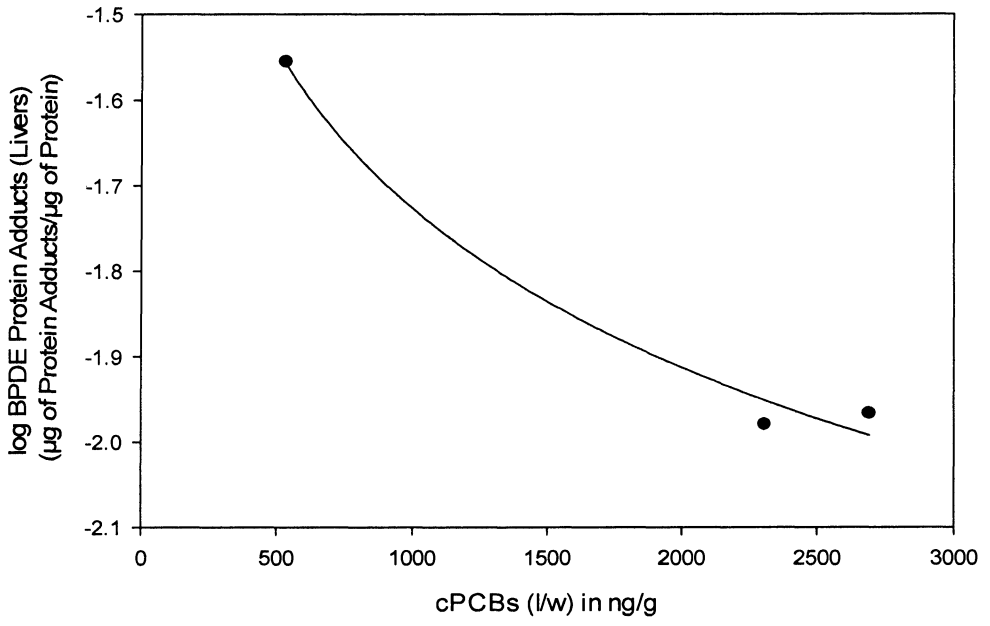

FIGURE 37. Log regression of cPCBs (l/w) versus the log of BPDE-protein adducts in livers. A slightly significant inverse correlation was found ($R = 0.99$, $R^2 = 0.99$, $p = 0.07$; $y = -0.268\ln(x) + 0.123$).

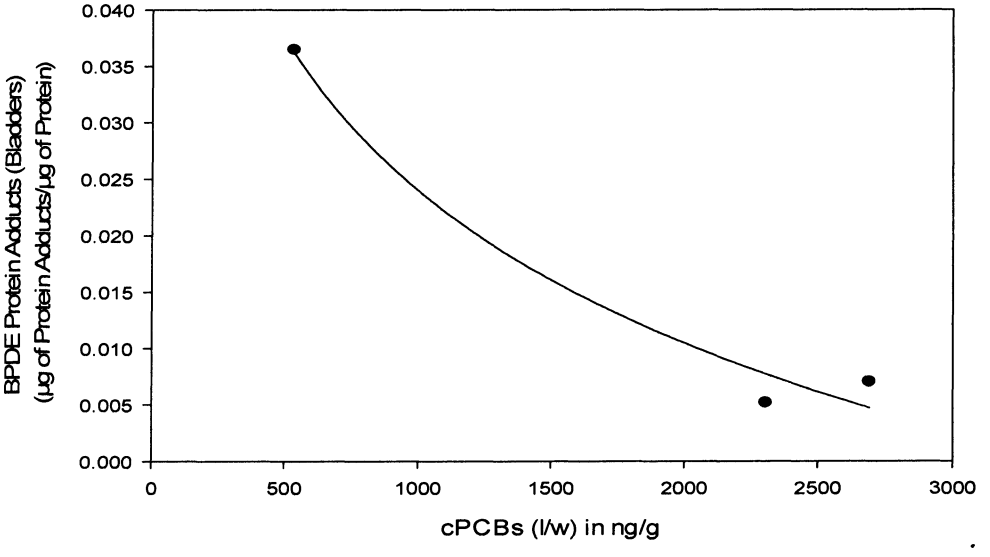

FIGURE 38. Log regression of cPCBs (in l/w) versus the BPDE-protein adducts in bladders. A slightly significant inverse correlation was found ($R = 0.99$, $R^2 = 0.98$, $p = 0.09$; $y = -0.019\ln(x) + 0.1582$).

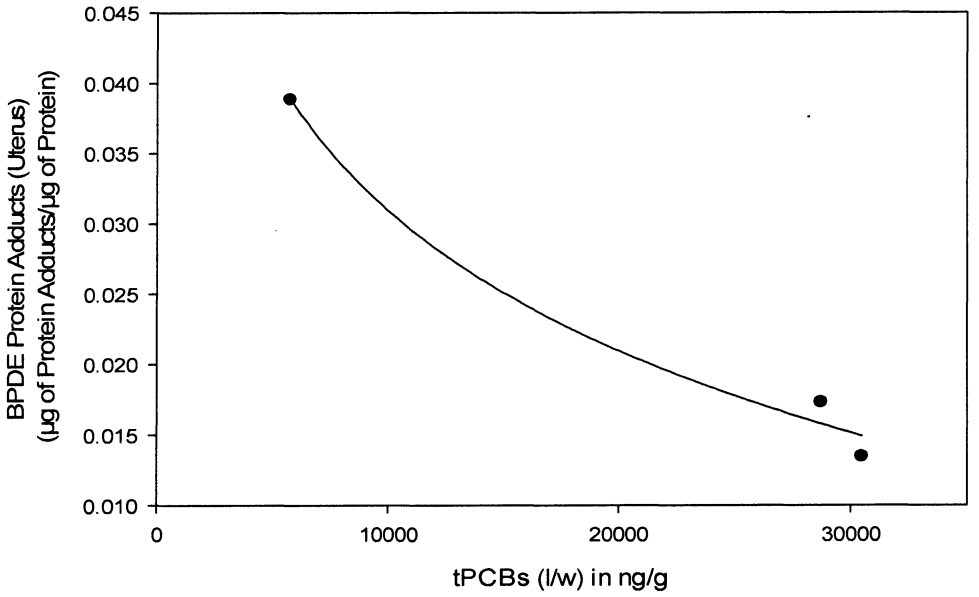

FIGURE 39. Log regression of tPCBs (in l/w) versus the BPDE-protein adducts in uterus. A slightly significant inverse correlation was found ($R = 0.99$, $R^2 = 0.99$, $p = 0.07$; $y = -0.014\ln(x) + 0.163$).

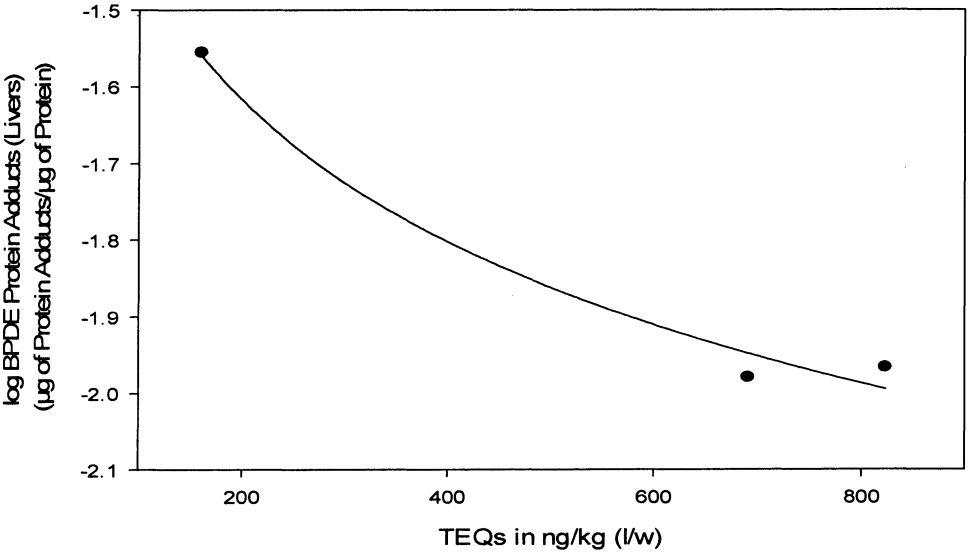

FIGURE 40. Log regression of TEQs versus the log of BPDE-protein adducts in livers. A slightly significant inverse correlation was found ($R = 0.99$, $R^2 = 0.98$, $p = 0.08$; $y = -0.266\ln(x) - 0.2065$).

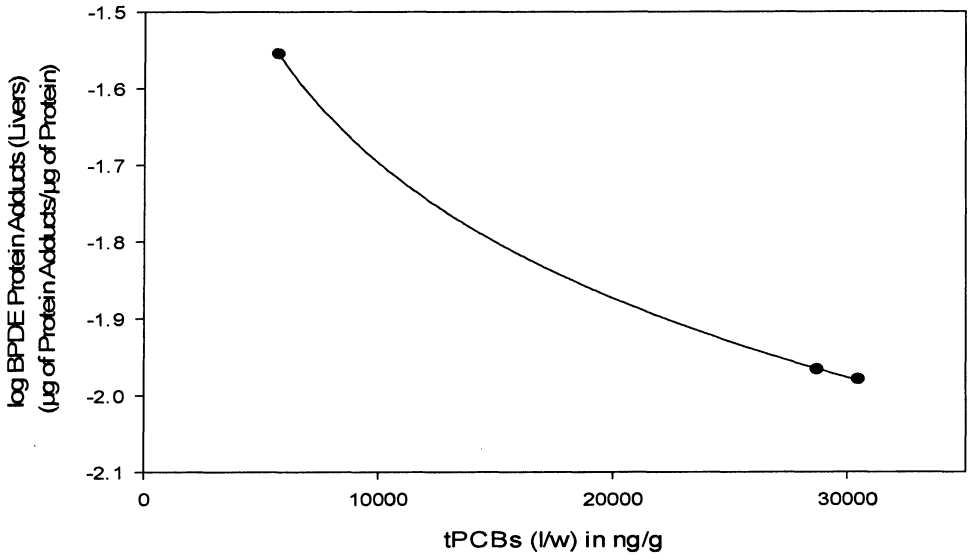

FIGURE 41. Log regression of tPCBs (in l/w) versus the log of BPDE-protein adducts in livers. A significant inverse correlation was found ($R = 1$, $R^2 = 1$, $p = 0.003$; $y = -0.254\ln(x) + 0.6441$).

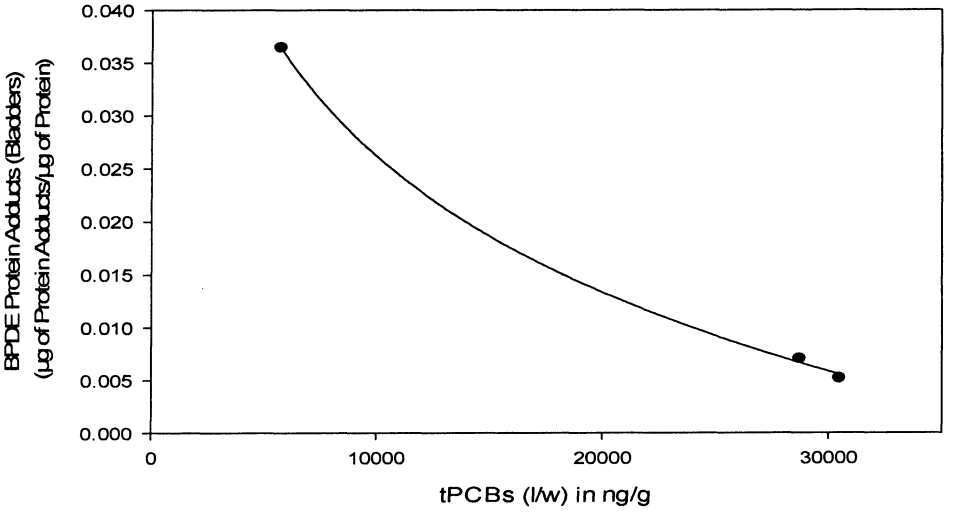

FIGURE 42. Log regression tPCBs (in l/w) versus the BPDE-protein adducts in bladders. A significant inverse correlation was found ($R = 1$, $R^2 = 1$, $p = 0.01$; $y = -0.018\ln(x) + 0.1965$).

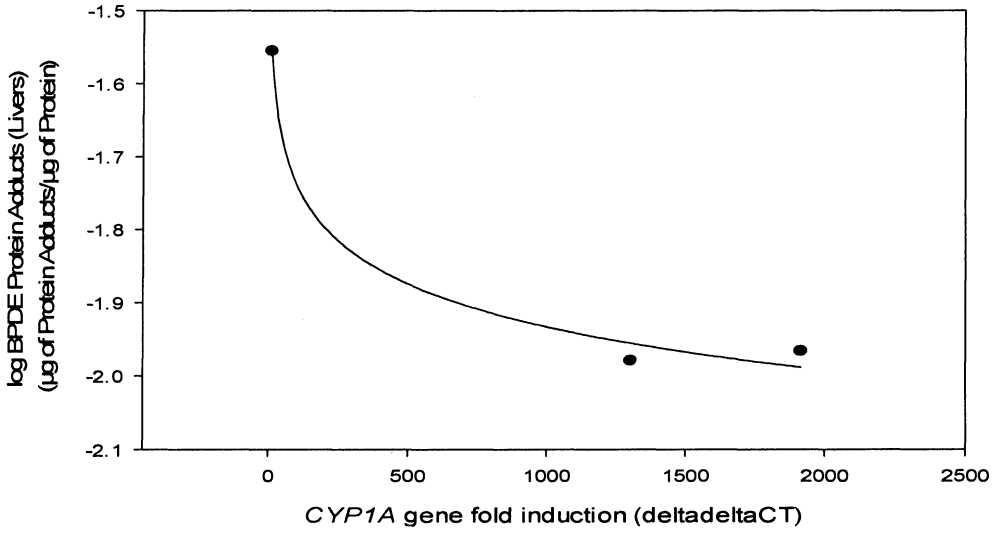

FIGURE 43. Log regression of the *CYP1A* gene induction versus the log of BPDE-protein adducts in livers. A slightly significant inverse correlation was found (R=0.996; R^2=0.99; p = 0.058; y = -0.085ln(x) - 1.3465).

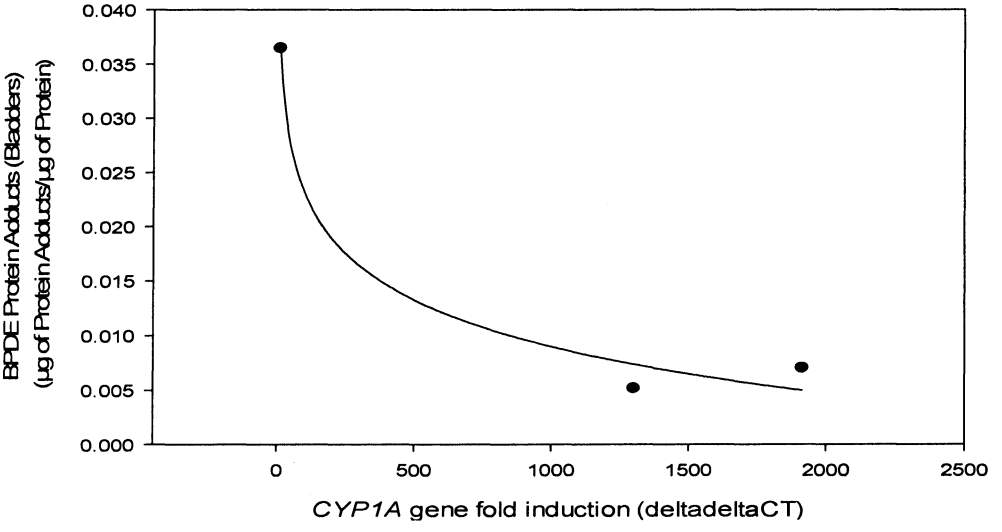

FIGURE 44. Log regression of the *CYP1A* gene induction versus BPDE-protein adducts in bladders. A slightly significant inverse correlation was found (R=0.99; R^2=0.99; p = 0.08; y = -0.006ln(x) + 0.0516).

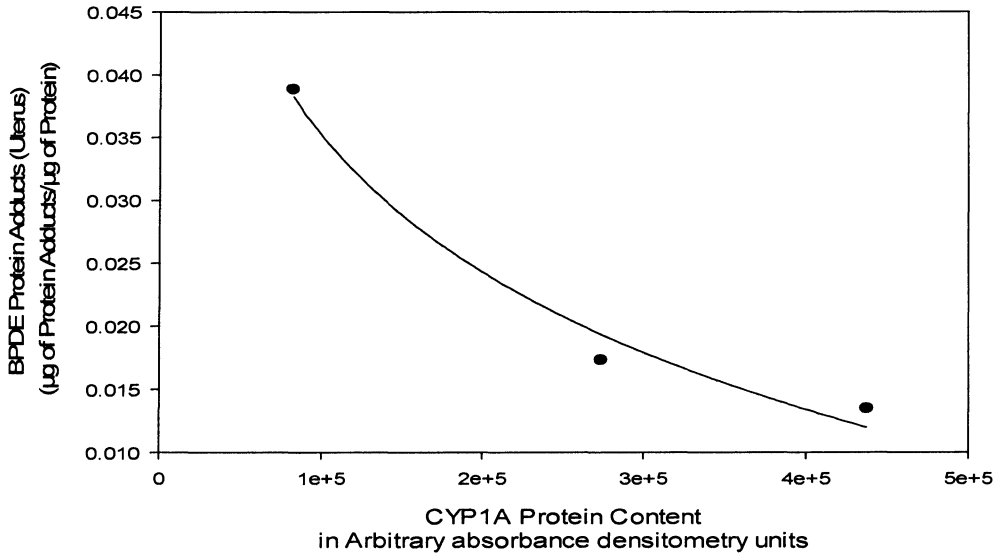

FIGURE 45. Log regression of CYP1A protein content versus BPDE-protein adducts in uterus. A slightly significant inverse correlation was found (R=0.99; R^2=0.98; p = 0.09; y = -0.016ln(x) + 0.216).

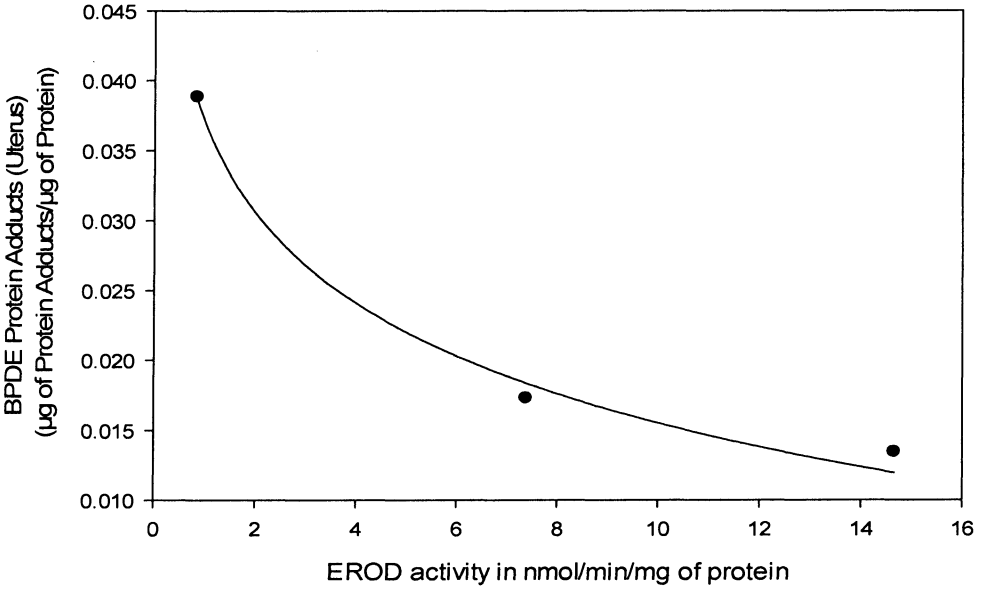

FIGURE 46. Log regression of CYP1A EROD activity versus BPDE-protein adducts in uterus. A slightly significant inverse correlation was found (R=0.996; R^2=0.99; p = 0.058; y = -0.009ln(x) + 0.0369).

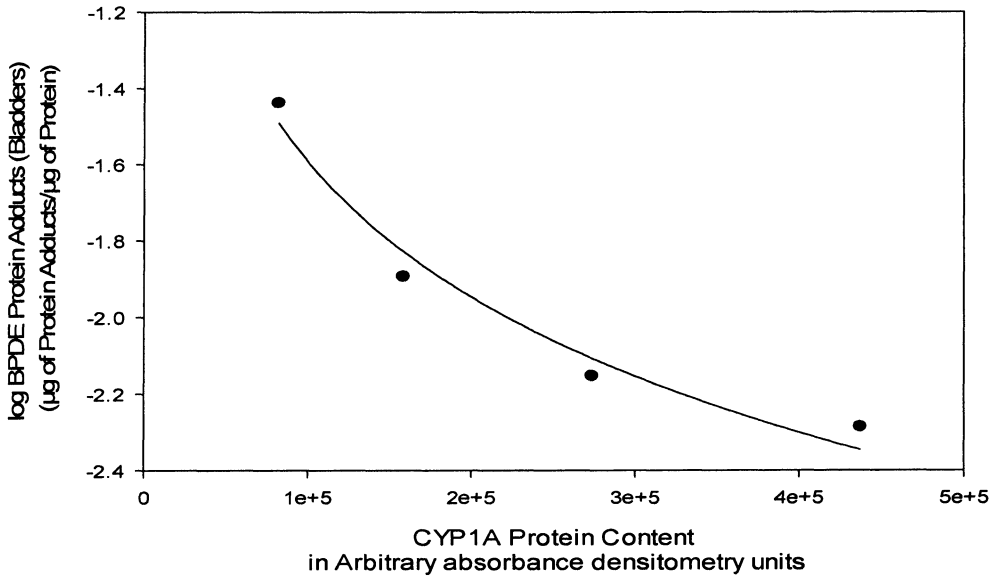

FIGURE 47. Log regression of CYP1A protein content versus BPDE-protein adducts in bladders. A significant inverse correlation was found ($R=0.99$; $R^2=0.97$; $p = 0.02$; $y = -0.511\ln(x) + 4.2873$).

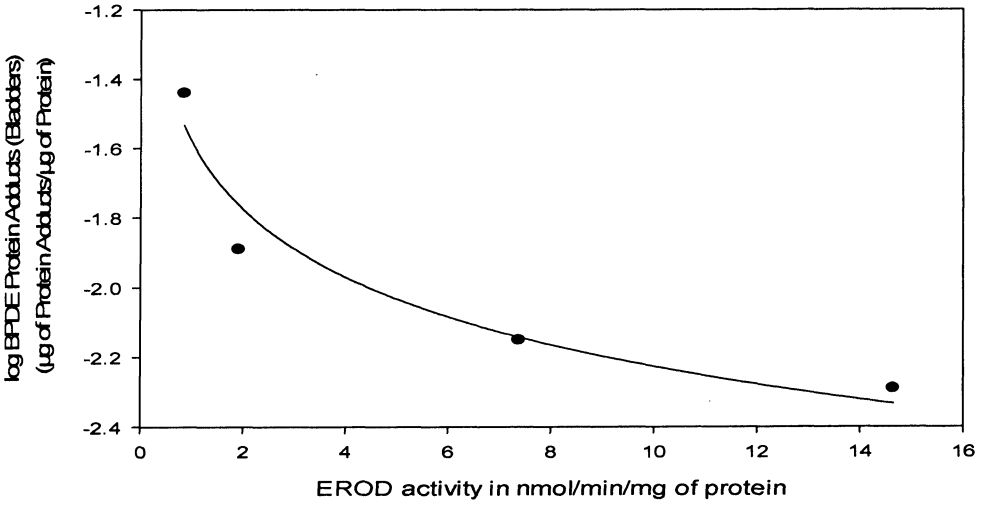

FIGURE 48. Log regression of EROD activity versus BPDE-protein adducts in bladders. A significant inverse correlation was found ($R=0.97$; $R^2=0.94$; $p = 0.03$; $y = -0.28\ln(x) - 1.5825$).

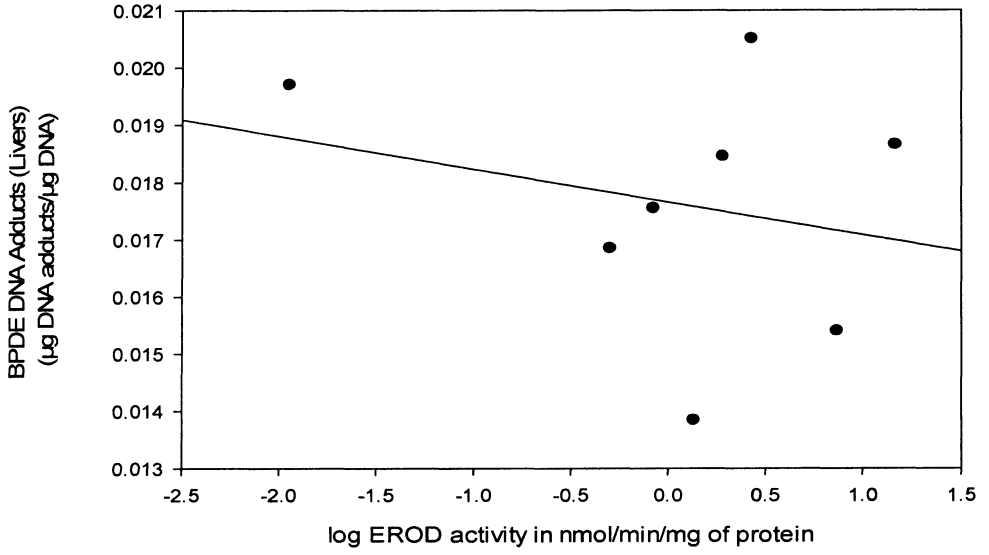

FIGURE 49. Log EROD activity levels versus log BPDE-DNA adduct levels in livers. No significant correlation was found ($R=0.25$; $R^2=0.06$; $p = 0.56$; $y = -0.0006x + 0.0177$). *Note.* Non detectable results were excluded.

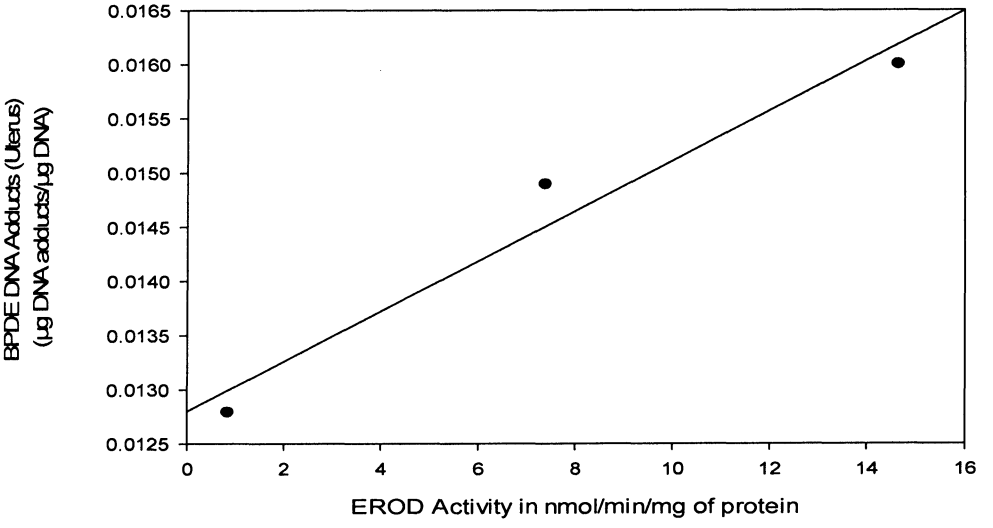

FIGURE 50. EROD activity levels versus BPDE-DNA adduct levels in uterus. No significant correlation was found ($R=0.98$; $R^2=0.96$; $p = 0.13$; $y = 0.0002x + 0.0128$).

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