

The good, the bad and the ugly: lessons learned from vitamins, persistent organic pollutants, and the interaction of the two in western Arctic beluga whales

by

Jean-Pierre Desforges
B.Sc., University of Ottawa, 2009

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of the Requirements for the Degree of

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University of Victoria

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Supervisory Committee

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Abstract

Many of the factors that shape contaminant accumulation profiles in marine mammals also strongly influence fat soluble vitamin accumulation. Vitamin A and E are essential fat soluble nutrients for numerous biological processes, including reproduction, growth, endocrine and immune function. Contaminants, such as polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs), can alter vitamin dynamics; as such these vitamins have been proposed as sensitive biomarkers of contaminant exposure in wildlife. In light of these considerations, the present thesis was aimed at better understanding the factors that influence the accumulation of lipophilic contaminants and vitamins in western Arctic beluga whales, and to determine if there was an interaction between the two.

Maternal offloading to neonates during gestation reduced overall contaminant (PCBs and PBDEs) and vitamin (A and E) concentrations in reproductively active female whales. The PCB and PBDE congener pattern in mothers changed during gestation as a result of preferential transfer of light-low Log K_{OW} congeners to the fetus. Overall, female beluga whales transferred approximately 11% of their PCB and PBDE blubber burden to their fetus. In terms of vitamins transfer, lower concentrations of tocopherols, retinol and retinyl esters were found in reproductively active females relative to males and reproductively inactive females. Metabolism was also found to be an important factor for contaminant and vitamin accumulation in beluga tissues. In a principal components analysis, PCBs clustered into metabolically-derived structure-activity groups, which separated along the first principal component according to its metabolic potential (metabolizable vs. recalcitrant). Contaminant-related up-regulation of metabolizing

enzymes, including cytochrome P450, likely explained changes in the concentration and pattern of PCB and PBDE congeners, as well as hepatic, plasma, and blubber vitamin A and E.

Since vitamins and lipophilic contaminants accumulated in beluga whales in the same way in relation to most biological processes, including sex, reproduction, size, condition, and feeding ecology, it was important to control and reduce the number of these confounding factors before claiming any tissue vitamin change was indeed the result of chemical exposure. In doing so, it was found that vitamin A and E homeostasis was influenced by PCBs in beluga whales, resulting in reduced hepatic storage and increased plasma and blubber concentrations. Overall, these results suggest that liver, plasma, and inner blubber vitamin A and E concentrations can be sensitive biomarkers of contaminant exposure only if major confounding effects are taken into consideration. The implications of altered vitamin dynamics on the health of beluga whales is unknown at this time; however, as Arctic marine mammals face continued stress related to climate change, increased human disturbance and emergence of infectious diseases, this study can serve as essential baseline data that can be used to monitor the health status of western Arctic beluga whales.

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List of Abbreviations

AhR – aryl hydrocarbon receptor	PCA – principal component analysis
AIC – Akaike Information Criteria	PCB – polychlorinated biphenyl
CYP – cytochrome P450	PDBE – polybrominated diphenyl ether
DDT - dichlorodiphenyltrichloroethane	POP – persistent organic pollutant
DeROH - dehydroretinol	PUFA – polyunsaturated fatty acid
FA – fatty acid	RBP – retinol binding protein
HPLC – high performance liquid chromatography	ROH - retinol
K _{OW} – octanol-water partition coefficient	SAG – structure activity group
MI – metabolic index	SEM – standard error of the mean
MUFA – monounsaturated fatty acid	SFA – saturated fatty acid
PBT – persistent, bioaccumulative and toxic	TOC - tocopherol
	TTR - transthyretin

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Chapter 1

Introduction

1.1 Western Arctic beluga whales (*Delphinapterus leucas*)

The beluga whale (*delphinapterus leucas*) is the most abundant odontocete, or toothed whale, in Arctic waters. Its distribution is circumpolar and covers most seasonally ice-covered waters in the northern hemisphere (Burns & Seaman 1985, Harwood & Smith 2002). Beluga whales typically spend the winter in ice covered offshore waters and migrate hundreds to thousands of kilometers to warmer coastal waters following pack-ice break-up presumably to molt, feed, and rear their young (Burns & Seaman 1985, O’Corry-Crowe et al. 1997). The Beaufort Sea stock of beluga whales share wintering grounds in the Bering Sea with several Alaskan beluga stocks as well as stocks that summer in Russian waters (Burns & Seaman 1985, O’Corry-Crowe et al. 1997). In the summer, the Beaufort Sea whales aggregate in nearshore waters of the Mackenzie estuary and the Beaufort Sea/Amundsen Gulf according to sex and life stage (Fig. 1.1). Beluga habitat use is characterized by differences in sea-ice and bathymetry, which are differential selected according to reproductive status and animal size, such that the following habitat groups have been defined: 1) shallow coastal waters selected by females (with and without calves) and small males (<4 m long); 2) sea-ice edge selected by large females (>3.7 m) and medium length males (3.8-4.3 m); and 3) closed sea-ice in deep offshore waters selected by large males (>4 m) (Loseto et al. 2006).

The diet of Beaufort Sea beluga whales is not well characterised due to the inherent difficulty of observing feeding behaviour in Arctic marine mammals and the absence of stomach contents of hunted whales. Dietary biomarkers, including fatty acids

and stable isotopes, have recently been used to describe beluga feeding ecology and have shown differences in diet that followed observed size-related habitat groupings (Loseto, Stern, Deibel, et al. 2008, Loseto et al. 2009). Furthermore, size and dietary biomarkers drove mercury uptake and biomagnification in beluga whales (Loseto et al. 2008, Loseto et al. 2008). As long lived, high trophic level predators, beluga whales are particularly vulnerable to changes in diet that result in altered food web dynamics (i.e., additional step in food web) and therefore the biomagnification of persistent, bioaccumulative pollutants. In light of the recent reports of accelerated warming and consequent reduction in sea-ice extent (Serreze et al. 2007), it is likely that food web dynamics will be altered for this ice-associated whale, which may lead to altered accumulation of contaminants.

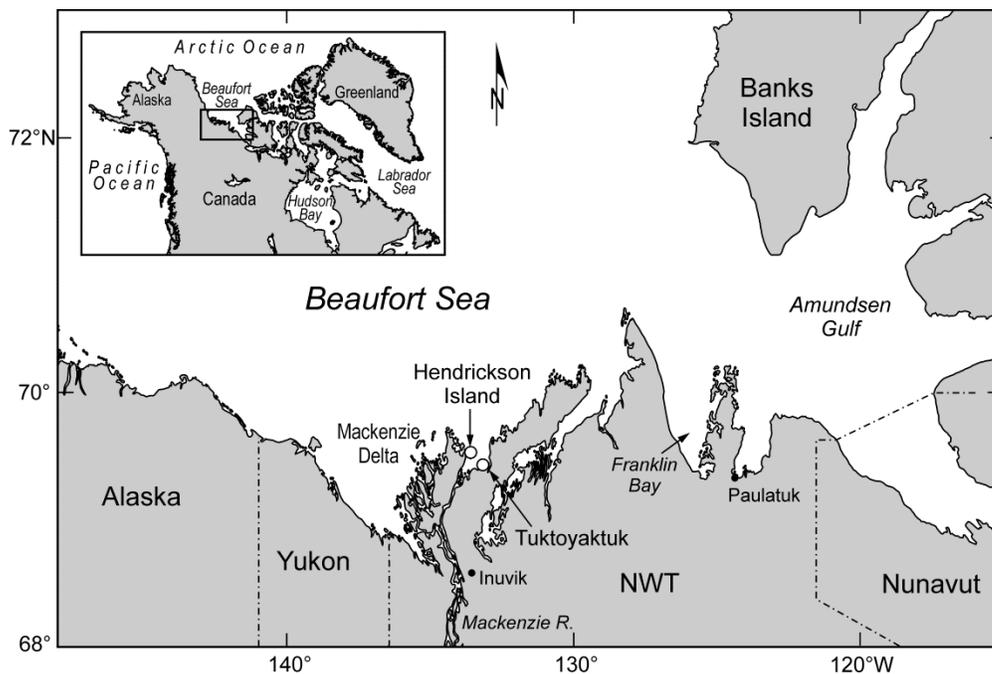


Figure 1.1 Beluga whales were harvested as part of the traditional Inuvialuit beluga hunt on Hendrickson Island, near the community of Tuktoyaktuk, Northwest Territories Canada.

1.2 Persistent organic pollutants (POPs)

There are thousands of anthropogenic compounds considered as pollutants and these are typically grouped together based on their similarity in characteristics such as molecular structure, physical and chemical properties, and biological activity (Jones & DeVoogt 1999, Newman & Unger 2003). Persistent organic pollutants (POPs) are a class of chemicals with a characteristic ability for transport over large geographical areas and bioaccumulation to high levels in biota. The first POPs produced on a large scale included dichlorodiphenyltrichloroethane (DDT), polychlorinated biphenyls (PCBs) and Chlordane, which were used extensively in industrial and agricultural practices (Macdonald et al. 2000). In light of the mounting evidence for the widespread distribution and toxic effects of POPs, the United Nations developed an international treaty to reduce or eliminate the release of toxic pollutants to the environment. The Stockholm Convention was thus adopted in 2001, and implemented in 2004, with specific goals to phase out the top 12 POPs (Ross 2006).

Polychlorinated biphenyls are a legacy contaminant, used as heat-resistant oils in electric transformers and capacitors and as industrial lubricants until they were banned in the late 1970's by most industrialized nations due to their toxicity and prevalence in global environments (Ross 2006). The biphenyl structure of PCBs results in the possible formation of 209 congeners defined by the number and position of chlorine atoms around the biphenyl (Fig.1.2). These structural characteristics also define the physicochemical and toxicological properties of PCBs; chlorine substitutions in the position nearest the biphenyl link (*ortho* position) forces the benzene rings to rotate out of the planar configuration (i.e., non-coplanar PCBs) whereas a planar configuration occurs in congeners lacking *ortho* chlorine substitutions (i.e., coplanar PCBs) (WHO 1993,

purely BDE 209) (Ross 2006, DeWit et al. 2009). Penta and octa formulations have been banned or restricted in most developed countries since 2004 while the deca formulation is currently being phased out in Europe and North America (Shaw et al. 2008).

The accumulation of contaminants into biota is a function of two processes, bioconcentration and bioaccumulation. Bioconcentration is defined as the accumulation of a contaminant in an organism from its surrounding environment (i.e. water or air), whereas bioaccumulation encompasses accumulation from the environment as well as from ingestion of food (Newman & Unger 2003). In aquatic environments, POPs enter at the bottom of the food web, via plankton and fish, and their concentrations are amplified at each trophic level in the food chain, a process referred to as biomagnification.

Accumulation in marine organisms can be represented by partitioning between water and lipid (organism), which can be estimated using the octanol-water partition coefficient (K_{OW}) (Mackay & Fraser 2000). Marine mammals are particularly vulnerable to biomagnification as they maintain large lipid stores and are typically long-lived, high trophic level predators with a relative inability to metabolize organic pollutants (Ross et al. 2000). Elevated concentrations of complex mixtures of POPs in marine mammals is troublesome as these toxic pollutants have been linked with a wide array of adverse health effects in laboratory, captive and free-range animals, including reproductive impairment, development toxicity, hepatic toxicity, carcinogenesis, dermal toxicity, immunosuppression, neurotoxicity, and endocrine dysfunction (Safe 1984, 1994, Ross, DeSwart, Addison, et al. 1996). Although causal relationships are difficult to prove, a “weight of evidence approach” can be applied in which results from several experiments are brought together to highlight unifying effects. This approach is particularly useful in

marine mammal toxicology due to the inherent logistical, ethical and legal constraints associated with sampling or capturing often endangered or at risk animals (Ross et al. 2000).

The accurate description of POP accumulation in marine mammals is extremely difficult due to complex life history of free-ranging animals. Numerous biological and ecological factors can influence contaminant concentrations and patterns in biota, and many of these factors can interact and confound each other through time (Borgå et al. 2004). The influence and interaction of sex and age on POP accumulation are the most frequently reported predictor variables in marine mammals as these have been shown to have important implications on contaminant tissue concentrations (Ross et al. 2000). Concentration of POPs tend to increase with age in males while they often decrease in females once they reach reproductive maturity due to maternal offloading during gestation and lactation (Borrell et al. 1995, Desforges et al. 2012). Since organic pollutants are strongly lipophilic, their accumulation can be significantly influenced by tissue lipid content and lipid dynamics (Krahn et al. 2004). The effect of lipid is typically addressed by lipid-normalizing contaminant data (Hebert & Keenleyside 1995). There are however many other confounding factors much more difficult to assess and interpret in wild animals, including body size and condition, disease, habitat use, migration, feeding ecology, and metabolism (Borgå et al. 2004).

Care must be taken in the design of marine mammal field studies to reduce the number or account for as many confounding factors as possible when addressing questions regarding specific biological processes. This is often difficult or logistically impossible, such that researchers have developed biological or chemical indicators

capable of summarizing complex natural processes. These “biomarkers” are invaluable tools to wildlife studies and can be applied to diverse biological /biochemical processes (Best & Schell 1996, Fossi 1998, Budge et al. 2008, Young et al. 2009).

1.3 Biomarkers of chemical exposure and effect

Though experiments can be easily devised to examine the acute or short term toxicity of a chemical on a laboratory rodent or fish, legal and ethical constraints prevent similar toxicity testing on large marine mammals. Furthermore, marine mammals are chronically exposed to a complex mixture of environmental pollutants, often at sub-acute concentrations, while undergoing natural biological phenomena throughout their life span (i.e., reproduction, migration, moulting, etc). In light of these complexities, biomarkers have been developed to evaluate the “health” of wild populations (Fossi et al. 1992). A biomarker can be defined as a biochemical, cellular, physiological, or behavioural change that can be measured in a biological system, providing evidence of exposure to, or toxic effects of, one or more contaminants (Depledge & Fossi 1994). In terms of population or ecosystem monitoring, the purpose of a biomarker is to detect an exposure-related change on a relatively small biological scale in order to provide an early warning signal of adverse effects at higher levels of biological organisation where damage can be significant and irreversible (Fossi 1998, Newman & Unger 2003).

Biomarkers can be useful tools to measure the integrated exposure of complex mixtures of environmental contaminants over space and time; however, a biomarker should meet all or most of the following criteria before it is developed and applied in ecological settings. First, it should be measured before any significant adverse effects at high levels of biological organization (i.e., population). Second, measurement should be

rapid, inexpensive and easily accomplished. Third, a biomarker should be highly specific and be based on a well-characterized mechanism of action. Fourth, a biomarker should be unaffected by confounding factors, thus representing a clear contaminant response. Fifth, a biomarker should have a strong dose-response relationship, whereby continued exposure leads to higher levels of ecologically relevant harm. Lastly, the ideal biomarker should be applicable to a wide range of sentinel species (Fossi 1994, Newman & Unger 2003). Despite the rigorous process for adequate biomarker selection, Fossi et al. (2012) noted that biomarkers rarely provide a specific or definite value of chemical exposure or severity of effect. Instead, the usefulness of biomarkers is in their unique ability to integrate the impact of multiple stressors and add to a “weight of evidence approach” in which the response of several biomarkers can more confidently indicate chemical exposure and/or effects (Fossi et al. 2012).

There are a number of biomarkers currently used for environmental monitoring of organic pollutants. Common biomarkers measure enzyme inhibition or induction and immunological and endocrine protein responses to chemical exposure (Fossi 1998). One of the most commonly used indicators of exposure is the induction of cytochrome P450 mixed function oxidases. This family of enzymes is important in the detoxification of xenobiotics, and their induction in wildlife occurs dose-dependently with chemical exposure (Fossi et al. 1992, Fossi 1998). Since cytochrome P450 enzymes relate specifically to organic pollutants, their activity have been used as indicators of response to PCB and other halogenated hydrocarbons (Fossi et al. 1992, Fossi 1994, Wolkers 1999). Another promising group of biomarkers for chemical exposure in wildlife are fat soluble vitamins.

1.4 Use of vitamin A and E as biomarkers of chemical exposure

Vitamin A is a collective term for a group of structurally similar lipophilic compounds possessing the biological activity of retinol, the parent vitamin A form. In mammals, vitamin A (also referred to as retinoids) is an essential nutrient and plays an important role in a wide variety of physiological processes including vision, growth and development, and the maintenance of reproductive, endothelial, endocrine and immune systems (Wolf 1984, Blomhoff 1994). Despite their importance in mammalian physiology, retinoids are not produced endogenously and must be acquired through diet. Retinoid imbalances, including deficiency and hypervitaminosis, have been associated with severe dysfunctions including reproductive impairment, embryonic mortality, growth retardation, bone deformities, and immunosuppression (Borrell et al. 2002, Blomhoff & Blomhoff 2005).

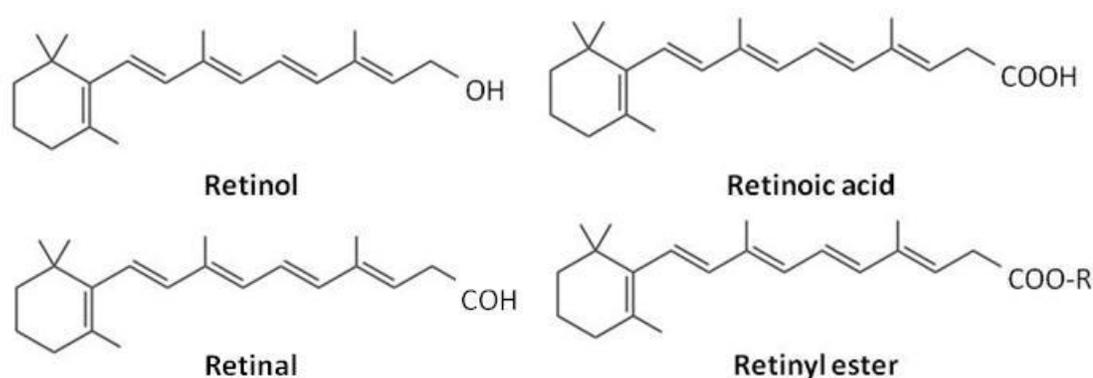


Figure 1.3 Basic structure of the four major vitamin A forms used for transport, storage and molecular action.

Vitamin A physiology, and therefore biological function, is defined by several different forms of retinoid compounds: retinol, retinal, retinyl esters and retinoic acid (Fig.1.3). Retinol is the parent compound and sustains most vitamin A functions. Retinol

circulates in blood bound to its transport protein (retinol binding protein (RBP)), which itself binds with the transport protein for thyroid hormone (transthyretin), forming a complex of retinol-RBP-transthyretin-thyroxine (Blomhoff 1994). This complex delivers vitamin A to target cells, in which retinol can be enzymatically converted to its active form, retinoic acid. Retinal is found in the retina of the eye and is an important component of the visual cycle (Wolf 1984). Retinoic acid is the active hormone form of vitamin A because once bound to its cellular binding protein it behaves as a transcription factor to activate specific nuclear retinoid receptors and thus regulate protein synthesis (Napoli 1996). Retinoids can also be delivered to tissues and stored as long chained fatty acid esters of retinol. Retinyl esters make up the largest fraction of total body vitamin A, with major storage sites found in liver and adipose tissue (Käkelä et al. 2002, Blomhoff & Blomhoff 2005). In marine mammals especially, adipose tissue (blubber) holds a large portion of total body retinoids (40-60% in pinnipeds) (Schweigert et al. 1987, Borrell et al. 1999, Mos & Ross 2002).

There have been a number of studies that show environmental contaminants can disrupt retinoid homeostasis in mammals. In laboratory animals, the effects of chemical exposure can be seen after a single dose (Brouwer et al. 1988) or chronic exposure (Bank et al. 1989), and can affect concentrations of multiple forms of vitamin A in several tissues (Kakela et al. 1999, Rolland 2000, Käkelä et al. 2002). Semi-field and free-range studies on marine mammals have demonstrated a link between POPs and tissue and plasma retinoid concentrations (see review by Simms and Ross 2001). Both the parent compound as well as its metabolic by-products (hydroxyl metabolites) can alter retinoid

dynamics. In this manner, all retinoid disruption can be classified as an AhR-mediated or a metabolite-mediated effect (Simms & Ross 2001).

In the AhR-mediated disruption, POPs bind to the AhR and induce the up-regulation of metabolic enzymes, many of which are important for retinoid metabolism. The general phase I and II metabolizing enzymes, including cytochrome P450 and uridine diphosphate glucuronyltransferase, have been found to oxidize and conjugate vitamin A metabolites (Brouwer et al. 1988, Besselink et al. 1998, Kelley et al. 2000). Enzymes related specifically to retinoid metabolism can also be affected by AhR induction; enzymes used to esterify retinol (lecithin retinol acyltransferases) and hydrolyze retinyl esters (retinyl ester hydrolases) as well as those used to catabolise retinoic acid (CYP26), all exhibit altered dynamics in laboratory animals exposed to PCBs (Hakansson & Ahlborg 1985, Hakansson et al. 1989, Mercier et al. 1990, Zile 1992).

Metabolite-mediated effects are due to hydroxyl-metabolite binding to the circulatory retinol transport complex. Hydroxylated metabolites of PCBs and PBDEs are structurally similar to thyroid hormone (thyroxine) and can bind to the thyroid hormone transport protein (transthyretin), displacing thyroxine. Once bound, the metabolite induces a conformational change in the transport protein, reducing its affinity to RBP-retinol (Brouwer et al. 1986). With no binding to transthyretin, the RBP-retinol complex is not large enough to prevent glomerular filtration and is excreted by the kidneys (Kelley et al. 1998). Overall, the exposure to organic pollutants can alter the storage, transport, metabolism and signalling of retinoids in mammals.

Vitamin E is also a group of structurally similar fat soluble compounds, but its structure is characterized by a chromanol head attached to a phytyl chain (Fig. 1.4). There are eight vitamin E compounds, divided into four tocopherols and four tocotrienols. Tocopherols differ from tocotrienols in the saturation of the phytyl chain; tocotrienols have a triple unsaturated side chain while the tocopherol side chain is completely saturated (Herrera & Barbas 2001). The four forms of each compound (α , β , γ , and δ) differ in the number and position of methyl groups on the chromanol ring (Fig. 1.4). The different forms of vitamin E are endogenously produced only in plants, though α -tocopherol is the most abundant form in mammals and has the highest biological activity (Hacquebard & Carpentier 2005).

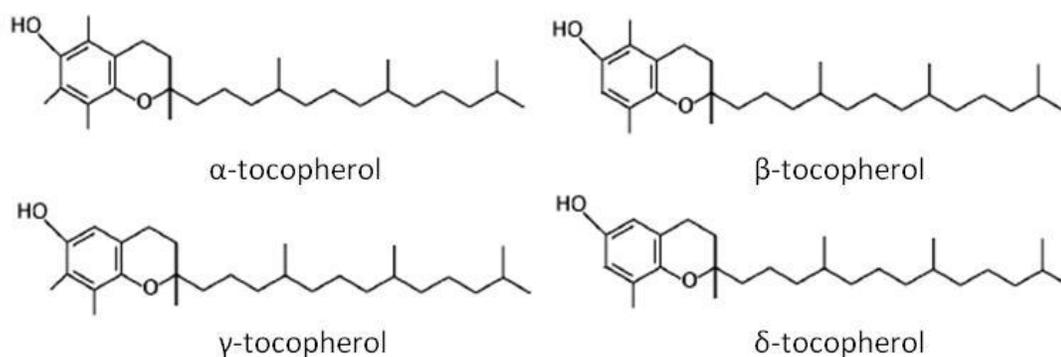


Figure 1.4 Structure of the four major vitamin E compounds found in mammals. The structures differ in the number and position of methyl groups on the chromanol ring.

Vitamin E is the most abundant and physiologically important lipid soluble antioxidant in the plasma and cells of most mammals (Rigotti 2007). Vitamin E functions as a chain-breaking antioxidant to prevent the propagation of free radical reactions in cell membranes and lipid rich environments; the phenolic hydrogen in tocopherol is donated to a peroxy radical (resulting from peroxidation of unsaturated lipids) creating a more

stable tocopheroxyl radical and reducing oxidative damage (Bramley et al. 2000, Herrera & Barbas 2001). Vitamin E concentrations are typically correlated to the quantity of unsaturated fatty acids in plants and animals, highlighting the importance of lipid dynamics for the biological requirement of vitamin E (Schweigert et al. 1990, Herrera & Barbas 2001). Because marine mammals accumulate high levels of unsaturated fatty acids from their diet, adequate tissue concentrations of vitamin E are particularly important to protect from lipid oxidation in their large blubber stores (Schweigert et al. 1990, Debier, Pomeroy, Baret, et al. 2002). In addition to its antioxidant role, there is increasing evidence that vitamin E plays an important role in other biological functions, including modulating cell signalling and proliferation, development and maintenance of the immune system, and modulating the activity of enzymes and the expression of genes (Zingg & Azzi 2004).

Persistent organic pollutants have been shown to affect the vitamin E status in several bird, fish and mammal species, including marine mammals (Saito 1990, Halouzka et al. 1994, Palace et al. 1996, Kakela et al. 1999, Nyman et al. 2003). Laboratory exposure to PCBs result in increased oxidative stress, likely resulting from AhR mediated induction of cytochrome P450 enzymes, leading to reduced hepatic vitamin E concentrations (Katayama et al. 1991, Kakela et al. 1999). Although few studies have examined vitamin E dynamics in marine mammals, the combined results indicate the potential for reduced hepatic concentrations, and increased plasma and blubber concentrations (Kakela et al. 1999, Nyman et al. 2003, Routti et al. 2005). Although the exact mechanism for increased plasma and blubber concentrations is unknown, it was

suggested that chronic exposure to PCBs leads to an adaptive response whereby oxidative stress increases the requirement for vitamin E (Nyman et al. 2003).

The combined results from laboratory, semi-field and free-ranging animal studies provide compelling evidence that PCBs and other organic pollutants can disrupt the dynamics of vitamin A and E in a wide variety of organisms. The extent of disruption is typically correlated to contaminant concentrations, suggesting a dose-response relationship (Novák et al. 2008). Vitamin A and E disruption is specific to contaminants that bind the AhR (i.e., halogenated hydrocarbons), biological effects occurs at relatively low levels of exposures, and analysis of their tissue or plasma concentration is rapid and relatively inexpensive. Furthermore, since these vitamins are important dietary hormones for biological functions such as reproduction, growth and development, significant alterations in their homeostasis may lead to population-level effects. For these reasons, vitamins A and E show promise as biomarkers of chemical exposure in wildlife. There remains, however, the question of sensitivity to confounding factors and the ability to identify a contaminant effect despite some level of natural variability.

1.5 Confounding factors limiting the use of vitamins as biomarkers

The variable results of vitamin-contaminant relationships in marine mammals highlight the caution that must be taken while interpreting tissue based contaminant effects. The differences between studies is likely the result of experimental design, whereby confounding factors such as species, age, sex, diet, condition, disease, reproductive status, moulting, trophic status and climate, are difficult to eliminate when sample sizes are low or when sampling is opportunistic (i.e., by-catch or strandings)

(Simms & Ross 2001, Borrell et al. 2002). There is therefore a need to better understand how natural processes affect vitamin dynamics in marine mammals.

Several studies have been undertaken in the past 15 years to better characterize the influence of biological processes on vitamin concentrations in marine mammals. Although these studies focus most often on retinoids, similar relationships are expected for vitamin E. A general increase of hepatic and blubber concentrations of vitamin A with age is the most common relationship observed in marine mammals (Rodahl & Davies 1949, Schweigert et al. 1987, Kakela et al. 1997, Borrell et al. 1999, Tornero et al. 2005, Rosa et al. 2007), though no trend or the opposite have also been reported (Rodahl & Davies 1949, Kakela et al. 1997). The positive age relationship with vitamin concentrations is suggested to result from a decrease in the circulatory clearance of retinoids with age, combined with continuous storage of retinyl esters due to excess vitamin intake via diet (Krasinski et al. 1989, Borrell et al. 2002).

Sex-related differences in marine mammals have been reported for several species (Rodahl & Davies 1949, Schweigert et al. 1987, Nyman et al. 2003, Tornero et al. 2005, Rosa et al. 2007), while others report no difference between males and females (Kakela et al. 1997, Borrell et al. 1999, Mos & Ross 2002, Tornero, Borrell, Forcada, & Aguilar 2004, Tornero, Borrell, Forcada, Pubill, et al. 2004). Though results vary, the most common trend appears to be higher tissue concentrations in males than females. The lower concentration in females is likely the result of the mobilization and transfer of a considerable portion of the mothers fat soluble vitamin burden to her offspring during lactation (Debier, Pomeroy, Baret, et al. 2002, Debier, Pomeroy, Wouwe, et al. 2002).

The high variation between studies may relate to species and sex differences in age, life-style and feeding ecology.

Vitamin A and E are strongly lipophilic, thus lipid dynamics are expected to have a strong influence on tissue levels of these vitamins. As with age and sex, vitamin concentrations have been found to be variable in relation to blubber lipid content (i.e., condition); condition correlated positively with blubber retinoids in some cases (Nyman et al. 2003, Tornero, Borrell, Forcada, & Aguilar 2004, Tornero et al. 2005), but not in others (Rodahl & Davies 1949, Borrell et al. 1999, Mos & Ross 2002, Rosa et al. 2007). It is possible that in healthy individuals, vitamin A and E status is more a reflection of age, sex and diet, while condition becomes more important in situations of lipid mobilisation (migration, lactation, food shortage, disease, etc) (Borrell et al. 1999, 2002).

As dietary vitamins, it would be expected that feeding ecology and diet strongly influence tissue vitamin concentrations. There are however very few studies that examine the influence of feeding ecology on vitamin levels in marine mammals. In a study of vitamin A supplementation in fur seals (*Callorhinus ursinus*), a fivefold increase in supplementation did not increase the vitamin disposal rate, suggesting that seals were accumulating excess vitamin A in storage tissues (Mazzaro et al. 1995). Tissue concentrations of retinol and dehydroretinol (found in freshwater fish) in ringed seals (*Phoca hispida* sp.) related most importantly to diet (Kakela et al. 1997). Similarly, liver tocopherol concentrations between seal populations was suggested to result mainly from dietary differences between marine and lacustrine seals (Kakela et al. 1997). In a study of mink, dietary levels of retinol and tocopherol were the main drivers of vitamin A and E concentrations in the liver of two different feeding groups (Kakela et al. 1999).

Furthermore, the percentages of several retinyl esters in the plasma of mink were found to relate to diet more so than contaminant exposure (Käkelä et al. 2003). In grey seals (*Halichoerus grypus*), hepatic vitamin A concentrations differed between Baltic and Sable island seals, a pattern which was reflected in the diet of each seal (Routti et al. 2005). In summary, it appears that dietary levels of fat soluble vitamins are a strong predictor of levels found in high trophic level predators.

Despite their variations in relationships with several biological processes, vitamin A and E fulfill most of the criteria for an ideal biomarker. These results do however reveal that research is needed to understand the dynamics and baseline levels of vitamin A and E in free-ranging marine mammals. In order to confidently establish contaminant-related effects on vitamin dynamics in studies of marine mammals, it is absolutely crucial to understand, minimize and account for natural physiological and ecological processes that may influence vitamin uptake and accumulation.

1.6 Research objectives

The overall goal of this thesis was to examine the accumulation and effect of PCBs and PBDEs in western Arctic beluga whales. The concentrations and pattern of persistent organic pollutants in marine mammals are the result of an integrated exposure through space and time. The contaminant profile at the time of sampling is therefore dependent on the life history of the animal, and it is only by a thorough examination of the many factors that can influence contaminant accumulation that it is possible to accurately describe the observed contaminant patterns. Coincidentally, many of the same factors that shape contaminant profiles also strongly influence fat soluble vitamin accumulation in marine mammals. In light of these considerations, the present thesis was

aimed at better understanding the factors that influence the accumulation of lipophilic contaminants and vitamins in beluga whales, and to determine if there is an interaction between the two. The specific objectives of this thesis were to:

- 1- Characterize the transplacental transfer of PCB and PBDE congeners in mother-fetus beluga pairs and to characterize the processes governing this partitioning;
- 2- Examine the role of dietary accumulation and metabolic elimination in influencing the blubber pattern of PCB and PBDE congeners in beluga whales;
and
- 3- Determine whether exposure to persistent organic pollutants and natural physiological and ecological factors affect tissue vitamin levels in beluga whales.

Chapter 2

Transplacental transfer of polychlorinated biphenyls and polybrominated diphenyl ethers in arctic beluga whales (*Delphinapterus leucas*)¹

Abstract

Arctic beluga whales (*Delphinapterus leucas*) transferred, on average, 11.4% (7.5 mg) and 11.1% (0.1 mg) of their polychlorinated biphenyl (PCB) and polybrominated diphenyl ether (PBDE) blubber burden to their near-term fetuses. A single physico-chemical parameter, Log K_{OW} , largely explained this transplacental transfer for PCBs ($r^2 = 0.79$, $p < 0.00001$) and PBDEs ($r^2 = 0.37$, $p = 0.007$), with congeners having a Log $K_{OW} < 6.5$ preferentially transferred to the fetus. Blubber concentrations of 257 ng/g lipid weight (lw) PCBs and 3.8 ng/g lw PBDEs in beluga fetuses highlights the exposure to endocrine disrupting compounds during a critical developmental stage. The implications of detecting these levels of legacy PCBs and the flame retardant PBDEs in unborn Arctic beluga are unclear.

¹ A modified version of this chapter is published in *Environmental Toxicology and Chemistry*:

Desforges J-PW, Ross PS, Loseto LL. 2012. Transplacental transfer of polychlorinated biphenyls and polybrominated diphenyl ethers in arctic beluga whales (*Delphinapterus leucas*). *Environmental Toxicology and Chemistry* 31(2):296–300.

Introduction

Persistent organic pollutants (POPs) such as polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) are ubiquitous in the environment and have a well-documented propensity to biomagnify in marine food webs (Muir & Norstrom 1994). Many marine mammals accumulate high levels of POPs, reflecting their often long lives, high trophic levels within aquatic food webs, and inability to readily eliminate these compounds. Exposure to lipophilic contaminants has been linked to immune dysfunction and neurotoxicity as well as disruption of endocrine and reproductive systems in marine mammals (Brouwer et al. 1989, Ross, DeSwart, Timmerman, et al. 1996).

Persistent organic pollutants are transferred from females to their offspring during gestation and lactation, thus exposing neonates to some of the highest levels of these endocrine disrupting compounds they will encounter during their lifetime (Addison & Brodie 1987). While there exists considerable variability in the degree to which POPs are transferred from female to offspring among species, lactational transfer in mammals during nursing commonly accounts for more than 80% of the total reproductive transfer (Borrell et al. 1995, Wolkers, Lydersen, et al. 2004). Nonetheless, POPs readily traverse placental membranes and may present a particular risk during gestation as thresholds for adverse effects are lowered during critical stages of fetal development (Borrell & Aguilar 2005).

The lactational transfer of POPs has been well described in marine mammals, but in utero exposure has rarely been documented because of difficulties in obtaining fetal samples from healthy pregnant females. Reports of prenatal exposure are almost

exclusively for PCBs and organochlorine pesticides (Tanabe et al. 1982, Aguilar & Borrell 1994, Borrell & Aguilar 2005, Greig et al. 2007), with only one report on transplacental transfer for PBDEs (Kajiwara et al. 2008). Furthermore, these studies often rely on opportunistic sampling of stranded and/or by caught animals of varying quality, and consider a limited dataset of congeners. Regardless, results from these reports indicate an inverse relationship between the degree of halogenation of contaminants and the mother-fetus transfer efficiency.

Through collaboration with Inuvialuit community subsistence hunters, we were able to take advantage of a unique opportunity to examine PCB and PBDE transfer dynamics in free-ranging healthy Arctic beluga whales (*Delphinapterus leucas*) during a critical juncture otherwise impossible to study in protected animals. The objective of the present study was to characterize the transplacental transfer of a full suite of PCB and PBDE congeners in two matched mother-fetus beluga pairs from Arctic Canada and to characterize the processes governing this partitioning.

Methods

Beluga samples were collected in 2008 and 2009 during the traditional harvest by Inuvialuit hunters at Hendrickson Island near the community of Tuktoyaktuk, in the Northwest Territories, Canada (69°30'N, 133°58'W) (Fig. 1.1). Full-depth blubber samples were taken from sites slightly dorsal of the pectoral flipper from the mothers and their near-term fetuses. Samples were wrapped in solvent rinsed foil, frozen at -20°C on site, stored in portable freezers, and shipped to Fisheries and Oceans Canada (Sidney BC, Canada) where they were stored at -80°C within two weeks of collection. Sub-samples

were taken to include all blubber layers and approximately 300 mg were analyzed for 205 PCB and 78 PBDE congeners by the Laboratory of Expertise in Aquatic Chemical Analysis (Fisheries and Oceans Canada) within 30 days of field collection. Extraction and clean-up procedures, instrumental analysis and conditions, and quality assurance/quality control criteria used for PCBs and PBDEs are described elsewhere (Ross et al. 2000). Because of varying degrees of moderate background contamination, all PBDE data were blank corrected. Because of analytical difficulties in reliably measuring highly brominated PBDEs, measurements of nona to deca congeners were not included. The percentage of lipid was determined from the remaining extract after drying under nitrogen flow. Congeners were quantified after resuspension in 1:1 dichloromethane-hexane by high resolution gas chromatography/high resolution mass spectrometry.

The mass of beluga whales and the blubber mass as percentage of total body weight was estimated from relationships described in Ryg et al. (1993). The total contaminant blubber burden, expressed in mg, was calculated by multiplying the body weight, the relative blubber mass (to total body weight), lipid content, and contaminant concentration (Borrell & Aguilar 2005). The contaminant transfer rate was defined as the ratio of fetal blubber burden to the combined fetal and maternal burden. Relationships between variables were determined by a linear regression analysis (Sigma Plot 10.0).

Results and Discussion

Analyzing these mother and near-term fetus pairs enabled us to evaluate the transplacental transfer of POPs prior to nursing during beluga's 10 to 14 month gestation period. Between 135 to 157 PCB congeners and 23 to 27 PBDE congeners were detected

in the four beluga samples. The dominant PCB and PBDE congeners were similar in all belugas; thus, the highest concentrated congeners were averaged and displayed in Table 2.1. The top 10 PCB congeners represented over 50% of the total concentration in the mother and fetus pairs. Similarly, BDE 47, 99, 100, 154, 49, 28/33, 66, and 71 accounted for over 95% of total PBDEs.

Table 2.1 Average blubber concentration (ng/g lw), estimated blubber burden (mg) and percent transfer from mother to fetus of the top 10 PCB and PBDE congeners in two mother-fetus beluga whale pairs.

		Mother concentration (ng/g lw)	Fetus concentration (ng/g lw)	Mother blubber burden (mg)	Fetus blubber burden (mg)	Transfer ^a (%)
PCBs	PCB 52	15.6	17.4	3.1	0.5	14.6
	PCB 95	11.7	12.1	2.3	0.4	13.9
	PCB 99	13.6	12.2	2.7	0.4	12.2
	PCB 101	16.6	17.7	3.3	0.5	14.0
	PCB 110	9.9	9.5	1.5	0.3	16.4
	PCB 118	13.5	13.7	2.6	0.4	13.6
	PCB 138/163	23.7	16.8	4.6	0.5	9.8
	PCB 149	12.1	10.1	2.4	0.3	11.3
	PCB 153	29.4	19.1	5.7	0.6	9.2
	PCB 187	11.4	3.6	2.2	0.1	4.6
	<i>ΣPCB</i>	<i>310</i>	<i>257</i>	<i>61</i>	<i>7.5</i>	<i>11.4</i>
PBDE	BDE 28/33	0.1	0.1	0.02	0.003	12.8
	BDE 47	3.4	2.5	0.7	0.07	13.4
	BDE 49	0.1	0.2	0.03	0.006	18.4
	BDE 66	0.09	0.06	0.02	0.002	10.7
	BDE 71	0.08	0.06	0.02	0.002	11.7
	BDE 99	0.7	0.4	0.3	0.02	7.7
	BDE 100	0.5	0.3	0.09	0.007	6.2
	BDE 153	0.05	0.01	0.009	0.0003	1.8
	BDE 154	0.2	0.05	0.04	0.001	2.9
	BDE 155	0.07	0.02	0.01	0.0005	3.8
	<i>ΣPBDE</i>	<i>5.5</i>	<i>3.8</i>	<i>1.1</i>	<i>0.1</i>	<i>11.1</i>

a- Transfer is defined as the ratio of fetal burden to the combined fetal and mother burden

The total concentration of PCBs and PBDEs were similar in the females and their prenatal offspring, with average values of 310 and 257 ng/g lw for PCBs and 5.5 and 3.8 ng/g lw for PBDEs in the mothers and fetuses, respectively (Table 2.1). Brominated diphenyl ether-47 represented 43 to 64% and 58 to 68% of Σ PBDE in the two mothers and two fetuses, respectively, consistent with the dominance of this congener in aquatic biota (DeWit et al. 2009). The concentration of top PCB and PBDE congeners in the mothers and fetuses differed by less than 10 ng/g lw in most cases (Table 2.1). Total PCB and PBDE concentrations in the mothers were considerably lower than the average level in six apparently non-reproductive adult females sampled during the same trip. Average female Σ PCB and Σ PBDE were 842 ± 503 and 21 ± 6.8 ng/g lw, respectively (L. Loseto, Fisheries and Oceans Canada, Winnipeg, Canada, Personal communication), with our beluga females representing on average 37% and 26% of these values. The lower values in these two females were consistent with their reproductive status, suggesting considerable transfer to their offspring; however, age cannot be ruled out as a driving factor as the mothers were considerably younger than the average age (23 and 30 vs 44). The Σ PBDE concentrations from the present study were similar to those found in western (9.3 ng/g) and eastern (12 ng/g) Canadian Arctic belugas, but far less than belugas from the St. Lawrence (535 ng/g) and Norway (72 ng/g) (Law et al. 2003, Tomy et al. 2009).

While congener-specific PCB and PBDE patterns appeared basically similar between mother and fetus, notable differences became evident when these concentrations were plotted as a function of the ratio of fetus to mother (Fig. 2.1). Because the partition trend was the same for both pairs, the values were averaged. The trends were similar for both PCBs and PBDEs, with a lower proportion of the heavier congeners appearing in the

fetus. For PCBs, congeners with five or fewer chlorine atoms were preferentially transferred to the fetus. The trend in the transfer rates calculated from total blubber burden clearly demonstrated the more ready transfer of lower weight congeners to the fetus compared to heavier congeners (Table 2.1). The transfer was highest for di-CBs (41%) and tri-BDEs (26%) and declined to low (1.2%) and null levels for nona-CBs and hepta- to octa-BDEs, respectively (results not shown). We calculated the overall average transfer rate as 11.4% for Σ PCBs and 11.1% for Σ PBDEs (Table 2.1).

To explore the transfer dynamics between mother and fetus, partition ratios were plotted against octanol-water partition coefficients (K_{OW}), which provides a measurement of lipid solubility (Fig. 2.2). We observed a significant negative correlation between $\log K_{OW}$ and the average partition ratio for PCBs ($r^2 = 0.79$, $p < 0.00001$) and PBDEs ($r^2 = 0.37$, $p = 0.007$). Greig et al. (2007) found a similar $\log K_{OW}$ -based partitioning between mother and fetus for 11 PCB congeners and 3 DDT isomers in California sea lions (*Zalophus californianus*). Our results indicate a similar mechanism of transfer of less lipophilic and lower molecular weight congeners to the fetus for both PCBs and PBDEs, with physico-chemical characteristics governing this transfer. Additionally, the point at which congener ratios diverged (i.e., at zero between mother and fetus) was $\log K_{OW}$ approximately 6.5 (corresponding to a molecular weight of ~ 350 Da), above which congeners were preferentially retained by the mother, while congeners below this value were readily transferred to the fetus.

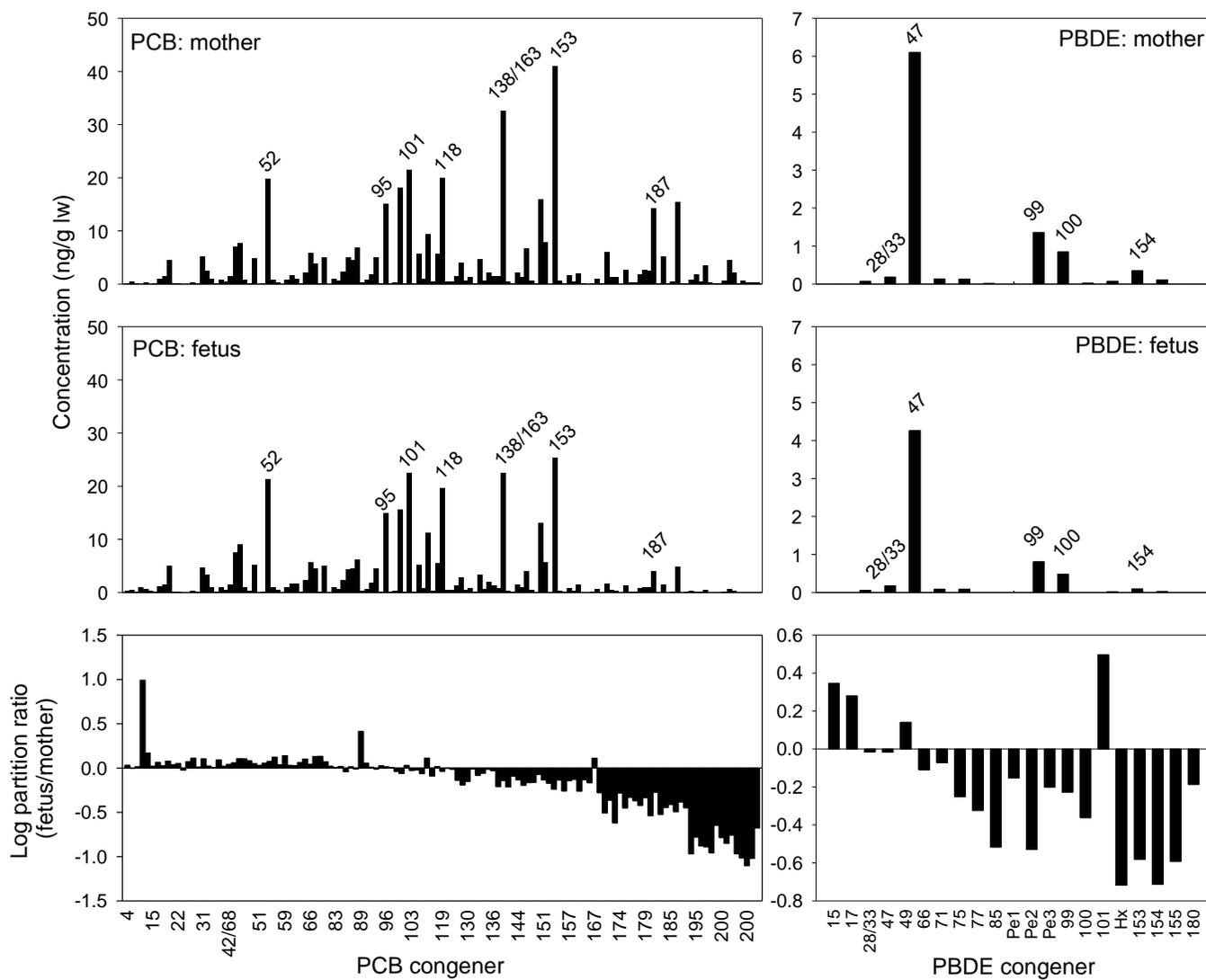


Figure 2.1 PCB and PBDE congener profile and partition ratios between mother and fetus beluga whales. Partition ratios were calculated as the blubber concentration in fetus divided by that in the mother, and were log transformed.

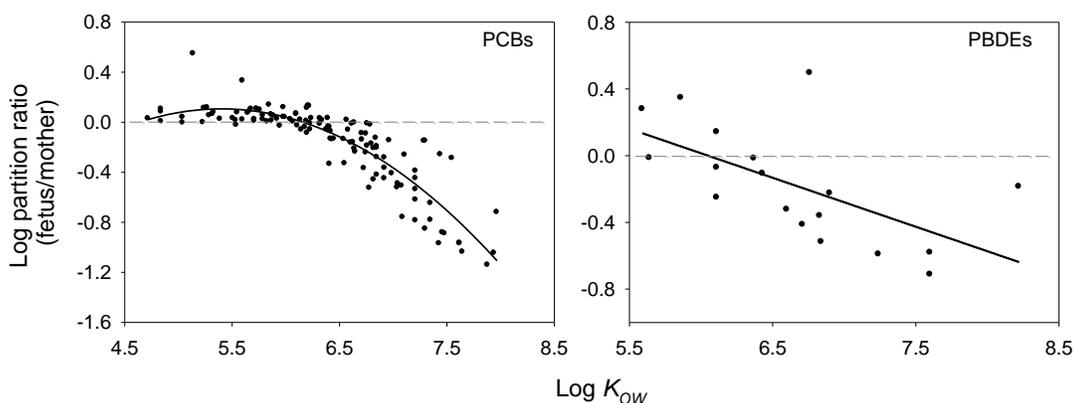


Figure 2.2 Average partition ratios plotted against logarithmic octanol/water partition coefficients ($\text{Log } K_{ow}$) for PCBs and PBDEs in two beluga mother-fetus pairs. $\text{Log } K_{ow}$ were taken from (Patil 1991, Makino 1998, Papa et al. 2009).

This relationship supports previous reports of a more efficient transplacental transfer of lighter congeners to the fetus as compared to high-molecular weight and more lipophilic congeners (Tanabe et al. 1982, Aguilar & Borrell 1994, Borrell & Aguilar 2005). In grey seals (*Halichoerus grypus*), a barrier between blubber and circulatory lipids was proposed, where transfer efficiency was inversely related to the degree of chlorination in PCBs (Addison & Brodie 1987). Tanabe et al. (Tanabe et al. 1982) described the transplacental transfer of organochlorines as being regulated by partitioning between mother and fetus blubber, whereby blood acts as a carrier. In this scenario, the lower affinity of high molecular weight congeners to polar lipids in the blood and placenta, as compared to nonpolar lipids in blubber (i.e., triglycerides), generates the partitioning trend observed between mother and fetus. These studies, as well as others describing blubber-blood or lactational transfer (Debieer et al. 2006, Ikonomidou & Addison 2008, Yordy et al. 2010), highlight the importance of basic physico-chemical properties in shaping the pharmacokinetics of POP partitioning in marine mammals.

While the transplacental transfer dynamics have been reported for PCBs in a number of marine mammals, to our knowledge, a study of the melon-headed whale (*Peponocephala electra*) is the only other that describes the transfer of PBDEs in a cetacean (Kajiwara et al. 2008). The results from that study agree with our findings describing the more efficient placental transfer of lower brominated PBDEs and complete resistance to transfer of hepta- to octa-BDEs. Our results add to the limited published reports on transplacental transfer of POPs; this information, together with lactational transfer, is useful in modeling the bioaccumulation of POPs in marine mammals. Because few studies are available, models of POP bioaccumulation often utilize non species specific transfer characteristics. Our results for transfer efficiency from mother to fetus for POPs are higher than those estimated in a St. Lawrence beluga model, which adapted placental transfer characteristics from Dall-type Dall's porpoise (*Phocoenoides dalli*) (Hickie et al. 2000). This highlights the importance of continued research into species-specific reproductive offloading of organic contaminants for the proper modeling of POP bioaccumulation in marine mammal populations.

Although the transport and fate of PCBs in the environment are reasonably well understood, PBDEs remain the subject of considerable attention. Lower-brominated PBDEs have been shown to exhibit similar long-range transport potential to PCBs; however, a full understanding of the bioavailability and trophic transfer of PBDEs remains somewhat elusive (Ross et al. 2009, DeWit et al. 2009). Our observation of the common governing role of Log K_{OW} for the transfer of both PCBs and PBDEs provides clear insight into the lipid-based transfer across cetacean placenta, an important component of the fate of persistent contaminants in marine mammals. This complements

those reports that describe the role of $\log K_{ow}$ in shaping the bioaccumulation and biomagnification of contaminants in marine food webs (Weijjs et al. 2009, DeWit et al. 2009).

The toxicological implications of the ready transplacental transfer of organic contaminants in the present study are unclear. Non-ortho PCBs (77, 126, and 169) and mono-ortho PCBs (105, 114, 118, 123, 156, 157, 167, and 189) were detected in the beluga fetuses, indicating potential toxicological risks through activation of the aryl-hydrocarbon receptor pathway (Ross et al. 2000). Although the Σ PCB toxic equivalents were below levels for adverse health effects as determined in harbour seal studies (Ross et al. 2000), perinatally exposed mammals are at a heightened risk of harmful effects compared to adults (Bleavins et al. 1984, Kihlstrom et al. 1992). This prenatal exposure to endocrine disrupting contaminants, at concentrations that are similar to those observed in adults, underscores the potential vulnerability of young Arctic beluga whales prior to exposure via nursing and subsequent feeding.

Chapter 3

Metabolic transformation shapes PCB and PBDE patterns in beluga whales (*Delphinapterus leucas*)

Abstract

While the accumulation of persistent contaminants in marine mammals can be attributed directly to their prey, the role of metabolism in shaping patterns is often overlooked. Here we investigate the role of metabolic transformation in influencing polychlorinated-biphenyl (PCB) and polybrominated diphenylether (PBDE) patterns in offshore and nearshore groups of beluga whales (*Delphinapterus leucas*) and their prey. Congener profiles and principal components analysis (PCA) revealed similar PCB and PBDE patterns in beluga whales feeding either offshore or nearshore, despite divergent contaminant patterns in the putative prey of these two feeding groups. The clustering of PCBs into metabolically-derived structure-activity groups (SAG), and the separation of metabolizable and recalcitrant groups along PC1 of the PCA, suggested an important role for metabolic transformation in shaping PCB patterns in beluga. Lack of metabolism for congeners with high *ortho*-chlorine content was revealed by metabolic slopes equal to or greater than 1.0. Metabolic slopes for all other structure-activity groups were <1.0 ($p < 0.001$), indicating metabolism of congeners with *ortho-meta* and *meta-para* vicinal hydrogens, suggestive of structure related induction of cytochrome-P450 enzymes (CYP1A/2B/3A). Metabolic indices <1.0 for PBDEs ($p < 0.001$) suggested that beluga are metabolizing these poorly understood flame retardants. The strikingly similar PCB patterns in a captive beluga and free-ranging beluga from the Beaufort Sea provide

additional support that metabolic transformation is a dominant driver of contaminant patterns in beluga.²

² A modified version of this chapter has been accepted for publication in *Environmental Toxicology and Chemistry*: Desforges J-PW, Ross PS, Dangerfield, N, Loseto LL. 2013. Metabolic transformation shapes PCB and PBDE patterns in beluga whales. *Environmental toxicology and chemistry*, in press.

Introduction

The wide range of physico-chemical properties exhibited by congeners of polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) presents a considerable challenge when studying these compounds; nevertheless, a number of studies have provided important insight into multi-media partitioning processes, environmental transport and fate, biological uptake, and food web structure (Wania & Mackay 1999, Macdonald et al. 2002, Krahn et al. 2007, Loseto, Stern, Deibel, et al. 2008). However, insight into physiological factors governing the uptake and loss of such persistent contaminants in marine mammals has been hampered by imprecise and incomplete knowledge of feeding ecologies for aquatic biota. Many studies have used contaminant concentrations and patterns, often in concert with stable isotopes and fatty acids, to identify discrete populations of marine mammals (Westgate & Tolley 1999, Krahn et al. 2007). Such studies document the influence of factors such as geographical separation and/or differences in diet in shaping contaminant patterns, but often fail to account for the role of metabolic transformation.

The importance of metabolic transformation of persistent organic pollutants in aquatic food webs has been inferred from an observed divergence in contaminant pattern between prey and predator (Tanabe et al. 1988, Boon et al. 1994). Boon et al. (1994) presented an early model whereby PCB patterns in marine mammals were compared to cod liver oil ('prey'). A differential bioaccumulation capacity was found for PCBs as some congeners were found to have increased susceptibility for enzymatic elimination. Numerous studies have since refined this model and confirmed the role of dietary exposure and subsequent biotransformation via metabolic cytochrome P450 enzymes (i.e. CYP1A/2B/3A) in shaping contaminant patterns in high trophic predators. In order to

build on such simplified 'prey' signals, studies of free-ranging marine mammals require information on the composition of diet in the real world. When more information is known, a food basket approach to diet which includes major known prey items can be a valuable tool to assess contaminant accumulation in marine predators (Cullon et al. 2005).

Polychlorinated biphenyls are legacy contaminants that were banned in the late 1970s by most industrialized nations due to their toxicity and prevalence in global environments. Polybrominated diphenyl ethers represent a more current-use flame-retardant in consumer products of which the manufacture and import are being phased out throughout Europe and North America (DeBoer 2009). Although similar in structure, slight differences in molecular structure and size between PCBs and PBDEs result in differences of important physico-chemical properties defining chemical transport and fate of their 209 congeners in the environment. For example, the higher range of octanol-water partition coefficients (Log K_{OW} : 5-10) and more rapid biological elimination ($t_{1/2}$ typically <500 days in fish) of PBDEs relative to the more persistent PCBs (Log K_{OW} : 4-8, $t_{1/2}$ up to >1000 days) illustrate important differences between these two contaminant classes (Niimi & Oliver 1983, Makino 1998, Papa et al. 2009, DiPaolo et al. 2010). Additionally, the environmental fate of PBDEs remains rather dynamic relative to PCBs due to their more current emission history (Ikonomou et al. 2002, Braune et al. 2005).

Through bioconcentration and bioaccumulation, PCBs and PBDEs enter the bottom of the food web and biomagnify to high levels in lipid tissues of top predators (Macdonald et al. 2002). Marine mammals are particularly vulnerable to biomagnification due to their long life span, high trophic level, and relative inability to

metabolize several organic contaminants. The increasing evidence of toxic injury associated with PCBs and related compounds in wildlife underscores the importance of carefully examining those factors that affect the accumulation and fate of such compounds. Free-range and 'semi-field' studies of marine mammals have provided strong evidence that contaminant exposure is linked to reproductive impairment, morphological deformities, immunotoxicity and endocrine disruption (Brouwer et al. 1989, Ross, DeSwart, Addison, et al. 1996, Ross 2000).

The beluga whale (*Delphinapterus leucas*) is the most abundant odontocete in the Arctic ocean, and as a high trophic level predator, plays an important role in the ecology of Arctic marine food webs (Loseto et al. 2009). Beluga whales are viewed as a sentinel of food web contamination in the Arctic because of their long life span, high trophic level feeding, and large fat reserves wherein lipophilic contaminants may be stored. During the summer months, beluga whales from the Beaufort Sea stock are known to segregate into habitat-use groups based on size and reproductive status, whereby females and small males (<4.2 m) select nearshore open-water and ice-edge habitats, while the larger males (>4.2 m) select closed sea-ice covered offshore waters (Loseto et al. 2006). This habitat segregation has been associated with divergent feeding ecologies, as evidenced by differences in stable isotopes, fatty acids and tissue mercury concentrations in the two major beluga habitat use groups (Loseto, Stern, & Ferguson 2008, Loseto, Stern, Deibel, et al. 2008, Loseto et al. 2009).

Our objective was to characterize the role of metabolic transformation in influencing the accumulation of PCBs and PBDEs in beluga whales. For this, we obtained samples of free-ranging adult male beluga whales and their putative prey from

the Beaufort Sea, in which we measured for 209 PCB and 78 PBDE congeners. By limiting our study to adult males, we avoided the confounding influences of losses associated with life history by adult females through reproduction, the preferential acquisition by neonates, and/or the influence of juvenile growth dilution (Hickie et al. 2000). The opportunity to examine contaminant patterns in a captive beluga whale and its diet provided a supplementary means of evaluating the role of metabolism in this cetacean species.

Methods

Sample collection

Beluga blubber samples were collected during the local Inuvialuit whale harvest at Hendrickson Island near the community of Tuktoyaktuk, in the Inuvialuit Settlement Region of the Northwest Territories Canada (Fig.1.1). A total of 59 adult male beluga were sampled in July 2007-2010, of which 18 were from 2007 (10 nearshore, 8 offshore whales), 19 from 2008 (10 nearshore, 9 offshore), 12 from 2009 (5 nearshore, 7 offshore) and 10 from 2010 (8 nearshore, 2 offshore). Full depth blubber samples were taken beginning at the skin and ending at the muscle layer from an area slightly dorsal of the flipper. Samples were immediately frozen on site at -20°C, stored in portable freezers and shipped to Fisheries and Oceans Canada (Sidney, BC) where they were stored at -80°C within two weeks of collection.

Prey samples were collected from various environments within the Beaufort Sea and surrounding area. Details on prey collection, morphometrics, age and stable isotope data are provided in Loseto et al. (Loseto, Stern, Deibel, et al. 2008). In brief, Arctic cod (*Boreogadus saida*) were harvested within 100 m of the ocean bottom in Franklin Bay

(offshore cod) and in the shallow waters of the Beaufort Sea Shelf north of the Mackenzie River outflow (nearshore cod). Only adults longer than 11 cm were selected for analysis. Arctic cisco (*Coregonus autumnalis*) and flounder (*Pleuronectes glacialis*) were harvested near the shoreline in the brackish waters of the Mackenzie Estuary. For both these species, only adult fish over 20 cm were selected. Finally, shrimp were collected within 60 cm of the sea floor and the decapod species *Eualus* and *Bythocaris* were identified and pooled for further analysis.

For a comparative analysis, a full depth blubber sample was taken from a deceased captive beluga whale from the Vancouver aquarium. The sample was taken from a juvenile female (3 years old) bred in captivity. This provided a useful case study whereby the exact diet of the whale was known throughout its lifetime. The beluga diet was unchanged during its lifetime, and prey were harvested once yearly from the same area in the Strait of Georgia, British Columbia. Diet consisted of Pacific herring (*Clupea pallasii*), capelin (*Mallotus villosus*) and California squid (*Loligo opalescens*), of which the whales' diet consisted of 66.5, 31 and 2.5% of each, respectively, over its lifetime.

Contaminant analysis

Beluga and prey samples were analyzed for PCB and PBDE congeners using high-resolution gas chromatography/high resolution mass spectrometry at the Laboratory of Expertise in Aquatic Chemical Analysis (Fisheries and Oceans Canada, Sidney, BC). Prey samples were pooled by species and homogenized so that the number of samples analyzed per species was one, but consisted of several individuals (offshore cod = 10, nearshore cod = 2, cisco = 4, flounder = 3, shrimp = 41). Reported concentrations here therefore reflect the results of pooled analyses for each species. Extraction and clean-up

procedures, instrumental analysis and conditions, and quality assurance/quality control criteria used for PCBs and PBDEs are described in detail elsewhere (Ikonomou et al. 2001). Of the possible 209 PCB and 78 PBDE congeners discernible by this analysis, 139 PCB and 30 PDBE individual or co-eluting congeners were detected consistently in beluga. All contaminant concentrations were blank corrected and lipid weight adjusted for more compatible comparisons between samples and species. Detection limit substitutions were made for congeners when 70% or more of samples had detectable congener concentrations. Where the 70% threshold was not reached, those congeners were not considered for further analysis.

Beluga food baskets

Previous reports have documented a difference in feeding ecology in beluga habitat use groups, suggesting a divergence in consumption of offshore and nearshore prey by large and small whales (Loseto, Stern, & Ferguson 2008, Loseto, Stern, Deibel, et al. 2008, Loseto et al. 2009). Here we use a food basket approach to diet as used in Cullon et al. (2005) as we believe it represents a more realistic picture of feeding behaviour than single prey models. For obvious reasons, detailed knowledge of the diet of individual whales is not known. The prey items that we selected are considered to be representative food types for beluga habitats; shrimp was used as proxy for invertebrates, flounder for benthic species, cisco for estuarine fish, and cod for marine and nearshore species. Based on the results of fatty acid analysis (Loseto et al. 2009), we estimated the relative proportion of prey in each food basket to be as follows: nearshore = 25% offshore cod, 40% nearshore cod, 25% cisco, 5% flounder and 5% shrimp; and offshore whales = 75% offshore cod, 15% nearshore cod, 5% flounder and 5% shrimp. Modifying

the proportions of the three dominant prey species did not result in any substantial difference in beluga metabolic potential (results not shown).

Structure-activity groups

PCB congeners were classified into structure-activity groups (SAGs) based on the original work of Boon et al. (Boon et al. 1994) and further expanded by Yunker et al. (Yunker et al. 2011). The SAGs are defined and characterized in terms of metabolic potential in marine mammals (Table 3.1). It is worth noting that congeners in SAG VI constitute a previously undefined group of remaining congeners with varying vicinal hydrogen placement and typically fewer than 2 *ortho*-Cl.

Metabolism

Principal component analysis (PCA) was used to characterize PCB patterns in beluga and their prey. Only congeners detected in 95% of whales were selected for PCA, and those congeners not detected were substituted with detection limits. The centered log ratio procedure was applied to the data in order to avoid negative bias in normalized data (Yunker et al. 2011). To remove concentration effects, data were normalized to total PCB concentration followed by geometric mean normalization of congener columns, and then log transformed and autoscaled before PCA. The results of the PCA were analyzed qualitatively and quantitatively by examining the plotting position of beluga (similarity of PCB profiles) and by correlating principal components to biological/chemical/physical parameters.

Table 3.1 PCB congeners are classified into structure activity groups (SAG) based on molecular structure and bioaccumulation potential in fish-eating marine mammals.

	Chlorine number	<i>Ortho</i> -Cl number	Vicinal H pairs		Metabolic potential ^a	
			<i>ortho-meta</i>	<i>meta-para</i>	pinnipeds	cetaceans
SAG I	5-10	1-4	0	0	low	low
SAG II	5-8	2-3	≥ 1	0	low	low
SAG III a	2-4	0-1	≥ 1	0	moderate-	moderate-
b	5-6	1	1-2	0	high ^b	high ^b
SAG IV	4-6	1-2	0	1-2	high ^c	low ^c
SAG V a	4-6	3-4	0	≥ 1	high ^c	low ^c
b	5-8	3-4	0	1		
SAG VI a	2-4	0-3	≥ 1	≥ 1	unknown ^{b,c}	unknown ^{b,c}
b	5-6	2-3	1-2	1-2		

a - based on work of Tanabe et al. (1988) and Boon et al. (1997)

b - congeners are biotransformed by CYP1A enzyme

c - congeners are biotransformed by CYP2B enzyme

Contaminant metabolism was estimated by comparing CB153 ratios ($R_{153} = \text{CB-X}$ or $\text{BDE-X} / \text{CB153}$) - CB153 representing a highly recalcitrant congener - for PCBs and PBDEs in beluga whale blubber with the same ratio in dietary items. The metabolic index (MI) was calculated as $R_{153 \text{ Beluga}} / R_{153 \text{ Prey}}$, thereby allowing values above one to signify accumulation exceeding that of CB153, and values below one to signify lesser accumulation. Metabolic slopes were also calculated as previously described in Bruhn et al. (1995). In brief, the R_{153} in beluga are plotted against the R_{153} in prey for congeners in each SAG group, with the slope of the linear regression line providing insight into metabolic processes. In this scenario, a slope above one indicates high accumulation and a slope below one indicates some level of metabolic elimination.

Table 3.2 Concentrations of the dominant PCB congeners in beluga whales, and these same congeners in their putative prey items from the Beaufort Sea. Concentrations are reported in ng/g lipid weight \pm standard error. Congener proportions of total PCB are reported in italics.

	CB 52	CB 95	CB 99	CB 101	CB 118	CB 138/163/164	CB 149	CB 153	Total PCB
Nearshore beluga	173 \pm 12	126 \pm 8	182 \pm 14	218 \pm 15	159 \pm 12	279 \pm 25	135 \pm 12	333 \pm 33	2813 \pm 210
	<i>6.1 \pm 2.5%</i>	<i>4.5 \pm 1.6%</i>	<i>6.5 \pm 2.8%</i>	<i>7.7 \pm 3.0%</i>	<i>5.6 \pm 2.5%</i>	<i>9.9 \pm 5.1%</i>	<i>4.8 \pm 2.4%</i>	<i>11.8 \pm 6.8%</i>	
Offshore beluga	246 \pm 13	180 \pm 11	287 \pm 21	340 \pm 19	245 \pm 17	454 \pm 40	204 \pm 15	507 \pm 43	4330 \pm 285
	<i>5.7 \pm 1.5%</i>	<i>4.2 \pm 1.3%</i>	<i>6.6 \pm 2.5%</i>	<i>7.8 \pm 2.3%</i>	<i>5.6 \pm 2.0%</i>	<i>10.5 \pm 4.7%</i>	<i>4.7 \pm 1.8%</i>	<i>11.7 \pm 5.1%</i>	
Offshore cod	8.77	4.08	4.33	8.24	6.29	9.17	5.83	10.5	162
	<i>5.4%</i>	<i>2.5%</i>	<i>2.7%</i>	<i>5.1%</i>	<i>3.9%</i>	<i>5.7%</i>	<i>3.6%</i>	<i>6.5%</i>	
Nearshore cod	3.76	1.42	1.75	3.82	2.57	3.56	2.52	3.92	54.5
	<i>6.9%</i>	<i>2.6%</i>	<i>3.2%</i>	<i>7.0%</i>	<i>4.7%</i>	<i>6.5%</i>	<i>4.6%</i>	<i>7.2%</i>	
Cisco	1.18	0.83	0.97	2.84	2.14	2.82	1.31	2.78	34.0
	<i>3.5%</i>	<i>2.4%</i>	<i>2.9%</i>	<i>8.4%</i>	<i>6.3%</i>	<i>8.3%</i>	<i>3.9%</i>	<i>8.2%</i>	
Flounder	0.55	0.62	1.29	2.02	1.65	4.59	2.11	7.81	40.0
	<i>1.4%</i>	<i>1.6%</i>	<i>3.2%</i>	<i>5.1%</i>	<i>4.1%</i>	<i>11.5%</i>	<i>5.3%</i>	<i>19.5%</i>	
Shrimp	1.34	0.40	2.66	2.33	3.70	5.75	1.24	7.06	53.7
	<i>2.5%</i>	<i>0.75%</i>	<i>5.0%</i>	<i>4.3%</i>	<i>6.9%</i>	<i>10.7%</i>	<i>2.3%</i>	<i>13.1%</i>	

Results and Discussion

PCBs and PBDEs in beluga prey

Concentrations of Σ PCBs in pooled beluga prey items ranged from 34 to 162 ng/g lw (Table 3.2). The congener pattern was dominated by CBs 153, 138, 118, 99, 101, 52, 149 and 110, but the relative importance of each congener varied between prey items. Offshore cod displayed a more even distribution of congeners compared to the more benthic/nearshore species (e.g. flounder and cisco), where the pattern was dominated by fewer, more halogenated congeners (Fig. 3.1). The lightest congeners (2-4 Cl) contributed a greater amount to the Σ PCB in the pelagic feeding offshore cod (~41%), compared to 30%, 29%, 17% and 3% in nearshore cod, shrimp, cisco and flounder, respectively.

Concentrations of Σ PBDEs in pooled beluga prey items ranged from 0.4 to 20.3 ng/g (Table 3.3). The predominant PBDE congeners were BDEs 47, 100, 99, 49 and 66, accounting for >90% of total PBDEs in all species (Fig. 3.2). Interestingly, BDE 99 was not detected in offshore cod and flounder. The more benthic/nearshore species (nearshore cod, cisco and flounder) had a greater number of detectable congeners and were characterized with an increased prevalence of penta and hexa homologs, specifically BDE congeners 100, 101, 153, 154 and 155.

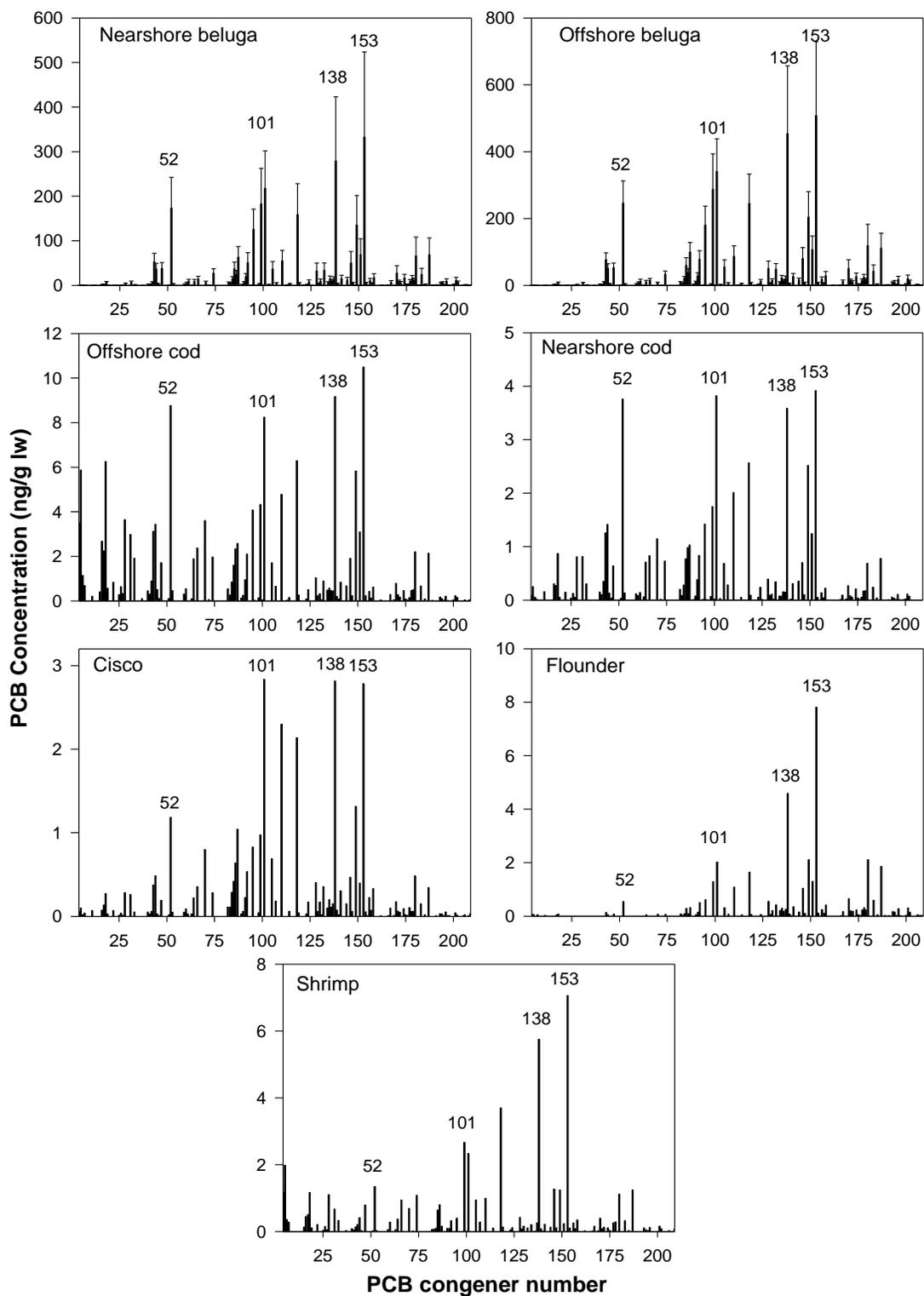


Figure 3.1 The concentration profiles of PCBs (ng/g lw) in nearshore and offshore beluga whales are noticeably different from those of their putative prey. Bars represent mean concentrations \pm standard deviation.

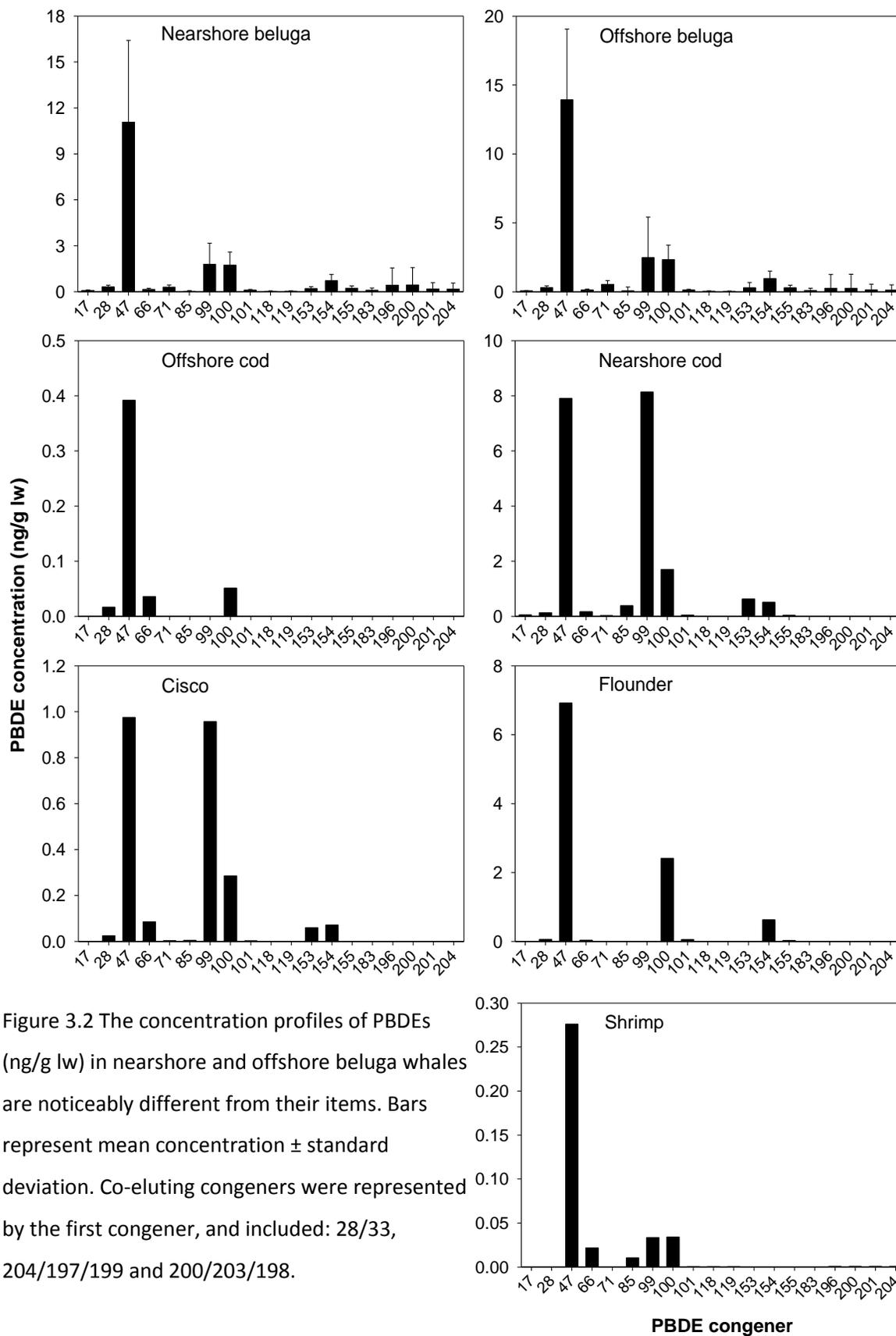


Figure 3.2 The concentration profiles of PBDEs (ng/g lw) in nearshore and offshore beluga whales are noticeably different from their items. Bars represent mean concentration \pm standard deviation. Co-eluting congeners were represented by the first congener, and included: 28/33, 204/197/199 and 200/203/198.

While the PCB and PBDE patterns in beluga prey items reflect a combination of many factors, the importance of the habitat in which they fed (i.e. nearshore vs offshore; benthic vs pelagic) was evident (Fig. 3.1& 3.2). Arctic cod caught in the Amundsen Gulf (offshore in the Beaufort Sea) was the prey item with the most marine/pelagic lifestyle among beluga prey; this corresponded with their high proportion of lower halogenated PCB and PBDE congeners. Conversely, heavier PCB and PBDE congener patterns were observed in the more benthic and nearshore feeding flounder and cisco. The divergent pattern between fish species is likely to be the result of feeding ecology as it relates to regional chemical partitioning between pelagic/marine and benthic/nearshore habitats; pelagic waters may have fewer highly halogenated and higher Log K_{OW} congeners, since these tend to partition more heavily onto sediment/particles and thus concentrate in benthic feeding species within nearshore areas (Scheringer et al. 2004). A similar divergent influence of marine vs terrigenous feeding regime was reported in British Columbia grizzly bears (Christensen et al. 2005). Bears feeding on a marine diet (salmon) reflected the generally lighter pattern associated with pelagic environments, while bears feeding more on terrigenous sources had PBDE patterns dominated by heavy congeners, including BDEs 209, 206, 207, and 208.

Table 3.3 Concentrations of the dominant PBDE congeners in beluga whales, and these same congeners in their putative prey. Concentrations are reported in ng/g lipid weight \pm standard error. Congener proportions of total PBDE are reported in italics.

	BDE 28/33	BDE 47	BDE 71	BDE 99	BDE 100	BDE 154	BDE 196	BDE 200/203/198	Total PBDE ^a
Nearshore beluga	0.31 +0.02 <i>1.7 \pm 0.6%</i>	11.1 +0.9 <i>60 \pm 29%</i>	0.29 +0.03 <i>1.6 \pm 0.8%</i>	1.78 +0.2 <i>9.7 \pm 7.5%</i>	1.74 +0.1 <i>9.4 \pm 4.6%</i>	0.72 +0.07 <i>3.9 \pm 2.2%</i>	0.42 +0.2 <i>2.3 \pm 6.1%</i>	0.44 +0.2 <i>2.4 \pm 6.2%</i>	17.2 +1.5
Offshore beluga	0.30 +0.02 <i>1.3 \pm 0.5%</i>	13.9 +1.0 <i>60 \pm 22.4%</i>	0.53 +0.05 <i>2.3 \pm 1.2%</i>	2.48 +0.6 <i>10.8 \pm 12.8%</i>	2.34 +0.2 <i>10.2 \pm 4.6%</i>	0.96 +0.1 <i>4.2 \pm 2.4%</i>	0.25 +0.2 <i>1.1 \pm 4.4%</i>	0.25 +0.2 <i>1.1 \pm 4.4%</i>	22.1 +1.8
Offshore cod	0.017 <i>2.5%</i>	0.39 <i>56.5%</i>	-- <i>--</i>	-- <i>--</i>	0.051 <i>7.4</i>	-- <i>--</i>	-- <i>--</i>	-- <i>--</i>	0.69
Nearshore cod	0.13 <i>0.64%</i>	7.90 <i>38.9%</i>	0.022 <i>0.11%</i>	8.13 <i>40.0%</i>	1.69 <i>8.3%</i>	0.51 <i>2.5%</i>	-- <i>--</i>	0.0048 <i>0.024%</i>	20.3
Cisco	0.024 <i>0.92%</i>	0.97 <i>37.3%</i>	0.0028 <i>0.11%</i>	0.96 <i>36.9%</i>	0.29 <i>11.2%</i>	0.071 <i>2.7%</i>	-- <i>--</i>	-- <i>--</i>	2.60
Flounder	0.055 <i>0.49%</i>	6.92 <i>61.2%</i>	-- <i>--</i>	-- <i>--</i>	2.41 <i>21.3%</i>	0.63 <i>5.6%</i>	-- <i>--</i>	-- <i>--</i>	11.3
Shrimp	-- <i>--</i>	0.28 <i>71.8%</i>	-- <i>--</i>	0.033 <i>8.5%</i>	0.034 <i>8.7%</i>	-- <i>--</i>	-- <i>--</i>	-- <i>--</i>	0.39

a- Total PBDE includes all di - octa brominated congeners

The degree to which metabolic transformation drives PCB and PBDE patterns in marine invertebrates and fish in our study is not clear. Although biotransformation processes for organochlorines are believed to be generally similar between fish and mammals, the reaction rates, contribution of different pathways, and metabolic products formed are different (Kleinow et al. 1987). Homeotherms have greater per-unit body weight energy requirements than poikilotherms, resulting in higher metabolic rates and caloric requirement, and effectively greater contaminant uptake and biotransformation (Braune et al. 2005). Moreover, studies of organochlorines in poikilotherms suggest that contaminant patterns relate more to exposure through water and diet than to metabolism (Braune et al. 2005, Yunker et al. 2011). Interestingly, the case may be different for PBDEs. For instance, a biomagnification model for PBDEs in an Arctic char (*Salvelinus alpinus*) food web was accurate only when metabolic debromination of BDEs -99, -100 and -153 to BDE-47 was considered (Gandhi et al. 2006). Furthermore, *in vivo* PBDE exposure in carp (*Cyprinus carpio*), chinook salmon (*Oncorhynchus tshawytscha*), rainbow trout (*O. mykiss*) and pike (*Esox lucius*) have illustrated the differential ability of fish to metabolize PBDE congeners (Stapleton, Letcher, & Baker 2004, Stapleton, Letcher, Li, et al. 2004, Roberts et al. 2011).

PCBs and PBDEs in beluga

The Σ PCB concentration in beluga ranged from 675 to 8306 ng/g lw. The average concentration was 3482 ng/g lw, however nearshore whales were less contaminated than offshore whales ($p < 0.001$). The recalcitrant congeners CB-153 and -138 dominated the PCB profiles in beluga, with the ranking of top PCB congeners being 153 > 138/163/164

> 101 > 99 > 52 > 118 > 149 > 95 (Table 3.2). These top congeners accounted for > 55% of Σ PCB.

The Σ PBDE concentration in beluga ranged from 7 to 48 ng/g lw, and did not differ between nearshore and offshore feeding groups ($p=0.08$). The predominant PBDE congeners in males were 47 > 99 > 100 > 154 > 49 > 71, accounting for >85% of Σ PBDEs (Table 3.3). As with prey, beluga were dominated by tetra (64%) and penta (22.4%) brominated congeners but had a higher contribution of heavy congeners relative to prey, particularly BDEs 153, 154, 155, 183, 196, and co-eluting congeners 200/203/198 and 204/197/199. Furthermore, the heaviest congeners showed a slightly higher contribution to total PBDE concentration in nearshore as compared to offshore whales.

At a glance, PCB and PBDE congener profiles are dissimilar between beluga and their putative prey; lighter congeners are proportionately less important in beluga relative to most prey species (Fig. 3.1 & 3.2). This provides the first indication of a preferential elimination of congeners from prey to predator, likely due to metabolic elimination. This is substantiated by the preferential trophic magnification of recalcitrant PCB congeners in the metabolic groups SAGI and SAGII, which together account for 28% of Σ PCB in prey but 47% in beluga whales, and the concurrent trophic elimination of metabolizable congeners in SAGIIIa and SAGVI groups (33% of Σ PCB in prey vs 16% in beluga).

PCB patterns in beluga

The robust PCB dataset (85 congeners) afforded the opportunity to use a PCA to explore congener patterns in this well studied contaminant class. An exploratory PCA was carried out using PCB data for all whales and prey items in order to compare patterns

between predator and prey. The clustering of beluga away from all prey items supported previous results of major pattern differences between prey and predator (Appendix 1, Fig. S1.1). A refined PCA model using only adult male beluga from the two feeding groups enabled a more detailed evaluation of the role of metabolism in shaping PCB patterns in whales. Fifty-five percent of the variance in PCB congener pattern was explained by the first two principal components (PC1: 41.9%, PC2:13.2%) (Fig. 3.3a,b). Despite the differences in PCB concentrations between the two beluga groups, the similar PCB histograms (Fig. 3.1) and the overlapping 95% confidence ellipses for PCA scores reveal remarkably similar congener patterns between these two different feeding groups (Fig. 3.3a). The considerable overlap of 95% confidence intervals (82% PC1 overlap) for beluga groups led us to pool data for all beluga whales for subsequent assessment of metabolism.

Highly chlorinated PCBs in SAGI and SAGII formed tight clusters projecting on the right side of the PCA loading plot while metabolizable congeners in SAGVI projected on the opposite side of the loading plot (Fig. 3.3b). Although projecting in more diffuse clusters, congeners with *m-p*-H and 4-6 Cl in SAGIV and SAGVa fell on the left side of the loadings plot and congeners with 5-8 Cl in SAGIIIb and SAGVb plotted on the right. There were only two congeners included in the PCA for SAGIIIa, such that 95% confidence ellipses were not calculated. The clustering of PCB congeners into predefined metabolic sub-groups (SAGs), combined with the clear distinction between metabolizable and recalcitrant groups along PC1, suggested a strong controlling effect of metabolic transformation in PCB accumulation.

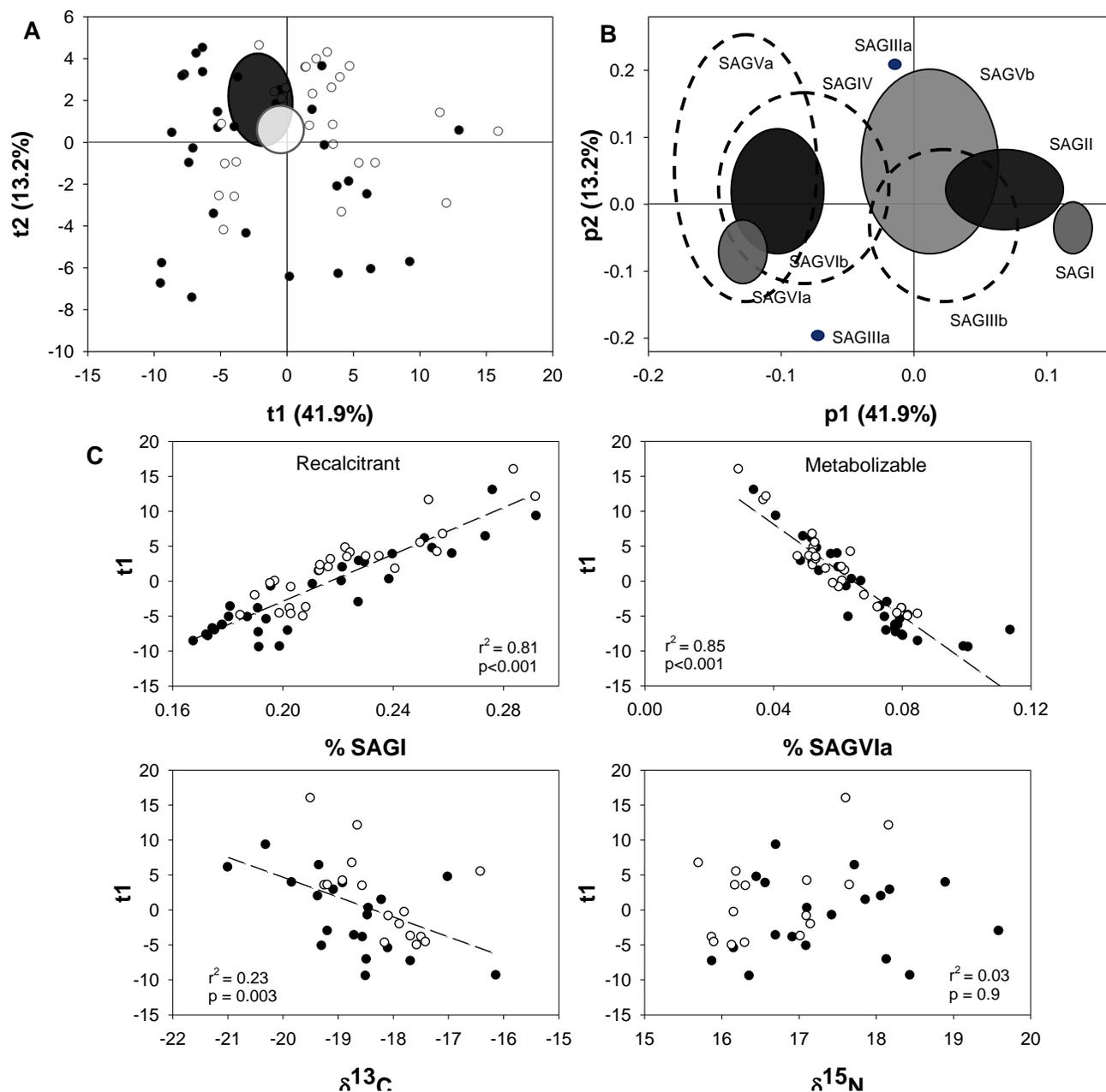


Figure 3.3 A principal component analysis (PCA) of 85 PCB congeners shows that metabolism shapes PCB patterns in adult male beluga. A) The PCA variable scores plot shows considerable overlap in the 95% confidence ellipses for nearshore (black filled circles) and offshore (white circles) whales. B) PCA factor loading was controlled by metabolic sub-groupings of PCBs into structure-activity groups, shown as 95% confidence ellipses. Only 2 congeners were included for SAGIIIa, so no ellipses were calculated. C) The first principal component (t1) was correlated with percent of total PCBs for Σ SAGI (recalcitrant) and Σ SAGVIa (metabolizable), stable isotopes of carbon but not stable isotopes of nitrogen.

Although metabolism likely represented a key driver of PCB patterns in beluga whales, several other chemical and biological factors clearly play a role in influencing the concentration of these contaminants. The elimination of certain compounds in beluga relative to prey may part result from differential absorption of congeners in the gut; however, absorption typically varies little between the lightest and heaviest congeners (Buckman et al. 2004, Thomas et al. 2005) such that the observed pattern change cannot be due to this alone (Fig. 3.1, Fig. 3.2, Fig. 3.3). Although contaminants typically accumulate in marine mammals with age (Ross 2000), age elicited no detectable effect on either concentration or patterns here ($p>0.05$, not shown). Trophic level did not influence PCB patterns in these whales as evidenced by the lack of correlation between the first principal component (t1) and $\delta^{15}\text{N}$ ($p=0.9$). Other factors such as $\delta^{13}\text{C}$ ($r^2=0.23$, $p=0.003$), length ($r^2=0.09$, $p=0.024$) and fatty acids ($r^2=0.13$, $p=0.007$) were correlated with t1 (Fig. 3.3c; Appendix 1, Fig. S1.1). The coefficients of determination for these relationships are low, suggesting that these biological variables explain a small proportion of the variation in PCB patterns among whales. While biological and ecological factors appear to play secondary roles in shaping PCB congener profiles, they do influence total concentrations in beluga whales (Loseto et al. in prep.).

The first principal component for the scores plot (t1) was positively correlated with ΣPCB ($r^2=0.62$, $p<0.001$; Appendix 1, Fig. S1.1). Similarly, t1 was positively correlated with the percent of total PCB for ΣSAGI ($r^2=0.81$, $p<0.001$), ΣSAGII ($r^2=0.82$, $p<0.001$), $\Sigma\text{SAGIIIb}$ ($r^2=0.21$, $p<0.001$) and ΣSAGVb ($r^2=0.81$, $p=0.37$) but inversely correlated for metabolizable $\Sigma\text{SAGIIIa}$ ($r^2=0.14$, $p=0.004$), ΣSAGIV ($r^2=0.85$, $p<0.001$), ΣSAGVa ($r^2=0.57$, $p<0.001$), ΣSAGVIa ($r^2=0.85$, $p<0.001$) and ΣSAGVIb ($r^2=0.69$,

$p < 0.001$) (Fig. 3.3c; Appendix 1, Fig. S1.2). Similar relationships were found between t_1 and SAG specific metabolic slopes (Appendix 1, Fig. S1.3). The divergent correlations for PCB congeners between the recalcitrant (positive) and metabolizable (negative) SAGs support our assertion that metabolism is explaining much of the variance in PCB patterns in beluga. Moreover, these correlations reveal a dose-response relationship whereby less recalcitrant congeners are increasingly eliminated as a function of contaminant concentration, resulting in an increased contribution of persistent congeners to the total concentration. This relationship is maintained despite eliminating the effect of concentration in the PCA data pretreatment, indicating a true pattern change with increasing dose. This suggests a concentration dependent activation of metabolic enzymes (i.e. CYP1A/2B/3A) that differentially metabolize contaminants based on molecular structure, as has been noted in marine mammals (White et al. 1994).

Structure-activity dependent metabolism in beluga

The metabolic index, calculated as the ratio of each congener to CB-153 in beluga relative to the same ratio in prey, has been used to assess dietary accumulation of contaminants in marine mammals (Wolkers, Van Bavel, et al. 2004). Values above parity ($MI > 1$) in our study were observed for numerous PCB congeners, of which the following were particularly high: CBs 182, 121, 181, 165, 112, 207 and 152 (Fig. 3.4). This quantitative dietary metabolic index clearly captures the preferential retention of more halogenated congeners in beluga relative to their prey.

Metabolic slopes did not differ between the two beluga groups for congeners of the more persistent SAGI, II, IV and Vb groups, but did for the more highly metabolizable SAG congeners (Fig. 3.5). Nevertheless, since the metabolic slope values

between SAGs were similar for both beluga groups, we combined results from the two feeding groups to reduce redundancy. The lack of metabolic transformation of congeners with high chlorine and low vicinal hydrogen content is reflected in beluga by metabolic slope values just above 1.0 for SAGI congeners (1.003 ± 0.008 ; $p=0.008$) and not different from 1.0 for SAGII congeners (0.978 ± 0.151 ; $p=0.27$) (Fig. 3.5). Metabolic slopes of 0.819 ± 0.189 , 0.718 ± 0.225 and 0.788 ± 0.143 in SAGIV, SAGVa and SAGVb were observed, indicating a relatively low metabolic transformation of congeners with *meta-para* hydrogen pairs (significantly different from 1.0 at $p < 0.001$ for all three groups). Furthermore, the higher metabolic slope as well as PCA projection near persistent PCBs (SAGI/II) for SAGVb congeners (5-8 Cl) relative to the lower slope of SAGVa (4-6 Cl) and its projection near metabolizable congeners (SAGIIIa/VI) illustrates the importance of the number of chlorines in determining metabolic potential in cetaceans. The highest metabolic capacity in beluga was observed for SAGIII and SAGVI congeners, which are characterized by a planar configuration and at least one *ortho-meta* hydrogen pair (Fig. 3.5). Within these PCB groups, the total number of Cl as well as the number of *ortho*-Cl strongly influenced metabolism; SAGIIIa (2-4 Cl) metabolism was greater than SAGIIIb (5-6 Cl), and metabolic slopes were significantly different between tri and tetra homologs within SAGVIb congeners (slopes = 0.065 and 0.515; $p < 0.001$).

Although we did not measure metabolic enzyme activity directly in this study, the metabolic slope results, corroborated by PCA, support previous reports on the presence and activity of cytochrome P450 enzymes. The low dietary accumulation of planar congeners with *ortho-meta* hydrogen pairs in this study is consistent with the relatively high CYP1A enzyme activity observed via *in vitro* hepatic microsomal protein assays for

beluga whales, as well as most marine mammals (White et al. 1994, McKinney et al. 2004). Similarly, McKinney et al. (2004) documented the presence of CYP2B proteins in beluga liver, albeit its activity was much lower than CYP1A enzymes. As congeners with *meta-para* hydrogen pairs are targeted by the CYP2B enzyme, the *in vitro* work appears to support the relatively low metabolic potential found in our beluga towards PCBs in SAGIV and SAGV.

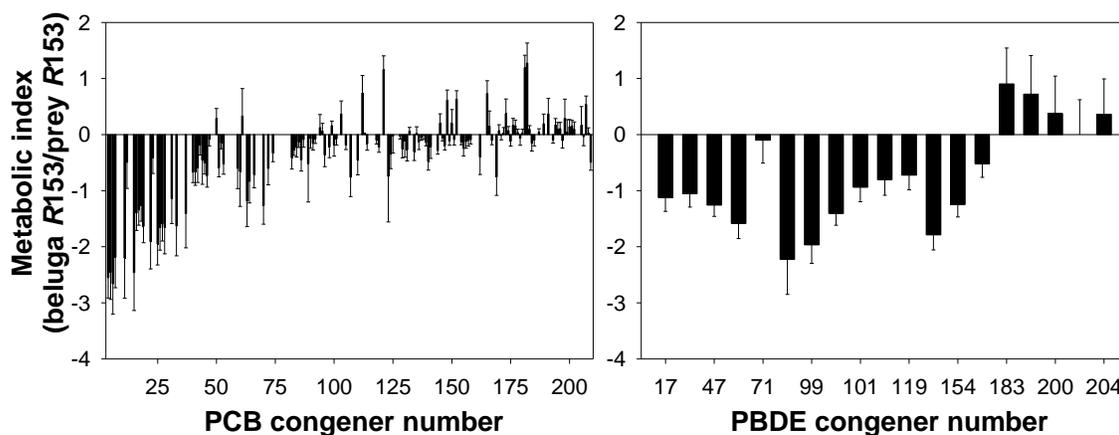


Figure 3.3 The average metabolic index of adult male beluga for PCBs and PBDEs shows increasing accumulation for more halogenated congeners. The metabolic index was calculated using specified food baskets based on feeding regimes for smaller nearshore and ice-edge associated whales and large offshore whales respectively. Values were log transformed.

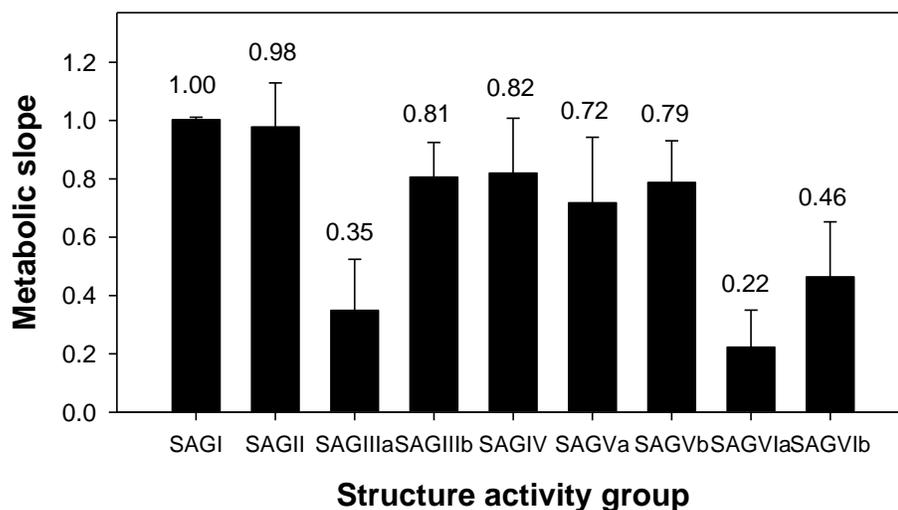


Figure 3.4 The metabolic slopes for PCBs in adult male beluga whales was strongly dependent on molecular structure and metabolic capacity in marine mammals, as summarized by grouping congeners into structure-activity groups (SAG). Values above each bar represent the mean slope for that SAG.

PBDE metabolism in beluga

The more limited congener dataset for PBDEs (19 congeners) prevented us from carrying out a similar pattern analysis using PCA to that carried out with the PCBs. Furthermore, since less information exists on the uptake and loss of PBDEs in marine mammals, an exploration of the role of metabolic transformation remained somewhat rudimentary. Nevertheless, the use of the MI approach did allow a basic exploration of dietary accumulation of PBDEs from prey to predator. Metabolic index values were below 1.0 for all PBDEs with the exception of hepta- and octa-brominated congeners; however high variation was observed in the accumulation of these congeners among whales (Fig. 3.4). Since no similar structure-activity grouping to PCBs currently exists for PBDEs, we calculated a metabolic slope by grouping all PBDE congeners together

and found it was an order of magnitude lower than slopes for PCBs ($r^2=0.67$, slope= 0.030 ± 0.019). Interestingly, BDE-47 and -99 contributed similarly in the food baskets ($R_{153}= 0.66$ vs 0.61 , respectively), but BDE-47 accumulated to a much greater extent in beluga than did BDE-99 ($R_{153}=0.03$ vs 0.005).

The more recent emission history of PBDEs renders it difficult to assess metabolism in Arctic biota because of its disequilibrium in food webs (Ikonomou et al. 2002, Braune et al. 2005). Our results for beluga suggested very little accumulation of PBDEs, which may be partly explained by high metabolic transformation. *In vitro* metabolism using beluga liver microsomes revealed significant elimination of BDEs 15, 28, 47 and 209, but none for BDE-49, -99, -100, -153, -154 and -183 (McKinney et al. 2006, 2011). Our MI results support the role for metabolism in eliminating BDE-28 and -47 and the lack of metabolism for BDE-183, but show a high metabolic capacity for most other congeners. Similar studies have consistently reported MI values below one for most PBDE congeners in marine mammals, including beluga, narwhal (*Monodon monoceros*), killer whales (*Orcinus orca*), ringed seals (*Pusa hispida*) and polar bears (*Ursus maritimus*) (Wolkers, Van Bavel, et al. 2004, Wolkers et al. 2006, 2007). This discrepancy suggests that either time constraints in the *in vitro* tests result in an underestimation of BDE metabolism or that estimating congener-specific metabolism of PBDEs in free-ranging marine mammals is problematic due to the complexities and non-steady state of this contaminant in arctic marine food webs. Nevertheless, there is evidence to suggest a decreased level of metabolism with increasing bromination of PBDEs in beluga. Further work is needed to understand and model the dynamics of PBDEs in aquatic food webs.

Metabolism in a captive beluga

PCB data from a captive aquarium beluga and its food items were included in the exploratory PCA with Beaufort Sea beluga in order to assess the extent to which patterns differed (Appendix 1, Fig. S1.1). Despite feeding on fundamentally different prey from different latitudes (i.e. arctic vs temperate environments), the captive whale PCB pattern was indistinguishable from those of wild Beaufort whales in the PCA plot. Furthermore, the aquarium food items clearly separated from all whales, underscoring once more that contaminant patterns in beluga reflect modification by metabolic transformation after dietary uptake.

The captive beluga provided a unique opportunity to evaluate metabolic transformation from prey to predator as exact dietary intake is known. The metabolic slope values for PCBs were considerably higher in the captive beluga relative to wild beluga for all SAGs with the exception of the highly recalcitrant SAG I and II congener groups, suggesting a much greater accumulation of all congeners from diet (Appendix 1, Fig. S1.4). Although the overall magnitudes were higher, the relative pattern between SAGs was similar in the captive and wild whales. For example, SAG IIIa and VIa, followed by VIb and Va were observed to be the most highly metabolized congener groups in the wild and captive whales alike. For PBDEs, the metabolic slope and metabolic index values for PBDEs were also considerably higher in the captive whale. Although higher, values remained below 1.0 for all PBDEs in the captive beluga, confirming our results in wild beluga suggesting significant metabolic elimination for all congeners.

The slight differences between captive and wild beluga could be due to several factors, including age, gender, captivity, and error associated in the assignment of prey to

the wild beluga. Age- and gender- related changes in hepatic cytochrome P450 expression have been reported in rats and fish (Perkins & Schlenk 1998, Yun et al. 2010), suggesting that the metabolic potential of the juvenile female beluga (age 3) may have been different from that of adults males (avg age =29). Captivity may also influence overall contaminant metabolism as the level of activity and diving behaviour, and therefore respiration and overall metabolic rates, may differ between captive and wild beluga. Finally, there is an inherent error in estimating metabolic capacities in free-ranging beluga as the exact dietary accumulation is unknown in this population. Despite these factors, the reasonable agreement in the profile of PCB and PBDE metabolism in wild and captive beluga supports our conclusion of an inferred activity of CYP1A/2B/3A in beluga whales which controls their accumulated (retained) PCB and PBDE profiles.

Conclusions

Divergent stable isotope ratios ($\delta^{13}\text{C}$, $\delta^{15}\text{N}$), Hg concentrations, fatty acid signatures, and overall PCB and PBDE concentrations observed in groups of beluga whales inhabiting nearshore vs offshore areas of the Beaufort Sea present a compelling picture of the implications of habitat selection on a marine mammal (Loseto, Stern, & Ferguson 2008, Loseto, Stern, Deibel, et al. 2008, Loseto et al. 2009). However, the remarkably similar PCB and PBDE congener patterns in the two feeding groups illustrate the very strong role played by metabolic enzymes in governing the overall accumulation of persistent contaminants. The direct evaluation of dietary accumulation and retention (MI and metabolic slopes) and the use of PCA provided strong support of the induction of CYP1A/2B/3A enzymes in beluga, something that was further corroborated in a captive whale with known diet. Since health risks to contaminant exposure in marine

mammals can be attributed to both parent contaminants and to their metabolites, our study provides a basis for the evaluation of the duality of risks attributed to the retained parent compounds and the inferred loss of reactive metabolites. Our findings should be considered as a cautionary note for researchers as they ponder the utility of using complex contaminant patterns as tracers of population segregation or divergent feeding ecologies.

Chapter 4

Persistent organic pollutants affect fat soluble vitamin profiles in Arctic beluga whales (*Delphinapterus leucas*) after accounting for effects of sex, age, condition and feeding ecology

Abstract

Beluga whales (*Delphinapterus leucas*) are long lived, high trophic level predators, and as such, biomagnify lipophilic contaminants to high levels in their tissues. Vitamins A and E are essential fat soluble nutrients and have been identified as potential biomarkers of contaminant exposure in wildlife. However, their use as biomarkers is limited without a thorough understanding of intrapopulation variation of vitamin dynamics in relation to natural conditions. We examined the influence of biological and ecological factors on vitamin A and E concentrations and patterns in liver, plasma and three layers of blubber from 66 healthy subsistence hunted beluga whales. Blubber was found to contain on average 77% (39.2 g) of the total body burden of vitamin A, but almost 99% (48 g) of total vitamin E in beluga whales. A gender effect was found whereby adult males had higher concentrations of vitamin A and E than reproductively active females of similar age. Principal component analysis (PCA) and Akaike information criteria selected multiple regressions revealed the importance of age, body condition and feeding ecology as predictors of tissue vitamin profiles. Total PCBs correlated with the first principal component ($r^2=0.13$, $p=0.014$) of a PCA of liver vitamins and was selected consistently as an important predictor of vitamin tissue concentrations in best fit multiple regression models, suggesting an important role for

contaminants in vitamin dynamics. A homeostatic effect of chemical exposure in beluga whales was further evidenced by significant correlations between PCBs and liver, plasma and blubber vitamin concentrations after eliminating the effect of confounding factors. Our findings are important as they indicate a likely toxic effect of PCBs in a relatively uncontaminated population of marine mammal and highlight the usefulness of tissue vitamin concentrations as a sensitive biomarker of chemical exposure.

Introduction

Beluga whales (*Delphinapterus leucas*) of the Beaufort Sea make-up the largest of at least 7 stocks in Canadian waters (Harwood & Smith 2002). The population migrates annually from wintering grounds in the Bering Sea to the warmer waters of the Mackenzie delta, Beaufort Sea and Amundsen Gulf, following the land-fast ice break-up (Norton & Harwood 1986). Beluga whales can live up to 60 years and weigh between 1500 - 2000 kg, though males grow to be considerable larger than females (DFO 2000). This size-dimorphism is associated with sea-ice and bathymetry dependent population segregation, whereby sex and life stage determine habitat use and feeding ecology (Loseto et al. 2006, 2008, 2009). Dietary biomarkers, including stable isotopes and fatty acids, have successfully described size-dependent feeding ecology in Beaufort beluga, which was found to have important implications for exposure to bioaccumulative contaminants (Loseto, Stern, & Ferguson 2008, Loseto, Stern, Deibel, et al. 2008). As long-lived, high trophic level predators, beluga whales are particularly vulnerable to the biomagnification of persistent, bioaccumulative, and toxic (PBT) pollutants, including polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs). Elevated exposure to complex chemical mixtures is of concern as these contaminants are associated with a wide-range of adverse effects, including reproductive impairment, developmental toxicity, neurotoxicity, and endocrine and immunologic dysfunction (Béland et al. 1993, Ross, DeSwart, Addison, et al. 1996, Ross 2002).

The use of biomarkers has emerged due to the need for diagnostic and prognostic tools to determine the exposure and effects of complex mixtures of environmental contaminants in humans and wildlife (Fossi 1998). Biomarkers have been defined several ways, but their general purpose is to provide a measure of biological change at the

individual or population level induced by one or more toxic contaminant (Fossi et al. 1992, Fossi 1994). Biomarker responses can range from genomic and immunologic toxicity to behavioural disturbance and altered growth and reproduction (Fossi et al. 2012). The use of this technique is particularly attractive for studies in marine mammals since the accumulation of complex contaminant mixtures is an integrated process through space and time, often via unknown dietary sources. Although biomarkers rarely provide a definitive value to describe the extent of chemical exposure or the severity of effect, they add to a “weight of evidence approach” which can be valuable to determine animal and ecosystem health (Fossi et al. 2012).

Fat soluble vitamins A and E are essential nutrients involved in several important biological processes, including growth, development, reproduction, protection against tissue damage, and proper immune and endocrine function (Blomhoff 1994, Debier & Larondelle 2005). Vitamin A is a collective term for a group of fat soluble molecules (also called retinoids) which include retinol, retinal, retinoid acid and retinyl esters. Specific roles have been identified for each retinoid, ranging from lipid storage (retinyl esters) to genomic transcription factors (retinoid acid) (Blomhoff 1994). Vitamin E is also a collective term and refers to several forms of tocopherols and Tocotrienols. Vitamin E is the most abundant antioxidant in vertebrates, where its function is to protect against oxidative stress in lipid rich environments (Palace & Werner 2006). Tissue levels of vitamin A and E have been shown to be affected by contaminants in laboratory and free-range animals (Bank et al. 1989, Katayama et al. 1991, Zile 1992, Simms & Ross 2001, Nyman et al. 2003). The important physiological function of these vitamins combined with the widespread evidence of contaminant disruption, supports the use of

vitamin A and E as biomarkers of chemical exposure in wildlife species. However, little is known about how natural physiological and ecological factors influence vitamin dynamics in free-ranging animals.

Studies in the past decade have begun to examine the influence of select biological parameters on vitamin concentrations in marine mammals. Animal condition, as described by blubber lipid content, was found to be a strong determinant of vitamin A in bottlenose dolphin (*Tursiops truncatus*), common dolphin (*Delphinus delphis*) and grey seals (*Halichoerus grypus*) (Nyman et al. 2003, Tornero, Borrell, Forcada, & Aguilar 2004, Tornero et al. 2005), but no effect was found in the bowhead whale (*Balaena mysticetes*), harbour porpoise (*Phocoena phocoena*), harbour seal (*Phoca vitulina*), harp seal (*Phoca groenlandica*) and hooded seal (*Cystophora cristata*) (Rodahl & Davies 1949, Borrell et al. 1999, Mos & Ross 2002, Rosa et al. 2007). Similarly, the effects of ageing on vitamin levels in marine mammal has been inconsistent; positive relationships were found in the blubber of Baltic ringed seals (*Phoca hispida*), freshwater grey seals, harbour porpoises, and bowhead whales, but no trend was found in bottlenose dolphins, Spitsbergen ringed seals, and marine grey seals (Schweigert et al. 1987, Kakela et al. 1997, Borrell et al. 1999, Rosa et al. 2007). The conflicting results of blubber lipid content and age on vitamin concentrations in marine mammals could be the result of several factors, including sex, diet, condition, disease, reproductive status, moulting, migration and other events. These results highlight the importance of understanding natural variations before applying vitamin A and E as biomarkers of chemical exposure.

The present study is aimed at addressing some of the issues surrounding the use of vitamins A and E as biomarkers of chemical exposure; namely, identifying and

describing the factors that are confounding contaminant-related effects. The goals were to characterize the influence of physiological and ecological factors on vitamin dynamics in western Arctic beluga whales and to determine if contaminant exposure caused a measurable effect on vitamin A and E dynamics. Biological parameters, including age, sex, blubber lipid content and thickness, whale size, stable isotopes and fatty acids, as well as tissue vitamin concentrations and blubber PCB and PBDE levels, were measured in 66 healthy, subsistence hunted beluga whale. Through a better understanding of the natural variations in whole body vitamin dynamics, we were able to account for and reduce the number of confounding factors, and more confidently explore contaminant-related vitamin A and E disruption in beluga whales.

Methods

Sample collection

Beluga tissue samples were collected during the yearly traditional beluga harvest by Inuvialuit hunters at Hendrickson Island, near the community of Tuktoyaktuk, in Northwest Territories Canada. A total of 66 whales were sampled over four years (2007-2010), of which 84% were adult males as hunters typically select for larger sized animals (Table 4.1). Blubber, liver and plasma samples were taken from each whale within hours of its death. Blood was collected directly from the jugular vein into heparinised plasma separation tubes (Becton-Dickinson, USA). Blood was centrifuged on site and plasma was collected and kept frozen at -80°C . Full depth blubber samples were taken slightly dorsal from the pectoral flipper. Liver and blubber samples were wrapped in solvent-rinsed foil, frozen at -20°C on site, stored in portable freezers, and shipped to Fisheries and Oceans Canada, where they were stored at -80°C within two weeks of collection.

Lower jaws were collected in order to age the whales by counting growth layer groups in dentine from a thin section of a tooth (1 GLG = 1 yr, Stewart et al. 2007). The number of whales harvested each year and select biological information are presented in Table 4.1.

Tissue vitamin analysis

Blubber and liver samples were analyzed for tocopherols (α -, γ - and δ -tocopherol), retinol, dehydroretinol and retinyl esters using high performance liquid chromatography (HPLC), according to the method described by Palace and Brown (1994). Stratification was evaluated in blubber by dividing the blubber sample into equally sized inner (closest to muscle), middle and outer (closest to skin) layers, which were analyzed separately. Briefly, approximately 100 mg of tissue was homogenised for 45 s in 2 ml of distilled deionised water. Proteins were denatured with HPLC grade ethanol containing retinol acetate, which was used as an internal standard. Vitamins were extracted with 500 μ l ethyl acetate:hexane (3:2 v/v), and a known volume was transferred to amber vials and evaporated under vacuum. The residue was reconstituted in mobile phase and immediately analyzed by HPLC.

Table 4.1 Biological chemical information for beluga whales captured near Tuktoyaktuk, NWT. Values represent mean \pm SEM.

	2007	2008		2009		2010
	males	males	female	males	females	males
n	18	15	3	12	7	10
age	25.4 \pm 1.8	33.9 \pm 2.6	38.3 \pm 11	29.3 \pm 2.4	44.2 \pm 4.9	24.3 \pm 1.5
Length (cm)	416.6 \pm 7.7	422.5 \pm 6.1	367.6 \pm 10	413.2 \pm 6.5	371.3 \pm 4.2	405.9 \pm 13
Blubber lipid (%)	93.1 \pm 1.2	88.7 \pm 1.7	81.8 ^a	89.0 \pm 0.7	88.0 \pm 1.7	90.3 \pm 1.3
$\delta^{13}\text{C}$ liver	n/a	-20.3 \pm 0.3	-21.2 \pm 0.9	-19.1 \pm 0.2	-19.3 \pm 0.3	-20.2 \pm 0.1
$\delta^{15}\text{N}$ liver	n/a	16.9 \pm 0.2	16.3 \pm 0.5	18.0 \pm 0.7	18.7 \pm 0.9	16.7 \pm 0.4
Total PCB (ng/g)	3607 \pm 325	3666 \pm 403	204 ^a	3570 \pm 440	766 \pm 180	3123 \pm 433

^a data available for only one of three whales

The quantification system used reversed-phased HPLC (Gilson model 322) equipped with a photodiode array (HP series 1100) and fluorescence detector (Shimadzu RF-10AXI). The diode array detector was set at 292 nm for tocopherols and 325 nm for retinoids. Fluorescence detection was set at an excitation wavelength of 330 nm and emission wavelength of 480 nm. Compounds were eluted isocratically with acetonitrile:methanol:water (70:20:10, v/v/v) at a constant flow rate of 1.0 ml/min on a Adsorbosphere HS C18 column (4.6 x 250 mm, 5 µm) with guard (4.6 x 7.5 mm, 5 µm). Separate calibration curves were obtained using serial dilutions of retinol, dehydroretinol, retinyl palmitate and α -tocopherol (Sigma–Aldrich, Canada) standard solutions that were quantified by absorption spectroscopy. All work was performed under red light, using HPLC-grade solvents.

Plasma retinol analysis

Plasma was analyzed for total circulatory retinol using methods adapted from Simms et al. (2000). Briefly, 250 µl of plasma was deproteinated using one part methanol and spiked with the synthetic retinol isomer all-*trans*-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8 - nonatetraen-1-ol (TMMP-OH; Hoffman-LaRoche, Switzerland), which was used as an internal standard. The sample was extracted twice using two parts hexane and the pooled extracts were evaporated using a gentle stream of nitrogen. The residues were then resuspended in 150 µl of methanol, and 50 µl was analyzed using reversed-phase HPLC (Beckman Coulter, California, USA) equipped with a Kinetex PFP column (4.6 x 100 mm, 5 µm) and guard column (4.6 x 75 mm, 5 µm). Retinol was eluted using a gradient of 90% methanol to 100% methanol over 5 min at a constant flow rate of 1.0 ml/min and detected via a photodiode array detector

(Beckman Coulter, model 166) set at a wavelength of 325 nm. All work was completed under yellow light and with HPLC grade solvents.

Stable isotope analysis

Carbon and nitrogen isotopes were analyzed at the Stable Isotope Laboratory at the University of Winnipeg; analytical details are described in Loseto et al. (2008b). In brief, liver tissue was freeze-dried (lipids were removed using chloroform/methanol extraction for carbon isotopes) and 1 mg was analyzed by continuous flow, ion-ratio mass spectrometry using a GV-Instruments IsoPrime attached to a peripheral, temperature-controlled, EuroVector elemental analyzer. Carbon and nitrogen isotope results are expressed using standard delta (δ) notation as described by deviations from a standard such as

$$\delta_{\text{sample}} \text{‰} = [(R_{\text{sample}} / R_{\text{standard}}) - 1] \times 1000$$

where R is the $^{13}\text{C}/^{12}\text{C}$ or $^{15}\text{N}/^{14}\text{N}$ ratio in the sample or standard. The standards used for carbon and nitrogen isotopic analyses are Vienna PeeDee Belemnite (VPDB) and IAEA-N-1 (IAEA, Vienna), respectively. Isotope results are reported in Table 4.1.

Fatty acid analysis

The exact diet of Beaufort Sea beluga whales is poorly characterised due to the inherent difficulty of studying foraging behaviour of an Arctic marine mammal. In light of this, fatty acids can be used as proxies for feeding ecology (Iverson et al. 2004, Loseto et al. 2009). Fatty acids were extracted from the inner blubber layer as this represents the most recent deposit from diet. The extraction method is described in detail in Loseto et al. (2008b). Briefly, lipids were extracted from blubber using chloroform:methanol (2:1 v/v)

and washed, filtered and evaporated before undergoing trans-esterification to form fatty acid methyl esters. The methyl esters were analyzed using gas chromatography-ion mass spectroscopy. A fatty acid dietary index was computed in Loseto et al. (in prep) by running a principal component analysis (PCA) using 38 of the 65 identified fatty acids in beluga that are known to transfer from prey to predator. The scores from the first two principal components are herein reported as fatty acid principal component 1 (FA-PC1) and fatty acid principal component 2 (FA-PC2).

Contaminant analysis

Full depth blubber samples were analyzed for 205 PCB and 78 PBDE congeners by the Laboratory of Expertise in Aquatic Chemical Analysis (Fisheries and Oceans Canada) according to their laboratory procedures (Ikonomou et al. 2001). Briefly, samples were ground with anhydrous sodium sulfate, packed onto a glass extraction column and extracted with hexane:dichloromethane (1:1 v/v). Following sample clean-up, contaminants were quantified by high-resolution gas chromatography-high resolution mass spectrometry. Details on the analytical conditions, criteria for identification and quantitation, and quality assurance/quality control are described in Ikonomou et al. (2001) and Ross et al. (2000).

Although 205 PCB and 78 PBDE congeners were analyzed, many congeners were not detected in every sample. Detection limit substitutions were made for congeners if they were detected in >70% of samples, while congeners detected in fewer than 70% of samples were not included in the analyses. All PCB and PBDE results are expressed on a lipid weight basis. Furthermore, because of relatively high background contamination,

PBDE results were all blank corrected, and because of analytical difficulties, nona and deca BDEs are not included in the calculation of total PBDE.

Data analysis

Differences between sexes for mean vitamin concentrations were determined with an independent samples t-test. Vitamin concentration differences between tissues were determined using an independent samples t-test when comparing two tissues or an analysis of variance (ANOVA) followed by pair-wise posteriori Tukey tests when comparing all blubber layers. Relationships between tissue vitamin concentrations and biological/ecological variables were tested using linear regression, with correlations considered significant if $p < 0.05$. All t-tests, ANOVAs and linear regressions were carried out using SPSS 16.0 (IBM, New York, US).

A PCA was used to examine variations in vitamin pattern among individual whales. The centered log ratio procedure was applied to the vitamin data before analysis in order to avoid negative bias in normalized data (Yunker et al. 2011). To remove concentration effects, data were normalized to total concentration followed by geometric mean normalization of vitamin columns, and then log transformed and autoscaled before PCA. PCA were run using Pirouette (Infometrix, Inc, Washington, US). Pattern analysis is used here as a qualitative method to highlights similarities and differences among whales based on the pattern of different vitamin compounds. The principal component scores can then be used quantitatively in bivariate regressions against biological, ecological and chemical variables.

Multiple regressions were run to compliment pattern analysis (PCA) with concentration specific results. Regressions were carried out on individual vitamin

compounds in liver, plasma, and inner and outer blubber layers in order to determine which variables were best describing vitamin levels. The best variables to describe vitamin concentrations were selected using the lowest Akaike Information Criteria (AIC) value (Ferguson et al. 2006, Loseto et al. 2008). Models were run combining biological and contaminant variables, and only the highest ranking AIC models are reported. Regressions were run using MYSTAT (Systat Software, Inc., Chicago, US).

Results

Temporal trends in beluga biological parameters

Whales captured in 2010 were significantly younger than in 2008 and 2009 (ANOVA, $p=0.004$) (Table 4.1). Blubber lipid content in 2007 whales was higher than in 2008 (ANOVA, $p=0.023$). Females were only captured in 2008 and 2009, and were older (t-test, $p<0.001$) and shorter (t-test $p<0.001$) than males. $\delta^{13}\text{C}$ varied temporally in beluga liver, where 2009 whales had significantly higher values than those in 2008 and 2010 (ANOVA, $p=0.003$). $\delta^{15}\text{N}$ also varied temporally, whereby values were highest in 2009 whales than 2008 (ANOVA, $p=0.035$).

Vitamin A and E in beluga whales

The concentration of vitamin A and E compounds in male beluga whale tissues are shown in Table 4.2. Three forms of vitamin E were detected in liver; α -tocopherol was the dominant form, with an average concentration of $12.4 \pm 1.5 \mu\text{g/g}$, followed by δ - and γ -tocopherol (4.5 ± 0.8 and $1.6 \pm 0.5 \mu\text{g/g}$, respectively). Both vitamin A1 (retinol) and vitamin A2 (dehydroretinol) were detected in liver samples, but retinol was the dominant alcoholic retinoid. Fifteen retinyl esters were quantified in beluga liver, of which palmitate was the most important, followed by oleate, linoleate and stearate.

As in liver, α -tocopherol was the dominant form of vitamin E in blubber (Table 4.2). The mean concentration of total vitamin E was $161.7 \pm 19.1 \mu\text{g/g}$ and $58.5 \pm 6.5 \mu\text{g/g}$ in inner and outer blubber, respectively. Retinol and dehydroretinol were detected in all blubber layers, and the average retinol-dehydroretinol ratio was several times higher in blubber than liver. There was a greater number of retinyl esters detected in inner compared to outer blubber, but both layers showed a similar pattern whereby retinyl linoleate was predominant, followed by oleate then myristate (Table 4.2).

Based on liver and blubber concentrations (tissues expected to contain the majority of vitamin A and E), the mean total body burden of vitamin A and E in adult beluga whales was estimated to be 49.9 g and 48.3 g, respectively (Table 4.3). Blubber had the highest vitamin E concentrations and this resulted in blubber containing approximately 98.5% of all tocopherol storage. Despite higher concentrations in liver, most of the body burden of vitamin A was contained in blubber ($77 \pm 2.6\%$).

Table 4.2 Vitamin A & E concentrations (ug/g fw \pm SEM) in male beluga whale tissues. Retinyl esters are presented using their fatty acid nomenclature. Values with different letter superscript have significantly different concentrations (ANOVA, $p < 0.05$).

	Liver	Inner blubber	Outer blubber
α -tocopherol	12.4 \pm 1.5 ^a	118.6 \pm 15.8 ^b	40.2 \pm 5.3 ^c
γ -tocopherol	1.6 \pm 0.5 ^a	23.3 \pm 5.6 ^b	8.8 \pm 1.9 ^b
δ -tocopherol	4.5 \pm 0.8 ^a	19.8 \pm 5.2 ^b	9.6 \pm 2.3 ^a
Retinol	32.7 \pm 2.9 ^a	6.1 \pm 0.6 ^b	4.7 \pm 0.4 ^c
Dehydroretinol	27.3 \pm 2.5 ^a	1.4 \pm 0.3 ^b	1.2 \pm 0.2 ^c
Capric (10:0)	24.2 \pm 3.9 ^a	0.04 \pm 0.04 ^b	1.2 \pm 0.3 ^c
Myristic (14:0)	9.2 \pm 1.6 ^a	11.8 \pm 1.6 ^b	13.9 \pm 1.2 ^b
Palmitic (16:0)	183.9 \pm 34.0 ^a	9.3 \pm 1.3 ^b	11.7 \pm 3.2 ^b
Margaric (17:0)	2.6 \pm 0.4	nd	nd
Stearic (18:0)	27.9 \pm 4.6 ^a	0.3 \pm 0.2	nd
Myristoleic (14:1n5)	7.3 \pm 1.5	nd	nd
Palmitoleic (16:1n7)	14.7 \pm 2.5	nd	nd
Oleic (18:1n9)	74.8 \pm 11.6 ^a	13.1 \pm 1.8 ^b	17.1 \pm 1.5 ^c
Gadoleic (20:1n9)	3.2 \pm 0.6	nd	nd
Erucic (22:1n11)	27.1 \pm 4.5 ^a	9.9 \pm 1.5 ^b	6.4 \pm 0.9 ^b
Linoleic (18:2n6)	51.9 \pm 9.5 ^a	20.4 \pm 2.4 ^b	21.7 \pm 1.9 ^b
Linolenic (18:3n)	13.4 \pm 2.2 ^a	3.7 \pm 0.6 ^a	5.1 \pm 0.6 ^a
Eicosadienoic (20:2n6)	8.5 \pm 1.2 ^a	0.3 \pm 0.1	nd
Arachidonic (20:4n6)	13.5 \pm 1.9 ^a	1.3 \pm 0.2 ^b	3.1 \pm 0.6 ^c
Timnodonic (20:5n3)	26.6 \pm 4.1 ^a	20.3 \pm 1.6 ^a	2.2 \pm 0.6 ^c
Total Tocopherol	18.4 \pm 2.4 ^a	161.7 \pm 19.1 ^b	58.5 \pm 6.5 ^c
Total Esters	488.6 \pm 77.0 ^a	90.3 \pm 7.9 ^b	82.7 \pm 6.4 ^b
Total Vitamin A	547.3 \pm 77.7 ^a	97.8 \pm 8.2 ^b	88.6 \pm 6.5 ^b

Table 4.3 Tissue burden of major vitamin compounds in beluga whales. Only blubber and liver concentrations were measured, thus total body burden is estimated as the addition of these two compartments.

	Total tocopherol^a	Retinol	Total Esters	Total Vitamin A^b
Liver (g)	0.36 + 0.048	0.64 + 0.063	9.54 + 1.57	10.69 + 1.59
% body burden	1.54 + 0.37	26.50 + 2.24	21.97 + 2.77	23.06 + 2.56
Blubber (g)	47.98 + 4.62	2.21 + 0.19	36.45 + 3.11	39.18 + 3.26
% body burden	98.46 + 0.37	73.50 + 2.24	78.03 + 2.77	76.84 + 2.56

a - includes α -, β -, γ -tocopherol

b - includes retinol, dehydroretinol and all retinyl esters

Sex differences

Tissue concentrations of vitamin A and E were compared between males and the average female (n=10, age=42 ±14). There were no differences between sexes for vitamin concentrations in liver (t-test, p>0.05), however, the average male-female ratio revealed higher retinol in males and higher tocopherols and esters in females (Fig. 4.1). Few concentrations were different between sexes in inner blubber, but the gender ratio revealed that α - and δ -tocopherol were higher in females while γ -tocopherol and most retinoids were higher in males (Fig. 4.1). In contrast, outer blubber was found to contain large sex differences in concentrations of vitamin A and E; females had higher tocopherols and retinyl esters but lower retinol than males. Comparing males to reproductively active females (<40years old; n=4, age=28.5 ± 3.9) revealed higher liver and inner blubber concentrations in males for tocopherols and almost all retinoids, while outer blubber showed higher retinol and similar or greater ester concentrations in males for outer blubber (not shown).

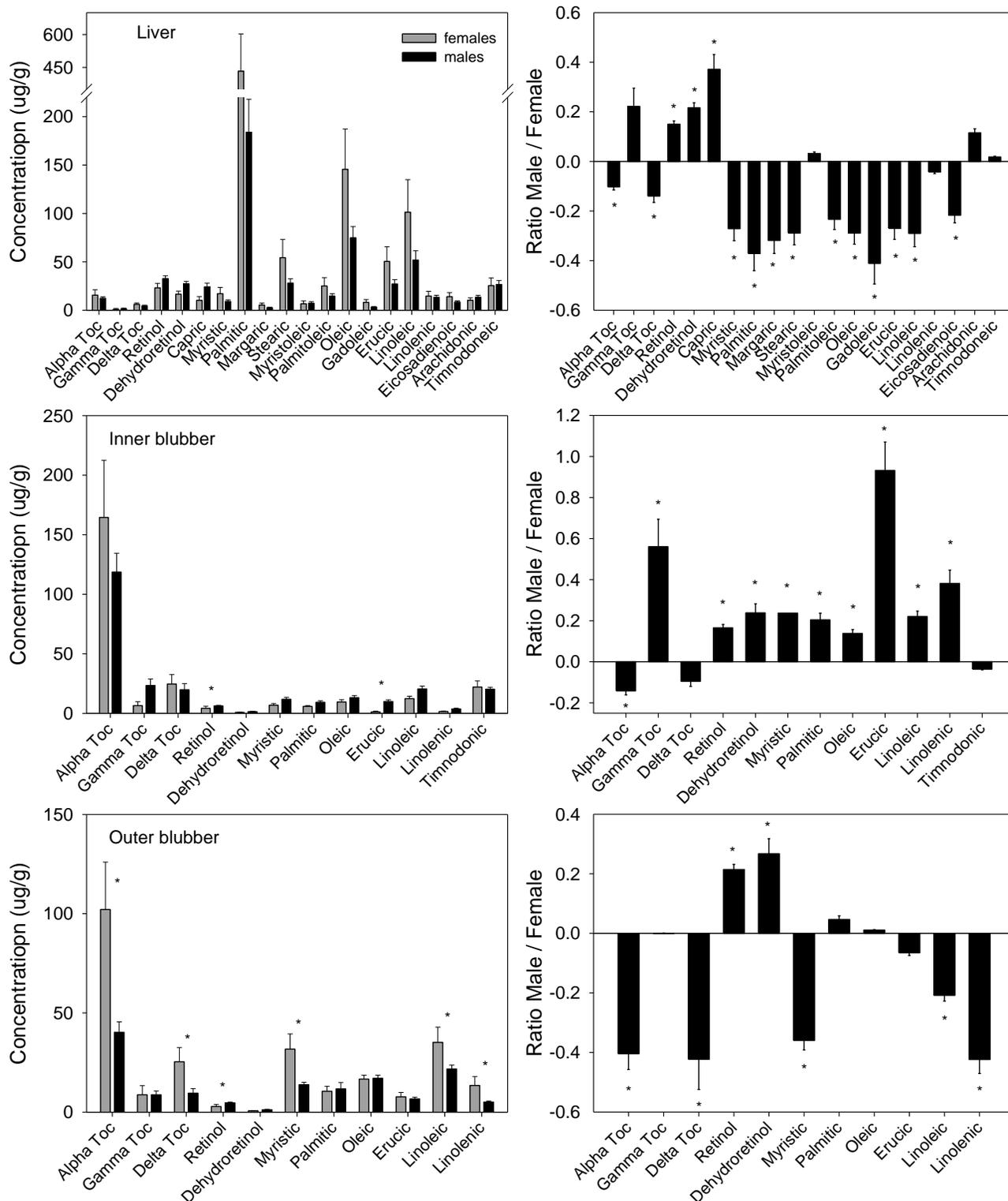


Figure 4.1 Sex differences for vitamin A and E in liver and blubber. Bars represent mean \pm sem. Sex ratios were calculated by dividing male values by the average female value, and were log transformed. * represents significant difference $p < 0.05$.

Tissue differences

Tocopherol concentrations in males were several times higher in blubber compared to liver, and blubber stratification was found to significantly influence tocopherol levels; α -tocopherol in inner blubber was higher than outer blubber (118.6 ± 15.8 vs 40.2 ± 5.3 $\mu\text{g/g}$) (Table 4.2). Retinoid profiles also differed among tissues; retinol was proportionally higher in liver compared to average blubber and greater in inner blubber compared to outer blubber, while esters showed the opposite trend (Table 4.2, Fig 4.1). Retinoids in liver negatively correlated with retinoids in inner blubber but did not relate to other blubber layers. No correlations were found for liver and blubber vitamin E. Total tocopherol, retinol and total vitamin A correlated strongly among blubber layers, while plasma retinol did not correlate with any tissue retinol or total vitamin A (not shown).

Beluga tissue vitamin patterns

An exploratory PCA was run using vitamin A and E concentrations for liver and separated blubber layers in beluga whales (Appendix 2, Fig. S2.1). The first two principal components accounted for 50% of the variance. The first PCA axis clearly separated liver from blubber vitamins while the second PCA axis distinguished retinoids from tocopherols. The first PCA axis related to age ($r^2=0.19$, $p=0.007$) and total PCB ($r^2=0.17$, $p=0.006$), while the second axis related to length ($r^2=0.10$, $p=0.04$) and $\delta^{15}\text{N}$ ($r^2=0.18$, $p=0.02$). In order to reduce the dominant effect of tissue differences, a refined PCA model was run specifically for liver concentrations of vitamin A and E (Fig. 4.2). The first two principal components accounted for 62% of the variance (PC1: 42%, PC2: 20%). The scores plot revealed loose clustering of whales based on sampling year as well

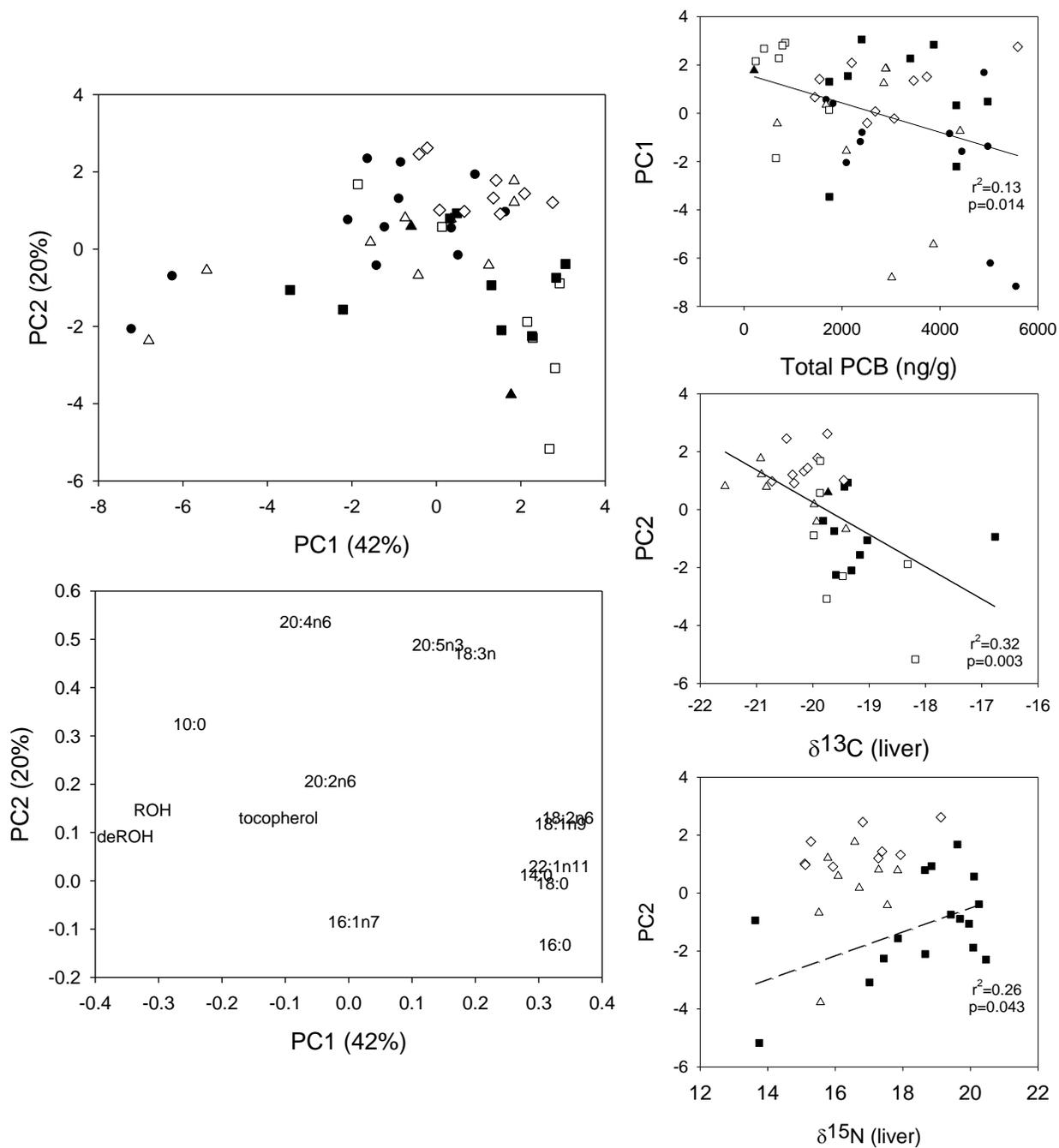


Figure 4.2 PCA of beluga whale retinoids and α -tocopherol in liver. Panels on the right show significant regressions of PC1 and PC2 with biological or chemical variables.

Symbols represent: ●=2007 males, Δ =2008 males, \blacktriangle =2008 females, \blacksquare =2009 males, \square =2009 females, \diamond =2010 males, ROH=retinol, deROH=dehydroretinol.

as a separate cluster of female whales. The loadings plot shows the active alcohol forms of vitamin A (retinol and dehydroretinol) on the negative side of the first axis and most of the retinyl esters on the positive side (Fig.4.2). Total PCB was the only variable that correlated to PC1 ($r^2=0.13$, $p=0.014$), while PC2 was related to $\delta^{13}\text{C}$ ($r^2=0.32$, $p=0.003$) and $\delta^{15}\text{N}$ (2009: $r^2=0.18$, $p=0.019$).

Multiple predictors of vitamin concentrations in beluga whales

Multiple regressions were run for the major vitamin A and E compounds (retinol, sum esters and α -tocopherol) in male beluga whales, and best fit models were selected using lowest AIC score (Table 4.4). Alpha-tocopherol in inner blubber was best described by positive relationships with age, stable isotopes and PCBs. Condition (blubber lipid content) also positively correlated with tocopherol in inner blubber. In outer blubber, condition and stable isotopes were identified as best the predictor variables for α -tocopherol. Total PCBs was the best predictor of liver tocopherol concentrations.

Outer blubber and liver retinol concentrations were best described by temporal variations and feeding ecology (Table 4.4). The best fit model in outer blubber selected year and length, while the model for liver retinol selected year, FA-PC1 and $\delta^{13}\text{C}$. The next best model in both tissues found contaminants to positively influence retinol levels. Year and contaminant levels best described retinol in inner blubber, while plasma retinol related best to age and PCBs (Table 4.4). Age and diet (FA-PC1) were important variables to describe inner and outer blubber retinyl ester levels, but trophic status and blubber condition (% lipid and thickness) complimented these in outer blubber whereas temporal variation and PCBs were selected in inner blubber. In liver, length, PCBs and temporal differences best explained ester concentrations (Table 4.4).

Table 4.4 Best fit multiple regressions of biological and ecology variables as well as contaminant concentrations for retinol, sum of retinyl esters and α -tocopherol in beluga whale tissues. Model variables were selected by lowest Akaike Information Criteria (AIC) values, where the model with the lowest AIC value is considered most appropriate. Vitamins were log transformed before analysis.

		R^2	p value	AICc
<i>Inner blubber</i>				
Retinol	* Year, Log PBDE	0.44	<0.001	-4.51
	* Year, Log PCB	0.42	<0.001	-2.59
	* Log PCB	0.08	0.046	18.75
Total esters	* Year, Age, FA-PC1, Log PCB	0.47	<0.001	8.86
	* Year, Length	0.34	<0.001	12.36
	* Log PCB	0.21	0.001	23.11
α -tocopherol	* Age, Log $\delta^{13}C_{liv}$, Log PCB	0.30	0.014	63.70
	* Age, Lipid, Log $\delta^{15}N_{liv}$	0.29	0.016	64.16
	* Log PCB	0.14	0.011	80.10
<i>Outer blubber</i>				
Retinol	* Year, Length	0.27	<0.001	12.81
	* Year, Log PBDE	0.28	<0.001	12.92
Total esters	* Age, FA-PC1, Log $\delta^{15}N_{liv}$	0.30	0.044	-21.48
	* Age, Blubber thickness	0.23	0.017	-20.76
	* Year, Blubber thickness, FA-PC1, Log PCB	0.27	0.036	-17.25
α -tocopherol	* Lipid, Log $\delta^{15}N_{liv}$, Log $\delta^{13}C_{liv}$	0.31	0.027	23.78
	* Lipid, Blubber thickness, Log $\delta^{15}N_{liv}$, Log $\delta^{13}C_{liv}$	0.37	0.038	24.47
<i>Liver</i>				
Retinol	* Year, FA-PC1, Log $\delta^{13}C_{liv}$	0.50	0.001	15.27
	* Year, FA-PC1, Log PCB, Log PBDE	0.58	<0.001	17.80
	* Year, FA-PC1, Log PCB	0.44	<0.001	21.69
Total esters	* Year, Length, Log PCB	0.29	0.008	58.64
	* Log PCB	0.10	0.047	74.91
α -tocopherol	* Log PCB	0.13	0.07	19.05
	* Year, Length, Log PCB	0.19	0.083	31.30
<i>Plasma</i>				
Retinol	* Age, Log PCB	0.39	0.02	-13.86
	* Year, Lipid, Blubber thickness	0.48	0.02	-13.08
	* Age, Log $\delta^{13}C_{liv}$, Log PCB	0.46	0.02	-12.57

The results of univariate correlation for major predictor variables identified through the PCA and multiple regressions with vitamin concentrations are shown in Table 4.5. Alpha-tocopherol and retinoids were found to decline with age in liver until approximately 35 years of age where concentrations would increase again. Age alone had little effect on blubber concentrations of most vitamins. Tocopherol concentrations in blubber increased exponentially with lipid content, but the slope differed with sampling year. Lipid content also correlated with inner blubber retinoids (Table 4.5). Length was positively related to most retinoids in blubber (inner total vit A: $r^2=0.21$, $p=0.001$; outer total vit A: $r^2=0.12$, $p=0.01$) and α -tocopherol in outer blubber ($r^2=0.14$, $p=0.006$). $\delta^{13}\text{C}$ correlated with few vitamins with the exception of retinol (liver: $r^2=0.16$, $p=0.032$; inner blubber: $r^2=0.27$, $p=0.007$), and inner blubber total vitamin A ($r^2=0.27$, $p=0.001$). $\delta^{15}\text{N}$ positively correlated with blubber tocopherols (inner: $r^2=0.18$ $p=0.01$; outer: $r^2=0.14$ $p=0.03$), and retinoids in outer blubber (total vit A: $r^2=0.15$, $p=0.02$) and liver (retinol: $r^2=0.14$, $p=0.05$).

Table 4.5 Univariate correlations of biological and ecological variables against major tissue vitamin concentrations. Numbers represent the pearson's correlation coefficient ($p<0.05$). Vit A represents total vitamin A.

	<i>Liver</i>			<i>Inner blubber</i>			<i>Outer blubber</i>		
	α -toc	retinol	vit A	α -toc	retinol	vit A	α -toc	retinol	vit A
Age	ns	ns	ns	ns	ns	ns	ns	ns	+0.32
Blubber lipid	ns	ns	ns	ns	+0.45	+0.37	ns	ns	ns
Length	ns	ns	ns	ns	+0.32	+0.46	+0.37	ns	+0.35
$\delta^{13}\text{C}_{\text{liver}}$	ns	+0.40	ns	ns	ns	ns	ns	ns	ns
$\delta^{15}\text{N}_{\text{liver}}$	ns	+0.37	ns	+0.42	ns	ns	+0.37	ns	+0.39

Toxicity effects on vitamin A and E

This large dataset afforded the exploration of the influence of several biological variables on vitamin concentrations; however, these factors are considered confounding when exploring contaminant-related changes. In light of this consideration, our dataset was reduced in order to minimize confounding effects by eliminating individuals who fell outside two standard deviations from the mean for each biological parameter. The reduced dataset consisted of 44 whales, which met the following range of characteristics: males, age=10-47 years, length=354-475 cm, blubber lipid=82-100%, and blubber thickness=4-13cm. Furthermore, to account for the effect of lipid, all vitamin concentrations were lipid corrected.

Using this dataset of reduced confounding influences, we found negative correlations between total PCBs and liver esters ($r^2=0.24$, $p=0.005$), total vitamin A ($r^2=0.19$, $p=0.001$), and the ester-retinol ratio ($r^2=0.20$, $p=0.008$) (Fig. 4.3). The opposite was found in inner blubber, where all retinoids (retinol: $r^2=0.22$, $p=0.001$; total: $r^2=0.43$, $p=0.001$) and tocopherol ($r^2=0.13$, $p=0.024$) were positively correlated with PCBs (Fig. 4.3). Plasma retinol was also positively related to PCBs ($r^2=0.30$, $p=0.009$; not shown). Outer blubber vitamin concentrations were not found to correlate with PCBs.

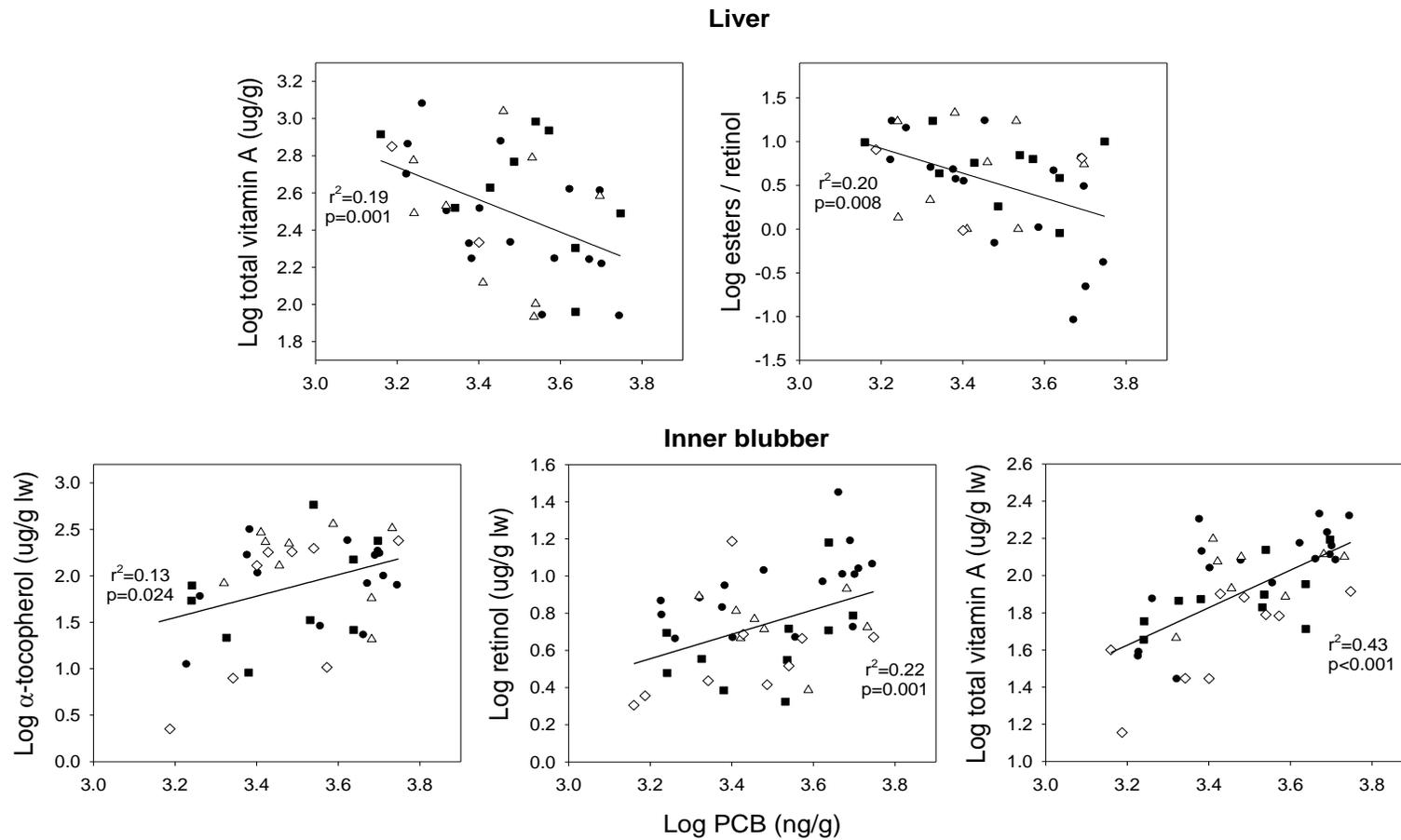


Figure 4.3 Vitamin A and E compounds correlate with PCB concentrations in liver and inner blubber after reducing confounding factors. The influence of confounding factors was reduced by limiting the dataset to whales falling within two standard deviations from the mean for each biological variable. Symbols represent: ●=2007 males, Δ =2008 males, \blacktriangle =2008 females, \blacksquare =2009 males, \square =2009 females, \diamond =2010 males, ROH=retinol, deROH=dehydroretinol.

Discussion

As vitamin A and E are accumulated through diet and stored in lipid rich tissues, many biological and ecological factors relating to physiology and feeding ecology can strongly influence their tissue concentrations (Borrell et al. 1999, Mos & Ross 2002, Routti et al. 2005, Rosa et al. 2007). A detailed understanding of how these natural factors influence retinoid and vitamin E concentrations is a necessary prerequisite to determine the usefulness of these essential nutrients as biomarkers for chemical exposure.

Sex and tissue selection influence vitamin A and E concentrations and patterns

Sex had a very strong influence on vitamin accumulation in beluga whales, but this relationship was found to differ between tissues and depend on female reproductive status. Young, reproductively active females, had lower tissue concentrations of most retinoids and tocopherols than adult males. Similar results were found for retinoids in bowhead whales (Rosa et al. 2007), and several pinniped species, including hooded, ringed, harp and grey seals (Rodahl & Davies 1949, Schweigert et al. 1987, Nyman et al. 2003). Marine mammals mobilise a large portion of their lipid reserves to produce high fat and energy-rich milk during lactation. During this process, fat-soluble vitamins are mobilised with lipids thus providing large amounts of vitamin A and E to suckling neonates (Schweigert et al. 1987, Debier, Pomeroy, Baret, et al. 2002, Debier, Pomeroy, Wouwe, et al. 2002, Debier et al. 2012) . Maternal offloading of vitamins may be contributing to the decreased tissue levels of retinoids and tocopherols in female beluga whales. Results from older females support these findings such that after reproductive senescence, tissue concentrations remained similar or become greater in females compared to males.

After removing the confounding effect of sex, concentrations and patterns of vitamin A and E varied between liver and blubber as well as between blubber layers. Hepatic vitamin E concentrations reported here were similar to those found in harp and grey seal livers, but an order of magnitude lower than Baltic ringed seals and Alaskan bowhead whales (Engelhardt 1977, Kakela et al. 1997, Nyman et al. 2003, Rosa et al. 2007). Blubber concentrations of tocopherol fall within the large range of values found in marine mammals (Kakela et al. 1997, Nyman et al. 2003, Rosa et al. 2007). Blubber stratification of vitamin E in beluga whales, with highest levels found in inner blubber, is in accordance with results found in bowhead whales (Rosa et al. 2007). The higher concentration of tocopherol in inner blubber is likely associated with the higher antioxidant demand in this layer due to its relatively higher metabolic activity and different fatty acid profile. Morphological and biochemical studies of marine mammal blubber have suggested that the layer nearest the skin (outer) is relatively inert and important for thermoregulation while the inner layer (nearest muscle) is the active site for lipid metabolism and dietary lipid incorporation (Koopman et al. 1996, Strandberg et al. 2008). Higher lipid metabolism combined with a higher proportion of unsaturated fatty acids, including PUFAs, in inner blubber (Koopman et al. 1996, Krahn et al. 2004) suggests the potential for higher oxidative stress and therefore tocopherol demand (Nacka et al. 2001, Debier & Larondelle 2005).

Retinoid concentrations in beluga were within the same range reported in other marine mammals (Kakela et al. 1997, Borrell et al. 1999, Nyman et al. 2003, Tornero, Borrell, Forcada, & Aguilar 2004, Tornero et al. 2005, 2006) with the exception of bowhead whales in which liver concentrations were an order of magnitude higher (7261

$\mu\text{g/g}$) and blubber concentrations were considerably lower (1-3 $\mu\text{g/g}$) (Rosa et al. 2007). Although retinoid concentrations were higher in liver, we found that blubber represented approximately three quarters of the total burden on a body mass basis, which was higher than what was calculated for pinnipeds (40-60%, (Schweigert et al. 1987, Mos & Ross 2002). This supports previous findings that blubber is an important depot for fat soluble vitamins in odontocetes and cetaceans (Borrell et al. 1999, Tornero, Borrell, Forcada, & Aguilar 2004, Rosa et al. 2007). In the present study, total vitamin A concentrations were not significantly different between inner and outer blubber, however the stratification of active and storage retinoids coincided with the aforementioned active and inert blubber layers; retinol was highest in inner blubber while most retinyl esters were proportionally higher in outer blubber. Similar results were found in Mos and Ross (2002) for harbour seals, but the opposite was found in bowhead whales (Rosa et al. 2007). In the latter, vitamin A was higher in outer blubber; however, saponified retinol was reported and high outer blubber levels likely reflected the higher concentration of esters rather than retinol. This discrepancy highlights the added benefit of analyzing the different forms of vitamin A when trying to understand vitamin dynamics in marine mammals.

Age, body condition, and feeding ecology predict tissue vitamin accumulation

Principal component analysis indicated tissue vitamin patterns in beluga whales varied with age and feeding ecology. Results from AIC selected multiple regressions corroborated results from pattern analysis; vitamin concentrations in blubber, liver and plasma were best predicted using age, feeding ecology and body condition (blubber lipid content). Temporal changes in tissue vitamin concentrations were also apparent.

Retinyl esters, total vitamin A and α -tocopherol increased with age in the blubber of beluga whales. Furthermore, this study is the first to show a relationship between age and tissue vitamin patterns, suggesting that not only concentrations of vitamins change with time. The positive influence of age on vitamin concentrations in beluga whales agrees with findings in other marine mammal, indicating a common physiological effect of increasing storage of vitamins over time (Schweigert et al. 1987, Kakela et al. 1997, Borrell et al. 1999, Rosa et al. 2007). Age related accumulation of retinoids is likely the result of higher than necessary dietary intake leading to a build-up of retinyl esters in lipid storage tissues (Borrell et al. 1999).

Blubber lipid content and thickness were strong predictors of blubber vitamin E, outer blubber retinoids, and plasma retinol concentrations. In all cases, vitamin concentrations increased with lipid content and thickness. The positive relationship between blubber lipid and tocopherol is likely related to the aforementioned antioxidant role these compounds play in lipid rich tissues. Lipid content relationships with retinoids in marine mammals are inconsistent in literature; some studies show a positive correlation (Tornero et al. 2005), while others find negative (Nyman et al. 2003, Tornero, Borrell, Forcada, Pubill, et al. 2004) or no correlation at all (Borrell et al. 1999, Mos & Ross 2002). Since retinoids are strongly lipophilic, the inconsistent relationship with lipid content may be related to seasonal and species related differences in diet, migration, moulting, disease or other confounding factors. For instance, beluga blubber lipid content in this study correlated with whale length ($r^2=0.29$, $p=0.03$), which also positively correlated with vitamin concentrations, suggesting a possible confounding effect.

Feeding ecology was a consistent predictor of vitamin patterns and concentrations in beluga whales. The second principal component (PC2) of both PCAs represented feeding ecology, as described by variations in length, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$. Since the loading plot of PC2 represented a shift from polyunsaturated fatty acyl esters to saturated /monounsaturated fatty acyl esters, the relationship with $\delta^{13}\text{C}$ may suggest that retinoid patterns in beluga whales are dependent in part on dietary carbon sources (i.e., pelagic vs benthic/nearshore). The relationship with $\delta^{15}\text{N}$ may suggest retinoid patterns also vary with trophic status, likely reflecting the difference in prey at different trophic levels. These relationships appear to be driven by yearly differences in stable isotopes, whereby 2009 whales had higher $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ than 2008 and 2010 whales. These annual differences are difficult to interpret, but may be in part related to temporal differences in feeding ecology due to changing sea-ice conditions (Loseto et al. 2013, in prep).

Multiple regression models and univariate correlations indicated that $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ as well as dietary fatty acids were important predictors of overall vitamin concentrations in beluga whales. Blubber tocopherol and retinyl esters increased with trophic status in whales, suggesting a possible food web magnification effect of vitamin concentrations. Results from carbon isotopes revealed higher blubber tocopherol and liver retinol levels in ^{13}C enriched whales, which may indicate a dietary effect whereby higher antioxidant levels (retinol and tocopherol) are found in benthic prey. Stable isotopes were found previously to be effective dietary biomarkers in Beaufort beluga whales where they successfully characterised size-related tissue mercury accumulation (Loseto, Stern, & Ferguson 2008, Loseto, Stern, Deibel, et al. 2008). Overall, it appears

that storage retinoids are influenced more importantly by trophic status, whereas retinol was predicted by carbon source.

Dietary fatty acids also related to retinoid tissue concentrations. Blubber esters and liver retinol concentrations increased in whales as their diet shifted away from prey rich in long-chained MUFAs to prey containing a higher proportion of PUFAs (20:5n3 and 22:6n3). In a study of fatty acids in beluga and their putative prey, it was found that beluga profiles rich in long-chained MUFAs (20:1n11, 22:1n11, 20:1n9, 22:1n9) corresponded to a higher consumption of pelagic Arctic cod, whereas a profile enriched in PUFAs (20:5n3, 22:4n3, 20:3n3, 20:4n6) suggested greater consumption of nearshore and brackish water fish, such as nearshore cod and Arctic cisco (Loseto et al. 2009). Taken together, these results suggest that the consumption of nearshore prey (high PUFAs) contributes positively to the overall retinoid storage depot in beluga blubber. Preliminary analyses of putative prey from the Beaufort Sea supported these findings in that higher total vitamin A levels were found in nearshore and benthic fish, including nearshore cod, cisco and flounder, relative to offshore cod (not shown). It is important to note however, that prey data are comprised of replicate averages of less than four fish per species. Further work is needed to better characterize retinoid concentrations and patterns in aquatic food webs.

Length was found to positively influence vitamin concentrations in beluga whales. Whale size in this beluga population has been shown to be one of the best predictors of habitat use, feeding ecology and consequently, accumulation of lipophilic contaminants (Loseto, Stern, & Ferguson 2008, Loseto, Stern, Deibel, et al. 2008, Loseto et al. 2009). These studies found that larger offshore whales accumulated higher mercury

and PCB concentrations, likely resulting from higher concentrations found in offshore prey. The positive relationship between length and vitamin concentrations here supports the general size-related accumulation of lipophilic compounds in beluga. This however contradicts the relationship between fatty acids and retinoid concentration previously described which showed increasing levels in whales consuming benthic/nearshore prey (i.e. typically smaller whales). This contradictory relationship is likely the results of several factors, including the confounding effect of blubber lipid content (higher in large whales) as well as higher food intake in larger whales. For instance, higher overall food intake in larger whales (Kastelein et al. 1994), may have contributed to the increased accumulation of retinoids over time, despite the slightly lowered prey retinoid concentrations. Furthermore, Arctic cod was found to have a lower caloric value than nearshore fish species, potentially resulting in a higher fish consumption rate in large offshore beluga relying on Arctic cod (L. Loseto, personal communication).

Contaminant associated effects on vitamin physiology

A combination of methods was used to examine the relationship between tissue vitamin concentrations and contaminants. First, a PCA indicated a contaminant-related pattern change in liver vitamins, whereby retinol increasingly dominated the retinoid pattern with increased PCB exposure. Second, multiple regression analyses for several tissues indicated reduced hepatic vitamins and increased plasma and blubber vitamins in relation to PCB concentrations. Lastly, using a reduced dataset to control for confounding factors, we found that persistent organic pollutants retained their significant relationship with liver, plasma and blubber vitamin concentrations.

Overall, our results suggested a contaminant effect on beluga whales resulting in increased mobilization of retinoids and tocopherol from the liver, leading to greater plasma and inner blubber vitamin levels. Our results are consistent with those found in other marine mammal studies, suggesting changes in retinoid homeostasis due to contaminant exposure in marine mammals (see Simms and Ross 2001 for review). Decreased hepatic retinoid storage is the most consistent and sensitive marker of contaminant exposure, and has been found in laboratory as well as free-ranging animals (Nilsson et al. 1996, Kakela et al. 1999, Käkälä et al. 2002, Nyman et al. 2003, Mos et al. 2007). The increased mobilisation of liver stores has been thought to result from the contaminant related up-regulation of hepatic enzymes involved in vitamin A metabolism, including retinyl ester hydrolases and cytochrome P450 enzymes (Bank et al. 1989, Nilsson et al. 1996). The elevated plasma retinol concentrations in contaminated whales is likely a reflection a hepatic mobilisation, and similar results have been found in laboratory and wild animals (Bank et al. 1989, Zile 1992, Murk et al. 1994, Simms et al. 2000, Nyman et al. 2003).

The increased adipose retinoid relationship with PCBs found here has been reported in mink (Kakela et al. 1999) and common dolphins (Tornero et al. 2006), while others have found the opposite effect (Kakela et al. 1999, Tornero et al. 2005, Mos et al. 2007). Tornero et al. (2006) proposed that the up- and down-regulation of blubber retinoids may depend on contaminant concentrations; they found reduced blubber concentrations in bottlenose dolphins but increased concentrations in common dolphins as a function of PCB exposure, with the difference suggested to result from the 2-8 times higher PCB levels in bottlenose dolphins (Tornero et al. 2005, 2006). In this manner,

exposure to high concentrations of organic pollutants may result in severe/acute liver depletion, leading to a drop in circulatory retinol and eventual depletion of adipose stores (Simms et al. 2000). In this study, beluga PCB concentrations were approximately 10 times lower than those in bottlenose dolphins and thus may not have led to the widespread body depletion of retinoid storage as seen in that study. Nonetheless, our results suggest a disruption in vitamin A dynamics in Arctic beluga whales exposed to relatively low contaminant concentrations.

Vitamin E dynamics were affected by PCBs similarly to retinoids in beluga whales. A reduction of hepatic stores followed by increased blubber concentrations occurred in relation to total PCB levels. Fewer studies have examined vitamin E in marine mammals, however similar results have been reported for mink and ringed and grey seals (Kakela et al. 1999, Nyman et al. 2003, Routti et al. 2005). Laboratory studies have shown that PCB exposure can lead to decreased hepatic and serum vitamin E concentrations and increased vitamin disposal via excretion through the kidney and spleen (Katayama et al. 1991). Nyman et al. (2003) proposed that elevated blubber vitamin E in contaminated seals was the result of the increased demand for antioxidants due to oxidative stress caused by PCBs. Oxidative stress caused by PCB activation of the aryl hydrocarbon receptor has been shown to be prevented by vitamin E (Slim et al. 1999, Yamamoto et al. 2001). Therefore, increased blubber tocopherol concentrations in beluga may in part reflect an adapted response to higher oxidative stress found in more highly contaminated whales.

Conclusion

Fat soluble vitamins and persistent organic pollutants share many accumulation characteristics; they both accumulate in lipid rich tissues, are maternally transferred to offspring via lactation, can accumulate with age, and the concentrations of each are strongly dependent on diet (Borrell et al. 1999, Debier, Pomeroy, Baret, et al. 2002, Debier et al. 2004, Tornero et al. 2006). It is therefore not surprising that studies of free-ranging marine mammals have reported inconsistent results of the effects of organic pollutants on tissue vitamin dynamics, especially when major confounding factors are not taken into account. We examined the influence of several biological and ecological factors, and found that vitamin A and E concentrations and pattern were tissue dependent and related to sex, age, condition, and feeding ecology. Contaminant concentrations were examined in concert with these variables and were found to be an important predictor of tissue vitamin profiles. After reducing our dataset by confining and accounting for confounding biological effects, our results further supported a homeostatic disruption of vitamin concentrations due to PCB exposure in beluga whales. Our findings are important as they suggest a toxic effect of PCBs in a relatively uncontaminated population of marine mammal. This highlights the usefulness of select tissue, but preferably multiple tissue, vitamin concentrations as a sensitive biomarker of chemical exposure.

Chapter 5

Conclusion

Since organic contaminants such as legacy PCBs and flame retardant PBDEs persist in the environment, bioaccumulate in organisms, and cause a wide-range of adverse effects, there is great importance for ecotoxicologists to understand the factors that lead to concentration differences within and between populations and species. This understanding is especially important for marine mammals as they are typically among the most contaminated animals on the planet (Ross et al. 2000). Unfortunately, research into these factors is particularly challenging in marine mammals as they accumulate contaminants through space and time, and often via unknown dietary sources. Nonetheless, captive research and well designed field studies can reduce the number of confounding factors to focus on the effect of specific biological events on contaminant dynamics. This information is useful to better predict which individuals, groups, populations and/or species are most vulnerable to contaminant accumulation and consequent toxic effects. In light of this, the purpose of this thesis was to characterize two of these important influencing factors (maternal offloading and metabolism) and examine the effect of contaminant exposure on fat soluble vitamin dynamics.

Female beluga whales transferred a considerable portion of their blubber PCB and PBDE burden to their offspring during gestation, resulting in comparable blubber concentrations in mother and fetus. Though the transfer process was selective (dependent on $\text{Log } K_{ow}$), neonates were still exposed to high levels of endocrine disrupting compounds during sensitive developmental stages. Maternal transfer was found to alter

PCB and PBDE concentrations and patterns in reproductively active females compared to males and reproductively inactive females; the preferential transfer of light-low Log K_{OW} congeners to offspring resulted in a heavier contaminant pattern in mothers. Metabolism was also shown to have a large influence on contaminant patterns. Despite different feeding ecologies between small and large whales, and completely different geographical location and diet in the case of a captive beluga, PCB patterns in all beluga converged into a similar profile. This profile related best to a metabolic elimination model whereby PCB congeners accumulated or were metabolized depending on the position and number of chlorine atoms.

As with persistent contaminants, tissue levels of vitamin A and E undergo natural variation due to biological and ecological factors. Although contaminant exposure in laboratory animals result in disrupted vitamin homeostasis, similar effects are difficult to determine in free-ranging marine mammals as they are affected by multiple factors that may confound a clear contaminant-relationship with vitamin levels. The effect of multiple biological factors on vitamin and PCB concentration in marine mammals is summarized in Table 5.1.

Although some variability exists, there is striking similarity in the influence of natural factors for the accumulation of lipophilic vitamins and contaminants. In our study alone, we found that reproduction, gender, blubber lipid content, $\delta^{13}C$, and metabolism affected PCB and vitamin levels in the same general way (i.e., increase or decrease). Furthermore, although PCBs were not correlated with $\delta^{15}N$ in beluga whales, trophic status is typically a strong predictor of contaminant concentrations in marine mammals, and was found to be important for vitamin accumulation in this study. Unfortunately, the

effect of many processes on vitamin dynamics is still unknown in marine mammals, including growth dilution, migration, and feeding ecology, all of which have great potential to alter vitamin accumulation (Table 5.1).

Table 5.1 The effect of biological factors on the concentrations of lipophilic vitamins and PCBs in marine mammals. Results report the most common effect, unless considerable variation has been found. The PCB effects are taken from Borgå et al. (2004). *indicate results found in this study.

	PCBs	Vitamin A	Vitamin E	references
Reproduction	↓*	↓*	↓*	1, 2, 3, 4
Gender	males ↑*	males ↑* / none	males ↑* / none	4, 5, 6, 7, 8, 9, 10, 11, 12, 13
Growth dilution	↓	none / unknown	unknown	5
Age	↑	↑* / none	↑* / none	4, 5, 6, 9, 10, 13
Blubber lipid	↑*	↑* or none	↑*	5, 7, 8, 10, 11, 13
Fasting	↑	↑ / ↓	none	14, 15
Migration	variable	unknown	unknown	none
Metabolism	↓*	↓* / ↑*	↓* / ↑*	8, 16, 17
δ ¹³ C	↓* / variable	↓* / unknown	↓* / unknown	none
δ ¹⁵ N	↑	↑* / unknown	↑* / unknown	none

1- Debier et al. 2002a, 2- Debier et al. 2002b, 3- Debier et al. 2012, 4- Schweigert et al. 1987, 5- Borrell et al. 1999, 6- Kakela et al. 1997, 7- Mos and Ross 2002, 8- Nyman et al. 2003, 9- Rodahl and Davies 1949, 10- Rosa et al. 2007, 11- Tornero et al. 2004a, 12- Tornero et al. 2004b, 13- Tornero et al. 2005, 14- Routti et al. 2010, 15- Simms and Ross 2000, 16- Kakela et al. 1999, 17- Routti et al. 2005

The common confounding effect of various biological factors on contaminant and vitamin accumulation highlights the difficulty in determining a contaminant-related change in vitamin dynamics among all the natural variability. Accordingly, we applied a multi-tiered approach combining complimentary results from principal component analysis, AIC selected multiple regressions, and univariate regressions of vitamin and contaminant concentrations in several tissues. In the latter, important confounding factors determined through PCA and multiple regression analysis were reduced or accounted for before analysis. Overall, we detected a potential homeostatic disruption of vitamin dynamics in response to PCB exposure, whereby PCBs caused increased mobilization of retinoids and tocopherol from the liver and greater plasma and inner blubber levels.

In summary, vitamin A and E were found to be sensitive biomarkers of contaminant exposure in beluga whales. We found that outer blubber was least sensitive to contaminant-related changes. This finding is relevant as blubber samples in free-ranging marine mammals are often taken using biopsy darts, a method which only captures the outer most blubber layer. If this result proves to be consistent in several marine mammals, vitamin concentrations in biopsy samples may not be useful biomarkers of chemical exposure. In contrast, vitamin dynamics in inner blubber, plasma and liver were strongly related to contaminant concentrations. Overall, our findings are important as they showed a contaminant effect on important biological endpoints (vitamins) in a relatively uncontaminated population of marine mammal.

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Appendix 1 – Metabolism

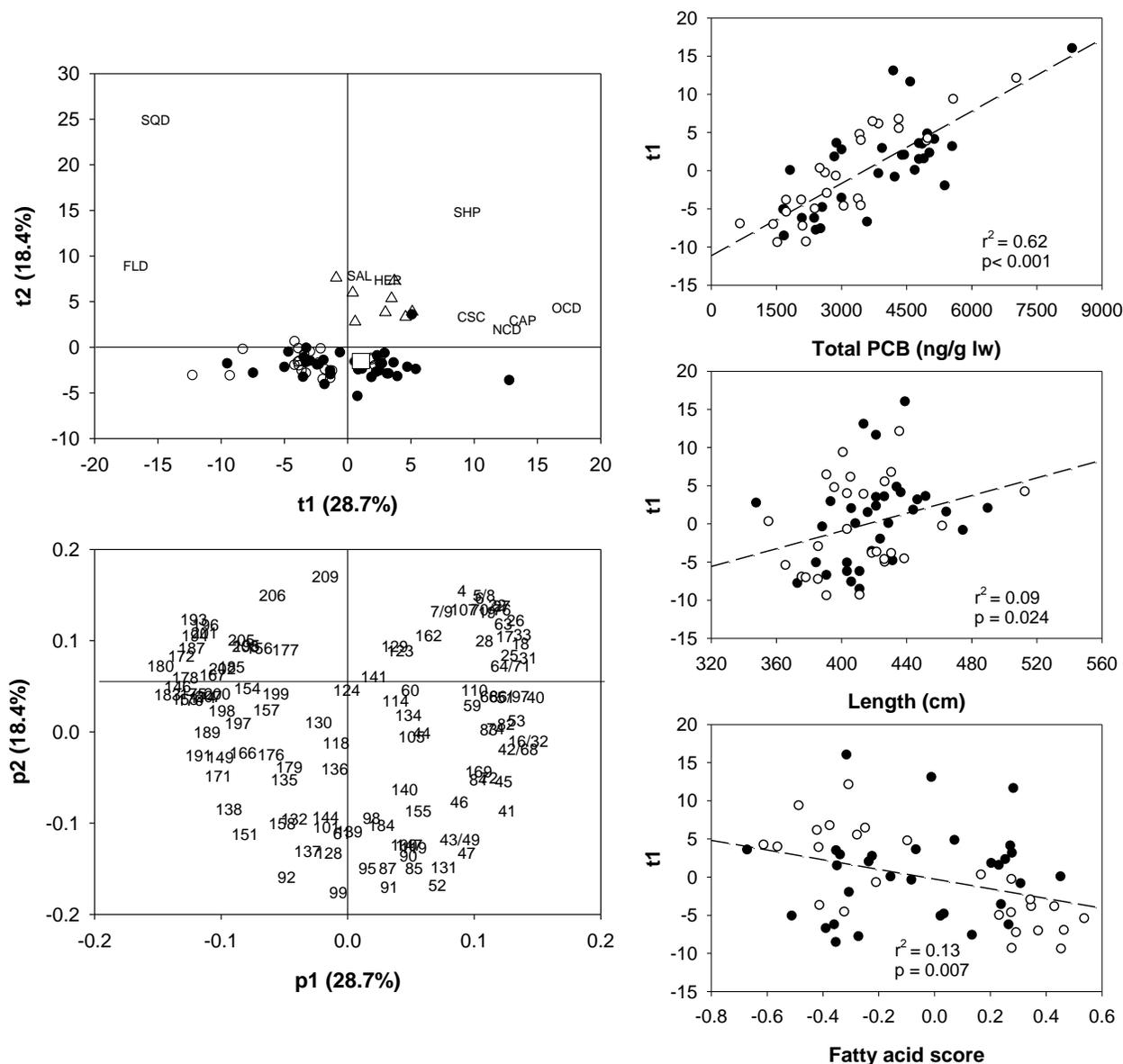


Figure S1.1 Principal component analysis (PCA) of PCBs for beluga whales and their putative as well as a captive beluga and its prey. Panels on the left show significant correlations of the first principal component with several PCBs, length and fatty acids. Symbols represent the following: Δ = females, \square = captive beluga, \circ = large offshore males, \bullet = small nearshore whales.

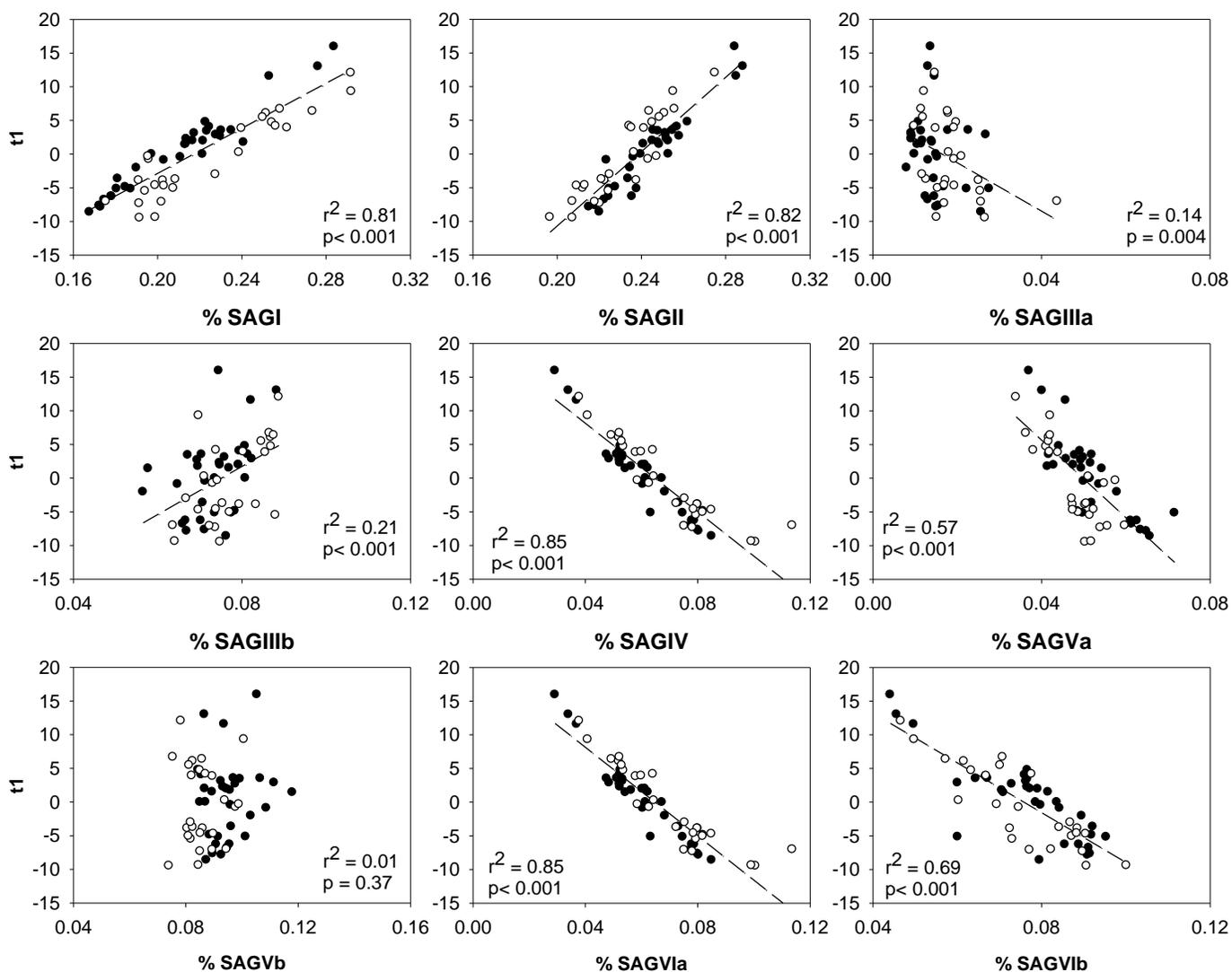


Figure S1.2 Linear regressions of the first principal component for the scores plot (t1) with the percent of total PCB for the sum of congeners in each structure-activity group (SAG). Small coastal and ice-edge associate whales (black) and larger offshore whales (white) are illustrated separately but the regression represents both groups together.

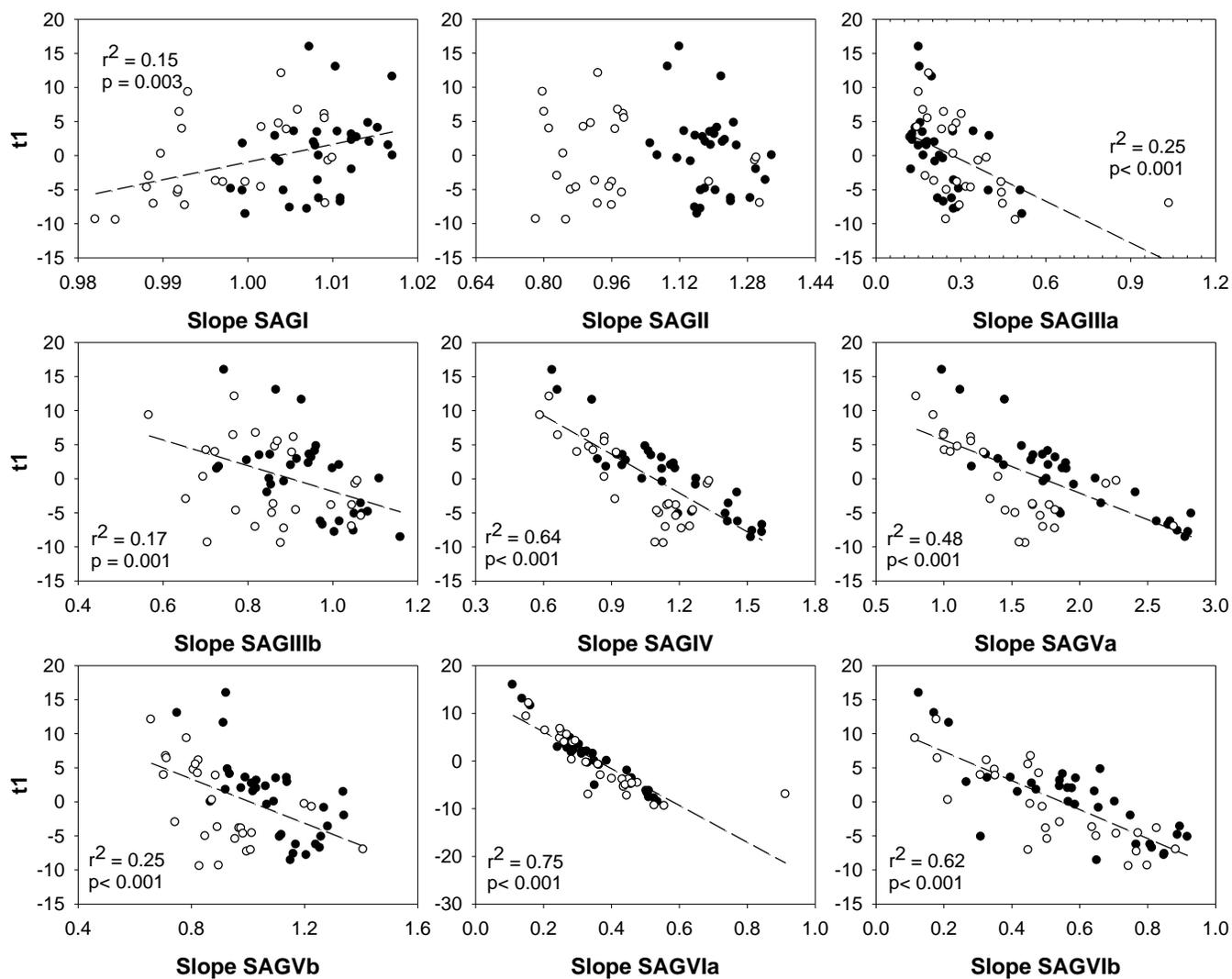


Figure S1.3 Linear regressions of the first principal component for the scores plot (t1) with the metabolic slope of individual beluga for each structure-activity group (SAG). Small coastal and ice-edge associate whales (black) and larger offshore whales (white) are illustrated separately but the regression represents both groups together.

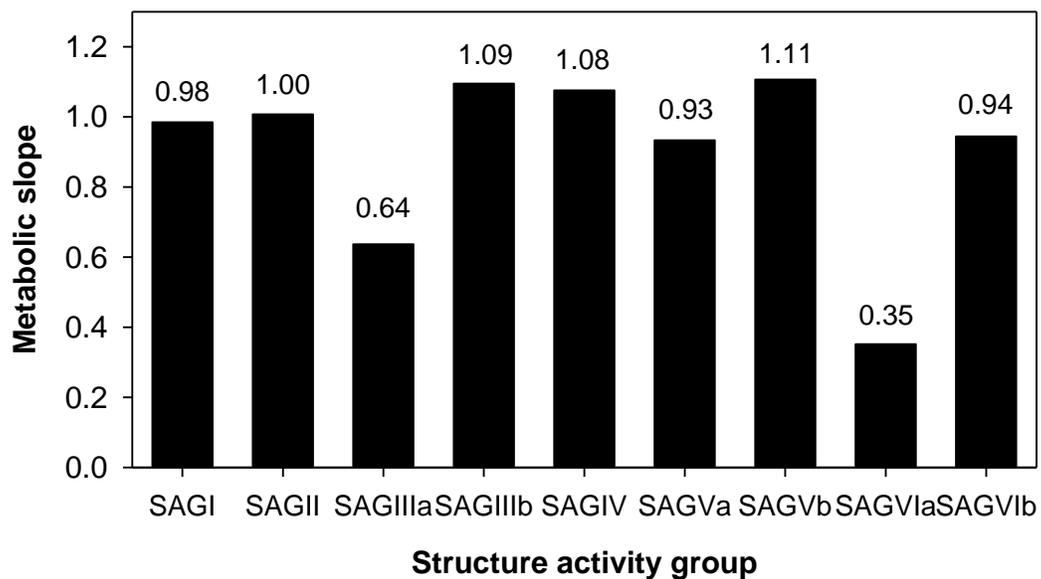


Figure S1.4 Metabolic slopes separated by PCB structure-activity groups (SAG) for the captive aquarium beluga whale. The exact diet of the whale was known and the prey signal represented the average diet of the whale over its lifetime, which did not vary significantly.

