EFFECTS OF LONG-TERM DIETARY EXPOSURE TO ORGANOHALOGEN CONTAMINANTS ON VITAMIN AND HORMONE STATUS IN THE GREENLAND SLEDGE DOG (*CANIS FAMILIARIS*)

PhD Thesis 2009

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Data sheet

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Abstract: The known overall dynamics of vitamin and endocrine disruption from organohalogen contaminants (OHCs) continue to be challenged by the constant change in complex mixtures of environmental chemical pollutants, as human desire to innovate continue. There is a great need for understanding threshold levels for each contaminant and its metabolites, but even more so the multifaceted toxicity of the complex mixtures of OHCs affecting the different animals and their organ systems. In order to mimic a long-term OHC mixture exposure situation, this study was performed as an exposure-control feeding study conducted on Greenland sledge dogs. This aimed at exploring how a diet rich in OHCs affects vitamin and hormone status in Arctic top predator mammals. The dogs were supplemented with fat containing high (exposed group: minke whale blubber) or low (control group: porcine fat) amounts of OHCs, polyunsaturated lipids and vitamins. The study showed negative correlations between e.g. vitamin A and DDT and PBDE. Also liver vitamin D (25OHD3) in exposed females was significantly lower, although they had received approximately 33% more vitamin D3 than controls. Despite the limited sample size, also testicular weights in male offspring were significantly lower in the exposed group, while plasma testosterone concentrations (and other male parameters) showed no significant difference between groups. The exposed females had significantly lower concentrations of thyroid hormone (Free T4) across all observations (6 to 18 months of age), and also for Free and Total T3 and T4, when sampled between 10 to 18 months of age. The present thesis supports the notion that wild mammals at the top of Arctic food chains are suffering from chronic non-lethal, subclinical symptoms due to exposure to complex environmental organohalogen contaminant mixtures.

Key words: Sledge dog, *Canis familiaris*, organohalogens, persistent organic pollutants, Arctic, vitamins, hormones, endocrine disruption, diet, exposure, effects

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Contents

THE PRESENT THESIS IS BASED ON THE FOLLOWING MANUSCRIPTS:

I. Kirkegaard M, Sonne C, Dietz R, Jenssen BM, Letcher RJ. Organohalogens in a whale blubber supplemented diet affects hepatic retinol and renal α-tocopherol concentrations in Greenland sled dogs (*Canis familiaris*) (in review, Environmental Research).

II. Kirkegaard M, Jacobsen J, Sonne C, Dietz R, Jensen AL, Letcher RJ. Altered 25-hyrdoxyvitamin D3 in liver of Greenland sledge dogs (*Canis familiaris*) dietary exposed to organohalogen polluted whale blubber (in review, Environmental Toxicology and Chemistry).

III. Kirkegaard M, Sonne C, Dietz R, Jenssen BM, Leifsson PS, Jensen J-E B, Letcher RJ. Testosterone concentrations and male genital organ morphology of Greenland sledge dogs (*Canis familiaris*) dietary exposed to organohalogen contaminants (in review, Journal of Toxicology and Environmental Health Part B).

IV. Kirkegaard M, Sonne C, Dietz R, Letcher RJ, Jensen AL, Jenssen BM, Grandjean P. Alterations in thyroid hormone status in Greenland sledge dogs (*Canis familiaris*) exposed to whale blubber contaminated with organohalogen compounds (to be submitted to Environmental Pollution).

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1 BACKGROUND

1.1 ORGANOHALOGEN CONTAMINANTS IN THE ARCTIC

1.1.1 Accumulation in food webs

Organohalogen contaminants (OHCs) are industrial chemicals (pesticides, flame retardants and chemical by-products). None of the OHCs occur naturally (de March et al. 1998). The basic structure of all OHCs is a phenyl ring, with attached "halogens" (hence the name organo*halogen* contaminants) like chlorine, bromine and fluorine groups. The physical-chemical properties of OHCs allow them to be transported far via atmospheric pathways (Barrie et al. 1992). Because these substances are volatile they enter the atmosphere where they persist until they condense and precipitate in cold Arctic pristine areas, remotely from the production and use (Macdonald et al. 2000). OHCs first enter the low trophic biota and are then biomagnified up the food chains, especially in the lipid rich Arctic marine food chains. Because OHCs are very persistent in the environment and are transferred from mother to offspring, they persist in the food chains for many years (AMAP 2004). Some OHCs are banned contaminants, but many are still in use. Examples are industrial chemicals and by-products like PCBs (polychlorinated biphenyls: now banned at least partially) and HCB (Hexacholorbenzene: also used in the 1960s as a fungicide). Other examples are chlorinated pesticides like DDTs (dichloro diphenyl trichloro-ethanes: used as an insecticide, banned in the western world, but used for malaria control in developing countries), CHLs (chlordanes: used as a insecticide and now banned), Dieldrin (used as a insecticide for termite control, no longer produced), Toxaphene (insecticide used on cotton crops, now banned but similar products are still in use) and HCHs (hexachlorocyclohexanes (γ-HCH = Lindane): in use as an insecticide, on crops, for headlice and scabies). New types of contaminants are BFRs (brominated flame retardants: some banned, many used in electronics and textiles), and PFCs (perflourochemicals: used as emulsifiers and additives in polymerization industry)(de March et al. 1998, Macdonald et al. 2000, AMAP 2004, Burniston et al. 2005, O'Shaughnessy 2008). Many OHCs are lipophilic and difficult to metabolize by the body, which is why they are accumulated in especially marine food webs, in the fatty tissues of top predators (de March et al. 1998, Fisk et al. 2001, de Wit et al. 2004, Verreault et al. 2005). Top mammal predators are first exposed to OHCs at the susceptible foetal stage and via lactation postnatally, and the OHCs are often retained in the body for many years. Top predators are therefore vulnerable species, as they accumulate higher concentration levels. Also, degradation and re-composition of adipose tissues, caused by fluctuating food supplies, cause pulse releases of OHCs into the blood stream and susceptible organ systems (Ellis et al. 2004, Hurley et al. 2004, Martin et al. 2004). The biochemical degradation time of OHCs and their toxic properties can vary substantially, and they are usually found in a complex mixture of many different substances and congeners in the body. Polar bears (*Ursus maritumus*), killer whales (*Orca orca*), seals (harp (*Pagophilus groenlandicus*) and fur seals (*Otariidae)),* minke whale (*Balaenoptera acuterostrata)*, pilot whale (*Globicephala*), narwhal (*Monodon monoceros*) and polar fox (*Vulpes lagopus*) are examples of top predators accumulating high amounts of OHCs through the Arctic food web (AMAP 2004). Arctic indigenous peoples are also highly exposed when they consume marine mam-

mal food products such as polar bears, seals and whales, where especially the blubber contains the highest concentrations of OHCs (AMAP 2003).

1.2 TOXICOLOGY OF ORGANOHALOGENS

1.2.1 Biotransformation of OHCs in mammals

The mammal body is equipped with enzyme mediated biotransformation processes which help detoxify and eliminate foreign substances such as OHCs by formation of more polar metabolites. These enzyme systems include the cytochrome P450 (CYP) monooxygenase systems, and they affect the individual's tissue profile and concentration levels of OHCs, in combination with their geographical region, migratory habits, diet choice, sex, age and species (e.g. Letcher et al. 2000, Verreault et al. 2009a). The CYP enzyme systems constitute a large family of different monooxygenases embedded in cell membranes, which can synthesize/metabolize reproductive hormones and vitamins as well as OHCs. Enzyme groups like CYP 1, 2 and 3 conduct primary (Phase I) pathways by e.g. inserting an (OH)-group to the OHC. This makes it a Phase I metabolite, which is then metabolized via a Phase II conjugation by other CYP enzymes. Examples of metabolites are OH-PCBs, $MeSO_2$-PCBs and OH-PBDEs, and laboratory studies have shown endocrine-related effects of these (Letcher et al. 2000, AMAP 2004, Verreault et al. 2009b). In polar bears, for example, OH-PCBs in blood are observed at concentrations several-fold higher than in liver, adipose, and brain tissue and furthermore, at concentrations far greater than those of their theoretical precursor PCB congeners (Verreault et al. 2009a).

1.3 EFFECTS OF ORGANOHALOGENS IN MAMMALS

1.3.1 Biomarkers of exposure and effects in wildlife

Biomarkers can be a tool to describe a change in an organism due to the exposure-related effects from OHCs. The OHC concentration in plasma is an exposure biomarker. In addition, effect biomarkers may include endocrine (hormone) or vitamin disruption in wildlife due to contaminants. They may encompass a number of physiological measurements like decreased or increased concentrations of vitamins and hormones, nutrients, enzymes, bone mineral density, immune parameters, certain gene expressions in the organism (genomic biomarkers) or they can reflect size, weight or histology of internal and sexual organs (Peakal et al. 1992, Tabuchi et al. 2006).

1.3.2 Combination effects of OHCs

Evidence is now available to suggest that the combined effects of different OHCs in some complex mixtures may occur even when all the individual mixture components are present at levels below doses that are known to cause observable effects (Kortenkamp 2007, 2008). One of the major difficulties in studying effects of OHCs in complex mixtures, is the uncertainty weather their effects are additive (simple summation, without enhancing or diminishing each other's action), antagonistic (less than

additive) or synergistic (effects greater than additive) (Daston et al. 2003, Kortenkamp 2007). One example is that mixtures of different classes of endocrine disrupting OHCs, such as estrogenic chemicals combined with anti-estrogenic chemicals or other contaminants, exert a "modulatory" influence on their respective effects. An example of this effect modulation is the inhibitory effect of co-planar PCBs on the formation of CYP 1A1 and 1A2, which are the steroid-metabolizing enzyme systems helping to detoxify and eliminate other OHCs. This modulation therefore disables the breakdown of OHCs, and greater amounts of co-planar PCBs in a complex mixture will cause a slow-down of the breakdown process of OHCs in the body and thereby promote their effects (Safe 1998; Chen et al. 2001, Reen et al. 2002).

1.3.3 Vitamin and endocrine disruption

Mechanisms underlying the toxic effects of OHCs show similarities in regard to processes that involve disruption of the endocrine and vitamin systems, especially vitamin A and the thyroid hormone dynamics. Vitamin A is essential for a wide range of physiological processes, including vision, growth and development, immune function, epithelial maintenance and reproduction, and it is needed in almost every tissue in the body (Novak et al. 2008). Thyroid hormones control foetal brain development, behaviour, growth, metabolism, and reproduction throughout life (Boas et al. 2006). Some of the effects on the thyroid system in wild animals are caused by metabolites rather than the precursor organohalogen congener. These metabolites (like OH-PCB, see Figure 1) attach to a protein complex that normally transports thyroid hormone and vitamin A in the body (AMAP 2004). They can also interact directly with the thyroid hormone receptor in different tissues and can influence a regulatory pathway of particular importance prenatally, thus affecting foetal brain development (AMAP 2004, Zoeller 2005). Because OHC metabolites thus occupy this transport complex and interact with thyroid hormone receptors, they may lead to imbalances of the thyroid hormone system or vitamin A dynamics or both. OHCs can also influence the levels of hormones by increasing or decreasing the normal hormone breakdown in the body. An example could be a greater production of specific CYP enzymes in the liver caused by some OHCs, which then increase breakdown of hormones, including testosterone in males (Guillette et al. 1999, Andric et al. 2000, Oskam et al. 2003, Hallgren and Darnerud 2002, You 2004, Kraemer et al. 2006). Also testis length, penis bone weight and length and penis bone mineral density in highly exposed polar bears has been shown to be affected by OHCs (Sonne et al. 2006a).

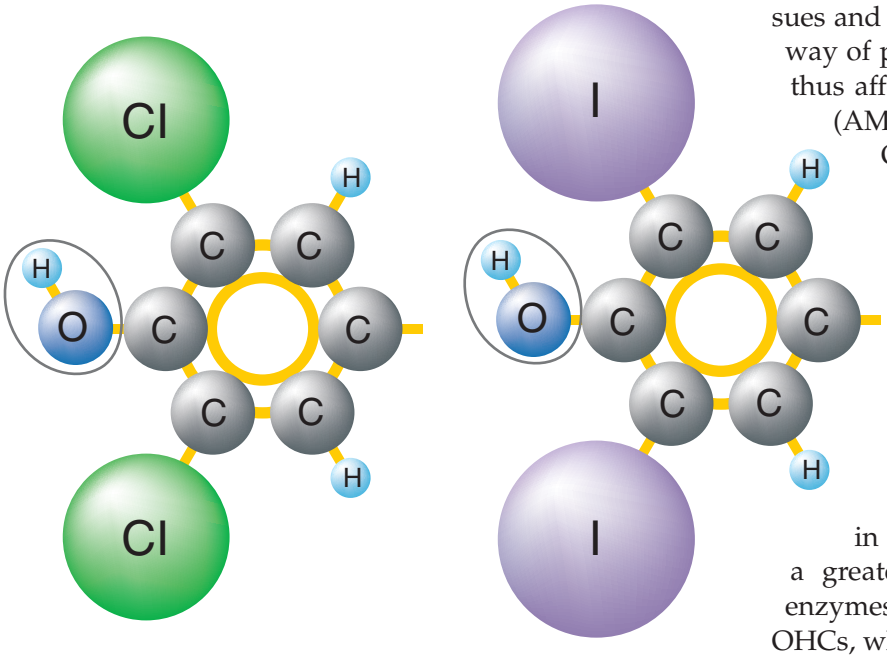


Figure 1. The figure shows the similarity between a hydroxyl metabolite of some PCBs (left) and the hormone thyroxin (right). This structural similarity allows OH-PCB to fit into a hormone receptor, which in turn can lead to a range of effects (AMAP 2004).

Vitamin A and Vitamin D_3 are very different in their chemical structure, but their receptors in cells show great similarities (Chatterjee 2001). Vitamin D_3 is actually a seco-steroid hormone with several important functions in the organism, including mineral homeostasis and, together with other endocrine hormones, is involved in calcium metabolism and bone mineralization (Haussler et al. 1998, Issa et al. 1998, Li et al. 2002, 2004). Other functions of the vitamin D_3 include regulation of blood pressure, immune functions, tissue proliferation, differentiation and apoptosis and cancer protection (Kinuta et al. 2000, Ylikomi et al. 2002, Li et al. 2004, Lou et al. 2004, Uitterlinden et al. 2004, Tuohimaa et al. 2005). The metabolite 25-hydroxyvitamin D_3 ($25OHD_3$) is the predominant form of vitamin D_3 (St-Arnaud 1999, Lou et al. 2004, Tuohimaa et al. 2005) while 1,25-dihydroxyvitamin D_3 ($1,25OHD_3$) is the most active hormonal form of vitamin D_3 (Kato 1999) (see Figure 2). These two types circulate the blood as complexes with vitamin D-binding protein, albumin and lipoproteins (Kalueff et al. 2004a, Chatterjee 2001). Experimental studies also link alterations in the vitamin D_3 and its receptor system to behavioural disorders in mice (Kalueff et al. 2004a-c), and contaminants have been suggested to depress $1,25OHD_3$ or lead to hyperthyroidism in seals (Routti et al. 2008). Alteration of the vitamin D_3 status by OHCs can therefore have severe adverse consequences.

Vitamin E also exists in different forms, with α-tocopherol as the most active form of vitamin E in mammals. It is found in most tissues, and is a powerful antioxidant, which protects cells from oxidation by free radicals that may occur as by-products of the organism's metabolism. Mammals mainly feeding on marine polyunsaturated lipids like blubber from seals or whales need more vitamin E, as these lipids may create free radicals in the body. When the body metabolises OHCs, the metabolites and/or by-products can also cause the production of oxygen radicals, or the specific OHC itself or a metabolite, might act as an oxygen radical (Boelsterli 2007). Vitamin E has no specific carrier protein in the bloodstream like thyroid hormones or vitamins A and D (Packer and Fuchs 1992), but several studies have reported alterations in vitamin E dynamics with exposure to PCBs in laboratory animals and wildlife, either as an increase or a reduction in levels in blubber, liver, spleen, and kidney (Katayama et al. 1991, Mäntylä and Ahotupa 1993, Käkelä et al. 1999, Nyman et al. 2003).

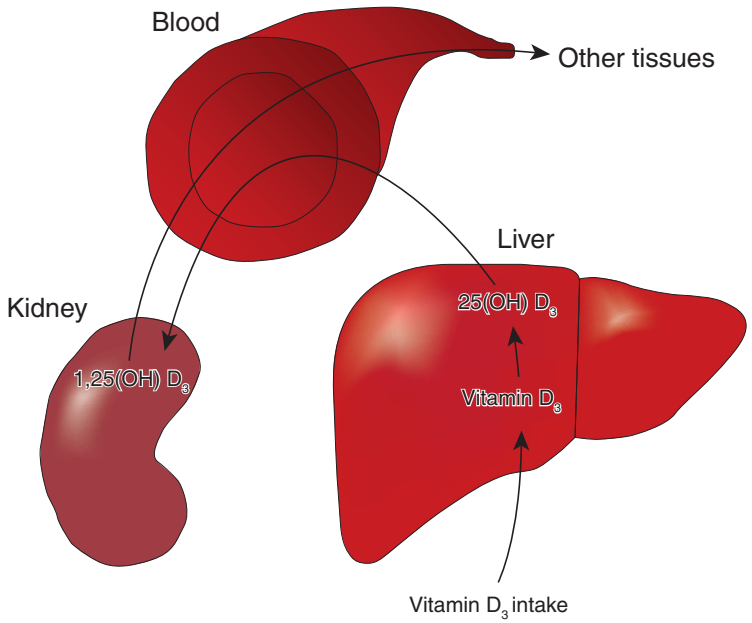


Figure 2. The figure shows how vitamin D3 from the diet is transformed to $25OHD_3$ in the liver, and distributed into the bloodstream, following transformation to the active $1,25OHD_3$ in the kidneys ($25OHD_3$ is also transformed to $1.25OHD_3$ in many other tissues, but only the kidneys also distribute this active form in the blood).

2 AIMS

The purpose of this thesis is to answer questions about the effects on hormonal and vitamin dynamics in Arctic top predators caused by organohalogen contaminants present in their diet. Many wildlife studies on effects of OHCs have been conducted, but cause-effect evaluations are difficult to investigate in observational studies of wild animals exposed to a complex mixture of OHCs. Because the exposure is not a matter of design, the unknown dietary intakes and type will lead to uncertainties. The aim of the diet experiment on sledge dogs (*Canis familiaris*) was to use this species as a potential surrogate Canoidea species for the East Greenland polar bear and to attempt to reproduce effects observed in free ranging wildlife (polar bear, Arctic fox and seal) under controlled circumstances. Furthermore, the aim was to evaluate the toxicity (effects) of an OHC complex mixture, carried out as a controlled study on sledge dogs and conducted in Greenland, using a moderate OHC dose within a natural diet matrix, which also reflects a local human dietary exposure source. This study attempted to mimic a real-life exposure situation, where an Arctic top predator consumes a diet containing a natural complex mixture of environmentally occurring OHCs in realistic doses originating from the Arctic marine mammal food web.

2.1 SPECIFIC AIMS

The specific hypotheses considered in this thesis are:

1. Vitamin A and vitamin E dynamics in Greenland sledge dogs are altered by dietary contaminant exposure from whale blubber

2. Vitamin D status in Greenland sledge dogs is altered by dietary contaminant exposure from whale blubber

3. Testosterone concentrations and genital organ morphology in sledge dog males is influenced by foetal and dietary contaminant exposure from whale blubber

4. Thyroid hormone dynamics or thyroid gland physiology in Greenland sledge dogs is altered by dietary contaminant exposure from whale blubber

3 METHODS

This section describes the designs, analyses and statistical methods of the four studies included in the thesis.

3.1 STUDY DESIGN

3.1.1 General study design

Eight pairs of female sister pups around two months of age were obtained from Inuit hunters in Aasiaat and Disko, Central West Greenland, in late 2003 through 2004. The feeding of the parents and their pups were recorded on the basis of oral questioning of the owners (pups were fed primarily oat meal or dog food pellets). These 14 pups became the P (Parent) generation bitches and the age span between them varied with 285 days (from 381 to 666 days) from the youngest to the oldest sister pair (see Figure 3). One sister in each pair was randomly allocated to an exposed group (fed whale blubber) or to a control group (fed porcine fat) immediately after entering the study at ca. two months of age. This was in order to minimize age and genetic differences among the two groups. When the females became sexually mature at an age of ca 6 months, the maternal generation (P) was mated with the same unexposed male sledge dog (again to harmonize and minimize genetic variability). Two exposed and four control dogs gave birth during the course of the study. The live offspring was the F (Foetal) generation, and was composed of 7 exposed siblings (6 male, 1 female) and 15 control siblings (8 male, 7 female), see Figure 3. After weaning at 6-8 weeks of age, they were fed the same diet as their mother. The offspring included in this thesis were selected to include animals monitored for at least 12 months, and preferably genetically related closely, resulting in 4 exposed (age 342 days, 3 males and 1 female) and 4 control (aged 388 days, 3 males and 1 female) siblings from the two litters of one sister pair (Figure 3). The dogs were kept in chains as locally required after an age of 6 months or in cages when whelping, and they were regularly exercised by dog sledge together with the other dogs. The dogs were euthanatized upon study completion at adulthood (age range for dogs used in this study: the maternal generation = 381-666 days and for offspring = 342-388 days). The animal experiments were performed on a licence granted by the Chief Veterinarian of the Home Rule Government in Greenland and conducted in accordance with national and institutional guidelines for the protection of animal welfare.

3.1.2 Diet and OHC exposure

All through the study period all the exposed dogs were fed blubber (naturally OHC contaminated) from a West Greenland minke whale (*Balaenoptera acuterostrata*), rich in polyunsaturated lipids and vitamins and low in mercury (Table 1). Control dogs were fed porcine fat (lard - low in OHCs and polyunsaturated lipids). The energy intake, controlled by the caretaker, was balanced to achieve comparable weights among siblings in the two groups, as controlled on a weekly basis. The dogs were fed 50–200 g/day of either blubber or porcine fat. Furthermore, all dogs were also fed equal amounts of standardized Royal Canine (RC) Energy 4300/4800 dry dog pellets (also 50-200 g/day) to cover basic nutrients and micro-

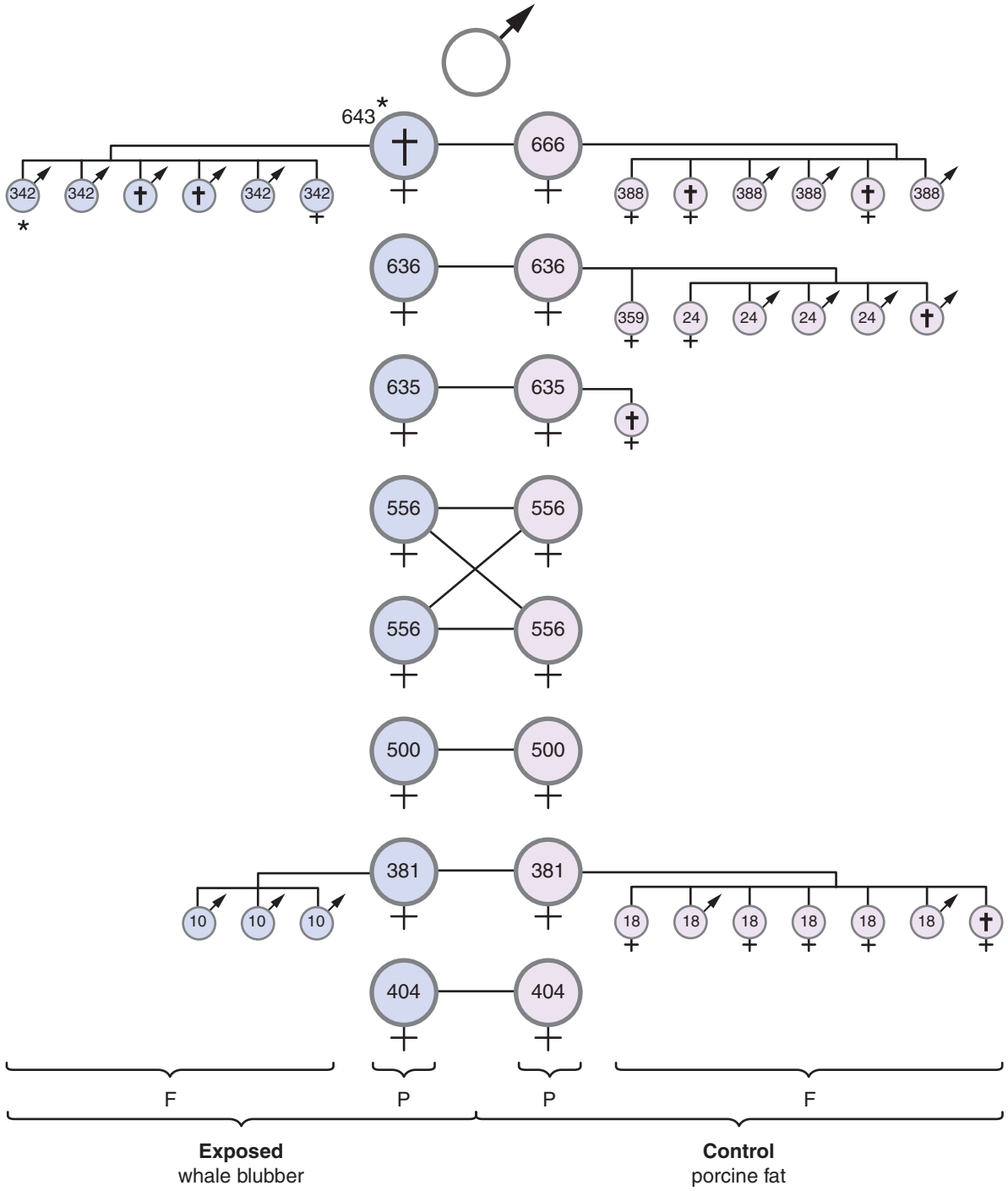


Figure 3. Generations and bloodline of 8 paired sisters mated with the same male, randomly placed in an exposed or a control group and their offspring. * †= death just prior to end of study, no tissue available. †= denotes offspring premature death. ♂=male and ♀= female. P= parental generation and F= foetal generation (offspring). Only offspring monitored for 12 months was included in the study. Number indicates age in days at study termination. Four bitches were sisters.

elements (www.royalcanin.com) (Figure 4). Food intake was increased in winter and decreased in summer to account for energy needs due to the large temperature differences between seasons. The minke whale blubber fed to the exposed group was obtained from subsistence hunting in south Greenland. The selection of whale blubber was chosen to secure large and homogenous amounts of a natural mixture of OHC. The blubber including epidermis was shipped frozen by the national distributor (NUKA A/S) responsible for providing human foodstuffs, including those from subsistence hunting, for local supermarkets throughout Greenland. Porcine fat fed to the control group was bought and shipped frozen to Greenland by

Figure 4. Sledge dogs being exercised by harness and sledge, kept in the locally required chains, and preparing and feeding dogs with whale blubber and dog food pellets (Photos: M. Kirkegaard, T.D. Rasmussen and S. Andersen).

the Danish company Dragsbæk (product trade name Blume). Whale blubber and porcine fat was stored in freezers at -18°C inside the mobile laboratory at the study location. The epidermis was removed from the blubber before feeding (see Figure 4). Royal Canin (RC type 4300 and 4800) dog pellets were bought from Royal Canin, Denmark, a company with a history of and experience in supplying dog food for dogs in Danish and international research projects. All collies of dog food were from one single batch, shipped to Greenland and also stored inside the mobile laboratory (and maintained below 0°C from October to May). All dogs were given fairly equal amounts of the two energy-different pellets to ensure an equal amount of nutrition (vitamins, minerals, protein and carbohydrates) in each group. The low calorie type (RC 4300) was given in summer and the high calorie type (RC 4800) in winter. The control group received 10% less pork fat than the amount of whale blubber provided to the exposed group, in order to equalize for available energy % in the two different fat types. All diet items were analysed for OHC content. In the Energy 4300 and 4800, only the ΣPCB congener CB-153 was detected. Table 1 shows OHC (Σ_{40}PCB and Σ_{11}PBDE, and the low levels of mercury), vitamin, nutrients and lipid content. The daily intake of 50-200g whale blubber (mean intake 112g) constituted approximately between 10.4-11.7 µg/kg bodyweight ΣOHC (or approximately between 4.6-6.1 µg/kg bodyweight ΣPCB). Vitamin A (retinol) was 18% higher, vitamin E (α-tocopherol) 22% higher and vitD_3 33% in the diet of the exposed dogs compared to controls (mean of a period of 4 weeks prior to sampling).

3.1.3 Field sampling

Daily feeding, daily weighing of diet items, weekly weighing, regular exercise, vaccination and sampling of blood plasma with different intervals

Table 1. Concentrations of vitamins, nutrients, lipids and OHCs in minke whale blubber, porcine fat and Energy4300 or Energy4800 Royal Canin (RC) diet. ΣPCB= Σ_{40}PCB and ΣPBDE = Σ_{11}PBDE.

Vitamins and Minerals	Whale blubber	Porcine fat	RC4300[a]	RC4800[a]
Vitamin A (mg/kg)	1.2	0.09	7.5	7.5
Vitamin C (mg/kg)	<10	<10	320	350
Vitamin D3 (mg/kg)	0.028	0.005	0.03	0.03
25OH D3 (mg/kg)	<0.001	0.002	.	.
Vitamin E (mg/kg)	75	15	650	700
Calcium (%)	.	.	1.3	1.5
Phosphorous (%)	.	.	0.95	1
Magnesium (%)	.	.	0.2	0.2
Iron (mg/kg)	<10	<10	250	250
Copper (mg/kg)	.	.	30	32
Manganese (mg/kg)	.	.	55	60
Zink (mg/kg)	0.6	0.1	190	200
Iodide (mg/kg)	.	.	3.5	3.5
Selenium (mg/kg)	0.15	<0.01	0.25	0.25
Sodium (%)	.	.	0.37	0.4
Chlorine (%)	.	.	0.73	0.8
Potassium (%)	.	.	0.62	0.7
Lipids (mg/g wet weight)				
Total lipid (mg/g)[b]	571	954	196	263
N–3 lipids (mg/g)	64.3	8.56	4.53	4.08
Lipid %	72	97	21	30
Contaminants (ng/g lw)				
ΣOHC[b]	2818	<0.01	1.9	2.7
ΣPCB[b]	1717	<0.01	1	0.5
ΣCHL	296	<0.01	<0.01	<0.01
ΣDDT	595	<0.01	<0.01	2.2
Dieldrin	94	<0.01	<0.01	<0.01
ΣHCH	20	<0.01	<0.01	<0.01
HCB	28	<0.01	<0.01	<0.01
ΣPBDE[c]	63	<0.01	0.9	0.4
Total Mercury	5	1	1	2

[a]RC4300 and RC4800: vitamins and nutrients are based on values from www.royalcanin.dk
[b]In the RC4300 and RC4800, only CB-153 was detected.
[c]ΣPBDE concentration in the pork fat was comprised of 51% BDE-99, 28% BDE-153 and 21% BDE-183. ΣPBDE concentration in the Energy 4300 and 4800 was comprised of 54% BDE-47 and 46% BDE-99.
Abbreviations: polychlorinated biphenyls, PCB; dichloro diphenyl tricloroethane, DDT; hexachlorobenzene, HCB; hexachlorocyclohexane, HCH; chlordanes, CHL; Polybrominated diphenyl ethers, PBDE.

during the dogs' life was performed by the daily caretaker (TDR) in the facilities of the laboratory housed in a mobile container. All dogs were regularly health examined by a field veterinarian, and immunized for canine distemper virus, parvovirus, hepatitis virus, parainfluenza virus and rabies (Duramune 4 Vet Scanvet®). The laboratory was heated and provided with electricity, an Eickemeyer centrifuge, storage room for dog food pellets and freezing facilities for storage of whale blubber and porcine fat. The blood samples were performed on fasting dogs (12-24 hours) and sampled using a Vacutainer™ blood collection system with an 18 gauge needle and sterile Lithium-Heparin Greiner 9 ml Vacuette and centrifuged at 3600 rpm for 10 min. Subsequently 4 mL blood plasma from each dog was sam-

pled in 10 mL sterile Nunc 348224 cryo tubes and frozen immediately to -18ºC. Thyroid glands and testes were sampled after euthanasia, weighed, measured and one either frozen or stored in formaldehyde until sectioning for histology. Penile bones (bacula) were frozen immediately to -18ºC after sampling. Adipose tissue, one thyroid gland, liver and kidney tissue was frozen immediately to -18ºC and stored this way until OHC analysis, and also liver and kidney samples were stored at -18ºC until arrival at the laboratory for vitamin analysis. All vials with tissue and plasma samples were wrapped immediately in aluminium foil to hinder vitamin degradation from UV light. One exposed bitch and her exposed pup was sampled for plasma during most of the study duration, but not sampled for tissues, and thus not analysed for OHCs, at the end of the study. This was due to unfortunate factors unrelated to the experiment (death due to apparent fighting and subsequent decay of tissues). Consequently, the numbers of plasma and tissue samples vary.

3.1.4 Effects on vitamins A, E and D (manuscripts I-II)

Study design I
Study I was designed to investigate vitamin A (retinol) and E (α-tocopherol) content in liver and kidney at the end of the study and plasma levels during the study. The number of animals included in this study was 16 P generation bitches (8 exposed and 8 control) and 8 F generation pups (4 exposed and 4 control) (Figure 5).

Plasma: The 8 pairs of P generation sister bitches were blood sampled at different intervals and frequencies during the study. Vitamins were measured in 53 exposed and 53 control blood plasma samples, taken between 3 months and 21 months of age. The 4 pairs of F generation pups (4 exposed and 4 control), were sampled between 3 months and 12 months of age and vitamins were measured in 19 exposed and 19 control plasma samples.

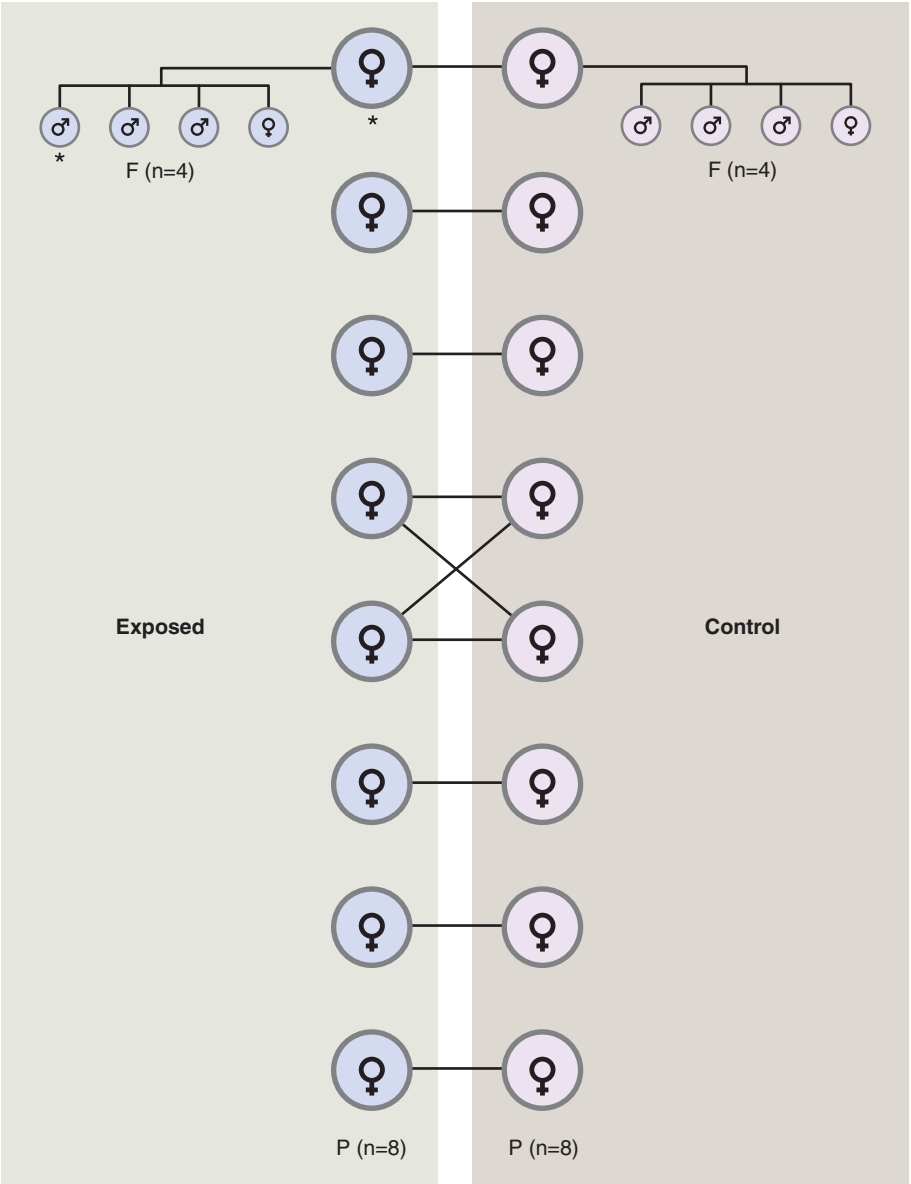
Tissues: Liver, kidney and adipose tissue were sampled from 7 sister bitch pairs (P) and from 3 sibling pairs (F). Thus, individual animals marked with * were included in the study with plasma samples only (see Figure 5), and no OHC analyses were performed.

OHCs: In all the other exposed individuals (P: n=7, F: n=3) the different OHCs measured in adipose tissue (and PCBs measured in liver, kidney and plasma) were correlated with concentrations of vitamin A and E.

Study design II
Study II was designed to investigate vitamin D_3 content in liver at the end of the study and plasma concentration levels during the study. The number of animals included in this study was 14 P generation bitches (7 exposed and 7 control) and 8 F generation pups (4 exposed and 4 control). Due to limited amounts of blood plasma, two P generation sister pairs were not part the study. Furthermore, one pair in the P generation was excluded, as vitamin values were unexplainably high in one dog (Figure 6).

Plasma: Samples from 6 pairs of P generation sister bitches were used, sampled at different intervals and frequencies during the study. $25OHD_3$ was measured in 40 exposed and 40 control blood plasma samples, taken between 3 months and 21 months of age. The 4 pairs of F generation pups (4 exposed and 4 control), were sampled between 3 months and 12 months of age and 25OHD3 in plasma was measured in 19 exposed and 19 control dogs.

Figure 5. The number of sledge dogs included in study I.



Tissues: Liver and adipose tissue was used from 6 sister bitch pairs (P) and from 3 sibling pairs (F). Thus, the individual animals marked with * were included with plasma samples only (see Figure 6), and no OHC analyses were performed.

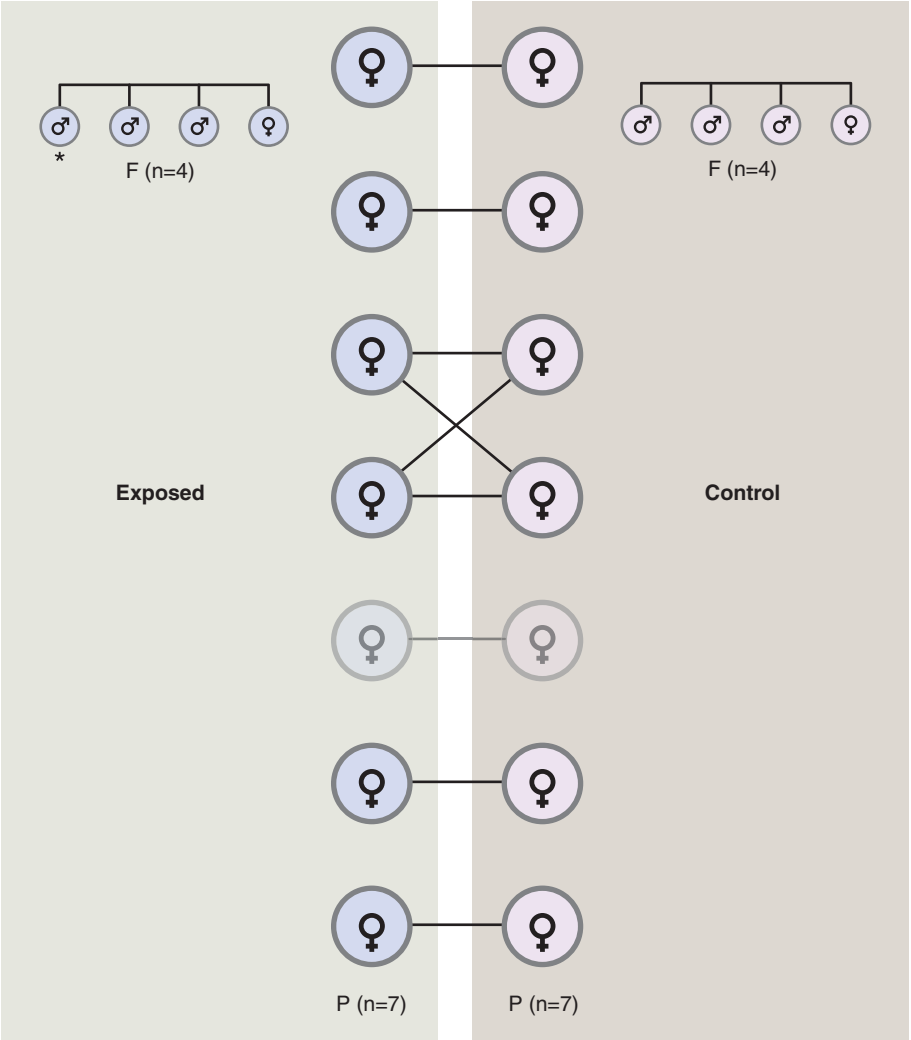
OHCs: In all the other exposed individuals (P: n=6, F: n=3) the different OHCs measured in adipose tissue (and PCBs measured in liver, kidney and plasma) were correlated with concentrations of vitamin D_3 and $25OHD_3$.

3.1.5 Effects on thyroid hormones, testosterone and male genital organs (manuscripts III and IV)

Study design III

The parameters chosen for this study were testosterone, testis and penile morphology and penile bone mineral density. One testicle was kept in neutral formaldehyde to evaluate histopathology, but unfortunately, the testes fixation was not successful due to cold temperature extremes in the

Figure 6. The number of animals included in study II.



field. The number of animals included in this study was 3 exposed males and 3 control males: they shared the same father and their mothers were sisters (Figure 7).

Plasma: The 3 pairs of F generation pups were sampled at different times between 3 months and 12 months of age and testosterone was measured in 15 exposed and 15 control plasma samples.

Tissues: It was possible to sample testis, penile bone and adipose tissue only from 2 of the 3 exposed male offspring at 12 months. Thus, the individual marked with * was included with plasma samples only (see Figure 7), and no OHC analyses were performed.

OHCs: OHCs were analysed in 2 of 3 exposed male dogs. Correlation analysis was not possible.

The general study design did not allow analysis of the number of male offspring born from the female cohort. However, it did allow an evaluation of the effect of *in utero* exposure to OHCs, as well as dietary exposure until 12 months of age, even when number of individuals was limited. True transgenerational effects were not a part of the study design, as this would require further generations of adult offspring. Although testes his-

Figure 7. The number of animals included in study III.

tology was also chosen as a parameter for this study, incorrect storage for a few hours after sampling resulted in tissue damage due to unexpected freezing, which made evaluation of testis histology impossible.

Study design IV

Study IV was designed to investigate thyroid hormones (free and total T3 and T4) in plasma samples during the study. The number of animals included in this study was 14 P generation bitches (7 exposed and 7 control) and 8 F generation pups (4 exposed and 4 control) (Figure 8).

Plasma: The P generation bitches were veinpunctured at different occasions between 6 to 18 months of age and a total of 22 blood plasma samples from 6 control and 6 exposed sister bitches provided results for this study. The 4 pairs of F generation pups (4 exposed and 4 control), were veinpunctured between 3 months and 12 months of age and thyroid hormones were measured in 20 exposed and 20 control plasma samples. Due to limited amounts of blood plasma, two P generation sister pairs were not part of TH assessment in plasma from 6 to 18 months. Also, analyses of only Total T3 and T4 were conducted in the F generation due to limited amounts of blood plasma.

Tissues: Adipose tissue (for OHC analysis), liver and kidney (for vitamin analysis) were analysed from 7 paired P generation bitches and from 3 exposed and 3 control F generation pups. The animal marked with * was included with plasma samples only (see Figure 8), and no OHC analyses were performed nor were thyroid glands sampled. Furthermore, it was possible to sample only the right thyroid gland in one control P bitch.

OHCs: The different OHCs measured in adipose tissue (and PCBs measured in thyroid gland, liver, kidney and plasma) were correlated with thyroid gland weights and canine Thyroid Stimulating Hormone (cTSH), sampled at the end of the study (P: n=6, F: n=3).

3.2 ANALYSES

3.2.1 Analysis of persistent organic pollutants in dog fatty tissue, liver and plasma and diet

Analyses of OHCs in sledge dog adipose tissue (and for some OHCs plasma, kidney, liver and thyroid glands) and in diet samples of pellets, whale blubber and porcine fat were carried out using a gas chromatograph–mass spectrometer (GC–MS) (Agilent 6890, GC-5973 MSD; Agilent Technologies, Palo Alto, CA, USA). The following contaminants were analysed

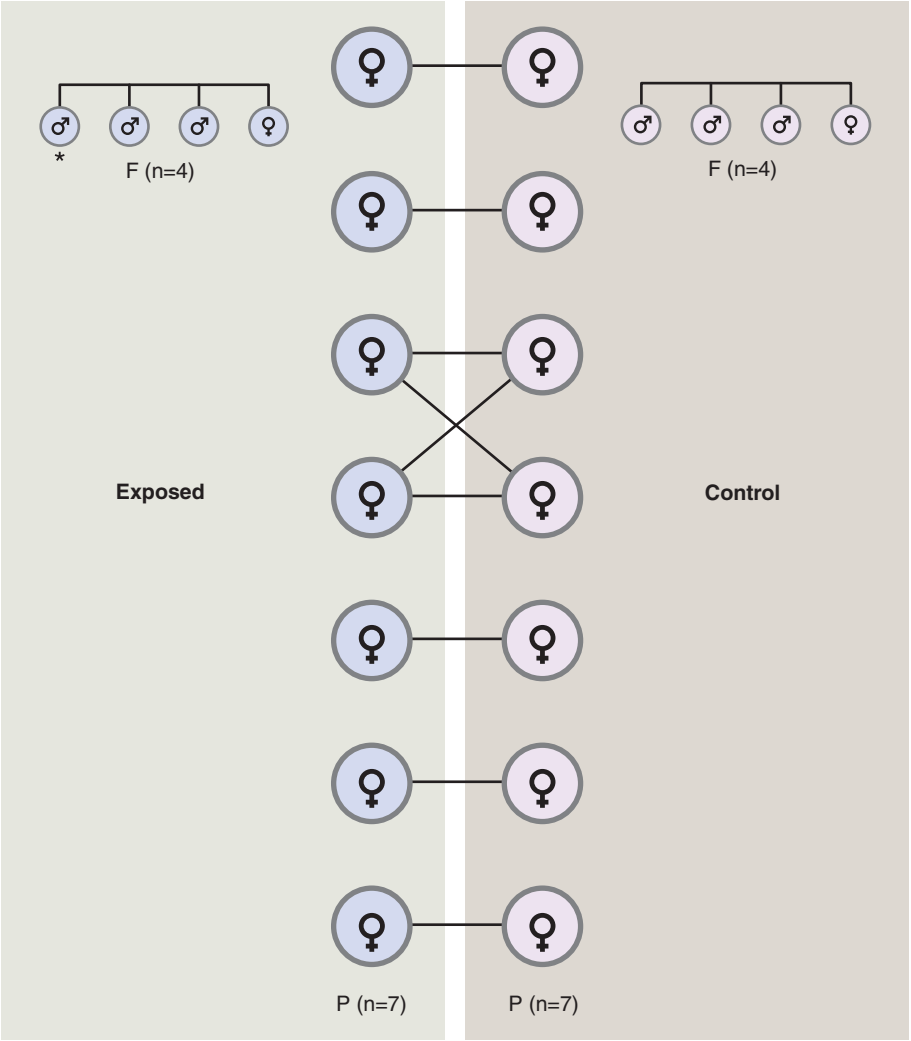


Figure 8. The number of animals included in study IV.

in dog tissue: PCBs; 59 congeners of PCB. OCs; 1,2,4,5-tetrachlorobenzene (TeClBz), pentachlorobenzene (PnClBz), hexachlorobenzene (HCB), -hexachlorocyclohexane (HCH), β-HCH, β-HCH, octachlorostyrene (OCS), the chlordanes and metabolites heptachlor epoxide, oxychlordane, *trans*-chlordane, *cis*-chlordane, *trans*-nonachlor, *cis*-nonachlor, *p,p'*-DDE, *p,p'*-DDD, *p,p'*-DDT, dieldrin, photomirex, mirex and tris(4-chlorophenyl)methane (TCPM). Also in fat, 36 PBDE congeners were monitored, as well as several other BFRs including 2,2,',4,4',5-pentabromobiphenyl (BB-101), pentabromotoluene (PBT), hexabromobenzene (HBB), total-(α)-hexabromocyclododecane (HBCD), bis(2,4,6-tribromophenoxy)ethane (BTBPE) and pentabromoethylbenzene (PBEB).

3.2.2 Analysis of testosterone

Plasma levels of testosterone were determined by a solid-phase radioimmunoassay (RIA) kit (Spectria®Testosterone RIA Coated Tube Radioimmunoassay, Orion Diagnostica, Espoo, Finland). The assay was validated with sledge dog heparin plasma and the standard method for analysis was used (Spectria 2005). The amount of the bound radioactive antigen was quantified using a gamma counter (Cobra Auto-Gamma: Pacard Instruments Company, Dowers Grove, IL, USA). Human serum controls in each kit (Immunoassay Plus Control, Level 1, 2 and 3, BIO-RAD Laboratories, Liquichek and Lyphchek, Hercules, CA, USA) were also assayed to test for repeatability.

3.2.3 Analysis of thyroid hormones

Analysis of total (T) and free (F) T3 and T4 in plasma samples from the P generation dogs were conducted using an automated electro-chemiluminescent immunoassay by Modular E-170 (Roche) in one single run. All assays were subjected to both daily internal and quarterly external quality control following RiliBäk and the EU ISO 17025 standards. Analysis of total T4 (TT4) and total T3 (TT3) from the F generation dogs was conducted by radio-immunoassay (RIA) with commercially available kits (Coat-A-Count; Diagnostic Product Corporation, LA, CA, USA) according to kit instructions. The amount of the bound radioactive antigen was quantified using a gamma counter (Cobra Auto-Gamma: Pacard Instruments Company, Dowers Grove, IL, USA). Human serum controls in each kit (Immunoassay Plus Control, Level 1, 2 and 3, Bio-Rad Laboratories, Liquichek and Lyphchek, Hercules, CA, USA) were also assayed to test for repeatability.

3.2.4 Analysis of albumin and Endogenous Canine Thyrotropin (cTSH)

Analyses of albumin and thyroid stimulating hormone in plasma (cTSH) were conducted using a chemiluminometer Immulite (DPC). The canine reference interval given is those currently used at the Central Laboratory at Department of Small Animal Clinical Sciences based on samples from healthy dogs send to the laboratory.

3.2.5 Analyses of vitamin A, E and C

Analyses of vitamins A (retinol) and E (α-tocopherol) in liver and kidney of the P and F generation dogs were analyzed using reverse-phase high-performance liquid chromatography (RP-HPLC) with fluorescence detection, according to Murvoll et al. 2005 with minor modifications. Vitamin A in plasma of both P and F generation of dogs and vitamin A in whale blubber was analyzed using reverse-phase high-performance liquid chromatography (RP-HPLC) with fluorescence detection (see manuscript I). Vitamin A in porcine fat (product trade name Blume) was based on literature studies (www.foodcomp.dk). Analyses of vitamin C and E in lard and whale blubber were performed at Eurofins Lab, Kolding, Denmark (www.eurofins.dk). Lipids in whale blubber and porcine fat were analysed using gas-chromatography and flame ionization (GC-FID) as described in Sonne et al. (2008a). All analyses for vitamins in the diet were performed accredited according to ISO 17025 (ISO, 2000).

3.2.6 Analyses of vitamin D_3 and 25OHD_3

Analyses of vitamin D_3 in liver and 25OHD_3 in plasma and liver of dogs, and vitamin D_3 and 25OHD_3 in diet items were analyzed using reverse-phase high-performance liquid chromatography (RP-HPLC) with fluorescence detection, as described in Jakobsen et al. (2007) and Jakobsen et al. (2004).

3.2.7 Analyses of other components in the diet

Metal analyses of food were performed at NERI-DAE Laboratory (Roskilde, Denmark; www.neri.dk) by Flow Injection Analyse System for mercury and selenium, and for zinc and iron by Atomic Absorption Spectrometry. Fatty acid analyses of food were conducted using gas-chromatography and flame ionization (GC-FID) as described in Sonne et al. (2008a).

3.3 STATISTICAL METHODS

Statistical analysis was performed with the SAS statistical software package (Enterprise Guide v4, SAS Institute Inc., Cary, NC, USA). The two-sided level of significance was set to $p \leq 0.05$, and in some cases $0.10 < p < 0.05$ was considered suggestive. Parametric statistics such as t-tests and Pearsons's product moment correlation coefficient were applied to data that complied with a normal distribution. The paired Students t-test was used with both generations, first to evaluate the paired P generation sisters randomly placed in each group at the beginning of the study. A Student's t-test was also used in the few cases where the number of individuals was low and differed between groups. The exposed and control sibling groups in the F generation were genetically related with the same father and mothers as sisters, and they were paired first according to sex and then by weight before paired statistical analysis. The data were designed for a paired test in order to eliminate differences from e.g. season, genetics or other factors between sister/sibling pairs. The study animals selected have a long generation time, and are costly to keep across a long time frame (more than 2 years), and thus no further attempts were made regarding breeding of new pups. Unfortunately, this design left a limited sample size of the offspring generation. However, a sample size of 6-8 parents in each (exposed and control) group was considered sufficient, with the duration of study and animal welfare in mind (Table 2).

Table 2. Overview of number of dogs included in each study

	STUDY I	STUDY II	STUDY III	STUDY IV
P generation				
Exposed	8	7	-	7
Control	8	7	-	7
F generation				
Exposed	4	4	3	4
Control	4	4	3	4

4 RESULTS

In this section the main results from the four papers are presented. A detailed description of the results can be found in the manuscripts I-IV. Appendix I provide a short description of the additional research studies conducted within the sledge dog project.

4.1 ORGANOHALOGEN CONTAMINANTS IN DOG TISSUE

Vitamin and hormone concentrations in dog tissues was evaluated by correlations with OHCs in all tissue types mentioned, except thyroid glands, which was only used in manuscript IV. Figure 9 and 10 show concentrations of the different OHCs in dog adipose tissue.

4.2 EFFECTS OF DIETARY CONTAMINANT EXPOSURE ON RETINOL AND α-TOCOPHEROL (MANUSCRIPT I)

In this study we investigated the effect on retinol and α-tochoperol concentrations from contaminant exposure concentration in different tissues across both generations of exposed dogs (P+F). A negative significant correlation between liver retinol and ΣBDE (r=-0.642, p=0.045) (Figure 11) and a non significant negative correlation between liver retinol and ΣDDT (r=-0.626, p=0.053) was observed.

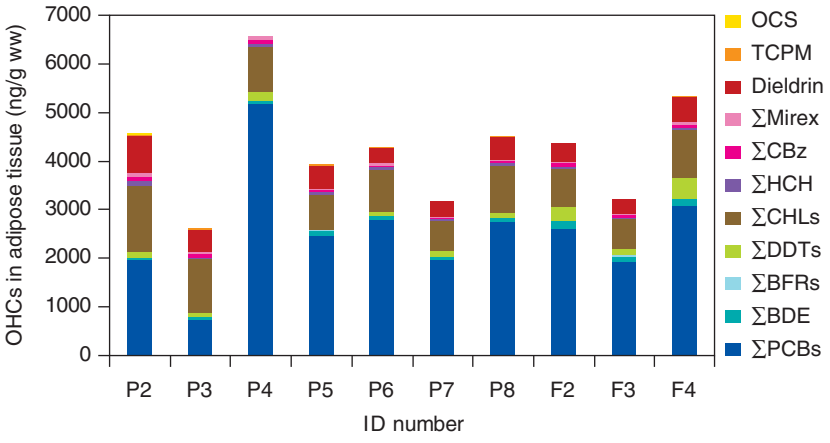


Figure 9. OHCs in exposed dogs (P: n=7 and F: n=3) measured in adipose tissue (ng/g ww). Age of P bitches decrease from left to right (636-381 days of age). Age of F pups was 342 days.
Abbreviations: polychlorinated biphenyls, PCB; Polybrominated diphenyl ethers, PBDE; Brominated flame retardents, BRFs; dichloro diphenyl tricloroethane, DDT; chlordanes, CHL; hexachlorocyclohexane HCH; hexachlorobenzene and tetra+ penta clorobenzene, CBz; tris(4-chlorophenyl) methane, TCPM; octachlorostyrene, OCS.

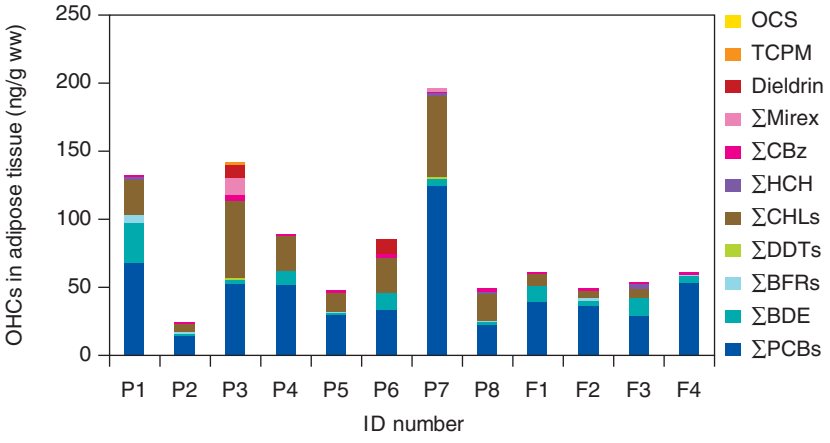


Figure 10. OHCs in control dogs (P: n=8 and F: n=4) measured in adipose tissue (ng/g ww). Age of P bitches decrease from left to right (666-381 days of age). Age of F pups was 388 days.
Abbreviations: polychlorinated biphenyls, PCB; Polybrominated diphenyl ethers, PBDE; Brominated flame retardents, BRFs; dichloro diphenyl tricloroethane, DDT; chlordanes, CHL; hexachlorobenzene, HCB; hexachlorocyclohexane, HCH; tetra+ penta clorobenzene, CBz; tris(4-chlorophenyl) methane, TCPM; octachlorostyrene, OCS.

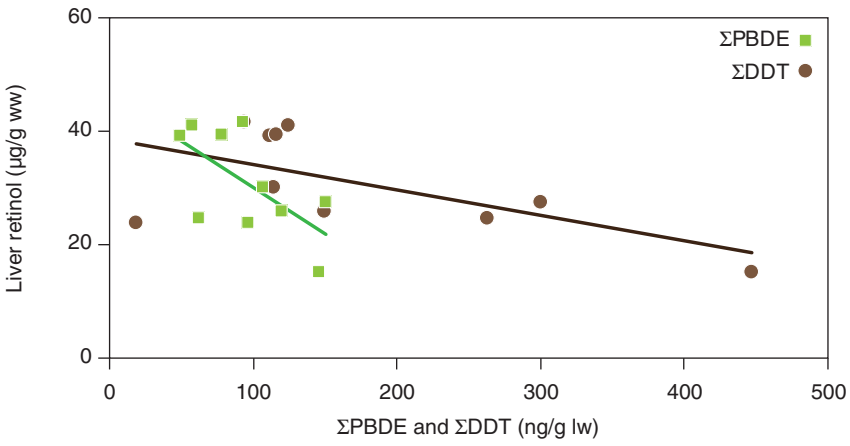


Figure 11. Liver retinol concentrations (µg/g ww) correlated with ΣDDT and ΣBDE (ng/g lw) in adipose tissue of 10 adult sled dogs (maternal and offspring) fed OHC contaminated whale blubber between 12-21 months. In the exposed group (P+F) a negative correlation was seen between liver retinol and ΣDDT (r=-0.626, p=0.053), and between liver retinol and ΣBDE (r=-0.642, p=0.045).

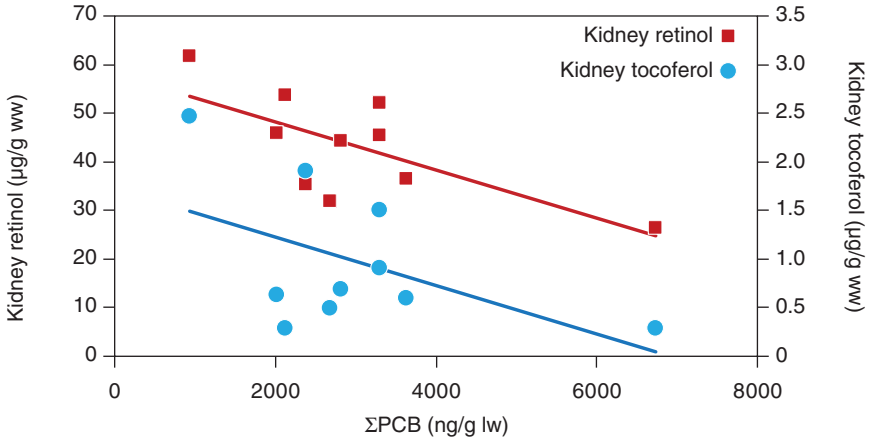


Figure 12. Kidney retinol and α-tocopherol concentrations (µg/g ww) correlated with ΣPCB (ng/g lw) in adipose tissue of 10 adult sled dogs (maternal and offspring) fed OHC contaminated whale blubber between 12-21 months. The negative correlation between kidney retinol and ΣPCB was only a trend (r=-0.514, p=0.13), whereas the negative correlation between kidney tocopherol and ΣPCB was significant (r=-0.702, p=0.024).

With respect to kidney retinol, there were positive correlations with ΣCHL (r=0.684, p=0.029) and dieldrin (r=0.642, p<0.046). Also, there was a negative trend between kidney retinol and ΣPCB in adipose tissue in the exposed P group (r=-0.514, p=0.13) (Figure 12). Kidney α-tocopherol and ΣPCB showed a significant negative correlation (r=-0.702, p=0.024) (Figure 12). No other correlation between retinol and α-tocopherol in liver and kidney, and ΣOHC in adipose tissue or ΣPCB in kidney, liver or plasma was observed. No significant correlations between α-tochoperol in plasma and OHCs were found in either generation.

With respect to plasma retinol of the P generation, no significant difference was found between the two groups (exposed n=8, 53 samples and control n=8, 53 samples) sampled between 3 and 21 months. However, the plasma α-tochoperol concentrations were significantly higher in the control group as compared to the exposed group. In the F-generation (exposed n=4, 19 samples and control n=4, 19 samples) sampled between 3 and 12 months, plasma retinol in the exposed group were significantly higher in the exposed than in controls (p<0.01) (Table 3). Also plasma α-tochoperol levels in the exposed group was significantly higher compared to control siblings (p<0.05).

There was an increase in hepatic retinol concentrations with age (r=0.70, p=0.01) for all control sled dogs (P+F), while no such age dependent effect was found among the exposed dogs.

Table 3. Liver (L), kidney (K) and plasma (P) retinol and α-tocopherol concentrations in a maternal (P) and an offspring (F) generation of the sled dogs after exposure for 12 months (F generation) or 12-21 months (P generation). P values (paired t-test) indicate which group (control or exposed) has highest values.

	RETINOL				TOCOPHEROL			
	Exposed (n=7)		*Control (n=7)*		*Exposed (n=7)*		*Control (n=7)*	
P generation	*mean*	*range*	*mean*	*range*	*mean*	*range*	*mean*	*range*
L (µg/g ww)	34.21	23.8-41.6	25.00	10.0-33.7	21.5	14.4-29.7	**34.8	23.1-56.0
K (µg/g ww)	1.00	0.30-2.50	1.10	0.20-5.60	43.3	26.5-61-7	54.5	28.1-73.6
P (µg/ml)	0.67	0.60-0.80	0.61	0.30-1.10	19.8	12.8-24.8	**30.0	22.0-38.9
	Exposed (n=3)		*Control (n=3)*		*Exposed (n=3)*		*Control (n=3)*	
F generation	*mean*	*range*	*mean*	*range*	*mean*	*range*	*mean*	*range*
L (µg/g ww)	**22.8	15.0-27.4	18.9	11.0-23.5	19.2	14.7-24.5	19.4	12.0-27.7
K (µg/g ww)	0.88	0.50-1.50	1.40	0.50-2.60	43.3	31.8-52.1	37.1	24.0-50.0
P (µg/ml)	**0.78	0.70-0.80	0.38	0.30-0.40	15.2	6.30-22.8	12.1	1.60-23.3

*=p<0.05, **=p<0.01

4.3 EFFECTS OF DIETARY CONTAMINANT EXPOSURE ON VITAMIN D STATUS (MANUSCRIPT II)

In manuscript II analyses of liver vitamin D_3 (vitD_3) in the P generation were evaluated pair-wise for six pairs (one control vs. one exposed sister). Significantly higher (p=0.004, n=12, paired t-test) concentrations of 25OHD_3 in liver of the control group was observed in the P generation. Vitamin D_3 in liver however, showed no difference between the two groups. Blood plasma 25OHD_3 was evaluated from 58 exposed and 58 control plasma samples taken from eight sister pairs at different intervals between 3 to 20 months of age. No overall significant difference between blood plasma concentrations of 25OHD_3 in control compared to exposed P dogs was observed in any maternal sister pairs. In this regard, total intake of vitamin D_3 in the diet from fat, blubber and dog pellets during the last 4 weeks of the study showed values significantly higher (p<0.01) in the exposed group (both P and F, separately) when compared with the control (see Table 4).

Offspring (F generation) liver tissue concentrations are presented in Table 5, and showed significantly higher levels of vitD_3 in exposed liver (p<0.05). The exposed F generation had also overall significantly higher concentrations of plasma 25OHD_3 (p=0.009) than the controls when evaluating 19 exposed and 19 control plasma samples taken at 3, 5, 7, 9 and 12 months.

Albumin in plasma showed a negative trend with increasing ΣPCB in liver (r=-0.583, p=0.0995, n=9) in the exposed bitches and pups combined (Figure 13).

4.4 DIETARY CONTAMINANT EXPOSURE RELATED TO TESTOSTERONE AND MALE SEXUAL ORGAN MORPHOLOGY (MANUSCRIPT III)

Table 6 shows testosterone, testes weight and length, baculum weight and body weight. There were no significant differences between groups (3 exposed and 3 control) when evaluating all testosterone concentrations at 3, 5, 7, 9 and 12 months of age. Also, no significant differences were seen for

Table 4. Vitamin D3 data from 12 Greenland sled dog bitches (6 exposed and 6 control) in diet and after up to 21 months of organohalogen contaminant exposure (maternal generation P). Notice liver $25OHD_3$ in the exposed group showed significantly lower levels compared to controls (p=0.004), and that liver tissue vitD3 is similar in the two groups, despite significantly higher dietary intake in the exposed group.

			DIET	FAT	LIVER		PLASMA
ID Exp/Con	Weight kg	Age days	VitD[a] µg/day	ΣPCB ng/g lw	VitD µg/100g	25OHD µg/100g	25OHD nmol/l
P1-Exp	25.7	636	10.2	2374	0.36	0.47	92.2
P2-Exp	21.5	635	9.2	936	0.28	0.31	90.7
P3-Exp	28.2	556	11.7	6740	0.19	0.35	68.4
P4-Exp	28.6	556	9.8	2822	0.21	0.36	84.8
P6-Exp	21.9	381	14.4	2131	0.61	0.52	105.4
P7-Exp	24.1	404	9.5	3633	0.28	0.20	43.5
Mean	**24.8**	**524**	***10.8**	****3106**	**0.32**	**0.39**	**81.0**
P1-Con	25.7	636	11.1	16	0.36	0.56	132.8
P2-Con	22.9	635	5.7	58	0.16	0.46	107.3
P3-Con	27.5	556	6.4	53	0.22	0.47	69.5
P4-Con	28	556	6.4	34	0.16	0.37	76.2
P6-Con	22.3	381	11.2	153	b1.042	0.64	90.9
P7-Con	24.1	404	6.2	26	0.22	0.29	43.9
Mean	**25.1**	**528**	**7.8**	**57**	**0.22**	***0.47**	**86.8**

[a]Total vitamin D3 in the diet (from blubber/porcine fat and RC dry food) from 4 weeks prior to sampling.
* = significantly higher (p<0.05), ** = significantly higher (p<0.01), Exp= exposed group, Con= control group.
#This value was left out of calculations of the mean and no difference in significance was then observed for $25OHD_3$ in liver

testes length (left), baculum length and weight (Table 6). At the termination of the this experiment when the animals were 12 months old, testes weights were significantly lower in the exposed group (n=2, mean weight of 4 testes 15.9 g) compared to the control (n=3, mean weight of 6 testes 19.2 g) (p<0.015). Figure 14 shows X-ray absorptiometry (DXA) as bone mineral density (calcium-phosphate; 131 hydroxyapatite) of bacula from a control and an exposed male sled dog. H=high density (white coloured pixels) and L=low density (coloured pixels). The $BMD_{baculum}$ mean value of the control dogs (0.13; n=3) was higher than in the exposed dogs (0.12; n=2) although not significantly (Figure 14). No significant difference in bodyweight was observed between the control and the exposed dogs.

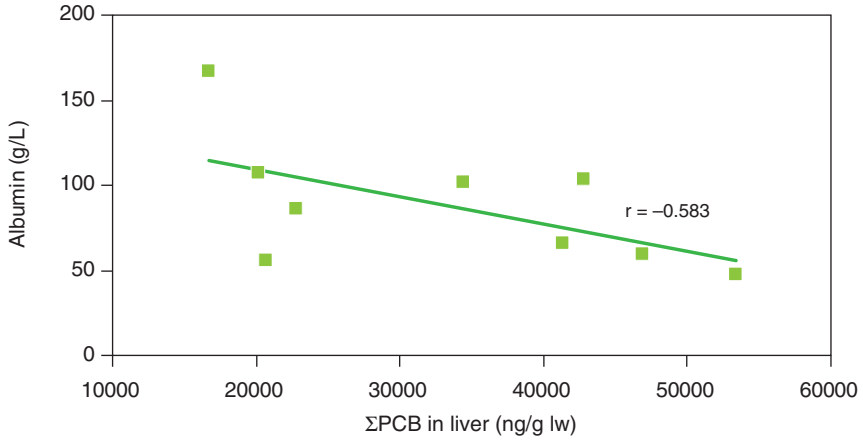


Figure 13. Pearson's correlation between albumin (g/L) in plasma and ΣPCB in liver (ng/g lw) in all exposed (P and F) Greenland sled dogs (r=-0.583, p=0.0995, n=9).

Table 5. Vitamin D3 data from 8 Greenland sled dogs (3 exposed and 5 control) in diet and after up to 12 months of organohalogen contaminant exposure (F generation). Notice that liver vitamin D3 was significantly higher in the exposed group.

			DIET	FAT	LIVER		PLASMA
ID Exp/Con	Weight kg	Age days	VitD3 µg/day	PCB ng/g lw	VitD µg/100g	25OHD µg/100g	25OHD nmol/l
F1-1 Exp	21.1	342	9.3	2686	0.24	0.41	61.9
F1-2 Exp	24.9	342	9.8	2025	0.34	0.52	71.0
F1-3 Exp	22.0	342	9.8	3303	0.44	0.49	74.9
Mean	**22.7**	**342**	****9.6**	***2671**	***0.34**	**0.47**	**69.3**
F1-1 Con	18.9	342	6.3	43	0.18	0.24	58.3
F1-2 Con	21.9	342	6.4	40	0.19	0.60	68.0
F1-3 Con	17.4	342	7.2	32	0.06	0.29	41.6
F1-4 Con	24.1	342	6.4	57	0.24	0.49	70.0
Mean	**20.6**	**342**	**6.6**	**43**	**0.17**	**0.41**	**59.5**

a Total vitamin D3 in the diet (from blubber/porcine fat and RC dry food) from 25 days prior to sampling.
* = significantly higher ($p<0.05$, student's t-test, paired), ** = significantly higher ($p<0.01$, student's t-test), Exp= exposed group, Con= control group.

Table 6. Testosterone (ng/ml), testes weight (g) and length (cm), baculum weight (g) and body weight (kg), and their mutual comparisons (T-test). M = months, TWM = testes weight (mean), TL = testes length, BaW = baculum weight, BaL = Baculum Length, BMD = body mineral density (g/cm^3), BW = body weight.

	Testosterone					Genital organs					
Group	3 M	5 M	7 M	9 M	12 M	TWM g	TL cm	BaW g	BaL cm	BMD g/cm^3	BW kg
Exposed 1	0.38	0.08	0.42	u.d.l.	0.6	-	-	-	-	-	-
Exposed 2	0.04	u.d.l.	1.4	1.9	2.9	14.76	3.9	1.55	8.2	0.13	21.1
Exposed 3	u.d.l.	0.17	1.04	6.6	4.9	17.09	3.6	2.52	9.4	0.11	22
Mean	**0.15**	**0.09**	**0.95**	**2.84**	**22.8**	**15.92**	**3.7**	**2.04**	**8.8**	**0.12**	**21.6**
Control 1	0.09	0.27	0.32	1.1	11	20.01	4.1	2.36	8.7	0.14	24.1
Control 2	u.d.l.	u.d.l.	0.43	2.6	1.1	17.76	3.9	1.8	8	0.13	18.9
Control 3	0.18	0.05	0.73	2.6	4.3	19.76	3.9	2.41	9	0.12	21.9
Mean	**0.1**	**0.11**	**0.49**	**2.1**	**5.47**	**19.18***	**4**	**2.19**	**8.6**	**0.13**	**21.6**

u.d.l. = Under detection limit, * Significantly higher (P= 0.015)

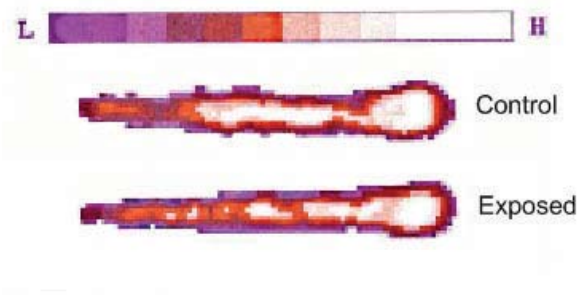


Figure 14. X-ray absorptiometry (DXA) as bone mineral density (calcium-phosphate; 131 hydroxyapatite) of bacula from a control and an exposed male sled dog. H = high density (white coloured pixels) and L = low density (coloured pixels).

4.5 EFFECTS OF DIETARY CONTAMINANT EXPOSURE ON THYROID HORMONE DYNAMICS (MANUSCRIPT IV)

A significant decrease in all the thyroid hormones, free and total T3 and T4, in the P generation (n=12) exposed dogs across 10 to 18 months of age ($p<0.005$) (mean % decrease: FT3=17; FT4=20; TT3=17; TT4=16), and for FT4 across all observations (6 to 18 months of age, $p=0.034$) was observed compared to controls (Figure 15). A significantly ($p=0.023$) higher TT4/FT4 ratio in exposed vs. control P generation dogs was also observed. Also, a decrease in thyroid gland weight with increasing ΣDDT (see Figure 16) and an increase in TT3 with increasing dieldrin in all exposed dogs (P and F, $p=0.002$, $p=0.001$, respectively, n=10) was observed. TT3 concentrations were significantly decreased (range: 13-29%) in the exposed offspring generation during all observed months (3 to 12 months, $p<0.001$, n=8, see Figure 17) compared to the control group of dogs, as well as significantly higher ratios of TT4/TT3 ($p=0.001$) were observed. No significant differences were seen between groups or generations for Thyroidea Stimulating Hormone (TSH) taken at the end of the study and no other correlations with OHCs were observed.

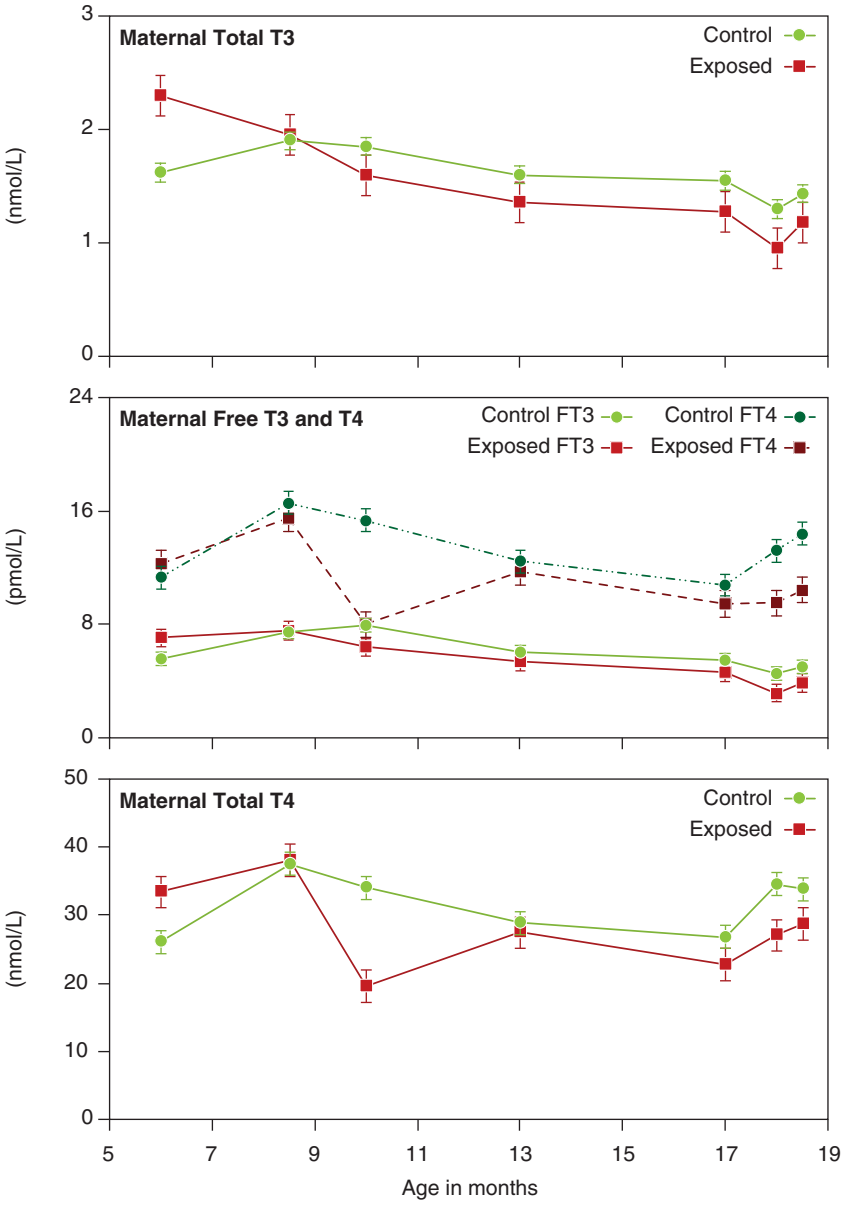


Figure 15. Mean ± SE of free T3, free T4, total T3 and total T4 in the exposed (n=6) and control (n=6) maternal P generation of Greenland sledge dogs (22 blood samples from exposed and 22 samples from control, taken at 6, 8.5, 10, 13, 17, 18, and 18.5 months). Overall concentration of free T4 was significantly ($p=0.034$) depressed in the exposed group.

Figure 16. Thyroid gland weights (left and right) and ΣDDT (ng/g lw) in the exposed sledge dogs (P +F generation: n=10, r= -0.653, p=0.002).

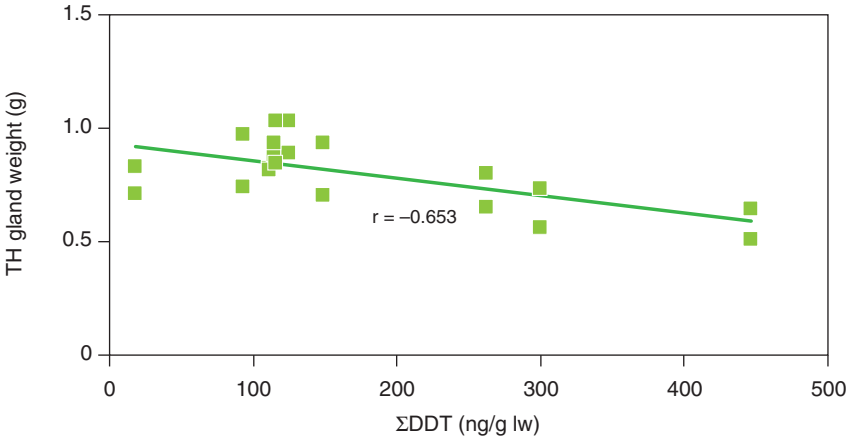
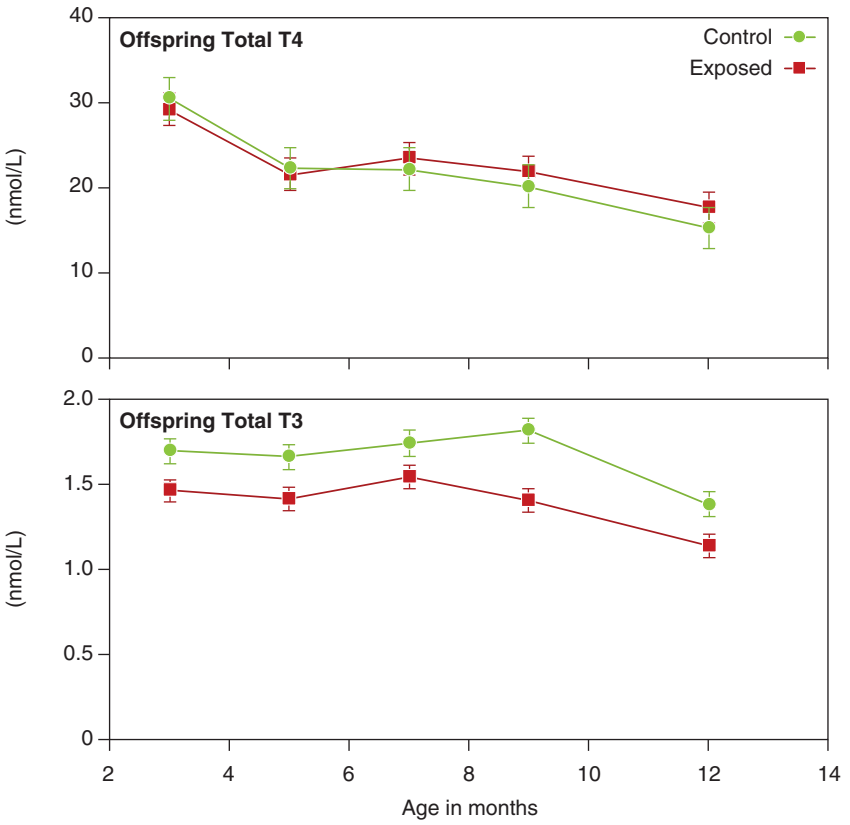


Figure 17. Mean ± SE of total T3 and total T4 in the exposed (n=4) and control group (n=4) of Greenland Sledge dogs (F offspring generation) taken at 3, 5, 7, 9 and 12 months of age (± up to 7 days in each group). Total T3 concentrations were significantly (p<0.001) depressed in the exposed group.



5 DISCUSSION AND CONCLUSIONS

5.1 STUDY VALIDITY

The advantages of the present exposure studies include 1) the duration of the studies of up to 12 to 21 months (as opposed to the typical short term laboratory studies), 2) a chronic low and realistic dose of exposure (as opposed to the typical high doses used in laboratory studies), 3) the dog as a large canine mammal model closely related to other Arctic top predators, 4) exposure to a naturally accumulated OHC mixture from minke whale blubber (as opposed to simple artificially constructed PCB or OHC mixtures), reflecting the general congener composition at high trophic levels of Greenland marine food webs, and 5) a study relevant to the real life dilemma for Arctic indigenous peoples, regarding the choice of traditional marine mammal food products vs. western imported products.

In addition the study involved genetically comparable individuals, studied over the same experimental period of age and environmental stressors, as well as a common basic feeding regime, except for the OHC exposure and other properties related to the different constitution of the fat component (discussed below). The studies present outcome variables that include concentrations of vitamins in plasma as well as organ tissue concentrations. Furthermore, it was possible to relate concentrations of hormones and vitamins to a large number of separate and total OHC concentrations in adipose tissue, as well as PCBs measured in several body compartments, e.g. plasma, liver, kidney and thyroid glands.

Almost all vitamin and hormone analyses planned with blood plasma were completed, although some analyses were prevented by insufficient amounts of plasma. OHC or other analyses in adipose tissue, liver, kidney or other tissues were completed in all dogs of the control group (8 bitches and 4 pups), and in 7 of 8 bitches and 3 of 4 pups in the exposed group.

Among the weaknesses, the small samples size and lack of OHC analyses in 2 exposed dogs constitutes a limitation in the studies. This was especially the case in study III where only males were used, thus resulting in weak statistical power to detect differences between groups, why results from this study should be interpreted with care. Furthermore, scientific arguments favours true trans-generational studies (up to an F3 generation) of endocrine disrupting effects when examining epigenetic alterations in the male germ line after *in utero* exposure (Anway and Skinner 2006). This could have been achieved by extending the study to include more offspring and/or sample existing animals over a longer period, but due to the unrealistic costs of including more animals and staff, this was not fesible. The lack of tissue for OHC and other analyses in the 2 exposed individuals was due to accidental death and destruction of tissues due to unattended fighting between dogs, but it is difficult to avoid such accidents while maintaining the dogs under conditions appropriate in the Arctic. Still, the sister/sibling pairing in exposed vs. control groups, the repeated blood sampling over many months from the same animals increased the number of samples considerably. In addition, the fact that OHCs and vitamins were measured in various tissues in addition to plasma, helps to compensate in part for the limited sample size.

The diet included a high percentage of polyunsaturated lipids from the whale blubber in the exposed group, but even though the caloric amounts were the same in the control group, it primarily consisted of saturated lipids from the porcine fat. Also, the vitamin content in the diet differed between the two groups. Retinol was about. 18% higher, α-tocopherol 22% higher and vitamin D_3 33% higher in the diet of the exposed dogs compared to control. Earlier investigations have stated that polyunsaturated lipids (n-3) have an unknown immuno modulating and suppressing effect (Martinez et al., 2000). Also, the very limited mercury exposure (Hg mean from the diet: exposed 8.5 ng/g ww and control 2.5 ng/g ww) between diets could theoretically be a potential contributor to effects between groups (see Table 1 for comparisons). This diet difference could have been overcome by chemically cleaning up whale blubber for OHCs for the control group but such an operation would also affect nutrients, which could have been compensated with artificial nutrients. Or, the different diets including whale blubber and pork fat, could have been constructed and homogenized for each group with added nutrients and fats to equalize energy and nutrient composition between groups, as was done in a study of blue foxes exposed to minke whale blubber (Sonne et al. In press). Nevertheless, this was not an option due to considerable unrealistic costs.

This type of experiment done using an OHC mixture of natural origin (whale blubber) similar to what Arctic top predators consume, is useful for studying complex mixtures, or on a case-by case basis, but leads to difficulties in extrapolating from one mixture to the other because small variations in composition of a complex mixture may lead to significant changes in its toxic effects. Furthermore, whole mixture approaches do not lead to an answer of whether OHCs act in an additive, antagonistic, or synergistic way (Kortenkamp 2007).

5.2 ORGANOHALOGEN CONTAMINANT INDUCED EFFECTS ON VITAMINS

5.2.1 Retinol and α-tocopherol

OHCs from the diet seemed to influence the concentration of vitamin A (retinol) and vitamin E (α-tocopherol) in liver and kidney. When looking at all exposed bitches and pups, ΣDDT and ΣPBDE showed significant negative correlations with liver retinol. Negative correlations were also observed between kidney α-tocopherol and ΣPCB. Additionally, two significant positive correlations were observed between kidney retinol and ΣCHL and dieldrin concentrations. Also, an age-related increase was observed in liver retinol in the control dogs, but not in the exposed dogs, indicating that the OHCs from the diet may have had a negative effect on the natural age related retinol stores in liver. Despite the fact that retinol was approximately 18% higher in the diet of the exposed dogs compared to controls, no significant difference was seen in plasma concentrations of retinol between the two groups of P generation bitches. The exposed F generation pups did show higher concentrations in plasma (mean values: ca 40% higher), but the reason for this difference between generations is unclear. These results suggest that dietary exposure from OHCs can, even in low concentrations, possibly affect retinol and α-tocopherol status in Arctic top predators.

5.2.2 Vitamin D_3 and 25OHD_3

How et al. (1994) observed that dogs do not adequately synthesize vitamin D_3 in the skin as the majority of other animals, so that they are mainly dependant on dietary supplementation. The exposed sledge dogs had received approximately 33% more vitamin D_3 in the diet than had the controls but despite this benefit, liver 25OHD_3 in the exposed P generation bitches was significantly lower. This finding indicates that OHCs may have negative effect on liver retention of 25OHD_3. No correlations with OHCs were observed in any generation, and vitamin D_3 concentrations in the liver and 25OHD_3 in plasma of both exposed bitches and pups, and also 25OHD_3 in the liver of exposed pups, were significantly higher than in the controls (see manuscript II for further details). Why a difference was observed between exposed bitches and their pups regarding 25OHD_3 content in liver is uncertain, but could be due to OHCs and associated age-related effects. In conclusion, a significantly higher amount of vitamin D_3 in the diet did not result in an overall significantly higher vitamin D_3 status in the exposed sledge dogs, and the retention level of liver 25OHD_3 in the exposed individuals may be due to dietary OHC exposure.

5.3 CONTAMINANT INDUCED EFFECTS ON HORMONES

5.3.1 Testosterone and male genital organs

Testes weights were significantly lower in the exposed group compared to the control. Although the observed difference between the groups was based on a limited number of individuals, it indicates an effect possibly caused by OHCs. The results are in accordance with previous studies reporting that length of adult polar bear male testes was negatively correlated with HCB and the weight of testes was negatively correlated with ΣCHLs, HCB and ΣPCBs (Sonne et al. 2006a). Our results are also in accordance with studies on *in utero* PCB exposed male rats which showed reduced testes weights at adulthood compared to controls (Hany et al. 1999; Kuriyama and Chahoud 2004), and reduced testes size in PCB exposed Rhesus monkeys (*Macaca mulatta*) (Ahmad et al. 2003). When considering the testosterone concentrations across age in months, there were no differences between the two groups. This is in accordance with a study showing no reduction in serum testosterone concentrations in 3 months old *in utero* PCB exposed male guinea pigs, although a reduction in testes weights was found in the same animals (Lundkvist 1990) and a study where no reduction in serum testosterone concentrations in 3 months old *in utero* OC mixture exposed male rats was observed (Anas et al. 2005). However, it should be noted that reduced testosterone concentrations, and to some extent also reduced testes weights, have been reported in OHC-exposed adult rats, monkeys, guinea pigs and wild polar bears (Faqi et al. 1998, Hany et al. 1999, Oskam et al. 2003; Kuriyama and Chahould 2004). The small number of male dogs available in this study makes any conclusions extrapolated to populations of wildlife or humans very uncertain. Nevertheless, an effect of OHC exposure on reduced testes weight is of very great importance and possibly indicates disruption of normal parameters in development of male sexual organs. The present study thus supports the hypothesis that a diet containing OHCs reduce the testicular size in East Greenland polar bears (Sonne et al. 2006a).

5.3.2 Thyroid hormones

The nearly two-year feeding regime conducted with P generation female sledge dogs from juvenile age to sexual maturity led to significantly depressed FT4 concentrations in the P generation exposed group (n=6) of dogs (during 6 to 18 months of age) across all age groups and the TT4/FT4 ratio was significantly higher than the controls. Furthermore, TT3, TT4, FT3, FT4 in the P generation exposed dogs were decreased across age groups from 10 to 18.5 months compared to controls. The mean % decrease ranged from 16-20% (increasing order: TT3, TT4, FT3, FT4). Also, TT3 from 3 to 12 months was reduced in the exposed F generation dogs. The mean % decrease in THs across month 3-12 of age in the exposed F generation ranged from 13 – 29% for TT3 (see manuscript IV for details). In addition, thyroid gland weights were significantly negatively correlated with ΣDDT in adipose tissue and TT3 concentrations were significantly positively correlated with dieldrin in all exposed sledge dogs (n=10, P+F). The decrease in T4 shown in the exposed dogs, is in accordance with previous reports that PCB exposure in beagle dogs caused a significant decrease in T4 after 12 weeks of dietary PCB exposure (25 ppm Aroclor 1248) (Fadden 1994). The reason for the higher plasma TH concentrations at 6 months, similar at 8.5 months and lower at 10 months in the exposed P group vs. control is uncertain. A possible explanation could be that time of OHC exposure affects TH metabolism, where short-term studies with 2-6 months of OHC exposure elevates TH concentrations, while chronic exposure causes depressed TH concentrations. A reason for the offspring generations' difference (depressed TT3 from 3 to 12 months, but no difference in TT4) from their parent generations is unknown, but could be ascribed to alterations in TH metabolism due to *in utero* exposure. Nevertheless, because TH metabolism is species specific, extrapolating results to other species should be done with caution.In conclusion, we observed a decrease in all THs measured (free and total T3 and T4) in the adult exposed dogs (P) from 10 to 18 months of age compared to controls. Also, a decrease in thyroid gland weight with increasing ΣDDT and an increase in TT3 with increasing dieldrin in all exposed dogs were observed. Furthermore, significantly depressed FT4 concentrations (P generation) and TT3 concentrations (F generation) during all observed months in the exposed compared to the control group of dogs were observed.

5.4 OHCs in the future

Many legacy contaminants, especially the most potent and persistent ones like PCBs, have been banned and concentrations in biota are slowly declining, more or less dependant on history of global output and/or persistence (AMAP 2004, French et al., 2006). Some, like DDT used against malaria, will probably still be used in smaller quantities for many years to come (O'Shaughnessy 2008). On the other hand, the temporal increases of many new contaminants will most likely continue (e.g. perflourinated contaminants (PCFs) and certain new brominated flame retardants) (AMAP 2004), and perhaps new types of contaminants will arise too, whereby the toxicokinetic pattern in different organisms and interactions between contaminant groups in complex mixtures can alter the current picture of health effects (Kortenkamp 2007, 2008). This way, compared to today, we may perhaps in the future see generally lower individual OHC congener concentration in each animal, but with a much more complex picture of different types of contaminants.

5.5 CONCLUSIONS ON HEALTH IMPLICATIONS OF CONTAMINANT INDUCED EFFECTS

One aim of the diet experiment on sledge dogs was to use this species as a potential surrogate *Canoidea* species for the polar bear and to reproduce some of the effects observed in free ranging wildlife (polar bear, Arctic fox and seal). Some of the results in the present study confirm these observed effects previously observed in wildlife. The present studies observed significant correlations between vitamin A and several OHCs, as well as a difference between groups in vitamin A concentrations with age. Also, a decreasing trend in liver $25OHD_3$, as well as a negative trend between PCBs and liver vitamin D_3 in all the exposed individuals was observed. Furthermore, higher amounts of vitamin D_3 in the diet did not result in a significantly higher vitamin D_3 concentration in the sledge dogs. While it has been observed that dogs are dependant on dietary supplementation of this vitamin, the adequacy of vitamin D_3 synthesis in the skin of wildlife like polar bear and seals has not been investigated. This underlines the importance of future studies of vitamin synthesis and disruption, which may be caused by OHCs, as it may have importance for the most susceptible individuals or in a worst case scenario, on a population scale. Regarding hormones and endocrine disrupting effects, the exposure was related to a reduction in testes weight in a small sample of male offspring exposed *in utero*, but not to any changes in testosterone concentrations. The present study thus supports the hypothesis that a diet containing OHCs reduce the testicular size in East Greenland polar bears. Also, significant decreases were seen in all thyroid hormones measured (Free and Total T3 and T4) in the adult exposed bitches compared to controls, and also a decrease in thyroid gland weight with increasing ΣDDT and an increase in Total T3 with increasing dieldrin in all exposed dogs was observed.

Endocrine glands and vitamin and hormone dynamics are therefore suggested to be influenced by low chronic doses of OHCs in Arctic top wildlife predators. The impacts on hormones and vitamin dynamics may interfere with bone metabolism and calcium homeostasis, and/or other important endocrine organ systems.

In conclusion, the present thesis supports the notion that wild mammals at the top of Arctic food chains are suffering from chronic non-lethal, subclinical symptoms and anomalities due to exposure to complex environmental organohalogen contaminant mixtures. This is likely to increase the risk of reduced reproductive rates and lifespan due to impaired fitness of populations. Despite these observations, it is still not possible to point to any increased mortality, reduced fecundity and potential population decline caused by effects of OHCs – especially in a situation, where a changing climate involves drastic disruptions in wildlife ecology.

6 PERSPECTIVES AND RECOMMENDATIONS

The known overall dynamics of vitamin and endocrine disruption from organohalogen contaminants continue to be challenged by the constant change in complex mixtures of chemicals, as the ever increasing human desire to innovate and prosper continue. There is a great need for understanding threshold levels for each contaminant and its metabolites, but even more so the multifaceted toxicity of the complex mixtures of OHCs affecting the different animals and their organ systems. Furthermore, if the temporal increases continue regarding new contaminants, new interactions between contaminant groups in the complex mixtures can alter the current picture of health effects. This may call for a shift of contaminant method research, as different approaches most likely will be needed to study future complex toxicity from environmental contaminants. Controlled semi-laboratory experiments may address these values, based on daily exposure and following OHC body burden and this knowledge can be essential in monitoring and assessing effects of contaminants on Arctic top predators. But many other ways exists to face these challenges.

Future research should focus on questions regarding contaminants and health parameters in Arctic top predators, including possible climate change scenarios. This may be possible by efficient circumpolar monitoring of the health of Arctic top predators and by integrating experimental, wildlife and human epidemiological studies, although an integration of these different fields of research is a great challenge. The effects of OHCs in relation to vitamin A have been monitored thoroughly in a number of wild species.

Due to species-specific properties of vitamin A dynamics, assessment of effects for each species in question is desirable, so that research in the future can focus on the species that is the most vulnerable. Also, further investigations concerning vitamin E alterations in liver, plasma and kidneys to target this as a general effect biomarker and elucidate further the dynamics of this vitamin in relation to environmental OHC exposure, would be desirable in several other species. Studies of vitamin D dynamics related to endocrine disruption during early developmental stages, in regard to OHCs, separate congeners and complex mixtures alike, are encouraged as well. As vitamin D metabolism and functions are not fully elucidated in many other species than humans and rats, general vitamin D dynamics experiments may lead to a better understanding of the effects of OHCs as well.

Relatively little information exists regarding the ways in which OHCs belonging to different classes may work together to produce combined effects, which is why research efforts should be encouraged to expand the knowledge in this field. As an example, anti-androgens, including androgen receptor (AR) antagonists, should be combined with estrogenic chemicals that also possess anti-androgenic properties in order to study possible impacts on disruption of male sexual development *in vivo*. PCBs are well-known disruptors of male sexual development, but very little is known about the way they act together with anti-androgens and estrogens, and it is urgent to fill this gap in order to understand more about effects of complex mixtures. Regarding future studies of effects from OHCs on the thyroid system, the present sledge dog study suggests that an OHC exposure time of at least 10 and preferably more than 18 months may be crucial in low-dose OHC exposure studies of larger mammals, and also

further *in utero* and true transgenerational studies are important, if true chronic effects on thyroid hormone dynamics are to be evaluated. Consequently, low-dose, semi-long term studies for less than 10 months already executed may not have provided a complete picture of thyroid hormonal effects from OHCs.

Evidence is available to show that joint effects occur even when all mixture components are present at levels below doses that cause observed clinical effects. In view of this evidence, the traditional chemical-by-chemical approach to risk assessment is hard to justify, and it is important to consider group-wise regulation of endocrine disruptors. We lack thorough understanding of interactions between categories of endocrine disruptors, and should therefore group these chemicals according to similar effects (not similar mechanisms) until a better understanding of mechanistic interactions exists. This should be done by an international, synchronized, targeted research effort aimed at improving our understanding of complex OHC mixtures.

Photo: S. Andersen

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8 DANSK RESUMÉ

Organohalogene kontaminanter (OHCer) er industrielle kemikalier (pesticider, bromerede flamme hæmmere og biprodukter fra kemikalie industrien) der har toksiske egenskaber for mange organismer. Andre studier viser at de kombinerede effekter af forskellige OHCer i komplekse blandinger sker, til trods for at de forskellige individuelle komponenter er til stede i små mængder hvor der ikke normalt ses effekter. Flere studier viser at mekanismerne bag toksiske effekter af de forskellige OHCer der involverer ændringer i hormon og vitamin systemet, sker f.eks. ved en blokering af transport komplekser der bærer hormoner og vitaminer og/eller interaktioner med de vævs receptorer vitaminet/hormonet benytter. For at efterligne en langtidseksponerings situation, blev et studie af 16 grønlandske slædehunde (*Canis familiaris*) og deres 8 afkom udført i Aasiaat, Grønland, i op til 21 måneder. Studiet blev udført for at undersøge hvordan hormoner og vitaminer hos arktiske pattedyr højt i fødekæden bliver påvirket af et højt indhold af OHCer i kosten. Det blev også undersøgt om der var ændringer i effekterne hvis dyrene var eksponerede kun i voksenlivet, eller både i fostertilstanden og via modermælk, og efterfølgende i voksenlivet. Hundene, der som søstre parvis var placeret tilfældigt i hver sin gruppe, blev fodret supplerende med fedt med et hhv. højt (vågehvals spæk, *Balaenoptera acuterostrata,* eksponeret gruppe) eller lavt (svinefedt, *Suis Scrofa,* kontrol gruppe) indhold af OHCer, flerumættede fedtsyrer og vitaminer.

Fedtvæv, lever, nyre, thyroidea kirtler, testikler og penis knogler blev indsamlet fra hunde i forskellige aldre (12 til 21 måneder) og analyseret ved studiets afslutning. Blodplasma blev indsamlet i forskellige intervaller undervejs i studiet. Eksempler på OHCer analyseret i fedtvæv inkluderede: polychlorerede biphenyler (PCBer, 59 congener), hexachlorobenzen (HCB), α-hexachlorocyclohexan (HCH), octachlorostyren (OCS), chlordaner (CHL, 6 metabolitter), *p,p'*-DDT (DDT, 2 metabolitter), dieldrin, mirex, polybromineret difenyl ether (PBDE, 36 congener) og andre bromerede flammehæmmere (BFRs). Analyserne viste at de eksponerede hunde havde 54 gange så højt indhold af OHCer i fedtvævet som kontrol hunde, og congener sammensætningen repræsenterede det typiske rovdyr øverst i den Grønlandske marine fødekæde.

Et studie af 12 eksponerede slædehunde (8 tæver og deres 4 hvalpe), samt deres 12 kontrol søstre/søskende (8 tæver og deres 4 hvalpe), blev lavet for at undersøge plasma, lever og nyre indhold af A vitamin (retinol) og E vitamin (α-tocoferol). Retinol var ca. 18% højere og α-tocoferol 22% højere i de eksponerede hundes foder end i kontrol foder. En signifikant stigning i lever retinol med alderen blev påvist for kontrol hunde, men ikke for eksponerede hunde. Lever retinol var signifikant højere i både eksponerede tæver og hvalpe end i kontrol hunde. Der var ingen forskel i eksponeret vs. kontrol tævers plasma retinol taget fra 3 måneders alderen til 21 måneder. De eksponerede hvalpes plasma retinol var signifikant højere end kontrol imellem 3 og 12 måneder. Lever α-tocoferol hos alle eksponerede hunde var signifikant lavere end kontrol. Studiet fandt en signifikant negativ korrelation hos eksponerede hunde mellem lever retinol og ΣDDT og ΣPBDE, samt mellem nyre α-tocoferol og ΣPCB. Derudover blev der påvist to signifikante positive korrelationer mellem nyre retinol og ΣCHL og dieldrin concentrationer.

Et andet studie blev gennemført for at sammenlignede vitamin D status imellem 6 eksponerede og 6 kontrol tæver i forskellige aldre samt deres 4 eksponerede og 4 kontrol hvalpe, fodret indtil mellem 12 og 21 måneder, for at undersøge om OHCer ville påvirke indholdet af vitamin D_3 og metabolitten $25OHD_3$ imellem de to grupper i relation til vitamin D indtag. Vitamin $25OHD_3$ i lever hos de eksponerede tæver var signifikant lavere, til trods for deres ca. 33 % højere indtag af vitamin D_3 i kosten i 4 uger inden prøvetagning. Vitamin D_3 i lever og $25OHD_3$ i plasma hos eksponerede tæver, samt $25OHD_3$ i lever hos eksponerede hvalpe, var signifikant højere end hos tilsvarende kontrol individer.

Et tredje studie blev gennemført på 6 hanhundehvalpe for at se effekter af OHCer på testosteron koncentrationer og hanlige kønsorganer, når hundene også var eksponeret pre-og postnatalt via moderens kost. 3 eksponerede hanhunde og 3 kontrol hanhunde, alle genetisk beslægtet med samme far og med mødre der var søstre, blev undersøgt for testosteronkoncentration ved 3, 5, 7, 9 og 12 måneder. Studiet fandt signifikant reducerede testikel vægte imellem de to grupper, dog med et begrænset antal individer. Der var ingen signifikante forskelle imellem penisknogle vægt og knogle mineral tæthed, testikel længde eller plasma testosteron koncentration.

For at undersøge sammenhængen mellem OHCer i kosten og thyroidea hormon status blev et studie af 7 eksponerede tæver og deres 4 afkom vs deres 7 kontrol søstre og deres 4 afkom udført. De eksponerede tæver havde signifikant lavere niveauer af alle thyroidea hormoner (frit og totalt T3 og T4) i alderen 10 til 18 måneder. Ydermere var frit T4 signifikant lavere for de eksponerede tæver i alle observerede måneder (måned 6 til 18), og her sås også en signifikant forhøjet total T4:frit T4 ratio. De eksponerede hvalpe havde en signifikant lavere koncentration af total T3 sammenlignet med kontroller fra måned 3 til 12, og en signifikant større total T4:total T3 ratio. Studiet fandt også en signifikant negativ sammenhæng mellem thyroidea kirtel vægt og ΣDDT, samt en signifikant positiv sammenhæng mellem total T3 og dieldrin for alle eksponerede hunde. Der var ingen sammenhænge mellem thyroidea stimulerende hormon (TSH) og OHC eksponering.

9 ENGLISH SUMMARY

Organohalogen contaminants (OHCs) are industrial chemicals (e.g., pesticides, flame retardants and chemical by-products) that exhibit toxic properties in many organisms. Recent evidence suggests that combined effects of different OHCs in complex mixtures may occur even when all the individual mixture components are present at levels below doses that are known to cause observable effects. Other studies also show that mechanisms of the different OHCs may involve disruption of the hormone and vitamin system and may occur by, e.g., interference with transport complexes carrying the hormones and vitamins and/or interaction with the vitamin or hormone receptors in target tissues. In order to mimic a long-term OHC mixture exposure situation, a study was performed as an exposure-control feeding study for 12 to 21 months of age conducted on 16 Greenland sledge dog (*Canis familiaris*) female bitches and their 8 offspring, in Aasiaat, Greenland. This model aimed at exploring how a diet rich in OHCs affects vitamin and hormone status in Arctic top predator mammals, and if the effects depend on whether the animals were exposed only during adulthood or also during foetal and early postnatal development via maternal transfer of OHCs. The dogs (paired sisters randomly placed in each group) were supplemented with fat containing high (exposed group: minke whale blubber, *Balaenoptera acuterostrata)* or low (control group: porcine fat, *Suis scrofa*) amounts of OHCs, polyunsaturated lipids and vitamins. Adipose tissue, liver, kidney, testes, thyroid gland and penile bone samples were sampled from dogs of different ages (12 to 21 months) at the end of the study. Plasma was sampled at different intervals during the study. Key OHCs analysed in adipose tissue included: polychlorinated biphenyls (PCBs, 59 congeners), hexachloro-benzene (HCB), α-hexachlorocyclohexane (HCH), octachlorostyrene (OCS), chlordanes (CHLs, 6 metabolites), *p,p'*-DDT (DDT, 2 metabolites), dieldrin, mirex, polybrominated diphenyl ether (PBDEs, 36 congeners) and other brominated flame retardants (BFRs). Analysis of adipose tissue in exposed dogs showed a 54 fold higher concentration of OHCs than in control dogs, and the complex mixture of OHCs showed moderate concentrations reflecting the general congener composition at higher trophic levels of Greenland marine food webs.

The first study of samples from 12 exposed sledge dogs (8 bitches and 4 pups) and their 12 porcine control sisters/siblings (8 bitches and 4 pups) were conducted to examine plasma, liver and kidney status of vitamins A (retinol) and E (α-tocopherol). Retinol was approximately 18% higher and α-tocopherol 22% higher in the diet of the exposed dogs compared to controls. An age-related increase was observed in hepatic retinol in the control dogs, but not in the exposed dogs. Hepatic retinol was highest in the exposed bitches and pups. Plasma retinol in bitches sampled between 3 and 21 months of age showed no significant difference between the two groups. Plasma retinol in exposed pups, sampled between 3 and 12 months, was significantly higher than in controls. In contrast, hepatic -tocopherol concentrations were significantly lower in exposed bitches and pups compared to the controls. Significant negative correlations within all exposed bitches and pups, between hepatic retinol and ΣDDT and ΣPBDE were observed, as well as between kidney α-tocopherol and ΣPCB. Additionally, two significant positive correlations were observed between kidney retinol and ΣCHL and dieldrin concentrations.

A second study compared vitamin D_3 status in samples from 11 exposed sledge dogs (7 bitches and 4 pups) and their 11 porcine control sisters/siblings (8 bitches and 4 pups), supplemented for 12-21 months of age, to examine whether the exposure from OHCs could deteriorate an expected increase in the circulating levels of vitamin D_3 and its metabolite 25-hydroxyvitamin D_3 ($25OHD_3$). Hepatic $25OHD_3$ in bitches was significantly lower in exposed individuals, although they had received approximately 33% more vitamin D_3 in the diet than had the controls. Vitamin D_3 in the liver and $25OHD_3$ in plasma of both exposed bitches and pups, and also $25OHD_3$ in the liver of exposed pups, was significantly higher than in the controls. A third study of 6 male offspring was conducted to examine testosterone concentrations and genital organs to illuminate the hypothesis that long-term dietary OHC exposure (including *in utero* and lactation exposure) would disrupt male reproductive hormones and development of sexual organs. The 3 exposed male and the 3 control male pups were all related (they shared the same father and their mothers were sisters). Plasma was sampled at month 3, 5, 7, 9 and 12 for testosterone analysis. Despite the limited sample size, the study showed that testicular weights were significantly lower in the exposed group. Baculum weight, bone mineral density, testicular length, and plasma testosterone concentrations were lower in the exposed group, but not significantly so.

A fourth study investigated if exposure to OHCs for up to 12 or 18 months of age is associated with altered circulating levels of thyroid hormones (THs) in 7 exposed and 7 control sister bitches of different ages, including 4 exposed and 4 control pups. The exposed bitches had significantly lower plasma concentrations of Free and Total T3 and T4 than the controls when sampled between 10 to 18 months of age, and significantly lower concentrations of Free T4 and a significantly higher Total T4/Free T4 ratio across all observations (6 to 18 months of age). The exposed pups had overall significantly lower plasma concentrations of Total T3 than the controls, when sampled from 3 to 12 months, and the ratio Total T4/Total T3 was significantly higher in the exposed pups. The study also found a decrease in thyroid gland weight with increasing ΣDDT and an increase in Total T3 with increasing dieldrin in all exposed dogs. The study found no difference between thyroid stimulating hormone between the groups.

10 GLOSSARY OF ACRONYMS

Σ	Sum of congeners
Ah	Aryl hydrocarbon
ALAT	Alanin transferase
ASAT	Aspartate aminotransferase
APP	Acute phase proteins
AR	Androgen receptor
BFR	Brominated flame retardant
BMD	Bone mineral density
CHLs	Chlordanes
CV	Coefficient of variance
CYP	Cytochrome isozymes
DDT	Dichloro diphenyl trichloroethane
DXA	Dual X-ray absorptiometry
FT4	Free Thyroxine
FT3	Free Triiodothyronine
HCB	Hexachloro benzene
HCH	Hexachlorocyclohexane
Hg	Mercury
HPLC	High performance liquid chromatography
lw	Lipid weight
NTNU	The Norwegian University of Science and Technology
OCS	Octachlorostyrene
OHCs	Organohalogen contaminants (OCs, PBDEs and PFOS)
OH-PCB	Hydroxylated PCB (metabolite)
PBDEs	Polybrominated diphenyl ethers
PCBs	Polychlorinated biphenyls
PFCs	Perfluoro compounds
PUFA	Polyunsaturate fatty acids
RBP	Retinol binding protein
TBG	Thyroxine-binding globulin
TT3	Total Triiodothyronine
T3	Triiodothyronine (active thyroid hormone)
TT4	Total T4 Thyroxine
T4	Thyroxine (T3 precursor)
TTR	Transthyretin
TTR	Transthyretin
RPM	Rounds per minute
SD	Standard deviation
UDPGT	Uridine diphosphoglucuronosyl transferase
ww	Wet weight

PAPER I

ORGANOHALOGENS IN A WHALE BLUBBER SUPPLEMENTED DIET AFFECTS HEPATIC RETINOL AND RENAL TOCOPHEROL CONCENTRATIONS IN GREENLAND SLED DOGS (*CANIS FAMILIARIS*)

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Organohalogens in a whale blubber supplemented diet affects hepatic retinol and renal tocopherol concentrations in Greenland sled dogs (*Canis familiaris*)

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Abstract
The aim of this study was to examine plasma, liver and kidney status of vitamin A (retinol) and vitamin E (α-tocopherol) in two groups of Greenland sledge dogs (*Canis familiaris*), with a total number of 16 bitches and 8 pups. The dogs were fed either minke whale (*Balaenoptera acuterostrata*) blubber (exposed dogs) or the same amounts of uncontaminated (control group) porcine fat for up to 12 to 21 months of age. The daily intake of 50-200g whale blubber (mean: 112g) constituted app. between 10.4-11.7 µg/kg bodyweight ∑OHC (or app. between 4.6-6.1 µg/kg bodyweight ∑PCB). Retinol was app. 18% higher and α-tocopherol 22% higher in the diet of the exposed dogs compared to controls. In adipose tissue, ΣOHC mean was 92 ng/g lw and 5005 ng/g lw for all control (n=12) and exposed dogs (n=10), respectively. Negative correlations between hepatic retinol and Σ-dichloro-diphenyldichloroethane (ΣDDT) ($p=0.053$), and between hepatic retinol and Σ-polybrominated diphenyl ethers (ΣPBDE) ($p=0.045$) were observed for all exposed animals. A negative correlation between kidney α-tocopherol and Σ-polychlorinated biphenyls (ΣPCB) concentrations was observed ($p=0.024$), whereas two positive significant correlations were observed between kidney retinol and Σ-chlordane related compounds (ΣCHL) and dieldrin concentrations ($p<0.05$). Hepatic α-tocopherol concentrations were significantly lower in the exposed group compared to controls, most likely due to a combination of OHC exposure and high dietary intake of unsaturated fatty acids. These results suggest that dietary exposure from OHCs can, even in low concentrations, possibly affect retinol and α-tocopherol status in Arctic top predators.

Key words: retinol, tocopherol, sledge dogs, dietary exposure, organohalogen contaminants, PCB

Introduction

Organohalogen contaminants (OHCs) in the environment are transported from lower latitudes to the Arctic where they persist and biomagnify in marine food chains resulting in high concentrations in top predators (Verreault et al., 2005; Muir et al., 2006; Jenssen et al., 2007). Because OHCs are lipophilic and because lipids are an important part of the diet of Arctic predators, these animals are exposed to large quantities of OHCs. Thus, there is a large concern for the effects that OHCs may cause on populations of Arctic mammals and seabirds (Jenssen, 2006). Several studies have shown that OHCs affects the homeostasis of several vitamins, such as vitamin A (retinol) and vitamin E (α-tochoperol) in wildlife species. In seals and polar bears there are several reports of negative associations between OHCs and plasma and hepatic retinol concentrations (Brouwer et al., 1989; Jenssen et al., 1995; de Swart et al., 1996; Bernhoft et al., 1997; Simms and Ross, 2000; Skaare et al., 2001; Nymann et al., 2002; Jenssen et al., 2003; Mos et al., 2007). The effects of OHCs on the retinol homeostasis have been confirmed in controlled laboratory studies on rats, mink (*Mustela vision*) and harbour seals (*Phoca vitulina*) (Brouwer and Vandenberg 1986; Chen et al., 1992; Morse and Brouwer 1995; Vanbirgelen et al., 1994; van der Plas et al., 2001; Brouwer et al., 1989; Martin et al., 2006; deSwart et al., 1996). OHCs and especially PCBs interfere with transport, storage, metabolism, and excretion of retinoids (de Swart et al., 1994; Simms and Ross, 2000; Jenssen et al., 2003). OHCs have also been demonstrated to affect vitamin E in laboratory and wildlife studies (Lilienthal et al., 2000; Murvoll et al., 2005; Murvoll et al., 2006; Banudevi et al., 2006; Routti et al., 2008). Vitamin E plays an essential role in preventing PCB-mediated oxidative stress (Yamamoto et al., 1994), liver lipid peroxidation (Oda et al., 1987; Stohs et al., 1984) and endothelial cell dysfunction (Slim et al., 1999). Vitamin A is necessary for vision and epithelial tissues, reproduction, bone structure, foetal development and immune system function of mammals (Machlin, 1991; McDowell, 2000). A state of deficiency or excess of this vitamin can constitute serious health threats to individuals or populations (Geraci, 1981; Machlin, 1991; McDowell, 2000). Insufficient vitamin E causes failure of oxidative stress protection (Debier et al., 2002), and affects e.g. reproductive, muscular, circulatory, nervous and immune systems (Hoekstra, 1975; Sheffy and Schultz, 1979; McDowell, 2000). Another important determinant of vitamin E requirements is the dietary concentration of polyunsaturated fatty acids (PUFAs, which whale blubber contains) as PUFAs are highly susceptible to auto-oxidation (Nacka et al., 2001; Banudevi et al., 2006), which subsequently may reduce levels of anti-oxidants such as α-tochoperol. Depression of vitamin status in Arctic animals can therefore be crucial in situations of very unpredictable food availability and adverse climate conditions. Marine mammals transfer high amounts of OHCs to their offspring during pregnancy and through lactation (Jenssen et al., 2003), and the periods of embryonic, foetal and postnatal development are remarkably susceptible to OHC exposure as compared to the adult organism (Grandjean et al., 2007). The aim of the present study was to examine how a diet rich on OHC affects vitamin status in Arctic top predator mammals, and if effects differ depending on if animals are exposed only during adulthood or if they prior to adulthood also have been exposed during fetal and postnatal development via maternal transfer of OHCs. To mimic a long-term exposure situation, the study was performed as a parallel feeding study for 12-21 months of age in sledge dogs (*Canis familiaris*) and their offspring, which were supplemented fat containing high or low amounts of the

constituents OHCs, PUFAs, retinol and α-tocopherol, respectively. Plasma, hepatic and renal samples were taken for analyses of retinol and α-tocopherol at the end of the study.

Materials and Methods

Experimental design

The maternal generation (P generation) was composed of 16 female sled dogs from Aasiaat (Disco Bay, West Greenland), obtained at two months of age. Eight were assigned to an exposed group and 8 to a control group, and the groups were composed of paired sisters, one in each group, in order to minimize age and genetic differences among the two groups. Two exposed and four control dogs gave birth during the course of the study. The F generation (F) in this study was composed of 4 exposed siblings (3 males, 1 female) and 4 control siblings (3 males, 1 female), related with the same father and mothers as sisters. The exposed pups were OHC exposed in utero and during lactation and then both groups were provided the respective diets after weaning (6-8 weeks) for 12 months before samples were taken. In one P sister pair and for one F generation pup, only plasma vitamin results between 3-18 months were used, as no results from liver, kidney and adipose tissue at the end of the study were available from these exposed individuals. The exposed dogs were fed blubber (naturally OHC contaminated) from a West Greenland minke whale for 12-21 months. The whale was obtained by native subsistence hunting. Control dogs were fed porcine fat made for human consumption. All dogs were fed 50–200 g/day of either blubber or porcine fat. Additionally, all dogs were fed equal amounts of standardized Royal Canine Energy 4300/4800 dry dog pellets (also 50-200 g/day) to cover basic nutrients and microelements (http://www.royalcanin.com/). All dogs were fed simultaneously on a daily basis. The two types of Royal Canin dog food given (E4800 more energy rich than E4300, but both with almost equal amounts of nutrients) was used to balance the body weights equally between sisters/siblings, and also appropriate for either a summer or winter diet. For further description and detailed discussion of concentrations of pollutants, lipids and nutrients in the diet, see Sonne et al. (2006, 2007, 2008) and Verrault et al. (2008, 2009a). According to local community requirements, all dogs were kept in chain, which also served to control food intake, and all dogs were exercised regularly. The dogs were immunized for the canine distemper virus, parvo virus, hepatitis virus, parainfluenza virus and rabies (Duramune 4 Vet Scanvet®) and were euthanatized upon experiment completion at adulthood (P generation: 12-21 months and for the F generation: 12 months). The present experimental design was performed on a license granted by the Official Chief Veterinarian from the Home Rule Government (Community of Nuuk, Greenland, 2003).

Field sampling

Adipose tissue, liver, kidney and plasma were obtained at the end of the study for analysis of OHCs. Plasma samples for analyses of retinol and α-tochoperol were taken at different intervals throughout the study period: a total of 106 blood samples from 8 sister pairs (8 control and 8 exposed) of the P generation, age 3 to 21 months, were obtained for vitamin analysis. The F generation, 4 sibling pairs (4 exposed and 4 control) were all blood sampled when they were 3, 5, 7, 9 and 12 months of age, in total 40 samples. Liver and kidney samples were taken from 10 exposed (P generation: 7, F generation: 3) and 12 control animals (P generation: 8, F generation: 4). In one P exposed bitch and for one F generation

pup no results from liver, kidney or adipose tissue were available due to logistic problems. Blood plasma (Lithium-Heparin) from each dog was sampled in sterilised cryo tubes and frozen at -18 º C immediately. Liver and adipose tissue was sampled after euthanasia and frozen at -18 º C immediately. All samples were stored at < -18 º C until arrival at the laboratory for analysis.

Analyses of retinol and α-tochoperol in plasma
A plasma sample of 200 µL was transferred to 2 mL plastic vials (Eppendorf Safe-Lock, www.ependorf.com) followed by mixing with 200 µL ethanol for precipitation of proteins. Subsequently, 600 µL pentan:ethylacetat (80:20) was added and the solution mixed for 10 min (MultiReax, Heidolph Instruments, Schwalbach, Germany) followed centrifugation at 2000 *g* at 4 °C for 10 min. The layer of organic solvent was transferred to a 10 ml glass vial. The extraction process with 600 µL pentan:ethylacetate (80:20) was repeated in total 3 times. The organic solvent was evaporated under nitrogen and dissolved in 200 µL mobile phase (2-propanol:*n*-heptane 1.5:98.5). A 100 µL aliquot was chromatographed by HPLC (Waters, Milford, MA, US) equipped with an auto-injector with a cooling device at 5°C (Waters 717plus) and a normal phase column of (Luna Amino, 3µm, 150*3 mm, Phenomenex,Torrance, CA, US). Retinol was detected by UV-detection at 325 nm (Waters, UV 2487) and α-tocopherol by fluorescence at 290 nm excitation and 327 nm emission (Waters 474). External calibration and peak area were used for quantification by HPLC-software (Waters, Empower 2). Quality control included the recovery of retinol and α-tocopherol in spiked plasma, and a housereference plasma. The recovery was 97.9 ± 6.5% and 103.9 ± 7.1% (mean±SD) for retinol (n=35) and α-tocopherol (n=35), respectively. The precision explained as CV for our housereference human serum was 6.8% and 6.0% for retinol (n=29) and α-tocopherol (n=32), respectively.

Analyses of retinol and α-tochoperol in liver and kidney
Retinol and α-tocopherol were extracted and analyzed according to Murvoll et al. (2005) with minor modifications. Crushed liver and kidney samples, two parallels of app. 7 mg each, were homogenized (Quiagen Tissue Lyser, Retsch, Germany) with Tungsten beads (d=5 mm). The samples were added 1000µL hexane containing internal standards, then sonicated for 2 min. (High-intensity ultrasonic processor, Sonics and Materials, USA) with an amplitude of 38% of maximum for this instrument (i.e., maximum allowed for microtip probe). After sonication the samples were vortexed and centrifuged for 3 min at 12 000 x̣ g (Eppendorf centrifuge 5415D, Eppendorf AG, Hamburg, Germany). The extraction process was repeated totally 3 times. The organic solvent was evaporated under nitrogen and dissolved in the mobile phase consisting of methanol:ultrapure water 98:2, then stirred and centrifuged for 3 min at 2000 x g (Megafuge 1.0, Tamro MedLab AS, Skårer, Norway). An aliquot of 20 µL was injected (Pharmacia LKB autosampler 2157) into a reversed phase column (Chrompack Intersil, ODS-3, 150 × 4.6 mm, 5 µm, Varian, Inc. (CA)) and the flow rate of the mobile phase was set to 1 ml/min. Retinol and α-tocopherol were detected by fluorescence detector set at exitation 330 nm and emission 408 nm (Pharmacia LKB Fluorescence Detector 2144). Peak area was used for quantification by use of external standard. The mobile phase was degassed with helium for 10 minutes in a 2.5 L flask before use. Retinol and α-tocopherol were quantified with a fluorescence detector (Pharmacia LKB Fluorescence Detector

2144) by peak integration in relation to standards of retinol and α-tochoperol (Sigma Chemical Co., St. Louis, MO). Concentrations in the highest standards were: $Liver_{RETINOL}$=1350 µg/L, $liver_{\alpha\text{-}TOCOPHEROL}$=25400 µg/L, $kidney_{RETINOL}$=1600 µg/L and $kidney_{\alpha\text{-}TOCOPHEROL}$=12700 µg/L. Quality assurance during analysis included seven-point linear calibration curves of the analyzed standard solutions. Duplicates with less than 25% coefficient of variance (%CV) were accepted. Data on internal reproducibility was determined by analyzing 10 parallels from the same liver or kidney (of one control sledge dog). In liver, the precision explained as CV was 8.4% for retinol and alfa-tocopherol at a level of 45 µg/g and 27 µg/g, respectively. In kidney, CV was 16.1% for retinol at 2.8 µg/g and for tocopherol 6.3% at 49µg/g.

Analyses of organohalogens
Organohalogen contaminant (organochlorines and brominated flame retardants) analysis in dog adipose tissue samples were conducted at the National Wildlife Research Centre, Carleton University, Ottawa, Canada, according to methods described in detail elsewhere (Gebbink et al., 2008a, 2008b; Verreault et al., 2008). Also ∑PCB in plasma, liver and kidney were made. The following contaminants were analysed: PCBs; sum of 59 congeners (CB16/32, 17, 18, 22, 31/28, 33/20, 42, 44, 47/48, 52, 56/60, 64/41, 66, 70/76, 74, 85, 87, 92, 95, 97, 99, 101/90, 105, 110, 114, 118, 128, 130, 137, 138, 141, 146, 149, 151, 153, 156, 157, 158, 170/190, 171, 172, 174, 176, 177, 178, 179, 180, 183, 187, 189, 194, 195, 196/203, 199, 200, 202, 206, 207 and 208), OCs; 1,2,4,5-tetrachlorobenzene (TeClBz), pentachlorobenzene (PnClBz), hexachlorobenzene (HCB), α-hexachlorocyclohexane (HCH), β-HCH, γ-HCH, octachlorostyrene (OCS), the chlordanes and metabolites heptachlor epoxide, oxychlordane, *trans*-chlordane, *cis*-chlordane, *trans*-nonachlor, *cis*-nonachlor, *p,p′*-DDE, *p,p′*-DDD, *p,p′*-DDT, dieldrin, photomirex, mirex and tris(4-chlorophenyl)methane (TCPM). Also in adipose tissue, sum of 36 PBDE congeners (BDE17, 25, 28, 47, 49, 54, 66, 75, 77, 85, 99, 100, 116, 119, 138, 139, 140, 153, 154, 155, 171, 180, 181, 183, 184, 190, 191, 196, 197, 201, 202, 203, 206, 207, 208 and 209), as well as several other BFRs including 2,2,',4,4',5-pentabromobiphenyl (BB-101), pentabromotoluene (PBT), hexabromobenzene (HBB), total-(α)-hexabromocyclododecane (HBCD), bis(2,4,6-tribromophenoxy)ethane (BTBPE) and pentabromoethylbenzene (PBEB). OHC data for blood and fat has been published elsewhere as well (Verreault et al., 2008, 2009). Analyses used in this study (for correlations in the exposed group including both P and F generation) include all mentioned OHCs in adipose tissue, as well as ∑PCB in plasma, liver and kidney. Analyses of OHCs in diet are described in Verreault et al. (2008).

Analyses of other vitamins, nutrients and fatty acids in diet
In the dietary fat supplements, analyses for vitamin C (ascorbic acid and dehydroascorbic acid) and E (α-tocopherol) were performed at Eurofins Lab (Kolding, Denmark) and for vitamin D (vitamin D_3 and 25-hydroxyvitamin D_3) and vitamin A (retinol) at National Food Institute (DTU, Søborg, Denmark) by use of HPLC. Methods and results of metal, nutrient and fatty acid analyses of food are presented in Sonne et al. (2008). Vitamin content in Royal Canin dog food pellets were described according to formula content by Royal Canin (www.royalcanin.com). All analyses for metal and vitamins were performed accredited according to ISO 17025 (ISO, 2000).

Statistical analyses

Statistical analysis was performed with the SAS statistical software package (SAS v9.1.3 Service Pack 4 for Windows and SAS Enterprise Guide v4.1, 2006), SAS Institute Inc., Cary, NC, USA). A Pearsons's product-moment correlation coefficient was used to assess all correlations of vitamins, albumin and OHCs in the exposed group ($p<0.05$ and as a trend $p<0.10$), consistently using all exposed animals (P+F, n=10). Linear regressions between age and vitamins were performed prior to Pearson's product moment correlation coefficient. A paired t-test was performed to test for differences between retinol and α-tochoperol in sister/sibling pairs at different ages in the exposed and control groups.

Results

Retinol

There was a negative correlation between liver retinol and ΣDDT in the exposed group just above 5% significance level ($r=-0.626$, $p=0.053$), whereas the negative correlation between liver retinol and ΣBDE was just below 5% significance level ($r=-0.642$, $p<0.045$) (Figure 1).

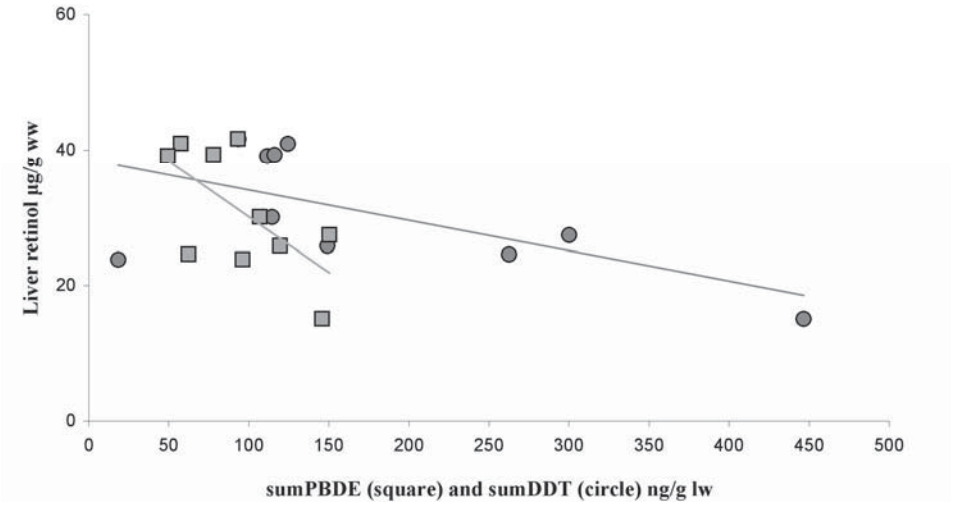

Figure 1. Liver retinol concentrations (µg/g ww) correlated with ΣDDT and ΣBDE (ng/g lw) in adipose tissue of 10 adult sled dogs (maternal and offspring) fed OHC contaminated whale blubber between 12-21 months. The negative correlation between liver retinol and ΣDDT in the exposed group was just above 5% significance level ($r = -0.626$, $p=0.053$), whereas the negative correlation between liver retinol and ΣBDE was just below 5% significance level ($r=-0.642$, $p<0.045$).

With respect to $retinol_{KIDNEY}$, there were positive significant correlations with ΣCHL ($r=0.684$, $p=0.029$) and dieldrin ($r=0.642$, $p<0.046$). Also, there was a negative correlation between $retinol_{KIDNEY}$ and $\Sigma PCB_{ADIPOSE}$ in the exposed P group ($r=-0.514$, $p=0.13$) (Figure 2). No other correlations were seen between $retinol_{LIVER}$, $retinol_{KIDNEY}$ or $retinol_{PLASMA}$ and $OHCs_{ADIPOSE}$ or ΣPCB_{LIVER}, ΣPCB_{KIDNEY}, ΣPCB_{PLASMA}.

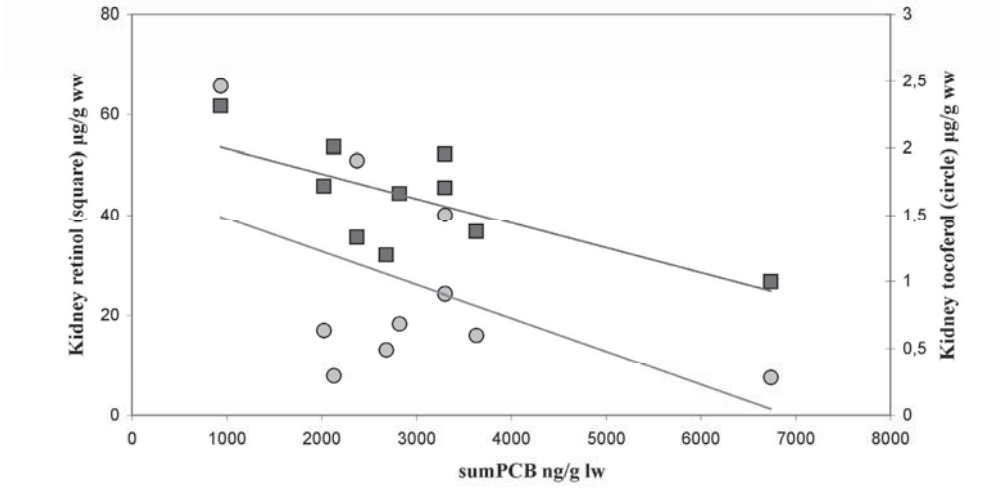

Figure 2. Kidney retinol and tocopherol concentrations (µg/g ww) correlated with ΣPCB (ng/g lw) in adipose tissue of 10 adult sled dogs (maternal and offspring) fed OHC contaminated whale blubber between 12-21 months. A negative, but not significant correlation between kidney retinol and ΣPCB was observed in the exposed group (r=-0.514, p=0.13), as well as a significant negative correlation between kidney tocopherol and ΣPCB (r=-0.702, p=0.024).

$Retinol_{LIVER, KIDNEY, PLASMA}$ mean concentrations at the end of the study (including range) for both generations are shown in Table 1. For the exposed P+F generation (n=10), $retinol_{LIVER}$ concentrations were higher than in the control generation (P+F, n=10) (p=0.023, paired t-test). With respect to $retinol_{PLASMA}$ of the P generation, no significant difference was found between the two groups (exposed=8, 53 observations and control= 8, 53 observations, sampled between 3 and 21 months of age). However, in the F-generation (exposed=4, 19 observations and control=4, 19 observations, sampled between 3 and 12 months of age), $retinol_{PLASMA}$ in the exposed group were significantly higher than in the control group (p<0.01, paired t-test) (Table 2). In the control group, there was an increase in $retinol_{LIVER}$ concentrations with age (r=0.70, p=0.01, P+F, n=12) for control sled dogs only. No such age dependent effect was found among the exposed dogs.

	RETINOL				**TOCOPHEROL**			
	Exposed (n=7)		*Control (n=7)*		*Exposed (n=7)*		*Control (n=7)*	
P generation	*mean*	*range*	*mean*	*range*	*mean*	*range*	*mean*	*range*
L (µg/g ww)	34.21	23.8-41.6	25.0	10.0-33.7	21.5	14.4-29.7	**34.8	23.1-56.0
K (µg/g ww)	1.00	0.30-2.50	1.10	0.20-5.60	43.3	26.5-61-7	54.5	28.1-73.6
P (µg/ml)	0.67	0.60-0.80	0.61	0.30-1.10	19.8	12.8-24.8	**30.0	22.0-38.9
	Exposed (n=3)		*Control (n=3)*		*Exposed (n=3)*		*Control (n=3)*	
F generation	*mean*	*range*	*mean*	*range*	*mean*	*range*	*Mean*	*range*
L (µg/g ww)	**22.8	15.0-27.4	18.9	11.0-23.5	19.2	14.7-24.5	19.4	12.0-27.7
K (µg/g ww)	0.88	0.50-1.50	1.40	0.50-2.60	43.3	31.8-52.1	37.1	24.0-50.0
P (µg/ml)	**0.78	0.70-0.80	0.38	0.30-0.40	15.2	6.30-22.8	12.1	1.60-23.3

**=p<0.05, **=p<0.01*

Table 1. Liver (L), kidney (K) and plasma (P) retinol and α-tocopherol concentrations in a maternal (P) and an offspring (F) generation of the sled dogs after exposure for 12 months (F generation) or 12-21 months (P generation). P values (paired t-test) indicate which group (control or exposed) has highest values.

Alpha-tochoperol

Alpha-tochoperol$_{LIVER, KIDNEY, PLASMA}$ concentrations (including range) for both generations are shown in Tabel 1. In the P-generation, the α-tochoperol$_{PLASMA}$ concentrations from 3-21 months (exposed: n=8, 53 observations and control: n=8, 53 observations) were significantly higher in the control group as compared to the exposed group ($p<0.001$, paired t-test) (Table 2). In the F generation (exposed: n=4, 19 observations and control: n=4, 19 observations), no difference was seen in α-tochoperol$_{PLASMA}$ levels from 3-12 months of age between the two groups. There was a negative correlation between α-tochoperol$_{KIDNEY}$ and $\Sigma PCB_{ADIPOSE}$ in the pooled exposed group ($r=-0.702$, $p=0.024$, Figure 2). No significant correlations between α-tochoperol$_{PLASMA}$ and OHCs were found in either generation.

	RETINOL			**TOCOPHEROL**		
Pair Nr.	*Exposed Obs.*	*Control Obs.*	*P (t-test)*	*Exposed Obs.*	*Control Obs.*	*P (t-test)*
M1	3	3	0.571	3	3	0.235
M2	3	3	0.611	3	3	0.529
M3	4	4	0.681	4	4	0.123
M4	10	10	**0.005	10	10	**0.001
M5	10	10	0.078	10	10	**<0.001
M6	8	8	0.993	8	8	**0.037
M7	6	6	0.062	6	6	**0.044
M8	9	9	0.799	9	9	**<0.001
Mean µg/ml	0.59	0.57	0.44	11,43	23,29	**<0.001
F1	4	4	0.181	4	4	0.443
F2	5	5	0.116	5	5	0.171
F3	5	5	*0.020	5	5	0.198
F4	5	5	*0.017	5	5	0.112
Mean µg/ml	0.56	0.33	**<0.001	15.1	17.68	0.242

**=p<0.05, **=p<0.01*

Tabel 2. Plasma retinol and α-tocopherol (µg/ml) in 12 exposed (53 observations) and 12 control (53 observations) sled dogs from 8 females (P) and 4 exposed (19 observations) and 4 control (19 observations) pups (F), showing if observations from one sibling (one exposed and one control in each pair) are significantly different from the other. Means indicate which group has highest values. Obs. refers to plasma samples taken between 3 and 21 months.

Organohalogen concentrations

Concentrations of OHCs in both adipose tissue and blood at the end of the study are more thoroughly described elsewhere (Sonne et al. 2008; Verrault et al. 2008, 2009). Significant differences were found between the exposed and control groups for most contaminants evaluated. When pooling the animals in the respective groups (P+F) total $\Sigma OHC_{ADIPOSE}$ was 86.4 ng/g lw (range: 27-241 ng/g lw) in the control animals (n=12), and 5005 ng/g lw (range: 3319-8565 ng/g lw) in the exposed animals (n=10). In the exposed P generation, $\Sigma OHC_{ADIPOSE}$ was 5430 ng/g lw (range: 3319-8565 ng/g lw) and in their F generation 4544 ng/g lw (range: 3385-5729 ng/g lw). The higher mean level of ΣPCB_{LIVER} of the exposed P generation was: 43 111 ng/g lw, whereas it was 20 065 ng/g lw in the exposed F generation. When pooling the P and F generations, $\Sigma PCB_{ADIPOSE}$ (n=12) was: mean 52 ng/g lw (range: 16-153 ng/g lw) in the control animals.

Organohalogens, vitamins, nutrients and other components in the diet
PCBs were dominating the OHC profile (ΣOHC =2812 ng/g lw) in the whale blubber given to the exposed group (P and F, n=10), showing the following concentrations of contaminants in decreasing order of presence; ΣPBC=1716 ng/g lw, ΣCHL=296 ng/g lw, ΣDDT=595 ng/g lw, dieldrin=94 ng/g lw, ΣPBDE=63 ng/g lw, HCB=28 ng/g lw, ΣHCH=20 ng/g lw. The exposed groups daily intake of 50-200g whale blubber (mean intake 112g) constituted app. between 10.4-11.7 µg/kg bodyweight ∑OHC (or app. between 4.6-6.1 µg/kg bodyweight ∑PCB)(Verreault et al. 2008, 2009; Sonne et al. 2008). Table 3 presents vitamins A, C, D and E in the diet. Vitamin A (retinol) was 18% higher, vitamin E (α-tocopherol) 22% higher and vitD_3 33% in the diet of the exposed dogs compared to controls (mean of a period of 4 weeks prior to sampling). Concentrations of mercury, zink, selenium, iron and fatty acids in all diet items are presented in Sonne et al. (2008).

	Whale blubber	**Porcine fat**	**RC4300***a*	**RC4800***a*
Vitamin A (mg/kg)	1.2 #	0.09	7.5	7.5
Vitamin C (mg/kg)	<10	<10	320	350
Vitamin D3 (mg/kg)	0.028	0.005	0.03	0.03
25OH D3 (mg/kg)	<0.001	0.002	.	.
Vitamin E (mg/kg)	75	15	650	700

 # Blubber from a minke whale caught randomly in South Greenland in 2004 was analyzed at National Food Institute, DTU, DK for content of retinol.

Table 3. Concentrations of vitamins A, C, D_3 (and $25OHD_3$) and E in minke whale blubber, porcine fat and Energy4300 or Energy4800 Royal Canin (RC) dog food.

Discussion
When all the exposed animals were pooled (P+F generation) the results showed a negative correlation between $retinol_{LIVER}$ and $\Sigma DDT_{ADIPOSE}$ and $\Sigma BDE_{ADIPOSE}$. These results are in accordance with results from experimental studies where rodents were fed standard laboratory diets with a penta-BDE mixture (18 mg/kg daily) showing decreased hepatic retinol levels (Hallgren et al., 2001; Ellis-Hutchings et al., 2006). To our knowledge, no other studies report negative correlations between $retinol_{LIVER}$ and ΣDDT. However, one study reports a positive correlation between DDE and $retinol_{LIVER}$ in common frogs (*Rana temporaria*) (Leiva-Presa and Jenssen, 2006). The apparent different effect of DDTs on $retinol_{LIVER}$ in frogs and dogs could be due to species specific differences in either the hepatic metabolism of OHCs or in the hepatic retinol regulation. No correlations between $retinol_{PLASMA}$ and $OHCs_{ADIPOSE}$ (or ∑PCB in liver, kidney and plasma) were found in the exposed dogs. This is in accordance with the results reported by Ellis-Hutchings et al. (2006), where dietary BDE exposed rats only responded with reduced $retinol_{PLASMA}$ if they had a marginal vitamin A status before exposure. Retinol transport in blood of most carnivore species is affected by the daily vitamin A supply and also the type of food given (Schweigert et al., 2000). Furthermore, plasma retinol levels are usually maintained within a normal range of concentrations as long as there is some minimal level of vitamin A in the liver and in extra-hepatic tissues (Blomhoff et al., 1991), and this seems especially to be the case for dogs as a species (Schweigert et al., 1990). Consequently, an alteration in plasma vitamin A level would indicate a severe toxic effect in the overall dynamics of vitamin A

homeostasis (Zile, 1992). Furthermore, contrary to most other species, dogs transport the majority of their vitamin A in blood as retinyl esters (retinyl stearate) bound to lipoproteins, a mechanism which is fundamentally different from other animals and man (Schweigert et al., 1990, McDowell, 2000). This difference in the retinyl ester pattern between plasma and liver, indicates that mobilisation of vitamin A from the liver involves a selective mechanism. Furthermore, canines seem to be the only mammal that excretes vitamin A as retinol and retinyl esters by a specific protein via the urine (Raila et al., 2002). Higher levels of vitamin A in organs and tissues, as well as the excretion of vitamin A with the urine, might be a consequence of the nonspecific binding of large amounts of retinyl esters to lipoproteins in blood (McDowell, 2000).

In dogs, hepatic retinol levels is much higher than in humans or foxes, but lower than in polar bears, and the kidney plays a specific function of the canine kidney contributing plasma retinol and retinyl esters in addition to the excretion of vitamin A with the urine (Raila et al., 2000). Wildlife studies have shown reduced $retinol_{PLASMA}$ concentrations in polar bears (*Ursus maritimus*) with high burdens of PCBs as well as HCB and HCHs (Skaare et al., 2000, 2001). Braathens et al. (2004), however, did not find decreased $retinol_{PLASMA}$ in polar bears correlated with PCBs. Generally, many studies on seals and sea lions have shown reduced $retinol_{PLASMA}$ with increased concentrations of PCBs and other OHCs (Brouwer et al., 1989; Jenssen et al., 1995, 2003; deSwart et al., 1996; Simms and Ross, 2000; Nymann et al., 2002; Debier et al., 2005). Thus, pinnipeds seem especially vulnerable to retinol depression by OHCs, although the mechanism is still unknown (Zile, 1992; Rolland, 2000).

A negative but not significant correlation was found between $retinol_{KIDNEY}$ and $\Sigma PCB_{ADIPOSE}$ in the exposed group ($p= 0.13$). However, two significant positive correlations between $retinol_{KIDNEY}$ and $\Sigma CHL_{ADIPOSE}$ and $dieldrin_{ADIPOSE}$ concentrations ($p<0.05$) were found. In rats, the kidneys respond to OHC exposure by increasing total amounts of vitamin A (Hakansson et al. 1987, 1994). A semi-field study of mink (*Mustela vision*) showed that a diet of contaminated carp (1.69 mg/kg PCBs) caused a significant decrease in $retinol_{KIDNEY}$ levels (Table 4, Martin et al., 2006). Hence, there appears to be species specific differences in the effects of OHC exposure on kidney vitamin A dynamics.

The results of the present study also show a significant increase in hepatic retinol concentrations with age in the control sled dogs. This is in accordance with other studies on retinoids in rats, explained by decreases in circulatory clearance of retinoids with increasing age, because higher tissue concentrations of retinol might be a defensive adaptation to protect against oxidative stress that becomes more prevalent with age (van der Loo et al., 2004). However, mammalian neonates receive appreciable amounts of vitamin A via milk during the nursing period (Debier et al., 2002). Consequently, lactation loss of retinol could be responsible for some reduction, although no such trend in reduction was observed in plasma or liver retinol for any of the lactating bitches compared to the non-lactating bitches. Lack of an age dependent increase in hepatic retinol in the exposed group may indicate that OHCs can interfere with the age related regulation of hepatic retinol. Although it is speculative, it could indicate that the pups become affected when they become older than 1 year.

Species	OHC type and PCB conc.	Tissue	Effects	References
VIT A				
Polar bear	PCB: 21-228 ng/lw (plasma)	PL	R ▼	Skaare et al. 2001
	PCB: 17-203 ng/g ww (plasma)	PL	R ◄►	Braathens et al. 2004
Gray seal	PCB: 181-2858 ng/g lw (plasma)	PL	R ▼	Jenssen et al. 1995
	PCB: 17-152 ng/g lw (liver)	PL	R ▲	Nyman et al. 2003
	PCB: 17-152 ng/g lw (liver)	LI	R ▼	Nyman et al. 2003
	PCB: 17-152 ng/g lw (liver)	LI, BL	RP ▼	
Harbour seal	PCB: 2506 +/- 594 µg/g lw (bl)	PL, BL	R ▼	Mos et al. 2007
	PCB: 2506 +/- 594 µg/g lw (bl)	PL	RP ◄►	Mos et al. 2007
	PCB: 2.1-17.6 mg/g lw (bl)	PL	R ▲	Simms and Ros 2000
Ringed seal	PCB: 17-152 ng/g lw (liver)	LI	RP ▼	Nyman et al. 2003
Mink	PCB: 19 mg/kg ww (liver)	PL	R ▼	Martin et al. 2006
	PCB: 19 mg/kg ww (liver)	PL	RS ▼	Martin et al. 2006
	PCB: 19 mg/kg ww (liver)	KI	R ▼	Martin et al. 2006
VIT E				
Ringed seal	PCB: 17-152 ng/g lw (liver)	PL, LI, BL	T ▲	Nyman et al. 2003
	DDT: 10-101 ng/g lw (liver)	PL, LI, BL	T ▲	Nyman et al. 2003
Gray seal	PCB: 17-152 ng/g lw (liver)	PL, BL	T ▲	Nyman et al. 2003
	DDT: 3-14 ng/g lw (liver)	PL, BL	T ▲	Nyman et al. 2003

Table 4. Associations between OHCs and vitamins A and E in wildlife, OHC type (and PCB congener), which tissue, which effect and the reference is given.

Hepatic α-tocopherol levels in the P generation and in the pooled group of exposed dogs together (P+F) were significantly lower than controls ($p<0.01$), even though diet concentrations of α-tochoperol in the exposed group were significantly higher. There were no correlations between $\alpha\text{-tocopherol}_{PLASMA}$ and $OHCs_{ADIPOSE}$ (or in $\sum PCB_{PLASMA,\ ADIPOSE,\ LIVER,\ KIDNEY}$) in the pooled group of the exposed dogs from both generations. The lower levels of $\alpha\text{-tochoperol}_{LIVER}$ in the exposed group may also be due to the high dietary concentration of PUFAs in the whale blubber based diet, and that hepatic α-tochoperol stores were depleted to prevent auto-oxidation (Nacka al et., 2001). Unfortunately, the effects of high OHC levels and high levels of PUFAs are confounding factors in this study, and we therefore cannot distinguish between the effects of these two factors. The results in this study do agree with similar studies on mink exposed to PCBs (Mantyla & Ahotupa, 1993; Kakela et al., 1999; Kakela et al., 2001).

In rats fed PCB, the absorption of vitamin E and lipids from the intestine was increased, thereby elevating the plasma lipid levels and the vitamin E concentration in plasma, kidney and spleen (Katayama et al., 1991). Therefore, Nyman et al. (2002) and Routti et al. (1995) proposed that elevated levels of vitamin E in blubber or plasma could be used as a biomarker of high OHC levels in Baltic seals. However, this is in contrast with our results, where we found no correlations between $\alpha\text{-tochoperol}_{PLASMA}$ and OHCs. This could be due to the effects of OHCs on canine vitamin E dynamics differ from that of the effects in rodent and pinniped species or the low sample size in the present study. However, the lack of effects of OHCs on $\alpha\text{-tochoperol}_{PLASMA}$ in the present study may simply also be due to lower body burdens of OHCs. In our study the mean level of ΣPCB_{LIVER} of the exposed P generation was: 43111 ng/g lw, whereas it was 20065 ng/g lw in the exposed F generation. The ΣPCB_{LIVER} concentrations in the two other studies by Nyman et al. and Routti et al. were higher: Baltic ringed seals: mean 34000 ±22000 ng/g lw and Baltic gray seals: mean 59000 ±40000 ng/g lw.

These results suggest that dietary exposure from OHCs can, even in low concentrations, possibly affect retinol and α-tocopherol status in Arctic top predators. The vitamin metabolism disruption caused by complex mixtures of naturally occurring organohalogen contaminants is important in both wildlife and humans. Further studies on vitamin E decrease in liver and increases in plasma and kidney to target this as a general biomarker in relation to environmental OHC exposure is encouraged in other species.

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PAPER II

ALTERED 25-HYRDOXYVITAMIN D3 IN LIVER OF GREENLAND SLEDGE DOGS (*CANIS FAMILIARIS*) DIETARY EXPOSED TO ORGANOHALOGEN POLLUTED WHALE BLUBBER

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Altered 25-hyrdoxyvitamin D3 in liver of Greenland sledge dogs (*Canis familiaris*) dietary exposed to organohalogen polluted whale blubber

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Abstract
This study compared vitamin D_3 status in Greenland sledge dogs either given minke whale (*Balaenoptera acuterostrata*) blubber high in organohalogen contaminants (OHC) or porcine fat low in OHCs, for a period of up to 12 to 21 months of age. Minke whale blubber is similar in OHC composition to part of the diet of Arctic top predators like polar bears (*Ursus maritimus*) and Arctic foxes (*Vulpes lagopus)* and therefore ideal to test as proxy source on model species like sledge dogs. Furthermore, it is rich in vitamin D_3, A and E and contains poly unsaturated fatty acids. In the present study, a group of 7 exposed and 7 control sister bitches (maternal generation) including 4 exposed and 4 control sibling pups were fed whale blubber (mean: 112g, app. between 10.4-11.7 µg/kg bodyweight ∑OHC or 4.6-6.1 µg/kg bodyweight ∑PCB) or porcine (*Suis scrofa*) fat daily. Tissue OHC residue concentrations were measured in dogs and their diet and related to $25OHD_3$ in blood plasma and $25OHD_3$ and vitamin D_3 in liver. The mean level of ∑PCB in adipose tissue of the exposed bitches (n=6) was 3658 ng/g lw and pups (n=3): 2671 ng/g lw. Mean concentrations of ∑PCB for all controls (n=11) were 53 ng/g lw. $25OHD_3$ in liver of the maternal generation was significantly lower in exposed individuals, although their diet contained app. 33% more vitamin D_3 than controls. Vitamin D_3 in liver and $25OHD_3$ in plasma of whale blubber-exposed groups, and also $25OHD_3$ in liver of exposed offspring dogs was significantly higher than porcine fat fed controls. In conclusion, a significantly higher amount of vitamin D_3 in the diet did not result in a significantly higher vitamin D_3 status. The lower level of $25OHD_3$ in liver of the exposed individuals may be due to the fact that the diet high in vitamin D_3 also had a natural dietary complex mixture of organohalogen contaminants.

Key words: Vitamin D_3, 25-hydroxyvitamin D_3, sledge dogs, Organohalogen contaminants, Polybrominated diphenyl ethers

Introduction

Vitamin D_3 ($vitD_3$) is categorized as a fat soluble seco-steroid hormone rather than a vitamin and has several important functions in the organism, including regulation of mineral metabolism, calcium metabolism and bone mineralisation. Other functions of the hormone include regulation of blood pressure, immune functions, cell proliferation and differentiation, gonadal functions, apoptosis and cancer protection [1-6]. It exists in two main forms: vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol) [6, 7]. $VitD_3$ is absorbed in the intestine or produced in skin from 7-dehydrocholesterol. It is metabolized first in the liver to 25-OH vitamin D_3 ($25OHD_3$) and then further to 1,25-dihydroxyvitamin D_3 ($1,25OHD_3$) mainly in the kidney but also in many other tissues. The latter is the active hormonal form of $vitD_3$ in tissues and binds to the nuclear vitamin D receptor (VDR) [7]. The liver hydroxylation of $vitD_3$ to $25OHD_3$ is, opposed to hydroxylation of $25OHD_3$ to $1,25OHD_3$, unregulated, and $25OHD_3$ is accepted as an indicator for $vitD_3$ status [8]. $25OHD_3$ and $1,25OHD_3$ circulate in the blood stream as complexes with Vitamin D-binding protein (DBP), albumin and lipoproteins [9]. Dogs (and cats) are not able to synthesize $vitD_3$ adequately in the skin following sunlight exposure [10]. The same is hypothesized for e.g. ringed seals, bearded seals, beluga whales and polar bears. Thus, for these animals $vitD_3$ may be an essential dietary vitamin [10,11]. Additionally, studies also link alterations in the vitD-vitD-receptor system to behavioural disorders in mice such as anxiety, motor behaviour, maze performance and exploration, suggesting that $vitD_3$ and its receptors are an important factor in the brain and imbalance may significantly affect emotional behaviour [9].

Only few studies have investigated effects of organohalogen contaminant (OHC) on the $vitD_3$ status in mammals. Lilienthal et al. [12] studied PCB exposed rat dams (5-40 mg PCBs/kg in the diet), and reported decreased levels of both $25OHD_3$ and $1,25OHD_3$ with OHCs reconstituted congener patterns similar to those found in human breast milk, even for the lowest dose. Routti et al. [13] studied wild grey and ringed seals from the Baltic region revealing a relationship between circulating $1,25OHD_3$, calcium, phosphate and thyroid hormone levels and hepatic PCB and DDT.

In order to mimic a long-term OHC mixture exposure situation, a study was conducted in a period of up to 12 to 21 months, where Greenland sledge dogs were given a diet containing minke whale blubber naturally contaminated with OHCs (exposed group) or a diet containing porcine (Sus scrofa) fat (control group). This study showed that animals in the OHC-exposed group experienced liver and kidney lesions, altered liver and kidney functions [14-16], insufficient immune response [15] and impaired cellular immunity [17]. Furthermore, concentrations of OHCs, calcium, phosphate, albumin, thyroxin, bone mineral density and hepatic activity of xenobiotic-metabolizing CYP enzymes have been reported previously [18-21]. In this study we report on the effects of the OHC spiked diet on levels of $vitD_3$ and $25OHD_3$ in liver and $25OHD_3$ in plasma in Greenland sledge dog bitches and their *in utero* exposed pups.

Materials and Methods

Experimental design

The maternal generation (P) was composed of 14 (7 pair-wise) female sledge dogs from Aasiaat (Disco Bay, in West Greenland), obtained at two months of age. One bitch from each sister-pair was assigned randomly to either an exposed

or control group in order to minimize age and genetic differences between the two groups. The bitches were mated with the same unexposed male. One and three of the exposed and control bitches, respectively, gave birth during the course of the study. The pup generation used in this study was composed of two litters from one exposed and one control sister bitch (8 dogs): 4 exposed siblings (3 male, 1 female) and 4 control siblings (3 male, 1 female) with the same father, and mothers that were sisters. After weaning at 6-8 weeks of age, they were fed the same diet as their mother. Organ tissue and OHC analyses from adipose tissue from one exposed pup were not available as part of this study, due to factors unrelated to the experiment. Furthermore, one sister pair (P) was excluded as outliers, as $25OHD_3$ values were unexplainably high in one animal. The exposed dogs were fed whale blubber without epidermis from a West Greenland minke whale, rich in OHCs, polyunsaturated lipids and vitamins and low in mercury, and obtained by native subsistence hunting. Control dogs were fed porcine fat for human consumption (low in OHCs and polyunsaturated fatty acids [14-19]). All dogs were fed 50–200 g/day of either blubber or porcine fat. Furthermore, all dogs were also fed equal amounts of standardized Royal Canine Energy 4300/4800 dry dog pellets (50-200 g/day) to cover basic nutrients and microelements (www.royalcanin.com). Furthermore, all dogs were fed simultaneously on a daily basis. The energy intake was balanced by achieving comparable weights among siblings in the two groups. Description and detailed discussion of concentrations of pollutants, lipids and nutrients in the diet has been published previously [18,19]. The dogs were subjected to various treatments during the study as described elsewhere [14-19], and were euthanized upon experiment completion at adulthood for the maternal generation at an age of 12 to 21 months while the pups were all 12 months. The animal experiment was performed on a licence granted by the Home Rule Government Chief Veterinarian in Greenland and conducted in accordance with national and institutional guidelines for the protection of animal welfare.

Blood and tissue sampling

The blood was sampled using a Vacutainer™blood collection system with an 18 gauge needle and sterile Lithium-Heparin Greiner 9 mL Vacuette and centrifuged at 3600 rpm for 10 min. Subsequently 4 mL blood plasma from each dog was sampled in 10 mL sterile Nunc 348224 cryo tubes and frozen to -18 º C immediately. The sister bitches were blood sampled at different intervals and frequencies during the study. 50 exposed and 50 control blood plasma samples were taken from 7 sister bitch pairs in the P generation between 3 months and 21 months of age. From the 4 F generation pups (4 exposed and 4 control) blood plasma samples from 19 exposed and 19 control plasma samples were taken between 3 months and 12 months of age. Liver and adipose tissue was sampled from 7 exposed and 7 control sister bitches (P) and from 3 exposed and 4 control pups (F) immediately after euthanasia, then transported and stored at the laboratory until analysis at -18 ºC.

Analysis of Vitamin D_3 and $25OHD_3$

These analyses were performed at the National Food Institute, DTU, Denmark. The analytical method and the equipment used to determine $vitD_3$ and $25OHD_3$ in blubber, lard, and liver are previously described in detail [22,23]. Briefly, the internal standards of vitamin D_2 and 25-hydroxyvitamin D_2 were added to the samples and saponified with ethanolic potassium hydroxid. After extraction of the unsaponifiable matter with diethylether:petroleumsether (1:1), the

purification of the extract included a solid-phase and a preparative HPLC step with silica and amino columns. Finally, the HPLC-system equipped with reversed phase columns, PDA and UV-detectors were adequate for the separation, detection and quantification of the vitamins. The detection and quantification levels were 0.02 and 0.1 µg vitamin D/100g, respectively. The analytical method and the equipment used to determine $25OHD_3$ in plasma are previously described [24]. Minor modifications were made as 25-hydroxyvitamn D_2 (Sigma-Aldrich, Buchs, Schwitzerland) was used as the internal standard. Briefly, plasma proteins were precipitated with ethanol and the supernatant was cleaned by a MFC18 solid-phase extraction. The 25OHD in the solution was separate, detected and measured by analytical HPLC equipped with a diode array detector (220-320 nm) and a UV-detector (265 nm) and quantification performed by internal standard method. Quantification limits for 25OHD3 and 25OHD2 were 6 nmol/l and 12 nmol/l, respectively, and no samples contained $25OHD_2$. All samples from each sledge dog was analysed in the same rum, while the inter-assay variation was 12.4% (n=62), and recovery rate 94,1%±6.7% (n=34). The analyses were performed in a laboratory accredited according to ISO17025.

Analyses of dietary vitamins, nutrients and fatty acids
Analysis of vitamins, minerals and fatty acids is presented elsewhere [19, 25]. All analyses for metal and vitamins were performed accredited according to ISO 17025 (ISO, 2000).

Analysis of organohalogens
Organohalogen contaminant (organochlorines and brominated flame retardants) analysis in blood plasma and dog adipose tissue samples were conducted at the National Wildlife Research Centre, Carleton University, Ottawa, Canada, according to methods described in detail elsewhere [19-20,26-29]. The following contaminants were analysed: PCBs; sum of 59 congeners (CB16/32, 17, 18, 22, 31/28, 33/20, 42, 44, 47/48, 52, 56/60, 64/41, 66, 70/76, 74, 85, 87, 92, 95, 97, 99, 101/90, 105, 110, 114, 118, 128, 130, 137, 138, 141, 146, 149, 151, 153, 156, 157, 158, 170/190, 171, 172, 174, 176, 177, 178, 179, 180, 183, 187, 189, 194, 195, 196/203, 199, 200, 202, 206, 207 and 208), OCs; 1,2,4,5-tetrachlorobenzene (TeClBz), pentachlorobenzene (PnClBz), hexachlorobenzene (HCB), α-hexachlorocyclohexane (HCH), β-HCH, γ-HCH, octachlorostyrene (OCS), the chlordanes and metabolites heptachlor epoxide, oxychlordane, *trans*-chlordane, *cis*-chlordane, *trans*-nonachlor, *cis*-nonachlor, *p,p'*-DDE, *p,p'*-DDD, *p,p'*-DDT, dieldrin, photomirex, mirex and tris(4-chlorophenyl)methane (TCPM). Also in adipose tissue, sum of 36 PBDE congeners (BDE17, 25, 28, 47, 49, 54, 66, 75, 77, 85, 99, 100, 116, 119, 138, 139, 140, 153, 154, 155, 171, 180, 181, 183, 184, 190, 191, 196, 197, 201, 202, 203, 206, 207, 208 and 209), as well as several other BFRs including 2,2,',4,4',5-pentabromobiphenyl (BB-101), pentabromotoluene (PBT), hexabromobenzene (HBB), total-(α)-hexabromocyclododecane (HBCD), bis(2,4,6-tribromophenoxy)ethane (BTBPE) and pentabromoethylbenzene (PBEB). OHC data for blood and fat has been published elsewhere as well [20]. Analyses used in this study (for correlations in the exposed group including both P and F generation) include all mentioned OHCs in adipose tissue, as well as ∑PCB in plasma, liver and kidney. Analyses of OHCs in diet are described elsewhere [20].

Statistical analyses

Statistical analysis was performed with the SAS statistical software package (SAS v9.1.3 Service Pack 4 for Windows and SAS Enterprise Guide v4.1, 2006), SAS Institute Inc., Cary, NC, USA). The level of significance was set to $p \leq 0.05$. A Student's paired t-test was performed to test for differences between plasma $vitD_3$ and 25OHD3 in the exposed and control groups at different ages (between 3-18 months), between paired P sister bitch observations. A Students t-test was used to evaluate F sibling observations (siblings related with the same father and mothers as sisters). Analyses were performed within the P and F generation, respectively. Linear regressions between age and vitamins in the exposed group (P+F) were performed prior to Pearson's product moment correlation coefficient, which was performed across generations between OHCs and vitamins.

Results

Maternal (P) Vitamin D_3 and 25OHD$_3$ in liver and plasma

Analyses of liver vitamin D_3 in the P generation were evaluated pair-wise for six pairs (i.e. control vs. exposed sisters) (Table 1). Significantly higher ($p=0.004$, $n=12$, paired t-test) concentrations of $25OHD_{3\ LIVER}$ of the control group was observed. $VitD_{3\ LIVER}$ showed no difference between the two groups. $25OHD_{3\ PLASMA}$ was evaluated from 40 exposed and 40 control plasma samples taken from 6 sister pairs from app. 3 months to 20 months of age.

			DIET	FAT	LIVER		PLASMA
ID	**Weight**	**Age**	**VitD** [a]	**∑PCB**	**VitD**	**25OHD**	**25OHD**
Exp/Con	*kg*	*days*	*µg/day*	*ng/g lw*	*µg/100g*	*µg/100g*	*nmol/l*
P1-Exp	25.7	636	10.2	2374	0.36	0.47	92.2
P2-Exp	21.5	635	9.2	936	0.28	0.31	90.7
P3-Exp	28.2	556	11.7	6740	0.19	0.35	68.4
P4-Exp	28.6	556	9.8	2822	0.21	0.36	84.8
P6-Exp	21.9	381	14.4	2131	0.61	0.52	105.4
P7-Exp	24.1	404	9.5	3633	0.28	0.20	43.5
Mean	24.8	524	*10.8	**3106	0.32	0.39	81.0
P1-Con	25.7	636	11.1	16	0.36	0.56	132.8
P2-Con	22.9	635	5.7	58	0.16	0.46	107.3
P3-Con	27.5	556	6.4	53	0.22	0.47	69.5
P4-Con	28	556	6.4	34	0.16	0.37	76.2
P6-Con	22.3	381	11.2	153	b1.042	0.64	90.9
P7-Con	24.1	404	6.2	26	0.22	0.29	43.9
Mean	25.1	528	7.8	57	0.22	*0.47	86.8

[a] *Total vitamin D_3 in the diet (from blubber/porcine fat and RC dry food) from 25 days prior to sampling.*
[b] *This value was left out of calculations of the mean and no difference in significance was then observed for 25OHD in live. * = significantly higher ($p<0.05$, student's t-test, paired), ** = significantly higher ($p<0.01$, student's t-test), Exp= exposed group, Con= control group*

Table 1. Vitamin D_3 data from 12 Greenland sled dog bitches (6 exposed and 6 control) in the diet and after up to 21 months of organohalogen contaminant exposure (P generation). Notice liver $25OHD_3$ in the exposed group showed significantly lower levels compared to controls ($p=0.004$), and that liver tissue $vitD_3$ is similar in the two groups, despite significantly higher dietary intake in the exposed group. Plasma was sampled at the end of the study.

No overall significant difference between concentrations of $25OHD_{3\ PLASMA}$ in control compared to exposed dogs was observed, when evaluating all paired plasma samples. Total intake of $vitD_3$ in the diet from fat, blubber and dog pellets during the 4 weeks prior to sampling showed values significantly higher ($p<0.01$) in the exposed group when compared with the control (see Table 1).

Offspring (F) vitamin D3 and 25OHD3 in liver and plasma

Offspring (F generation) liver tissue concentrations are presented in Table 2, and showed significantly higher levels of $vitD_{3\ LIVER}$ in the exposed group ($p<0.05$). Again total intake of $vitD_3$ in the diet from fat, blubber and dog pellets during the study period showed values significantly higher ($p<0.0001$) in the exposed group (see Table 2). No significant difference was observed between the two groups for either $25OHD_{3\ LIVER}$ or $25OHD_{3\ PLASMA}$ with samples taken at the end of the study. The exposed F generation group had overall significantly higher concentrations of plasma $25OHD_{3\ PLASMA}$ ($p=0.009$) than the controls when evaluating 19 exposed and 19 control plasma samples taken at 3, 5, 7, 9 and 12 months.

			DIET	FAT	LIVER		PLASMA
ID	**Weight**	**Age**	**VitD3**	**PCB**	**VitD**	**25OHD**	**25OHD**
Exp/Con	*kg*	*days*	*µg/day*	*ng/g lw*	*µg/100g*	*µg/100g*	*nmol/l*
F1-1 Exp	21.1	342	9.3	2686	0.24	0.41	61.9
F1-2 Exp	24.9	342	9.8	2025	0.34	0.52	71.0
F1-3 Exp	22.0	342	9.8	3303	0.44	0.49	74.9
Mean	22.7	342	**9.6	*2671	*0.34	0.47	69.3
F1-1 Con	18.9	342	6.3	43	0.18	0.24	58.3
F1-2 Con	21.9	342	6.4	40	0.19	0.60	68.0
F1-3 Con	17.4	342	7.2	32	0.06	0.29	41.6
F1-4 Con	24.1	342	6.4	57	0.24	0.49	70.0
Mean	20.6	342	6.6	43	0.17	0.41	59.5

*[a] Total vitamin D3 in the diet (from blubber/porcine fat and RC dry food) from 25 days prior to sampling. * = significantly higher ($p<0.05$, student's t-test, paired), ** = significantly higher ($p<0.01$, student's t-test), Exp= exposed group, Con= control group*

Table 2. Vitamin D_3 data from 7 Greenland sled dogs (3 exposed and 4 control) in the diet and after up to 12 months of organohalogen contaminant exposure (F generation). Notice liver $vitD_3$ was significantly higher in the exposed group.

Relationships between $vitD_3$ in liver and $25OHD_3$ in liver/plasma

$25OHD_{3\ PLASMA}$ tended to increase with increasing $25OHD_{3\ LIVER}$ ($n=9$, $r=0.555$, $p=0.121$) when pooling all exposed animals in both generations. For all pooled controls, a similar almost significant trend was observed with the same parameters ($n=10$, $r=0.634$, $p=0.052$). A positive trend was also observed for $25OHD_{3\ PLASMA}$, which increased with increasing $vitD_{3\ LIVER}$; exposed (both generations): $r=0.558$, $p=0.116$ ($n=9$), controls (both generations): $r=0.356$, $p=0.312$ ($n=10$).

OHCs and relationships with $vitD_3$ and albumin

Significant differences were found between the exposed and control groups for most contaminants evaluated. The mean level of ∑OHCs in adipose tissue of the P generation exposed group ($n=6$) was 5496 ng/g lw (range: 3319-8565 ng/g lw, mean age 528 days), which was in the same magnitude as the F generation ($n=3$): 4544 ng/g lw (range: 3385-5789 ng/g lw, mean age 342 days). Concentrations of ∑OHCs for all controls (all animals pooled, $n=11$) ranged between 27-241 ng/g lw. ∑PCB in the adipose tissue for exposed dogs were 55 (pups, F) and 60 (bithces, P) fold higher compared to the respective control groups, and these differences were significant for both the P and the F generations (Table 1 and 2). In the exposed group (all animals pooled, $n=9$), negative, though not significant, correlations were found for $vitD_{3\ LIVER}$ and ∑PCB in liver ($r=-0.588$, $p=0.096$) and for $25OHD_{3\ PLASMA}$ and $\sum PBDE_{\ ADIPOSE}$ ($r=-0.52$, $p=0.153$). Concentrations of organohalogens in fat and blood at the end of the study are published in detail elsewhere [19-21]. Albumin in plasma showed a negative trend with increasing $\sum PCB_{LIVER}$ ($r=-0.58$, $p=0.099$, $n=9$) in the exposed group (Figure 1).

Concentrations of albumin and alkaline phosphatase, inorganic phosphate, iron, thyroxin, calcium and magnesium in plasma at the end of the study have been published previously [18], and no other significant correlations with OHCs were seen for these parameters.

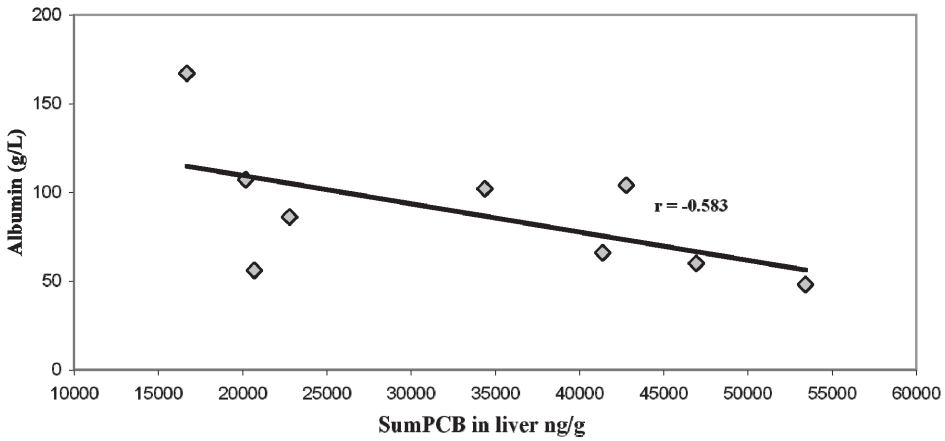

Figure 1. Pearson's product moment correlation between albumin (g/L) in plasma and ∑PCB in liver (ng/g lw) in all exposed (P and F) Greenland sled dogs (n=9, r=-0.583, p=0.0995)

OHCs, vitamins, nutrient and fatty acids in the diet

PCBs were dominating the OHC profile (ΣOHC=2812 ng/g lw) in the whale blubber given to the exposed group (maternal and offspring), showing the following concentrations of contaminants in decreasing order of presence; ΣPBC=1716 ng/g lw, ΣCHL=296 ng/g lw, ΣDDT=595 ng/g lw, dieldrin=94 ng/g lw, ΣPBDE=63 ng/g lw, HCB=28 ng/g lw, ΣHCH=20 ng/g lw. The mean daily intake of minke whale blubber or porcine fat was 112g, for the exposed group approximately between 10.4-11.7 µg/kg bodyweight ∑OHC or 4.6-6.1 µg/kg bodyweight ∑PCB. Detailed information on OHCs in the sledge dogs have been published previously [20]. The two groups were fed equal amounts of normal dog Royal Canin (RC) pellets (www.royalcanin.com) to cover basic nutrient needs. Vitamins, nutrients and lipid concentrations in the different diet items are presented elsewhere [19-20]. Vitamin A (retinol) was 18% higher, vitamin E (α-tocopherol) 22% higher and vitD_3 33% (Table 1 and 2) in the diet of the exposed dogs compared to controls (mean of a period of 4 weeks prior to sampling).

Discussion

The sledge dog, and possibly the polar bear, ringed and bearded seal as well, have evolved thick skin and fur at the expense of a sub-cutaneous ability to produce $vitD_3$ in adequate amounts by means of sunlight [10,11]. Thus, a dietary intake of $vitD_3$ is essential. Via the diet, the exposed sledge dogs (P+F) received app. 33% more $vitD_3$ vs. controls in a period of 4 weeks prior to sampling, based on recorded daily food intake. Despite the above mentioned higher $vitD_3$ intake in the exposed group, concentrations of $25OHD_{3\ LIVER}$ was significantly lower and $vitD_{3\ LIVER}$ similar to controls for the P generation. Furthermore, $vitD_{3\ LIVER}$ showed a declining but not significant trend with increasing $\sum PCB_{LIVER}$ in the exposed dogs ($r=-0.588$, $p=0.096$). This agrees partly with a study on ringed seals (*Phoca hispida)* which showed that persistent organic pollutants like PCBs disrupt $vitD_3$ metabolism (1,25OHD) [13]. The reason for the similar concentration of $vitD_{3\ LIVER}$ and lower $25OHD_{3\ LIVER}$ in the exposed P generation dogs despite their higher intake compared to controls remains speculative. One suggestion could be that transport proteins, e.g. albumin and Vitamin D binding protein (DBP), are affected by OHCs. Thus, $vitD_3$ may not be adequately transported to the liver, consequently resulting in a lesser transformation of $vitD_3$ to plasma $25OHD_3$ in the liver. In humans, DBP, albumin and lipoproteins are the principle carriers of $vitD_3$, and DBP and albumin are both members of the albumin gene family and synthesized in the liver [30,31]. A declining trend was seen between albumin and increasing $\sum PCB_{LIVER}$ of the exposed sledge dogs ($p=0.099$) (DBP was not measured). This agrees somewhat with another study, where co-planar PCBs were shown to induce kinetic changes in albumin levels of rats exposed *in utero* [32]. The mechanism of an albumin and $vitD_3$ correlation is not clearly understood as yet [33], but in humans, the affinity of albumin for $25OHD_3$ is substantially lower than that of the DBP.

Routti et al. [13], proposed that bone lesions in Baltic gray seals may be associated with contaminant mediated $vitD_3$ and thyroid disruption, as a clear relationship between circulating $1,25OHD_3$, calcium, phosphate and thyroid and hepatic PCB and DDT load was observed. We observed no relationships with $vitD_3$ and $25OHD_3$ in liver or $25OHD_3$ in plasma and concentrations of alkaline phosphatatse, iron, calcium, magnesium, thyroxin and bone mineral density at the end of the study (concentrations published previously [19]). However, $1.25OHD_3$ was not measured in the present study and the female seals studied by Routti et al. exhibited uterine occlusions and infertility, presumably because of higher concentrations of accumulated OHCs ($\sum PCB_{LIVER}$: Baltic ringed seals, mean 34000 ±22000 ng/g lw and Baltic gray seals, mean 59000 ±40000 ng/g lw and for the present dog study ΣPCB_{LIVER}: exposed P mean 43111 ng/g lw and exposed F mean 20065 ng/g lw). Furthermore, low sample size or species specific $vitD_3$ dynamics could suggest the difference between the studies.

Another suggestion to the low or similar $vitD_3$ in liver between the groups of P generation dogs despite different dietary levels could be related to a so-called "drug-drug interaction". $VitD_3$ is hydroxylated to $25OHD_3$ in the microsomes and mitochondria in liver by the CYP, 25-hydroxylation enzymes. One suggested CYP enzyme mediating the hydroxylation of $vitD_3$ in microsomes is CYP2R1, which is preferentially expressed in the liver, and is not sexually dimorphic. Furthermore, CYP2R1 is highly conserved among species ranging from fish to human [34,35]. The ability of a xenobiotic substrate that up-regulates CYPs (e.g. OHCs in liver) by affecting the activity of a natural substrate (e.g. $vitD_3$ in liver)

is a drug-drug interaction [36]. Vitamin D_3 is a seco-steroid hormone, and Verreault et al. [21] have shown that testosterone hydroxylation activity resulting in the formation of various hydroxylated testosterone metabolites in vitro (and thus mediated by various CYPs) was greater in dogs from the present exposed generations relative to the controls. This suggests that the induction of various CYPs, and perhaps CYP2R1 as well, may explain the increased $vitD_3$ to $25OHD_3$ metabolism, which subsequently resulted in low levels of $vitD_3$ in the liver of the exposed P generation animals. Furthermore, there may be increased metabolism of $25OHD_3$ in plasma to $1,25OHD_3$ in tissues and kidney, as $25OHD_{3\ LIVER}$ was lower in the exposed group (relative to controls). This indicated a greater need for $1,25OHD_3$ in tissues of the OHC exposed bitches.

Another aspect of $vitD_3$ transport disruption could be fatty acids in the diet. One study concluded that n-3 polyunsaturated (in our case whale blubber) fatty acids, opposed to saturated (in our case porcine) fatty acids, are capable of decreasing the binding of $25OHD_3$ to DBP in humans [37]. Another study conclude that DBP possesses relatively weaker binding to fatty acids than albumin, so when albumin is depressed, DBP does not take over it in terms of fatty acid scavenging and transportation [38]. Whether this possible inhibitory potency is the same for canine DBP and the n-3 polyunsaturated fatty acids from whale blubber given to the exposed group of dogs in this study is unknown.

Furthermore, the adequate daily intake of $vitD_3$ in the diet is for adult dogs suggested to be 0,36 µg per kg bodyweight pr day [39], in our case, for a sledge dog of 25 kg this would be 9 µg pr day. This recommended level is met for the exposed group (both generations, app. 10 µg pr day) but higher than the intake of the controls (app. 6-8 µg pr day). A potential confounding factor could be that adequate amounts of $vitD_3$ in general for sledge dogs in the Arctic has been met in all sledge dogs of the present study and the difference between groups might naturally be unrecognizable, if excess $vitD_3$ is naturally excreted. Although this is less likely, as significant differences in vitamin concentration were found between groups in the F generation.

In conclusion, a significantly higher amount of $vitD_3$ in the diet did not result in a significantly higher $vitD_3$ status in the sledge dogs, and the lower level of $25OHD_{3\ LIVER}$ of the exposed individuals may be due to dietary OHC exposure. Because most Arctic top predators have evolved with thick skin and fur at the expense of adequate synthesis of $vitD_3$ in the skin following sunlight exposure, a stable dietary $vitD_3$ intake is of extreme importance. This may especially be the case for the polar bear, which in addition has evolved heavily pigmented skin for thermoregulatory beneficial purposes [11]. Hypothetically, a small risk of population declines in these Arctic species may exist due to organohalogen contaminant mediated/influenced disruption of various endogenous vitamin and hormone systems, which may be amplified by other stressors such as climate change and energetic stress from depleted sea ice conditions. For example, OHC temporal trends and thus exposure in western Hudson Bay polar bears has been shown to be related to changes in sea ice (earlier ice break-up date) as a function of regional climate warming and dietary deviations from their main prey, the ringed seal, to seal (bearded seal) who feed at a lower trophic level [40]. This underlines the importance of future studies of systemic vitamin D disruption in wildlife, which may be caused by OHCs, as it may have importance for the most susceptible individuals or in a worst case scenario, on a population scale. Future

studies targeting OHCs and circulating metabolites of vitamin D_3 in wildlife is encouraged.

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PAPER III

TESTOSTERONE CONCENTRATIONS AND MALE GENITAL ORGAN MORPHOLOGY IN GREENLAND SLED DOGS (*CANIS FAMILIARIS*) DIETARY EXPOSED TO ORGANOHALOGEN CONTAMINANTS

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Testosterone concentrations and male genital organ morphology in Greenland sled dogs (*Canis familiaris*) dietary exposed to organohalogen contaminants

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Abstract
Arctic top predators accumulate high amounts of environmental organohalogen contaminants (OHCs) in their fat tissues. The aim of this study was to investigate if a low-level, long-term *in utero* and postnatal exposure to organohalogen pollutants disrupts the development of male reproductive organs and testosterone production in Greenland Sled dogs (*Canis familiaris*), as a model of Arctic top predators feeding on marine mammals. Six male dogs were followed for one year and testosterone concentrations, testes/baculum morphology and bone mineral density (BMD) was determined. The exposed group (n=3) was exposed to OHCs *in utero* from maternal dietary intake of minke whale blubber (*Balaenoptera acutorostrata*) and had a post-weaning intake of 10.4-11.7 µg/kg/day resulting in an adipose tissue range of ∑OHC 4518-5729 ng/g lw (∑PCB of 2686-3303 ng/g lw) after one year. The control group (n=3) was exposed to very low concentrations of OHCs through pork fat. No significant difference was seen in plasma testosterone concentrations between the 6 male dogs (control: n=3 and exposed n=3) measured at 3, 5, 7, 9 and 12 months of age or between baculum weight, BMD or testicular length. Testicular weights were significantly lower in the exposed group (p=0.015, n=2). Although this study has a limited number of animals, it suggests that *in utero* and following 12 months of chronic exposure to a complex mixture of contaminants in the form of naturally accumulated OHCs does not affect testosterone levels, but possible affects testicular weights in sledge dogs.

Keywords: PCB, organohalogen contaminants, dog, testosterone, testis

1. Introduction

The polar bear (*Ursus maritimus*) and the Arctic fox (*Vulpes lagopus*) are top predators in Arctic ecosystems, and have been reported to have high body burdens of long-range transported lipophilic OHCs (organohalogenated contaminants) (Derocher et al., 2003; Dietz et al., 2004; Fuglei et al., 2007). Because Arctic organisms such as polar bears and polar foxes have large natural variations in diet availability and climate stress, they undergo seasonal fasting periods where they utilize stored adipose tissue as their energy source (Dietz et al., 2004; Prestrud and Nilssen 1992). Such loss of adipose tissue is associated

with high levels of OHCs becoming (rapidly) bio-available, exposing vital organs to increased OHC exposure and thus increasing the risk of negative health effects (Gebbink et al., 2008; Polischuk et al., 1995; Sonne et al., 2006a). OHCs and other contaminants occurring in the environment have been shown to interfere with the male reproductive system in mammals (Andric et al., 2000; Dallinga et al., 2002; Oskam et al., 2003). Also mechanisms associated with metabolism of OHCs have been shown to disrupt *in vitro* metabolism of testosterone in the sled dog (C*anis familiaris*) cohort studied herein (Verreault et al., 2009b). This increases the potential endocrine disrupting effects of OHCs. Such disruption may have critical adverse effects on the principal androgen, testosterone, which is necessary for (1) differentiation of the male urogenital tract during foetal life, (2) the development of the secondary male appearance and sexual behaviour, and (3) stimulation and maintenance of spermatogenesis and therefore the capacity for fertilization (Lincoln, 1981). Parameters of reproductive disruption due to a chronic long-term dietary exposure with environmentally occurring OHCs have been studied in a few species like e.g. common seal (*Phoca vitulina*) (Reijnders et al., 1986) and mink (*Mustela vison*) (Restum et al.,1998). Furthermore, reproductive disruption has been reported in highly contaminated polar bears (*Ursus maritimus*) from East-Greenland and Svalbard (Sonne et al., 2006a, Oskam et al., 2003). Greenland sled dogs are usually provided with marine mammals as a food source (Born 1981), and are thus exposed to dietary concentrations of OHCs similar to that of other Arctic top predators. Therefore they serve as a unique environmental toxicological model of wild Arctic top mammalian predators, as studies on these wild animals are impossible to conduct in the same way. Examinations of sled dogs fed a naturally OHC contaminated diet consisting of the blubber of a minke whale (Balaenoptera acutorostrata) harvested in the waters off West Greenland have shown that relative to control individuals fed pork fat, the exposed sled dogs exhibited liver and kidney lesions (Sonne et al., 2007a, 2008a), altered liver and kidney functions (Sonne et al., 2007a, 2008a,b), insufficient immune response (Sonne et al., 2007b) and impaired cellular immunity (Sonne et al., 2006b). The aim of the present study was to investigate the hypothesis put forward by Sonne et al., (2006a), suggesting that a long term dietary exposure (including *in utero* and lactation exposure) to a naturally occurring mixture of organohalogens, disrupt male reproductive hormones and development of sexual organs in polar bears. To examine this, plasma testosterone concentrations sampled at 3, 5, 7, 9 and 12 months of age, baculum length, weight and bone mineral density (BMD), and testes weight were studied in two groups of Greenland sled dogs, one group that was given a diet containing blubber from minke whales (*Balaenoptera acuterostrata*), and one control group that was given a diet containing low contaminated pork fat. Additionally, the males in this study were exposed to the mentioned diets *in utero* and via lactation, as the maternal generation was given the same respective diets from 2 months of age.

2. Materials and methods

2.1. *Experimental Design*

The present study is part of a larger two-generation study on the health effects caused by OHC exposure in Greenland sled dogs. The animal experiments were performed on a license granted by the Home Rule Government in Greenland. The experimental design was conducted on Greenland sled dogs in Aasiaat, Disco Bay, in West Greenland. The parent generation (P) was composed of two sister bitches: one exposed and one control, mated with the same unexposed

male. The pup generation (F) investigated here was composed of 6 male pups, 3 from the exposed bitch and 3 from the control bitch, born 20 days apart. The pups were, after weaning, given the same diet as their mother. During the gestation and lactation (6-8 weeks), the two mothers were kept at approximately the same body weight and condition to avoid confounding from energy intake. This was also the case for the pups after weaning. The mothers were given the exposed and control diet, respectively, immediately after entering the project at an age of two months. Furthermore, a substantial amount of the mother's contaminant burden was passed on to the exposed pups *in utero* and via suckling (Verreault. et al. 2009a). However, because it was not possible to sample milk, the exposure could not be estimated. The control mother bitch was fed porcine fat. The 3 control male pups were fed equal amounts (∑OHCs <0.01 ng/g lw) of porcine fat to maintain bodyweights similar to the exposed group. Furthermore, all dogs were fed equal amounts of standardized Royal Canine Energy 4300/4800 dry dog pellets (50-200 g/day) to cover basic nutrients and microelements (www.royalcanin.com) and feeding occurred simultaneously on a daily basis. ∑PCBs in the whale blubber was 1716 ng/g lw and in pork fat <0.01 ng/g lw. Vitamin A (retinol) was 18% higher, vitamin E (α-tocopherol) 22% higher and vitD_3 33% in the diet of the exposed dogs compared to controls (mean of a period of 4 weeks prior to sampling). See further description, analyses and detailed discussion of all concentrations of pollutants, lipids and nutrients in the diet, in Sonne et al. (2006a, 2007b). The 6 male pups in this study were followed for up to one year of age, and blood samples for testosterone analyses were drawn at 3, 5, 7, 9 and 12 months. For one of the exposed males it was not possible to sample other organic matrixes than blood plasma for testosterone analysis. The dogs were subjected to various treatments during the study as described elsewhere (Sonne et al., 2006a, 2007a, 2007b, 2008a), and were euthanatized upon experiment completion.

2.2. *Sampling*

All pups underwent a 24 h fast before sampling of blood from the caval vein using an 18 gauge needle and 9 mL VACUETTE® Heparin tubes (Lithium-Heparin, Greiner). The blood was centrifuged at 3600 rpm for 10 minutes (plasma) and subsequently 4 mL blood plasma from each dog was transferred to 10mL Nunc 348224 cryo tubes. Plasma was immediately frozen at -18 º C until analysis of testosterone at the Norwegian University of Science and Technology. Subcutaneous adipose tissue, penile bones and blood samples for chemical analysis were also collected from each individual, and kept at −20 °C or lower temperatures until laboratory analyses. Testes were dissected within 30 min. following euthanasia. The weight of both testes was recorded while the length of the left testes without epididymidis was assessed. The penile bones (bacula) were thawed, carefully cleaned and air dried before weighing and Bone Mineral Density evaluation.

2.3. *Testosterone analysis*

Plasma testosterone was measured using a commercially available radioimmunoassay kit (Spectria® Testosterone RIA Coated Tube Radioimmunoassay, Orion Diagnostica, Espoo, Finland). The assay was validated with sled dog heparin plasma and the standard method for analysis was used (Spectria 2005). Validation showed that serial dilutions of sled dog plasma with high testosterone concentration produced an antibody binding curve parallel to the standard curve. To survey inter- and intra-assay variability, BIO-RAD,

Lyphochek®Immunoassay Plus Control Level 1, 2 and 3 was used. The intra-assay CV (coefficient of variation) was 1.5 - 8.6% and the maximal inter-assay CV was 8.1% (n=12). This was considered as satisfactory. The inter-assay CV for control samples with high testosterone concentrations was 9.88% (n=11; mean 10.81 nm/L). To test for recoveries of testosterone, known amounts of testosterone were added to normal samples (n=4). Mean recovery was 95.7% ± 9.2 (SD), range was 83.9 – 107.8%. The detection limit, defined as twice the standard deviation of the 0-binding value, was approximately 0.03 ng/ml (0.1 nmol/l), and the optimal measurement range was 0.144 – 14.4 ng/ml (0.5-50 nmol/l). The repeatability of the assays was also tested by running samples in duplicates, and three low values had a CV > 20%, where one was just above and two under the optimal measurement range level; 0.425 ng/ml (52 % CV), 0.083 ng/ml (35% CV) and 0.050 ng/ml (56% CV).

2.4. *Analysis of organohalogens*
Analyses of organohalogens
Organohalogen contaminant (organochlorines and brominated flame retardants) analysis in dog adipose tissue samples were conducted at the National Wildlife Research Centre, Carleton University, Ottawa, Canada, according to methods described in detail elsewhere (Gebbink et al., 2008a, 2008b; Verreault et al., 2008). The following contaminants were analysed: PCBs; sum of 59 congeners (CB16/32, 17, 18, 22, 31/28, 33/20, 42, 44, 47/48, 52, 56/60, 64/41, 66, 70/76, 74, 85, 87, 92, 95, 97, 99, 101/90, 105, 110, 114, 118, 128, 130, 137, 138, 141, 146, 149, 151, 153, 156, 157, 158, 170/190, 171, 172, 174, 176, 177, 178, 179, 180, 183, 187, 189, 194, 195, 196/203, 199, 200, 202, 206, 207 and 208), OCs; 1,2,4,5-tetrachlorobenzene (TeClBz), pentachlorobenzene (PnClBz), hexachlorobenzene (HCB), α-hexachlorocyclohexane (HCH), β-HCH, γ-HCH, octachlorostyrene (OCS), the chlordanes and metabolites heptachlor epoxide, oxychlordane, *trans*-chlordane, *cis*-chlordane, *trans*-nonachlor, *cis*-nonachlor, *p,p'*-DDE, *p,p'*-DDD, *p,p'*-DDT, dieldrin, photomirex, mirex and tris(4-chlorophenyl)methane (TCPM). Also in adipose tissue, sum of 36 PBDE congeners (BDE17, 25, 28, 47, 49, 54, 66, 75, 77, 85, 99, 100, 116, 119, 138, 139, 140, 153, 154, 155, 171, 180, 181, 183, 184, 190, 191, 196, 197, 201, 202, 203, 206, 207, 208 and 209), as well as several other BFRs including 2,2,',4,4',5-pentabromobiphenyl (BB-101), pentabromotoluene (PBT), hexabromobenzene (HBB), total-(α)-hexabromocyclododecane (HBCD), bis(2,4,6-tribromophenoxy)ethane (BTBPE) and pentabromoethylbenzene (PBEB) were performed. Analyses of OHCs in diet is described in Verreault et al. (2008).

2.5. *X-ray osteodensitometry*
Bacula were thawed, prepared and dried before weight, length and BMD were assessed. Bacula BMD was determined by X-ray osteodensitometry in order to detect osteopenia (osteoporosis) using a Norland XR 26 X-ray bone densitometer (Norland Corporation, Wisconsin, USA). X-ray osteodensitometry determined bone mineral density (calcium-phosphate; hydroxyl-apatite) during a DXA scan. $BMD_{baculum}$ was measured in 5 individual sled dogs (3 controls, 2 exposed). All bacula were scanned in "*Small subject*" mode (Resolution: 1.0x1.0mm; Scan width: 10.0cm; Scan Speed: 40mm/sec) and analysed in XR software revision 2.4®, which generated a picture of the bone segment and calculated the bone mineral density of hydroxyl-apatite ($BMD_{baculum,}$ $g*cm^{-2}$). The DXA-scanner was daily calibrated using a phantom with known mineral density showing

reproducibility >99% and accuracy within ±2SD. Based on an 11-fold rescanning of BMD_{femur} CV was calculated to 1.3%.

2.6. *Statistical Analysis*

Statistical analyses were performed with the SAS statistical software package (SAS V9.1 and SAS Enterprise Guide V4.0, SAS Institute Inc., Cary, NC, USA). P values of <0.05 were considered significant and values of 0.1<p<0.05 were considered a trend. Testosterone concentrations at different ages (3-12 months) were evaluated using a Students t-test (paired). A Students t-test (one-tailed, unequal variance), with testes and baculum variables as dependent variable and group (exposed vs. control) as explanatory variable, was applied to test for group differences.

3. Results

3.1. *Exposure*

Adipose tissue concentrations of ∑OHCs (including all measured organohalogen contaminants) in the exposed group averaged 5123 ng/g lw [range: 4518-5729 ng/g lw] (see Table 1 for details on individual OHCs). There was a significantly lower level (P< 0.05) of the different OHCs in the control group. The ∑OHC content in whale blubber was 2818 ng/g lw, ∑PBDEs 63 ng/g lw, ∑DDTs 595 ng/g lw, HCH 20 ng/g lw, ∑CHLs 88.7 ng/g lw, HCB 28 ng/g lw, dieldrin 94 ng/g lw and Mirex 7.1 ng/g lw (details published in Sonne et al. 2008c).

The exposed males' mother was given the contaminated diet for 240 days (from age two months) until the time of weaning. During this time she was fed a total of ca. 20 kg organohalogen contaminated blubber, adding up to a total of approximately 120 mg ∑OHCs (7.5 µg/kg bodyweight/day, including PCBs and all other contaminants measured). During a period of 9 months (from weaning at 8 weeks until study termination), the 3 exposed male pups were fed approximately 64 mg ∑OHCs, of which ca. 39 mg were ∑PCBs. This is equivalent to 10.9 µg/kg bodyweight/day for ∑OHCs and 6.6 µg/kg bodyweight/day for ∑PCBs. ∑OHC data has been published elsewhere regarding dog tissue (excluding individual values in Table 1; Verreault et al., 2009a) and whale blubber (Sonne et al. 2008c).

	CONTROL				**EXPOSED**		
Mean OHC	*F1*	*F2*	*F3*	*mean*	*F2*	*F3*	*mean*
∑OHCs	65.1	66.2	53.9	61.7	4518	5729	5124
∑PCBs	56.9	42.7	40.2	46.6	2686	3303	2995
∑DDTs	0	0	0	0.0	300.1	808.6	554
∑CHLs	0	9.1	6.8	5.3	808.6	1070.6	940
∑HCH	0	0	0	0.0	44.5	46.8	46
∑CBz	2.4	2	1.2	1.9	66.1	66.2	66
∑Mirex	0	0	0	0.0	27.3	43.5	35
Dieldrin	0	0	0	0.0	425	566.3	496
TCPM	0	0	0	0.0	0	20.6	10
OCS	0	0	0	0.0	0	6	3
∑BDEs	5	12.3	2.9	6.7	150.4	145.8	148
∑BRFs	0.8	0	2.8	1.2	5.7	13.8	10

Table 1. Organohalogen contaminant (OHC) concentrations in fat tissue from Greenland sled dog (exposed to minke whale blubber or control pork fat). Sum of OHCs include all monitored OHCs, sum of Brominated Flame Retardents (∑BFRs) include brominated diphenyl ethers (BDEs).

3.2. *Testosterone*

Testosterone in all male pups increased significantly with age from 3 months until the end of the study at 12 months (early adulthood) (p=0.049, paired t-test). The concentrations of testosterone (ng/ml) in blood plasma at age 3, 5, 7, 9 and 12 months showed no significant difference between the two groups (1>p>0.22)(Table 2). No significant difference was found between exposed and control groups when combining all ages (p=0.72).

	Testosterone					**Genital organs**					
Group	*3 M*	*5 M*	*7 M*	*9 M*	*12 M*	*TWM g*	*TL cm*	*BaW g*	*BaL cm*	*BMD g/cm3*	*BW kg*
Exposed 1	0.38	0.08	0.42	u.d.l.	0.6	-	-	-	-	-	-
Exposed 2	0.04	u.d.l.	1.4	1.9	2.9	14.76	3.9	1.55	8.2	0.13	21.1
Exposed 3	u.d.l.	0.17	1.04	6.6	4.9	17.09	3.6	2.52	9.4	0.11	22
Mean	0.15	0.09	0.95	2.84	22.8	15.92	3.7	2.04	8.8	0.12	21.6
Control 1	0.09	0.27	0.32	1.1	11	20.01	4.1	2.36	8.7	0.14	24.1
Control 2	u.d.l.	u.d.l.	0.43	2.6	1.1	17.76	3.9	1.8	8	0.13	18.9
Control 3	0.18	0.05	0.73	2.6	4.3	19.76	3.9	2.41	9	0.12	21.9
Mean	0.1	0.11	0.49	2.1	5.47	19.18*	4	2.19	8.6	0.13	21.6

*u.d.l. = Under detection limit, * = (P= 0.015)*

Table 2. Testosterone (ng/ml), testes weight (g) and length (cm), baculum weight (g) and body weight (kg), and their mutual comparisons (T-test). M = months, TWM = testes weight (mean), TL = testes length, BaW = baculum weight, BaL = Baculum Length, BMD = body mineral density, BW = body weight.

3.3. *Testes and baculum*

Table 2 shows testosterone, testes weight and length, baculum weight and body weight and their mutual comparisons by T-tests (marked by *). M = months, TWM = testes weight mean, TL = testes length, BaW = baculum weight, BaL = Baculum Length, BMD = body mineral density (g/cm3), BW = body weight. At the termination of the experiment when the animals were 12 months old, testes weights were significantly lower in the exposed group (n=2, mean weight of 4 testes 15.9 g) compared to the control (n=3, mean weight of 6 testes 19.2 g) (Students t-test: p<0.015; Table 2). Testes length (left), baculum length and weight were also lower in the exposed group but not significantly (Table 2). Figure 1 shows X-ray absorptiometry (DXA) as bone mineral density (calcium-phosphate; 131 hydroxyapatite) of bacula from a control and an exposed male sled dog. No significant difference in BMD or bodyweight was observed between the control and the exposed dogs.

Figure 1. X-ray absorptiometry (DXA) showing bone mineral density (calcium-phosphate; 131 hydroxyapatite) of bacula from a control and an exposed male sled dog. The $BMD_{baculum}$ mean value of the Control dogs (n=3) was not significantly higher (1.6 fold) than in the Exposed dogs (n=2). H = high density (white coloured pixels) and L = low density (coloured pixels).

4. Discussion

4.1. *Study design and OHC exposure*

The design of the study allowed us to compare two groups of siblings with same age and genetics but with different OHC exposures. The exposed group was exposed to OHCs *in utero* (6 weeks), through lactation (6-8 weeks) and afterwards via the diet up to 12 months of age. The control group was exposed to significantly lower OHC levels (Table 1). All six males had the same father, and within the group the same mother. Furthermore the mothers of the dogs in the two groups were sisters. This ensured that the genetic variability of the pups in the two groups was relatively similar, and differences in response due to genetic variation between the dogs in the two groups could be assumed to be relatively small. Furthermore, animals in the two groups were born only 20 days apart, and this ensured that the two groups were exposed to similar environmental conditions throughout their neonatal and juvenile development. Thus, the design gave us the opportunity to compare two groups with relative identical ages for the terminal parameters. Verrault et al. (2008) discussed the potential of the sled dogs in this study as a surrogate species for polar bears, suggesting that OHC accumulation, metabolite formation and retention distinguish the two species to some extent. However, according to Verrault et al. 2008, both exposed sled dogs from this study and free-ranging East Greenland polar bears possibly exhibit a general lack of relationship between adipose tissue OHC concentrations and ages, suggesting that the important disparities in blubber intake and age between dogs and polar bears in this case had minimal impact on the OHC concentration variation (further discussion on this topic and important dietary-related effects, see Verrault et al., 2008, 2009a).

4.2. *Testosterone*

Testosterone in all male dogs increased significantly with age from 3 months until the end of the study when they were 12 months old ($p=0.05$, t-test). This is in accordance with the results from another study which investigated testosterone concentrations in young domestic dogs (Mialot et al., 1988). Overall, when considering the temporal trend in testosterone levels, there were no differences between the two groups. This is in accordance with studies showing 1) no reduction in serum testosterone concentrations in 3 months old *in utero* PCB exposed male guinea pigs, although a reduction in testes weights was found in the same animals (Lundkvist 1990) and 2) no reduction in serum testosterone concentrations in 3 months old *in utero* OC mixture exposed male rats (Anas et al., 2005). However, it should be noted that reduced testosterone concentrations, and to some extent also reduced testes weights, have been reported in OHC exposed adult rats, monkeys, guinea pigs and wild polar bears (Faqi et al., 1998, Hany et al., 1999, Kuriyama and Chahould 2004, Oskam et al., 2003). In this study we measured total concentrations of testosterone, and consequently there could be a potential difference in measured testosterone concentration due to a difference in amounts of testosterone binding protein in the individual dogs. Measuring the free fraction of testosterone would have given a more detailed description. Furthermore, Verrault et al., 2008a studied the formation rates of metabolic products when testosterone is hyrdoxylated by liver microsomes *in vitro*, and showed that formation of 6 - and 16 -OH-testosterone and androstenedione were on average 23 27% higher in the exposed sled dogs relative to the control dogs of the present study. This suggests that a greater metabolism of testosterone could take place in the exposed sled dogs compared

to the control, although this can be compensated for by complex hormone interactions, to maintain overall homeostasis of testosterone in the body.

4.3. *Testes*

Testes weights were significantly lower in the exposed group compared to the control. Although the observed difference between the groups was based on a limited number of individuals, it could indicate an effect caused by OHCs. Furthermore, the results are in accordance with previous studies: 1) Sonne et al. (2006a) observed a significant negative correlation between the length of adult polar bear male testes and HCB, a significant negative correlation between the weight of subadult testes and ΣCHLs and ΣPCBs, and a significant negative correlation between the weight of adult male testes and ΣPCBs and HCB. 2) *In utero* PCB exposed male rats experienced significantly reduced testes weights at adulthood compared to controls (Hany et al., 1999, Kuriyama and Chahoud 2004). 3) In a controlled study on Resus monkeys (*Macaca mulatta*), 6 months exposure to 200 µg/kg/day Arochlor 1242 caused significant reduced testes size (Ahmad et al., 2003) (compared to this study which had a daily ∑OHC intake of 10.4- 11.7 µg/kg/day). In addition, several other studies where rats and rabbits have been exposed in utero, have shown that contaminants cause other related effects on male reproductive morphological traits, such as reduced anogenital distance, hypospadias, cryptorchidism, and epididymal agenesis (Gray et al., 2001; Palmer et al., 2000; Wolf et al., 2000; You et al., 1998). Such effects, including reduced testes size, are symptoms of male reproduction dysfunction, and in human medical terms it is suggested to be part of the Testicular Dysfunction Syndrome (TDS), which is linked to pre- and post natal exposure to OHCs as well as genetics (Skakkebaek et al., 2001). Furthermore, other human studies link more TDS symptoms like testicular cancer, sperm cell morphology, motility and offspring sex ratio to OHC pre- and post natal exposure, suggesting that the large number of chemicals that humans are exposed to may collectively cause reproductive problems (Skakkebaek et al., 2001, Toft et al., 2004, Andersen et al. 2008). The lower testes weight of the OHC exposed sled dog pups as compared to the control pups in this study may, as previously suggested by Palmer et al. (2000), be caused by effects on androgen dependent events. Thus, the effects may be caused by chronic occupancy of androgen receptors by OHCs (pesticides), their metabolites, or by down regulation of the androgen receptor (Bonefeld-Jorgensen et al., 2001; Colciago et al., 2006; Schrader and Cooke 2003). Furthermore, it may be due to estrogenic/anti-androgenic endocrine interactions. Although not studied here, this can also result in a lower number of mature and fertile spermatozoa, reduce sperm quality and if exposure occurs during embryonic development, hence have a trans-generational (epigenetic) effect on male reproduction, causing potential hazards on a population level (van Duursen et al., 2003, Skinner and Anway 2006. Fielden et al., 2001, Gray et al., 2001, Pfleiger-Bruss et al., 1999, Rune et al., 1991a,b).

4.4. *Baculum*

In the present study, baculum weight, length and BMD did not differ between the exposed and control groups, even though baculum size was slightly higher in the controls than in the exposed dogs. Effects of OHCs on baculum size were evaluated in a similar study where domestic Arctic blue fox were exposed to a diet containing blubber from minke whale for 16 months (Sonne et al., 2009). In accordance with the present study, that particular study revealed no difference between control and exposed groups of foxes. In contrast, Bone Mineral Density

(BMD) of East Greenland male polar bear bacula was negatively associated with concentrations of chlordane, DDTs in subadults and HCB in adults (Sonne et al 2006a). Since negative associations between OHC burdens and baculum size have been shown in wild East Greenland polar bears, it is possible that the lack of a significant difference in the present study could be due to limited sample size or that the dogs were fed commercial feed which contained sufficient basic amounts of nutrients and microelements. It is therefore likely that the nutritional quality of the feed plays a role in the association reported between OHC and baculum size in wild polar bears, ascribed to factors such as differences in dietary composition of vitamins (D, E, A), n3/n6 fatty acids and micronutrients (minerals).

4.5. *Limitations*

The small number of male dogs available in this study makes any conclusions extrapolated to populations of wildlife or humans very uncertain. Nevertheless, a potential effect of OHC exposure of reduced testes weight is of very great importance and should be considered investigated in future chronic, low dose exposure studies of environmentally relevant complex mixtures.

5. Conclusion

We conclude that a peri- and postnatal exposure for 12 months of age to an average *of ca.* 100 g minke whale blubber per day containing an estimated total of 120 mg ∑OHCs (7.5 µg/kg bodyweight/day) in an environmentally relevant complex mixture does not affect testosterone values, but possibly affects testicular weights in male sledge dogs.

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PAPER IV

ALTERATIONS IN THYROID HORMONE STATUS IN GREENLAND SLEDGE DOGS (*CANIS FAMILIARIS*) EXPOSED TO WHALE BLUBBER CONTAMINATED WITH ORGANOHALOGEN COMPOUNDS

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Alterations in thyroid hormone status in Greenland sledge dogs (*Canis familiaris*) exposed to whale blubber contaminated with organohalogen compounds

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Abstract
As a model of high trophic level carnivors, sledge dogs (*Canis familiaris*) were fed for 12-18 months of age with minke whale (*Balaenoptera acutorostrata*) blubber that contained organohalogen compound (OHC) contamination corresponding to a daily dose of polychlorinated biphenyls of 128 µg. Controls were fed uncontaminated porcine (*Suis scrofa*) fat. Changes in thyroid hormone levels were assessed in 7 exposed and 7 control sister bitches and their 4 exposed and 4 control pups. Significantly lower free and total T3 and T4 were seen in exposed vs. control bitches toward the end of the observation period, and free T4 was lower throughout observations at 6-18 months. The totalT4/freeT4 ratio was significantly higher in exposed vs. controls. A negative correlation with thyroid gland weight was significant for the tissue concentration of ΣDDT, as was the positive association with total T3 for dieldrin. This controlled study therefore supports observational data that OHCs may adversely affect thyroid functions, and it suggests that OHC exposure time of 10 months or more is required for current OHC contamination levels to result in detectable adverse effects on thyroid hormone dynamics.

Key words: Canis familiaris, T3, T4, thyroxine, TSH, PCB, Organohalogen contaminants, sledge dog

Introduction

Environmental organohalogen contaminants (OHCs) may interfere with thyroid hormone (TH) action in mammals (Brucker-Davis et al., 1998; Boas et al., 2006; Braathen et al., 2004). Among prevalent OHCs, polychlorinated biphenyls (PCBs) and organochlorine pesticides (OCPs) such as dichlorodiphenyl trichloroethane (DDT) and its metabolites, and chlordanes are known to possess endocrine disrupting properties in exposed biota (Jenssen 2006). Some OHCs and their metabolites have a structural resemblance to the THs thyroxine (T4) and triiodothyronine (T3). Therefore, they can affect the biological action in mammals by disrupting the thyroid homeostasis at the receptor level or by affecting the metabolism of THs, e.g. by occupying the binding sites on the transport proteins, so that TH cannot bind (or is displaced), and THs may then be excreted at a higher rate (Gauger et al. 2007, van der Plas et al. 2001, Hallgren et al 2001, Hallgren & Darnerud 2002, Zhou et al 2001, Stoker et al 2004, Fowles et al 1994, Boas et al 2006). This property may also involve certain metabolites such as the hydroxy-PCB metabolites that may mimic the thyroid hormone T4, when the OH group is in one of the two para positions, with chlorine in the vicinal ortho position similar to that of iodine in T4 (Letcher et al. 2000). For environmentally relevant PCBs and polybrominated diphenyl ether (PBDE) flame retardants, and their hydroxy metabolites , there is a high competition for both T3 and T4 on the transthyretin (TTR) transport protein both in humans and in gulls (Ucán-Marín et al 2009).

Alterations in thyroid function may have significant behavioural, neurologic, neuropsychologic and thermoregulational consequences throughout the life cycle. Particularly during gestation TH disruption can permanently alter normal neurodevelopment (Sher et al. 1998, McNabb 1992, Boas et al. 2006). Fadden (1994) reported that in beagle dogs, a dietary exposure to PCB (25 ppm PCB, Aroclor 1248) reduced thyroid gland weights and caused structural changes such as lower number of mature follicles and hypertrophic perifollicular C cells. Furthermore, a significant decrease in thyroxine (T4) and an increase in THS levels after 12 weeks of dietary PCB exposure (25 ppm as Aroclor 1248) were reported. Thyroid gland disruption due to a chronic exposure with naturally biomagnified OHCs in the diet have also been observed in foxes fed OHC contaminated minke whale blubber, and they exhibited significantly larger prevalence of thyroid glandular histopathological lesions (Rogstad pers.comm.), Sonne et al. in press). In conjunction with other stressors, exposure to environmental contaminants during gestation, can expose the growing foetus to hazardous and fluctuating concentrations of TH disrupting OHCs. This period is a very sensitive stage with respect to such toxicity, and even subtle maternal hypothyroidism can adversely affect postnatal offspring brain development (Zoeller et al. 2002).

In order to mimic a long-term OHC mixture exposure situation, a study was conducted during a period of 21 months, where Greenland sledge dogs were given a diet containing minke whale blubber known to be contaminated with OHCs (exposed group) or a diet containing porcine (*Sus scrofa*) fat (control group). Previous results of this study has shown that animals in the OHC-exposed group experienced liver and kidney lesions and altered functions (Sonne et al. 2007a,b and 2008a,b), deficient immune response (Sonne et al. 2007a) and impaired cellular immunity (Sonne et al., 2006). In this report we document the effects of the OHC spiked diet on levels of free and total T3 and T4 in plasma of

Greenland sledge dogs. Thyrotropin (thyroid stimulating hormone, TSH), was also evaluated. As T4 in canine plasma binds to albumin, the binding protein concentration was also evaluated, as was thyroid gland weight and histology.

Materials and Methods

Experimental design

The maternal generation (P) was composed of 14 female sledge dogs from Aasiaat (Disco Bay, West Greenland), obtained at two months of age. Seven females were assigned to an exposed group and 7 to a control group. To minimize age and genetic differences between the two groups, the two groups were composed of paired sisters, one in each group. The P generation was mated with the same unexposed male to further harmonize genetic homogeneity. One and three of the exposed and control dogs, respectively, gave birth during the course of the study. The offspring generation (F) was composed of 4 exposed siblings (3 male, 1 female) and 4 control siblings (3 male, 1 female): they shared the same father and their mothers were sisters. After weaning at 6-8 weeks of age, they were fed the same diet as their mother. The exposed dogs were fed blubber from a West Greenland minke whale, rich in polyunsaturated lipids, vitamins and OHCs and low in mercury, obtained via native subsistence hunting. Control dogs were fed porcine fat (low in OHCs, mercury and polyunsaturated fatty acids). All dogs were fed 50–200 g/day of either blubber or porcine fat throughout the duration of the study period. The daily exposure to whale blubber (mean: 112g) constituted about 10.4-11.7 µg/kg body weight ∑OHC (corresponding to a PCB intake of 4.6-6.1 µg/kg body weight). All dogs were also fed equal amounts of standardized Royal Canine Energy 4300/4800 dry dog pellets (50-200 g/day) to cover basic nutrients and microelements (www.royalcanin.com). Feeding occurred simultaneously on a daily basis. The energy intake was balanced to achieve comparable body weights among siblings in the two groups. Further description and detailed discussion of dietary intakes of pollutants and nutrients has been publisehd elsewhere (Sonne et al. 2006, 2007, 2008b). The dogs were subjected to various immune treatments during the study (Sonne et al., 2006) and were euthanized upon completion of the study at adulthood (age for P: 12-18 months and F: 12 months). The animal experiment was performed on a licence granted by the Home Rule Government in Greenland and conducted in accordance with national and institutional guidelines for the protection of animal welfare.

Field sampling

All dogs were fasted for 12-24h before sampling. From the P generation blood was sampled at the end of study and also at different age intervals during the study, thereby allowing samplings to be compared at several specific ages. Twenty-two blood samplings from 6 pairs (6 exposed and 6 control) of the P generation were taken for thyroid hormone analysis at approximately 6 (n=10), 8.5 (n=4), 10 (n=4), 13 (n=10), 17 (n=8), 18 (n=4) and 18.5 months (n=4), within an interval of up to ±8 days of age at each occasion. As the sister pairs were born in different seasons, samples were not taken at the same season at comparable ages. A total number of 40 blood samples were obtained from the F generation (4 exposed and 4 controls). These were sampled at about 3 (n=8), 5 (n=8), 7 (n=8), 9 (n=8) and 12 months (n=8), within an interval of up to ±7 days at each occasion, at the same time of the year. Blood plasma from each dog was sampled in sterile 10 ml lithium heparin tubes and frozen immediately to -18 º C. Thyroid

glands were sampled and weighed after euthanasia; one was frozen immediately to -18 º C for OHC analysis and one kept in formaldehyde until sectioning for histology. In addition, adipose tissue for OHC analysis was sampled and frozen immediately to -18 º C. All frozen samples were stored at-18 º C until arrival at the laboratory for analysis.

Thyroid hormone analysis

P generation

Analysis of total (T) and free (F) T3 and T4 in heparin plasma samples was conducted at IDEXX Vet Med Lab (Ludwigsburg, Germany). All plasma samples were defrosted at room temperature and analysed in the laboratory in a single run. FT3, TT3, FT4 and TT4 were conducted using an automated electro-chemiluminescent immunoassay by *Modular E-170* (Roche). All assays were subjected to both daily internal and quarterly external quality control following RiliBäk and EU ISO 17025 standards. All results originated from accepted analytical runs. The canine reference intervals were used (Lammerer, 2003). Analyses of samples of the P-generation taken at the completion of the study were analyzed using the methods described below for the F generation.

F generation pups

Analysis of TT4 and TT3 from all F pups was conducted at the Norwegian University of Science and Technology, Institute of Biology, Norway, according to Verrault et al 2004. Radio-immunoassay (RIA) was used to determine concentrations of TT3 and TT4 with commercially available kits (Coat-A-Count; Diagnostic Product Corporation, LA, CA, USA). The amount of the bound radioactive antigen was quantified using a gamma counter (Cobra Auto-Gamma: Packard Instruments Company, Dowers Grove, IL, USA). Validation showed that serial dilutions of sledge dog plasma with high hormone concentration produced an antibody binding curve parallel to the standard curve. To survey inter- and intra-assay variability, BIO-RAD, Lyphochek®Immunoassay Plus Control Level 1, 2 and 3 was used. The intra-assay CV (coefficient of variation) was 0.3-4.1% for TT3 and 0.5-8.0% for TT4. The maximal inter-assay CV was 7.3% (n=8) for TT3 and 7.2% (n=6) for TT4. The inter-assay CV for control samples with high hormone concentrations was 5.1% (n=8; mean, 4.33 nm/L) for TT3 and 3.7% (n=9; mean 168.7 nm/L) for TT4. Mean recovery was 88.5% ± 11.6 (SD) (range 75.09 – 103.5%, n=4) for TT3 and 121.6% ± 22.2 (SD) (range 100.2 – 162.6.8%, n=4) for TT4. The detection limits (analytical sensitivity) for TT3 and TT4 were 0.11 nmol/L and 3.23 nmol/L, respectively. These quality control data were considered as satisfactory. In the F generation, specimens contained plasma volumes that were too small for FT4 and FT3 to be analyzed. Plasma samples were analysed in duplicate, and mean values were used in the statistical treatment of the results. Analytic results under the detection limit were set to half of the limit. TH concentrations with CVs>15% for duplicate analyses were omitted. Control samples composed of plasma from one individual dog and plasma from one individual cow were also run to test the variation of the analyses. Human serum controls in each kit (Immunoassay Plus Control, Level 1, 2 and 3, Bio-Rad Laboratories, Liquichek and Lyphchek, Hercules, CA, USA) were also assayed to test for repeatability. Results were in the acceptance range of the kits and met the laboratory's requirements for precision.

Endogenous canine thyrotropin (cTSH) and albumin

Analyses were conducted at the Central Laboratory, Department of Small Animal Clinical Sciences, Faculty of Life Sciences, University of Copenhagen, Denmark. The analyses were routinely employed at the laboratory and conducted using a chemiluminometer Immulite (DPC). The assay is subjected to both daily internal and quarterly external quality control and only results from accepted analytical runs were reported. The canine reference interval given is the one currently used at the Central Laboratory at Department of Small Animal Clinical Sciences based on samples from healthy dogs supplied to the laboratory.

Thyroid gland histology

A single and entire lobe of the thyroid gland was taken within 30 minutes after euthanasia from each individual and preserved in phosphate buffered formaldehyde. After gross examination in the laboratory, a mid-longitudinal representative subsection was excised from all thyroid glands and the tissue was trimmed, processed conventionally, embedded in paraffin and sectioned at about 4 μm. Haematoxylin (Al-Haematein)-Eosin (HE) staining was applied for routine diagnostics. Thyroid tissue was blindly evaluated in low and high power Leica microscope fields (5-40x magnification).

Analyses of organohalogen contaminants

Analysis of organohalogen contaminants (organochlorines and several brominated flame retardants) in dog adipose tissue samples was conducted at the Letcher Research Laboratory at the National Wildlife Research Centre, Carleton University, Ottawa, Canada, according to methods described in detail elsewhere (Gebbink et al., 2008a, 2008b; Verreault et al., 2008; Gauthier et al 2009). Also sumPCB in plasma, liver and kidney were made. The total amount of PCB (∑PCB) in plasma, liver and kidney was based on the sum of the following 59 congeners (CB16/32, 17, 18, 22, 31/28, 33/20, 42, 44, 47/48, 52, 56/60, 64/41, 66, 70/76, 74, 85, 87, 92, 95, 97, 99, 101/90, 105, 110, 114, 118, 128, 130, 137, 138, 141, 146, 149, 151, 153, 156, 157, 158, 170/190, 171, 172, 174, 176, 177, 178, 179, 180, 183, 187, 189, 194, 195, 196/203, 199, 200, 202, 206, 207 and 208). The analyses also included 1,2,4,5-tetrachlorobenzene (TeClBz), pentachlorobenzene (PnClBz), hexachlorobenzene (HCB), α-hexachlorocyclohexane (HCH), β-HCH, γ-HCH, octachlorostyrene (OCS), the chlordanes and metabolites heptachlor epoxide, oxychlordane, *trans*-chlordane, *cis*-chlordane, *trans*-nonachlor, *cis*-nonachlor, *p,p'*-DDE, *p,p'*-DDD, *p,p'*-DDT, dieldrin, photomirex, mirex and tris(4-chlorophenyl)methane (TCPM). Also in adipose tissue, sum of 36 PBDE congeners (BDE17, 25, 28, 47, 49, 54, 66, 75, 77, 85, 99, 100, 116, 119, 138, 139, 140, 153, 154, 155, 171, 180, 181, 183, 184, 190, 191, 196, 197, 201, 202, 203, 206, 207, 208 and 209), as well as several other BFRs including 2,2,',4,4',5-pentabromobiphenyl (BB-101), pentabromotoluene (PBT), hexabromobenzene (HBB), total-(α)-hexabromocyclododecane (HBCD), bis(2,4,6-tribromophenoxy)ethane (BTBPE) and pentabromoethylbenzene (PBEB) were monitored. Details on the OHC data for blood and fat has been published elsewhere (Verreault et al., 2008, 2009). Analyses used in this study (for correlations in the exposed group including both P and F generation) include all mentioned OHCs in adipose tissue, as well as ΣPCB in plasma, liver, kidney and thyroid gland tissue. Analyses of OHCs in diet are described in Verreault et al. (2008).

Vitamin and nutrient analyses in the diet

Analysis of vitamins, minerals and fatty acids is presented elsewhere (Sonne et al. 2008c, Kirkegaard et al., Submitted). All analyses for metal and vitamins were performed accredited according to ISO 17025 (ISO, 2000).

Statistical analyses

Statistical analysis was performed with the SAS statistical software package (SAS v9.1.3 Service Pack 4 for Windows and SAS Enterprise Guide v4.1, 2006, SAS Institute Inc., Cary, NC, USA). The level of significance was set to $p \leq 0.05$. A Student's paired t-test was performed to test for differences between thyroid hormones (FT3, FT4, TT3 and TT4) and their ratios in the exposed and control groups at different ages (between 3-18 months), between paired P sister or F sibling observations (siblings related with the same father and mothers as sisters). These analyses were performed within the P and F generation, respectively. In the above mentioned tests, no thyroid hormone statistical analyses mixing the two generations (P vs. F) were made in order to avoid inter-laboratory confounding and no assumptions were made between results from the two generations for these tests. Thyroid gland weights and Endogenous Canine Thyrotropin (cTSH) between exposed and control groups were evaluated using a Student's paired t-test. Linear regressions between THs, cTSH or thyroid gland weights, and age or bodyweight were performed prior to Pearson's product moment correlation coefficient, which was performed between OHCs and THs, cTSH or thyroid gland weights (the values analysed in the P and F generations for this test were performed in the same laboratory).

Results

Thyroid hormones

P generation bitches

At 8.5 months of age (i.e. after 6.5 months of exposure) the TH levels in the exposed group were comparable to the control group. However, when the dogs aged (10-18.5 months of age), the TH levels (FT3, FT4, TT3, TT4) in the exposed dogs were significantly lower (mean % decrease: FT3=17; FT4=20; TT3=17; TT4=16) than in the control dogs, when comparing paired sisters in each group (paired t-test, $p<0.005$)(Figure 1). At this age the % decrease showed no temporal trend across months of examination. Furthermore, all observed values for FT4 in the exposed group (n=6, 22 samples across 6-18 months) was significantly lower than matched samples in the control group ($p=0.023$, paired t-test) (Table 1). The TT4/FT4 ratio in the exposed maternal group was significantly higher ($p=0.013$, paired t-test) than the controls, thus suggesting that TT4 was elevated relatively to FT4 in the exposed dogs, as compared to the controls. There were no significant differences between exposed and control groups for the ratios TT3/FT3, TT4/TT3 and FT4/FT3 (Table 1). From age 6-21 months, Free and Total T3 significantly declined with age in the exposed P generation ($r= -0.65$ and -0.75, respectively, $p<0.001$). FT4 and TT4 showed a similar trend, but not significantly so. In the control P generation, correlations ranged from $r=0.15$ to $r=-0.27$ during the same months for all THs, and FT3 and TT3 showed a negative trend, but again not significantly so.

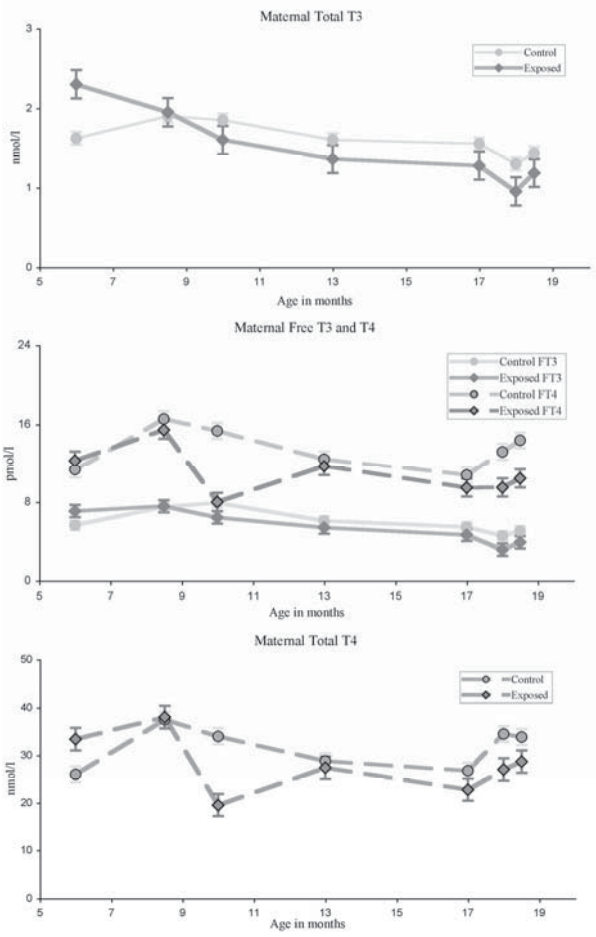

Figure 1. Mean ± SE of Free T3, Free T4, Total T3 and Total T4 in the exposed (n=6) and control (n=6) maternal P generation of Greenland sledge dogs (22 blood samples from exposed and 22 samples from control, taken at 6, 8.5, 10, 13, 17, 18, and 18.5 months). Overall concentrations of Free T4 were significantly ($p=0.034$) depressed in the exposed group.

	THYROID HORMONES AND RATIOS					
	Mean ± SE		Range		Median	
	Ex	*Con*	*Ex*	*Con*	*Ex*	*Con*
FT3	5.62 ± 0.41	5.95 ± 0.37	3.1 - 9.4	3.2 - 8.5	4.40	6.00
TT3	1.59 ± 0.12	1.61 ± 0.07	0.9 - 3.3	1.0 - 2.2	1.80	1.70
FT4	11.07 ± 0.64	*12.82 ± 0.59	7.0 - 15.8	8.5 - 17.4	10.6	13.5
TT4	27.90 ± 1.65	30.0 ± 1.27	16.0 - 41.0	19.0 - 41.0	28.0	30.5
TT4/FT4	* 2.55 ± 0.07	2.36 ± 0.05	2.12 - 3.52	2.04 - 3.02	2.51	2.35
TT3/FT3	0.29 ± 0.01	0.28 ± 0.01	0.21 - 0.49	0.22 - 0.38	0.29	0.29
TT4/TT3	19.03 ± 1.23	19.30 ± 1.04	10.4 - 28.9	12.4 - 30.0	18.1	18.6
FT4/FT3	2.15 ± 0.14	2.27 ± 0.12	0.99 - 3.12	1.60 - 3.70	2.16	2.26

**=significantly higher (p<0.05).*

Table 1. Concentrations of Free (pmol/L) and Total (nmol/L) T3 and T4 and ratios of Total (T) T4: Free (F) T4, TT3/FT3, TT4/TT3, FT4/FT3 (mole ratios) for sledge dogs (*Canis familiaris*) in the P generation (22 samples exposed n= 6 and 22 samples control, n=6 taken at 6, 8.5, 10, 13, 17, 18, and 18.5 months).

F generation pups

For TT3, the exposed group (n=4, 19 blood samples) had overall significantly lower concentrations than the control group (n=4, 19 blood samples) ($p<0.0001$, paired t-test) (Figure 2). For TT4, the exposed group and the control group did not differ significantly over the entire period. The ratios TT4/TT3 were significantly higher in the exposed group ($p=0.001$, paired t-test), thus TT4 was elevated relatively to TT3 in the exposed group (Table 2). TT4 significantly declined with age (3-12 months) in both exposed and control pups ($r=-0.44$ and $r=-0.58$, $p<0.006$). TT3 showed a negative trend, but not significantly so.

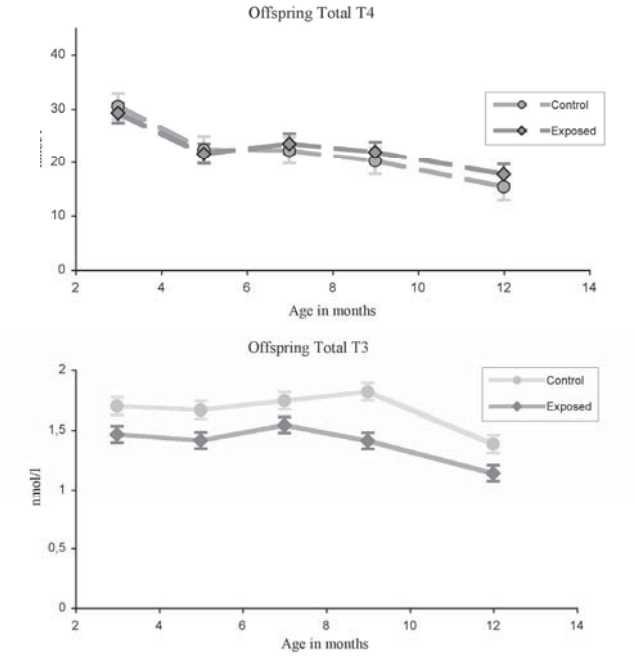

Figure 2. Mean ± SE of total T3 and total T4 in the exposed (n=4) and control group (n=4) of Greenland Sledge dogs (F generation) taken at 3, 5, 7, 9 and 12 months of age (± up to 7 days in each group). Total T3 concentrations were significantly ($p<0.001$) depressed in the exposed group.

THYROID HORMONES AND RATIOS						
	Mean ± SE		Range		Median	
	Ex	*Con*	*Ex*	*Con*	*Ex*	*Con*
TT3	1.44 ± 0.04	** 1.63 ± 0.05	0.67 - 2.00	1.00 - 2.02	1.48	1.70
TT4	23.00 ± 1.16	22.34 ± 1.28	9.67 - 38.68	9.14 - 43.65	23.4	21.7
TT4/TT3	**15.89 ± 0.57	13.40 ± 0.62	7.90 - 22.77	8.28 - 22.36	15.7	13.4

**=p<0.01, **=p<0.001*

Table 2. Concentrations of total (nmol/L) T3 and T4 and ratios of Total (T) T4:Total (T) T3 (mole ratios) for sledge dogs (*Canis familiaris*) in the F (offspring) generation (19 samples exposed, n=4 and 19 samples control, n=4, taken at 3, 5, 7, 9, and 12 months).

Endogenous Canine Thyrotropin (cTSH) and thyroid gland weight and histology

Endogenous Canine Thyrotropin (cTSH levels) in the P generation at the end of study did not differ between the exposed group (mean 0.12 ng/mL, range 0.04-0.21, n=6) and the control group (mean 0.11 ng/mL, range 0.06-0.15, n=7). cTSH levels in the F generation at the end of the study in the exposed (0.12 ng/mL, range 0.08-0.14, n=4) and control (0.09 ng/mL, range 0.05-0.12, n=5) groups were not significantly different. There were also no difference between exposed and control groups across generational pooled samples (paired t-test). There was no significant difference in thyroid gland weight between groups of dogs (Table 3) and no macroscopic or histopathological changes were found in any individuals.

THYROID GLANDS AND cTSH						
	Mean ± SD				Range	
TH gland (g)	*n*	*EXP*	*n*	*CON*	*EXP*	*CON*
P right	7	0.887 ±0.14	7	0.947 ±0.46	0.65-1.03	0.49-1.91
P left	7	0.817 ±0.08	6	0.707 ±0.10	0.71-0.93	0.58-0.83
F right	3	0.723 ±0.21	3	0.777 ±0.10	0.51-0.93	0.67-0.87
F left	3	0.633 ±0.07	3	0.850 ±0.18	0.56-0.70	0.64-0.98
cTSH (ng/mL)						
P	7	0.121 ±0.07	7	0.109 ±0.04	0.04-0.21	0.06-0.15
F	3	0.120 ±0.03	3	0.077 ±0.10	0.08-0.14	0.05-0.11

Tabel 3. Thyroid hormone (TH) gland weights and canine Thyroid Stimulating Hormone (cTSH) in the maternal generation (P) and their offspring (F). No significant differences were observed between groups.

OHCs, vitamins and nutrient in the diet

The OHC profile (ΣOHC =2812 ng/g lipid weight) of the whale blubber given to the exposed group revealed the presence of the following contaminants in decreasing order of concentrations; ΣPBC =1716 ng/g lw, ΣCHL =296 ng/g lw, ΣDDT =595 ng/g lw, dieldrin=94 ng/g lw, ΣPBDE =63 ng/g lw, HCB =28 ng/g lw, ΣHCH =20 ng/g lw. The exposed groups mean daily intake of minke whale blubber was 112g, containing 128 µg ΣPCBs (for further details, see Verreault et al. 2008). The two groups were fed equal amounts of normal dog Royal Canin (RC) pellets (www.royalcanin.com) to cover basic nutrient needs. Vitamin A (retinol) was 18% higher, vitamin E (α-tocopherol) 22% higher and vitD_3 33% in the diet of the exposed dogs compared to controls (mean of a period of 4 weeks prior to sampling). Concentrations of vitamins, nutrients and lipids in all diet items have been described elsewhere (Sonne et al., 2008c, Kirkegaard et al., Submitted).

Organohalogen contaminants in dog tissue

Concentrations of organohalogens in adipose tissue and blood plasma are described more thoroughly elsewhere (Verrault et al. 2008, 2009; Sonne et al. 2008c). Significant differences were found between the exposed and control groups for most contaminants evaluated. For all animals (both P and F) the mean ΣOHC concentration in adipose tissue was 87 ng/g lw (26-241, n=11) for the control group and 5005 ng/g lw (3319-8565, n=10) for the exposed group, with a higher men in the exposed P generation of 5430 ng/g lw (range: 3319-8565, n=7) than in the F generation (mean, 4544 ng/g lw; range, 3385-5729, n=3). The mean ΣPCB concentration in adipose tissue for all dogs in the control group (n=11) was 50 ng/g lw (range 16 -153) and for all exposed dogs 2995 ng/g lw (range 936-6740, n=10). The mean ΣPCB concentration in the thyroid gland of the exposed group was 5129 ng/g lw for the P generation (n=5) and4161 ng/g lw (n=3) for the F generation, whereas the overall mean ΣPCB concentration in controls (P+F) was 331 ng/g lw (n=9).

Relationships between OHCs and response variables

Correlations between OHCs and THs, thyroid gland weights and canine TSH in all exposed sledge dogs were performed (P generation: n=7 + F generation: n=3), and age/bodyweight were accounted for before analysis. Significant negative correlations were observed between thyroid gland weights (left + right) and ΣDDT concentrations (r=-0.653, p=0.002), and a significant positive correlation between TT3 and dieldrin (r=0.867, p=0.001) for all exposed dogs (P+F), see Figure 3 and 4. Other correlations with OHCs were in the same direction, but did not achieve statistical significance.

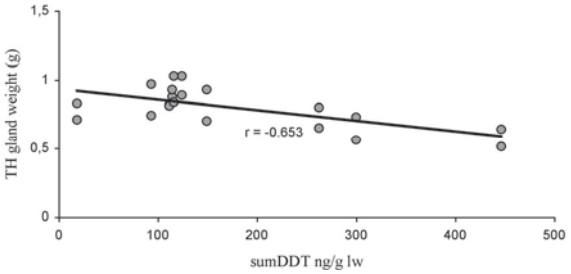

Figure 3. Thyroid gland weights (left and right) and ∑DDT (ng/g lw) in the exposed sledge dogs (P +F generation: n=10, r= -0.653, p=0.002).

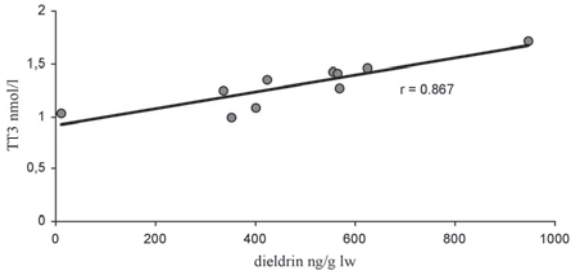

Figure 4. Total T3 (nmol/l) and dieldrin (ng/g lw) in the exposed sledge dogs (P +F generation: n=10, r= 0.867, p=0.001, Pearson's correlation)

Discussion

The nearly two-year feeding regime conducted with P generation sledge dogs bitches from juvenile age to sexual maturity led to significantly depressed FT4 concentrations in the P generation exposed group (n=6) of dogs (during 6 to 18 months of age) across all age groups. Likewise, the FT4:TT4 ratio was significantly highest in the controls. Furthermore, FT3, FT4, TT3 and TT4 in P generation exposed dogs were decreased across age groups from 10 to 18.5 months compared to controls. Also, TT3 from 3 to 12 months was reduced in the exposed F generation dogs. In addition, thyroid gland weights were negatively correlated with ΣDDT in adipose tissue at the time of autopsy (r=-0.653, p=0.002) and TT3 concentrations were positively correlated with dieldrin also measured at the end of the observation period (r=0.867, p=0.001) in all exposed sledge dogs (n=10, P+F). The decrease in T4 shown in the exposed

dogs is in accordance with previous reports that PCB exposure in beagle dogs caused a significant decrease in T4 after 12 weeks of dietary PCB exposure (25 ppm Aroclor 1248) (Fadden 1994).

T4 in canine plasma binds to certain proteins besides the major TH transport proteins in dogs (Thyroxine-binding prealbumin, albumin and thyroxine-binding globulin), but the affinity for these is much lower than in other species (Daminet & Furguson 2003). Canines also have a lower TT4 concentration and a larger FT4 fraction. Therefore, the rate of turnover and loss of T4 in urine and faeces can be much higher in dogs than in other species (Schuurman et al., 1991; Daminet & Furguson 2003). The mechanism or pathways leading to decreased THs in the OHC exposed group is unclear. One explanation could be that the rate of excretion of THs in the exposed dogs may be enhanced because dogs are better at metabolising PCBs to hydroxylated metabolites (OH-PCBs) than other species. These PCB metabolites appear to displace T4 from transport proteins in other species, specifically on human and gull TTR (Ucan-marin et al 2009), but apparently not in the dog. When THs are displaced from their transport proteins, excretion rates of the free hormone increase, thus potentially leading to reduced THs levels (Graves et al. 1990, Brouwer et al. 1986, Daminet & Furguson 2003, Lans et al. 1994).

Glucuronidation is known to be a rate limiting step in the biliary (faecal) excretion of T4 (Graves et al. 1990). However, Verreault et al. 2009 showed that liver uridine diphosphate glucuronyltransferase (UDPGT) activity in the exposed sledge dogs from this study was not different from the controls, indicating that the OHC complex mixture in this case did not up-regulate this particular enzyme, so that increased excretion of T4 in the faeces by this pathway would seem unlikely. A reason for this observation could be that sledge dogs or greyhounds are less efficient metabolizers of PCBs compared to beagle and mongrel dogs, due to different genetic polymorphisms (Verreault et al. 2009, Hay-Kraus et al. 2000).

The ranges of TT4 and FT4 in the dogs in the present study agree with general reference values for sledge dogs (Lee et al. 2004). The reason for the higher plasma TH concentrations at 6 months, similar at 8.5 months and lower at 10 months in the exposed P group vs. control is uncertain at the moment. A possible explanation could be that OHC exposure has an effect on TH metabolism that changes over time, so that short-term studies with 2-6 months of OHC exposure cause elevated TH concentrations, whereas chronic exposure above 10-12 months leads to depressed TH concentrations. The mean % decrease in THs across 3-12 months of age in the exposed F generation ranged from 13 to 29% for TT3. The difference in the offspring generations with depressed TT3 from 3 to 12 months, but no difference in TT4, which is at variance with observations in their parent generations, is unknown, but could possibly be ascribed to alterations in TH metabolism due to *in utero* exposure.

The mean % decrease in THs across 10-18 months of age in exposed vs. control bitches ranged from 16 to 20% (increasing order: TT3, TT4, FT3, FT4). The extent of potential physiological effects associated with the observed decreases of THs in the exposed dogs (and possibly wildlife with similar exposure) are unclear, as is also the observed decrease in THs. In humans, even mild TH insufficiency (pre- and early postnatally) can produce measurable deficits in very

specific neuropsychological functions such as visual attention, visual processing, subnormal contrast sensitivity, visuospatial skills, gross and fine motor skills, slower response speeds and language and memory skills (Zoeller and Rovet 2004). These developmental effects can potentially impact wild animals in regard to predation or escape success, and ultimately population fitness. Nevertheless, because TH metabolism is very species specific, extrapolating these results to other species should be done with caution.

It has been observed that body size and age has an effect on TT4 in dogs, where TT4 is the highest in nursing pups and lowest in older individuals, but the general effect of age and body size on serum FT4 and TSH concentrations in dogs is unknown (Paradis et al. 2003). Fore some drugs, specific differences in regard to sex (Nishibe et al. 1998) and age (Tanaka et al. 1998) have been observed for hepatic microsomal protein induction, expression, and activity of CYP isoenzymes. The depression of TT3 and increase in the TT4/TT3 ratio in the F exposed group (in the absence of results for Free T3 and T4) could be a result of Type 1 deiodinase (D1) inhibition in the exposed group, because D1 converts T4 to T3 in liver tissue (Zoeller and Crofton 2005).

Other evidence on TH disruption from OHCs has been reported in wildlife: 1) polar bears show negative correlations between PCBs and FT4, TT4, FT3, and the TT4/TT3 ratio, and positive correlations with the TT3/FT3 ratio (Braathen et al. 2004). 2) Seals (*Halichoerus grypus*) from the Baltic Sea show that blubber concentrations of PCBs and DDTs correlate negatively with plasma TT3 and FT3 (Sørmo et al. 2005). 3) Seals (*Phoca largha* and *Phoca fasciata*) from Hokkaido show that PCB, DDT and chlordanes correlate negatively with plasma total T3 and free T3 concentrations (Shiba et al. 2001). 4) Sea lions (*Zalophus californianus*) show concentrations of ΣPCBs negatively correlated with T3 (Debier et al. 2005). For Arctic wildlife, relationships between OHC levels and THs and other hormones have recently been summarized in Letcher et al (2009). These studies reveal somewhat different associations between OHCs and TH concentrations, and it is of course possible that these associations could be spurious. However, it is also possible that the environmental mixtures to which different populations are exposed, as well as species specific differences, can produce slightly different effects (Zoeller et al. 2002). In humans, PCBs can reduce the carrying capacity of the blood for T4, reduce the serum half-life of T4, and also reduce the ability of TSH to increase serum T4 *in vivo*. Thus, PCBs may directly interfere with the ability of the thyroid gland to respond to TSH (Zoeller et al. 2002). A lower number of mature follicles and hypertrophic perifollicular C cells in the thyroid gland were observed in Arctic foxes exposed to minke whale blubber (Sonne et al. in press). Likewise, it has been reported that in beagle dogs, PCB exposure caused similar structural changes and also an increase in thyroid hormone levels were seen after 12 weeks of dietary exposure (25 ppm Aroclor 1248) (Fadden 1994). In that particular study no differences were seen on thyroid gland weights. In the present study, no difference were found in TSH levels, thyroid weights or histology, although thyroid gland weights of all exposed dogs were negatively correlated with ΣDDT in adipose tissue. This may indicate that the levels of OHCs were not potent enough to cause a direct and significant response between groups in thyroid gland weights, histology or TH levels, but sufficient enough to decrease THs following a long-term dietary exposure to OHCs (across many months) for both generations of dogs. However, in interpreting these data, the timing of exposure may also need

consideration. While contaminant concentrations were assessed at the termination of the study, interference with thyroid development may have been caused by (unmeasured) concentrations present at some previous time during the study. The fact that some contaminants showed better correlations than others may therefore be a reflection of the degree to which the tissue accumulation levels reflect the causative concentrations.

In conclusion, we observed a decrease in all THs measured (Free and Total T3 and T4) in the adult exposed dogs (P) from 10 to 18 months of age compared to controls. Also, a decrease in thyroid gland weight with increasing ΣDDT and an increase in total T3 with increasing dieldrin in all exposed dogs were observed. Furthermore, significantly depressed free T4 concentrations (P generation) and total T3 concentrations (F generation) during all observed months in the exposed compared to the control group of dogs were observed. We suggest that an OHC exposure time of minimum 10 to 18 months is needed in low dose OHC exposure studies on larger mammals, if true chronic effects on thyroid hormone dynamics are to be detected. This period of life is also likely to be critical for the health of exposed animals.

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APPENDIX

OTHER RESEARCH PROJECTS ON THE SLEDGE DOG STUDY

Until now, ten scientific articles have been published on data from the sledge dog study (paper no. 1-6 are co-authored by M. Kirkegaard). The following is a short description of each of the ten studies. No thorough literature inclusion has been conducted as these effect studies do not directly relate to the vitamin and hormone studies which the present Ph.D. has been built on.

1) Sonne C, Dietz R, Larsen HJS, Loft KE, Kirkegaard M, Letcher RJ, Shahmiri S, Møller P. Impairment of cellular immunity in West Greenland sledge dogs (*Canis familiaris*) dietary exposed to polluted minke whale (*Balaenoptera acutorostrata*) blubber. Environmental Science and Technology 2006, 40: 2056–2062

To determine if immunotoxicity from OHCs in Svalbard and East Greenland polar bears (*Ursus maritimus*) is a true cause-effect relationship, we conducted a generational controlled study on domestic West Greenland sledge dogs. To characterize the effect of OHC exposure on the cell-mediated immunity, intra-dermal *in vivo* stimulation (delayed hypersensitivity; type IV) with mitogens (PHA and Con A; unspecific induced lymphocyte proliferation) was applied to 16 bitches (8 exposed and 8 control), and the same mitogens and an additional antigen (KLH; specific Tlymphocyte response following immunization) was applied to their 9 pups (4 exposed and 5 control), see Figure 1. Four weeks prior to the intradermal test, all pups were immunized (age span 14-17 weeks) subcutaneously with 1 mL of Keyhole Limpet Hemocyanin. NaCl was used as a negative control. Wheal size (diameter, height, and erythema) was measured at 24 and 48 h delayed type hypersensitivity. When pooling all exposed (n=12) and control (n =13) generations in the statistical analyses, exposed individuals responded more weakly than control individuals for *all* PHA and Con A intra-dermal reactions measured. An impairment of this part of the immune system by polluted minke whale blubber may hence lead to decreased resistance to infections caused by microbial pathogens. Earlier investigations have stated that polyunsaturated fatty acids (n-3) have an unknown immuno modulating and suppressive effect, which is used prior to organ transplantations for minimizing the risk of tissue rejection. We could therefore not distinguish among the relative immunosuppressive effects from the high concentrations of OHCs and polyunsaturated fatty acids (n-3) when evaluating whale blubber vs porcine fat immunopathy.

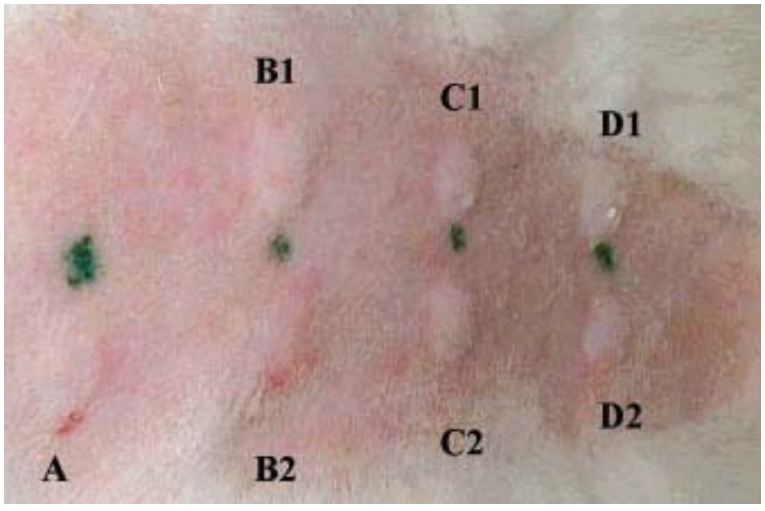

Figure 1. Order of intradermal application of control, PHA, Con A, and KLH in a 21-week-old puppy from the control group at time) 0 using 1-mL, 27G, U-100 Insulin Myjectors (Terumo, Europe N. V., Leuven, Belgium). A, Negative control (0.9% NaCl); B1, PHA, 50 ppm; B2, PHA, 200 ppm; C1, Con A, 50 ppm; C2, Con A, 250 ppm; D1) KLH, 20 ppm; D2) KLH, 200 ppm. Green marks are for the orientation.

2) Sonne C, Fonfara S, Dietz R, Kirkegaard M, Letcher RJ, Shahmiri S, Andersen S, Møller P. Multiple cytokine and acute phase protein gene transcription in West Greenland sledge dogs (*Canis familiaris*) dietary exposed to organic environmental pollutants. Archives of Environmental Contamination and Toxicology 2007, 53:110–118

A study on liver tissue and ethylenediaminetetraacetic acid (EDTA) blood cytokine and acute-phase protein (APP) mRNA expressions using reverse transcriptionase–polymerase chain reaction was conducted in sledge dogs with contaminated minke whale blubber as dietary pollutant source. Immune system cells, such as macrophages, monocytes and also endothelial cells, produces pro- and anti-inflammatory cytokines and are responsible for the initiation and progression of immune responses. Cytokine expressions are used to assess the status and progression of the immune response, whereas pro-inflammatory cytokines initiate the immune response. Seven mRNA cytokine and five APP expressions were analysed in EDTA blood and liver tissue samples by real-time RT-PCR. Two of seven blood cytokine expressions (IL-6 and IL-12) and three of five APP expressions (haptoglobin [HP], heat shock protein, and fatty acid–binding protein [FABP]) were lowest in the exposed group, whereas the remaining five blood cytokine expressions (IL-2, IL-10, IFN-c, TNF-a, and TGF-b) and two APP expressions (MT1 and MT2) were highest in the exposed group. In liver tissue, three cytokine expressions (IL-10, IFN-c, and TNF-a) and two APP expressions (MT1 and MT2) were highest in the exposed group, and the remaining cytokine and APP expressions were lowest in the exposed group. Of these, the liver tissue expression of haptoglobin and fatty acid–binding was significantly lowest in the exposed group (both $p<0.05$, Fig. 2). The OHC exposure was hence associated with a genotoxic decrease in HP and FABP gene expression in the liver of sledge dogs, indicating a restricted acute-phase reaction and insufficient immune response.

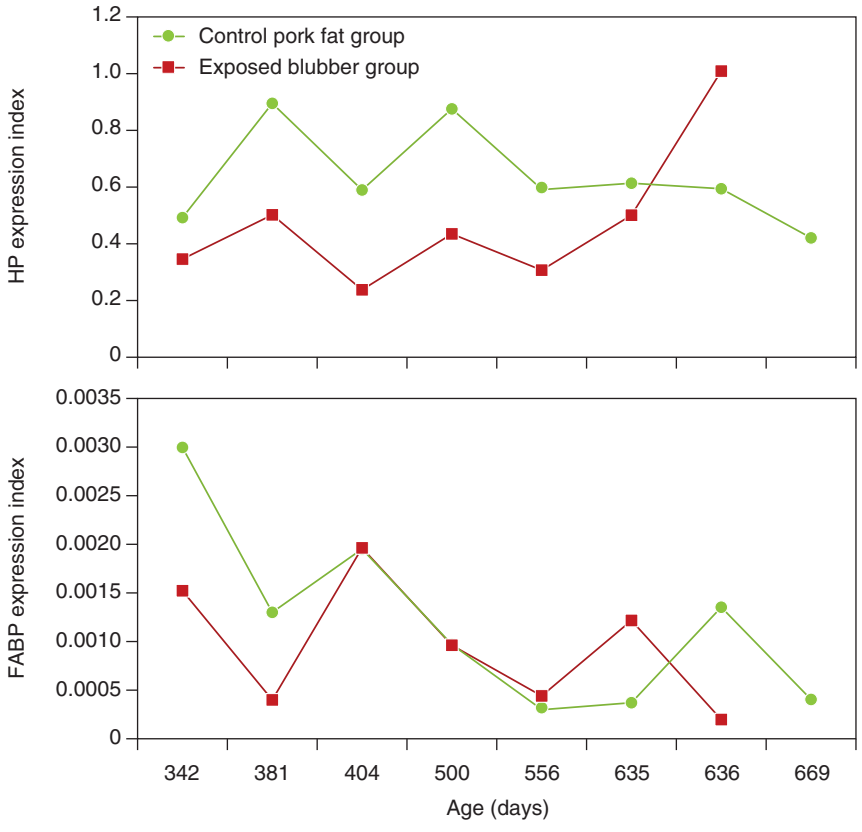

Figure 2. Liver haptoglobin (top panel) and fatty acid-binding protein expression index (bottom panel) vs. age (in days).

3) Sonne C, Leifsson PS, Dietz R, Kirkegaard M, Møller P, Jensen AL, Letcher RJ, Shahmiri S. Renal lesions in Greenland sledge dogs (*Canis familiaris*) exposed to a natural dietary cocktail of persistent organic pollutants. Toxicology and Environmental Chemistry 2007, 89:563–76

Diffuse thickening of the glomerular capillary wall is described in domestic animals, wildlife and humans due to immune deposits on the epithelial side of the glomerular capillary basement membrane, and this type of lesion was associated with drug abuse and exposure to toxic substances, such as OHCs and heavy metals, as well as chronic recurrent infections from micro organisms (bacteria, virus, and parasites). In this study, the impact from dietary intake of minke whale blubber high in organohalogen and other chemical contaminants on renal morphology and function was investigated. Our results showed significantly higher frequencies of glomerular, tubular, and interstitial lesions in the exposed group (Figure 3). Furthermore, higher urine protein: creatinine ratio and plasma urea levels were found in the exposed group, which indicated a negative impact on kidney function via tubular and glomerular dysfunctions. The histopathological changes found in glomeruli, tubules, and interstitium were similar to those reported in Baltic grey seal and ringed seal sampled between 1977–1996, and shown to be contaminated with OHCs (PCBs, DDT) and heavy metals. Similar lesions were also reported in East Greenland polar bears sampled during 1999–2002 having a comparable exposure to OHCs and heavy metals as the sledge dogs in the present study.

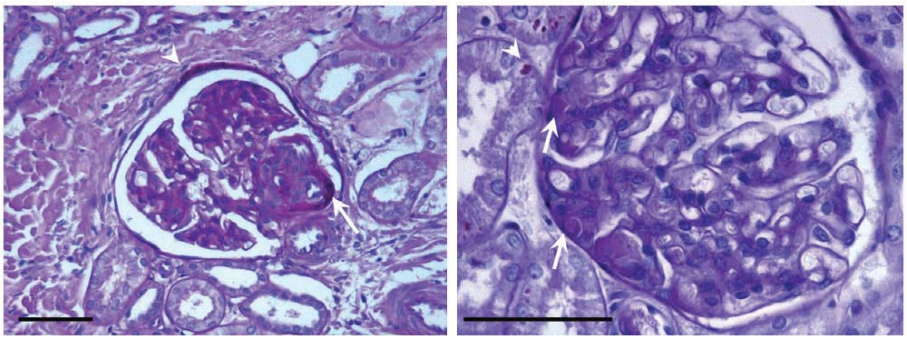

Figure 3. Left: Diffuse thickening of glomerular capillary wall and Bowman's basement membranes as well as mesangial deposits in the kidney of a 336 day old Greenland sledge dog exposed to minke whale blubber as OHC source for 336 days. Note the PAS positive deposits in the glomerular basement membrane (arrow) and the segmental thickening of Bowman's basement membrane (arrow head). Periodic acid–Schiff staining, magnification x20, bar=50 mm. Right: Glomerular arteriosclerosis (arrow) and pigments (arrow head). Periodic acid–Schiff staining, magnification x40, bar=50 mm.

4) Sonne C, Leifsson PS, Dietz R, Kirkegaard M, Møller P, Jensen AL, Shahmiri S, Letcher RJ. Greenland sledge dogs (*Canis familiaris*) exhibit liver lesions when exposed to low-dose dietary organohalogen contaminated minke whale (*Balaenoptera acutorostrata*) blubber. Environmental Research 2008, 106: 72–80

We assessed the relationship between exposure to organohalogen polluted minke whale blubber and liver morphology and function in a generational controlled study of 28 Greenland sledge dogs. In general, the prevalence of liver lesions was highest in the exposed group. Specifically, the prevalence of portal fibrosis was 15% in the exposed group and 0% in the control, which was a significant difference. Furthermore, the prevalence of mild bile duct hyperplasia (Figure 4), and vascular leukocyte infiltrations was significantly higher in the exposed group (all $p<0.05$). In case of granulomas, the frequency was significantly highest in the P generation (bitches), while the prevalence of portal fibrosis was highest in the F generation (pups) ($p<0.05$). No significant difference between exposed and controls were found for bile acid, ALAT, and ALKP, while ASAT and LDH were significantly highest in the control group ($p<0.05$).

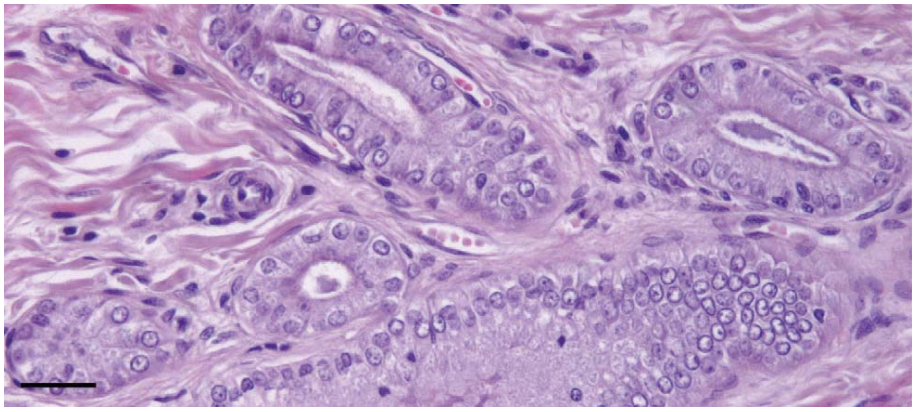

Figure 4. Mild bile duct proliferation accompanied by portal fibrosis in a 336-day-old Greenland sledge dog exposed to minke whale blubber as OHC source for 336 days. HE staining, magnification 10x, bar = 50 mm.

5) Sonne, C., Dietz, R., Kirkegaard, M., Letcher, R.J., Shahmiri, S., Andersen, S., Olsen AK, Jensen AL. Effects of organohalogen pollutants on haematological and urine clinical–chemical parameters in Greenland sledge dogs (*Canis familiaris*). Ecotoxicology and Environmental Safety 2008, 69; 381–390

In this study blood plasma and urine clinical–chemical parameters were measured and compared between the 14 bitches and 8 pups form the control and exposed cohorts. Based on existing reference intervals, Arctic mammals may have blood clinical–chemical endpoint levels that differ from comparable species at lower latitudes. The cortisol:creatinine ratio (Figure 5), protein:creatinine ratio, alkaline phosphatase, cholesterol and inorganic phosphate were significantly highest (all p<0:05) in the pup generation. The cortisol:creatinine ratio, cholesterol, lactate dehydrogenase and creatinine kinase were significantly higher (all p<0:05) in the control group, while glucose was significantly highest (p<0:05) in the exposed group. Furthermore, the blood cholesterol levels indicate that exposure via the diet to marine mammal blubber has a preventive effect on the development of cardiovascular diseases.

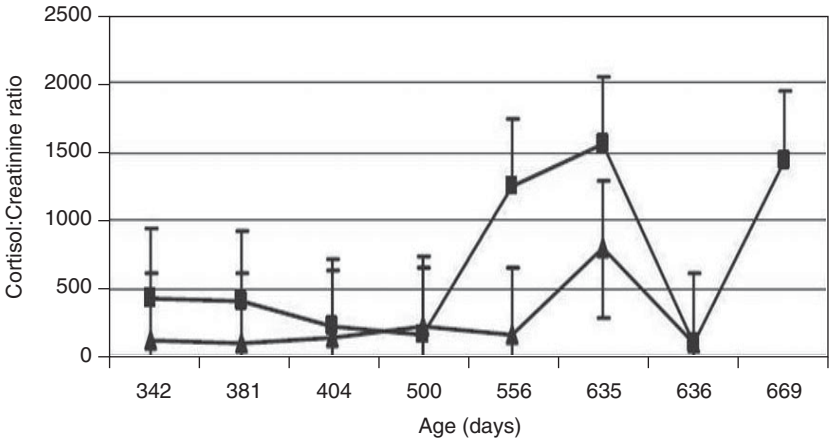

Figure 5. Cortisol:creatinine ratio vs. age (days) in blood plasma and urine from exposed and control Greenland sledge dog bitches and pups (pups represents the individuals of age 342 days). ▲, individuals feed OHC contaminated minke whale blubber (exposed group) and ■, individuals feed pork fat (control group) for up to 669 days. Error bar of ±1 SD included.

6) Sonne S, Rigét FF, Jensen J-EB, Hyldstrup L, Teilmann J, Dietz R, Kirkegaard M, Andersen S, Letcher RJ, Jakobsen J. Does the nutrition profile of vitamins, fatty acids and microelements counteract the negative impact from organohalogen pollutants on bone mineral density in Greenland sledge dogs (*Canis familiaris*)? Environment International 2008, 34: 811–820

There is a great need for understanding the impact from dietary OHCs on bone mineral composition – and thereby osteoporosis – in especially arctic wildlife such as polar bears as well as humans. For that purpose, we measured BMD (bone mineral density, Figure 6) by DXA scanning (g/cm^{-2}) in 15 age and weight normalized sledge dog bitches and their 26 pups divided into a control group (n=26) given 50–200 g/day clean pork fat and a treated group (n=15) given OHC polluted minke whale blubber as main lipid sources. The results showed that BMD increased significantly with age (linear regression: $p<0.0001$, $r^2=0.83$, n=41) while no sex difference was found in the F-generation (all $p>0.3$). No differences in BMD_{femur} or $BMD_{vertebrae}$ between exposed and control individuals in the bitch generation were found (both $p>0.38$). Likewise, no difference between exposed and control subadults and juveniles in the F-generation were found (all $p>0.33$). Correlation analyses between BMD_{femur}, $BMD_{vertebrae}$ and groups of OHCs, respectively, did not show any statistically significant relationships nor a clear or decreasing trend (p= 0.07–0.78; r: –0.2-0.59; n: 10–18).

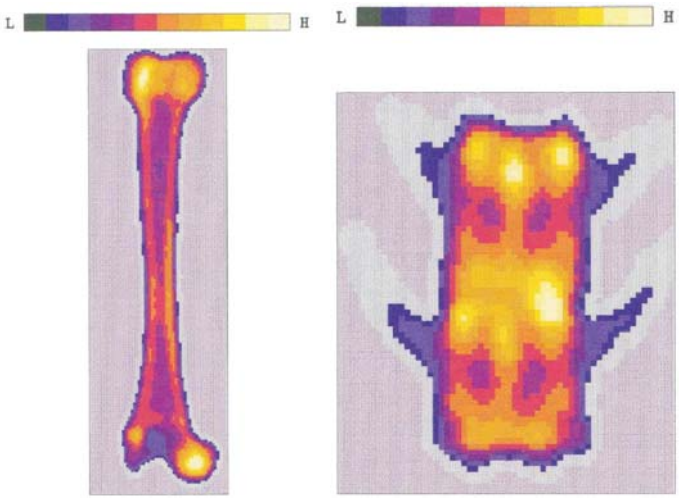

Figure 6. DXA scanning image of femur (left) and two vertebrae (right) from a 342 day old female sledge dog fed pork fat (control). Note the light high density areas (H) of cortical bone tissue (light) and the lower dark density areas (L) of trabecular bone tissue (dark).

7) Verreault J, Dietz R, Sonne C, Gebbink WA, Shahmiri S, Letcher RJ. Comparative fate of organohalogen contaminants in two top carnivores in Greenland: Captive sledge dogs and wild polar bears. Comparative Biochemistry and Physiology, Part C 2008, 147: 306–315

In the present comparative study, the authors used the captive sledge dog as surrogate species for the East Greenland polar bears to investigate some factors that may influence the bioaccumulation and biotransformation of major chlorinated and brominated OHCs in adipose tissue and blood (plasma) of control (fed commercial pork fat) and exposed (fed West Greenland minke whale (Balaenoptera acutorostrata) blubber) adult female sledge dogs. Furthermore, we compared the patterns and concentrations of OHCs and their known or suggested hydroxylated (OH) metabolites (e.g., OH-PCBs) in sledge dogs were compared with those in adipose tissue and blood (plasma) of East Greenland adult female polar bears, and blubber of their main prey species, the ringed seal (Pusa hispida). The two-year feeding regime conducted with sledge dogs led to marked differences in overall adipose tissue (and plasma) OHC residue accumulation between the control and exposed groups. Characteristic prey-to-predator OHC bioaccumulation dynamics for major PCB and PBDE congeners (patterns and concentrations) and biotransformation capacity with respect to PCB metabolite formation and OH-PCB retention distinguished, to some extent, captive sledge dogs and wild polar bears (see Figure 7).

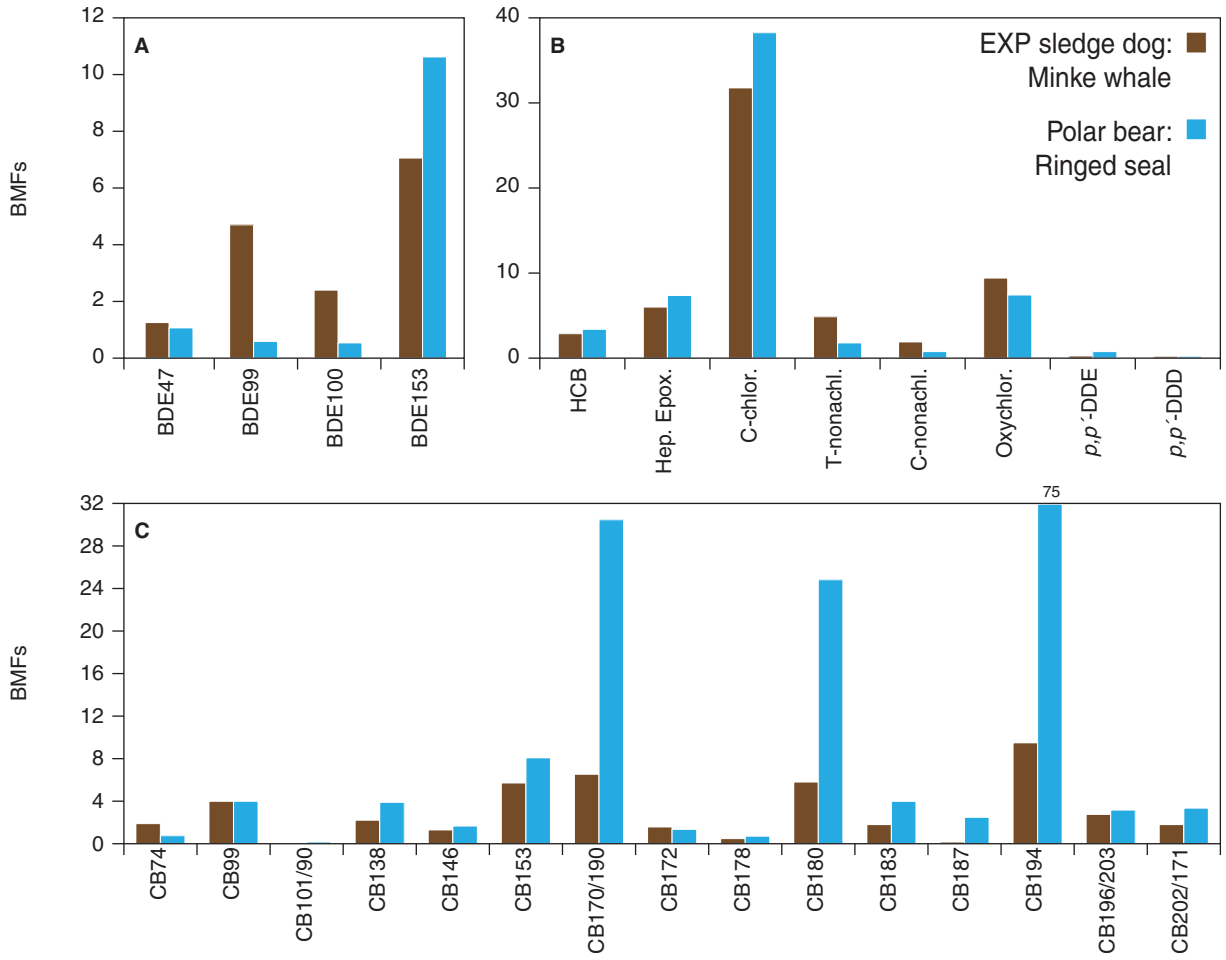

Figure 7. Biomagnification factors (BMFs) of PBDE (A), organochlorine (B) and 15 major PCB (C) congeners/compounds (detected in 60% or more of the samples) based on adipose tissue/blubber sample concentrations from minke whale to exposed (EXP) sledge dog, and from ringed seal to polar bear.

8) Verreault J, Maisonneuve F, Dietz R, Sonne C, Letcher RJ. Comparative hepatic activity of xenobiotic-metabolizing enzymes and concentrations of organohalogens and their hydroxylated analogues in captive Greenland sledge dogs. Environmental Toxicology & Chemistry 2009, 28: 162-172

The catalytic activity of major xenobiotic-metabolizing phase I and II hepatic microsomal enzymes was assessed on Greenland sledge dogs fed a naturally organohalogen contaminated diet (Greenland minke whale blubber; exposed group) or a control diet (pork fat; control group). Relative to control dogs, ethoxyresorufin-*O*-deethylase (EROD) activity in exposed dogs was twofold higher (p=0.001). Testosterone hydroxylation yielded 6β- and 16β-hydroxy (OH) testosterone and androstenedione, with higher rates of production (23–27%; $p \leq 0.03$) in the exposed individuals. In the exposed dogs, epoxide hydrolase (EH) activity was 31% higher (p=0.02) relative to the control dogs, whereas uridine diphosphoglucuronosyl transferase (UDPGT) activity was not different (p=0.62). When the exposed and control dogs were combined, the summed (Σ) plasma concentrations of OH-polychlorinated biphenyl (PCB) congeners were predicted by plasma ΣPCB concentrations and EROD activity ($p \leq 0.04$), whereas testosterone hydroxylase, EH, and UDPGT activities were not significant predictors of these concentrations. Consistent results were found for individual OH-PCB congeners and their theoretical precursor PCBs (e.g., 4-OH-CB-187 and CB-183, and 4-OH-CB-146 and CB-146) and for EROD activity. No association was found between ΣOH–polybrominated diphenyl ether (PBDE) and ΣPBDE plasma concentrations, or between potential precursor-metabolite pairs, and the enzyme activities.

9) Verreault J, Letcher RJ, Sonne C, Rune Dietz. Dietary, age and trans-generational effects on the fate of organohalogen contaminants in captive sledge dogs in Greenland. Environment International 2009, 35: 56–62

The study of Verreault et al. (2009) investigated the influence of age, dietary and trans-generational factors on the fate of major lipophilic chlorinated and brominated OHCs in adipose tissue of a potential surrogate captive species for the polar bear, the sledge dog in West Greenland. Adult female sledge dogs (P) and their sexually-mature (F1) and/or pre-weaning pups (F1-MLK) were divided into an exposed group (EXP) fed blubber from a Greenland minke whale and a control group (CON) given commercially available pork fat. Large dietary treatment-related differences in summed and individual congener/compound adipose tissue concentrations of polybrominated diphenyl ethers (PBDEs), hexachlorobenzene (HCB), chlordanes (CHLs) and polychlorinated biphenyls (PCBs) were found between the EXP and CON groups for all the sledge dog cohorts (Figure 8). However, among the F1-MLK, F1 and P dogs in both of the EXP and CON groups, little or no difference existed in PBDE, HCB, CHL and PCB concentrations, suggesting higher state of equilibrium in adipose tissue concentrations from a very early stage of life. In contrast, the distribution pattern (proportions to the summed concentrations) of OHC classes, and the major congeners/ compounds constituting those classes, varied on a dietary group- and/or cohort-dependent manner.

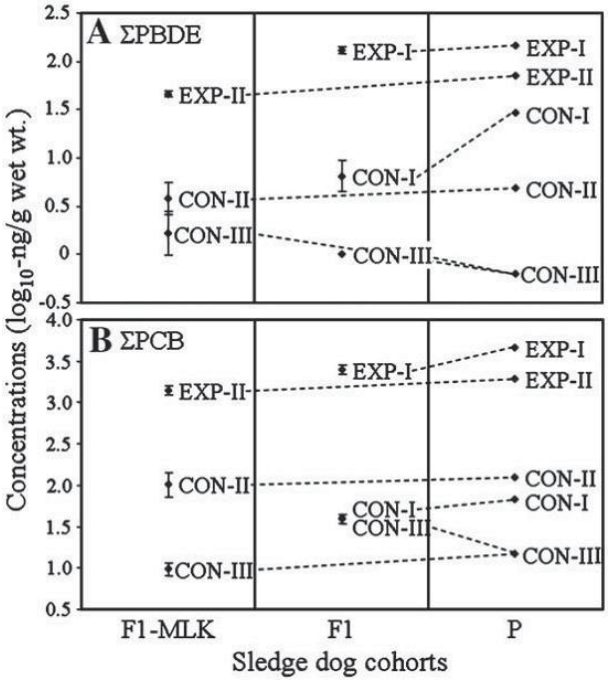

Figure 8. Unadjusted mean (±1 standard error as vertical bars) or individual concentrations (log10-transformed ng/g wet wt.) of ΣPBDE (**A**) and ΣPCB (**B**) in adipose tissue of control (CON) and exposed (EXP) adult female sledge dogs (P) and their first- and secondgeneration pups, including sexually mature (F1) and pre-weaning pups that received maternal milk exclusively (F1-MLK). Pup-mother pairs are identified by stippled lines.

10) Verreault J, Robert J. Letcher RJ, Sonne C, Dietz R (in press). In vitro metabolism of polychlorinated biphenyls and cytochrome P450 monooxygenase activities in dietary-exposed Greenland sledge dogs. Comparative Biochemistry and Physiology, Part C

The in vitro metabolism of a polychlorinated biphenyl (PCB) mixture was examined using hepatic microsomes of dietary-exposed Greenland sledge dogs to an organohalogen-rich diet (Greenland minke whale blubber: EXP cohort) or a control diet (pork fat: CON cohort). The associations between in vitro PCB metabolism, activity of oxidative hepatic microsomal cytochrome P450 (CYP) isoenzymes and concentrations of PCBs and hydroxylated metabolites were investigated. The CON dogs exhibited a 2.3-fold higher depletion percentage for the PCB congeners having at least two pairs of vicinal meta-para Cl-unsubstituted carbons (PCB-18 and -33) relative to the EXP dogs (Figure 9). This depletion discrepancy suggests that there exist substrates in liver of the organohalogen-contaminated EXP dogs that can competitively bind and/or interfere with the active sites of CYP isoenzymes, leading to a lower metabolic efficiency for these PCBs. Testosterone (T) hydroxylase activity, determined via the formation of 6β-OH-T, 16β-OH-T, 16β-OH-T and androstenedione, was strongly correlated with the depletion percentages of PCB-18 and -33 in both cohorts. Based on documented hepatic microsomal CYP isoenzyme substrate specificities in canines, present associations suggest that primarily CYP2B/2C and CYP3A were inducible in sledge dogs and responsible for the in vitro metabolism of PCB-18 and -33.

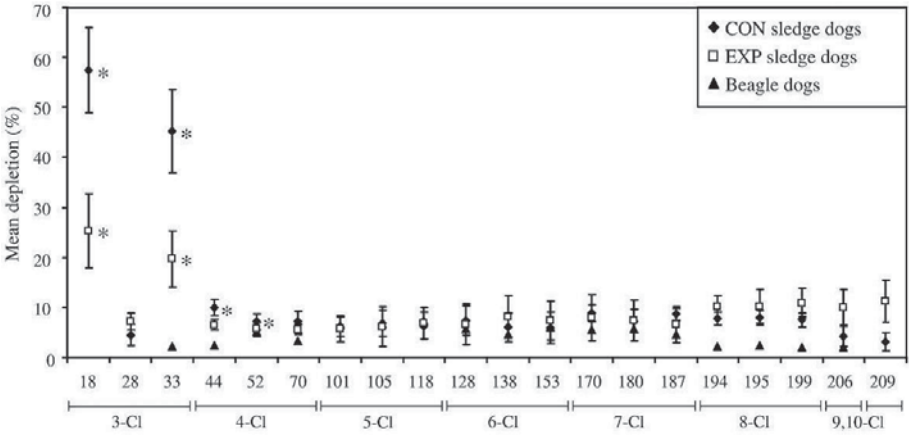

Figure 9. Mean depletion percentages (±1 standard error) of twenty PCB congeners in a hepatic microsomal in vitro assay including captive control (CON) (n=5) and exposed (EXP)(n=5) adult female Greenland sledge dogs as well as adult female beagle dogs (n=3, pooled sample). The PCB congeners that were significantly depleted are indicated with an asterisk (*).