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Cellular responses induced by environmental stress factors in arctic seabird chicks

Antioxidant defense system dynamics and autophagic lysosomal processes related to contaminant exposure and food restriction

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Cellular responses induced by environmental stress factors in arctic seabird chicks

Responses of the antioxidant defense system and autophagic lysosomal processes related to contaminant exposure and food restriction

By

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"The marvelous richness of human experience would lose something of rewarding joy if there were no limitations to overcome. The hilltop hour would not be half so wonderful if there were no dark valleys to traverse."

Helen Keller

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Preface

In the past 50 years, Arctic territories have obtained status as the litmus test for the world's environmental condition regarding pollution and climate. Scientifically documented changes in polar areas make the warning signals clang in influential offices world-wide, and new policies usually follow in their wake. Polar research activities have therefore intensified during the last decade. Long term monitoring of contaminants in arctic biota have resulted in excellent data series which characterize the nature of the exposure both with respect to chemical composition, distribution and levels in various species. However, studies of physiological responses in the exposed organisms are scarce.

Although the arctic is no longer inaccessible, it remains logistically tricky for biological studies, so crude sampling methods have been the rule and the resulting tissue samples have been exclusive and much sought after. Studies of physiological effects have therefore been challenging to accomplish, and have not received much focus until recent years. This has particularly been the case for vertebrates at higher trophic levels, Recently, however, responses linked directly to exposure to xenobiotics, such as activities of detoxification enzymes or activation of specific cellular receptors, have been targets of investigations in different species. Also, well known effects on the endocrine system found in model research animal species such as rodents or zebrafish (*Danio rerio*) have been studied in arctic organisms.

Lately, as both methods and logistics have improved, the scope has broadened to new research fields and analytic methodology. These studies have revealed novel information about genetics, immunology and mechanistic responses on the sub-cellular level in animals that inhabit some of the climatically most hostile areas on the planet and at the same time are exposed to a cocktail of contaminants. However, this field still has a long way to go before relevant cellular stress responses in arctic animals are fully characterized.

Normally, the most appropriate way of obtaining valuable toxicological effect data is through exposure experiments. Experimental studies on arctic animals in the wild, however, present both ethical and practical challenges, which is the main reason why these animals have not been used much in experimental studies.

In the work presented in this doctoral thesis, we have sought to fill some of the gaps in our knowledge about cellular responses to environmental stress in some of the most abundant arctic seabird species. We chose to focus on stress related cellular mechanisms and processes, dynamics of the antioxidant defense system and the autophagic lysosomal processes. Furthermore, we have done this by including both field samples from seabird chicks living in the wild, and samples from an experimental set-up with seabird chicks in captivity.

Marit Nøst Hegseth
Tromsø, August 2011

List of papers

Paper 1:

Hepatic antioxidant responses related to levels of PCBs and metals in chicks of three Arctic seabird species.

Marit Nøst Hegseth, Lionel Camus, Lisa Bjørnsdatter Helgason, Raffaella Bocchetti, Geir Wing Gabrielsen, Francesco Regoli.
Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 2011; 154: 28-35.

Paper 2:

Effects of exposure to halogenated organic compounds combined with dietary restrictions on the antioxidant defense system in herring gull chicks.

Marit Nøst Hegseth, Lionel Camus, Stefania Gorbi, Francesco Regoli, Geir Wing Gabrielsen.
Science of the Total Environment, 2011, Jun 15; 409 (14):2717-24

Paper 3:

Impact of Halogenated Organic Contaminant Exposure and Fasting on Antioxidant Defence System on the Kidney of Herring Gull Chicks

Marta K. Jansen, Marit Nøst Hegseth, Raffaella Bocchetti, Francesco Regoli, Geir W. Gabrielsen and Lionel Camus
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Paper 4:

Lysosomal and lipid-associated parameters in the livers of three species of arctic seabird chicks: Species differences and relationships with contaminant levels

Marit Nøst Hegseth, Francesco Regoli, Stefania Gorbi, Raffaella Bocchetti, Geir Wing Gabrielsen Lionel Camus.
Marine Pollution Bulletin, 2011, Aug, 62(8): 1652-60.

Paper 5:

Effects of contaminant exposure and food restriction on hepatic lysosomal and lipid associated parameters in Herring gull chicks.

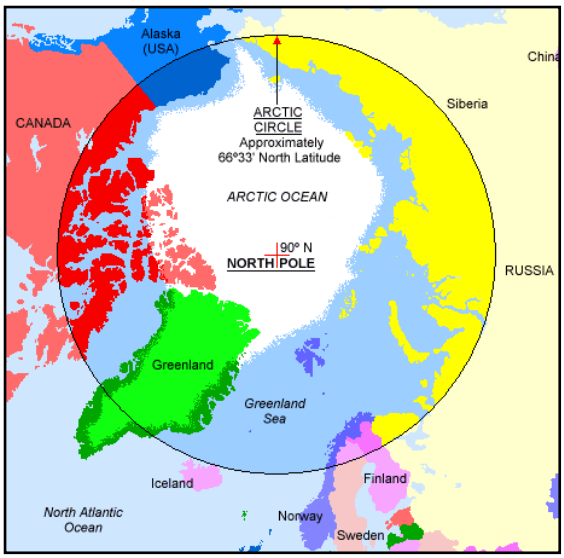
Marit Nøst Hegseth, Stephania Gorbi, Raffaella Bocchetti, Geir Wing Gabrielsen, Francesco Regoli, Lionel Camus
Manuscript, submitted to Environmental Research.

Introduction

The Arctic

The Arctic is considered as the circumpolar part of the northern hemisphere that is located north of the Arctic Circle (66° 33'N). Arctic is also defined as the area north of the 10ºC isotherm, which means the areas where the average temperature of the warmest month of the year is below 10ºC. This is the area that surrounds the North Pole, and countries that have land areas within the region include the United States, Canada, Russia, Denmark (Greenland), Sweden, Finland, Iceland and Norway. However, of the Arctic is covered by the Arctic Ocean, which includes rim shelf seas such as the Beaufort, Greenland, Kara and Barents Seas, and is
partly covered by ice covered year-round[1].

The arctic climate is characterized by strong seasonality within the frigid range of the thermometer, with short cool summers and long cold winters when the midnight sun and the darkness of the polar night, respectively, define the seasons[2].



Picture 1:
Map of the arctic region indicated as the area within the Arctic Circle. Source: http://athropolis.com/

The arctic Svalbard is a Norwegian archipelago located at 74-81° north in the Barents Sea and is the geographical area around which this doctoral work has centered. Some of the issues addressed are related to the special climatic conditions of this region.

Picture 2: Map of the Svalbard archipelago, with the largest island Spitsbergen marked in red. Source http://commons.wikimedia.org/wiki/File:Spitsbergen.png

Birds in the polar region

There are several "true" arctic bird species, such as the northern fulmar (*Fulmarus glacialis*) and glaucous gull (*Larus hyperboreus*), which stay in the arctic climate zone all year round, including the winter. In addition, there are tenfold as many bird species that migrate back to the arctic regions in spring for socializing, mating, breeding and nesting[3;4]. Due to its relatively mild climate, the Svalbard area is particularly densely populated by these arctic migratory birds, a majority of which are seabird species. Estimates based on databases at the Norwegian Polar Institute and the Norwegian Institute of Nature Research indicate that the total population of seabirds in the Barents Sea numbers approximately 16 million individuals. The most abundant species are Brünnich's guillemot (*Uria lomvia*), Atlantic puffin (*Fratercula arctica*), little auk (*Alle alle*) and black-legged kittiwake (*Rissa tridactyla*), which together constitute almost 90% of the total number of individuals[4].

Barnacle geese (*Branta leucopsis*) flying across Kongsfjorden near Ny-Ålesund, Spitsbergen. August 2007.
Photo: Marit Nøst Hegseth

The diet of seabirds in the Svalbard area consists of different fish species, such as polar cod (*Boreogadus saida*), herring (*Clupea harengus*), capelin (*Mallotus villosus*) and Atlantic cod (*Gadus morhua*) in addition to invertebrates like amphipods, crustaceans and squid. Some species, such as the northern fulmar and little auk, have a diet where the main constituents are invertebrates, but most seabirds have fish as their main food[5;6].

Adaptations to cold climate and food scarcity

Harsh climate conditions, particularly during the winter season, pose physiological challenges to arctic organisms. The animals that live in this region have adapted to this unforgiving habitat through various physiological mechanisms. For example, most arctic animals deposit

thick layers of subcutaneous fat during summer which will help them through periods of food scarcity, breeding, molting or migration and serve as insulation during the cold winter[3]. Consequently seabird species, like other arctic animals, have lipid reserves that vary with the seasons: from 2-5% of their body mass during the summer months to 15-20% in the beginning of the winter season. As in arctic mammals, the highest lipid content in seabirds is normally found in mid winter, and the lowest in April and May, when they return to their colonies, and again during the chick rearing period, when they stay on their nests for long periods of time without foraging[4].

Hence, the arctic conditions place extreme demands on the life forms that exist here, and even though the animals are well adapted to their way of life, particularly stressful periods or additional stress factors may be physiologically challenging.

Environmental stress factors: Contaminant exposure and food restriction

Seabirds have traditionally been used as sentinel organisms in monitoring changes within the marine ecosystem. A wide range of environmental stress factors may affect the seabird population directly or indirectly, such as industrial fisheries, pollution and even climate change[7]. Also arctic seabirds encounter environmental stress factors such as constant exposure to xenobiotics and repeated periods of food restriction (food deprivation) during their lifetime. Previous investigations have shown that these two environmental stress factors constitute significant challenges for arctic animals[3;8-12]; hence, the focus in this doctoral work is directed towards contaminant exposure and food deprivation.

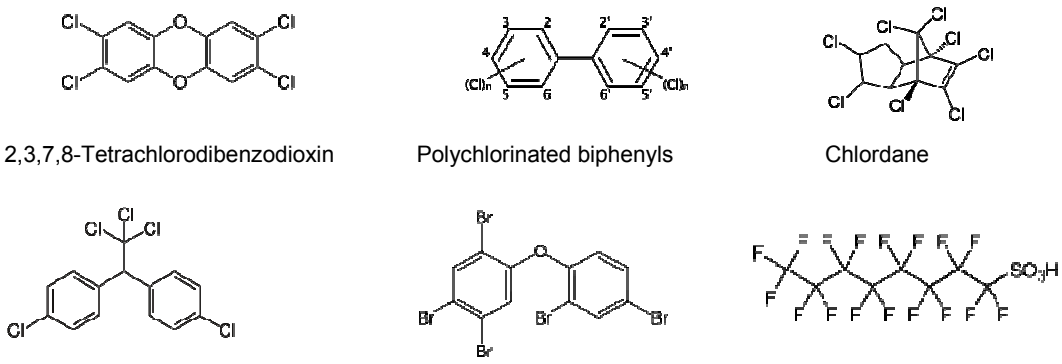
Organic contaminants

Ever since Rachel Carson published her book "Silent Spring" in 1962[13] halogenated organic compounds have had a place on the agenda of political decision makers, environmentalists and scientists. From the early 70's scientific papers that reported the occurrence of these compounds in arctic biota were published[14-16], and since then the body of research regarding sources, fate and effects of these compounds has been growing continually[9;17;18]. These contaminants originate from products used for industrial or agricultural purposes or in

consumer products in industrialized parts of the world, but are nevertheless found in arctic biota, far away from the original source of discharge.

The continuous development of novel chemical analyzing techniques has enabled detection of an increasing number of pollutants in biological samples, including samples from arctic animals[9]. The compounds that are classified as persistent organic pollutants (POPs) have been, and are still, of major concern. POPs are a group of organic substances with similar chemical characteristics[17]. They are resilient to degradation through chemical, physical or biological decomposition mechanisms, they are lipophilic, and despite their large molecular size, they are semi-volatile. These are inherent properties that make them toxicologically relevant.

A large share of the POPs are organohalides, such as haloalkanes or haloaromatics. These are alkane or aromatic structured hydrocarbons with halogen substituents, primarily chlorine, bromine or fluorine. This substitution makes the halogenated organic compounds (HOCs) especially persistent because the halogen substituents hinder enzymatic degradation.



Picture 3: The structure of some of the most commonly found halogenated organic compounds

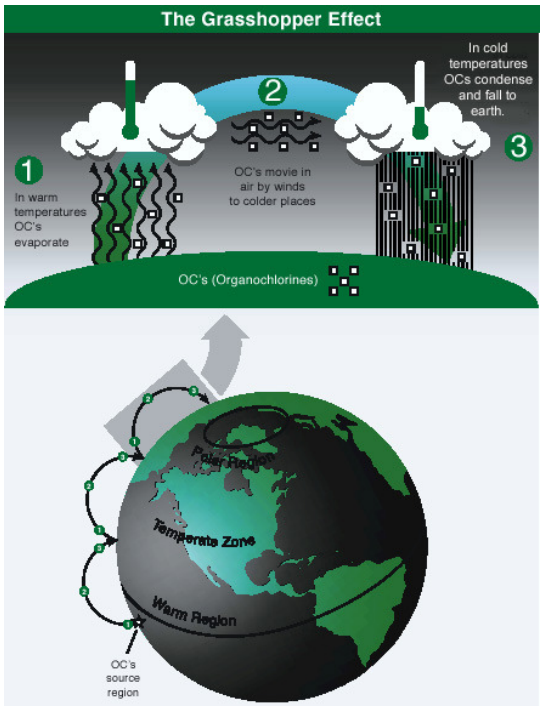
Some of the most common environmental contaminants belong to this group, for instance the polychlorinated biphenyls, polychlorinated dibenzodioxins, pesticides such as DDT and chlordane, brominated flame retardants and the more recent contribution to the group, the fluorinated compounds which often are used as non-stick surface treatments on consumer products or in fire-fighting foam[19].

Pathways to the Arctic for halogenated organic contaminants

The semi-volatile property makes it possible for these compounds to evaporate from the site of discharge.

Long range atmospheric transportation, probably through the seasonally dependent "grasshopper" effect, ultimately deposits compounds in arctic regions[20].

When these compounds have reached the Arctic, borne by either atmospheric or ocean currents, they can enter the arctic marine food chain[18]. The lipophilic properties of these substances make it plausible that this happens through passive diffusion over membranes into phytoplankton or zooplankton at the lowest trophic levels.



Picture 4:
The Grasshopper effect, POPs repeatedly evaporate to the atmosphere and are deposited on the ground where they again evaporate to the atmosphere, moving closer and closer to the polar regions where the most volatile compounds are eventually deposited. Source: Environment Canada-Ontario region, http://www.on.ec.gc.ca/laws/coa/atmospheric-e.html

These organisms are eaten by animals higher up in the food chain, and due to low water solubility the contaminants are not easily excreted, but rather stored in lipids in the animal. The contaminant concentration increases with each trophic level, and the highest concentrations of HOCs are generally found in top predators[16;17;21]. This biomagnification mechanism is not specific for the arctic, but due to the importance of lipids as energy source for the animals that inhabit this region, lipophilic contaminants become significant in the context of exposure.

Biological effects related to HOC exposure

The toxic effects of halogenated organic compounds have been investigated in both humans and animals. The toxic potential of a HOC is often related to its structure and the degree of resemblance with intrinsic molecules with important physiological functions in the organisms, such as hormones. With regard to human health, the documented effects of HOC exposure vary according to the nature of the exposure. Hence, the HOC may add to or block

the intended effect of the original substance and may have severe consequences for the organism. Acute effects caused by exposure to large doses of one or more of these compounds have mainly been observed in cases of accidents in workplaces, large industrial discharge accidents or occasional episodes of poisoning. Dermal effects such as chloracne, low birth weight, hyperpigmentation, liver pathology and unspecific neurological effects have been observed after high levels of exposure in such cases[22;23]. Effects of chronic exposure to lower doses of HOCs have been investigated in occupational and epidemiological studies, but there is little consensus regarding the risk related to such low-grade exposure. Some individual compounds like dioxin are known to cause immunological and endocrine impairment and cancer, even at low doses[24], but the actual impact of chronic, low level exposure to other HOCs is more controversial and not unanimously recognized[23]. However, most people are exposed to a cocktail of HOCs, and the combined effects of this mixed exposure over a lifetime are hard to predict, which is clearly illustrated by the lack of consistent results in effect studies on humans. The risk of developing breast cancer, obesity and diabetes are examples of issues that are currently debated in relation to human HOC exposure[25;26].

Many of the known effects of HOC exposure stem from laboratory studies of animals where various adverse effects such as cancer, immunotoxicity, growth reduction, endocrine disruption, dermal effects, enzyme inhibition and reproductive toxicity have been shown[23;27-31]. However, in most laboratory studies the animals have been exposed to much higher doses than those that occur under natural conditions. The animals are often exposed only to one or a few compounds at the same time and the exposure period has often been short (i.e. days or weeks).

The realistic situation for wild animals, including those in the Arctic, is that the contaminant exposure is continuous during the entire lifetime of the animal. The exposure starts as early as in prenatal life or just after birth due to maternal transfer of contaminants via the placenta or to the egg, and through lactation[32-34]. During their young and adult lives the animals are exposed to contaminants mainly through the diet, and the levels vary depending on the source of feed, but are still considerably lower than the doses given in most laboratory experiments[17;23]. In contrast to the controlled exposure in the laboratory, the contaminant exposure that wild arctic animals experience varies in amount and consists of a cocktail of halogenated or other organic compounds, such as polycyclic aromatic hydrocarbons (PAHs), heavy metals and their metabolites[17;18;35]. Exposure encompasses possibly thousands of compounds about which little is known regarding potential antagonistic or synergistic effects,

and it is almost impossible to separate the effect from one compound from the other. For these reasons, effect studies in wild animals are challenging.

Still, numerous excellent studies on contaminant induced effects in arctic animals have been published the last three decades and particularly since the beginning of the 21st century[9]. Exposure to HOCs has, for example, been associated with endocrine alterations in polar bears, arctic foxes and seals[10;11;36;37], and hormone-related changes in the size and morphology of genitalia of sledge dogs and polar bears have been connected with high levels of HOCs[38;39]. Immunological dysfunctions, possibly caused by HOC exposure, have been documented in polar bears and seals[9]. In addition, neurological and behavioral alterations and changes in biotransformation mechanisms have been associated with HOC exposure in arctic mammals[9;17].

Arctic seabirds have been the subject of a number of investigations regarding HOC exposure and biological effects[9;18;19;40-42]. For example, effects on the endocrine system have been investigated in the glaucous gull, and high HOC levels have been associated with disrupted endocrine functions, particularly related to thyroid hormones[40;43]. This gull is an apex species in the food chain, and tends to carry high concentrations of HOCs. It is therefore one of the arctic seabird species most frequently studied in relation to toxic effects of contaminant exposure. Adverse effects on the gull's immune system have also been reported, and high HOC levels have been associated with increased parasite burdens[44;45]. Behavioral and genetic abnormalities, and enzymatic, reproductive, and metabolic impairment are other effects related to HOC exposure that have been found in glaucous gulls and other arctic seabird species, such as greater black-backed gulls (*Larus marinus*), northern fulmars, guillemots (*Uria sp*.) and shags (*Phalacrocoracidae sp.*)[9]. Cytochrome P450 enzymes in the liver play key roles in biotransformation and detoxification of HOCs[46;47], and the biotransformation capacity has been shown to differ between arctic seabird species both with regard to efficiency and classes of chemicals that are metabolized[32;48;49]. Variation in biotransformation properties between species may therefore have implications for their vulnerability toward specific compounds.

Hence, the scientific evidence for adverse biological effects of HOCs in arctic organisms, including seabirds, is continuously being strengthened. However, there are still huge knowledge gapsn as only a few species have been investigated, and glaucous gull is the only species for which extensive data has been collected[42]. We still know little about differences in sensitivity between species, between individuals and between life stages.

Earlier studies have touched on some important physiological systems and features, but still there are many important organs, cellular mechanisms and processes for which we lack information regarding how these processes are affected by HOC exposure.

Heavy metals

In this work the main focus has been directed towards HOCs. Heavy metals are present at relatively low levels in the arctic, and are therefore not generally considered a significant threat to animal health, at least in comparison with organic compounds. However, there are exceptions, and some of these metals (primarily cadmium, mercury and lead) cause concern in regard to ecosystem health[18;35]. We do not know how sensitive seabird chicks may be towards compounds containing these potentially toxic heavy metals. Hence, we have included heavy metals as a secondary stress factor in our field investigations.

"Heavy metals" is a rather vague term used to describe a group of elements with some properties in common. Which elements are included varies depending on the context and the exact meaning of the term has never been defined by official organs such as IUPAC[50]. Nonetheless, for scientific purposes, the term "heavy metals" is often used to describe metals of relevance to contamination and ecotoxicology or occupational toxicity. The group often includes metallic elements that are found in excessive amounts in the environment due to anthropogenic activities and in addition do not have any known functions in the organisms. Examples of such elements are mercury (Hg), cadmium (Cd) and lead (Pb), and in this work chromium (Cr), Cd and Pb have been included.

Pathways to the Arctic for heavy metals

Heavy metals occur naturally in the arctic environment, but the levels of several metals started to increase steadily after 1850 due to human activities, and most reached peak levels around 1970. Since then levels of many heavy metals have declined, but for some, for instance mercury, there is still continuous input and the levels are increasing even today[18]. Metals are transported to the Arctic mainly through air and ocean currents, but rivers, ground water, sea ice and biotic transport are also important routes of transportation[35]. The input to the Arctic is connected with the discharge of the element elsewhere. Levels of mercury are currently increasing mainly due to increased discharges in Asia, while lead concentrations are continuously decreasing due to reduced discharges worldwide (particularly after leaded fuels were phased out[18]).

Arctic animals are exposed to the metals mainly through their food and drinking water[35]. The uptake of an element depends on its chemical properties, the uptake mechanisms and often on the animal's nutritional status. Many metals, such as iron and selenium, are essential to physiological processes, but may be harmful in excess doses. Other elements have no natural functions in the organism, but may resemble essential metals that the organism needs to maintain its normal physiological processes and are readily taken up in the digestive system through the same uptake mechanisms. Some heavy metals bioaccumulate over time in an organism due to slow excretion, but others have short biological half lives. Some metals also exist in an organic form (like methyl mercury)[35]. Such compounds are efficiently absorbed due to their lipophilic properties, and may biomagnify[51]. Methyl mercury exposure was not included in our analysis, however.

Biological effects related to heavy metal exposure

Depending on the element, its chemical form and the target organ, heavy metal exposure is associated with several different biological effects in exposed humans and animals[35]. For example, organic mercury and lead have been associated with neurological dysfunctions and neurobehavioral effects in indigenous arctic peoples who are exposed to high levels of these elements [52], and chronic cadmium exposure is nephrotoxic, may cause skeletal disorders and is suspected to be carcinogenic. Lead can also inhibit important enzymes in the heme synthesis pathway, thus leading to anemia, and can cause liver and renal damage and reproductive disorders. Many arctic mammals such as polar bears, whales and seals accumulate heavy metals to levels above the threshold for toxic effects in other species[35]. Interestingly, few adverse effects on potential target organs have been observed in the arctic animals[53;35].

Seabirds that live in the Arctic are also exposed to heavy metals, but the levels vary both spatially and between the species, depending on the diet. For example, northern fulmars feed on invertebrates and have high cadmium and mercury levels compared to little auk, that eat plankton and has low levels. Lead exposure has declined in recent years, and levels in seabirds are generally low[54-57]. The mercury levels in some arctic seabirds have been sufficiently high that one might suspect harmful effects, and kidney and liver damage has been found in seabirds and aquatic birds outside the Arctic exposed to Hg and Cd[54-57]. Generally, as with arctic marine mammals, arctic seabirds tolerate high concentrations of heavy metals such as Hg compared to terrestrial birds[7;18]. However, associations between Hg and lipid peroxidation and antioxidant decline have been observed in glaucous gulls in the Canadian

arctic[58] and renal lesions have been disclosed in northern fulmars from the UK with high renal Cd levels[7].

We have included a small number of heavy metal analyses in our investigations as very few investigations have been done on the effects of heavy metal exposure in arctic seabirds.

Food deprivation and metabolic stress

Fasting, or abstaining from food, is a situation which affects biochemical processes in the body. Normally, glucose obtained from the diet through aerobic respiration is the primary energy source for most organisms[59]. However, some hours after the last meal the blood glucose levels decline and glycogen stored in the liver is converted to glucose which is released into the blood stream. When the glycogen reserves in the liver are empty the body turns to alternative energy supplies. Fat is then the primary source of energy. Triglycerides are broken down into fatty acids and glycerol and the fatty acids are transformed to acetyl-CoA through β–oxidation in the mitochondria or peroxisomes. Acetyl-CoA enters the citric cycle and generates energy as ATP molecules[59]. This fat catabolism will continue as long as there is fat available in the organism. If the food deprivation continues even after the fat reserves are used, the body turns to proteins as energy source. At this point the fasting has turned into starvation, and the processes that follow can lead to serious physiological conditions, ranging from electrolyte imbalance to renal failure and ultimately death[60]. Seabirds experience recurring periods of emaciation in their high-latitude habitat but little is known about how this affects the function of important intracellular defense mechanisms.

Contaminant exposure combined with food deprivation

All halogenated organic compounds are lipophilic and are stored in lipid-rich tissues, such as liver, brain and blubber. Here they are metabolized to a certain extent, but even though the metabolites are more water soluble than the mother compound, most of them are still retained in the fat. This means that as long as the energy source of the animal is dietary glucose or hepatic glycogen the contaminants stay in the fat. But as soon as body lipids start being mobilized, the stored compounds are released to the circulatory system in the body, giving them potential to cause harm once again in new target organs[10;12;61]. Changes in contaminant concentrations in different tissues after fasting and emaciation have previously been examined

in some arctic animals: ringed seals (*Pusa* [*Phoca*] *hispida*)[10], black-legged kittiwake[8] and Arctic charr (*Salvelinus alpinus*)[62].

Cellular responses to contaminant exposure and food deprivation

The previously described environmental stress factors have been found to induce various effects in arctic animals. However, few investigations have examined the impact of contaminant exposure and fasting on the antioxidant defense system in seabirds and no investigations of the autophagic lysosomal responses have previously been reported in these species. These two cellular systems have evolved to handle various forms of cellular stress induced by chemicals, food deficiency or other environmental factors. The actions of these cell protection systems are essential for cellular well being, and the animal's health and longevity.

The antioxidant defense system: Protection against oxidative stress

Without an extensive antioxidant defense system, life on earth as we know it today would have been impossible. These defense mechanisms are therefore vital to all organisms, but still there are only a handful of reports on these mechanisms in seabirds[58;63-67]. Characterization of the antioxidant defense system in seabirds offers vital knowledge about the basic physiology of these species and expands our understanding of how environmental stress may impact the birds.

Oxygen, the necessary evil

Shortly after the earth's formation 4.9 billion years ago there was basically no atmosphere, and later as a stable gaseous layer was established around the planet, this first atmosphere did not contain free oxygen. The first organisms that utilized photosynthesis to generate energy were cyanobacteria, which appeared approximately 2.5 billion years ago. O_2 is a by-product of the photosynthesis, and slowly the atmospheric oxygen content started to rise, reaching the present concentration of 21% approximately 500 million years ago, at the beginning of the Cambrian period. At this time in the earth's history the major phyla that exist today appeared, and this evolutionary progress has partly been explained by the increased levels of free oxygen in the atmosphere[68;69].

Aerobic organisms are dependent on oxygen to maintain the cellular energy production. However, O_2 is a toxic gas due to its strong oxidative properties. Oxygen easily takes up

electrons that are generated in the cell[70] and consequently, free radicals or other reactive chemical species are produced[71]. Free radicals are atoms or molecules that are capable of independent existence despite having one or more unpaired electrons. This makes some of these species highly reactive and capable of oxidizing other compounds. Radicals are formed when a non-radical loses or gains an electron, or when a covalent bond is broken such that each atom gets one electron from the bonding pair (homolytic fission). Reactive species may also include reactive molecules or atoms that are not radicals, and they are not exclusively oxygen species but also species that include nitrogen, sulfur or halogens[68].

In aerobe cells, free radicals and reactive species are constantly generated due to the presence of oxygen, particularly through the mechanisms and processes involved in oxygen dependent metabolism; hence, the mitochondria are a major source.

The constituents of the antioxidant defense system

To control the oxidation hazards all aerobic cells are equipped with an extensive antioxidant defense system. The simplest form of defense is basically to avoid oxygen as much as possible, as seen in some bacteria species which move away when the O_2 levels are high enough to trigger a warning signal. However, for most organisms the antioxidant defense system exists in both intra- and extracellular compartments and consists of enzymes and non-enzymatic molecules that cope with the reactive species. Antioxidant enzymes catalytically remove reactive species. The other molecules serve as "decoys" being directly attacked and oxidized by the radicals, thereby protecting important biomolecules from oxidation. Other antioxidant actions may involve chaperone activities, reduction in intracellular radical generation or direct quenching of radicals by some substances[68]. Hence, "antioxidant" is a broad term, and includes numerous compounds. However, some antioxidant molecules have been attributed more important and specific roles in the total antioxidant defense system, and are often used as biomarkers or targets in toxicological studies that involve oxidative stress.

Antioxidant enzymes

The antioxidant enzymes described below are those that are most important and most regularly studied in a wide range of organisms and are also included in the analysis in the seabird chicks in the present work.

- Superoxide dismutases

In 1969 McCord and Fridovich discovered the function of superoxide dismutases (SODs[72]). They found that these proteins were capable of removing the superoxide anion ($O_2^{\bullet-}$) catalytically, and this discovery initiated today's extensive antioxidant research[73].

The SOD enzymes exist in some form in all living aerobe organisms, and are divided into two main classes with different protein folds and catalytic metal ions: Cu/Zn SODs and Mn SOD/Fe SOD which exist in eukaryotes and some prokaryotes[74]. SODs are unevenly distributed between tissues in different organisms, for example in humans where Cu/Zn SOD activity is high in liver, but not detectable in cardiac muscle or testis[68].

All SODs catalyze the same reaction:

$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \longrightarrow H_2O_2 + O_2$$

The reaction is extremely efficient. SODs are important enzymes in an organism, and defects in the expression or function of these enzymes are linked to aging and serious pathological conditions, such as the fatal neurodegenerative disease amyotrophic lateral sclerosis (ALS)[74].

- Catalase

The H_2O_2 generated by dismutation of $O_2^{\bullet-}$ and other oxidative enzymatic reactions, can be removed by two different types of enzymes. One of them is catalases, which are some of the most important antioxidant enzymes. Catalases are highly conserved and exist in most aerobic organisms, although a few bacteria, parasites and algae do not contain this enzyme[68]. Catalase is mainly restricted to peroxisomes, but is also present in smaller amounts in other cellular compartments. All animals have catalase activity and it is expressed in all tissues, but similarly to SODs, catalase is often highly expressed in liver. The net reaction of the enzyme is:

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

However, also other small molecules such as formate, methanol or ethanol may act as substrates[75].

- Glutathione peroxidases

Similar to catalases, glutathione peroxidases (GPXs) remove H_2O_2. However, the reaction differs from that catalyzed by catalase in that GPXs' reduction of H_2O_2 is coupled to the oxidation of reduced glutathione (GSH), which in itself is an antioxidant:

$$H_2O_2 + 2GSH \longrightarrow GSSG \text{ (oxidized glutathione)} + 2H_2O$$

GPXs may also reduce other organic peroxides, and the general reaction is:

$$ROOH + 2GSH \longrightarrow GSSG + ROH + H_2O$$

GPX activities are, in accordance with SOD and catalase, also highest in the liver, but high GPX activity is also found in kidneys in humans. Several types of GPXs exist, and at least 8 different types have been detected in mammals[76]. Most of them require selenium in their active sites to execute the enzymatic reactions, but some of the GPXs do not contain selenium. Se-dependent GPXs are normally the dominating form of GPXs in mammals, and have also been characterized in several non-mammalian vertebrates such as fish and birds. The Se-independent GPXs are usually predominant in invertebrates[76]. Some of these enzymes with GPX activity that do not contain selenium catalyze reduction of most organic peroxides but not H_2O_2.

- Glutathione reductase

Glutathione reductase (GR) does not function directly as an antioxidant enzyme by acting upon reactive species, but is still an important constituent of the total antioxidant defense system. This enzyme reduces oxidized glutathione (GSSG) back to its reduced state (GSH), which is the form of glutathione that acts as an antioxidant. This reduction is dependent on NADPH, which is provided mainly by the pentose phosphate pathway[68]. GR catalyzes this reaction:

$$GSSG + NADPH + H^+ \longrightarrow 2GSH + NADP^+$$

GR normally has a high capacity, and is not the rate-limiting enzyme in the glutathione system[76].

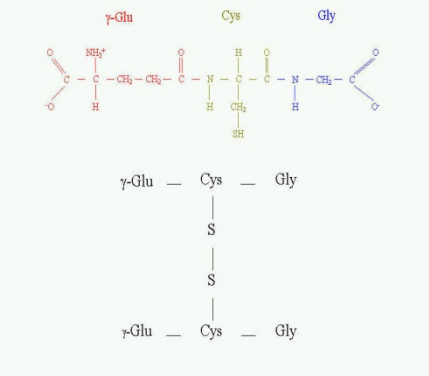
Non-enzymatic antioxidants

- Glutathione

Reduced glutathione (GSH) is a tripeptide consisting of glutamate, cysteine and glycine residues. In its oxidized form (GSSG) two GSH molecules are joined together through oxidized –SH groups that form a disulphide bridge. It is synthesized in the cytosol of most cells, but the highest generation is in the liver.

Glutathione does not function solely as a cofactor in GPX-catalyzed reactions, but is also involved in cell communication, metabolic and detoxifying processes and also functions directly as an antioxidant, in addition to being involved in a wide range of other physiological mechanisms and processes. It is hence an important

molecule not only for antioxidant purposes, and exists intracellularly in high concentrations of 5-10 mM[68].



Glutathione molecule. Source: http://www.cryst.bbk.ac.uk/pps97/assignments/projects/leluk/glutat.jpg

As a direct antioxidant GSH can react with most radical species, but not efficiently with $O_2^{\bullet-}$. Individuals that have deficiencies in the GSH metabolism may suffer from neurological defects, hemolysis or early onset of cataract, and decreased GSH levels have been connected with several pathological conditions, such as alcoholic liver disease, Parkinson's disease and anorexia nervosa. Cigarette smokers usually have significantly lower GSH concentrations in red blood cells than non-smokers[68]. The ratio between oxidized and reduced glutathione is often suggested as a suitable biomarker of oxidative stress. Normally only a small portion (10%) of the total glutathione in a cell is in the oxidized state. In case of oxidative stress this ratio may be shifted towards less reduced and more oxidized glutathione. Several studies of metal-induced oxidative stress in birds have shown that the GSSG/GSH ratio is a sensitive biomarker[77].

Other non-enzymatic antioxidants

Several other small non-enzymatic molecules are assumed to have important antioxidant properties. Some of them are synthesized *in vivo*, such as bilirubin, the hormone melatonin, uric acid and coenzyme Q. Other low-molecular antioxidants are obtained through the diet. Typical examples are vitamin C (ascorbic acid), vitamin E (α-tocopherol), plant-derived pigments such as the vitamin A (retinol) precursors carotenoids (which are also found in colorful animals such as goldfish and in bright bird plumage). Other antioxidants include polyphenols such as flavonids present in wine, tea and onions, or other phenolic compounds found in food items such as carnosic and rosmaric acids in spices and herbs[68]. Within the rather broad definition of antioxidants one can include a wide range of compounds. The uptake and effects of the thousands of potential antioxidants that come from internal or external sources are not always known, and many of these compounds have primary physiological functions other than antioxidant activity. For logistical and economical reasons

we have not included analyses of other non-enzymatic antioxidants besides glutathione in the present work.

Hence, the total antioxidant defense system includes a wide range of known and unknown compounds. Measurement of only one or two parameters in this complicated system will therefore not depict the pro- and antioxidant state in the cell or organ satisfactorily. Methods to measure the total antioxidant capacity have been developed which allow more acurate assessment of the oxidative status than can be achieved through measurement of single parameters[78;79]. These methods can capture the contribution from unidentified antioxidants in addition to the main constituents, and such assays were applied in the analyses of the bird tissue in the present studies.

Oxidative stress

The presence and activity of antioxidants is normally regulated according to the amount of reactive species present. If, however, the amount of reactive species exceeds the capacity of the antioxidant defense system, the cell experiences what is called oxidative stress. External influences such as ionizing radiation, UV light or xenobiotics may induce excess reactive species generation. In such situations DNA, membranes and proteins are at risk of oxidative damage which may be detrimental to the cell and eventually cause cell death. Extensive repair mechanisms exist, however, and damage can be repaired. This is particularly true of DNA damage. Unrepaired damage to the DNA molecule may lead to mutations and can eventually induce cancer[68].The antioxidant system is therefore a crucial part of the defense mechanisms that protects an organism against the possible detrimental effects of externally induced stress.

Contaminant induced oxidative stress
- Mechanisms

Exposure to xenobiotics such as redox cycling compounds, PAHs, HOCs, metals and air pollutants may induce increased generation of reactive species in cells of the exposed animal[80]. Basically, there are several mechanisms through which this can occur, either separately or in combination. Many environmental exposures, such as tobacco smoke or HOC mixtures, contain a cocktail of compounds and can act through several of these mechanisms simultaneously[68]:

- The xenobiotic compound is a reactive species in itself.
- The xenobiotic compound is metabolized to a reactive species.

- The xenobiotic compound undergoes aerobe biotransformation and reactive species are generated as by-products of the enzymatic reactions.
- The xenobiotic compound undergoes redox cycling, where the compound is reduced, the reduction product is reoxizided by oxygen in a process which generates $O_2^{\bullet-}$ and re-generates the mother compound. The cycle can then repeat itself, generating more $O_2^{\bullet-}$. The pesticide paraquat is an example of a xenobiotic compound that undergoes redox cycling.
- The xenobiotic compound may interfere with the antioxidant defense system, for example by inhibiting enzyme activity, or deplete GSH through conjugation reactions during biotransformation.
- The xenobiotic compound may stimulate endogenous generation of reactive species, for example through affecting mitochondrial electron transport.
- The xenobiotic compound can bind to biomolecules and thereby act as antigens. This provokes the immune system, and may increase reactive species generation.

Halogenated organic compounds and oxidative stress

Numerous previous studies have suggested that HOC exposure may generate reactive species and that the exposure causes oxidative stress[80-82]. However, the specific mechanisms have not yet been extensively investigated. Some HOCs, such as pentachlorophenol (PCP), are metabolized to compounds that can form adducts with DNA which may cause mutations and eventually cancer[68]. Dioxin, or TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) has been shown to induce mitochondrial reactive species generation, oxidative DNA damage and lipid peroxidation. The exact mechanisms are unknown, but reduced antioxidant enzyme activities have been found in liver from chicken (*Gallus gallus*) after TCDD exposure[83]. Other mechanisms such as increased biotransformation through CYP enzymes, redox cycling of metabolites, induction of the immune system and inhibition of antioxidant enzymes have also been suggested as causes of TCDD-induced oxidative stress[84]. Uncoupling of the catalytic cycle of CYP1A and release of reactive species has been suggested as a mechanism behind non-ortho-PCB toxicity[85].

Many HOCs, such as some PCBs and PBDEs have structures similar to TCDD (planar, non-ortho substituted congeners), and these dioxin-like compounds may induce oxidative stress through similar mechanisms as mentioned above. Some previous investigations have found such effects in free-living animals. For example, PCB exposure decreased the total antioxidant capacity in bivalves [86], and PBDE metabolites were suggested to affect vitamin A status, and

thereby cause oxidative stress in American kestrels (*Falco sparverius*)[28]. Also, exposure to PCB 126 (which resembles TCDD in structure) generated oxidative stress in American kestrels[87], and pesticides such as HCB and DDTs have been associated with decreased total oxyradical scavenging capacity in herons[88]. The oxidative stress inducing potential of HOCs depends on the features of the individual compounds, such as their chemical structure or metabolite generation.

Metals and oxidative stress

Metals are of great significance both in the antioxidant defense system and the generation of reactive species. Metals such as iron, copper, zinc, selenium and magnesium act as cofactors in antioxidant enzymes, and are therefore essential for optimal antioxidant defense[73;89]. However, some of these metals also act as pro-oxidants. Transition metals, such as copper, manganese and iron, contain one unpaired electron and actually qualify as radicals. Excess amounts of ions of these metals in the cytosol may catalyze unwanted free radical reactions. The Fenton reaction is one of the most influential free radical reactions, where Fe^{2+} reacts with H_2O_2 and generates $OH^{\bullet}$.

The generation of $OH^{\bullet}$ through the Fenton reaction is thought to be one of the contributing factors in lipid peroxidation. Lipid peroxidation is the oxidative deterioration of polyunsaturated lipids (PUFA), which may damage cell membrane integrity and function, as biological membranes contain large amounts of PUFA side-chains. This reaction can be initiated by radicals formed through the Fenton reaction[90]. Excess amounts of Fe^{2+} may therefore cause great harm to the cell. Lipid peroxidation is a much-used biomarker of oxidative stress in ecotoxicological studies, and previous investigations have shown that birds that are exposed to metals have increased levels of lipid peroxidation residues (e.g. malondialdehyde)[58;91;92].

Autophagic lysosomal responses

Autophagy is an evolutionarily highly conserved cellular mechanism and a well regulated catabolic process in which essential cellular components, like proteins, are broken down. The monomeric units of the degraded macromolecules, for example amino acids, are later exported to the cytosol for reuse. Various stress conditions like food restriction, oxidative stress or pathogen infection can initiate the autophagic processes which plays important roles in maintaining cellular homeostasis[93;94]. Impairment of autophagic processes is associated with

the development of several pathologies, such as tumorigenesis and neurodegenerative conditions such as Alzheimer's disease[94].

Several studies have shown a strong association between exposure to environmental contaminants and autophagic dysfunctions, and parameters that indicate such dysfunctions are regularly used as biomarkers of xenobiotic-induced stress in wild animals, particularly marine invertebrates and fish[95;96].

Lysosomes

Lysosomes are spherical intracellular organelles of various size and shape which were discovered by Christian de Duve and colleagues in the 1950s. They are separated from the cytosol by a lipoprotein membrane, and the lumenal environment in the organelle is strongly acidic with a pH of approximately 4.8. The lysosomes function as the cells' waste managers and contain more than 50 different enzymes, most of them acid hydrolases such as proteases and lipases[97-99]. They can fuse with different cellular membrane-bound compartments or organelles to receive macromolecules destined for degradation. Bacteria, viruses or other foreign particles that have entered the cell are degraded in these organelles[100]. Additionally, cytoplasmic components are delivered and digested through autophagy in the lysosomes.

The lysosomal membrane

The lysosomal membrane has many of the same permeability properties as other biological membranes. Compounds with certain chemical properties can diffuse across the membrane while most substances can only enter or leave the organelle through active transport. Specific porters for the transport of a wide range of metabolites such as amino acids and sugars are located in the membrane and facilitate the efflux of these compounds bound for the cytosol[101]. The lysosomal membrane can, however, be damaged by xenobiotics that enter the cell. Some xenobiotic compounds possess properties that make them able to enter the lysosomes and perturb lysosomal function and integrity [102]. Reactive oxygen species, generated by intracellular mechanisms or induced by xenobiotics both inside and outside of the lysosome, can harm the lysosomal membrane by increasing its susceptibility to permeabilization[103]. Alterations of the lysosomal membrane properties may cause various detrimental cellular effects such as cell death by apoptosis or necrosis [104;105].

Several of the environmental contaminants that are currently causing concern possess properties which potentially allow them to enter the lysosome and perturb its functions, for

example by destabilizing the lysosomal membrane, and such effects have been shown after exposure to environmental pollutants in previous studies of marine animals[96;106-110]. However, investigations of lysosomal membrane stability related to contaminant exposure in seabirds have not previously been reported.

Lipofuscin

Lysosomes break down large molecules to amino acids, fatty acids and simple sugars which later are used in anabolic processes. Over time, a non-degradeable brown-yellow pigment called lipofuscin or age pigment will accumulate as a result of imperfections in the lysosome activities[111]. Lipofuscin consists mainly of various oxidized protein residues and lipid components, such as triglycerides, free fatty acids, cholesterol and phospholipids, in addition to some carbohydrates and small amounts of metals[111]. Lipofuscin is primarily located in secondary lysosomes, also called residual bodies, which are vacuoles formed by the fusion of a lysosome with a phagosome or organelle[112]. In the microscope, these can be seen as electron dense masses surrounded by lysosome membranes[113;114]. Several mechanistic dysfunctions in lysosomes are proposed to contribute to the accumulation of lipofuscin, including inactive or lost enzymes, loss of important non-enzymatic proteins or disruption of trafficking to and from the lysosome. Moreover, dysfunctions in the autophagic pathway have been shown to increase with age and contribute to the accumulation of lipofuscin[111;112;115]. Oxidation of proteins and lipids is another mechanism involved in the generation and accumulation of lipofuscin. The acidic lumen of the lysosome allows degradation of cellular components like mitochondria, which contain metallo-proteins such as ferritin and cytochromes. In this process ferrous iron can be released[116]. In the presence of hydrogen peroxide originating from various cellular oxidative reactions, hydroxyl radicals are generated through the Fenton reaction. In the presence of unsaturated fatty acids the hydroxyl radicals initiate a chain reaction leading to the generation of aldehydes such as malondialdehyde[90;117]. These aldehydes can react with and cross-link protein residues, creating the polymeric material which constitutes the bulk of the lipofuscin granule[111].

Generation of malondialdehyde (MDA) as a feature of the generation of lipofuscin in the lysosomes is a result of the presence of hydrogen peroxide and iron in proximity of unsaturated fatty acids. This process is not specific to lysosomes, but can also happen in other compartments in the cell[90;117]. An increased cellular ROS generation caused by contaminants can, under the same conditions, lead to lipid peroxidation and generation of MDA. MDA levels

have therefore been used as a biomarker of oxidative stress in relation to contaminant exposure in both birds and other animals[27;91;92;118].

Neutral lipids

Lipids are loosely described as a heterogeneous group of naturally occurring small hydrophobic or aliphatic molecules derived from or consisting of fatty acids or components biosynthetically or functionally related to these. In organisms, lipids serve important roles as membrane building blocks, signaling substances and for energy storage[119]. Triacylglycerols and cholesteryl esters are examples of lipids that are classified as *neutral lipids*[120]. Intracellular unsaturated neutral lipids can be associated with secondary lysosomes or be present as cytoplasmic lipid droplets[121;122]. Changes in intracellular content of neutral lipids have been associated with various stress responses. For example, neutral lipids have important roles in energy homeostasis[121] and dietary changes or other stress factors have been shown to mobilize lipids from lipid droplets[106;107;120;123]. However, observations of stress-induced *increases* of neutral lipid levels and induction of lipidosis-like conditions associated with increased autophagy and impaired lysosomes have also been reported[109;124]. Hence, levels of neutral lipids change in response to cellular stress, and may be used as an indicator of cellular well-being.

Due to environmental conditions, arctic seabirds go through cycles of "fattening" and emaciation, and are exposed to HOCs throughout their lifetimes. Lysosome associated responses to similar types of environmental stress in other marine animals have shown to be effective predictors of cell injury and animal health[96]. These biomarkers may therefore also be applicable for arctic seabirds.

Main objective and secondary goals

The main objective of this doctoral thesis was to relate cellular responses to environmental stress factors (contaminant exposure and food deprivation) in arctic seabird chicks

Specific aims were to investigate responses of the antioxidant defense system and the autophagic lysosomal processes in liver from arctic seabird chicks, and to relate these responses to stress induced by contaminant exposure and food deprivation.

Secondary goals

- Characterize and compare the antioxidant defense system in livers from black-legged kittiwake (BK), herring gull (HG) and northern fulmar (NF) chicks in relation to contaminant levels (field study; paper 1).

- Carry out a feeding and exposure experiment with newly hatched HG chicks and accomplish realistic HOC exposure and fasting resembling natural conditions (papers 2, 3 and 5).

- Assess the effect of a realistic HOC exposure and fasting on the hepatic antioxidant defense system (single antioxidant parameters and total oxyradical scavenging capacity) in HG chicks (experimental study, paper 2).

- Assess the effect of a realistic HOC exposure and fasting on the total renal oxyradical scavenging capacity in HG chicks (experimental study, paper 3).

- Characterize and compare hepatic lysosomal autophagic parameters (lipofuscin, neutral lipids, lysosomal membrane stability, lipid peroxidation) in BK, HG and NF chicks in relation to contaminant levels (field study, paper 4).

- Assess the effect of realistic HOC exposure and fasting on hepatic lysosomal autophagic parameters (lipofuscin, neutral lipids, lysosomal membrane stability, lipid peroxidation) in HG chicks (experimental study, paper 5).

Materials and Methods

Samples

This thesis is based on analyses of liver samples collected from wild-living chicks of three of the most common seabirds on the northern hemisphere, BK, HG and NF. In addition, liver and kidney samples from captured and reared HG chicks which were experimentally exposed to contaminants and/or fasted were analyzed.

Study species

Herring gull, Larus argentatus

HG is a member of the laridae family, which comprise the bird species informally called "seagulls". It is one of the most abundant gull species and has a circumpolar distribution[125]. In Western Europe it is found breeding from the south of France to the northern part of Norway and sporadically on Spitsbergen, mainly along the coast. The HG is a large gull species which can weigh as much as 1200 g and have a wingspan of up to 155 cm[125]. It is an omnivorous, opportunistic feeder with occasionally necrophagous, predatorial and kleptoparasitic foraging behavior. Consequently, in addition to being a common sight along the shores, it is also a frequent visitor at garbage dumps and trash cans in populated areas, particularly along the coast[126;127]. Due to its varied diet which includes anthropogenic waste and its high trophic position in the food web, this species risks exposure to environmental contaminants and xenobiotic substances.

Photo: Juvenile herring gull, Marit Nøst Hegseth

This gull was chosen as a study species due to its distribution across the northern hemisphere, trophic position and foraging behavior. In addition, HG chicks are semi-precocial, meaning that they are hatched with down and open eyes and although they are mobile, they stay close to the nest and are fed by their parents. This property makes the chicks robust and suitable for experimental work, and was the main reason why herring gulls were chosen.

Black-legged kittiwake, Rissa tridactyla

The BK is also a member of the laridae family, but is smaller than HG. It has a wingspan of 90-100 cm and weighs no more than 450 g[128]. It is the most numerous gull species in the world, has a circumpolar distribution and breeds in arctic and temporal zones across the northern hemisphere. BKs are common in Svalbard, with an estimated 270,000 breeding pairs. It is the only gull species that hatches in cliffs, and the chicks are therefore bound to the nest until they fledge. The chicks are fed regurgitated food from their parents[128]. The BK is a pelagic species outside of the breeding season with a diet mainly consisting of small fish and invertebrates. The BK is therefore positioned at a slightly lower trophic position than the herring gull, and the potential for exposure to dietary contaminants depends on the contaminant level in the prey they catch[4;6].

Tissue from the BK has generally been shown to have lower contaminant levels than the herring gull, indicating less exposure[17]. Due to the close phylogenetic relationship the HG and BK can be expected to have a similar physiology. However, obvious differences in diet, life history and behavior make it interesting to compare these two species to reveal possible differences also on the physiological level.

Photo: Black-legged kittiwake on an ice floe outside the Brøgger peninsula in Kongsfjorden, Spitsbergen, August 2007. Photo: Marit Nøst Hegseth

Northern fulmar, Fulmarus glacialis

NFs are members of the procellariidae family which includes petrels and shearwaters, and is the only member of this family that breeds in Svalbard. They are found in the same areas as HG and BK, but are strictly pelagic and will only stay on land for breeding. They are slightly bigger than BK, with a wingspan of up to 120 cm, but are stockily built and considerably heavier, weighing up to 1000 g[128]. Due to their pelagic lifestyle their diet is tightly connected to the arctic-pelagic food web and consists of small fish in addition to a wide range of invertebrates such as crustaceans, squid and polychaetes[6;129]. Another habit of the northern fulmar is to follow boats, fishing vessels in particular. A part of its diet therefore often consists of waste from ships, and can vary from fish offal and food scraps originating from the kitchen to unfortunate objects such as plastic waste, which has been known to kill many NFs after ingestion[130]. Fulmars are otherwise known for longevity. Because of their varied diet they place themselves at a higher trophic level than BK, but lower than the HG, and are known to have moderate contaminant body burden[6;129].

Photo: Northern fulmar in Kongsfjorden, Spitsbergen, August 2007. Photo: Marit Nøst Hegseth

However, previous studies have shown that NFs have a less efficient biotransformation capacity towards some classes of xenobiotics than other examined seabird species[131], and may therefore be at greater risk of harmful effects than bird species with better metabolic capability. Another particular feature of the fulmar is an oil-like substance which they keep in their proventriculus, or craw, an expanded and muscular pouch which is basically an enlarged part of the esophagus that normally is used in birds for storage of food before it reaches the ventricle. The oil consists mainly of triglycerides and wax esters, and it is used both as a defense mechanism (when it is spat at predators) and as an energy supplement for the bird itself and its chicks[132]. These features are among the physiological factors that separate the

fulmar from the gulls and this, in addition to the similarities in habitat and diet, makes it an interesting species to compare to the two gull species.

Field samples

Papers 1 and 4 are based on samples from wild BK, NF and HG chicks. Sampling details are given in these two papers. BK chicks were sampled from their nests at the colony called "Krykkjefjellet" in Kongsfjorden in Svalbard (78°55′N 11°56′E). The NF chicks were also sampled in Kongsfjorden, but had left the nest and were therefore caught at sea. The HG chicks had also left the nest at the time of sampling, and were caught at sea near Sommarøya outside Tromsø, Norway (69° 37′ 55.07″ N, 18° 1′ 11.16″ E).

Experimental samples

Papers 2, 3 and 5 are based on liver samples from 40 HG chicks that went through a feeding and fasting experiment during the summer of 2008. This experiment is described briefly in paper 2 and 3, but will be more elaborately presented in the following section.

The HG chicks were collected in the end of June 2008 at Sommarøy outside Tromsø, Norway (69° 37′ 55.07″ N, 18° 1′ 11.16″ E). The chicks were 2-5 days old when collected, and were transported immediately after collection to outdoor cage facilities located at Kårvika aquaculture research station at Kårvik, Ringvassøy (outside Tromsø).

The cage facility was a 48 m^2 (approximately 4x12 m) building roofed with corrugated plates, walls consisting of chicken wire and wooden floors built on low pillars. The inside was divided in four cage rooms separated by one solid wooden wall between the two middle rooms, and chicken wire walls between the other compartments. A passage alongside the rooms provided access to the separate rooms, and was used for food preparation and equipment storage (see figure 2).

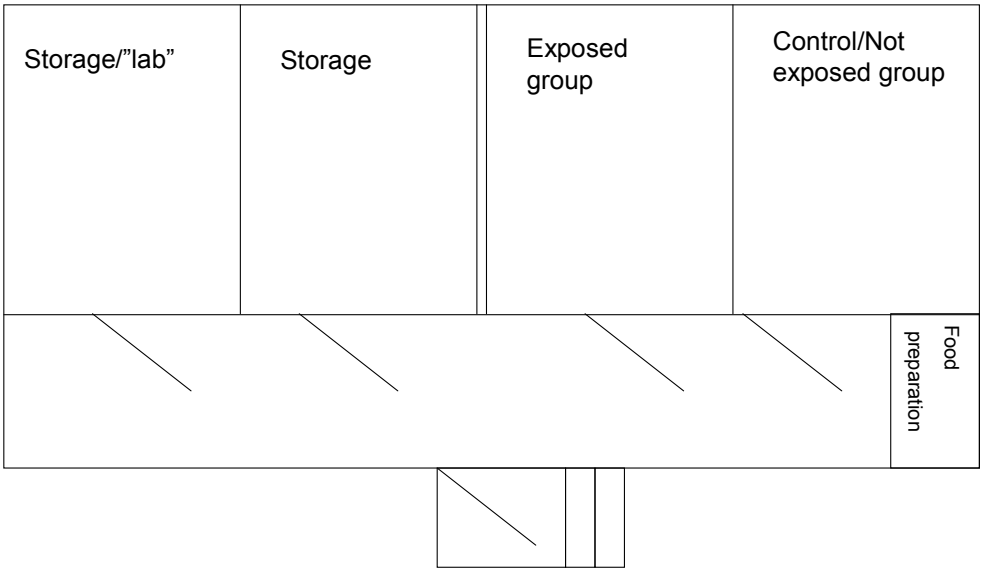


Figure 1

Sketch of the cage facility used in the herring gull experiment

The bottom 60 cm of the walls was covered with solid wooden plates to preclude visual contact between the neighboring cage rooms to prevent stress, and as shelter from wind and rain. Using outdoor cages ensured that the light and temperature conditions matched the natural situation during the northern Norwegian summer for HG chicks. Heating lamps were used on particularly cold and windy days throughout the whole experimental period.

The first 18 days the chicks were kept in a common 9 m^2 cage room for acclimatization. During this period they were provided heating lamps and cardboard boxes filled with paper bedding for extra shelter. The floor was covered with thick paper which was changed several times daily, but after two weeks, blisters and other afflictions started to appear on the chicks' feet due to the lack of the toughening of the sole of the foot that normally occurs when the chicks walk on rock. The paper cover was therefore removed, and the chicks walked on the wooden floor with occasional strips of roofing felt and rocks scattered around. They were also given tilted ladder-like installations for climbing, perching and hiding. Plants, seaweed and sticks were distributed as amusement and recreation. Drinking water was available in several large plastic cups mounted on the walls in head-high positions, and fresh water for bathing was offered in several low plastic vats up to 4 times a day.

The first period the chicks were fed a diet consisting solely of herring fillets twice a day complemented with two pills each week containing food supplements (minerals and

vitamins) suited for marine animals in captivity, and they had unlimited access to drinking water. The food was distributed to the birds *ad libitum* at an approximate amount of 150 g per bird daily, but individuals with unsatisfactory growth rate were additionally hand fed. The last weeks they received fish entrails and small amounts of haddock, salmon and commercial dog- and catfood in addition to the herring filets to vary the diet and avoid anorexia due to monotonous food.

Growth (weight, tarsus size, wing length) of each individual was measured every second day the first weeks, and weight was monitored every second day throughout the experimental period.

Herring gull chicks in the cage facilities. Left: approximately 14 days old. Right: approximately 1 month old. Photo: Marit Nøst Hegseth

After 18 days the chicks were separated in two groups, one control and one exposed group, with 20 individuals in each, in two neighboring 9 m^2 cages (see figure 2). Exposure started at day 19. The control group was given clean cod liver oil without extra additives or supplements (provided by Möller's/Axellus AS, Postboks 4293 Nydalen, NO-0402 Oslo). Commercially sold cod liver oil goes through a cleaning process in which organic contaminants are separated in a particular fraction which is removed and normally discarded. The exposed group was given this particular fraction of the cod liver oil, containing a natural mix of halogenated organic contaminants. By using this exposure regime we were able to study the effect of a naturally occurring mix of organic contaminants that had gone through biotransformation on their way through the food chain, and not an artificial mix or single compounds. Both groups got approximately 12 ml of oil daily which was fed to the birds individually using a 20 ml syringe connected to a silicon tube. The chicks were exposed for 43 days before sampling.

At the first sampling, 43 days after the start of the contamination regime, nine individuals each from the control and the exposed groups were sedated by inhalation of oxygen-driven isoflurane before blood samples were taken from the wing. The birds were then sacrificed by decapitation and organs were sampled. The liver was dissected out, divided into smaller pieces. Liver pieces for biochemical analyses were immediately frozen in liquid nitrogen and stored at -80°C. The liver samples for contaminant analysis were stored at -20°C until analyses were conducted.

The remaining nine individuals in the two groups were then exposed to food restriction for one week by reducing the daily food rations approximately 70% before the birds were sacrificed and samples taken. This was done to simulate a natural fasting situation with a drastic reduction in energy intake resulting in an average weight loss of approximately 10% before sampling.

Three chicks were humanely sacrificed during the experiment due to untreatable self-inflicted injuries. The experiment was approved by the Experimental Animal Welfare Committee of Norway.

Biochemical and histological analysis

The methods that were chosen for sample preparation and analysis of the antioxidant parameters are well described and commonly used in similar studies of cell systems in tissue from other species. The methods were optimized for use on bird liver tissue in the laboratory at Akvaplan-NIVA AS in Tromsø, and the details are described in papers 1 and 3.
The histological work and analyses of lipid peroxidation were performed in the laboratory of Prof. Francesco Regoli at the Polytechnic University of Marche, Ancona, Italy. The measurements of neutral lipids, lysosomal membrane stability and MDA levels were done according to standard methods and are described briefly in papers 2 and 4 and in greater detail in Bocchetti *et al*[106]and Regoli *et al*[110]. For determination of lipofucsin content, the cryostat sections were mounted in a glycerol-gelatin mounting medium and analyzed microscopically (lipofuscin fluorescent spectrum: excitation maximum at 340-390 nm, emission maximum at 430-490 nm, using a DAPI filter). The measurement of lipofuscin autofluorescence in normally not the preferred method to detect lipofuscin granules due to possible interference from other intracellular fluorescent compounds that emit light at the same wave length. Nonetheless, this approach was chosen for these studies because Schmorl's reaction, which is

is used to detect lipofuscin, did not stain the bird liver tissue. In Schmorl's reaction lipofuscin reduces ferric ferrocyanide to prussian blue, but this reaction was not apparent in this case. This could be due to a lack of lipofuscin in the samples, or that the lipofuscin in our samples was not in such an oxidative state that it was capable to reduce the ferric ferrocyanide, or that the tissue itself was not able to retain the stain. The last mentioned was probably the case, as attempts to stain with eosin and hematoxylin were also unsuccessful. In any case, the approach of examining the tissue for lipofuscin through autofluorescence was chosen to avoid further uncertainties related to unsuccessful staining.

Table 1

Overview of the methodology and original references for the biochemical analyses that have been used in the studies included in this thesis.

Measured parameter	**Method description**	**Reference**
Catalase activity	Enzyme activity assessed spectrophotometrically by measuring the decrease of H_2O_2.	[133]
Glutathione reductase activity	Enzyme activity assessed spectrophotometrically by measuring the rate of NADPH oxidation.	[134]
Se-dependent/Se-independent glutathione peroxidase activity	Enzyme activity assessed spectrophotometrically by measuring the rate of NADPH oxidation in the presence of glutathione reductase and H_2O_2 or cumene hydroperoxide as substrates.	[135]
Superoxide dismutase activity	Enzyme activity assessed spectrophotometrically by measuring the rate of inhibition of the generation of the formazan dye WST-1 through reaction with $O_2^{\cdot -}$ generated from the conversion of hypoxanthine by xanthine oxidase	[136]
Glutathione concentration and GSH/GSSG ratio	Assessed by an enzymatic recycling method using glutathione reductase and Ellman's reagent (5,5'-dithiobis-2-nitrobenzoic acid, DTNB). For the measurement of GSSG, samples were previously treated with 2-vinylpyridine	[137;138]
Total Oxyradical Scavengicg Capacity against OH•/ROO•/ONOOH	Quantification the efficiency of cellular antioxidants to inhibit the reaction between artificially generated radicals and the substrate α–keto- γ-methiolbutyric acid by measuring the amount of ethylene gas generated. Respectively, fenton chemistry/ homolysis of 2-2'-azo-bis-(2-methylpropionamidine)-dihydrochloride/ decomposition of 3-morpholinosydnonimine were used to generate the three radical types.	[78;79]
Lipid peroxidation/Malonedialdehyde	Spectrophotometric measurment of the adduct formed between malondialdehyde and thiobarbituric acid	[90]

Contaminant analyses

Halogenated organic compounds

The HOCs were analyzed by the laboratories of the Norwegian Institute of Air Research (NILU) in Tromsø and at Kjeller. Details about the methods for HOC analysis in the present study are given by Nøst (2009)[139] and Helgason et al. (2010)[131]. In brief, approximately 2 g of liver tissue was homogenized and dried in sodium sulfate. The homogenate mix was transferred to a glass column where it was extracted with cyclohexane/acetone. An additional cleanup and fractionation step based on a method by Sandanger *et al.* (2007)[140] was carried out on a Zymark Rapidtrace Automated SPE workstation (Caliper Life Sciences, Inc.) using columns packed with 1 g florisil. $MeSO_2$- and OH-metabolites were additionally cleaned up on acidic silica columns. Three fractions were collected: fraction 1 was 7.2 mL of dichloromethane/n-hexane (1/4 v/v) as the eluting solvent containing neutral compounds, fraction 2 was 9 mL of acetone/n-hexane (1/10 v/v) containing $MeSO_2$-PCBs/$MeSO_2$-DDE, and finally fraction 3 was 15 mL metanol/dichloromethane (1/5 v/v) containing the OH-PCBs. The third fraction was evaporated to dryness and derivatized using 1 mL of diazomethane in n-hexane. A final clean-up was performed on the Rapidtrace using a column of 0.7 g of 25% sulfuric acid silica with 0.2 g neutral silica on top and extracted using 8 mL of dichloromethane. Evaporation steps were performed on a Labconco evaporator (Labconco Corp., Kansas City, MO). The amount of extractable organic material (EOM) was determined gravimetrically. All plasma extracts were transferred to gas chromatograph (GC) vials with 150 µL inserts, and isooctane was added before a gentle evaporation with nitrogen gas to the final volume. Finally, 10 µL of octachloronaphthalene was added as recovery standard. The analysis of the compounds was performed by high-resolution gas chromatography (HRGC) on an Agilent 7890A GC equipped with an Agilent 7683B automatic injector and an Agilent 5975C mass spectrometer (MS) (Agilent, Folsom, CA). The GC was fitted with a 30 m DB-5 MS column (5% phenyl-methylpolysiloxane; 0.25 mm i.d., 0.25 mm film thickness) from J&W Scientific (CA, USA). A 1 µL aliquot of the sample extract was injected without splitting and helium used as a carrier gas at a constant flow of 1.5 mL/min. Temperature program: initial temperature 70°C (2 min), 15°C/min to 180°C, 5°C/min to 280°C (5 min). The MS was running in the negative ion chemical ionization mode (NICI) for metabolites. The MS was operated in single ion monitoring mode (SIM). All chemical analyses followed international requirements for quality and control (QA/QC), e.g., recommendations of the

Arctic Monitoring and Assessment Programme (AMAP) and the requirements in the European quality norm EN 17049. For each batch of 10 liver samples one standard reference (NIST Cod oil 1588a) and one blank were prepared.

These halogenated organic compounds were detected in one or more samples: PCB 18, 28, 31, 33, 37, 47, 52, 66, 74, 99, 101, 105, 114, 118, 123, 128, 138, 141, 149, 153, 156, 157, 167, 170, 180, 183, 187, 189, 194, 206, 209, pesticides: o,p'-DDE, p,p'-DDE, p,p'DDD, p,p'DDT, α-hexachlorocyclohexane (HCH), β-HCH, pentachlorobenzene (PeCB), hexachlorobenzene (HCB), pentachlorophenol (PCP), tribromoanisole (TBA), polybrominated diphenyl ether (PBDE)-28, 47, 71/49, 99, 100, 119, 154, hydroxylated PCB and PBDEs: 4OH-PCB 107, 146, 187, 3OH-PCB153, 2-OH-BDE 68, methylsulphone-PCB/DDTs: 3/4$MeSO_2$-PCB 49, 52, 91, 87, 101, 110, 132, 141, 149, 174, 3-$MeSO_2$-DDE, 5-methoxy-BDE 47.

Metal analyses

The analysis of metals (Cd, Cr, Pb, Se, Fe) has previously been described in detail in a publication from Fattorini et al.[141] Briefly, the liver samples were dried (60°C) until the weight was constant, and then digested under pressure with nitric acid and H_2O_2 in a microwave digestor system (Mars CEM, CEM Corporation, Matthews, NC, USA). The metal concentration was assessed by atomic absorption spectrophotometry with flame and flameless atomization (SpectrAA 220FS and SpectrAA-240Z Zeeman, Varian, Mulgrave, VIC, Australia). Quality assurance and quality control was done by processing blank samples and reference standard materials (lyophilized mussel tissue, Standard Reference Material NIST-2977, National Institute of Standards and Technology, Gaithersburg, MD, USA). Concentrations obtained for standard reference materials were always within the 95% confidence interval of certified values.

Summary of results

Paper 1

Hepatic antioxidant processes related to levels of PCBs and metals in chicks of three Arctic seabird species.

In paper 1 we investigated the properties and species differences of antioxidant defenses in BK, NF and HG chicks and the putative relationship between the antioxidant responses and liver concentrations of the essential metals selenium and iron in addition to halogenated organic compounds, represented by polychlorinated biphenyls (PCBs).

The results showed significant differences in contaminant levels and antioxidant activity and antioxidant defense capacity between the species. The levels of antioxidant defense parameters were in the same range as previous findings in adult terrestrial birds, although glutathione levels in BK and HG were higher than what had been reported in previous studies and CAT activity was considerably lower in BK chicks than in other bird species. The data set consistently showed a pattern of huge differences between BK and NF chicks, whereas HG chicks in general had intermediate responses. The contrasting pattern applied to both antioxidant responses and levels of metals and PCB. BK had the lowest levels of contaminants. PCBs, selenium and iron levels were positively related to the responses of antioxidants with potential to reduce $HO^{\bullet}/H_2O_2$ (Se-dependent GPX, CAT and TOSC against $HO^{\bullet}$) while the opposite trend was observed for Se-independent GPX, TOSC against peroxyl radicals (ROO·) and peroxynitrite (ONOOH). However, direct causal relationships between antioxidant responses and PCB concentrations could not be shown on an individual level.

Paper 2

Effects of exposure to halogenated organic compounds combined with dietary restrictions on the antioxidant defense system in herring gull chicks.

In paper 2 we investigated the effects of exposure to HOC, particularly PCBs, and fasting on the hepatic antioxidant defense system in HG chicks that were exposed to contaminated cod-liver oil

for 6 weeks and then fasted for 1 week. We assessed the hepatic total oxyradical scavenging capacity (TOSC) against peroxynitrite, hydroxyl and peroxyl radicals, and measured levels of glutathione (reduced: GSH, and oxidized: GSSG) and the enzymatic activities of catalase (CAT), glutathione peroxidase (GPX), glutathione reductase and superoxide dismutase.

The results showed that fasting significantly increased the HOC levels in the HG chick livers (ΣPCB; Control group, fed = 20.6 ng/g ww, fasted = 41.9 ng/g ww ($p<0.05$), Exposed group, fed = 448.5 ng/g ($p<0.0005$), fasted = 2296.0 ng/g ww ($p<0.0005$)). Limited effects were observed on antioxidant responses; significant effects were only found for CAT activity, Se-dependent GPX activity and the GSH/GSSG ratio in the exposed, fasted group. CAT and Se-dependent GPX activities correlated negatively with the PCB concentrations within this group, and a non-linear relationship between glutathione and contaminant levels was also found. These effects were generally not observed after exposure or fasting alone.

Paper 3

Impact of Halogenated Organic Contaminant Exposure and Fasting on Antioxidant Defense System in the Kidney of Herring Gull Chicks

In paper 3 we investigated the effects of PCB exposure and fasting on the total renal oxyradical scavenging capacity (TOSC) in HG chicks that were exposed to contaminated cod-liver oil for 6 weeks and then fasted for 1 week. TOSC against three reactive species were measured: peroxynitrite, peroxyl, and hydroxyl radicals.

We found a similar trend for the TOSC against all three radical types. Fasting alone reduced the TOSC value, and exposure reduced it further, but interestingly, the combination of fasting and exposure led to an increased TOSC value compared to the other treatment groups. At an individual level positive correlations between high ΣPCB and TOSC against ROO and HNOOH were found.

Paper 4

Lysosomal and lipid-associated parameters in the livers of three species of arctic seabird chicks: Species differences and relationships with contaminant levels

In paper 4 lysosomal membrane stability, lipofuscin (LF), malondialdehyde (MDA) and neutral lipid (NL) levels were analyzed in liver of wild BK, HG, and NF chicks. Possible relationships between parameters associated with lysosomal autophagy and concentrations of halogenated organic compounds (HOCs) and Cr, Cd, Pb and Fe were investigated.
There were significant species differences in the levels of NL, LF and lysosomal membrane stability, but these parameters were not directly associated with the respective HOC concentrations (BK: 25.56 ± 7.4 ng/g ww, HG: 71.80 ± 51.7 ng HOCs/g ww and NF: 193.19 ± 80.6 ng/g ww). However, accumulation of both LF and NL in the three species was positively associated with the respective Cr, Cd and Pb concentrations. HG chicks presented the lowest lysosomal membrane stability in addition to the highest levels of LF, which indicated that there was a tendency towards impaired lysosomes in this species compared to NF and BK. Lipid peroxidation was associated with HOC and Fe^{2+} levels, and specific HOCs showed positive and significant correlations with MDA levels on an individual level in HG chicks.

Paper 5

Effects of contaminant exposure and food restriction on hepatic lysosomal and lipid associated parameters in herring gull chicks.

In paper 5 lysosomal autophagic responses such as lysosomal membrane stability, neutral lipids (NL), lipofuscin (LF), and malondialdehyde (MDA) levels were analyzed and related to levels of halogenated organic contaminants (HOCs) in HG chicks experimentally exposed to halogenated organic compounds through the diet, and fasted. NL stores were depleted and lysosomal membranes were destabilized after exposure to HOCs and food deprivation, and after the combination of both stressors. These responses were not related specifically to one type of stress or its magnitude. Synergistic or additive effects of HOC exposure and fasting were not observed. LF accumulated and MDA levels increased as a result of fasting, but were unaffected by HOC

exposure. LF accumulation was strongly associated with percent weight change, such that large weight loss was associated with high LF levels, and slight weight gain was associated with low LF levels. Hence, lysosomal autophagic processes in livers of herring gull chicks were affected by the stress that was imposed upon them, and food deprivation caused more directly effects than HOC exposure.

General discussion

Contaminant levels in the chicks

Field samples from free ranging chicks

Publications that report contaminant levels in chicks of BK, NF and HG from the Barents Sea area are scarce. However, a number of investigations have analyzed ΣPCB levels in adult individuals of these three species, and a handful of studies have reported levels of Cd and Pb. Hence, we have been able to compare our findings to contaminant levels reported by others. All concentrations and references to where these data were found are listed in table 2.

As expected, ΣPCB levels in the liver of BK, NF and HG chicks in our studies were considerably lower than what others had found in livers of adult individuals of the same species. HOCs such as PCBs accumulate over a lifetime and adults are thus expected to have higher levels of these compounds than chicks. Very young chicks can also have high contaminant concentrations due to maternal transfer of contaminants through the egg[34;172;173]. The concentrations are expected to decrease due to dilution during the chick's rapid growth in its first weeks of life, and has previously been shown in HG chicks[174]. It is, however, difficult to relate the contaminant levels in the HG chicks in our studies to the levels that were reported in HG chicks from Finland[174]. The degree of exposure to contaminants in these two colonies might be very different. Moreover, our investigations did not allow us to assess HOC kinetics in the population as we had only one sampling of chicks at the same age. Hence, our knowledge of the contamination level of the Tromsø population of HGs is limited to 55-day-old chicks.

Species and location	ΣHOCs ng/g ww	ΣPCBs ng/g ww	Cd ug/g dw	Cr ug/g dw	Pb ug/g dw	Ref.
Black-legged kittiwake chicks, wild	**25.56**	**16.8**	**0.207**	**0.047**	**0.126**	**175;176**
Adult Black-legged kittiwake, Jan Mayen	---	*378.5*	---	---	---	*17*
Adult Black-legged kittiwake, Bjørnøya	---	*520*	---	---	---	*17*
Adult Black-legged kittiwake, Svalbard	---	*480*	---	---	---	*17*
Adult Black-legged kittiwake, Barents sea	---	*492*	---	---	---	*9*
Juvenile Black-legged kittiwake, Hornøy	---	---	*0.18*	---	---	*35*
Adult Black-legged kittiwake, Hornøy	---	---	*14.4*	---	---	*35*
Adult Black-legged kittiwake, Prince Leopold is., Canada	---	---	*39.4*	---	*<0.08*	*177*
Adult Black-legged kittiwake, Bjørnøya	---	---	*16.2*	---	---	*178*
Adult Black-legged kittiwake, Hornøya	---	---	*27.8*	---	---	*178*
Adult Black-legged kittiwake, Ny-Ålesund	---	---	*48*	---	---	*178*
Northern fulmar chicks, wild	**193.19**	**73.5**	**0.301**	**0.121**	**0.157**	**175;176**
Adult Northern fulmar, Baffin bay	---	*158*	---	---	---	*17*
Adult Northern fulmar, Jan Mayen	---	*686*	---	---	---	*17*
Adult Northern fulmar, Alaska	---	*622*	---	---	---	*9*
Adult Northern fulmar, Faroe Islands	---	---	*16.4*	---	---	*35*
Adult Northern fulmar, Prince Leopold is., Canada	---	---	*24.2*	---	*<0.14*	*177*
Adult Northern fulmar, Bjørnøya	---	---	*36.6*	---	---	*178*
Adult Northern fulmar, Ny-Ålesund	---	---	*109*	---	---	*178*
Herring gull chicks, wild	**71.8**	**45.3**	**0.526**	**0.154**	**0.703**	**175;176**
Herring gull chicks, experiment, control group	**30.4**	**20.6**	**---**	**---**	**---**	**179;180**
Herring gull chicks, experiment, fasted group	**61.5**	**41.9**	**---**	**---**	**---**	**179;180**
Herring gull chicks, experiment, exposed group	**646.4**	**448.5**	**---**	**---**	**---**	**179;180**
Herring gull chicks, experiment, exp. and fasted group	**2937.6**	**2296**	**---**	**---**	**---**	**179;180**
Newly hatched herring gulls, Finland	---	*4300*	---	---	---	*181*
Adult herring gulls, Great Lakes, USA	---	*1000-3000*	---	---	---	*182*
Adult herring gulls, lowest contaminated colonies, Great Lakes	---	*1800-5000*	---	---	---	*183*
5-day-old herring gull chicks, Finland	---	*620**	*0.054***	---	*0.165***	*174*
Adult herring gull, Siberia	---	---	*26.3*	---	---	*35*
Adult herring gull, Hornøya	---	---	*2.18*	---	---	*178*
Adult herring gull, Ainov island, Russia	---	---	*0.53*	---	---	*178*

Table 2: Concentrations of HOCs, PCBs, Cr, Cd and Pb in livers from adult and juvenile BK, NF and HG from arctic areas. Observations in bold are from our studies, italic notations are observations published by others. References are given in the table. *= recalculated from lipid weight ** = recalculated from wet weight based on a 3x loss of weight during tissue drying

Published data on hepatic trace metal levels in arctic seabirds in general are scarce, and we have not been able to find reports on Cr levels in these species. One other investigation has presented Pb concentrations in liver of BK and NF chicks[177]. These samples were collected in Canada and the Pb levels were below the detection limit of the analysis. Hence, the Pb levels in BK and NF chick livers sampled in Ny-Ålesund, Svalbard, were higher than in the Canadian chicks. In the case of Cd, several studies present data from BK, NF and HG liver. The Cd levels

in BK and NF chick liver in our studies are approximately 100 times lower than what has been found in adult individuals of the same species in the same areas. One study included BK chicks from Hornøya, Norway, and reported Cd levels very similar to what we observed in the BK chicks from Svalbard[35]. Additionally, HG chicks in our study had approximately the same hepatic Cd level as adult HGs from Ainov Island in Russia, one-fourth of the level that was reported in adult HGs from Hornøya, and 10-fold higher Cd levels than 5-day-old HG chicks from Finland.

Mercury is the only trace metal that has been extensively investigated also in arctic seabirds[19]. However, we excluded this substance from our investigations because of limited resources. Therefore, despite its biological relevance, methyl mercury was not measured. We did not find any connections between the hepatic levels of inorganic mercury (Hg) and the effect parameters in our studies, and the liver concentrations of this metal were therefore not reported in our publications.

To summarize, our findings indicate that BK, NF and HG chicks generally have lower hepatic concentrations of contaminants – both organic and metals – than adult individuals of the same species in the same geographical areas. We assume that the concentrations of contaminants that are given in our publications reflect the levels in the chicks in the specific colonies at the time of sampling in the Svalbard and Tromsø areas.

The effects on the antioxidant defense system and autophagic lysosomal responses in the livers of the chicks in our studies have thus been observed at relatively low contaminant levels. As adult birds of these species have been found to have considerably higher contaminant levels than the chicks, it is recommended to investigate the antioxidant defense system and autophagic lysosomal responses also in adult birds.

Contaminant levels in the experimentally exposed HG chicks

In the HG chicks from the experimental study only ΣHOCs were analyzed. As seen in table 2, PCBs were found in the two groups which were not intentionally exposed to HOCs. Maternal transfer of contaminants[34] and contaminants in the food items (herring) were likely the primary causes for the presence of ΣHOCs (ΣPCBs) in these chicks. However, the ΣPCB concentrations in the control group were low compared to those in the wild ranging HG

fledglings from the same colony[175]. The wild ranging HG chicks had ΣHOC levels comparable to those in the control birds after fasting. The exposed HG chicks in the experiment had 10-50 times higher ΣPCB levels than the wild ranging chicks. This indicated that the contaminant exposure imposed during the experiment was higher than the natural exposure at the sampling site. However, the highest ΣPCB concentrations measured in the HG chicks from the experiment (the mean value of the 'Exp. & fasted' group) were approximately 50% lower than values detected in the livers of newly hatched HG chicks from Finland[181] and similar to the levels reported in the least contaminated HG colonies from the Great Lakes region[182;183]. These observations indicated that the exposure levels in the experiment were environmentally relevant, albeit higher than what was found in wild ranging HG chicks at similar age in the same colony. Additionally, the PCB levels in the "exposed" and "exposed and fasted" groups were within the same range as PCB levels reported in liver of other arctic animals at a high trophic level such as the polar bear and the arctic fox (see figure 4).

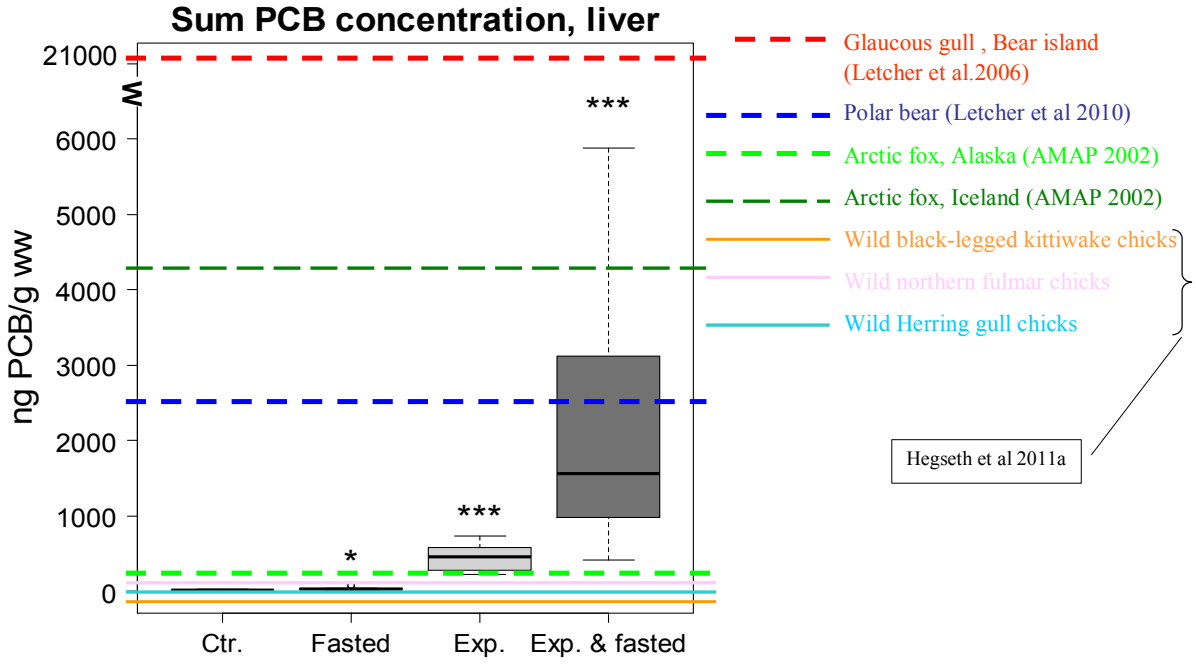


Figure 3: Liver PCB levels (ng/g wet weight) in HG chicks in the four treatment groups from the experiment (boxes with error bars). Previously reported PCB levels in Arctic animals at high trophic positions in addition to the HOC levels reported in wild BK, NF and HG chicks are indicated with stippled lines on the figure.

Some of the effects that we observed on the antioxidant defense system and autophagic lysosomal responses in the HG chicks in our experimental studies were dose-dependent. Hence, effect studies with older HG individuals with higher contaminant concentrations could possibly reveal more severe effects than what we found in the HG chicks.

Species differences in the antioxidant defense system in seabirds

– The influence of physiological factors

So far only a handful of investigations have examined antioxidant defenses in seabird species[58;63-67]. Similar to what we found in paper 1 these studies revealed species differences in antioxidant capacity in seabirds[142]. For example Corsolini et al. (2001) showed that penguins have significantly higher blood plasma TOSC against peroxyl radicals than other Antarctic seabird species[63]. Cohen and colleagues (2009) showed that species per se was an important explanatory factor with regard to antioxidant variations in different bird species[143]. Nevertheless, care must be taken when comparing levels of antioxidants between studies due to factors related to methodological differences or inter-laboratory variations. Bearing these uncertainties in mind, the results in paper 1 showed that the levels of hepatic antioxidant defense parameters found in BK, HG and NF chicks were in the same range as previous findings in livers of adult glaucous gulls and other terrestrial birds (great tits (*Parus major*), pied flycatchers (*Ficedula hypoleuca*), eider ducks (*Somateria mollissima*), Canada goslings (*Branta Canadensis*) and mallard ducklings (*Anas platyrhyncos*))[58;91;144-149]. This observation is interesting in an evolutionary perspective and indicates that antioxidants are highly conserved physiological features. The observed similarities also imply that the analytical methodology gives comparable results and that standardized methods used for laboratory studies of humans and other mammals are suitable also for various species of birds in the wild.

Life stage

The work included in this thesis has focused on nestlings and recent fledglings of three seabird species. In the studies cited above, the sampled birds were mainly adults, and the age difference complicates direct comparisons of antioxidant levels between the species. Indeed, Constantini and Bonadonna (2010) found that antioxidant capacity differed between nestling and adult blue petrels (*Halobaena caerulea*)[64] and that adults had higher antioxidant capacity than nestlings. The study by Wayland *et al*. (2010) of adult glaucous gulls reported hepatic GPX activities in the same range as we observed in the chicks in paper 1[58]. This suggests that the difference between the antioxidant capacity of chicks and adults of arctic seabird species is moderate. However, at present we are not familiar with the antioxidant levels in adult BK, NF or HG and there may be large inter-species differences in the adult populations.

In their study of blue petrels, Constantini and Bonadonna (2010)[64] suggested that the low antioxidant levels in nestlings compared to adults were due to underdeveloped antioxidant defense system or depleted antioxidant levels due to growth-induced increase in metabolic activities. Accordingly, we found in paper 1 that the youngest chicks (BK in our studies) had the lowest antioxidant capacity, particularly towards $OH^{\bullet}$ and H_2O_2 which are reactive oxygen species that are strongly related to metabolism. We argued that slight differences in life stage of the sampled chicks from the three seabird species may have influenced the activity of the antioxidant defense system, in addition to dietary influences. The BK chicks were still nestlings at the time of sampling and HG and NF chicks had already fledged. As pointed out by Constantini and Bonadonna (2010), energy expenditure and oxygen consumption increase during the nesting period towards the time of fledging due to muscle mass growth and higher metabolism[64;150-152]. Moreover, the period after fledging places extreme energy demands on newly fledged chicks[153]. Increased basal metabolic rate has been associated with increased antioxidant levels to meet the demand for antioxidant capacity when generation of reactive species increases[154]. We therefore find it reasonable to assume that the older chicks (NF and HG) experienced more extensive ROS formation than the younger chicks due to increased energy expenses, and consequently, that antioxidant scavenging capacity towards $OH^{\bullet}$ and H_2O_2 was augmented in HG and NF. Hence, we do not believe that BK had the highest metabolic rate of the three seabird species. However, this issue was not investigated as it was beyond the scope of

this thesis. Nor do we believe that BK chicks had the lowest antioxidant scavenging capacity towards $OH^{\bullet}$ and H_2O_2 due to depletion, as suggested for the blue petrel nestlings[64], but rather that the low antioxidant levels reflected low levels of ROS, a theory which is supported by our findings in paper 4.

In paper 4 we investigated autophagic lysosomal parameters such as accumulation of lipofuscin and neutral lipids, and decreased lysosomal membrane stability. These parameters are associated with increased autophagic rate and autophagy is induced when there is an increased demand for nutrients, for example as a result of increased metabolism or short term starvation[93;94;96;103]. Additionally, increased autophagic activities are associated with increased generation of ROS[96]. The older chicks, HG in particular, had short lysosomal membrane labilization time and elevated levels of lipofuscin compared to BK. These findings indicated that autophagic processes were less pronounced in BK chicks compared to NF chicks and HG chicks. Hence, differences in both autophagic lysosomal parameters and antioxidant defense against OH• and H_2O_2 point towards higher metabolic activity and higher generation of ROS in HG and NF compared to BK chicks.

Longevity

Aging and longevity was not a primary focus of our studies. However, due to the close relationship between oxidative damages, autophagic mechanisms and aging it is appropriate to address this topic as well. Lipofuscin accumulation and lysosomal dysfunction have both been related to aging[155]. However, we do not think it is relevant to discuss parameters such as lipofuscin accumulation or lysosomal membrane stability in the context of this thesis. The rationale behind this evaluation is primarily the very young age of the sampled chicks but also because we have only one sampling time and, hence, no basis for temporal comparison in our investigations. In addition, we do not have any other data from birds to compare our results with. Antioxidant capacity, on the other hand, is a frequently investigated topic in relation to aging, also in birds. Hence, we believe that it is relevant to examine our data from this perspective.

Oxidative stress has been extensively studied in birds, particularly during the past two decades[77;142]. A large share of these investigations has focused on oxidative stress in relation to

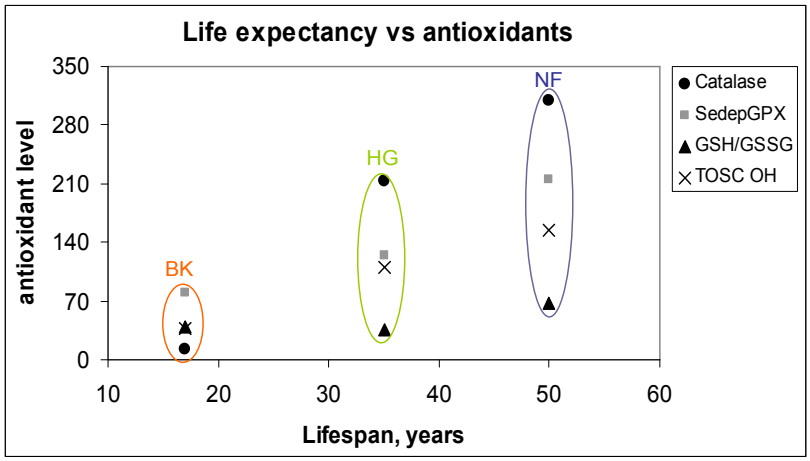
longevity and aging[142;156;157]. Birds in general have very long lifespan compared to mammals and may live up to three times longer than a mammal with the same body weight. In addition, seabirds generally have longer life expectancies than terrestrial bird species. In mammals, physiological factors such as body size, heart rate, metabolic rate and body temperature are strongly correlated with the expected duration of life[158]. Birds do not comply with these norms, as they generally have higher body temperature, higher metabolic rate and smaller body size compared to mammals with corresponding life expectancies[142].

One of the most widespread theories regarding why birds live so long concerns oxidative stress, or rather the absence of it[142;159]. Low levels of oxidative stress can be achieved through different strategies: reducing radical generation, increasing the efficiency of the antioxidant defense system or having greater resistance towards oxidative damage in cellular components such as membranes. The latter seem to be the case for some seabird species: it has been shown that their cellular membranes consist of a higher proportion of monounsaturated fatty acids than some terrestrial species, and are thus less likely to undergo lipid peroxidation and consequently sustain cell damage[160]. Another investigation implies that radical generation in mitochondria is low in birds compared to mammals, and that the DNA in birds is more resistant to oxidative damage[161]. Studies of the relationship between life expectancy and antioxidant levels across vertebrate species have shown diverging and contradictory results[162-164]. This indicates that, despite several attempts to find direct connections between antioxidant status and longevity, there is still no consensus regarding these factors.

Hepatic antioxidant capacity of three different seabird species was investigated in paper 1 in this thesis, and the most striking finding was the consistent patterns of species differences with large contrasts between the antioxidant levels in BK and NF chicks throughout the data set. These two species presented the highest and the lowest values, but neither of them had consistently high or low values for all the measured parameters. This has been further elaborated in the discussion in paper 1. HG chicks in general presented intermediate antioxidant levels. The expected life span of these three species differ in accordance with the contrasts in antioxidant capacity, since BK has the shortest life expectancy (15-20 years)[165], HG is expected to live approximately twice as long[166-168], while NF may reach the age of 50 years or older[169]. These differences in life expectancy are positively associated with the inter-species patterns of catalase and Se-dependent GPX activities, TOSC against $OH^{\bullet}$ and GSH/GSSG ratio, such that BK has the

lowest expected lifespan and the lowest levels of these antioxidant parameters, while NF with the longest life expectancy had the highest levels of these particular antioxidants. The opposite pattern was observed for non-Se-dependent GPX and TOSC against $ROO^{\bullet}$ and HNOOH, which adds to the divergence and inconsistencies reported in previous investigations[162-164].

a)



b)

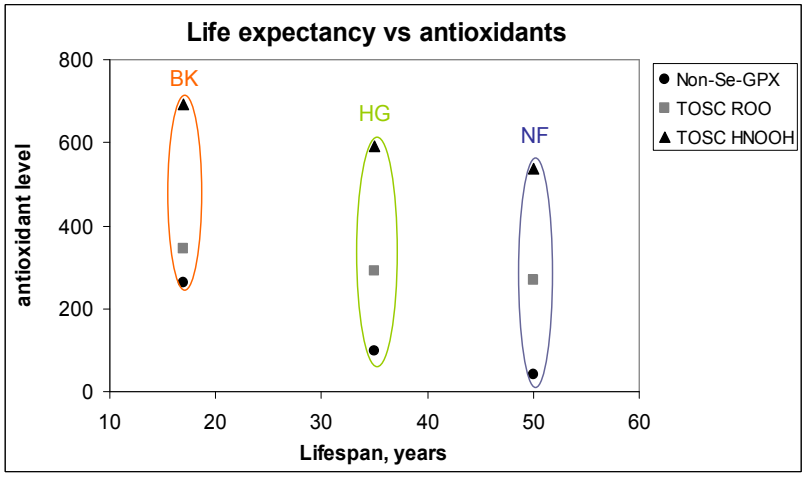


Figure 2 a) and b): Illustrations of the relationships between average levels of different antioxidants and theoretical life expectancy for black-legged kittiwake (BK), herring gull (HG) and northern fulmar (NF). 3a) Positive associations between the life expectancy of the species and average levels of catalase and Se-dependent GPX activites, GSH/GSSG ratio and TOSC-OH values. 3b) Negative associations between the life expectancy of the species and average levels of non-Se-dependent GPX activity, TOSC-ROO and TOSC-HNOOH values.

Constantini (2008)[142] argues that maximum life span in birds is not predicted by their respective antioxidant defense systems, but that the antioxidant defense system nonetheless is important in increasing the mean life span, such that manipulation of antioxidants by factors like diet may prevent diseases and pathological conditions. In addition, previous studies in birds have

implied that environmental factors, such as diet, influence the antioxidant capacity to a larger extent than genetic ones[67;143;170]. In paper 1 we explain the observed inter-species antioxidant patterns as responses to dietary differences which are reflected in the differences in levels of metals and PCBs. This is in accordance with Constantini's reasoning but there are, nevertheless, interesting connections between the antioxidant patterns and the life expectancies of the three species, as shown in figure 1. More specifically, the antioxidant defense mechanisms against $OH^{\bullet}$ and H_2O_2 were positively associated with life expectancy in BK, HG and NF chicks. Sohal (1992)[171] suggested that generation of $OH^{\bullet}$ and H_2O_2 could be a causal factor in aging, and hence that an extensive antioxidant defense against these reactive species might prevent DNA and membrane damage that would promote aging. The extensive defense against $OH^{\bullet}/H_2O_2$ that was observed in NF and correspondingly weak defense in BK may thus contribute to the contrasting lifespan of these two species.

Hence, according to our findings in paper 1 the antioxidant defense capacities were related to dietary differences and as shown here, antioxidant defense, particularly against $OH^{\bullet}/H_2O_2$, could play a role in determining the life expectancies of the three species. However, the seabirds in our study were chicks and relatively few individuals were sampled, so further studies that include larger samples, adult individuals and more species would contribute to elucidate the relationship between antioxidant defense and longevity in seabird species.

Effects of contaminant exposure on the hepatic antioxidant defense system in seabirds

Previous investigations have shown that exogenous factors such as organic contaminants and metals can affect the antioxidant defense system in birds[28;29;77;87;91;184]. The aim of the investigation presented in paper 1 was to describe possible associations between contaminant levels and antioxidant defense capacity in the three seabird species. No direct causal relationships between contaminant body burden and antioxidant responses were found. However, we did find associations between the average concentrations of metals and PCBs and the mean levels of the antioxidant capacities in the three species. The inter-species variation in PCB levels could mirror variation in other factors with potential impact on antioxidant levels. As diet is the main source of metals and halogenated organic contaminants such as PCBs in birds, food is an

important factor in explaining the variation in metal and PCB levels and, possibly, in antioxidant levels.

Indeed, diet has earlier been shown to be a major influential factor for the maintenance and function of the antioxidant defense system[143] as micronutrients from food function as non-enzymatic antioxidants or are essential for enzymatic processes. Selenium (Se) is required for proper functioning of Se-dependent GPX and iron is important for catalase activity[68;75;144]. Accordingly, we observed correspondence between the hepatic concentrations of these metals and activity levels of Se-dependent GPX and catalase in the three species. Furthermore, our observations in BK, NF and HG chicks were in accordance with relationships between Se concentration and Se-dependent GPX activity revealed in adult glaucous gulls[58], as shown in figure 2. Our NF and HG fledglings presented Se-dependent GPX activities and Se levels at the same magnitude and with the same response pattern as in adult glaucous gulls. Our BK nestlings had significantly lower levels of both Se and enzyme activity, but still in accordance with the relationship seen in glaucous gulls.

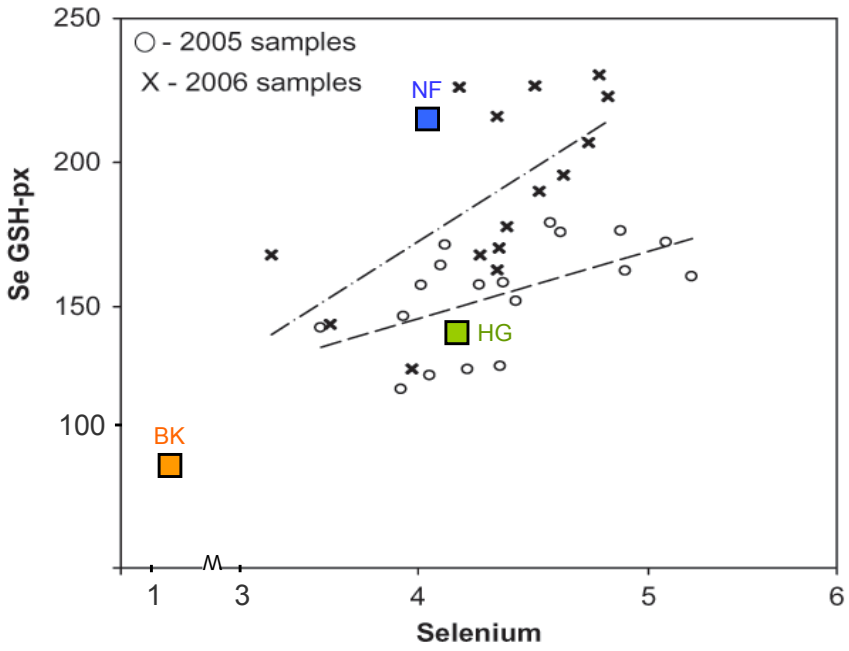


Figure 5 Modified from original figure (Wayland et al. 2010)[58]. Relationship between hepatic Se (µg/g dw.) and hepatic Se-dependent GPX activity (U/mg protein) in adult glaucous gulls sampled in 2005 and 2006 in the Canadian arctic (○ and ×, respectively[58]) including observations for average Se concentration and Se-dependent GPX activities[175] in BK, HG and NF chicks showed as orange, green and blue squares, respectively.

Hence, Se concentration appears as an important predictor of Se-dependent GPX activity in seabirds, also in chicks. A relationship between GPX activity and Se-concentration has previously been observed in terrestrial and aquatic bird species. Both high levels of Se and Se deficiency have been associated with increased oxidative stress[144;145;185], albeit through different mechanisms. Selenium in specific chemical states may oxidize thiols such as glutathione and reduce oxygen generating $O_2^{\bullet-}$; hence excess amounts of selenium may induce oxidative stress[186]. However, Se deficiency may also lead to oxidative stress through decreased Se-dependent GPX activity. Either way, selenium has a central role in regulating the antioxidant status in birds, and we have shown that this may also be the case in chicks of BK, HG and NF.

The experimental study presented in papers 2 and 3 was designed to reveal possible effects of HOCs on the antioxidant defense system in seabird chicks. The lack of direct PCB-inflicted effects on the antioxidant defense system found in paper 1 was assumed to be related to the moderate levels of ΣPCBs, which were approximately 30-150 times below the LOAELs (Lowest observed adverse effect level) and NOELs (No observed effect level) for end points previously indicated in arctic bird species and also below the recommended PCB concentration limit set in the Canadian guidelines for protecting birds[17]. However, the HOC exposure in the experiment led to considerably higher (5-80 fold) ΣPCB concentrations in the exposed HG groups compared to what was found in the wild chicks.

Particularly high hepatic ΣPCB concentrations were found in the group that had been fasted after the exposure period. In the most severely exposed individuals we did indeed detect changes in some of the antioxidants, as presented in paper 2. Catalase activity and Se-dependent GPX activity decreased in a dose-dependent manner when liver ΣPCB concentrations exceeded about 500 ng/g ww. In addition, a nonlinear, polynomial relationship between glutathione levels and ΣPCB concentrations was revealed in the chicks with the highest ΣPCB concentrations. This model suggested that HOC exposure induced a biphasic response with a counteractive, or compensatory, increase in glutathione levels up to a certain ΣPCB concentration (approximately 1500 ng/g ww). This was then followed by a toxic response, observed as a dose-dependent decrease in glutathione levels with higher PCB concentrations. Hence, we did observe effects of HOC exposure on the antioxidant responses in the liver of the exposed HG chicks, but to a limited extent and only in the individuals with the highest contaminant concentrations.

Antioxidant capacity in different tissues

In paper 3 we found that the antioxidant capacity in liver and kidneys responds differently to HOC exposure and food deprivation. The response to fasting per se was similar in the two tissues, with decreased average TOSC values for all three radical types. However, in the group that was both exposed and fasted there was a marked difference between the antioxidant capacities in the two organs. In the liver the TOSC values decreased with increasing HOC levels while in the kidneys the TOSC values were significantly higher in this group compared to the other groups. This effect was particularly pronounced for TOSC against ROO• and $ONOO^-$. Similar changes in antioxidant capacity have been observed in birds exposed to stress hormones, where total antioxidant capacity was slightly reduced in liver but significantly increased in kidneys[187]. In addition, the individual antioxidant scavenging capacity towards these radical types in the kidneys was positively correlated with PCB concentrations in the livers of chicks in the exposed groups, which indicated that this effect was mainly governed by HOCs, possibly through release of stress hormones (e.g. cortisol) in response to the increase in HOC levels during fasting.

Birds excrete the ammonia that is formed during protein catabolism as uric acid, as opposed to urea in mammals. Uric acid is an antioxidant which is particularly efficient as a scavenger of $ONOO^-$ [68]. Uric acid synthesis occurs partly in the kidneys and uric acid is filtered through the kidneys for excretion[188]. Hence, bird kidneys are constantly in contact with uric acid. Furthermore, stressful conditions may affect protein or amino acid turnover, and fasting has been shown to induce protein catabolism, resulting in increased excretion of uric acid[188]. The combination of high contaminant concentration and fasting may therefore have exerted significant stress on the birds. Stress induced protein catabolism may have caused an increased excretion of uric acid, resulting in increased renal TOSC values, particularly TOSC against $ONOO^-$. It would have been interesting to examine the concentration of uric acid, enzymatic activity of important biotransformation enzymes and potential oxidative damage such as lipid

peroxidation in the renal tissue to illuminate the mechanisms behind the TOSC responses, but such analyses were not done in this study.

The increase in TOSC against ROO• and $ONOO^-$ that we observed in the kidneys was not seen in the liver of the same HG chicks, which illustrates that antioxidant capacity is not uniform in the entire organism. The antioxidant defense system is dynamic and changes according to the particular requirements; at present we do not know which organ is the most sensitive towards oxidative stress in seabird chicks. We have concentrated mainly on the liver in our work due to practical and logistical considerations, but analysis of antioxidant capacity in other vital organs such as the brain or heart muscle in the seabird chicks would have been of great value in an extensive characterization of the antioxidant defense system in seabird species.

Oxidative damage, lipid peroxidation

One consequence of oxidative stress is lipid peroxidation. In paper 4 we described significant species differences in MDA levels that were consistent with the HOC concentrations in the species, and MDA levels in the individual wild HG chicks correlated with specific HOCs. As presented in paper 1 the highest antioxidant scavenging capacities towards $OH^{\bullet}$ and H_2O_2 were found in NF and HG chicks, the two species that also had the highest lipid peroxidation levels. We earlier suggested that increased ROS generation due to higher energy expenditure may have led to the greater production of antioxidants in HG and NF compared to BK chicks. Hence, increased ROS generation may also have caused increased lipid peroxidation in HG and NF chicks, which actually implies that the antioxidant capacities in the wild HG and NF chicks were overstrained. In paper 5 we reported that HOC exposure alone did not induce lipid peroxidation in the HG chicks, but increased levels of lipid peroxidation were found in both fasted groups (i.e. exposed and non-exposed to HOC). Hence, food restriction was a stronger inducer of lipid peroxidation than HOC exposure, and when combined, the two stressors did have synergistic effects. These results contradict what we reported in paper 4, where we found clear relationships between HOC concentrations and lipid peroxidation. Previous investigations confirm that relationships between organic contaminant exposure and lipid peroxidation in seabirds is complex and merely indicative[58;66]. Perez and colleagues (2010)[66] reported slightly

(but not significantly) elevated lipid peroxidation in blood plasma of yellow-legged gulls (*Larus michahellis*) experimentally exposed to crude oil (PAHs), and Wayland et al. (2010)[58] found weak relationships between hepatic mercury concentration and lipid peroxidation levels in glaucous gulls, and no significant associations between HOC/PCBs and lipid peroxidation. Hence, HOC exposure in itself is probably not a strong factor in inducing lipid peroxidation in seabird liver. Other stress factors that alone, or in combination with HOC exposure, impair the antioxidant defense system may on the other hand induce lipid peroxidation. Our data showed a clear difference in lipid peroxidation between the wild HG chicks and the chicks in the experiment; the wild chicks had significantly higher MDA levels than all the HG chicks in the treatment groups in the experiment. Most certainly the wild chicks experienced physiological challenges above and beyond the factors the chicks in our experimental set-up encountered, and faced challenges that were not measured in our limited studies. The nutrient intake and contaminant exposure of the captive chicks was thoroughly controlled, in contrast to the wild living chicks. These factors may have influenced the antioxidant capacity in the HG chicks in the experiment. Indeed, the GSH/GSSG ratio was significantly higher in the HGs in the experiment than in the wild living chicks. High GSH/GSSG ratio indicates a healthy antioxidant defense system, and the low GSH/GSSG ratio in the wild living HG chicks imply that these chicks were experiencing oxidative stress which may have induced lipid peroxidation.

Lipid peroxidation is a much used biomarker of contaminant-induced oxidative stress in studies of birds[58;91;92;149;189;190]. Our investigations illustrate that the contaminant compounds are not always the main reason for lipid peroxidation, but that other stress factors such as food deprivation or ROS generation induced by increased metabolism may be contributing or explanatory factors for oxidation of lipids.

Autophagic lysosomal responses

Autophagic lysosomal responses are valuable measures of early onset cellular effects of various forms of stress, including contaminant exposure. In papers 4 and 5 lysosomal parameters that have frequently used in studies of marine and terrestrial animals were investigated in seabird species. To our knowledge these were the first reported studies on lysosomes in arctic seabird species.

Neutral lipids

In paper 4 we found significantly higher levels of neutral lipids in wild HG chicks than in wild NF and BK chicks. In this paper we speculated that this lipidosis-like condition could be a consequence of impaired lysosomal function caused by xenobiotic exposure as shown in previous studies in marine invertebrates[124]. The associations between Cr, Cd and Pb concentrations and neutral lipid levels in the three species of wild seabird chicks indicated that metal exposure could induce neutral lipid accumulation. The experimental study was designed to address whether HOC exposure affected neutral lipid dynamics, and our results showed that HOC exposure depleted the neutral lipid droplets. Contradictory to our findings, previous investigations have found that neutral lipids accumulate as a consequence of exposure to organic contaminants[124;191,109;192;193]. However, the cited studies examined invertebrates and fish and species differences may therefore explain the differences in neutral lipid response.
Neutral lipids are important energy sources for cells; they are rapidly depleted during fasting and accumulate equally rapidly after a meal is ingested[122;124]. The results from the experiment reported in paper 5 illustrated this process, as the fasted HG chicks had significantly lower neutral lipid levels than the chicks in the control group. Hence, the variation in neutral lipid levels that we found in the wild chicks was possibly related to the nutritional status of the chicks rather than being caused by contaminant exposure. HG chicks, the species that had the highest neutral lipid levels, were fed fish entrails just before the sampling. BK chicks, which had the lowest neutral lipid levels, were still nestlings and were dependent on their parents for food, which may have led to brief fasting periods between feedings, and lower intracellular fat reserves. In seabird liver tissue, depletion of neutral lipids appeared as a general response to various types of stress, such as HOC exposure or food deprivation, rather than a specific biomarker of contaminant exposure.

No previous studies on how neutral lipid storage in birds responds to contaminant exposure have been reported. Other marine organisms such as gastropods, bivalves and fish have on the other hand frequently been examined, although without consistent results. Moore and colleagues (1998 and 2007) reported that exposure to contaminants such as PAHs, PCBs and copper was associated with lipidosis-like conditions and accumulation of NLs in lysosomes in bivalves[124;191]. These results have been confirmed in other studies of bivalves, as well as fish and

terrestrial gastropods exposed to organic contaminants[109;192;193]. Contradictory results were reported in yet other investigations in bivalves and gastropods, where depletion of neutral lipids after exposure to both metals and organic contaminants were found[194;195]. Additionally, Regoli (1992) found that variations in sediment metal concentration did not influence neutral lipid levels in bivalves, but that stress induced by transplantation to other areas caused depletion of neutral lipids [110]. Consequently, the usefulness of neutral lipid levels as a biomarker of contaminant-induced stress is continuously being debated. The results of our work contribute to this debate, and offer new information regarding which factors may affect intracellular neutral lipid status in seabird species.

Lysosomal membrane stability

Destabilization of the lysosomal membrane has been associated with augmented autophagic degradation as a consequence of various types of environmental stress[96;109;191]. Lysosomal membrane stability is often used as a biomarker of cell injury and animal health status in environmental sentinel species[96]. Numerous studies of invertebrates and fish have illustrated the connection between exposure to several classes of contaminants and decreased lysosomal membrane stability[109;192;196;197], and our results show that lysosomal membranes are destabilized as a result of contaminant exposure also in seabird chicks. High concentrations of Cr, Cd and Pb were associated with low lysosomal membrane stability in the wild chicks (paper 4). Exposure to Cd has been strongly associated with impairment of lysosomal integrity and function consistently across the phyla, from slime molds to bivalves and fish[96;109;110;192;197-200]. Oxidation of membrane lipids, inactivation of enzymes[201;202] or exceeded lysosomal storage capacity due to engulfed Cd-metallothionein complexes[199] have been postulated as underlying causes. Furthermore, the HG chicks that were exposed to HOCs in our experiment had lower lysosomal membrane stability than the control group (paper 5). However, food deprivation and HOC exposure destabilized the lysosomal membrane in the HG chicks to the same extent, and the combined stress with consequent elevated HOC concentration in the "exposed and fasted" group did not result in significant additional destabilization of the lysosomal membrane. Hence, the response that we observed was specific towards neither HOC exposure nor fasting, and the lysosomal membrane integrity did not respond to an increased amount of stress.

Destabilized lysosomal membranes are associated with impaired cell health, and have for example been related to liver damage and tumor progression in fish liver[109]. Consequently, stress factors that lead to reduced lysosomal membrane stability may cause cell damage and potentially liver pathology. Complete pathological examination of the seabird livers was outside the scope of our investigations, and we therefore do not know whether the destabilized lysosomal membranes in the HG chicks were associated with additional health issues.

Lipofuscin

In paper 4 we reported significant species differences in lipofuscin accumulation. However, we found no associations between HOC concentration and degree of lipofuscin accumulation, and concluded that lipofuscin accumulation was not related to HOC concentrations. The results from the experimental study presented in paper 5 confirm this finding, as HOC exposure did not induce lipofuscin accumulation in the HG chicks. We have not succeeded in finding information from previous studies regarding lipofuscin accumulation in bird species. Hence, we do not know whether our findings are representative for other bird species. However, lipofuscin accumulation in relation to contaminant exposure has previously been examined in aquatic invertebrates[110;191;195] and fish[203-205]. All these investigations showed that contaminant exposure induced lipofuscin accumulation; however, the responses in invertebrates and fish appeared to be somewhat different with regard to specificity towards the different classes of pollutants. In fish, lipofuscin accumulated mainly after exposure to organic compounds[203-205], and in invertebrates, mainly after exposure to metals[110;191;195]. This apparent discrepancy between fish and invertebrates may merely reflect the choice of study design, but may also imply that there are variations in the lysosomal responses in different animal classes. Broeg and colleagues (2008) investigated the effects of both metal and PAH exposure in the fish species corkwing wrasse (*Symphodus melops*), and found that accumulation of lipofuscin was specific for exposure to organic contaminants and was not seen after metal exposure[203]. On the other hand, exposure to metals, and copper in particular, has been associated with lipofuscin accumulation in invertebrates[110;191;195;206]. These findings imply that lysosomes in different classes of animals may respond differently towards the same pollutant, and that different classes of pollutants cause different effects on the lysosomes in the same animal. As previously mentioned, HOC exposure

was not associated with lipofuscin accumulation in the seabird chicks in either of our investigations, in contrast to the responses reported in fish species[203-205]. However, associations between mean values of Cd, Cr and Pb and the levels of lipofuscin were disclosed in the wild chicks that were investigated in paper 4. These findings indicated that increased levels of these metals could lead to accumulation of lipofuscin also in seabirds, in accordance with the previous mentioned studies of lysosomes in marine invertebrates[110;191;195;206]. Although we found no previous studies of lipofuscin in bird species, other aspects of lysosomal pathology were investigated in kidneys of Peking duck (*Anas platyrynchos*) exposed to Cd and Pb[56]. In this study metal exposure induced swelling of lysosomes, and the authors suggested that this was caused by increased lysosomal degradation of dysfunctional proteins, lipids and organelles that had been damaged by Cd or Pb. Terman and Brunk (2004) previously described that when the rate of lysosomal degradation processes increases, lipofuscin generation increases accordingly[155]. The metals may thereby have caused impairment of cellular components and consequently increased the rate of lysosomal degradation. Hence, the species difference in lipofuscin levels that we observed in the seabird chicks was possibly related to different concentrations of Cr, Cd and Pb in the species.

Nevertheless, the most evident cause of lipofuscin accumulation in the seabird chicks was food deprivation. As shown in paper 5, the two groups that had been fasted had significantly higher lipofuscin levels than the control group. Furthermore, the individual body mass change (%) of the chicks correlated significantly with the lipofuscin levels, regardless of treatment group. Chicks with the greatest loss of body mass had the highest lipofuscin levels, while chicks that gained body mass had the lowest lipofuscin levels. Lysosomal autophagic processes generate lipofuscin and are known to be augmented by food deprivation[96;111;115]. Prior to our investigation, this had not been shown in birds, but increased lipofuscin accumulation has been reported in emaciated reindeer (*Rangifer tarandus tarandus*)[207]. Additionally, lipid peroxidation was significantly more prominent in the fasted HG chicks than in the HOC exposed chicks. Lipid peroxidation is associated with lipofuscin, and may, like lipofuscin generation, be induced by increased autophagy in situations of food restriction.

No information is available regarding how food deprivation and accumulation of lipofuscin or lipid peroxidation affects arctic seabird health over time. However, accumulation of lipofuscin in humans and other mammals is associated with aging and several pathological

conditions. These include heart failure, neurodegeneration and macular degeneration[111]. Stress imposed on lysosomes by periods of fasting may therefore contribute to premature aging and promotion of the lipofuscin-associated pathologies in individuals that lose a large proportion of their body mass. Accordingly, individuals that lose less body mass are probably less likely to experience negative consequences due to lipofuscin accumulation, such as premature aging. As previously discussed, seabirds have remarkably long life expectancies in comparison with mammals of similar size. How lysosomes function in adult and elderly seabirds is not known. There are, however, investigations that show that periods of food deprivation may have positive health effects[208], for example through mechanisms where autophagic processes "clean up" the cell interior through breakdown of impaired proteins or other damaged cellular components. Our investigations do however show that fasting of HG chicks for a short period of time (1 week) results in increased lipofuscin accumulation, which is generally considered to have a negative influence on cellular health. The effects of long term fasting or repeated cycles of fasting over time on the health of adult HGs have yet to be investigated.

The potential of autophagic lysosomal responses as biomarkers of environmental stress in seabirds

Autophagic lysosomal responses are widely used as biomarkers to assess risks posed by environmental stress in studies using aquatic invertebrates such as bivalves and gastropods as model organisms. Many investigations in fish species have already been done, and the amount of information on autophagic lysosomal responses in this group of animals is growing. It may be possible to implement these biomarkers as tools in health status monitoring also in other organisms. However, thorough characterization of the physiological properties of each individual species and its response to xenobiotics is needed. Our investigations are the first studies of autophagic lysosomal responses in arctic seabird chicks, and offer some starting points in the process towards characterization of these properties in seabird species.

In accordance with our findings in seabird chicks, previous investigations of neutral lipid levels in different animals give inconsistent results, as discussed in the main discussion. Because of their role as energy storage in the cell, neutral lipids are mobilized in several different situations.

In addition lipidosis-like conditions may occur when lysosomal functions are impaired, such that neutral lipids cannot be released on demand and therefore accumulate. Hence, the levels of neutral lipids are affected by several mechanisms which may act in synergy or antagonistically, and changes in neutral lipid levels are consequently difficult to predict. A vast amount of work is needed to characterize neutral lipid dynamics and how these are affected by different stress factors in seabird species.

Because of their unpredictable and complex dynamics, neutral lipid levels are probably not well suited as a biomarker of specific stress types in seabirds. To include neutral lipid analyses in addition to other related parameters could, however, offer useful information regarding cellular health status and stress responses, also in seabirds.

Lipofuscin accumulation is used as a biomarker of xenobiotic-induced lysosomal stress in a wide range of organisms. Lipofuscin accumulation is also strongly associated with aging. We observed no effects of HOC exposure on lipofuscin levels in seabird chicks. Increasing Cr, Cd and Pb concentrations were, however, associated with lipofuscin accumulation. The concentrations of these metals in the livers of the seabird chicks were very low, 30-300 fold lower than what had previously been reported in adult birds of the same species[35] and 2-20 times lower than what had been found in studies of lysosomal effects caused by heavy metal exposure in other marine and terrestrial animals[108;110;192].This observation implies that lipofuscin accumulation may be a sensitive marker for early cellular effects of exposure to heavy metals such as Cr, Cd and Pb in arctic seabirds. Nevertheless, body mass loss caused lipofuscin accumulation in a significant, dose-dependent manner in our experiment.

We suggest that lipofuscin accumulation is a good biomarker of food deprivation and possibly heavy metal exposure in seabird chicks. However, a natural situation will always present several stress factors simultaneously, and further investigations should be performed on complex stress situations for seabirds including contaminant exposure, fasting and other challenges.

Based on the observations in our studies, lysosomal membrane stability may be applied as an indicator of cellular well-being in seabirds, but it appears as a marker of general environmental stress rather than a specific marker of food deprivation, heavy metal or HOC exposure. A brief

overview of previous studies of lysosomal membrane stability in different animals confirms the impression of lysosomal membrane stability as an unspecific stress marker. Decreased lysosomal stability has been linked to increased autophagic sequestration of impaired cellular components induced by a multitude of stress factors, such as hypoxia, salinity, hyperthermia, organic contaminants, heavy metals, pH, food deprivation and reproductive challenges[108-110;191-193;200;203;209;210].

Hence, lysosomal membrane stability is a good indicator of cellular stress across the phyla, including in seabird chicks, but does not appear appropriate as a biomarker of any particular type of stress. In any case, further characterization of this response in seabird liver is recommended.

Population perspective

The main findings in this doctoral work imply that the two investigated cellular defense systems in seabird livers are affected at high levels of contaminants and/or during periods of food deprivation. For seabird colonies that are exposed to high levels of contaminants and go through frequent periods of fasting, impairment of the antioxidant defense system or autophagic lysosomal processes is a possible consequence. Weakened cellular defense- and repair mechanisms may make the seabirds susceptible to more severe health effects such as infections, neurological disorders or possibly cancer, as discussed previously. If a large share of the individuals in a population have impaired health this may have severe consequences for the population. The glaucous gulls on Bjørnøya have been investigated thoroughly in that regard[42]. This species is a top predator and accumulates high levels of organic contaminants. Severe effects on the immune system, endocrine system, reproduction, behavior, development and survival have been associated with contaminant exposure[9]. These health effects threaten the population, and some of these physiological impairments may have been initiated by reduced cellular defense systems. Hence, monitoring of the antioxidant defense system and autophagic lysosomal processes may provide useful early warning signals of environmental stress-induced health effects in seabird populations. No such investigations have been done on the glaucous gull population on Bjørnøya. It would be of great interest to characterize the antioxidant defense system and autophagic lysosomal processes in this highly contaminated and otherwise extensively studied seabird population. Such studies might reveal

connections between antioxidant defense and autophagic lysosomal responses and physiological responses or pathological conditions in arctic seabirds and the possible impact on the population can be assessed.

Summary

Only a handful of previous studies of the antioxidant defense system in seabird species exist, and this doctoral thesis is the first to describe the antioxidant defense system in herring gull (*Larus argentatus*) (HG), northern fulmar (*Fulmarus glacialis*) (NF) and black-legged kittiwake (*Rissa tridactyla*) (BK) chicks. Furthermore, we are as far as we know the first to investigate hepatic autophagic lysosomal responses in seabird species. The investigations have generated new information regarding important cellular stress responses in seabird chicks.

The first aim was to characterize and compare the antioxidant defense system in livers from BK, HG and NF chicks in relation to contaminant levels:

- **Significant species differences between wild HG, NF and BK chicks' antioxidant defense systems were found. A consistent pattern of species differences that applied to both the HOC levels and the antioxidant responses showed large contrasts between BK and NF chicks throughout the data set.**
- The HOC levels were not correlated with antioxidant responses in the wild seabird chicks. We concluded that exposure to HOCs was assumed not to be a major explanation for the differences in levels and activity of the antioxidant parameters.
- Contrasts in metabolism, life stage, species-specific diet composition, life history and life expectancies could be related to the species differences in antioxidant capacity.
- Dietary differences were the most likely explanation for the inter-species variation in contaminant concentration, and the different diets of the three species also influenced the antioxidant capacity accordingly.
    - Nutritional factors such as selenium and iron were found to affect parts of the hepatic antioxidant defense system in the chicks.
- The antioxidant responses were in the same order of magnitude as previous reports from investigations of bird species, and well known methodology from human and mammal research could easily be adapted to seabird tissue.

Furthermore, we carried out a feeding and exposure experiment with newly hatched HG chicks and accomplished realistic HOC exposure and fasting resembling natural conditions.

Through this experiment we assessed the effect of a realistic HOC exposure and fasting on the hepatic antioxidant defense system (single antioxidant parameters and total oxyradical scavenging capacity) in HG chicks:

- High hepatic PCB levels (above 500 ng/g ww) affected some constituents of the antioxidant defense system in the exposed HG chicks (catalase and Se-dependent GPX activity, GSH/GSSG ratio). However, *total* antioxidant capacity was not affected in the HG chick livers, indicating that compensatory mechanisms may have been involved.
- **The antioxidant defense system in seabird chicks is dynamic and resilient towards contaminant exposure at ecologically relevant levels, but effects were seen at high HOC concentrations.**

Through the same experiment the effect of HOC exposure and fasting on the renal total oxyradical scavenging capacity in HG chicks was investigated:

- The liver and kidneys in the HG chicks responded differently to the combined stress of HOC exposure and food deprivation.
- Increase in TOSC against ROO• and $ONOO^-$ was observed in the kidneys but not in the liver of the same chicks. This finding may have been related to the high levels of uric acid that bird kidneys filtrate, particularly in periods of stress.
- **Antioxidant capacity is not uniform in the entire organism, and organs respond differently according to their basic physiological properties.**

Lipid peroxidation was included as an endpoint of oxidative damage in the liver.

- HOC concentration was linked to the level of lipid peroxidation in wild HG chicks.
- HOC exposure alone did not cause increased MDA levels in HG chicks. Fasting, on the other hand, induced lipid peroxidation.
- **HOC exposure per se did not appear to induce lipid peroxidation in seabird chick liver. However, in combination with other stress factors it appeared to influence the occurrence of such damage.**

Additionally, we have for the first time investigated autophagic lysosomal responses in seabird species. Hepatic lysosomal autophagic parameters (lipofuscin, neutral lipids, lysosomal membrane stability) were characterized and compared in wild BK, HG and NF chicks in relation to contaminant levels. Through the exposure experiment the effects of HOC exposure and fasting on these parameters were assessed in HG chicks.

- Hepatic neutral lipid and lipofuscin levels in the seabird chicks differed between species.

- We observed that the neutral lipid dynamics differed according to the different contaminant types: HOC exposure depleted neutral lipids and there was a possible association between accumulation of neutral lipids and exposure to Cr, Cd and Pb.
- Cr, Cd and Pb levels were also associated with lipofuscin accumulation in the wild chicks but HOC exposure did not affect their lipofuscin levels.
- Strong correlations between change in body mass and lipofuscin levels were found in the HG chicks. Hence, the most evident cause of lipofuscin accumulation in the HG chicks was food deprivation.

- Low lysosomal membrane stability was associated with increased concentrations of Cr, Cd and Pb in the wild BK, NF and HG chicks.
- The HG chicks that were exposed to HOCs in the experiment had lower lysosomal membrane stability than the control group.
- Food deprivation and HOC exposure destabilized the lysosomal membrane in the HG chicks to the same extent.

- **Food deprivation appeared a strong driving force for autophagic lysosomal activities.**
- **Interestingly, HOC exposure induced almost exactly the same response as fasting, with the exception that HOCs apparently did not affect lipofuscin accumulation.**
- **Even more surprisingly, the combined stress of fasting and HOC exposure did not cause an additive or synergistic effect.**
- **Different HOC concentrations did not influence the magnitude of the response, hence, no dose-response pattern could be observed.**
- **In addition, we found that heavy metals probably influenced autophagic lysosomal responses.**
- **We concluded that autophagic lysosomal parameters respond to cellular stress of different origin. However, lack of dose-dependent response and specificity makes them suboptimal for identifying the source of stress in seabird chicks.**

To summarize, our investigations illustrated that the hepatic antioxidant system and autophagic lysosomal responses in seabird chicks differ between species. Factors such as species-specific physiological characteristics, diet composition and contaminant exposure, in addition to food restriction, may influence these physiological systems. Negative health effects are associated with some of these responses; however, given the short time-span of this investigation such effects are difficult to predict. Additionally, the hepatic contaminant levels in the seabird chicks in our investigations were considerably lower than what is normal in adult individuals in the wild. Some of the effects we observed were dose-dependent. Hence, more pronounced effects may be expected in seabirds with higher contaminant burden than the chicks in our investigations.

Recommendations and future perspectives

This doctoral work investigated previously unstudied aspects of the physiological responses of arctic seabirds to various types of stress. In research, however, all new answers lead to new questions. To follow through on some of the loose ends that our work has left behind, several recommendations can be given:

- Scientific work with unstandardized, wild animals will always create variation in the results, but to improve the statistical power and reliability of the results **at least 10 individuals should be included in the analysis.**

- Cellular responses, particularly responses that involve the antioxidant defense system, are dynamic features that change constantly. All the samples in our studies were taken at a single time-point, and the values we report are merely snap-shots of the situation at the moment of sampling. To illuminate the dynamics of the cellular responses and to find any temporal trends, **more sampling points should be included.**

- **Other organs than liver and kidneys should also be investigated** so that the correct target organ or the most sensitive organ can be identified. This is important if the aim is to incorporate antioxidant defense and autophagic lysosomal parameters in environmental monitoring of bird health and its relation to environmental challenges.

- Antioxidant defense mechanisms and autophagic lysosomal processes are closely related to aging and longevity and therefore are particularly interesting issues with regard to seabirds. In order to better understand how these cellular defense mechanisms develop over time, **individuals of several age groups within a species should be studied.**

- More detailed investigations of the impact of specific contaminants or contaminant mixtures should be preformed. Information on d**ose-response relationships would be of great value**, and additional **effect parameters** within the two cellular defense systems

should be included. Levels of non-enzymatic antioxidants such as uric acid, ascorbic acid and α–tocopherol in liver and kidneys would offer new and useful information and add pieces to the puzzle of understanding the dynamics of the antioxidant defense system in seabirds.

- **Other methods, such as molecular or genetic characterization, should be included** to better understand the mechanisms underlying the responses.

- **Additional species should be investigated** to gain a better understanding of the species differences and which factors determine the observed variation.

- Although experimental studies offer many advantages in toxicological research, they also raise a long range of challenging practical and ethical issues. If one nevertheless decides that an experiment is the only solution to the scientific problem, one should strive to **be extremely well prepared, to have thorough plans, and last but not least; to run a pilot study.** It is impossible to foresee all potential critical issues before the experiments have been tried, and a pilot study will both improve the likelihood of success and reduce the number of animals that may be sacrificed without a just cause.

- Seasonality is an important factor for animals in the Arctic. Our results indicated that fasting had considerable impact on both the antioxidant defense system and the autophagic lysosomal responses in seabird chicks. Hence, **sampling both during the feeding season and during periods of fasting would be crucial** for basic characterization of these cellular mechanisms in seabirds throughout the year.

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