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# METAL-INDUCED OXIDATIVE STRESS AND ANTIOXIDANT DEFENCE IN SMALL PASSERINE BIRDS

by

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# LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, referred to in the text by their Roman numerals:

**I** Koivula*, M.J. and Eeva, T. (2010) Metal-related oxidative stress in birds. Environmental Pollution, 158, 2359-2370.

**II** Koivula*, M.J., Kanerva, M., Salminen, J.-P., Nikinmaa, M. and Eeva, T. (2011) Metal pollution indirectly increases oxidative stress in great tit (*Parus major*) nestlings. Environmental Research, 111, 362-370.

**III** Rainio, M.J., Kanerva, M., Salminen, J.-P., Nikinmaa, M. and Eeva, T. (2013) Oxidative status in nestlings of three small passerine species exposed to metal pollution. Science of the Total Environment, in press.

**IV** Berglund, Å.M.M., Rainio, M.J., Kanerva, M., Nikinmaa, M. and Eeva, T. Oxidative status in relation to age, condition, reproductive performance and pollution in three passerine species. (Submitted manuscript)

**V** Rainio, M.J., Kanerva, M., Wahlberg, N., Nikinmaa, M. & Eeva, T. (2012) Variation of basal EROD activities in ten passerine bird species – relationships with diet and migration status. PLOS ONE, 7 (3), e33926.

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# 1. INTRODUCTION

Following the major increase in the amount of oxygen in the atmosphere ca. 2.4 billion years ago (Lyons & Reinhard, 2009), living organisms have evolved to use oxygen for efficient energy production by means of electron transport chains that donate electrons to $O_2$, such as those in the mitochondria of eukaryotic cells (Halliwell & Gutteridge, 2007, Pamplona & Costantini, 2011). However, at the same time reduction-oxidation (redox) reactions have led to increased production of reactive oxygen species (ROS), which are highly reactive oxygen-containing molecules produced as a by-product of normal cellular metabolism (Halliwell & Gutteridge, 2007). These molecules play a role in normal cell signalling and control of the redox status of organisms, but at high levels they are harmful (Jackson, 2005, Thannickal & Fanburg, 2000). Consequently, organisms have evolved mechanisms to minimize the effects of oxidative metabolism, such as ROS production, and have developed effective antioxidant defence systems to prevent harmful pro-oxidant effects (Halliwell & Gutteridge, 2007). When the production of ROS is increased above normal, and cannot be handled by antioxidant defence, it can cause the biochemical stress on cells called oxidative stress, and consequent damage to the organism, including oxidation of biomolecules such as DNA, proteins and lipids (Halliwell & Gutteridge, 2007, Bae et al., 2009, Beckman & Ames, 1998, Harman, 1956, Sies, 1991). Oxidative stress is a continuous process; the on-going production of pro-oxidants in the system is probably to some extent always present, thus causing continuous oxidative damage at some level (Pamplona & Costantini, 2011). In a recent review, Pamplona and Costantini (2011) concluded that oxidative stress could have worked as a modulator of phenotypic development, a constraint of life-history evolution in animals, as well as a physiological correlate of behavioural differences between individuals. Thus animals have evolved a considerable number of different antioxidant mechanisms to regulate the redox balance, in order to deal with varying amounts of reactive oxygen species (Pamplona & Costantini, 2011). Along with normal metabolism, the processing of many toxic compounds can also produce several reactive species, which may increase the oxidative stress in species living in contaminated environments (Valko et al., 2005).

Since the start of industrialization, the human impact has become a major threat to the environment, causing large amount of anthropogenic pollution for example through mining and smelting, increased traffic load, fossil fuel combustion and the chemical industry (Walker et al., 2005). Although industrial emissions have declined considerably over the decades with improved technology and increased environmental awareness, a number of harmful compounds are still present in the environment. One important category of anthropogenic pollution consists of metals, which are ubiquitous in the environment. They originate in part from industry, but also from natural processes (Walker et al., 2005). While

metals appear naturally in the environment and many of them are essential micronutrients for organisms, in large amounts they have toxic effects on organisms. The most important sources of metal emission are mining activities and metal smelters, producing metals such as copper (Cu), nickel (Ni), lead (Pb), arsenic (As), iron (Fe), mercury (Hg), zinc (Zn) and cadmium (Cd). The most likely source of metal contamination in animals is via their diet, but high concentrations can also be found in soil and water, whence metals can accumulate in animals. Metals can also become concentrated through food webs, where high levels of metals can accumulate in species at the top of food chains (Burger, 1993, Hernandez et al., 1999). A number of metals participate in important metabolic and signalling pathways, causing detrimental effects on animals exposed to high environmental metal concentrations; these effects may be direct or indirect (Valko et al., 2005).

## 1.1. Metals

Metals can appear in different forms in the environment. Their toxicity is related to their oxidative state and their reactivity with other compounds (Scheuhammer, 1987, Valko et al., 2005, Walker, 1995). Metals can also interact with each other, preventing, neutralizing or increasing their toxic effects. Metals can be divided into redox-active and redox-inactive metals according to their function. Redox-active metals, such as iron (Fe), copper (Cu), chromium (Cr) and vanadium (V), catalyze Fenton reactions. Fenton reactions generate reactive hydroxyl radicals, and are commonly associated with membranous fractions such as mitochondria, microsomes and peroxisomes (Valko et al., 2005, Stohs & Bagchi, 1995). The Fenton reaction is one of the most powerful oxidizing reactions involving hydrogen peroxide and a ferrous iron catalyst. In the reaction, peroxide is broken down into a hydroxide ion and a hydroxyl radical (Stohs & Bagchi, 1995). Examples of Fenton reaction of metals are the following:

$Fe^{2+} + H_2O_2 \rightarrow Fe\ (III) + OH^{\bullet} + OH^{-}$

$Cu^{+} + H_2O_2 \rightarrow Cu^{2+} + OH^{\bullet} + OH^{-}$

Redox-inactive metals, including lead (Pb), cadmium (Cd), arsenic (As) and mercury (Hg), can deplete the major antioxidants of cells, such as glutathione and other thiol-containing antioxidants and protein-bound thiol groups, being most probably the primary route for their toxicity. Thus metals can either increase ROS production or reduce antioxidant defence (Ercal et al., 2001, Valko et al., 2005, Stohs & Bagchi, 1995).

The concentration of a given metal in the body depends on the level and duration of metal exposure. Metals have both direct and indirect effects on organisms. In birds, for example, they directly affect health, morphology, egg quality and number, growth rate and nestling condition (Nyholm, 1994, Janssens et al., 2003a, Bel´skii et al., 2005).

Indirect effects of metal pollution on wildlife are related to habitat alteration, food chain, community structure and ecological relationships between species (Kiikkilä, 2003, Heliövaara & Väisänen, 1993). Studies with great tits, for example, have shown lower breeding performance with poorer-quality diet in polluted areas, emphasizing the importance of secondary environmental changes, such as food quality, as an indicator of indirect metal pollution (Eeva et al., 2005b, Eeva et al., 2009b). In studying pollution effects on animals, it is important to emphasise that individuals are often exposed to a mixture of pollutants, in sublethal individual concentrations, that may have synergistic, antagonistic or additive effects (Walker et al., 2005). Environmental factors, such as weather, food availability or pathogens, may also enhance the effects of pollutants, thus making it difficult to relate the concentration of a particular environmental pollutant to changes at the individual or population level (Walker et al., 2005). In addition, metals have been shown to induce oxidative stress by increasing the production of ROS, unbalancing the cellular redox status and making antioxidants insufficient for defence against increased free radical formation (Halliwell & Gutteridge, 2007, Ercal et al., 2001). Metals are able to form reactive oxygen and reactive nitrogen species, including the superoxide ($O_2^-$) and hydroxyl radical (•OH), nitric oxide (NO), hydrogen peroxide ($H_2O_2$) and other endogenous oxidants that may be harmful to the organisms (Valko et al., 2005). Most important in biological and toxicological terms is the hydroxyl radical, because of its oxidative potential and its reactivity with biomolecules (Ercal et al., 2001).

## 1.2. Antioxidant defence

Effective protection against oxidative damage is crucial for the functioning of organisms. There are many environmental factors associated with oxidative damage, such as weather, food availability, intra- or interspecific competition and predation risk, all of which can vary across habitats and in different life-stages (e.g. reproduction, hibernation, moulting and migration), placing animals under continuous pressure (Pamplona & Costantini, 2011). Many organisms have therefore evolved multi-layered enzymatic and non-enzymatic systems (e.g. glutathione, carotenoids, vitamins and glutathione-related enzymes) to defend themselves against ROS-derived damages (Sies, 1993). The maintenance of antioxidant defence, however, can be costly to the individual, since energy is needed to keep these defences up-regulated and to activate endogenous repair systems. In defending themselves against oxidative stress, animals can thus face a number of trade-offs, which may affect their life-history strategies (Pamplona & Costantini, 2011). Trade-offs can be defined as negative correlations between life-history traits, where the increased benefit of one trait reduces the benefit of another because of limitations in the total resources available to be allocated to them (Begon et al., 1996). In most cases trade-offs are related to effects on reproductive success, such as the number of offspring and their fitness or parental investment in reproduction (Begon et al., 1996). Recently many studies have

investigated the impact of oxidative stress on life-history strategies and trade-offs (e.g. survival and reproduction) (Costantini, 2008, Devevey et al., 2008, Monaghan et al., 2009). Likewise carotenoids, immune defence and stress hormones have been the subject of active research in many animal groups, notably birds (Alonso-Alvarez et al., 2004b, Romero, 2004, Costantini & Dell'Omo, 2006, Isaksson et al., 2007). Less attention has been paid, however, to the function of different antioxidant enzymes and antioxidant levels in different bird species as indicators of oxidative stress in polluted environments (Norte et al., 2009b, Berglund et al., 2007, Isaksson et al., 2005, Hegseth et al., 2011b).

## 1.3. Biomarkers for metal-related oxidative stress in birds

### *1.3.1. Glutathione and glutathione-related enzymes*

Both non-enzymatic and enzymatic antioxidants can be used to determine the effects of metal exposure on the oxidative status of the birds (Koivula et al., 2011). Glutathione is one of the most important small antioxidant molecules, controlling the oxidative status in almost all organisms examined to date (Andrews, 2000, Pinto et al., 2003). It functions by binding metals at sulfhydryl groups, thereby preventing them from creating ROS (Andrews, 2000, Pinto et al., 2003). The regulation of oxidative status by glutathione involves its cycling between reduced (GSH) and oxidized (GSSG) form (Halliwell & Gutteridge, 2007) (Fig. 1). The ratio of GSH:GSSG gives the overall oxidative state of cells; consequently, changes in this ratio are often used as an indicator of oxidative stress (Halliwell & Gutteridge, 2007, Hoffman, 2002, Isaksson et al., 2005, Stephensen et al., 2002).

Glutathione cycling is catalyzed by several enzymes, such as glutathione peroxidase (GP), glutathione-S-transferase (GST) and glutathione reductase (GR) (Fig. 1). The ROS level is directly regulated by reactions catalyzed by the enzymes catalase (CAT) and superoxide dismutase (SOD) (Ercal et al., 2001, Pinto et al., 2003) (Fig. 1). In this thesis, I use the term 'oxidative state' to refer to antioxidant GSH and the ratio of GSH:GSSG; the term 'oxidative status' is used to include both antioxidant enzyme activities and the level of GSH and the GSH:GSSG ratio. The efficiency of redox regulation, and consequently of defence against oxidative stress, depends both on the activities of antioxidant enzymes and on the levels of non-enzymatic antioxidants and their cycling, which in turn is affected by their uptake, production and excretion (Halliwell & Gutteridge, 2007). In studies evaluating disturbances of oxidative status of organisms caused by environmental pollution, it is therefore important to measure both enzymatic and non-enzymatic antioxidants.

Other methods, such as the measurement of total antioxidant activity (TAA) and the evaluation of plasma antioxidant power (OXY), together with the dROM test, which measures reactive oxygen metabolites, have also been commonly used in studies of oxidative stress in different animals (Isaksson et al., 2007, Markó et al., 2011, Costantini et al., 2010). The ideal approach

in studies of oxidative status and antioxidant defence would be to find the best possible biomarkers and the best combination of different methods for each species. Using the most prominent biomarkers for each species and the optimal combination of markers for that particular study environment will yield more accurate results and conclusions (Hõrak & Cohen, 2010). In addition to determining the relevant biomarkers for each species, it is also important to keep in mind the technical requirements and repeatability of the techniques applied. Different biomarkers measure different aspects of redox balance, and careful planning is therefore essential in designing the study set-up (Hõrak & Cohen, 2010).

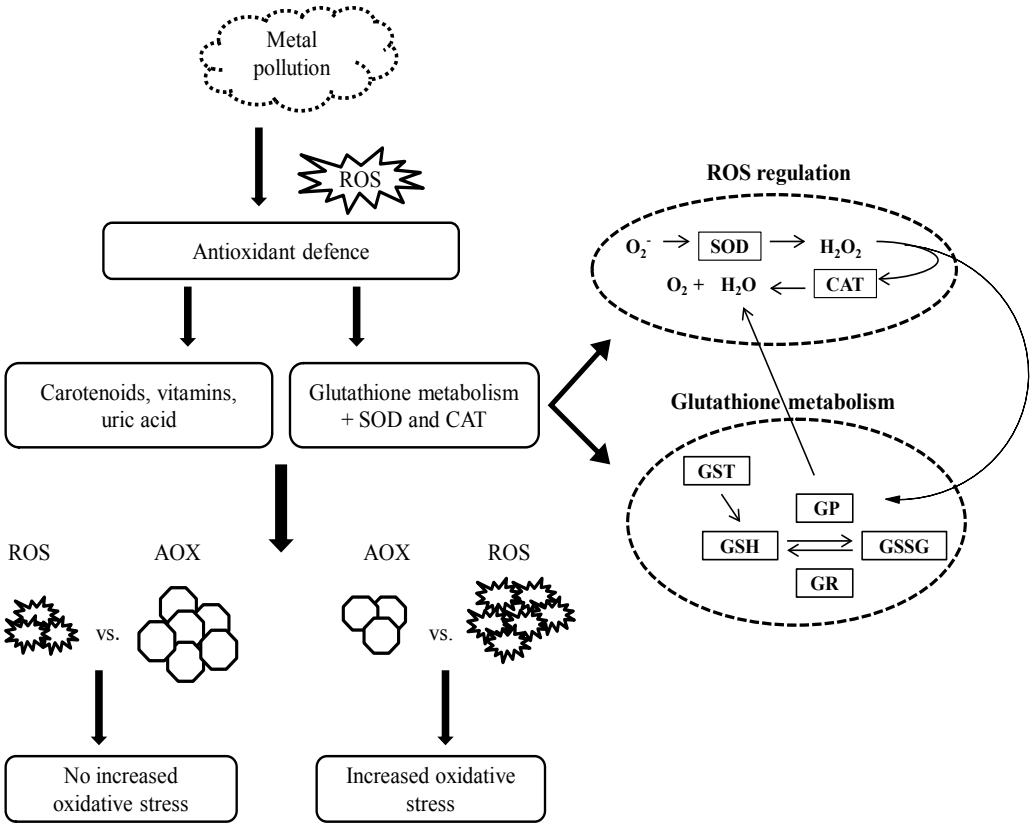


**Fig. 1.** Antioxidant defence in metal-polluted environment. Antioxidant defence consist mainly of antioxidants (AOX), such as carotenoids, vitamins, uric acid and glutathione as well as glutathione-related enzymes (GP, GR, GST). GP (glutathione peroxidase) participate in the glutathione metabolism by oxidizing GSH (reduced glutathione) to GSSG (oxidized glutathione), which is then reduced back to GSH by GR (glutathione reductase) (Halliwell & Gutteridge, 2007). GST conjugates GSH to electrophiles (Ketterer et al., 1983), and also breakdown products of lipid peroxides to GSH (Stephensen et al., 2002). SOD (superoxide dismutase) and CAT (catalase) are the major enzymes related to ROS regulation. SOD transforms superoxides to $H_2O_2$, which is further catalyzed by CAT to $H_2O$ and molecular oxygen. Alternatively, GP can also convert $H_2O_2$ to harmless by-products by using $H_2O_2$ to oxidize other substrates (e.g. GSH to GSSG). Antioxidants are able to cope with ROS production caused by normal metabolism, but if antioxidant defence is disturbed by increased ROS formation due to xenobiotics, oxidative stress may occur (Halliwell & Gutteridge, 2007).

### *1.3.2. Carotenoids and vitamins*

Carotenoids are a group of small-molecule antioxidants of plant origin, appearing in the blood as circulating lipoproteins and in tissues within the hydrophobic interior of membranes. Animals are unable to synthesize carotenoids, but need to get them from their diet (Monaghan et al., 2009, Halliwell & Gutteridge, 2007). Plasma carotenoid levels have been shown to vary in relation to environmental pollution, due to pollution-related changes in food webs (Eeva et al., 2008, Geens et al., 2009, Eeva et al., 2009b). Carotenoids are also suggested to act as antioxidants against oxidative damage (Alonso-Alvarez et al., 2004a, Lozano, 1994, von Schantz et al., 1999), although recent studies have shown that they may play a more important role in sexual signalling and immune defence (Costantini & Dell'Omo, 2006, Costantini & Møller, 2008, Hõrak, 2007, Isaksson et al., 2005, Koivula et al., 2011, Tummeleht et al., 2006, Isaksson & Andersson, 2008). One commonly known carotenoid is β-carotene, but in birds the most important and abundant carotenoids are lutein and zeaxanthin (McGraw et al., 2003). Carotenoids are closely linked to vitamins, since these (e.g. vitamins E and C) are able to reduce oxidized carotenoids for re-use and vice versa (Catoni et al., 2008). Vitamin A is also related to carotenoids; over fifty carotenoids can be used as a source of vitamin A (Surai, 2002). The mechanism of carotenoids in defence against ROS is based on their ability to reduce free radicals by becoming oxidized themselves (Catoni et al., 2008).

The lipid-soluble vitamin E (α-tocopherol) is considered to be the main antioxidant involved in membrane defence, protecting membranes and lipoproteins from oxidation (Halliwell & Gutteridge, 2007). The antioxidant activity of vitamin E is associated with its capacity to reduce the hydroxyl group of its chromanol ring (Pamplona & Costantini, 2011). Vitamins also interact with each other to improve the antioxidant protection, but high levels of vitamin E can also have pro-oxidant effects in the absence of vitamin C. Vitamin C (ascorbate) also has an antioxidant capacity, being able to scavenge free radicals by transferring electrons and prevent oxidative damage by inhibiting lipid peroxidation induced by free radicals (Halliwell & Gutteridge, 2007). The role of vitamin C in wild birds has received less attention, but it has been suggested to improve avian immunity (Catoni et al., 2008).

### *1.3.3. Detoxification capacity in birds*

In addition to antioxidant defence, animals have also developed molecular mechanisms for detoxifying harmful compounds, including toxic compounds of anthropogenic origin. Animals are able to modulate their enzyme activities and detoxification systems in response to pollution levels, thereby improving their chances of survival in contaminated environments. An important enzyme group participating in these detoxification processes is that of mixed function oxidases (MFO), which are the members of cytocrome P450

enzymes, found in all organisms examined so far (Bernhardt, 1996). Their function is based on their ability to metabolize exogenous and endogenous harmful compounds to a more polar form, which can be more easily excreted from the organs (Bernhardt, 1996, Tanhuanpää et al., 1999). P450 enzymes are mainly concentrated in the liver, where toxic compounds are modified to more excretable forms by phase I (involving MFO enzymes) and phase II biotransformation reactions (Andersson & Förlin, 1992). One of the most important enzymes of the P450 group is enzyme CYP1A, which participates in the detoxification process of xenobiotics (Guengerich & Liebler, 1985). The induction of CYP1A can be detected by measuring ethoxyresorufin-*O*-deethylase (EROD) activity (a catalytic measurement of CYP1A induction), which is a sensitive biomarker for several contaminants (Whyte et al., 2000). Species-specific differences in detoxification capacity have been found in different animal groups; the variation has been usually related to differences in their MFO system (Newman, 2010, Fossi et al., 1995a). Species are able to develop a unique spectrum of cytochrome P450 enzymes for detoxifying harmful compounds, such as xenobiotics or plant secondary compounds, from their organs. The P450 enzymes also appear to be the most abundant drug-metabolizing enzymes, with the broadest range of substrate specificity. A single P450 protein can metabolize several diverse chemicals, enabling very effective detoxification of large numbers of substances in the diet and environment (Gonzalez & Nebert, 1990). Detoxification capacity is often associated with diet composition, especially in birds (Ronis & Walker, 1989, Fossi et al., 1995a, Sinclair & Sinclair, 1993), but has also sometimes been connected with the metabolic rate (Ronis & Walker, 1989, Sinclair & Sinclair, 1993).

## 1.4. Metal-induced oxidative stress in birds

Birds have been used as biomarkers of metal pollution in many ecotoxicological studies. The impact of metal pollution has been widely studied among passerine birds located close to a variety of metal industries (Bel'skii, 2003, Berglund et al., 2010, Berglund et al., 2011, Dauwe et al., 2006, Eeva & Lehikoinen, 2000, Janssens et al., 2003a, Nyholm, 1994, Swiergosz et al., 1998, Eens et al., 1999). Previous studies have reported a reduction in survival and breeding success, genetic alterations, paler plumage colour and lower carotenoid levels, and food depletion in polluted areas (Berglund et al., 2010, Eeva et al., 2006, Eeva & Lehikoinen, 1996, Eeva et al., 2003, Eeva et al., 1998, Geens et al., 2010, Janssens et al., 2003b). Information as to the physiological effects of metal pollution in small passerines, especially in relation to oxidative damage, is nevertheless still limited (Bel'skii & Stepanova, 1995, Berglund et al., 2007, Geens et al., 2009, Koivula et al., 2011). Birds have certain unique molecular mechanisms (e.g. low rates of mitochondrial oxygen radical production and high blood glucose levels) compared to other vertebrates, allowing effective defence against free radicals and oxidative stress (Costantini, 2008, Pamplona & Costantini, 2011); this adds to the interest of using birds as study species. In

addition, birds are well suited to this type of study, being relatively long-lived compared to many mammals of similar size (except for bats). They have also developed strategies for coping with much higher metabolic rates and energy expenditure than mammals (Costantini, 2008).

Studies concerning oxidative stress have yielded contradictory results, some of them indicating increased oxidative stress in relation to pollution levels (Berglund et al., 2007, Isaksson et al., 2005, Kamiński et al., 2009), while others have not found a direct association between pollution exposure and oxidative stress (Isaksson et al., 2009, Koivula et al., 2011). It would therefore be useful to expand our knowledge of the mechanisms causing oxidative stress, since there are a number of factors related to antioxidant defence; these include habitat, temperature, climate and diets, as well as intracellular conditions, to mention but a few. Studies related to pollution also need to use multiple biomarkers to detect oxidative stress and damage to the system, since many biomarkers have been shown to interact with each other. Species-specific variation in the biomarkers used also needs to be taken into account, since it may notably affect the interpretation of the results.

It is often unclear whether the direct effects of environmental pollution are the primary cause of oxidative stress, or whether the indirect effects are perhaps even more harmful, especially in long-term exposure to pollution. Further studies are thus needed to determine the exposure levels of different contaminants causing increased oxidative stress in different species, and to enable us to distinguish between natural variation and that caused by pollution exposure. Better information as to species-specific oxidative status and detoxification capacity will help to identify the species most vulnerable to environmental pollution, and to plan conservation strategies for species at risk.

## 1.5. Aims of the thesis

The first purpose of my thesis was to draw up an extensive review concerning the effects of metal pollution on oxidative stress in birds. The second was to study the oxidative status in three free-living passerine bird species close to a metal smelter complex. *In vitro* studies in birds have shown increased oxidative stress when exposed to certain metals, but it is unknown how well they reflect the effects in nature. In studying natural populations, it is possible to take into account several natural stress factors, which cannot always be included in studies *in vitro*. So far only few studies have been conducted in the field to study pollution effects in terrestrial free-living bird species (Isaksson et al., 2005, Berglund et al., 2007, Congiu et al., 2000, Hõrak et al., 2007) and they have arrived at contradictory results. One of my aims was therefore to achieve a better understanding of the metal-related oxidative status of free-living passerine birds by studying the

nestlings and adult females of three species – the great tit (*Parus major*), the blue tit (*Cyanistes caeruleus*) and the pied flycatcher (*Ficedula hypoleuca*) – along a pollution gradient (Fig. 2). My third purpose was to determine the detoxification capacity of ten passerine species in relation to their diet and migration status, using EROD activity as the biomarker for detoxification capacity.

**Fig. 2.** Nestlings of the great tit, blue tit and pied flycatcher, used in studies of oxidative status close to a metal smelter.

The first article deals with metal-related oxidative stress in general, providing a broad perspective on the toxic effect of metals and on the mechanisms whereby metals cause oxidative stress in birds via increased ROS production (I). Birds were chosen as model species because a great deal is known about their antioxidant defences (most specifically carotenoids and vitamins); at the same time, the mechanisms of metal-related oxidative stress in free-living populations are still quite poorly understood. The review also provides an overview of the methods used in studies of avian oxidative status, including various antioxidants, antioxidant enzymes and metallothioneins.

The next three articles focus on the effects of metal pollution on oxidative status in the vicinity of a metal smelter, using great tit, blue tit and pied flycatcher nestlings (II, III) and adult females (IV) as model animals. Article II deals with the question whether great tit nestlings face increased oxidative stress when exposed to metal pollution. I also used experimental methods to determine whether carotenoid supplementation affects oxidative stress by improving antioxidant defence against increased ROS production due to metal pollution. The spectrum of plumage colour was measured to find out whether carotenoid-based plumage colouration is related to plasma carotenoid concentration and oxidative status in great tit nestlings. I further explored the effects of oxidative stress on nestling growth, survival and fledging success.

The focus in articles III and IV is on interspecific variation in oxidative status in a metal-polluted environment, in order to determine the extent of interspecific variation in oxidative status and the ability of each species to maintain their oxidative status and regulation of enzyme activities in such an environment. The article III considers nestlings of the great tit, blue tit and pied flycatcher and article IV adult females of the

same species, sampled from the same nests as the offspring; this enables a comparison of the oxidative status of the offspring and their mothers along the pollution gradient. The reproductive performance of adult females, defined in terms of brood size and fledging success of their nestlings, was also assessed in relation to metal pollution and oxidative status (IV). In III the association of carotenoids with oxidative status was further studied in the offspring.

In the last article (V), I studied the detoxification capacity of ten free-living passerine species in relation to their diet and migration status. Variation between passerine species in their detoxification capacity has so far been only poorly understood. My purpose was therefore to provide new information as to the levels of EROD activities in migratory and resident birds, and to compare EROD activities between insectivores and granivores. I also combined, for the first time, the relative basal metabolic rate and relative liver mass with EROD activities, to find out whether they are associated with the detoxification process of harmful compounds.

# 2. MATERIALS AND METHODS

## 2.1. Study area and study species at smelter site (II-IV)

The studies of this thesis were conducted in the summers of 2004 and 2008 in the vicinity of a non-ferrous metal smelter in the town of Harjavalta (61°20' N, 22°10' E), an area with one of the highest rates of metal pollution in Finland (Fig. 3). The main pollutants in the smelter area are Ni and Cu, but As, Zn, Pb and sulphuric oxides are also emitted in appreciable amounts (Kiikkilä, 2003). Bird studies have been conducted in this area for the past 22 years, and birds have been shown to suffer from metal pollution throughout this period (Eeva et al., 2009a, Koivula et al., 2011, Eeva et al., 1998), although pollution levels have declined significantly from the 1990s to the present (Kubin et al., 2000). I used thirteen study sites (only twelve of which were used in 2004) established along the pollution gradient: seven sites (six in 2004) in the polluted area and six in the unpolluted one. The polluted area, referred to as zone 1, was situated < 2 km (2004) or < 2.5 km (2008) from the smelter; the unpolluted area, referred to as zone 2, was situated > 5 km (2004) or > 2.5 km (2008) from the smelter, where pollution levels are already close to background levels (Eeva et al., 2008) (Fig. 3). The differences in distance between the study years do not affect the interpretation of the results, since there were no study sites at a distance of 2-5 km. The habitat type in all study areas was forest dominated by barren Scots pine (*Pinus sylvestris*), thus avoiding habitat-related variation between the areas (Fig. 4).

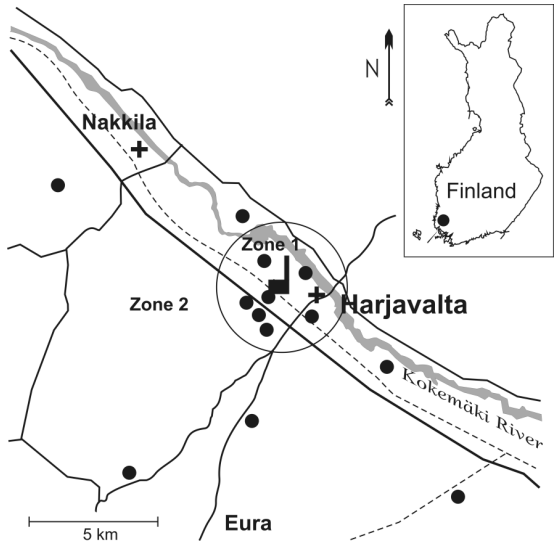


**Fig. 3.** Map of the study areas in the vicinity of Harjavalta. Black dots represent study areas; crosses indicate town centres. The circle around the smelter represents the polluted area, less than 2.5 km from the smelter.

The great tit and the blue tit were used as a study species, for three reasons: they are both abundant in these study areas, they breed in nest boxes, and there is plenty of background information as to the effect of metal pollution on these species (Burger, 1993, Eens et al., 1999, Janssens et al., 2003b, Dauwe et al., 2005, Eeva et al., 1998, Eeva & Lehikoinen, 1995). Both species also have carotenoid based colouration, making them ideal study objects for studies related to carotenoid availability and antioxidant defence. A third species used in the studies was the pied flycatcher, a small migratory insectivore which is also abundant in the study areas. Metal pollution has been shown to lessen breeding success and nestling growth in both Parid species (Eeva et al., 2009a) and to cause food depletion in the great tit (Eeva et al., 2003), while pied flycatchers have shown smaller egg size, clutch size and hatchability as well as thin eggshells caused by decreased Ca levels in their diet close to the smelter (Fig. 5) (Eeva & Lehikoinen, 2004).

**Fig. 4.** Study area close to the pollution source.

**Fig. 5.** Pied flycatcher nest in polluted area.

## 2.2. Study area and study species in a detoxification capacity study (V)

The study concerning the detoxification capacity of birds was conducted between August and October 2008 near the city of Turku (60°26' N, 22°11' E) in SW Finland, where I used three sampling sites. The habitats included a grove dominated by the common alder (*Alnus glutinosa*), reed beds (*Phragmites*) and garden habitats (Fig. 6). Barn swallows (*Hirundo rustica*) were collected in 2007 from reed beds near Turku (60°17'N, 22°11'E).

A.  B. 

**Fig. 6.** Bird trapping in common alder dominated grove (A) and in reed bed habitat (B) (photos: Markku Saari).

The study species were the great tit, blue tit, chaffinch (*Fringilla coelebs*), greenfinch (*Carduelis chloris*), house sparrow (*Passer domesticus*), willow warbler (*Phylloscopus trochilus*), reed warbler (*Acrocephalus scirpaceus*), sedge warbler (*Acrocephalus schoenobaenus*) reed bunting (*Emberiza schoeniclus*) and barn swallow. All species are common in the area and are relatively easy to catch with mist nets. The species were chosen to represent different migratory statuses and feeding habits. The birds were divided into four groups according to their migratory behaviour and diet, based on published information on each species (Cramp & Perrins, 1994a, Cramp, 1992, Cramp & Perrins, 1994b, Cramp & Perrins, 1993): 1) migratory insectivores, 2) migratory granivores, 3) non-migratory/ partly migratory insectivores, and 4) non-migratory/partly migratory granivores (Table 1). The division, however, is quite rough; some granivores (e.g. the reed bunting) sometimes also eat insects, especially during the breeding season. Since some of the species (the great tit, blue tit and greenfinch) classified here as non-migratory species may also show partial migratory behaviour, they were grouped together.

**Table 1.** Division of birds into four groups based on their migratory status and diet (photos: Kalle Rainio).

| | **Migratory birds** | **Non-migratory birds/ partial migrants** |
|---|---|---|
| **Insectivores**   | Willow warbler<br>Sedge warbler<br>Reed warbler<br>Barn swallow | Great tit<br>Blue tit |
| **Granivores**   | Chaffinch<br>Reed bunting | Greenfinch<br>House sparrow |

## 2.3. Sampling methods – studies of oxidative status (II-IV)

### *2.3.1. Sampling in the field*

All nestlings and adult females were tagged with aluminium rings for individual identification. Morphological measurements (weight and wing length) and blood samples were taken from two randomly selected nestlings per brood (II, III) and from adult females from the same nests (IV). Since young birds are thought to be sensitive to the detrimental effects of pollutants (Scheuhammer, 1987), low body mass and small wing length can be used as indicators of a poor-quality environment. Those morphometric measures are also commonly used as indicators of health status and condition in birds (Dauwe et al., 2006), which can further be used in determining survival and reproductive success (Tinbergen & Boerlijst, 1990). The body condition index for adult females was calculated using body mass and wing length, by the formula 'body mass (g)/wing length (mm)'. Females were aged by their plumage characters in two age-classes: one year old or older (Svensson, 1992), since the low return rate of the females does not allow more accurate ring-based age determination. Fledging success (%) was calculated for each species by the formula '(number of fledglings / number of hatchlings) × 100', and analysed statistically with general linear mixed models (II). In the carotenoid supplementation study (II) one nestling was randomly chosen for blood sampling from each treatment group (supplemented vs. un-supplemented). Blood samples were taken by brachial venipuncture using 75 µl heparinized capillary tubes (Fig. 7.), which were immediately centrifuged for 5 min at 4000 r/min to separate the plasma and red blood cells (Fig. 8.). The plasma and red blood cells were placed in liquid nitrogen until permanent storage at -80°C.

**Fig. 7.** Blood sampling from the brachial vein (photo: Markku Saari).

**Fig. 8.** Travelling laboratory used in the field studies (photo: Markku Saari)

The carotenoid treatment, conducted in 2004, was started when the nestlings were three days old (II). The nestlings were weighed, after which each brood was divided into two comparable groups according to body mass, with each group including nestlings of similar weight. These body-mass groups were then randomly divided between a treatment and a control group. The carotenoid-supplemented group was treated with water-dispersed carotenoid beadlets (lutein 5% CWS, Roche, Basel, Switzerland), containing 5% lutein and 0.25% zeaxanthin, diluted in distilled water to yield a lutein concentration of 5 mg/ml (the dose given was an oral volume of 0.1 ml per nestling). The control group was treated by giving the same amount of distilled water (for a more detailed description see Eeva et al. 2008). The plasma lutein level increased 2.1-fold in the treated nestlings compared to the natural levels, but was still within the range of natural variation (Eeva et al., 2008).

### *2.3.2. Carotenoid analyses*

The plasma carotenoids, lutein, zeaxanthin and β-carotene, were determined with high performance liquid chromatography (HPLC). A known volume of plasma (10-35µl) was extracted 3 × with 100% acetone. The solvent was evaporated from the combined extract under a vacuum and the residue was dissolved in a small volume of 80% acetone. The carotenoid compositions of the extracts (lutein, zeaxanthin, β-carotene) were analysed with HPLC at 450nm using a Merck Purospher STAR RP-18 (55x2mm, i.d., 3µm) column (Darmstadt, Germany). β-carotene was quantified as such and other carotenes as lutein equivalents (II-III).

### *2.3.3. Plumage colour measurements*

The plumage colour of the great tits was measured in 2004 from two randomly selected nestlings per brood (one treatment, one control), when they were 16 days old (II). The nestlings were photographed side by side with a digital camera, with a uniform grey cardboard as background, and a yellow reference card (C2, M17, Y86 and K0) was used in determining the colour of each nestling. The nestlings were placed in a plastic holder to keep them immobile while photographing the plumage on the ventral side of the body (for a more detailed description see Eeva et al. 2008). Digital imaging has proved to be a sensitive way to measure colour variation as long as the ambient light can be controlled (Villafuerte & Negro, 1998, Montgomerie, 2006). The main interest was in the plumage yellowness, thus focusing on the intensity of lutein, which is the main determinant of yellow colour in the great tit plumage. The proportion (%) of the yellow component (Yc), which is the corrected value for the plumage colour (calculated using the reference colour value), was used as a measure of plumage yellowness (Eeva et al., 2008).

### *2.3.4. Faecal metal analyses*

Faeces were collected from two nestlings per brood (II-IV), after which the faecal sacs were combined within the same brood and dried at 50°C for 72 h. Samples were weighed in a range of 0.15-0.20g and 2 ml of Supra-pure $HNO_3$ and 0.5 ml of $H_2O_2$ were added to the samples in Teflon bombs for digestion with a microwave system (Milestone High Performance Microwave Digestion Unit mls 1200 mega). The samples were then diluted to 50 ml with de-ionized water. The samples were prepared according to the method instructions, and the concentrations of metals (Cu, Ni, As, Cd, Pb, Zn and Ca) were determined with ICP-MS (Elan 6100 DRC, PerkinElmer-Sciex, Boston, USA) (for details see Eeva et al. 2008) (II-IV). Since all the faecal metal concentrations were positively correlated with each other, principal components were calculated from the metal data (Ni, Cu, As, Cd, Pb) for both study years (2004 and 2008) and for each species. As the first principal component (PC1) explained most of the variation in the data sets, it was used in the models as an explanatory variable, describing the general level of metal exposure (II-III).

### *2.3.5. Biomarker analyses*

Red blood cells from the nestlings were used to measure oxidative status biomarkers (i.e. GSH, GSH:GSSG, GP, GR, GST, CAT and SOD) (II-IV). These biomarkers were chosen as indicators of oxidative status, since glutathione is one of the most important antioxidants and the most abundant non-protein thiol in cells. The ratio of GSH:GSSG gives the overall oxidative state of the cells and is an important measure of oxidative stress (Valko et al., 2005, Halliwell & Gutteridge, 2007). Glutathione metabolism also plays an essential role in metal-induced oxidative stress, as the functional groups of glutathione serve as binding sites for several metals (Andrews, 2000, Pinto et al., 2003).

The samples were analysed using a microplate reader (Envision, Perkin-Elmer Wallac, Turku, Finland). All enzyme activities were measured in triplicate (intra-assay coefficient of variability [CV] < 10% in all cases) using 96- (CAT) or 384-well (GP, GR, SOD) microplates. Three control samples were used with each plate, so as to be able to correct inter-assay precision with the ratio specific to the particular plate (range 0.8-1.2). As we were attempting to minimize sample volume, we also used correspondingly smaller reagent volumes than those recommended by the analytical kits. GP, GR, GST and CAT were measured using the Sigma kit (Sigma Chemicals, St. Louis, Missouri, USA) and SOD with the Fluka kit, according to the method instructions (Fluka, Buchs, Germany) (II-IV). Total GSH was measured with OxisResearch kit (OxisResearch, Foster City, California, USA) (II). Total GSH and the ratio of GSH:GSSG were measured with the ThioStar® glutathione detection reagent (Arbor Assays, Ann Arbor, Michigan, USA), using reduced glutathione as the standard (Sigma Chemicals, St. Louis, Missouri, USA)

(III, IV). The protein concentration was measured according to the Bradford method (Bradford, 1976) using BioRad stock (BioRad, Espoo, Finland) diluted to $dH_2O$ (1:5) and BSA (1 mg/ml) (Sigma Chemicals, St. Louis, Missouri, USA) as a standard, with an Envision microplate reader at an absorbance of 595 nm (II-IV).

#### *2.3.6. Temperature data*

Temperature data were collected by the Finnish Meteorological Institute, using the station within the study area in Harjavalta (meteorological station at Peipohja, Kokemäki, 61°16' N, 22°15' E) (II-IV). The nestlings of the study species can be considered as ectothermic during early development. This means that variation in the ambient temperature is likely to influence the nestling's body temperature, its metabolic rate, and quite possibly its oxidative stress response. We therefore calculated for each nestling the mean (daily) temperature during the first nine days of life (for the pied flycatcher) or the first eleven days (for the great tit and the blue tit), for use as a covariate in our analyses.

### 2.4. Sampling methods – study of detoxification capacity (V)

#### *2.4.1. Sampling in the field*

The birds collected in this study (V) were all young females (born during the summer of 2008), 1222 individuals per species, except in the case of reed warblers and sedge warblers, for which sex determination in the field was impossible. The sample size of these two species was 30 individuals, and they were sexed later in the laboratory. The birds were trapped with mist nets, which were checked every half hour. By collecting young birds I was able to exclude the possible influence of migration and of the diet consumed at their wintering grounds, and to reduce potential variation in their life-time pollution exposure and age-related pollutant accumulation. By concentrating on one sex, the possible confounding effect of sex on detoxification capacity was avoided. The birds were sacrificed by decapitation and immediately dissected for the liver tissue, which was separated and stored in liquid nitrogen to prevent further changes in enzyme activities.

#### *2.4.2. Laboratory analyses*

In the detoxification capacity study (V), EROD activity was measured from liver samples. EROD activity was used as a measure of detoxification capacity, since it is a good indicator of CYP1A induction and has been commonly used in detoxification capacity studies in several animal groups (Whyte et al., 2000, Fossi et al., 1995a, Marsili et al., 1996, Walker & Ronis, 1989).

The frozen liver samples were homogenized with TissueLyser (Qiagen, Austin, USA) at +4°C to prevent the loss of enzymatic activity prior to measurement. Centrifugation (10 000 g, 15 min) was conducted at +4°C; the homogenizing buffer was kept ice-cold as well (Burke & Mayer, 1974). The complete supernatant was used in EROD activity analyses. The enzyme CYP1A converts 7ethoxyresorufin to a fluorescent product resorufin, which can be detected with a fluorometer (Whyte et al., 2000). The amount of resorufin grows linearly as a function of time; thus the higher the resorufin yield versus time unit, the higher the EROD activity in the sample. To make the samples comparable, the activity was proportioned to the total protein concentration. The unit for EROD activity is thus ηmol/min/mg protein. EROD activity was measured according to Burke and Mayer (1974), with adaptations to microplate. The measurements were carried out in triplicate (intra-assay coefficient of variability [CV] < 10% in all cases), using 96-well plates with an Envision microplate reader (Perkin-Elmer Wallac, Turku, Finland). Three control samples were used in each plate, to allow correction of inter-assay precision with the ratio specific to a particular plate (range 0.8-1.2). A blank sample (plain EROD-reaction mix) was used as a negative control. The protein assay was conducted with the Bradford method (Bradford, 1976); the protein concentration was measured with an Envision microplate reader at an absorbance of 595 nm.

### *2.4.3. Phylogenetic analyses*

The phylogeny of the species studied was taken into account in the analyses, since closely related species are more likely to share similar evolutionary traits, such as the MFO system. The data on basal metabolic rates (McNab, 2009, McKechnie et al., 2006) and on the liver masses of the species (unpublished data, courtesy of A.P. Møller) were combined with my own data to determine the relationship of EROD activity to the relative basal metabolic rate (BMR; W/body mass (g)) and relative liver mass (liver mass (g) x 100/ body mass (g)) (average for the species). The phylogenetic relationships of the species were taken from Treplin et al. (2008) and branch lengths of the topology were estimated using DNA sequence data from six gene regions (a more specific description is given in article V and references therein). The resulting phylogenetic tree with branch lengths (substitutions per site per time unit) was input into Mesquite phylogenetic software (Maddison & Maddison, 2010), for the independent contrasts analysis (EROD activity, relative BMR and relative liver mass). Phylogeny, however, was not included in the analyses of diet and migration status, because these variables had only two categories, and diet in particular was so strongly dependent on phylogeny (insectivores vs. granivores) that the analysis would have no power to detect phylogenetically independent variation with this number of species. The phylogenetically corrected (OLS) regression (with 95% confidence intervals (CI) and predicted intervals (PI) mapped onto the original data space) and correlations between the variables were

analyzed with Felsenstein´s independent contrasts method in the Mesquite program after ensuring the normality of distributions (V). Felsenstein´s correlations ($r_F$) are based on phylogenetically independent contrasts (PIC), enabling correlations among the character sets for species that are not statistically independent, due to a common ancestor in their phylogenetic history.

# 3. MAIN RESULTS AND DISCUSSION

## 3.1. Oxidative status in metal polluted environment (II-IV)

None of the species studied showed increased oxidative stress caused by direct metal pollution (II-IV). The species varied, however, in their regulation of antioxidant enzyme activities when exposed to metals (III, IV). The earlier literature indicates an overall increase in oxidative stress when natural populations of organisms are exposed to anthropogenic pollution (Isaksson, 2010), although the results have varied depending on the species used and the level of pollution exposure. Common to earlier studies has been the strongest response of glutathione to pollution exposure, followed by glutathione-related enzymes GP and GST (Isaksson, 2010), indicating that they are the most prominent biomarkers for metal-induced oxidative stress. Elevated enzyme activities in polluted areas have been reported for example for the white stork (*Ciconia ciconia*) (Kamiński et al., 2009), the pied flycatcher (Berglund et al., 2007), the great tit (Isaksson et al., 2005, Norte et al., 2010) and several waterfowl species (Hoffman et al., 1998, Hoffman, 2002), while some studies with great tits have not found a direct association between pollution exposure and oxidative stress (Isaksson et al., 2009, Koivula et al., 2011).

While metal pollution did not directly affect the oxidative status of the birds in my studies, it is possible that metal pollution can indirectly affect oxidative status via a deficiency of high quality food in polluted areas, as was also suggested in the study with great tit nestlings (II). It has been suggested that indirect pollution effects may be even more common in nature than direct ones, and may also occur at lower contaminant levels (Eeva et al., 2003). Metal concentrations were not measured in the adult birds, but the small difference in oxidative status between the polluted and unpolluted areas indicates no direct link with metal pollution in adult birds either (IV). However, while adult females showed no direct pollution effects on their oxidative status, their brood size and the fledging success of the offspring were significantly lower at higher metal concentrations (IV).

The results indicate that the moderate pollution levels (though among highest found in Finland) occurring in this study area are probably not high enough to cause major effects on birds' intracellular physiology, as indicated by the relatively low oxidative profiles in the study species. The lack of major pollution effects on the birds' physiological parameters may be due to their effective antioxidant defence, which is able to cope with the additional ROS induced by metal pollution without showing major effects on particular antioxidant enzyme activities or glutathione levels. On the other hand, metal

concentrations in the surroundings of the smelter may still be within the range that birds are able to tolerate for a certain time period without showing major intracellular toxic effects. In addition to the time spent foraging in a metal-contaminated environment, the concentration of metals in their food items may also be critical for birds' survival in the polluted environment. For example caterpillars, which are the main food of great tit nestlings (50-70%) (Cramp & Perrins, 1993), contain relatively low amount of metals such as copper (Eeva et al. 2005). To date relatively little is known as to the extent to which small free-living birds can tolerate certain metals without showing increased oxidative damage. Thus experimental dose-response studies are needed, not only under laboratory conditions but also in the field, in the natural habitats of birds, to uncover the specific dose-response relationship between certain metals and oxidative damage.

Of course, it is also worth considering whether we are using the best possible biomarkers to study metal-induced oxidative stress in wild birds in their natural habitats; more generally, whether methods developed for *in vitro* studies can be applied in the field. In order to answer those questions, more comparative studies are needed to see whether laboratory studies are directly applicable to field studies, and whether free-living birds show similar effects in laboratory circumstances compared to their natural habitats, where birds are exposed to a wide range of external factors.

Different tissues contain variable amounts of antioxidant enzymes and accumulate different amounts of contaminants, and thus are not directly comparable. The choice of the proper tissue to measure oxidative status biomarkers is essential, but it is also important to consider the ethical problems of tissue sampling, especially in the case of free-living animals. Both liver tissue and blood have been commonly used in oxidative stress studies. However, the use of blood to measure oxidative status biomarkers has recently been questioned, since the lifespan of avian erythrocytes is no longer than 28-45 days (Thrall et al., 2012). It is thus possible that antioxidant protection in erythrocytes is less pronounced than in other tissues, meaning that even though oxidative damage is likely to occur, it may have less of an effect on overall oxidative stress at the level of the organism as a whole. This may cause some problems in sampling adult birds; in small nestlings, which have not yet undergone red blood cell regeneration, blood may still be a relatively safe, non-destructive way to measure different biomarkers.

### *3.1.1. Interspecific variation in oxidative status (III-IV)*

While we found no difference in the overall oxidative state of our three study species, in either nestling or adults (III, IV), there was substantial variation in the manner in which this oxidative state was achieved in nestlings of the three species (III), which differed significantly in their antioxidant enzyme activities (GP, GST, CAT and SOD) (III). The main difference was observed between nestlings of pied flycatchers and Parids in their

SOD and CAT activities, pied flycatchers having significantly higher SOD activity but lower CAT activity than either of the Parids (III-IV). A similar trend was also found in adult females in SOD and CAT activities, but not in the glutathione-related enzymes GP and GST (III-IV). The interpretation of CAT activities in pied flycatchers, however, needs to be considered with caution, since most of the activities (72.6% in nestlings and 88.0% in adult females) were below the detection limit (0.97 nmol/min/mg). These results mean that although the species studied had a similar glutathione metabolism, they differed in their regulation of SOD and CAT activities, which are the major enzymes regulating ROS levels. SOD activity plays an important role in defence against oxidative stress by keeping the $O_2^-$ concentration at a low level. $O_2^-$ is further catalysed by CAT into water and molecular oxygen (Fridovich, 1997, Halliwell & Gutteridge, 2007). Interspecific differences in those enzymes may thus indicate different regulation of them in antioxidant defence. Variation in the regulation of enzyme activities even in closely related species suggests that glutathione regulation may not be strictly phylogenetically determined. This, however, remains to be confirmed in future studies.

The oxidative status of the different species also varied between the study areas. Adult females in all species studied showed significantly lower total GSH levels in the polluted area, but this was not the case for nestlings (III, IV). The difference may be due to differences in glutathione metabolism between adults and nestlings. The metal accumulation rate may also vary with age. In nestlings only blue tits showed higher GP activity and lower GSH:GSSG ratio in the polluted area, but the GSH:GSSG ratio of blue tits in this area was not smaller than that of the other species. These results suggest that although blue tit had up-regulated enzyme activities in the polluted area, they may not suffer oxidative stress more than the other species. Since the species studied in general showed only minor differences in their oxidative status when exposed to pollution, the regulation of oxidative status can be seen as species-specific rather than as related to metal pollution alone (III). This is in agreement with the findings of Costantini et al. (2010), who have concluded that the relationship between oxidative physiology and life-history traits may be species-specific rather than general across avian species.

To conclude, while only small effects were observed in the species' oxidative state (i.e. GSH:GSSG ratio), the regulatory mechanisms underlying these values differed between species. Interestingly, adult females had somewhat higher total GSH level and enzyme activities, indicating a higher oxidative profile of adults compared to nestlings. Adult females, on the other hand, had lower GSH:GSSG ratios than nestlings (III-IV). While adults may encounter accumulated effects caused by metals (Sawicka-Kapusta et al., 1986), nestlings too may suffer a variety of detrimental effects if exposed to pollutants during a critical time during development (Isaksson, 2010, Scheuhammer, 1987). Breeding adults may also face additional stressors, such as parasites, predators, poor nutrition, adverse weather conditions or certain physical strains related to reproduction,

which may increase their oxidative status compared to small nestlings (Isaksson et al., 2005). Isaksson (2010) concluded that adult individuals in general had a higher oxidative status than juveniles when living in a polluted environment. For example, adult great tits had higher total GSH levels than their nestlings in both rural and urban habitats (Isaksson et al., 2005). Likewise adult blue petrels (*Halobaena caerulea*) have shown higher serum antioxidant capacities (OXY) compared to nestlings (Costantini & Bonadonna, 2010).

Metabolic differences may also explain the difference in oxidative status between the small nestlings and adult females. Nestling metabolic rates (measured as mass-specific resting metabolic rate, RMR) have been shown to be low in newly hatched chicks (Ricklefs, 1989, Klaassen & Drent, 1991, Weathers & Siegel, 1995), but to increase quickly during development (Olson, 1992, Choi et al., 1993, Visser & Ricklefs, 1993, Bech & Østnes, 1999), the peaks being even higher than in adult birds with similar body masses (Klaassen & Bech, 1992, Olson, 1992). Aged individuals, instead, often show reduced basal metabolic rates (BMR) (Moe et al., 2009, Broggi et al., 2010), which may be a consequence of functional failures (e.g. damage to biomolecules and telomere shortening) caused by the aging process (Finkel & Holbrook, 2000, Monaghan & Haussmann, 2006, Broggi et al., 2010). The higher oxidative status in adult females in my study (IV) may thus be related to a lowered BMR caused by increased oxidative damage. However, since BMR was not measured, this remains to be confirmed in future studies. It is also possible that other environmental factors and the costs of reproduction may further enhance those effects in adult birds compared to nestlings.

My results show that antioxidant enzyme activities in one species may not be comparable with those in others, even among close relatives and at the same study sites. Thus species-specific oxidative status needs to be taken into account, especially in pollution-related studies. This study highlights the importance of understanding the mechanisms whereby oxidative status is regulated in different species, since without this information it is difficult to assess whether a given environmental disturbance causes oxidative stress or not. So far there have been only few studies comparing the oxidative status of a number of free-living species using the same study set up. Some of these studies have found a similar oxidative status between species (Costantini et al., 2007), while others have shown differences in species' oxidative balance under the same environmental conditions (Costantini & Bonadonna, 2010, Costantini et al., 2010). Interspecific studies related to the regulation of antioxidant enzyme activities in polluted environments are even scantier (Hoffman et al., 1998, Hegseth et al., 2011b, Martinez-Haro et al., 2011). Most studies have also concentrated on one species at a time (Berglund et al., 2007, Isaksson et al., 2009, Koivula et al., 2011, Hegseth et al., 2011a); thus comparisons among different species or studies are difficult because of varying measurement techniques or differences in the tissues (blood, liver, kidney) used in the studies. Since different tissues accumulate different amounts of metals (Dmowski, 1993), direct comparisons among

them are difficult. Furthermore, different environmental conditions need to be taken into account, since many environmental factors may affect species' oxidative status (Norte et al., 2009a). Thus more comparable studies under the same environmental conditions are necessary to understand interspecific variation in the regulation of oxidative status.

### *3.1.2. Metal-induced effects on oxidative status of the species (II-IV)*

#### 3.1.2.1. Great tits

Of the species studied, the great tit (both nestlings and adults) turned out to be least susceptible to oxidative stress in relation to direct metal pollution (III, IV). Great tit nestlings did show increased GP activities in the polluted area, but the difference was related to nestling body mass rather than to direct metal pollution (II). The result suggests that up-regulated GP activities are related to poorer nestling condition in metal-polluted areas (II). The same trend was also found in 2008 (III), although the effect was not as strong as in 2004. CAT activity, in contrast, was higher in heavier nestlings than in smaller ones in 2008 (III). Parallel to my studies, birds have also shown increased GP activities in areas close to a pulp mill (Norte et al., 2010) and to another copper smelter (Kamiński et al., 2009), suggesting that GP is sensitive to pollution exposure. Likewise elevated GP activities have been found in common coots *(Fulica atra)* exposed to lead (Martinez-Haro et al., 2011). GP activity also increased with warmer developmental temperature, which may suggest higher oxidative status in nestlings born later in the breeding season, when the temperature is higher (II). This could be due to a higher metabolic rate of ectothermic nestlings at warmer temperatures (Eeva et al. 2003), which may in turn increase oxidative status. In 2008, however, there was no significant relationship between GP activity and temperature (III). Likewise the total GSH level was lower at warmer temperatures in 2008 (III). These contradictory results may be related to a difference in ambient temperatures between the years, since the nestling period in 2004 was colder than that in 2008 (ranges 7.5-12.5 and 10.3-16.3°C respectively). Nestling age at the time of sampling also differed between the years (9 and 11 days in 2004 and 2008 respectively). This may cause variation in enzyme activities, since the metabolic rate, which may also affect enzyme activities, is dependent on temperature, and the relationship between metabolic rate and temperature in nestlings may change with age. It should be kept in mind, however that there may also be other factors affecting the result, along with temperature alone. The study in 2008 (III), for example, showed a strong positive correlation between hatching day and temperature in all three species, indicating that later hatched nestlings also faced higher temperatures. It is possible that some environmental factors, such as precipitation or food quality, change over time, thus affecting birds hatched later in the summer. This in turn could be reflected in enzyme activities or total GSH levels. More specific studies linked to food availability are thus needed to confirm the connection between glutathione metabolism and temperature.

The activity of GST, the enzyme related to biotransformation processes, increased in nestlings with brood size in 2004 (cold breeding season), suggesting increased nestling competition for food and space, which in turn may increase oxidative stress in nestlings in larger broods. This effect was not found in 2008 (warm breeding season), possibly due to better food availability or some other factors affecting the condition of nestlings in that year. Brood size affects many condition-related variables, such as body mass, growth rate and immune responses, as well as certain physiological parameters, including plasma carotenoids and red blood cell resistance to free radicals (Dijkstra et al., 1990, Mock & Parker, 1997, Alonso-Alvarez et al., 2006).

Although great tit nestlings were studied in two different years, the absolute enzyme activities may not be comparable between years because of the different storage period of the blood samples at 80°C before the laboratory analyses. It is possible that enzyme activities may decrease with longer storage periods, even when otherwise stored properly. Thus the yearly variation needs to be interpreted with caution. The different measurement techniques used for glutathione levels in the two years also make direct comparisons impossible. Normal seasonal and yearly variation in antioxidants and enzyme activities cannot be excluded either, since environmental conditions, such as temperature, food quantity and quality, season, physiological stress during nestling growth, or anthropogenic stressors, may all have an impact on the oxidative balance. However, based on the parallel results between the two study years, I can conclude that metal pollution, at the levels prevailing in this study area, has no direct effect on the oxidative status of great tit nestlings, but rather indirect effects via diet and condition. Since GP activity showed similar results in both study years, it can be used as one potential biomarker for oxidative stress in great tit nestlings, especially in describing condition-related effects of metals. GP activity has also been suggested to be a reliable indicator of health, since it seems to be relatively constant for long time periods, compared to many other physiological components of health (Norte et al., 2008).

#### 3.1.2.2. Blue tits

Blue tits, studied only in 2008, seemed to react more strongly to metal pollution in their nestling phase compared to the other species studied (III). The nestlings showed increased GP and GST activities relative to increased metal concentration, suggesting a higher response to metals. Individual variation, however, was relatively high in both study areas. The increased activities of antioxidant enzymes can be considered as protective responses against metal pollution (Martinez-Haro et al., 2011), reflecting the activation of defence mechanisms (Halliwell & Gutteridge, 2007, Koivula & Eeva, 2010). Thus the up-regulation of GP and GST may provide evidence of a protective response against metal exposure, reflecting the activation of antioxidant defence. However, if oxidative enzymes such as GP prove insufficient in combating metal exposure, the result may

be increased oxidative stress. As oxidative damage (e.g. lipid peroxidation) was not measured in this study, I cannot say whether there was increased cellular damage caused by metals. However, since the GSH:GSSG ratio did not show a significant change in relation to metal concentrations, increased oxidative stress caused directly by metals is unlikely to occur. The induction of GST activity has been shown to be an evolutionary response of cells in protection against metabolites and degenerative disorders related to oxidative stress (Hayes et al., 2005, Raza, 2011). Increased GST activities have earlier been found for example in pied flycatchers close to a sulphide ore smelter (Berglund et al., 2007). In contrast to nestlings, adult blue tit females showed no associations between oxidative status and metal exposure (IV). Similarly, we observed no effect on female age, condition, clutch size or nestling age at sampling time, the latter ranging between 11 and 17 days (IV). The lack of significant associations, however, may be due to the small sample sizes of blue tit females, and needs to be confirmed in further studies.

In addition to direct pollution effects on enzyme activities, blue tit nestlings showed also condition-related effects on their oxidative status. CAT activity and GSH:GSSG ratio were higher in heavier nestlings, suggesting that nestlings in better condition may have a more effective defence against oxidative stress. The CAT activity and GSH:GSSG ratio may also be interrelated. Since CAT is involved in the transformation of hydrogen peroxide to water and oxygen, an increase in its activity lessens hydrogen-peroxide–induced oxidation, thereby favouring the maintenance of glutathione in its reduced state. The lower GSH:GSSG ratio found in blue tit nestlings in the polluted area may also be related to the lower GSH:GSSG ratio in lighter nestlings, since nestlings in the polluted area were slightly lighter than in the unpolluted one, indicating indirect pollution effects.

Blue tit nestlings had also increased GST activities and total GSH levels at warmer temperatures. The collinearity in total GSH level and GST activity may be related to their function, since GSH acts as a co-substrate for the GST enzyme (Kaplowitz, 1980). In 2008 variation in the ambient temperature during the nestling period was quite high, ranging from 10.3 to 16.3°C. The increased GST activity may thus indicate higher activity of the phase two detoxification system at warmer temperatures. In great tits the effect of temperature on total GSH level was the opposite, although both species had similar temperature conditions during the nestling period. Again, it is possible that some environmental factors have changed over time, thus affecting the later hatched birds. However, the reason for the opposite results remains unknown and calls for experimental studies in glutathione metabolism and temperature variation. So far little is also known about seasonal variation in oxidative status in free-living birds, further confirming the importance of including temperature variation in studies of oxidative status. Cold-acclimated Wistar rats have shown increased lipid peroxidation and GSH levels in brown adipose tissue, indicating that GSH is an important antioxidant involved in adaptation of the tissue to higher peroxidative damage (Dequiroga et al., 1991)antioxidant enzymes and lipid-peroxidation in brown adipose

tissue</IDText><DisplayText>(Dequiroga et al., 1991. Long-term cold exposure has also increased CAT and GP activities in tissues of field voles (*Microtus agrestis*), suggesting better protection against ROS damage (Selman et al., 2000). In general, temperature-related studies have shown that the effects of temperature on enzyme activities are tissue-specific and dependent on the extent of the cold exposure (Buzadžić et al., 1999).

3.1.2.3. Pied flycatchers

Neither nestlings nor adult pied flycatchers showed any direct association between metal concentrations and antioxidant enzyme regulation. The total GSH level, on the other hand, was higher in nestlings exposed to higher metal concentrations close to the pollution source. Earlier studies have indicated both an increase (Ji et al., 2006, Mateo & Hoffman, 2001) and a decrease (Martinez-Haro et al., 2011, Gurer & Ercal, 2000, Ercal et al., 2001, Reglero et al., 2009) in total GSH levels in polluted environments, suggesting that pollution may impair glutathione production. This may be related to dose-dependent effects of glutathione or to the duration of metal exposure (Henny et al., 2002, García-Fernández et al., 2002, Thomas & Wofford, 1984). Thus it is possible that the increased total GSH levels found here are related to the relatively low metal concentrations and short duration of metal exposure of nestlings in my study area.

Pied flycatcher nestlings also showed condition-dependent effects on oxidative status, with higher GP and GST activities found in lighter nestlings. If the higher total GSH levels at higher metal concentrations are also taken into account, the up-regulated enzyme activities of lighter nestlings may be the consequence of indirect metal pollution (III). These findings, together with the higher ratio of GSH:GSSG in heavier nestlings, suggest that heavier nestlings may be less vulnerable to oxidative stress than lighter ones, especially when we take into account the fact that smaller nestlings are often found in polluted areas. Up-regulation of enzyme activities is also energy demanding. Thus, energy allocated to antioxidant defence may leave a smaller proportion of energy to be allocated to other functions, such as growth. Therefore, the lower body mass of the nestlings may also reflect trade-off between growth and effective antioxidant defence.

Earlier studies in the same smelter area have found lower fledging success and lower food availability closer to the pollution source (Eeva et al., 2005a, Eeva et al., 2003, Eeva et al., 2009b, Eeva et al., 2008, Sillanpää et al., 2008). In the study carried out in 2004 (II), lighter great tit nestlings showed higher GP activity, which is consistent with the result for pied flycatchers. Pied flycatcher nestlings, on the other hand, showed lower GST activity with larger brood size (III), while the opposite was found in great tits in 2004 (II). This may be related to the generally smaller brood size of the pied flycatcher (ca. 6-7) compared to the great tit (ca. 8-10), although great tits tend to match their brood size to the prevailing food availability (Decker et al., 2012, Perrins & McCleery,

1989). Contrary to blue tits, pied flycatcher nestlings had lower GST activities and total GSH levels at warmer temperatures. The opposite orientation of those biomarkers may be due to the relatively low variation in temperature during the nestling period in pied flycatchers: only 1.6°C, compared to 6.0°C for the blue tits. The small range of nestling time temperatures in the case of the pied flycatcher suggests that the association observed between temperature and enzyme activity may be irrelevant.

### *3.1.3. Interspecific variation in metal profile and fledging success*

In spite of the close phylogenetic relationship between great tits and blue tits, they responded differently to metal pollution (III). Although adult birds did not show much variation in their oxidative status relative to metal exposure, blue tit nestlings showed a stronger response to increased metal levels, with up-regulated enzyme activities, than great tit nestlings. This may be related to differences in their metabolic mechanisms, since in my study areas the two species consume quite similar diets and occupy the same kind of breeding habitat. Blue tits, however, have been shown to feed their nestlings with smaller food items (e.g. aphids (Homoptera)), while the great tit favours proportionally more spiders (Araneae), Diptera, and Lepidopteran larvae (Cowie & Hinsley, 1988, Cramp & Perrins, 1993, Eeva et al., 2005b, Eeva et al., 1997). Blue tits are also smaller than great tits (Cramp & Perrins, 1993), with the ensuing higher metabolic rate per mass unit (Svensson et al., 1998, Nilsson & Raberg, 2001); together with rapid feeding rates, this may affect metal accumulation (Eeva et al., 2009a, Root, 1990). The metal profile, on the other hand, turned out to be relatively similar in Parids, while Ca levels were significantly lower in blue tits (III). It is thus possible that the blue tit is more vulnerable to metal-related detrimental effects with a low Ca level in their diet (Eeva et al., 2009a). Pied flycatchers, on the other hand, had higher Cu and Cd concentrations compared to Parids, probably due to dietary differences between these species (Eeva et al., 2005b). Spiders and isopods, which are often found in pied flycatcher nests in polluted areas, have been shown to contain high concentrations of Cu and Cd in contaminated environments (Hunter et al., 1987). The Ca levels of pied flycatchers were even lower than in blue tits. Low Ca levels have been shown to cause thinner eggshells in the same smelter area (Eeva & Lehikoinen, 1995, Eeva & Lehikoinen, 2004).

The fledging success of great tit nestlings was significantly affected by pollution exposure (II). Nestlings in the polluted area had significantly lower fledging success and lower body mass than those in the unpolluted area (II), suggesting a lower food supply and poorer-quality food in the polluted area. A similar trend was also observed in blue tit and pied flycatcher nestlings in 2008. Pollution has been shown to reduce certain important food sources for birds, hence indirectly affecting their breeding performance and survival (Eeva et al., 1997, Eeva et al., 2003, Eeva et al., 2005b, Bengtsson & Rundgren, 1984, Perrins, 1991). To conclude, in spite of only minor direct pollution

effects on the birds' intracellular physiology, fledging success was significantly lower in polluted areas than in unpolluted ones, indicating pollution-mediated effects on nestling condition. The absence of direct pollution effects contributing to oxidative stress may be due to the relatively low metal concentrations in the study area used in this thesis, compared to some other avian studies carried out close to pollution sources (Bel'skii et al., 1995, Janssens et al., 2003a, Berglund et al., 2007, Dmowski, 1993).

### *3.1.4. Oxidative status, age and reproductive success of adult females (IV)*

Adult females of all species studied showed only one direct effect of metal pollution on oxidative status, i.e. decreased total GSH levels in the polluted areas. On the other hand, their oxidative status seemed to be more closely related to life-history traits, such as brood size and fledging success of their nestlings (IV). Females of the great tit and pied flycatcher differed in their breeding effort and in the use of antioxidant enzymes, whereas the small sample size of blue tit females did not allow such comparison. In the great tit brood size was not directly associated with pollution levels, age or the condition of females during the late nestling period, but females rearing larger broods had a lower GSH:GSSG ratio, indicating increased use of GSH as an antioxidant. Since GST activity also increased with larger clutch size (number of eggs laid), the results suggest a potential trade-off in the great tit between reproduction and oxidative status. Fledging success, on the other hand, was higher in older females; the females with higher fledging success also had higher GP and CAT activities, both of which are involved in catalysing $H_2O_2$ into water and molecular oxygen. The up-regulation of those enzyme activities thus indicates costs for older females with better reproductive success. It is possible that this greater reproductive success in older females may be an effect of the selective disappearance of females of poor quality. Alternatively, it may be linked to the greater experience of older females.

In contrast to the great tit, the fledging success of pied flycatcher females was not associated with any of the oxidative status biomarkers studied, but brood size was negatively associated with the body condition index and with the level of metal exposure. In other words, females with larger broods were leaner at the end of the nestling period and had fewer offspring in the polluted environment. At the end of the nestling period pied flycatcher females also had higher CAT activity with larger broods, which may indicate costs of reproduction. It has been suggested that parents rearing large broods may not be constrained by the actual laying and incubation, but rather by their chick-feeding abilities (Costantini, 2010, Deerenberg et al., 1995, Alonso-Alvarez et al., 2004b). The present study shows that feeding large broods is costly for females and may induce higher antioxidant enzyme activities. Several studies have linked reproduction with oxidative stress (Alonso-Alvarez et al., 2004b, Wiersma et al., 2004, Monaghan et al., 2009, Bize et al., 2008, van de Crommenacker et al., 2011, Losdat et al., 2011, Markó et al., 2011), with contradictory results. Higher reproductive investment has sometimes been found

to result in reduced oxidative protection (Alonso-Alvarez et al., 2004b, Wiersma et al., 2004, Losdat et al., 2011) and to promote oxidative damage (Alonso-Alvarez et al., 2010), while other studies have not found any relationship between reproduction and antioxidant defence (Markó et al., 2011, Bize et al., 2008).

Oxidative stress has often been connected with aging (Finkel & Holbrook, 2000), although this relationship has lately been questioned (Speakman & Selman, 2011). Avian studies have shown contradictory results: either aged females have been more resistant to oxidative stress (Markó et al., 2011), or they have experienced higher oxidative stress and lipid peroxidation (Alonso-Alvarez et al., 2010). Interestingly, female age was an important factor affecting the oxidative status of birds in my studies as well (IV). In great tits, young females (1 year old) had higher GP activity and GSH:GSSG ratio than older females (2 years or older); young pied flycatcher females, in contrast, had higher CAT activities than older ones, indicating that antioxidant enzyme regulation is species-related. These results suggest that older females of both species have reduced antioxidant defence in terms of lower enzyme activities and GSH:GSSG ratio, but then again older females had better reproductive success (fledging success in the great tit and brood size in the pied flycatcher) in combination with higher enzyme activities (IV). However, the division of females into only two age classes, as in my studies, makes a more detailed interpretation of the effect of age difficult, as age-related effects can be asymptotic (Bize et al., 2008). Previous studies related to age have shown higher total antioxidant activities (TAA) (Isaksson et al., 2007) and plasma antioxidant capacities (OXY) (Markó et al., 2011) in older females compared to younger ones. Older red-legged partridges (*Alectoris rufa*), on the other hand, have been found to have higher GSSG levels and end-products of lipid peroxidation (TBARS) than younger individuals (Alonso-Alvarez et al., 2010).

### *3.1.5. Relationship of carotenoids, plumage colour and oxidative status in metal-polluted environment (II-III)*

Carotenoid supplementation increased the plasma lutein concentration in birds treated with carotenoids compared to the control birds, and the concentration was higher in polluted sites compared to unpolluted sites. On the other hand, there was no interaction on lutein concentration between carotenoid supplementation and study area, indicating a similar response in plasma carotenoid levels in both study areas, polluted and unpolluted (II). There was likewise no association between plasma carotenoid level and metal concentration, suggesting that the metal levels, in this study, did not affect carotenoid levels. Carotenoid availability in great tit nestlings has also been found to be similar in urban and rural habitats in the study of Isaksson and Andersson (2007), although their results showed lower carotenoid concentrations in caterpillars in urban areas, but then again a higher abundance of caterpillars as well as a higher feeding frequency in these areas. In earlier studies in my study area, plasma carotenoid concentrations have been

shown to vary considerably between years (Eeva et al., 2005b, Eeva et al., 2009b); the levels have also varied nonsynchronously among breeding seasons between polluted and unpolluted areas (Eeva et al., 2012). In 2004 carotenoid availability was relatively good in both study areas, which may explain the non-existent difference between the study areas.

Carotenoid treatment likewise had no effect on the oxidative profile of great tit nestlings, suggesting no increased antioxidant capacity in treated birds (II). Carotenoid profiles were relatively similar in all species independent of age (nestlings vs. adults) in 2008 as well (III, IV). Pied flycatchers, however, had significantly lower carotenoid levels in the polluted area, but this was not the case in either of the Parids (Eeva et al., 2012), suggesting different carotenoid profiles between pied flycatchers and Parids.

Yellow plumage colour has been shown to vary in relation to metal exposure, birds being paler in more polluted environments (Eeva et al., 1998, Isaksson et al., 2005, Hõrak et al., 2001, Geens et al., 2009). So far only few studies concerning oxidative stress and plumage colour have been conducted, yielding contradictory results. Plumage colour has either been paler with a shift in the ratio of reduced and oxidized glutathione towards the oxidized form (Isaksson et al., 2005), or there has been no association between plumage colour and oxidative status biomarkers (measured as antioxidant capacity) (Geens et al., 2009). I did not find any effect on plumage colour between polluted and unpolluted areas, but the yellowness of the plumage was positively correlated with the plasma carotenoid concentration. The lutein treatment also produced brighter yellow plumages (II). Plumage colour also increased with brood size, indicating that a good availability of carotenoid-rich food enables bigger broods with brighter yellow colour of their breast feathers. As I found no relationship between plumage colour, metal exposure and oxidative status biomarkers, it can be concluded that moderate pollution levels do not directly affect variation in plumage colouration (see also Eeva et al. 2008), and that plumage colour alone is not a good indicator of oxidative stress in great tit nestlings. Isaksson et al. (2007) found that plumage colouration and plasma carotenoid concentrations in the great tit did not reflect total (non-enzymatic) antioxidant activity (TAA) of plasma, suggesting that carotenoids are most likely not used as antioxidants in the plasma. Similar results have also been arrived at with the Eurasian kestrel (*Falco tinnunculus*) (Costantini et al., 2006) and the blue tit (Larcombe et al., 2010), although using different measurement techniques.

## 3.2. Variation of basal EROD activities in relation to diet and migration status (V)

### *3.2.1. Diet and migration status*

The induction of CYP1A, an enzyme belonging to the MFO group, is an important measure in studies investigating detoxification capacity in animals. The induction of the

CYP1A enzyme was measured using EROD activity analysis. The study of interspecific variation in basal liver EROD activity showed that species differ in their detoxification capacity and that the variation was significantly related to diet, but not directly to migration status. Diet and migration status, however, showed significant interaction on EROD activity. Migratory insectivores had the highest EROD activities, while migratory granivores had lower or similar EROD activities compared to non-migratory granivores (V). Migratory species are likely to use more variable food, with higher amounts of secondary compounds, in both their breeding and their overwintering habitats, and have therefore developed higher detoxification capacities, making them better adapted to natural compounds but also to those of anthropogenic origin. The feeding habits of species with a narrow diet, in contrast, have often led to low microsomal mono-oxygenase activity towards many xenobiotics, since their food items have contained only relatively limited amounts and varieties of those compounds in the course of evolution (Fossi et al., 1995a).

Interspecific variation in detoxification capacity, found in several animal groups (Fossi et al., 1995b), has been linked with species-specific differences in the MFO system, the function of which is based on their ability to detoxify harmful compounds into less toxic forms (Newman, 2010). Earlier studies have shown that omnivorous species, using a mixed diet (plant and animal food), have higher detoxification capacities than species with narrow diets (Fossi et al., 1995a, Fossi et al., 1995b). Ronis and Walker (1989) and Sinclair and Sinclair (1993) in their review articles, have also showed a strong relationship between hepatic microsomal mono-oxygenase activities and diet, as found in studies with in thirty species of birds in ten different orders. Liukkonen-Anttila et al. (2003) compared EROD activities in Galliformes using different diets, showing higher EROD activity in species consuming variable food items compared to those with a more narrow diet, indicating better detoxification capacity in the omnivorous birds. In general, EROD activity levels seemed to be relatively low in Galliformes compared to the Passeriformes of my study (Liukkonen-Anttila et al., 2003).

### *3.2.2. Relationship between relative BMR, relative liver mass and EROD activity*

Relative BMR (basal metabolic rate per mass unit) was included in my studies in order to determine the effects of metabolic rates on detoxification capacity, since metabolic rate is thought to affect the production of harmful metabolites. Mass-specific BMR was used because the avian BMR is negatively related to body mass (McNab, 2008). However, at the species level, after taking species phylogenies into account, I did not find any significant association between EROD activity and relative BMR, although EROD activity increased slightly with a higher relative BMR (V). In this study set, long-distance migrants had quite high relative BMR coupled with a high detoxification capacity, which may be related to different physiological adjustments between migratory

and resident species. Jetz et al. (2008) also found a higher BMR in migratory birds than in non-migratory ones. Since diet has been shown to be related to BMR in birds, suggesting an effect on the species' detoxification capacities, EROD activity was combined with the birds' BMR and diet as well. Studies on the food-habit hypothesis (FHH) have shown contradictory results among different species with regard to the relationship between BMR and dietary habits (Cruz-Neto et al., 2001, McNab, 2002, Bozinovic et al., 2007). The FHH predicts that species consuming food with low energy, low digestibility and unpredictable availability have a low, mass-independent BMR. Some studies have shown omnivores to have a higher BMR than specialist birds eating only insects, seeds or aquatic vertebrates (McNab, 2009), while others have found no connection between diet and BMR in passerine birds (Foley & McArthur, 1994). In my study, insectivores had both a higher relative BMR and higher EROD activity than granivores. The higher BMR of insectivores could be related to the size of the birds, since the insectivores were slightly smaller than the granivores. The lower detoxification capacity of granivores suggests that secondary plant compounds, which in some cases are thought to increase BMR because of an activated detoxification pathway (Sabat et al., 2009, Cork & Foley, 1991, Silva et al., 2004), may not have raised the BMR in my study.

To investigate the relationship between liver mass and detoxification capacity, relative liver mass was also included in the analyses, since higher liver mass could indicate better detoxification capacity. EROD activity correlated positively with relative liver mass in my study when species phylogeny was taken into account, suggesting that a proportionally larger liver also functions more efficiently. Of the species studied, the willow warbler and reed warbler for example had large relative liver masses. Relative liver mass also correlated positively with relative BMR, indicating that birds with large relative liver masses have higher relative BMR. Daan et al. (1990) showed that birds with relatively high BMR for their body mass also had relatively large kidneys and hearts. Due to a lack of similar studies combining relative liver mass and EROD activity, however, I cannot compare my own results to those of others.

# 4. CONCLUSIONS

This thesis shows that direct metal pollution has no major effect on the intracellular physiology of the great tit, the blue tit or the pied flycatcher, more specifically on their oxidative status (II-IV). This may be due to the moderate levels of pollution in my study area, even close to the metal smelter in Harjavalta. Indirect pollution effects via diet, on the other hand, indicate that metals have detrimental effects on birds close to the pollution source, including lowered fledging success, body condition and body mass, as well as up-regulated enzyme activities (II, III). The results were similar in nestlings and adult females, although nestlings responded more strongly to metal exposure with increased antioxidant enzyme activities. Adult females, on the other hand, had somewhat higher oxidative profiles than nestlings, which may be due to additional stressors faced by females during the breeding season (III, IV). In addition to metal exposure, also the condition of the birds affected to their oxidative status, causing up-regulation of antioxidant enzyme activities and a shift in the GSH:GSSG ratio towards more GSSG, the oxidized form of glutathione. Of the oxidative status biomarkers studied, GP and GST activities, the level of total GSH and the ratio of GSH:GSSG showed any association with metal contamination, but the association was species-specific. My results foreground the importance of conducting studies on natural populations, as additional stressors (e.g. weather, parasites, and food quantity and quality), can affect the oxidative status of species via different pathways. This thesis also highlights the importance of combining ecological, toxicological and physiological methods in studies concerning antioxidant defence, in order to more fully understand the complexity of this process in free-living birds.

Another important finding was that species differ from each other in their oxidative status, which needs to be acknowledged in studies of oxidative stress (II, III). Results with one species cannot be generalized to others because basal non-enzymatic and enzymatic antioxidant levels may vary considerably, not just between taxa but also between species within the same taxonomic group. Information as to basal levels of antioxidants and antioxidant enzymes is important in determining whether a certain environmental disturbance causes oxidative stress for a particular species or not.

This thesis shows that carotenoids cannot be considered very effective antioxidants against metal-induced oxidative stress, which is in agreement with some recent studies concerning carotenoids in antioxidant defence. However, this may be related to the fact that birds in polluted areas did not show elevated oxidative stress either. Earlier studies in the same smelter area have shown lower plasma carotenoid levels in the polluted areas, due to variable diet between the polluted and unpolluted areas. Since I did not observe increased oxidative stress in birds exposed to metals either, it is possible that carotenoids are not important in the defence against oxidative stress. Plumage colouration was

likewise not a good indicator of oxidative stress, since no association was found between plumage colour and the oxidative status of great tit nestlings.

It needs to be kept in mind, however, that species differ in their antioxidant defences and that the combination and amount of antioxidants may vary between species, making studies of antioxidant defence more challenging. The use of both enzymatic and non-enzymatic biomarkers in detecting oxidative stress together with measurements of oxidative damage (e.g. lipid peroxidation, the amount of reactive species, telomere shortening, DNA adducts) are important in gaining an overall picture of antioxidant defence in certain study species. However, the precise exposure levels of particular pollutants that can significantly increase oxidative stress in free-living animals remain an open question in this thesis, and need further study in the future. Likewise the combinations of various pollutants and other environmental factors affecting oxidative status in birds need to be taken into account in future studies, in order to understand the mechanisms of antioxidant defence.

In addition to antioxidant defence, birds also have other mechanisms for coping with toxic compounds in their environment. This thesis has shown that species differ from each other in their basal hepatic EROD activities, suggesting differing detoxification capacities in the species studied. Migratory insectivores had the highest EROD activity, while migratory granivores had either lower or similar EROD activities compared to non-migratory/partial migratory granivores. Non-migratory/partial migratory insectivores, on the other hand, had higher EROD activity than granivores in general. These results indicate that granivores are likely to be more vulnerable to environmental contaminants than insectivores and that migratory birds are likely to be less susceptible to contamination than resident birds. However, the strong relationship between diet and migration status needs to be stressed, as the diet varies considerably between breeding and overwintering habitats. It is thus important to find out whether the wintering grounds explain the difference in EROD activities between the migratory groups and whether diet-based variation in EROD activity occurs when migration is excluded.

Relative BMR and relative liver mass were also combined in this thesis for the first time with EROD activity. When species phylogeny was taken into account, no association was found between relative BMR and EROD activity. Species with higher EROD activities, however, also had relatively large livers, indicating that a proportionally large liver also functions more efficiently. However, the small sample size within each study species needs to be taken into account in interpreting these results. Further studies, with larger numbers of species, are urgently needed to confirm the relationships between detoxification capacity, BMR and organ size at both the ecotoxicological and the physiological level. An understanding of basal liver EROD activities in each species is important to improve the use of EROD activity as a possible biomarker in ecotoxicological studies. Greater understanding of tolerance and detoxification ability will also help in identifying those species that are most sensitive to environmental contamination.

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