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# CONSEQUENCES OF POLLUTION-RELATED CAROTENOID LIMITATION IN BIRDS

by

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*Valo kaareutuu valoon,*
*pimeys pimeyteen.*
*Olen nähnyt molemmat*

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# LIST OF ORIGINAL ARTICLES

This thesis is a summary of the following publications and a manuscript, referred to in the text by their Roman numerals:

I. Sillanpää, S., Salminen, J.-P., Lehikoinen, E., Toivonen, E. and Eeva, T., (2008): Carotenoids in a food chain along a pollution gradient. Science of the Total Environment 406: 247-255.

II. Eeva, T., Sillanpää, S., Salminen, J.-P., Nikkinen, L., Tuominen, A., Toivonen, E., Pihlaja, K. and Lehikoinen, E. (2008): Environmental pollution affects the plumage color of Great tit nestlings through carotenoid availability. EcoHealth 5: 328-337.

III. Sillanpää, S. Salminen, J.-P. and Eeva, T. (2009): Breeding success and lutein availability in Great tit (*Parus major*). Acta Oecologica 35: 805-810.

IV. Eeva, T., Sillanpää, S. and Salminen, J.-P. (2009): The effects of diet quality and quantity on plumage colour and growth of Great tit nestlings: a food manipulation experiment along a pollution gradient. Journal of Avian Biology 40: 1-9.

V. Sillanpää, S., Salminen, J.-P. and Eeva, T. (2010): Fluctuating asymmetry in Great tit nestlings with different diet quality, calcium availability and pollution exposure. Science of the Total Environment 408: 3303-3309.

VI. Sillanpää, S., Salminen, J.-P. and Eeva, T.: Female quality and maternal effects in Great tit along an air pollution gradient. Manuscript.

# 1. INTRODUCTION

## 1.1. Air pollution and carotenoids in a food chain: plant-insect-bird

In insectivorous birds, carotenoid levels are affected through many different pathways at each level of a food chain. Air pollution affects plants, insects and birds, directly or indirectly. Various studies, using different plant species (see below, section 1.2) have shown different responses; in some studies air pollution has increased carotenoid concentration, in some it has reduced them and in some studies had no impact. These discrepancies may be due to differences in air pollutants, in pollution levels, in environmental conditions and/or in study species.

Herbivorous insects accumulate carotenoids from their diet (see Ahmad 1992). The only exception known so far is that aphids can produce carotenoids by themselves (Moran and Javik 2010). Different carotenoid levels at the base part of the food chain, in plants, determine the extent to which carotenoids are available for phytophagous insects, the accumulation of carotenoids in insects may be affected by air pollution, which may lower the carotenoid levels in some insect species (Isaksson and Andersson 2007); in other groups of insects, pollution may have no effect on carotenoid concentrations (Eeva et al. 2010). It is also possible that at least some insect species may actually accumulate more carotenoids in response to air pollution, since carotenoids function as antioxidants against oxidative stress in insects (see Ahmad 1992), and such stress may be caused by pollution (see e.g. Migula et al. 2004).

Insectivorous passerine birds, adults and nestlings, accumulate both pollutants and carotenoids from their diet (Lindqvist 1992, Lindqvist and Block 1997, Partali et al. 1987, Isaksson and Andersson 2007). Female birds further deposit both pollutants and carotenoids in the egg yolk (Maedgen et al. 1982, Tsipoura et al. 2008, Saino et al. 2002a, Isaksson et al. 2008). Carotenoid concentrations and their profile in the yolk may show high variability both among and within species (Hargitai et al. 2006), and the amount of carotenoids allocated to the egg yolk may be reduced, either directly or indirectly, by air polltion (Hõrak et al. 2002), although this is not always the case (see Isaksson et al. 2008). Lutein and zeaxanthin have been found to be the most abundant carotenoids in great tit plasma (Isaksson 2008). Isaksson et al. (2008) found no difference in the carotenoid concentration of plasma in great tit females between an urban (polluted) and a rural (unpolluted) area. Only lutein and zeaxanthin, without structural modifications, are transferred to the plumage of great tit (Partali et al. 1987), and carotenoid-based coloration in birds may be weakened by air pollution, either directly or indirectly, via the availability of carotenoid-rich prey (Eeva et al. 1998, Eeva et al. 2005, Isaksson et al. 2007a, Dauwe and Eens 2008, Geens et al. 2009). Eeva et al. (2010) suggest that the paler carotenoid-based plumage coloration of great tit (*Parus major*) nestlings in a polluted area is due to varying dietary proportions of lutein-rich food items, rather than to pollution-related variation in insect carotenoid levels. Eeva et al. (2010) also suggest that in the unpolluted area great tit nestlings received more carotenoids from their diet than pied flycatcher (*Ficedula hypoleuca*), primarily due to the much higher proportion of carotenoid-rich lepidopteran larvae in the great tit diet.

The relationship between air pollutants and carotenoids has been studied in various plant species (most recent studies: El-Khatib 2003, Miteva et al. 2005, Rai et al. 2005, Rajput and Agraval 2005, Deniz and Duzenli 2007, Polonikova and Voskresenskaya 2008, Rafiq et al. 2008, Ganesh et al. 2008, Sharma and Tripathi 2009). Investigation of the relationship between air pollutants and carotenoids in insects has been virtually lacking to date (but see Isaksson and Andersson 2007, Eeva et al. 2010). In birds the relationship between air pollutants and carotenoids has been studied in many aspects (Bortlotti et al. 2003, Eeva et al. 2005, Bustnes et al. 2007, Isaksson and Andersson 2007, Isaksson et al. 2008, Geens et al. 2009, Isaksson et al. 2009), but, tritrophic- and/or two-trophic approaches have been quite scarce (Eeva et al. 2005, Isaksson and Andersson 2007, Isaksson 2009, Eeva et al. 2010).

Air pollution is a widespread phenomenon including anthropogenic emissions for instance from industry and traffic (Eeva 1996, Kiikkilä 2003, Akoto et al. 2008, Rafiq et al. 2008, Fenger 2009, Ngayila et al. 2009). The main anthropogenic air pollutants affecting the soil, vegetation, insects and birds, and their ecological interactions are ozone, sulphuric oxide, nitrogen oxides, and heavy metals such as As, Cu, Ni, Pb and Zn (Hughes 1988, Koricheva 1994, Eeva 1996, El-Khatib 2003, Kiikkilä 2003, Rajput and Agraval 2005, Akoto et al. 2008, Cape 2008, Rafiq et al. 2008, Fenger 2009, McFrederick et al. 2009). Air pollution gives rise to continuous abiotic environmental stress in plants and animals (Heinrichs 1988, Koricheva 1994). In addition to studying the effect of air pollution on single organisms, it is also important to investigate their impact on processes of involving entire food chains.

Carotenoids are hydrocarbons with a linear hydrocarbon skeleton of conjugated double bonds, which can exist alone or can be cyclised at one or both ends. Carotenoids containing only carbon and hydrogen are referred to as carotenes (e.g. β-carotene). Since carotenes lack functional groups, they are quite nonpolar and lipid soluble. Carotenoids that contain oxygen are termed xantophylls (e.g. zeaxanthin, lutein, astaxanthin); these can be further subdivided into hydroxycarotenoids (e.g. lutein) and ketocarotenoids (e.g. astaxanthin) (McGraw 2006). Carotenoids have many important physiological functions at all trophic levels (Krinsky 1994, Olson and Owens 1998, Fraser and Bramley 2004, Škerget et al. 2005, Carrol and Berenbaum 2006). Plants are able to produce carotenoids in chloroplasts; animals are unable to synthesize carotenoids *de novo*, and therefore have to obtain carotenoids from their diet. Thus carotenoids are thought to be a limited resource for organisms higher up in the food chain (Young and Britton 1993, Ahmad 1992, Olson and Owens 1998, McGraw and Hill 2001, McGraw 2006; but see Moran and Javik 2010).

## 1.2. Air pollutants, carotenoids and plants

Carotenoids protect plants from endogenous and exogenous oxidative stress (Bungard et al. 1999). Carotenoids have light-harvesting properties in plants, and protect chlorophyll from photo-oxidative destruction and cell membranes from reactive oxygen species caused by anthropogenic pollution. Hence their reduction would have serious consequences for chlorophyll pigments (Sieferman-Harms 1987, Ekmecki and Terzioglu 2005). Heavy metal related stress can reduce the amount of carotenoids in plants, due perhaps to interference by heavy metals with pigment metabolism, chromium, for example, caused damage in photosynthetic tissue where the formation of free oxygen radicals was abundant in chlorophyll (Ganesh et al. 2008). The synergetic effects of sulphuric oxide and nitrogen oxides emitted by traffic reduced the amount of carotenoids in *Crocus sativus* compared to a control site (Rafiq et al 2008). In an experimental study of the effects of cadmium on *Phyllanthus amarus,* Rai et al. (2005) reported that increasing cadmium concentrations gradually decreased the amount of carotenoids. Miteva et al. (2005) used two stress factors on tomato plants simultaneously, namely arsenic and the Cucumber mosaic virus (CMV). They found a lowered carotenoid concentration in plants inoculated with CMV combined with two arsenic treatment levels compared to the controls. They suggested that the result was due to chloroplast number reduction and structural injuries, leading to pigment synthesis reduction and their increased destruction. Polonikova and Voskresenskaya (2008), on the other hand, found that with increasing anthropogenic pollution from industry and traffic (e.g. heavy metals, nitrogen oxides), causing oxidative stress, the amount of carotenoids actually increased in two grass species. They suggested that the accumulation of carotenoids was part of natural defence mechanism against anthropogenic pollution, aimed at protecting of intracellular components sensitive to damaging agents. Deniz and Dunzelly (2007) compared the effects on four evergreen plants of industrial pollution (sulphuric oxide, nitrogen oxides and heavy metals) at a polluted site near an oil refinery and a control site. They found that carotenoid concentrations were higher at the polluted than the control site. Carotenoids paralleled elevated sulphur oxide and carbon dioxide levels. They suggested that high concentrations of both carbon dioxide and sulphur dioxide may lead to increases in carotenoid concentrations. El-Khatib (2003) studied the effects of two levels of ozone exposure in four plant species. In two of the species, the higher ozone level caused significant increases in the percentage inhibition of carotenoids. The opposite trend was recorded in the other two species, which appeared to loose a higher amount of pigments at the lower level of ozone. Wen et al. (2004) studied the response to air pollution in three woody species: in one species carotenoid content in leaves was reduced by air pollution, while in the other two it was increased. Sharma and Tripathi (2009) found a reduction of carotenoids in two tree species due to air pollution. Isaksson (2009) found that in both birch (*Betula verrucosa)* and oak (*Quercus robur*) the total carotenoid concentration and lutein concentration were lower in an urban (i.e. polluted) than in a rural (unpolluted) area, but there was no difference in zeaxanthin concentrations. She suggested that the lower carotenoid concentrations of urban trees reflected environmental stress. To sum up, existing studies suggest that in response to air pollution carotenoids can either decline or increase, the latter suggesting a protective role.

## 1.3. Air pollutants and carotenoids and insects

Pollutants may cause the oxidative destruction of carotenoids (Bačkor and Vaczi 2002); they may also increase or decrease the amount of primary (e.g. nutrients) and secondary compounds (e.g. phenolics) of plants, thereby altering nutritive value of plants for herbivorous insects (Heliövaara and Väisänen 1993, Riemer and Whittakker 1989, Loponen et al. 2001). Heavy metals accumulate in herbivorous insects at high doses, because the insects have to process large quantities of plant material to meet their nutritional needs for adequate development and growth (Hodkinson and Hughes 1982, Linqvist and Block, Kozlov et al. 2000). The direct effects on herbivorous insects of air pollutants, especially heavy metals, are usually negative, i.e. toxic, and may result in a decline in population density. Indirect effects are achieved via diet quality, predators and/or parasitoids, and the impact on population density can be either negative or positive (Alstad et al. 1982, Riemer and Whittakker 1989, Ruohomäki et al. 1996, Koricheva et al. 1998). Eeva et al. (2005) studied copper and lead concentrations in invertebrates in three different groups; i.e. spiders, beetles and caterpillars; the concentrations were higher in the polluted than in the unpolluted area. Insects have an important role in the transfer of metals in the food chain, since they are important primary consumers of plants and an important source of food for many organisms at higher trophic levels (Lindqvist 1992, Lindqvist and Block 1997). The carotenoid content of an insect depends on the carotenoids in its diet, but many insect species selectively absorb one carotenoid, lutein, over the others (Ahmad 1992). Carotenoids act in the pigmentation of insects, protecting them for instance from predation and light damage (Rotchild 1978, Carrol and Berenbaum 2006). Carotenoid depletion may further delay the hatching of insect larvae (Sakamoto et al. 2003). In insects, carotenoids function as antioxidants against endogenous and exogenous oxidative stress (Ahmad 1992). Lepidopteran larvae are a rich source of carotenoids for organisms higher up in the food chain (Partali et al. 1987, Eeva et al. 2010). To my knowledge, to date only two studies have addressed air pollution and carotenoids simultaneously in the invertebrate diet of birds. Isaksson and Andersson (2007) studied four Lepidoptera genera (caterpillars) in urban and rural areas. The most common carotenoid in all genera was lutein, followed by zeaxanthin. In urban caterpillars carotenoid concentration was significantly lower than in rural caterpillars. Moreover, all but one of the caterpillar genera had a higher lutein/zeaxanthin ratio in urban areas than in rural ones. Eeva et al. (2010) studied the carotenoid concentrations in six invertebrate groups: adult moths and butterflies (Lepidoptera), lepidopteran and sawfly caterpillars (Symphyta), spiders (Aranea), beetles (Coleopteran), ants (Formicidae) and cockroaches (Ectobiidae). They found that the groups differed significantly in their mean carotenoid concentrations, with lepidopterans showing the highest carotenoid concentrations, but found no differences between the polluted and the unpolluted area.

## 1.4. Air pollutants, carotenoids and birds

Air pollutants are maternally deposited to bird eggs during egg formation (e.g. Maedgen et al. 1982, Swaileh and Sansur 2006, Tsipoura et al. 2008), reflecting the pollution load of females (circulating and tissue levels of pollutants) (e.g. Burger and Gochefeld 1991, Tsipoura et al. 2008). However, different heavy metals may correlate between females and their laid eggs either positively or negatively, depending on the pollutant in question (Burger and Gochefeld 1996). Heavy metals cause oxidative stress to the developing embryo of many organisms, including birds (e.g. Thompson and Bannigan 2008, Rahman et al. 2009). Carotenoids, on the other hand, have an antioxidant capacity in the egg yolk (Surai 2002, Hargitai et al. 2006), and carotenoid limitation may be especially crucial to the avian embryo in need of antioxidant protection (see. Surai et al. 2001), as the embryo's development relies completely on maternally deposited resources (Hõrak et al.

2002). Females may vary the amount of carotenoids deposited in the yolk in relation to environmental and social factors and/or their own condition (Hõrak et al. 2002, Saino et al. 2002a, Saino et al. 2004, Verboven et al. 2005, but see Török et al. 2007).

Pollution can reduce the survival of both adult birds and nestlings. This decrease may also be species and sex-specific, females for example being more vulnerable to pollutants than males. The lower survival rates of birds in polluted areas may also be due to higher emigration from the low- quality and unproductive habitat rather than to the direct mortality of adult birds for instance from heavy metal poisoning (Belskii et al. 1995, Eeva and Lehikoinen 1998, Eeva et al. 2009a, but see Eeva et al. 2008 for dispersal distances). Heavy metal pollution loads in birds may differ between different tissues and organs and in different age stages (juveniles vs. adults) or between females and males (Hutton and Goodman 1980, Janiga and Žemberyová 1998, Swaileh and Sansur 2006). Pollution may cause environmentally mediated stress effects in nestling and adult birds either directly or indirectly, via reduced food availability that affects a birds' condition and phenotype, such as carotenoid based plumage coloration (Eeva et al. 1998, Belskii et al. 2005, Eeva et al. 2005, Berglund et al. 2007, Isaksson et al. 2007a, Dauwe and Eens 2008, Geens et al. 2009). The ability of parents to provide nestlings with carotenoid-rich diet has many benefits for nestlings, such as antioxidant protection, better body condition and immunocompetence, and may thus enhance overall fitness of nestlings (Biard et al. 2005, Møller et al. 2000, Koutsos et al. 2003).

Heavy metals promote the production of free radicals and may cause oxidative stress (see Koivula and Eeva 2010 for review). Cells under oxidative stress display variable dysfunctions due to lesions caused by reactive oxygen species (ROS) in lipids, proteins and DNA. Thus oxidative stress may be partly responsible for the toxic effects of heavy metals to different organisms (Ercal et al. 2001). Carotenoids may have antioxidant functions in vertebrates (Krinsky 2001), but in the case of birds proof of mobilization of carotenoids (i.e. from plasma) or of their antioxidant capacity in recent studies and meta-analysis is weak or absent (Isaksson et al 2007b, Constantini and Møller 2008, Isaksson and Andersson 2008, Cohen and McGraw 2009, Geens et al. 2009). Cohen and McGraw (2009) further suggest that assuming a single antioxidant system is an oversimplification, and that ecological studies attempting to examine antioxidant function should use multiple measures. In addition to the direct effects of heavy metals, their indirect effects affect birds via the reduced availability of carotenoid-rich insects or the lower carotenoid concentrations in insects, in polluted/urban areas compared to unpolluted/rural areas (Eeva et al. 1998, Eeva et al. 2005, Isaksson et al. 2007a; but see Eeva et al. 2010 for carotenoid concentrations).

## 1.5. Aims of the thesis

In my thesis I have studied the relationships between heavy metal pollution and carotenoids in woody plants, insects and an insectivorous passerine bird (*Parus major*). The first aim of the thesis was to analyze tri-trophic interaction (plant – insect – bird) between heavy metal pollution (from a copper smelter) and carotenoids (**I**). In article **I** I demonstrated that the carotenoid concentration of birch leaves was higher in the polluted area and differed in two birch species: the silver birch (*Betula pendula*) and the downy birch

(*Betula pubescens*). The larvae of birch-leafeating insect species differed markedly in their carotenoid concentrations, the autumnal moth accumulating more lutein than β-carotene, while the opposite was true for sawflies (Hymenoptera: Symphyta). In addition, sawflies had higher lutein concentrations close to pollution source than further away, while in autumnal moths there was no corresponding change in lutein concentration between the polluted and the unpolluted area. The lutein concentration in the plasma and plumage of great tit (*Parus major*) nestlings did not differ along the pollution gradient. In article **II** two hypotheses were tested experimentally, to explain earlier findings that breast feathers of birds exposed to heavy metals were less yellow than those in unpolluted environments. The first hypothesis posited a deficiency of carotenoids in the diet; the second involved pollution-related changes in the transfer of carotenoids to feathers. The results support the first hypothesis: that pollution-related variation in the plumage colour of the great tit is due to a carotenoid deficiency in the diet. The relationships among temporal variation in the availability of carotenoid-rich food, tissue carotenoid levels and breeding success are poorly known. Article **III** investigated the effect of temporal changes in diet quality and quantity on the carotenoid profile of great tit nestlings along a pollution gradient. There was a declining seasonal trend in the lutein concentration of caterpillars, probably due to a negative relationship between the dry mass of larvae and their lutein concentration. The declining concentration of lutein in caterpillars may explain the declining trend in nestlings' plasma lutein concentration during breeding season found in article **III**, despite the increase in caterpillar biomass. In articles **IV** and **V,** food manipulation experiments (two food treatments with abundant carotenoids, one food treatment with scanty carotenoids and one an unsupplemented control) were used to study the effects of diet quality and quantity on plumage colour and growth (**IV**) and the effect of diet quality on fluctuating asymmetry (FA) in tail feather length and mass of great tit nestlings along a pollution gradient (**V**). Nestlings in the polluted area grew better with the carotenoid-rich food, while no such effect was found in the unpolluted area. Nestlings also showed higher plasma lutein levels and higher carotenoid chroma values in the unpolluted than the polluted area (**IV**). Carotenoid-rich food increased FA in tail feather length, but not in tail feather mass. The results suggest that a high carotenoid concentration and low calcium availability in the diet may lead to higher FA in tail feather length. FA in tail feather mass, on the other hand, was higher in the polluted than in the unpolluted area in the control group suggesting indirect effect of pollution on this measure of FA, mediated by lower food availability. Finally, in article **VI** I studied female quality (body condition, size, plumage colour, age) and possible maternal effects mediated by female quality in the great tit in relation to the lutein concentration of the egg yolk, egg mass and nestling carotenoid chroma. The carotenoid chroma of females and nestlings was lower in the polluted than in the unpolluted area, although the difference for females was so small that it probably has no biological significance. Variation was found in yolk lutein concentration, mediated by female carotenoid chroma and body condition, which may relate to the condition of females and/or environmental effects experienced by them. The effect on the yolk lutein concentration of combined female body condition and carotenoid chroma may indicate a trade-off between allocation of lutein to the yolk and the female body condition. More chromatic females produced more chromatic nestlings, indicating a possible maternal effect mediated by female quality. Egg mass was not affected by the quality of females.

# 2. MATERIALS AND METHODS

## 2.1. Study area

The study was carried out during the field season in the summers of 2004 and 2005 at a well-known pollution gradient of point source pollution, a copper-smelter, in the town of Harjavalta in SW Finland (61˚20’N,22˚10’E) in twelve nest box areas at different distances (from 0.5 km to 12 km) from the factory (see Eeva 1996). The main pollutants in the area are sulphuric oxide and heavy metals (especially As, Cu, Zn, Ni and Pb), arising from long-lasting industrial emissions (from 1945 onwards) (Eeva 1996, Kiikkilä 2003). Although the emissions have decreased, the soil, vegetation, insects and birds are affected by direct and/or indirect effects of heavy metal contamination in the polluted area due to current and historical depositions (Eeva and Lehikoinen 2000, Kiikkilä 2003, **I**, **II, III, IV, V, VI**). Heavy metal concentrations diminish exponentially from the point source of pollution, approaching background levels approximately five kilometres from the smelter (Eeva 1996, Eeva et al. 1997). Based on this information nest box sites were accordingly divided to the polluted and the unpolluted ones. In articles **V** and **VI,** the division was based on distance less than five from the smelter or further away. In artcles **I** ,**II, III** and **IV** the division was based on a distance less than two kilometres; background areas were located more than five kilometres from the smelter. This difference among the studies does not affect the results, as there were no study sites at distance of 2 – 5 km. Dividing the study sites in these two ways resulted in six sites in the polluted area and six sites in the unpolluted area. To avoid additional variation in the results due to varying habitat quality, the study areas were selected of the same habitat type, i.e. the forest typical of the study area, dominated by relatively barren pine (*Pinus sylvestris*) (Fig. 1). The collection of data took place by permissions of the Animal Care & Use Committee of Turku University and the Regional Environmental Centre.

**Figure 1.** Forest type typical for study sites, dominated by relatively barren bine (*Pinus sylvestris*).

## 2.2. Study organisms

### 2.2.1. Birches

The trees used as study organisms were two closely related deciduous birch species: the silver birch (*Betula pendula*) and the downy birch (*Betula pubescens*) (**I**) Birches are good objects for studying pollution effects, in that they tolerate pollution exposure relatively well and readily colonize polluted areas (Denny and Wilkins 1987, Eltrop et al 1991). In 2004 carotenoid profiles, i.e. lutein, β-carotene and total carotenoids, were measured in both silver birch and downy birch. In the study area these two common birch species form mixed stands with barren pine (*Pinus sylvestris*) and spruce (*Picea abies*). Leaf samples were collected from both birch species in the middle of the nestling period of the great tit (1st – 4th June) in each territory. At the time of leaf sampling autumnal moth were in late developmental stage (from 4th to 5th instar), while sawflies were approximately in the middle of their larval development stage. In each territory we sampled two silver birches (31 territories) and two downy birches (78 territories) of the same height, if present. Leaves were collected evenly from different parts of the canopy. The leaves were cut from the branch without damaging the leaf itself. The leaves were stored at –20˚C and protected from light until the carotenoid analyses.

### 2.2.2. Caterpillars

In 2004 the number of folivorous caterpillars was estimated from birch trees in each great tit territory (290 trees in 58 territories). Birches of 1 – 4 m of height were shaken in a standardized manner and the fallen larvae were collected from a white plastic sheet (2m x 2m) underneath of each birch. The collection of larvae was repeated four times during the great tit nestling period. We divided the larvae into three different groups: autumnal moth larvae (*Epirrita autumnata*), sawfly larvae (Hymenoptera: Symphyta), and other larvae. Sawfly larvae consisted of early season species. The larvae were weighed and stored at –20˚C protected from light until the carotenoid analyses. Lutein and β-carotene were analysed from 67 autumnal moth and 48 sawfly larvae. Larvae, whose dry mass was too small to perform carotenoid analyses, were excluded from the carotenoid analyses (**I, II, III**).

### 2.2.3. Great tit

The great tit is a territorial and resident species that is susceptible to pollution in the study area throughout the year (see Gosler 1993, Eeva 1996). Although the great tit is an omnivorous species, it prefers insects, especially when feeding nestlings. Depending on their availability, phytophagous caterpillars make up 60-95% of the nestling diet (Gosler 1993, Eeva 1996)

In 2004 and 2005 nest boxes were visited daily from the beginning of the breeding season. After the female had completed the clutch, the ninth egg was collected from each nest for later lutein concentration analyses of the yolk. We standardized our sampling by taking the ninth egg in the sequence, or the last-laid egg, if the clutch size was less than nine. If carotenoid availability is restricted, differences between polluted and unpolluted areas as well as among territories should appear more clearly in eggs near the end of the laying sequence (see Hørak et al. 2002). The eggs were weighed and the yolks separated from the albumen and stored protected from the light at –22˚C until the lutein analyses of the yolk (**II, VI**). To determine the precise hatching date of the remaining eggs, the nests were checked daily, starting two days before the estimated hatching date (the approximate incubation time of females in our study area is 13-14 days).

#### *2.2.3.1. Within-brood carotenoid manipulation*

At an age of three days the nestlings were weighed and divided within each brood into two comparable groups according to their body mass. These groups were randomly assigned to the treatment (water-dispersed carotenoid supplementation or control group). The nestlings were marked with black, non-toxic ink on the right or left tarsus according to treatment. At six days of age each nestling was ringed with an individual aluminium ring (**I, II, III**).

Carotenoid/water supplementation started at day 3 from hatching for a total of 71 broods (polluted area 33, unpolluted area 38). We avoided disturbing the birds just after hatching, but an early start was important; carotenoid-based plumage coloration in the great tit is probably primarily determined during the first six days after hatching (Fitze et al. 2003). Carotenoid beadlets (Lutein 5% CWS, Roche, Base, Switzerland), containing 5% lutein and 0.25% zeaxanthin, were dispersed in distilled water to achieve a lutein concentration of 5mg/ml. The supplement was given to the nestlings in an oral dose of 0.1 ml of the supplement per nestling daily from day 3 to day 8. The supplemented dose ( =0.5 mg lutein/day/nestling) was approximately three to eight times higher than the estimated natural daily lutein intake (Fitze et al. 2003). Despite the relatively high dosage, however, circulating lutein levels were not exceptionally high in the experiment; only two of the supplemented nestlings showed plasma lutein concentrations (139 and 149 μg/ml) above the maximum found among the unsupplemented nestlings (121 μg/ml). This may mean that if dose is high substantial part of ingested carotenoid is not absorbed (McGraw et al. 2001). From day 8 onwards, 0.2 ml of supplement was given every other day until day 14. During the time that carotenoid supplementation was given to the treatment group, the control nestlings received the same amount of distilled water (**II**).

The nestlings were weighed at days 8 and 16. Nestling body mass and subsequent survival, as measured by recruitment to breeding populations, are correlated in many bird species, including the great tit (Perrins 1965). Body mass and fledging probability (the probability of that hatchling will fledge) was used as an overall response to pollution stress (**I, II**) and carotenoid treatment (**II**). Two nestlings per brood (1 treatment + 1control) were selected for blood sampling at day 9. We avoided sampling exceptionally small nestlings, but otherwise the selection was random. Blood samples were collected using sterile needles and heparinised micro-capillaries from the brachial vein (Fig. 2) and centrifuged immediately for 5 minutes at 4000r/min (Fig. 3). The hematocrit level (the proportion of packed red blood cell volume) was measured with a ruler; the plasma was separated, preserved in ice during transport, and kept protected from light at –20˚C until the carotenoid analyses (**I, II, III**). The hematocrit level is commonly used as a general measure of condition in wild birds, although it may be affected by many other factors in addition to nutritional condition (Fair et al. 2007). In the study area of this thesis, one such factor might be heavy metal pollution, which could cause anaemia in birds (Fair et al. 2007). In nestlings, hematocrit also increases with age due to increased erythropoiesis (Fair et al. 2007). The age of sampled nestlings, however, was standardized.

**Figure 2.** Blood sampling of great tit (*Parus major*) nestlings at day 9 in the breeding season of 2004.

**Figure 3.** Centrifuge with collected blood samples.

At day 16, two nestlings (1 treatment + 1 control per brood) were randomly selected (Fig. 4) and photographed side by side on uniform gray cardboard with a digital camera. Direct sunlight was avoided during photographing and the reference card was preserved protected from the light. Two images were taken of each pair of nestlings to assess the repeatability of the measurements (**II**). Digital imaging has been shown to be a sensitive and repeatable way of measuring colour variation, as long as inter-photograph variation in ambient lighting can be controlled (Villafuerte and Negro 1998, Montgomerie 2006). Digital image analyses are based on colour variation in the range of human colour vision and cannot measure variation in the UV-range, which birds can see (Cutchill 2006). The main interest, however, was in the intensity of one carotenoid pigment (lutein) within the human range of vision. After photographing, samples of the yellow breast feathers were taken from the same two nestlings for lutein determination. The feathers were kept protected from light at –22˚C until the analyses (**I, II**).

**Figure 4.** 16-day old great tit nestlings (control nestling on the left side, lutein-supplemented nestling on the right side).

### *2.2.3.2. Between-brood manipulation*

In 2005 the last-laid egg in a clutch was collected with the same protocol as in 2004; egg mass was determined and the eggs stored in –22˚C for yolk lutein concentration analyses. A feeding experiment was conducted with great tit nestlings along a pollution gradient. Experimental nests were evenly distributed among different feeding manipulation and control groups according to hatching dates, to avoid the confounding effects of different hatching dates on the results. Otherwise the nests were divided randomly by turn among different feeding manipulation and control groups at different sites in polluted and unpolluted areas (**IV, V, VI**). Extra food was offered to broods in plastic cups inside the nest box (Fig. 5.). Parents readily fed their nestlings with the food offered; this was tested in advance by videotaping nests with supplemented food in plastic cups inside the nest box. Placing the extra food inside the nest box prevents other birds from utilising the food source. The feeding experiment started when the nestlings were three days old and was continued until they were 13 days old. The nestlings were offered extra food every other day as follows; group 1: mixture of lutein-rich autumnal moth larvae and mealworm larvae (*Tenebrio molitor* L.) 50% of each and distilled water; group 2: mealworms and distilled water; group 3: mealworms and lutein dispersed in distilled water; and group 4: distilled water (control group with an empty food cup).

**Figure 5.** Extra food was offered to great tit nestlings from the third to the thirteenth day of age.

During each feeding, the broods were given 1 g of larvae/nestling and 0.2 ml of water-dispersed lutein or distilled water/nestling. The lutein and water were administred orally. The lutein originated from carotenoid beadlets (Lutein 5% CWS, Roche, Basel, Switzerland) containing 5% lutein and 0.25% zeaxanthin. The supplemented dose of lutein was 0.5 mg lutein/d/nestling.The details of dosage were the same as in 2004 (see above). Larvae of the autumnal moth contain approximately 98 µg/g, d.w. lutein (mean) while mealworms are almost lutein-free (0.06 µg/g, mean). Blood samples were taken from two randomly picked nestlings from each brood (exceptionally small nestlings were not sampled), with the same protocol as described above.

In 2005 when nestlings were 15 days old, yellow breast feathers were collected from each nestling for colour determination. Breast feathers were also collected when the nestlings were 13 days old for colour determination from females (yearlings and adult) (**IV, V, VI**). Female wing length (mm) was measured with a ruler. To measure fluctuating asymmetry (FA), both outermost tail feathers were plucked from all nestlings in a nest at an age of 15 days. The feathers were placed in paper bags and stored in a dry place at room temperature. FA was measured by mounting and straightening each feather with insect needles before measurement with a digital calliper (±0.01 mm). Feathers were measured twice in random order to assess measurement error in relation to FA (Merilä and Björklund 1995). After the first measurement the feathers were removed from the paper bed and measured a second time, blindly and in random order. The measurements were made by one person and the repeatability of measures was calculated afterwards following Lessels and Boag (1987). The repeatability of feather length measures was high, 99.95%. Tail feathers were weighed once in random order with an electronic balance with accuracy of ±0.01 mg. FA in tail feather length and mass was calculated for each individual as the absolute difference between the numerical values of each side's measurements (e.g. Palmer and Strobeck 1986). We assumed the absence of directional asymmetry in tail feathers based on the study by Eeva et al. (2000), (see also Cuervo and Møller 1999). Both the FA in tail feather mass and length were size-corrected before the analyses (by dividing the FA by the mean mass or length of tail feathers), since although nestlings were of same age they differed in developmental stage. FA values in tail feather length and mass were transformed to percent values (**V**).

## 2.3. Carotenoid, heavy metal, and calcium analyses of different samples

In 2004, the extraction and analyses of carotenoids in birch leaves, insect larvae, egg yolks, plasma and yellow breast feathers were performed with high-performance liquid chromatography (HPLC). The heavy metal concentrations (As, Cd, Cu, Ni, Pb, Zn) of nestling faeces were analysed with ICP-Mass Spectrometry (Elan 6100 DRC, PerkinElmer-Sciex) (**I, II, III**).

The same methods were used in 2005 for the extraction and analyses of lutein in egg yolks and plasma and the analysis of calcium and heavy metal concentrations (As, Cd, Cu, Ni, Pb, Zn) of nestling faeces (**IV, V, VI**).

In the analyses of carotenoid concentrations, all extraction procedures were separately optimized for feathers, plasma etc to ensure that the extractions were quantitative, i.e. that no carotenoids were left unextracted in the extraction residue. It can be estimated that the extraction plus subsequent HPLC analysis resulted in a maximum of 5% deviation in the quantitative results, the typical deviation being closer to 2-3% (Salminen, unpublished data).

## 2.4. Plumage colour analyses

In 2004, digital photographs were taken of nestlings in a plastic holder to keep them in the same position. The pictures were taken from the ventral side, and the same yellow reference card (C2, M17, Y86, K10) was induced in each picture (**II**). CMYK coloration refers to the tristimulus colour space, which has been developed to describe the colours visible to humans (Montgomerie 2006). Plumage yellowness was analysed from digital images with Corel Photo-Paint 12 Software. A positive correlation between plumage yellowness and feather lutein concentrations showed that the relevant colour parameter was measured (**II**).

In 2005, the carotenoid chroma of nestlings, yearlings and adult females breast feathers was analysed with an AvaSPec-2048 spectrometer, AvaLight-DHS halogen light source and FCR-7UV400-2ME reflection probe. As a measure of yellowness, i.e. carotenoid concentration of breast feathers, the value of carotenoid chroma (R700-R450)/R700 was used, where R is percentual reflectance (Andersson and Prager 2006) (**IV, V VI**).

In 2004 spectrometer was not yet available for colour determination; therefore digitized images were used for the colour determination of nestling breast feathers. In addition, the breast feathers were also analysed by the HPLC technique for their lutein concentration. Many aspects of human colour technology and computational colour vision can be quite useful if other techniques are not available (Andersson and Prager 2006), although spectrometric quantification of reflectance, and in this case of carotenoid chroma (spectral purity/saturation) of feathers, is a much more precise technique. The most common method of measuring bird colouration in use today is to determine a complete reflectance spectrum in the bird-visible range, using a spectrometer and then to calculate certain indices of hue, saturation (chroma), and brightness from these spectral data (Montgomerie 2006). However, human colour discrimination may be a quite reasonable indication of signal variation perceived by birds, even though it lacks the ultraviolet (UV) portion on the spectra, which is visible to birds (Andersson and Prager 2006). Digitized photographs can be analysed using graphic software applications to give mean values of hue, saturation and brightness for the colour patch (Montgomerie 2006). The limitations of using photography are that it cannot quantify reflectance below 400 nm and may lack both precision and accuracy. While for several reasons reflectance spectrometry is a better method for the measurement of bird colouration than digital photography, there are some situations in which photography can still be more practical (Montgomerie 2006). Digital photography provides a means of quantifying colours in very small or very large patches, compared to spectrometer's measure (approximately 3 $mm^2$) (Montgomerie 2006).

# 3. MAIN RESULTS AND DISCUSSION

## 3.1. Carotenoid profiles of birches and caterpillars along a pollution gradient

Total carotenoid concentrations were higher in the polluted than the unpolluted area in silver birch (*Betula pendula*) but not in downy birch (*Betula pubescens*); and β-carotene concentrations were higher in both birches studied in the polluted area than in the unpolluted area. With respect to lutein concentration there was no difference between the two areas in either of the birch species studied (**I**). Contrary to study **I**, Isaksson (2009) found lower total carotenoid and lutein concentrations in birch (*Betula verrucosa*) and oak (*Quercus robur*) in an urban (polluted) than in a rural (unpolluted) area but no difference in zeaxanthin concentrations. The contradiction between the results of Isaksson (2009) and study **I** may be due the different tree species studied and to the differences in pollutants and pollution levels. Isaksson (2009) suggested that the lower carotenoid concentrations of urban trees reflect environmental stress. I suggest that the higher concentrations of total carotenoids and β-carotene in birch leaves in the polluted area found in study **I** may be an adaptive response of birches near the pollution source. Increased antioxidant levels may protect birches under potentially stressful conditions. For example, the increased β-carotene concentration can probably be explained by the importance of β-carotene as an antioxidant; β-carotene is known to play a protective role in cells against oxidative stress and it is relatively resistant to degradation (see e.g. Young and Britton 1993, Chouldhury and Behera 2001, Winiewski and Dickinson 2003). Carotenoids also function in photoprotection (Demming-Adams et al. 1996). As the forest canopy is more open in the polluted than in the unpolluted area, it can thus be expected that birches in the polluted area will also need more photoprotection (**I**). By producing zeaxanthin, for instance by metabolizing β-carotene, higher plants may respond to light-mediated environmental stress (Young 1991, Davison et al. 2002). Both birch species studied are also shade-intolerant (Porsmuth and Niimets 2006), which may partly account for the results.

The higher β-carotene concentrations in silver and downy birch and the higher total carotenoid concentration silver birch in the polluted area suggest that more of these carotenoids is available to the next trophic level, i.e. to the herbivorous larvae of autumnal moths and sawflies. Sawfly larvae indeed accumulated more β-carotene in the polluted than in the unpolluted area. In autumnal moths the higher availability of β-carotene in the polluted area was not reflected in a higher β-carotene concentration, suggesting selective absorption. With respect to the lutein concentration of birches, the same amount of lutein was available to leaf-chewing caterpillars in the polluted and the unpolluted area. Though the same amount of lutein was available for next trophic level in both study areas, sawflies had higher concentrations of lutein in the polluted than the unpolluted area, while the lutein concentrations of autumnal moths did not differ between the areas. Smaller larvae generally showed higher lutein and β-carotene concentrations, although sawflies in the polluted area showed an opposite trend (**I**). Isaksson and Andersson (2007) found lower concentrations of total carotenoids and relatively more zeaxanthin in various lepidopteran caterpillars in an urban than in a rural environment. In study **I** carotenoid concentrations either showed no association with pollution or were higher in the polluted area, depending on the insect species in question. With respect to the lutein and β-carotene concentrations there seems to be a difference between autumnal moth and sawfly larvae in the efficiency and/or need to accumulate these carotenoids in the polluted area. Various secondary metabolites may increase, decrease and/or remain constant in birches due to pollution (Loponen et al. 2001); and leaf-chewing insects have to cope with plant-derived allelochemicals, which may also function as pro-oxidants (Ahmad 1992), and the sensitivity of autumnal moths and sawflies to these pro-oxidants may differ. Secondary metabolites, such as phenolics, also vary during the growing season (Haukioja et al. 2002). This temporal variation may affect the carotenoid profiles of leaf-chewing insects, since the later-peaking sawflies may be exposed to larger amounts of secondary chemicals than the earlier-peaking autumnal moths (**I, III**).

The comparison of autumnal moths and sawflies showed that sawflies accumulated β-carotene over lutein while autumnal moths accumulated lutein over β-carotene.While insects have often been found to selectively accumulate lutein over other carotenoids (Ahmad 1992, Isaksson and Andersson 2007), this preference is not universal, since sawflies accumulated β-carotene over lutein (**I**). In conclusion, the different carotenoid profiles of autumnal moths and sawflies suggest a preference for different carotenoids, and may also indicate a different sensitivity to oxidative stress, whether caused by pollution, ambient light conditions or various allelochemicals.

In addition to the different carotenoid profiles of autumnal moths and sawfly larvae, their biomass during the breeding season of the great tit also differed. The biomass of autumnal moths peaked later in the polluted area than the unpolluted one. In the polluted area there were fewer autumnal moth larvae during the first sampling period than during the second or third sampling period. During the fourth sampling period there was no autumnal moth larvae present in the sampled trees. In the unpolluted area the autumnal moth biomass was highest during the first sampling period (17 - 21 May) and the second sampling period (24 - 27 May) and it then decreased towards the third sampling period (31 May - 4 June) and was zero during the fourth sampling period (14 -17 June). The mean biomass of sawfly larvae did not differ between the polluted and the unpolluted area over the four sampling periods. The biomass of sawflies increased in both study areas during the sampling periods; it was lowest in the first sampling period and at highest during the fourth sampling period (**III**). In both sawflies and autumnal moths the lutein concentration of larvae decreased during the breeding season The negative association found between caterpillar dry mass and lutein concentration may indicate carotenoid depletion in the cource of larval development (**III**). Since the sampling of birch leaves did not cover the full larval periods of autumnal moths and sawflies ( **I**; see also Isaksson 2009), it is difficult to draw any general conclusions as to temporal changes in the carotenoid concentration of birches or their effects on the carotenoid concentrations of phytophagous caterpillars (**III**).

## 3.2. Carotenoid availability and plasma and feather lutein concentration and plumage colouration

### 3.2.1. Natural availability of caterpillars and plasma and feather lutein concentration and plumage colouration of great tit nestlings

The temporally decreasing lutein concentration of sawflies and lutein-rich autumnal moths was probably also reflected as the decreasing plasma lutein concentration of great tit nestlings between early and late broods during the breeding season of the great tit. The biomass of lutein-rich autumnal moth larvae also decreased during the breeding season of great tit, while the biomass of sawflies increased. In terms of total caterpillar biomass, food availability was higher for late than for early great tit nestlings. On the other hand, the food composition, in terms of lutein availability, was better for early than for late nestlings in both the polluted and the unpolluted area (**III**). The decreasing temporal trend in the plasma lutein concentration of nestlings found in study **III** contradicts study **IV**, where the temporal trend in lutein concentration of plasma was positive. Mols et al. (2005) found that the proportion of caterpillars in great tit nestling diet decreased with advancing date. The different results in the same study area but in different years (**III, IV**) suggest that the probable explanation for the opposite trends in plasma lutein concentration was the difference in the total amount of lutein-rich caterpillars available and their peaking time during the whole two breeding seasons of the great tit. Caterpillar availability for the great tit in my study area seems to vary considerably among years (Eeva et al. 2005).

In the summer 2004 neither the plasma nor the feather lutein concentration were lower in the polluted than the unpolluted area (**I, II, III**), unlike the following year, when plasma lutein concentration was lower in the polluted than in the unpolluted area (**IV**). The caterpillar biomass showed a positive association with feather lutein concentration, suggesting that caterpillar abundance is an important determinant of tissue carotenoid concentration in great tit nestlings (**I**). There was also a positive correlation between the plasma and the feather lutein concentrations, and plasma carotenoid concentration has been shown to predict the plumage concentration of carotenoids (Møller et al. 2000). Lutein is the main pigment determining the yellow coloration in great tit breast feathers (Partali et al. 1987). The intensity of yellow colour in nestling plumage was higher in the unpolluted area than the polluted area, and plumage yellowness correlated positively with the feather lutein concentration. The measurement of yolk lutein concentration confirmed that pollutant levels were not related to the amount of lutein available via the egg yolk for developing nestlings. In addition, the higher the caterpillar biomass the more yellow the nestlings. Although plumage yellowness was lower in the polluted area, faecal heavy metals were not directly related to the yellowness of breast feathers. This result suggests that the primary reason for the pale plumage coloration of nestlings in the polluted sites of the study area was lutein deficiency in the diet and not changes in carotenoid deposition caused by environmental pollutants (**II**). Likewise, plasma lutein concentrations were not directly related to dietary heavy metal exposure, suggesting that the difference between the areas arose primarily via the indirect effects of pollution (**IV**), with changes in invertebrate food composition as a probable explanation (Eeva et al. 2005).

### 3.2.2. Carotenoid and food supplementations and their effects on lutein concentration of plasma and feathers and plumage colouration.

In 2004, lutein supplementation led to higher plasma and feather lutein concentrations than in the control nestlings; plasma lutein concentrations were also higher in the polluted than in the unpolluted area. Likewise, plumage yellowness was 13 % higher in the lutein-supplemented group than in control group. Feather lutein concentrations were higher in the lutein-supplemented group than in the control group but there was no difference between the polluted and the unpolluted area. Plumage yellowness was 13% higher in the lutein-supplemented group than in the control group as well as 4.8% higher in the unpolluted area than the polluted area. All these results suggest that tissue levels of lutein are constrained by the natural availability of carotenoid-rich prey. The finding that even lutein-supplemented nestlings were paler in polluted than the unpolluted area may indicate that the difference between the study areas in feather lutein concentrations and plumage yellowness is due to pollution-related changes in feather microstructure and/or differences in the naturally available diet. Nestlings in a polluted area may produce feathers that are lower in quality, and thus less yellow, than in an unpolluted area (**II**). Along with the carotenoid concentration of feathers, their structural components may also contribute to the intensity of yellow coloration (Shawkey and Hill 2005, Shawkey et al. 2006). Another possible reason for the paler plumage coloration in the polluted area compared to the unpolluted area may be that, independent of the carotenoid concentration, the expression of carotenoid coloration can be affected by nutritional condition and nutritional stress (**II**, Hill 2006). In addition to lutein supplementation, the breast feathers in the lutein-supplemented group were the more yellow the higher the natural biomass of caterpillars in the environment (**II**). The yellowness of nestling breast feathers was enhanced by the lutein supplementation combined with a caterpillar-rich diet.

In 2005, a four-level food manipulation experiment was run. Food manipulation groups in both the polluted and in the unpolluted area: autumnal moth+mealworm, mealworm, mealworm+lutein, and control group. Contrary the previous year, plasma lutein concentrations in the control group, which relied on natural food resources alone, were higher in the unpolluted than the polluted area. This inter-year variation, found in studies carried out in the same study area and at the same study sites, is probably due to to yearly variation in the timing and abundance of caterpillars (**I**, **II**, **IV**). Plasma lutein levels were higher in the mealworm+lutein supplemented group than in the other groups (**IV**).

In 2005, the wing length and body mass of nestlings in the polluted area increased in the autumnal moth+mealworm and in lutein+mealworm group compared to other groups. No corresponding change in wing length was found in the unpolluted area and the control group had a higher body mass than the lutein+mealworm group. In the polluted area, additional lutein may stimulate nestling growth, when natural food resources are lutein-deficient. In green finches (*Carduelis chloris*), Hõrak et al. (2006) found that lutein supplementation increased the birds' fat reserves, possibly indicating that carotenoids interfere with the lipid metabolism (**IV**). The plasma lutein concentration was also higher in the unpolluted than in the polluted area in lutein+mealworm group.

In the control group carotenoid chroma of nestling breast feathers was higher in 2005 in the unpolluted than polluted area, paralleling the yellowness of breast feathers in 2004 (**II, IV**). Carotenoid chroma was also higher in the lutein+mealworm supplement group compared to the other groups, and higher for late broods than early ones (**IV**). The nestling growth rate**,** plasma lutein concentration and carotenoid chroma were not directly related to faecal heavy metal levels, measured as a proxy for dietary heavy metal exposure. Rather, the colour intensity of great tit nestlings has been found to correlate positively with the availability of herbivorous larvae (Eeva et al. 1998, **II**). The differences observed in growth rate, plasma lutein concentration and carotenoid chroma between the polluted and the unpolluted area could be due primarily to the indirect effects of pollution, i.e.

prey composition and availability during the breeding season (Eeva et al. 2005, **III, IV**). Carotenoid availability may enhance growth, and the poorer availability of carotenoid-rich food may limit nestling growth in the polluted area (**IV**). The response of plumage colour to lutein supplementation may depend on natural carotenoid availability (**IV**) as was the case in study **II.** The natural diet still seems to have important effect over additional supplementation of carotenoids in plumage colour of nestlings (**II, IV**). This may be due to the fact that a substantial part of the ingested carotenoid may not be absorbed if the dosage is high (McGraw et al. 2001). In nestlings, the regular carotenoid dose they receive from parental provisioning with caterpillars, could explain why natural variation in carotenoid availability is still important, even when nestlings are provided with additional carotenoids during the nestling phase. The carotenoid content of the food provided by the parents affects the plasma levels of lutein and this thereby via the circulation of lutein to growing feathers contributes to the plumage coloration of nestlings (**IV**).

## 3.3. Effect of carotenoids and calcium on developmental stability of nestling tail feathers

Developmental stability is influenced by both genotype and environment, and may be used as an indirect measure of environmental stressors, such as food deficiency or pollutants (Palmer and Strobeck 1986, Møller 1990, Watson and Thornhill, 1994, Clarke 1995). Fluctuating asymmetry (FA), i.e. small non-directional and random deviations from perfect symmetry in paired bilateral characters, is a measure of developmental instability (Palmer and Strobeck 1986, Watson and Thornhill 1994, Clarke 1995). The effects of food quality and quantity on developmental stability in outermost tail feather length and mass were studied in a food manipulation experiment along a pollution gradient. The experiment consisted of food manipulation groups: autumnal moth+mealworm, mealworm, mealworm+lutein and a control group. The effects of faecal calcium and heavy metal levels (as a proxy of dietary levels) on developmental stability of tail feathers were also studied (V).

Since the immuno-challenge of birds may result in higher morphological asymmetry (Amat et al. 2007) and, since carotenoid depletion may also affect the individual's ability to mount an immune response (Møller et al. 2000), we first hypothesized that carotenoid depletion might further enhance the FA of tail feathers (**V**). Contrary to this hypothesis, FA in tail feather length was lower in birds provided with low-carotenoid (mealworm) diet, indicating that a carotenoid-rich diet and higher plasma lutein concentration may actually enhance the FA in this character either directly or indirectly via the immune response (**V**). The increase in FA in tail feather length in nestlings receiving a carotenoid-rich diet is not a consequence of enhanced growth due to increased lutein availability.

Calcium is an essential element for birds and an important component of feathers (Scheuhammer 1991, Niecke et al. 2003), and calcium may strengthen feathers structurally (Niecke et al. 1999). A high faecal calcium concentration, a proxy for dietary calcium levels, decreased FA in tail feather length suggesting that calcium availability may be an important determinant of developmental stability of tail feather length. Although heavy metal levels were higher in the polluted than the unpolluted area, dietary heavy metal levels did not result in higher FA in tail feather length. This may indicate that nestlings are able to cope with dietary heavy metals (see also Thomas 1993) in this respect, or that heavy metal exposure was not strong enough to increase FA in tail feather length. On the other hand, control nestlings showed higher FA in tail feather mass and lower feather mass in the polluted than the unpolluted area; this suggest that nestlings in the polluted area were unable to grow symmetrical tail feathers relative to their mass, probably because of a deficiency of naturally occurring nutritionally high-quality food items. The effects of feeding manipulation on the developmental stability of tail feather mass were less straightforward to interpret. In the unpolluted area FA in tail feather mass was apparently higher in the mealworm and mealworm+lutein manipulated groups, but a corresponding effect was not observed in the polluted area. This suggests that in unpolluted area providing mealworms as extra food for nestlings may have partially replaced the naturally occurring high-quality diet, as also suggested in study **IV**, while in the polluted area providing nestlings with mealworms parallelled the overall low-quality diet available there. The result also suggests that in the polluted area the quantity or quality of extra food offered to nestlings may have been insufficient to override the effect of the general lack of food on the developmental stability in tail feather mass. In the unpolluted area, the higher FA in tail feather mass in the mealworm and mealworm+lutein groups suggests that while mealworms are rich in protein they may lack other nutrients essential for symmetrical feather growth. While FA in tail feather length seems to be enhanced by a carotenoid-rich diet, FA in tail feather mass seems to be affected by the indirect effects of pollution probably via the structural composition of tail feathers. These results suggest that FA in tail feather mass and length is affected by different factors and thus reflects two different aspects of the developmental stability of growing tail feathers. The developmental stability of tail feather length and mass are apparently two distinct features (**V**).

## 3.4. Female quality and maternal effects in relation to heavy metal pollution exposure

The relationship between female quality (age, size, body condition index, plumage colour) and maternal effects (yolk lutein concentration, egg mass and nestling plumage colour) was studied in relation to heavy metal pollution exposure. Female body size or condition index did not differ between the polluted and the unpolluted area. In the polluted area, however, larger females, measured in terms of wing length, were less yellow than smaller ones, while in the unpolluted area there was no relationship between size and yellowness. This could suggest a trade-off between the self-maintenance of larger females and the lower availability of carotenoid-rich food items in the polluted area during the autumn moult. That larger females had lower carotenoid chroma in the polluted area could be an indirect effect of pollution, mediated by food quality (**VI**, Eeva et al. 1998, Eeva et al. 2005). The lack of relationship between body size and carotenoid chroma in the unpolluted area could be due to the higher amount of carotenoid-rich food available there (Eeva et al. 2005, **III, VI**). There was no difference between the carotenoid chroma of yearlings and adult females (**VI**); this is contrary the result of Hõrak et al. (2001), who found that breast feathers in the great tit were less yellow in adult females than in yearlings. This difference between the results of study **VI** and Hõrak et al. (2001) may be partly due to the different methods used to calculate the yellowness of feathers (hue vs. carotenoid chroma). While plumage coloration is often a reliable signal of age in male birds, there have been only few studies on the relationship between female coloration and age, and the results have been mixed (see Amundsen and Pärn 2006), thus it is difficult to draw any conclusions (**VI**).

In study **VI,** yolk lutein allocation was dependent on the interaction between female carotenoid chroma and body condition index. More chromatic females with a low body condition index deposited more lutein in the egg yolks, while those with high body condition index deposited less. With respect to body condition, this result partly contradicts the studies of Hargitai et al. (2009) and Navara et al. (2006), who found a positive association between female condition and carotenoid allocation to yolks. Our result may indicate a trade-off between body condition and the allocation of lutein to the egg yolk. Different temporal and spatial environmental conditions, such as ambient temperature and/or breeding habitat, may affect both the concentration and the profile of yolk carotenoids (see Hargitai et al. 2006). The amount of carotenoids available to females increases the carotenoid concentration of yolks (Blount et al. 2002, Biard et al. 2005). It may also be that females with high plumage carotenoid chroma but with low body condition index have had higher levels of circulating carotenoids. Isaksson et al. (2008) found a strong positive correlation between total yolk carotenoid investment and the female plasma carotenoid concentration; this was suggested to be due to individual variation in female nutrition. In addition, lutein allocation to eggs may be affected by other physiological factors, such as immunostatus (see Saino et al. 2002a). Isaksson et al. (2008) found no difference in the lutein concentrations of the egg yolks between an urban (polluted area) and a rural (unpolluted area) great tits, while in study **VI** the greater amount of heavy metals in the environment the less lutein females deposited in the yolk. On the other hand, females in the polluted area deposited on average more lutein in the yolk than in the unpolluted area. This discrepancy may be due to variation in territory quality within and between areas (**VI**).

There was no relationship between female carotenoid chroma or body size and egg mass. Females with a higher body condition index laid heavier eggs than those with a lower body condition index (**VI**). Egg production is costly and incurs costs for female fitness (survival); these costs may be dependent on environmental conditions at the time of laying (Visser and Lessels 2001). In passerine birds the average egg volume produced in lifetime is also generally positively related to body condition of the female (e.g. Potti 1999). In addition to environmental effects, egg volume is apparently also heritable (Potti 1999). Isaksson et al. (2008) found no differences in egg mass between urban (polluted) and rural (unpolluted) areas. Similarly, we found no difference in egg mass between the polluted and the unpolluted area (**VI**).

The carotenoid chroma of great tit nestlings was lower in the polluted than the unpolluted area, but heavy metal levels (PC1) were not directly related to the plumage colour. The lower carotenoid chroma of nestling great tits in the polluted area suggests that the ability of mothers/parents to provide carotenoid-rich food items for their nestlings may be lower due to lower caterpillar abundance in the polluted area; this in turn may affect the carotenoid chroma and thus the plumage coloration of nestlings (see Eeva et al. 1998, Eeva et al. 2005, **I, III, VI**). The yolk lutein concentration was not related to the carotenoid chroma of nestlings. There was, however, a positive relationship between the plumage carotenoid chroma of females and the plumage carotenoid chroma of nestlings, which may suggest a possible maternal effect on nestling phenotype. Contrary to study **VI**, Isaksson et al. (2006), in a cross-fostering study, found no relationship between the chroma of great tit nestlings and their rearing or genetical female parent. Study **VI** is correlational; thus maternal and environmental effects on the carotenoid chroma of nestlings cannot be disentangled. The results, however, emphasize the role of post-hatching maternal/parental care and/or the effect of territory quality on nestling plumage coloration (**VI**).

Yearlings with a high body condition index laid larger clutches than those with a low body condition index, while the opposite was true for adult females. The finding that yearlings in good condition laid larger clutches than those in poor condition low may indicate that they have better access to resources, such as better territory quality and foraging areas, and are therefore in better physiological condition, which may in turn lead to higher reproduction effort measured as clutch size. The finding that adult females with a higher body condition index laid smaller clutches than those in poorer body condition may be due to a relationship between reproductive effort, age, and future reproduction and survival prospects (see Visser and Lessels 2001 for survival) (**VI**). In addition, clutch size may partly reflect conditions at the time of laying (Visser and Lessels 2001).

The higher the heavy metal levels (PC1) in the environment, the smaller the clutches females laid. This is likely to be an indirect (e.g. nutritional condition of females during laying period) effect of heavy metal pollution on females and further on clutch size (**VI**). Eeva et al. (2009b) found that the clutch size of the great tit decreased in the most polluted area. In addition, the fittest individuals will most likely occupy the best territories and lay the largest clutches (Ahola et al. 2009). Contrary to the results of study **VI,** Janssens et al. (2003) found no difference in the clutch size of the great tit along a heavy metal pollution gradient. Study **VI** also found that long-winged and intensively coloured females laid, independently, larger clutches than females with lower carotenoid chroma. This suggests that female size and carotenoid chroma are related to clutch size through different pathways (**VI**). Yellower females may be healthier or have a higher immune capacity through the higher amount of carotenoid available (McGraw 2006), and may thus be in better overall condition to lay larger clutches. In addition, larger females may be able to lay larger clutches due to physiological, social behaviour (e.g. better competitors over food than smaller ones), and/or environmental reasons (e.g. better territory quality) (**VI**).

Female age may affect the reproduction outcome of birds; the experience and effort hypotheses (Curio 1983) predict that adult females are in better position with respect to reproduction than yearling birds. Study **VI** yielded no support for the experience and/or effort hypotheses in terms of relationship between female age (yearlings vs. adult females) and the carotenoid chroma of the nestlings, yolk lutein concentrations, egg mass or clutch size. That nestlings of different-aged females did not differ in their plumage carotenoid chroma suggests that the experience of parenting which is achieved with older age (Newton 1989) did not affect post-hatching maternal care over the ambient variation in the availability of carotenoid-rich food (**VI**). Saino et al. (2002b) found in their study with barn swallows that nestling phenotype actually deteriorated with parental age (yearlings, and two years old vs. three years old). Body size and feather development in nestlings declined with both paternal and maternal age; T-cell mediated immunity also declined with maternal age (Saino et al. 2002b).

# 4. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

*Birches*

The elevated β-carotene concentrations in the polluted area compared to the unpolluted one found in study **I** may suggest an adaptive response to pollution stress in silver and downy birch. Alternatively the results might be explained by a need for photoprotection in the polluted area, where the canopy is more open than in the unpolluted area, or as a response to shading. There have been only few studies of carotenoid profiles and concentrations during the growing season in the leaves of different birch species (**I**, Marakaev et al. 2006, Isaksson 2009). These three studies have produced partly mixed results; it is therefore difficult to draw any general conclusions about the relative importance and possible interactions of different environmental factors affecting carotenoid concentrations in birch species studied so far, although studies **I** and Marakaev et al. (2006) suggest that the elevation of carotenoid concentrations is due to heavy metal pollution. Long-term data on different birch species are needed to assess the effects of different environmental factors and/or their interactions on carotenoid profiles and concentrations in the leaves of different birch species over the entire growing season.

*Autumnal moth and sawflies*

The different carotenoid profiles and concentrations in autumnal moths and sawflies suggest different preferences and/or needs for the accumulation of different carotenoids, and may also indicate a different sensitivity to oxidative stress, whether caused by pollution or by different allelochemicals present in birch leaves during the growing season. At least some sawfly species seem to respond to pollution with elevated lutein and β-carotene concentrations, while no such response was found in the autumnal moth. Variation in secondary chemicals in birches during growing season (Haukioja et al. 2002) may also affect carotenoid profiles of leaf-chewing insects peaking at different times, later-peaking insects being more exposed to larger amounts of secondary chemicals. A lutein preference seems to be common among lepidopteran caterpillars (**I**, Isaksson and Andersson 2007, Eeva et al. 2010). This lutein preference is not ubiquitous, however; since the larvae of at least some sawfly species accumulate β-carotene over lutein (**I**; but see Eeva et al. 2010).

To disentangle the effects of heavy metal pollution and plant-derived secondary metabolites or their possible interactive effects on carotenoid profiles of autumnal moth and different sawfly species would need an experimental approach: caterpillars could be grown on birch leaves and the carotenoid concentrations of larvae could be assessed at different developmental stages with the simultaneous sampling of birch leaves for carotenoid profiles, phenolic compounds, heavy metals and their concentrations, to cover the whole larval period of different species.

*The great tit*

Thesis was that although the lutein concentration in the plasma and feathers of control nestlings and in feathers of lutein-supplemented nestlings did not differ between the polluted and the unpolluted area (**I, II**) the yellowness in the breast feathers in both groups was lower in the polluted than in the unpolluted area. Articles **IV** and **VI** also found that nestlings were paler in the polluted than in unpolluted area. These results support the hypothesis that pollution-related variation in plumage colour in great tit nestlings is due to carotenoid deficiency in the diet (**II**), and that natural carotenoid availability in the diet affects yellow plumage coloration in addition to the nestling response to carotenoid supplementation (**IV**). There is also yearly variation in caterpillar abundance. Furthermore, different insect species also peak at different times during the the nestling phase of the great tit; this may affect early and late broods differently in different years, due to variation in carotenoid composition and availability of caterpillars (**I, III**, **IV;** see also Eeva et al. 2005, Isaksson and Andersson 2007). The lutein concentration of feathers seems not to be the only factor predicting the yellowness of breast feathers (**II**). The feathers may also be of poorer quality in the polluted than the unpolluted area. Carotenoid deficiency in the diet and the overall nutritive value of the diet, along with feather quality, can be seen as indirect effects of pollution (**I, II, III, VI**). Future research on the carotenoid status of birds should take two-trophic approach, since such an approach has been scarce at least with respect to pollution exposure. Studies with insectivorous birds should deal simultaneously not only with the carotenoid concentration and quantity of different food items in the diet, but also with the dietary concentrations of different nutrients and pollutants to determine their possible direct and/or interactive effects on the circulation and different storage levels of various carotenoids as well as their possible effects on plumage coloration with respect to pollution exposure.

While carotenoid availability increased the carotenoid chroma of great tit nestlings and in the polluted area enhanced their growth (**II, IV**), carotenoid-rich diet seems to have a negative effect on the developmental stability of tail feather length either directly or indirectly. On the other hand, mealworm supplementation led to higher fluctuating asymmetry (FA) in tail feather mass in the unpolluted area, which may suggest that mealworm supplementation in part replaced naturally occurring high quality diet (**IV, V**). Calcium seems to be important in maintaining the developmental stability of feather length. Heavy metal pollution seems to have an indirect negative effect on the developmental stability of tail feather mass in nestlings due to natural food availability. FA in tail feather length and mass are two distinct features, affected by different factors, and foreground the importance of measuring several morphological parameters in studying the effects of environmental stress with respect to FA, since different morphological traits may be affected by different factors (**V**).

Lutein deposition in the egg yolk is a maternal effect, found in study **VI**. This may indicate a trade-off between female body condition and the ability to allocate lutein to the yolk. Females with high plumage carotenoid chroma but with a low body condition index may have had higher levels of circulating carotenoids and thus were able to transfer more lutein to the yolks. If, on the other hand, females with high carotenoid chroma deposit less lutein in the yolk, they may be able to maintain a high body condition. The lower the heavy metal load in the environment, the more lutein allocated by females to the yolk indicating possible indirect effect of pollution on laying females. The positive relationship found between carotenoid chroma in females and nestling may suggest a possible maternal effect on offspring phenotype, but in a correlational study it is impossible to disentangle this result from possible environmental effects (**VI**). In future studies of maternal effects on offspring, several measures of female quality should be applied simultaneously in cross-fostering studies in relation to pollution exposure to uncover the possible direct and interactive effects of different aspects of female quality and pollution on maternal effects.

The central results of this thesis have to do with pollution effects at different trophic levels in a terrestrial food chain, plant – insect – bird, with respect to various carotenoid profiles and concentrations, differences between them and their manifestation by different mechanisms and outcomes in different organisms. These findings brings new knowledge to the growing area of carotenoid research with respect to pollution in different organisms. Tri-trophic and two-trophic approaches in this topic in terrestrial food chains are scarce, further research is needed in the future. The thesis has shown that heavy metal pollution affects carotenoid concentrations and carotenoid profiles at all three trophic levels: the plant, the insect and the bird. In birches the increase in carotenoid concentration may be be an adaptive response to pollution. Folivorous caterpillars of different species showed different preferences for different carotenoids, while in at least some sawfly species heavy metal pollution seems to increase the amount of carotenoids directly and/or indirectly via elevated carotenoid availability from their diet in the polluted area. At the top of the food chain, in birds, the effects of heavy metal pollution appeared as the paler plumage colour of great tit nestlings. The pollution effects in great tit nestlings were mediated indirectly by the availability of carotenoid-rich food and by food quality. The effects of pollution at base part of the food chain, in birches, are probably not directly reflected at the top of the food chain, i.e. in great tit nestlings.

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