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Carotenoid Composition of Invertebrates Consumed by Two Insectivorous Bird Species

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Abstract Dietary carotenoids are important pigments, antioxidants, and immune-stimulants for birds. Despite recent interest in carotenoids in bird ecology, we know surprisingly little about the carotenoid content of invertebrates consumed by birds. We compared carotenoid (lutein, β-carotene, and total) concentrations in invertebrates brought to nestlings by two insectivorous passerines, the great tit, *Parus major* and the pied flycatcher, *Ficedula hypoleuca*. We also compared carotenoid levels between environments that were either polluted by heavy metals or were not polluted, because the carotenoid-based plumage color of *P. major* nestlings is affected by environmental pollution. Lepidopterans were the most carotenoid-rich food items and contained the largest proportion of lutein. There were no differences in carotenoid concentrations in the food items of the two bird species but *P. major* nestlings obtained more carotenoids from their invertebrate diet than *F. hypoleuca* nestlings because the *P. major* diet had a higher proportion of lepidopteran larvae. In polluted areas, *P. major* nestlings consumed lower levels of dietary carotenoids than in unpolluted areas because of temporal differences in caterpillar abundance between polluted and unpolluted sites. Our study suggests that pollution-related difference in nestling plumage color in *P. major* is related to varying dietary proportion of lutein-rich food items rather than pollution-related variation in insect carotenoid levels.

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Introduction

Carotenoid pigments have been studied intensively in avian ecology during the last decade (Hill and McGraw 2006). Besides their important role in visual signaling, plant-derived carotenoids have essential physiological functions as antioxidants, immunostimulants, and pro-vitamins in birds (Surai et al. 2001). Birds acquire all of their carotenoids through their diet (Goodwin 1986; Brush 1990). For example, some herbivorous invertebrates that are rich in plant-derived carotenoids are important sources of carotenoids for insectivorous birds, such as the great tit, *Parus major* (Partali et al. 1985; Slagsvold and Lifjeld 1985; Tummeleht et al. 2006; Isaksson and Andersson 2007; Eeva et al. 2008). While carotenoid concentrations have been documented for one important food item, herbivorous caterpillars (Partali et al. 1985; Isaksson and Andersson 2007; Sillanpää et al. 2008), there is little information on other important invertebrate groups. A better understand of dietary carotenoids will elucidate the sources of variation in carotenoid levels in wild birds.

We compared carotenoid (lutein, β-carotene, unidentified carotenoids, and total) concentrations in dietary invertebrates for two insectivorous passerines, the great tit and the pied flycatcher, *Ficedula hypoleuca*. To get the most relevant information about the bird's diet, we collected the food items that birds brought to their nestlings. Sampling invertebrates collected by birds ensures that we are sampling just those species that are important for birds during breeding. Furthermore, with this sampling procedure we could estimate the mass proportions of the

main invertebrate groups in the diets of birds, which enabled us to calculate species-specific dietary carotenoid profiles. Because plasma carotenoid levels and plumage color of *P. major* are related to environmental pollution (Eeva et al. 1998, 2009), we collected data at two separate sites, one polluted by heavy metals and the other a relatively unpolluted reference site.

Methods and Materials

Study Site The data were collected from 12 study sites in the summers 2000 and 2002, in the pollution gradient of a copper smelter in the town Harjavalta (61°20′ N, 22°10′ E), SW Finland. Elevated heavy metal concentrations occur in soil, vegetation, insects, and birds in the polluted area (Kiikkilä 2003), and metal contents decrease exponentially with increasing distance to the smelter, approaching background levels at sites further than 5 km from the smelter (Jussila and Jormalainen 1991). A more detailed description of the study area and the pollution gradient has been given in Eeva et al. (1997). Six study sites in the proximity (<2 km) of the pollution source and six more distant (>5 km) sites were established with 40–50 nest boxes at each site.

Food Samples We collected food samples (N=338 individual food items) from *F. hypoleuca* and *P. major* parents feeding their nestlings at the age of 6–11 or 8–13 d, respectively. Since *P. major* nestlings hatched, on average, 16 d earlier than *F. hypoleuca*, the sampling of invertebrates also took place about 16 d later in the latter species. Birds were captured with a shutter trap at the entrance hole of a nest-box. A piece of plastic transparency above the begging nestlings prevented parents from giving the food items to nestlings. When an adult bird entered the nest-box we took the food item. Maximum duration of disturbance per brood was 20 min. Invertebrates were identified and classified into eight groups as follows: spiders (Aranae), beetles (Coleoptera), ants (Formicidae), adult moths, and butterflies (Lepidoptera), lepidopteran caterpillars, sawfly caterpillars (Symphyta), cockroaches (Ectobiidae), and other invertebrates (including Diptera, adult Hymenoptera, Isopoda, Lithobiidae, Myriapoda, and Gastropoda). Food items were weighed (mg) and preserved at −20°C in the dark until analysis. Sampling was conducted following the Finnish guidelines for ethical treatment of vertebrate experimental subjects.

Carotenoid Analyses For carotenoid analyses, 1–9 individuals from the same taxonomic group were put together, freeze-dried, and ground into fine powder. The total number of samples was 63 from *F. hypoleuca* diets and 23 from *P. major* diets. The powder (15 mg) was extracted three times in dark at 10°C with 400 μl of acetone, and the residue was then washed with 100 μl of acetone. The combined acetone extract (1300 μl) was concentrated for 50 min with an Eppendorf concentrator and was freeze-dried. The freeze-dried residue was dissolved in 300 μl of acetone, and 60 μl were injected into the HPLC system. The carotenoid composition was analyzed at 450 nm using a YMC C-30 column (250× 4 mm, i.d., 5 μm). β-carotene was quantified as β-carotene equivalents and the other carotenoids as lutein equivalents, using commercial lutein and β-carotene as standards (Extrasynthese, France). In some of the samples, the concentrations were below the detection limit (0.5 μg/g) and were replaced with a value of $0.5/\sqrt{2}$ in the statistical analyses (Hornung and Reed 1990).

Although the degradation of carotenoids is relatively slow at −20°, it is likely that some degradation occurred during the relatively long sample storage (7–9 yr). The first-order degradation kinetics of total carotenoids in wheat flour at −20° is described by the rate equation $\ln C = \ln C_0 - kt$; C = concentration, k = reaction rate constant, 0.15×10^{-3} day^{-1}, t = time in days (Hidalgo and Brandolini 2008). From that rate equation, we estimate that when we analyzed our samples they contained 61–68% of the original carotenoid concentration. The exact degradation rate is unknown since it may depend on the matrix (Hidalgo and Brandolini 2008). Therefore, absolute concentrations found in this study cannot be compared directly with levels found in fresh invertebrates. The main interest in our study, however, was to compare relative concentrations among different invertebrate groups.

Statistical Analyses We analyzed carotenoid concentrations (lutein, β-carotene, unidentified carotenoids, and total carotenoids) with separate generalized linear models with a negative binomial error distribution and log link function by using the GLIMMIX procedure of SAS 9.1 (SAS Institute 2003). The independent factors in the models were: bird species, year, area (polluted vs. unpolluted), and invertebrate group. Due to the small number of samples in most invertebrate groups, we did not consider the interactions between the main effects. Statistical interpretation of the group-differences was based on the 95% confidence intervals of the means. For example, if the 95% confidence intervals of means overlap half the length of one arm, this corresponds approximately to statistical significance at p= 0.05 (Cumming 2009).

Results

Carotenoid concentrations in different invertebrate groups are shown in Table 1. There were no statistically significant

Table 1 Mean concentrations (μg/g, d.w.) of lutein, β-carotenoid, unidentified carotenoids, and total carotenoids in invertebrate groups normally consumed by *Parus major* and *Ficedula hypoleuca* nestlings in polluted and unpolluted areas

Group	n	Lutein mean (±SE)	β-carotenoid mean (±SE)	Unidentified mean (±SE)	Total mean (±SE)
Aranae	9	1.7 (±0.27)	1.2 (±0.17)	3.3 (±0.82)	6.1 (±1.3)
Coleoptera	16	1.5 (±0.51)	2.4 (±0.79)	8.6 (±4.6)	12.4 (±5.9)
Formicidae	7	<0.5[b]	0.69 (±0.12)	0.86 (±0.20)	1.8 (±0.35)
Lepidoptera adults	13	6.4 (±1.8)	7.9 (±3.1)	13.6 (±4.9)	27.8 (±9.5)
Lepidoptera larvae	22	14.0 (±2.0)	4.7 (±0.81)	10.1 (±3.2)	28.5 (±5.2)
Symphyta larvae	5	3.0 (±1.6)	1.9 (±0.68)	6.1 (±2.5)	10.7 (±4.4)
Blattaria	4	<0.5[b]	1.0 (±0.20)	1.3 (±0.67)	2.4 (±0.78)
Others	10	0.70 (±0.17)	0.77 (±0.39)	0.79 (±0.44)	1.8 (±1.0)
Among group effect[a]		$F_{7,75}$=15.7 P<0.001	$F_{7,75}$=5.6 P<0.001	$F_{7,75}$=4.4 P<0.001	$F_{7,75}$=8.5 P<0.001

[a] GLM with negative binomial error distribution. Independent factors were: species, year, pollution zone and invertebrate group

[b] All values below the detection limit (0.5 μg/mg)

differences in mean concentrations of carotenoids in dietary invertebrates consumed by the two bird species, nor between years or study areas (for lutein, β-carotene, unidentified carotenoids, and total: P>0.05 in all cases). Invertebrate groups, however, differed significantly in their mean carotenoid concentrations (Table 1). The highest lutein, β-carotene, and total carotenoid concentrations were found in lepidopterans (Fig. 1). Moderate total concentrations were found in Coleoptera, Symphyta, and Aranae (Fig. 1). Ants, cockroaches, and the group "other" invertebrates showed the lowest total concentrations (Fig. 1). The proportion of lutein from total carotenoids was highest in lepidopterans (mean±SE proportions calculated over individual samples: 56±4.9% for caterpillars; 40±6.5% for adults). The proportion of β-carotene was highest in cockroaches (44±18.9%), and the proportion of unidentified carotenoids was highest in beetles (45±7.4%) and spiders (44±6.7%).

Although there were no differences in the concentration of carotenoids in food items used by the two bird species, the birds used different invertebrate groups in different proportions (Table 2). For example, in the unpolluted area, the biomass-based proportion of carotenoid-rich lepidopteran larvae in diet was 3.4 times higher in *P. major* than in *F. hypoleuca* (Table 2). We calculated the carotenoid profile for the nestling diet of each species, taking the varying dietary proportions of different invertebrate classes into account (Fig. 2). *Parus major* nestlings received 1.4 and 5.7 times more carotenoids than *F. hypoleuca* nestlings in the polluted and unpolluted areas, respectively (Fig. 2). This

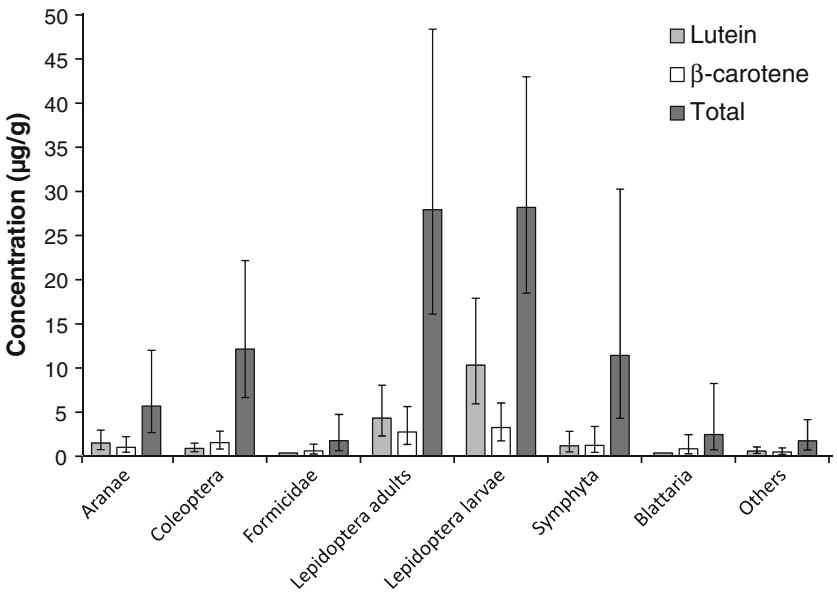

Fig. 1 Mean concentrations (μg/g, d.w.) of lutein, β-carotenoid, and total amount of carotenoids in invertebrate groups consumed by *Parus major* and *Ficedula hypoleuca* nestlings. Values are model based expected marginal means backtransformed from a log-scale. Error bars indicate the 95% confidence interval. N=86

Table 2 Proportions (% of total fresh mass) of invertebrate groups in the diet of *Parus major* and *Ficedula hypoleuca* nestlings in polluted and unpolluted areas

Group	*P. major*		*F. hypoleuca*	
	Polluted	Unpolluted	Polluted	Unpolluted
Aranae	8.1	3.9	2.5	10.8
Coleoptera	12.4	1.4	29.0	10.1
Formicidae	3.5	1.5	2.5	13.5
Lepidoptera adults	14.4	9.0	15.3	21.5
Lepidoptera larvae	50.8	77.1	37.9	22.8
Symphyta larvae	3.6	0.0	3.9	2.0
Blattaria	0.0	0.3	1.2	5.8
Others	7.2	6.9	7.8	13.7

Calculated from total biomass of collected food items (*P. major*: *N*=121; *F. hypoleuca*: *N*=217)

was due primarily to the much higher proportion of lepidopteran larvae in diet of *P. major* (Table 2). In *P. major*, dietary carotenoid content was lower in the polluted area, while in *F. hypoleuca* it was higher in the polluted area (Fig. 2). For both polluted and unpolluted areas the proportion of dietary lutein was somewhat higher in *P. major* (48%) than in *F. hypoleuca* (37%).

Discussion

Carotenoid concentrations differed among the main invertebrate food groups in the nestling diets of *P. major* and *F. hypoleuca*. Lepidopterans were the most carotenoid-rich food items, with lepidopteran caterpillars (e.g., *Operophtera brumata*, *Epirrita autumnata*) containing the largest amount of lutein on a mass and proportional basis. Relatively high carotenoid concentrations also were found in adult Lepidoptera (e.g., *Bupalus piniarius, Thera obeliscata*), which are an important source of carotenoids especially for *F. hypoleuca*. Beetles, sawfly larvae, and spiders contained moderate total carotenoid concentrations, but with a much lower proportion of lutein than in lepidopterans. Some invertebrate groups that were important in the diet of *F. hypoleuca*, such as ants and cockroaches, had relatively low carotenoid concentrations.

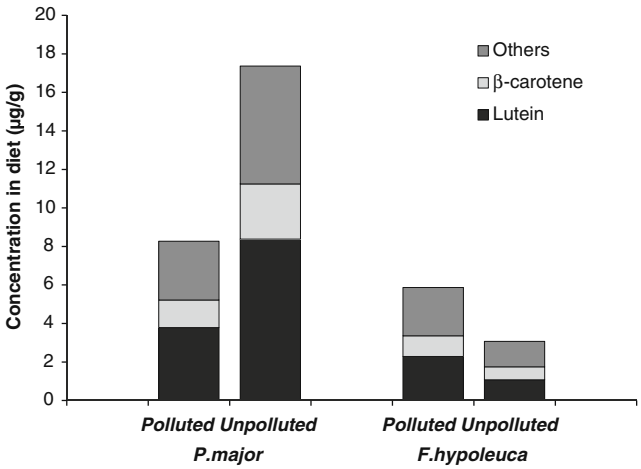

Fig. 2 Concentrations of lutein, β-carotenoid and other carotenoids in the invertebrate diet of *Parus major* and *Ficedula hypoleuca* nestlings. The values take account the concentration in each invertebrate group (Table 1) and the proportion of each group in the diet (Table 2)

The levels of carotenoids in the insects in our study, were lower than previously published values. Sillanpää et al. (2008) found lutein concentrations of 80–90 μg/g (d.w.) in a sample of autumnal moth (*Epirrita autumnata*) larvae, while in our sample the average caterpillar lutein concentrations was only 14 μg/g (d.w.). Isaksson and Andersson (2007) reported lutein concentrations of 16–26 μg/g (w.w.) in lepidopteran caterpillars, equivalent to 123–200 μg/g (d.w.) assuming that caterpillar water content is 87% (Sillanpää et al. 2009). Lutein concentration of sawfly larvae was 15–18 μg/g (d.w.) in a study of Sillanpää et al. (2008), while it was 3 μg/g (d.w.) in our sample. The relatively large differences most likely are due to degradation of carotenoids during the relatively long storage of our samples (see Methods). However, the rather diverse species composition in our samples also may partly explain these differences. For example, lepidopteran caterpillars include species of Geometridae, Noctuidae, Arctiidae, Lasiocapidae, and Nymphalidae, and there is likely variation in carotenoid concentrations among the species within this group (Isaksson and Andersson 2007).

The carotenoid profiles of the invertebrates normally consumed by *P. major* and *F. hypoleuca* nestlings were diverse. In all, 34% of total carotenoids was lutein, 21% β-carotene, and 46% unidentified carotenoids. The large number of species in our samples suggest that the unidentified carotenoids may include numerous compounds. For example, astaxanthin and lycopene are important carotenoids in insects, and likely are represented in our samples (Goodwin 1986). Carotenoid profiles of our samples also varied within the same insect group, possibly due to highly variable species composition. For example,

within Coleoptera, the highest carotenoid concentrations (up to 98 μg/g) occurred in samples containing only ladybirds (Coccinellidae). *Coccinella septempunctata*, which was the primary ladybird species in our sample, has a very diverse carotenoid profile (Britton et al. 1977). These unpalatable beetles, which are especially numerous in the polluted area, frequently were observed in the nestling diet of *F. hypoleuca,* while *P. major* seems to avoid them (Eeva et al. 2005).

Invertebrate carotenoid profiles differ from plasma carotenoid profiles of *P. major* and *F. hypoleuca* nestlings. In plasma, lutein is the dominant carotenoid, comprising up to 57% (*F. hypoleuca*) and 75% (*P. major*) of the total carotenoids (Biard et al. 2006; T. Eeva, unpublished data). Birds absorb different carotenoids disproportionally, and xanthophylls (like lutein) generally are absorbed relatively efficiently (Goodwin 1986; Surai et al. 2001; McGraw 2005). Xanthophylls also are the primary carotenoids in the integument coloration of many bird species, including *P. major* (McGraw 2006; Eeva et al. 2008). Many bird species can modify dietary carotenoids chemically, but in *P. major* the main feather pigments (lutein and zeaxanthin) are transported from food to feathers without modification (Brush 1990; McGraw 2006).

We found no significant differences in carotenoid concentrations of food items collected by the two bird species. This is not surprising since the diets of *P. major* and *F. hypoleuca* include some of the same species (Cramp and Perrins 1993; Eeva et al. 2005). Furthermore, due to a large variation in concentrations among samples and relatively small sample size (especially in *P. major*), our analysis could not have detected small differences in carotenoid concentrations. The normal diet of *P. major* nestlings, however, contains considerably more carotenoids than the diet of *F. hypoleuca* because *P. major* consumes a much larger proportion of carotenoid-rich lepidopterans (Eeva et al. 2005). In agreement with our study, Sillanpää et al. (2008) showed that there was no pollution-related difference in concentrations of lutein and β-carotene in caterpillars of one important dietary lepidopteran species, the autumnal moth (*Epirrita autumnata*).

Temporal differences in caterpillar abundance between polluted and unpolluted sites are an important determinant of dietary carotenoid concentration and carotenoid intake in our study species (Eeva et al. 2005; Sillanpää et al. 2009). Heavy metal rich food is known to retard growth and increase mortality of herbivorous insect larvae (Heliövaara and Väisänen 1990; Martens and Boyd 1994; Ruohomäki et al. 1996). At least in some years, the peak abundance of lepidopteran caterpillars occurs later in the polluted area (Sillanpää et al. 2009). As a consequence, the diet of *P. major* nestlings in the polluted sites contains lesser amounts of carotenoids than the diet in the unpolluted area. In contrast, the nestlings of later-breeding *F. hypoleuca* may consume more carotenoids in the polluted area than their conspecifics in the unpolluted area.

Carotenoid concentrations differ markedly among the main invertebrate taxa in the diet of *P. major* nestlings. Lepidopterans are the most carotenoid-rich food items, and contain a high proportion of lutein. Dietary variation in carotenoid levels may have important consequences for bird species that use carotenoid pigments for their feather coloration. Lutein is the most important feather color pigment in *P. major*, the pale yellow coloration of *P. major* nestlings in a polluted area is a manifestation of low dietary carotenoid levels (Eeva et al. 1998). Our study suggests that this difference in nestling plumage color is due to varying dietary proportion of lutein-rich food items rather than due to pollution-related variation in insect carotenoid levels.

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