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The chemical pathway of carotenoids: from plants to birds

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Carotenoids are interesting in avian ecology due their dietary origin and the resulting environmental dependence of certain functions and traits such as plumage pigmentation and the immune system. Carotenoids are frequently assumed to be limited for birds, but data on availability is rarely collected. Thus, to understand the role of the carotenoids as mediators of trade-offs, additional information on the whole chemical pathway of carotenoids is needed. In conjunction with previous studies on carotenoid ecology and physiology of the Great Tit *Parus major* living in Swedish urban vs. rural environments, I here present data on 1) carotenoid concentration in two primary carotenoid producers, birch *Betula verrucosa* and oak *Quercus robur* and 2) vegetation cover on Great Tit territories. Previous studies found lower carotenoid concentrations in caterpillars and plumage pigmentation in urban compared to rural areas. Correspondingly, urban trees had significantly lower total carotenoid concentration in comparison to rural trees, but there was no environmental difference in the relative amount of lutein and zeaxanthin (Lut:Zx ratio) in sun-exposed leaves. Second, urban Great Tits had significantly more shade in their territories than rural birds, which may affect the leaves Lut:Zx composition. The results, taken together, show that the environmental difference in carotenoid concentration of caterpillars and Great Tit yellow breast coloration, may occur because urban trees synthesise less or have used up more carotenoids in response to environmental stress compared to rural trees. Thus, tracking carotenoid content in different steps of the food chain raises questions about how fine-scale environmental heterogeneity can affect optimal foraging behaviour and individual variation in carotenoid-dependent traits.

Key words: carotenoid availability, lutein, *Parus major*, plumage coloration, zeaxanthin

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Avian carotenoid ecology and physiology has experienced a dramatic increase in attention over the last decade (Hill & McGraw 2006). The focus on carotenoids arises from a number of reasons relating to their link between proximate and ultimate levels of explanation. For example, carotenoids have both optical and physiological properties that make them widely used as plumage pigments, pre-cursors of vitamin A, cell-membrane stabilizers and, possibly, as antioxidants (Britton 1995). Consequently, variation in carotenoid intake, uptake and metabolism may constitute causal explanations for trade-offs and individual variation in natural populations. Carotenoids are synthesized by plants, algae, and some bacteria, while birds and other vertebrates need to obtain carotenoids from their diet

(Goodwin 1984). External carotenoid availability for birds therefore depends on two factors; (1) carotenoid concentration in the primary carotenoid producers (i.e. plants), and (2) carotenoid uptake and metabolism of food items (e.g. leaf eating invertebrates such as caterpillars). Considering how fundamental assumptions of carotenoid availability have been in studies of the evolutionary ecology of carotenoid-dependent traits (e.g. sexual signalling, Hill & McGraw 2006), studies of external carotenoid availability for insectivorous birds are surprisingly scarce. Thus, despite the wealth of information on variation in carotenoid-dependent traits in birds and its link to mate choice, health and competitive ability, information on actual carotenoid availability and its dependence on external factors is to my knowl-

edge limited to a couple of studies (Partali *et al.* 1987, Isaksson & Andersson 2007). In addition to the analytic approaches of the above studies, Olson (2006) used a comparative broad-scale approach (e.g. insects vs. fruit vs. seeds) to evaluate carotenoid limitations. Other studies that have addressed the importance of dietary carotenoids have looked at plasma levels or gut content in relation to plumage or skin colour (Hill *et al.* 1994, Grether *et al.* 2002, Hill *et al.* 2002). Furthermore, only one study has presented the whole carotenoid cycle (vegetation → caterpillars → birds, i.e. plasma, yolk and feathers) (Partali *et al.* 1987). The pioneering study by Partali *et al.* (1987) is limited, however, since they used data from only one population. Therefore, they were unable to look at variation between populations and also between plant and caterpillar species. We have conducted extensive studies over the last five years in two contrasting environments (urban and rural) in southwestern Sweden, and our work has revealed variation in carotenoid storage and allocation, both among and within populations (e.g. Isaksson *et al.* 2007a,b, 2008, Isaksson & Andersson 2007). Here I extend these studies by addressing the final missing link within the Great Tit system – the primary producers – and provide an overview of our results in an attempt to evaluate how important the different levels are for birds in natural environments and their potential implications for natural variation in carotenoid-dependent traits.

Methods

Recently emerged, but fully matured leaves from birch and oak trees were collected in 2006 from the urban and rural areas on two consecutive days (19 and 20 June; $n = 5$ for each combination of species and habitat), at a time when leaf eating caterpillars, a preferred food source for Great Tits, are abundant. Two leaves were collected from each tree for High Performance Liquid Chromatography (HPLC) analysis. The urban area (Slottsskogen) was located within Gothenburg's city limits in southwestern Sweden and the rural population (Gräppås) was located approximately 40 km south of Gothenburg (further details see Isaksson *et al.* 2005, 2007b). All leaves were growing on an open location (i.e. with free access to sunlight) on a height of 1.5–1.7 m, and of similar size (within species). Leaves were immediately weighed (wet weight) and stored in a dark envelope. Before extraction, the dry weight of the leaves was also recorded.

CAROTENOID EXTRACTION AND HPLC ANALYSIS

Leaves were homogenized with an electric homogenizer in ethanol and both carotenoid extraction and HPLC

analysis followed the protocol published in Isaksson & Andersson (2007). All carotenoid concentrations were calculated on the basis of leaf dry weight ($\mu\text{g/g}$). Identified stereo isomers (9-*cis*-lutein, 9-*cis* and 13-*cis*-zeaxanthin) were pooled with the respective parent carotenoid, and referred to as total lutein and total zeaxanthin, respectively. The measure of total carotenoid concentration also included beta-carotene, violaxanthin, and neoxanthin. Other minor carotenoids with peak areas less than 1% were excluded from the analysis (these small peaks added up to $0.97\% \pm 0.40$ SE of the total carotenoid amount).

VEGETATION COVER IN GREAT TIT TERRITORIES

The ecology and biology of the Great Tit populations in the present urban and rural environment has been described in detail in several publications (see for example Isaksson *et al.* 2005, 2007a,b, 2008, Isaksson & Andersson 2007). In 2005 one digital photo was taken from each of 39 Great Tit territories ($n_{\text{urban}} = 20$ and $n_{\text{rural}} = 19$) in the same urban and rural populations as described above. All photos were taken in a standardized way by the same person. All nest boxes were at a height of 1.5–1.7 m and the photo was taken by placing the camera on the nest box roof pointing up at the canopy. Vegetation cover was estimated by using selection tools in Adobe Photoshop CS3 for Windows NT.

STATISTICAL ANALYSIS

Except for two trees (where only one leaf was collected, one urban and one rural birch), the mean from two leaves from each tree was used in the statistical analysis (see Table 1). Two factor ANOVA with interaction was used in all analyses with leaf carotenoids. Simple ANOVA was used to analyze vegetation cover in territories among the two environments. The significance level was set to $P < 0.05$ and all values are presented as means \pm SE. Statistical analyses were performed in JMP 5.1 (SAS Institute Inc., 2003).

Results

There was no difference in leaf water content between habitats or tree species (habitat: $F_{1,16} = 0.08$, $P = 0.779$; species: $F_{1,16} = 0.97$, $P = 0.339$; habitat \times species: $F_{1,16} = 0.15$, $P = 0.706$). Urban had significantly lower total carotenoid concentration than rural leaves (mean urban leaves = 186.6 ± 9.47 , rural = 214.9 ± 9.47 $\mu\text{g/g}$, see Fig. 1 and Table 1). Similarly, absolute concentration of lutein was significantly lower in urban leaves (mean urban = 91.36 ± 5.52 , rural = 110.61 ± 5.52 $\mu\text{g/g}$, Table 1), but there was no difference in zeaxanthin concentrations (mean urban = 23.48 ± 2.07 ,

Table 1. Two-way ANOVA of carotenoid concentrations in leaves by habitat (urban and rural), species (birch and oak) and their interaction. Bold *P*-values indicate a significant effect, $df = 1$ and $df_{\text{model}} = 16$ for all analyses.

	Total carotenoid		Lutein		Zeaxanthin		Lut:Zx		Xanthophyll cycle carotenoids	
	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
Habitat	4.46	0.050	6.09	0.025	0.22	0.646	0.60	0.451	2.80	0.114
Species	0.93	0.348	0.99	0.336	0.15	0.708	0.60	0.452	0.36	0.556
Habitat x Species	2.47	0.135	1.69	0.212	0.54	0.475	0.23	0.635	1.45	0.247

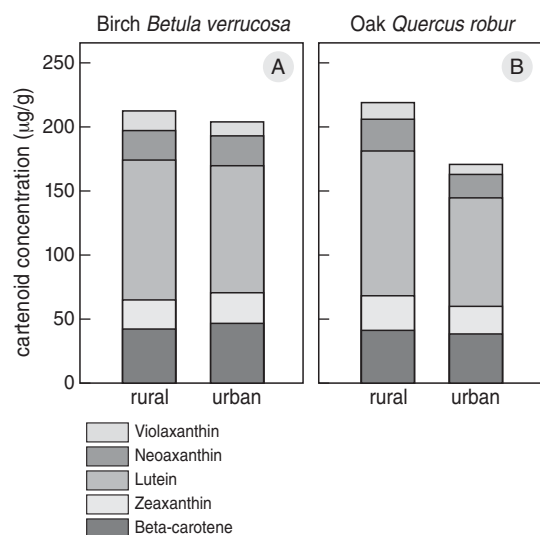


Figure 1. Carotenoid concentrations in Swedish rural and urban birch and oak leaves. Components of total carotenoid concentrations are indicated.

rural = $24.85 \pm 2.07 \mu\text{g/g}$, Table 1), or in the two carotenoids included in the xanthophyll cycle (zeaxanthin plus violaxanthin; Table 1, see Demming-Adams *et al.* 1996). The relative composition of lutein and zeaxanthin (Lut:Zx) was not significantly different between the environments (Table 1).

Finally, Great Tit territories in the urban environment had significantly more cover (i.e. less sun-exposure) than territories in the rural environment ($84\% \pm 4.24$ vs. $75\% \pm 2.04$, $F_{1,38} = 19.49$, $P < 0.0001$).

Discussion

Previous research by our group has shown that there are urban/rural differences in both the total carotenoid concentration and/or Lut:Zx ratio of caterpillars and in different parts of the Great Tit system (e.g. Isaksson *et al.* 2005, 2007a,b, 2008, Isaksson 2007, Isaksson & Andersson 2008). Here I extend these findings and show that there are differences in leaf carotenoid con-

tent and vegetation cover in territories, but not in carotenoid composition (Lut:Zx ratio), between the same urban and rural environments. In plants, carotenoids are crucial components for protecting the chlorophyll from photo-oxidation, but also to protect cell membranes from reactive oxygen species (ROS) caused by anthropogenic pollution and pesticides (Ekmekci & Terzioğlu 2005). Plants exposed to oxidative toxic compounds show a decrease in carotenoid concentration (Ekmekci & Terzioğlu 2005), which suggests that the lower carotenoid content of urban trees reflects environmental stress. A similar pattern was recently found in caterpillars from the same urban and rural habitats (Isaksson & Andersson 2007). Thus, the variation in carotenoid content of caterpillars probably directly reflects the lower carotenoid concentration of leaves, the primary food for caterpillars. If carotenoids were limited for birds, a similar among-habitat variation should exist in avian systems. In line with this, urban Great Tits have a paler carotenoid-based breast (estimated as carotenoid chroma; Isaksson *et al.* 2005). Furthermore, the environmental variation in carotenoid concentration of leaves and caterpillars is not reflected in the internal carotenoid availability in Great Tits (e.g. as reflected by levels in yolk and plasma) and thus seems to be overridden by variation in other parts of the carotenoid physiology (e.g. uptake, transportation, and allocation) and annual and small-scale spatial and temporal variation (Isaksson *et al.* 2007a,b, 2008). However, it is currently unknown to what extent annual variation in carotenoid acquisition and allocation supersedes among- and within-habitat variation (but see Isaksson *et al.* 2007b).

The two major carotenoids, lutein and zeaxanthin, that can be followed throughout the food chain (leaves → caterpillars → birds) are worth discussing separately because of their difference in structure and function. In plants, the main function of lutein is to pass on light energy to the chlorophyll, whereas zeaxanthin is involved in the xanthophyll cycle, which

protects the chlorophyll by channelling the light energy away (Demming-Adams *et al.* 1996). Thus, the Lut:Zx ratio is expected to vary more between sun-exposed and shaded leaves within a tree than between trees or environments from similar light exposure. Since the sampling was standardised for sun-exposure, the lack of environmental difference in Lut:Zx ratio is consistent with this similarity, however, in absolute concentration, lutein was higher in rural leaves compared to urban leaves. Interestingly, the territories established by Great Tits in the urban area had significantly more cover (i.e. less sun-exposure) than the rural territories. Since this difference was controlled for in the collected leaves the potential Lut:Zx difference between the areas may indeed be significant between the two environments. Generally, zeaxanthin is proposed to be a better antioxidant *in vitro* and have higher absorption maxima (i.e. more orange) than lutein (Mortensen *et al.* 1997). Thus, a lower Lut:Zx (i.e. relatively more zeaxanthin) would be preferable for birds, because it provides better antioxidant protection and better pigmentation if the colour trait is under positive selection. Consequently, either selective foraging in sun-exposed parts of the tree and/or habitat, or selective uptake and allocation of zeaxanthin would be adaptive. Interestingly, rural caterpillars, Great Tit egg yolks, and feathers contain relatively and absolutely more zeaxanthin compared to the urban environment (Isaksson & Andersson 2007, Isaksson *et al.* 2008). The adaptive value, if any, of these results is unknown. However, it is possible that small-scale heterogeneity in sun-exposure of leaves is important for carotenoid acquisition via the carotenoid content of caterpillars. Such heterogeneity may both explain, and be of importance for the interpretation of, individual variation in carotenoid-dependent traits, and may have consequences for, for example, signal honesty and reproductive allocation decisions. Studies of the ecology and physiology of carotenoids in the Great Tit (Partali *et al.* 1987, Isaksson 2007) suggest that a greater insight into variation in carotenoid production, uptake, metabolism and storage in different parts of the food chain is of importance to better understand signal constraints and honesty. These results provide strong evidence for environmental variation in carotenoid production and availability, but only limited evidence that this variation is sufficient to cause trade-offs among carotenoid-dependent traits, as is commonly assumed in evolutionary ecology.

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