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Levels of antioxidants in rural and urban birds and their consequences

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Abstract Numerous animals have successfully invaded urban habitats, although the factors associated with invasion success remain poorly understood. Urban areas are characterized by warmer microclimates, higher levels of primary productivity, longer breeding seasons and higher levels of pollutants. All these factors should cause oxidative stress, favoring invasion by species that have access to high levels of antioxidants. We analyzed concentrations of two categories of dietary, fat-soluble antioxidants (total carotenoids, total vitamin E) in the liver, the main storage organ in birds. Individuals killed by cats had lower levels of vitamin E than individuals that died for other reasons, showing natural selection on stored antioxidants. Bird species that had successfully colonized urban areas had significantly higher levels of vitamin E and total carotenoids than species that did not succeed, and rural populations had higher concentrations of vitamin E and total carotenoids than urban populations of the same species. Interspecific differences in concentrations of fat-soluble antioxidants, and differences between rural and urban populations of the same species, were accounted for by diet, but also by time since urbanization and number of generations since urbanization. These findings suggest that antioxidants, and by implication the ability to cope with oxidative stress, have contributed to successful invasion of urban areas by birds, and that the concentration of these antioxidants has changed in response to the urban environment.

Keywords Carotenoids · Condition · Diet · Invasion · Predation

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Introduction

Urban habitats are expanding rapidly due to conversion of farmland, forest and other habitats into cities (e.g., Shochat et al. 2006). This conversion has important implications for plants and animals because they differ in their ability to cope with the specific features of urban environments. At least six factors distinguish urban and rural habitats. Urban areas generally have higher temperatures than the surroundings (Klausnitzer 1989). Urban areas have higher levels of CO_2 and a wide range of pollutants (Klausnitzer 1989; Shochat et al. 2006). As a consequence of higher temperatures, urban areas have longer growing seasons than rural areas (Klausnitzer 1989; Jokimäki et al. 1996). Higher temperatures and higher levels of CO_2 result in higher primary productivity and higher food abundance (Klausnitzer 1989; Stephan 1999; Jokimäki et al. 1996). As a consequence of higher food abundance, urban areas have higher population densities than rural areas (Luniak and Mulsow 1988; Klausnitzer 1989; Gliwicz et al. 1994; Stephan 1999). Finally, proximity to humans in urban areas poses a problem for species with low thresholds of fear (Blumstein 2006; Møller 2008).

Given these characteristics of urban habitats, what constitutes a successful invader of urban environments? While many studies have proposed specific features being responsible for successful urbanization (e.g., Bonier et al. 2007), only a single study has compared the characteristics of urbanized and closely related, non-urbanized species. Møller (2009) showed that bird species that had successfully colonized urban habitats had greater dispersal propensity, a higher rate of feeding innovations, a larger bursa of Fabricius, a history of low predation risk as revealed by short flight distances when approached by a potential predator and difficulty of losing feathers when attacked by a predator, higher fecundity and higher adult survival rate than closely related sister taxa that did not succeed. Furthermore, in a comparison of pairs of urban and nearby rural populations of blackbirds *Turdus merula*, urban populations had lower abundances of ticks and blood parasites than rural populations (Evans et al. 2009).

All activities produce free radicals as a by-product of metabolism, and such reactive biochemicals result in oxidative stress and damage to DNA, other molecules and cell membranes unless they are neutralized by enzymatic and non-enzymatic antioxidants (Halliwell and Gutteridge 2007). Fat-soluble antioxidants like carotenoids and vitamins A and E are prime agents for controlling oxidative stress (e.g., Møller et al. 2000; Surai 2002; Halliwell and Gutteridge 2007). While the antioxidant function of vitamin E is well supported by experiments (e.g., Surai 2002), evidence for an antioxidant function of carotenoids is equivocal at best, with a recent meta-analysis of all published studies of birds showing no significant overall effect (Costantini and Møller 2008). However, there is considerable evidence for carotenoids having other physiological functions such as immuno-stimulation (e.g., Bendich 1989; Chew 1993; Møller et al. 2000; Kim et al. 2000; Pérez-Rodríguez et al. 2008; Saino et al. 2003). Animals living in urban environments are likely to more often suffer from oxidative stress than rural conspecifics because the growing season and hence the reproductive season is longer, temperatures are higher, and pollution levels are elevated. The reproductive season is considerably longer in urban than in rural populations of the same species (e.g., Batten 1973; Gliwicz et al. 1994; Stephan 1999; A. P. Møller et al. unpublished data), causing individuals in urban populations to have higher levels of metabolism for extended periods of time than rural individuals. Temperatures are also generally higher in urban areas although this factor is unlikely to affect birds that have very high thermoneutral zones and well-regulated production of free radicals at the mitochondrial level (Brown et al. 2009). Many studies have shown that pollution in urban habitats is a cause of oxidative stress in humans (e.g., Brauner et al. 2007), although the only available study of birds did not show a significant effect (Isaksson et al. 2009). Urban great tits *Parus major* had lower levels of carotenoids as evidenced by their pale coloration than rural individuals (Slagsvold and Lifjeld 1985; Eeva et al. 1998; Hõrak et al. 2001; Isaksson et al. 2005). Total antioxidant capacity in old great tits from urban habitats was elevated compared to the level in rural individuals (Isaksson et al. 2007). Adult urban great tits had higher current levels of oxidative stress measured as the ratio between oxidized and reduced glutathione than rural adults, while there was no difference for nestlings (Isaksson et al. 2005). Furthermore, urban great tits had lower levels of at least some carotenoids and vitamin A and E in their eggs than rural birds (Hõrak et al. 2002; Isaksson et al. 2008). Finally, urban birds had smaller clutches, more clutches per year, and higher adult survival rates than rural birds (Batten 1973; Gliwicz et al. 1994; Hõrak and Lebreton 1998; Stephan 1999; Hõrak et al. 2002). These observations suggest that urban birds may differ from rural birds in terms of antioxidant status and level of oxidative stress, although the small number of species studied does not allow any firm conclusions.

The objectives of the present study were to test for differences in levels of fat-soluble antioxidants in the liver (which is the main storage organ) in relation to successful invasion of urban habitats by birds. More specifically, we investigated the following questions:

1. How is body condition related to antioxidant levels? If poor condition increases the risk of infection, or causes a re-lapse of chronic infection, we should expect individuals in poor condition to decrease intake of antioxidants while increasing mobilization of stored antioxidants to compensate for reduced intake. This should result in a positive relationship between antioxidants stored in the liver and condition.
2. Is predation risk related to antioxidant levels? If antioxidants stored in the liver are essential for survival, we predicted that individuals captured by cats would have lower levels of antioxidants than individuals dead for other reasons. This provided a crucial test of whether antioxidants have fitness consequences, and whether there is natural selection on antioxidant levels.
3. Do antioxidant levels differ between urbanized and non-urbanized species? If dietary antioxidants have played a role in successful colonization of urban habitats, we should expect urbanized species to have higher levels of antioxidants than related species that have not been urbanized.

4. Do antioxidant levels differ between urban and rural populations of the same species? Longer growing seasons, longer reproductive seasons, and higher pollution levels should tend to decrease levels of antioxidants in urban compared to rural populations.
5. Are antioxidant levels in urban and rural populations related to diet and time since urbanization? An urban environment should increase the level of oxidative stress, implying that urban populations should have more depressed levels of antioxidants than rural populations. Furthermore, if the cause of differences in antioxidant levels between urbanized and non-urbanized species and between urban and rural populations is dietary, differences in antioxidants should be predicted by diet because some diets (such as fruits and ants) contain high levels of antioxidants, while others do not. In contrast, if there were physiological adaptations to urban environments, so that urban populations had evolved improved means of acquiring antioxidant-rich food, absorbing antioxidants from the food, and subsequently metabolizing these antioxidants, we would expect the difference in antioxidant levels between urban and rural populations to diminish by time since initial urbanization.
6. Are there trade-offs between antioxidants? If carotenoids reflect levels of other antioxidants such as vitamin E (Hartley and Kennedy 2004), then we should expect a positive correlation between concentrations of carotenoids and vitamin E. In contrast, if there was a trade-off in terms of physiological function between carotenoids and vitamin E, we should expect a negative correlation between concentrations, although only experimental studies could rigorously test for a trade-off. These expectations should particularly apply when controlling for differences in diet that may mask or obscure any trade-off. Biard et al. (2009) have shown in comparative analyses that the concentration of carotenoids and vitamin E are positively correlated among species of birds, but that only carotenoid concentration is positively correlated with sexual plumage coloration in males, showing that carotenoids and vitamin E can be positively correlated but still covary independently with other phenotypic characters.

Because vitamin E has a clear antioxidant function, while that is not the case for carotenoids (Costantini and Møller 2008), the questions listed above should particularly apply to vitamin E rather than carotenoids. We tested these predictions using an extensive database derived from analyses of concentrations of total carotenoids and vitamin E in the liver of 660 individuals belonging to 153 species of birds.

Materials and methods

Study samples

J. E. received specimens for taxidermy and collected a sample of fresh liver from each for biochemical analyses. For all specimens J. E. also recorded date, year, site and cause of death upon receipt.

Body condition

We recorded body mass and liver mass to the nearest 0.1 g for all specimens studied. J. E. scored all individuals for body condition using an index based on the shape of the body over the sternum (Møller and Erritzøe 2000). This index with increments of one varies from −3, i.e., very lean, to 0, i.e., normal, to +3, i.e., very fat.

Cause of death

All Danish taxidermists are by law enforced to record the cause of death of all specimens that they receive, and this information is entered into an official protocol. Here we distinguished between specimens that had been killed by cats and all other specimens, but as a control group we also used all specimens that had been killed by colliding against windows.

Antioxidant analyses

Fat-soluble antioxidants were estimated in microgram per gram. Livers were frozen immediately after collection, and then maintained at −20°C until analysis. Any livers that were not absolutely fresh were discarded from the present study. Vitamin E concentration was determined by a Shimadzu Prominence full HPLC system (Sil-20A autosampler, LC-20AD solvent delivery system, RF-10 A_{XL} spectrofluorometric detector, CBM-20Alite system controller, CTO-100AS*vp* column oven) fitted with a 3-μm C-18 reverse phase HPLC column (type S30DS2, 15 cm × 4.6 mm; phase separation; Spherisorb, UK). Chromatography was performed using a mobile phase of methanol/distilled water (97:3, v/v) at a flow rate of 1.05 ml/min. Fluorescence detection of vitamin E used excitation at 295 nm and emission at 330 nm. Peaks of δ-, μ- and α-tocopherol were identified by comparison with the retention time of standards of tocopherols (Sigma, Poole, UK). All sampled livers were analyzed for vitamin E concentration. Vitamin E was calculated as the summed concentrations of δ-, γ- and α-tocopherol. Concentrations and not quantity of vitamin E were used as the variable of interest in statistical analysis because concentration is the main factor in determining physiological action of antioxidants at the level of tissues (Surai 2002). The inter-assay coefficients of variation for α-tocopherol determination are typically 3.9% (Surai et al. 1999).

Total carotenoid concentration of liver was determined using the same HPLC system, with a diode array detector set at 444 nm, fitted with a Waters Spherisorb type NH2 column (25 cm × 4.6 mm; phase separation) with a mobile phase of methanol/distilled water (97:3), at a flow rate of 1.5 ml/min as described by Hõrak et al. (2002). The HPLC was calibrated using lutein standards (Sigma). All analytic detections were performed at 30°C in column oven and constant temperature in room temperature at 24°C controlled by air-conditioning.

There was statistically significant consistency in estimates of total carotenoids and total vitamin E among individuals of the same species (carotenoids, $F = 4.03$, $df = 152, 506$, $P < 0.0001$; vitamin E, $F = 1.65$, $df = 152, 506$, $P < 0.0001$).

Urbanization

We adopted the approach for defining urbanization proposed by Møller (2009). Specifically, a species being classified as urbanized had to fulfil two criteria: breeding populations occur inside towns and cities, population densities in towns and cities are higher than in nearby rural habitats. Based on these criteria we made a list of 63 urbanized species of breeding birds out of the 521 species recorded in the Western Palearctic (Cramp and Perrins 1977–1994), fulfilling the two criteria listed above. We note explicitly that many species are urbanized in some areas, but not in others because urbanization is an ongoing process.

Timing of urbanization

We adopted an approach suggested by Møller (2008) to estimate timing of urbanization. In the following we assume that colonization of urban environments can be approximated from observations by keen ornithologists that habitually follow changes in number and distribution of birds closely. Any heterogeneity in colonization processes or increase in population size will cause noise in the data and ultimately make it more difficult to discern any patterns. We estimated the year when different species became urbanized in Denmark using two different approaches. First, we asked two keen amateur ornithologists with more than 60 years' field experience (W. C. Årestrup and E. Flensted-Jensen) living in Denmark to state when different species of birds were first recorded breeding in urban areas. An approximate year of urbanization was recorded, with a value of 1950 assigned to species that were known to breed in urban habitats before the two observers started watching birds. We also independently recorded these years for all species before asking for estimates from the two independent observers. These three data sets were highly consistent in assignment of year of urbanization (all three Pearson $r > 0.96$, $n = 44$ species).

Second, we recorded the timing of invasion of urban environments in Copenhagen, Denmark from old published records (Gram 1908; Fløystrup 1920, 1925). If the year of urbanization was prior to those recorded and reported in these sources, we assigned 1850 as the year of urbanization (because Gram's observations date back to 1858). Although urbanization is likely to have occurred much earlier for many species such as house sparrow *Passer domesticus* and rock pigeon *Columba livia*, these estimates are conservative. This data set provided independent estimates of the time of urbanization, but these were still strongly positively correlated with the estimates of A. P. M. from Northern Jutland (Pearson $r = 0.72$, $n = 41$ species). We used our own estimates combined with those from Copenhagen for species that became urbanized before 1950 in the analyses.

Number of generations

We extracted information on adult survival rate (S) and age at first reproduction (A), using Cramp and Perrins (1977–1994) as a source, and if information was missing, we used Glutz von Blotzheim and Bauer (1966–1997). We estimated generation time (G) as $A + S/(1 - S)$, where A is age at first reproduction and S is adult survival rate. Thus, the number of generations since urbanization was estimated as the number of years since urbanization divided by generation time.

Diet

We classified the diet of all species with respect to eight food categories, using Cramp and Perrins (1977–1994) as a source. These were regular presence (scored as 1) or absence (scored as 0) of seeds, fruit, other plant material, vertebrates, insects, ants, other aquatic invertebrates, and other terrestrial invertebrates.

Summary statistics for the data set are reported in Appendix 1.

Statistical analyses

All statistical tests were made with JMP (2000). We $\log_{10}$-transformed concentration of carotenoids and vitamin E to achieve distributions that did not significantly differ from normal distributions.

Because concentrations of carotenoids and vitamin E were positively correlated, when we used one as a response variable, we included the other as a predictor variable. The correlation between carotenoids and vitamin E was sufficiently weak not to cause a problem of collinearity.

We analyzed the relationship between concentrations of carotenoids and vitamin E by including species as a factor. We tested for an association between antioxidants and predation by comparing the phenotype of prey and non-prey of the same species in a paired design to increase the statistical power of the test. We weighted these interspecific differences within species by sample size to account for the fact that sampling effort varied among species.

We tested whether successful urbanization was related to concentrations of the two categories of antioxidants in a logistic regression, using estimates of carotenoids and vitamin E from rural populations only. Because concentration of carotenoids and vitamin E may be determined by diet, urbanization and time since urbanization, we produced a full model that included all these factors as predictors. This model was reduced to the minimum, best fit model using Akaike's information criterion (AIC) as a guideline, with the final model containing all factors with a change in delta AIC > 2.0 (Burnham and Anderson 2001). We constructed a similar model to the model investigating the relationship between urbanization and antioxidants at the interspecific level by analyzing differences in concentration of carotenoids and vitamin E between rural and urban populations of the same species. For this model we estimated the difference in concentration as $\log_{10}$-transformed mean concentration in rural birds minus $\log_{10}$-transformed mean concentration in urban birds.

Finally, we tested in a paired design (to improve statistical power) whether urbanization was associated with consistent differences in concentration of carotenoids and vitamin E by comparing mean concentrations for the two habitat categories as described above.

We estimated effect size as Pearson's product moment correlation coefficient because it is easily interpretable, as the squared value provides an estimate of the amount of variance explained. Cohen (1988) suggested that a small effect had an $r = 0.10$, accounting for 1% of the variance, an intermediate effect an $r = 0.30$, accounting for 9% of the variance, and a large effect an $r = 0.50$, accounting for 25% of the variance.

Comparative analyses

Species are not statistically independent observations in comparative analyses because apparent phenotypic correlations among species may result from species sharing a common ancestor rather than convergent evolution.

We controlled for similarity in phenotype among species due to common descent by calculating standardized independent linear contrasts (Felsenstein 1985), using the software CAIC (Purvis and Rambaut 1995). Because information for the composite phylogeny originated from different studies using different molecular and phylogenetic methods, consistent estimates of branch lengths were unavailable. Therefore, branch lengths were transformed assuming a gradual model of evolution with branch lengths being proportional to the number of species contained within a clade. We tested the statistical and evolutionary assumptions of the comparative analyses (Garland et al. 1992) by regressing absolute standardized contrasts against their SDs. In order to test for effects of problems of heterogeneity in variance: (1) we excluded outliers (contrasts with Studentized residuals >3) in a second series of analyses (Jones and Purvis 1997), and (2) analyses were repeated with the independent variable expressed in ranks. These analyses are conservative tests of the null hypothesis, explicitly investigating the robustness of the conclusions. In neither case did these new analyses change any of the conclusions, and they are therefore not reported here.

The composite phylogeny used in the comparative analyses was based on Sibley and Ahlquist (1990), combined with information from other sources (Hackett et al. 2008; Jønsson and Fjeldså 2006; Appendix 2). The results from the phylogenetic analyses were qualitatively similar to those found when making calculations using the taxonomy of Sibley and Monroe (1990).

A common underlying assumption of most statistical approaches is that each data point provides equally precise information about the deterministic part of total process variation, i.e., the SD of the error term is constant over all values of the predictor variables (Sokal and Rohlf 1995). The standard solution to violations of this assumption is to weight each observation by sampling effort in order to use all data, by giving each datum a weight that reflects its degree of precision due to sampling effort (Draper and Smith 1981; Neter et al. 1996). Comparative analyses may be confounded by sample size if sampling effort is important, and if sample size varies considerably among taxa (Garamszegi and Møller 2009). In order to weight regressions by sample size in the analysis of contrasts, we calculated weights for each contrast by calculating the mean sample size for the taxa immediately subtended by that node (Møller and Nielsen 2006).

Regressions of standardized linear contrasts were forced through the origin because the comparative analyses assume that there has been no evolutionary change in a character when the predictor variable has not changed (Purvis and Rambaut 1995).

Results

Liver antioxidants, body condition and predation

Liver mass did not differ significantly between urban and rural populations of the same species [urban mean

(SE) = 3.57 g (0.69), rural 3.71 g (0.71), $n = 35$ species; analysis of covariance (ANCOVA) with body mass as a covariate, effect of population: $F = 0.76$, $df = 1, 33$, $P = 0.67$]. The concentration of vitamin E was positively correlated with the concentration of total carotenoids in a model that included species as a factor [$F = 122.53$, $df = 1, 506$, $r^2 = 0.19$, $P < 0.0001$, slope = 1.14 (0.10)], and this was also the case in an analysis of species [$F = 19.38$, $df = 1, 123$, $r^2 = 0.14$, $P < 0.0001$, slope = 0.40 (0.09)] and contrasts [$F = 44.99$, $df = 1, 123$, $r^2 = 0.28$, $P < 0.0001$, slope = 0.33 (0.05)]. The concentration of vitamin E in the liver was elevated in birds in poor body condition, in a model that controlled for differences among species and concentration of total carotenoids (Fig. 1; Table 1). In contrast, there was no significant relationship between total carotenoid concentration and body condition in a model that also included species and concentration of vitamin E (Table 1).

Table 1 Fat-soluble antioxidants in relation to body condition, species and the concentration of the other antioxidant

Variable	Sum of squares	*df*	*F*	*P*	Slope (SE)
Total carotenoids					
Body condition	0.18	1	0.98	0.32	–0.02 (0.02)
Species	109.76	139	4.17	<0.0001	
Total vitamin E	11.96	1	63.13	<0.0001	0.14 (0.02)
Error	82.77	437			
Vitamin E					
Body condition	13.34	1	10.77	0.0011	–0.15 (0.05)
Species	264.22	139	1.53	0.0006	
Total carotenoids	78.19	1	63.13	<0.0001	0.91 (0.11)
Error	293.24	115			

The two models had the statistics: $F = 5.23$, $df = 141, 437$, $r^2 = 0.63$, $P < 0.0001$ and $F = 2.34$, $df = 141, 437$, $r^2 = 0.43$, $P < 0.0001$, respectively

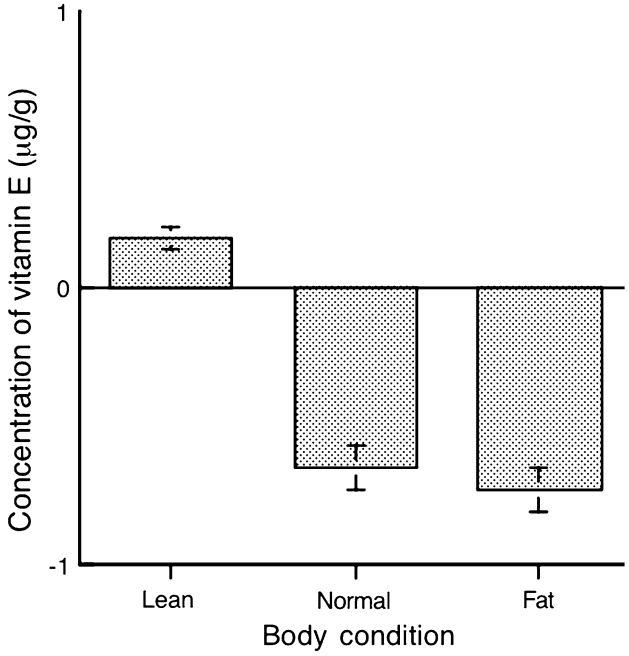

Fig. 1 Mean (±SE) concentration of total vitamin E (µg/g) in lean birds, birds with normal condition, and fat individuals

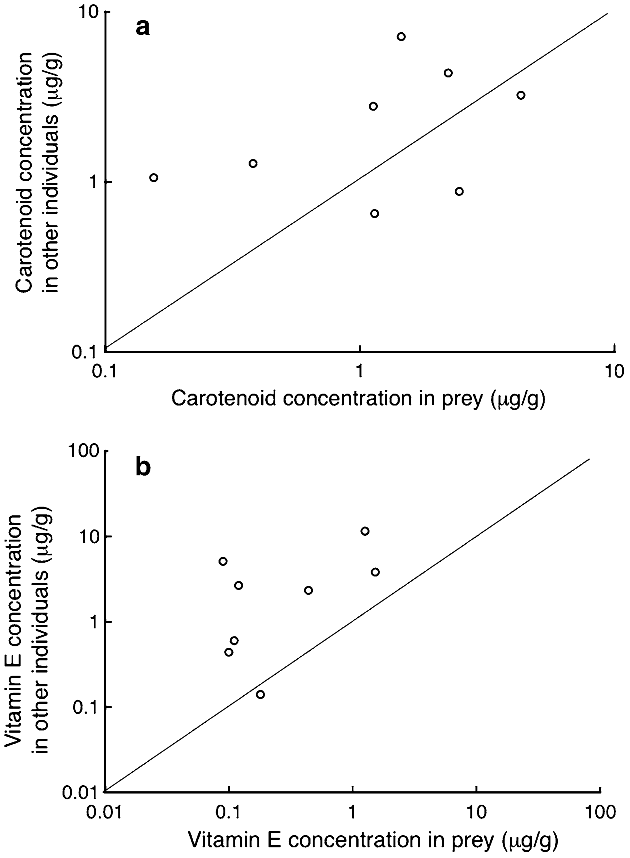

Fig. 2 Mean (SE) concentration of **a** total carotenoids (µg/g) and **b** total vitamin E (µg/g) in prey and other individuals of the same species. Each observation represents an estimate for prey and non-prey of a single species. *The line* shows similar concentrations in the two categories of birds

We had information on antioxidant concentration in eight species for which we had 16 prey and 46 non-prey individuals. Individuals that fell prey to cats had significantly lower contents of vitamin E than individuals of the same species that died for other reasons (Fig. 2; paired *t* test, $t = 3.54$, $df = 7$, $P = 0.0095$), while the effect for total carotenoids was not statistically significant (Fig. 2; paired *t* test, $t = 1.47$, $df = 7$, $P = 0.19$). In contrast, there was no significant difference in vitamin E or carotenoid concentration for the same eight species between individuals that had collided against windows or had died for other reasons ($t < 0.91$, $df = 7$, $P > 0.40$). There was no significant difference in body condition between individuals that had been killed by cats or had died for other reasons ($t = 0.84$, $df = 7$,

Table 2 Fat-soluble antioxidants in relation to diet, urbanization, time since urbanization, and number of generations since urbanization

Variable	Sum of squares	df	F	P	Slope (SE)
Total carotenoids					
Species					
Ants	51.02	1	105.12	<0.0001	0.94 (0.09)
Seeds	6.84	1	14.09	0.0003	0.10 (0.03)
Vitamin E	22.13	1	45.59	<0.0001	0.37 (0.05)
Error	58.25	120			
Contrasts					
Ants	2.99	1	22.09	<0.0001	1.30 (0.28)
Vitamin E	11.03	1	81.37	<0.0001	0.41 (0.05)
Error	15.85	117			
Vitamin E					
Species					
Vertebrates	16.93	1	19.20	<0.0001	0.18 (0.04)
Ants	17.08	1	19.37	<0.0001	–0.69 (0.16)
Carotenoids	43.44	1	49.27	<0.0001	0.71 (0.10)
Error	105.80	120			
Contrasts					
Ants	8.01	1	26.45	<0.0001	–2.10 (0.41)
Other terrestrial invertebrates	2.29	1	7.57	0.0069	–0.38 (0.14)
Number of generations	1.80	1	5.94	0.016	0.17 (0.07)
Carotenoids	22.34	1	73.81	<0.0001	0.93 (0.11)
Error	34.82	115			

The four models had the statistics: $F = 56.40$, $df = 3, 120$, $r^2 = 0.59$, $P < 0.0001$; $F = 44.28$, $df = 2, 117$, $r^2 = 0.27$, $P < 0.0001$; $F = 21.90$, $df = 3, 120$, $r^2 = 0.35$, $P < 0.0001$; and $F = 28.06$, $df = 4, 115$, $r^2 = 0.20$, $P < 0.0001$, respectively

$P = 0.43$). Finally, there was no significant difference in spleen mass for the same eight species between individuals that had been killed by cats or had died for other reasons ($t = 0.39$, $df = 7$, $P = 0.71$).

Liver antioxidants and urbanization

Urbanized bird species had significantly higher levels of total carotenoids than species that had not colonized urban areas, even when restricting the analysis to individuals from rural populations [Wald $\chi^2 = 4.57$, $P = 0.033$, slope = 0.45 (0.21)], while there was no significant difference for vitamin E (Wald $\chi^2 = 0.03$, $P = 0.86$). However, both carotenoid and vitamin E concentration were significantly higher in urbanized than in non-urbanized species in an analysis of contrasts [vitamin E, $F = 6.01$, $df = 1, 123$, $r^2 = 0.05$, $P = 0.016$, slope = 0.16 (0.07); carotenoids, $F = 5.01$, $df = 1, 123$, $r^2 = 0.04$, $P = 0.027$, slope = 0.23 (0.10)].

The concentration of total carotenoids was predicted by diet, but not significantly by urbanization, time since urbanization or number of generations since urbanization (Table 2). A diet containing ants and seeds increased the concentration of total carotenoids in the liver (Table 2). In this model, the concentration of carotenoids was positively correlated with the concentration of vitamin E (Table 2). In an analysis of contrasts there was a significant effect of a diet consisting of ants, a marginal effect of vertebrates, and a significant positive correlation with vitamin E (Table 2).

Species with high levels of total vitamin E in the liver had a diet that contained vertebrates, but was deficient in ants (Table 2). In addition, there was a significant positive correlation between the concentration of vitamin E and carotenoids (Table 2). In an analysis of contrasts a diet of ants and other terrestrial invertebrates was associated with low levels of vitamin E, and there was a significant positive correlation with carotenoid concentration (Table 2). Finally, the concentration of vitamin E increased with the number of generations since urbanization (Table 2). If the analyses were restricted to species that were classified as being urbanized, the conclusions remained qualitatively similar (results not shown).

Liver antioxidants in rural and urban populations

Birds from rural populations had higher levels of total carotenoids than birds from urban populations, with a mean difference of 12% (Fig. 3a; paired t test, $t = 2.47$, $df = 35$, $P = 0.019$). The extreme value in Fig. 3a was caused by the greater spotted woodpecker *Dendrocopos major* that, like all woodpeckers, has a high level of doradexantin (Stradi 1995, p. 112). Likewise rural populations had higher levels of total vitamin E than urban populations, with the mean

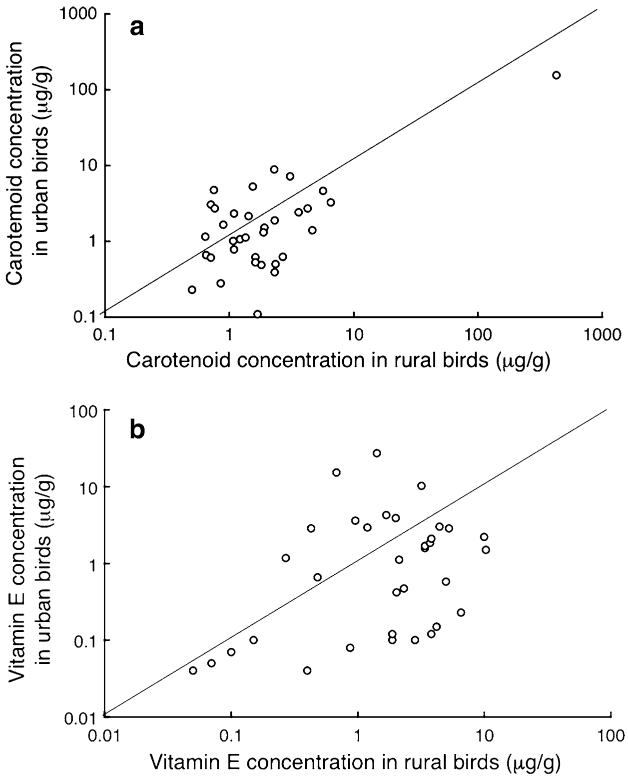

Fig. 3 Concentration of **a** total carotenoids (μg/g) and **b** total vitamin E (μg/g) in different bird species from rural and urban areas. Each observation represents an estimate for an urban and a rural population of a single species. The *line* shows similar concentrations in the two environments

difference amounting to more than a factor 2 (Fig. 3b; paired *t* test, $t = 2.11$, $df = 35$, $P = 0.0006$).

The differences in antioxidants between rural and urban populations could partly be accounted for by diet and time since urbanization (Table 3). Total carotenoid concentration in rural populations was lower in species eating other terrestrial invertebrates than in urban populations of the same species (Table 3). Total carotenoids tended to be larger in urban compared to rural populations of species that had been urbanized and with the number of generations since urbanization (Table 3). Species that had higher concentrations of vitamin E in rural than in urban populations had lower concentrations of carotenoids in rural than in urban populations (Table 3).

Total vitamin E concentration in the liver in urban populations was larger than in rural populations in species eating other plant material and ants (Table 3). In addition, the concentration of vitamin E in the liver in urban populations was larger than in rural populations in species that had been urbanized for a long time, and in species that had lived a larger number of generations in urban habitats (Table 3). Finally, small species had higher concentrations of vitamin E in the liver in urban than in rural populations than had large species (Table 3).

Discussion

The main findings of this study were that the concentration of two kinds of fat-soluble antioxidants in the liver differed with respect to successful invasion of urban environments by birds. The concentration of vitamin E was negatively correlated with body condition, while that was not the case for total carotenoids. Prey of cats had lower concentrations of vitamin E than non-prey, while this was not the case for total carotenoids. Bird species that had successfully invaded urban environments had higher concentrations of vitamin E and carotenoids than unsuccessful invaders. Furthermore, rural populations had higher concentrations of carotenoids and vitamin E than urban populations of the same species. Interspecific differences in antioxidants and intraspecific differences among populations of the same species were partly explained by diet,

Table 3 Difference in concentration of fat-soluble antioxidants between rural and urban populations in relation to diet, time since urbanization and number of generations

Variable	Sum of squares	*df*	*F*	*P*	Slope (SE)
Total carotenoids					
Other terrestrial invertebrates	13.65	1	8.36	0.0072	–0.24 (0.08)
Number of generations	5.34	1	3.27	0.081	0.21 (0.12)
Vitamin E	9.14	1	5.60	0.025	–0.02 (0.01)
Error	52.67	30			
Vitamin E					
Other plant material	7,983.25	1	53.05	<0.0001	–8.85 (1.22)
Ants	2,695.94	1	17.91	0.0002	–7.73 (1.83)
Time since urbanization	933.50	1	6.20	0.019	0.07 (0.03)
Number of generations	1,102.03	1	7.32	0.012	5.71 (2.11)
Body mass	1,538.84	1	10.23	0.0035	4.56 (1.43)
Error	4,063.37	27			

The two models had the statistics: $F = 7.51$, $df = 3, 29$, $r^2 = 0.44$, $P = 0.0007$ and $F = 20.18$, $df = 5, 27$, $r^2 = 0.79$, $P < 0.0001$, respectively time since urbanization and number of generations since urbanization.

While vitamin E is known to be a powerful antioxidant (Surai 2002), only little information links vitamin E to life history. For example, de Ayala et al. (2007) provided the first experimental test of the effects of vitamin E under field conditions, showing that body mass, body condition and feather growth of nestlings improved with supplementation. Møller et al. (2005, 2008) showed for birds in Chernobyl that levels of vitamin E were positively correlated with the frequency of sperm with normal morphology and hatching success. Here we tested the prediction that liver antioxidants predicted risk of predation by investigating carotenoids and vitamin E in birds captured by cats and in conspecifics that had died for other reasons. Survivors had higher concentrations of vitamin E than non-survivors, while this was not the case for carotenoids. Previous studies have shown that spleen size is larger in birds falling prey to cats (Møller and Erritzøe 2000). Here we excluded the possibility that selection acted on other factors such as spleen size or body condition. Furthermore, there was no consistent difference in concentration of vitamin E with respect to mortality due to collision with windows. These findings suggest that viability selection acted on vitamin E concentration, or a correlate thereof. Another possibility is that acute stress caused by cats reduced levels of vitamin E, although we are unaware of a possible mechanism. Furthermore, higher predation rates in rural than in urban populations of the same species (Møller 2008) should produce the opposite result to that observed here. Low concentrations of vitamin E in the liver of urban birds would render these relatively susceptible to predation, although low levels of predation in urban birds (Møller 2008) would reduce selection due to predation in urban relative to rural habitats.

Vitamin E concentration, but not carotenoid concentration, was significantly negatively related to body condition. We expected that sick or parasitized individuals in poor condition should decrease intake of antioxidants while mobilizing stored antioxidants to compensate for reduced intake, resulting in a positive relationship between antioxidants stored in the liver and condition. The observed negative correlation between vitamin E and condition is inconsistent with the hypothesis that individuals in good condition also have large stores of fat-soluble antioxidants. One possible explanation for the negative correlation between vitamin E and condition is that individuals in prime condition store more vitamin E in sites other than the liver.

Urbanized bird species had significantly higher concentrations of vitamin E and carotenoids than non-urbanized species, even when restricting the analyses to individuals from rural areas. Furthermore, concentrations of carotenoids and vitamin E were higher in urban than in rural populations of the same species. These findings suggest that levels of vitamin E and carotenoids predisposed certain bird species to successful invasion of urban habitats, and that urban populations subsequently had reduced levels of carotenoids and vitamin E compared to rural populations. Urbanization is a cause of oxidative stress due to pollution (Brauner et al. 2007), high levels of activity due to extended breeding seasons, and high transmission rates for parasites that cause activation of the immune system and hence produce oxidative stress (Costantini and Møller 2009). Because the concentration of vitamin E was higher in urbanized than non-urbanized species and in urban than in rural populations of the same species, it appears that low levels of vitamin E prevented or delayed successful colonization of cities by birds. Furthermore, vitamin E concentration subsequently changed in urban environments as a function of time since urbanization and the number of generations spent in urban environments. Higher levels of vitamin E and carotenoids in rural compared to urban populations of the same species may account for the observation that urban populations of great tits often have pale carotenoid-based coloration (Slagsvold and Lifjeld 1985; Eeva et al. 1998; Hõrak et al. 2001; Isaksson et al. 2005). Carotenoids only have little if any effect on antioxidant status of birds (review in Costantini and Møller 2008), and carotenoids may be common pigments in sexual signals because they signal physiological functions such as immuno-stimulation (Bendich 1989; Chew 1993; Møller et al. 2000; Kim et al. 2000; Pérez-Rodríguez et al. 2008; Saino et al. 2003). It is well known that immune responses and activation of the immune system cause oxidative stress (Costantini and Møller 2009). Because interspecific and intraspecific patterns of concentrations of carotenoids were similar, we can conclude that the physiological functions of carotenoids have affected invasion success of urban habitats, but may furthermore have negatively affected urban populations once established. Tentatively, low levels of parasitism in urban birds (e.g., Hõrak et al. 2001; Evans et al. 2009) may have prevented carotenoids from being a limiting resource for efficient immune function in urban environments.

There were consistent differences in antioxidants between populations of the same species. Previous studies of single pairs of populations have indicated that antioxidants including carotenoids differ between urban and rural habitats (Slagsvold and Lifjeld 1985; Eeva et al. 1998; Hõrak et al. 2001, 2002; Isaksson et al. 2005, 2008). The present study constitutes a significant step forward by showing, for 36 species, that these differences are large and can be generalized. There was a significant effect of time since urbanization and number of generations since urbanization on the difference in concentration of fat-soluble antioxidants between rural and urban populations, suggesting that differences in local adaptation play a role in determining concentrations of vitamin E and carotenoids. The fact that the differences between populations and species were partly accounted for by differences in diet suggests that some species may be pre-adapted to live in urban environments. Different components of diet accounted for intraspecific and interspecific differences in concentration of vitamin E and carotenoids, suggesting that different aspects of the diet are associated with an initial predisposition to invade urban environments and account for subsequent establishment in urban environments. The present study was based on fat-soluble antioxidants, but water-soluble antioxidants such as uric acid are likely to differ with respect to diet, although such effects must await future studies.

Numerous studies have shown that carotenoids play an important role in sexual signals of birds and other classes of animals (e.g., Møller et al. 2000), although the information contained in such signals remains unknown. One possibility is that the concentration of carotenoids reflects the concentration of important antioxidants like vitamin E (Hartley and Kennedy 2004), and a positive correlation would then be expected. We found a significant positive correlation between concentrations of vitamin E and carotenoids, even when we included diet in the model, consistent with the hypothesis proposed by Hartley and Kennedy (2004).

In conclusion, consistent patterns in the concentration of fat-soluble antioxidants were found in the liver of urban and rural birds. A mammalian predator selected avian prey with a low concentration of stored vitamin E, and vitamin E was significantly related to body condition. Bird species that became urbanized had different levels of vitamin E and carotenoids than non-urbanized species, with similar differences between urban and rural populations of the same species. These findings suggest that successful invasion of urban environments was facilitated by the presence of antioxidants, and that urban populations experienced reduced concentrations of vitamin E and carotenoids following urbanization, with the difference between populations depending on time since urbanization and number of generations since urbanization.

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