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SHORT COMMUNICATION

Does urban life change blood oxidative status in birds?

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ABSTRACT

Cities may expose wild animals to new types of selection pressures, potentially leading to differentiation among urban and rural populations. One cellular mechanism likely important in determining the viability of vertebrate populations is resistance to oxidative stress, as tissue degradation resulting from oxidative stress may decrease reproductive performance and survival. We hypothesized that city-thriving Eurasian blackbirds (*Turdus merula*) would be more resistant to oxidative stress when exposed to stressful conditions than rural conspecifics. Hand-raised city and rural blackbirds kept under common garden conditions indeed differed in blood oxidative status when exposed to chronic stress: city birds had lower oxidative damage during stressful conditions compared with rural birds, but also tended to generally maintain lower levels of non-enzymatic and enzymatic antioxidants than rural birds. These findings show that individuals from urban and rural areas differ intrinsically in their blood oxidative status physiology, possibly as an adaptation to city life.

KEY WORDS: Antioxidants, Oxidative stress, Population, Stress response, Urbanization, Vertebrates

INTRODUCTION

Urban areas have grown rapidly in the last decades at the expense of natural habitats. It is therefore pivotal to understand whether and how environmental changes induced by human activities determine new selection regimes and influence the viability of natural populations of plants and animals. Certain species have been rather successful in exploiting the new ecological opportunities offered by urban habitats (Bonier et al., 2007). The novel and potentially stressful conditions generated by cities may also represent a force that generates intraspecific variation. For example, avian studies have provided evidence for population differentiation in reproductive biology (Partecke et al., 2004), corticosterone stress physiology (Partecke et al., 2006) or parasite infection (Giraudeau et al., 2014).

To further our understanding of how animal populations respond to the environmental conditions imposed by urban habitats, we need to investigate major physiological processes that may differ between urban and rural populations. For example, the ability to detoxify the body from pollutants might play an important role in the adaption to urban life (Herrera-Dueñas et al., 2014). Another mechanism thought to be particularly important is susceptibility to oxidative stress, which can be induced by various environmental, social and internal conditions, given its links with reproductive performance, growth, cellular senescence and survival in free-living animals (Costantini, 2014).

Eurasian blackbirds (*Turdus merula* Linnaeus 1758) are one of the most common songbird species in European cities. Hand-raised urban blackbirds show an attenuated hormonal stress response following exposure to a stressor than rural blackbirds (Partecke et al., 2006), suggesting that they may be less susceptible in endocrine traits to stressors. Here, we tested the hypothesis that blackbirds from an urban population would also show a lower intrinsic susceptibility to oxidative stress following chronic exposure to stressors (Fig. 1) than rural conspecifics. We therefore exposed hand-raised urban and rural blackbirds in a common garden setup (to remove any confounding variables from birds being exposed to different sources of pollution) to repeated immune and disturbance stressors for over 1 year ('stressed' group), comparing their blood oxidative status with that of a non-stressed ('control') group.

RESULTS AND DISCUSSION

After 11 months of repeated immune and chronic disturbance challenges, oxidative damage significantly increased in rural blackbirds compared with their oxidative damage values at the beginning of the experiment and with those of control rural, control urban or stressed urban blackbirds at the end of the experiment (all $P<0.01$). At the end of the experiment, control rural birds, and control and stressed urban birds had oxidative damage levels significantly lower than those they had at the beginning of the experiment (all $P<0.01$; Table 1, Fig. 2). Levels of plasma non-enzymatic antioxidant capacity (OXY) and activity of the antioxidant enzyme glutathione peroxidase (GPX) in red blood cells changed over time irrespective of treatment group or population; however, (i) stressed rural blackbirds had higher OXY than control rural blackbirds ($P=0.024$) and (ii) rural blackbirds had higher GPX than urban blackbirds ($P=0.046$) (Table 1, Fig. 2).

Hence, long-term stress exposure differentially affected blood oxidative status in hand-raised blackbirds, supporting our hypothesis that urban birds appear to be more resistant than rural birds to a long-term stress exposure.

In previous work, hand-raised rural Eurasian blackbirds exposed to a standardized stressor responded with a greater secretion of stress hormones than urban conspecifics (Partecke et al., 2006). As stress hormones can increase oxidative damage in multiple tissues (Costantini et al., 2011), our findings imply that the higher endocrine responsiveness of rural birds to stressors might result in greater oxidative damage than in urban birds. Hence, urban birds might be better able to handle environmental stressors.

While showing more oxidative damage at the end of the experiment, rural blackbirds also generally maintained higher antioxidant defenses when compared with urban birds. Such a difference might be due to rural birds having higher levels of stored non-enzymatic antioxidants than urban birds. Although this

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Received 10 April 2014; Accepted 3 June 2014

The Journal of Experimental Biology (2014) doi:10.1242/jeb.106450

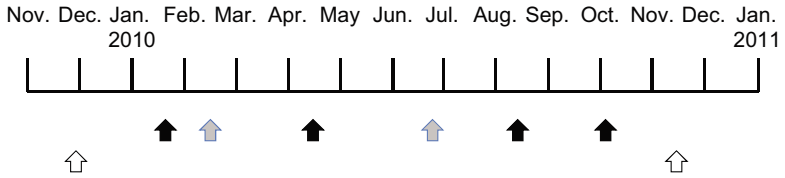

Fig. 1. Timeline of the experiment. White arrow indicates bleeding; black arrow indicates lipopolysaccharide (LPS) injection (immune challenge); gray arrow indicates chronic disturbance. Birds did not breed during the experiment because males and females were kept in separate cages.

hypothesis has not been tested in our blackbird populations, a previous comparative study on birds found that rural populations had higher concentrations of vitamin E and carotenoids in the liver than urban conspecifics, likely caused by differences in their diet (Møller et al., 2010). Our captive birds were maintained on identical diets throughout their lives. However, we cannot exclude that urban and rural birds differ in their ability to assimilate dietary antioxidants, or that their antioxidant status already differed when we collected them as nestlings from the wild. A study on captive zebra finches (*Taeniopygia guttata*) found that individuals fed a poor quality diet in early life had significantly lower concentrations of circulating carotenoids and vitamins A and E in adulthood (Blount et al., 2003). In addition to non-enzymatic antioxidants, rural birds in our study also had a higher baseline activity of GPX, possibly to cope with the higher production of hydroperoxides (oxidative damage). Given that the activity of GPX is dependent on the expression of genes coding for this particular enzyme, these results suggest that city and rural birds may also differ in the environmentally induced expression of genes regulating GPX.

In conclusion, our study provides evidence for differentiation in blood oxidative status between urban and rural individuals of a bird species. Overall, our results showed that rural birds may (i) experience higher blood oxidative damage than urban ones when exposed to challenging conditions and (ii) maintain higher baseline blood antioxidant defenses compared with urban birds, possibly serving an adaptive function as they are more likely to be exposed to oxidative stress. Our results also highlight the importance of assessing the oxidative status of individuals both under baseline conditions and after exposure to challenging conditions.

Like previous studies on differentiation between urban and rural birds that sampled only two populations (e.g. Partecke et al., 2004; Partecke et al., 2006), our study may be suffering from pseudoreplication because the urban blackbirds in our sample may derive from a single colonization event. Hence, even though our results are consistent with findings in other taxa (Lucas and French, 2012), additional studies on multiple urban and rural populations or meta-analyses of existing publications across taxa are needed to confirm our findings.

Further studies are also required to unravel the mechanisms underlying the possible differentiation between conspecific populations, for example in behavioral, hormonal and/or immune traits. It also remains to be tested how genetic differentiation and physiological priming in early life through prevailing environmental conditions (e.g. contaminants, food quality) may interact to shape the organization of the redox system of urban versus rural birds and whether that confers selective advantages for life in urban areas.

MATERIALS AND METHODS

Animals and description of experiment

This study was authorized by the Regierungspräsidium of Freiburg, Germany (permit number: 35/9185.81/G-08/97). Eurasian blackbirds were collected in 2007 at an age of 5–11 days from nine urban and nine rural nests. Urban birds, which hatched between 21 April and 17 June (median hatching date 11 May), were collected in the city center of Munich (48°07′N, 11°34′E; 518 m.a.s.l.), and rural birds, which hatched between 11 April and 12 June (median hatching date 11 May), were collected in a managed forest (47°53′N, 11°04′E; 553 m.a.s.l.) ca. 40 km southwest of Munich. The nestlings were hand-raised and kept under common-garden conditions. Birds were housed in individual home cages, and at 2.5 years of age exposed to a year-long experiment during

Table 1. Statistical output of linear mixed models

Dependent variable	Source	Full model			Final model		
		d.f.	*F*	*P*	d.f.	*F*	*P*
OD	Population	1,9.69	3.5	0.093	1,9.69	3.5	0.093
	Treatment group	1,32.4	32.4	0.035	1,32.4	32.4	0.035
	Period	1,38.0	92.3	<0.001	1,38.0	92.3	<0.001
	Population × treatment group	1,32.4	7.0	0.012	1,32.4	7.0	0.012
	Population × period	1,38.0	15.9	<0.001	1,38.0	15.9	<0.001
	Treatment group × period	1,38.0	18.0	<0.001	1,38.0	18.0	<0.001
	Population × treatment group × period	1,38.0	15.4	<0.001	1,38.0	15.4	<0.001
OXY	Population	1,42.0	1.6	0.209	1,39.9	2.7	0.110
	Treatment group	1,42.0	3.5	0.070	1,39.9	4.9	0.032
	Period	1,66.9	13.3	0.001	1,70.0	15.9	<0.001
	Population × treatment group	1,42.0	5.5	0.023	1,39.9	5.7	0.022
	Population × period	1,66.9	1.7	0.198			
	Treatment group × period	1,66.9	1.5	0.227			
	Population × treatment group × period	1,66.9	0.034	0.854			
GPX	Population	1,13.7	8.3	0.012	1,13.0	6.5	0.024
	Treatment group	1,30.1	10.6	0.003	1,29.9	7.9	0.009
	Period	1,35.0	107.5	<0.001	1,38.0	101.8	<0.001
	Population × treatment group	1,30.1	0.5	0.505			
	Population × period	1,35.0	1.4	0.246			
	Treatment group × period	1,35.0	2.9	0.096			
	Population × treatment group × period	1,35.0	0.03	0.875			

Models tested the long-term (11 months) effects of our disturbance regime on plasma oxidative damage (OD), plasma non-enzymatic antioxidant capacity (OXY) and activity of the antioxidant enzyme glutathione peroxidase (GPX) in red blood cells.

The Journal of Experimental Biology (2014) doi:10.1242/jeb.106450

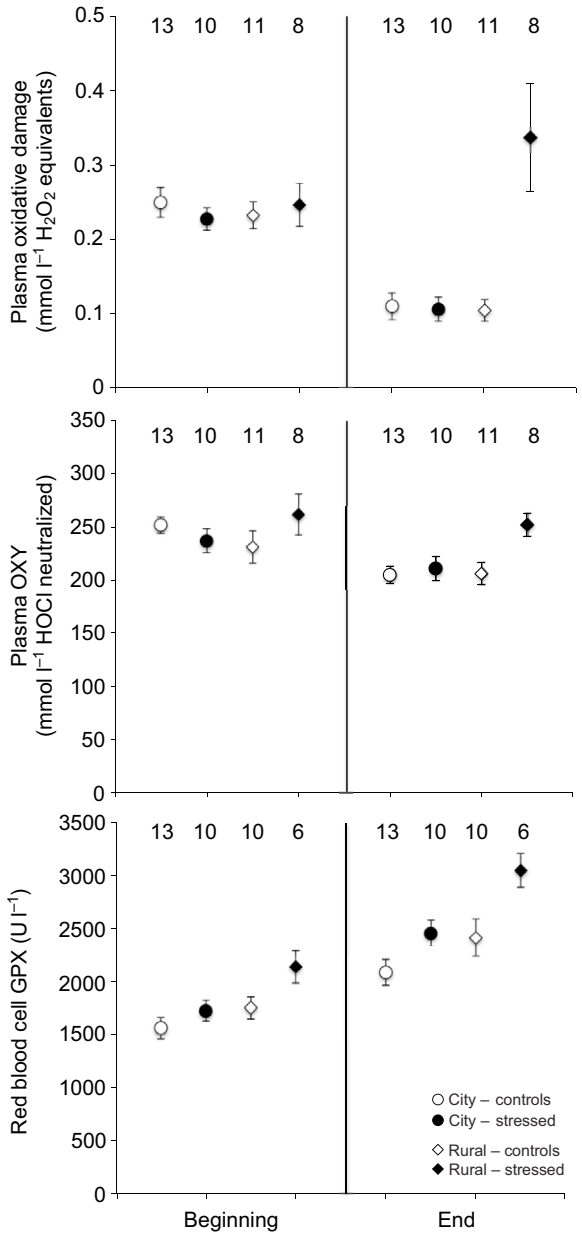

Fig. 2. After 11 months of repeated immune challenge and chronic disturbances, oxidative damage increased in rural blackbirds from the stressed group compared with the other experimental groups. Rural birds (especially those from the stressed group) had higher antioxidant defenses than other birds, although those were not significantly influenced by the treatment. Data are presented as means ± standard error. Sample sizes are given in the upper part of each figure.

which half of the birds were used as the stressed group, while the other half were used as the control group. The birds were exposed to a simulating natural photoperiod, so they underwent annual cycles of reproductive activity (large gonads in spring and summer and small in autumn and winter when they became photorefractory) and molt. However, birds did not breed during the experiment because males and females were kept in separated cages. Birds were provided with food and water *ad libitum*. Urban and rural individuals and the sexes were roughly equally distributed across the two experimental groups (stressed group: five urban males, six rural males, six urban females, two rural females; control group: six urban males, six rural males, seven urban females, five rural females). Furthermore, we tried to balance siblings across treatments too. One stressed rural female and one stressed urban male died over the course of the experiment, hence they were not counted in the above sample sizes. In total, stress-exposed birds were subjected to four immune challenges (injection of lipopolysaccharide, LPS; Sigma L2880) and two chronic disturbance stress periods according to the timeline in Fig. 1. Our chronic disturbance regime represented a milder version of existing chronic stress protocols for small passerines [i.e. fewer disturbances per day and fewer days with disturbances (cf. Rich and Romero, 2005)]. Every day, for 10 consecutive days, we applied each of the following four treatments to birds in the stressed group, in a random order and at random times, but always during daylight hours: 30 min of chasing (waving a catching net with a yellow plastic bag attached in front and over the top of the cage for a conspicuous and noisy disturbance), 30 min of crowding (adding two to three other birds to a single cage), 60 min of restraint (putting an individual into a cloth bag) and 60 min of a radio playing loudly in the room. For the immune treatment, we injected a dose of 2.0 µg LPS diluted in phosphate-buffered saline (PBS) per gram body mass into the breast muscle. Control birds were injected with PBS only. The concentration of LPS used successfully induces an acute phase response including fever and sickness behavior in other songbird species (M.H., Mark F. Haussmann, T.J.G., Christa Matlack, D.C., Michael Quetting, James S. Adelman, Ana Catarina Miranda and J.P., unpublished).

All (control and stress-exposed) birds were sampled for biomarkers at the same time periods. Blood samples were collected from the wing vein in heparinized capillary tubes and stored on ice until centrifugation. Plasma and red blood cells were stored at −80°C until laboratory analyses. Blood samples were taken in December 2009 and November 2010 to assess long-term (comparing the beginning versus the final part of the long-term experiment) effects of repeated stressors.

Laboratory analyses

Oxidative damage products were measured in plasma using the d-ROMs assay (Diacron International, Grosseto, Italy). It mostly measures hydroperoxides, which are intermediate oxidative damage compounds and precursors of several end-products of lipid peroxidation, such as malondialdehyde, hydroxynonenal and isoprostanes. The reaction of a dilution series of cumene hydroperoxide with the d-ROMs reagents was highly linear (range 0–4.5 µmol l^{-1}, R^2=0.9996; physiological values in vertebrates). The non-enzymatic antioxidant capacity was measured in plasma using the OXY-adsorbent assay (Diacron International). It quantifies the *in vitro* reaction of circulating antioxidants with HOCl (an oxidant of pathologic relevance in biological systems). The activity of GPX was measured in red blood cells using the Ransel assay (RANDOX Laboratories, Crumlin, UK). GPX is an antioxidant enzyme that detoxifies cells from hydrogen peroxide and hydroperoxides. The absorbance was read with a Thermo Scientific Multiskan Spectrum (ThermoFisher, Vantaa, Finland) at 505 nm for oxidative damage products, 490 nm for OXY and 340 nm for GPX. All analyses were run in duplicate and the intra- and inter-assay coefficients of variation were, respectively: 5.9% and 11.4% for oxidative damage products; 5.6% and 8.9% for OXY; 3.3% and 5.9% for GPX.

Statistical analyses

Statistical analyses were carried out using SAS Version 9.3 (Cary, NC, USA). Linear mixed models with a repeated measures design were used to test the effects of our long-term stress protocol on blood oxidative status. Response variables were oxidative damage, OXY and GPX. In each model, we included population, treatment group and sampling period as fixed factors; individuals (nested within brood) and brood (nested within population) were included as random factors. We also included two- and three-way interactions among fixed factors. We used a backward elimination process to exclude independent variables with P>0.05, starting from the three-way interaction. *Post hoc* comparisons were performed using the Tukey test when we found a statistically significant interaction effect. Sample size varies across analyses (see Fig. 2). Oxidative damage was rank-transformed in order to achieve normality of residuals.

Acknowledgements

We thank two anonymous reviewers for providing comments that helped us to improve presentation and interpretation of results. For crucial assistance during experiments, we thank J. Lodde, E. Fricke, H. Schmid, G. Beltrami, M. Romero, D. Santos, D. Dominoni, K. H. Siebenrock, M. Echeverry, K. Mortega, N. Perfito, G.

Bentley, A. Schmidt, F. Wikelski and M. van Toor. Excellent bird care was provided by T. Vogler, K. Greeven, E. Jäckle, A. Falk, P. Sigwart, M. Schoenwald and R. Dunz. Many thanks to M. Romero and the BEEP lab group for helpful discussions.

Competing interests
The authors declare no competing financial interests.

Author contributions
M.H., T.J.G. and J.P. designed the study; M.H., T.J.G. and J.P. collected samples; D.C. analysed samples and data; D.C. wrote the manuscript with contributions from T.J.G., M.H. and J.P.

Funding
Funding was provided by the Max Planck Society to M.H.; by the Volkswagen Foundation (Initiative 'Evolutionary Biology') to J.P.; and by US National Science Foundation (NSF) International Research Fellowship Program (IRFP) no. 0852986 to T.G.

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