TECHNICAL COMMENT

Covariation in Oxidative Stress Markers in the Blood of Nestling and Adult Birds

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ABSTRACT

Interest in the imbalance between the production of reactive oxygen species and the state of the antioxidant machinery—that is, oxidative stress—has recently grown among comparative physiologists and evolutionary/behavioral ecologists. The number and types of markers used to estimate oxidative stress is, however, under debate. The study of covariation among these markers is necessary to better interpret the information content of each independent variable. Here, the covariation in levels of 10 blood parameters in a group of zebra finches (*Taeniopygia guttata*) as nestlings and adults was analyzed across a large data set. Total glutathione levels in erythrocytes were negatively correlated with plasma carotenoid values in nestlings only, supporting the implication of carotenoids in the antioxidant machinery during a particularly stressful period of life. Plasma lipid levels (triglycerides [TRGs]) as well as plasma antioxidant capacity—the latter tested with and without control for uric acid levels—showed individual consistency with age. Plasma TRG and uric acid levels were strongly correlated with plasma lipid peroxidation and antioxidant capacity, respectively, suggesting an influence of recent intake or mobilization of energy stores on these variables. The meaning of oxidative stress markers, whether corrected or uncorrected for levels of nutritional metabolites, remains to be explored. Experiments manipulating diet composition and oxidative stress are necessary to confirm or reject the hypothesized causalities.

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Introduction

Oxidative stress is usually defined as the imbalance between the state (levels, integrity, or activity) of the antioxidant machinery and the rate of production of reactive oxygen species (ROS) by the organism (Halliwell and Gutteridge 2007; Metcalfe and Alonso-Alvarez 2010). Such an imbalance leads to oxidative damage in principal biomolecules. The term, nonetheless, remains controversial as certain levels of oxidative stress are required for important cell signaling pathways (Dröge 2002; Jones 2006). Furthermore, the ways in which oxidative stress should be assessed is also debated.

The degree of oxidative stress in an individual cannot be inferred merely by quantifying a single arm of the oxidative balance (frequently antioxidants), as the other arm may be equilibrating any possible effects (Costantini and Verhulst 2009). This has led to the claim that many different markers of oxidative stress should be assessed simultaneously (e.g., Costantini 2008; Cohen and McGraw 2009), although how many and what procedures must be used are not established. The information derived from a number of parameters may reveal functional emergent properties (Cohen et al. 2012) but also result in redundant information (Costantini et al. 2011). In fact, a plethora of oxidative stress markers has been proposed, mostly derived from biomedicine (e.g., Hensley and Floyd 2003; Halliwell and Gutteridge 2007; Monaghan et al. 2009). Comparative physiologists have used many of them for years (e.g., Perez-Campo et al. 1998; Hermes-Lima and Zenteno-Savín 2002), but evolutionary and behavioral ecologists have only recently begun to use the simplest ones to answer questions related to sexual signaling and life-history theories (Monaghan et al. 2009; Metcalfe and Alonso-Alvarez 2010). However, many techniques have limitations. For instance, studies analyzing the covariation of oxidative stress markers were initially focused on testing the antioxidant properties of pigments producing colorful ornaments (i.e., carotenoids; Pérez-Rodríguez 2009). Many studies (e.g., Cohen et al. 2008; Costantini and Møller 2008 and references therein) tested the correlation between circulating carotenoid levels and the trolox-equivalent antioxidant capacity or total antioxidant status of plasma (Monaghan et al. 2009). Studies of such indexes, however, revealed that a

large part of the variability was due to the amount of nitrogen waste (i.e., uric acid [UA]) in plasma (Cohen et al. 2007; Costantini 2011), which is a potent antioxidant (Stinefelt et al. 2005; Halliwell and Gutteridge 2007). Nevertheless, whether plasma UA levels are actively regulated in response to oxidative challenges is still unclear (Sautin and Johnson 2008), with variability usually explained by protein catabolism or inflammation (Martinon et al. 2006; Sautin and Johnson 2008). Other authors have highlighted a key role for the intracellular antioxidant glutathione (GSH; e.g., Alonso-Alvarez et al. 2008, 2010; Galván and Solano 2009). A common procedure involves determining the imbalance between the levels of reduced and oxidized GSH fractions (GSH : GSSG), which may reveal intracellular oxidative stress given that it represents the rate at which the antioxidant is oxidized (Pastore et al. 2003; Owen and Butterfield 2010). However, the GSH : GSSG ratio is also a crucial regulatory redox couple involved in many cellular processes (e.g., Dröge 2002; Kemp et al. 2008), and GSH even acts in the cell nucleus to influence gene expression (e.g., Markovic 2010).

Alternatively to the assessment of antioxidants, other researchers have proposed the prioritization of oxidative damage measurements, as they indicate the final result of the imbalance (e.g., Simons et al. 2012). The most used and probably easiest technique for assessing oxidative damage is the estimation of levels of a product of lipid peroxidation named malondialdehyde (MDA) by quantifying spectrophotometrically thiobarbituric acid–reactive substances (e.g., Aust 1985). However, MDA can be present in food (e.g., Papastergiadis et al. 2012). Furthermore, levels of blood lipids are strongly influenced by food intake, which could affect the variability of the measurement and interpretations (Brown et al. 1995; Halliwell and Gutteridge 2007). In this regard, to improve accuracy and reduce the influence of food, other authors have proposed the use of high-precision liquid chromatography (HPLC) instead of spectrophotometry for specifically quantifying MDA (Halliwell and Gutteridge 2007; Monaghan et al. 2009).

Despite all these limitations, comparative physiologists and evolutionary/behavioral ecologists continue to use many of these techniques. However, as far as we know only a handful of these studies have explored covariation among a relevant number of oxidative stress markers, that is, including some index of oxidative damage. All of them have used birds as model species and blood as the experimental tissue (Costantini et al. 2011, 2013; Sepp et al. 2012; Alan and McWilliams 2013).

Here, several oxidative stress markers were analyzed in zebra finches (*Taeniopygia guttata*). MDA values in plasma and erythrocytes, plasma carotenoid levels, and a spectrophotometric measure of overall plasma antioxidant capacity were measured because all of them are broadly used in comparative physiology evolutionary and ecology studies (see above). The GSH : GSSG ratio and total GSH (tGSH) levels in erythrocytes were also determined, as they represent the functional levels of a key intracellular antioxidant and the overall redox state of the cell (Halliwell and Gutteridge 2007). Parameters related to protein and lipid metabolism (UA and TRGs, respectively) were also assessed in plasma. The zebra finch is a classical model species among birds, and many studies of it have addressed the oxidative stress question (e.g., Wiersma et al. 2004; Alonso-Alvarez et al. 2006; Costantini et al. 2013). A large data set (>120 birds) including values of 10 variables assessed in erythrocytes and/or plasma was analyzed. Bivariate correlations among variables were tested on the same individuals as nestlings and adults. Previous studies have revealed that both positive and negative relationships between antioxidant levels and oxidative damage markers can be expected in blood, as antioxidants may be exhausted because of their involvement in protection or, rather, their mobilization to fight off free radicals (Calabrese 2007; Costantini et al. 2010). We may also predict a positive relationship between plasma UA levels and a measure of antioxidant capacity, as well as a positive link between the levels of circulating lipids (TRGs) and lipid oxidative damage (see above). Our aim was to provide a better picture of the measurements used to estimate oxidative stress, allowing researchers to choose the most appropriate tools for future studies.

Methods

The data analyzed here are part of a study in which GSH levels of nestling zebra finches were experimentally reduced. Seventy-eight pairs of adult birds were placed in breeding cages (0.6 m × 0.4 m × 0.4 m) including a nest box. Straw, a commercial mix of seeds for exotic birds (KIKI, Callosa de Segura, Spain), and water were provided ad lib. The pairs bred over a 5-month period. Temperature (mean: 22° ± 1°C) and light-dark daily cycles (16L : 8D) were controlled. Half of the nestlings in a brood were randomly assigned to a treatment receiving DL-buthionine-S,R-sulfoximine (BSO; Sigma, B2640) diluted in sterilized distilled water (50 mg/mL) or distilled water only (controls). BSO specifically inhibits the synthesis of GSH into blood cells (see Gálvan and Alonso-Alvarez 2008). A volume of 0.06 mL of the solution/water was intraperitoneally injected every 2 d from 4 to 10 d of age (i.e., four injections). The dose was calculated from a pilot experiment on 26 nestlings and previous work in other passerines (Galván and Alonso-Alvarez 2008). The experimental design was reviewed and approved by the Committee for Ethics in Animal Experimentation of the Universidad de Castilla–La Mancha (approval 1201-8).

A blood sample was collected for each 12-d-old nestling (±1 d). Males and females were separately housed in different rooms (2.80 m × 3 m × 2.50 m for each one) when they reached 35–40 d of age. A second blood sample was collected for each adult (mean: 126 d old; range: 64–192 d old). All blood samples were immediately stored at 4°C and centrifuged at 5,000 *g* within 4 h. The plasma was then removed, and the buffy coat was discarded by pipetting. Plasma and erythrocyte fractions were separately stored at −80°C.

GSH Levels in Erythrocytes

GSH was quantified following Griffith (1980) with some modifications. Briefly, the blood pellets were thawed and erythro-

cytes pipetted avoiding the pellet surface (which could still contain white blood cells). Erythrocytes were immediately diluted (1 : 10 w/v) and homogenized in a stock buffer (0.01 M phosphate-buffered saline [PBS] and 0.02 M ethylenediaminetetraacetic acid), working on ice to avoid oxidation. Three working solutions were created in the same stock buffer as follows: 0.3 mM nicotinamide adenine dinucleotide phosphate (NADPH; solution 1), 6 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB; solution 2), and 50 U of GSH reductase/mL (solution 3). An aliquot (0.5 mL) of homogenate of blood cells was vortexed with 0.5 mL of diluted trichloroacetic acid (10% in H_2O) three times for 5 s each time within a 15-min period. In the meantime, samples were protected from light and refrigerated to prevent oxidation. The mixture was then centrifuged (1,125 *g* for 15 min at 6°C), and the supernatant was removed. Subsequent steps were performed in an automated spectrophotometer (A25 autoanalyzer; Biosystems, Barcelona). Solutions 1 and 2 were mixed at a ratio of 7 : 1 v/v, respectively, and 160 μL of this new mixture was automatically added to 40 μL of sample (i.e., supernatant) in a cuvette. Then, 20 μL of solution 3 was added after 15 s, and absorbance at 405 nm was monitored after 30 and 60 s. The change in absorbance was used to determine tGSH levels by comparing the output with the results from a standard curve generated by serial dilution of GSH from 1 to 0.031 mM. Results are given in millimoles per gram of pellet.

To determine oxidized GSH (GSSG) levels, an aliquot (400 μL) of the supernatant obtained for tGSH measurement was adjusted to 7.5 pH by adding 6 N NaOH. Then, 2-vinilpiridine (8 μL) was added to the aliquot, and the mixture was vigorously shaken at room temperature and in the dark to promote GSH derivatization. The mixture was centrifuged (1,125 *g* for 10 min), and the change in absorbance of the supernatant was assessed at 405 nm as described for the tGSH assay. Previous studies in other bird species reported that GSSG and tGSH levels assessed twice are highly repeatable, following Lessells and Boag (1987; $r = 0.92$ and 0.89, respectively; $n = 25$; Alonso-Alvarez et al. 2010). To attain an index of GSH oxidation rate, reduced GSH (named as GSH) was calculated by subtracting GSSG from tGSH levels. The GSH : GSSG ratio was obtained afterward (Owen and Butterfield 2010).

Lipid Peroxidation

The protocol of Agarwal and Chase (2002) with modifications by Nussey et al. (2009) was followed to assess MDA. Plasma and red blood cell (RBC) levels were assessed in separate laboratory sessions. The standard curve for calibration was prepared using a 1,1,3,3-tetraethoxypropane stock solution (5 μM in 40% ethanol) serially diluted using 40% ethanol. Fifteen microliters of a butylated hydroxytoluene (BHT) solution (0.05% w/v in 95% ethanol), 120 μL of a phosphoric acid solution (0.44 M), and 30 μL of a thiobarbituric acid (TBA) solution (42 mM) were added to 15 μL of plasma or standards. For blood pellets, 50 μL of BHT, 400 μL of phosphoric acid solution, and 100 μL of TBA were added to 50-μL aliquots of homogenized RBC samples (diluted in a stock buffer; see the GSH protocol above) or standards. All samples (plasma, homogenized RBC samples, and standards) were capped and vortexed for 5 s. Then the standards and samples were heated at 100°C for 1 h in a dry bath incubator to allow formation of MDA-TBA adducts. The reaction was stopped by placing samples and standards on ice for 5 min. Subsequently, *n*-butanol was added to each tube to extract the MDA-TBA complex. To plasma samples and their standards, 75 μL of *n*-butanol was added, whereas 250 μL was added to homogenized RBC samples and their standards. Subsequently, tubes were vortexed for 60 s and centrifuged at 18,000 *g* at 4°C for 3 min. Fifty microliters of the plasma, RBC samples, and standards of the upper (*n*-butanol) phase were moved to HPLC vials, which were immediately saturated with N_2 to avoid oxidation. Samples and standards were injected into an Agilent 1100 series HPLC system (Agilent, Waldbronn, Germany) fitted with a fluorescence detector set and a 5-μm ODS-2 C-18 4.0 × 250-mm column maintained at 37°C. The mobile phase was MeOH : KH_2PO_4 (50 mM; 40 : 60 v/v), running isocratically for 10 min at a flow rate of 1 mL/min. Data were collected at 515 nm (excitation) and 553 nm (emission). A subsample of RBCs assessed twice produced high intrasession ($r = 0.92$, $n = 20$, $P < 0.001$) and intersession ($r = 0.87$, $n = 20$, $P < 0.001$) repeatabilities. A subsample of plasma samples assessed twice produced very high intrasession ($r = 0.97$, $n = 20$, $P < 0.001$) and intersession ($r = 0.98$, $n = 20$, $P < 0.001$) repeatabilities.

Plasma Antioxidants

A technique often called total antioxidant status was assessed to estimate the availability of nonenzymatic antioxidants. Since the term "total antioxidants" is questionable, only the general term "antioxidants" was used. We will thus use only the abbreviation AOX. The procedure is based on Miller et al. (1993), modified by Cohen et al. (2007). Briefly, a standard was made by dissolving a water-soluble α-tocopherol derivative, Trolox (Aldrich), in PBS buffer to 1.2 mM. Metmyoglobin was generated by mixing equal volumes of 400 μM myoglobin (from equine skeletal muscle; Sigma) and 740 μM potassium ferricyanide, then passing the mixture through a column of sephadex (G15-120, Sigma). The chromogen, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS, Sigma), was mixed in buffer to 153 μM. The assay was run in 96-well microplates on a Bio-Tek reader (PowerWave XS2; Bio-Tek Instruments, Winooski, VT), temperature was maintained at 37°C, and readings were performed at 734 nm. Five microliters of standard (Trolox), blank (PBS only), and samples were put separately into the wells. Next, 15 μL of metmyoglobin and 250 μL of ABTS were sequentially added to each well. A multichannel pipette was used to simultaneously add 50 μL of 200 μM H_2O_2 to all the wells, starting the reaction. All samples were assessed twice and produced high repeatabilities ($r = 0.86$, $n = 394$, $P < 0.0001$), and the mean was used in data analyses.

UA and TRGs in Plasma

TRG and UA levels were assessed in 5 μL of plasma each, using a Bio-Tek microplate reader (PowerWave XS2; Bio-Tek Instruments) fixed at 505 and 520 nm, respectively. The glycerol phosphate oxidase/peroxidase method and the uricase/peroxidase method were used for measuring TRG and UA levels, respectively (kits from Biosystems). Repeatabilities in 45 samples assessed twice were high (TRGs: $r = 0.94$, $P < 0.001$; UA: $r = 0.99$, $P < 0.001$).

Total Carotenoids

Plasma aliquots (5 μL) were diluted in ethanol (1 : 10) in 0.6-mL tubes. Tubes were vortexed for 3 min. The flocculent protein was precipitated by centrifuging at 1,500 g for 10 min at 4°C. The absorbance of the supernatant was determined at 446 nm in a Biotek microplate reader device (see above). A standard curve of lutein (Sigma) was used for quantifying total carotenoids. A subsample assessed twice produced high repeatabilities ($r = 0.87$, $n = 26$, $P < 0.001$).

Statistical Analyses

To attain the highest sample size available, all individuals in the experiment were included in this study. We first ran mixed models (PROC MIXED, SAS ver. 8e; SAS Institute 2006; Littell 2006) to obtain residuals removing variability due to the experimental procedure (i.e., it had the expected outcome, that is, reduced tGSH values in BSO-treated birds compared with controls; see the appendix, available online), sex, environment (nest effects), parental effects, and laboratory session. This allowed the comparison of all the variables in a similar state, as some may have been more sensitive to certain influences than others. We ran models for each blood variable (i.e., GSH : GSSG, tGSH, erythrocyte MDA, plasma MDA, AOX, UA, TRGs, and carotenoids), which were separately tested as dependent variables. Models were performed for nestlings and adults separately. The treatment and the sex of the bird were added as fixed factors. Brood size, laying date (Julian calendar), hatching order, and the age of the bird (the latter only for adults; see above) were tested as potential confounding covariates. Since UA and TRG levels were strongly correlated with AOX and plasma MDA, respectively (see "Results"), we also tested UA- and TRG-independent variability for these variables by adding UA and TRGs as covariates in alternative models. The identity of the brood nested into the cage identity and the laboratory session were always maintained as random factors. We removed all nonsignificant terms ($P > 0.05$) in a backward stepwise procedure to obtain the best fit model and residuals. The procedure was compared with the Akaike Information Criterion and always agreed. All the models used to obtain the residuals for blood variables are reported in the appendix (tables A1, A2; tables A1–A3 are available online).

Residuals for each blood variable were correlated separately for nestlings and adults. Residuals obtained from the models were always normally distributed. Hence, Pearson's correlation coefficients were used. To attain a similar power for detecting significant correlations among individuals, only those birds reporting data for each blood variable were tested. Significant results were checked for potential outliers. One nestling and five adults (outliers for different parameters) were removed. This also implied the removal of the full data set for the outlier to maintain an equal statistical power among birds. The elimination of outliers did not alter the main results. Final sample sizes were 169 nestlings and 122 adults. Principal component analyses did not provide additional information on connections established from bivariate analyses. A contingency table was analyzed to know whether the number of significant correlations as well as those showing a trend toward significance ($P < .10$) was higher in some age group. The statistical power of the tests was analyzed by using GPOWER freeware (ver. 3.1.6; Faul et al. 2009), following Cohen's (1992) conventions.

Results

Correlational Study

GSH : GSSG ratios and tGSH levels were strongly and positively correlated at both ages. We must in any case consider that GSSG and tGSH were not independent variables, as the first is determined after tGSH quantification (see "Methods").

Taking this into account, 10 other significant correlations were detected at the nestling stage (table 1; fig. 1). Carotenoid levels were negatively correlated with tGSH. A trend toward a significant negative relationship between plasma carotenoid levels and GSH : GSSG ratios was also found. The levels of MDA in RBC and plasma were not significantly correlated. RBC MDA was positively correlated with AOX. UA was positively correlated with AOX, as expected, but it was also positively correlated with TRG and RBC MDA levels. Moreover, plasma MDA was strongly correlated with TRGs, which in turn was positively correlated with carotenoids and AOX. Finally, TRG-controlled plasma MDA levels showed a trend toward a negative significant link to tGSH levels ($P = 0.063$).

In adulthood, the number of significant links decreased. Adults again showed a strong, significantly positive relationship between GSH : GSSG and tGSH values and between AOX and UA (table 1). TRGs were, again, positively correlated with plasma MDA and UA. Plasma MDA was positively linked to both UA and tGSH, although the latter showed only a trend (table 1, right side; $P = 0.057$) that became significant after controlling for TRGs ($P = 0.016$; fig. 2).

Correlation coefficients with the same sign were observed when testing control birds only (i.e., 77 nestlings and 57 adults), although three tests in nestlings (AOX vs. RBC MDA, AOX vs. TRGs, and carotenoids vs. TRGs) and two tests in adults (UA vs. plasma MDA and UA vs. TRGs) lost their significance (figs. 1, 2; $P > 0.10$ for all).

Individual consistency between measures of AOX, UA-independent AOX, and TRGs at both ages was detected (all positively correlated: respectively $r = 0.26$, $P = 0.022$; $r = 0.38$, $P = 0.001$; and $r = 0.34$, $P = 0.003$). A trend toward a significant

Table 1: Pearson's correlation tests for blood variables in nestling (left side) and adult (right side) zebra finches

	RBC GSH : GSSG		RBC tGSH		RBC MDA		Plasma MDA		Plasma TRGs		Plasma TRG-indep MDA		Plasma AOX		Plasma UA-indep AOX		Plasma UA		Plasma carotenoids	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*
RBC GSH : GSSG	...	...	**.658**	**<.001**	−.068	.458	.062	.500	−.074	.417	.104	.256	.096	.292	.092	.311	.043	.637	.102	.265
RBC tGSH	**.450**	**<.001**	...	...	−.079	.388	.173	.057	.031	.736	**.218**	**.016**	−.066	.473	−.008	.933	−.076	.408	.008	.929
RBC MDA	.100	.197	.092	.234	...	...	−.076	.405	−.064	.483	−.043	.638	.120	.189	.144	.114	.079	.388	−.060	.510
Plasma MDA	.003	.974	−.045	.561	.012	.878	...	...	**.532**	**<.001**	**.810**	**<.001**	.100	.273	−.106	.246	**.209**	**.021**	.066	−.471
Plasma TRGs	.098	.214	.112	.148	.038	.622	**.428**	**<.001**	...	...	.023	.800	.099	.280	−.121	.183	**.211**	**.020**	.018	.845
Plasma TRG-indep MDA	−.055	.477	−.143	.063	.005	.945	**.852**	**<.001**	−.059	.443	...	...	.068	.456	−.012	.892	.097	.289	−.066	.471
Plasma AOX	.010	.897	.024	.762	**.175**	**.023**	.121	.117	**.157**	**.042**	.023	.767	...	...	**.551**	**<.001**	**.824**	**<.001**	.019	.839
Plasma UA-indep AOX	−.034	.665	.001	.991	.021	.788	.024	.755	−.123	.111	.079	.305	**.708**	**<.001**	...	...	.017	.853	.045	.625
Plasma UA	.053	.490	.011	.883	**.215**	**.005**	.137	.075	**.348**	**<.001**	−.052	.498	**.699**	**<.001**	.005	.951	...	...	−.005	.959
Plasma carotenoids	−.130	.093	**−.183**	**.017**	.063	.416	.122	.114	**.182**	**.018**	.047	.541	.080	.301	−.003	.964	.119	.124	...	...

Note. Data are residuals obtained from models controlling for different variables (see "Methods"). Significant tests ($P < 0.05$) are in boldface. For nestlings, $N = 169$; for adults, $N = 122$. For raw data showing concentrations, see table A1 (available online). AOX = nonenzymatic antioxidants, GSH : GSSG = reduced versus oxidized glutathione ratio, indep = independent, MDA = malondialdehyde, RBC = red blood cell, tGSH = total glutathione, TRG = triglyceride, TRG-indep MDA = MDA values corrected for TRG variability, UA = uric acid, UA-indep AOX = AOX values corrected for UA variability.

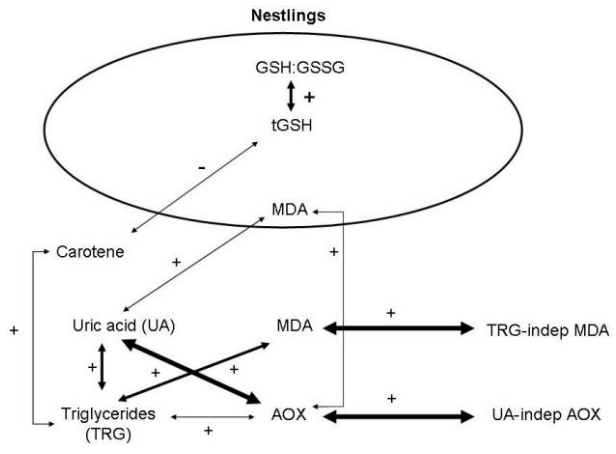

Figure 1. Significant correlations between markers of oxidative stress, nitrogen, and lipid metabolites in the blood of nestling zebra finches. Variables in the circle represent those analyzed in the erythrocyte fraction, whereas those outside the circle were assessed in plasma. The thickness of the arrows is proportional to Pearson's coefficient (table 1). AOX = nonenzymatic antioxidants, GSH : GSSG = reduced versus oxidized glutathione ratio, MDA = malondialdehyde, tGSH = total glutathione, TRG-indep MDA = MDA values corrected for TRG variability, UA-indep AOX = AOX values corrected for UA variability. Also see "Methods."

positive correlation was also found in UA ($r = 0.20$, $P = 0.087$). Other variables were not correlated ($P > 0.10$ for all).

Differences in the Number of Links between Data Sets

The nestling data set reported a higher number of tests showing significance or a trend toward significance (nestlings: $n = 14/45$, 31%; adults: $n = 9/45$, 20%; $\chi^2 = 17.52$, $P < .001$).

Power Analyses

The nestling data set ($N = 169$) provided very high power (>97.9%) to detect medium or large effect sizes ($r \geq 0.3$; i.e., Cohen 1992), although it provided low power (25.4%) to detect small effects ($r \leq 0.1$). Similarly, the adult data set ($N = 122$) provided high power (>92.4%) to detect medium or large effect sizes and low power (19.5%) to detect small effects. When testing control nestlings ($n = 77$) or individual consistency ($n = 78$), the conventional 80% power to detect medium effect sizes was almost attained (>76%–77%), but power to detect small effects was only 14%. Finally, medium (54.5% power) and small (10%) effect sizes were poorly detected when testing control adults only.

Discussion

A number of links among oxidative stress markers and metabolites were detected. In nestlings, RBC tGSH levels were negatively correlated with plasma carotenoid values, a connection that disappeared in adults. Plasma levels of parameters associated with lipid and protein metabolism (TRGs and UA, respectively) were correlated with plasma lipid peroxidation (MDA) and AOX, in that order, showing strong links at both ages. Other significant connections were also revealed. Individual consistency in AOX, UA-independent AOX, and TRG values was also observed. In cases were no significant relationship was found, we can at least conclude that no strong links (medium or large effect sizes) were indeed present. It should be considered that 783 samples would have been required to detect small effects (sensu Cohen et al. 1992).

A higher number of links were detected in nestlings. This may be due to (1) a higher (though not so different) statistical power in the nestling data set (see "Results"), (2) the fact that the nestling data set would include nestlings enduring oxidative challenges that will not survive to adulthood (i.e., mortality would have reduced variability among adults), and/or (3) a higher pressure of oxidative stress on the antioxidant machinery during development (see below).

Following the order in table 1, we can first note that GSH : GSSG values were strongly and positively correlated with tGSH levels. This is quite obvious, as the ratio is constructed from tGSH. In turn, tGSH was negatively correlated with plasma carotenoid levels only among nestlings. The medical literature shows that carotenoid supplementation in the diet increases tGSH levels in mammalian tissues, including blood (e.g., Imamura et al. 2006; Kamiloglu et al. 2006; Serpeloni et al. 2010; Gao et al. 2011). However, in greenfinches (*Carduelis chloris*; Sepp et al. 2012) and zebra finches (Costantini 2011, 2013) significant correlations between plasma carotenoid levels and RBC tGSH or total thiols (a proxy of tGSH) were not detected. Nonetheless, the sample sizes in the two studies were smaller than those here used, and their analyses did not include nestlings. Also in adult greenfinches, experimental inhibition

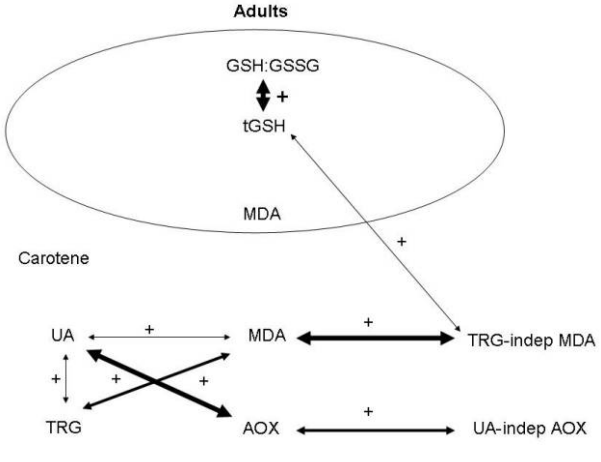

Figure 2. Significant correlations between markers of oxidative stress, nitrogen, and lipid metabolites in the blood of adult zebra finches. Variables in the circle are those analyzed in the erythrocyte fraction, whereas those outside the circle were assessed in plasma. The thickness of the arrows is proportional to Pearson's coefficient (table 1). Abbreviations are the same as in figure 1.

of tGSH synthesis did not significantly affect circulating carotenoid levels, and carotenoid supply in the diet did not influence RBC tGSH (Hõrak et al. 2010). On the contrary, in developing red-legged partridges (*Alectoris rufa*) whose oxidative stress was manipulated by adding a prooxidant compound (diquat), both RBC tGSH and plasma carotenoid levels declined (Galván and Alonso-Alvarez 2009; Alonso-Alvarez and Galván 2011). Thus, some experimental works in different species may support a positive relationship between these variables. Our correlational analyses, in contrast, show the opposite. They suggest that growing birds underwent a sort of balance between plasma and erythrocyte antioxidants to maintain redox status. We must remember that higher oxidative stress during growth is predicted and supported by several studies (reviewed in Metcalfe and Alonso-Alvarez 2010). Accordingly, our results and avian studies (see above) suggest that the integration of carotenoids in the antioxidant system would be stronger for nestlings. A higher carotenoid availability due to food intake or mobilization from liver stores (McGraw 2006) might have allowed a reduction of tGSH synthesis in erythrocytes among growing birds.

The results also support a general implication of carotenoids in the avian antioxidant machinery, an idea previously questioned (Costantini and Møller 2008; Isaksson et al. 2008). In adult zebra finches, nonetheless, Costantini et al. (2013) have recently described negative correlations between plasma values of carotenoids and two markers of oxidative damage (protein carbonyls and hydroperoxides), which suggests that carotenoids were bleached by oxidation and/or involved in antioxidant defense (i.e., Hartley and Kennedy 2004; see also Simons et al. 2012 and Gao et al. 2013 for some new support for the role of carotenoids as antioxidants). In our nestling and adult zebra finches, significant links between carotenoids and markers of oxidative damage were, however, not detected.

Costantini et al. (2011, 2013) also found a negative correlation between total thiols in RBCs and a measure of plasma antioxidant status (termed as OXY; Costantini et al. 2011). We did not find a similar link when testing RBC tGSH and AOX, but, in addition to age-related differences (see above), this could be due to differences in procedures. The OXY variable represents the capacity of antioxidants to cope with the in vitro prooxidant activity of hypochlorous acid (HOCl) and is apparently independent from UA variability (Costantini 2011; but see Alan and McWilliams 2013).

The correlation between plasma MDA values and TRG levels was highly significant at both nestling and adult ages. Some authors have argued that plasma MDA levels are influenced by diet, as MDA is also present in food (Halliwell and Gutteridge 2007 and references therein). Nevertheless, MDA may also be influenced by the amount of circulating lipids susceptible to oxidation. Biomedicine has focused on this from the perspective that a high-fat diet is responsible for a number of modern lifestyle diseases. Hypertriglyceridemia has been associated with atherosclerosis, higher circulating levels of those lipoproteins more vulnerable to oxidation (low-density lipoproteins [LDLs]), and higher levels of serum MDA-modified LDLs (Kondo et al. 2001; Tanaga et al. 2002). A positive correlation between TRG and MDA levels in plasma has also been described in those with diabetes (Griesmacher et al. 1995; Lee 2001; Vidya et al. 2011), diabetes being in turn linked to atherosclerosis and other vascular disorders (DeFronzo and Ferrannini 1991).

For medical researchers, the correlation between plasma TRG and MDA levels merely reveals a lipid-mediated risk factor for vascular diseases (e.g., Rumley et al. 2004) but no problem in interpreting plasma MDA variability. Lipid-controlled MDA variability could, however, provide substantially different information, likely not reflecting recent lipid intake. Indeed, when plasma MDA was controlled for TRG levels a significant positive correlation with RBC tGSH arose in adults (fig. 2). This suggests some compensatory mechanism (Calabrese 2007; Costantini et al. 2010) in RBCs against oxidative insults originating in other parts of the organism. Such a hormetic response could require the maturation of antioxidant mechanisms, which may explain the trend toward a contrary relationship between the same parameters in nestlings ($P = 0.063$). Accordingly, studies in other taxa have shown that GSH metabolism takes some time to develop (Metcalfe and Alonso-Alvarez 2010 and references therein).

TRG levels in blood may not only be influenced by recent intake but by mobilization of lipid stores as well. In birds, plasma TRG and UA values are inversely related during starvation because the body fat is mobilized as much as the protein stores (muscles) are preserved, and vice versa (Jenni-Eiermann and Jenni 1994; Alonso-Alvarez and Ferrer 2001). However, in our study TRG and UA levels were positively correlated at both ages. Since plasma UA levels in birds increase a few hours after feeding (Okumura and Tasaki 1969; Voss and Siems 2006), our results suggest that TRG values were influenced by recent intake. Nonetheless, individual consistency with age in both TRG and UA levels also suggests that some variability in these parameters was intrinsic. Along these lines, hereditary hypertriglyceridemia and hyperuricemia have been described in poultry (Cole and Austic 1980; Cho et al. 1987). In adult greenfinches, Sepp et al. (2012) showed individual consistency in AOX (similar to our results), RBC tGSH, and carotenoids but not in UA (TRGs were not assessed), although only 30 birds over an interval of a few days were studied.

That TRGs were uncorrelated with RBC MDA also suggests that the plasma MDA measure is more sensitive to circulating lipids than cell-based measures. Furthermore, RBC and plasma MDA values were unrelated, indicating that the same marker of oxidative damage to lipids could provide different information. Variability in RBC MDA should indicate damage to the cell membrane of erythrocytes, whereas plasma MDA should represent damage to lipids arriving from any tissue, intestinal absorption (diet), or fat stores.

AOX (also trolox-equivalent antioxidant capacity or total antioxidant status) mostly represent the state of hydrophilic antioxidants (see Cao and Prior 1998). This may explain the lack of correlation between AOX and lipophilic carotenoids (i.e., xanthophylls) and the strong influence of hydrophilic UA (Cohen et al. 2007; Costantini 2011). UA is a powerful anti-

oxidant (Halliwell and Gutteridge 2007), although it is unknown whether plasma UA levels are actively upregulated in the face of oxidative stress (Sautin and Johnson 2008). As mentioned, plasma UA concentration increases quickly after feeding and during fasting (Okumura and Tasaki 1969; Alonso-Alvarez and Ferrer 2001). Therefore, physiological factors not strictly related to the antioxidant response may explain a large part of AOX variability. Nevertheless, RBC MDA was positively correlated with plasma UA in nestlings, supporting an active mobilization of UA under situations of high oxidative stress, such as those associated with growth (Metcalfe and Alonso-Alvarez 2010 and references therein).

Other significant correlations support a role for recent intake. TRGs were positively linked to both AOX and carotenoids in the nestling plasma. The first link disappeared when AOX was corrected for UA, the latter likely being influenced by recent feeding. On the other hand, carotenoids are only obtained from the diet (McGraw 2006), and commercial food contains carotenoids. Since carotenoids are lipophilic compounds, high plasma TRG levels could be a consequence of high amounts of dietary lipids that could in turn favor carotenoid absorption by intestines (Surai 2002). Similarly, in adults the positive correlation between plasma UA and MDA became nonsignificant when MDA was controlled for TRGs.

The links described here reveal the complexities of the antioxidant system. They suggest that no universal marker of oxidative stress can convincingly be obtained, and discussions on results based on such markers must be carefully interpreted. Results also point to a relevant influence of the recent intake on values of some blood markers. This presents the question of the meaning of corrected or uncorrected values for variability in levels of nutritional metabolites. In the case of MDA, although apparently an end product of lipid peroxidation, it may still induce damage to DNA and proteins (Halliwell and Gutteridge 2007). Uncorrected MDA values would thus serve as an index of future damage, whereas TRG-corrected MDA may serve to estimate the level of damage that has already taken place. Experiments manipulating diet composition and oxidative stress are now required to disentangle, confirm, or reject the hypothesized causalities.

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Appendix from A. A. Romero-Haro and C. Alonso-Alvarez, "Covariation in Oxidative Stress Markers in the Blood of Nestling and Adult Birds"

(Physiol. Biochem. Zool., vol. 87, no. 2, p. 353)

Supplementary Tables

Table A1. Oxidative stress and metabolic parameters from nestling ($N = 169$) and adult ($N = 122$) zebra finches

	Mean	SE	Range
Nestlings:			
GSSG (μmol/g)	1.02	.27	.29–1.96
tGSH (μmol/g)	3.45	.10	1.64–15.17
RBC MDA (μM)	110.9	4.44	35.86–362.03
Plasma MDA (μM)	4.45	.12	2.07–11.28
Plasma TRGs (mg/dL)	278.6	7.76	42.02–527.44
Plasma AOX (mM)	.78	.02	.44–1.33
Plasma UA (mg/dL)	11.09	.29	4.51–21.56
Carotenoids (μg/mL)	6.06	.17	1.87–11.44
Adults:			
GSSG (μmol/g)	1.30	.22	.61–1.70
tGSH (μmol/g)	3.59	.06	2.21–5.25
RBC MDA (μM)	171.6	7.02	82.85–470.50
Plasma MDA (μM)	4.98	.21	1.85–14.09
Plasma TRGs (mg/dL)	230.8	6.84	64.83–493.35
Plasma AOX (mM)	.86	.02	.29–1.84
Plasma UA (mg/dL)	9.74	.34	2.76–22.01
Carotenoids (μg/mL)	7.76	.22	2.96–14.37

Note. Concentrations (raw data) were not used in correlation tests (see "Methods"). AOX = nonenzymatic antioxidants, GSSG = oxidized glutathione, MDA = malondialdehyde, RBC = red blood cell, tGSH = total glutathione, TRG = triglyceride, UA = uric acid.

Table A2. Generalized mixed models used to obtain residuals for blood variables of nestling zebra finches

	Treatment			Sex			Brood size			Laying date			Hatching order		
Dependent variable	*F*	df	*P*	*F*	df	*P*	*F*	df	*P*	*F*	df	*P*	*F*	df	*P*
GSH : GSSG	**8.69**	**1, 163**	**.003**	.51	1, 204	.475	**4.14**	**1, 116**	**.044**	1.53	1, 69	.221	**4.05**	**1, 158**	**.045**
tGSH	**15.79**	**1, 175**	**<.001**	.05	1, 208	.816	**4.17**	**1, 107**	**.043**	.53	1, 94.3	.466	.38	1, 168	.537
Cell MDA	**5.55**	**1, 159**	**.020**	.01	1, 193	.939	96.8	1, 1.27	.263	.26	1, 101	.613	.19	1, 155	.664
Plasma MDA	.28	1, 175	.600	**2.70**	**1, 216**	**.099**	**5.97**	**1, 104**	**.016**	**5.95**	**1, 99.2**	**.016**	1.82	1, 174	.179
TRGs	.35	1, 152	.556	.37	1, 186	.544	.00	1, 137	.970	1.07	1, 106	.303	**7.04**	**1, 182**	**.009**
TRG-indep plasma MDA	.35	1, 146	.557	.87	1, 188	.351	**5.96**	**1, 98.6**	**.016**	**4.06**	**1, 83.8**	**.047**	.15	1, 157	.704
AOX	.01	1, 164	.978	**4.35**	**1, 210**	**.038**	1.03	1, 125	.312	**6.08**	**1, 86.5**	**.015**	**7.49**	**1, 203**	**.006**
UA	.73	1, 164	.394	.53	1, 210	.465	1.61	1, 130	.206	**3.11**	**1, 92.5**	**.081**	**7.65**	**1, 201**	**.006**
UA-indep AOX	.81	1, 157	.370	**6.63**	**1, 197**	**.010**	.08	1, 127	.782	**3.35**	**1, 94.9**	**.070**	**3.12**	**1, 191**	**.079**
Carotenoids	**5.11**	**1, 168**	**.025**	.01	1, 200	.935	**2.65**	**1, 123**	**.098**	**13.76**	**1, 96.2**	**.001**	**8.93**	**1, 163**	**.003**

Note. Statistics for each factor and covariate in the final model (boldface) or at the last step before being removed ($P > 0.10$) are reported. Satterthwaite degrees of freedom (df) are reported. Uric acid (UA)–independent (indep) AOX also included UA as a covariate ($F_{1, 209} = 193.77$, $P < 0.0001$), whereas triglyceride (TRG)–independent plasma malondialdehyde (MDA) included TRGs ($F_{1, 204} = 62.44$, $P < 0.0001$). Brood identity nested into cage identity (*P* values: 0.001–0.212) and laboratory session (*P* values: 0.013–0.300) were always included as random factors. AOX = nonenzymatic antioxidants, GSH : GSSG = reduced versus oxidized glutathione ratio, tGSH = total glutathione.

Table A3. Generalized mixed models used to obtain residuals for blood variables of adult zebra finches

	Treatment			Sex			Brood size			Laying date			Hatching order			Age at sampling		
Dependent variable	*F*	df	*P*	*F*	df	*P*	*F*	df	*P*	*F*	df	*P*	*F*	df	*P*	*F*	df	*P*
GSH : GSSG	**7.44**	**1, 105**	**.007**	.03	1, 131	.870	.57	1, 66	.452	.26	1, 66	.611	.07	1, 99.3	.792	.01	1, 64	.913
tGSH	**4.64**	**1, 92.1**	**.033**	**4.76**	**1, 123**	**.031**	1.16	1, 86.2	.284	1.87	1, 127	.173	1.76	1, 108	.187	**4.13**	**1, 69**	**.045**
Cell MDA	**4.74**	**1, 132**	**.031**	.02	1, 127	.880	**4.61**	**1, 132**	**.033**	2.42	1, 132	.122	.31	1, 128	.576	2.00	1, 132	.160
Plasma MDA	**12.69**	**1, 96.8**	**.001**	**12.69**	**1, 96.8**	**.001**	2.08	1, 60.6	.154	2.32	11, 23.3	.141	.36	1, 71.1	.551	.22	1, 73.3	.641
TRGs	.41	1, 95.2	.521	**4.26**	**1, 122**	**.041**	.72	1, 73.5	.397	**3.29**	**1, 6.73**	**.098**	.12	1, 104	.731	**4.67**	**1, 15.5**	**.046**
TRG-indep plasma MDA	.44	1, 59.7	.510	**6.86**	**1, 88.4**	**.010**	1.84	1, 55.3	.180	1.07	1, 66.3	.304	.43	1, 58.4	.514	.01	1, 67.9	.909
AOX	.52	1, 135	.470	0.17	1, 130	.678	.01	1, 127	.994	.13	1, 102	.715	.32	1, 133	.574	.23	1, 51.9	.631
UA	.13	1, 129	.720	0.25	1, 130	.621	.72	1, 131	.396	.01	1, 128	.922	1.66	1, 132	.200	.04	1, 126	.837
UA-indep AOX	.17	1, 98.9	.678	**3.71**	**1, 126**	**.056**	.51	1, 73.8	.478	.48	1, 81.3	.491	.70	1, 103	.405	1.66	1, 118	.200
Carotenoids	.32	1, 114	.571	**5.04**	**1, 139**	**.026**	.18	1, 107	.669	**5.04**	**1, 139**	**.026**	**3.68**	**1, 139**	**.057**	**7.67**	**1, 145**	**.006**

Note. Statistics for each factor and covariate in the final model (boldface) or at the last step before being removed ($P > 0.10$) are reported. Satterthwaite degrees of freedom (df) are reported. Uric acid (UA)–independent (indep) AOX also included UA as a covariate ($F_{1, 125} = 284.21$, $P < 0.0001$), whereas triglyceride (TRG)–independent plasma malondialdehyde (MDA) included TRGs ($F_{1, 115} = 52.40$, $P < 0.0001$). Brood identity nested into cage identity (*P* values: 0.010–0.382) and laboratory session (*P* values: 0.018–0.473) were always included as random factors. AOX = nonenzymatic antioxidants, GSH : GSSG = reduced versus oxidized glutathione ratio, tGSH = total glutathione.