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RESEARCH ARTICLE

Biochemical integration of blood redox state in captive zebra finches (*Taeniopygia guttata*)

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SUMMARY

Integration is a property of biological systems that refers to the extent to which their components are correlated through functional, structural, developmental or evolutionary interdependency. This study examined patterns of functional integration among different molecular components of the blood redox system (both plasma and red blood cells) in zebra finches (*Taeniopygia guttata*). Our results show a two cluster organization of the six measured variables: one cluster includes glutathione peroxidase in both red blood cells and plasma, thiol concentration in red blood cells and plasma hydroperoxides; the other cluster comprises a measure of the non-enzymatic antioxidant capacity in red blood cells and plasma. The interaction network amongst these variables shows (i) a strong positive connectivity among hydroperoxides, glutathione peroxidase and thiols, and (ii) negative connectivity between non-enzymatic and enzymatic antioxidants. Overall, our results also suggest strong and significant integration between the oxidative state of red blood cells and plasma.

Key words: oxidative stress, vertebrates, oxidative damage, antioxidants, modularity, network.

INTRODUCTION

All biological systems exhibit an integration of morphological, metabolic, biochemical and/or genetic components, such that these are correlated through functional, structural, developmental or evolutionary interdependency (Klingenberg, 2007; Klingenberg, 2008; Ravasz et al., 2002; Mitteroecker and Bookstein, 2007; Bruner et al., 2010). The degree of integration is mostly inferred from the analysis of correlation or covariance matrices of multiple variables (Schlosser and Wagner, 2004; Mitteroecker and Bookstein, 2007; Klingenberg, 2008). Under this analytical framework, the strength of the correlations among measured variables reflects their degree of interdependency; it also potentially reveals the existence of semi-independent units, called modules, so helping us to understand how such systems are regulated and controlled (Cheverud, 1996; Mitteroecker and Bookstein, 2007; Klingenberg, 2008).

The biochemical machinery offering protection against oxidation of body tissues involves a large number of molecular components and pathways. It is the collective action of these defence systems that protects cells against oxidative damage caused by reactive chemical species, so avoiding increases in oxidative stress (Sies, 1991; Halliwell and Gutteridge, 2007; Costantini and Verhulst, 2009). Redox mechanisms have traditionally been studied by means of *in vitro* or *ex vivo* systems, which focus on the description of specific components, often in isolation from each other. However, the effectiveness of the contribution of one antioxidant component to protection is not totally independent of that of other components, as a variable degree of biochemical integration may be observed among different antioxidants (Halliwell and Gutteridge, 2007). Moreover, antioxidants may react with one or more kinds of pro-oxidants, depending on how specific they are (Halliwell and Gutteridge, 2007). The degree of specificity of an antioxidant will also therefore determine the level of integration between particular pro-oxidants and antioxidants. Quantification of the strength of this integration could provide information on the functional or developmental relationships among these components, as well as on whether different components of the antioxidant defences have followed common or independent evolutionary trajectories.

The relationships between different antioxidants could influence the efficiency and effectiveness of their response to pro-oxidants. For example, if two hypothetical antioxidants A and B strongly depend on each other, an impaired functioning of A reduces the efficacy of B, generating a constraint on the antioxidant response itself. This kind of interdependency might limit the rate of evolutionary change, and could be a constraint to phenotypic plasticity as suggested by several authors for other traits (Schlichting, 1986; Pigliucci et al., 1995; Gianoli, 2001), with selection acting to optimize the functioning of the whole system rather than of a single component.

In addition to its intrinsic value, knowledge of the connectedness and integration of the antioxidant defence system might be very useful because it might facilitate selection of the most suitable sets of redox biomarkers that provide complementary rather than redundant information on the oxidative balance of a particular study system. We therefore measured six molecular biomarkers (three each in both plasma and red blood cells) in the zebra finch (*Taeniopygia guttata*) in order to (1) describe the patterns of functional integration among different molecular components of the blood redox system, (2) define the integration between the redox state of plasma and that of red blood cells (RBCs), (3) quantify the interaction network of the connections among components of the redox state, and (4) produce a subset of biomarkers that provide a comprehensive measure of the blood redox state.

MATERIALS AND METHODS

Sampling

All experimental work was conducted under UK Home Office Project Licence PPL 60/3447 and 60/4109. A sample of blood was taken from the brachial vein of 78 subadult zebra finches *Taeniopygia guttata*, Vieillot 1817 (43–45 days old) by venipuncture, and collected using microhaematocrit heparinized capillary tubes (Vetlab Supplies Ltd, Broomers Hill Park, Pulborough, West Sussex, UK). Blood samples were maintained on ice and then centrifuged to separate plasma from RBCs. Body mass (0.01 g) of the birds was measured using an electronic balance, and tarsus length (0.01 mm) was measured with a digital calliper. The samples of plasma and RBCs were snap frozen and stored at –70°C. Laboratory analyses were carried out within 1 month of collection. In addition to biomarkers of blood redox state, we measured total proteins in both plasma and RBCs, and expressed values of biomarkers per mg of proteins occurring in the matrix if the uncorrected biomarker correlated positively with the protein concentration. This may be important for several reasons: (i) oxidation of proteins increases the level of hydroperoxides, so a high protein content may lead to a high level of hydroperoxides; (ii) many kinds of proteins have functional thiol groups, so protein levels could influence thiol levels; (iii) a high protein availability may be associated with a high synthesis of enzymes.

Laboratory analyses

Plasma hydroperoxides

Plasma hydroperoxides (compounds produced in the early phases of the oxidative cascade) were measured by colorimetric determination using the d-ROMs test (Diacron International, Grosseto, Italy). The procedure was carried out as in previous studies (Costantini and Dell'Omo, 2006; Costantini et al., 2008) with some slight modifications. Preliminary analyses showed the presence of some deposit (e.g. lipids) on the bottom of wells. Therefore, incubation of the reaction was done in small tubes; after 75 min of incubation at 37°C, the solutions were centrifuged (13,000 r.p.m. for 2 min) and the supernatant (190 µl) pipetted into the well of a fresh microplate. The same procedure was applied to the reference standard and blank. The absorbance was read with a Thermo Scientific Multiskan Spectrum (ThermoFisher, Vantaa, Finland) at a wavelength of 505 nm. Measurements are expressed as mmol l^{-1} of H_2O_2 equivalents. Analyses were run in duplicate and the mean coefficient of variation was 4.48%.

Plasma and RBC antioxidant capacity

The OXY-Adsorbent test (Diacron International) quantifies the ability of antioxidant compounds present in a solution to cope with the *in vitro* oxidant action of hypochlorous acid (HOCl; an endogenously produced oxidant). The procedure was carried out as in previous studies (Costantini and Dell'Omo, 2006; Costantini et al., 2008) (see also Costantini, 2010) with some slight modifications. Plasma and RBC samples were diluted 1:100 and 1:800 with distilled water, respectively. Then RBC solutions were centrifuged at 3000 r.p.m. for 1 min in order to remove any residues derived from RBC lysis. A 200 µl aliquot of HOCl solution was incubated with 2 µl of the diluted plasma or RBC for 10 min at 37°C. The same relative volumes were used for the reference standard and blank (i.e. water). At the end of the incubation, 2 µl of the chromogen *N*,*N*-diethyl-*p*-phenylenediamine was added. An alkyl-substituted aromatic amine dissolved in the chromogen is oxidized by the residual HOCl and transformed into a pink derivative. The intensity of the coloured complex is inversely related to the antioxidant capacity of the sample. The absorbance was read with the Multiskan Spectrum at a wavelength of 490 nm. Measurements are expressed as mmol l^{-1} of HOCl neutralized according to the following formula:

$$\frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}} - A_{\text{std}}} \times OXY_{\text{std}} , \quad (1)$$

where A indicates absorbance and std the reference standard. Similar results were obtained using a calibration curve as a reference. Analyses were run in duplicate and the mean coefficient of variation was 4.02 and 5.80% for plasma and RBC OXY (non-enzymatic antioxidant capacity), respectively.

Plasma and RBC activity of glutathione peroxidase

The activity of glutathione peroxidase (GPX) in plasma and RBCs was quantified using the Ransel assay (Randox Laboratories, Crumlin, UK). This assay is based on the original method of Paglia and Valentine (Paglia and Valentine, 1967). The principle of the assay is that GPX present in the sample catalyses the oxidation of glutathione (GSH) by cumune hydroperoxide. In the presence of glutathione reductase and NADPH the oxidized glutathione is then converted to the reduced form with a concomitant oxidation of NADPH to $NADP^+$. This generates a decrease in absorbance that is followed for 3 min by reading at 340 nm. Both plasma and RBC samples were diluted 1:40 with diluting agent provided with the assay. RBC solutions were centrifuged at 3000 r.p.m. for 1 min in order to remove any residues derived from RBC lysis. Then, 200 µl of reagent (glutathione 4 mmol l^{-1}; glutathione reductase ≥0.5 U l^{-1}; NADPH 0.34 mmol l^{-1}), previously reconstituted with buffer (PBS 0.05 mol l^{-1}, pH 7.2; EDTA 4.3 mmol l^{-1}), was pipetted into a well. Subsequently 4 µl of sample and 8 µl of cumene hydroperoxide were added, and absorbance was read at 340 nm after 1, 2 and 3 min using the Multiskan Spectrum. The temperature inside the plate reader was maintained at 37°C. Absorbance of the reagent blank was subtracted from that of the sample. GPX concentration was calculated using the following formula: $(\Delta A\,\text{m}^{-1})\times 15{,}873$. Values are expressed as U GPX l^{-1} of plasma or U GPX l^{-1} of haemolysate. Analyses were run in duplicate and the mean coefficient of variation was 5.54 and 4.96% for plasma and RBC GPX, respectively.

RBC thiols

The concentration of total thiols (e.g. glutathione, thioredoxin) present in RBCs was quantified using the –SHp test (Diacron International). RBCs were first diluted 1:200 with distilled water. Then samples were centrifuged at 3000 r.p.m. for 1 min in order to remove any residues derived from RBC lysis. Then 250 µl of a buffer phosphate solution (pH 7.6) was pipetted into the well of a microplate and 12.5 µl of the haemolysate was added. The absorbance was read with the Multiskan Spectrum at a wavelength of 405 nm. This absorbance was then subtracted from the absorbance of the same sample read at 405 nm after 5 min of reaction with 5 µl of 5,5′-dithiobys-2-nitrobenzoic acid (DTNB; chromogen). The DTNB reacts with the thiols present in the solution, generating a coloured complex whose intensity is directly proportional to the concentration of thiols. Thiol concentrations were calculated using a standard solution of L-cysteine purchased with the kit (Diacron International) and were expressed as µmol l^{-1} of –SH groups. Analyses were run in duplicate and the mean coefficient of variation was 7.55%.

Plasma and RBC proteins

Proteins were measured using the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Plasma and RBC samples were

diluted 1:60 and 1:120, respectively. RBC solutions were centrifuged at 3000 r.p.m. for 1 min in order to remove any residues derived from RBC lysis. Then 5 µl of each sample, reference standard (bovine serum albumin) or reagent blank (water) was pipetted into the well of a microplate and 200 µl of Bradford dye reagent was added. The plate was incubated at room temperature and absorbance was read after 10 min using the Multiskan Spectrum. Protein concentration was expressed as mg protein ml^{-1} of plasma or mg protein ml^{-1} of haemolysate. Analyses were run in duplicate and the mean coefficient of variation was 4.78% for plasma proteins and 1.84% for RBC proteins.

Statistical analyses

To avoid pseudoreplication, a mean value for brood (N=31) was used for each variable in all analyses. Correlation matrices based on individual values were, however, extremely similar to those based on brood mean values, as shown by the significant high correlation between them (Mantel test, r=0.89, P=0.0028). Preliminary bivariate correlational analyses were performed to evaluate whether values of each redox biomarker correlated with protein levels and so whether they needed to be expressed per mg protein present in the sample. Bivariate correlational analyses were also performed to evaluate whether values of each biomarker varied with sampling hour, body mass or tarsus length of the individual. The only significant correlations were between RBC proteins and RBC GPX (r=0.404, P=0.012), sampling hour and plasma OXY (r=–0.523, P=0.001), and tarsus length and plasma GPX (r=0.461, P=0.005). Therefore in the next analyses, we expressed RBC GPX activity as U GPX mg^{-1} protein ml^{-1} haemolysate. Plasma OXY and plasma GPX values were included as residuals of a linear regression on sampling hour and tarsus length, respectively. Fitting a quadratic curve did not improve the relationships between any of the variables (data not shown). Correlation matrices including original or transformed variables were, however, not significantly different. We used correlation matrices because the variables were measured in different units, so there was no need to standardize the variables (Jolliffe, 2002).

We then performed the Kaiser–Meyer–Olkin (KMO) test of sampling adequacy and Bartlett's test of sphericity to evaluate whether the dataset was appropriate for factor analysis. The KMO test quantifies whether the partial correlations among variables are strong enough (≥0.5) to support the use of factor analysis. Bartlett's test evaluates the null hypothesis that the correlation matrix is an identity matrix (i.e. each variable correlates perfectly with itself, but has no correlation with the other variables) – rejection of the null hypothesis indicates that the variables are sufficiently interrelated to justify factor analysis. Given that both tests satisfied the criteria (KMO test=0.60; Bartlett's test=42.25, P<0.0001), a principal components approach to factor analysis (version 15, SPSS Inc., Chicago, IL, USA) was used to quantify the degree and sign of association among variables. We used an Equimax rotation with Kaiser normalization, which minimizes the number of variables that load highly on a factor and the number of factors needed to explain a variable, although similar results were obtained using the Varimax method (data not shown). To determine how redox variables clustered with each other, cluster analysis (unweighted pair-group method with arithmetic average; UPGMA) was performed on the correlation matrix using PAST version 1.94 (Hammer et al., 2001). A bootstrap analysis with 1000 replications was performed to evaluate the robustness of each node of the generated tree, while a cophenetic correlation coefficient was calculated as a measure of how faithfully the dendrogram preserved the pairwise distances between the original data points.

Table 1. Loadings of variables onto the first two principal components

Variable	PC1	PC2
RBC GPX	**0.800**	–0.243
Plasma GPX	**0.790**	–0.200
RBC thiols	**0.697**	–0.002
Plasma hydroperoxides	**0.579**	0.418
RBC OXY	0.034	**0.834**
Plasma OXY	–0.362	**0.794**

The highest loadings are shown in bold type.
RBC, red blood cell; GPX, glutathione peroxidase; OXY, non-enzymatic antioxidant capacity; PC, principal components.

A two-block partial least squares regression was then performed using PAST to test the covariance (i.e. integration) between the redox status of the plasma and that of the RBCs, using linear combinations of the three measures for each matrix (OXY, GPX and hydroperoxides for plasma, and OXY, GPX and thiols for RBCs). Finally, a network diagram was generated using the correlation matrix. Each variable was represented by a node and each link between two nodes represented the biochemical interaction between them. The aim of the network is to visualize whether two variables A and B are directly connected to each other or connected through a third variable C, but it is not meant to define the direction of the interaction.

RESULTS

The principal components analysis (PCA) shows that the combination of the first two axes explained 63.7% of the total variance. PC1 and PC2 had eigenvalues of 2.4 and 1.4, respectively, while all the other components had eigenvalues less than 1 and explained little of the variation, so were not considered further. PC1 (39.9% of the total variance) was associated with a high and positive loading of plasma hydroperoxides, RBC thiols, plasma GPX and RBC GPX (Table 1). In contrast, PC2 (23.8% of the total variance) had a high and positive loading of RBC OXY and plasma OXY (Table 1).

In agreement with the PCA, UPGMA cluster analysis identified two clusters of variables (Fig. 1): one cluster included RBC GPX, plasma GPX, RBC thiols and plasma hydroperoxides while the

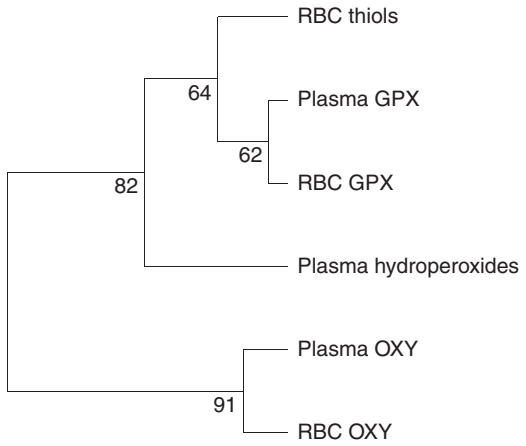

Fig. 1. Dendrogram generated by cluster analysis (unweighted pair-group method with arithmetic average; UPGMA) performed on the correlation matrix. The percentage of replicates where each node was still supported is given on the dendrogram (bootstrap with 1000 replicates). RBC, red blood cell; GPX, glutathione peroxidase; OXY, non-enzymatic antioxidant capacity.

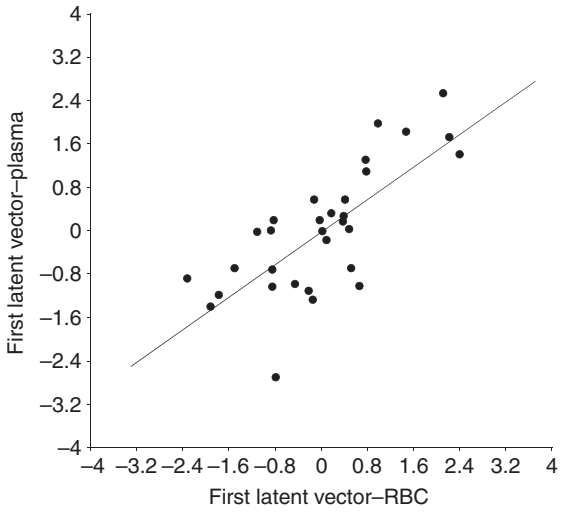

Fig. 2. Two-block partial least squares regression showing the covariation between linear combinations of the variables reflecting the redox status of plasma (N=3 variables) and RBCs (N=3 variables), respectively.

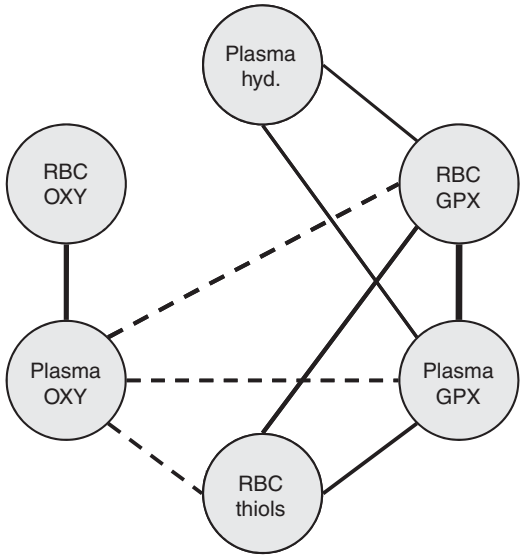

Fig. 3. Network model drawn to show the connections between the six redox variables measured in the blood of young zebra finches. Each node of the network refers to one variable. Two nodes are connected when significantly correlated. The thickness of the link is directly proportional to the coefficient of correlation (range: 0.29 to 0.63 and –0.33 to –0.39 for the positive and negative correlations, respectively) Continuous and dashed lines indicate positive and negative correlations, respectively. Each link is assumed to mirror a reciprocal biochemical interaction between the two connected nodes (hyd., hydroperoxides).

second included RBC OXY and plasma OXY. Bootstrap analysis showed a high level of robustness of each node of the generated tree, in particular for the separation of the two main clusters, while the nodes within the GPX–thiols–hydroperoxides cluster were also supported (Fig. 1). The cophenetic correlation coefficient of the dendrogram was 0.948.

The partial least squares regression between first latent vectors summarizing the redox status of plasma and RBCs (based on the three measurements per matrix) was highly significant, explaining 88.6% of the covariance between the two sets of variables (Fig. 2). The oxidative balance of the two blood compartments therefore varied in parallel between individuals.

The redox network (Fig. 3) showed a good connectivity amongst the measured variables, having a total of 9 of the maximum 15 potential links among nodes. The mean number of connections for each node was k=3. RBC GPX, plasma GPX and plasma OXY had the highest number of nodes (k=4 each), while RBC OXY was the least connected node (k=1). The network shows that the two nodes representing the concentration of the GPX enzyme in both plasma and RBCs were positively connected to hydroperoxides and thiols. Non-enzymatic antioxidant activity in the plasma (plasma OXY) was positively connected to that in RBCs, but negatively connected to RBC GPX, plasma GPX and RBC thiols.

DISCUSSION

Our data show that, in subadult zebra finches, blood levels of GPX, thiols and hydroperoxides are highly integrated, suggesting that levels of GPX and thiols are upregulated on demand with an increase in circulating hydroperoxides. A separate component of the antioxidant defence system in the blood is formed by the non-enzymatic antioxidants, with the capacity of these to cope with HOCl being positively correlated in plasma and RBCs. The network diagram (Fig. 3) further shows that the activity of GPX is directly connected to the level of hydroperoxides, while levels of thiols are related to hydroperoxide concentrations indirectly through the activity of GPX.

GPX is an antioxidant enzyme synthesized by animals to detoxify the tissues from hydroperoxides and hydrogen peroxide, which are reduced to their corresponding alcohols and water, respectively (Halliwell and Gutteridge, 2007). The forms of GPX occurring in plasma and RBCs are structurally different, and it is also uncertain whether plasma GPX is able to function properly because of the low levels of thiol cofactors (such as glutathione and thioredoxin) occurring in the plasma (Brigelius-Flohé, 1999; Halliwell and Gutteridge, 2007). Our analysis shows, however, that levels of GPX in plasma and RBCs are tightly and positively correlated with each other, even if their absolute concentrations differ, with both being related to the concentration of their substrate (hydroperoxides). Although RBC GPX does not directly interact with plasma hydroperoxides, it is likely that levels of plasma hydroperoxides reflect to some extent the concentration of peroxides in RBCs. In contrast, thiols (e.g. glutathione) participate directly in these detoxification reactions, working as enzymatic cofactors of GPX, and so are crucial for GPX to be able to remove hydroperoxides from the body. The active role of thiols in the removal of hydroperoxides is well exemplified by the network diagram, where the thiol node was connected directly to those of plasma GPX and RBC GPX. As expected, thiols are only indirectly connected to plasma hydroperoxides because there is no direct biochemical interaction between them (Halliwell and Gutteridge, 2007).

One limit to our capacity to infer the biological meaning of the cluster formed from the two (plasma and RBC) biomarkers of antioxidant capacity is that they are themselves already integrated measures of part of the non-enzymatic antioxidant molecules present in the sample. Our results suggest, however, that the non-enzymatic antioxidant machinery present in plasma and RBCs might be strongly functionally integrated. Similarly, our results suggest a strong connection between the activity of the enzyme GPX in plasma and the same enzyme in RBCs.

The second principal component also indicates that the levels of plasma and RBC non-enzymatic antioxidants are positively related

to plasma hydroperoxides, suggesting that zebra finches may also upregulate components of non-enzymatic antioxidant machinery other than GPX in response to levels of oxidative damage. This result, however, needs further exploration because their association is generally low and not statistically significant, as shown by the network model. Moreover, the network model suggests that the interaction between plasma hydroperoxides and non-enzymatic antioxidants could be indirect rather than direct, through the activity of GPX. Finally, a negative association was found between these biomarkers of antioxidant capacity and GPX. This finding suggests that in our study non-enzymatic and enzymatic antioxidants are differentially regulated, giving some flexibility to the overall antioxidant system. Consistent with this interpretation is the finding that any positive benefits of vitamin C supplementation in mice were offset by compensatory reductions in endogenous protection mechanisms, leading to no net reduction in accumulated oxidative damage (Selman et al., 2006). This has implications for the selection of biomarkers to quantify the antioxidant status of a tissue: our results suggest that measuring both antioxidant capacity and GPX activity may produce a better estimate of blood antioxidant status than measurements of just a single component.

Although our analyses suggest some significant biological signals that warrant further investigation, it is also important to highlight the limitations of our study. First, our investigation was restricted to subadult animals (around 45 days old), fully independent but not yet fully sexually mature. Studies on gene and metabolic networks suggest that these systems can become less integrated as the individual ages, so becoming noisier and less stable because of a decrease in the effectiveness of communication among functional units (Csermely and Soti, 2006; Xue et al., 2007; Southworth et al., 2009; Soltow et al., 2010). So, in future studies it will be important to evaluate network robustness in older individuals to see whether any changes in the degree of integration of the network mirror a signal of senescence. Second, our study considered only baseline values of redox variables. Some evidence suggests that phenotypic integration increases with environmental stress, possibly because of the convergence of the various response mechanisms when there is an increased need to promote self-maintenance and survival (e.g. Schlichting, 1986; Waitt and Levin, 1993; Kawano and Hara, 1995; Badyaev, 2005). Also, the degree of correlation among redox variables strongly increases under stressful conditions (Dotan et al., 2004). Therefore, in future studies, it will also be important to look at the effect of external stressors on the degree of integration of the blood redox system.

Another challenge will be to take into account possible non-linear functional interactions among components of the antioxidant system, and to incorporate relationships among components from tissues other than the blood. This last approach could prove useful for the identification of redox modules, being units of tightly integrated molecules that function almost independently from each other. It will be necessary to test whether the molecules that form an identifiable redox module in one tissue also operate in the same integrated way in other tissues. A logical extension of these studies would be to explore the links with the mechanisms underlying ageing; note that Kowald and Kirkwood (Kowald and Kirkwood, 1996) called for a synthesis of formerly distinct hypotheses into a network theory of ageing, with the advantage of integrating diverse mechanisms and their interactions.

In conclusion, multivariate statistical analyses of a range of biochemical measures of redox variables can reveal the level of system integration and can identify clusters of molecules that covary. In particular, our study identified a tight association between GPX and hydroperoxide levels in the plasma and GPX and thiol levels in RBCs. Our study also suggests that antioxidant systems present in plasma and RBCs may be highly integrated.

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REFERENCES

Badyaev, A. V. (2005). Stress-induced variation in evolution: from behavioural plasticity to genetic assimilation. *Proc. R. Soc. B Biol. Sci.* **272**, 877-886.
Brigelius-Flohé, R. (1999). Tissue-specific functions of individual glutathione peroxidases. *Free Radic. Biol. Med.* **27**, 951-956.
Bruner, E., Martin-Loeches, M. and Colom, R. (2010). Human midsagittal brain shape variation: patterns, allometry and integration. *J. Anat.* **216**, 589-599.
Cheverud, J. M. (1996). Developmental integration and the evolution of pleiotropy. *Am. Zool.* **36**, 44-50.
Costantini, D. (2010). On the measurement of circulating antioxidant capacity and the nightmare of uric acid. *Methods Ecol. Evol.* doi: 10.1111/j.2041-210X.2010.00080.x
Costantini, D. and Dell'Omo, G. (2006). Environmental and genetic components of oxidative stress in wild kestrel nestlings (*Falco tinnunculus*). *J. Comp. Physiol. B* **176**, 575-579.
Costantini, D. and Verhulst, S. (2009). Does high antioxidant capacity indicate low oxidative stress? *Funct. Ecol.* **23**, 506-509.
Costantini, D., Fanfani, A. and Dell'Omo, G. (2008). Effects of corticosteroids on oxidative damage and circulating carotenoids in captive adult kestrels (*Falco tinnunculus*). *J. Comp. Physiol. B* **178**, 829-835.
Csermely, P. and Soti, C. (2006). Cellular networks and the aging process. *Arch. Physiol. Biochem.* **112**, 60-64.
Dotan, Y., Lichtenberg, D. and Pinchuk, I. (2004). Lipid peroxidation cannot be used as a universal criterion of oxidative stress. *Progr. Lipid Res.* **43**, 200-227.
Gianoli, E. (2001). Lack of differential plasticity to shading of internodes and petioles with growth habit in Convolvulus arvensis (Convolvulaceae). *Int. J. Plant Sci.* **162**, 1247-1252.
Halliwell, B. H. and Gutteridge, J. M. C. (2007). *Free Radicals in Biology and Medicine*, 4th edn. Oxford: Oxford University Press.
Hammer, Ø., Harper, D. A. T. and Ryan, P. D. (2001). PAST: Paleontological Statistics Software Package for Education and Data Analysis. *Palaeontol. Electronica* **4**, 9.
Jolliffe, I. T. (2002). *Principal Component Analysis*, 2nd edn. New York: Springer.
Kawano, S. and Hara, T. (1995). Optimal balance between propagule output, propagule size, and cost of propagule production in plants with special reference to its evolutionary ecological implications. *Plant Species Biol.* **10**, 119-125.
Klingenberg, C. P. (2007). Analyzing modularity in complex morphological structures. *J. Morphol.* **268**, 1094-1094.
Klingenberg, C. P. (2008). Morphological Integration and Developmental Modularity. *Annu. Rev. Ecol. Evol. Syst.* **39**, 115-132.
Kowald, A. and Kirkwood, T. B. L. (1996). A network theory of ageing: the interactions of defective mitochondria, aberrant proteins, free radicals and scavengers in the ageing process. *Mutat. Res.* **316**, 209-236.
Mitteroecker, P. and Bookstein, F. (2007). The conceptual and statistical relationship between modularity and morphological integration. *Syst. Biol.* **56**, 818-836.
Paglia, D. E. and Valentine, W. (1967). Studies on quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *J. Lab. Clin. Med.* **70**, 158-169.
Pigliucci, M., Schlichting, C. D. and Whitton, J. (1995). Reaction norms of Arabidopsis 2. Response to stress and unordered environmental variation. *Funct. Ecol.* **9**, 537-547.
Ravasz, E., Somera, A. L., Mongru, D. A., Oltvai, Z. N. and Barabasi, A. L. (2002). Hierarchical organization of modularity in metabolic networks. *Science* **297**, 1551-1555.
Schlichting, C. D. (1986). The evolution of phenotypic plasticity in plants. *Annu. Rev. Ecol. Syst.* **17**, 667-693.
Schlosser, G. and Wagner, G. P. (2004). *Modularity in Development and Evolution*. Chicago: The University of Chicago Press.
Selman, C., McLaren, J. S., Meyer, C., Duncan, J. S., Redman, P., Collins, A. R., Duthie, G. G. and Speakman, J. R. (2006). Life-long vitamin C supplementation in combination with cold exposure does not affect oxidative damage or lifespan in mice, but decreases expression of antioxidant protection genes. *Mech. Ageing Dev.* **127**, 897-904.
Sies, H. (1991). Oxidative stress: oxidants and antioxidants. London: Academic Press.
Soltow, Q. A., Jones, D. P. and Promislow, D. E. L. (2010). A network perspective on metabolism and aging. *Integr. Comp. Biol.* **50**, 844-854.
Southworth, L. K., Owen, A. B. and Kim, S. K. (2009). Aging mice show a decreasing correlation of gene expression within genetic modules. *PLoS Genet.* **5**, e1000776.
Waitt, D. E. and Levin, D. A. (1993). Phenotypic integration and plastic correlations in *Phlox Drummondii* (Polemoniaceae). *Am. J. Bot.* **80**, 1224-1233.
Xue, H., Xian, B., Dong, D., Xia, K., Zhu, S., Zhang, Z., Hou, L., Zhang, Q., Zhang, Y. and Han, J.-D. H. (2007). A modular network model of aging. *Mol. Syst. Biol.* **3**, 147.