Loss of integration is associated with reduced resistance to oxidative stress

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Running title: Oxidative stress and integration
SUMMARY

One cellular mechanism thought to be particularly important as a constraint on lifespan and life-history strategies is oxidative stress. Susceptibility to oxidative stress is influenced by a number of antioxidant defences, whose effectiveness depends on the synergistic and competitive interactions among them (biochemical integration). It is generally assumed that exposure to oxidative stress is detrimental, but it is also possible that low level oxidative stress has a positive effect on integration, and therefore carries some benefits. Using three experimental groups of zebra finches (control, mild and high flight activity), we tested whether exercise-induced oxidative stress altered the integration of the pro-oxidant/antioxidant system by manipulating levels of flight activity, known to generate oxidative stress in birds. We show for the first time that a short-term high level of physical activity leads to a reduction in integration among components of the blood antioxidant defences, associated with a reduced resistance to oxidative stress. We found no evidence of improved integration in the antioxidant defences at low levels of oxidative stress exposure, suggesting that improved integration is not the route whereby any benefits of low level stress exposure occur. These findings point to a reduction in biochemical integration as a potential mechanism explaining a reduced resistance to oxidative stress induced by short-term stressors.

Key words: antioxidants, birds, flight effort, oxidative damage, vertebrates.
INTRODUCTION

Understanding the physiological processes underlying variation in lifespan is of central importance to both evolutionary ecology and biomedical research (Ricklefs and Wikelski, 2002; Romero, 2004). One cellular mechanism thought to be particularly important in this regard is oxidative stress (Halliwell and Gutteridge, 2007; Costantini, 2008; Monaghan et al., 2009), caused by generation of oxidative damage to biomolecules (Sies, 1991; Halliwell and Gutteridge, 2007; Costantini and Verhulst, 2009) and overoxidation of thiol groups (Jones, 2006; Sohal and Orr, 2012). Accumulation of such damage is thought to contribute to cell senescence and loss in organ performance (Weinert and Timiras, 2003; Kirkwood, 2005).

Susceptibility to oxidative stress is influenced by a number of antioxidants (Surai, 2002; Halliwell and Gutteridge, 2007; Pamplona and Costantini, 2011). However, the effectiveness of the defence machinery depends not only on how good the antioxidants are at preventing oxidative damage, but also on the synergistic and competitive interactions among different antioxidants and on how changes in levels of oxidative damage are accompanied by parallel changes in antioxidants. This last aspect is particularly important because this might reflect the organism’s response to the oxidative challenge and because many products of oxidative damage (e.g., hydroperoxides, malondialdehyde, hydroxynonenal) can be still active as pro-oxidants so must be degraded to less noxious compounds (Halliwell and Gutteridge, 2007). Such interdependency is referred to as biochemical integration of the redox system (Papin et al., 2004; Costantini et al., 2011; Pamplona and Costantini, 2011). Generally speaking, all biological systems exhibit an integration of morphological, metabolic and/or genetic components, such that these are
intercorrelated through functional, structural, developmental or evolutionary interdependency (Klingenberg, 2007, 2008; Ravasz et al., 2002; Mitteroecker and Bookstein, 2007; Cohen et al., 2012). The degree of integration of a system is mostly inferred from the analysis of correlation or covariance matrices (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 (Cheverud, 1996; Mitteroecker and Bookstein, 2007; Klingenberg, 2008).

There is evidence that integration of a metabolic or genetic system may change with exposure to environmental stressors, possibly depending on the degree of prioritization of processes that promote self-maintenance and survival or reproduction (e.g., Giuliani et al., 2004; Csermely and Soti, 2006; Tun et al., 2006; Parter et al., 2007; Southworth et al., 2009). The extent to which exposure to stressors is detrimental for the organism depends on the nature and strength of the stressor. Mild stress exposure can actually have a stimulatory and, possibly, beneficial effect through a hormetic response to the stressful stimulus (Southam and Ehrlich, 1943). Hormesis occurs when the dose-response relationship is bi-phasic, where high intensities of a stressor cause inhibition of homeostatic mechanisms but low intensities cause their stimulation (Mattson and Calabrese, 2010). Hormesis has been documented across a wide range of organisms (from bacteria to vertebrates), in response to exposure to at least 1,000 different chemical and environmental stressors (Mattson and Calabrese, 2010).

A unifying feature that may underlie the qualitatively similar hormetic response to this diverse array of stressors could be that many can induce oxidative stress (Costantini et
al., 2010; Mattson and Calabrese, 2010). Exposure to free radicals generated by a mild stressor may stimulate the antioxidant defence system, rendering the organism better able to cope with that stressor (Radak et al., 2008, Ristow et al., 2009). However, it is not known whether this involves any change to the level of integration among components of the redox system. For example, a decrease in integration might be expected whether a disruption of homeostatic mechanisms (e.g., decrease in molecular communication, deregulation of redox balance control by thiols) makes the cells less capable of efficiently controlling the activity of free radicals (Dröge, 2002; Jones, 2006). The strength of correlations (and hence integration) among measures of oxidative status may change, depending on whether they reflect measurements made under baseline or stressful conditions (Dotan et al., 2004). An important question therefore is whether the level of integration changes linearly with the increase of a stressor intensity or shows a biphasic response (showing greater levels of integration after mild exposure to the stressor), indicating a link with hormesis.

Here we manipulate the intensity of flight (an activity which is known to generate oxidative stress in birds (Nikolaidis et al., 2012)) in zebra finches (Taeniopygia guttata, Vieillot 1817) to test whether there is a link between the degree of oxidative stress induced by exercise and the integration of the different components of the blood redox system. Moreover, by inducing intermediate as well as high levels of flight activity we test for evidence of hormesis in both the level of oxidative stress and the degree of redox integration.

**MATERIALS AND METHODS**

Study birds were produced by pairs of stock zebra finches. Manipulation of flight effort
was carried out using a simple automated system fitted into a modified bird cage with motorized hinged perches (for more details see Costantini et al., 2012). Briefly, cages (l × h × d: 200 × 95 × 83 cm) were equipped with automatic perches, whose movement was controlled by a pre-set timing system and manual speed control, by which it was possible to regulate the frequency of movement of perches (from 1 to every 60 seconds). When an occupied horizontal perch slowly dropped downwards to the vertical position, the birds on it had to fly to the far end of the cage where the other perch was now available. The cage for control birds was the same size as that for birds undergoing flight manipulations, but had fixed perches. The work was conducted under license from the UK Home Office.

On day 0 of the experiment, a sample of blood was taken from the brachial vein by venipuncture and collected using microhaematocrit heparinised capillary tubes (Vetlab Supplies Ltd, Broomers Hill Park, Pulborough, West Sussex, UK). Blood samples were maintained on ice and then were centrifuged to separate plasma from red blood cells (RBCs). Birds were then allocated to three experimental groups that differed in the intensity of flight activity: Control Flight (12 males, 15 females), Mild Flight (12 males, 15 females), High Flight (12 males, 14 females). Birds were 66.5 ± 1.1, 53-98 (mean ± standard error, range) days old at the time of the experiment. There were no differences among experimental groups in terms of average age (N=26-27 birds per group, P=0.76) and homogeneity of variances (Levene's test, P=0.43), showing that the age distribution did not differ among groups. Moreover, in preliminary analyses, the inclusion of age as a covariate in all the models did not alter the statistical outcomes obtained from models performed without the inclusion of age, nor did age covary significantly with any of the variables (P≥0.064). Sexes were assigned to the treatment groups to give a balanced sex ratio among
groups. Each bird was randomly assigned to a particular experimental group, and the birds from different treatments were kept together in single-sex common cages (8–10 birds per cage, following UK Home Office recommendations; balanced number among treatment groups) in order to rule out confounding cage effects. On day 1 of the experiment, Mild Flight and High Flight birds were put in two flight cages, respectively, and were allowed to become accustomed to the new environment, with both perches being left fixed in the horizontal position for one hour. After this one hour settling period, the system was set in operation so that the two perches folded away alternately, causing the birds to fly the length of their aviary to the other perch every 30 seconds for a duration of two hours. On both days 2 and 3, Mild Flight and High Flight birds were again placed in the flight cages and allowed to settle with fixed perches for 10 minutes. After this settling period, the system was set in operation so that the two perches folded away every 15 seconds for 10 minutes for both groups, and then again every 15 seconds for 3 hours for Mild Flight birds, and every 1 second for 3 hours for High Flight birds. A sample of blood was taken soon after the end of the trial. On each of the three days Control Flight birds were put inside a cage of the same size as that of treated birds, but with fixed perches; their flight activity was thus minimal (average distance covered in a 3-h trial by control birds was 165.8 m as estimated in Costantini et al., 2012) compared to that of the treatment birds. On days 1, 2 and 3, Mild Flight birds were induced to fly 315 (210 takeoffs), 939 (626 takeoffs) and 939 (626 takeoffs) meters, while High Flight birds were induced to fly 315 (210 takeoffs), 2,733 (1,822 takeoffs) and 2,733 (1,822 takeoffs) meters. On the latter two days the Mild and High Flight birds were therefore flying around 6 and 16 times more than the distance of the control birds as estimated in Costantini et al. (2012). The protocol was designed to induce
strong exercise in the High group without being excessive, so the distances chosen were well within the known capability of captive zebra finches (e.g. birds induced to fly 7,200 m in 2 hours by Bauchinger et al., 2010). None of the birds were exhausted during the exercise manipulation, all continuing to fly back and forth between perches for the duration of each trial. Moreover, all the birds were still alive several months after the end of the experiment, showing that none of the birds experienced any adverse effects afterwards. As in the study by Nudds and Bryant (2000) that used a similar apparatus, neither controls nor treatment birds received water or food during the three hour flight trials, but had access to *ad libitum* food and water in their holding cages at all other times.

### Evaluation of blood oxidative status

In order to measure the level of oxidative stress and the integration of the redox system we measured two biomarkers of oxidative damage (hydroperoxides and protein carbonyls), three antioxidant enzymes (glutathione peroxidase GPX; superoxide dismutase SOD; catalase CAT) and three non-enzymatic antioxidants (thiols, uric acid, carotenoids) in the blood samples taken before and after the 3-day period of exercise. A Thermo Scientific Multiskan Spectrum (ThermoFisher, Vantaa, Finland) was used to read the absorbance of the reaction solutions.

Hydroperoxides (R-OOH; intermediate oxidative damage products) were measured in plasma using the d-ROMs assay (Diacron International, Grosseto, Italy). Hydroperoxides derive from oxidation of several biomolecular substrates, such as polyunsaturated fatty acids, cholesterol, proteins and nucleic acids, and are precursors of end-products of lipid peroxidation, such as malondialdehyde, hydroxynonenal and isoprostanes (Halliwell and...
Gutteridge, 2007; Lajtha et al., 2009). The specificity of the assay for hydroperoxides is higher than 90% (Alberti et al., 2000; Buonocore). The absorbance was read at 505 nm. Hydroperoxides were expressed as mM of H₂O₂ equivalents and values were calculated according to a calibration curve run in each assay. Analyses were run in duplicate and the mean coefficients of intra- and inter-assay variation were 3.50 and 7.22 %, respectively. Intra- and inter assay repeatabilities *sensu* Lessells & Boag (1987), were 0.952 and 0.911, respectively.

The method from Levine et al. (1990; see also Cao and Cutler, 1995; Montgomery et al., 2011) was used to quantify the concentration of protein carbonyls in plasma. Protein carbonyls indicate protein oxidative damage. Carbonyls (C=O) are introduced into proteins from free radicals or via reactions with lipid peroxidation products (malondialdehyde and hydroxynonenal) or carbohydrates; protein carbonylation is mostly irreversible (Halliwell and Gutteridge, 2007). All plasma samples were first diluted with distilled water in order to have a concentration of 1 mg of proteins per ml, as measured by the Bradford protein assay (Bio-Rad Laboratories, Hercules, USA) using albumin as a reference standard. Nucleic acids were removed by adding 1 volume of a 10 % solution of streptomycin sulfonate to 9 volumes of sample. Protein carbonyls were derivatized to 2,4-dinitrophenylhydrazone by reaction with 2,4-dinitrophenylhydrazine (DNPH) according to Levine et al. (1990; see also Cao and Cutler, 1995; Montgomery et al., 2011). The pellet was precipitated with cold trichloroacetic acid at 20% and then washed three times with a solution 1:1 of cold ethanol-ethyl acetate. The pellet was finally re-suspended in 350 μl of guanidine hydrochloride 6 M. The absorbance was read at 370 nm. The mean absorbance of the control tubes was subtracted from the mean absorbance of the sample tubes and the extinction coefficient for
DNPH (0.022/µM/cm) was used to calculate the protein carbonyl concentration, which was expressed as nmol PC mg\(^{-1}\) protein. Analyses were run in duplicate and the mean coefficients of intra- and inter-assay variation were 9.98 and 13.60 %, respectively. Intra- and inter assay repeatabilities were 0.897 and 0.836 respectively.

The Ransel assay (RANDOX Laboratories, Crumlin, UK) was used to quantify the concentration of GPX in haemolysate (red blood cells diluted 1:40 with diluting agent provided with the assay). This assay is based on the original method of Paglia and Valentine (1967) and analyses were carried out according to previous studies (e.g., Costantini et al., 2011). The kinetic reaction was followed for 3 minutes by reading at 340 nm. A blank reaction was subtracted from the sample absorbance. Values were expressed as U l\(^{-1}\) of haemolysate. Analyses were run in duplicate and the mean coefficients of intra- and inter-assay variation were 3.24 and 5.70 %, respectively. Intra- and inter assay repeatabilities were 0.979 and 0.938 respectively.

The Ransod assay (RANDOX Laboratories, Crumlin, UK) was used to quantify the concentration of SOD in haemolysate (red blood cells diluted 1:600 with distilled water). The assay was performed following the manufacturer’s instructions (see also Woolliams et al., 1983). The absorbance was read at 505 nm. Concentrations were calculated using a calibration curve run for each assay and were expressed as U ml\(^{-1}\) of haemolysate. Analyses were run in duplicate and the mean coefficients of intra- and inter-assay variation were 8.29 and 13.39 %, respectively. Intra- and inter assay repeatabilities were 0.901 and 0.848 respectively.

The OxiSelect Catalase activity assay (Cell Biolabs, San Diego, USA) was used to quantify the concentration of CAT in haemolysate (red blood cells diluted 1:50 with...
distilled water). The assay was performed following the manufacturer’s instructions. The absorbance was read at 520 nm. Concentrations were calculated using a calibration curve run for each assay and were expressed as U ml\(^{-1}\) of haemolysate. Analyses were run in duplicate and the mean coefficients of intra- and inter-assay variation were 8.65 and 12.31 %, respectively. Intra- and inter assay repeatabilities were 0.923 and 0.854 respectively.

The -SHp test (Diacron International, Grosseto, Italy) was used to quantify the concentration of total thiols (e.g., glutathione, thioredoxin) in haemolysate (red blood cells diluted 1:200 with distilled water) according to Costantini et al. (2011). The assay is based on the reaction of thiols with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB; chromogen). The DTNB reacts with the thiols present in the solution generating a colored complex, whose intensity is directly proportional to the concentration of thiols (Ellman et al., 1959). The absorbance was read at 405 nm. Concentrations were calculated using a standard solution of L-cysteine purchased with the kit (Diacron International, Grosseto, Italy) and were expressed as µmol l\(^{-1}\) of -SH groups. Analyses were run in duplicate and the mean coefficients of intra- and inter-assay variation were 5.91 and 7.34 %, respectively. Intra- and inter assay repeatabilities were 0.965 and 0.922 respectively.

The concentration of carotenoids in plasma was calculated according to Alonso-Alvarez et al. (2004). The absorbance was read at 450 nm. Concentrations were calculated using a calibration curve of lutein (alpha-carotene-3,3'-diol; Sigma-Aldrich, U.K.) run for each assay and were expressed as µg ml\(^{-1}\) of plasma. Analyses were run in duplicate and the mean coefficients of intra- and inter-assay variation were 2.83 and 4.85 %, respectively. Intra- and inter assay repeatabilities were 0.987 and 0.946 respectively.
The Uric Acid assay (RANDOX Laboratories, Crumlin, UK) was used to quantify the concentration of uric acid in plasma. The assay was performed following the manufacturer’s instructions. The absorbance was read at 520 nm. Concentrations were calculated using a calibration curve run for each assay and were expressed as mg dl\(^{-1}\) of plasma. Analyses were run in duplicate and the mean coefficients of intra- and inter-assay variation were 3.83 and 5.64 \%, respectively. Intra- and inter assay repeatabilities were 0.980 and 0.933 respectively.

**Statistical analyses**

We first analysed the response to treatment (defined as the percentage within-individual change over the experimental period) of each single variable separately, using General Linear Mixed Effect Models in STATISTICA (Version 10, StatSoft, Inc., Tulsa, OK, USA). Each model included Treatment, Sex, and their interaction as fixed factors. Brood of origin of each juvenile was entered as a random factor to control for pseudoreplication. Non-significant terms were sequentially removed from the models starting from the interaction between Sex and Treatment and the analyses were repeated until we obtained a model with only significant terms. The brood was always retained in the models to control for pseudoreplication. Assumptions of normality of residuals (Kolmogorov-Smirnov test) and homogeneity of variances (Levene’s test) were respected. Post-hoc comparisons were carried out using the Fisher LSD test. Pre-treatment values did not differ among groups (p-values ≥ 0.11). Similar results (data not shown; hydroperoxides, protein carbonyls, thiols and uric acid were again the only variables to be significant) were obtained by, instead of using percentage changes, analysing the response to treatment of each measure of oxidative status.
by including in each model the pre- and post-treatment values of the variable as covariate
and dependent variable respectively; Treatment, Sex, and their interaction were again
included as fixed factors, and brood as a random factor.

In order to test how the degree of integration of the blood redox state changed in
response to treatment we first compared correlation matrices of normalized variables (mean
= 0, standard deviation = 1) of within-individual change values among groups, by fitting
Multivariate Analysis of Variance (MANOVA) with Treatment, Sex, and their interaction
as fixed factors. In preliminary analyses, we also ran Multivariate Generalised Linear
Mixed Models using Markov chain Monte Carlo methods (MCMCglmm package) in R
(Version 2.15.0, Vienna, Austria) in order to control for brood identity, which was included
as a random factor. Given that results were qualitatively similar to those of the MANOVA,
we only present the outcomes of the MANOVA for easy of presentation and calculation. As
with the univariate models, non-significant terms were sequentially removed from the
models starting from the higher order interactions and the analyses were repeated until we
obtained a model with only significant terms. Post-hoc comparisons were performed using
the Hotelling test in PAST version 1.94 (Hammer et al., 2001). The integration of blood
redox state was then estimated using the relative standard deviation of eigenvalues ($SD_{rel}(\lambda) = \sqrt{\text{Var}(\lambda)}/\sqrt{N-1}$) of the correlation matrix according to Pavlicev et al. (2009) and Haber
(2011): it is calculated as the ratio of the square root of the variance of eigenvalues ($\lambda$) to
the square root of the number of variables ($N$) minus 1, and varies between zero
(corresponding to no relationships among the variables) and one (perfect correlation
between all variables). Eigenvalues were calculated by running a Principal Component
Analysis on the correlation matrix for each experimental group separately. Finally, a two-
block partial least squares regression was performed using PAST for each experimental
group, separately, to test the covariance (i.e. integration) between the redox status change
of the plasma and that of the red blood cells (Costantini et al., 2011).

RESULTS

Univariate response to flight regime

In Fig. 1, we show the response to treatment (percentage within-individual change over the
experimental period) of each single redox variable separately, while in the Supplementary
Materials we report the raw values as mean ± standard error to show the pre- and post-value
treatments of measures of oxidative status. Statistical outcomes of the final models and
effect size of treatment differences are reported in Table 1. Groups did not differ in any of
the pre-treatment measures of oxidative status (p-values ≥ 0.11).

Compared to Control Flight (CF) birds, High Flight (HF) birds had a greater
increase in oxidative damage (both hydroperoxides and protein carbonyls) and decrease in
thiol concentration over the course of the flight trials. HF birds had also a greater increase
in protein carbonyls and a decrease in thiols than Mild Flight (MF) birds; hydroperoxides
were only marginally higher in HF than MF birds (Fisher test, P = 0.08). The change in both
oxidative damage biomarkers and thiols did not differ between CF and MF birds (Fisher
test, P ≥ 0.22).

The concentration of plasma uric acid in CF birds significantly decreased over the
trials compared to both MF and HF birds. The change in Catalase, GPX, SOD and
carotenoids did not differ among groups. Finally, we found that, regardless of treatment,
hydroperoxides increased more in females (+45.8%) than males (+21.2%), while GPX decreased more in females (+2.2%) than males (+8.3%).

**Multivariate response and integration of response to flight regime**

The Multivariate Analysis of Variance showed a significant effect of treatment (Wilks' lambda = 0.572, \(P=0.0006\)), while sex and the interaction between sex and treatment were not significant (data not shown). Post-hoc comparisons showed that the multivariate response significantly differed between HF and both CF (\(P=0.0005\)) and MF (\(P=0.005\)) birds; CF and MF birds did not differ. Such differences reflect a differential index of integration of the physiological response, being similar in CF (SD_{rel}(\lambda) = 0.276) and MF (0.259) birds, but lower in HF (0.205) birds (Fig. 2). A similar pattern was found for the covariance in changes in redox state of plasma and red blood cells: while the covariance was significant in all three experimental groups, it was lower in HF birds (55.65%) than in the other two groups (CF = 72.95%, MF = 69.42%).

**DISCUSSION**

The results of this study clearly show that, when exposed to a short-term major increase in physical activity, individuals suffer more oxidative stress. Our results also show that oxidative stress and integration increased and decreased, respectively, with the intensity of the flight effort, and there was no evidence of a hormetic response. In parallel with this, our study shows that integration between the changes in redox state of plasma and of red blood cells decreased with the increase in flight effort.
Previous studies that looked at genetic or metabolic networks have found that their integration is not a steady-state trait, but may change over time (e.g., Csermely and Soti, 2006; Southworth et al., 2009; Soltow et al., 2010). For example, genes work within semi-independent functional groups, such as metabolic pathways or regulatory networks. One effect of aging is a reduction in the correlation in gene co-expression pathways (Southworth et al., 2009). Therefore, by comparing how the correlation in, say, gene expression or metabolite concentrations differs between two states, it is possible to make inferences about changes in functional interconnectedness of those variables. In our study, the effect of the flight treatment was to cause a reduction in the correlations among the collected biochemical variables (expressed as percentage within-individual change over the experimental period) in the High Flight birds. This resulted in a lower redox integration index in these individuals. A reduced correlation among biochemical measures in the High Flight compared to the Control and Mild Flight would suggest that in those individuals the change in one component of blood oxidative status was less dependent on that of other components. Such a reduction in integration was also apparent in the covariation between the changes of oxidative status of plasma and that of red blood cells. While the covariation between plasma and blood cells in control birds was high and comparable to a previous study (Costantini et al., 2011), that study looked only at baseline measures of oxidative status (i.e. those in non-exercising birds), and the effect of high levels of exercise in the present study was to reduce that covariation (see value for HF birds).

There are at least three alternative (and not mutually exclusive) explanations for the reduction in integration in HF birds and consequent increase in oxidative stress. Firstly, the loss in biochemical integration may reflect a failure in the homeostatic mechanisms
regulating the oxidative status of the blood. The short-term increase in flight activity was particularly acute for High Flight birds, which could have perceived it as an unpredictable and so stressful event. Although in the wild zebra finches can fly tens of kilometers per day, under our captive conditions the physical activity of birds is usually low (over our experimental period Mild and High Flight birds were flying around 6 and 16 times respectively the distance of Control Flight birds, who were probably as active as all birds had been in their holding cages prior to the experiment). Therefore the increase in the level of exercise experienced by the High Flight birds may have been too great for them to have adjusted their homeostatic mechanisms sufficiently to control their oxidative status, especially if thiol concentrations also dropped (Jones, 2006; Sohal and Orr, 2012). Consequently, an increase in production of free radicals might not have been tackled by the antioxidant response, leading to an increase in oxidative damage. It is also worth highlighting that oxidation of molecules involved in redox regulation and control might have altered their functionality, hence reducing their capacity of interacting with other molecules.

A second explanation is that High Flight birds deprioritised compensatory mechanisms for oxidative stress in the short-term because for some reason it was too costly to upregulate these under the circumstances of the trials. Zebra finches could, for example, be programmed to sacrifice protection against oxidative stress during periods where allocation of resources to other specific functions (such as flight) is given priority. It is noteworthy that zebra finches whose flight activity was experimentally increased (by increasing the number of chicks that they had to feed) showed a decrease in circulating levels of antioxidant enzymes (Wiersma et al., 2004). Although our experimental birds
were not breeding at the time of the experiment, it could be that our manipulation of flight activity might have simulated similar conditions as in that experiment, where birds responded to an increased energetic demand by apparently re-allocating resources away from antioxidant defences (Wiersma et al., 2004). This would imply that mechanisms regulating blood oxidative status are conserved across those different stages of the life-cycle (e.g., chick-rearing period, migration) that demand strong increases in physical activity. The fact that in our experiment enzymatic antioxidant defenses did not decrease as in Wiersma et al. (2004) might also suggest that the lack of an additional effort (i.e. chick-rearing as in Wiersma et al., 2004) or the younger age of our birds could have made the overall effort less demanding for the birds.

Finally, since our results also show that there was not only an overall reduction in integration, but also a change in the specific connections among variables, this could suggest that the decrease in integration was the result of an increase in modularity, i.e., the molecular groups we measured were part of different pathways that under stressful conditions work almost independently from each other. A module is a discrete unit of a number of elements interacting in a tightly integrated way that performs a specific task, separable from the functions of other modules (Hartwell et al., 1999). Parter et al. (2007) showed that metabolic networks of bacteria living in variable environments are more modular than networks of bacteria living in more stable conditions. The authors suggested that unstable environmental conditions promote modular organization because each module deals with a specific task. Given that in an unstable environment an organism can be faced with several environmental pressures, it could be more functionally efficient to separate the
processing of the stimulus and the response to it among different modules, according to the nature of the stimulus itself.

In our present study, the integration-modularity explanation might be to some extent supported by the fact that the HF birds appears to have two independent modules connecting redox components rather than a single one (see Fig. 2), and that the links between levels of hydroperoxides, glutathione peroxidase and protein carbonyls became significant only in the High Flight birds. Glutathione peroxidase is an antioxidant enzyme that reduces the hydroperoxides to their corresponding alcohols, hence indirectly limiting the carbonylation of proteins generated by (i) the degradation products of hydroperoxides (i.e., malondialdehyde and hydroxynonenal), or (ii) the free radicals generated by the cleavage of hydroperoxides through the Fenton reaction (Halliwell and Gutteridge, 2007).

However, this explanation is not supported by other aspects. For example, it is unclear why concentrations of thiols (which are important co-factors of activity of glutathione peroxidase; see pp. 110-117 in Halliwell and Gutteridge, 2007) were not linked to those of glutathione peroxidase as previously found in zebra finches for baseline concentrations (Costantini et al., 2011). This might be because the reduction in thiols we observed was not enough to compromise the activity of glutathione peroxidase, hence resulting in a lack of significant correlation between them. It is also unclear why the three antioxidant enzymes (GPX, SOD, Catalase), whose functions are related to each other (Pamplona and Costantini, 2011), did not cluster together. It is also worth mentioning that in both the Control and Mild Flight birds, changes in both biomarkers of oxidative damage (hydroperoxides and protein carbonyls) were correlated with those in uric acid, but such connections disappeared in the High Flight birds. In birds, flight can increase protein catabolism of muscle fibres.
(Bordel and Haase, 2000; Jenni et al., 2000), which in turn influences the production of uric acid, a waste product of nitrogen metabolism. However, uric acid is also well known to have antioxidant properties (Iqbal et al., 1999; Klandorf et al., 1999; Tsahar et al., 2006). It could be that in the High Flight birds, the plasma concentration of uric acid did not decrease over the trials as it did in the Control (and to a lesser extent Mild Flight) birds, because it was replenished through a higher rate of protein catabolism induced by the more intense flight activity (e.g. Bordel and Haase, 1993; Schwilch et al., 1996). Therefore, this would make the interpretation of the connections of uric acid with oxidative damage more complicated because changes in plasma uric acid concentration might be influenced by both the excretory pathway of uric acid and its links to protein turnover (proteins are substrates of oxidation) and by antioxidant demands. Further studies will therefore be needed (i) to test the explanations we provide for the differences in biochemical integration of responses to flight activity and (ii) to clarify the role of uric acid in the regulation of blood oxidative status.

We observed a positive link between catalase and uric acid in CF and MF (but not HF) birds. The positive link between catalase and uric acid might reflect higher need of catalase to remove hydrogen peroxide produced by oxidation of uric acid (Halliwell and Gutteridge, 2007). Moreover, uric acid was also negatively linked to hydroperoxides in both CF and MF groups, which might reflect its capacity to scavenge singlet oxygen and hydroxy radicals (Halliwell and Gutteridge, 2007), hence reducing hydroperoxide formation. However, we do not know why such a link was absent in HF birds. As stated above, this might be because changes in plasma uric acid concentration might be influenced
by both protein catabolism and antioxidant demands. However, further and more specific studies will be needed to unravel the mechanistic bases of these links.

Our results showed no evidence of a hormetic response to flight activity, since Mild Flight birds did not experience any reduction in damage nor increase in antioxidant defenses or integration index. That physical activity may induce hormetic responses is well established (Mattson and Calabrese, 2010). Depending on the type of flight (e.g., short with repeated takeoffs, steady-state), as well as its duration and intensity, the flight effort can be either stimulatory or detrimental in terms of energy expenditure or stress. An increase in energy expenditure has been previously demonstrated in captive zebra finches engaged in short and repeated flights accompanied by around 500 takeoffs in eight hours (Nudds and Bryant, 2000). However, changes in metabolic rate were also affected by the training level of the individual bird, with trained finches showing a decrease rather than an increase in metabolism compared to untrained finches (Nudds and Bryant, 2001). Similarly, plasma concentrations of malondialdehyde (an end-product of lipid peroxidation) in captive budgerigars *Melopsittacus undulatus* were significantly lower after 9 weeks of flight training than after a single exercise session (Larcombe et al., 2010). Therefore, in our case, the duration of the treatment (3 days) could have been too short to allow the detection of a stimulatory effect on the oxidative stress response.

In conclusion, we show that a reduction in biochemical integration is associated with a reduced resistance to oxidative stress induced by a short-term increase in physical activity, but found no evidence that this exercise regime induced a hormetic response. We also highlight that it will be important to look at integration in a comparative framework because differences in correlations among antioxidant levels among species might exist.
(e.g., Cohen and McGraw 2009; Sepp et al., 2012), hence the degree of integration might be subject to different selective pressures. Finally, it will be also important to look at how increases or decreases in biochemical integration of the oxidative stress response impinge on Darwinian fitness.

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REFERENCES


Levine, R.L., Garland, D., Oliver, C.N., Amici, A., Climent, I., Lenz, A.G., Ahn, B.W.,


and feeding in birds during migration: a nutritional and physiological ecology

Mitteroecker, P. and Bookstein, F. (2007). The conceptual and statistical relationship

Monaghan, P., Metcalfe, N. B. and Torres, R. (2009). Oxidative stress as a mediator of
life history trade-offs: mechanisms, measurement and interpretation. *Ecol. Lett.* **12**, 75-
92.


Nikolaidis, M.G., Kyparos, A., Spanou, C., Paschalis, V., Theodorou, A.A. and

Biol.* **203**, 1561-1572.

Nudds, R.L. and Bryant, D.M. (2001). Exercise training lowers the resting metabolic rate


**Figure captions**

Figure 1. Response to the experimental treatments, expressed as percentage within-individual change over the experimental period. Values are shown as mean ± standard error. Post-hoc comparisons are, however, based on least square means. Significant differences among groups are highlighted as follows: * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$. CF = Control Flight; MF = Mild Flight; HF = High Flight. GPX = glutathione peroxidase; SOD = superoxide dismutase; CAT = catalase.

Figure 2. Network model drawn to show the connections between the eight redox variables measured in the blood; networks are presented separately for the three treatment groups (CF = Control Flight, MF = Mild Flight, HF = High Flight). Each node of the network refers to one variable. Significant Pearson correlations between standardised within-individual change values (calculated for each treatment group separately) are indicated by continuous (positive) or dashed (negative) lines between nodes; note the progressive reduction in the number of lines and increasing dissociation between nodes moving from CF through MF to HF.

**Table Captions**

Table 1. Results of Linear Mixed Effect models of the response to the flight regime (n = 80 birds). Outcomes in bold refer to variables included in the final model (the rest were sequentially excluded from the model because they were not significant). GPX = glutathione peroxidase; SOD = superoxide dismutase; CAT = catalase.
Figure 1
Hydroperoxides
Protein carbonyls
Glutathione peroxidase
Catalase
Superoxide dismutase
Thiols
Carotenoids
Uric acid

Integration index
CF = 0.276
MF = 0.259
HF = 0.205
Table 1. Results of Linear Mixed Effect models of the response to the flight regime (n = 80 birds). Outcomes in bold refer to variables included in the final model (the rest were sequentially excluded from the model because they were not significant). GPX = glutathione peroxidase; SOD = superoxide dismutate; CAT = catalase.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Brood</th>
<th>Treatment</th>
<th>Sex</th>
<th>Treatment × Sex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroperoxides</td>
<td>F29,47 = 2.88, p = 0.0006</td>
<td>F2,47 = 4.26, p = 0.020, effect size = 0.72</td>
<td>F1,47 = 6.68, p = 0.013</td>
<td>F2,45 = 1.59, p = 0.21</td>
</tr>
<tr>
<td>Protein carbonyls</td>
<td>F29,48 = 1.10, p = 0.37</td>
<td>F2,48 = 4.83, p = 0.012, effect size = 0.77</td>
<td>F1,47 = 0.29, p = 0.60</td>
<td>F2,45 = 1.13, p = 0.33</td>
</tr>
<tr>
<td>GPX</td>
<td>F29,49 = 1.34, p = 0.18</td>
<td>F2,49 = 1.40, p = 0.26</td>
<td>F1,49 = 4.32, p = 0.043</td>
<td>F2,45 = 2.50, p = 0.09</td>
</tr>
<tr>
<td>SOD</td>
<td>F29,50 = 1.45, p = 0.12</td>
<td>F2,50 = 0.40, p = 0.67</td>
<td>F1,47 = 0.15, p = 0.70</td>
<td>F2,45 = 1.10, p = 0.34</td>
</tr>
<tr>
<td>CAT</td>
<td>F29,50 = 1.32, p = 0.19</td>
<td>F2,50 = 0.48, p = 0.62</td>
<td>F1,49 = 0.38, p = 0.54</td>
<td>F2,45 = 0.04, p = 0.96</td>
</tr>
<tr>
<td>Thiols</td>
<td>F29,48 = 1.42, p = 0.14</td>
<td>F2,48 = 7.16, p = 0.002, effect size = 0.92</td>
<td>F1,47 = 0.12, p = 0.73</td>
<td>F2,45 = 0.69, p = 0.51</td>
</tr>
<tr>
<td>Uric Acid</td>
<td>F29,48 = 2.21, p = 0.007</td>
<td>F2,48 = 4.89, p = 0.012, effect size = 0.78</td>
<td>F1,47 = 0.01, p = 0.92</td>
<td>F2,45 = 1.65, p = 0.20</td>
</tr>
<tr>
<td>Carotenoids</td>
<td>F29,68 = 3.87, p &lt; 0.001</td>
<td>F2,68 = 0.14, p = 0.87</td>
<td>F1,68 = 1.07, p = 0.30</td>
<td>F2,45 = 0.90, p = 0.41</td>
</tr>
</tbody>
</table>