RESEARCH ARTICLE

Loss of integration is associated with reduced resistance to oxidative stress

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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.

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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 the 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; Klingenberg, 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 Sőti, 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 1000 different chemical and environmental stressors (Mattson and Calabrese, 2010).

A possible unifying feature that may underlie the qualitatively similar hormetic response to this diverse array of stressors is 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 manipulated the intensity of flight (an activity that 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 tested 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 (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 s). 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 licence 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 heparinized capillary tubes (Vetlab Supplies Ltd, Broomers Hill Park, Pulborough, West Sussex, UK), and subsequently stored on ice. Blood samples 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 (CF, 12 males, 15 females), mild flight (MF, 12 males, 15 females), high flight (HF, 12 males, 14 females). Birds were 66.5±1.1 days old (mean ± s.e.m., range 53–98 days) at the time of the experiment. There were no differences among experimental groups in terms of mean 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 ($P\geq0.064$). 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, MF and HF birds were put into two separate flight cages and were allowed to become accustomed to the new environment, with both perches being left fixed in the horizontal position for 1 h. After this 1 h 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 s for a duration of 2 h. On both day 2 and day 3, MF and HF birds were again placed in the flight cages and allowed to settle with fixed perches for 10 min. After this settling period, the system was set in operation so that the two perches folded away every 15 s for 10 min for both groups, and then again every 15 s for 3 h for MF birds, and every 1 s for 3 h for HF birds. A sample of blood was taken soon after the end of the trial. On each of the 3 days CF 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 previously (Costantini et al., 2012)] compared with that of the treatment birds. On days 1, 2 and 3, MF birds were induced to fly 315 m (210 takeoffs), 939 m (626 takeoffs) and 939 m (626 takeoffs), while HF birds were induced to fly 315 m (210 takeoffs), 2733 m (1822 takeoffs) and 2733 m (1822 takeoffs). On days 2 and 3 the MF and HF birds were therefore flying around 6 and 16 times more than the previously estimated distance of the control birds (Costantini et al., 2012). The protocol was designed to induce strong exercise in the HF group without being excessive, so the distances chosen were well within the known capability of captive zebra finches [e.g. birds were induced to fly 7200 m in 2 h in a previous study (Bauchinger et al., 2010)]. None of the birds were exhausted during the exercise manipulation, with 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 a previous study (Nudds and Bryant, 2000) that used a similar apparatus, neither controls nor treatment birds received water or food during the 3 h flight trials, but they 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 the 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 et al., 2000). The absorbance was read at 505 nm. Hydroperoxides were expressed as mmol l^{-1} of H$_2$O$_2$ 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 and Boag, 1987) were 0.952 and 0.911, respectively.

A previously published method (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 protein ml^{-1}, as measured by the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA) using albumin as a reference standard. Nucleic acids were removed by adding 1 volume of a 10% solution of streptomycin sulphate to 9 volumes of sample. Protein carbonyls were derivatized to 2,4-dinitrophenylhydrazone by reaction with 2,4-dinitrophenylhydrazine (DNPH) (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 1:1 solution of cold ethanol:ethyl acetate. The pellet was finally re-suspended in 350 μl of guanidine hydrochloride 6 mol l^{-1}. 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 μmol l^{-1} cm^{-1}) was used to calculate the protein carbonyl (PC) 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 (RBCs diluted 1:40 with diluting agent provided with the assay). This assay is based on the original method of Paglia and Valentine (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 min 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) was used to quantify the concentration of SOD in haemolysate (RBCs 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, CA, USA) was used to quantify the concentration of CAT in haemolysate (RBCs 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 (RBCs diluted 1:200 with distilled water) as previously (Costantini et al., 2011). The assay is based on the reaction of thiols with 5,5′-dithiobis-2-nitrobenzoic acid (DTNB; chromogen). DTNB reacts with the thiols present in the solution, generating a coloured complex, whose intensity is directly proportional to the concentration of thiols (Ellman, 1959). The absorbance was read at 405 nm. 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 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 as elsewhere (Alonso-Alvarez et al., 2004). The absorbance was read at 450 nm. Concentrations were calculated using a calibration curve of lutein (α-carotene-3,3′-diol; Sigma-Aldrich) 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) 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 ($P \geq 0.11$). Similar results (data not shown; hydroperoxides, protein carbonyls, thiols and uric acid were again the only variables to be significant) were obtained by 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, instead

of using percentage changes; 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, s.d.=1) of within-individual change values among groups, by fitting multivariate (M)ANOVA with treatment, sex and their interaction as fixed factors. In preliminary analyses, we also ran multivariate generalized linear mixed models using Markov chain Monte Carlo methods (MCMCglmm package) in R (R Development Core Team, 2012) 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 [s.d.$_{rel}$(λ)=$\sqrt{Var(\lambda)}/\sqrt{N-1}$] of the correlation matrix (see Pavlicev et al., 2009; Haber, 2011): it is calculated as the ratio of the square root of the variance of eigenvalues (λ) 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 RBCs (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 supplementary material Table S1 we report the raw values as means ± s.e.m. to show the pre- and post-treatment values 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 ($P \geq 0.11$).

Compared with CF birds, 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 decrease in thiols than MF birds; hydroperoxides were only marginally higher in HF than in 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, $P \geq 0.22$).

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

Multivariate response and integration of response to flight regime
The MANOVA 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 [s.d.$_{rel}$(λ)=0.276] and MF [s.d.$_{rel}$(λ)=0.259] birds, but lower in HF [s.d.$_{rel}$(λ)=0.205] birds (Fig. 2). A similar pattern was found for the covariance in changes in redox state of plasma and RBCs: 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 RBCs 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 Sőti, 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 ageing 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 the percentage within-individual change over the experimental period) in the HF birds. This resulted in a lower redox integration index in these individuals. A reduced correlation among biochemical measurements in the HF compared with the CF and MF 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 RBCs. While the covariation between plasma and blood cells in control birds was high and comparable to findings of a previous study (Costantini et al., 2011), that study looked only at baseline measurements 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. First, 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 HF birds, which could have perceived it as an unpredictable and so stressful event. Although in the wild, zebra finches can fly tens of kilometres per day, under our captive conditions the physical activity of birds is usually low (over our experimental period MF and HF birds were flying around 6 and 16 times, respectively, the distance of CF 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 HF birds may have been too great
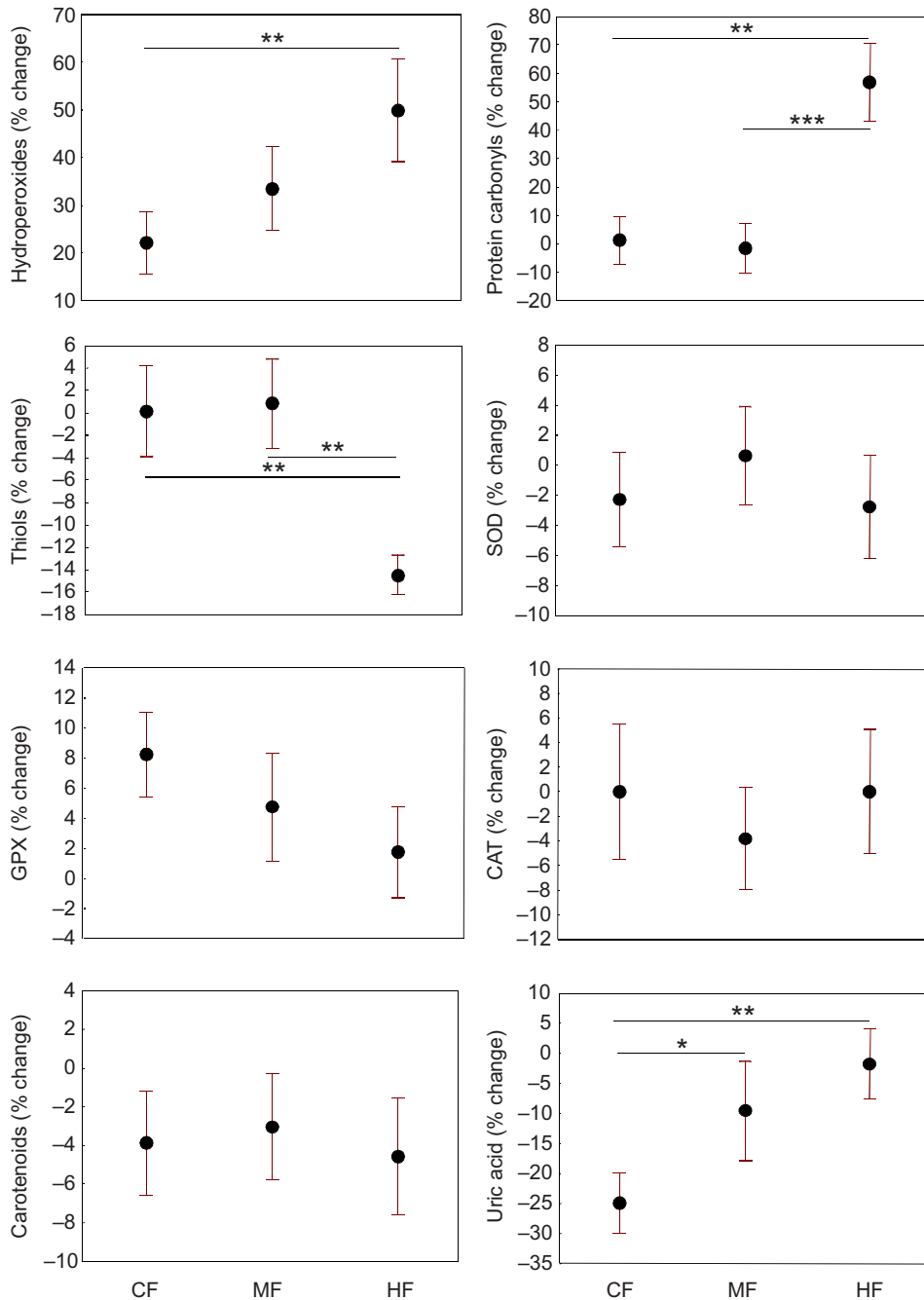
Fig. 1. Response to the experimental treatments, expressed as the percentage within-individual change over the experimental period. Values are shown as means ± s.e.m. However, *post hoc* comparisons are based on least-squares means. Significant differences among groups are indicated by asterisks: *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$. CF, control flight; MF, mild flight; HF, high flight; GPX, glutathione peroxidase; SOD, superoxide dismutase; CAT, catalase.

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 the 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 to interact with other molecules.

A second explanation is that HF birds deprioritized 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 the 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 simulated similar conditions to those 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 defences did not decrease as in Wiersma and colleagues' study (Wiersma et al., 2004) might also suggest that the lack of an

Table 1. Results of linear mixed effect models of the response to the flight regime (*N*=80 birds)

Variable	Brood	Treatment	Sex	Treatment × sex
Hydroperoxides	$F_{29,47}$=2.88, *P*=0.0006	$F_{2,47}$=4.26, *P*=0.020, effect size=0.72	$F_{1,47}$=6.68, *P*=0.013	$F_{2,45}$=1.59, *P*=0.21
Protein carbonyls	$F_{29,48}$=1.10, *P*=0.37	$F_{2,48}$=4.83, *P*=0.012, effect size=0.77	$F_{1,47}$=0.29, *P*=0.60	$F_{2,45}$=1.13, *P*=0.33
GPX	$F_{29,49}$=1.34, *P*=0.18	$F_{2,47}$=1.40, *P*=0.26	$F_{1,49}$=4.32, *P*=0.043	$F_{2,45}$=2.50, *P*=0.09
SOD	$F_{29,50}$=1.45, *P*=0.12	$F_{2,48}$=0.40, *P*=0.67	$F_{1,47}$=0.15, *P*=0.70	$F_{2,45}$=1.10, *P*=0.34
CAT	$F_{29,50}$=1.32, *P*=0.19	$F_{2,47}$=0.48, *P*=0.62	$F_{1,49}$=0.38, *P*=0.54	$F_{2,45}$=0.04, *P*=0.96
Thiols	$F_{29,48}$=1.42, *P*=0.14	$F_{2,48}$=7.16, *P*=0.002, effect size=0.92	$F_{1,47}$=0.12, *P*=0.73	$F_{2,45}$=0.69, *P*=0.51
Uric acid	$F_{29,48}$=2.21, *P*=0.007	$F_{2,48}$=4.89, *P*=0.012, effect size=0.78	$F_{1,47}$=0.01, *P*=0.92	$F_{2,45}$=1.65, *P*=0.20
Carotenoids	$F_{29,50}$=3.87, *P*<0.001	$F_{2,47}$=0.14, *P*=0.87	$F_{1,49}$=1.07, *P*=0.30	$F_{2,45}$=0.90, *P*=0.41

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.

additional effort [i.e. chick rearing as in Wiersma et al. (Wiersma et al., 2004)] or the younger age of our birds could have made the overall effort less demanding for the birds.

Finally, as 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 of 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 and colleagues (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 is to some extent supported by the fact that the HF birds appear to have two independent modules connecting redox components rather than a single one (see Fig. 2), and that the links between levels of hydroperoxides, GPX and protein carbonyls became significant only in the HF birds. GPX 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 the activity of GPX; see pp. 110–117 of Halliwell and Gutteridge (Halliwell and Gutteridge, 2007)] were not linked to those of GPX 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 GPX, hence resulting in a lack of significant correlation between them. It is also unclear why the three antioxidant enzymes (GPX, SOD, CAT), whose functions are related to each other (Pamplona and Costantini, 2011), did not cluster together. It is also worth mentioning that in both the CF and MF 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 HF birds. In birds, flight can increase the protein catabolism of muscle fibres (Bordel and Haase, 2000; Jenni et al., 2000; McWilliams et al., 2004), 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 HF birds, the plasma concentration of uric acid did not decrease over the trials as it did in the CF (and to a lesser extent MF) birds, because it was
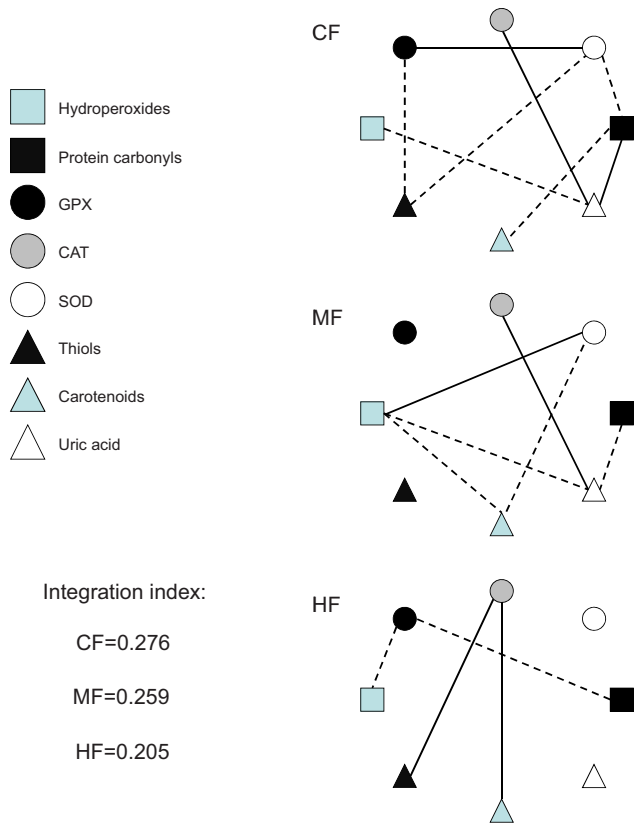

Fig. 2. Network model drawn to show the connections between the eight redox variables measured in the blood; networks are presented separately for the three treatment groups (CF, control flight; MF, mild flight; HF, high flight). Each node of the network refers to one variable. Significant Pearson correlations between standardized 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. GPX, glutathione peroxidase; SOD, superoxide dismutase; CAT, catalase.

Hydroperoxides

Protein carbonyls

GPX

CAT

SOD

Thiols

Carotenoids

Uric acid

Integration index:

CF=0.276

MF=0.259

HF=0.205

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 CAT and uric acid in CF and MF (but not HF) birds. The positive link between CAT and uric acid might reflect the higher need of CAT 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 hydroxyl 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, it is possible that this is 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, as MF birds did not experience any reduction in damage or increase in antioxidant defences 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 previously been demonstrated in captive zebra finches engaged in short and repeated flights accompanied by around 500 takeoffs in 8 h (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 with 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 have shown 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 also be important to look at how increases or decreases in biochemical integration of the oxidative stress response impinge on Darwinian fitness.

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Table S1. Descriptive statistics of biochemical variables collected. Units of measurements: protein carbonyls as nmol mg^{-1} protein; uric acid as mg dl^{-1} of plasma; carotenoids as µg ml^{-1} of plasma; thiols as µmol l^{-1} of –SH groups; hydroperoxides as mmol l^{-1} of H$_2$O$_2$ equivalents; glutathione peroxidase as Units l^{-1} of haemolysate; superoxide dismutase as U ml^{-1} of haemolysate; catalase as U ml^{-1} of haemolysate.

	Pre-Treatment value mean	Pre-Treatment value standard error	Post-Treatment value mean	Post-Treatment value standard error
	Protein carbonyls	Protein carbonyls	Protein carbonyls	Protein carbonyls
Control Flight	6.88	0.67	6.88	0.76
Mild Flight	8.25	1.00	7.94	1.26
High Flight	7.66	0.75	10.53	0.92
	Uric acid	Uric acid	Uric acid	Uric acid
Control Flight	12.85	0.67	9.01	0.44
Mild Flight	13.16	0.72	10.90	0.72
High Flight	12.40	0.64	11.73	0.68
	Carotenoids	Carotenoids	Carotenoids	Carotenoids
Control Flight	32.17	1.94	30.31	1.64
Mild Flight	29.62	2.04	28.05	1.80
High Flight	31.97	2.42	30.03	2.29
	Thiols	Thiols	Thiols	Thiols
Control Flight	20888.50	803.94	20525.79	884.79
Mild Flight	22014.07	995.83	21571.77	808.05
High Flight	24022.20	951.26	20453.60	832.13
	Hydroperoxides	Hydroperoxides	Hydroperoxides	Hydroperoxides
Control Flight	0.53	0.03	0.64	0.05
Mild Flight	0.50	0.03	0.66	0.05
High Flight	0.48	0.04	0.65	0.05
	Glutathione peroxidase	Glutathione peroxidase	Glutathione peroxidase	Glutathione peroxidase
Control Flight	1447.50	50.23	1551.45	52.31
Mild Flight	1517.38	54.92	1567.06	58.70
High Flight	1543.89	46.33	1554.25	46.18
	Superoxide dismutase	Superoxide dismutase	Superoxide dismutase	Superoxide dismutase
Control Flight	445.28	12.23	429.93	13.47
Mild Flight	447.06	14.01	442.74	12.47
High Flight	426.91	11.09	411.79	14.16
	Catalase	Catalase	Catalase	Catalase
Control Flight	429.10	60.04	410.61	56.10
Mild Flight	483.50	55.98	456.15	51.07
High Flight	417.39	32.41	413.33	39.96