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RESEARCH ARTICLE

Acute effects of intense exercise on the antioxidant system in birds: does exercise training help?

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ABSTRACT

The acute effects of an energy-intensive activity such as exercise may alter an animal's redox homeostasis, although these short-term effects may be ameliorated by chronic exposure to that activity, or training, over time. Although well documented in mammals, how energy-intensive training affects the antioxidant system and damage by reactive species has not been investigated fully in flight-trained birds. We examined changes to redox homeostasis in zebra finches exposed to energy-intensive activity (60 min of perch-to-perch flights twice a day), and how exercise training over many weeks affected this response. We measured multiple components of the antioxidant system: an enzymatic antioxidant (glutathione peroxidase, GPx) and non-enzymatic antioxidants (measured by the OXY-adsorbent test) as well as a measure of oxidative damage (d-ROMs). At no point during the experiment did oxidative damage change. We discovered that exposure to energy-intensive exercise training did not alter baseline levels of GPx, but induced exercise-trained birds to maintain a higher non-enzymatic antioxidant status as compared with untrained birds. GPx activity was elevated above baseline in trained birds immediately after completion of the second 1 h flight on each of the three sampling days, and non-enzymatic antioxidants were acutely depleted during flight after 13 and 44 days of training. The primary effect of exercise training on the acute response of the antioxidant system to 2 h flights was increased coordination between the enzymatic (GPx) and non-enzymatic components of the antioxidant system of birds that reduced oxidative damage associated with exercise.

KEY WORDS: Reactive species, Glutathione peroxidase, Non-enzymatic antioxidants, Zebra finch, Flight, Hormesis

INTRODUCTION

Animals must engage in energy-intensive activities (e.g. migration, escape from predators, reproduction) to enhance their survival and fitness. These activities increase metabolic rate, which can potentially lead to an increase in reactive species (RS) generation, and an increased need for antioxidant defenses to protect against damage to cells, tissues and organs (Beaulieu et al., 2011; Cooper-Mullin and McWilliams, 2016; Halliwell and Gutteridge, 2007; Morosinotto et al., 2018; Pingitore et al., 2015). Antioxidant defenses are multifaceted, and include enzymatic antioxidants, micromolecular sacrificial molecules and dietary antioxidants, and are ubiquitous across most taxa (Cohen and McGraw, 2009; Cooper-Mullin and McWilliams, 2016; Halliwell and Gutteridge, 2007). However, studies that assess how the antioxidant system reacts to energy-intensive activities have demonstrated a wide variety of responses, even when examining the same type of energy-intensive activity (Hõrak and Cohen, 2010). For example, activation of the immune system via an injection of phytohemagglutinin increased lipid peroxidation and total antioxidant defenses in greenfinches (*Chloris chloris*) (Hõrak et al., 2007), whereas challenging the immune system by injecting a bacterial lipopolysaccharide reduced the antioxidant barrier in the blood of zebra finches (*Taeniopygia guttata*) (Bertrand et al., 2006). These antioxidant defenses may respond differently depending on which aspect of the antioxidant system is measured, the type, duration or fuel used for an activity, the type of damage, and an animal's physiological state (Cohen and McGraw, 2009; Costantini et al., 2011a; Halliwell and Gutteridge, 2007; Jenni-Eiermann et al., 2014; Skrip and McWilliams, 2016). Particularly lacking are studies that identify which key components of the antioxidant system [i.e. sacrificial molecules such as uric acid, endogenous antioxidant enzymes such as glutathione peroxidase (GPx) and non-enzymatic antioxidants (OXY)] predictably respond to, interact with or are unresponsive to certain challenges and activities (Cooper-Mullin and McWilliams, 2016).

On a short time scale, energy-intensive activities may alter an animal's redox homeostasis, although these acute effects may be alleviated by chronic exposure to that activity, or training, over a longer period of time. Numerous studies in mammals have demonstrated that intense exercise can induce acute oxidative stress, but regular exercise training can reduce exercise-induced damage by increasing endogenous antioxidant capacity (Gomes et al., 2012; Gomez-Cabrera et al., 2008; Jackson, 2005; Nikolaidis et al., 2012; Pingitore et al., 2015; Smarsh and Williams, 2016; Sun et al., 2010; Vaanholt et al., 2010). In contrast, only a few studies have examined the effects of exercise training in other taxa. Southern corroboree frogs (*Pseudophryne corroboree*) given dietary carotenoids had improved exercise endurance during initial aquatic and terrestrial escape-response trials, but not after five consecutive weeks of repeated escape-response trials (McInerney et al., 2017; Silla et al., 2016). A comparison of several species of elasmobranch and teleost fishes that differed in their swimming ability and activity revealed that those capable of reaching faster swimming speeds had higher antioxidant enzyme activities than species with lower swimming capacities (Vélez-Alavez et al., 2015). These studies indicate that exercise training can beneficially stimulate multiple components of the antioxidant system of at least a few species of running or swimming vertebrates, although no such studies on antioxidant responses to flight training have been conducted to date on volant vertebrates.

Flying birds offer a particularly interesting suite of species for investigating the immediate effects of exercise on the antioxidant system and the potential benefits of exercise training. Flapping

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flight often increases metabolic rate up to 30 times basal metabolic rate (Nudds and Bryant, 2000; Tatner and Bryant, 1986; Wikelski et al., 2003), with associated increases in acute RS production (Costantini, 2014; Costantini et al., 2010; Jenni-Eiermann et al., 2014). In addition, passerines primarily use fat as fuel during flights (Bairlein, 1990; Hambly et al., 2002; Jenni and Jenni-Eiermann, 1998), particularly during migration, when the capacity for fat metabolism is highest (DeMoranville et al., 2019; Gerson and Guglielmo, 2013; Jenni-Eiermann et al., 2002; McWilliams et al., 2004; Price et al., 2011). Relying on fat as fuel may also increase RS production as fats are catabolized (Costantini et al., 2007; Skrip et al., 2015). Studies of free-living migratory birds have demonstrated that the antioxidant system of birds is quite dynamic during the alternating bouts of flying and fasting, resting and feeding that are typical for most migrating birds (Cooper-Mullin and McWilliams, 2016; Costantini, 2008). For example, protein oxidative damage was high in red blood cells from European robins (*Erithacus rubecula*) captured during a nocturnal migratory flight as compared with resting or foraging individuals, but actively migrating robins also had higher levels of GPx, an antioxidant enzyme (Jenni-Eiermann et al., 2014). In contrast, serum non-enzymatic antioxidant capacity did not differ between freshly arrived and rested garden warblers (*Sylvia borin*) at a spring stopover site (Skrip et al., 2015) and did not change before or after flights in northern bald ibis (*Geronticus eremita*) trained to follow an ultra-light aircraft during migration (Bairlein et al., 2015). In the only study to directly test the relationship between training and oxidative damage, Larcombe et al. (2010) found that takeoff flight training reduced circulating lipid damage in captive budgerigars (*Melopsittacus undulates*) compared with a single bout of exercise. Thus, the immediate effect of flight on the antioxidant system remains equivocal, the effect of flight training on the antioxidant system is largely untested, and how the collective action and integration of key components of the antioxidant system responds to the production of RS during acute and trained flight remains an open question.

In this study, we exercised zebra finches, *Taeniopygia guttata* (Vieillot 1817), for 2 h each day for ≥6 weeks to determine how key components of the circulating antioxidant system respond to the acute challenge of such daily flight, as well as how such exercise training over many weeks influences this response. We evaluated the following hypotheses and predictions. Hypothesis 1 – training effect: exercise training alters baseline levels of enzymatic and non-enzymatic antioxidants. Hypothesis 2 – acute exercise effect: short-term intense exercise alters the antioxidant system, and the magnitude or direction of the acute change associated with a given exercise bout will be affected by training. Hypothesis 3 – inevitable damage: short-term intense exercise causes oxidative damage in part because the pace of the antioxidant system response is too slow. Hypothesis 4 – coordinated antioxidant response: within individuals, different aspects of the antioxidant system should work in concert and acute changes in enzymatic antioxidants should be correlated with changes in non-enzymatic antioxidants.

MATERIALS AND METHODS

Exercise training

The zebra finches used in this experiment were part of a larger experiment on lipid turnover rate in exercised and non-exercised birds (Carter et al., 2018, 2019), and were acquired from a known-age captive population of birds at Sacred Heart University, CT, USA. All care and experimental procedures were reviewed and approved by the University of Rhode Island's Institutional Animal Care and Use Committee under protocol AN11-12-009. Zebra finches are nomadic in the wild, may travel long distances across the interior of Australia to find suitable breeding conditions (Griffith and Buchanan, 2010; Mariette and Griffith, 2012) and are relatively easily trained to fly in small groups within aviaries (Carter et al., 2018, 2019; Griffith and Buchanan, 2010; Skrip and McWilliams, 2016). Prior to exercise training, 65 zebra finches were kept in same-sex aviaries (2.1×0.9×1.8 m L×W×H) for 8 weeks on a 14 h:10 h light:dark cycle under full-spectrum light, with lights on at 06:00 h. Birds were banded with an aluminium numbered band on their right leg for individual recognition, with one color band on the left leg to indicate treatment (trained, red; untrained, green). During these 8 weeks, birds were acclimated to the aviaries and to a standard mixed-seed diet (Hagen #B2405, Mansfield, MA USA) primarily composed of millet (*Pennisetum glaucum*) supplemented with fresh kale, a source of ascorbic acid and α-tocopherol, once weekly (Podsędek, 2007).

Birds were randomly assigned to an exercise-trained treatment group (n=33: 16 males, 17 females) or an untrained, sedentary group (n=32: 15 males, 17 females). The exercise-trained zebra finches were subjected to two 1 h periods of stop-and-go perch-to-perch flights (11:00 h–12:00 h and 13:30 h–14:30 h) in a 6×3×2 m (L×W×H) flight arena (for further details, see Bauchinger et al., 2010; Skrip et al., 2016; Carter et al., 2018). The birds flew to and from two perches 4 m apart in opposite corners of the arena, with a cloth wall partially dividing the arena in the middle. A handler walked clockwise around the arena during flight training sessions for 300 laps per hour while tracking the number of circuits with a hand counter. This resulted in about 1200 flights of at least 4 m per bird each day, or approximately 4.8 km day^{-1} (or 2.4 km h^{-1}). Short-burst flights of this type incur energetic costs that are approximately 3 times higher than sustained flight in small songbirds (Nudds and Bryant, 2001), and respiratory quotients for zebra finches exposed to perch-to-perch flights were indicative of primarily burning fats (0.75±0.01; Nudds and Bryant, 2001), a fuel type that increases the potential for oxidative damage (Skrip and McWilliams, 2016). However, short-burst exercise training was previously demonstrated to decrease oxidative damage in the plasma of captive budgerigars (Larcombe et al., 2008). During the two 1 h exercise periods, we removed food and water from the cages housing untrained birds to ensure both treatment (exercise-trained) and control (untrained sedentary) birds were similarly fasted. Although not subjected to perch-to-perch flight training, untrained birds were allowed to voluntarily move and fly unrestricted in their same-sex aviaries (2.1×0.9×1.8 m L×W×H). All birds were measured at the beginning of the experiment (tarsus, wing chord, mass and fat), and condition indices (fat and mass) were measured once a week to monitor their health. Trained birds in this study were exercised every day for 44 days.

Blood sampling

To examine changes in the oxidative status of finches before, during and after the 44 days of exercise training, we randomly selected 10 male zebra finches from the untrained group and 10 male zebra finches from the trained group for blood sampling. Using blood allowed us to take repeated measures from the same individual over time, and previous work in mammals has indicated that although the magnitude of the effect of exercise on the antioxidant system likely varies depending on the tissue measured, acute exercise still alters redox homeostasis in almost every fluid, blood cell, tissue and organ regardless of large differences in basal rate of RS generation among tissues (Nikolaidis et al., 2012). We sampled only males to control

for any sex differences (Isaksson, 2013; López-Arrabé et al., 2018), and to avoid confounding problems with reproduction, as females can deposit antioxidants into their eggs (Skrip et al., 2016). We obtained blood samples (200 µl) at days 0–1, 12–13 and 43–44 of exercise training from the same individuals prior to training in the morning (08:00 h) to obtain baseline measurements of antioxidant status, and again within 10 min after training in the afternoon (14:30 h) to examine acute changes in antioxidants and damage. The untrained and trained birds were sampled on two subsequent days so that all birds could be bled within 10 min of entering the room to control for possible short-term increases in blood metabolites associated with our activities. On days 0–1 and 12–13 of the exercise regime, we took blood samples from the same 10 untrained and trained zebra finches before and immediately after exercise. On days 43–44, we took blood samples from 5 of the original 10 trained and untrained birds, as the other 5 birds from each group had been randomly selected to be sampled as part of a larger experiment (Carter et al., 2018). All blood samples were centrifuged within 10 min of collection to separate the red blood cells from the plasma and were stored at −80°C until further analysis.

Analysis of oxidative status

We measured several indicators of antioxidant status and oxidative damage in blood of these birds including non-enzymatic antioxidant capacity (OXY), GPx (an important enzymatic antioxidant) (Cooper-Mullin and McWilliams, 2016; Costantini et al., 2011b; Masaki et al., 1998) and reactive oxygen metabolites (ROMs). Non-enzymatic antioxidant capacity was measured with the OXY-adsorbent test (OXY) in the plasma (concentration unit: mmol l^{-1} of HClO neutralized; Diacron International, Grosseto, Italy). OXY directly measures the ability of a plasma sample to quench the oxidant hypochlorous acid and provides an index of non-enzymatic antioxidant capacity, without being complicated by inclusion of uric acid (Alan and McWilliams, 2013; Costantini, 2011; Skrip and McWilliams, 2016). GPx activity in red blood cells was measured indirectly via a coupled reaction with glutathione reductase following the manufacturer's protocol (concentration unit: nmol min^{-1} ml^{-1}; Cayman Chemical glutathione peroxidase assay kit). Oxidized glutathione produced upon reduction of hydroperoxides by GPx is recycled to its reduced state by glutathione reductase and NADPH. The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm. The rate of decrease in A_{340} is directly proportional to the GPx activity (Arazi et al., 2017; Celi et al., 2013).

Oxidative damage was measured using the d-ROMs test (concentration unit: mmol l^{-1} H_2O_2 equivalents; Diacron International). This test works by first decreasing the pH of the plasma to release metal ions from proteins to cleave circulating ROMs through incubation with a solution of 0.01 mol l^{-1} acetic acid/sodium acetate buffer. The subsequent products react with a chromogen (*N*,*N*-diethyl-*p*-phenylenediamine) which has a color intensity that is proportional to the concentration of ROMs in the plasma and was measured at 505 nm (Costantini, 2016; Costantini et al., 2007). ROMs measured in this test are primarily hydroperoxides, which are produced when RS interact with many different biological macromolecules (Costantini, 2016), but in plasma, are primarily produced during lipid oxidation events (Davies, 2016; Ito et al., 2017), and are correlated with levels of circulating isoprostanes, an end product of lipid peroxidation events (Lubrano et al., 2002). As hydroperoxides are precursors of lipid peroxidation products such as malondialdehyde or isoprostanes (Halliwell and Gutteridge, 2007), they are more likely to reflect a whole-animal response to changes in RS as they occur earlier in the oxidative cascade than those end products.

OXY and d-ROM tests were run in duplicate and the GPx test was run in triplicate. Sample sizes reported in the figure legends indicate individuals with sufficient repeatability among samples to be included in the analysis (coefficient of variation, CV<9%).

Statistics

We used R version 3.6.0 (http://www.R-project.org/) with the nlme package (https://CRAN.R-project.org/package=nlme) to perform linear mixed effects analyses for longitudinal data to determine the influence of treatment group (trained versus untrained), blood sampling stage and their interaction for each of the three oxidative parameters: plasma non-enzymatic antioxidant capacity (OXY), GPx activity in red blood cells and plasma oxidative damage (d-ROMs). The best model for each oxidative parameter was chosen based on Akaike's information criterion, and then fitted with restricted maximum likelihood (Bolker et al., 2009). We first inspected whether measures of condition (mass or fat score) changed within individuals and among flight groups over the experiment. We then examined the change in baseline (pre-flight levels) across the experiment to determine the effect of training on all the baseline oxidative parameters. We also examined the change from baseline within a training day (post-flight minus pre-flight levels). As random effects, we included intercepts for the individual bird. As our sampling time points were not evenly spaced, we used the autocorrelation structure of order 1, with a continuous time covariate (corCAR1). We visually inspected residual plots, and this did not reveal any noticeable deviations from homoscedasticity or normality. We used Pearson correlations to examine how changes in GPx during a given flight were associated with changes in non-enzymatic antioxidants, and how that relationship was affected by exercise training.

RESULTS

Changes in mass and fat score over time were not different among untrained and trained zebra finches throughout the experiment (mass: flight day, $F_{8,59}$=5.22, P=0.01; training group, $F_{1,15}$=0.46, P=0.51; interaction, $F_{8,59}$=0.87, P=0.55; fat score: flight day, $F_{8,59}$=2.00, P=0.06; training group, $F_{1,15}$=0.35, P=0.56; interaction, $F_{8,59}$=1.46, P=0.19). Additionally, including these factors in our models of antioxidant capacity or oxidative damage did not change our results. Therefore, we excluded these condition measures as covariates in our final analyses of oxidative status.

Hypothesis 1 – training effect

Baseline GPx activity does not change during training

Baseline GPx activity did not change over time in untrained or trained zebra finches throughout the experiment (Fig. 1A: flight day, $F_{2,15}$=2.42, P=0.122; training group, $F_{1,5}$=0.02, P=0.97; interaction, $F_{2,15}$=1.63, P=0.227). Baseline GPx prior to exercise training varied among individuals (range: 935.83–2572.39 nmol min^{-1} ml^{-1}) but was not different among the trained or untrained zebra finches (lsmeans±s.e.m.: trained=1597.30±205.14 nmol min^{-1} ml^{-1}, untrained=1586.17±191.96 nmol min^{-1} ml^{-1}, t=|0.040|, P=0.98).

Baseline OXY is maintained in trained birds

Baseline OXY decreased over time in untrained birds, while it remained unchanged in trained zebra finches throughout the experiment (Fig. 1B: flight day, $F_{2,25}$=5.51, P=0.010; training group, $F_{1,18}$=0.01, P=0.893; interaction, $F_{2,25}$=4.26, P=0.026). Baseline OXY prior to exercise training varied among individuals (range: 198.48–361.76 $mmol^{-1}$ HClO neutralized) but was not

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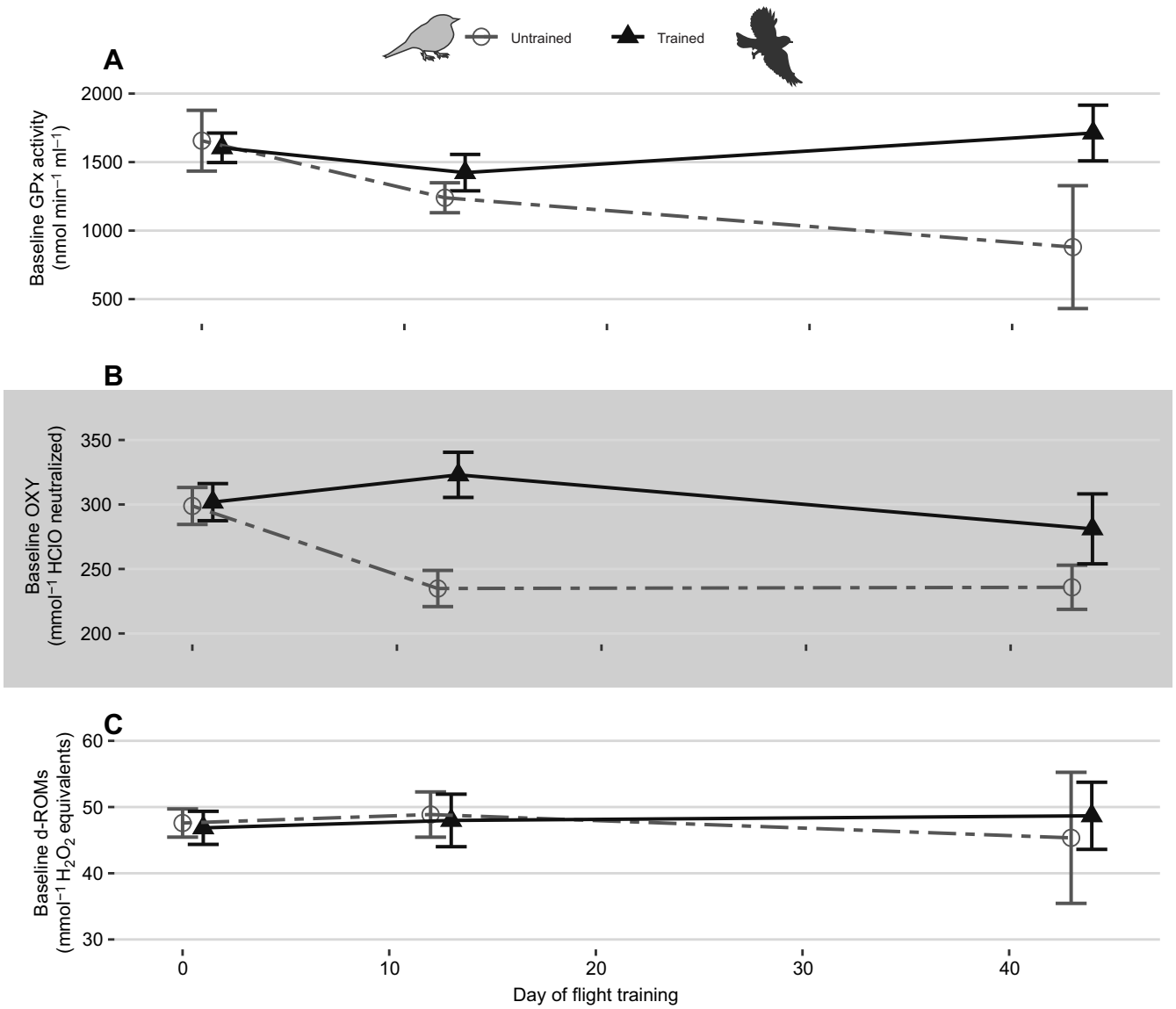

Fig. 1. Baseline changes to the antioxidant system in response to daily exercise training. (A) Baseline enzymatic activity (glutathione peroxidase, GPx) in red blood cells of zebra finches prior to training on day 0–1 (n=10 untrained, n=8 trained), prior to exercise after 12–13 days (n=10 untrained, n=9 trained) and prior to exercise after 43–44 days of training (n=5 untrained, n=5 trained; flight day P=0.122, training group P=0.97, interaction P=0.227). (B) Baseline non-enzymatic antioxidant capacity (OXY) from plasma of zebra finches prior to training on day 0–1 (n=10 untrained, n=10 trained), prior to exercise after 12–13 days (n=10 untrained, n=10 trained) and prior to exercise after 43–44 days of training (n=5 untrained, n=5 trained; flight day P=0.010, training group P=0.893, interaction P=0.026). (C) Baseline reactive oxygen metabolites (d-ROMs) were not significantly different for trained or untrained zebra finches over time (n=10 untrained, n=10 trained; flight day P=0.969, training group P=0.898, interaction P=0.945).

different among trained and untrained birds (lsmeans±s.e.m.: trained=301.81±15.51 mmol^{-1} HClO neutralized, untrained=298.86±15.30 mmol^{-1} HClO neutralized, t=|0.14|, P=0.99).

Baseline ROMs do not change during training

Baseline ROMs (d-ROMs) did not change over time in untrained and trained zebra finches throughout the experiment (Fig. 1C: flight day, $F_{2,17}$=0.03, P=0.969; training group, $F_{1,15}$=0.017, P=0.898; interaction, $F_{2,15}$=0.057, P=0.945).

Hypothesis 2 – acute exercise effect

GPx activity is elevated immediately after flight in trained birds

GPx activity was similarly elevated above baseline in trained zebra finches immediately after completion of the second 1 h flight on each of the three sampling days (Fig. 2A: flight day, $F_{2,13}$=0.54, P=0.593; training group, $F_{1,15}$=23.17, P=0.002; interaction, $F_{2,13}$=3.05, P=0.082). GPx activity did not change during this same time in untrained birds (Fig. 2A).

OXY decreases with acute exercise after training

By 12–13 days of exercise training, non-enzymatic antioxidant capacity was on average lower than baseline in trained zebra finches immediately after completion of the second 1 h flight while it was consistently positive in untrained birds (Fig. 2B: flight day, $F_{2,13}$=1.27, P=0.300; training group, $F_{1,15}$=4.36, P=0.028; interaction, $F_{2,13}$=4.15, P=0.041).

Hypothesis 3 – inevitable damage

ROMs do not change during acute exercise

Reactive oxygen metabolites did not change immediately after flights compared with baseline, or between exercised-trained and

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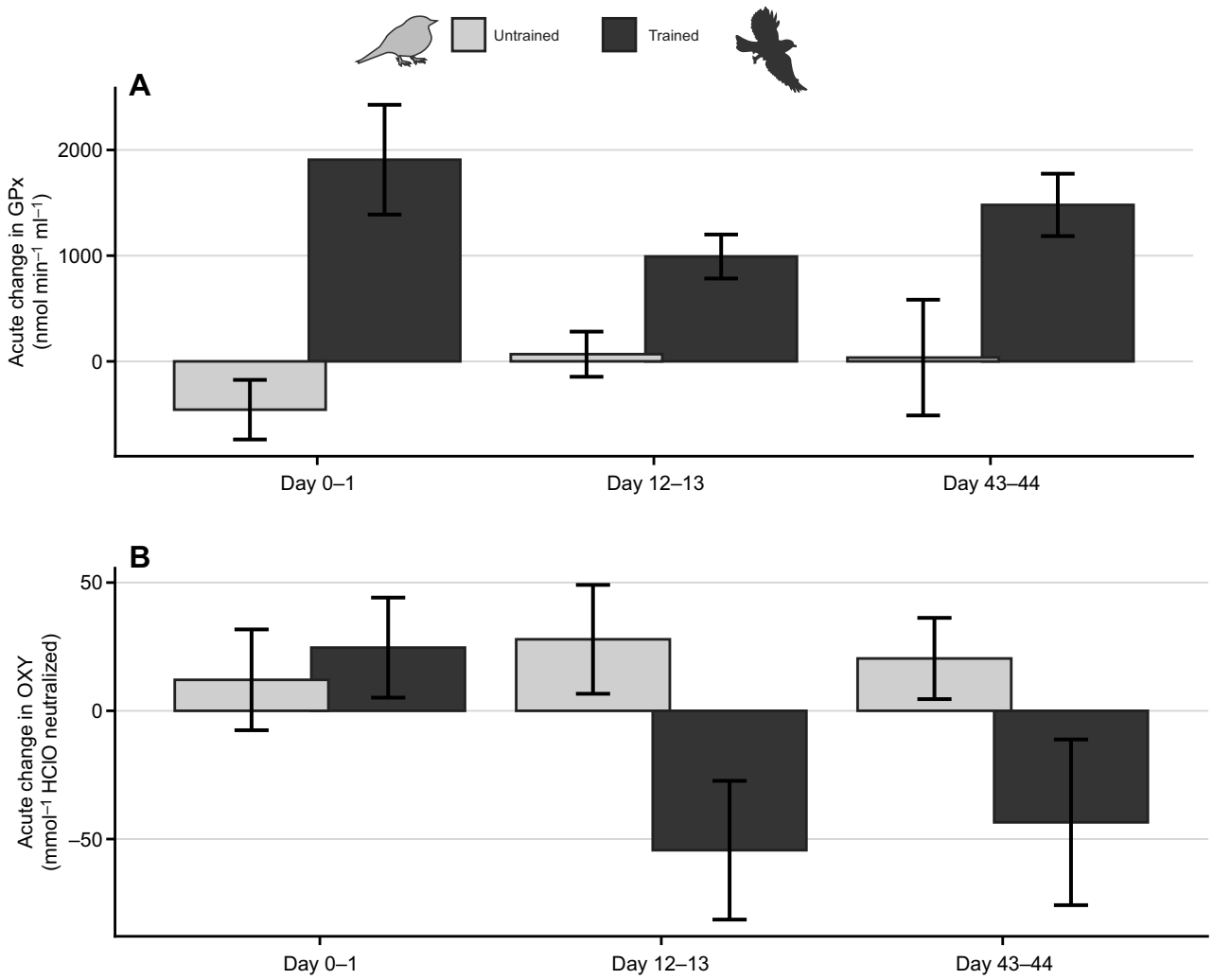

Fig. 2. Acute antioxidant responses to daily exercise training over time. (A) Change in GPx activity in red blood cells immediately after the 2 h flight training compared with baseline on day 0–1 (n=10 untrained, n=8 trained), day 12–13 (n=10 untrained, n=9 trained) and day 43–44 of exercise training (n=5 untrained, n=5 trained; flight day P=0.593, training group P=0.002, interaction P=0.082). (B) Change in non-enzymatic antioxidant capacity in plasma immediately after the 2 h flight training compared with baseline on day 0–1 (n=10 untrained, n=10 trained), day 12–13 (n=10 untrained, n=10 trained) and day 43–44 (n=5 untrained, n=5 trained; flight day P=0.300, training group P=0.028, interaction P=0.041).

untrained sedentary zebra finches (flight day, $F_{2,13}$=0.33, P=0.722; training group, $F_{1,15}$=0.48, P=0.72; interaction, $F_{2,13}$=0.23, P=0.797).

Hypothesis 4 – coordinated antioxidant response

GPx and OXY are negatively correlated after exercise training

Prior to the start of exercise training (day 1), the change in GPx activity immediately after the second 1 h flight compared with baseline was not significantly correlated with the change in non-enzymatic antioxidant capacity (Fig. 3; training day 1, Pearson R^2=0.002). However, after 13 days and 44 days of training, the changes in GPx activity associated with acute flight were negatively correlated with non-enzymatic antioxidant capacity (Fig. 3; training day 13: Pearson R^2=0.763, training day 44: Pearson R^2=0.645).

DISCUSSION

This experiment is one of very few studies of birds to examine changes in an individual's antioxidant system over time, and the first to examine the effects of long-term flight training on multiple aspects of the antioxidant system. We found that zebra finches avoided potential damage associated with acute flight by rapidly and reversibly increasing the activity of GPx and utilizing their non-enzymatic antioxidant capacity during exposure to two 1 h flight bouts per day. Exercise training (≥6 weeks of such daily flights) did not alter baseline levels of these enzymatic (GPx) and non-enzymatic (OXY) components of the antioxidant system. The primary effect of exercise training was an increased coordination of these two key components of the antioxidant system; specifically, the acute increase in GPx associated with acute flight became negatively correlated with that of OXY but only after a couple weeks of training.

Hypothesis 1 – training effect

Our first hypothesis was that energy-intensive training alters baseline levels of enzymatic and non-enzymatic antioxidants. The results for enzymatic antioxidants (GPx) were not consistent with hypothesis 1 whereas those for non-enzymatic antioxidants provided some support for this hypothesis. Daily exercise training did not alter the baseline levels of GPx in individual zebra finches throughout our 44 day experiment. However, trained birds had

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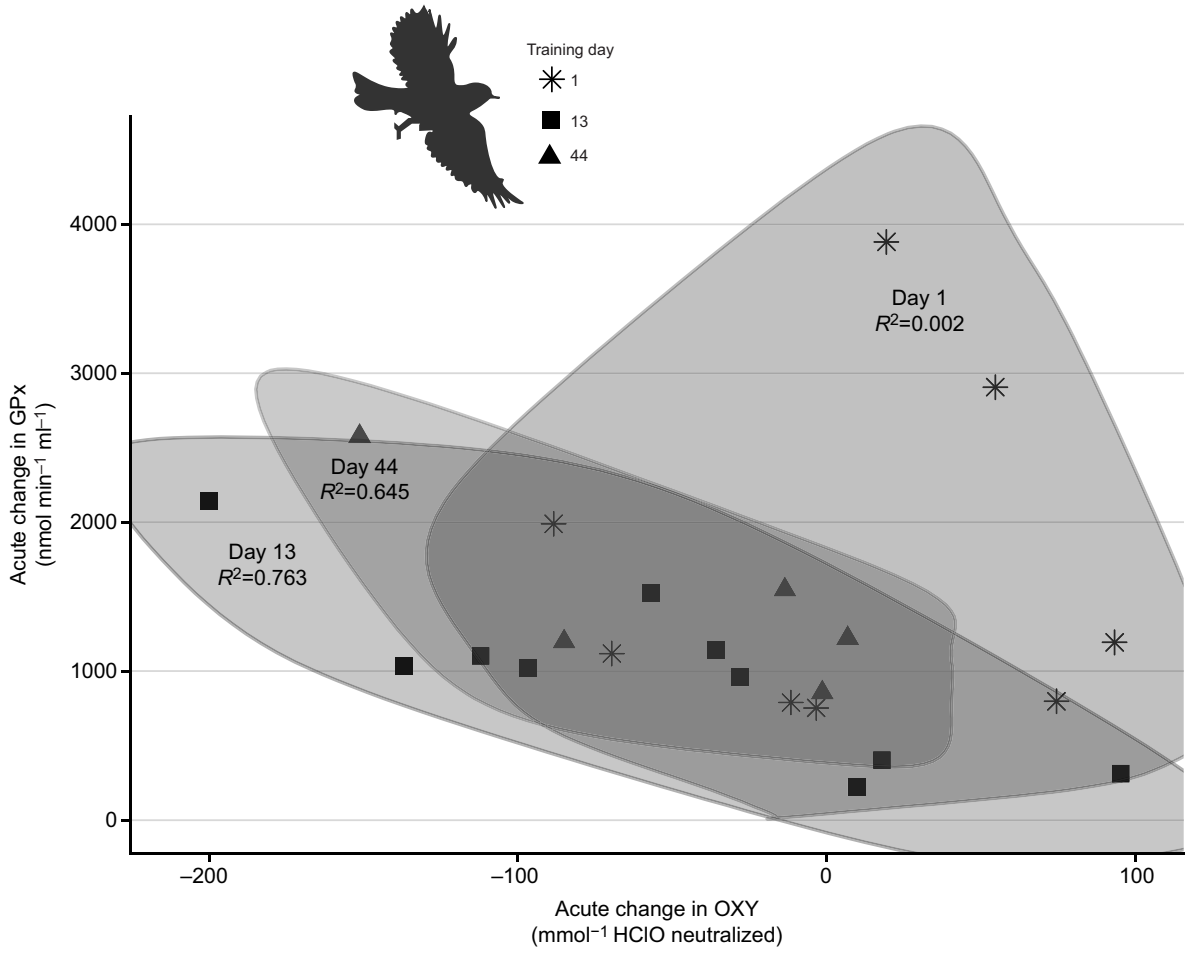

Fig. 3. Correlation between acute changes in GPx activity and OXY over time for zebra finches actively trained in a flight arena. Each symbol represents an individual zebra finch: day 1, stars (n=8); day 13, solid squares (n=9); and day 44, solid triangles (n=5). R^2 values indicate Pearson correlation coefficients. Acute change represents baseline values subtracted from those measured after the 2 h flight. For OXY, positive values indicate an increase in non-enzymatic antioxidant capacity during the 2 h flight, whereas negative values indicate a decrease.

higher levels of OXY compared with untrained birds on day 13 and day 44 of training, indicating that training caused birds to maintain levels of circulating non-enzymatic antioxidants. The drop in baseline OXY in untrained birds was most likely due to natural temporal changes in antioxidant activity, as has been demonstrated in wild birds (Cohen et al., 2008a; Norte et al., 2009; Raja-aho et al., 2012). Diet-independent changes in circulating non-enzymatic antioxidants recorded across 10 species of birds in the wild from June to July in Michigan likely reflect changes in physiology (e.g. circulating hormones, oxidative costs or changes in how antioxidants are stored) among breeding and non-breeding stages (Cohen et al., 2008a). It is possible that the untrained zebra finches in this experiment experienced similar physiological changes even when not exposed to changes in photoperiod or temperature, and that subjecting birds to daily training suppressed those changes. Birds that were trained for more than 1 day maintained their non-enzymatic antioxidant capacity by consistently replenishing it after exercise. These results suggest that birds preserve non-enzymatic antioxidant capacity, but the time scale of this response is relatively slow as birds did not invest in or use OXY until day 13.

Hypothesis 2 – acute exercise effect

Our second hypothesis was that the antioxidant system in birds responds to short-term intense activity and that training changes the magnitude or direction of this response relative to baseline. Consistent with this hypothesis, trained zebra finches increased the activity of their enzymatic antioxidant system (GPx) during exposure to two 1 h flight bouts each day, and as baseline GPx did not change over time, this seemingly returned to baseline by the next morning. Furthermore, training did not alter the magnitude of this acute GPx response. European robins caught during flight also had higher levels of GPx activity compared with birds resting on stopover (Jenni-Eiermann et al., 2014). The rapid up- and down-regulation of GPx activity during flight and after rest suggests that maintaining high levels of GPx may be costly, perhaps as a result of the selenium co-factors needed for GPx synthesis and function (Cockell et al., 1996; Franson et al., 2011; Halliwell and Gutteridge, 2007; Zigo et al., 2017). Broiler chicks reared under constant cold conditions (12–14°C), and therefore unable to rest, increased oxygen requirements by 185%, and had higher GPx activity initially (<3 weeks) followed by a decrease (>3 weeks) (Pan et al., 2005), further supporting the idea that maintaining up-regulated GPx activity was too costly after a certain amount of time.

The consistent upregulation of enzymatic antioxidants (GPx) in zebra finches in response to acute exercise throughout training was different from that documented in exercising mammals (Gomez-Cabrera et al., 2008). Exercise training promotes mitochondrial biogenesis in skeletal muscle of mammals, which results in an

enhanced upregulation of superoxide dismutase and GPx activity with training (Powers and Criswell, 1996; Vilela et al., 2018). In contrast, many wild birds are able to physiologically adjust to changes in exercise levels without the need for training (Dietz et al., 1999; Vézina et al., 2007; Zúñiga et al., 2016). Our results extend this to adjustments associated with a bird's antioxidant system, which can be modulated without the need for exercise training, and are in agreement with previous studies (Jenni-Eiermann et al., 2014). Racing pigeons exposed to a simulated flight by inducing muscle contractions (standardized electric muscle simulation) acutely increased GPx activity (Schoonheere et al., 2009), although whether the pigeons were able to return to pre-exercise levels quickly during rest was not measured. Additionally, reactive species production is generally higher in songbird mitochondria than in rat mitochondria (Barja, 1998; Herrero and Barja, 1997; Perez-Campo et al., 1998), which may in turn have led to different levels and time scales of response of the endogenous antioxidant system to intense exercise.

The time scale and intensity of exercise training are important to consider when examining the functional links among training, metabolic changes and oxidative parameters (Costantini, 2019). Zebra finches that were experimentally manipulated to fly at a pace of $\geq$911 m h^{-1} had increased damage to lipids and proteins, higher uric acid, depleted thiols and no change to enzymatic antioxidants compared with birds flying at a pace of $\leq$55.2 m h^{-1} (Costantini et al., 2013). Our birds experienced a faster pace of exercise during daily flight (2.4 km h^{-1}) and underwent training for a much longer period of time. We found that the time scale on which the antioxidant system responded to exercise training was different for OXY as compared with GPx. Non-enzymatic antioxidant capacity decreased relative to baseline (pre-flight levels) after birds were exposed to two 1 h flight sessions within 13 days of the start of exercise training, while untrained birds increased non-enzymatic capacity over the same time period. Although there may be circadian rhythms associated with antioxidant capacity (Cohen et al., 2008b; Norte et al., 2009), increased non-enzymatic antioxidant capacity from morning to afternoon in untrained zebra finches was likely a byproduct of the two 1 h bouts of fasting, although more work to determine how time of day, food intake and fasting affect circulating non-enzymatic antioxidants in passerines is needed. Our test for non-enzymatic antioxidant capacity (OXY) quantifies the contribution of any antioxidants that react with hypochlorous acid, which includes proteins, thiols, ascorbate, tocopherols and carotenoids (Costantini, 2011), and a decrease in OXY or thiols during flight perhaps indicates that those antioxidants were used and not yet recycled or replaced (Costantini et al., 2013; Skrip and McWilliams, 2016).

Hypothesis 3 – inevitable damage

Our third hypothesis was that energy-intensive activities cause oxidative damage to lipids in birds. Counter to this hypothesis, exposure to two 1 h flight sessions each day did not cause oxidative damage to lipids in zebra finches at any point during training. There are several plausible explanations for why we failed to see changes in oxidative damage: (1) the antioxidant capacity of these birds sufficiently responded to protect against oxidative damage, (2) exposure to two 1 h flights per day was not sufficiently strenuous to cause an increase in RS and thus damage, (3) mitochondrial RS production did not increase proportionally with an increase in oxygen consumption traditionally associated with flight, and (4) an increase in oxygen consumption (state 3 to state 4 energy transition) instead caused a decrease in RS production (Barja, 1998, 2007; Herrero and Barja, 1997; Perez-Campo et al., 1998). However, given that zebra finches increased enzymatic antioxidant capacity during flight training, the first explanation seems most likely although the other three possibilities cannot be fully tested until direct measures of RS production are available and validated for birds (Logan et al., 2014; Salin et al., 2015). Tissues of animals with high rates of RS production should have a high capacity for enzymatic antioxidants to protect against damage from RS (Barja, 2007; Cooper-Mullin and McWilliams, 2016; Costantini, 2014; Skrip and McWilliams, 2016), and any change in antioxidant enzymes reflects a disruption of oxidative status (Costantini, 2019). Our zebra finches increased GPx over a day of flight training, indicating they were likely exposed to a concomitant increase in RS. Although trained and untrained zebra finches had similar body mass and fat reserves at each stage of the experiment, we did not measure changes in body condition over a single day of flight training. Therefore, it is possible that trained birds increased protein catabolism during flight, leading to a concomitant increase in circulating uric acid, an important non-enzymatic antioxidant (Tsahar et al., 2006). An increase in uric acid along with the measured increase in GPx could further explain why we observed no differences in oxidative damage associated with flight.

In contrast to our findings, the few studies to examine oxidative challenges in wild migratory passerines have found increased oxidative damage associated with flight (Jenni-Eiermann et al., 2014; Skrip et al., 2015). However, migratory birds undergo seasonal, photoperiod-induced, physiological changes to enhance fat catabolism and, ultimately, their metabolic output (DeMoranville et al., 2019; Dick, 2017; Guglielmo et al., 2002; Jenni and Jenni-Eiermann, 1998; Price et al., 2011) that may result in an especially high oxidative challenge not experienced by our non-migratory zebra finches. Additionally, how quickly a bird shifts to using fat as fuel may inherently affect the risk of lipid peroxidation associated with flight. In general, birds use glycogen and proteins during the early stages (i.e. in the first ~15–20 min) of a longer-duration flight as lipid metabolism is upregulated (McWilliams et al., 2004). Birds rapidly (<20 min) increase lipid metabolism during these long-duration flights and lipids account for 85–95% of fuel use (Jenni-Eiermann, 2017; McWilliams et al., 2004). Although pigeons exhibit a more gradual shift (1–2 h) to fat oxidation during flight (Schwilch et al., 1996), American robins (*Turdus migratorius*) in migratory condition were primarily burning fat as fuel within 20 min of continuous flight in a wind tunnel (Gerson and Guglielmo, 2013). Importantly, lipid fuels even short-duration hovering flights, measured as peak metabolic rate (Pierce, 2005), and non-migratory, captive zebra finches were relying primarily on fat with some protein catabolism for short-burst flights (Hambly et al., 2002; Nudds and Bryant, 2000, 2001). Therefore, the risk of damage by RS during flight may vary based on the species measured or migratory state, although more studies are needed to explore these impacts.

Hypothesis 4 – coordinated antioxidant system response

Our fourth hypothesis was that different aspects of the antioxidant system should work in concert, and acute changes in enzymatic antioxidants should be correlated with changes in non-enzymatic antioxidants. We found that novel intense exercise (day 1) increased the enzymatic (GPx) but not the non-enzymatic antioxidant system relative to baseline in zebra finches. However, after 13 and 44 days of training, the acute changes in GPx activity and non-enzymatic antioxidant concentration associated with flights became strongly and negatively correlated. Our interpretation of this coordinated response requires making some assumptions about (1) the time

scale over which each antioxidant parameter responds, and (2) the potential prioritization of each response. We found that GPx activity increased immediately after exercise on all days during training and, as baseline GPx did not change over time, seemingly returned to baseline by the next morning. In contrast, trained birds maintained baseline non-enzymatic antioxidants on all days during training, but non-enzymatic antioxidant capacity did not respond to acute exercise until day 13. These results suggest that exercise induces a rapid and prioritized upregulation of GPx activity relative to OXY.

Careful scrutiny of Fig. 3 reveals that after a couple of weeks of exercise training the coordinated response of GPx and OXY to 2 h of flying occurs because individuals that more strongly upregulate GPx also 'use up' more of their non-enzymatic antioxidant capacity, while individuals that less strongly upregulate GPx in response to acute exercise use less of their non-enzymatic antioxidant system. As GPx can be localized inside cells near the mitochondria where most RS are produced (Brigelius-Flohé, 1999; Cooper-Mullin and McWilliams, 2016), it may be beneficial for a bird to invest briefly in a costly enzymatic antioxidant as an immediate response to an energy-intensive activity. After many days of exercise training, birds exposed to the 2 h daily flights apparently invested in this consistent, short-term GPx upregulation and used more non-enzymatic antioxidant capacity, perhaps in response to RS diffusing out of the cells, interacting with other molecules to form lipid peroxidation cascades and accumulating damage.

This experiment is one of very few studies in birds to examine changes in the antioxidant system over multiple time points, and the first to examine the effect of long-term flight training on multiple components of the antioxidant system. Therefore, our experiment provides several insights into how the antioxidant system in songbirds responds to a given energy-intensive activity (120 min of perch-to-perch flights), and how exercise training over many weeks affects this response. The primary antioxidant response to a given energy-intensive activity was, unlike for mammals, to rapidly and reversibly increase antioxidant enzyme (GPx) activity and utilize non-enzymatic antioxidants (OXY). Exercise training for 44 days did not alter pre-flight, baseline levels of GPx, while baseline levels of OXY were maintained in exercise-trained birds compared with untrained birds. The principal effect of exercise training was an increased coordination of these two key components of the antioxidant system; specifically, the acute increase in GPx associated with acute flight became negatively correlated with that of OXY but only after a couple weeks of training.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: C.C.-M., S.R.M.; Methodology: C.C.-M., W.A.C.; Formal analysis: C.C.-M.; Investigation: C.C.-M., W.A.C.; Resources: S.R.M.; Data curation: C.C.-M.; Writing - original draft: C.C.-M.; Writing - review & editing: W.A.C., S.R.M.; Visualization: C.C.-M.; Supervision: S.R.M.; Project administration: S.R.M.; Funding acquisition: S.R.M.

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