

RESEARCH ARTICLE

Flight muscle protein damage during endurance flight is related to energy expenditure but not dietary polyunsaturated fatty acids in a migratory bird

Morag F. Dick^{*,‡} and Christopher G. Guglielmo

ABSTRACT

Migration poses many physiological challenges for birds, including sustaining high intensity aerobic exercise for hours or days. A consequence of endurance flight is the production of reactive oxygen species (ROS). ROS production may be influenced by dietary polyunsaturated fatty acids (PUFA), which, although prone to oxidative damage, may limit mitochondrial ROS production and increase antioxidant capacity. We examined how flight muscles manage oxidative stress during flight, and whether dietary long-chain PUFA influence ROS management or damage. Yellow-rumped warblers were fed diets low in PUFA, or high in long-chain n-3 or n-6 PUFA. Flight muscle was sampled from birds in each diet treatment at rest or immediately after flying for up to a maximum of 360 min in a wind tunnel. Flight increased flight muscle superoxide dismutase activity but had no effect on catalase activity. The ratio of glutathione to glutathione disulphide decreased during flight. Oxidative protein damage, indicated by protein carbonyls, increased with flight duration (Pearson $r=0.4$). Further examination of just individuals that flew for 360 min ($N=15$) indicates that oxidative damage was related more to total energy expenditure (Pearson $r=0.86$) than to flight duration itself. This suggests that high quality individuals with higher flight efficiency have not only lower energy costs but also potentially less oxidative damage to repair after arrival at the destination. No significant effects of dietary long-chain PUFA were observed on antioxidants or damage. Overall, flight results in oxidative stress and the degree of damage is likely driven more by energy costs than fatty acid nutrition.

KEY WORDS: Migration, Oxidative stress, Antioxidants, Songbird, Flight, Wind tunnel, Polyunsaturated fatty acids

INTRODUCTION

Migratory flight requires birds to sustain high metabolic rates for prolonged periods of time. Research into the seasonal changes in the flight muscle that enable birds to accomplish these feats has generally focused on increasing fatty acid transport and oxidation capacity (Guglielmo, 2010). However, migratory flights also pose an oxidative challenge that may shape migration (Jenni-Eiermann et al., 2014). Reactive oxygen species (ROS) and the damage they

can produce are negative consequences of exercise (Ji, 1999; Costantini et al., 2008). Mitochondrial respiration is one source of reactive species, with approximately 0.15% of oxygen consumed being converted into superoxides (St-Pierre et al., 2002). Additional ROS are produced during muscle contraction from NADPH oxidases in the sarcoplasmic reticulum, sarcolemma and transverse tubules (Zuo et al., 2011; Sakellariou et al., 2013; Goncalves et al., 2015). ROS produced during exercise can be important regulators of contractile force, cell signalling pathways and gene expression (Powers and Jackson, 2008; McClung et al., 2010; Powers et al., 2010). However, excessive ROS production can lead to oxidative stress, where the pro-oxidant:antioxidant ratio favours pro-oxidants, causing damage to macromolecules, impaired redox control and changes in cell signalling (Sies and Jones, 2007). Birds must manage ROS produced during flight in order to make non-stop travel lasting up to several days possible. Failure to do so could impair the functioning of contractile machinery and mitochondrial function needed to sustain flight, impacting flight efficiency, endurance capacity and post-flight recovery.


Birds can use endogenous antioxidant enzymes, such as catalase, superoxide dismutase (SOD) and glutathione peroxidase, to defend against ROS (Powers and Jackson, 2008). They may also limit the production of ROS from the mitochondria (Brand, 2000), or produce antioxidants such as glutathione (GSH) or uric acid, a metabolic by-product produced during flight (Tsahar et al., 2006). Dietary antioxidants can also contribute to ROS defenses and may include anthocyanins found in berries eaten by migrating birds, particularly in fall (Schaefer et al., 2008), or excess protein intake from animal prey, which may increase plasma uric acid (Smith and McWilliams, 2009; Alan and McWilliams, 2013; Eikenaar et al., 2016). Birds likely use a mixture of these mechanisms to manage ROS in preparation for and during migratory flight.

Similar to the seasonal upregulation of aerobic and oxidative capacity in the muscle during migration (McFarlan et al., 2009; Zajac et al., 2011), increasing antioxidant capacity may be a key element to migratory preparation. In the flight muscle, Mn-SOD protein content increases during migration (Banerjee and Chaturvedi, 2016). At stopover, birds increase circulating antioxidants in preparation for the next migratory flight as part of the refuelling process (Skrip et al., 2015; Eikenaar et al., 2016). Furthermore, frugivorous birds may preferentially select berries rich in antioxidants (Schaefer et al., 2008). During a migratory flight, glutathione peroxidase activity increases in blood cells (Jenni-Eiermann et al., 2014), and plasma uric acid concentrations may increase in some birds (Schwilch et al., 1996; Gerson and Guglielmo, 2011; Guglielmo et al., 2017). Despite the increase in antioxidant capacity in preparation for flight and further alterations during flight, an overall decrease in plasma antioxidant capacity occurs during flight (Costantini et al., 2007, 2008). The effects of

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oxidative stress during flight are observed from increased protein damage in blood cells (Jenni-Eiermann et al., 2014) and increased concentration of plasma creatine kinase, a marker of muscle damage (Guglielmo et al., 2001). Perturbations to flight muscle mitochondrial function also occur and may decrease ATP production capacity (Gerson, 2012). After flight, birds repair the damage accrued to their mitochondria (Gerson, 2012), replenish their fuel stores and may increase circulating non-enzymatic antioxidant capacity (Eikenaar et al., 2016). Thus, the refuelling process may concurrently aid in the repair of oxidative damage from the previous flight (Skrip et al., 2015). Whether flight performance influences oxidative damage during flight is unknown. In cycling humans, increasing exercise duration and intensity increases protein damage (Goldfarb et al., 2002; Bloomer et al., 2007), which suggests that longer flights or a higher rate of energy expenditure in flight may also increase oxidative damage in birds.

Molecules differ in susceptibility to oxidative damage, depending on their structure and location within the cell. Although polyunsaturated fatty acids (PUFA) have been highlighted for their potential to enhance migratory performance (Price and Guglielmo, 2009; Weber, 2009; Pierce and McWilliams, 2014), they are also prone to oxidative damage because of the lower energy threshold to remove hydrogen from carbons with double bonds. Once a fatty acid loses an electron to a free radical, it becomes a lipid radical and can form a lipid peroxy radical with oxygen, which can then react with another PUFA and further increase damage (Skrip and McWilliams, 2016). Additionally, PUFA are incorporated into mitochondrial membranes, which puts them in close proximity to the site of ROS production (Price, 2010; Skrip and McWilliams, 2016).

Feeding a high PUFA diet to white-throated sparrows (*Zonotrichia albicollis*) increased plasma oxidative damage markers (Alan and McWilliams, 2013). Conversely, in the flight muscle, high PUFA diets decreased the net effect of mitochondrial ROS either through decreased ROS production in the mitochondria via alterations to membrane composition, or through increased antioxidant capacity, resulting in decreased oxidative damage during flight (Gerson, 2012). Although the mechanisms for the effect of PUFA during flight are unknown, oxidative damage to PUFA could initiate cellular signalling. Lipid peroxide products can interact with transcription factors, such as nuclear factor (erythroid-derived 2)-like 2 (Nrf2), causing a signalling cascade that results in the upregulation of endogenous antioxidants (Parvez et al., 2018). In rats, n-3 PUFA supplementation increases basal lipid oxidative damage and the upregulation of endogenous antioxidant enzyme capacity in hearts (Abdukeyum et al., 2016). The increased antioxidant capacity in the supplemented rats at rest meant that during an acute oxidative challenge, the rats were better able to manage ROS production and accrued less damage (Abdukeyum et al., 2016). Potentially, increasing dietary PUFA may increase lipid damage at rest but also increase antioxidant capacity and, as such, lower oxidative damage during endurance flight.

The type of PUFA may also influence this response; the n-3 PUFA docosahexaenoic acid (DHA, 22:6 n-3) increases the mRNA abundance of glutathione peroxidase and SOD in skeletal muscle compared with the n-6 PUFA arachidonic acid (ARA, 20:4 n-6) (Hashimoto et al., 2016). This could be related to the structure of the fatty acid itself, with DHA being more prone to oxidative damage, and differences in the signalling of the lipid peroxide products (Delmastro-Greenwood et al., 2014; Parvez et al., 2018). Interestingly, despite differences in antioxidant enzyme mRNA

abundances, no difference was detected in lipid peroxidation compared with the control (Hashimoto et al., 2016). During migration, increasing dietary PUFA could elicit a complex response. At rest, birds fed high long-chain PUFA diets may have greater oxidative damage, but also greater antioxidant capacity. However, because ROS production is elevated during endurance flight, birds eating a high long-chain PUFA diet may have lower net damage because of the higher endogenous antioxidant capacity.

The majority of studies assessing oxidative balance in migratory birds have used blood samples. Changes in blood markers may not fully represent how birds manage ROS during flight, and the response could differ among tissues. Furthermore, assessing these factors under controlled conditions, where metrics of flight performance can be measured, is critical to understanding the interactions between ROS and energy expenditure and fuel mixture. In the present study, we examined whether dietary DHA and ARA influence antioxidant enzymes and oxidative damage in the flight muscles of migratory yellow-rumped warblers (*Setophaga coronata coronata*) at rest or after simulated migratory flight. We also examined whether antioxidant systems or oxidative damage were related to endurance flight performance (flight duration, energy cost and fuel composition). We predicted that long-chain PUFA would increase antioxidant enzyme activity and damage at rest, but this would help lower oxidative damage during endurance flight. Additionally, we predicted that oxidative damage would increase with the rate of energy expenditure during flight.

MATERIALS AND METHODS

Birds and experimental design

Sixty yellow-rumped warblers [myrtle warblers, *Setophaga coronata coronata* (Linnaeus, 1766)] were caught in October 2013 at Long Point, Ontario, Canada, and brought to the Advanced Facility for Avian Research at the University of Western Ontario. The yellow-rumped warbler was chosen for the study because it is an abundant, boreal-breeding, long-distance migratory species that acclimates very well to captivity, and will eat a wide variety of synthetic diets (Afik et al., 1997; Guglielmo et al., 2017). Crucially, for our study, they have a high individual success rate in wind-tunnel endurance flights, which requires fewer birds being brought into captivity to achieve appropriate sample sizes (Guglielmo et al., 2017). The birds were kept initially on a 12 h:12 h light:dark (12L:12D) photoperiod and switched to a short-day photoperiod (9L:15D) in later November to keep the birds in a non-migratory condition. The birds were fed a synthetic high carbohydrate diet [16.3% dextrose, 3.6% casein (Affymetrix USB, Cleveland, OH, USA), 1.6% agar (Affymetrix USB), Briggs-N-Salt mix (MP Biomedicals, Solon, OH, USA), 1.6% AIN-76 vitamin mix (MP Biomedicals), 72.4% water, 3.1% oil and 0.9% cellulose (MP Biomedicals)]. Before the start of the trial, the diet was made with canola oil. All animal capture, care and procedures followed Canadian Council on Animal Care guidelines and were approved by the University of Western Ontario Animal Use Subcommittee (protocol 2010-216), and by the Canadian Wildlife Service (permit CA-0256).

The experimental phase of the study began in late February 2014 while the birds were on a short-day photoperiod in a non-migratory condition. To standardize the amount of time birds consumed the experimental diets and time on a long-day photoperiod (trigger for migratory condition), we randomly grouped the birds into 10 blocks of six birds, with two birds per diet in each block. One block would start the experimental protocol each week. In week 1, the block of birds was switched onto the three experimental diets differing in fatty

acid composition. We carefully formulated oil blends to enrich diets in n-3 PUFA (DHA, a long-chain n-3 PUFA), n-6 PUFA (ARA, a long-chain n-6 PUFA) or monounsaturated fatty acids (MUFA) (Table 1). At the start of week 3, the birds were switched to long day photoperiod (16L:8D) to trigger a spring migratory condition. In week 6, flight assessments and tissue sampling were conducted on the flown and control birds. Ideally, for each diet in a block, one bird would be sampled as an unflown control, and the other sampled immediately after the flight assessment (flown). Flight assessments were conducted in a specially designed wind tunnel for birds at the Advanced Facility for Avian Research. The birds were individually and voluntarily flown at 8 m s^{-1} , 15°C and 70% relative humidity for a minimum of 54 min and up to 360 min (wind tunnel description in Gerson and Guglielmo, 2011). Full flight energetic results and details are reported in Dick and Guglielmo (2019). Briefly, birds were measured immediately pre and post flight using quantitative magnetic resonance (ECHO-MRI; Guglielmo et al., 2011) to determine body fat and wet lean mass. The loss of fat and lean mass during the flight was used to calculate the energy cost of flight using the conversion factors of 39.6 kJ g^{-1} for fat and 5.3 kJ g^{-1} for wet lean mass (Gerson and Guglielmo, 2011; Jenni and Jenni-Eiermann, 1998). Power (J s^{-1}) was calculated from total energy expenditure divided by flight duration. Relative protein contribution was calculated as the percent of total energy from lean mass catabolism.

The control birds were food-deprived for 2 h before lights out and sampled at the start of the dark period (19:00 h). Because flying in a wind tunnel is voluntary for the birds, if neither bird flew they would both be sampled 2–3 days after a flight attempt. We defined a failed flight as any flight under 30 min in duration. Flights ended when the bird stopped three times within a 5-min period or reached the 360 min mark. In total 24 birds successfully completed wind tunnel flights (flown: n-3 PUFA=7, n-6 PUFA=8, MUFA=9), with five 360-min flights in each group. The remaining birds were sampled as controls (n-3 PUFA=9, n-6 PUFA=12, MUFA=11). Over the course of the study, 11 birds failed their scheduled flight attempt (n-3 PUFA=4; n-6 PUFA=4; MUFA=3). These failed fliers were reassigned to the control group and sampled after a minimum

recovery period of two to three additional days on their experimental diet. Additionally, four preselected control birds were switched into the flown group and successfully completed flights (n-3 PUFA=2; n-6 PUFA=1; MUFA=1). We were unable to identify any physical or physiological factor that could have contributed to the failed flights. There were no significant differences ($P>0.4$) between control birds that were originally designated as a control or that had failed a flight and were switched into the control group. Additionally, no significant differences ($P>0.4$) were observed between flown birds and those that had failed a flight for variables that were not affected by flight.

Immediately at the start of the dark period (control, 19:00 h) or after flight (flown, 21:00–02:30 h), the birds were anaesthetized using inhaled isoflurane (^{PR}Florane) and killed by decapitation. Samples of the adipose, flight muscle, brain and liver were flash-frozen in liquid nitrogen and stored at -80°C for this and additional studies. Flight muscle phospholipid fatty acid profiles were analyzed following Price et al. (2009) and Thomas et al. (2012). A summary of the flight muscle membrane fatty acid profiles is presented in Fig. 1. Full details of the fatty acid analysis and results are reported in Dick and Guglielmo (2019).

Antioxidant enzyme activity

For the catalase and SOD assays, approximately 100 mg of flight muscle tissue was homogenized in 1 ml of cold 20 mmol l^{-1} Hepes buffer (pH 7.2, with 1 mmol l^{-1} EGTA, 210 mmol l^{-1} mannitol and 70 mmol l^{-1} sucrose). The homogenate was centrifuged at $10,000 \text{ g}$ for 15 min at 4°C , and the supernatant was split into two microcentrifuge tubes and stored at -80°C until assayed. Homogenates were diluted with Hepes buffer to approximately 0.1 mg ml^{-1} on the day of assay. Catalase was measured in duplicate using a method modified from Weydert and Cullen (2009). Briefly, $10 \mu\text{l}$ of homogenate was added to $810 \mu\text{l}$ phosphate buffer in a cuvette, and absorbance at 240 nm was measured for 2 min to obtain a background rate (Cary 100, Varian, Inc. Palo Alto, CA, USA). The reaction was initiated by the addition of $180 \mu\text{l}$ of 30 mmol l^{-1} hydrogen peroxide and absorbance was measured for a further 2 min. Catalase activity was calculated from the molar extinction of hydrogen peroxide ($34.9 \text{ l mol}^{-1} \text{ cm}^{-1}$) and corrected for background hydrogen peroxide degradation levels [intra-assay coefficient of variability (CV) 4.26%]. Activity is expressed per milligram of protein, using the protein content of the homogenate measured using the Bradford assay (BioRad Laboratories, Mississauga, ON, Canada) with bovine serum albumin (BSA) as a standard.

SOD activity was measured using a colorimetric SOD assay kit (706002, Cayman Chemical Co., Ann Arbor, MI, USA). The SOD activity assay utilizes a tetrazolium salt to detect the capacity of SOD to dismutate superoxide radicals produced by xanthine oxidase, which also results in the secondary conversion of the tetrazolium salt into formazan dye. The sample homogenates were diluted with the sample buffer (50 mmol l^{-1} Tris-HCl, pH 8.0) to approximately $0.2\text{--}0.25 \text{ mg protein ml}^{-1}$, and $10 \mu\text{l}$ of the diluted homogenate was used per reaction with the samples run in duplicate (inter-assay CV=2.95%, intra-assay CV=2.90%). The prepared plates were incubated with the assay buffer (50 mmol l^{-1} Tris-HCl, pH 8, with 0.1 mmol l^{-1} diethylenetriaminepentaacetic acid and 0.1 mmol l^{-1} hypoxanthine), the tetrazolium salt solution and xanthine oxidase on a shaker for 30 min at room temperature ($\sim 20^\circ\text{C}$) and then read at 460 nm to detect the formazan dye. SOD activity was calculated using the linear regression of the standard curve and expressed per mg of protein.

Table 1. Composition of the dietary oil blends used in the experimental semi-synthetic diets fed to the yellow-rumped warblers

% of dietary oil	MUFA	n-3 PUFA	n-6 PUFA
Olive oil ^a	72	57	58
Coconut oil ^b	28	15	30
ARASCO (20:4 n-6) ^c	0	0	12
DHASCO (22:6 n-3) ^c	0	28	0
Net fatty acid profile ($\text{g } 100 \text{ g}^{-1}$ oil)			
SFA	39.4	39.8	39.8
MUFA	53.8	49.1	47.1
PUFA	6.74	10.2	11.2
n-3 PUFA	0.50	5.02	0.42
DHA (22:6 n-3)	0	4.62	0
n-6 PUFA	6.25	5.14	10.8
18:2 n-6	6.05	4.89	6.74
ARA (20:4 n-6)	0.15	0.25	4.06

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; DHA, docosahexaenoic acid; ARA, arachidonic acid.

^aLoblaws Inc., Toronto, ON, Canada.

^bSpectrum Naturals, Delta, BC, Canada.

^cDSM Nutritional Products Ltd, Kaiseraugst, Switzerland.

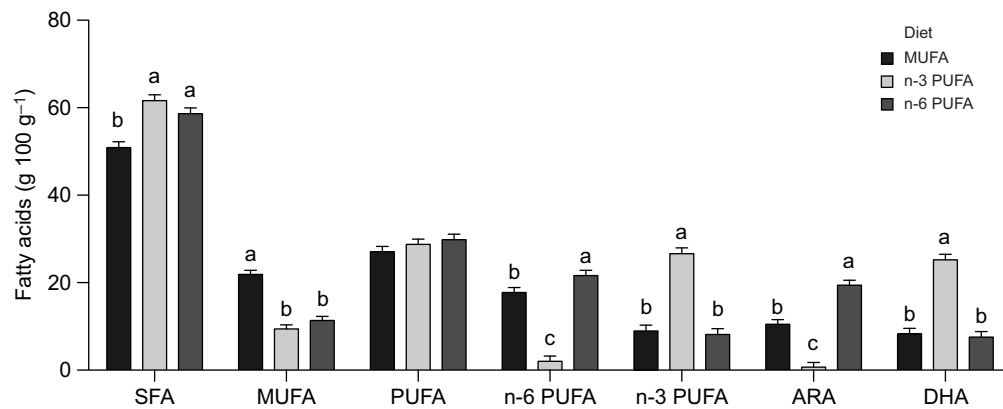


Fig. 1. Fatty acid composition of flight muscle phospholipids of yellow-rumped warblers (*Setophaga coronata coronata*) fed the experimental diets. Values are means \pm s.e.m. Fatty acids with different lowercase letters differ significantly ($P < 0.05$). SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; ARA, arachidonic acid; DHA, docosahexaenoic acid.

Glutathione

The reduced form of GSH and the oxidized disulphide dimer (GSSG) were measured using a glutathione assay kit (Cayman Chemical Co., 703002). Here, total GSH is measured by the reaction of GSH with DTNB, yielding TNB and GSTNB, and GSTNB is recycled back to GSH by glutathione reductase producing another TNB. The inclusion of glutathione reductase also reduces GSSG to GSH, resulting in the measurement of total GSH. To measure GSSG only, 2-vinylpyridine is added to the samples to derivatize GSH and allow only GSSG to be measured. Approximately 60–65 mg of flight muscle tissue were homogenized in nine volumes of 50 mmol l⁻¹ PBS with 1 mmol l⁻¹ EDTA (pH 7.4) and centrifuged at 10,000 g for 15 min at 4°C. A volume of 500 μ l of the supernatant was removed and deproteinated with the addition of 500 μ l of 10% meta-phosphoric acid (MPA). The mixture was allowed to sit at room temperature for 5 min, and then centrifuged for 2 min at 2000 g. The deproteinated supernatant was collected, stored at -20°C and assayed the next day. Prior to assaying, 50 μ l of a 4 mol l⁻¹ triethanolamine was added to the sample. To quantify total GSH concentration, 50 μ l of the deproteinated sample was diluted in 950 μ l MES buffer [pH 6.0, 0.2 mol l⁻¹ 2-(*N*-morpholino)ethanesulphonic acid, 0.1 mol l⁻¹ phosphate, and 2 mmol l⁻¹ EDTA]. For GSSG, 500 μ l of the deproteinated sample was diluted with 250 μ l of MES buffer and incubated with 7.5 μ l of 1 mol l⁻¹ 2-vinylpyridine in ethanol for 60 min to derivatize the GSH in the sample, and GSH standards were treated in the same manner. Samples were run in duplicate according to the kit instructions, and after the addition of the GSH reductase, glucose-6-phosphate dehydrogenase and enzyme co-factors, the microplates were incubated in the dark on a shaker for 25 min and absorbance was measured at 405 nm. Total GSH and GSSG concentrations were calculated using standard curves. GSH concentration was calculated from the difference in total GSH and GSSG concentrations. The intra- and inter-assay CVs were 2.21% and 3.98%, respectively, for total GSH, and 1.11% and 3.07% for GSSG.

Protein carbonyl quantification

Protein carbonyls were measured using a modified method from Levine et al. (1990) with the addition of a chromophore removal step (Barreiro et al., 2005). Briefly, 60 mg of tissue was homogenized in 600 μ l of buffer (50 mmol l⁻¹ PB buffer with 10 mmol l⁻¹ EDTA, pH 7). The homogenate was centrifuged at 10,000 g for 10 min at 4°C, and 500 μ l supernatant was collected and stored at -80°C until assay the next day. To remove chromophores in the homogenate, the 500 μ l supernatant was added to 1.4 ml HCl-acetone (3:100 v/v) and the protein was precipitated. The precipitate was washed twice with 1.6 ml of HCl-acetone, followed by two washes of 1.8 ml 10%

trichloroacetic acid (TCA). Between washes, the samples were centrifuged at 10,000 g for 10 min at 4°C. The pellet was resuspended in 500 μ l of 50 mmol l⁻¹ phosphate buffer with 6 mol l⁻¹ urea. Next, the dechromophored homogenate was divided into two 200 μ l aliquots, and 500 μ l of 10 mmol l⁻¹ 2,4-dinitrophenylhydrazine (DNPH) in 2.5 mol l⁻¹ HCl was added to one aliquot and 500 μ l of 2.5 mol l⁻¹ HCl to the other aliquot to serve as a blank. The samples were incubated in the dark at room temperature for 45 min, and vortexed every 15 min. Following incubation, 1 ml of 20% TCA solution was added and the samples were placed on ice for 10 min before centrifuging at 10,000 g for 10 min at 4°C. The samples were then washed with 1 ml of 10% TCA and placed on ice for 5 min and centrifuged again with the supernatant discarded. The pellet was then washed twice with 1 ml of ethanol-ethyl acetate (1:1 v/v) to remove any residual DNPH and centrifuged at 10,000 g for 10 min at 4°C, discarding the supernatant each time. Finally, the pellet was resolubilized in 500 μ l of 6 mol l⁻¹ guanidine hydrochloride, placed in a 37°C water bath for 20 min, and vortexed. The samples were centrifuged at 700 g for 10 min to remove any debris. The absorption of the samples was measured at 380 nm, in duplicate, with 200 μ l of the labelled or blank sample. The carbonyl content was measured using an absorbance coefficient of 22,000 l mol⁻¹ cm⁻¹. Protein content of the final samples was calculated from the absorbance of a BSA standard curve in 6 mol l⁻¹ guanidine hydrochloride at 280 nm.

Statistics

To establish whether any of the measured variables changed during flight, we first examined whether flight duration influenced antioxidants or damage as either a main effect or an interaction with diet. If flight duration did not influence a variable in the flown group, flown and control birds were analyzed using a two-way ANOVA to test for an effect of flight, diet and its interaction. If flight duration did influence a variable, we compared the control groups with birds that completed 360 min flights to test for an effect of flight, diet and its interaction. This was followed with an analysis of the effect of diet on the flown birds including flight duration as a main effect and as an interaction with diet. Body mass, sex and the start date blocking factor were tested as potential covariates but were not significant factors at $P \leq 0.05$, and thus were not included in the analyses.

Correlations between flight performance [duration, energy used and relative protein contribution (RPC) to fuel mixture] and antioxidants and protein carbonyls were determined using Pearson correlation analysis. To test effects of flight duration, all flights were used. However, as flight duration influences flight costs and RPC, only the 360 min flights were used to test for correlations with these two variables. Statistical analysis was performed using SAS (v 9.4).

Values are presented as means±s.e.m., except when flight duration was a significant factor, in which case least squares means are presented instead for the flown groups. Significance was accepted at $\alpha < 0.05$.

RESULTS

Body composition and flight muscle fatty acid profile

Final body mass did not significantly differ between diet group (n-3 PUFA: 13.53±0.36 g; n-6 PUFA: 13.54±0.34 g; MUFA: 13.55±0.33 g; $F_{2,54}=0.001$, $P=0.99$). Similarly, body composition did not differ between the diets, with no significant differences in fat mass (n-3 PUFA: 2.85±0.23 g; n-6 PUFA: 2.54±0.24 g; MUFA: 2.80±0.23 g; $F_{2,54}=0.48$, $P=0.62$) or wet lean mass (n-3 PUFA: 8.53±0.15 g; n-6 PUFA: 8.66±0.16 g; MUFA: 8.52±0.15 g; $F_{2,54}=0.23$, $P=0.79$).

In the flight muscle phospholipids, the MUFA group had higher proportions of MUFA and lower proportions of saturated fatty acids (SFA; Fig. 1). Total PUFA levels did not differ among diet groups. Birds in the n-6 PUFA and n-3 PUFA treatments had higher proportions of ARA and DHA, respectively, and the MUFA group was intermediate for ARA and had similar proportions of DHA as the n-6 PUFA group. Overall, the muscle phospholipids in the dietary treatments were enriched in the targeted fatty acids (ARA and DHA) with minimal differences in the other fatty acid classes.

Antioxidant enzymes

Within the flown birds, there was no effect of flight duration or interaction between duration and diet on catalase activity (duration: $F_{1,18}=0.65$, $P=0.65$; diet×duration: $F_{2,18}=1.83$, $P=0.19$). Combining the analysis with the control birds, there was no effect of diet, flight or their interaction (diet: $F_{2,50}=0.07$, $P=0.94$; flight: $F_{1,50}=1.10$, $P=0.30$; diet×flight: $F_{2,50}=0.53$, $P=0.59$; Fig. 2A). SOD activity in the flown birds was not influenced by flight duration or its interaction with diet (duration: $F_{1,18}=0.33$, $P=0.57$, diet×duration: $F_{2,18}=2.22$, $P=0.13$). When combined with control birds, there was an effect of flight ($F_{1,50}=5.05$, $P=0.03$) and SOD activity was 17% higher in the flown birds compared with control birds (Fig. 2B). Total SOD activity was not influenced by diet in either the control or flown birds, nor was there a significant diet by flight interaction (diet: $F_{2,50}=0.71$, $P=0.50$; diet×flight: $F_{2,50}=2.57$, $P=0.09$). Higher SOD activity during flight may have been a result of the lower SOD activity in the control n-6 PUFA diet group. Although the interaction between diet and flight was not significant ($P=0.09$), within the control birds the n-6 PUFA group had lower SOD activity than the MUFA group, with the n-3 PUFA group being intermediate in activity. Only the n-6 PUFA diet group had significantly greater SOD activity during flight when examining the diet by flight interaction.

Glutathione

Total GSH concentrations were \log_{10} transformed to meet the normality assumption. Total GSH concentrations were not influenced by flight duration or an interaction between flight and diet (duration: $F_{1,18}=0.95$, $P=0.34$, duration×diet: $F_{2,18}=0.63$, $P=0.44$). Total GSH concentration did not differ between control and flown birds, nor between dietary treatments (diet: $F_{2,50}=2.41$, $P=0.10$, flight: $F_{1,50}<0.01$, $P=0.96$; diet×flight: $F_{2,50}<0.01$, $P=0.99$; Fig. 3A). GSH in the flown birds was not influenced by flight duration ($F_{1,18}=0.68$, $P=0.42$) or its interaction with diet ($F_{2,18}=0.02$, $P=0.98$). There was no difference in GSH concentrations between the control and flown birds, or among diets, and there was no interaction (flight: $F_{1,50}=0.18$, $P=0.68$; diet: $F_{2,50}=2.29$, $P=0.11$, diet×flight: $F_{2,50}=0.14$, $P=0.87$; Fig. 3B). GSSG concentration in the flown birds

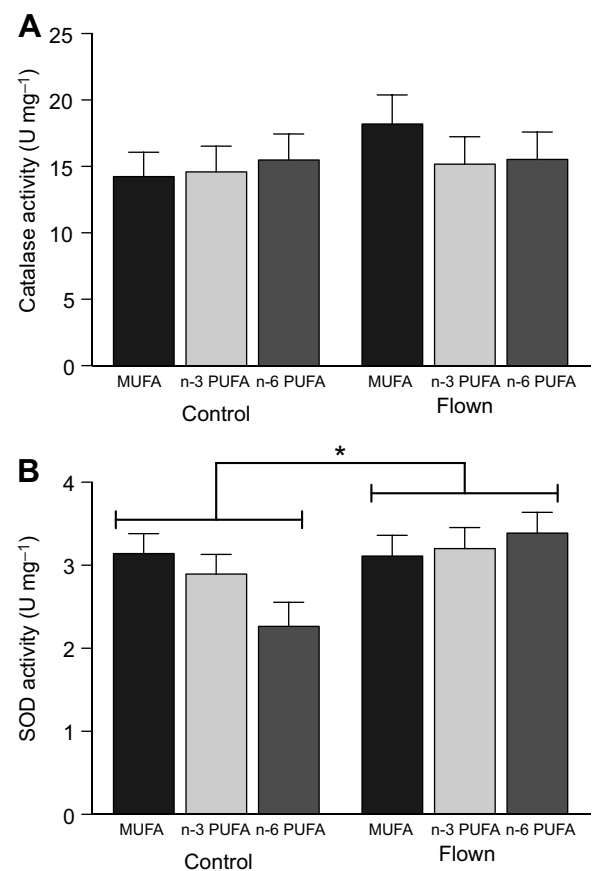


Fig. 2. Flight muscle antioxidant enzyme activity per milligram of protein of yellow-rumped warblers (*Setophaga coronata coronata*) fed diets enriched in monounsaturated (MUFA), n-3 polyunsaturated (n-3 PUFA) or n-6 polyunsaturated (n-6 PUFA) fatty acids. (A) Catalase; (B) superoxide dismutase (SOD). Values are means±s.e.m. Asterisk indicates a significant difference between control and flown birds (* $P < 0.05$).

was not influenced by flight duration ($F_{1,18}=2.21$, $P=0.16$) or its interaction with diet ($F_{2,18}=2.05$, $P=0.16$). GSSG concentrations significantly increased by 27% during flight, and diet did not affect GSSG concentrations (diet: $F_{2,50}=1.48$, $P=0.24$, flight: $F_{1,50}=17.52$, $P=0.0001$; diet×flight: $F_{2,50}=0.36$, $P=0.70$; Fig. 3C). The GSH:GSSG ratio was not influenced by flight duration ($F_{1,18}=1.00$, $P=0.33$) or its interaction with diet ($F_{2,18}=2.05$, $P=0.16$). Compared with the control birds, flown birds had a lower GSH:GSSG ratio (diet: $F_{2,50}=3.04$, $P=0.06$, flight: $F_{1,50}=24.05$, $P < 0.0001$, diet×flight: $F_{2,50}=0.80$, $P=0.45$; Fig. 3D), indicating that flown birds were under oxidative stress.

Protein carbonyls

Compared with the control group, birds that flew for 360 min had significantly higher protein carbonyl concentrations (control: 0.039 ± 0.006 ; flown: 0.095 ± 0.004 nmol mg⁻¹), but no effect of diet was detected (diet: $F_{3,38}=0.62$, $P=0.61$, flight: $F_{1,38}=32.74$, $P < 0.0001$; diet×flight: $F_{2,38}=2.23$, $P=0.13$). Within the flown birds, flight duration increased protein carbonyls, but there was no effect of diet or the diet×duration interaction (duration: $F_{1,18}=4.73$, $P=0.04$; diet: $F_{2,18}=0.18$, $P=0.84$; duration×diet: $F_{2,18}=0.88$, $P=0.43$; Fig. 4A).

Flight performance, antioxidant defence and oxidative damage

No significant correlations between the antioxidants and flight performance (duration, energy costs and inferred RPC) or body

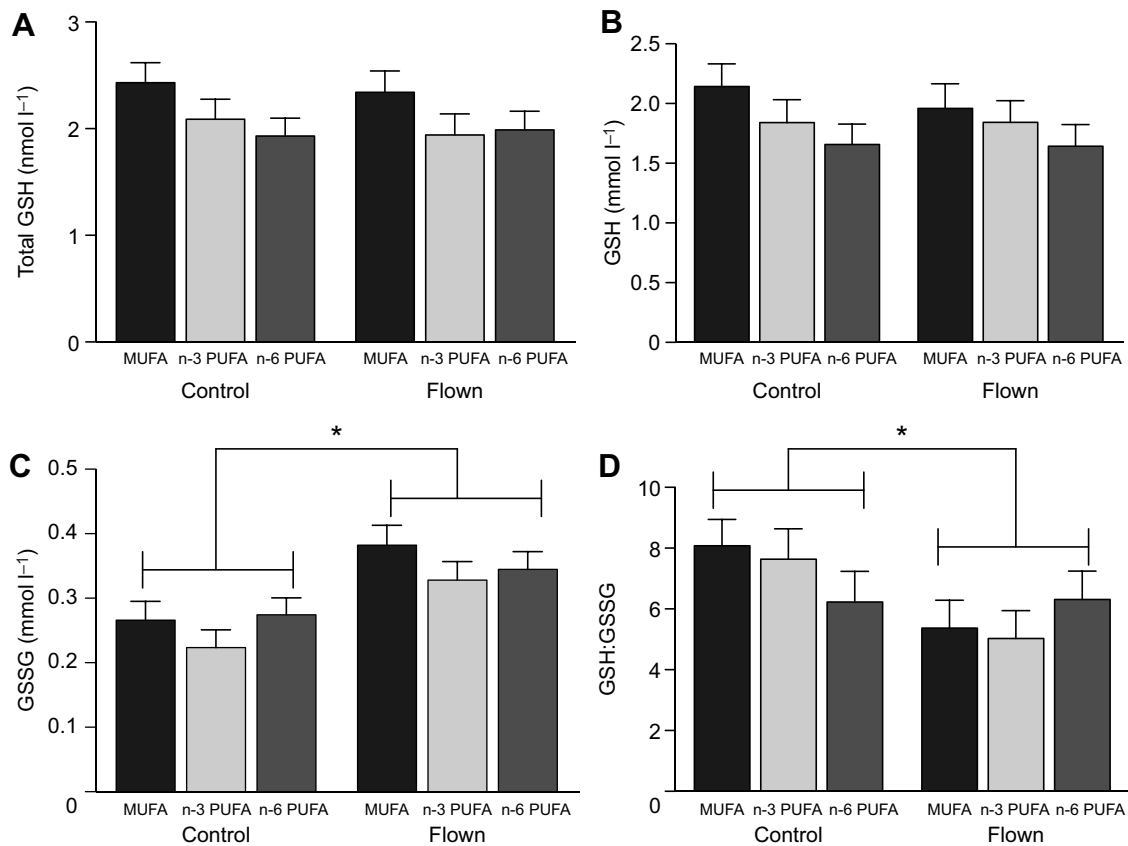


Fig. 3. Flight muscle concentrations of glutathione in yellow-rumped warblers (*Setophaga coronata coronata*) fed diets enriched in monounsaturated (MUFA), n-3 polyunsaturated (n-3 PUFA) or n-6 polyunsaturated (n-6 PUFA) fatty acids. (A) Total GSH, (B) GSH, (C) GSSG and (D) GSH:GSSG ratio. Values are means \pm s.e.m. Asterisks indicate a significant difference between control and flown birds (* $P < 0.05$).

mass were observed when testing all flights combined (Table 2), or in the 360 min flights alone (Table 3). Protein carbonyls were significantly correlated with flight duration ($r=0.40$, $P=0.05$; Fig. 4B); however, the relationship between protein carbonyls and total energy costs was stronger ($r=0.58$, $P=0.003$). The effect of flight duration was removed by examining the 360 min flights only, and this made the correlation between energy use and protein carbonyls even stronger ($r=0.86$, $P=0.0002$; Fig. 4C). Furthermore, in the 360 min flights, protein damage was also correlated with the fuel mixture used, with protein damage decreasing with increasing RPC ($r=-0.53$, $P=0.05$; Fig. 4D).

DISCUSSION

Oxidative damage during migratory flight could be an important physiological challenge during migration for birds. We found potential evidence for increased enzymatic antioxidant capacity during flight, but also evidence of oxidative damage in the flight muscle. Furthermore, protein damage was strongly linked to energy expenditure and flight efficiency at the individual level. Although the fatty acid manipulation successfully created membranes rich in either DHA or ARA, we found no effect of diet on antioxidants or damage. To our knowledge, this is the first study to examine directly oxidative stress in the flight muscle of birds after endurance flight.

Changes during flight in antioxidant enzyme activity differed between catalase and SOD. Catalase activity did not change during flight, but SOD activity increased. It is currently not clear whether catalase activity changes with acute exercise, as the response does not appear uniform across studies in human models (Powers and Jackson, 2008). During exercise, catalase activity may decrease

during the initial stage before returning to initial activity level in mice (Wang et al., 2015). We found no effect of flight duration on catalase activity, suggesting that catalase activity remains constant throughout migratory flight in yellow-rumped warblers. In contrast, high-intensity and long-duration exercise results in the largest increases in SOD activity in mammals (Powers et al., 1994). Similarly, we found that SOD activity increased with flight in the current study. It should be noted that flight activity did not influence blood endogenous antioxidants in zebra finches (*Taeniopygia guttata*), including SOD and catalase (Costantini et al., 2013). Additionally, flight in migratory Nathusius' bats (*Pipistrellus nathusii*) did not significantly alter blood SOD activity but did increase blood glutathione peroxidase activity (Costantini et al., 2018). This suggests that tissues differentially regulate SOD activity during flight. In red-headed buntings (*Emberiza bruniceps*), the Mn-SOD (a mitochondria-specific SOD isomer) increases in abundance in the flight muscles during the migratory season (Banerjee and Chaturvedi, 2016). A similar pattern has been observed in migrating Atlantic salmon (*Salmo salar*) muscles during spawning, where SOD activity increases, but catalase activity remains unchanged (Bombardier et al., 2010). These findings suggest that SOD may be an important and dynamic endogenous antioxidant in the muscles of migrating animals.

Total GSH concentrations and GSH levels did not change during flight, and this is a similar response to other oxidative muscles (Ji et al., 1992). This suggests that there was no major increase the synthesis or transport of GSH into the muscle to increase cellular concentrations. GSH can react directly with radicals to donate a pair of electrons, or it can serve as the substrate for glutathione

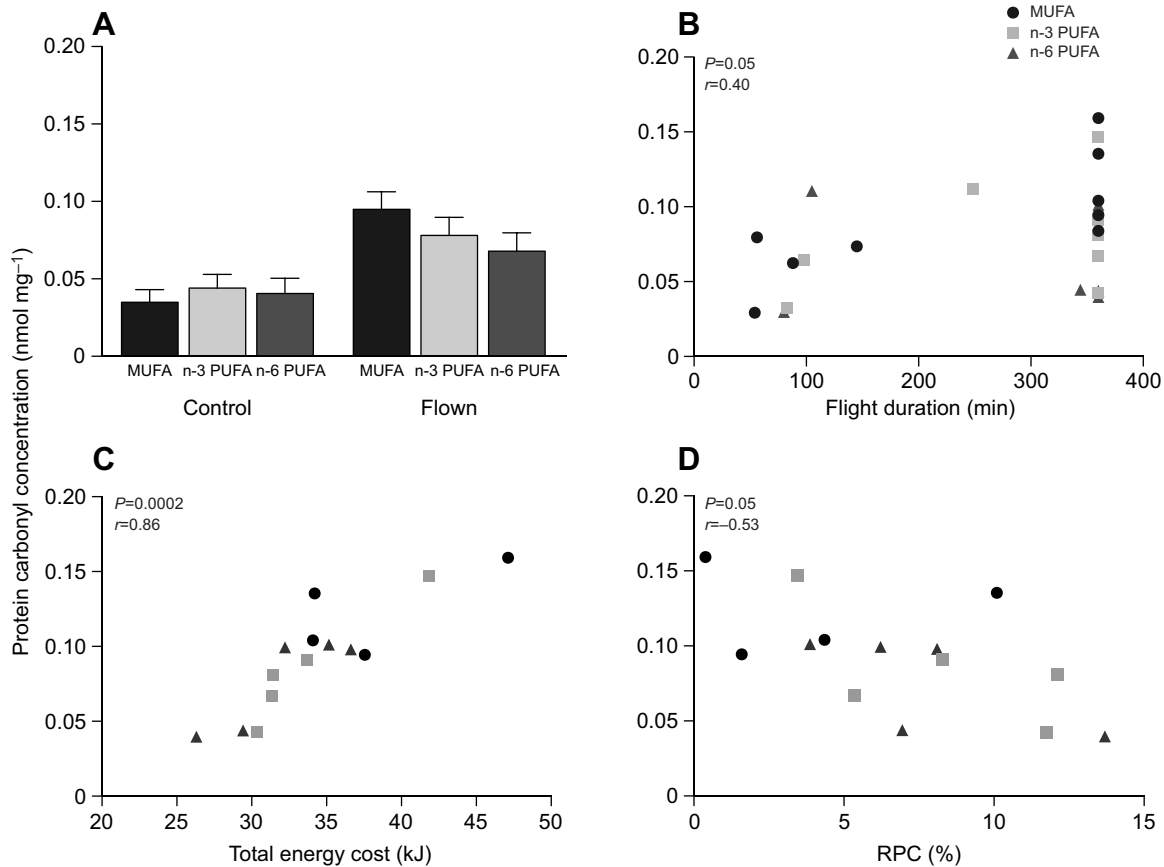


Fig. 4. Factors affecting flight muscle protein carbonyl concentrations in yellow-rumped warblers (*Setophaga coronata coronata*). (A) Protein carbonyl concentration in control (means \pm s.e.m.) and flown birds (flight duration included as a covariate, least-squares means \pm s.e.m.). (B) Effect of flight duration and diet on protein carbonyls. (C) Relationship between protein carbonyl concentration and total energy cost in the 360 min flights only. (D) Relationship between protein carbonyl concentration relative protein contribution (RPC) in the 360 min flights only.

peroxidase, with both yielding GSSG (Powers and Jackson, 2008). GSSG concentrations increased during flight, and this drove a decrease in the GSH:GSSG ratio. Because flight duration did not significantly influence this ratio, flying birds may have a different redox homeostasis balance than at rest, and this could be beneficial for optimizing muscle force contraction or for cell signalling.

Endurance flight may cause oxidative stress and damage and is detectable in the blood of birds during or recently after flight (Costantini et al., 2007, 2008; Jenni-Eiermann et al., 2014). For example, in our experiment, flight significantly increased flight muscle protein carbonyl concentrations. The damage accrued by

individual birds during flight was positively correlated not only with the duration of flight, but also very strongly with flight energy expenditure. This relationship suggests that birds that have increased flight costs as a result of either longer flight duration or higher energy costs will have greater oxidative protein damage. Flight duration is significantly correlated with energy expenditure ($r=0.88$, $P<0.0001$; Dick and Guglielmo, 2019). The effect of energy expenditure on protein damage is especially evident within the 360 min flights, where all birds flew the same duration and simulated distance (288 km) but varied in energy expenditure by nearly 100%. Body mass was strongly correlated with total energy

Table 2. Correlations between measures of flight performance and measures of antioxidant status and protein damage for all flights (56–360 min) made by yellow-rumped warblers

	Duration (min)		Total energy (kJ)		Body mass (g)	
	<i>r</i>	<i>P</i> -value	<i>r</i>	<i>P</i> -value	<i>r</i>	<i>P</i> -value
SOD	−0.15	0.24	−0.06	0.77	−0.07	0.76
Catalase	−0.22	0.30	−0.27	0.20	0.14	0.53
Total GSH	−0.13	0.52	−0.15	0.47	0.17	0.39
GSH	−0.12	0.54	−0.16	0.44	0.10	0.62
GSSG	0.31	0.14	0.36	0.08	0.35	0.09
GSH:GSSG	0.05	0.79	0.007	0.97	−0.24	0.26
Protein carbonyls	0.40	0.05	0.58	0.003	0.28	0.17

SOD, superoxide dismutase; GSH, glutathione; GSSG, glutathione disulphide. Bold values indicate significant correlations ($P<0.05$).

Table 3. Correlations between measures of flight performance and measures of antioxidant status and protein damage in 360 min flights made by yellow-rumped warblers

	Body mass (g)		Total energy (kJ)		RPC (%)	
	<i>r</i>	<i>P</i> -value	<i>r</i>	<i>P</i> -value	<i>r</i>	<i>P</i> -value
SOD	0.002	0.99	0.41	0.18	−0.51	0.08
Catalase	−0.15	0.60	−0.42	0.14	0.22	0.48
Total GSH	−0.67	0.81	−0.19	0.48	0.11	0.68
GSH	−0.17	0.55	−0.24	0.38	0.17	0.52
GSSG	0.48	0.07	0.21	0.44	0.27	0.34
GSH:GSSG	−0.42	0.11	−0.28	0.30	0.26	0.33
Protein carbonyls	0.39	0.17	0.86	0.0002	−0.53	0.05

SOD, superoxide dismutase; GSH, glutathione; GSSG, glutathione disulphide. Bold values indicate significant correlations ($P<0.05$).

expenditure ($r=0.75$, $P=0.0012$; Dick and Guglielmo, 2019). Interestingly, body mass was not significantly correlated with protein carbonyl concentrations ($r=0.39$, $P=0.17$), suggesting that a combination of factors influence protein damage. Flying in the wind tunnel was voluntary, and all birds that flew for 360 min transitioned into a stable exercising state within 30 min of starting their flight. We did not notice any overt feather damage or differences in flight behaviour in the wind tunnel that may have influenced energy expenditure. Our findings suggest that variation in muscle protein damage could be attributed to the metabolic cost of flight rather than just duration itself. Thus, protein damage may be affected by additional factors that influence flight energy requirements, such as winds and turbulence, or individual wing shape, flight kinematics and flight speed. Conversely, it could also be that birds that accrue more protein damage may be metabolically less efficient flyers.

Protein damage during flight was also correlated with the fuel mixture as increasing RPC correlated with lower protein carbonyls. This relationship could be an artefact of a negative relationship we found between flight power and RPC in the same birds (Dick and Guglielmo, 2019). However, catabolizing more protein could also convey benefits in terms of managing oxidative stress. Firstly, uric acid (the waste product of amino acid catabolism) is a potent antioxidant (Powers and Jackson, 2008). During flight, an increase in plasma uric acid may occur (George and John, 1993; Schwilch et al., 1996), and the non-enzymatic oxidation of uric acid to allantoin can be used as a plasma marker of oxidative stress (Tsahar et al., 2006). A greater RPC may reflect an increase in the production of uric acid and an increase in antioxidant capacity during flight. Alternatively, birds that have a higher RPC during flight may have higher protein turnover during flight. Protein carbonyls can serve as a tag for degradation (Radak et al., 2013). If protein turnover is higher as a result of a higher RPC, the net accumulation of carbonyls may be potentially lower. Regardless of the cause, it is not just how far a bird flies that predicts oxidative damage. The amount of fuel and oxygen used, and the composition of the fuel also influence the amount of oxidative damage to the flight muscles.

PUFA are vulnerable to ROS owing to their proximity to radical production in mitochondrial membranes, particularly with increasing chain length and number of double bonds (Ng et al., 2005; Skrip and McWilliams, 2016). The incorporation of PUFA into mitochondrial membranes may also decrease ROS production, and help preserve mitochondrial function during endurance flight (Gerson, 2012). Our study showed no significant effect of dietary long-chain PUFA on antioxidant enzymes or oxidative damage at rest or after endurance flight, nor any effect on flight performance (duration, costs or RPC; Dick and Guglielmo, 2019). This suggests that dietary intake of DHA and ARA, and their incorporation into flight muscle membranes, does not alter ROS defenses or net damage in yellow-rumped warblers during migration. This differs from white-throated sparrows (*Zonotrichia albicollis*), where dietary PUFA increased plasma oxidative damage (Alan and McWilliams, 2013). Furthermore, the type of long-chain PUFA also did not influence antioxidant enzyme activity or damage. DHA increases the mRNA expression of antioxidant enzymes in muscle compared with ARA, but this may not translate in to any difference in muscle damage (Hashimoto et al., 2016). The increased vulnerability from PUFA has been highlighted as a concern in migratory birds (Price, 2010; Alan and McWilliams, 2013; Skrip and McWilliams, 2016), but empirical support is lacking. Our study suggests that a prolonged high intake of long-chain PUFA may not be a large oxidative burden or alter endogenous antioxidants during

migration. However, we were unable to assess lipid oxidative damage in this study, and acknowledge the possibility of differences in lipid damage caused by dietary long-chain PUFA. Furthermore, all our groups had similar proportions of total PUFA in their flight muscle membranes, which may also limit our ability to assess the effect of PUFA on antioxidants and oxidative damage.

Overall, endurance flight leads to an increase in SOD activity. However, the flight muscle is also in a temporary state of oxidative stress, and oxidative damage is an unavoidable hazard. The time frame for resolving ROS damage occurring in flight appears to be relatively rapid. Within a few days, the redox balance in the blood returns to normal (Skrip et al., 2015), and mitochondrial function returns to pre-flight levels (Gerson, 2012). The relationship between oxidative damage and flight energy costs suggests that factors that influence energetics – such as body mass, feather condition and environmental conditions – may also influence oxidative damage, and this warrants further investigation. Potentially, optimizing flight performance may not only reduce the energy costs but also limit flight-related damage. We did not find any evidence that oxidative damage limited flight duration, and it is currently unknown whether the severity of oxidative damage limits migration in terms of migratory flight duration or overall speed of migration. If an oxidative stressor is great enough, it potentially could negatively influence metabolic flight efficiency, flight duration or recovery time at stopover. All of these factors influence the migratory performance and should be included in assessments of migratory performance.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: M.F.D., C.G.G.; Methodology: M.F.D.; Formal analysis: M.F.D., C.G.G.; Investigation: C.G.G., M.F.D.; Resources: C.G.G.; Writing - original draft: M.F.D.; Writing - review & editing: M.F.D., C.G.G.; Visualization: M.F.D.; Supervision: C.G.G.; Project administration: M.F.D., C.G.G.; Funding acquisition: C.G.G.

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