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RESEARCH ARTICLE

Controlled expression of the migratory phenotype affects oxidative status in birds

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ABSTRACT

High caloric intake can increase production of reactive oxygen and nitrogen species. We examined whether the emergence of the migratory phenotype, primarily signalled by increased food intake and fuelling, is accompanied by changes in oxidative status. We induced autumn migration followed by a non-migratory wintering phase in common quails (*Coturnix coturnix*). We compared three markers of oxidative status – oxidative damage to lipids expressed as thiobarbituric acid reactive substances (TBARS); superoxide dismutase (SOD); and glutathione peroxidase (GPx) – between birds sampled during the migratory and non-migratory phase. We found that the emergence of the migratory phenotype was associated with: (i) higher levels of TBARS in the liver; (ii) lower levels of SOD in red blood cells and, marginally, in the liver; (iii) higher levels of GPx in the pectoral muscle; and (iv) sex-specific changes in red blood cells and liver. We found no link between food intake and variation in markers of oxidative status in any of the tissues examined, despite food intake being higher in the migratory birds. However, the increase in body mass was positively correlated with muscle GPx activity as birds entered the pre-migratory fattening phase, while the amount of decrease in body mass was negatively correlated with muscle GPx as birds transitioned to the non-migratory phase. Such correlations were absent in red blood cells and liver. Our work suggests that during the emergence of the migratory phenotype, birds might strategically displace oxidative costs on the liver in order to safeguard the pectoral muscles, which have a fundamental role in successfully completing the migratory flight.

KEY WORDS: Avian migration, Refuelling, Oxidative stress, Oxidative damage, Lipid peroxidation, Enzymatic antioxidants

INTRODUCTION

The continuous consumption of oxygen as a result of aerobic metabolic processes leads to the production of reactive oxygen and nitrogen species (RONS) (Halliwell and Gutteridge, 2015). It is well recognised that RONS can damage vitally important molecules, including nucleic acids, proteins and lipids (Martindale and Holbrook, 2002; Birben et al., 2012). Aerobic organisms have various defence pathways characterised by both exogenous and endogenously produced antioxidants to buffer and counteract RONS-induced damage (Halliwell and Gutteridge, 2015). Overproduction of RONS, RONS-generated molecular damage, or alterations in the antioxidant machinery can all represent evidence of biochemical oxidative stress (see Costantini, 2019 for a discussion on the definition of oxidative stress). Studies across different vertebrate species show that changes in oxidative status, either naturally occurring (e.g. with age) or experimentally induced, can have fitness consequences, for example, influencing probability of survival and reproductive behaviour (Salmon et al., 2010; Herborn et al., 2015; Costantini et al., 2016; Marasco et al., 2017).

Two key processes that are associated with changes in oxidative status, often increasing RONS production, are increased caloric intake and intense physical exercise (Masoro, 2000; Costantini et al., 2008, 2012; Boden et al., 2015). Both of these processes are fundamental features of many life-history stages, such as animal migration. The exceptional physiological adaptations that birds have evolved to accommodate the demands of long-distance migratory flights have attracted intense research over the last decades (see Cornelius et al., 2013 for a comprehensive review on this topic). In preparation for migratory flights, birds increase food consumption (hyperphagia) and show rapid increases in lipogenesis in the liver, volume of fat stores and flight muscle, as well as body mass (Jenni-Eiermann and Jenni, 1992; Bairlein, 2002; Jenni and Schaub, 2003). Migrating birds are capable of maintaining remarkably high metabolic rates during their endurance non-stop flights compared with mammalian migrants (Jenni-Eiermann and Jenni, 2012; Guglielmo, 2018). Therefore, it has been hypothesised that the increased metabolic demands associated with the expression of the migratory phenotype, from the preparatory fuelling stages to the migratory flights, expose birds to increased RONS production and, hence, to an increased risk of oxidative stress (Costantini et al., 2007; Eikenaar et al., 2016).

To date, studies examining the links between markers of oxidative status and migratory behaviour provide only partial support for the above hypothesis. In fact, although endurance flight is generally associated with increased levels of oxidative damage and shifts in the antioxidant machinery (birds: Jenni-Eiermann et al., 2014; Dick and Guglielmo, 2019; mammals: Costantini et al., 2018; but see Bairlein et al., 2015), the extent of changes in oxidative stress during the preparatory fuelling phase – that is, the emergence of the migratory phenotype – is still surprisingly understudied. Most of the work assessing the links between migration and oxidative stress has been carried out in birds caught at stopover sites. Results from these studies are mixed; while some studies support the existence of functional correlations between body energy stores and circulating antioxidants or levels of oxidative damage (Costantini et al., 2007; Skrip et al., 2015; Eikenaar et al., 2016, 2020), others report no associations among these variables (Skrip et al., 2015; Ferretti et al., 2020). However, all of these studies targeted a specific life-history stage of migration, as all birds were sampled far from their pre-migratory

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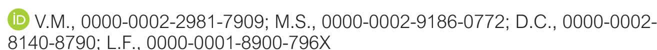
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regions and in the midst of flight–refuel cycles. Consequently, the subjects of these studies likely had a physiological status that differed from that shown by birds during the pre-migratory stages. To the best of our knowledge, the only study so far that has specifically examined oxidative status dynamics in relation to pre-migratory fuelling has been conducted in the long-distance migrant the Hudsonian godwit (*Limosa haumastica*) by Gutiérrez and colleagues (2019). Surprisingly, those authors found that the birds increased plasma non-enzymatic antioxidant capacity and reduced oxidative damage to lipids as the pre-migratory spring season progressed (Gutiérrez et al., 2019). It therefore remains unclear how pre-migratory fuelling and hyperphagia affect the oxidative status of migratory birds.

Another aspect that deserves attention in the context of migration eco-physiology is that the majority of studies in birds have measured markers of the oxidative status in a single tissue – blood. To better understand potential dynamics of oxidative status in relation to the expression of the migratory phenotype, it is important to investigate whether changes in oxidative status occur in the pre-migratory preparatory phase and to rely on a multi-tissue approach because metabolic activity varies across tissues in migratory birds (Price et al., 2008). It is indeed well recognised that migratory birds undergo substantial changes in body composition as they prepare to initiate migration, including increased size of flight muscles, gut and liver (Landys-Ciannelli et al., 2003). A suite of metabolic and proteome changes has also been observed during the pre-migratory fattening, and these changes generally involve increased *de novo* fatty acid production capacity and transport in the liver, and augmented pectoralis muscle fatty oxidation (Banerjee and Chaturvedi, 2016; see also Guglielmo, 2018 for a mini-review on this aspect). However, to what extent such remarkable organ metabolic reorganisation causes tissue-specific changes in the oxidative status in migratory birds remains to be determined.

Furthermore, very few studies to date have assessed whether the potential associations between migratory behaviour and oxidative status might vary between the two sexes, or between the time of day at sampling (i.e. day versus night). These aspects could be particularly relevant because: (i) oxidative status markers can vary between the two sexes (Marasco et al., 2013; Eikenaar et al., 2017, 2020); (ii) sex-specific differences in migratory strategies are known to exist in several species (Kokko et al., 2006; Debeffe et al., 2019; Gutiérrez et al., 2019); and (iii) circadian rhythmicity in RONS generation and scavenging have been described across a variety of living organisms (reviewed in Wilking et al., 2013), and circadian rhythms are critical for the expression of the migratory phenotype (Berthold, 1993; Gwinner, 1996). Here, we experimentally controlled the migratory status of young adult common quails [*Coturnix coturnix* (Linnaeus 1758)] originated from a captive breeding population by simulating autumn migration followed by a non-migratory stage. The common quail is the only long-distance migrant of the western Palearctic Phasianidae. The species breeding area is broadly distributed in Eurasia, while the overwintering distribution ranges from sub-Saharan to Central and North Africa (Cramp and Simmons, 1980). As previously shown (Boswell et al., 1993), the common quail undergoes repeated cycles of migratory fattening in captive conditions, making this species a good model for studying the physiology of migratory fuelling. Our experimental design enabled us to assess potential differences in the individual's oxidative status in relation to the activation and de-activation of migratory fuelling while minimising, or controlling for, potential confounding factors associated with photoperiod changes, circadian rhythms, sex and age-related differences of experimental animals. We examined three different markers of oxidative status: a marker of oxidative damage to lipids [thiobarbituric acid reactive substances (TBARS)], and two markers of enzymatic antioxidant activity [superoxide dismutase (SOD) and glutathione peroxidase (GPx)] in birds that were sampled at a standardised time during the day or at night. The measurements were performed in red blood cells, liver and pectoral muscle of the same individual birds. As increased food intake can lead to increased exposure to RONS (see references above), we predicted that levels of oxidative damage would be higher in the fattened migratory birds compared with the birds sampled during the non-migratory phase. We also predicted that the changes in oxidative status in relation to the expression of the migratory phenotype would vary among tissues owing to known changes in metabolic activity of different organs associated with migratory behaviour (references above).

MATERIALS AND METHODS

Study subjects

Eggs of common quail used in this experiment were obtained from our breeding stock kept at the Konrad Lorenz Institute of Ethology (Vetmeduni, Vienna, Austria). The stock birds originated from wild founders captured during the spring migration/breeding season (April/May) on the coast of Sicily (southern Italy) near Palermo in 2008, 2009 and 2010. As previously shown (Smith et al., 2018), microsatellite and mtDNA screening excluded the presence of admixture with Japanese quails released for hunting practices within our study population. The eggs were incubated at 37.5°C and approximately 55% humidity while being turned twice hourly (incubator: MG 70/100 F, FIEM srl, Italy). Fourteen days after the start of incubation, eggs were transferred into the hatchers within the same incubator, and humidity was increased to 70–75%. After hatching, all experimental birds (female=34, male=34, total n=68) were reared in sex-mixed groups under a 16 h:8 h light:dark cycle until approximately 7 weeks of age. The birds were housed in sex-mixed groups of 11–12 in each pen (six pens in total). Food (turkey starter, Lagerhaus, Austria) and water were provided *ad libitum* throughout the course of the experiment. All birds were housed within a single room and the temperature was maintained between 20 and 24°C.

Ethical statement

All animal procedures were performed in compliance with the Austria legislation with approval of the Ethics Committee of the University of Veterinary Medicine Vienna, and the Federal Ministry of Science, Research and Economy (BMWFW-68.205/0037-WF/V/3b/2017).

Experimental manipulation of the migratory phenotype

When the birds were on average 7 weeks of age (mean±s.e.m.: 47.9±0.4 days old, indicated as experimental day 0), they were exposed to a gradual reduction of day length (30 min week^{-1}) until the photoperiod reached 12 h:12 h light:dark (experimental day 49), after which the photoperiod was maintained constant until the end of the experiment. This light:dark schedule simulated autumn migration to (sub)tropical regions followed by a sedentary stage at the wintering grounds. To ascertain the effectiveness of the photoperiod manipulation in inducing the expected seasonal physiological changes of migratory birds, we regularly monitored body mass and subcutaneous fat stores. We performed a total of 10 consecutive measurements over the period of the experiment (Fig. 1). Body mass was measured using an electronic balance (0.01 g precision); fat stores were estimated from the furcular, scapular and abdominal depots on a scale from 0 to 5 following previous work in the common

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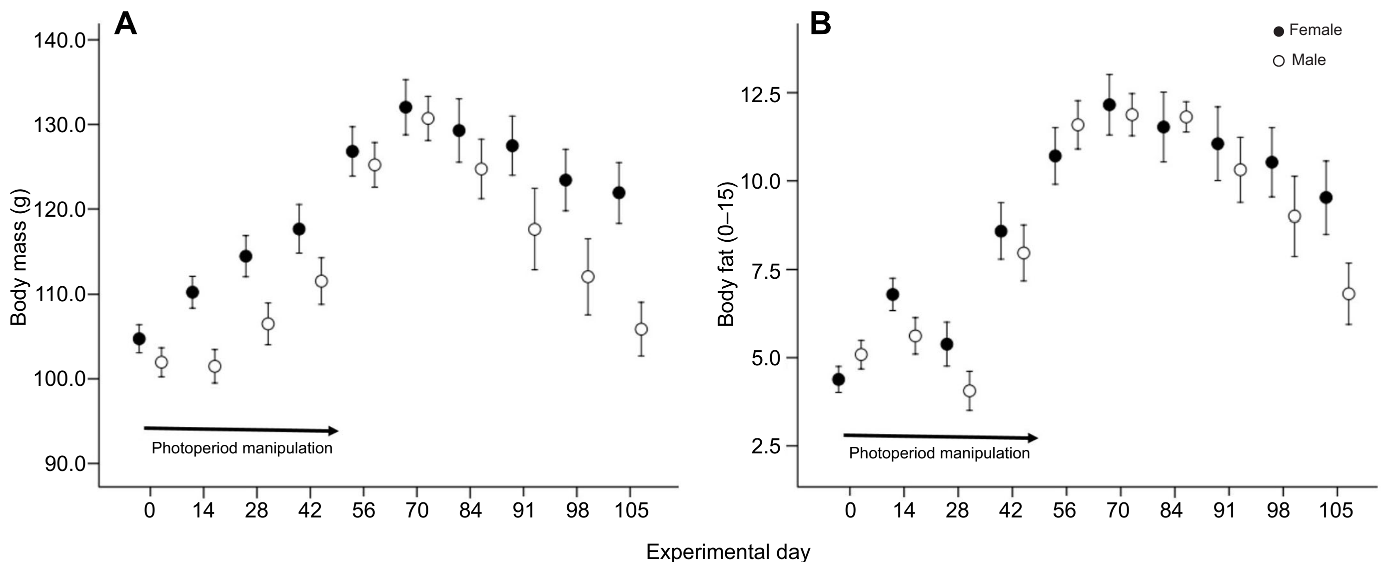

Fig. 1. Dynamic changes in body mass and total body fat stores in a captive population of pure-bred common quails subjected to a gradual decrease in daylight, separated by sex. (A) Body mass and (B) total body fat stores (scale: 0–15). Data are shown as means±s.e.m. Sample size from experimental days 0–56: 68 birds; sample size from experimental days 70–105: 35 birds.

quail (Boswell et al., 1993). Fat scores from these three areas were summed together to produce total body fat scores of 0–15, which have been shown to correlate well with body fat measured using composition analyses (Boswell et al., 1993).

Sampling schedule

All sampling procedures were performed under the same photoperiod conditions (i.e. 12 h:12 h light:dark). Eight weeks after beginning the reduction of the photoperiod (experimental day 56), 33 out of 68 birds housed within three separate pens were randomly chosen and allocated to the migratory sampling group (October 2017, experimental days 56–70); of these, 16 birds (7 females and 9 males) were sampled at 09:00 h (3 h after lights on), and 17 birds (8 females and 9 males) were sampled at 21:00 h (3 h after lights off). The remaining birds (n=35) housed in the other three enclosures were thus allocated to the non-migratory sampling group (December 2017, experimental days 105–119); of these, 18 birds were sampled at 09:00 h (9 females and 9 males) and 17 birds were sampled at 21:00 h (10 females and 7 males). For both groups, sampling procedures were performed over a period of 9 days in three separate batches (over three consecutive days per batch). Two days prior to the sampling procedures, the birds housed within the same pen were transferred into single cages (100×100×100 cm). For every individual bird, we measured daily food consumption upon the start of the single housing (10:00–11:00 h) until the following 24 h by weighing the remaining food in the bowl and any food pellet that had been scattered around the cage by the bird. Food intake was calculated as the difference between the mass of the remaining food from the mass of the food provided at the beginning of the experiment. As the food was relatively dry, we did not need to adjust food intake values for evaporative water loss, as this was negligible. We tested measurement repeatability for daily food intake within a subset of 12 experimental birds (6 birds for each sampling group) over two consecutive days from the start of the single housing (the second measurement was performed 22–23 h after the first measurement). Within-individual repeatability for daily food intake was very high (r=0.93, P<0.0001; Lessells and Boag, 1987), thus this measure is a suitable indicator of daily food consumption over the short-term. We collected tissues from a maximum of two birds at the same sampling time point in order to collect samples as close to the defined collection time as possible (i.e. 09:00 h and 21:00 h, 3 h after lights on and off, respectively) and in order to obtain baseline physiological measurements. No birds were sampled before 46–47 h from the start of the single housing to minimise potential effects of the changes in the social environment, which are unlikely as the birds were always in visual and acoustic contact with their conspecifics.

Tissue collection

A blood sample (up to 500 µl) was taken from each individual bird within 3 min of entering the experimental room (mean±s.e.m.: 66.85±8.11 s) and placed on ice. As soon as blood sampling was complete, birds were weighed using an electronic balance to the nearest 0.01 g and immediately euthanised via intraperitoneal injection of 1 ml of sodium pentobarbital (200 mg ml^{-1}). Within a few minutes post mortem, tissues (pectoral muscle and liver) were dissected out, immediately flash-frozen in liquid nitrogen, and stored at −80°C until laboratory analysis. Collected blood samples were spun (2000 rpm) within 15 min of collection to separate plasma from red blood cells, and stored at −80°C until laboratory analyses. Tissue collection during the night was performed using a head lamp with red light in order to minimise sudden hormonal changes associated with light exposure (recently reviewed by Ouyang et al., 2018).

Laboratory analyses

Oxidative status markers were analysed in red blood cells and muscle and liver tissues. Hemolysates were prepared by adding 300 µl of PBS [supplemented with 20% (v/v) of glycerol and with 0.2 mmol l^{-1} of phenylmethylsulfonyl fluoride as an inhibitor of proteases] to 20 mg of red blood cells. Samples were then left for 5 min on ice, and then successively vortexed for 10 s and centrifuged for 10 min at 20,000 ***g***. The supernatant was then recovered and split into four different tubes to be used for the analyses. To prepare tissue homogenates of liver or muscle, 1 ml of PBS (i.e. supplemented PBS, see above) was added to 50 mg of tissue. The tissue was then homogenised using a pestle in

a 1.5 ml tube. After complete homogenisation (i.e. the tissue sample was reduced to very small fragments and there was no visible trace of non-homogenised pieces of tissue), tubes were vortexed for 10 s and sonicated for 10 min. After sonication, samples were again vortexed for 10 s and then centrifuged at 20,000 ***g*** for 10 min. Finally, the supernatant was collected and split into four different aliquots for each tissue to be used for analyses of oxidative status markers.

Lipid peroxidation was quantified using the TBARS assay. Samples (5 µl of haemolysates or homogenates) were mixed with 0.5 ml of TBA reagent [0.5% (w/v) thiobarbituric acid (TBA) in 20% TCA], vortexed for 30 s and incubated at 90°C for 45 min. To protect samples from potential oxidation caused by heating, we added 0.01% of the synthetic antioxidant butylated hydroxyl toluene (BHT) to the extraction buffer (Esterbauer and Cheeseman, 1990). After incubation, samples were cooled in a bath of ice to stop the reaction. Samples were then centrifuged at 2500 ***g*** for 1 min and the supernatant was transferred in a plate in duplicate. Absorbance was measured at 532, 600 and 450 nm, and the amount of malondialdehyde (MDA) equivalents was calculated using the formula $6.45\times(A_{532}–A_{600})–0.56\times A_{450}$. Values were expressed as nmol MDA equivalents ml^{-1} (Hodges et al., 1999). We expressed values as an equivalence instead of a concentration because the TBARS method measures a number of aldehydes generated by lipid peroxidation, so that MDA is not the only lipid peroxidation compound measured (Halliwell and Gutteridge, 2015). TBARS values are strongly correlated with lipid peroxidation values obtained using HPLC methods (e.g. Moselhy et al., 2013; Kil et al., 2014; Reitznerova et al., 2017), indicating that the formation of aldehydes at working conditions is generally negligible. Samples were run in duplicate, and the intra-assay coefficients of variation for TBARS were 7.8%, 5.6% and 4.2% for red blood cells, liver and muscle, respectively.

The activity of GPx was determined using a commercially available kit (Ransel assay, RANDOX Laboratories, Crumlin, UK). Briefly, GPx activity was measured in 4 µl of diluted erythrocyte haemolysate or of tissue homogenates by measuring the decrease of nicotinamide adenine dinucleotide phosphate (NADPH) absorbance at 340 nm. The kinetic reaction was followed for 3 min and a blank reaction was subtracted from the sample absorbance. Values were expressed as units mg^{-1} protein. The activity of SOD was also determined using a commercially available kit (Ransod assay, RANDOX Laboratories) in 6 µl of diluted erythrocyte haemolysate and tissue homogenates. SOD activity was determined by measuring the inhibition of 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) reduction at 505 nm. Concentrations were calculated using a calibration curve run for each assay and were expressed as units mg^{-1} protein. The activities of GPx and SOD were expressed by the amount of proteins in the sample measured with the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA), which uses albumin as a reference standard. Samples were run in duplicate, and the intra-assay coefficients of variation for GPx were 4.4%, 3.9% and 4.1% for red blood cells, liver and muscle, respectively, while for SOD they were 9.4%, 9.6% and 18.5%. Owing to limited amounts of collected tissue for some birds, we were unable to measure all oxidative status markers in all experimental birds – full details on sample sizes for each oxidative status marker are provided in Fig. 1.

Statistical analyses

Statistical analyses were performed in R v3.6.2 (https://www.r-project.org/) integrated in RStudio v1.2.5042 (http://www.rstudio.com/) using general linear models (GLMs) or generalised linear mixed models (GLMMs) with a Gaussian distribution error. To assess the effects of the photoperiod manipulation on food intake and fat scores, we performed two separate analyses. The first analysis was performed using the data collected from experimental days 0 to 56, which represents the time period in which day length was gradually reduced from a 16 h:8 h light:dark cycle to a 12 h:12 h light:dark cycle, simulating autumn migration. The second analysis was performed using the data collected from experimental days 70 to 105, that is, the time period in which the photoperiod remained constant in order to simulate the transition to the non-migratory phase. For both analyses, we used GLMMs (R package lmes4; Bates et al., 2015) with time, sex and their interaction as fixed factors; individual bird identity was entered as random factor in order to control for the presence of within-individual repeated measurements. We entered in the model the interaction of time and sex to assess potential differences in morphological changes over time between males and females; when significant (P<0.05), the term was further explored by performing simple slopes analysis (R package interactions; https://cran.r-project.org/package=interactions). We used GLM to assess whether food intake differed in relation to sampling phase (migratory versus non-migratory), sex and the two-way interaction sampling phase×sex to check for sex-specific differences in food intake between the two sampling phases.

Covariation of each marker of oxidative status among tissues was low (0.17<Pearson r<−0.21). Thus we performed separate GLMs, by tissue (i.e. red blood cells, liver and muscle) and oxidative status markers (i.e. TBARS, SOD and GPx), to assess for differences in oxidative status associated with the sampling phase (migratory versus non-migratory), sex and time of day at sampling (day versus night), along with the two-way interactions sampling phase×sex and sampling phase×time. Significant interaction terms were further explored using pairwise *post hoc* contrasts (R package emmeans; Lenth, 2016). We then performed distinct GLMs on each oxidative status marker, for each tissue and sampling phase with respect to food intake, sex and time of day at sampling. As food intake is only one aspect of fuelling, we used similarly structured GLMs to assess whether within-individual changes in body mass, or fat stores, during the migratory phase (between experimental day 0 and the day in which birds allocated in the migratory group were individually sampled) or the non-migratory phase (between experimental day 70 and the day in which birds allocated in the non-migratory group were individually sampled) explained variation with any measures of oxidative status. Results of the models including the change in fat stores as a covariate did not differ from those that included the change in body mass as a covariate instead. Thus, we reported only outputs of the GLMs including the change in body mass as a covariate. These additional models tested whether the amount of fuel stored or consumed explained whether oxidative status changed in relation to activation or de-activation of the migratory phenotype, respectively. This approach circumvented inevitable collinearity between the sampling phase and the aforementioned covariates (see Results). In all models, non-significant interactions (P>0.05) were sequentially dropped from the final models. We evaluated the magnitude of the biological effect of interest by calculating the effect size (Hedges' g or partial η^2) along with 95% confidence intervals (CIs) for significant main factors as recommended in Nakagawa and Cuthill (2007). All models met the assumptions of normality and homogeneity, which were assessed via graphical diagnostics of the residuals (Zuur et al., 2009). Unless otherwise specified, descriptive statistics are provided as means±s.e.m.

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RESULTS

Effects of photoperiod manipulation on body mass, fat stores and food intake

From experimental days 0 to 56, the body mass of experimental quails steeply increased as days became shorter over time (full statistics in Table 1, Fig. 1A). The body mass increase was substantial (i.e. on average 22%) and it did not differ between males and females (Table 1). Regardless of the experimental day, females were, as expected, heavier than males (Table 1). The steep increase in body mass was mainly due to accumulation of body fat stores over time (Table 1, Fig. 1B). We found no effect of sex as a main factor or in interaction with time (Table 1). From experimental days 70 to 105, when the photoperiod was maintained constant at 12 h:12 h light:dark, the body mass of the birds gradually decreased over time, on average by 13% (Table 1, Fig. 1A). Although such a decrease occurred in both sexes, it was stronger in the males compared with the females (slope analysis – male: −0.72±0.07, t=−10.96, P<0.001; female: −0.30±0.06, t=−4.99, P<0.001; Fig. 1A). The decrease in body mass was associated with a reduction of subcutaneous fat scores over time (Table 1, Fig. 1B). Once again, such a reduction was detected in both sexes, though it was stronger in the males than the females (slope analysis – male: −0.14±0.02, t=−7.52, P<0.001; female: −0.07±0.02, t=−4.12, P<0.001; Fig. 1B).

As expected, birds sampled during the migratory phase consumed more food compared with the birds sampled during the non-migratory phase (7.52±0.58 versus 5.43±0.53 g, Hedges' g=0.64, 95% CI=0.15, 1.12; full statistics in Table 2). There was no effect of sex as a main factor or in its interaction with sampling phase (Table 2).

Effects of photoperiod manipulation on markers of oxidative status

Levels of lipid peroxidation measured in the red blood cells and pectoral muscle were not influenced by sampling phase, sex or time of day (full statistics in Table 3, Fig. 2A,C). In the liver, birds sampled during the migratory phase showed higher levels of lipid peroxidation compared with the birds sampled during the non-migratory phase (103.4±1.6 versus 99.3±1.2 nmol TBARS ml^{-1}, Hedges' g=0.49, 95% CI=0.01, 0.97; Table 3, Fig. 2B). We found no effects of either sex or time of day as main factors, or of their interaction with sampling phase (Table 3).

Migratory birds had lower SOD in the red blood cells than non-migratory birds (1.22±0.06 versus 1.68±0.09 units SOD mg^{-1} protein, Hedges' g=−1.06, 95% CI=−1.57, −0.55; Table 3, Fig. 2D). This difference was consistent between sexes and between sampling times (Table 3). There was no effect of sex or sampling time as main factors (Table 3). Similarly, and regardless of sex and times at sampling, we found that SOD levels measured in the liver were lower during the migratory phase compared with the non-migratory phase (24.16±1.63 versus 28.86±1.66 units SOD mg^{-1} protein), although this difference was only marginally significant (Hedges' g=−0.53, 95% CI=−1.05, 0.00; Table 3, Fig. 2E). Contrary to the other two tissues, we found no effect of sampling phase, sex or time of day at sampling on SOD levels in the muscle (Table 3, Fig. 2F).

GPx in red blood cells differed between the migratory compared with the non-migratory phase in a sex-specific fashion (Table 3). Pairwise *post hoc* contrasts within the sexes indicated that migratory males had lower levels of GPx compared with non-migratory males (*post hoc*: P<0.0001), whereas GPx activity did not differ between migratory and non-migratory females (*post hoc*: P=0.3) (Fig. 2G). In the liver, the effects of sampling phase were also male-driven (Table 3). Opposite to what we observed in the red blood cells, however, such an interaction effect was due to migratory males showing higher levels of GPx compared with non-migratory males (*post hoc*: P=0.02); no differences were found between migratory and non-migratory females (*post hoc*: P=0.2) (Fig. 2H). Regardless of sex and time of day at sampling, GPx levels in the muscle were higher in migratory birds compared with non-migratory birds (0.12±0.006 versus 0.10±0.005 units GPx mg^{-1} protein, Hedges' g=0.59, 95% CI=0.10, 1.08; Table 3, Fig. 2I).

Effects of changes in body mass or food intake on markers of oxidative stress in relation to activation or de-activation of the migratory phenotype

The changes in body mass observed in preparation to migration, or during the transition to the non-migratory phase, did not covary with lipid peroxidation levels in any of the tissues examined (full statistics in Table S1A–C). Similarly, lipid peroxidation was not associated with variation in food intake within either the migratory or the non-migratory group (P>0.3 for all, full statistics in Table S1A–C).

Table 1. Results of generalised linear mixed models (GLMMs) with a Gaussian distribution error to assess the effects of the photoperiod manipulation on body mass and subcutaneous fat scores performed in a captive population of common quail in order to simulate autumn migration followed by a non-migratory life-history stage

	Body mass					Fat scores				
	Estimate	s.e.	d.f.	*t*	*P*	Estimate	s.e.	d.f.	*t*	*P*
Experimental days 0–56										
Bird ID (r)	100.410					4.291				
Residual	95.750					10.403				
Intercept	104.474	2.155	112.242	48.485	<0.0001	4.282	0.557	163.320	7.694	<0.0001
Day	0.369	0.038	270.000	9.727	**<0.0001**	0.103	0.012	270.000	8.256	**<0.0001**
Sex (male)	−6.449	3.047	112.242	−2.116	**0.037**	−0.488	0.787	163.320	−0.620	0.536
Time:Sex	0.035	0.054	270.000	0.662	0.509	0.007	0.018	270.000	0.369	0.713
Experimental days 70–105										
Bird ID (r)	184.580					10.955				
Residual	50.410					4.218				
Intercept	153.888	6.294	166.397	24.450	<0.0001	17.413	1.755	170.917	9.924	<0.0001
Day	−0.302	0.060	138.000	−4.993	**<0.0001**	−0.072	0.018	138.000	−4.117	**<0.0001**
Sex (male)	29.043	9.309	166.397	3.120	**0.002**	5.393	2.595	170.917	2.078	**0.039**
Time: Sex	−0.420	0.089	138.000	−4.699	**<0.0001**	−0.071	0.026	138.000	−2.755	**0.007**

Fixed factors estimates are indicated in parentheses. r indicates a random factor and its associated variance. Significant terms (P<0.05) are in bold.

Table 2. Results of a general linear model (GLM) assessing whether food intake differed in relation to relevant study factors

	Estimate	s.e.	*t*	*P*
Intercept	7.720	0.714	10.818	<0.0001
Group (non-migratory)	−2.125	0.794	−2.676	**0.009**
Sex (male)	−0.364	0.794	−0.459	0.648
Group:Sex*				1.00

Fixed factor estimates are indicated in parentheses. Significant terms (*P*<0.05) are in bold; *non-significant interaction term was removed from the final model.

We found no relationships between changes in body mass within each sampling phase and enzymatic SOD activity in any of the tissues examined (Table S1D–F). Analysis suggested a negative correlation between liver SOD and food intake among the non-migratory birds (P=0.04; Table S1E), though the magnitude of such effect was relatively low given the high variation in the estimated effect size (partial η^2=0.15, 95% CI=0.03, 0.30); there was no covariation between these two variables within the migratory birds (Table S1E). SOD in red blood cells and in muscle did not covary with daily food consumption (Table S1D,F).

Variation in enzymatic GPx activity in red blood cells and in liver was not associated with changes in body mass in either sampling phase (Table S1G–I). On the contrary, the changes in body mass we observed during the activation and de-activation of the migratory phenotype did covary with muscle GPx (Table S1I). Specifically, the amount of fuelling recorded as birds shifted into the migratory state was positively correlated with muscle GPx (partial η^2=0.18, 95% CI=0.02, 0.38; Fig. 3A); in contrast, the amount of loss in body mass within the birds sampled during the non-migratory phase was negatively correlated with muscle GPx (partial η^2=0.24, 95% CI=0.06, 0.44; Fig. 3B).

DISCUSSION

We found novel evidence that the emergence of the migratory phenotype (i.e. pre-migratory fuelling) in the common quail is associated with marked tissue-specific changes in oxidative status irrespective of the increased food intake. Specifically, we found that the emergence of the migratory phenotype was associated with: (i) higher levels of lipid oxidative damage in the liver; (ii) lower activity of SOD in the red blood cells and, marginally, in the liver; (iii) higher activity of GPx in the pectoral muscle; (iv) sex-specific changes in GPx activity within the red blood cells and the liver; and (v) associations between changes in body mass consequent to the activation and de-activation of the migratory phenotype and muscle GPx activity.

The increase in liver oxidative damage to lipids, coupled with the tissue-specific changes detected in the enzymatic antioxidant machinery within the birds sampled during the migratory phase than the non-migratory phase, supports, at least to a certain extent, our first prediction – that the emergence of migratory behaviour increases RONS production. The increase in liver oxidative damage to lipids in the migratory birds might also be the result of reduced antioxidant capacity, as SOD activity was also marginally reduced within this tissue compared with non-migratory birds. Thus, reduced antioxidant protection coupled with intense fat storage in the liver might have determined such increased levels of lipid damage. Our data are somewhat in contrast with the study by Gutiérrez et al. (2019), as godwits reduced red blood cell oxidative damage to lipids and increased plasma non-enzymatic antioxidant capacity as the pre-migratory season progressed (i.e. at pre-departure relative to prior wintering and fuelling stages), despite having higher basal metabolic rates before departure. Gutiérrez et al. (2019)

Table 3. Results of GLMs assessing the effects of sampling phase, time of day at sampling, and sex on three markers of oxidative status [thiobarbituric acid reactive substances (TBARS), superoxide dismutase (SOD) and glutathione peroxidase (GPx)] separately by tissues examined (red blood cells, liver and pectoral muscle)

	Estimate	s.e.	*t*	*P*
Lipid peroxidation (i.e. TBARS)				
Red blood cells				
Intercept	126.570	3.505	36.110	<0.0001
Group (non-migratory)	−5.744	3.359	−1.710	0.092
Time (night)	−5.556	3.344	−1.662	0.102
Sex (male)	−3.274	3.362	−0.974	0.334
Group:Sex*				0.8
Group:Time*				0.4
Liver				
Intercept	105.164	2.101	50.059	<0.0001
Group (non-migratory)	−4.299	1.997	−2.153	**0.035**
Time (night)	−1.189	1.992	−0.597	0.553
Sex (male)	−2.171	1.999	−1.086	0.282
Group:Sex*				0.9
Group:Time*				0.9
Muscle				
Intercept	95.733	1.993	48.037	<0.0001
Group (non-migratory)	−1.358	1.907	−0.712	0.479
Time (night)	0.023	1.903	0.012	0.990
Sex (male)	−0.340	1.907	−0.178	0.859
Group:Sex*				0.9
Group:Time*				0.7
SOD				
Red blood cells				
Intercept	1.226	0.112	10.992	<0.0001
Group (non-migratory)	0.473	0.108	4.396	**<0.0001**
Time (night)	−0.097	0.107	−0.908	0.367
Sex (male)	0.073	0.108	0.682	0.498
Group:Sex*				0.1
Group:Time*				0.7
Liver				
Intercept	25.271	2.481	10.185	<0.0001
Group (non-migratory)	4.663	2.344	1.990	0.052
Time (night)	−2.786	2.361	−1.180	0.243
Sex (male)	0.553	2.364	0.225	0.823
Group:Sex*				0.4
Group:Time*				0.7
Muscle				
Intercept	5.340	0.462	11.568	<0.0001
Group (non-migratory)	−0.202	0.441	−0.459	0.648
Time (night)	0.406	0.441	0.920	0.361
Sex (male)	0.595	0.441	1.350	0.182
Group:Sex*				0.5
Group:Time*				0.6
GPx				
Red blood cells				
Intercept	0.147	0.024	6.100	<0.0001
Group (non-migratory)	0.034	0.029	1.191	0.238
Time (night)	0.020	0.020	0.960	0.341
Sex (male)	−0.025	0.029	−0.875	0.385
Group:Sex	0.089	0.041	2.189	**0.032**
Group:Time*				0.2
Liver				
Intercept	0.348	0.024	14.363	<0.0001
Group (non-migratory)	0.035	0.029	1.195	0.237
Time (night)	0.003	0.021	0.121	0.904
Sex (male)	0.052	0.030	1.739	0.087
Group:Sex	−0.103	0.041	−2.486	**0.016**
Group:Time*				1.0
Muscle				
Intercept	0.122	0.008	15.885	<0.0001
Group (non-migratory)	−0.018	0.007	−2.431	**0.018**
Time (night)	0.005	0.007	0.741	0.461
Sex (male)	−0.008	0.007	−1.063	0.292
Group:Sex*				0.3
Group:Time*				0.7

Fixed factor estimates are indicated in parentheses. Significant terms (*P*<0.05) are in bold; *non-significant interaction terms were removed from the final models.

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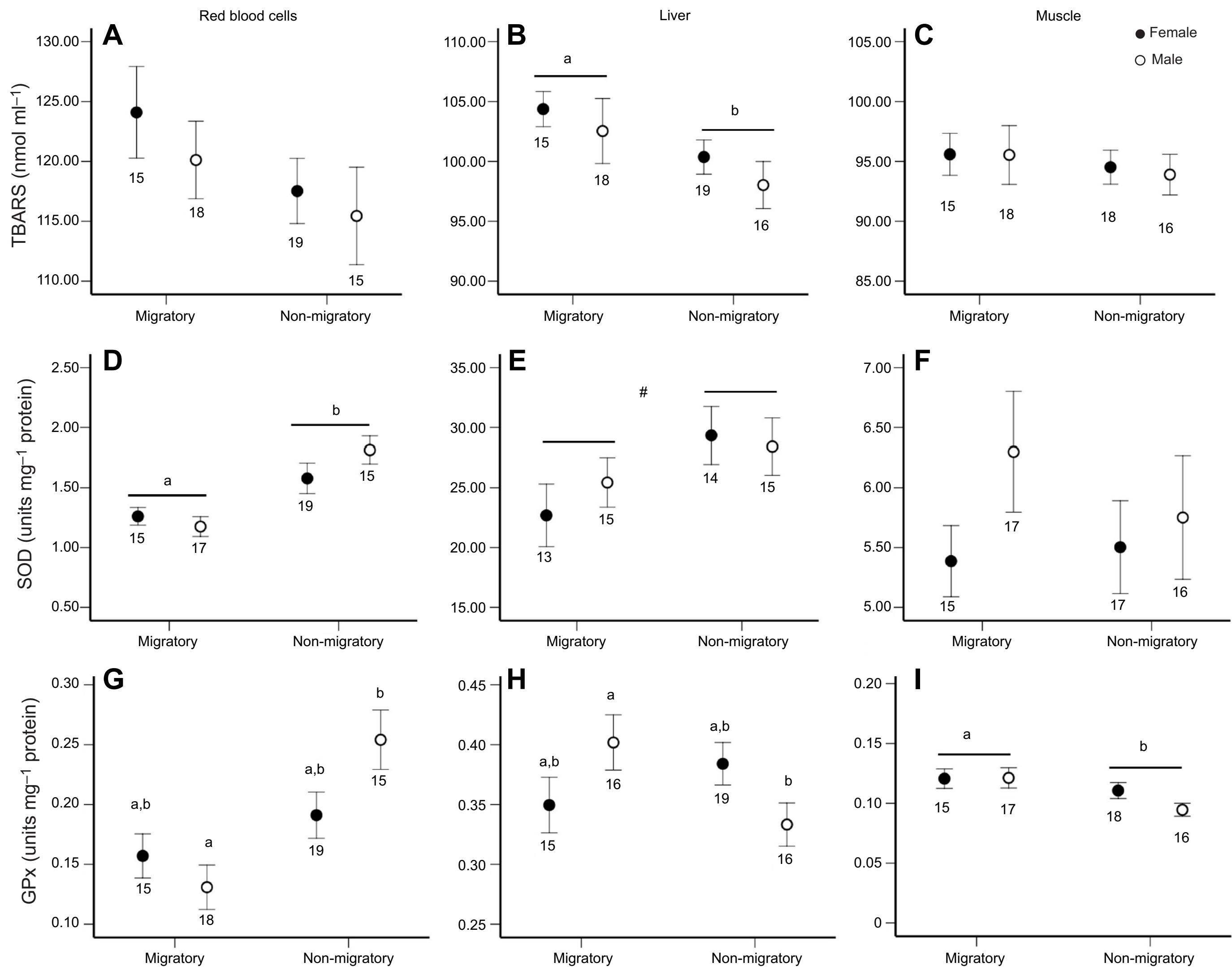

Fig. 2. Oxidative stress biomarkers measured in the red blood cells, liver and pectoral muscle tissues in female and male common quails sampled during the migratory or the non-migratory phase. (A–C) Thiobarbituric acid reactive substances (TBARS, values reported to ml of homogenate); (D–F) superoxide dismutase (SOD); and (G–I) glutathione peroxidase (GPx). Numbers indicate sample sizes separately by tissue, sampling phase and sex; minor differences across the different markers of oxidative status are due to missing measurements; different letters indicate significant differences (P<0.05) between experimental groups; $^{\#}P$=0.05. Data are shown as means±s.e.m.

examined changes in oxidative status during the period preceding spring migration, while in our study we experimentally controlled autumn migration. The difference in the results between our study and the godwit study might be due to season-specific changes in oxidative status in relation to migration (Skrip et al., 2015), but also to differences in migratory life-history strategies, or in diet between the two species as both studies relied on measurements of MDA, the end product of damage to polyunsaturated fatty acids and not other types of fat.

As expected, we found that the experimental birds sampled during the migratory phase consumed more food than the birds sampled during the non-migratory phase. However, our data do not support the hypothesis that hyperphagia itself increases oxidative damage in migratory birds, as within the migratory birds we found no associations between food intake and any markers of oxidative status among the three tissues examined. These findings are in line with previous work in captive northern wheatears (*Oenanthe oenanthe*) in which the experimental manipulation of food intake via consecutive fasting and re-feeding cycles did not alter plasma lipid peroxidation, nor were its levels explained by food intake (Eikenaar et al., 2016). While that study simulated stopover refuelling, which occurs after the birds have gone through a catabolic fasting phase, in our study the birds were sampled during the anabolic fuelling phase. Consequently, we could generalise that higher food intake in migratory birds does not lead to oxidative stress regardless of the migratory phase. These data deserve further attention as they highlight clear differences with mammals, in which high caloric intake often increases RONS production (Masoro, 2000). Increased caloric intake is one important adaptation to migration because fat accumulated before departure will provide the energy needed to support the strenuous migratory flight. In fact, we did find marked associations between changes in energy stores and muscle GPx activity as a function of the activation and de-activation of the migratory phenotype. During pre-migratory fuelling, the birds showing higher increases in body mass (and fat scores) also had higher muscle GPx activity; in contrast, during the transition to the non-migratory phase, the birds showing higher losses in body mass had lower muscle GPx activity. Interestingly, however, we found no

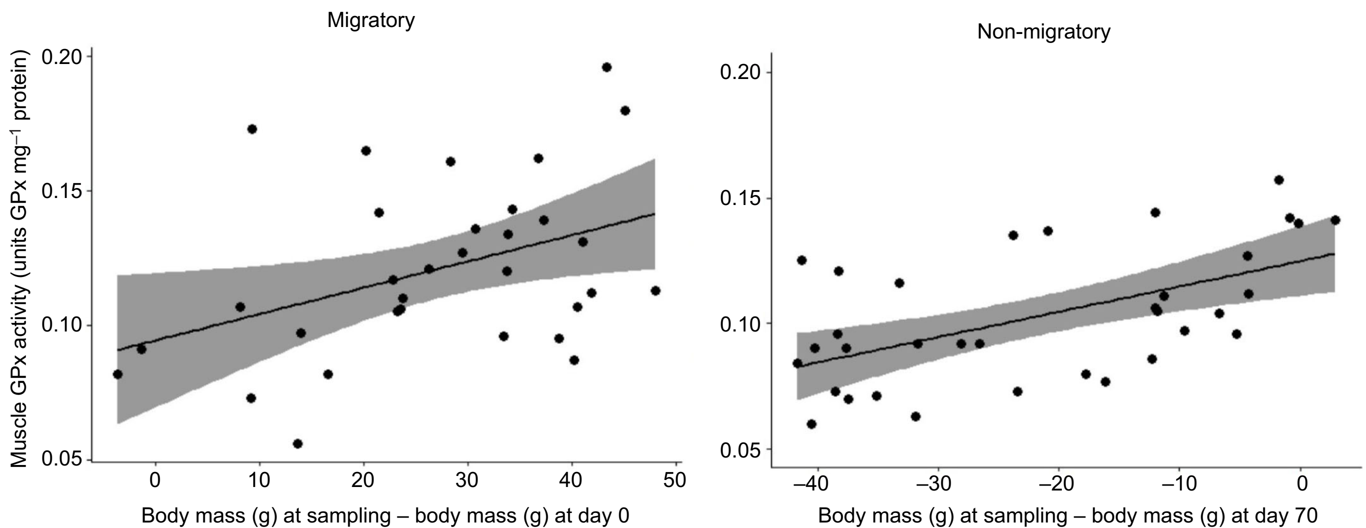

Fig. 3. Correlation plots of muscle GPx activity and within-individual changes in body mass detected within the birds sampled during the migratory phase (activation of the migratory phenotype) or the non-migratory phase (de-activation of the migratory phenotype). Grey area represents 95% confidence intervals.

link between muscle SOD activity and the change in body mass. GPx represents the secondary line of enzymatic antioxidant defences. It detoxifies the cell from hydrogen peroxide derived from SOD activity and early peroxidation compounds such as hydroperoxides (Halliwell and Guttreridge, 2015). Thus, we propose that the marked increase in body mass and subcutaneous fat scores during activation of the migratory phenotype might have exposed the birds to higher production of intermediate damaging compounds in the muscle that were efficiently quenched by prioritising upregulation of GPx. As we discuss further in the paragraph below, the upregulation of muscle GPx might be an evolved tissue-specific metabolic adaptation to avoid potential detrimental side effects of increased food intake. A comparison with species that did not evolve migratory habits would be valuable to increase our understanding of the metabolic mechanisms enabling migratory birds to avoid pathological consequences of fat intake and accumulation.

Tissue-specific changes in oxidative status in relation to the expression of the migratory phenotype were in line with our second prediction. Different tissues are expected to be differentially susceptible to oxidative damage depending on their function (Lopez-Torres et al., 1993; Pamplona et al., 2004), but also on species' life histories and developmental stages (Costantini et al., 2011; Marasco et al., 2013). As the liver has a primary metabolic function, the higher levels of oxidative damage to lipids within this tissue in the migratory birds could be linked to the increased metabolic rates and fat storage accompanying the pre-migratory stages (Gutiérrez et al., 2019). However, the inference that increased metabolic rates lead to higher RONS production is not supported by recent experimental work *in vivo* in the brown trout (*Salmo trutta*), as individuals with higher oxygen consumption rates actually had lower levels of hydrogen peroxide in the liver (Salin et al., 2015). The remarkable seasonal changes in metabolic rates exhibited by migratory birds represent an invaluable model to carry out further *in vivo* studies to experimentally test the direct effects of energy metabolism on oxidative stress. The lack of an increase in lipid peroxidation in the muscle is an intriguing result. The 'sparing' of the muscle could be an adaptation to protect this tissue from excessive accumulation of RONS. Muscles can be prone to oxidative damage, as intense exercise increases oxidative damage within this tissue (Wang et al., 2015; Dick and Guglielmo, 2019). Increased production of RONS during migratory flights could have negative consequences on flight performance (Dick and Guglielmo, 2019), and therefore on fitness outcomes. Thus, it is plausible that the changes in enzymatic defences we found in birds during the fuelling stage might represent physiological adaptations in preparation to endurance migratory flights. At least to some extent, this possibility finds some support as: (1) SOD activity levels did not differ between the two sampling phases in the muscle, as opposed to what was found in the blood and in the liver, where SOD activity was reduced in the birds sampled during the migratory phase (the reduction was marginally significant in the liver); (2) muscle GPx activity was increased during the migratory phase compared with the non-migratory phase; and, importantly, (3) the upregulation of muscle GPx was stronger in the migratory birds that accumulated larger fat stores, and such associations were absent in the other two tissues examined. Further studies in this bird species would be needed to experimentally test the validity of a 'sparing of vital tissues for migration' hypothesis in relation to oxidative stress and to determine whether the tissue-specific upregulation of enzymatic defences is linked with fitness outcomes.

Our data on GPx suggest the presence of sex-specific differences in relation to the migratory phase in the red blood cells and liver. Sexual dimorphism in glutathione biology and GPx enzymes have been relatively well characterised in mammalian models and could be attributable to sex hormones and to differences in the reproductive life cycles between sexes (recently reviewed by Wang et al., 2020). Although background information in birds is limited, our data suggest that male quails, at least during the fuelling stage, may show stronger differences in oxidative status than female quails, and possibly be better protected against oxidative challenges during the migratory period than females. However, we also cannot exclude that females might potentially tolerate more damage than males in this species. We note that our photoperiod protocol to experimentally control expression of the migratory phenotype produced sex-specific effects on the fuelling cycle. In fact, although fattening dynamics did not differ between sexes, males showed later, steeper decreases in body mass and fat stores compared with the females. However, it is unlikely that this difference explains the sex-specific results we

observed in GPx activity as there was no interaction effect between sex and food intake on GPx. We cannot exclude that sex-specific effects were linked to reproductive factors (Boswell et al., 1993), although all our experimental birds had regressed gonads throughout the experiment. Further studies would be needed to explore whether sex-specific differences in migratory behaviour may be functionally linked with markers of oxidative stress in this species.

In conclusion, our study in a captive population of pure-bred common quails provides novel evidence that birds undergo marked tissue-specific changes in oxidative status as they shift into the migratory and the non-migratory states. Our results suggest that migratory birds displace oxidative costs in the liver to strategically safeguard flight muscles from detrimental increases in oxidative damage. Future studies in different bird species (e.g. migratory versus non-migratory), ideally under a naturally varied diet in the wild, are needed to validate this possibility, and thus to directly test the links between changes in oxidative status and fitness outcomes in relation to migratory events.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: V.M., L.F.; Methodology: V.M., M.S., D.C., G.P., L.F.; Formal analysis: V.M.; Investigation: V.M., M.S.; Resources: V.M., M.S., D.C., L.F.; Data curation: V.M., M.S.; Writing - original draft: V.M.; Writing - review & editing: V.M., M.S., D.C., L.F.; Visualization: V.M., M.S., D.C., L.F.; Project administration: V.M.; Funding acquisition: V.M., L.F.

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Data availability
Data are available from the Mendeley Data Repository: doi:10.17632/8kcnh4kcd8.1.

Supplementary information
Supplementary information available online at
https://jeb.biologists.org/lookup/doi/10.1242/jeb.233486.supplemental

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Table S1. Results of General Linear Models (GLMs) to assess the impact of variation in food intake or in the within-individual changes in body mass detected within the birds sampled during the migratory period, or non-migratory period with measures of oxidative status (full details on data analysis are provided in the Materials and Methods). Fixed factors estimates are indicated in parenthesis, in bold significant terms ($p < 0.05$).

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