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Migratory refueling affects non-enzymatic antioxidant capacity, but does not increase lipid peroxidation

Cas Eikenaar [a,*], Johanna Jönsson [b], Anna Fritzsch [a], Hong-Lei Wang [b], Caroline Isaksson [b]

[a] Institute of Avian Research, An der Vogelwarte 21, 26386 Wilhelmshaven, Germany
[b] Department of Biology, Lund University, SE-22362 Lund, Sweden

HIGHLIGHTS

- Migrating birds engage in over-eating, which in mammals causes oxidative stress.
- We experimentally tested if over-eating causes oxidative stress in migratory birds.
- Individuals over-eating had higher antioxidant capacity than control individuals.
- There was no difference between these individuals in oxidative damage to lipids.
- Migrants may use the antioxidant properties of uric acid to forego oxidative damage.

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ABSTRACT

All aerobic organisms are to some degree affected by oxidative stress, which is an imbalance between pro-oxidants and antioxidants in favor of the former. Pro-oxidants can damage DNA, proteins and lipids, and as such oxidative stress can carry considerably fitness costs. In mammals excessive calorie intake is a known cause of oxidative stress. We investigated whether in migrant birds, which typically engage in over-eating in between flights (refueling), high food intake causes oxidative stress. In an experiment we compared levels of plasmatic total non-enzymatic antioxidant capacity (AOX) and oxidative damage (lipid peroxidation) between migrants repeatedly fasted and refed (simulating the flight-refuel cycle of wild migrants), and migrants on ad libitum food. We found that refueling increased AOX, an effect mainly attributable to an increase in uric acid level, an antioxidant that is produced during protein metabolism. Accordingly, variation in AOX was mainly explained by the refueling birds' food intake. However, food intake in migrants on ad libitum food did not explain any variation in AOX. Refueling did not affect lipid peroxidation, nor were its levels explained by food intake. We propose that over-eating migrants retain uric acid, which might be a very low cost mechanism to forego oxidative damage.

1. Introduction

Metabolic processes that consume oxygen continuously produce a wide variety of reactive oxygen species (ROS), which can damage DNA, proteins and lipids [5,11,25]. To prevent or minimize oxidative damage, aerobic organisms have several lines of defense, in which specific exogenous and endogenous sources of antioxidants detoxify ROS [15,22,37]. Oxidative stress occurs when the antioxidant defenses cannot prevent the damaging actions of ROS [55]. Chronic oxidative stress may be a causal factor in aging and certain diseases [29,57,59], and as such carries considerably fitness costs [31,58]. Examples of processes that have been associated with an increase in oxidative stress are intense physical activity (e.g. [14,45]) and high caloric intake (e.g. [42,57,65]). These two processes are key elements of the bi-annual migrations made by billions of birds between their breeding and wintering sites [28]. After a period of pre-migratory fueling, most migrants alternate flight bouts with stopover periods, that serve to refuel and to rest. Migratory endurance flight is carried out at very high metabolic rates [10], and to fuel migratory flights migrants engage in over-eating, i.e. repeatedly go through periods of very high caloric intake [4,39,41]. Therefore, migrating birds are likely to face increased ROS production, which they would have to counteract by enhancing their antioxidant defenses.

The few studies that have related migratory behaviour in birds to oxidative stress indeed suggest that this is happening. First, nocturnal migratory endurance flight of European robins (*Erithacus rubecula*) is associated with enhanced activity of an antioxidant enzyme (i.e., glutathione peroxidase), but also increased accumulation

* Corresponding author.
E-mail address: cas.eikenaar@ifv-vogelwarte.de (C. Eikenaar).

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of protein carbonyls, which is an oxidative damage to proteins [34]. Second, at stopover sites migrant birds consume fruit, especially fruit rich in antioxidants [9]. Finally, in migrants at stopover, fat stores were observed to be positively correlated with plasmatic non-enzymatic antioxidant capacity [13,56] or oxidative damage to lipids [56]. The positive correlation between fat stores and antioxidant capacity was interpreted as migrants building up a prophylactic antioxidant capacity prior to flight [56]. Taken together, these studies support the idea that migrant birds face increased ROS production.

What has not received any attention so far is whether high food intake during (repeated) migratory refueling at stopover sites has an effect on components of the oxidative stress status. This is surprising, as during migration birds spend far more time and energy at stopover sites than they do flying [54,66]. To fill this knowledge gap, we experimentally tested whether (repeated) over-eating during migratory refueling affects components linked to oxidative stress. More specifically, we compared plasmatic total non-enzymatic antioxidant capacity (AOX) and plasmatic malondialdehyde (MDA) level between captive migrants that were repeatedly fasted and refed, and captive migrants that had continuous access to ad libitum food. Fast-refeeding experiments are commonly used to simulate stopover refueling in captive migrants (e.g. [6,8,27]). AOX and MDA level were measured both after the first fast-refeeding bout and after four fast-refeeding bouts. Additionally, we related food intake during refeeding to both AOX and MDA level. As study subjects we used northern wheatears (*Oenanthe oenanthe*). Northern wheatears are long-distance migrants and spend a considerably part of their lives at stopover sites [53,54] where they, by overeating, accumulate fuel at high rates [17,18,51]. Consequently, if high food intake would cause oxidative damage, the fitness costs could be substantial. Therefore, we expected that, in response to high food intake during refueling, northern wheatears increase their antioxidant capacity to forego oxidative damage.

2. Methods

2.1. Experimental set-up

Late August 2014, 37 northern wheatears, all born in captivity, were transferred from their outdoor breeding aviaries to three indoor rooms (with comparable numbers of males and females in each room). The wheatears used in the experiment ranged from the 1st to 3rd generation of captive bred birds, and stemmed from wheatears taken as nestlings from Norwegian and Icelandic populations in 2006 and 2008. In the rooms the birds were housed individually in cages of $40 \times 40 \times 50$ cm with ad libitum access to food and water. Food, presented as a mash, was a mixture of water (50%), dried insect parts (10%), sunflower oil (8.7%), casein (10.3%), cellulose (14%), sugars (4.5%), and minerals (2.5%). Each bird's wing length (maximum chord) was measured to the nearest 0.5 mm. Wing length was used to calculate lean body mass, employing a linear regression based on 220 'lean' northern wheatears with fat score <2 [35] and muscle score <2 [3]: lean body mass [g] = 0.29 g mm^{-1} × wing length [mm] − 6.85 g (linear regression: $n = 220$, $F_{1,218} = 95.07$, $adj\text{-}R^2 = 0.30$, $p < 0.0001$, after [52]). The birds were weighed bi-weekly to determine their fuel reserves: fuel load = (body mass − lean body mass) / lean body mass. Fuel load thus represents the estimated amount of fuel a bird carries relative to its lean body mass. To stimulate migratory fueling, on 7 September the photo-period in the rooms was switched from long days (14L:10D) to shorter days (12L:12D). This was effective as the birds' mean and SD increase in body mass over the following two and a half weeks was 3.34 ± 2.68 g (corresponding to an increase in fuel load of 0.16 ± 0.13). On 25 September, when all birds were carrying fuel reserves large enough to sustain several days of fasting (fuel load >0.3), we started to measure their daily food intake and weigh them (both to the nearest 0.1 g) each day at lights on until the end of the experiment. Daily food intake (wet mass) was determined by subtracting food mass on day X + 1 from food mass on day X. Spilled food, collected by placing the food trays in bird baths, was added to the food mass on day X + 1. Prior to the actual experiment, on 26 September all birds were blood-sampled (ca. 80 μl) within a few minutes from entering the room. Blood samples were taken approx. six hours after lights in the rooms went on. We were able to do so because the lights in the 3 rooms went on (and off) with a 30 min difference between the rooms, and blood-sampling was done with four people. The plasma was separated by centrifugation immediately afterwards and stored at −80 °C for approx. 9 months until assaying. On 29 September in each room the birds were randomly assigned to one of two treatment groups. Birds from the first group (refueling birds, $n = 18$) underwent four consecutive fast-refeeding bouts. In these bouts, the food provided was reduced to 2 g a day (hereafter referred to as a fast), followed by days on ad libitum food, allowing the birds to refuel (after [19,20]). Each bird always ate its 2 g of food. The first fast-refeeding bout consisted of 5 days of fasting, followed by six refeeding days, and the next three bouts each consisted of three fasting and three refeeding days. The reason for the longer first fast was to make sure that also the birds that at the start of the experiment were carrying very large fuel stores (fuel load >0.5) were motivated to refuel. From each bird, a blood-sample was taken on the fifth refeeding day of the first fast-refeeding bout and on the third refeeding day of the fourth fast-refeeding bout. Blood-sampling procedures were as described above. To exclude a possible effect of handling stress on AOX and MDA, the birds were not weighed on the three blood-sampling days. One bird that fell ill was taken out of the experiment reducing the sample size to 17 refueling birds. Birds from the second group (control birds, $n = 19$) underwent the same treatment, with the only difference that they had continuous access to ad libitum food. Throughout the experiment, the temperature in the rooms was held at approx. 20 °C and all birds had ad libitum access to water. During the experiment we recorded the focal birds' nocturnal migratory restlessness (i.e., Zugunruhe). Zugunruhe was recorded automatically with motion-sensitive microphones, one of which was attached to the right wall of each cage. Each time a bird moved, an impulse was transmitted to a converting device (developed by R Nagel, Wilhelmshaven, Germany). To avoid the recording of occasional non-migratory activity, we set a threshold of three impulses per second before it was recorded as an activity count. All birds showed Zugunruhe in nearly each of the nights of the experiment (see Supplementary material), supporting our premise that birds were in a migratory condition. The experiment was conducted at the Institute of Avian Research, Wilhelmshaven Germany. All procedures were approved by the Lower Saxony State Office for Consumer Protection and Food Safety, Germany.

2.2. AOX

Total non-enzymatic antioxidant capacity of the plasma was measured using the ferric reducing antioxidant power (FRAP) assay, which gives the overall reducing potential i.e. non-enzymatic antioxidant potential of the sample [7]. Briefly, 5 μl plasma was diluted (1:8 with ddH_2O), 20 μl of the diluted plasma sample was then incubated with 150 μl working solution (sodium acetate trihydrate + 2, 4, 6-Tris (2-pyridyl)-s-triazibe (TPTZ) + Iron (III) chloride hexahydrate (FeCl3–6H2O); 10:1:1) for 20 min at room temperature. Immediately following incubation, the colour generated from the reduction of Fe^{3+} (ferric) to Fe^{2+} (ferrous) was measured on a FLUOstar OMEGA (BMG LABTECH) plate reader at 593 nm. The data obtained were compared with a standard curve made from standards of known Fe^{2+} concentration (Iron (II) sulphate heptahydrate ($FeSO_4$–$7H_2O$)) that were prepared freshly each assaying day. All samples were run in duplicate and the inter- and intra-assay coefficients of variance were 8.17 and 4.9, respectively. All chemicals used were purchased from Sigma-Aldrich (Stockholm, Sweden).