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Long flights and age affect oxidative status of homing pigeons (*Columba livia*)

David Costantini[1,2,*], Gaia Dell'Ariccia[1] and Hans-Peter Lipp[1]

[1]Division of Neuroanatomy and Behaviour, Institute of Anatomy, University of Zurich, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland and [2]Dipartimento di Biologia Animale e dell'Uomo, University La Sapienza, Viale dell'Università 32, I-00185 Roma, Italy

*Author for correspondence (e-mail: david.costantini@uniroma1.it)

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SUMMARY

Flying is an energy demanding activity that imposes several physiological challenges on birds, such as increase in energy expenditure. Evidence from sports medicine shows that exhausting exercise may cause oxidative stress. Studies on avian flight have so far considered several blood parameters, such as uric acid, corticosteroids, or circulating free fatty acids, but only one study has analysed markers of oxidative stress in flying birds. In this study, we evaluated, for the first time, how different flight efforts affect the oxidative status using homing pigeons (*Columba livia*) as a model species. Two groups of pigeons flew for around 60 and 200 km, respectively. Pigeons that flew for 200 km had a 54% increase in oxidative damage as measured by serum reactive oxygen metabolites (ROMs), a 19% drop in total serum antioxidant capacity (OXY) and an 86% increase of oxidative stress (ROMs/OXY×1000). Older pigeons depleted more serum antioxidants regardless of the release distance. Among pigeons that flew the longer distance, heavier ones depleted less serum antioxidants. The results of the study suggest that long flights may cause oxidative stress, and that older individuals may experience higher physiological demands.

Key words: aging, antioxidants, free radicals, migration, oxidative stress.

INTRODUCTION

Flapping flight is an energy demanding activity (e.g. Pennycuick, 1968; Pennycuick, 1990; Tucker, 1968; Schmidt-Nielsen, 1984; Masman and Klaassen, 1987; Ward et al., 2001; Videler, 2006). Metabolic rate during flight may actually increase by as much as 12 times the resting values (Raveling and Lefebvre, 1967; Rayner, 1982; McWilliams et al., 2004; Videler, 2006). It is also known that metabolic demands may change from one species to another (Hails, 1979).

Long-term fasting flights carried out during migration impose severe physiological challenges (Wikelski et al., 2003; McWilliams et al., 2004; Costantini et al., 2007a) (but see Hasselquist et al., 2007), but also relatively short flights may demand considerable energy (Nudds and Bryant, 2000). Several studies on homing pigeons have shown that none of the investigated blood parameters (e.g. uric acid, haematocrit, osmolality, Na^+ and K^+) show drastic changes during flights lasting around 1 h (John et al., 1988; Bordel and Haase, 1993; George and John, 1993). During flights of about 1–2 h, the metabolism of pigeons switches from using carbohydrates to fats in order to produce energy (John et al., 1988). Indeed, circulating free fatty acids increase in pigeons after 1 h of flight. Similar changes in nutrient consumption for energy production during flight have also been found in several other species (Jenni et al., 2000; McWilliams et al., 2004). Given that, in general, lipids and, in particular, unsaturated fatty acids are quite susceptible to free radical damage (Bielski et al., 1983; Porter et al., 1995) and that birds mainly accumulate unsaturated (mostly 16:1, 18:1 and 18:2 fatty acids) rather than saturated fatty acids (reviewed by McWilliams et al., 2004), such metabolic changes during long flights could expose birds to an oxidative challenge (Costantini et al., 2007a).

Metabolic activity produces reactive oxygen and nitrogen species (Leffler, 1993; Beckman and Ames, 1998). These chemicals are responsible for a plethora of oxidative damages to lipids, proteins and nucleic acids. To overcome pro-oxidants and to maintain redox homeostasis (i.e. balance between pro-oxidants and antioxidants), organisms have evolved numerous ways to protect themselves, such as enzymatic and low molecular mass antioxidants, or specific cellular components that repair oxidatively damaged molecules (Yu, 1994). The balance between pro-oxidants and antioxidants is considered the degree of oxidative stress (Finkel and Holbrook, 2000).

Studies on avian flight have so far considered several blood parameters, such as biomacromolecules, electrolytes or hormones [e.g. circulating proteins, uric acid and ions (George and John, 1993); haematocrit, plasma free fatty acids and ions (Bordel and Haase, 1993); arginine vasotocin (Giladi et al., 1997); corticosterone and uric acid (Jenni et al., 2000); proteins (Schwilch et al., 2002); and haematocrit (Jenni et al., 2006)]. However, only one recent study analysed pro-oxidants and plasma antioxidants in flying birds (Costantini et al., 2007a). The study provided indirect evidence that long migratory flights may shift the redox balance toward more oxidative conditions.

Sports medicine studies indicate that exercise may increase oxygen consumption and pro-oxidant production (Alessio, 1993; Ji, 1999). Also, some recent studies on birds have shown that the physical activity, such as breeding effort (Alonso-Alvarez et al., 2004; Wiersma et al., 2004) or exercise in a hop/hover wheel (Tsahar et al., 2006), may affect the oxidative status.

This study sought to evaluate how the redox system responds to different flight efforts in homing pigeons (*Columba livia*), a bird species in which flight physiology is well known (e.g. Pennycuick, 1968; Rothe et al., 1987; John et al., 1988; Bordel and Haase, 1993; Bordel and Haase, 2000; George and John, 1993; Schwilch et al., 1996). To quantify the oxidative status of this species, we determined, (1) the oxidative damage as serum reactive oxygen metabolites (ROMs; primarily hydroperoxides) and (2) the total

serum antioxidant capacity (OXY). In addition, we used the ratio between ROMs and OXY as a measure of the oxidative status with higher values, meaning higher oxidative stress (OS) (Costantini et al., 2006; Costantini et al., 2007a; Costantini et al., 2007b). Given that high activity levels may increase free radical production, we expected to find a positive association between flight time and oxidative stress.

MATERIALS AND METHODS

Study area and field study

Pigeons (*Columba livia* Gmelin 1789) were housed in former Swiss Army mobile lofts obtained from the Swiss Homing Pigeon Foundation. Lofts were located in Testa di Lepre (20 km north-west of Rome). All pigeons were bred locally and belonged to a line adapted to the location. Food (seed mixture for pigeons: corn, wheat, peas, vetch, rice, sunflower, millet; Agricola Aranova, Fiumicino, Roma, Italy) and water were provided *ad libitum*.

For the experiments, birds of both sexes were used. They were 1–5 years old, and all individuals had undergone numerous training flights before being used for the study. Body mass (to the nearest 5 g) was recorded for both control and flying birds on the morning of the release.

Pigeons (19, 10 and 17) were released, respectively, from Lake Vico (linear distance between the release site and the loft: 45 km; short distance, SD), and from Ardea (linear distance between the release site and the loft: 39 km; short distance, SD), and Arezzo (linear distance between the release site and the loft: 172 km; long distance, LD). We chose these distances because given a flight speed of around 60–70 km h^{-1}, we expected pigeons released from Arezzo to fly for more than 2 h. This is important because within about 1–2 h of flight, the metabolism of pigeons switches from carbohydrates to fats (John et al., 1988).

All birds included in a same release were released together. Two more pigeons were released along with the other for the releases from Ardea and Arezzo with a miniaturised global position system (GPS) data logger attached on their backs to track the pigeons' flight paths (Steiner et al., 2000). These birds were not included in the oxidative stress analyses.

On all release days, there were sunny conditions and wind was absent or weak. On the day of release, pigeons had been put in transport cages at sunrise and transported to the site of release. Meanwhile, one of the investigators manipulated the control birds in a similar manner. Control birds were also kept in transport cages, were released in the loft at the same time of release of flying pigeons, and were not allowed to access food or water until bleeding. Control birds for the release from Vico were not included in the analyses because they were manipulated in a different and slightly more stressful way as were those released from Ardea and Arezzo. This itself caused changes in the redox status that will be reported elsewhere. Both control and flying pigeons were bled 2 days before the release in order to avoid pre-release stress. These values were considered baseline values. Post-treatment blood samples were taken within 15 min of the birds' return to the loft. We bled only specimens that returned on experimental days (5, 3 and 7 from Vico, Ardea, and Arezzo, respectively). Control pigeons (N=7) were bled concomitantly with birds that flew. Both pre- and post-treatment blood samples (300 μl) were drawn from the tarsal vein and kept on ice until centrifugation. The serum was stored at –20°C.

Lab analyses

ROMs and OXY were measured by the d-ROMs test and the OXY-adsorbent test, respectively, as previously described (Costantini and Dell'Omo, 2006a; Costantini and Dell'Omo, 2006b; Costantini et al., 2006; Costantini et al., 2007a; Costantini et al., 2007b). Briefly, reactive oxygen metabolites (primarily hydroperoxides, ROOH) are early peroxidation products of the exposure of biological macromolecules (mainly lipids, but also proteins and nucleic acids) to reactive oxygen species. ROMs were determined by diluting the serum (20 μl) with 200 μl of a solution containing 0.01 mol l^{-1} acetic acid and sodium acetate buffer (pH 4.8), and *N*,*N*-diethyl-*p*-phenylenediamine as chromogen, followed by incubation for 75 min at 37°C. The acidic pH favours the release of iron and copper from serum proteins that catalyse the cleavage of hydroperoxides in two different free radicals. When these compounds react with an alkyl-substituted aromatic amine solubilized in the chromogen, they produce a complex of a colour intensity that is directly proportional to its concentration. ROMs are expressed as mmol l^{-1} H_2O_2 equivalents. The total serum antioxidant capacity was quantified as the ability of the serum antioxidant barrier to cope with the oxidant action of hypochlorous acid (HOCl; oxidant of pathologic relevance in biological systems). The serum (10 μl) was diluted 1:100 with distilled water. A 200 μl aliquot of a titred HOCl solution was incubated with 5 μl of the diluted serum for 10 min at 37°C. Then, 5 μl of the same chromogen solution used for the ROMs determination was added. An alkyl-substituted aromatic amine dissolved in the chromogen is oxidized by the residual HOCl and transformed into a pink derivative. The intensity of the coloured complex is inversely related to OXY. Measurements are expressed as mmol l^{-1} HOCl neutralised. The repeatability tested on 35 duplicates was significantly high for both markers of oxidative stress (intraclass correlation coefficient: ROMs, r=0.94, P<0.001; OXY, r=0.99, P=0.001) (see Lessells and Boag, 1987).

Statistical analyses

Statistical analyses were performed using the STATISTICA package (version 7.0, StatSoft, Inc. 2004, Tulsa, OK, USA). Generalized linear/non linear models (GLZ) (McCullagh and Nelder, 1989; Dobson, 2001) with normal error function and an identity-link function (all dependent variables are normally distributed) were used to evaluate whether flight duration affected ROMs and OXY values. A backward removal with a critical P<0.05 was used to build the minimum model, significantly explaining the observed variance. In both models, we included, as dependent variables, post-flight values of ROMs and of OXY, respectively, with their pre-flight values included as covariates. Two more models were tested to evaluate effects of flight on the relative balance between ROMs and OXY (i.e. degree of oxidative stress), including in one model as dependent variable the ROMs/OXY (×1000) ratio and in the other one the post-flight values of ROMs as dependent variable and the post-flight values of OXY as covariate, according to previous studies (Costantini et al., 2007a). Age and body mass were always included as covariates (Pearson correlation between age and body mass: r=–0.21, P=0.36). Two-way interactions between distance and age or body mass were also included in all models. Values are shown as mean ± s.e.m.

RESULTS

Samples from both short-distance (SD) groups (i.e. Vico and Ardea) were pooled together since both had similar physiological responses to flight effort (Mann-Whitney U-test: change in ROMs, P=0.57; change in OXY, P=0.14; change in OS, P=0.57). SD and long-distance (LD) pigeons returned home, respectively, in 80.0±14.5 min

Table 1. Descriptive statistics of experimental pigeons

	ROMs		OXY		OS				
Group	Pre	Post	Pre	Post	Pre	Post	Age (years)	Body mass (g)	*N*
C	0.52±0.05	0.46±0.07	383.9±14.5	371.0±17.3	1.36±0.15	1.27±0.19	3.3±0.4	417.1±14.3	7
SD	0.64±0.07	0.44±0.04	328.2±13.6	312.9±23.5	1.98±0.21	1.48±0.19	2.8±0.5	419.4±12.6	8
LD	0.50±0.09	0.77±0.13	407.9±17.4	330.1±22.5	1.23±0.22	2.29±0.33	3.4±0.4	440.7±6.9	7

Values are mean ± s.e.m.

C, control; SD, short distance; LD, long distance; pre, pre-flight; post, post-flight; ROMs, reactive oxygen metabolites (mmol l^{-1} H_2O_2 equivalents); OXY, serum anti-oxidant capacity (mmol l^{-1} HOCl neutralised); OS, oxidative stress (ROMs/OXY×1000).

and 309.6±31.1 min (*t*-test: t=–6.96, P<0.001). Data logged by GPS and extracted using the software, WINTRACK, show that pigeons of both SD and LD groups flew slightly further than the linear distance between the release site and the loft. Pigeon pairs with GPS released from Ardea and from Arezzo flew 57 and 58 km and for 187 and 190 km, respectively, so hereafter we refer to values of around 60 and 200 km, respectively, for the SD and LD groups.

The three experimental groups (Table 1) did not differ in age ($F_{2,19}$=0.75, P=0.49) or in body mass ($F_{2,19}$=1.18, P=0.33).

Reactive oxygen metabolites showed a significant (54%) increase in LD pigeons (Wald=10.54, d.f.=2, P=0.005) All other terms were removed at P values ⩾0.83. By contrast, all three experimental groups showed decreased levels of OXY. However, this was mainly evident in LD pigeons that had a 19% drop in antioxidant capacity (Wald=15.86, d.f.=2, P=0.0004; Fig. 1) that was as much as five to six times that of control or SD pigeons. The lose in serum antioxidant capacity was higher in older individuals (Wald=27.03, d.f.=1, P<0.001; Fig. 2). Finally, the covariation between body mass and change in OXY was different across groups (experimental group×body mass: Wald=14.03, d.f.=2, P=0.0009). Specifically, the sign of the covariation was positive for LD pigeons and negative for control and SD pigeons. All other terms were removed at P values ⩾0.73.

The ratio between ROMs and OXY (×1000) showed an 86% increase in LD pigeons (Wald=11.23, d.f.=2, P=0.004). All other terms were removed at P values ⩾0.49. Similar results were obtained for the second model of oxidative stress (experimental group: P=0.005).

DISCUSSION

We found that flights lasting more than 3 h impair redox homeostasis of homing pigeons, shifting the redox status, i.e. balance between pro-oxidants and antioxidants, toward more oxidative conditions. Pigeons flying a short distance (around 60 km) showed no significant change in ROMs or OXY compared to control birds. By contrast, pigeons flying around 200 km had increased levels of ROMs (54%) and decreased levels of serum antioxidant capacity (19%) compared to pre-flight levels. The balance between ROMs and OXY indicates that LD pigeons had an 86% increase in oxidative stress. This suggests that pigeons flying long distances deplete many antioxidants to compensate for increased levels of free radical production resulting from flight effort. Unexpectedly, the change in serum antioxidants was modulated by age, with older pigeons depleting more serum antioxidants, perhaps mirroring to some extent the use of antioxidants for maintaining the redox homeostasis. This was evident for the LD pigeons, as well as for the SD and control pigeons. Although these latter two groups experienced no strong stressful challenge as did LD pigeons, they, indeed, experienced a phase of fasting, which may have contributed to the slight changes of the antioxidant profile and may have been more stressful for the older pigeons. Actually, dietary antioxidants (e.g. vitamins) are an important component of the serum antioxidant capacity (Costantini and Dell'Omo, 2006b). Finally, among LD pigeons, heavier individuals depleted less serum antioxidants.

Studies from sports medicine show that strenuous exercise may increase reactive oxygen species production in skeletal muscle and, to a lesser extent, in the heart, jeopardizing body redox homeostasis (e.g. Alessio, 1993; Ji, 1999). In birds, endurance flight is known to increase protein catabolism of muscle fibres (Bordel and Haase, 2000; Jenni et al., 2000; Schwilch et al., 2002). This is in turn translated into increased levels of uric acid, a waste product of nitrogen metabolism. We did not measure uric acid, but it is known that flight increases its concentration in the blood of homing pigeons (Bordel and Haase, 1993; Schwilch et al., 1996). Uric acid is well known as an important antioxidant (Ames et al., 1981; Iqbal et al., 1999; Klandorf et al., 1999; Tsahar et al., 2006), but serum antioxidant capacity decreased in LD pigeons. This result may suggest that the uric acid levels did not increase enough to buffer the high depletion of other serum antioxidants, such as vitamins.

Studies on pigeons flying in a wind tunnel (Rothe et al., 1987) or in the wild (Schwilch et al., 1996) showed a shift to a high and stable lipid-based metabolism within 1–2 h of flight. Prolonged flights caused increased hydrolysis of triglycerides from adipose tissues to free fatty acids and glycerol and oxidation of free fatty acids by muscle activity (Schwilch et al., 1996). To some extent, such metabolic changes may be determined by the time since feeding, which is an important determinant of the fuels pigeons use in flight (Gannes et al., 2001). Overall, a lipid-based metabolism may have further contributed to increasing oxidative stress in LD pigeons. Unsaturated fatty acids are the main form of lipids stored in avian tissues (McWilliams et al., 2004), and are quite susceptible to free radical damage. These findings may suggest that pigeons and, in general, birds might need to balance energy gain and oxidative cost, both derived from metabolising lipids.

Unexpectedly, we found that older pigeons depleted more serum antioxidants in order to maintain redox homeostasis. This pattern held for controls and both experimental treatments. Mechanisms that regulate and maintain body homeostasis are known to lose functionality as a consequence of aging (Martin and Grotewiel, 2006; Kregel and Zhang, 2007). Also, studies on birds show a loss in physiological function with time (reviewed by Vleck et al., 2007).

In the present study, the higher depletion of serum antioxidant capacity of older pigeons has two possible explanations: a declining capability of the organism to respond to stress or an increasing susceptibility to stress. Actually, our data suggest that, by depleting more serum antioxidants, older pigeons were able to maintain levels of oxidative damage, as measured by ROMs, similar to those in younger pigeons.

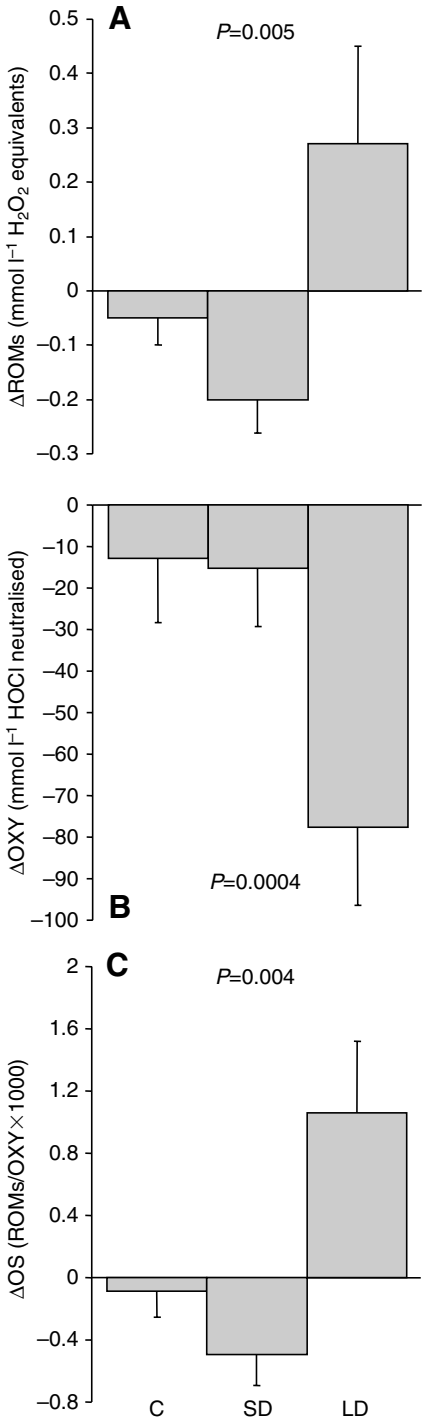

Fig. 1. Effects of flight duration on oxidative status. Pigeons that flew around 200 km (LD, long distance; *N*=7) showed (A) increased levels of oxidative damage as measured by serum reactive oxygen metabolites (ROMs), (B) decreased total serum antioxidant capacity (OXY), and (C) increased levels of oxidative stress (OS) as measured by the ratio between ROMs and OXY (×1000) compared with both controls (C; *N*=7) or pigeons that flew around 60 km (SD, short distance; *N*=8). Values are shown as mean ± s.e.m. of post- minus pre-treatment values. *P* values of the generalized linear/non-linear models are shown.

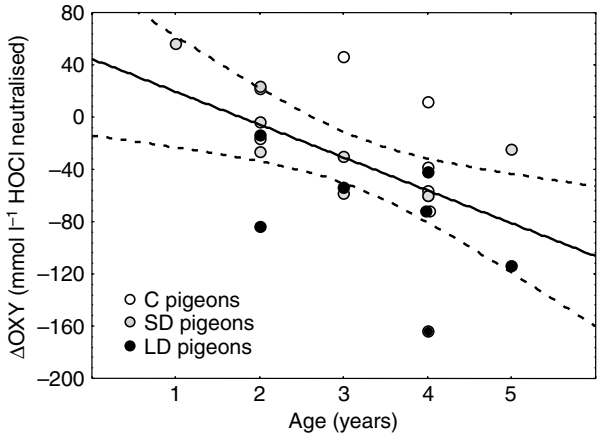

Fig. 2. The effect of age on total serum antioxidant capacity. The change (post- minus pre-flight values) in the total serum antioxidant capacity (OXY) is negatively correlated with age (*r*=–0.55, *N*=22, *P*=0.008). Regression line and 95% confidence interval (broken lines) are shown. C, control; SD, short distance; LD, long distance.

A pigeon's lifespan is from 3–5 years in the wild to a maximum of 35 years in a managed loft (Johnston and Janiga, 1995). The average lifespan of pigeons included in the present study (3.1 years) is therefore about 9% of the maximum life-span that pigeons should attain when kept in a loft. It is known that pigeons may show signs of declining body function with age. For example, declines with age in choroidal blood flow and choroidal vascularity have been actually observed at 3–5 years (Fitzgerald et al., 2001). This fact, along with the findings of the present study, suggests that a loss in body function might already be evident when pigeons are 4–5 years old. Yet, our data do not allow us to choose between these two explanations.

It is not clear why heavier LD pigeons depleted less serum antioxidants. Given that age and body mass were not correlated, age does not seem to explain this result. A study on red knots (*Calidris canutus*) showed that flight muscle efficiency increases with body mass because of a higher fuel load (Kvist et al., 2001). This could suggest that somehow heavier birds might have higher metabolic efficiency, produce fewer pro-oxidants, and have less oxidative damage. However, further research is warranted to test this hypothesis.

Conclusions

Our study showed for the first time that long flights may cause oxidative stress, and that older pigeons deplete more serum antioxidants. Possibly, heavier pigeons may deplete less serum antioxidants during a long-distance flight. Given that our study was not specifically designed for evaluating whether age or body mass affects redox status of flying pigeons, future research should analyse in detail how these two factors modulate the oxidative cost of long flights. Finally, future studies of our group will be aimed also at evaluating to what extent increase in distance remains correlated with changes in redox status.

LIST OF ABBREVIATIONS

GPS	global position system
LD	long distance
OS	oxidative stress
OXY	total serum antioxidant capacity
ROM	reactive oxygen metabolite
ROOH	hydroperoxide
SD	short distance

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