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Effects of immune supplementation and immune challenge on oxidative status and physiology in a model bird: implications for ecologists

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SUMMARY

One route to gain insight into the causes and consequences of ecological differentiation is to understand the underlying physiological mechanisms. We explored the relationships between immunological and oxidative status and investigated how birds cope physiologically with the effects of immune-derived oxidative damage. We successively implemented two experimental manipulations to alter physiological status in a model bird species: the homing pigeon (*Columba livia*). The first manipulation, an immune supplementation, was achieved by oral administration of lysozyme, a naturally occurring and non-specific antimicrobial enzyme. The second manipulation, an immune challenge, took the form of an injection with lipopolysaccharide, a bacterial endotoxin. Between groups of lysozyme-treated and control birds, we compared lipopolysaccharide-induced changes in reactive oxygen metabolites, total antioxidant capacity, haptoglobin, oxygen consumption, body mass and cloacal temperature. Lysozyme supplementation intensified the lipopolysaccharide-induced inflammatory response and generated short-term oxidative and metabolic costs. We identified significant interactions between immune supplementation and immune challenge in terms of reactive oxygen metabolites, haptoglobin and oxygen consumption. Our study provides alternative interpretations of differences in oxidative and immunological indices and demonstrates that these indices can also fluctuate and interact across very short time scales, reflecting something akin to current 'health status' or 'physiological condition'. These ephemeral effects highlight the need to broadly consider current physiological condition when drawing conclusions that relate physiology to ecology and evolution.

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INTRODUCTION

One goal of ecology is to identify the proximate causes of ecological differentiation. To this end, detailed knowledge of physiological mechanisms is often useful. Numerous new assays that promote this type of mechanistic understanding have become available in tandem with a proliferation of studies of physiological and immunological ecology (e.g. Matson et al., 2005; Tieleman et al., 2005; Costantini and Dell'Omo, 2006; Millet et al., 2007) (reviewed in Lee, 2006). However, clear-cut ecologically relevant interpretations of the data from these new assays often remain elusive. This problem is most obvious and pervasive in comparative studies, which sometimes lead to tenuous conclusions based on ecophysiological correlates (Matson, 2006; Salvante, 2006) (reviewed in Lee, 2006). One solution is to strengthen the ecological context of this new class of physiological and immunological assays. Studies that integrate newer experimental manipulations and responses with well-established ones are likely to build the strongest foundation for future research.

Studies of both wild and captive animals are important when unravelling the relationships between ecology and physiology. However, the results of field studies tend to be complicated to interpret mechanistically, particularly if confounding factors are inadequately controlled. Regarding physiological functions, confounding factors are likely to be numerous, diverse and interrelated. For example, indices of immune function and antioxidant capacity may vary with life history stage (Norris and Evans, 2000; Monaghan et al., 2009), age (Lavoie, 2005; Cohen et al., 2009a), stress levels (McEwen, 1998), energy use (Saino et al., 1998; Cohen et al., 2008), nutritional state (Cohen et al., 2009b) and health status (Costantini and Møller, 2009; Monaghan et al., 2009). By controlling for such confounding factors, studies that experimentally manipulate the physiological status of captive individuals living under standardized conditions are invaluable. These studies can fill gaps in our ecological understanding of physiological assays. To this end, we explored the relationship between immunological and oxidative status in captive pigeons.

Organisms must deal with diverse threats posed by pathogens, parasites and other sources of antigenic stimulation. The immune system defends against these threats, but this system also generates reactive oxygen species (ROS). During inflammatory immune responses, the cytotoxic effects of free oxygen radicals are exploited by phagocytes to destroy pathogens. Lysosomes release free radicals intended to kill pathogens (Klebanoff and Clark, 1978), but these free radicals may also harm host cells and contribute to oxidative damage (Halliwell and Gutteridge, 1985; Dowling and Simmons, 2009). Aside from the direct release by phagocytes, mitochondria produce ROS as a by-product of oxygen consumption and normal metabolic activity (Finkel and Holbrook, 2000). Some immune responses, most notably fever, have direct energetic repercussions (Demas et al., 1997; Ots et al., 2001); immune-associated increases

in metabolic activity may result in further oxidative damage. Thus, oxidative damage is an unavoidable cost of some immune defences (Ames et al., 1993; Beckman and Ames, 1998), and from a physiological trade-off perspective, the costs of immune-derived oxidative damage may limit investment in these defences (Råberg et al., 1998; Bertrand et al., 2006).

Regardless of the source, ROS require counteraction. Oxidative damage caused by ROS can lead to accelerated ageing, pathologies and early death (Finkel and Holbrook, 2000). Organisms can minimize these harmful effects through antioxidant barriers, which comprise a range of endogenous and exogenous compounds that impede pro-oxidant chain reactions by directly neutralizing ROS (Halliwell and Gutteridge, 1999). Allocation of resources to antioxidant systems probably occurs at the expense of other fitness-relevant investments such as reproduction and growth (Monaghan et al., 2009). The ROS–antioxidant balance, and consequently the rate at which oxidative damage is generated when the balance favours the former, is equated to oxidative stress (Finkel and Holbrook, 2000; Costantini and Verhulst, 2009).

A clear understanding of the relationship between immunological and oxidative status – specifically how animals cope physiologically with the harmful effects of immune-derived oxidative damage – is currently lacking. Recent reviews emphasize the need for experimental studies to elucidate mechanisms of oxidative stress and to facilitate interpretation of field-study results (Costantini, 2008; Monaghan et al., 2009). Thus far, experimental investigations of links between immune function and oxidative stress have arrived at mixed conclusions. Some studies show that immune challenges can increase oxidative damage (Bertrand et al., 2006; Costantini and Dell'Omo, 2006; Hõrak et al., 2007; Torres and Velando, 2007) and decrease antioxidant barriers (Bertrand et al., 2006; Costantini and Dell'Omo, 2006), while others show no relationship (Alonso-Alvarez et al., 2004; Hõrak et al., 2006; Cohen et al., 2007; Pérez-Rodríguez et al., 2008; Costantini and Møller, 2009). These equivocal conclusions may result from the quantification of different components of oxidative stress: antioxidants in some cases (e.g. Hõrak et al., 2006; Tummeleht et al., 2006) and pro-oxidants in others (e.g. Torres and Velando, 2007). The balance of oxidative status involves many different compounds; measuring only one component of the balance may affect interpretation (Costantini and Møller, 2009; Costantini and Verhulst, 2009; Monaghan et al., 2009).

In this study we sought to gain a greater understanding of the relationships between immune status, oxidative stress and energy balance. We explored how modifying baseline levels of a non-specific immune defence alters the physiological effects of an inflammatory immune challenge. Specifically, our approach was to successively implement two immunological manipulations in a single flock of captive pigeons.

The first manipulation, an immune supplementation, was achieved by orally administering lysozyme, an enzyme involved in non-specific immunity. Lysozyme is characterized by its ability to lyse susceptible bacteria, but the molecule also possesses numerous other anti-inflammatory, antibacterial and antiviral functions (reviewed in Sava, 1996). Lysozyme concentrations can vary naturally (among eggs/chicks within clutches and through time in adult birds) (Saino et al., 2002), and can positively affect immune function, health status and growth when orally administered (Sava, 1996; Siwicki et al., 1998; Humphrey et al., 2002; Brundige et al., 2008).

The second manipulation, an immune challenge, took the form of an injection with lipopolysaccharide (LPS). LPS is an endotoxin present in the outer membrane of Gram-negative bacteria and has the capacity to interact with a range of host target cells and molecules (Raetz, 1990). Mimicking bacterial infections, LPS challenges can elicit acute phase responses (APR) (Adler et al., 2001). APRs peak between 12 and 24 h after challenge and encompass diverse physiological effects, including fever and changes in acute phase protein concentrations (Exton, 1997; Adler et al., 2001). These proteins have various functions relating to systemic inflammation. For example, haptoglobin (Hp) sequesters iron and is an antioxidant (Gutteridge, 1987).

We hypothesized that lysozyme supplementation would minimize the detrimental effects and costly consequences of an LPS challenge. We predicted that, compared with controls, lysozyme-treated birds would exhibit weaker APRs and smaller shifts to oxidative stress following the challenge. We also predicted that LPS challenge would cause birds to acutely shift into oxidative stress, characterized by greater pro-oxidant production and deficient antioxidant capacity. Specifically, we examined the effects of the LPS challenge and lysozyme supplementation on oxidative stress, Hp concentration, oxygen consumption, body mass and cloacal temperature.

MATERIALS AND METHODS

Study subjects

We studied 16 adult homing pigeons (*Columba livia*, Gmelin 1789; eight females, eight males) that lived year-round in single-sex groups of four in outdoor aviaries (2 m×3 m×2 m). Food (seed/pellet mix, grit, vitamins) and water were available *ad libitum*. All birds were hatched in captivity and similarly aged (~32 months). All had identical sampling and measurement histories; none had prior exposure to experimental immune manipulations. During the August 2008 experimental period, birds were transitioning from breeding season to moult. This period had mean low/high temperatures of 13/21°C and sunrise/sunset times of ~06:15 h/21:00 h (at 53°11′N, 6°36′E).

Biological and logistical considerations influenced our study-species choice. Pigeons are known to endogenously produce lysozyme (Gavilanes, 1982) and exhibit energetically expensive febrile responses to lipopolysaccharide (Nomoto, 1996). The large difference between sample volume (<1 ml) and total blood volume, the relatively high erythrocyte turnover rate, and the intransience of haematocrit after repeated blood draws in pigeons (Rodnan et al., 1957; Bond and Gilbert, 1958), all suggest our blood collection protocol minimally impacted our study subjects. All animal experimental protocols were approved by the Animal Experimentation Committee of the University of Groningen (DEC licence no. 5095).

For the experiment, we assigned birds to eight 'focal pairs'. These same-sex pairs consisted of cage-mates. One member of each pair was randomly assigned to either the lysozyme group or the control group (four females, four males per group in total). Pairs underwent the same 7 day experimental routine. Routines were staggered in time, so data and sample collection spanned 14 days in total.

Experimental timeline

On each of the first 6 days of the experimental routine (see supplementary material Fig. S1 for illustration), we orally administered lysozyme (180 mg; L6876, Sigma, St Louis, MO, USA) in 1 ml phosphate-buffered saline (PBS; P4417, Sigma) per bird per day or PBS alone as a control (1 ml per bird per day). During the first three experimental days, focal pairs remained in their aviary after receiving their assigned supplement.

On day 4 following supplement administration, we removed focal pairs from their aviary for physiological measurements. Each member of the pair was placed singly into a dark box

(30 cm×25 cm×28 cm) at room temperature to acclimatize and fast for 4 h (from t=–4 until t=0 h). At t=0 (~17:00 h), we measured 'pre-metabolic' body mass, a baseline for calculating changes over the metabolic measurement periods. We then placed the birds into individual 13.5 l metabolic chambers sealed with air-tight lids. The two metabolic chambers sat inside the same climatic chamber, which was pre-set at 25±0.5°C (thermoneutral for pigeons) (Calder and Schmidt-Nielsen, 1967). Oxygen consumption was recorded throughout the night. In the morning on day 5 (t=18 h), each bird was removed from the chamber. We collected 'post-metabolic' data and samples in a sequence that reflected sensitivity to handling: first, cloacal temperature; second, blood sample; third, body mass. Cloacal temperature was recorded <2.5 min (1.77±0.29 min) after removing a bird from its metabolic chamber. Blood samples were drawn immediately after recording cloacal temperature (<5 min after removal). Birds were given their day 5 supplement and returned to their aviaries.

On days 6 and 7, we repeated procedures from days 4 and 5, but we added the LPS challenge. On day 6, the challenge was administered to both members of every pair after the birds had received their final oral supplement and rested/fasted for 4 h. The challenge was a single intraperitoneal injection of 2 ml kg^{-1} body mass of a 1.25 mg ml^{-1} solution of LPS (L7261, Sigma) dissolved in PBS. After injection, the birds were placed in the metabolic chambers. The collection of measurements and samples following the immune challenge was done in accordance with the procedures and timeline described above. The experimental protocol ended on day 7 when the birds were returned to their aviary.

The birds used in this experiment were part of a long-term study, which involved identical handling and sampling procedures for all study subjects. The experiment described here was situated approximately mid-way between two sample collection time points. We used physiological data from these points to determine whether the lysozyme and control groups differed by chance outside of the experimental period. Specifically, reactive oxygen metabolites (ROMs), total antioxidant capacity (TAC) and Hp were measured in samples collected approximately 6 weeks before and 4 weeks after the experiment. We also measured body mass and cloacal temperature when birds were being assigned to groups, approximately 2 weeks before the experiment began. All birds were re-weighed at the first long-term sample collection time point after the experiment.

Additional details about lysozyme supplementation

Most of what is known about the therapeutic effects of lysozyme is rooted in agricultural animal sciences (e.g. Humphrey et al., 2002; Brundige et al., 2008) and human medicine (e.g. Sava, 1996). If ecologists and others studying diverse free-living taxa wish to incorporate lysozyme supplementation treatments into their research programmes, then species-specific validation or dose–response studies may be required.

Most ecological and evolutionary questions relating to lysozyme involve egg albumin concentrations, but some work has also been done on plasma concentrations. Plasma lysozyme concentrations are known to change naturally on the order of days in birds (Saino et al., 2002). Therefore, we sought to manipulate lysozyme on this time scale in advance of the immune challenge. We wanted to provide our treatment group with more than a single supplement but also avoid a long-term regimen. Ultimately, we favoured a time course that could be applied in field-based experiments, particularly those focused on breeding birds. The *in vivo* half-life of intravenously injected lysozyme is fairly short, but a slow re-release of the labelled lysozyme from tissues extends the biological half-life to almost one day in humans and permits levels to be detected even after several days (Hansen et al., 1971; Hansen et al., 1972). Thus, we chose to supplement the birds daily.

The uniform status and condition of the captive study subjects and the balanced nature of the supplementation groups minimized the likelihood of systematic differences between groups in this study. In future studies, particularly ones involving multiple levels of variation (e.g. age class, sex and condition) in wild animals, taking all factors into consideration in advance might be unfeasible. Still, repeated-measures protocols could prove useful. For example, additional explanatory power might be gained from determining initial lysozyme levels before tailoring individual- or group-specific supplementation doses and regimens (more difficult) or from simply determining the effects of a standard supplementation *ex post facto* (less difficult).

We supplemented birds *via* the oral route, which was expected to result in a more gradual distribution of lysozyme among tissues than would occur following intravenous injection. Moreover, administration *via* injection, especially under non-sterile outdoor conditions, runs the risk of eliciting an unwanted immune response (e.g. from contamination), which would complicate interpretations. We integrated among a variety of different studies relating to plasma chemistry and oral administration of lysozyme [e.g. nutrition (Humphrey et al., 2002), ecology (Saino et al., 2002; Millet et al., 2007) and toxicology (Speijers and Van Apeldoorn, 1998; Cerven et al., 2008)] in determining an appropriate therapeutic dose of lysozyme. Because orally administered lysozyme must interact with the gastrointestinal tract and be absorbed to fully exert an effect, we used a dose based more on pharmacological effects than on naturally occurring physiological concentrations. Specifically, the dose we used was equal to the higher of two concentrations determined to be non-toxic in rats (Cerven et al., 2008), in line with the highest level provided to growing chicks (Humphrey et al., 2002), and approximately an order of magnitude higher than the dose provided to growing pigs (Brundige et al., 2008).

Blood collection

We drew small blood samples by brachial venipuncture using heparinized syringes. We collected and froze the plasma for future analyses. Pre-challenge blood samples were collected on day 5, after 22 h of fasting and before that day's supplement. Post-challenge blood samples were collected on day 7, after 22 h of fasting and 18 h after the LPS injection.

Physiological measurements

Body mass and cloacal temperature

We measured body mass using a digital balance (PB3002, Mettler-Toledo, Greifensee, Switzerland) and cloacal temperature using a thermocouple thermometer (450-ATT, Omega Engineering, Stamford, CT, USA).

Oxygen consumption

We used standard flow-through respirometry methods to calculate mean night-time oxygen consumption (Gessaman, 1987). Compressed ambient air was dried and pumped through a positive pressure open-flow system. Air passed through calibrated mass-flow controllers (model 5850S, Brooks Instrument, Hatfield, PA, USA) at 2 l min^{-1} into the metabolic chambers. After leaving the chambers, the air passed through silica gel filters to remove the moisture added by the birds, and then the percentages of O_2 and CO_2 were measured with gas analysers (O_2: Servomex Xentra 4100, Crowborough, East

Sussex, UK and CO_2: Uras 10E, Hartmann & Braun, Frankfurt, Germany). The reference stream, analysed once every 2 h (from minutes 1 to 18), went directly to the gas analysers. Between reference periods, air leaving the two metabolic chambers was analysed in alternating 17 min bouts.

We calculated O_2 consumption and CO_2 production rates based on the air flow rate and the differences in the percentages of both gasses between the reference stream and the metabolic-chamber streams using an equation adapted from Hill (Hill, 1972). In general, CO_2 production paralleled O_2 consumption (see supplementary material Tables S1–S3); thus the CO_2 results are not discussed further. We obtained qualitatively similar results after analysing mass-specific rates and whole-animal rates with body mass included as a covariate (see supplementary material Tables S1–S3); thus we only consider mass-specific rates.

We included a 4 h acclimatization period (see supplementary material Fig. S1) to ensure that all birds experienced consistent conditions in advance of the measurement period. Specifically, this period was used to minimize and standardize any possible effects of capture stress and digestion (*sensu* Tieleman et al., 2009) on oxygen consumption. Birds were also allowed to rest in the sealed, temperature-controlled metabolic chambers for an additional hour (59±5 min) before we collected the first measurements that were included in the nightly means (Lasiewski et al., 1966).

ROMs

We used the d-ROMs test (Diacron, Grosseto, Italy) to measure the level of hydroperoxides, ROMs that signal lipid and protein oxidative damage (Alberti et al., 2000; Iamele et al., 2002). We diluted 40 µl of plasma in 400 µl of the provided acidic buffered solution and followed the 'end-point mode' instructions provided by the manufacturer. We measured absorbance at 505 nm (spectrophotometer DU-720; Beckman Coulter, Brea, CA, USA), and we calculated ROMs concentration (in mmol l^{-1} of H_2O_2 equivalents) from the absorbance. Duplicate measurements of samples over two separate assays and within the same assay showed an inter-assay variation of 1.84% and an intra-assay variation of 1.34%.

TAC

TAC was measured using the OXY-Adsorbent test (Diacron, Grosseto, Italy), which measures the effectiveness of the blood antioxidant barrier by quantifying its ability to cope with the oxidant action of hypochlorous acid (HClO). We diluted 10 µl of plasma in 500 µl of distilled water; we then mixed 10 µl of this diluted plasma with 500 µl of the HClO solution provided and continued by following the manufacturer's instructions. We measured absorbance at 505 nm and calculated total antioxidant capacity (in mmol l^{-1} of HClO neutralized). Inter- and intra-assay variations were 5.12% and 3.27%, respectively. For more details regarding the ROMs and TAC assays see Costantini and Dell'Omo (Costantini and Dell'Omo, 2006).

Hp

Haptoglobin and its functional equivalents are acute phase proteins found in a range of taxa including birds (Delers et al., 1988; Matson, 2006; Matson et al., 2006). Hp normally circulates at low concentrations, but concentrations can increase rapidly in response to infection, inflammation or trauma. Thus elevations in Hp may indicate an immune response. Hp has also been demonstrated to function as an antioxidant (Gutteridge, 1987). We measured concentrations of Hp (mg ml^{-1}) by using a commercially available assay kit (TP801; Tri-Delta Diagnostics, Morris Plains, NJ, USA) and following the 'manual method' instructions provided by the manufacturer. In essence, this functional assay measures the haem-binding capacity of plasma (Matson 2006; Matson et al., 2006).

Statistics

Individuals were measured repeatedly over time, so we used multilevel mixed models [MLWiN 2.02 (Rasbash et al., 2004)] with 'individual' included as a random effect. We did not include either 'cage' or 'parentage' as random effects because neither significantly improved the models (cage: $\chi^2_1<0.41$, $P>0.53$; parentage: $\chi^2_1<2.49$, $P>0.11$). We began all analyses of the LPS-induced physiological responses (Δ_{LPS}) by examining the main effect of sex and the interaction between sex and supplementation group. Neither of these had a significant effect on any of the physiological responses ($P>0.101$), except for sex in Hp ($\beta_{females}=-0.10\pm0.03$, $\chi^2_1=12.54$, $P<0.001$). With Hp, including or excluding sex did not impact on the significance of the lysozyme supplementation (both $P<0.001$). Thus, we never included sex or the interaction between sex and supplementation group in our final models. Final models included only two explanatory variables: supplementation group (control or lysozyme) and a covariate comprising baseline (pre-challenge) values of the dependent variable under consideration. We used a bivariate general linearized mixed model (GLMM) with ROMs and TAC as response variables. This approach assesses the covariance between the two response variables and allows for determination of the extent to which oxidative damage is counteracted by the antioxidant barrier. In all models, significance of explanatory variables was determined from the Wald statistic, which approximates the χ^2 distribution. Statistical significance level was set at $\alpha=0.05$. Full statistics, including group means, are provided in the supplementary material Tables S1–S3.

RESULTS

Inflammatory effects of the LPS challenge

We first examined the consequences of the LPS challenge in our control pigeons. We found clear effects with most of the measured physiological parameters (open bars in Fig. 1, see supplementary material Tables S1–S3 for full statistics). In control birds, the plasma concentrations of ROMs and Hp both showed highly significant increases (ROMs: $\chi^2_1>121$, $P<0.001$ and Hp: $\chi^2_1>34$, $P<0.001$) following the LPS challenge. TAC decreased, but not significantly ($\chi^2_1=3.03$, $P=0.08$). The LPS challenge led to significantly increased mass-specific oxygen consumption ($\chi^2_1=8.99$, $P=0.003$). It also resulted in significantly greater mass loss (over the 18 h metabolic measurement periods; $\chi^2_1=5.7$, $P=0.02$) and significantly elevated cloacal temperature ($\chi^2_1=4.64$, $P=0.03$).

Effects of lysozyme supplementation on LPS-induced inflammation

Having established that the LPS challenge perturbed pigeon physiology, we examined the effects of lysozyme supplementation on the magnitude of the LPS-induced changes (Δ_{LPS}, Fig. 1; see supplementary material Tables S1–S3 for full statistics). LPS responses were stronger in the lysozyme than the control groups for three of the six measured parameters: ROMs concentration ($\beta=0.20\pm0.08$, $\chi^2_1=6.59$, $P=0.010$), Hp concentration ($\beta=0.12\pm0.04$, $\chi^2_1=11.76$, $P<0.001$) and oxygen consumption ($\beta=0.03\pm0.01$, $\chi^2_1=5.75$, $P=0.016$). Lysozyme supplementation did not significantly affect LPS-induced reductions in TAC ($\beta=7.88\pm5.70$, $\chi^2_1=1.91$, $P=0.167$). Likewise, lysozyme supplementation did not significantly affect responses to LPS in terms of mass loss ($\beta=-0.03\pm0.33$,

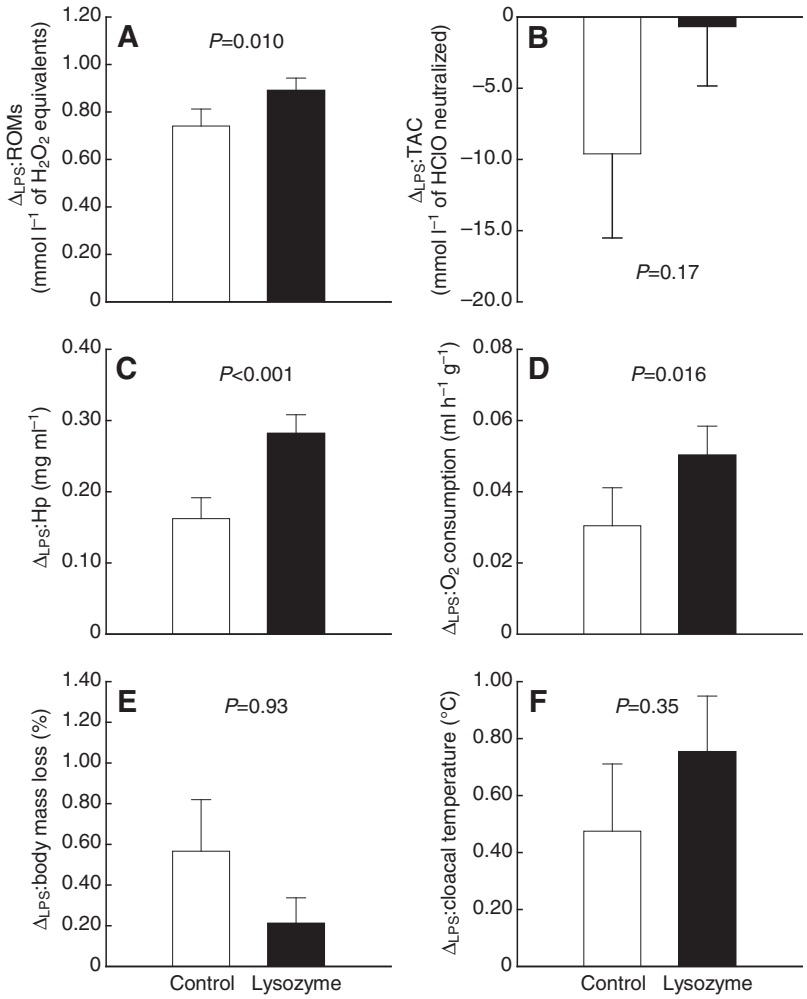

Fig. 1. The effect of lysozyme supplementation on the magnitude of the lipopolysaccharide (LPS)-induced changes in (A) reactive oxygen metabolites (ROMs), (B) total antioxidant capacity (TAC), (C) haptoglobin (Hp) concentration, (D) oxygen consumption, (E) body mass loss and (F) cloacal temperature in pigeons. Bars indicate group means and s.e.m. (N=8), and differences between the groups are indicated following the analyses in which the baseline (pre-challenge) values were included as a covariate.

χ^2_1=0.01, P=0.929) and cloacal temperature (β=0.23±0.24, χ^2_1=0.89, P=0.345).

In analyses of all six parameters, we always included baseline (pre-challenge) values of the parameter under consideration as a covariate. With three parameters, this covariate was significant (ROMs: χ^2_1=4.33, P=0.037; TAC: χ^2_1=6.64, P=0.010; cloacal temperature: χ^2_1=6.12, P=0.013; see supplementary material Tables S1–S3 for full statistics).

Effects of lysozyme supplementation only

Prior to their random assignment to either the control or lysozyme group, birds did not differ in any pre-measured physiological parameter (ROMs, TAC, Hp, body mass, cloacal temperature; all P>0.1). After 4 days of supplementation (pre-challenge time point), we found significant differences between the lysozyme and control groups in two of the six experimental parameters: lysozyme-treated birds consumed more oxygen (χ^2_1=7.62, P=0.006) and lost more body mass over the metabolic measurement period (χ^2_1>12, P<0.001; see supplementary material Tables S1–S3 for full statistics). After the full supplementation period (6 days) and the LPS challenge (post-challenge time point), we found significant differences between the lysozyme and control groups in three of the six experimental parameters: lysozyme birds had higher ROMs (χ^2_1=6.48, P=0.011), higher Hp concentrations (χ^2_1=11.48, P<0.001) and consumed more oxygen (χ^2_1=5.79, P=0.016) (see supplementary material Tables S1–S3 for full statistics). Pre-metabolic masses did not differ between supplementation groups at either experimental time point (P>0.14), but did decline over the 48 h period preceding LPS challenge (lysozyme: P<0.080; control: P<0.001). Four weeks after the experiment, control and lysozyme groups did not differ in any measured physiological parameter (ROMs, TAC, Hp, body mass; all P>0.2).

Relationships between pro-oxidants, antioxidants and oxygen consumption

The ROMs response to the LPS challenge was significantly stronger in the lysozyme-treated birds than in the control birds (Table 1). We found no group difference in the TAC response. Overall lysozyme supplementation also significantly strengthened (χ^2_2=6.73, P=0.03) the combined ROMs/TAC response (i.e. treated statistically as one response variable). This effect seemed to result primarily from dissimilar ROMs responses between the two groups (also see Fig. 2A). The ROMs response did not correlate with the TAC response, as the covariance between the ROMs and TAC responses was not significant (Table 1).

LPS challenge and lysozyme supplementation significantly increased both oxygen consumption and ROMs (Fig. 2B). The positive correlation between oxygen consumption and ROMs approached significance (χ^2_1=3.54, P=0.06; after correction for LPS challenge and lysozyme supplementation effects). We found no relationship between oxygen consumption and TAC (β=–36.55±119.61, χ^2_1=0.09, P=0.76).

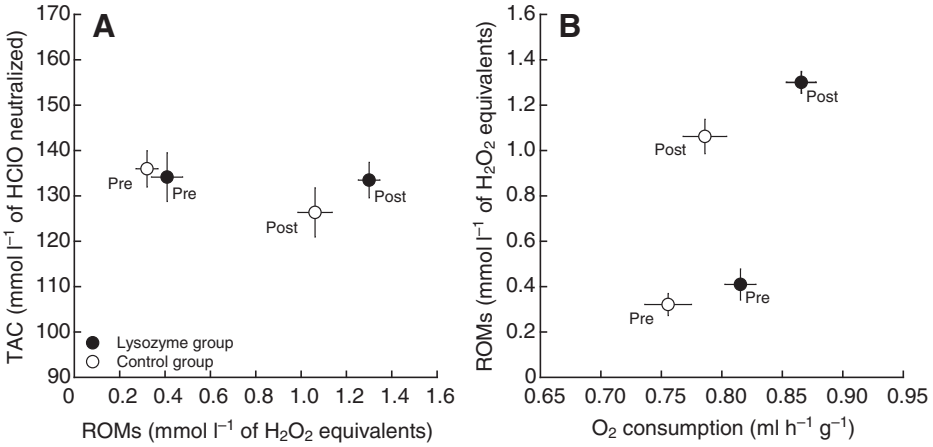

Fig. 2. Relationships between ROMs and (A) TAC and (B) oxygen consumption within treatment groups pre- and post-LPS challenge. Group means are shown ±s.e.m. (all *N*=8).

DISCUSSION

We examined the interaction between an immune supplementation and immune challenge in captive pigeons and measured the effects on multiple physiological parameters. The LPS immune challenge increased ROMs, oxygen consumption, Hp and cloacal temperature (in both lysozyme-treated and control birds), and caused body mass loss (control birds only). In some cases, we found that the magnitude of the LPS-induced responses differed between the lysozyme and control groups, suggesting that lysozyme heightened physiological responses and induced some costs.

Lysozyme supplementation, LPS challenge and their interaction

We successfully created two groups that differed in physiological status through lysozyme supplementation. Compared with the controls, the lysozyme-treated birds showed a greater response to the LPS challenge. Lysozyme has been reported to bind *in vitro* to LPS and dampen its detrimental effects (Ohno and Morrison, 1989) and to reduce oxidative stress (Liu et al., 2006). We found no *in vivo* evidence for these effects. Rather, lysozyme-supplemented pigeons exhibited stronger inflammatory responses to LPS with greater increases in oxidative damage and oxygen consumption.

Previous studies suggest that preventative and therapeutic administration of lysozyme has net positive effects in terms of growth and health (Sava, 1996; Humphrey et al., 2002; Brundige et al., 2008). In young pigs, consumption of lysozyme-supplemented milk improves gastrointestinal health and protects against bacterial infections without limiting growth (Brundige et al., 2008). In chicks, lysozyme supplementation exerts similar effects to the preventative administration of antibiotics (Humphrey et al., 2002), perhaps by limiting the energy diverted towards background inflammation (*sensu* Roura et al., 1992).

In our adult pigeons we found lysozyme supplementation augmented immune function. However, the supplementation appeared to bear short-term oxidative and metabolic costs, which are previously unreported and which seem to conflict with lysozyme's role in growth promotion. *In vitro* studies further expose this apparent contradiction. Lysozyme directly enhances phagocytic activity of human polymorphonuclear leukocytes (Klockars and Roberts, 1976), including macrophages (reviewed in Jollès and Jollès, 1984). Some *in vivo* studies even reflect this finding: lysozyme injections stimulate cellular and humoral immunity in fish, thereby reducing mortality rates (Siwicki et al., 1998). During immune responses, leukocytes increase oxygen uptake and generate free oxygen radicals that are used to destroy bacteria (Klebanoff and Clark, 1978; Halliwell and Gutteridge, 1999). The inconsistency of lysozyme being beneficial to energetically demanding growing animals on the one hand, but apparently amplifying energetic costs of an immune response on the other, may be partly explained by developmental or ontogenetic differences in immune system architecture and antigen exposure. Completely reconciling this contradiction will probably require both

Table 1. Bivariate GLMM examining the effect of lysozyme supplementation on the magnitude of the response to the LPS challenge of ROMs and TAC in pigeons, and accounting for the levels before the challenge

	ROMs response to LPS challenge			TAC response to LPS challenge			ROMs and TAC response combined		
	Estimate ± s.e.	χ^2_1	*P*	Estimate ± s.e.	χ^2_1	*P*	Estimate ± s.e.	χ^2_2	*P*
Final model									
Intercept	0.90±0.09			45.77±31.97					
Lysozyme treatment*	0.18±0.07	5.73	0.017	–	–	–	–	6.73	0.035
ROMs pre-challenge	–0.47±0.23	4.06	0.044	34.40±17.27	3.97	0.046	–	9.08	0.011
TAC pre-challenge	–	–	–	–0.47±0.22	4.66	0.031	–	4.98	0.083
Covariance							0.38±0.40	0.91	0.340
Rejected terms									
Lysozyme treatment*	–	–	–	5.44±5.43	1.00	0.317			
TAC pre-challenge	0.00±0.00	0.00	1.000	–	–	–			

GLMM, general linearized mixed model; LPS, lipopolysaccharide; ROMs, reactive oxygen metabolites; TAC, total antioxidant capacity.
*Reference category is 'control group'.
Significant explanatory variables were left in the minimal adequate model after stepwise removal of non-significant variables.
Covariance between the two response variables indicates the individual correlation between the ROMs and TAC response.

in vitro and *in vivo* studies of the same species at different life history stages and under different conditions.

When analysing the effects of lysozyme supplementation on the magnitude of the LPS-induced changes, we always included baseline (pre-challenge) values of the parameter under consideration as a covariate. For most parameters, this covariate was an important predictor of the LPS response strength. With antioxidants, baseline levels have been shown to predict response strength following an energetic challenge (Cohen et al., 2008), suggesting that physiological status affects the capacity to further increase antioxidant defences. Similarly, a ceiling may exist that limits further generation of antioxidants, even in the face of a pro-oxidant challenge. Antioxidants are thought to have costs, as the resources involved with antioxidant defences may have alternative roles within the body, such as growth or coloration (Monaghan et al., 2009). Investments in antioxidants should therefore not exceed the critical limit at which other physiological systems are detrimentally affected.

Oxidant–antioxidant balance: the relationship between ROMs and TAC

Recent reviews of oxidative stress analyses emphasize the importance of measuring both pro-oxidant and antioxidant variables (Costantini and Verhulst, 2009; Monaghan et al., 2009). Some previous studies (Costantini and Dell'Omo, 2006; Costantini et al., 2007) measure both variables but presented the results separately. Sometimes ratios (e.g. ROMs/TAC×1000) were computed, but their interpretive value is murky because ROMs and TAC have different units (Costantini et al., 2007; Costantini et al., 2008). However, careful examination of the oxidant–antioxidant balance and understanding within-individual covariance between pro-oxidants and antioxidants can provide additional insight into the combined action of the different components (Costantini and Møller, 2009; Costantini and Verhulst, 2009; Monaghan et al., 2009).

Combining the two oxidative stress variables (Table 1, Fig. 2A), we found that both supplementation groups experienced oxidative stress after the LPS challenge. This result was mainly attributable to increases in ROMs and not to changes in TAC. As similarly reported by Cohen and colleagues (Cohen et al., 2007), the LPS challenge that we implemented did not impair total antioxidant capacity in any apparent manner. Rather, individuals shifted acutely into oxidative stress as more ROMs were produced, a result that is in accord with various other avian studies (Bertrand et al., 2006; Costantini and Dell'Omo, 2006; Hõrak et al., 2007; Torres and Velando, 2007). The apparent stability of antioxidant defences before and after challenge may be explained in part by counteracting physiological responses to LPS (Cohen et al., 2007). For example, simultaneous release and consumption of an expendable subset of antioxidant defences would appear as no net change in TAC. Likewise, because we measured total antioxidant capacity, we know little about the composition of defences, which may or may not have changed following the LPS challenge.

Another explanation of the observed ROMs and TAC responses relates to the acute nature of the employed challenge. A rapid surge of pro-oxidants may at first exceed the deployment rate of antioxidant defences. In contrast, a gradual elevation of oxidant levels over longer time intervals may result in less oxidative stress, as antioxidant defences are concomitantly up-regulated (reviewed in Monaghan et al., 2009). Such chronic elevations might lead to reallocation of resources away from other functions that rely on antioxidants and towards defences against oxidative damage. In this context, a challenge-dependent response hierarchy might exist. During acute immune challenges, quick (oxidant-generating) responses may be most important, leaving the effects of pro-oxidants for later concern. The pigeons studied here responded rapidly to the acute LPS challenge despite the risks associated with increased pro-oxidants that were not immediately inactivated by antioxidant defences. In contrast, when facing chronic or less-severe immune challenges, responses that minimize oxidative damage may be favourable.

Hp

Hp concentration increased after LPS challenge. Concentrations of acute phase proteins generally increase within hours of an inflammatory challenge (Adler et al., 2001), and in our pigeons Hp remained elevated 18 h post-challenge. As a marker of inflammation, Hp concentration indicated that lysozyme-treated birds experienced stronger responses to the LPS challenge than control birds. Three days of lysozyme supplementation alone did not affect Hp concentration. Thus, the effects of supplementation on LPS challenge were unlikely to be due simply to pre-challenge reductions in inflammation. Such reductions have been posited to drive the growth-promoting properties of lysozyme (Humphrey et al., 2002).

The total antioxidant barrier did not cope effectively with the sudden overproduction of pro-oxidants, even though Hp was produced in high concentrations. The total antioxidant barrier comprises an array of components that act at different stages in the oxidative reaction chain and that are generated in different locations and at different rates. Some antioxidants may be rapidly generated to excess, whereas the provision rate of others may not be sufficient to cope with the increasing pro-oxidant levels. Though Hp can act as a strong antioxidant (Gutteridge, 1987; Dobryszycka, 1997) and inhibit the pro-oxidants that accompany respiratory burst (Wagner et al., 1996), our results show that Hp alone was insufficient to counteract the oxidizing power of all newly formed oxygen radicals. This result highlights the functional specificity inherent to individual antioxidants. Such specificity can shape interpretation and limit inference when quantifying single antioxidants (Costantini and Møller, 2009; Monaghan et al., 2009).

Mechanistically, Hp functions by complexing with free haem and reducing the availability of plasma iron. Changes in plasma trace metal availability are common features of APRs (Exton, 1997). By complexing with haem, Hp prevents the molecule both from participating in free radical chain reactions (Gutteridge, 1987; Dobryszycka, 1997) and from serving as a nutrient for invading bacteria (Exton, 1997). Other mechanisms can also lead to reductions in plasma iron [e.g. anorexic behaviour (Exton, 1997)], but these mechanisms also stem from inflammatory challenges, relate to APRs, and therefore do not affect our interpretations of elevated Hp concentration. Moreover, plasma samples were collected from all pigeons at nutritionally comparable time points (i.e. fasted for 22 h), so differences in feeding behaviour did not account for differences in plasma Hp or iron. With or without inflammation, other releases of haem (e.g. from haemolytic anaemia) lead to decreased Hp concentration (Körmöczi et al., 2006), which we did not observe.

Oxygen consumption

Mass-specific oxygen consumption increased after the immune challenge. This finding supports the existing indications of direct energetic costs of some immune responses (Demas et al., 1997; Ots et al., 2001). We found a positive relationship between oxygen consumption and ROMs (Fig. 2B). The majority of pro-oxidant radicals are by-products of respiration (Finkel and Holbrook, 2000), and their production is thought to be directly linked to the

consumption of oxygen as a function of metabolism (Cohen et al., 2008). Higher metabolic activity and elevated oxygen consumption are therefore expected to correlate positively with the production of oxidants and with the demand for antioxidant defences. Support for this hypothesis comes from studies which show acute exercise in unaccustomed animals leads to the generation of more oxidants than the antioxidant barrier can neutralize (Ji, 1999; Leeuwenburgh and Heinecke, 2001) (reviewed in Monaghan et al., 2009). Yet, care must be taken before simply attributing metabolic rate as a proxy for free radical production. Elevated oxygen consumption might result in the down-regulation of free radical production by mitochondria (Brand, 2000; Speakman et al., 2004), and free radical generation may differ between taxa with different life histories (Herrero and Barja, 1998). For instance, the rate of mitochondrial pro-oxidant production per unit of oxygen consumed in pigeons was relatively low compared with that in rats (Barja, 2007).

Improving interpretations of physiological measurements

Incorporating physiological and immunological measurements into field studies is increasingly popular among ecologists, but there is a tendency to interpret differences solely within the framework of ecology and evolution and to ignore physiological considerations. Our study reveals that some of the complexity associated with measuring physiological parameters extends beyond individual physiological systems (Matson et al., 2006; Cohen and McGraw, 2009) to interactions between related systems. Physiological parameters not only vary on ecological and evolutionary time scales (e.g. Tieleman et al., 2005; Lee, 2006; Cohen et al., 2008) but also can fluctuate and interact across very short time scales, as our study demonstrates, reflecting something similar to current ‘health status’ or ‘physiological condition’.

Variation in health status or physiological condition can be thought of as the full range of values that individuals might experience in a lifetime, from homeostatic to pathologic. In this sense, health status or physiological condition does not necessarily depend on individual quality and may be strongly affected by stochastic events such as a disease epidemic. This variation, sometimes overlooked by ecologists, can potentially have profound effects on the interpretation of field-measured physiological parameters within and among populations. Within populations, variation caused by differences in status is likely to be interpreted as statistical noise and can be seen as a nuisance. Interactions between individual quality and status, however, would clearly complicate interpretation. Likewise, if differences in ecology and current status are confounded among study populations, then unwarranted conclusions might be drawn.

Field studies often rely on single point measurements, and conducting experiments on free-living animals is often unfeasible. Nevertheless, some approaches can minimize the risks of misinterpreting the effects of current status. Measurements across multiple years or periods, among a greater number of populations, or repeated within individuals can all assist in identifying ephemeral effects that are limited in time or space. In the absence of these approaches, potential differences in unmeasured aspects of current status should be contemplated when interpreting results. Baseline levels may also be important for understanding physiological responses; in field studies, however, establishing baseline or response levels is sometimes impossible. In these cases, experiments on captive individuals of the study species or a model species may be valuable for understanding how competing physiological demands play out. Taking full advantage of both experimental and correlative approaches is necessary for unravelling the many levels of variation associated with defences against disease and oxidative stress.

LIST OF ABBREVIATIONS

APR	acute phase response
Hp	haptoglobin
LPS	lipopolysaccharide
ROMs	reactive oxygen metabolites
ROS	reactive oxygen species
TAC	total antioxidant capacity

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REFERENCES

Adler, K. L., Peng, P. H., Peng, R. K. and Klasing, K. C. (2001). The kinetics of hemopexin and α1-acid glycoprotein levels induced by injection of inflammatory agents in chickens. *Avian Dis.* **45**, 289-296.

Alberti, A., Bolognini, L., Macciantelli, D. and Caratelli, M. (2000). The radical cation of N,N-diethyl-para-phenylendiamine: A possible indicator of oxidative stress in biological samples. *Res. Chem. Intermed.* **26**, 253-267.

Alonso-Alvarez, C., Bertrand, S., Devevey, G., Gaillard, M., Prost, J., Faivre, B. and Sorci, G. (2004). An experimental test of the dose-dependent effect of carotenoids and immune activation on sexual signals and antioxidant activity. *Am. Nat.* **164**, 651-659.

Ames, B. N., Shigenaga, M. K. and Hagen, T. M. (1993). Oxidants, antioxidants, and the degenerative diseases of aging. *Proc. Natl. Acad. Sci. USA* **90**, 7915-7922.

Barja, G. (2007). Mitochondrial oxygen consumption and reactive oxygen species production are independently modulated: implications for aging studies. *Rejuvination Res.* **10**, 215-224.

Beckman, K. B. and Ames, B. N. (1998). The free radical theory of aging matures. *Physiol. Rev.* **78**, 547-581.

Bertrand, S., Criscuolo, F., Faivre, B. and Sorci, G. (2006). Immune activation increases susceptibility to oxidative tissue damage in Zebra Finches. *Funct. Ecol.* **20**, 1022-1027.

Bond, C. F. and Gilbert, P. W. (1958). Comparative study of blood volume in representative aquatic and nonaquatic birds. *Am. J. Physiol.* **194**, 519-521.

Brand, M. D. (2000). Uncoupling to survive? The role of mitochondrial inefficiency in ageing. *Exp. Gerontol.* **35**, 811-820.

Brundige, D. R., Maga, E. A., Klasing, K. C. and Murray, J. D. (2008). Lysozyme transgenic goats’ milk influences gastrointestinal morphology in young pigs. *J. Nutr.* **138**, 921-926.

Calder, W. A. and Schmidt-Nielsen, K. (1967). Temperature regulation and evaporation in the pigeon and the roadrunner. *Am. J. Physiol.* **213**, 883-889.

Cerven, D., DeGeorge, G. and Bethell, D. (2008). 28-Day repeated dose oral toxicity of recombinant human apo-lactoferrin or recombinant human lysozyme in rats. *Regul. Toxicol. Pharmacol.* **51**, 162-167.

Cohen, A. A. and McGraw, K. J. (2009). No simple measures for antioxidant status in birds: complexity in inter- and intraspecific correlations among circulating antioxidant types. *Funct. Ecol.* **23**, 310-320.

Cohen, A. A., Klasing, K. C. and Ricklefs, R. E. (2007). Measuring circulating antioxidants in wild birds. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **147**, 110-121.

Cohen, A. A., Hau, M. and Wikelski, M. (2008). Stress, metabolism, and antioxidants in two wild passerine bird species. *Physiol. Biochem. Zool.* **81**, 463-472.

Cohen, A. A., Mauck, R. A., Wheelwright, N. T., Huntington, C. E. and McGraw, K. J. (2009a). Complexity in relationships between antioxidants and individual life-history parameters in a seabird and a songbird. *Oikos* **118**, 1854-1861.

Cohen, A. A., McGraw, K. J. and Robinson, W. D. (2009b). Serum antioxidant levels in wild birds vary in relation to diet, season, life history strategy, and species. *Oecologia* **161**, 673-683.

Costantini, D. (2008). Oxidative stress in ecology and evolution: lessons from avian studies. *Ecol. Lett.* **11**, 1238-1251.

Costantini, D. and Dell’Omo, G. (2006). Effects of T-cell-mediated immune response on avian oxidative stress. *Comp. Biochem. Physiol. A Physiol.* **145**, 137-142.

Costantini, D. and Møller, A. P. (2009). Does immune response cause oxidative stress in birds? A meta-analysis. *Comp. Biochem. Physiol. A Physiol.* **153**, 339-344.

Costantini, D. and Verhulst, S. (2009). Does high antioxidant capacity indicate low oxidative stress? *Funct. Ecol.* **23**, 506-509.

Costantini, D., Coluzza, C., Fanfani, A. and Dell’Omo, G. (2007). Effects of carotenoid supplementation on colour expression, oxidative stress and body mass in rehabilitated captive adult kestrels (*Falco tinnunculus*). *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* **177**, 723-731.

Costantini, D., Dell'Ariccia, G. and Lipp, H. P. (2008). Long flights and age affect oxidative status of homing pigeons (*Columba livia*). *J. Exp. Biol.* **211**, 377-381.
Delers, F., Strecker, G. and Engler, R. (1988). Glycosylation of chicken haptoglobin: isolation and characterization of three molecular variants and studies of their distribution in hen plasma before and after turpentine-induced inflammation. *Biochem. Cell Biol.* **66**, 208-217.
Demas, G. E., Chefer, V., Talan, M. I. and Nelson, R. J. (1997). Metabolic costs of mounting an antigen-stimulated immune response in adult and aged C57BL/6J mice. *Am. J. Physiol.* **273**, R1631-R1637.
Dobryszycka, W. (1997). Biological functions of haptoglobin – new pieces to an old puzzle. *Eur. J. Clin. Chem. Clin. Biochem.* **35**, 647-654.
Dowling, D. K. and Simmons, L. W. (2009). Reactive oxygen species as universal constraints in life-history evolution. *Proc. R. Soc. Lond. B. Biol. Sci.* **276**, 1737-1745.
Exton, M. S. (1997). Infection-induced anorexia: active host defence strategy. *Appetite* **29**, 369-383.
Finkel, T. and Holbrook, N. J. (2000). Oxidants, oxidative stress and the biology of ageing. *Nature* **408**, 239-247.
Gavilanes, J. G. (1982). Pigeon egg white lysozyme. *Int. J. Pept. Prot. Res.* **20**, 238-245.
Gessaman, J. A. (1987). Energetics. In *Raptor Management Techniques Manual* (ed. G. A. Giron Pendeleton, B. A. Millsap, K. W. Cline and D. M. Bird), pp. 289-320. Washington, DC: National Wildlife Federation.
Gutteridge, J. M. C. (1987). The antioxidant activity of haptoglobin towards haemoglobin-stimulated lipid peroxidation. *Biochim. Biophys. Acta* **917**, 219-223.
Halliwell, B. and Gutteridge, J. M. C. (1985). Oxygen radicals and the nervous system. *Trends Neurosci.* **8**, 22-26.
Halliwell, B. and Gutteridge, J. M. C. (1999). *Free Radicals in Biology and Medicine*. Oxford: Oxford University Press.
Hansen, N. E., Karle, H. and Andersen, V. (1971). Lysozyme turnover in the rat. *J. Clin. Invest.* **50**, 1473-1477.
Hansen, N. E., Karle, H., Andersen, V. and Ølgaard, K. (1972). Lysozyme turnover in man. *J. Clin. Invest.* **51**, 1146-1155.
Herrero, A. and Barja, G. (1998). H_2O_2 production of heart mitochondria and aging rate are slower in canaries and parakeets than in mice: sites of free radical generation and mechanisms involved. *Mech. Ageing Dev.* **103**, 133-146.
Hill, R. W. (1972). Determination of oxygen consumption by use of the paramagnetic oxygen analyzer. *J. Appl. Physiol.* **33**, 261-263.
Hõrak, P., Zilmer, M., Saks, L., Ots, I., Karu, U. and Zilmer, K. (2006). Antioxidant protection, carotenoids and the costs of immune challenge in greenfinches. *J. Exp. Biol.* **209**, 4329-4338.
Hõrak, P., Saks, L., Zilmer, M., Karu, U. and Zilmer, K. (2007). Do dietary antioxidants alleviate the cost of immune activation? An experiment with greenfinches. *Am. Nat.* **170**, 625-635.
Humphrey, B. D., Huang, N. and Klasing, K. C. (2002). Rice expressing lactoferrin and lysozyme has antibiotic-like properties when fed to chicks. *J. Nutr.* **132**, 1214-1218.
Iamele, L., Fiocchi, R. and Vernocchi, A. (2002). Evaluation of an automated spectrophotometric assay for reactive oxygen metabolites in serum. *Clin. Chem. Lab. Med.* **40**, 673-676.
Ji, L. L. (1999). *Antioxidants and oxidative stress in exercise. Exp. Biol. Med.* **222**, 283-292.
Jollès, P. and Jollès, J. (1984). What's new in lysozyme research? *Mol. Cell. Biochem.* **63**, 165-189.
Klebanoff, S. J. and Clark, R. A. (1978). *The Neutrophil: Function and Clinical Disorders*. Amsterdam: Holland Publishing Company.
Klockars, M. and Roberts, P. (1976). Stimulation of phagocytosis by human lysozyme. *Acta Haematol.* **55**, 289-295.
Körmöczi, G. F., Säemann, M. D., Buchta, C., Peck-Radosavljevic, M., Mayr, W. R., Schwartz, D. W. M., Dunkler, D., Spitzauer, S. and Panzer, S. (2006). Influence of clinical factors on the haemolysis marker haptoglobin. *Eur. J. Clin. Invest.* **36**, 202-209.
Lasiewski, R. C., Acosta, A. L. and Bernstein, M. H. (1966). Evaporative water loss in birds-I. Characteristics of the open flow method of determination, and their relation to estimates of thermoregulatory ability. *Comp. Biochem. Physiol.* **19**, 445-457.
Lavoie, E. T. (2005). Avian immunosenescence. *Age* **27**, 281-285.
Lee, K. A. (2006). Linking immune defenses and life history at the levels of the individual and the species. *Integr. Comp. Biol.* **46**, 1000-1015.
Leeuwenburgh, C. and Heinecke, J. W. (2001). Oxidative stress and antioxidants in exercise. *Curr. Med. Chem.* **8**, 829-838.
Liu, H., Zheng, F., Cao, Q., Ren, B., Zhu, L., Striker, G. and Vlassara, H. (2006). Amelioration of oxidant stress by the defensin lysozyme. *Am. J. Physiol.* **290**, E824-E832.
Matson, K. D. (2006). Are there differences in immune function between continental and insular birds? *Proc. R. Soc. Lond. B. Biol. Sci.* **273**, 2267-2274.
Matson, K. D., Ricklefs, R. E. and Klasing, K. C. (2005). A hemolysis-hemagglutination assay for characterizing constitutive innate humoral immunity in wild and domestic birds. *Dev. Comp. Immunol.* **29**, 275-286.
Matson, K. D., Cohen, A. A., Klasing, K. C., Ricklefs, R. E. and Scheuerlein, A. (2006). No simple answers for ecological immunology: relationships among immune indices at the individual level break down at the species level in waterfowl. *Proc. R. Soc. Lond. B. Biol. Sci.* **273**, 815-822.
McEwen, B. S. (1998). Protective and damaging effects of stress mediators. *N. Engl. J. Med.* **338**, 171-179.
Millet, S., Bennett, J., Lee, K. A., Hau, M. and Klasing, K. C. (2007). Quantifying and comparing constitutive immunity across avian species. *Dev. Comp. Immunol.* **31**, 188-201.
Monaghan, P., Metcalfe, N. B. and Torres, R. (2009). Oxidative stress as a mediator of life history trade-offs: mechanisms, measurements and interpretation. *Ecol. Lett.* **12**, 75-92.
Nomoto, S. (1996). Diurnal variations in fever induced by intravenous LPS injection in pigeons. *Pflügers Arch. Eur. J. Physiol.* **431**, 987-989.
Norris, K. and Evans, M. R. (2000). Ecological immunology: life history trade-offs and immune defense in birds. *Behav. Ecol.* **11**, 19-26.
Ohno, N. and Morrison, D. C. (1989). Lipopolysaccharide interaction with lysozyme. Binding of lipopolysaccharide to lysozyme and inhibition of lysozyme enzymatic activity. *J. Biol. Chem.* **264**, 4434-4441.
Ots, I., Kerimov, A. B., Ivankina, E. V., Ilyina, T. A. and Hõrak, P. (2001). Immune challenge affects basal metabolic activity in wintering great tits. *Proc. R. Soc. Lond. B. Biol. Sci.* **268**, 1175-1181.
Pérez-Rodríguez, L., Mougeot, F., Alonso-Alvarez, C., Blas, J., Vinuela, J. and Bortolotti, G. R. (2008). Cell-mediated immune activation rapidly decreases plasma carotenoids but does not affect oxidative stress in red-legged partridges (*Alectoris rufa*). *J. Exp. Biol.* **211**, 2155-2161.
Råberg, L., Grahn, M., Hasselquist, D. and Svensson, E. (1998). On the adaptive significance of stress-induced immunosuppression. *Proc. R. Soc. Lond. B. Biol. Sci.* **265**, 1637-1641.
Raetz, C. R. H. (1990). Biochemistry of endotoxins. *Annu. Rev. Biochem.* **59**, 129-170.
Rasbash, J., Steele, F., Browne, W. and Prosser, B. (2004). *A User's Guide to Mlwin Version 2.0.* London: Institute of Education.
Rodnan, G. P., Ebaugh, F. G., Jr, Fox, M. R. S. and Chambers, D. M. (1957). The life span of the red blood cell and the red blood cell volume in the chicken, pigeon and duck as estimated by the use of Na2Cr51O4: with observations on red cell turnover rate in the mammal, bird and reptile. *Blood* **12**, 355-366.
Roura, E., Homedes, J. and Klasing, K. C. (1992). Prevention of immunologic stress contributes to the growth-permitting ability of dietary antibiotics in chicks. *J. Nutr.* **122**, 2383-2390.
Saino, N., Calza, S. and Møller, A. P. (1998). Effects of a dipteran ectoparasite on immune response and growth trade-offs in barn swallow, *Hirundo rustica*, nestlings. *Oikos* **81**, 217-228.
Saino, N., Dall'Ara, P., Martinelli, R. and Møller, A. P. (2002). Early maternal effects and antibacterial immune factors in the eggs, nestlings and adults of the barn swallow. *J. Evol. Biol.* **15**, 735-743.
Salvante, K. G. (2006). Techniques for studying integrated immune function in birds. *Auk* **123**, 575-586.
Sava, G. (1996). Pharmacological aspects and therapeutic applications of lysozymes. In *Lysozymes: Model Enzymes in Biochemistry and Biology* (ed. P. Jollès), pp. 433-449. Basel: Birkhauser Verlag.
Siwicki, A. K., Klein, P., Morand, M., Kiczka, W. and Studnicka, M. (1998). Immunostimulatory effects of dimerized lysozyme (KLP-602) on the nonspecific defense mechanisms and protection against furunculosis in salmonids. *Vet. Immunol. Immunopathol.* **61**, 369-378.
Speakman, J. R., Talbot, D. A., Selman, C., Snart, S., McLaren, J. S., Redman, P., Krol, E., Jackson, D. M., Johnson, M. S. and Brand, M. D. (2004). Uncoupled and surviving: individual mice with high metabolism have greater mitochondrial uncoupling and live longer. *Aging Cell* **3**, 87-95.
Speijers, G. J. A. and Van Apeldoorn, M. M. E. (1998). Lysozyme. In *Clinical Safety Information.* IPCS Inchem. Bilthoven, The Netherlands: Laboratory for Toxicology.
Tieleman, B. I., Williams, J. B., Ricklefs, R. E. and Klasing, K. C. (2005). Constitutive innate immunity is a component of the pace-of-life syndrome in tropical birds. *Proc. R. Soc. Lond. B. Biol. Sci.* **272**, 1715-1720.
Tieleman, B. I., Versteegh, M. A., Fries, A., Helm, B., Dingemanse, N. J., Gibbs, H. L. and Williams, J. B. (2009). Genetic modulation of energy metabolism in birds through mitochondrial function. *Proc. R. Soc. Lond. B. Biol. Sci.* **276**, 1685-1693.
Torres, R. and Velando, A. (2007). Male reproductive senescence: the price of immune-induced oxidative damage on sexual attractiveness in the blue-footed booby. *J. Anim. Ecol.* **76**, 1161-1168.
Tummeleht, L., Mägi, M., Kilgas, P., Mänd, R. and Hõrak, P. (2006). Antioxidant protection and plasma carotenoids of incubating great tits (*Parus major* L.) in relation to health state and breeding conditions. *Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol.* **144**, 166-172.
Wagner, L., Gessl, A., Parzer, S. B., Base, W., Waldhausl, W. and Pasternack, M. S. (1996). Haptoglobin phenotyping by newly developed monoclonal antibodies. Demonstration of haptoglobin uptake into peripheral blood neutrophils and monocytes. *J. Immunol.* **156**, 1989-1996.