RESEARCH ARTICLE

Determinants and Short-Term Physiological Consequences of PHA Immune Response in Lesser Kestrel Nestlings

JOURNAL OF EXPERIMENTAL ZOOLOGY
PART A
ECOLOGICAL GENETICS AND PHYSIOLOGY
A Journal of Integrative Biology

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ABSTRACT

Individual immune responses are likely affected by genetic, physiological, and environmental determinants. We studied the determinants and short-term consequences of Phytohaemagglutinin (PHA) induced immune response, a commonly used immune challenge eliciting both innate and acquired immunity, on lesser kestrel (*Falco naumanni*) nestlings in semi-captivity conditions and with a homogeneous diet composition. We conducted a repeated measures analyses of a set of blood parameters (carotenoids, triglycerides, β-hydroxybutyrate, cholesterol, uric acid, urea, total proteins, and total antioxidant capacity), metabolic (resting metabolic rate), genotypic (MHC class II B heterozygosity), and biometric (body mass) variables. PHA challenge did not affect the studied physiological parameters on a short-term basis (<12 hr), except plasma concentrations of triglycerides and carotenoids, which decreased and increased, respectively. Uric acid was the only physiological parameter correlated with the PHA induced immune response (skin swelling), but the change of body mass, cholesterol, total antioxidant capacity, and triglycerides between sessions (i.e., post—pre treatment) were also positively correlated to PHA response. No relationships were detected between MHC gene heterozygosity or resting metabolic rate and PHA response. Our results indicate that PHA response in lesser kestrel nestlings growing in optimal conditions does not imply a severe energetic cost 12 hr after challenge, but is condition-dependent as a rapid mobilization of carotenoids and decrease of triglycerides is elicited on a short-term basis. *J. Exp. Zool. 9999A: XX–XX, 2014.* © 2014 Wiley Periodicals, Inc.

How to cite this article: Rodríguez A, Broggi J, Alcaide M, Negro JJ, Figuerola J. 2014. Determinants and short-term physiological consequences of PHA immune response in lesser kestrel nestlings. J. Exp. Zool. 9999:1–11.

The vertebrate immune system is a complex array of different components that function as a defence against pathogens threatening the organism (Owen et al., 2010). Individual immune responses, and the way they interact with other vital parameters are highly variable, often involving allocation conflicts between other physiological or life-history traits (Lochmiller and Deerenberg, 2000; Hasselquist and Nilsson, 2012). Even non-pathogenic immune challenges can induce relevant changes in traits as diverse as growth (van der Most et al., 2011), metabolic rate (Eraud et al., 2005), reproduction (Knowles et al., 2009), and other competing immune functions (Forsman et al., 2008) or life-history

Grant sponsor: I3P pre-doctoral grant from the National Spanish Research Council; Juan de la Cierva postdoctoral grant by the Spanish Ministry of Science and EU FEDER program; research project numbers: CGL2009-10652 CGL2009-11445 CGL2012-38262.

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Received 4 October 2013; Revised 7 April 2014; Accepted 11 April 2014
DOI: 10.1002/jez.1868
Published online XX Month Year in Wiley Online Library (wileyonlinelibrary.com).

traits (Velando et al., 2006). However, the specific costs and currency mediating such trade-offs still remain obscure (Ardia et al., 2011).

Energy has been claimed to be one of such currencies mediating tradeoffs between immunity and other traits like growth, reproduction, or thermoregulation (Nilsson et al., 2007). However, recent evidence highlights the fact that while energetic costs of an immune response can be significant, these are dependent on the different components of the immune response being elicited, and according to the particular environmental and physiological context (Hasselquist and Nilsson, 2012). Energy expenditure can have indirect effects on immunity by means of changes in the oxidative balance. Oxidative stress is generated as a by-product of aerobic metabolism damaging cell macromolecules, and has been linked to diverse selective pressures on survival and reproduction (Monaghan et al., 2009; Garratt and Brooks, 2012). Organisms counteract oxidative stress by acquiring and producing antioxidants, and while most antioxidative activity is enzymatic, non-enzymatic antioxidants also play a relevant role in maintaining oxidative balance, particularly in blood (Pamplona and Costantini, 2011). Therefore, oxidative balance is a complex synergistic trait dependent on the antioxidative enzymatic capacity, the diverse antioxidant levels and the oxidative stress being generated by the individual metabolic activity, which may rapidly change in time and in different tissues (Cohen and McGraw, 2009). Oxidative stress has been suggested to mediate the relationship between immunity and other vital components (Dowling and Sommons, 2009), and recent evidence suggests a link between oxidative stress and immunity, albeit its significance is still under discussion (Costantini and Møller, 2009). Immune activation increases susceptibility to oxidative damage (Bertrand et al., 2006), but such relation may change according to the different components of the immune response being elicited (Costantini and Møller, 2009). Furthermore, carotenoids (CAR) are a diverse group of lipophilic molecules important for the immunity and individual fitness, and since they cannot be synthesized de novo by animals, necessarily need to be ingested, or acquired during embryogenesis through maternal transfer (Pérez-Rodríguez, 2009). Carotenoids have been claimed to underlie honest-signalling due to their dual role as pigments and antioxidants/immuno-stimulants, and although the antioxidative function of CAR has lately been questioned (Pérez-Rodríguez, 2009), their active role as mediators of both the immune system and the oxidative balance remains undisputed (Simons et al., 2012).

Individual variation in immune investment may arise from a variety of factors, not only adaptive adjustments but also constraints resulting from resource or genetic based trade-offs (Ardia et al., 2011). Furthermore, individual energy expenditure and physiological condition can affect both oxidative and immunological indices (van de Crommenacker et al., 2010). Therefore, given the interactive nature of all physiological traits, it is important to set up baseline measurements of relevant parameters, to interpret the precise relationships among traits potentially involved in trade-offs with immunity (Ardia et al., 2011). Immunity is a fitness-related trait that is affected by environmental and individual physiological conditions, although variation in diverse immune components are genetically determined. For example, genetic diversity at the functionally important genes belonging to the major histocompatibility complex (MHC) is acknowledged to play a central role in the immune system of vertebrates (reviewed by Sommer, 2005). However, genetic contributions to the phenotypic variability of immune response have rarely been demonstrated in wild bird species (see Sepil et al., 2013 and references therein).

Phytohaemagglutinin (hereafter PHA) is a mitogen of vegetal origin that when injected intradermally induces an immune response, which has been often used as a proxy of the cell-mediated immune capacity (Kennedy and Nager, 2006). However, recent studies have challenged this idea (Martin et al., 2006), and shown that PHA-induced immune response is a multifactorial process involving both innate and acquired cell-mediated elements, and reflecting an individuals' ability to mount an inflammatory response (Kennedy and Nager, 2006; Vinkler et al., 2010). PHA test is the most widely used in-vivo measure of immunocompetence in avian ecological studies (Kennedy and Nager, 2006), and has been linked to various traits such as fitness (Cichon and Dubiec, 2005; López-Rull et al., 2011), growth conditions (Hõrak et al., '99), oxidative stress or plasma CAR levels (Simons et al., 2012). Furthermore, variation in PHA response has been found in selected chicken lines, implying a significant genetic variation for this response (Sundaresan et al., 2005). In fact, MHC genes have previously been found to be associated with the phenotypic variability of this response in chickens and in a wild passerine (Taylor et al., '87; Bonneaud et al., 2005; but see Bonneaud et al., 2009). Alternatively, other studies on PHA response heritability highlight the fact that environmental and early-maternal effects may override genetic effects (Pitala et al., 2007).

Up to now, most of our knowledge about the variability and dynamics of the immune responses comes from model species (Lazzaro and Little, 2009). However, non-model species can offer a valuable insight on ecological and evolutionary immunology (Matson et al., 2006; Pedersen and Babayan, 2011). Determining individual sources of variation of the immune response can yield important insights into the mechanistic and evolutionary factors shaping these responses, and how these are related to other physiological or life-history variables.

In this paper, we studied the determinants and short-term consequences of PHA injection on a non-model species, the lesser kestrel *Falco naumanni*. We analyzed resting metabolic rate, several physiological plasma metabolites related to the oxidative balance and nutritional condition, and MHC heterozygosity to evaluate the physiological cost of mounting a PHA response. We used lesser kestrel nestlings in semi-captivity conditions and with a homogeneous diet composition.

MATERIALS AND METHODS

Birds and Experimental Design

Captive bred lesser kestrel nestlings were brought from the DEMA facilities (Almendralejo, Spain, more info at www.demaprimilla.org) at an age of ~20 days to Estación Biológica de Doñana (CSIC) building (Seville, Spain), in where a reintroduction project was being carried out. Nestlings were released into outdoor hacking nest boxes simulating a natural breeding colony, in where they came in contact with adult birds, both captive irrecoverable individuals that fed them as adoptive parents, and feral birds (see Rodríguez et al., 2013). Birds remained 8.4 ± 2.6 (mean ± SD) days undisturbed for acclimatization purposes, and then subjected to two consecutive and identical night-time respirometry sessions, each one lasting around 12 hr (20.00 p.m. to 08.00 a.m) (see below). The birds were weighted (to the nearest 0.1 g) at the start and the end of each session. After each respirometry session, birds were blood sampled and returned to the hacking nest boxes, where they were fed ad libitum with dead laboratory mice and 3-day old chicken (see Rodríguez et al., 2013 for details). Individuals did not receive any food during respirometry sessions. Before the start of the second respirometry session, birds were either challenged with PHA or sham-controlled with PBS (see below). At the end of the second respirometry session, the response to PHA challenge was measured (see below), and birds were returned to the hacking nest boxes where remained until they freely fledged.

Measurements and Sample Collection

Plasma Biochemical Parameters. All individuals were sampled for blood (0.35 mL) from the brachial vein after each respirometry session (~08:00 a.m.), avoiding undesired variation resulting from circadian rhythms (Rodríguez et al., 2011). Blood samples were kept cool (~4°C) until they were centrifuged (4,000 rpm during 20 min) within 1 hr of sampling. Plasma was separated and stored at −80°C, and the cellular fraction was stored at −20°C and later employed for genetic analyses.

Plasma was analyzed for eight biochemical parameters (CAR, total antioxidant capacity, triglycerides (TRG), βHB, cholesterol (CHL), uric acid (UAC), urea (URE), and total proteins (PRT). Carotenoid concentration in plasma was measured by means of N-1000 NanoDrop spectrophotometer at 450 nm as in Bortolotti et al. (2000). Total antioxidant capacity (TAC) is a measure of the capacity of plasma to neutralize reactive oxygen species, and was measured as described in Erel (2004). Recent studies point out that TAC is mostly representative of the water soluble components of the antioxidative system (Cohen and McGraw, 2009). However, it is commonly agreed that measurement of TAC in combination with other fat-soluble antioxidants may provide a more complete image of the antioxidant system (Monaghan et al., 2009). The remaining plasma metabolites are related to the nutritional state of bird (McCue, 2010). Triglycerides, βHB, and CHL are all involved in fat metabolism. TRG are the storage form of lipids, and are good indicators of fat deposition, βHB is an indicator of the catabolism of fatty acids, whereas CHL is known to be a good predictor of general nutritional condition and body mass. Total proteins (PRT), URE, and UAC are all involved in protein catabolism (Jenni-Eiermann and Jenni, '98). Further, UAC is a common circulating hydrophilic antioxidant that accounts for an important portion of the antioxidant capacity in blood (Cohen et al., 2007). All plasma metabolites (except CAR) were measured according to standard methods implemented on a Cobas INTEGRA 400 plus Chemistry autoanalyser (Roche Diagnostics Ltd. Burgess Hill, West Sussex, UK).

DNA Extraction and MHC Genotyping. DNA was extracted from blood samples following the HotSHOT protocol (Truett, 2006) and used for molecular sex determination (Rodríguez et al., 2011). The second exon of a single and highly polymorphic MHC class II B gene was PCR-amplified and sequenced following Alcaide et al. (2008). Direct sequencing chromatograms were carefully inspected by eye and edited in BIOEDIT v7.0.5.3 (Hall, '99), and IUPAC nucleotide degenerate codes were introduced for each heterozygous site. MHC diploid genotypes were then resolved into individual haplotypes using the Bayesian PHASE platform implemented in DNASP v5 (Librado and Rozas, 2009) following Alcaide et al. (2011). Then, we translated each allele into amino acids and we counted the number of different amino acids between the two alleles of each bird, as a measure of MHC heterozygosity (MHC). Furthermore, we categorized nestlings according to the number of most frequent alleles in South West Spain, that is, 1 = individuals holding at least one of the most frequent alleles; 0 = individuals holding infrequent alleles. *Fana*2 (20%) and *Fana*19 (12%) were the most frequent alleles in South West Spain (Alcaide et al., 2008).

Respirometry. Resting metabolic rate (RMR) was measured as the average minimal oxygen consumption under post-absorptive digestive conditions during the resting phase of the daily cycle in an open circuit respirometer (McNab, '97). Birds were individually placed in an air-sealed chamber (3L) inside a climate cabinet at 27°C, within the thermoneutral zone of similarly sized falcons as the lesser kestrel (Shapiro and Weathers, '81; Bush et al., 2008). The respirometer consisted of two independent sets consisting of 4 and 8 channels, respectively. Both respirometers had exactly the same components except for the number of channels. Outdoor air was pushed towards each chamber through independent mass-flow controllers (Flow-bar-8) adjusted to 700 mL/min. A valve system controlled by gas-flow multiplexer (RM4-8) conducted outcoming air in 10 min cycles from each chamber towards a water vapor analyzer (RH 300) and then to the CO_2-Oxygen analyzer FOXBOX-C Field gas analysis system (Sable systems international, Las Vegas, NV, USA) before being released. Each set up had an empty chamber that allowed calibration for any possible setup bias. Differences in measurements between both set ups due to the cycle

Table 1. Mean values (±SD) and sample sizes of the physiological parameters measured after each respirometric session (8:00 a.m.), before (pre-treatment), and after (post-treatment) the experimental treatment in lesser kestrel nestlings (see Methods for details).

			Pre-treatment		Post-treatement			
					Control		PHA	
Parameter	Abbrev.	Units	Mean ± SD	*n*	Mean ± SD	*n*	Mean ± SD	*n*
Resting metabolic rate	RMR	mLO_2/min	4.03 ± 0.46	20	3.91 ± 0.40	7	3.97 ± 0.84	14
Body mass	MASS	g	136. 6 ± 6.9	36	132.3 ± 6.6	12	132.6 ± 7.51	23
β-hydroxybutyrate	βHB	μmol/L	1396.3 ± 585.3	36	1596.8 ± 512.6	11	1816.1 ± 633.1	23
Cholesterol	CHL	mg/dL	194.54 ± 32.67	36	184.75 ± 27.25	11	184.96 ± 30.73	23
Total antioxidant capacity	TAC	μmol/L	930.06 ± 115.79	36	827.95 ± 185.99	11	836.33 ± 89.54	23
Total proteins	PRT	g/dL	2.23 ± 0.25	36	2.12 ± 0.29	11	2.24 ± 0.23	23
Triglycerides	TRG	mg/dL	88.00 ± 18.78	36	89.13 ± 20.50	11	62.16 ± 17.98	23
Uric acid	UAC	mg/dL	6.84 ± 2.27	36	5.95 ± 1.84	11	5.59 ± 1.33	23
Urea	URE	mg/dL	11.64 ± 10.99	36	11.02 ± 10.84	11	8.167 ± 5.86	23
Carotenoids	CAR	PPM	0.96 ± 0.32	36	0.81 ± 0.20	11	0.99 ± 0.21	23

length were unlikely to bias our results (Cooper and Withers, 2010). The value of oxygen consumption (mLO_2/min) was taken as the lowest value of running 10 min averages during a measurement session and was calculated according to Hill ('72).

PHA test. PHA challenge consisted of a subcutaneous injection in the left patagia of 0.1 mL of PHA-P (L-8754 Sigma–Aldrich, St. Louis, MO, USA) diluted in saline solution PBS (Sigma P-5119) at 2.5 mg/mL or just PBS, following (Smits et al., '99). Patagia width was measured at the point of injection (to the nearest 0.01 mm) three times just prior to and 12 hr after injection, using a pressure sensitive micrometer (Baxlo Precision, Polinyà, Spain). The micrometer was removed completely from the wing between each measurement and all measurements were taken by the same person (A.R.). Measures of patagium thickness were highly repeatable (intra-class correlation = 0.957, $F_{34,\ 70} = 67.604$, $P = <0.001$), and average values were used thereafter.

Statistical Analyses

We evaluated the inter-correlations (Pearson coefficients) among 10 physiological parameters measured on pre-treatment session (RMR, MASS, βHB, CHL, TAC, PRT, TRG, UAC, URE, and CAR; Tables 1 and 2), and tested for potential gender differences. Furthermore, repeated measures General Linear Models were used to test for treatment effect (PHA vs. control), on the previously mentioned variables as measured during pre- and post-treatment sessions. The selection of explanatory variables was based on their co-variation with response variables on pre-treatment session in order to reduce variance (see Table 2). Gender was also introduced as a factor when significant differences were reached on the

Table 2. Correlations between the metabolic, biochemical, and phenotypic variables measured in this study.

	RMR	MASS	βHB	CHL	TAC	PRT	TRG	UAC	URE	CAR
RMR	–	**0.675**	−0.320	−0.107	−0.049	−0.188	−0.208	0.143	0.102	0.319
MASS	**0.001**	–	**−0.434**	−0.111	0.255	0.053	0.048	0.307	−0.045	0.253
βHB	0.169	**0.008**	–	0.220	−0.199	−0.280	0.134	−0.188	−0.134	−0.255
CHL	0.652	0.518	0.197	–	**−0.417**	0.242	**0.420**	−0.239	0.100	−0.046
TAC	0.837	0.134	0.245	**0.011**	–	0.253	0.254	**0.766**	0.277	0.048
PRT	0.429	0.758	0.099	0.155	0.136	–	**0.434**	**0.427**	**0.368**	−0.170
TRG	0.378	0.780	0.435	**0.011**	0.134	**0.008**	–	0.244	**0.530**	0.056
UAC	0.547	0.069	0.272	0.160	**<0.001**	**0.009**	0.151	–	**0.343**	0.017
URE	0.670	0.794	0.436	0.561	0.102	**0.027**	**0.001**	**0.040**	–	0.067
CAR	0.170	0.136	0.134	0.789	0.779	0.322	0.745	0.923	0.700	–

Correlation coefficients (*r*-values) are given above diagonal and *P*-values below diagonal. Significant correlations ($P < 0.05$) are in bold.

pre-treatment values of the response variable. Although some explanatory variables were correlated (see Table 2), multicollinearity was not an issue according to variance inflation factors (range = 1.060–1.493). Finally, considering only PHA challenged birds, we analyzed the correlations between PHA response and (a) the variables measured on pre- and post-treatment sessions, and (b) the change in these variables between post and pre-treatment sessions. Statistical analyses were conducted using SPSS v.19 package (IBM Company, Chicago, IL, USA). Samples sizes varied among sessions.

RESULTS

Among the parameters explored, we found ten significant correlations (see Table 2). RMR was positively related to body mass, whereas mass was in turn negatively related to βHB. TAC was positively related to UAC, but negatively to CHL. UAC was in turn positively related to PRT and URE. URE was additionally related to PRT and TRG in a positive way. And TRG was positively related to PRT, and CHL. Finally, CAR was independent of any other physiological parameters considered. Only TRG concentration differed by sex ($t_{34} = 2.851$, $P = 0.007$), being larger in females (101.1 ± 15.7 mg/dL; mean ± SD) than in males (82.9 ± 17.6 mg/dL) during the pre-treatment session.

PHA challenge did not affect most of the studied physiological parameters (RMR, body mass, βHB, CHL, PRT, UAC, or URE), even when considering the significantly correlated variables as covariates. Only plasma concentrations of TRG and CAR were significantly affected by the treatment (Table 3). TRG concentration significantly decreased as a result of PHA challenge, and remained stable in the control treatment (Fig. 1a). CAR concentration increased as a result of the PHA challenge and decreased in the control treatment (Fig. 1b).

When considering PHA challenged individuals, UAC concentration on pre-treatment session was the only physiological parameter correlated with the PHA variation, and the positive relationship persisted on post-treatment session, although the slope decreased (Fig. 2; note that 95% confidence intervals only overlap at low values of PHA response and UAC, and they do not include the regression lines). The other physiological parameters on pre and post-treatment session did not correlate with PHA (*P*-values >0.05 in all cases). Finally, only changes between sessions in MASS, CHL, TAC, and TRG were positively correlated to PHA immune response (Fig. 3), the rest of physiological parameters remaining non-significant (*P*-values >0.05 in all cases). MHC heterozygosity, measured as the number of amino acid differences, did not correlate with PHA response ($r = -0.017$, $P = 0.939$). In addition, mean PHA induced immune response did not vary between individuals holding frequent or infrequent MHC alleles ($t_{21} = -0.564$, $P = 0.579$).

DISCUSSION

Treatment Effects (PHA vs. control)

Lesser kestrel nestlings growing in ad libitum food conditions experienced limited short-term (12 hr after-challenge) physiological consequences of a PHA challenge. All nutritional parameters, both related to protein (PRT, URE, and UAC) and lipid metabolism (CHL, TRG, βHB) showed little variation throughout the experiment, and only TRG and CAR concentrations in plasma were affected by PHA challenge. As blood samples were collected after nocturnal fasting (12 hr after PHA challenge), variation in blood metabolites were more likely the result of an active mobilization between blood and other tissues than because of differences in feeding or nutrient absorption. TRG concentration in plasma

Table 3. Results for the repeated measures' GLM on the interaction between intra-group factor (pre- and post- treatment order) and treatment (PHA or control).

Response variable	Explanatory variables	F	d.f.	*P*
Resting metabolic rate	Treatment + MASS	0.045	1.13	0.835
Body mass	Treatment + RMR + βHB	0.016	1.15	0.900
β-hydroxybutyrate	Treatment + MASS	0.425	1.31	0.519
Cholesterol	Treatment + TAC + TRG	1.022	1.30	0.320
Total antioxidant capacity	Treatment + CHL + UAC	0.319	1.30	0.577
Total proteins	Treatment + UAC + TRG + URE	0.058	1.29	0.812
Triglycerides	Treatment + PRT + CHL + URE + sex	12.508	1.27	**0.001**
Uric acid	Treatment + TAC + URE + PRT	3.214	1.29	0.083
Urea	Treatment + PRT + TRG + UAC	1.856	1.29	0.184
Carotenoids	Treatment	9.704	1.32	**0.004**

The selection of explanatory variables was based on their co-variation with response variables (see main text). Note that positive signs among model terms do not refer to the relationship among variables.
Significant *P*-values ($P < 0.05$) are in bold.

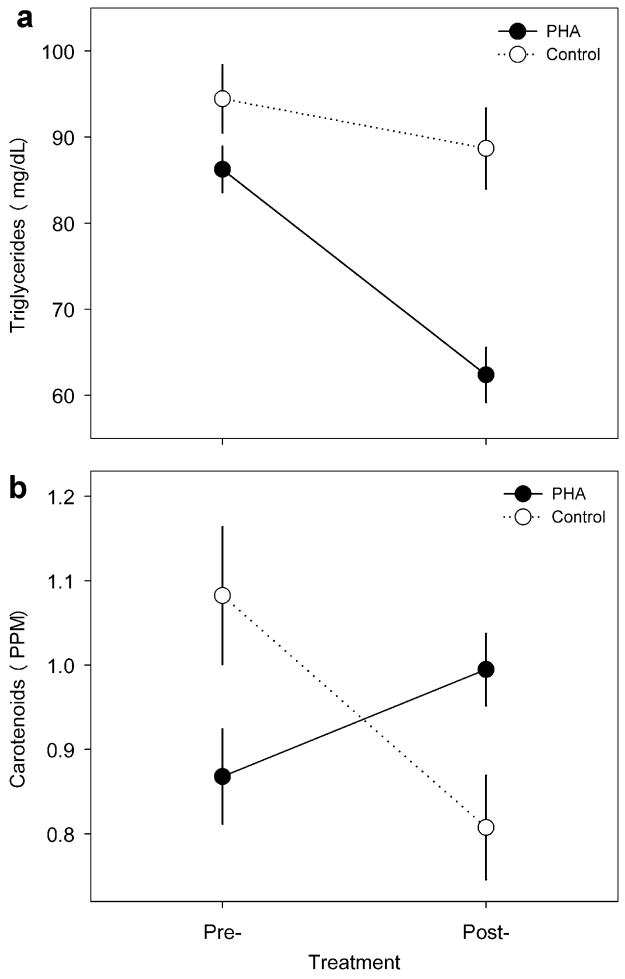

Figure 1. Mean (±SE) variation of triglyceride (a) and carotenoid (b) concentrations in plasma of lesser kestrel nestlings treated with PHA (filled circles) or PBS sham-control (open circles) on pre and post-treatment sessions.

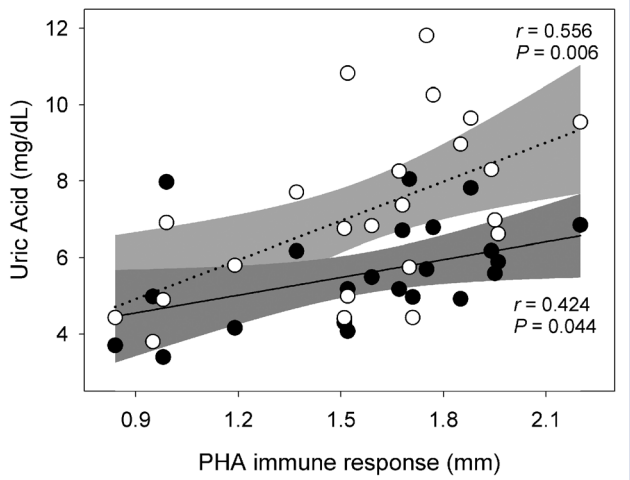

Figure 2. Relationship between uric acid concentration in plasma pre-treatment (open circles, dotted line, and grey area represent the values of each case, the regression line and 95% confidence intervals) and post-treatment (filled circles, black line, and dark grey area), and PHA induced immune response measured post-treatment in lesser kestrel nestlings. Pearson correlation coefficients (*r*) with the corresponding *P*-values are included for each session.

dropped within the 12 hr from challenge, suggesting that TRG were used as energy source more in PHA-challenged individuals than in controls (although it was not accompanied by an increased metabolic rate-see below). Furthermore, the observed trends in βHB, that is, higher levels in PHA-challenged birds than in control birds and pre-treatment values (Table 1), support the hypothesis of TRG as energy source, as the depletion of TRG together with increased βHB are common outcomes of lipid catabolism (Jenni-Eiermann and Jenni, '98). TRG depletion could be also explained by other factors, as they are actively involved in the immune response (see examples in Barcia and Harris, 2005; Radovic et al., 2012).

Carotenoids play a subtle but significant role as immune-modulators (Costantini and Møller, 2009; Simons et al., 2012), and they have often been found to decrease in immune challenged organisms, suggesting a relevant role in the development of the immune response (Pérez-Rodríguez et al., 2008; Vinkler and Albrecht, 2010). We found CAR to be significantly affected by the immune challenge, increasing in PHA-challenged birds. This apparently counterintuitive result has been previously recorded in wild nestlings of Eurasian kestrel (*Falco tinnunculus*), and was interpreted as a remobilization from skin, fat reserves and liver to blood stream (Costantini and Dell'Omo, 2006). Furthermore, in a previous study on wild lesser kestrel nestlings TGR were positively related to body mass and to CAR-based tarsus coloration, indicating that better nourished nestlings had higher TGR levels, and CAR allocated to signalization (Rodríguez et al., 2011). Thus, the depletion of TRG and the increase of circulating CAR suggest that mounting a PHA immune response entails a physiological cost for lesser kestrel nestlings detectable from a few hours after the immune challenge.

RMR of the lesser kestrel was independent of PHA challenge, suggesting that energy does not play a relevant role in this immune response, at least under the studied conditions and the time-scale considered. Favorable conditions for growth necessarily required in a conservation project (including in our case ad libitum fresh and sanitated food) may buffer the potential energetic costs of the experimental treatment. In addition, the time-scale considered in this study (<12 hr) may not be sufficient for detecting metabolic adjustments induced by PHA response, which can last over 3 days (Navarro et al., 2003). In line with this,

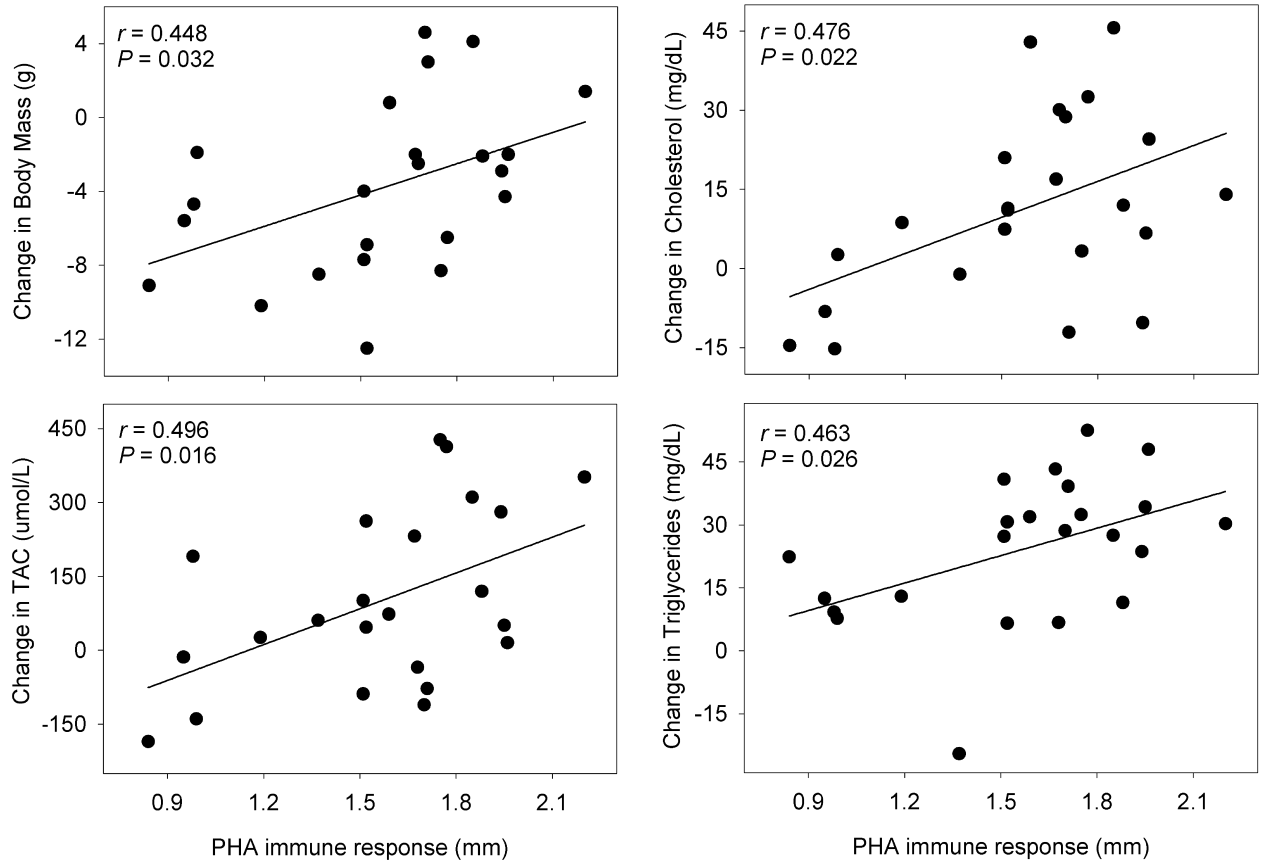

Figure 3. Change (post–pre treatment) in body mass and three plasma metabolite concentrations with PHA induced immune response in lesser kestrel nestlings. Pearson correlations (*r*) with the corresponding *P*-values are included.

Gutiérrez et al. (2011) found that metabolic effects of a PHA challenge were only detectable after 48 hr, and these only changed in food-restriction conditions. Alternatively, certain immune responses can be prioritized over other energy expensive activities (Hegemann et al., 2012), implying that energetic costs of an immune response are species and context dependent (Lee et al., 2005). Although it is commonly agreed that the activation and maintenance of an immune response incurs energetic costs (Martin et al., 2003; Burness et al., 2010; Abad-Gómez et al., 2013; King and Swanson, 2013), most studies conclude that these costs are not important enough to make energy the currency of the assumed trade offs between immunity and other traits (Eraud et al., 2005; Nilsson et al., 2007). However, in combination with other costly activities, the energetic costs of mounting an immune response may become biologically relevant for the individual (Ots et al., 2001; Hawley et al., 2012).

Correlates of PHA Immune Response

When studying the variation in PHA response (i.e., taking into account only PHA-challenged birds), only UAC appeared as a relevant predictive parameter. Pre- and post-treatment levels of UAC were positively related to PHA response, and nestlings with a higher PHA response exhibited a larger decrease in UAC (Fig. 2). As far as we know, our study is the first one reporting this link (see Hõrak et al., 2007). In birds, high circulating UAC levels could be consequence of amino acid or purine catabolism rather than up-regulation of antioxidant protection (Cohen et al., 2007). However, the difference between slopes suggests that UAC levels could be depleted by bleaching of the reactive oxygen species produced during the immune response to PHA (Cohen et al., 2007). Another non-mutually exclusive explanation is that PHA could induce depletion of UAC due to its incorporation to purine biosynthesis. In human T-lymphocytes, PHA activated purine biosynthesis while catabolism remained unaffected (Barankiewicz and Cohen, '87). Furthermore, individuals with higher PHA response exhibited higher increase in TAC between sessions. Considering that TAC and UAC are highly interrelated hydrosoluble components of the antioxidative system (Table 2; Cohen and McGraw, 2009), the positive relationship between the change in TAC and PHA response (Fig. 3), and the depletion of UAC (Fig. 2)

suggest that PHA response affected the antioxidant balance of birds by shifting the levels of the different antioxidants.

Change in body mass between sessions together with CHL and TRG paralleled the degree of PHA response, suggesting that this immune response is condition dependent. Plasma metabolites have provided robust information on the physiological condition, mass change or growth rates on a variety of wild avian species (Seaman et al., 2005; Dietz et al., 2009; Albano et al., 2011). Our results add to evidence found by previous studies on wild populations of Eurasian kestrel and American kestrel (*Falco sparverius*) that confirmed the importance of environmental conditions in explaining nestling's PHA response (Tella et al., 2000; Martínez-Padilla, 2006; Martínez-Padilla and Viñuela, 2011).

If MHC gene variability is correlated with the diversity of lymphocyte receptors, it could be anticipated that most heterozygous individuals may trigger a more efficient immune response as they are capable to bind and present a higher diversity of antigens (see Sommer, 2005). Natural selection plays a relevant role in shaping MHC variability in lesser kestrels (e.g., Alcaide et al., 2008, 2012), and therefore we assumed that PHA immune response could be affected by the presence of certain alleles in the genome. However, in this study PHA-response was unrelated to MHC class II heterozygosity or to the presence of certain alleles in the genome of lesser kestrel nestlings. Although our results should be considered with caution due to sample size constraints, they are in agreement with Bonneaud et al. (2009), who found PHA heritability to be independent from MHC variation. The lack of association between PHA-response and MHC II variability could stem from the time frame considered, as MHC genotypes had been significantly associated with PHA in chicken, but only after 72 hr post-injection (Taylor et al., '87). Certain MHC supertypes have shown to confer resistance to specific parasites in songbirds (e.g., Radwan et al., 2012; Sepil et al., 2013 and references therein), and in the case of PHA test, an association between PHA response and a single MHC allele was found in house sparrows (Bonneaud et al., 2005). Given that PHA response does not mirror T-cell function (Vinkler et al., 2010), this association is unlikely to reflect a direct involvement of MHC in the physiological processes elicited by PHA (Licastro et al., '93; Bonneaud et al., 2005). In accordance with this hypothesis, responsiveness to PHA can be attributed to other immune genes than MHC II, for example Toll-like receptors, which are sentinels of the innate immune system responsible of triggering the inflammatory response and the regulation of the acquired immunity (reviewed by Iwasaki and Medzhitov, 2010).

CONCLUSIONS

Lesser kestrels nestlings growing in optimal conditions (ad libitum food supply) experienced slight physiological changes to PHA challenge, while RMR remained constant on a short-term basis (<12 hr). PHA-response induced a rapid depletion of TRG, and paralleled changes in body mass and other plasma metabolites suggesting a condition-dependent nature of this immune response. Furthermore, a rapid mobilization of CAR and changes in the hydrophilic components of the antioxidative balance indicate that PHA-response elicits significant changes in metabolic pathways related to nutritional and oxidative balance, while keeping the overall energy budget unaffected.

ACKNOWLEDGMENTS

We are grateful to Antonio Rivera and Carlos Moreno for their help during the field work and to Francisco Miranda and Olaya García for their work at the Ecophysiology and Molecular Ecology labs at the EBD-CSIC. We are indebted with Esa Hohtola for his valuable advice. Two anonymous reviewers provided useful comments on earlier drafts. A.R. was supported by an I3P pre-doctoral grant from the National Spanish Research Council. J.B. was funded by Juan de la Cierva postdoctoral grant by the Spanish Ministry of Science and EU FEDER program. This study was funded by the projects CGL2009-10652, CGL2009-11445, and CGL2012-38262.

LITERATURE CITED

Abad-Gómez JM, Gutiérrez JS, Villegas A, et al. 2013. Time course and metabolic costs of a humoral immune response in the little ringed plover *Charadrius dubius*. Physiol Biochem Zool 86:354–360.

Albano N, Masero JA, Villegas A, Abad-Gómez JM, Sánchez-Guzmán JM. 2011. Plasma metabolite levels predict bird growth rates: a field test of model predictive ability. Comp Biochem Physiol A 160: 9–15.

Alcaide M, Edwards SV, Negro JJ, Serrano D, Tella JL. 2008. Extensive polymorphism and geographical variation at a positively selected MHC class IIB gene of the lesser kestrel (*Falco naumanni*). Mol Ecol 17:2652–2665.

Alcaide M, Rodríguez A, Negro, JJ. 2011. Sampling strategies for accurate computational inferences of gametic phase across highly polymorphic Major Histocompatibility Complex loci. BMC Res Notes 4:151.

Alcaide M, Rodríguez A, Negro JJ, Serrano D. 2012. Male transmission-ratio distortion support MHC-linked cryptic female choice in the lesser kestrel. Behav Ecol Sociobiol 66:1467–1473.

Ardia DR, Parmentier HK, Vogel LA. 2011. The role of constraints and limitation in driving individual variation in immune response. Funct Ecol 25:61–73.

Barankiewicz J, Cohen A. 1987. Purine nucleotide metabolism in phytohemagglutinin-induced human T lymphocytes. Arch Biochem Biophys 258:167–175.

Barcia AM, Harris HW. 2005. Triglyceride-rich lipoproteins as agents of innate immunity. Clin Infect Dis 41:S498–S503.

Bertrand S, Criscuolo F, Faivre B, Sorci G. 2006. Immune activation increases susceptibility to oxidative tissue damage in Zebra Finches. Funct Ecol 20:1022–1027.

Bonneaud C, Richard M, Faivre B, Westerdahl H, Sorci G. 2005. An Mhc class I allele associated to the expression of T-dependent immune response in the house sparrow. Immunogenetics 57:782–789.

Bonneaud C, Sinsheimer JS, Richard M, Chastel O, Sorci G. 2009. Mhc polymorphisms fail to explain the heritability of phytohaemagglutinin induced skin swelling in a wild passerine. Biol Lett 5:784–787.

Bortolotti GR, Tella JL, Forero MG, Dawson RD, Negro JJ. 2000. Genetics, local environment and health as factors influencing plasma carotenoid levels in wild American Kestrels (*Falco sparverius*). Proc R Soc Lond B 267:1433–1438.

Burness G, Armstrong C, Fee T, Tilman-Schindel E. 2010. Is there an energetic-based trade-off between thermoregulation and the acute phase response in zebra finches? J Exp Biol 213:1386–1394.

Bush NG, Brown M, Downs CT. 2008. Effects of short-term acclimation on thermoregulatory responses of the rock kestrel, *Falco rupicolus*. J Thermal Biol 33:425–430.

Cichon M, Dubiec A. 2005. Cell-mediated immunity predicts the probability of local recruitment in nestling blue tits. J Evol Biol 18:962–966.

Cohen AA, McGraw KJ. 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 AA, Klasing K, Ricklefs R. 2007. Measuring circulating antioxidants in wild birds. Comp Biochem Physiol B 147:110–121.

Cooper CE, Withers PC. 2010. Effect of sampling regime on estimation of basal metabolic rate and standard evaporative water loss using flow-through respirometry. Physiol Biochem Zool 83:385–393.

Costantini D, Dell'Omo G. 2006. Effects of T-cell immune response on avian oxidative stress. Comp Biochem Physiol A 145:137–142.

Costantini D, Møller AP. 2009. Does immune response cause oxidative stress in birds? A meta-analysis. Comp Biochem Physiol A 153:339–344.

Dietz MW, Jenni-Eiermann S, Piersma T. 2009. The use of plasma metabolites to predict weekly body-mass change in red knots. Condor 111:88–99.

Dowling DK, Sommons LW. 2009. Reactive oxygen species as universal constraints in life-history evolution. Proc R Soc B 276:1737–1745.

Eraud C, Duriez O, Chastel O, Faivre B. 2005. The energetic cost of humoral immunity in the collared dove, *Streptopelia decaocto*: is the magnitude sufficient to force energy-based trade-offs? Funct Ecol 19:110–118.

Erel O. 2004. A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. Clin Biochem 37:277–285.

Forsman AM, Vogel LA, Sakaluk SK, Grindstaff JL, Thompson CF. 2008. Immune-challenged house wren broods differ in the relative strengths of their responses among different axes of the immune system. J Evol Biol 21:873–878.

Garratt M, Brooks RC. 2012. Oxidative stress and condition-dependent sexual signals: more than just seeing red. Proc R Soc B 279:3121–3130.

Gutiérrez JS, Masero JA, Abad-Gómez JM, Villegas A, Sánchez-Guzmán JM. 2011. Metabolic consequences of overlapping food restriction and cell-mediated immune response in a long-distance migratory shorebird, the little ringed plover *Charadrius dubius*. J Avian Biol 42:259–265.

Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acids Symp Ser 41:95–98.

Hasselquist D, Nilsson JÅ. 2012. Physiological mechanisms mediating costs of immune responses: what can we learn from studies of birds? Anim Behav 83:1303–1312.

Hawley DM, DuRant SE, Wilson AF, Adelman JS, Hopkins WA. 2012. Additive metabolic costs of thermoregulation and pathogen infection. Funct Ecol 26:701–710.

Hegemann A, Matson KD, Versteegh MA, Tieleman BI. 2012. Wild skylarks seasonally modulate energy budgets but maintain energetically costly inflammatory immune responses throughout the annual cycle. PLoS ONE 7:e36358.

Hill RW. 1972. Determination of oxygen consumption by use of the paramagnetic oxygen analyzer. J Appl Physiol 33:261–263.

Hõrak P, Tegelmann L, Ots I, Møller AP. 1999. Immune function and survival of great tit nestlings in relation to growth conditions. Oecologia 121:316–322.

Hõrak P, Saks L, Zilmer M, Karu U, Zilmer K. 2007. Do dietary antioxidants alleviate the cost of immune activation? An experiment with greenfinches. Am Nat 170:625–635.

Iwasaki A, Medzhitov R. 2010. Regulation of adaptive immunity by the innate immune system. Science 327:291–295.

Jenni-Eiermann S, Jenni L. 1998. What can plasma metabolites tell us about the metabolism, physiological state and condition of individual birds? An overview. Biol Cons Fauna 102:312–319.

Kennedy MW, Nager RG. 2006. The perils and prospects of using phytohaemagglutinin in evolutionary ecology. Trend Ecol Evol 21:653–655.

King MO, Swanson DL. 2013. Activation of the immune system incurs energetic costs but has no effect on the thermogenic performance of house sparrows during acute cold challenge. J Exp Biol 216:2097–2102.

Knowles SCL, Nakagawa S, Sheldon BC. 2009. Elevated reproductive effort increases blood parasitaemia and decreases immune function in birds: a meta-regression approach. Funct Ecol 23:405–415.

Lazzaro BP, Little TJ. 2009. Immunity in a variable world. Phil Trans R Soc B 364:15–26.

Lee KA, Martin LB, II Wikelski M. 2005. Responding to inflammatory challenges is less costly for a successful avian invader, the house sparrow (*Passer domesticus*), than its less-invasive congener. Oecologia 145:244–251.

Librado P, Rozas J. 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25:1451–1452.

Licastro F, Davis L-J, Morini MC. 1993. Lectins and superantigens—membrane interactions of these compounds with t-lymphocytes affect immune-responses. Intl J Biochem 25:845–852.

Lochmiller RL, Deerenberg C. 2000. Trade-offs in evolutionary immunology: just what is the cost of immunity? Oikos 88:87–89.

López-Rull I, Celis P, Salaberria C, Puerta M, Gil D. 2011 Post-fledging recruitment in relation to nestling plasma testosterone and immunocompetence in the spotless starling. Funct Ecol 25:500–508.

Martin LB, II Scheuerlein A, Wikelski M. 2003. Immune activity elevates energy expenditure of house sparrows: a link between direct and indirect costs? Proc R Soc Lond B 270: 153–158.

Martin LB, II Han P, Lewittes J, et al. 2006. Phytohemagglutinin-induced skin swelling in birds: histological support for a classic immunoecological technique. Funct Ecol 20:290–299.

Martínez-Padilla J. 2006. Daytime variation in T-cell-mediated immunity of Eurasian kestrel *Falco tinnunculus* nestlings. J Avian Biol 37:419–424.

Martínez-Padilla J, Viñuela J. 2011. Hatching asynchrony and brood reduction influence immune response in Common Kestrel *Falco tinnunculus* nestlings. Ibis 153:601–610.

Matson KD, Cohen AA, Klasing KC, Ricklefs RE, 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 B 273:815–822.

McCue MD. 2010. Starvation physiology: reviewing the different strategies animals use to survive a common challenge. Comp Biochem Physiol A 156:1–18.

McNab BK. 1997. On the utility of uniformity in the definition of basal rate of metabolism. Physiol Zool 70:718–720.

Monaghan P, Metcalfe NB, Torres R. 2009. Oxidative stress as a mediator of life history trade-offs: mechanisms, measurements and interpretation. Ecol Lett 12:75–92.

Navarro C Marzal A, De Lope F, Møller AP. 2003. Dynamics of an immune response in house sparrows *Passer domesticus* in relation to time of day, body condition and blood parasite infection. Oikos 101:291–298.

Nilsson J-A, Granbom M, Raberg L. 2007. Does the strength of an immune response reflect its energetic cost? J Avian Biol 38:488–494.

Ots I, Kerimov AB, Ivankina EV, Ilyina TA, Hõrak P. 2001. Immune challenge affects basal metabolic activity in wintering great tits. Proc R Soc Lond B 268:1175–1181.

Owen JP, Nelson AC, Clayton DH. 2010. Ecological immunology of bird-ectoparasite systems. Trends Parasitol 26:530–539.

Pamplona R, Costantini D. 2011. Molecular and structural antioxidant defenses against oxidative stress in animals. Am J Physiol Regul Integr Comp Physiol 301:R843–R863.

Pedersen AB, Babayan SA. 2011. Wild immunology. Mol Ecol 20:872–880.

Pérez-Rodríguez L. 2009. Carotenoids in evolutionary ecology: re-evaluating the antioxidant role. BioEssays 31:1116–1126.

Pérez-Rodríguez L, Mougeot F, Alonso-Alvarez C, et al. 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.

Pitala N, Gustafsson L, Sendecka J, Brommer JE. 2007. Nestling immune response to phytohaemagglutinin is not heritable in collared flycatchers. Biol Lett 3:418–421.

Radovic B, Aflaki E, Kratky D. 2012. Adipose triglyceride lipase on immune response, inflammation, and atherosclerosis. Biol Chem 393:1005–1011.

Radwan J, Zagalska-Neubauer M, Cichoń M, et al. 2012. MHC Diversity, malaria and Lifetime reproductive success in collared flycatchers. Mol Ecol 21:2469–2479.

Rodríguez A, Negro JJ, Figuerola J. 2011. Sources of variation for nutritional condition indices of the plasma of migratory lesser kestrels in the breeding grounds. Comp Biochem Physiol A 160:453–460.

Rodríguez A, Negro JJ, Bustamante J, Antolín J. 2013. Establishing a lesser kestrel colony in an urban environment for research purposes. J Raptor Res 47:214–218.

Seaman DA, Guglielm CG, Williams TD. 2005. Effects of physiological state, mass change and diet on plasma metabolite profiles in the western sandpiper *Calidris mauri*. J Exp Biol 208:761–769.

Sepil I, Lachish S, Hinks AE, Sheldon BC. 2013. Mhc supertypes confer both qualitative and quantitative resistance to avian malaria infections in a wild bird population. Proc R Soc B 280:20130134.

Shapiro CJ, Weathers WW. 1981. Metabolic and behavioral responses of American kestrels to food deprivation. Comp Biochem Physiol A 68:111–114.

Simons MJP, Cohen AA, Verhulst S. 2012. What does carotenoid-dependent coloration tell? Plasma carotenoid level signals immunocompetence and oxidative stress state in birds—a meta-analysis. PLoS ONE 7:e43088.

Smits JE, Bortolotti GR, Tella JL. 1999. Simplifying the phytohemagglutinin skin-testing technique in studies of avian immunocompetence. Funct Ecol 13:567–572.

Sommer S. 2005. The importance of immune gene variability (MHC) in evolutionary ecology and conservation. Front Zool 2:16.

Sundaresan NR, Ahmed KA, Saxena VK, et al. 2005. Differential expression of inducible nitric oxide synthase and cytokine mRNA in chicken lines divergent for cutaneous hypersensitivity response. Vet Immunol Immunopathol 108:373–385.

Taylor RL, Cotter PF, Wing TL, Briles WE. 1987. Major histocompatibility (b) complex and sex effects on the phytohemagglutinin wattle response. Anim Genet 18:343–350.

Tella JL, Bortolotti GR, Forero MG, Dawson RD. 2000. Environmental and genetic variation in T-cell-mediated immune response of fledgling American kestrels. Oecologia 123:453–459.

Truett GE. 2006. Preparation of genomic DNA from animal tissues. In: Kieleczawa J, editor. DNA sequencing II: optimizing preparation and cleanup. Sudbury, UK: Jones and Bartlett. p 33–46.

van de Crommenacker J, Horrocks NPC, Versteegh MA, et al. 2010. Effects of immune supplementation and immune challenge on oxidative status and physiology in a model bird: implications for ecologists. J Exp Biol 213:3527–3535.

van der Most PJ, de Jong B, Parmentier HK, Verhulst S. 2011. Trade-off between growth and immune function: a meta-analysis of selection experiments. Funct Ecol 25:74–80.

Velando A, Drummond H, Torres R. 2006. Senescent birds redouble reproductive effort when ill: confirmation of the terminal investment hypothesis. Proc R Soc B 273:1443–1448.

Vinkler M, Albrecht T. 2010. Carotenoid maintenance handicap and the physiology of carotenoids-based signalisation of health. Naturwissenschaften 97:19–28.

Vinkler M, Bainova H, Albrecht T. 2010. Functional analysis of the skin-swelling response to phytohaemagglutinin. Funct Ecol 24: 1081–1086.