| 1 | Determinants and short-term physiological consequences of PHA immune response in |
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| 2 | lesser kestrel nestlings |
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| 18 | Running Head: PHA immune response on lesser kestrel nestlings |
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20 Abstract

21 Individual immune responses are likely affected by genetic, physiological, and 22 environmental determinants. We studied the determinants and short-term consequences of 23 Phytohaemagglutinin (PHA) induced immune response, a commonly used immune challenge 24 eliciting both innate and acquired immunity, on lesser kestrel (Falco naumanni) nestlings in 25 semi-captivity conditions and with a homogeneous diet composition. We conducted a 26 repeated measures analyses of a set of blood parameters (carotenoids, triglycerides, β -27 hydroxybutyrate, cholesterol, uric acid, urea, total proteins, and total antioxidant capacity), 28 metabolic (resting metabolic rate), genotypic (MHC class II B heterozygosity) and biometric 29 (body mass) variables. PHA challenge did not affect the studied physiological parameters on 30 a short-term basis (<12 hours), except plasma concentrations of triglycerides and carotenoids, 31 which decreased and increased, respectively. Uric acid was the only physiological parameter 32 correlated with the PHA induced immune response (skin swelling), but the change of body 33 mass, cholesterol, total antioxidant capacity and triglycerides between sessions (i.e. post - pre 34 treatment) were also positively correlated to PHA response. No relationships were detected 35 between MHC gene heterozygosity or resting metabolic rate and PHA response. Our results 36 indicate that PHA response in lesser kestrel nestlings growing in optimal conditions does not 37 imply a severe energetic cost 12 hours after challenge, but is condition-dependent as a rapid 38 mobilization of carotenoids and decrease of triglycerides is elicited on a short-term basis. 39 40 Keywords: Carotenoids, Falco naumanni, Immune response, Major histocompatibility

41 complex, Phytohaemagglutinin, Resting metabolic rate, Total antioxidant capacity,

42 Triglycerides

43 Introduction

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45 The vertebrate immune system is a complex array of different components that 46 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 47 48 variable, often involving allocation conflicts between other physiological or life-history traits 49 (Lochmiller and Deerenberg, 2000; Hasselquist and Nilsson, 2012). Even non-pathogenic 50 immune challenges can induce relevant changes in traits as diverse as growth (van der Most 51 et al., 2011), metabolic rate (Eraud et al., 2005), reproduction (Knowles et al., 2009) and 52 other competing immune functions (Forsman et al., 2008) or life-history traits (Velando et al., 53 2006). However, the specific costs and currency mediating such trade-offs still remain 54 obscure (Ardia et al., 2011).

55 Energy has been claimed to be one of such currencies mediating tradeoffs between 56 immunity and other traits like growth, reproduction or thermoregulation (Nilsson et al., 57 2007). However, recent evidence highlights the fact that while energetic costs of an immune 58 response can be significant, these are dependent on the different components of the immune 59 response being elicited, and according to the particular environmental and physiological 60 context (Hasselquist and Nilsson, 2012). Alternatively, energy expenditure can have indirect 61 effects on immunity by means of changes in the oxidative balance. Oxidative stress is 62 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., 63 64 2009; Garratt and Brooks, 2012). Organisms counteract oxidative stress by acquiring and 65 producing antioxidants, and while most antioxidative activity is enzymatic, non-enzymatic 66 antioxidants also play a relevant role in maintaining oxidative balance, particularly in blood 67 (Pamplona and Costantini, 2011). Therefore, oxidative balance is a complex synergistic trait 68 dependent on the antioxidative enzymatic capacity, the diverse antioxidant levels and the 69 oxidative stress being generated by the individual metabolic activity, which may rapidly 70 change in time and in different tissues (Cohen and McGraw, 2009). Oxidative stress has been 71 suggested to mediate the relationship between immunity and other vital components 72 (Dowling and Sommons, 2009), and recent evidence suggests a link between oxidative stress 73 and immunity, albeit its significance is still under discussion (Costantini and Møller, 2009). 74 Immune activation increases susceptibility to oxidative damage (Bertrand et al., 2006), but 75 such relation may change according to the different components of the immune response 76 being elicited (Costantini and Møller, 2009). Furthermore, carotenoids are a diverse group of

77 lipophilic molecules important for the immunity and individual fitness, and since they cannot

- 78 be synthesized *de novo* by animals, necessarily need to be ingested, or acquired during
- 79 embryogenesis through maternal transfer (Pérez-Rodríguez, 2009). Carotenoids have been
- 80 claimed to underlie honest-signalling due to their dual role as pigments and
- 81 antioxidants/immuno-stimulants, and although the antioxidative function of carotenoids has
- 82 lately been questioned (Pérez-Rodríguez, 2009), their active role as mediators of both the
- 83 immune system and the oxidative balance remains undisputed (Simons et al., 2012).

84 Individual variation in immune investment may arise from a variety of factors, not 85 only adaptive adjustments but also constraints resulting from resource or genetic based trade-86 offs (Ardia et al., 2011). Furthermore, individual energy expenditure and physiological 87 condition can affect both oxidative and immunological indices (van de Crommenacker et al., 88 2010). Therefore, given the interactive nature of all physiological traits, it is important to set 89 up baseline measurements of relevant parameters, to interpret the precise relationships among 90 traits potentially involved in trade-offs with immunity (Ardia et al., 2011). Immunity is a 91 fitness-related trait that is affected by environmental and individual physiological conditions, 92 although variation in diverse immune components are genetically determined. For example, 93 genetic diversity at the functionally important genes belonging to the major 94 histocompatibility complex (MHC) is acknowledged to play a central role in the immune 95 system of vertebrates (reviewed by Sommer, 2005). However, genetic contributions to the 96 phenotypic variability of immune response have rarely been demonstrated in wild bird 97 species (see Sepil et al., 2013 and references therein).

98 Phytohaemagglutinin (hereafter PHA) is a mitogen of vegetal origin that when 99 injected intradermally induces an immune response, which has been often used as a proxy of 100 the cell-mediated immune capacity (Kennedy and Nager, 2006). However, recent studies 101 have challenged this idea (Martin II et al., 2006), and shown that PHA-induced immune 102 response is a multifactorial process involving both innate and acquired cell-mediated 103 elements, and reflecting an individuals' ability to mount an inflammatory response (Kennedy 104 and Nager, 2006; Vinkler et al., 2010). PHA test is the most widely used in-vivo measure of 105 immunocompetence in avian ecological studies (Kennedy and Nager, 2006), and has been 106 linked to various traits such as fitness (Cichon and Dubiec, 2005; López-Rull et al., 2011), growth conditions (Hõrak et al., 1999), oxidative stress or plasma carotenoid levels (Simons 107 108 et al., 2012). Furthermore, variation in PHA response has been found in selected chicken 109 lines, implying a significant genetic variation for this response (Sundaresan et al., 2005). In 110 fact, MHC genes have previously been found to be associated with the phenotypic variability 111 of this response in chickens and in a wild passerine (Taylor et al., 1987; Bonneaud et al.,

112 2005; but see Bonneaud et al., 2009). Alternatively, other studies on PHA response

113 heritability highlight the fact that environmental and early-maternal effects may override

114 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.

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129 Materials and methods

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131 Birds and experimental design

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133 Captive bred lesser kestrel nestlings were brought from the DEMA facilities 134 (Almendralejo, Spain, more info at www.demaprimilla.org) at an age of ~20 days to Estación 135 Biológica de Doñana (CSIC) building (Seville, Spain), in where a reintroduction project was 136 being carried out. Nestlings were released into outdoor hacking nest boxes simulating a 137 natural breeding colony, in where they came in contact with adult birds, both captive 138 irrecoverable individuals that fed them as adoptive parents, and feral birds (see Rodríguez et 139 al., 2013). Birds remained 8.4 ± 2.6 (mean \pm SD) days undisturbed for acclimatization 140 purposes, and then subjected to two consecutive and identical night-time respirometry 141 sessions, each one lasting around 12h (20.00 p.m. to 08.00 a.m) (see below). The birds were 142 weighted (to the nearest 0.1 g) at the start and the end of each session. After each 143 respirometry session, birds were blood sampled and returned to the hacking nest boxes, 144 where they were fed ad libitum with dead laboratory mice and three-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.

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151 Measurements and sample collection

- 152
- 153 Plasma biochemical parameters
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All individuals were sampled for blood (0.35mL) 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 (4000 rpm during 20 min) within 1 hour 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.

161 Plasma was analyzed for eight biochemical parameters (carotenoids, total antioxidant 162 capacity, triglycerides, β -hydroxybutyrate, cholesterol, uric acid, urea and total proteins). 163 Carotenoid (CAR) concentration in plasma was measured by means of N-1000 NanoDrop 164 spectrophotometer at 450nm as in Bortolotti et al. (2000). Total antioxidant capacity (TAC) is 165 a measure of the capacity of plasma to neutralize reactive oxygen species, and was measured 166 as described in Erel (2004). Recent studies point out that TAC is mostly representative of the 167 water soluble components of the antioxidative system (Cohen and McGraw, 2009). However, 168 it is commonly agreed that measurement of TAC in combination with other fat-soluble 169 antioxidants may provide a more complete image of the antioxidant system (Monaghan et al., 170 2009). The remaining plasma metabolites are related to the nutritional state of bird (McCue, 171 2010). Triglycerides (TRG), β -hydroxybutyrate (β HB) and cholesterol (CHL) are all involved 172 in fat metabolism. TRG are the storage form of lipids, and are good indicators of fat 173 deposition, β HB is an indicator of the catabolism of fatty acids, whereas CHL is known to be 174 a good predictor of general nutritional condition and body mass. Total proteins (PRT), urea 175 (URE) and uric acid (UAC) are all involved in protein catabolism (Jenni-Eiermann and Jenni, 176 1998). Further, UAC is a common circulating hydrophilic antioxidant that accounts for an 177 important portion of the antioxidant capacity in blood (Cohen et al., 2007). All plasma 178 metabolites (except CAR) were measured according to standard methods implemented on a

179 Cobas INTEGRA 400 plus Chemistry autoanalyser (Roche Diagnostics Ltd. Burgess Hill,
180 West Sussex, UK).

- 181
- 182 DNA extraction and MHC genotyping
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184 DNA was extracted from blood samples following the HotSHOT protocol (Truett, 185 2006) and used for molecular sex determination (Rodríguez et al., 2011). The second exon of 186 a single and highly polymorphic MHC class II B gene was PCR-amplified and sequenced 187 following Alcaide et al., (2008). Direct sequencing chromatograms were carefully inspected by eye and edited in BIOEDIT v7.0.5.3 (Hall, 1999), and IUPAC nucleotide degenerate 188 189 codes were introduced for each heterozygous site. MHC diploid genotypes were then 190 resolved into individual haplotypes using the Bayesian PHASE platform implemented in 191 DNASP v5 (Librado and Rozas, 2009) following Alcaide et al., (2011). Then, we translated 192 each allele into amino acids and we counted the number of different amino acids between the 193 two alleles of each bird, as a measure of MHC heterozygosity (MHC). Furthermore, we 194 categorized nestlings according to the number of most frequent alleles in South West Spain, 195 i.e. 1 = individuals holding at least one of the most frequent alleles; 0 = individuals holding 196 infrequent alleles. Fana2 (20%) and Fana19 (12%) were the most frequent alleles in South 197 West Spain (Alcaide et al., 2008).

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199 Respirometry

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201 Resting metabolic rate (RMR) was measured as the average minimal oxygen 202 consumption under post-absorptive digestive conditions during the resting phase of the daily 203 cycle in an open circuit respirometer (McNab, 1997). Birds were individually placed in an 204 air-sealed chamber (3L) inside a climate cabinet at 27 °C, within the thermoneutral zone of 205 similarly sized falcons as the lesser kestrel (Shapiro and Weathers, 1981; Bush et al., 2008). 206 The respirometer consisted of two independent sets consisting of 4 and 8 channels 207 respectively. Both respirometers had exactly the same components except for the number of 208 channels. Outdoor air was pushed towards each chamber through independent mass-flow 209 controllers (Flow-bar-8) adjusted to 700mL/min. A valve system controlled by gas-flow 210 multiplexer (RM4-8) conducted outcoming air in 10 min cycles from each chamber towards a 211 water vapour analyzer (RH 300) and then to the CO₂-Oxygen analyzer FOXBOX-C Field gas 212 analysis system (Sable systems int.) 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 length were unlikely to bias our results (Cooper and Withers,

215 2010). The value of oxygen consumption (mLO₂/min) was taken as the lowest value of

running 10 min averages during a measurement session and was calculated according to Hill

217 218

219 PHA test

(1972).

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221 PHA challenge consisted of a subcutaneous injection in the left patagia of 0.1 mL of 222 PHA-P (L-8754 Sigma-Aldrich) diluted in saline solution PBS (Sigma P-5119) at 2.5 mg/mL 223 or just PBS, following (Smits et al., 1999). Patagia width was measured at the point of 224 injection (to the nearest 0.01 mm) three times just prior to and 12 h after injection, using a 225 pressure sensitive micrometer (Baxlo Precision S.L.). The micrometer was removed 226 completely from the wing between each measurement and all measurements were taken by 227 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. 228 229

230 Statistical analyses

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232 We evaluated the inter-correlations (Pearson coefficients) among ten physiological 233 parameters measured on pre-treatment session (RMR, MASS, β HB, CHL, TAC, PRT, TRG, 234 UAC, URE and CAR; Table 1 and 2), and tested for potential gender differences. 235 Furthermore, repeated measures General Linear Models were used to test for treatment effect 236 (PHA vs. control), on the previously mentioned variables as measured during pre- and post-237 treatment sessions. The selection of explanatory variables was based on their co-variation 238 with response variables on pre-treatment session in order to reduce variance (see Table 2). 239 Gender was also introduced as a factor when significant differences were reached on the pre-240 treatment values of the response variable. Although some explanatory variables were 241 correlated (see Table 2), multicollinearity was not an issue according to variance inflation 242 factors (range = 1.060-1.493). Finally, considering only PHA challenged birds, we analyzed 243 the correlations between PHA response and a) the variables measured on pre- and post-244 treatment sessions, and b) the change in these variables between post and pre-treatment 245 sessions. Statistical analyses were conducted using SPSS v.19 package (IBM Company, 246 Chicago, IL, USA). Samples sizes varied among sessions.

- 247 **Results**
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249 Among the parameters explored, we found ten significant correlations (see Table 2). 250 RMR was positively related to body mass, whereas mass was in turn negatively related to 251 β HB. TAC was positively related to UAC, but negatively to CHL. UAC was in turn 252 positively related to PRT and URE. URE was additionally related to PRT and TRG in a 253 positive way. And TRG was positively related to PRT, and CHL. Finally, CAR was 254 independent of any other physiological parameters considered. Only TRG concentration 255 differed by sex ($t_{34} = 2.851$, P = 0.007), being greater in females ($101.1 \pm 15.7 \text{ mg/dL}$; mean 256 \pm SD) than in males (82.9 \pm 17.6 mg/dL) during the pre-treatment session. 257 PHA challenge did not affect most of the studied physiological parameters (RMR, 258 body mass, β HB, CHL, PRT, UAC or URE), even when considering the significantly 259 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).

264 When considering PHA challenged individuals, UAC concentration on pre-treatment 265 session was the only physiological parameter correlated with the PHA variation, and the 266 positive relationship persisted on post-treatment session, although the slope decreased (Fig. 2; 267 note that 95% confidence intervals only overlap at low values of PHA response and UAC, 268 and they do not include the regression lines). The other physiological parameters on pre and 269 post-treatment session did not correlate with PHA (*P*-values > 0.05 in all cases). Finally, only 270 changes between sessions in MASS, CHL, TAC and TRG were positively correlated to PHA 271 immune response (Fig. 3), the rest of physiological parameters remaining non-significant (P-272 values > 0.05 in all cases). MHC heterozygosity, measured as the number of amino acid 273 differences, did not correlate with PHA response (r = -0.017, P = 0.939). In addition, mean 274 PHA induced immune response did not vary between individuals holding frequent or 275 infrequent MHC alleles ($t_{21} = -0.564$, P = 0.579). 276

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- 281 Discussion
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283 Treatment effects (PHA vs. control)

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285 Lesser kestrel nestlings growing in *ad libitum* food conditions experienced limited 286 short-term (12 hours after-challenge) physiological consequences of a PHA challenge. All 287 nutritional parameters, both related to protein (PRT, URE and UAC) and lipid metabolism 288 (CHL, TRG, β HB) showed little variation throughout the experiment, and only TRG and 289 CAR concentrations in plasma were affected by PHA challenge. As blood samples were 290 collected after nocturnal fasting (12 hours after PHA challenge), variation in blood 291 metabolites were more likely the result of an active mobilization between blood and other 292 tissues than because of differences in feeding or nutrient absorption. TRG concentration in 293 plasma dropped within the 12 hours from challenge, suggesting that TRG were used as 294 energy source more in PHA-challenged individuals than in controls (although it was not 295 accompanied by an increased metabolic rate -see below). Furthermore, the observed trends in 296 β HB, i.e. higher levels in PHA-challenged birds than in control birds and pre-treatment 297 values (Table 1), support the hypothesis of TRG as energy source, as the depletion of TRG 298 together with increased β HB are common outcomes of lipid catabolism (Jenni-Eiermann and 299 Jenni, 1998). TRG depletion could be also explained by factors other than nutrition, as they 300 are actively involved in the immune response (see examples in Barcia and Harris, 2005; 301 Radovic et al., 2012).

302 Carotenoids play a subtle but significant role as immune-modulators (Costantini and 303 Møller, 2009; Simons et al., 2012), and they have often been found to decrease in immune 304 challenged organisms, suggesting a relevant role in the development of the immune response 305 (Pérez-Rodríguez et al., 2008; Vinkler and Albrecht, 2010). We found CAR to be 306 significantly affected by the immune challenge, increasing in PHA-challenged birds. This 307 apparently counterintuitive result has been previously recorded in wild nestlings of Eurasian 308 kestrels (Falco tinnunculus), and was interpreted as a remobilisation from skin, fat reserves 309 and liver to blood stream (Costantini and Dell'Omo, 2006). Furthermore, in a previous study 310 on wild lesser kestrel nestlings TGR were positively related to body mass and to CAR-based 311 tarsus coloration, indicating that better nourished nestlings had higher TGR levels, and CAR 312 allocated to signalisation (Rodríguez et al., 2011). Thus, the depletion of TRG and the 313 increase of circulating CAR suggest that mounting a PHA immune response entails a

314 physiological cost for lesser kestrel nestlings detectable from a few hours after the immune315 challenge.

316 RMR of the lesser kestrel was independent of PHA challenge, suggesting that energy 317 does not play a relevant role in this immune response, at least under the studied conditions 318 and the time-scale considered. Favourable conditions for growth necessarily required in a 319 conservation project (including in our case *ad libitum* fresh and sanitated food) may buffer 320 the potential energetic costs of the experimental treatment. In addition, the time-scale 321 considered in this study (<12 hours) may not be sufficient for detecting metabolic 322 adjustments induced by PHA response, which can last over three days (Navarro et al., 2003). 323 In line with this, Gutiérrez et al. (2011) found that metabolic effects of a PHA challenge were 324 only detectable after 48 hours, and these only changed in food-restriction conditions. 325 Alternatively, certain immune responses can be prioritized over other energy expensive 326 activities (Hegemann et al., 2012), implying that energetic costs of an immune response are 327 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 II et al., 328 329 2003; Burness et al., 2010; Abad-Gómez et al., 2013; King and Swanson, 2013), most studies 330 conclude that these costs are not important enough to make energy the currency of the 331 assumed trade offs between immunity and other traits (Eraud et al., 2005; Nilsson et al., 332 2007). However, in combination with other costly activities, the energetic costs of mounting 333 an immune response may become biologically relevant for the individual (Ots et al., 2001, 334 Hawley et al., 2012).

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336 *Correlates of PHA immune response*

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338 When studying the variation in PHA response (i.e. taking into account only PHA-339 challenged birds), only UAC appeared as a relevant predictive parameter. Pre- and post-340 treatment levels of UAC were positively related to PHA response, and nestlings with a higher 341 PHA response exhibited a larger decrease in UAC (Fig. 2). As far as we know, our study is 342 the first one reporting this link (see Hõrak et al., 2007). In birds, high circulating UAC levels 343 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 344 345 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 346 347 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, 1987). 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.

355 Change in body mass between sessions together with CHL and TRG paralleled the 356 degree of PHA response, suggesting that this immune response is condition dependent. 357 Plasma metabolites have provided robust information on the physiological condition, mass 358 change or growth rates on a variety of wild avian species (Seaman et al., 2005; Dietz et al., 359 2009; Albano et al., 2011). Our results add to evidence found by previous studies on wild 360 populations of Eurasian kestrel and American kestrel (Falco sparverius) that confirmed the 361 importance of environmental conditions in explaining nestling's PHA (Tella et al., 2000; 362 Martínez-Padilla, 2006; Martínez-Padilla and Viñuela, 2011).

363 If MHC gene variability is correlated with the diversity of lymphocyte receptors, it 364 could be anticipated that most heterozygous individuals may trigger a more efficient immune 365 response as they are capable to bind and present a higher diversity of antigens (see Sommer, 366 2005). Natural selection plays a relevant role in shaping MHC variability in lesser kestrels 367 (e.g. Alcaide et al., 2008, 2012), and therefore we assumed that PHA immune response could 368 be affected by the presence of certain alleles in the genome. However, in this study PHA-369 response was unrelated to MHC class II heterozygosity or to the presence of certain alleles in 370 the genome of lesser kestrel nestlings. Although our results should be considered with caution 371 due to sample size constraints, they are in agreement with Bonneaud et al. (2009), who found 372 PHA heritability to be independent from MHC variation. The lack of association between 373 PHA-response and MHC II variability could stem from the time frame considered, as MHC 374 genotypes had been significantly associated with PHA in chicken, but only after 72 hours 375 post-injection (Taylor et al., 1987). Certain MHC supertypes have shown to confer resistance 376 to specific parasites in songbirds (e.g. Radwan et al., 2012; Sepil et al., 2013 and references 377 therein), and in the case of PHA test, an association between PHA response and a single 378 MHC allele was found in house sparrows (Bonneaud et al., 2005). Given that PHA response 379 does not mirror T-cell function (Vinkler et al., 2010), this association is unlikely to reflect a 380 direct involvement of MHC in the physiological processes elicited by PHA (Licastro et al., 381 1993; Bonneaud et al., 2005). In accordance with this hypothesis, responsiveness to PHA can

- 382 be attributed to other immune genes than MHC II, for example Toll-like receptors, which are
- 383 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).
- 385
- 386 Conclusions
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388 Lesser kestrels nestlings growing in optimal conditions (*ad libitum* food supply) 389 experienced slight physiological changes to PHA challenge, while resting metabolic rate 390 remained constant on a short-term basis (<12h). PHA-response induced a rapid depletion of 391 triglycerides, and paralleled changes in body mass and other plasma metabolites suggesting a 392 condition-dependent nature of this immune response. Furthermore, a rapid mobilization of 393 carotenoids and changes in the hydrophilic components of the antioxidative balance indicate 394 that PHA-response elicits significant changes in metabolic pathways related to nutritional and 395 oxidative balance, while keeping the overall energy budget unaffected.

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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).

| Parameter | Abbrev. Units | | Pre-treatment | | Post-treatement | | | | |
|----------------------------|---------------|-----------------------|--------------------|----|--------------------|----|--------------------|----|--|
| | | | | | Control | | РНА | | |
| | | | Mean \pm SD | n | Mean \pm SD | п | Mean \pm SD | п | |
| 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 | |

Table 2

618 Correlations between the metabolic, biochemical and phenotypic variables measured in this study. Correlation coefficients (*r*-values) are given

619 above diagonal and *P*-values below diagonal. Significant correlations (P < 0.05) are in bold.

| | 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 |
| β HB | 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 | - |

623 **Table 3**

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

625 control). The selection of explanatory variables was based on their co-variation with response variables (see main text). Note that positive signs

- 626 among model terms do not refer to the relationship between variables.
- 627

| Response Variable | Explanatory Variables | F | d.f. | Р |
|----------------------------|-----------------------------------|--------|-------|-------|
| Resting metabolic rate | Treatment + MASS | 0.045 | 1, 13 | 0.835 |
| Body mass | Treatment + RMR + β HB | 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 |

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



Fig. 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.



- 641 **Fig. 3.** Change (post pre treatment) in body mass and three plasma metabolite
- 642 concentrations with PHA induced immune response in lesser kestrel nestlings. Pearson



643 correlations (r) with the corresponding P-values are included.