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ORIGINAL PAPER

# The influence of diet on nestling body condition of an apex predator: a multi-biomarker approach

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**Abstract** Animal body condition refers to the health and physiological state of individuals, and multiple parameters have been proposed to quantify this key concept. Food intake is one of the main determinants of individual body condition and much debate has been generated on how diet relates to body condition. We investigated this relationship in free-living Bonelli's eagle (*Aquila fasciata*) nestlings sampled at two geographically distant populations in Spain. Nestlings' main prey consumption was estimated by isotopic analyses. A multi-biomarker approach, including morphometric and blood biochemical measures (i.e. hematocrit, plasma biochemistry and oxidative stress biomarkers), enabled us to integrate all the body condition measures taken. A greater consumption of a preferred prey [i.e. the European rabbit (*Oryctolagus cuniculus*)] improved nestling body condition, as indicated by lower levels of cholesterol in plasma, greater activity of enzymes mediating in protein catabolism, higher levels of tocopherol and glutathione, and less glutathione peroxidase activity, which also suggested lower degree of oxidative stress. On the other hand, increased diet diversity was positively correlated with higher levels of oxidized glutathione, which suggests that these nestlings had poorer body condition than those with a higher frequency of preferred prey consumption. Several factors other than diet [i.e. altitude of nesting areas, nestling sex and age, sampling time (before or after midday) and recent food ingestion] had an effect on certain body condition measures. Our study reveals a measurable effect of diet on a predator's body condition and demonstrates the importance of considering the potential influence of multiple intrinsic and extrinsic factors when assessing animal body condition.

**Keywords** Blood biochemistry · Body condition · Optimal foraging theory · Oxidative stress · Raptors · Stable isotope analysis

**Abbreviations**

| | |
|---|---|
| SIA | Stable isotope analyses |
| AO | Antioxidant |
| OS | Oxidative stress |

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| | |
|---|---|
| ROS | Reactive oxygen species |
| RBC | Red blood cell |
| H' | Shannon–Weaver index |
| SMI | Scaled mass index |
| GLMMs | Generalized linear mixed models |
| $AIC_c$ | Akaike's information criterion adjusted for sample size |
| $AIC_{cw}$ | Akaike weights |
| $R^2_m$ | Marginal coefficient of determination |
| $R^2_c$ | Conditional coefficient of determination |
| OC | Rabbit consumption |
| AR | Consumption of partridges |
| CP | Consumption of wood pigeons |
| CL | Consumption of domestic pigeons |
| PAS | Consumption of passerines |

## Introduction

Body condition is a key concept used in studies of animal biology to quantify the health and physiological state of individuals (Brown 1996; Stevenson and Woods 2006; Labocha and Hayes 2012). Internal and external factors influence animal body condition (e.g. Zera and Harshman 2001; Clinchy et al. 2004; Bonal and Aparicio 2008) and the potential influence of both should be considered when attempting to disentangle the complexity underlying this concept.

Diet is one of the most important determinants of body condition and may affect individual growth rate and survival, and, ultimately, individual fitness (Pothoven et al. 2001; Sorensen et al. 2009; Harrison et al. 2011). The diet-body condition association has been studied in several species differing in their feeding strategies and under a variety of ecological scenarios (Stevenson and Woods 2006; Lefcheck et al. 2013). For instance, the relationship between individual body condition and generalist versus specialist diets (i.e. diets composed of many versus few food types—e.g. species, respectively) has been much debated in recent decades (see Bowen et al. 1995; Lefcheck et al. 2013). Most of the current scientific knowledge on this topic is, however, based on microcosm studies performed in laboratories under controlled conditions. Thus, our knowledge of how trophic composition and diversity relates to the body condition of wild animals is still at a preliminary stage. In this regard, the difficulty in assessing the food intake of free-living organisms, especially at individual level, may have hampered research on this topic. Fortunately, recently developed techniques based on stable isotope analyses (SIA) allow for individual diet reconstruction by converting isotopic composition (most frequently the carbon and nitrogen in consumer tissues and main food resources) into dietary proportions (Parnell et al. 2010). Therefore, SIA offer wildlife ecologists a useful tool for performing applied studies of animal nutritional and functional ecology. This is particularly interesting in the case of endangered species, in which the study of individual body condition in natural populations can help to detect potential threats to population viability and, at the same time, provide a guide for management actions (see Ferrer and Dobado-Berrios 1998; Balbontín and Ferrer 2002; Hernández and Margalida 2010).

Despite the importance of the individual body condition concept in ecological and evolutionary studies, its assessment is not straightforward. A range of indices—from morphological to biochemical and physiological—that measures different levels of biological organization can be used to estimate animal body condition (Brown 1996; Stevenson and Woods 2006; Labocha and Hayes 2012). Nevertheless, the large number of factors that influence individual body condition makes the interpretation of results difficult, particularly when only one or a few body condition parameters are analyzed. In this regard, assessments of body condition based on integrated analyses of multiple body condition parameters are recommendable, although this type of analysis is still uncommon.

Traditionally, morphological indices have been used to assess animal body condition (Brown 1996; Stevenson and Woods 2006). These indices are based on the premise that once corrected for the structural size of an individual, its body mass is indicative of the amount of non-structural energy reserves (e.g. fat and proteins) and, ultimately, individual body condition (Stevenson and Woods 2006). Thus, to establish a meaningful comparison between individuals that differ in size, morphological indices must account for the effects of growth, development and sex differences on body mass-morphometric relationships (see Peig and Green 2009, 2010). This critical point has generated discussion since the violation of the basic assumptions on which indices are based (e.g. that mass increases linearly with size) will lead to inaccurate estimates of body condition (Green 2001; Peig and Green 2009; Labocha and Hayes 2012). Despite controversy, numerous studies have associated morphological indices with fitness parameters such as survival rates or reproductive success, thereby offering ecologists a practical methodology for addressing animal fitness-related hypotheses (Brown 1996; Schulte-Hostedde et al. 2005).

Measuring blood parameters is another common procedure for estimating animal body condition (Milner et al. 2003; Owen 2011). Most studies, however, use captive animals in rehabilitation or research centers and reference values for free-living organisms are much scarcer. Plasma constituents are among the most common blood parameters analyzed. For instance, some plasma components are indicative of the activity in or damage to specific

tissues (Harrison and Harrison 1986), while other plasma metabolites such as iron, carbohydrate, fat, total amount of protein and nitrogen waste concentrations may be more directly related to diet. These metabolites can be synthesized and obtained from ingested food, or mobilized from endogenous body reserves in tissues (Hochleithner 1994; Alonso-Alvarez 2005; Stevenson and Woods 2006). Apart from diet, factors such as age, sex and recent food ingestion may also influence plasma biochemistry (see Hochleithner 1994; Dobado-Berrios et al. 1998; Alonso-Alvarez 2005) and thus hinder parameter interpretation. For instance, uric acid increases after food intake, especially in species with high protein diets (Hochleithner 1994). Yet, uric acid is also a by-product of protein catabolism, which can increase with food scarcity once lipid reserves have been consumed, and so high uric acid levels may also be indicative of trophic stress and muscle damage (Hochleithner 1994). Therefore, the proper interpretation of plasmatic parameters for each individual requires an integrated assessment of multiple body condition parameters.

In recent years, analyses of antioxidant (AO) defenses and oxidative stress (OS) have received considerable attention in studies of animal ecology as proxies of individual fitness (Monaghan et al. 2009; Costantini et al. 2010). Aerobic organisms generate reactive oxygen species (ROS) as a by-product of normal metabolic processes (Balaban et al. 2005). ROS can damage key biomolecules such as lipids, proteins and DNA due to their high reactivity. OS occurs as a result of an imbalance between the production of ROS and the body's ability to mitigate their harmful effects through AO defenses (Finkel and Holbrook 2000; Monaghan et al. 2009). Antioxidants include diet-derived (e.g. vitamins and carotenoids) and endogenous molecules that prevent or minimize oxidative damage through interrelated mechanisms. Because animals obtain some of their AO defenses from their diet, which also determines energy and nutrient income, an individual's nutritional state can potentially affect the delicate balance that generally exists between ROS production and AO defenses (see Catoni et al. 2008; Monaghan et al. 2009). It has been observed, however, that growing individuals facing temporary food restrictions are capable of adjusting their metabolism and growing rate to enhance resistance to oxidative stress during their lifetime (Alonso-Alvarez et al. 2006; Noguera et al. 2011). Therefore, measures of AO defenses alone are not enough to make inferences regarding OS, as these substances can be regulated by an animal's exposure to ROS (Costantini and Verhulst 2009; Monaghan et al. 2009). Overall, although AO and OS biomarkers offer a powerful tool for assessing individual body condition and for detecting developmental problems or stress in growing individuals, their interpretation requires the integration of the presence of AO defenses with measures of oxidative damage.

Bonelli's eagle (*Aquila fasciata*) is a long-lived raptor distributed from south-east Asia and the Middle East to the western Mediterranean (del Hoyo et al. 1994). The European population is listed as endangered (BirdLife International 2004) in light of a marked decline in number and range in recent decades, related to unnaturally high mortality rates, habitat degradation and the decline of its main prey species (Real 2004; Hernández-Matías et al. 2011, 2013). In Western Europe, Bonelli's eagle mainly predates on European rabbits (*Oryctolagus cuniculus*), red-legged partridges (*Alectoris rufa*), pigeons (*Columba* spp.), other birds and lizards (Real 1991; Moleón et al. 2009). In this geographical area, rabbits and, to a lesser extent, partridges are considered to be the preferred prey item for this raptor and are preferentially consumed wherever they are abundant, thereby reducing this eagle's diet diversity (Real 1991; Moleón et al. 2009, 2012). Greater consumption of preferred prey is assumed to provide more efficiency in terms of energy intake in this species and so it is expected to benefit eagles' body condition. For instance, recent studies have shown that Bonelli's eagle productivity at both territorial and local population levels is positively and negatively influenced, respectively, by greater preferred prey consumption and greater diet diversity (Resano-Mayor et al. 2014a, 2016). Nevertheless, how diet affects nestling body condition is almost unknown in this and most other long-lived birds.

By using Bonelli's eagle as a study model, here we explore the relationships between nestling diet and body condition, estimated, respectively, by isotopic analyses and a multi-biomarker approach including morphometric and blood biochemical measures. The specific objectives were: (1) to provide reference values for body condition measures (morphometric index, plasmatic biochemistry and antioxidant metabolism) in free-living Bonelli's eagle nestlings, (2) to test whether or not the consumption of preferred prey and diet diversity correlate with nestling body condition, and (3) to assess the potential influence of intrinsic and extrinsic factors other than prey consumption on body condition estimates. Our main prediction is that greater consumption of preferred prey will positively correlate with nestling body condition, while greater diet diversity (to be expected when the preferred prey is not abundantly consumed) will have the opposite effect.

## Methods

### Study area

During 2010–2011 we monitored a total of 43 Bonelli's eagle breeding territories located in two local populations in Spain: 33 different territories in Catalonia (41°20′N,

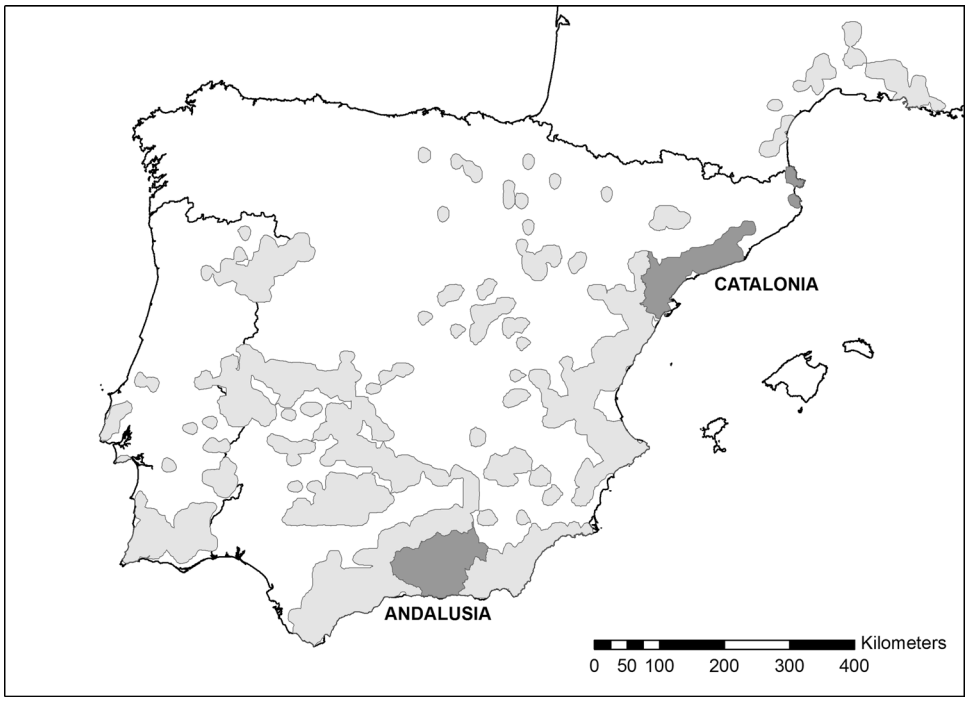


**Fig. 1** Distribution map of Bonelli's eagle breeding areas in Western Europe (modified from Hernández-Matías et al. 2013). The studied populations are shown in *dark grey*: Catalonia and Andalusia

01°32′E; northeast Spain; $n = 23$ and 24 in 2010 and 2011, respectively) and 10 in Andalusia in 2011 (37°76′N, 03°85′W; southern Spain) (Fig. 1). Breeding nests were located on cliffs; the altitude of nesting areas ranged from 30 to 790 m asl in Catalonia and from 500 to 1500 m asl in Andalusia. All territories are typical Mediterranean landscapes (see Gil-Sánchez et al. 2004; Carrascal and Seoane 2009; Bosch et al. 2010) characterized by marked variation in habitat coverage, habitat structure and prey abundances, especially in Catalonia.

## Sample collection

Breeding territories were monitored between January and March to check for breeding activity (i.e. incubation behavior). In late March and April, occupied nests were observed to detect the presence, number and age of nestlings, which were estimated by feather development and backdating from laying dates (Real 1991; Gil-Sánchez 2000). To avoid disturbance, observations were always carried out at long distance from the nests using 10 × binoculars and 20–60 × spotting scopes. Once nestlings were around 35–45 days old, experienced climbers accessed the nests to capture the chicks, which were then carefully transported to the top of the cliff for morphometric measurements and tissue collection. Body mass was then recorded to the nearest 25 g and tarsus length measured to the nearest 0.01 mm using an electronic caliper; these measurements were subsequently used to estimate nestling body condition based on morphometrics (see below). We also measured the length of the seventh primary to accurately estimate nestling age in days (see Mañosa et al. 1995). Four mantle feathers were then sampled from each chick for individual diet estimates via isotopic analyses (see below) as feather isotopic composition indicates nestling diet at the time of tissue development (i.e. the previous few weeks). Finally, before nestlings were returned to the nest, we sampled 2.2 ml of blood from the brachial vein with a 20-gauge needle. Blood was collected in two heparinized tubes of 1 ml, from which two hematocrit capillaries were filled. The remaining blood was collected in an Eppendorf tube with 0.5 ml of absolute ethanol for molecular sex determination, which was performed following the method described in Fridolfsson and Ellegren (1999). All blood samples were immediately stored at 4 °C in a portable fridge until processed in the laboratory within 12 h. Once in the laboratory, hematocrit capillaries were centrifuged at 9000 rcf for 5 min and the hematocrit was measured as the percentage of packed red cell volume in relation to the total column height (plasma plus packed cell volume) using the same electronic caliper. Heparinized tubes were centrifuged at 10,000 rcf for 5 min to separate the plasma (supernatant) from the red blood cell (RBC) fraction. Then, RBC samples were washed with a cold saline solution followed by centrifugation at 10,000 rcf for 5 min to remove the supernatant. Finally, the four vials (two with plasma and two with RBC; an aliquot for each fraction) were frozen in liquid nitrogen and then stored a few days later at −80 °C until analysis. The blood samples in ethanol were directly frozen at −20 °C until sex determination.

### Individual diet estimates

Nestlings' main prey consumption was estimated by analyzing the isotopic ratios of carbon ($^{13}C$:$^{12}C$; $\delta^{13}C$) and nitrogen ($^{15}N$:$^{14}N$; $\delta^{15}N$) in feathers following the procedure described by Resano-Mayor et al. (2014b). Isotopic measurements were conducted at the *Centres Científics i Tecnològics* in the University of Barcelona using the methods in Resano et al. (2011).

The Bayesian mixing model SIAR (Parnell et al. 2010) was used to estimate the relative contribution of the main prey categories in the diet of each chick. The main prey categories included in the SIAR were European rabbits, red-legged partridges, wood pigeons (*Columba palumbus*), domestic pigeons (*C. livia* dom.) (distinguishing between pigeons that forage on crops in the wild and those that are associated with dovecotes and are fed with corn (*Zea mays*); see Resano-Mayor et al. 2014a), passerines (Corvidae, Sturnidae and Turdidae), Eurasian red squirrels (*Sciurus vulgaris*), ocellated lizards (*Timon lepidus*) and yellow-legged gulls (*Larus michahellis*) (only in Catalonia given that this prey was not consumed in Andalusia). Prey isotopic values were obtained from the most recently published studies (see Resano-Mayor et al. 2014a). The trophic discrimination factors included in the SIAR were 2.1 ‰ ± 0.08 for $\delta^{13}C$ and 2.7 ‰ ± 0.5 for $\delta^{15}N$, described for feathers from peregrine falcons (*Falco peregrinus*) fed on the flesh of Japanese quails (*Coturnix japonica*) (Hobson and Clark 1992). Mean prey consumption estimates from SIAR were selected for subsequent analyses. These prey consumption percentages were also used to estimate the diet diversity by means of the Shannon–Weaver index (1949) (*H'*), including all pigeon groups as a single prey category.

### Morphometric body condition index

To obtain an estimate of body condition based on nestling morphometric, we calculated the scaled mass index (SMI), which scales each individual's body mass to the value expected if all nestlings had the same body size, by using the inherent power relationship between mass and size modeled from the data (Peig and Green 2009). The SMI was estimated separately for males and females given morphometric sexual dimorphism. We scaled the body masses of each chick to the mean tarsus length (110.60 and 113.10 mm for males and females, respectively) using a secondary major axis slope of 2.78 for males and 2.50 for females (see Peig and Green 2009). Tarsus length was significantly and positively correlated with other morphometric measurements (e.g. bill length, tarsus diameter, central nail and claw length, primary length and tail length; Spearman correlations all with $p < 0.01$). Moreover, a detailed study on Bonelli's eagle nestling growth also showed similar growth curves when considering the abovementioned measurements (see Mañosa et al. 1995). Therefore, tarsus length should be representative of the overall skeletal size.

### Plasma biochemistry

The plasma fraction stored at −80 °C was used to measure calcium, magnesium, phosphorus, glucose, cholesterol, triglycerides, creatinine, urea, uric acid, total proteins and the activities of alkaline phosphatase (ALP), aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and creatine kinase (CK), as described in Martinez-Haro et al. (2011). Plasma biochemistry was measured spectrophotometrically using an A25 autoanalyser and commercial kits from BioSystems S.A. (Barcelona, Spain).

Levels of vitamin A (free retinol in alcoholic form) and their esterified forms with fatty acids (retinyl palmitate), vitamin E ($\alpha$-tocopherol) and carotenes (lutein and zeaxanthin) were determined in plasma by high-pressure liquid chromatography (HPLC, Agilent Technologies 1100 Series), as described in Rodríguez-Estival et al. (2011). All these plasmatic components are important antioxidant defenses obtained from diet and their levels indicate individual capacity in the event of oxidative stress.

### Red blood cell analysis

Several oxidative stress biomarkers were analyzed in RBC after homogenization (1:10 w/v) in a stock buffer (1.15 % KCl in 0.01 M PBS—pH 7.4—with 0.02 M EDTA), as previously described in Mateo et al. (2003) and Reglero et al. (2009). Firstly, the activities of two antioxidant enzymes, glutathione peroxidase (GPx) and superoxide dismutase (SOD), were determined spectrophotometrically with an A25 autoanalyzer and using Ransel and Ransod kits, respectively (Randox Laboratories, Crumlin, UK). Homogenized samples were diluted by 1:20 and 1:25 (v:v) with Ransel diluting agent and Ransod sample diluents for GPx and SOD determinations, respectively. Enzyme activities were expressed relative to milligrams of protein in the homogenates calculated spectrophotometrically. Membrane lipid peroxidation in erythrocytes was estimated as thiobarbituric acid-reactive substances (TBARS). Determination of TBARS was performed colorimetrically with a spectrophotometer (Ultrospec2100pro, UV/vis, Amersham Biosciences). Levels of total glutathione (tGSH) and GSH in oxidized form (oxGSH; 2 oxGSH = 1 GSSG) were obtained by a reaction coupled to GSH reductase as described by Reglero et al. (2009) with an A25 autoanalyzer. The oxGSH was expressed as a molar concentration and as a percentage of the tGSH (%oxGSH).

### Data analysis

Descriptive parameters of all body condition measures (i.e. scaled mass index, hematocrit, plasma biochemistry, antioxidants and oxidative stress biomarkers) were calculated as the mean, standard deviation (±SD) and minimum and maximum values. Descriptive parameters are shown for nestlings for each local population-year (Catalonia 2010: $n = 33$ nestlings; 2011: $n = 41$; and Andalusia 2011: $n = 18$), as well as for all the monitored nestlings ($n = 92$).

To test the effect of diet on nestling body condition we applied generalized linear mixed models (GLMMs), which allowed us to account for the potential non-independence of clustered observations from the same territory, year and population. Each body condition measure was modeled as the response variable using the identity link function and errors were assumed to be normally distributed. Rabbit consumption (OC), diet diversity (*H*') and the sum OC + *H*' were selected as explanatory variables in all the models in order to test our main prediction that greater consumption of preferred prey (i.e. OC) and an increase in diet diversity correlate positively and negatively, respectively, with nestling body condition. Given that other variables (diet-related or otherwise) may have a relevant effect on our body condition measures, previous GLMMs were used to select the most feasible variables for each body condition measure, that is, those which reduced the null model $AIC_c$ value by 2 or more points. Potential explanatory variables included in the previous GLMMs were as follows: the consumption of partridges (AR), wood pigeons (CP), domestic pigeons (CL) and passerines (PAS), brood size (categorical variable: 1 vs. 2), nestling age, sex, the additive effects of age + sex, altitude of nesting areas, sampling time (categorical variable: whether nestlings were sampled before or after midday), and nestling food ingestion (categorical variable: whether nestlings had been recently fed—i.e. had a full crop or there was fresh prey in the nest—or not). When assessing the scaled mass index as a response variable, all models included sex as a fixed factor to control for sexual size dimorphism. GLMMs were fitted using the *lmer* function from the *lme4* package of R (Bates et al. 2012). Model selection was based on Akaike's Information Criterion adjusted for sample size ($AIC_c$), the Akaike weights ($AIC_{cw}$) being computed to assess the probability that each candidate model was the best of the proposed set (Burnham and Anderson 2002). The goodness-of-fit of each model was estimated from marginal ($R^2_m$) and conditional ($R^2_c$) coefficients of determination, following (Nakagawa and Schielzeth 2013). The $R^2_m$ value shows the proportion of the variance in the raw data explained by the fixed effects only, while the $R^2_c$ value shows the proportion of the variance explained by the full model, including both fixed and random effects. All figures were created by using SigmaPlot version 10.0.

## Results

### Reference values

Descriptive values of body condition measures (i.e. scaled mass index, hematocrit, plasma biochemistry, antioxidants and oxidative stress biomarkers) for nestlings from each local population-year, and for the overall 92 monitored nestlings (40 males and 52 females), are given in Table 1.

### Effects of diet and other parameters on body condition

A summary of the main effects of diet composition and diversity, brood size, nestling age, nestling sex, altitude of nesting areas, sampling time and nestling recent food ingestion on body condition measures is given in Table 2. All these interactions are described below (see Tables A1–4, available online, for details of the models used for each body condition measure).

#### *Scaled mass index*

The best-fitted model for the SMI included the consumption of rabbits, *H*' and nestling sex (Online Resource Table A1), although the relationship between nestling diet and this index was weak and showed no clear pattern.

#### *Hematocrit*

We did not find any effect of diet on nestlings' hematocrit, which was, however, highly and positively correlated with the altitude of nesting areas (Fig. 2). The $R^2_m$ of the best-fitted model indicated that the 41.4 % of the variance in hematocrit was explained by altitude, while random effects (i.e. territory, year and population) slightly improved the model explanatory power (Online Resource Table A2).

#### *Plasma biochemistry*

We found no clear effect of diet on either calcium or phosphorus levels. Despite the fact that the best-fitted models for these parameters included the consumption of passerines and pigeons, these prey categories had little explanatory power, i.e. very low $R^2_m$. No diet effect was detected for magnesium levels since the null model was the best fit (Online Resource Table A3).

Although glucose levels tended to increase with age, the main factor explaining plasmatic glucose was nestling sex, with males having higher levels than females (Fig. 3a). Despite being included in the best-fitted model, neither rabbit consumption nor *H*' had any strong effect on glucose levels, as suggested by the low $R^2_m$ of this model (Online Resource Table A3).

**Table 1** Descriptive values of nestling body condition in Catalonia 2010 ($n = 33$) and 2011 ($n = 41$), Andalusia 2011 ($n = 18$), and the overall monitored individuals ($n = 92$)

| Body condition measures | Catalonia 2010 | Catalonia 2011 | Andalusia 2011 | Overall |
|---|---|---|---|---|
| Scaled mass index (g) | 1666.81 ± 212.60 | 1690.75 ± 186.42 | 1611.29 ± 224.25 | 1666.62 ± 203.55 |
| | 1287.56/2021.90 | 1382.70/1956.46 | 1355.12/2085.33 | 1287.56/2085.33 |
| Hematocrit (%) | 29.66 ± 2.30 | 29.45 ± 2.18 | 32.34 ± 2.37 | 30.04 ± 2.48 |
| | 23.16/35.27 | 25.10/33.53 | 28.71/37.26 | 23.16/37.26 |
| *Plasma biochemistry* | | | | |
| Calcium (mg/dl) | 10.61 ± 1.26 | 10.11 ± 1.00 | 10.96 ± 1.29 | 10.45 ± 1.19 |
| | 6.78/13.20 | 5.68/12.15 | 7.68/12.51 | 5.68/13.20 |
| Magnesium (mg/dl) | 1.37 ± 0.24 | 1.37 ± 0.39 | 1.49 ± 0.44 | 1.39 ± 0.35 |
| | 1.01/1.88 | 0.83/2.77 | 0.95/2.92 | 0.83/2.92 |
| Phosphorus (mg/dl) | 6.52 ± 0.88 | 6.61 ± 1.24 | 6.76 ± 0.90 | 6.61 ± 1.05 |
| | 5.34/9.69 | 5.14/12.59 | 5.20/8.36 | 5.14/12.59 |
| Glucose (mg/dl) | 317.30 ± 29.95 | 319.88 ± 19.69 | 319.28 ± 22.60 | 318.84 ± 24.14 |
| | 266/421 | 278/371 | 277/347 | 266/421 |
| Cholesterol (mg/dl) | 183.82 ± 27.81 | 178.76 ± 19.60 | 184.72 ± 32.97 | 181.74 ± 25.52 |
| | 110/236 | 132/238 | 141/241 | 110/241 |
| Triglycerides (mg/dl) | 90.88 ± 70.44 | 104.76 ± 106.29 | 122.94 ± 85.29 | 103.34 ± 90.57 |
| | 6/324 | 14/595 | 5/272 | 5/595 |
| Creatinine (mg/dl) | 0.33 ± 0.10 | 0.35 ± 0.10 | 0.34 ± 0.06 | 0.34 ± 0.09 |
| | 0.06/0.50 | 0.13/0.80 | 0.25/0.44 | 0.06/0.80 |
| Urea (mg/dl) | 18.35 ± 7.46 | 12.31 ± 4.83 | 20.91 ± 9.36 | 16.16 ± 7.69 |
| | 5.50/34.80 | 5.80/25.80 | 12.20/53.90 | 5.50/53.90 |
| Uric acid (mg/dl) | 13.13 ± 5.41 | 11.85 ± 4.34 | 13.61 ± 4.99 | 12.65 ± 4.88 |
| | 6.84/28.73 | 6.01/26.87 | 6.71/23.00 | 6.01/28.73 |
| Total proteins (g/l) | 32.30 ± 3.08 | 31.82 ± 3.03 | 34.49 ± 2.50 | 32.52 ± 3.09 |
| | 23.20/38.30 | 26.40/42.70 | 28.80/39.60 | 23.20/42.70 |
| Alkaline phosphatase (U/l) | 785.88 ± 139.19 | 897.39 ± 162.08 | 1024.06 ± 270.56 | 882.17 ± 198.71 |
| | 400/1001 | 629/1359 | 610/1602 | 400/1602 |
| Aspartate aminotransferase (U/l) | 222.03 ± 26.69 | 214.24 ± 25.59 | 209.61 ± 28.06 | 216.13 ± 26.61 |
| | 184/318 | 173/280 | 178/291 | 173/318 |
| Lactate dehydrogenase (U/l) | 2207.88 ± 882.50 | 2352.51 ± 455.56 | 2382.56 ± 409.70 | 2306.51 ± 634.09 |
| | 597/4780 | 1797/3786 | 1935/3657 | 597/4780 |
| Creatine kinase (U/l) | 4233.45 ± 781.71 | 4274.83 ± 698.68 | 4352.11 ± 1844.40 | 4275.11 ± 1032.84 |
| | 3068/5784 | 2916/5988 | 2172/7868 | 2172/7868 |
| *Antioxidants and oxidative stress biomarkers* | | | | |
| Retinol (μM) | 4.72 ± 0.96 | 5.30 ± 0.58 | 7.56 ± 1.43 | 5.53 ± 1.39 |
| | 2.88/6.53 | 3.82/6.29 | 4.61/10.18 | 2.88/10.18 |
| Retinyl palmitate (μM) | 0.17 ± 0.19 | 0.15 ± 0.19 | 0.33 ± 0.50 | 0.19 ± 0.28 |
| | 0.00/0.89 | 0.00/1.06 | 0.00/2.17 | 0.00/2.17 |
| Tocopherol (μM) | 17.39 ± 3.86 | 21.51 ± 4.61 | 22.27 ± 4.66 | 20.18 ± 4.81 |
| | 7.78/27.53 | 13.79/35.77 | 13.81/28.80 | 7.78/35.77 |
| Lutein (μM) | 2.72 ± 0.78 | 3.03 ± 0.85 | 3.72 ± 1.25 | 3.05 ± 0.97 |
| | 1.27/4.12 | 1.02/4.47 | 0.77/5.91 | 0.77/5.91 |
| Zeaxanthin (μM) | 1.23 ± 0.42 | 1.33 ± 0.42 | 1.47 ± 0.52 | 1.32 ± 0.45 |
| | 0.48/2.22 | 0.43/2.30 | 0.18/2.05 | 0.18/2.30 |
| GPx (U/mg protein) | 0.28 ± 0.09 | 0.28 ± 0.09 | 0.32 ± 0.10 | 0.29 ± 0.09 |
| | 0.12/0.48 | 0.08/0.51 | 0.15/0.54 | 0.08/0.54 |
| SOD (U/mg protein) | 1.35 ± 0.21 | 1.07 ± 0.30 | 1.55 ± 0.25 | 1.26 ± 0.32 |
| | 1.01/1.98 | 0.62/2.02 | 1.18/2.10 | 0.62/2.10 |

**Table 1** continued

| Body condition measures | Catalonia 2010 | Catalonia 2011 | Andalusia 2011 | Overall |
|---|---|---|---|---|
| TBARS (μmol/g pellet) | 0.06 ± 0.02 | 0.06 ± 0.02 | 0.06 ± 0.01 | 0.06 ± 0.02 |
| | 0.04/0.09 | 0.02/0.09 | 0.04/0.09 | 0.02/0.09 |
| tGSH (mmol/g pellet) | 4.79 ± 0.90 | 5.03 ± 0.83 | 4.61 ± 0.60 | 4.87 ± 0.83 |
| | 3.18/6.54 | 3.35/7.71 | 3.45/5.59 | 3.18/7.71 |
| oxGSH (μmol/g pellet) | 838.86 ± 504.68 | 938.52 ± 597.56 | 598.53 ± 512.63 | 836.25 ± 558.13 |
| | 98.58/1914.65 | 148.16/1979.97 | 28.89/1611.68 | 28.89/1979.97 |
| %oxGSH | 8.47 ± 4.61 | 9.47 ± 5.83 | 6.40 ± 5.33 | 8.51 ± 5.39 |
| | 1.17/19.24 | 0.96/19.56 | 0.27/17.36 | 0.27/19.56 |

The parameters shown are the scaled mass index, hematocrit, plasma metabolites (calcium, magnesium, phosphorus, glucose, cholesterol, triglycerides, creatinine, urea, uric acid and total proteins), plasma enzyme activities (alkaline phosphatase, aspartate aminotransferase, lactate dehydrogenase and creatine kinase), plasma antioxidants (retinol, retinyl palmitate, tocopherol, lutein and zeaxanthin), and oxidative stress biomarkers analyzed in red blood cells (antioxidant enzymes GPx, glutathione peroxidase; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substances; tGSH, levels of total glutathione; and oxGSH and %oxGSH, GSH in oxidized form). The mean, standard deviation (±SD), minimum and maximum (min/max) values are shown for each measure; units of measurement are specified

Cholesterol and triglyceride values increased as rabbit consumption fell and *H'* rose (Fig. 4a, b). In the case of triglycerides, however, nestlings that had recently ingested food had considerably higher levels than nestlings that had not recently fed (Fig. 3b). In fact, this factor explained triglyceride levels better than any other dietary parameter as shown by the higher $R^2_m$ of the model (Online Resource Table A3).

Creatinine was not affected by diet. However, it was the only parameter that showed a strong variation in relation to sampling time, as nestlings sampled before midday had lower creatinine levels than those sampled after midday (Fig. 3c) (Online Resource Table A3).

Urea levels slightly increased with lower *H'* (Fig. 4c), although the $R^2_m$ of the best-fitted model that included the consumption of rabbits and $H'$ reveals the weak explanatory power of these dietary parameters (Online Resource Table A3). Nestling food ingestion had the same effect on uric acid as described above for triglycerides, with higher uric acid levels in nestlings that had recently ingested food than in those that had not (Fig. 3d). Despite the fact that the best-fitted models for total proteins included the consumption of partridges and domestic pigeons, we detected no clear relationship between these dietary parameters and nestlings' total protein levels (Online Resource Table A3).

The plasmatic activities of ALP and CK tended to be higher in males than in females (Fig. 3e, f), although nestling age was the main determinant, as shown by the increase in levels of these enzymes with age (Fig. 4d, g). Rabbit consumption and $H'$ were the main dietary parameters affecting the activity of AST and increased with greater consumption of rabbits and lower $H'$. The same pattern was found for LDH activity, although in this case the relationship was weaker than for the AST (Fig. 4e, f) (Online Resource Table A3).

*Antioxidants and oxidative stress*

When detected, the effects of diet on plasmatic antioxidant defenses varied according to the parameter. We detected no clear effect of diet on either retinol or lutein. Retinyl palmitate, however, increased in nestlings that had recently ingested food (Fig. 5). Tocopherol levels were slightly higher in nestlings with more rabbit consumption and lower diet diversity (Fig. 6a); the greater consumption of wood pigeons tended to increase the levels of zeaxanthin (Fig. 6d), although the $R^2_m$ values in these cases were low (Online Resource Table A4).

The activity of the enzyme GPx increased with greater consumption of passerines and decreased with greater rabbit consumption (Fig. 6b). In the case of the SOD, greater consumption of wood pigeons reduced this enzyme's activity, which increased with lower diet diversity (Fig. 6c); rabbit consumption showed no clear effect on the activity of this enzyme. We detected no effect of diet on TBARS. In contrast, tGSH increased with greater consumption of rabbits (Fig. 6e), but diet diversity had no effect. Both oxGSH and %oxGSH increased with greater $H'$ (Fig. 6f); rabbit consumption showed no clear effect (Online Resource Table A4).

## Discussion

The assessment of individual body condition is crucial in studies of animal ecology and conservation biology because animal health may determine fitness parameters and can be used to anticipate and develop actions for conservation efforts (Brown 1996; Stevenson and Woods 2006). In this study, we describe the effects of diet on the body condition of free-living Bonelli's eagle nestlings as

**Table 2** Summary of the main effects of diet composition and diversity, brood size, nestling age, nestling sex, altitude of nesting area, sampling time and nestling recent food ingestion (first column) on body condition measures (first line)

| Explanatory parameters | Scaled mass index | Hematocrit | Calcium | Magnesium | Phosphorus | Glucose | Cholesterol | Triglycerides | Creatinine | Urea | Uric acid | Total proteins | Alkaline phosphatase | Aspartate aminotransferase | Lactate dehydrogenase | Creatine kinase | Retinol | Retinyl palmitate | Tocopherol | Lutein | Zeaxanthin | GPx | SOD | TBARS | tGSH | oxGSH | %oxGSH |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Rabbits | = | | | | | = | − | = | | = | | | | + | = | | | | + | | | − | = | | + | = | = |
| Partridges | | | | | | | | | | | | = | | | | | = | | = | = | | | | | | | |
| Wood pigeons | | | = | | = | | | | | | | | | | | | = | | = | = | + | | − | | | | |
| Dom. pigeons | | | | | = | | | | | | | = | | | | | | | = | | | | | | | | |
| Passerines | | | = | | = | | | | | | | | | | | | = | | = | = | | + | | | | | |
| Diet diversity | = | | | | | = | + | = | | = | | | | − | = | | | | − | | | | − | | = | + | + |
| Brood size | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Nestling age | | | | | | = | | | | | | | + | | | + | | | | | | | | | | | |
| Nestling sex | = | | | | | * | | | | | | | = | | | = | | | | | | | | | | | |
| Altitude nesting area | | + | | | | | | | | | | | | | | | | | | | | | | | | | |
| Sampling time | | | | | | | | | * | | | | | | | | | | | | | | | | | | |
| Recent food ingestion | | | | | | | | * | | | * | | | | | | | * | | | | | | | | | |

Diet parameters analyzed were the consumption of rabbits, partridges, wood pigeons, domestic pigeons, passerines and diet diversity. Body condition measures are the same as described in Table 1: the scaled mass index, hematocrit, plasma metabolites (calcium, magnesium, phosphorus, glucose, cholesterol, triglycerides, creatinine, urea, uric acid and total proteins), plasma enzyme activities (ALP, alkaline phosphatase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase, CK, creatine kinase), plasma antioxidants (retinol, retinyl palmitate, tocopherol, lutein and zeaxanthin), and oxidative stress biomarkers analyzed in red blood cells (antioxidant enzymes GPx, glutathione peroxidase; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substances, tGSH, levels of total glutathione; oxGSH and %oxGSH, GSH in oxidized form). Symbols denote whether the parameter had an effect on body condition estimates; either a strong "*", distinguishing between positive "+" or negative "−"for continuous variables, or a weak "=" effect

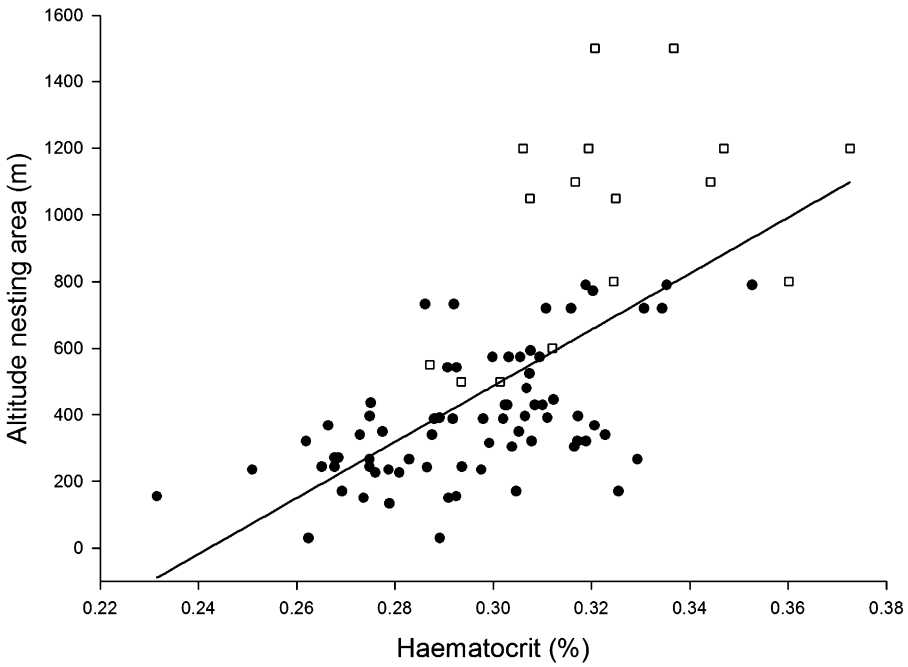


**Fig. 2** Relationship between hematocrit values (%) in Bonelli's eagle nestlings and the altitude of nesting areas (m a.s.l.). The trend line of the relationship between the variables is shown. *Black dots* refer to nestlings from Catalonia and *white squares* to nestlings from Andalusia

estimated by morphometric and blood biochemical indicators. We also analyzed whether or not nestling body condition was influenced by other intrinsic or extrinsic factors such as sex, age, time of day at sampling, or recent food intake. Additionally, our study provides reference values for multiple body condition measures in nestlings from two geographically distant populations (Catalonia and Andalusia; see also Balbontín and Ferrer 2002), which, in the case of the antioxidant metabolism, constitutes the first such data for this species and contributes to the hitherto scarce literature on the subject in other raptors (see e.g. Galván et al. 2010; Sternalski et al. 2010; Casagrande et al. 2011).

### Effects of diet and other parameters on the scaled mass index and hematocrit

Morphological indices are among the most commonly used methods to assess body condition in birds since they offer an easy and straightforward way of estimating an animals' nutritional status (Brown 1996; Stevenson and Woods 2006). Their usefulness, however, depends on how accurately they can compensate for variation in mass due to body size; if this is not accomplished, results may be misleading (Brown 1996; Green 2001; Peig and Green 2009). In this study, we scaled nestling body mass to the mean tarsus length of all individuals (accounting for sexual dimorphism) as if they were of identical skeletal size. This allowed us to obtain body condition estimates that were uncorrelated with nestling body size (i.e. tarsus length) and so we are confident that the scaled mass index did not reflect differences in nestling body size. Even so, we found no relationship between the scaled mass index and dietary parameters. This result suggests that body condition parameters based on nestling biometry are not sensitive enough to detect diet effects during the rearing period in Bonelli's eagle, especially in comparison with results from the blood biochemical level (see below). Nevertheless, it is worth to mention that the scaled mass index integrates a longer time period of food intake in comparison with the biochemical measurements.

The traditional use of hematocrit values as an indicator of body condition in wild birds has recently been questioned due to the fact that multiple factors such as age, sex, geographical elevation, energy expenditure, parasitism, dehydration, nutrition and genetics may influence these values (see Fair et al. 2007). In our study, nestling hematocrit was mainly explained by the altitude of nesting areas; on the other hand, neither age nor dietary parameters showed any clear effect on this parameter. Several studies have shown that hematocrit is either independent of or increases with elevation (Carpenter 1975; Borras et al. 2010). Accordingly, we found that Bonelli's eagle nestlings raised in more elevated nests had higher hematocrit values, which will enable them to ensure adequate oxygen delivery to tissues at lower partial oxygen pressures. The wide altitudinal range of nestling sites in our study area, which ranges from coastal cliffs to mountain ranges up to 1500 m asl, possibly facilitated the detection of this pattern. In general, breeding territories in Andalusia were located at higher altitude than in Catalonia and so most nestlings in the former region had higher hematocrit values. We thus recommend that the effect of altitude should be accounted for in studies using hematocrit as an indicator of nestling body condition.

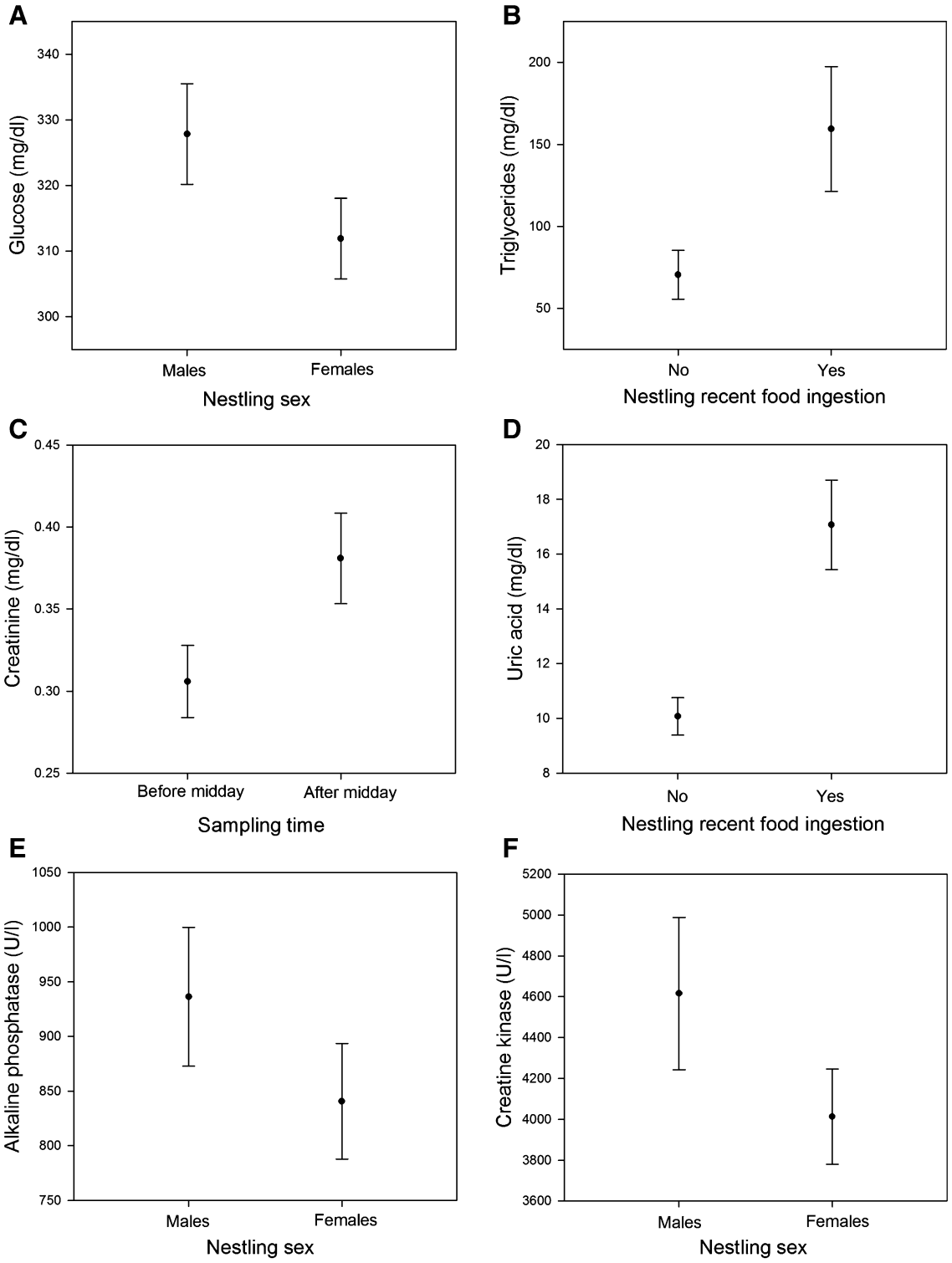


**Fig. 3** Effects of Bonelli's eagle nestling sex, recent food ingestion and sampling time on plasma biochemical parameters: glucose (**a**), triglycerides (**b**), creatinine (**c**), uric acid (**d**), alkaline phosphatase (**e**) and creatine kinase (**f**)

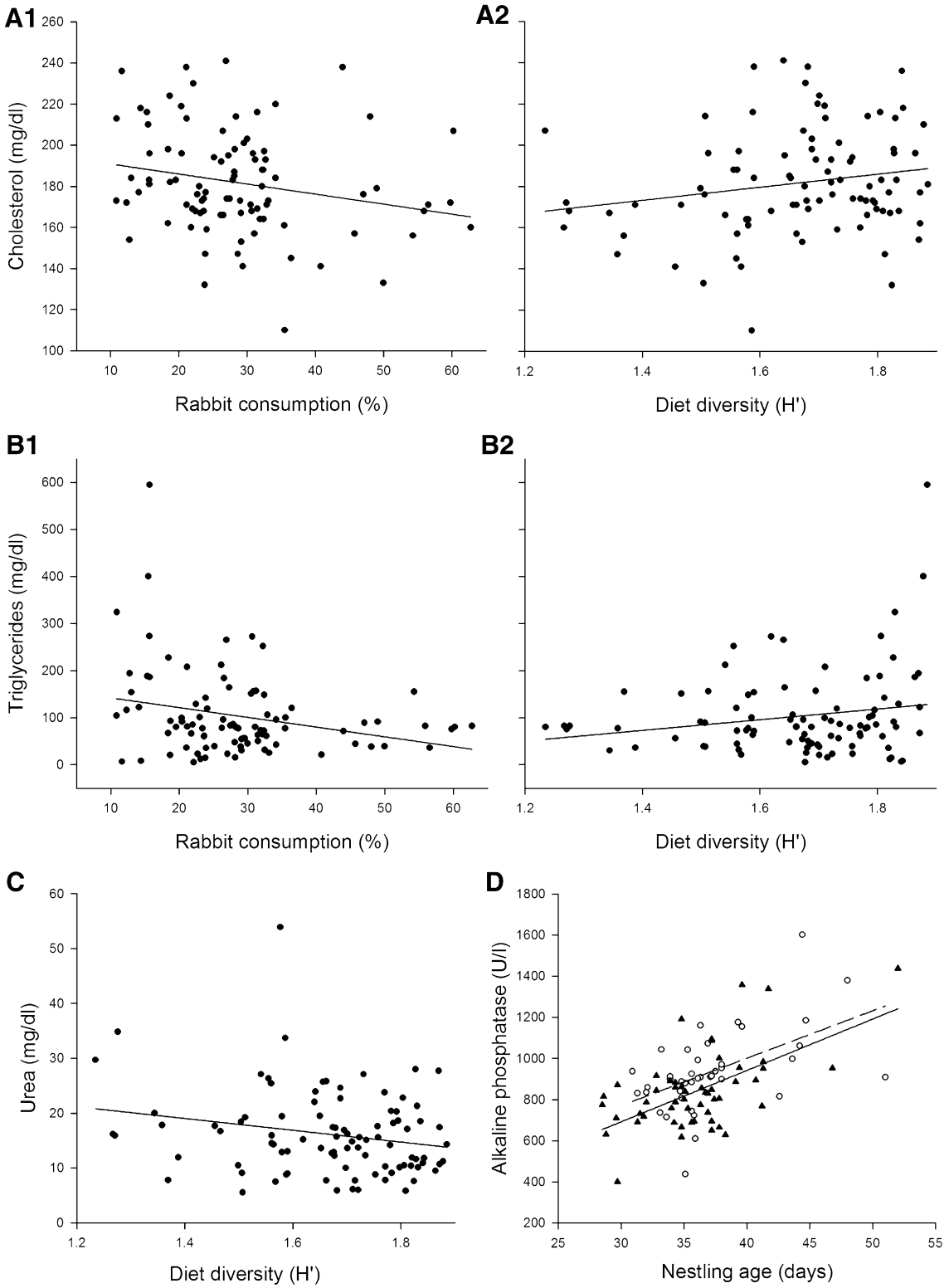


**Fig. 4** Effects of rabbit consumption (%), diet diversity (*H'*) and Bonelli's eagle nestling age (days) on nestling plasma biochemical parameters: cholesterol (**a1**, 2), triglycerides (**b1**, 2), urea (**c**), alkaline phosphatase (**d**), aspartate aminotransferase (**e1**, 2), lactate dehydrogenase (**f1**, 2) and creatine kinase (**g**). In the case of the relationships shown in **d** and **g**, *open circles* denote males and *filled triangles* females. Trend lines are shown

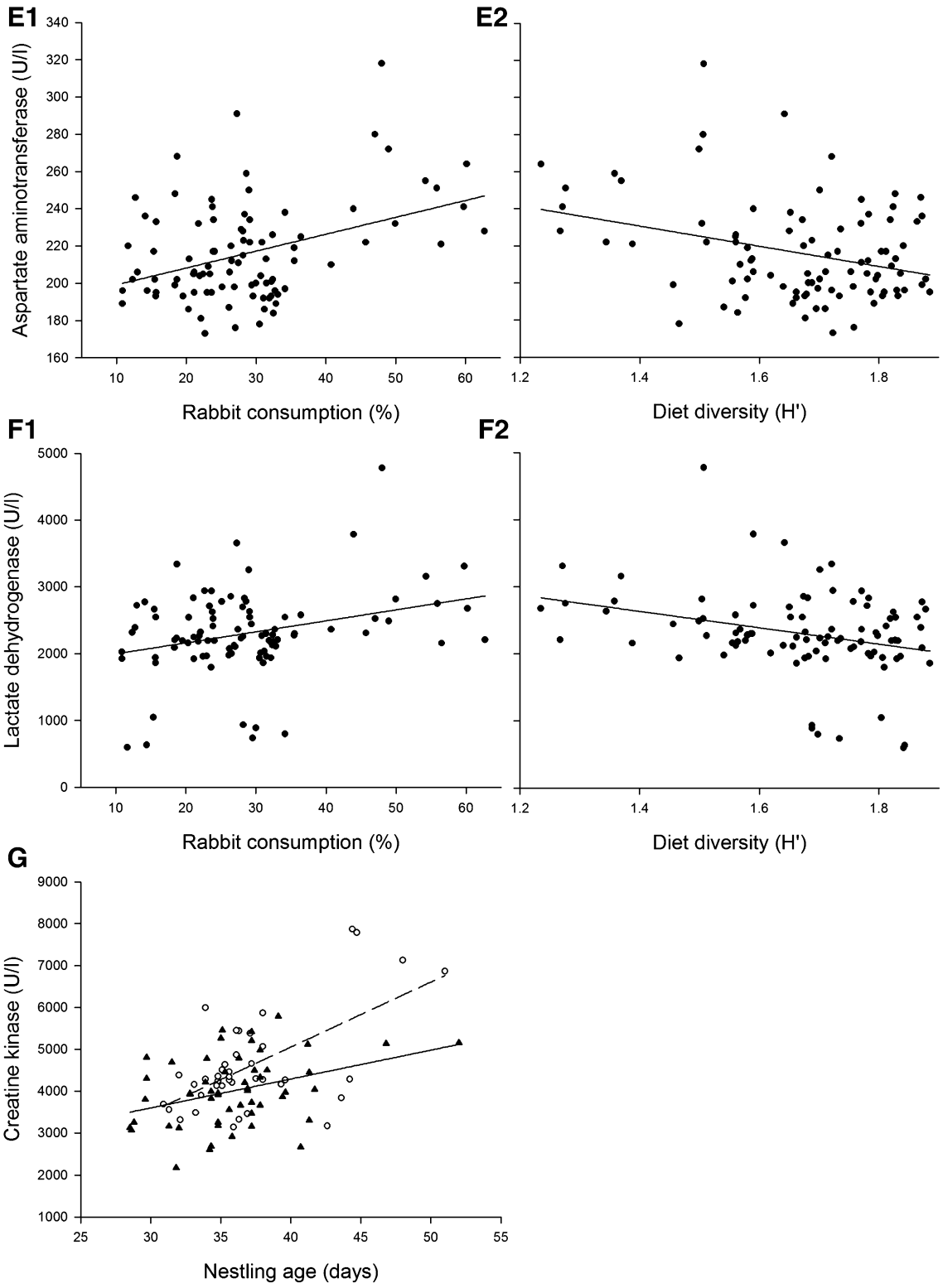


**Fig. 4** continued

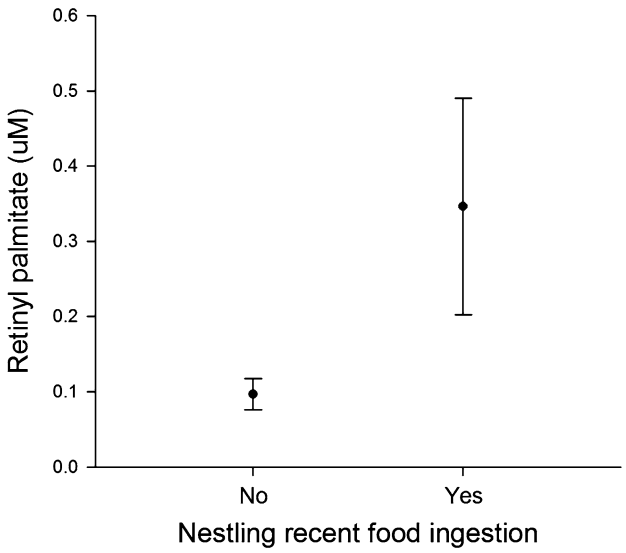


**Fig. 5** Effects of recent food ingestion by Bonelli's eagle nestlings on levels of plasma retinyl palmitate

### Bonelli's eagle reference values of plasma metabolites

Descriptive reference values of plasma metabolites are available for numerous raptor species, including Bonelli's eagle, and provide insights into the health of both captive and free-living individuals (Polo et al. 1992; Ferrer and Dobado-Berrios 1998; Balbontín and Ferrer 2002; Hernández and Margalida 2010). Overall, the Bonelli's eagle nestlings in our study showed similar plasma parameter values to free-living nestlings analyzed in western Andalusia by Balbontín and Ferrer (2002), albeit with a higher range for most parameters. This was probably because we analyzed a considerably larger number of nestlings ($n = 92$ vs. 28) from two geographically distant populations (Catalonia and eastern Andalusia), which, in the case of nestlings from Catalonia, had important dietary differences between territories (see Resano-Mayor et al. 2014a). We also detected slightly higher values in phosphorus, glucose and triglycerides in nestlings in our study; nevertheless, the mean and range of values for alkaline phosphatase reported by Balbontín and Ferrer (2002) were much higher. When comparing our results with published results for free-living nestlings of other raptor species in Spain, Bonelli's eagle nestlings had (a) higher mean glucose concentration than nestlings of either Spanish Imperial eagle (*Aquila adalberti*), Booted eagle (*Aquila pennata*), Egyptian vulture (*Neophron percnopterus*) or Bearded vultures (*Gypaetus barbatus*); (b) similar cholesterol and triglycerides to the eagle species but slightly lower values than the vultures; (c) similar total protein concentration to Imperial eagles and the two vulture species (nestlings of Booted eagle had very low plasma protein values); and (d) similar urea and uric acid to eagles, higher values for these two metabolites than Egyptian vultures, and similar urea levels but much higher uric acid than Bearded vultures (Dobado-Berrios et al. 1998; Ferrer and Dobado-Berrios 1998; Casado et al. 2002; Hernández and Margalida 2010). All these differences in blood parameters may be explained by, among other factors, specific characters such as growth patterns, metabolism and diet, as well as variations in sampling procedures, so comparisons should be considered with caution.

### Effects of diet and other parameters on plasma biochemistry, antioxidants and oxidative stress biomarkers

Although avian blood parameters (including plasma biochemistry and antioxidant metabolism) provide information about individual nutritional status, few studies have integrated a multiple blood parameter approach with individual diet estimates into an interpretation of bird body condition. In this study, we hypothesized that greater consumption of rabbits (i.e. preferred prey) and greater diet diversity (i.e. a proxy of low availability of preferred prey) would positively and negatively affect, respectively, Bonelli's eagle nestling body condition. Both greater rabbit consumption and lower diet diversity reduced cholesterol and triglycerides in plasma. In birds, an increase in these lipid reserves has been related to starvation due to the mobilization of endogenous fats during fasting (García-Rodríguez et al. 1987a; Hochleithner 1994; Rubio et al. 2014); although the opposite can also be true, that is, greater levels of plasma triglycerides have been measured in well-fed birds (Castellini and Rea 1992). Thus, high rabbit consumption possibly indicates that nestlings' ever-increasing energetic demands are fulfilled, while an increase in diet diversity could lead to an energy shortage that would trigger the use of endogenous lipid reserves. Greater diet diversity was also related with lower urea levels. Suitable ingestion rates of protein-rich diets can increase the urea levels in birds (Koutsos et al. 2001; Salahuddin et al. 2012) as protein breakdown into amino acids releases urea (Hochleithner 1994). Thus, nestlings with a more diverse diet could have an overall lower protein intake leading to a subsequent reduction in urea levels. This idea is supported by lower AST activity (an enzyme involved in amino acid catabolism) in nestlings with greater diet diversity and lower rabbit consumption (see Das and Waterlow 1974). Therefore, based on conventional plasma parameters, nestlings with greater diet diversity seem to have poorer nutritional status as they have to mobilize endogenous fat reserves and also receive a lower intake of protein-rich food (see Parker et al. 2005). On the other hand, nestlings with greater rabbit consumption have overall better body condition, thereby supporting our assumption that this is an optimal prey item for the species.

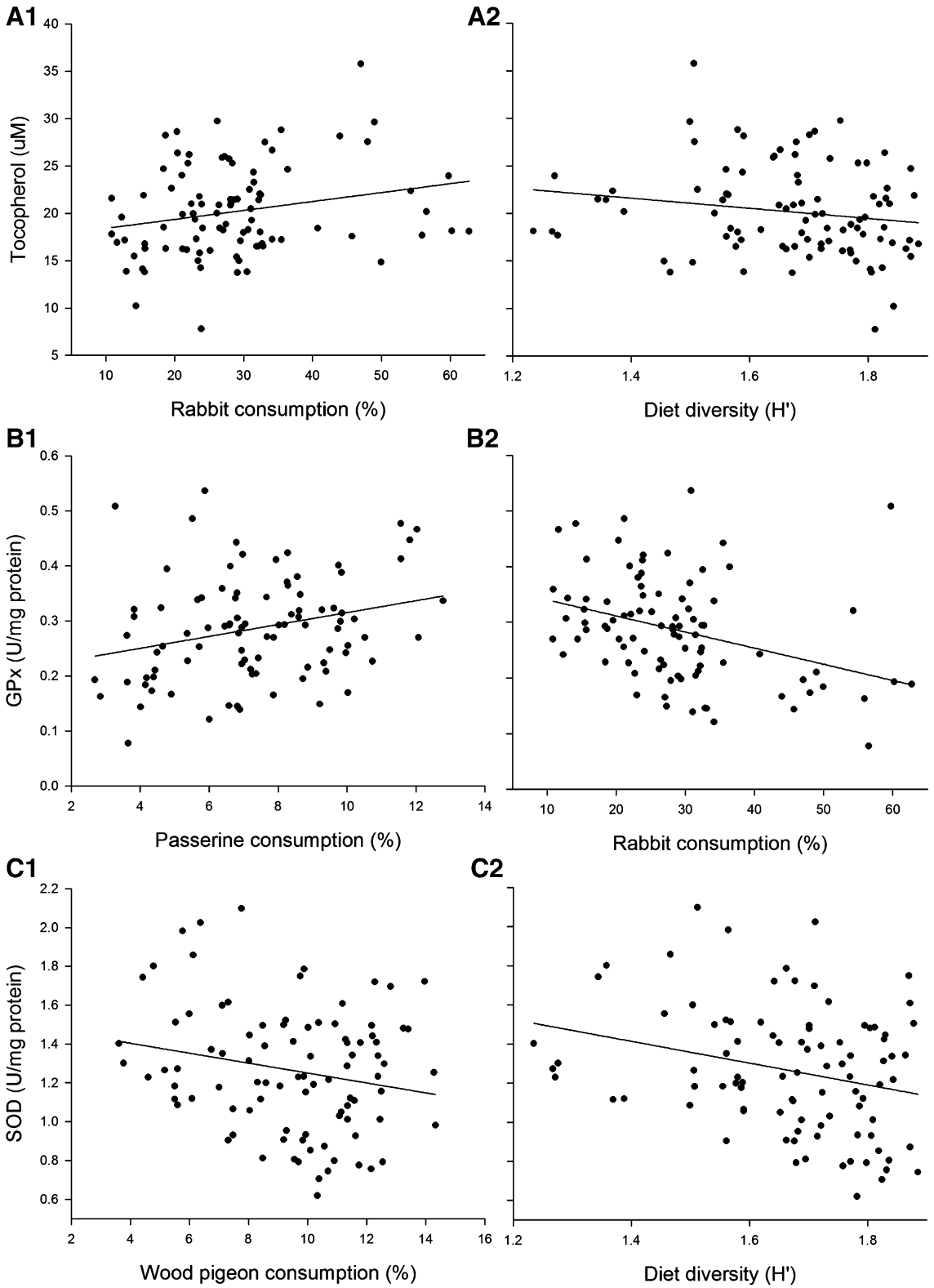


**Fig. 6** Effects of rabbit consumption (%), passerines consumption, wood pigeon consumption and diet diversity (*H'*) on plasma antioxidants and oxidative stress biomarkers: tocopherol (**a1**, 2), glutathione peroxidase (GPx) (**b1**, 2), superoxide dismutase (SOD) (**c1**, 2), zeaxanthin (**d**), total glutathione (tGSH) (**e**), oxidized glutathione (oxGSH) (**f1**) and the percentage of oxGSH (%oxGSH) (**f2**). Trend lines are shown

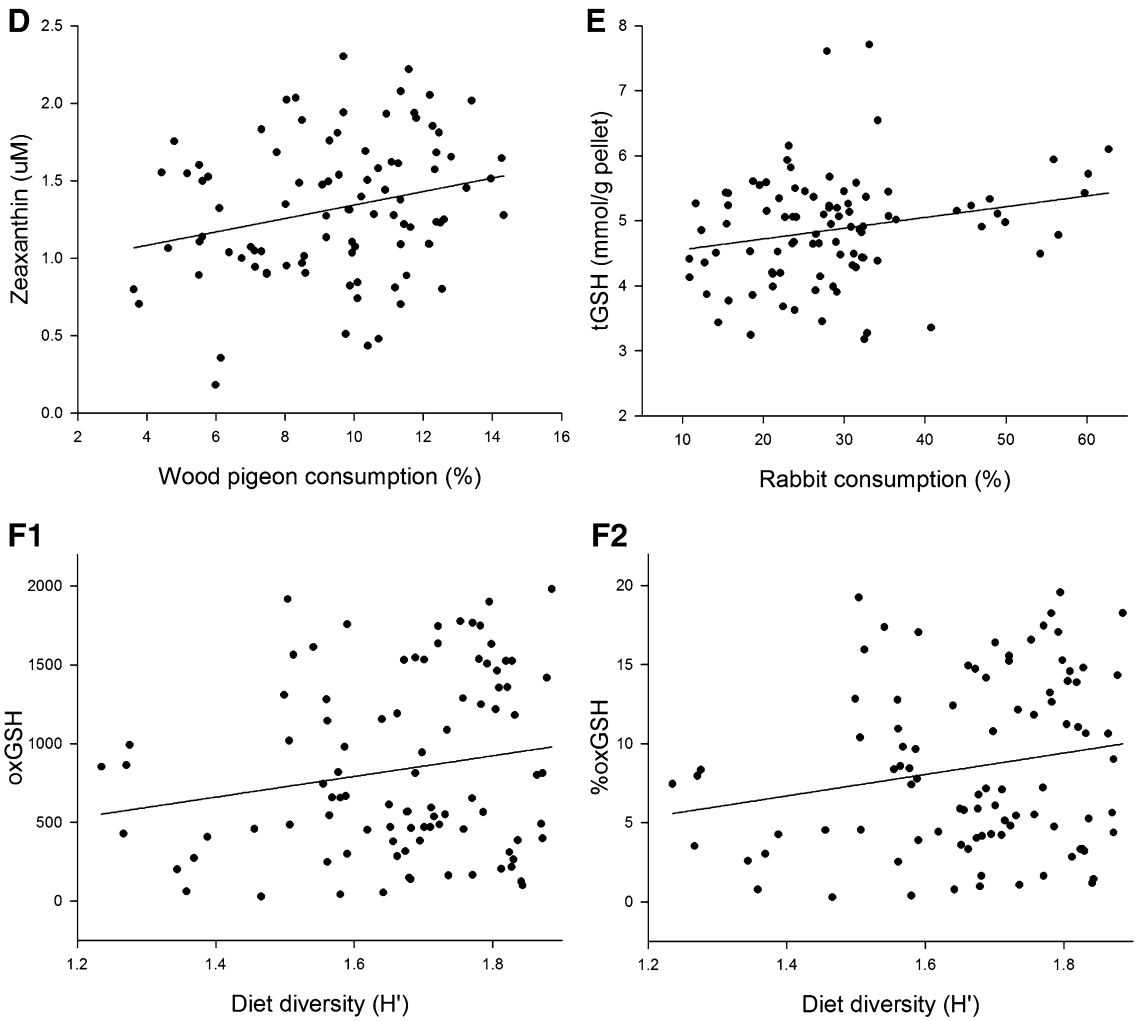


**Fig. 6** continued

The analyses of AO defenses and OS biomarkers in an organism can be informative of the physiological functioning of the body and individual redox status. Vitamins and carotenoids are important plasmatic AO obtained from diet (Meydani et al. 1995; Møller et al. 2000). In our study, the Bonelli's eagle nestlings that consumed more rabbits had higher tocopherol levels, which fell as diet diversity increased. Tocopherol co-occurs with chlorophyll in chloroplasts and is abundant in leafy wild vegetables in our study area (Morales et al. 2012); thus, the greater consumption of a herbivorous prey (rabbits) will lead to higher tocopherol levels. Moreover, greater wood pigeon consumption increased nestlings' zeaxanthin levels, which is expected in a granivorous prey item given the high content of this carotenoid in grain (Liu 2007). Given that both these AO substances prevent cell membranes from lipid peroxidation (Burton and Ingold 1981; Sujak et al. 1999), the greater consumption of these prey items could enhance nestling AO capacity. On the other hand, GSH is a key intracellular AO peptide that has vital functions in animals (Wu et al. 2004). Protein malnutrition and OS can markedly reduce GSH concentrations (Lu 2009). In our study, nestling tGSH increased with greater rabbit consumption, suggesting that this prey item enhanced nestlings' ability to scavenge ROS since it supplies higher protein levels (and GSH precursors) and also other AO such as tocopherol. This result supports our previous interpretation that an optimal diet enhances nutritional body condition, thereby suggesting that nutritional and antioxidant states are closely related. Nevertheless, an integrative approach for assessing animal OS should also measure the activity of AO enzymes such as GPx and SOD, which interact with the AO substances and regulate the overall ROS levels to maintain physiological homeostasis (Finkel and Holbrook 2000). Nestling GPx activity increased with greater passerine consumption and decreased with greater rabbit consumption. Given that GPx catalyzes the glutathione-dependent reduction of hydrogen peroxide ($H_2O_2$) and other peroxides (Wu et al. 2004),

increased enzyme activity could be related to greater abundance of ROS. Thus, greater passerine consumption may imply nestling food shortage and increasing ROS levels, and may ultimately activate GPx to avoid OS. On the other hand, greater rabbit consumption not only increased nestling tGSH (i.e. AO capacity to directly scavenge ROS) but also reduced the activity of GPx, suggesting an overall better redox balance (i.e. less ROS to be scavenged) associated with greater consumption of this preferred prey. Moreover, in this enzyme reaction GSH becomes oxidized (oxGSH), which, if measured relative to the tGSH (%oxGSH), provides an estimate of oxidative damage. Both the oxGSH and the %oxGSH increased with greater diet diversity, suggesting greater OS in nestlings with more diverse diets. In the case of the SOD, greater consumption of wood pigeons and greater diet diversity reduce this enzyme's activity. SOD speeds the conversion of superoxide anion ($O_2^{-\cdot}$) to $H_2O_2$, which subsequently serves as a substrate of GPx and catalase. Thus, contrary to expectations, we did not detect any increase in this enzyme's activity with lower rabbit consumption or greater diet diversity; probably because other routes aside from the activation of SOD synthesis were used to cope with the increase in ROS (e.g. AO defenses could play an important role in scavenging ROS before activating SOD synthesis). Finally, TBARS did not correlate with any dietary parameter, suggesting that nestling diet had no effect on the oxidative damage of the erythrocyte membrane, probably due to the existence of a compensatory mechanism aimed at avoiding lipid peroxidation and hence OS damage (see Dimova et al. 2008).

Hematological values, however, are influenced by many factors other than individual nutritional condition. For instance, age-related differences have been described for several blood parameters (Dobado-Berrios et al. 1998; Alonso-Alvarez 2005). In our study, the levels of plasmatic enzymes ALP and CK increased with nestling age. An increase in ALP activity with age has been reported in the nestlings of other raptor species (Hoffman et al. 1985; Viñuela et al. 1991) as this enzyme is related with osteogenesis (Dobado-Berrios and Ferrer 1997). For instance, Viñuela et al. (1991) found that ALP levels in nestlings of both red and black kites (*Milvus milvus* and *Milvus migrans*, respectively) increased until an age of approximately 34 and 38 days, respectively, and then decreased. We found, however, a continuous increase in ALP with age, which may indicate that Bonelli's eagle nestlings progressively increase their ossification until they fledge. Despite being also involved in osteogenesis, in our study inorganic phosphorus was uncorrelated with nestling age and levels of ALP. The positive relationship between CK and nestling age was explained by an increase in muscle formation while growing, along with greater physical activity. Given that CK mediates in muscle contraction and increases with exercise-related muscle damage (Hochleithner 1994), we expect the oldest nestlings to have the highest enzyme levels due to increased wing-flapping activity before fledging. Sex differences in blood parameters have been also reported (Hochleithner 1994). Here, we found that male nestlings had higher plasmatic levels of glucose than females; nevertheless, despite the fact that males tended to have greater ALP and CK activity than females, sex differences were not as evident as in the case of glucose. Glucose differences could be explained by different metabolic rates between sexes due to sexual size dimorphism. Females are larger and may allocate more glucose from plasma for tissue formation than males. This idea has been previously suggested by Balbontín and Ferrer (2002) and Casado et al. (2002), who, respectively, reported higher plasmatic glucose levels in both Bonelli's and Booted eagle male nestlings and suggested that this was because males have lower energy demands for tissue growth than females. Blood parameter concentrations can also change after food ingestion (Hochleithner 1994). In this regard, Bonelli's eagle nestlings had a postprandial increase in plasma triglycerides, uric acid and retinyl palmitate. Given that triglyceride and uric acid levels may increase either shortly after food consumption (Lumeij and Remple 1991; Hochleithner 1994) or due to severe tissue damage or starvation (García-Rodríguez et al. 1987a; Hochleithner 1994), taking into account the recent food intake helped us appreciate that the high levels of these parameters were indicative of recent food intake rather than the consequence of a pathology or disease. Creatinine was the only blood parameter that varied with sampling time, being higher in nestlings sampled after than before midday. This result contrasts with the absence of diurnal rhythms for this plasmatic parameter in either Common buzzards (*Buteo buteo*) or Eagle owls (*Bubo bubo*) (García-Rodríguez et al. 1987b). Overall, our results highlight the importance of considering factors such as individual age, sex, time of last food intake and sampling time when interpreting avian blood parameters in order to avoid reaching misleading conclusions regarding body condition.

## Conclusions

By controlling for the potential influence of other intrinsic and extrinsic factors, our study reveals the usefulness of a multi-biomarker approach in assessing the body condition of Bonelli's eagle nestlings in relation to diet. Different body condition measures supported our prediction that greater consumption of a preferred prey item (rabbit) improved nestling body condition, whereas an increase in diet diversity had the opposite effect. In this regard, the most informative parameters were plasmatic levels of

cholesterol, AST activity, tocopherol and zeaxanthin, as well as several oxidative stress biomarkers such as GPx, tGSH, oxGSH and %oxGSH. On the other hand, a single body condition measure such as the scaled mass index provided little information. Overall, these results highlight the importance of a multi-parameter approach when attempting to reach a deeper understanding of the relationships between diet and animal body condition, even though such an approach may preclude the possibility of a straightforward interpretation. The assessment of the relationships between nestling diet and body condition are crucial in endangered species such as the Bonelli's eagle because they can guide and focus management actions (e.g. improving local environmental characteristics as a means of enhancing the abundance of preferred prey items such as rabbits) in territories in which nestlings are in a poor health status. The abundance of key Bonelli's eagle prey such as rabbits is mainly related to habitat characteristics, disease outbreaks and hunting practices (Delibes-Mateos et al. 2007; Moleón et al. 2009). On the other hand, higher diet diversity probably reflects the inability of breeding individuals to find sufficient high quality prey (Resano-Mayor et al. 2016). The further application of multi-biomarker approaches to these and other populations within the distribution range of this species might help test optimal foraging theory assumptions (MacArthur and Pianka 1966; Pyke et al. 1977) and ascertain the contribution of nestlings' body condition to local (territories and populations) demographic fates. Finally, our study may serve as a practical starting point for future monitoring programs aiming to evaluate nestling health in Bonelli's eagles and other long-lived raptors, which could also be used as indicators of environmental changes (i.e. prey abundances and assemblages) in ecosystems highly influenced by human activities such as many of those in the Mediterranean Basin.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** All applicable international, national, and institutional guidelines for the care and use of animals were followed.

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