Physiological effects of toxic elements on a wild nightjar species

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Abstract

Nightjars are considered human-tolerant species due to the population densities reached in strongly managed landscapes. However, no studies have been done evaluating metal-related effects on physiology, condition or fitness in any nightjar species. The main aim of this study was to evaluate how metal exposure affects physiology and condition in red-necked nightjar (*Caprimulgus ruficollis*) populations inhabiting three different environments in southeastern Spain: agricultural-urban area (n = 15 individuals), mining area (n = 17) and control area (n = 16).

Increased plasma mineral levels (magnesium and calcium) and alkaline phosphatase (ALP) activity were observed in breeding females, and ALP was significantly higher in young birds due to bone growth and development. In the mining-impacted environment, nightjars showed decreased retinol (17.3 and 23.6 μM/ml in the mining area and control area), uric acid (28.8 and 48.6 mg/dl in the mining area and control area) and albumin (16.2 and 19.6 g/l in the mining area and control area), probably impaired by a combination of toxic metal exposure and low prey quantity/quality in that area. Moreover, they showed increased plasma tocopherol levels (53.4 and 38.6 μM/ml in the mining area and control area) which may be a response to cope with metal-induced oxidative stress and lipid peroxidation. Blood concentrations of toxic metals (As, Pb, Cd and Hg) were negatively associated with calcium, phosphorus, magnesium, ALP, total proteins and body condition index. This could lead to metal-related disorders in mineral metabolism and ALP activity that may potentially increase the risk of skeletal pathologies and consequent risk of fractures in the long term, compromising the survival of individuals. Further studies need to be carried out to evaluate potential metal-related effects on the antioxidant status and bone mineralization of nightjars inhabiting mining environments.

Keywords: metal exposure; toxic effects; ecophysiology; nightjars; *Caprimulgus ruficollis* **Capsule:** Metal-related effects on physiology and condition of nightjars

Introduction

The avian order Caprimulgiformes comprises a group of insectivorous birds, commonly known as nightjars, nighthawks and their allies, including more than 120 species distributed worldwide. However, they are poorly studied due to their nocturnal habits, cryptic plumage and elusive behavior (Braun and Huddleston, 2009). Even though they are supposed to show a marked tolerance to habitat alterations from human activities (Camacho et al., 2014), to the best of our knowledge, no studies have been conducted to evaluate metal-related effects on physiology, condition or fitness in any caprimulgiform species, with the only exception of a recent investigation describing blood metal concentrations in red-necked nightjar (*Caprimulgus ruficollis*) (Espín et al., 2020).

Toxic metals may induce a broad range of pathological effects in organisms. Plasma biochemistry is particularly relevant in bird species, as they usually show minimal clinical signs of disease (Harr, 2005). The analysis of different biochemical parameters in plasma provides an overview of the nutritional and health status, and can help identifying health problems in wild populations. For this reason, in the past years a few publications provided reference values for plasma biochemistry in different avian species (e.g. Agusti Montolio et al., 2018; Casado et al., 2002; Gómez-Ramírez et al., 2016; Han et al., 2016; Harr, 2002). However, we are unaware of any study having analyzed plasma biochemistry in nightjars or their allies.

Different authors have studied biochemical alterations related to metal exposure, such as elevation in liver function biomarkers (alanine aminotransferase and aspartate aminotransferase) and reduction of total proteins and albumin, evidencing a perturbation of liver synthetic function in different organisms (Alya et al., 2015; Javed et al., 2017; Martinez-Haro et al., 2011; Stout et al., 2010). Effects of metals and metalloids in plasma vitamin and carotenoid concentrations have also been evaluated (Geens et al., 2009; Martinez-Haro et al., 2011; Ortiz-Santaliestra et al., 2015; Ruiz et al., 2016). Carotenoids and vitamins are micronutrients obtained from the diet with different essential roles in growth and development. For example, tocopherol is the most common form of vitamin E, an antioxidant protecting cell membranes against lipid peroxidation by neutralizing lipid radicals (Traber and Atkinson, 2007); and carotenoids and retinol, the active antioxidant form of vitamin A, play important roles in cell differentiation and proliferation, immune response, and antioxidant protection (Britton, 1995; Chew and Park, 2004; Tanumihardjo, 2011; Zile, 2001). Retinol is known to have antioxidant

properties, and it is involved in the reduction of oxidized tocopherol into its useful form (Wang and Quinn, 1999), while the antioxidant capacity of some carotenoids in birds has been questioned (Costantini and Møller, 2008; Koch et al., 2018).

The main aim of this study is to evaluate how metal exposure affects physiology and condition in red-necked nightjars inhabiting three different environments in southeastern Spain: agricultural-urban area, mining area and control area. Differences in biochemical and condition parameters between sexes and age groups are also evaluated. Based on previous studies in different bird species, we expect to find altered biochemistry in the mining area characterized by a higher metal exposure (As, Cd, Pb and Mn) in these individuals (reported in Espín et al., 2020). Moreover, blood Pb concentration in the mining-impacted site was of special concern since it was in the range of blood concentrations related to subclinical/clinical effects in other species, and associated with notably decreased hematocrit values (up to 44% hematocrit depression at blood concentrations >1000 ng/ml w.w., Espín et al., 2020). Therefore, other physiological effects related to metal cocktail exposure in the mining area are expected.

Material and methods

Study area and species

The study area is situated in Murcia, southeastern Spain (37º 45´N, 0º 57´W) (Figure 1). The main characteristics of the area can be found in Espín et al. (2020). Briefly, three scenarios with different metal pollution were selected according to the main land use as follows: mining area, agricultural-urban area and control area. The mining area corresponds to a closed mine site (Cartagena-La Unión Mining District) with extraction activity since Phoenicians times until 1992 (Conesa et al., 2008). Toxic metals in this area are still spread impacting on surrounding ecosystems (Conesa and Schulin, 2010). Consequently, significant concentrations of toxic metals and metalloids have been and are still found in blood samples of wild birds in this area, mainly for Pb, As, Cd and Hg (Espín et al., 2014a, 2014b, 2020; García-Fernández et al., 1995). A geographical barrier (mainly mountain ranges) separates the mining area from the other study areas preventing aerial dispersion of metals from the mine site. The agricultural-urban area is occupied by large extensions of irrigation agriculture (mainly vegetable, alfalfa and cotton crops) next to one of the largest industrial parks in Spain (2.5 km far from the agricultural zone) and a densely populated city (2950 inh/km^2, 3.5 km far from the agricultural zone). Finally,

the control area (Escalona and Altaona mountains) has low human occupancy and is dominated mainly by small tree crops (citric, almond and olive trees) scattered in a shrubland and forest matrix (León-Ortega et al., 2017). In this background area, wild bird biomonitoring studies show that the exposure to metals is low (Espín et al., 2014a, 2014b).

The red-necked nightjar (henceforth "nightjar") is a long-distance migrant breeding in temperate regions of the Iberian Peninsula and northern Africa, whose numbers increase considerably from early spring (Aragonés, 2003; Camacho, 2013). Even though the exact range of its wintering grounds are not well-known today, available observational data suggest Western Sahara as the main wintering region (Cleere et al., 2013). Its diet is mainly composed by nocturnal aerial insects, with moths (Noctuidae and Thaumetopoeidae families) representing the main prey species in Spain (Sáez and Camacho, 2016). Moreover, other insect families (e.g. Gryllidae and Cicadidae) can occasionally be important part of the nightjar diet in population peak periods (authors' unpublished data). The suitability of nightjar as a model species to explore the study aims lies in its high tolerance to anthropogenic habitat disturbances (Camacho et al., 2014), which allows the occurrence of nightjar populations in different scenarios under contrasting contamination degrees. Moreover, this species shows very high fidelity to the breeding site, with almost all birds composing a population breeding annually in the same area (Camacho et al., 2016). Indeed, data from an intensive nest monitoring conducted in our study region showed that the same breeding pairs place the nests in the same small plot every year (interannual mean distance among nest: 129 ± 118 m, n=16) (author´s unpublished data). Furthermore, high natal philopatry has been described for young birds, with males (94%) and females (73%) being recruited into the breeding population in their first year of life (Camacho et al., 2014). Therefore, even though nightjar is a migrant species, individuals are probably exposed to a similar pollution degree every year in the breeding grounds due to their habitat fidelity.

Sampling and measurements

A total of 48 nightjars were captured in seven nocturnal sampling visits to the investigated areas during early summer (June-July) in 2017: 15, 17 and 16 birds were caught in agricultural-urban, mining and control area, respectively. To trap the birds, transects were conducted by driving a vehicle at constant speed (15-20 km/h) from dusk to dawn over gravel roads in the study areas. All nightjars were spotted while feeding on gravel roads,

except four nesting adults which were detected incubating or brooding chicks. When detected, nightjars were trapped by using a LED torch and handheld net with 10 mm mesh-size (Jackson, 2003). When captured, the body mass, wing and keel length (±0.1 mm), fat score and stomach volume were recorded for each bird. Fat accumulation and stomach content were measured following Camacho et al. (2014). In order to determine fat stores, we used a modified scale to assign visually values ranging from 0 (not visible fat) to 4 (full fat-covered belly). Stomach volume was recorded by means of an abdomen palpation (scored as full, ¾, ½, ¼ or empty), which provides an estimate of the amount of food contained (Table S1 shown as Supplementary Material). Moult details were used to determine age and sex of the captured nightjars (n=19 males and n=29 females; Table S1), according to previous studies (Alonso and Caballero, 2003; Forero et al., 1995; Gargallo, 1994; Tornero and Sanchís, 2017). Nightjars were grouped into three age classes: juveniles (young birds in their first summer – EURING age code III, n=5), breeding subadults (birds in their second summer – EURING age code V, n=8) or breeding adults (older birds – EURING age code VI, n=35) (Table S1). At the time of the capture, brood patch signs indicated that most birds were on nestling (n=21) or incubation period (n=12), while the other birds were young birds or adults which had probably finished the first clutch.

A veterinarian performed the clinical exploration of each individual in the field before blood sampling, and all the individuals were considered clinically healthy. Blood samples (ca. 1 mL) were collected from the brachial vein using 30G needles and 1 mL-syringes, and were stored in heparinized Eppendorf tubes under refrigerated conditions until processed in the laboratory. Hematocrit was recorded using a capillary tube reader after centrifugation of blood at 2200 g for 5 min. One Eppendorf tube with whole blood (ca. 200 µl) was frozen at −80°C for element analysis (see results in Espín et al., 2020) and other measurements. Another Eppendorf tube with blood (ca. 800 µl) was centrifugated at 10000 g for 5 min, and plasma and red blood cell were divided in different tubes frozen at −80°C for biochemical analysis. The handling process per nightjar ranged 10−15 minutes and all birds were released in the same location where they were caught.

Biochemical analyses

An overview of the plasma biomarkers analyzed and their major physiological function is shown in Table S2 (Supplementary Material).

An A25 BioSystems spectrophotometer autoanalyser (BioSystems S.A., Barcelona, Spain) was used to determine plasma biochemistry with commercial kits from BioSystems S.A. The plasma enzyme activities analyzed were alkaline phosphatase (ALP; Enzyme Commission (EC) no. 3.1.3.1), aspartate aminotransferase (AST; EC 2.6.1.1), creatine kinase (CK; EC 2.7.3.2) and lactate dehydrogenase (LDH; EC 1.1.1.27). The plasma constituents analyzed were albumin, total protein, cholesterol, glucose, magnesium (Mg), uric acid, calcium (Ca) and phosphorus (P). The spectrophotometer autoanalyser was calibrated using the Biochemistry Calibrator (BioSystems S.A.) and quality control was checked by analyzing the Biochemistry Control Serum (BioSystems S.A.), obtaining results within the range recommended in the instructions of the commercial control for all the biochemical parameters evaluated. Blanks were included in the beginning of each batch of samples. A preanalytical dilution of plasma samples with purified water was needed (1:1 ratio) to maximize sample volume (Johns et al., 2018). Four avian samples were diluted and both the original and the x2-diluted samples were analyzed to check the dilution-induced effect. Mean bias was within the acceptable limits from Westgard QC's website on CLIA Requirements for Analytical Quality for all the parameters evaluated (2-10% except for LDH with 12% and CK with 17% variation, being the acceptable values ranging 10-30% depending on the parameter) (Westgard Consulting, 2019). Differences between results obtained at different dilution factors may confound comparisons, but comparison of data obtained at a single dilution factor is highly recommended (Johns et al., 2018).

Retinol and α-tocopherol concentrations were measured in plasma samples by high-pressure liquid chromatography (HPLC) coupled to diode array (DAD) and fluorescence (FLD) detection according to Rodríguez-Estival et al. (2010). Plasma samples (100 µL) were added to an eppendorf tube to which 200 µL of water and 150 µL of ethanol were added. Then, 50 µL of retinyl acetate (58 mM) and α-tocopheryl acetate (1.04 mM) in ethanol were added as internal standards. The head-space of each tube was flushed with N_2 and immediately capped to avoid oxidation of vitamins during the extraction process. Then, samples were vortexed for 5 min and sonicated for 1 min, and then extracted twice with 1 mL of hexane using vortex mixing for 15 min each time. Hexane phases were recovered after centrifuging for 5 min at 14,000 rcf (4 ºC). These hexane extracts were combined and evaporated to dryness with N_2 flow. Residues were immediately redissolved in 200 µL of methanol and injected into the HPLC-DAD-FLD system (Agilent 1200 Series). Vitamins were separated using a Agilent ECLIPSE XDB-C18 4.6mm x 150mm 5um. Samples were eluted isocratically using 80% acetonitrile

(Hipersolv Chromanorm HPLC LC-MS grade, Prolabo), 19% methanol (Hipersolv Chromanorm, Gradient Grade, Prolabo) and 1% water. This starting proportion was maintained for 15 min; the acetonitrile was then increased to 100% in 15 min period, was held at this level for 1 min, and then returned to initial conditions over 2 min. The flow rate was 1 mL/min and the injection volume were 20 µL. Data were collected using (DAD) and FLD simultaneously. The DAD wavelength used for free retinol was 325 nm; in FLD, the excitation and emission wavelengths for α-tocopherol were 295 nm and 325 nm, respectively. Calibration curves were prepared with standards of free retinol and α-tocopherol (Sigma). The percentage recovery was 90% for both vitamins.

Statistical procedures

We first tested differences in the levels of biochemical parameters and morphological measures among the three areas and between sexes. Because body mass depends not only on the energy storages but also on its structural size and stomach volume at the time of measurement, we calculated the residual masses of birds from a linear model using wing length (a measure of structural size) and stomach volume as explanatory factors. These residual masses are used as a body condition index (BCI) in the following analyses. Most of the variables were normally distributed (by visual inspection of histogram and Kolmogorov-Smirnov test for normality) and five biochemical parameters (lutein, Ca, LDH, AST, ALP) were $\log_{10}$ transformed to make them better conform normal distribution. After that, we analyzed differences among sampling areas by linear models (LM) with area, sex and their interaction as explanatory factors. Model estimates (least squares means and 95% confidence limits) for $\log_{10}$-transformed values were back-transformed to the original scale for tables and figures. For the elements that showed significant differences among the three areas, we further ran Tukey's test as a *post hoc* analysis.

A second set of linear models were ran to test the associations of toxic metals with biochemical parameters and some of the morphological variables. Twelve elements (As, Ba, Cd, Co, Cr, Cu, Hg, Mn, Pb, Se, Sr, Zn) included in the ATSDR's list of toxic elements (ATSDR, 2017) were selected in these analyses. In few cases the element levels were below the limit of quantification (see Espín et al., 2020) and for those we substituted <LOQ values by a random number between 0 and LOQ. Copper followed a normal distribution while all the other elements were $\log_{10}$ transformed for the analyses. Because element levels were largely intercorrelated, a principal component analysis was performed to reduce the number of variables and to avoid collinearity problems in our

models. First three principal components (PC1 − PC3) explained 75% of the variation in the metal data (eigenvalues: PC1 5.7, PC2 1.8, PC3 1.4) and were used as explanatory factors in the following models. Overall value for Kaiser's measure was 0.75, indicating adequate sampling. PC1 got positive loadings from all the elements, and highest loadings from essential trace elements such as Cu and Zn, but also Ba. PC2 got highest positive loadings from non-essential toxic elements As, Cd, Hg and Pb. PC3 got highest positive loading from Cr and Se. In the LMs, we used sex, age, PC1 − PC3, wing length and BCI as explanatory factors for biochemical parameters, and sex, age and PC1 − PC3 for morphological variables. Finally, associations among biochemical parameters were inspected with a hierarchical cluster analysis by using Pearson correlation matrix with average linkage method for clustering. Because there were a few (1 for Ca and Mg, 9 for creatine kinase) missing values in the data, we used a Markov chain Monte Carlo (MCMC) method for imputing the missing values for the cluster analysis. Alpha level was set to 0.05 in all analyses. All the analyses were ran with a statistical software SAS 9.4 (SAS Institute Inc., 2013).

Results

Differences in the levels of plasma biochemical parameters and morphological measures among the three areas and between sexes are shown in Table 1. Retinol, tocopherol, uric acid and albumin levels differed among sampling areas. Nightjars in the mining area showed lower levels for these parameters than those in the control and/or urban areas, except for tocopherol, where nightjars in the mining area showed higher levels. In addition, nightjars in the control area showed shorter wings compared to the mining and agricultural-urban areas. Because in many bird species young individuals have shorter wings than old ones, we added age in this model to check if differences among areas could be explained by varying age distribution. Despite that the wing length slightly increased along the age (overall age effect: $F = 28.7_{2,40}$, $p < 0.0001$, $n = 48$) the control area still showed 2.4% shorter wing lengths as compared to the urban one (Tukey's test: $t = -3.08_{40}$, $p = 0.010$), but did not statistically differ from the birds of the mining area (Tukey's test: $t = -0.89_{40}$, $p = 0.65$). Few sex-related differences were found, females showing lower retinol and higher Mg, Ca and ALP levels than males. An interactive effect area × sex was found for uric acid and P, males showing lower levels than females in the control and mining area, respectively.

The PC2 component describing the levels of toxic metals (As, Cd, Hg and Pb) in blood was positively associated with tocopherol levels and negatively associated with Ca, P, ALP, Mg, total proteins and BCI (Table 2, Figure 2). In addition, PC1 component, mainly describing the levels of the essential elements Cu and Zn but also the toxic element Ba, was negatively associated with retinol and uric acid and positively associated with lutein, P, Mg, Ca and ALP (Table 2). The PC3 component describing concentrations of Cr and Se was positively associated with retinol, uric acid and albumin, while negative associations were found between PC3 and P, Ca, ALP and BCI (Table 2).

Age-specific differences were observed for blood plasma retinol, total proteins and BCI, young birds showing lower values than adults, and for ALP, where young birds showed higher activity (Table 2, Figure 3).

The biochemical parameters Ca, Mg, P, ALP and lutein were grouped in a cluster analysis, as well as cholesterol, total proteins and albumin, creatine kinase and glucose, or uric acid and retinol (Figure 4), indicating similar behavior (i.e. positive associations) among them.

Discussion

Nightjars inhabiting the mining-impacted area showed decreased retinol, uric acid and albumin and increased tocopherol levels in plasma compared to those in the agricultural-urban and control areas. The ancient mining area is a highly impacted and degraded ecosystem (Conesa and Schulin, 2010). As previously reported, nightjars in that area had higher As, Cd, Pb and Mn levels in blood than their conspecifics in agricultural-urban and control environments (Espín et al., 2020). In addition, metal-polluted environments generally have lower abundance and diversity of terrestrial insects (Eeva et al., 2005; Heliövaara and Väisänen, 1990), which may affect breeding success of insectivorous birds (Eeva et al., 1997), such as the nightjar. In this sense, decreased albumin generally reflects decreased protein synthesis due to poor diet, although it may show liver disease, or increased protein loss due to e.g. enteropathies or nephropathies (Doneley, 2016). In addition, exposure to Cd and Pb reduced albumin level in experimental studies, which could indicate liver dysfunction as this organ is the main site of plasma protein (mainly albumin) synthesis (Alya et al., 2015; Andjelkovic et al., 2019). Retinol is involved in growth, cell differentiation and immune function (Tanumihardjo, 2011; Zile, 2004), and carotenoids obtained through the diet are the most important precursors of vitamin A

(Koivula and Eeva, 2010). Moreover, previous studies have reported that metals and metalloids such as Pb and As may cause disturbances in retinol metabolism leading in decreased plasma levels in wild birds (Martinez-Haro et al., 2011; Ortiz-Santaliestra et al., 2015). This vitamin A-depletion has been associated with Pb-related alterations in bone composition and mineralization in wildlife species (Rodríguez-Estival et al., 2013). Uric acid is an abundant and strong antioxidant and the main form of nitrogen excretion in birds (Koivula and Eeva, 2010), and its plasma levels may be increased due to normal physiologic processes such a protein consumption or ovulation (Hochleithner, 1994; Kolmstetter and Ramsay, 2000). Therefore, these biochemical parameters in nightjars from the mining area may be impaired by a combination of toxic metal exposure and insufficient food intake or protein-rich items due to poor food quantity and quality in these environments (Birnie-Gauvin et al., 2017).

Vitamin E is a potent antioxidant that inhibits lipid peroxidation by scavenging lipid peroxyl radicals (Koivula and Eeva, 2010; Traber and Atkinson, 2007). Thus, the increased plasma tocopherol levels in birds facing elevated toxic metal concentrations in blood may be a response to cope with metal-induced oxidative stress and lipid peroxidation in individuals from the mining area, as observed in previous studies in wild birds (Martinez-Haro et al., 2011; Ruiz et al., 2016). Accordingly, PC2 describing blood concentrations of As, Pb, Cd and Hg was positively associated with tocopherol levels. In general, increased vitamin E and decreased vitamin A in the polluted environment could be expected on the basis of some earlier results in birds (Hargitai et al., 2016; Martinez-Haro et al., 2011; Ruiz et al., 2016).

Calcium, P and Mg are essential elements closely related since they are critical for several physiologic functions such as skeletal development, mineral metabolism, energy transfer, cell membrane function, and cell signaling, among others (Moe, 2008). Alkaline phosphatase is essential for the mineralization process, and it is a marker of bone turnover that may reflect pathologic and normal processes related to bone cell activity on the skeleton (Hochleithner, 1994; Tilgar et al., 2004), so it is closely related with Ca, P and Mg. Those elements and ALP in nightjars were grouped in a cluster analysis indicating their positive association. In this study, Ca, P and Mg were positively associated with PC1 component describing the essential elements Cu and Zn, while PC2 component describing blood concentrations of As, Pb, Cd and Hg was negatively associated with Ca, P, ALP, Mg, total proteins and BCI. Animals inhabiting polluted environments may show

poorer condition due to the toxic effects themselves or because of the diversion of energy towards detoxification (Frid and Caswell, 2017). These metals/metalloid are also known to decrease the expression of ALP and plasma mineral levels (Ca, Mg and P) by affecting mineral metabolism due to decrease intestinal absorption of those elements (resulting in hypocalcemia and hypophosphatemia), increase excretion, competition and substitution of cations (Andjelkovic et al., 2019; Brzóska and Moniuszko-Jakoniuk, 2005; Mateo et al., 2003; Rodríguez and Mandalunis, 2018; Yachiguchi et al., 2014). As a consequence, different studies have reported malformations, improper mineralization of bones and skeletal thinning, decreased extremity length or reduced growth in birds exposed to metals/metalloids (Albert et al., 2008; Álvarez-Lloret et al., 2014; Eeva and Lehikoinen, 1996; Gangoso et al., 2009; Larison et al., 2000; Sánchez-Virosta et al., 2018).

No difference was found in the current study for structural size (keel length, wing length) between the mining and control areas. However, nightjars in the agricultural-urban area showed slightly longer wings than in the control area, which may be explained by phenotypic plasticity in response to resource availability. Even though we did not record data on food quantity or quality, the availability of foraging sites (mainly roads; Camacho et al., 2014; Jackson, 2003) is considerably higher in the agricultural-urban area (highly patched) than in the control area (more natural). Therefore, a higher food supply may explain the larger size observed in the agricultural-urban area. This hypothesis has been already proposed to explain the larger body size showed by nightjars in managed areas against natural ones (Camacho et al., 2016). On the other hand, metal-related disorders in mineral metabolism and ALP activity in the mining area could lead to increased risk of bone pathologies such as osteoporosis or skeletal weakness in a long-term basis, and consequent risk of fractures compromising the survival of nightjars (Álvarez-Lloret et al., 2014; Gangoso et al., 2009). Further studies are needed to evaluate potential metal-related effects on bone mineralization of nightjars inhabiting mining-impacted environments.

Uric acid and retinol concentrations have been directly associated in different studies (Choi et al., 2012; Ford and Choi, 2013), which could be related to the shared role of xanthine oxidase, which is used to convert xanthine to uric acid, and retinol to retinoic acid (Choi et al., 2012; Mawson, 1984). In this study, those biochemical parameters in plasma were grouped in a cluster analysis showing their association, and they were negatively associated with PC1 component (describing general level of all metals with strongest loading for Cu, Zn and Ba) and positively associated with PC3 (describing Cr

and Se levels). On the other hand, Mg, P, Ca and ALP were positively associated with PC1 component, while P, Ca and ALP were negatively associated with PC3. Nutrient interrelationships are complex, since they are directly or indirectly connected and any movement in one of them will result in the movement of the others. Moreover, both an excess or a lack of essential elements due to nutrition deficiencies or environmental pollution can lead in detrimental effects compared to the positive effects of their physiological concentrations (Zofkova et al., 2017). Some of these nutrient interrelationships could explain part of the associations found in nightjars, such as the direct metabolic relationship between some elements functioning in concert in the organism (Cu-Ca, Zn-Mg, Zn-P and Se-retinol) or the indirect relationship between Cu-retinol at physiological levels (Watts, 1990). In addition, Zn and Mg are co-factors of ALP (Lowe and John, 2018), which likely explains their associations.

Regarding the main sex- and age-related differences in biochemistry, females showed higher Mg, Ca and ALP levels than males, while young birds showed higher ALP and lower total protein than breeding subadult and adults. Increased mineral levels and ALP activity have been observed in breeding females due to the egg formation (Hochleithner, 1994). Therefore, we suppose that gender differences in our data primarily reflect breeding-phase related physiological adjustments. In this sense, it would be interesting to compare sex differences between young and breeding birds, but our sample contained too few young birds for such comparison. ALP activity is significantly higher in juvenile birds due to bone growth and development, and advancing age is associated with higher total protein levels (Hochleithner, 1994).

Conclusions

This is the first study evaluating metal-related effects on physiology and body condition in any nightjar species. We did not find visible clinical signs of disease and morphological parameters, including body mass, were not affected by the mining-impacted environment. However, nightjars showed decreased plasma retinol, uric acid and albumin in the mining site, probably impaired by a combination of toxic metals exposure and potential poor food quantity and quality in that environment. Moreover, they reflected increased plasma tocopherol levels which may be a response to cope with metal-induced oxidative stress and lipid peroxidation. In addition, blood concentrations of toxic metals (As, Pb, Cd and Hg) were negatively associated with plasma Ca, P, Mg, ALP, total proteins and BCI. Those negative effects could lead to metal-related disorders in mineral metabolism and

ALP activity that may potentially increase the risk of bone pathologies in metal-impacted areas, compromising the survival.

One of the main mechanisms of metal toxicity is their ability to produce oxidative stress. Therefore, further studies need to be carried out to evaluate potential metal-related effects on the antioxidant status and bone mineralization of nightjars inhabiting mining environments.

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Tables

Table 1. Least squares means and 95% confidence limits (CL) of the biochemical plasma variables and condition parameters of the red-necked nightjar (*Caprimulgus ruficollis*) populations at three sampling environments (agricultural-urban, mining and control area) and two sexes. Linear models for comparison of means. Tukey's test among areas: means with the same letter are not statistically different. Significant effects marked in bold.

		Area (CL)			*Sex (CL)*		*F_{dfs} p*		
	n	*Agricultural-Urban*	*Mining*	*Control*	*Female*	*Male*	*Area*	*Sex*	*Area × Sex*
Retinol (μM/ml)	48	21.4 [ab] (17.5−25.2)	17.3 [a] (14.1−20.5)	23.6 [b] (20.3−26.9)	18.3 (15.8−20.8)	23.3 (20.1−26.4)	**3.88 2,42 0.029**	**6.24 1,42 0.017**	1.55 2,42 0.22
Tocopherol (μM/ml)	48	38.8 [a] (29.8−47.8)	53.4 [b] (45.9−60.9)	38.6 [a] (30.8−46.4)	44.7 (38.9−50.5)	42.5 (35.1−49.9)	**4.85 2,42 0.013**	0.22 1,42 0.64	0.71 2,42 0.50
Lutein (μM/ml) °	48	0.278 (0.143−0.542)	0.601 (0.345−1.05)	0.283 (0.159−0.503)	0.503 (0.328−0.77)	0.26 (0.15−0.45)	2.37 2,42 0.11	3.67 1,42 0.062	0.55 2,42 0.58
Uric acid (mg/dl)	48	51.8 [a] (43.9−59.7)	28.8 [b] (22.3−35.4)	48.6 [a] (41.8−55.4)	44.9 (39.9−49.9)	41.2 (34.8−47.7)	**13.2 2,42 <0.0001**	0.80 1,42 0.37	**5.02 2,42 0.011**
Phosphorus (mg/dl)	48	10.7 (7.89−13.5)	9.66 (7.33−12)	10.7 (8.28−13.1)	11.4 (9.60−13.2)	9.32 (7.01−11.6)	0.25 2,42 0.78	2.04 1,42 0.16	**5.21 2,42 0.0095**
Magnesium (mg/dl) °	47	9.8 (9.29-10.4)	10.3 (9.88-10.8)	9.96 (9.51-10.4)	10.4 (10.1-10.8)	9.64 (9.22-10.1)	1.25 2,41 0.30	**8.14 1,41 0.0067**	0.74 2,41 0.49
Calcium (mg/dl) °	48	11.2 (9.23−13.5)	12.9 (11−15.1)	12.5 (10.6−14.7)	14.1 (12.5−15.9)	10.5 (8.96−12.3)	0.68 2,42 0.51	**8.91 1,42 0.0047**	1.33 2,42 0.28
Lactate dehydrogenase (U/l) °	48	1570 (1160−2110)	1350 (1050−1730)	1180 (912−1530)	1180 (972−1430)	1560 (1220−2000)	1.02 2,42 0.37	3.30 1,42 0.077	0.50 2,42 0.61
Glucose (mg/dl)	48	502 (455−549)	483 (444−522)	522 (482−563)	500 (470−530)	505 (467−544)	0.98 2,33 0.38	0.05 1,33 0.82	0.87 2,33 0.43
Creatine kinase (U/l)	39	2970 (2280−3660)	2950 (2240−3660)	2850 (2220−3470)	3190 (2670−3700)	2660 (2080−3250)	0.05 2,42 0.96	1.86 1,42 0.18	1.55 2,42 0.22
Cholesterol (mg/dl)	48	320 (264−376)	293 (246−340)	311 (262−360)	310 (274−346)	306 (260−352)	0.30 2,42 0.75	0.02 1,42 0.90	0.04 2,42 0.96
Aspartate transaminase (U/l) °	47	408 (343−487)	393 (338−458)	407 (350−474)	426 (381−477)	381 (329−441)	0.07 2,41 0.93	1.50 1,41 0.23	1.19 2,41 0.31
Alkaline phosphatase (U/l) °	48	139 (65.9−295)	105 (56.2−196)	211 (110−403)	229 (142−369)	92.6 (50.0−171)	1.24 2,42 0.30	**5.47 1,42 0.024**	0.95 2,42 0.40
Albumin (g/l)	48	20.5 [a] (18.6−22.4)	16.2 [b] (14.6−17.8)	19.6 [a] (17.9−21.2)	18.5 (17.3−19.7)	19.0 (17.4−20.6)	**7.03 2,42 0.0023**	0.26 1,42 0.61	0.46 2,42 0.63
Total protein (g/l)	48	34.4 (30.6−38.1)	31.4 (28.2−34.5)	34.0 (30.8−37.3)	34.9 (32.5−37.3)	31.6 (28.5−34.7)	1.03 2,42 0.37	2.89 1,42 0.096	0.04 2,42 0.96
Body mass (g)	48	90.5 (85.9−95.1)	94.3 (90.5−98.2)	91.9 (87.9−95.8)	93.7 (90.7−96.6)	90.8 (87−94.5)	0.91 2,42 0.41	1.52 1,42 0.23	0.05 2,42 0.95
Wing length (mm)	48	208 [a] (205−212)	208 [a] (205−211)	203 [b] (200−206)	207 (205−210)	206 (203−209)	**3.36 2,42 0.044**	0.36 1,42 0.55	1.46 2,42 0.24
Keel length	44	33.7 (33.0−34.3)	34.0 (33.4−34.5)	33.0 (32.4−33.7)	33.3 (32.9−33.8)	33.8 (33.2−34.4)	2.44 2,42 0.10	1.63 1,42 0.21	1.62 2,42 0.21
Fat score	48	0.477 (−0.067−1.02)	0.715 (0.263−1.17)	0.754 (0.284−1.22)	0.744 (0.397−1.09)	0.554 (0.107−1.00)	0.34 2,42 0.71	0.46 1,42 0.50	2.03 2,42 0.14
Body condition index °°	48	−2.60 (−6.64−1.43)	2.07 (−1.28−5.43)	−0.258 (−3.74−3.22)	0.571 (−2.01−3.15)	−1.10 (−4.41−2.22)	1.04 2,42 0.36	1.23 1,42 0.27	0.02 2,42 0.98

° Variable $\log_{10}$ transformed for the analysis and back transformed for the table.

°° Body mass controlled for structural size (wing length) and stomach content.

Table 2. Linear models for explaining variation in the biochemical plasma variables and body condition index (BCI) of the red-necked nightjar (*Caprimulgus ruficollis*). Three principal components (PC1, PC2 and PC3) describe variation in concentration of toxic metals in blood (see methods). Terms in the reduced models are shown in bold.

		F_{dfs} *(estimate)*						
	n	*Sex*	*Age*	*PC1*	*PC2*	*PC3*	*Wing*	*BCI*
Retinol (µM/ml)	48	**$10.8_{1,42}$ ****	**$13.2_{2,42}$ *****	**$5.29_{1,42}$ * (−0.79)**	$0.27_{1,40}$ (0.42)	**$23.9_{1,42}$ *** (3.26)**	$1.31_{1,41}$ (−0.20)	$0.16_{1,39}$ (0.061)
Tocopherol (µM/ml)	48	**$3.48_{1,43}$**	**$0.92_{2,43}$**	$2.21_{1,42}$ (1.50)	**$7.0_{1,43}$ * (6.02)**	$2.15_{1,40}$ (−3.21)	$1.98_{1,39}$ (−0.77)	$0.95_{1,41}$ (−0.37)
Lutein (µM/ml) °	48	**$4.25_{1,42}$ ***	**$3.05_{2,42}$**	**$5.57_{1,42}$ * (0.069)**	$0.02_{1,40}$ (−0.011)	$1.63_{1,41}$ (−0.080)	$0.01_{1,39}$ (−0.0014)	**$6.21_{1,42}$ * (0.026)**
Uric acid (mg/dl)	48	**$3.11_{1,42}$**	**$0.95_{2,42}$**	**$7.66_{1,42}$ ** (−2.83)**	$3.07_{1,40}$ (−3.96)	**$6.47_{1,42}$ * (5.06)**	$3.46_{1,41}$ (0.94)	$0.01_{1,39}$ (0.041)
Phosphorus (mg/dl)	48	**$2.16_{1,41}$**	**$0.81_{2,41}$**	**$8.03_{1,41}$ ** (0.72)**	**$16.4_{1,41}$ *** (−2.39)**	**$8.09_{1,41}$ ** (−1.38)**	$1.85_{1,40}$ (0.18)	$0.56_{1,39}$ (0.084)
Magnesium (mg/dl) °	47	**0.01 $_{1,40}$**	**0.65 $_{2,40}$**	**7.71 $_{1,40}$ ** (0.0068)**	**8.71 $_{1,40}$ ** (−0.014)**	9.03 $_{1,40}$ (0.013)	0.64 $_{1,38}$ (0.00093)	2.30 $_{1,39}$ (−0.0014)
Calcium (mg/dl) °	48	**1.13 $_{1,41}$**	**2.22 $_{2,41}$**	**26.3 $_{1,41}$ *** (0.029)**	**38.5 $_{1,41}$ *** (−0.081)**	**21.5 $_{1,41}$ *** (−0.050)**	1.55 $_{1,39}$ (0.0038)	1.25 $_{1,40}$ (0.0027)
Lactate dehydrogenase (U/l) °	48	**2.53 $_{1,44}$**	**1.76 $_{2,44}$**	2.05 $_{1,43}$ (−0.020)	0.61 $_{1,40}$ (0.026)	0.95 $_{1,41}$ (−0.027)	0.21 $_{1,42}$ (0.0077)	0.05 $_{1,39}$ (0.0014)
Glucose (mg/dl)	48	**0.05 $_{1,44}$**	**2.68 $_{2,44}$**	2.02 $_{1,42}$ (−6.93)	0.68 $_{1,40}$ (9.55)	2.34 $_{1,43}$ (14.3)	1.45 $_{1,41}$ (−3.02)	0.01 $_{1,39}$ (0.26)
Creatine kinase (U/l)	39	**2.57 $_{1,35}$**	**1.36 $_{2,35}$**	1.62 $_{1,34}$ (105)	0.40 $_{1,33}$ (129)	0.31 $_{1,32}$ (144)	0.01 $_{1,30}$ (−4.58)	0.03 $_{1,31}$ (6.19)
Cholesterol (mg/dl)	48	**0.13 $_{1,44}$**	**0.11 $_{2,44}$**	1.09 $_{1,41}$ (6.55)	0.80 $_{1,43}$ (−12.9)	0.00 $_{1,39}$ (−0.79)	0.31 $_{1,40}$ (1.91)	0.99 $_{1,42}$ (−2.34)
Aspartate transaminase (U/l) °	47	**1.31 $_{1,42}$**	**0.27 $_{2,42}$**	0.04 $_{1,38}$ (0.0018)	0.29 $_{1,40}$ (−0.011)	0.09 $_{1,39}$ (0.0053)	1.24 $_{1,41}$ (0.049)	**4.89 $_{1,42}$ * (0.0063)**
Alkaline phosphatase (U/l) °	48	**0.56 $_{1,41}$**	**7.05 $_{2,41}$ ****	**5.61 $_{1,41}$ * (0.055)**	**19.9 $_{1,41}$ *** (−0.24)**	**6.10 $_{1,41}$ * (−0.11)**	0.00 $_{1,39}$ (0.00017)	0.07 $_{1,40}$ (0.0027)
Albumin (g/l)	48	**0.15 $_{1,43}$**	**1.11 $_{2,43}$**	1.22 $_{1,40}$ (−0.24)	2.47 $_{1,42}$ (−0.75)	**14.0 $_{1,43}$ *** (1.55)**	3.02 $_{1,41}$ (0.20)	0.08 $_{1,39}$ (−0.027)
Total protein (g/l)	48	**0.01 $_{1,43}$**	**3.93 $_{2,43}$ ***	1.03 $_{1,41}$ (0.39)	**9.65 $_{1,43}$ ** (−2.60)**	0.25 $_{1,40}$ (0.37)	1.67 $_{1,42}$ (0.26)	0.06 $_{1,39}$ (0.042)
BCI °°	48	**3.34 $_{1,42}$**	**5.81 $_{2,42}$ ****	1.38 $_{1,41}$ (0.45)	**5.81 $_{1,42}$ * (−1.98)**	**8.82 $_{1,42}$ ** (−2.08)**	N.A.	N.A.

° Variable was $\log_{10}$ transformed for the analysis.

°° Body mass controlled for structural size (wing length) and stomach content.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Figures

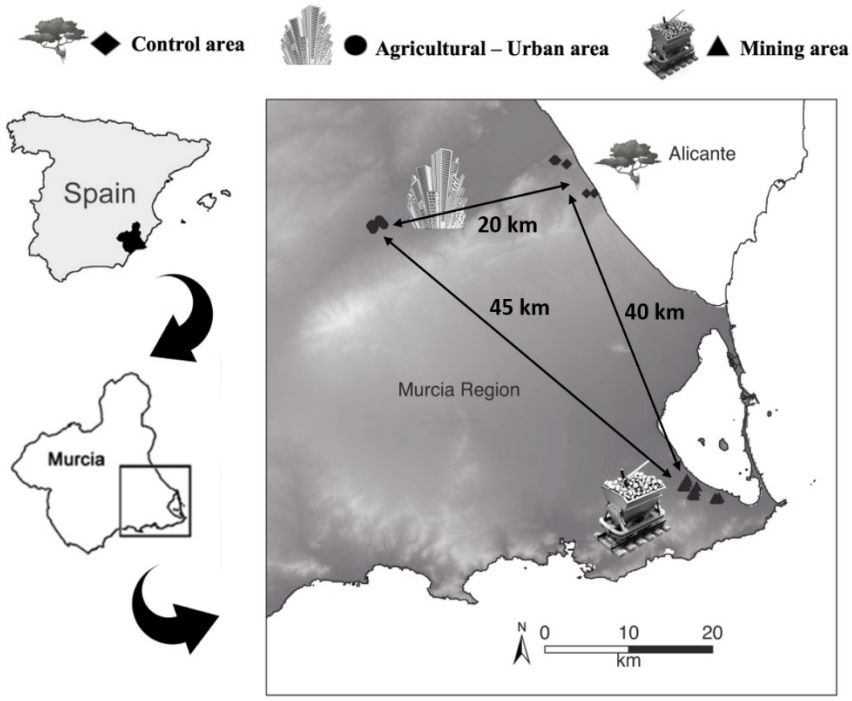

Figure 1. Geographical locations of the three studied areas. Symbols indicate the exact position where red-necked nightjars were captured.

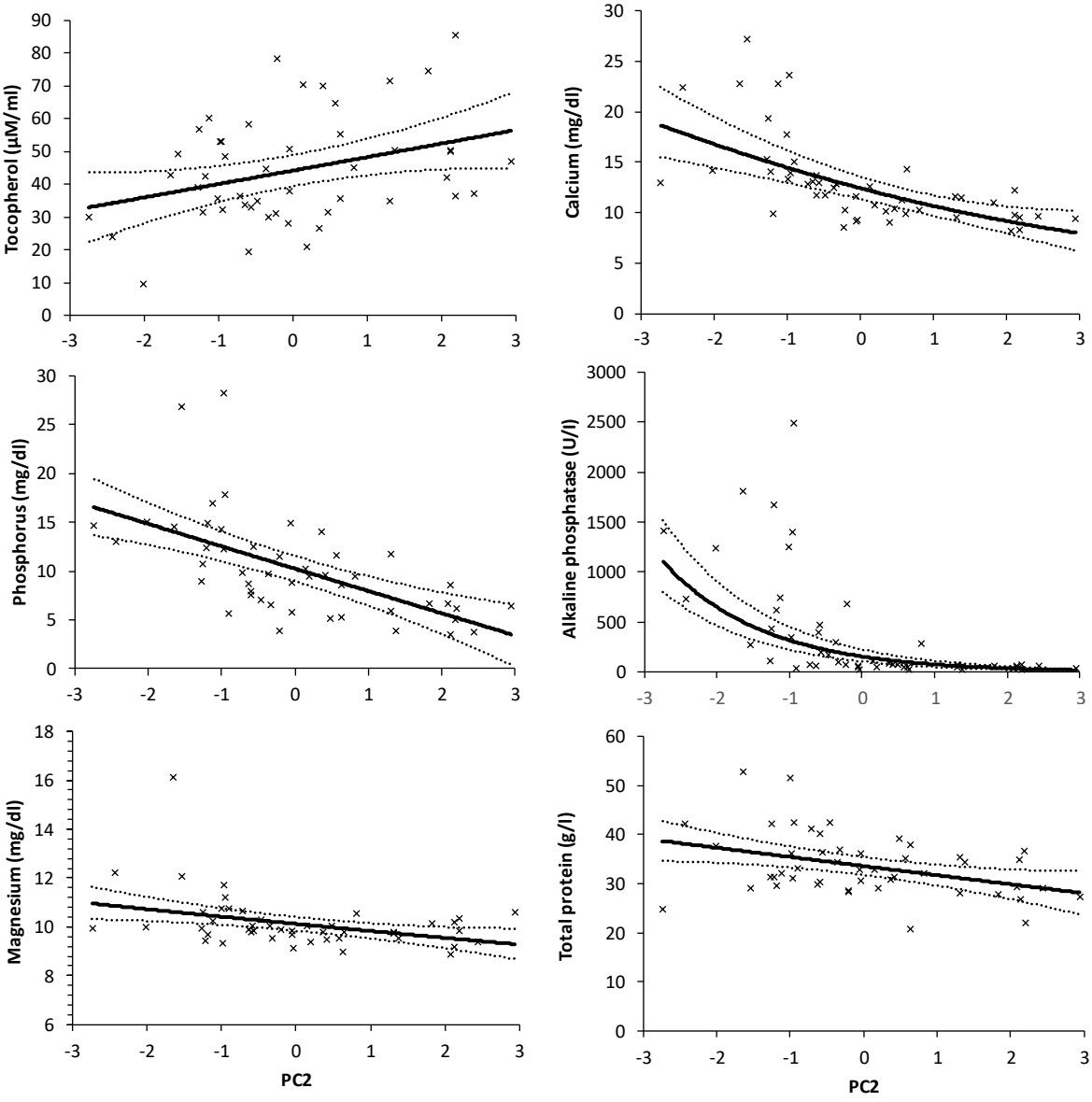

Figure 2. Biochemical markers in the red-necked nightjar (*Caprimulgus ruficollis*) plasma, relative to the second principal component describing the levels of toxic metals (As, Cd, Hg and Pb) in blood. Prediction line and 95% confidence limits come from the models of Table 2 (log-transformed values are transformed back to the original scale). Crosses denote the original data points.

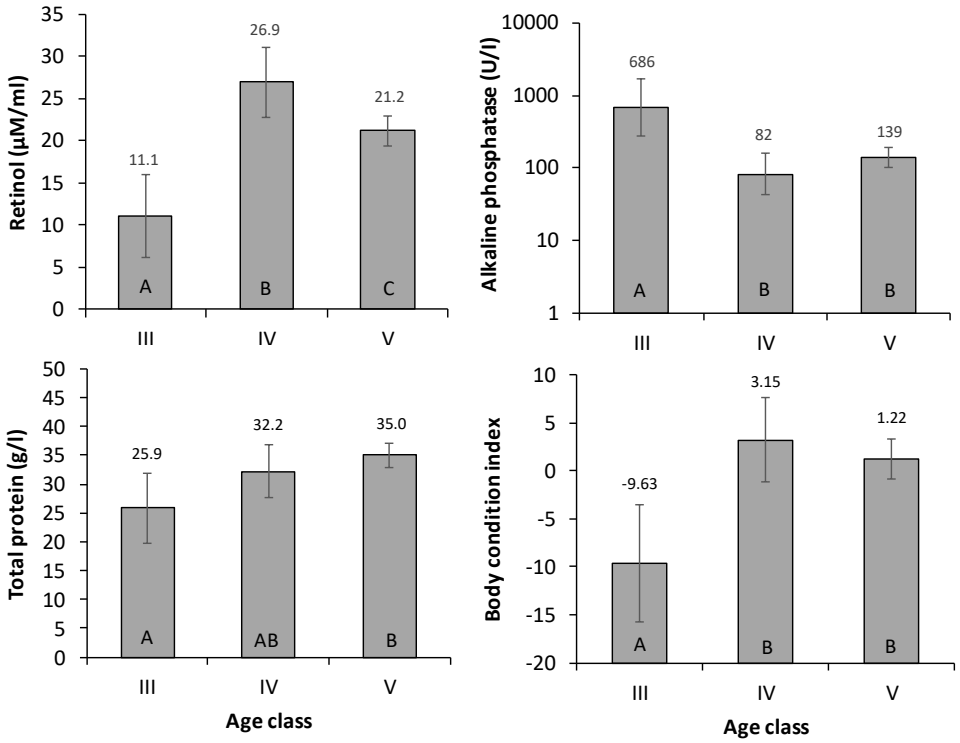

Figure 3. Means (±95% confidence limits) of four biomarkers showing age-specific differences in blood plasma of the red-necked nightjar (*Caprimulgus ruficollis*). N = 48 (for age classes: III = 5, V = 8, VI = 35). Tukey's test: means with the same letter are not statistically different.

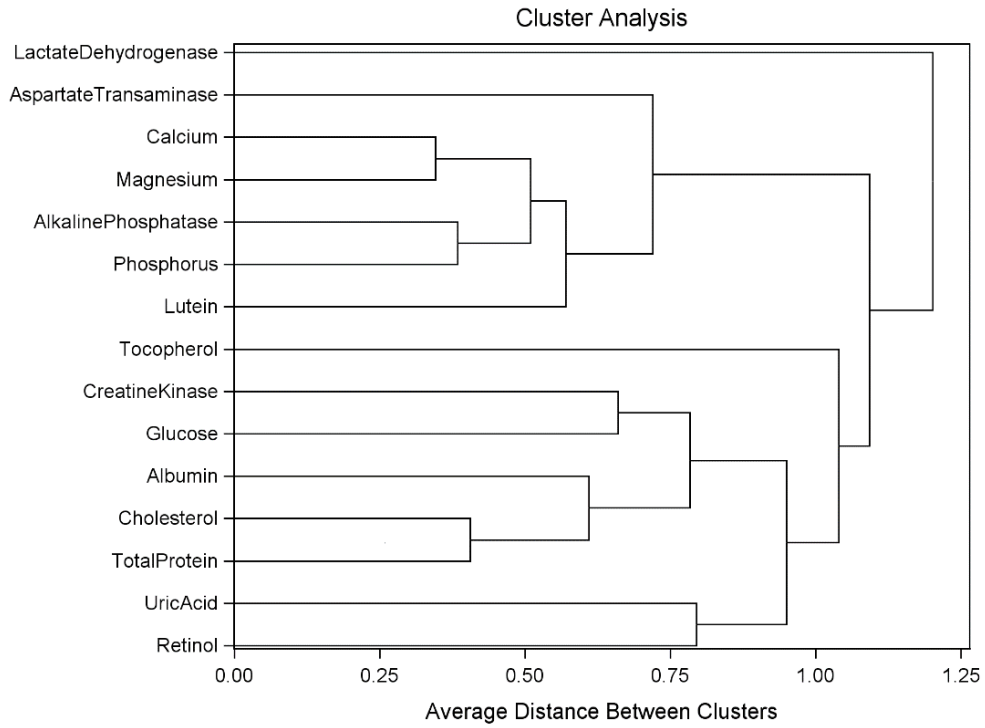

Figure 4. Hierarchical clustering of biochemical markers in blood plasma of the red-necked nightjar (*Caprimulgus ruficollis*) based on their Pearson correlation matrix. N = 48.