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ASSESSMENT OF CORTISOL AND DHEA CONCENTRATIONS IN GRIFFON VULTURE (*GYPS FULVUS*) FEATHERS TO EVALUATE ITS ALLOSTATIC LOAD

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Abstract

The use of a non-invasive approach to collect biological samples from natural populations represents a great means of gathering information while avoiding handling animals. Even if corticosterone is the main glucocorticoid investigated in birds, there has been observed a proportional direct link between corticosterone and cortisol concentrations. Dehydroepiandrosterone (DHEA) can be produced by the adrenal cortex and should have prominent antiglucocorticoid properties also in birds. The aim of this study was to verify if there is any difference in the cortisol and DHEA feather concentrations between clinically normal and physiologically compromised Griffon vultures (*Gyps fulvus*) through the non-invasive approach of collecting moulted feathers without having to pluck them from the bird. The study was carried out using 8 physiologically compromised (PC) Griffons and 9 clinically normal Griffons considered as the control (CTRL) group that were necropsied or from the wildlife rehabilitation centre. Primary and secondary covert feathers were either collected directly from the birds' cage floors, or, in the case of dead Griffons, they were plucked off the animals. The results, obtained by RIA, revealed that both cortisol ($P<0.01$) and DHEA ($P<0.05$) feather concentrations were higher in the PC than in the CTRL group. No difference was observed by comparing the cortisol/DHEA ratio between the two evaluated groups ($P=0.15$). Pearson's correlation coefficients showed no correlation between feather hormone concentrations in the PC group ($r=0.01$, $P=0.96$) while a positive correlation in the CTRL group ($r=0.65$, $P=0.006$) was observed. In conclusion, our study reveals that moulted feathers can be a non-invasive and an interesting tool to evaluate the allostatic load of wild birds and they allowed better understanding the relationship between hormones of the hypothalamic–pituitary–adrenal axis and the physiological status of the birds.

Key words: wild birds, raptors, HPA axis, steroids, coverts

In the course of their lives, birds continuously face several challenges, which may threaten the stability of their physiological functions in terms of emotional, metabolic and health conditions, and ultimately compromise their performance and survival (Romero and Fairhurst, 2016). These circumstances typically trigger a cascade of endocrine secretions involving the hypothalamic–pituitary–adrenal (HPA) axis, and result in the release of glucocorticoid hormones (GCs) in blood circulation (McEwen, 2007). The release of GCs helps the animal counteract stressors by adopting some energy-saving behaviours that enable it to cope with the situation (Wingfield, 2013; Wingfield and Sapolsky, 2003). While temporarily high concentrations of these hormones have the benefit of mobilizing energy stores and glucose to deal with stressors, they also represent the main component of vertebrate chronic stress increasing over prolonged or repetitive negative stimuli responses (Landys et al., 2006; Sapolsky et al., 2000). Chronic activation of the HPA axis has been implicated in a decline in growth rate, immune defence, body condition and survival functions (Kitaysky et al., 2003; Sapolsky et al., 2000).

Although in birds, as in rodent species, the key stress hormone is corticosterone, a proportional direct link exists between corticosterone and cortisol concentrations. Concentrations in feathers of house sparrows (*Passer domesticus*) of both GCs, corticosterone and cortisol, do not differ statistically, suggesting that both hormones are associated with survival outcomes, showing a similar response to the different physiological conditions of the birds (Koren et al., 2012). Furthermore, comparison of corticosterone and cortisol values in mule ducks (*Anas platyrhynchos* × *Cairina moschata*) indicate that cortisol can be considered a reliable acute stress indicator in routine examinations (Flament et al., 2012).

Dehydroepiandrosterone (DHEA) and its sulphate metabolite are endogenous steroids secreted by the adrenal cortex in response to adrenocorticotropic hormones (ACTH) (Kroboth et al., 1999). In several animal models as well as in humans, DHEA showed neuro-protective, anti-oxidative, anti-inflammatory and anti-glucocorticoid effects (Blauer et al., 1991; Maninger et al., 2009; Wright et al., 1992), playing a significant role in protecting against the negative consequences of stress (Hu et al., 2000). At present, DHEA effects are well documented in mammal species, but far less is known in birds (Newman et al., 2013). High circulating concentrations of DHEA have been recorded in non-breeding song sparrows (Newman et al., 2008; Newman and Soma, 2009) while plasma DHEA concentrations found in the gregarious species of brent geese (*Branta bernicla bernicla*) were negatively correlated with the dominance score (Poisbleau et al., 2009). Since in mammals DHEA has anabolic and anti-glucocorticoid effects and, thus, protects against the catabolic effects of cortisol (Kalimi et al., 1994; Labrie et al., 2006), a common procedure used to simultaneously test the impact of both hormones is the ratio between cortisol and DHEA (Goodyer et al., 1998; Hechter et al., 1997; Qiao et al., 2017). It has been observed in different studies (Gallagher et al., 2006; Wolkowitz et al., 2001) that under acute stress stimulus concentrations of DHEA increase as those of cortisol rise. Under conditions of chronic stress DHEA remains unchanged or progressively diminishes while cortisol increases or does not change. This condition results in an elevated cortisol/DHEA ratio (Wolkowitz et al., 2001) that, in a scenario of allostatic

overload, when demands exceed the resources available to meet the needs (McEwen and Wingfield, 2003), could be expected to be found.

The use of a non-invasive approach to collect samples of biological material from natural populations represents a great means in advancing biological knowledge while avoiding handling animals. Analysis performed on tissues that have accumulated hormones over a relatively long period, like feathers do, offers an alternative to blood samples, providing a retrospective window on plasma hormone exposure (Bortolotti et al., 2008; Romero and Fairhurst, 2016). The measurement of hormones in feathers is a particularly powerful approach in conservation biology because also moulted feathers can be analysed and provide information about individuals or colonies. Moreover, it is relatively easy to carry out because collected feathers require no special conditions for storage and transport. Steroid hormones stored in feather tissues show systemic hormone concentrations over a longer time period. This characteristic of mirroring physiological conditions over the duration of feather growth offers the opportunity to evaluate feather hormone concentrations in a variety of contexts, from sexual selection to environmental enrichment (Fairhurst et al., 2011; Kennedy et al., 2013; Kouwenberg et al., 2013; Harms et al., 2015). Concentrations of GCs in feathers have been observed to vary in response to life history events including health status (Harriman et al., 2014; Meitern et al., 2013; Mougeot et al., 2010; Sild et al., 2014). While DHEA effects are well described in mammal species, far less is known on birds (Newman et al., 2008). Circulating concentrations of DHEA have been evaluated in regard to bird reproductive behaviours (Newman and Soma, 2009; Newman et al., 2013; Poisbleau et al., 2009) while feather DHEA has been used in a study of ecotoxicology (Monclús et al., 2018). Since hormones in feathers are supplied from the capillary around the follicle and reflect the blood hormonal concentration of the period when the feathers grew (Bortolotti et al., 2008), also chronic stress can be assessed using feather hormone concentrations (Jenni-Eiermann et al., 2015).

The aim of this study was to verify if there is any difference in the cortisol and DHEA feather concentrations between the clinically normal and the physiologically compromised Griffon vultures through the non-invasive approach of collecting moulted feathers.

Material and methods

Animal selection

All procedures performed on the animals were in strict accordance with the guidelines of the Ethics Committee of the University of Sassari (Italy). For this study we chose the Griffon vulture (*Gyps fulvus*, Hablizl, 1783), a gregarious cliff-nesting raptor also known as Eurasian griffon, which became extinct between the 19th and 20th centuries throughout Italy with the exception of the island of Sardinia, where it is now considered Critically Endangered by IUCN (2012). In north-western Sardinia, near the town of Bosa, a small population is still present, with 40–42 nesting pairs divided into 6 colonies (2017, personal monitoring data).

This study comprised 17 adult Griffon vultures (8 females and 9 males): four of them necropsied at the Department of Veterinary Medicine of the University of Sassari and 13 of them from the Wildlife Rehabilitation Centre (Bonassai, Agenzia Fo.Re. S.T.A.S., Italy). The latter were individually caged and provided with food and water *ad libitum*. The diet consisted of sheep died for accidental causes at Sardinian farms and given as whole carcasses to griffons. They had been at the Centre for at least 6 month, showed trust towards humans and were accustomed to routine activities. Plumage was used to age individuals (Zuberogoitia et al., 2013) and the sex was determined as described by Griffiths et al. (1998).

The Griffon vultures included in the study were divided in two groups according to their known health condition: one group comprised Griffons that were physiologically compromised (PC group; $N = 8$; 4 females and 4 males) while the other group consisted of clinically normal Griffons (CTRL group; $N = 9$; 4 females and 5 males). The decision of introducing an animal in either the PC or in the CTRL group was taken by qualified personnel on the basis of the specimen's clinical history, clinical examinations and, in case of clinical signs, biomedical laboratory investigations for the animals kept in captivity or the necroscopic examination for the deceased animals. The physiologically compromised animals were animals showing pathologies, physical as well as behavioural, that could be also related with chronic exposure to distress (wing lesions, virus infections and feeding disorders).

Feather sampling

Intending a future application of feather analyses on field studies the feathers used in this study were those that are more commonly found on the ground (personal observations).

A total of 34 primary and secondary covert feathers were collected from the two groups of animals during the summer period. At the beginning of the study all feathers were removed from the cage floors so that the covert feathers could be subsequently collected directly from the floor of the cages over a one-month period, while in case of dead Griffons, the same type of feathers were directly plucked from the animals. Preliminarily, following the Houston's suggestions (1975), feathers have been collected from three dead adult Griffon vultures for the length evaluation and classification of the primary coverts and secondary coverts. Thus, on the basis of the measurement a covert feather can be either primary or secondary unless directly plucked from the wing. The samples were kept in paper envelopes and stored at room temperature until the analysis was performed.

Feather cortisol assay

The calamus was removed and all the feather vanes were minced into pieces of <5 mm with scissors. The obtained samples were washed twice in 3 ml isopropanol for one minute and approximately 35 mg of minced feather vanes were extracted in a glass vial with 4 ml of methanol. The vials were incubated at 37°C for 16 h. Next, the liquid in the vial was evaporated to dryness at 37°C under an airstream suction hood. The remaining residue was dissolved in 0.8 ml of phosphate-buffered saline (PBS), 0.05 M, pH 7.5 (RIA buffer). The feather cortisol was measured using

a solid-phase microtitre RIA procedure. In brief, a 96-well microtitre plate (Opti-Plate, Perkin-Elmer Life Science, Boston, MA, USA) was coated with goat anti-rabbit γ-globulin serum, diluted 1:1,000 in 0.15 mM sodium acetate buffer, pH 9, and incubated overnight at 4°C. The plate was washed twice with RIA buffer, pH 7.4, and incubated overnight at 4°C with 200 µL of the anti-cortisol serum diluted 1:12,000. The rabbit anti-cortisol antibody used was obtained from Biogenesis (Poole, UK). The cross-reactivities of this antibody with other steroids are as follows: cortisol 100%, corticosterone 1.8% and aldosterone <0.02%. After washing the plate with RIA buffer, standards (5–300 pg per well), a quality control extract, the test extracts and tracer (Hydrocortisone [Cortisol, (1,2,6,7-3H [N])-], Perkin-Elmer Life Sciences, Boston, MA, USA) were added, and the plate was incubated overnight at 4°C. Bound hormone was separated from free hormone by decanting the extract and washing the wells in RIA buffer. After the addition of 200 µL scintillation cocktail, the plate was counted on a beta-counter (Top-Count, Perkin-Elmer Life Sciences, Boston, MA, USA). The intra- and inter-assay coefficients of variation were 3.7 and 9.4%, respectively. The assay sensitivity (defined as the hormone concentration resulting in a displacement of the labelled hormone at least 2 standard deviations from maximal binding) was 0.98 pg per well.

To evaluate assay accuracy, any possible interference of the components within the extract with antibody binding was analysed through the recovery of exogenous cortisol added to pooled feather extracts. Each of four reconstituted feather extracts were divided into three independent aliquots and spiked with three different known cortisol concentrations, mixed, and assayed. The percentage of recovery was determined as follows: amount observed/amount expected × 100, where the amount observed is the value obtained from the spiked sample and the amount expected is the calculated amount of standard hormone added plus the amount of endogenous hormone in the unspiked sample. Recovery rate was 100.7±7.3% (mean ± SD). The measured hormone concentrations in the spiked samples correlated with the expected concentrations: r was 0.99 and the model was given by the equation y=1.125x–1.744.

To determine the parallelism between cortisol standards and endogenous cortisol in feathers, samples containing high concentrations of endogenous cortisol (100 µL) were serially diluted in 0.05 M PBS, pH 7.5, to obtain volumes of 50, 25, and 10 µL. The relationship between feather cortisol concentrations and the standard cortisol curve determined through linear regression was linear: the correlation coefficient (r) was 0.99 and the model was given by the equation y=0.976x+2.253.

Feather dehydroepiandrosterone (DHEA) assay

The extracts obtained from feathers as described above were also used to measure dehydroepiandrosterone (DHEA) concentrations using the solid-phase microtiter RIA previously mentioned for feather cortisol analyses. The 96-well microtiter plate coated with goat anti-rabbit γ-globulin serum diluted 1:1,000 in 0.15 mM sodium acetate buffer, pH 9, and incubated overnight at 4°C, after the double washing with RIA buffer (pH 7.4) were incubated overnight at 4°C with 200 µL of the antibody serum diluted 1:2,000 for DHEA. The rabbit anti-DHEA antibody used was obtained from Sigma-Aldrich (Darmstadt, DE). The cross-reactivities of the anti-DHEA antibody

with other steroids were as follows: DHEA, 100%; pregnenolone, 0.1%; androstenediol, 0.08%; dihydrotestosterone, 0.05%; sulphate DHEA, 0.02%; testosterone, <0.01%; 5α-androstane-diol-3β,3 α, <0.01%; 5β-androstane-3α, <0.01%; estradiol, <0.01%; progesterone, <0.01%; estrone, <0.01%; cholesterol, <0.01%. The plate was treated and the hormone analyses were carried out as detailed above for feather cortisol analyses but the tracer was DHEA [1,2,6,7-^{3}H (N)]. The plate was then counted on the β-counter.

The intra- and inter-assay coefficients of variation were 4.3 and 10.1% for DHEA. The sensitivity of the assay was 0.41 pg/well for DHEA.

The recovery rate for DHEA was 89.8 ± 15.9% (mean ± SD). The measured hormone concentrations in the spiked samples correlated with the expected concentrations of DHEA: *r* was 0.99 and the model was given by the equation y=0.838x+1.812.

The relationship between feather DHEA and its respective standard curve, determined through linear regression, was linear with the correlation coefficient of *r*=0.99. The model was described by the equation y=1.017x–0.200.

Statistical analyses

The data were log 10 transformed to achieve normal distributions and the statistical analyses were performed using Minitab® 17 Statistical Software (2010) with ANOVA. The significance was considered at P<0.05 with a 95% interval confidence. Pearson's correlation coefficients (r) were calculated to evaluate the strength and the direction of a linear relationship between the cortisol and DHEA concentrations recorded in the two groups (Hinkle et al., 2003).

Results

The statistical analyses revealed no difference between the sexes within the two groups of Griffons considered.

Table 1 shows the mean and standard deviation and the median of the first and third interquartile ranges (IQR) for cortisol, DHEA and the cortisol/DHEA ratio in both the CTRL and the PC groups of Griffon vultures.

Table 1. Feather hormone concentrations (means and standard deviations (SD), medians along with the first and third interquartile ranges (IQR)) for cortisol, DHEA and the cortisol/DHEA ratio in the CTRL (N = 9) and the PC (N = 8) groups of Griffon vultures. Before analysis values were logarithmically transformed

	Cortisol (pg/mg)				DHEA (pg/mg)				Cortisol/DHEA			
	mean	SD	median	IQR	mean	SD	median	IQR	mean	SD	median	IQR
CTRL	0.63	0.18	0.66	0.55–0.73	0.84	0.34	0.86	0.64–1.12	–0.21	0.24	–0.19	(–0.40)–(–0.02)
PC	0.89	0.18	0.90	0.79–0.99	0.96	0.30	1.00	0.90–1.08	–0.07	0.26	–0.08	(–0.26)–(0.05)

The results of this study showed higher feather cortisol concentrations in the PC than in the CTRL group (P<0.01) and the same was observed for the DHEA concentrations (P<0.05). The cortisol/DHEA ratio between the two evaluated groups showed no statistically significant differences (P=0.15). The box plots of cortisol, DHEA, and cortisol/DHEA ratio are shown in Figure 1.

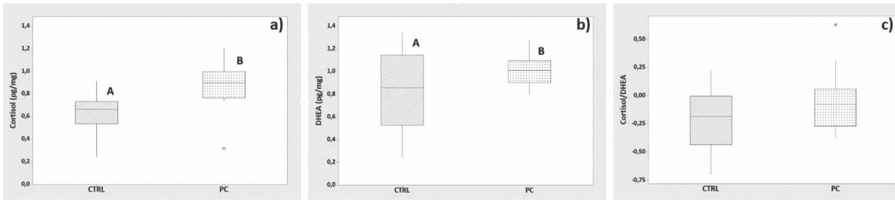

Figure 1. Feathers concentrations in the CTRL (N = 9) and the PC (N = 8) groups of Griffon vultures for a) cortisol, b) DHEA and c) cortisol/DHEA ratio. The box represents the interquartile range, in which the horizontal line represents the median, the lower and upper vertical lines are the upper and lower 25% of the distribution respectively, the asterisk symbol identifies outliers. Different uppercase letters show strong statistical differences (P<0.001). Before analysis values were logarithmically transformed

The Pearson's correlation coefficients were used to examine the associations between cortisol and DHEA: the results showed no correlation between cortisol and DHEA feather concentrations in the PC group (r=0.01, P=0.96) and a positive correlation in the CTRL group (r=0.65, P=0.006) (Figure 2).

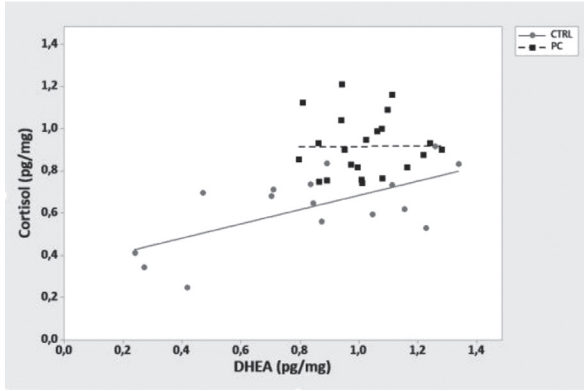

Figure 2. Correlation of cortisol concentrations with DHEA concentrations in feathers. In the CTRL group the Pearson's correlation coefficient showed a positive correlation (r=0.65; P=0.006) (y=1.3247x+0.0465); in the PC group no correlation between the cortisol and DHEA feather concentrations was found (r=0.01; P=0.96). Before analysis values were logarithmically transformed

Discussion

Although stress response is a fundamentally adaptive mechanism, there are maladaptive consequences associated with prolonged or frequent stress stimuli (Sapolsky et al., 2000).

Feathers give, in terms of hormones evaluation, a large time window, ranging from days to months depending on the growth rate of the type of feather. Thus it would appear that the analysis of feather hormone concentrations is the only method available to obtain a long-term and retrospective measure of the HPA axis activity during moulting. Given that the overstimulation of the HPA axis activity can develop in a physiologically compromised status of the animal as well some physiologically compromised statuses can overstimulate the HPA axis activity, we evaluated the allostatic load by assessing the feather hormone concentrations in two groups of Griffon vultures, also taking into account their known health conditions.

In this paper, we use a non-invasive and helpful tool to study the endocrine activity of a threatened species such as the Griffon vulture. The results of this current study show that both cortisol and DHEA concentrations can be measured in feathers of this wild bird species. The sample processing, the hormone extraction method and the analytical technique described in this paper allowed the detection of cortisol and DHEA concentrations in all the samples of Griffon vulture feathers collected. In addition, the detection of this hormone was biochemically validated, demonstrating the suitability of our RIA assay in the quantification of cortisol and DHEA in samples processed through the above described methodology. This step is of primary importance as new techniques to measure steroid hormones are being developed.

There is an increasing body of research that, in analysing another non-invasive biological samples such as hair, shows significantly higher cortisol concentrations associated with clinical and subclinical diseases when compared with those recorded in clinically healthy individuals. The above mentioned studies have been carried out both in human patients and in different animal species (Comin et al., 2013; Galuppi et al., 2013; Novak et al., 2014; Van Uum et al., 2008). Consistent with this, our study also showed significantly higher feather cortisol concentrations for the PC group compared to the CTRL group. This suggests that the feather had recorded the higher HPA axis activity of the animals belonging to the PC group that had been included in this group earlier on the basis of the evaluation made by qualified personnel assessing the specimen's clinical status or the necroscopic outcome. The physiologically compromised griffons showed pathologies, both physical and behavioural, that could be related to chronic exposure to distress and, thus, to a higher HPA axis activity, with a consequent marked release of GCs in order to cope with the situation. The organism releases GCs indicating the mobilization of resources to support adaptation, but this catabolic way is exerted at the expense of anabolism-based activity potential such as the defence against disease (Huber, 2018; Rauw, 2012). Different authors had already observed that another GC, corticosterone, shows higher feather concentrations in relation to the animal clinically unhealthy status (Bortolotti et al., 2008; Harriman et al., 2014; Sild et al., 2014). Further, blood corticosterone levels allowed predicting a host superspreader phenotype in the West Nile virus system (Gervasi et al., 2017).

Interestingly, the highest feather cortisol concentrations were found in the two animals that had suffered a long starvation period as a result of serious physical problems, which had negatively affected the quality of their lives for over 2 months (personal observations). High cortisol concentrations were observed in the case of

the vulture with a wing bone lesion (Figure 3) that partially compromised its flight capacities. Animals recovering from virus infections showed higher feather cortisol concentrations than the mean value recorded for the CTRL group. In the CTRL group, the highest value of feather cortisol concentration was found in a specimen that had suddenly died after the hormonal evaluation, although it had previously appeared in good physical conditions to the qualified personnel.

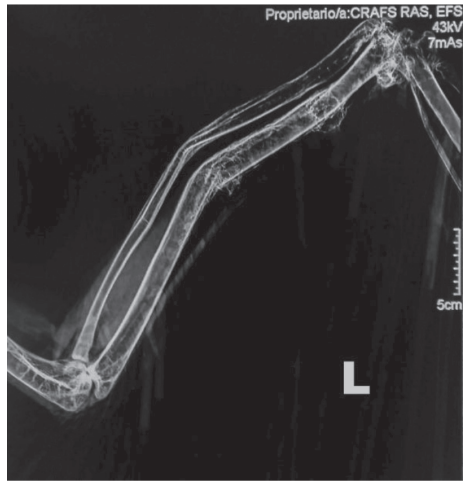

Figure 3. Radiological image of the left wing of the Griffon vulture (in the PC group) showing the radio-ulna fracture. The ossified lesion does not result perfectly aligned

While cortisol assay in feathers is catching on in the research field, to our knowledge only one other study in the field of ecotoxicology evaluated feather DHEA (Monclús et al., 2018). Our study showed statistical difference between the mean values recorded in the two studied groups. The higher DHEA concentrations recorded in the PC group could be part of the adaptive response of the endocrine to ameliorate the potential damaging effects of the higher circulating cortisol (Hu et al., 2000; Kalimi et al., 1994) even if, later in time, the DHEA concentrations could have dropped as an early sign of adrenal exhaustion and allostatic overload. On the other hand, the diversity in DHEA concentrations between the two groups examined did not impact on the cortisol/DHEA ratio given that a difference between the CTRL and the PC group was not found. Moreover, a low mean value of the cortisol/DHEA ratio was recorded in our study in both groups. Thus, in view of the literature available on other species (Hu et al., 2000; Kalimi et al., 1994), the low cortisol/DHEA ratio could be ascribed to animals that even being part of the PC group were still resilient and able to cope with the stressors at the time of feather moulting thanks to the DHEA ability to mitigate negative effects caused by chronic elevations of glucocorticoids. The correlation found between the feather cortisol and the DHEA concentrations in the PC group could support these considerations about the biologic relationship between cortisol and DHEA.

Interestingly, the lowest DHEA and the highest cortisol/DHEA ratio were recorded in the two animals that had suffered a long starvation period, along with high cortisol concentrations as already described above. On the other hand, an animal resilient to the infection of both the Avian influenza and the West Nile virus, showed high DHEA concentrations and low cortisol/DHEA ratio. Both the DHEA concentrations and the cortisol/DHEA ratio were high in the specimen of the CTRL group that, as previously mentioned, suddenly died after the hormonal evaluation. This can suggest a role of DHEA in protecting against the negative consequences of stress as described in humans by Hu et al. (2000) even if, later in time, DHEA concentrations can drop as a sign of allostatic overload, when demands exceed the resources available (McEwen and Wingfield, 2003).

In conclusion, the results showed the different clinical status of the studied animals as a potential biological source of variation of feather steroids and the feather analyses could be the only method available to obtain a long-term and retrospective measurement of hormonal concentrations in wild birds. Thus, the assessment of cortisol and DHEA concentrations in feathers could be used as an index of the allostatic load constituting a tool offering key data that could be used in planning strategies for threatened species conservation. Future researches could allow getting threshold values to evaluate an overloaded HPA axis for the Griffon vulture species.

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