*The impact of captivity on some haematological parameters of griffon vultures (*Gyps fulvus*)*

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Summary
Haematological analysis is an essential field of veterinary medicine that provides inexpensive and reliable support to determinate animal health. The knowledge of how different factors affect the normal mean values of blood parameters is key to understand and improve animal health. In order to investigate how captivity can affect the haematological profile of birds of prey, the erythrocyte count, haemoglobin concentration, haematocrit, mean corpuscular volume, total leukocytes count of 123 griffon vultures (*Gyps fulvus*) were analysed. The birds were divided into 4 groups according to their life conditions: a control group of free-living griffons, 2 semi-captive groups held in an aviary for 15 and 30 days respectively, representing short-term captivity, and a captive group that had lived in cage for about 2 years. Our results showed that long-term captivity could influence haematocrit value and haemoglobin concentration. Furthermore, discriminant analysis highlighted significant separation between the captive birds on one hand and the control group and the semi-captive birds on the other. Instead, short-term captivity did not seem to affect prominently haemocytometric profile.

*Effetti della cattività su alcuni parametri ematologici del grifone (*Gyps fulvus*)*

Parole chiave
Cattività,
Conta cellulare,
Ematologia,
Fauna selvatica,
Uccelli predatori.

Riassunto
L'analisi emocitometrica rappresenta un campo fondamentale della medicina veterinaria, in quanto fornisce un supporto economico ed attendibile per la determinazione della salute degli animali. La conoscenza riguardo come diversi fattori possano influenzare i valori medi dei parametri ematici risulta quindi essere uno strumento indispensabile. Al fine di valutare come lo stato di cattività possa influenzare il profilo ematologico dei rapaci, è stato condotto uno studio sulla valutazione di alcuni parametri emocitometrici in 123 grifoni (*Gyps fulvus*). Nello specifico sono stati valutati: la conta eritrocitaria, la concentrazione di emoglobina, l'ematocrito, il volume medio corpuscolare e la conta dei leucociti totali. Gli uccelli sono stati suddivisi in 4 gruppi in base alle loro condizioni di vita: un gruppo controllo di grifoni in libertà, 2 gruppi di grifoni in semi-cattività tenuti in una voliera rispettivamente per 15 e 30 giorni (rappresentanti la cattività a breve termine) e un gruppo di grifoni in cattività tenuti in voliera per circa 2 anni. I nostri risultati hanno mostrato che la cattività a lungo termine potrebbe influenzare i valori di ematocrito e la concentrazione di emoglobina. Inoltre, l'analisi discriminante ha sottolineato una separazione significativa degli uccelli in cattività sia rispetto al gruppo controllo che ai gruppi in semi-cattività. Al contrario, la cattività a breve termine sembra non influenzare in maniera significativa il profilo emocitometrico.

Introduction

Haematological investigation is an inexpensive and simple method of analysis, which ensures objective and reliable results. It is an important diagnostic tool in veterinary and aviary medicine, since the levels of haematological values can provide useful information on the health and nutritional conditions of the individuals and on the adaptation to their habitat (Cooper 1975, Campbell 1988, Hernandez *et al.* 1990, Averbeck 1992, Ferrer 1993). Furthermore, the knowledge of the normal concentration of blood constituents in birds of prey is an essential step in wildlife conservation and re-introduction projects of endangered species breeding programs (Lepoutre *et al.* 1983).

However, data on normal ranges of many haematological parameters are scarce or not available at all for several species of birds-of-prey (Ferrer 1993).

Over the last few years, a growing interest on the haematology of raptors has led many authors to describe baseline haematological and blood chemistry for some species. Nevertheless, most of these studies are based on a small number of individuals, usually held in captivity (Ferrer *et al.*1987, Hernandez *et al.* 1990, Polo *et al.* 1992) with rare exceptions (de le Court 1995, Dobado-Berrìos *et al.* 1998, Bowerman *et al.* 2000). Moreover, these studies do not often consider the variables that can influence the levels of haematological values. As a matter of fact, blood parameters may be affected by diet quality, age, sex, circadian rhythms, activity, and captivity (Perry *et al.* 1986, Ferrer *et al.* 1994, de le Court *et al.* 1995, Dobado-Berrìos *et al.* 1998), so it may be useful to investigate the correlation between the changes in level of haematological constituents and the different factors listed above.

Erythrocyte count and haematocrit are commonly used indices, since their levels may reflect nutritional stress condition (LeResche *et al.* 1974, Morton 1994, Dowson and Bortolotti 1997) and variation of erythropoiesis. These 2 parameters can be integrated with information about the mean corpuscular volume measurement. Indeed, similar levels of haematocrit may not reflect similar physiological conditions because haematocrit variations may depend on 2 factors: cell size and cell density; while mean corpuscular volume can represent a more objective indicator (Bearhop *et al.* 1999).

White blood cell count is an important index because values higher than 15 K/µl are indicative of stress in birds (Campbell 1984). In any case, it is generally reported that vultures and eagles tend to have higher total leukocyte counts than hawks, falcons, and owls (Monks and Forbes 2007).

The aim of this study is to investigate the influence of short-term and long-term captivity on some blood parameters. We reported a study of 5 haemocytometric parameters in free-living, semi-captive, and captive Eurasian Griffon vultures (*Gyps fulvus*) in order to evaluate the effect of captivity on normal haematological ranges. The analysed parameters were: red blood cell (RBC) count, haemoglobin (HGB) concentration, haematocrit (HCT), mean corpuscular volume (MCV), and white blood cell (WBC) count.

Materials and methods

Animals

For this study, a total of 123 griffon vultures (*Gyps fulvus*) were sampled. The sampled individuals were all sub-adults (3-4 year old) and adults (≥ 5 year old), apparently healthy.

Fifty-four free-living individuals were caught and sampled in North Eastern Spain, Castellón Province – Cinctorres municipality (40º 34′ N 0º 12′ W). Animals had an average body weight of 8.86 ± 0.68 Kg. After being sampled, they were immediately released. These free-living griffons represent the control group.

Another smaller sample of 18 wild griffons was captured in the same place and kept in an aviary for 30 days. Blood samples were taken after 15 days (semi-captive group 1) and after 30 days (semi-captive group 2). They were fed on meat (mainly pork) and water *ad libitum* twice a week. Griffons had average body weights of 8.93 ± 0.63 and 9.10 ± 0.54 kg after 15 and 30 days in the cage, respectively.

A group of 51 sub-adults, coming from the Centre of the Grupo de Rehabilitación de la Fauna Autóctona y su Hábitat (G.R.E.F.A.) in Spain, was held in an aviary in South Italy, Alcara Li Fusi - Rocche del Crasto (Sicily, Italy) for about 2 years. At the end of this period, blood samples were taken for each individual before the griffons were released in Parco dei Nebrodi in Sicily (Italy), as part of a reintroduction project started in 1998. The animals in captivity were fed on meat scraps *ad libitum* twice a week and they had an average body weight of 9.20 ± 0.41 kg.

Haematology

Blood was drawn without anaesthesia from the brachial vein using 2 ml heparinized syringes and then immediately collected into 3 ml tubes (VACUETTE®) containing Tripotassium Ethylene Diamine Tetra-acetic Acid (K_3EDTA), an anticoagulant used for haemocytometric determinations. All tubes were kept and carried to the laboratory at a temperature of 4°C and immediately analysed,

since erythrocytes may lyse within 24-48 hours of exposure to Ethylene Diamine Tetra-acetic Acid (EDTA). Other anticoagulants, such as heparin, can cause cell clumping, which may interfere with cell counts (Campbell 1988, Hawkey and Dennett 1989).

All haematological analyses, with the exception of WBC, were analysed by means of an automated cell counter with veterinary software, Cell-Dyn 3700 (Abbott Laboratories, Abbott Park, Illinois, USA). Total erythrocyte can be obtained with quite good precision by means of automated cell counters (Steel *et al.* 1976, Bounous and Stedman 2000, Naidoo *et al.* 2008). On the contrary, automated analysis of differential leukocyte counts is not an accurate method for some leukocyte parameters (Lilliehöök *et al.* 2004).

Total leukocyte count was evaluated using a Neubauer haemocytometer. The blood samples used for manual count had been previously stained with Natt-Herrick solution (Natt and Herrick 1952). This method requires a 1:200 dilution of blood samples in methyl violet 2B buffer. We counted the cells inside the 9 large squares of the haemocytometer. We obtained WBC value per µl using the formula (total leukocytes in 9 squares + 10%) x 200 (Campbell 1994).

Statistical analyses

We processed the data of the total leukocyte count and the erythrocyte parameters separately.

All the statistical analyses were performed using "Statistica" (Statsoft) software. The discriminant function analysis was used to determine whether the groups differ with respect to the mean of a variable.

As for the erythrocyte parameters, a matrix of total variances and covariances was obtained in order to determine which variables contributed to the discrimination among the groups. A multivariate F test among matrices was first performed to determine the existence of significant differences (with regard to all variables) among the groups. In case of statistically significant matrices, the variables with significantly different means were further analysed. In addition to that, in order to examine the correlation between 2 sets of variables, canonical correlation analysis was performed for a multiple group of discriminant analysis.

ANOVA analyses, followed by post-hoc Tukey-Kramer tests, were used to assess differences among all the investigated parameters for free-living, semi-captive, and captive griffon vultures. A probability level of $p < 0.05$ was considered significant.

Results

Comparative analysis of the haemocytometric data on free-living, semi-captive, and captive griffons

Table I summarises the data on haemocytometric values obtained for the free-living, semi-captive, and captive griffon vultures in this study.

The ANOVA analysis shows significant differences ($p < 0.01$) in WBC count ($p = 0.0034$) among the groups. The highest WBC value was found in captive birds (12.17 ± 3.88 K/µl). The Tukey-Kramer test confirmed statistical differences between the captive group and semi-captive group 1 (8.27 ± 1.76 K/µl; $q = 4.042$; $p < 0.05$) and between the captive group and semi-captive group 2 (7.09 ± 0.84 K/µl; $q = 4.856$; $p < 0.01$), but not between the captive and the free-living griffons (10.45 ± 4.93 K/µl).

In terms of erythrocyte parameters, ANOVA test was not statistical significant for RBC ($p = 0.5336$).

As for the other investigated parameters, ANOVA analysis resulted extremely significant ($p < 0.001$) for HGB, HCT, and MCV values. Moreover, post-hoc analysis points out a very significant variance in HGB concentration among captive group (18.86 ± 1.53 g/dl) and free-living (15.35 ± 1.04 g/dl; $q = 18.913$; $p < 0.001$),

Table I. *Haemocytometric values (mean ± Standard Deviation) of the* Gyps fulvus. Samples from free-living and semi captive groups were collected in North Eastern Spain, Castellón Province - Cinctorres municipality from April to October 2007; samples from the captive group were collected at Alcara Li Fusi - Rocche del Crasto (Sicily, Italy) on August 2011.

	Free-living (a) N=54		Semi-captive1 (b) N=18		Semi-captive 2 (c) N=18		Captive (d) N=51		p (all)
	X ± SD	p	X ± SD	p	X ± SD	p	X ± SD	p	
WBC (K/µl)	10.45 ± 4.93		8.27 ± 1.76	d*	7.09 ± 0.84	d**	12.17 ± 3.88	b*,c**	**
RBC (M/µl)	2.41 ± 0.33		2.73 ± 0.16		2.87 ± 0.13		2.56 ± 0.20		ns
HGB (g/dl)	15.35 ± 1.04	d***	15.2 ± 0.97	d***	15.53 ± 0.98	d***	18.86 ± 1.53	a-c***	***
HCT (%)	49.33 ± 4.28	d***	49.33 ± 3.20	d**	51.75 ± 2.80	d***	43.50 ± 3.40	a-c***	***
MCV (fl)	207.37 ± 22.18	b-d***	180.56 ± 5.77	a***	181.25 ± 5.79	a***	170.19 ± 3.77	a***	***

Differences among captivity related groups according to ANOVA (p-all). When appropriate, a multiple comparison test Tukey-Kramer was performed: different letters in p columns show significant differences among groups. a = Free-living; b = Semi-captive1; c = Semi-captive2; d = Captive. *p < 0.05; **p < 0.01; ***p < 0.001; ns = non-significant.

semi-captive group 1 (15.20 ± 0.97 g/dl; q = 13.889; p < 0.001), semi-captive group 2 (15.53 ± 0.98 g/dl; q = 11.898; p < 0.001).

Similarly, Tukey-Kramer test was extremely significant (p < 0.001) for the comparison of HCT in captive (43.50 ± 3.40%) and free-living (49.33 ± 4.28%; q = 8.894; p < 0.001), and between semi-captive 1 (49.33 ± 3.20%; q = 6.704; p < 0.001) and semi-captive 2 (51.75 ± 2.80%; q = 7.898; p < 0.001). It was also very significant for the comparison of HCT in captive and semi-captive birds (48.83 ± 3.69%; q = 5.440; p < 0.01).

Mean corpuscular volume in free-living vultures was higher than the erythrocyte size in the other sampled animals, with a mean value of 207.37 ± 22.18 fl. Tukey-Kramer test showed extremely significant differences between free-living and semi-captive -1 (180.56 ± 5.77 fl; q = 4.843; p < 0.001), and between semi-captive -2 (181.25 ± 5.79 fl; q = 4.547; p < 0.001) and captive birds (170.19 ± 3.77 fl; q = 15.472; p < 0.001).

Haemocytometric discriminant analysis among the groups

Figure 1 shows the plot of canonical axes, corresponding to the data for erythrocyte parameters, with regard to the living conditions of the birds. As shown, the group of captive griffon vultures formed a cluster independent from both the control group and the semi-captive groups.

The squares in Figure 1 represent the canonical correlation 0.900 (p < 0.001) and show a significant separation among the group of captive griffons and both the control and the semi-captive groups. On the contrary, the 2 semi-captive groups did not differ significantly from one another and from wild birds. So captivity seems not to have significant short-term effects on the investigated haematological values. Along root 1 (X-axis) in Figure 1, especially the values of haemoglobin and haematocrit were discriminant for the effect of captivity. Along root 2 (Y-axis) haematocrit and mean corpuscular volume intensely contribute to separate the groups.

Discussion

The application of raptors haematology evaluation could be involved on wildlife conservation and reintroduction projects or endangered species breeding programs (Lepoutre *et al.* 1983), but so far a limited number of studies have focused on

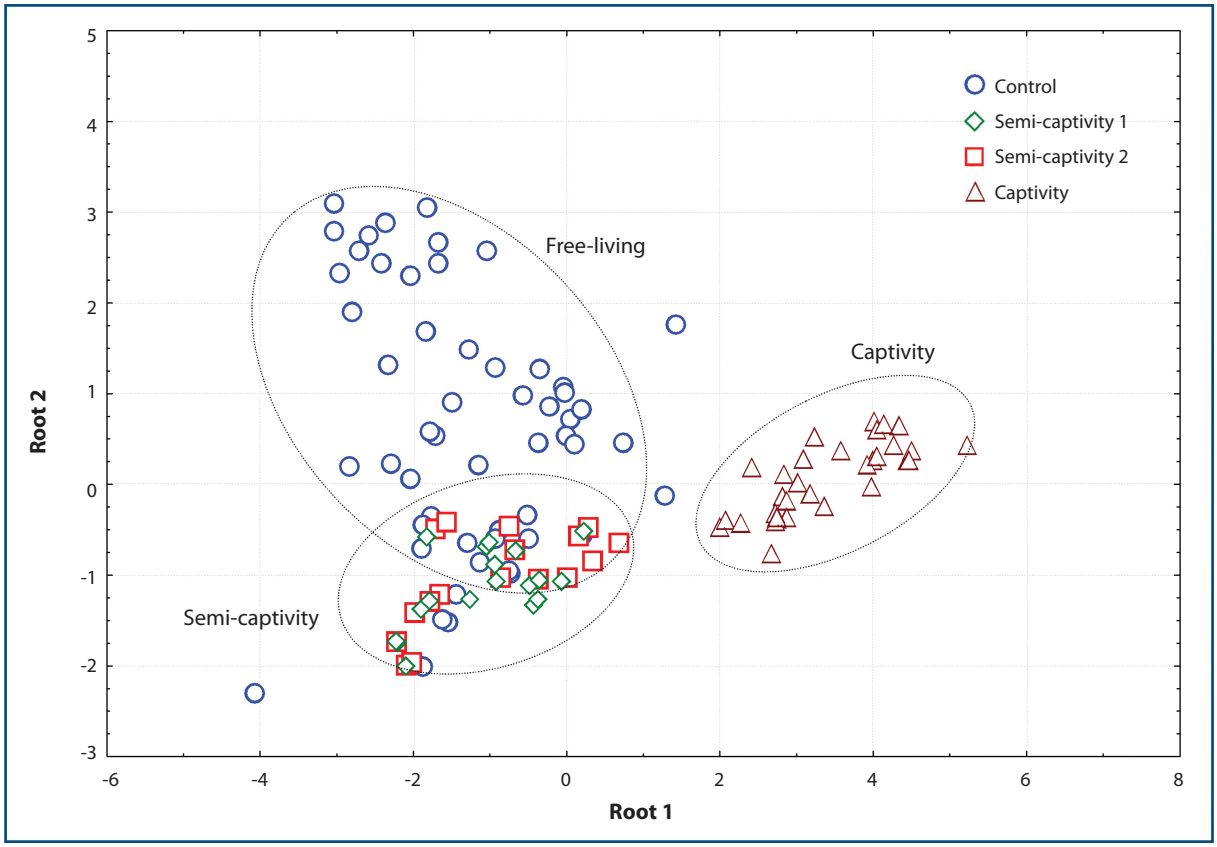

Figure 1. *Effect of the* Gyps fulvus *captivity on erythrocyte parameters.* Scatter plot of the canonical discriminant analysis of erythrocyte parameters. Samples from free-living and semi captive groups were collected in North Eastern Spain, Castellón Province - Cinctorres municipality from April to October 2007; samples from the captive group were collected at Alcara Li Fusi - Rocche del Crasto (Sicily, Italy) on August 2011.

haematological and blood chemistry baseline. The majority of these studies are based on a small number of individuals, usually held in captivity (Ferrer *et al.*1987, Hernandez *et al.* 1990, Polo *et al.* 1992) with rare exceptions (de le Court 1995, Dobado-Barrios 1998). In this study, we showed that captivity might have long-term effect on certain haematological values, especially regarding erythrocyte parameters.

The common range of WBC count assumed for the majority of avian species fell within 15-30 k/µl (Sturkie, 1986). In particular, for *Gyps fulvus*, Polo and colleagues present a range of 5-24 k/µl with a mean value of 13.19 ± 7.32 k/µl (Polo *et al.* 1992). In this study, all the WBC counts overlap with previous ranges, but the ANOVA analysis underlined significant differences among the groups in accordance with the length of their captivity. Lower values recorded for WBC in semi-captive groups are consistent with findings of Azeez and colleagues (Azeez *et al.* 2013) on the changes of haematology of birds in captivity. In their study, Azeez and colleagues (Azeez *et al.* 2013) observed a decrease in WBC count from 4 to 8 weeks in cage, and authors reported that captivity imposes immunological challenges different from those seen in free-living. Indeed in the present study, WBC values for free-living were higher than the values for semi-captive groups, even if we did not detect statistical difference. An elevated WBC count may be attributed to higher stress levels, social interactions, capture, and growth rate in younger animals (Marco *et al.* 1997), it is not necessarily indicative of diseases. Higher values in free-living could be due to capture, since it is difficult to measure normal WBC count accurately in raptors since these birds are unavoidably stressed when handled and especially when captured in the wild (Powers *et al.*, 1994). Increased WBC values in captive group could instead indicate that long-term captivity exposed the animals to higher stress levels, compared to a short period in cage.

Red blood cell count values for griffon vultures in this study were similar to the mean value of 2.55 ± 0.22 M/µl obtained in captive white-backed vultures by Naidoo and colleagues (Naidoo *et al.* 2008). Moreover, we did not find significant differences of RBC mean values among the experimental groups related to the captivity.

The HCT levels obtained were similar to the accepted haematocrit range for raptors, which means 35-55 l/l (0.35-0.55%) (Fudge 2000, Cooper 2002). However free-living and semi-captive individuals in this study presented significant higher values than griffons held in captivity and they were significantly separated from captive group also from discriminant analysis. In fact, HCT may increase because of the stress caused by capture and immobilization, to which wild species are particularly sensitive. Some studies agree that higher HCT could be normal if related to the greater activity of flight in free-living birds as compensatory mechanism for oxygen requirement (Hawkey *et al.* 1984). Thus, reduced activity associated with captivity may cause these differences in HCT levels (Dawson and Bortolotti 1997). Furthermore, HCT can be affected by dehydration and chronic undernourishment (Ferrer *et al.* 1987, Cooper 2002). Therefore, higher values in wild griffons could be also indicative of their greater risk for dehydration than captive individuals, which were fed *ad libitum* (Dawson and Bortolotti 1997).

Erythrocyte dimensions are very homogeneous among birds, particularly when compared with other vertebrate classes (Hartman and Lessler 1964). Mean values of MCV obtained for semi-captive and captive griffon vultures are similar to those reported by Polo and colleagues (170.0 ± 15.5 fl) (Polo *et al.* 1992). On the contrary, free-living individuals show higher red blood dimensions. In other non-mammalian vertebrate, an increase in RBC size has often been associated with anaesthesia or hypoxia, but increased levels of MCV are also normally considered to be a consequence of stress (Nussey *et al.* 1995). Therefore, the high values (207.37 ± 22.18 fl) obtained for wild griffons could be a consequence of an excessive exposure of these birds to stress factors.

Haemoglobin concentration in vulture is higher than in other Falconiformes species (Balasch *et al.* 1976). In accordance with previous investigations on haemoglobin values in accipitrides, which reported a mean concentration of 15.1 ± 1.9 g/dl (Polo *et al.* 1992), HGB of griffon vultures in this work average 15.35 ± 1.04 g/dl in free-living individuals, 15.20 ± 0.97 g/dl and 15.53 ± 0.98 g/dl in vultures after 2 weeks and 30 days in an aviary, respectively. Instead, data regarding griffons from Alcara Li Fusi in Sicily (Italy) display that long-term captivity causes significant variation on haemoglobin concentration, with a mean value of 18.86 ± 1.53 g/dl. In addition, different diets could have contributed to create this substantial difference in HGB.

In conclusion, our results demonstrate that there is a correlation between some haematological values and the life conditions of griffon vultures. In fact, a prolonged captivity period makes these raptors less subject to food and water deficiency, resulting in significant decrease in HTC and increase in HGB values. Nevertheless, the environment of the aviary may expose birds to different types of other stress factors. In regard of the duration of captivity period, with the only exception of MCV parameter, we did not find any significant difference in blood parameters in animals caged for 1 month. Similarly,

no relevant differences were found between free-living and semi-captive individuals. Therefore, we can conclude that haematological values can be influenced only by enduring captivity.

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