Social rank affects the hematologic profile in red deer hinds

Francisco CEACERO^{1,*}, Enrique GASPAR-LÓPEZ^{2,3}, Tomás LANDETE-CASTILLEJOS^{2,3,4}, Laureano GALLEGÓ³, Andrés J. GARCÍA^{2,3,4}.

¹ Department of Animal Science and Food Processing. Faculty of Tropical AgriSciences. Czech University of Life Sciences Prague. 165 21, Prague 6 - Suchbát, Czech Republic.

² Departamento de Ciencia y Tecnología Agroforestal y Genética. Universidad de Castilla-La Mancha (UCLM). 02071, Albacete, Spain.

³ Sección de Recursos Cinegéticos y Ganaderos. Instituto de Desarrollo Regional (IDR). 02071, Albacete, Spain.

⁴ Instituto de Investigación en Recursos Cinegéticos (IREC). 13071, Ciudad Real, Spain.

* Corresponding author: Dr. Francisco Ceacero. Mail: ceacero@ftz.czu.cz; Phone: +420 733450473; Fax: +420 234381801.

ABSTRACT

We studied the effects of social rank on the hematologic profile in a herd of twenty-four female Iberian red deer hinds. Animals were blood sampled (6 times per animal) and samples were analyzed with ADVIA® A120. Social rank hierarchy was determined by analyzing aggressive interactions. After controlling for age and body mass, dominance rank significantly explained WBC count, hemoglobin and hematocrit with a negative effect (*i.e.*, lower values in dominant hinds). Results are similar to those reported for stressed individuals due to physical immobilization, but do not support the predicted enhanced erythropoiesis due to higher levels of androgens. Results for WBC may also reflect that subordinate hinds must allocate a higher amount of resources to immunity as a results of injuries produced by dominant hinds, while simultaneously facing a worst access to food sources. For RBC, results may reflect that subordinate hinds need increased hematocrit and hemoglobin likely to be ready for fast flight responses. Even if the mechanisms need to be further studied, our results show that social rank influences hematologic profile, and thus it should be considered for a correct interpretation of blood analyses in social Cervid species.

Keywords: *Cervus elaphus*; immunity; social rank; dominance; stress effects on immunity.

INTRODUCTION

The hematologic profile of cervids is relatively well known. However, relatively little information is available about how the hematologic profile is influenced by a variety of factors, with age, sex, reproductive status, seasonality, and disease being the most important factors proposed (Boes, 2010). The influence of stress in blood parameters is also well known, but in Cervids it has been mainly studied related to capture, handling and immobilization procedures: RBC count, packed cell volume, Hgb, WBC or lymphocyte counts values are significantly higher in excited deer than in resting or chemically restrained ones (Marco and Lavin, 1999; Boes, 2010). Thus, the hematologic profile reported in previous studies on Cervids show a wide variability (see Boes, 2010 for an exhaustive summary), since it is almost impossible to obtain normal resting blood values. According to Wilson and Pauli (1982) the excitable nature of deer may be the main reason behind these effects.

However, there are other stressful situations which may affect the hematologic profile which have not been well studied in Cervids, like the stress related to dominance relationships. Is there any physiological mechanism which may suggest an effect of social rank in hematologic profile? Most social mammals establish hierarchies, and it has been reported an increase in WBC count in submissive animals (Hjarvard et al., 2009 for pigs). Social rank may also affect RBC count: on one hand, submissive animals are socially stressed (Thouless, 1990), and it is well known that other stressful situations like physical immobilization increases RBC count, hematocrit (Hct) and Hgb (Boes, 2010). On the other hand, androgen levels and social rank are closely related both in males and females. In female ungulates treatment with androgens consistently raises social rank (Bouissou, 1983), and these increased testosterone levels in dominant animals may also have positive effects in the hematologic profile, since it has been

observed that testosterone stimulates erythropoiesis in female mice (Gordon et al., 1970; Gorshein and Gardner, 1970). Thus, it can be predicted that social rank influences hematologic profile in several ways: increasing (stress mediated) WBC, and increasing (stress mediated) or decreasing (androgen mediated) RBC in submissive hinds. These predictions have not been studied in Cervids.

We studied the influence of social rank in the hematologic profile in a captive herd of Iberian red deer hinds (*Cervus elaphus hispanicus*). This is a good animal model since hinds create stable hierarchies and are subjected to social stress. We focused our study during lactation. This is the most demanding stage of reproduction (Ofstedal, 1985), and it has been pointed out that immune system needs are covered only after other needs for maintenance, lactation or reproduction have been fulfilled (Coop and Kyriazakis, 1999; Houdik et al., 2001). Thus, this competition between lactation and immunity may help to find stronger patterns of variability on the hematologic profile.

MATERIAL and METHODS

Study animals

The study was performed on a group of 24 Iberian red deer hinds (*Cervus elaphus hispanicus*) kept at the Experimental Farm of Castilla-La Mancha University in Albacete, Spain (38°57'10''N, 1°47'00''W, 690m altitude). Hinds were 2 to 12 years old, individually identified by ear tags and collars. The group was maintained in a fenced enclosure of 15,000 m², and received *ad libitum* food based on barley straw and meal from barley, alfalfa, oat and sugar beets (16% crude protein, 11% humidity).

Hinds were weighed at the blood sampling days, as part of the routine handling activities in the farm.

Blood sampling

Blood samples were obtained from the right jugular vein, always at the same moment of the day (between one and three hours after dawn), in order to avoid variations because of the circadian rhythm (Ingram et al., 1999). None of the animals showed clinical signs of disease at the moment of the extraction and no sedation was required. Blood samples were taken from the jugular vein while animals were standing inside a small handling box (2m x 2m x 0.6m). Sampling of all animals was performed on six dates in the middle of the lactation period (weekly from July to middle August). Blood was collected into evacuated tubes for hematology containing an anticoagulant (EDTAk3). The samples were refrigerated (4-8 °C) within the first 15 min after collection and analyzed in the first 2-3 h after the extraction. Analyses were performed with ADVIA® 120 Hematology System (Siemens) at the Complejo Hospitalario de Albacete, using Advia 120 MultiSpecies System Software. The principles of this automated hematology analyzer are photometric measurement for hemoglobin, optical laser-light scattering for cell enumeration, myeloperoxidase staining coupled with flow cytometry–nuclear globularity analysis for WBC differential, flow cytometry, and laser diffraction for RBC and platelet count (Lippi et al., 2005). This instrument has counted and identified blood cells in human and animal samples since 1998. Calibrated commercial controls were run daily prior to analyzing samples. Blood samples were analyzed to determine red blood cell count, hemoglobin concentration, hematocrit, mean corpuscular volume (MCV), red cell distribution width (RDW), mean corpuscular haemoglobin (MCH), mean cell hemoglobin concentration (MCHC), white blood cell count, lymphocyte count, non-

lymphoid leukocyte count, platelet count, and mean platelet volume (MPV). Since deer has a lack of the myeloperoxidase enzyme (Knoll, 2000) the differentiate count only could classify leukocytes into two types: lymphocytes and non-lymphoid leukocytes. Each datum was the mean of 2 measures performed for every sample, with intra-assays coefficients that varied between 0.78 and 2.93 %. Mean values after the six sampling days were used in further statistical analyses. Handling and sampling procedures were designed to reduce the stress and health risks for the subjects, according to the European and Spanish laws and current guidelines for ethical use of animals in research (ASAB, 2006), and considering that the herd is well habituated to routine handling (Ceacero et al., 2014). All experimental procedures were conducted under the approval of the Universidad de Castilla-La Mancha Ethics Committee.

Dominance Index

Interactions to establish social rank were monitored during the same weeks as the blood sampling. Although social hierarchy is considered to be linear and highly stable in red deer hinds (Ceacero et al., 2007), small changes may occur due to changes in weight, body condition or injuries. Thus, the observation period was extended for a few weeks to obtain an adequate amount of data during several days; but not for longer, to avoid variations in the hierarchy during the period of blood collection. A total of 14 observation hours were carried out in 2 h periods during those moments with higher social activity (Ceacero et al., 2007). All interactions were registered avoiding interferences in the behavior of the animals, according to the focal group sampling method (Altmann, 1974). The observer stayed hidden from the animals outside the enclosure, with optimal observation conditions. Following Thouless and Guinness (1986), agonistic interactions were considered as occasions when one hind physically

118 attacked another one, or made a ritualized gesture associated with attacks that led to the
119 other animal moving away. Threats included one or more of the following behaviors:
120 butting with the forehead against the other's body; biting (usually directed towards the
121 back or ears); kicking with the forelegs and chasing; all of which varied in intensity,
122 sometimes being reduced to a mere intention movement in which no actual contact was
123 made (Hall, 1983). Dominance rank for each individual was calculated as a linear
124 hierarchy by winner–loser outcome of interactions on Matman 1.1.4 matrix
125 manipulation and analysis program (Noldus, Wageningen, The Netherlands) as
126 explained by (de Vries, 1998). This method was chosen respect to other proposed ones
127 because it can be applied to a small sample size, as we had, with significant results. To
128 determine the statistical significance of the linearity (h') of the dominance hierarchy, a
129 sampling process using 10000 randomizations was performed (de Vries, 1995).
130 Dominance hierarchy was reorganized by a two-step iterative procedure (1000
131 sequential trials) to order individuals by first minimizing the number of inconsistencies,
132 and thereafter the strength of the inconsistencies. Linear hierarchy was transformed
133 according to the formula $1 - (\text{rank}/n)$ [$n = 24$]. Therefore, dominance index varied in the
134 range [0, 1] (0=submissive; 1=dominant).

136 *Statistical Analyses*

137 In descriptive values, mean $\pm$ SD is indicated throughout the text. In the initial analysis
138 of the data, Pearson correlations were performed to understand the relationships of the
139 explanatory variables (Dominance index, Body mass and Age) among themselves and
140 with the studied hematologic variables. Since these were usually correlated with any or
141 with all the three explanatory variables, General Linear Models disentangled which of

the independent variables better explained the hematologic profile observed. Analyses were performed using IBM® SPSS® Statistics (version 20.0 for Windows, IBM, USA).

RESULTS

During the study, the mean age of the animals studied was 6.1 ± 3.3 years old (range: 2-12), and the mean body mass was 107.4 ± 14.9 Kg (range: 75.1-136.9). Dominance index, body mass and age were positively correlated with MCV and MCH, and negatively with RBC, Hgb, Hct and Lymphocytes. WBC was negatively correlated with dominance index and body mass, but not with age (Table 1).

General Linear Models clarified these relationships (Table 2). Dominance index was the main variable explaining Hgb ($R^2 = 34.4\%$), Hct ($R^2 = 34.6\%$) and WBC ($R^2 = 32.0\%$). Body mass was the main variable explaining RBC ($R^2 = 56.2\%$) and MCH ($R^2 = 45.9\%$). Age was the main variable explaining MCV ($R^2 = 46.8\%$), count of lymphocytes ($R^2 = 44.8\%$) and platelet ($R^2 = 20.8\%$). Finally, count of not lymphocytes was explained by dominance index and age ($R^2 = 29.9\%$).

All the hematologic parameters measured were in the range reported for adult hinds of the species (Teare, 2016; not shown for concision purposes). Nevertheless, since this study is focused on the effect of social rank (dominance index) on the hematologic profile, the correlations calculated in the initial analyses are shown in Figure 1 (Hgb), Figure 2 (Hct) and Figure 3 (WBC), together with the most relevant means and ranges published (Teare, 2016; Marco and Lavin, 1999).

DISCUSSION

Determining the hematologic profile is a useful tool for monitoring the health status of deer herds (Klein et al., 2002; Zomborsky et al., 1997; Boes, 2010), but it is also necessary to determine the factors that affect them. Our study confirmed several relationships which have been previously reported, but also confirmed our hypothesis: social rank affects hematologic profile. The influence of age has been well studied, and effects on RBC count, Hgb (Chapple, 1989; Maede et al., 1990) MCV (Chapple, 1989; Maede et al., 1990; Peinado et al. 1999) and lymphocyte count (negative effect reported by Upcott and Herbert, 1965 and Mohri et al., 2000; positive effect reported by McAllum, 1978) seems clear across Cervid species. In general, our results confirmed those previously reported. However, our data also showed a negative effect on Hct and lymphocytes count, and positive on MCH; although models showed that age is the main factor affecting only lymphocytes count and MCV. It has been recently pointed out that there is an unclear relationship between age and white blood cell parameters in deer (Boes, 2010). Most Cervids are social animals and generally there is a correlation between age and social rank (Table 1; Ceacero et al., 2007). Our results clearly showed that the effect of social rank and WBC count is stronger than that between age and WBC count, which suggests that previous results may have been misinterpreted because they were confounded with the effect of social rank, but having age lower explanatory power.

On the other hand, few relationships of hematologic profile with body mass have been described (only with RBC; Hawkey and Hart, 1985). Our data show that RBC, WBC and lymphocyte count, Hgb and Hct are negatively affected by body mass, whereas it influences positively MCV and MCH. However, body mass is the main factor explaining the variability observed only in RBC count and MCH. Thus, we may

conclude that hinds with greater body mass have lower RBC count, but with greater MCH.

Nevertheless, the main goal of this study was to highlight the relationship between hematologic profile and social rank. About the erythrocytes, we proposed two physiological mechanisms how they may be affected by social rank: First, predicts greater RBC count in dominant animals due to increased erythropoietic activity mediated by greater androgen levels (Gordon et al., 1970; Gorshein and Gardner, 1970). This should happen even during lactation, when hinds have low RBC count (Zomborszky et al., 1997). However, this relationship was negative, and negative correlations also appeared with Hgb and Hct and positive with MCV and MCH. But the most important relationships according to models are those with Hgb (Fig. 1) and Hct (Fig. 2). Thus, this physiological mechanism was not supported, since the relationship was negative. The second proposed mechanism was lower RBC count in submissive animals due to increased stress and glucocorticoids level. Our results fit well with those described when comparing chemically restrained deer (low stress) with physically restrained ones (high stress). RBC, WBC count, Hgb and Hct are consistently higher in physically restrained (Marco and Lavin, 1999; Boes, 2010). It has been also observed that routine handling may reduce this “stress-effect” by a 10-20% in these values (Chapple et al., 1991), which are similar to the differences observed among our more dominant and more submissive animals. We may thus conclude that social stress in submissive animals may have a chronic effect as long term stressor on the hematologic profile, similar to that found in physically restrained deer. The effect on WBC count has not been reported previously in Cervids, but it has been well studied in pigs (de Groot et al., 2001) and other vertebrates (reviewed in Engler, 2004).

Our results suggest that social stress lead to increased RBC count, WBC count, Hgb and Hct values in submissive hinds. These responses are similar to those observed in other stressful situations like physical restraining. As can be observed in the figures, our study animals showed values within the currently accepted reference values (Teare, 2016); WBC count values are similar to those reported for physically restrained Iberian red deer; and Hgb and Hct are in mean very similar to those reported for chemically restrained Iberian red deer, and much smaller than expected for physically restrained (Marco and Lavin, 1999). Our animals were subjected to weekly handling as part of the routine in the experimental facilities, which has been reported to lead to habituation to handling and low stress measured by behavioral indicators (Ceacero et al., 2014). Altogether, that means that our results and conclusions are not affected by other stress factors, and further confirms social stress as the main explanation for the results obtained.

Adaptive and ecological implications

In general, our results agree with those previously reported in other groups of mammals. That suggests an underlying mechanism that has been conserved during evolution (Engler et al., 2004). For being conserved, these effects of stress on complete blood count may be beneficial to the different ecological needs within the animals in the herd. About Hgb and Hct, it can be argued that submissive hinds must be more ready to flee at high speed, which requires more supply of oxygen to the cells; on the contrary, dominant hinds rarely have to use reactions requiring burst of oxygen supply. About WBC, the effect found may be mediated (or reinforced) by nutrition: body condition interact with immunocompetence in the way that immune system needs are covered only after other needs such as maintenance or reproduction have been fulfilled (Coop

and Kyriazakis, 1999). That means that diet protein is used for milk production with higher priority than for immune system, especially under food restriction situations (Houdik et al., 2001). Social rank not only clearly determines the access to food in hinds, but also allows them to select feeds with greater nutritional value (in particular, energy: Ceacero et al., 2012), which means that subordinate hinds may have less resources for immune barriers, thus being forced to use internal immunity (white blood cells, immunoglobulins, etc.) against opportunistic pathogens which may enter the body through the wounds generated by bites and injuries after the interaction with dominant hinds (similar results were found for restricted food experiments in Landete-Castillejos et al., 2002). Thus, dominant hinds will benefit of a lower energy expenditure in WBC, while submissive will benefit of enhanced defenses. All these predictions need to be further studied in the future.

Conclusions and future steps

In summary, since WBC count, Hgb and Hct are clearly affected by social rank, but also MCV, MCH, and Lymphocytes count to some extent, we propose that social rank may be considered in the interpretation of hematologic profile of red deer, and probably also for other social cervids and ungulates. Similar studies must also be conducted in males, for which hierarchies are based on α -male structures much more intense during rut than outside the mating season. Increase of RBC count mediated by testosterone levels must be also tested in males especially during rut, even if this hypothesis was discarded in hinds.

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353

Table 1. Pearson correlations among Dominance index, Body mass and Age and the hematologic profile in a herd of 24 Iberian red deer hinds (*Cervus elaphus hispanicus*) during lactation.

	Dominance index	Body mass	Age
Body mass	0.571 **		
Age	0.752 **	0.682 **	
Red Blood Cell Count (RBC)	-0.585 **	-0.727 **	-0.621 **
Hemoglobin (Hgb)	-0.637 **	-0.577 **	-0.586 **
Hematocrit (Hct)	-0.615 **	-0.584 **	-0.533 **
Mean Corpuscular Volume (MCV)	0.460 *	0.714 **	0.623 **
Red Blood Cell Distribution Width (RDW)	ns	ns	ns
Mean Corpuscular Hemoglobin (MCH)	0.453 *	0.738 **	0.595 **
Mean Cell Hemoglobin Concentration (MCHC)	ns	ns	ns
White Blood Cell Count (WBC)	-0.545 **	-0.456 *	ns
Lymphocytes count	-0.472 *	-0.662 **	-0.649 **
Not Lymphocytes count	ns	ns	ns
Platelet count	ns	ns	ns
Mean Platelet Volume (MPV)	ns	ns	ns

Table 2. General Linear Models showing the influence of Dominance index, Body mass and Age on the hematologic profile of Iberian red deer hinds (*Cervus elaphus hispanicus*) during lactation.

Dependent variable	Independent variables ^a	R ²	β	t	p
Red Blood Cell Count (RBC)	<i>Intercept</i>	56.2%	15.390 $\pm$ 1.216	12.651	
	Body mass		-0.058 $\pm$ 0.011	-5.195	<0.001
Hemoglobin (Hgb)	<i>Intercept</i>	34.4%	16.513 $\pm$ 0.348	47.439	
	Dominance Index		-1.907 $\pm$ 0.575	-3.315	0.003
Hematocrit (Hct)	<i>Intercept</i>	34.6%	43.301 $\pm$ 0.891	48.601	
	Dominance Index		-4.905 $\pm$ 1.472	-3.332	0.003
Mean Corpuscular Volume (MCV)	<i>Intercept</i>	46.8%	39.757 $\pm$ 1.402	28.348	
	Age		0.874 $\pm$ 0.203	4.299	<0.001
Red Blood Cell Distribution Width (RDW)	-				
Mean Corpuscular Hemoglobin (MCH)	<i>Intercept</i>	45.9%	9.512 $\pm$ 1.832	5.192	
	Body mass		0.071 $\pm$ 0.017	4.218	<0.001
Mean Cell Hemoglobin Concentration (MCHC)	-				
White Blood Cell Count (WBC)	<i>Intercept</i>	32.0%	7.079 $\pm$ 0.379	18.699	
	Dominance Index		-1.967 $\pm$ 0.626	-3.145	0.005
Lymphocytes count	<i>Intercept</i>	44.8%	3.226 $\pm$ 0.248	12.999	
	Age		-0.149 $\pm$ 0.036	-4.129	<0.001
Not Lymphocytes count	<i>Intercept</i>	29.9%	3.763 $\pm$ 0.364	10.326	
	Dominance Index		0.177 $\pm$ 0.070	2.527	0.011
	Age		-2.131 $\pm$ 0.758	-2.810	0.020
Platelet count	-				

^a Dashes indicate that no significant model was obtained.

Figure 1. Correlation between dominance index and hemoglobin (g/dL) concentration in a herd of Iberian red deer hinds (*Cervus elaphus hispanicus*). Reference values are: Straight red line - mean for *C. elaphus* hinds according to Teare (2016); Grey area - reference interval for *C. elaphus* hinds according to Teare (2016); Broken red line - mean for physically restrained females of *C. e. hispanicus* according to Marco and Lavin (1999); Dotted red line - mean for chemically restrained *C. e. hispanicus* according to Marco and Lavin (1999).

Figure 2. Correlation between dominance index and hematocrit (%) concentration in a herd of Iberian red deer hinds (*Cervus elaphus hispanicus*). Reference values are: Straight red line - mean for *C. elaphus* hinds according to [Teare \(2016\)](#); Grey area - reference interval for *C. elaphus* hinds according to [Teare \(2016\)](#); Broken red line - mean for physically restrained females of *C. e. hispanicus* according to [Marco and Lavin \(1999\)](#); Dotted red line - mean for chemically restrained *C. e. hispanicus* according to [Marco and Lavin \(1999\)](#).

Figure 3. Correlation between dominance index and white blood cell count ($\times 10^9/\text{L}$) concentration in a herd of Iberian red deer hinds (*Cervus elaphus hispanicus*).

Reference values are: Straight red line - mean for *C. elaphus* hinds according to Teare (2016); Grey area - reference interval for *C. elaphus* hinds according to Teare (2016); Broken red line - mean for physically restrained females of *C. e. hispanicus* according to Marco and Lavin (1999); Dotted red line - mean for chemically restrained *C. e. hispanicus* according to Marco and Lavin (1999).