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Short communication

EFFECT OF SEASON ON HEMATOLOGIC, BIOCHEMICAL, AND HORMONAL ANALYTES IN RAMS OF TWO BREEDS

OIKONOMIDIS Ioannis L.[1*], KIOSSIS Evangelos[2], BROZOS Christos[2], KRITSEPI-KONSTANTINOU Maria[1]

[1]Diagnostic Laboratory, School of Veterinary Medicine, Faculty of Health Sciences, Aristotle University of Thessaloniki, Thessaloniki, Greece; [2]Farm Animal Clinic, School of Veterinary Medicine, Faculty of Health Sciences, Aristotle University of Thessaloniki, Thessaloniki, Greece

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Clinicopathological investigations are essential for the evaluation of the health status of ruminants. Apart from species-specific reference intervals, the effect of common biological factors should be considered for an accurate interpretation of laboratory data. The aim of this study was to evaluate the effect of season on hematologic and biochemical analytes, and serum total thyroxine and cortisol in adult rams of two breeds. Four blood samples (one every season) were collected from each ram. Complete blood count was performed on the Advia 120 (Siemens Healthcare Diagnostics, USA), while the differential leukocyte count was manually conducted. Biochemical and hormonal analyses were performed on Flexor E (Vital Scientific, The Netherlands), AVL 9180 (Roche Diagnostics, Belgium), and Immulite 1000 (Siemens Healthcare Diagnostics, USA), respectively. Linear mixed effects models (R language) were employed for statistical analyses. Forty-three (26 Chios, 17 Florina), adult, clinically healthy rams were included. Statistically significant (p<0.05), mostly breed-independent seasonal differences were observed in almost all of the analytes. However, when assessing these differences in view of the respective reference intervals, only a few of them were considered biologically important. Specifically, mild hyperglycemia and mild decrease in the concentration of total calcium and inorganic phosphorus were detected in winter, while a mild increase in thyroxine concentration (autumn) and creatine kinase activity (spring and summer) was also noted. In conclusion, seasonal effects should be considered when evaluating laboratory results in rams; however, season does not appear to have an essential effect on the clinicopathological profile of rams reared in the Mediterranean region.

Keywords: biochemistry; endocrinology; hematology; reference interval; sheep; variation

*Corresponding author: e-mail: ioannis.oikonomidis@ed.ac.uk

INTRODUCTION

Clinicopathological investigations are an integral part of the evaluation of health status of ruminants, while they are also considered an objective method to evaluate animals welfare [1]. However, the interpretation of laboratory data is strongly dependent not only on appropriate and accurate species-specific reference intervals (RIs), but also on the understanding of the effect that common biological factors have on clinicopathological analytes.

The effect of several biological factors, such as age, breed, physiological state, and season on clinicopathological analytes has been considerably studied in female sheep [2-7]. Nonetheless, similar studies in rams have not drawn particular scientific attention. However, small ruminant farms only sporadically employ artificial insemination; therefore, rams enact a major role in productivity of farms. Additionally, the great discrepancies in morphometry, physiological states, and management between male and female sheep further underline the need for such sex-specific studies.

The objective of this study was to evaluate the effect of season on hematologic, biochemical, and selected hormonal analytes in adult rams. Moreover, given the reported breed-dependent differences in some of those analytes [8], we hypothesized that an interplay between breed and season is plausible. Therefore, the second objective was to assess whether the effect of season is breed-dependent using rams belonging to two essentially different (in terms of origin, morphometry, and productivity) Greek breeds, namely Chios and Florina.

MATERIALS AND METHODS

This study included 43 clinically healthy rams of Chios (n=26) and Florina (n=17) breeds reared in the Animal Science Institute, Giannitsa, Central Macedonia, Greece. The mean age of the rams was 2.2 years, ranging from 1.5 to 4 years. The experimentation period comprised a whole year during which the rams' health status and welfare were regularly monitored in compliance with the relevant European Union directives. The rams were currently vaccinated and dewormed and were not under any kind of treatment during the month that preceded each blood sampling. Blood samples were collected four times from each ram; in summer and winter solstice and in autumn and spring equinox.

Jugular venipunctures using an 18-gauge needle and a holder were performed in the morning (between 9.00 and 11.00), with the rams being lightly restrained in a standing position. Blood was collected directly into 10mL evacuated tubes containing K_3EDTA and clot activator (BD Vacutainer, BD, Plymouth, UK). The samples in the clot activator containing tubes were allowed to clot for 30 minutes at room temperature, then after centrifugation at 1,800 g for 15 minutes, sera were yielded and were transferred into plain tubes. The samples were placed in a cooler bag and were transported to

the Diagnostic Laboratory, School of Veterinary Medicine, Aristotle University of Thessaloniki, Greece. The hematologic analyses were performed within 4 hours after blood sampling, while serum samples were stored at 4°C and were assayed within 8 hours after blood sampling. Sera for hormonal analyses were stored at -20°C and were examined within 3 days after blood sampling.

Routine maintenance, preparation, adjustment, assay and quality control procedures were conducted as defined in the respective manuals for all analyzers used in this study. Moreover, the same reagent lots were used throughout the study. Complete blood count was performed on the Advia 120 automated hematology analyzer (Siemens Healthcare Diagnostics, Deerfield, USA) selecting the ovine setting of the multispecies software. Manual differential white blood cell counts based on 200 cells were conducted on Giemsa-stained blood smears by two independent observers (I.L.O, M.K.K.). The biochemical analyses were performed using an automated spectrophotometric biochemical analyzer (Flexor E, Vital Scientific N.V., Dieren, The Netherlands), except for globulins and electrolytes. The latter were measured using an electrolyte analyzer (AVL 9180, Roche Diagnostics, Vilvoorde, Belgium), while serum globulins concentration was calculated by subtracting the albumin concentration from the total protein concentration. More information about the methods employed can be found in a recently published article [8]. Serum thyroxine and cortisol concentrations were measured on the Immulite 1000 (Siemens Healthcare Diagnostics, Deerfield, USA), which has been previously validated for thyroxine and cortisol measurement in sheep [8].

In order to study the effect of factors Breed and Season on the mean values of the examined parameters, the Linear Mixed Effects (LME) modeling was used [9]. The optimal fixed component structure was defined through a backward elimination procedure. Graphical validation was used to assess the underlying assumptions of homoscedasticity and normality of residuals of the selected models. All statistical analyses were conducted using the statistical language R (R Foundation for Statistical Computing, Vienna, Austria) and the function lmer from package lme4. In addition, the function step from package lmerTest was used in order to perform backward elimination of all effects of the examined LME. The p-values for the fixed component of the model were calculated from F test based on Kenward-Roger approach in order to get approximate degrees of freedom. For post-hoc analysis, Bonferroni correction (α/m, where m is the number of hypotheses tested) was used in order to derive conclusions. All the tests conducted were two-tailed (non-directional) and a difference was considered as statistically significant when p-value was less than 0.05.

RESULTS

Due to the small variation of basophils and total bilirubin measurements, we decided not to fit an LME model since we could not have extracted meaningful findings. Breed-independent statistically significant seasonal differences were detected in

the mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, lymphocytes, monocytes, platelets, mean platelet volume, plateletcrit, total proteins, glucose, cholesterol, triglycerides, total calcium (tCa), inorganic phosphorus (Pi), potassium, urea, creatinine, alanine transaminase, aspartate transaminase, alkaline phosphatase, γ-glutamyltransferase, and creatine kinase (CK) (Table 1). Conversely, breed-dependent statistically significant differences were observed in red blood cells, hemoglobin, hematocrit, white blood cells, neutrophils, eosinophils, platelet distribution width, albumin, globulins, sodium, lactate dehydrogenase, and thyroxine (Table 2).

Table 1. Mean and standard deviation (SD) of the analytes, for which the effect of season was breed-independent. Rams of Chios (n=26) and Florina (n=17) breeds were used. Values within a row that do not share a common letter differ significantly ($p<0.05$). The bold font indicates the changes that were considered biologically significant either because the mean values were outside the respective reference intervals or because they were arithmetically pronounced. ALT, alanine transaminase; ALP, alkaline phosphatase; AST, aspartate transaminase; CK, creatine kinase; GGT, γ-glutamyltransferase; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MPV, mean platelet volume; PCT, plateletcrit; RDW, red cell distribution width.

Analyte	Unit	Mean (SD)					Reference interval
		Spring	Summer	Autumn	Winter	Year	
MCV	fL	30.5 (2.1) [a]	28.5 (1.9) [b]	28.7 (2.0) [b]	30.6 (2.6) [a]	29.6 (2.4)	25.4 - 34.1
MCH	pg	10.8 (0.9) [a]	10.3 (0.7) [b]	10.5 (0.8) [b]	11.0 (0.8) [c]	10.6 (0.8)	9.0 - 11.9
MCHC	g/dL	35.3 (2.0) [a]	36.2 (1.6) [b]	36.5 (2.1) [b]	35.9 (2.6) [b]	36.0 (1.8)	32.5 - 39.7
RDW	%	18.1 (1.5) [a]	17.7 (1.0) [a]	18.0 (0.9) [a]	18.2 (2.1) [a]	18.0 (1.4)	15.9 - 20.3
Lymphocytes	$10^3/\mu L$	4.8 (1.2) [a]	5.7 (1.4) [b]	5.2 (1.1) [c]	4.4 (1.0) [d]	5.1 (1.3)	2.8 - 9.3
Monocytes	$10^3/\mu L$	0.15 (0.14) [a]	0.49 (0.27) [b]	0.19 (0.17) [a]	0.28 (0.17) [d]	0.28 (0.23)	0 - 0.7
Platelets	$10^3/\mu L$	507 (137) [a]	575 (176) [b]	523 (153) [a]	488 (151) [a]	523 (157)	229 - 823
MPV	fL	6.0 (1.4) [a]	6.0 (1.4) [a]	5.4 (1.3) [b]	6.4 (2.4) [a]	6.0 (1.7)	4.1 - 9.9
PCT	%	0.30 (0.07) [a]	0.33 (0.09) [b]	0.27 (0.07) [c]	0.29 (0.08) [a,c]	0.30 (0.08)	0.16 - 0.49
Total proteins	g/dL	7.3 (0.5) [a]	7.5 (0.7) [b]	7.3 (0.6) [a,b]	6.9 (0.5) [c]	7.2 (0.6)	6.2 - 8.6
Glucose	mg/dL	95 (19) [a]	86 (16) [b]	**112 (22)** [c]	**129 (29)** [d]	105 (27)	64 - 112
Cholesterol	mg/dL	47 (10) [a]	55 (11) [b]	50 (7) [c]	35 (9) [a,d]	46 (12)	28 - 77
Triglycerides	mg/dL	24 (9) [a]	31 (19) [b]	27 (10) [b,c]	24 (13) [a,b]	26 (13)	12 - 56
Total calcium	mg/dL	11.0 (0.6) [a]	10.6 (0.6) [b]	10.4 (0.6) [c]	**9.4 (0.6)** [d]	10.4 (0.8)	8.5 - 12.0
Inorganic phosphorus	mg/dL	8.5 (1.9) [a,b,c]	8.3 (1.6) [b]	8.8 (1.4) [c]	**7.0 (1.6)** [d]	8.1 (1.8)	5.5 - 12.6
Potassium	mEq/L	5.4 (0.6) [a]	5.5 (0.5) [b]	6.1 (0.9) [a,b]	5.2 (0.6) [c]	5.5 (0.7)	4.4 - 7.2
Urea	mg/dL	35 (8) [a]	52 (15) [b]	41 (9) [b]	34 (11) [c]	41 (13)	22 - 65
Creatinine	mg/dL	1.0 (0.1) [a]	1.5 (0.2) [b]	1.6 (0.2) [b]	1.6 (0.3) [b]	1.4 (0.3)	0.8 - 2.0
ALT	U/L	28 (13) [a]	34 (19) [b]	29 (10) [c]	26 (11) [a]	29 (14)	13 - 48
AST	U/L	129 (45) [a]	150 (87) [b]	134 (61) [c]	122 (61) [a]	134 (66)	75 - 203
ALP	U/L	281 (131) [a]	352 (134) [b]	294 (154) [a]	217 (91) [c]	286 (137)	87 - 760
GGT	U/L	52 (11) [a]	56 (13) [b]	53 (14) [a]	49 (12) [c]	52 (13)	17 - 76
CK	U/L	**519 (539)** [a]	**404 (751)** [b]	293 (306) [b]	227 (195) [c]	361 (504)	120 - 395

Table 2. Mean and standard deviation (SD) of the analytes, for which the effect of season was breed-dependent. Rams of Chios (n=26) and Florina (n=17) breeds were used. C: Chios breed; F: Florina breed. Values within a row that do not share a common letter differ significantly ($p<0.05$). The bold font indicates the changes that were considered biologically significant either because the mean values were outside the respective reference intervals or because they were arithmetically pronounced. LDH, lactate dehydrogenase; PDW, platelet distribution width; RBCs, red blood cells; WBCs, white blood cells.

Analyte	Unit	Mean (SD)					Breed-specific reference interval
		Spring	Summer	Autumn	Winter	Year	
RBCs (C)	10^6/μL	10.72 (0.92) [a]	10.94 (0.79) [a]	11.57 (0.85) [b]	10.57 (0.87) [a]	10.95 (0.93)	9.20 - 12.63
RBCs (F)	10^6/μL	11.03 (0.86) [a]	12.47 (1.05) [b]	12.40 (0.87) [a,c]	10.53 (1.41) [b]	11.61 (1.35)	9.01 - 14.24
Hemoglobin (C)	g/dL	11.6 (1.5) [a]	11.4 (0.7) [a]	12.3 (1.1) [b]	11.7 (1.0) [a]	11.8 (1.2)	9.3 - 13.8
Hemoglobin (F)	g/dL	11.7 (0.9) [a]	12.6 (1.0) [b]	12.6 (1.1) [b]	11.2 (0.8) [a]	12.0 (1.1)	9.4 - 14.7
Hematocrit (C)	%	32.7 (3.4) [a]	30.9 (2.2) [b]	33.2 (3.0) [a]	32.2 (2.7) [a,b]	32.3 (2.9)	25.8 - 36.8
Hematocrit (F)	%	33.4 (2.8) [a]	35.7 (2.9) [b]	35.4 (2.5) [b]	31.9 (2.7) [a]	34.1 (3.1)	26.5 - 41.8
WBCs (C)	10^3/μL	9.4 (1.9) [a]	11.4 (1.9) [b]	10.6 (1.6) [c]	9.0 (1.7) [a]	10.1 (2.0)	6.1 - 14.8
WBCs (F)	10^3/μL	8.8 (2.4) [a]	9.8 (2.4) [b]	8.9 (1.8) [a,b]	9.8 (2.5) [b]	9.3 (2.3)	4.2 - 13.9
Neutrophils (C)	10^3/μL	3.7 (1.0) [a]	4.4 (1.3) [b]	4.3 (1.0) [a,b]	3.8 (1.4) [a,b]	4.1 (1.2)	1.3 - 6.9
Neutrophils (F)	10^3/μL	4.0 (1.7) [a]	4.2 (1.2) [a]	4.1 (1.0) [a]	5.5 (1.8) [b]	4.4 (1.5)	0.7 - 6.5
Eosinophils (C)	10^3/μL	0.34 (0.17) [a]	0.27 (0.16) [b,c]	0.33 (0.19) [a,b]	0.17 (0.11) [c]	0.28 (0.17)	0 - 0.64
Eosinophils (F)	10^3/μL	0.24 (0.16) [a]	0.18 (0.13) [a]	0.21 (0.17) [a]	0.08 (0.09) [b]	0.18 (0.15)	0 - 0.48
PDW (C)	%	72.7 (14.9) [a]	72.8 (14.7) [a]	69.7 (18.4) [a]	69.2 (15.4) [a]	71.1 (15.8)	36.8 - 101.3
PDW (F)	%	67.1 (17.6) [a]	76.5 (19.9) [b]	68.5 (15.3) [a,b]	79.8 (17.3) [b]	73.0 (18.0)	37.3 - 111.1
Albumin (C)	g/dL	3.7 (0.2) [a]	3.6 (0.2) [a]	3.7 (0.4) [a]	3.6 (0.2) [a]	3.7 (0.3)	3.2 - 4.1
Albumin (F)	g/dL	3.8 (0.2) [a]	3.8 (0.2) [a]	3.3 (0.8) [b]	3.8 (0.2) [a]	3.7 (0.5)	3.0 - 4.4
Globulins (C)	g/dL	3.5 (0.5) [a]	3.7 (0.6) [a]	3.5 (0.6) [a]	3.2 (0.5) [b]	3.5 (0.6)	2.1 - 4.8
Globulins (F)	g/dL	3.5 (0.5) [a]	3.9 (0.5) [b]	4.2 (0.8) [c]	3.3 (0.4) [a]	3.7 (0.6)	2.2 - 4.7
Sodium (C)	mEq/L	147 (2) [a]	146 (2) [b]	149 (3) [c]	141 (3) [d]	146 (4)	139 - 153
Sodium (F)	mEq/L	146 (1) [a]	147 (2) [a]	150 (2) [b]	144 (3) [c]	147 (3)	139 - 153
LDH (C)	U/L	1292 (257) [a]	1232 (209) [a]	1018 (189) [b]	915 (160) [c]	1116 (256)	760 - 1553
LDH (F)	U/L	1277 (226) [a]	1256 (412) [a]	1154 (123) [a]	1051 (161) [b]	1185 (266)	765 - 1614
Thyroxine (C)	μg/dL	5.7 (1.4) [a]	6.4 (1.6) [a]	**9.2 (2.1)** [b]	7.5 (2.8) [c]	7.2 (2.4)	2.3 - 10.5
Thyroxine (F)	μg/dL	7.6 (1.7) [a,b]	7.9 (1.6) [b]	**9.6 (1.3)** [c]	6.6 (1.8) [a]	7.9 (1.9)	4.0 - 11.6
Cortisol (C)	μg/dL	2.6 (0.9) [a]	3.4 (2.6) [a]	3.9 (1.4) [a]	2.7 (2.5) [a]	3.2 (2.0)	0.9 - 4.7
Cortisol (F)	μg/dL	2.9 (0.8) [a]	2.6 (1.1) [a]	4.9 (1.5) [a]	7.3 (13.7) [a]	4.4 (7.0)	0.8 - 5.1

DISCUSSION

The present study is the first extensive investigation of the effect of season on clinicopathological and selected hormonal analytes in rams. Statistically significant, mostly breed-independent, seasonal differences were detected in almost all of the studied analytes. However, the vast majority of the observed differences were devoid of any clinical significance since the mean values remained well within the respective

RIs throughout the year. On the contrary, the seasonal differences in glucose, CK, tCa, Pi, and thyroxine can be considered clinically relevant given that the mean values were outside the respective RIs (glucose, CK) or, if not, arithmetically pronounced changes were observed (tCa, Pi, thyroxine).

Mean glucose concentration reached and exceeded the upper reference limit in autumn and winter, respectively. The observed mild hyperglycemia in an otherwise healthy population is likely due to catecholamine or corticosteroid-mediated stress. The latter is further justified by the concurrently detected two-fold increase in mean cortisol concentration. Our results are in agreement with those previously published for goats and they are possibly attributed to photoperiod alterations [10,11].

A minimal increase in the mean values of CK was observed during spring and summer. This is a non-specific finding of limited importance, since CK can be greatly affected even by minimal muscle damage [12], and probably reflects an increase in physical activity (e.g. walking, running, fighting) of rams as the daytime is increasing. Interestingly, mild, acute muscle damage attributed to increased physical activity has been previously reported in rams during a controlled reproductive period [13].

The mean values of tCa and Pi remained within the RIs, but they were considerably lower in winter. This is likely related to the reduced daytime since ultraviolet light is necessary for the conversion of 7-dehydrocholesterol to cholecalciferol (vitamin D_3), which, in its active form, promotes hypercalcemia and hyperphosphatemia [14]. Our findings are in agreement with those of previous studies on female sheep reared in similar climate conditions [6,7]. In fact, it has been suggested that supplementation with vitamin D may be needed during winter [7].

Although, seasonal differences in serum thyroxine were breed-dependent, the most striking finding was the higher serum thyroxine concentration (approaching the upper reference limit) observed in both breeds in autumn. This is likely related to ambient temperature [7,15,16] and/or photoperiod alterations [17]. Additionally, in Chios rams, serum thyroxine concentration remained high in winter, whereas in Florina rams returned to spring and summer values. Such breed-dependent differences in thyroid gland responses have already been reported in male sheep [18].

Blood samplings were performed once during every season and we appreciate that as a major limitation of this study. However, we preferred to study a considerable number of analytes in the cost of having a smaller number of repeated measurements. The relatively small sample size might be considered as a second limitation; nonetheless, a substantially larger group of rams reared under controlled conditions is almost infeasible to be found, given the very low ratio of male-to-female sheep (usually 1:50 to 1:100) in contemporary farms.

In conclusion, although statistically significant differences were detected in the vast majority of the studied analytes, a few of them were considered to be of some clinical importance. Therefore, seasonal effect should be considered when evaluating the laboratory results in rams, but it does not appear to essentially influence the

clinicopathological profile of rams reared in the Mediterranean region under similar conditions to our population.

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Authors' contributions

OI was involved in blood sampling and study design, performed the laboratory examinations and wrote the final manuscript. KE and BC were involved in blood sampling, study design, and manuscript review. KKM was involved in study design and supervision, and manuscript review. All authors have approved the final version of the manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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SEZONSKI UTICAJ NA HEMATOLOŠKE, BIOHEMIJSKE I HORMONSKE PARAMETRE KOD OVNOVA DVE RASE

OIKONOMIDIS Ioannis L., KIOSSIS Evangelos, BROZOS Christos, KRITSEPI-KONSTANTINOU Maria

Ispitivanja u kliničkoj patologiji su ključna za evaluaciju zdravstvenog statusa preživara. Pored referentnih parametara koji su specifični za vrstu, efekti uobičajenih bioloških faktora treba da se uzmu u obzir za pravilnu procenu i interpretaciju laboratorijskih nalaza. Cilj studije je bio da se obavi evaluacija sezonskih uticaja na hematološke i biohemijske parametre kao i rezultate ispitivanja tiroksina i kortizona kod odraslih ovnova dve rase. Od svakog ovna uzimano je po četiri uzorka krvi; u svim godišnjim dobima (sezonski) po jedan uzorak. Kompletna analiza krvnih elemenata obavljena je primenom Advia 120 (Siemens Healthcare Diagnostics, USA) aparata, a diferencijalna

leukocitna formula je urađena manuelno. Biohemijska i hormonska analiza je obavljena primenom Flexor E (Vital Svientific, The Netherlands), AVL 9180 (Roche Diagnostics, Belgium) odnosno Immulite 1000 (Siemens Healthcare Diagnostics, USA) aparata. U cilju statističke obrade i analize podataka linearnim modelom (R efakat) , ispitivanje je obavljeno na 43 (26 Chios i 17 Florina) odrasla, klinički zdrava ovna. Kod skoro svih parametara koji su ispitivani, uočene su statistički značajne ($P<0,05$) sezonske razlike koje nisu bile povezane sa rasom. Međutim, kada je obavljana procena ovih razlika u smislu referentnih intervala za svaki parametar, samo za mali broj je moglo da se zaključi da imaju neki biološki značaj. Tačnije rečeno, uočena je umerena hiperglikemija i smanjenje koncentracija ukupnog kalcijuma i neorganskog fosfora tokom zimskog perioda. Isto tako, uočeno je blago povećanje u koncentracijama tiroksina (jesen) i aktivnosti kreatin kinaze (proleće i leto). Na osnovu dobijenih rezultata, može se zaključiti da je potrebno da se pri evaluaciji laboratorijskih nalaza kod ovnova u obzir uzmu sezonski efekti. Međutim, sezonski efekti u toku godine izgleda da nisu izraženi kod ovnova koji se uzgajaju u regionu Mediterana.