# Veterinarija ir Zootechnika

**Volume 78(100)**
**2020**

# Veterinarija ir Zootechnika

LITHUANIAN UNIVERSITY OF HEALTH SCIENCES

**Volume 78(100)**
**2020**

## Aims and Scope

An international scientific journal **"Veterinarija ir Zootechnika"** since 1924 publishes original research and review papers on all aspects of veterinary medicine, food safety and animal sciences. From 1952 to 1994 journal was published under the title: „Acta of Lithuanian Veterinary Academy". After decision of the Research Council of Lithuania under the auspices of the Government of Republic of Lithuania from year 1995 scientific journal „Veterinarija ir Zootechnika" (Vet Med Zoot) was re-established as the Official Organ of the Veterinary Academy (VA) in collaboration with Veterinary Academy, Veterinary Institute, LVA Animal Science Institute, Lithuanian University of Agriculture and Immunology Institute of Vilnius University.

**Issues per Year** – 4. From 2016 - 2.

**The Guide for Authors** can be found of the journal's website https://vetzoo.lsmuni.lt/directions-to-authors

## Submission of Manuscripts

The manuscripts are registered in the Editorial Office of LSMU VA "Veterinarija ir zootechnika" after filling the Manuscripts Registration Form (*Supplement 1*).
One copy of the manuscript should be addressed to the Editorial Office or e-mailed to the following address: evaldas.slyzius@lsmuni.lt
The manuscripts are refereed (*Supplement 2*) at least by two members of the Editorial Board or appointed referees. The authors are expected to take into consideration the remarks and before the final submission make correction or give motivated explanations of discount of the remarks. The amended paper should be sent back to the Editorial Office in two weeks after the submission of reviews to the author.
If two reviews are negative the article is rejected. If one review is negative, the article should be reviewed by additional referee. If the review of the third referee is positive, the final decision is made by the Editorial board.
The final corrected manuscript should be submitted to the Editorial Board together with the signed letter that authors have approved the paper for release and in agreement with its publication (*Supplement 3*).
The paper should include reference that scientific research has been carried out in accordance with the EU and Lithuanian legal acts.

## Publication Information:

„VETERINARIJA ir ZOOTECHNIKA"
A scientific journal and the Official Organ of the Veterinary Academy,
Lithuanian University of Health Sciences (LSMU VA).

ISSN 1392-2130 (Print)
ISSN 2669-2511 (Online)

evaldas.slyzius@lsmuni.lt



Layout Saulius Medžionis
Printed by UAB "Igmovila", Laisvės al. 38A-12, LT-44240 Kaunas, Lithuania. Edition of 10 copies.

# Veterinarija ir Zootechnika

LITHUANIAN UNIVERSITY OF HEALTH SCIENCES

**Volume 78(100)**
**2020**

# Veterinarija ir Zootechnika

LITHUANIAN UNIVERSITY OF HEALTH SCIENCES

**Volume 78(100)**
Pages 1–60
2020

## CONTENTS

# Canine *Trypanosoma evansi* Infection (Surra) in Lahore, Pakistan-Short Communication

**Muhammad Asif[1, 2], Kamran Ashraf[1], Tariq Jamil[3], Nisar Ahmad[1], Sami Ullah[2], Shakeel Hussain[1], Mudassar Iqbal[4], Muhammad Nasrullah[5], Abdul Hameed Khan[6] and Muhammad Usman Ghani[6]**

*[1]Department of Parasitology, University of Veterinary and Animal Sciences, Pakistan*
*[2]Livestock and Dairy Development Department, Government of Punjab, Pakistan*
*[3]Department of Microbiology, University of Veterinary and Animal Sciences, Pakistan*
*[4]University College of Veterinary and Animal Sciences, The Islamia University of Bahawalpur, Pakistan*
*[5]Department of Clinical Medicine and Surgery, University of Veterinary and Animal Sciences, Pakistan*
*[6]Department of Pathology, University of Veterinary and Animal Sciences, Pakistan*

***Keywords:*** *Trypanosoma evansi; surra; canine; Punjab; Pakistan*

***Abstract.*** *Surra caused by Trypanosoma evansi is a well-known infection in camel and equines and to a lesser extent in other domestic and wild animals in Pakistan. The purpose of this study was to evaluate the burden and the precise cause of surra in dogs. A total of 160 blood samples were collected, and 3.75% (6/160) were found positive by microscopic examination and 2.5% (4/160) by PCR. One female (25%) and 3 male (75%) dogs were found positive by PCR. Among them, the 2 positive dogs were from Pakistani bully breed and the other 2 from local/non-descriptive breeds. All 4 dogs were between 2–4 years of age. A more precise study is needed for determination of the associated risk factors with this infection. To the best of our knowledge, this is the first-ever report of specific Trypanosoma evansi detection in dogs in Pakistan.*

## Introduction

*Trypanosoma evansi* is a blood borne parasite belonging to the family *Trypanosomatidae*. It causes the surra disease in domestic and wild animals and is widely present in south Asia, Africa and South America (Aregawi et al., 2019; Luckins, 1988). It rarely causes disease in humans (Joshi et al., 2005). The disease is mechanically transmitted by hematophagous flies from genus *Stomoxys*, *Tabanus* and *Glossina* spp. (Desquesnes et al., 2013). Dogs are highly susceptible to surra depicting sever clinical signs that may lead to death. The diagnostic techniques include direct microscopy of a peripheral blood smear coupled with a serological, e.g., immunofluorescence test (IFAT), an enzyme linked immunosorbent assay (ELISA) and a card agglutination test (CATT) and/or molecular biological based detection and differentiation, e.g., polymerase chain reaction (PCR) (OIE, 2019; Tehseen et al., 2015).

Surra was first discovered in 1880 by Griffith Evans at Dera Ismail Khan district of Khyber Pakhtunkhwa (KPK), Pakistan (Luckins, 1988). It is a well-known disease in camels and equines in Pakistan (Bhutto et al., 2010; Hasan et al., 2006; Hussain et al., 2016; Shah et al., 2004; Shahzad et al., 2010; Tehseen et al., 2017; Tehseen et al., 2015). Recent reports in wildlife have also emerged (Muhammad et al., 2007; Shahid et al., 2013). Canine trypanosomiasis has also been previously reported in the country but identification of the aetiology at a species level have been ignored (Gadahi et al., 2008; Rashid et al., 2008). Lahore is the second largest, cultural and provincial capital in the country and a significant population owns dogs as pet animals (Jafri and Rabbani, 1999). Keeping in view the importance and the increasing trend of dog owning, the current study was designed to investigate infection load, precise aetiology, and update the existing knowledge of trypanosomiasis in dogs in Lahore, Pakistan.

Correspondence to Muhammad Asif and Tariq Jamil, Livestock and Dairy Development Department, Government of Punjab, Lahore 54000, Pakistan and Department of Microbiology, University of Veterinary and Animal Sciences, Lahore 54100, Pakistan
Email: asifvet460@gmail.com and drtariqjamil01@gmail.com

## Materials and Methods

### *Study duration and collection of blood samples*

A total of 160 blood samples were collected from clinically suspected dogs (⩾ 4 months of age) for trypanosomiasis presented at Pet Center outdoor and Department of Parasitology, University of Veterinary and Animal Sciences, Lahore, Pakistan from March 01 till April 30, 2015. The infection was suspected based on a history of intermittent fever, anaemia, fascial and pharyngeal oedema, hind limb paralysis and corneal opacity. Blood (3 mL) was drawn for each sample by a sterile syringe through cephalic or saphenous vein puncture and immediately transferred into sterile EDTA (Ethylenediaminetetraacetic acid) coated blood collection tubes to avoid blood clotting.

### *Microscopic examination*

The blood samples were processed by Giemsa staining followed by a microscopic examination at 1000x for *Trypanosoma* spp. examination as per standard procedures of Department of Parasitology, University of Veterinary and Animal Sciences, Lahore, Pakistan.

***DNA extraction and amplification***

DNA was isolated as described by Britto et al. (1993). Specific primers targeting transferrin protein genes 6 and 7 (ESGA 6/7) were used to amplify a 237 bp fragment. The set composed of a 21-mer forward primer 5′-ACA TTC CAG CAG GAG TTG GAG-3' and a 21-mer reverse primer 5'-CAC GTG AAT CCT CAA TTT TGT-3' (Holland et al., 2001). PCR conditions were as follows: first, a denaturation step of 4 minutes at 94 °C followed by 35 cycles consisting of 1-minute denaturation at 94 ºC, 1-minute primer-template annealing at 55 °C and 1-minute polymerization at 72 ºC. The last extension step lasted for 5 minutes at 72 °C. The PCR products were analyzed on a 1.5 % agarose gel (1 h at 90 V) along with a 100 bp marker stained with ethidium bromide (2 µL/50 mL gel) on an ultraviolet (UV) trans-illuminator (Dolphin-Doc, Wealtec, USA).

**Results**

Of the total 160 samples, 103 (64.4%) were male dogs and 57 (35.5%) were females. The dogs recruited in the study belonged to Afghan kochi (n = 1), Barbarian (n = 1), Boxer (n = 1), Bull dog (n = 1), Bull terrier (n = 1) Pakistani bully (n = 19), Cross breed (n = 10), Doberman (n = 4), German shepherd (n = 52), Labrador (n = 37), Pointer (n = 11), Pug (n = 1), Rottweiler (n = 8), Russian husky (n = 7) and local/non-descript (n = 6). Of these 160 blood samples, only 6 samples (3.75%) showed positive by microscopic examination (Fig. 1). By PCR, only 4 (2.5%) samples were found positive (Fig. 2). Among 4 PCR positive dogs, 3 (75%) were males and 1 (25%) was female. Two dogs were from Pakistani bully breed (1.25%) and the other 2 from local/non-descript (1.25%) breeds. These positive dogs were between 2–4 years of age and were raised as pet dogs at homes separate from other domestic animals. These dogs were fed on commercial pet food and received routine vaccination schedule against infectious diseases (e.g., canine distemper, leptospirosis, infectious canine hepatitis, canine parvovirus infection and rabies).

**Discussion**

Surra is frequently reported in camels and equines and less frequently in bovines, small ruminants, dogs and wildlife in Pakistan. We used microscopy coupled with PCR for species specific detection of trypanosomes. Domestic dogs could possibly get infected and pose threat for zoonotic disease transmission.

In our study, we identified 3.75% (6/160) suspected cases by direct microscopy and 2.5% (4/160) by PCR. Regionally, surra has been reported in dogs as well as other domestic animals from India (up to 19.8%), Iran and Afghanistan (Aref et al., 2013; Hosseininejad et al., 2007; Ravindran et al., 2008). This might be because of hematophagous flies, e.g., *Stomoxys*, *Tabanus* and *Glossina* spp. prevalent in this region (Luckins, 1988). Of the several species of trypanosomes, only *Trypanosoma evansi* has been reported in this region (Ravindran et al., 2008). Infection has not been associated with sex in dogs so far (Chowdhury et al., 2005; Prasad et al., 2015). The 4 PCR positive dogs were between 2–4 years of age, and it is in accordance with previous reports (Lakshmi et al., 2007; Nazifi et al., 2004; Rashid et al., 2008). However, opposing results do exist (Prasad et al., 2015). The other 2 samples might have lost detectable amounts of DNA during the extraction procedure. A higher prevalence of *Trypanosoma evansi* infection was found in Pakistani bully dogs and local/non-descript breeds compared with other selected 13 breeds of dogs. The

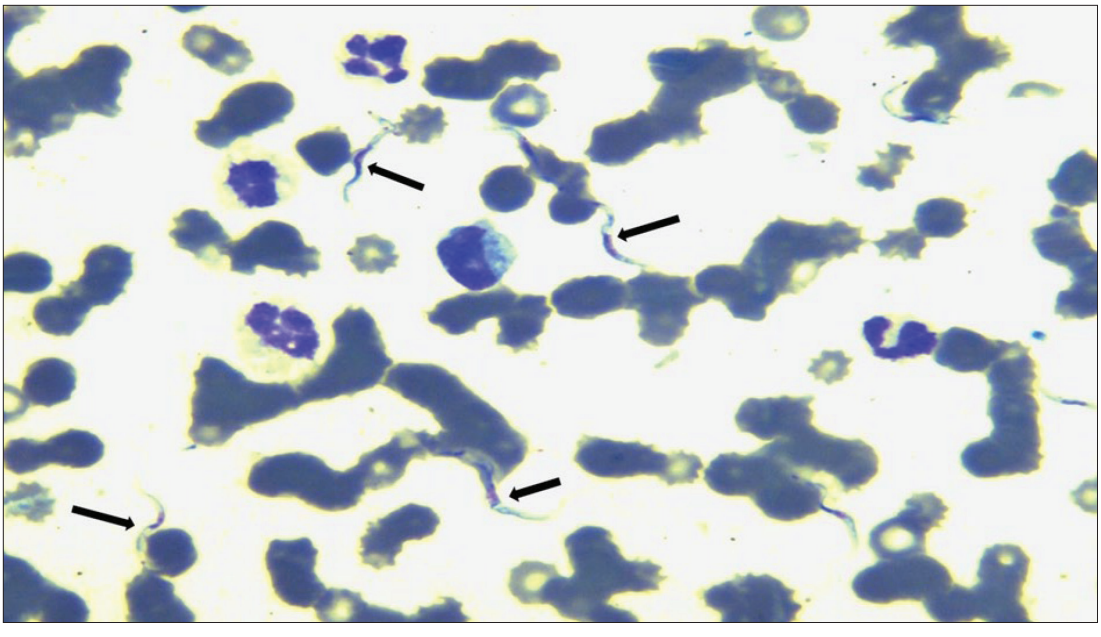


*Fig. 1. Trypanosoma* in a Giemsa-stained blood smear (Arrows showing *Trypanosoma*)

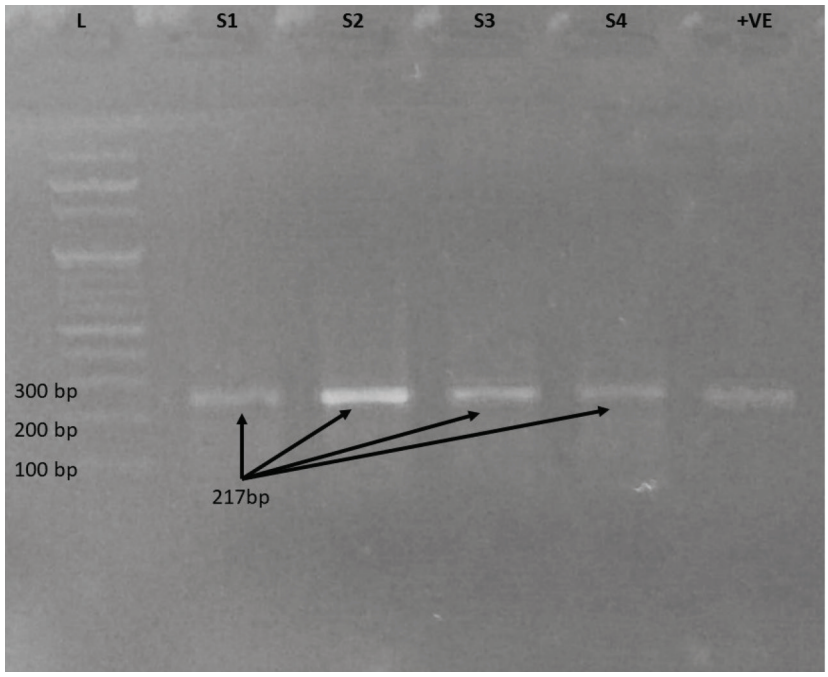



*Fig. 2.* PCR-based detection of *Trypanosoma evansi*

breed of the dog has not been associated with the infection (Chowdhury et al., 2005). A more precise study would be needed for clarification.

We conclude that *Trypanosoma evansi* infection is present in dogs in Pakistan. Dogs between 2–4 years of age can be more susceptible. Breed does not seem to play a role in susceptibility. The present investigation emphasizes the need for the routine screening of blood samples in dogs with a precise data collection of biologically plausible variables to determine the statistical association and the risk for this infection in future. To the best of our knowledge, this report first presents the prevalence of this infection specifically in dogs in Pakistan.

**Ethical statement**

The blood samples were collected as per ethical standards of the Research Board of the University of Veterinary and Animal Sciences, Lahore, Pakistan. Verbal consent was taken from the owners before participation in the study.

**Competing interests**

Authors declare no conflict of competing interests.

**Funding**

Not applicable.

**References**

1. Aref M., Yasin S. M., Bahear W., Ghulam Z., Hastie L., Dennison T., Schauwers W., Büscher P., Deborggraeve S. Canine *Trypanosoma evansi* infection in Afghanistan. Veterinary Parasitology. 2013. 197. P. 638-641.
2. Aregawi W. G., Agga G. E., Abdi R. D., Büscher P. Systematic review and meta-analysis on the global distribution, host range, and prevalence of *Trypanosoma evansi*. Parasites & Vectors. 2019. 12. P. 67.
3. Bhutto B., Gadahi J., Shah G., Dewani P., Arijo A. Field investigation on the prevalence of trypanosomiasis in camels in relation to sex, age, breed and herd size. Pakistan Veterinary Journal. 2010. 30. P. 175-177.
4. Britto C., Cardoso M. A., Wincker P., Morel C. M. A simple protocol for the physical cleavage of *Trypanosoma cruzi* kinetoplast DNA present in blood samples and its use in polymerase chain reaction (PCR)-based diagnosis of chronic Chagas disease. Memórias do Instituto Oswaldo Cruz. 1993. 88. P. 171-172.
5. Chowdhury P., Biswas U., Guha C., Jana P. S. Prevalence of canine trypanosomosis in and around Kolkata city. Indian Veterinary Journal. 2005. 82. P. 797-798.
6. Desquesnes M., Dargantes A., Lai D.-H., Lun Z.-R., Holzmuller P., Jittapalapong S. *Trypanosoma evansi* and surra: A review and perspectives on transmission, epidemiology and control, impact, and zoonotic aspects. BioMed Research International 2013. 321237.
7. Gadahi J., Arijo A., Abubakar M., Javaid S., Arshed M. Prevalence of blood parasites in stray and pet dogs in Hyderabad area: Comparative sensitivity of different diagnostic techniqes for the detection of microfilaria. Veterinary World. 2008. 1. P. 229-232.
8. Hasan M. U., Muhammad G., Gutierrez C., Iqbal Z., Shakoor A., Jabbar A. Prevalence of *Trypanosoma evansi* infection in equines and camels in the Punjab region, Pakistan. Annals of the New York Academy of Sciences. 2006. 1081. P. 322-324.
9. Holland W., Claes F., My L., Thanh N., Tam P., Verloo D., Büscher P., Goddeeris B., Vercruysse J. A comparative evaluation of parasitological tests and a PCR for *Trypanosoma evansi* diagnosis in experimentally infected water buffaloes. Veterinary Parasitology. 2001. 97. P. 23-33.
10. Hosseininejad M., Shirani D., Nabian S., Nassiri S. M., Mazaheri R. *Trypanosoma evansi* in three dogs in Iran. Comparative Clinical Pathology. 2007. 16. P. 69-71.
11. Hussain R., Khan A., Abbas R. Z., Ghaffar A., Abbas G., Ali F. Clinico-hematological and biochemical studies on naturally

infected camels with trypanosomiasis. Pakistan Journal of Zoology. 2016. 48. P. 311-316.
12. Jafri S. A., Rabbani M. Prevalence of canine diseases in Lahore area. Pakistan Veterinary Journal. 1999. 19. P. 40-42.
13. Joshi P. P., Shegokar V. R., Powar R. M., Herder S., Katti R., Salkar H. R., Dani V. S., Bhargava A., Jannin J., Truc P. Human trypanosomiasis caused by *Trypanosoma evansi* in India: the first case report. The American Journal of Tropical Medicine and Hygiene. 2005. 73. P. 491-495.
14. Lakshmi R., Suresh K., Srinivas M., Sreenu M. Canine trypanosomiasis. Indian Veterinary Journal. 2007. 84. P. 186-187.
15. Luckins A. G. *Trypanosoma evansi* in Asia. Parasitology Today. 1988. 4. P. 137-142.
16. Muhammad G., Saqib M., Sajid M., Naureen A. *Trypanosoma evansi* infections in Himalayan black bears (*Selenarctos thibetanus*). Journal of Zoo and Wildlife Medicine. 2007. 38. P. 97-100.
17. Nazifi S., Razavi S., Molazem M. Fatal trypanosomiasis in a dog a case report. Indian Veterinary Journal (India). 2004. 81. P. 824.
18. OIE. *Trypanosoma evansi* infection (Surra). In: Manual of diagnostic tests and vaccines for terrestrial animals. Paris, France. 2019. P. 660-674.
19. Prasad K. L., Kondaiah P., Rayulu V., Srilatha C. Prevalence of canine trypanosomiasis in certain areas of Andhra Pradesh. Journal of Parasitic Diseases. 2015. 39. P. 238-240.
20. Rashid A., Rasheed K., Hussain A. Trypanosomiasis in dog; a case report. Journal of Arthropod-Borne Diseases. 2008. 2. P. 48-51.
21. Ravindran R., Rao J. R., Mishra A. K., Pathak K. M. L., Babu N., Satheesh C. C., Rahul S. *Trypanosoma evansi* in camels, donkeys and dogs in India: Comparison of PCR and light microscopy for detection-short communication. Veterinarski Arhiv. 2008. 78. P. 89-94.
22. Shah S., Phulan M., Memon M., Rind R., Bhatti W. Trypanosomes infection in camels. Pakistan Veterinary Journal. 2004. 24. P. 209-210.
23. Shahid M., Janjua S., Burbach J. S. PCR-Based Detection of *Trypanosoma evansi* infection in semi-captive asiatic black bears (*Ursus thibetanus*). Pakistan Veterinary Journal. 2013. 33. P. 442-445.
24. Shahzad W., Munir R., Khan M. S., Ahmad M. D., Ijaz M., Ahmad A., Iqbal M. Prevalence and molecular diagnosis of *Trypanosoma evansi* in Nili-Ravi buffalo (*Bubalus bubalis*) in different districts of Punjab (Pakistan). Tropical Animal Health and Production. 2010. 42. P. 1597-1599.
25. Tehseen S., Jahan N., Desquesnes M., Shahzad M. I., Qamar M. F. Field investigation of *Trypanosoma evansi* and comparative analysis of diagnostic tests in horses from Bahawalpur, Pakistan. Turkish Journal of Veterinary and Animal Sciences. 2017. 41. P. 288-293.
26. Tehseen S., Jahan N., Qamar M. F., Desquesnes M., Shahzad M. I., Deborggraeve S., Büscher P. Parasitological, serological and molecular survey of *Trypanosoma evansi* infection in dromedary camels from Cholistan Desert, Pakistan. Parasites & Vectors. 2015. 8. P. 415.

*Received 1 June 2020*
*Accepted 23 September 2020*

# Paired left-right asymmetries of the hoof surface in the Pyrenean Catalan yearlings are less marked among hindlimbs

**Pere M. Parés-Casanova[1], Noelia López-Navarro[1]**
*[1]Department of Animal Science, ETSEA, University of Lleida, Lleida, Catalonia, Spain*

**Keywords:** *Cavall Pirinenc Català, equine forelimb, hoof conformation, mechanical hoof stress.*

**Abstract.** *Most published researches that describe an equine hoof form are based on lineal and angular measurements. Here we apply geometric morphometric methods to study symmetries between fore and hind pairs in a pure horse breed. For this purpose, we studied a sample of 27 right-left pairs of distal limbs (12 forelimb and 15 hindlimb pairs) from young Pyrenean Catalan Horses ("Cavall Pirinenc Català"), a meat local breed from Catalonia (Spain), managed under semi-extensive conditions at NE Pyrenees. The outline of the hoof was represented by a set of 2 landmarks and 88 semi-landmarks, which were studied by means of geometric morphometric methods. Surfaces were similar for all limbs but significant shape variations appeared in fluctuating asymmetry, with less contra-paired differences in hindlimbs than between forelimbs. The detected asymmetry of the solar surface in this study may provide, in our opinion, and indicator of mechanical, not environmental, stress, as hooves, acting as robust but malleable pieces, can change shape according to pressure forces. So, if the hoof surface in the Pyrenean Catalan Horse exhibits asymmetry, it may be just a mere plasticity due to pressure forces. This asymmetry would be expressed less markedly in the hind pair, which represents the "motor" part of the equine body and so, much linked to a perfect symmetrical functional oerforming.*

## Introduction

Bilateral symmetry among animals is rarely perfect, i.e., left and right parts, areas, lengths or widths do not measure perfectly equal (Adams et al., 2013). In a population, bilateral asymmetry can occur in three general patterns (Auffray et al., 1999), *viz*: fluctuating asymmetry (FA), directional asymmetry (DA) and antisymmetry (AS). FA is a random deviation from bilateral symmetry, DA involves repeatable deviations from symmetry towards the same side, and AS is bimodal asymmetry that is random with respect to side (Auffray et al., 1999) (Pither & Taylor, 2000) (Mancini et al., 2005).

Geometric morphometrics (GM) is of superior statistical power than traditional morphometric approaches (Reyment, 2010) (Adams et al., 2013). As GM is based on sets of Cartesian coordinates of landmarks (measurement points that are homologous), it preserves the geometry and, thus, can represent shape deformation studies better than linear morphometrics (Reyment, 2010) (Adams et al., 2013). Semi-landmarks are points along such smooth outlines that are initially placed at approximately corresponding positions (Gower, 1975) (Webster & Sheets, 2010)shape variation, and covariation of shape with other biotic or abiotic variables or factors. The resulting graphical representations of shape differences are visually appealing and intuitive. This paper serves as an introduction to common exploratory and confirmatory techniques in landmark-based geometric morphometrics. The issues most frequently faced by (paleo. Their exact locations are ultimately estimated statistically in order to create geometrically homologous landmarks that can be used as if they were anatomical landmarks (Gower, 1975) (Webster & Sheets, 2010).

In the present paper, we apply GM methods to study paired size and shape asymmetries of the hoof outline in a sample of a horse breed.

Correspondence to Pere M. Parés-Casanova, Department of Animal Science. ETSEA. University of Lleida Av. Alcalde Rovira Roure 191. E-25198 Lleida, Catalonia, Spain.
E-mail: peremiquelp@ca.udl.cat

## Materials and methods

### *Study population*

The Pyrenean Catalan Horse ("*Cavall Pirinenc Català*") is a compact, broad-built, predominantly chestnut horse with rather short limbs with a small population (< 4,600) located in the North Eastern part of the Pyrenees, along the Catalan-French border (Infante Gil, 2011). Genetic analysis suggests that it is closely related to the Breton and Comtois breeds (Infante Gil, 2011). Today mainly managed for meat production, it is reared outdoors throughout the year in a free all year-round grazing lifestyle, normally without receiving additional food beside some low-quality straw in winter (Infante Gil, 2011). When selected for sacrifice, yearlings are gathered in paddocks and receive additional feeding with hay and concentrates during the last 2–3 months before slaughter, at 10–12 months of age ("poltres", average body weight 350 kg) (Parés-Casanova, 2011). Animals of this breed do not receive any hoof care, trimming, or shoeing; therefore, their hooves must be considered "normally" shaped (Parés-Casanova, 2011). In the breed, hoof wall problems are rarely found, being the "splay foot", i.e., the hoof wall flaring outwards, the most frequently found non-clinics abnormality (*pers. obs.*).

### *Sampled limbs*

At a commercial abattoir, 27 pairs of distal limbs (12 from forelimbs and 15 from hindlimbs) were obtained from 15 different young unshod Pyrenean Catalan yearlings (< 12 months) immediately after normal slaughter for commercial purposes. The healthy and sound sampled yearlings were unshod; no hooves had received trimming or other podal interventions. At the abattoir, the limbs were disarticulated at the level of the basipodium, being rinsed with water before measurements were performed. Sex and exact days age were not registered. The lack of three forelimb pairs was due to a sampling loss in the abattoir for reasons other than authors' procedures.

### *Extraction of size and shape*

The outline of each hoof was drawn on a sheet and the contour was digitized by means of 88 semi-landmarks and two anatomical landmarks located at the two axial-most positions (most dorsal on the wall and most posterior on the bulb of heel) (Fig. 1). This procedure was repeated for two replicas of each image. Finally, all landmark configurations were superimposed by a Generalized Procrustes Analysis to standardize for position, size, and orientation of the configurations (Adams et al., 2013). The resulting Procrustes shape coordinates were used for further statistical analysis. The reader could directly consult primer references, as (Klingenberg, 2015) and (Savriama & Klingenberg, 2011).

The software TPSUtil v. 1.70 (Rohlf, 2015) was used to prepare and organize the images. Landmarks were digitized twice, using TPSDig v. 1.40 (Rohlf, 2015), by one of the authors (Noelia) in two different blind sessions. In order to compare Procrustes to tangent space distances between individuals, we applied a previous analysis with TPSSmall v. 1.33 (Rohlf, 2015), which reflected a high degree of approximation of shapes in the sample (i.e., shape space) in relation to the reference shape (i.e., tangent space) ($r = 0.999$). For each limb, we examined size and shape variation separately. Size (interpreted as hoof surface) was computed as centroid size (CS, the square root of the sum of squared distances from the landmarks to their centroid) (Webster & Sheets, 2010). A regression of CS (log-transformed) *versus* shape (regression scores) was done to verify if allometry (size-related shape changes) existed. Differences in CS between right-left pairs and between limbs were analysed by a two-way NPMANOVA (Non-Parametric Multivariate Analysis of Variance) using Euclidean distances with 9,999 permutations, and "side" (right/left) and limb (fore and hind) as factors. This Procrustes ANOVA indicated the degrees of freedom, means of squares, F and *p* values for the effects from individuals, sides (expressing DA), individuals* sides (expressing FA) and measurement error (expressing differences between replicas).

Finally, a Canonical Variate Analysis (CVA) was done to compare shapes among four limbs, expressing their differences as Mahalanobis distances (Md). The major purpose of CVA is to maximize differences between groups by producing weighted variables, referred to canonical variates. Typically, the first canonical variates account for most of the variation present. All morphometric analyses were done with MorphoJ v. 1.06c (Klingenberg, 2011) and PAST v. 2.17c software (Hammer et al., 2001). The confidence level was established at 95%.

## Results

### *Allometry*

Significative regression of centroid size *versus* shape appeared with a 2.7% and 3.4% of shape variation explained by size variation for fore and hindlimbs, respectively ($p < 0.001$).

### *Hoof size for each limb*

Sizes were similar among all limbs ($p > 0.05$) (Table 1 and Fig. 2).

### *Hoof size symmetry for fore and hindlimbs*

For hoof size, Procrustes ANOVA showed highly significant variations in symmetry within individuals and sides*individual interaction (FA), but no for sides (DA) (Table 2).

### *Hoof shape symmetry for fore and hindlimbs*

For hoof shape, Procrustes ANOVA showed highly significant variations in symmetry within individuals and sides*individual interaction (FA), but not for sides (DA) (Table 3), with a 2.15% and 23.8% of the variance for FA for fore and hindlimbs, respectively. CVA reflected statistical differences between all

*Table 1.* Results of the two-way NPMANOVA (Non-Parametric Multivariate Analysis of Variance) using Euclidean distances for shape coordinates, and "side" and fore or hindlimb as factors. There appeared no differences among limbs.

| Source | Sum of squares | Degrees of freedom | Mean square | F | *p* |
|---|---|---|---|---|---|
| Fore/hind | 3841 | 1 | 3841.0 | 1.1335 | 0.2949 |
| Right/left | 5124.8 | 1 | 5124.8 | 1.5123 | 0.2262 |
| Interaction | –13727 | 1 | –13727 | –4.0508 | 0.8371 |
| Residual | 3.52E + 05 | 104 | 3388.8 | | |
| Total | 3.48E + 05 | 107 | | | |

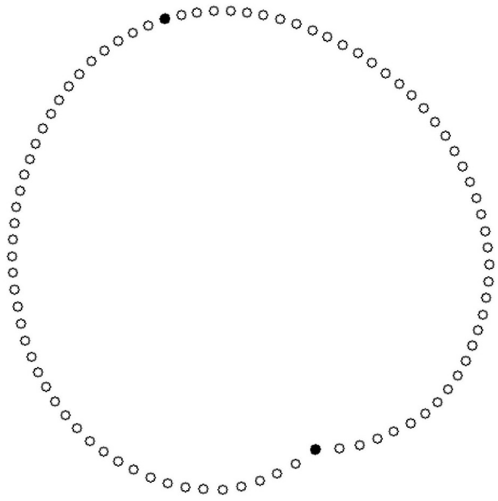


*Fig. 1.* Hoof outline, digitized by a set of 88 semi-landmarks and two anatomical landmarks (black dots) located at the two axial-most positions (most dorsal and most posterior).

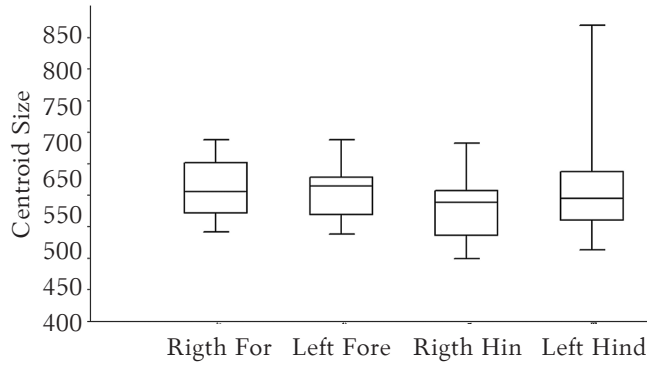



*Fig. 2.* A box plot for centroid sizes (interpreted as hoof surface) for 12 right forelimbs, 12 left forelimbs, 15 right hindlimbs and 15 left hindlimbs in Pyrenean Catalan Horse ("*Cavall Pirinenc Català*").

For each sample, the 25–75% quartiles are drawn using a box. The median is shown with a horizontal line inside the box. The minimal and maximal values are shown with short horizontal lines ("whiskers"). Centroid sizes were statistically similar among all limbs.

*Table 2.* Procrustes ANOVA of hooves size and shape in matching symmetry for 12 forelimb pairs for Pyrenean Catalan Horse ("Cavall Pirinenc Català"). Sums of squares and mean squares are in units of Procrustes distances (i.e. dimensionless).

1/Size

| Effect | Sum of squares | Degrees of freedom | Mean square | F | *p* |
|---|---|---|---|---|---|
| Individual | 73818.127911 | 6710.738901 | 11 | 17.34 | < .0001 |
| Sides | 36.894910 | 36.894910 | 1 | 0.10 | 0.7633 |
| Individual*sides | 4256.736665 | 386.976060 | 11 | 2.69 | 0.0208 |
| Error | 3456.387249 | 144.016135 | 24 | | |

2/Shape

| Effect | Sum of squares | Degrees of freedom | Mean square | F | *p* |
|---|---|---|---|---|---|
| Individual | 0.15618727 | 0.0000806752 | 1936 | 2.12 | < .0001 |
| Sides | 0.00692511 | 0.0000393472 | 176 | 1.04 | 0.3627 |
| Individual*sides | 0.07351831 | 0.0000379743 | 1936 | 1.54 | < .0001 |
| Error | 0.10406824 | 0.0000246374 | 4224 | | |

Note: sides = directional asymmetry (DA); individual*sides = fluctuating asymmetry (FA).

*Table 3.* Procrustes ANOVA of hooves size and shape in matching symmetry for 15 hindlimb pairs from Pyrenean Catalan Horse ("*Cavall Pirinenc Català*"). Sums of squares and mean squares are in units of Procrustes distances (i.e., dimensionless).

1/Size

| Effect | Sum of squares | Degrees of freedom | Mean square | F | *p* |
|---|---|---|---|---|---|
| Individual | 182141.272015 | 13010.090858 | 14 | 2.86 | 0.0293 |
| Sides | 10297.793839 | 10297.793839 | 1 | 2.27 | 0.1545 |
| Individual*sides | 63649.296804 | 4546.378343 | 14 | 22.10 | < .0001 |
| Error | 6172.944814 | 205.764827 | 30 | | |

2/Shape

| Effect | SS | MS | df | F | *p* |
|---|---|---|---|---|---|
| Individual | 0.16326655 | 0.0000662608 | 2464 | 1.55 | < .0001 |
| Sides | 0.00761430 | 0.0000432631 | 176 | 1.01 | 0.4411 |
| Individual*sides | 0.10524435 | 0.0000427128 | 2464 | 1.36 | < .0001 |
| Error | 0.16521947 | 0.0000312916 | 5280 | | |

Note: sides = directional asymmetry (DA); individual*sides = fluctuating asymmetry (FA).

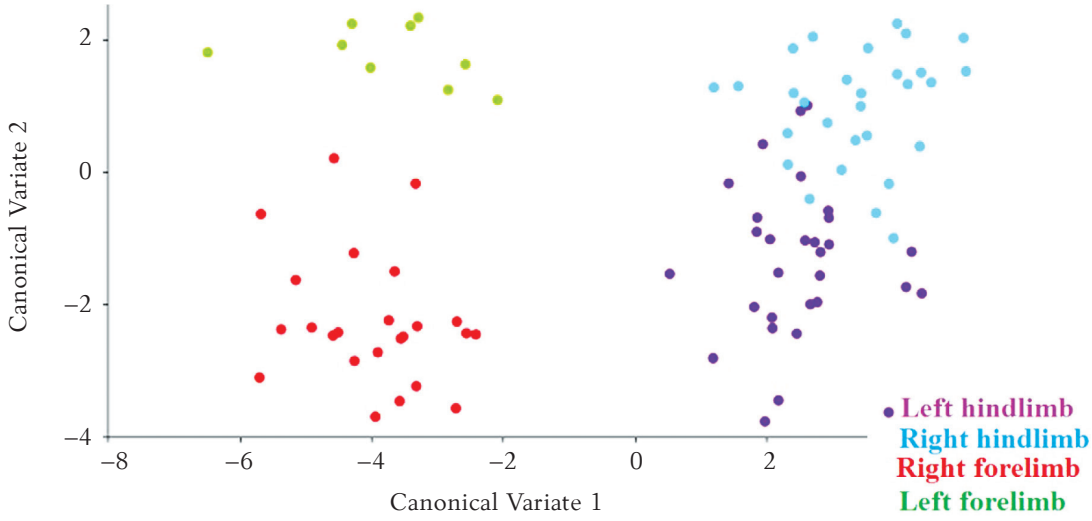



*Fig. 3.* Canonical Variate Analysis for 12 forelimb pairs and 15 hindlimb pairs shape in Pyrenean Catalan Horse ("*Cavall Pirinenc Català*").
It reflected statistical differences between pairs ($p < 0.05$), and left and right hindlimbs appeared less asymmetrical between them.

limbs, although hindlimb pair showed less asymmetry (Md = 2.735) than forelimb pair (Md = 3.651) ($p$ values from 10,000 permutation rounds < 0.001) (Fig. 3).

## Discussion

The study aim was to assess hoof size and shape asymmetries on the solar surface in a local yearling breed maintained under extensive management. Having not care of feet, conclusions of this research can express natural horse hoof wearing. We applied geometric morphometric methods to study asymmetries.

Few studies have evaluated asymmetries using geometric morphometric methods in horses. The more symmetrical certain characters are, the fitter the individual is said to be; thus, increased levels of stress supposedly disrupt developmental processes and correlate with increased levels of asymmetry (de Coster et al., 2013). Detected fluctuating asymmetry of the solar surface in our study may provide, in our opinion, and indicator of mechanical, not environmental, stress. In fact, hooves are rigid pieces that, as a robust but malleable tissue, can change shape according to pressure forces. So, if hoof surface in the Pyrenean Catalan Horse exhibits asymmetry, it may be just a mere plasticity due to pressure forces. This plasticity would be expressed more uniformly in the hind pair, the "motor" part of the horse body. Moreover, larger solar surfaces (measured by centroid size) had different shapes, something logical as horse mass influences hoof morphology (Leśniak et al., 2019).

In conclusion, horses' hooves are not perfectly symmetric and actually do not form mirrored pairs with their opposing hoof, at least in yearlings of Pyrenean Catalan Horse. Among other age group or elite performance breed, data could be totally different. It has to be studied in further studies.

## Conflicts of interest

The authors declare no conflicts of interest.

## Acknowledgements

We thank MAFRISEU SA for providing access to the legs. We are also grateful to anonymous reviewers for their insightful recommendations.

## Supporting information

The contents of all supporting data are the sole responsibility of the authors.

## References

1. Adams, D. C., Rohlf, F. J., & Slice, D. E. (2013). A field comes of age: geometric morphometrics in the 21st century. Hystrix, 24(1), 7–14. https://doi.org/10.4404/hystrix-24.1-6283
2. Auffray, J. C., Debat, V., & Alibert, P. (1999). Shape asymmetry and developmental stability. In J. C. M. Mark A.J. Chaplain, G.D. Singh (Ed.), On growth and form: spatio-temporal pattern formation in biology (Issue 1, pp. 309–324). John Wiley and Sons Ltd.
3. de Coster, G., van Dongen, S., Malaki, P., Muchane, M., Alcántara-Exposito, A., Matheve, H., & Lens, L. (2013). Fluctuating Asymmetry and Environmental Stress: Understanding the Role of Trait History. PLoS ONE, 8(3), 1–9. https://doi.org/10.1371/journal.pone.0057966
4. Gower, J. C. (1975). Generalized procrustes analysis. Psychometrika, 40(1), 33–51.
5. Gunz, P., & Mitteroecker, P. (2013). Semilandmarks: A method for quantifying curves and surfaces. Hystrix, 24(1), 103–109. https://doi.org/10.4404/hystrix-24.1-6292
6. Hammer, Ø., Harper, D. A. T., & Ryan, P. D. (2001). PAST v. 2.17c. Palaeontologia Electronica, 4(1), 1–229.
7. Infante Gil, N. (2011). Caracterización y gestión de los recursos genéticos de la población equina de carne del Pirineo

Catalán (Cavall Pirinenc Català): interrelacion con otras razas cárnicas españolas. Universitat Autònoma de Barcelona.
8. Klingenberg, C. P. (2011). MorphoJ: An integrated software package for geometric morphometrics. Molecular Ecology Resources, 11(2), 353–357. https://doi.org/10.1111/j.1755-0998.2010.02924.x
9. Klingenberg, C. P. (2015). Analyzing fluctuating asymmetry with geometric morphometrics: concepts, methods and applications. Symmetry, 7, 843–934. https://doi.org/10.3390/sym7020843
10. Leśniak, K., Whittington, L., Mapletoft, S., Mitchell, J., Hancox, K., Draper, S., & Williams, J. (2019). The Influence of Body Mass and Height on Equine Hoof Conformation and Symmetry. Journal of Equine Veterinary Science, 77, 43–49. https://doi.org/https://doi.org/10.1016/j.jevs.2019.02.013
11. Mancini, S., Sally, S. L., & Gurnsey, R. (2005). Detection of symmetry and anti-symmetry. Vision Research, 45(16), 2145–2160. https://doi.org/10.1016/j.visres.2005.02.004
12. Parés-Casanova, P. M. (2011). A Nonlinear Model for Estimating Hoof Surface Area in Unshod Meat-type Horses. Journal of Equine Veterinary Science, 31(7), 379–382. https://doi.org/10.1016/j.jevs.2011.03.020
13. Parés-Casanova, P. M., Samuel, O. M., & Pelegrín, J. (2016). Weight equality in euthyroid young heavy horses-a postmortem pilot study. Veterinarija Ir Zootechnika, 74(96).
14. Pither, J. P., & Taylor, D. (2000). Directional and fluctuating asymmetry in the black winged damselfly Calopteryx maculata (Beauvois) (Odonata: Calopterygidae). Canadian Journal of Zoology, 78, 1740–1748.
15. Reyment, R. A. (2010). Morphometrics: An Historical Essay. In Morphometric for Nonmorphometricians (Vol. 124, pp. 9–25). https://doi.org/10.1007/978-3-540-95853-6
16. Rohlf, F. J. (2015). The tps series of software. Hystrix, 26(1), 9–12. https://doi.org/doi:http://dx.doi.org/10.4404/hystrix-26.1-11264
17. Roland, E., Stover, S. M. S. M., Hull, M. M. L., & Dorsch, K. (2003). Geometric symmetry of the solar surface of hooves of thoroughbred racehorses. American Journal of Veterinary Research, 64(8), 1030–1039. https://doi.org/10.2460/ajvr.2003.64.1030
18. Savriama, Y., & Klingenberg, C. P. (2011). Beyond bilateral symmetry: geometric morphometric methods for any type of symmetry. BMC Evolutionary Biology, 11(1), 280. https://doi.org/10.1186/1471-2148-11-280
19. Webster, M., & Sheets, H. D. (2010). A practical introduction to landmark-based geometric morphometrics. In J. A. and G. Hunt (Ed.), Quantitative Methods in Paleobiology (pp. 163–188). The Paleontological Society.
20. Zelditch, M. L., Swiderski, D. L., & Sheets, H. D. (2004). Geometric morphometrics for biologists: a primer. Elsevier Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-778460-1.X5000-5

*Received 27 August 2020*
*Accepted 11 October 2020*

# Temperature-humidity index – an indicator for prediction of heat stress in dairy cows

**Dimo Dimov[1], Toncho Penev[1] and Ivaylo Marinov[2]**
[1]*Department of Applied Ecology and Animal Hygiene, Faculty of Agriculture, Trakia University, Bulgaria*
[2]*Department of Animal Husbandry – Ruminants and Dairy Farming, Faculty of Agriculture, Trakia University, Bulgaria*

***Keywords:*** *temperature-humidity index; heat stress; dairy cows*

***Abstract.*** *The growing interest in the thermal comfort of dairy cows is justified, not only in countries located in tropical zones, but also in zones with temperate climate where high ambient temperatures are becoming a problem. The temperature-humidity index (THI) is a value representing the combined effect of air temperature and humidity associated with the level of heat stress. THI values lower than 72 mean that the cow's body is in favourable environmental conditions and is not subject to heat stress. At THI values of 75 to 78, the animal organism is under heat stress, but the mechanisms of thermoregulation still manage to cope, while at THI over 78 it is assumed that the stress is so high that it is impossible to maintain the thermoregulatory mechanisms or normal body temperature.*

## Introduction

Climate change, defined as a long-term imbalance of normal climatic conditions such as temperature, wind and rainfall specific for a particular region, is likely to be one of the major challenges facing humanity this century (Bertocchi et al., 2014). In the course of climate change, it is suggested that, even in regions traditionally characterized by less extreme climatic conditions, cows will face temperatures beyond their "comfort zone" (Brügemann et al., 2012). The growing interest in the thermal comfort of farm animals is justified, not only in countries located in tropical zones, but also in zones with temperate climate where high ambient temperatures are becoming a problem (Segnalini et al., 2013; Nardone et al., 2010). In addition to climate change, the problem of thermal comfort in dairy cows is exacerbated by an increase in their sensitivity to heat stress due to increased milk yield, thereby reducing the temperature threshold at which cows respond by reducing milk yield (Berman, 2005). This is due to the fact that the released metabolic heat increases when the productivity of animals increases.

Heat stress directly and indirectly affects feed intake, cow's body temperature, metabolic processes, feed utilization efficiency, milk productivity, reproductive function, cow behavior, and disease risk (Kadzere et al., 2002; West, 2003; Jordan, 2003; Cook et al., 2007; Rhoads et al., 2009; Bernabucci et al., 2010).

Most studies of thermal stress in animal husbandry are based on temperature and relative humidity (Igono and Johnson, 1990; Bouraoui et al., 2002; St-Pierre et al., 2003; West, 2003; Correa-Calderon et al., 2004), since data on the amount of thermal radiation received from animals, wind speed and rainfall are not publicly available. On the other hand, temperature and humidity data can be taken from ordinary weather stations located near the site.

Correspondence to Toncho Penev, Department of Applied Ecology and Animal Hygiene, Faculty of Agriculture, Trakia University, 6000 Stara Zagora, Bulgaria. E-mail: tonchopenev@abv.bg

## Temperature-humidity index

The tolerance of animals to high air temperatures depends on the amount of water vapour in the air as this affects the rate of heat loss by evaporation. For the study of heat stress in animal husbandry, the THI is a commonly used bioclimatic index (Hahn et al., 2003). Conceptually, it is difficult to determine whether the THI is best suited to measure the heat stress in dairy cows. Other indices are also formulated empirically and often without reference to the body temperature of cattle. Nevertheless, the original THI and several variations of it have been widely used to assess the degree of heat stress in dairy cows (Mader et al., 2006; Bohmanova et al., 2007; Morton et al., 2007). The temperature-humidity index is expressed as a single value representing the combined effect of air temperature and humidity, which is usually used to estimate the degree of thermal discomfort in dairy cows (Armstrong, 1994). Different species of animals and humans, on the other hand, have different sensitivity to ambient temperature and humidity (Yousef, 1985).

Initially, this index was used only for humans (Thom, 1959), but it was quickly adopted and began to be used in different species of animals (Lendelova and Botto, 2011). Over the last 50 years, this index has undergone numerous modifications in terms of the measurement range to respond adequately to the level of heat stress in dairy cows. There is a significant difference between perceptions of human and cattle in particular. This also applies with respect to the THI. The different variants of this index rely on different weights of the individual components included in its determination. For example, in humans the effect of a temperature measured with a wet thermometer on comfort is almost 6 times greater than that measured with a dry thermometer, whereas in cattle this effect

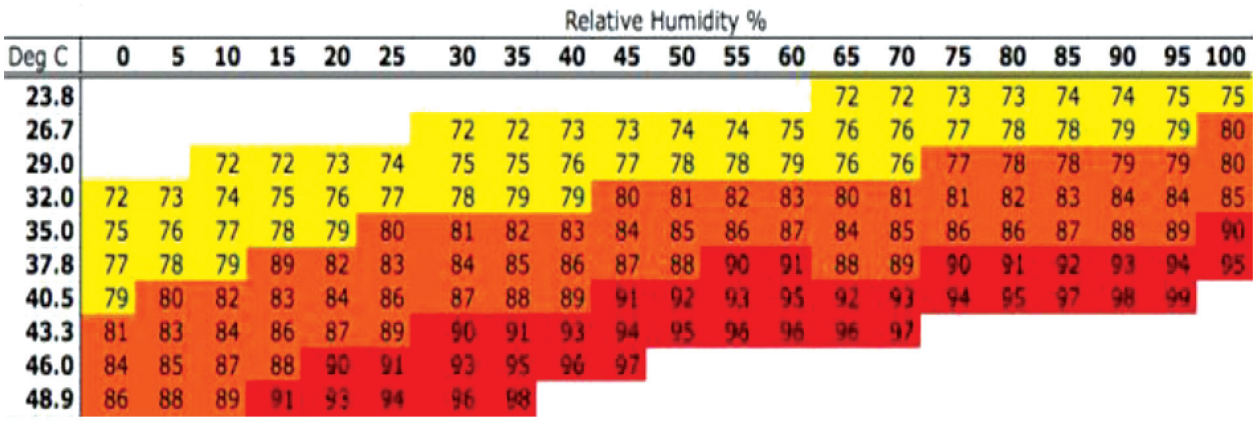
is only about twice as large. This difference is reflected in the evaporation capacity. Humans can dissipate about 190% of their metabolic heat by evaporation, while cattle can dissipate only 105% (Bianca, 1962). According to Collier et al. (2006), this index is used to evaluate the environmental conditions that affect animals. The disadvantage of this index according to the authors is that it does not include the influence of solar radiation. Mahdy et al. (2014), however, consider this index to be one of the most important indicators showing the overall comfort of dairy cows. According to them, the THI is a good tool for determining the occurrence of heat stress.

When dairy cows are subjected to heat stress, they respond by increasing respiration rate and rectal temperature (Omar et al., 1996); in addition, panting, slowed heart rate, and profuse sweating are observed (Blazquez et al., 1994) as well as reduced feed intake (National Research Council, 1989), all of which lead to reduced milk production (Abdel-Bary et al., 1992). Heat stress is associated with several important changes in the behaviour of cattle. When the air temperature is increased from 25 to 40 °C, feed intake is decreased by 46%, rumination is decreased by 22%, standing is increased by 34%, drinking is increased by 30% and locomotion activity is decreased by 19% (Tapki and Sahin, 2006). In dairy cows subjected to heat stress, changes in milk composition, somatic cells count and mastitis incidence have been observed (Du Preez et al., 1990). Studies also show that levels of thyroid hormones and cortisol are affected by heat stress (Wise et al., 1988; Muller et al., 1994). Decreased dry matter intake and subsequent decline in milk productivity are signs of heat stress in lactating cows (Beede and Collier, 1986). Under heat stress, metabolic acidosis and metabolic alkalosis associated with bicarbonates are observed, as well as respiratory acidosis and respiratory alkalosis associated with partial pressure of carbon dioxide ($CO_2$) (Dale and Brody, 1954). Heat stress increases the partial pressure of oxygen in the blood ($O_2$) due to increased alveolar ventilation (Hales and Findlay, 1968) and urinary creatinine concentrations (Thompson, 1973), suggesting muscle catabolism. Mitra et al. (1972) found that the plasma concentration of growth hormone and its rate of secretion decrease at high temperatures (35 °C). Igono et al. (1988) indicate that growth hormone concentrations in the milk of low, medium and high productive cows decrease when the temperature-humidity index exceeds 70 and suggest that this is due to inhibition of growth hormone production in order to reduce production of metabolic heat. McGuire et al. (1991) found that the reduction in plasma concentrations of growth hormone were not observed in cows at thermoneutral conditions, fed with the same one ration under the two different environmental conditions. In an experiment with lactating cows, Johnson et al. (1988) reported a decrease in thyroid hormones triiodothyronine and thyroxine in response to heat stress, which they attributed to attempts to reduce the production of thermal energy in the cow. Earlier, Alvarez and Johnson (1973) reported that in dairy cows exposed to heat stress a higher concentration of epinephrine and norepinephrine into the blood plasma were observed. Heat stress affects both energy and water metabolism (Silanikove, 1992), increasing plasma and extracellular fluid. Cows under heat stress conditions tend to have an increased water content in the rumen, as a result of accelerated rumen exchange of liquid fractions (Silanikove, 1989). Heat stress reduces the mobility of the reticulum and slows down the rumination process (Attenberry and Johnson, 1968). Berman et al. (1985) reported a decrease in thermoregulatory ability under heat stress in dairy cows, which increased seasonal depression in the birth rate (Al-Katanani et al., 1998). Wilson et al. (1998) state that heat stress leads to a decrease in peripheral estradiol-17β concentrations.

Badinga et al. (1993) found that heat stress at the beginning of ovulation reduced the diameter and volume of the dominant follicle. Wolfenson et al. (1997) found that heat stress from day 3 to day 5 of the estrous cycle increased androstenedione and decreased estradiol-17β concentrations in the follicular fluid of the dominant follicle. Hansen and Arechiga (1999) attributed to heat stress physical lethargy, difficulty in detecting oestrus, and a reduction in the number of cows eligible for embryo transfer. In addition, Hansen (1997) reported that heat stress in summer in hot regions was the cause of deteriorating fertility of breeding sires. Ingraham et al. (1974) argued that to optimize conception, heat stress must be minimized at least 12 days before conception. Heat stress also adversely affects the ovum, sperm in the reproductive tract, embryo development, and leads to a change in hormonal balance (Thatcher, 1974).

The THI reports the combined effect of temperature and relative humidity on physiological, productive, etc. indicators in cows. Based on the reported effects of different THI values in dairy cows, a scale was developed representing different zones with THI values associated with different degrees of risk of temperature stress (Fig. 1).

Fig. 1 shows that THI values lower than 72 mean that the cow's body is in favourable environmental conditions and is not subjected to heat stress. At THI values of 75 to 78, the animal organism is under heat stress, but the mechanisms of thermoregulation still manage to cope, while at THI values over 79 it is assumed that the stress is so high that it is impossible to maintain the thermoregulatory mechanisms or normal body temperature. The prevailing view is that milk production begins to decline when THI reaches a value above 72 (this corresponds to a temperature of 25 °C and a humidity of 50%) and this index value is taken as an upper limit (Igono et al., 1992; Ravagnolo et al., 2000). Upadhyay et al. (2009) conclude that high THI values have a negative impact on cow's milk



| Deg C | Relative Humidity % | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 5 | 10 | 15 | 20 | 25 | 30 | 35 | 40 | 45 | 50 | 55 | 60 | 65 | 70 | 75 | 80 | 85 | 90 | 95 | 100 |
| 23.8 | | | | | | | | | | | | | | 72 | 72 | 73 | 73 | 74 | 74 | 75 | 75 |
| 26.7 | | | | | | | 72 | 72 | 73 | 73 | 74 | 74 | 75 | 76 | 76 | 77 | 78 | 78 | 79 | 79 | 80 |
| 29.0 | | | 72 | 72 | 73 | 74 | 75 | 75 | 76 | 77 | 78 | 78 | 79 | 76 | 76 | 77 | 78 | 78 | 79 | 79 | 80 |
| 32.0 | 72 | 73 | 74 | 75 | 76 | 77 | 78 | 79 | 79 | 80 | 81 | 82 | 83 | 80 | 81 | 81 | 82 | 83 | 84 | 84 | 85 |
| 35.0 | 75 | 76 | 77 | 78 | 79 | 80 | 81 | 82 | 83 | 84 | 85 | 86 | 87 | 84 | 85 | 86 | 86 | 87 | 88 | 89 | 90 |
| 37.8 | 77 | 78 | 79 | 89 | 82 | 83 | 84 | 85 | 86 | 87 | 88 | 90 | 91 | 88 | 89 | 90 | 91 | 92 | 93 | 94 | 95 |
| 40.5 | 79 | 80 | 82 | 83 | 84 | 86 | 87 | 88 | 89 | 91 | 92 | 93 | 95 | 92 | 93 | 94 | 95 | 97 | 98 | 99 | |
| 43.3 | 81 | 83 | 84 | 86 | 87 | 89 | 90 | 91 | 93 | 94 | 95 | 96 | 96 | 96 | 97 | | | | | | |
| 46.0 | 84 | 85 | 87 | 88 | 90 | 91 | 93 | 95 | 96 | 97 | | | | | | | | | | | |
| 48.9 | 86 | 88 | 89 | 91 | 93 | 94 | 96 | 98 | | | | | | | | | | | | | |

*Fig. 1.* Temperature-humidity index.
Source: Pennington and van der Deven (2010).

productivity. The data obtained from the University of Arizona show that high-yielding cows lower their milk yield at the THI value of around 68 (Zimbelman et al., 2009). According to Bouraoui et al. (2002), the productivity of dairy cows is not affected by THI values from 35 to 72, but when raised above these values, the authors found that a decrease in milk protein was observed. Ravagnolo et al. (2000), in their study, found a decrease in the milk yield and milk fat by 0.012 kg and 0.2 kg, respectively, at an increase of the THI with each unit above value of 72, and the time that animals spend in lying and resting is reduced by up to 3 h per day when the THI remains at a value above 72 more than 10 h per day (Cook et al., 2007). According to Bouraoui et al. (2002), an increase in the THI from 68 to 78 leads to a decrease in the daily milk yield per cow by 0.41 kg for each increase of the THI with a unit over 69. Geers et al. (2014) summarize that THI values between 60 and 65 should be taken as the lower limit of heat stress, as above these values an adverse effect on milk productivity and conception rate are being reported. Silanikove (2000) considers that THI values above 80 represent an upper critical limit for the survival of ruminants. Based on a study conducted in Scotland, high THI values influence the quantity and composition of milk produced, depending on whether the cows were housed outdoors or indoors. The intensity of solar radiation also has affected productivity, while moderate winds have helped to mitigate the negative effects of heat stress (Hill and Wall, 2015). In environmental conditions of South Bulgaria in a one-year study, Dimov et al. (2017) found that in the summer season values specific for heat stress occurrence in dairy cows were reported to be average daily above 75. A known risk of such conditions in spring is daily average over 69. The daytime THI averages were highest in the resting area during the summer in all three studied farms with small differences from 73.86 to 74.48, followed by spring season, where variations in daily averages were from 66.79 to 68.85. Keown and Grant (1999) found that the lethal limit for dairy cows starts at 38° C and a relative air humidity of 80%. According to these authors, even at relatively low air temperatures, cows are under heat stress at a high relative humidity. An air temperature of 31 °C and 40% relative humidity are equivalent to 27 °C and 80% relative humidity (corresponding to THI 78) according to Rao et al. (2014). Vitali et al. (2009) consider that values 77 and 87 should be taken as the upper minimum and upper maximum critical THI values. Above these values, they report increased mortality in cows housed under conditions of an intensive production system. Mader et al. (2006) include both air velocity and solar radiation to THI. The authors found that for each 1 m/s increase in air velocity, the THI decreased by 1.99 units, and when the solar radiation intensity decreased by 100 W/m, the THI decreased by 0.68 units.

Table 1 shows the THI values that indicate the stress levels at which cows respond to and the symptoms observed (Chase, 2006).

The author believes that the severity of heat stress is conditioned by many factors, but the key ones are:

- air temperature and humidity;
- the length of time cows are subjected to heat stress;
- the degree of temperature drop during the night to cool animals;
- airflow and ventilation condition;
- the size of the cow, the breed and the colour of the coat;
- availability and accessibility to water.

The sensitivity of cattle to heat stress increases as milk yield increases (Berman, 2005). This is due to the fact that the metabolic heat released increases as the productivity of animals increases. Studies have shown that as milk yield increases from 35 to 45 kg per day, the heat stress threshold decreases by 5 °C (Berman, 2005). Even a slight increase in ambient temperature can cause an increase in the standing time (Smith et al., 2012). Reducing the resting time leads to reduced milk production (Grant, 2007; Bach et al., 2008).

*Table 1.* Effect of heat stress on dairy cows (Chase, 2006)

| THI | Level of stress | Comments |
|---|---|---|
| < 72 | None | |
| 72–79 | Mild | Dairy cows will adjust by seeking shade, increasing respiration rate and dilation of blood vessels. The effect on milk production will be minimal. |
| 80–89 | Moderate | Both saliva production and respiration rate will increase. Feed intake may be depressed and water consumption will increase. There will be an increase in body temperature. Milk production and reproduction will be decreased. |
| 90–98 | Severe | Cows will become very uncomfortable due to high body temperature, rapid respiration (panting) and excessive saliva production. Milk production and reproduction will be markedly decreased. |
| > 98 | Danger | Potential cow deaths can occur. |

Herbut and Angrecka (2012) recommended the setting of THI values for different zones separately, and not for the entire building, which would make it possible to provide more suitable zones for animals during heat waves. In addition, this will help to determine the requirements for the construction of new buildings with appropriate ventilation systems, both in technical terms and localization of these facilities within the building. Dimov et al. (2017) found significant differences in the values of the THI outdoor and in the rest area of animals in the building. The differences were in the range of 2.38 °C to 0.09 °C, respectively, for spring and winter. This indicates that the semi open free-stall barns for dairy cows, especially the area above the stalls, do not provide comfortable temperature conditions. In reporting the comfort indices, it has been found that the percentage of cows lying in stalls (cow comfort index (CCI)) decreased by 12.45% at values of the THI of ⩾ 58 to ⩽ 68 to values above 75. More indicative is the SUI (stall usage index, showing how many cows use boxes), which shows that of all cows in the group that are not feeding only 35.43% prefer to lie in stalls at the values of the THI above 75. The rest of cows prefer to stand upright not only in stalls (CCI – 64.98%), but also in other technological zones. In the same study, negative and statistically significant regressions were found between the THI, the CCI and the SUI, which means that an increase in THI values with 1 above 68 would result in a decrease in SUI values of 1.41% and, in the CCI, by 0.84%. An increase in the THI above 68 results in a slight tendency for an increase in the percentage of cows standing upright in stalls. Cows prefer to stay in other zones where they possibly can get cooler (Dimov et al., 2017). Grant et al. (2012) indicate that dairy cows have a strong behavioural need for complete rest. Lactating cows are highly motivated to stand lying for about 12 hours per day (Cook et al., 2005; Drissler et al., 2005; Gomez and Cook, 2010). Cook et al. (2007) noted that when the THI increased from 56 to 74, lying time decreased from 10.9 to 7.9 hours per day, and standing in alleys increased from 2.6 to 4.5 hours per day. Lameness and hoof lesions increased significantly with the extension of time standing upright. Like Collier et al. (2011), they report that cow activity has shifted around THI 68, necessitating the use of more aggressive strategies to reduce heat stress than traditionally used. To solve this problem, it is advisable to provide cooling facilities over the more important areas of the building, additionally to over feeders, stalls or resting areas for cows. To save electricity when using cooling equipment in livestock buildings, they can be set for operation only in periods reaching risky levels of the THI.

## Conclusion

Given that global temperature of earth is increasing on an annual basis and the trend is to increase further in the future, heat stress will become a permanent obstacle for dairy cattle farming and not only. Predicting heat stress and responding appropriately and timely to this problem can significantly reduce its negative effects. The temperature-humidity index is an excellent aid to dairy cattle farmers. All the necessary data to determine this index are readily available and no special skills are needed to calculate it, as any farmer could, if desired, get this data and determine the temperature-humidity index for their farm.

## References

1. Abdel-Bary H.H., Mahmoud M.M., Zaky H.I., Mohamed A.A. Effects of season and month of calving on estrous performance, services conception and milk yield of Friesian cows in Egypt. Egypt. J. Anim. Prod. 1992, 29 (2): 229–253.
2. Al-Katanani Y.M., Webb D.W., Hansen P.J. Factors affecting seasonal variation in non-return of lactating dairy cows. J. Dairy Sci. 1998, 81: (Suppl), 217, Abstract.
3. Alvarez M.B., Johnson H.D. Environmental heat exposure on cattle plasma catecholamine and glucocorticoids. J. Dairy Sci. 1973, 56: 189–194.
4. Armstrong D.V. Heat stress interaction with shade and cooling. J. Dairy Sci., 1994, 7: 2044-2050.
5. Attenberry J.T., Johnson H.D. Effect of environmental temperature, controlled feeding and fasting on rumen motility. J.

Anim. Sci. 1968, 29: 734–737.
6. Bach A., Valls N., Solans A. and Torrent T. Associations between nondietary factors and dairy herd performance. J. Dairy Sci., 2008, 91:3259-3267.
7. Badinga L., Thatcher W.W., Diaz T., Drost M., Wolfenson D. Effect of environmental heat stress on follicular development and steroidogenesis in lactating Holstein cows. Theriogenology 1993, 39: 797–810.
8. Beede D.K., Collier R.J. Potential nutritional strategies for intensively managed cattle during thermal stress, J. Anim. Sci. 1986, 62: 543–554.
9. Berman A. J. Estimates of heat stress relief needs for Holstein dairy cows. J. Anim. Sci. 2005, 83: 1377-1384.
10. Berman A., Folman Y.M., Kaim M., Mamen Z., Herz D., Wolfenson A., Graber Y. Upper critical temperatures and forced ventilation effects for high-yielding dairy cows in a tropical climate. J. Dairy Sci. 1985, 68: 488–495.
11. Bernabucci U., Lacetera N, Baumgard L.H., Rhoads R.P., Ronchi B. and Nardone A. Metabolic and hormonal acclimation to heat stress in domesticated ruminants. Animal. 2010, 4: 1167–1183.
12. Bertocchi L., Vitali A., Lacetera N., Nardone A., Varisco G. and Bernabucci U. Seasonal variations in the composition of Holstein cow's milk and temperature–humidity index relationship. Animal, 2014, 8: 4, 667–674.
13. Bianca W. Relative importance of dry- and wet-bulb temperatures in causing heat stress in cattle. Nature 1962, 195: 251–252.
14. Blazquez N.B., Long S.E., Mayhew T.M., Perry G.C., Prescott N.J., Wathes C.M.,. Rate of discharge and morphology of sweat glands in the perineal, lumbodorsal and scrotal skin of cattle. Res. Vet. Sci. 1994, 57: 277–284.
15. Bohmanova J., Misztal I., and Cole J.B. Temperature-Humidity Indices as Indicators of Milk Production. Losses due to Heat Stress. J. Dairy Sci. 2007, 90:1947–1956.
16. Bouraoui R., Lahmar M., Majdoub A., Djemali M., Belyea R. The relationship of temperature-humidity index with milk production of dairy cows in a Mediterranean climate. Anim. Res. 2002, 51: 479–491.
17. Bouraoui R., Majdoub A., Djemali M., Belyea R. The relationship of temperature-huminity index with milk production of dairy cows in a Mediterranean climate. Anim. Res. 2002: 51, 479-491.
18. Brügemann K., Gernand E., von Borstel U. K. and König S. Defining and evaluating heat stress thresholds in different dairy cow production systems. Archiv Tierzucht, 2012: 55, 1, 13-24.
19. Chase L.E. Climate Change Impacts on Dairy Cattle. Climate Change and Agriculture: Promoting Practical and Profitable Responses. Department of Animal Science Cornell University, Ithaca, NY, 2006, 14853,.
20. Collier R.J., Dahl G.E., and VanBaale M.J. Major Advances Associated with Environmental Effects on Dairy Cattle. J. Dairy Sci., 2006, 89:1244–1253.
21. Collier R.J., Zimbelman R.B., Rhoads R.P., Rhoads M.L., and Baumgard L.H. A Re-evaluation of the Impact of Temperature Humidity Index (THI) and Black Globe Humidity Index (BGHI) on Milk Production in High Producing Dairy Cows. Proc. of the Western Dairy Management Conference, March 9-11 Reno, NV, Pages, 2011, 113-126.
22. Cook N.B., Bennett T.B., and Nordlund K.V. Monitoring indices of cow comfort in free-stall housed dairy herds. J. Dairy Sci., 2005, 88:3876-3885.
23. Cook N.B., Mentink R.L., Bennett T.B., and Burgi K. The effect of heat stress and lameness on time budgets of lactating dairy cows. J. Dairy Sci., 2007, 90: 1674-1682.
24. Correa-Calderon A., Armstrong D., Ray D., DeNise S., Enns M., and Howison C. Thermoregulatory responses of Holstein and Brown Swiss heat-stressed dairy cows to two different cooling systems. Int. J. Biometeorol., 2004, 48:142–148.
25. Dale H.E., Brody S., Thermal stress and acid–base balance in dairy cattle. Missouri Agric. Exp. Stat. Res. Bull. 1954, No. 562.
26. Dimov D., Marinov I., Penev T., Miteva Ch., Gergovska Zh. Influence of temperature-humidity index on comfort indices in dairy cows. Sylwan, 2017, 161 (6): 68-85.
27. Drissler M., Gaworski M., Tucker C.B., and Weary D.M. Freestall maintenance: effects on lying behavior of dairy cattle. J. Dairy Sci., 2005, 88: 2381-2387.
28. Du Preez J.H., Hatting P.J., Giesecke W.H., Eisenberg B.E. Heat stress in dairy cattle and other livestock under Southern African conditions. III. Monthly temperature-humidity index mean values and their significance in the performance of dairy cattle, Onderstepoort J. Vet. Res. 1990a, 57: 243–248.
29. Geers R., Vermeulen L., Snoeks M. & Permentier L. Do Dairy Cattle Need Protection against Weather in a Temperate Climate? A Review. Journal of Agricultural Science, 2014, 6, 12: 9-21.
30. Gomez A., and Cook N.B. Time budgets of lactating dairy cattle in commercial freestall herds. J. Dairy Sci., 2010, 93: 5772-5781.
31. Grant R. Economic Benefits of Improved Cow Comfort. William H. Miner Agricultural Research Institute. Chazy, NY 12921., 2012, www.novusint.com
32. Grant R. Taking advantage of natural behavior improves dairy cow performance. Pages 225- 236 in Proc. Western Dairy Management Conf., Reno, NV., 2007.
33. Hahn G.L., Mader T.L., Eigenberg R.A. Perspective on development of thermal indices for animal studies and management. EAAP Technical Series, 2003 7: 31–44.
34. Hales J.R.S., Findlay J.D. The oxygen cost of thermally induced and CO -induced hyperventilation in the ox. Respir. Physiol. 1968, 4: 353–356.
35. Hansen P.J. Effects of environment on bovine reproduction. In: Youngquist, R.S. (Ed.), Current Therapy in Large Animal Theriogenology.W.B. Saunders, Philadelphia, 1997, pp. 403–415.
36. Hansen P.J., Arechiga C.F. Strategies for managing reproduction in heat-stressed dairy cows. J. Dairy. Sci. 1999, 82 (Suppl.), 36–50.
37. Herbut P., Angrecka S. Forming of temperature-humidity index (THI) and milk production of cows in the free-stall barn during the period of summer heat. Animal Science Papers and Reports 2014, 4, (30), 363-372.
38. Hill D.L. and Wall E. Dairy cattle in a temperate climate: the effects of weather on milk yield and composition depend on management. Animal, 2015, 9, (1): 138-149.
39. Igono M.O. and Johnson H.D. Physiological stress index of lactating dairy cows based on diurnal pattern of rectal temperature. J. Interdiscip. Cycle Res., 1990, 21: 303–320.
40. Igono M.O., Bjotvedt G., and Sanford-Crane H.T. Environmental profile and critical temperature effects on milk production of Holstein cows in desert climate. Int. J. Biometeorol., 1992, 36: 77–87.
41. Igono M.O., Johnson H.D., Steevens B.J., Hainen W.A., Shanklin M.D. Effect of season on milk temperature, milk growth hormone, prolactin, and somatic cell counts of lactating cattle. Int. J. Biometeorol. 1988, 32: 194–200.
42. Ingraham R.H., Gillette D.D., Wagner W.D. Relationship temperature and humidity to conception of Holstein cows in tropical climate. J. Dairy Sci. 1974, 54: 476–481.
43. Johnson H.D., Katti P.S., Hahn L., Shanklin M.D. Shortterm heat acclimation effects on hormonal profile of lactating cows. In: Research Bulletin No. 1061, 1988. University of Missouri, Columbia.
44. Jordan E.R. Effects of heat stress on reproduction. Journal of Dairy Science, 2003, 86: 104–114.
45. Kadzere C.T, Murphy MR, Silanikove N, Maltz E. Heat stress in lactating dairy cows: a review. Livest Prod Sci., 2002, 77: 59-91.
46. Keown J.F., and Grant R.J. How to Reduce Heat Stress in Dairy Cattle. University of Missouri-Columbia, agricultural publication, 1999, G3620. USA.
47. Lendelova J., Botto L. Evaluation of thermal-humidity index in animal housing. Bioclimate: In: Source and Limit of Social Development International Scientific Conference. Topoľčianky, Slovakia, 2011.
48. Mader T.L., Davis M.S., Brown-Brandl T.M. Environmental factors influencing heat stress in feedlot cattle. J. Anim. Sci., 2006, vol. 84, p. 712-719.

49. Mahdy C.El., Popescu S., Borda C., Boaru A. Aspects of the Welfare of Dairy Cows in Farms with Tied-Stall Maintenance System and Action of the Upstream Factors. Part I. Bulletin UASVM Animal Science and Biotechnologies, 2014, 71(2): 159-167.
50. McGuire M.A., Beede D.K., Collier R.J., Buonomo F.C., DeLorenzo M.A., Wolcox C.J., Huntington G.B., Reynolds C.K. Effects of acute thermal stress and amount of feed intake on concentrations of somatotropin, insulin-like growth factor (IGF)-I and IGF-II, and thyroid hormones in plasma of lactating Holstein cows. J. Anim. Sci. 1991, 69: 2050–2056.
51. Mitra R.G., Christison G.I., Johnson H.D. Effect of prolonged thermal exposure on growth hormone (GH) secretion in cattle. J. Anim. Sci. 1972, 34: 776–779.
52. Morton J.M., Tranter W.P., Mayer D.G., and Jonsson N.N. Effects of environmental heat on conception rates in lactating dairy cows: Critical periods of exposure. J. Dairy Sci., 2007, 90: 2271–2278.
53. Muller C.J.C., Botha J.A., Smith W.A. Effect of shade on various parameters of Friesian cows in a Mediterranean climate in SouthAfrica. 1. Feed and water intake, milk production and milk composition, S. Afr. J. Anim. Sci. 1994: 24, 49–55.
54. Nardone A., Ronchi B., Lacetera N., Ranieri M.S. and Bernabucci U. Effects of climate changes on animal production and sustainability of livestock systems. Livestock Science, 2010, 130: 57–69.
55. National Research Council. In: 6th Revised Edition Update. Nutrient Requirements of Dairy Cattle. National Academy Press, Washington, DC, 1989.
56. Omar E.A., Kirrella A.K., Soheir A., Fawzy A., El-Keraby F. Effect of water spray followed by forced ventilation on some physiological status and milk production of post calving Friesian cows. Alex. J. Agric. Res. 1996, 4: 71–81.
57. Pennington & van der Deven, Heat Stress in Dairy Cattle, University of Arkansas www.document www.uaex.edu / Other_Areas/publications/ PDF/FSA-3040.pdf (accessed 22 March 2010).
58. Rao T.K.S., Chauhan I.S., Fulsoundar A.B., Gamit V.V., Parveen K. Improving comfort and welfare to mitigate stress in dairy animals- a review. Wayamba Journal of Animal Science, 2014, P1070-P1084.
59. Ravagnolo O., Misztal I., and Hoogenboom G. Genetic component of heat stress in dairy cattle, development of heat index function. J. Dairy Sci., 2000, 83: 2120–2125.
60. Rhoads M.L., Rhoads R.P., VanBaale J.J., Collier R.J., Sanders S.R., Weber W.J., Crooker B.A., and Baumgard L.H. Effects of heat stress and plane of nutrition on lactating Holstein cows: I. Production, metabolism, and aspects of circulating somatropin. J. Dairy Sci. 2009, 92: 1986-1997.
61. Segnalini M., Bernabucci U., Vitali A., Nardone A., Lacetera N. Temperature humidity index scenarios in the Mediterranean basin. Int J Biometeorol., 2013, 57: 451–458.
62. Silanikove N. Effects of heat stress on the welfare of extensively managed domestic ruminants. Livest. Prod. Sci., 2000, 67: 1–18.
63. Silanikove N. Effects of water scarcity and hot environment on appetite and digestion in ruminants: a review. Livest. Prod. Sci. 1992, 30: 175–194.
64. Silanikove N., Tadmore A. Rumen volume, saliva flow rate and systemic fluid homeostasis in dehydrated cattle. Am. J. Physiol. 1989, 256, 809–815.
65. Smith J.F., Collier R.J., Harner J.P. and Bradford B.J. Strategies to reduce heat stress in dairy cattle Proc. 27th Annual Southwest Nutrition and Management Conf. http:// animal.cals.arizona.edu/swnmc/Proceedings/ 2012, 65–84.
66. St-Pierre N.R., Cobanov B., Schnitkey G. Economic loses from heat stress by US livestock industries. J. Dairy Sci., 2003, 86(E Suppl.): 52–77.
67. Tapki I., Sahin A. Comparison of the thermoregulatory behaviours of low and high producing dairy cows in a hot environment. Appl. Anim. Behav. Sci. 2006, 99: 1-11.
68. Thatcher W.W. Effects of season, climate and temperature on reproduction and lactation. J. Dairy Sci. 1974, 57: 360–368.
69. Thom E.C. The discomfort index. Weatherwise, 1959, 12: 57–59.
70. Thompson G.E. Review of the progress of dairy science: climate physiology of cattle. J. Dairy Res. 1973, 40: 441–473.
71. Upadhyay R.C., Ashutosh A.K., Gupta S.K., Singh S.V., Rani N. Inventory of methane emission from livestock in India. In: Aggarwal PK (ed) Global climate change and Indian agriculture. ICAR, New Delhi, 2009: 117–122.
72. Vitali A., Segnalini M., Bertocchi L., Bernabucci U., Nardone A., Lacetera N. Seasonal pattern of mortality and relationships between mortality and temperature-humidity index in dairy cows. J. Dairy Sci., 2009, 92: 3781–3790.
73. West J.W. Effects of heat-stress on production in dairy cattle. J. Dairy Sci. 2003, 86: 2131–2144.
74. Wilson S.J., Kirby C.J., Koeningsfield A.T., Keisler D.H., Lucy M.C. Effects of controlled heat stress on ovarian function of dairy cattle. 2. Heifers. J. Dairy Sci. 1998, 81: 2132–2138.
75. Wise M.E., Armstrong D.V., Huber J.T., Hunter R., Wiersma F. Hormonal alterations in the lactating dairy cow in response to thermal stress, J. Dairy Sci. 1988, 71: 2480–2485.
76. Wolfenson D., Lew B.J., Thatcher W.W., Graber Y., Meidan R.. Seasonal and acute heat stress effects on steroid pro duction by dominant follicles in cows. Anim. Reprod. Sci. 1997, 47: 9–19.
77. Yousef M.K. Stress physiology in Livestock. CRC Press, Boca Raton, FLA, USA, 1985.
78. Zimbelman R.B., Rhoads R.P., Rhoads M.L., Duff G.C., Baumgard L.H., and Collier R.J. A re-evaluation of the impact of temperature humidity index (THI) and black globe humidity index (BGHI) on milk production in high producing dairy cows. Pages 158–169 in Proceedings of the Southwest Nutrition Conference. R. J. Collier, ed. Accessed Feb., 2009, 2. http://cals.arizona.

*Received 21 April 2020*
*Accepted 1 October 2020*

# The Correlation between Localization of Skin Changes and Risk Factors Associated with Atopic Dermatitis

Senka Babić[1], Šemso Pašić[1], Amir Zahirović[2]
[1]*Department of Microbiology and Infectious Disease, Veterinary Faculty, University of Sarajevo, Bosnia and Herzegovina*
[2]*Department of Internal Diseases, Veterinary Faculty, University of Sarajevo, Bosnia and Herzegovina*

***Keywords:*** *dogs, atopic dermatitis, skin changes, localization.*

***Abstract.*** *The aim of this study was to investigate the frequency of localization of skin changes in dogs with atopic dermatitis (AD) with regard to some risk factors for development of atopic dermatitis in dogs (age, sex, breed, living conditions, seasonality, washing/bathing, Malassezia infections and intradermal testing results). Among 50 dogs with clinical signs compatible with atopic dermatitis, pruritus was observed in 37 (74%), and alopecia in 19 (38%) of dogs ($P < 0.05$). Pruritus was commonly noted in purebred dogs compared with crossbreed dogs ($P < 0.05$). Malassezia yeasts were noted in 25 (50%) dogs by cytological examination, most commonly from ear samples ($P < 0.05$); there was no correlation between the cytology presence of yeast and positive IDT to M. pachydermatis allergen or pruritus. The skin changes were located on legs/paws (n = 23; 46%) abdomen/chest/axillae (n = 12; 24%), back/sacral area/tail (n = 12; 24%), head (n = 11; 22%), inguinal/genital area (n = 11; 22%) and hips/groin (n = 9; 18%); otitis was noted in 13 (26%) dogs. Most dogs had changes on the skin of legs/paws ($P < 0.05$). Female dogs and dogs over 3 years of age were predisposed to otitis, while male dogs, dogs up to 3 years of age, dogs kept indoor as well as dogs with a positive intradermal test to house dust and the house dust mite allergen group were predisposed to skin changes on legs/paws ($P < 0.05$). In frequently washed/bathed dogs, skin changes on leg/paws were more commonly noted, while in rarely washed/bathed dogs, skin changes were more common on abdomen/chest/axillae ($P < 0.05$). Also, the correlation was found between the seasonality onset of AD signs and the localization of skin changes.*

## Introduction

Atopic dermatitis (AD) is one of the most common dermatoses in dogs (Favrot, 2009). It is defined as a genetic predisposed inflammatory and pruritic disease with characteristic clinical features associated with IgE, mostly, against environmental allergens (Halliwell, 2006).

Clinical signs in dogs with AD were described in the last century. From the first description to date, clinical signs differ significantly in the prevalence of the features such as age, sex, breed, localization and description skin visible changes, as well as extent and pruritus distribution (Griffin and DeBoer, 2001; Bruet et al., 2012). Depending on included allergens, clinical signs of AD can be seasonal (e.g., hypersensitivity to pollen) and non-seasonal (e.g., hypersensitivity to house dust mite) (Zur et al., 2002; Brar et al., 2017). Also, there is a possibility that breed predisposition and clinical signs may vary depending on geographical regions (Jaeger et al., 2010.). Pruritus without lesions is a common primary clinical sign in CAD (Olivry, 2012; Griffin and DeBoer, 2001), or if lesions are present, then they are in the form of erythema (Griffin and DeBoer, 2001; Favrot, 2009; Olivry, 2012). In the acute form, skin lesions are characterized by intensive pruritus with excoriations and/or salivary staining (Nagata, 2000). However, AD is often diagnosed as a chronic form characterized by alopecia, hyperpigmentation and lichenification, particularly at predilection areas (Nagata, 2000; Griffin and DeBoer, 2001). Pruritus and consequently lesions usually involve face, ears, limbs, abdomen, axilla, groin and perineum (Nagata, 2000; Griffin and DeBoer, 2001; Favrot, 2009). Any one or any combination of those areas can be affected (Nagata, 2000; Griffin and DeBoer, 2001; Olivry, 2012). In some cases, mild pruritus can be unrecognized by the owner, but indirect proofs of pruritus such as excoriation or saliva-dyed hair may be present (Griffin and DeBoer, 2001; Favrot, 2009). Bacterial and *Malassezia* infections are common complications (Zur et al., 2002), and low percentage (about 5%) of dogs may show chronic otitis externa as the main clinical sign (Nagata, 2000).

The diagnosis of CAD is difficult, and requires patience, time and effort, so it is a time-consuming and complicated process (Hensel et al., 2015; Gedon and Mueller, 2018; Harvey et al., 2019). Since no clinical signs or manifestations are pathognomonic, a definitive diagnosis is not possible based on an interview with the owner and a clinical examination (DeBoer and Hillier, 2001). Dogs can exhibit different clinical signs. Many of them may be caused by other skin conditions, and body areas and intensity of affection may be different (Hensel et al., 2015; Gedon and Mueller, 2018; Harvey et al., 2019).

Corresponding author: Senka Babić, Department of Microbiology and Infectious Disease, Veterinary Faculty, University of Sarajevo, Zmaja od Bosne 90, Sarajevo, Bosnia and Herzegovina; senka.babic@vfs.unsa.ba

The differential diagnosis of AD is based on age onset, breed and clinical signs. Ectoparasites and flea bite hypersensitivity must be ruled out by ectoparasite control, as well as sarcoptes mange and food allergy. Up to date, there is no single test that can distinguish atopic from non-atopic dogs (DeBoer, and Hillier, 2001; Gedon and Mueller, 2018).

The aim of this study was to show frequency of localization (distribution) of skin changes in dogs with AD, and to determine if there is any correlation of risk factors for development of AD (age, sex, breed, living conditions, seasonality, washing/bathing, *Malassezia* infections and intradermal testing results) on their localization.

## Material and methods

In this study, 50 dogs (26 female and 24 male dogs) with a history and clinical signs compatible with AD were included. The average age was 4.2 years (20 dogs up to 3 years and 30 dogs over 3 years of age). Forty dogs were purebred and 10 dogs were crossbreed. A detailed history was followed by clinical and dermatological examination of the dogs. Intensity of pruritus was evaluated by the owners (Rybncek et al., 2008) and grouped as no pruritus, mild, moderate and severe pruritus. Also, the owners assessed the dog's activity (scratching, chewing, licking or rubbing) as normal, mild, moderate and severe (Bruet et al., 2012). All information obtained from the owners was recorded. Skin changes at any site on the body were documented as absent or present (Graham et al., 2019). For data analysis, the distribution of skin changes was systematized in seven areas: ears (presence of otitis); legs and/or paws; hips and/or groins; abdomen and/or chest and/or axillae; back and/or sacral area and/or tail; and inguinal and/or genital area.

For AD diagnosis, criteria according to Prelaud (Prelaud et al., 1998) and positive intradermal tests were used. Food allergy, ectoparasites and other pruritic diseases were ruled out. For the detection of *Malassezia* yeasts, samples from ear external canals and skin with changes were collected from all dogs using sterile cotton swabs. Gram's stained slide smears were used for microscopic examination. Five random fields were examined under an oil immersion objective (x1000 high power field). Yeast cells were characterized according to their morphology compatible to *Malassezia* yeast. Absence of yeast cells per field was considered negative, while one and more cells per field were considered positive (Nascente et al., 2015).

An intradermal test (IDT) was performed by 15 allergens according to manufacturer's instructions (Greer, Lenoir, USA). For data analysis, allergens were grouped into six groups: 1) house dust and house dust mites; 2) grass and weeds (plantain/sorrel mix, 7 grass mix, and ragweed); 3) tree pollens (pine mix and 7-east tree mix); 4) fungi (*Trichophyton mentagrophytes*, *Malassezia pachydermatis* and mould mix); 5) insects (*Culicoides*, house fly and flea antigen); 6) epithelia and feathers (cat epithelia and feather mix). The influence of age, sex, seasonality onset of signs, living conditions, washing/bathing, *Malassezia* infections and IDT results to lesion distribution in dogs with AD were examined.

***Statistical analysis.*** $\chi^2$ and Fisher's exact test were used for comparison of examined parameters and localization of skin changes. A probability value of $\leqslant 0.05$ was considered statistically significant.

## Results

According to the data obtained from histories, the highest percentage of dogs lived indoors (86%), had AD signs in spring and summer (52%), and washed/bathed 1–4 times per a month (58%).

The owners noticed pruritus in 74% of dogs. The intensity of pruritus was evaluated as follows: mild in 2 dogs, moderate in 14 dogs, severe in 14 dogs. The owners could not determine intensity of pruritus in 7 dogs. According to the owners, 13 dogs showed no signs of pruritus. The activity of these dogs (scratching, chewing, licking or rubbing) was assessed by the owners as moderate in 8 dogs. For 5 dogs, the owners could not determine intensity of activity. Skin lesions and/or pruritus were observed in this group of dogs as indirect signs of pruritus. In the examined dogs, pruritus was a more common sign than alopecia (38% of dogs) ($P < 0.05$). Pruritus was more commonly noted in purebred than in crossbreed dogs ($P < 0.05$), while no significant difference was noted for occurrence of alopecia regarding all compared parameters. Pruritus compared with alopecia was more common in both sexes and age groups, in pure breed dogs, as well as in dogs with the spring and summer onset of signs ($P < 0.05$).

*Malassezia* yeasts were detected in 25 dogs by cytology (in ears in 19 dogs, skin in 4 dogs, ears and skin in 2 dogs). Yeast was more commonly detected in ears ($P < 0.05$). Among 19 dogs with positive cytological examination in ears, 8 dogs had otitis externa. Seven dogs with a positive cytological test and 10 dogs with a negative cytological test had a positive IDT to *M. pacydermatis* antigen. There was no correlation between the cytology presence of yeasts and a positive IDT to *M. pachydermatis* allergen or pruritus.

Skin changes were present in the form of erythema/urticaria, hyperpigmentation, rash, macules/papules, crusts, lichenification, seborrhea, oedema and alopecia. Not all areas were affected simultaneously in the same dogs. The greatest number of dogs had leg and/or paw skin changes ($P < 0.05$), while otitis externa was noted in 13 dogs (Fig. 1). Pruritus and skin changes were commonly noted in dogs with a positive IDT to house dust and house dust mite, the grass and weed pollen allergen group and the tree pollen allergen group.

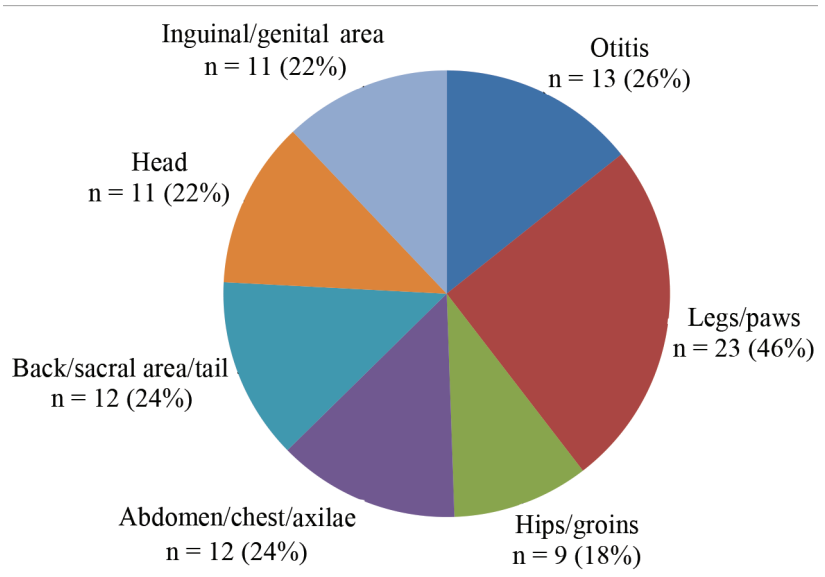



*Fig. 1.* Distribution of skin changes in 50 dogs with atopic dermatitis

Otitis externa was commonly observed in female dogs and dogs over 3 years of age ($P < 0.05$). In female dogs as well as in dogs over 3 years of age and in purebred dogs, more changes were noted on legs/paws than on hips/groins, abdomen/chest/axillae, head and inguinal/genital area ($P < 0.05$). On the other hand, the skin changes on legs/paws were more common than otitis in male dogs and dogs up to 3 years of age ($P < 0.05$) (Table 1).

Significant statistical differences ($P \leqslant 0.05$) were found for compared parameters as follows (Table 2 and Fig. 2):

Dogs living indoor commonly had leg/paw skin changes. Frequently washed/bathed dogs had a greater number of leg/paw skin changes regarding other body areas (except inguinal/genital area). Generally, the largest number of rarely washed/bathed dogs had skin changes on abdomen/chest/axillae. On the other hand, in this group of dogs, skin changes localized on abdomen/chest/axillae were frequently observed than on hips/groins.

In dogs with the spring and summer onset of AD signs, changes on leg/paw skin were noted more often than otitis and hip/groin skin changes, while in dogs with the non-seasonal onset of AD signs, skin changes on legs/paws were more frequently noted than on the inguinal/genital area. In dogs with the spring and summer onset of AD signs, changes on hip/groin skin were rarely noted compared with the back/sacral area/tail and the inguinal/genital area.

Otitis was frequently observed among dogs with an IDT positive to the tree pollen allergen group than among dogs with an IDT positive to the epithelia and feather allergen group. In dogs with an IDT positive to house dust and house dust mite allergen group, changes were more frequently localized on legs/paws than on abdomen/chest/axillae skin. Among dogs with an IDT positive to the fungi allergen group, skin changes on legs/paws were most often observed compared with all other examined body areas, except otitis.

*Table 1.* Distribution of skin changes regarding breed, sex and age of dogs

| Distribution of changes (number of dogs with skin changes) | Number of dogs with skin change | | | | | |
|---|---|---|---|---|---|---|
| | Breed | | Sex | | Age | |
| | Cross breed | Pure breed | Male | Female | Up 3 years | Over 3 years |
| Otitis (n = 13) | 1 | 12 | 3 | 10 | 2 | 11 |
| Legs/paws (n = 23) | 4 | 19 | 9 | 14 | 8 | 15 |
| Hips/groins (n = 9) | 1 | 8 | 4 | 5 | 3 | 6 |
| Abdomen/chest/axillae (n = 12) | 2 | 10 | 4 | 8 | 6 | 6 |
| Back/sacral area/tail (n = 12) | 1 | 11 | 7 | 5 | 4 | 8 |
| Head (n = 11) | 3 | 8 | 6 | 5 | 6 | 5 |
| Inguinal/genital area (n = 11) | 3 | 8 | 4 | 7 | 4 | 7 |

*Table 2.* Distribution of skin changes regarding living conditions, washing/bathing and seasonality of AD onset in dogs

| History parameters (number of dogs) | | Number of dogs with skin change | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Otitis n = 13 | Legs/paws n = 23 | Hips/groins n = 9 | Abdomen / chest/axillae n = 12 | Back/sacral area/ tail n = 12 | Head n = 11 | Inguinal/genital area n = 11 |
| Living conditions | Indoor (n = 43) | 11 | 21 | 8 | 9 | 9 | 9 | 10 |
| | Outdoor (n = 5) | 1 | 1 | 0 | 2 | 2 | 1 | 1 |
| | Box (n = 2) | 1 | 1 | 1 | 1 | 1 | 1 | 0 |
| Washing/ bathing | 1–4 times per a month (n = 29) | 6 | 14 | 6 | 3 | 6 | 6 | 7 |
| | 1–5 times per a year (n = 18) | 5 | 7 | 2 | 8 | 5 | 4 | 3 |
| | Unknown (n = 3) | 2 | 2 | 1 | 1 | 1 | 1 | 1 |
| Seasonality | Spring-summer (n = 26) | 5 | 13 | 2 | 7 | 8 | 7 | 8 |
| | Autumn/winter (n = 9) | 3 | 4 | 3 | 2 | 4 | 1 | 2 |
| | Non-seasonally (n = 15) | 5 | 6 | 4 | 3 | 2 | 3 | 1 |

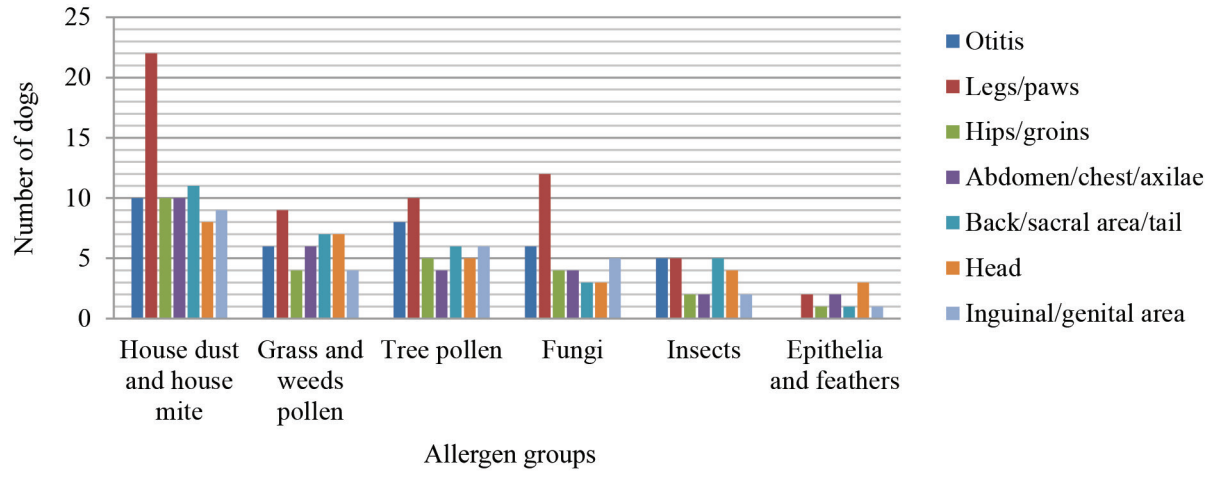



*Fig. 2.* Distribution of changes regarding IDT results

## Discussion

Atopic dermatitis in dogs is a lifelong disease with variable clinical presentation (Nuttal et al., 2013; Hensel et al., 2015). It is a chronic clinical syndrome with a complex pathogenesis (Marsella, 2012). There are no pathognomonic clinical signs, and a definitive diagnosis cannot be made based on history and clinical examination (DeBoer and Hillier, 2001). The diagnosis of CAD is based on the fulfilment of clinical criteria, excluding other possible cases with similar clinical signs, skin scraping and cytology, and allergy testing should be performed to identify potential allergen causes that can be avoided or treated with allergen-specific immunotherapy (Hensel et al., 2015). Therefore, every case should be treated individually (Nuttal et al., 2013), and it must be kept in mind that dogs that do not fulfil diagnostic criteria may actually be atopic (Khoshnegah and Pakzad, 2009). In addition to pruritus, as the most common signs of CAD, particularly on face, ears, paws, extremities and/or ventrum (Griffin and DeBoer, 2001; Hillier, 2002), secondary skin lesions often occur at the pruritus site as a consequence of self-trauma, secondary infections and chronic inflammations (Griffin and DeBoer, 2001; Favrot, 2009; Hensel et al., 2015). The individual threshold for pruritus and a threshold for AD development of each patient play a very important role in development of clinical signs of disease (Marsella and Sousa, 2001); and the skin reaction pattern as well as the distribution of skin lesions vary according to breed, individual and clinical course (Nagata, 2000). Furthermore, dogs

living outdoors can be less carefully followed for allergic signs than indoor dogs; therefore, the signs may not be easy to determine (Hakanen et al., 2018).

Depending on the allergens involved, pruritus and clinical signs may initially be seasonal and non-seasonal (Zur et al., 2002; Favrot, 2009; Brar et al., 2017). On the other hand, some dogs may develop seasonal and non-seasonal pruritus, or pruritus present year-round that may worsen during particular seasons (Hillier, 2002). According to our data, the highest percentage (52%) of dogs had the spring-summer onset of signs (Table 2), which is in consistence with data suggesting the seasonal character of CAD (Griffin and DeBoer, 2001; Zur et al., 2002; Favrot et al., 2009). Although pruritus is the main clinical sign of CAD, it must be kept in mind that mild forms of pruritus may be unrecognized by the owner (Griffin and DeBoer, 2001; Favrot, 2009), and the mild disease form or the form associated with minimal pruritus may be unreported (Griffin and DeBoer, 2001). According to literature data, pruritus is present in 69% (Jang et al., 2013) to 100% (Brar et al., 2017) dogs with AD. In this study, pruritus was noted by the owners in 74% dogs. It should be mentioned that it was more commonly observed in purebred dogs. Activities related to pruritus such as scratching, chewing, licking or rubbing (Bruet et al., 2012) were not recognized by the owners as a sign of pruritus, which they considered as normal dog behaviour. The owners rated this activity as moderate in 8 dogs; and in 5 dogs, they could not determine the intensity of these activities. In dogs without evidence of pruritus by the owners, skin changes and/or alopecia due to pruritus have been noted (Favrot, 2009; Jaeger et al., 2010). Alopecia as one of the most common signs of AD, especially in chronic cases (Nagata, 2000; Brar et al., 2017), was noted in 38% of dogs in our study. This is in accordance with literature data stating the presence of alopecia in dogs with AD from 34% (Sung and Huang, 2009) to 78.26% (Jyothi at al., 2013). We also noted pruritus more commonly than alopecia in both sexes and age groups, as well as in purebred dogs and dogs with the spring and summer onset of AD signs.

In animals with overgrowth of *Malassezia* yeast, or in individuals that are predisposed to allergic sensitization, the consequent inflammatory response can cause clinical signs such as dermatitis and pruritus (Bond et al., 2020). Infections caused by this yeast are common in dogs with atopic dermatitis. In the present study, *Malassezia* yeasts were noted in 25 (50%) dogs by cytology examination. This is in accordance with previous studies (Zur et al., 2002; Jang et al., 2013). In our study, there was no correlation between the cytology presence of yeasts and a positive IDT to *M. pachydermatis* allergen or pruritus. This is in contrast to previous findings where atopic dogs with *Malassezia* dermatitis more frequently had a positive IDT response then without *Malassezia* dermatitis or otitis (Farver et al., 2005). In our study, among 19 dogs with cytological evidence of *Malassezia* in ears but not on the skin, 5 dogs had a positive IDT to *M. pachydermatis* allergen; there was no significant difference in a positive IDT to *M. pachydermatis* allergen between dogs with and without cytological presence of the yeast in the ears. In previous research (Farver et al., 2005), all dogs with *Malassezia* otitis but without dermatitis (MD-MO+) reacted with a positive reaction. On the other hand, in research by Layne et al. (2016), there was no significant difference in the concentrations of *Malassezia*-specific IgE between dogs with recurrent *Malassezia* otitis and dogs with healthy ears, suggesting that hypersensitivity is not always involved in such infections. So, proteins from *Malassezia* yeasts can act as allergens in dogs predisposed to the development of atopic dermatitis (Bond et al., 2020).

Any one or any combination of the body areas can be affected (Griffin and DeBoer, 2001). Lesions are most commonly present on face, and flexural and friction areas; most commonly affected areas are face (particularly periocular and periorbital skin), inside aspect of the pinnae, dorsal and ventral interdigital areas, flexural aspects of joints and extremities (cubital, tarsal, carpal and metatarsal flexures), axillae, abdomen, groin, perineum and ventral tail); and otitis externa is commonly present (Olivry, 2012). Although, most publications suggest legs and paws as the most commonly affected areas, there are some studies where lesions are more commonly noted on the ventral part of the body (ventral abdomen and chest, inguinal area, axillae, ventral neck) than on paws (Zur et al., 2012). In this study, we noted the presence of skin changes on one or a combination of areas as follows: legs/paws (n = 23; 46%), abdomen/chest/axillae (n = 12; 24%), back/sacral area/tail (n = 12; 24%), head (n = 11; 22%), inguinal/genital area (n = 11; 22%) and hips/groin (n = 9; 18%), and otitis was noted in 13 (26%) of dogs (Fig. 1). The distributions of lesions are in accordance with literature data (Khoshnegah and Pakzad, 2009; Jaeger et al., 2010; Brar et al., 2017; Graham et al., 2019).

Our data show that the highest percentage (58%) of dogs with AD were washed/bathed 1–4 times per month; skin changes on legs/paws were more common than on any other body area (except inguinal/genital area). While in the group of rarely washed/bathed dogs (1–5 times per year), changes were most commonly noted on the skin of the abdomen/chest/axillae. Comparing frequently and rarely washed/bathed dogs, we found that skin changes on the abdomen/chest/axillae were more common in frequently washed/bathed dogs (Table 2). Although, washing/bathing helps remove allergens from the coat (Marsella, 2012), according to Meury et al. (2011), washing/bathing of dogs once or more per week is strongly correlated with development of CAD, because washing the dogs is an element of normal

treatment of allergic dogs. Also, it is possible that frequent washing/bathing removes sebum affecting the epidermal lipid layer, thus compromising the function of the skin barrier (Meury et al., 2011).

In research by Chanthic et al. (2008), it was concluded that most of the skin reactions to each allergen group had no significant association with a skin lesion location. However, they found a correlation between a positive IDT to the pollen group and skin lesions on the perineum and the tail area. On the other hand, they noted that dogs with a positive IDT to house dust and the house dust mite group more likely had skin lesions of the feet, but there is no statistical significance (Chanthic et al., 2008). According to our data, pruritus and skin changes were frequently noted in dogs with a positive IDT to house dust and the house dust mite group, the grass and weed pollen group and the tree pollen group of tested allergens. Furthermore, skin changes on legs/paws were more frequently observed in dogs with a positive IDT to the groups of tested allergens as follows: house dust and house dust mite, tree pollen as well as fungi (Fig. 2). Additionally, dogs included in this study in the highest percentage (86%) were kept indoors. In these dogs, the most common localization of skin changes was in the area of the legs/paws (Table 2). This could be explained by the presence of house dust mite indoors, because the ventral parts of the body were more often affected in dogs kept indoors (Wilhem et al., 2011), and prolonged exposure to this allergen may trigger or worsen clinical signs of CAD (Favrot, 2009). According to Marsella et al. (2006), the epicutaneous route of allergen exposure may play an important role in CAD, for the purposes of both the sensitization and the perpetuation of CAD (Pucheu-Haston et al., 2008). Consequently, skin barrier dysfunctions may lead to increased allergen penetration and an increased risk for allergic sensitization (Marsella, 2012). In addition, Marsella et al. (2006) consider that an allergen exposure route does not determine the distribution of lesions, but continuous epicutaneous exposure to allergens probably may play the most important role.

We noted that skin of legs/paws was affected in the highest percentage (46%) of dogs. This is in accordance with other research. The skin of legs and paws was more commonly affected, and the percentage ranges from 21.9% (Chanthick et al., 2008) to 72% (Khoshnegah and Pakzad, 2009). Possible explanations may be direct percutaneous allergen resorption or high density of cutaneous mast cells on paws (Jaeger et al., 2010). Mast cells directly participate in CAD pathogenesis and their number can vary depending on the body area (Jaeger et al., 2010). Besides, according to Auxilia and Hill (2000), their number is highest in the medial and lateral pinna and in the ventral interdigital skin of the hind and fore feet. Auxilia and Hill (2000) suggest that cutaneous mast cell distribution may be involved in the frequent occurrence of ear and foot pruritus, but also suggest that differences in mast cell counts, epidermal thickness or hair follicle density do not adequately explain the predilection sites of CAD.

Atopic dermatitis is one of the primary causes of the development of otitis externa in dogs (Saridomichelakis et al., 2007), and if the otitis externa occurs for the first time in middle or older age of dogs, allergy cannot be ruled out as the primary cause (Paterson, 2016). We recorded otitis externa in 26% of dogs. That is in accordance with literature data that otitis externa has been reported in a wide range from 28% (Chanthick et al., 2008) to 79% (Sung and Huang, 2009). A possible contributing factor to its development in dogs with AD is the increased number of cutaneous mast cells in the lateral and medial pinna (Auxilia and Hill, 2000; Jaeger et al., 2010). We noted sex and age predisposition to develop otitis externa in dogs with AD; female dogs and dogs over 3 years of age were predisposed. Although the highest number of dogs with otitis externa was recorded in purebred dogs, we did not find significant differences regarding crossbreed dogs (Table 1). Contrary to our data, Zur et al. (2002) did not find sex and age predisposition to otitis externa in dogs with AD, but they noted that crossbreed dogs had the lowest risk for otitis externa development. On the other hand, in the research by Saridomichelakis et al. (2007), the higher prevalence of otitis externa associated with AD was in female dogs with a history of pruritic skin diseases. In a study conducted on dogs with AD, a significant correlation was found between a positive IDT to cultivated plant pollen and otitis externa (Zur et al., 2002). The correlation of a positive IDT and otitis externa was also found in our study. Otitis externa was more common in dogs with a positive IDT to the tree pollen allergen group than in dogs with a positive IDT to the epithelia and feather allergen group. Similar to our results, in research by Zur et al. (2012), dogs with otitis externa were less allergic to feathers, but had more positive IDT reactions to house dust and house dust mite allergens than to other tested groups of allergens.

## Conclusion

Based on the data from this study, we can conclude that dogs with atopic dermatitis were prone to leg/paw skin changes, and pruritus was the most dominant sign. The skin changes on legs/paws were more common in female dogs, dogs over 3 years of age and dogs with a positive IDT to house dust and the house dust mite allergen group. The correlation was also found between localization of skin changes and frequencies of washing/bathing of dogs, as well as the seasonality onset of signs of AD. There was no correlation between the cytology presence of yeasts and a positive IDT to *M. pachydermatis* allergen or pruritus.

## References


1. Auxilia S.T., Hill P.B. Mast cell distribution, epidermal thickness and hair follicle density in normal canine skin: possible explanations for the predilection sites of atopic dermatitis. Veterinary Dermatology. 2000. T. 11 (4). P. 247-254.
2. Bond R., Morris D. O., Guillot J., Bensignor E. J., Robson D., Mason K. V., Kano R., Hill P. B. Biology, diagnosis and treatment of *Malassezia* dermatitis in dogs and cats Clinical Consensus Guidelines of the World Association for Veterinary Dermatology. Veterinary Dermatology. 2020. T. 31. P. 27–e4.
3. Brar K.R., Dhaliwal P.S., Kumar A., Chhabra S., Uppal S.K. Clinico-pathological studies on atopic dermatitis in dogs. Journal of Animal Research. 2017. T. 7 (3). P. 507-513.
4. Bruet V., Bourdeau P.J., Roussel A., Imparato L., Desfontis J-C. Characterization of pruritus in canine atopic dermatitis, flea bite hypersensitivity and flea infestation and its role in diagnosis. Veterinary Dermatology. 2012. T. 23 (6). P. 487-e93. doi: 10.1111/j.1365-3164.2012.01092.x
5. Chanthick C., Anaman S., Buathet K. The prevalence of positive intradermal allergy tests in 114 dogs with atopic dermatitis in the Bangkok metropolis, Thailand. Veterinary Immunology and Immunopathology. 2008. T. 126 (3-4). P. 256-262.
6. DeBoer D.J., Hillier A. The ACVD task force on canine atopic dermatitis (XV): fundamental concepts in clinical diagnosis. Veterinary Immunology and Immunopathology. 2001. T. 81 (3-4). P. 271–276.
7. Farver K., Morris D. O., Shofer F., Esch B. Humoral measurement of type-1 hypersensitivity reactions to a commercial *Malassezia* allergen. Veterinary Dermatology. 2005. T. 16. P. 261–268.
8. Favrot C. Clinical signs and diagnosis of canine atopic dermatitis. European Journal of Companion Animal Practice. 2009. T. 19. (3). P. 219-222.
9. Gedon N.K.Y., Mueller R.S. Atopic dermatitis in cats and dogs: a difficult disease for animals and owners. Clinical and Translational Allergy. 2018. T. 8: 41. doi:10.1186/s13601-018-0228-5
10. Graham M., Chan W.Y., Hill P. Lesion distribution in cases of canine atopic dermatitis in South Australia. Australian Veterinary Journal. 2019. T. 97 (8). P. 262-267.
11. Griffin C.E., DeBoer D.J. The ACVD task force on canine atopic dermatitis (XIV): clinical manifestations of canine atopic dermatitis. Veterinary Immunology and Immunopathology. 2001. T. 81. P. 255-269.
12. Hakanen E., Lehtimaki J., Salmela E., Tiira K., Anturaniemi J., Hielm-Björkman A., Ruokolainen L., Lohi H. Urban environment predisposes dogs and their owners to allergic symptoms. Scientific Reports. 2018. T. 8: 1585. doi:10.1038/s41598-018-19953-3
13. Halliwell R. Revised nomenclature for veterinary allergy. Veterinary Immunology and Immunopathology. 2006. T. 114. P. 207–208.
14. Harvey N.D., Shaw S.C., Blott S.C. Vazquez-Diosdado J.A., England G.C.W. Development and validation of a new standardised data collection tool to aid in the diagnosis of canine skin allergies. Scientific Reports. 2019. T. 9: 3039. doi: 10.1038/s41598-019-39630-3.
15. Hensel P., Santoro D., Favrot C., Hill P., Griffin C. Canine atopic dermatitis: detailed guidelines for diagnosis and allergen identification. BMC Veterinary Research. 2015. T. 11: 196 doi: 10.1186/s12917-015-0515-5
16. Hillier A. Definitively diagnosing atopic dermatitis in dogs. Veterinary Medicine. 2002. T. 97 (3). P. 198-209.
17. Jaeger K., Linek M., Power H.T., Bettenay S.V., Zabel S., Rosychuk R.A.W., Mueller R.S. Breed and site predispositions of dogs with atopic dermatitis: a comparison of five locations in three continents. Veterinary Dermatology. 2010. T. 21 (1). P. 119-123.
18. Jang H-M., Kang B-T., Jung D-I. A retrospective study for allergic dermatitis in 35 dogs. Journal of Biomedical Research. 2013. T. 14 (4). P. 201-205.
19. Jyothi J., Satish K.K., Nagaraj P., Chandrasekhar R. Diagnostic and therapeutic management of canine atopic dermatitis. Intas Polivet. 2013. T. 14 (II). P. 245-251.
20. Khoshnegah J., Pakzad S.M. Canine atopic/allergic dermatitis in Mashhad (North-East of Iran): clinical observations. Iranian Journal of Veterinary Research. 2009. T. 10 (4, 29). P. 352-359.
21. Layne E.A., DeBoer D.J. Serum *Malassezia*-specific IgE in dogs with recurrent *Malassezia* otitis externa without concurrent skin disease. Veterinary Immunology and Immunopathology. 2016. T. 176. P. 1–4.
22. Marsella R. An update on the treatment of canine atopic dermatitis. Veterinary Medicine: Research and Reports. 2012. T. 3. P. 85–91.
23. Marsella R., Nicklin C., Lopez J. Studies on the role of routes of allergen exposure in high IgE-producing beagle dogs sensitized to house dust mites. Veterinary Dermatology. 2006. T. 17 (5). P. 306-12.
24. Marsella R., Sousa C.A. The ACVD task force on canine atopic dermatitis (XIII): threshold phenomenon and summation of effects. Veterinary Immunology and Immunopathology. 2001. T. 81. P. 251-253.
25. Meury S., Molitor V., Doherr M.G., Roosje P., Leeb T., Hobi S., Wilhelm S., Favrot C. Role of the environment in the development of canine atopic dermatitis in Labrador and golden retrievers. Veterinary Dermatology. 2011. T. 22 (4). P. 327-334.
26. Nagata M. Diagnosis of atopic dermatitis in dogs. Waltham Focus. 2000. T. 10 (2). P. 4-9.
27. Nascente P. S., Meinerz A. R. M., Faria R. O., Nobre M. O., Escareno J. J. H., Mendes J. F., Meireles M. C. A., Mello J.R.B. *Malassezia pachydermatis* presence in canine external otitis and dermatopathies. Journal of Yeast and Fungal Research. 2015. T. 6 (3). P. 25-30.
28. Nuttall T., Uri M., Halliwell R. Canine atopic dermatitis - what have we learned?. Veterinary Record. 2013. T. 172 (8). P. 201-207.
29. Olivry T. What can dogs bring to atopic dermatitis research?. Chemical Immunology and Allergy. 2012. T. 96. P. 61-72.
30. Paterson S. Discovering the causes of otitis externa. Practice. 2016. T. 38. P. 7-11.
31. Prelaud P., Guaguere E., Alhaidari Z., Faivre N., Heripret D. Reevalution of diagnostic criteria of canine atopic dermatitis. Revue de Medecine Veterinaire. 1998. T. 149. P. 1057-1064
32. Pucheu-Haston C.M., Jacksonw H.A., Olivry T., Dunston S.M., Hammerberg B. Epicutaneous sensitization with *Dermatophagoides farinae* induces generalized allergic dermatitis and elevated mite-specific immunoglobulin E levels in a canine model of atopic dermatitis. Clinical and Experimental Allergy. 2008. T. 38. P. 667-79.
33. Rybnicek J., Lau-Gillard P.J., Harvey R., Hill P.B. Further validation of a pruritus severity scale for use in dogs. Veterinary Dermatology. 2009. T. 20 (2). P. 115-22.
34. Saridomichelakis M.N., Farmaki R., Leontides L.S., Koutinas A.F. Aetiology of canine otitis externa: a retrospective study of 100 cases. Veterinary Dermatology. 2007. T. 18 (5). P. 341-347.
35. Sung T. Y., Huang H.P. The incidence of positive intradermal skin test reactions in dogs with atopic dermatitis. JVCS. 2009. T. 2 (2). P. 31-36.
36. Wilhem S., Kovalik M., Favrot, C. Breed-associated phenotypes in canine atopic dermatitis. Veterinary Dermatology. 2011. T. 22 (2). P. 143-149.
37. Zur G., Ihrke P.J., White S.D., Kass P.H. Canine atopic dermatitis: a retrospective study of 266 cases examined at the University of California, Davis, 1992-1998. Part I. Clinical features and allergy testing results. Veterinary Dermatology. 2002. T. 13 (2). P. 89–102.
38. Zur G., Skorinsky I., Bdolah-Abram T. Canine atopic dermatitis in the Middle East: clinical signs, signalment and common allergens. Veterinarni Medicina. 2012. T. 57. P. 410–419.


*Received 11 November 2020*
*Accepted 16 December 2020*

# Short communication: An ultrasound study of healthy digital flexor tendons on the metapode in meat calves

**Pere M. Parés-Casanova[1]; Júlia Palos-Vila[1]**
*[1]Department of Animal Science, ETSEA, University of Lleida, Lleida, Catalonia (Spain)*

***Keywords:*** *Bruna dels Pirineus, Cattle, Digits, Limbs, Ultrasonography*

***Abstract.*** *Until now, there has been little investigation of the ultrasonographic appearance of normal anatomical structures of distal limbs and normal dimensions of digital flexor tendons in calves. Studies on the descriptive and topographic anatomy of soft tissue structures in limbs are essential before ultrasonographic signs of injury can be recognized. Ultrasound measurements of the normal size of tendons and ligaments allow recognition of abnormalities. The aim of this study is to provide ultrasonographic measurements of digital flexor tendons in the metapodial region in the meat calf. For this purpose, 44 limbs (right and left forelimbs and right and left hindlimbs) from 11 healthy Bruna dels Pirineus and their F1 crosses calves (345–672 days of age and 160.0–331.5 kg carcass weight) were studied post mortem. Cross transverse sections for the structures of three metapodial zones were studied echographically at 7.5 MHz with an ExaGo machine. Although some studies of morphometric measurements by ultrasound in cattle have been reported, no survey of the measurements and proportions of all flexor tendons and ligaments in the metapodial region of calves was found in the literature. The results of this descriptive study allow the establishment of thickness of healthy digital flexor tendons and provide with echographic reference data in young bovines.*

## Introduction

The tendon structures located on the palmar area of the cow are (Berlingieri and Artoni 2011) (International Committee on Veterinary Gross Anatomical Nomenclature 2017):

- tendons of the digital flexor muscles: superficial (*flexor digitorum [digitalis] superficialis*) and deep (*flexor digitorum [digitalis] profundus*);
- *lig. intertendineum;*
- interosseous tendons II and IV (*tendo dorsalis abaxialis* and *tendo plantaris abaxialis*).

The incidence of cattle lameness has been extensively studied, although the prevalence of tendon disorders is unknown (Anderson, Desrochers, and St. Jean 2008), with ultrasonographic studies much less applied than in horses. At the same time few studies have proved the correctness of ultrasonographic inspections of tendons and ligaments by comparing them with necropsy findings. Ultrasound is a safe, easy, non-invasive and effective technique for analysis of soft tissue injuries and currently accessible to any veterinarian clinician (Martínez Martínez 2005) (Gonçalves et al. 2014). Therefore, comprehensive values of ultrasonographic images of digital flexors are required to describe accurately the anatomy of tendons. Moreover, ultrasound measurements of the normal size of tendons and ligaments help to recognize abnormalities.

The purpose of this study was to map the distal limb region in healthy *Bruna dels Pirineus* (Pyrenean Brown) calves in order to obtain ultrasonographic sectional measurements of flexor tendons and determine if age, sex, carcass weight limb and cutting area have any effect over normal measurements. As provided values are for non-pathologic flexor tendons for this breed, the study can provide the practising veterinarian on the racetrack with reference values, taking age of an animal into account, as this work focuses on structures on calves.

Corresponding author: Correspondence to Pere M. Parés-Casanova, Department of Animal Science. ETSEA. University of Lleida Av. Alcalde Rovira Roure 191. E-25198 Lleida, Catalonia (Spain). E-mail: peremiquelp@ca.udl.cat

## Materials and methods

A sample of 44 limbs (right and left forelimbs and right and left hindlimbs) from 11 healthy *Bruna dels Pirineus* and their F1 crosses calves (345–672 days of age and 160.0–331.5 kg carcass weight) was collected. The limbs were randomly collected from a slaughterhouse in a commercial slaughterhouse in Catalonia, Spain. The animals were not lame and were clinically healthy according to *ante mortem* abattoir official veterinary inspection and with no previous trimming. Individual information was not possible for samples; therefore, sex and carcass weight for each animal could not be considered, although there were no castrated animals. After slaughter, the limbs were isolated proximal to the carpus and tarsal joints. The samples were transferred to the University of Lleida, Department of Animal Science, where they were stored at −18°C until processing. After thawing at room temperature, the limbs were cleaned in order to eliminate artifacts during ultrasomnography (US).

US was done at room temperature. The contact acoustic gel was applied on the surface of the skin of the area to be inspected and on the surface of the probe to be in contact with the skin, in order to avoid the interposition of air between the probe and the

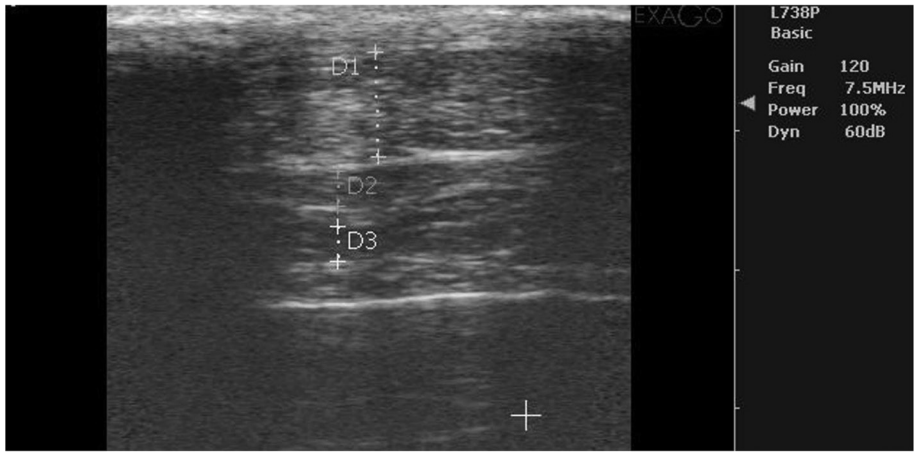



*Fig. 1.* An echographical transverse view (7.5 MHz) of tendons of the digital flexor muscles: superficial (*flexor digitorum [digitalis] superficialis*) (D1), deep (*flexor digitorum [digitalis] profundus*) (D3) and *lig. intertendineum* (D2).

skin. An US device was equipped with a linear type probe which had a pad adapted. Images were obtained with the US device at 7.5 MHz with an ExaGo machine and transverse images were obtained (Fig. 1). To obtain them, we rotated the probe 90º until it was perpendicular to the transversal axis of the limb. We moved the transducer around the area trying not to press too much anatomical structures to avoid possible artifacts. Measurements were performed on three levels determined by dividing the palmar metapodial region into three equidistant sections, starting from the proximal border of the metacarpal bone (level 1) to the proximal border of the proximal sesamoid bones (level 3). Therefore, tendons/ligaments of calves with different metapodial lengths were measured at an equal ratio. US was performed for both fore and hind limbs and the data were digitally recorded. All ultrasonographic examinations and measurements were performed by the second author.

As some of the values were not normally distributed, non-parametric tests were applied. A one-way NPMANOVA (non-parametric multivariate analysis of variance) analysed differences between genders (6 ♂ and 5 ♀) and fore and hind pairs, and a two-way NPMANOVA analysed differences using "limb" and "level" as factors and 9,999 permutation rounds. For variance tests, Gower's distances were used as they can handle continuous and categorical variables. Linear multivariate regressions with log-transformed values were performed using "age" and "carcass weight" as independent variables.

The obtained data were statistically analysed with statistical software program PAST v. 2.17c (Hammer, Harper, and Ryan 2001). Differences between means at $p < 0.05$ were considered significant.

## Results

All parts of the palmar structures were seen on the obtained pictures: skin, subcutaneous tissues, deep digital flexor tendon and superficial digital flexor tendon, distal accessory ligament and interosseous tendon. The surface of the third metapodial bone was clearly detected as a hyperechoic line.

As there appeared no differences between genders ($p = 0.075$), sexes were pooled for ulterior analysis. There were no regressions neither with age ($p = 0.204$, Wilk's $\lambda = 0.975$, $F_{2,126} = 1.61$) nor carcass weight ($p = 0.092$, Wilk's $\lambda = 0.963$, $F_{2,126} = 2.42$). Limbs presented statistical differences ($p = 0.006$), as well as measurements w according to level ($p = 0.0001$) but their interaction was not significant ($p = 0.783$). Measurements for fore and hindlimbs (but not for right-left pairs) were statistically different ($p = 0.0023$), being those of the latter higher. Table 1 reflects the main descriptive statistics for each tendon and level.

## Discussion

Ultrasound is a widely used technique to evaluate tendon lesions on equids and much less in cattle. There is no report describing the ultrasonographic appearance of distal limbs in the *Bruna dels Pirineus* breed. The most commonly used ultrasound criteria for examining tendon and ligament injuries are the size (area) and echogenicity of tendons. However, in order to apply them in the bovine clinic, we must first know their normal values (Berlingieri and Artoni 2011). So, this work focuses on structures that make up the face of bovine flexor digital tendons, in order to add information about these anatomical structures.

There are few studies on cattle, most researches having been done in equids. In our study, the fore and hind limbs had similar ultrasonographic appearances. The flexor tendons were easily identified as having linear, uniformly intense echogenicity. But measurements for the thoracic and pelvic limbs were different, the latter being bigger, something expected as hindlimbs have biomechanics more stressed during locomotion. Interestingly, in Nellore and Girolando cattle, there have been found no significant differences when comparing thoracic/pelvic limbs at any age

*Table 1.* Morphometric measurements (thickness) for the two flexor tendons and ligament for 44 ultrasonographied limbs from meat calves belonging to *Bruna dels Pirineus* breed and their F1 crosses.

A/ Forelimbs*

| | Proximal level | | Middle level | | Distal level | |
|---|---|---|---|---|---|---|
| | FDS | FDP | FDS | FDP | FDS | FDP |
| Minimal value | 2.6 | 4.2 | 3.7 | 4.6 | 2.5 | 3.6 |
| Maximal value | 9.4 | 11.1 | 8.5 | 12.5 | 5.9 | 14.1 |
| Mean | 5.6 | 8.0 | 6.1 | 7.4 | 3.9 | 8.9 |
| Standard deviation | 1.865 | 1.920 | 1.280 | 2.010 | 0.863 | 2.936 |
| Coefficient of variation | 33.1 | 24.0 | 21.0 | 27.0 | 22.0 | 32.8 |

B/ Hindlimbs*

| | Proximal level | | Middle level | | Distal level | |
|---|---|---|---|---|---|---|
| | FDS | FDP | FDS | FDP | FDS | FDP |
| Minimal value | 4.0 | 3.0 | 4.2 | 3.1 | 2.7 | 3.8 |
| Maximal value | 11.6 | 14.4 | 9.2 | 12.2 | 9.0 | 17.5 |
| Mean | 7.5 | 8.8 | 7.1 | 7.6 | 4.5 | 9.3 |
| Standard deviation | 2.340 | 2.479 | 1.411 | 2.308 | 1.594 | 3.240 |
| Coefficient of variation | 31.0 | 28.0 | 19.9 | 30.3 | 35.2 | 35.0 |

*Measurement in mm, except for coefficient of variation, in %. Thickness is expressed as palmo/planto-dorsal measurement. Results for fore and hindlimbs are presented separately, these latter being higher.
FDS: *Flexor digitorum superficialis*; FDP: *Flexor digitorum profundus*.

(Gonçalves et al. 2014). Differences appear between proximal, middle and distal sections.

Although some studies of morphometric measurements by ultrasound in cattle have been reported, only a survey ofmeasurements and proportions of all flexor tendons in the metapodial region of Nellore and Girolando breed was found in the literature (Gonçalves et al. 2014). Values for these breeds are clearly lower than those found by us. We think that differences in tendon/ligament dimensions could be due to breed, age, body weight, height, exercise programme and accuracy of ultrasound equipment. Thus, more information on variations for more breeds would be needed.

## Conclusions

Ultrasonographic morphometric measurements (thickness) for flexors, at proximal, middle and distal levels, for calves belonging to *Bruna dels Pirineus* breed and their F1 crosses are offered. The values were different between thoracic and pelvic limbs, the latter being bigger. Differences also appear between proximal, middle and distal sections. The results of this study establish important ultrasonographic reference data of normal structures of distal limbs and normal dimensions of flexor tendons in meat calves for use in clinical practice.

## Conflict of interest

The authors declare that there are no competing interests regarding the publication of this paper. There were no funders in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The *postmortem* use of non-edible parts did not require an approval from the Ethics Committee.

## References

1. Anderson, D.E., A. Desrochers, and G. St. Jean. 2008. "Management of Tendon Disorders in Cattle." *Veterinary Clinics of North America - Food Animal Practice* 24(3): 551–66.
2. Berlingieri, M.A., and S.M.B. Artoni. 2011. "Morphological Aspects of Flexor Face of Digits of Crossbred Nelore Heifers." *Ars Veterinaria* 27(2): 66–72.
3. Gonçalves, P.V.R. et al. 2014. "Ultrasonography of the Distal Limbs in Nellore and Girolando Calves 8 to 12 Months of Age." *BMC Veterinary Research* 10.
4. Hammer, Ø., D.A.T. Harper, and P.D. Ryan. 2001. "PAST v. 2.17c." *Palaeontologia Electronica* 4(1): 1–229.
5. International Committee on Veterinary Gross Anatomical Nomenclature. 2017. *Nomina Anatomica Veterinaria*. World Association of Veterinary Anatomist.
6. Martínez Martínez, M. de los Llanos. 2005. "Estudio Ecográfico (Ecogenicidad y Área) de Los Tendones Flexores de La Extremidad Anterior Del Caballo Pura Raza Español." Universidad de Murcia. http://nadir.uc3m.es/alejandro/phd/thesisFinal.pdf.

*Received 7 October 2020*
*Accepted 24 November 2020*

# Factors that Influence Personal Perceptions and Reactions to Animal Cruelty

**Gergana Nikolova Balieva[1], Ivanka Lazarova[1]**
*[1]Department of Hygiene, Technology and Control of Food Products of Animal Origin, Veterinary Legislation and Management, Faculty of Veterinary Medicine, Trakia University, Bulgaria*

**Keywords:** *attitudes toward animals; animal cruelty; perceptions of animal welfare and animal rights.*

***Abstract.*** *People's perceptions on animal welfare issues like animal emotions and rights and human-animal interactions appeared as a subject of our scientific interest with the aim to investigate the public awareness on animal cruelty. An anonymous written questionnaire was distributed among respondents with different demographics throughout Bulgaria. Results showed that female respondents (57.66%) strongly agreed on the ability of animals to experience feelings (P = 0.000), with significant differences for the respondents aged 19–24 and those who strongly agreed on animal rights (P = 0.000). Public understanding on animal sentience was significantly influenced by the participants' relationship with pets and farm animals and their urban residence (P < 0.05).*

*The study found a positive correlation between the groups of those who strongly agreed on animal rights and those who were fully aware on the nature of animal abuse (r = 0.39; P <0.05). The majority of the respondents (42.04%) recognized physical abuse, but emotional and sexual abuse were not well recognized. In order to protect the abused animal, the majority of the participants in the study (45.65%) stated that they would respond with a combination of physical and verbal interaction with the offender and a call to the authorities.*

## Introduction

The sensitiveness of the wide public to the animal welfare issues has increased in the last decades as research has provided scientific evidence on animal sencience (Duncan, 2004; Duncan, 2006; Boissy et al., 2007; Sneddon et al., 2014). The understanding of the ability of non-human animals to feel pain and distress (Proctor et al., 2013; Cornish et al., 2018) has led to development and implementation of a comprehsive legislative framework on animal protection which at international level clearly defines the requirements for humane treatment of animals throughout their life-span (breeding, transport, slaughter, experiments, etc.), ensuring that the core "five freedoms" are met. Still, some issues as animal rights and emotions appear to be disputable among people due to their varying attitudes to animals. A range of studies have suggested that personal attitudes towards animals have been shaped by opportunities for human-animal interaction and relationship (Coleman, 2008; Kupsala et al., 2015; Mariti et al., 2018) facilitated by some demographic factors as gender, age, education, occupation, urban or rural background and even nationality (Philips & McCulloch, 2005; Philips et al., 2012; De la Fuente et al., 2017; Cornish et al., 2018; Tamioso et al., 2018).

Correspondence to Gergana Balieva, Department of Hygiene, Technology and Control of Food Products of Animal Origin, Veterinary Legislation and Management, Faculty of Veterinary Medicine, Trakia University, 6000 Stara Zagora, Bulgaria.
E-mail: gnikolova.vet@gmail.com

At the same time, people's perceptions of animal sentience and wellbeing have shaped their understanding on animal maltreatment and abuse. In the scientific literature, animal abuse has been clearly defined into four main types (Rowan, 2006; Munro & Munro, 2008; Mogbo et al., 2013) with a direct connection with interpersonal violence and antisocial behavior (Madfis & Arluke, 2014; Vinas et al., 2018; Hoffer et al., 2018a, 2018b; Richard & Reese, 2019). However, the legislation on animal protection differs among the countries (Sankoff & White, 2009; Shaffner, 2011; Takacova et al., 2013; Balajty et al., 2018) as in some states acts of animal cruelty are not subject to mandatory reporting (Alleyne et al., 2019), although liability towards all acts perceived as animal abuse is legally determined (Babcock & Neihsl, 2006; Lamparello & Boyd, 2013; Solarova, 2019; Kirov et al., 2019). Scholars have argued that animal-care providers and especially professionals like veterinarians have the duty to promote positive animal welfare and try to prevent acts of animal cruelty (Morris, 2010; Lachance, 2016; Englar, 2018, Joo et al., 2020). When the wider public is considered it becomes clear that the individual intention to intervention in controversial abusive situations is influenced by a number of factors as gender, age, occupation, personal interaction with animals, etc. (Arkow, 2015; Ostovic et al., 2016; Mikuš et al., 2020). Meanwhile, people's reaction could vary from emotional disturbance to reporting to the authorities or physical and verbal response to protect the animal victim (Sienauskaite, 2017; Scott-Park, 2019; Pręgowski & Cieślik, 2020).

**Objective**

The objective of this study was to investigate people's perceptions on animal welfare issues like animal emotions and animal rights with emphasis on their understanding on cruelty acts towards animals. The study hypothesized that public perceptions on animal abuse varried significantly due to a complex of heterogenous characteristics, thus predisposing people's reactions to violent behaviour directed to non-human animals.

**Materials and methods**

The study was carried out under the form of an anonymous written questionnaire among veterinary students at Trakia University, Stara Zagora, and other respondents throughout Bulgaria (n = 333 in total) in the period of March 2019 throughout May 2019. At the beginning of the course in forensic veterinary medicine in the fifth year, veterinary students were asked for their willingness to participate in the survey and those with a positive answer were given five paper questionnaires each. One questionnaire had to be filled in personally. The rest four questionnaires per student were given for distribution among their relatives and friends; thus, respondents with different demographics were included in the survey. Before completing the questionnaire, the respondents were informed in written (a top paragraph of the questionnaire) about the aim of the study, anonymity, and that participation in this study was voluntary. This study did not need ethics approval.

The questions were distributed in several sections. Briefly, the first section (questions 1–7) contained questions on the participant's demographic data, such as age, gender, residence (capital city, city-administrative centre, small town, village), occupation, education, previous experience with pet animals and with farm animals. The second section (questions 8–11) focused on the perception of the respondents about the ability of animals to experience emotions and pain, as well as on the participants' position regarding animal rights, knowledge on animal abuse, opinion on the public attention to animal welfare and protection seen as too excessive. The five-grade Likert scale was used for answering the questions from this section (ranged as strongly agree, agree, neither agree nor disagree, disagree, strongly disagree).

The third section (questions 12–13) contained statements and open-ended questions that aimed at determining the participants' awareness on acts related to animal cruelty and their personal reaction in animal cruelty situation.

All results from the questionnaires returned to the authors were coded with numerical values and each text answer was given a number (presented on the legend of the survey). Thus, long statements were converted for easier data analysis. After coding, the data were statistically processed (Statistica v. 7 software, StatSoft, Inc.). The Kolmogorov-Smirnov test was used for verification of the normality of data distribution. The study parametres were analyzed through descriptive statistics (frequency distribution tables), correlation analysis (Pearson correlation coefficient) and the Student t test (t test for independent samples). P values less than 0.05 were considered statistically significant. The results afterwards were presented on diagrams (Excel, Windows 10).

**Results**

The demographic characteristics of the participants in the survey varied in age, education, occupation and residence (Table 1). The data showed that most of the respondents were women (57.66%), aged 19–24 (63.67%), graduated from a high school (77.48%) and studying for their university degree (72.07%). The majority of the respondents were from urban residence, including the capital city and administrative cities throughout the country (89.79% in total), while only 10.21% of them came from rural settings.

It appeared that the demographic profile of the participants influenced their attitudes towards animal welfare and protection issues as the Student t test found significant differences in favour of the women and

*Table 1.* Demographic characteristics* of the respondents' profiles

| Respondents' Demographics | Count | Percentage |
|---|---|---|
| Gender | | |
| Male | 140 | 42.04 |
| Female | 192 | 57.66 |
| Age (years) | | |
| ⩽ 18 | 5 | 1.50 |
| 19–24 | 212 | 63.67 |
| 25–29 | 61 | 18.32 |
| 30–60 | 44 | 13.21 |
| 61–64 | 2 | 0.60 |
| 65+ | 3 | 0.90 |
| Residence | | |
| Capital city | 19 | 5.70 |
| City–Regional administrative centre | 190 | 57.06 |
| City–Municipal administrative centre | 87 | 26.13 |
| Town | 3 | 0.90 |
| Village | 34 | 10.21 |
| Occupation | | |
| High school student | 5 | 1.50 |
| University student | 240 | 72.07 |
| Employed | 5 | 1.50 |
| Unemployed | 77 | 23.13 |
| Retired | 6 | 1.80 |
| Education | | |
| Primary school | 1 | 0.30 |
| Middle school | 5 | 1.50 |
| High school | 258 | 77.48 |
| Bachelor degree | 33 | 9.91 |
| Master degree | 35 | 10.51 |
| PhD | 1 | 0.30 |

*Values may not total 100% for each category because of non-responder and rounding of values.

those who strongly agreed on the ability of animals to experience feelings (t[332] = 8.054, P = 0.000). Such significant differences were found also for the respondents aged 19–24 and those who strongly agreed on animal rights (t[327] = 15.419, P = 0.000) and animal feelings respectively (t[327] = 25.801, P = 0.000). People from an urban background, living in regional administrative centres, were significantly more aware of animal sentience as they strongly agreed that animals had rights (t[333] = 14.569, P = 0.000) and could feel emotions (t[333] = 23.125, P = 0.000).

Regarding the participants' relationship with animals, the study found that the majority of them had cared for pets previously or at the present moment (91.89% in total). At the same time, a smaller share of the respondents had experience with farm animals in the past or present (51.35% in total) (Fig. 1). However, this kind of interaction with companion or productive animals was found to affect significantly the participants' understanding of animal sentience: pet keepers strongly agreed on animal rights (t[333] = 6.870, P = 0.000) and feelings (t[333] = 18.471, P = 0.000), and so did owners of farm animals (t[332] = 11.048, P = 0.000 for animal rights; t[332] = 20.260, P = 0.000 for animal feelings).

The personal attitudes of the participants in the survey towards the statements "Animals have rights" and "Animals have feelings" were investigated (Fig. 2). The majority of the respondents strongly agreed that animals were able to experience emotions (94%) and had rights (69.97%), with a positive correlation between the two groups (r = 0.40; P < 0.05). The survey also recorded a positive correlation between the respondents who strongly agreed on animal rights (69.97%) and those who stated to be fully aware (86.19%) of the nature of animal cruelty acts (r = 0.39; P < 0.05).

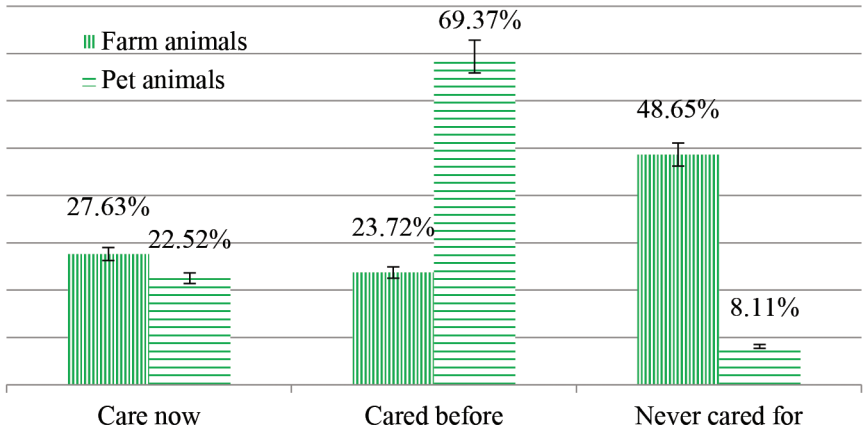



*Fig. 1.* Respondents' distribution regarding their relationship with farm animals and pets

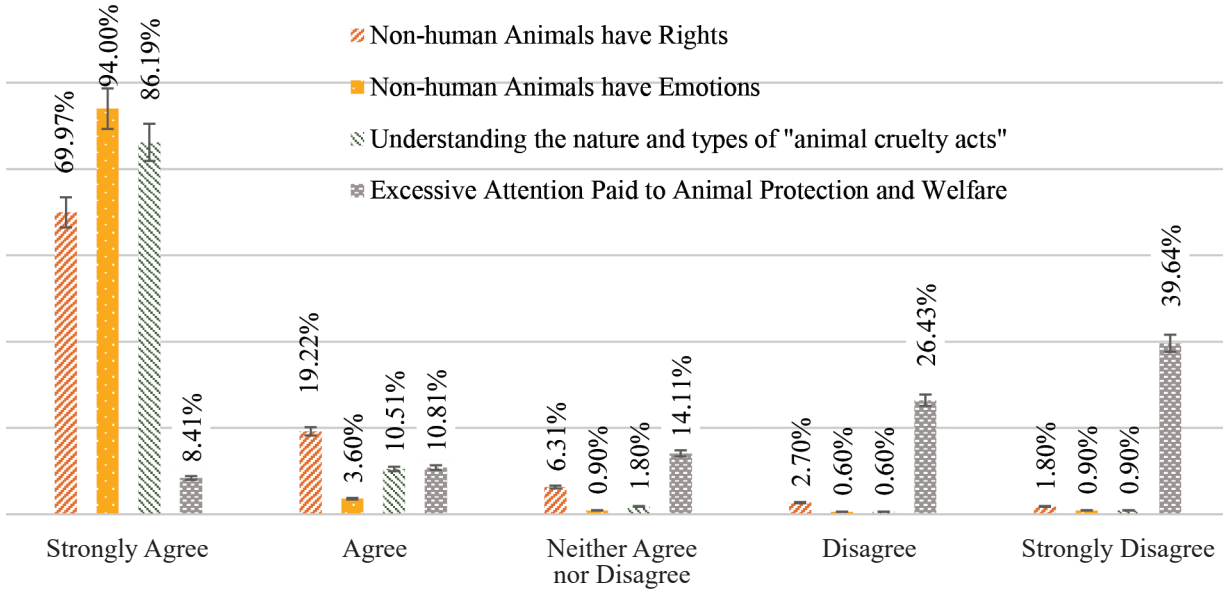



*Fig. 2.* Respondents' attitudes towards animal rights, feelings, animal cruelty and welfare issues
*Values may not total 100% for each category because of non-responder.

The rate of the participants in the survey who strongly disagreed that excessive public attention was paid to animal protection and welfare (39.64%) increased with the decrease of the respondents who recognized the animal emotions and rights (1.80% for rights and 0.90% for emotions) (Fig.2), with a negative correlation established ($r = -0.31$ for emotions and $r = -0.26$ for rights; $P < 0.05$). The Student t test found a significant difference between the group of the respondents who strongly agreed on their high awareness of animal abuse acts and those who disagreed that too much attention had been put on the animal welfare and protection issues ($t[333] = -33.122$, $P = 0.000$).

Asked to identify the acts that appeared to be abusive towards the non-human animals, most of the respondents pointed out physical actions like beating, dragging, etc. (42.04%). Of all other types of violence, only neglect was perceived as cruelty (0.90%). In fact, half of the participants stated that animals were subjected to more than one type of abusive human behaviour (51.05%), combining physical and emotional abuse (Fig. 3). It appeared that the respondents probably did not recognize emotional or sexual abuse as single acts of aggresive behaviour to animals. However, the study found significant differences between the respondents' unawareness of sexual abuse of animals and the group of pet keepers ($t[333] = -11.912$, $P = 0.000$) and farm animal owners ($t[332] = -8.342$, $P = 0.000$) as well.

In a hypothetic situation with demonstrated animal cruelty, the participants in the survey were eager to approach the offender in several ways: 23.12% would call the competent authorities to sanction the perpetrator, 11.11% would physically interfere, 9.91% would try verbally to interrupt the offender and 1.80% would not react to protect the abused animal (but feel emotionally disturbed) (Fig. 4). The majority of the respondents (45.65%) stated they would try more than one approach (a combination of physical and verbal interaction with the offender and a call to the authorities) in order to protect the abused animal.

The respondents' perceptions on the sentience of non-human animals (recognition of animal rights and feelings) appeared to be among the factors determining their personal reactions in situations with expressed violence against animals, in favour of a verbal approach towards the offender ($t[333] = 6.912$,

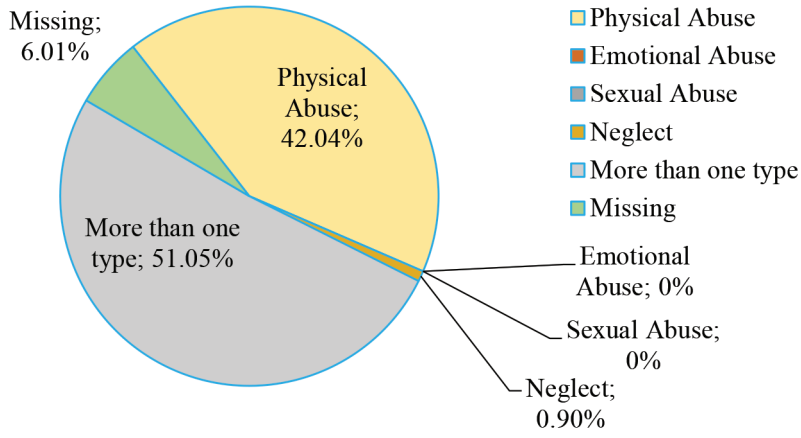



*Fig. 3.* Respondents' distribution regarding their recognition of types of animal cruelty

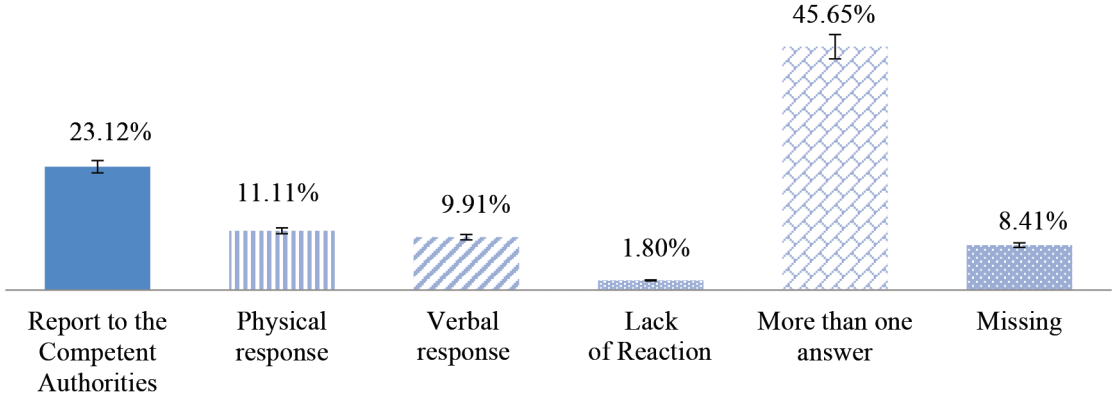



*Fig. 4.* Respondents' personal reactions in an animal cruelty situation, frequency distribution

P = 0.000). The same response to protect abused animals was preferred by the group of respondents who declared full awareness on the nature of animal cruelty acts (t[333] = –21.816, P = 0.000). In fact, women were found to show a significant difference in favour of the verbal interaction with the offender (t[332] = –19.841, P = 0.000).

**Discussion and conclusions**

Attitudes of the wide public towards non-human animals have been supposed to be formed by various factors like demographics, animal use, human-animal interaction and professional occupation. Studies in different regions of the world have compared the sensitivity of professionals as veterinarians, biologists, animal scientists, ordinary citizens and students on welfare issues like pain and feelings in animals (Ostovic et al., 2016; Tamioso et al., 2018; Menor-Campos et al., 2019; Mikuš et al., 2020) with evidence on the higher awareness on their ability to experience emotions and suffering perceived by women, aged 18–29 years old, with higher education. In consistence with these findings, our study found significant differences towards the sentience of animals and their rights and emotions, strongly agreed by female respondents, aged 19–24 years old and studying for their university degree. Generally, women were related with greater concern to non-human animals, compared with men (Knight et al., 2004; Herzog, 2007), although some authors did not find associations between animal welfare issues awareness and respondents' gender (Johnstone et al., 2019) or age (Zalaf and Egan, 2015).

Professional occupation in the field of human-animal interaction and animal care also plays a role in the formation of people's perception on wellbeing and sentience of animals. Specialists like veterinarians, animal scientists and even farmers have showed higher perceptions of sentience of productive animals (Tamioso et al., 2018, Ostovic et al., 2016) although a lower level of welfare seems to be tolerated for farm animals compared with the wellbeing of pets on the whole (Mariti et al., 2018; Howell et al., 2016; Wolfensohn and Honess, 2007). The participants in our survey who had previous or current experience in raising companion or farm animals were found to be equally and significantly sensitive (P < 0.05) towards the rights and emotions of animals. This close contact with animals has been confirmed to take part in the formation of positive attitudes towards animals by people in more rural countries (Zalaf and Egan, 2015). On the contrary, other authors have argued that urban context affects much more the public perceptions on animal sentience, cognition and overall welfare as urban citizens consider morally the animals kept with their mental capacities and use for entertainment, not for food (Jasper & Nelkin, 1992; Bratanova et al., 2011). In consistence with this position, we found significant differences in favour of the respondents from an urban background, living in cities-administrative centres, towards the ability of animals to experience emotions and suffering (P = 0.000).

Public awareness on abusive behaviour towards animals was also investigated in the light of human-animal interaction. The respondents in our study appeared to be mostly aware of physical abuse of animals (42.04%) demonstrated like beating, shooting, dragging, drowning, etc., which were pointed out by Newberry (2018) like the main methods used in animal cruelty offences, most commonly on dogs. Regarding the other types of abuse, like emotional and sexual abuse, our study showed that they appeared to be unrecognized by the public. In fact, significant differences between the respondents' unawareness of sexual abuse of animals and the group of pet keepers (P = 0.000) and farm animal owners (P = 0.000) were found. We could suggest that personal interaction with animals in the livestock sector predisposed a more utilitarian view of animal values which other authors (Taylor & Signal, 2006; Coleman, 2008; Verbeke, 2009) argued to be a factor for considering improper or abusive treatment of animals.

Regarding individual approaches in an abusive situation including animal victims, research has showed that public attitudes to animal cruelty were dependent on demographics, culture, values and beliefs, etc. (Ascione et al., 2003; Henry, 2004; Baldry, 2005; Hensley & Tallichet, 2005; Gullone & Robertson, 2008; Gullone, 2014; Hawkins & Williams, 2020) and varied among different employment sectors in society like ordinary citizens and professionals (Taylor and Signal, 2006; Joo et al., 2020).

As awareness towards animal abuse increased in the wide public, Tiplady et al. (2013) reported that 98% of people experienced at least one of the possible immediate reactions after encountering animal cruelty situations. This fact coincided with our results which showed that at least five types of reactions were defined by the respondents in animal cruelty cases – a call to competent authorities, a physical approach to the offender, a verbal approach, lack of reaction (but including emotional disturbance) and a combination of the mentioned, e.g., a physical or verbal response and a report, the last one being chosen by the majority of the respondents (45.65%). Emotional disturbance, like feeling pity for animals, sadness, anger or hatred, has been argued by Tiplady et al. (2013) and Sinclair et al. (2018) to be detected as a form of compassion which appeared to be an impulse toward both human and animal violence. Ethical beliefs and intention to protect the animal have been found to be reasons for response in abusive cases (Kogan et al., 2017; Pręgowski & Cieślik, 2020). At the same time, Taylor and Signal (2006) defined personal intervention like a physical approach to be seen as an inappropriate action in animal cruelty acts with the group with such an inappropriate response to be much less likely to report deliberate animal cruelty overall compared with any other group in their survey. However,

approximately half of the respondents in our study (45.65%) indicated the personal response as a possibility to interrupt the offender in combination with verbal interaction and a call to the authorities.

The emotional response to animal cruelty, like compassion and empathy, has been found by Tiplady et al. (2013) to be demonstrated more likely by women than men. In our study, women were found to show a significant difference in favour of the verbal interaction with the offender (P = 0.000). At the same time, Taylor and Signal (2006) have reported that both genders indicated a willingness to report incidents of violence toward animals (women = 4.40%, men = 4.06%). When veterinary professionals were considered about their propensity to report animal cruelty, Joo et al. (2020) have found that female veterinarians had a much stronger intention of reporting animal abuse cases to police (P = 0.01). However, only a minority of veterinarians have reported the suspected cases (Stolt et al., 1998; Kogan et al., 2017; Milroy et al., 2018; Pręgowski & Cieślik, 2020).

In conclusion, the present study found that young women in their university undergraduate degrees, as well as residents with an urban profile from cities-administrative centres demonstrated high awareness on the abilities of animals to feel pain, have emotions and rights. Such high sensitiveness on animal welfare issues was declared by the respondents, i.e., pet keepers and farm animal owners, whose understanding on animal sentience was significantly affected by the past or present personal interaction with their non-human companions.

The respondents' awareness of animal sentience and wellbeing was found to shape their perceptions on the nature of animal cruelty. It appeared that the majority of the participants in the study recognized physical abuse towards animal victims but were not fully aware of emotional or sexual abusive behaviour. However, although disregarding certain types of animal cruelty, the respondents indicated a significantly important willingness to respond in controversial abusive situations, mainly through a personal approach to the offender (physically or verbally) with a propensity to report and call the competent authorities on the scene. Based on the different demographic profile of the respondents, especially the specific group of veterinary students, it could be said that the findings at this stage did not represent the general public opinion.

Further studies would be necessary to identify people's knowledge to whom to report in order to assist the animal health and protection services and to improve the state of prevention of animal cruelty.

**Acknowledgment**

The authors wish to thank all the participants who filled in the questionnaire.

This research received no external funding.

## References

1. Alleyne E, Sienauskaite O, & Ford J. To report, or not to report animal abuse: The role of perceived self-efficacy in veterinarians' decision-making. Veterinary Record 2019, 185, 538.
2. Arkow P. Recognizing and responding to cases of suspected animal cruelty, abuse, and neglect: what the veterinarian needs to know. Veterinary Medicine: Research and Reports 2015, 6: 349-359.
3. Ascione FR, Friedrich WN, Heath J, & Hayashi K. Cruelty to animals in normative, sexually abused, and outpatient psychiatric samples of 6- to 12- year-old children: Relations to maltreatment and exposure to domestic violence. Anthrozoos, 2003, 16, 194-212
4. Babcock SL, & Neihsl A. Requirements for mandatory reporting of animal cruelty. J Am Vet Med Assoc 2006; 229: 685-689.
5. Balajty R, Takacova D, Ruffo G, Fossati P. Differences between the Italian and Slovak legal systems with respect to animal protection. Veterinarni Medicina, 2018, 63: 292-297.
6. Baldry AC. Animal abuse among preadolescents directly and indirectly victimized at school and at home. Criminal Behaviour and Mental Health, 2005, 15, 97-110.
7. Boissy A, Arnould C, Chaillou E, Désiré L, Duvaux-Ponter C, Greiveldinger L, Leterrier C, Richard S, Roussel S, Saint-Dizier H, Meunier-Salaün MC, Valance D, Veissier I. 2007. Emotions and cognition: a new approach to animal welfare. Animal Welfare, 16 (1): 37-43
8. Bratanova B, Loughnan S, Bastian B. The Effect of Categorization as Food on the Perceived Moral Standing of Animals. Appetite 2011, 57, 193–196.
9. Coleman G. Public Perceptions of animal pain and animal welfare. In Scientific Assessment of Management of Animal Pain; Mellor, D., Thornber, P.M., Bayvel, D., Kahn, S., Eds.; OIE: Paris, France, 2008.
10. Cornish A, Wilson B, Raubenheimer D, McGreevy P. Demographics Regarding Belief in Non-Human Animal Sentience and Emotional Empathy with Animals: A Pilot Study among Attendees of an Animal Welfare Symposium. Animals, 2018, 8, 174.
11. De la Fuente MF, Souto A, Caselli CB, Schiel N. People's perception on animal welfare: Why does it matter? Ethnobiology and Conservation, 2017, 6:18.
12. Duncan IJH. A concept of welfare based on feelings. In: Benson, G.J., Rollin, B.E. (Eds.), The Well-Being of Farm Animals: Challenges and Solutions. 2004. Blackwell, Ames, Iowa, 85–101.
13. Duncan IJH. The changing concept of animal sentience. Applied Animal Behaviour Science 2006. 100: 11–19
14. Englar R. Using a Standardized Client Encounter in the Veterinary Curriculum to Practice Veterinarian-Employer Discussions about Animal Cruelty Reporting. Journal of Veterinary Medical Education, 2018, 45(4)
15. Gullone E. Risk factors for the development of animal cruelty. Journal of Animal Ethics, 2014, 4, 61-79.
16. Gullone E, & Robertson N. The relationship between bullying and animal abuse in adolescents: The importance of witnessing animal abuse. Journal of Applied Developmental Psychology, 2008, 29, 371-379.
17. Hawkins RD & Williams JM. Children's attitudes towards animal cruelty: Exploration of predictors and socio-demographic variations. Psychology, Crime & Law, 2020, 26:3, 226-247.
18. Henry BC. The relationship between animal cruelty, delinquency, and attitudes toward the treatment of animals. Society & Animals, 2004, 12, 185-207.
19. Hensley C & Tallichet SE. Animal cruelty motivations: Assessing demographic and situational influences. Journal of Interpersonal Violence, 2005, 20, 1429-1443.
20. Herzog HA. Gender differences in human-animal interactions: A Review. Anthrozoos. 2007; 20: 7-21.
21. Hoffer TA, Hargreaves-Cormany H, Muirhead Y, Meloy JR. The Relationship Between Family Violence and Animal

Cruelty. In book: Violence in Animal Cruelty Offenders, p. 35-37. 2018a. Springer.
22. Hoffer TA, Hargreaves-Cormany H, Muirhead Y, Meloy JR. Motivations of Animal Cruelty Acts and Other Violence. In book: Violence in Animal Cruelty Offenders, p. 43-45. 2018b. Springer.
23. Howell TJ, Mornement K, Bennett PC. Pet dog management practices among a representative sample of owners in Victoria, Australia. Journal of Veterinary Behavior, 2016, 12: 4-12.
24. Jasper J, Nelkin D. The Animal Rights Crusade; The Free Press: New York, NY, USA, 1992.
25. Johnstone ECS, Frye MA, Lord LK, Baysinger AK, Edwards-Callaway LN. Knowledge and Opinions of Third Year Veterinary Students Relevant to Animal Welfare Before and After Implementation of a Core Welfare Course. Frontiers in Veterinary Science 2019, 6:103
26. Joo S, Chun MS, Jung Y. An Analysis of Veterinary Practitioners' Intention to Intervene in Animal Abuse Cases in South Korea. Animals, 2020, 10, 802
27. Kirov V, Kostov I, Kirova S, & Balieva GN. Contemporary development of legislation on criminalization of animal cruelty. Knowledge International Journal, 2019, 32(1), 201 – 206 (In Bulgarian)
28. Knight S, Vrij A, Cherryman J, Nunkoosing K. Attitudes towards animal use and belief in animal mind. Anthrozoos. 2004; 17: 43-62.
29. Kogan LR, Schoenfeld-Tacher RM, Hellyer PW, Rishniw M, Ruch-Gallie RA. Survey of attitudes toward and experiences with animal abuse encounters in a convenience sample of US veterinarians. Journal of the American Veterinary Medical Association. 2017, 250(6):688-696.
30. Kupsala S, Vinnari M, Jokinen P, Räisänen P. Citizen Attitudes to Farm Animals in Finland: A Population-Based Study. J. Agric. Environ. Ethics. 2015. 28:601–620.
31. Lachance M. Breaking the silence: The veterinarian's duty to report. Animal Sentience 2016; 6: 1.
32. Lamparello A & Boyd M. Vulnerable Victims: Increasing Animal Cruelty Sentences to Reflect Society's Understanding of the Value of Animal Lives. Connecticut Law Review, 2013, Vol. 45, 31-40
33. Madfis E & Arluke A. Animal Abuse as a Warning Sign of School Massacres. Homicide Studies, 2014, 18(1):7-22.
34. Mariti C, Pirrone F, Albertini M, Gazzano A, Diverio S. Familiarity and Interest in Working with Livestock Decreases the Odds of Having Positive Attitudes towards Non-Human Animals and Their Welfare among Veterinary Students in Italy. Animals. 2018; 8:150.
35. Menor-Campos DJ, Diverio S, Sánchez-Muñoz C, López-Rodríguez R, Gazzano A, Palandri L, Mariti C. Attitudes toward Animals of Students at Three European Veterinary Medicine Schools in Italy and Spain. Anthrozoos 2019, 32(3):375-385.
36. Mikuš T, Ostović M, Sabolek I, Matković K, Pavičić Ž, Mikuš O, Mesić Ž. Opinions towards Companion Animals and Their Welfare: A Survey of Croatian Veterinary Students. Animals 2020, 10(2):199.
37. Milroy KE, Whiting M, Abeyesinghe S. Reporting of suspected dog fighting to the police, Royal Society for the Prevention of Cruelty to Animals and equivalents by veterinary professionals in the UK. Veterinary Record, 2018, 183, 567.
38. Mogbo TC, Oduah FN, Okeke JJ, Ufele AN, Nwankwo OD. Animal Cruelty: A Review. Journal of Natural Sciences Research. ISSN 2225-0921 (Online), 2013, Vol.3, No.8, 94-98.
39. Morris A. Reporting animal abuse: a vet's responsibility to society? Vet Record, 2010, 167 (17), 638
40. Munro R, Munro HMC. Animal abuse and unlawful killing: Forensic veterinary pathology. London: Elsevier, 2008
41. Newberry M. Associations between different motivations for animal cruelty, methods of animal cruelty and facets of impulsivity. Psychology Crime and Law, 2018, 24(1):52-78.
42. Ostovic M, Mesić Ž, Mikuš T, Matković K, Pavičićet Ž. Attitudes of veterinary students in Croatia toward farm animal welfare. Animal Welfare 2016, 25: 21-28.
43. Phillips C, Izmirli S, Aldavood J, Alonso M, Choe B, Hanlon A, Handziska A, Illmann G, Keeling L, Kennedy M, Lee G, Lund V, Mejdell C, Pelagic V and Rehn T. Students' attitudes to animal welfare and rights in Europe and Asia. Animal Welfare 2012, 21: 87-100
44. Phillips CJC, McCulloch S. Student attitudes on animal sentience and use of animals in society. J. Biol. Educ. 2005. 40:17–24.
45. Pręgowski MP & Cieślik S. Attitudes to Animal Abuse in Veterinary Practice in Poland, Anthrozoös, 2020, 33:3, 427-440.
46. Proctor HS, Carder G, Cornish AR. Searching for Animal Sentience: A Systematic Review of the Scientific Literature. Animals 2013, 3, 882-906.
47. Richard C & Reese LA. The Interpersonal Context of Human/Nonhuman Animal Violence. Anthrozoos, 2019, 32(1):65-87.
48. Rowan AN. Animal Cruelty: Definitions and sociology. Behavioral and Brain Sciences, 2006, 29 (3), 238-239
49. Sankoff P & White S. Animal Law in Australasia: A New Dialogue. 2009. Federation Press. ISBN 186287719X, 9781862877191.
50. Schaffner J. Anti-Cruelty Laws. In book: An Introduction to Animals and the Law. 2011, 22-70.
51. Scott-Park F. Recognising and reporting animal abuse. Veterinary record, 2019, 185(17):402-404
52. Sienauskaite O. Reporting animal abuse incidents. Veterinary record, 2017, 181(1)
53. Sinclair M, Derkley T, Fryer C, Phillips CJ. Australian Public Opinions Regarding the Live Export Trade before and after an Animal Welfare Media Exposé. Animals 2018, 8, 106.
54. Sneddon LU, Elwood RW, Adamo SA, Leach MC. Defining and assessing animal pain. Anim. Behav. 2014. 97:201–212.
55. Solarova D. Criminal liability for animal abuse in Ukraine and foreign countries. "Molody Vcheny", ISSN (Online): 2313-2167, 2019, 3(67), 402-404. (In Ukrainian)
56. Stolt LB, Johnson-Ifearulundu YJ, & Kaneene JB. Attitudes of veterinarians, animal control directors, and county prosecutors in Michigan regarding enforcement of state animal cruelty legislation. J Am Vet Med Assoc 1998; 211: 1521-1523.
57. Takacova D, Bugarský A, Hečko R, Mašlej M, Süli J. Animal as subject of law. Magyar Allatorvosok Lapja, 2013, 135(1):57-59.
58. Tamioso PR, Rucinque DS, Miele M, Boissy A, Molento CFM. Perception of animal sentience by Brazilian and French citizens: The case of sheep welfare and sentience. PLoS ONE, 2018, 13(7): e0200425.
59. Taylor N & Signal TD. Community Demographics and the Propensity to Report Animal Cruelty. Journal of Applied Animal Welfare Science, 2006, 9:3, 201-210.
60. Tiplady CM, Walsh DA B, Phillips CJ. Public Response to Media Coverage of Animal Cruelty. J Agric Environ Ethics (2013) 26:869–885.
61. Verbeke W. Stakeholder, citizen and consumer interests in farm animal welfare. Animal Welfare, 2009, 18, 325-333.
62. Viñas NQ, Randour ML, Arkow P. Animal Cruelty as a Gateway Crime. 2018. Publisher: COPS, Dep. of Justice, USA
63. Wolfensohn S, Honess P. Laboratory animal, pet animal, farm animal, wild animal: which gets the best deal? Animal welfare, 2007, 16(1):117123.
64. Zalaf A & Egan V. A new questionnaire examining general attitudes toward animals in Cyprus and the United Kingdom. Journal of Veterinary Behavior 10 (2015), 111-117.

*Received 10 August 2020*
*Accepted 7 December 2020*

# Genotype Influence on the Consumption and Use of Fodder Nutrients by Pure-Breed and Cross-Breed Bull Calves

**Tursun Satymbaevich Kubatbekov[1], Vladimir Ivanovich Kosilov[2], Ekaterina Olegovna Rystsova[3], Marina Vladimirovna Bolshakova[3], Anna Valievna Tadzhieva[3], Evgeniya Igorevna Simonova[3]**

*[1]Department of Morphology and Veterinary Sciences, Russian State Agrarian University – Moscow Timiryazev Agricultural Academy, Russia;*

*[2]Department of Production Technology and Processing of animal origin produce, Orenburg State Agrarian University, Russia;*

*[3]Department of Veterinary Medicine, Peoples' Friendship University of Russia, Russia*

***Keywords:*** *Simmentals, crossbred bulls, Russian Black Pied cattle, nutrients, consumption*

***Abstract.*** *The research was conducted to assess the positive influence of the bull calf genotypes on nutrient use efficiency in their diet to increase meat productivity. The aim of this study was to perform an analysis of the efficiency of consumption and use of nutrients in the diet by purebred Simmental calves and their first-generation crosses (F1) with Red Steppe and Russian Black Pied cattle.*

*The results of the study show that crossbred bulls ½ Simmental x ½ Russian Black Pied cattle exceeded thoroughbred calves of the same age of the Simmental breed and crossbred youngsters ½ Simmental x ½ Red Steppe breed in terms of nutrient consumption by 8.4-1030.5 g (2.0–11.70%), the amount of digested nutrients by 10.7–948.7 g (3.5–16.0%) and the value of the digestibility coefficient by 0.28–3.24%. Crossbred calves ½ Simmental x ½ Red Steppe breed showed minimal differences in the analyzed indicators. This study shows that the targeted selection of animals from different breeds for crossbreeding results in better nutrient intake and productivity, which opens new perspectives for the meat industry.*

## Introduction

Currently, there is a shortage of meat production, especially beef production, in the Russian Federation. It is necessary to develop and implement a program for the accelerated development of cattle breeding to fulfill the missing amounts of meat (Sedykh, 2018) to solve this problem. Therefore, the industry's primary direction is the rational use of genetic resources in Russian selection (Kayumov, 2019). In the Russian Federation, the foremost amounts of beef are obtained from the rearing of the super-replaced young herd and rejected animals from the main herds of dairy and mixed breeds such as Red Steppe, Russian Black Pied cattle, and Simmental.

Recently, the breeders have paid much attention to the Simmental cattle breed. The performance potential of the Simmental fattening bulls has been improved by selective breeding during the past decades, resulting in changes in fattening and slaughter traits of bulls (Honig, 2020). This breed has plenty of economically useful qualities, such as a high level of meat productivity and others. Simultaneously, the valuable properties of the Simmental animals are inherited not only during purebred breeding but also during crossbreeding (D'Occhio, 2019).

The purpose of this study was to assess the efficiency of consumption and use of nutrients in the diet by purebred Simmental calves and their first-generation crosses (F1) with Red Steppe and Russian Black Pied cattle.

Correspondence to Ekaterina Olegovna Rystsova, Department of Veterinary Medicine, Peoples' Friendship University of Russia, Miklukho-Maklaya, 8/2, 117198, Moscow, Russia.
E-mail: rystsova-eo@rudn.ru

## Materials and Methods

The studies were carried out in 2016–2018 in the Limited liability company (LLC) "Zailechye" in the Orenburg region. During this scientific experiment, three groups of 6-month-old bulls (15 animals in each group) were formed according to the following genotypes: group I – Simmental breed, group II – ½ Simmental x ½ Red Steppe, and group III – ½ Simmental x ½ Russian Black Pied cattle.

Animals in the groups differed only in the breed. Other indicators such as age and weight were balanced in all three groups and did not significantly differ.

Throughout the experiment, young animals were kept at the feedlot under year-round stall maintenance. The conditions of keeping and feeding corresponded to the Russian Federation's standards and were the same for all three groups (GOST 32855-2014 Requirements in raising and feeding cattle young livestock for meat production food-stuffs for children. Standard technological process). Feed consumption was promoted monthly for two adjacent days according to the difference in the mass of the given feed and uneaten residues. Young animals of all groups were provided with a complete, balanced feeding, which contributed to the manifestation of the genetic potential of meat productivity.

During the balance (physiological) experiment, feed consumption was assessed daily for three animals from the group. The consumption and use of nutrients were determined according to the balanced

experiment results, considering the diet's chemical composition. Three animals were selected from each group, composing balanced groups according to the principle of a mini-herd (from each already formed group of 15 animals, three bulls were selected, only for the balanced experiment) to carry out digestibility balance experiments, since they are costly.

The data were proceeded using the Statistica Statgraf software package. Arithmetic means with standard deviations are presented in Tables 1–3. The Student's t-test compared the mean values for independent variables (Tables 1–2). The significance of differences between means was reported at P 0.01 and P 0.05. In the physiological experiment, the number of animals was reduced. Only this experiment was conducted using the mini-herd principle due to the high complexity and cost of this study. It was not possible to carry out an accurate statistical analysis due to the small amount of data. However, it is planned to repeat this experiment with more animals in the future.

**Results**

The analysis results in the balanced experiment show the influence of genotype on the consumption of individual nutrients in the diet (Table 1).

The leading position in the consumption of all types of feed nutrients was occupied by crossbred bulls (½Simmental x ½ Russian Black Pied cattle.) of group III. Purebred bulls of the Simmental breed (group I) and their first-generation crossbreds (½ Simmental x ½ Red Steppe) of group II were inferior to them in dry matter consumption by 200.0 g (2.1%, $P < 0.05$) and 1030.0 g (11.7%, $P < 0.01$), organic matter by 189.4 g (2.1%, $P < 0.05$) and 968.6 g (11.6%), crude protein by 24.6 g (2.1%) and 126.7 g (11.7%, $P < 0.01$), crude fat by 8.4 g (2.0%, $P < 0.05$) and 43.2 g (11.6%, $P < 0.01$), crude fiber by 44.8 g (2.1%, $P < 0.05$) and 230.7 g (11.7%, $P < 0.01$ ), and nitrogen-free extractive substances (NFE) by 111.6 g (2.1%, $P < 0.05$) and 568 g (11.6%, $P < 0.01$).

Crossbred calves (½ Simmental x ½ Red Steppe) of group II showed the smallest consumption of all types of nutrients in feed. Purebred calves of the Simmental breed of group I surpassed them in dry matter consumption by 830.0 g (9.4%, $P < 0.01$), organic matter by 779.2 g (9.3%, $P < 0.01$) , crude protein by 102.1 g (9.4%, $P < 0.01$), crude fat by 34.8 g (9.3%, $P < 0.05$), crude fiber by 185.9 g (9.4%, $P < 0.01$), nitrogen-free extractives (NFE) by 456.4 g (9.3%, $P < 0.01$). It is known that nutrients received from food are digested and absorbed only partially. Then they are included in metabolic processes taking place in the animal's body. Undigested nutrients are excreted in the feces (Van Gastelen, 2019).

It should be taken into account that the efficiency of digestion of feed nutrients is significantly influenced by a complex of factors, both phenotypic and genotypic (Oss, 2017). This is confirmed by our study results, which show the influence of calf genotype in the experimental groups on the digestibility of nutrients (Table 2).

At the same time, the crossbred bulls (½ Simmental x ½ Russian Black Pied cattle) of group III showed the maximum amount of digested substances. They surpassed the purebred peers of the Simmental breed of group I and crossbred young animals (½ Simmental x ½ Red Steppe) of group II in terms of the amount of digested dry matter, respectively, by 244.8 g (3.7%, $P < 0.05$) and 948.7 g (16.0%, $P < 0.01$), or-

*Table 1.* Amount of nutrients taken with feed by experimental young animals (on average for one animal per day), g

| Characteristic | Group | | | | | | Significance | | |
|---|---|---|---|---|---|---|---|---|---|
| | I | | II | | III | | I and II | I and III | II and III |
| | Statistical indicator | | | | | | | | |
| | X ± Se | Cv | X ± Se | Cv | X ± Se | Cv | | | |
| Dry matter | 9662.5 ± 56.42 | 2.14 | 8832.5 ± 52.34 | 2.02 | 9862.5 ± 64.21 | 2.16 | ** | * | ** |
| Organic matter | 9102.1 ± 46.46 | 2.01 | 8322.9 ± 47.12 | 2.14 | 9291.5 ± 52.19 | 2.33 | ** | * | - |
| Crude protein | 1188.5 ± 15.64 | 1.88 | 1086.4 ± 14.21 | 1.7 | 1213.1 ± 15.92 | 1.88 | ** | - | ** |
| Crude fat | 405.8 ± 4.81 | 2.1 | 371.0 ± 4.24 | 1.66 | 414.2 ± 5.12 | 2.18 | * | * | ** |
| Crude fiber | 2164.4 ± 29.22 | 3.14 | 1978.5 ± 28.06 | 3.01 | 2209.2 ± 30.01 | 3.23 | ** | * | ** |
| NFE | 5343.4 ± 34.11 | 3.2 | 4887.0 ± 36.18 | 3.32 | 5455.0 ± 38.71 | 3.31 | ** | * | ** |

*Mean values in rows differ at $P \leqslant 0.05$; ** Mean values in rows differ at $P \leqslant 0.01$.
«-» inaccurate data. X – mass (g); Se – standard error; Cy – digestibility coefficient (%)

*Table 2.* The number of nutrients digested by the experimental young animals (on average per one animal per day), g

| Characteristic | Group | | | | | | Significance | | |
|---|---|---|---|---|---|---|---|---|---|
| | I | | II | | III | | I and II | I and III | II and III |
| | Statistical Indicator | | | | | | | | |
| | X ± Se | Cv | X ± Se | Cv | X ± Se | Cv | | | |
| Dry matter | 6632.3 ± 40.31 | 1.3 | 5928.4±39.61 | 1.22 | 6877.1 ± 41.62 | 1.33 | *** | * | ** |
| Organic matter | 6424.3 ± 28.16 | 1.16 | 5762.8 ± 24.84 | 1.08 | 6610.0 ± 26.23 | 1.1 | *** | * | *** |
| Crude protein | 801.2 ± 6.28 | 1.9 | 719.5 ± 5.85 | 1.88 | 832.6 ± 6.18 | 1.82 | ** | ** | *** |
| Crude fat | 293.6 ± 4.13 | 2.16 | 260.6 ± 3.09 | 2.04 | 304.3 ± 3.21 | 2.1 | *** | ** | *** |
| Crude fiber | 1225.9 ± 17.42 | 2.33 | 1095.3 ± 15.21 | 2.1 | 1268.5 ± 19.43 | 2.52 | ** | ** | *** |
| NFE | 4103.6 ± 28.12 | 1.9 | 3,687.4 ± 26.21 | 1.81 | 4204.6 ± 29.32 | 2.02 | ** | * | ** |

*Mean values in rows differ at $P \leqslant 0.05$; **Mean values in rows differ at $P \leqslant 0.01$.
***Mean values in rows differ at $P \geqslant 0.001$.
X – mass (g); Se – standard error; Cy – digestibility coefficient (%).

ganic matter by 186.0% g (2.9%, $P < 0.05$) and 847.2 g (14.7%, $P < 0.001$), crude protein by 31.4 g (3.9%, $P < 0.01$) and 113.1 g (15.7%, $P < 0.001$), crude fat by 10.7 g (3.6%, $P < 0.01$) and 43.7 g (16.8%, $P < 0.001$), crude fiber by 42.9 g (3.5%, $P < 0.01$) and 173.2 g (15.8%, $P < 0.001$), and nitrogen-free extractives (NFE) by 101.0 g (2.5%, $P < 0.05$) and 517.2 g (14.0%, $P < 0.01$).

Purebred bulls of the Simmental breed of group I surpassed the crossbred bulls (½ Simmental x ½ red steppe) of group II in terms of the amount of digested dry matter by 703.9 g (11.9%, $P < 0.001$), organic matter by 661.5 g (11.5%, $P < 0.001$), crude protein by 81.7 g (11.4%, $P < 0.01$), crude fat by 33.0 g (12.7%, $P < 0.001$), crude fiber by 130.6 g (11.9%, $P < 0.01$), and nitrogen-free extractives (NFE) by 416.2 g (11.3%, $P < 0.01$).

In the process of digestion of nutrients in the gastrointestinal tract of animals, they undergo significant structural changes. At the same time, they acquire the ability to take part in metabolic processes and the formation of organs and tissues.

The value of the digestibility coefficient characterizes the diet effectiveness in the animal's body, expressed as a percentage. Moreover, in certain types of nutrients, its level has significant differences. Besides, the value of the digestibility coefficient depends on the genetic characteristics of animals. This position is confirmed by the results of our research (Table 3).

The leading position in terms of the digestibility coefficient of all types of nutrients of the ration was occupied by crossbred bulls (½ Simmental x ½ Russian Black Pied cattle) of group III. Their advantage over purebred animals of the Simmental breed of group I and crossbred young animals (½ Simmental x ½ Red Steppe) of group II in terms of the digestibility coefficient was as follows: 1.09% and 2.61%, respectively, for dry matter; 0.56% and 1.90% for organic matter; 1.22% and 2.40% for crude protein; 1.12% and 3.24% for crude fat; 0.78% and 2.06% for crude fiber; and 0.28% and 1.63% for nitrogen-free extractive substances (NFE). Crossbred animals (½ Simmental x ½ Red Steppe) of group II slightly differed in the digestibility coefficient in all

*Table 3.* Digestibility coefficients by experimental young animals, %

| Characteristic | Group | | | | | |
|---|---|---|---|---|---|---|
| | I | | II | | III | |
| | Statistical indicator | | | | | |
| | X ± Se | Cv | X ± Se | Cv | X ± Se | Cv |
| Dry matter | 68.64 ± 0.12 | 0.30 | 67.12 ± 0.14 | 0.31 | 69.73 ± 0.17 | 0.36 |
| Organic matter | 70.58±0.24 | 0.81 | 69.24 ± 0.25 | 0.84 | 71.14 ± 0.23 | 0.80 |
| Crude protein | 67.41 ± 0.15 | 0.43 | 66.23 ± 0.12 | 0.40 | 68.63 ± 0.16 | 0.45 |
| Crude fat | 72.35 ± 0.22 | 2.30 | 70.23 ± 0.21 | 2.26 | 73.47 ± 0.24 | 2.33 |
| Crude fiber | 56.64 ± 0.30 | 1.33 | 55.36 ± 0.26 | 1.30 | 57.42 ± 0.29 | 1.30 |
| NFE | 76.80 ± 0.31 | 1.26 | 75.45 ± 0.28 | 1.23 | 77.08 ± 0.34 | 1.30 |

*X – mass (g); Se – standard error; Cy – digestibility coefficient (%).

types of nutrients. They were inferior to the purebred animals of the Simmental breed of group I in terms of the analyzed indicator to dry matter by 1.52%, organic matter by 1.34%, crude protein by 1.18%, crude fat by 2.13%, crude fiber by 1.28%, and nitrogen-free extractive substances (NFE)- by 1.35%.

## Discussion and Conclusions

Animals receive nutrients with the consumption of feed, which contributes to the vital activity of their body. Nutrients entering the animal's body with feed become material for organ and tissue development and involve in all body metabolic processes (Broadhead et al., 2019).

In this study, the leading position in the consumption of all types of feed nutrients was occupied by crossbred bulls (½ Simmental x ½ Russian Black Pied cattle) of group III.

Crossbreeding is an up-and-coming method in animal husbandry. Crossbred bulls result in better nutrient intake and productivity, which opens new perspectives for the meat industry (Van Raden, 2020). The use of beef breed sires, especially of late-maturing breeds, in dairy herds improves the carcass characteristics and carcass gain of the slaughtered progeny, most noticeable in young bulls (Eriksson, 2020).

Three breed-sex types of cattle were examined within a similar study for their growth performance and carcass quality in an organic production setting (Murphy, 2017, 2018). It was concluded that combined use of genetically superior crossbred beef breed x Holstein bulls and heifers may be an alternative to purebred Holstein bulls in organic beef production of young cattle because of their improved carcass weight and carcass conformation, similar growth performance and lower total feed intake (Vestergaard, 2019).

In this study, young bulls of all experimental groups were distinguished by a high level of consumption and use of nutrients. However, the leading position was occupied by the first generation crossbred animals of Simmentals with Russian Black Pied cattle.

## References


1. Broadhead D., Mulliniks T.J., Funston R.N. Developmental Programming in a Beef Production System. Veterinary Clinics of North America: Food Animal Practice. 2019. T. 35. P. 379-390.
2. D'Occhio M.J., Baruselli P.S., Campanile G. Influence of nutrition, body condition, and metabolic status on reproduction in female beef cattle: A review. Theriogenology. 2019. T.125. P.277-284.
3. Eriksson S., Ask-Gullstrand P., Fikse W.F., Jonsson E., Eriksson J-A., Stalhammar H., Wallenbeck A., Hessle A. Different beef breed sires used for crossbreeding with Swedish dairy cows - effects on calving performance and carcass traits. Livestock Science. 2020. T. 232. 103209.
4. GOST (Russian National Standard) 32855-2014. Requirements in raising and feeding of cattle young livestock for the production of meat food-stuffs for children. Standard technological process. Standartinform. 2019. P. 1-15.
5. Honig A. C., Inhuber V., Spiekers H., Windisch W., Götz K-U., Ettle T. Influence of dietary energy concentration and body weight at slaughter on carcass tissue composition and beef cuts of modern type Fleckvieh (German Simmental) bulls. Meat Science. 2020. T. 169. 108209.
6. Kayumov F.G., Kosilov V.I., Gerasimov N.P., Bykova O.A. The effect of SNP polymorphisms in growth hormone gene on weight and linear growth in crossbred red Angus × Kalmyk heifers. Digital agriculture - development strategy Proceedings of the International Scientific and Practical Conference (ISPC 2019). Advances in Intelligent Systems Research. 2019. P. 325-328.
7. Oss D.B., Machado F.S., Tomich T.R., Pereira L.G.R., Campos M.M., Castro M.M.D., Da Silva T.E., Marcondes M.I. Energy and protein requirements of crossbred (Holstein × Gyr) growing bulls. Journal of Dairy Science. 2017. T. 100 N.4. P. 2603-2613.
8. Morales R, Phocas F, Solé M, Demyda-Peyrá S., Menéndez-Buxadera A., Molina A. Breeding beef cattle for an extended productive life: Evaluation of selection criteria in the Retinta breed. Livestock Science. 2017. T. 204. P. 115-121.
9. Murphy, B., Crosson, P., Kelly, A.K., Prendiville, R. Animal performance and economic implications of alternative production systems for dairy bulls slaughtered at 15 months of age. Irish J. Agric. Food Res. 2017. T. 56. P. 93–103.
10. Murphy, B., Kelly, A., Prendiville, R. Alternative finishing strategies for Holstein- Friesian bulls slaughtered at 15 months of age. Agric. Food Sci. 2018. T. 27. P. 28–37.
11. Schwarz, F.J., Röpke, R., Schams, D., Kirchgessner, M. Effects of sex and growth on plasma concentrations of growth hormone, insulin-like growth factor-I and insulin in fattening Simmental cattle. J. Anim. Physiol. Anim. Nutr. 1992. T. 68. P. 263–271.
12. Sedykh T.A., Gizatullin R.S., Kosilov V.I., Chudov I.V., Andreeva A.V., Giniyatullin M.G., Islamova S.G., Tagirov Kh. Kh., Kalashnikova L.A. Adapting Australian Hereford cattle to the conditions of the southern Urals. Research Journal of Pharmaceutical, Biological, and Chemical Sciences. 2018. T. 9. No 3. P. 885-898.
13. Van Gastelen S., Dijkstra J., Bannink A. Are dietary strategies to mitigate enteric methane emission equally effective across dairy cattle, beef cattle, and sheep? Journal of Dairy Science. 2019. T. 102. N. 7. P. 6109-6130.
14. Van Raden P.M., Tooker M.E., Chud T.C.S., Norman H.D., Megonigal J.H., Haagen I.W., Wiggans G.R. Genomic predictions for crossbred dairy cattle. Journal of Dairy Science. 2020. T. 103. N. 2. P. 1620-1631.
15. Vestergaard M., Jørgensen K.F., Çakmakçı C., Kargo M., Therkildsen M., Munk A., Kristensen T. Performance and carcass quality of crossbred beef x Holstein bull and heifer calves in comparison with purebred Holstein bull calves slaughtered at 17 months of age in an organic production system. Livestock Science. 2019. T. 223. P.184-192.


*Received 20 October 2020*
*Accepted 15 December 2020*

# Improved quality of traditional East European soured milk produced with wild-type *Lactococcus lactis* and fortified with local dill (*Anethum graveolens*)

**Justina Milerienė[1], Kristina Kondrotienė[1], Loreta Šernienė[1], Vaida Andrulevičiūtė[2], Neringa Kašėtienė[1], Dalia Sekmokienė[1], Lina Laučienė[1], Mindaugas Malakauskas[1]**

[1]*Department of Food Safety and Quality, Veterinary Academy, Lithuanian University of Health Sciences, Kaunas, Lithuania*
[2]*Department of Biochemistry, Medicine Academy, Lithuanian University of Health Sciences, Kaunas, Lithuania*

***Keywords:*** *antioxidant properties, fermentation, wild-type Lactococcus lactis, dill extract, soured milk.*

***Abstract.*** *Recently, main product development and research in the dairy industry have been targeted to the enrichment of products with local natural preservatives and wild-type starters leading to enhanced product safety and sustainability. The objective of the present study was to enhance the safety, quality, sensory acceptability, and sustainability of traditional East European soured milk by adding wild-type Lactococcus lactis bacteria and dill (Anethum graveolens) $CO_2$ extract. All fortified samples showed lower pH (0.05–0.1 points) at Days 1–14, lower D (–) lactate content (8–25%) throughout the whole storage, higher phenolic content (1–2%) and slightly higher overall acceptability at the end of storage compared with unfortified samples. Due to these properties and little impact on the industrial starter and wild L. lactis, dill $CO_2$ extract could be incorporated to soured milk production. Both tested wild-type Lactococcus lactis strains were able to reduce the pH of the milk to standard value 4.46–4.64 in 8 h and produced mainly L (+) lactic acid during storage time. Wild-type L. lactis maintained LAB counts at 1 log unit higher than the control sample until Day 14. Wild-type strains showed high viability during storage time, and actively acidified milk creating acceptable flavor, so they could be promising starters as a single strain or co-starter cultures.*

## Introduction

Consumers seek dairy foods for a healthier lifestyle, so synthetic preservatives are now being replaced by natural various plant extracts (Burt, 2004; Khorshidian et al., 2018), and local wild lactic acid bacteria (LAB) cultures are being investigated as possible starters (Sarao and Arora, 2017; Yerlikaya, 2019) .

The most important research area in the dairy industry (Sekmokiene, 2018) is the enrichment of dairy products with active plant components with aromatic, anti-oxidizing and antimicrobial properties, but it is still insufficiently investigated (Veiga et al., 2020). Many various biologically active substances (mainly bioflavonoids) are found in plant extracts and they are known to have a huge positive impact on the human body and to change the main characteristics of most dairy products they are used in (Gabriel-Danut et al., 2009).

Soured milk denotes a dairy product produced by the acidification of milk, giving a tart taste. This product is made at home commonly or produced commercially and consumed in Europe, especially in Eastern Europe. Traditionally, fresh milk is left to sour by keeping it in a warm place for a day. Naturally occurring acid causes milk to coagulate in this process and at the same time inhibits the growth of harmful bacteria and improves the product's shelf life (Pophaly et al., 2018).

Modern commercial soured milk may differ from milk that has become sour naturally. Soured milk produced by fermentation with different strains introduces the consumer to new flavors. In Eastern Europe, it can be widely used in different food recipes, like cold summer soured milk soups, which are often spiced up with local herbs. Dill (*Anethum graveolens*) is the most common among them, due to its pleasant and distinct flavor, availability of the plant throughout the summer period and emotional impact on traditional East European consumer. Recently, dill has found its place in the recipes of ready to use niche commercial soured milk products. The shelf life of these products is shorter than the traditional plain soured milks. Thus, the novelty of this study is in exploring the possibilities of fortifying soured milk with dill extracts. The effect of various aqueous dill extracts (Amirdivani and Baba, 2011; Marhamatizadeh et al., 2012; Abbas et al., 2013) and essential dill oils (Hassanien et al., 2014) has been widely analyzed. The main active bio-components in dill extract are carvone, limonene, dill apiol and α-phellandrene (Jianu et al.; Chahal et al., 2017). The properties of $CO_2$ dill extract in various dairy matrixes are less explored. *Lactococcus lactis* is a well-known starter culture used in large-scale manufacture of a vast range of fermented dairy products (fermented milk, sour cream, butter, soft and hard cheeses, etc.) due to de-

Correspondence to Justina Milerienė, Department of Food Safety and Quality, Veterinary Academy, Lithuanian University of Health Sciences, Kaunas, Lithuania, Tilžės 18, Kaunas LT-47181, Lithuania. E-mail: justina.mileriene@lsmu.lt

sirable properties such as acid production, flavor development, and bacteriophage resistance (Kelly et al., 2010)including wild-type isolates and dairy starter cultures, were screened on the basis of their phenotype and the macrorestriction patterns produced from pulsed-field gel electrophoresis (PFGE. However, specialized dairy starters have evolved to become essential components of industrial processes and are no longer fit to survive outside the dairy environment (Kandasamy et al., 2018)preservation and sensory qualities. These foods turn out to play a central role in the diet of several cultures because of its enriched health benefits that are known to possess antimicrobial, antidiabetic, anti-atherosclerotic, antioxidant and anti-inflammatory activities. Consequently, fermentable microorganisms, fermentation process and its products draw scientific interest. Currently fermented food production is mainly carried out using starter cultures for a precise and expectable fermentation. Lactic acid bacteria (LAB. Therefore, studies on technological, biochemical and organoleptic properties of newly isolated wild-type LAB, which may have genes that can be used to enhance the metabolism of dairy strains, are emerging (Nuryshev and Stoyanova, 2016; Yerlikaya, 2019; Fusieger et al., 2020)important substances that add buttery flavor notes in dairy products. Twenty-three L. lactis subsp. lactis isolates were obtained from dairy products (milk and cheese. Consumption of locally sourced strains isolated from food and subsequently used in the production of fermented milk products can significantly improve the condition and adaptation of the intestinal microflora (Cavanagh et al., 2015).

Wild-type LAB are a demanding microorganism and requires various nutrients and special environmental conditions for growth and propagating. Supplementation of carrier foods with nutritious and/or protective/sustaining ingredients such as plant extracts can, therefore, improve its viability having a potential synergistic effect on spoilage bacteria as well (Abdollahzadeh et al., 2018)physicochemical, rheological, and sensory characteristics of probiotic fermented milk was investigated. DE was added to milk at the level of 0–12 g/100 mL; the mixtures were then fermented with Lactobacillus acidophilus La-5. The initial probiotic concentrations ranged between 8.16 and 8.77 log10 CFU/g. Although the highest DE concentration led to a significant count reduction (from 8.16 to 6.44 log10 CFU/g. The application of plant extracts and wild LAB is a promising technology, which has been successfully used for bio preservation and functionality of milk products (Sarao and Arora, 2017).

In a recent study, we analyzed technological potential of *Lactococcus lactis* strains naturally present in raw and fermented milk (Kondrotiene et al., 2018). Therefore, to conduct this study, we selected two wild *Lactococcus lactis* subsp. *lactis* strains (LL16 and LL76) possessing most potential properties. Soured milk produced with newly isolated wild-type strains from Lithuanian raw and naturally fermented milk may be able to introduce the new desirable flavors, and their abilities as a potential starter were tested in this study. The study was undertaken to determine the suitability of locally produced dill $CO_2$ extract (*Anethum graveolens*) together with wild-type *Lactococcus lactis* strains for formulation of traditional East European soured milk. Fortification of traditional soured milk with locally produced dill $CO_2$ extract is an innovation that might be more commercially attractive and thus worth being tested.

## Materials and methods

### *Lactic acid bacteria and plant extracts*

Two wild *Lactococcus lactis* strains were isolated from raw (LL16) and fermented (LL76) small scale local farm milk samples and stored at –80°C in M17 broth (Merck, Germany) in the presence of 30% glycerol until further analysis (Kondrotiene et al., 2018). Before conducting any experiments, strains were revitalized in MRS broth (Biolife, Milano, Italy) by growing for 18 h at 30°C.

Dill (*Anethum graveolens*) $CO_2$ extract was purchased from a small-scale organic farm in Kaunas region. The extract demonstrated antimicrobial activity against *Bacillus cereus, Listeria monocytogenes,* and *Brochothrix thermosphacta.* The total phenolic count of the dill $CO_2$ extract was 7.15 ± 0.8 GAE mg/g of extract and antioxidant activity (ABTS) was 53.21 ± 0.12 μmol TEA/g, respectively.

### *Preparation of experimental soured milk*

Soured milk was prepared according to traditional soured milk preparation technology (Walstra et al., 1999). Standardized (2.52% fat, 3.03% protein, 4.54% lactose, 8.12% non-fat solids, pH 6.69 ± 0.02, determined with FoodScan (Foss, Denmark)), homogenized and pasteurized cow's milk was collected from local dairy factory and kept refrigerated (4°C) until analysis. After warming up milk to 30°C, it was distributed into three 400 mL vats and individually inoculated with 2% (approx. 4 log CFU bacteria per vat) of wild *L. lactis* strains (LL16 and LL76, respectively) and a commercial starter as a control (C). The commercial starter (F-DVS) for milk fermentation was purchased from CHR Hansen, Denmark. Deep-frozen bulk granules of mesophilic LD-type culture of mixed *L. lactis, L. cremoris, Leuconostoc sp,* and *St. thermophiles* (LAB concentration > $1\times10^{10}$ CFU/mL) were stored and prepared according to manufacturer's instructions to reach approx. 4 log CFU bacteria per vat. After inoculation, the content of each vat was divided into 2 parts (200 mL each) again, one portion was fortified with dill extract (10 μL/100 mL milk) and the other one remained unfortified (C-D, LL16-D, LL76-D). The samples were incubated for 8 h at 24°C, cooled to 4°C and stored for 28 days.

***Analysis of experimental sour milk samples***

Soured milks were sampled in triplicate for pH, D/L lactates and microbial examination at the beginning of storage (Day 1) and after 7, 14, 21 and 28 days of storage at 4 °C.

pH of samples was measured directly with a pH-meter (Sartorius Professional meter for pH Measurement, Germany).

The concentrations of L(+) and D(–) lactates (g/100 g) were determined using Megazyme assay Kit (Megazyme International Ireland, Bray, Ireland) and following manufacturer's instructions.

The changes of lactic acid bacteria were determined after 7, 14, 21 and 28 days of storage at 4°C. Soured milk samples (10 g) were aseptically taken from each triplicate, placed into a sterile stomacher bag (VWR Blender bag, US), diluted (1:10, w/v) in sterile Peptone water (Liofilchem) and homogenized with Stomacher 400 Circulator (Seward, UK) for 2 min. Decimal dilutions were prepared according to ISO 6887-5 (2010) with sterile Peptone water (Liofilchem) and plated on corresponding media. Quantification of microbiological counts was carried out using the pour plate technique. LAB counts ($\log_{10}$ CFU/mL) were enumerated on MRS agar (Biolife, Milano, Italy) and incubated under aerobic conditions at 30°C for 72 h.

Sensory analysis of milks was performed on Day 1, 14, and 28 by a panel of tasters comprising 10 to 12 participants, both men and women, with ages ranging from 22 to 50 years old. The panelists were selected and instructed to work according to ISO 8586 (2012) and had practical skills to evaluate milk products. General acceptability of samples (20 g) was analyzed using a 9 mm lineal rating scale ranging from no or very low acceptability (score 0–1) to an excellent one (8–9). The samples were coded with 3-digit randomized numbers and served at room temperature, before the evaluation. Panelists were exposed to each sample in random order and were asked to assess general acceptability. Two evaluation sessions were performed.

Antioxidant activity was determined by the 2.2´-azinobis-(3-ethylbenzthiazoline)-6-sulfonate (ABTS) method as described earlier with minor modification (Re et al., 1999). The ABTS+ solution was prepared mixing ABTS (7 mM) and potassium persulfate (2.45 mM) and stored in the dark for 16 h before use. To determine scavenging activity of different supercritical $CO_2$ extracts, the ABTS radical solution was diluted with distilled water to an absorbance of 0.800 at 734 nm. The absorbance of 20 µL of extracts with 3 mL of ABTS solution was measured after 1 h storing in the dark. A series of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) solutions (100–500 mg/L) were used for calibration. The Trolox equivalent antioxidant capacity (TEAC) values were calculated from the calibration curve and the radical scavenging capacity (RSC) values were expressed in µmol Trolox equivalents (TE) per product g (µmol TE/g).

Total phenolic content (TPC) of the samples was determined using the Folin-Ciocalteau method (FC) and expressed in milligrams of the Gallic acid equivalents (GAE) per 100 g of sample (Lotito and Frei, 2004)and flavonoid-rich foods may help protect against chronic diseases by antioxidant mechanisms. In the present study we investigated: (1. Fermented milk (3 g) was extracted with methyl alcohol (3 mL) for 10 min using a vortex. The tubes were centrifuged at 700 g for 10 min and the upper phase layer was collected and analyzed immediately.

***Statistical analysis***

The data analysis was performed by SPSS statistical package (Chicago, SPSS Inc., SPSS 24). Data were analyzed using descriptive statistics (Explore) and one-way analysis (ANOVA) methods. Means were compared using Bonferroni's multiple range tests, and statistical significance was standardized by ANOVA at $P < 0.05$.

**Results**

The study revealed significant differences of the acidity parameters of the soured milk samples during storage time (Table 1). Both of our tested wild-type *Lactococcus lactis* strains were able to reduce the pH of milk from 6.6 ± 0.03 to a standard pH for fermented milks (4.46–4.64) after an 8-h fermentation process.

Interestingly, all fortified samples showed significantly lower pH on Day 1 compared with unfortified samples. No such differences among fortified and plain samples were detected later throughout the storage period. pH values significantly increased on Day 7 and decreased to the Day 1 level on Day 14, during the storage. In all extract-free samples, it remained at the Day 14 level, increasing significantly in dill fortified samples at the end of storage.

LAB counts were monitored in soured milks during the whole storage time (Table 1). LAB counts remained stable during the first 2 weeks of storage and then gradually decreased till the end of the storage period (28 days) in all samples. On Day 14, there was a significant drop in LAB counts detected in control sample C and all samples fortified with dill extract. The same drop was spotted in the plain samples with wild-type starters a week later – on Day 21. The lowest viability of LAB was monitored in the sample with wild LL76, and the highest was observed in the sample with wild LL16 ($P < 0.05$) and in control samples.

Reduction of pH is caused by the metabolic process of lactic acid bacteria which produce organic acids. In our samples, LAB produced mainly L (+) lactate during storage time (Table 2). The amounts of undesirable D lactate increased during storage in all samples. Significantly lower concentrations of D lactate were detected in all the samples fortified with dill extract. This tendency was especially prominent in the samples with wild-type strains LL16 and LL76.

*Table 1.* Counts of lactic acid bacteria (LAB, $\log_{10}$ CFU/mL) and pH in sour milk samples without extract (C, LL16, LL76) and fortified with dill extract (C-D, LL16-D, LL76-D) during storage time at 4°C

| Storage | Day 1 | Day 7 | Day 14 | Day 21 | Day 28 |
|---|---|---|---|---|---|
| Samples | Lactic acid bacteria (log10 CFU/mL) | | | | |
| C | 7.67 ± 0.01a | 7.43 ± 0.01a | 5.96 ± 0.02bA | 6.00 ± 0.03bA | 6.26 ± 0.02bA |
| C-D | 7.49 ± 0.02a | 7.49 ± 0.02a | 5.91 ± 0.01bA | 6.23 ± 0.02bA | 5.49 ± 0.01bB |
| LL16 | 7.18 ± 0.01a | 7.45 ± 0.03a | 7.04 ± 0.01aB | 5.57 ± 0.03bA | 4.85 ± 0.01bC |
| LL16-D | 7.22 ± 0.02a | 7.11 ± 0.01a | 6.28 ± 0.03bA | 4.60 ± 0.02bB | 4.11 ± 0.03bD |
| LL76 | 7.00 ± 0.01a | 7.08 ± 0.02a | 7.08 ± 0.01aB | 5.68 ± 0.02bA | 4.74 ± 0.01cC |
| LL76-D | 7.20 ± 0.03a | 6.90 ± 0.03a | 6.60 ± 0.02bA | 4.70 ± 0.03bB | 3.86 ± 0.02cD |
| Samples | pH | | | | |
| C | 4.52 ± 0.03aA | 4.69 ± 0.07B | 4.54 ± 0.01aA | 4.52 ± 0.01aB | 4.57 ± 0.01aB |
| C-D | 4.49 ± 0.01 bA | 4.64 ± 0.01B | 4.50 ± 0.01bA | 4.52 ± 0.01aB | 4.61 ± 0.01aB |
| LL16 | 4.43 ± 0.04 A | 4.66 ± 0.12 B | 4.42 ± 0.01 bA | 4.47 ± 0.01 bB | 4.47 ± 0.01aB |
| LL16-D | 4.36 ± 0.12 bA | 4.62 ± 0.04 B | 4.43 ± 0.03 bA | 4.46 ± 0.02 bB | 4.47 ± 0.01aB |
| LL76 | 4.44 ± 0.05 A | 4.69 ± 0.07 B | 4.49 ± 0.03 bA | 4.50 ± 0.08 aB | 4.50 ± 0.01aB |
| LL76-D | 4.40 ± 0.01 bA | 4.58 ± 0.01 B | 4.52 ± 0.01 bA | 4.53 ± 0.01aB | 4.51 ± 0.01aB |

Values presented are means of three replicates ± standard deviation.
Means in the same row with different lowercase letters indicate significant differences ($P < 0.05$) among storage days.
Means in the same column with different capital letters indicate significant differences ($P < 0.05$) among strains.

*Table 2.* The concentrations of L(+) and D(–) lactates (g/100 g) in sour milk samples without extract (C, LL16, LL76) and fortified with dill extract (C-D, LL16-D, LL76-D) during storage at 4°C

| Storage | Day 1 | Day 7 | Day 14 | Day 21 | Day 28 |
|---|---|---|---|---|---|
| Samples | L (+) lactate (g/100 g) | | | | |
| C | 1.53 ± 0.00Aa | 0.87 ± 0.00Ab | 1.23 ± 0.00Ac | 1.34 ± 0.02Ad | 1.13 ± 0.00Ae |
| C-D | 0.91 ± 0.00Ba | 0.72 ± 0.00Bb | 1.68 ± 0.00Bc | 1.94 ± 0.02Bd | 1.81 ± 0.02Be |
| LL16 | 2.17 ± 0.00Ca | 0.83 ± 0.01Cb | 1.95 ± 0.02Cc | 2.26 ± 0.02Cd | 2.94 ± 0.02Ce |
| LL16-D | 1.69 ± 0.01Da | 0.65 ± 0.00Db | 2.43 ± 0.00Dc | 1.54 ± 0.02Dd | 3.28 ± 0.01De |
| LL76 | 2.76 ± 0.00Ea | 0.94 ± 0.00Eb | 2.90 ± 0.01Ec | 2.60 ± 0.01Ed | 2.05 ± 0.04Ee |
| LL76-D | 2.98 ± 0.01Fa | 1.31 ± 0.01Fb | 3.85 ± 0.00Fc | 3.49 ± 0.01Fd | 3.48 ± 0.01Fd |
| Samples | D (–) lactate (g/100 g) | | | | |
| C | 0.19 ± 0.04Aa | 0.60 ± 0.03Ab | 0.31 ± 0.02Ac | 0.59 ± 0.01Ab | 0.26 ± 0.02Ad |
| C-D | 0.03 ± 0.03Ba | 0.05 ± 0.00Ba | 0.06 ± 0.01Ba | 0.08 ± 0.02Bb | 0.10 ± 0.02Bb |
| LL16 | 0.17 ± 0.03Aa | 0.54 ± 0.01Cb | 0.36 ± 0.04Cc | 0.91 ± 0.03Cd | 0.81 ± 0.01Ce |
| LL16-D | 0.18 ± 0.02Aa | 0.12 ± 0.00Db | 0.06 ± 0.03Bc | 0.06 ± 0.02Bc | 0.05 ± 0.04Dc |
| LL76 | 0.22 ± 0.03Ca | 0.57 ± 0.00Eb | 0.38 ± 0.01Cc | 0.37 ± 0.04Dc | 0.42 ± 0.05Ed |
| LL76-D | 0.08 ± 0.04Da | 0.09 ± 0.03Fa | 0.12 ± 0.02Db | 0.09 ± 0.00Ba | 0.05 ± 0.03Da |

Values presented are means of three replicates ± standard deviation.
Means in the same row with different lowercase letters indicate significant differences ($P < 0.05$) among storage days.
Means in the same column with different capital letters indicate significant differences ($P < 0.05$) among strains.

We detected no significant differences in general acceptability among samples (Table 3) during storage. Storage affected the acceptability negatively; all samples demonstrated a slight decrease in acceptability on Day 28. Nevertheless, the samples fortified with dill extract showed the tendency to slightly reduce the sensory acceptability of control and experimental samples on Day 1 and enhance it at the end of the experiment.

Table 3 shows that the process of fermentation and storage (Day 1 versus 28) influenced the total phenolic content (TPC) with a slight decrease over the storage time, with the highest values found in the samples fortified with dill on Day 1 ($P \geqslant 0.05$). Significantly higher contents of TPC were found in all plain samples on Day 14 and Day 28.

Antioxidant activity also varied depending on the strain (Table 3). Between wild type strains, the high-

*Table 3.* Total phenolic content (GAE mg/100 g), and antioxidant activity (μmol TE/g), and overall acceptability (1-9) of sour milk samples without extract (C, LL16, LL76) and fortified with the dill extract (C-D, LL16-D, LL76-D) during storage at 4°C

| Sample | Total phenolic content (GAE mg/100 g) | | | Antioxidant activity (μmol TE/g) | | |
|---|---|---|---|---|---|---|
| | Day 1 | Day 14 | Day 28 | Day 1 | Day 14 | Day 28 |
| C | 1.46 ± 0.01$^{a}$ | 1.25 ± 0.04$^{bA}$ | 1.26 ± 0.08$^{bA}$ | 0.36 ± 0.08$^{aA}$ | 1.02 ± 0.03$^{bA}$ | 0.06 ± 0.07$^{c}$ |
| C-D | 1.53 ± 0.09$^{a}$ | 1.61 ± 0.06$^{bB}$ | 1.30 ± 0.08$^{cA}$ | 0.05 ± 0.06$^{aB}$ | 0.59 ± 0.01$^{bB}$ | 0.05 ± 0.02$^{a}$ |
| LL16 | 1.39 ± 0.06$^{a}$ | 1.53 ± 0.06$^{bB}$ | 1.16 ± 0.08$^{cB}$ | 0.55 ± 0.01$^{aA}$ | 0.60 ± 0.03$^{aB}$ | 0.08 ± 0.08$^{b}$ |
| LL16-D | 1.41 ± 0.08$^{a}$ | 1.26 ± 0.06$^{bA}$ | 1.09 ± 0.02$^{cB}$ | 0.08 ± 0.05$^{aB}$ | 0.14 ± 0.07$^{bC}$ | 0.04 ± 0.08$^{a}$ |
| LL76 | 1.50 ± 0.02$^{a}$ | 1.48 ± 0.02$^{bB}$ | 1.49 ± 0.04$^{bA}$ | 0.21 ± 0.02$^{aA}$ | 0.34 ± 0.02$^{bB}$ | 0.01 ± 0.05$^{a}$ |
| LL76-D | 1.52 ± 0.06$^{a}$ | 1.31 ± 0.03$^{bA}$ | 1.20 ± 0.02$^{cA}$ | 0.46 ± 0.01$^{aA}$ | 0.26 ± 0.05$^{aC}$ | 0.02 ± 0.03$^{b}$ |
| Sample | Overall sensory acceptability (1–9) | | | | | |
| | Day 1 | Day 14 | Day 28 | | | |
| C | 6.97 ± 2.96$^{a}$ | 6.12 ± 2.56 | 5.67 ± 1.26$^{b}$ | | | |
| C-D | 6.41 ± 2.57$^{a}$ | 6.21 ± 1.75 | 5.91 ± 2.27$^{b}$ | | | |
| LL16 | 7.03 ± 2.49$^{a}$ | 6.48 ± 1.95 | 5.73 ± 1.29$^{b}$ | | | |
| LL16-D | 6.73 ± 3.24$^{a}$ | 6.53 ± 2.31 | 5.83 ± 2.32$^{b}$ | | | |
| LL76 | 7.73 ± 2.77$^{a}$ | 6.62 ± 2.12 | 5.73 ± 1.27$^{b}$ | | | |
| LL76-D | 7.46 ± 1.66$^{a}$ | 6.72 ± 1.96 | 5.96 ± 2.23$^{b}$ | | | |

Values presented are means of three replicates ± standard deviation.
Means in the same row with different lowercase letters indicate significant differences ($P < 0.05$) among storage days.
Means in the same column with different capital letters indicate significant differences ($P < 0.05$) among strains.

est antioxidant activity was determined in strain LL16 on Day 1 and the lowest in LL76. The antioxidant capacities obtained in this study increased on Day 14 and decreased on Day 28 in all samples, but they are significantly lower comparing with the samples fortified with dill extract. No differences in antioxidant activity were detected among the samples at the end of the storage.

## Discussion and conclusions

One of the most important properties of a starter in dairy fermentation is the ability to produce acid rapidly (Bello et al., 2013). At the beginning of the storage (before Day 14), herbal soured milks had faster rates of pH reduction than the plain ones ($P < 0.05$). Similar pH tendencies in dill fortified fermented products were detected by Amirdivani and Baba (2011). In a study of yoghurts fortified with thyme, grape and green tea extracts, Alwazeer et al. (2020) concludes that addition of herbal extracts into the formulations of fermented milk can affect the metabolism of starters, especially their metabolic flux, acidification rate and reducing activity.

LAB starters must demonstrate multiple benefits in foods such as delaying spoilage and producing bioactive metabolites. Bioactive metabolites that are specifically the result of LAB are increasingly identified in foods. Ensuring the presence of these metabolites in products is contributing to health functionality (Champagne et al., 2018). Lactic acid as a LAB metabolite is important not only as a bio-preservation agent (Corsetti et al., 2015), it also comes in two bioactive lactic acid forms: L (+) and D (–). High concentrations of D (–) lactic acid are harmful to humans and should be avoided in food, whereas L (+) lactic acid is the preferred isomer in food products (Reddy et al., 2008). D-lactate can cause health complications, and the expected median lethal dose (LD50) per orally poisoned rats is around 4.5 g/kg (Pohanka, 2020). Wild *L. lactis* strains produced mainly L (+) lactic acid in our samples during storage time. Most of it was produced in a LL76 sample fortified with dill extract ($P < 0.05$). Interestingly, dill extract added to the control and experimental products significantly decreased D (–) lactic acid concentration. This is in agreement with other studies (Marhamatizadeh et al., 2012).

One of the criteria for a wild-type LAB to be regarded as acceptable starters is consumer acceptance and survival of microorganisms through the processing and storage of a final product (Sarao and Arora, 2017). In this study, the ability of the wild *L. lactis* strain to maintain viable counts at the same level as an industrial mix of starter cultures shows promising technological potential. Such isolates could be considered for replacement or supplementation of commercial starters used to produce fermented products. However, the fortification of soured milk samples with the dill $CO_2$ extract had a negative impact on the viability of LAB at the end of the storage. The results of our study revealed the significant decrease of LAB counts in all samples with the dill extract: on Day 14 in the samples with wild-type strains, and on Day 28 in control samples. At the end of the storage, wild-type strains were at 1 log lower counts than

those of the control sample. The same tendency appeared in the fortified samples – all samples with dill expressed 1 log less viable cells than their unfortified counterparts. The decrease in the LAB count was less noticeable in control samples with dill containing a commercial starter. The main component in the dill extract is carvone (Chahal et al., 2017), which can disrupt a pH gradient and membrane potential of cells (Champagne et al., 2018). In 1995, Oosterhaven et al. announced that carvone reduced the growth of *S. thermophilus* and *L. lactis* through interrupting the metabolic energy status of the cells. The use of dill essential oil *in vitro* was not effective on the growth of some lactic acid bacteria: *L. delbrueckii* ssp. *bulgaricus* and *St. thermophilus* (Abbas et al., 2013). Nevertheless, the results regarding its effect on LAB are controversial. It has been reported that addition of some aromatic and essential oils to yoghurt during its manufacture had a stimulatory effect on lactic acid bacteria by enhancing their growth and acid production (Abou Ayana, 2011). It can be stated that the impact of the main antimicrobial agent on non-target organisms highly depends on the method of extraction, dosage, and dairy matrix and starter interactions (Nadal et al., 2010).

Flavor is one of the most important attributes for consumers that can be negatively affected by putting more attention to bio-preservation of dairy produce (Khorshidian et al., 2018), such as choosing strong unpleasant volatile and non-volatile metabolites producing *L. lactis* strains and applying too high concentrations of plant extracts thus increases the off-flavor in the final bouquet. In our study, there were no significant differences among samples regarding sensory evaluation, i.e., the samples with wild-type strains were equally acceptable and dill fortification by the end of the 28 days of storage was able to slightly enhance the sensory acceptance of soured milk samples.

There were significant differences in antioxidant activity among strains in our study, indicating that the antioxidant activity of *L. lactis* can vary by source and strain (Ozdogan et al., 2012)namely its resistance to bile salt, pepsin, pancreatin, acid and antibiotics. Moreover, the ability of L. lactis to inhibit the adhesion of Escherichia coli ETEC and Salmonella Typhimurium SL1344 to Caco-2 cells were examined and also the hydrophobicity, iron-ion chelating ability, and determination of α,α-diphenyl-β-picrylhydrazyl (DPPH. The antioxidant capacities were storage dependent: increasing at the beginning and decreasing at the end of the storage period. Since plain-samples contain no plant extracts, the TPC values in sour milk reflect phenolic compounds related to milk protein breakdown by LAB. Surprisingly, less antioxidant activity was detected in the fortified samples. Contrary results were found in the similar studies by Amirdivani and Baba (2011). TPC and antioxidant capacity per g of a pure dill extract (7.15 GAE mg /100g, ABTS 53.21 μmol TE/g, respectively) were quite high, so other factors may have interfered in the colorimetric determination of TPC and ABTS in soured milks.

In conclusion, development of dairy products with local wild-type starters and new flavors has potential benefits thereby increasing sales and consumer's satisfaction. Traditional preparation of soured milk may be beneficial by including plant extracts to enhance the flavor as well as product quality. In view of its organoleptic properties, the dill extract could most readily be incorporated in manufactured East European fermented dairy products that are traditionally associated with herbs (savory dishes such as herbal sour milk or soft white curd cheese) or spices (dried and baked/smoked varieties of curd cheese). Due to the prominent phenolic content, ability to reduce D (–) lactate in fermented milk products and acceptable aroma, the dill extract can be proposed to dairy producers as a natural, safe, biodegradable flavor enhancer. Antagonism between the extract and food ingredients is undesirable and further research is needed, so it could be avoided in practical applications.

Industrial dairy *L. lactis* strains have undergone a significant genome decay and are considered to be unable to survive outside of this niche (Kelly et al., 2010)including wild-type isolates and dairy starter cultures, were screened on the basis of their phenotype and the macrorestriction patterns produced from pulsed-field gel electrophoresis (PFGE. With the emergence of wild-type *L. lactis* with promising sensory and technological properties, such isolates could be used producing traditional and novel fermented products with the potential to sustain the natural diversity of gut microbiota. Combinations of probiotic *L. lactis* and the dill extract can be exploited to maximize the phenolic content in the product and to minimize the concentrations required to achieve a particular D (–) lactate decreasing effect.

**Conflict of interest**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Acknowledgments**

This research was funded by the European Regional Development Fund according to the supported activity 'Research Projects Implemented by World-class Researcher Groups' under Measure No. 01.2.2-LMT-K-718.

## References


1. Abbas H. M., Mohamed S. H. S., Zaky, W. M., Kassem J. M., Salem, M. M. E., Said- H. A. H., et al. Impact of Antimicrobial Properties of Some Essential Oils on Cheese Yoghurt Quality. World Applied Sciences Journal. 2013. T. 27. P. 497–507. DOI:10.5829/idosi.wasj.2013.27.04.13623.
2. Abdollahzadeh S. M., Zahedani M. R., Rahmdel S., Hemmati F., and Mazloomi S. M. Development of Lactobacillus acidophilus-fermented milk fortified with date extract. LWT - Food Science and Technology. 2018. T. 98. P. 577–582. DOI: 10.1016/j.lwt.2018.09.042.
3. Abou Ayana I. Improvement of the Properties of Goat's Milk Labneh using some Aromatic and Vegetable Oils. International Journal of Dairy Science. 2011. T. 2. P. 112-123. DOI:10.3923/ijds.2011.112.123.
4. Alwazeer D., Bulut M., and Tunçtürk Y. Fortification of milk with plant extracts modifies the acidification and reducing capacities of yoghurt bacteria. International Journal of Dairy Technology 2020. T. 73. P. 117–125. DOI:10.1111/1471-0307.12643.
5. Amirdivani S., and Baba A. S. Changes in yogurt fermentation characteristics, and antioxidant potential and in vitro inhibition of angiotensin-1 converting enzyme upon the inclusion of peppermint, dill and basil. LWT - Food Science and Technology. 2011. T. 44 P. 1458–1464. DOI:10.1016/j.lwt.2011.01.019.
6. Bello B. D., Zeppa G., Bianchi D. M., Decastelli L., Traversa A., Gallina S., et al. Effect of nisin-producing *Lactococcus lactis* starter cultures on the inhibition of two pathogens in ripened cheeses. International Journal of Dairy Technology. 2013 T. 66. P. 468-477. DOI:10.1111/1471-0307.12064.
7. Burt S. Essential oils: Their antibacterial properties and potential applications in foods - A review. International Journal of Food Microbiology. 2004. T. 94. P. 223–253. DOI:10.1016/j.ijfoodmicro.2004.03.022.
8. Cavanagh D., Fitzgerald G. F., and McAuliffe O. From field to fermentation: the origins of Lactococcus lactis and its domestication to the dairy environment. Food Microbiology. 2015. T. 47. P. 45–61.
9. Chahal K., Kaur R., Kumar A., and Bhardwaj U. Chemistry and biological activities of Anethum graveolens L. (dill) essential oil: A review. Journal of Pharacognosy and Phytochemistry. 2017. T. 6. P. 295-306.
10. Champagne C. P., Gomes da Cruz A., and Daga M. Strategies to improve the functionality of probiotics in supplements and foods. Current Opinion in Food Science. 2018. T. 22. P. 160–166. DOI:10.1016/j.cofs.2018.04.008.
11. Corsetti A., Perpetuini G., and Tofalo R., "Biopreservation effects in fermented foods," in Advances in Fermented Foods and Beverages: Improving Quality, Technologies and Health Benefits (Elsevier Inc.). 2015. P. 311–332. DOI:10.1016/B978-1-78242-015-6.00013-X.
12. Fusieger A., Martins M. C. F., de Freitas R., Nero, L. A. and de Carvalho, A. F. Technological properties of Lactococcus lactis subsp. lactis bv. diacetylactis obtained from dairy and non-dairy niches. Brazilian Journal of Microbiology. 2020. T. 51. P. 313–321. DOI:10.1007/s42770-019-00182-3.
13. Gabriel-Danut M., Gabriela R., Elisabeta B., Aida V., Liliana G., Doina A., et al., Research concerning the production of a probiotic dairy product with added medicinal plant extracts. in The Annals of the University of Dunarea de Jos of Galati. 2009. P. 32-37.
14. Hassanien M. F. R., Mahgoub S. A., and El-Zahar K. M. Soft cheese supplemented with black cumin oil: Impact on food borne pathogens and quality during storage. Saudi Journal of Biological Science 2014. T. 21. P. 280–288. DOI:10.1016/j.sjbs.2013.10.005.
15. International Organization for Standardization 2010: ISO 6887-5:2010 Microbiology of food and animal feeding stuffs. Preparation of test samples, initial suspension and decimal dilutions for microbiological examination Specific rules for the preparation of milk and milk products. 13. Available at: https://www.iso.org/standard/50306.html [Accessed March 16, 2020].
16. International Organization for Standardization 2012: ISO 8586:2012 Sensory analysis — General guidelines for the selection, training and monitoring of selected assessors and expert sensory assessors. 28. Available at: https://www.iso.org/standard/45352.html.
17. Jianu C., Mişcă C., Stoin D., Bujancă G., and Alexandra Teodora L.-G., Section Advances in Biotechnology chemical composition and antioxidant properties of dill essential oil. 2018. DOI:10.5593/sgem2018/6.4.
18. Kandasamy S., Kavitake D., and Shetty P. H. "Lactic Acid Bacteria and Yeasts as Starter Cultures for Fermented Foods and Their Role in Commercialization of Fermented Foods," in Innovations in Technologies for Fermented Food and Beverage Industries (Springer International Publishing). 2018. P. 25–52. DOI:10.1007/978-3-319-74820-7_2.
19. Kelly W. J., Ward L. J. H., and Leahy S. C. "Chromosomal Diversity in Lactococcus lactis and the Origin of Dairy Starter Cultures," in Genome Biology and Evolution. 2010. P. 729–744. DOI:10.1093/gbe/evq056.
20. Khorshidian N., Yousefi M., Khanniri E., and Mortazavian A. M. Potential application of essential oils as antimicrobial preservatives in cheese. Innovative Food Science and Emerging Technologies. 2018. T. 45. P. 62–72. DOI:10.1016/j.ifset.2017.09.020.
21. Kondrotiene K., Kasnauskyte N., Sernien, L., Gölz G., Alter T., Kaskoniene V., et al. Characterization and application of newly isolated nisin producing Lactococcus lactis strains for control of Listeria monocytogenes growth in fresh cheese. LWT - Food Science and Technology. 2018. T. 87. P. 507–514. DOI:10.1016/j.lwt.2017.09.021.
22. Lotito S. B., and Frei B. Relevance of apple polyphenols as antioxidants in human plasma: Contrasting in vitro and in vivo effects. Free Radical Biology and Medicine. 2004. T. 36. P. 201–211. DOI:10.1016/j.freeradbiomed.2003.10.005.
23. Marhamatizadeh M. H., Jaafari F., Rezazadeh S., Ehsandoost E., and Mohammadi M. Effects of dill extract (Anethum-graveolens L.) on growth and survival of Lactobacillus acidophilus and Bifidobacterium bifidum in probiotic milk and yoghurt. Global Veterinaria. 2012. T. 9. P. 252–257. DOI:10.5829/idosi.gv.2012.9.3.6543.
24. Nadal E. S., Sayas-Barberá E., Fernández-López J., and Pérez-Alvarez J. A. "Food Formulation to Increase Probiotic Bacteria Action or Population," in Bioactive Foods in Promoting Health (Elsevier Inc.). 2010. P. 335–351. DOI:10.1016/B978-0-12-374938-3.00022-0.
25. Nuryshev M. Z., and Stoyanova L. G. New Probiotic Culture of Lactococcus lactis ssp. lactis: Effective Opportunities and Prospects. Journal of Microbiology and Biochemical Technology. 2016. T. 8. DOI:10.4172/1948-5948.1000299.
26. Oosterhaven K., Poolman B., and Smid E. J. S-carvone as a natural potato sprout inhibiting, fungistatic and bacteristatic compound. Industrial Crops and Products. 1995. T. 4. P. 23–31. DOI:10.1016/0926-6690(95)00007-Y.
27. Ozdogan D. K., Akcelik N., Aslim B., Suludere Z., and Akcelik M. Probiotic and antioxidative properties of L. Lactis ll27 isolated from milk. Biotechnology and Biotechnological Equipment. 2012. T. 26, P. 2750–2758. DOI:10.5504/bbeq.2011.0091.
28. Pohanka M. D-Lactic Acid as a Metabolite: Toxicology, Diagnosis, and Detection. BioMed Research International. 2020. Volume 2020. P. 1 - 9. DOI: 10.1155/2020/3419034
29. Pophaly S. D., Chauhan M., Lule V., Sarang P., Tarak J., Thakur K., et al. "Functional Starter Cultures for Fermented Dairy Products," in Microbial Cultures and Enzymes in Dairy Technology (IGI Global). 2018. P. 54–68. DOI:10.4018/978-1-5225-5363-2.ch003.
30. Re R., Pellegrini N., Proteggente A., Pannala A., Yang, M., and Rice-Evans C., Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Biology and Medicine. 1999. T. 26, P. 1231–1237. DOI:10.1016/S0891-5849(98)00315-3.
31. Reddy G., Altaf M., Naveena B. J., Venkateshwar M., and Kumar E. V. Amylolytic bacterial lactic acid fermentation -

A review. Biotechnology Advances. 2008. T. 26. P. 22–34. DOI:10.1016/j.biotechadv.2007.07.004.
32. Sarao L. K., and Arora M. Probiotics, prebiotics, and microencapsulation: A review. Critical Reviews in Food Science Nutrition. 2017:. T. 57, P. 344–371. DOI:10.1080/10408398.2014.887055.
33. Sekmokiene, D. The influence of aqueous extracts on curd-type cheese quality. researchgate.net. 2018. DOI:10.6001/zemesukiomokslai.v25i2.3754.
34. Veiga M., Costa E. M., Silva S., and Pintado M., Impact of plant extracts upon human health: A review. Critical Reviews in Food Science Nutrition. 2020. T. 60. P. 873–886. DOI:10.1080/10408398.2018.1540969.
35. Walstra P., Geurts T. J., Noomen A., Jellema A., and Boekel M. A. J. S. van. Dairy technology: principles of milk properties and processes. Dairy Technology– Principles of Milk Properties and Processes. 1999
36. Yerlikaya, O. Probiotic potential and biochemical and technological properties of Lactococcus lactis ssp. lactis strains isolated from raw milk and kefir grains. Journal of Dairy Science. 2019. T. 102. P. 124–134. DOI:10.3168/jds.2018-14983.

*Received 5 November 2020*
*Accepted 28 December 2020*

# Sperm Motility and Viability of Chilled Ram Semen Collected by Artificial Vagina and Electroejaculation

**Stanimir Yotov[1], Anatoli Atanasov[1], Ivan Fasulkov[1], Manol Karadaev[1], Anton Antonov[1], Plamen Georgiev[1], Elena Kistanova[2]**

*[1]Department of Obstetrics, Reproduction and Reproductive Disorders, Faculty of Veterinary Medicine, Trakia University, Stara Zagora, Bulgaria*

*[2]Bulgarian Academy of Sciences, Institute of Biology and Immunology of Reproduction, Sofia, Bulgaria*

**Keywords:** *ram semen, chilling, artificial vagina, electroejaculation*

*Abstract. The current study aimed to evaluate motility and viability of chilled ram semen collected by artificial vagina and electroejaculation during the non-breeding season. A total of 18 ejaculates from clinically healthy rams in a non-breeding season were collected by artificial vagina (AV; n = 9) and electroejaculation (EE; n = 9) and submitted to preliminary evaluation. After that all ejaculates were diluted through a Tris-based extender containing low concentration (5%) of glycerol and egg yolk and stored at 5°C for 48 hours. Motility and viability of semen samples were evaluated at 0, 6, 24 and 48 h of storage. Estimation of motility was carried out by a microscopic digital system, and viability was assessed by the one-step eosin-nigrosin staining technique. Until 6 h of storage, the differences between motility and viability of semen collected by AV or EE were non-significant, while at 24 and 48 h the parameters were higher (P < 0.05) in semen collected by AV. The increasing time of storage correlated negatively with the evaluated parameters (P < 0.05). In conclusion, the chilled semen from ram in the non-breeding season collected by AV demonstrated better motility and viability until 48 h, compared with the semen collected by EE, and could be recommended for artificial insemination up to 24 h after storage at 5°C. The time of storage had a negative effect on sperm motility and viability (P < 0.05).*

## Introduction

The intensive sheep farming requires artificial insemination of animals with liquid semen preserved at 0–5°C for an extended period or use of frozen semen (Salmon and Maxwell, 2000; Joshi et al., 2001; Abulizi et al., 2012). Maintenance of the spermatozoa biological potential and their genetic information after storage in low temperatures is crucial for sheep breeding practice (Gundogan, 2009). Many reasons can affect the quality of ejaculates intended to chilling or freezing but one of the most important is the method of semen collection (Maxwell and Watson, 1996; Maxwell et al., 2007; Jimenez-Rabadan et al., 2012; Maksimović et al., 2018).

In ram, the main semen collection techniques are artificial vagina (AV) and electroejaculation (EE). The semen collection with AV is similar to a natural service and is easy to apply, but requires an extended training period of rams and not all animals can be successfully trained (Wulster-Radclife et al., 2001). Electroejaculation has some advantages because it is a quick method, appropriate for use in non-trained males or those with problematic sexual behaviour, and the collected semen has an increased volume compared with ejaculates collected by AV (Mattner and Voglmayr, 1962; Marco-Jimenez et al., 2005; Jimenez-Rabadan et al., 2012).

Correspondence to Stanimir Yotov, Department of Obstetrics, Reproduction and Reproductive Disorders, Faculty of Veterinary Medicine, Trakia University, Student campus, 6000 Stara Zagora, Bulgaria. E-mail: stanrad@abv.bg

Different studies have presented the effects of both methods on small ruminant semen quality (Matthews et al., 2003; Marco-Jimenez et al., 2005; Ledesma et al., 2015). Electroejaculation is very variable and the collected semen is often contaminated with urine, and thus had poor motility (Fennessy et al., 1990). In a study by Marco-Jimenez et al. (2005), electroejaculation resulted in a lower recovery efficiency, as a consequence of a lack of response to the electrical stimulation and the fresh semen quality was not significantly different between recovery methods, except for the concentration of spermatozoa. However, a higher number of stable and functional spermatozoa were found for frozen-thawed spermatozoa collected by electroejaculation than by artificial vagina. In contrast, other authors (Matthews et al., 2003; Bopape et al., 2015) recorded better percentage of motile and live sperm cell semen collected by AV compared with EE, and Jiménez-Rabadán et al. (2012) indicated a higher sperm quality after thawing of cryopreserved semen obtained by artificial vagina.

Ram spermatozoa are sensitive to extreme temperature changes during cooling and freezing which induce damage to the sperm plasma membrane or structural changes leading to a capacitation process (Hammerstedt et al., 1990; Salamon and Maxwell, 1995; Watson, 1990). The cryopreserved sperm cells, used for artificial insemination, have to be eight times increased to achieve normal fertilization rates because of the lower viability, reduced motility and increased abnormal apical ridge (Shannon and Vishwanath, 1995; Gillan et al., 1997). Also, a significant seasonal

variation in the semen quality of small ruminants has been reported in different studies (Ritar, 1993; Roca et al., 1992; Maxwell et al., 2007). In spite of all abovementioned, the data for an effect of a semen collection method on the sperm quality during storage at low temperature regimens in still debatable.

The aim of this study was to evaluate the motility and viability of chilled semen from Pleven Blackhead rams collected by artificial vagina and electroejaculation during the non-breeding season.

## Material and Methods

### *Experimental animals and management*

The study was carried out in 18 Pleven Blackhead rams with an average age of 1.8 ± 0.9 years, body weight 65 ± 4.9 kg, reared in individual boxes at a small ruminant unit, located at N 42.25 and E 25.38. The animals were housed in the uniform technology, feeding, immunoprophylaxis regimen and drinking of water *at libitum*. Investigation was conducted during the non-breeding season. The study was performed in accordance with the recommendations of Animal Ethics Committee and regulations for human attitude and animal protection.

### *Semen collection, processing and evaluation*

Prior to the semen collection, an abstinence period of 30 days was provided and a physical examination was performed. The rams were separated in two groups according to the semen collection method: group I (n = 9, AV) and group II (n = 9, EE). A total of 18 ejaculates were used for this study. In group I, semen was collected by the artificial vagina method in presence of a teaser sheep, while in group II the ejaculates were collected by electro-ejaculator for small ruminants (Minitübe, Germany). All semen samples were obtained between 9.00 and 10.00 a.m., then transported to the laboratory within 5 minutes, placed on a water bath at 35°C and submitted to a primary assessment. The volume was measured by a graduated pipette and mass motility was evaluated on the base of wave motion observed under the microscope at 10× magnification (scale 0–5, Evans and Maxwell, 1987). The sperm concentration ($\times 10^9$/mL) was determined by a Photometer SpermaCue (Minitüb, Germany), calibrated for small ruminant semen. Only semen with a normal color and transparency, volume > 1 mL, concentration > $1\times10^9$/mL and wave motion > 3.5 and abnormal sperms were included in the experiment.

The semen was diluted with a Tris-glucose-glycerol-egg yolk (TGGY) extender, adapted to Evans and Maxwell's (1987) prescription. All chemicals were purchased from *Alfa Aesar (Thermo Fisher Scientific GmbH, Germany)*. TGGY stock solution included Tris-hydroxymethyl aminomethane 3.63 g, glucose 0.5 g, citric acid 1.99 g, glycerol 5 mL, gentamycin 50 μg/mL and aqua bidestillata up to 100 mL. The stock solution was prepared one day before semen collection and stored at 5°C. The completed extender included adding egg-yolk 5% (v/v) to a stock solution before semen collection and the extenders were placed in a water bath at 35°C.

After the primary assessment, each ejaculate was diluted at a ration 1:1 and kept on a water bath 5 minutes for adaptation of semen to the extender. Additional dilution until adjustment of the sperm concentration to $200\times10^6$ cells per mL was performed. The three samples of equal amounts of diluted semen of each ejaculate were stored at 5°C in a refrigerator for 48 hours.

### *Semen evaluation*

The motility and viability of the spermatozoa were evaluated at 0, 6, 24 and 48 hours after storage at 5°C in a refrigerator.

### *Motility evaluation*

The motility was estimated by microscopic examination using Motic Image Plus Digital System (Motic China Group Ltd, 2001–2004), including a microscope, objectives with different magnification, a digital camera and relevant software. Immediately before examination, the semen samples were gently mixed and a 5 μL drop was placed on a slide warmed at 37°C, covered with a 20 mm × 20 mm cover slip and observed at 200–400× by a qualified operator. The average value of three consecutive observations at least of five different microscopic fields was calculated as a final motility (Ax et al., 2000).

### *Viability evaluation*

The sperm viability was assessed by one-step eosin-nigrosin staining technique (Mortimer, 1994). The smear was prepared by mixing 2 equal drops of semen and staining solution (0.67% eosin -Y and 10% nigrosin dissolved in 0.9% sodium chloride in distilled water). After incubation of the mixture at room temperature (20 degrees Celsius) for 30 seconds, it was placed on a warm slide, spreading with a second slide and dried on air. The viability was assessed by counting 200 cells under a microscope at magnification of 400×. Sperm cells that were unstained (white) were accepted as alive, whereas stained (pink or red coloration) were considered to be dead.

### Statistical analysis

The results were processed by statistical program Statistica version 7.0 (Stat-Soft.,1984–2000 Inc., Tulsa, OK, USA). The semen motility and viability were expressed as mean ± standard deviation (Mean ± SD). Analysis of variance (ANOVA) and Tukey`s post hoc test were used for comparison of the motility and viability for the different methods of semen collection. The effect of time of storage on evaluated parameters was determined by correlation analysis. Statistical significance was accepted at $P < 0.05$.

## Results and Discussion

The primary evaluation showed a significantly greater volume of ejaculate after using electroejaculation ($P < 0.05$) which confirmed the results of previous research in this topic (Table 1). The sperm concentration and wave motion of spermatozoa for both methods were close. There was no significance between motility and viability of the spermatozoa at 0 h after dilution (Fig. 1). The primary motility and viability of semen collected by AV (78 ± 2.9% and 80.5 ± 2.1%) and EE (72 ± 6.1% and 76.3 ± 4.5%) decreased slowly until 6 h of storage. A similarity in sperm motility for fresh semen collected by these methods was also stated by Marco-Jimenez et al. (2005) and Jimenez-Rabadan et al. (2012). However, the evaluated parameters negatively correlated with the increased time of storage in both groups. All correlation coefficients for motility ($R = –0.97$ (AV); $R = –0.99$ (EE) and viability ($R = –0.98$ (AV); $R = –0.95$ (EE) were highly negative ($P < 0.05$). It was in accordance with other reports for a decreased motility in ram semen during liquid storage (Kheradmand et al., 2006; Azizunnesa et al., 2014). Significant differences ($P < 0.05$) were detected among the values registered at 24 and 48 h of storage. Between abovementioned intervals, the sperm motility of spermatozoa collected by artificial vagina decreased by 8%, but still was in an acceptable range, whereas in semen collected by EE, this reduction was 21%. A similar relationship was registered for the viability (Fig. 2). The values of this parameter also differed significantly ($P < 0.05$), at 24 h and 48 h of storage at 5°C in a refrigerator (AV – 76.4 ± 2.4% and 73.4 ± 3.4% vs. EE – 69.2 ± 6.4% and 65.1 ± 5.8%). In this aspect, Maksimović et al. (2018) observed that some of the ram semen samples collected by EE almost completely lost sperm cell activity at 24 h after storage. Semen samples from buck ejaculates collected by AV with greater values for most assessed sperm parameters than those obtained by EE have been revealed by Jimenez-Rabadan et al. (2012) and Bopape et al. (2015). In contrast, Ledesma et al. (2015) reported for more resistant sperm cells to cryodamage in ram semen collected by EE compared with sperm collected by AV. It was evidenced by the higher percentage of sperm with intact and functional plasma membrane, intact acrosome and a greater in vitro fertilizing potential observed after thawing. The determined slower reduction of the values of semen collected by AV was in support of the assertion for better resistance of spermatozoa to a low temperature.

The current study is in agreement with the hypothesis of other investigations for the influence of the semen collection method on resistance of sperm to low temperatures for an extended period. Jimenez-Rabadan et al. (2012) accepted that the EE procedure changes the chemical composition of the seminal plasma connected with sperm cell freezing capacity. Marco-Jiménez et al. (2008) showed differences in the protein profile in samples obtained by both methods, which can

*Table 1.* Parameters of ejaculates recorded in primary evaluation according to the method of semen collection

| Method of semen collection | Volume (mL) | Concentration ($\times 10^9$/mL) | Wave motion (Scale 0–5) |
|---|---|---|---|
| | Mean ± SD | Mean ± SD | Mean ± SD |
| Electroejaculation (n = 9) | 1.8 ± 0.35[a] | 2.36 ± 0.64[a] | 3.64 ± 0.12[a] |
| Artificial vagina (n = 9) | 1.5 ± 0.24[a] | 2.84 ± 0.42[a] | 3.72 ± 0.20[a] |

The values in a column marked with a different superscript differ at $P < 0.05$.

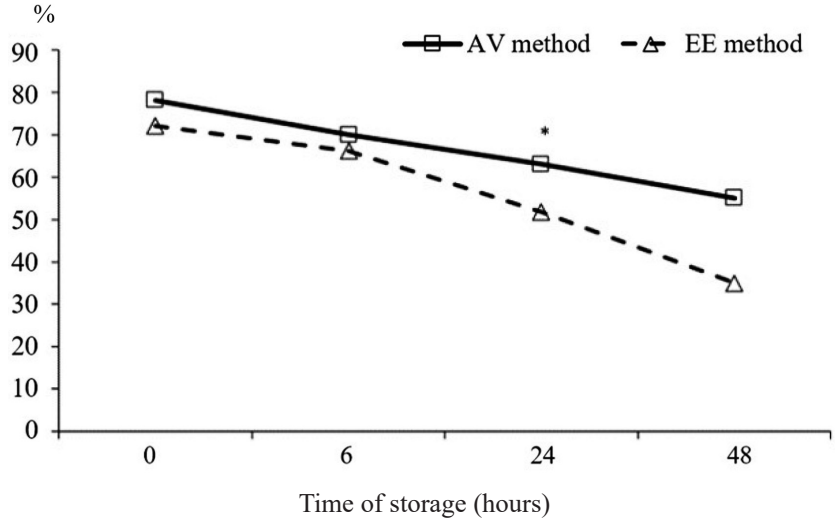



*Fig. 1.* Sperm motility of chilled semen during storage at a temperature of 5°C for 48 hours according to the method of semen collection

The values in the intervals marked with an asterisk differ at $P < 0.05$.

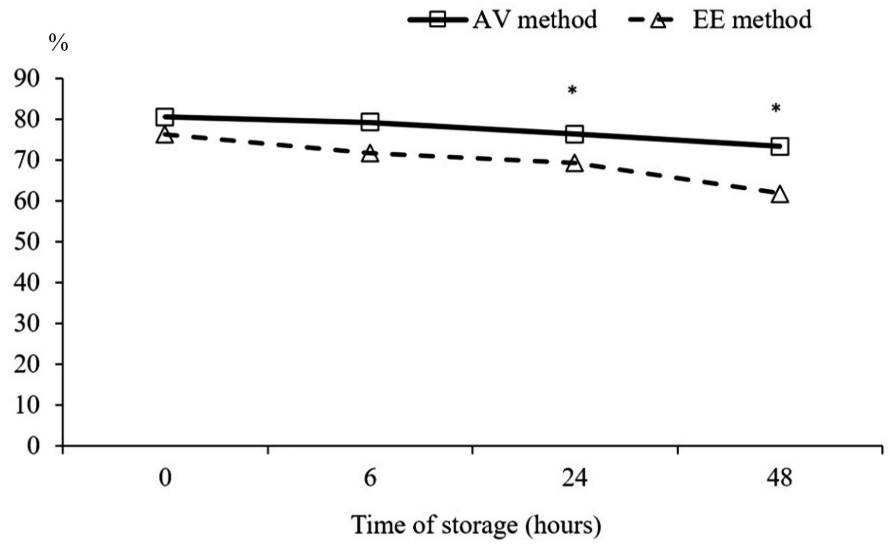



*Fig. 2.* Sperm viability of chilled semen during storage at a temperature of 5°C for 48 hours according to the method of semen collection

The values in the intervals marked with an asterisk differ at $P < 0.05$.

be attributed to prevention of the sperm against cold-shock damage (Barrios et al., 2005). In addition, the better motility and viability of spermatozoa collected by AV than EE at 24 of storage indicated that it can be recommended for artificial insemination.

In conclusion, the chilled semen from ram in the non-breeding season collected by artificial vagina and diluted through the abovementioned semen extender demonstrated better motility and viability until 48 h, compared with semen collected by electroejaculation, and could be recommended for artificial insemination up to 24 h after storage at 5°C. The time of storage had a negative effect on sperm motility and viability ($P < 0.05$). This data will be useful when ram semen has to be stored for a short time in a refrigerator or transported in a longer distance.

**Acknowledgements**

The authors thank the research staff of the Laboratory of Reproduction, Faculty of Veterinary Medicine, Trakia University for their help during the investigation.

**References**

1. Ax R.L., Dally M.A., Lenz R.W., Love C.C., Varner D.D., Hafez B., Bellin M.E. Semen evaluation. In: Hafez, B., Hafez, E.S.E., Eds. Reproduction in Farm Animals. 7th ed., Lippincott Williams and Wilkins, Philadelphia. 2000, Pp. 365-375.
2. Azizunnesa Zohara B.F., Bari F.Y., Alam M.G.S. Effects of proportion of egg yolk and preservation time on chilled semen from indigenous rams. GSTF Journal of Veterynary Sciences. 2014. T 1. P. 18-26.
3. Barrios B., Fernández-Juan M., Mui˜no-Blanco T., Cebrián-Pérez J.A. 2005. Inmunocytochemical localization and biochemical characterization of two seminal plasma proteins that Project ram spermatozoa. Journal of Andrology. T. 26. P. 539-549.
4. Bopape M.A., Lehloenya K.C., Chokoe T.C., Nedambale, T.L. Comparison of electro ejaculator and artificial vagina on semen collection from South African indigenous goat following assessment by computer aided sperm analysis. Open Journal of Animal Sciences. 2015. T. 5. P. 210-218.
5. Fennessy P.F., Mackintosh C.G., Shackell, G.H. Artificial insemination of farmed red deer Cerõus.elaphus. Animal Production. 1990. T. 51. 613-621.
6. Gillan L., Evans G., Maxwell W.M. Capacitation status and fertility of fresh and frozen–thawed ram spermatozoa. Reproduction Fertility and Development. 1997. T. 9. P. 481-487.
7. Gundogan M. Short term preservation of ram semen with different extenders. 2009. Kafkas University Veterinary Faculty Dergisi. T. 15. P. 429-435.
8. Hammerstedt R.H., Graham J.K., Nolan J.P. Cryopreservation of mammalian sperm: what we ask them to survive. 1990. Journal of Andrology. T. 11. P. 73-88.
9. Jiménez-Rabadán P., Ramón M., García-Álvarez O., Maroto-Morales A., Del Olmo E., Pérez-Guzmán M.D., Bisbal A., Fernández-Santos M.R., Garde J.J., Soler A.J. Effect of semen collection method (artificial vagina *vs.* electroejaculation), extender and centrifugation on post-thaw sperm quality of Blanca-Celtibérica buck ejaculates. 2012. Animal Reproduction Science. T. 132. P. 88-95.
10. Joshi A., Bag S., Naqvi S.M.K., Sharma R.C., Rawat P.S., Mittal J.P. Effect of short-term and long-term preservation on motion characteristics of Garole ram spermatozoa: A prolific micro sheep breed of India. Asian-Australas Journal of Animal Sciences. 2001. T. 14. P. 1527-1533.
11. Kheradmand A., Babaei H., Abshenas J. Comparative evaluation of the effect of antioxidants on the chilled-stored ram semen. 2006. Iranian Journal of Veterinary Research. T. 7. P. 40-45.
12. Ledesma A., Manes J., Ríos G., Aller J., Cesari A., Alberio R., Hozbor F. Effect of seminal plasma on post-thaw quality and functionality of corriedale ram sperm obtained by electroejaculation and artificial vagina. 2015. Reproduction in Domestic Animals. T. 50. P. 386-392.
13. Marco-Jiménez F., Puchades S., Gadea J., Vicente J.S., Viudes-de-Castro M.P. Effect of semen collection method on pre-and post-thaw Guirra ram spermatozoa. 2005. Theriogenology. T. 64. P. 1756-1765.
14. Marco-Jiménez F., Vicente J.S., Viudes-de-Castro M.P. Seminal plasma composition from ejaculates collected by artificial vagina and electroejaculation in Guirra ram. 2008. Reproduction in Domestic Animals. T. 43. P. 403-408.

15. Matthews N., Bester N., Schwalbach L.M.J. A comparison of ram semen collected by artificial vagina and electro-ejaculation. 2003. SA-Animal Sciences, T. 4. P. 28-30.
16. Mattner, P.E., Voglmayr, J.K. A comparison of ram semen collection by the artificial vagina and by electroejaculation. 1962. *Australian Journal* of *Experimental Agriculture* and *Animal* Husbandry. T. 2. P. 78-81.
17. Maxwell W.M., de Graaf S.P., Ghaoui Rel-H., Evans G. Seminal plasma effects on sperm handling and female fertility. 2007. Society for Reproduction and Fertility. Suppl. 64. P. 12-38.
18. Maxwell W.M.C., Watson P.F. Recent progress in the preservation of ram semen. Animal Reproduction Science. 1996. T. 42. P. 55-65.
19. Mortimer D. Practical Laboratory Andrology. Oxford University Press, New York, USA. Practical Laboratory Andrology. Oxford University Press, New York, USA. 1994. Pp. 66-69.
20. Ritar A.J. Control of ovulation, storage of semen and artificial insemination of fibre-producing goats in Australia: A review. *Australian Journal* of *Experimental Agriculture* and *Animal* Husbandry. 1993. T. 33. P. 807-820.
21. Roca, J., Martínez, E., Sanchez-Valverde, M.A., Ruiz, S., Vazquez, J.M., 1992. Seasonal variations of semen quality in male goats: study of sperm abnormalities. Theriogenology. T. 38. 115-125.
22. Salamon S., Maxwell W.M.C. Frozen storage of ram semen I. Processing, freezing, thawing and fertility after cervical insemination. Animal Reproduction Science. 1995. T. 37. P. 185-249.
23. Salomon S., Maxwell W.M.C. Storage of ram semen. 2000. Animal Reproduction Science. T. 62. P. 77-111.
24. Shannon P., Vishwanath R. The effect o optimal and suboptimal concentration of spermatozoa on the fertility of fresh semen and frozen bovine semen and theoretical model to explain the fertility differences. 1995. Animal Reproduction Science. T. 39. P. 1-10.
25. Watson P.F. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. 1995. Reproduction Fertility and Development, T. 7. P. 871-891.
26. Wulster-Radcliffe, M.C., Wiliams, M.A., Stellflug, J.N., Lewis, G.S., 2001. Technical note: Artificial vagina vs. a vaginal collection vial for collecting semen from rams. Journal of Animal Science. T. 79. P. 2964-2967.

*Received 30 November 2020*
*Accepted 28 December 2020*

# Histopathological changes in kidney and liver with oxidative stress and protection by plant extracts

**Ahmed Sami Farhan[1] and Mohammed Tareq Mohammed[2]**
[1]*Department of Biology, College of Science, University of Anbar, Iraq*
[2]*Department of surgery, College of Medicine, University of Anbar, Iraq*

***Keywords***: *Hibiscus, kidney, liver, Malva, mice, and potassium permanganate.*

***Abstract.*** *Potassium permanganate ($KMnO_4$) is utilized to cleanse pathogenic microbes in vegetables and fruits. Humans may be exposed to this compound from vegetables and fruits. This study aims to evaluate the impact of oxidants such as potassium permanganate in kidney and liver tissues and assess protective roles of aqueous extracts of H. sabdariffa flowers and M. parviflora leaves against the oxidizing substance. For this purpose, 28 female Swiss albino mice were distributed into four groups: each group contained seven animals. Group I recruited control animals. Group II received a daily $KMnO_4$ (0.5 mg/kg/BW) dose. Group III received a daily oral dose containing $KMnO_4$ and an aqueous extract of H. sabdariffa flowers (0.5 mg/kg/BW + 500 mg/kg/BW). Group IV received a daily $KMnO_4$ and an aqueous extract of M. parviflora leaves (0.5 mg/kg/BW + 300 mg/kg/BW). The treatment in all groups lasted for 30 consecutive days. Mice exposed to $KMnO_4$ showed severe histopathological changes in the kidney and the liver. The treatment with aqueous extracts of H. sabdariffa flowers and M. parviflora leaves prevented the damage of tissues induced by $KMnO_4$ and exhibited a better protective role.*

## Introduction

Potassium permanganates ($KMnO_4$) are oxidizing factors utilized in aquaculture (Franca et al., 2013). KMnO4 is utilized to wash vegetables and fruits for its ability to sterilize pathogenic microbes (Subramanya et al., 2018). Free radicals induce damage that become the main concern because of its serious consequences. Free radicals may result in different degenerative diseases such as rheumatoid arthritis, aging and tumors. There are two species of free radicals which are formed in the organisms, i.e., reactive oxygen species and reactive nitrogen species. These are released in the human body through pathophysiological pathways. Normally, free radicals are scavenged by antioxidants, but this neutralization is not completed leading to a phenomenon called oxidative stress that is responsible for the damage. Free radicals affect lipids, proteins, and DNA. Together with endogenous antioxidants, food components are excellent sources of natural antioxidants. Most vegetables and fruits are rich sources of antioxidants (Dhaliwal and Singh, 2015). $KMnO_4$ is known by its oxidant and irritant properties and by the acute toxicity of manganese. Swallowing a little dose (4–20 mg/kg) of $KMnO_4$ can lead to gastrointestinal ordeal; however, bolus ingestion causes respiratory arrest followed by coagulative necrosis and hemorrhage in the esophagus, the stomach, and the liver (Willhite et al., 2013). Potassium permanganate is seldom utilized for suicidal trail. Swallowing it can cause positional and systemic toxicities like damage and necrotic tissues that result from permanganate toxicity. A patient with a lethal dose of $KMnO_4$ has narrowing of the superior airway, bringing about complicated intubation in addition to liver inhibition and coagulopathy as systemic manifestation (Agrawal et al., 2014).

*Hibiscus sabdariffa* (Family: malvaceae) is a perennial and herbal plant distributed in Iraq. It is used in various foods in addition to herbal medicine (Balarabe, 2019). Different therapeutic benefits are found in the hibiscus plant, including anti-positive and gram-negative bacteria, anti-tumor, anti-apoptosis and anti-oxidant activity (Puro et al., 2017). This plant is a rich source of antioxidants and prevents DNA breakdown (Adeoye et al., 2019). It works to increase the capacity of antioxidants inside the cell and reduce oxidative stress (Soto et al., 2016). It protects the kidney tissue and improves its functions against toxins such as lead (Okonkwo, 2020). The ethanolic extract of *Hibiscus sabdariffa* calyces has hypolipidemic effects that reduce serum cholesterol and triglycerides (Umoren et al., 2020; Gaffer and Mustafa, 2019). Many natural compounds are presented in calyx extract of *Hibiscus sabdariffa*, such as anthocyanin, flavonoid, carotenoid, phenol, and vitamin C (Jamini et al., 2019).

*Malva parviflora* L. (malvaceae family) is a perennial herb distributed in all regions of Iraq. It has a pharmaceutical importance containing phenolics, flavonoids, flavonols and fatty acids (Rasheed et al., 2017). The polyphenols in the leaves of this plant show high antioxidant activity (Abd El-Salam and Morsy, 2019). Certain compounds, such as oleanolic acid, scopoletin and tiliroside, have the ability to lower blood pressure and prevent oxidative damage and inflammation in the kidneys (Lagunas-Herrera et al., 2019). Other compounds in this plant have

Correspondence to Ahmed Sami Farhan, Department of Biology, College of Science, University of Anbar, Ramadi, Anbar, Iraq. E-mail: ahmed.sami@uoanbar.edu.iq

anti-bacterial activity, especially bacteria that infect the urinary tract (Kidane et al., 2019). *M. parviflora* extracts possess analgesic and anti-inflammatory compounds (Ramirez-Serrano et al., 2019). *M. parviflora* has shown its pharmacological activities in various sicknesses. Leaves are utilized in the arrangement of wounding and bulging. A lotion manufactured from leaves is utilized to treat trauma and broken limbs. These leaves are utilized to draw bulging, inflamed festering wounds. *M. parviflora* has various activities like antidiabetic, antifungal, hepatoprotective, neuroprotective, anti-irritant, antioxidant, and anti-ulcerogenic activity (Singh and Navneet, 2017).

## Materials and methods

This study was approved by the ethical committee at the University of Anbar. The granted authorization No. 1 was issued in 11/1/2021. The flowers of *H. sabdariffa* and leaves of *M. parviflora* were collected from the area (Anbar-Iraq), were washed with water and dried, and then stored in tight polythene bags.

### *Hibiscus and Malva extract preparation*

Dried *H. sabdariffa* flowers and *M. parviflora* leaves were ground to obtain powder. A magnetic stirrer device was used to mix the powder of each plant with 10 volumes (w/v) of pure water for 2 hours. This 10% solution was then clarified by gauze, sanitized via filtration on 0.1-micron filters (Millipore), and the drying was carried out at 50°C using an incubator. This dried extract was suspended in water to the final concentration for *H. sabdariffa* 50 mg/mL (Okonkwo, 2020) and for *M. parviflora* 30 mg/mL (Rasheed et al., 2017).

### *Stock solution production of $KMnO_4$*

Potassium permanganate used in this study was obtained from the Department of Chemistry, College of Science, University of Anbar. The stock solutions were acquired and serial dilutions were done.

### *Animals*

Twenty-eight (28) Swiss albino mice (female) aged between 2 and 3 months were purchased from the Iraqi Center for Cancer Research and Medical Genetics, Al-Mustansiriyah University. They were kept in cages for 4 weeks for environmental adaptation. Twenty-eight mice weighing between 25–30 g were divided into 4 groups, I, II, III, and IV of 7 mice per group. Group I was control, group II had KMnO4 (0.5 mg/kg/BW) daily, group III had an aqueous extract of *H. sabdariffa* flowers (500 mg/kg BW) and $KMnO_4$ (0.5 mg/kg/BW) daily, and group 4 had an aqueous extract of *M. parviflora* leaves (300 mg/kg/BW) and $KMnO_4$ (0.5 mg/kg/BW). The experiment lasted until the end of thirty days.

### *Histological section*

The tissue specimens of kidneys and the liver were abstracted from the mice and located in formalin. Pieces of the organs were prepared for microscopic examination. The tissues were dehydrated for 2 hours in a graduated level of alcohol (ethanol 50% to absolute) in the ascending stage, and then they were submerged in xylene for 30 minutes. The tissue was impregnated with wax of paraffin and cut at 4-micron thickness. The tissue sections were floated on a water bath at 50°C less than the melting grade of paraffin wax. They were dried (20–30 minutes) and dyed with hematoxylin and eosin stain, hydrated, cleared and mounted (DPX) in a mountant, averting blebs of air. Pictures were taken by an electronic camera at 40X and 10X magnification powers in a light microscope.

## Results

The histopathological changes in the mice organs were microscopically examined. The results indicated various stages of structural changes in the kidney and the liver.

### *Kidney*

The kidneys of the control group (I) demonstrated intact renal corpuscles and tubules in cortex and medulla (Fig. 1). The kidneys in the second group (administered with $KMnO_4$) showed blood vessel congestion, destruction of renal tubules, cellular infiltration and necrosis, large urinary space in renal corpuscles (Fig. 2). However, the kidneys in groups III and IV showed normal renal corpuscles and tubules (Figs. 3 and 4) compared with damaged kidney tissues as demonstrated in Fig. 2.

### *Liver*

The control liver (group I) shows intact hepatic lobules, central veins and sinusoidal capillaries that ordinarily conserve the normal architecture viewed in this organ (Fig. 5); meanwhile, animals of group II show edema, blood vessel congestion, cellular necrosis, chromatin condensation in the nucleus, cellular infiltration, and nucleus like a ring (Fig. 6). There are no plausible changes in the hepatic cells, micro- and macro-vasculature, and hepatic plates in the mice (group III and IV) treated with $KMnO_4$ and with aqueous extracts of plants (Figs. 7 and 8).

## Discussion

The mechanism of action of $KMnO_4$ in the body is through the generation of strong oxidative free radicals that attack the building blocks inside the cell, such as proteins, fats and nucleic acids (Dhaliwal and Singh, 2015). The histopathological observations of kidney sections of mice in the current study showed blood vessel congestion, destruction of renal tubules, cellular infiltration and necrosis, and a large urinary space in renal corpuscles. The histopathological observations of liver sections of mice in the current study showed edema, blood vessel congestion, cellular necrosis, chromatin condensation in the nucleus, cellular infiltration, and nucleus like a ring (circular

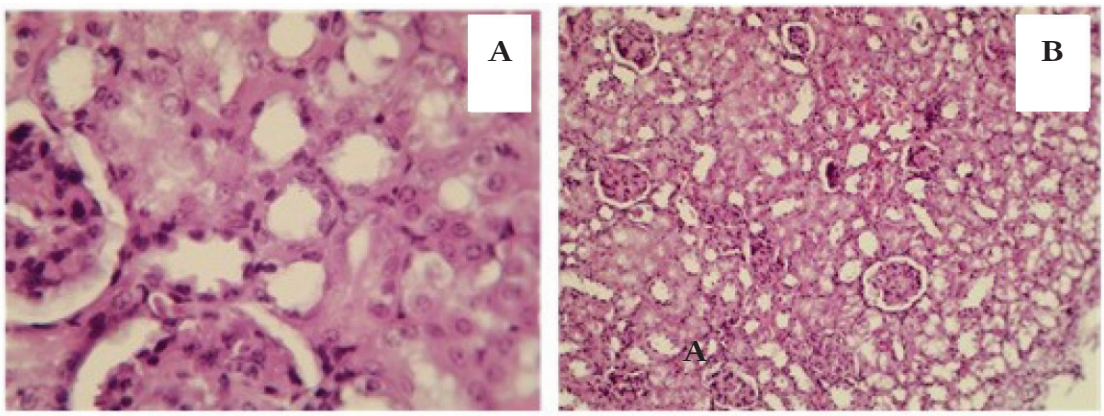



*Fig. 1.* Cross-sections of the kidney in control mice.
A: 10X and B: 40X (H & E).

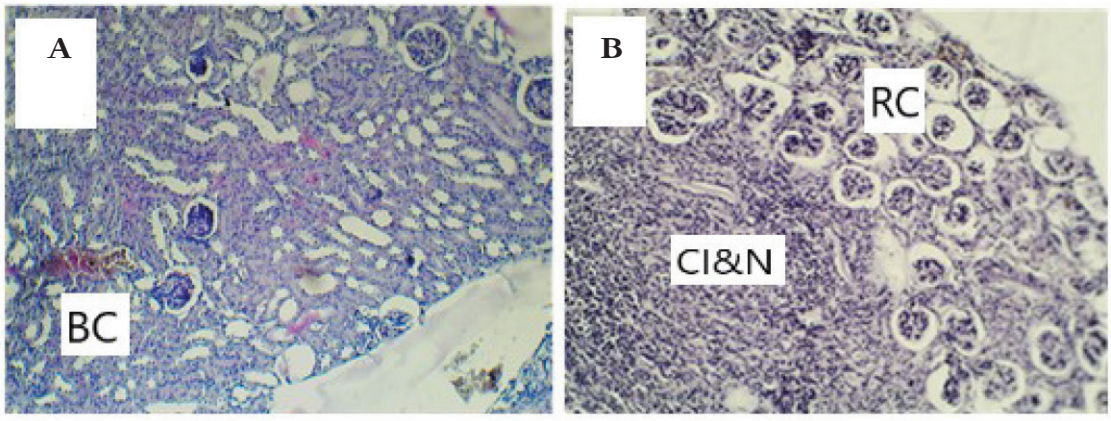



*Fig. 2.* Cross-sections of the kidney in mice exposed to $KMnO_4$.
(A) BC (blood vessel congestion) and (B) RC (renal corpuscle),
CI&N (cellular infiltration and necrosis). A: 10X and B: 40X (H & E).

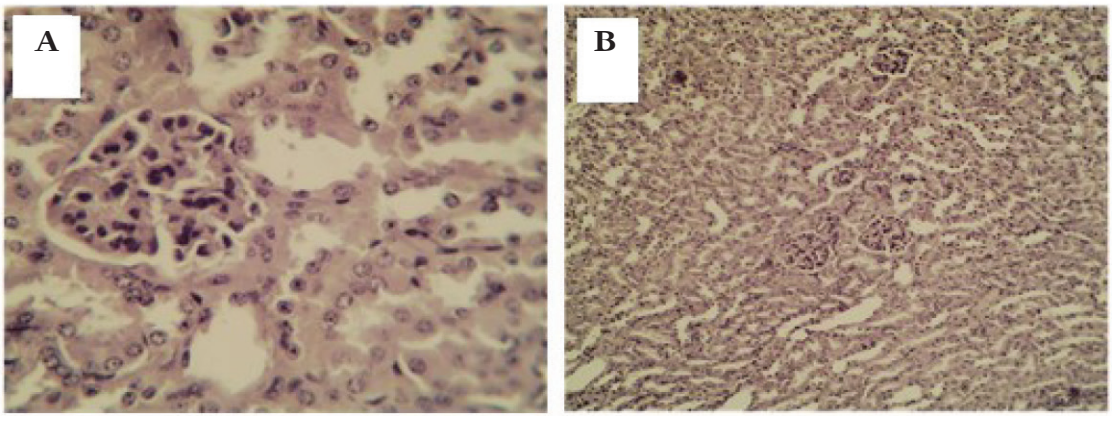



*Fig. 3.* Cross-sections of the kidney in mice administrated $KMnO_4$
and treated with an aqueous extract of *Hibiscus*.
A: 40X and B: 10X (H & E).

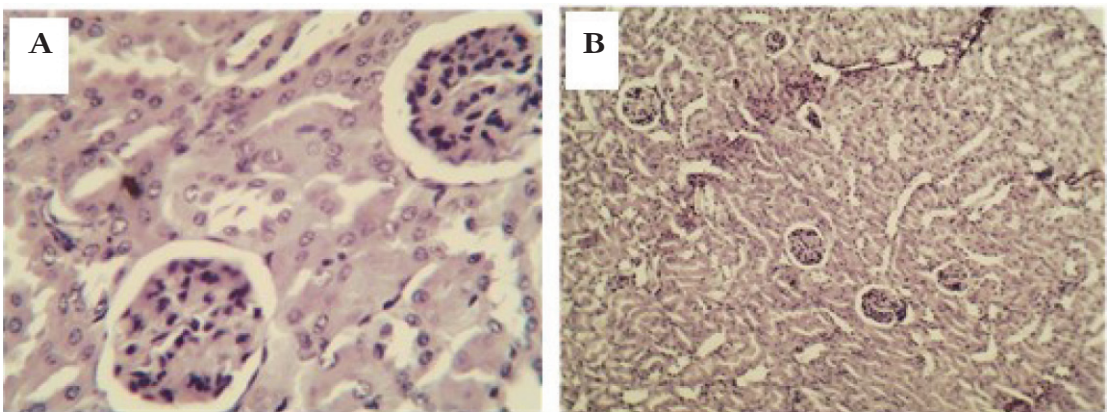



*Fig. 4.* Cross-sections of the kidney in mice administrated $KMnO_4$ and treated with an aqueous extract of *Malva*.
A: 40X and B: 10X (H & E).

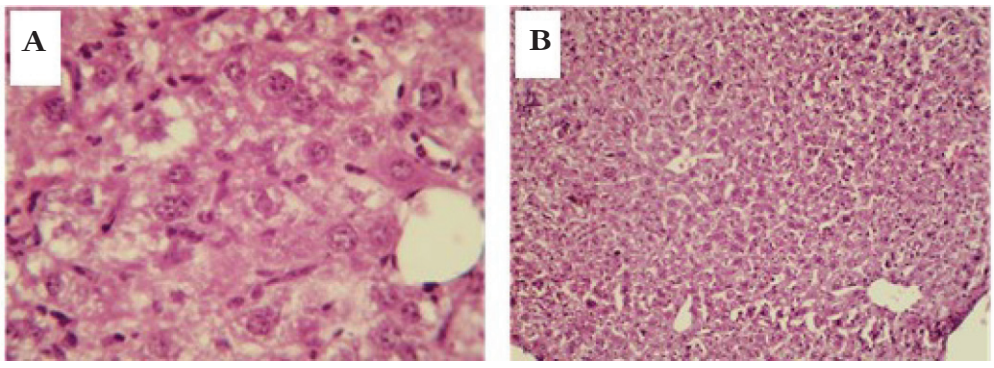



*Fig. 5.* Cross-sections of the liver in control mice.
A: 40X and B: 10X, (H & E).

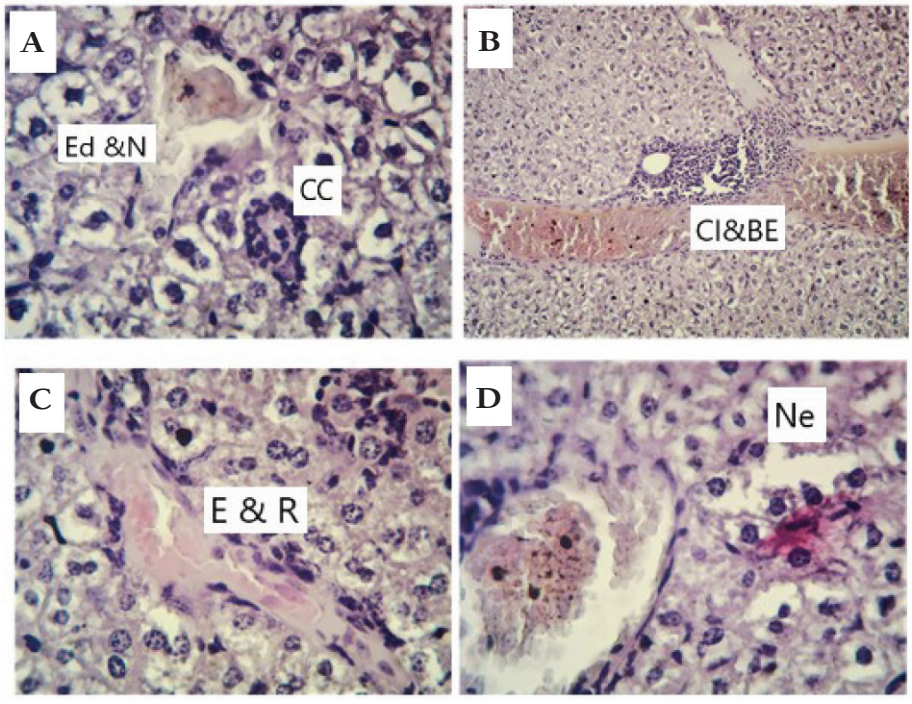



*Fig. 6.* Cross-sections of the liver in mice exposed to $KMnO_4$.
(A) Ed (edema), N (necrosis) and CC (chromatin condensation); (B) CI (cellular infiltration), BE (blood vessel edema); (C) E (edema), R (nucleus like a ring or circle); and (D) Ne (necrosis).
A, C, and D: 40X and B: 10X (H & E).

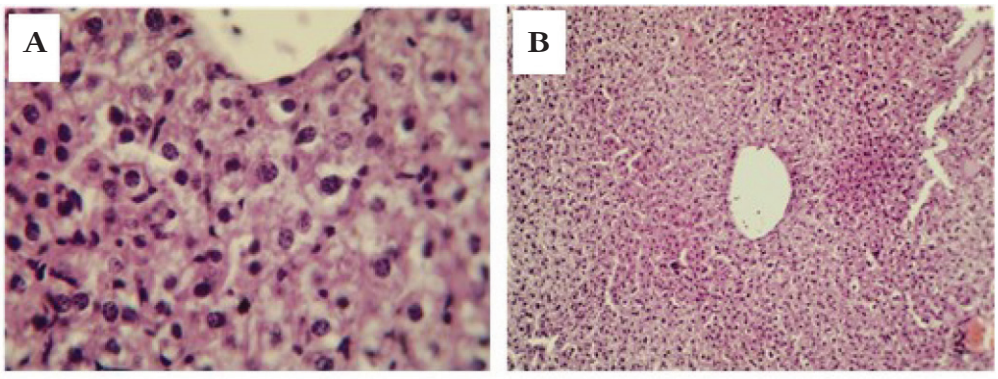



*Fig. 7.* Cross-sections of the liver in mice administrated $KMnO_4$ and treated with an aqueous extract of *Hibiscus*.
A: 40X and B: 10X (H & E).

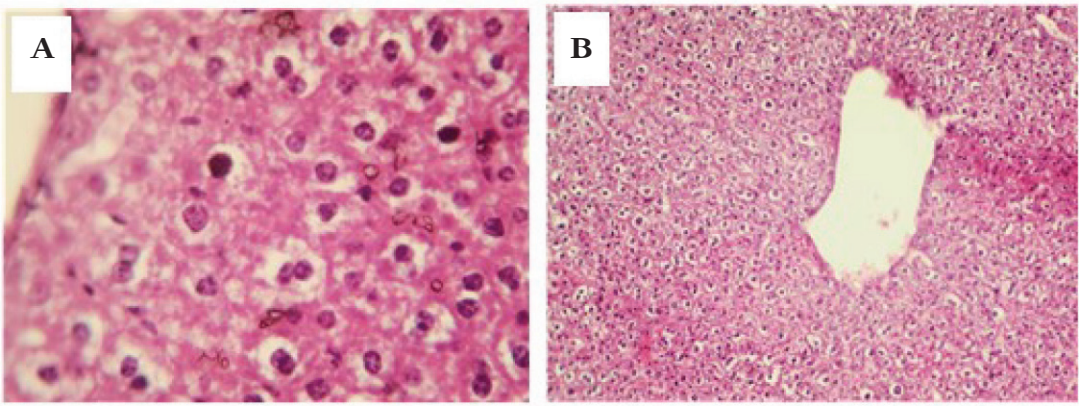



*Fig. 8.* Cross-sections of the liver in mice administrated $KMnO_4$ and treated with an aqueous extract of *Malva*.
A: 40X and B: 10X (H & E).

shape). These pathological changes may act as an indicator to kidney and liver toxicity, and oxidative stress with $KMnO_4$. The oxidant materials lead to the development and progression of kidney and liver damage, so it is considered the major pathological mechanism (Marins et al., 2020). Ejikeme et al. (2016) have found that $KMnO_4$ leads to a histopathological change and injuries in the tissues of the kidney (like cystic spaces, necrotic tubules, and destruction of renal tubules) and the liver (like enlargement of the sinusoids, disintegration of hepatic chords, and liver steatosis); besides, $KMnO_4$ affects urea and creatinine levels in the body. Al- Zwean (2017) has found that $KMnO_4$ induced significant changes in hepatic enzymes (like ALP, AST, and ALT) and proteins. Ali (2017) has observed various histopathological changes in the renal tissue (like aggregations of mononuclear cells and congestion of blood vessels), and coagulative necrosis in the hepatic tissue. Another study has shown that $KMnO_4$ affected biochemical molecules in the kidneys and the liver of mice (Hussein and Kata, 2008).

Medicinal plants are considered sources of natural productions and the most remarkable of the functional compounds are antioxidants. Antioxidant compounds finish the chain reactions resulting from free radicals (Rani et al., 2015). The kidneys in the groups treated with $KMnO_4$ and with aqueous extracts of plants showed normal renal corpuscles and tubules being intact without any structural damages. There are no plausible changes in the hepatic cells, micro- and macro-vasculature, and hepatic plates in the mice treated with $KMnO_4$ and with aqueous extracts of plants. Pacome et al. (2014) have shown that the components that form the petals of *H. sabdariffa* like alkaloids, anthocyanins, phenols, flavonoids, saponins, steroids, sterols and tannins contribute to the antioxidative effectiveness and have a scavenging ability (around 97%). However, the extracts of *H. sabdariffa* are possible sources of natural antioxidants, and this substantiates their utilities in herbal medicine. The results of improving the liver from the effect of hibiscus agree with Adeyemi et al. (2014). Therefore, it can be concluded that antioxidants in the *H. sabdariffa* extract, especially the anthocyanins, preserve the kidneys and the liver against oxidative factors.

*Malva parviflora* prevents inflammation, oxidative damage, and hypertension in the kidneys of mice because it contains some compounds such as oleanolic acid, scopoletin and tiliroside (Lagunas-Herrera et al., 2019). Another study has shown that the extracts of *M. parviflora* protect the liver from toxins (Mallhi et

al., 2014). Lowering tissue damage is exhibited in the histopathologic valuations of the current study. Treating diabetic rats with *M. parviflora* reduces oxidative stress and fat oxidation and protects the kidneys and the liver (Gutierrez, 2012). The results of the *M. parviflora* that has antioxidant activity agree with those of Farhan et al. (2012) and Ridh et al. (2018). Using the *M. parviflora* extract leads to improvement of all these injuries, which shows the protective effects of *M. parviflora* against histopathologic damage due to $KMnO_4$. Because this damage is caused by activated inflammatory and oxidative factors following $KMnO_4$ usage, it appears that *M. parviflora* decreases the histopathologic damage by lowering oxidative stress and inflammation. In advowson of these findings, it is exhibited that the utilization of antioxidants can weaken renal and hepatic injuries due to $KMnO_4$ (Ejikeme et al., 2016).

## Conclusion

The current study explains for the first time that using aqueous extracts of *H. sabdariffa* and *M. parviflora* can prevent strongest oxidants ($KMnO_4$) that induce renal and hepatic injuries. The histological changes in the kidney and the liver may be a direct cause of renal toxicity and hepatotoxicity at high $KMnO_4$; and in other cases, *H. sabdariffa* and *M. parviflora* have to some extent protect the kidneys and the liver as well. The protective mechanisms of plant extracts may be due to reduced oxidative stress, reduced inflammation, or increased effectiveness of antioxidants in the body, or possibly other unknown mechanisms, which require further research studies. These herbal extracts can also be utilized as food additives for aquaculture.

## Acknowledgment

The researchers would like to thank Dr. Suzan Al-Ani for all the information provided and assistance in the work. Thanks to Mr. Ahmed Diaa for helping us with the animal anatomy.

## Conflict of interests

Authors declare no conflict of interest.

## References

1. Abd El-Salam EA, Morsy NFS. Optimization of the extraction of polyphenols and antioxidant activity from Malva parviflora L. leaves using Box–Behnken design. Preparative Biochemical Biotechnology Journal, 2019. P. 1-8.
2. Adeoye BK, Oyewole OB, Idowu MA, Ademuyiwa O, Obadina AO, Adaramola FB, Ani IF and Ngozi EO. Protection against Oxidative Damage Properties of Hibiscus sabdariffa Drink (Zobo) and Added Natural Food Additives Oxidative Damage Properties of Hibiscus Sabdariffa Drink and Additives. European Journal of Scientific Research, 2019. T. 152(2). P. 153-162.
3. Adeyemi DO, Ukwenya VO, Obuotor EM, Adewole SO. Anti-hepatotoxic activities of Hibiscus sabdariffa L. in animal model of streptozotocin diabetes-induced liver damage. BioMedicalCenter Complementary and Alternative Medicine, 2014. T. 14:277. P. 1-11.
4. Agrawal VK, Bansal A, Kumar R, Kumawat BL, Mahajan P. Potassium permanganate toxicity: A rare case with difficult airway management and hepatic damage. Indian Journal of Critical Care Medicine, 2014. T. 18 (12). P. 819-821.
5. Al- Zwean DHY. The effect of water treated with potassium permanganate (KMnO4) and chlorine on some hygienic and physiological parameters in the local rabbits. Journal of Entomology and Zoology Studies, 2017. T. 5(3). P. 1554-1559.
6. Ali HA. Hematological and Histopathological Adverse Effects of kmno4 on mice. Mirror of Research in Veterinary Sciences and Animals Journal, 2017. T. 6 (1) P. 1-8.
7. Balarabe MA. Nutritional Analysis of *Hibiscus sabdariffa* L. (Roselle) Leaves and Calyces. Plant Journal, 2019. T. 7(4) P. 62-65.
8. Dhaliwal JS, Singh H. Free Radicals and Anti-oxidants in Health and Disease. International Journal of Oral Health Medical Research, 2015. T. 2(3). P. 97-99.
9. Ejikeme OG, Juliana AE, Felicia E, Bright IC, Osita EC. Renal and hepatic histopathology of intraperitoneally administered potassium permanganate on Clarias gariepinus juveniles. African Journal of Biotechnology, 2016. T. 15(41). P. 2332-2343.
10. Farhan H, Rammal H, Hijazi A, Hamad H, Daher A, Reda M, Badran B. In Vitro Antioxidant Activity of Ethanolic and Aqueos Extracts from Crude Malva parviflora L.Grown in Lebanon. Asian Journal Pharmaceutical and Clinical Research, 2012. T. 5. P. 234-238.
11. França JG, Ranzani-Paiva MJT, Lombardi JV, Carvalho S, Filipak-Neto F, Oliveira-Ribeiro CA. Toxic effect of potassium permanganate on Oreochromis niloticus based on hematological parameters and biomarkers of oxidative stress. International Journal of Fisheries and Aquaculture, 2013. T. 5(1). P. 1-6.
12. Gaffer EG and Mustafa HA. The Hypolipidaemic Effect of the Ethanolic Extract of Hibiscus sabdariffa L. Calyces on Induced Hyperlipidaemia in Albino Rats. EC Veterinary Science Journal, 2019. T. 4(6). P. 379-386.
13. Gutierrez RMP. Evaluation of hypoglycemic activity of the leaves of Malva parviflora in streptozotocin-induced diabetic rats. Food and Function Journal, 2012. T. 3. P. 420–427.
14. Hussein MA, Kata FS. Some hematological and biochemical effects of potassium permanganate (KMnO4) on female mice (Mus Musculus L.). Journal Basrah Researches (Sciences), 2008. T. 34(3). P. 9-14.
15. Jamini TS, Aminul Islam AKM, Mohi-ud-Din M and Hasan Saikat MM. Phytochemical Composition of Calyx Extract of Roselle (Hibiscus sabdariffa L.) Genotypes. Journal of Food Technology and Food Chemistry Introduction, 2019. T. 2(1). P. 1-6.
16. Kidane A, Rezene A, Michael M, G/Hannes O, G/Michael S, Mehreteab S, Yohannes E, Jyoti J, Tesfalem M and Andom H. Anti-Bacterial Activities of Selected Traditional Medicinal Plants Against Urinary Tract Infection Causing Microorganisms. International Journal of Medicinal Plants and Natural Products, 2019. T. 5(1). P. 16-22.
17. Lagunas-Herrera H, Tortoriello J, Herrera-Ruiz M, Martinez-Henandez GB, Zamilpa A, Santamaria LA, Lorenzana MG, Lombardo-Earl G, and Jimenez-Ferrer E. Acute and Chronic Antihypertensive Effect of Fractions, Tiliroside and Scopoletin from Malva parviflora. Biological and Pharmaceutical Bulletin Journal, 2019. T. 42(1). P. 18–25
18. Mallhi TH, Abbas K, Ali M, Qadir MI, Mohammad Saleem M, Khan YH. Hepatoprotective activity of methanolic extract of Malva parviflora against paracetamol-induced hepatotoxicity in mice. Bangladesh Journal Pharmacology, 2014. T. 9. P. 342-346.
19. Marins E, Silva JS, Pasinato AZ, Rosa PC, Oliveira VA, Moretto JG, Vargas JE, Soares FAA, Barcelos RP. Caramel dye IV increases hepatic and renal oxidative stress injuries. Integrative Food, Nutrition and Metabolism, 2020. T. 7. P. 1-6.

20. Okonkwo, Joy C, Chuka BD, Monago-Ighorodje C, Comfort, Joseph OC, Oforibo and Clement E. Nephroprotective Effects of Aqueous Extracts of Hibiscus sabdariffa Calyces and Jatropha curcas Leaves against Lead Toxicity. International Journal of Biochemistry and Biophysics, 2020. T. 8(1). P. 9-16.
21. Rani PS, Vunguturi S, Ali MR. Evaluation of antioxidant activity of some medicinal plants using oxidizing agent in acidic medium -By Colorimetry. International Journal of Innovative Science, Engineering & Technology, 2015. T. 2(5). P. 643-647.
22. Ridh DAM, Sarheed NM, Kokaz OF. Activity of Ethanolic Extraction of Malva parviflora and liqourice as Antifungal and Antioxidant in male rats. Journal of Pharmaceutical Science and Research, 2018. T. 10(4). P. 777-781.
23. Pacome OA, Bernard DN, Sekou D, Joseph DA, David NJ, Mongomake K, and Hilaire KT. Phytochemical and Antioxidant Activity of Roselle (Hibiscus Sabdariffa L.) Petal Extracts. Research Journal of Pharmaceutical, Biological and Chemical Sciences, 2014. T. 5(2). P. 1453-1465.
24. Puro K, Aochen C, Ghatak S, Das S, Sanjukta R, Mahapatra KP, Jha AK, Shakuntala I, Sen A. Studies on the therapeutic properties of Roselle (Hibiscus sabdariffa) calyx: A popular ingredient in the cuisine of North East India. International Journal of Food Science and Nutrition, 2017. T. 2(5). P. 01-06.
25. Ramirez-Serrano CE, Jimenez-Ferrerb E, Herrera-Ruiz M, Zamilpa A, Vargas-Villa G, Ramirez-Carreto RJ, Chavarria A, Tortoriello J, Pedraza-Alva G and Perez-Martinez L. A *Malva parviflora*´s fraction prevents the deleterious effects resulting from neuroinflammation. Biomedicine & Pharmacotherapy Journal, 2019. T. 118. P. 1-9.
26. Rasheed HU, Nawaz H, Rehman R, Mushtaq A, Khan S, Azeem MW. Little Mallow: A review of botany, composition, uses and biological potentials. International Journal of Chemical and Biochemical Sciences, 2017. T. 12. P. 157-161.
27. Singh A, Navneet. Ethnomedicinal, Antimicrobial and Pharmacological aspects of Malva parviflora Linn.: A review. The Journal of Phytopharmacology, 2017. T. 6(4). P. 247-250.
28. Soto ME, Zuniga-Munoz A, Lans VG, Duran-Hernandez EJ and Perez-Torres I. Infusion of Hibiscus sabdariffa L. Modulates Oxidative Stress in Patients with Marfan Syndrome. Mediators of Inflammation Journal, 2016. P. 1-12.
29. Subramanya SH, Pai V, Bairy I, Nayak N, Gokhale S, Sathian B. Potassium permanganate cleansing is an effective sanitary method for the reduction of bacterial bioload on raw *Coriandrum sativum*. Journal of Bio Medical Center Research Notes, 2018. T. 11:124 P. 1-5.
30. Umoren EB, Ekpenyong JF, Oyama OE and Obembe AO. The Hypolipidemic Effect of Aqueous Extract of Hibiscus sabdariffa on Paracetamol-induced Hepatotoxicity in Albino Wistar Rats. European Journal of Medicinal Plants, 2020. T. 31(7). P. 54-60.
31. Willhite CC, Bhat VS, Ball GL, McLellan CJ. Emergency Do Not Consume/Do Not Use concentrations for potassium permanganate in drinking water. Human and Experimental Toxicology, 2013. T. 32(3). P. 275–298.

*Received 1 December 2020*
*Accepted 31 December 2020*

# Short communication: Evaluation of alternative diluting media for cryopreservation of goat semen

**Medardo Moreno[1], Juan Chavarría[1], José Guevara[1], Byron Flores[2]**
*[1]School of Agricultural Sciences, Catholic University of the Dry Tropic (UCATSE), Nicaragua*
*[2]Centro Veterinario de Diagnóstico e Investigación (CEVEDI), Departamento de Veterinaria y Zootecnia, Escuela de Ciencias Agrarias y Veterinarias, Universidad Nacional Autónoma de Nicaragua-León (UNAN-León), Nicaragua*

**Keywords:** *thermoresistance, semen, goat, conservation, motility*

**Abstract.** *Goat farming is an important source of food production in developing countries, where artificial insemination is difficult due to the low quality of semen cryopreservation. In the present investigation, four diluents, namely cow's milk (T1), goat's milk (T2), egg yolk (T3) and commercial (Minutube, USA) Triladyl diluent (T4), were evaluated for the cryopreservation of semen from a Saanen goat buck. Each medium was assessed during 4 repetitions and each repetition performed 10 aliquots for a total of 160 aliquots in study. The color and sperm concentration were revised and counted in the Neubauer chamber from fresh semen; the motility and the thermoresistance were evaluated every 24 hours for a total of 96 hours post-freezing at −174°C. Post-thaw motility was similar in the 4 mediums in the first 24 hours ($p \geqslant 0.05$). After 96 hours, post-thaw motility in T1 (2.25 ± 0.50) and T2 (2.00 ± 0.51) was lower than in T3 (3.00 ± 0.81) and T4 (3.75 ± 0.50), ($p < 0.05$). The thermoresistance was similar for all treatments up to 48 hours ($p \geqslant 0.05$), but after 72 hours T4 (1.75 ± 0.50) and T3 (1.25 ± 0.50) showed better results ($p < 0.05$) than T1 (0.50 ± 0.50) and T2 (0.25 ± 0.5). Egg yolk and Triladyl diluents showed a better effect (Kruskal Wallis, $p < 0.05$) of cryopreserved Saanen goat semen viability in vitro compared with cow's milk and goat's milk.*

## Introduction

Goat farming is important as a food production source for economically deprived populations, especially in developing countries, where the economic resources of small producers are limited and artificial insemination is still insipient, mainly due to the low quality of semen cryopreservation (Ferreira et al., 2014).

In 2019, the Nicaraguan Institute of Agricultural Technology (INTA) created research and innovation farms (INTA, 2019) to study milk and meat production, and reproduction among different breeds in sheep and goat herds, making the last effort in the development to genetic improvement.

Artificial insemination is an important tool for genetic improvement, using stallions with superior productive characteristics, although the improvement success depends largely on the development of satisfactory diluents for semen, which should protect the sperm during freezing and increase its life span with a minimal effect on fertility (Palomino et al., 2014).

Cryopreservation of sperm is an important tool for breeding programs and conservation of breeds of various species, including small ruminants. However, its effectiveness depends on the type of semen extenders and additives used to stabilize sperm cells during the freezing and thawing processes (Ismail et al., 2020) vitality, and fertility of spermatozoa after freezing. Different diluents have been studied to increase sperm quality, prevent intracellular ice crystal formation and reduce damage to the membrane during and after cryopreservation (Jiménez-Rabadán et al., 2016) electroejaculation. The thermoresistance test (TRT) simulates the time of the sperm persisting in the female genital tract by exposure to 38°C for a long time and concomitantly the motility of the sperm after measuring heat stress (Talini et al., 2019).

There is an expansive range of diluents and methods used in the preservation of goat semen (Cuevas & Kevin, 2018; Sun et al., 2020). Mediums used for this purpose extend the viability of the sperm cell for a limited period (refrigeration) or indefinitely (freezing), making the most of the number of doses obtained per ejaculate. Among the natural diluents, egg yolk and skimmed or ultra-pasteurized milk are usually used to preserve goat semen (Amiridis & Fthenakis, 2012). The objective of this study was to evaluate the effect of extenders based on cow's milk, goat's milk, egg yolk and Triladyl diluents for *in vitro* viability of cryopreserved Saanen goat semen.

## Materials and methods

The study was carried out in the research facility of the Universidad Católica del Trópico Seco, located at 13°14'39" N, 86°22'29" W, and 865 m above sea level, in the department of Estelí, Nicaragua. Seminal fluid was obtained with an artificial vagina from a Saanen buck. The animal, 4 years old and weighing 70 kg, was in a good general health condition

Correspondence to Byron Flores, Centro Veterinario de Diagnóstico e Investigación (CEVEDI), Departamento de Veterinaria y Zootecnia, Escuela de Ciencias Agrarias y Veterinarias, Universidad Nacional Autónoma de Nicaragua-León (UNAN-León), Carretera a La Ceiba 1 Km al Este, León, Nicaragua.
E-mail: byronfloressomarriba@gmail.com

according to clinical examination. Its physiological parameters were normal with respiratory rate of 13/min, heart rate of 75/min, and rectal temperature of 39°C. A complete blood count (CBC) was also performed and the normal values were obtained: hematocrit 35%, hemoglobin 11 g/dL, eosinophils 2%, neutrophils 35%, lymphocytes 60%, and monocytes 3%. Additionally, semen color, sperm concentration, motility and heat stress resistance were evaluated, according to previously described methods (Alomar, 2019; Malejane et al., 2014)the formation of hydrogen peroxide (H2O2. Four semen samples were collected at 7-day intervals using an artificial vagina at 37°C, obtaining an average of 4 mL per extraction. From the last extraction, 1:20 dilutions were made in each case, obtaining 160 straws of 0.5 mL, which were prepared with four mediums (T1, UTH cow's milk; T2, goat's milk; T3, egg yolk; and T4, Triladyl) realizing four repetitions (24 h, 48 h, 72 h, and 96 h) post-freezing at –174°C, with 10 straws each. Every 24 h post-freezing, the motility was evaluated, thawing the cryopreserved straws in a water bath at 37°C for 26 s, placing 10 µL on a pre-warmed slide (at 37°C); subsequently, rotating the slide on its vertical axis and observing in a microscope with a 10× objective. Sperm movements were registered on a scale of 0 to 5 (Luna-Orozco et al., 2019). In each evaluation, 10 microscopic fields were analyzed to include at least a total of 300 sperms, according to previously described recommendations (Cardoso et al., 2003). Moreover, a thermoresistance test was performed by incubating an aliquot of 0.5 mL at 38°C in an aerated water bath, as previously described (Schulze et al., 2017). After the incubation period (300 min), motility was determined on a scale of 0 to 5. Table 1 shows the proportion of each ingredient in cryopreserved semen in 100 mL of distilled water.

To compare motility and thermoresistance between the mediums, a non-parametric test (Kruskal-Wallis) was applied to evaluate the statistical significance $p < 0.05$.

*Table 1.* Proportion of each ingredient in semen cryopreservatives in 100 mL of distilled water

| Ingredients | Egg yolk | UHT cow's milk | Goat's milk | Triladyl |
|---|---|---|---|---|
| Natural diluting | 20% | 10% | 10% | 20% |
| Glycerol | 6% | 6% | 6% | Include |
| Glucose | 0.9% | 0.9% | 0.9% | Include |
| Penicillin | 100,000 UI | 100,000 UI | 100,000 UI | Include |
| Streptomycin | 100 mg | 100 mg | 100 mg | Include |
| Sodium citrate | 0.9% | 0.9% | 0.9% | Include |

**Results and discussion**

The extracted semen was yellowish in color with a creamy appearance, a similar result obtained by Memon et al. (2011), describing fresh goat semen with yellowish and milky color. This is a satisfactory result since a red color indicates the presence of blood, a gray color indicates the presence of an infection or abnormalities and yellow indicates the presence of urine (Bravo et al., 2002).

The sperm count performed resulted in 6.5 billion sperms per mL of semen, similar data to the parameters proposed by other authors who recommend that the counted sperm range should be between 2 and 6 billion per mL (Cueto, 2016).

The comparison of the post-thaw motility showed significant differences between the four mediums after 48 hours ($p < 0.05$), with the lowest motility in the samples preserved with goat's milk (2.50 ± 0.52) and cow's milk (2.75 ± 0.57) and the highest in the semen preserved with Tryladil (4.00 ± 0.20) and egg yolk (3.50 ± 0.57) (Fig. 1). The results after 96 hours showed that the most effective treatments were egg yolk (3.0 ± 0.81) and Tryladil (3.75 ± 0.50), with similar results for both ($p \geqslant 0.05$), but they were different from those obtained with cow's milk (2.25 ± 0.50) and goat's milk (2.00 ± 0.51), ($p < 0.05$). Similar results were reported by researchers who describe that citrated egg yolk has been shown to function as a non-permeable cryoprotectant for semen, finding sperm motility of 3.6 ± 0.2 in semen after 2 hours at –196°C (Luna-Orozco et al., 2019). Most cryopreservatives contain egg yolk, as it acts as a reservoir for phospholipids and cholesterol that helps to protect the plasma membrane and acrosome against temperature-related injuries (Swelum et al., 2018) pigeon (P. After 96 hours, it was observed that the egg yolk had a better result than goat's milk and cow's milk. Similar results were found in a study carried out in Peru, where the viability of the sperm prepared with skimmed milk was only visible 6 hours after refrigeration vs 48 hours when egg yolk was used (Palomino et al., 2014).

Our data differ from those described by authors who indicate that, although egg yolk or skimmed milk are the most common semen diluents for goats, their interaction between goat seminal plasma is harmful to spermatozoa, a condition which is not seen with other mammalian seminal plasmas (Daramola et al., 2016; Purdy, 2006). Also, bulbourethral enzymes have been reported to react with egg yolk in goat and sheep semen, causing hydrolysis of lecithin and triglycerides present in egg yolk, resulting in high semen toxicity (Luna-Orozco et al., 2019). To address this problem, natural additives such as coconut water (Bottini-Luzardo et al., 2013; Luna-Orozco et al., 2019), or liposome diluents free of animal origin proteins, which enhance fertility, especially in small ruminants (Elodie Pillet et al., 2012) addition of egg yolk in extenders is not without disadvantages and the demand to find cryoprotective alternatives is strong. The objec-

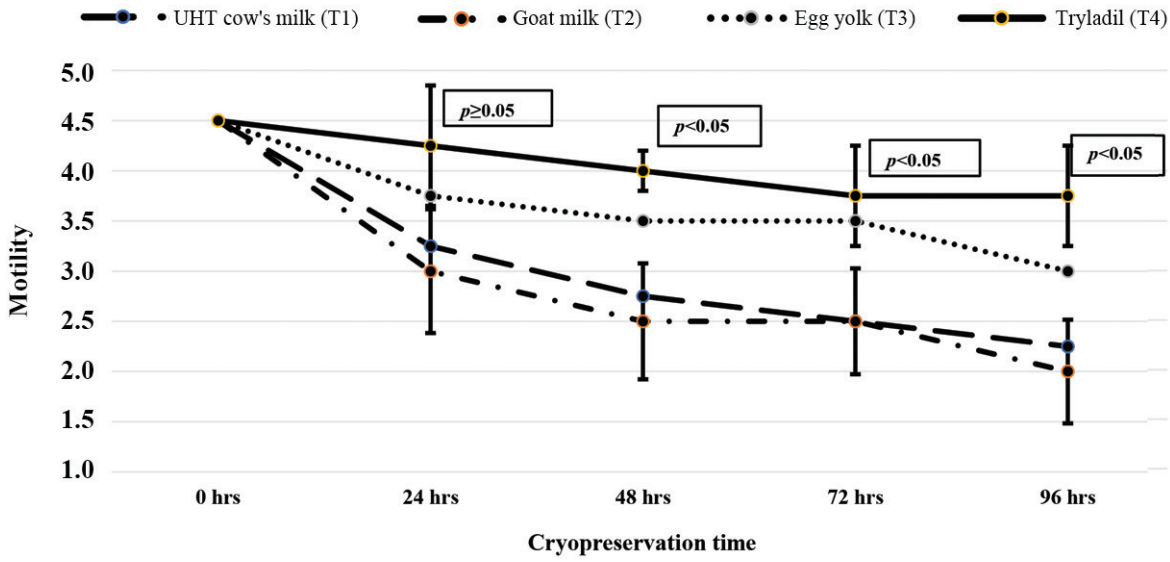



*Fig. 1.* Sperm motility for the different cryopreservation times.
Only the error bars are shown for the treatments with higher (T4) and lower (T2) motility.
The p values correspond to the comparison between the 4 groups according to the Kruskal Wallis test.

tive of this study was to test the cryoprotective capacities of liposomes composed of egg yolk phospholipids. Two experiments were conducted: 1, were used, despite of their higher cost. Another recommendation is to dilute the goat semen sample in a buffered diluent and then separate the seminal plasma from the sperm, by washing the cells either once or twice, each for 10–15 min at 550–950 g (Purdy, 2006).

The thermoresistance test, by analysis of variance according to Kruskal Wallis, and sperm motility showed significant differences ($p < 0.05$) between the different diluents after 76 hours. Better results were observed in egg yolk (1.25 ± 0.50) and commercial Triladyl diluent (1.75 ± 0.50) compared with cow's milk (0.5 ± 0.57) and goat's milk (0.25 ± 0.50) (Fig. 2). These findings coincide with those described by researchers who observed better motility after the thermal resistance test compared with the skimmed milk diluent (Ferrari & Barnabe, 1999). In addition, a study carried out in Brazil reported better results as the concentration of egg yolk increased; they attributed this result to the fact that egg yolk can provide more protection for cryopreservation of sperm increase osmotic pressure, which results in cell dehydration with consequent reduction of intracellular ice formation (Ferreira et al., 2014). A study testing treolase in goat semen cryopreservatives showed similar results when they managed to maintain high sperm motility in the thermoresistance test during the first hours of cryopreservation (Aboagla & Terada, 2003). Meanwhile, other researchers found that the presence of disaccharide in cryopreservatives did not increase

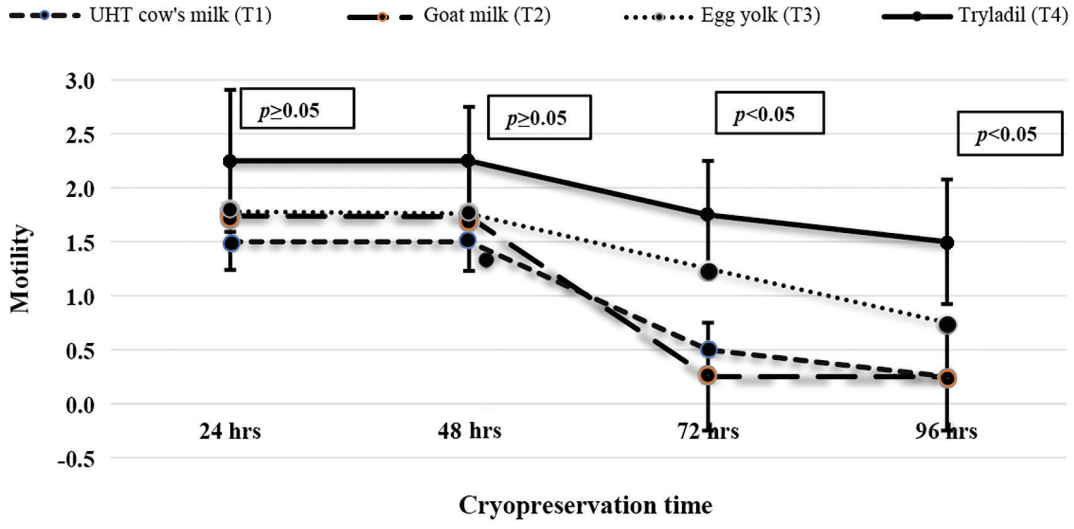



*Fig. 2.* Sperm motility after different cryopreservation times in the thermoresistance test
Only the error bars are shown for the treatments with higher (T4) and lower (T2) motility.
The p values correspond to the comparison between the 4 groups according to the Kruskal Wallis test.

heat resistance or protect the membrane integrity of frozen goat sperm (Quan et al., 2012).

With the post-thaw semen thermoresistance test, the integrity of the sperm is evaluated during artificial insemination; after the test, the thawed semen must have at least 15% motility to be considered of good quality (Talini et al., 2019). The results of this study showed that both Tryladil and egg yolk prepared semen-maintained motility above 1, even after 96 hours of cryopreservation, thus meeting this requirement.

## Conclusions

Sperm motility in cryopreserved Saanen buck semen was higher with Tryladil and egg yolk diluents compared with cow's milk and goat's milk (Kruskal Walli, $p < 0.05$). This difference was marked after 48 hours post-freezing at −170°C, while the sperm motility comparison between Tryladil and egg yolk was similar even after 96 hrs (Kruskal Wallis, $p \geqslant 0.05$). The thermoresistance was also higher in semen cryopreserved with Tryladil and egg yolk diluents compared with cow's milk and goat's milk (Kruskal Wallis, $p < 0.05$). However, this difference was observed until after 72 hours post-freezing. The results of this study revealed that Tryladil diluents and egg yolk were effective in the cryopreservation of Saanen buck semen.

## Acknowledgements

We are very thankful to Álvaro Chávez, José Luis Balcazar and Christiane Düttmann for English editing of this manuscript.

## References

1. Aboagla EM-E, Terada T. Trehalose-Enhanced Fluidity of the Goat Sperm Membrane and Its Protection During Freezing. Biol Reprod. 2003. Vol. 69(4). P. 1245–1250.
2. Alomar M. Formation of Hydrogen Peroxide by Chilled Goat Spermatozoa and the Effects of Dead Spermatozoa on Motility Characteristics. Iranian Journal of Applied Animal Science. 2019. Vol. 9(1). P. 79–85.
3. Amiridis GS, Fthenakis GC. Reproductive health management of sheep and goats. Preface. Anim Reprod Sci. 2012. Vol. 130(3–4). P. 125.
4. Bottini-Luzardo M, Centurión-Castro F, Alfaro-Gamboa M, Aké-López R, Herrera-Camacho J. Effect of addition of coconut water (Cocos nucifera) to the freezing media on post-thaw viability of boar sperm. Trop Anim Health Prod. 2013. Vol. 45(1). P. 101–106.
5. Bravo PW, Moscoso R, Alarcon V, Ordoñez C. Ejaculatory process and related semen characteristics. Arch Androl. 2002. Vol. 48(1). P. 65–72.
6. Cardoso R de CS, Silva AR, Uchoa DC, da Silva LDM. Cryopreservation of canine semen using a coconut water extender with egg yolk and three different glycerol concentrations. Theriogenology. 2003. Vol. 59(3). P. 743–751.
7. Cueto M. Obtención, procesamiento y conservación del semen ovino. Segunda edición. INTA; 2016.
8. Cuevas M, Kevin A. Estudio comparativo de tres diluyentes (Andromed, Triladyl y Citrato de Sodio con yema de huevo) en la preservación de semen caprino. 2018;
9. Daramola JO, Adekunle EO, Oke OE, Onagbesan OM, Oyewusi IK, Oyewusi JA. Effects of coconut (Cocos nucifera) water with or without egg-yolk on viability of cryopreserved buck spermatozoa. Anim Reprod. 2016. Vol. 13(2). P. 57–62.
10. Elodie Pillet, Labbe C, Batellier F, Duchamp G, Beaumal V, Anton M, Desherces S, Schmitt E, Magistrini M. Liposomes as an alternative to egg yolk in stallion freezing extender. Theriogenology. 2012. Vol. 77(2). P. 268–279.
11. Ferrari S, Barnabe VH. Efeito de dois tipos de diluidores e dois métodos de congelação na qualidade do sêmen criopreservado de caprinos. Braz J Vet Res Anim Sci. 1999. Vol. 36(4).
12. Ferreira V da S, Mello MRB de, Fonseca CEM da, Dias ÁCF, Cardoso JM, Silva RB, Martins Júnior WP, Ferreira V da S, Mello MRB de, Fonseca CEM da, Dias ÁCF, Cardoso JM, Silva RB, Martins Júnior WP. Effect of seminal plasma and egg yolk concentration on freezability of goat semen. Rev Bras de Zootec. 2014. Vol. 43(10). P. 513–518.
13. INTA. Agro-innovación Nicaragua 2019 | INTA. 2019.
14. Ismail AA, Abdel-Khalek A-KE, Khalil WA, Yousif AI, Saadeldin IM, Abomughaid MM, El-Harairy MA. Effects of mint, thyme, and curcumin extract nanoformulations on the sperm quality, apoptosis, chromatin decondensation, enzyme activity, and oxidative status of cryopreserved goat semen. Cryobiology. 2020. Vol. 97. P. 144–152.
15. Jiménez-Rabadán P, Soler AJ, Ramón M, García-Álvarez O, Maroto-Morales A, Iniesta-Cuerda M, Fernández-Santos MR, Montoro V, Pérez-Guzmán MD, Garde JJ. Influence of semen collection method on sperm cryoresistance in small ruminants. Anim Reprod Sci. 2016. Vol. 167. P. 103–108.
16. Luna-Orozco JR, González-Ramos MA, Calderón-Leyva G, Gaytán-Alemán LR, Arellano-Rodríguez F, Ángel-García O, Véliz-Deras FG. Comparison of different diluents based on liposomes and egg yolk for ram semen cooling and cryopreservation. Iran J Vet Res. 2019. Vol. 20(2). P. 126.
17. Malejane CM, Greyling JPC, Raito MB. Seasonal variation in semen quality of Dorper rams using different collection techniques. S Afr J Anim Sci. 2014. Vol. 44(1). P. 26–32.
18. Memon AA, Wahid H, Rosnina Y, Goh YM, Ebrahimi M, Nadia FM, Audrey G. Effect of butylated hydroxytoluene on cryopreservation of Boer goat semen in Tris egg yolk extender. Anim Reprod Sci. 2011. Vol. 129(1). P. 44–49.
19. Palomino T. L, Camacho S. J, Huanca L. W, Falcón P. N. Conservación de semen caprino en los dilutores citrato-yema y leche-descremada yema. Rev investig vet Perú. 2014. Vol. 12(1). P. 1–7.
20. Purdy PH. A review on goat sperm cryopreservation. Small Rumin Res. 2006. Vol. 63(3). P. 215–225.
21. Quan GB, Hong QH, Lan ZG, Yang HY, Wu SS. Comparison of the Effect of Various Disaccharides on Frozen Goat Spermatozoa. Biopreserv Biobank. 2012. Vol. 10(5). P. 439–445.
22. Schulze M, Ammon C, Schaefer J, Luther A-M, Jung M, Waberski D. Impact of different dilution techniques on boar sperm quality and sperm distribution of the extended ejaculate. Anim Reprod Sci. 2017. Vol. 182. P. 138–145.
23. Sun L, Fan W, Wu C, Zhang S, Dai J, Zhang D. Effect of substituting different concentrations of soybean lecithin and egg yolk in tris-based extender on goat semen cryopreservation. Cryobiology. 2020. Vol. 92. P. 146–150.
24. Swelum AA-A, Saadeldin IM, Alanazi MB, Ba-Awadh H, Afifi M, Alowaimer AN. Effects of adding egg yolks of different avian species to Tris glycerol extender on the post-thawing quality of buck semen. Anim Reprod Sci. 2018. Vol. 195. P. 345–354.
25. Talini R, Kozicki LE, Gaievski FR, Polo G, Lima LGF, Santiago J, Segui MS, Weiss RR, Galan TGB, Talini R, Kozicki LE, Gaievski FR, Polo G, Lima LGF, Santiago J, Segui MS, Weiss RR, Galan TGB. Bovine semen thermoresistance tests and their correlation with pregnancy rates after fixed-time artificial insemination. Arq Bras Med Vet Zootec. 2019. Vol. 71(6). P. 2085–2092.

*Received 4 December 2020*
*Accepted 31 December 2020*