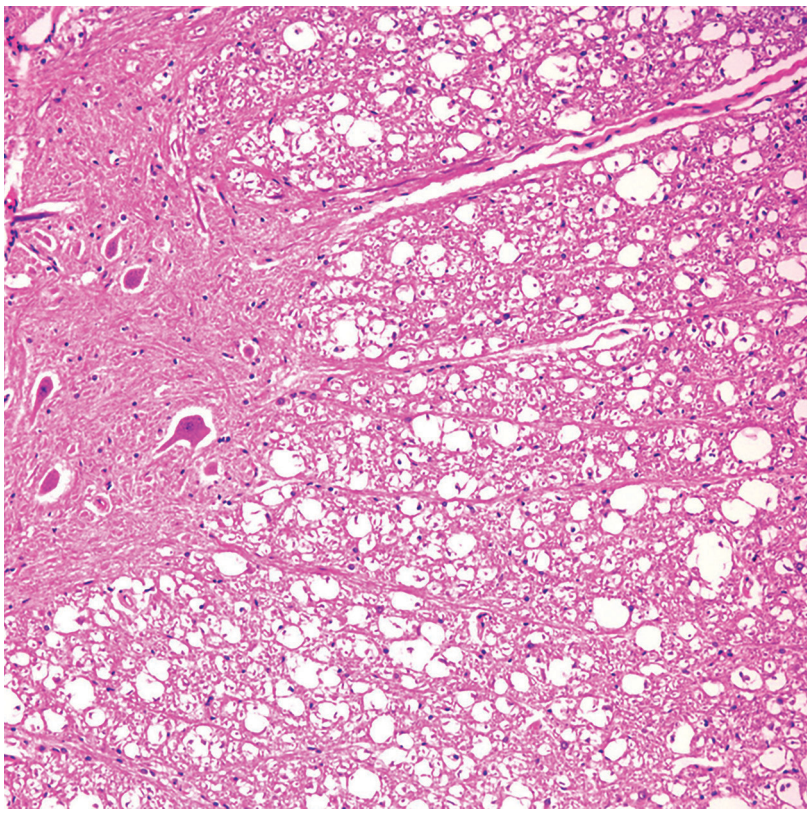
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Cover illustration: Hypomyelinated spinal cord white matter in a goat with enzootic ataxia. (Fontes et al., p.966)

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Original Article
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Chemical and structural composition of black pigmented supragingival biofilm of bovines with periodontitis[1]

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ABSTRACT.- Saraiva J.R., Buso-Ramos M.M., Borsanelli A.C., Schweitzer C.M., Gaetti-Jardim Jr. E., Höfling J.F., Ramos T.N.M. & Dutra I.S. 2019. **Chemical and structural composition of black pigmented supragingival biofilm of bovines with periodontitis.** *Pesquisa Veterinária Brasileira 39(12): 933-941*. Departamento de Apoio, Produção e Saúde Animal, Faculdade de Medicina Veterinária de Araçatuba, Universidade Estadual Paulista "Júlio de Mesquita Filho", Rua Clóvis Pestana 793, Cx. Postal 533, Jardim Dona Amélia, Araçatuba, SP 16050-680, Brazil. E-mail: iveraldo.dutra@unesp.br

Bovine periodontitis is a multifactorial disease primarily associated with a potentially pathogenic microbiota housed in the oral biofilm of animals. Biofilms are organized structures, in which the constituents coexist in symbiosis, already described as a predisposing factor to periodontitis in other species. The objective of the present study was to characterize the structure and chemical aspects of the bovine black pigmented supragingival biofilm using scanning electron microscopy (SEM) and energy dispersive spectroscopy (EDS), respectively, and determine its relationship with bovine periodontitis. Eleven premolar teeth from different animals were evaluated; five non-pigmented samples and six samples with black pigmented biofilms were initially submitted to SEM, and three areas of these samples were selected for EDS. The structure of the pigmented biofilm was more complex and irregular because of a higher content of mineral elements. The semi-quantitative EDS data indicated an association of iron ($p<0.014$) and magnesium ($p<0.001$) with the occurrence of periodontitis, whereas carbon, phosphorus, calcium, manganese, sodium, and potassium were not associated with the disease. Carbon ($p<0.039$), manganese ($p<0.007$), and iron ($p<0.015$) were associated with pigmentation, whereas phosphorus, calcium, and magnesium were not associated with it. Spearman correlation test showed the relationships between calcium and phosphorus, and iron and silicon. The strong association of iron in the pigmented supragingival biofilm and with the occurrence of periodontitis suggests the presence of microorganisms that use this element in their metabolism and that are also associated with bovine periodontitis. This study suggests that the pigmented deposits in the crown of the teeth of cattle are an true

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biofilm with the deposition of iron, and it indicates that the presence of iron and magnesium in these formations may be involved in the metabolism of some microorganisms associated with the etiology of bovine periodontitis.

INDEX TERMS: Supragingival biofilm, bovine, periodontitis, scanning electron microscopy, energy dispersive spectroscopy.

RESUMO.- [**Composição química e estrutural do biofilme supragengival pigmentado de bovinos com periodontite.**] A periodontite bovina é uma infecção multifatorial associada primariamente à microbiota potencialmente patogênica presente no biofilme bucal. Biofilmes são estruturas organizadas, nas quais os constituintes convivem em simbiose, descritos em outras espécies como um fator predisponente à periodontite. O objetivo do presente estudo foi caracterizar estrutural e quimicamente o biofilme supragengival pigmentado de preto em bovinos, utilizando-se as técnicas de microscopia eletrônica de varredura (MEV) e espectroscopia de dispersão de energia (EDS), respectivamente, correlacionando os elementos identificados à ocorrência de periodontite e pigmentação. Foram avaliados 11 dentes primeiro-molares; cinco amostras sem pigmentação visível e seis amostras com biofilme pigmentado de preto, que foram submetidas inicialmente à MEV; posteriormente foram selecionadas três áreas aleatórias de cada dente para realização da EDS. A estrutura do biofilme pigmentado revelou formações irregulares e mais complexas, provavelmente devido ao maior acúmulo de elementos minerais. Os resultados semi-quantitativos da EDS apontaram associações entre a presença de ferro ($p<0,014$) e magnésio ($p<0,001$) com a ocorrência de periodontite. Carbono, fósforo, cálcio, manganês, sódio e potássio não apresentaram associação com a periodontite. Em relação à pigmentação, carbono ($p<0,039$), manganês ($p<0,007$) e ferro ($p<0,015$) foram os elementos estatisticamente significantes, enquanto fósforo, cálcio e magnésio não apresentaram associação com a pigmentação. O teste de correlação de Spearman demonstrou associações entre os elementos cálcio e fósforo, e ferro e silício. A forte associação do ferro presente no biofilme supragengival com a ocorrência de periodontite, sugere a presença de micro-organismos que utilizam este elemento em seu metabolismo e que possivelmente tenham envolvimento com o desenvolvimento da periodontite bovina. Os resultados inéditos do presente trabalho sugerem que os depósitos pigmentados que se formam na coroa dos dentes de bovinos são um biofilme verdadeiro com deposição de ferro, e indicam que a presença de ferro e magnésio nestas formações pode estar envolvida no metabolismo de alguns dos principais micro-organismos associados à etiologia da periodontite bovina.

TERMOS DE INDEXAÇÃO: Composição química, biofilme supragengival, bovinos, periodontite, microscopia eletrônica de varredura, espectroscopia de dispersão de energia.

INTRODUCTION

Dental biofilms are dynamic and complex structures that are formed in dental enamel through the organized colonization of multiple microbial species of the buccal environment (Kolenbrander et al. 2002). These biofilms can be classified as subgingival, when they are formed in the gingival sulcus or periodontal pocket, or supragingival, when they are formed on the clinical crown of the teeth (Kolenbrander et al. 2010). Besides acting on oral health maintenance, some bacterial species present in biofilms play an important role in the etiology of periodontal diseases in humans (Hojo et al. 2009, Akcali & Lang 2017), and animals (Elliott et al. 2005, Rober et al. 2008, Gaetti-Jardim Junior et al. 2012, Borsanelli et al. 2016, Agostinho 2017, Campello 2017).

Periodontitis is a multifactorial infection associated with peculiar bacterial complexes, capable of interacting with host tissues and cells resulting in the release of cytokines, chemokines, and inflammatory mediators responsible for causing destruction of periodontal structures (Holt & Ebersole 2005, Hajishengallis 2015). These microbial groups are amphibiotic, establishing different types of ecological relationships (Marsh et al. 2011), and occupying ecological niches in healthy gingival sulcus and periodontal pockets (Bosshardt 2018). The interaction between these specific microbial groups, especially Gram-negative anaerobic rods, and environmental conditions is still poorly understood, since cattle periodontitis is more prevalent in animals that remain in newly formed areas or have gone through pasture reform process (Dutra et al. 1986, Dutra & Döbereiner 2001). In calf periodontal lesions, the mean percentage of pigmented black bacteria is considerably higher when compared to that of the same animals after being transferred to indene areas and showing remission of periodontitis clinical symptoms (Dutra et al. 2000). The disease does not occur without common microorganisms constituents of the microbiota, which are mostly found in the oral biofilm of these animals.

Pseudomonas, *Burkholderia*, and *Actinobacteria* are the most prevalent taxa in the subgingival microbioma of healthy cattle, whereas *Pophyromonas*, *Prevotella*, and *Fusobaterium* are more frequent in animals with periodontitis (Borsanelli et al. 2018). The diversity of the microbial biofilm increases with periodontitis, but the factors that lead to tissue changes and the possible structural biochemical characteristics of the oral biofilm in animals with different periodontal conditions are not clear.

The process of accumulation and mineralization of the dental biofilm results in calculus formation. In ruminants, this calculus is generally visualized as an accumulation with brown to black pigmentation strongly adhered to the dental surface. In cows, it was described as covering varying extensions of the masticatory teeth. However, at the time, little was known about the role played by dental calculus in the establishment and progression of periodontitis (Ingham 2001). In small ruminants, black pigmented supragingival calculus was described as a common finding and associated with the occurrence of gingival recession (Agostinho 2017, Campello 2017).

In humans, the main components of dental calculus are calcium, magnesium, fluorine, and carbon dioxide (Gron et al. 1967,

Fialová et al. 2017), which may be associated with environmental compounds, diet, and habits (Fialová et al. 2017). In cattle, the physical properties of dental enamel have been well characterized; the main constituents of the hydroxyapatite crystals, oxygen, calcium, and phosphorus, appear in high concentrations (Nogueira et al. 2014). Nevertheless, studies on the structure and chemical composition of the supragingival biofilm in cattle and their correlation with periodontitis are quite limited.

Understanding the ecology of supragingival biofilm and its relationship with periodontitis is an important step for advancing the knowledge on the etiology, pathogenesis, control, and prophylaxis of this disease in cattle. The aim of this study was therefore to characterize the structure and chemical aspects of the black pigmented supragingival biofilm using scanning electron microscopy (SEM) and energy dispersive spectroscopy (EDS), respectively, and understand its possible relationship with bovine periodontitis.

MATERIALS AND METHODS

Sample collection. Eleven maxillary first molar teeth from different bovines, five with no visible pigmentation and six with a visually pigmented black biofilm, were collected (Fig.1). These samples were collected from animals with a mean age of 36 months from a slaughterhouse in the region of Araçatuba, São Paulo, Brazil. Data such as sex, place of origin, diet, dental changes, and approximate age of each animal were obtained. Periodontal condition was established after probing the gingival margin of each tooth. Animals with a periodontal pocket deeper than 5mm were considered to present periodontitis. For dental extraction, the area between the third premolar and the second molar teeth was isolated and the gingival mucosa of the first molar was removed with a Molt periosteal elevator. The teeth were extracted from the alveolar bone using a simple bisel chisel and a Mead hammer. Sample collection was performed up to 30 minutes after slaughter to preserve the biofilm structure formed on the buccal surface of the upper first molar.

Preparation of the samples. After extraction, each dental root was sectioned; the samples clinical crowns had a maximum width of 12mm and a height and length of less than 30mm. Then, the organic matter debris was removed from each sample. Samples were washed twice with 300µL of physiological solution (NaCl 0.9%), and then, the crown biofilm was fixed with 1ml of 2% glutaraldehyde for 30 minutes at room temperature. The biofilm was then dehydrated in sequential ethanol baths (1mL) at concentrations 50%, 70%, 90%, and 100% for 10 minutes at each concentration. After dehydration and drying, the samples were stored in a container with silica for moisture control (modified from Santana et al. 2013).

Structural characterization by scanning electron microscopy (SEM) and mineral composition analysis by energy dispersive spectroscopy (EDS). For the semi-quantitative analysis of mineral composition of bovine biofilm using EDS, the first molars were fixed with a carbon double-face tape, mounted on a colorless acrylic resin stub, and received a carbon cover (Denton Vaccum Desk II). Images of the biofilms with and without pigmentation on the cervical region of the vestibular face of the first molars were captured using SEM JSM-5600 (Jeol, Tokyo, Japan) with magnifications of 100x and 3000x. Using the adjustment of Jeol's parameters (spot-size between 28-32nm and PHA ideal dead time between 20-25%) at 3000x magnification, the SEM images were transferred to the EDS micro-analysis (Energy-dispersive X-ray analysis; Vantage, Noran Instruments, Middleton/WI, USA). In the images captured with a magnification of 3000x, three areas of 81µm^2 each on the cervical surface of the samples were randomly selected for the semi-quantitative analysis; these areas were bombarded with high energy electrons allowing the detection of dispersive X-rays and evaluation of the characteristic absorption profile of each chemical element. Each absorption spectrum was captured for 100 seconds at 15kV voltage and 32mm working distance. Mean and standard deviation of each registered mineral were calculated.

Statistical analysis. The semi-quantitative values of the mineral elements were correlated with the clinical condition (periodontitis or healthy periodontum) and with the presence of pigmentation using the Student's t-test and the Mann-Whitney test. The possible associations between the different composite elements present in the samples were evaluated using the Spearman correlation test. The predicted significance level was $p<0.05$. Descriptive evaluations were made based on the SEM images.

Research ethics commission. The experiment was approved by the Ethics Committee on Animal Use (CEUA) of the Faculty of Agrarian and Veterinary Sciences-Unesp, Campus Jaboticabal/SP (Process FCAV-Unesp No. 013966/17).

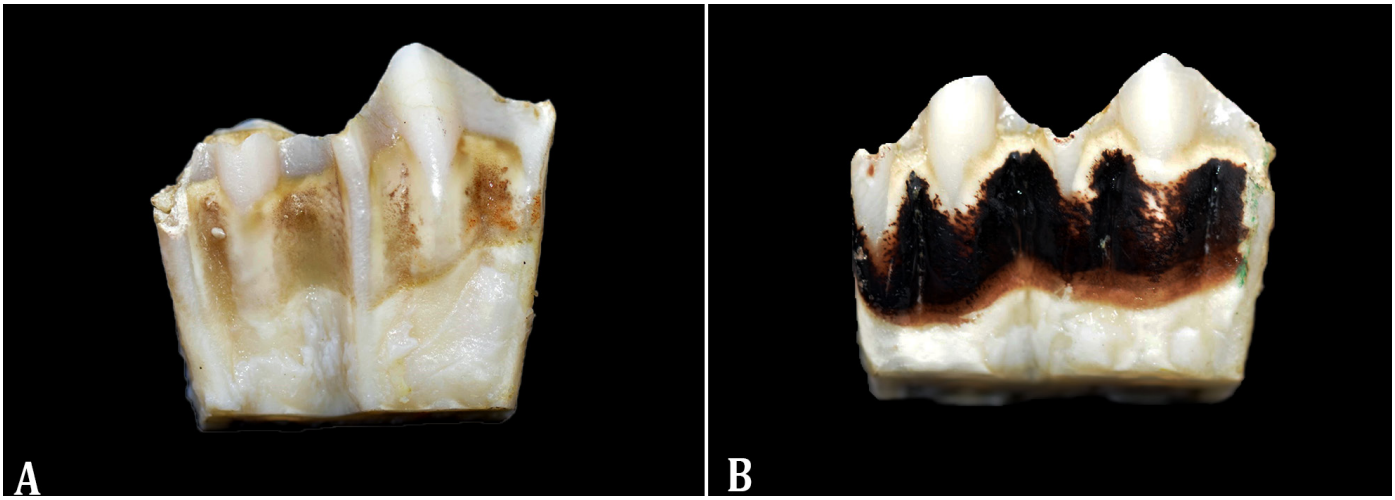


Fig.1. (**A**) Bovine upper first-molar vestibular face without visible pigmentation. (**B**) Bovine upper first-molar vestibular face with dark pigmentation.

RESULTS

Scanning electron microscopy

Of the 11 dental arches evaluated, 4 had periodontal pockets at one or more sites. The SEM images of both groups revealed calculus-like formations covered by a biofilm, evidencing the existence of irregular areas with structures that resemble the deposition of oxides and hydroxides, such as calcium hydroxide, and other elements. When the images of the calculus and biofilm without pigmentation were compared with the black pigmented structures, it was observed that these pigmented structures had greater structural complexity, with a more irregular surface and greater abundance of the deposits (Fig.2 and 3). Irregular, coccoid, and bacillary images surrounded by organic and inorganic matrix, suggestive of microbial content, could also be observed.

Energy dispersion spectroscopy

The Student's t-test showed that iron ($p<0.014$) and magnesium ($p<0.001$) were associated with the occurrence of periodontitis in the arches of the evaluated animals (Table 1). These results were confirmed by the Mann-Whitney test ($p<0.025$ and $p<0.001$, respectively). Likewise, the carbon content inside the pigmented calculus and biofilm was higher than that observed in non-pigmented samples (t-Test, $p<0.039$). Other elements such as manganese (Mann-Whitney test, $p<0.007$) and iron (Mann-Whitney test, $p<0.015$) were also associated with the presence of pigmented biofilm (Table 2). The Spearman's correlation test showed that calcium content increased with increased phosphorus levels, and iron content increased with increased silicon levels, irrespective of the presence of pigmentation in the calculus and dental biofilm or periodontal condition (Table 3).

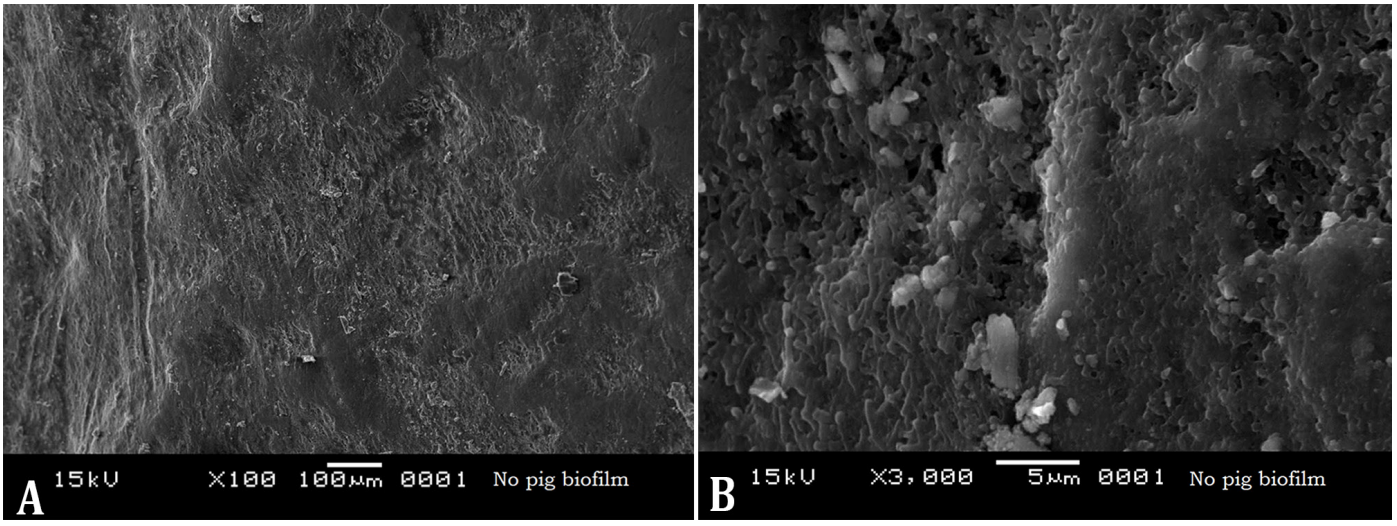


Fig.2. (**A**) Ultrastructure of the non-pigmented supragingival biofilm of bovine. Magnification = 100x. (**B**) Surface structure of the biofilm with irregular areas similar to mineral deposition (calcium hydroxides and other elements). Magnification = 3000x.

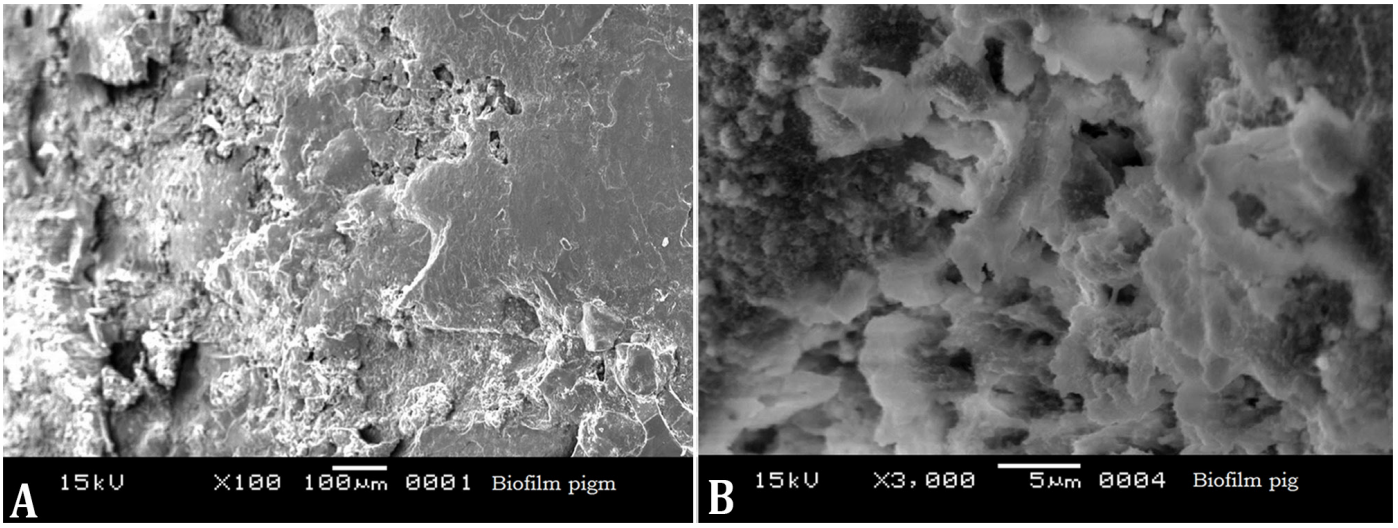


Fig.3. (**A**) Ultrastructure of pigmented supragingival biofilm of bovine. Magnification = 100x. (**B**) Surface structure of the pigmented biofilm with irregular areas, high mineral abundance (hydroxides of calcium and other elements), and high structural complexity. Magnification = 3000x.

Table 1. Significance level of the main chemical elements identified by the energy dispersion spectroscopy technique in the supragingival biofilm of bovines with periodontitis, calculated using the Student's t-test

Element	Mean and stardard deviation		Level of significance
	Animals with periodontitis	Animals periodontally healthy	
C	37.0 ± 13.92	34.02 ± 14.36	0.643
P	18.14 ± 3.87	19.59 ± 5.61	0.438
Ca	35.55 ± 15.29	47.25 ± 16.88	0.057
Mn	1.67 ± 0.87	6.38 ± 17.17	0.517
Na	1.12 ± 0.27	3.79 ± 5.06	0.490
Fe*	28.7 ±24.22	6.64 ± 9.09	0.014
Mg*	3.78 ± 1.28	0.75 ± 0.46	0
K	1.39 ± 0.48	10.87 ± 13.37	0.148

* Statistically significant elements.

Table 2. Significance level of the main chemical elements identified by the energy dispersion spectroscopy technique in bovine teeth with black pigmented supragingival biofilm, calculated using the Mann-Whitney test

Element	Mean		Level of significance
	Non-pigmented teeth	Pigmented teeth	
C*	122.00	88.00	0.039
P	244.00	284.00	0.895
Ca	297.00	264.00	0.129
Mn*	67.00	123.00	0.007
Fe*	8.00	163.00	0.015
Mg	33.00	87.00	0.194

* Statistically significant elements.

Table 3. Major chemical elements detected by the energy dispersion spectroscopy technique in the bovine supragingival biofilm and their correlations with each other, calculated by the Spearman correlation test

Elements	C	P	Ca	Mn	Na	Fe	Mg	K	Si	Tl
C	1.00	-0.63	-0.63	0.13	0.31	-0.64	0.83	-0.37	-0.65	0.46
P	-0.63	1.00	**1.00**	-0.85	-0.93	-0.19	-0.96	-0.48	-0.18	-0.98
Ca	-0.63	**1.00**	1.00	-0.85	-0.93	-0.20	-0.96	-0.49	-0.18	-0.98
Mn	0.13	-0.85	-0.85	1.00	0.98	0.68	0.66	0.87	0.67	0.94
Na	0.31	-0.93	-0.93	0.98	1.00	0.53	0.79	0.77	0.52	0.99
Fe	-0.64	-0.20	-0.20	0.68	0.53	1.00	-0.10	0.95	**1.00**	0.39
Mg	0.83	-0.96	-0.96	0.66	0.79	0.10	1.00	0.21	-0.11	0.88
K	0.37	-0.48	-0.49	0.87	0.77	0.95	0.21	1.00	0.95	0.65
Si	0.65	0.18	-0.18	0.67	0.52	**1.00**	-0.11	0.95	1.00	0.38
Tl	0.46	-0.98	-0.98	0.94	0.99	0.39	0.88	0.65	0.38	1.00

Values in bold: maximum correlation = 1.00.

DISCUSSION

The development of the microbial biofilm is a relevant step in the implantation and evolution of periodontitis in ruminants (Borsanelli et al. 2015a, 2015b, 2017, Agostinho 2017, Campello 2017), a cause that limits productivity and animal welfare (Borsanelli et al. 2016). Although the current advances in the microbiological studies of periodontal diseases are significant, little is known about the nature, structure, and composition of the biofilm and calcified formations associated with it, such as the dental calculus in cattle.

Biofilm formation and development process, as described in human patients (Marsh et al. 2011, Bosshardt 2018, Marsh & Zaura 2017), depends on the nonspecific interaction between the bacterial cell surface and the components of the acquired film and the dental surface (Marsh et al. 2011), creating conditions for more intense and specific interactions between microbial adhesins and their receptors in the host (Socransky & Haffajee 2002, Marsh et al. 2011). In addition to these microbial and host cellular components, the composition of the liquid environment is also relevant (Malamud 1985), and oral conditions imply salivary composition, which results from the diet (Kolenbrander et al. 2010, Fialová et al. 2017). It is particularly relevant to highlight that in ruminant animals the chemical and microbial content of the rumen interacts

with the oral counterparts (Myer et al. 2015). Among the microbial adhesins, the role played by fimbriae and other appendicular structures deserves attention (Marsh et al. 2011), especially among the main periodontal pathogens, such as the genus *Porphyromonas* (Socransky & Haffajee 2005, Nagano et al. 2018).

The absorption of calcium and phosphate from the saliva and the consequent mineralization of the biofilm result in the formation of the supragingival calculus, which manifests itself in layers that are differentiated by mineral content (Friskopp & Isacsson 1984). Structurally, dental calculus is covered by a layer of metabolically active microorganisms that accumulate in both periodontally healthy and diseased teeth (Friskopp & Hammarström 1980). In humans, the association of dental calculus with periodontal diseases is well established (Akcali & Lang 2017).

Calcium phosphate can be considered one of the most important components of dental tissue formation. Irregular crystallization of these inorganic constituent in biological tissues results in pathological conditions such as dental calculus formation (Dorozhkin & Epple 2002). In addition to the salivary flow rate, salivary supersaturation with calcium phosphate salts is a factor that favors calculus formation (Jin & Yip 2002). In the present study, calcium and phosphorus were correlated regardless of the animal's clinical condition or presence of pigmentation, as observed between iron and silicon (Table 3).

In humans, silicon is present in the saliva, biofilm, and dental calculus. A large part of this element is found in the form of silica in food (Jin & Yip 2002), and, at concentrations of 0–2mg/mL, it acts as a stimulant factor for the precipitation of calcium phosphate (Damen & Ten Cate 1989). When incorporated into the diet, silica may increase the rate of dental calculus formation (Gaare et al. 1989), probably because of the ability of calcium to bind to silicic acids and silica (Jin & Yip 2002). The food factor was not evaluated in the present study, and the findings associated with this element need to be further investigated.

SEM images of the supragingival calculus in human patients are characterized by the presence of heterogeneous content covered by filamentous microorganisms (Friskopp & Hammarström 1980). In bovines, the images of the calculus covered by supragingival biofilm (pigmented and non-pigmented) indicated the presence of structural formations with irregular areas, possibly resulting from mineral deposits similar to those described by Salles et al. (2012). These irregular deposits appear in greater abundance in pigmented samples. Cocoid and bacillary morphology structures were also observed, which suggests the presence of microbial content covering the supragingival calculus.

During its initial development, biofilm increases in thickness and microbial co-aggregation networks and physiological interconnections are established between the bacterial components of the biofilm, independently of the host species and even in *in vitro* studies (Sanguansermsri et al. 2017, Neilands et al. 2019). Favorable conditions are therefore created for the establishment of complex microbial communities (Lang et al. 2010). According to Jin & Yip (2002), the mineralization of this biofilm is a natural consequence of the physical and chemical aspects present in the mouth, in which saliva and diet provide mineral elements such as calcium and phosphorus, which are a large part of the deposited mineral content (Table 2).

The semi-quantitative analysis using EDS indicated a strong correlation of the presence of iron and magnesium with the occurrence of periodontitis. Iron is an essential element for the growth and metabolic function of most living organisms, acting at different stages of cellular catabolism and anabolism, such as redox reactions, oxidative radical transport, DNA synthesis, and free radical scavenging mechanisms (Lewis 2010). However, the focus of the present study was the role of iron as an element that controls the expression of numerous microbial virulence factors, potentiating damage to host tissues (Schaible & Kaufmann 2004). In this context, some of the major oral microorganisms, such as the genera *Porphyromonas* and *Prevotella*, accumulate iron derived from gingival and periodontal bleeding as little water-soluble compounds with dark pigmentation (Smalley et al. 2003) as was observed in the bovine microbial biofilms, especially in animals with periodontitis. This relationship between iron, calculus pigmentation and oral biofilm, and presence of bone loss in cattle was also observed in the present study (Table 1 and 2).

With the establishment of the inflammatory framework in the insertion periodontal tissues, the amount of iron bound to blood proteins present in the gingival fluid increases significantly, so bleeding related to periodontal destruction provides hemin resulting from the hemolysis process and may modify the microbiota interaction (Lewis 2010) to act as a protective molecule in oxidative stress (Smalley et al. 1998).

Since 76% of iron available to mammals is stored as heme groups in oxygen-carrying cells such as hemoglobin and myoglobin, the major microorganisms associated with periodontal infections eventually associate the expression of proteases and peptidases with the presence of iron (Lewis 2010, Byrne et al. 2013), as well as surface receptors and transport proteins (Shoji et al. 2010). This constitutes a strategy to obtain iron besides acquiring the amino acids that will be used in its fermentative metabolism, which ends up releasing several toxic compounds (Olczak et al. 2005); capable of inhibiting cell proliferation and tissue repair, besides presenting direct toxic action on host cells (Lewis 2010).

As the oral biofilm of different host species presents bacteria genera capable of intense proteolytic metabolism and iron deposition, besides being involved with the periodontal destruction (Lewis 2010, Byrne et al. 2013), it can be assumed that these microorganisms are involved in the formation of these deposits and in the observed periodontal destruction.

Previous studies by the same research group have shown that bacteria of the genera *Porphyromonas* and *Prevotella*, such as *Porphyromonas gingivalis*, *Porphyromonas endodontalis*, *Prevotella melaninogenica,* and *Prevotella buccae*, which require hemin supplementation for *in vitro* growth, are among the main microorganisms detected in periodontal pockets of cattle and other ruminants (Dutra et al. 1986, Borsanelli et al. 2015b, 2017, 2018). This association between the genera *Porphyromonas* and *Prevotella* and periodontitis in cattle has been discussed, and Borsanelli et al. (2015b) showed that the presence of *P. endodontalis, P. melaninogenica*, and *Prevotella intermedia* was much higher in animals with periodontal bone loss than in periodontally healthy animals.

In addition to iron, the semi-quantitative analysis using EDS revealed an association between magnesium and periodontitis, already reported as one of the elements in human dental calculus (Fialová et al. 2017). Magnesium is an essential element for bacterial metabolism, which directly interferes with cell multiplication rates of a wide variety of species (Gadd 1992). Magnesium deficiency can lead to degradation of the ribonucleoprotein complex and it is associated with loss of cell viability (Walker 1994). However, the metabolic activity of magnesium in the dental biofilm and its relation to periodontitis in cattle is still unknown.

The semi-quantitative analysis also showed that carbon, manganese, and iron were significantly correlated with tooth pigmentation. Most of the pathogenic bacteria are heterotrophic and use carbon, which is present in a large variety of substrates, as a source of energy (Muñoz-Elías & McKinney 2006). It is possible that the iron deposits present in the pigmented calculus and biofilm samples are linked to organic compounds, rich in carbon, which would justify the association of these elements, as noted in the present study.

Manganese is an important element in bacterial metabolism that, as well as iron, also acts to protect oxidative stress. In addition, this element is involved in other functions during infection, for example, in regulating the expression of genes associated with virulence. In its non-enzymatic form, manganese is important to maintain bacterial viability in aerobic environments, among other activities (Zaharik & Finlay 2004, Lewis 2010). Like magnesium, the function of manganese in the dental biofilm of cattle and other species is little known.

Black pigmented supragingival biofilm has been associated with a higher degree of gingival recession in dairy goats. In the same study, the sites with major lesions were in the masticatory teeth (Campello 2017). Agostinho (2017) reported similar findings between presence of supragingival biofilm and gingival recession in sheep and also noted that older animals presented larger amounts of these microbial deposits. Although these deposits may also occur in young animals and not always linearly, these associations may indicate that the accumulation of supragingival biofilm acts as an important risk factor for the development and progression of periodontitis in small ruminants.

In the subgingival biofilm of bovines with periodontitis, it was possible to demonstrate similarities in the identification of some periodontopathogens already recognized in human periodontitis (Borsanelli et al. 2015a, 2015b, Borsanelli 2017). Little is known about the microbiological composition of the supragingival biofilm of cattle, but the associations found in the present study may indicate an important role of the microbiota residing in these formations and of their chemical metabolites in the triggering and pathogenesis of this disease in cattle.

CONCLUSIONS

The structures formed on bovine dental surfaces corroborate the existence of a biofilm of microbial origin, which mineralizes and is structured into a supragingival calculus.

The dark pigmentation on the dental surface of cattle probably results from the deposition of iron in the supragingival biofilm of these animals, since some oral microorganisms have their metabolism deeply linked to the metabolism of this element.

The association between the presence of iron and magnesium in the supragingival biofilm and the occurrence of periodontitis in cattle may indicate an important role of the supragingival microbiota and its metabolites in the etiology of bovine periodontitis.

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Welfare and locomotor system disorders in active draft horses (cart horses)[1]

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ABSTRACT.- Prado V.C.M., Hage M.C.F.N.S & Dória R.G.S. 2019. **Welfare and locomotor system disorders in active draft horses (cart horses).** *Pesquisa Veterinária Brasileira 39(12):942-948.* Departamento de Medicina Veterinária, Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo, Avenida Duque de Caxias Norte 225, Pirassununga, SP 13635-900, Brazil. E-mail: vivian.cmprado@gmail.com

Horses performing tasks is a common scene in urban centers, evidenced by the significant number of animals pulling carts along the streets (cart horses). Despite the rising concern of the population for animal welfare, as well as the creation of laws that prohibit traction activity in several Brazilian municipalities, these attitudes must be based on the impact of such activity on equine health. Locomotor system disorders are commonly observed in animals of this species, and become even more frequent when they are submitted to unhealthy working conditions. In this context, we conducted a survey on locomotor system disorders in 11 active draft horses. To this end, we performed lameness assessments and radiographic and ultrasound examinations. Information on management regarding the horses' hooves, type of harness used, and type of work performed was also obtained through the application of questionnaires to the owners. The collected data showed that, although most of the animals in the study presented critical orthopedic conditions, they were not treated and the horses did not rest for adequate time. We observed that the disorders, which are often chronic, may be directly associated with incorrect management of the animals. We emphasize the importance to disseminate information that values the welfare of traction animals in order to tackle mistreatment.

INDEX TERMS: Animal well-being, locomotor system, draft horses, cart horses, lameness assessment, horses.

RESUMO.- [**Bem-estar e afecções do aparelho locomotor de equinos de tração (carroceiros) em atividade.**] Cavalos desempenhando tarefas para o homem é ainda uma cena marcante nos centros urbanos, evidenciada pela grande quantidade de animais que tracionam carroças pelas ruas (carroceiros). Apesar da crescente preocupação da população com o bem estar animal, assim como a criação de leis que proíbem a atividade de tração em diversos municípios brasileiros, é imprescindível que estas atitudes estejam embasadas no impacto de tal atividade sobre a saúde dos equinos. Nesta espécie, afecções do aparelho locomotor são comuns, tornando-se ainda mais frequentes quando os animais são submetidos a condições insalubres de trabalho. Por conseguinte, este estudo promoveu um levantamento das afecções que acometem o aparelho locomotor de 11 equinos de tração em atividade, por meio de exames de claudicação, radiográficos e ultrassonográficos. Informações sobre manejo realizado pelos proprietários quanto aos cascos de seus animais, tipos de arreios utilizados e tipo de trabalho ao qual eram submetidos foram obtidas através de questionários. Verificou-se ao relacionar dados coletados, que a maioria dos animais do estudo apresentava afecções ortopédicas importantes, e que, entretanto, não eram tratados ou mantidos em repouso por tempo adequado. Observou-se que as afecções encontradas, muitas vezes crônicas, podem ter relação direta com o manejo incorreto dos animais, e ressalta-se a importância da disseminação de informações que prezem pelo bem estar do animal de carroça, a fim de combater maus tratos nesta categoria animal.

TERMOS DE INDEXAÇÃO: Bem estar animal, afecções de aparelho locomotor, equinos de tração, carroceiros, exame de claudicação, cavalos.

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INTRODUCTION

Since their domestication, horses have been used by humankind in various types of work and athletic activities. They are often demanded beyond their natural limits, despite growing awareness and claims for improvement in equine welfare (Fraser 2001, Goodship & Birch 2001). In particular, horses used as traction forces for carts laden with rubbish and rubble produced by the population within urban centers should be monitored for their well-being and health, as they still represent an essential work tool in society (Rezende 2004).

The athletic characteristics presented by an animal are the product of several factors to which it is subjected, such as climate, management, training, type of harness, work surface and genetics (Jones 1987), and the performance of animals also reflects the excellent condition of its locomotor apparatus (Maranhão et al. 2006). Locomotor system disorders were one of the leading clinical findings in the draft horses involved in recent animal studies conducted in Pakistan (Hameed et al. 2016) and Ethiopia (Mekonnen & Channe 2016), in which almost all of the animals studied, active traction mules, presented limitations of use in relation to their orthopedic disorders.

Inflammatory and degenerative orthopedic disorders lead to functional disability in horses, reducing their performance, which combined with the high costs of therapeutic procedures, impact mainly the poorest population, who cannot afford veterinary assistance to their horses, considered an essential work tool (De Bari et al. 2001, Souza 2006). Although the quality of trimming and shoeing is vital in active horses, in the case of draft horses (cart horses), a high prevalence of foot imbalance has previously been demonstrated, probably due to inadequate techniques used in the manufacture of rubber horseshoes, whose non-uniform thickness causes unequal distribution of limb forces during movement (Maranhão et al. 2007). In addition, the excess load to which a horse is subjected causes poor weight distribution between the limbs and hooves, favoring the onset of locomotor system disorders, since there is no adequacy of traction vehicles to the horse's gait (Faria et al. 2014, Mariz et al. 2014).

Currently, the use of draft horses in rural areas as an economic alternative has been widely discussed (Maranhão et al. 2006), but there are few reports on the use of these animals in the urban environment the literature, and the existing ones do not assess the correlation between the health and working conditions of these animals. Rio de Janeiro was the first Brazilian state to prohibit the use of animal traction vehicles in urban centers (State Law no. 7,194; January 7, 2016) (Rio de Janeiro 2016). Other localities have also followed the same principle, prohibiting, regulating or reducing the number of animal traction vehicles through legislation such as Municipal Law no. 11,468 of December 29, 2011 (Londrina 2011), Municipal Law no. 10,531 of September 10, 2008 (Porto Alegre 2008), Municipal Law no. 13,908 of December 19, 2011 (Curitiba 2011), Municipal Law no. 8,122 of October 17, 2018 (Araçatuba 2018), Municipal Law no. 9,411 of December 18, 2018 (Belém 2018), among others.

This study aimed to evaluate the locomotor system disorders in active draft horses (cart horses) in the municipality of Pirassununga, Sao Paulo state, and observe their relationship with the type of work performed and trimming, shoeing and harnessing in this population with a concern for animal welfare.

MATERIALS AND METHODS

The study sample was composed of 11 lame horses, six geldings and five mares, aged 15 years (±7.28) on average, with mean weight of 331kg (±28.87), which performed physical activity pulling carts in the municipality of Pirassununga, Sao Paulo state, Brazil. All animals were linked to the "Projeto Carroceiro" (Wagoner Project) of the "Faculdade de Zootecnia e Engenharia de Alimentos", "Universidade de São Paulo" (FZEA-USP).

After conducting physical examination and lameness assessment, anamnesis data were collected through questionnaires applied to the horses' owners. Radiographic and ultrasound examinations of the musculoskeletal structures were performed, as indicated by the lameness examination. All lameness assessments were performed by the same veterinarian with experience in the field. Diagnostic imaging examinations were performed by an experienced examiner (M.C.F.N.S.H.) and observed by an inexperienced evaluator (V.C.M.P.).

The lameness examination was performed according to Stashak (2002), and consisted of observation of the animal at rest and during exercise, palpation and systematic manipulation of the limbs, followed by flexion tests and perineural anesthetic blocks. Any changes in anatomy, painful response to palpation/manipulation, as well as worsening of lameness in response to flexion tests were recorded. The radiographs were taken as recommended by O'Brien (2006) and were obtained using 100mA, 90kV portable X-ray equipment with a rotating ampoule. Ultrasonography was performed according to the examination protocol described by Genovese et al. (1986) using an Esaote® MyLab™ ClassC Vet device equipped with a high-frequency linear transducer (LA 435 6-18MHz).

The questionnaire applied to owners included questions regarding the type of work performed by the animal, traction load, work frequency, hoof handling such as trimming and shoeing (who harnesses the horse, how often it is harnessed, and what material is used in shoeing), and type harness (neck collar or breast strap) used. Information on previous lameness and treatment was also collected.

RESULTS

All horses examined were lame and fully performing working activities. Ten different locomotor system disorders were identified and divided by similarity into five groups: Group 1, tissue lesions such as tendonitis and firing, effusion and adhesion of the sheath; Group 2, degenerative joint disease (DJD) and joint effusion; Group 3, conditions caused by excess load on the metacarpophalangeal and/or metatarsophalangeal joints, such as hyperextension and proximal sesamoid bone lesions; Group 4, fractures; Group 5, laminitis with distal phalanx rotation.

The following percentages of locomotor system disorders were observed: 63.6% (7/11), Group 1; 45.4% (5/11), Group 2; 45.4% (5/11), Group 3; 18.2% (2/11), Group 4; 9% (1/11), Group 5. Thus, 36.4% of the animals (4/11) were included in one lesion group, 45.4% (5/11) were in two injury groups, and 18.2% (2/11) were in three lesion groups (Table 1; Fig.1).

None of the owners claimed to notice lameness, medicate their animals, or dismiss them from work. The vast majority of the owners associated lameness with the type of gait of their animals.

Among the draft horse owners that responded to the questionnaire (n=9), 22.2% (2/9) did not provide their animals with any type of trimming or shoeing and 77.8% (7/9) of them did. According to the owners, 44.4% (4/9)

Table 1. Equine locomotor system disorders according to group and diagnostic tool used (ultrasound or x-ray)

Animal	Group	Diagnostic tool	Locomotor system disorder
A*	1/3	US	(TLs) Chronic tendonitis reactivated in flexor tendons. (RTL) Acute tendonitis in SDFT. Periostitis in MPSB. (LTL) Adhesion between SDFT and tendon sheath with sheath effusion.
B	1/3	XR	(LPL) LPSB and MPSB periostitis.
		US	(LPL) Chronic tendonitis reactivated in flexor tendons and sheath effusion.
C	1/2/4	XR	(LPL) Tibia avulsion fracture. Degenerative joint disease (DJD) in intertarsal joints.
		US	(RTL) Chronic tendonitis reactivated in flexor tendons and sheath effusion. Chronic tendonitis in DDFT. (LTL) Chronic tendonitis reactivated in SDFT. Annular ligament desmitis. (RPL) Adhesion between SDFT and DDFT and tendon sheath effusion. (LPL) Adhesion between SDFT and DDFT and tendon sheath effusion. Annular ligament desmitis.
D	2	XR	(LPL) Osteophyte in the insertion of the medial collateral ligament of the proximal interphalangeal joint (DJD) and joint effusion.
E	2	XR	(RPL) Osteophyte in the 3rd metatarsal bone (DJD).
F*	5	XR	(LTL, RPL) Rotating laminitis of the 3rd phalanx.
G	1	US	(RTL) Laceration of the radial carpal extensor tendon and carpal sheath, with sheath effusion.
H	1/3	US	(RPL) Acute tendonitis in SDFT and tendon sheath effusion. Periostitis in LPSB and MPSB. (LPL) Acute tendonitis in SDFT and tendon sheath effusion.
I	1/2	XR	(LPL) Osteophyte in proximal interarsal joint (DJD) and joint effusion.
		US	(RTL) Chronic tendonitis reactivated in flexor tendons with sheath effusion. Annular ligament desmitis.
J	2/3/4	XR	(LTL) Osteophytes in the insertion region of the 1st phalanx sesamoid ligaments (DJD). Flexural deformity (hyperextension) of the metacarpophalangeal and distal interphalangeal joints. Periostitis in the 3rd and 4th metacarpal bones. (RTL) Periosteal reaction in the radius, 3rd metacarpal, ulnar, intercarpal and carpal bones (DJD). Joint effusion. (LPL) LPSB avulsion fracture. Flexural deformity (hyperextension) in the metatarsophalangeal joint.
K	1/3	US	(RTL) Chronic tendonitis reactivated in SDFT and DDFT. Ligament check desmitis. Periosteal reaction in MPSB. (LTL) Chronic tendonitis reactivated in flexor tendons. Periostitis in MPSB and LPSB.

* Animals whose owners did not respond to the questionnaire; 1 = tendonitis, desmitis, adhesion and effusion of the tendon sheath, 2 = degenerative joint disease and joint effusion, 3 = hyperextension and injuries to proximal sesamoid bones, 4 = fractures, 5 = laminitis, US = ultrasound, XR = x-ray, TLs = thoracic limbs, RTL = right thoracic limb, LTL = left thoracic limb, RPL = right pelvic limb, LPL = left pelvic limb, SDFT = superficial digital flexor tendon, DDFT = deep digital flexor tendon, MPSB = medial proximal sesamoid bone, LPSB = lateral proximal sesamoid bone, DJD = degenerative joint disease.

of the draft horses underwent only manual trimming. The horseshoes, made from truck rubber tires, were fixed to the hooves of the horses by their owners, using nails and a hammer. The handmade horseshoes were fixed back into place when they detached from the hooves, and were replaced with new ones only when thoroughly worn. Also according to the owners, 33.3% (3/9) of the draft horses were provided with proper farrier trimming and conventional metal horseshoes, which were refit when detached from the hooves and replaced when worn. All owners reported that in the time interval between horseshoe (rubber or metal) detachment and refit or replacement, the animals remained active, with some hooves shoed and others not.

Anamnesis showed that the animals worked 29h (±16.1h) per week (five working days) on average. Percentage distribution by weekly working hours was as follows: 22.2% (2/9), 10h; 11.1% (1/9), 20h; 22.2% (2/9), 30h; 22.2% (2/9), 40h; 22.2% (2/9), over 40 h. Common loads carried by the animals included rubble (6/9, 66.7%) and people (3/9, 33.3%). Regarding the type of harness, 77.8% (7/9) used neck collar and 22.2% (2/9) used breast strap.

DISCUSSIONS

According to the animals' history, lameness assessments and images examinations, it was possible to identify different locomotor disorders in the draft horses of this study, and they could be associated with the data obtained on the type of work performed, type of harness used, and hoof care. It is worth noting that all animals were under full work activity despite the disorders that affected them - a finding also observed in similar studies (Hameed et al. 2016, Mekonnen & Channe 2016).

Tendonitis, especially chronic tendonitis, was the disease most commonly found among the animals, accounting for 63.6% (7/11) of those examined, suggesting that acute tendon injuries were not correctly treated and the rest period was not respected, making the injuries chronic. Resting for at least two months is recommended for the treatment of horses with tendonitis at any stage of evolution, according to Thomassian (2005). Even with traditional therapies, the healing of a tendon injury can extend over long periods of a horse's life until it is resolved entirely (Maia 2008). Tendon injuries are justified in the animals under study, as they are associated with great efforts (McIlwraith 2006), as found by

the questionnaires, which also showed that the work of these animals is intense, pulling heavy loads for long hours daily (6±3h per day, five days per week, on average), as observed by Pritchard et al. (2005).

Synovial fluid effusion in tendon sheaths was associated with lesions in equine tendon or ligament structures, characterizing tenosynovitis, diagnosed in 45.4% (5/11) of the evaluated animals. This may be the result of injuries caused by excessive exercise that sometimes causes hyperextension of the metacarpo/metatarsophalangeal joints, especially when they are subjected to excess load (Gomide et al. 2004), as in the case of the animals under study. Likewise, as a consequence of tendon injuries, 18.2% (2/11) of adhesion between tendons and adjacent structures and 9% (1/11) of annular firing were found, with pain and severe lameness (Dik et al. 1995, McIlwraith 2006). Treatment of these conditions is usually

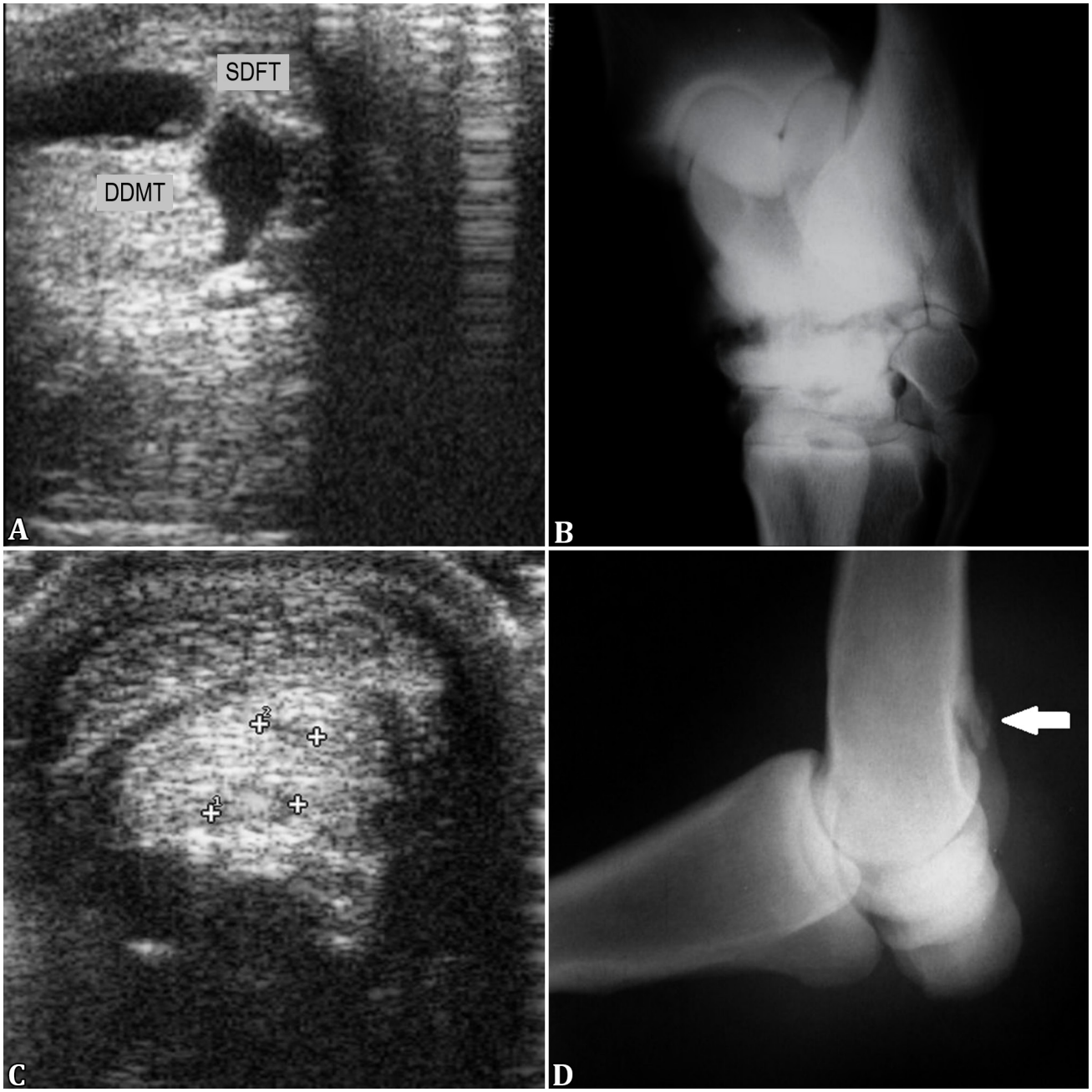


Fig.1. Illustration of injuries found in the locomotor system of the draft horses studied. (**A**) Adhesion and effusion between the superficial digital flexor tendon (SDFT) and deep digital flexor tendon (DDFT) of the left pelvic limb, visualized in the transverse ultrasound image of the proximal plantar region. (**B**) Decrease in radiographic interline caused by osteolysis of the subchondral bone that composes the proximal and distal intertarsal joints, with adjacent sclerosis visualized in dorsolateral-plantaromedial radiograph. (**C**) Two chronic (inter-crossed) cross-sectional lesions in the DDFT of the right thoracic limb in the transverse ultrasound image. (**D**) Bone fragment with rounded contours (arrow) in the distal third of the 3rd left metatarsal bone, observed in lateral radiograph, suggestive of avulsion fracture of the lateral proximal sesamoid bone and flexural deformity in the metacarpophalangeal joint.

surgical, followed by intensive postoperative physiotherapy, which was not performed in any of the animals under study considering that that they were active.

Lesions to the proximal sesamoid bones were found in 45.4% (5/11) of the animals under study, and an avulsion apex fracture of the proximal sesamoid bone and four other periosteal reactions in these bones were diagnosed at the point of insertion of the lateral and medial suspensory ligament branches. The suspensory apparatus consists of the suspensory ligament of the metacarpal bone and its branches, in addition to the proximal sesamoid bones and their ligaments (Denoix 1994). These structures are subject to injuries, mainly due to overload from exhaustive physical activities, such as pulling excess load (Pool & Meagher 1990), as well as hoof imbalance (Gibson & Steel 2002). If treated, apex fractures of the proximal sesamoid bones may have a good prognosis (Woodie et al. 2000), as well as firing of the lateral and medial branches of the suspensory ligament, usually associated with periostitis at its insertion site in the proximal sesamoid bones (Gibson & Steel 2002). However, in the animals of this study,, no treatment had been performed, and they remained active.

As a consequence of local trauma, failure in joint stability and chronic osteoarthritis (McIlwraith 2002, Park 2014), it was possible to verify the presence of degenerative joint disease (DJD) in 54.5% (6/11) of the animals; this disorder is almost always associated with findings such as osteophytes, periosteal reaction, and joint effusion. Upright defects, angular and flexural deformities, unbalanced and firing sheath, and overweight or joint overload are the causes of DJD, which most often progresses to loss of joint function and pain (Veiga 2006). Considering the data obtained from the questionnaires, it is evident that the draft animals (cart horses) physically exercise under inadequate conditions, which resulted in the numerous injuries observed.

Laminitis was diagnosed in one animal, which presented 4 and 5 degree rotation in the distal phalanx of the left thoracic and right pelvic limbs, respectively. Pathogenesis of this condition involves mechanical overload of the hooves when subjected to intense impact from exercise on hard soil (Baxter 1994). This condition leads to intense pain, which makes it impossible for the animal to develop any physical activity with effort (O'Grady 2010). In this case, although the animal was limping, it was still active.

The data obtained showed that the pelvic limbs of the studied animals presented larger number of lesions (14) compared with the thoracic limbs (10). Thus, it is evident that there is greater stress on the pelvic limbs of draft horses, which are overloaded when they have to pull carts with heavy loads, because its center of balance is usually displaced closer to the thoracic limbs (Ross 2003, Moyer & Carter 2007). This fact corroborates the evidence that draft horses (cart horses) are submitting their locomotor system to activities that they are not physiologically adapted, predisposing the onset of injuries, which opposes ethical and animal welfare aspects.

As for trimming and shoeing, responses to the questionnaires showed that 66.7% of the horses were not submitted to the proper procedures and two animals worked without horseshoes. It is worth noting that handmade horseshoes manufactured by animal owners using tire rubber do not follow the basic principles of trimming and shoeing, which consider the biomechanics of the locomotor system and hoof balance in order to minimize orthopedic injuries and lameness (Stashak et al. 2006). Owners claim they use this material, which can be easily obtained and handled, in order to prevent slipping on urban asphalt caused by lack of adherence of traditional metal horseshoes (Yxklinten 1996). These findings stress the need for studies addressing the benefits of trimming followed by shoeing with horseshoes made of rubber, respecting the anatomy and biomechanics of equine limbs.

Moreover, fitting with handmade horseshoes by owners who are unaware of hoof anatomy and shoeing techniques favors technical errors such as puncture wound, that is, perforation by misplaced nails in vascularized hoof tissue, which can lead to severe and potentially fatal conditions, such as tetanus (Cullinane et al. 1999).

It was also observed that the draft horses assessed lack periodic hoof trimming and had their horseshoes replaced much less frequently than recommended, which are factors that predispose orthopedic injuries because of growing unbalance of the hooves, compromising the movement of the animals (Stashak et al. 2006). Furthermore, there was the fact that the animals remained working with some hooves shoed and others not when detachment occurred. Sporting animals with unbalanced hooves are predisposed to conditions such as arthritis, osteitis, desmitis and tendonitis due to irregular distribution of forces between bone, tendon, and ligament structures (Balch et al. 1995).

Although animals are used as a means of work, special attention is not paid to maintaining the health of the locomotor system, which is essential to prevent the occurrence of orthopedic injuries, ensuring the welfare of horses in service.

Assessment of the type of harness showed that the animals that used neck collar presented a larger number of orthopedic injuries (average of two conditions per animal) compared with those that used breast strap (average of 1.57 conditions per animal). According to Hovell (1998), a good harness is one that allows the animal to apply its full tractive force to itself, whether a neck collar or a breast strap. However, it is known that some types of equipment, such as neck collars, can be designed to compress the jugular veins of the animal, rendering ineffective in its function because it does not allow full utilization of the horse's tractive force (Proust 1991), which unnecessarily tries harder to perform its tasks. Therefore, the use of breast straps should be encouraged among wagoners.

Regarding working time, it was observed that most animals worked continuously and intensively 6 (±3) hours per day on average, five days a week, and that 44% of them work 8 h a day or more, five days a week. This finding includes cart horses in the category of sport animals (Ross 2010), justifying hoof health care and use of proper harness, and emphasizes the need to evaluate the full load carried so that it is not excessive for the working time performed. Likewise, aspects such as adequate nutrition, body score, age, and hygiene of these draft horses should be considered and the type and time of work performed should be identified. This should be the individual suitability required for a group of animals to perform their activities, that is, the welfare of each animal, which would reflect in smaller occurrence of disorders, including orthopedic conditions.

Results of this study showed that most of the observed locomotor system disorders were associated with excessive physical effort, inadequate hoof management, and lack of early treatment of orthopedic conditions, that is, a lame animal should be immediately evaluated by a veterinarian, dismissed from its activities during treatment, and return to work only after discharge. This behavior would prevent acute disorders from becoming chronic and draft horses from working under pain and suffering, as evidenced in this study. Similarly, Ross (2010) reported the neglect of top-class horse owners and trainers, who often spare no competitions even when their horses clearly show lameness, reinforcing the enormous mental as well as physical strength that these animals should have in order to perform their tasks under non-ideal health conditions.

Horses with orthopedic injuries should not undergo intense and continuous work, as observed in this study. A horse in pain does not eat properly (Price et al. 2003), which favors weight loss and, consequently, decreased performance (Kronfeld 1993). This study highlights the need for improvements in the welfare of draft horses, which work inappropriately and are kept in service in poor health, negatively affecting their productivity. Prioritizing the welfare of these animals will considerably improve the quality of their work, leading to more significant revenue to their owners (Hameed et al. 2016).

Often, as governmental organizations are not structured to identify problems associated with animal traction, their data result unrepresentative or inadequate to actual animal conditions (Goe & McDowell 1980). We suggest that municipalities register and supervise wagoners in urban activity, and offer veterinary services that would be responsible for adequacy of the nutritional, sanitary and specific service capacity of each horse, as well as for offering medical care, authorizing the use of the animal only after discharge.

CONCLUSIONS

Traction horses (cart horses) perform daily physical activity even with major orthopedic injuries, especially chronic ones. Hard work associated with inadequate hoof management is the predisposing factor for the various orthopedic disorders found.

It is important to disseminate information that values the welfare of draft horses and emphasize the need of a competent veterinarian in the supervision of this activity in order to tackle common mistreatment to in this animal species.

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Spontaneous poisoning by *Talisia esculenta* in cattle[1]

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ABSTRACT.- Melo J.K.A., Soares G.S.L., Ramos T.R.R., Almeida V.M., Nascimento A.L.O., Silva Filho G.B., Chaves H.A.S. & Mendonça F.S. 2019. **Spontaneous poisoning by *Talisia esculenta* in cattle.** *Pesquisa Veterinária Brasileira 39(12):949-953.* Laboratório de Diagnóstico Animal, Universidade Federal Rural de Pernambuco, Rua Dom Manoel de Medeiros s/n, Dois Irmãos, Recife, PE 52171-900, Brazil. E-mail: fabio.mendonca@pq.cnpq.br

Talisia esculenta, commonly known as pitombeira, is a tree which fruits are widely consumed by human beings in northeastern Brazil. The aim of this work is to describe the epidemiological, clinical and pathological aspects of two outbreaks of spontaneous poisoning by *T. esculenta* in cattle in the dry region of Pernambuco, northeastern Brazil. The cases occurred in the municipalities of São Bento do Una and Belo Jardim. From a total of 25 adult cattle, eight become sick after ingest *T. esculenta* leaves and fruits. Four cattle died until 72 hours after the first clinical signs; which consisted in ataxia, reluctance to walk, tottering, head tremors muscle spasms in the limbs, rigidity of the pelvic limbs with wide base stance position, ruminal atony and, when stressed, presented falls and remained in abnormal positions. Two cattle were necropsied; the only significant finding was the presence of partially digested leaves, barks and seeds of *T. esculenta* in ruminal contents. Microscopically no lesions were observed. There is no specific therapy for poisoning by *T. esculenta* leaves. Prophylaxis consists in preventing cattle from gaining access to pasture areas containing the plant.

INDEX TERMS: Spontaneous poisoning, *Talisia esculenta*, cattle, poisonous plants, plant poisoning, ataxia, ruminants.

RESUMO.- [**Intoxicação espontânea por *Talisia esculenta* em bovinos**]. *Talisia esculenta,* popularmente conhecida como pitombeira, é uma árvore cujos frutos são amplamente consumidos por seres humanos no nordeste do Brasil. O objetivo deste trabalho é descrever os aspectos epidemiológicos, clínicos e patológicos de dois surtos de intoxicação espontânea por *T. esculenta* em bovinos no Agreste de Pernambuco, Nordeste do Brasil. Os casos ocorreram nos municípios de São Bento do Una e Belo Jardim De um total de 25 bovinos adultos, oito adoeceram após consumirem as folhas e frutos de *T. esculenta.* Quatro morreram em até 72 horas após a observação dos primeiros sinais clínicos; que consistiam em ataxia, relutância em caminhar, andar cambaleante, tremores de cabeça, espasmos musculares nos membros, rigidez dos membros pélvicos com posição de ampla base, atonia ruminal e, quando excitados, apresentavam quedas e permaneciam em posições anormais. Dois bovinos foram necropsiados e o único achado significante foi a presença de folhas, cascas e sementes parcialmente digeridas de *T. esculenta* no conteúdo ruminal. Microscopicamente não foram observadas lesões. Não existe terapia específica para a intoxicação pelas folhas de *T. esculenta*. A profilaxia consiste em evitar que bovinos tenham acesso às áreas de pastagem contendo a planta.

TERMOS DE INDEXAÇÃO: Intoxicação espontânea, *Talisia esculenta*, bovinos, plantas tóxicas, intoxicação por plantas, ataxia, ruminantes.

INTRODUCTION

Talisia esculenta (A. St.-Hil.) Radlk., commonly known as "pitomba" or "pitombeira", is a tree from 5 to 15 meters high (Fig.1) of the Sapindacea family, which produces fruits

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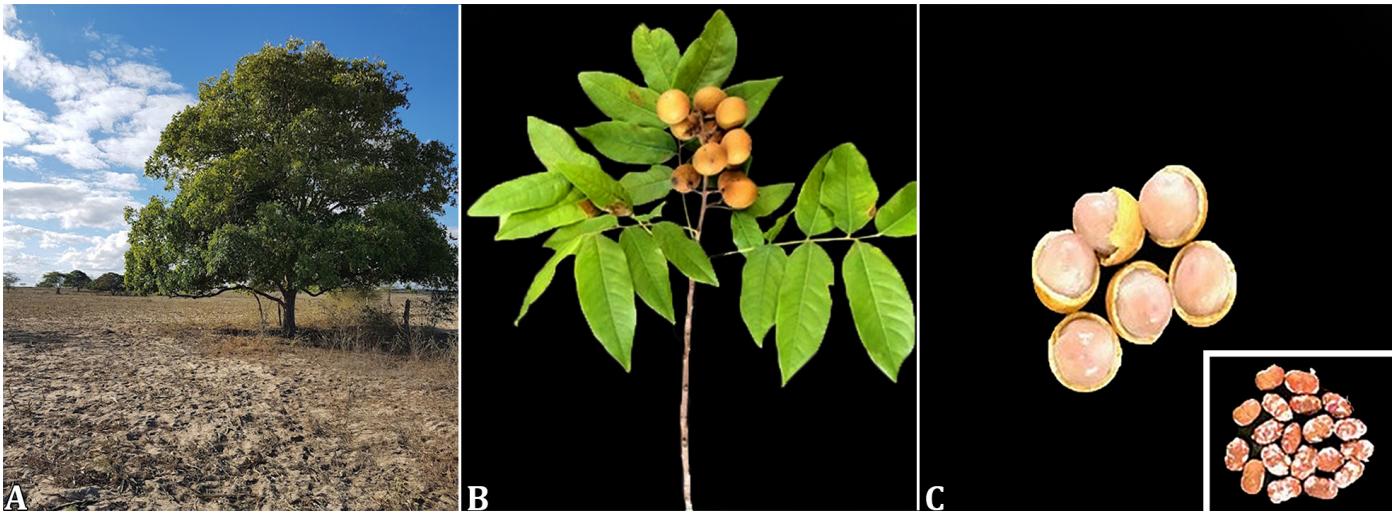


Fig.1. (**A**) *Talisia esculenta*, a tree in a dry grazing area of cattle. São Bento do Una, Pernambuco, northeastern Brazil. (**B**) Detail of branch and fruits. (**C**) Part of the barks were removed to show the pulp. Inset: brown seeds without pulp.

with up to 2,5cm in length, almost globular with oval brown seeds, which pulp is widely consumed in northeastern Brazil and other regions of the country. This species is found in almost all Brazilian territory, from the Amazon, from where it originates, to Rio de Janeiro and can also be found in other countries such as Paraguay and Bolivia (Guarim-Neto et al. 2003). It has flowering period from August to October with fruit ripening, in some regions, between January and March (Lorenzi 2002).

The use of the pitomba seed tea is used in Brazilian folk medicine as an astringent and the leaves are used with the intent to heal diarrhea, dehydration, and joint and kidney problems in humans (Guarim-Neto et al. 2003). However, despite consumption and use in folk medicine, there are no records of human poisoning because the seeds and leaves are not ingested *in natura*; just the pulp is normally consumed. However, outbreaks of poisoning in cattle, sheep and dogs that consumed seeds and/or leaves have recently been described (Riet-Correa et al. 2014, Mota et al. 2016, Almeida et al. 2018). The poisoning was also reproduced experimentally in sheep (Riet-Correa et al. 2014). The main toxin of *T. esculenta* seeds and leaves is still unknown, however, seeds and leaves contain talisin, a lectin that has hemagglutinating erythrocyte activity (Freire et al. 2003).

The aim of this work is to describe the epidemiological, clinical and pathological aspects of two outbreaks of spontaneous poisoning by *T. esculenta* in cattle in the dry region of Pernambuco, Northeast of Brazil.

MATERIALS AND METHODS

Epidemiological and clinical data of *Talisia esculenta* (Sapindaceae) poisoning in cattle were obtained during technical visits in the municipalities of São Bento do Una and Belo Jardim, Pernambuco, in northeastern Brazil.

In São Bento do Una municipality, eight crossbred dairy cattle presented an acute neurological clinical picture. In Belo Jardim, five cattle presented similar clinical neurological signs. From these outbreaks, three cattle were selected for a systematized nervous clinical exam, performed according to Riet Correa et al. (2002). Their general condition, appetite, color of mucous membranes, rectal temperature, heart and respiratory rates, form of abdomen, and rumen/reticulum motility were recorded. Rumen fluid was also collected to perform laboratory examinations. Examination of rumen fluid was performed according to Dirksen et al. (1993). The pH of the rumen fluid samples was measured at the time of sampling using pH indicator strips. The color, odor, appearance, sedimentation-flotation, reduction of methylene blue and protozoa activity was analyzed. Density, motility, live-dead ratio and predominance of protozoa were evaluated by direct microscopy (Dehority 1993).

To perform blood count tests, blood samples from each cattle were collected using a vacuum system through puncture of the jugular vein. These samples were stored in two 10 mL tubes, one with the anticoagulant ethylenediaminetetraacetate acid (EDTA) in a 10% aqueous solution and the other without anticoagulant. The serum was separated by centrifugation at 2.500rpm for 10 minutes and maintained at -20°C until analysis.

Two cattle were necropsied after spontaneous death. Samples of the CNS were obtained from the cerebrum, brainstem, cerebellum, diencephalon and spinal cord. Moreover, fragments of liver, kidney, heart, lung, spleen, rumen, reticulum, omasum, abomasum and intestines were collected, fixed in 10% formalin, processed routinely, stained with hematoxylin and eosin (HE).

Additionally, the cattle grazing areas were inspected in order to find poisonous plants and watch the feeding behavior of the herds.

RESULTS

The outbreaks occurred in São Bento do Una (8°31'20"S 36°26'37" W) and Belo Jardim (08º20'08"S 36º25'27" W), in the Ipojuca Valley, dry region of Pernambuco State, northeastern Brazil. The region has a semiarid climate type, with a mean annual rainfall of 890mm, an average annual temperature of 23.0°C and the rainy season is from March to July. The vegetation is composed by hyperxerophilic caatinga and subcaducifolia tropical forest.

The farms where the outbreaks occurred had less than 20ha and had similar management. The herds were left in a non-cultivated feedlot to graze because in the previous years, the region had a long period of drought and the farmers did

not prepared pastures for cattle feeding. In the morning the herds were left in areas with fresh water and containing *Talisia esculenta* trees, which were used for the cattle shading and in the afternoon, they were removed to stalls to receive chopped grass and commercial ration.

In São Bento do Una, from a total of ten adult crossbred Holstein cows, three were poisoned from May to June and from these, two died after presenting anorexia, head tremors and in less than 24 hours lateral recumbency and death. One cow with less severe poisoning presented anorexia, adipsia, instability to walk, head and limbs tremors, muscular spams, lateral gait, reluctance to walk and when forced to move presented falls; and after arising remained in wide base stance (Fig.2). The period of evolution until the total recovery was 7 days after removal of the pasture area. During the inspection of the grazing area a total of 15 *T. esculenta* trees in fructification period were found. The herd consumed the fruits directly from the ground and/or the leaves from the low branches of some trees (Fig.3).

In Belo Jardim five adult crossbred Nelore cattle, from a total of 15 animals in the herd, presented similar neurological clinical signs consisted in congested episcleral vessels, anorexia, ataxia, intense muscles tremors, mainly the head and ears, and constant muscular spasms of the hindlimbs, unusual stance positions and lateral gait followed by fallings to the ground. There was also ruminal atony with moderate bloat. After 24-72 hours two cattle died and were necropsied. At gross examination, the only finding was the presence of seeds and partially digested leaves in the rumen. No microscopically lesions were observed.

The main changes observed in ruminal fluid were decreased of methylene blue activity, and slight reduction of the density and motility of rumen microfauna. No hematological abnormalities were present.



Fig.2. (**A**) Cattle spontaneously poisoned by *Talisia esculenta* showing ataxia, mild bloat, (**B**) difficulty in walking and (**C**) remaining in wide base stance.



Fig.3. (**A**) Dairy cow consuming *Talisia esculenta* leaves and (**B**) seeds in a grazing area of cattle.

DISCUSSION

São Bento da Una and Belo Jardim are municipalities with a dry climate and like other municipalities in the region usually have prolonged drought periods, a factor predisposing to cases of poisoning due to toxic plants ingestion (Tokarnia et al. 2012). However, *Talisia esculenta* fruits are palatable and are easily consumed by ruminants; if disponible for consuming under the trees, when falling on the ground during fructifying period can cause poisonings outbreaks in ruminants.

Epidemiological and clinical data were similar those previously reports in sheep at Pernambuco (Almeida et al. 2018) and in cattle and sheep at Paraíba (Riet-Correa et al. 2014). The poisoning had acute evolution and cattle presented neuromotor clinical signs after 12 hours after ingestion of fruits and or leaves. However, the bloat with decreased ruminal movements, decreased methylene blue reduction test, and death of rumen protozoa as described in this study, suggest that the digestive system is also affected (Riet-Correa et al. 2014).

In a previous study, the poisoning was reproduced experimentally in five sheep by the administration of 30-60g of leaves/kg body weight and in two sheep with doses of 5 and 10g of seeds/kg (Riet-Correa et al. 2014). Toxic doses for cattle remain unknown. The toxic doses of the seeds were smaller than the toxic doses of the leaves, suggesting that the toxic compound is more concentrated in the seeds than in the leaves (Riet-Correa et al. 2014).

No gross or histologic lesions were reported in the sheep or cattle spontaneous or experimentally poisoned by the seeds or leaves of *T. esculenta* (Riet-Correa et al. 2014, Almeida et al. 2018). The diagnosis should be based on the history of ingestion of seeds or leaves of the plant and the absence of significant gross or histologic lesions. In another hand, the presence of seeds in the rumen content associated to neurological clinical signs has diagnostic value and must be considerate in the diagnosis of this poisoning.

Toxic principles of *T. esculenta* seeds remains unknown but several reports have shown that reserve proteins act as plant defense against insects and herbivores (Macedo et al. 2011). *T. esculenta* seeds contain talisin, a reserve protein that act as a storage protein, presenting lectin-like activities i.e. caracterized by a weakly hemagglutination inhibition of human and animal erythrocytes in high concentrations (starting from 500μg mL^{-1}) (Freire et al. 2003). In addition, talisin also displays protease-inhibitor properties, as bovine trypsin, and induces neutrophil and mononuclear cell inflammatory response by a mechanism related to specific protein-carbohydrate interactions (Freire et al. 2003, Macedo et al. 2011). These interactions could serve as major signals in some cells causing the release of proinflammatory mediators such as cytokines, nitric oxide and leukotrienes. Additionally, it was reported that talisin has an inhibitory effect of the carbohydrate's glucose and mannose in mice neutrophils and these activities may be related to different affinities of talisin for the glycosyl groups present on the cells surfaces (Freire et al. 2003).

The main differential diagnoses of *T. esculenta* poisoning are rabies, listeriosis, and botulism, which are common diseases in ruminants in Pernambuco (Riet-Correa et al. 2014). Other neurological poisonous plants, such as *Ipomoea* spp. and *Solanum paniculatum* are endemic in northeastern Brazil and should be considerate in the differential diagnosis (Antoniassi et al. 2007, Guaraná et al. 2011, Carvalho et al. 2014, Mendonça et al. 2018).

CONCLUSIONS

The seeds of *Talisia esculenta* are palatable and if consumed by cattle cause acute nervous signs.

The leaves are ingested by cattle due to scarcity of food and cause identical clinical signs.

Farmers should avoid planting this plant species in grazing areas of ruminants due to its toxicity.

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Conflict of interest.- The authors have no conflicts of interest to declare.

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Antimicrobial activity of propolis extract fractions against *Staphylococcus* spp. isolated from goat mastitis[1]

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ABSTRACT.- Dos Santos H.C., Vieira D.S., Yamamoto S.M., Costa M.M., Sá M.C.A., Silva E.M.S. & Silva T.M.S. 2019. **Antimicrobial activity of propolis extract fractions against *Staphylococcus* spp. isolated from goat mastitis.** *Pesquisa Veterinária Brasileira 39(12):954-960.* Universidade Federal do Vale do São Francisco, Rodovia BR-407, Lote 543, Projeto de Irrigação Nilo Coelho s/n, Petrolina, PE 56300-000, Brazil. E-mail: sandramariy@gmail.com

The indiscriminate use of antibiotics in the treatment of caprine mastitis causes the appearance of resistant microorganisms, besides leaving residues in milk, putting at risk to human health. In this way, propolis is an alternative in the treatment of diseases because it has antimicrobial activity, mainly because of the presence of flavonoids in its composition. The aim of this study was to evaluate the antimicrobial potential of propolis to *Staphylococcus* spp. Isolated from cases of goat mastitis and qualify the crude ethanoic extract by high performance liquid chromatography (HPLC). In this study, the minimum bactericidal concentration values of propolis extracts in ethanol, ethyl acetate and hexane showed that the best concentrations capable of promoting the highest mortality of the isolates of *Staphylococcus* spp. from mastitis in goats, were 6250, 3125 and 1562.5µg/mL, respectively. By the microplate adherence test, it was found that 20.78% isolates were not able to form biofilm, 14.70% were classified as moderate and 64.70% were weak and none as a strong biofilm producer. Propolis in its different diluents was able to affect the formation of biofilm and showed a pronounced marked antimicrobial activity against *Staphylococcus* spp. strains and may be indicated for use in *in vivo* studies.

INDEX TERMS: Antimicrobial activity, propolis extract, *Staphylococcus* spp., isolated, goat mastitis, caprine, mastitis, solvents.

RESUMO.- [**Atividade antimicrobiana de frações do extrato de própolis frente à *Staphylococcus* spp. isolados de mastite caprina.**] O uso indiscriminado de antibióticos no tratamento de mastite caprina leva ao desenvolvimento de micro-organismos resistentes que poderão estar presentes em alimentos, colocando em risco a saúde humana. Dessa forma, a própolis surge como uma alternativa para o tratamento de doenças por possuir uma ação antimicrobiana, principalmente pela presença de flavonoides em sua composição. O objetivo desse estudo foi avaliar o potencial antimicrobiano da própolis frente à *Staphylococcus* spp. isolados de casos de mastite caprina e qualificar o extrato etanoico bruto por cromatografia líquida de alta eficiência (CLAE-DAD). Neste estudo, os valores de concentração bactericida mínima (CBM) dos extratos de própolis em álcool etílico, acetato de etila e hexano nos isolados foram de 6250, 3125 e 1562,5µg/mL, respectivamente. Pelo teste de aderência à microplacas, observou-se que 20,78% dos microorganismos, não foram capazes de formar biofilme, 14,70% foram classificados como moderados, 64,70% em fracos e nenhum como forte produtor de biofilme. A própolis em seus diferentes diluentes foi capaz de afetar a formação de biofilme e apresentou significativa atividade antimicrobiana frente a cepas de *Staphylococcus* spp., podendo ser indicada para utilização em estudos *"in vivo"*.

TERMOS DE INDEXAÇÃO: Atividade antimicrobiana, extrato de própolis, *Staphylococcus* spp., isolados, mastite caprina, cabra, própolis, solventes, caprinos.

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INTRODUCTION

Mastitis, mainly in the subclinical form, lead to damages in milk production, which directly affects milk quality and reduces the industrial yield of dairy products. There is a wide range of microorganisms that can cause mastitis, and the most common pathogens found are *Staphylococcus* spp. (Sampimon et al. 2010, Sá et al. 2011, Leitner et al. 2012, Peixoto et al. 2015, Acosta et al. 2016).

Defense mechanisms of these microorganisms include the production of biofilm or slime, an extracellular matrix of polysaccharides which allows the interaction between microorganisms, making difficult the action of allopathic products (Clutterbuck et al. 2007, López et al. 2010). Biofilm formation thus contributes to the resistance to the products used in routine mastitis treatment. Therefore, the search for new therapeutic alternatives is extremely important. Beekeeping products have gained space in the therapy of various diseases (Pereira et al. 2002), such as propolis, that plays a fundamental role in combating resistant microorganisms. This ability may be related to its chemical composition (Dias et al. 2012, Silva et al. 2012), as well as lower toxicity rates associated to synthetic antimicrobials (Marcucci 1995).

Propolis may have considerable concentrations of phenolic compounds, such as flavonoids and aromatic acids, as well as different types of terpenoids, justifying several beneficial potentials like antimicrobial, antioxidant, anti-inflammatory and antitumor activities (Bankova et al. 2000, Sforcin & Bankova 2011, Valente et al. 2011). Some authors argue that the products present in propolis may change according to the season and the raw material obtained by bees (Bankova 2005, Popova et al. 2011). As an alternative product, some studies have compared the best solvent for its use and diversified applications. In this case, some of them indicated solvents are water, ethanol, combinations of two and products such as ethyl acetate and hexane (Miyataka et al. 1998, Vargas et al. 2004, Mello et al. 2010).

In this context, this study aimed to evaluate the antimicrobial effects of propolis in different solvents and fractions against *Staphylococcus* spp., from cases of mastitis in goats and qualify the crude ethanolic extract.

MATERIALS AND METHODS

Experiment location. The experiment was conducted at the Laboratory of Animal Microbiology and Immunology of the "Universidade Federal do Vale do São Francisco" (Univasf). Crude propolis was obtained from apiaries in the municipality of Casa Nova, state of Bahia. Subsequently, and sent to the Laboratory of Phytochemical Bioprospection of the "Universidade Federal Rural de Pernambuco" (UFRPE) for phytochemical analysis.

Phytochemical analysis of propolis extract. The propolis extract was subjected to a preliminary phytochemical analysis, aiming at the characterization of the main secondary metabolites. To analyze the potential of different fractions of propolis extract, 79.4g propolis was mixed with ethanol in a beaker for extraction in an ultrasonic bath. Then, the propolis extracts in ethanol extract (EtOH) was obtained, stock solution from which the partitions were made to obtain ethyl acetate (AcOEt) and hexane extracts from propolis, as shown in Figure 1.

Thus, the EtOH extract (41.7g) was suspended in MeOH: H_2O (1:1) and extracted with hexane and AcOEt. After separation,

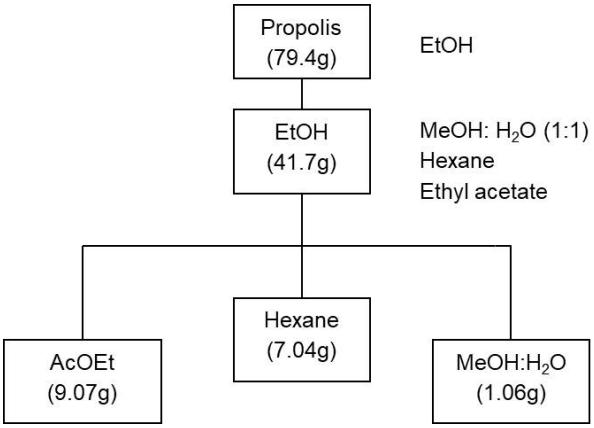


Fig.1. Extraction of propolis and propolis extracts in ethanol, ethyl acetate and hexane.

9.07g of the AcOEt fraction and 7.04g of the hexane fraction were obtained. The EtOH extract and the fractions were subjected to the total phenolic content tests (Folin-Ciocalteu), conducted in triplicates. The results were expressed as gallic acid equivalents, calculated by means of a curve constructed with concentrations ranging from 5 to 200µg/mL. The fractions were analyzed in high performance liquid chromatography (HPLC) and identification of the constituents of the samples was performed by comparison with previously isolated flavonoid standards of beekeeping/meliponic products.

Bacterial samples. Isolates of *Staphylococcus* spp. (n=34) obtained from cases of subclinical mastitis in goats were used in this study, which were present in the bacterial library of the Laboratory of Animal Microbiology and Immunology. These isolates were initially identified as belonging to the genus *Staphylococcus*, based on the biochemical and staining characteristics (Jacques et al. 2010) and two reference strains were used, ATCC 25923 (*S. aureus*) and ATCC 12228 (*S. apidermidis*).

Preparation of stock solution. The determination of the Minimum Bactericidal Concentration (MBC) was performed based on document M07-A9 (CLSI 2012). We weighed 0.25g of the extract diluted in ethanol, ethyl acetate and hexane, which were suspended in 10mL of the same solvents to obtain a stock solution at a concentration of 25,000µg/mL.

Preparation of inoculum. To prepare the bacterial inoculum, the 0.5 McFarland scale equivalent was turbid in 5mL of saline, then 0.1mL was withdrawn and added in 9.9mL of Mueller Hinton broth (MH), which was then final suspension of use in the microdilution test.

Broth microdilution assay. 96-well plates were used and 200µL of MH and 200µL of the extract were added to each well by serial dilution, starting from the stock solution up to eighth dilution and discarding the final portion. After this dilution process, 10µL of the bacterial suspension microplates were added to the wells.

The material was incubated at 37°C for 24h under aerobic conditions. From all wells, an aliquot was taken, seeded on the surface of MH agar and again brought to the oven at 37°C for 24 hours. The MBC was defined as the lowest concentration of ethanolic extract capable of causing the death of the inoculum. As a positive control, we used the strain *S. aureus* ATCC 25923 due to its considered pathogenicity.

A control of the broth was carried out to evaluate if contamination and extractors occurred, in order to assess whether the effect demonstrated was related to the product or propolis. All assays were performed in triplicate.

Assay for measurement of biofilm. The quantification of biofilm formation of the isolates was performed by means of the microplate adhesion test, with adaptations (Merino et al. 2009).

The isolated colonies were inoculated in 3mL of TBS (tryptic soy broth) with glucose (0.25%) and incubated at 37°C for 24h, to prepare the inoculum to be used *in vitro.* From each sample, 200µL were withdrawn where they were inoculated into 96 well plates and again incubated at 37°C for 24h. After 24h, three washes were performed with 200µL of distilled water. The wells were then stained with 100µL of gentian violet crystal (0.25%) for 3 minutes and again washed with distilled water. To dissolve the dye, 200µL of the alcohol-acetone (80:20) was used, after this process to evaluate the biofilm formation was performed reading the absorbance in a reader of microplates of Elisa Easys® and measured in filter of 620nm.

Measurement of biofilm production by optical density. It was possible to determine the biofilm production by the following classification according to the standards obtained by optical density (OD): without biofilm production (OD sample ≤OD negative control), weak biofilm production (OD negative control <OD sample ≤2.OD negative control), moderate biofilm production (OD negative control <OD sample≤ 4.OD negative control) and strong biofilm production (OD sample <4.OD negative control). The ODm (average optical density) observed for the negative control, *S. epidermidis* was 0.07, so that samples with OD values equal to or less than 0.07 are classified as without biofilm production (negative). Values of 0.07 to 0.14 are weak biofilm production, above 0.14 to 0.28 are moderate production and values above 0.28 will be classified as strong biofilm production.

Interference of the propolis extract with biofilm formation. Bacterial inocula were grown in 10mL TSB with 1% glucose at 37°C, for 24h. Of these, 100µl were added to the wells of the microplate, which had previously been added with 100µl propolis extract or 100µl of culture medium in the controls.

The concentration of the extract used was equivalent to half of the MBC value for each isolate. Thus, after 24h of incubation at 37°C, the plates were subjected to gentian violet staining, as described in the previous topic. The efficacy of the extract in interfering with the biofilm formation was defined by the equation: mean OD of treated wells/mean OD of the control wells x 100, using data evaluation according to the methodology adapted from Nostro et al. (2007).

Interaction between propolis extract and consolidated biofilm. After the incubation period and biofilm formation, the wells were washed three times with distilled water to remove non-adhered cells and then added 200µl propolis extract (0.5, 0.25, 0.125 MBC), (20). The OD was determined immediately after addition of the extract (0h) and 24h after. The interference of the extract with the consolidated biofilm was defined by the equation: mean OD_{0h}/mean OD_{24h} x 100.

Statistics. The data were processed and submitted to analysis of variance, and the differences between means were determined by Tukey's test with 1% level of significance (SAS 2002). The mean MBC of each bacterial isolate was considered to be the response variable. The optical densities of the isolates in the presence of the propolis extracts, as well as the means related to the capacity of the extract to interfere with the consolidated biofilm were compared by the means test.

RESULTS

Phytochemical analysis of propolis extract

On the total phenolics content, the AcEOt fraction compared to the others was the one that presented the highest amount of these metabolites (Table 1), but the statistical analysis of these data was not performed. With regard to the analysis of the chromatogram obtained by HPLC and comparison with the retention times of standard flavonoids and ultraviolet spectra, it was possible to identify the substances as naringenin, kanferol and isoramnetin, the latter two being the main flavonoids of propolis.

Antimicrobial activity of the propolis extracts against the isolates

The ethanolic extract of propolis showed better efficacy (72.67%) than the ethyl acetate extract (56.49%) and hexane extract (10.29%) on the *Staphylococcus* spp. bacteria (P<0.01), as shown in Table 2. However, there was no interaction (P>0.01) between the fractions (ethyl alcohol, ethyl acetate and hexane) as the result of MBC. In order to obtain these results, the mean MBC of all the extracts was considered for all the isolates. However, there were differences between the concentrations in which the best concentrations that caused the highest percentage of sensitivity were those of 6250, 3125 and 1562.5µg/mL, these concentrations being intermediate (Table 3).

Measurement of biofilm production

The results of the biofilm production profile of the 34 isolates are shown in Figure 2, and the majority 22/34 (64.70%) were considered weak biofilm production according to the optical density standard delimited in the methodology.

Interference of the propolis extract with biofilm formation

Only the bacterial isolates classified as moderate biofilm producers (5 isolates) were used in this assay, these isolates were characterized *as* coagulase-positive *Staphylococci* (CoPS) and all were tested for the three types of extract evaluated in the present study (Table 4). Thus, the ethanolic extract and the hexane extract were able to reduce the production profile

Table 1. Total phenolic compounds present in the propolis extract obtained from apiaries in the municipality of Casa Nova in different chemical products, state of Bahia

Extract	Total phenolics (mg EAG/g)[a]
Ethanol	36.21±0.08
Ethyl acetate	63.91±0.04
Hexane	12.63±1.08

[a] Gallic acid equivalents per gram of extract.

Table 2. Efficacy profile of the diluted propolis extract in different compounds (EtOH, AcOEt and hexane) against the isolates of *Staphylococcus* spp. obtained from cases of caprine mastitis

Extract	Efficacy (%)*
Ethanol	72.67[a]
Ethyl acetate	56.49[b]
Hexane	10.29[c]

* The efficacy profile was obtained according to the average effect of the extract at all dilutions in the different extracts; [a,b,c] mean values followed by different lowercase letters in the same column are significantly different by Tukey test at 1% probability.

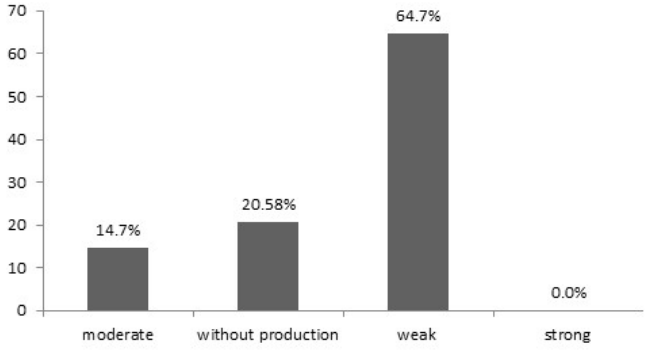


Fig.2. Measurement of biofilm production of all isolates of *Staphylococcus* spp.

Table 3. Sensitivity and resistance profile relationship of all isolates with CBM standards of all extracts (EtOH, AcOEt and hexane) used

Concentrations µg/mL	% Resistant	% Sensitive
97.6	85.62[a]	14.37[a]
195.3	71.89[ab]	27.77[ab]
390.6	62.74[ab]	37.25[abc]
781.3	46.73[b]	53.26[bc]
1562.5	36.92[c]	63.07[c]
3125	35.62[c]	64.37[c]
6250	34.31[c]	65.68[c]
12500	53.92[b]	46.07[bc]

[a,b,c] Mean values followed by different lowercase letters in the same column are significantly different by Tukey test at 1% probability.

of three and two isolates, respectively, being considered no longer a moderate producer, but rather a weak biofilm producer.

The ODm observed for the negative control was 0.066, so that samples with DOm values equal to or less than 0.066 are classified as non-producing (negative), values between 0.066 and 0.132 are classified as weak producers, between 0.132 and 0.264 as moderate and values above 0.264 are classified as strong producers.

Interaction between propolis extract and consolidated biofilm

All extracts presented an anti-biofilm profile according to what is present in Table 5, but the ethanolic extract presented the best activity (P<0.01), in relation to the other extracts, since it showed a reduction of 24 hours of action.

DISCUSSION

Phytochemical analysis of propolis extract

There was a higher content of phenolic compounds in the ethyl acetate fraction (AcOEt), not discarding the other fractions that also presented significant extraction (Table 1), being the bioactive compounds identified as naringenin, kanferol and isoramnetin. Kumazawa et al. (2004) reported that when analyzing propolis from different places, materials from China and Europe contained higher levels of flavonoids

Table 4. Profile of the extracts on the biofilm in the formation of the isolates (five) considered previously moderate biofilm products

Identification (SCP)	Extract		
	Ethanol extract	Ethyl acetate extract	Hexane extract
97 RM	Weak	Strong	Strong
98 LM	Weak	Strong	Weak
101 LM	Strong	Strong	Weak
102 RM	Weak	Strong	Strong
104 RM	Strong	Strong	Strong
Mean values	489.586000[ab]	613.832000[a]	263.202000[b]

SCP = *Staphylococcus* coagulase positive, RM = right mammary half, LM = left mammary half; [a,b] mean values followed by different lowercase letters are significantly different by Tukey's test at 1% probability.

Table 5. Average activity profile of the extracts on the consolidated biofilm of *Staphylococcus* coagulase positive

	Extract		
	Ethanol extract	Ethyl acetate extract	Hexane extract
0 hour	0.624[aA]	0.34[bB]	0.517[abA]
24 hours	0.423[bB]	1054[aA]	0.57[BA]

[a,b,A,B] Mean values followed by different lowercase letters, in the same row, and uppercase letters, in the same column, are significantly different by Tukey's test at 1% probability.

and phenolic acid esters. Thus, there is a medicinal potential in propolis and that this may vary according to the origin of products used to manufacture it by bees.

Several authors have reported that propolis has a range of compounds, such as flavonoids, alkaloids, terpenoids, and coumarins that reflect benefits to human and animal health, because these compounds have antimicrobial, antitumor, antioxidant activity (Cottica et al. 2011, Ortega et al. 2011, Valente et al. 2011, Chasset et al. 2016, Mouhoubi-Tafinine et al. 2016). The proportions of the various substances present in propolis depend on their place and time of collection (Wagh 2013), but not only that, the question of the locality in which the propolis was produced may be related to the presence of metabolites for therapeutic purposes.

We know that propolis is a complex mixture made by different compounds and some would be derived from plants (Wagh 2013). In general, crude propolis is composed of a mixture of several products, most of which is the resin, and the other components are waxes in addition to the essential oils (about 10% of the composition) and there is even the presence of pollen (Pietta et al. 2002, Wagh 2013). The main essential compounds responsible for biological activities are polyphenols, aromatic acids and diterpene acids (Wagh 2013) and there are not many disparities between the presence of these compounds and the different types of propolis.

Sensitivity of the isolates to the propolis extract

The ethanolic extract of propolis showed better efficacy (72.67%) against the isolates of *Staphylococcus* spp., however the other extract used also obtained efficacy (Table 2). In this

way all the products used were able to expose the bioactive compounds of propolis, highlighting the antimicrobial activity of this product. Dias et al. (2012) when studying different types of propolis in a MRSA strain, observed that all had antimicrobial activity against this strain, but the best activity was observed in the propolis of vines, which also had a greater amount of flavonoids, as well as this work an activity stands out due to the presence of this compound.

Mean MBC values of the fractions were from 12500 to 781.3µg/mL, with a higher sensitivity observed in intermediate values of 3125, 1562.5 and 781.3µg/mL, ranging from 64% to 53% values that can be justified considering that there was a better exposure of the bioactive compounds in these concentrations, favoring the bioactivity of the products. According to Aligiannis et al. (2001), values of MBC below 500µg/mL are considered to be strongly inhibited, while Ríos & Recio (2005) suggest results below 1000µg/mL, so the results of this work are in agreement with the results described by the authors cited, highlighting again the antimicrobial potential of propolis. We observed that in the highest concentration (12500µg/mL), when we talked about microbial sensitivity, a lower sensitivity of the bacterial strains was observed (Table 3) in relation to the other dilutions, something that could be related to this would be the complexity of the extract in this dilution, because due to the great concentration and the size of its particles, the contact with the bacterial cells can be diminished, affecting the final effect of the product, which perceives an increase, as it is diluted in concentrations capable of killing the bacteria.

When it is sought to develop new drugs, the ideal would be those that have an action against a greater range of groups of microorganisms. The difference in sensitivity between Gram-positive and Gram-negative bacteria against propolis is explained by the characteristics of the cell wall composition between these groups (Najmadeen & Kakamand 2009). However, in this work, propolis was shown to have significant activity against Gram-positive organisms, converging with studies by Dias et al. (2012) and Fernandes et al. (2015). Wagh (2013) reports that components with medicinal potential are constantly present in propolis, thus there is reinforcement that there is an antimicrobial potential in propolis due to its components.

Anti-biofilm activity: consolidated and in formation

According to the results shown in Table 3, the extract in ethyl acetate did not show any efficiency in decreasing the biofilm formation on the evaluated isolates (P>0.05). Also, according to Doern & Brecher (2011), the results of in vitro tests related to antimicrobial activity do not translate as expected to that observed *in vivo*, herefore tests involving the use of animals become indispensable for the best evaluation of the antimicrobial potential of natural products. Bacterial resistance is associated with both the low penetration of the antimicrobial compounds in the biofilm and the phenotypically protected state induced in the bacteria that compose the biofilm, where the microbial metabolism is extremely low (Costerton et al. 1995, Altieri et al. 2013). Another factor is that bioactive products capable of affecting both biofilm formation and bacterial survival have not been completely exposed (Table 2-4), which could cause the propolis diluted in ethyl acetate to present a profile of activity near or better than that of the extract diluted in ethanol.

It was also observed that both the ethanolic extract and the hexane extract were able to interfere with the first stages of biofilm formation, with the ethanolic extract having the best effect at zero time (P<0.05), thus conferring a possibility of acting as a prophylactic agent at the beginning of infections. When using natural products against the production of biofilm, they are reported to act better in the initial stages of biofilm formation (Chusri et al. 2012, Salta et al. 2013). Thus, the inhibition of the biofilm in formation may be related to phenomena linked to enzymatic inhibition of protease or to the interference of bacterial quorum sensing (González-Ortiz et al. 2014). It is possible to consider that action of propolis may be related to these mechanisms, but no analysis was performed to determine this activity profile.

Thereby, biofilms are considered structures intimately associated with microbial survival in both the environment and in the host (Doern & Brecher 2011, González-Ortiz et al. 2014). Once contained in the biofilm, bacterial cells are able to resist the host defenses and even the action of 38 antimicrobials agents, once the concentration required to cause bacterial death can be up to 1000x greater (Walters et al. 2003, Fux et al. 2004, Hall-Stoodley & Stoodley 2009).

Therefore, in the search for alternative sources to the antimicrobial treatment, much attention has been directed to substances capable of interfering with the bacterial biofilm (Issac Abraham et al. 2011, Budzyńska et al. 2011). The biofilm interferes in the mode of action of the antimicrobials, due to their low penetration, as well as to the phenotypically protected state in the bacteria that make up this structure (Jacques et al. 2010, Altieri et al. 2013). Thus, natural products such as propolis may appear as an alternative for the prevention of bacterial diseases interfering in the formation of the microbial biofilm.

CONCLUSIONS

The ethanol extract of propolis showed better efficacy against the isolates of *Staphylococcus* spp. and the fractions used were able to interfere both with the consolidated biolfim and during its formation.

Propolis can be considered for *in vivo* studies, in order to better understand its effects in uncontrolled environments.

Conflict of interest statement.- The authors have no competing interests.

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Original Article
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Outbreak of enzootic ataxia in goats and sheep in the state of Bahia[1]

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ABSTRACT.- Fontes T.N., Carvalho J.S., Mendonça M.F.F., Farias S.S., Mori C.S., Silva D.N., Madureira K.M. & Peixoto T.C. 2019. **Outbreak of enzootic ataxia in goats and sheep in the state of Bahia.** *Pesquisa Veterinária Brasileira 39(12):961-969.* Laboratório de Patologia Veterinária, Escola de Medicina Veterinária e Zootecnia, Universidade Federal da Bahia, Av. Adhemar de Barros 500, Salvador, BA 40170-110, Brazil. E-mail: tcpeixoto@ufba.br

The objective of this study was to describe for the first time the clinical-epidemiological, laboratory and pathological aspects of different forms of enzootic ataxia in goats and lambs in the state of Bahia, as well as to propose an emergency oral treatment with copper sulfate in an individualized way for neonates. Three outbreaks of enzootic ataxia were studied. The history, clinical signs and epidemiological data were obtained from the owners and checked during the visits. The first outbreak (FO) occurred in 2013 in a property located in the municipality of Itaberaba and affected a herd composed of 90 goats and 130 sheep. The second outbreak (SO) occurred in 2014 in the municipality of Santa Luz and affected a herd of 90 goats and 110 sheep. The third outbreak (TO) occurred in 2018, in a property located in the same municipality of the SO, being the herd constituted by 80 sheep. Samples of blood were collected from all goats and sheep treated for serum copper dosage. Nine animals (five goats and four sheep) that presented a more severe clinical picture and unfavorable prognosis were necropsied for diagnostic confirmation. During the necropsy of the FO, SO and TO animals, liver samples were collected for copper dosage as well as fragments of several organs for histopathology. At the site where the SO occurred, samples of soil and the main forages where the animals were kept were collected to determine copper, iron, molybdenum, sulfur and zinc contents. The clinical and histopathological findings in the three outbreaks studied were characteristic of enzootic ataxia. In the outbreaks studied, the frequency of enzootic ataxia was higher in goats (52) than in lambs (39). In the goats the mean values of serum copper (0.05mg/kg) and hepatic (2.48mg/kg) of the FO and SO were well below their respective reference values, 12 times lower than serum levels and eight times lower in relation to liver contents. On the other hand, TO sheep presented mean values of serum copper (0.015mg/kg) reduced by 40 times. The levels of iron and sulfur were high in the fodder of the property where the SO occurred, already in the soil, iron levels were high and copper levels were reduced. The treatment tested in neonates and lambs with two doses of copper sulphate solution orally at a dose of 20mg/kg for sheep and 35mg/kg for goats during the first and second week of life was effective in prevention of enzootic ataxia in neonates and can be used for emergency control of the disease. The occurrence of enzootic ataxia (congenital and late form) in goats and sheep was first reported in Bahia. It is also worth noting that this disease caused by copper deficiency has caused great damage to the breeders of small ruminants, mainly due to the high mortality of the animals affected.

INDEX TERMS: Outbreak, enzootic ataxia, goats, sheep, Bahia, Brazil, copper, small ruminants, mineral deficiency, microelement.

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RESUMO.- [**Surto de ataxia enzoótica em caprinos e ovinos no estado da Bahia**.] Objetivou-se com esse estudo descrever pela primeira vez os aspectos clínico-epidemiológicos, laboratoriais e patológicos de diferentes formas de ataxia enzoótica em cabritos e borregos no estado da Bahia, bem como propor um tratamento oral emergencial com sulfato de cobre de forma individualizada para neonatos. Foram estudados três surtos de ataxia enzoótica. O histórico, sinais clínicos e dados epidemiológicos foram obtidos com os proprietários e verificados durantes as visitas técnicas. O primeiro surto (PS) ocorreu em 2013 em uma propriedade localizada no município de Itaberaba e acometeu um rebanho composto por 90 caprinos e 130 ovinos. O segundo surto (SS) aconteceu em 2014 no município de Santa Luz e afetou um rebanho formado por 90 caprinos e 110 ovinos. O terceiro surto (TS) ocorreu 2018, em uma propriedade localizada no mesmo município do SS, sendo o rebanho constituído por 80 ovinos. Amostras de sangue foram colhidas de todos os caprinos e ovinos atendidos, para dosagem de cobre sérico. Nove animais (cinco caprinos e quatro ovinos) que apresentavam quadro clínico mais grave e prognóstico desfavorável foram necropsiados para confirmação diagnóstica. Durante a necropsia dos animais do PS, SS e TS amostras de fígado foram coletadas para dosagem de cobre, bem como amostras de diversos órgãos para exame histopatológico. Na propriedade onde ocorreu o SS, foram coletadas amostras de solo e das principais forragens onde os animais eram mantidos, a fim de se determinar os teores de cobre, ferro, molibdênio, enxofre e zinco. Os achados clínicos e histopatológicos verificados nos três surtos estudados foram característicos de ataxia enzoótica. Nos surtos estudados, a frequência da ataxia enzoótica foi maior em cabritos (52) do que em cordeiros (39). Nos caprinos os valores médios de cobre sérico (0,05mg/kg) e hepático (2,48mg/kg) do PS e SS encontravam-se muito abaixo dos respectivos valores de referência, sendo 12 vezes menor em relação aos níveis séricos e oito vezes menor em relação aos teores hepáticos. Já os ovinos do TS apresentavam valores médios de cobre sérico (0,015mg/kg) reduzidos cerca de 40 vezes. Os teores de ferro e enxofre encontravam-se elevados nas forragens da propriedade onde ocorreu o SS, já no solo, os níveis de ferro apresentavam-se elevados e os de cobre reduzidos. O tratamento testado nos cabritos e borregos neonatos, com duas administrações individualizadas com solução de sulfato de cobre, por via oral, na dose de 20mg/kg para ovinos e 35mg/kg para caprinos, na primeira e segunda semana de vida, foi eficaz na prevenção da ataxia enzoótica nos neonatos e pode ser utilizado para controle emergencial da doença. Comprovou-se pela primeira vez na Bahia a ocorrência da ataxia enzoótica (forma congênita e tardia) em caprinos e ovinos. Ressalta-se ainda que, esta doença causada por deficiência de cobre, tem cursado com grandes prejuízos aos criadores de pequenos ruminantes, sobretudo, devido à alta mortalidade dos animais acometidos.

TERMOS DE INDEXAÇÃO: Surto, ataxia enzoótica, caprinos, ovinos, estado da Bahia, cobre, pequenos ruminantes, deficiência mineral, microelemento.

INTRODUCTION

Enzootic ataxia may be due to primary (simple) copper deficiency in the diet, or secondary (Tokarnia et al. 2010), (conditioned) due to the presence of antagonists in the diet. Antagonists interfere with the use or absorption of copper by the animal (Tokarnia et al. 1966, Riet-Correa et al. 2001, Suttle 2010), such as molybdenum, sulfur, zinc and iron (Gould & Kendall 2011). Copper's interactions with other elements can make difficult to establish dietary requirements of this microelement for the animals of a herd, which emphasizes the importance of evaluating the epidemiological characteristics of each property, in an attempt to determine the predisposing regional factors for the occurrence of cupric deficiency.

The disease manifests itself in two forms: congenital and late. The congenital form presents a primary lesion localized in the cerebral white matter that occurs in the first days after birth. This can lead to stillbirths or weak animals, unable to stay in season and tetraparesis, who usually die during the first week of life. In the late form, the primary lesion occurs in the motor tract of the spinal cord and brainstem (Riet-Correa et al. 2001, Santos et al. 2006, Zatta & Frank 2007), one to two weeks after birth and manifests itself with ataxia and paresis of the pelvic limbs (Lewis et al. 1974).

In Brazil, the first outbreaks of enzootic ataxia were identified in Piauí from 1954 to 1958 by Tokarnia et al. (1966) in 12 lambs up to six months old. The disease, until then of unclear etiology, was known as "escancho". After evaluating clinical, necroscopic, histopathological histories and liver copper levels, the authors concluded that the disease is associated with copper deficiency, similar to known enzootic ataxia, in countries such as Australia, England and New Zealand. Later, Santos et al. (2006) reported an outbreak of late enzootic ataxia in 46 goats and 35 lambs in the wild region of Pernambuco state (PE), which was attributed to a large amount of iron in forage and soil. Subsequently, other studies were performed in PE using a serum and hepatic copper dosages, one in the Araripina region. A primary marginal copper deficiency was found in 141 goats and 141 sheep (Marques et al. 2011) and another in backcountry (Sertão region) of the São Francisco river valley (Silva Junior et al. 2015). However, in this last study, the dosages performed in 80 goats and 80 sheep did not show a copper deficiency. In 2008, in the municipality of Mossoró (RN), there was an outbreak of late enzootic ataxia affecting five lambs in which high liver levels were observed iron (Sousa et al. 2012). Recently, Silva et al. (2014) reported four outbreaks of late enzootic ataxia and one congenital outbreak in goats in the semiarid region of Paraíba state. Recent studies in Bahia evaluated mineral levels in 80 goats and 80 sheep raised in the municipalities of Casa Nova and Juazeiro. However, the mean serum and hepatic values of copper and zinc found did not indicate mineral deficiency, although serum iron levels were found high (Silva et al. 2016).

No descriptions of copper deficiency outbreaks were found in ruminants in the state of Bahia. Thus, this study aimed to describe, for the first time, outbreaks of copper deficiency in goats and sheep in the municipalities of Itaberaba and Santa Luz/BA.

MATERIALS AND METHODS

First outbreak. The first outbreak (FO) occurred in February 2013, in a property located in the municipality of Itaberaba (12°31'40" S, 40°18'25" W), Chapada Diamantina region, in two herds, one consisting of 90 crossbred goats Boer and another by 130 Dorper crossbred sheep. The herds were reared on an extensive pasture-based diet, predominantly grass, supplemented with corn and

protein mineral salt for cattle. The water supplied to the animals in drinking fountains was piped from the river. According to the owner only young animals (45 days to eight months) were affected by the disease caused by sex. The animals showed cambial gait, weakness and chronic incoordination of pelvic limbs with progression to decubitus, followed by death. However, an evolution of the clinical picture was individual for each animal, starting between the first and second month of life. There were no previous histories of this disease on the farm and in the region. Inspection of the installations revealed that the iron pipes supplying running water to the drinking fountains were highly oxidized. On this occasion, one male, crossbred, castrated (eight months old) goat (goat1-FO), and one sheep (sheep1-FO) presented clinical manifestations of the disease and were referred to CDP-UFBA for clinical follow-up and treatment. The sheep1-FO showed clinical improvement, with significant gait and balance recovery, and after 21 days was discharged. The goat1-FO remained hospitalized for 40 days, did not respond to the treatment instituted, and, due to the worsening of the clinical condition (progressing to permanent decubitus) and unfavorable prognosis, the owner opted for euthanasia and necropsy.

Second outbreak. The second outbreak (SO) occurred in August 2014, in the municipality of Santa Luz, northeastern Bahia, at a farm with a herd of 90 goats and another herd of 110 sheep of no defined breed. The animals were bred extensively with feed based on native Caatinga pasture and supplemented with goat mineral salt and pond water. According to history, four years ago, the disease affected goats and, to a lesser extent, sheep, and there was an increase in the number of cases each year. The disease evolved chronically and progressed with incoordination of the pelvic limbs, which manifested shortly after birth or between one and two-month-old goats and worsened over the weeks, with more frequent falls and difficulty walking (Fig.1). During the visit, 14 live goats (of the 34 initially affected) presented clinical signs. Among these, we selected three castrated male goats aged between three and five months (goat1-SO, goat2-SO, and goat3-SO) for physical evaluation, as they presented more pronounced clinical signs. Due to the unfavorable prognosis, the owner opted for euthanasia and necropsy for a diagnostic conclusion. We visited the farm again in June 2018. During the inspection of the herd, a recently calved goat (goat4-SO) exhibited clinical signs characteristic of the congenital form of enzootic ataxia. The newborn goat presented weakness, inability to get up and stay stationary (Fig.2), paresthesia of the pelvic limbs, as well as tachycardia, lethargy, wheezing and inability to ingest colostrum. Goat4-SO was followed for about six hours, and due to worsening of the clinical picture with unfavorable prognosis, it was euthanized *in extremis* and necropsied.

Third outbreak. The third outbreak (TO) occurred in June 2018, in a property located in the same municipality of the SO (Santa Luz, 11°15′21″ S/39°22′30″ W), with the herd consisting of 80 non-breed sheep. The herd was bred extensively on native pasture and received vitamin supplementation with mineral salt for sheep and water from the pond. According to the breeder, the disease had affected sheep for seven months, was chronic and affected only young animals up to 90 days old, and usually had a fatal outcome. Adult animals showed no clinical changes, and there was no sexual predilection. The animals presented incoordination of the pelvic limbs (Fig.3), weakness, staggering gait, frequent falls when moving, with progression to decubitus and death. At the time of the visit, six sheep presented clinical signs of the disease, we selected the four most affected animals for physical evaluation (sheep1-TO, sheep2-TO, sheep3-TO, and sheep4-TO), three males and one female,

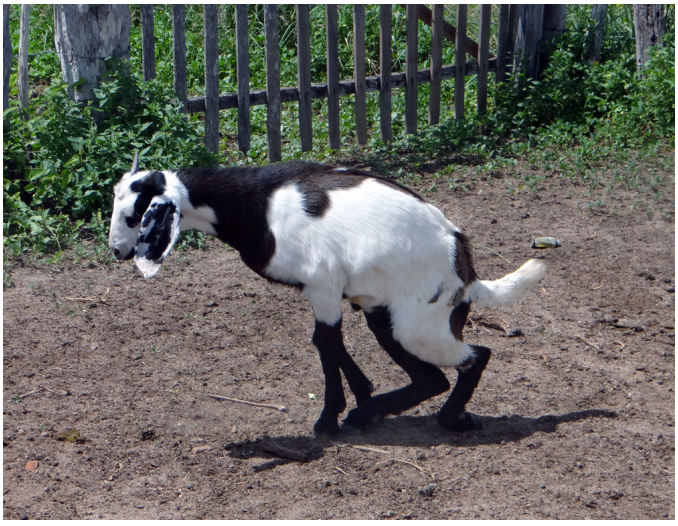


Fig.1 Incoordination of the pelvic limbs and difficulty in locomotion. Late enzootic ataxia, Goat 2 of the second outbreak.



Fig.2. Newborn goat with weakness and inability to stay in season. Congenital enzootic ataxia, Goat 4 of the second outbreak.



Fig.3. Incoordination of the pelvic limbs' moments before the fall. Late enzootic ataxia, Sheep 2 and 3 of the third outbreak.

aged three to eight months. These four sheep were euthanized by the owner's choice and then necropsied for diagnostic elucidation.

Biochemical exam. We collected blood samples from the evaluated animals (goat1-FO, goat1-SO, goat2-SO, goat3-SO, goat4-SO, sheep1-TO, sheep2 -T, sheep3-TO and sheep4-TO) by puncture of the jugular veins using 5mL syringes and needles (Descarpack®). Serums for biochemical analysis were obtained after clot retraction, centrifuged at a speed of 1200rpm for ten minutes, and then stored in a polyethylene microtube (Eppendorf), protected from light and stored at -20 °C (Lopes et al. 2007). The samples collected from goat1-FO, goat1-SO, goat2-SO and goat3-SO were sent to the Faculty of Veterinary Medicine and Animal Science of the University of São Paulo (USP) and the animals goat4-SO, sheep1-TO, sheep2-TO, sheep3-TO and sheep4-TO were sent to Axys Analyzes Laboratory (Porto Alegre, Rio Grande do Sul) for serum levels of Cu.

Necropsy. A total of nine animals (five goats and four sheep), together with the three outbreaks, were necropsied for the diagnostic conclusion. During necropsy, fragments of organs from the abdominal and thoracic cavity, brain, spinal cord, skin, and femoral biceps muscle were collected. This material was fixed in 10% phosphate-buffered formalin and sent to the Veterinary Pathology Laboratory of the Federal University of Bahia (LPV-UFBA).

Histopathology. After fixation, these materials were cleaved, routinely processed for histopathology, and stained with hematoxylin-eosin (HE). For better evidence of demyelination, some spinal cord fragments of the nine animals and goat4-SO brain embedded in paraffin were referred to the Veterinary Pathology Sector (SPV) of the Federal University Rio Grande do Sul(UFRGS) to perform the method Klüver-Barrera, using Luxol Fast Blue staining (Klüver & Barrera 1953).

Hepatic copper dosage. Liver fragments were collected from all nine autopsied animals using clean stainless steel razors, stored in 50mL polypropylene tubes and frozen at -20°C. The material referring to goats goat1-FO and goat1-SO, goat2-SO and goat3-SO, was sent to the Faculty of Veterinary Medicine and Animal Science of the University of São Paulo (USP) for hepatic Cu dosage. The samples were placed in trays and in an automated LIOTOP® device (model L101), then ground and pistil crushed (Pinheiro et al. 2011). Mineral dosing was performed according to the technique previously described by Nomura et al. (2005), using the technique of induced plasma optical emission spectrometry (ICP-OES) in the VARIAN Vista-MPX simultaneous axial CCD equipment in an automatic sampling system.

Soil and plant analysis. We collected soil samples and the central pasture plants and forages from two paddocks of the property where the SO occurred, where the animals were kept. These samples were sent to the Agricultural and Environmental Analysis Laboratory in Linhares (ES), for the determination of Cu, Fe, Mo, S, and Zn contents. We also collected samples of the following plants: malva (*Malva sylvestris*), native grass, signal grass (*Urochloa mosambicensis*), buffel grass (*Cenchrus ciliaris* L.), guinea grass (*Panicum maximum* cv. Green Panic) and "icó" (*Capparis yco*). Soil samples were collected from ten different pasture points of each paddock analyzed. After homogenization and quartering, two 150mg samples were obtained, which were sent for analysis, according to recommendations from Embrapa (1997).

Treatment. Prophylactic treatment in goats and neonatal loans, with two individual administrations with oral copper sulfate solution, 35mg/kg dose for goats, and 20mg/kg for sheep, in the first and second week of life.

Statistical analysis. All data obtained from the study were tabulated, collected, ordered, and analyzed using descriptive statistics in order to obtain possible correlations between the various clinical factors, soil, water, and pastures.

RESULTS AND DISCUSSION

The diagnosis of the three outbreaks of enzootic ataxia in small ruminants occurred in two municipalities of Bahia, based on epidemiological data, history, clinical signs, and necroscopic examination, confirmed by histopathological evaluation and serum and hepatic copper dosages. Additionally, the copper deficiency was also confirmed by the analysis of mineral contents in plants and soil from the property where the SO occurred.

Clinical aspects

The main clinical signs found in the five goats and four sheep of the three outbreaks studied are shown in Table 1. Similar findings were described in goats (Cordy & Knight 1978, Banton et al. 1990, Riet-Correa et al. 2001, Dinev et al. 2005, Santos et al. 2006, Sousa et al. 2012, Silva et al. 2014) and sheep (Tokarnia et al. 1966, Riet-Correa et al. 2001,

Table 1. Clinical signs presented by goats and sheep of the three outbreaks of enzootic ataxia diagnosed in Bahia

	Goat 1 FO	Goat 1 SO	Goat 2 SO	Goat 3 SO	Goat 4 SO	Sheep 1 TO	Sheep 2 TO	Sheep 3 TO	Sheep 4 TO
Sacral loin hyperesthesia	x	x	x	x	x	x	x	x	x
Ataxia	x	x	x	x	x	x	x	x	x
Motor incoordination	x	x	x	x	x	x	x	x	x
Muscle tremors	x	x	x	x	x	x	x	x	x
Frequent falls	x	x	x	x	x	x	x	x	x
Abnormal postures	x	x	x	x	x	x	x	x	x
Achromotrichia	x	x	x	x	x	x	x	x	x
Bad nutritional status	-	x	x	x	-	-	-	-	-
Dry cough	x	-	-	-	-	x	x	x	x
Dyspnea	x	-	-	-	-	-	-	-	-
Dry sterols	x	-	-	-	-	-	-	-	-
Sacral loin hyperesthesia	x	-	-	-	-	-	-	-	-

FO = first outbreak, SO = second outbreak, TO = third outbreak.

Santos et al. 2006, Sousa et al. 2012, Oliveira Filho 2016) with cupric disability. Additionally, one goat (goat1-FO) presented respiratory changes such as dry cough, dyspnea, and bilateral diffuse dry rales secondary to prolonged decubitus.

Periocular achromotrichia identified in the SO goats is related to melanin deficiency since copper participates in the synthesis of the enzyme polyphenol oxidase, which transforms tyrosine into melanin and dopamine, and leads to more severe depigmentation of the hair around the eyes in animals with cupric deficiency. In these cases, brown, red, or black fur may be clarified and reddish or whitish, which can be seen in the SO goats (Quiroz-Rocha & Bouda 2001). Nutritional deficiency in the SO animals may be related to food scarcity, since the outbreak occurred during the dry season, coupled with the difficulty of locomotion and, consequently, the search for food (Tokarnia et al. 1966). The respiratory alterations found (goat1-FO) are probably due to the animal's low immunological condition and prolonged decubitus, also described by Santos et al. (2006), who reported cough and catarrhal nasal discharge, as well as conjunctivitis and contagious ecthyma.

When evaluating the occurrence of enzootic ataxia in all herds studied in the Northeast, there was a higher occurrence of the disease in goats (52) than in lambs (39). The higher number of affected goats may be explained by the higher serum and hepatic copper concentrations in this species when compared to sheep (Riet-Correa et al. 2001). This finding indicates that the nutritional requirements of goats are higher than in sheep, making them more susceptible (Riet-Correa et al. 2001). However, such differences were found in a study conducted in small ruminants raised in Pernambuco, where serum and hepatic copper concentrations were found higher in goats (11.37±2.57µmol/L and 152.46±79.58ppm) than in sheep (9.85±2.71µmol/L and 158.45±83.05ppm) considered healthy and sent for slaughter (Marques et al. 2011). Santos et al. (2006), emphasize that there is no difference in serum and copper contents (11.7µmol/L±1.6), but highlighted the importance of new studies to confirm their exposure. On the other hand, Silva et al. (2016) found differences in the serum concentration (12.2±0.4µmol/L sheep and 13.8±0.3µmol/L goats) and hepatic (238±14µmol/L sheep and 220±13µmol/L goats) averages of Cu in goats and sheep in the state of Bahia.

In the FO, the frequency indexes of enzootic ataxia were 36.67% in goats (33/90) and 7.69% in sheep (10/130); For the SO, the frequency was of 38.89% (34/90) in goats and in the TO there was 37.5% (30/80) in sheep. These indexes were similar to those described by Santos et al. (2006), in the wild region of Pernambuco State, which found a prevalence of 46.3% and 24.2% in goats (25/54) and lambs (15/62), respectively. Regarding mortality, mortality rates were 35.6% (32/90) for goats and 7.69% (10/130) for sheep. In the SO, mortality was 68.6% (24/35) in goats, and in the TO, it was 45% (36/80). On the other hand, Sousa et al. (2012) observed mortality rates around 75% in a property composed of 56 sheep in the municipality of Mossoró/RN. Santos et al. (2006) observed mortality of 44.4% for goats and 22.6% for sheep in a property located in Surubim/PE. Several factors can influence these rates, such as time of year, animal susceptibility, the concentration of copper antagonists in soil and pasture, mineralization performed and outbreak duration without technical intervention (Peixoto et al. 2005, Marques et al. 2011), which could justify the differences between the obtained indexes.

Regarding the animals' age in the outbreaks studied, we found that the disease affected newborns to four months old animals. Signs of enzootic ataxia may vary in animals of different ages due to the fact that goats and lambs born from mothers with less severe copper deficiency have normal myelination at birth and develop demyelination in the postnatal period (Radostitis et al. 2007). Also, the fetal myelination process begins in late pregnancy and first occurs in the brain, extending centrifugally to the spinal cord when it is completed only 30 days after birth (Gambling et al. 2011).

Thus, considering the myelination period and the age of the animals affected in the outbreaks studied, we determined that the late form of the disease is common, corroborating the outbreaks reported in other studies (Santos et al. 2006, Sousa et al. 2012, Silva et al. 2014). However, on the property of the SO, the birth of an animal (goat4-SO) with neurological signs was observed, compatible with the congenital presentation of the disease, which was confirmed by serum copper dosage (0.011mg/kg), as well as lesions macro and microscopic features.

In the case of sudden death, the diagnosis of enzootic ataxia can be made by assessing the liver or serum levels of the mineral; however, the assessment of deaths on the property is especially important to rule out poisoning by toxic plants (Riet-Correa et al. 2001). It is noteworthy that, in the present study, a differential diagnosis was made with plant poisoning with neurological changes and sudden death, cerebellar hypoplasia, meningitis, intestinal parasitism (Radostitis et al. 2007), soft goat syndrome (Riet-Correa et al. 2004), caprine arthritis encephalitis (Riet-Correa et al. 2001), polioencephalomalacia (Lima et al. 2005) and oropharyngeal and neurological lesions associated with the use of metering guns (Sant'Ana et al. 2007). For such differentiation, we performed pasture inspections, clinical and epidemiological data evaluation, macroscopic and histopathological examinations, as well as copper dosages.

Biochemical

The results of the biochemical measurements of copper are shown in Table 2. In goats, the mean serum (0.05mg/kg) and hepatic (2.48mg/kg) copper values of the FO and SO were well below their respective values (0.6-1.5mg/kg and <20mg/kg)

Table 2. Blood and liver copper levels in goats and sheep from enzootic ataxia outbreaks in the state of Bahia

Animal	Serum (mg/kg)	Hepatic (mg/kg)
goat1-FO	0.045	1.911
goat1-SO	0.056	0.083
goat2-SO	0.083	4.440
goat3-SO	0.052	3.489
goat4-SO	0.011	-
sheep1-TO	0.012	-
sheep2-TO	0.016	-
sheep3-TO	0.013	-
sheep4-TO	0.018	-
Reference values	0.6-1.5	<20

FO = first outbreak, SO = second outbreak, TO = third outbreak.

(Underwood 1981, Radostitis et al. 2007), 12 times lower than serum levels, and eight times lower than liver levels. The TO sheep had mean serum copper values (0.015mg/kg) reduced about 40 times. These mean values were lower than those found by Marques et al. (2011) in Bahia in serum and hepatic dosages of sheep (9.58±2.52µmol/L - 158.45±83.05mg/kg) and goats (11.55±2.68µmol/L - 152.46±79.58mg/kg) healthy for slaughter.

Macroscopic and microscopic findings

No significant macroscopic lesions were found in any of the goats and sheep that presented the late form of enzootic ataxia in the outbreaks studied. Goat4-SO, which developed the congenital form of the disease, showed marked brain lesions in the white matter, especially in the parietal and temporal lobes characterized by bilateral symmetric hydranencephaly (Fig.4) without white matter, similar to that described by Radostitis et al. (2007). Additionally, in some cases, nonspecific macroscopic changes and/or secondary to prolonged decubitus were observed, such as areas of pulmonary consolidation in the ventral cranial portions and purulent exudate in the bronchial lumen (goat1-FO), mild pulmonary edema, mild pulmonary emphysema and focal atelectasis (goat1-SO, goat2-SO, goat3-SO), small bowel mucosa hyperemia with brownish mucosal content (goat1-FO), mild parasitic abomasitis, mild splenomegaly, mild to moderate regional lymphadenomegaly and mild mesenteric, moderate multifocal crusted chronic dermatitis (goat1-SO, goat2-SO, and goat3-SO). Histopathological evaluation showed the spinal cord, mild to moderate demyelination/degeneration of myelin in brain white matter (goat4-SO), and cervical and lumbar parts, mainly in separate funicles (Fig.5) and multi-stage chromatolysis (goat1- FO, goat1-SO, goat2-SO, and goat3-SO). Also, a special Luxol Fast Blue stain on goat1-FO, goat1-SO, goat2-SO, goat3-SO, ov1-TO, sheep2-TO, sheep3-TO, and sheep4-TO evidenced the cervical, thoracic, and lumbar parts of the spinal cord multifocal areas of white matter hypomyelination (Fig.6) in the dorsal, ventral lateral region, and ventral medial fissure, in addition to multistage chromatolysis and hypomyelination of goat4-SO cerebral white matter.

The histological lesions found were characteristic of enzootic ataxia caused by copper deficiency, showing intense vacuolization of white matter, axonal spheroids associated with axonal swelling due to demyelination (Dinev et al. 2005). There was an intense inflammatory infiltrate composed of macrophages, neutrophils, and lymphocytes within the alveoli, bronchi, and bronchioles (bronchopneumonia secondary to prolonged decubitus), as well as moderate hepatocyte vacuolization, predominantly periportal, small foci of necrosis, hemorrhage, and accumulation of debris, cells in the liver of goat1-FO. No histological lesions were observed in the gray matter, which corroborates the fact that the affected animals did not have a lack of appetite, fever, and thirst, since in the gray matter, mainly, the hypothalamus is responsible for regulating such functions (Zachary 2013). Recently Silva et al. (2014) used as a complementary diagnostic method radiographic evaluations in goats, showing irregular patterns of radiolucency of long bones, resulting from the failure of endochondral ossification, which causes growth retardation, decrease in weight gain and gear changes.

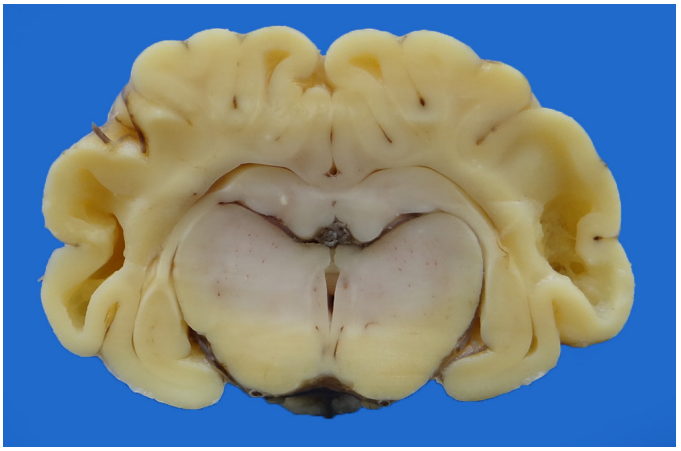


Fig.4. Brain cross-section showing bilateral symmetrical hydranencephaly. Congenital enzootic ataxia, parietal and temporal lobes, newborn goat (goat4-SO).

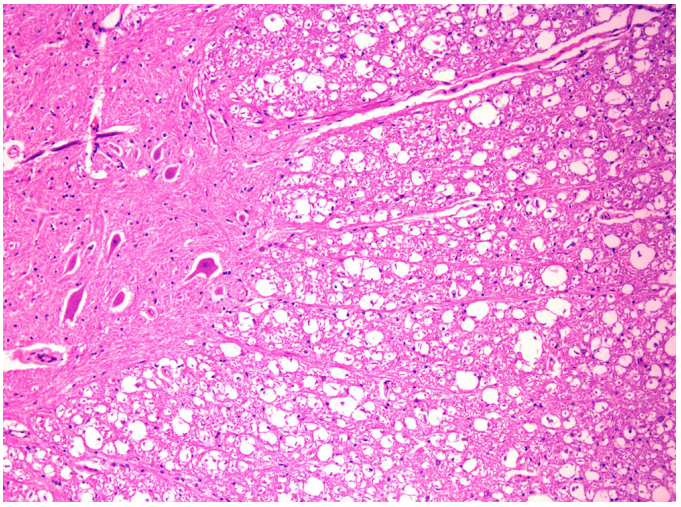


Fig.5. Marked multifocal hypomyelination of the white matter of the spinal cord. Late enzootic ataxia, Goat 2 of the second outbreak. HE, obj.10x.

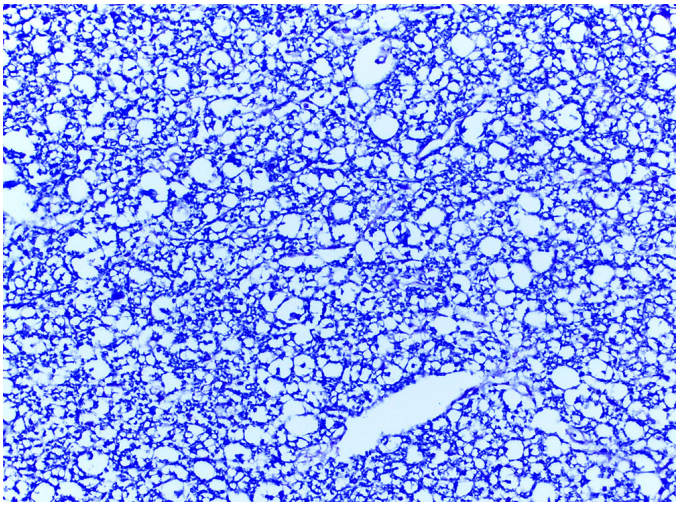


Fig.6. Marked diffuse hypomyelination of the white matter of the spinal cord. Congenital enzootic ataxia, newborn goat (goat4-SO). Luxol Fast Blue, obj.20x.

Soil and pasture analysis

In farms where the FO and SO occurred, it could be determined that enzootic ataxia resulted from secondary (conditioned) copper deficiency. In the FO, although it was not possible to evaluate the mineral levels in the soil and pastures to confirm the triggering factor of deficiency, it was found that the iron pipe used to direct the water to the drinkers had very high oxidation, which strongly suggested high iron content in drinking water. In the SO, we prove that iron antagonism in the intestinal absorption of copper was the cause of the deficiency, considering the high levels of iron in the evaluated plants and the adequate levels of copper. Iron levels in the soil were also very high and copper levels low. The Cu, Fe, Mo, S, and Zn contents found in the pasture plant and soil samples used by the SO herd are shown in Table 3 and 4, respectively. The levels of copper required in the pastures (suitable for diet) are species-specific. In sheep, the appropriate copper value is 5ppm, while goats require twice as high levels (10ppm). Levels of 3-5ppm are marginal and may cause clinical or subclinical deficiency. Secondary deficiency can occur in pastures with copper levels above 5ppm, but with 3-10ppm molybdenum levels. In pastures with molybdenum levels below 3ppm and adequate copper levels, there is no risk of copper deficiency (Riet-Correa et al. 2001).

Treatment

Due to the suspicion of enzootic ataxia, goat1-FO and sheep1-FO were treated with oral copper sulfate solution at a dose of 35mg/kg in a single dose and a 10mL/vo SID poly vitamin tonic (Poten Fort B12®), during all days of hospitalization. Sheep1-FO showed clinical improvement and was discharged 21 days after the start of treatment. Goat1-FO remained hospitalized for 40 days, and although receiving the same treatment, no clinical improvement was obtained. We evaluated the efficacy of emergency prophylactic administration of an individually administered oral copper sulfate solution in animals from the herd of all three farms where outbreaks occurred (FO, SO, and TO). The first dose was administered shortly after birth and the second dose repeated after one week. This treatment was adapted from the few data found in other studies with goats and sheep (Radostitis et al. 2007, Pugh & Baird 2012), given the scarcity of studies on the treatment of copper deficiency in these species. The evaluated treatment proved to be active and prevented the occurrence of new cases of late enzootic ataxia in all three properties. According to the farmers, after the beginning of prophylactic treatment, the disease only occurred when the newborn animals were mistakenly not treated prophylactically. No citations of prophylactic treatments of this nature were found in the literature. Such treatment also avoids the intoxication of sheep as they should not consume mineral mixtures with more than 0.4%-0.8% copper sulfate (Riet-Correa et al. 2004). However, for proper prophylaxis, soil correction/fertilization and supplementation of pregnant females is recommended to prevent the occurrence of the congenital form of the disease (Tokarnia et al. 1971, Peixoto et al. 2005).

We found significant direct and indirect economic losses on the visited properties due to the high mortality rates of the animals affected by enzootic ataxia. These facts were responsible for the dissatisfaction of the owners who claimed to be about to give up their creations and move to large cities if the problem was not solved. It is noteworthy that misconceptions about the real cause of the disease persist in the field and result in unnecessary treatments and the wrong combat of plants considered toxic and the perpetuation of the disease. Curiously, in the SO, the owner claimed that the producers in the region believed that the disease was genetically transmitted and had a hip bone malformation; while in the TO, the owner attributed the disease to plant poisoning. The CDP-UFBA extension work is essential for the dissemination of knowledge and technologies to small breeders of the state of Bahia (Santos et al. 2016), aiming at the consolidation of goat farming in the Brazilian semiarid, an activity that has an essential economic and socio-cultural role for the generation of income for small farmers, livestock production for subsistence and the establishment of man in the field.

CONCLUSIONS

The results obtained in this study confirmed for the first time the occurrence of enzootic ataxia (congenital and late form) in goats and sheep in the state of Bahia. This disease

Table 3. Copper, iron, manganese, sulfur and zinc contents in mg/kg in the samples of plants collected from the pasture of the farm where the second outbreak occurred (Santa Luz/BA)

Micro minerals	Malva	Native grass	Signal grass (urucloa)	Buffel grass	Gripan grass	"Icó" (*Capparis yco*)	Average	Reference values
Cu	5.0	5.0	5.0	5.0	4.0	5.0	4.8	4.0-14.0
Fe	158.0	209.0	140.0	143.0	136.0	250.0	172.6	100.0-150.0
Mn	0.8	0.8	1.6	0.8	0.8	0.8	0.9	0.1-1.4
S	10.5	5.6	1.6	1.2	2.3	6.7	4.6	1.0-3.0
Zn	25.0	18.0	18.0	20.0	45.0	19.0	24.1	20.0-50.0

Table 4. Copper, iron and sulfur contents in mg/dm³ in the soil samples of the paddocks of the farm where the second outbreak occurred (Santa Luz/BA)

Micro minerals	Paddock 1	Paddock 2	Average	Reference values
Cu	0.30	0.20	0.25	0.60-1.5
Fe	87.0	71.0	79.0	21.0-31.0
S	6.0	4.0	5.0	5.0-10.0

caused by copper deficiency has been causing significant damage to small ruminant breeders, mainly due to the disease's high mortality.

Enzootic ataxia occurred in the studied farms due to secondary (conditioned) copper deficiency triggered by iron antagonist action. Oral prophylactic treatment with copper sulfate solution for newborn goats was effective in preventing the late form of enzootic ataxia in properties where outbreaks occurred and can be used as an emergency method for outbreak control.

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Impact of high-energy diets on the rumen environment and digital cushion in confined cattle[1]

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ABSTRACT.- Tavares N.C., Barbosa A.A., Bermudes R.F., Rechsteiner S.M.E.F., Cruz L.A.X., Bruhn F.R.P., Silva P.M. & Martins C.F. 2019. **The impact of high-energy diets on the rumen environment and digital cushion in confined cattle.** *Pesquisa Veterinária Brasileira 39(12):970-977.* Universidade Federal de Pelotas, Campus Capão do Leão, Av. Eliseu Maciel s/n, Capão do Leão, RS 96900-010, Brazil. E-mail: martinscf68@yahoo.com.br

This study aimed to identify gross and microscopic changes, caused by high-energy diets, in the rumen environment and hoof of confined beef cattle. The study sample comprised 40 confined heifers (*Bos taurus*) with no disease history divided into four experimental groups using different diets: Group 1 (D1, control), 48:52 forage:concentrate ratio; Group 2 (D2), 30:70 forage:concentrate ratio; Group 3 (D3), 30:70 forage:concentrate ratio + sucrose; Group 4 (D4), 100% concentrate. All animals underwent clinical examination, assessment of ruminal fluid pH and lameness, and sample collection after slaughter for histopathology of the hoof laminae and digital cushion and ruminal tissue. All dependent variables of the study were compared using the SPSS 20.0 statistical software. The variables that did not show normality (HR, RM, ST, and CRT) were compared with application of the Kruskal-Wallis test followed by the Dunnet's multiple comparison test. All other variables were submitted to analysis of variance (ANOVA) followed by Tukey's test. The different diets had an impact on the rumen environment ($p<0.05$) of the heifers assessed, with momentary general depression in the first 12 h after sucrose induction (D3), as well as mild clinical signs in D4. The animals in D3 and D4 presented lower motility ($p<0.05$) and ruminal pH ($p<0.01$) than those in D1. Of the 40 heifers, 27.5% (n=11) showed gross lesions in the epithelium of ruminal pillars, whereas 22.5% (n=9) of those in D3 and D4 presented these lesions. Sole corium thickness varied between heifers in D3 compared with those in D1 and D2 ($p<0.05$). Therefore, high-energy diets, as used in this study, alter some clinical parameters and the rumen environment, causing lesions in the rumen mucosa, and of lesser intensity, in the hoof corium and laminae, suggestive of laminitis.

INDEX TERMS: High-energy diets, rumen environment, digital cushion, confined cattle, acidosis, hoof, histology, laminitis, confinement.

RESUMO.- [**O impacto de dietas de alto valor energético sobre o ambiente ruminal e o tecido digital em bovinos confinados.**] O objetivo deste trabalho foi identificar alterações macroscópicas e microscópicas provocadas por dietas de alto valor energético no ambiente ruminal e casco de bovinos de corte (*Bos taurus*) em confinamento. Foram utilizadas 40 novilhas mantidas em confinamento, sendo divididas em 4 grupos experimentais: Grupo D1(controle) - 48% de volumoso e 52% de concentrado; Grupo D2 - 30% de volumoso e 70% de concentrado; Grupo D3 - 30% de volumoso e 70% de concentrado + sacarose; Grupo D4 - 100% de concentrado. Foi realizado exame clínico dos animais, avaliação do pH ruminal, da claudicação, além de coletas de amostras post-mortem para análises histopatológicas da região laminar do casco, coxim digital e do rúmen. Todas as variáveis dependentes do estudo foram comparadas por meio do software estatístico

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SPSS 20.0. As variáveis sem normalidade (FC, MR, Tc e TPC) foram comparadas pelo teste de Kruskal Wallis, seguido pelo teste de comparação múltipla de Dunnet. Todas demais foram submetidas a análise de variância (ANOVA), seguida pelo teste de Tukey. Foi observado impacto das distintas dietas ao ambiente ruminal (p<0,05). Os grupos D3 e D4 exibiram menor motilidade (p<0,05) e pH ruminal (p<0,01). 27,5% (n=11) dos animais tiveram registros macroscópicos de lesões no epitélio dos pilares ruminais enquanto que os submetidos às dietas 3 e 4 foi observada ocorrência de 22,5% (n=9) de lesões macroscópicas no rúmen. As espessuras do córium solear variaram entre os grupos que receberam a indução de sacarose em relação ao grupo controle e novilhas alimentadas com a dieta 2 (p<0,05). Sendo assim dietas com elevada densidade energética, na condição em que foi empregada, alteraram alguns parâmetros clínicos e o ambiente ruminal, provocando lesões na mucosa do rúmen e de pouca intensidade no córium e tecido laminar, sugestiva de laminite.

TERMOS DE INDEXAÇÃO: Dietas, alto valor energético, ambiente ruminal, tecido digital, bovinos confinados, acidose, casco, histologia, laminite, confinamento.

INTRODUCTION

Beef cattle raising is an activity of great importance in the Brazilian economy. Concerning variable costs, the intensive system presents considerably higher expenses, mainly because of investment in animal feed. While providing concentrate feed increases production costs, increasing the dietary concentrate ratio improves animal performance as it increases daily weight gain and reduces termination time. Thus, confinement enables larger production in the same physical space, ensuring high system profitability (Missio et al. 2009).

Inclusion of carbohydrate-rich foods that are easily metabolized in cattle diet may lead to development of metabolic changes such as sub-acute rumen acidosis (SARA) (Ortolani et al. 2010). This metabolic disorder, in addition to causing a sharp decline in ruminal motility, ingestion and rumination, can also affect, secondarily, the locomotor system (Mulling & Greenough 2006).

The precise etiology and pathogenesis of foot disorders in ruminants are not fully known. Dietary energy density may be a predisposing factor as a cause of locomotion disorders. Knowledge of the mechanisms that lead to these disorders has been mainly described empirically, and is often extrapolated from equine species. However, there are anatomo-functional differences between the two species, such as absence of secondary dermis and epidermis in bovines, with the laminar region occupying only the distal half of the third phalanx, being smaller when compared with that of horses (Greenough 2007). Thus, the area of adherence of the phalanx to the coronary band through the laminar region is much smaller in cattle compared with horses (Lischer et al. 2002). Moreover, the fact that horses are monogastric and cattle are polygastric (ruminants) does not allow a perfect comparison between the two species.

To date, scientific studies have addressed the impact of nutrition on the cervical/laminar region, but there is lack of information on the impact on the health of the digital cushion, especially when inflammatory systemic events are present in the laminae and corium of the digital cushion of cattle submitted to increased-grain diets. This information is important and needs to be further studied, as digital cushion depletion is associated with body condition score, as well as with the incidence of sole ulcers and white line disease (Bicalho et al. 2009).

Although there is abundance of studies seeking the understandings of hoof structures, digital cushion, and associations between ruminal acidosis and lameness (Lean et al. 2013) on dairy cattle, there are relatively few studies addressing confined beef cattle, in which episodes of SARA occur (Plaizier et al. 2009). Therefore, it is possible that variations in the starch proportions in the diet of beef cattle may promote ruminal damage, with systemic deleterious effects on other tissues, including digit constituents such as the digital cushion, which are associated with pathological events occurring in the corium and laminar tissues. These structural changes in the hoof may predispose cattle to lameness and development of hoof injuries, leading to economic losses (Souza et al. 2006). In this sense, different carbohydrate sources have been used to induce ruminal acidosis with the purpose of studying its dynamics and effects on animal health, providing knowledge about preventive measures that may benefit hoof health. Thus, exogenous sucrose supplementation has been shown to induce rumen acidosis (Ortolani 1995, Maruta & Ortolani 2002, Krause & Oetzel 2006).

Understanding the relationship between increased-grain diets with integrity of the hoof wall and the support function offered by the digital fat pad through clinical criteria and gross and microscopic anatomical changes may be relevant in maintaining the hoof health of confined beef cattle. Thus, based on what has been previously exposed, this study focused on identifying the gross and microscopic changes caused by high-energy diets in the rumen environment and hoof of confined beef cattle.

MATERIALS AND METHODS

The experiment was conducted in the municipality of São Lourenço do Sul, Rio Grande do Sul state, in a commercial farm and slaughterhouse that raised of beef cattle under intensive management. All study procedures were approved by the Animal Experimentation Ethics Commission (CEEA) of the "Universidade Federal de Pelotas" (UFPel) under protocol no. 0105185/2017. The cattle were confined in barns, in 6x7m collective stalls, totaling 42m^2 (4.2m^2/head), with ceiling height of 6m.

Forty heifers (*Bos taurus taurus*), 80% Angus and 20% Hereford, homogeneously distributed between groups, aged approximately 18 months, with average initial weight of 341±57.13kg composed the study sample. The following inclusion criteria were used: I) no history of disease; II) body condition score (BCS) between three and four on a scale from zero to five (Nicholson & Butterworth 1986).

The animals were divided into four experimental groups of 10 animals each using different diets, and all of them were clinically healthy with no history of systemic disease or hoof lesions: Group 1 (D1, Control) 48:52 forage:concentrate ratio; Group 2 (D2) 30:70 forage:concentrate ratio; Group 3 (D3) 30:70 forage:concentrate ratio + sucrose; Group 4 (D4) 100% concentrate (Table 1). The amount of sucrose supplied was calculated based on the technique described by Ortolani (1995), according to equation Y=1057+43.1 x LW 0.75, where: Y = amount of sucrose expressed in grams, LW = live weight in kilograms, and LW$^{0.75}$ = metabolic weight. The result obtained from this formula was decreased by 15%, as

performed by Maruta & Ortolani (2002), aiming to minimize risks to animal health.

The heifers underwent an adaptation period of 15 days prior to study commencement (except those in D4). After this period, the cattle received the treatment diets for 45 days, totaling two months of confinement. The feed provided was weighed daily and always adjusted to allow 5-10% leftovers, which were later weighed and recorded daily before the first meal of the next day (Campos et al. 2016).

Animal requirements were determined according to the National Research Council (NRC 1996). Dietary samples were collected weekly for bromatological analysis (Table 2).

At the end of confinement, the animals were sent to pre-slaughter fasting, underwent general clinical examination, and had the ruminal fluid pH measured individually in a squeeze chute. In animals receiving sucrose in the diet (D3), the examinations were performed 24 h after induction.

General clinical examination of the animals included the measuring of heart rate (HR), respiratory rate (RR), capillary refill time (CRT), rectal temperature (T°C), skin turgor (ST), mucosal staining (MS), and ruminal motility (RM) as described by Feitosa (2004). Animals that presented results within the reference values according to Feitosa (2004) in the general clinical examination were considered healthy.

A portable pH meter (Noro et al. 2013) was used for the measurement of ruminal fluid pH in a single collection by the ruminocentesis dorsomedial procedure, conducted in all animals 3 to 4 hours after the last feeding (pre-slaughter fasting) in the afternoon. Animal lameness was evaluated daily throughout the study period in a fenced area located next to the confinement stalls according to the locomotion score characterized by Ferreira et al. (2005).

All animals were weighed and had their body condition score (BCS) evaluated at the beginning and end of the experiment through weighing and visual assessment, respectively, after a 12-hour fasting.

After opening of the digits, the complete digital cushion, the laminar tissue in the distal proximal dorsal region of the claw, the coronary dermis, and the sole corium were collected from all digits in the four limbs. The digital cushion fragments were weighed on a precision scale and the volume was determined by immersing it in a beaker containing 20 mL of water. All digital cushion samples collected were identified using numbered seals previously used as a means of identifying the animals and limbs. The samples were fixed in 10% formalin solution for further histological processing (Luna 1968).

After cutting the samples in order to remove the areas to be manipulated using histological forceps, the laminar tissue, digital cushion, and rumen samples were processed following the traditional histology methodology, with subsequent embedding in paraffin blocks according to routine technique. Subsequently, 5 µm-thick histological sections were placed on histological slides and stained using the hematoxylin and eosin (HE) and periodic acid-Schiff (PAS) techniques (Luna 1968).

All dependent variables of the present study were compared between the different treatments (diets) using the SPSS 20.0 statistical software. Variables with normal distribution (T°C, RR, pH, percentage of ulcerated ruminal areas, sole corium thickness, BCS, average daily gain intake, carcass yield, and weight, volume and proportion of connective, adipose and vascular tissues of the digital cushion) were submitted to analysis of variance (ANOVA) followed by Tukey's test. The variables that did not show normality (HR, RM, ST, and CRT) were compared by Kruskal-Wallis test followed by the Dunnet's multiple comparison test.

Table 1. Characterization of animal diets

Feed	Treatment (forage:concentrate ratio)			
	D1 (48:52)	D2 (30:70)	D3 (30:70)	D4 (0:100)
Corn silage (kg/MS)	3.750	2.240	2.240	0.00
Concentrate (kg/MS)	4.050	5.440	5.440	7.425
Dry matter (kg)	7.800	7.680	7.680	7.425

Table 2. Bromatological composition of the experimental diets (%)

Components	Diet (forage:concentrate ratio)		
	D1 (48:52)	D2 and D3 (30:70)	D4 (0:100)
DM	100	100	100
OM	1.20	0.75	1.75
NDF	43.25	37.73	28.54
ADF	22.35	19.42	14.54
CP	8.90	9.84	11.32
EE	4.71	4.96	5.37
TDN	71.07	74.90	81.29

DM = dry matter, OM = organic matter, NDF = neutral detergent fiber, ADF = acid detergent fiber, crude CP = protein, EE = ether extract, and TDN = total digestible nutrients.

RESULTS

The different diets used in this study affected the rumen environment (p<0.05) of the assessed heifers. Diarrhea was observed in the first 12 h after sucrose solution administration (Table 3), as well as reduced rumination and momentary general depression in the heifers in D3 and mild clinical signs in those in D4 (Table 4).

Acute and subacute attacks to the digital cushion derived from the consumption of increased-grain diets were not clinically observed, since none of the heifers that received the different diets showed lameness and postural abnormalities.

Differences between the clinical variables tested were observed only between the ruminal clinical parameters (RM and pH). Heifers inD3 and D4 showed lower motility (p<0.05) and ruminal pH (I<0.01) than those in D1 (Table 4).

As the forage:concentrate ratio was changed in the increased-grain diets, more acidic ruminal environments were formed, changing the organ color (Fig.1). Of the 40 beef heifers fed different diets, 27.5% (n=11) showed gross lesions in the epithelium of ruminal pillars compatible with

Table 3. Percentage (%) and number (n) of inconsistent stools in the different diets

Diet	Diarrhea	
	Presence	Absence
D1	20.0 (2)[a]	80.0 (8)[a]
D2	50.0 (5)[ab]	50.0 (5)[ab]
D3	100.0 (10)[c]	0.0 (0)[c]
D4	70.0 (7)[b]	30.0 (3)[b]
Total % (n)	60.0 (24)	40.0 (16)

[a,b,c] Values followed by superscript lower-case letters in the same column statistically differ (p≤0.05) by the Fisher's exact test.

Table 4. Clinical profile 12 h prior to slaughter of the different diet groups

Variable	D1	D2	D3	D4
T (°C)*	39.8 (0.11)[a]	39.6 (0.08)[a]	39.9 (0.10)[a]	39.8 (0.11)[a]
RR (mov/min)*	49.8 (5.96)[a]	50.8 (4.49)[a]	60.0 (5.01)[a]	65.5 (4.18)[a]
HR (beat/min)**	106.5 (27.00)[a]	100.0 (20.00)[a]	110.0 (11.25)[a]	105.0 (16.00)[a]
CRT (s)**	2.0 (0.25)[a]	2.0 (1.00)[a]	2.0 (0.00)[a]	2.0 (0.25)[a]
ST**	0.0 (0.00)[a]	0.0 (0.00)[a]	0.0 (0.25)[a]	0.0 (0.00)[a]
RM (mov/min)**	3.0 (0.25)[a]	3.0 (1.00)[ab]	2.0 (0.00)[b]	2.0 (0.25)[b]
pH*	6.4 (0.17)[c]	5.4 (0.17)[b]	4.5 (0.09)[a]	5.1 (0.05)[b]

T (°C) = rectal temperature, RR = respiratory rate, HR = heart rate, CRT: capillary refill time, ST = skin turgor, RM = ruminal motility, pH = ruminal fluid pH; * variables with normal distribution described as mean (standard error) compared by ANOVA and Tukey's test, ** variables without normal distribution of means (interquartile interval) compared by Kruskal-Wallis and Dunnet's tests; [a,b,c] values followed by different letters in the same line are statistically different ($p \leq 0.05$).

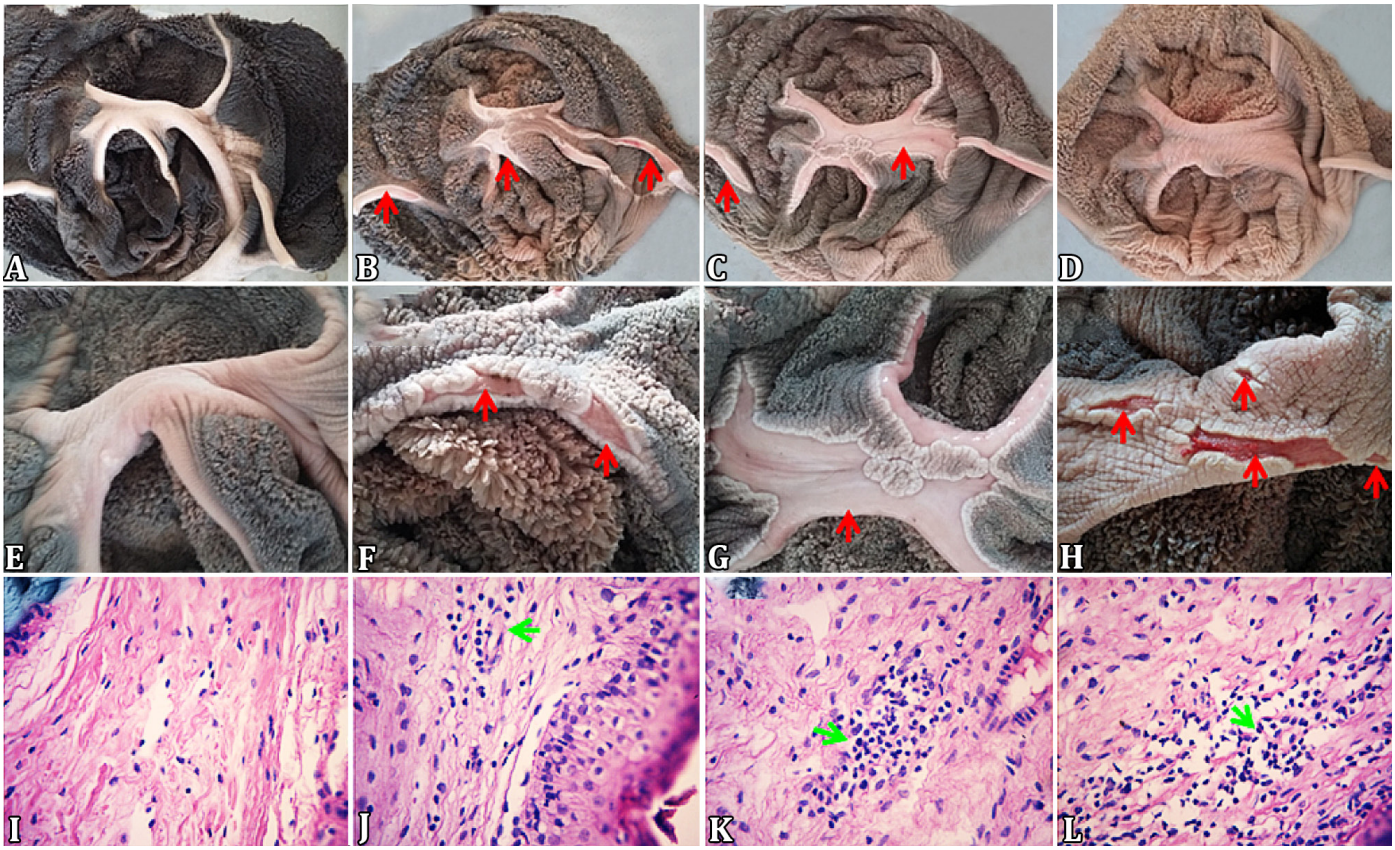


Fig.1. Histology of (**A**-**D**) rumen environment, (**E**-**H**) rumen pillars, and (**I**-**L**) ruminal epithelium. Diets: (**A**,**E**,**I**) control, (**B**,**F**,**J**) unbalanced, (**C**,**G**,**K**) unbalanced with sucrose induction, and (**D**,**H**,**L**) only concentrate. Ulcers in the ruminal pillars (red arrows) and inflammatory infiltrates (green arrows). (**I**-**L**) HE, obj.40x.

development of ruminitis, whereas 22.5% (n=9) of those in D3 and D4 presented these lesions. Approximately 40 (D3) to 50% (D4) of the animals presented ulceration in the ruminal pillars, with average of 5.23 and 2.0% of the affected area, respectively (Fig.1G,H). Discreet papillary lesion was observed in only one animal in D3.

Histopathology showed preserved rumen papillae, with slight degeneration of keratinized stratified squamous epithelium cells associated with discrete lymphocyte infiltrates in *the lamina propria* and some papillary vessels with inflammatory cells. The results of this study showed a mild inflammatory reaction of the papillary region in animals in D3 and D4 (Fig.1K,L) compared to the papillary region of those in the other groups. Analysis of lymphocyte infiltration in the supportive connective tissue of the ruminal mucosa indicated a chronic effect of the inflammatory response, with no difference between diets ($p>0.05$).

Histopathologic differences in the digital tissues ($p<0.05$) were evident, characterizing a milder form of aggression to the corium and laminar tissue (Table 7, Fig.2). None of the animals, regardless of the diet received, showed congestion, hemorrhage, and detachment of the basement membrane from digital laminar tissue.

A small number of animals (n=5, 12.5%) had epidermal cells within physiological standards, with predominance of oval cells perpendicular to the basement membrane. Although 87.5% (n=35) of the heifers presented secondary epidermal cells with rounded and flattened nuclei, the variation in laminar histology characteristics observed between individuals receiving different levels of dietary concentrate did not contribute to variability between them ($p>0.05$, Table 5).

Differences were found ($p<0.05$) when the digits were analyzed separately for cell morphometry. In the left medial front digit, the data presented approximate statistical significance ($p<0.07$) for epidermal cell morphometry between the groups

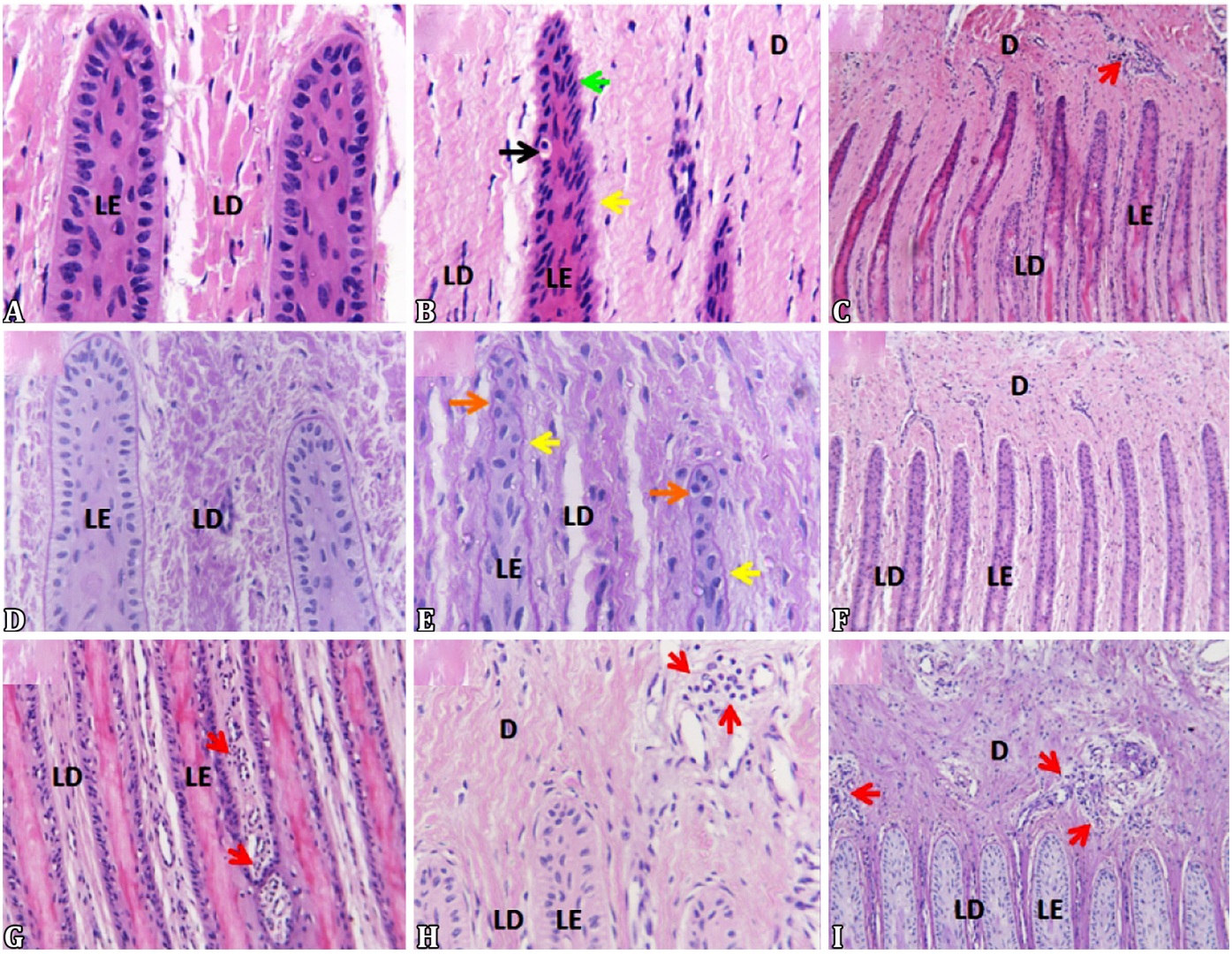


Fig.2. Histology of laminar tissue with dermis (D), dermal (DL) and epidermal (EL) laminae. Inflammatory infiltrates (red arrows), rounded nuclei (orange arrows), elongated and flattened nuclei (green arrows), individual keratinized epidermal cells (black arrows), irregular basal membrane (yellow arrows). (**B**,**C**,**E**) Normal disposition and (**A**,**D**,**F**,**H**,**I**) middle third of the dermal and epidermal slides. (**A**,**B**,**C**,**G**) HE, obj.10x. (**D**,**E**,**F**,**H**,**I**) PAS, obj.40x.

Table 5. Cell morphometry on epidermal laminae according to the diet groups

Diet	Changes of epidermal cells							
	MLFD		LLFD		MLHD		LLHD	
	No	Yes	No	Yes	No	Yes	No	Yes
D1% (n)	70 (7)[a]	30 (3)[a]	80 (8)[a]	20 (2)[a]	40 (4)[a]	60 (6)[a]	40 (4)[a]	60 (6)[a]
D2% (n)	20 (2)[a]	80 (8)[a]	60 (6)[a]	40 (4)[a]	40 (4)[a]	60 (6)[a]	20 (2)[a]	80 (8)[a]
D3% (n)	20 (2)[a]	80 (8)[a]	50 (5)[a]	50 (5)[a]	20 (2)[a]	80 (8)[a]	20 (2)[a]	80 (8)[a]
D4% (n)	20 (2)[a]	80 (8)[a]	50 (5)[a]	50 (5)[a]	20 (2)[a]	80 (8)[a]	10 (1)[a]	90 (9)[a]
Total	32.5 (13)	67.5 (27)	60.0 (24)	40.0 (16)	30.0 (12)	70.0 (28)	22.5 (9)	77.5 (31)

MLFD = Medial left front digit, LLFD = lateral left front digit, MLHD = medial left hind digit, LLHD = lateral left hind digit; [a] values followed by different letters in the same column statistically differ ($p\leq0.05$) by the Fisher's exact test.

Table 6. Occurrence of tapering and stretching in laminar tissue

Diet	Laminar tapering and stretching							
	MLFD		LLFD		MLHD		LLHD	
	No	Yes	No	Yes	No	Yes	No	Yes
D1% (n)	90 (9)[a]	10 (1)[a]	90 (9)[a]	10 (1)[a]	90 (9)[a]	10 (1)[a]	70 (7)[a]	30 (3)[a]
D2% (n)	70 (7)[ab]	30 (3)[ab]	90 (9)[a]	10 (1)[a]	60 (6)[ab]	40 (4)[ab]	50 (5)[a]	50 (5)[a]
D3% (n)	30 (3)[b]	70 (7)[b]	70 (7)[a]	30 (3)[a]	50 (5)[ab]	50 (5)[ab]	30 (3)[a]	70 (7)[a]
D4% (n)	40 (4)[b]	60 (6)[b]	50 (5)[a]	50 (5)[a]	30 (3)[b]	70 (7)[b]	40 (4)[a]	60 (6)[a]
Total	57.5 (23)	42.5 (17)	75.0 (30)	25.0 (10)	57.5 (23)	42.5 (17)	47.5 (19)	52.5 (21)

MLFD = Medial left front digit, LLFD = lateral left front digit, MLHD = medial left hind digit, LLHD = lateral left hind digit; [a,b] values followed by different letters in the same column statistically differ ($p \leq 0.05$) by the Fisher's exact test.

Table 7. Means of sole corium thickness (CT) in centimeters (standard error)

Digit CT	D1	D2	D3	D4
MRFD	0.35 (0.02)[a]	0.33 (0.02)[a]	0.31 (0.03)[a]	0.34 (0.03)[a]
LRFD	0.39 (0.02)[a]	0.37 (0.02)[a]	0.32 (0.03)[a]	0.33 (0.04)[a]
MLFD	0.39 (0.03)[b]	0.33 (0.02)[ab]	0.28 (0.02)[a]	0.33 (0.03)[ab]
LLFD	0.38 (0.03)[a]	0.39 (0.02)[a]	0.22 (0.02)[a]	0.34 (0.02)[a]
MRHD	0.29 (0.03)[a]	0.31 (0.27)[a]	0.25 (0.02)[a]	0.29 (0.03)[a]
LRHD	0.32 (0.02)[ab]	0.35 (0.03)[b]	0.26 (0.02)[a]	0.29 (0.02)[ab]
MLHD	0.29 (0.02)[a]	0.32 (0.02)[a]	0.24 (0.01)[a]	0.28 (0.02)[a]
LLHD	0.36 (0.02)[b]	0.30 (0.02)[ab]	0.26 (0.02)[a]	0.32 (0.03)[ab]

MRFD = Medial right front digit, LRFD = lateral right front digit, MLFD = medial left front digit, LLFD = lateral left front digit, MRHD = medial right hind digit, LRHD = lateral right hind digit, MLHD = medial left hind digit, LLHD = lateral left hind digit; [a,b] values followed by different letters in the same column statistically differ ($p \leq 0.05$) by the Fisher's exact test.

that received sucrose solution (D3) and fast fermentation (D4) and the Control Group (D1) (Table 6, Fig.2).

The percentage of tapering and stretching of the secondary epidermal laminae varied between the groups with different levels of dietary concentrate ($p<0.05$). Heifers in D3 and D4 showed higher tapering and stretching of the epidermal laminae in the medial left front digit compared with those in D1 and in the medial left hind digit between heifers in D4 and those in D1 (Table 6, Fig.2).

Histopathology of the laminar tissue (Table 6) identified presence of mild to moderate stretching and tapering lesions in the epidermal laminae. Sole corium thickness varied between heifers in D3 and D1 and those D2 ($p<0.05$, Table 7). When considering the parallelism between the dorsal surface of the distal phalanx and the innermost line of the epidermal region, no differences were observed between the groups ($p>0.05$).

DISCUSSION

The different diets used in the present study had an impact on the rumen environment of the heifers assessed, and those that received the fast fermentation diet (D4) presented mild clinical signs. Gressley et al. (2011) emphasized that integrity of the ruminal epithelium may be relevant for controlling the relation between acidosis and laminitis, as a potential for absorption of rumen toxins. This fact may explain the absence of clinical signs in the digits of the heifers evaluated, as lesions were only observed in the ruminal pillars, a site with lower toxin absorption potential compared with the papillary region.

Sucrose-induced animals (D3) and exclusively concentrate-treated animals (D4) showed lower ruminal motility and fluid pH compared with those in the control group (D1). Reduction ruminal fluid pH was due to rumen acid accumulation, which resulted from the fermentation of non-fibrous carbohydrate, especially highly-degradable starch - the main constituent of the cereal grains used in this study (corn and soybean residue). Variation in these ruminal characteristics to levels below 5.6 (ruminal fluid pH) and hypomotility are a key features for sub-acute ruminal acidosis (SARA) testing (Krause & Oetzel 2006). The animals in D1 and D2 received sufficient effective fiber to stimulate chewing and rumination, increasing salivation and, consequently, ruminal buffer, thus reducing fluctuations of ruminal characteristics (RM and pH).

The lower thickness observed in the digits of the heifers that received sucrose, despite the evident ruminal and histological parameters, did not characterize phalanx displacement (sinking) in the individuals that showed greater reduction in ruminal pH and motility, because of the distance between the horizontal planes of the band. The coronary artery and the apex of the distal phalanx extensor process did not vary. These clinical characteristics are expected when there are aggressive episodes of ruminal acidosis confirmed by the presence of lameness, a locomotion condition not evidenced in this study.

Acute and subacute attacks to the digitals resulting from the consumption of increased-grain diets were not clinically observed. Thoefner et al. (2004) validated the experimental induction technique for acute laminitis in cattle based on oligofructose overload. During this experiment, the animals developed signs of acute laminitis confirmed by histopathology; however, the degree of lameness was poorly perceived.

In another clinical trial using the same laminitis induction technique, the animals presented mild to moderate lameness, and the monitoring time and observers' experience were considered for the highest recorded dynamic assessment sensitivity (Danscher et al. 2009). Therefore, when seeking to identify variations in locomotion in confined animals with mild lameness by observational, interpretive and subjective methods, individual interpretation factors with different criteria are faced, affecting the results and hindering judicious measurements.

The presence of lesions in the epithelium of ruminal pillars due to changes in the forage:concentrate ratio was a very relevant finding, which is compatible with ruminal acidosis. These results were higher than those reported by Vechiato (2009) who analyzed cattle raised under intensive management system and identified a frequency of 11.8% ruminal acidosis. Although, numerically, the animals in D3 and D4 presented higher percentage of ruminitis, differences between the diets were not observed. It is speculated that if the increase of the sample was considered, significant results could be established, mainly taking into account differences between the ruminal clinical parameters (RM and pH) detected (Table 4, Fig.1).

Lymphocyte infiltrates in the supportive connective tissue of the ruminal mucosa indicate that the mononuclear response was not specific to any diet. This characterizes a stage of inflammatory reaction that usually follows the acute process, indicating recovery, especially when considering the close aggression of the rumen to the slaughter of at least seven days.

Integrity of the ruminal epithelium seems to be relevant to control the association between ruminal acidosis and laminitis. Although neither the acute and sub-acute forms were evidenced in this study for the different diets, histopathological differences in the digital tissues characterized a milder form of aggression to the corium and laminar tissue.

Based on these results, it is beneficial to explain how specific gross lesions occurred in the ruminal pillars considering the generalized acidity in the rumen environment. Some factors may have contributed to the occurrence of more aggressive pillar lesions. Indeed, the region of the rumen pillars presents morphometric and contraction characteristics that allowed more significant contact with acid diet, causing the mechanical damage evidenced. Van Soest et al. (1991) described that the size and number of rumen papillae per mucosal area influence the absorption and neutralization of acids in the rumen. In this context, the region of the ruminal pillars presents smaller morphometric characteristics than the papillary region (size and number), with reduced absorption and neutralization of acids, thus favoring formation of ulcers by acids produced in the rumen (D3 and D4) in contrast with the region covered by papillae.

Regarding cell morphometry, the results of the present study were similar to those reported by Mendes et al. (2013), who compared the dermis-epidermis junction of culling cows with and without lesions indicative of laminitis and found similar percentages in both cases. These results corroborate the pathological findings of the present study because, although the animals in D1 presented changes in cell percentage, relatively higher indices were observed in the laminar tissues of the heifers in D3 and D4, though without statistical difference in the comparison between groups. These changes in cell morphometry reveal that the pathophysiological mechanisms of laminitis may have been grouped into an imbalance of local cell metabolism and the internal ultrastructural integrity of the digit through a moderate predisposing vascular disorder, not resulting in marked breakdown of cellular integrity, and events that culminate in loss of laminar integrity (Stokes 2005).

Higher tapering and stretching of the epidermal laminae in the medial left front digit of animals in D3 and D4, compared with those in D1 and in the medial left hind digit between heifers in D4 and those in D1, are interpretative of chronic variations in laminar tissue. Therefore, by histopathology of the laminar tissue, it was possible to identify presence of mild to moderate stretching and tapering lesions of the epidermal laminae, which are consistent with the laminar histopathologic records described by Thoefner et al. (2004), who identified them in the first 24 h after the clinical attack of experimental laminitis induction in cattle and after 24 and 72 h of induction by oligofructose overload (Danscher et al. 2009).

According to Obel (1948), the decrease in the onychogenic substance (nail-forming) would be the cause of stretching of the epidermal laminae, a site that would first suffer the damage caused by laminitis. For Obel, the destabilization caused by stretching lesions could also contribute to the displacement of the distal phalanx inside the hoof, a condition not evidenced in the digits of the heifers in this study.

Parallelism between the dorsal surface of the distal phalanx and the innermost line of the epidermal region did not differ between the groups. In other words, beef cattle fed different diets did not show rotational phalangeal misalignment in any of their digits. These observations corroborate the absence of clinical signs of lameness in the beef heifers throughout the experimental period. According to Pollitt (2008), the dorsal surfaces of the wall and the distal phalanx should form parallel lines in healthy animals. In cases of laminitis, with the fragility of the hoof-phalanx junction, a positive distal angle is formed between these two lines, which increases as the phalanx rotates, pulled by the deep digital flexor tendon. In the studied population, all heifer digits presented zero angulations, which characterize parallelism.

These results point to variation of laminar characteristics between digits, characterizing clinically relevant information that should be considered. Possibly, variation in these morphometric characteristics between digits is associated with biomechanical stress and/or distinct systemic inflammatory response between the individuals in the aforementioned groups. Future studies are needed in order to analyze the differences between digits of confined cattle, taking into consideration the use of systemic biological markers, where the explanation for this may possibly be found.

Presence of low intensity lymphocytes in the laminar tissues characterized inflammatory and degenerative process of low intensity, not compromising the architecture of the heifers regardless of the diet, which certainly would be different in the presence of a large number of erythrocytes and leukocytes, leading to loss of laminar architecture (Paes Leme et al. 2010).

These significant morphometric differences (CT) between the digits of the heifers in this study were not related to the acidosis/laminitis complex, and may be associated with intrinsic (genetic and metabolic) and extrinsic (environmental) factors, which can influence skeleton formation and the process of growth and development.

CONCLUSIONS

Although diets with sucrose solution and increased-grain change the clinical profile of confined beef cattle, they promote mild aggression to the corium and laminar tissue and to the integrity of the epithelium, with histological changes suggestive of laminitis.

Ruminal papillary tissue seems to be relevant to control the association between acidosis and laminitis. Therefore, high-energy diets, as used in this study, alter some clinical parameters and the rumen environment, causing lesions in the rumen mucosa and, of lesser intensity, in the hoof corium and laminae, suggestive of laminitis.

Observation of animals submitted to this type of feeding management is relevant and deserves further studies.

Conflict of interest statement.- The authors have no competing interests to declare.

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Poisoning by *Brachiaria* spp. in various lamb breeds at increasing levels of supplementation during growth[1]

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ABSTRACT.- Melo G.K.A., Ítavo C.C.B.F., Silva J.A. Ferelli K.L.S.M., Silva P.C.G., Pupin R.C. & Lemos R.A.A. 2019. **Poisoning by *Brachiaria* spp. in various lamb breeds at increasing levels of supplementation during growth.** *Pesquisa Veterinária Brasileira 39(12): 978-982.* Laboratório de Anatomia Patológica, Faculdade de Medicina Veterinária e Zootecnia, Universidade Federal de Mato Grosso do Sul, Avenida Senador Felinto Muller 2443, Vila Ipiranga, Campo Grande, MS 79074-460, Brazil. E-mail: ricardo.lemos@ufms.br

The study evaluate the frequency of poisoning by *Brachiaria* spp. at the rearing, growth and termination stages in various lamb breeds at increasing supplementation levels. Forty-five lambs were used in the growth phase in pastures of *Brachiaria* spp. with a history of having induced poisoning. The lambs were distributed in nutritional treatments: lambs receiving mineral supplementation (MS), energy/protein supplementation (EPS) at 0.8% of body weight (bw), EPS at 1.6% bw and EPS at 2.4% bw. The lambs were allotted two flocks (F1 and F2) of 21 and 24 lambs each. Clinical signs of poisoning were observed in all treatments. All MS lambs died. The frequency of poisoning were highest in the 0.8% EPS and 1.6% EPS treatments. All lambs in the 2.4% EPS treatment recovered. F1 lambs had a higher frequency of poisoning than F2 lambs regardless of nutritional treatment. Morbidity rates for the F1 and F2 lambs were 52.3 and 16.7%, respectively. Supplementation was not sufficient to decrease the frequency of poisoning in lambs at the termination stage, which was dependent on the genetic origin of the lambs. EPS of 2.4% bw treatment, was an efficient nutritional strategy to minimize the effects of poisoning in lambs fed on *Brachiaria* spp.

INDEX TERMS: Poisoning, *Brachiaria* spp., lamb, supplementation, growth, hepatogenic photosensitization, toxic plants, small ruminants, plant poisoning.

RESUMO.- [**Intoxicação por *Brachiaria* spp. em várias raças de cordeiros com incremento nos níveis de suplementação durante o crescimento.**] O objetivo deste estudo foi avaliar a frequência da intoxicação por *Brachiaria* spp. nas fases de recria, crescimento e terminação em várias raças de cordeiros em níveis crescentes de suplementação. Quarenta e cinco cordeiros foram utilizados na fase de crescimento em pastagens de *Brachiaria* spp. com história de intoxicação, distribuídos em diferents tratamentos nutricionais: cordeiros recebendo suplementação mineral (SM), suplementação energética/protéica (SEP) a 0,8% do peso vivo (pv), SEP a 1,6% pv e SEP a 2,4% pv. Vinte e um cordeiros vieram do lote F1 e 24 cordeiros foram do lote F2. Sinais clínicos de intoxicação foram observados em todos os tratamentos. Todos os cordeiros com MS morreram. As frequências de intoxicação foram maiores nos tratamentos com 0,8% e 1,6% de SEP. Todos os cordeiros no tratamento com 2,4% de SEP se recuperaram. Cordeiros F1 apresentaram maior frequência de intoxicação que os cordeiros F2, independentemente do tratamento nutricional. As taxas de morbidade para os cordeiros F1 e F2 foram de 52,3 e 16,7%, respectivamente. A suplementação não foi suficiente para diminuir a freqüência de intoxicação em cordeiros na fase de terminação, dependente da origem genética dos cordeiros. O SEP de 2,4% pv, no entanto, foi eficiente para minimizar os efeitos da intoxicação em cordeiros alimentados com *Brachiaria* spp.

TERMOS DE INDEXAÇÃO: Intoxicação, *Brachiaria* spp., cordeiros, suplementação, crescimento, fotossensibilização hepatógena, plantas tóxicas, pequenos ruminantes.

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INTRODUCTION

Most cultivated pastures in Brazil contain grasses of the genus *Brachiaria* (Oliveira et al. 2014). The Brazilian Cerrado, a large savanna, has an estimated 60 million ha of cultivated pastures, 85% of which contain *Brachiaria* (Macedo 2005). Poisoning by these grasses, however, causes direct and indirect losses in sheep flocks (Mustafa et al. 2012, Porto et al. 2013). Outbreaks have been described in weaned lambs in the growth phase (Riet-Correa et al. 2011, Mustafa et al. 2012, Pupin et al. 2016) and in suckling lambs (Melo et al. 2018), demonstrating that these categories are more vulnerable than others, being susceptible to poisoning from the beginning of the consumption of the forage (Melo et al. 2018).

The influence of genetics on the sensitivity to poisoning (Pupin et al. 2016, Castro et al. 2018) indicates that the selection of animals genetically resistant to poisoning is an efficient means of prevention. This selection, however, is a slow process, because methodologies for the identification of resistant animals have not been defined. Alternatives such as controlled grazing (Castro et al. 2018) reduce the time of grazing and consequently the amount of grass eaten by sheep. This method, although efficient under experimental conditions, requires the cultivation of forages other than *Brachiaria* spp., limiting factor in properties that are constituted only by foragers of this genus.

Dietary supplementation is a promising and easily applied alternative for suckling lambs (Melo et al. 2018) but has not been evaluated in other categories of sheep.

The aim of this study was to evaluate the frequency of poisoning by *Brachiaria* spp. in various lamb breeds during the growth phase submitted to increasing levels of energy/protein supplementation (EPS).

MATERIALS AND METHODS

The experiment was approved by the "Universidade Federal de Mato Grosso do Sul" (UFMS) ethics committee on the use of animals, protocol no. 481/12. It was conducted at the experimental farm of the Faculty of Veterinary Medicine and Animal Science, (20°26'34.31"S, 54°50'27.86"W), UFMS.

We tested 45 lambs with a mean age of 80 days and a mean weight of 19.50kg from two flocks, F1 and F2. We tested 45 lambs with a mean age of 80 days and a mean weight of 19.50kg from two flocks, Flock 1 (F1) and Flock 2 (F2). The breed composition of the herds was as follows: F1 lambs were the product of crosses between mixed-breed ewes raised from birth on pastures of *Brachiaria* spp. with an Ile de France ram. F2 lambs were the product of crosses between naïve (not previously exposed to *Brachiaria*) Dorper x Santa Inês ewes with a White Dorper ram. Both rams that originated F1 and F2 herds were naïve and were adapted to the consumption of *Brachiaria* spp., as previously described (Melo et al. 2018).

The experiment comprised two trials. The first trial began on August 16 and ended on November 26, 2013, and the second trial began on July 11 and ended on September 19, 2014. The termination phase (weaning to slaughter) was used as the experimental period.

Weaned lambs were distributed in four treatments (Table 1) and began receiving different levels of EPS. The lambs were allocated in pens containing *Brachiaria* grasses with a history of poisoning. EPS was provided daily at 8:00 a.m., and lambs had free access to food, water and mineral supplementation (MS) regardless of treatment. The pens were mixed pastures of *Brachiaria humidicola*, *B. decumbens* and *B. brizantha*, with a predominance of *B. brizantha*. Twelve 0.4-ha pens (three replicates per treatment) were used. The animal load used per pen was determined by the supply of leaf dry matter - 10% of body weight (bw) -, with variable stocking, and control sheep were used to adjust the animal load when necessary.

The concentrations of protodioscin in *Brachiaria* spp. were determined from 10 samples collected from representative areas in each plot every 28 days using metal quadrats of 0.5×0.5m. The grasses were cut close to the soil (McMeniman 1997), and the leaves were subsequently separated. The leaves were dried in a forced-ventilation oven at 55°C for 96h and ground in a mill with 1-mm sieves. The concentration of protodioscin was determined by the method described by Ganzera et al. (2001).

Lambs were weighed at the beginning and end of the experimental period, with fasting of solids for 16h, and weighed every 14 days during the experimental period. The experimental period ended when the animals in the group with the highest nutritional intake (2.4% EPS) reached 32kg bw.

Parasitological follow-up and anthelmintics treatment were performed every 14 days. Fecal samples were collected directly from the rectal ampulla for quantifying the number of eggs per gram (EPG) of feces (Gordon & Whitlock 1939 - modified), with a sensitivity of 1:25. Lambs with EPG counts ≥500 were dewormed with monepantel.

The lambs were monitored daily for signs of poisoning by *Brachiaria* spp. The expected lesions for determining poisoning in the lambs were restricted to specific sites of non-pigmented skin, mainly in the face and ears. Clinical signs consisted of edema, alopecia, erythema, formation and detachment of scabs, apathy, anorexia, jaundice, and weight loss (Mustafa et al. 2012, Porto et al. 2013, Pupin et al. 2016, Melo et al. 2018).

Lambs presenting clinical signs were separated from the flock and placed in shelters protected from solar radiation. They were fed with Tifton-85 hay (*Cynodon dactylon*), EPS, MS and water ad libitum. The lambs were reintroduced to their treatments when they had completely recovered. The lambs that died were necropsied, and fragments of various organs were sampled, fixed in 10% formalin, routinely processed and stained with hematoxylin-eosin for histological examination.

The experimental design consisted of randomized blocks based on the flock of origin and the distribution of lambs in the treatments

Table 1. Distribution of lambs by supplementation level

Treatment[a]	Number of animals (F1/F2)[b]	Description
MS	11 (4/7)	Lambs in continuous grazing, receiving MS
0.8% EPS	12 (5/7)	Lambs in continuous grazing, receiving MS and 0.8% body weight (bw[c]) EPS
1.6% EPS	10 (7/3)	Lambs in continuous grazing, receiving MS and 1.6% bw EPS
2.4% EPS	12 (5/7)	Lambs in continuous grazing, receiving MS and 2.4% bw EPS

[a] MS = mineral supplementation, EPS = energy/protein supplementation, [b] F1 = flock 1, F2 = flock 2, [c] bw = body weight.

(MS, 0.8% EPS, 1.6% EPS or 2.4% EPS). The frequency of poisoning was assessed by a chi-square test at $P<0.05$.

RESULTS

The concentrations of protodioscin (% dry matter) ranged from 1.04 to 2.17 (mean±SD: 1.59±1.33) in the experimental pens. Clinical signs of poisoning were observed at all levels of supplementation. The epidemiology, clinical evolution, genetic origin and nutritional treatment at the termination stage of the lambs submitted to pasture supplementation are described in Table 2.

The frequencies of poisoning were highest at the 0.8 and 1.6% EPS supplementation levels. Five lambs in the 0.8% EPS treatment became ill but recovered. Four lambs in the 1.6% EPS treatment presented clinical signs; three survived and one died. Three lambs in each of the MS and 2.4% EPS treatments became ill. All three lambs in the MS treatment died, and all three lambs in the 2.4% EPS treatment recovered (Table 2).

The frequency of poisoning did not differ significantly ($P>0.05$) between the supplementation levels (Table 3). The frequency of poisoning, however, differed significantly ($P<0.05$) between the two flocks. The morbidity rates of F1 and F2 lambs were 52.3% (11/21) and 16.7% (4/24), respectively.

Clinical, necropsy and histopathological findings differed in intensity but were similar between treatments. The main clinical signs included hepatogenic photosensitization, with lesions restricted to specific sites of non-pigmented skin, mainly on the face, and symptoms consisted of edema, alopecia, erythema, formation and detachment of scabs, apathy, anorexia, and weight loss. The necropsy findings were similar among the treatments and consisted of alopecia and crust formation around the eyes and ears, with scar retraction of the ears. Livers had rounded edges and an evident lobular pattern and were diffusely light brown. The kidneys were blackened, and the cut pelvis was yellowish. Histological lesions were present in the liver and the skin of the ears and eyelids.

DISCUSSION

The concentrations of protodioscin (1.59±1.33%) were similar to those previously considered toxic for lambs. Concentrations ranging from 0.74 to 2.56% have been reported in weaned lambs (Mustafa et al. 2012, Pupin et al. 2016) and from 0.62 to 1.15% in suckling lambs (Melo et al. 2018).

Table 2. Epidemiology, genetic origin, clinical evolution and history of *Brachiaria* spp. poisoning in the rearing stage of the lambs

Treatment[a]	Flock	Age[b] (d)	OCS[c] (d)	Month of poisoning	Clinical evolution (d)		History of poisoning
					Recovery	Death	
MS	F1	115	20	July		12	Yes
MS	F2	88	11	July		25	Yes
MS	F1	105	18	July		93	Yes
0.8% EPS	F2	123	24	August	43		Yes
0.8% EPS	F2	123	24	August	43		No
0.8% EPS	F1	97	33	August	28		No
0.8% EPS	F1	126	21	July	23		Yes
0.8% EPS	F1	110	21	July	24		Yes
1.6% EPS	F1	113	31	October	14		Yes
1.6% EPS	F1	107	14	July		56	Yes
1.6% EPS	F1	117	28	August	44		No
1.6% EPS	F1	115	26	July	52		Yes
2.4% EPS	F1	109	62	November	13		Yes
2.4% EPS	F2	167	120	January	31		No
2.4% EPS	F1	68	12	July	15		Yes

[a] MS = mineral supplementation, 0.8% EPS = lambs receiving 0.8% bw EPS, 1.6% EPS = lambs receiving 1.6% bw EPS, 2.4% EPS = lambs receiving 2.4% of bw EPS, [b] age at onset of clinical signs, [c] onset of clinical signs after the start of the experimental period (after weaning), [d] bw = body weight.

Table 3. Frequency of poisoning of F1 and F2 lambs in the growth phase at increasing levels of supplementation

Treatment	Flock		*P*
	F1[a]	F2[b]	
	Positive cases (%)		
MS	50.0 (2/4)[a]	14.3 (1/7)[b]	0.8204
0.8% EPS	60.0 (3/5)[a]	28.6 (2/7)[b]	0.8220
1.6%EPS	57.1 (4/7)[a]	0.0 (0/3)[b]	0.8450
2.4% EPS	40.0(2/5)[a]	14.3 (1/7)[b]	0.8320
P	0.0202	0.0222	-

[a] Lambs of the F1 flock are SRD sheep raised on *Brachiaria* spp. pastures and an Ile de France breeder adapted to the consumption of *Brachiaria* spp., [b] lambs of the F2 flock are offspring of Santa Inês × Dorper sheep and a White Dorper ram adapted to the consumption of *Brachiaria* spp.; different lowercase letters within a row indicate significant differences by a χ^2 test ($P<0.05$).

The frequency of poisoning was higher in F1 than F2 regardless of the level of supplementation. A genetic component was thus likely responsible for the resistance or sensitivity of the animals to poisoning. The role of genetics in the sensitivity to poisoning has previously been described by Pupin et al. (2016), who reported a morbidity of 73.33% and varying degrees of sensitivity in offspring of susceptible parents, but lambs from resistant parents presented no clinical signs. Fifteen lambs presented clinical signs during the experimental period, 11 of which had already had signs of poisoning in the breeding phase, with recurrence in the termination phase. Of these 11, seven (63.63%) survived and four (36.36%) died. Four other lambs presented signs of poisoning only at the termination stage, and all survived. The sheep therefore had adapted to the consumption of *Brachiaria* spp. to different degrees. The mechanisms of this adaptation remain unknown, but changes may occur in the ruminal microbiota, altering the transformation of the toxin (Albernaz et al. 2010, Riet-Correa et al. 2011). The transfer of ruminal content of adult sheep adapted to grazing *B. brizantha* to susceptible lambs can reduce the morbidity rate relative to lambs that do not receive transfaunation (Castro et al. 2018).

The genetic origin of the lambs was a determinant for the occurrence of poisoning. Three of the four deaths were in F1. The severity of the clinical signs, however, differed among the levels of supplementation. Five, four and three animals at the 0.8, 1.6 and 2.4% EPS supplementation levels, respectively, presented clinical signs. Only one animal died in the 1.6% EPS treatment. Three animals in the MS treatment became ill, all of which subsequently died. EPS minimized the effects of poisoning, influencing mortality rates at different levels of supplementation.

In another study, EPS efficiently prevented the effects of poisoning in suckling lambs reared on *Brachiaria* spp. and supplemented with creep feeding, with only four of 34 lambs presenting clinical signs of poisoning, but 17 of 34 lambs reared under the same grazing conditions but receiving only MS presented clinical signs (Melo et al. 2018).

We did not determine the mode of action of the supplement but presumed that the ruminal microbiota had been modified by the ingestion of the concentrated feed (Miles et al. 1991), altering the sequence of chemical changes of lithogenic steroid saponins in the rumen and consequently damaging liver tissue (Miles et al. 1994, Meagher et al. 1996, Albernaz et al. 2010). The macro- and microscopic lesions were characteristic of poisoning by *Brachiaria* forages (Mustafa et al. 2012, Porto et al. 2013, Pupin et al. 2016, Melo et al. 2018).

CONCLUSION

Increasing levels of EPS were not sufficient to decrease the frequency of poisoning, which depended on the genetic origin of the lambs. EPS of 2.4% bw, however, was an efficient nutritional strategy to minimize the effects of poisoning in lambs fed on *Brachiaria* spp.

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Statement of animal rights.- All procedures performed with the animals of the present experiment were in accordance with the ethical standards established by the Ethics Committee on the Use of Animals (CEUA) of the "Universidade Federal de Mato Grosso do Sul" (UFMS).

Conflict of interest.-The authors declare that there are no conflicts of interest

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Original Article
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Oral trichomoniasis in raptors in Southern Brazil[1]

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ABSTRACT.- Echenique J.V.Z., Soares M.P., Bruni M., Farias N.A., Moretti V.D., Bandarra P.M., Albano A.P.N. & Schild A.L. 2019. **Oral trichomoniasis in raptors in Southern Brazil**. *Pesquisa Veterinária Brasileira 39(12):983-988.* Laboratório Regional de Diagnóstico, Faculdade de Veterinária, Universidade Federal de Pelotas, Campus Capão do Leão s/n, Pelotas, RS 96010-900, Brazil. E-mail: gmpsoares@gmail.com

A retrospective study of oral trichomoniasis cases in raptors was carried out at "Laboratório Regional de Diagnóstico" of "Faculdade de Veterinária" of "Universidade Federal de Pelotas" (LRD-UFPel) from December 2014 to August 2017. Seven necropsy reports were reviewed. All raptors including Falconiformes and Strigiformes orders were from periurban zones. Four birds were adults, and there was no apparent sex predisposition. Clinical signs included dysphagia, regurgitation, and anorexia. Gross lesions were characterized by multifocal yellow to coalescent caseous nodules adhered to the oral cavity, which extended to the entrance of the esophagus. Microscopically, the lesions were characterized by severe focally extensive heterophilic granulomatous stomatitis. *Trichomonas gallinae* was isolated in modified Diamond medium of all samples collected from birds. It is believed the transmission occurred by the predation of domestic pigeons (*Columbia livia domestica*) contaminated with *T. gallinae* that agglomerate in patios of grain processing complexes.

INDEX WORDS: Oral trichomoniasis, raptors, Brazil, *Trichomonas gallinae*, *Caracara plancus*, *Asio clamator,* oral trichomoniasis, spillover, wildlife animals.

RESUMO.- [**Tricomoníase oral em rapinantes no sul do Brasil.**] Foi realizado um estudo retrospectivo de casos de tricomoníase oral em rapinantes recebidos no Laboratório Regional de Diagnóstico da Faculdade de Veterinária da Universidade Federal de Pelotas (LRD-UFPel) no período de dezembro de 2014 a agosto de 2017. Foram revisados os protocolos de necropsia encontrando-se sete casos. Os animais eram todos de zonas periurbanas e foram afetadas a ordem Falconiformes e a ordem Strigiformes. Das aves afetadas 4/7 eram adultas e não houve aparente predisposição por sexo. Os sinais clínicos incluíram disfagia, regurgitação e anorexia. As lesões se caracterizaram por nódulos amarelados multifocais a coalescentes aderidos a cavidade oral, que se estendiam até a entrada do esôfago. Histologicamente as lesões caracterizaram-se por estomatite heterofílica granulomatosa focalmente extensiva acentuada. Foi isolado em meio Diamond modificado *Trichomonas gallinae* de todas as amostras coletadas das aves. Acredita-se que a fonte de transmissão tenha ocorrido a partir da predação por estes rapinantes de pombas domésticas (*Columbia livia domestica*) portadoras com *T. gallinae* que se aglomeram em pátios de complexos de beneficiamento de grãos.

TERMOS DE INDEXAÇÃO: Tricomoníase oral, rapinantes, Brasil, *Trichomonas gallinae*, *Caracara plancus, Asio clamator*, tricomoníase oral, spillover, aves silvestres.

INTRODUCTION

Oral trichomoniasis in prey is a cosmopolitan disease that has been described in several bird orders from various countries (Peppler & Oettié 1992, McKeon et al. 1997, Krone et al. 2005) and islands (Harmon et al. 1987, Padilla et al. 2004,

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Forzán et al. 2010). In Brazil, the disease has been described in birds of prey in São Paulo (Joppert 2007) and in Minas Gerais in *Ramphastidae* family, birds of prey, and passerines (Ecco et al. 2012, Andery et al. 2013). In Rio Grande do Sul there are reports of trichomoniasis in domestic pigeons (Tasca & De Carli 1999), and cases in prey are not described. Studies have shown that it is a disease with high potential for dissemination and a severe factor in decreasing wild bird populations worldwide. This is mainly due to the severity of the lesions and the destruction of oral cavity tissues and surrounding areas (Cole 1999, Forrester & Foster 2008). The disease is caused by *Trichomonas gallinae*, a mobile protozoan, which has four typical free flagella arising from a basal granule at the anterior pole of the organism and an undulating membrane (Stabler 1941, BonDurant & Honigberg 1994). This protozoan has a wide variety of hosts (Forrester & Foster 2008) and the domestic pigeon (*Columba livia domestica*) is the main reservoir and source of infection for birds of prey and can be a limiting factor for the conservation of these species (Tasca & De Carli 1999). *T. gallinae* has a direct life cycle, having no cyst or resistance form, and can be transmitted from parents to nestlings by regurgitation (crop milk), infected feeders and/or acquired in contaminated water (Kocan 1969, De Carli et al. 1979, Cole 1999, Purple & Gerhold 2015).

Birds of prey are carnivorous and comprise several orders including: Accipitriforms orders represented by hawks and eagles such as *Parabuteo unicincus* (Harris's hawk) and *Rupornis magnirostris* (Roadside hawk); Falconiformes, with 63 species that are mainly represented by "Carcarás" (genera *Daptrius*, *Milvalgo* and *Caracara*); and Strigiformes which comprises owls and has 223 described species (Piacentini et al. 2015). Birds of prey can be found in Brazilian biomes, are often submitted in wildlife rehabilitation centers politraumatized (Joppert 2007). In addition, these birds are affected by a wide variety of diseases, including oral trichomoniasis (Work & Hale 1996, Joppert 2007, Park 2011). This disease has been described in wildlife rehabilitation centers, especially affecting birds from periurban areas, with a 10% prevalence rate (Joppert 2007, Ecco et al. 2012). In private clinics in the Middle East, in falconry raptors the disease prevalence ranges from 8% to over 30% (Samour et al. 1995, Bailey et al. 1996). Cases of oral trichomoniasis in free-range birds of prey have not been described in Rio Grande do Sul State.

The aim of the present study was to describe the clinical, epidemiological, gross and histological aspects of oral trichomoniasis in free range birds of prey submitted for necropsy at Regional Diagnostic Laboratory of the Veterinary Faculty of the "Universidade Federal de Pelotas" (LRD-UFPel). As well suggest the form of contamination of these birds in the southern region of Rio Grande do Sul.

MATERIALS AND METHODS

From 2014 to 2017, seven cases of birds of prey suspected of oral trichomoniasis were received at the LRD-UFPel. These birds were sent from December 2014 to August 2017 by the Wildlife Rehabilitation Center (NURFS-CETAS-UFPel), and the clinical records were obtained with the responsible veterinarian. All birds were necropsied and samples from all organs were collected and fixed in 10% buffered formalin, routinely processed for histology and stained with hematoxylin and eosin (HE). Fragments of tongue and palate lesions were stained with periodic acid-Schiff (PAS) and Masson's trichrome (MT). Two swab samples were collected from oral lesions for parasitological analysis. One was and immediately examined between slide and coverslip with physiological solution in light microscope. The second sample was collected for parasitological culture and inoculated in modified Diamond media. The samples were inoculated in a falcon tube containing bovine fetal serum, plus 0.3mg/mL meropenem and 0.2mg/mL amphotericin B and incubated at 37°C for 24 to 48 hours (Diamond 1957). The tube was centrifuged at 1500rpm for 10 minutes, and an aliquot of the pellet was placed between slide and coverslip for viewing the trophozoites under light microscopy.

RESULTS

Clinical signs and epidemiology

From December 2014 to August 2017 were submitted seven raptors from six different species, belonging to two families, *Strigidae* (4/7) and *Falconidae* (3/7). The birds came from periurban areas of Rio Grande, Pelotas, and Camaquã municipalities and were sent submitted to NURFS-CETAS-UFPel, presenting oral lesions compatible with oral trichomoniasis. The bird's category was juvenile (3/7), and adult (4/7) with three males and four females. The cases were registered between October and February, and one case was observed in August. The disease progressed from one to nine days. One bird was dead on arrival, and two others were euthanized due to severity lesions and poor prognosis. The summary of epidemiological data is presented in Table 1. All seven birds had poor body condition being 4/7 lean and 3/7 cachectic. Clinical signs observed in all seven birds were characterized by dysphagia, regurgitation, and anorexia. Signs of respiratory distress and rales, orthopnea position, and open beak were observed in one bird. In three birds, the feathers were covered with regurgitation remains.

Pathology

All animals received were presenting the chronic form of the disease, and there were lesions in the oral cavity that ranged from small nodules to yellowish plaques measuring aproximally 5mm, to large, well-adhered, multifocal nodules that occupied almost the entire palate. (Fig.1). Also, brownish velvet plaque lesions were observed. These lesions could be observed on the hard palate and choanae. In two birds, part of the jugal and maxillary bones was affected, with tissue loss and necrosis (Fig.2). In Bird Number 5 was also observed bone involvement with sinusitis and impairment of the cribriform laminae.

Histologically the lesions were characterized by multifocal to coalescent severe heterophilic ulcerative stomatitis and/or esophagitis. It was observed a hipeosinophilic center surrounded by macrophages and giant cells, degenerated heterophils and lymphocytes with a discrete fibroblast and connective tissue proliferation within the peripheral areas. Around the granulomas, there was also an abundant inflammatory infiltrate of lymphocytes, plasmocytes, and macrophages associated with necrotic areas (Fig.3). In some fragments, it was possible to observe hyperplasia of the epithelium adjacent to the lesions. In PAS staining it was not possible to observe the trophozoites. In cases where bone involvement was observed, there was bone loss, necrotizing osteomyelitis, osteolysis, and bone remodeling with areas of fibrosis best observed on MT staining.

Table 1. Epidemiological data and macroscopic lesions of the eight cases of oral trichomoniasis in prey received at LRD-UFPel from 2014 to 2017

Case no.	Common name/ species	Date	Municipality of origin	Gender and age	Clinical course	Macroscopic lesions	Concomitant diseases
1	American kestrel/ *Falco sparverius*	12/2014	Pelotas	Adult female	1 day	Extensive yellow erosive lesion from the tip of the beak until the jugal arch on the lower right side of the oral cavity affecting only soft tissue. Near the jugal arch the subcutaneous tissue is swollen forming a 1.5cm diameter nodule.	No
2	Striped owl/ *Asio clamator*	2/2015	Camaquã	Young female	Arrived dead	Yellowish nodule approximately 1cm in diameter adhered to the soft palate, which partially obstructed the glottis.	Dislocation of the right tibiotarsometatarsal joint with associated myiasis
3	American barn owl/ *Tyto furcata*	12/2016	Camaquã	Adult male	1 day	Friable yellow-brown plaque extending across the soft palate and half of the hard palate on the left side only. Multifocal necrotic nodules with caseous appearance, whitish and soft to the cut, measuring 3mm in diameter around the tongue and ulcers in the right hard palate about 5mm in diameter.	Severe multifocal pulmonary aspergillosis
4	Southern caracara/ *Caracara plancus*	2/2016	Rio Grande	Young male	4 days	5cm yellowish nodule extending from the right jugal arch to the mandible branch. Velvety necrotic plaques about 7mm, diffuse around the enlarged tongue.	No
5	Striped owl/ *Asio clamator**	10/2016	Pelotas	Adult female	0 days	Caseous, yellow oral plaque lesion occupying the right half of the hard palate with bone involvement and extended to the esophageal entrance. There was necrosis of the cribriform skull blades, sinusitis and palpebral edema.	No
6	Southern caracara/ *Caracara plancus*	11/2016	Rio Grande	Young male	7 days	Ulcerative yellow lesion affecting the mandible, maxilla, pterygoid region and palatal region with bone involvement of the jugal arch and exposure of the right choanae.	Marked diffuse fibrin heterophilic pneumonia
7	Great horned owl/ *Bubo virginianus*	8/2017	Rio Grande	Adult female	0 days	Yellowish plaque affecting only the left side, affecting two thirds of the hard palate and the entire soft palate forming a large mass that caused obstruction of the esophageal entrance.	No

* Animals arrived at NURFS with extensive lesions and were euthanized.

Parasitology

On direct examination, no parasites were found. In the culture of the lesions, mobile organisms were observed 24 hours after incubation. The identification of *Trichomonas gallinae* was performed by observing the morphological aspects described by Stabler (1941) and Abraham & Honigberg (1964). The organisms had a piriform shape, rounded at both ends with 10.1-14.8μm in length x 7.3-8.0μm wide, well-developed undulating membrane, not reaching the posterior end of the parasite, with accessory filament, clearly evident axostyle about 1/3 the size of the cell and exposed at the posterior portion; four uneven anterior flagella, absence of the fifth free flagella in the posterior portion and oval nucleus (Fig.4).



Fig.1. Bird Number 7. *Bubo virginianus*. Oral cavity. Caseous yellowish focally extensive plaque, occupying the left half of the hard palate, compromising the choanae and extending to the esophageal entrance.

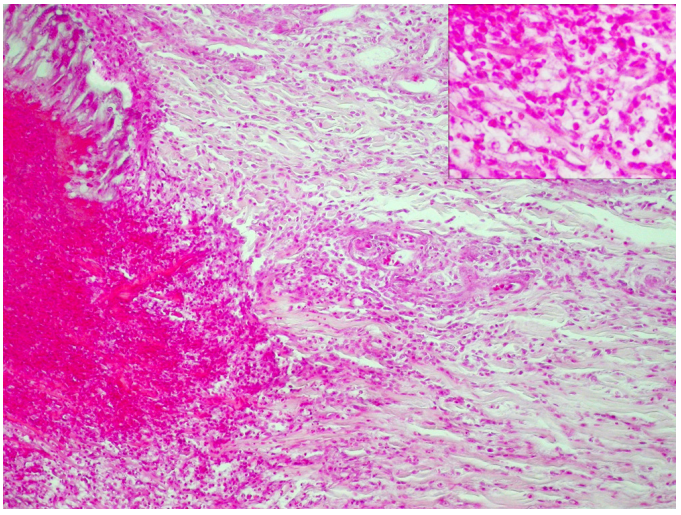


Fig.3. Bird Number 6. *Caracara plancus*. Hard palate. Hypereosinophilic area surrounded by macrophages, giant cells with discrete fibroblast proliferation (upper left) and inflammatory infiltrate containing degenerate heterophils and macrophages in the periphery and center of the tissue. HE, obj.10x. Inset: more massive enlargement of the lower-left corner where degenerate heterophils and macrophages are observed. HE, obj.40x.



Fig.2. Bird Number 6. *Caracara plancus*. Beak and oral cavity. Ulcerative yellow- brown lesion with detachment and jugal bone, mandible, maxilla, pterygoid region, and palatal region necrosis with exposure of the bottom of the eye.

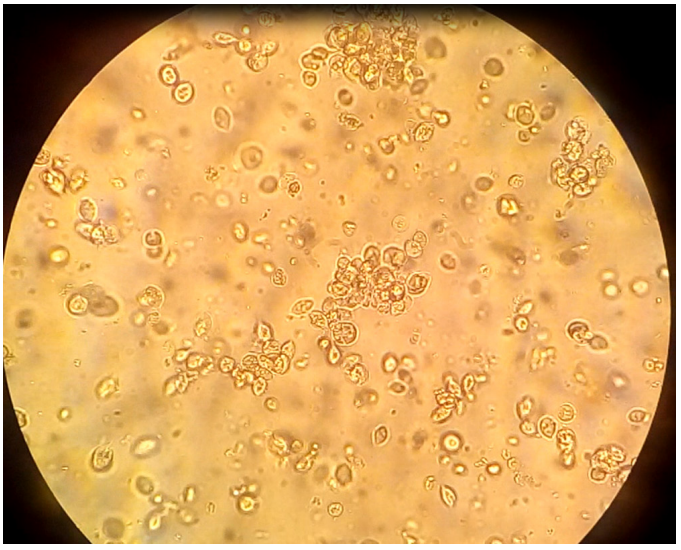


Fig.4. Bird Number 3. *Tyto furcata*. Diamond media, direct smear, 100x. Innumerous trophozoites of *Trichomonas gallinae* after 24 hours incubation. It can be notices the pyriform and/or rounded shaped organisms.

DISCUSSION

The diagnosis of oral trichomoniasis by *Trichomonas gallinae* was based in clinical signs, macroscopic and histological lesions and morphological identification of the agent described by other authors (Stabler 1941, Abraham & Honigberg 1964).

Birds with trichomoniasis in the present study presented variations in severity and lesions extension, and in two cases, there was involvement of the esophagus and crop. It has been reported that the disease is more severe in young animals (Cole 1999). In this study, this fact can be observed and confirmed since 3/7 were adult birds, and juvenile birds had more severe lesions. On the other hand, it has also been mentioned that the lesions are even more severe in birds from naïve populations. This fact is highlighted by nearby extinction of islands endemic bird populations due to introduction of *T. gallinae* reservoir species (Rosenfield et al. 2002, Park 2011). In the present report, the affected animals probably belonged to disease-free populations and/or were

immunosuppressed due to lack of food. The hypothesis of immunosuppression is reinforced by the occurrence of a case of aspergillosis among the affected prey.

The cases occurred mainly in the warmer months (from October to February). It has been suggested that high temperatures associated with accumulated moisture in grains dispersed in grain plants yards may be an initial source of contamination and spread among domestic pigeons inhabiting periurban areas (Purple & Gerhold 2015) and consequently contamination of their predators.

Histologically in all birds, coalescing multifocal granulomatous heterophilic ulcerative stomatitis was observed and only two animals had bone involvement. Although PAS staining is recommended (Mesa et al. 1961, Narcisi et al. 1991), in the present study there was no positive histochemical staining for the agent, as found by other authors (Amin et al. 2011). Other special stains can also be used to facilitate visualization of *T. gallinae* in tissues, such as Warthin-Starry staining (Araujo et al. 2018). Direct examination of the lesions was also not efficient in identifying the agent after swab collection from the birds' oral cavity. The Diamond culture was the most efficient diagnostic method (Boal et al. 1998, Boal & Mannan 1999, Amin et al. 2014). This media was developed for the detection of *Trichomonas* sp. in women and has been used as the gold diagnostic standard for the identification of this parasite (Diamond 1957).

Oral trichomoniasis is considered an emerging disease in many parts of the world and is affecting wild species in which there are no previous reports of the disease, characterizing the spillover (Bunbury et al. 2007, Forzán et al. 2010, Lawson et al. 2011). The disease should be differentiated from oral candidiasis, fowlpox (pox virus) and hypovitaminosis A, since in these diseases oral lesions are similar to those described in trichomoniasis (Cole 1999, Joppert 2007, McDougald 2013).

In the present study, it is believed that the affected raptors were contaminated by their prey (domestic pigeons). Most came from the Rio Grande municipality, where, due to the existence of one of the largest bulk carriers complex in Brazil, there is a large agglomeration of birds, including the domestic pigeon; and from Camaquã and Pelotas where there are grain-processing complexes. All animals came from those periurban areas where those plants are located. Although pigeons are not normal food items in raptors diet, it is likely that due to insufficiency of food because of environmental degradation, these raptors are changing their consumption habits and may have started to prey pigeons, which are important in the *T. gallinae* maintenance life cycle as reservoirs (Tasca & De Carli 1999). The pigeons may feed on the loose grains within this area. *T. gallinae* protozoan has already been isolated from wet grains, becoming viable for up to five days (Kocan 1969, Purple & Gerhold 2015), which would lead to contamination of pigeons and maintain the agent in the environment and its life cycle.

Besides, the all raptors present in this study had a low body condition and half had associated diseases, which probably reduced their hunting capacity of birds, forcing them to look for easy and probably infected prey. Some studies have shown that domestic and wild Columbiformes harboring *T. gallinae* have lower body condition, even clinically healthy, making them more susceptible to predation (Villanúa et al. 2006). In other countries the source of pigeon contamination are garden feeders and waterers, which lead to bird crowding (Cole 1999, Lawson et al. 2011, 2012). Four female birds were affected in the reproductive season (spring/summer). This can be explained because these birds have a tendency to look for easier preys in order to no stay away from the nest.

CONCLUSIONS

Trichomoniasis occurs in the southern region of Rio Grande do Sul in different species of raptors and its contamination is probably due to the predation of reservoir pigeons that feed on loose grains in the bulk terminals of periurban areas of the state municipalities of Camaquã, Pelotas and Rio Grande and can be considered a spillover event. The disease affected both young and adult animals and was characterized by all cases of a long evolution with granulomatous lesions.

It was also concluded that PAS is not suitable for staining the agent, and *Trichomonas gallinae* proliferates in a modified Diamond culture media.

Conflict of interest statement. The authors declare that they have no conflict of interest.

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Ultrasound monitoring of the uterus and ovaries of dominant and subordinate females of yellow-breasted capuchin (*Sapajus xanthosternos*) and robust tufted capuchin (*Sapajus robustus*) in captive colonies during the ovarian cycle and anestrus periods[1]

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ABSTRACT.-Pissinatti T.A., Ribas J.A.S., Maróstica E., Pissinatti A. & Ferreira A.M.R. 2019. **Ultrasound monitoring of the uterus and ovaries of dominant and subordinate females of yellow-breasted capuchin (*Sapajus xanthosternos*) in robust tufted capuchin (*Sapajus robustus*) in captive colonies during the ovarian cycle and anestrus periods.** *Pesquisa Veterinária Brasileira 39(12):989-996*. Departamento de Fisiologia e Farmacologia, Instituto Biomédico, Universidade Federal Fluminense, Rua Professor Hernani Melo 101, São Domingos, Niterói, RJ 24210-130, Brazil. E-mail: ribasjas@vm.uff.br

The yellow-breasted capuchin (*Sapajus xanthosternos*) and robust tufted capuchin (*Sapajus robustus*) are endangered species due to destruction of their natural habitat and predatory chase. However, it is still necessary to elucidate some details of their reproductive physiology in order to obtain better indices in the assisted reproduction of these species. This study aimed to evaluate the ovarian cycle of 13 dominant and subordinate females of *S. xanthosternos* (n=8) and *S. robustus* (n=5) using sagittal and transversally scanned ultrasound of their uterus and ovaries. Sonograms were performed every seven days for two months. The ovarian cycle phase and anestrous condition were confirmed by colpocytology. Our results showed different uterine parameters (craniocaudal diameter, dorso-ventral diameter, and transverse diameter) (P<0.05) between anestrous subordinate females and other ovarian cycle phases and social classes. The mean of uterine volume was higher in dominant females than subordinate females in all cycle phases (P<0.05), except in follicular phase. During anestrus, endometrial width was smaller in subordinate females than in dominant females (P<0.05). Subordinate females showed differences in endometrial measures (P<0.05) between anestrous period and follicular and luteal periods. Ovarian measures in dominant females were higher than in subordinate females only during anestrus (P<0.05). In the subordinate females, ovarian parameters were different (P<0.05) between anestrus and follicular and luteal phases. Dominant females showed higher volume of right ovary compared to volume of the left ovary during anestrus and follicular phase (P<0.05). Follicles and corpus luteum were distinguished by ultrasonography in most exams (86.11%). During anestrus, measurable ovarian structures were not observed in both ovaries in dominant and subordinate females. In conclusion, the methodology used in this study allowed to evaluate the ovarian cycle in *S. xanthosternos* e *S. robustus* females and that cycle phase/anestrus and social class of the female influenced the size of the uterus and ovaries.

INDEX TERMS: Ultrasound, monitoring, uterus, ovaries, dominant female, subordinate female, yellow-breasted capuchin, *Sapajus xanthosternos*, robust tufted capuchin, *Sapajus robustus*, captive colonies, ovarian cycle, anestrus period, neotropical primates, reproductive system, female, reproduction, wildlife animals.

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RESUMO.- [**Acompanhamento ultrassonográfico do útero e ovários de fêmeas dominantes e subordinadas de macacos-prego–do–peito–amarelo (*Sapajus xanthosternos*) e macacos-prego-de-crista (*Sapajus robustus*) em colônias cativas, durante o ciclo ovariano e períodos de anestro.**] O macaco-prego-do-peito-amarelo (*Sapajus xanthosternos*) e o macaco–prego-de-crista (*Sapajus robustus*) encontram-se em risco de extinção devido a destruição do seu habitat e a caça predatória. Porém, ainda necessita-se elucidar alguns detalhes de sua fisiologia reprodutiva, para obterem-se melhores índices por meio de reprodução assistida. Sendo assim, o objetivo deste trabalho foi acompanhar o ciclo ovariano de 13 fêmeas dominantes e subordinadas de *S. xanthosternos* (n=8) e *S. robustus* (n=5) por meio de cortes ultrassonográficos sagitais e transversais do útero e dos ovários. Estas fêmeas foram examinadas uma vez por semana durante dois meses. A fase do ciclo ovariano/anestro foi confirmada pela colpocitologia. Os resultados da pesquisa demonstrou diferença significativa (P<0,05) relacionada ao diâmetro crânio-caudal, diâmetro dorso-ventral e diâmetro transversal entre as fêmeas subordinadas em anestro com todas as outras fases do ciclo ovariano e classes sociais. A média do volume uterino foi maior nas fêmeas dominantes que nas subordinadas em todas as fases do ciclo (P<0,05) a exceção da fase folicular. A largura endometrial, durante o período de anestro, foi menor nas fêmeas subordinadas quando comparada às dominantes (P<0,05). Nas fêmeas subordinadas, houve diferenças (P<0,05) nas mensurações endometriais entre o período de anestro e das fases folicular e lútea. Quanto aos ovários, as médias das medidas observadas nas fêmeas dominantes foram superiores as das subordinadas durante o anestro (P<0,05). As médias das medidas das variáveis ovarianas das fêmeas subordinadas apresentaram diferenças entre o anestro e as fases folicular e lútea (P<0,05). Nas fêmeas dominantes o volume do ovário direito foi maior que o do ovário esquerdo durante o anestro e na fase folicular (P<0,05). Folículos e corpos lúteos foram diferenciados pela ultrassonografia na maioria das coletas (86,11%). Durante o anestro não foram observadas estruturas ovarianas mensuráveis nos ovários em ambas as classes sociais. Concluiu-se que a metodologia empregada neste estudo permitiu o acompanhamento do ciclo ovariano das fêmeas *S. xanthosternos* e *S. robustus* e que as fases do ciclo/anestro e a classe social das fêmeas influenciaram as medidas do útero e ovários.

TERMOS DE INDEXAÇÃO: Ultrassonografia, útero, ovários, fêmeas dominantes, fêmeas subordinadas, macacos-prego-do-peito-amarelo, *Sapajus xanthosternos*, macacos-prego-de-crista, *Sapajus robustus*, colônias cativas, ciclo ovariano, anestro, primatas neotropicais, sistema reprodutivo, ultrassom, reprodução, animais selvagens.

INTRODUCTION

Brazil has the most abundant diversity of apes in the world (Mittermeier et al. 1994). A thorough understanding of these primates, both *in situ* and *ex situ*, is of vital importance to conservationist projects aimed at multiplying endangered specimens and preserving them of its genetic variety (Domingues et al. 2007). These include several species of capuchin monkeys (*Cebus* spp.) and hooded capuchin monkeys (*Sapajus* spp.) (Alfaro et al. 2012), such as the *Sapajus xanthosternos* and *Sapajus robustus*, which have their habitats very restricted due to deforestation caused by the need for expansion of cities or farms, leading them almost to extinction (Aurichio 1995, Domingues et al. 2004). The genus *Sapajus* is characterized by living in groups of six to eight individuals usually formed by a dominant couple (alpha) and some of their infant females, which normally do not reproduce, are characterized as subordinate (Linn et al. 1995). The ovarian cycle of the genus *Sapajus* can vary from 16 to 24 days. Ovulation occurs from day 8 to 12 of the cycle, and in case of fertilization, the gestation lasts between 150 and 162 days, usually producing a baby (Nagle & Denari 1983, Nagle et al. 1994). The interval between births is 576 to 613 days, which leads to low annual population growth (Domingues & Caldas-Bussiere 2002, Domingues et al. 2005).

The correct understanding of the reproductive physiology of these primates is of vital importance for new conservation proposals (Monteiro et al. 2009), having a geometric growth from the moment when non-invasive diagnostic aid methods were started as instruments for research on this conservation purpose (Ortiz et al. 2005, Domingues et al. 2007, Guimarães 2007). Ultrasonography stands out because it allows a good evaluation of the reproductive organs of both females and males, and the monitoring of reproductive cycles, pregnancies, and detection of pathologies that would lead to reduced fertility or serious health damage (Yearger & Concannon 1996, Seier et al. 2000, Monteiro et al. 2006, Domingues et al. 2007).

Ultrasound follow-up of the uterus and ovaries should be performed by longitudinal and transverse scans, assessing the echogenicity, dimensions, contours, position, and shapes (Sauerbrei et al. 2000, Alves et al. 2007). For the uterus, echogenicity should be homogeneous, i.e., the parenchyma should not have texture variations (Ortiz et al. 2005). Uterine dimensions should be measured in longitudinal and sagittal planes. There is no considerable variation in contour, position, and shape of the uterus in animals of the same species. In the ovaries, echogenicity, size, and contour vary according to the phase of the cycle and the ovarian structures present at the time of examination. The position may change according to the fullness of the bladder and bowel loops, so the uterus is used as a reference for ovarian identification (Monteiro et al. 2009).

Given the importance of a better understanding of the reproductive physiology of endangered primates, this research aimed to ultrasonically evaluate the uterus and ovaries of dominant and subordinate females of captive colonies of *S. xanthosternos* and *S. robustus* in different phases of the world ovarian cycle and periods of anestrus.

MATERIALS AND METHODS

This study was approved by "Comitê de Ética de Pesquisas em Animais (Animal Research Ethics Committee)" (CEPA) of "Universidade Federal Fluminense" under protocol number 051207.

Initially, all females used in the study were weighed and underwent a clinical examination. Soon after, those who were healthy had their sexual organs examined by trans-abdominal ultrasound, excluding pregnant females. Thirteen non-pregnant *Sapajus* females (eight *Sapajus xanthosternal*, five *Sapajus robustus*) of reproductive age (from two to seven years old), kept in captivity at the "Centro de Primatologia do Rio de Janeiro" (CPRJ), geographically located between the parallels 22°27' and 22°32' of latitude S and the meridians 42°50 'and 42°56' of longitude WGr, in an almost central position of the state territory of Rio de Janeiro. Its perimeter comprises an estimated area of 259.54 hectares in the municipality of Guapimirim, Rio de Janeiro, Brazil, with the climate of the AW by Köppen-Geiger Climate

classification region. All females were required to be in direct contact with the alpha male in their groups. Their distributions regarding occupation and social classes were: Nursery 1 - a dominant female and three subordinates; Nursery 2 - two subordinate females (the dominant one did not enter the research because she was pregnant); Nurseries 3, 4 and 5 - one dominant female in each; Nursery 6 - one dominant and three subordinate females. When we divided the animals by social class, there were five dominant females and eight subordinate females.

Capture and immobilization methods. In an attempt to reduce the stress of the animals and prevent accidents and escapes, they were sedated in the enclosures and taken to the Veterinary Hospital of the CPRJ, where all necessary research procedures were performed. Sedation was performed with 5.0% ketamine hydrochloride at a dosage of 11.00mg/kg associated with diazepam at a dosage of 0.25mg /kg and atropine sulfate 0.04mg/kg.

Gynecological ultrasound examinations and vaginal cytology collection. Ovarian cycles were followed once a week for two months (which would correspond to three or four ovarian cycles) by ultrasound and vaginal cytology, and data were recorded on individual records. During the study period, females remained in the nurseries with their respective family groups.

Transabdominal ultrasound examination was performed using Carbogel® and a Toshiba Just Vision 200 B-mode device with a 5.0 to 8.0MHz adjustable linear transducer. Soon after chemical containment, the animals were positioned on the examination table in dorsal decubitus, and the uterus was examined as suggested by Monteiro et al. (2009), taking into account the contour, shape, echogenicity, and dimensions. In order to evaluate the dimensions, sagittal and transverse ultrasound sections were obtained. Through the sagittal section was obtained the length or craniocaudal diameter of the uterus (CCD), which represents the distance from the bottom of the uterus to the cervix and dorsal-ventral diameter (DVD) that indicates the depth of the uterus and is obtained perpendicular to the CCD. The transverse section obtained the transverse diameter (TD), which represents the width of the uterus (Monteiro et al. 2009), as well as the endometrial width (EW) (Ortiz et al. 2005). Uterus volume (UV) was calculated by the formula (π/6 [DCCxDDVxDCC]) (Domingues et al. 2007).

Using the uterus as a reference point, rotate it or transducer to the transverse plane to locate and identify the right and left ovaries. These were subject to taxation as to contours, shape, echogenicity, dimensions, and presence or absence of ovarian structures (follicles and corpus luteum). The sagittal section obtained the length and height variables, and the width was obtained by the transverse plane - these were always measured at the maximum ovarian parenchyma diameter. When detected, ovarian structures were measured using their largest internal diameters (Domingues et al. 2007).

Ovarian volume (OV) was calculated using the formula (π/6 [length x width x height]), as described by Domingues et al. (2007). A single observer measured all dimensions.

For diagnostic complement of the ovarian cycle of the monkeys, colpocytology was performed, and the slides stained by the Panotic® technique following the manufacturer's recommendations. Samples were analyzed, as indicated by Gompel & Koss (1997), based on the morphological characteristics of vaginal epithelial cells. After identification of the cells, the phase of the cycle in which the female was found was determined using the classification of Nagle & Denari (1983).

Statistical analysis. The results of ultrasound measurements of the uterus, endometrium, and ovaries were recorded on individual files, and after all, collections were recorded in charts. The basic parameters of descriptive statistics (mean, variance, and standard deviation) were calculated for all variables analyzed. After a preliminary analysis of data variance, no differences in uterine and ovarian measurements were observed between *S. xanthosternos* and *S. robustus* species. It was then decided to work with a single group, formed by the two species. The results were submitted to ANOVA (analysis of variance) to compare the characteristics submitted. The Tukey's test compared individual averages. The results were analyzed considering the anestrus, cycle phases, and social class, as well as the interaction between these variables. The paired Student's t-test was used to compare the means of measurements between right and left ovary at each phase of the ovarian and anestrus cycle, within the same social classes. P values <0.05 were considered statistically significant. GraphPad Prisma 5.0 software (GraphPad Software., California, USA) was used to perform statistical analysis.

RESULTS

A transabdominal ultrasound examination was efficient in evaluating and measuring the uterus and ovaries throughout the study (Fig.1). However, the measurement of the endometrium could not be performed in 70.0% of the evaluations of female anestrus, due to its small size. The means (± standard

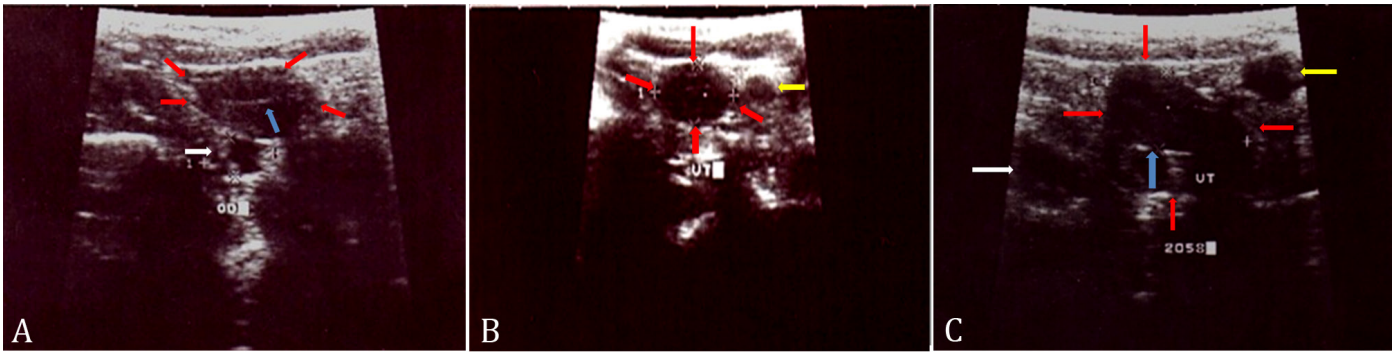


Fig.1. Representative ultrasound images of the uterus, endometrium, and ovary (s) of female *Sapajus xantosthernos* and *Sapajus robustus*. (**A**) Sagittal section of uterus (between red arrows), endometrium (blue arrow), right ovary (white arrow) of a cyclic subordinate female. (**B**) Cross-section of the uterus (between the red arrows) and left ovary (yellow arrow) of a subordinate subordinate female in the anestrus. Note the absence of endometrium and ovarian structures. (**C**) Sagittal section of uterus (between red arrows), endometrium (blue arrow), right ovary (white arrow), and left ovary (yellow arrow) of a cyclic dominant female.

deviations of the mean) of the uterine and ovarian variables at each phase of the cycle and in the anestrus are shown in Table 1 and 2, respectively.

We observed significant differences (P<0.05) related to all uterine variables (craniocaudal diameter-CCD), dorsal-ventral diameter-DVD, transverse diameter-TD, and uterine volume-UV) between the anestrous subordinate females with all other phases of the ovarian cycle and social classes. However, there was no variation in CCD, DVD, and TD between the phases of the ovarian cycle (follicular, luteal and menstrual), nor between social classes (dominant and subordinate) (P>0.05) (Table 1). The mean uterine volume was higher in dominant females than in the subordinate females in all phases of the cycle (P<0.05), except for the follicular phase (Table 1).

The smallest and largest uterine measurements in their respective diameters (cm) and volume (cm^3) were: CCD=0.49 and 2.75, DVD=0.31 and 2.01, TD=0.23 and 1.81 and UV=0.11 and 1.12. The smallest measurements were observed in the anestrus of the subordinate females and the largest in the follicular phase of the dominant females. In dominant females, the smallest diameters (cm) and volume (cm^3) were: CCD=0.77, DVD=0.49, TD=0.61, and UV=0.31, they are observed in the anestrus. In the subordinate females, the largest diameters (cm) and volume (cm^3) were measured in the follicular phase, being respectively CCD=2.18, DVD=1.98, TD=1.17, and UV=1.25.

Endometrial width during the anestrus period was smaller in subordinate females when compared to dominant females (P<0.05) (Table 1). Subordinate females revealed differences in endometrial measurements between the anestrus period and the follicular and luteal phases (P<0.05). Regarding the width of the endometrium, the maximum measured was 1.35cm in a dominant female in the early luteal phase and the smallest of 0.20cm in an anestrus subordinate female. It is recalled that, as already mentioned, the width of the endometrium could not be measured on some occasions, especially in the subordinate females, due to the small width. In dominant females, the smallest measurement was 0.38cm in the anestrus. In the subordinate females, the largest measured was 1.03cm in the follicular phase.

Regarding the ovaries, the average measurements observed in the dominant females were higher than those of the subordinate only during the anestrus (P<0.05). The average measurements of the ovarian variables of the subordinate females showed differences between the anestrus and the follicular and luteal phases in both ovaries (P<0.05) (Table 2). Dominant females had higher volume of the right ovary compared to the left ovary during the anestrus and in the follicular phase (P<0.05) (Table 2).

Table 1. Mean ± standard deviation of uterine variables of dominant (α) and subordinate (β) non-pregnant *Sapajus xanthosternos* and *Sapajus robustus* females, obtained by transabdominal ultrasound examination during the ovarian and anestrus phases

	Anestrus		Menstrual phase		Follicular phase		Luteal phase	
	α	β	α	β	α	β	α	β
CCD (cm)	1.42±0.07	0.91±0.18*	1.66±0.39	1.50±0.38	1.74±0.31	1.70±0.52	1.77±0.35	1.61±0.35
DVD (cm)	0.86±0.28	0.56±0.19*	1.02±0.24	0.74±0.27	1.10±0.17	1.01±0.33	1.06±0.26	0.92±0.28
TD (cm)	0.95±0.33	0.54±0.25*	1.05±0.23	0.81±0.31	1.05±0.16	1.04±0.27	1.04±0.25	0.88±0.23
UV (cm^3)	0.63±0.35^{a}	0.27±0.11^{b}*	0.94±0.22^{a}	0.49±0.28^{b}	1.06±0.43^{a}	0.97±0.55^{a}	1.02±0.56^{a}	0.68±0.55^{b}
EW (cm)	0.70±0.25	0.41±0.05^{A}**	0.73±0.18	0.56±0.20^{AB}	1.00±0.49	0.70±0.21^{B}	0.76±0.23	0.71±0.14^{B}

CCD = craniocaudal diameter, DVD = dorsal-ventral diameter, TD = transverse diameter, UV = uterus volume, EW = endometrial width; * values differ (P<0.05, ANOVA) from other values in the same row, ** values differ (P<0.05, ANOVA) from the value of dominant females in the anestrus; values with different letters indicate significant differences (P<0.05) by the variance test (ANOVA): ^{a,b} lower case letters on the same line indicate differences between social classes at each phase of the ovarian cycle and in the anestrus, ^{A,B} capital letters indicate differences between the subordinate females at different stages of the cycle and in the anestrus.

Table 2. Mean ± standard deviation of ovarian variables of non-pregnant, dominant (α) and subordinate (β) non-pregnant *Sapajus xanthosternos* and *Sapajus robustus* females, obtained by transabdominal ultrasound examination during the ovarian cycle and anestrus phases

	Anestrus		Menstrual phase		Follicular phase		Luteal phase	
	α	β	α	β	α	β	α	β
ROL (cm)	0.76±0.33^{a}	0.55±0.14^{bA}	0.77±0.16	0.64±0.31^{AB}	0.91±0.22	0.77±0.20^{B}	0.87±0.22	0.68±0.21^{B}
ROH (cm)	0.67±0.11^{a}	0.47±0.07^{bA}	0.71±0.05	0.50±0.16^{AB}	0.83±0.11	0.67±0.14^{B}	0.76±0.09	0.63±0.14^{B}
ROW (cm)	0.57±0.16^{a}	0.39±0.11^{bA}	0.52±0.13	0.47±0.26^{AB}	0.69±0.19	0.57±0.16^{B}	0.63±0.19	0.55±0.17^{B}
ROV (cm^3)	0.18±0.02^{a}*	0.06±0.01^{bA}	0.17±0.03	0.09±0.02^{AB}	0.25±0.12*	0.17±0.07^{B}	0.22±0.09	0.14±0.02^{B}
LOL (cm)	0.72±0.20^{a}	0.54±0.18^{bA}	0.81±0.17	0.67±0.20^{AB}	0.88±0.26	0.84±0.37^{B}	0.85±0.26	0.76±0.21^{B}
LOH (cm)	0.60±0.11^{a}	0.46±0.09^{bA}	0.69±0.12	0.53±0.17^{AB}	0.72±0.18	0.69±0.13^{B}	0.75±0.15	0.61±0.20^{B}
LOW (cm)	0.52±0.17^{a}	0.37±0.11^{bA}	0.41±0.03	0.48±0.14^{AB}	0.59±0.15	0.49±0.15^{AB}	0.60±0.19	0.55±0.22^{B}
LOV (cm^3)	0.13±0.02^{a}**	0.05±0.03^{bA}	0.15±0.03	0.10±0.03^{AB}	0.20±0.11**	0.15±0.05^{B}	0.19±0.08	0.13±0.03^{B}

ROL = right ovary length, ROH = right ovary height, ROW = right ovary width, ROV = right ovary volume, LOL = left ovary length, LOH = left ovary height, LOW = left ovary width, LOV = left ovary volume; * ** Indicate P<0.05 by the paired Student's t-test in the comparison between the right and left ovary, in the same variable, in the same column; values with different letters indicate significant differences (P<0.05) by the variance test (ANOVA): ^{a,b} lower case letters indicate different values between social classes during anestrus, ^{A,B} capital letters indicate differences in values of the same social class at each phase of the ovarian and anestrus cycle.

The ovaries presented minimum measurements of length, height, and width (cm) of 0.43, 0.22, and 0.17 observed in the anestrus of subordinate females and a maximum of 1.23, 0.98, and 0.87 observed in the follicular phase of dominant females. The minimum measurements (cm) measured in the ovaries of the dominant females for length, height, and width (cm) were 0.51, 0.39, and 0.28, respectively, also observed in the anestrus while the maximum measurements (cm) obtained in the ovaries of subordinate females were observed follicular phase, being 1.12, 0.91, and 0.82 in length, height, and width (cm), respectively. The smallest and largest ovarian volumes were observed respectively in the left ovary of a subordinate subordinate female in the anestrus (0.03cm^3) and the right ovary of a dominant female in the follicular phase (0.32cm^3). Regarding this parameter, in the case of dominant females, the lowest observed was 0.10cm^3 in the left ovary in the anestrus, and in the subordinate females, the largest was 0.26cm^3 in the follicular phase.

Measurable ovarian structures were present in 69.23% (n=72/104) of the examinations and ranged from 0.10 to 0.62cm in diameter, with structures larger than 0.40cm (dominant follicles and corpus luteum) being present in 49.01% (n=51/104) of the comments. When we separated by classes, in dominant females, ovarian structures were observed in 87.50% (35/40) of the ultrasound exams, and 55.00% (22/40) had diameters greater than 0.40cm. In the subordinate females, ovarian structures were observed in 57.81% (37/64) of the examinations, and those with a diameter greater than 0.40cm represented 48.57% (29/64) of the observations.

As for the side in which the structures were present, we observed 40.27% (29/72) of them in the right ovary, 43.06% (31/72) in the left, and 16.67% (12/72) in both ovaries. Structures more significant than 0.40cm showed a distribution of 49.02% (25/51) in the right ovary and 50.98% (26/51) in the left ovary. When we separated classes, we observed that dominant females had structures larger than 0.40 cm in diameter in the right ovary in 59.09% (13/22) of the examinations and the left ovary in 40.91% (9/22). Already in the class of subordinate females, when also considered structures of this same diameter, they were observed in the right ovary in 41.38% (12/29) of the examinations while in the left ovary represented 58.62% (17/29) of the observations.

Follicles and corpus luteum were differentiated by ultrasound in most collections (86.11%). Cystic corpus luteum (probably early-stage luteal bodies) were not differentiated from follicles in 90.0% of cases, as evidenced by vaginal cytology. During anestrus, no measurable ovarian structures were observed in both ovaries, either in dominant females or in the subordinate females. However, it should be noted that in the dominant females, during the anestrus, it was observed that the ovarian echotexture was more heterogeneous when compared to the ovarian echotexture of the subordinate females. This difference was due to the many anechoic points present in both ovaries that structurally resembled tiny immeasurable follicles (<0.08cm).

DISCUSSION

Although this study involved two species of *Sapajus* (*Sapajus xanthosternos* and *Sapajus robustus*), the data were analyzed together as a single group, since measurements of the uterus, endometrium, ovaries and ovarian structures did not differ ($p>0.05$). The animals are phylogenetically close and have a geographical distribution with intercession between their endemic territories, and there may be natural hybridization of individuals, as already observed by Martins et al. (2017). They are also similar in size and weight, and this is relevant since the individual's weight directly interferes with uterine and ovarian measurements and volume (Coutinho et al. 2011). The most important - that could bring variations to the study -would be different breeding conditions, management, and environment, a fact that did not occur, since the groups were kept in the same place, under the same conditions.

In these primate species, as well as in *Sapajus apella* (Ortiz et al. 2005, Domingues et al. 2004, Domingues et al. 2005, Alves et al. 2007), in *Macaca mulatta* (Bishop et al. 2009) and in *Aotus azarae infulatus* (Monteiro et al. 2009) the ultrasound examination was efficient in the evaluation and measurement of the uterus and ovaries during the ovarian cycle phases. The ultrasound examination was also efficient in the evaluation and measurement of the uterus and ovaries during anestrus periods that interspersed with the recorded ovarian cycles in some females during the research.

The uterine and ovarian measurements of the subordinate females of *S. xanthosternos* and *S. robustus* of the present study are similar to those of *S. apella* described by Ortiz et al. (2005). However, dominant females, showed higher measurements than those of Ortiz et al. (2005) and were closer to those of Domingues et al. (2007) also in *S. apella*. This fact may be related to the category of females used in both studies, i.e. Ortiz et al. (2005) probably also used nulliparous and/or subordinate females, while Domingues et al. (2007), monkeys that had already calved and/or dominant that were always cyclical. Both authors kept females separated in individual cages during their research, i.e., there was no establishment of dominance among individuals as occurs in a flock, which is an important point in articles involving uterine measurements in primates. These measures can also be very useful when we are unfamiliar with the previous reproductive life of captive or free-living females, providing insight into whether the female has already given birth or not, and providing insight into her social class.

It is noteworthy the lack of citations in the scientific literature on uterine and ovarian ultrasound measurements of the species in this study, being necessary to compare with other species of the genus *Sapajus* to discuss the results obtained. It should also be taken into account that different ultrasound equipment, methodologies used by the researchers and the equipment operator, may provide small differences between the measures, requiring careful comparison of the results of various researches (Mercadante et al. 2010).

The uterine diameters and volume of the subordinate females in the anestrus were lower ($P<0.05$) than the other values observed in this research. This is explained by cyclic-interspersed anestrus periods more frequently recorded in the subordinate females compared to dominant females (unpublished data), which highlighted the differences between uterine measurements in these periods, as hormonal influence is directly related the variation of uterine volume (Mauad Filho et al. 2001). Dominant females, which generally presented more constant cycles and rare periods of anestrus intercalated between them, revealed greater and more constant hormonal stimulus, maintaining uterine measurements in

the anestrus and in the next phases of the cycle, not differing between each other (P>0.05).

Regarding uterine volume, we observed differences (P<0.05) between dominant and subordinate females in the anestrus and all phases of the cycle except for the follicular phase. This is due to the multiple pregnancies through which the dominant females have passed through their reproductive life, making their womb larger, while the subordinates were nulliparous. The number of births is the most critical factor for increased uterine measurements in sexually mature females (Coutinho et al. 2011). In the follicular phase, due to the edema caused by the estrogen hormone (Ortiz et al.2005), which increased uterine measurements in both dominant and subordinate females, this difference in volume was "masked", which led to similar measurements between social classes (P<0.05).

There were no significant changes in uterine measurements between dominant females during different phases of the cycle, as observed in other neotropical primates (Ortiz et al. 2005, Domingues et al. 2007, Monteiro et al. 2009). Uterine measurements of subordinate females also did not vary during the phases of the cycle, a fact not commented on in other studies.

The smallest uterine measurements observed during the research occurred in the anestrus, due to the total absence of hormonal stimuli that characterizes this period. The largest uterine measurements occurred during the follicular phase, due to the action of the estrogen produced by the ovarian follicles on the uterus, as previously explained. However, when evaluating the smallest uterine measurements measured during the phases that make up the ovarian cycle of females *Sapajus* spp. (i.e., menstrual, follicular and luteal phases), these were observed during the menstrual phase (Table 1). During this phase, endometrial flaking occurs, and edema resolves, leading to shrinkage of the uterus as a whole (Dixson 2012).

The mean endometrial measurement during the anestrus period was higher in the dominant females when compared to the subordinate females (P<0.05) (Table 1). This is probably due to the longer periods without hormonal stimulation that the latter experienced, as the gonadal hormones are the same responsible for stimulating the proliferative and secretory phases and cause significant morphological changes in the endometrium (Dixson 2012). Subordinate females showed differences in endometrial measurements between the anestrus period and the follicular and luteal phases (P<0.05). As in anestrus measurements were extremely low, remembering that in some females the endometrium could not be measured during anestrus, the difference between this period and the measurements of cyclic females became more evident. In dominant females, even with smaller anestrus measurements, no measurable differences were observed between the cyclic and acyclic periods. In these females it was also possible to measure it even in the anestrus, i.e., the endometrium did not suffer significant metric and visual changes. Such a fact can be explained by the most constant hormonal stimulus observed in this social class during the research (unpublished data).

There were no significant differences in endometrial measurements between ovarian cycle phases in both alpha and subordinate females, as mentioned in other studies (Ortiz et al. 2005). The largest endometrial measurement was observed in a female at the beginning of the luteal phase, which still had edema caused by the estrogen hormone from the follicular phase and already had a small influence of progesterone, which stimulates stromal water retention and endometrial gland secretion (Dixson 2012). The lowest endometrium measurement was observed in the anestrus due to the absence of hormonal stimulation.

Regarding the ovaries, the average ovarian measurements of dominant females were higher than those of subordinates during anestrus, probably due to a larger population of preantral follicles observed in this social class, as already observed in *Leonthopithecus* spp. (Brasil 2008). Besides, the more extended periods of anestrus observed in the subordinate females, when compared to the dominant ones occurred during the experiment (unpublished data), i.e., the most prolonged periods in which their ovaries did not suffer hormonal stimulation. Ovarian volume is known to decrease significantly over long periods without hormonal action (Wallace & Kelsey 2004, Nichols et al. 2005). Mean ovarian measurements did not vary between short anestrus periods and cycle phases in alpha females. However, in the subordinate females, differences (P<0.05) were observed in these measurements between the acyclic periods (anestrus) and all other phases of the ovarian cycle. Such difference between the dominant and subordinate females is explained: even in the periods when they were in anestrus, confirmed by vaginal cytology, homogeneous echotexture of their ovaries and absence of ultrasound measurable structures, a greater amount was observed of tiny anechoic points when compared to the ovaries of the subordinate females, which contributed positively to the ovarian measurements. Perhaps these small structures are several early/early antral/antral follicles. According to a report by Brasil et al. (2007) in *Leonthopitecus* spp., dominant females have a lower rate of follicular atresia than subordinate females.

Right ovarian volume was greater than left ovarian volume in dominant females during anestrus and follicular phase (P<0.05), probably because there is a larger follicular population in this ovary as observed in *S. apella* (Domingues et al. 2004) and/or preantral follicles as reported in *Leonthopithecus* spp. (Brasil et al. 2006). Another fact to be considered is that this difference may be related to a larger increase of interstitial glandular tissue in the right ovary when compared to the contralateral as observed in *A. azarae infulatus* (Monteiro et al. 2009). As for the difference between the volume of the right and left ovaries observed in the dominant females in the follicular phase, it may be associated with a larger number of dominant follicles and consequently of ovulation occurring in the right ovary during the research period, as observed by Domingues et al. (2007) in *S. apella*. However, it is known that the development of dominant follicles/ovulation in *S. apella* does not alternate between right and left ovary in consecutive cycles, ovulation in the same ovary being observed for up to five consecutive cycles (Ortiz et al. 2005). It can, therefore, be assumed that, during the study period, there was a greater formation of dominant follicles and ovulation in the right ovary of the dominant females, which caused the volume difference between them during the follicular phase (P<0.05). Subordinate females showed greater alternation regarding the side of follicular dominance and ovulation,

which provided ovaries with similar volumes also during the follicular phase (P>0.05).

It was not possible to differentiate follicles from cystic corpus luteum, which were probably observed at the beginning of luteogenesis, using 2D ultrasound, without daily monitoring of the development of the structures. Although vaginal cytology specimens clearly determined the luteal phase, the sonographic image of the ovarian structure was not elucidative for the definition of the structure as the corpus luteum, mainly due to the presence of fluid inside it. Differentiation between these structures might be possible with the use of 4D Power Doppler, as performed by Bishop et al. (2009).

This differentiation would also be possible if one follows the cycles daily as performed by some authors (Domingues et al. 2005, Ortiz et al. 2005), although even so, initial corpus luteum is usually difficult to identify structures (Morgan et al. 1987). However, the daily manipulation of the monkeys in the present study could increase their stress level, causing hormonal changes and consequently in the ovarian cycles, besides interfering even more in the behavior of the colony, modifying these results.

CONCLUSIONS

The methodology used in this study allowed following the ovarian cycle of dominant and subordinate infants of *Sapajus* spp. efficiently, quickly and noninvasively.

The social class influenced the uterus and ovarian measures during the ovarian cycle and not anestrus.

However, many aspects of the sonographic anatomy, reproductive cycle, and behavior of several New World primate species still need further study to clarify issues that improve the results of conservation programs for these species.

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Original Article
Animal Morphophysiology
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Morphological description of ovary and uterus of the nurse shark (*Ginglymostoma cirratum*) caught off at the Fortaleza coast, Northeast Brazil[1]

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ABSTRACT.- Rêgo M.G., Araujo M.L.G., Barros M.E.G., Aires, L.A., Oliveira, P.G.V., Hazin F.H.V., Fitzpatrick J.L. & Evêncio-Neto J. 2019. **Morphological description of ovary and uterus of the nurse shark (*Ginglymostoma cirratum*) caught off Fortaleza coast, Northeast Brazil**. *Pesquisa Veterinária Brasileira 39(12):997-1004.* Departamento de Morfologia e Fisiologia Animal, Área de Histologia, Universidade Federal Rural de Pernambuco, Rua Dom Manoel de Medeiros s/n, Dois Irmãos, Recife, PE 52171-900, Brazil. E-mail: mari_rego03@hotmail.com

The nurse shark, *Ginglymostoma cirratum* (Bonnaterre, 1778) is one of the most studied species of elasmobranchs. However, the knowledge of their reproductive biology is still relatively rare, particularly in the western South Atlantic. This study aimed to describe the morphology of the uterus and the ovary of *G. cirratum*, based on specimens caught off at the Fortaleza/CE coast, northeast Brazil. Samples were collected from September 2012 to June 2013, from regular landings of artisanal fishing, which commercialize this species freely. A total of ten females were collected. The methodologies followed for analyzing the ovaries and uterus of those females included both macroscopic and histological analysis. *G. cirratum* has internal type ovary morphology, with invaginations of connective tissue, which defines compartments and separate oocyte groups in ovigerous lots. The epithelium lining the ovary changes from simple columnar ciliated in the area without ovigerous lots, which turns into a simple cubic epithelium in the coating portion of the epigonal organ where ovarian tissue is absent. The uterine mucosa has secretory cells denoted by Alcian Blue staining, indicating the production of mucopolysaccharides, even in immature individuals. This lecithotrophic shark has a uterine vascularized mucosa that is one characteristic of viviparous elasmobranch species.

INDEX TERMS: Morphology, ovary, uterus, nurse shark, *Ginglymostoma cirratum*, Fortaleza, Brazil, female, reproduction, histology, sharks.

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RESUMO.- [**Descrição morfológica de ovário e útero de Tubarões-lixa (*Ginglymostoma cirratum*) capturados na costa de Fortaleza, Nordeste do Brasil.**] O tubarão-lixa, *Ginglymostoma cirratum* (Bonnaterre, 1778) é uma das espécies mais estudadas de elasmobrânquios. O conhecimento de sua biologia reprodutiva, no entanto, ainda é relativamente raro, particularmente no Oeste do Atlântico Sul. Este estudo objetivou a descrição morfológica do útero e ovário de *G. cirratum*, baseado em espécimes capturados na costa de Fortaleza/CE, no Nordeste do Brasil. Um total de dez fêmeas foi coletado de setembro de 2012 a junho de 2013, nos desembarques regulares da pesca artesanal, que comercializam

essa espécie livremente. A metodologia foi de acordo com as analises macroscópica e histológica dos ovários e útero. *G. cirratum* tem o tipo de morfologia de ovário interno, com invaginações de tecido conjuntivo, que define compartimentos e separa grupos de oócitos em lotes ovígeros. O epitélio que reveste o ovário varia de epitélio simples colunar ciliado na área que não apresenta lotes ovígeros para epitélio simples cúbico na porção de revestimento do órgão epigonal, onde o tecido ovariano é ausente. A mucosa uterina possui células secretoras, evidenciadas pela coloração em Alcian Blue, indicando a produção de mucopolissacarídeos, mesmo em indivíduos imaturos. Este tubarão lecitotrófico apresenta a mucosa uterina vascularizada, sendo esta uma característica de espécies de elasmobrânquios vivíparos.

TERMOS DE INDEXAÇÃO: Morfologia, ovário, útero, tubarão-lixa, *Ginglymostoma cirratum*, Fortaleza, Brasil, fêmea, reprodução, histologia, tubarões.

INTRODUCTION

The nurse shark, *Ginglymostoma cirratum* (Bonnaterre, 1778) is an abundant species in tropical and subtropical waters of the Atlantic Ocean, being present along the entire coast of northeast Brazil. It is a coastal species, widely used in aquariums around the world, because of its docility and resistance (Cervigón & Alcalá 1999, Compagno et al. 2005, Garla et al. 2009, Santander-Neto et al. 2011). According to the International Union for Conservation of Nature's (IUCN) Red List, the nurse shark is listed as vulnerable in Brazil. It is also included in the national list of endangered species (IN445/2015 - MMA) and, therefore, its capture is prohibited in Brazil. However, there are several reports of illegal fishing and usage of these animals in aquariums without the necessary license from the responsible bodies (Arthaud 1999, Gruber & Sundström 2000, Rosa et al. 2005, Cunha et al. 2016). For instance, in the Port of Fortaleza, Ceará, Brazil, the species accounts for 15% to 20% of annual shark landings (Arthaud 1999).

According to Castro (2000), this species, which may reach up to 4m long, is viviparous lecithotrophic, with a litter size from 10 to 20 embryos per uterus and a gestation period ranging from 131 days to 240 days (Castro 2000, Carrier et al. 2004). The reproductive cycle is biennial, with an estimated vitellogenesis period of 18 months (Castro 2009).

The reproductive system of *G. cirratum* has the same anatomical features shared by other elasmobranchs, given the conservative nature of the morphology of female reproductive tract in this group (McMillan 2007). The ovaries of the nurse shark are asymmetric (Castro 2000), being only the right ovary functional. The gonad is 5% to 10% the weight of the epigonal organ in immature specimens (Fänge & Mattisson 1981). The other components of the reproductive system correspond to the pair of oviducal glands and uteri, both functional.

The nurse shark is one of the most studied species of elasmobranchs, with regard to several aspects of their biology, such as their immune system (Fidler et al. 1969, Ross & Jensen 1973, Fänge & Mattisson 1981, Rumfelt et al. 2002, Dooley & Flajnik 2005). Knowledge of their reproductive biology, however, is still relatively rare (Carrier & Luer 1990, Castro 2000, Pratt Junior & Carrier 2001, Saville et al. 2002), particularly in the western South Atlantic (Castro & Rosa 2005, Garla et al. 2015, Rêgo et al. 2015). This study, therefore, aimed to describe the morphology macroscopic and microscopic of the uterus and the ovary of *G. cirratum*, based on specimens caught off at the Fortaleza/CE coast, northeast Brazil.

MATERIALS AND METHODS

Ethics statement. The authorization for keeping these samples was part of our scientific collector's permit granted by ICMBio ("Instituto Chico Mendes de Conservação à Biodiversidade") number 40674-4. The method adopted in this study was approved by the Ethics Committee on Animal Experimentation (CEUA) at the "Universidade Federal Rural de Pernambuco" (UFRPE), under the license number 058/2013.

Collection area. Ten female nurse sharks were collected at Mucuripe port, Fortaleza/CE (lat: 03°43'00"S; long: 038°28'07"W) (Fig.1), from September 2012 to June 2013, from regular landings by fishing boats, which routinely sell them freely. Before being marketed, the total length (TL) and pre-caudal length (PC) were measured. After being eviscerated by the fishermen, the ovaries and uterus were collected and then fixed in 10% buffered formalin.

Macroscopic description and characterization of the ovary and uterus. The ovaries and uteri were measured in length and width (cm) and classified macroscopically, according to The International Council for the Exploration of the Sea (ICES 2013). Females were classified as: immature, developing, and capable of reproduction, in early pregnancy, in middle pregnancy, in the end of pregnancy, post-birth and in regeneration.

Histological characterization. After 24 hours of fixation, the organs were cleaved, replaced in 10% buffered formalin for 24 hours, and then transferred to 70% ethanol. Subsequently, the samples were sent to the UFRPE, were processed them using standard histological protocol. The ovary and uterus fragments were dehydrated through successive washing in alcohol solutions with gradually increased concentrations, diaphanized, impregnated in butyl alcohol and embedded in paraplast. The paraplast blocks were then cut into 5µm-thick sections, which were placed on slides, kept at 37°C for 24 hours for drying. The sections were then stained with hematoxylin eosin-phloxine (HE) and Gomori trichrome, periodic acid–Schiff (PAS) and Alcian Blue to 2.5%. A trinocular microscope NIKON 50i, equipped with an imaging system to capture microscopic images, captured the images of histological sections.

RESULTS

The nurse shark ovaries are internal and suspended by the mesentery in the anterior abdominal cavity, with only the right ovary being functional (Fig.2). Of the ten females examined, eight were immature, while the remaining two were in the development stage. Immature females varied in their total length (TL) from 85 and 135cm. The reproductive tract showed a whitish ovary with width ranging from 1.5 to 2.5cm, and the presence of undifferentiated ovarian follicles. The ovary was embedded in the epigonal organ with its cranial morphology being similar to a network (Fig.2A). At this stage, females present extremely thin and slender uteri and reduced oviducal glands.

Females in developing stage had a total length (TL) ranging from 152 to 164cm. The ovaries had a width ranging from 3 to 4cm, with thicker walls and more yellowish aspect (Fig.2B) than in immature females. The ovarian follicles were evident

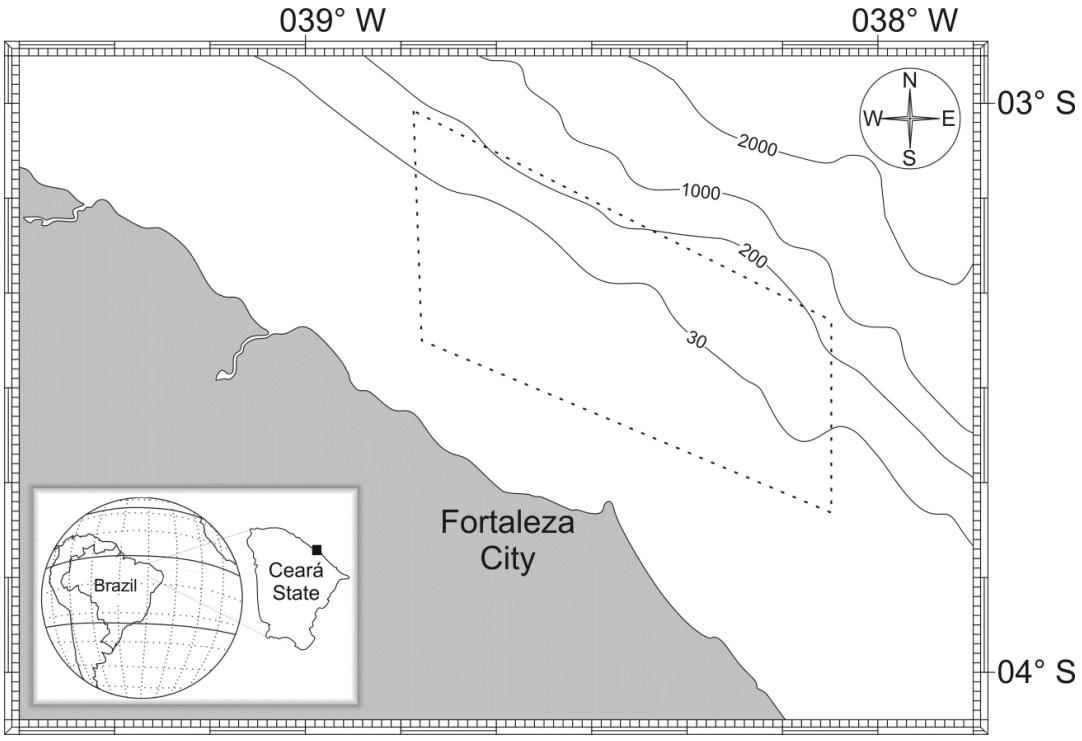


Fig.1. Map indicating the location of the sampling area, port of Mucuripe, Fortaleza/CE (Santander-Neto 2014).

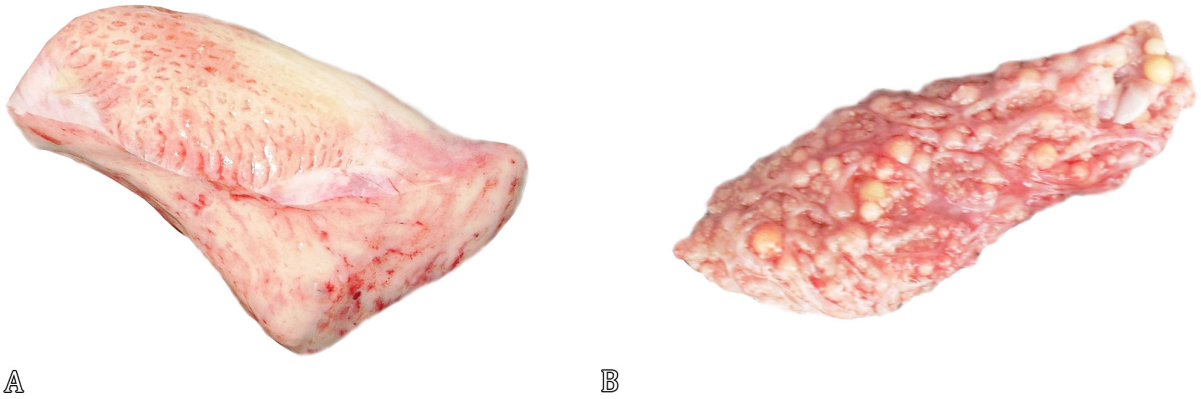


Fig.2. Macroscopic and microscopic photos of shark ovary evidencing (**A**) immature ovary (arrow), and (**B**) ovary in development (arrow).

with oocytes at different stages of vitellogenesis. The uteri from females in this stage had thicker walls than in immature females, and the oviducal glands were more developed as well.

Ovary

Histologically, the ovaries were coated by a simple columnar ciliated epithelium in the area without ovigerous lots, which turns into a simple cubic epithelium in the coating portion of the epigonal organ where ovarian tissue is absent (Fig.3A). Although it is an internal ovary, there is an area of connective tissue that makes up the ovarian stroma and separates the ovigerous portion from the limphomieloid portion of the epigonal body. The connective tissue creates compartments with isolated ovigerous lots (Fig.3B). The epithelial tissue in these places becomes pleated (Fig.3C).

In the ovary parenchyma of immature individuals, the presence of previtellogenic follicles type I and type II were identified by an increase in oocyte and by the polymorphism in the follicular layer, which consists initially of a layer of squamous cells, progressing to a layer of columnar and cuboidal cells. At this stage of follicular development, the teak and the pellucid zone are visible. The larger previtellogenic follicles show an increase in vascularization network between theca (Fig.4A,B).

Females in the developing stage had ovaries in stages I and II of vitellogenesis. Around the vitellogenic oocytes, there is the pellucid zone, the stratified follicular epithelium and the teak, with evident vascularisation. Atresia was observed in all follicular development stages (Fig.4C). In this phase, the epigonal organ is already proportionally small. The ovarian

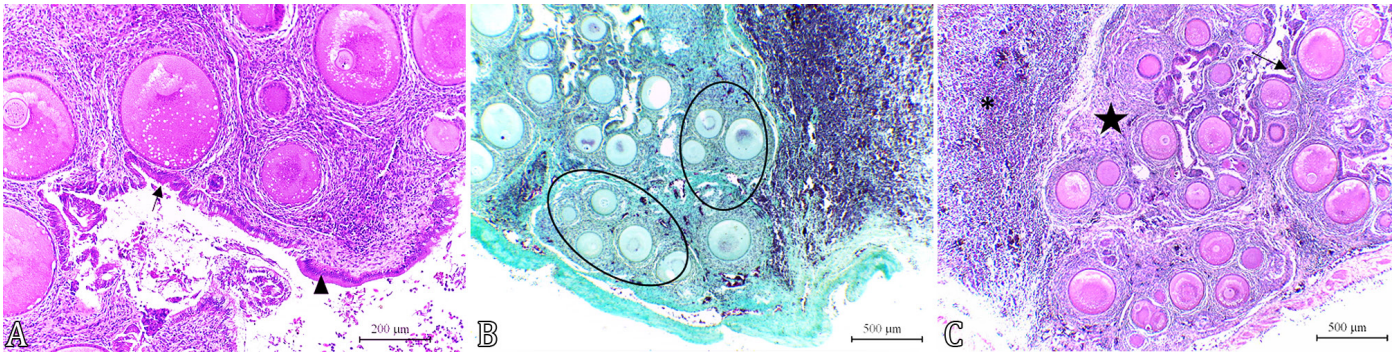


Fig.3. Nurse shark ovary evidencing (**A**) the modification of the ovarian epithelium which is simple columnar epithelium in the region without the ovigerous lots (arrow) and simple cubic epithelium in the epigonal organ lining portion (arrowhead). HE, bar = 200µm. (**B**) Different ovigerous lots (circulated). Trichrome Gomori, bar = 500µm. (**C**) Epithelial tissue at these sites becomes pleated (arrow). It is observed that there is a portion of connective tissue (star) that composes the ovarian stroma and that separates the ovum portion of the portion of the epigonal organ (asterisk). HE, bar = 500µm.

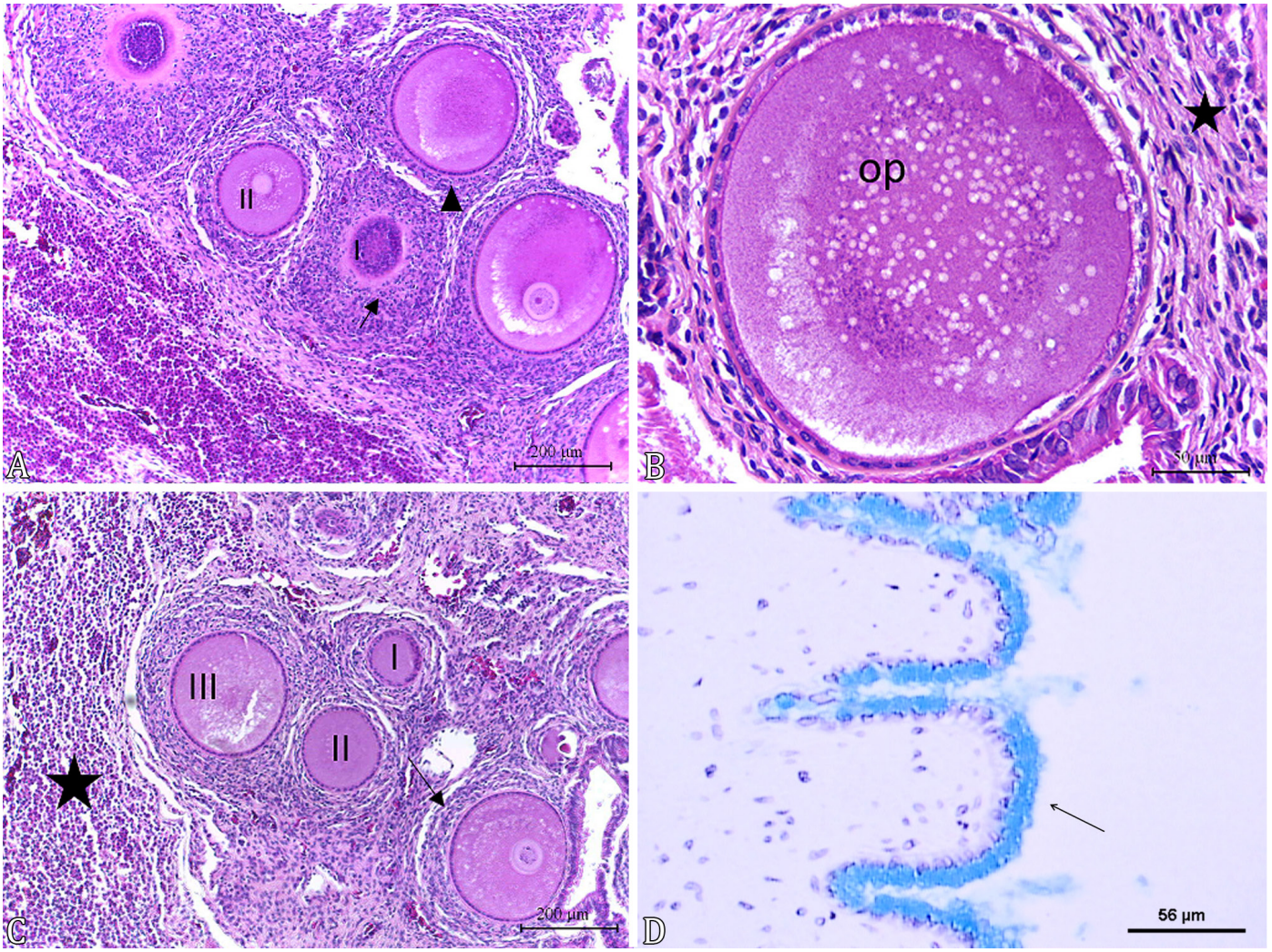


Fig.4. Nurse shark ovary. (**A**) In the immature ovary, the presence of type I and type II pre-vitellogenic follicles was observed, identified by the oocyte enlargement and polymorphism of the follicular layer, which initially consists of a layer of pavement cells (arrow), and progressing to a layer of cubic and columnar cells (arrowhead). HE, bar = 200µm. (**B**) Major pre-vitellogenic follicles present a layer of polymorphic stratified cells (star). HE, bar = 50µm. (**C**) The ovary of females in the developing stage had follicles in stages I and II of vitellogenesis. Around the vitellogenic oocytes, the zona pellucida, stratified follicular epithelium (arrow), and teak with evident vascularization are observed. The epigonal organ is already in small proportion (star). HE, bar = 200µm. (**D**) The columnar ovarian epithelium presented secretory cells with reactivity to Alcian Blue 2.5% (arrow). Alcian Blue 2.5%, bar = 56µm.

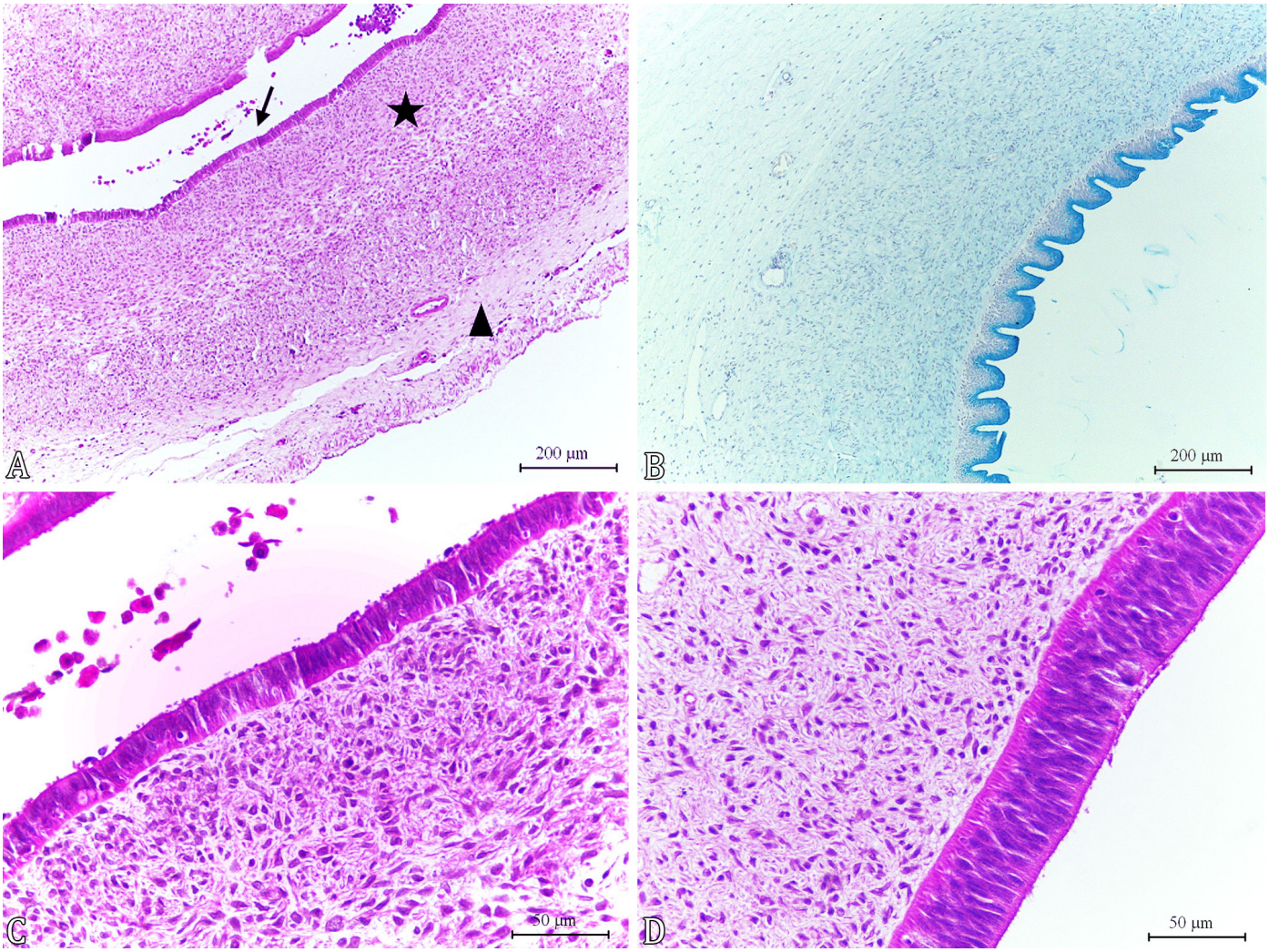


Fig.5. Nurse shark uterus. (**A**) Showed that the nurse shark uterus is constituted by three (3) layers: mucous (star), muscular (arrowhead) and serosa (arrow). HE, bar = 200µm. (**B**) It was evident an acellular layer that was positive to trichromatic Gomori and negative AB to 2.5% between the mucosa and submucosa suggesting that the formed layer of collagen. Alcian Blue, bar = 200µm. (**C**) In the middle region, a decrease in cell layer of the epithelium and the basal portion was observed with invagination of connective blood vessels into the columnar epithelium (arrow). HE, bar = 50µm. (**D**) The distal segment of uterus is lining with the epithelium stratified prismatic ciliary. HE, bar = 50µm.

developmental stage of the columnar epithelium showed the presence of secreting cells with reactivity to Alcian Blue 2.5% (Fig.4D).

Uterus

The results showed that the uterus of the nurse shark is constituted by three layers: mucosa, muscular and serosa (Fig.5A). The mucosa consists of epithelium, which varies according to the uterine region. Just below the epithelium coating is the undeveloped lamina propria, composed of loose connective tissue with blood and lymphatic vessels. In this area, the uterine glands are not visible. The muscle layer consists of two layers of well-developed muscular tissue. The serosa is a thin layer formed of loose connective tissue, covered externally by a single layer of squamous epithelial tissue (Fig.5A). An acellular layer that was positive to trichrome Gomori and negative Alcian Blue to 2.5% between the mucosa and connective tissue was evident, suggesting a collagenous nature (Fig.5B).

The epithelium of uterine mucosa ranged from simple to stratified columnar. In the cranial region of the uterus, the epithelium has stratified columnar appearance with the mucosal cells Alcian Blue 2.5% reactive (Fig.5B). In the middle region, occurs a decrease in cell layer of the epithelium. In the basal portion, an invagination of connective blood vessels into the columnar epithelium was observed (Fig.5C). Stratified prismatic ciliary epithelium was present in the distal segment of uterus (Fig.5D). In the uterine body region, vascularization of the mucosa was further developed. The oviduct mucosa had a well-developed invagination covered by stratified epithelium, with superficial secreting cells with reactive secretion to the Alcian Blue to 2.5% (Fig.6A,B).

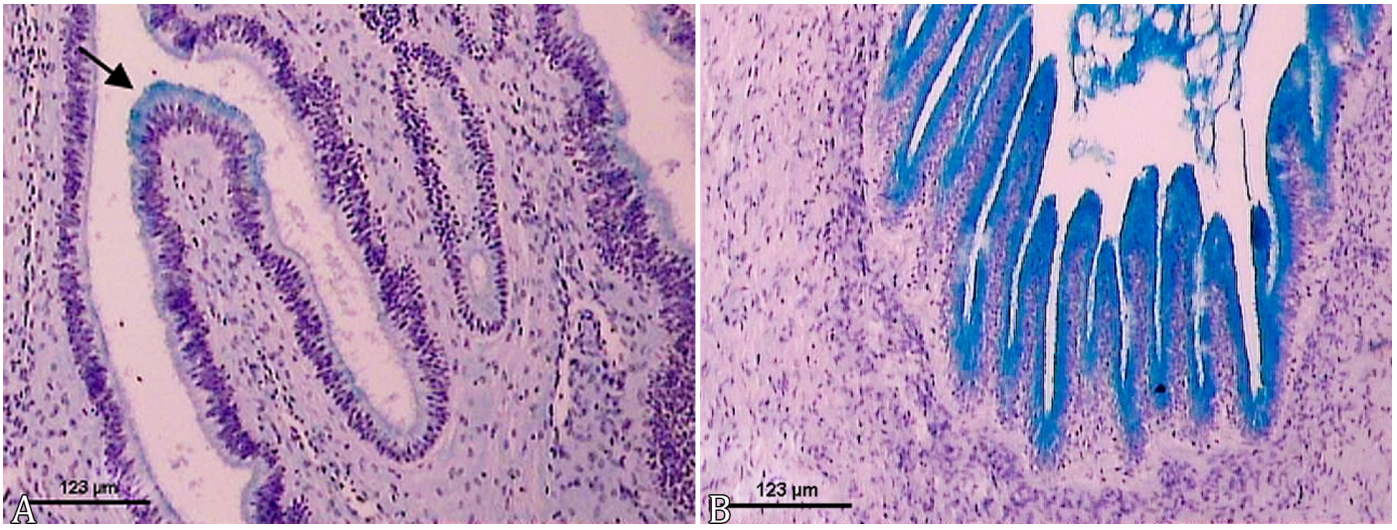


Fig.6. Nurse shark uterus. (**A**,**B**) The oviductal featured by presenting a mucosa with well developed invagination covered by stratified epithelium, with apical portion of superficial secreting cells reactive to the AB 2.5%. (**A**) Trichrome Gomori, bar = 123µm. (**B**) Alcian Blue, bar = 123µm.

DISCUSSION

According to Carrier et al. (2004) and Hamlett (2007), sharks have two ovaries that are paired and located at the front end of the abdominal cavity. Typically, both ovaries are functional, though in some species of sharks (e.g. members of the genera *Scyliorhinus*, *Carcharhinus*, *Mustelus* and *Sphyrna*) only the right ovary is functional, and the left ovary is absent or non-functional (Pratt Junior 1988). In *Ginglymostoma cirratum* only the right ovary is functional, with a structure similar to that described for *Cetorhinus maximus* (Matthews 1950) and *Rhincodon typus* (Nozu et al. 2015).

Pratt Junior (1988) classified the ovaries of sharks as internal and external, based on the morphological interactions between the ovary and the epigonal body. Nurse sharks have the same ovarian characteristic as the Lamnidae family (Pratt Junior 1988) and Cetorhinidae family (Matthews 1950), which have an internal ovary. This type of ovary is embedded in the epigonal body, where invaginations of connective tissue define separate groups of oocyte compartments, as found here for *G. cirratum* (Fig.3C).

The lining of the ovary identified in the nurse shark (Fig.3A) has the same morphological characteristics of the epithelium described for the ovary of the *Zearaja chilensis* (Wehitt et al. 2015) and *Sympterygia acuta* (Díaz-Andrade et al. 2009). Both species are oviparous and have the ciliated epithelium lining the ovary.

Chatchavalvanich & Visuttipat (1997) described the ovary of *Dasyatis bleekeri* as a simple epithelium composed of cells varying from squamous to cubic, without the presence of cilia. However, Hamlett et al. (1999) described the *Stingaree jamaicensis* ovary and identified epithelial cells lining the organ with apical microvilli by using electron microscopy. Considering the conservative profile of elasmobranch ovaries (McMillan 2007), we can infer that the epithelium of *G. cirratum* ovarian coating consists of epithelial cells with apical microvilli and no ciliated cells. The elucidation of this question, however, depends on future studies of the ultrastructure of the ovary of that species.

Gračan et al. (2013) observed a predominance of smaller previtellogenic (type I) and few average previtellogenic follicles (type II) in the ovaries of immature *Squalus acanthias*. The same was observed for *G. cirratum* here, which had a higher incidence of follicles type I in immature females and an increase of type II in the ovaries of developing females. The presence of oocytes in the beginning of vitellogenesis occurred in developing ovaries of *R. typus* (Nozu et al. 2015) and of *S. acanthias* (Gračan et al. 2013). Like in the present study, Castro (2000) also reported vitellogenesis occurrence in immature and developing female ovaries.

The variation in shape and number of layers of follicle cells, based on follicular development, was recorded for various elasmobranch species (Guraya 1986, Pratt Junior 1988, Díaz-Andrade et al. 2009, Gračan et al. 2013). As recorded by Lutton et al. (2005), granulosa cells of immature batoid *Leucoraja erinacea* may vary from simple to cubic form and while follicular genesis and oogenesis take progress the cells develop into a columnar structure. In *G. cirratum*, the morphology of vitellogenic follicles is similar to that described for *S. acanthias* (Gračan et al. 2013), but different from *Z. chilensis* (Wehitt et al. 2015) and *D. bleekeri* (Chatchavalvanich & Visuttipat 1997). These discrepancies can be attributed to the existing differences between sharks and rays (Dodd 1983).

The uterus of *G. cirratum* agrees with the general pattern described by Hamlett et al. (1998) for elasmobranch species. The morphological constitution of the uterine lining presents variations depending on the reproduction mode of elasmobranchs (Matthews 1950, Storrie et al. 2009, Wehitt et al. 2015). *G. cirratum* has secretory cells in the endometrium denoted by Alcian Blue staining (Fig.5B), indicating the production of mucopolysaccharides, even in immature individuals. This is also observed in other lecitotrophic species, such as *Mustelus antarcticus* (Storrie et al. 2009).

The presence of an underlying laminate layer containing vessels of different dimensions (Fig.5C) reinforces the vascularization role of the uterus mucosa in viviparous species (Hamlett & Koob 1998).

Recently, a study on the shark *R. typus* showed that there is an increase in the uterine vasculature to assist in gas exchange with embryos (Joung et al. 1996). In aplacentary viviparous elasmobranchs, it has been assumed that the endometrium is not specialized to secrete nutrients to the embryo, participating only in the breathing process and osmoregulation (Hamlett et al. 1999). However, various evidences have indicated that some lecitotrophic species, such as *Centrophorus granulosus* (Cotton et al. 2015), *M. antarcticus* (Storrie et al. 2009) and *Galeocerdo cuvier* (Castro et al. 2016) do present some level of matrotrophy.

The term mucoid histotrophy (Hamlett et al. 2005, Musick & Ellis 2005) indicates a form of matrotrophy, where the embryo nourishment is complemented with the mucus produced by the uterine epithelium. The distinction between lecitotrophy and mucoid histotrophy is given by the weight ratio between the newly fertilized egg and the embryo brought to term. In *S. acanthias* there is a considerable loss (15-55%) in organic matter content, from egg to embryo (Ranzi 1932, 1934, Needham 1942), indicating a lecithotrophic species. *C. granulosus* (Cotton et al. 2015) and *M. antarcticus* (Storrie et al. 2009) has a loss of less than 20%, which suggests maternal nutritional contribution to the developing embryo (Hamlett et al. 2005).

Due to the presence of structures in the uterine epithelium with high secretory activity in the early and late gestation, additional differences between these reproduction modes become evident by histological analysis (Hamlett et al. 2005).

Castro (2000) observed a 42% loss in weight during embryonic development in *G. cirratum*, indicating that it is a lecitotrophic species. The presence of AB + secreting cells in the uterine epithelium of the specimens studied in this work is, therefore, likely related to the secretion production to the uterine epithelium lubrication and not for embryo nutrition, as also identified in *M. antarcticus* (Storrie et al. 2009). However, further studies are needed to prove this statement. The acellular layer shown between the mucosa and submucosa, with a positive reactivity to Gomori Trichrome, and negative for Alcian Blue, indicates the presence of collagen and fibroblasts bundles. A single layer of squamous epithelium constituting the serosa layer delimits a layer of smooth muscle in *Mustelus schmitti* (Galíndez et al. 2010). The same morphology was observed here in the nurse sharks examined.

CONCLUSIONS

This study demonstrates how necessary the understanding of mucoid histotrophy contribution is for embryonary development of elasmobranch species which has the lecithotrophy as the mode of reproduction.

The next step seems to be identifying and examine the presence of blood vessel in uterine epithelium and the advantage of having ovigerous lots to reproductive potential of *Ginglymostoma cirratum*.

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Conflict of interest.- The authors declare no conflicts of interest.

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Colégio Brasileiro de Patologia Animal

Em 15 de agosto de 2019

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a) O **TÍTULO** deve ser conciso e indicar o conteúdo do artigo; pormenores de identificação científica devem ser colocados em MATERIAL E MÉTODOS.
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h) **MATERIAL E MÉTODOS** deve reunir a totalidade dos dados que permitam o desenvolvimento de trabalho semelhante por outros pesquisadores.

i) Em **RESULTADOS** devem ser apresentados concisamente os dados obtidos.

j) Na **DISCUSSÃO** devem ser confrontados os resultados diante da literatura. Não convém mencionar artigos em desenvolvimento ou planos futuros, de modo a evitar uma obrigação do autor e da revista de publicá-los.

k) **CONCLUSÕES** devem basear-se somente nos resultados obtidos e devem ser apresentados em diferentes parágrafos (uma Conclusão somente deve ser apresentada em parágrafo único).

l) Os **Agradecimentos** não devem aparecer no texto ou em notas de rodapé; devem ser sucintos e colocados antes da Declaração de conflito de interesse e da Lista de Referências.

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a) Fonte **Cambria, corpo 10, entrelinha simples; página formato A4, com 2cm de margens** (superior, inferior, esquerda e direita), texto corrido em uma coluna justificada, com as Legendas das Figuras no final (logo após a Lista de REFERÊNCIAS) sem repetir as legendas junto com as Figuras.

b) ABSTRACT e RESUMO serão escritos em um só parágrafo corrente e não devem conter citações bibliográficas.

c) A redação dos artigos deve ser concisa, com a linguagem, tanto quanto possível, no passado e impessoal.

d) Os nomes científicos usados no manuscrito devem ser apresentados por extenso (p.ex. *Palicourea marcgravii*), no início de cada capítulo (**TÍTULO, ABSTRACT, RESUMO, INTRODUÇÃO, etc.**), quando aparecem pela primeira vez, seguido da abreviação do gênero (p.ex. *P. marcgravii*).

e) Nos títulos dos Quadros e nas Legendas das Figuras os nomes científicos devem ser apresentados por extenso, já que estes são independentes do texto.

f) No texto, os sinais de chamada para notas de rodapé devem ser números arábicos colocados em sobrescrito após a palavra ou frase que motivou a nota. Essa numeração será contínua por todo o artigo; as notas deverão ser lançadas ao pé da página em que estiver o respectivo número de chamada, sem o uso do "Inserir nota de fim", do Word.

Notem: para evitar a separação em duas linhas, os numerais devem ser apresentados junto com suas unidades, ou seja, sem espaçamento, por exemplo: 100ppm, 10mm, 50cm, 18x10cm, (P<0,05), 15h. A abreviação de número é "nº" e não "nº"; grau Celsius é "ºC" e não "ºC".

g) Os Quadros (não usar o termo Tabela) e as Figuras devem ser citados no texto, pelos respectivos números, em ordem crescente e devem ser submetidos separadamente do texto!

h) Siglas e abreviações das instituições, ao aparecerem pela primeira vez, deverão ser colocadas entre parênteses, após o nome da instituição por extenso;

i) Citações bibliográficas serão feitas pelo sistema "autor e ano", p.ex. (Caldas 2005); artigos de até dois autores serão citados pelos nomes dos dois (Pedroso & Pimentel 2013); e com mais de dois, pelo nome do primeiro, seguido de "et al.", mais o ano (Brito et al. 2015); se dois artigos não se distinguirem, a diferenciação será feita através do acréscimo de letra minúscula ao ano (Barros 2017a, 2017b). A ordem de citação deve ser cronológica (Barbosa et al. 2003, Armién et al. 2004).

j) **Recomenda-se consultar na íntegra todos os artigos citados**; se isto não for possível, deve-se colocar no texto a referência original (não consultada na íntegra) seguida do ano, p.ex. (Bancroft 1921); na Lista de Referências deve ser incluída a referência original como: Bancroft 1921. ..... título. ... periódico. (Apud Suvarna & Layton 2013). A referência consultada também deve ser incluída na Lista de Referências.

k) O uso de "comunicação pessoal" e de "dados não publicados" deve ser feito apenas em casos excepcionais; no texto com citação de Nome e Ano, e na Lista de Referências como: Barbosa 2016. Comunicação pessoal (Universidade Federal do Pará, campus Castanhal).

l) As **Legendas das Figuras** devem conter informações suficientes para sua compreensão (independente do texto); e devem ser precedidas de "Fig." seguida do número sem espaço, p.ex. "Fig.8. ...". Para elaboração das legendas sugerimos consultar exemplos nos últimos fascículos (www.pvb.com.br).

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m) O Título dos **Quadros** devem ser em **negrito**, sem ponto, e a "garganta" (título das colunas) deve ser escrita em claro e separada por dois traços longos horizontais; o Título dos Quadros e da "garganta" devem ser escritas em caixa alta e baixa. Os Quadros (não usar o termo Tabela) devem conter os resultados mais relevantes. Não há traços verticais, nem fundos cinzentos; excepcionalmente pode conter traços horizontais. Os sinais de chamada serão alfabéticos, recomeçando, com "a" em cada Quadro. As chamadas de rodapé deverão ser lançadas logo abaixo do Quadro respectivo, do qual serão separadas por um traço curto à esquerda; e devem evitar números arábicos. Os títulos não têm ponto no final, ao passo que as legendas terminam com um ponto. Os Quadros devem ser apresentados em Word e ser editáveis, a fim de inserirmos eventuais alterações de apresentação, dentro das normas da revista.

n) Dados complexos devem ser expressos por Gráficos (devem ser chamados de **Figuras**). Os gráficos devem ser produzidos em 2D, sem fundo e sem linhas horizontais. Em gráficos contendo texto a fonte deve ser Cambria tamanho 10.

3. **Apresentação das Figuras:**

a) As figuras devem ser salvas em 300dpi, arquivo TIF.

b) Enviar cada figura separadamente.

c) Identificar as figuras em ordem conforme a menção no texto.

d) As figuras solitárias devem ter seus arquivos identificados como (Fig.1, Fig.2 ...)

e) As figuras que serão destinadas a formar uma prancha devem ter seus arquivos identificados como (Fig.1A, Fig.1B ...). As pranchas devem ser compostas por múltiplas subfiguras. Imagens destinadas a uma prancha devem ser de mesmo tamanho.

f) Para micrografias usar, de preferência, barras de escala para indicar o aumento; apresentar na legenda sempre o método de coloração e a objetiva, p. ex.: HE, obj.40x.

g) As legendas de figuras devem conter inicialmente o que se observa na imagem, seguida das informações adicionais (Formato típico da legenda: Fig.1. (**A**) Descrição da imagem. Diagnóstico, órgão ou tecido, espécie animal, número do caso. Método de coloração e objetiva.).

h) As legendas de figuras devem ser apresentadas junto com o texto do artigo, após as Referências.

4. **Todas as referências citadas no texto devem ser incluídas na Lista de Referências e vice-versa**; **na revisão final do artigo pelos autores,** antes da submissão, **isto deve ser conferido criteriosamente, para evitar discrepâncias (o sistema ScholarOne bloqueia automaticamente artigos com discrepâncias).**

Exemplos de Referências

➢ Artigos publicados em periódicos:

Martins K.P.F., Fonseca T.R.S., Silva E.S., Munhoz T.C.P., Dias G.H.S., Galiza G.J.N., Oliveira L.G.S. & Boabaid F.M. 2018. Bócio em bovinos. Pesq. Vet. Bras. 38(6):1030-1037.

Rondelli L.A.S., Silva G.S., Bezerra K.S., Rondelli A.L.H., Lima S.R., Furlan F.H., Pescador C.A. & Colodel E.M. 2017. Doenças de bovinos no Estado de Mato Grosso diagnosticadas no Laboratório de Patologia Veterinária da UFMT (2005-2014). Pesq. Vet. Bras. 37(5):432440.

Hooiveld M., Smit L.A., Wouters I.M., Van Dijk C.E., Spreeuwenberg P., Heederik D.J. & Yzermans C.J. 2016. Doctor-diagnosed health problems in a region with a high density of concentrated animal feeding operations: a cross-sectional study. Environ. Health 17:15-24.

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(Notem: Os iniciais dos autores devem ser colocados sem espaço. O sinal "&" é usado para separar o penúltimo do último autor. As primeiras letras das palavras do título de artigos publicados em periódicos científicos devem ser de preferência minúsculas. A palavra "Revista" deve ser abreviada como "Revta" em diferença a "Rev.", do inglês "Review". Deve-se indicar o número do respectivo volume do periódico e, se possível, também do fascículo. Somente abreviações tem um ponto, exceto as que terminam com a última letra da palavra em extenso. O traço entre as páginas é curto (-) e não comprido. Não devem ser usados "pontovírgulas" (;) em lugar de vírgulas.

- Livros:

Tokarnia C.H., Brito M.F., Barbosa J.D., Peixoto P.V. & Döbereiner J. 2012. Plantas Tóxicas do Brasil para Animais de Produção. 2ª ed. Helianthus, Rio de Janeiro, p.305-348.

Marsh P. & Martin M. 1992. Oral Microbiology. 3rd ed. Chapman and Hall, London, p.167-196.

(Notem: A primeira letra de termos do título de livros deve ser maiúscula. Devem ser mencionadas as páginas que foram consultadas, em vez do total de páginas do livro.

- Capítulos de livros:

Barros C.S.L. 2007. Doenças víricas: leucose bovina, p.159-169. In: Riet-Correa F., Schild A.L., Lemos R.A.A. & Borges J.R.J. (Eds), Doenças de Ruminantes e Equídeos. Vol.1. 3ª ed. Pallotti, Santa Maria.

Tokarnia C.H., Brito M.F., Barbosa J.D., Peixoto P.V. & Döbereiner J. 2012. Plantas que afetam o funcionamento do coração, p.27-94. In: Ibid. (Eds), Plantas Tóxicas do Brasil para Animais de Produção. 2ª ed. Helianthus, Rio de Janeiro.

(Notem: As primeiras letras das palavras do título de capítulos de livros são minúsculas, mas as de livros são maiúsculas.)

- Dissertações e Teses:

Rech R.R. 2007. Alterações no encéfalo de bovinos submetidos à vigilância das encefalopatias espongiformes transmissíveis. Tese de Doutorado, Universidade Federal de Santa Maria, Santa Maria. 228p.

(Notem: (1) Deve-se evitar citações de Dissertações ou Teses; deve-se preferir citar artigos baseados nas mesmas e publicados em periódicos científicos que são de mais fácil acesso. (2) Não deve-se tentar de publicar o texto de Dissertação ou Tese praticamente na íntegra sem escrever um artigo conciso de seus resultados.

- Resumos publicados em eventos:

Mendonça F.S., Almeida V.M., Albuquerque R.F., Chaves H.A.S., Silva Filho G.B., Braga T.C., Lemos B.O. & Riet Correa F. 2016. Paralisia laríngea associada à deficiência de cobre em caprinos no semiárido de Pernambuco (IX Endivet, Salvador, BA). Pesq. Vet. Bras. 36(Supl.2):50-51. (Resumo)

Pierezan F., Lemos R.A.A., Rech R.R., Rissi D.R., Kommers G.D., Cortada V.C.L.M., Mori A.E. & Barros C.S.L. 2007. Raiva em equinos. Anais XIII Encontro Nacional de Patologia Veterinária, Campo Grande, MS, p.145-146. (Resumo)

(Note: Evitar na consulta o uso de Resumos ao invés de artigos na íntegra!)

Colégio Brasileiro de Patologia Animal

Em 15 de agosto de 2019

GUIDE FOR AUTHORS

Papers to "Pesquisa Veterinária Brasileira" (PVB), a Brazilian Journal of Veterinary Research, are submitted in Word online through ScholarOne, link <https://mc04.manuscriptcentral.com/pvb-scielo>

The authors should submit their papers in English, with a Portuguese Summary. To prove the quality of the English, a certificate of the English language is required, with exception of authors native in English.

With the communication of acceptance of the paper, the author for correspondence will be asked for payment of a Paper Charge of US$ 480.00 (*R$ 1.500,00*) for each article submitted in English.

As of 2019, **all authors should register in the ORCID** (Open Researcher and Contributor ID https://orcid.org/register) and link it to their ScholarONE profile. The link can be done by editing the user profile on ScholarONE in the option **Associate your existing ORCID iD**. The ORCID identifiers contribute to the singular identification of the authors and to the interoperational and bibliometric processes. Using an ORCID, researchers are easily and correctly connected with their research results, publications and affiliations.

Papers should be prepared in all details according to the style of the journal (www.pvb.com.br), in order to be peer reviewed. Tables and Figures should be submitted separately from the text.

PVB publishes Original Articles, but also Critical Literature Reviews and Topics of General Interest; no Short Communications are accepted.

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The opinions and concepts emitted are of the responsibility of the authors. The Editorial Board of the journal, assisted by the peer review, may suggest or ask for modification of the text.

The author rights of the accepted papers are preserved.

1. **The submitted article should be organized in TITLE, ABSTRACT, RESUMO (the last when authors are from a Portuguese speaking country), INTRODUCTION, MATERIALS AND METHODS, RESULTS, DISCUSSION, CONCLUSION(S) (the last three preferably as separate chapters), Acknowledgements, Conflict of interest statement and REFERENCES:**

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b) **Authors with several first and family names should shorten their names for scientific publication,** as for example: Cláudio Severo Lombardo de Barros writes Cláudio S.L. Barros or Barros C.S.L., and Franklin Riet-Correa Amaral writes Franklin Riet-Correa or Riet-Correa F. **The papers should not have more than 8 (eight) authors.** Corresponding author should be one who guarantees the contact with the Editorial Board of PVB. Asterisks for call to the footnotes should be elevated once more, in order to appear larger.

c) The **heading of the ABSTRACT** should contain the shortened and inverted names of the authors, the year, the Title (in brakes when translated), and the postal address of the laboratory or institution where the main part of the research was done (Always compare the authors of the paper and their shortened and inverted in the heading of the Abstract to avoid discrepancies).

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e) The **ABSTRACT** should be a well explained version of the Portuguese RESUMO, followed by "INDEX TERMS" which should include terms of the title, as they are not only Additional Index Terms.

f) The **RESUMO** should contain (1) what have been investigated, indicating (2) materials and methods used, (3) the most important results, and (4) the conclusion, followed by "TERMOS DE INDEXAÇÃO" (which include also words of the title, as they are not only Additional Index Terms).

g) The **INTRODUCTION** should be short, with citation of the specific literature without assuming main importance, followed by the objective of the research.

h) In **MATERIALS AND METHODS** should be given all data necessary for other research workers to repeat the research.

i) In **RESULTS** are presented the data obtained in a concise form.

j) In **DISCUSSION** the results should be confronted with the literature. Research in development or future planning should not be mentioned, to avoid the obligation for the journal to publish the results.

k) The **CONCLUSIONS** should be based only on the results obtained.

l) **Acknowledgements** should not be mentioned in the text or in footnotes.

m) **Conflict of interest or none** should be mentioned.

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b) **ABSTRACT** and **RESUMO** are written in only one paragraph and should not contain references.

c) The articles should be concise, always when possible in past tense and impersonal.

d) The scientific names should be presented in full (p.ex. *Palicourea marcgravii)* at the beginning of each chapter (Title, Abstract, Resumo, Introduction, etc.) when they appear for the first time, followed with abbreviation of the genus (p.ex. *P. marcgravii*).

e) In the Title of Tables and in Figure captions the scientific names are written in full.

f) In the text, calls to footnotes are given in Arabic numbers, in crescent order through the whole paper, without use of "Insert final note" of Word.

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The abbreviation for number is "nº" and not "nº"; for degree Celsius "ºC" and not "ºC".

g) Tables and Figures should be cited in the text with their respective numbers in crescent order.

h) Abbreviations of institutions when presented in the first place should be put within parentheses, after the full name of the institution.

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j) **All cited articles should be consulted in full text**; if not possible, the original reference is put into the text as p.ex. Bancroft (1921); but in the List of References this should appear as: Bancroft 1921. ..... title. ... journal .... (Apud Suvarna & Layton 2013). The consulted reference should be also included in full in the List.

k) The use of "personal communication" and "non-published data" should be exceptional and cited in the text as Author and Year, and in the List of References as p.ex. Barbosa 2016. Personal Communication (Universidade Federal do Pará, campus Castanhal, Brazil).

l) **Figure captions** (p.ex. "Fig.3. ……..") should be sufficiently informative for understanding (because Figures are independent from the text).

m) The **Title of Tables** should be written in **bold** and the **Heading** (titles of the columns) should be in clear (not bold), written in capital and small letter and separated by two long horizontal lines. There are no vertical lines and no grey bottom; exceptionally can exist horizontal lines. The calls for footnotes should be in small letters or other signs, but not in Arabic numbers. Tables should be submitted in Word (not as images) to allow corrections according to the style of the journal.

n) Complex data should be presented as **graphics (but named Figures)** in 2D without grey bottom and horizontal lines. Graphics including text should be written with Cambria at 10 pitch.

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a) Save images at 300 dpi, TIF files.

b) Send each figure separately.

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d) Individual figures must have their files named as (Fig.1, Fig.2, …).

e) Images that will compose a plate must have their files identified as (Fig.1A, Fig.1B,). Plates should be comprised by multiple images, and all images must have the same dimensions.

f) Use preferably scale bars for micrographs. For optical micrographs indicate at the legend finally the staining method and the objective used, for example: HE, obj.40x.

g) Figure legends should contain initially what is seen on the image, followed by additional information (Legend example: Fig.1. (**A**) Sentence description. Diagnosis, organ or tissue, animal species, case number. Staining method and objective used.).

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Exemples for References:

➢ Articles published in scientific journals:

Ubiali D.G., Cruz R.A., De Paula D.A., Silva M.C., Mendonça F.S., Dutra V., Nakazato L., Colodel E.M. & Pescador C.A. 2013. Pathology of nasal infection caused by *Conidiobolus lamprauges* and *Pythium insidiosum* in sheep. J. Comp. Pathol. 149(2/3):137-145.

Hooiveld M., Smit L.A., Wouters I.M., Van Dijk C.E., Spreeuwenberg P., Heederik D.J. & Yzermans C.J. 2016. Doctor-diagnosed health problems in a region with a high density of concentrated animal feeding operations: a cross-sectional study. Environ. Health 17:15-24.

(Note: The first letters of the words in the title of papers published in journals are small. It is preferable to indicate the number of the respective issue.)

➢ Books:

Marsh P. & Martin M. 1992. Oral Microbiology. 3rd ed. Chapman and Hall, London, p.167-196.

Tokarnia C.H., Brito M.F., Barbosa J.D., Peixoto P.V. & Döbereiner J. 2012. Plantas Tóxicas do Brasil para Animais de Produção. 2ª ed. Helianthus, Rio de Janeiro, p.305-348.

(Note: The first letter in the words of the title of books should be capital.)

➢ Chapters of books:

Uzal F.A., Plattner B.L. & Hostetter J.M. 2016. Alimentary system, p.1-257. In: Maxie M.G. (Ed.), Jubb, Kennedy and Palmer´s Pathology of Domestic Animals. Vol.2. 6th ed. Elsevier, St Louis, Missouri.

Barros C.S.L. 2007. Doenças víricas: leucose bovina, p.159-169. In: Riet-Correa F., Schild A.L., Lemos R.A.A. & Borges J.R.J. (Eds), Doenças de Ruminantes e Equídeos. Vol.1. 3ª ed. Pallotti, Santa Maria, RS.

Tokarnia C.H., Brito M.F., Barbosa J.D., Peixoto P.V. & Döbereiner J. 2012. Plantas que afetam o funcionamento do coração, p.27-94. In: Ibid. (Eds), Plantas Tóxicas do Brasil para Animais de Produção. 2ª ed. Helianthus, Rio de Janeiro.

➢ Dissertations and Theses:

Rech R.R. 2007. Alterações no encéfalo de bovinos submetidos à vigilância das encefalopatias espongiformes transmissíveis. Tese de Doutorado, Universidade Federal de Santa Maria, Santa Maria. 228p.

(Note: Use articles which originated from dissertations or theses instead of these).

➢ Abstracts published in Events:

Massa A.T., Potter K.A. & Bradway D. 2016. Epizootic bovine abortion outbreak in Eastern Nevada cattle. Annual Meeting American College of Veterinary Pathologist (ACVP), New Orleans, Lousiana. (Abstract D-50)

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(Note: Consult entire papers instead of only Abstracts)

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