UNIVERSIDADE FEDERAL DO PARANÁ

SETOR DE CIÊNCIAS AGRÁRIAS

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS VETERINÁRIAS

GABRIELLE ADAD FORNAZARI

INVESTIGAÇÕES OFTÁLMICAS EM ESPÉCIES SELECIONADAS DE UNGULADOS ARTIODÁTILOS

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências Veterinárias, do Setor de Ciências Agrárias, da Universidade Federal do Paraná, como requisito parcial para obtenção de título de Mestre em Ciências Veterinárias.

Orientador: Professor D.Sc. Ivan Roque de Barros Filho
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Curitiba, 24 de março de 2015

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RESUMO

A presente dissertação engloba três artigos distintos que contribuem para o conhecimento científico atual da oftalmologia de ungulados artiodátilos selvagens e domésticos. Cada artigo científico tornou-se um capítulo da dissertação. Todos os estudos foram conduzidos pela autora e por colaboradores do Laboratório de Oftalmologia Comparada (LABOCO), da Universidade Federal do Paraná (UFPR). O primeiro capítulo relata uma estrutura anatômica jamais descrita em camelídeos do Velho Mundo, uma espinha óptica localizada no osso esfenóide. No que se refere à importância deste novo achado, a observação da estrutura óssea na órbita de camelos e dromedários (Artiodactyla), quando combinada com a constatação anterior de uma estrutura anatômica semelhante em morcegos (Chiroptera), pode fornecer apoio adicional quanto à proximidade dessas duas ordens animais, aparentemente muito distintas na árvore filogenética. A função exata da espinha óssea descrita deverá ser mais investigada no futuro, porém aparenta servir como suporte para músculos extraoculares. O segundo capítulo descreve as características anatômicas normais do olho, biometria ocular e parâmetros de testes oftálmicos para o aoudad (*Ammotragus lervia*). Seus olhos localizam-se lateralmente na cabeça, são proeminentes e possuem diversas características anatômicas especializadas provenientes de adaptações evolutivas para o pastejo, as quais já foram previamente observadas em outras espécies de ungulados como cavalos, bois e ovelhas. Foram estabelecidos os valores normais para testes oftálmicos comumente realizados: Teste Lacrimal de Schirmer (TLS), microbiota bacteriana normal da superfície ocular, estesiometria corneal, tonometria, espessura da córnea, ultrassonografia em modo B do bulbo ocular, análise de fotografia do fundo de olho e, por fim, mensuração da fissura corneana e palpebral. O conhecimento das variações anatômicas normais, resultados biométricos e parâmetros normais para testes diagnósticos oftálmicos nunca antes explorados nesta espécie podem auxiliar médicos veterinários e particularmente oftalmologistas veterinários no diagnóstico de várias doenças oculares que eventualmente ocorrem. O aoudad é um capríneo selvagem considerado ameaçado de extinção em seu habitat natural, entretanto está presente em diversos parques zoológicos ao redor do mundo e também em parques de caças legalizados. O terceiro e último capítulo consiste em um estudo histopatológico do carcinoma espinocelular ocular bovino (CEOB), uma importante doença neoplásica de ocorrência mundial, que impacta no bem-estar animal e que também traz sérios efeitos deletérios na produção de gado bovino. Trata-se da primeira investigação realizada exclusivamente com casos de CEOB diagnosticados no Estado do Paraná e em Santa Catarina. Foi realizada a classificação histológica de 10 casos diagnosticados de CEOB no LABOCO, sendo que esta foi uma tentativa pioneira de produzir um esquema de graduação histológica plausível em estudo brasileiro de CEOB. Curiosamente os nossos resultados de análise fenotípica estão em desacordo com dados de investigações anteriores, visto que seis de dez casos de CEOB foram observados em animais com olhos bem pigmentados. O parâmetro de anaplasia não se correlacionou bem com invasão tumoral mais profunda e índice mitótico, isso ocorreu com a maior parte das amostras analisadas. Uma associação entre a classificação histológica e técnicas moleculares pode ser alternativa viável para investigar e prever o comportamento do CEOB no futuro.

PALAVRAS-CHAVE: Oftalmologia. Ungulados artiodátilos. Espinha óptica. Osso esfenóide. Carneiro-da-barbária. Carcinoma espinocelular ocular.

ABSTRACT

The present dissertation encompasses three different texts contributing to the current scientific ophthalmology knowledge of ungulate artiodactyls. Each of these papers became a chapter in the present dissertation. All studies were conducted by the author and collaborators from Laboratory of Comparative Ophthalmology (LABOCO), Federal University of Paraná (FUPR). The first chapter reports a previously undescribed anatomic structure, an optic spine on the sphenoid bone in Old World camelids. Concerning the importance of this novel finding, the observation of this osseous structure on the bony orbit of camels and dromedaries (Artiodactyla) when combined with the previous finding of a similar anatomic structure in a bat (Chiroptera) may provide further support to the close proximity of these two apparently very distinct animal orders in the phylogenetic tree. The exact function of this osseous spine remains to be further investigated but seems to serve as an attachment for extraocular muscles. The second chapter describes normal anatomic eye features, biometry and ophthalmic test parameters for the aoudad (*Ammotragus lervia*). Its eyes are large and laterally placed in the head with several specialized anatomic features attributed to be evolutionary adaptations for grazing, which were also previously observed in other prey species of ungulates, such as horses, sheep and cattle. Normal values for commonly used ophthalmic tests were established, namely: Schirmer tear test (STT), normal ocular surface bacterial microbiota, corneal esthesiometry, tonometry, corneal thickness, B-mode ultrasonography of the globe, fundus photography analysis and lastly corneal and palpebral fissure measurements. Knowledge of the normal anatomic variations, biometric findings and normal parameters for ocular diagnostic tests may assist veterinarians and particularly veterinary ophthalmologists in the diagnosis of several ocular diseases that eventually occur in this previously unexplored wild caprid species, which is considered under threat of extinction in its natural habitat but rather widespread in zoological parks throughout the world and also used as game in legalized hunting parks. The third and last chapter consists of a histopathological study of the bovine ocular squamous cell carcinoma (OSSC), a worldwide prevalent and serious neoplastic disease, while having negative effects on animal welfare also have profoundly deleterious effects on cattle production. This was the first investigation performed exclusively in OSCC cases diagnosed in the State of Paraná and Santa Catarina. A histologic classification of 10 OSCC cases referred to LABOCO was performed. This is a first attempt to produce an acceptable histological grading scheme in a Brazilian OSCC study. Curiously our findings are in disagreement with previous investigations since six out of ten OSCC cases were observed in animals with heavily pigmented eyes. Awkwardly, anaplasia parameters did not correlate well with tumor invasion of deeper tissues and mitotic indexes in most samples analyzed. Possibly, an association between histological grading and molecular techniques such as in situ hybridization and immunohistochemistry would be a feasible route to investigate and predict OSCC behavior in the future.

KEYWORDS**:** Ophthalmology. Artiodactyla. Optic spine. Sphenoid bone. Barbary sheep. Ocular squamous cell carcinoma.

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1 GENERAL INTRODUCTION

Ophthalmology of large mammals, particularly ungulates (order Artiodactyla) represent a substantial lacuna in the scientific literature of vision research. Wild and domestic large mammal ophthalmology falls rather awkwardly in an intersection of three different specialty areas: veterinary ophthalmology, wild animal medicine and large animal medicine. The majority of veterinary ophthalmologists involve themselves predominantly in small animal, equine and very sporadically in small exotic pet medicine. On the other side, most wild animal clinicians and large animal practitioners do not specialize in ophthalmology. Thus, most ocular diseases in production animals and large wild animals are dealt with by farm animal and wild animal veterinarians, respectively. Discrete eye diseases of cows, sheep and pigs are commonly unnoticed. The same happens with large wild mammals. The truth is that referral veterinary ophthalmology specialists rarely diagnose and treat common eye conditions of either large wild mammals, domestic ruminants and, certainly camelids. Accordingly, the same pattern is followed in the research field. While the progress of investigative ophthalmology of small animals is certainly outstanding, ophthalmic investigations involving camelids, wild and domestic ruminants are very uncommon and often are underfunded. This is very unfortunate because studying the eyes of these creatures is nothing short of fascinating. Several diseases can be studied in these species and its eyes can even serve as a model for ophthalmic diseases that affect human beings, including ocular squamous cell carcinoma studied here. Therefore, in an attempt to contribute specifically in this information-deficient area, we have produced a series of investigations with anatomy, clinic and histopathology of wild and domestic large mammals. Data contained herein will be of value for ophthalmic investigators worldwide and large and wild animal practitioners, hopefully filling part of the scientific lacuna.

2 A DESCRIPTION OF AN OPTIC SPINE ON THE SPHENOID BONE OF CAMELS AND DROMEDARIES

Descrição de uma espinha óptica no osso esfenóide de camelos e dromedários

2.1 ABSTRACT

Objective: To describe the presence of an intraorbital cylindrical osseous structure (a spine) in two animal species: camel (*Camelus bactrianus*) and dromedary (*Camelus dromedaries*). A homologous osseous structure was previously observed in the large fruit-eating bat (*Artibeus lituratus*).
Procedures: The bony anatomy of the orbital cavity was studied and quantified on macerated skulls of 3 camels and 2 dromedaries. Additionally, one macerated skull of a large fruit-eating bat (*Artibeus lituratus*) was used for comparative purposes.
Results: The anatomic description of these unique intraorbital spine was made while studying the bony orbit of macerated skulls, and was considered homologous to that of the bat based on the same anatomic position (at the bone bridge that separates the optic canal and the sphenorbital fissure) and similarities in shape. We suggest the name optic spine of the sphenoid bone.
Discussion: The novel observation of an optic spine on the sphenoid bone in camels and dromedaries (Artiodactyla), when combined with the previous finding of a similar anatomic structure in a bat (Chiroptera) suborder Microchiroptera, may provide further support to the close proximity of these two apparently very distinct animal orders in the phylogenetic tree, and may contribute to the understanding of bat evolution and provide new directions for future research. The function of this osseous spine remains to be investigated, although we hypothesize that the optic spine of the camelids may serve as an attachment site for extraocular muscles.

KEY-WORDS: Anatomy. Bony orbit. *Camelus bactrianus*. *Camelus dromedaries*. Optic spine. Sphenoid bone. Comparative studies. Mammals.

2.2 RESUMO

Objetivo: Descrever a presença de um processo ósseo intraorbital cilíndrico (uma espinha) em duas espécies animais: camelo (*Camelus bactrianus*) e dromedário (*Camelus dromedaries*). Uma estrutura óssea homóloga foi previamente observada no morcego-das-frutas (*Artibeus lituratus*).

Procedimentos: Foi estudada a anatomia óssea da cavidade orbital de crânios macerados de três camelos e dois dromedários. Além disso, um crânio macerado do morcego-das-frutas (*Artibeus lituratus*) foi utilizado para fins comparativos.
Resultados: A descrição anatômica desta espinha intraorbital óssea inédita em camelídeos foi feita a partir do estudo da órbita de crânios macerados sendo que foi considerada homóloga com aquela do morcego, tendo como base a mesma posição anatômica (na ponte óssea que separa o canal óptico e a fissura esfeno-orbital) além de similaridades em seu formato. Sugerimos o nome de espinha óptica do osso esfenóide.
Discussão: Uma observação científica nova da espinha óptica no osso esfenóide em camelos e dromedários (Artiodátilos), quando combinada com a constatação anterior de estrutura anatômica semelhante em um morcego (Quiróptero) subordem dos Microquirópteros, pode fornecer um apoio adicional da proximidade dessas duas ordens animais aparentemente distintas na árvore filogenética. Além disso, pode contribuir para a compreensão do processo evolutivo do morcego e proporcionar novas direções para pesquisas futuras. A função da espinha óssea continua a ser investigada, embora acreditamos que possa servir como um sítio de inserção para os músculos extra-oculares.

PALAVRAS-CHAVE: Anatomia. Órbita óssea. *Camelus bactrianus*. *Camelus dromedaries*. Espinha óptica. Osso esfenóide. Estudos comparativos. Mamíferos.

2.3 INTRODUCTION

Evolutionary relationships among several different orders of the animal tree of life have proven difficult to determine or have received little support in the vast majority of phylogenomic studies of mammalian systematics, and thus remain unresolved at best. Among those mammals with significant knowledge gaps are the bats (Chiroptera), despite representing one of the largest and most diverse radiations of mammals, and accounting for one-fifth of extant species. Found worldwide, bats are also the only mammals to have achieved true self-powered flight, and they play a major ecological role as pollinators and insect predators (Patterson et al. 2003; Simmons et al. 2008).

Currently the position of bats in the evolutionary tree of life is considered conflicting or incomplete, thus the phylogenetic and geographic origin of bats (and the entire order Chiroptera) remains unclear. A plausible reason for this fact is that bats are not well represented in the fossil record (Altringham, 1996; Nowak, 1999; Springer et al., 2001; Patterson et al., 2003; Van Den Bussche & Hoofer, 2004; Eick et al., 2005;

Gunnell & Simmons, 2005; Simmons et al., 2008). There are some possible reasons for the lack of fossil evidence. One is that bats have small, delicate skeletons that do not fossilize very well. Another is that most species live in tropical forests, where conditions are usually unfavorable for the formation of fossils (Carroll, 1988). Despite a poorly represented fossil record, even the earliest fossil bats dating back 45 to 50 million years ago have an outstanding resemblance to modern microbats, and intriguingly no fossil bats have yet been identified that are in any way intermediate in form between modern microbats and early tree-living ancestors (Altringham, 1996; Simmons et al., 1998; Nowak, 1999; Springer et al., 2001; Eick et al., 2005; Teeling et al., 2005). Thus, according to Altringham (1996), modern microbats may have made their appearance about 65–100 million years ago. If so, they amazingly shared the world with the dinosaurs, and watched their extinction at the end of the Cretaceous period. Historically, the most common assumption about the evolutionary history of bats has been based on morphological evidence, grouping bats with primates, flying lemurs, and tree shrews to form the Archonta (Szalay, 1977; Novacek, 1992; Gunnell & Simmons, 2005).

New genomic evidence demonstrates an unexpected sister relationship between Chiroptera and Cetartiodactyla (Hallström & Janke, 2008; Nery et al., 2012, Zhang et al. 2013). The curious and unusual phylogenetic position and consequent evolutionary proximity between Chiroptera and Artiodactyla has received genomic but no real morphological support until now.

The skull has also been used as a major skeletal structure to determine taxonomic affiliations as it is subject to phenotypic changes because of selective breeding (Bruenner et al., 2002)

The objective of this study is to report the presence of an intraorbital cylindrical osseous structure, a spine, in two animal species: camel (*Camelus bactrianus*) and dromedary (*Camelus dromedaries*). A homologous osseous structure in the bony orbit was previously only observed in a bat (the large fruit-eating bat *Artibeus lituratus*) (Machado et al., 2007). The observation of the same anatomic feature in the bony orbit of both Artiodactyla (Old World camelids) and Chiroptera (bats, specifically of the suborder Microchiroptera) may provide further support towards the growing body of evidence suggesting close proximity of these two apparently very distinct animal orders within the evolutionary tree.

2.4 MATERIALS AND METHODS

A thorough examination of the bony orbit from 5 previously macerated skulls of Old World camelids (3 adult camels and 2 adult dromedariess) was performed, including anatomic description and gross specimen morphometry. From the three camel skulls studied, two (from one 35 year-old male and one 21 year-old female) belonged to the collection of Capão da Imbuia – Museum of Natural History (MHNCI) and one from a 34 year-old male belonged to the collection for environment education of the Curitiba Zoo, both institutions located in Curitiba-PR, Brazil. The two dromedarian skull samples (one from a 23 year-old male and the other from an 18 year-old female) belonged to the Veterinary Anatomy Museum of the University of Contestado, located in Canoinhas-SC, Brazil. One previously macerated skull of an adult large fruit-eating bat (*Artibeus lituratus*) was used in this investigation for morphologic and photographic comparisons. This skull belonged to the private collection of one of the authors (MM).

The camel skulls where previously naturally cleaned by a decomposition process while the dromedarian skulls where prepared by a laboratorial maceration technique. The skin and most of the soft tissues and eyes were removed to initially clean the skulls, and then a maceration technique consisting of a boiling process followed by cold water immersion in a closed recipient for two weeks was performed. After maceration the skulls were immersed in 50% hydrogen peroxide for approximately 24 h for bleaching. Following this step they were washed in distilled water and air dried. The nomenclature used for skull osteology follows previously published work on osteology and camel anatomy (Neumani, 1911; Smuts & Bezuidenhout, 1987, Olsen, 1988; Shahid & Kausar, 2005; Yahaya et al., 2012 a,b).

The macerated skulls and optic spines were measured with a measuring tape, a ruler, and a digital pachymeter, and were then digitally photographed. Selected osteometric parameters were measured, according to Sarma (2006), Karimi (2011) and Yahaya (2012 a,b) and orbital indexes calculated according to Kaur et al. (2012).

Morphometric analysis of the skull included: The skull length, i.e. the interincisive space to the most caudal aspect of the occipital bone (the intersection point between the sagittal and nuchal crest); Skull width, i.e. the distance between the two most lateral points of the frontal bones (the most lateral parts of the dorsal margin of the orbit) (Figure 1); The intraorbital bony spine width at the base and at the tip, dorsal and ventral lengths (Figure 2 C) bilaterally (Figure 3); The orbital horizontal and vertical diameters (Figure 4). Additionally, orbital indexes were calculated as follows: Orbital index = Vertical diameter of the orbit × 100/Horizontal diameter of the orbit.

2.5 RESULTS

The overall shape of the skull of camels and dromedaries is very similar (Fig. 1). Both when viewed from above are roughly pentagonal in shape, elongated towards the maxilla and mandible. Both are wider in the frontal bone region (skull width) than between the zygomatic bones. The orbits are nearly circular and enclosed (complete) situated laterally and slightly cranially (Figs. 1 and 2). The rim of the frontal bone is serrated (Fig.2 A). An irregular transverse elevation separates the parietal and nuchal surfaces (Fig. 1). The occipital bone formed the entire nuchal surface and invaded upon the dorsal surface. It joined the parietal bone at the transverse suture. The sagittal and occipital crests on the dromedary are considerably more pronounced or developed than those found in the camel skull.

FIGURE 1 - DORSAL VIEWS OF THE SKULLS OF A REPRESENTATIVE ADULT CAMEL (A) AND AN ADULT DROMEDARY (B). LEGEND: ARROWHEADS REPRESENTS THE POINTS TO MEASUREMENT OF THE SKULL LENGTH (INTERINCISIVE SPACE AND THE INTERSECTION POINT BETWEEN THE SAGITTAL AND NUCHAL CREST), AND ARROWS REPRESENTS THE POINTS TO MEASUREMENT OF THE INTERORBITAL LENGTH. NOTE THAT AN IRREGULAR TRANSVERSE ELEVATION SEPARATES THE PARIETAL AND NUCHAL SURFACES. BAR: 10 CM.

Inside the bony orbit we have observed an unusual osseous spine that is slender and elongated in shape, directed rostrolaterally, and also slightly ventrally in camels (*Camelus bactrianus*) (Fig. 2. A) and dromedaries (*Camelus dromedaries*) (Fig. 2 B). It is located bilaterally (Fig. 3) on the bone bridge that separates the optic canal and the sphenorbital fissure on the sphenoid bone complex (Fig. 2 C and Fig. 4) of these species. This bony spine is thin, cylindrical in shape, and tapers at its free rostral end in both species (Fig.2 C). It is slightly wider at its sphenoid bone base in camels than in dromedaries. In camels, the spine is camel is slightly irregular at its free end (Fig. 1 A).

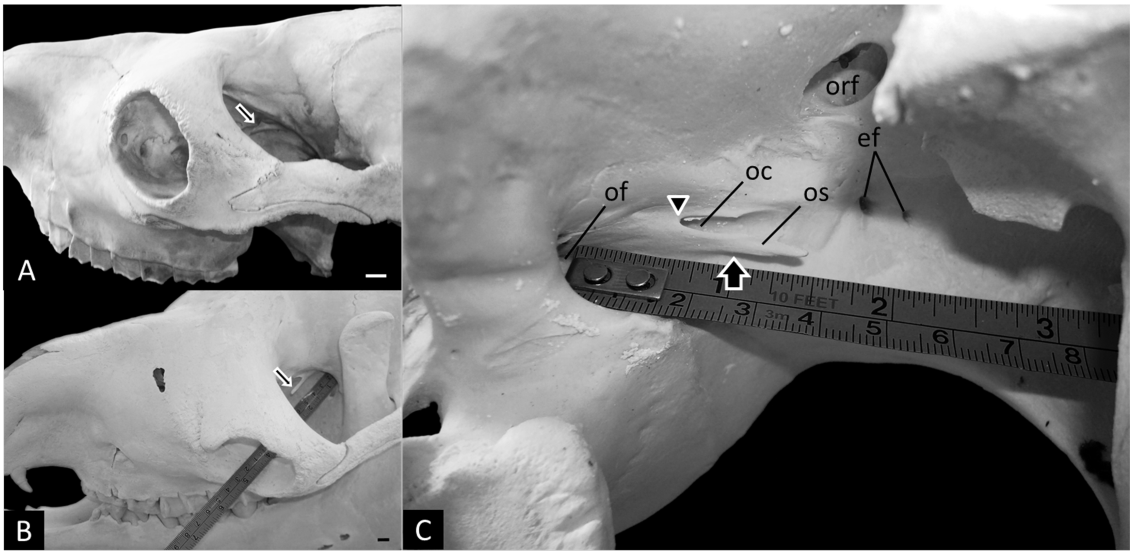

FIGURE 2 - GENERAL TOPOGRAPHY AND FORM OF THE OPTIC SPINE OF THE SPHENOID BONE IN OLD WORLD CAMELIDS. OBLIQUE DORSOCAUDAL VIEW OF THE LEFT OPTIC SPINE OF THE SPHENOID BONE OF A CAMEL (A), DORSOLATERAL VIEW OF THE LEFT OPTIC SPINE OF A DROMEDARY (B), AND VENTROLATERAL VIEW OF THE MEDIAL AND CAUDAL WALLS OF THE BONY ORBIT OF A REPRESENTATIVE ADULT DROMEDARY SKULL (C). THE OPTIC SPINE OF THE SPHENOID (ARROW) IS CLEARLY SEEN IN THESE MACERATED SKULLS WITHOUT THE AID OF ANY MAGNIFYING DEVICE. NOTE THAT THE ORBIT IS COMPLETE AND THE OPTIC SPINE OF THE CAMEL IS SLIGHTLY IRREGULAR AT ITS FREE END, COMPARED WITH THE DROMEDARIES. VENTRAL (ARROW HEAD) AND DORSAL (ARROW) BASES OF THE OPTIC SPINE OF THE SPHENOID (OS) CAN BE SEEM. LEGEND: ETHMOIDAL FORAMINA (EF), OPTIC CANAL (OC), ORBITAL FISSURE (OF); ORBITOROTUNDUM FORAMEN (ORF). BARS: 1CM.

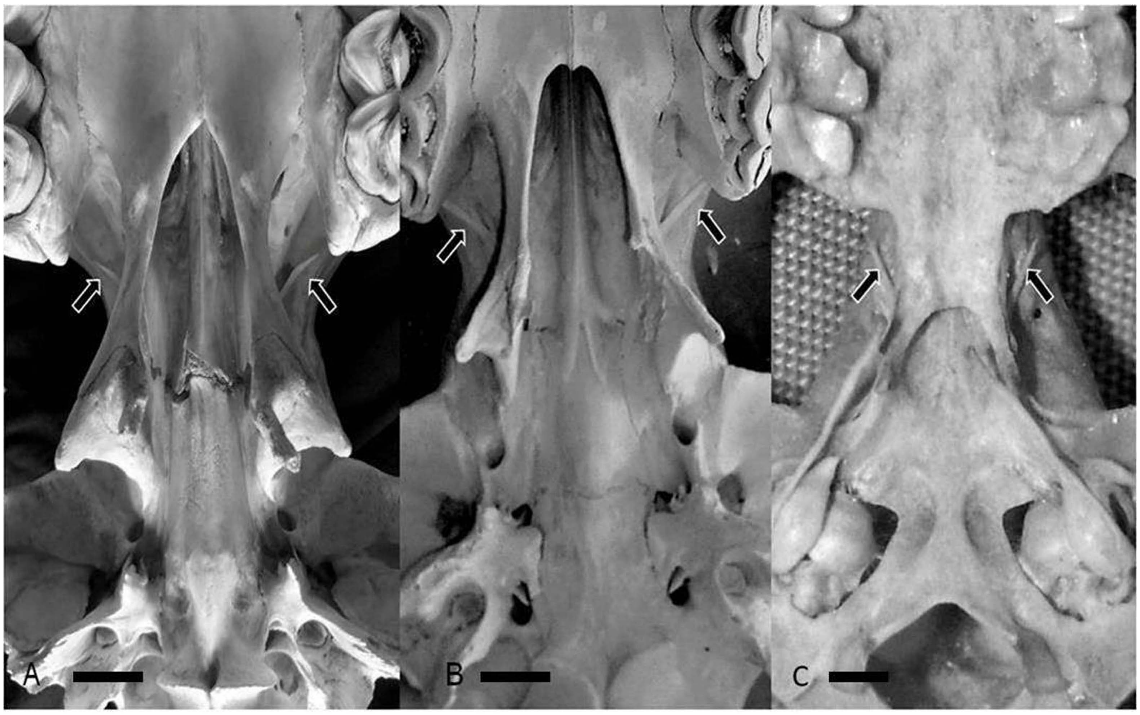

FIGURE 3 - DETAIL OF THE BILATERAL ARRANGEMENT OF THE OPTIC PROCESSES (ARROWS) OF THE SPHENOID BONE ON A VENTRAL VIEW OF THE BASE OF THE SKULLS OF A REPRESENTATIVE ADULT CAMEL (A) BAR: 2 CM, AN ADULT DROMEDARY (B) BAR: 2CM, AND AN ADULT LARGE FRUIT-EATING BAT (*ARTIBEUS LITURATUS*) (C) BAR: 2 MM. NOTE THAT ALTHOUGH BOTH BONY ELEMENTS ARE ROSTRALLY ORIENTED, THE OPTIC SPINE IN THE CAMEL AND IN THE DROMEDARY ARE MORE LATERALLY ORIENTED THAN THE OPTIC SPINE OF THE LARGE FRUIT-EATING BAT.

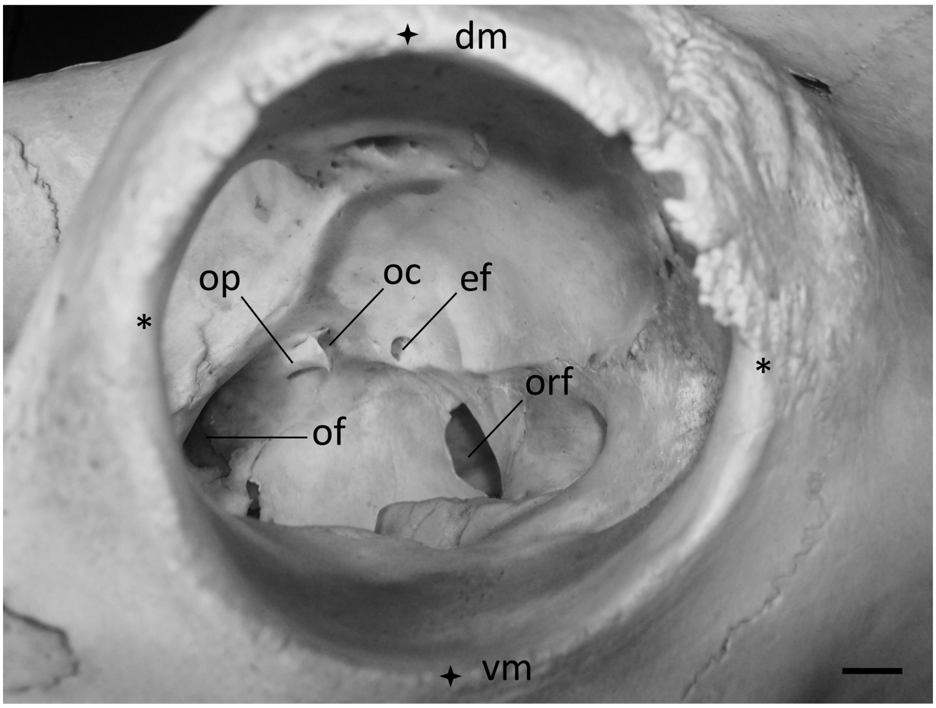

FIGURE 4 - ROSTROLATERAL VIEW OF THE BONY ORBIT THROUGH THE ORBITAL ADIT OF A REPRESENTATIVE ADULT CAMEL SKULL. LEGEND: DORSAL MARGIN OF THE ORBIT (DM); ETHMOIDAL FORAMEN (EF), OPTIC CANAL (OC), ORBITAL FISSURE (OF); ORBITOROTUNDUM FORAMEN (ORF); OPTIC SPINE OF THE SPHENOID (OS); VENTRAL MARGIN OF THE ORBIT (VM). STARS REPRESENT THE POINTS TO MEASUREMENT OF THE ORBITAL HEIGHT (DORSOVENTRAL AXIS), AND ASTERISKS REPRESENTS THE POINTS TO MEASUREMENT OF THE ORBITAL DIAMETER (MEDIOLATERAL AXIS). BAR: 1 CM.

Morphometry on dromedary skulls

The mean skull width was 24.75 ± 1.8 cm and the mean skull length was 41.33 ± 03.44 cm. The mean intraorbital bony optic spine length from the ventral base to tip was 2.10 ± 1.06 cm and the length from the dorsal base to tip was shorter, measuring 1.25 ± 0.94 cm. The mean width at the base was 0.34 ± 0.42 cm at the base and 0.21 ± 0.03 cm at the tip of the spine. The mean orbital horizontal diameter measured 6.26 ± 0.79 cm. The mean orbital vertical diameter was 6.11 ± 0.73 cm. The mean orbital index was 102.45.

Morphometry on camel skulls

The mean skull width was 27,4 ± 2,7 cm and the mean skull length was 53.10 ± 2.82 cm. The mean intraorbital bony optic spine length from the ventral base to tip was 2.11 ± 0.81 cm and the length from the dorsal base to tip was shorter, measuring 1.40 ±

0.30 cm. The mean width at the base was 0.41 ± 0.56 cm and 0.27 ± 0.09 cm at the tip of the spine. The mean orbital horizontal diameter was 6.15 ± 0.44 cm. The mean orbital vertical diameter was 5.92 ± 0.50 cm. The mean orbital index was 103.88.

2.6 DISCUSSION

General osteology and osteometry of camel and dromedary skulls have been published elsewhere (Neumani, 1911; Olsen, 1988, Yahaya et al., 2012). Mean orbital horizontal diameter found in dromedaries investigated in the present work was similar to the one (6.01 ± 0.07 cm) reported by Yahaya et al. (2012)b. Mean orbital vertical diameter in dromedaries parallels results from Yahaya et al. (2012)b, which varied from 5.74 ± 0.12 cm to 6.12 ± 0.21 cm. Additionally, mean dromedary skull length found in our investigation also was comparable to the data from Monfared (2013), which was 46.2 ± 2.74 cm and Yahaya (2012), which varied from 45.50 ± 0.65 cm to 49.44 ± 0.86 cm. Nevertheless, none of these previous studies described the presence of the optic spine of the sphenoid bone. Orbital indexes of both species were considerably large. Both were larger than the goat 86.11 to 92.14 (Sarma, 2006) but smaller than the Mehraban sheep, which varied from 108.38 from 109.07 (Karimi et al., 2011)

Despite being rather inconspicuous, these spines have remained undescribed in Old World camelids until now, possibly because of the limited research available in the literature regarding morphological features of their eye, adnexa and orbit (Neumani, 1911; Tayeb, 1951; Abdalla et al., 1970; Awkati & Al-Bagdadi, 1971; Smuts & Bezuidenhout, 1987; Olsen, 1988; Abuel-Atta et al., 1997; Wang JL. 2002; Cui et al., 2004; Shahid & Kausar, 2005; Yahaya et al., 2012 a,b; El-Tookhy et al., 2012). Even detailed studies of the cranioencephalic structures of dromedaries using diagnostic imaging techniques such as radiography (Saber, 1990), computed tomography (Alsafy et al., 2014) and magnetic resonance (Arencibia et al., 2005) failed to detect and describe the spines of the sphenoid bone.

Notwithstanding the scant information known about the evolutionary history of bats, evidence suggests that bats may have originated in the northern supercontinent of Laurasia, possibly in North America (Teeling et al., 2005) as part of a large group of placental mammals (Laurasiatheria) including shrews, hedgehogs, pangolins, whales, carnivorans, and most hoofed mammals such as camels, among others. Several questions still remain regarding how the different orders of several mammalians in the supraordinal group Laurasiatheria evolved.

Traditionally bats were placed along with primates, flying lemurs, and tree shrews, forming the Archonta Anatomical (Szalay, 1977; Novacek, 1992). However, in more recent phylogenetic analyses of the complete mitochondrial genome of the Jamaican fruit bat (*Artibeus jamaicensis*), it appeared that bats may be more closely related to “cetferungulates”, a clade including Cetacea, Artiodactyla, Perissodactyla, and Carnivora (Pumo et al., 1998). Phylogenetic analyses from the c-myc gene sequences also support this relationship (Miyamoto, 2000). Other phylogenetic investigations using relationships with genome data started to place bats near cows (Hallström & Janke, 2008). Posteriorly, phylogenetic analyses investigating a very large amount of genomic sequence data have provided even greater and clearer support for the sister relationship between Chiroptera and Cetartiodactyla (Nery et al., 2012; Zhang et al., 2013). Cetartiodactyla is the clade in which whales and even-toed ungulates are currently placed. The term was coined by merging the name for the two orders, Cetacea and Artiodactyla, into a single word. Cetacea includes whales and dolphins. Artiodactyla includes pigs, peccaries, hippopotamuses, camel, dromedary, llamas, chevrotains (mouse deer), deer, giraffes, pronghorn, antelopes, sheep, goats, and cattle.

Here, taking the current description into account and following the results from Machado et al. (2007) in a bat, we provide anatomical evidence for the support of a possible sister relationship between Chiroptera and Cetartiodactyla in the form of intraorbital osseous spines. The first intraorbital osseous spines, observed in an animal was on the *Artibeus lituratus* (a large fruit-eating bat) (Machado et al., 2007). The spine is a slender and elongated bony spine, directed rostrolaterally and slightly ventrally. It is located bilaterally on the bone bridge that separates the optic canal and the sphenorbital fissure on the alisphenoid bone (from the sphenoid complex) (Figure 1 c). The group of researchers suggested the name optic spine of the alisphenoid bone. These equivalent bony optic spines described here in Old World camelids are similar in anatomical position and general shape, but are more rostrolaterally oriented and slightly less ventral than the spines of the large fruit-eating bat. Nevertheless, the anatomic feature was thus far exclusively reported to Old World Camelids and the large fruit-eating bat and was considered homologous based on the same anatomic position (at the bone bridge that separates the optic canal and the sphenorbital fissure) and shape similarities, which surpasses angular differences (Fig. 3).

Function of this osseous spine as well as potential differences in immature animals remains to be investigated. We hypothesize that the optic process of the camelids may serve as an attachment site for extraocular muscles in a similar manner to the optic spine of the alisphenoid bone in bats (Machado et al., 2007). In order to prove this assumption, future studies should perform a careful dissection in fresh or fixed camelid skulls, paying special attention to the delicate attachments of the extraocular muscles. The observation of an optic spine on the sphenoid bone in camels and dromedaries (Artiodactyla), when combined with the previous finding of a such anatomic component in a bat (Chiroptera, suborder Microchiroptera), may provide further support to the close proximity of these two apparently very distinct animal orders in the phylogentic tree, and contribute to the understanding of bat evolution and perhaps provide new directions for future research.

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3 THE EYE OF THE AOUDAD (*Ammotragus lervia*): REFERENCE VALUES FOR SELECTED OPHTHALMIC DIAGNOSTIC TESTS

O olho do aoudad (Ammotragus lervia): valores de referência para testes oftálmicos selecionados

3.1 ABSTRACT

The purpose of this study was to describe normal anatomic eye features and establish reference values for ophthalmic tests in the aoudad *(Ammotragus lervia)*. Aoudads possess eyes are large and laterally placed in the head with several specialized anatomic features attributed to be evolutionary adaptations for grazing. Normal values for commonly used ophthalmic tests were established: Schirmer tear test (STT) - 27.22 ± 3.6 mm/min; Predominant ocular surface bacterial microbiota - Staphylococcus sp.; Corneal esthesiometry- 1.3 ± 0.4 cm; Rebound tonometry- 19.47 ± 3.9 mmHg; Corneal thickness- 630.07 ± 20.67 μm, B-mode ultrasonography of the globe- axial eye globe length 29.94 ± 0.96 mm, anterior chamber depth 5.03 ± 0.17 mm, lens thickness 9.4 ± 0.33 mm, vitreous chamber depth 14.1 ± 0.53 mm; Corneal diameter- horizontal corneal diameter 25.05 ± 2.18 mm, vertical corneal diameter 17.95 ± 1.68 mm; Horizontal palpebral fissure length- 34.8 ± 3.12 mm. Knowledge of these normal anatomic variations, biometric findings and normal parameters for ocular diagnostic tests may assist veterinary ophthalmologists in the diagnosis of several ocular diseases.

KEY-WORDS: Barbary sheep. Wild caprid. Ocular parameters. Biometry.

3.2 RESUMO

O objetivo deste estudo foi descrever as características oculares anatômicas normais e estabelecer valores de referência para testes oftalmológicos para o aoudad (*Ammotragus lervia*). Seus olhos localizam-se lateralmente na cabeça, são grandes e possuem diversas características anatômicas especializadas, provenientes de adaptações evolutivas para o pastejo. Foram estabelecidos os valores normais para testes oftálmicos comumente realizados: teste da lacrimal de Schirmer (TSS) - 27,22 ± 3,6 mm/min; Microbiota bacteriana normal da superfície ocular - Staphylococcus sp .; Estesiometria corneal - 1,3 ± 0,4 cm; Tonometria de rebote - 19,47 ± 3,9 mmHg; Espessura da córnea - 630,07 ± 20,67 mm, Ultrassonografia em modo B (comprimento axial do bulbo ocular - 29,94 ± 0,96 mm, profundidade da câmara anterior - 5,03 ± 0,17 mm, espessura da lente - 9,4 ± 0,33 mm, profundidade da câmara vítrea - 14,1 ± 0,53 mm, diâmetro da córnea horizontal 25,05 ± 2,18 mm, diâmetro da córnea vertical - 17,95 ± 1,68 mm, comprimento da fenda palpebral - 34,8 ± 3,12 mm. O conhecimento das variações anatômicas normais, resultados biométricos e parâmetros normais para testes diagnósticos oftálmicos pode auxiliar oftalmologistas veterinários no diagnóstico de várias doenças oculares.

PALAVRAS CHAVE: Carneiro-da-Barbária. Capríneo selvagem. Parâmetros oculares. Biometria.

3.3 INTRODUCTION

The aoudad (*Ammotragus lervia*) is a species of wild caprid (goat-antelope) that naturally occurs in northern Africa in Algeria, Tunisia, northern Chad, Egypt, Libya, northern Mali, Mauritania, Morocco, Niger and Sudan (west of the Nile, and in the Red Sea Hills east of the Nile). It is also known as Barbary sheep, waddan, arui, and arruis (Cassinello, 1998; Wacher et al., 2002; Cassinello et al., 2004). The binomial name *Ammotragus lervia* derives from the Greek ammos ("sand", referring to the sand-coloured coat) and tragos ("goat"). The species name *lervia* derives from the wild sheep of northern Africa (Cassinello, 1998; Wacher et al., 2002). In its native distribution in northern Africa the aoudad was classified as “vulnerable” species by the 2012 Red List of the International Union of Conservation of Nature (IUCN) due to natural habitat loss and pouching (Alados & Shackleton, 1997; Hilton-Taylor, 2000; Cassinello et al., 2008). It has, however, been successfully introduced to North America, Europe and elsewhere primarily for trophy-hunting purposes. These populations have a large number of individuals and are free-ranging, commonly competing with the native mammals for resources (Cassinello et al., 2008). The aoudad is a stocky, heavily built wild ruminant, with short legs and a rather long skull (Kingdon, 1997; Stuart & Stuart, 1997). Both sexes have horns that sweep backwards and outwards in an arch; those of the male are much thicker and reach up to 50 cm. Aoudads weight can vary from 40 to 140 kg. Males also differ from females by their significantly heavier weight, up to twice that of females (Kingdon, 1997), and the notably longer curtain of hair that hangs from the throat, chest and upper part of the forelegs (Kingdon, 1997; Stuart & Stuart, 1997; Cassinello, 1998). The coat is woolly during the winter, but moults to a finer, sleek coat for the hot summer months. It has a sandy-brown color, darkening with age, with a slightly lighter underbelly and a darker line along the back (Kingdon, 1997; Stuart & Stuart, 1997). The eyes of the aoudad are bright and apparently large in relation to its body size, reminding a cervid-like or antilocaprid-like morphology more than a caprid one. Concerning aoudads in the scientific literature, hormonal parameters and studies about applied reproductive techniques has been published (Hamon & Heap, 1990; Crenshaw et al., 2000; Abáigar et al., 2012; Santiago-Moreno et al., 2013). In the same way genetic studies (McLelland et al., 2005; Manca et al., 2006; Mereu et al., 2008), epidemiologic surveys and reports of specific infectious diseases (Yeruham et al., 2004; Candela et al., 2009; Pirastru et al., 2009; Portas et al., 2009; Münster et al., 2013; Morikawa et al., 2014) and parasitosis (Pence & Gray, 1981; Cho et al., 2006; Mayo et al., 2013) were investigated. Additionally, a case report of pemphigus foliaceus has been published in this species (Brenner et al., 2009). However, we could find no ophthalmic investigations or even reports of ocular diseases in the species, possibly because baseline values for diagnostic tests have not been established yet for aoudads. Knowledge of baseline values is evidently required for both appropriate diagnosis and treatment of ocular diseases in zoo and exotic animals. Important parameters to be established in wild animals include Schirmer tear test (STT) and intraocular pressure (IOP), echobiometric findings as well as normal conjunctival bacterial microbiota (Prado et al., 2005; Kudirkiene et al., 2006; Montiani-Ferreira et al., 2006; Martins et al., 2007; Montiani-Ferreira et al., 2008a; Wang et al., 2008; Ribeiro et al., 2009; Lima et al., 2010; Ghaffari et al., 2012). These normal ophthalmic parameters in domestic, exotic and zoo animals become important references for the veterinary clinician and other researchers after published. The purpose of this study was to describe normal ophthalmic parameters in aoudads, including morphological features, biometry of anatomical structures, corneal ultrasonic pachymetry, globe echobiometry, Schirmer´s Tear Test (STT), intraocular pressure (IOP), corneal sensitivity, bacterial conjunctival microbiota, and fundus photography.

3.4 MATERIALS AND METHODS

All ophthalmic procedures using live aoudads were conducted in accordance with UFPR's Animal Use Committee, protocol 045/2013 and with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Eighteen adult captive aoudads (11 males and 7 females) of different ages (varying from 1.5 to 7 years of age, mean 4 ± 2.04 years) belonging to Curitiba´s Zoo (Zoológico de Curitiba), Curitiba-PR, Brazil (25°25′S and 49°16′W) were captured for clinical evaluation as part of a health survey by the park authority (Fig. 1) during the winter of 2014 in three different occasions. A detailed ophthalmic health evaluation including all the tests cited here was performed in this survey. Physical examinations, including a complete blood count panel, were performed before ocular examinations to exclude animals with indications of systemic disease. Aoudads with evidence of ocular or systemic diseases were excluded. Procedures and tests necessary to produce this work were split between the investigators. However, to avoid discrepancies related to inter-observer repeatability, the same person always performed the same ocular test on each occasion.

Ophthalmic tests

Clinical tests were performed while the aoudads were physically restrained by two experienced handler using ropes, taking care to keep the animal comfortable. When the head was manually stabilized for taking measurements special attention was given to avoid applying pressure to the neck region with hands or ropes, to prevent iatrogenic alterations in IOP. The sequence of procedures performed in this study was: (i) ocular inspection (including photography), (ii) Schirmer tear test (STT), (iii) collection of material for bacterial culture analysis, (iv) corneal esthesiometry, (v) tonometry, (vi) central corneal thickness (CCT) measurement with an ultrasonic pachymeter, (vii) B-mode ultrasonography of the globe, (viii) fundoscopy and lastly (ix) corneal and palpebral fissure measurements (Figure 2).

Ocular inspection

A total of 36 eyes, from 18 healthy adult aoudads were selected and used in this investigation. The anterior ocular structures were evaluated using a Finoff transilluminator (3.5 V halogen fiber optic, Welch Allyn, Skaneateles Falls, NY, USA) and a slit lamp biomicroscope (Hawk Eye; Dioptrix, L'Union, France) and photographed with a 7.2 megapixel reflex digital camera with a Carl ZeissTM lens and 12x of optical zoom (DSC-H5; Sony TM, Minato, Tokyo, Japan) (Fig. 3a).

Schirmer tear test

Sterile standardized STT strips (Schering Plough Animal Health, Union, NJ, USA) were used to perform the Schirmer type I test (Fig. 2a), which measures the basal plus a portion of the reflex tear secretion.

Microbiological analysis

For the microbiological analysis, samples were obtained by carefully touching the conjunctival sac and ocular surface (cornea and bulbar conjunctiva) with a cotton swab (Fig 2e). No topical anesthetic was used prior to sample collection as this may interfere with the growth of organisms (Mullin & Rubinfeld, 1997). Aerobic bacterial culture of the microorganisms was performed in BHI broth (brain–heart infusion), and on 5% sheep blood agar and MacConkey plates, which were incubated at 37°C in an aerobic environment for 24–48 h. The same bacterial growth media used in this research was also used elsewhere to establish normal conjunctival microbiota of the opossum, raccoon, ferret and chinchilla in other investigations (Pinard et al., 2002; Manca et al., 2006; Montiani-Ferreira et al., 2006; Montiani-Ferreira et al., 2008a). Bacterial colonies were identified by Gram's stain and standard procedures.

Corneal esthesiometry

For the normal corneal sensitivity analysis, all aoudads were manually restrained, and a Cochet-Bonnet esthesiometer (Luneau Ophtalmologie, Chartres Cedex, France) was used (Fig. 2b). This instrument contains an adjustable nylon filament with a defined diameter, length and surface (0.12 mm diameter, 60 mm length, and 0.0113 mm^2

surface), which was applied in different lengths to the center of the cornea. A stimulus produced by the instrument's nylon monofilament that reaches the corneal touch threshold induces a corneal reflex, consisting of prompt eyelid closure, and discrete retraction of the globe. In this study only the center of the cornea was analyzed for corneal touch threshold, which was repeated five times using the same length of the nylon filament. The length of the nylon filament was then decreased at 5-mm increments until each aoudad responded with a corneal blink reflex. The corneal touch threshold was then quantified in mm length of the filament necessary to cause a blink reflex. The length of the filament, indicating a corresponding pressure, at which the corneal blink reflex was positive, was deemed the central corneal sensitivity or central corneal touch threshold.

Intraocular pressure

Intraocular pressure (IOP) was measured in 36 eyes, using a veterinary rebound tonometer (Tonovet, Veterinary Division of S&V Technologies AG, Henningsdorf, Germany) (Fig. 2d) with the P setting, which was a preset for other animals except dogs and horses. Six measurements were taken and averaged by the tonometer´s internal software.

Central corneal thickness

Central corneal thickness measurements were taken after the instillation of sterile topical anesthetic (proparacaine hydrochloride 0.5% ophthalmic solution USP; Alcon Laboratories, Forth Worth, TX, USA). CCT was measured using an ultrasonic pachymeter (Model 200P+; Micropach, Sonomed, Lake Success,NY, USA), with the speed of sound in the cornea preset at 1640 m/s (Fig. 2c).

B-mode ultrasonographic biometry

B-mode scan ultrasonography was performed using a Sonix SP High Performance B-mode System (Ultrasonix, Richmond, BC, Canada). The B-scan 14-MHz probe was gently placed on the corneal surface perpendicular to the center of the cornea using ultrasonic transmission gel (Aquasonic-100; Parker Laboratories Inc., Fairfield, NJ, USA). Care was taken during probe placement to avoid corneal indentation. Reflected ultrasonic waves were captured. Optimal positioning was confirmed when the posterior wall of the eye globe could be clearly visualized on the B-scan ultrasonogram and the image appeared symmetrical and the reflections from the four principal landmarks (cornea, anterior lens surface, posterior lens surface and retinal surface) along the optic axis were perpendicular. The optimal image was frozen on the screen and then all echobiometric measurements were taken (Fig. 2f).

Fundoscopy

After B-mode ultrasonographic biometry the aoudads´ eyes were gently rinsed twice with 0.9 % saline solution in order to remove the ultrasonic transmission gel. Subsequently the aoudad´s funduses were examined using an indirect ophthalmoscope (Heine Omega 180 Headworn Binocular Indirect Ophthalmoscope, Dover, NH) and photographed using the topical endoscopy fundus imaging technique (TEFIT) (Fig. 3a) or a slit lamp containing a built-in indirect ophthalmoscopy lens (Digital 1.0x Imaging Lens, Hawk Eye, Dioptrix, L'Union, France) (Fig. 3b). For the TEFIT procedure a rigid, 8-mm-diameter laparoscope with a 0 degree angle and a crescent-shape illumination tip (WeckTM, Pilling Weck, Markham, ON, Canada) was used. Both the rigid arthroscopy probe and the rigid laparoscope were connected to an adapter of a 7.2 megapixel reflex digital camera with a Carl ZeissTM lens and 12x of optical zoom (the same previously cited). The light source was a 175W xenon lamp (Karl StorzTM, Tuttlingen, Germany) linked to the arthroscopy probe and the rigid laparoscope by a flexible fiber optic. Pupillary dilation for fundoscopy and fundus photography was performed following instillation of the following eyedrops: tropicamide 1% and phenylephrine 10% (Frumtost, São Paulo, SP, Brazil) one drop of each in each eye, with approximate 3-min intervals, every 10 min three times.

Corneal and palpebral fissure biometry

Palpebral fissure length, vertical and horizontal corneal diameters were measured using a stainless steel caliper ruler with an LCD display and an accuracy of ±0.02 mm (Neiko Tools, Klamath Falls, OR, USA).

Statistical analyses

The obtained data were submitted to a Kolmogorov-Smirnov Goodness-of-Fit Test. Unpaired *t*-tests were used for data comparison between, right and left eyes and males and females. *P*-values < 0.05 were deemed significant. JMP (SAS Institute, Inc., Cary, NC, USA) software was used to perform both descriptive and inferential statistical analyses.

FIGURE 1 - (A) PART OF THE GROUP OF AOUDADS (*Ammotragus lervia*) FROM CURITIBA´S ZOO INVESTIGATED IN THIS STUDIED. THE PICTURE SHOWS A MIXED-AGED GROUP BUT ONLY THE ADULT ANIMALS WERE INVESTIGATED. (B) A REPRESENTATIVE EXAMPLE OF THE GENERAL EXTERNAL APPEARANCE OF THE EYE OF THE AOUDAD. IT IS POSSIBLE TO OBSERVE TRUE CILIA (LONGER AND THICKER AT THE UPPER EYELID). BELOW THE LOWER EYELID MARGIN IS POSSIBLE TO SEE TWO ROWS SPARSELY DISTRIBUTED LONGER HAIRS (ASTERISK). NOTE IN THE ANTERIOR UVEA THE EXTENSIVE IRIS COLLARETTE AND THE PRESENCE OF AN UPPER AND A LOWER (MORE DISCRETE) CORPORA NIGRA (ARROWS). THE PUPILLARY APERTURE SHAPE WAS OVAL WITH THE LONG AXIS HORIZONTAL. THE LIMBUS IS RELATIVELY LARGE AND HEAVILY PIGMENTED.

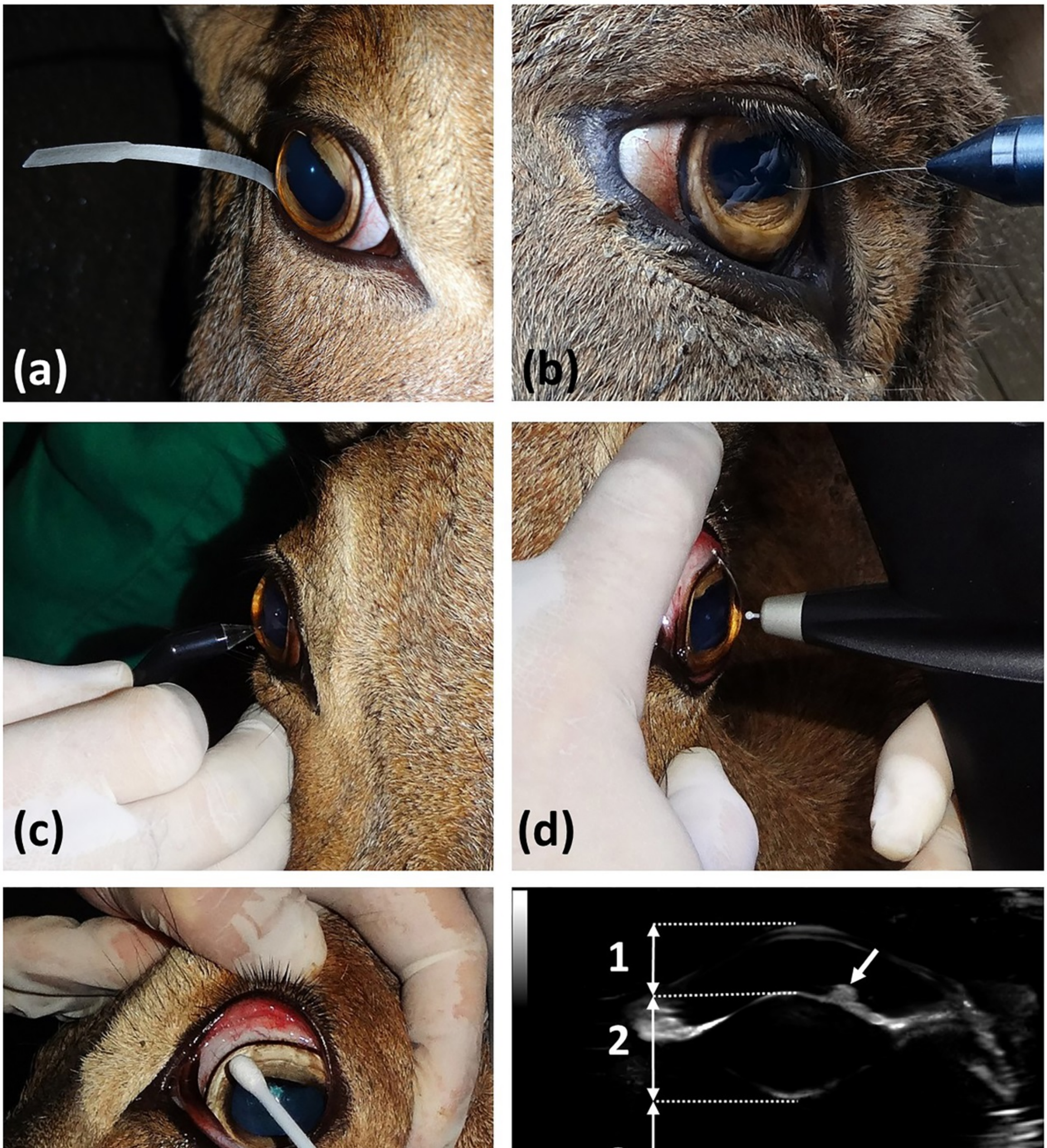

FIGURE 2 - PHOTOGRAPHS OF SELECTED OCULAR TESTS PERFORMED IN AOUDADS. (A) SCHIRMER TEAR TEST; (B) ESTHESIOMETRIC ANALYSIS OF CENTRAL CORNEA; (C) CORNEAL PACHYMETRY; (D) REBOUND TONOMETRY; (E) SWABBING THE CONJUNCTIVA AND EYELID MARGINS; AND (F) B-MODE OCULAR ECHOBIOMETRY. BESIDES THE GLOBE AXIAL LENGTH, THE FOLLOWING ECHOBIOMETRIC MEASUREMENTS WERE PERFORMED: 1- ANTERIOR CHAMBER DEPTH (AXIAL ANTERIOR CHAMBER LENGTH); 2- LENS THICKNESS (AXIAL LENS LENGTH); 3- VITREOUS CHAMBER DEPTH (AXIAL VITREOUS CHAMBER LENGTH). NOTE THE SUPERIOR CORPORA NIGRA (ARROW).

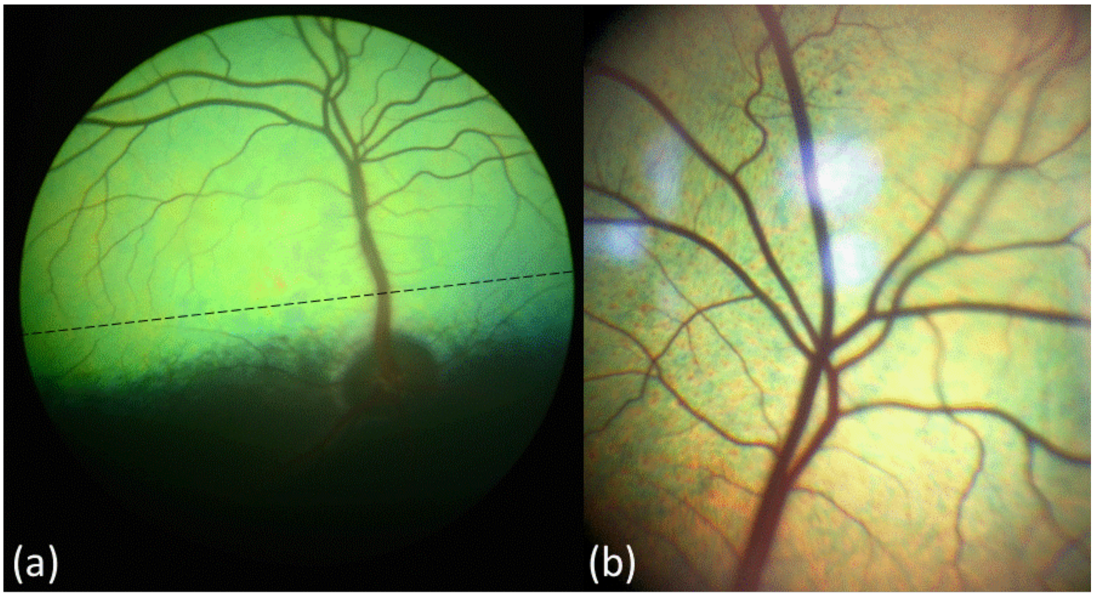

FIGURE 3 - AOUDAD´S FUNDOSCOPIC APPEARANCE CAPTURED USING TEFIT (A) AND AN INDIRECT LENS COUPLES WITH A SLIT LAMP BIOMICROSCOPE (HAWK EYE; DIOPTRIX, L'UNION, FRANCE). NOTE THE EXTENSIVE *TAPETUM LUCIDUM* (A) THE HOLANGIOTIC RETINAL VASCULAR PATTERN - (A) AND (B). THE *TAPETUM* HAS A GRANULAR OR SPECKLED APPEARANCE (B). THE OPTIC DISC IS GRAYISH IN COLOR, OVAL IN SHAPE, LOCATED JUST INFERIOR TO THE INFERIOR BORDER OF THE *TAPETUM LUCIDUM* (A). FROM THE CENTER OF THE OPTIC NERVE RADIATES THE MAJOR BLOOD VESSELS OF THE AOUDAD´S RETINA (A). BLOOD VESSELS ARISING FROM THE DORSAL AND VENTRAL QUADRANTS TAPER TOWARDS A REGION JUST ABOVE THE INFERIOR BORDER OF THE *TAPETUM LUCIDUM*. AT THIS REGION NO BLOOD VESSELS ARE PRESENT AND AN IMAGINARY LINE CAN BE TRACED CREATING A STREAK WHERE THIN RETINAL BLOOD VESSELS ARE RARE OR ABSENT (A).

3.5 RESULTS

All continuous numeric data obtained for all ophthalmic tests in the population used in this investigation were normally distributed according to the Kolmogorov-Smirnov Goodness-of-Fit Test. Table 1 contains the condensed results of the descriptive statistical analyses.

Morphological features of the normal aoudad eye

Ophthalmic examinations revealed that the normal anterior ocular structures in the aoudad include dorsal and ventral puncta. Additionally, aoudads possess true cilia (eyelashes) at the upper (Fig. 1b) and lower eyelid margins; with the lower cilia being thinner and sparsely distributed. Aoudads have a third eyelid (nictitating membrane) which moves across the surface of the cornea from the nasal canthus to the temporal canthus. The margin of the third eyelid is pigmented in the shape of a dark line along the free edge of the third eyelid. Above the upper eyelid margin and below the lower eyelid margins, two rows of modified-sparsely distributed longer hairs, resembling vibrissae, also called “tactile hair”, were found in all individuals (Fig. 1b), being approximately 16 to 18 pairs located above and 6 to 8 pairs below. The iris colors varied from a yellowish-brown to a grayish-brown according to each individual. The iris collarette had no Crypts of Fuchs visible, being somewhat plain and comprised the largest region of the iris, separating the pupillary portion from the ciliary portion (Fig. 1b). At the ciliary margins *corpora nigra* were found. The lower *corpora nigra* was considerably more discrete. The pupillary aperture shape was oval with the long axis horizontal. The presence of corpora nigra makes the pupil gain a rectangular appearance when observed from a distance.

Table 1- Summary of the main results

Ophtalmic Test or Parameter	Unit	Mean	Standard Deviation	95% Confidence Interval
Schirmer tear test	mm/min	27.22	3.6	26.04 - 28.4
Esthesiometry	cm	1.3	0.4	1.18 - 1.43
Intraocular pressure	mmHg	19.47	3.9	18.2 - 20.74
Central corneal thickness	µm	630.07	20.67	623.32 - 636.82
Axial globe length	mm	28.43	0.88	26.65 - 28.43
Anterior chamber depth	mm	5.03	0.17	4.7 - 5.4
Lens thickness	mm	9.4	0.33	8.73 - 10.06
Vitreous chamber depth	mm	14.1	0.53	12.93 - 15.06
Palpebral fissure length	mm	34.8	3.12	33.77 - 35.82
Corneal horizontal length	mm	25.05	2.18	24.34 - 25.77
Corneal vertical length	mm	17.95	1.68	17.40 - 18.50

TABLE 1 - A SUMMARY OF THE MAIN RESULTS OBTAINED IN THE DESCRIPTIVE STATISTICAL ANALYSIS PERFORMED ON OCULAR DIAGNOSTIC TESTS AND OPHTHALMIC BIOMETRY.

Schirmer tear test (STT)

No significant STT differences were determined between right and left eyes or between genders. Mean STT results for both eyes was 27.22 ± 3.6 mm/min.

Microbiological analysis

Bacteria were isolated in microbiological samples from 33 out of 36 eyes. Five different genera Gram-positive bacterial species were identified. The genera of the isolates were: *Corynebacterium*, *Micrococcus*, *Bacillus*, *Streptococcus* and *Staphylococcus* sp. Four different genera of Gram-negative bacteria were isolated. The genera of the isolates were: *Escherichia*, *Acinetobacter*, *Enterobacter* and *Citrobacter* sp. A single genus of bacteria was isolated from 11 eyes. Two genera of bacteria were isolated from 20 eyes. Three genera of bacteria were isolated from two eyes. *Staphylococcus* sp. was the most common bacteria isolated, being present in 13 eyes (prevalence of 36.1%). *Micrococcus* sp. and *Bacillus* sp. were the second most common bacteria isolated, being present in 9 eyes each (prevalence of 25%). Lastly, *Corynebacterium* sp. was present in 5 eyes (prevalence of 13.88%).

Corneal esthesiometry

There were no significant differences between males and females or between left and right eyes. The mean SD central corneal sensitivity was 1.3 ± 0.4 cm.

Intraocular pressure (IOP)

The mean SD value for IOP was 19.47 ± 3.9 mmHg. There was no significant difference in IOP between males and females and no significant differences between left and right eyes.

Central corneal thickness (CCT)

The mean SD CCT was 630.07 ± 20.67 μm. There was no significant difference in CCT between males and females and no significant differences between left and right eyes.

B-mode ultrasonographic biometry

No significant biometric differences were determined between right and left eyes or between genders. The mean axial eye globe length was 29.94 ± 0.96 mm. Mean anterior chamber depth (axial anterior chamber length) was 5.03 ± 0.17 mm. Mean lens thickness (axial length) was 9.4 ± 0.33 mm. Mean vitreous chamber depth (axial chamber length) was 14.1 ± 0.53 mm.

Fundus examination and fundus photography

As viewed by the ophthalmoscope, it was possible to observe that the aoudad retina possess an extensive *tapetum lucidum* usually of a greenish-yellow to a yellowish-green in color with a typical holangiotic retinal vascular pattern (Fig. 3). The *tapetum* has a granular or speckled appearance (Fig. 3). The optic disc was oval in shape and located just inferior to the inferior border of the *tapetum lucidum*. From the center of the optic nerve radiates the major blood vessels of the retina (Fig. 3). Blood vessels arising from the dorsal and ventral quadrants taper towards a region just above the inferior border of the *tapetum lucidum*. At this region no blood vessels are present and an imaginary line can be traced creating a streak where thin retinal blood vessels are rare or absent (Fig. 3a).

Corneal and palpebral biometry

The transition between cornea and the sclera (limbus) is relatively large and heavily pigmented. It appears as a dense thick band in this area (Fig. 1b). Mean horizontal corneal diameter of both eyes was 25.05 ± 2.18 mm and the mean vertical corneal diameter of both eyes was 17.95 ± 1.68 mm. The mean horizontal palpebral fissure length of both eyes was 34.8 ± 3.12 mm.

3.6 DISCUSSION

This study reports the means and confidence intervals of several ophthalmic tests and biometric data found in the eyes of a group of clinically normal aoudads (*Ammotragus lervia*), which was previously unavailable in the scientific literature.

The eyes of the aoudad are relatively large for the size of its head and body, and are therefore prominent. The eyelashes are long and add to the distinctive appearance in addition to the long eyelid vibrissae. In other species already investigated vibrissae are considered to be true sensory organs located in anatomical areas where protective reflexes are important such as around the eye, or where light is limited (McGreevy, 2004). Aoudads have a fairly elongated head and their eyes are placed laterally and quite far back. These features together are similar to the horse head morphology (McGreevy, 2004) and are probably evolutionary adaptations to prevent tall grass from obstructing the view when grazing in both species.

The presence of an elongated horizontally oval pupil observed here in the aoudad but also in other ungulates such as horses (Murphy & Arkins, 2007), cows, sheep and goats (Walls, 1943) indicates the presence of a wide lateral vision (Murphy & Arkins, 2007). This type of pupil alternatively called “rectangular” (Prince, 1956) also is present on the deer, camel, hyrax, goats, sheep and horse. Optical analyses show that the pupillary elongation expands field of view horizontally allowing terrestrial prey animals to see objects near the ground plane both in front of and behind them (Sprague et al., 2013).

Another evolutionary adaptation found in the eye of the aoudad is the corpora nigra, which are pigmented projections found on the upper and lower margins of the pupillary aperture. This anatomic structure already described in ungulates (Walls, 1943) is known to have many functions such as to contribute to pupillary constriction, to prevent actinic damage during grazing and possibly to work as an anti-glare device (Davidson, 1991). In the eye of the aoudad the upper corpora nigra is considerably larger than the lower one. The authors believe that this feature accentuate information from the inferior visual field (Davidson, 1991).

The horizontal palpebral fissure length of the aoudad (34.8 mm) is only a bit smaller than that of the cow (44.4 mm) and that of the horse (39.5 mm) (Wieser et al., 2013), being both noticeably larger and heavier animals. It is however, considerably bigger than the one reported for animals with similar sizes and weight such as the sheep (27.0 mm), goat (28.8 mm) (Wieser et al., 2013), dwarf goat (21.6 mm) (Olopade & Onwuka, 2004) and that of the Red Sokoto goats (25.0 mm) (Olopade & Onwuka, 2003). The cornea also follows this same trend and can be considered absolutely and relatively large. Its curvature was not evaluated but its external appearance is very prominent. Like in other ungulates the horizontal (transverse) corneal diameter is invariably considerably larger than the vertical (Henderson, 1950; Grinninger et al., 2010). The horizontal and vertical lengths (diameters) were similar to the ones reported for of the miniature horse, which was 25.8 mm and 19.4 mm respectively (Plummer et al., 2003).

The greenish *tapetum lucidum* observed is similar to the typical *tapetum fibrosum* found in cow, sheep, goat and horse (Ollivier et al., 2004). The dark specks visible in the tapetal fundus of all aoudads investigates are identical to previously describe structures called “Stars of Winslow” (stelullae of Winslow) in other ungulates such as sheep, goats and horses. These structures represent deep choroidal vessels communications with other blood vessels from the choriocapillaris layer and the specks are the sites of tapetal penetration (Galán et al., 2006). The presence of this normal anatomic feature was not previously described in aoudads.

The band or linear region in the aoudad fundus with the absence of blood vessels seems to represent a retinal specialization, with a higher photoreceptor density and visual acuity called “visual streak”, similar to an area centralis in dogs and cats or macula in human beings. This feature also never was described in the aoudad eye. It was however documented in other ungulates, including sheep. Cattle and sheep eyes, as many other mammalian eyes, have a retina with a ‘visual streak’ (Hebel, 1976). This is an elongated area of high ganglion cell density (Hebel, 1976; Heffner & Heffner, 1992). It determines visual acuity in a particular part of the visual field (a horizontal line). Probably this visual streak (or retinal specialization) of the aoudad eye runs in parallel to the oval-shaped pupillary aperture and thus both characters might work together.

STT is considered the gold standard of the available diagnostic test used to diagnose keratoconjunctivitis sicca (KCS) in domestic and in most wild animals. It is therefore important to perform a STT in all aoudads with ocular disease to rule out KCS as a cause of chronic eye disease such as corneal ulcers, conjunctivitis, keratitis and ocular discharge (Brooks, 2010; Trbolova et al., 2012). Although there has been no report of KCS in aoudads to date, it certainly occurs in the species as it is a common ocular disease in most animals and even human beings. It may be that the disease is underreported in aoudads due to the lack of knowledge of normal values for this test. When comparing STT values found in available studies of other species of ruminants, STT results in aoudads are quite high, similar but even higher to those already found in sheep (26.40 ± 17.70 mm/min) (Wieser et al., 2013), higher than the normal values found for the llama (17.3 ± 1.1 mm/min) (Trbolova et al., 2012) and considerably higher than the ones found for goats (14.50 ± 3.78 mm/min) (Wieser et al., 2013) and for pigmy goats (15.8 ± 5.7 mm/min) (Broadwater et al., 2007).

Normal conjunctival bacterial microbiota has been studied in several wild mammals such as the opossum (Pinard et al., 2002), bison (Davidson et al., 1999), deer (Dubay et al., 2000), and elephant (Tuntivanich et al., 2002). In the vast majority of these reports, Gram-positive bacteria were the most common isolates and the present report is no exception. Both pathogenic and nonpathogenic bacteria were found in this investigation. *Escherichia coli*, *Enterobacter* sp and *Citrobacter* sp were isolated from the eyes of aoudads in this study. The finding of these Gram-negative bacteria suggests possible eye contamination with fecal material during physical restraint and/or maybe these bacteria represent a transient agent of the conjunctiva. Nonetheless, *Escherichia coli* was also isolated from normal conjunctival microbiota of dogs (Prado et al., 2005; Wang et al., 2008) and horses (Pisani et al., 1997; Andrew et al., 2003). *Enterobacter* sp and *Citrobacter* sp were already isolated from the conjunctiva of clinically normal eyes of horses and human beings working as health professionals in a hospital environment (Pisani et al., 1997; Trindade et al., 2000). Additional studies are still necessary to try to determine whether or not some of these Gram-negative bacteria are normal inhabitants of the aoudad's ocular microbiota. The label pathogenic versus non-pathogenic is misleading because it is known that in some cases of bacterial conjunctivitis, a formerly nonpathogenic conjunctival bacterium can overgrow and cause an imbalance of the ocular surface microbiota population, becoming pathogenic (Samuelson, 1999).

The Cochet–Bonnet esthesiometer estimates the degree sensitivity of the cornea by evaluating the corneal touch threshold (Chan-Ling, 1989; Barret et al., 1991). The mean corneal touch threshold obtained in this investigation was similar to the one of the foal (1.4 cm) (Brooks et al., 2000), chinchilla (1.24 cm) (Lima et al., 2010), Guinea pig

(1.35 cm) (Wieser et al., 2013) and rabbit (1.47 cm) (Wieser et al., 2013), demonstrating that aoudads possess a less sensitive cornea compared to other species such as the adult horse, cat and cows (Wieser et al., 2013). These results should be interpreted with caution because of the well-known low precision of the Cochet–Bonnet esthesiometer in the 0.5- to 2.0-cm filament length range (Wieser et al., 2013). The aoudad´s corneal sensitivity encountered in this investigation was exactly within that range. The pressure applied to the surface of the cornea by the examiner also can vary. It is known that these parameters affect this test results significantly (Boberg-Ans, 1956). In the present study, the temperature and air humidity were not assessed in order to be able to correct the corneal sensitivity measurements with the nylon filament. Unfortunately, no formula or correction table exists at this time for the nylon filament currently used and the temperature or humidity conditions, which imposes a challenge for extrapolating corneal sensitivity data obtained with the Cochet-Bonnet esthesiometer. In light of all these possible variables and interferences produced by the examiner some authors claim that a new esthesiometer, which can display the pressure applied to the surface of the cornea, should be created in order to make the measurement of the CTT more sensitive and comparisons between investigations more precise.

Tonometry is a fundamental part of a complete ophthalmic evaluation in any animal species. The main value of tonometry lies in the ability to detect pressure increases as an important clinical sign of glaucoma. However, a normal range of values for each species needs to be established. IOP measurements in the aoudads using Tonovet resulted in means and ranges that were a bit higher than those reported for most other wild and domestic ungulates (Ofri et al., 2000; Willis et al., 2000; Ofri et al., 2001). For instance, normal reported mean IOP for sheep was 16.36 mm Hg (Pigatto et al., 2011), which was considerably lower than the one found for the aoudad. The aoudad´s IOP seems to be similar to other ungulates with higher IOP such as the zebra (Ofri et al., 1998) and dairy cattle (Gum et al., 1998). However, comparison is difficult since most of the normal ranges for IOP previously reported in ungulates were obtained with applanation tonometers, some even with indentation tonometry (Ofri et al., 1998). Before comparing and extrapolating IOP data from one study to other researchers need to make sure if the tonometry method was the same. It was shown that Tonovet rebound tonometer may significantly overestimate the IOP values from the applanation tonometer, at least in one study using normal Eurasian Eagle owls (Jeong et al., 2007). Another study conversely, showed that results for the TonoVet-D calibration are similar to those obtained for dogs (Knollinger et al., 2005). Even though the rebound tonometer is tolerated well by most animal species because of the rapid and minimal stress-inducing method of tonometry that it is, another factor to be considered when establishing IOP in wild and exotic species is stress. It is known that IOP values increase if the animal is firmly restrained specially in wild animal species (Jeong et al., 2007). Granting the animals were examined in their typical housing environment, all animals were physically restrained it is possible that stress could have influenced our results, yet care was taken in order to avoid neck pressure.

Ultrasonic corneal pachymetry is an accurate and reliable *in vivo* method currently available to measure corneal thickness in animals and human beings (Korah et al., 2000). It was shown that ultrasonic pachymetry set at a standard velocity of 1636 m/s overestimates CCT as compared to optical coherence tomography (Alario & Pirie, 2014). However, correlation between the two mentioned modalities is excellent. Mean central corneal thickness (CCT) acquired with an ultrasonic pachymeter has been the subject of a number of reports investigating the cornea of human beings (Korah et al., 2000), several domestic (Stapleton & Peiffer, 1979; Gilger et al., 1991; Gilger et al., 1998), exotic and wild animals (Montiani-Ferreira et al., 2006; Montiani-Ferreira et al., 2008a; Montiani-Ferreira et al., 2008b; Lima et al., 2010). Even so, CCT has never been measured in the normal aoudad eye. In our investigation mean CCT of the aoudad was not significantly different between males and females. The aoudad CCT is a bit thicker than the adult dogs (598.54 μm) (Alario & Pirie, 2014) and slightly thinner than the horse one (785.60 μm) (Plummer et al., 2003). It is similar to those values found for adult Saanen goats using a high-resolution 20-MHz A- and B-mode ultrasonography transducer (Ribeiro et al., 2009).

Echobiometric data of the eye globe obtained using A- and B-mode ultrasonography were reported in children (Kurtz et al., 2004) several domestic (Schiffer et al., 1982; Rogers et al., 1986; Cotrill et al., 1989; Gilger et al., 1998; Tuntivanich et al., 2002; Plummer et al., 2003; Ribeiro et al., 2009), exotic and wild mammal species

(Fernandes et al., 2003; Hernández-Guerra et al., 2007; Montiani-Ferreira et al., 2008; Lima et al., 2010; Ruiz et al., 2015), but this feature was not previously investigated in the aoudad. The aoudad´s axial globe length, lens thickness, and chamber depths were not significantly different according to the eye (left or right) studied or gender. This lack of difference was also observed in dog eyes and eyes of most other wild and exotic animals studied using B-mode ultrasonography. The eye of the aoudad is large in both ways, absolutely and relative to its body size. The axial globe length found adult for aoudads is larger than the one obtained in other large mammals including cadaveric eyes of sheep Ramboullet (El-maghrabmy et al., 1995) and Ile de France Sheep (Brandão et al., 2004) and Saneen goats (Ribeiro et al., 2009). The dimension of the internal structures such as anterior chamber depth, lens thickness and vitreous chamber depth follow the same pattern, being all comparable but larger than the sheep (Brandão et al., 2004), goat (Ribeiro et al., 2009). Only the bovine eye (Potter et al., 2008) and buffalo (*Bos bubalis*) (Kassab, 2012; Assadnassab & Fartashvan, 2013) and the dromedary eye (Osuobeni & Hamidzada, 1999; Kassab, 2012) demonstrated similar echobiometric dimensions, with equivalent lens thickness and vitreous chambers depth even though these are considerable larger ungulates in terms of body size.

In conclusion, this study provides novel data for normal reference ranges for several ophthalmic tests and ocular biometric parameters in healthy aoudads. The eyes are large and laterally placed in the head with several anatomic features in the eye of the aoudad are being here attributed to be evolutionary adaptations for grazing, which was also previously observed in other prey species of ungulates, such as horses, sheep and cattle.

Often a complete ocular examination of zoo animals is not routinely performed (Townsend, 2010) due to limitations such as lack of appropriate instruments (ophthalmoscopes, tonometers), disposable diagnostic test material (such as STT strips, fluorescein strips and eyedrops) and proper facilities (safe, large dark rooms). Nevertheless, the results of this study may assist veterinarians and veterinary ophthalmologists in particular in the diagnosis of several ocular diseases in aoudads.

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4 A HISTOPATHOLOGICAL STUDY OF OCULAR SQUAMOUS CELL CARCINOMA IN HOLSTEIN AND HOLSTEIN-CROSSES COWS FROM THE STATE OF PARANÁ AND SANTA CATARINA, BRAZIL

Estudo histopatológico do carcinoma espinocelular ocular em vacas holandesas e holandesas mistas do estado do Paraná e Santa Catarina, Brasil

4.1 ABSTRACT

A histopathological study was performed in ten cases of bovine ocular squamous cell carcinoma (OSSC). All cases were diagnosed in Holstein or Holstein-crosses cows. In a nationwide publicity campaign veterinarians were urged to send potential samples to participate in a research. This was the first investigation performed exclusively in OSCC cases diagnosed in the State of Paraná and Santa Catarina. The prognostic values of currently accepted histopathological grading of squamous cell carcinomas (SCC) vary considerably. Thus, in the present study an effort to develop an acceptable system of histological grading systems for routinely prepared 10% formalin-fixed samples that were embedded in paraffin, cut in 5 µm sections and stained with HE, based on a combination of two previously existing classifications systems. However, anaplasia parameters did not correlate well with tumor invasion of deeper tissues and mitotic indexes in all samples analyzed. Curiously, six out of the ten OSCC cases were observed in animals with heavily pigmented eyes. An association between histological grading and molecular techniques such as *in situ* hybridization and immunohistochemistry would be a feasible route to better investigate, classify and predict OSCC behavior in the future.

KEY-WORDS: Bovine. Cancer. Histologic classification. Ophthalmology. Tumor behavior.

4.2 RESUMO

Um estudo histopatológico de vacas com diagnóstico clínico de carcinoma espinocelular ocular bovino (CEOB) foi realizado em dez casos de vacas holandesas e mestiças da raça holandesa em diversos graus acometidas. Por meio de uma chamada publicitária nacional, médicos veterinários foram convidados a enviar amostras participantes para a pesquisa. Esta foi a primeira investigação realizada exclusivamente em casos de CEOB diagnosticados no Estado do Paraná e em Santa Catarina. Os valores prognósticos atualmente aceitos da classificação histopatológica de carcinomas espinocelulares variam consideravelmente. Assim, o presente estudo empenhou-se no desenvolvimento de um sistema de classificação histológico aceitável, baseada em uma combinação de dois sistemas de classificações previamente existentes, para amostras fixadas rotineiramente com formol a 10%, embebidos em parafina, cortados em secções de 5 µm e coradas com HE. Contudo, em todas as amostras analisadas, os parâmetros de anaplasia não se correlacionaram adequadamente com a invasão tumoral e índices mitóticos. Curiosamente, seis dos dez casos de CEOB foram observados em animais de olhos fortemente pigmentados. No futuro, uma categorização baseada na associação entre a classificação histológica e técnicas moleculares, como a hibridização *in situ* e imuno-histoquímica, poderia se tornar uma alternativa viável para melhor investigar e prever o comportamento do CEOB.

PALAVRAS-CHAVE: Bovinos. Câncer. Classificação histológica. Comportamento tumoral. Oftalmologia.

4.3 INTRODUCTION

Ocular squamous cell carcinoma (OSCC or “cancer eye”) has been recognized in the literature since the latter part of the nineteenth century (Miller & Gelatt, 1991). It is a primary neoplasm of epithelial origin that may occur in different ocular and periocular tissues, such as palpebral skin, epithelial surfaces of the cornea and conjunctiva, third eyelid and limbus. OSCC occurs with high frequency in cattle all over the world (Reed, 1982; Tsujita & Plummer, 2010) and it is the leading cause of enucleation among all other ocular diseases in the species (Schulz & Anderson, 2010). The incidence of OSCC European countries is lower than in Africa and the Americas (Russell et al., 1976; Spadbrow & Hoffmann, 1980).

In many countries OSCC was found to be the most common form of all kinds of neoplasia affecting cattle (Plummer, 1956; Brandly & Migaki, 1963; Murray, 1968; Priester & Mantel, 1971; Naghshineh et al., 1991). There is an obvious lack of comprehensive investigations concerning the rate of occurrence of tumors in the Brazilian cattle in general. In fact, regardless of the country, OSCC is generally considered a very common neoplasm in cattle and has significant economic importance (Miller & Gelatt, 1991). European breeds of cattle and their crosses, particularly those with unpigmented skin on the face, are commonly observed to have OSCC (Stewart et al., 2006). Ocular squamous cell carcinoma does occur in other farm animal species such as sheep, swine, goats but all with a lower incidence (Priester & Mantel, 1971).

In several epidemiological surveys from Africa (Murray, 1968; Bastianello, 1982), England (Cotchin, 1960), Israel (Yeruham et al., 1999), Canada (Plummer, 1956; Dukes et al., 1982), Netherlands (Misdorp, 1967), United States of America (Russell et al., 1956; Anderson and Skinner, 1961; Anderson, 1970) and United Kingdom (Anderson & Sandison, 1968) OSCC is listed as the most common or at least among the top three most common neoplastic disease diagnosed in farm animals. In the United States, the prevalence of OSCC varies with geography and is higher in the southwestern region and in lower latitudes with higher levels of sunlight (Russell et al., 1956; Anderson & Skinner, 1961; Anderson, 1970). There are no general OSCC epidemiologic studies and retrospective investigations considering the whole country of Brazil. There are however, some surveys from few specific Brazilian states, such as Pará (De Sousa et al., 2011), Paraíba (Carvalho et al., 2012) and some Brazilian studies focused on the caseload of general bovine tumors in specific veterinary pathology laboratories (Ramos et al., 2007; Keller et al., 2008; Lucena et al., 2011) or ocular tumors (Werner et al., 1998). Additionally, there are few Brazilian case reports of OSCC in literature (Galera & Martins, 2001; De Barros et al., 2006; Alvim et al., 2007; Parra & Toledo, 2008).

The prognostic values of currently accepted histopathologic grading of squamous cell carcinomas (SCC) vary considerably. For instance, Broder´s grade was created in 1927 initiated quantitative grading in cancer studies. The method was well accepted and used for many years but later demonstrated as of no prognostic value (Bhargava et al., 2010).

The objective of this investigation was to study OSCC cases referred to the Laboratory of Comparative Ophthalmology (LABOCO), Federal University of Paraná (UFPR) exclusively from samples of affected Holstein and Holstein-crosses cows from the State of Paraná and Santa Catarina, in the south of Brazil and apply a combination of histologic classifications.

4.4 MATERIALS AND METHODS

A targeted publicity campaign was created involving the production of an electronic announcement posted on a social networking site in the internet urging Brazilian veterinarians and veterinary ophthalmologists to send tissue samples of ocular squamous cell carcinoma in cattle to be part of a histopathological investigation concerning the occurrence and histopathological classification of bovine OSCC, being developed at the Comparative Ophthalmology Laboratory (LABOCO), Federal University of Parana (UFPR). In return, participants would receive a free histopathological analysis of the case. Additionally, an educational illustrative guide to help identifying ocular squamous cell carcinoma cases was developed to be used by the employees working in abattoirs.

Samples

Squamous cell carcinoma samples from milk-producing breeds Holstein and Holstein-crosses, being all females (cows) were evaluated in the LABOCO-UFPR from March 2013 to December 2014. The samples studied in this investigation originated from three different sources of collaboration that responded to the publicity campaign and personal communications.

Histopathological processing and grading

Samples were fixed in 10% formalin, embedded in paraffin and cut in 5 µm sections were then stained with hematoxylin and eosin for routine histopathological diagnosis in glass slides. All ocular squamous cell carcinomas were analyzed classified according to clinical (when possible), macroscopic and microscopic features (Table 1 and 2). The degree of anaplasia (undifferentiation), tissue invasion and mitotic index were classified using ordinal categorical variables according to a combination of Broder’s System, in which tumors are graded on the basis of degree of keratinization and island formation of tumor cells (Peiffer & Simons, 2002; Bhargava et al., 2010) and the classification proposed by Carvalho et al. (2005) that includes differentiation, mitotic index and degree invasion. In the present proposed system, three parameters reflecting tumor features are analyzed together adding the two aforementioned systems and including degree of anaplasia (degree of keratinization combined with cell features such as squamous differentiation, cytoplasm size and nuclear pleomorphism), tissue invasion and number of mitotic figures per higher field (400x) (Table 2). A minimal anaplasia score (1) would be attributed to well differentiated neoplasms (low degree of anaplasia), usually cells with abundant cytoplasm, classically with concentric laminated masses of keratin (keratin pearls). A moderate anaplasia score (2) would referred to neoplasms exhibiting moderate degree of keratinization, small- to medium-sized keratin pearls, presence of few neoplastic cell islands, still with spinous differentiation but with increased number of poorly differentiated cells. The maximal score (3) would be attributed to neoplasms containing cells with poor cellular differentiation (highly anaplastic), showing practically no keratin formation, only individual cell keratinization, several islands and trabeculae. Tissue invasion was classified according to the extension into adjacent tissues, as follows: none to minimum extension (1); extension to immediately adjacent tissues (i.e. eyelid dermis, corneal stroma, conjunctival substantia propria) (2) and extension to deeper tissues (i.e. muscle, uvea). Regarding the mitotic index: If an average of 0-1 mitotic figure was observed per high-power field (400x), the score would be considered minimal (1), if 2-3 mitotic figures were observed the score would be intermediate (2) and then, if more than 3 mitotic figures were observed, the score would be maximal (3) (Table 2, Figure 3c). After each of these factors was analyzed individually a median (anaplasia, invasion and mitotic rate, dubbed AIM) score was then calculated for each case. Additionally, the presence and degree of inflammatory infiltrate was evaluated and scored as well.

4.5 RESULTS

A total of 97 bovine eye samples with suspicious lesions were received by the laboratory. Ten (10.31%) of those samples were diagnosed as OSCC. The remaining samples received ended up being diagnosed as non-neoplastic, mixed conditions, such as anomalies (such as a dermoid), traumatic (corneal perforations, eyelid lacerations), infectious (bovine infectious keratoconjunctivitis) and inflammatory (uveitis) diseases.

A summary of the clinical history and OSCC clinical appearance is presented on Table 1. Representative examples of samples received from clinical cases in which an excisional biopsy was performed are presented in Figure 1. Correspondingly, representative examples of tissue samples macroscopically analyzed and processed at LABOCO (eyeballs and eyelid tissues cut and measured) are presented in Figure 2.

From the resulting ten samples, six (Bov12, Bov16, Bov21, Bov26, Bov27, Bov30) were collected by a large animal veterinary practitioner working in the city of Guarujá do Sul, State of Santa Catarina, Brazil (26°23'07"S 53°31'40"W). All these cases from Guarujá do Sul were surgically managed by excisional biopsies. Three samples (Bov4, Bov15, Bov17) were donated by an abattoir (Argus Ltd., SIF 1710) located at the city of São José dos Pinhais in the State of Paraná, Brazil (25°32'06"S 49°12'21"W) (Table 1). During this period all eyes with suspicious lesions were identified and collected by an abattoir employee and subsequently submitted to transpalpebral enucleation and sent to LABOCO. Lastly, one case (Bov9) was diagnosed and collected at the Canguiri Experimental Station, located in Pinhais, in the State of Parana, Brazil (25° 26' 41"S 49° 11' 33" W), which is a farm owned by the Federal University of Parana. This last case was identified and surgically managed by excisional biopsy. Unintentionally, all samples received were from females (cows) and from Holstein or Holstein-crosses, from the State of Paraná and Santa Catarina.

Table 1 - Clinical data and tumor features observed macroscopically before the histopathological analysis

Case ID	Age (years)	Lesion localization	Affected eye	Surface appereance	Eyelid color	Breed	Origin
Bov04	3	Limbal	Right	Corneoconjunctival congested round plaque	Beige	Holstein/Mix	PR
Bov09	7	Third eyelid	Right	Verrucous, hyperemic (with purulent discharge)	Black	Holstein	PR
Bov12	4	Third eyelid	Left	Small nodules, hyperemic	Black	Holstein	SC
Bov15	3	Limbal	Right	Corneoconjunctival congested plaque	White	Holstein/Mix	PR
Bov16	6	Inferior eyelid	Left	Hyperemic plaque, multiple crustsy lesions	Black	Holstein	SC
Bov17	3	Limbal	Left	Verrucous corneoconjunctival mass	White	Holstein/Mix	PR
Bov21	8	Third eyelid	Right	Small nodules, congested, purulent discharge	White	Holstein/Mix	SC
Bov26	6,7	Third eyelid	Left	Nodular, congested	Black	Holstein	SC
Bov27	3	Third eyelid	Right	Nodular, congested	Black	Holstein	SC
Bov30	5	Third eyelid	Right	Nodular, ulcerated, congested	Black	Holstein	SC

TABLE 1 - CLINICAL DATA AND TUMOR FEATURES OBSERVED MACROSCOPICALLY BEFORE THE HISTOPATHOLOGICAL ANALYSIS.

Ten OSCC from different cows were macroscopically analyzed (Figure 2) histologically diagnosed (Figure 3) and classified during the investigation (Table 2). Regarding the pigmentation of the eyelid, six were pigmented (black) (Figures 1a, 1b and 1d), three were non-pigmented (white) and one was partially pigmented (white with dark patches) (Figure 1c). The mean age of the animals affected was 5 years, the minimum age observed was 3 years and the maximum age was 8 years (Table 1). The third eyelid was the most common location (Figure 1). Of these 10 tumors, six were present in the third eyelid, three in the corneoconjunctival junction (limbus) and one in the inferior eyelid. Six tumors affected the right eye and four the left eye. Six of ten tumors were clinically classified as variants of nodular, two were considered plaques and two were verrucous. Regarding the breed, six animals were pure Holstein and four were products of crosses between Holstein and other breeds. Regarding the origin of the samples, six were from the state of Santa Catarina and four from the state of Paraná (Table 1).

No clear association was found between anaplasia, invasiveness and mitotic index, local aggressiveness and inflammation in the 10 tumors analyzed (Table 2). Regarding the degree of anaplasia, only one tumor (Bov12, Figure 1b) demonstrated a high degree, but with minimum invasion and mitotic index (Table 2). Only one sample showed a high degree of tissue invasion (Bov15, Figure 3a) but correspondent low degree of anaplasia. Another two samples showed the maximum degree of mitotic indexes (Bov16 and 17) but low (Bov16, Figure 2c) to moderate (Bov17, Figure 2a) degrees of differentiation and tissue invasion (Table 2). Similarly, the highest inflammation score observed in a sample (Bov27) did not correlate well with any other high scores of malignancy features observed in the histopathological classification (Table 2).

Table 2 - Results of the proposed tumor grading system according to selected histopahological features

Case ID	Degree of anaplasia	Tissue invasion	Mitotic index	Median "aim" score	Inflammation
Bov04	2	1	1	1	1
Bov09	1	2	1	1	2
Bov12	3	1	1	1	2
Bov15	1	3	2	2	1
Bov16	1	2	3	2	1
Bov17	2	2	3	2	1
Bov21	1	2	2	2	2
Bov26	2	1	1	1	2
Bov27	2	2	1	2	3
Bov30	1	2	2	2	1

TABLE 2 - RESULTS OF THE PROPOSED TUMOR GRADING SYSTEM ACCORDING TO SELECTED HISTOPATHOLOGICAL FEATURES

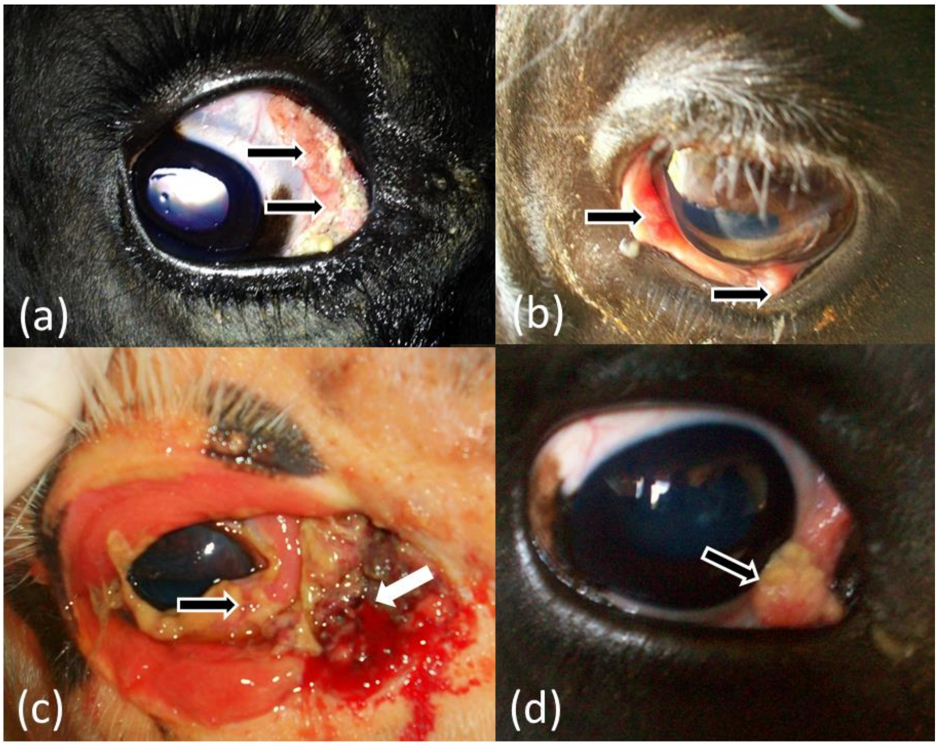

FIGURE 1 - REPRESENTATIVE EXAMPLES OF THE CLINICAL APPEARANCE OF SELECTED OSCC CASES IN WHICH TISSUE SAMPLES WERE COLLECTED AFTER SURGICAL EXCISIONAL BIOPSY. A) THIS SEVEN-YEAR-OLD HOLSTEIN COW (BOV09) HAD CHRONIC THIRD EYELID MARGIN MASSES (ARROWS) IN THE RIGHT EYE, WHICH WAS DISCRETELY VERRUCOUS, HYPEREMIC (WITH DISCRETE PURULENT DISCHARGE). THIS COW BELONGED TO A MILK-PRODUCING HERD FROM UFPR´S CANGUIRI EXPERIMENTAL STATION. NOTE THAT THE EYELID AND UVEAL TISSUE WERE HEAVILY PIGMENTED. B) THIS FOUR-YEAR-OLD HOLSTEIN COW (BOV12) SHOWED SMALL, HYPEREMIC NODULAR LESIONS IN THE LEFT THIRD EYELID MARGIN (ARROWS). THIS COW WAS PART OF A MILK-PRODUCING HERD IN THE CITY OF GUARUJÁ DO SUL, SC. NOTE THAT THE EYELID AND UVEAL TISSUE ALSO ARE PIGMENTED IN THIS ANIMAL. C) THIS EIGHT-YEAR OLD COW (BOV21) HAD A COLLECTION OF SMALL CONGESTED NODULES IN THE RIGHT THIRD EYELID (ARROW). NOTE THE SUBSTANTIAL PURULENT DISCHARGE DUE TO LOCAL PARASITIC INFESTATION BY FLY LARVAE (MAGGOTS) (WHITE ARROW) AND THE FACT THAT IN THIS ANIMAL THE EYELID WAS NOT PIGMENTED. THIS ANIMAL ALSO WAS PART OF A MILK-PRODUCING HERD IN THE CITY OF GUARUJÁ DO SUL, SC. D) THIS FIVE-YEAR OLD COW (BOV30) SAME AS IN FIGURE 2D HAD A CONGESTED SOLITARY NODULAR LESION WITH A DISCRETELY VERRUCOUS SURFACE (ARROW). THIS ANIMAL ALSO WAS PART OF A MILK-PRODUCING HERD IN THE CITY OF GUARUJÁ DO SUL, SC. NOTE THE PIGMENTED EYELIDS.

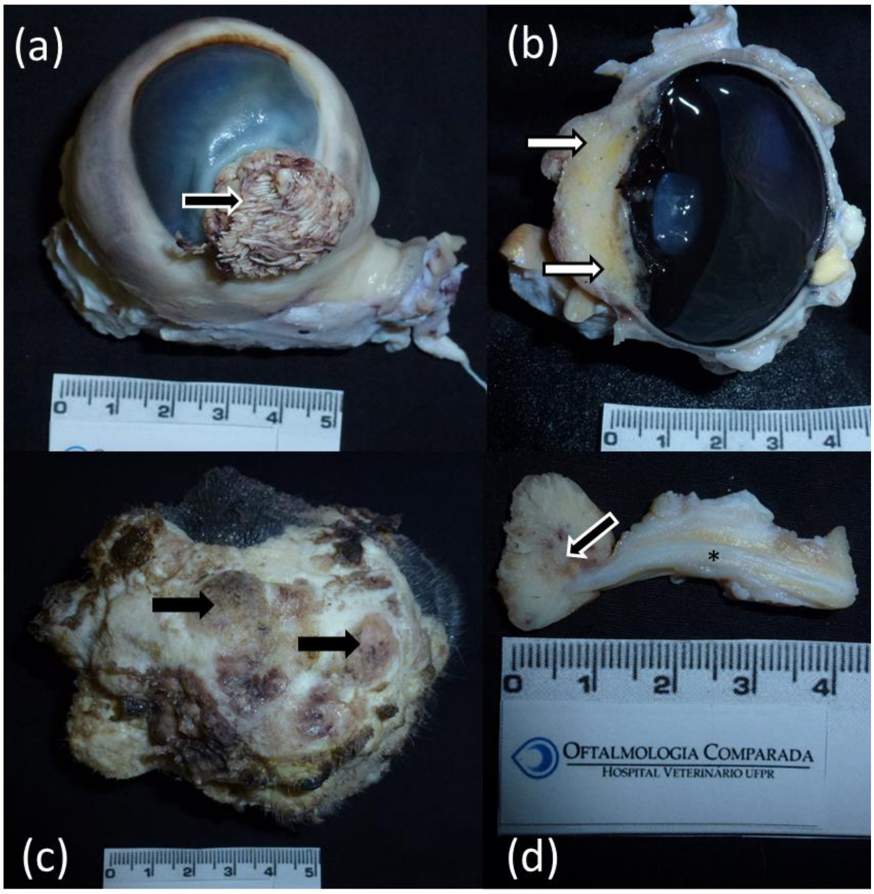

FIGURE 2 - MACROSCOPIC DETAILS REPRESENTATIVE OSCC CASES INVESTIGATED. A) AN OSCC FROM A HOLSTEIN-CROSS COW (BOV17) THAT WAS SENT BY A LOCAL ABATTOIR. THE CASE SHOWED A CONSPICUOUSLY VERRUCOUS CORNEOCONJUNCTIVAL (LIMBAL) MASS OF ABOUT 2.3 CM IN HORIZONTAL DIAMETER (ARROW). B) AN UNCOMMON TYPE OF PRIMARY CORNEAL SQUAMOUS CELL CARCINOMA. THE TUMOR, WHICH STARTED IN THE CORNEAL EPITHELIUM (ARROWS), SPREAD TO THE ADJACENT OCULAR SURFACE TISSUES AND EVEN INVADED INTRAOCULAR STRUCTURES. THE CASE WAS FROM A THREE-YEAR OLD HOLSTEIN-CROSS COW SENT TO A LOCAL ABATTOIR (BOV15). THIS ANIMAL HAD UNPIGMENTED EYELIDS. C) THIS OSCC CASE WAS DIAGNOSED IN A SIX-YEAR-OLD MILK-PRODUCING COW (BOV16) THAT DEMONSTRATED A SERIES OF CRUSTY HYPEREMIC PLAQUE-LIKE STRUCTURES IN THE INFERIOR EYELID OF THE LEFT EYE (ARROWS). IT WAS COLLECTED BY EXCISIONAL SURGICAL BIOPSY IN CITY OF GUARUJÁ DO SUL, SC. D) THIS IS MACROSCOPIC VIEW OF A LONGITUDINALLY CUT THIRD EYELID. IT IS POSSIBLE TO SEE THE INTERNAL CARTILAGINOUS “T” (ASTERISK). THE ARROW SHOWS AN OSCC MASS THAT AROSE AT THE MARGIN OF THE THIRD EYELID. THE CASE WAS DIAGNOSED IN A FIVE-YEAR-OLD MILK-PRODUCING COW (BOV30) AND WAS COLLECTED BY EXCISIONAL SURGICAL BIOPSY IN CITY OF GUARUJÁ DO SUL, SC.

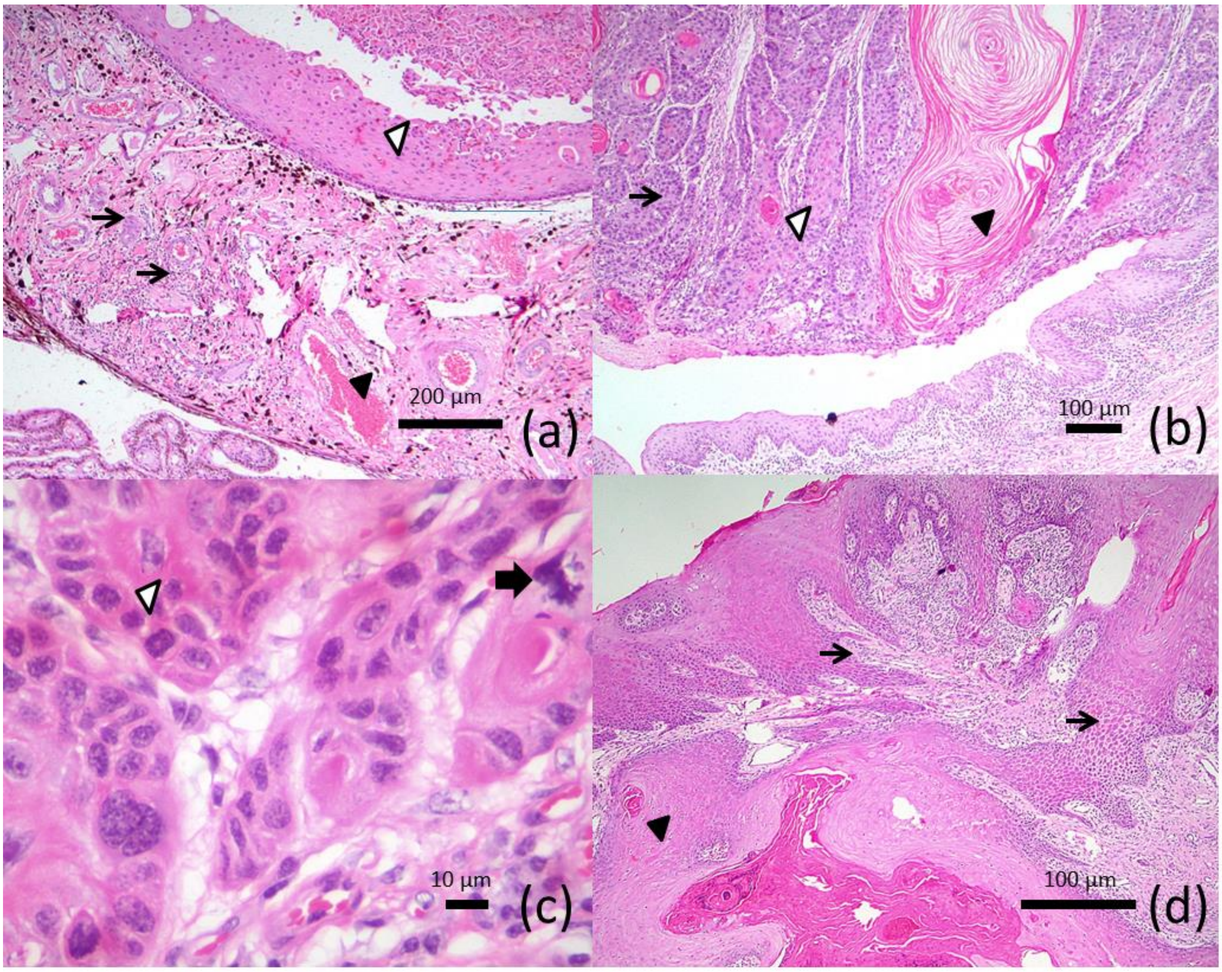

FIGURE 3 - PHOTOMICROGRAPHS OF SELECTED CASES OF OSCC INVESTIGATED. A) BOV15- DETAIL OF NEOPLASTIC CELLS INVADING THE IRIS STROMA FORMING SEVERAL SMALL ISLANDS (ARROWS) AND VASCULAR CONGESTION (BLACK ARROWHEAD). IN THIS CASE TUMOR CELLS INVADED THE ANTERIOR CHAMBER (WHITE ARROWHEAD) AND UVEAL TISSUE CAUSING SEVERE UVEITIS; B) BOV30- OSCC ON THE THIRD EYELID SHOWING NEOPLASTIC CELLS FORMING SMALL ISLANDS (ARROWS), KERATIN PERALS (WHITE ARROWHEAD) AND A HORN CYST (BLACK ARROWHEAD). THERE IS SEVERE SUBEPITHELIAL INFLAMMATORY CELL INFILTRATE IN THE ADJACENT CONJUNCTIVA OF THE THIRD EYELID; C) BOV17- DETAIL OF THE CELLULAR AND NUCLEAR ATYPIA PRESENT IN THE OSCC NEOPLASTIC CELLS. ONE OF THE VISIBLE MITOTIC FIGURES IS ABERRANT (BLACK ARROW). IN THIS PHOTOMICROGRAPHY IT IS POSSIBLE TO SEE THAT THESE CELLS ARE EPITHELIAL CELLS (DUE TO THE PRESENCE OF DESMOSOMES – WHITE ARROWHEAD); D) BOV04 - INTRAEPITHELIAL NEOPLASTIC IRREGULAR HYPERPLASIA, DYSPLASIA AND NEOPLASTIC TRANSFORMATION (BLACK ARROWS) AND KERATIN PEARLS (BLACK ARROWHEAD) AND INFLAMMATORY CELL INFILTRATE.

4.6 DISCUSSION

The etiology of OSCC is probably multifactorial, with genetic/phenotypic (lack of pigment within the epidermis at the site of the tumor), environmental (prolonged exposure to ultraviolet light) (Goldschmidt & Hendrick, 2002) and possibly viral factors (Heeney & Valli, 1985) playing roles, such as bovine papilloma virus (Rutten et al., 1992) and more feasibly bovine herpesvirus (Anson et al., 1982).

Regarding the age the affected animals, our results disagree with the average age of cattle with ocular squamous cell carcinoma previously published as being 8 years (Cordy, 1990) but agrees with previous reports that state that the tumors are hardly ever seen in cattle younger than 3 years (Radostits et al., 2000).

An association between eyelid pigmentation “white eye” and occurrence of cancer eye lesions in cattle was established several decades ago (Anderson et al., 1957a). In Zimbabwe, European breeds of cattle and their crosses, particularly those with unpigmented skin on the face, are commonly observed to have OSCC (Murray, 1968; Stewart et al., 2006). In the state of Florida, USA, the frequency of OSCC is particularly high in the white-faced Hereford. This breed is commonly kept in an environment favorable for the development of OSCC and has a hereditary inclination towards facial hypopigmentation (Tsujita & Plummer, 2010). A similar observation was made in Italy (Pugliese et al., 2014). Regarding a possible relation of the presence of OSCC with lack of pigmentation at the site of the lesion, our findings disagree with these previous investigations since six out of 10 cases were observed in animals with pigmented eyelids and uveal tissues.

It is well established that UV radiation is a complete carcinogen by itself that is capable of inducing and promoting neoplastic changes. UV radiation initiates tumorigenesis by inducing mutations in the p53 tumor suppressor gene (Epstein & Epstein, 1962; Blum, 1969). As a tumor promoter, UV radiation induces cell proliferation by stimulating the production of various growth factors and cytokines as well as the activation of their receptors (De-Metys et al., 1995; Rosette & Karin, 1996). The relationship between bovine ocular squamous cell carcinoma and ultraviolet radiation was studied. Experimental procedures were devised to irradiate cattle with predetermined quantities of ultraviolet beta. Irradiation induced a preneoplastic ocular growth in one of four irradiated cattle (Kopecky et al., 1979). This pattern is similar to that observed with human skin-cancer cases (Heeney & Valli, 1985). Lightly pigmented human skin when chronically exposed to sunlight may undergo a series of changes, commencing with the development of keratoses and frequently progressing to basal cell epitheliomas or squamous cell carcinomas (SCCs) (Tsujita & Plummer, 2010). In the United States, the prevalence of OSCC varies with geography and is higher in the southwestern region and in lower latitudes with higher levels of sunlight (Anderson & Skinner, 1961; Anderson, 1970; Russell et al., 1976; Anderson & Badzioch, 1991). Even though there are Brazilian regions, mainly the tropical ones with much higher ultraviolet indexes (UVI), the animals investigated here lived in a still considerably sunny subtropical location where the ultraviolet index varies from medium (3.5 UVI) during the winter to extreme (13.4 UVI) during the summer months (Corrêa et al., 2003). Thus, in our investigation, it is also conceivable that ultraviolet radiation has contributed as a carcinogen to the development of these 10 OSCC cases regardless of the eyelid pigmentation. We believe the already proven susceptibility of certain breeds such as Holstein and Hereford (Tsujita & Plummer, 2010) has more to do with UV radiation exposure combined with a genetic characteristic present in both breeds of cattle than the phenotype of eyelid pigmentation *per se*. Although OSCC forms only part of the "skin cancer" complex in man, the epidemiologic similarities of the condition in cattle and man living in the same tropical environment are remarkable (Silverstone & Searle, 1970; Scott & Straf, 1977).

According to our results (Table 1), the main site of OSCC were third eyelid (60%) followed by the corneoconjuntival junction (*limbus*) (20%), cornea (10%) and eyelids (10%). Our findings are similar to those by Gharagozlou et al. (2007) that identified 70% of OSCC in the third eyelid and adjacent conjunctiva in dairy cattle. Our findings for OSCC site predilection strongly disagreed however with those of Russell et al. (1956) and Anderson et al. (1957b). These reports have indicated that approximately 75% of ocular carcinomas and precursor lesions were found on the limbus and cornea and only the remainder was observed in the conjunctiva and third eyelid. Since there are different possible causes and predisposing factors, the authors believe that tumor location may vary according to the cause, for instance those OSCC related with UV exposure might occur more commonly whereas viral triggered OSCC might occur more frequently in the limbal tissue.

Curiously, no association was found between anaplasia, invasiveness and mitotic index and local aggressiveness in the OSCC analyzed, using a combination of two well-established grading systems. In other words, undifferentiated OSCCs were not more invasive than well-differentiated OSCCs. In the same manner undifferentiated OSCCs were not more invasive or possessed a higher mitotic index than well-differentiated ones. One possible explanation for this finding might be that the different tumors investigated had different causes or predisposing factors and the main histologic features may conceivably vary according to the etiology. However, it is important to bear in mind that the animals were not thoroughly examined for metastasis in distant sites of the body. Thus, the correlation of a maximum histological AIM score might still correlate with the presence of metastasis in a distant site. Conceivably, the histologic grade of the squamous cell carcinoma reflects characteristics individual neoplasm that can vary independently and there is no clear relationship between the grade itself and cure rate, stage of disease and metastatic involvement (Bhargava et al., 2010). Another factor missing from the clinical history is time of development. Since tissue invasion and overall tumor aggressiveness requires at least some time to occur, knowing how long it took to develop would be an interesting variable to have knowledge of in order to establish possible relations.

The presence of inflammation also varied considerably. The explanation for this finding might be that inflammation was a confounding variable, possibly caused by external factors such as exposure conjunctivitis, presence of dirt, auto-traumatism, parasitic infestation by fly larvae (maggots) (Figure 1c) and possibly due to the host response to tumor antigens (Wilcock, 1993).

OSCC is certainly regarded as the most common form of bovine tumor worldwide (Plummer, 1956; Brandly & Migaki, 1963; Priester & Mantel, 1971; Naghshineh et al., 1991). Because of this fact, in our investigation, the number of cases received was not as high as expected by the authors. Even though a wide publicity campaign was executed urging Brazilian veterinarians and veterinary ophthalmologists to send tissue samples of OSCC for a histopathological investigation, only 10 cases were received after two years. Several possible explanations arise here: 1) There are just as many cases as in other countries in Brazil but veterinarians tend to not collaborate in investigations due to lack of interest in the histopathological diagnosis; 2) There are just as many cases as in other countries in Brazil but veterinarians in the field and in the abattoir do not give medical attention when applicable or do not recognize the tumors; lastly 3) There are not as many OSCC cases in Brazil or in the Brazilian regions investigated, compared to other countries and regions. There are some evidences in the literature that reinforce this latter possibility. In the south of Brazil Lucena et al. (2011) previously reported OSCC not in first place in terms of rate occurrence of tumors, but in fourth place, behind tumors of the gastrointestinal tract, skin, hemopoietic tissue, being just more common than squamous cell carcinomas of the urinary system. One possible explanation for this difference is that the south of Brazil is certainly a region rich of rural areas where bracken fern (*Pteridium aquilinum*) is abundant in the pastures. It is well known that when cattle ingest less than 10 g/kg body weight per day for longer periods (1 year or more), a chronic disease characterized by intermittent hematuria is observed. This form of toxicity is known as enzootic hematuria and is related to the development of tumors in the urinary bladder. Haemangioma also is frequently found in these cases, but several other types of benign and malignant neoplasms may be seen, including papillomas, haemangiosarcomas and transitional cell carcinomas (Gabriel et al., 2009) and squamous cell carcinoma in the upper digestive tract (Souto et al., 2006). It is presumed that the development of gastrointestinal squamous cell carcinomas occurs when cattle ingest small amounts of bracken fern for extended periods (years) of time (Souto et al., 2006). Conversely, in other parts of the world the occurrence of the occurrence of squamous cell carcinoma in the digestive system and urinary bladder of cattle is reportedly rare (Plummer, 1956; Bastianello, 1982) and is almost not observed in cattle grazing pastures where bracken fern is absent.

Even though treatment was not the subject of our investigation, many therapeutic protocols have been described for OSCC with varying levels of difficulty, required equipment and results. These options include surgical excision (as performed here), cryotherapy, radiofrequency hyperthermia, immunotherapy, chemotherapy, and radiation therapy (Tsujita & Plummer, 2010).

4.7 CONCLUSION

In summary, although a common tumor, the genesis of OSCC has not been fully investigated and several theories have been advanced to explain its development. It is conceivable that the etiology changes in different regions of the world and also varies significantly within each region. The histologic parameters used in the classification were logical and easy to apply. However, anaplasia parameters did not correlate well with invasion of deeper tissues and mitotic indexes in the samples analyzed. This probably has something to do with the etiology of the tumor on each sample. Thus it was not possible to predict local tumor behavior based on the main histological features investigated here alone. Nevertheless, we still believe that histologic classification is an important detail that needs to be studied in more detail in order to unveil OSCC etiology. Possibly, an association between histological grading and molecular techniques such as *in situ* hybridization and immunohistochemistry would be a feasible route to investigate and predict OSCC behavior. This is a first attempt to produce an acceptable histological grading scheme in a Brazilian OSCC study. Lastly, this is the first OSCC investigation performed exclusively in OSCC cases diagnosed in the State of Paraná and Santa Catarina.

4.8 REFERENCES

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SUPPLEMENT 1 - ANIMAL USE COMMITTEE

Os Testes oftálmicos foram efetuados como parte do exame físico durante o projeto "Ocorrência de doenças infecciosas e zoonoses nos Aoudads (*Ammotragus lervia*) do Zoológico Municipal de Curitiba"

UFPR

Universidade Federal do Paraná
Setor de Ciências Agrárias
Comissão de Ética no Uso de Animais – CEUA SCA

CERTIFICADO

Certificamos que o protocolo no. 045/2013, referente ao projeto "Ocorrência de doenças infecciosas e zoonoses nos Aoudads (*Ammotragus lervia*) do Zoológico Municipal de Curitiba", sob a responsabilidade de Vivien Midori Morikawa, na forma em que foi apresentado (uso de 17 amostras de sangue de Aoudads), foi aprovado pela Comissão de Ética no Uso de Animais do Setor de Ciências Agrárias, em reunião realizada dia 15 de maio de 2014.

CERTIFICATE

We certify that the protocol number 045/2013, regarding the project "Occurrence od infectious diseases and zoonoses in Aoudads (*Ammotragus lervia*) from the Curitiba Zoo", under Vivien Midori Morikawa's supervision, in the terms it was presented (use of 17 blood samples of Aoudads), was approved by the Animal Use Ethics Committee of the Agricultural Sciences Campus of the Universidade Federal do Paraná (Federal University of Paraná, Brazil) during session on May 15th, 2014.

Curitiba, 18 de agosto de 2014.

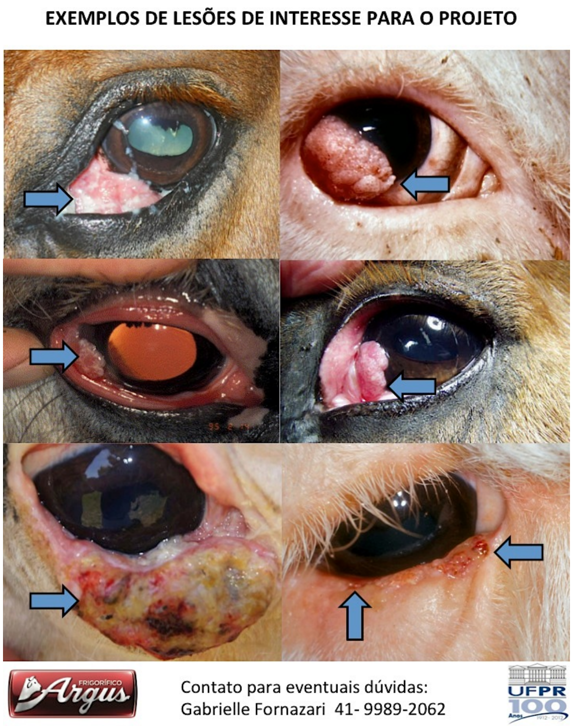
Ricardo Guilherme D'Otaviano de Castro Vilani
Presidente

Ananda Portella Félix
Vice-Presidente

Comissão de Ética no Uso de Animais
Setor de Ciências Agrárias
Universidade Federal do Paraná.

SUPPLEMENT 2 - ELECTRONIC ANNOUNCEMENT POSTED ON A SOCIAL NETWORKING SITE

SUPPLEMENT 3 - EDUCATIONAL ILLUSTRATIVE GUIDE TO HELP IDENTIFYING OCULAR SQUAMOUS CELL CARCINOMA CASES TO BE USED BY THE EMPLOYEES WORKING IN ABATTOIRS



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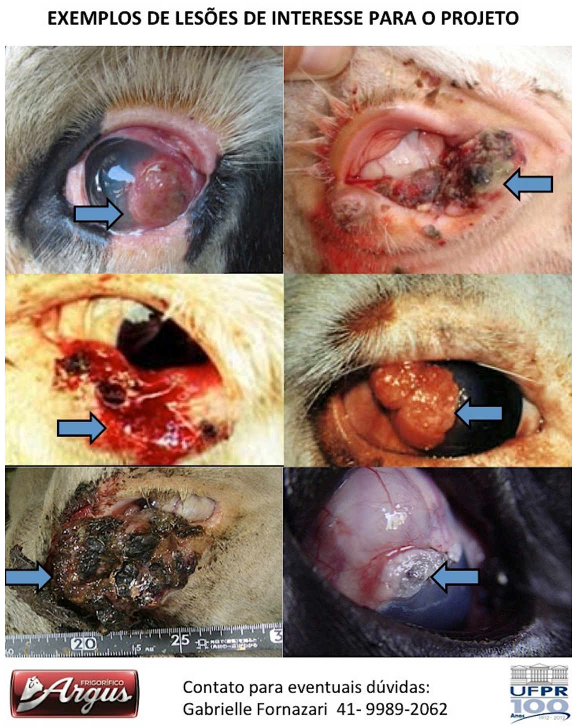

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