UNIVERSIDADE FEDERAL DO PARANÁ

MARIZA BORTOLINI

THE OCULAR EFFECT OF 0,2% CANNABIDIOL OPHTHALMIC SOLUTION IN BEAGLE DOGS AND CHARACTERIZATION OF A NOVEL FORM OF PROGRESSIVE RETINAL ATROPHY IN THE GERMAN SPITZ DOG: A CLINICAL, ELECTRORETINOGRAPHIC, AND BREEDING STUDY

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Orientador: Professor Dr. Fabiano Montiani-Ferreira

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, sob orientação do Prof. Dr. FABIANO MONTIANI FERREIRA, que após terem inquirido a aluna e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

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RESUMO

A presente dissertação de mestrado é composta de dois capítulos e buscou contribuir com o conhecimento para a oftalmologia comparada, integrando temas pertinentes e atuais às áreas da clínica e da pesquisa na oftalmologia de animais domésticos, também relevantes na oftalmologia de seres humanos. O primeiro capítulo tem como objetivo investigar o potencial terapêutico do canabidiol, um componente já regularmente utilizado no tratamento de diversas doenças em seres humanos, mas com poucas informações sobre seus efeitos em animais domésticos. Tal estudo avalia o efeito na produção lacrimal, pressão intraocular e estesiometria de cães da raça Beagle após instilação de colírio de canabidiol na concentração de 0,2%. O Capítulo 2 trata da caracterização de uma nova forma de distrofia de retina em cães da raça Spitz Alemão, incluindo suas características clínicas, eletrorretinográficas, achados em tomografia de coerência óptica, teste do reflexo pupilar à luz cromática e seu caráter hereditário. O estudo de tais doenças hereditárias de retina nos cães é de grande interesse para os avanços nos estudos sobre o caráter clínico, genético e terapêutico do grupohomólogo e de grande prevalência de retinopatias degenerativas em seres-humanos, visto que o cão é um modelo experimental de estudo elucidativo e terapêutico destas doenças decorrente as inúmeras similaridades interespécies.

Palavras-chave: Oftalmologia comparada, retina, atrofia, ERG, Spitz Alemão, canabidiol, Beagle, PIO, STT, KCS.

ABSTRACT

The present dissertation is composed of two chapters and sought to contribute to the knowledge of comparative ophthalmology, integrating important and current themes to clinical and research areas in domestic animal ophthalmology, which are also relevant in human ophthalmology. The first chapter aims to investigate the therapeutic potential of cannabidiol, a component already regularly used in the treatment of several diseases in humans, but with little information about its effects in domestic animals. This study evaluates the effect on tear production, intraocular pressure and esthesiometry of Beagle dogs after instillation of 0.2% cannabidiol eye drops. Chapter 2 deals with the characterization of a new form of retinal dystrophy in dogs of the German Spitz breed, including its clinical, electroretinographic, findings in optical coherence tomography, pupillary chromatic light test, and its hereditary character. The study of such inherited retinal diseases in dogs has special interest for the advances in studies on the clinical, genetic and therapeutic character of the homologous group and the high prevalence of degenerative retinopathies in humans, since the dog is an experimental model of such diseases because of their numerous interspecies similarities.

Key-words: comparative ophthalmology, retinal, atrophy, ERG, German Spitz, cannabidiol, Beagle, IOP, STT, KCS.

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CHAPTER ONE - THE OCULAR EFFECT OF 0,2% CANNABIDIOL OPHTHALMIC SOLUTION IN BEAGLE DOGS

1.1 ABSTRACT

The genus Cannabis is in the family Cannabaceae in the major group Angiosperms (Flowering plants). Its main components of pharmaceutical interest are cannabidiol (CBD) and Δ9-tetrahydrocannabinol (THC). Several of its therapeutic effects have already shown to activate or modulate the activity of the endocannabinoid system. In this context, the aim of the study is to investigate a possible effect of 0.2% CBD eye drops on tear production and intraocular pressure (IOP). This study included 14 healthy beagle dogs from the Canine Nutrition Laboratory at UFPR (LENUCAN). The objective of this randomized, blind study was to evaluate the effects of a 0.2% cannabidiol eye drops every 12hs in one eye against control eye drops containing only the pharmaceutical vehicle in the contralateral eye. Tear production and intraocular pressure were significantly increased when compared to the control eye, corroborating other previous authors. Therefore, further studies are needed so that the use of 0.2% CBD in eye disorders resulting from low tear production could be a treatment option, since it also increases the IOP considerably.

1.2 INTRODUCTION

The plant Cannabis sativa is one of the most used drugs in the world and contains >100 different cannabinoids. The most abundant of which is the main psychoactive component,Δ9-tetrahydrocannabinol (THC), and the non-intoxicating cannabinoid, cannabidiol (CBD). In addition, anandamide and arachidonylethanolamide are produced endogenously by animals [5, 6]. In some countries, THC is already used to treat disorders that cause acute pain, while CBD is commercially sold in the United States as an herbal supplement for chronic and inflammatory pain relief[12]. The clinical interest of these two substances in ophthalmology is still under scrutiny, as the main receptors for these molecules are found in several animal species and in almost all ocular structures[6,7]. Several of its effects on ocular tissue have already

been published[7]. In veterinary medicine, the effects of cannabinoids have been studied especially during the last decades, as the research of these compounds was suppressed for a long time due to the controversial status of cannabis as a drug[13]. The aim of the study is to investigate a possible effect of 0.2% CBD eye drops on two important ocular parameters: tear production and intraocular pressure(IOP).

1.3 MATERIALS AND METHODS

Fourteen healthy Beagle dogs from the Canine Nutrition Laboratory (LENUCAN) of UFPR were used in this investigation. The study was authorized by the Animal Use Ethics Committee (CEUA) of the agricultural sciences sector through protocol 044/2019. It was conducted in a randomized, blind study, in which 0.2% cannabidiol eye drops in lipophilic medium chain fatty acid (MCFA) vehicle, were instilled every twelve hours for five days in one eye and only the vehicle drug in the contralateral eye of these animals (figure 1). Ocular parameters and photographs were taken before the application of the drops at three times: 48 hours (pr 3), 24 hours (pr 2) and 1 hour before installation (pr 1). Subsequently, the procedures were repeated 1 hour (p1), 4 hours (p2), 48 hours (p3) and 96 hours (p4) after the beginning of the experiment, with the exception of STT, which was measured at p3 (48 hours) and p4 (96 hours).

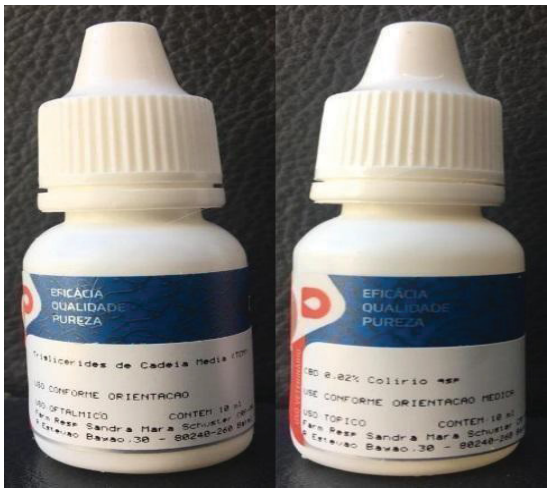


Figure 1. Ophthalmic solutions used in this study (0.2% cannabidiol – left; control – right).

All the animals included were evaluated by a specialized veterinarian in the veterinary ophthalmology service to exclude the ones with any ophthalmic conditions. STT was performed in both eyes of the animals (figure 2). After one minute, the STT strip was removed and the number corresponding to the tear absorption of the strip in the respective animal's eye was registered.

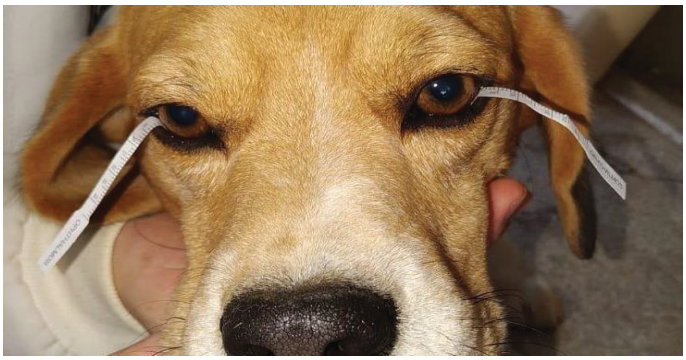


Figure 2 Schirmer's tear test in a beagle dog.

Tonometry also was performed with the minimal restraint using a rebound tonometer. The average of six measurements taken perpendicular to the central portion of the cornea was performed for each eye.

Photographs of both eyes of all animals were taken and compared before each measurement of the previously mentioned parameters, in order to evaluate for the presence of conjunctival hyperemia or ocular secretion. In case of intense congestion, the animal would be removed from the study and a treatment to promote comfort would be instituted.

To minimize the possibility of sampling errors or subjectivity of the exams the same person performed all procedures. Starting with photography, followed by STT and finally tonometry, with all animals examined in the same place, sitting on a table and with the minimum necessary restraint.

The results of the tested eyes were compared with the control eyes using the paired t-Student's test, divided into pre-, post-test, and control eye drops. When the parameter compared had a significant value, p-value <0.05, a paired comparison was performed between each moment of the measurements described above.

1.4 RESULTS

1.4.1 SCHIRMER'S TEAR TEST

Schirmer's tear test showed an overall significant increase ($p<0.03$) in tear production in the treated eye when compared to the control group, especially at 48 and 96 hours (figures 3 and 4).

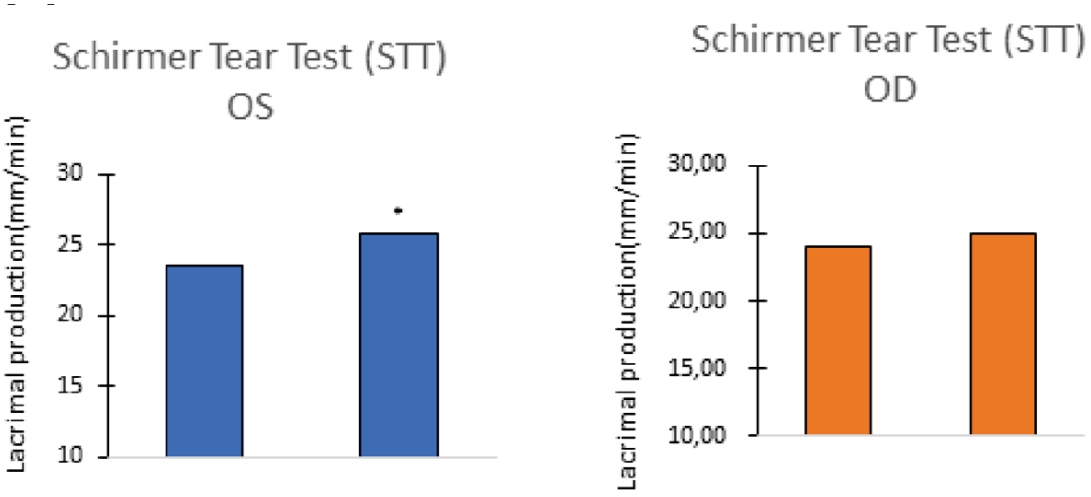


Figure 3 - Overall STT mean values from pre and post treatment for both eyes. Note that in the left eye (OS) a significant increase was observed.

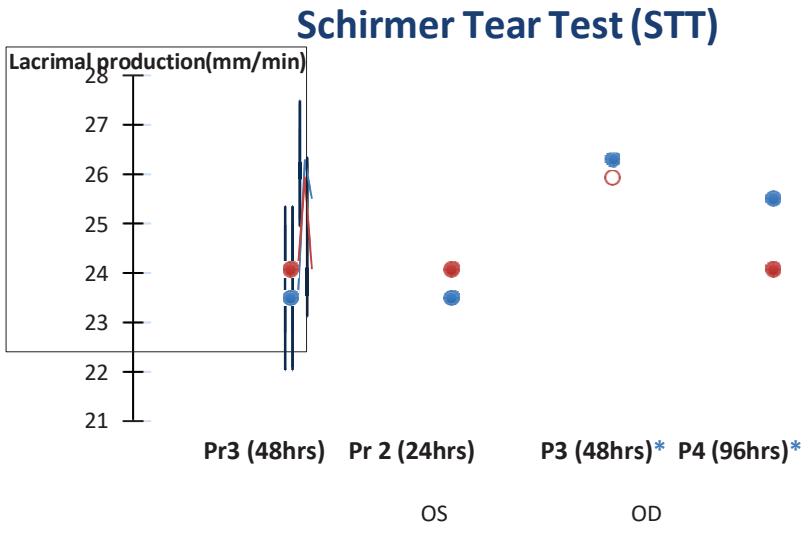


Figure 4 – Mean STT values at each time point, for both eyes

Table 1 Mean and standard deviation of the values found in the animals at each time evaluate

Schirmer tear test (mm/min)				
Tested groups				
	OS		OD	
Pr 3	23.5	±1.46	24.07	±1.27
Pr 2	23.5	±.1.46	24.07	±1.27
P3	25.5	±0.83	25.92	±0.98
P4	25.5	±0.83	24.07	±0.95
P value	0.03		0.36	

1.4.2 Tonometry

The tonometry of the animals showed a significant increase ($p<0.001$) in the intraocular pressure of the treated group (figure 5) compared to the control group ($p<0.11$).

Figure 5 Tonometry´s mean values from pre and post treatment for both eyes. Note the increase in IOP in the treated eye (OS)

A progressive IOP increase was evidenced at all times after the start of treatment (figure 6).

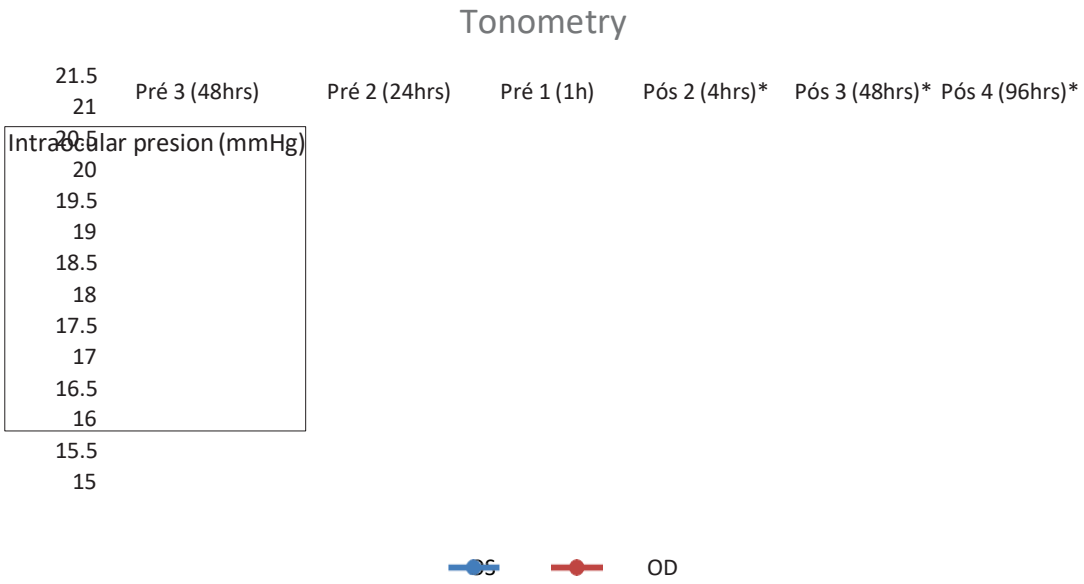
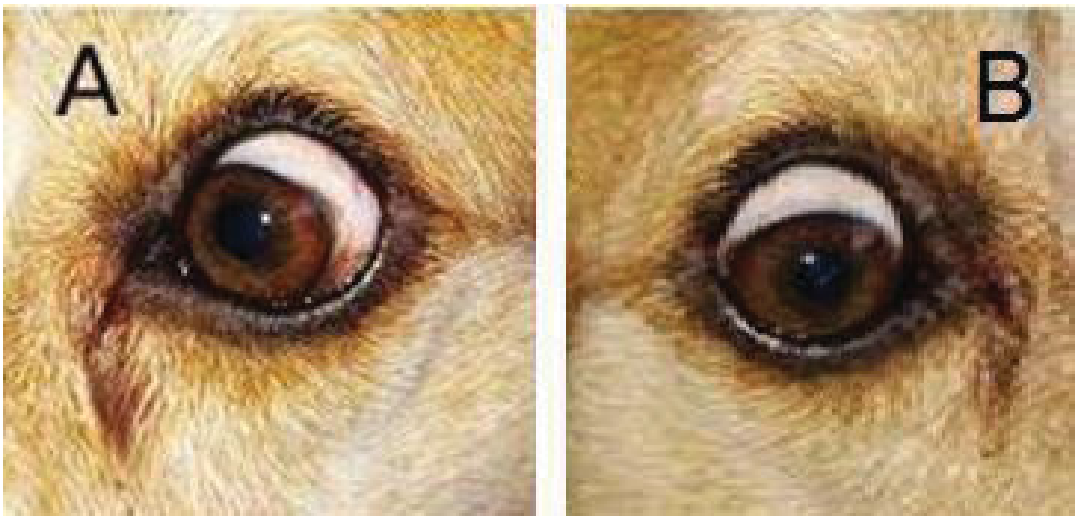


Figure 6 Comparison of means and standard deviation obtained by rebound tonometry in each respective evaluated time. * After each time point represent statistically significant values

Table 2 Mean and standard error of the values found in the animals at each time evaluated. *significant difference when compared to the control group by the paired Student's t-test.

Intraocular pressure (mmHg)				
Tested groups				
	OS		OD	
Pr 3	17.1	±0.69	17.35	±0.89
Pr 2	16.35	±0.90	17.85	±1.04
Pr1	18.07	±0.62	17.71	±1.05
P2	18.85	±1.0	18.14	±1.17
P3	18.57	±1.13	18.14	±0.99
P4	19.71	±1.07	19	±1.09
P value	0.001		0.119	



The general ocular condition of all animals remained unchanged, with no evidence of hyperemia, secretion or lesions on the eyelids due to pruritus. (figure 7).

Figure 7 General appearance of the of left (A) and right eye (B) of a Beagles that received CBD. Note absence of any hyperemia or epiphora in both photos

1.5 DISCUSSION

The main data obtained in this study was the statistically significant difference in increased tear production and intraocular pressure. Schirmer's tear test has a reference value between 18.64 ± 4.47 mm/min to 23.90 ± 5.12 mm/min, used mainly

to diagnose keratoconjunctivitis sicca, with values below 5 mm/min being diagnostic for this disease[16]. Keratoconjunctivitis sicca is due to decreased tear film production or quality, which is most often immune-mediated. For the treatment of this disease, immunomodulators eye drops, tear stimulants, tear replacements or surgeries are used as a last resort (17 et al, 1977). Since the eyes treated with CBD obtained significant values (Table 1) in normal dogs, it would be convenient to carry out studies comparing the effect of using cannabidiol for treatment or as an adjunct in the treatment of keratoconjunctivitis sicca. After all, studies on the potential therapeutic use of these substances in the treatment of this disease are still not documented. Studying in depth the therapeutic potential of this substance is interesting as the chronic use of Cannabis in humans would be related to the increase in tear production[17], whereas synthetic cannabinoid substances used to treat anxiety and insomnia, such as nabilone, keratoconjunctivitis sicca is described as a side effect of the drug in humans[18.] Tonometry is commonly used for the diagnosis of glaucoma or as a complementary data in other ophthalmic conditions. Values considered normal for measuring IOP range from 15-25mmHg in most animals, with values commonly found between 15 and 18 mmHg in dogs[15]. Over the years, interest in the use of cannabinoids for the treatment of glaucoma has grown exponentially, as studies correlate the decrease in IOP with the activation ofCB1R-type receptors by cannabinoid molecules[1,14]. However, in this work it was possible to observe that CBD had the opposite effect, causing an increase in IOP when compared to the control group, corroborating the findings of Miller et al (2018),in which it is mentioned that IOP would be influenced by at least three cannabinoid receptors (CB1, GPR18 and GPR19). Thus, CBD would have its supposed mechanism of action indirectly acting on the CB1 type receptor as a negative allosteric modulator[4]. Therefore, the use of eye drops containing an exclusive formulation of CBD would be contraindicated as an option in the treatment of eye disorders that aim to reduce IOP.

Cannabinoid receptors (CB1R and CB2R) constitute essential members of the endocannabinoid system. CB1Rs are responsible for most of the psychotropic effects and are found predominantly in presynaptic neurons of the nervous system, in the ocular trabecular meshwork, ciliary muscles, ciliary epithelium and in the retina. They modulate the activity of postsynaptic neurons by regulating the release

of neurotransmitters such as gamma-aminobutyric acid (GABA), glutamate, dopamine and noradrenaline[1,2,9,11]. Currently, this modulation has been studied with greater emphasis for the treatment of glaucoma by increasing the drainage of aqueous humor. Studies using rat models for diabetic retinopathy, which were treated with cannabidiol, showed a significant reduction in oxidative stress, neurotoxicity and drastic prevention of retinal cell death[2,6,9]. CB2Rs are highly expressed in immune system cells, retinal pigment epithelium, amacrine, horizontal and ganglion cells. In the anterior segment of the eye tissue, its expression is commonly low in non-pathological conditions, increasing considerably in inflammatory situations [1, 8]. In studies promoting the induction of experimental uveitis[8], the activation of these receptors via cannabinoids triggered anti-inflammatory responses. There was a reduction in the production of pro-inflammatory mediators, such as tumor necrosis factor alpha (TNFα), vascular endothelial growth factors (VEGF) and leukocyte recruitment, when compared with the use of anti-inflammatory drugs already commonly used in the treatment of this illness [1,6,8,9]. Glaucoma is a group of ocular disorders, secondary to an abnormal increase in intraocular pressure (IOP), leading to progressive retinal ganglion cell death, optic nerve damage and blindness if not effectively treated[3,9]. Currently, the treatment of glaucoma consists of several drugs or surgical procedures to control the increase in IOP in dogs. The main pharmacological classes used are beta-adrenergic blockers and alpha-adrenergic agonists, carbonic anhydrase inhibitors, prostaglandin analogues, hyperosmotic and parasympathomimetic agents[9]. The maintenance of IOP is dependent on the balance of the rate of aqueous humor production by the ciliary body and its drainage through the trabecular meshwork and uveoscleral pathway[3] together with the extracellular excess of glutamate responsible for one of the induction mechanisms of cytotoxicity in glaucoma through excessive production of peroxynitrite and increase in intracellular calcium[2,6]. As described by Miller et al (2018), the use of THC in normotensive rats was responsible for reducing the IOP considerably for at least 8 hours through the activation of two cannabinoid receptors. It was also observed that the application of CBD together with THC causes the modulation of cannabinoid receptors, increasing the IOP[4]. With this in mind, the use of cannabinoids becomes interesting, as there are studies showing that CB1R and CB2R agonists together increase aqueous

humor drainage and reduce the formation of intraocular cytotoxic compounds [1,2,3,6.] Chronic and painful corneal lesions trigger inflammatory responses that involve the production of proinflammatory cytokines, algesic neuropeptides, leukocyte recruitment and neovascularization. Currently, pharmacological therapy for patients with eye pain is through anti-inflammatory drugs, corticoids, tricyclic antidepressants, GABAergic drugs and opioids. These commonly fail to provide complete pain relief and may trigger unwanted side effects[8]. Vanilloid 1 receptors (TRPV1), present in the epithelial and endothelial cells of the cornea of rodents and primates are responsible for causing the sensitization of afferent nociceptive fibers to chronic stimuli. In studies, it is reported that iní experimental models, drugs that act as CB1R agonists could produce analgesia on the ocular surface through the inactivation of TRPV1. The use of endocannabinoid system agonists for the treatment of ocular pain is interesting, as these drugs have promising potential for the treatment of corneal injuries by reducing pain, ocular inflammation, repair time and scar diameter [1,6,8,10]

1.5.1 CONCLUSION

The 0.2% CBD formulation significantly increased tear production and IOP of the evaluated eyes compared to the control. Therefore, this study suggests that it is an interesting active ingredient to be used in other studies with the aim of elucidating the therapeutic potential in the treatment of diseases such as keratoconjunctivitis sicca. However, its ability to increase IOP in healthy animals is an indication that this medication would be contraindicated in animals suspected of or diagnosed with glaucoma. Further studies in the area of cannabinoid compounds, both natural and synthetic, are recommended, as many of their effects are not yet described in detail.

1.6 ACKNOWLEDGMENTS

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1 CHAPTER TWO - CHARACTERIZATION OF A NOVEL FORM OF PROGRESSIVE RETINAL ATROPHY IN THE GERMAN SPITZ DOG: A CLINICAL, ELECTRORETINOGRAPHIC, AND BREEDING STUDY

1.1 ABSTRACT

Objective: To describe a clinical presentation, electroretinographic and optical coherence tomographic findings of a distinct progressive retinal atrophy (PRA) in German Spitz and a pedigree analysis.

Animals: Thirty-three owners and breeders of German Spitz dogs were examined by the Comparative Ophthalmology Service at Universidade Federal do Paraná during this study.

Procedures: The thirty-three dogs were subjected to a routine ophthalmic examination, including behavioral and visual testing, dazzle and pupillary light reflexes (PLRs), indirect ophthalmoscopy with fundus photography and bilateral electroretinography (ERG). OCT and chromatic pupillary light reflex (cPLR) tests were performed in eleven animals.

Results: Sixteen dogs were identified with PRA with ERG recordings showing an extinguished rod activity (flat line) and almost all cone activity even in 2 month old dogs clinically blinds. Despite all dogs with PRA had failed to avoid objects under scotopic and photopic conditions, whereas light reflexes and pupillary reflexes (PLRs) were preserved. Funduscopy of affected animals showed small alterations during the initial examination (2 to 4 months of age), and initial signs included a pale optic nerve head and slight vascular attenuation.. Oscillatory nystagmus was detected in eight young affected animals Multifocal retinal bullae developed in six affected animals at 7 months of age which can no longer be noticeable during fundoscopy at approximately 12 months of age. Slowly deterioration of the PLR´s and a resting mydriasis are recorded at 36 months. Pedigree analysis proposed an autosomal recessive mode of inheritance. Lack of a pupil response after red light

illumination, with normal response after blue light illumination was observed. *Conclusion*: Here, a phenotype of an early onset type of inherited progressive retinal degeneration is reported in the German Spitz dogs with an extinguished ERG rod response associated with multifocal retinal bullae and distinguished dorsal retinal degeneration at 36 months of age. This study suggests a possible new variation of the PRA in German Spitzes and proposes more studies to elucidate the changes seen in these individuals.

Key Words: progressive retinal atrophy, electroretinography, retina, OCT, bullae, German Spitz, chromatic pupillary light reflex

2.2 INTRODUCTION

Progressive retinal atrophy (PRA) in dogs is an umbrella term that is used to name a group of inherited retinal disorders that possess a considerable degree of genetic heterogeneity within and between domestic dog breeds [1,2]. The condition is analogous to retinitis pigmentosa (RP) and Leber's congenital amaurosis (LCA), characterized by profound loss of visual function early in life in humans[3-5], The condition has been recognized in more than 100 breeds, several other breeds are suspected of presenting a form of this inherited retinopathy. Affected animals, however, may present profound differences regarding the age of onset, rate of progression, mode of inheritance, genetic features and molecular etiology. In most affected animals, a bilateral progressive loss of scotopic and peripheral vision precedes the loss of photopic vision and total blindness [2, 6-8]. The diagnosis is based on clinical history, ophthalmologic and complementary examinations such as fundoscopy[9], electroretinography (ERG)[10-13] and genetic testing,[14-24] when available. The most common funduscopic changes observed in dogs with PRA are bilateral and symmetrical, including tapetal hyper-reflectivity in the early stages followed by vascular attenuation, pigmentary changes[10] and atrophy of the optic nerve head in the later stages of disease[, 2,9, 10,13]. These canine inherited retinopathies can be further classified as dysplasia or as a degenerative disease, according to several factors such as the age of onset, affected cells, mode of inheritance, genetic and molecular pathogenesis[3],[5,14-24]. Dysplastic disease occurs

before the complete development of the retinal structures, and commonly are of an early-onset. In this type of PRA the loss rods and cones may occur simultaneously and the rate of progression is usually fast [5,14].In degenerative retinopathies the retinal cells develop normally but then degenerate at some point during the animal's life [1,2,22] The most common degenerative retinal disorder is progressive rod-cone degeneration (PRCD). These are typically diagnosed in dogs older than 3 years, although it may also be diagnosed in animals that are much older. This is why these conditions are usually referred to as of a late-onset [15]. Most forms of PRA are inherited as autosomal recessive traits[10-14]. However, X-linked forms and autosomal dominant forms have also been documented [6].Mutations in several different genes have been identified as the underlying cause for canine PRA [2,8,11, 13-23]. Some of these genes are the same ones implicated in the analogue human condition [24,25] . Thus, new discoveries regarding this condition in animals may represent great opportunities for comparative studies and for better understanding normal visual function itself [23]. Clinical characterization of the different specific forms of PRA is fundamental to obtain a reliable model for future genetic and therapeutic investigations [23-26]. The objective of this investigation is to describe a novel form of PRA in German Spitz dogs through clinical, electroretinographic, OCT, genetic and pedigree analyses.

2.3 MATERIALS AND METHODS

2.3.1 Animals

Thirty-three German Spitz dogs were examined by the Ophthalmology Service at the Veterinary Teaching Hospital at The Federal University of Paraná (UFPR). A signed informed consent was obtained from the participant owners. All procedures were conducted in accordance with the Association for Research in Vision and Ophthalmology's (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research, and the institution´s own Animal Use Committee. All dogs included in the study and investigations performed on each one are shown in Table 1. Pedigree information available was collected for the examined dogs and study was performed for identification of mode of inheritance of this PRA.

2.3.2 Clinical examination

All dogs underwent a complete physical examination and a complete blood panel before ocular examinations to exclude animals with indications of systemic disease. Vision testing was performed including ability to track falling cotton wool balls and to negotiate an obstacle course test in both bright and dim light. Ophthalmic examination included assessment of the menace response, dazzle reflex, and pupillary light reflex. The anterior segment was examined by slit-lamp biomicroscopy (Hawk Eye, Dioptrix, L'Union, France). The fundus was examined by indirect ophthalmoscopy (Eyetec Equipamentos Oftálmicos, São Carlos, Brazil). Additionally, several fundus photographs (ClearView ®, Optibrand Ltd., Fort Collins, CO, USA) were obtained from all affected dogs and selected age-matched clinically unaffected dogs. Several dogs were reexamined to monitor disease progression (Table 1).

Table 1. Age and sex of the German Spitzs included in the study and tests performed

Dog number	**Sex**	**Age at first examination (months)**	**Age at diagnosis (months)**	**Reexamination age (months)**	**ERG age (months)**	**Repeated ERG age**	**OCT age (months)**	**cPLR age (months)**
Affected dogs								
#1	M	3	3		3			
#2	M	3	3	7,12,24	3	24	32	36
#3	M	3	3	7,12,24	3	24	36	36
#4	F	5	5		5			

#5	F	22	22	9

#6	M	9	9	9	9		
#7	M	2	3	5	3	16	
#8	M	2	3	4,5,7,8	4	8	
#9	F	2	3	4,5,7,8	4	8	15
#10	M	3	3	4	4	13	
#11	M	4	4		4		
#12	M	15	15		15		
#13	F	1,5	2	4	2	4	2
#14	F	1,5	2		2		2
#15	F	1,5	2		2		2
#16	M	1,5	1.5				

Unnaffected dogs

#17	F	3	3		3		
#18	M	12	12		12	12	
#19	F	15	15		15		
#20	M	9	9		9		
#21	F	60	60		60		
#22	M	9	9		9		
#23	M	15	15		15		

#24	F	39	39	39		
#25	F	14	14	14		
#26	F	73	73	73		
#27	F	4	4	4		
#28	M	4	4	4		
#29	F	8	8	8		
#30	F	17	17	17	18	18
#31	M	36	36	36	36	
#32	M	7	7	7	7	
#33	F	10	10	10		

2.3.3 Electroretinography

Electroretinography (ERG) was performed after pupillary dilation using 1% tropicamide eye drops (Mydriacyl, AlconTM, São Paulo, SP, Brazil) associated with 10% phenylephrine eye drops (Frumtost, São Paulo, SP, Brazil). ERGs were performed without anesthesia in all dogs. In six dogs (four affected and two unaffected) were examined under anesthesia with acepromazine (0.03 mg/Kg; Acepran, 0.2, Vetnil Ltda, São Paulo, SP, Brazil), propofol for induction (5 mg/kg; Propovan 1%, Cristalia Ltda, Itapira, SP, Brazil), and they were intubated and maintained with isoflurane (1- 1.5% Isoforine, Cristalia Ltda, Itapira, SP, Brazil) delivered in oxygen (30 mL/kg/min) using a semi-closed system. A Jackson Rees modified Ayre's T-piece with fresh gas flow of 250 ml/kg/min was used in dogs weighing less than 4 kg. Heart and respiratory rates, electrocardiographic trace, noninvasive blood pressure, end-tidal CO2, pulse oximetry, and esophageal temperature were continuously evaluated by a multiparameter monitor (LifeWindow LW9xVet,

Digicare Animal Health, Boynton Beach, Florida, USA). To record the electrical responses of the retina, a portable ERG unit containing a Mini Ganzfeld flash photostimulator with white LED's (HMsERG VET System, OcuScience®, Henderson, NV, USA) was positioned at 1 cm from the corneal surface, within the eyes placed centrally in the palpebral fissure and the animals were evaluated under scotopic and photopic conditions. A topical corneal anesthetic was applied (proparacaine hydrochloride 0.5% ophthalmic solution USP; Alcon Laboratories, Forth Worth, TX, USA), and eyes were positioned using a lid speculum, followed by the placement of an active corneal contact recording electrode (ERG-Jet, Fabrinal SA, La Chaux-de-Fonds, Switzerland), and platinum subdermal needles (Model E2, Grass Technologies, Warwick, USA) were used as reference and ground electrodes, positioned in 2 cm from the lateral canthus and cervical dorsal region, respectively. Electrode impedance was maintained at <5 kΩ, and bandpass was 0.3–300 Hz (SOMMA, 2017). After 20 minutes of dark-adaptation a simplified scotopic ERG protocol consisting of a combined rod-cone response to a standard intensity (average of four flashes, 0.1 Hz, 0.47 log cds/m^2) and a high-intensity (average of four flashes, 0.05 Hz, 1 log cds/m^2) flashes under scotopic condition was performed in all animals. ERGs in the six dogs (with 3 months being the earliest age) that were anesthetized, a preprogrammed protocol previously used in two investigations [2, 17] was performed, consisting of the following stimuli: (i) Rod function was tested every 4 min during the period of 20 min of dark adaptation using a dim stimulus (average of 10 flashes, 0.5 Hz, 2 log cds/m^2); (ii) combined rod-cone response to a standard intensity (average of four flashes, 0.1 Hz, 0.47 log cds/m^2) and a high-intensity (average of four flashes, 0.05 Hz, 1 log cds/m^2) flashes under scotopic conditions; (iii) cone function following light adaptation (10 min at 30 cd/m^2) using a standard intensity flash (average of 32 flashes, 2 Hz, 1 log cds/m^2) and a cone flicker test (128 flashes, 31 Hz, 1 log cds/m^2).

2.3.4 Optical Coherence Tomography (OCT)

Spectral domain optical coherence tomography (SDOCT, Spectralis HRA+OCT; Heidelberg Engineering Inc.,Heidelberg, Germany) was

performed to obtain in vivo high-resolution cross-section images of the retina and optic nerve head [29](ONH) of 12 dogs. Seven clinically affected animals at 4-month old (1), 8-month-old affected (2), 13-month-old (1), 16-month-old (1), 32-month-old (1) and 36-month-old (1); and four clinically unaffected animals at 7-month-old (1), 12-month-old (1), 18-month-old (1), 36-month-old (1); #13, #8, #9, #10, #7, #3, #4, and #32, #30, #31 respectively) (Table 1). This procedure was performed at separate examination sessions. Anesthesia protocol was the same as previously described for the ERGs. The pupils were dilated, and the eyes were positioned using a lid speculum. In sequence, 10 retinal cross-sectional images were obtained for each eye examined, including tapetal, non tapetal, and papillary areas. Total retinal thicknesses were compared between affected and clinically unaffected dogs at similar ages at matching areas.

2.3.5 Chromatic pupillary light reflex test

In 11 dogs (four clinically unaffected and seven clinically affected dogs) pupil light reflex after chromatic with a red and blue light was tested (cPLR unit, Vision Biomedical Solutions, Apatin, Serbia). The test was performed by positioning the instrument 3 cm in front of the left eye with a red light beam (630 nm) for 10 seconds after dark adaptation of 10 seconds, and for the same 10 seconds in front of the right eye after 30 seconds of pupil adaptation. In sequence, the same procedure was executed with the blue light. As for infants and younger children[30], a grading chart (pupilometer) provided by the equipment manufacturer was held by the experimenter in front of the eye to estimate the pupillary diameter (or pupil response).

2.4 RESULTS

2.4.1 Animals

During the study period, sixteen dogs were identified as affected with a novel form or retinal dystrophy, of which 10 dogs (62.5%) were male and 6 (37.5%) female, with ages varying from 1.5 to 36 months.

2.4.2 Clinical examination

As soon as the affected dogs open their eyelids, signs of severe visual

impairment are already present. All affected animals failed to respond to menace response, dazzle reflex and visually-track falling cotton wool balls. When challenged by obstacle course tests, all affected dogs failed to avoid objects under scotopic and photopic conditions, whereas light reflexes and pupillary reflexes (PLRs) were initially preserved but gradually getting slower but still present even in the oldest animals examined (36 months of age). Oscillatory nystagmus was detected in eight young affected animals (from 3- 6 months of age), slowly fading out with age and disappearing after 1 year of age.

2.4.3 Funduscopic aspects

Funduscopy of affected animals showed small alterations during the initial examination of dogs, between 2 to 4 months of age. Initial signs included a pale optic nerve head and slight vascular attenuation. Zones of hyperreflectivity started to be detected in animals at 4 months of age, which became more evident at 15 months of age (Figure 1) followed by a slow deterioration of the PLR´s and a resting mydriasis at 36 months.

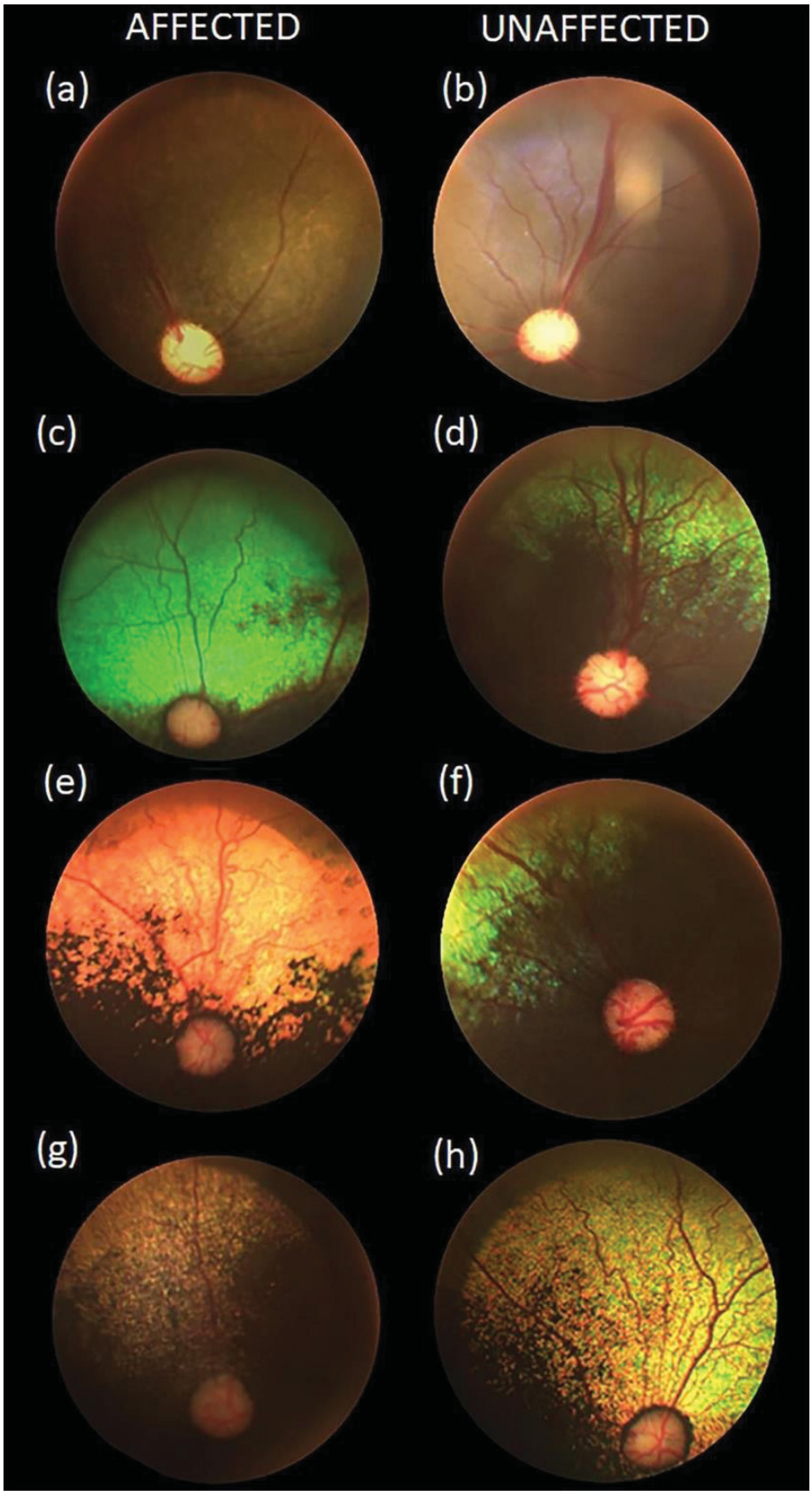
AFFECTED
UNAFFECTED
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)

Figure1. Selected fundus photographs of age-matched of clinically affected (a, c, e and g) and unaffected (b,d,f and h) dogs of the following ages: 2 months (a and b - dogs #13 and #17); 3 months (c and d – dogs #9 and #28); between 5 and 7 months (e and f – dogs #8 and #20); between 36 and 39 months (g and h – dogs #2 and #24). Note that the fundus of the affected dog (diagnosed by ERG) appears ophthalmoscopically close to normal at 2 months of age. At 3 months of age, mild/early vascular attenuation and slight pallor of the optic nerve head are visible when compared to an age-matched dog. At 5 to 7 months of age, retinal bullae (in this picture more noticeable in the retinal periphery), some tapetal hyper-reflectivity and more advanced vascular attenuation also were present. Between 36 and 39 months of age, affected dogs already presented a marked progressive generalized retinal degeneration and severe retinal vascular attenuation typical of end-stage retinal atrophy

Multifocal retinal bullae developed in six affected animals at 7 months of age. These areas of detachments can no longer be noticeable during fundoscopy at approximately 12 months of age (Figure 2).

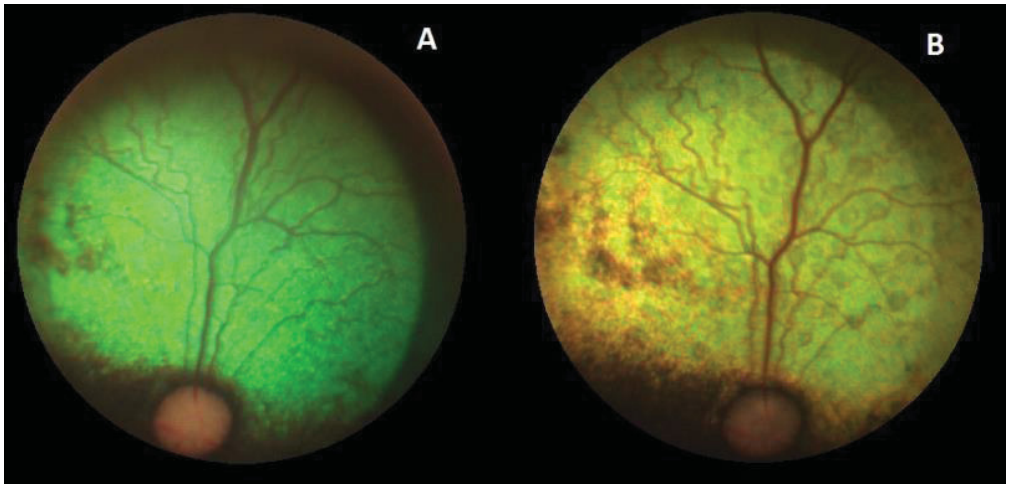

Figure 2. Fundus photographs of the same affected female. A: At 3 months of age, there is no evidence of bullae and the general aspect of the fundus is close to normal. B: At 7 months of age, Multifocal retinal bullae had developed, mainly in the dorsal aspect of the tapetal area. Although these fundus pictures were taken in slightly different angles there were no perceptible retinal bullae in A

2.4.5 Electroretinography

ERG recordings showed extinguished rod activity (flat line) and almost all cone activity (except a reminiscent photopic and flicker response) in clinically blind dogs (Figure 3) at 3 months of age.

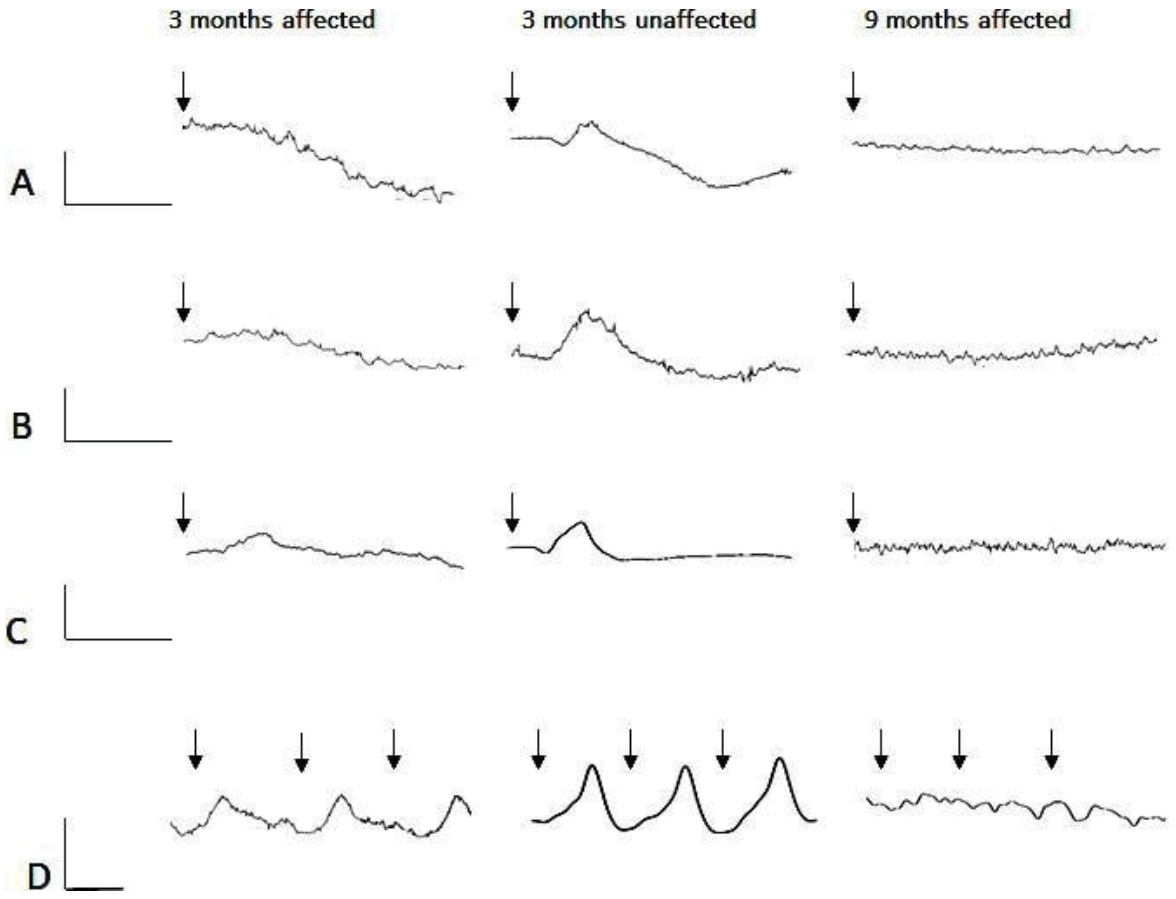

Figure 3. Representative standard ERGs recorded from normal and affected German Spitzes. In A the dogs were dark-adapted for 20 min and stimulated with a flash of -2 log cds/m^2 (rod-driven response); In B the dogs were stimulated with a flash of 0.47 log cds/m^2 (combined rod-cone driven response); In C the animals were light-adapted for 10 min and stimulated with a higher intensity flash of 1 log cds/m^2. In D the dogs were stimulated with a 30 Hz flicker with the intensity of 1 log cds/m^2. Note the absence of a scotopicERG response in the affected dogs in all ages. A very decreased response compared to unaffected controls was obtained during the cone driven and flicker responses, which became completely extinguished by 9 months of age. Size bars: vertical = 100 IV (a-d); horizontal = 50 ms (a-c) and 20 ms (d). Arrows = stimulus flash.

2.4.6 Optical Coherence Tomography (OCT)

OCT imaging of the retina of 4 dogs one 4 month old, two 7,5 months

old, and one 16 months old (animals #13, #8, #9 and #7) detailed the presence of multiple retinal bullae in tapetal fundus. OCT imaging clearly shows a separation between the photoreceptors and the retinal pigment epithelium (Figure 4). At approximately 24 months of age, uneven areas of advanced retinal degeneration were noticed. The dorsal aspect of the retina was more atrophied than the ventral areas of fundus.

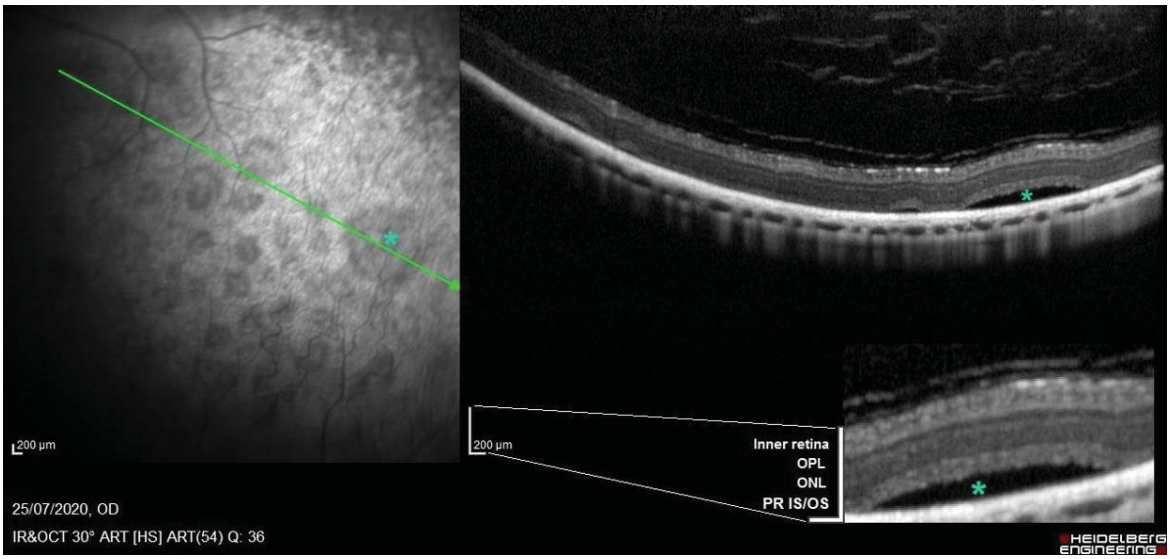

Figure 4. A cSLO infrared image of the tapetal region showing the multiple bullae in the central area of the tapetal fundus in a female affected German Spitz. B. A SD-OCT image across the largest bulla indicated in A with a blue asterisk. In this area, separation between the photoreceptors and the retinal pigment epithelium can be seen

OCT´s full retinal thickness comparison from two age-matched dogs (36

months) both central and peripheral retina revealed decrease in retinal thickness values in affected dogs in the advanced stage of the retinal degeneration (Figure 5).

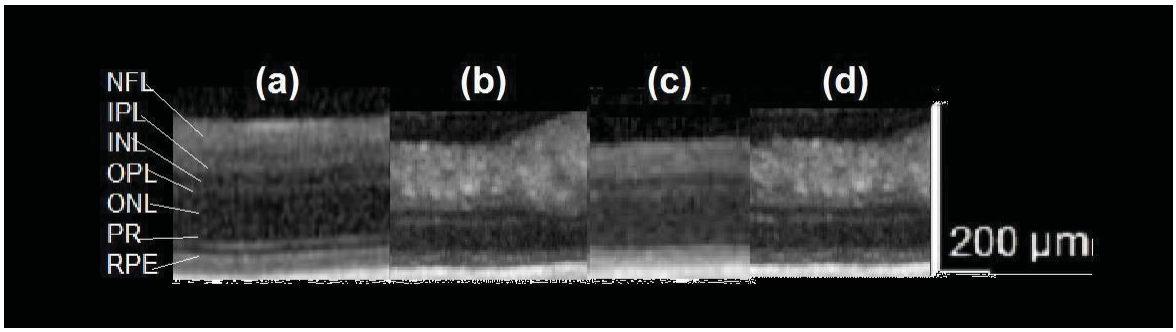

Figure 5. Distinction between total central retinal thickness of the clinically unaffected (#31; a) and affected 36 month-old dog (#3, b) is evident and accentuated in the outer nuclear layer. The same distinction is possible to notice between the peripheral retina of an affected dog (#3, d) and unaffected (#31, c).

2.4.5 Chromatic pupillary light reflex test

In all affected animals from 1.5 to 22 months, a lack of a pupil response after red light illumination, with normal response after blue light illumination was observed (Figure 6). After this age, dogs had a slower and weaker pupillary response after blue light illumination. However, it was still present in the oldest dog examined submitted to this test (36 months).

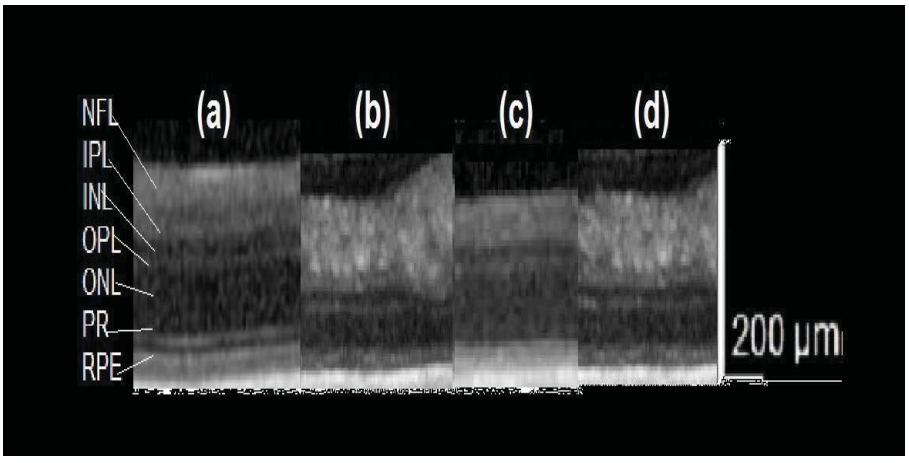

Figure 6. cPLR is an affected dog at 1,5 months old. A. Lack of a pupil

response after red light (630 nm). B. Normal pupil response to 480 nm).

2.4.6 Breeding analysis

Results of the analysis of the pedigree (Figure 7) suggests an autosomal recessive mode of inheritance for the disease. Considering all families analyzed, equivalent numbers of affected males and females have been identified with the disease, and affected offspring were produced from the mating of two clinically normal dogs.

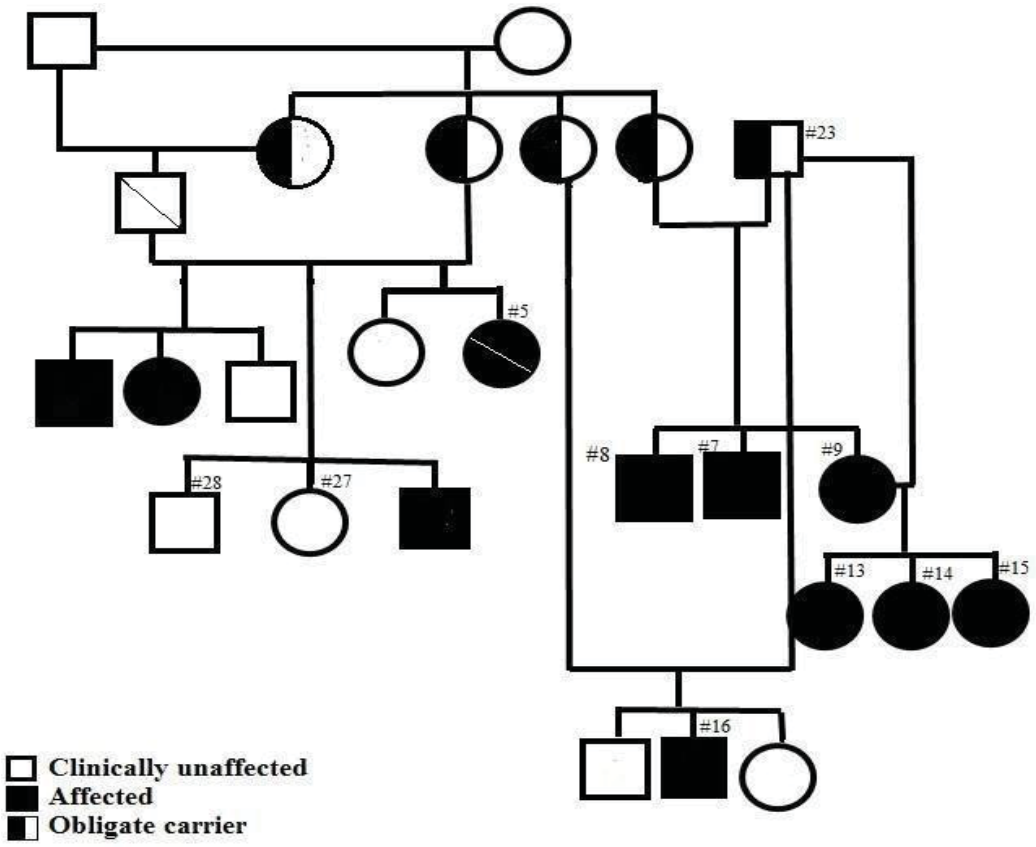

Figure 7. Pedigree of one of the German Spitz families investigated in which PRA was segregating, suggesting an autosomal recessive inheritance mode. All dogs classified as clinically unaffected (carriers) and affected were examined at least by indirect ophthalmoscopy and had ERGs performed. The pedigree suggests an autosomal recessive mode of inheritance with both males and females being affected, and in this lineage, an affected dog is produced from the mating of two phenotypically normal dogs.

2.5 DISCUSSION

Here, a phenotype of an early onset type of inherited progressive retinal degeneration is reported in the German Spitz dogs. Retinal degeneration already has been cited to occur in German Spitz dogs [2]. Kelawala et al. 2017 recognized an important variation in age of onset of PRA in a few dogs of the German Spitz breed, and found that certain families were affected early with a more rapid progression of disease, compared to other dogs that developed the inherited retinal disorder at later time [15]. However, the condition was not further characterized in this breed. The German Spitz breed can be genetically tested for one mutation of the PRCD gene that accounts for PRA and it is available commercially (https://www.wisdompanel.com/en-us). It was reported by Miyadera[1] that early-onset PRA/CRD manifests during the postnatal retinal differentiation period, between 2 and 6 weeks, and results from abnormal or interrupted retinal development or progressive degeneration occurring during or immediately after retinogenesis. They also observed that the early onset of these diseases typically leads to a relatively rapid progression toward late-stage retinal degeneration and can be clinically evident in young dogs.

Nystagmus has been cited in other forms of retinal degenerations [13, 24] and seems to be a common clinical feature in canine early-onset retinal degenerations [22,23,32].

Progressive bilateral hyper-reflectivity, hypopigmentation of non tapetum and attenuation of retinal arteries are all common signs seen in dogs affected with other forms of PRA[1,2,13,15,16]. All sixteen dogs affected included in this study showed very mild fundoscopy abnormalities during the first months of age, similarly to other early-stage retinal atrophies [13]. More consistent fundus abnormalities started to be observed by the age of 3 months, as vascular attenuation and hyperreflexivity became more evident and more typical of other forms of progressive retinal atrophy [6]. The difference in the retinal sensitivity to blue and red stimuli was suggestive of a photoreceptor dysfunction in humans and animals[19, 29-31]. Vision was

reduced initially (detectable from as early as 1.5 months of age). Three owners noticed this fact, reporting that the affected dogs used to sit and “look up”, while unaffected dogs would usually “look down” and investigate their surroundings. A few other owners reported that non-affected animals have a tendency to be more active and explore their surroundings. Other owners did not usually notice it until the dogs were four to even 6 months of age. This is consistent with a reduction of photoreceptor function, which was also detected in the ERG of the affected dogs, especially rod function. It appears that rod function is compromised earlier than cone function in this phenotype, since a discrete response was still recordable in light-adapted-affected animals stimulated with a higher intensity flash and 30 Hz flicker (cone driven response). This feature is a consequence of an early degeneration and loss of the rods photoreceptor followed by the loss of cones of the retina observed in the majority of the retinal degeneration phenotypes [6,8]. Reduced photoreceptor function was detected by ERG before fundus changes developed[11].

Multifocal retinal bullae had developed in six PRA affected animals at 7 months of age and were not detected in older dogs with end-stage retinal atrophy. A similar feature has been observed in Whippet dogs affected with another early onset inherited retinopathy [13]. Some of the dogs had not been examined ophthalmoscopically at a younger age. Thus, it was not possible to tell whether similar lesions had been present. At about 2 years of age, a considerably more advanced degeneration in the dorsal area compared to the ventral fundus could be observed in oppose to what was found in Papillon dogs[11].

Pedigree analysis suggests an autosomal recessive mode of inheritance. However, establishment of a breeding colony wouldallow confirmation of the suspected mode of inheritance, as well provide subjects for further electrophysiologic and histopathological characterization.

The identification of mutations that cause PRA in unique dog

breeds allows the development of DNA tests as a tool for breeders to use to eradicate known PRA mutations[23-26,] , . Those studies are an invaluable source of possible therapeutic treatment for the affected dog population and for the equivalent human disease. This study suggests a possible new variation of the PRA in German Spitzes and proposes more studies to elucidate the changes seen in these individuals.

2.6 Acknowledgments

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UNIVERSIDADE FEDERAL DO PARANÁ
SETOR DE CIÊNCIAS AGRÁRIAS
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

CERTIFICADO

Certificamos que o protocolo número 044/2019, referente ao projeto **"O efeito ocular da solução oftálmica de canabidiol a 0,2% em cães da raça Beagle",** sob a responsabilidade **Fabiano Montiani Ferreira –** que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino – encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de Outubro, de 2008, do Decreto nº 6.899, de 15 de julho de 2009,e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) DO SETOR DE CIÊNCIAS AGRÁRIAS DA UNIVERSIDADE FEDERAL DO PARANÁ - BRASIL, com grau 1 de invasividade, em reunião de 07/08/2019.

Vigência do projeto	Agosto/2019 até Agosto/2019
Espécie/Linhagem	*Canis lupus familiaris* (canino)/Beagle
Número de animais	14
Peso/Idade	12,8 Kg/4 anos
Sexo	Macho e fêmea
Origem	Laboratório de Estudos em Nutrição Canina da Universidade Federal do Paraná, Curitiba/PR, Brasil.

CERTIFICATE

We certify that the protocol number 044/2019, regarding the project **"The ocular effect of 0,2% cannabidiol ophthalmic solution in beagle dogs"** under **Fabiano Montiani Ferreira** supervision – which includes the production, maintenance and/or utilization of animals from Chordata phylum, Vertebrata subphylum (except Humans), for scientific or teaching purposes – is in accordance with the precepts of Law nº 11.794, of 8 October, 2008, of Decree nº 6.899, of 15 July, 2009, and with the edited rules from Conselho Nacional de Controle da Experimentação Animal (CONCEA), and it was approved by the ANIMAL USE ETHICS COMMITTEE OF THE AGRICULTURAL SCIENCES CAMPUS OF THE UNIVERSIDADE FEDERAL DO PARANÁ (Federal University of the State of Paraná, Brazil), with degree 1 of invasiveness, in session of 07/08/2019.

Duration of the project	August/2019 until August/2019
Specie/Line	*Canis lupus familiaris* (canine)/Beagle
Number of animals	14
Wheight/Age	12.8 Kg/4 years
Sex	Male and female
Origin	Canine Nutrition Studies Laboratory of the Federal University of Parana, Curitiba/PR, Brazil.

Curitiba, 07 de agosto de 2019

Chayane da Rocha

Coordenadora CEUA-SCA

UNIVERSIDADE FEDERAL DO PARANÁ
SETOR DE CIÊNCIAS AGRÁRIAS
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

CERTIFICADO

Certificamos que o protocolo número 084/2019, referente à pesquisa **"Caracterização clínica e genética de uma nova forma de atrofia progressiva de retina em cães da raça spitz alemão"**, sob a responsabilidade de **Fabiano Montiani-Ferreira** – que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino – encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de Outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) DO SETOR DE CIÊNCIAS AGRÁRIAS DA UNIVERSIDADE FEDERAL DO PARANÁ - BRASIL, com grau 2 de invasividade, em 11/12/2019.

Finalidade	Pesquisa científica
Vigência da autorização	Janeiro/2020 até Julho/2020
Espécie/Linhagem	*Canis familiaris* (canino)
Número de animais	10
Peso/Idade	2 – 30 kg/1 – 3 anos
Sexo	Macho e fêmea
Origem	Sob tutela.

CERTIFICATE

We certify that the protocol number 084/2019, regarding the research **"Clinical and genetic characterization of a novel form of progressive retinal atrophy in German spitz dogs"** under **Fabiano Montiani-Ferreira** supervision – which includes the production, maintenance and/or utilization of animals from Chordata phylum, Vertebrata subphylum (except Humans), for scientific or teaching purposes – is in accordance with the precepts of Law nº 11.794, of 8 October 2008, of Decree nº 6.899, of 15 July 2009, and with the edited rules from Conselho Nacional de Controle da Experimentação Animal (CONCEA), and it was approved by the ANIMAL USE ETHICS COMMITTEE OF THE AGRICULTURAL SCIENCES CAMPUS OF THE UNIVERSIDADE FEDERAL DO PARANÁ (Federal University of Paraná, Brazil), with degree 2 of invasiveness, in session of 11/12/2019.

Puporse	Cientific research
Validity	January/2020 until July/2020
Specie/Line	*Canis familiaris* (canine)
Number of animals	10
Wheight/Age	2 – 30 kg/1 – 3 years
Sex	Male and female
Origin	Under guardianship.

Curitiba, 06 de janeiro de 2020

Chayane da Rocha
Coordenadora CEUA-SCA