




Canine *POMC* deletion (P187fs) allele frequency in Labrador Retrievers in Brazil¹

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The Labrador Retriever is among the main breeds with the greatest predisposition to obesity. Several factors, especially the interrelationships between food management, exercise and social factors; influence the likelihood of a dog becoming obese. Furthermore, genetic factors are also responsible for obesity in dogs, and in Labrador Retriever, a frameshift mutation (P187fs) in *pro-opiomelanocortin (POMC)* gene is strongly associated with obesity. There is no knowledge of studies that have previously evaluated the prevalence of the canine *POMC* deletion (P187fs) in Brazilian Labrador Retriever. Therefore, the objective of this study was to investigate this mutation in Labrador Retriever dogs in Brazil. Of the 108 Labrador Retrievers that were assessed in this study, 59 were from a previous study, composed by animals assisted in a veterinary hospital with unknown lineage, and 49 were from a prospective study, composed of 19 pet and 30 assistance/rescue Labrador Retriever dogs. The obesity risk and appetite questionnaire were applied, with some modifications, to tutors of the animals used in the prospective study. Fragments of the DNA, containing the mutation, were amplified by PCR and submitted to direct gene sequencing. The allele frequency of the mutation was 21.3% and was out of Hardy-Weinberg equilibrium ($P < 0.05$). Using only the data of animals with known lineage, the presence of the mutated allele was higher in the Assistance/rescue Group than Pet Group ($P < 0.01$), furthermore, the allele frequencies observed in Assistance Group (31.7%) was out of Hardy-Weinberg equilibrium ($P < 0.05$), while that in the Pet Group (18.4%) was in equilibrium ($P > 0.05$). Although the mutation has increased the food-motivation in the assistance/rescue dogs, other variables, especially frequent exercising, favored that these animals maintained the ideal body weight (body condition score = 5). In summary, the Hardy-Weinberg disequilibrium observed in the allele distribution of the deletion *POMC_P187fs* in this study, independently of the Labrador Retriever group assessed, suggesting the possibility of positive selection of the mutated allele, which may lead to the maintenance of this deleterious allele in the studied population.

INDEX TERMS: Canine *POMC*, P187fs, allele frequency, Labrador Retriever, Brazil, gene, mutation, dogs, obesity, prevalence.

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RESUMO.- [Frequência de alelos mutados (P187fs) no *POMC* de cães Labrador Retriever no Brasil.] O Labrador Retriever é uma das principais raças caninas com maior predisposição à obesidade. Vários fatores, especialmente as interações entre a alimentação, exercício e fatores sociais,

influenciam a probabilidade de um cão se tornar obeso. Além disso, fatores genéticos são também responsáveis pela obesidade em cães, e no Labrador Retriever a mutação “frameshift” P187fs no gene pró-opiomelanocortina (*POMC*) está fortemente associada à obesidade. Não existem estudos prévios de prevalência da deleção P187fs no gene *POMC* em cães Labrador Retriever no Brasil. Portanto, o objetivo deste estudo foi investigar esta mutação em cães da raça Labrador Retriever no Brasil. Dos 108 Labradore Retrievers avaliados neste estudo, 59 eram de um estudo retrospectivo (composto por animais atendido no hospital veterinário e sem linhagem conhecida) e 49 eram de um estudo prospectivo (composto por 19 cães pet e 30 cães de assistência/resgate). Um questionário de risco de obesidade modificado foi aplicado nos tutores dos animais usados no estudo prospectivo. Fragmentos de DNA, contendo a mutação, foram amplificados por PCR e submetidos ao sequenciamento gênico direto. A frequência alélica da mutação foi de 21,3% e estava fora do equilíbrio de Hardy-Weinberg ($P < 0,05$). Usando somente os dados dos animais de linhagem conhecida, a presença do alelo mutado foi maior no Grupo de cães de Assistência/resgate que no Grupo de Pets ($P < 0,01$), além disso, as frequências alélicas nos Grupos de Assistência/resgate (31,7%) e no de pets (18,4%) estavam fora e em equilíbrio de Hardy-Weinberg ($P < 0,05$), respectivamente. Embora a mutação tenha aumentado a motivação pelo alimento em cães Labrador Retriever do Grupo de Assistência/resgate, outras variáveis, especialmente o frequente exercício, favoreceu a manutenção o peso corporal ideal (peso corporal = 5). Em resumo, o desequilíbrio de Hardy-Weinberg observado na distribuição do alelo *POMC*_P187fs observado neste estudo, independentemente do grupo de Labrador Retriever avaliado, sugere a possibilidade de uma seleção positiva para o alelo mutado, o qual poderá levar a manutenção desse alelo deletério nesta população.

TERMOS DE INDEXAÇÃO: Alelos mutados, *POMC*, caninos, P187fs, Labrador Retriever, Brasil, gene, mutação, cães, obesidade, prevalência.

INTRODUCTION

Obesity or excessive overweight is characterized by the accumulation of excessive amounts of adipose tissue in the body and is the most common nutritional disorder in companion animals (German 2006). As in humans, several disorders may be due to obesity in dogs, especially, metabolic and endocrine abnormalities, orthopedic disease, cardiorespiratory disease (Pöppel & González 2005, German 2006, Lund et al. 2006, Clark & Hoenig 2016, Frye et al. 2016). Obesity in dogs is affected by the interrelationships between food management, exercise and social factors (Bland et al. 2009, Aptekmann et al. 2014, Debastiani 2018). The higher prevalence of canine obesity seen in recent years may be also influenced by the increase in human obesity (Raffan et al. 2016).

Furthermore, genetic factors are also responsible for obesity in dogs (Skorczyk et al. 2007, Grzes et al. 2011, Skorczyk et al. 2011, Mankowska et al. 2015, Miyabe et al. 2015) and specifically in Labrador Retriever a frameshift mutation (P187fs) in *pro-opiomelanocortin* (*POMC*) gene is strongly associated with obesity (Raffan et al. 2016). This mutation provokes a 14bp deletion in the *POMC* gene, which causes loss of production of β -MSH (melanocyte-stimulating hormone) and β -endorphin (Raffan et al. 2016). Although, the

mechanism by which reduced β -MSH and β -endorphin due to the mutation causes behavioral and weight phenotypes remains to be precisely elucidated, Raffan et al. (2016) observed that *POMC*_P187fs mutation is associated with increased body weight (per allele effect of 0.33 SD), adiposity, and greater food motivation. The *POMC*_P187fs mutation was found closely related to Flat-Coated Retriever, however, it was absent from dogs of 38 other diverse breeds (Raffan et al. 2016).

Obesity is the second major primary disorder observed in Labrador Retrievers in the United Kingdom (McGreevy et al. 2018). As in other countries (Payan-Carreira et al. 2015, McGreevy et al. 2018), the Labrador Retriever is among the main breeds with the greatest predisposition to obesity in Brazil (Braos et al. 2009, Aptekmann et al. 2014, Debastiani 2018). In addition, there is no knowledge of studies that have previously evaluated the prevalence of the canine *POMC* deletion (P187fs) in Brazilian Labrador Retriever. Therefore, the objective of this study was to investigate this mutation in Labrador Retriever dogs in Brazil.

MATERIALS AND METHODS

Ethics statement. All procedures were approved by the Board of Ethics and Animal Experimentation of the institution (Protocol no. 220/2016 - CEUA).

Experimental animals and samples. For the present molecular study, blood or buccal swab was randomly collected from 108 Labrador Retrievers mainly from states of São Paulo (89/108), followed by Santa Catarina (13/108), Minas Gerais (3/108), and Pernambuco (3/108), based on informed consent. Labrador Retriever cohort was composed of 69 females (64%) and 39 males (36%), with ages ranging from ten months to 15 years (average age of 6.6 years old). Of the 108 dogs assessed in this study, 59 were Labrador Retrievers that had been submitted to the Veterinary Hospital at FMVZ-Unesp, Botucatu, for medical examination, and blood samples from these dogs were used for genomic DNA purification. The prospective study used the remaining 49 Labrador Retrievers, where a buccal swab was collected from these animals and the Dog Obesity Risk and Appetite (DORA) questionnaire was applied, with some modifications, to tutors of these animals (Raffan et al. 2015).

According to age, the animals were divided into three groups: young animals (age under 5 years), adult animals (age ranging from 5 to 10 years), and old animals (age over 10 years). The body condition score (BCS) of all dogs was attributed by veterinary practitioners using previously validated standard chart of the WSAVA (World Small Animals Veterinary Association) (Laflamme 1997, Kealy et al. 2002, Mawby et al. 2004, German et al. 2010, Jeusette et al. 2010), who had representative images and accompanying descriptors. The dogs were classified on a scale of 1-9 (1 to 3-BCS under ideal; 4 to 5-BCS ideal; and 7 to 9-BCS over ideal), and the animals with BCS of 7 to 9 were considered obese.

DNA purification and genotyping analysis. Genomic DNA was purified from blood or buccal swab samples using the GenElute™ Genomic Blood DNA Kit (Sigma-Aldrich®) according to the manufacturer’s instructions. The DNA obtained was used to genotype the deletion (P187fs) in the *POMC* gene previously associated with obesity in Labrador Retrievers (Raffan et al. 2016). Two specific primers (JPPOMC-F3 5'-AAGCCGGTGGGCAAGAA-3', and JPPOMC-R3 5'-GGAAGTGCTCCATCTTGTAGGG-3') were designed with Primer Express software (Applied Biosystems, Grand Island, NY) to genotype the *POMC*_P187fs (244-bp). The specificity of the PCR primers was evaluated in silico with the Basic Local Alignment

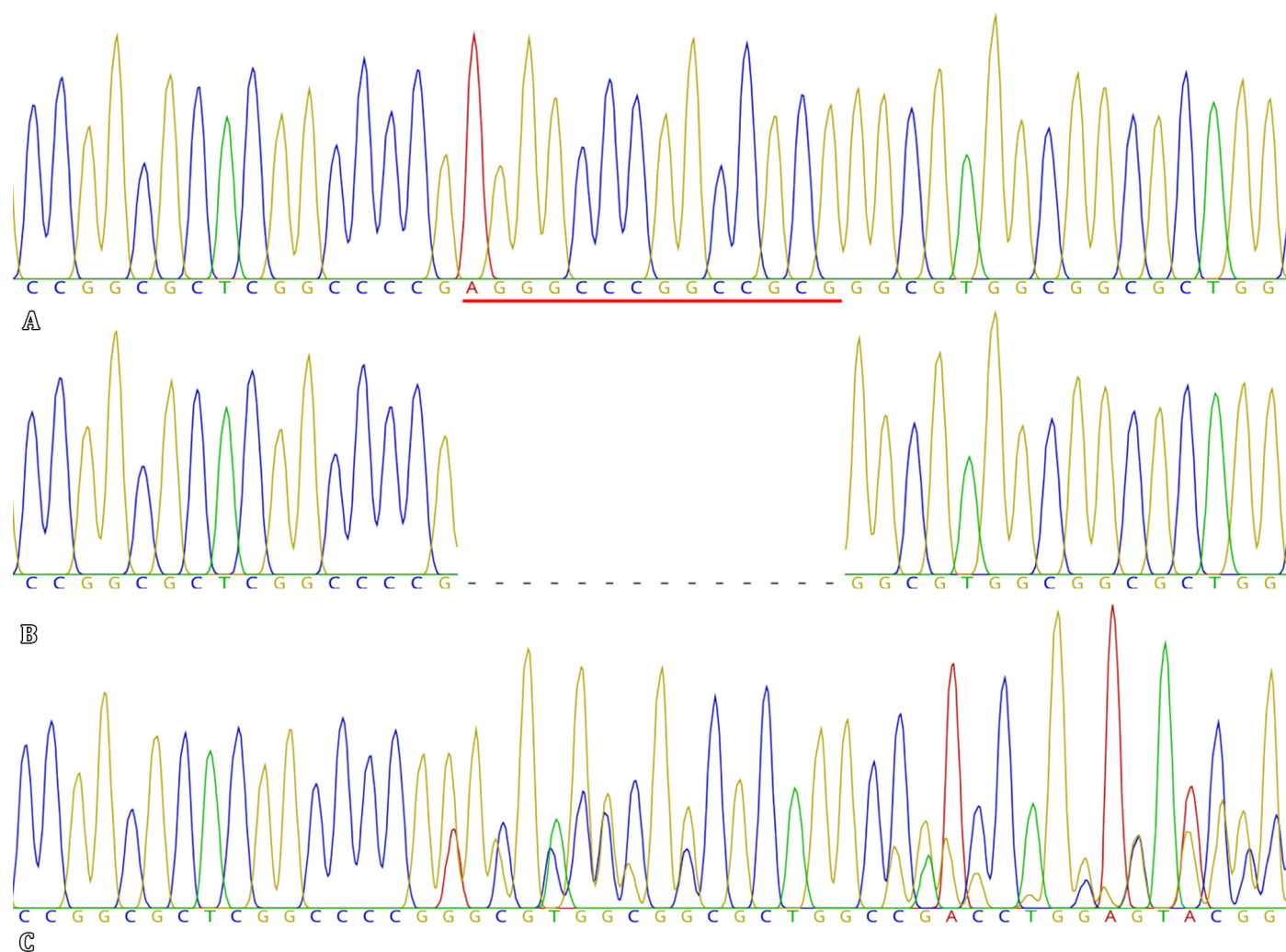


Fig.1 Partial chromatogram showing (A) capillary sequencing results from wild-type, (B) homozygous deletion, and (C) heterozygous Labrador Retriever dogs. (A) It is possible to observe the 14bp (AGGGCCCCGCCGCG) of the deletion *POMC*_P187fs (underlined in red), while at (B) these 14 bases are absent. (C) The double peaks may be observed from the beginning of the deletion *POMC*_P187fs, i.e., the 14bp are only deleted in one allele. Image obtained in the Geneious® 10.0.9 software (Biomatters Ltd, Auckland, New Zealand).

Search Tool (National Center for Biotechnology Information, USA). Polymerase chain reactions were performed in a total volume of 25µL, which contained 2.5µL of template DNA, 0.3µM each forward and reverse primer, 1.25µL of DMSO (5% v/v), 12.5µL of GoTaq® Green PCR Master Mix (Promega), and 7.5µL of nuclease-free water q.s.p. In addition, a no-template control reaction was performed to check for the possible presence of contamination in the PCR preparations. The amplification conditions were as follows: initial denaturation at 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 63.5°C for 1 minute, and extension at 72°C for 1 minute, and final extension at 72°C for 5 minutes. Amplicons were analysed by 1.5% agarose gel electrophoresis, purified, and subjected to direct sequencing. The obtained PCR products were analyzed via 1.5% agarose gel electrophoresis (Invitrogen™, Carlsbad, CA), stained with the Sybr® Safe DNA Gel Stain (Invitrogen™), purified using the QIAquick® PCR Purification Kit (Qiagen®), and then subjected to direct sequencing.

Sequencing analysis. To sequence the DNA, 10µL of each PCR product, 5µL of the purified forward primer and BigDye® Terminator

Cycle Sequencing Kit were used (Life Technologies™ CA, USA). The sequences were determined using the ABI 3500 Genetic Analyzer (Life Technologies™ CA, USA). The obtained sequences and the electropherograms (Fig.1) were analyzed using Geneious® 10.0.9 (Biomatters Ltd, Auckland, New Zealand). The sequences were compared with the *Canis lupus familiaris POMC* sequence (XM_022404519.1), deposited in GenBank™, using BLAST¹.

Data analysis. Chi-square or Fisher tests were used to compare the proportion of obesity and genotype versus age, gender, and coat colour. The prospective of Assistance/rescue Group and Pets Group Labrador Retrievers were compared using Chi-square or Fisher tests, in order to analyse proportion of obesity and genotype versus exercise frequency, type of food (house feeding or commercial dog food), and behaviour features (excitement, unremitting interest, snack training, speed of intake, and selective behaviour). Statistical analyses were conducted using GraphPad Prism® version 7 for Windows and

¹ Basic Local Alignment Search Tool. Available at <<http://blast.ncbi.nlm.nih.gov/Blast.cgi>>

the level of statistical significance was set at 0.05. The genotypic frequency was estimated and the data were analyzed descriptively. The chi-square test was also used to test whether alleles were in Hardy-Weinberg equilibrium within different groups.

RESULTS AND DISCUSSION

The samples used in the previous study (n=59) belonged to the DNA bank of the Biomol Laboratory of the Department of Veterinary Clinical Science of the FMVZ-Unesp and were collected during 2015 and 2016, while the samples used in the prospective study (n=49) were collected during 2017 and 2018. Of the 108 Labrador Retrievers tested, 16 dogs (15%) were identified as homozygous for deletion *POMC_P187fs*, 14 dogs (13%) were identified as heterozygous, and 78 (72%) were homozygous for the wild-type (WT) allele (Table 1). Therewith, in the present study the allele frequency of the mutant allele (21.3%) was higher than that observed in UK and USA (12%; 2% homozygous for deletion, 20% heterozygous, and 78% WT) (Raffan et al. 2016). Differently from what was observed in these two countries (Raffan et al. 2016), the allelic distribution of the deletion *POMC_P187fs* was out of Hardy-Weinberg equilibrium in the population evaluated in the present study ($P < 0.01$). Furthermore, when analysing separately the groups of animals of the previous study (composed of 59 Labrador Retrievers of unknown lineage.) and prospective (composed of 19 pet and 30 assistance/rescue Labrador Retriever dogs) were observed frequencies of 19.5% and 23.5%, respectively, and both frequencies were out of Hardy-Weinberg equilibrium ($P < 0.01$). However, using only the data of animals with known lineage, the presence of the mutated allele was statistically higher ($P = 0.0423$) in the assistance/rescue dogs than pets dogs, furthermore, the allele frequencies observed in this group (31.7%) was out of Hardy-Weinberg equilibrium ($P < 0.01$), while the allele frequencies (18.4%) observed in the Pet Group was in equilibrium ($P = 0.122$). Therefore, these results were similar to those of Raffan et al. (2016), which observed that the mutation was significantly more common in Labrador Retrievers selected to become assistance dogs than pets (allele frequencies, 45% vs. 12%), and the allelic distribution of the deletion *POMC_P187fs* in the assistance dogs was out of Hardy-Weinberg equilibrium, while in the Pet Group was in equilibrium. These authors believed that temperament and trainability are the main drivers for selection of assistance dogs, and positive reinforcement with food reward is a mainstay of puppy training, and with this, dogs carrying the *POMC* deletion may be more likely to be selected as assistance dogs.

Of the 69 females evaluated, nine (13%) were identified as homozygous for deletion *POMC_P187fs*, nine (13%) were identified as heterozygous for the deletion *POMC_P187fs*, and 51 (74%) were homozygous for the wild-type (WT) allele. While of the 39 males evaluated, seven (18%) were identified as homozygous for deletion *POMC_P187fs*, five (13%) were identified as heterozygous for the deletion *POMC_P187fs*, and 27 (69%) were homozygous for the WT allele. According to age, 29 (27%) Labrador Retrievers were young, 60 (55%) were adult, and 19 (18%) were old. The allele mutated distribution in this age group was as follows: young dogs were 4% homozygous for deletion, 17% heterozygous, and 79% WT; adult dogs were 23% homozygous for deletion, 12% heterozygous, and 65% WT; and old dogs were 5% homozygous for deletion, 11% heterozygous, and 84% WT. In the present study, there was no relationship between gender ($P = 0.7861$) or age ($P = 0.0886$) with the genotype of the animals. This was expected since the deletion *POMC_P187fs* is an autosomal mutation, not related to sex chromosomes and age of the animals (Raffan et al. 2016).

In the present study, more obese females (49%, 34/69) were observed than obese male (36%, 14/39), however, there was no statistical difference ($P = 0.2297$) in the proportion of obese and lean Labrador Retrievers between males (14 obese and 25 leans) and females (34 obese and 35 leans). On the other hand, obesity was statistically more frequent in old animals than in young and adult animals ($P = 0.0408$). Although, the correlation between age or gender with canine obesity is well established, being females and old dogs more susceptible to obesity (Braos et al. 2009, Aptekmann et al. 2014, Debastiani 2018), there are other variables that may increase the risk of a dog being obese.

Forty-seven percent of dogs assessed in the present study were yellow (51/108), 29% black (31/108), and 24% chocolate/liver (26/108). Although chocolate Labrador Retrievers had a significantly shorter lifespan than non-chocolate Labrador Retrievers (McGreevy et al. 2018), in the present study, no relationship was observed between the coat colour and obesity ($P = 0.8949$) or genotype ($P = 0.0682$).

In order to verify the relation between of the deletion *POMC_P187fs* and obesity versus other variables, the DORA questionnaire (Raffan et al. 2015) was applied, with some modifications, to tutors of the dogs of the prospective study. As previously stated in the present study, the presence of the mutated allele was statistically higher ($P = 0.0423$) in the assistance/rescue dogs than pets Labrador Retrievers, nevertheless, the dogs of the assistance/rescue lineage (seven obese and 23 non-obese) were statistically lean ($BCS \leq 5$) than

Table 1. Genotype prevalence, allele frequency, and Hardy-Weinberg analysis of the deletion *POMC_P187fs* in Labrador Retrievers dogs

Group	Genotype			Allele frequency	Hardy-Weinberg equilibrium ^a
	Wild-type	Heterozygous for deletion	Homozygous for deletion		
Total	72% (78/108)	13% (14/108)	15% (16/108)	21.3%	Out P = 0.0000007
Retrospective	76% (45/59)	9% (5/59)	15% (9/59)	19.5%	Out P = 0.00000004
Prospective	67% (33/49)	19% (9/49)	14% (7/49)	23.5%	Out P = 0.00002
Pet	69% (13/19)	26% (5/19)	5% (1/19)	18.4%	In P = 0.12230
Assistance/rescue	60% (18/30)	17% (5/30)	23% (7/30)	31.7%	Out P = 0.000000007

^a Chi-square test, if $P < 0.05$, not consistent with Hardy-Weinberg equilibrium.

the pets (ten obese and nine non-obese) ($P=0.0062$). Raffan et al. (2016), observed the positive influence of the *POMC* deletion with higher body weight ($p<0.0001$; mean effect size 1.9kg per deletion allele), and that assistance/rescue dogs were more affected by it. The controversial findings observed in the present study may be explained according to other aspects observed in the assistance/rescue dogs, i.e., these dogs were submitted to a greater frequency of exercise (three or more times a week) than the pets ($P=0.0021$), and the supply of human food only occurred in the Pet Group ($P=0.0002$). On the other hand, assistance/rescue dogs, influenced by the genotype (Raffan et al. 2016), were more excited ($P=0.0183$) and had an incessant interest in the food ($P=0.0035$); and received more snacks ($P=0.0378$) than pets. It is known that obesity in dogs is influenced by the interrelationship between food management, exercise and social factors (Bland et al. 2009, Aptekmann et al. 2014, Debastiani 2018). However, the higher frequency of exercise and the single supply of animal feed overrides the food-motivation observed in the Assistance/rescue Group, making them less obese.

CONCLUSIONS

In summary, the present study showed the existence of the deletion *POMC*_P187fs in Labrador Retriever in Brazil.

The Hardy-Weinberg disequilibrium observed in the allelic distribution of the deletion *POMC*_P187fs in this study, independently of the Labrador Retriever group assessed, suggesting the possibility of positive selection of the mutated allele, which may lead to the maintenance of this deleterious allele in this population.

The presence of the mutated allele was significantly more common in Labrador Retrievers selected to become assistance/rescue dogs. However, although the mutation has increased the food-motivation in these group of the Labrador Retrievers, other variables, especially the frequent exercise, favored that these animals maintained the ideal body weight ($BCS=5$).

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