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ABSTRACT

This study aimed to verify the body condition parameters that can be used to characterise possible cases of neglect in dogs with visceral leishmaniasis. Fifty dogs were used in the study. The control group contained 11 dogs. Of the 39 infected dogs, 25 and 14 were included in the multisymptomatic and oligo/asymptomatic groups, respectively. The parameters evaluated included body score, body mass index, bone marrow fat percentage (Soxhlet method), bone marrow supernatant fat content (mm in a 15-ml tube), and visceral fat content. We observed that most euthanised dogs with canine leishmaniasis were multisymptomatic, implying that they were unwell and had a low body condition score. This condition is associated with low bone marrow fat content and maintenance of visceral fat reserves. Therefore, the assessment of body score and bone marrow fat content associated with visceral fat content can be used as evidence of neglect in dogs with visceral leishmaniasis.

1. Introduction

Canine leishmaniasis (CanL) is an anthropozoonosis that affects humans and domestic and wild animals. A related species, *Leishmania donovani* complex, comprises *L. donovani* and *L. infantum* (*chagasi*) [1]. They are transmitted primarily by sandflies of the genus *Lutzomyia*, and dogs are the main reservoirs of the parasite in urban areas [2–4].

In Brazil, the Ministry of Health mandates that all dogs with a positive visceral leishmaniasis (CanL) diagnosis must be euthanised [5]. Furthermore, the treatment of dogs with CanL using drugs for human use or drugs not registered within the Ministry of Agriculture is not allowed [6]. Recently, miltefosine was licenced for the treatment of VL in dogs [7]. Euthanasia of dogs with VL is controversial and is frequently not accepted by owners [8,9]. This problem has been exacerbated by the high cost of miltefosine treatment. For this reason, these dogs are often maintained by the owners without any treatment until they present precarious health body conditions, characterised by disseminated lesions and severe body wasting. Subsequently, they are sent to a zoonosis control centre for euthanasia. Keeping a sick animal without a specific recommended treatment is considered abusive and is an environmental crime in Brazil [10].

In many cases of suspected neglect, animals are emaciated, i.e., they demonstrate a severe and diffuse loss of fat and skeletal muscle mass [11,12], which may result from two chronic pathophysiological mechanisms: cachexia and starvation. Cachexia is a multifactorial syndrome that leads to the loss of body weight and loss of muscle mass and fat and is associated with increased protein catabolism attributed to underlying disease(s) [13]. Starvation can be attributed to the prolonged deprivation of food and its morbid effects. Starvation can be reversed with the resumption of food consumption, whereas in cachexia, wasting does not respond well to food consumption [14].

Progressive weight loss in leishmaniasis can be attributed to

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cachexia. The catabolic nature of the disease leads to the atrophy of myocytes and adipocytes, with consequent release of free fatty acids. As the disease progresses, food consumption also decreases, resulting in weight loss, which is exacerbated in dogs whose owners have low incomes and who generally do not provide food with an adequate nutritional level [15–18]. Therefore, with the aim of characterising possible cases of neglect in dogs with VL, we evaluated body condition parameters including body score, body mass index, visceral fat content, and bone marrow fat content in dogs with VL.

2. Materials and methods

2.1. Ethical approval

This study was approved by the Animal Ethics and Experimentation Committee (Protocol FOA 01084–2015).

2.2. Animals

Fifty (33 male and 17 female) mixed-breed adult dogs aged one to five years were obtained from the Zoonosis Control Centre of Araçatuba, São Paulo, Brazil. Samples from all dogs in this study were subjected to enzyme-linked immunosorbent assay (ELISA) for anti-*Leishmania* antibody detection (cut-off 0.270) and quantitative polymerase chain reaction (qPCR) to determine the DNA and parasite load in spleen samples. To assess the general health status of the dogs clinical staging was defined according to Solano-Gallego et al. [22] using clinical, haematological, biochemical, and serological parameters. Screening for *Ehrlichia canis* and *Babesia* spp. was performed using ELISA [19,20] and indirect immunofluorescence analysis for *Toxoplasma gondii* and *Neospora caninum* were performed [21].

The dogs were divided into three groups, namely, control (CONT), oligo/asymptomatic (OLIGO), and multisymptomatic (MULT) groups. The control group included 11 dogs that showed negative ELISA and qPCR results for VL. The 39 dogs with positive ELISA and qPCR results were divided into two groups according to the signs presented. In the OLIGO group, 11 dogs with mild signs of seborrheic dermatitis, lymphadenomegaly, and mild weight loss or absence of signs of the disease were included. For the MULT group, 25 dogs presenting with moderate-to-severe skin lesions and other signs such as onychogryphosis, anaemia, and severe weight loss (generalised muscular loss and/or cachexia) were included.

Blood samples were aseptically collected from the jugular vein in tubes with and without anticoagulants. All dogs in the study were euthanised at the Zoonosis Control Centre of Araçatuba, following the Ministry of Health's CanL Surveillance and Control Manual [23] and Resolution 1000/2012 of the Federal Council of Veterinary Medicine (CFMV), which details the procedures and methods for euthanasia of animals [24].

2.3. Parasite load

DNA extraction from the spleens of dogs was performed with 10 mg of tissue using a commercial DNeasy Blood & Tissue Kit (QIAGEN) according to the manufacturer's recommendations. Parasite load was quantified via real-time qPCR with a final reaction volume of 20 μL using ITS1 primers (forward 5' AGCTGGATCATTTTCCGATG 3' and reverse 5' TATGTGAGCCGTTATCCACGC 3'), PCR Master Mix Power SYBR Green (Applied Biosystems), and 50 ng of the DNA sample. The amplification conditions were as follows: Initial incubation for 12 min at 95 °C, followed by 40 cycles at 95 °C for 15 min, 60 °C for 20 s and 72 °C for 20 s each. The dissociation curve of the amplified fragment was determined at 95 °C for 15 s, 60 °C for 15 s, followed by 20 min until reaching 95 °C for 15 s. In each reaction, a standard curve with *L. infantum* promastigote DNA (MHOM/BR00/MER02) was constructed with a serial dilution of the parasite DNA from 10^7 to 10^1 [25].

2.4. Necroscopic evaluation and sampling

Routine necropsy was performed to collect tissue samples. During necropsy, special attention was paid to changes that could be related to emaciation, such as the amount of visceral fat deposits, muscle mass loss, and stomach content, as suggested by Gerdin et al. [12]. Additionally, gross leishmaniasis-related lesions were observed. Spleen samples were collected, frozen, and stored for qPCR analysis. A pool of bone marrow from both femurs and humeri was collected through longitudinal cuts performed using a band saw.

2.5. Body condition assessment

The body score was calculated using a classification system defined by Royal Canin ™ [26], where a score of one implies a very thin body type, two implies a thin body type, three implies an ideal body type, four implies a fat body type, and five implies a very fat body type. Body mass index was calculated by dividing the animal's weight (kg) by the length of the spine (m). Measurements were obtained from the atlanto-occipital to the sacroiliac joint [27].

2.6. Visual scoring of visceral adipose tissue

All quantifications were performed by three independent observers with no prior knowledge of the clinical data of the dogs. To assess visceral fat reduction, the perirenal, pericardial, and omental fat reserves were evaluated during necropsy by a veterinarian, and the photo-documented images were evaluated by two veterinary pathologists. The amount of visceral fat was classified according to a semi-quantitative score, where (+) implies mild, (++) implies moderate, and (+++) indicates a marked reduction in the amount of adipose tissue (Supplementary figure 1: AB (mild), CD (moderate), EF (marked)). To determine the average score of each group, the average score determined by all three evaluators for each evaluated area/dog was used.

2.7. Quantification of bone marrow fat

Bone marrow samples from both femurs and humerus collected during the microscopic examination were homogenised in a porcelain crucible and stored in Falcon tubes. Two quantitative methods were used to evaluate bone marrow fat. The supernatant fat content was evaluated in 3 g aliquots of bone marrow homogenate, and the aliquots were mixed with 8 ml of water in measuring tubes of 15 ml capacity and then centrifuged at 4000 rpm for 5 min. After this step, the height of the supernatant fat in the tube was measured in mm [28]. Fat percentage was evaluated in 2 g aliquots via the lipid extraction method based on exhaustive leaching using an organic solvent (ethyl ether) in a complete Soxhlet® extractor, followed by solvent removal by evaporation [29–31].

2.8. Statistical analysis

The D'Agostino–Pearson test was used to assess the normality of the data. The difference between the parasite loads of the infected groups was evaluated using the Mann-Whitney test. Differences between groups were determined using analysis of variance (ANOVA) and Tukey's test for data with residuals of normal distribution and homoscedastic variances, as observed in the Bartlett's test. KruskalWallis and Dunn tests were used for data that did not fit these parameters. Categorical data were evaluated using the Fisher's exact test. Statistical significance was set at $P < 0.05$. Spearman's correlation coefficient was used to determine the correlation between parameters. All statistical analyses were performed using Prism software (v8.0.1, GraphPad, La Jolla, CA, USA).

3. Results

3.1. Diagnosis of CanL and co-infections

For the diagnosis of *L. infantum*, the dogs were subjected to ELISA and qPCR. All dogs in the infected groups demonstrated anti-*Leishmania* antibodies (ELISA OD, mean ± standard deviation, MULT 0.94 ± 0.34, OLIGO 0.84 ± 0.48 and CONT 0.008 ± 0.06; cut-off: 0.27). The qPCR for quantifying parasite load showed an efficiency of 0.83, a slope of −3.831, and an R^2 of 0.957. *Leishmania* DNA was detected in the spleens of 35 of the 50 dogs. In 23 dogs from the MULT group and 12 dogs from the OLIGO group, the parasite load varied from 2.54×10^1 to 1.35×10^5 ng of DNA. The average load was 1.69×10^4 (± 3,20 × 10^4) in the MULT group and 1.05×10^4 (± 2.26×10^4) in the OLIGO group, with no significant difference observed between groups.

Screening for other pathogens showed positive results for at least one parasite, in addition to *Leishmania* spp., in all 50 dogs. In the ELISA, anti-*Ehrlichia* antibodies were detected in 80%, anti-*Neospora* antibodies in 76%, and anti-*Babesia* antibodies in 72% of the dogs, regardless of the group evaluated. In the indirect immunofluorescence assay, 42% of dogs had anti-*Toxoplasma* antibodies. Ectoparasitism by fleas and/or ticks was observed in 90% of the dogs (Table 1). Comparison between groups showed no statistical difference in serological positivity for *Ehrlichia* spp., *Babesia* spp., and *Neospora* spp.

By evaluating the clinical staging of CanL [22], we found that 8% (3/39) of the dogs were in stage I of the disease, 67% (26/39) in stage II, 15% (6/39) in stage III, and 10% (4/39) in stage IV. We did not find any correlation between clinical staging and parameters of body condition, bone marrow fat, visceral fat, or serous atrophy.

3.2. Clinical pathology

Regarding clinicopathological changes, the occurrence of thrombocytopenia, hyperproteinaemia, and azotaemia did not differ between groups. Hypoalbuminaemia was present in 88% of dogs in the MULT group (mean = 1.34 g/dL), and it was more frequent than in the CONT (p = 0.0003) and OLIGO (p = 0.0013) groups. In the CONT and OLIGO groups, the mean of hypoalbuminaemia occurrence was 2.08 and 1.76 g/dL, respectively. Likewise, anaemia was more frequently noted in the MULT group than in the CONT (p = 0.0001) and OLIGO (p = 0.0051) groups. Also in the OLIGO group, aenemia was more frequent than in the CONT group (p = 0.0300). These and other findings are presented in Table 2.

As for the severity of anaemia, we saw that in the MULT group, moderate anaemia was the most frequent (54.17%), whereas in the OLIGO group, moderate and severe anaemia presented with the same frequency (42.86%). In the CONT group, only two dogs had anaemia; one of them presented with mild anaemia and the other demonstrated moderate anaemia (Table 3) [32]. Regarding the classification by erythrocyte volume (MCV) and haemoglobin (HCCM), we observed a higher frequency of normochromic normocytic anaemia in dogs in the MULT group (58.33%), whereas hypochromic normocytic anaemia was the most frequent (71.43%) in the OLIGO group. Two dogs with anaemia in the CONT group demonstrated normocytic normochromic anaemia (Table 3) [32].

Table 1
Observation of co-infection and ectoparasitosis in MULTI, OLIGO, and CONT groups.

	GROUPS							
	MULT (n = 25)		OLIGO (n = 14)		CONT (n = 11)		TOTAL (n = 50)	
Aetiological agent	N	%	N	%	N	%	N	%
Toxoplasma spp.	10	40.0	8	32.0	3	27.3	21	42
Neospora spp.	23	92.0	10	40.0	5	45.5	38	76
Ehrlichia spp.	23	92.0	9	36.0	8	72.7	40	80
Babesia spp.	19	76.0	12	48.0	5	45.5	36	72
Tick infestation	22	88.0	13	52.0	10	90.9	45	90
Flea infestation	14	56.0	7	28.0	5	45.5	26	52

Table 2
Laboratory alteration observed in various groups.

	GROUPS							
	MULT (n = 25)		OLIGO (n = 14)		CONT (n = 11)		TOTAL (n = 50)	
Laboratory alteration	N	%	N	%	n	%	N	%
Anaemia (Erythrocytes <5.5 106/μL; haematocrit <37%; haemoglobin <12 g/dL) [32]	24	96.0	8	32.0	2	18.2	34	68
Leucocytosis (>17,000 mm^3)[32]	6	24.0	5	20.0	2	18.2	13	26
Lymphopenia (<1000 mm^3)[32]	13	52.0	5	20.0	4	36.4	22	44
Thrombocytopenia (<200 mm^3)[32]	4	16.0	4	16.0	2	18.2	10	20
Alkaline phosphatase increase (>156 UI/L)[33]	1	4.0	0	0.0	0	0.0	1	2
Alanine aminotransferase increase (>86 UI/L)	2	8.0	0	0.0	0	0.0	2	4
Uraemia (59.92 mg/dL) [34]	10	40.0	4	16.0	1	9.1	15	30
Azotaemia (≥1.4 mg/dL) [34]	6	24.0	6	24.0	2	18.2	14	28
Hyperproteinaemia (>7.1 g/dL)[33]	19	76.0	11	44.0	6	54.5	36	72
Hypoalbuminaemia (<2.6 g/dL)[33]	22	88.0	9	36.0	3	27.3	34	68
Hyperglobulinaemia (>4.4 g/dL)[33]	17	68.0	10	40.0	4	36.4	31	62

3.3. Necroscopic evaluation

Among the macroscopic findings observed in the dogs in this study, we highlight in the MULT, OLIGO and CONT groups respectively, the presence of skin lesions in 96% (24/25), 92.9% (13/14) and 81.8% (9/11), splenomegaly in 84% (21/25), 71.4% (10/14) and 72.2% (8/11), and hepatomegaly in 56% (14/25), 50% (7/14) and 9.1% (1/11) of the dogs. These and other findings from macroscopic examination are presented in Table 4.

In addition, generalized muscle wasting was observed in 56% (14/25) of dogs in the MULT group, in 7.1% (1/14) of the OLIGO group and 18.1% (2/11) of the CONT group. Muscle wasting was statistically more frequent in the MULT group than in the OLIGO (p = 0.026) and CONT (p = 0.0311) groups (Fig. 1A; Table 4).

Temporal muscle mass loss was observed in 48% (12/25) of MULTI, and 9.1% (1/11) of CONT dogs. In the OLIGO group this change was not observed. This sign was significantly more frequent in the dogs of MULT group than in the OLIGO (p = 0.0112) and CONT (p = 0.0173) groups (Fig. 1B; Table 4).

Dog food was most frequently detected gastric content in the MULT and OLIGO groups present in 60% and 71.4% of the stomachs, respectively. In the CONT group, no food was found in the majority of dogs (63.6%). In 36% of the dogs in this study, the stomachs were empty, and gastric foreign bodies were found in 14% of dogs. These and other findings from macroscopic examination are presented in Table 5.

3.4. Body condition and fat parameters

To evaluate the body condition of each dog, the following parameters were investigated: body score, body mass index, visceral fat reserve (pericardial, perirenal, and omental), presence of serous atrophy, and bone marrow fat content.

The body score calculated using the Royal Canin™ [26] classification

Table 3
Classification of the severity of anaemia and erythrocyte volume (MCV) and haemoglobin (MCHC).

		GROUPS						
		MULT (n = 25)		OLIGO (n = 14)		CONT (n = 11)		Total (n = 50)
Classification		N	%	N	%	N	%	N
Severity	Mild	10	41.67	2	28.57	1	50.0	12
	Moderate	13	54.17	3	42.86	1	50.0	17
	Severe	1	4.17	3	42.86	0	0	4
Volume/ haemoglobin	Normocytic hypochromic	9	37.50	5	71.43	0	0	14
	Normocytic normochromic	14	58.33	2	28.57	2	100	18
	Microcytic normochromic	1	4.17	1	14.29	0	0	2

Table 4
Prevalence of lesions observed on macroscopic examination in MULTI, OLIGO, and CONT groups.

Sign Category	Individual sign	GROUPS							
		MULT (n = 25)		OLIGO (n = 14)		CONT (n = 11)		TOTAL (n = 50)	
		N	%	N	%	N	%	N	%
S	Temporal muscle mass loss	14	56.0	1	7.1	2	18.2	17	34.0
	Generalised muscle mass loss	12	48.0	0	0.0	1	9.1	13	26.0
	Pale mucous membranes	5	20.0	3	21.4	0	0.0	8	16.0
	Icteric mucous membranes	1	4.0	0	0.0	0	0.0	1	2.0
	Total	15	60.0	3	21.4	4	36.4	22	44.0
RE	Lymphadenomegaly	14	56.0	7	50.0	1	9.1	22	44.0
	Splenomegaly	21	84.0	10	71.4	8	72.7	39	78.0
	Total	23	92.0	10	71.4	9	81.8	42	84.0
GI	Hepatomegaly	18	72.0	8	57.1	4	36.4	30	60.0
	Parasites in the gastrointestinal tract	3	12.0	1	7.1	0	0.0	4	8.0
	Foreign body	1	4.0	0	0.0	0	0.0	1	2.0
	Total	20	80.0	8	57.1	4	36.4	32	64.0
O	Ocular secretion	5	20.0	2	14.3	1	9.1	8	16.0
IT	Ear tip lesion	14	56.0	6	42.9	8	72.7	28	56.0
	Alopecia focal/diffuse	17	68.0	6	42.9	3	27.3	26	52.0
	Hyperkeratosis	3	12.0	0	0.0	0	0.0	3	6.0
	Ulcers	21	84.0	6	42.9	0	0.0	27	54.0
	Onychogryphosis	20	80.0	2	14.3	1	9.1	23	46.0
	Total	24	96.0	13	92.9	9	81.8	46	92.0

S: Systemic signs. RE: Reticuloendothelial signs. GI: Gastrointestinal.
O: Ophthalmic. IT: Integument.

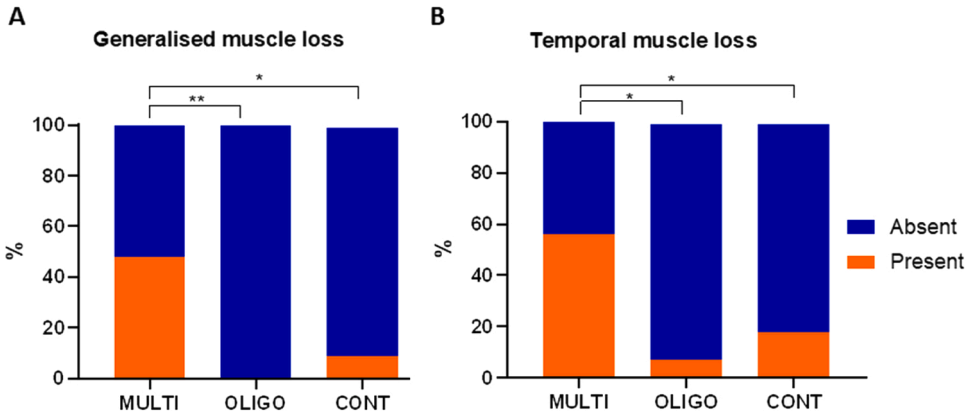

Fig. 1. Comparison of the frequency of muscle mass loss among control (CONT), oligo/asymptomatic (OLIGO) and multisymptomatic (MULT) groups. A: Generalised muscle mass loss; MULT showed greater frequency than OLIGO ** p = 0.026; CONT * p = 0.0311. B: Temporal muscle mass loss; MULT showed greater frequency than OLIGO, * p = 0.0112; CONT * p = 0.0173 (Fisher's test).

showed low scores in the MULT group compared to those observed in the OLIGO ($p \leq 0.0001$) and CONT (p = 0.0001) groups (Fig. 2A, B; Fig. 3B; Table 6).

The body mass index was calculated by dividing the animal's weight (kg) by the length of the spinal column (m) [27]. The average body mass index of the CONT group did not differ from that of the other groups (Table 6). However, the OLIGO and MULT groups (p = 0.0188) differed with a lower mean observed with the MULT group (Fig. 3B; Table 6).

The mean score attributed to pericardial fat was lower in the MULT group than that in the CONT (p = 0.0241) and OLIGO (p = 0.0436) groups (Fig. 4A). However, omental and perirenal fat contents showed similar average values in all groups (Fig. 4B, C). Serous atrophy was observed in only six dogs. However, no statistical difference was observed between the groups infected with *Leishmania* and the CONT

Table 5
Stomach content observed in various groups.

	GROUPS							
	MULT (n = 25)		OLIGO (n = 14)		CONT (n = 11)		TOTAL (n = 50)	
Stomach contents	N	%	N	%	N	%	N	%
Empty	7	28.0	4	28.6	7	63.6	18	36.0
Dog food	15	60.0	10	71.4	3	27.3	28	56.0
Human food	2	8.0	0	0.0	1	9.1	3	6.0
Foreign material	4	16.0	3	21.4	0	0.0	7	14.0

group (Fig. 4D).

The amount of bone marrow fat was assessed using two methods, namely, tube supernatant (mm) and bone marrow extraction (%). The amount of fat per extraction (%) was lower in the MULT group than that in the OLIGO ($p = 0.0003$) and CONT ($p = 0.0022$) groups. (Fig. 5A; Table 6). Similarly, the mean bone marrow fat content in the tube (mm) was lower in the MULT group than that in the OLIGO ($p = 0.0081$) and CONT ($p = 0.0009$) groups (Figs. 5B, 6A, and B; Table 6).

In addition, the body score was positively correlated with the bone marrow fat content obtained both by chemical extraction ($p = 0.0002$; $r = 0.6041$) (Fig. 7D) and by the supernatant in the tube after centrifugation ($p = 0.0003$; $r = 0.5542$) (Fig. 7A, B).

4. Discussion

This study aimed to examine parameters of body condition, visceral fat reserve, and bone marrow fat content in dogs with CanL as a possible way to identify cases of neglect. For this purpose, 50 dogs of the same origin, namely, the Zoonoses Control Centre in the municipality of Araçatuba, State of São Paulo, Brazil, an area endemic for CanL, were evaluated. Of these, 39 were diagnosed with CanL using ELISA and qPCR and were separated into two groups, namely, oligo/asymptomatic and multisymptomatic according to clinical signs. The other 11 dogs (ELISA- and PCR-negative for *L. infantum*) were included in the control group.

Co-infection is very frequent in dogs naturally infected with *L. infantum* [35–38], which was also observed in this study (Table 1), where co-infections were monitored by serology. Dogs infected with *E. canis* can maintain high antibody titres for years [39,40]. Therefore, we did not interpret this result as the presence of active disease; we assumed that these dogs were seropositive for the agent. Moreover, the seroprevalence of *Neospora caninum* in clinically normal dogs has been reported in several studies [41–43]. The positive serology for other infectious agents is the reality of most infected animals; we could not select dogs with *Leishmania* infection only to observe neglected parameters in untreated chronic patients.

Table 6
Effect of leishmaniasis based on the parameters of body condition and bone marrow and visceral fat reserve.

	GROUPS					
	CONT		OLIGO		MULT	
Parameter	Mean	SD	Mean	SD	Mean	SD
Fat (%)	64.10^{a}	15.54	58.47^{b}	15.92	21.64^{ab}	9.183
Fat (mm/tube)	$5{,}50^{a}$	5.486	4.00^{b}	3.93	1.60^{ab}	1.42
Body score	3.273^{a}	0.646	2.929^{b}	0.474	1.880^{ab}	0.526
Body mass index	22.92	11.06	28.59^{a}	8.524	18.33^{a}	7.382
Pericardial fat (+)	2.286^{a}	0.611	1.760^{b}	0.597	2.400^{ab}	0.516
Omental fat (+)	2.080	0.702	2.000	0.554	2.000	0.667
Perirenal fat (+)	1.960	0.735	2.429	0.514	2.500	0.527

SD: Standard deviation; groups with the same letter on the line differ from each other ($p < 0.05$). Fat (%); body mass index = ANOVA + Tukey's test. Fat (mm/tube); body score; pericardial, perirenal, and omental fat = Kruskal-Wallis + Dunn's test.

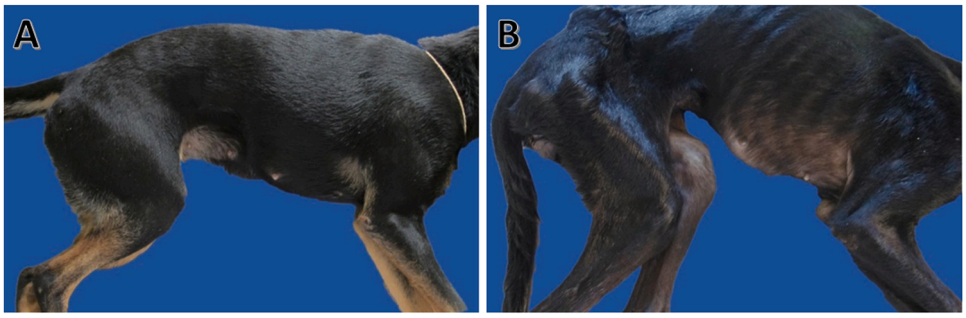

Fig. 2. Demonstration of the body score of the dogs. A: Photographic image of a study dog classified with body score (III). B: Photographic image of a study dog classified with body score (I).

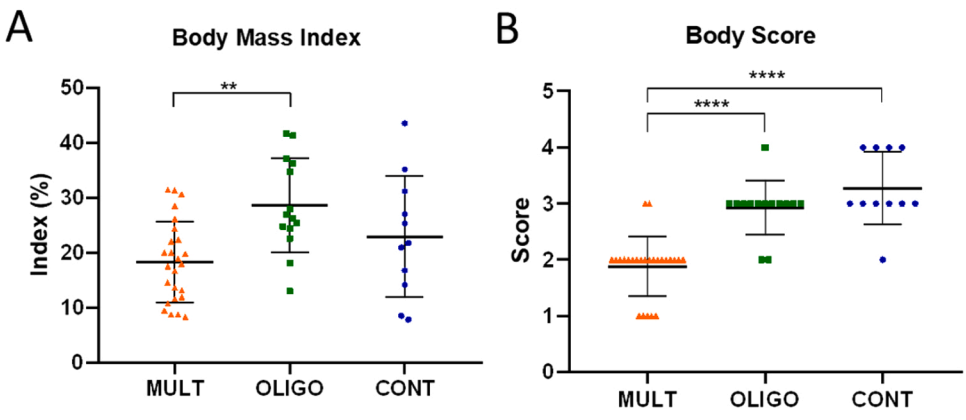

Fig. 3. Comparison of the results of the body score and body mass index, mean and standard deviations among the control (CONT; blue circle), oligo/asymptomatic (OLIGO; green square), and multisymptomatic (MULT; red triangle) groups. A: Photographic image of a study dog classified with body score (III). B: Photographic image of a study dog classified with body score (I). C: The average body mass index in the MULT group was less than that in the OLIGO group ** $p = 0.0023$ (ANOVA + Tukey's test). D: The average body score in the MULT group was less than that in the OLIGO group **** $p < 0.0001$; CONT **** $p < 0.0001$ (Kruskal–Wallis + Dunn's tests).

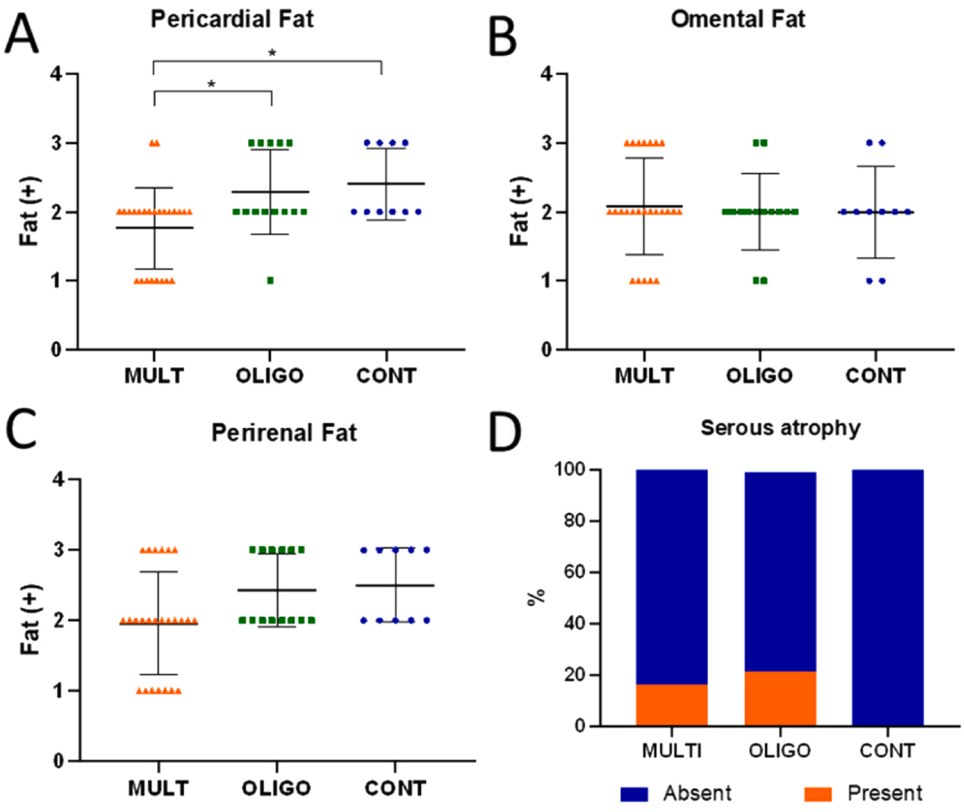

Fig. 4. Comparison of results, mean and standard deviation among control (CONT; blue circle), oligo/asymptomatic (OLIGO; green square), and multisymptomatic (MULT; red triangle) groups of the perirenal, pericardial, and omental fats, and the frequency of serous atrophy observed on microscopic examination. A: The average of the pericardial fat (+) index in the MULT group was less than that observed in the OLIGO group * $p = 0.0436$; CONT * $p = 0.0241$ (Kruskal–Wallis + Dunn's tests). B: omental fat and C: perirenal fat did not differ among groups (Kruskal–Wallis + Dunn's tests) ($p > 0.05$). D: Serous atrophy did not show differences among groups (Fisher's exact test) ($p > 0.05$).

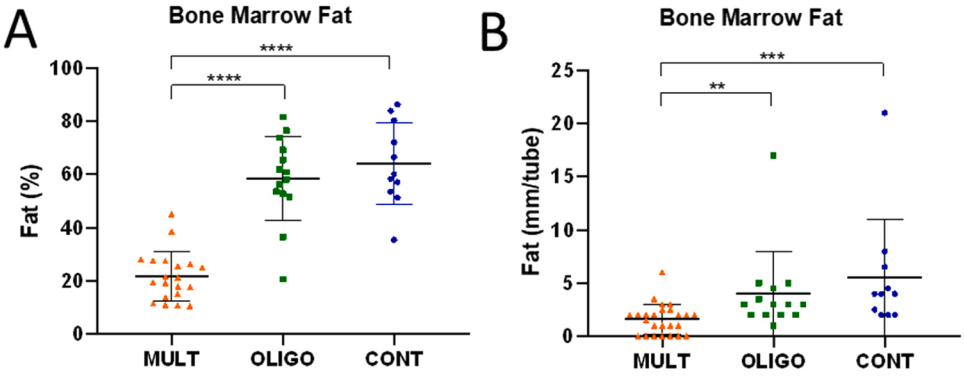

Fig. 5. Comparison of the mean and standard deviation of bone marrow fat content among control (CONT; blue circle), oligo/asymptomatic (OLIGO; green square), and multisymptomatic (MULT; red triangle) groups. A: Fat extracted from bone marrow (%) in the MULT group was less than that in the OLIGO and CONT groups $p = ** ** < 0.0001$ (ANOVA + Tukey's test). B: Bone marrow fat content (mm/tube) in the MULT group was less than that in the OLIGO group $p = ** 0.0081$; CONT *** 0.0009 (Kruskal–Wallis + Dunn's tests).

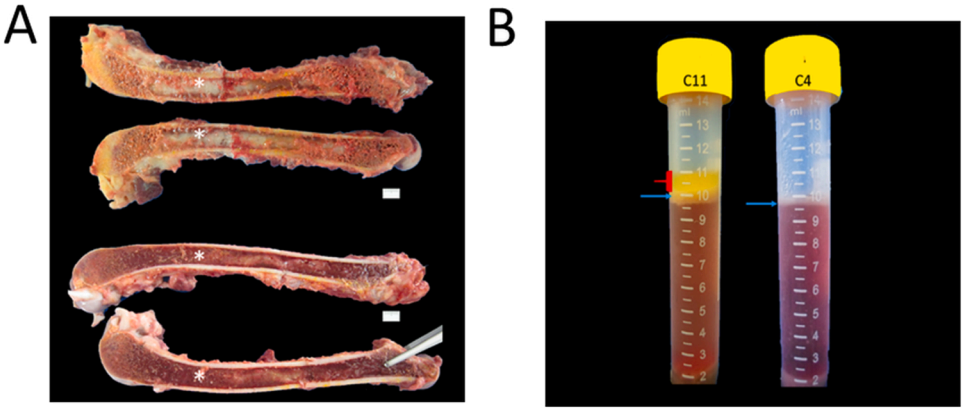

Fig. 6. Demonstration of the visualisation of bone marrow fat content. A: Longitudinal sections of the femur showing bone marrow fat (*) in oligo/asymptomatic dogs (upper) and multisymptomatic dogs (bottom). B: Supernatant fat content in mm in tubes with homogenised bone marrow samples derived from oligo/asymptomatic (OLIGO - C11) and multisymptomatic (MULTI - C4) dogs, respectively. The highest amount of supernatant fat was observed in oligo/asymptomatic dogs (red line); Supernatant fat is invisible in multisymptomatic dog. The blue arrow corresponds to the precursors of the medullary white blood cells.

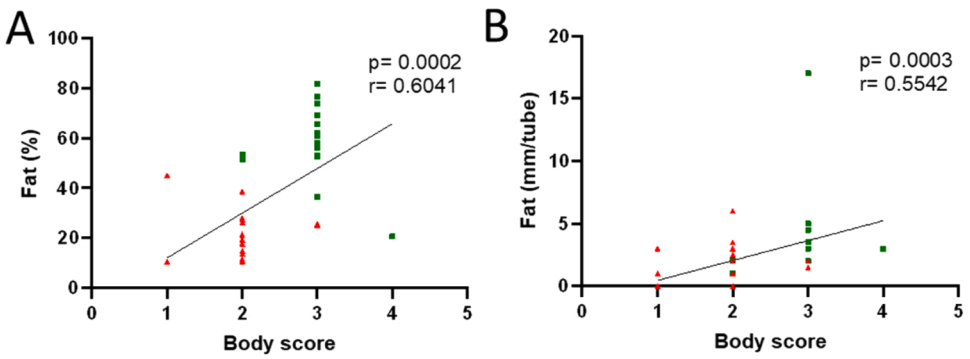

Fig. 7. Correlation between body condition parameters including bone marrow and perirenal fat content in dogs with leishmaniasis (n = 39) from oligo/asymptomatic (OLIGO; green square) and multisymptomatic (MULT; red triangle) groups. In each graph, the values of the correlation coefficients r and p are shown. A: Observed moderate correlation between body score and amount of bone marrow fat per extraction (%). B: Observed moderate correlation between body score and the amount of bone marrow fat in the tube (mm) (Spearman test).

The parasite load of dogs belonging to the infected groups was determined using qPCR. Although previous studies have reported a correlation between symptomatology and parasite load [44,45], in this study, no difference was observed between the parasite loads in the OLIGO and MULT groups. However, the relationship between symptomatology and parasite load is controversial, as asymptomatic animals can have a high parasite load and vice versa [46].

Clinical pathological changes (Table 2) were all compatible with those observed in dogs with CanL [47–50]. We highlight the percentage of dogs with anaemia (68%), hypoalbuminaemia (68%), or hyperproteinaemia (72%). In general, anaemia in CanL is hyporegenerative and often normocytic and normochromic [47,48]. Anaemia observed in studies examining starvation also presented these characteristics [51]. Hypochromic microcytic anaemia usually results from chronic iron deficiency; however, iron stores and/or availability can be affected by chronic diseases, such as CanL [32,52]. Anaemia in CanL may be attributed to several mechanisms, such as anaemia related to chronic disease, spleen and hepatic haemolysis, bone marrow disorders, and renal dysfunction [52–56]. Hyperproteinaemia in CanL results from an increase in the levels of β-globulins and γ-globulins. It is accompanied by hypoalbuminaemia, characterising plasma dysproteinaemia. Hypoalbuminaemia observed in infected dogs is a result of renal failure and decreased albumin production by liver cells [57].

Skin lesions and splenomegaly were observed in most dogs in this study. Changes that are commonly seen in dogs with CanL [45,47,48]. Also we observed generalized and temporal muscle wasting was statistically more frequent in the MULT group than in the OLIGO and CONT groups (Table 4; Fig. 1A; B). Muscle mass loss during CanL results from a combination of local immune-mediated inflammation and protein catabolism, and it is most frequently observed in association with several other clinical signs. Also is one of the alterations that outlines the chronicity of the disease [47,58,59].

Despite the lack of a strict definition of cachexia, involuntary weight loss, muscle mass loss, and increased inflammatory response are key factors in its diagnosis [60,61]. Involuntary loss of body weight and muscle mass are the most common features of cachexia and are considered diagnostic factors for this syndrome [60]. Tumour necrosis factor-α, interleukin (IL)−1β, IL-6, and interferon-γ are among those cytokines whose expression is most consistently upregulated in cachexia [62]. These cytokines are also produced during CanL [63] and may contribute to the pathogenesis of muscle mass loss. Both muscle atrophy and cachexia are used to describe muscle mass loss in infected dogs [58,64].

Most of the sick dogs in this study ingested food (Table 5), although no statistical differences were observed between the groups. This finding differs from that of a study with emaciated dogs suspected of starvation, in which a higher frequency of empty stomachs was observed in sick animals. This suggests that CanL does not affect the appetite of most dogs. The fact that digesta were found in the stomachs of dogs on macroscopic examination suggests that they were fed a few hours before death [12]. The dogs in this study were selected from the Zoonoses Control Centre that provides the dogs with food after collection before euthanasia. Therefore, we do not have information regarding the quality or quantity of the diet provided prior to collection.

Considering that the evolution of clinical signs of canine CanL culminates in progressive deterioration of the general state of the body, we used the Royal Canin™ system's classification scale, which allocates a body score ranging from one to five [26]. The mean score of the dogs in the MULT group was two, significantly lower than that of the other two groups, OLIGO and CONT with a mean score of three (Fig. 3A; Table 6). A score of two implying a thin body type is considered below the ideal score, whereas a score of three is considered normal or ideal [26].

In the present study, we found that the body mass index did not differ between the control and infected dogs; however, it did differ between the MULT and OLIGO groups (Fig. 3B), which does not agree well with the results of the body score observed in the dogs in this study. Previous studies performed by Mawby et al. [65] and Jeusette et al. [66] also concluded that body mass index does not correlate well with body fat (Fig. 3B). Body weight and BMI disregard differences in body composition arising from the variety of body proportions in different dog breeds [65]. For this reason, weight was not discussed in this study. However, the mean weight in kg of the MULT Group (Mean ± Standard Deviation= 8744 ± 4822) was significantly lower than that of the OLIGO (14.76 ± 6142), but did not differ from the CONT group (11.47 ± 7247). For BMI calculation, we used the spine length, which can give a better idea of animal size. The average column length did not differ between groups (Supplementary table 1).

The mean visceral fat reserve did not differ between groups (Fig. 4A, B), with the exception of pericardial fat, which was macroscopically significantly reduced in the MULT group (Fig. 4C), which may indicate an initial process of mobilisation of visceral fat stores in these dogs. Gerdin et al. [12] reported that emaciated dogs show loss of visceral fat reserve and bone marrow fat. However, MULT dogs showed more muscle mass loss than the other groups and a lower body condition score, which takes into account both fat and muscle mass [67]. Despite of the low score of MULT dogs, fat deposits were slightly affected and only pericardial fat was decreased. Furthermore, loss of muscle mass prior to the depletion of visceral and medullar reserves suggests cachexia. In cachexia, a disproportionate loss of lean mass, compared to that of fat mass, is observed [13,68,69].

The mean percentage of bone marrow fat content in the CONT group (64.1%) was similar to the normal value (59.96%) described in dogs with an ideal body score in the study by Lamoureux et al. [31] (Table 6). However, it differed somewhat from the mean value described by Meyerholtz et al. [29], who found a mean body fat content of 82% in dogs with normal scores ranging from 65% to 98%. The OLIGO group had values close to those of normal dogs described in the literature [31]. The MULT group had a mean bone marrow fat content of 21.64%, similar to previously reported values in dogs with a low body score [29]. The amount of bone marrow fat determined in millimetres in the tube was compared with that of the commonly used quantitative fat extraction method with solvent. Tube fat measurement is a quick and

accessible method that shows a moderate positive correlation with solvent extraction fat values. Furthermore, it correlated with body score in the same way as fat extracted with the solvent (Fig. 7B).

The bone marrow fat stores of MULT dogs were reduced despite the maintenance of visceral fat stores. This result excludes starvation as the cause. of bone marrow fat reduction in the dogs in this study. This is because in the cases of starvation and emaciation, the body first mobilises subcutaneous and visceral fat stores, such as pericardial, omental, and perirenal fat, before mobilising bone marrow fat [11,12,70–72].

Diffuse granulomas have been described in dogs with a large number of intracytoplasmic parasites in the bone marrow of the macrophages. In addition, these dogs presented a significant reduction in the bone marrow cell populations (pancytopenia), with a predominance of adipose tissue, characterising medullary aplasia [56]. We observed a reduction in the medullary adipose fat content in dogs with CanL. These results differ from those of Momo et al. [56], which can be explained by differences in the time of disease evolution in naturally infected dogs. In addition, unlike Momo et al. [56], who studied most asymptomatic and oligosymptomatic dogs, we selected a group of dogs (MULTI) with clinical signs that varied from moderate to severe to examine the parameters of neglect. Therefore, the bone marrow fat content reduction observed in this study may indicate a later stage of the disease, since a reduction in the bone marrow fat content was noticed mostly in the MULT group.

The pathogenesis of decreased medullary fat content in dogs with CanL may include a medullary metabolic change due to the presence of parasites and/or the production of inflammatory chemical mediators in the bone marrow milieu. The lipid content of bone marrow adipose tissue is mostly used as an energy source for populations of osteoblasts, osteoclasts, and hematopoietic cells [73,74]. Bone marrow fat content reduction detected in the late stages of CanL may affect haematopoiesis and contribute to worsening of the clinical presentation.

A high percentage of dogs with tick-borne diseases was observed in this study, and recent research on co-infection in dogs with CanL confirmed the worsening of symptoms and disease progression in dogs with CanL [75]. This fact can reinforce the neglect situation of dogs since internal or external parasitism is a physical manifestation of inadequate husbandry and it is considered a sign of negligence [11].

Most dogs included in this study were multisymptomatic dogs. In a cross-sectional study of the CanL predictor factor, symptomatic dogs had owners with lower incomes, whereas asymptomatic dogs had owners with higher incomes [76]. Additionally, the only drug allowed to treat dogs with leishmaniasis in Brazil is expensive. Because of this, some owners refuse to euthanize the animal and do not administer any type of treatment. The disease progresses chronically as the dogs become increasingly debilitated and evolve to very poor body conditions (multisymptomatic). The owner accepts euthanasia only at this point [8]. Unfortunately, animals in such precarious health condition can be characterised as those who have been neglected. Neglect is defined as failure to provide physical and mental well-being. The lack of adequate veterinary treatment is considered a type of neglect [77]. Our study showed poor body health indicators in dogs chronically infected with *L. infantum*, such as a low body score and reduced medullary adipose tissue content, whereas visceral fat stores remained within the normal range.

5. Conclusion

In summary, most canine CanL dogs that were euthanised showed apparent signs of disease in addition to a low body score and reduced bone marrow fat content. Parameters such as body score and bone marrow fat content associated with visceral fat stores can be used as evidence of neglect in dogs with VL. Furthermore, these parameters can provide evidence that differentiates neglect in terms of veterinary care from those who have suffered from hunger or malnutrition.

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Declaration of Conflicting Interest

The authors (s) declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.fsiae.2022.100049.

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