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Myostatin serum levels depends on age and diet in athletic and no athletic dogs

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ABSTRACT

Myostatin is a growth factor related to muscular mass atrophy via mTOR pathway inhibition. Mutations in this gene have been correlated with high muscular mass development in different species of mammals, including human and dogs. Different studies have shown that sport practice increases myostatin gene expression. Some of them were conducted in canine breeds selected for different sport practices, including mushing sports. In this study, body weight, muscular mass, and serum levels of myostatin were analysed in different canine breeds, selected, and not selected for sprint and middle-distance racing, and the effect on epidemiological factors was evaluated. Sex, reproductive status, and canine breed affects body weight and muscular mass, being higher in males, and in sled canine breed. Age has an effect in body weight and myostatin serum levels, being lower in elder dogs. Sport practice and type of diet had an effect in muscular mass development but not in myostatin serum levels. Results showed a high positive correlation between muscular mass and body weight but not with myostatin levels. These results suggest that independent-myostatin mechanisms of mTOR pathway regulation could be related to muscular mass development in dogs.

Introduction

Myostatin (MSTN) is a growth factor (also known as growth and differentiation factor 8 or GDF8), which regulates skeletal muscle development (Shelton et al., 2019). The gene encoding MSTN protein is highly conserved in mammalian species and mutations in this gene have been described in different species, including human (Schuelke et al., 2004), livestock such as cattle (Grobet et al., 1997), sheep (Kijas et al., 2007), or pig (Stinckens et al., 2008), and companion animals such as horses (Hill et al., 2010) or dogs (Mosher et al., 2007). In livestock, these mutations have been related to the double muscle phenotype, a consequence of less expression of *MSTN* gene, low levels of MSTN protein production and increased proliferation of myoblasts (Aiello et al., 2018). In fact, studies *in vivo* in mice have demonstrated that treatment with an MSTN inhibitor increases muscle mass (Arounleut et al., 2013). Changes in muscular mass development and muscle strength due to sport practice is mediated by MSTN in human. For example, an increase in levels of *MSTN* expression has been observed after ten days of sport detraining (Jespersen et al., 2011), suggesting a relevant role of MSTN protein in the negative regulation of skeletal muscular mass development, and short-term resistance training diminished the myostatin serum levels (Jaworska et al., 2020), which seems to indicate that resistance training modified muscle strength through modulation of MSTN concentrations in humans.

In dogs, a mutation in *MSTN* gene caused gross muscle hypertrophy in whippets (Shelton and Engvall, 2007) and studies in Golden retriever have shown that MSTN inhibition in the long term increased muscle mass (Bish et al., 2011; Kornegay et al., 2016). However, only two studies have been conducted in this species related to sport activity and MSTN. Mosher et al. (2007) described one mutation in the *MSTN* gene present in racing whippets that conferred a competitive edge to these animals, related to greater muscle development, and Shiomitsu et al. (2022) reported that serum MSTN levels decreases with sport and age in Alaskan sled dogs. This canine breed is one of the most popular used for "mushing", a sport in which dogs (*Canis lupus familiaris*) pull a means of transport (usually a bicycle) carrying their owner (Leggieri et al., 2019).

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This sport has two main variants, and the selection of dogs varies between them, although it has always been carried out on a sled dog basis. Thus, for the middle distance (races up to 40 km) the most common canine breed used is Alaskan sled dog (Huson et al., 2010), whereas for the sprint variant (races up to 6 km) the Greyster sled dog is used. However, no study has been carried out on these canine breeds regarding their MSTN levels compared to other breeds, or muscle development or other factors that could influence these levels.

The main objective of this study is to analyse the serum levels of MSTN in these breeds used for mushing and to compare these results with those obtained in other canine breeds. In addition, the factors related to these serum levels will be studied in both athletic and non-athletic dogs.

Material and methods

Animals and epidemiological data

Animal experiments were conducted in accordance with the Declaration of Helsinki ethical principles and approved by the Animal Experimentation Ethics Committee of the Universidad Cardenal Herrera-CEU, with code 2024-VSC-PEA-0102. Informed consent was obtained from the owners of all participating healthy dogs. For all animals included, a structured questionnaire was applied to gather epidemiological data. The data obtained for all dogs were sex (male or female), reproductive status (castrated or intact), age (young, less than four years old; adult, between four and ten years old; and elder, more than ten years old), canine breed, body weight (recorded to the nearest 0.1 kg using a calibrated scale), feeding (feed with or without meat) and athletic dogs (sport practice) or not. All dogs had a standard body condition score (BCS) between 5 and 9, within normal parameters (Laflamme, 1997).

A total of 102 dogs were included in this study: 50 males (49.02 %) and 52 females (50.98 %); 36 were castrated (35.29 %), before the first year of age. All the animals were in Spain, were similar mean of temperature and humidity. Of the total animals, 27 were young (26.47 %) while 56 were adults (54.90 %) and 19 elder animals (18.63 %). The age ranged from one to fifteen years old. Regarding breed purity, 34 dogs were purebred non-sled dog (33.33 %) and 68 were sled dog breed (58 Alaskan sled dog and 10 Greyster) (supplementary file 1), of which 63.73 % practiced sport (mushing) at the time of the study. All athletic dogs had similar exercise routines, with between one and two hours of physical exercise daily during the training period, when the study was carried out. In relation to feeding, 50 dogs were fed exclusively on commercial feed (49.02 %), whereas 8 were fed only meat (7.84 %) and the rest of the animals fed on commercial feed and meat simultaneously. All dogs feed one a day, first thing in the morning. Summarised epidemiological data on the dogs included in the study are shown in Table 1. The complete epidemiological information was in the supplementary file 2.

Blood sample collection and muscular mass measure

Five millilitres of whole blood samples were collected by cephalic venipuncture using Vacutainer tubes without anticoagulant. In sport dogs, the samples were collected within 1 h of finishing exercise. Samples were kept at room temperature to obtain serum aliquots, which were stored at −20ºC until processing.

Three veterinarian raters measured the muscular mass as the mean of muscular circumferences of two quadriceps, by a calibrated tape rather. For this, the middle diaphysis of the femur was taken as a reference (Cardona Ramirez et al., 2015)

Serological analysis

Myostatin was measured in serum samples using quantitative sandwich ELISA (GFG-8/Myostatin, R&D systems, Inc; Minneapolis, USA), following the manufacturer's instructions. Briefly, samples, standard and controls were diluted 1:2 and added to each previously coated plate well. After two hours of incubation at room temperature and washing, the conjugate antibody was added and incubated at room temperature on the shaker for two hours. Subsequently, substrate solution was incorporated and incubated for 30 min protected from light. Finally, the stop solution was added and the absorbance at 450 nm was measured. The calibration curve was carried out with concentrations between 0 and 2000 pg/mL.

Table 1
Epidemiological data of animals included in the study.

Variable			n (%)
Sex	Male		50 (49.0 %)
	Female		52 (51.0 %)
Reproductive status	Castrated		36 (35.2 %)
	Intact		66 (64.7 %)
Age	Young		27 (26.5 %)
	Adult		56 (54.9 %)
	Elder		19 (18.6 %)
Breed	No sled dog (n=34, 33.33 %)	Pointer	14 (13.7 %)
		Breton	2 (2.0 %)
		Griffon	6 (5.9 %)
		English setter	2 (2.0 %)
		Spanish Alano	4 (3.9 %)
		Border Collie	2 (2.0 %)
		Pitbull	2 (2.0 %)
		Husky	2 (2.0 %)
	Sled dog (n=68, 66.67 %)	Greyster	10 (9.8 %)
		Alaskan	58 (56.9 %)
Sport	Athletic dogs		65 (63.7 %)
	No athletic dogs		37 (36.3 %)
Feeding	Fed		50 (49.0 %)
	Fed + Meat		44 (43.1 %)
	Meat		8 (7.8 %)

Statistical analysis

Normality and homoscedasticity of body weight, muscular mass, muscular mass corrected by body weight and myostatin serum levels were tested by Shapiro-Wilks and Levene tests, respectively. Data were analysed by General Lineal model including myostatin serum levels, muscular mass, body weight, and muscular mass corrected by body weight (calculated as the average quadriceps divided by body weight) as dependent variables and sex (male or female), reproductive status (castrated or intact), age (young, adult or elder), canine breed, sled canine breed or not, practice sport or not (athletic or non-athletic dog), and nutrition (fed, meat or both), as independent variables. A univariable analysis was carried out first and subsequently a multivariable analysis, including all variables analysed and interactions between these variables. The interactions between variables have been analyzed. Correlation between quantitative variables was analysed by Pearson's test. The statistical program SAS (North Carolina State University, Cary, CA, USA) were used for the statistical analysis and the statistical significance was set at $\alpha < 0.05$.

Results

The mean body weight was 24.31 ± 5.59 Kg, whereas the mean muscular mass measured as mean of quadriceps circumferences was 36.93 ± 3.78 cm, and the mean of myostatin serum levels was 2853.31 ± 769.31 pg/mL. Statistical analysis did not reveal statistical significance for interaction between variables included in multivariate analysis. Sex, reproductive status, age, and canine breed had a statistical effect on both body weight (Table 2). Males and castrated dogs have

Table 2
Body weight according to epidemiological groups based in the multivariable analysis. Body weight data show as adjusted mean ± standard error. Different superscript in the same column means statistical differences.

Variable			Body weight (kg)	*p*-value
Sex	Male		26.13 ± 5.56	**< 0.0001**
	Female		22.13 ± 4.62	
Reproductive status	Castrated		21.33 ± 4.13	**< 0.0001**
	Intact		25.94 ± 5.64	
Age	Young		23.18 ± 6.20^{a}	**0.0140**
	Adult		26.13 ± 4.92^{b}	
	Elder		20.57 ± 4.39^{a}	
Breed	No sled dog	Pointer	19.14 ± 0.72^{ab}	**0.0319**
		Breton	17.00 ± 0.00^{ab}	
		Griffon	20.33 ± 3.14^{ab}	
		English setter	19.0 ± 0.00^{a}	
		Spanish Alano	18.00 ± 9.24^{ab}	
		Border Collie	12.00 ± 0.00^{a}	
		Pitbull	18.00 ± 0.00^{ab}	
		Husky	25.50 ± 0.00^{b}	
	Sled dog	Greyster	28.25 ± 3.27	0.3050
		Alaskan	26.76 ± 4.34	
Sport	Athletic dogs		27.09 ± 4.16	0.3949
	No athletic dogs		19.43 ± 4.31	
Feeding	Fed		23.33 ± 6.12	0.0680
	Fed + Meat		26.20 ± 4.63	
	Meat		20.00 ± 2.73	

more body weight than females and intact animals, whereas adult dogs have more body weight than young and elder.

Canine breed had influence body weight. In fact, sled dog breeds presented greater body weight (26.98 ± 4.21 kg) and muscular mass (39.01 ± 2.67 cm) than non-sled dog breeds (18.97 ± 3.97 Kg for body weight, and 32.76 ± 1.58 cm for muscular mass, respectively). In non-sled dogs, canine breed had an effect on body weight and on muscular mass, whereas in sled dogs, Greyster dogs (selected for sprint) presented higher muscular mass than Alaskan dogs (selected for middle race). Muscular mass depends on sex, reproductive status, and sport practice, so males, intact dogs, and athletic had more muscular mass than females, castrated and non-athletic dogs. Nutrition also affects muscular mass, being lower in dogs who only ate meat than dogs who ate fed or fed and meat simultaneously (Table 3).

The analysis of quadriceps average corrected by body weight reveals similar effects on variables analyzed, with the difference that this parameter depends on the canine breed only in no sled dogs, and the age is a statistically significant factor, being higher this parameter in young and elder animals (Table 4).

Serum levels of myostatin are only related to age, being higher in young and adult dogs than elder (2910.39 ± 602.01 pg/mL for young dogs, 2925.50 ± 821.00 pg/mL for adult, and 2559.42 ± 790.92 pg/mL for elder dogs, respectively; *p*-value<0.05). The mean of serum myostatin levels by gender and age show in Table 5. No differences were found between sex, reproductive status, canine breed, sport practice, or different type of nutrition, and the interaction between sex and age were not statistically significant.

Myostatin serum levels were less correlated with body weight (Fig. 1, r = 0.03307), muscular mass (Fig. 2, r = 0.14201) and muscular mass corrected by body weight (r = 0.06722), whereas body weight and muscular mass had a high and positive correlation (Fig. 3, r = 0.83708).

Table 3
Muscular mass (measured as mean of quadriceps perimeter) according to epidemiological groups based in the multivariable analysis. Muscular mass data show as adjusted mean ± standard error. Different superscript in the same column means statistical differences.

Variable			Muscular mass (cm)	*p*-value
Sex	Male		37.78 ± 3.91	**0.0214**
	Female		36.11 ± 3.50	
Reproductive status	Castrated		34.90 ± 3.27	**< 0.0001**
	Intact		38.04 ± 3.60	
Age	Young		37.10 ± 3.47^{a}	0.2259
	Adult		38.00 ± 3.51^{a}	
	Elder		33.54 ± 3.11^{b}	
Breed	No sled dog	Pointer	33.21 ± 1.10	**0.0151**
		Breton	33.00 ± 0.00	
		Griffon	33.17 ± 0.26	
		English setter	32.00 ± 0.00	
		Spanish Alano	31.88 ± 3.03	
		Border Collie	29.25 ± 0.00	
		Pitbull	32.50 ± 0.00	
		Husky	34.50 ± 0.00	
	Sled dog	Greyster	40.73 ± 2.17	**0.0219**
		Alaskan	38.72 ± 2.66	
Sport	Athletic dogs		39.08 ± 2.72	**< 0.0001**
	No athletic dogs		33.16 ± 2.04	
Feeding	Fed		36.41 ± 4.05^{a}	**< 0.0001**
	Fed + Meat		38.27 ± 3.11^{a}	
	Meat		32.88 ± 0.58^{b}	

Table 4
Muscular mass corrected by body weight (measured as mean of quadriceps perimeter divided by body weight) according to epidemiological groups based in the multivariable analysis. Muscular mass data show as adjusted mean ± standard error. Different superscript in the same column means statistical differences.

Variable			Muscular mass corrected (cm/kg)	*p*-value
Sex	Male		1.69 ± 0.33	**<0.0001**
	Female		1.46 ± 0.22	
Reproductive status	Castrated		1.68 ± 0.24	**0.0163**
	Intact		1.53 ± 0.33	
Age	Young		1.70 ± 0.42^{b}	**0.0015**
	Adult		1.48 ± 0.20^{a}	
	Elder		1.69 ± 0.31^{b}	
Breed	No sled dog	Pointer	1.73 ± 0.06	**0.0099**
		Breton	1.94 ± 0.00	
		Griffon	1.66 ± 0.24	
		English setter	1.68 ± 0.00	
		Spanish Alano	2.13 ± 0.92	
		Border Collie	2.44 ± 0.00	
		Pitbull	1.81 ± 0.00	
		Husky	1.35 ± 0.00	
	Sled dog	Greyster	1.45 ± 0.13	0.7264
		Alaskan	1.47 ± 0.19	
Sport	Athletic dogs		1.46 ± 0.18	**< 0.0001**
	No athletic dogs		1.78 ± 0.38	
Feeding	Fed		1.65 ± 0.37^{b}	**0.0111**
	Fed + Meat		1.49 ± 0.20^{a}	
	Meat		1.67 ± 0.21^{b}	

Discussion

This study analyses the factors related to body weight, muscular mass and myostatin serum levels in dog selected for the mushing sport

Table 5
Serum levels of myostatin (pg/mL) in the different epidemiological groups of dogs. Data shows as adjusted mean ± standard error.

		Sex		
		Male	**Female**	**Overall**
Age	**Young**	3036.72 ± 653.54	2774.34 ± 532.80	2910.39 ± 602.01
	Adult	3062.71 ± 791.20	2814.84 ± 840.59	2925.50 ± 821.00
	Elder	2446.10 ± 696.29	2715.22 ± 931.70	2559.42 ± 790.92
Overall		2919.78 ± 763.92	2789.39 ± 776.46	2853.31 ± 769.31

and other canine breeds. The results showed that males and intact dogs had greater body weight and muscular mass than females and castrated animals. Adult dogs had higher body weight than young and elder dogs, whereas no effect was found in muscular mass according to age. Diet and sport practice had an effect on muscular mass. Dogs fed on feed and meat simultaneously had higher muscular mass than animals who fed only meat. Regarding sport practice, athletic dogs (dogs that regularly practiced sport) presented greater muscular mass. Canine breed had an influence on body weight in non-sled dogs, with this variable being higher in Husky dogs than other canine breeds included in this study, although the low sample size in some of the dog breeds included in our study means that this result must be verified with a larger number of animals per dog breed. In sled dogs, Greyster breed (used for sprint) exhibited more muscular mass than Alaskan breed (used for middle distance). MSTN serum levels only depends on age, being higher these levels in adult dogs. Finally, body weight and muscular mass manifested high positive correlation, whereas MSTN serum levels did not present high correlations with either body weight or muscular mass.

According to our results, body weight depends on the canine breed and its development (Salt et al., 2017; Strandberg et al., 2023). In all canine breeds studied, the growth rate was higher in males than females

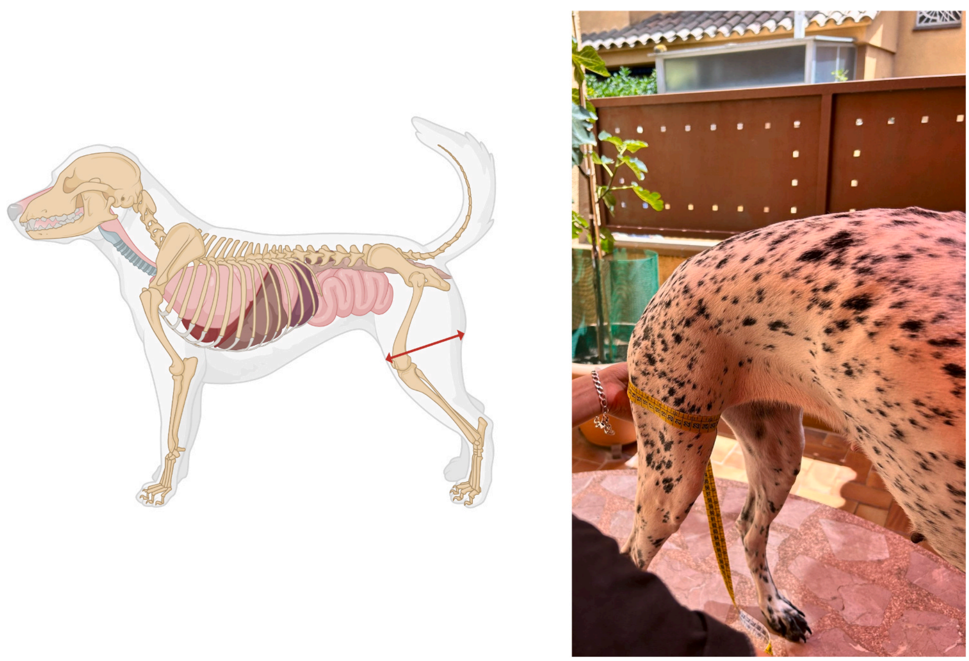

Fig. 1. Squematic diagram (left) and picture (right) of the quadriceps average measurement.

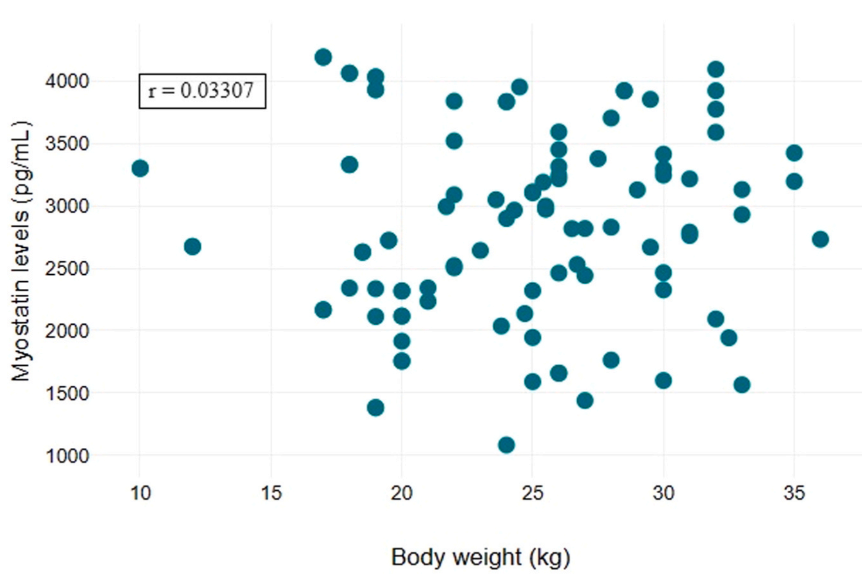

Fig. 2. Scatter diagram between myostatin serum levels (pg/mL) and body weight (Kg) in dogs analyzed.

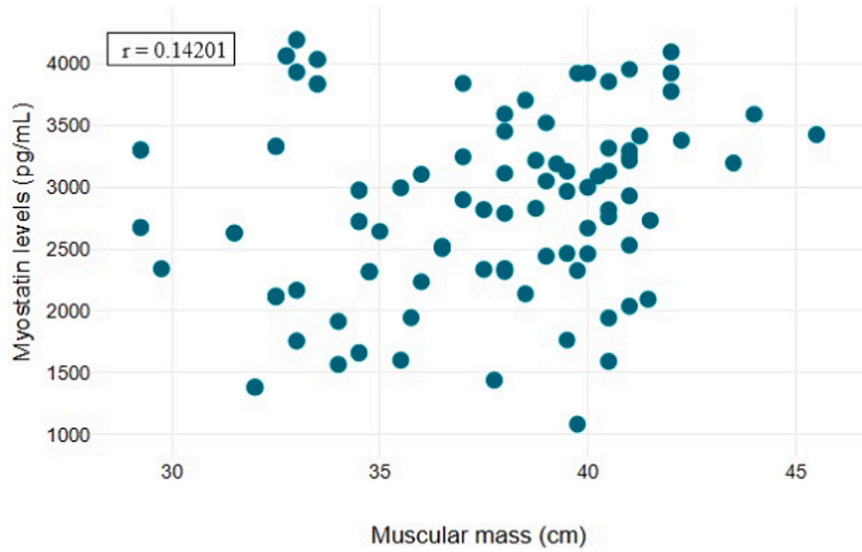

Fig. 3. Scatter diagram between myostatin serum levels (pg/mL) and muscular mass (measured as mean of quadriceps perimeter, cm) in dogs analyzed.

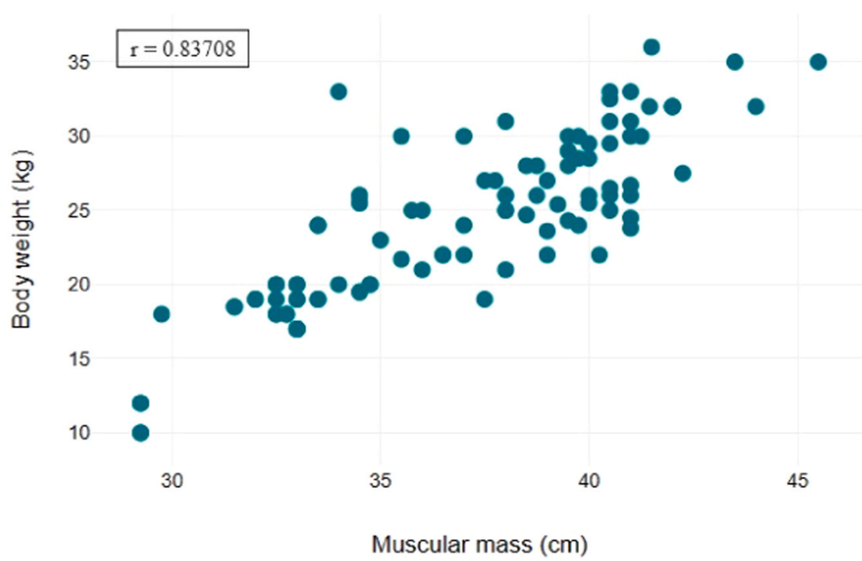

Fig. 4. Scatter diagram between body weight (Kg) and muscular mass (measured as mean of quadricepts perimeter, cm) in dogs analyzed.

and, subsequently, the body weight was greater in males, taking the same age into consideration (Hawthorne et al., 2004; Trangerud et al., 2007). These high body weights are positively correlated with muscular mass development, higher in males than in females (Bassett et al., 2020). In this regard, several studies have been conducted in human, demonstrating the effect of testosterone in muscular development, specifically in the development of skeletal muscle hypertrophy and synthesis of muscle fibres (O'Reilly et al., 2021). High levels of testosterone explain the higher body weight and muscular mass found in intact dogs included in our study.

Genetic selection of sled dogs has been carried out by crossing different canine breeds to obtain certain characteristics related to the specific breed type. Genomic principal components studies with sled canine breeds demonstrated genetic substructure related to selection for sprint or middle distance competition (Huson et al., 2010; Thorsrud and Huson, 2021). Our results showed that higher body weight (correlated to muscular mass) and muscular mass in sled dogs than in non-sled dogs, and within the sled dog breeds evaluated, greater muscular development in the Greyster breed, selected for sprint.

The genetic selection in Alaskan sled dogs has resulted in animals with a particular metabolism, which gives them the ability to postpone fatigue, increasing their ability to perform middle distance races (Tosi et al., 2021). Until today, studies related to the development of muscular mass and running type had not been carried out in dogs, but different studies in human have demonstrated that muscular composition and development depends on the type of training that runners do, differentiating the metabolism and muscular composition between sprint and medium-long distance runners (Miyamoto et al., 2019; Stafilidis and Arampatzis, 2007; Yamazaki et al., 2022).

In both the sled and non-sled canine breeds analysed, training increased muscular mass. The practice of physical exercise has been shown to be one of the most determining factors of muscular development in all mammalian species (Flück, 2006). In contrast to our results, Lee et al. (2021) found no differences in muscular mass in Beagle dogs after training. However, that study measured the muscular mas after only 12 weeks of training, which might not be enough time to observe changes in muscular development associated with exercise practice, as skeletal muscle plasticity depends on different molecular pathways, including regulation pre- and posttranscriptional processes, as well as translation and protein degradation, which would need more time to carried out (Coffey and Hawley, 2007; Egan and Sharples, 2023). As the dogs included in our study had been trained from a young age, these processes had been able to take place and, therefore, their muscular mass was greater than in non-athletic dogs.

Pourabbas et al. (2021) observed an increase in muscular mass with high-protein diet in athletic men regarding a diet rich in carbohydrates. In the same study, the high levels of protein in the diet are correlated with a low level of myostatin in serum. Similar results were reported by Binns et al. (2017) in a population of older adults. However, our results showed that dogs fed with feed and meat simultaneously showed greater muscular development than dogs fed only feed or only meat. These results could be explained by different protein metabolism in dogs. In fact, the effect on diet in skeletal muscle has been studied in dogs, concluding that high animal protein diet grain-free decreases the taurine but not carnitine plasma levels (McCauley et al., 2024). Carnitine has relevant role in fatty acid oxidation (Lin and Odle, 2003), essential in dogs, especially in working dogs (Menchetti et al., 2019). In this regard, Zoran (2021) published a relevant review, where differences between nutritional metabolism between human and dogs shows, and importance of diet selection according to canine work.

Regarding myostatin serum levels, only age influence these levels. In fact, elder dogs had low levels of MSTN, according to previous results obtained by Shiomitsu et al.(2022) in Alaskan sled dogs. Our results indicated that these differences are present not only in sled canine breeds, but also in the others canine breeds studied. The low sample size for some of the dog breeds included in the study does not allow us to reach conclusions in this regard, so more studies are necessary to know if the dog breed influences myostatin levels. The loss of MSTN serum levels in elderly could be a physiological mechanism to compensate the sarcopenia, although more studies in sarcopenic and non-sarcopenic dogs are necessary to demonstrate this effect.

Our results do not indicate a strong correlation between myostatin levels and muscular mass development. Effect on MSTN in muscular mass development is a consequence of inhibition of the rapamycin (mTOR) pathway (Joulia-Ekaza and Cabello, 2007; Kim et al., 2005). Protein consumption increases mTOR signalling, which is related to muscular mass regulation (Hulmi et al., 2009). But the mTOR pathway could be inhibited by other mechanisms, not only myostatin. For example, studies in mice have shown that chronic stress increases *REDD1* expression, an inhibitor of mTOR pathways and, in consequence, leads to muscular atrophy (Fushimi et al., 2023; Hain et al., 2021). Recently, Leduc-Gaudet et al. (2023) demonstrated that the FoxO-dependent gene *MYTHO* is related to mTOR pathway regulation. In fact, *MYTHO* overexpression causes muscular atrophy by mTOR inhibition. In accordance with that and in line with our results, alternative mechanisms to mTOR regulation and muscular mass development could be involved in the dogs included in this study. The increase in muscular mass correlated with protein diet but not with differences in MSTN could indicate that the regulation of muscular mass development is dependent on the mTOR pathway, and the mTOR inhibition could be related to other regulators.

Further studies related to muscular mass development regulation and the role of myostatin and other molecules that regulate mTOR pathways are necessary to elucidate the physiology of muscular mass increase, protein diet and the effect of exercise in canine breeds.

Conclusions

Body weights are positively correlated with muscular mass in all animals included in this study, and depend on sex, reproductive status, sport practice and levels of protein diet. Different canine breed exhibited different myostatin serum levels, which are related to diet but not to muscular mass. The results could indicate myostatin-independent mechanisms of mTOR pathway regulation in sled and non-sled dogs. Further studies are necessary to elucidate the effect of canine breed selection for racing on muscular mass development and its regulation according to exercise practice and dietary protein levels.

Ethics approval and consent to participate

The experimental protocol was approved by the Ethics Committee of Universidad Cardenal Herrera CEU (protocol code 2024-VSC-PEA-0102). The experimental protocols complied with the guidelines of the Ethic Committee. Blood samples were collected by the researchers (veterinarians) after getting informed consent from the owners of the horses. All efforts were made to minimize animal suffering during sample collection. Oral and written informed consent was obtained from all people who participated in the study.

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Consent for publication

Not applicable.

CRediT authorship contribution statement

Lola Llobat: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis. **Guillem Ruvira:** Data curation. **Pablo Jesús Marín-García:** Formal analysis, Conceptualization. **Sandra Bendig:** Writing – original draft, Methodology, Data curation. **Juan José Ramos:** Data curation. **Ana Lesta:** Methodology, Investigation.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.tvjl.2024.106207.

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