POST-MORTEM ANALYSIS IN THREE MUSCLES OF RED NECK OSTRICH (*STUTHIO CAMELUS MASSAICUS*)

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ABSTRACT
A description of the pelvic limb musculature of ostrich (Red Neck) is given. Muscle tissues were obtained from muscle iliotibialis lateralis pars preacetabularis, muscle femorotibialis medius and muscle fibularis longus. The histochemical results revealed the presence of two types of fibres: slow-twitch fibres (type I) and fast-twitch intermediate fibres (type IIA). A higher incidence of type I compared to type IIA fibres in muscle iliotibialis lateralis pars preacetabularis and muscle femorotibialis medius was noticed. The immunohistochemical results revealed that in this species calpain/calpastatin system was localized in various parts of the muscles, although μ-calpain and m-calpain totally disappeared at 4 days post-mortem. Calpastatin and p-94 were present during all the period considered (0, 2 days, 4 days and 6 days post-mortem). In this study changes in enzymatic activities of μ and m-calpain were observed, particularly the activity of μ and m-calpain decrease rapidly during post mortem storage.

INTRODUCTION
The ostrich is the largest living bird in the world; flightless, as the emu, rhea and cassowary. Between the three ostrich groups (African Black, Red Neck and Blue Neck), the Red Neck is the largest; its meat represents an excellent alternative to other sources of meat. The calpain proteolytic system plays a key role in the tenderization process and therefore it is a relevant factor for meat quality. Particularly, in the skeletal muscle this system is thought to be involved in selective degradation of a number of intracellular proteins, including some myofibrillar proteins. Calpains are a family of calcium-dependent cytosolic cysteine proteases. This family comprises μ and m-calpain, and their natural specific inhibitor, calpastatin. p94, or calpain 3, is a skeletal muscle-specific calpain and is probably closely related to the specific functions of the organs in which it is predominantly expressed (1). Also myofibre type and myofibre size of specific muscles are important factors for meat quality, because they influence several peri- and post-mortal biochemical processes in muscles (2). Some aspects of meat quality such as tenderness and juiciness are influenced by the distribution of different fibre types, their number and their area. Avian skeletal muscles typically consist of three basic fibre types: type I or SO (red, slow-twitch oxidative), type IIA or FOG (intermediate, fast-twitch oxidative glycolytic fibres), and type IIB or FG (white, fast-twitch glycolytic). The aims of this study were:

- to analyze the histochemical and morphometrical characteristics of three pelvic limb muscles;
- to evaluate the localization of the calpain and calpastatin by immunohistochemical analysis;
- to evaluate the enzymatic activity of the calpain/calpastatin system at different post-mortem time periods by calpain activity assay.

MATERIALS AND METHODS
Fourteen clinically healthy male ostriches (Red Neck) from a farm located in Potenza (Italy), weighing 100 kg (±10), were slaughtered by electrical stunning at 14 months of age. Animals were treated according to the guidelines of the European Community on the treatment of experimental animals (Reg. CE 1/2005; directives 74/577/EEC; Law 439 2 August 1978). The slaughter house had EEC approved with reference to rules 852/853/854/2004; 2076/2005.

The following muscles were used in the study (particularly the deep layers): muscle iliotibialis lateralis pars preacetabularis, muscle femorotibialis medius, muscle fibularis longus. From each muscle, samples were withdrawn at different times (0, 2, 4 and 6 days post-mortem). At day 0 post mortem samples were withdrawn and immediately frozen in liquid nitrogen (-196°C), and successively stored at -80°C until histochemical and immunohistochemical analyses were conducted. After 2 days, 4 days and 6 days post-mortem samples were withdrawn from refrigerated carcasses (4°C) to perform immunohistochemical analyses.

Muscle Histochemistry
Identification of fibre types was performed on 8μ transversal sections of the samples using a cryostat and histochemical staining. Sections were stained histochemically for myosin ATPase and succinic dehydrogenase simultaneously on the same muscular fibres (3, 4, 5, 6). The method used for the combined histochemical staining (acid myosin ATPase

and succinic dehydrogenase/ m-ATPase + SDH) consisted of different phases. Acid pre-incubation at pH of 4.5 was performed at room temperature for 15 minutes and was always followed by two, 1-minute rinses in $CaCl_2$ 0,18 M in tris hydroxymethyl aminomethane buffer rinse solution. Nitro blue tetrazolium (NBT) incubation was performed for the detection of SDH activity at 37° for 40 minutes followed by two rinses in distilled water. For the myofibrillar (acid) ATPase portions, the procedure was performed at 37°C at a pH of 9.4 for 50 minutes along three 30-s rinses in $CaCl_2$ solution and incubation for 3 minutes in $CoCl_2$ solution. Finally, ammonium sulphide staining of the acid ATPase procedure was performed. Cover slips were placed over the stained tissue sections and fixed in place using glycerol jelly. Additional serial sections were also histochemically stained for detection of basic m-ATPase and SDH activities. The basic m-ATPase method was used for the first control procedure and consisted of different phases. Sodium-cacodylate and sucrose solution were used for incubation for 5 minutes followed by two 1-minute rinses in $CaCl_2$ in tris-hydroxymethyl aminomethane buffer rinse solution. Sigma 221 and $CaCl_2$ solution was performed for 10 minutes at pH 10 followed by two 1-minute rinses in $CaCl_2$ and tris hydroxymethyl aminomethane buffer (Merck, USA) rinse solution. For the myofibrillar ATPase portions, the procedure was performed at 37°C at a pH of 9.4 for 50 minutes along three 30-s rinses in $CaCl_2$ solution and incubation for 3 minutes in $CoCl_2$ solution. Finally, ammonium sulphide staining of the acid ATPase procedure was performed. Cover slips were placed over the stained tissue sections and fixed in place using glycerol jelly. The SDH method was used for the second control procedure and consisted of different phases. Incubation in NBT at 37°C was performed for 40 minuts followed by two rinses in distilled water. Finally formaldehyde solution was applied for 10 minutes. Cover slips were placed over the stained tissue sections, and fixed in place using glycerol jelly.

Muscle immunohistochemistry

Immunohistochemistry was performed using the Universal DakoCytomation Labelled Streptavidin-Biotin2 System, Horseradish Peroxidase (LSAB2 System, HRP). Transverse serial sections of the samples were obtained using a cryostat at -30°C. Acetone was successively used as fixative at 4°C for 4 minutes and incubation in PBS (phosphate-buffered saline) was performed for 5 minutes at a pH of 7.2-7.4. Sections were incubated with 3% of H_2O_2 and methanol for 30 minutes. After a brief rinse in PBS, an incubation for two hours with primary antibody was performed. The following monoclonal antibodies were used:

1. Anti-Calpain-94: Anti-Calpain-94 (DOMAIN I) (Sigma). Rabbit monoclonal antibody against p94 (large subunit) was produced against the corresponding human antigen (Rabbit-anti-human).
2. Anti-μ-Calpain: Anti-μ-Calpain (DOMAIN IV) (Sigma). Rabbit monoclonal antibody against-μ-calpain (large subunit) was produced against the corresponding human antigen (Rabbit-anti-human).
3. Anti-m-Calpain: Monoclonal Anti-m-Calpain (DOMAIN III/IV) mouse IgG1 (Sigma). Mouse monoclonal antibody against m-calpain was produced against the corresponding bovine antigen (Mouse-anti-bovine).
4. Anti-Calpastatin: Monoclonal Anti-Calpastatin antibody mouse IgG1 (Sigma). Mouse monoclonal antibody against m-calpastatin was produced against the corresponding bovine antigen (Mouse-anti-bovine).

Both a 1:100 dilution and a 1:200 dilution of monoclonal anti-Calpain-94, anti-μ-Calpain, anti-m-Calpain, and anti-Calpastatin antibodies preparations were used. Sections were then rinsed twice for 5 minutes in PBS, incubated for 20 minutes with biotinylated secondary antibody (YELLOW) (Dako, Glostrup, Denmark) and again rinsed twice for 5 minutes in PBS. Streptavidin-HRP (RED) (Dako) was used for incubation for 15 minutes followed by two rinses for 5 minutes in PBS. A DAB (3,3'-diaminobenzidine) chromogen was added for 5 minutes followed by rinse in distilled water. After 5 minutes staining, the sections were rinsed with water, counterstained with Haematoxylin (Sigma, The Netherlands) and mounted with Kaiser's glycerol gelatin (Merk, The Netherlands). The specificity of immunostaining was verified: (i) by incubating sections with phosphate-buffered saline (PBS) instead of the specific primary antisera; (ii) by incubating sections with pre-immune sera instead of primary antisera; (iii) by incubating sections with PBS instead of secondary antibodies; and (iv) by absorption of the antisera with excess of synthetic peptide before incubation with sections. The results of these control tests were negative, the controls showing elimination of staining.

Calpain activity assay

For proteins extraction 300 mg of tissue samples from the muscles were homogenized using a Polytron (Brinkman Instruments, Westbury, NY) in 0.9 mL of post-rigor extraction buffer containing 100 mM Tris base, 10 mM EDTA, 0.05% 2-mercaptoethanol, adjusted with HCl to pH 8.3 and a cocktail of protease inhibitors (Protease Inhibitor Cocktail Tablets; Roche). After centrifugation at 8.800 g for 30 minutes, the supernatant (containing soluble proteins) was collected.

Immunoprecipitation was carried out as follows. 100 μl of a 1:1 slurry of protein A-Sepharose beads was incubated for 1 hour at 4°C with 5 μg of anti-μ-Calpain (DOMAIN IV) (Sigma) or anti-m-Calpain (DOMAIN III/IV) mouse IgG1 (Sigma). The beads were washed three times with lysis buffer (NaCl 150mM, Hepes 20mM, glycerol 10 %, triton 1%), and incubated overnight at 4°C with 100 μg of protein extract. Immunoprecipitated proteins were washed three times with 1 ml of lysis buffer. Cell lysates were used to detect calpain activity by means of the Calpain-Glo protease assay (Promega, WI, USA) that furnish specific calpain luminogenic substrates (7). The Calpain-Glo Reagent was prepared as indicated in the Promega protocol. 25 μl of Calpain-Glo Reagent and 25 μl of sample were mixed and incubated for 30 minutes, and the luminescence was recorded with a luminometer (GloMax™20/20 Luminometry System GloMax™ 96 Microplate Luminometer).

Fibre size

Fibre size was determined from the same section used to determine myofibre number. For each fibre type, using an image-analysing system LAS (Leica Application Suite Interactive measurement), the area, the perimeter, and the

maximum and minimum diameter were measured. For each muscle, not less than 200 fibres, belonging to eight random fields, were measured.

Statistical Analysis

Data were processed by the analysis of variances and means which were estimated by following the general linear model (Proc GLM; SAS Institute, 1992), in which the factors considered are fixed and the effect of the other factors is expressed as deviation from the general average (μ).

The model used was:

$y_{ijk}=\mu+Mu_i+Ft_j+(Mu*Ft)_{ij}+\varepsilon_{ijk}$

y_{ijk} = value of k^{th} relative observation to the j^{th} fibre type of the i^{th} muscle;

Mu_i = fixed effect of the i^{th} muscle (i=1,2,3).

Ft_j = fixed effect of the j^{th} fibre type (j=1,2).

$(Mu*Ft)_{ij}$ = fixed effect of the i^{th} muscle with the j^{th} fibre type.

ε_{ijk} = residual error.

The significance between the mean values was evaluated using the Student's t-test.

Calibration Curve

A calibration curve was created for our results. The percentage of the analyte and the relative response for each standard were obtained using linear progression analysis. This yielded a model described by the equation y=1.15x+2.8 with R^2=0.98. A series of solutions of Calpain-Glo Reagent were prepared with different percentages in order to obtain this line (Figures 1, 2, 3).

RESULTS

The muscles, the object of the study, were dissected following the standards of the gross anatomical methodology. Anatomical nomenclature was taken from "Nomina Anatomica Avium". In the examined muscles, the presence of two fibre types was noticed: type IIA, pink (or intermediate), with moderate m-ATPase activity and intermediate oxidative activity and type I, red, with low m-ATPase activity and high oxidative activity. The type IIB fibres were absent. M. iliotibialis lateralis originates from the postacetabular part of the ilium and inserts via aponeurosis on lateral surface of knee. It is a hip extensor and abductor. Particularly we considered the pars preacetabularis (deep layer). In this muscle, the fibres type I were 56%, and the fibres type IIA 44%. M. femorotibialis medius originates from the lateral femoral trochanter and inserts on the proximal tibio-tarsus via tendofascial sheet of stifle. It consisted of type I fibres (58%) and type IIA fibres (42%). M. fibularis longus originates from the lateral tendofascial sheet of the knee and proximal fibula and inserts on the proximal tarsometatarsus. In the M. fibularis longus, type IIA fibres were 61% and type I fibres 39%. The results of the analysis of variance showed significant muscle x fibre interactions (Table 1) for the morphometric characteristics evaluated.

The differences between M. femorotibialis medius and M. fibularis longus, with regard to fibre area, were 9% for type IIA (4572.55 vs. 4170.83 P <0.001) and 19% for type I fibres (4136.6 vs. 3332.15 P <0.001). The differences between M. femorotibialis medius and M. iliotibialis lateralis pars preacetabularis were 29% for the IIA fibres (4572.55 vs. 3232.70, P <0.001) and 24% for type I fibres (3332.15 vs. 2536.50, P <0.001) (Tables 2 and 3).The other morphometric parameters also had the same trend.

Calpastatin

The immunohistochemical results showed the presence of calpain/calpastatin system in the muscles object of study. At day 0 post-mortem, calpastatin gave a moderately positive reaction at the perinuclear zone and sarcolemma in all the muscular fibres (Figure 5).

At 2 days post-mortem, calpastatin gave a moderately positive reaction as previously, while at 4 and 6 days post-mortem calpastatin gave a positive reaction (Figure 6).

m-Calpain

At day 0 post-mortem, m-calpain gave a moderately positive reaction close to the connective tissue and sarcolemma in all muscular fibres (Figure 7). At day 2 post-mortem, m-calpain gave a positive reaction, while it totally disappeared at 4-6 days post-mortem.

μ-Calpain

At day 0 post-mortem, a marked positive reaction and a clear distribution of the μ-calpain was found near the sarcoplasmatic and nuclear membranes in all the muscular fibres (Figure 8).

At day 2 post-mortem, μ-calpain was clearly localized in the cytosol, while it disappeared by 4 and 6 days post-mortem.

p94

At day 0 post-mortem p94 gave a positive reaction in all muscular fibres (Figure 9).

At 2, 4 and 6 days post-mortem the isoform gave a positive reaction against the peri-nuclear membrane and surrounding cytosol, showing slight variations at interfibrillar connective level (Figure 10).

The negative control is shown in figures 11 and 12; particularly such negative tests show no specific immunoreactions for μ- and m-Calpain in the control section.

The enzymatic activity of μ and m-calpain were seen to change during post-mortem storage in the muscles. M-calpain activity was 44% at 0 days post-mortem, 83% at 2 days post-mortem, 31% at 4 days post-mortem and 8% at 6 days for the M. iliotibialis lateralis pars preacetabularis (Figure 1).

The other muscles considered show a similar trend (Figures 2, 3). However μ-calpain activity decreased more rapidly than that of m-calpain activity. Particularly μ-calpain activity which was 90% at 0 days post-mortem, 52% at 2 days post-mortem, 20% at 4 days post-mortem and 4% at 6 days for the M. iliotibialis lateralis pars preacetabularis (Figure 1). The other muscle considered show a similar trend (Figures 2, 3).

DISCUSSION

Comparison of the pelvic limb musculature of the ostrich with that of other ratites is difficult due to the lack of up-to-date literature. In agreement with the study of Patak and Baldwin (8), we noticed that the pelvic limb musculature of many ratites is similar. In fact, similarly to our study, the origin of M. iliotibialis lateralis in the Brown Kiwi is from the dorsal edge of the ilium over most of its length. However, in this species M. fibularis longus does not form a conjoined tendon with that of M. flexor perforatus digiti III (9). In agreement with Gangl *et al.* (10), we found that in the ostrich the origin of M. femorotibialis medius appears similar to that of the Emu. In the Emu this muscle originates on the cranioproximal surface of the corpus femoris and on the medial side of the crista cnemialis cranialis (8).

Consistently with previous study on four muscles of the Blue Neck ostrich (4), the present study shows that type IIB fibres are totally absent in M. femorotibialis medius, M. iliotibialis lateralis pars preacetabularis and M. fibularis longus. These fibres are mainly recruited during short bursts of activity, when high power output is required. The absence of such fibres in these three muscles could be related to a conversion of type IIB fibres to type IIA fibres, characterized by more versatile oxide-glycolytic metabolism and correlated with differences in energetic demands between flapping and glinding modes of flight (11). Our study shows that M. femorotibialis medius and M. iliotibialis lateralis pars preacetabularis have similar percentage of type IIA (42% vs. 44%) and type I (58% vs. 56%) fibres, and in particular the prevalence type I fibres possibly relating to their functional role in the leg to maintain posture. In a study on the muscle of chicken, Iwamoto *et al*. (12) observed that the M. iliotibialis lateralis is composed of IIA and IIB fibres. Sakakibara *et al*. (13) using adult Silkie and White Leghorn chickens, compared the fibre composition of M. iliotibialis lateralis and M. Pectoralis and they noticed that M. iliotibialis lateralis was composed of two fibre types, IIR and IIW, showing strong and weak reduced nicotinamide adenine dinucleotide dehydrogenase activities, respectively. Torrella et al. (14) noticed that four wing muscles of wild yellow-legged gulls (*Larus cachinnans*) (M. scapulotriceps, M. pectoralis, M. scapulohumeralis, and M. extensor metacarpi) exhibited higher percentages of fast oxidative glycolytic fibres (>70%) and lower percentages of slow oxidative fibres (<16%) than the muscles involved in nonflight locomotion (M. gastrocnemius and M. iliotibialis). The leg muscles studied included a considerable population of slow oxidative fibres (>14% in many regions), which suggests that they are adapted to postural activities. In agreement with the study of Velotto and Crasto (4) our study shows that the M. fibularis longus is a good indicator for the area of type I fibres, while M. femorotibialis medius is good for the area of type IIA fibres. Torrella *et al.* (15) noticed that it is possible that greater surface area to volume ratios of smaller fibres may influence their metabolic functions, as proposed by Gleeson and Harrison (16) who found an inverse relationship between fibre cross-sectional area and enzyme activities in reptilian muscle.

Regarding the calpain/calpastatin system, our study in agreement with that of Geesink and Koohmaraie (17), showing a moderately positive reaction of the m-calpain in the early hours after death. A marked localization of μ-calpain near the sarcoplasmatic and nuclear membranes mainly in the early hours of post-mortem was noticed. In agreement with Koohmaraie (18), we deduce that the μ-calpain, in contrast to the m-calpain, is mainly responsible of the meat tenderness in the first stages after the death when pH values are still close to neutrality, at a temperature of 15 °C and at a Ca^{2+} concentration of approximately 5-6.5 μM (19).

The proteolysis extension induced by the calpains is modulated by the calpastatin, which inhibits the calpains. Calpastatin gave a moderately positive reaction after 2 days post-mortem; this is mainly due to an excessive concentration of Ca^{2+} that affects its degradation. In the early hours after death a light calpastatin localization in the cytosol was noticed. In agreement with De Tullio *et al.* (20) we believe that subsequently to the Ca^{2+} increase, the associated form of calpastatin, localized around the nucleus, undergoes a dephosphorylation performed by a phosphoprotein phosphatase, and distributes itself in the cytosol like soluble protein. p94, does not require calcium for its activation and is not inhibited by calpastatin (1). Moreover our study shows that, also in this species, calpain/calpastatin system is present and acts as in other studied mammals, however a large part of the enzymatic activity ends at 48 hours after death. Boehm *et al.* (21) showed that in mammals such as bovines, extractable m-calpain activity changed slightly during the first 7 days after death (decreased to 63% activity after 7 days), whereas extractable calpastatin activity decreased substantially (to 60% after 1day and to 30% after 7 days of postmortem storage) during this period. This suggests that, although ostrich shows similar characteristics of muscular fibres (colour and diameter) to those of the mammals, the activation and deactivation times of the calpain/calpastatin system are briefer. Other authors confirmed the short time of calpain activity in birds, in particular Lee et al. (22) showed that in chicken muscle the μ-calpain activity had disappeared by 6 h after postmortem, showing an activation by calcium. Finally our study highlights that in ostrich muscles, the rapid maximum tenderness reached could be explained by a greater activation of the m-calpain and μ-calpain systems.

The results suggest that the calpain/calpastatin system in ostriches show a particular trend for different fibres types. Also there is a lack of data on the role and function of the calpain/calpastatin system in the tenderization process in ostrich skeletal muscle, particularly pertaining to the ageing process. The immunohistochemical localization and tissue distribution of the calpain/calpastatin system could be the first step to obtain important information on the function of this system in this species. Finally, as fibre type seems to be responsible of meat quality, future work should be undertaken to study this important aspect on ostrich meat.

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TABLES

Table 1. - *F* value relative to morphometric parameter of muscle fibre

Parameter	Muscle	Fibre type	Interaction muscle*fibre type
Area	183.5***	177.5***	26.3***
Perimeter	199.6***	213.2***	19.5***
Minimum Diameter	75.00***	146.3***	12.5***
Maximun Diameter	193.4***	161.2***	18.5***
*** *P< 0.001*			

Table 2. Mean value (avg.) and variation coefficient (v.c., %) of morphometric characteristics of fibre types in the considered muscles.

Muscle	Fibre type			
	Type IIA		Type I	
	Avg	v.c., %	Avg	v.c., %
	Area/μm²			
FL	4170.83	24	4136.6	35
FM	4572.55	27	3332.15	26
IL	3232.7	25	2536.5	33
	Perimeter/μm			
FL	252.34	16	242.01	20
FM	325.11	16	225.28	20
IL	222.73	15	200.67	19
	Minimum Diameter/μm			
FL	60.31	19	57.85	25
FM	62.21	17	53.05	20
IL	55.05	18	49.59	20
	Maximum Diameter/μm			
FL	90.34	19	85.03	22
FM	96.6	18	79.05	22
IL	80.12	15	75.26	21

FL: *M. Fibularis longus*
FM: *M. Femorotibialis medius*
IL: *M. Iliotibialis lateralis pars preacetabularis*

Table 3. Significant (* P<0.05; ***P<0.001) comparison among muscles related to fibre types

	Fibre Type			
	Type IIA			
			Diameter/μm	
Difference	Area/μm²	Perimeter/μm	Minimum	Maximum
FL-FM	-401.72***	-12.77***	-1.9*	-6.26
FM-IL	1339.85***	42.38***	7.16***	16.48***
FL-IL	938.13***	29.61***	5.26***	79.78***
	Type I			
			Diameter/μm	
Difference	Area/μm²	Perimeter/μm	Minimum	Maximum
FL-FM	804.45***	-16.73***	4.8***	5.98***
FM-IL	795.65***	24.61***	3.46***	3.79***
FL-IL	1600.1***	41.34***	8.26***	9.77***

FL: *M. Fibularis longus*;
FM: *M. Femorotibialis medius*;
IL: *M. Iliotibialis lateralis pars preacetabularis*

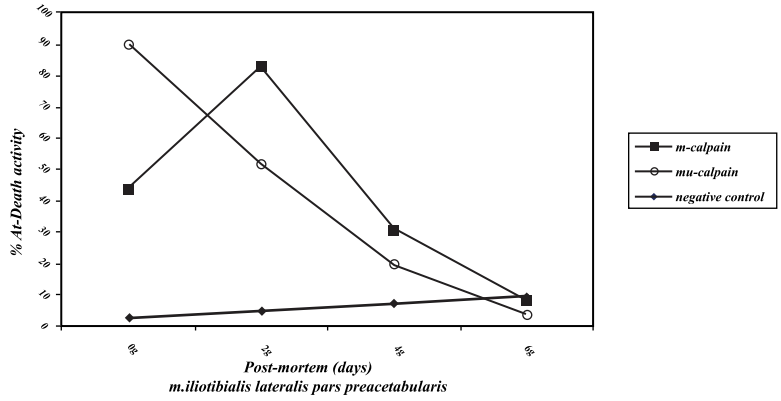

Fig.1. M- and μ– calpain activity at 0,2,4,6 days post-mortem for the **M. Iliotibialis lateralis pars preacetabularis**.

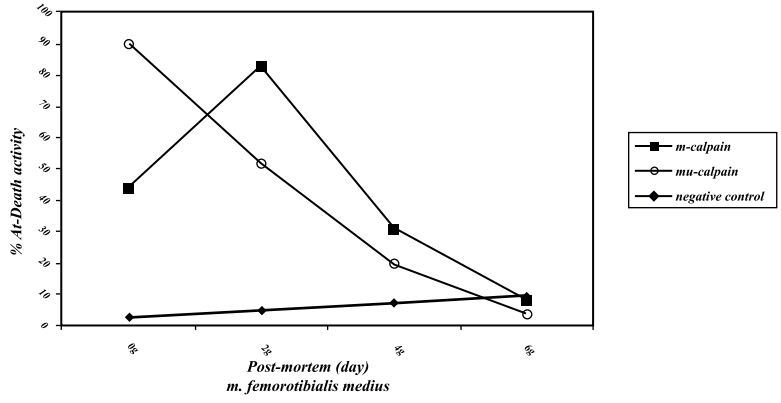

Fig.2. M- and μ –calpain activity at 0,2,4,6 days post-mortem for the **M. Femorotibialis medius**.

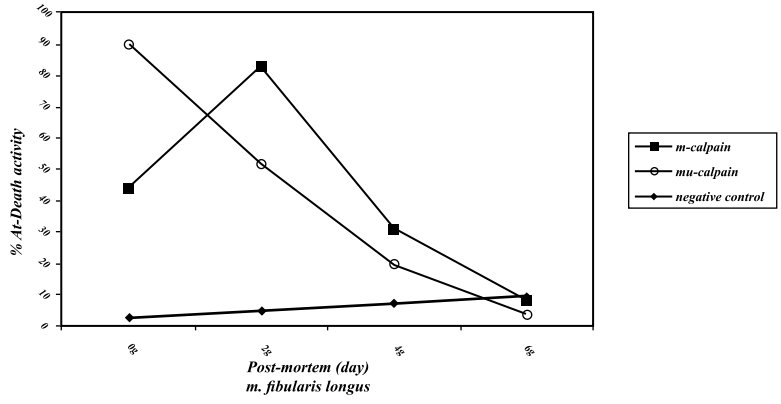

Fig.3. M- and μ –calpain activity at 0,2,4,6 days post-mortem for the **M. Fibularis longus**.

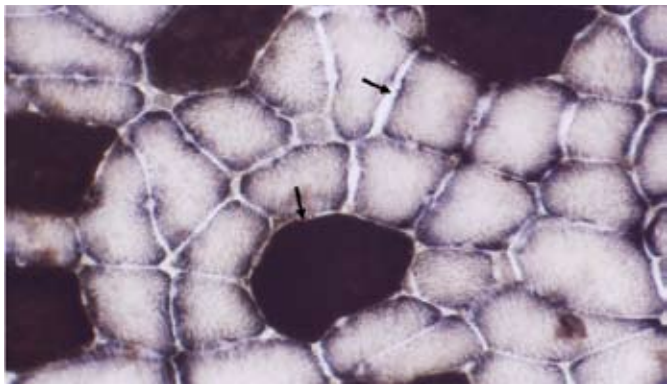

Fig.4. M .Fibularis longus (160x) A light micrograph showing histochemical staining of two types of fibres: slow oxidative, SO (darkest area) and fast oxidative glycolytic, FOG (intermediate area). Cross-section (8-μ) of muscle treated for ATPase + SDH combined reaction after pre-incubation at pH 4.6.

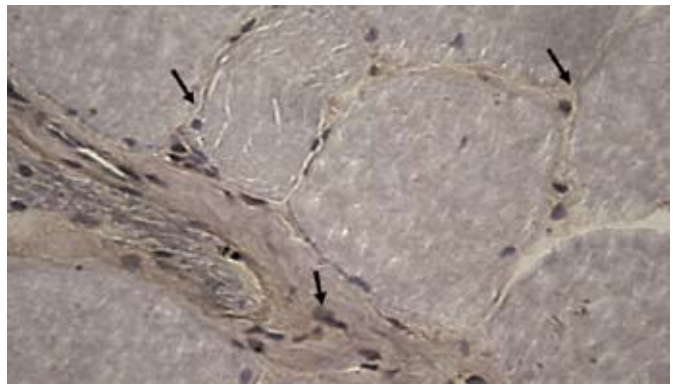

Fig.7. M. Iliotibialis lateralis pars preacetabularis (250x) A light micrograph showing immunohistochemical staining of m-calpain (DOMAIN III/IV) on 8μ serial sections. Fibers integrity was judged by haematoxylin-eosin staining. The arrow indicates m-calpain positive reaction. At day 0 *post mortem* m-calpain gives a moderately positive reaction close to the connective tissue and sarcolemma.

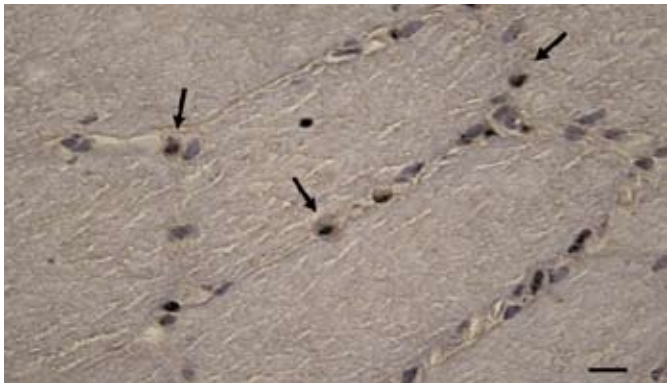

Fig.5. M. Fibularis longus (250x) A light micrograph showing immunohistochemical staining of calpastatin on 8μ serial sections. Fibers integrity was judged by haematoxylin-eosin staining. The arrow indicates calpastatin positive reaction. At day 0 *post mortem* calpastatin gives a moderately positive reaction against the perinuclear zone, and near the membrane.

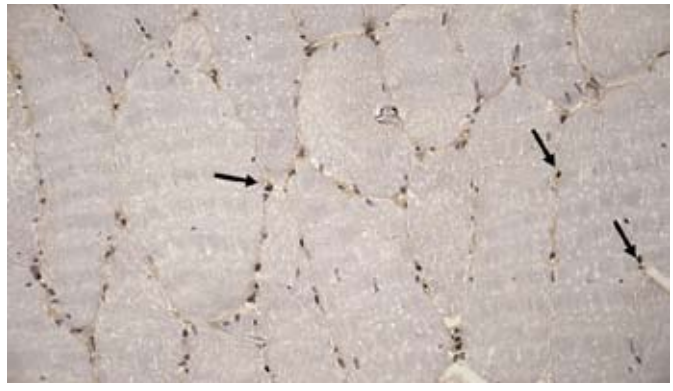

Fig.8. M. Femorotibialis medius (160x) A light micrograph showing immunohistochemical staining of μ-calpain (DOMAIN IV) on 8μ serial sections. Fibers integrity was judged by haematoxylin-eosin staining. The arrow indicates μ-calpain positive reaction. At day 0 *post mortem* μ-calpain gives a marked positive reaction near the sarcoplasmatic and nuclear membranes.

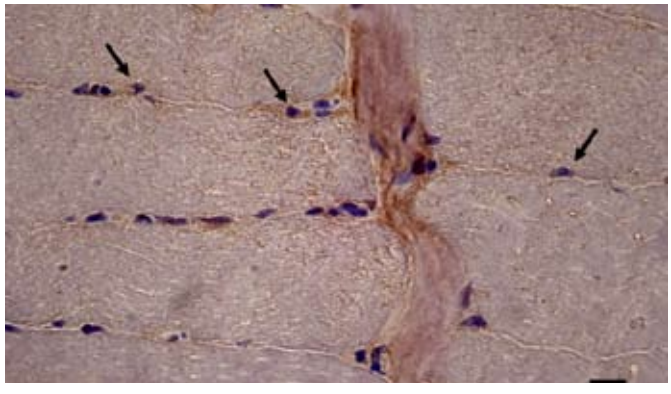

Fig.6. M. Fibularis longus (250x) A light micrograph showing immunohistochemical staining of calpastatin on 8μ serial sections. Fibers integrity was judged by haematoxylin-eosin staining. The arrow indicates calpastatin positive reaction. At 4 days *post mortem* calpastatin gives positive reaction.

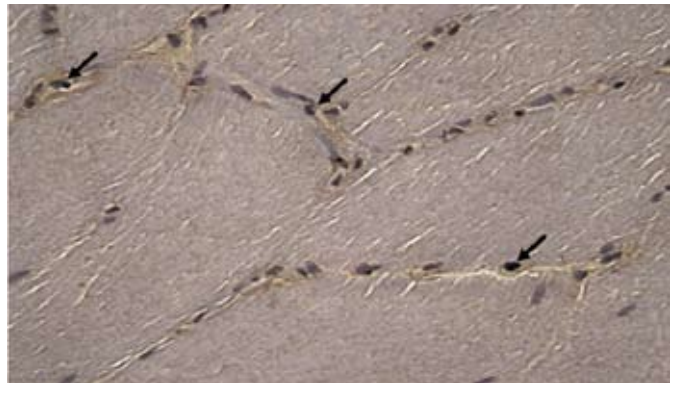

Fig.9. M. Fibularis longus (400x) A light micrograph showing immunohistochemical staining of p94 (DOMAIN I) on 8μ serial sections. Fibers integrity was judged by haematoxylin-eosin staining. The arrow indicates p94 positive reaction. At day 0 *post mortem* p94 gives positive reaction.

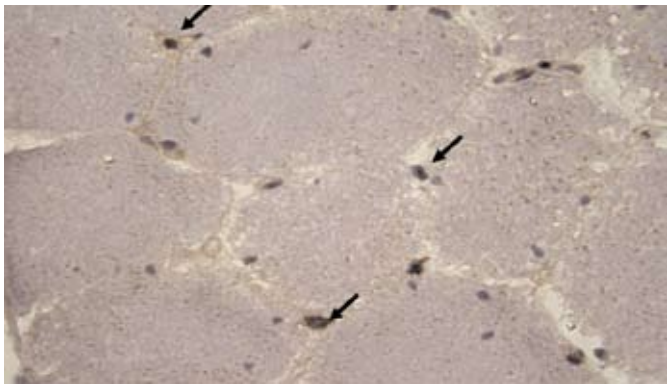

Fig.10. M. Iliotibialis lateralis pars preacetabularis (250x) A light micrograph showing immunohistochemical staining of p94 (DOMAIN I) on 8μ serial sections. Fibers integrity was judged by haematoxylin-eosin staining. The arrow indicates p94 positive reaction. At 4 days *post mortem* p94 gave a positive reaction against the perinuclear membrane and surrounding citosol, showing slight variations at intrafibrillar connective level.

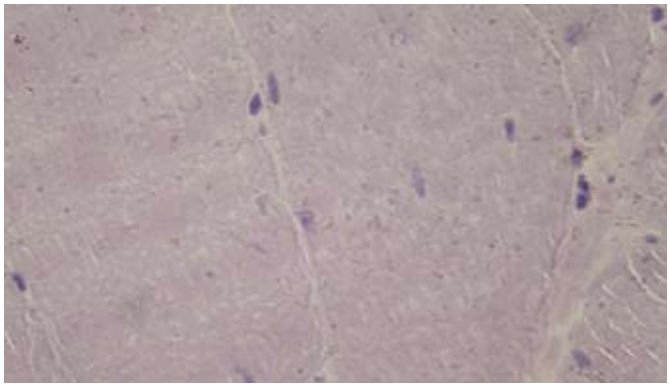

Fig.11. Negative control of -μ-Calpain immunoreaction in thin section of **M. Iliotibialis lateralis pars preacetabularis.** After incubation in the absence of primary antibody against -μ-calpain (large subunit) was produced against the corresponding human antigen (*Rabbit-anti-human*).

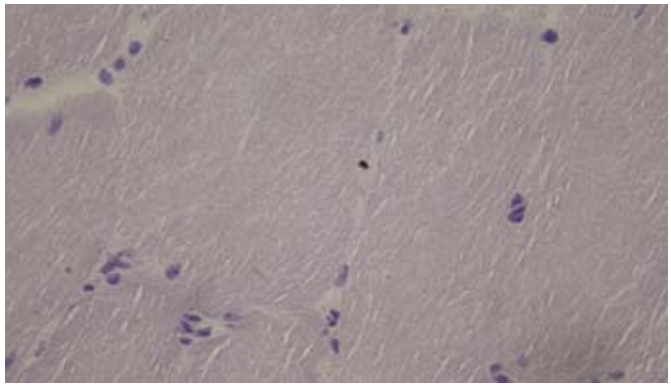

Fig.12. Negative control of -m-Calpain immunoreaction in thin section of **M. Fibularis longus.** After incubation in the absence of primary antibody against m-calpain was produced against the corresponding bovine antigen (*Mouse-anti-bovine*).