Histochemical and morphometric study of the cricoarytenoideus lateralis muscle in the horse

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Summary. Histochemical and morphometric parameters of the cricoarytenoideus lateralis muscle of the horse are presented. Using myosin ATPase staining after acid preincubation, 3 fibre types (I, IIA and IIC) were identified. Using NADH-TR staining, type I fibres showed high oxidative capacity, whereas type II fibres had high or low oxidative capacity. The type I to type II ratio was of 35:65. This ratio remained constant in the age range examined. Statistically significant (p<0.01) differences were found in values for fibre size between groups of horses weighing more than 500 kg and less than 400 kg. Mean area of type II fibres was greater (p<0.001) than that of type I fibres. There were no significant differences in mean area between left and right muscles in the group of animals with less weight. In contrast, significant differences (p<0.05) in mean area between left and right muscles were found for type I fibres in the group of animals exhibiting a higher weight. The histographical distribution of fibre type areas was unimodal. Most adult horses showed muscle fibre type grouping in the left muscle.

Key words: Horse, Muscle fibres, Histochemistry, Cricoarytenoideus lateralis

Introduction

Studies on normal histochemical and morphometric features in intrinsic laryngeal muscles in domestic mammals are scarce (Mascarello and Vegetti, 1979; Sautet et al., 1987; Braund et al., 1988). Such data should be useful as reference values when compared with pathological changes in these parameters secondary to neuropathic disorders (Braund et al., 1982). The idiopathic laryngeal hemiplegia (ILH) is a relatively frequent disease in the horse, produced by changes in the recurrent laryngeal nerve (RLN). In this condition, early

changes including fibre type grouping and atrophied and hypertrophied fibres appear in the adductor muscles of the larynx (Cahill and Goulden, 1986; Duncan et al., 1991). The purpose of the present study was to define normal histochemical and morphometric parameters in one of the laryngeal adductor muscles, the cricoarytenoideus lateralis (CAL), against which abnormal muscle parameters may be compared.

Materials and methods

Twenty-two crossbreed horses in apparent good health, between 4 months and 18 years of age, were used in this study (Table 1). The ages of the horses were determined by examination of the teeth, or obtained from known records. All the animals were derived from abattoir sources. Due to variation in body weight of horses in this study, the population was divided into two groups for quantitative studies: group A (less than 400 Kg body weight) and group B (greater than 500 kg body weight). From these animals the left and right CAL were removed post-mortem from the laryngeal cartilages. From each muscle, a block was taken for frozen transverse sectioning. Within 30 minutes of death of the animal, the muscle was frozen by immersion in isopentane which was standing in liquid nitrogen. The frozen block was mounted on a chuck, using an embedding medium for frozen tissue specimens. Transverse serial sections were cut from each block at a thickness of 10 μm using a cryostat-microtome maintained at -20 °C, and stained for myofibrillar myosin adenosine triphosphatase (ATPase) after preincubation in alkaline (pH 9.4) and acid (pH 4.6 and 4.3) buffer (Dubowitz and Brooke, 1973). Myofibres were classified into types I, IIA, IIB and IIC according to the ATPase staining patterns (Dubowitz and Brooke, 1973). In addition, serial sections were stained for NADH-tetrazolium reductase (NADH-TR) and menadione-linked alpha glycerophosphate dehydrogenase (aGPD), as described by Dubowitz and Brooke (1973).

The mean area (± SD) of type I and type II fibres was

Table 1. Data on horses studied.

HORSE NUMBER	SEX	AGE (years)	BODY WEIGHT (kg)
1	F	0.5	150
2	F	0.8	376
3	M	1.66	254
4	M	1	254
5	M	1	314
6	F	1	328
7	F	1.25	376
8	M	0.3	386
9	F	0.4	384
10	M	0.4	342
11	M	1.25	542
12	M	3	636
13	M	1.5	502
14	F	10	354
15	M	10	330
16	F	10	520
17	F	11	728
18	F	11	708
19	M	12	502
20	F	12	726
21	M	16	606
22	F	18	388

determined from a random fibre count of 100 for each fibre type/muscle specimen using a Leitz-ASM image analyser. A magnification of x 400 was used for muscle area measurements. Percentages of type I and type II fibres were determined from a random count of 500 muscle fibres/muscle specimen from randomly selected fields. Atrophy and hypertrophy factors were calculated from histograms of muscle fibre areas from all horses, as described by Dubowitz and Brooke (1973). In calculating these factors for horses in group A, a fibre area range of 300 to 2300 μm^2 was chosen for type I fibres, and of 1100 to 3500 μm^2 for type II fibres. These numbers were chosen because approximately 85 to 90% of fibres were within this range. Due to the overall greater fibre areas, for group B horses a fibre area range of 300 to 2700 μm^2 was selected for type I fibres, and of 1100 to 3900 μm^2 for type II fibres. A weighting system was used to calculate the atrophy and hypertrophy factors. For example, in type II fibres of group A horses, the number of fibres between 700 to 1100 μm^2 was multiplied by 1, the number of fibres between 300 to 700 μm^2 was multiplied by 2, and so on. These products were added together and then divided by the total number of fibres in the histogram to put the result on a proportional basis. The resulting number was multiplied by 1000, and this was the atrophy factor. The hyper-trophy factor was similarly derived to express the proportion of fibres larger than 3500 μm^2.

Routine statistical methods were used to calculate the mean and standard deviation (SD). Statistical analysis of data was done using a one-way analysis of variance (ANOVA). A level of significance of $p < 0.05$ was accepted as statistically significant. Comparison between the different groups of data was carried out using the least significant difference (LSD) method.

Results

The histochemical reaction for ATPase at pH 9.4 revealed dark-staining type II and light-staining type I fibres (Fig. 1a). This reaction reversed after pre-incubation in the acid pH range. In the great majority of type II fibres this reversal occurred at approximately pH 4.6 (Fig. 1b), and there was little evidence for diversity within type II fibres with regard to the extent of inactivation of ATPase at different points in the acid reversal sequence. For this reason, this group of type II fibres was considered as a homogeneous population of type IIA fibres. A small percentage of type II fibres, less than 2% of the total population, stained darkly at pH 9.4 and moderately at pH 4.3, and were classified as type IIC fibres. The NADH-TR-stained sections showed two distinct intensities of high and low, with high and low corresponding to type II fibres, and high intensity corresponding to type I fibres (Fig. 2a,b). The histochemical reaction for aGPD showed two intensities, with high intensity corresponding to type II fibres and low corresponding to type I fibres (Fig. 1c).

Values for fibre type percentage, mean fibre area and atrophy and hypertrophy factors are given in Table 2. There were no statistically significant differences in fibre type percentage between the two weight groups, between young (less than 3 years old) and adult (more than 10 years old) horses and between left and right muscles. Mean type II fibre area was significantly ($p < 0.001$) greater than that of type I fibres. Mean fibre area of type I and type II fibres was significantly ($p < 0.01$) smaller in group A horses as compared with group B horses. Mean fibre area of type I and type II fibres was significantly ($p < 0.05$) smaller in young horses (less than 3 years old) as compared with adult horses (more than 10 years old). In group B horses, the mean fibre area in left muscle was greater than that of right muscle, and this difference was significant ($p < 0.05$) in type I fibres.

Frequency distribution analysis of fibre areas revealed a unimodal distribution for type I and for type II muscle fibres. Distribution curves are shown in figure 3 for group A horses. Due to the difference between left and right muscles, fibre area distribution curves for group B horses are given separately for left and right side (Fig. 4).

Examination of the distribution of muscle fibres was performed. In left and right CAL from younger horses (less than 3 years old), type I and type II fibres manifested a mosaic pattern (Fig. 5a). In contrast, muscle fibre type grouping was present in left CAL of adult horses (moren than 10 years old) in cases no. 15-21 (Fig. 5b).

Discussion

ATPase staining in this study revealed two major fibre types in CAL: type I and type IIA fibres, and an insignificant number of type IIC fibres. However, type IIB fibres have not been identified with this staining
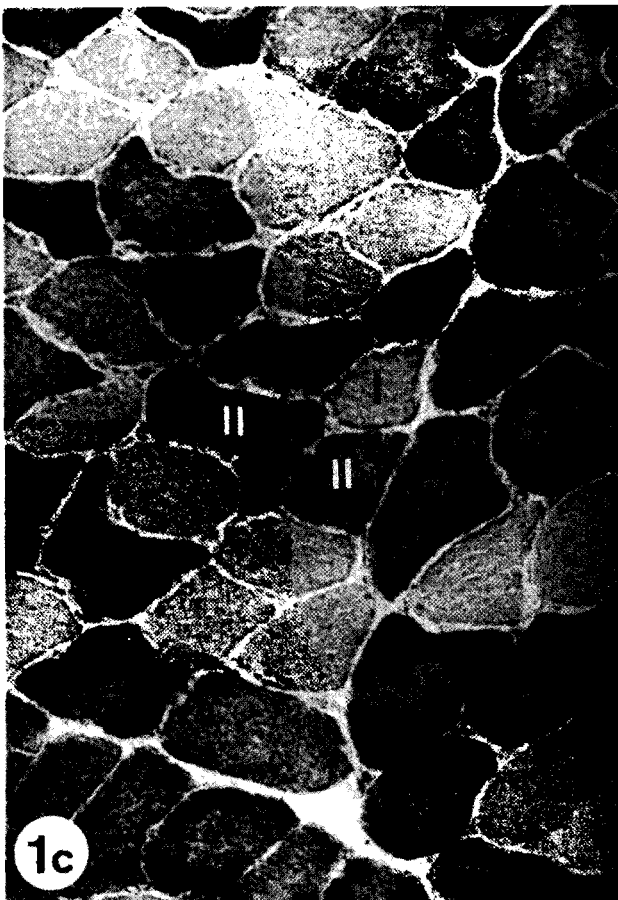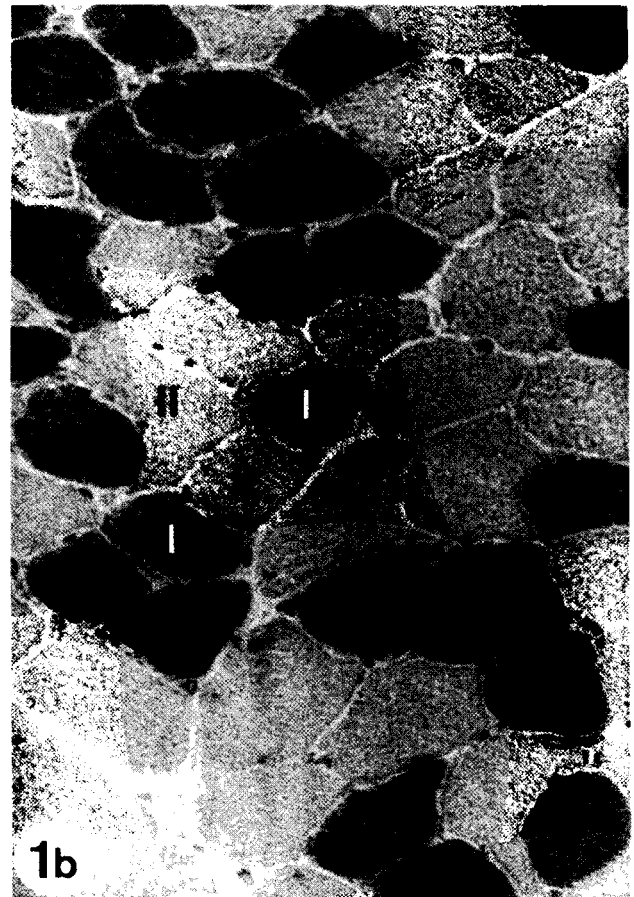
Table 2. Percentages, mean fibre areas and atrophy and hypertrophy factors in the cricoarytenoideus lateralis muscle.

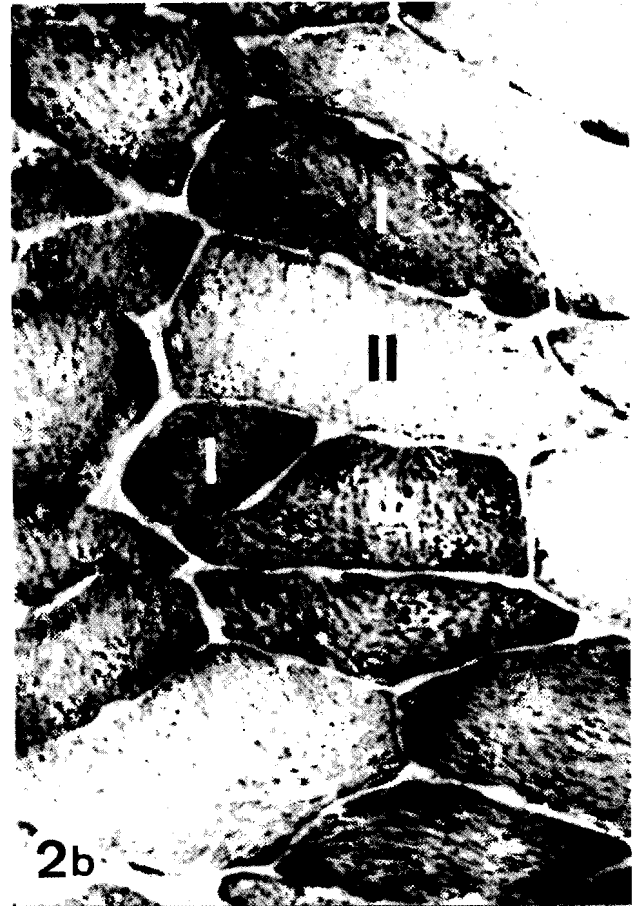|		TYPE I	TYPE II
Group A horses (n= 13)	Fibre percentage (%±SD)	34±6	66±6
	Mean area (μm²±SD)	1529±572	2326±714
	Atrophy factor	21	101
	Hypertrophy factor	157	109
Group B horses (right side) (n=9)	Fibre percentage (%±SD)	36±12	64±12
	Mean area (μm²±SD)	1840±707	2776±857
	Atrophy factor	5	51
	Hypertrophy factor	208	229
Group B horses (left side) (n=9)	Fibre percentage (%±SD)	34±7	66±7
	Mean area (μm²±SD)	2317±944	3031±1048
	Atrophy factor	3	32
	Hypertrophy factor	720	475

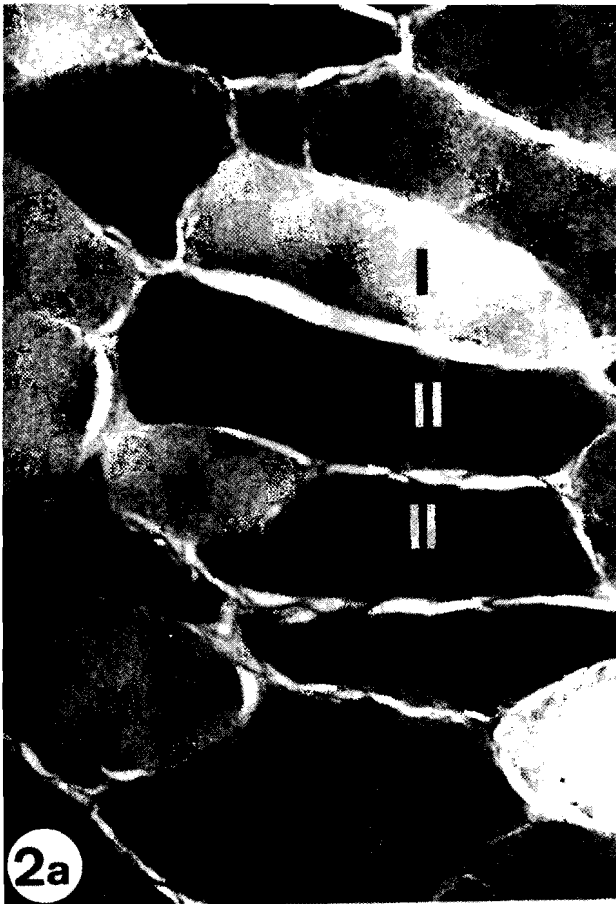**Fig. 1.** Transverse serial sections of cricoarytenoideus lateralis muscle stained for (a) myosin ATPase following preincubation at pH 9.4, (b) myosin ATPase following preincubation at pH 4.6, (c) menadione-linked alpha glycerophosphate dehydrogenase. I: type I fibres; II: type II fibres. x 300
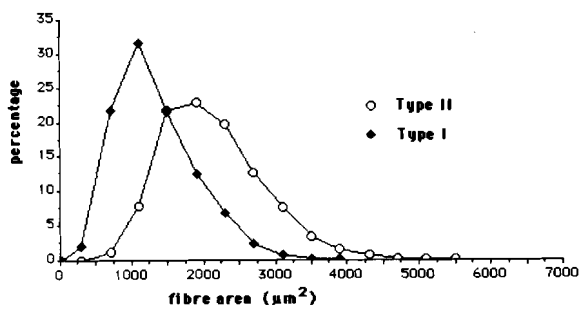
Fig. 2. Transverse serial sections of cricoarytenoideus lateralis muscle stained for **(a)** myosin ATPase following preincubation at pH 9.4 **(b)** NADH-tetrazolium reductase. I: type I fibres; II: type II fibres. x 450

Fig. 3. Histogram (fibre area distribution curve) of the cricoarytenoideus lateralis muscle from group A horses, showing the distribution of type I and type II fibres.
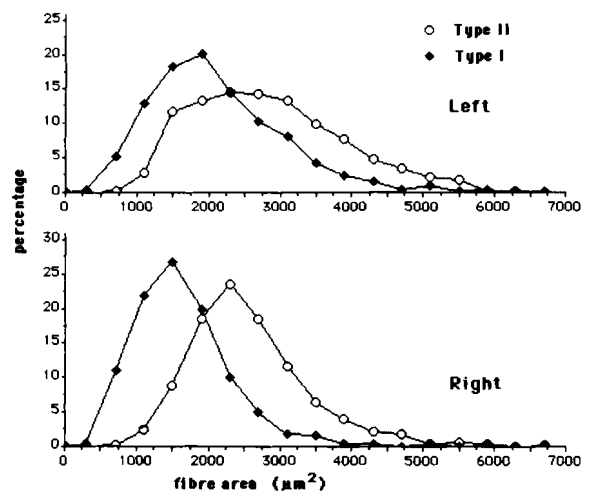
Fig. 4. Histograms (fibre area distribution curves) of the left and right cricoarytenoideus lateralis muscle from group B horses, showing the distribution of type I and type II fibres.

procedure. Type IIB fibres stain intensely at an alkaline pH, moderately at pH 4.6 and lightly at pH 4.3 (Dubowitz and Brooke, 1973). Although we have detected in some cases gradual inactivation of ATPase activity in type II fibres within 4.6-4.5 pH range, the major inactivation of type II fibre ATPase occurred at pH 4.6. For that reason we consider that the population of type II fibres in our study corresponds to type IIA fibres. Type IIB fibres are represented in selected equine appendicular muscles (Andrews and Spurgeon, 1986). However, other studies have shown an absence of type IIB fibres in equine biceps brachii (Hermanson et al., 1991) and in canine appendicular muscles (Braund et al., 1978; Snow et al., 1982). On the other hand, type IIB fibres have not been identified in laryngeal muscles of man (Teig et al., 1978; Malmgren and Gacek, 1981) and of different animal species (Mascarello and Veggetti, 1979; Braund et al., 1988).

The low percentage of type IIC fibres observed in CAL is in agreement with other studies in laryngeal muscles of the dog (Braund et al., 1988) and in limb muscles of the horse (Andrews and Spurgeon, 1986).

Type I fibres stained intensely with the oxidative

stain, NADH-TR. This histochemical profile is similar to that mentioned for equine appendicular muscles (Lindholm and Piehl, 1974; Aberle et al., 1976; Snow and Guy, 1976, 1980; Essen et al., 1980; Snow et al., 1981; Snow, 1983; Essen-Gustavsson and Lindholm, 1985). In our study, results from NADH-TR staining suggest the existence of two fibre populations within type IIA fibres, one of them exhibiting high oxidative activity and another with low oxidative activity. Muscle fibres with low oxidative activity have also been identified by some authors in laryngeal muscles of cat and man (Edström et al., 1974; Lacau-Saint Guily and Fardeau, 1983), but not in the horse (Mascarello and Veggetti, 1979). Our results may seem contradictory, since in equine appendicular muscles fibres with low oxidative activity, as determined by means of NADH-TR staining, are, in general, type IIB fibres which have not been found in this study. However, it is worthwhile to bear in mind that contractile capacity of muscle fibre, as indicated by its myosin ATPase reaction, does not determine its oxidative profile because the contractile and metabolic properties of fibre muscle are regulated independently (Pette, 1985). The oxidative activity of
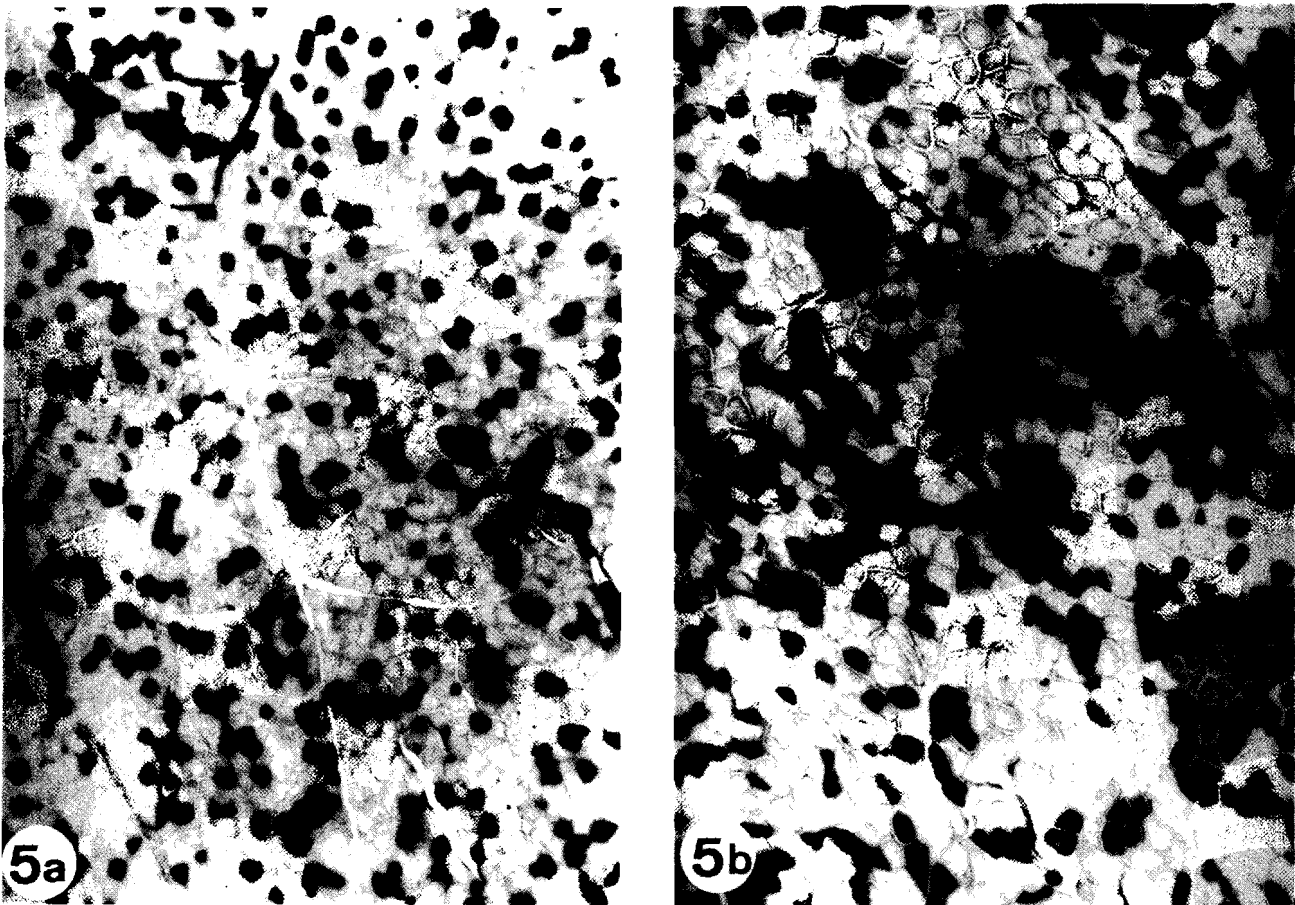

Fig. 5. Transverse sections of left cricoarytenoideus lateralis muscle in a young **(a)** and in an adult horse **(b)**. A normal mosaic pattern is present in a, whereas fibre type grouping occurs in b. Mysoin ATPase at pH 4.3 preincubation. x 50

equine muscle fibres is directly related to their mitochondrial density (Hoppeler, 1983). In contrast, the contractile activity of muscle fibre strictly depends on the molecular structure of the contractile proteins (Gollnick et al., 1973).

Our results for the glycolytic enzyme, aGPD, indicate that type II fibres show a homogeneously high anaerobic capacity as compared with type I fibres. Similar data have been reported in intrinsic laryngeal muscles in different animal species (Edström et al., 1974; Mascarello and Veggetti, 1979; Malmgren and Gacek, 1981; Lacau-Saint Guily and Fardeau, 1983).

It is believed that type II fibres belong to fast-contracting motor units, that are involved in phasic contraction. Although in this study the percentage of type II fibres in CAL was smaller than 82%, a figure previously reported in the horse (Mascarello and Veggetti, 1979), predominance of type II fibres probably reflects the contribution of this muscle to the sphincter function of the larynx, since the necessity for rapid contraction in this mechanism of protection is evident (Teig et al., 1978). Furthermore, the high number of type II fibres in CAL may be related to the fact that, as indicated by electromyographic studies, this muscle shows phasic activity during the expiratory phase of respiratory cycle (Goulden et al., 1976).

In our study, fibre type percentages did not vary between young (less than 3 years old) and adult (more than 10 years old) horses. It is generally accepted that the type I to type II ratio is determined genetically (Komi et al., 1977). Thus, absence of age-related variation in the ratio of type I to type II fibres has been observed in appendicular muscles of dog (Braund et al., 1982), where adult fibre percentages are attained by three months of age (Braund and Lincoln, 1981). In the horse, most authors report that the percentage of type I fibres remains constant throughout postnatal development (Lindholm and Piehl, 1974; Essen et al., 1980; Henckel, 1983). In contrast, an increase in type I fibres with age has been reported in the gluteus medius muscle in thoroughbred horses (Ronéus et al., 1991).

Our studies on fibre size in this study reveal that mean fibre areas of type I and type II fibres in CAL were significantly (p<0.01) smaller in horses weighing less than 400 kg as compared with horses over 500 kg in weight. Although fibre size varies in limb muscles according to body weight (Braund et al., 1982), this relation has not been found in intrinsic laryngeal muscles of the dog (Braund et al., 1988). In our results, type I and type II fibres were significantly (p<0.05) larger in adult horses as compared with young horses. Nevertheless, this difference may be due to a high number of horses over 500 kg in weight among the adult group in our study. In this context, age-related variation of muscle fibre size is not clear. A decrease in fibre size has been reported in adult dogs as compared with younger dogs (Braund et al., 1982). Type I and type II fibre size in CAL was smaller than that reported by some authors in limb muscles of horses (Lindholm and Piehl, 1974;

Essen-Gustavsson and Lindholm, 1985; Valberg et al., 1988; López-Rivero et al., 1990a,b). A similar feature has been recorded in intrinsic laryngeal muscles of the dog (Braund et al., 1988). Small fibre size provides an advantage for oxidative metabolism due to a smaller area necessary for diffusion of oxygen and energetic substrates (Essen-Gustavsson et al., 1984; Essen-Gustavsson and Lindholm, 1985). As a result, this feature as well as the high oxidative capacity observed in intrinsic laryngeal muscles as compared with that of limb muscles (Mascarello and Veggetti, 1979), would facilitate the independence of anaerobic metabolism during muscular contraction and simultaneously provide a higher resistance to prolonged activity. As in equine limb muscles (Lindholm and Piehl, 1974; Aberle et al., 1976; Essen et al., 1980; Snow, 1983; Essen-Gustavsson and Lindholm, 1985), the mean area of type II fibres in CAL was significantly (p<0.001) greater than that of type I fibres. A similar feature has been reported in canine laryngeal muscles (Braund et al., 1988).

Atrophy and hypertrophy factors are a measure of the proportion of muscle fibres outside the normal range (Dubowitz and Brooke, 1973). The low values of atrophy factor in animals of groups A and B in our study reflected few abnormally small fibres in CAL. In a similar way, the low values of hypertrophy factor in group A horses reflected few abnormally large fibres. In contrast, left CAL in group B horses exhibited a high hypertrophy factor and was remarkably higher than that of right CAL. Most horses in group B also showed extensive muscle fibre type grouping in left CAL. These findings might be related with the high percentage of horses subclinically affected by equine laryngeal neuropathy (Cole, 1946; Gunn, 1972; Duncan et al., 1974). A characteristic feature of chronic peripheral neuropathies, as is the case of ILH, is the appearance of a wide variability in frequency distribution of fibre size (Dubowitz and Brooke, 1973). In particular, hypertrophy of muscle fibres can be observed during the early stages of ILH (Duncan et al., 1974). On the other hand, muscle fibre type grouping, a process originated by denervation and subsequent reinnervation of motor units (Karpati and Engel, 1968; Edström and Kugelberg, 1969), appears in the laryngeal muscles of horses subclinically affected by laryngeal neuropathy (Duncan et al., 1974; Cahill and Goulden, 1986). Although ILH is a condition that presents clinical signs in young animals (Cook, 1965), pathological changes in laryngeal muscles become more severe with age (Duncan et al., 1974). In our study, group B horses were mainly animals older than 10 years, which seems consistent with this interpretation.

In the present study, histochemical and morphometric characteristics have been described for the crico-aryteneoideus lateralis muscle of the horse. These data may provide information about muscle fibre composition, morphometry and enzyme characteristics, and might be used as a reference for comparison in disease. Because of the high incidence of equine

laryngeal neuropathy, most adult animals exhibited neurogenic changes in left CAL. For that reason, these animals cannot be used in establishing a range of incidence of normal morphometric features in CAL. The use of young animals not yet exhibiting neurological changes in CAL precludes this problem, but in that case the influence of body weight in muscle fibre size must be taken into consideration.

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