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Immunohistochemical analysis of laryngeal muscle of horses clinically affected with recurrent laryngeal neuropathy

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Abstract

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Background: As myosin heavy chain (MyHC) profile of muscle fibres is heavily influenced by neural input, changes in MyHC expression are expected in horses clinically affected with recurrent laryngeal neuropathy (RLN) yet this has not been thoroughly investigated.
Objectives: To describe changes in MyHC and fibre diameter in left cricoarytenoideus dorsalis muscle (L-CAD) of horses with clinical signs of RLN.
Study design: Observational cohort study.
Methods: Immunohistochemistry was used to assess the MyHC-based fibre-type proportion, size and grouping in the L-CAD of ten Thoroughbred horses, five clinically affected with RLN and five unaffected controls based on resting endoscopic examination. The Mann-Whitney U test was used to compare the two groups.
Results: Compared to controls (of mean age 3.0±1.7 years) which only expressed type I, IIA and IIX MyHC, the L-CAD of affected horses (of mean age 2.8±0.8 years) had obvious fibre-type grouping, and despite apparent compensatory hypertrophy of a small number of fibres, a decrease in overall fibre diameter (median difference -35.2 μm, 95% CI -47.4 to -7.9, P =0.02) and diameter of type IIA fibres (median difference -46.8 μm, 95% CI -52.1 to -5.0, P=0.03). Anti-fast MyHC (MY32) cross-immunoreacted with embryonic-MyHC. Whereas MY32-positive fibres were identified as type IIX in controls, in affected horses these fibres were less than 50 μm diameter with internal nuclei and were MYH3-positive for embryonic myosin indicating depletion of type IIX fibres yet active regeneration and fibre renewal.
Main limitations: Small sample size that did not include subclinical cases. Fibre size and appearance rather than staining colour were relied upon to differentiate embryonic from type IIX MyHC.
Conclusions: Horses clinically affected with RLN have overall atrophy of fibres, loss of IIX fibres and expression of embryonic myosin indicating regenerative capacity. Despite hypertrophy of some remaining fibres, the overall decline in the bulk of fibres including those most fatigue resistant may be the critical change that results in failure to maintain arytenoid abduction during exercise although direct comparison to subclinical cases is needed to confirm this.

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Introduction

Recurrent laryngeal neuropathy (RLN) is an important performance limiting disease of horses characterised by progressive loss of large diameter myelinated axons most severe in the distal

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portions of the left recurrent laryngeal nerve (rLN)[1-3]. Changes in the laryngeal muscles are characteristic of neurogenic disease that is chronic and repetitive in nature with cyclic denervation and reinnervation [2; 4]. Although adductor muscles are also affected [5], dysfunction of the cricoarytenoideus dorsalis muscle (CAD), the only abductor, is clinically most important. Failure of abduction of the left arytenoid and vocal cord results in respiratory impairment during inspiration, with resultant decreased athletic performance and abnormal respiratory noise during exercise.

With RLN, changes in the laryngeal muscles are evident to varying degrees and include fibre-type grouping, atrophy of single fibres or groups of muscle fibres, compensatory hypertrophy of some remaining fibres, and eventual fibrosis and fat replacement [2; 4]. Neuropathologic changes within the rLN and laryngeal muscle [2; 6] and reduced conduction velocity in the left rLN [7] also occur in many endoscopically normal horses and these animals are considered sub-clinically affected.

The functional demands of muscles are met by a variety of muscle fibre types that have a range of speed, endurance and power characteristics. Fibre type is determined by expression of different isoforms of myosin heavy chain (MyHC) [8]. Normal laryngeal muscle of mature horses is comprised of three fibre types, slow (type I), IIA and IIX [9; 10], expected to provide motor units of this muscle with a tenfold range of speeds [11]. Embryonic-MyHC is not normally present in adult muscle yet is transiently re-expressed during muscle regeneration following injury or disease and can be used to identify regenerating muscle fibres [12-14]. As altered neural input occurs with RLN and MyHC profile is heavily influenced by neural input [15], changes in MyHC profile occur in this disease [9]. Knowledge of fibre type distribution is essential for our understanding of laryngeal function, RLN and adaptive mechanisms that enable subclinical horses to remain asymptomatic. In the left CAD muscle (L-CAD) of horses considered sub-clinically affected with RLN there was virtual elimination of type IIX fibres, a lower proportion of slow fibres, greater abundance of IIA fibres, and hypertrophy of many remaining slow and IIA fibres [9; 10]. Loss of type IIX, the fastest fibres present in equine laryngeal muscle, is expected to be associated with a reduction of the speed range of laryngeal muscles from 10-fold to only 4-fold [11] and may explain some of the alterations in movement of the left arytenoid cartilage observed in horses with partial paralysis. Yet as these horses can maintain laryngeal abduction during exercise, loss of IIX fibres appears tolerated and we assume hypertrophy of remaining fatigue resistant

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fibres allows horses to remain subclinical. Whilst this suggests that nerve fibres innervating IIX muscle fibres are more susceptible to damage incurred by RLN, the sub-clinically affected horses reported by Rhee and others [9] were at least 10 years of age and horses clinically affected with RLN were not included.

We aimed to identify changes in MyHC expression and fibre diameter in the L-CAD muscle of horses with clinical signs of RLN and hypothesised that these horses would have loss of IIX fibres, a reduction in the bulk of slow (type I) fibres and expression of embryonic-MyHC.

Materials and Methods

A section of muscle, 5-10 mm in diameter and approximately 10 mm in length, was dissected from the mid-belly of the lateral compartment of the L-CAD from a convenience sample of five clinically affected (RLN group) and five endoscopically normal Thoroughbred horses (controls) in race training. Samples from the RLN group were collected during prosthetic laryngoplasty surgery from horses with Havemeyer grade III.2 or greater [16] on resting laryngoscopic examination and a history of exercise intolerance. Control samples were collected from horses with normal laryngeal function (Havemeyer grade I) on resting endoscopic examination immediately after euthanasia for unrelated reasons. Endoscopic examination was performed within 24 hours of sample collection in all horses and laryngeal function was graded by the same observer. Samples were identified by number only to ensure blinding when obtaining the measurements.

Immediately following collection, muscle samples were coated with OCT compound, snap frozen in isopentane quenched liquid nitrogen and stored at -80°C until immunofluorescent staining. Frozen muscle samples were mounted using OCT compound to allow cutting of 10um transverse sections in a cryostat at -20°C. Staining was performed immediately following sectioning using a methodology based on that reported by Tulloch *et al*. [17]. All antibodies were directly conjugated to ALEXA dyes using the Zenon labelling technology. Type I fibres (slow) were sequentially labelled with NOQ7.5.4D (ALEXA350) and type IIA (fast) with N2.261 (ALEXA488). MY32 antibody was used (which identifies all type 2 fibres) labelled with ALEXA594. With this staining regime under fluorescence type I fibres will appear green, type IIA fibres yellow/orange and remaining fast fibres (type IIX) appear red. Separate transverse muscle cryosections (10μm) were stained for embryonic myosin using a mouse anti-MYH3 antibody (clone F1.652) in combination with ALEXA594 labelled

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MY32 as described above to confirm cross reactivity of the two antibodies. These sections were blocked in 10% (v/v) donkey serum (Millipore, Billerica, Massachusetts, USA) in wash buffer (0.1%Tween, 0.5%BSA in 1xPBS) for one hour, and then incubated with the primary antibody anti-MYH3 (Santa Cruz Biotechnology, 1:200), for 60 minutes at room temperature. The sections were washed in PBS and then incubated with the fluorescent secondary antibody, donkey anti-rat IgG Alexa Fluor 488 (Life Technologies, 1:250) in the dark for 90 minutes. Nuclei were stained in all sections with 1µg/µl Hoechst (Life Technologies) in PBS for one minute before mounting with polyvinyl alcohol with glass coverslips.

Digital images were captured on a Zeiss Axio Imager M1 upright fluorescent microscope with an AxioCam MRm camera running AxioVision software V4.8.2.0 (Carl Zeiss, Oberkochen, Germany). A minimum of five images were collected per section at 100x magnification to allow the analysis of a minimum of 250 fibres. Analysis of images was performed by the same observer using Image J (National Institute of Health, version 1.8.0_1121) with each individual fibre being traced, categorised based on myosin staining pattern and the minimum Feret's diameter (defined as the closest possible distance between two parallel tangents of the muscle fibre) automatically calculated by the software.

Prism 8.3.1 (Graphpad Software, San Diego, CA) was used for statistical analysis. The coefficient of variation (CV%) for each horse and fibre type was defined as (standard deviation of fibre diameters/mean of fibre diameters) x 100. The Shapiro-Wilk test was used to test for normality of values for each group of five control horses and each group of five affected horses. Three of these 22 groups were not normally distributed and each of these three groups had a statistical outlier. These groups were proportion of type IIX/embryonic fibres in the control group, fibre diameter of type IIA fibres in the affected group and CV% of type IIX/embryonic fibres in the affected group. The distributions of the affected and control groups were compared using the Mann-Whitney U test. The Hodges-Lehmann (H-L) estimate of the difference between two population medians was estimated as the median of the set of 25 (5 by 5) differences between each value in the affected group and each value in the control group. A p-value <0.05 was considered significant.

Results

Ten Thoroughbred horses including five clinically affected with RLN mean age 2.8 [SD 0.8] years and with resting endoscopic grade [16] of III.2 (2 horses) or III.3 (3 horses) and five

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endoscopically normal (grade I) mean age 3.0 [SD 1.7] years were used (Table 1). The L-CAD muscle of affected horses (RLN group) had obvious fibre-type grouping with groups of small diameter fibres of the same type and hypertrophy of some remaining fibres (Figure 1A) while control horses had a normal mosaic fibre pattern (Figure 1B). Although the proportion of each fibre type did not appear to differ in the L-CAD muscle of clinically affected horses compared to controls (Table 2), anti-fast MyHC antibody cross-reacts with developmental MyHC forms [18] and so the double staining technique used did not differentiate embryonic from IIX fibres. There was a significant decrease in overall fibre diameter in affected horses ([p =0.02]; Table 3). This included a significant decrease in median fibre diameter of type IIA (p=0.03) and embryonic/IIX (p=0.008) fibres in clinically affected horses, although the distribution in fibre sizes did indicate the presence of a small number of larger fibres suggesting some potential hypertrophic compensation (Figure 2). The smaller diameter (less than 50 μm) of most of the fibres stained positive for type IIX/embryonic and internally placed nuclei indicated active regeneration and fibre renewal consistent with embryonic myosin and depletion of IIX fibres. Importantly, in the additional sections stained using anti-MYH3 (anti-embryonic), embryonic-MyHC was only observed in the affected animals with a total absence in controls (Figure 3).

In control samples considered to be normal the extent of variation in myofibre diameter through calculating a coefficient of variation or CV% (Table S1) was consistent across all fibre types and when all fibre diameters were considered as a group. In comparison, the extent of variation in clinically affected samples was consistently higher indicating changes most likely related to processes such as denervation, compensatory hypertrophy and regenerative myogenesis. However, in samples from clinically affected horses, the distribution of embryonic/IIX fibre sizes showed a distinct pattern. This was attributed to regenerative myogenesis (embryonic MyHC positive) with a tight peak (with little variation) of fibre diameters less than 50uM and less than the smallest type IIX fibres in controls.

Discussion

Despite compensatory hypertrophy of some remaining fibres, we identified an overall decrease in muscle fibre diameter, loss of IIX fibres and presence of embryonic fibres in Thoroughbred racehorses clinically affected with RLN. There was a shift to smaller diameter type IIA fibres with disease yet for type I fibres the reduction in median diameter was not significant. We previously reported loss of type IIX fibres, no change in the proportion yet

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significantly fewer type I and IIA fibres per microscopic field in the L-CAD of four horses with advanced RLN [10]. Indeed, gross atrophy of the L-CAD muscle is usual in clinically affected animals. A left to right ratio <0.8 of CAD muscle thickness measured using transoesphageal ultrasound correlated with muscle volume and predicted dynamic collapse (exercising grade C)[19]. Furthermore, an increase in type I fibres was observed in laryngeal muscle of Thoroughbred racehorses in training (E. Walmsley, personal communication). On these bases we theorised that clinically affected horses might have lost a critical volume of the most fatigue-resistant slow (type I) fibres in addition to faster type IIA and fastest type IIX fibres. Although loss of the aforementioned fibre-types might be the critical factor in determining whether horses are clinically affected with RLN with failure to maintain arytenoid abduction during exercise we were unable to confirm this when comparing this small sample size of horses. Even minor loss of slow fibres would drastically reduce dynamic range of contraction speeds and reduce the capacity of the L-CAD muscle to endure sustained periods of abduction. Such functional loss is sometimes detected on endoscopic examination during high speed exercise in horses with partial laryngeal paralysis. Further evaluation of the impact of RLN on the bulk of type I fibres with advancing disease required.

Embryonic myosin was only identified in clinically affected horses, all with obvious or marked abductor deficit and failure to achieve and maintain abduction yet not complete immobilisation of the arytenoid cartilage and vocal fold (i.e. Havemeyer grade III.2 and III.3) [16]. This indicates that affected horses maintain regenerative capacity, an important finding as it suggests that surgical reinnervation likely remains possible unless extensive muscle fibrosis occurs. This regenerative capacity may also preserve some muscle function for a period of time following onset of disease. We had predicted that with advanced denervation in clinically affected horses muscle fibre regeneration would be stimulated [20], that denervated fibres would express embryonic-MyHC isoforms [20-22] and as surviving nerve fibres would no longer be able to maintain a functionally sufficient population of remaining muscle fibres that marked atrophy would occur. Indeed, this was observed in clinically affected horses in the current study. In earlier work by the first author and others, a significant increase in the number of myonuclei per muscle fibre, central nuclei, and activation of muscle satellite cells was observed in the L-CAD muscle from clinically affected horses regardless of age, degree of atrophy or duration of disease. The latter finding suggests that the muscle is attempting to regenerate by intrinsic muscle satellite cell activity[10].

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As discussed earlier some horses with normal laryngeal function have subclinical disease. Rhee *et al.* described loss of the fastest (type IIX) fibres in laryngoscopically normal horses (all >10 years of age) with no history of exercise intolerance and considered sub-clinically affected based on fibre-type grouping [9]. Based on these findings it appears that loss of IIX fibres may be well tolerated. This earlier study also provided insight into adaptive mechanisms that enable subclinical horses to remain clinically unaffected as these horses also demonstrated a decrease in the proportion of slow fibres suggesting some loss of slow fibres follows the loss of type IIX fibres, yet an increase in the proportion of type IIA fibres and hypertrophy of remaining slow and type IIA fibres [9]. The present study considered clinically normal horses compared to those with diagnosed clinical disease. In unaffected horses in both studies type IIA fibres predominated in the L-CAD followed by slow (type I) fibres and relatively few type IIX. The virtual absence of type IIX fibres in the LCAD of sub-clinical horses [9] is consistent with our results in clinically affected samples in that the central position of the nucleus, the small diameters (vast majority were smaller than the smallest fibres in the control samples) and the cross reactivity with embryonic MyHC indicate an absence of IIX fibres. While sub-clinical samples show overall hypertrophy of remaining type IIA and slow (type I) fibres [9] our data shows a shift to smaller fibres. This is probably related to severity of disease and the chronic nature of the denervation.

While the exact prevalence of subclinical disease is unknown, pathologic changes in the rLN and laryngeal muscles are present in 30% or more of horses [1; 5; 7; 23-25]. As a result, we expected to encounter subclinical disease in some of the control horses in the current study, yet fibre-type grouping was not observed in this group. We propose that this may have been due to the age of horses (6 years or less) and the small sample size. Similarly, muscles without fibre-type grouping reported by Rhee *et al*. were from horses aged 2 and 6 years (and from a third horse of unknown age) [9]. Study of a larger number of horses would be necessary to identify fibre-type grouping in young horses without clinical signs of RLN. Then, the direct comparison of L-CAD muscle samples from subclinical and clinically affected, age and training status matched, young horses stained using the same method may be undertaken to improve our understanding of the critical point at which subclinical disease becomes clinical.

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As expected based on findings in horses with subclinical disease [9], the loss of type IIX fibres identified in clinically affected horses in the current study was also predicted. Loss of the largest myelinated axons occurs in RLN with loss of the fastest conduction velocity nerve impulses expected. As these nerve fibres innervate type IIX muscle fibres (the fastest fibres present in the equine CAD muscle[9]), it follows that type IIX fibres are more susceptible to damage incurred by equine RLN, or their signals more easily corrupted than those innervating type IIA or type I (slow) fibres. It should be noted, however, that an antibody that specifically reacts with MyHC-IIX was not used to identify type IIX fibres in the current study. Attempts in our laboratory to use a specific MyHC-IIX monoclonal antibody have been unsuccessful. With the double staining method used, both type IIX and embryonic fibres stain red, yet the smaller diameter (less than 50 μm) of the majority of fibres and internally placed nuclei indicating active regeneration and fibre renewal identified positive stained fibres as expressing embryonic myosin in affected horses. This was supported by positive staining for embryonic fibres using anti-MyH3 (anti-embryonic) on additional sections.

As expected there was obvious fibre-type grouping in clinically affected horses in addition to scattered angular fibres, groups of atrophied fibres and some remaining hypertrophied fibres with central nuclei [26]. Fibre-type grouping is a diagnostic sign of early neuropathy and an indicator of partial denervation followed by reinnervation of muscle fibres by intact nerve terminals of neighbouring fibres [27]. Clusters of muscle fibres acquire the same histochemical properties since neural influence determines fibre-type and changes represent ongoing, continual or intermittent nerve injury with repeated attempts at reinnervation. In comparison, although surgical transection of the rLN results in immediate laryngeal paralysis, it does not result in fibre-type grouping and changes in MyHC profile differ from those seen with naturally occurring disease [10; 28].

We only included Thoroughbred horses of racing age. This is likely important as ageing-related changes in the expression of MyHC fibres have been observed in aged horses [29-32]. Furthermore, we only included horses in race training as the intensity and duration of training may influence MyHC fibre type not only in equine locomotor muscles [29; 33] but also in laryngeal muscle. On this basis, as mentioned earlier, our preliminary investigations of gene expression for MyHC fibre type in laryngeal muscle of trained compared to untrained Thoroughbreds suggests that there is upregulation of slow MyHC with training (i.e. a shift

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towards a slower fibre type occurs as a training adaptation in laryngeal muscle as it does in gluteal muscle) (E. Walmsley *et al*., personal communication).

A limitation of the current study is that the sample size might not have been large enough to detect any decrease in the proportion and mean diameter of type I fibres in affected horses compared to controls. In addition, none of the affected horses had complete immobility of the left arytenoid and vocal cord on resting endoscopy. The total bulk of type I fibres may be critical in maintaining abduction during exercise and this might not be reflected by considering the proportion of type 1 fibres or their mean diameter in isolation. A further limitation of the current study is that laryngeal function was assessed at rest but not by dynamic endoscopy during exercise and although resting laryngeal function is reasonably sensitive and highly specific for predicting laryngeal function at exercise [34] we cannot be entirely sure that all control horses had normal laryngeal function during exercise. However, we only included horses with a known history of normal exercise tolerance in the control group and with exercise intolerance in the affected group.

In conclusion, horses clinically affected with RLN have an overall decrease in muscle fibre diameter attributed to a reduction in size of IIA fibres, depletion of IIX fibres and expression of embryonic myosin indicating remaining potential for regeneration. Although there is hypertrophy of some remaining fibres, we propose that the overall loss of bulk of more fatigue resistant muscle fibres and not simply loss of IIX fibres, which appears tolerated as it occurs in subclinical horses[9], results in the failure to maintain arytenoid abduction during exercise. Longitudinal studies are required to identify changes over time and to identify cut-off points for L-CAD muscle volume and more specifically for type I fibre bulk that differentiate dynamic collapse from horses able to maintain adequate abduction during exercise.

Authors' declaration of interests

No competing interests have been declared.

Ethical animal research

This study was approved by the University of Melbourne Animal Ethics Committee.

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Owner informed consent

Client consent was obtained for inclusion in the study.

Data accessibility statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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None.

Authorship

C. Steel, E. Walmsley, B. Ahern and J. White contributed to study design. C. Steel, E. Walmsley, C. Coles and J. White contributed to study execution. C. Steel, G. Anderson and J. White contributed to data analysis and interpretation. C. Steel, E. Walmsley, J. White and G. Anderson contributed to preparation of the manuscript. All authors gave their final approval of the manuscript. G. Anderson and J. White had access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Figure legends

Figure 1A: Transverse sections of the left cricoarytenoideus dorsalis muscle of a 3-year-old Thoroughbred entire male with recurrent laryngeal neuropathy (grade III.2). A sequential double-staining immunofluorescent technique was used (type I fibres are labelled with NOQ7.5.4D [ALEXA350], type IIA with N2.261 [ALEXA488] and all remaining type II fibres and embryonic with MY32 antibody [ALEXA594]). Type I fibres are stained green, type IIA yellow-orange and small red staining fibres are considered embryonic fibres due to their small size and internal nuclei. Additional immunolabelling with 1µg/µl Hoechst identifies all nuclei (blue staining). Fibre-type grouping and marked reduction in the diameter of type IIA and some type I fibres is evident.

Figure 1B: Transverse cryostat section of the left cricoarytenoideus dorsalis muscle of a 3-year-old Thoroughbred gelding with normal (grade I) laryngeal function on endoscopic

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examination at rest. The staining protocol used was the same as for figure 1A. There is a normal mosaic pattern with a predominance of type IIA fibres (yellow orange), fewer type I (green), and a small number of type IIX (red) staining fibres. Nuclei are stained blue. Scale bar = 100 μm.

Figure 2: Distribution of muscle fibres by diameter for type I (a), type IIA (b) and type IIX or embryonic-MyHC in the left cricoarytenoideus dorsalis muscle of horses clinically affected with recurrent laryngeal neuropathy (affected) compared to endoscopically normal horses (controls). Note that although the double staining technique used could not differentiate embryonic from IIX fibres, the smaller diameter (<50 μm) of most red-staining fibres together with internally placed nuclei evident on stained sections (not shown) indicated active regeneration and fibre renewal consistent with embryonic myosin in affected but not control horses.

Figure 3: Transverse sections of biopsy samples of left cricoarytenoideus dorsalis muscle from an unaffected (control) horse (A and C) and a horse clinically affected with recurrent laryngeal neuropathy (B and D). There is a lack of positively stained fibres in the negative control samples (IgG only) from the unaffected (A) and affected (B) horse. Lack of staining is also evident with embryonic MyHC staining of a sample from an unaffected horse (C) but in the affected horse (D) many regenerating fibres reactive for embryonic myosin (green staining) and demonstrating internal nuclei are evident. Sections are stained using anti-MYH3 as the primary antibody and a secondary fluorescent (green) antibody. Additional labelling identifies nuclei (blue staining). Scale bar = 100 μm.

Table 1: Age, sex and resting grade of laryngeal function of Thoroughbred racehorses

Group	Age (years)	Sex	Havemeyer grade* of resting laryngeal function
Control	2.0	Male entire	1
	2.0	Male castrate	1
	2.0	Male castrate	1
	3.0	Male castrate	1
	6.0	Female	1
RLN Group	2.0	Male castrate	3.2

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2.5	Male castrate	3.2
2.5	Male entire	3.3
3.0	Male entire	3.3
4.0	Male castrate	3.3

RLN = Recurrent Laryngeal Neuropathy. *From Robinson (2004) Havemeyer grade 1: All arytenoid cartilage movements are synchronous and symmetrical and full arytenoid cartilage abduction can be achieved and maintained; grade 3.2: Obvious arytenoid abductor deficit and arytenoid asymmetry and full abduction is never achieved; grade 3.3 Marked but not total arytenoid abductor deficit and asymmetry with little arytenoid movement. Full abduction is never achieved.

Table 2: Percentage of each muscle fibre type in the left cricoarytenoideus dorsalis muscle based on double-staining immunofluorescent technique (type I fibres are labelled with NOQ7.5.4D [ALEXA350], type IIA with N2.261 [ALEXA488] and all remaining type II and embryonic fibres with anti-MY32 [ALEXA594]).

Fibre	Group	N	Median	Min	Max	H-L Diff	95% CI	p-value
Type I	Control	5	26.18	11.36	35.25			
						-0.02	-19.44 to	
	Affected	5	15.91	12.04	58.96		32.78	>0.9
Type IIA	Control	5	64.51	56.91	87.88			
						-8.61	-37.02 to	
	Affected	5	61.93	33.96	86.11		21.60	0.5
Type IIX/embryonic	Control	5	2.84	0.38	22.65			
						6.71	-9.58 to	
	Affected	5	13.07	1.85	23.85		21.01	0.2

H-L Diff: Hodges-Lehmann estimate of the difference between two population medians. CI: Confidence interval

Table 3: Muscle fibre size (Feret's minimum diameter, μm) in the left cricoarytenoideus dorsalis muscle of horses clinically affected with recurrent laryngeal neuropathy compared to unaffected control horses

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Fibre	Group	n	Median	Min	Max	H-L Diff	95% CI	p-value
Type I	Control	5	78.03	68.97	94.80			
	Affected	5	69.47	50.67	94.79	-11.10	-31.84 to 16.76	0.2
Type IIA	Control	5	83.48	74.76	86.36			
	Affected	5	36.64	33.08	78.50	-46.84	-52.12 to -4.98	0.03
Type IIX/embryonic	Control	5	64.86	34.73	89.99			
	Affected	5	20.99	20.46	30.26	-43.44	-69.00 to -13.74	0.008
All Fibres	Control	5	82.70	73.80	88.25			
	Affected	5	45.42	35.42	74.80	-35.22	-47.44 to -7.90	0.02

H-L Diff: Hodges-Lehmann estimate of the difference between two population medians. CI: Confidence interval

Supporting Information

Table S1: Coefficient of variation (CV%) of fibre diameter in the left cricoarytenoideus dorsalis muscle of horses clinically affected with recurrent laryngeal neuropathy compared to unaffected control horses.

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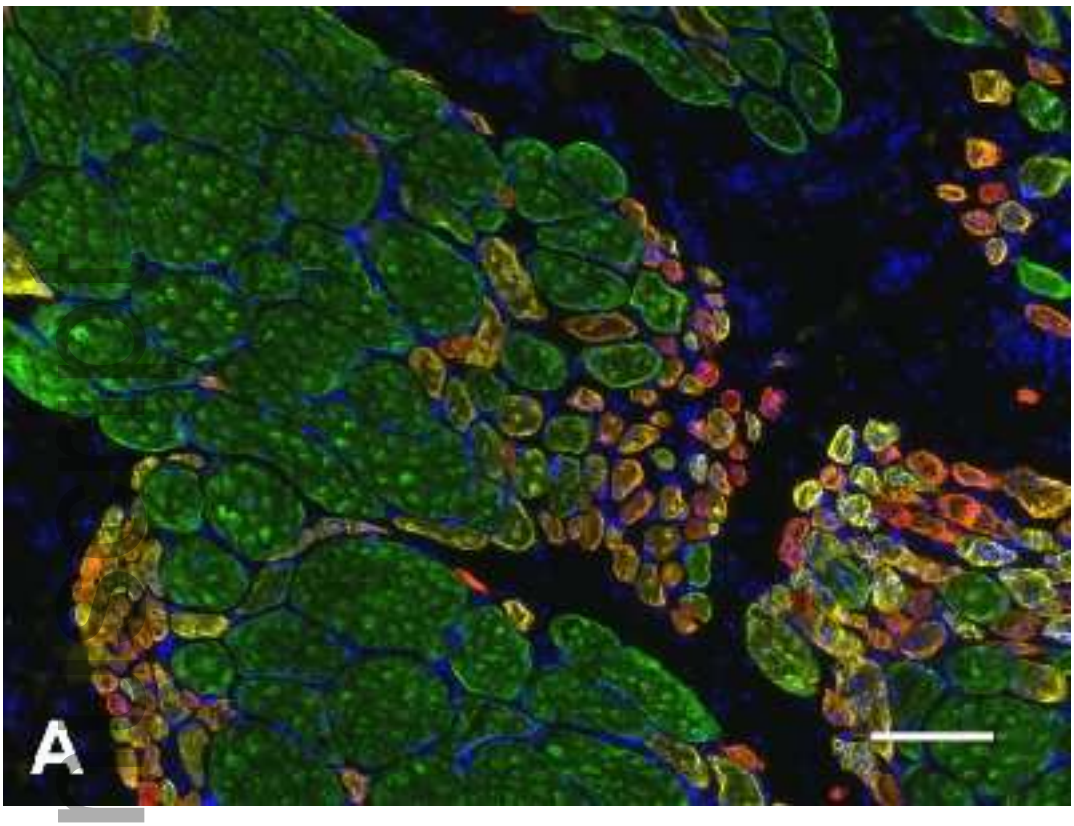

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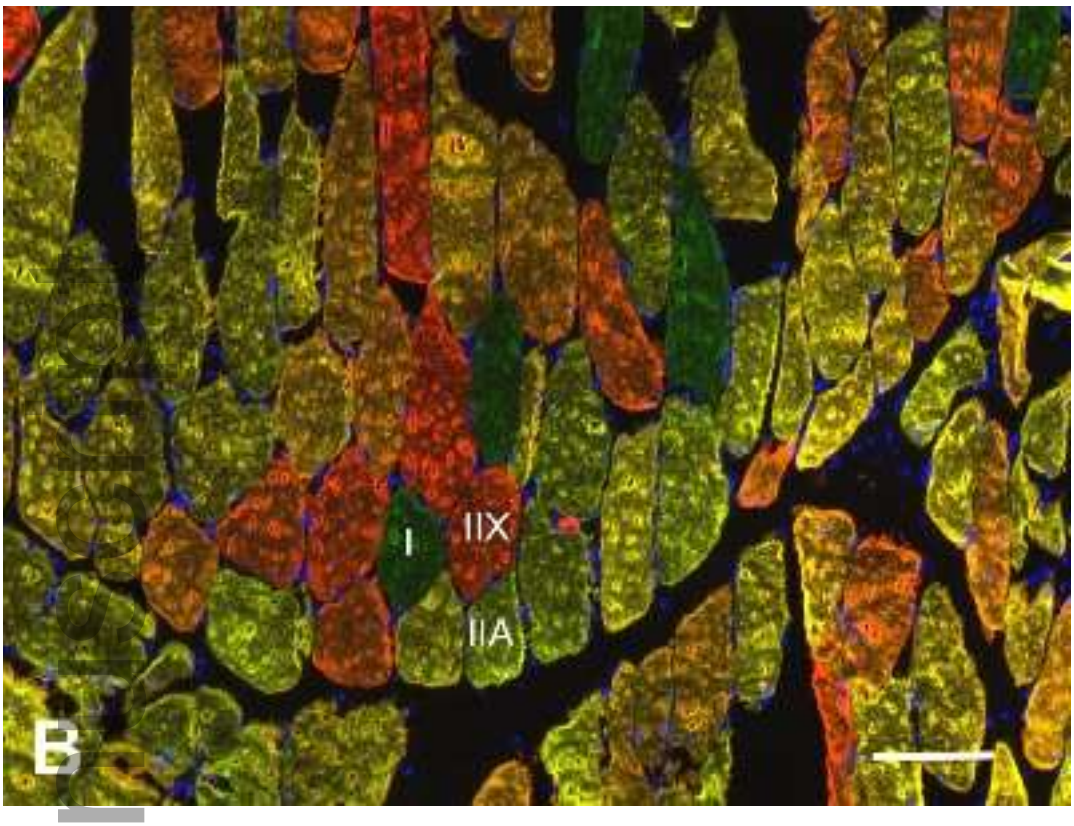

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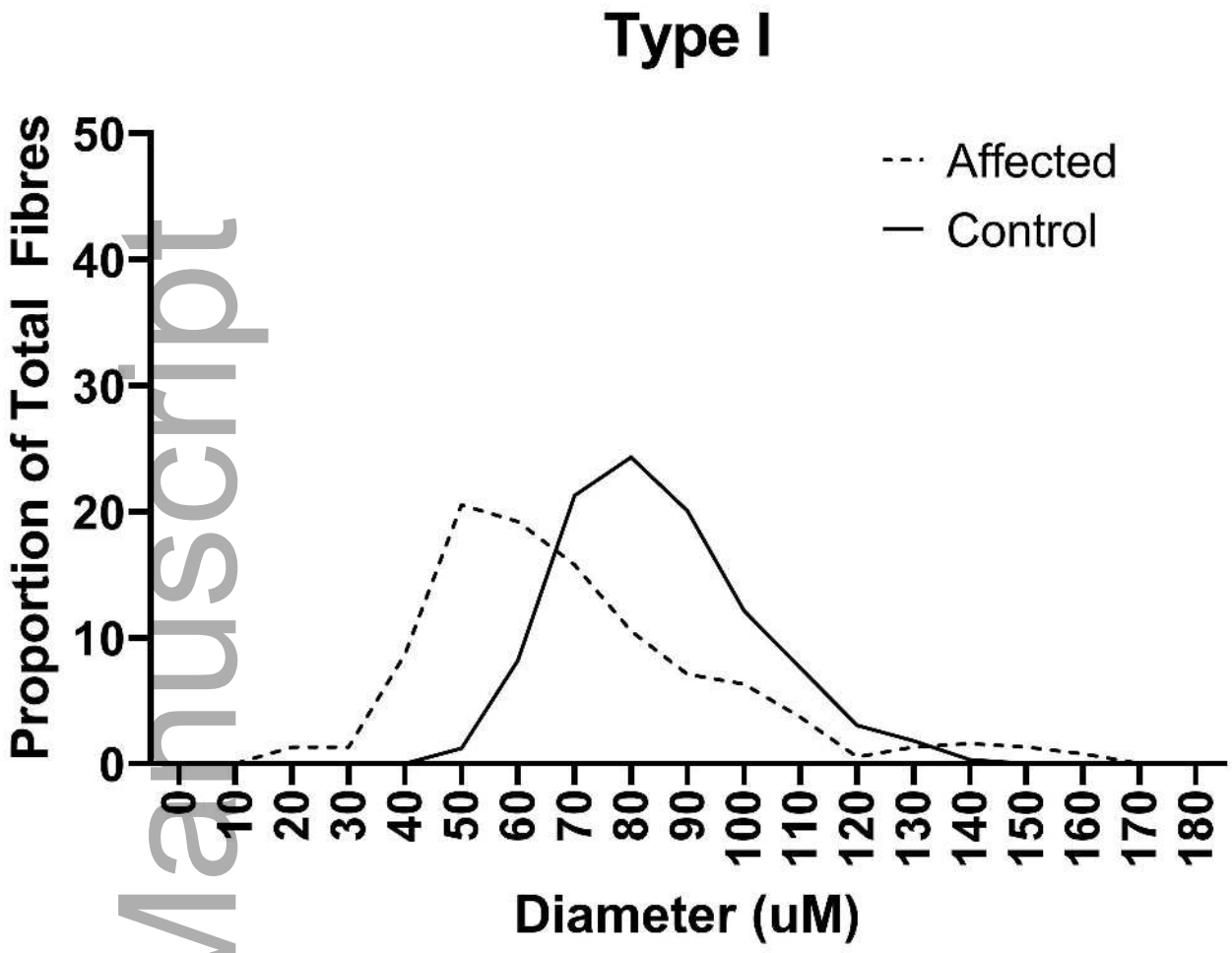

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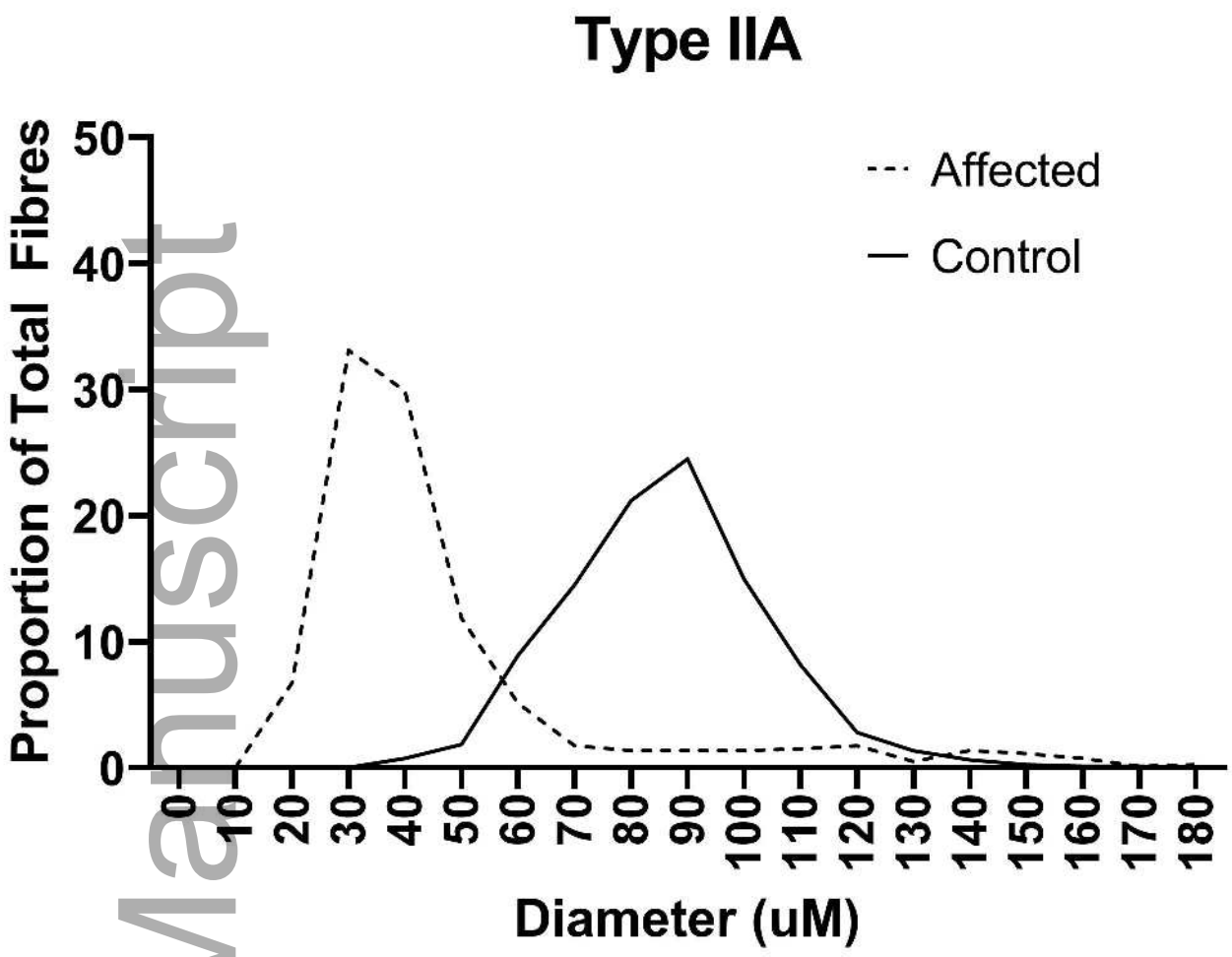

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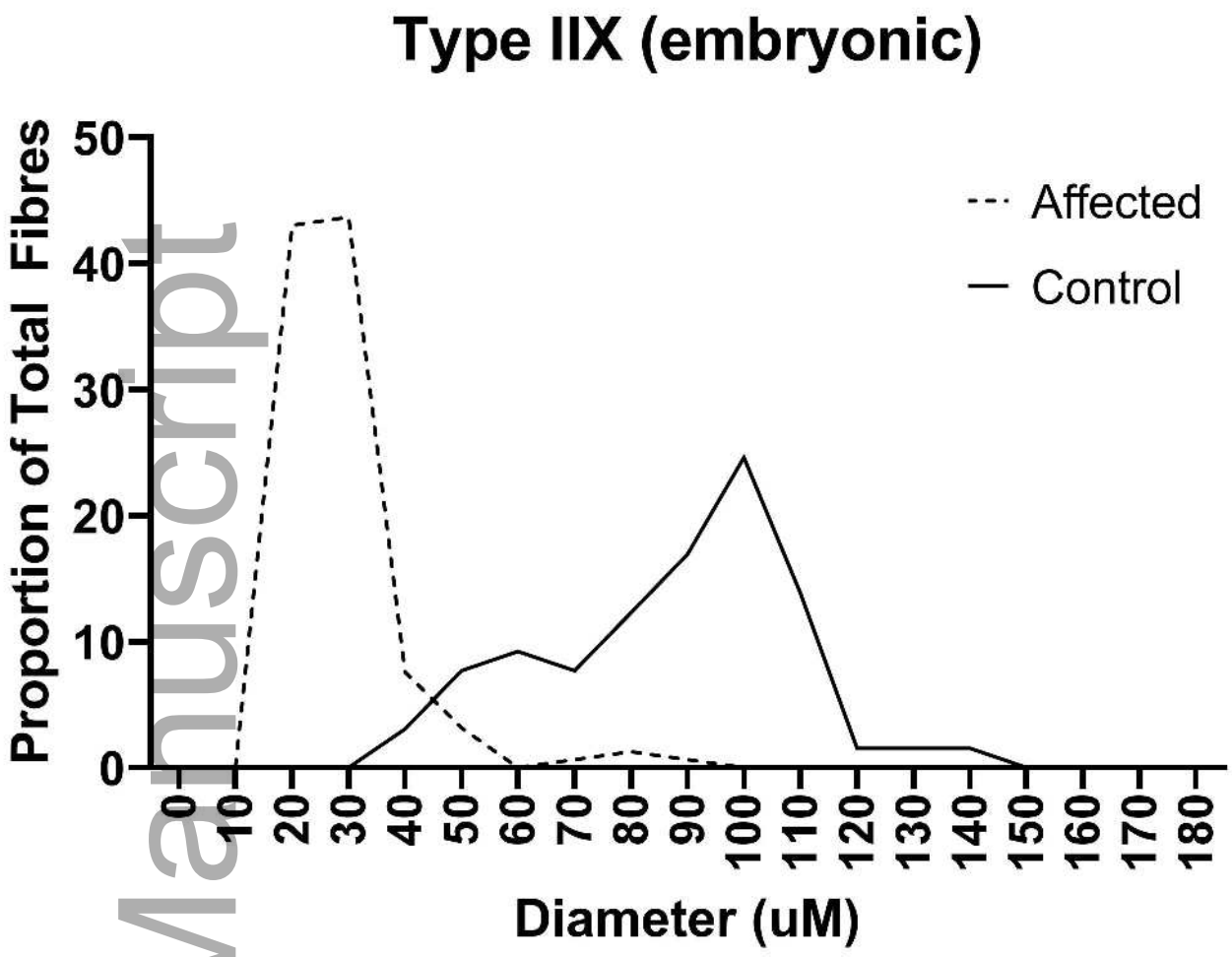

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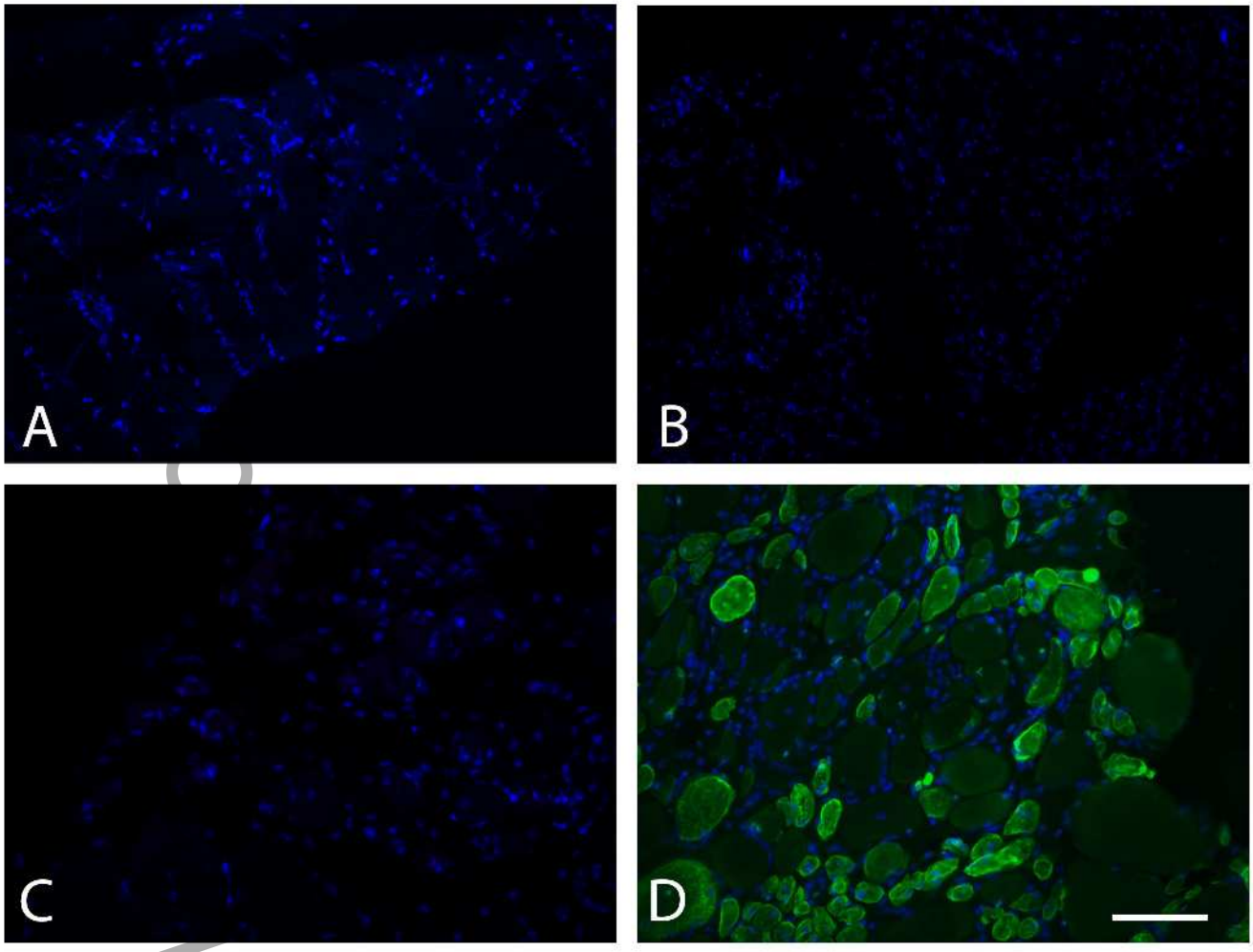

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