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2   DR. CATHERINE STEEL (Orcid ID : 0000-0001-7106-7884)

3   DR. BENJAMIN AHERN (Orcid ID : 0000-0001-5527-8964)

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10   **Immunohistochemical analysis of laryngeal muscle of horses clinically affected with**

11   **recurrent laryngeal neuropathy**

12

13   C. M. Steel*[1], E. A Walmsley[1], G. A. Anderson[1], C. A. Coles[2], B. Ahern[3] and J. D. White[4]

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15   [1]University of Melbourne, School of Veterinary and Agricultural Sciences, Werribee, VIC

16   3030 Australia;

17   [2]Murdoch Children's Research Institute, The Royal Children's Hospital, Parkville, VIC 3052

18   Australia;

19   [3]University of Queensland, School of Veterinary Science, Equine Specialist Hospital, Gatton,

20   QLD 4343 Australia and

21   [4]Office of the Pro Vice Chancellor Research and Innovation, Charles Sturt University, Wagga

22   Wagga, NSW 2678, Australia.

23

24   *Corresponding author email: csteel@unimelb.edu.au

25

27   **Running title:** Immunohistochemistry of laryngeal muscles of horses with recurrent

28   laryngeal neuropathy

29

30   **Abstract**

31   **Background:** As myosin heavy chain (MyHC) profile of muscle fibres is heavily influenced

32   by neural input, changes in MyHC expression are expected in horses clinically affected with

33   recurrent laryngeal neuropathy (RLN) yet this has not been thoroughly investigated.

34   **Objectives:** To describe changes in MyHC and fibre diameter in left cricoarytenoideus

35   dorsalis muscle (L-CAD) of horses with clinical signs of RLN.

36   **Study design:** Observational cohort study.

37   **Methods:** Immunohistochemistry was used to assess the MyHC-based fibre-type proportion,

38   size and grouping in the L-CAD of ten Thoroughbred horses, five clinically affected with

39   RLN and five unaffected controls based on resting endoscopic examination. The Mann-

40   Whitney U test was used to compare the two groups.

41   **Results:** Compared to controls (of mean age 3.0±1.7 years) which only expressed type I, IIA

42   and IIX MyHC, the L-CAD of affected horses (of mean age 2.8±0.8 years) had obvious fibre-

43   type grouping, and despite apparent compensatory hypertrophy of a small number of fibres, a

44   decrease in overall fibre diameter (median difference -35.2 μm, 95% CI -47.4 to -7.9, P

45   =0.02) and diameter of type IIA fibres (median difference -46.8 μm, 95% CI -52.1 to -5.0,

46   P=0.03). Anti-fast MyHC (MY32) cross-immunoreacted with embryonic-MyHC. Whereas

47   MY32-positive fibres were identified as type IIX in controls, in affected horses these fibres

48   were less than 50 μm diameter with internal nuclei and were MYH3-positive for embryonic

49   myosin indicating depletion of type IIX fibres yet active regeneration and fibre renewal.

50   **Main limitations:** Small sample size that did not include subclinical cases. Fibre size and

51   appearance rather than staining colour were relied upon to differentiate embryonic from type

52   IIX MyHC.

53   **Conclusions:** Horses clinically affected with RLN have overall atrophy of fibres, loss of IIX

54   fibres and expression of embryonic myosin indicating regenerative capacity. Despite

55   hypertrophy of some remaining fibres, the overall decline in the bulk of fibres including those

56   most fatigue resistant may be the critical change that results in failure to maintain arytenoid

57   abduction during exercise although direct comparison to subclinical cases is needed to

58   confirm this.

59

60

61   **Introduction**

62   Recurrent laryngeal neuropathy (RLN) is an important performance limiting disease of horses

63   characterised by progressive loss of large diameter myelinated axons most severe in the distal

64 portions of the left recurrent laryngeal nerve (rLN)[1-3]. Changes in the laryngeal muscles

65 are characteristic of neurogenic disease that is chronic and repetitive in nature with cyclic

66 denervation and reinnervation [2; 4]. Although adductor muscles are also affected [5],

67 dysfunction of the cricoarytenoideus dorsalis muscle (CAD), the only abductor, is clinically

68 most important. Failure of abduction of the left arytenoid and vocal cord results in respiratory

69 impairment during inspiration, with resultant decreased athletic performance and abnormal

70 respiratory noise during exercise.

71

72 With RLN, changes in the laryngeal muscles are evident to varying degrees and include fibre-

73 type grouping, atrophy of single fibres or groups of muscle fibres, compensatory hypertrophy

74 of some remaining fibres, and eventual fibrosis and fat replacement [2; 4]. Neuropathologic

75 changes within the rLN and laryngeal muscle [2; 6] and reduced conduction velocity in the

76 left rLN [7] also occur in many endoscopically normal horses and these animals are

77 considered sub-clinically affected.

78

79 The functional demands of muscles are met by a variety of muscle fibre types that have a

80 range of speed, endurance and power characteristics. Fibre type is determined by expression

81 of different isoforms of myosin heavy chain (MyHC) [8]. Normal laryngeal muscle of mature

82 horses is comprised of three fibre types, slow (type I), IIA and IIX [9; 10], expected to

83 provide motor units of this muscle with a tenfold range of speeds [11]. Embryonic-MyHC is

84 not normally present in adult muscle yet is transiently re-expressed during muscle

85 regeneration following injury or disease and can be used to identify regenerating muscle

86 fibres [12-14]. As altered neural input occurs with RLN and MyHC profile is heavily

87 influenced by neural input [15], changes in MyHC profile occur in this disease [9].

88 Knowledge of fibre type distribution is essential for our understanding of laryngeal function,

89 RLN and adaptive mechanisms that enable subclinical horses to remain asymptomatic. In the

90 left CAD muscle (L-CAD) of horses considered sub-clinically affected with RLN there was

91 virtual elimination of type IIX fibres, a lower proportion of slow fibres, greater abundance of

92 IIA fibres, and hypertrophy of many remaining slow and IIA fibres [9; 10]. Loss of type IIX,

93 the fastest fibres present in equine laryngeal muscle, is expected to be associated with a

94 reduction of the speed range of laryngeal muscles from 10-fold to only 4-fold [11] and may

95 explain some of the alterations in movement of the left arytenoid cartilage observed in horses

96 with partial paralysis. Yet as these horses can maintain laryngeal abduction during exercise,

97 loss of IIX fibres appears tolerated and we assume hypertrophy of remaining fatigue resistant

98   fibres allows horses to remain subclinical. Whilst this suggests that nerve fibres innervating

99   IIX muscle fibres are more susceptible to damage incurred by RLN, the sub-clinically

100   affected horses reported by Rhee and others [9] were at least 10 years of age and horses

101   clinically affected with RLN were not included.

102

103   We aimed to identify changes in MyHC expression and fibre diameter in the L-CAD muscle

104   of horses with clinical signs of RLN and hypothesised that these horses would have loss of

105   IIX fibres, a reduction in the bulk of slow (type I) fibres and expression of embryonic-MyHC.

106

107   **Materials and Methods**

108   A section of muscle, 5-10 mm in diameter and approximately 10 mm in length, was dissected

109   from the mid-belly of the lateral compartment of the L-CAD from a convenience sample of

110   five clinically affected (RLN group) and five endoscopically normal Thoroughbred horses

111   (controls) in race training. Samples from the RLN group were collected during prosthetic

112   laryngoplasty surgery from horses with Havemeyer grade III.2 or greater [16] on resting

113   laryngoscopic examination and a history of exercise intolerance. Control samples were

114   collected from horses with normal laryngeal function (Havemeyer grade I) on resting

115   endoscopic examination immediately after euthanasia for unrelated reasons. Endoscopic

116   examination was performed within 24 hours of sample collection in all horses and laryngeal

117   function was graded by the same observer. Samples were identified by number only to ensure

118   blinding when obtaining the measurements.

119

120   Immediately following collection, muscle samples were coated with OCT compound, snap

121   frozen in isopentane quenched liquid nitrogen and stored at -80°C until immunofluorescent

122   staining. Frozen muscle samples were mounted using OCT compound to allow cutting of

123   10um transverse sections in a cryostat at -20°C. Staining was performed immediately

124   following sectioning using a methodology based on that reported by Tulloch *et al*. [17]. All

125   antibodies were directly conjugated to ALEXA dyes using the Zenon labelling technology.

126   Type I fibres (slow) were sequentially labelled with NOQ7.5.4D (ALEXA350) and type IIA

127   (fast) with N2.261 (ALEXA488). MY32 antibody was used (which identifies all type 2

128   fibres) labelled with ALEXA594. With this staining regime under fluorescence type I fibres

129   will appear green, type IIA fibres yellow/orange and remaining fast fibres (type IIX) appear

130   red. Separate transverse muscle cryosections (10μm) were stained for embryonic myosin

131   using a mouse anti-MYH3 antibody (clone F1.652) in combination with ALEXA594 labelled

132 MY32 as described above to confirm cross reactivity of the two antibodies. These sections

133 were blocked in 10% (v/v) donkey serum (Millipore, Billerica, Massachusetts, USA) in wash

134 buffer (0.1%Tween, 0.5%BSA in 1xPBS) for one hour, and then incubated with the primary

135 antibody anti-MYH3 (Santa Cruz Biotechnology, 1:200), for 60 minutes at room temperature.

136 The sections were washed in PBS and then incubated with the fluorescent secondary

137 antibody, donkey anti-rat IgG Alexa Fluor 488 (Life Technologies, 1:250) in the dark for 90

138 minutes. Nuclei were stained in all sections with 1μg/μl Hoechst (Life Technologies) in PBS

139 for one minute before mounting with polyvinyl alcohol with glass coverslips.

140

141 Digital images were captured on a Zeiss Axio Imager M1 upright fluorescent microscope

142 with an AxioCam MRm camera running AxioVision software V4.8.2.0 (Carl Zeiss,

143 Oberkochen, Germany). A minimum of five images were collected per section at 100x

144 magnification to allow the analysis of a minimum of 250 fibres. Analysis of images was

145 performed by the same observer using Image J (National Institute of Health, version

146 1.8.0_1121) with each individual fibre being traced, categorised based on myosin staining

147 pattern and the minimum Feret's diameter (defined as the closest possible distance between

148 two parallel tangents of the muscle fibre) automatically calculated by the software.

149

150 Prism 8.3.1 (Graphpad Software, San Diego, CA) was used for statistical analysis. The

151 coefficient of variation (CV%) for each horse and fibre type was defined as (standard

152 deviation of fibre diameters/mean of fibre diameters) x 100. The Shapiro-Wilk test was used

153 to test for normality of values for each group of five control horses and each group of five

154 affected horses. Three of these 22 groups were not normally distributed and each of these

155 three groups had a statistical outlier. These groups were proportion of type IIX/embryonic

156 fibres in the control group, fibre diameter of type IIA fibres in the affected group and CV% of

157 type IIX/embryonic fibres in the affected group. The distributions of the affected and control

158 groups were compared using the Mann-Whitney U test. The Hodges-Lehmann (H-L) estimate

159 of the difference between two population medians was estimated as the median of the set of

160 25 (5 by 5) differences between each value in the affected group and each value in the control

161 group. A p-value <0.05 was considered significant.

162

163 **Results**

164 Ten Thoroughbred horses including five clinically affected with RLN mean age 2.8 [SD 0.8]

165 years and with resting endoscopic grade [16] of III.2 (2 horses) or III.3 (3 horses) and five

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

183

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

193

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

200   significantly fewer type I and IIA fibres per microscopic field in the L-CAD of four horses

201   with advanced RLN [10]. Indeed, gross atrophy of the L-CAD muscle is usual in clinically

202   affected animals. A left to right ratio <0.8 of CAD muscle thickness measured using

203   transoesphageal ultrasound correlated with muscle volume and predicted dynamic collapse

204   (exercising grade C)[19]. Furthermore, an increase in type I fibres was observed in laryngeal

205   muscle of Thoroughbred racehorses in training (E. Walmsley, personal communication). On

206   these bases we theorised that clinically affected horses might have lost a critical volume of

207   the most fatigue-resistant slow (type I) fibres in addition to faster type IIA and fastest type

208   IIX fibres. Although loss of the aforementioned fibre-types might be the critical factor in

209   determining whether horses are clinically affected with RLN with failure to maintain

210   arytenoid abduction during exercise we were unable to confirm this when comparing this

211   small sample size of horses. Even minor loss of slow fibres would drastically reduce dynamic

212   range of contraction speeds and reduce the capacity of the L-CAD muscle to endure sustained

213   periods of abduction. Such functional loss is sometimes detected on endoscopic examination

214   during high speed exercise in horses with partial laryngeal paralysis. Further evaluation of the

215   impact of RLN on the bulk of type I fibres with advancing disease required.

216

217   Embryonic myosin was only identified in clinically affected horses, all with obvious or

218   marked abductor deficit and failure to achieve and maintain abduction yet not complete

219   immobilisation of the arytenoid cartilage and vocal fold (i.e. Havemeyer grade III.2 and III.3)

220   [16]. This indicates that affected horses maintain regenerative capacity, an important finding

221   as it suggests that surgical reinnervation likely remains possible unless extensive muscle

222   fibrosis occurs. This regenerative capacity may also preserve some muscle function for a

223   period of time following onset of disease. We had predicted that with advanced denervation

224   in clinically affected horses muscle fibre regeneration would be stimulated [20], that

225   denervated fibres would express embryonic-MyHC isoforms [20-22] and as surviving nerve

226   fibres would no longer be able to maintain a functionally sufficient population of remaining

227   muscle fibres that marked atrophy would occur. Indeed, this was observed in clinically

228   affected horses in the current study. In earlier work by the first author and others, a

229   significant increase in the number of myonuclei per muscle fibre, central nuclei, and

230   activation of muscle satellite cells was observed in the L-CAD muscle from clinically

231   affected horses regardless of age, degree of atrophy or duration of disease. The latter finding

232   suggests that the muscle is attempting to regenerate by intrinsic muscle satellite cell

233   activity[10].

234

235　As discussed earlier some horses with normal laryngeal function have subclinical disease.

236　Rhee *et al.* described loss of the fastest (type IIX) fibres in laryngoscopically normal horses

237　(all >10 years of age) with no history of exercise intolerance and considered sub-clinically

238　affected based on fibre-type grouping [9]. Based on these findings it appears that loss of IIX

239　fibres may be well tolerated. This earlier study also provided insight into adaptive

240　mechanisms that enable subclinical horses to remain clinically unaffected as these horses also

241　demonstrated a decrease in the proportion of slow fibres suggesting some loss of slow fibres

242　follows the loss of type IIX fibres, yet an increase in the proportion of type IIA fibres and

243　hypertrophy of remaining slow and type IIA fibres [9]. The present study considered

244　clinically normal horses compared to those with diagnosed clinical disease. In unaffected

245　horses in both studies type IIA fibres predominated in the L-CAD followed by slow (type I)

246　fibres and relatively few type IIX. The virtual absence of type IIX fibres in the LCAD of sub-

247　clinical horses [9] is consistent with our results in clinically affected samples in that the

248　central position of the nucleus, the small diameters (vast majority were smaller than the

249　smallest fibres in the control samples) and the cross reactivity with embryonic MyHC

250　indicate an absence of IIX fibres. While sub-clinical samples show overall hypertrophy of

251　remaining type IIA and slow (type I) fibres [9] our data shows a shift to smaller fibres. This is

252　probably related to severity of disease and the chronic nature of the denervation.

253

254　While the exact prevalence of subclinical disease is unknown, pathologic changes in the rLN

255　and laryngeal muscles are present in 30% or more of horses [1; 5; 7; 23-25]. As a result, we

256　expected to encounter subclinical disease in some of the control horses in the current study,

257　yet fibre-type grouping was not observed in this group. We propose that this may have been

258　due to the age of horses (6 years or less) and the small sample size. Similarly, muscles

259　without fibre-type grouping reported by Rhee *et al*. were from horses aged 2 and 6 years (and

260　from a third horse of unknown age) [9]. Study of a larger number of horses would be

261　necessary to identify fibre-type grouping in young horses without clinical signs of RLN.

262　Then, the direct comparison of L-CAD muscle samples from subclinical and clinically

263　affected, age and training status matched, young horses stained using the same method may

264　be undertaken to improve our understanding of the critical point at which subclinical disease

265　becomes clinical.

266

267 As expected based on findings in horses with subclinical disease [9], the loss of type IIX
268 fibres identified in clinically affected horses in the current study was also predicted. Loss of
269 the largest myelinated axons occurs in RLN with loss of the fastest conduction velocity nerve
270 impulses expected. As these nerve fibres innervate type IIX muscle fibres (the fastest fibres
271 present in the equine CAD muscle[9]), it follows that type IIX fibres are more susceptible to
272 damage incurred by equine RLN, or their signals more easily corrupted than those
273 innervating type IIA or type I (slow) fibres. It should be noted, however, that an antibody that
274 specifically reacts with MyHC-IIX was not used to identify type IIX fibres in the current
275 study. Attempts in our laboratory to use a specific MyHC-IIX monoclonal antibody have
276 been unsuccessful. With the double staining method used, both type IIX and embryonic fibres
277 stain red, yet the smaller diameter (less than 50 μm) of the majority of fibres and internally
278 placed nuclei indicating active regeneration and fibre renewal identified positive stained
279 fibres as expressing embryonic myosin in affected horses. This was supported by positive
280 staining for embryonic fibres using anti-MyH3 (anti-embryonic) on additional sections.
281
282 As expected there was obvious fibre-type grouping in clinically affected horses in addition to
283 scattered angular fibres, groups of atrophied fibres and some remaining hypertrophied fibres
284 with central nuclei [26]. Fibre-type grouping is a diagnostic sign of early neuropathy and an
285 indicator of partial denervation followed by reinnervation of muscle fibres by intact nerve
286 terminals of neighbouring fibres [27]. Clusters of muscle fibres acquire the same
287 histochemical properties since neural influence determines fibre-type and changes represent
288 ongoing, continual or intermittent nerve injury with repeated attempts at reinnervation. In
289 comparison, although surgical transection of the rLN results in immediate laryngeal paralysis,
290 it does not result in fibre-type grouping and changes in MyHC profile differ from those seen
291 with naturally occurring disease [10; 28].
292
293 We only included Thoroughbred horses of racing age. This is likely important as ageing-
294 related changes in the expression of MyHC fibres have been observed in aged horses [29-32].
295 Furthermore, we only included horses in race training as the intensity and duration of training
296 may influence MyHC fibre type not only in equine locomotor muscles [29; 33] but also in
297 laryngeal muscle. On this basis, as mentioned earlier, our preliminary investigations of gene
298 expression for MyHC fibre type in laryngeal muscle of trained compared to untrained
299 Thoroughbreds suggests that there is upregulation of slow MyHC with training (i.e. a shift

300   towards a slower fibre type occurs as a training adaptation in laryngeal muscle as it does in

301   gluteal muscle) (E. Walmsley *et al*., personal communication).

302

303   A limitation of the current study is that the sample size might not have been large enough to

304   detect any decrease in the proportion and mean diameter of type I fibres in affected horses

305   compared to controls. In addition, none of the affected horses had complete immobility of the

306   left arytenoid and vocal cord on resting endoscopy. The total bulk of type I fibres may be

307   critical in maintaining abduction during exercise and this might not be reflected by

308   considering the proportion of type 1 fibres or their mean diameter in isolation. A further

309   limitation of the current study is that laryngeal function was assessed at rest but not by

310   dynamic endoscopy during exercise and although resting laryngeal function is reasonably

311   sensitive and highly specific for predicting laryngeal function at exercise [34] we cannot be

312   entirely sure that all control horses had normal laryngeal function during exercise. However,

313   we only included horses with a known history of normal exercise tolerance in the control

314   group and with exercise intolerance in the affected group.

315

316   In conclusion, horses clinically affected with RLN have an overall decrease in muscle fibre

317   diameter attributed to a reduction in size of IIA fibres, depletion of IIX fibres and expression

318   of embryonic myosin indicating remaining potential for regeneration. Although there is

319   hypertrophy of some remaining fibres, we propose that the overall loss of bulk of more

320   fatigue resistant muscle fibres and not simply loss of IIX fibres, which appears tolerated as it

321   occurs in subclinical horses[9], results in the failure to maintain arytenoid abduction during

322   exercise. Longitudinal studies are required to identify changes over time and to identify cut-

323   off points for L-CAD muscle volume and more specifically for type I fibre bulk that

324   differentiate dynamic collapse from horses able to maintain adequate abduction during

325   exercise.

326

327   **Authors' declaration of interests**

328   No competing interests have been declared.

329

330   **Ethical animal research**

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

332

350

351

352 **Figure legends**

353 **Figure 1A:** Transverse sections of the left cricoarytenoideus dorsalis muscle of a 3-year-old

354 Thoroughbred entire male with recurrent laryngeal neuropathy (grade III.2). A sequential

355 double-staining immunofluorescent technique was used (type I fibres are labelled with

356 NOQ7.5.4D [ALEXA350], type IIA with N2.261 [ALEXA488] and all remaining type II fibres

357 and embryonic with MY32 antibody [ALEXA594]). Type I fibres are stained green, type IIA

358 yellow-orange and small red staining fibres are considered embryonic fibres due to their small

359 size and internal nuclei. Additional immunolabelling with 1μg/μl Hoechst identifies all nuclei

360 (blue staining). Fibre-type grouping and marked reduction in the diameter of type IIA and some

361 type I fibres is evident.

362

363 **Figure 1B:** Transverse cryostat section of the left cricoarytenoideus dorsalis muscle of a 3-

364 year-old Thoroughbred gelding with normal (grade I) laryngeal function on endoscopic

365 examination at rest. The staining protocol used was the same as for figure 1A. There is a

366 normal mosaic pattern with a predominance of type IIA fibres (yellow orange), fewer type I

367 (green), and a small number of type IIX (red) staining fibres. Nuclei are stained blue. Scale

368 bar = 100 μm.

369

370 **Figure 2:** Distribution of muscle fibres by diameter for type I (a), type IIA (b) and type IIX

371 or embryonic-MyHC in the left cricoarytenoideus dorsalis muscle of horses clinically

372 affected with recurrent laryngeal neuropathy (affected) compared to endoscopically normal

373 horses (controls). Note that although the double staining technique used could not

374 differentiate embryonic from IIX fibres, the smaller diameter (<50 μm) of most red-staining

375 fibres together with internally placed nuclei evident on stained sections (not shown) indicated

376 active regeneration and fibre renewal consistent with embryonic myosin in affected but not

377 control horses.

378

379 **Figure 3:** Transverse sections of biopsy samples of left cricoarytenoideus dorsalis muscle

380 from an unaffected (control) horse (A and C) and a horse clinically affected with recurrent

381 laryngeal neuropathy (B and D). There is a lack of positively stained fibres in the negative

382 control samples (IgG only) from the unaffected (A) and affected (B) horse. Lack of staining

383 is also evident with embryonic MyHC staining of a sample from an unaffected horse (C) but

384 in the affected horse (D) many regenerating fibres reactive for embryonic myosin (green

385 staining) and demonstrating internal nuclei are evident. Sections are stained using anti-

386 MYH3 as the primary antibody and a secondary fluorescent (green) antibody. Additional

387 labelling identifies nuclei (blue staining). Scale bar = 100 μm.

388

389

390 **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

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

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

410

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

412

413

414 **Supporting Information**

415 **Table S1:** Coefficient of variation (CV%) of fibre diameter in the left cricoarytenoideus

416 dorsalis muscle of horses clinically affected with recurrent laryngeal neuropathy compared to

417 unaffected control horses.

418

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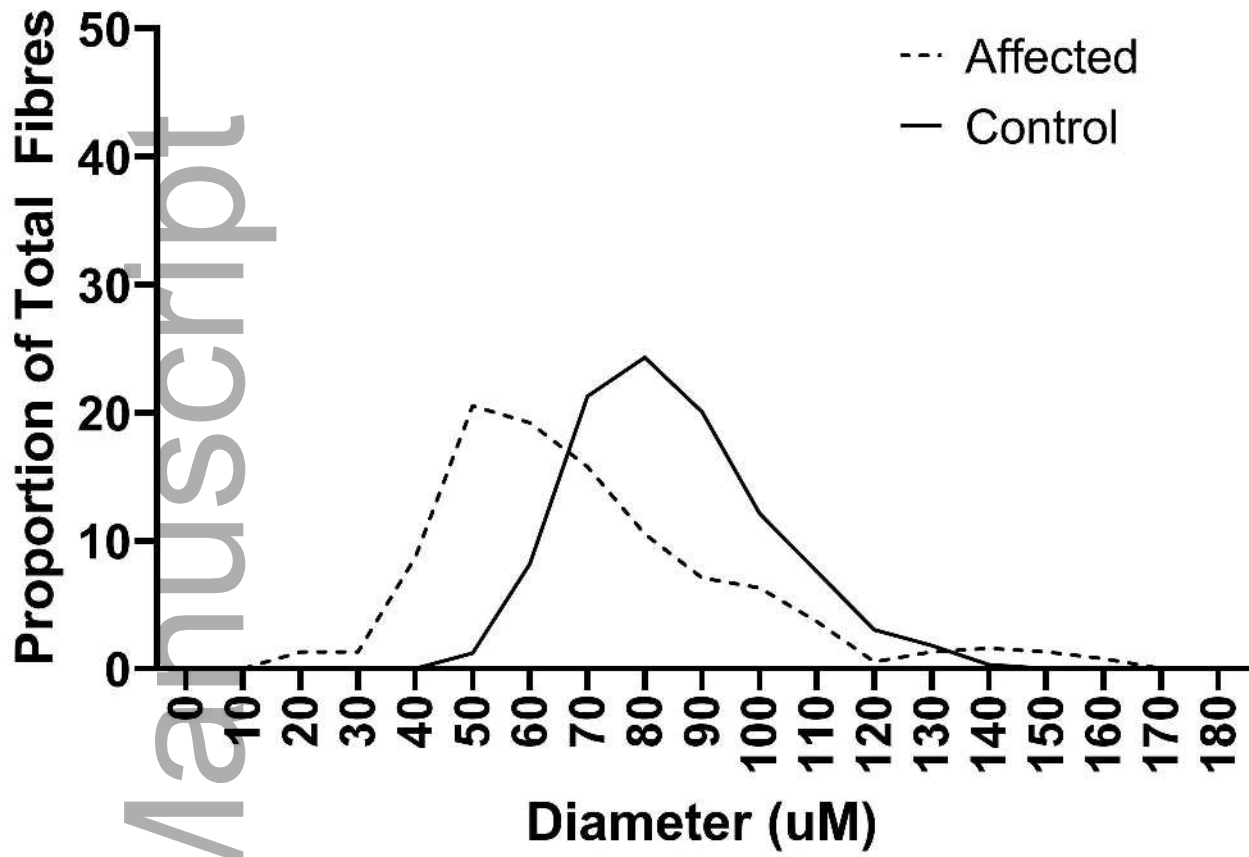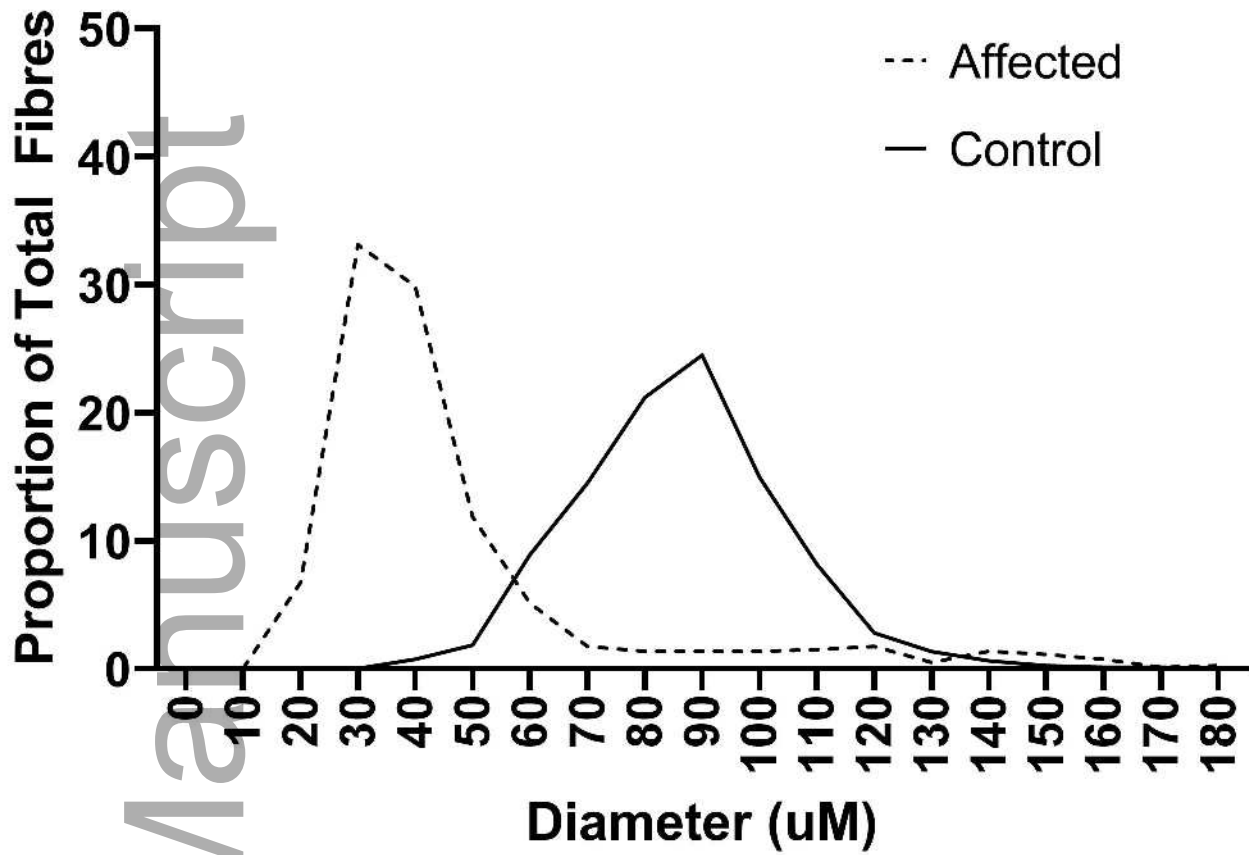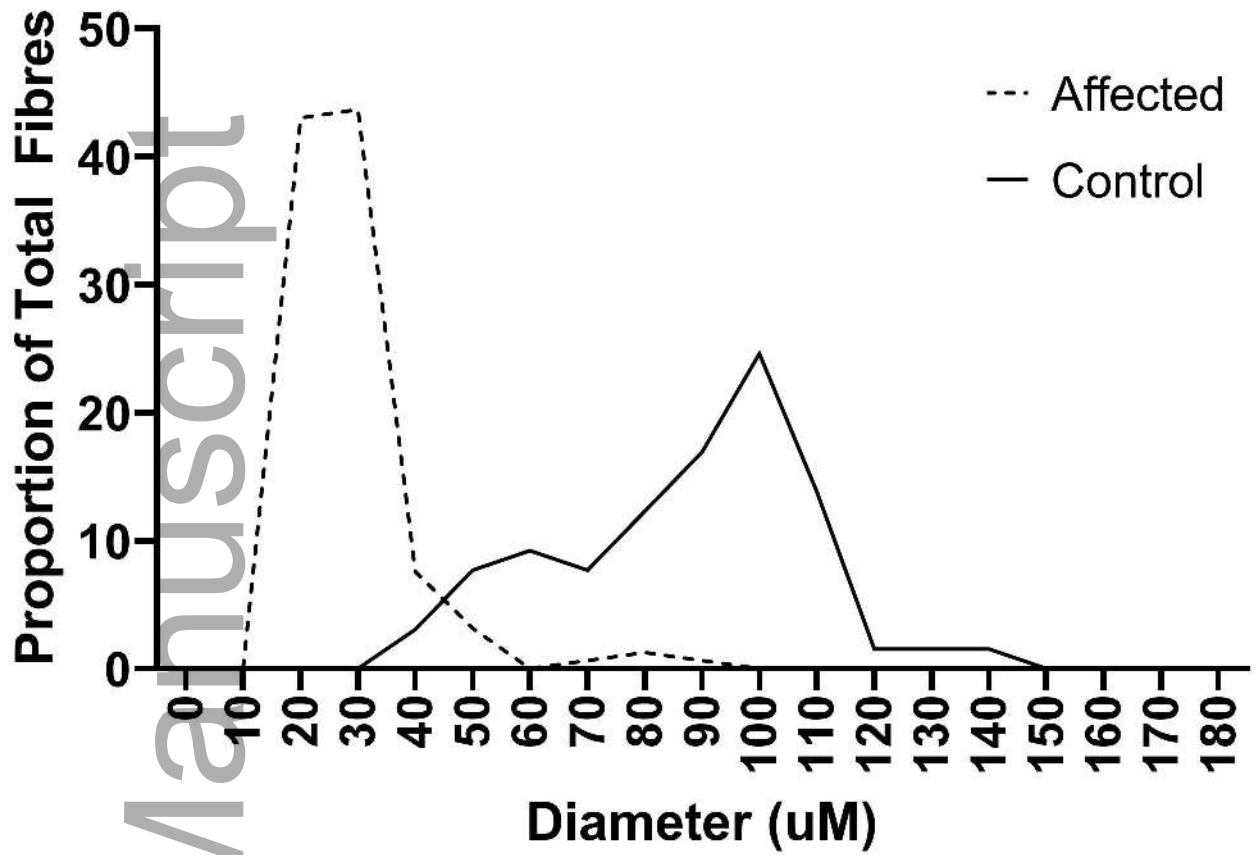
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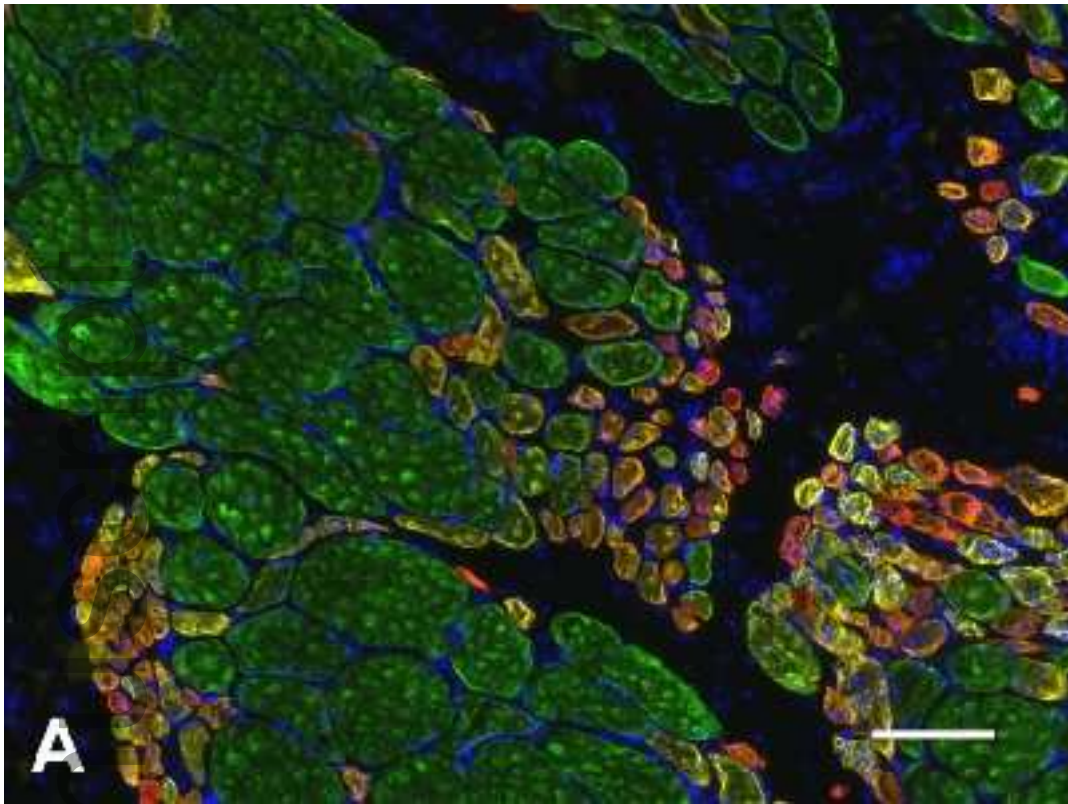
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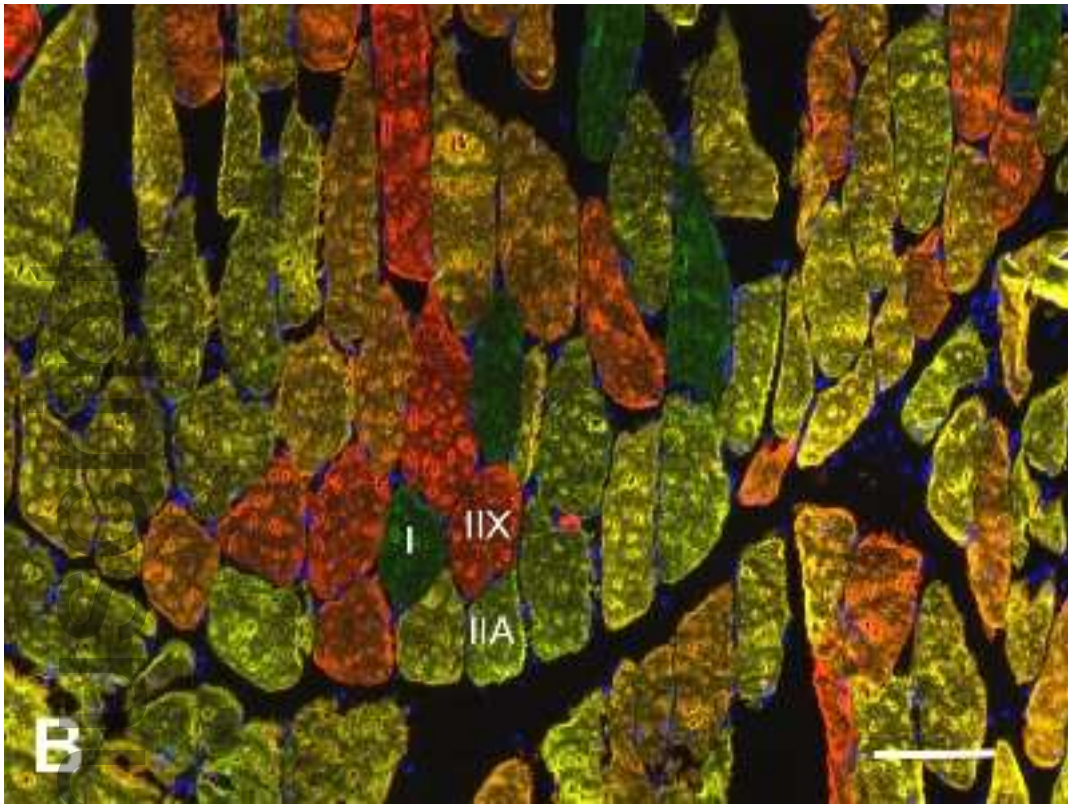
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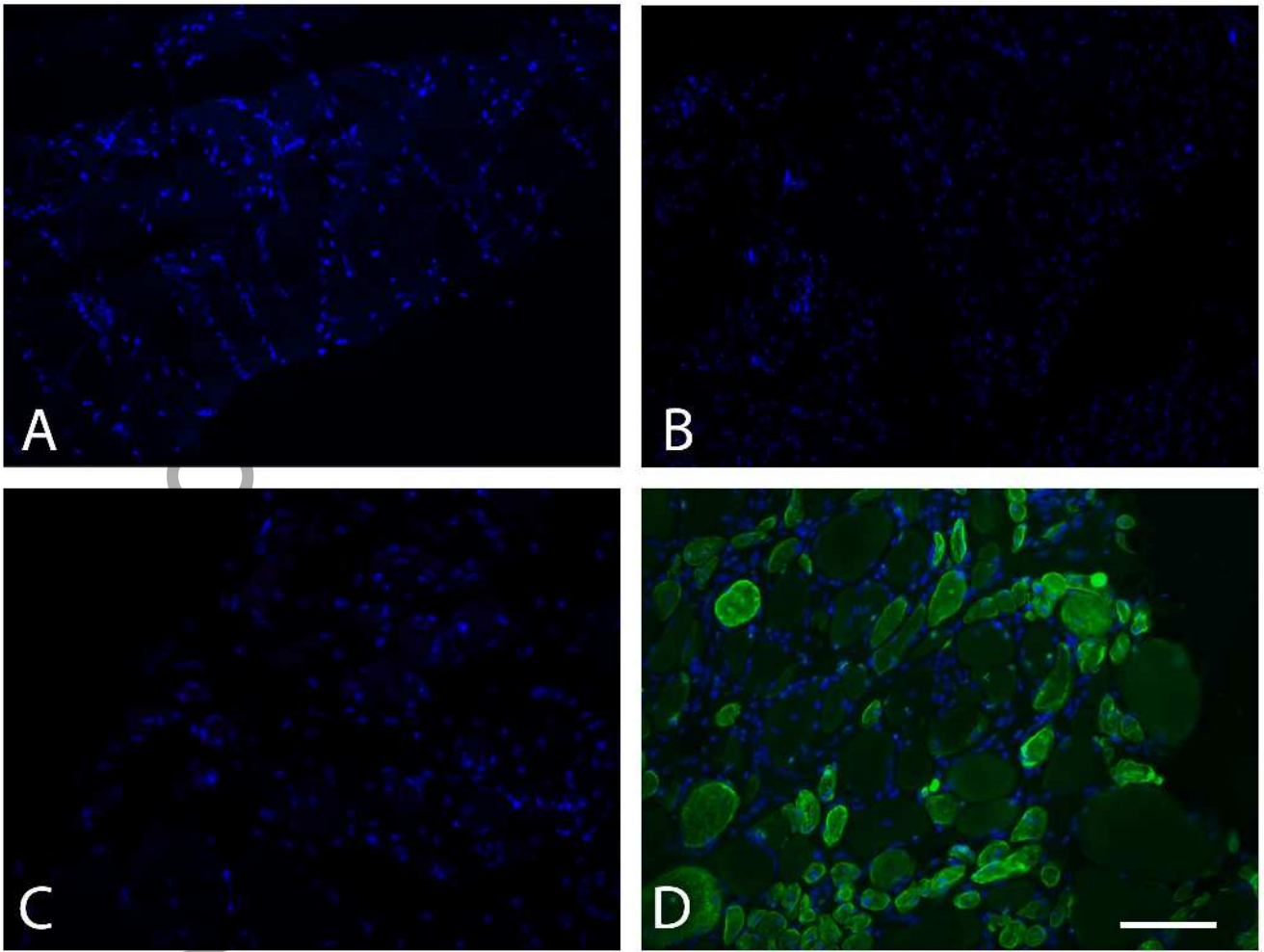
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Author/s:
Steel, CM; Walmsley, EA; Anderson, GA; Coles, CA; Ahern, B; White, JD

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