

1 **Effects of Functional Electrical Stimulation on Denervated Laryngeal Muscle in a**
2 **Large Animal Model**

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20

21 **Abstract**

22 Bilateral Vocal Fold Paralysis (BVCP) is a life threatening condition that follows injury
23 to the Recurrent Laryngeal nerve (RLn) and denervation of the intrinsic laryngeal
24 musculature. Functional electrical stimulation (FES) enables restoration and control of a
25 wide variety of motor functions impaired by lower motor neuron lesions. Here we
26 evaluate the effects of FES on the sole arytenoid abductor, the posterior cricoarytenoid
27 muscle (PCA) in a large animal model of RLn injury. Ten horse were instrumented with
28 two quadripolar intra-muscular electrodes in the left PCA muscle. Following a twelve
29 week denervation period, the PCA was stimulated using a once daily training session for
30 eight weeks in seven animals. Three animals were used as unstimulated controls.
31 Denervation produced a significant increase in rheobase ($p < 0.001$). Electrical stimulation
32 produced a 30% increase in fiber diameter in comparison with the unstimulated control
33 group ($33.9 \pm 2.6 \mu\text{m}$ FES+, $23.6 \pm 4.2 \mu\text{m}$ FES -, $p = 0.04$). A trend towards a decrease in
34 the proportion of type 1 (slow) fibers and an increase in type 2a (fast) fibers was also
35 observed. Despite these changes improvement in PCA function at rest was not observed.
36 These data suggest that electrical stimulation using a relatively conservative set of
37 stimulation parameters can reverse the muscle fiber atrophy produced by complete
38 denervation while avoiding a shift to a slow (type 1) fiber type.
39

40 **Introduction**

41 Bilateral Vocal Fold Paralysis (BVCP) is a life threatening condition that follows injury
42 to the Recurrent Laryngeal nerve (RLn), most commonly from trauma during thyroid
43 surgery or anterior cervical approaches to the spine ¹⁻⁴. In the case of complete RLn
44 transection without spontaneous recovery, the intrinsic laryngeal musculature is
45 chronically denervated and the vocal folds remain immobile in a closed position
46 producing dysphonia, stridor, aspiration and dyspnoea ⁵. Functional electrical stimulation
47 (FES) is a well-established treatment option for lower motor neuron deficits that enables
48 restoration and control of a wide variety of motor functions impaired by lower motor
49 neuron lesions, from locomotion to breathing. Despite the technical limitation
50 represented by low excitability of muscle undergoing atrophy and fibrotic degeneration
51 following permanent motor neuron damage, FES has also been used clinically to provide
52 direct electrical activation of long term denervated muscle ^{6,7}. Animal models of RLn
53 injury have been previously used to evaluate the use of FES to stimulate a denervated
54 laryngeal musculature. These models have predominantly focused on the dog ⁸⁻¹⁰, sheep
55 ^{11,12}, cat ¹³, pig ¹⁴⁻¹⁶, and more recently, horse ^{17,18}. Confounding features of these animal
56 studies have been variation in the parameters used for stimulation including number of
57 pulses, daily activation rate and rest periods between stimulation, in addition to varying
58 rates of denervation across species and both positive and negative effects of stimulation
59 on muscle integrity and the process of reinnervation have been reported ¹⁹⁻²²
60 . In this study we evaluate the effects on muscle size and function of direct intramuscular
61 stimulation in denervated laryngeal muscle using relatively long stimulation pulses at a
62 low daily activation frequency. We use an equine model as the large size of the posterior

63 cricoarytenoid muscle (PCA) reduces some of the technical difficulties associated with
64 electrode placement in other species²³. We focus on the PCA as the sole arytenoid
65 abductor and because restored or returning PCA function is a requirement for
66 tracheostomy removal in patients with BVCP⁹

67

68

69 **Material and Methods**

70 This study was performed in accordance with the PHS Policy on Humane Care and Use
71 of Laboratory Animals, federal and state regulations, and was approved by the local
72 Institutional Animal Care and Use Committee (IACUC). Animals were brought into the
73 research unit and given a 7-day acclimatization period prior to any procedure. Daily
74 record logs of medical procedures were maintained.

75 *Experimental design:* Ten adult horses (Age range 5-7years, weight range 440-550kg)
76 with normal arytenoid function (Havemeyer grade 1,²⁴) were instrumented with two
77 quadripolar intra-muscular electrodes (5 French) in the left posterior cricoarytenoid
78 (PCA) muscle. Implanted electrodes were connected to a custom connector placed
79 subcutaneously on the left side of the neck that facilitated FES using an external
80 multichannel stimulating system (Master-8, AMPI, Israel).

81 *Surgical procedure:* Electrodes were implanted as previously described under general
82 anesthesia¹⁸. Briefly, an open approach to the lateral compartment of the left PCA
83 muscle was made and electrodes (quadripolar, K5-P4, Osypka AG, Rheinfelden,
84 Germany, figure 1) with a spiraling cathode at their tip were inserted using an insertion
85 cannula (Pajunk 18G needle, Pajunk GmbH, Germany). The lateral neuromuscular

86 compartment was selected as stimulation in this location produces the greatest arytenoid
87 abduction²⁵. Following a three week recovery period, and collection of baseline data,
88 each horse was anesthetized again and the left RLn was approached through a 10cm mid
89 cervical incision ventral to the jugular vein. Correct identification of the RLn was
90 confirmed using direct stimulation with an insulated needle (Stimuplex Insulated Needle;
91 Braun Medical, Bethlehem, PA) and 1 mA, 2 Hz, 0.15 ms duration pulses (Innervator
92 232; Fisher & Paykel Healthcare, Auckland, New Zealand): arytenoid abduction twitches
93 were verified endoscopically. The RLn was transected and the proximal and distal ends
94 ligated with 2/0 Ethilon. All horses received broad-spectrum antibiotics (trimethoprim-
95 sulfadiazine 30mg/kg, PO, BID) and Phenylbutazone (1mg/kg, PO, BID) for 5 to 7 days.
96 The effects of denervation were allowed to proceed without further intervention for
97 twelve weeks and then the left PCA muscle was stimulated using a once daily training
98 session for eight weeks (50Hz, 2ms pulse width, 2 s on 2s off 10 volts, for 48 min/day) to
99 produce a total of 72,000 impulses/day (daily frequency equivalent 0.83 Hz). Daily
100 activation below 1 Hz was used to minimize any shift to type I (slow) fibers²⁶. All
101 stimulation pulses used were biphasic with the negative (cathodic) phase occurring before
102 the positive phase. Seven animals were stimulated using these parameters (referred to as
103 the FES+ group) and three were implanted but not stimulated (controls, referred to as the
104 FES- group)). The other intrinsic laryngeal muscles were not stimulated and served as
105 internal controls.

106

107 Outcome measures:

108 *Overview:* The effects of denervation and stimulation were determined using quantitative
109 assessments of muscle excitation (strength-duration curve), muscle contraction judged by
110 arytenoid abduction response to increasing stimulation, computed-tomography based
111 reconstruction of PCA volume and, standardized exercise tests to determine laryngeal
112 function under increasing inspiratory loads. These observations were made immediately
113 after surgery, after the 12 week period of denervation and after a subsequent eight weeks
114 of FES. At the end of the study, immunohistochemistry of the left and right PCA and
115 Lateral Cricoarytenoid muscles (LCA) was performed to determine fiber type
116 distributions and minimum fiber (Feret's) diameters (MFD).

117 *Strength-duration trials:* To assess the excitation characteristics of the PCA muscle
118 following denervation, rheobase - the minimum amplitude needed to elicit a threshold
119 response at infinitely long pulse duration of the electrical stimulation and chronaxie -the
120 pulse duration at twice the rheobase were determined ²⁷. The procedure was performed
121 under light sedation with videoendoscopic observation of the left arytenoid cartilage ¹⁸.
122 Single biphasic balanced pulses were applied to the left PCA muscle with pulse durations
123 from 0.5 ms to 8ms. Pulse amplitude was increased from zero in 0.1mA steps until a
124 perceptible abduction twitch of the left arytenoid cartilage was observed via the
125 endoscope (threshold). The test stimuli were applied during expiration hold (the short
126 hesitation at the end of expiration) and the sequence of pulse durations was randomized.
127 Threshold was readily observed endoscopically.

128 The PCA response to increasing levels of electrical stimulation was determined at rest by
129 increasing the stimulation voltage (0, 0.5,2,..10 volts) of a short 10 second burst using
130 fixed pulse duration (2ms) and frequency (50Hz). Thirty seconds rest was allowed

131 between bursts and the sequence of stimulation was randomized. These parameters were
132 chosen as 50Hz is above the fusion frequency for the equine PCA¹⁸. The PCA response,
133 measured by the degree of arytenoid abduction, was determined at each stimulation level
134 using a flexible videoendoscope (Olympus GIF-140) passed into the nasopharynx via the
135 right ventral nasal meatus (figure 2). Briefly, a line was drawn to connect the dorsal and
136 ventral-most points of the rima glottidis and extending dorsally for a distance of one third
137 of the dorsoventral height of the rima glottidis. Lines were then drawn from the dorsal
138 most end of this line, tangential to each of the arytenoid cartilages. Finally, the angles
139 between the dorsoventral line and the tangents were measured^{28,29}

140

141 *Determination of PCA volume using Computed Tomography:* PCA volume was
142 estimated in vivo using a validated quantitative Computed Tomography (QCT) technique
143 [WM, this paper is currently in review how would you like me to reference it?]. Briefly,
144 under general anesthesia, QCT (Aquilion LB16, Toshiba) with reconstruction was used to
145 determine volume of the left and right PCA muscles. The cricoid cartilage was used as a
146 volume standard throughout the study. To improve spatial resolution, scan spacing and
147 thickness were set to minimum levels to minimize in-plane pixel size (0.25-0.50 mm),
148 scan spacing (1 mm), and scan thickness (1 mm). DICOM sets were imported into CT
149 reconstructive software (Mimics™, Materialise, Belgium) using Lossless compression.
150 A semi-automated segmentation tool, 3D LiveWire™, was used to outline the muscle of
151 interest³⁰. In-plane segmentation was repeated along the entire length of each PCA
152 muscle, and the tool was used to segment each portion. A gradient magnitude of 60%
153 with 0 attraction was used to best outline the muscle body. Mimics settings were further

154 adjusted to optimize smoothness without shrinkage (100 iterations, 1.00 smooth factor,
155 shrinkage compensation *on*). Triangle reduction using an advanced edge reducing mode
156 (0.0370mm tolerance, 15° edge angle, and 100 iterations) was also applied. Other
157 parameters remained at their default settings. From these operations, a custom three-
158 dimensional (3D) reconstruction of each muscle was generated with associated
159 geometrical properties. The volume of the cricoid cartilage was obtained at each time
160 point to provide a known standard.

161

162 *Exercise Protocol and Instrumentation:* All horses were exercised on a treadmill (0°
163 incline) under control conditions to verify normal upper respiratory tract at exercise
164 (laryngeal grade IA) ²⁴ prior to denervation. PCA function under increasing levels of
165 inspiratory load was determined using a standardized exercise test ^{18,31}. Each horse was
166 subsequently evaluated at an exercise intensity corresponding to 50,80,90 and 100% of
167 their maximum heart rate (HRmax) ³² to standardize the load experienced by the PCA
168 muscle at increasing levels of exercise intensity. After a warm up period, one minute was
169 spent at each level before increasing to the next. After the period of denervation, during
170 each one minute interval, the PCA was unstimulated in the first 30 seconds to determine
171 native function and the PCA was transiently stimulated in the second 30 seconds (50Hz,
172 2ms, 10V) to determine the response to stimulation. Horses were evaluated under these
173 conditions before RLn transection (baseline), after the period of denervation and after the
174 period of stimulation. No stimulation was applied at the baseline timepoint. Data
175 recorded during exercise trials included heart rate, electrocardiogram and accelerometer
176 measurements. Nasopharyngeal and laryngeal movements were recorded using a flexible

177 videoendoscope (Olympus GIF-140) passed into the nasopharynx via the right ventral
178 nasal meatus and secured. Heart rate was measured by an on-board monitor (Hippocard
179 Systems, Lexington, KY). Data were recorded onto DVD disks for subsequent analysis.

180

181 Immunohistochemistry:

182 Following euthanasia, the left and right PCA and LCA muscles were isolated and mid-
183 body samples (6mm³ approximately) embedded in cutting medium (Tissue-Tek OCT
184 Compound, Sakura Finetek, Netherlands) so that the fibers were orientated vertically and
185 frozen in melting isopentane precooled in liquid nitrogen (-156°C). Thereafter, 7µm
186 cryosections were air-dried onto glass slides (Superfrost plus microscope slides, VWR
187 International Ltd, Lutterworth, UK) and stored at -80°C. Routine Hematoxylin and Eosin
188 staining was performed on two sections from each muscle.

189 All mature skeletal muscle fiber types (1, 2a and 2x), hybrid fibers and collagen V were
190 identified in single equine muscle cryosections using a multiple immunofluorescence
191 labelling technique with 4 different primary antibodies.³³

192 Images were obtained using a Leica DMRA2, or DM4000 florescent microscope (Leica
193 Microsystems (UK) Ltd, Buckinghamshire, U.K.) via the x10 objective and LEICA A4,
194 L5, TX2, Y5 filters. The fluorescent camera used was a Zeiss MRm camera (ZEISS
195 microscopy), with a dedicated software program (Axiovision 4.8, www.zeiss.co.uk).

196 Background correction was performed in Volocity (Version 6, PerkinElmer,
197 Massachusetts 02451, USA) and was performed by imaging the even field in all filters
198 using Fluor-Ref (www. MicroscopyEducation.com). For determination of MFD by
199 individual fiber type or hybrid fiber designations, merged images were imported into

200 Image J (www.imagej.nih.gov) and fibers ($n > 400$) were manually measured and
201 subjectively assigned a fiber type or hybrid fiber designation by relative fluorescence.

202

203 Separate 7 μm muscle sections were used to determine the percentage collagen V
204 (severity of fibrosis) per unit area. A goat anti-collagen V antibody (1:20 diluted in
205 phosphate buffered saline) (Goat anti-type V collagen, Southern Biotechnology,
206 Birmingham, Alabama, USA) was used with a fluorescent secondary antibody (1:1000
207 diluted in phosphate buffered saline, Alexafluor 488 rabbit antigoat IgG,
208 www.invitrogen.com). Two images were obtained from each muscle sample using the
209 x10 objective and background corrected as described. Images were thresholded and
210 analysed in Volocity (Version 6, PerkinElmer, Massachusetts 02451, USA) for
211 objective quantification of collagen V expression per unit area.

212

213 Data Analysis

214 Strength duration data were subjected to curve fitting with the exponential function $V =$
215 $a * e^{b+c*\tau} + d$ whereas $V =$ Threshold voltage, $\tau =$ Stimulus pulse width and a, b, c, d
216 being constants that are evaluated for every SDC Curve, using custom software (Matlab
217 Curve-Fitting toolbox). Robust bisquare fitting and the trust region algorithm were used
218 to optimize curve fit with a maximum of 500 iterations and 750 evaluations. Lower
219 boundaries were 0.1, -0.1, -100 and 0, upper boundaries were 100, 100, -0.15 and 100
220 and starting points were 1.5, 1, -1.7 and 1 for a, b, c and d respectively. A logarithmically
221 spaced weighing vector was used to assign the first and last measured values the weight
222 1. The minimum weight was 0.3 for the middle value.

223

224 Endoscopic images of the *rima glottidis* were captured from the DVD recordings using

225 editing software (Video Wizard, Womble Multimedia, CA, USA). The degree of

226 arytenoid abduction was measured using a previously validated technique³⁴

227 with commercially available software (Able Image Analyser). Briefly a line is drawn

228 connecting the dorsal- and ventral- most points of the *rima glottidis*. This line was then

229 extended dorsally for a distance of one third of the dorsoventral height of the *rima*

230 *glottidis*. A tangential line to the arytenoid cartilages was drawn, and the angle between

231 the dorsoventral line and the tangential line measured¹⁸

232 . Minimum fiber diameters obtained for each fiber type within each muscle were

233 allocated into 5um bins and plotted as histogram envelopes.

234

235 For continuous outcome measures (arytenoid abduction response, rheobase, chronaxie,

236 PCA volume, fiber type proportion) a mixed effect model was fitted to the data to

237 determine the relationship between the outcome variable and relevant fixed effects using

238 horse as a random effect; interaction terms were included as appropriate. Minimum fiber

239 diameter data was fitted with a mixed effect model with muscle nested within horse

240 identity. To determine PCA response at increasing levels of inspiratory load, separate

241 models were fitted to the data for each time period (baseline, post denervation, post FES)

242 and each condition during testing under load (native function or FES applied

243 (stimulated)). Tukey's *post hoc* tests and linear contrasts were used as appropriate.

244 Statistical analysis was performed using JMP (SAS Institute, Cary, North Carolina,

245 USA). Significance was set at $p < 0.05$ throughout.

246

247 **Results**

248 All horses recovered uneventfully from surgery. Postoperative swelling and discomfort
249 were minimal and resolved within 10-14 days. Appropriate healing was confirmed in all
250 horses at suture removal. Horses remained comfortable throughout the study.

251

252 *Muscle Excitation characteristics:* The algorithm used to fit the strength duration curves
253 fit the raw data well with an R^2 (mean \pm sd) of 0.97 ± 0.022 . Evaluating changes in
254 rheobase, there was no significant interaction between time point and animal group (FES
255 + or control, $p=0.11$). Controlling for animal group (FES+ or FES-) there was a
256 significant increase in rheobase following denervation (baseline 0.91 ± 0.44 mA, post
257 denervation 4.96 ± 0.54 mA and post FES 3.64 ± 0.79 mA, $p<0.001$). There were no
258 differences between the FES + and FES – groups. Controlling for animal group, mean
259 chronaxie also slightly increased following denervation (1.01 ± 0.54 ms, 1.42 ± 0.44 ms,
260 1.32 ± 0.60 ms), although these changes were not significant ($p=0.24$).

261

262 *Response to increasing stimulation:* Prior to denervation, the PCA response to increasing
263 stimulation, measured using left arytenoid abduction reached a plateau, with maximal
264 arytenoid abduction of 50 degrees, above 3 volts of stimulation (figure 3). The response
265 to increasing stimulation following denervation and FES was markedly damped. No
266 significant differences were observed between FES+ and control horses.

267

268 *Volumetric changes:* Denervation produced a profound and significant 30-40% decrease
269 in left PCA volume in both FES+ and control (FES-) groups over the twelve week
270 denervation period (figure 4). In the FES + group, this atrophy did not proceed after the
271 onset of stimulation, in contrast atrophy did proceed in the control group during this
272 period. Left PCA volume was not significantly different between control and FES +
273 groups at the post FES time point (linear contrast, $p=0.22$). CT determination of cricoid
274 volume remained very stable throughout the time course with estimated volumes of 31.9
275 $\pm 3.5 \text{ cm}^3$, $31.6 \pm 4.0 \text{ cm}^3$, $29.6 \pm 3.4 \text{ cm}^3$ at each time point and no significant difference
276 between FES + and FES- groups.

277

278 *Function under load:* PCA function under increasing levels of inspiratory load (negative
279 inspiratory pressure) was markedly reduced after denervation (figure 5). No significant
280 differences were observed between FES + and control (FES -) groups either pre-
281 denervation (baseline) or post-denervation in native function or function during transient
282 electrical stimulation (stimulated). However, after the period during which the FES+
283 group received daily stimulation, native function was significantly improved at high
284 levels of inspiratory load (90 and 100 HRmax) in FES+ animals compared to controls
285 ($p=0.05$ and $p<0.01$ respectively, linear contrasts). No significant differences were
286 observed between FES + and control animals during transient stimulation at exercise at
287 this time point, although the means for function during transient stimulation showed the
288 same trend as the means for the native function.

289

290 *Immunohistochemistry:*

291 Controlling for other factors in the model, denervation of the left PCA produced a
292 37% reduction in minimum diameter (mean \pm se) for all fibers (23.6 ± 4.2 μ m) compared
293 to the innervated right PCA muscle (37.4 ± 4.2 μ m, $p < 0.05$). Denervation produced a
294 similar decrease in the mean minimum fiber diameter of the left LCA (23.9 ± 4.2 μ m)
295 compared to the innervated right LCA (40.4 ± 4.2 μ m, $p < 0.05$).

296 Electrical stimulation of the left PCA muscle produced a 30% increase in fiber
297 diameter in comparison with the unstimulated control group (33.9 ± 2.6 μ m FES+, 23.6
298 ± 4.2 μ m FES -, $p = 0.042$, linear contrast). The modal fiber diameter remained at
299 approximately 25 μ m, but a much higher proportion of fibers had hypertrophied to
300 between 40-60 μ m in the stimulated left PCA compared to the control left PCA (figure 6).

301 Electrical stimulation in the left PCA muscle produced a trend towards a decrease
302 in the proportion of type 1 (slow) fibers (linear contrast, $p = 0.09$) and a trend towards an
303 increase in type 2a (fast) fibers (linear contrast, $p = 0.052$, figure 7). No other significant
304 differences in type 1 or type 2a fibers were observed. Overall there was a significant
305 increase in the proportion of hybrid fibers in the denervated (left PCA and LCA) muscles
306 compared to the innervated (right PCA and LCA) muscles (linear contrast, $p = 0.0004$)
307 with no hybrid fibers detected in the innervated muscles. There was also a significant
308 decrease in the proportion of hybrid fibers in the stimulated PCA compared with the
309 unstimulated PCA ($p = 0.003$). There were no significant differences in any fiber type
310 proportion between horses in the stimulated and unstimulated groups in any of the
311 muscles to which FES was not applied – for example left LCA (L LCA stimulated) in the
312 horses which received FES in the left PCA and the left LCA in the horses which did not
313 receive FES in the left PCA (L LCA control). Overall model fits were good for each

314 fiber type (Model: Proportion \sim Horse_{random} + muscle+ group (stimulated or control) +
315 muscle*group; adjusted $R^2 = 0.63, 0.54, 0.98, 0.56$ for types 1, 2a, 2x and hybrid fibers
316 respectively). Unlabeled fibers summed to 0.54% across all animals and muscles and
317 were excluded from subsequent analysis.

318 Denervation produced an increase in the proportion of collagen V expression per
319 unit area (figure 8). Controlling for other factors in the model the denervated left PCA
320 and LCA ($56.4 \pm 3.6\%$, $59.9 \pm 5.6\%$) had significantly more collagen than the innervated
321 right PCA and LCA ($23.6 \pm 3.6\%$, $21.6 \pm 3.6\%$, $p=0.001$). There was no effect of FES in
322 the left PCA muscle ($p=0.72$, linear contrast) and no significant difference between other
323 unstimulated muscles in the FES + or FES – groups ($p=0.73$).

324

325 **Discussion**

326 These data demonstrate arrest of, and partial recovery from the consequences of complete
327 denervation using electrical stimulation in a large animal model. We identify increased
328 muscle volume and fiber cross-sectional area within the the posterior cricoarytenoid
329 muscle with stimulation We also identify a 30% increase in the muscle fiber diameter
330 with electrical stimulation and a trend towards a shift in an increasing proportion of type
331 2a fibers with electrical stimulation. These changes were produced using a relatively
332 conservative set of stimulation parameters [72,000 pulses/day, a daily activation rate of
333 1.83% (0.83 Hz) and a daily rest period of 23 hours]. These parameters were selected to
334 avoid a shift to increasing proportions of type 1 fibers seen at daily stimulation
335 frequencies above 1Hz²⁶ and achieved this goal.

336 The decrease in PCA volume of 30-40% over a 20 week denervation period,
337 suggests that slow rather than rapid atrophy occurs after denervation in this model. This
338 is consistent with slow atrophy and absence of necrosis found after denervation in other
339 large animal ^{14,22,35} rather than rodent models of denervation. ^{36,37} The equine model may
340 represent a good system in which to study the effects of electrical stimulation on
341 laryngeal muscle as the larger size of the equine PCA reduces some of the technical
342 considerations for electrode implantation, and endoscopic and volumetric reconstruction
343 techniques allow longitudinal assessment of structure and function.

344 Denervation produced a 37% reduction in fiber diameter as anticipated. The
345 relative proportion of collagen V also increased with denervation as fiber diameter
346 decreased. This increase in the proportion of collagen V is a consequence of both the
347 smaller fibers (producing an increase in circumference relative to cross-sectional area)
348 and an increased amount. The changes in fiber diameter were partially reversed with
349 electrical stimulation with a 30% increase in the shortest cross-sectional diameter
350 (minimum fiber diameter, MFD) when FES was applied to the left PCA muscle. These
351 changes are similar the 25% increase in MFD identified in other large animal models
352 evaluating the effects of FES ³⁸ in completely denervated laryngeal muscle. One
353 limitation of this study is that no assessment of muscle recruitment by FES was made and
354 additional optimization of electrode placement may improve the observed response. In
355 addition, longer pulses ²² or a longer period of stimulation ³⁹ may be required to optimize
356 direct intramuscular stimulation of the denervated PCA muscle

357 The trend towards a shift in an increasing proportion of type 2a fibers with
358 electrical stimulation suggests that the parameters used met the required goal of avoiding

359 a shift towards type 1 (slow) fibers which are characterized by reduced contraction force,
360 and time to peak contraction, characteristics that are particularly important in the PCA
361 which maintains airway patency during inspiration ¹¹

362 The increase in the proportion of type 1 fibers with denervation is anticipated as
363 these fibers are more resistant to denervation ^{36,40-42}

364

365

366 The response of the PCA muscle under load, imposed by increasing levels of
367 exercise intensity and so increasing the inspiratory pressure against which the PCA must
368 contract to maintain airway patency, is interesting. FES has been shown to improve
369 resting muscle tone even in the presence of complete denervation ⁴³

370 and increased tonicity in the PCA muscle will prevent complete collapse of the arytenoid
371 cartilage into the airway. This increase in tonicity is particularly important under high
372 inspiratory loads (90 and 100% HRmax) and may explain the significant differences in
373 native function at these levels, despite the absence of a detectable difference at lower
374 levels of exercise intensity (50 and 80% HRmax). Testing of intramuscular stimulation
375 during exercise subjects the arytenoid to high inspiratory negative pressures and may be a
376 more strenuous requirement than restoration of abduction in humans and dogs ^{10,38,44}

377 .

378 These data also demonstrate prevention of progression of the effects of
379 denervation in the stimulated animals (figure 5) with equivalent function in the stimulated
380 group after the denervation period and after FES. In contrast, the control group
381 demonstrated considerable worsening in native function after the additional denervation
382 period of eight weeks during which the FES+ animals were stimulated. This may be due

383 to increased resting muscle tone or shortened resting length in the stimulated CAD
384 muscles. An alternative explanation would be additional innervation provided by the
385 cranial laryngeal nerve. Function during stimulation was improved compared to native
386 function and so although a similar trend to prevent progression during stimulated function
387 was observed, this was less marked than the trend in changes in native function.

388 The prevention of progression identified by evaluating native function was
389 reflected in volumetric changes of the PCA muscle suggesting that FES at these relatively
390 low stimulation levels may reverse the ongoing atrophy produced by denervation. The
391 use of cricoid cartilage volume provided a useful known standard over time. This
392 technique has not been used in other animal models of laryngeal denervation will be used
393 in future experiments evaluating the effects of FES on the rate of reinnervation after
394 nerve injury. These techniques could also be applied in human patients to track the
395 response to electrical stimulation or reinnervation.

396 The increase in rheobase following denervation is consistent with ongoing
397 denervation and loss of neuromuscular junctions within the first 72 hours after
398 denervation. This algorithm was practical to fit to the data and provides an unbiased
399 method to obtain both chronaxie and rheobase without subjective curve fitting. It is
400 therefore recommended for future studies evaluating the effects of electrical stimulation
401 on reinnervation after nerve injury. The excitability of the PCA muscle improved only
402 slightly with once-daily activation. This demonstrates that there was no evidence of
403 reinnervation of the PCA.

404 In summary, these data suggest that electrical stimulation using a relatively
405 conservative set of stimulation parameters can reverse the muscle fiber atrophy produced

406 by complete denervation while avoiding a shift to a slow (type 1) fiber type, and
407 improving function under load. This study also describes the use of an effective
408 algorithm to evaluate muscle excitation and the use of muscle reconstruction using
409 computed tomography data sets to longitudinally track the response to muscle
410 stimulation. These approaches will be used in future work to evaluate the effects of more
411 aggressive stimulation parameters, with a shorter interval between training, on denervated
412 muscle and to evaluate the effects of electrical stimulation on reinnervation in a large
413 animal model of nerve injury.

414 Figure Legends

415 Figure 1. Illustration of K5-P4 electrode. Note: 3 anodes each 1.5mm long, screw length
416 2.5 mm with 2.5 turns; screw diameter 1.7 mm; screw surface area 8 mm². Screw
417 material is Platinum-Iridium (80%-20%). Total electrode length 300 mm. Insertion tool
418 shown in place (left)

419

420 Figure 2. Quantification of right PCA muscle function using videoendoscopic assessment
421 of arytenoid abduction. Right arytenoid abduction at rest (A); and during stimulation at
422 rest (B). Images are obtained from the same horse at the same time point prior to
423 denervation.

424 Figure 3. PCA response to increasing levels of stimulation at rest. Response determined
425 as abduction of the left arytenoid in response to biphasic pulses with duration 2ms, 50Hz.
426 Data shown are mean and standard deviation.

427

428 Figure 4. Changes in left PCA volume muscle in response to denervation and subsequent
429 stimulation. Data shown are mean and standard deviation. Different letters denote
430 significant differences between groups (Tukey's post hoc test, $p < 0.05$).

431

432 Figure 5. PCA response to increasing levels of inspiratory load and increasing exercise
433 intensity (%HRmax). Native function significantly improved at high levels of inspiratory
434 load (90 and 100 HRmax) after FES+ animals compared to controls (*, $p = 0.05$ and **,
435 $p < 0.01$, linear contrasts).

436

437 Figure 6. Fiber diameter distribution (%) in the denervated left PCA and LCA muscles
438 compared to contralateral innervated controls (right PCA and LCA). Bin size is 5 μ m.
439 FES was only applied to the left PCA in stimulated animals (solid line). Other muscles
440 served as internal controls and FES was not applied. Dotted lines describe animals in
441 which FES was not applied. Denervation produced a left shift in fiber diameter
442 distribution ($p < 0.001$). Within the left PCA muscle, electrical stimulation produced an
443 increase in fiber diameter (*, $p = 0.042$).

444

445 Figure 7. Proportion of fiber types 1, 2a and 2x within each PCA and LCA muscles.

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447 Figure 8. Representative immunohistochemistry images of intrinsic laryngeal
448 musculature labeled simultaneously for type 1, 2a and 2x fibers. Note increasing
449 collagen V proportion with decreasing fiber diameter.

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