

# Ultrasonographic detection of early atrophy of the intrinsic laryngeal muscles of horses

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## OBJECTIVE

To describe the ultrasonographic changes in the cricoarytenoideus dorsalis (CAD) and cricoarytenoideus lateralis (CAL) muscles of horses before and at various times during the 32 weeks after unilateral neurectomy of the right recurrent laryngeal nerve.

## ANIMALS

28 healthy Standardbreds.

## PROCEDURES

For each horse, the appearance of the CAD and CAL muscles on the right (neurectomized) and left (control) sides was serially monitored ultrasonographically by percutaneous (CAD and CAL) and transesophageal (CAD) approaches. The ultrasonographic images were assessed to determine the mean pixel intensity, muscle thickness, and appearance grade, and comparisons were made between the muscles of the neurectomized and control sides.

## RESULTS

The muscle appearance grade and mean pixel intensity for the CAL and CAD muscles on the neurectomized side were significantly increased by 2 and 4 weeks, respectively, after the neurectomy. The transesophageal approach enhanced the ultrasonographic visibility of the CAD muscle and allowed us to detect a significant decrease in the thickness of the CAD muscle on the neurectomized side over time, compared with thickness of the CAD muscle on the control side.

## CONCLUSIONS AND CLINICAL RELEVANCE

Results suggested ultrasonography can be used to successfully assess the CAL and CAD muscles of horses. A qualitative grading scheme was sufficient for successful detection and monitoring of muscle atrophy and reduced the need for image standardization. The transesophageal approach described for assessment of the CAD muscle warrants further investigation. (*Am J Vet Res* 2015;76:426–436).

In horses, RLN is a common disease of the laryngeal muscles in which primary axonopathy of the recurrent laryngeal nerve results in secondary neurogenic atrophy and dysfunction of the intrinsic laryngeal muscles that it innervates.<sup>1–4</sup> Recurrent laryngeal neuropathy primarily manifests on the left side, although horses with advanced disease occasionally have histologic lesions consistent with early neurogenic atrophy in the right intrinsic laryngeal muscles.<sup>4,5</sup>

The clinical signs associated with RLN include upper airway obstruction that worsens during exercise and are primarily caused by the progressive deterioration of the action of the CAD muscle. However, histologic evidence suggests that RLN affects the CAL

muscle, an adductor of the arytenoid cartilage, earlier and more severely than it does the CAD muscle.<sup>4,5</sup> In horses, ultrasonographic assessment of the CAL muscle is a reliable technique for detection of disease.<sup>6–8</sup> Most of the CAL muscle belly can be ultrasonographically assessed from the lateral acoustic window of the larynx.<sup>7</sup> Percutaneous ultrasonographic assessment of the CAD muscle is limited and includes mainly the musculotendinous junction at the cranial insertion of the muscle on the muscular process of the arytenoid cartilage.<sup>6,8</sup> Although real-time ultrasonography is inferior to traditional endoscopy for the assessment of laryngeal movements,<sup>9</sup> the use of ultrasonography to assess the CAL muscle is more accurate than is resting endoscopy for the diagnosis of RLN.<sup>6,8,9</sup> On the basis of the results of those studies,<sup>6,8,9</sup> it appears that ultrasonography is useful for the detection of abnormal muscle morphology induced by neurogenic atrophy despite its inability to accurately quantify laryngeal muscle function. Furthermore, the use of quantitative

## ABBREVIATIONS

bpm	Beats per minute
CAD	Cricoarytenoideus dorsalis
CAL	Cricoarytenoideus lateralis
CI	Confidence interval
RLN	Recurrent laryngeal neuropathy

ultrasonography improves the accuracy of RLN detection and might be useful for classification of disease severity.<sup>9</sup>

Suspicion is increasing that horses with RLN develop histologic abnormalities in the left intrinsic laryngeal muscles prior to the onset of the functional deficits that characterize clinical disease. This implies that it might be possible to use ultrasonography prior to the onset of clinical signs to detect underlying neurogenic atrophy. To our knowledge, the nature and extent of histologic lesions that are necessary to induce a detectable change in the ultrasonographic appearance of the intrinsic laryngeal muscles have not been explored, yet such information is fundamental to the development of ultrasonography as a screening tool for RLN in horses.

To date, ultrasonographic assessment of the CAD muscle has been limited to a small portion of the muscle that is visible from the percutaneous lateral acoustic window of the larynx. In the study reported here, a transesophageal ultrasonographic approach to augment the assessment of the CAD muscles was introduced. We anticipated that this approach would provide a more complete assessment of the CAD muscle, which would allow for more accurate measurements of muscle size, echo intensity, and appearance. The purpose of the study was to describe the ultrasonographic changes in the intrinsic laryngeal muscles of horses before and at various times during the 32 weeks after unilateral neurectomy of a recurrent laryngeal nerve. Our hypotheses were that the echo intensity of muscles innervated by the recurrent laryngeal nerve would increase over time following neurectomy, that the changes in muscle appearance described by quantitative techniques would be superior to those described by qualitative techniques, and that ultrasonographic assessment of the CAD muscle by a transesophageal approach would be superior to that by a percutaneous approach.

## Materials and Methods

### Animals

Twenty-eight Standardbred racehorses acquired from an auction were used for the study. Because the horses were acquired from an auction, additional history regarding health and performance was not available for any of the horses. The study population included 17 mares and 11 geldings with a mean age of 4.2 years (range, 2 to 8 years). Each horse was determined to be clinically normal on the basis of results of a physical examination, lameness examination at a walk and a trot, CBC, serum biochemical analysis, and endoscopic examination of the upper airways conducted at rest and during high-speed exercise on a treadmill. Horses were individually housed in box stalls with 8 to 10 hours of access to an outside paddock (weather permitting) daily for the duration of the study. The horses had *ad libitum* access to hay and water and were occasionally fed grain concen-

trate. The body weight and body condition score were monitored monthly for each horse, and feeding regimens were modified when necessary to ensure that none of the horses had substantial alterations in body weight during the study. All study protocols were approved by the Institutional Animal Care and Use Committee of the University of Guelph.

### Study design

Four horses were assigned to each of 7 groups in a nonrandom manner on the basis of logistic factors that included stall availability and personnel and equipment availability for data collection. The 7 groups were identified on the basis of the length of time following neurectomy that the horses were ultrasonographically evaluated. This included 4, 8, 14, 18, 24, 28, and 32 weeks.

### Endoscopic evaluation of the upper airway

Prior to study enrollment, each horse underwent an endoscopic examination of the upper airway while at rest and during high-speed exercise on a treadmill. Each horse was instrumented with a heart rate monitor that tracked heart rate during the treadmill exercise and was acclimatized to the treadmill 2 to 4 times prior to the endoscopic evaluation. The high-speed exercise test on the treadmill consisted of a 5-minute warm-up period without the endoscope in place during which the treadmill speed was adjusted to achieve a target heart rate of 130 to 180 bpm for each horse. The endoscope was then positioned in the left nostril, and the treadmill speed was incrementally increased each minute by 1 m/s until a target heart rate of 220 bpm was achieved. That speed was maintained for 2 minutes, after which the treadmill speed was gradually decreased over 30 seconds to a walking speed. The horse was walked until the heart rate decreased to < 100 bpm and then removed from the treadmill and walked and hosed with cold water as necessary on the basis of its vital signs. Horses were excluded from the study if they had evidence that a laryngoplasty surgery was previously performed or another abnormality was identified.

### Neurectomy procedure

The right recurrent laryngeal nerve was transected in each horse. The right recurrent laryngeal nerve was selected to avoid the potential presence of subclinical RLN on the left side. Each horse was anesthetized, and an approximately 5-cm skin incision was made dorsal and parallel to the right jugular vein in the midcervical area of the neck. The incision was bluntly extended until the omohyoid muscle was identified. The omohyoid muscle was bluntly separated to expose the right carotid artery. The carotid sheath was incised, and the recurrent laryngeal nerve was isolated. Identification of the nerve was confirmed by simultaneous stimulation with a nerve stimulator (settings: 1 Hz, 1 millisecond, and 2 to 3 mA) and an

endoscopic laryngeal examination. Once the nerve was identified, it was transected and a 2-cm section was removed. The nerve stimulator was applied to the vagus nerve to confirm the absence of arytenoid cartilage movement following transection of the recurrent laryngeal nerve. The *in vivo* cut ends of the recurrent laryngeal nerve were ligated with 2-0 polypropylene suture to prevent axonal regrowth. The incision was closed by apposition of the subcutaneous tissue with 2-0 synthetic absorbable suture<sup>a</sup> in a simple continuous pattern and apposition of the overlying skin with staples. After each horse recovered from anesthesia, an endoscopic examination of the upper airway was performed to confirm the presence of complete right laryngeal hemiplegia. Following surgery, each horse was administered phenylbutazone (1 g, PO, q 12 h) for 3 days and trimethoprim sulfa (22 mg/kg, PO, q 12 h) for 7 days. The incision site was monitored daily for evidence of heat, swelling, discharge, or signs of pain. Skin staples were removed 14 days after surgery.

### Ultrasonographic evaluation

Following neurectomy, ultrasonographic assessment of the larynx focused on the CAL and CAD muscles. Serial ultrasonographic imaging of the CAL and CAD muscles was achieved by the use of both a percutaneous approach, which allowed complete assessment of the CAL muscle and limited assessment of the CAD muscle in a longitudinal plane from the lateral acoustic window of the larynx, and a transesophageal approach, which allowed complete assessment of the CAD muscle but was not used to assess CAL muscle. Ultrasonographic evaluation of the larynx by the percutaneous approach was available for the entire study period and was used to assess the CAL and CAD muscles of all 28 horses. The equipment necessary for evaluation of the larynx by the transesophageal approach became available after the study began, and this approach was used to assess the CAD muscle of only 21 horses.

The percutaneous approach was performed with an ultrasound machine<sup>b</sup> and 8.5-MHz curvilinear probe<sup>c</sup> as described<sup>6-9</sup> through the lateral acoustic window of the larynx, which allowed nearly complete assessment of the CAL muscle and limited assessment of the CAD muscle. During each assessment, standardized images of the CAL and CAD muscles were obtained bilaterally with equipment parameters fixed for gain, power, depth, and gray map. Additional images of the CAL muscles were obtained by the use of operator-defined individualized gain settings without altering the depth, gray map, or power.

For each horse, ultrasonographic laryngeal examination by the percutaneous approach was performed within 24 hours before unilateral neurectomy (baseline). Because the rate of neuropathic change in the laryngeal muscles following neurectomy was unknown at the beginning of the study, percutaneous ultrasonographic examination of the larynx was performed every 48 hours for 8 weeks after surgery for the first

8 horses enrolled in the study. The objective and subjective measures of the mean echogenicity of the CAL muscle within each week did not differ significantly for those 8 horses; therefore, ultrasonographic examination of the larynx was performed weekly on all subsequent horses to reduce horse handling. For the 8 horses that had multiple ultrasound examinations performed within the same week, only the first ultrasound examination of each week was used for further analysis.

A novel transesophageal approach was used to expand the imaging capabilities for the CAD muscles of 21 of the 28 study horses. This approach used a commercially available transesophageal ultrasound probe, which is a small ultrasound probe situated on the end of a flexible cable similar to that of an endoscope. The probe was designed to be passed into the esophagus to facilitate imaging of adjacent structures through the esophageal wall and is widely used for imaging the heart. The CAD muscles are located directly ventral to the esophagus on the dorsal aspect of the larynx; therefore, our rationale was that a transesophageal approach could provide an appropriate acoustic window to assess the thickness of the CAD muscles.

Because use of a transesophageal ultrasonographic probe to assess the CAD muscles through a transesophageal acoustic window had not been described prior to this study, we first performed the technique in cadaveric horses and used concurrent laryngeal palpation and dissection to validate the ultrasonographic findings. Specifically, the transesophageal ultrasound probe was positioned in the cranial portion of the esophagus just past the cricopharyngeus muscle and upper esophageal sphincter. At this location, there are no other anatomic structures between the esophageal wall and the cricoid cartilage of the larynx, and the ultrasound probe was rotated by the operator until the cricoid cartilage was observed ultrasonographically, which indicated that it was directed toward the ventral esophageal wall. The cricoid cartilage is easily recognizable because of the hypoechoic appearance of the cartilage with a well-defined hyperechoic wall and the characteristic shape of the sagittal ridge on its dorsal aspect. Given that the CAD muscles have broad contact with the dorsal aspect of the cricoid cartilage and are the only anatomic structures in that location, the cricoid cartilage can be used as an anatomic landmark for locating them. With the transesophageal probe positioned in the cranial aspect of the cervical portion of the esophagus and directed ventrally, the CAD muscles can be observed bilaterally in a paramedian location with the sagittal ridge of the cricoid cartilage used to indicate the midline. The CAD muscles span the dorsal aspect of the cricoid cartilage and have visible striations. In most of the cadavers, the probe angle was rotated throughout the examination so that the entire thickness of the CAD muscles could be observed.

In the study horses, the transesophageal approach was performed by the use of an ultrasound machine<sup>d</sup>

with a pediatric transesophageal probe.<sup>c</sup> The probe was placed in the left nostril and passed into the caudal portion of the nasopharynx, where it was gently manipulated until the horse was stimulated to swallow. In some instances, video endoscopy of the upper airway was performed concurrently to aid in probe placement; however, this was rarely necessary as the operator gained experience. Once the probe was positioned in the cranial portion of the esophagus, representative images of the CAD muscle were acquired bilaterally. Effort was made to obtain images at the rostral-caudal midpoint of the CAD muscle, which was identified by sliding the probe from the caudal to rostral aspects of the muscle and then sliding it back approximately midway. During this procedure, the ultrasound machine parameters for gain were not standardized; instead, each image was optimized by the operator, although the probe, depth, gray map, and power parameters were held constant. The images obtained at the midpoint were used to determine the thickness of the CAD muscle, which was measured as the length of a line drawn parallel to the vertical plane located abaxial to the sagittal ridge of the cricoid cartilage (ie, midline). Ultrasonographic laryngeal examination by the transesophageal approach was performed at baseline and at 1-week intervals after neurectomy for the 4 horses that were evaluated for 4 weeks and at baseline and at 4-week intervals after neurectomy for the remaining 17 horses on which it was performed.

### Ultrasonographic image analysis

Following image acquisition from both the percutaneous and transesophageal approaches, the images of the CAD and CAL muscles were stored and exported for further processing. All identifying data (eg, patient name, date, and side) were removed from the images, and they were converted to JPEG format and randomized. An experienced observer (HJC), who was knowledgeable about the study but was unaware of the group of horses from which the images were obtained, assessed the images retrospectively during a 6-week period. Each image was evaluated 3 times, with at least a 1-week interval between observations so that intraobserver variation could be assessed. A 4-point grading scale was developed to qualitatively assess laryngeal muscle appearance. Briefly, grade 1 indicated that the muscle had a normal appearance and echotexture that was similar to the ipsilateral thyrohyoid muscle; grade 2 indicated that the overall echogenicity of the muscle was slightly increased, compared with that of the ipsilateral thyrohyoid muscle; grade 3 indicated that the muscle had a heterogeneous and nonuniform echogenicity, although the overall increase in echogenicity was not a prominent feature; and grade 4 indicated that the muscle had a heterogeneous and nonuniform echogenicity and the overall increase in the echogenicity was readily apparent.

For the quantitative grayscale analysis, the images obtained with the standardized machine settings were used to evaluate the CAL muscle; however, images of

the CAD muscle were not obtained with standardized machine settings, so the images used for the qualitative grading of laryngeal muscle appearance were used for the grayscale analysis. The images were imported into a commercially available software program<sup>f</sup> and converted into grayscale images. A standardized, round region of interest was overlaid on the CAL and CAD muscles, and the mean  $\pm$  SD and range of the pixel intensity within that area were determined.

### Statistical analysis

The echo intensity data were modeled as an autoregression model, and a logit transformation was used because the dependent variable had a finite range (ie, 0 to 255). A generalized linear mixed model was used to assess the muscle appearance grades. Residual analyses were performed to test the ANOVA model assumptions (ie, the errors are normally distributed, homoscedastic [equal variance], and independent). The assumption of normality was assessed with 4 tests (Shapiro-Wilk, Kolmogorov-Smirnov, Cramer-von Mises, and Anderson-Darling) and the plotting of residuals against predicted values. Intra-observer repeatability was calculated with the  $\kappa$  statistic, and when disagreement was present, the most commonly assigned grade from the 3 assessments was used for further analysis. The effect of neurectomy over time was assessed by the use of a mixed model, which included side (right or left) as a fixed effect and a random effect to account for multiple observations from the same horse. For all statistical models, the model assumptions were tested, and transformations were applied to the data when necessary to satisfy those assumptions. All analyses were performed with commercially available statistical software,<sup>g</sup> and values of  $P < 0.05$  were considered significant.

## Results

### Horses

No clinically relevant abnormalities were detected during physical examination of any of the 28 horses. Incidental findings included skin abrasions or scars ( $n = 23$ ), an aural growth (1), lameness (4), and cardiac arrhythmia (1). Results of all CBCs and serum biochemical analyses were within the reference ranges established for horses at our institution. For all horses, results of the assessment of the video endoscopic examinations while at rest and during high-speed exercise on the treadmill were considered clinically normal. The mean age and body weight, median body condition score, and sex distribution did not differ significantly among the 7 groups of horses. Neither body weight ( $P = 0.35$ ) nor body condition score ( $P = 0.68$ ) changed significantly for any of the horses during the study period.

All horses had complete laryngeal paralysis of the right side immediately following neurectomy, which indicated that transection of the right recurrent laryngeal nerve was successful. None of the horses developed postoperative complications, and all incisions healed as expected.



Both the percutaneous and transesophageal ultrasound approaches were well tolerated by all horses without sedation. Initially, concurrent use of a video endoscope positioned in the contralateral nostril was useful to aid in the placement of the transesophageal ultrasound probe, but it became unnecessary as the ultrasonographer gained experience with the technique. In fact, the transesophageal probe was placed without endoscopic assistance for most of the examinations.

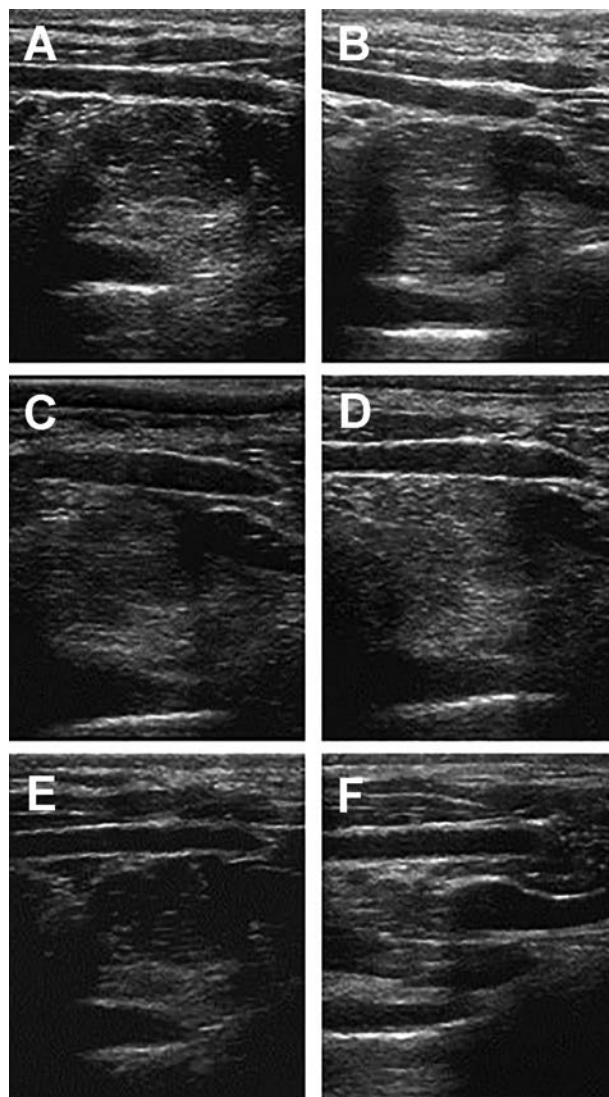
### CAL muscle

The CAL muscles could be assessed in both the transverse and longitudinal planes by the percutaneous approach. Occasionally, pinpoint mineralization within the thyroid cartilage created an acoustic shadow, but this was always mild and did not prohibit CAL muscle assessment in any of the horses. The use of standardized ultrasound machine settings facilitated acquisition of a comparable set of images for quantitative image analysis; however, it frequently did not result in optimal CAL muscle image appearance, and the images were considered of substandard quality from a diagnostic perspective. The standardized images were always used for quantitative image analysis (ie, mean pixel intensity), but were not always used for grading muscle appearance. Serial ultrasonographic images of the CAL muscle from one of the study horses are provided (**Figure 1**).

The mean pixel intensity over time, as determined from the standardized ultrasonographic images of the CAL muscles on the neurectomized (right) and control (left) sides, was summarized (**Figure 2**). The mean pixel intensity did not differ significantly ( $P = 0.234$ ) between the neurectomized and control sides prior to neurectomy (baseline). However, following neurectomy, the mean pixel intensity for the CAL muscle on the neurectomized side increased significantly ( $P < 0.001$ ) from baseline over time, whereas that for the CAL muscle on the control side did not change significantly ( $P = 0.278$ ) from baseline over time. The odds that the mean pixel intensity of the CAL muscle on the neurectomized side would be greater than that for the CAL muscle on the control side attained significance ( $P = 0.034$ ) at 2 weeks after neurectomy (OR, 1.21; 95% CI, 1.02 to 1.46) and continued to increase for the remainder of the observation period. The variance of the mean pixel intensity for the CAL muscle on the neurectomized side was significantly ( $P = 0.049$ ) greater than the variance of the mean pixel intensity for the CAL muscle on the control side beginning 3 weeks after neurectomy (OR, 1.47; 95% CI, 1.14 to 1.89).

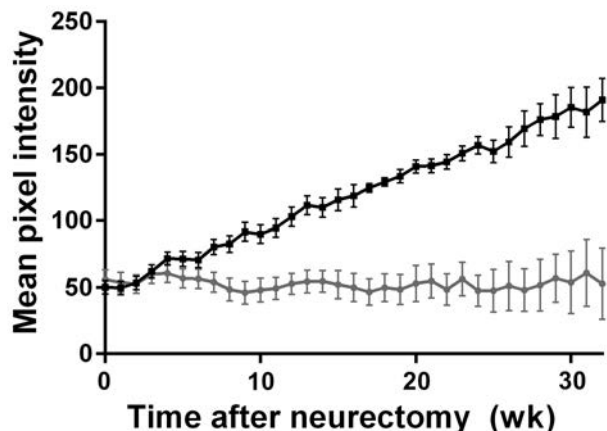
The muscle appearance grades over time for the CAL muscles on the neurectomized side were summarized (**Figure 3**). The mean muscle appearance grade did not differ significantly ( $P = 0.238$ ) between the CAL muscles of the neurectomized and control sides at baseline. Compared with the mean muscle appearance grades at baseline, the mean muscle appearance

grade increased significantly ( $P < 0.001$ ) over time for the CAL muscle on the neurectomized side, but did not change significantly ( $P = 0.638$ ) over time for the CAL muscle on the control side. The odds that the mean muscle appearance grade for the CAL muscle on the neurectomized side would be greater than that for the CAL muscle on the control side attained significance ( $P = 0.009$ ) at 2 weeks after neurectomy (OR, 3.44; 95% CI, 1.35 to 8.74) and continued to increase



**Figure 1**—Serial ultrasonographic images obtained from a lateral acoustic window of the control (left; A, C, and E) and neurectomized (right; B, D, and F) CAL muscles of a 4-year-old Standardbred at 4 (A and B), 18 (C and D), and 28 (E and F) weeks after unilateral neurectomy of the right recurrent laryngeal nerve. Notice that the echogenicity of the CAL muscle on the neurectomized side is increased, compared with that of the CAL muscle on the control side at each time. At 4 weeks after neurectomy, the boundaries of the CAL muscle on the neurectomized side (B) are still well-defined despite the increase in muscle echogenicity. At 18 weeks after neurectomy, the boundaries of the CAL muscle on the neurectomized side (D) are obscured, and the muscle remains hyperechoic. At 28 weeks after neurectomy, the CAL muscle on the neurectomized side (F) is ill defined with increased echogenicity and a nonuniform appearance.

until 16 weeks following neurectomy, after which the odds remained fairly stable for the remainder of the observation period.

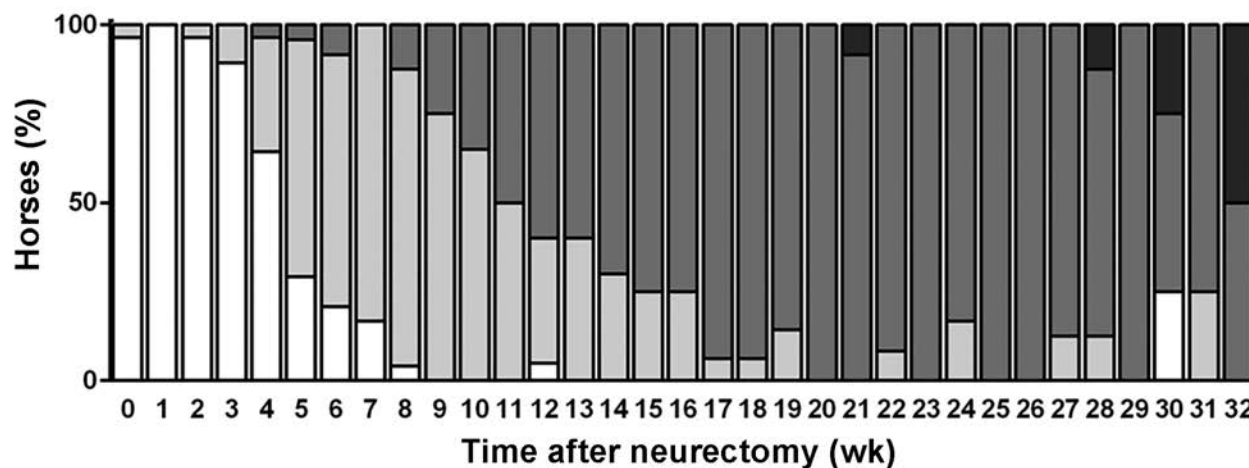


**Figure 2**—Mean  $\pm$  SEM pixel intensity for the CAL muscles on the neurectomized (right; black line) and control (left; gray line) sides of adult Standardbreds as determined by ultrasonography performed by a percutaneous approach through a lateral acoustic window of the larynx at 1-week intervals after neurectomy of the right recurrent laryngeal nerve. Four horses were assigned to each of 7 groups in a nonrandom manner, and the groups were identified on the basis of the number of weeks (4, 8, 14, 18, 24, 28, and 32) after neurectomy that the horses were evaluated. Thus, the values for weeks 1 through 4 represent the mean for 28 horses, the values for weeks 5 through 8 represent the mean for 24 horses, the values for weeks 9 through 14 represent the mean for 20 horses, the values for weeks 15 through 18 represent the mean for 16 horses, the values for weeks 17 through 24 represent the mean for 12 horses, the values for weeks 25 through 28 represent the mean for 8 horses, and the values for weeks 29 through 32 represent the mean for 4 horses. The values for week 0 represent the baseline measurements for images obtained immediately before the neurectomy was performed.

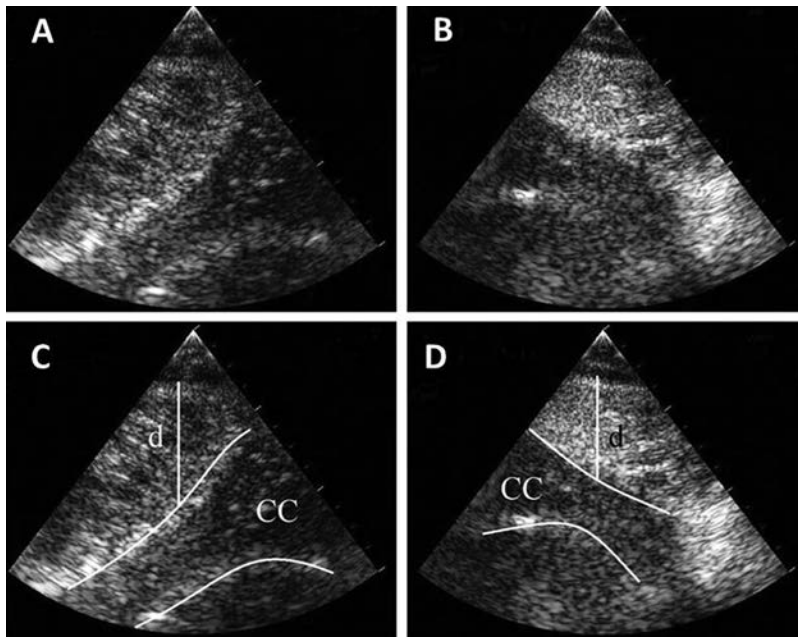
## CAD muscle

The percutaneous approach provided limited visibility of the CAD muscles in the longitudinal plane at the muscular process of the arytenoid cartilage. The location of the larynx relative to the angle of the mandibles and the amount of flesh around the throat region hindered the ability to observe the insertion of the CAD muscle on the muscular process. Concurrent manual pressure on the contralateral side of the larynx made its dorsal aspect more assessable from the lateral acoustic window and facilitated imaging of the CAD muscle. Use of standardized ultrasound machine settings facilitated acquisition of a comparable set of images for quantitative image analysis but frequently resulted in substandard images for diagnostic purposes. Consequently, the standardized images were always used for quantitative image analysis but were not always used for muscle appearance grading. This situation was substantially more apparent for the CAD muscles, compared with the CAL muscles. The mean muscle appearance grade, mean pixel intensity, and size of the CAD muscle as determined by the percutaneous approach did not differ significantly between the neurectomized and control sides over time.

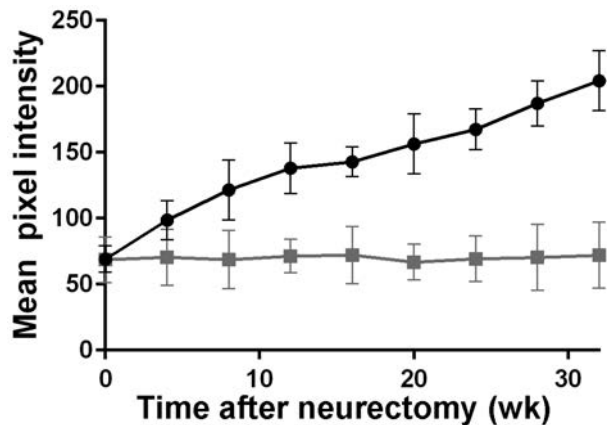
The transesophageal approach enhanced the visibility of the CAD muscles (**Figure 4**). The mean pixel intensity of the CAD muscles from both the neurectomized and control sides, as determined from images obtained by the transesophageal approach, was summarized (**Figure 5**). The mean pixel intensity did not differ significantly ( $P = 0.174$ ) between the CAD muscles of the neurectomized and control sides prior to neurectomy. Following neurectomy, the mean pixel intensity of the CAD muscle on the neurectomized side increased significantly ( $P < 0.001$ ) from baseline over time, whereas that for the CAD



**Figure 3**—Summary of muscle appearance grades assigned to the CAL muscle on the neurectomized side for the horses of Figure 2. A 4-point grading scale was developed to qualitatively assess laryngeal muscle appearance. Grade 1 (white bars) indicated that the muscle had a normal appearance and echogenicity that was similar to the ipsilateral thyrohyoid muscle; grade 2 (light gray bars) indicated that the overall echogenicity of the muscle was slightly increased, compared with that of the ipsilateral thyrohyoid muscle; grade 3 (dark gray bars) indicated that the muscle had a heterogenous and nonuniform echogenicity, although the overall increase in echogenicity was not a prominent feature; and grade 4 (black bars) indicated that the muscle had a heterogenous and nonuniform echogenicity and the overall increase in the echogenicity was readily apparent. The bars represent the percentage of horses with that score at each time. See Figure 2 for remainder of key.



**Figure 4**—Ultrasonographic images obtained by a transesophageal approach of the CAD muscles on the control (A and C) and neurectomized (B and D) sides of a 4-year-old Standardbred 14 weeks after neurectomy of the right recurrent laryngeal nerve. Panels C and D are identical to panels A and B, respectively, except that in panels C and D, the cricoid cartilage (CC) has been delineated by curved white lines and the thickness of the CAD muscle (d) has been delineated by a straight white line. Notice that the CAD muscle on the neurectomized side has increased echogenicity (ie, appears brighter), compared with the CAD muscle on the control side.



**Figure 5**—Mean  $\pm$  SEM pixel intensity for the CAD muscles on the neurectomized (right; black line) and control (left; gray line) sides for 21 of the 28 horses of Figure 2 as determined by ultrasonography performed by a transesophageal approach at 4-week intervals after neurectomy of the right recurrent laryngeal nerve. The CAD muscle of only 21 horses was evaluated because the equipment necessary for evaluation of the larynx by the transesophageal approach did not become available until after the study began. Thus, the values for weeks 1 through 8 represent the mean for 21 horses, the values for weeks 9 through 14 represent the mean for 20 horses, the values for weeks 15 through 18 represent the mean for 16 horses, the values for weeks 17 through 24 represent the mean for 12 horses, the values for weeks 25 through 28 represent the mean for 8 horses, and the values for weeks 29 through 32 represent the mean for 4 horses. See Figure 2 for remainder of key.

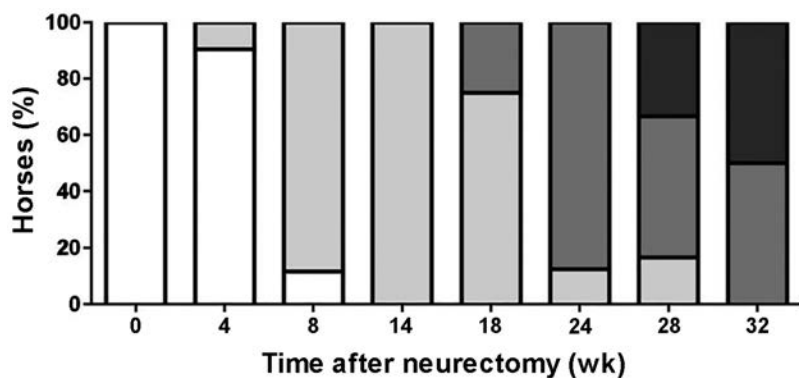
muscles on the control side did not change significantly ( $P = 0.791$ ) from baseline at any time. The odds that the mean pixel intensity for the CAD muscles on the neurectomized side would be greater than the mean pixel intensity for the CAD muscles on the control side were significant ( $P = 0.005$ ) beginning 4 weeks after neurectomy (OR, 1.21; 95% CI, 1.089 to 1.349) and continued to increase for the remainder of the observation period. The variance of the mean pixel intensity of the CAD muscle did not differ significantly ( $P = 0.861$ ) between the neurectomized and control sides prior to neurectomy. However, the variance of the mean pixel intensity for the CAD muscle on the neurectomized side was significantly ( $P = 0.013$ ) greater than that for the CAD muscle on the control side beginning at 12 weeks after neurectomy, although the magnitude of the difference was small ( $F$  ratio, 1.14; 95% CI, 1.03 to 1.26).

The mean muscle appearance grades as determined from images obtained by the transesophageal approach did not differ significantly ( $P = 0.775$ ) between the CAD muscles of neurectomized and control sides at baseline. Al-

though the mean muscle appearance grades for the CAD muscle on the control side did not change significantly ( $P = 0.954$ ) from baseline over time, those for the CAD muscle on the neurectomized side increased significantly ( $P < 0.001$ ) from baseline and were summarized (**Figure 6**). The odds that the mean muscle appearance grade for the CAD muscle on the neurectomized side would be greater than the median muscle appearance grade for the CAD muscle on the control side did not quite reach significance ( $P = 0.052$ ) at 4 weeks after neurectomy (OR, 11.43; 95% CI, 0.98 to 133.76) but was significant ( $P < 0.001$ ) at 8 weeks after neurectomy (OR, 500.8; 95% CI, 62.29 to 4,026.66).

The thickness of the CAD muscle, as measured on images obtained by the transesophageal approach on the neurectomized side, decreased significantly ( $P < 0.001$ ) over time, compared with the thickness of the CAD muscle on the control side. At baseline, the CAD muscle on the control side was slightly but significantly ( $P = 0.017$ ) thicker than the CAD muscle on the neurectomized side. The CAD muscle on the control side was significantly ( $P < 0.001$ ) thicker than the CAD muscle on the neurectomized side at 4 weeks after neurectomy and remained so for the duration of the observation period. The ratio of the thickness of the CAD muscle on the neurectomized side to the thickness of the CAD muscle on the control side decreased by 99.3% each week, such that it was approximately 85.2% of its baseline value by week 24.





**Figure 6**—Summary of muscle appearance grades assigned to the CAD muscle on the neurectomized side for the horses of Figure 5. See Figures 3 and 5 for remainder of key.

### Repeatability of muscle appearance grades

Each of 540 CAL muscle images was assessed 3 separate times, and the intraclass correlation coefficient for the muscle appearance grade was 0.86. The same muscle appearance grade was consistently assigned to 492 of the 540 (91.1%) images. For the remaining 48 images, 1 of the 3 observations differed from the other 2 (ie, no image was assigned 3 different grades), although it never differed by > 1 grade. The majority (36/48 [75%]) of disagreement was between grades 2 and 3.

Similarly, each of 91 CAD muscle images was assessed 3 separate times, and the intraclass correlation coefficient for the muscle appearance grade was 0.72. The same muscle appearance grade was consistently assigned to 72 of the 91 (79.1%) images. One observation differed from the other 2 for 18 images, and a different muscle appearance grade was assigned at each observation for 1 image. The most common disagreement was between grades 1 and 2 (9/19) and between grades 2 and 3 (9/19).

### Discussion

Results of the present study indicated that ultrasonography could be used to identify and monitor neuropathic changes over time in the intrinsic laryngeal muscles (CAL and CAD muscles) of horses. Assessment of ultrasonographic images of the CAL and CAD muscles by both quantitative (mean pixel intensity) and qualitative (muscle appearance grade) methods was successful for monitoring muscle changes during the development of neurogenic atrophy, with significant changes detected in the CAL and CAD muscles as early as 2 and 4 weeks, respectively, after neurectomy. These findings implied that the amount of tissue alteration needed to significantly affect the overall echo intensity of the muscle develops soon after denervation in both the CAL and CAD muscles.

We expected that the quantitative measurement of mean pixel intensity would provide the earliest detection of neurogenic muscle atrophy, and indeed, use of this method identified significant differences in

the CAL muscle on the neurectomized (right) side at 2 weeks after neurectomy. However, the qualitative measurement of muscle appearance grade also identified significant differences in the CAL muscles at 2 weeks after neurectomy. Additionally, the magnitude of the odds that the CAL muscles on the neurectomized side would be assigned an abnormal muscle appearance grade, compared with the CAL muscles on the control (left) side, was greater than the magnitude of the odds that the mean pixel intensity for the CAL muscles on the neurectomized side was greater, compared with the mean pixel intensity for the CAL muscles on the control

side throughout the observation period. Although this finding might appear to promote the use of the qualitative grading of muscle appearance rather than the quantitative measurement of mean pixel intensity to assess or monitor neurogenic muscle atrophy, the differences in the magnitude of the ORs between the 2 assessment methods might have been attributable to differences between dichotomized and continuous variables. The statistical differences identified during the weeks immediately after neurectomy were not sufficiently robust to be diagnostically reliable.

During assessment of the CAD muscle images obtained by the transesophageal approach, the mean pixel intensity on the neurectomized side was significantly greater than that on the control side beginning at 4 weeks after neurectomy, whereas the muscle appearance grade did not differ significantly between the neurectomized and control sides until 8 weeks after neurectomy. This finding should be interpreted cautiously because the CAD muscle was measured only every 4 weeks instead of every week as the CAL muscle was; therefore, it is possible that a difference in muscle appearance grade could have been detected earlier had the CAD muscle been assessed more frequently. This supposition is partially supported by the fact that the OR that the muscle appearance grade for the CAD muscle on the neurectomized side would be greater than that for the CAD muscle on the control side was close to our cut off for significance ( $P = 0.052$ ) at 4 weeks after neurectomy. Also, only 21 of the 28 study horses were evaluated with the transesophageal approach, which reduced the power of the analyses for the CAD muscle. Regardless of the approach used, ultrasonographic changes associated with neurogenic muscle atrophy were detected in the CAL muscle earlier than in the CAD muscle. This may be an indication that, following neurectomy, atrophy proceeds more rapidly in the CAL muscle than it does in the CAD muscle in a manner similar to that observed in naturally occurring RLN<sup>4</sup>; however, prior to the present study, the rate of atrophy development in the CAL and CAD muscles had not been assessed in horses with induced RLN. Alternatively, this finding could indicate



that ultrasonographic assessment of the CAL muscle is more accurate than that of the CAD muscle because the CAL muscle is more readily assessable than is the CAD muscle for ultrasonographic examination, or the ultrasonographic equipment used for assessment of the CAL muscle by the percutaneous approach was more sensitive for detection of early changes associated with atrophy than was the equipment used for assessment of the CAD muscle by the transesophageal approach.

In addition to the mean pixel intensity, we also analyzed the mean variance of pixel intensity within the designated region of interest because we expected the variability of pixel intensity to increase as muscle atrophy progressed. For the CAL muscle, the mean variance of pixel intensity differed significantly ( $P = 0.026$ ) between the neurectomized and control sides beginning at 3 weeks after neurectomy, and the magnitude of that difference continued to increase for the remaining duration of the observation period. For the CAD muscle, the mean variance of pixel intensity did not differ significantly between the neurectomized and control sides until 12 weeks after neurectomy, and although the magnitude of the difference was small, it remained significant throughout the remaining duration of the observation period. It was evident that there were substantial differences between the CAL and CAD muscles when the variance of pixel intensity was used to assess muscle atrophy. It is unclear whether those differences were caused by a nonuniform response of the CAL and CAD muscles to the neurectomy procedure, differences in the quality of the ultrasonographic images obtained by the percutaneous (CAL muscle) and transesophageal (CAD muscle) probes, or a combination of those factors.

Although the use of quantitative ultrasonography is frequently superior, compared with other diagnostic imaging modalities for the subjective assessment of many conditions,<sup>10-12</sup> it remains underutilized, most likely because of practical limitations. Quantitative ultrasonography requires the use of a single ultrasound machine with exact standardization of equipment settings and protocols for each examination. Furthermore, for a quantitative measurement such as mean pixel intensity to be useful, a reference range for clinically normal muscle must be established so that results can be interpreted. The establishment of reference ranges for quantitative ultrasonographic assessment of skeletal muscle in humans required the use of highly controlled equipment.<sup>10-12</sup> In addition to the standardized gain, probe, and power settings like we used in the present study, those investigators<sup>10-12</sup> extrapolated the raw ultrasound data to circumvent the proprietary processing algorithms that are inherent to the final ultrasound image, which requires advanced hardware and software capabilities that are impractical for most clinical settings. Furthermore, reference ranges would need to be established for each ultrasound machine and theoretically could not be shared between centers. Even though establishment of refer-

ence ranges for individual ultrasound machines might be achievable in research settings, it remains a major limiting factor for the application of quantitative ultrasonography in clinical practice.

Ultrasonographic images of the laryngeal muscles obtained by the percutaneous approach with standardized machine settings were frequently considered to be of inferior diagnostic quality for clinical assessment of muscles. Patient factors such as skin cleanliness, oil content, and pigmentation; hair length; body composition; and fat distribution within the tissues all contribute to variation in the ultrasonographic appearance of muscles that the ultrasonographer cannot compensate for when the machine settings are standardized. To our knowledge, inconsistent image quality has not been an issue in studies<sup>10-12</sup> that involved human subjects, and it is unclear whether it did not occur or was not identified. It is possible that there is less variation in the ultrasonographic appearance of the muscles of humans or that factors affecting the ultrasonographic appearance of muscles can be readily controlled by preparation of human patients.

Use of a subjective or qualitative ultrasonographic measurement such as the muscle appearance grade developed for the present study requires high-quality, representative images of the muscle. Subjective ultrasonographic techniques do not require the use of standardized machine settings. Instead, experienced ultrasonographers optimize the quality of the images during acquisition, and this is how most diagnostic ultrasonography is performed in both human and veterinary medicine. High-quality images obtained by operator optimization of machine settings facilitate the diagnostic usefulness of those images in clinical settings. Because the quality of the ultrasonographic images obtained with the standardized machine settings was frequently compromised in the present study, we recommend that ultrasonographic images of the intrinsic laryngeal muscles of horses be obtained with both standardized and optimized techniques to ensure that sufficient diagnostic information is available during image interpretation.

The challenges associated with quantitative ultrasonography can be avoided by the use of a subjective measurement such as the muscle appearance grade described in this study instead of an objective measurement such as the mean pixel intensity. With proper training and experience, the muscle appearance grading system can be used to assess ultrasonographic images of muscles regardless of location or equipment used to obtain the images. An advantage of the muscle appearance grading system is that it assesses various characteristics of the muscle, including its echo intensity, echotexture, and uniformity of appearance, and allows comparison with adjacent control tissues, whereas quantitative ultrasonography measures only the echo intensity of muscle. An ultrasonographic grading system<sup>13</sup> described for assessment of appendicular skeletal muscle was not used in the present study because it uses the intensity of the adjacent long

bone echo as a referent for the muscle appearance grades and a bone echo is not available for reference in the laryngeal region.

Results of the present study suggested that ultrasonographic assessment of the CAD muscle by a percutaneous approach is limited. Indeed, reports of percutaneous approaches for the ultrasonographic assessment of the larynx in humans,<sup>14</sup> dogs,<sup>15</sup> and horses,<sup>6-9</sup> do not include a description of the CAD muscles (termed the posterior cricoarytenoid muscles in humans). On the basis of the anatomic location of the CAD muscles and our clinical experience with ultrasonographic evaluation of the intrinsic laryngeal muscles of horses, we hypothesized that a percutaneous approach would not be useful for accurate quantification of the CAD muscle. Because serial evaluation of the CAD muscle is clinically important for monitoring the response of horses with RLN to neuromuscular treatments, we attempted to use a percutaneous window to assess the CAD muscle appearance in the present study. This approach failed to identify any quantitative or subjective changes in the CAD muscle following neurectomy. Therefore, we consider a percutaneous approach unsuitable for the ultrasonographic assessment of the CAD muscles, and caution should be used during the interpretation of CAD muscle images obtained by this approach.

Conversely, the transesophageal approach provided an extremely useful method for evaluation of the CAD muscles. Because the CAD muscles are located on the dorsal aspect of the larynx, a transesophageal probe positioned in the upper portion of the esophagus can be directed ventrally to assess the bellies of both CAD muscles. In the present study, the transesophageal approach was efficient and well tolerated by the horses and provided images that were useful for the quantitative measurement of pixel intensity and the subjective evaluation of the appearance of the CAD muscle.

The main challenge encountered with the transesophageal approach was the proximity of the CAD muscle belly to the near field of the ultrasound image. This limited our ability to obtain an image of the entire muscle belly in 1 section, and because the CAD muscle has a pennate shape (ie, the thickness of the muscle is not uniform from the cranial to caudal ends), use of this technique to measure the thickness of the CAD muscle warrants further investigation. Validation of the transesophageal approach for measurement of the CAD muscle thickness may require correlation with a cross-sectional imaging modality such as CT. Such validation is important because accurate measurement of CAD muscle size has the potential to contribute to the detection and monitoring of horses with RLN. In the present study, the thickness of the CAD muscle on the neurectomized side as determined by ultrasonographic images obtained by the transesophageal approach decreased over time, compared with that at baseline (ie, immediately prior to neurectomy) and the thickness of the CAD muscle on the control side. However,

the accuracy of the measurements of CAD muscle thickness was not verified in the present study, and additional research into the use of a transesophageal approach for the ultrasonographic assessment of the CAD muscle is necessary before this technique is used for horses with RLN.

Interestingly, at baseline, the thickness of the CAD muscle on the control side was significantly less than the thickness of the CAD muscle on the neurectomized side. The reason for this difference was unknown, but subclinical RLN on the control side might have contributed to it, or the difference might have been an incidental finding. Again, further validation of the accuracy of transesophageal ultrasonographic measurements of the CAD muscles is warranted.

Knowledge obtained from the use of unilateral neurectomy of a recurrent laryngeal nerve to induce neurogenic laryngeal muscle atrophy in clinically normal horses should be extrapolated cautiously to horses with naturally occurring RLN or other neuropathic conditions. In the present study, ultrasonography successfully detected changes in the echogenicity of the CAL and CAD muscles following neurectomy, even when those changes were subtle. We expect that the implementation of serial ultrasonographic evaluation of the intrinsic laryngeal muscles will aid in treatment planning and disease monitoring of horses with RLN and may also have translational applications in human medicine. Elucidation of the amount of tissue alteration necessary for ultrasonographic detection of disease is warranted to better determine the applicability of ultrasonography for detecting and monitoring horses with RLN.

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## Footnotes

- a. Polysorb, Covidien, Mansfield, Mass.
- b. Logic 5 ultrasound machine, GE Healthcare Inc, Bothell, Wash.
- c. 8.5-MHz curvilinear ultrasound probe, GE Healthcare Inc, Bothell, Wash.
- d. Vivid 9 ultrasound machine, GE Healthcare Inc, Bothell, Wash.
- e. 9T pediatric transesophageal ultrasound probe, GE Healthcare Inc, Bothell, Wash.
- f. Image Pro Premiere, Media Cybernetics Inc, Rockville, Md.
- g. SAS, version 9.2, SAS Institute Inc, Cary, NC.

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