ULTRASOUND GUIDED BIOPSY OF THE *CRICOARYTENOIDEUS LATERALIS* IN THE HORSE

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ABSTRACT

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Recurrent laryngeal neuropathy (RLN) is a distal axonopathy of the recurrent laryngeal nerve. Current diagnostics rely upon disease recognition in the clinically affected horse, with no ante-mortem test yet available to detect subclinical muscle fiber changes. The purpose of the present study was to validate a novel method of muscle retrieval from the equine larynx in the standing horse.

First, an immunohistochemical comparison of muscle fiber types was made between the left *cricoarytenoideus lateralis* and the *cricoarytenoideus dorsalis* in six endoscopically normal horses. Subsequently, ultrasound guided biopsy of the left *cricoarytenoideus lateralis* was performed in six, sedated horses, which were then monitored for complications. The procedure was well tolerated and biopsy specimens suitable for immunohistochemical analysis were successfully obtained in all horses. No major complications were noted following the procedure.

Ultrasound guided biopsy provided a minimally invasive and effective method to obtain *cricoarytenoideus lateralis* muscle samples in horses.

To my parents, Odran and Patricia,
For their magnificent devotion to our family
To my brother Andrew, and sisters Catherine and Amy,
For all their support throughout my life

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
ATM	*Arytenoideus transversus* muscle
CADM	*Cricoarytenoideus dorsalis* muscle
CALM	*Cricoarytenoideus lateralis* muscle
H	Hour
Mab	Monoclonal antibody
MHz	Megahertz
MyHC	Myosin heavy chain
RLN	Recurrent laryngeal neuropathy

INTRODUCTION

Recurrent laryngeal neuropathy (RLN) is one of the more commonly encountered obstructive lesions to involve the equine upper airway. The disease is caused by a failure of the *cricoarytenoideus dorsalis* muscle (CADM) to abduct the arytenoid cartilage, with the left side being more severely affected than the right. The consequent obstruction to air movement results in diminished performance, particularly at speed, due to the limitations of lung oxygenation and subsequent hypoxemia. Although the specific etiology of the disease has yet to be configured, studies have classified it as a bilateral mononeuropathy with the primary changes occurring in the distal axon of the recurrent laryngeal nerve (primarily the left). Clinical disease in the horse is confirmed by upper airway endoscopy, but signs are generally not recognized until the horse is several years old, so horse enthusiasts may purchase an endoscopically normal horse only to have it develop performance-limiting RLN later in life. However, post-mortem studies have shown that neuromuscular alterations occur much earlier in life when the disease is still in the subclinical stages. Also, changes are found to occur much earlier in the adductor muscles, such as the *cricoarytenoideus lateralis* muscle (CALM), than in the CADM. Therefore, a diagnostic test that is able to identify horses likely to develop RLN is needed. Given the superficial location of the equine larynx and the advancements made in both biopsy instrumentation and medical imaging, it seems plausible that the intrinsic laryngeal muscle tissue can be harvested in the standing horse for analysis. This thesis describes the development and testing of a safe method to biopsy the CALM. Also included are the results of preliminary experiments that I conducted to compare muscle fiber typing in the CALM and CADM.

The thesis is divided into 4 chapters. The first is a literature review that will facilitate an understanding of RLN, the disease process, the current diagnostic methods used in assessing clinical disease and provide information on our current understanding of subclinical changes that occur in the laryngeal muscle fiber types. Also, a general overview of muscle fiber types and methods of biopsy will be given. At the end of the chapter I explain my conclusions regarding the potential usefulness of laryngeal muscle biopsy in horses.

In chapter 2, an immunohistochemical comparison is made between the left CALM and the CADM. The objective was to validate that subclinical changes of RLN occurred either concurrently or earlier in CALM compared to the CADM. This was important, as although clinical expression of RLN is due to failure of the CADM, this muscle is not as accessible as the CALM for biopsying.

In chapter 3, the technique and safety of ultrasound guided biopsy of the left CALM is assessed in six healthy adult horses.

In chapter 4, I draw conclusions from my work and make a number of recommendations as to how this new technique can be adopted and implemented.

CHAPTER 1

LITERATURE REVIEW

Recurrent laryngeal neuropathy (RLN) is a commonly encountered upper respiratory condition of the horse, caused by a failure of the *cricoarytenoideus dorsalis* muscle (CADM) to contract and abduct the arytenoid cartilage during times of increased airflow requirements. Consequently, the condition causes both an abnormal respiratory noise and limits maximal performance. Most cases are idiopathic in nature, although the primary changes are known to occur in the distal axon of the recurrent laryngeal nerve (primarily the left), with subsequent atrophy of the CADM as the disease progresses. Upper airway endoscopy is the gold standard method of diagnosis in clinically overt cases, but post-mortem studies have shown that neuromuscular alterations can occur much earlier in life when the disease is still at a subclinical stage. Thus, having the ability to analyze the intrinsic laryngeal musculature throughout a horse's life might provide a better understanding of the pathogenesis and clinical onset of RLN and hopefully, a tool for prediction of clinical RLN. For these reasons I investigated in conscious horses the feasibility of harvesting sufficient quantities of laryngeal muscle tissue suitable for analysis.

In this Literature Review, I describe the basic anatomy of the larynx, review the current understanding of RLN, the disease process and the diagnostics used in assessment for clinical disease. A detailed review of the muscle fiber isoforms comprising the intrinsic laryngeal musculature is also covered, with specific reference to the alterations occurring during subclinical RLN disease. A general overview of muscle biopsy methods and tissue preservation

is also given. At the end of the review, I explain my conclusions regarding the potential usefulness of laryngeal muscle biopsy in horses.

Anatomy of the larynx

The equine larynx is located at the junction of the head and proximal cervical region, protected in part by the rami of the mandibles laterally, the cervical vertebrae and occiput dorsally, and by the paired *sternothyrohyoideus* muscles and skin ventrally [1]. It forms a hollow, cylindrical structure that connects the pharynx to the trachea. The larynx is responsible for performing a number of vital functions. Firstly, it regulates the volume of airflow from the nasal passages into the lower respiratory tract such that during times of increased demand, the dilation of the laryngeal lumen permits a greater delivery of oxygen to the lungs. Secondly, with the shared passage of food and air through the nasopharynx, closure of the laryngeal *aditus* prevents contamination of the lower airway. Other functions of the larynx include phonation and coughing. In order to perform such a complex range of duties, it is composed of a mix of cartilage, ligaments, muscular tissue and mucous membrane.

Cartilages

The larynx is comprised of 3 paired (cuneiform, corniculate and arytenoid) and 3 unpaired cartilages (cricoid, thyroid and epiglottis) [1], intimately connected to form the basic framework of the larynx and ensuring rigidity during pressure changes of respiration. All are comprised of hyaline cartilage, with the exception of the apices and vocal processes of the arytenoid, the epiglottic, cuneiform and corniculate cartilages, which are composed of elastic

cartilage. Hyaline cartilage has the capacity to ossify over time and thus add further rigidity to the laryngeal structure, a feature not seen at any stage with elastic cartilage.

Of all the cartilages, the cricoid is most caudally located and connects the larynx to the first tracheal ring. It differs from the other laryngeal cartilages by being uniquely shaped in a signet ring fashion. Dorsally, the ring extends in a rostrocaudal direction forming a broad lamina that is separated in two by a median ridge. The laminae have a slight dorsal concavity that gives rise to the respective origins of the left and right CADM. Each side has two separate surfaces for articulation with the arytenoid and thyroid cartilages. The laminae begin to taper ventrally forming the lateral arches of the cricoid, and unify at its ventral most prominence – a landmark that easily palpable. Stabilization of the cricoid occurs through the action of a number of ligaments. The cricotracheal ligament connects the caudal aspect of the cricoid with the rostral aspect of the first tracheal ring. The rostral aspect of the cricoid, which is slightly thicker and more rounded in appearance than the adjacent caudal area, connects with the thyroid via ligamentous and muscular attachments. The cricothyroid membrane extends from the rostral aspect of the ventral arch onto the ventral aspects of the laminae and body of the thyroid cartilage.

The thyroid is the largest of all the laryngeal cartilages, making contact with the epiglottic, cricoid and arytenoid cartilages. The body of the thyroid occupies a rostrocaudal location, unifying both left and right components. Coming off each lateral margin of the body, the thyroid is continued in a dorsocaudal direction forming two sagittally orientated laminae. These laminae form the lateral borders of the cricothyroid space, with the body of the thyroid forming the rostral border and the cricoid completing the caudal component of the arrangement. The dorsal borders of the thyroid laminae possess rostral and caudal cornual extensions. The

caudal cornu contacts the cricoid cartilage though a synovial articulation. The rostral cornu forms a fissure with the main body of the lamina – with time it converts into a foramen as a connective tissue band develops on the rostral border. This foramen allows the cranial laryngeal nerve and artery access to the axial margins of the thyroid cartilage. The rostral cornu also articulates with the thyrohyoid bone, which along with the other soft tissue attachments allows the hyoid apparatus and larynx to move in unison. Rostrally, the body of the thyroid contacts the base of the epiglottis, with the elastic thyroepiglottic ligament stabilizing the attachment.

The epiglottis is the most rostral cartilage within the larynx. It has a broad attachment to the rostral aspect of the thyroid body, and then extends in a rostrodorsal direction, tapering to end in a pointed apex. The lateral margins of the cartilage have a distinctly crenated appearance. The lingual portion of the cartilage is convex in a sagittal plane, and concave in the transverse plane. Its elastic cartilage composition allows it to freely move - an important feature for protecting the lower airway. During deglutition, the epiglottis retroverts about its base and occludes the *rima glottis* ensuring the food bolus only enters into the esophagus. The paired cuneiform cartilages represent two caudal extensions from the epiglottis, which are situated just rostral to the thyroid cartilage.

The paired arytenoid cartilages are pyramidal shaped and lie on the medial aspect of the thyroid laminae. Their caudal aspects overlap the cricoid cartilage, forming the synovial cricoarytenoid articulation. Each arytenoid consists of a caudodorsal base and a rostral apex. A prominent muscular process on the dorsal border serves as the site of attachment for the CADM and *cricoarytenoideus lateralis* muscle (CALM). Rostrally, the arytenoids have a cartilaginous joint with the corniculate cartilages. The ventral border also has a distinct prominence, the vocal

process, to which the vocal ligament attaches. The arytenoid and epiglottic cartilages have the greatest range of motion of all the laryngeal cartilages.

Ligaments

Numerous broad fibro-elastic ligaments provide extensive connections between the cartilages of the larynx. These include the vocal, the vestibular, the transverse arytenoid and cricotracheal ligaments. The vocal ligament spans between the vocal process of the arytenoid cartilage and the body of the thyroid cartilage. The vocal fold (comprising the vocal ligament and muscle) and medial surface of the arytenoid cartilage outlines the narrowest luminal diameter of the larynx – the *rima glottidis*. The vestibular ligament extends from the cuneiform cartilages and lateral aspect of the epiglottis, to the ventral aspect of the arytenoid cartilages. The transverse arytenoid ligament is relatively short, connecting the medial aspects of the opposing arytenoids. The function of the cricotracheal ligament has been previously discussed.

Musculature

Extrinsic

Four paired muscles primarily control spatial movement of the larynx with respect to the head and neck. They include the *hyoepiglottis*, the *thyrohyoideus*, *sternothyroideus* and the *omohyoideus*. The hypoglossal nerve (cranial nerve XII) supplies efferent branches to the *thyrohyoideus* and the *hyoepiglottis* muscles, whereas the ventral branches of the first and second cervical nerve innervate the *sternothyrohyoideus* and *omohyoideus* muscles. Coordinated activity ensures that the larynx moves as a whole.

Intrinsic

The intrinsic musculature of the larynx can be classified into two groups based on their action upon the arytenoid cartilage. Those muscles that adduct the cartilage include the CALM, the *transversus arytenoideus*, the *cricothyroideus*, the *vocalis* and *ventricularis*. The CADM is the sole abductor of the arytenoid.

The CALM, which is the muscle that I propose to biopsy, is a pyramidally shaped muscle tapered towards it ventral aspect, originating from the rostral border of the cricoid cartilage and inserting onto the muscular process of the arytenoid cartilage. The laminar portion of the thyroid cartilage lies on the immediate lateral aspect of the muscle, with the main body of the arytenoid cartilage on the corresponding medial surface. This effectively protects the CALM between two cartilaginous layers, the outer one of which must be traversed to accomplish muscle biopsy. The *transversus arytenoideus* is a narrow muscle that connects the dorsomedial aspects of the arytenoid cartilages. The *cricothyroideus* muscle spans between the lateral aspect of the cricoid laminae and the adjacent medial surface of the thyroid laminae. This muscle tenses the vocal ligaments by moving the thyroid cartilage caudally, causing adduction of the vocal folds and increased dorsoventral diameter of the *rima glottidis*. The combined actions of the *vocalis* and *ventricularis* muscles adduct the vocal process of the arytenoid cartilage and the attaching vocal fold.

The sole abductor of the vocal fold, the CADM, is the largest of all the intrinsic muscle groups. It is divided into 2 muscle bellies – a lateral and medial portion [2]. Both originate off the dorsal laminae of the cricoid cartilage and share a common insertion onto the muscular portion of the arytenoid cartilage.

Neurovascular supply

Neural innervation to the intrinsic musculature comes from the cranial and recurrent laryngeal nerves, both of which originate from the *nucleus ambiguus* of the brain. The cranial laryngeal nerve passes down over the lateral pharyngeal wall and through the foramen below the rostral cornu of the thyroid lamina. Deep to the thyroid, the nerve branches in two – an internal branch that innervates the mucosal lining of the larynx, esophageal entrance and the pharyngeal floor, and an external branch that innervates only the *cricothyroideus* muscle [1].

The left and right recurrent laryngeal nerves descend the neck into the thorax, sharing a common trunk with their respective vagus nerve. The nerve fibers consist mostly of medium sized, myelinated fibers contained by individual fascicles within the vagus nerve. On making the about-turn in the thorax to continue back up the neck, the left recurrent nerve differs from the right by passing around the aortic arch, over the *ligamentum arteriosum* rather than around the subclavian artery. This differing of pathway adds an extra 20-30cm to the overall length of the left [1].On approaching the larynx, the abductor and adductor fibers begin to partition into separate fascicles having remained mixed throughout the neck. The recurrent laryngeal nerves are responsible for innervation of the intrinsic laryngeal musculature, with the exception of the *cricothyroideus*.

Taking into consideration the relative sizes of the intrinsic laryngeal muscles, only the CADM and CALM have enough substance that would be amenable to biopsy. However, the dorsal location of the CADM, its horizontal orientation and positioning adjacent the esophagus could result in a greater risk of inadvertent damage to surrounding structures than if the CALM were targeted. This latter muscle is conveniently situated between the thyroid and arytenoid cartilages, facilitating ultrasound imaging of the muscle. Biopsy access to this muscle will

require negotiation through the thyroid cartilage, and as it is susceptible to mineralization later in life, will require an instrument strong enough to penetrate the laminar portion of the thyroid, yet retain enough sharpness to sample the tissue.

Etiology of recurrent laryngeal neuropathy

Over the years, numerous theories have been proposed for the etiology of RLN. Whilst a small number of cases arise for identifiable reasons (e.g. external trauma to the cervical region, perivascular injections of irritant substances, or iatrogenically following cervical surgery), most are idiopathic. The disease is overrepresented in large breeds [3], with proposed theories of causation ranging from mechanical factors such as stretch to the recurrent laryngeal nerve and its blood supply during neck movement, growth, or the caudal shift of the heart during embryological development, to environmental factors such as toxins or vitamin deficiencies [4]. Despite all of the theories, no definitive cause has yet been identified.

Attempts have been made to distinguish RLN as either a mononeuropathy or polyneuropathy. Although early work analyzing other long peripheral nerves in the horse documented axonal changes suggestive of a polyneuropathy [5, 6], these studies were limited by sampling relatively small numbers, being uncontrolled and not taking into account the normal age-related pathological changes that can be demonstrated in distal limb nerves of horses [7]. Also, it is uncommon for a horse affected with RLN to present with clinical signs of polyneuropathy such as megaoesophagus, bilateral stringhalt, tetraparesis, muscle atrophy and hyporeflexia. More recent work has examined multiple, long peripheral nerves and their innervated muscles in RLN-affected horses [8]. These investigators were unable to demonstrate

primary axonal lesions in any other nerves, thus officially classifying the disease as a bilateral mononeuropathy.

The presence of hereditary factors contributing towards the pathogenesis of RLN has substantial evidence. Studies have shown that offspring of affected stallions are more likely to be affected than offspring of unaffected stallions [9, 10]. One must be cautious however of the findings of such organized breeding studies, as the widespread nature of RLN make selection of an adequate control population difficult.

In man, neurodegenerative conditions are complex and poorly understood with the etiology in many instances unknown [11]. Neuronal compartments often crucially depend upon one another for survival, and molecular defects in one compartment can trigger cellular degeneration in distant parts of the neuron – thus, the site of degeneration is not a reliable indicator of where the initial defect took place. A classic example is the transected motor axon, where neuromuscular junctions that are many centimeters away from the lesion degenerate first, yet axons adjacent to the lesion retain continuity for two to three times longer [12]. A closely studied peripheral neuropathy in humans is the condition Charcot-Marie-Tooth disease [13]. This is an inherited condition, known to exist in multiple forms and affect multiple levels of both lower motor and sensory neurons. Molecular genetic approaches to the investigation of heritable diseases generally involve either linkage or candidate gene studies. However, both methods require accurate phenotyping of affected individuals of the disorder. To date, the gold standard method for RLN diagnosis is histopathology of laryngeal muscles for evidence of neurogenic atrophy, but this can only be performed post mortem making it unsuitable for extensive population studies. If a method were developed to biopsy the intrinsic laryngeal muscles in the

live horse, long-term genetic studies into the disease would become a possibility, and even prediction of early disease onset in subclinical horses.

Prevalence of recurrent laryngeal neuropathy

All horses may be affected with RLN, although clinical evidence suggests that larger breeds are principally affected [14]. Attempts have been made to quantify the incidence of RLN in the population, but many studies have been biased by sampling populations of horses which may have been inherently screened already for pre-existing airway disease. For example, studies of actively racing Thoroughbred horses may be overlooking a percentage of individuals unable to make it into training or through the auction due to early onset disease, thus falsely lowering the overall incidence. Also, RLN has been considered to have a broad clinical spectrum, ranging from neuromuscular changes in the asymptomatic horse to complete hemiplegia associated with overt clinical signs. Thus, the reported prevalence varies with the manifestation of the disease and the diagnostic criteria used.

The prevalence of RLN in North American draft breeds has been estimated to be 35% [3], with a higher percentage of Percheron (42%) and Belgian (31%) horses being affected than Clydesdales (17%). These authors also found that in the same population of horses, there was a significant affect of height on the prevalence of RLN in the Percheron and Belgian breeds, but not the Clydesdales. In a different study examining 48 Clydesdales in New Zealand, 46% were found to be affected with the disease [15]. This would suggest that even for a given breed population, there is considerable within-breed variability worldwide. In the Thoroughbred population, results of endoscopic gradings of adult horses in training estimate the disease incidence to be 2.6-8.3% [16, 17].

A gender predilection has also been reported for RLN in a number of studies, with males over-represented for all breeds [14, 18]. The condition is more commonly diagnosed in Thoroughbred horses between 2 and 3 years of age, but in other breeds, particularly the draught horses, there is an increased rate of diagnosis over 3 years of age, with the peak age of diagnosis in draught animals ranging 7 to 9 years; for the Standardbred and the American Quarter Horse, it is 4 to 6 years [14]. It is difficult to assess whether these age ranges truly represent the age of disease onset, or reflect the age these breeds engage their first bout of strenuous work and thus expose previously covert symptoms. Work conducted by Harrison *et al.* [19] provided evidence that draft breed foals as young as two weeks of age can have extensive histological evidence of fiber-type grouping, suggesting the onset of clinical disease could be much earlier than described for this breed type.

Pathogenesis of recurrent laryngeal neuropathy

The characteristic pathological lesions of horses affected with RLN have been well documented using both light and electron microscopy techniques [5, 6, 20-25]. The disease is now accepted as a degenerative axonopathy, preferentially affecting the large, myelinated nerve fibers of the left recurrent laryngeal nerve but also involving the right to a lesser extent. Pathological changes are noted in the more distal segments of the nerve initially, and show a gradual progression proximally with increasing disease severity. The lesion is thought to occur within the axon itself, as indicated by collapsed myelin sheaths without an axis cylinder, increased myelin sheath thickness, regenerating Schwann cell membrane clusters and paranodal and internodal accumulations of axonal debris and organelles [8].

The intrinsic laryngeal muscular lesions, in both clinical and subclinical cases are typical of chronic neurogenic atrophy. Denervation of the adductor muscles precedes the abductor involvement and typical changes include scattered angular fibers, groups of atrophied fibers adjacent to hypertrophied fibers with central nuclei, and regional replacement of muscle fibers with fat and connective tissue [21, 25]. Of the adductor group, the CALM has been shown to be amongst the earliest and most severely affected muscle, as evident from early muscle fiber type regrouping studies using ATPase staining [26]. Fiber type grouping occurs when denervated muscle fibers in the vicinity of a surviving nerve fiber are reinnervated by collateral sprouts from this fiber, and thus adopt the metabolic and phenotypic characteristics of the innervating nerve. As a single nerve recruits more fibers, the population of contiguous muscle fibers becomes more homogenous and the normal mosaic pattern of the muscle is lost.

In an attempt to explain the preferential loss of muscle fibers from the adductors, it was suggested that the adductor muscles may be innervated by a greater percentage of large myelinated nerve fibers [21]. This has been shown not to be the case, with equal distribution of large and small fibers amongst all the groups [27]. Also, it has been proposed that preferential adductor muscle denervation is due to the fibers innervating the CALM dividing off the parent nerve more distally than those to the CADM. This theory would also support the early degeneration of the left recurrent laryngeal nerve compared to the right due to its greater length [8]. To date, conclusive evidence for this has not been provided.

Evidence for pathological changes of the recurrent laryngeal nerve within the central nervous system have also been sought but with limited success [24]. One of the major obstacles has been in accurately identifying the neuronal cell bodies of the nerve fibers within the *nucleus ambiguus*, which has rather indistinct boundaries in the horse. Further ultra-structural

examination of this area in clinical cases is needed to rule out the possibility of a central located lesion.

Diagnostics

History and physical examination

The presenting history of an RLN-affected horse varies depending on a number of factors. In some instances, the disease may only be detected on a routine endoscopic examination as part of a pre- or post-purchase examination without prior overt clinical signs. In others, the failure of full arytenoid abduction will result in impeded airflow to the lower respiratory tract and thus can present as a cause of poor performance.

A reduced ability to fully achieve and maintain arytenoid abduction during exercise consequentially results in turbulent airflow through the laryngeal lumen, inspiratory dyspnea and a characteristic stridor or "roaring/whistling" sound during inspiration [28, 29]. This noise is often one of the earliest signs detected by the owner and is created by air turbulence across the *aditus* of the laryngeal ventricle, which is now enlarged due to atrophy of the surrounding muscles and flaccidity in the vocal fold. For horses not competing in endurance events, the performance impedance may not be the concerning issue, but the presence of a noise during competition can be undesirable and lead to disqualification or loss of potential points and/or prize money.

Atrophy of the CADM alters the contours of the larynx in a manner that can be appreciated by external palpation; it hollows out the space dorsal to the arytenoid and cricoid cartilage such that the muscular process is more prominent. Although such atrophy is

pathognomonic for RLN, muscle wastage must be advanced and experience is necessary for the examiner to be comfortable with such findings.

Endoscopy

Endoscopy of the upper airway has been widely accepted as the gold standard method for the diagnosis of RLN in the live horse. An examination is best performed with the horse at rest in a quiet environment, using a nose twitch to provide restraint. The administration of sedatives as part of the examination is now discouraged, as xylazine has been shown to lead to an increased number of false positive diagnoses via laryngeal muscle paresis and subsequent asynchrony of the arytenoid cartilages [30]. With the *rima glottidis* in full view, the movements of the larynx should be observed for a period of time under normal quiet breathing. The endoscopic appearance of the larynx may vary depending through which nostril the endoscope is passed, and also on the degree of CADM atrophy and loss of function. Thus, for an observer to make consistent examinations between horses, an identical procedure should be used in all. In horses with laryngeal hemiplegia, there is an obvious asymmetry of the *rima glottidis*, failure of the arytenoid and corniculate cartilage to abduct normally, a kinking of the aryepiglottic fold and dilation of the laryngeal saccule on the left side [31]. It is unlikely that a horse will voluntarily fully abduct the arytenoid and corniculate cartilages under resting conditions. To achieve this, the horse may be encouraged to swallow by flushing water into the nasopharynx or touching the tip of the epiglottis with the endoscope. Alternatively, the nostrils may be occluded for a sufficient time to observe whether or not full abduction can be achieved and maintained [32].

In an attempt to objectively grade the movements of the arytenoid cartilages, a plethora of classification systems have been proposed over the years including a 4-point [33, 34], 5-point

[35] and 6-point scale [36]. However, a consensus grading system was recently agreed upon [37] and has been shown to correlate well with histopathological changes in the laryngeal muscles of affected horses [38]. The scale uses a 7-grade system, with four main grades and a number of subcategories.

Grade

1 All arytenoid cartilage movements are synchronous and symmetrical and full arytenoid cartilage abduction can be achieved and maintained.

2 Arytenoid cartilage movements are asynchronous and/or larynx asymmetric at times but full arytenoid cartilage abduction can be achieved and maintained.

   a. Transient asynchrony, flutter or delayed movements are seen

   b. There is asymmetry of the *rima glottidis* much of the time due to reduced mobility of the affected arytenoid and vocal fold but there are occasions, typically after swallowing or nasal occlusion when full symmetrical abduction is achieved and maintained.

3 Arytenoid cartilage movements are asynchronous and/or asymmetric. Full arytenoid cartilage abduction cannot be achieved and maintained.

   a. There is asymmetry of the *rima glottidis* much of the time due to reduced mobility of the affected arytenoid and vocal fold but there are occasions, typically after swallowing or nasal occlusion when full symmetrical abduction is achieved but not maintained.

   b. Obvious arytenoid abductor deficit and arytenoid asymmetry. Full abduction is never achieved.

c. Marked but not total arytenoid abductor deficit and asymmetry with little arytenoid movement. Full abduction is never achieved.

4 Complete immobility of the arytenoid cartilage and vocal fold,

The controversy surrounding the interpretation and clinical significance of endoscopic findings at rest is confounded by the fact that evaluation is based entirely on subjective assessments. A study by Perkins *et al.* [39] looked at the inter- and intra-observer agreement of grading arytenoid movement in horses during resting endoscopic examination. This study found a good agreement between both observers, and also between repeat observations made by each individual. Of more concern was that even on a daily basis, horses showed considerable variation between consecutive examinations with only 42% being assigned the same grade. This highlights the limitation of resting endoscopic examination in the definitive diagnosis of RLN.

Dynamic endoscopy

In a small percentage of horses, the larynx may appear endoscopically normal at rest but during and immediately after exercise, evidence of RLN will only become apparent. Conversely, a few horses will also display asymmetrical/asynchronous arytenoid movement at rest and yet be able to achieve perfect functioning during maximal performances [40]. Strenuous exercise, sufficient to reveal exercise intolerance and respiratory noise as described by the owner, immediately followed by endoscopic examination may also be able to demonstrate incomplete arytenoid abduction. However, with the rapid recovery of respiratory rates and airflows back to normal there may be too long a delay between completion of exercise and observation of the larynx. For this reason, the equine treadmill [41] and the dynamic overground endoscope [42, 43] have become invaluable as they allow for simultaneous observation of laryngeal movements

during exercise of the subject. Comparisons have been made between the laryngeal movements of horses during resting and treadmill endoscopic examinations [44], with a significant correlation found between both. However, these authors found that 7% of horses that had normal grades assigned at rest developed some form of dynamic laryngeal collapse during exercise. Likewise, 61% of horses with grade 3 laryngeal asymmetry at rest (5 point grade system) and 19% with grade 4, went on to complete the treadmill examination and maintained full abduction throughout. This study particularly highlights the need for additional testing procedures to compliment upper airway endoscopy. Given the broad spectrum of clinical disease, muscle fiber analysis from a biopsy sample and correlation with the observed endoscopic findings may give a more accurate classification of the disease status of the horse.

Ultrasound examination

Ultrasonography of the equine larynx has been recently described [45]. The technique was developed initially to assist in the diagnosis of arytenoid chondritis and dorsal displacement of the soft palate, although replications of results for the latter have been somewhat disappointing. However, the authors noted marked disparity between the echogenicity of the left and right CALM with clinical signs of RLN. Specifically, they found an increased echogenicity of the left side compared to the right, and this lead them to speculate that sonographic examination of the CALM might prove to be a sensitive diagnostic tool for RLN.
Healthy muscle has an anechoic appearance due to its highly organized internal structure consisting of repeating links of identical structural proteins [46]. This arrangement provides minimal interfaces for sound reflection giving a dark, hypoechoic appearance. In neuromuscular disorders, changes in the muscle architecture alter its sonographic appearance giving it an

increased echogenicity and loss of heterogeneity. These changes result from underlying alterations in muscle histology. As fat, fibrosis, and inflammation increase, the numbers of reflective interfaces in muscle increase as well. The loss of muscle mass tends to increase echogenicity of the remaining tissue by collapsing the existing fibrous tissue into a smaller volume [46]. In man, pathologic ultrasound changes have been identified in muscle as early as 10 days following acute denervation [47], with effected muscles showing a progressive increase in echogenicity with disease chronicity.

Since the original laryngeal ultrasound study [45], the technique has been validated for prediction of abnormal arytenoid movement during maximal exercise as observed during treadmill endoscopy [40]. By comparing the echogenicity of left and right CALM, the technique had a sensitivity of 90% and specificity of 98% when compared to the treadmill findings. This was superior to resting endoscopic examination, which had a sensitivity and specificity of 80% and 81%, respectively.

In developing methods for my MS research, it has been my experience that ultrasonography of the equine larynx can be readily performed with minimal preparation of the horse. Longitudinal and transverse images of the CADM and CALM can be obtained, albeit with some difficulty for the CADM. Provided that mineralization of the cartilages has not occurred, the intrinsic musculature and cartilage can be readily assessed. It must be cautioned that despite the apparent superior results of using ultrasound to predict the movements of the arytenoid cartilage, it should not be used as the sole predictive test of RLN as many other conditions of the upper airway can occur coincidentally.

In the present study, I propose to biopsy the left CALM. Should iatrogenic denervation injury occur following the procedure, it is anticipated that histological changes in the CALM should become sonographically apparent within the follow-up period of 8 weeks.

Predicting the development of clinical recurrent laryngeal neuropathy

Numerous histological studies of the intrinsic laryngeal musculature have shown that evidence of fiber-type grouping and neurogenic atrophy occurs in horses that have no clinical signs of RLN [6, 21, 48, 49]. These horses have been referred to in the studies as subclinically affected. It is unclear whether these changes are normal for the aged horse, or truly represent the early stages of a progressive disease. All studies have been limited by harvesting muscle tissue following euthanasia, so long-term follow up of endoscopic changes in the laryngeal movements have so far been impossible. Whilst endoscopic progression of RLN is known to occur [50], absolute confirmation of disease progression would require serial histological examinations of laryngeal musculature or nervous tissue – something which has previously been unable to do. It is anticipated that with the development of a muscle biopsy technique of the left CALM, many unanswered questions regarding the onset and progression of the disease will be answered.

Muscle

Composition and contraction

During embryological development, muscle tissue originates from the mesodermal cell layer. Mononucleated myocytes, derived from this layer fuse together and form independent, multinucleated skeletal muscle fibers, each of which have their own basal lamina (endomysium). Adjacent muscle fibers aggregate to form muscle bundles or fascicles, with each bundle encased

in a connective tissue layer called the perimysium. Individual muscle fibers rarely run the entire length of the muscle bundle, particularly if the muscle is very long. In a study looking at feline *sartorius* muscle, only 7 of approximately 1000 cells ran the whole length of the bundle [51]. The muscle is then completed by the aggregation of lots of muscle bundles, with the whole group being encompassed in a third connective tissue layer called the epimysium. Interspersed between individual muscle fibers are small, quiescent stem cells (satellite cells), which respond in times of injury or exercise by dividing and fusing with either injured fibers or forming completely new muscle fibers.

Each muscle fiber is composed of a basement membrane, referred to as the sarcolemma, and its sarcoplasmic contents, which are akin to the cytoplasmic contents of other cells. Normal muscle fibers do not possess centrally located nuclei, but instead are positioned peripherally beneath the sarcolemma. Most of the sarcoplasmic volume is taken up with myofibrils, each of which is composed of numerous individual myofilaments. Located between the myofibrils are the mitochondria and the sarcoplasmic reticulum. Whilst the mitochondria are the main source of adenosine tri-phosphate (ATP) providing energy for contraction, the sarcoplasmic reticulum replaces the role of smooth endoplasmic reticulum, which is absent in muscle fibers but found elsewhere in the body. The sarcoplasmic reticulum serves as a reservoir of calcium ions, which are essential to the contraction of the muscle unit. Periodically, it has dilated end sacs known as terminal cisternae, which can span from one side of the muscle fiber to the other. The space created between two adjacent cisternae allows invaginations of the sarcolemma, perpendicular to the long axis of the muscle fiber, thus creating tubular folds (T-tubules). These tubules are vital in allowing a depolarizing action potential to rapidly penetrate deep within the cell. One T-tubule, together with two terminal cisternae is functionally known as a triad.

As mentioned previously, myofibrils within the sarcoplasm are composed of numerous tubular myofilaments. These are protein units, composed mainly of actin and myosin arranged in a repeating unit called a sarcomere. Actin is the thinner of the two proteins, and consists of two g-actin chains arranged together in a helix formation to give a single, microfilament called f-actin. Numerous actin microfilaments are joined end-to-end in one long continuous strand, with multiple strands then stacked on one another. Interspersed between the actin microfilaments in an ordered fashion is the second protein myosin. This is the predominant protein in skeletal muscle, and it makes up the largest portion of the contractile apparatus of muscle fibers. Each myosin unit is comprised of four light chains and two heavy chains. The regular arrangement of actin and myosin in the sarcomere gives skeletal muscle its striated appearance on microscopic examination

In addition to the actin and myosin components that constitute the sarcomere, skeletal muscle also contains the proteins troponin and tropomyosin, which assist with muscle contraction. These two proteins are associated with actin and cooperate to ensure timely contact with myosin. Under the influence of acetylcholine released at the neuromuscular junction by motor neurons, calcium is released from the sarcoplasmic reticulum and interacts with troponin. Calcium-bound troponin then undergoes a conformational alteration that leads to the movement of tropomyosin, thus exposing the myosin binding sites on the actin microfilament. This in turn allows for myosin and actin ATP-dependent cross bridge cycling to occur, in turn shortening the muscle.

Muscle fiber isotypes

Rather than being composed of a homogenous fiber composition with identical physiological and metabolic properties, skeletal muscle has evolved into a heterogeneous mixture of fiber types. This diversity is the basis of the flexibility that allows the same muscle to be used for various tasks from continuous low-intensity activity, to repeated submaximal contractions, and to fast and strong maximal contractions. The speed at which a muscle contracts is related to its myosin adenosine triphosphatase (ATPase) activity, the specific isoforms of the myofibrillar contractile proteins present, as well as the isoforms of the calcium sequestering enzymes of the sarcoplasmic reticulum [52]. To complicate matters further, virtually every contractile protein possesses a number of isoforms, each of which have a slightly different property from the other.

Research into myosin ATPase activity demonstrated that different myosin isoforms displayed different acid and alkaline labilities, forming the basis for the first type of histochemical staining method and means of differentiating fiber types [53]. ATPase activity in faster contracting muscle fibers is alkali-stable and acid-labile, that is, muscle showed a high ATPase activity after alkaline preincubation and low ATPase activity after acid preincubation. Conversely, more slowly contracting muscle fibers were alkali-labile and acid-stable. Further work by Brooke *et al.* [54] using a range of preincubation pH solutions was able to classify muscle fibers into three basic types - type 1 (slow fibers) and types 2a & 2b (fast fibers). With better understanding of muscle isoforms, it has now been recognized that this classification into three muscle types is over simplistic. There are now at least 16 different myosin heavy chain (MyHC) isoforms recognized in the various muscles of mammals.

Modern techniques using immunohistochemistry and gel electrophoresis have identified a total of four muscle fiber types in mammalian skeletal muscle: Type 1 (slow), 2a, 2x and 2b, with increasing speeds of contraction in the order listed [55]. These fibers have been found to exist in either a pure or hybrid MyHC state according to the scheme: 1⇔ 1/2a ⇔ 2a ⇔ 2a/2x ⇔ 2x ⇔ 2x/2b ⇔ 2b. However, this pattern of MyHC gene expression is not obligatory; for example polymorphic fibers co-expressing type 1 and 2x but not 2a MyHCs have been detected in normal diaphragm muscle [56].

The four major fiber type groups vary in their distribution throughout the body, depending on the species, the location of harvested muscle and even the position within the muscle. The *masseter* muscle in the equine species contains exclusively type 1 fibers [57], while in rats it is composed of all four fiber types, with a predominance of fast 2x and 2a fibers [58]. It would seem plausible that differences in the frequency and speed of jaw closure between the two species would account for the differing fiber profiles.

Equine muscle isoforms

The muscle fiber population and metabolic properties of skeletal muscles from the horse have been extensively studied, with composition consisting of Type 1, Type 2a and Type 2x fibers (Rivero, Talmadge et al. 1996). Although one older study using traditional histochemical staining methods have reported Type 2b fibers [59], more recent studies using immunohistochemistry have failed to detect this fiber type in the skeletal muscle of either young or old horses [60-62]. These studies have also shown the presence of a small percentage of hybrid fibers coexpressing two kinds of MyHC isoforms (type 1/2a and type 2a/x). In an attempt to quantify the distribution of MyHC isoforms around the whole equine skeleton, Kawai *et al.*

[61] looked at 46 different muscle samples taken from the same location in each of six Thoroughbred horses. Representative tissue was taken from five areas; the hindlimb muscles; the thoracic and trunk muscles; the forelimb muscles; the head and neck muscles; and the respiratory-related muscles. Overall, most of the muscles had a predominance of type 2a fibers. The forelimb muscles had a higher percentage of type 2a fibers and a significantly lower percentage of type 2x fibers than the hindlimb muscles, whereas the muscles of the thorax and trunk shared a similar fiber distribution with those of the hindlimb. It is speculated that both age and training related factors play a significant role in fiber type composition for an individual horse, with exercise increasing the percentage of type 2a fibers [63].

Laryngeal muscle isotyping

Overview

Laryngeal muscles have evolved to serve the highly specialized and complex functions of moving the intrinsic cartilage needed for airway protection, respiration and phonation. The diverse range in expression of various MyHC isoforms is no different from that of skeletal muscle. Studies have identified the four basic MyHC isoforms in varying proportions (type 1, 2a, 2x and 2b arranged in increasing order of contractile speeds) in a range of species, along with an additional MyHC isoform – extraocular (EO) [64]. The latter fiber type displays accelerated contractile properties compared to the previous four and is found predominantly in avian species. In general, fast contracting muscle fiber types disappear from the laryngeal muscle population as animal size increases, which would appear a logical response given the decreasing rate of respiration with increasing animal size. In the rat and rabbit, expression of the two fastest MyHC

(EO and 2b) have been noted [64, 65], with the EO isoform expression being lost in the dog [66] and cat [67], and neither EO or 2B being noted in the human [68].

Equine laryngeal muscle isotyping

Early studies of equine laryngeal musculature revealed the presence of both type 1 and type 2 fibers [25]. Further work [69] using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) identified types 1 and 2B MyHC isoforms in sections of muscle tissue taken from *thyroarytenoideus* and CADM of horses with and without gross evidence of muscle atrophy. However, the conclusions of this study remain in doubt, given the advances made in immunohistochemical staining methods and the complete absence of Type 2b fibers noted further studies of the CADM and *arytenoideus transversus* muscles (ATM) [48].

A recent study [48] has provided a valuable insight of the MyHC isoforms present in some of the equine laryngeal musculature. Investigators looked at the fiber types within both muscles of abduction (CADM and ATM) in six, non-RLN-affected horses, based on resting endoscopic examination prior to euthanasia. Firstly, only three MyHC isoforms were expressed in laryngeal muscles (types 1, 2a and 2x), either singularly or co-expressed as hybrid fibers (1/2a and 2a/2x). The percentage of hybrids accounted for less than 10% of the overall fiber type population, concurring with the percentage found in the rest of the equine skeleton [61]. Secondly, subclinical disease was noted immunohistochemically in 50% of horses, as evident from fiber-type regrouping bilaterally in the ATM, but only in the left CADM. Changes were always more pronounced in the left musculature compared to the right counterpart. These results also concur with early ATPase studies which indicated that the left-sided laryngeal musculature are more severely affected in RLN than the right side, and that the adductors are more affected

than the abductor [25]. Thirdly, in muscle from horses with sub-clinical disease, there was a virtual elimination of type 2x fibers in the left and right ATM, and a reduced abundance of these fibers in the left CADM compared with the right CADM. This decrease was accompanied by a significant increase in the percentage of 2a and slow fibers, with concurrent hypertrophy of these fiber types also noted. However, the authors also documented that there was a reduced proportion of slow fibers with an increase in 2a fibers in left-sided muscles compared to the right. This shift in MyHC isoform from 1⇒2a and 2a⇐2x signifies a dramatic loss in the dynamic range of contractile speeds of the laryngeal musculature; furthermore, the loss of slow, fatigue-resistant fibers the larynx would cause reduced ability to endure sustained periods of function. These authors concluded that multiple pathophysiological mechanisms are at work in early RLN, including selective denervation and reinnervation of 2X muscle fibers, corruption of neural impulse traffic that regulates 2X and slow muscle fibers, and compensatory hypertrophy of remaining fibers. The authors suspect that horses affected with possible early stage RLN are able to remain subclinical by compensatory hypertrophy of surviving muscle fibers.

Muscle fiber transformation

One of the unique features of muscle tissue is the ability of individual fibers to undergo adaptive changes in response to use and disuse, including changes in fiber size (hypertrophy and atrophy) and fiber type (fast to slow fiber type switches), with associated changes in muscle force and resistance to fatigue. This plasticity has been attributed to several factors, in particular mechanical loading, nerve activity patterns, and levels of circulating hormones [70].

The regulation of muscle morphology by its neural input was first demonstrated in feline muscle [71], where slow muscle fibers became faster when innervated by a fast nerve and fast

muscles became slower when innervated by a slow nerve. Such responses have also been noted in other species, including the rat [72]. Studies looking at the electrical stimulation of muscle fibers using different electrical impulse patterns to reproduce the firing pattern of fast and slow motor neurons have shown that the effect of the nerve is mediated primarily by specific impulse patterns. Thus, a phasic, high-frequency electrical impulse can stimulate muscle to transform into faster contracting fibers (a switch from type 1 to type 2 fibers), whereas transition in the opposite direction is induced by tonic, low frequency waveforms [70]. In order for a muscle fiber to undergo transition, all compartments of the cell must adapt including the components of the excitation-contraction coupling, cell metabolism and contractile machinery. Research into the exact chronological order of changes has only recently begun, with the process yet to be determined. It is thought the transition from fast to slow fibers starts with sustained, elevated levels of cytoplasmic calcium being released from the sarcoplasmic reticulum under prolonged neural stimulation. Calcium then binds with the messenger protein calmodulin, which in turn leads to an increase in the activated levels of the enzyme calcineurin. This is a phosphatase enzyme that acts on the transcription factors of the nuclear factor of activated T-cells (NFAT), which enter the nucleus and bring about the necessary alterations in gene expression [73].

Muscle biopsy

Overview

Muscle biopsy is a relatively safe and repeatable technique that can be performed in most species. In horses, histopathological analyses of biopsies are being used increasingly to assist in the investigation of exertional myopathies, unexplained muscle atrophy and poor performance. With significant advances being made in equine diagnostics, muscle biopsy has now become a

routinely performed technique for equine polysaccharide storage myopathy [74], equine motor neuron disease [75], immune mediated myositis [76], atypical myoglobinuria [77] and myotonic dystrophy [78].

Muscles that are commonly biopsied in the horse include the *semimebranosus*, *semitendinosus, sacrocaudalis dorsalis medialis* [75, 79] and the *gluteus medius* [80]. Muscle can be retrieved using either a Bergstrom biopsy needle or via an open surgical approach. An open approach affords the opportunity to retrieve a larger sample size, but requires a longer procedure time and carries the risk of increased wound dehiscence. Critical to either technique is ensuring proper, transverse or longitudinal orientation of the muscle sample retrieved onto a secured mounting board, as an incorrectly positioned muscle sample will be subject to contraction artifact and non-diagnostic, obliquely-sectioned fibers under microscopic examination. Complications associated with the techniques have been limited to superficial infection following wound dehiscence [79] or mild hemorrhage responsive to digital pressure [80].

Histological analysis of muscle biopsy samples allow for the assessment of fiber types, measurements of fiber cross-sectional area, differentiation of cell infiltrates in myositis and assessment of the degree of atrophy and replacement of muscle with fat and connective tissue. Although no exact number of muscle fibers for analysis is recognized, previous studies have relied upon a count of ~800 fibers in order to accurately differentiate the percentages of various fiber types [61]

Needle biopsy

The procedure is generally well tolerated by most patients, but it is advisable to restrain the horse in stocks and sedate to effect. After selecting a suitable muscle for biopsy, the overlying skin is clipped and aseptically prepared as for a routine surgical procedure. Local anesthetic infiltrated either directly over the proposed site, or remotely in an inverted "L" pattern provides anesthesia of the area. Cutaneous desensitization is only required and deeper infiltration is unnecessary and can create artifacts in the muscle sections. A small stab incision through the skin is made with a number 15 blade, permitting access to the muscle with the Bergstrom biopsy needle [74]. The needle is comprised of three parts – a hollow 6mm diameter outer needle portion with a pointed tip and a biopsy window opening on the side of the needle shaft near the tip; an inner, sharp-edged cutting cylinder, fitted tightly within the needle; and a stylet to enable removal of the specimen from the needle. With the stylet removed, the biopsy needle is placed perpendicularly through the skin incision into the muscle to a depth of approximately 8cm. Optimizing the yield of harvested muscle can be achieved in two ways. Firstly, the needle should be orientated such that the biopsy window is position perpendicular to the direction of the fiber pattern. Secondly, negative pressure with a syringe connected to the needle will encourage muscle into the needle. With the outer needle finally positioned, the inner cutting cylinder is placed into its centre and pushed down fully towards the biopsy window, thereby sectioning the muscle within the needle from the parent muscle. The whole assembly is then removed as one from the horse, and the muscle extracted from the needle with the use of the stylet. If the incision has been kept to a minimum, skin closure is generally unnecessary otherwise a single suture or staple is sufficient to close the defect. The advantages of the technique are that it is minimally invasive, quick to perform and the cost of equipment is relatively inexpensive. Disadvantages

include the inconsistencies in sample sizes, difficulty in insuring accurate fiber alignment to allow a true transverse and longitudinal plane and the inability of the side-mounted biopsy window to harvest muscle tissue directly at the end of the needle [80], making it difficult to utilize in an ultrasound guided biopsy procedure.

Open surgical biopsy

Surgical biopsy of a muscle requires a more invasive technique than use of the Bergstrom needle. Initial preparatory steps are the same, including patient restraint and surgical site asepsis. Using a number 10-scalpel blade, an 8-10cm long incision is made over the proposed biopsy site. Using blunt dissection, the overlying connective tissue is removed from the muscle. Curved hemostats are used to tunnel through the muscle at either end of the incision, such that a sample size 3-5cm long and 1cm wide is isolated and elevated [79]. With a firm grasp of the biopsy sample, the tissue is removed by incising either end. It is critical that the portion of muscle be undermined prior to incision of either the proximal or distal ends, as failure to do so will result in muscle contraction and inability to correctly orientate the sample for fixation. Any excess hemorrhage from the parent muscle bed can be controlled by digital pressure for a few minutes, prior to closure. The epimysium should be closed in a simple continuous fashion using absorbable suture, and the skin apposed in a simple interrupted or vertical mattress pattern using a nonabsorbable suture. Protective covering of the incision is unnecessary. This technique requires no additional specialized instruments, and offers the advantage of harvesting a larger and more consistent sample of tissue than with the biopsy needle. However, the approach is more invasive and has the potential for a greater cicatrix formation, which may prove an unacceptable risk for certain owners.

Frozen muscle biopsy

A variety of automated, semi-automated and manual biopsy devices marketed for human breast tissue sampling are also suited to muscle harvesting. Side-sampling, Trucut® needles operate on the same principle as the Bergstrom needle but when considering the applicability to sampling the CALM, the distance from the needle tip to the biopsy chamber is too long (>10mm), resulting in the majority of the sampling chamber lying outside the relatively narrow muscle (~10mm thick) rather than within it. Also, the flaccid muscle has a tendency to deform when sampled and yields were small (< 800 fibers) and unpredictable in my early trials. In an attempt to provide some rigidity to the muscle core prior to sampling, I investigated the possibility of providing a temporary freeze to the area of interest with the biopsy device. Although pre-freezing does produce some ice-crystal formation within the sarcoplasm, it has been found not to interfere with histological or immunohistochemical analysis [81]. An end-on sampling device (Cassi II) device is fully automated with a reusable hand piece and disposable needles of 10- and 12–gauge. The instrument has a patented Stick-Freeze technology™, where CO_2 is released from a canister in the handle around the 19-gauge guide needle positioned in the target tissue. This temporarily freezes and secures the tissue adjacent to the needle, allowing the larger rotational cannula to advance down over the guide needle cutting the core biopsy in the process. The advanced cannula protects the sample on removal from the patient, thereby minimising the risk of sample loss. In my preliminary studies, this instrument reliably provided cores of muscle tissue with fiber numbers in excess of my target figure (>800 fibers).

Storage of muscle samples

Harvested muscle being sent for laboratory analysis is usually transported either fresh, frozen or formalin fixed [81], and selecting an appropriate method will depend upon the specifications required by the diagnostic laboratory, and the intended method of tissue analysis. The fixing of tissue in formalin solution is convenient for most veterinary practitioners, especially as muscle biopsy is frequently performed in the field and therefore often delayed in being packaged and shipped to the laboratory. Tissue specimens are best reduced in size to no larger than 0.5 – 1cm in diameter, otherwise the formalin will have difficulty penetrating the central portion [80]. The technique introduces artifact, particularly when the formalin solutions are not buffered appropriately. Preparation of formalin-fixed muscle requires a high temperature for vacuum wax embedding that may contribute to muscle fiber shrinkage of up to 30%, and reduces or eliminates enzymatic activity and epitope recognition for immunohistochemical applications [81]. Also, cytoplasmic glycogen and lipid stores are not optimally maintained and it is impossible to identify metabolic or contractile fiber types [82]. Studies have compared the histological preparations of formalin-fixed, chilled and frozen muscle tissue stained with hematoxylin and eosin (H&E), periodic acid-Shiff (PAS) and amylase-PAS in attempt to determine the optimal method of preservation [82]. In formalin-fixed sections, investigators were unable to distinguish variation in fiber size, anguloid atrophy, central nuclei and intracytoplasmic-rimmed vacuoles, compared to the sections frozen immediately or chilled. This inability to clearly identify the cell membranes in H&E sections compared to the other techniques, resulted in the authors concluding that formalin fixation of tissues is not ideal for either characterizing myopathies in the horse, or immunohistochemical analysis.

When performed correctly, freezing of muscle tissue avoids the artifacts seen with formalin-fixation. Because freshly frozen muscle preserves both architecture and histochemical activity, it is the technique of choice for muscle fiber isotyping [81]. Snap freezing fresh muscle tissue in isopentane precooled in liquid nitrogen provides the best samples for analysis [81]. Practically this is difficult, as few equine premises possess the necessary equipment to handle isopentane. Thus tissues are often sent in a chilled state to a specialist laboratory for processing. Studies in dogs and humans have assessed the adverse effects of delayed processing on muscle specimens, and there is a general consensus that muscle fiber diameter increases above baseline values with increasing duration of storage [83, 84]. The cause of fiber swelling in stored muscle samples is thought to be due to disruption of transmembrane ionic gradients, possibly associated with decreased concentrations of ATP in muscle cells and concurrent altered activity of Na^+-K^+ ATPase within the sarcolemmal membrane. For equine skeletal muscle, it has been determined that in the absence of facilities to immediately freeze muscle tissue, samples should be wrapped in a dry gauze, placed in a plastic container and shipped on ice packs in a polystyrene box, arriving at the final destination within 24 hours of sampling [81]. Enclosing the sample in dry as opposed to damp gauze induces less storage related artifacts, while providing sections suitable for histopathological analysis. Tissue samples stored under conditions replicating a delay in delivery (i.e. ~72 hours storage, prolonged exposure to ambient temperature) show artifacts, autolysis and muscle fiber degradation. Tissue samples stored in excessively wet gauze become over-hydrated, resulting in great difficultly preparing suitable cryosections from specimens.

Overall, fresh or chilled muscle frozen in isopentane remains the gold standard method of preservation for performing immunohistochemical analysis in all species [81].

For the purposes of my biopsy technique, harvested muscle samples will be stored on a plastic sampling tray, specifically designed for use with the selected biopsy instrument. The tray will be placed in a polystyrene box pre-filled with ice for transport to a laboratory within 15 minutes of sampling. In accordance with best practice for immunohistochemical analysis, my muscle samples will be frozen in isopentane cooled in liquid nitrogen for future processing.

Summary

Whilst our understanding of the various muscle fiber types present within the larynx has advanced in recent times, there are still a number of unanswered questions. First, characterization of muscle fiber types within the CALM*, vocalis, ventricularis* and *cricothyroideus* muscles using immunohistochemical techniques has yet to be documented. Second, the comparative distribution of immunohistochemically-defined MyHC isoforms in the CALM and CADM of the same subject is unknown. This would determine if changes occur in the CALM fiber type population and fiber cross sectional area, earlier and/or more severely in either clinical or subclinical cases of RLN. If the latter were to be true, then biopsy of the CALM might be used to predict changes in the CADM and therefore development of clinical RLN.

It is worth noting that all subclinical RLN-affected horses in a recent paper [48] were greater than 11 years of age. Given that muscle fiber denervation and reinnervation occurs in rats and humans in late life [85], the significance of fiber regrouping in such horses has yet to definitively attributed as a consequence of RLN development, or simply a result of normal and age-related changes. Hence, having the ability to biopsy laryngeal muscle *in vivo* and compare the fiber type composition with the results of serial, endoscopic examinations, would provide better information about RLN- and age-related changes that occur in the laryngeal musculature.

Currently, laryngeal muscle specimens have only been taken at post mortem or from RLN-affected horses during a routine prosthetic laryngoplasty [38]. Thus, there is a clear need for a minimally invasive, laryngeal muscle biopsy technique that can be readily performed in the horse.

CHAPTER 2

A COMPARATIVE STUDY OF THE MUSCLE FIBER-TYPE EXPRESSION IN THE LEFT DORSAL AND LATERAL CRICOARYTENOID MUSCLES

Summary

Reasons for performing the study: Biopsy of the *cricoarytenoideus lateralis* muscle (CALM) would provide a convenient method to investigate the molecular basis of equine recurrent laryngeal neuropathy (RLN). However, the myosin heavy-chain isoform (MyHC) composition of the CALM is unknown, and it is unclear (a), if MyHC distribution varies within the muscle, (b) if variations of MyHC within regions of CALM, (c) if the MyHC distribution within CALM is similar to that in the other intrinsic laryngeal muscles.

Objectives: To compare MyHC isoform populations between the left CALM and *cricoarytenoideus dorsalis* (CADM), and assess regional distributions of isoform populations from a variety of locations within each muscle.

Methods: Larynges were harvested from six horses that had upper airway endoscopic examinations performed 24hr before euthanasia. The left CALM and CADM were removed and each divided into three regions (CALM – dorsal, middle, ventral; CADM – cranial, middle, caudal). Immunochemistry of muscle sections was performed using a battery of monoclonal antibodies for types 1, 2a and 2x fibers. Counts of each fiber type were performed manually using montages of photomicrographs of serial sections for a

minimum total of 800 fibers, using the uptake pattern of monoclonal antibodies for isoform identification. Muscle fiber distribution between regions and muscles was analysed using a repeated measures ANOVA, first with all data from horses grouped together, and then repeated with horses divided into subclinical-RLN and normal groups. Sub-categorization was based on the presence or absence of fiber-type regrouping on immunohistochemistry.

Results: The fiber types within the left CALM were similar to those within other intrinsic laryngeal muscles. However, the CALM had significantly more type 2a and less type 1 fibers than the CADM. The presence or absence of fiber-type grouping could not distinguish between grade I or II endoscopic scores. In horses with fiber-type regrouping, the percentage of type 1 fibers within the CALM progressively decreased from dorsal to ventral, a trend not apparent in normal horses. There was no regional distribution of fiber types in the CADM.

Conclusions and potential relevance: The CALM reflected muscle fiber-type changes occurring within other intrinsic laryngeal muscles, making it suitable for investigation of RLN pathogenesis by biopsy. The regional changes within the muscle may reflect selective axonopathy of the recurrent laryngeal nerve branches. Further neuroanatomical investigations of the CALM are warranted.

Introduction

The laryngeal muscles provide coordinated movements of the intrinsic cartilages necessary for respiration and airway protection. Studies of mammalian larynges have identified five basic myosin heavy chains (MyHC) isoforms in varying proportions (types 1, 2a, 2x, 2b and extraocular arranged in increasing order of contractile speeds) [64, 86]. Early studies of equine laryngeal musculature revealed the presence of both types 1 and 2 fibers [25]. Further work using gel electrophoresis identified types 1 and 2b MyHC isoforms in sections of the *thyroarytenoideus* and *cricoarytenoideus dorsalis* muscle (CADM) [69]. However, the presence of 2b fibers conflicts with more recent evidence using immunohistochemistry, which confirmed the complete absence of this fiber, type in the equine CADM and *arytenoideus transversus* muscles (ATM) [48]. Indeed, this latter study by Rhee *et al.* [48] identified three MyHC isoforms (types 1, 2a and 2x), either singularly or co-expressed as hybrid fibers (1/2a and 2a/2x) in the CADM and ATM. Also noted was subclinical disease in 50% of their study horses as evident from fiber-type regrouping bilaterally in the ATM, but only in the left CADM. These results concur with earlier ATPase studies by Duncan *et al.* [25], which indicated that the left laryngeal musculature is more severely affected in recurrent laryngeal neuropathy (RLN) than the right, and that the adductors are more affected than the abductor. Thirdly, in muscle from horses with subclinical disease, there was a virtual elimination of type 2X fibers in the left and right ATM, and a reduced abundance of these fibers in the left CADM compared with the right CADM.

In order to fully investigate the RLN disease process, long-term molecular genetic studies are necessary. For this, accurate phenotyping of horses is required and to date, the only method of doing so is by histological studies of nerve or muscle samples obtained from the larynx at post-mortem. Therefore, a method of biopsying the intrinsic laryngeal musculature in live horses

is needed for such studies. Pilot work has shown that the *cricoarytenoideus lateralis* muscle (CALM) is a more convenient muscle for sampling than the CADM, but confirmation of its accuracy in reflecting muscle fiber-type changes within the CADM are needed.

The CALM is an adductor of the arytenoid cartilage, receiving innervation from the recurrent laryngeal nerve. The work by Duncan *et al.* [25] identified two basic fiber types within the muscle, types 1 and 2, but a complete MyHC mapping of the left CALM has yet to be reported. Also, it is unclear whether regional differences within the intrinsic laryngeal muscles have any effect on the muscle fiber population. If such differences were to occur, this could reflect selective axonopathy within branches of the recurrent laryngeal nerve supplying the muscles.

The purpose of this study was to compare the MyHC isoform composition of the left CALM and CADM from a variety of locations within each muscle using a battery of selective monoclonal antibodies. We hypothesize that endoscopically normal horses can have evidence of denervation-reinnervation injury, with more advanced changes occurring in the CALM, and secondly, that isoform populations are similarly distributed throughout each muscle.

Materials and methods

Subjects

Six Thoroughbred horses of varying age (mean 13 years; range 5 - 25 years), weight (mean 472 kg; range 442-495 kg) and gender (4 geldings, 2 mares) were used in the study. Horses were examined to ensure no palpable evidence of laryngeal muscle atrophy or prior laryngoplasty, laryngotomy or myotomy. The study protocol was approved by the Institutional Animal Care and Use Committee at Michigan State University.

Endoscopy

Within the 24hr before euthanasia, horses underwent resting endoscopic examination. Exams were performed with the horse restrained in stocks, a nose twitch applied and the endoscope passed via the right ventral meatus. Water was instilled into the horse's pharynx three times to observe arytenoid action during swallowing. Horses also were subjected to three periods of forced nasal occlusion that was maintained until it was determined if the horse could or could not fully abduct the arytenoid cartilages and maintain this abduction for several seconds. Digital recordings were made, coded and randomised for grading by an ACVS Diplomate (J.A.S.), experienced in airway evaluations and blinded as to the identity of the videos. An established 4-grade classification system was used [87].

Tissue collection

Within 30 minutes of euthanasia, the left CALM and CADM were removed from the larynx and each was sharply divided into 3 approximately equal sections. The CALM was divided into dorsal, middle and ventral regions and the CADM into cranial, middle and caudal regions. Each of the 6 samples were trimmed and mounted on cork with Tissue-Tek O.C.T. (Sakura)[1], frozen in isopentane cooled in liquid nitrogen and then stored at –80°C until processed.

Immunohistochemistry

Serial sections of muscle samples were cut at 6 μm in a cryotome maintained at –20°C, mounted onto slides, and stained using monoclonal antibodies (MAbs) against MyHCs as the primary antibody. Initial MAbs used included NOQ7-5-4D (anti-type 1)(Abcam)[2], SC-71 (anti-

type 2a), and 6H1 (anti-2x) (Hybridoma bank)[3]. Secondary antibodies used Vectastain Elite ABC kit Universal (mouse IgG) and Vectastain ABC kit (mouse IgM)(Vector)[4]. However, in trials using the 6H1 (anti-2x) MAb, the stain could not be optimised for accurate fiber determination and was thus discarded from all further analysis. Counts of each fiber type were performed manually using montages of photomicrographs from serial sections for a minimum total of 800 fibers. Only those fibers whose complete sarcolemma could be seen were included in calculations. Isoform identification was performed using the uptake pattern of MAbs; type 1 and type 2a were readily distinguished using their specific MAb; hybrid type 1/2a fibers were simultaneously stained with both NOQ7-5-4D and SC-71 MAbs; type 2x fibers failed to stain with either MAb. We acknowledge that the lack of a specific 2x antibody may have resulted in an inability to distinguish 2x and hybrid 2a/2x fibers, but we presume the latter were counted as type 2a fibers.

Preliminary microscopic examination of muscle sections confirmed variations in the grouping of fiber-types amongst horses, with evidence of both normal mosaic fiber pattern and fiber-type regrouping. These grouping patterns allowed classification of horses as either "normal" or "subclinical," respectively. Given that the changes were expected to occur earlier and more severely in the left CALM than the CADM, slides from all regions of CALM were used for classification. Horses were categorized based on the consensus of 2 observers (H.O.N., H.D.F.), using the distribution of type 1 fibers identified with NOQ7-5-4D as the grading criterion (Figures 1 and 2).

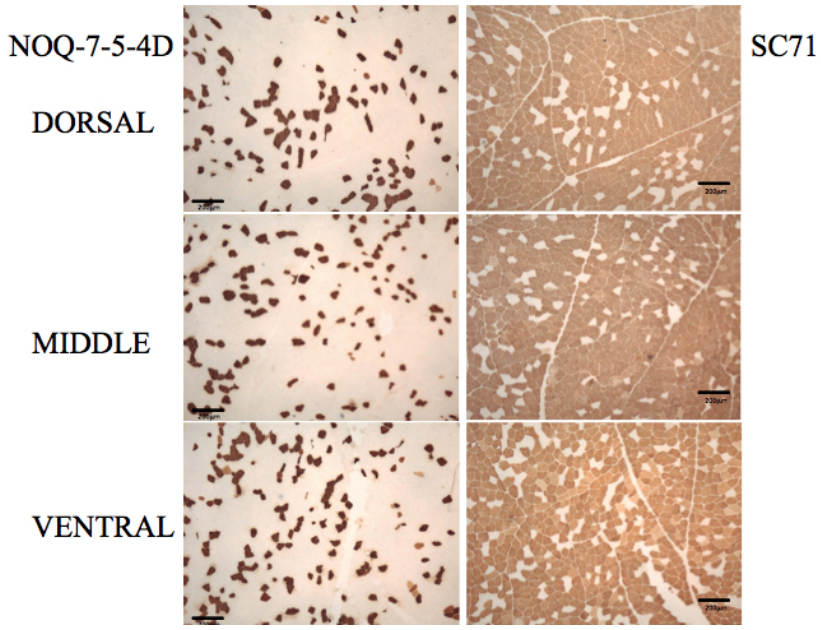

Figure 1: Photomicrograph of NOQ7-5-4-D- (type 1 fibers stain dark) and SC-71-stained (type 2a fibers stain dark) fibers taken from the three regions of the left cricoarytenoideus lateralis muscle of a normal horse. Type 1 fibers are uniformly scattered in a mosaic pattern throughout all fields. Magnification 10X. Bar = 200μm. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this thesis.

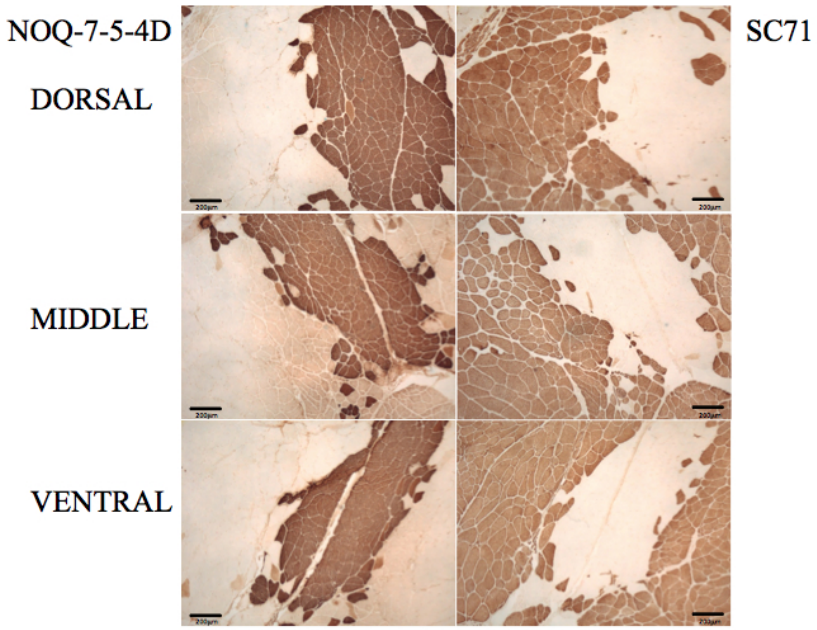

Figure 2: Photomicrograph of NOQ7-5-4-D- (type 1 fibers stain dark) and SC-71-stained (type 2a fibers stain dark) fibers taken from the three regions of the left cricoarytenoideus lateralis muscle of a subclinical horse. Type 1 fibers are grouped rather than uniformly distributed throughout all fields. Magnification 10X. Bar = 200μm.

Statistical analysis

For each horse, numerical values of fiber counts were converted into percentages for each region within each muscle. Data were analysed by mixed model ANOVA. For all tests, significance was set at $P < 0.05$.

Results

Categorization based on isoform distribution

Two horses had a mosaic pattern and were classified as normal; the remainder had fiber-type grouping and were classified as subclinical (Table 1).

Horse	Sex	Age (yrs)	Endoscopy Grade	Classification
1	G	10	1	Subclinical
2	G	13	1	Normal
3	M	18	1	Subclinical
4	G	25	2	Subclinical
5	M	5	2	Normal
6	G	9	2	Subclinical

Table 1: Horse details including endoscopic grades and immunohistochemical classification. G = gelding; M = mare.

Endoscopy

All horses were able to achieve full arytenoid abduction during airway examination, but three horses showed some degree of resting arytenoid asynchrony (Grade 2). There was no relationship between the degree of asynchrony and horse age, with grade 1 horses having a

median age of 13 yrs, (range 10 – 18 yrs) and grade 2 horses a median age of 9 yrs (range 5 – 25 yrs). There was no relationship between endoscopy score and the immunohistochemical sub-categorization; both normal and subclinical horses had both grade 1 and 2 endoscopy scores (Table 1).

All horses

Thirty-six muscle samples were analyzed, with three each taken from the left CALM and CADM from each of the 6 horses. A total of 51807 fibers were counted, averaging 1439 fibers per region thus exceeding our target of ~800 fibers [61]. Muscle isoforms corresponding to types 1, 2a, 1/2a hybrid were identified in all of the muscle sections and 2x in all but one. ANOVA analysis (fixed factor horse, variable factors muscle and region) showed significant differences in percentages of muscle fiber types between CADM and CALM but no significant effect of muscle region and no significant muscle region interaction. The CALM had significantly fewer type 1 fibers ($P = 0.002$) and significantly more type 2a fibers ($P = 0.015$) than did the CADM. There were no differences in the percentage of type 1/2a or 2x types, both of which represented only a small percentage (Figure 3).

Normal horses

Although there were only two horses with a mosaic fiber-type distribution, both of these had 2x fibers in all regions of both the CADM and CALM. Also, subjectively, it appeared that in both horses, the CADM possessed a greater percentage of type 1 fibers and fewer type 2a fibers in all regions compared to the CALM. There were no obvious regional differences for fiber-type percentages (Figure 4).

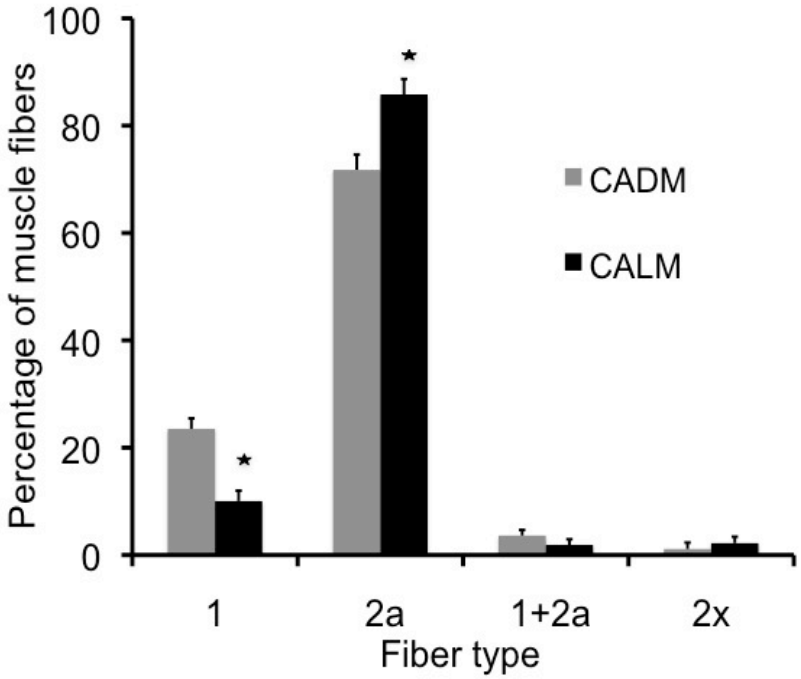

Figure 3: The percentage of muscle fibers types in the *cricoarytenoideus dorsalis* (CADM) and *cricoarytenoideus lateralis* muscle (CALM). For each fiber type, significant differences between muscles are indicated (✶).

Subclinical horses

The initial mixed model ANOVA was repeated in the subclinical group. This confirmed the significant differences between the muscles in type 1 and type 2a fiber types. Inspection of the data and ANOVA results suggested the presence of regional differences in percentage of fiber types in CALM but not CADM; therefore, the data for each muscle were analyzed separately by one-way ANOVA. For the CADM, there were no significant differences in the percentages of muscle isoforms between the cranial, middle and caudal regions of the muscle (Figure 5). By contrast, the CALM showed marked regional differences, with a significant decline in the percentage of type 1 fibers from dorsal to ventral ($P = 0.04$) (Figure 6). Type 2a fibers showed the converse distribution pattern, increasing in percentage from dorsal to ventral,

although no significant differences between regions were detected (P = 0.059). Three of the four subclinical horses had a smaller percentage of type 2x fibers than did the normal horses.

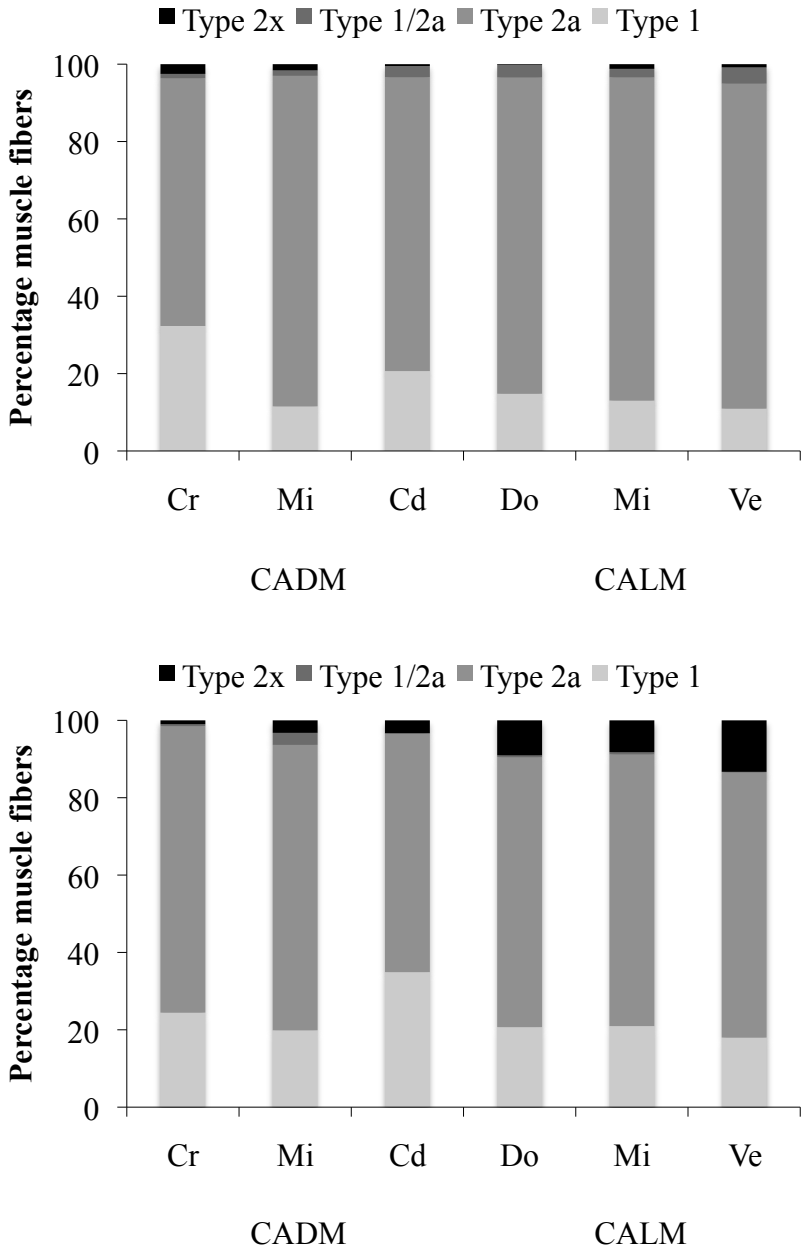

Figure 4: Muscle fiber type distribution in two normal horses in each of the three regions of the *cricoarytenoideus dorsalis* (Cr = cranial, Mi = middle, Cd = caudal) and *cricoarytenoideus lateralis* muscle (Do = dorsal, Mi = middle, Ve = ventral). Top = horse 2, bottom = horse 5.

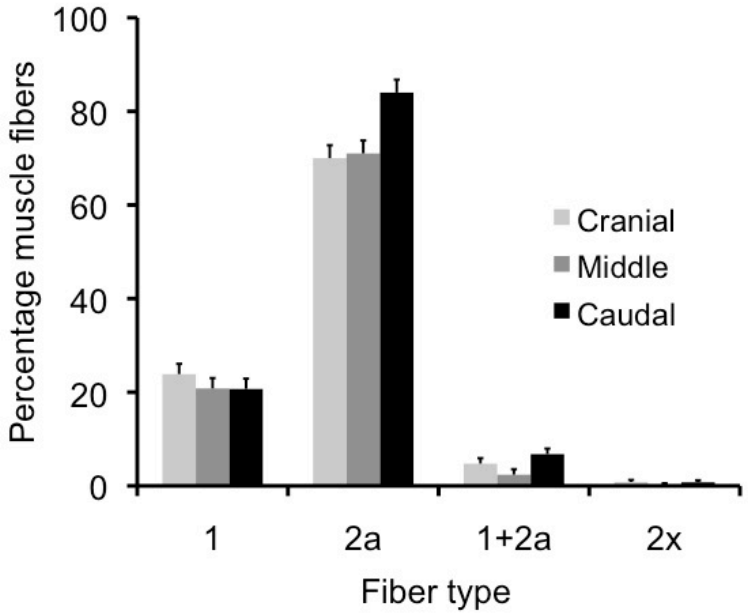

Figure 5: The percentage of muscle fiber types distributed across each of the three regions of the *cricoarytenoideus dorsalis* muscle from subclinical horses.

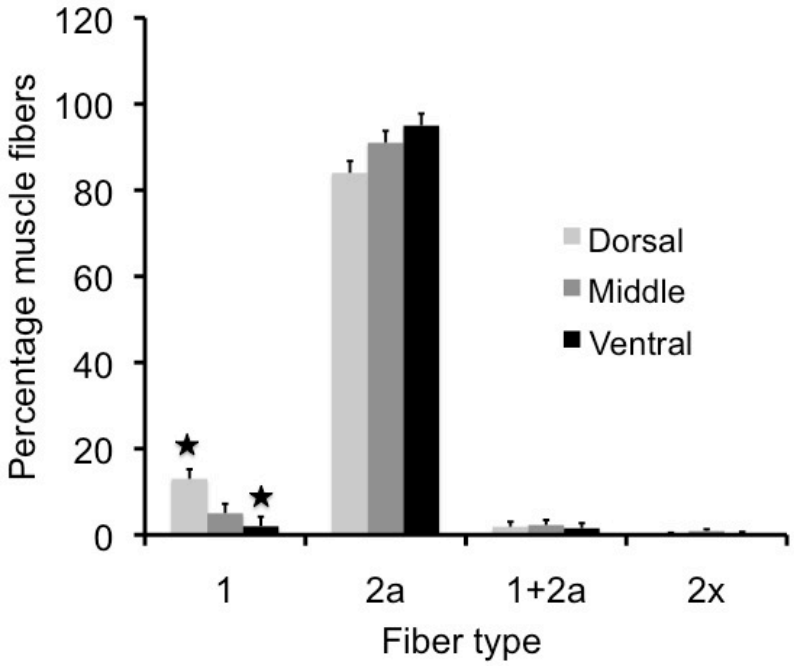

Figure 6: The percentage of muscle fiber types distributed across each of the three regions of the *cricoarytenoideus lateralis* muscle from subclinical horses. For type 1 muscle fibers, regions that differ significantly from each other are indicated (★).

Discussion

The need for this investigation came about during the design of an ultrasound-guided biopsy technique of the laryngeal muscles. Due to its anatomical location, the CALM is easier to biopsy than CADM, but it is unclear whether muscle fiber populations within the former accurately reflect changes in the latter. This investigation therefore, had three major purposes. First, to conduct a complete immunohistochemical characterization of the MyHC isoforms found within the CALM; second, to determine if isoform populations within the CALM and CADM were comparable; and third, to investigate regional variations of fiber type populations within the two muscles. The latter was necessary to determine what part of the CALM should be selected for biopsy. Our results show that the CALM shares a similar population of MyHC isoforms to the CADM and other intrinsic laryngeal muscles studied [48], but that the CADM and CALM differ significantly in the percentages of type 1 and type 2a fiber types. Furthermore, there is a significant regional variation in the percentage of muscle fiber types within the CALM.

In our pilot work, we were unable to replicate the use of the 6H1 MAb (anti-2x) as used in previous studies (Personal communication C.M. Steel, University of Melbourne) [48, 88]. Consequently, the identity of type 2x isoforms could only be detected by their complete lack of stain uptake across all photomicrographs and not by a specific antibody uptake. Whilst the authors acknowledge the methodology limitations of such an analysis, the technique has been used before [62, 89, 90]. The authors speculate an overestimation of type 2a fibers due to the inclusion of type 2a/2x fibers, with a concomitant underestimation of type 2x fibers. A technique for multiple immunofluorescence labeling of all equine muscle fiber isoforms has been recently developed [91]. This method has the advantage of positively staining types 1, 2a and 2x fibers,

along with all hybrids and does not rely on the negative stain uptake for type 2x fiber as reported here, thus could increase the accuracy of fiber type detection.

Both the CADM and the CALM contained type 1, 2a, 1/2a and 2x fibers. However, the CALM contained fewer type 1 fibers and more type 2a fibers than the CADM. This shift in fiber-type pattern reflects changes seen in other equine laryngeal adductors of RLN-affected horses [48]. It has been suggested that during the development of RLN, the slow contracting type 1 and faster contracting type 2x fibers are transformed into the intermediate-contracting type 2a fibers, with changes occurring earlier and more severely in the adductor muscles than in the CADM. If this is the case, the results of the present study indicate that even in horses with normal arytenoid function, muscle denervation-reinnervation is more advanced in the CALM than the CADM. This is in agreement with the observations of Duncan *et al*. [25] who found more extensive fiber-type regrouping in the CALM than the CADM of horses with clinical RLN. In agreement with Rhee *et al*. [48], type 2x fibers were found in all regions of CADM and CALM of normal horses, and in smaller percentages in three of the four subclinical horses. However, despite muscle fiber-type regrouping present in the CALM of one of the subclinical horses, type 2x fibers were still present in percentages similar to those of normal horses. Thus, confirmation of the loss of type 2x fibers in subclinical and clinical RLN-affected horses awaits an investigation of a larger population of animals.

Even though all six horses in the study were adjudged to have normal arytenoid movements (Grades I or II) based on upper airway endoscopy [87], four of these had evidence of fiber-type regrouping which is indicative of denervation-reinnervation by the recurrent laryngeal nerve. This finding of subclinical disease is in keeping with other studies [48]. The lack of agreement between endoscopy grading and histopathological changes was not surprising for two

reasons. First, although there is broad correlation between endoscopic grade and histopathological score, there can be considerable variation of the latter within each endoscopic grade [38]. Second, endoscopic grade can vary between daily or weekly examinations [36, 39, 92]. In the present study, although efforts were made to use consistent technique in the examinations, grading could have been improved by averaging a greater number of observations over several days.

The progressive decrease in the percentage of type 1 muscle fibers in subclinical horses proceeding from dorsal to ventral in the CALM is a new observation. Based on our two horses with mosaic fiber-type pattern, the same regional distribution of type 1 muscle fibers does not appear to occur in normal horses. Further investigation using a larger sample population is needed to confirm this. The neuroanatomy of the CADM has been shown to consist of 2 distinct neuromuscular components, each receiving separate branches of the recurrent laryngeal nerve [2]. However, the exact division of the nerve within the CALM is unknown. If separate branches of the nerve were found to innervate different regions of the CALM muscle, differing rates of axonal degeneration within the branches of the recurrent laryngeal nerve might explain the regional variations of muscle isoform transformation. Further studies detailing the neuroanatomy of the CALM are therefore warranted.

This study supports our hypothesis that the CALM can act as a suitable muscle for biopsy in the study of RLN. The distribution of fiber-types in the CALM is either similar to that of the CADM (normal horses), or at a stage more indicative of RLN (subclinical horses). The CALM is a convenient muscle for sampling compared to the CADM, due the ease with which it can be sonographically imaged, and also accessed with biopsy instruments. During development of an ultrasound-guided biopsy technique of the CALM, the middle region of the muscle proved to be

the best location for maximizing biopsy yields compared to the dorsal and ventral regions. The present investigation supports use of the middle region for biopsy. Subclinical changes at this location appear to indicate an intermediate stage of disease progression within the CALM. It is also clear that the left CALM muscle can have normal muscle morphology, i.e. a mosaic fiber pattern. What remains to be determined is whether the changes we have observed in the CALM are truly a reflection of subclinical disease, or simply a consequence of normal aged-related axonal changes occurring within the left recurrent laryngeal nerve. This question could be answered by a long-term study comparing serial endoscopic examinations of arytenoid movements in a large group of horses with analysis of CALM muscle fiber-type morphology, obtained by repeated biopsy.

Sources of funding

Supported by the Freeman Funds, Michigan State University College of Veterinary Medicine and by the Equine Respiratory Disease Endowment provided by the Matilda R. Wilson Fund.

Manufacturers' addresses

1 Sakura Finetek, Torrance, California, USA.

2 Abcam, Cambridge, Massachusetts, USA.

3 Developmental Studies Hybridoma Bank, University of Iowa, Iowa, USA.

4 Vector Laboratories, INC. Burlingame, California, USA.

CHAPTER 3

ULTRASOUND GUIDED BIOPSY OF THE *CRICOARYTENOIDEUS LATERALIS* MUSCLE: TECHNIQUE AND SAFETY IN HORSES.

Summary

Reasons for performing the study: Current diagnosis of recurrent laryngeal neuropathy (RLN) depends upon disease recognition in the clinically affected horse. Biopsy of the intrinsic laryngeal muscles may provide a method to identify the changes in fiber-type composition that occur in RLN before clinical signs become apparent.

Objective: To develop an ultrasound-guided biopsy technique of the left *cricoarytenoideus lateralis* muscle (CALM), and evaluate its efficacy and safety *in vivo*.

Methods: Six standing horses underwent ultrasound-guided biopsy of the left CALM. Frozen muscle cores were obtained with a breast biopsy tool. Serial endoscopic, ultrasonographic and physical examinations before and for eight weeks after the biopsy were assessed for iatrogenic trauma. Histology of representative muscle core cross-sections were analyzed for the total number of muscle fibers obtained with each biopsy.

Results: All horses tolerated the procedure well, and the left CALM was harvested in all instances. Biopsy samples had an average weight of 0.043 grams (range = 0.023-0.077 grams) and 3418 fibers in cross-section (range = 711 - 7143). Laryngeal endoscopic

grade did not change significantly throughout the 8-week follow up. The left CALM had a significantly increased echogenicity compared to the right throughout the study ($P < 0.001$), but there was no difference between the pre-biopsy echogenicity and that at completion of the study.

Conclusions and potential relevance: Ultrasound guided biopsy of the left CALM is safe and well tolerated, providing a minimally invasive method to obtaining muscle from healthy horses. This new technique may be applicable to early diagnosis of and research into RLN.

Introduction

Recurrent Laryngeal Neuropathy (RLN) is a distal axonopathy of the recurrent laryngeal nerve that results in the partial or total collapse of the left side of the larynx of affected horses during inhalation [4, 8, 93]. The consequent obstruction to air movement results in diminished performance, particularly at speed. The disease presents clinically because of dysfunction of the *cricoarytenoideus dorsalis* muscle, yet ipsilateral adductor muscles (*cricoarytenoideus lateralis, transversus arytenoideus, vocalis* and *ventricularis*) undergo denervation-reinnervation much earlier in the disease course [19, 25, 48].

In RLN, denervated muscle fibers are reinnervated by intact adjacent nerve endings of contiguous muscle fibers, resulting in identical histochemical properties in place of fiber-type heterogeneity. This process, known as fiber-type grouping, is a histological characteristic of early neuropathy [94]. Evidence of subclinical changes has been shown to occur in foals as young as 6 wks [19]. However, clinical expression of RLN generally does not occur until a horse is several years old [50], so an apparently normal horse may be purchased only to have it develop performance-limiting RLN later in life.

The gold standard for RLN diagnosis is upper airway endoscopy, either at rest or during exercise [34, 44]. Recent work by Garrett *et al.* [40] comparing the echogenicity of the *cricoarytenoideus lateralis* muscle (CALM) to endoscopic findings of abnormal arytenoid movement found that the modality compared favorably, both in terms of sensitivity and specificity. However, no ante mortem test is yet available that will detect early subclinical changes in muscle fiber types in clinically apparent normal horses.

The deep location of the *cricoarytenoideus dorsalis* muscle between the larynx and esophagus makes it difficult to image sonographically [40, 45], and relatively inaccessible to

biopsy safely. In contrast, the CALM has a more superficial location between the arytenoid and thyroid cartilages making it an ideal candidate for biopsy. As the CALM shows earlier histopathological changes compared to the *cricoarytenoideus dorsalis* muscle when horses are affected with RLN [25], ante mortem analysis of the CALM fiber-type composition would provide a technique for studying the disease progression, allowing comparisons of muscle morphology with endoscopic findings, and possible prediction of clinical disease.

The purpose of this study was to develop a safe biopsy technique of the left CALM in the standing, sedated horse. We hypothesized that by using ultrasound guidance, muscle samples can be accurately retrieved for histological analysis, whilst avoiding inadvertent trauma to surrounding structures.

Materials and Methods

Horses

Six mares (mean age 7.3 years, range 3 – 14 years) with a mean weight of 489 kg (range 449 – 516 kg) and height of 162cm (range 157.5 – 172.7 cm) were used in the study. For inclusion, horses had a normal upper respiratory system based on endoscopy, and palpation of the larynx to ensure no evidence of laryngeal muscle atrophy or prior laryngoplasty, laryngotomy or myotomy. The study protocol was approved by the Institutional Animal Care and Use Committee at Michigan State University.

Endoscopy

Endoscopic examination of the larynx was performed with the horse restrained in stocks, a nose twitch applied and the endoscope passed via the right ventral meatus. The duration of

examinations were classified as either long or short depending upon whether the airway was being assessed for arytenoid movements only (long), or evidence of iatrogenic trauma post-biopsy (short). For long examinations, horses were subject to three periods of forced nasal occlusion and three swallowing actions following the instillation of water into the nasopharynx. Occlusion was continued until it was determined if the horse could or could not fully abduct the arytenoid cartilages and maintain this abduction for several seconds. Short examinations were performed to assess the larynx for intraluminal penetration or haemorrhage, submucosal haematomas or perilaryngeal swelling in the immediate post-biopsy period. The timing of endoscopic examinations is provided (Table 2). Digital recordings were made, coded and randomised, with long examinations graded using a standardised scale [34] by an ACVS Diplomate (J.A.S.), experienced in airway evaluations and blinded as to the identity of the videos.

	Wk 1				Wk 2-8
	Day 1	Day 2		Day 3-7	
		Pre-B	15,30,120,240 mins PB		
Endoscopic Exam	LE	N	SE	SE	LE
Ultrasound Exam	Y	Y	Y	Y	Y
Physical Exam	Y	Y	Y	Y	Y
Biopsy Site Evaluation	-	N	Y	Y	Y

Table 2: Timeline of biopsy events: Wk = week; Pre-B = pre-biopsy; PB = Post biopsy; LE = long endoscopic examination; SE = Short endoscopic examination; N = not performed; Y = procedure performed.

Ultrasonography

Laryngeal ultrasound examinations were performed at a variety of time points by a single operator (H.O.N.) using an established technique [45] (Table 2). Horses were restrained in stocks and sedated using detomidine (0.001 mg/kg bwt, i.v.) and butorphanol (0.001 mg/kg bwt, i.v.), with the head and neck supported in extension. Left and right laryngeal regions were clipped from the second tracheal ring rostral to the ramus of the mandible, and from the ventral midline to a level approximate with the transverse processes of the cervical vertebrae. The clipped area was cleansed and saturated with 70% isopropyl alcohol as an acoustic coupling agent. Examinations were performed using the same ultrasound machine (P5 Logic)[1] throughout the study with fixed frequency, depth and gain settings, and a 10 MHz linear transducer used for acquiring serial images and an 8 MHz microconvex transducer for performing the biopsy.

A lateral window was used to assess the CALM, both in transverse and longitudinal planes. In transverse, the left CALM was imaged between the thyroid, arytenoid and cricoid cartilages. Three transverse views were obtained from the dorsal, middle and ventral thirds of the muscle. With the probe in longitudinal orientation, a fourth image of the central CALM was obtained. Comparable images were acquired from the right CALM. Images were digitally recorded, coded, randomised to the left versus right orientation on the screen, and stored for evaluation by an ACVR Diplomate (E.A.B.) blinded to the identity of the individual and timing of image acquisition. Echogenicity of the CALM was measured objectively using software analysis (McKesson)[2], where a numerical value of echogenicity was assigned to the largest elliptical region of interest that best represented the muscle.

Muscle biopsy technique

Biopsy of the left CALM was performed with the horse positioned as for laryngeal ultrasonography. The use of a head collar with a detachable throatlatch provided unobstructed access to the biopsy site. No additional clipping to the area prepared for ultrasonography was required. The skin of the left side was anaesthetized using 5ml mepivicaine hydrochloride (Carbocaine-V)[3] in an inverted "U" pattern, centred immediately dorsal to the muscular process of the arytenoid cartilage. It was necessary to aseptically prepare both right and left laryngeal areas, as intraprocedural palpation of the right side was required. All horses received perioperative phenylbutazone (Phenylbutazone 20% injection)[4] (2.2mg/kg bwt, B.I.D., i.v.) for 24hr.

The microconvex transducer was placed in a sterile glove prefilled with 5mls of ultrasound gel, and then in contact with the skin using 70% isopropyl alcohol as a contact agent. In the event of suboptimal transducer positioning, improvements were made by either extending the head and neck, or lateral flexion of the neck to the right. The location for biopsy was invariably at the junction of the dorsal and middle thirds, centred between the cranial and caudal borders of the muscle. Care was taken to identify the CALM between both thyroid and arytenoid cartilages, as the latter acted as a physical barrier, preventing the biopsy instrument from penetrating the laryngeal lumen.

With the probe longitudinally orientated, a 20-gauge 1.5-inch hypodermic needle (Precision Glide Needle)[5] was placed under ultrasound guidance through the skin, thyroid cartilage and into the CAL muscle at the ventral limit of the proposed biopsy. Advancement continued until contact was made with the arytenoid cartilage (Figure 8).

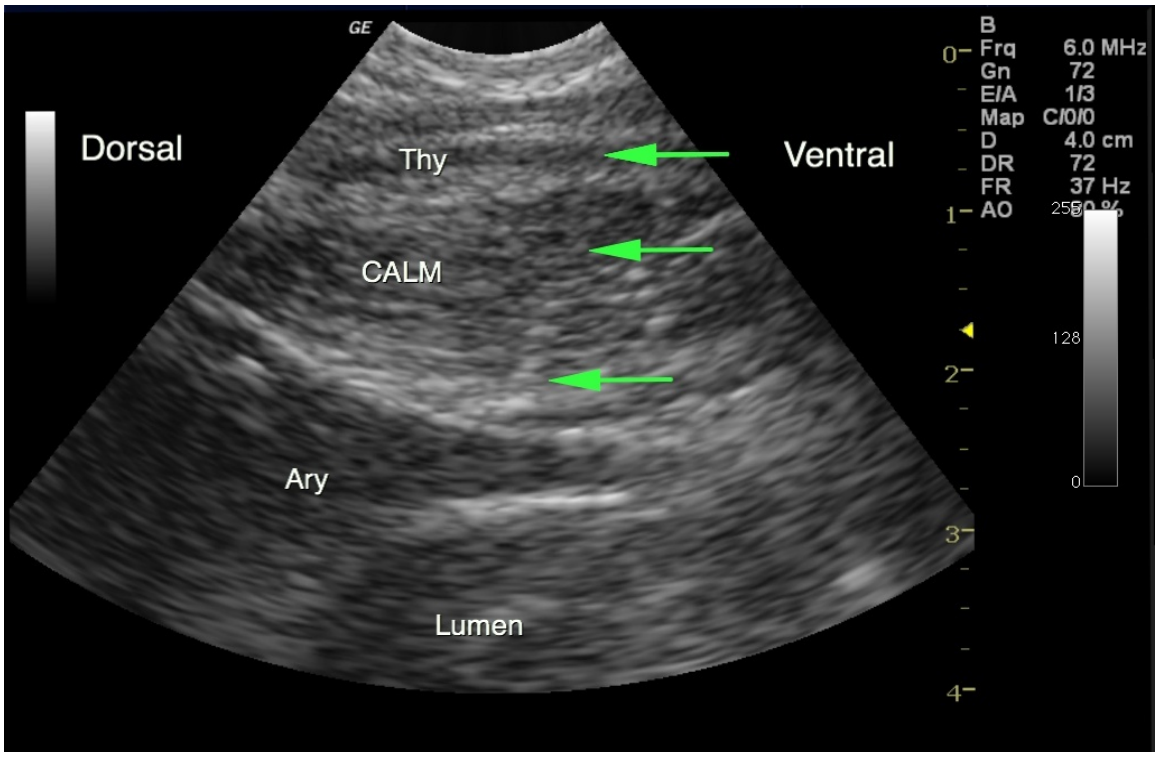

Figure 7: Longitudinal sonographic appearance of the left equine larynx with the 20-gauge guide needle (green arrows) in place through the thyroid cartilage (Thy), *cricoarytenoideus lateralis* muscle (CALM) and stopping at the arytenoid cartilage (Ary). The lumen of the larynx (Lumen) lies deep to the arytenoid cartilage.

Rotating the probe in a transverse plane confirmed appropriate needle position in the centre of the CALM. A 5mm skin incision was made at the dorsal aspect of the needle using a number 11 scalpel blade orientated in a dorsoventral direction. A 10-gauge coaxial cannula and introducer (Vacora)[6] was inserted through the skin and advanced onto the thyroid cartilage immediately along the dorsal aspect of the pre-placed needle, ensuring correct trajectory of the assembly. Oscillating the introducer facilitated passage through the entire wing of the thyroid cartilage. Complete advancement through the thyroid was confirmed by a marked loss of resistance. It was important to place the operator's index finger on the shaft of the introducer ~10mm from the point as a stopper in the event of unexpected resistance loss.

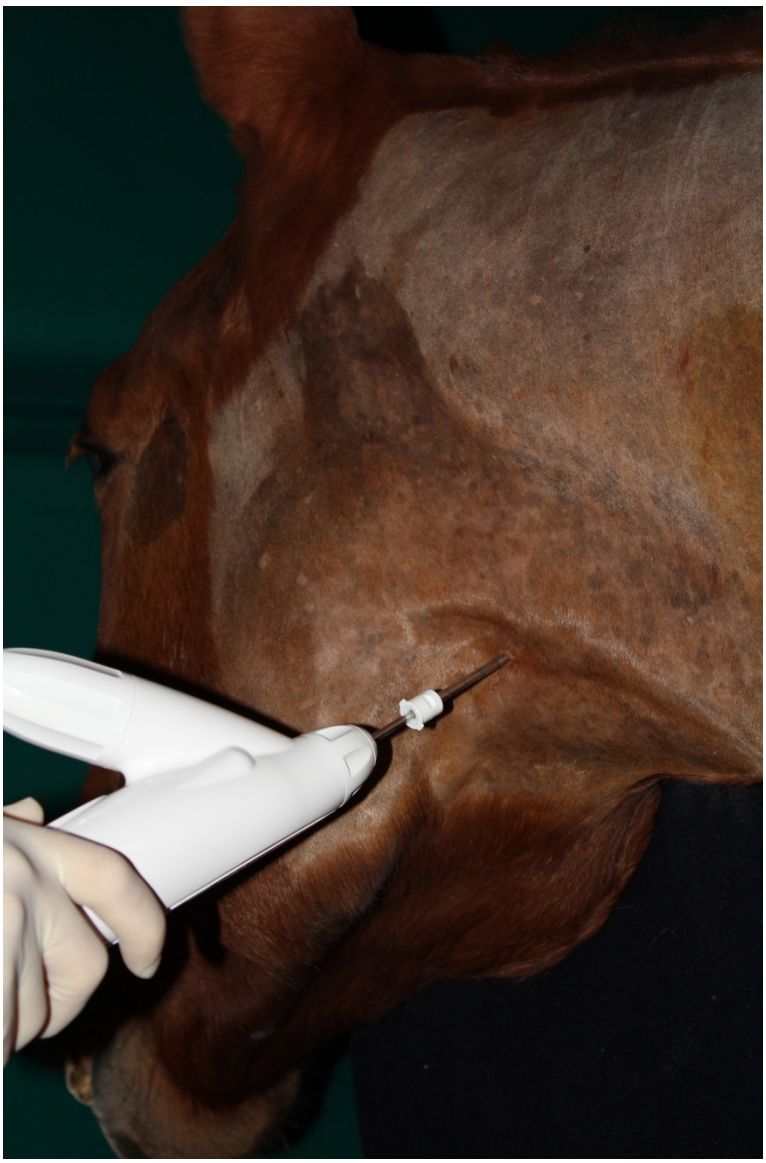

Figure 8: Performing a biopsy of the left *cricoarytenoid lateralis* muscle, with the instrument positioned though the introducer cannula.

Ideal placement of the assembly had the point of the introducer just exiting the axial margin of the thyroid cartilage. The outer cannula was unlocked and advanced so that it was firmly embedded within the wing of the thyroid cartilage, allowing removal of the introducer. The 10-gauge biopsy instrument (Cassi II)[7] was inserted until the 19-gauge guide needle made contact with the abaxial surface of the arytenoid cartilage (Figure 8). A full thickness, core

biopsy was then taken using the automated device. The instrument has a patented Stick-Freeze technology™, where CO_2 is released from a canister in the handle around the 19-gauge guide needle positioned in the target tissue. This temporarily freezes and secures the tissue adjacent to the needle, allowing the larger rotational cannula to advance down over the guide needle cutting the core biopsy in the process. The advanced cannula protects the sample on removal from the patient, thereby minimising the risk of sample loss. The biopsy was placed onto a cassette (Cassi II biopsy tray)[7] ensuring that the correct dorsoventral orientation was maintained, and then onto ice and stored in a polystyrene box until transported to a laboratory for processing (<15 minutes).

A coaxial introducer specifically for the needle was not available for purchase from the same company, so an alternative device was sought (Vacora)[6]. After sampling the first 2 horses, the outer cannula was customised by shortening the shaft length because an insufficient amount of biopsy needle was exiting the cannula into the tissue (Figure 9).

Figure 9: The factory supplied cannula and introducer (1st & 2nd left) where customized by shortening the length of the cannula (far right) by 20mm to allow a greater length of biopsy instrument to enter into the *cricoarytenoid lateralis* muscle.

The incision was closed with a single skin staple and an iodine-based surgical applied (Duraprep)[8]. An ice pack was held on the area for 30 minutes to limit any potential swelling. No additional incision protection was provided. Details of post biopsy examinations are provided (Table 2).

Muscle biopsy analysis

Biopsies were weighed immediately upon retrieval. For processing, muscle cores were mounted on cork with Tissue-Tek O.C.T. (Sakura)[9] and frozen in isopentane cooled in liquid nitrogen and then stored at -80^{0}C. Serial sections were cut at 6μm in a cryotome maintained at -20^{0}C and stained with haematoxylin and eosin. Slides were examined microscopically and total numbers of intact muscle fibers for each biopsy were counted manually using montages of digital photomicrographs.

Statistical analysis

Endoscopic grades were assessed using a 1-way, repeated measures ANOVA with horse and time as variables. Ultrasound data were assessed using a 2-way, repeated measures ANOVA with horse, side and time as variables. For all tests, significance was set at $P < 0.05$.

Results

Horses

All horses tolerated the procedure well. No major complications occurred either during or following the technique. Intraluminal penetration was suspected to occur in one horse with the first use of the shortened cannula as indicated by a trace amount of mucosal hemorrhage adjacent to the ventral aspect of the left arytenoid cartilage. It was suspected that an excessively ventral approach below the arytenoid had been made. No additional antibiotics or anti-inflammatories were provided to the horse and no complications were noted. Evidence of haemorrhage resolved within 4h of the procedure. All skin incisions healed well and staples were removed within 3-7 days following the procedure.

Endoscopic examination

There was no significant difference in the assigned endoscopic grades for any of the horses over the duration of the study ($P = 0.69$). The mean grade throughout the period was 1.5 (Range 1 – 2.5).

Ultrasound examination

In the 24h following biopsy, image quality from the left larynx was severely attenuated and measurements were inconsistent and sometimes impossible due to the presence of gas and thus discarded from final analysis. Throughout the study, there was a significant difference noted in the increased echogenicity of the left CALM compared to the right ($P < 0.001$) (Figure 10). Although the echogenicity of the left CALM increased for the first week following biopsy, values returned to near baseline levels thereafter with significant differences noted in the echogenicity at three different time points (Figure 10). Critically, there was no difference in values taken at the start and end of the study.

Muscle analysis

The average weight of muscle specimen was 0.043 g (Range 0.023 – 0.077 grams) and the average number of fibers per biopsy was 3418 (Range 711 - 7143). Average fiber count for the first 2 horses using the non-modified cannula was 1165, compared with 4544 fibers on average for the last 4 horses. Some areas of the muscle biopsies had evidence of freeze artifact, but this did not hinder fiber calculations (Figure 11).

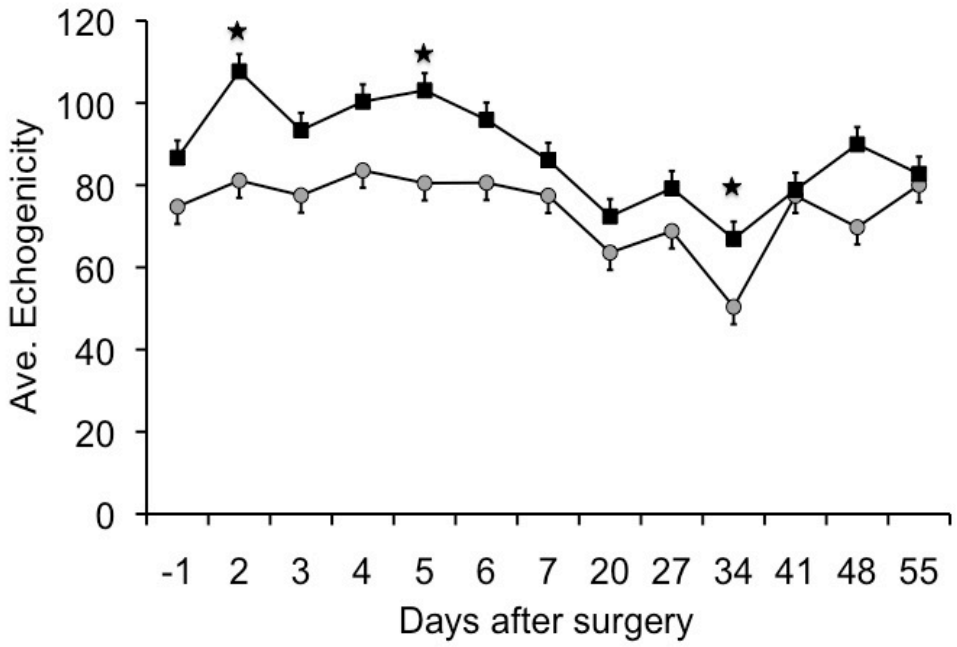

Figure 10: Average echogenicity scores assigned to the left (■) and right (○) *cricoarytenoid lateralis* muscles throughout the study period. The time points when echogenicity of the left side varied from their baseline values (day -1 after surgery) are indicated (★).

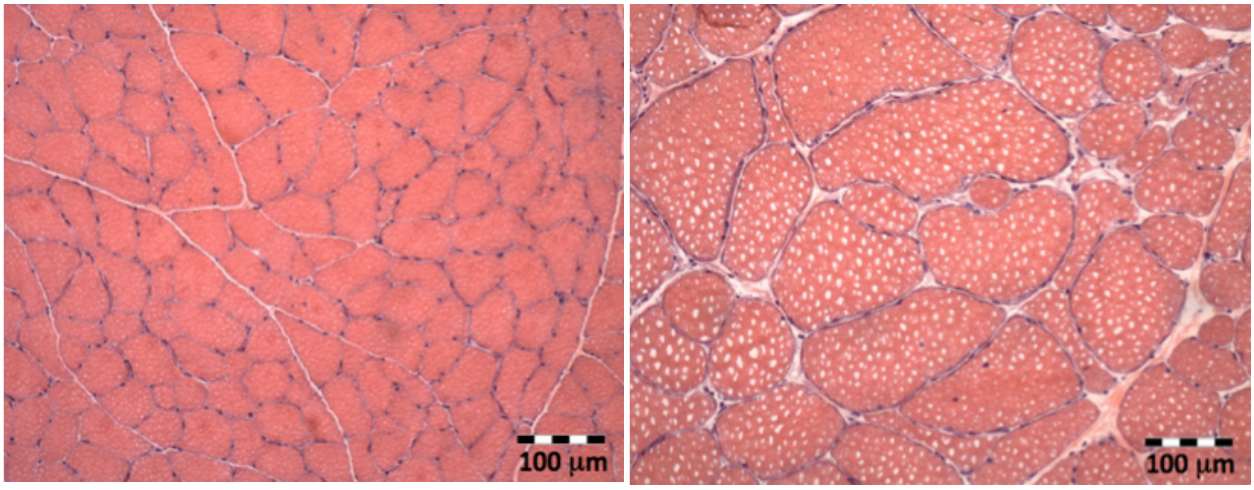

Figure 11: H&E stained muscle sections taken from two different biopsy samples showing the absence (left) and presence (right) of ice-artifacts within the muscle fibers. Both sections were still suitable for performing immunohistochemical analysis. Magnification 10X. Bar = 100µm

Discussion

Ultrasound guided biopsy of the left CALM proved to be a repeatable and safe technique in the sedate horse, yielding samples suitable for histological analysis [61]. No major intra- or post procedural complications occurred, with only minor intraluminal hemorrhage noted endoscopically in a single case. The use of ultrasound proved critical in locating the CALM muscle and positioning of the guide needle through the thyroid cartilage and into the muscle.

Equine muscle biopsy has been used to provide information on various neuromuscular disease cases [79, 80]. Traditional retrieval methods use a Bergstrom biopsy needle [80], but an open surgical approach has been used on the equine larynx to retrieve muscle coincidentally during a laryngoplasty procedure [38]. Various biopsy devices were tested on cadavers during our initial pilot work. Side-sampling, Trucut® needles were not suited as the distance from the needle tip to the biopsy chamber was often too long, resulting in the majority of the sampling chamber lying outside the muscle rather than within it. Also, the flaccid muscle had a tendency to deform when sampled and yields were often small (< 800 fibers) and unpredictable. An end-on sampling device (Cassi II)[7] used for human breast biopsy was selected as it was the only instrument that reliably supplied > 800 muscle fibers needed for analysis [61]. This device is fully automated with a reusable hand piece and disposable needles of 10- and 12–gauge, but for the purposes of the study the former was used as it provided a superior muscle yield. The 19-gauge guide needle is not suited to penetrating through the laminar portion of the thyroid cartilage, hence the need for a separate introducer and cannula system that could accommodate the 10-gauge instrument. This sharp, trochar-pointed introducer easily penetrated through the thyroid of all horses, even those with sonographic evidence of cartilage mineralisation. The outer cannula was secured within the thyroid cartilage so that the end of the shaft just exited the axial

margin of the thyroid, and thus provided a direct tunnel through the superficial tissue directly over the CALM for instrument passage. The ability of the instrument to sample all tissue directly in contact with the shaft of the needle up to and including tissue at the needle tip was advantageous. The CO_2 freezing of muscle tissue adjacent to the needle produced some post-processing artifacts, but this did not inhibit our histological analysis (Figure 11). Also, immunohistochemistry was successfully performed on the biopsy samples as part of a separate study.

Ultrasound of the CALM demonstrated no significant differences in the overall muscle echogenicity as a result of the procedure. Carbon dioxide and room air infiltration during biopsy severely attenuated image quality for the successive 24h, but potential complications could still be monitored endoscopically during the interim. Since this study was performed, Garrett et al. [40] have demonstrated agreement between abnormal arytenoid movements based on the endoscopy and the relative echogenicity of the CALM, with those horses more severely affected having increased echogenicity of the muscle. In man, pathologic ultrasound changes have been identified in muscle as early as 10 days following acute denervation [47], with affected muscles showing a progressive increase in echogenicity with disease chronicity. It was anticipated that detrimental side effects to the CALM, or adjacent nervous tissue would be detectable as an increased echogenicity within the 8-week study period, however, this was found not to be the case.

Upper airway endoscopy demonstrated no significant difference in the grade of laryngeal movements at the start or end of the procedure. It was noteworthy that some horses did show subtle variations in grade during the study, often varying by a grade between weekly examinations. This variation has been shown to be normal finding in other long [17, 36] and

short-term evaluations [92]. Post mortem evaluation of the biopsy sites 18 months later was possible in 2 horses after they were euthanized as part of an unrelated study. No gross abnormalities were noted in the CALM in either horse, although a small, residual hole was still present in the thyroid cartilage in a single case.

Our data demonstrate that ultrasound guided biopsy of the left CALM is a repeatable and safe technique that can be readily performed in the standing sedate horse. It is anticipated that the procedure may be beneficial in long-term, muscle characterization studies of RLN. Success with the procedure relies on familiarity with cross-sectional anatomy of the larynx and the coordination of the imaging probe with the guide needle.

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Manufacturers' addresses

1 GE Healthcare, Waukesha, Wisconsin, USA

2 Medical imaging, San Francisco, California, USA

3 Pharmacia & Upjohn Company, New York, USA

4 Sparhawk laboratories, Lenexa, Kansas, USA

5 Becton Dickinson & CO, Franklin Lakes, New Jersey, USA

6 Bard Biopsy Systems, Temple, Arizona, USA.

7 Scion Medical Technologies, LLC, Newton, Massachusetts, USA

8 3M Medical, St Paul, Minnesota, USA

9 Sakura Finetek, Torrance, California, USA.

CHAPTER 4

SUMMARY, CONCLUSIONS AND FUTURE INVESTIGATIONS

Despite the significant progress made in medical diagnostic imaging and molecular genetic techniques, our understanding of recurrent laryngeal neuropathy (RLN) has failed to advance at a similar rate. Although much is known about the signalment, clinical signs and disease impact, our understanding of the etiology is still limited. Regarding diagnostics, clinical disease recognition using upper airway endoscopy both at rest and during exercise still remains the gold standard, although recent work using ultrasonography appears to have promise in detecting early evidence of muscle atrophy in the intrinsic laryngeal musculature.

This thesis has demonstrated that ultrasound-guided biopsy of the left *cricoarytenoideus lateralis* muscle provides a safe, repeatable, minimally invasive method to obtaining muscle specimens in standing, sedated horses. The information gained from such biopsy samples provides investigators with a new opportunity to study RLN. Until now, fiber-type analysis has been limited to samples harvested either at post-mortem or intraoperatively during surgical correction. A number of studies have classified horses with a normal ante-mortem upper airway endoscopy but evidence of muscle fiber type regrouping on post-mortem analysis as being subclinically affected. The authors of these papers have assumed that any histological evidence of muscle denervation-reinnervation is an indication of early onset RLN. However, muscle fiber regrouping is a normal age-related process in all species so questions remain as to whether these subclinical horses are being correctly classified. A future, long-term study that could be performed which would help address this issue would be a comparative study where endoscopic examinations of the upper airway are compared with a muscle fiber-type analysis on an annual

basis throughout the entire lifetime of a cohort population. Thus, serial endoscopic examinations could be matched with muscle fiber samples obtained using a biopsy technique. Changes in the population of muscle fiber types, fiber sizes and fiber shapes could be correlated with onset of the disease.

In conclusion, ultrasound guided biopsy of the CALM has been shown to be a safe and effective technique for specimen retrieval in the horse, and it is anticipated that this new procedure will allow advances in understanding of RLN pathogenesis and development of an early diagnostic test for RLN.

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