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Partitioning of pulmonary lung resistance in horses: Ex-vivo.

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Partitioning of pulmonary lung resistance in horses: *Ex-vivo*.

by

James Alexander Nicol

A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Partitioning of pulmonary lung resistance in horses: Ex-vivo". submitted by James Nicol in partial fulfillment of the requirements of the degree of Master of Science.

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Abstract

Horses can be affected by two non-infectious inflammatory lung diseases, namely heaves and inflammatory airway disease (IAD). Heaves is characterized by episodes of bronchoconstriction and has many similarities with asthma. It is challenging to treat, but before new therapies can be proposed it is necessary to understand which airway size is most affected by bronchoconstriction and is the best target for a new treatment. The aim of the present study is to dissect the lung resistance to airflow so that the “bottleneck” between lower airways of various sizes can be identified. The specific objectives were 1) to partition total lobar resistance (from the cranial lobes of horses’ lungs) using ex-vivo lungs ventilated in physiological conditions and 2) to assess the effect of increasing respiratory rate and negative chest pressure on lung mechanics at the level of different airway sizes. To do this, we used 13 healthy ex-vivo horse lungs and ventilated them in an airtight negative pressure box under 9 different conditions of pressure and respiratory rates, while partitioning of resistances were performed using the alveolar capsule and retrograde catheter techniques. Total lobar resistance ($R_L$) partitioned into 4 component resistances: Large airway resistances ($R_{\text{large}}$), middle airway resistances ($R_{\text{middle}}$), small airway resistances ($R_{\text{small}}$), and resistances contributed by the lung tissue ($R_{\text{tissue}}$). We found that $R_{\text{small}}$ was the airway size contributing the most resistance to $R_L$, and that the relative contributions of $R_{\text{large}}$ and $R_{\text{small}}$ to $R_L$ increased when box pressure decreases. The relative contribution of $R_{\text{tissue}}$ to $R_L$ did the opposite.
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<tr>
<td>AIC</td>
<td>Akaike information criterion</td>
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<tr>
<td>ANOVA</td>
<td>ANalysis Of VAriance</td>
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<tr>
<td>ASM</td>
<td>Airway Smooth Muscle</td>
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<td>BAL</td>
<td>Bronchoalveolar Lavage</td>
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<tr>
<td>BIC</td>
<td>Bayesian information criterion</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic Obstructive Pulmonary Disorder</td>
</tr>
<tr>
<td>DPPC</td>
<td>Dipalmitoyl phosphatidylcholine (lung surfactant)</td>
</tr>
<tr>
<td>E_L</td>
<td>Lung Elastance</td>
</tr>
<tr>
<td>E_t</td>
<td>Tissue Elastance</td>
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<tr>
<td>GLMM</td>
<td>Generalized linear mixed model</td>
</tr>
<tr>
<td>IAD</td>
<td>Inflammatory Airway Disease</td>
</tr>
<tr>
<td>ID</td>
<td>Internal Diameter</td>
</tr>
<tr>
<td>IOS</td>
<td>Impulse Oscillometry System</td>
</tr>
<tr>
<td>FOT</td>
<td>Forced Oscillation Technique</td>
</tr>
<tr>
<td>P_a</td>
<td>Alveolar pressure</td>
</tr>
<tr>
<td>P_airway</td>
<td>Airway pressure</td>
</tr>
<tr>
<td>P_middle</td>
<td>Middle airway pressure</td>
</tr>
<tr>
<td>P_p large</td>
<td>Peripheral airway pressure from the large retrograde catheter</td>
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<td>P_p small</td>
<td>Peripheral airway pressure from the small retrograde catheter</td>
</tr>
<tr>
<td>P_p small</td>
<td>Peripheral airway pressure</td>
</tr>
<tr>
<td>PE</td>
<td>Polyethylene (used in tubing)</td>
</tr>
<tr>
<td>P_tp</td>
<td>Transpulmonary pressure (also sometimes called Δ P_{tp})</td>
</tr>
<tr>
<td>R_airway</td>
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<td>R_tissue</td>
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<tr>
<td>RAO</td>
<td>Recurrent Airway Obstruction (aka. heaves)</td>
</tr>
<tr>
<td>V</td>
<td>Airflow (Used interchangeably with dV/dt)</td>
</tr>
<tr>
<td>V_C</td>
<td>Vital Capacity</td>
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<tr>
<td>V_E</td>
<td>Minute Ventilation</td>
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<tr>
<td>V_T</td>
<td>Tidal Volume</td>
</tr>
<tr>
<td>ZA</td>
<td>Alveolar impedance</td>
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</table>
1. GENERAL INTRODUCTION.

1.1. The respiratory system.

The mammalian respiratory system consists of two multi-lobed lungs and associated vasculature. Ventilation is achieved by means of maintaining negative pressure inside a sealed thoracic cavity with the help of a muscular diaphragm, intercostal muscles and accessory muscles. During inspiration, contraction of the diaphragm forces the abdominal contents down, thereby increasing the vertical dimension of the chest cavity by 1 cm during normal tidal breathing and up to 10 cm during forced inhalation in humans (Campbell 1970). Contraction of the external intercostal muscles pull the ribs upward and forward thereby increasing the lateral and anteroposterior dimensions of the thorax while accessory muscles such as the scalene and sternomastoids elevate the first 2 ribs and sternum respectively (Campbell 1970). These accessory muscles play greater roles during exercise than during quiet breathing. Air follows the negative pressure gradient created by the aforementioned increase in thoracic volume and enters the elastic lungs, prior to which it passes across the structures of the upper respiratory tract; the nose, mouth, sinus cavities, structures of the pharynx. It enters the lower respiratory tract first in the trachea and then throughout the branched distribution structure known as the bronchial tree, finally ending up in the acini where gas exchange takes place in the alveoli.

Passive expiration is facilitated by the lung, the chest wall and diaphragm returning to equilibrium following expansion resulting from inspiration. Muscles assisting in voluntary expiration and during exercise are those of the abdominal wall; the rectus abdominus, internal and external obliques and the transverses abdominus (Campbell 1970). However it should be noted there is significant differential recruitment of individual accessory muscles over a wide range of respiratory effort (Yokoba, Abe et al. 2003) and over changes in posture (Abe, Yamada
et al. 1999) or during hypercapnea (Abe, Kusuhara et al. 1996). Internal intercostals have an opposite effect than the externals by pulling the ribs down and inward, thereby reducing thoracic volume (Campbell 1970), however these concepts have been somewhat challenged as of late. Recent studies in anesthetized dogs have suggested that muscle spindles of the external intercostals have relatively little impact on the mechanical behavior of the respiratory system (Easton, Hawes et al. 1999). In awake canine studies by Easton et al. (Easton, Hawes et al. 1999) it was found that parasternal intercostal activity ceased during inspiratory flow, however external intercostals activity continued following inspiration, and even persisted through expiration. It would seem muscle spindles play a more important role in the mechanics of breathing than previously thought.

1.1.1. **The conducting system.**

The bronchial tree or lower respiratory tract is a branched distribution structure conducting airways in the mammalian lung that carries air from the trachea, across the carina, through the bronchi, through the smaller bronchioles and into the acini where gas exchange can take place across the alveolar epithelium, basement membrane and capillary endothelia. In humans, the length, diameter and number of bifurcations found among daughter airways down to the level of the alveoli are important and influence how air is distributed throughout the lung. At each generation, the branching is essentially dichotomous with each airway being divided into two smaller daughter airways of similar size (Horsfield and Cumming 1968; Yeh 1979). In contrast, equine lung airways are longer along the tracheal axis than they are along the lateral branches leading off the main bronchi (Phalen and Oldham 1983). In addition smaller airway segments branch from a main larger parent bronchus, known as monopodal branching (Yeh
In addition, the anatomy of the lungs are species-specific; The equine lung has 2 main lobes, 2 cranial lobes and 1 cardiac lobe (Smith, Aguilera-Tejero et al. 1994).

Figure 1. Map of the right and left lung of the horse. Branches are labelled using modification of the binomial numbering system, RCLB = right caudal lobe bronchus, LCLB = left caudal lobe bronchus. Image adapted from Smith et al. (1994). Equine Vet J 26(4): 283-290.
While Smith et al. developed a numbering system for ease of *in-vivo* bronchoscopic examination, they addressed only the caudal lobes because endoscopic examination of cranial lobes proved difficult (Smith, Aguilera-Tejero et al. 1994). Based on the above, the new extrapolated numbering system described by Smith et al. was applied specifically to the cranial lobes because they are the lung lobes of interest used in this project (Figure 2).
Figure 2. Diagram of adapted airway nomenclature described by Smith et al. for lung cranial lobe binomial numbering. Image adapted from Smith et al. (1994) Equine Vet J 26(4): 283-290.
The left and right bronchi of the equine cranial lobes (branches 1.1 and 2.1) arise respectively from the dorsolateral sides of the left and right main caudal lobar bronchi (Nakakuki 1993) and curve nearly 180° off in the cranial direction (Smith, Aguilera-Tejero et al. 1994). As such they are subject to atelectasis and poor ventilation while in-vivo, a condition also seen in the cranial lobes and middle lobes of cattle (Robinson, Ingersoll et al. 1984) and dogs (Robinson and Milar 1980).

Nakakuki developed a different numbering system than the binomial one based on compartmentalization of the equine lung (Figure 2). They proposed the following scheme: dorsal, lateral, ventral and medial bronchiole systems that arise respectively from the dorsal, lateral, ventral and medial bronchus on either side. Furthermore, they proposed that cranial lobe bronchioles be comprised of the first bronchiole of the dorsal bronchiole system or the two bronchioles arising from the lateral sides of the trachea (Nakakuki 1993). The authors proposed cranial lobe bronchioles be composed of either 2 of the above situations depending on anatomical convention, but did not address the smaller branches with respect to the cranial lobe only. Additionally the bronchi from which equine cranial lobes arise appear to be unique compared to those of pigs, cows, goats or dogs with exception to the right cranial lobe of which the bronchi does originate similarly to that of the dog (Nakakuki 1993).

Importantly, *in-vivo* equine cranial lobes are oriented near the vertical, a condition that was replicated in our experiments by means of suspending the lungs from the top of the box.

**1.1.2. Resistance to airflow.**

As air flows through a tube, one can measure a difference in pressure from one end of the tube to the other. For a given airflow, the greater the pressure drop, or pressure difference between the two sites, the greater the resistance to airflow in the tube. Halving the radius or
doubling the length of a tube increases resistance by 16 fold and 2 fold respectively (West 2012) according to Poiseuille’s equation:

\[ R = \frac{8\eta l}{\pi r^4} \]

Where \( R \)=resistance to airflow, \( \eta \)=viscosity, \( l \)= length, and \( r \)=radius.

The characteristics of the air flow, being laminar or not, have an effect on the resistance to its movement through the tubes. Laminar airflow is most often seen at low pressure and/or flow rates, but can change as these variables increase, resulting in airway turbulence. Recent computer simulations may validate laminar airflow for small airways but not larger airways where resistance increases due to the effects of turbulence, and partially account for the differences in resistance seen during inhalation and exhalation (Majumdar, Alencar et al. 2005). Compared to laminar flow, turbulent airflow seen in large airways has different properties in that pressure is not proportional anymore to flow rate \((dV/dt)\), but rather to its square:

\[ P = K(dV/dt)^2 \]

In other words, turbulence is most likely to occur where airflow velocity is high and tube diameter is large as illustrated by the Reynolds equation \((Re)\):

\[ Re = \frac{(2rvd)}{\eta} \]

where \( d \)= density, \( v \)= velocity, \( r \)= radius and \( \eta \)= viscosity.

A Reynolds number \(> 2000\) means turbulence is probable in a closed system, however the mammalian airway tree rapidly branches from larger primary bronchi hence a combination of laminar and turbulent airflow is most probable (Pedley 1977). Due to a decrease in airflow velocity, less turbulence occurs in smaller airways as a result of the large number of parallel airways.
In static human anatomical models of the airway tree (Figure 4), it is the medium size bronchi that contribute the most to airflow resistance. As in many other species, the very small bronchi (beyond generation 7) contribute very little to airflow resistance (Pedley, Schroter et al. 1970). Because of a greater cumulative functional diameter for very small airways (i.e. the diameter obtained by adding each small airway next to each other), this result is what would naturally be expected. The total cross sectional area of the branching airways arranged in parallel increases which translates into a decreased air velocity and an overall decrease in resistance to airflow. The enormous sum of the cross sectional area of parallel airways from about 2.5 cm$^2$ in the human trachea to 180cm$^2$ at about generation 16 not only affects airway resistance, but also has an impact on airway resistance to airflow. Other investigations into areas of greatest postnasal resistance in humans also support airway generations six to eight contributing most to the resistance (Ingram and McFadden 1977), while others have predicted airways of 7 mm inner diameter, classified as medium sized airways, to be chiefly responsible (Horsfield and Cumming 1968). This model however negates elasticity of the airways (especially those of the small bronchioles lacking cartilage rings), hemodynamic effects and possible effects presented by different mammalian lung varieties.
Figure 4. Location of the chief site of airway resistance in humans. Note that the intermediate size bronchi contribute most to the airflow resistance and that very little is located in the very small airways. Image adapted from Pedley et.al. 1970. Respi Physiol 9:387.

1.1.2.1. Confounding factors for anatomical airway resistance.

It should cautiously be noted that airway resistance is determined not only by morphological changes of the airways themselves, but also by neural and humoral mechanisms as well (Nadel and Barnes 1984). As a result the distribution of airway resistances might differ between *in vivo* and *ex-vivo* lungs, and further consideration would have to be taken if one was to study the effects of pharmaceuticals on pathological conditions such as heaves, also called equine Recurrent Airway Obstruction (RAO) and formally known as Chronic Obstructive Pulmonary Disorders (COPD). In addition, anatomical predictions in static models do not take
into account the effect of changing lung volumes and their influences on airway diameters and airway resistance during dynamic breathing (Macklem, Woolcock et al. 1969). Furthermore, cholinergic receptors (Barnes, Basbaum et al. 1983) and vagal fibers (Woolcock, Macklem et al. 1969; Hoppin, Green et al. 1978) are located with the greatest density in large and medium sized airways with the least density found in the peripheral airways. However, Fuller and Freed concluded that most cholinergic reactivity resides in the peripheral lung which is largely responsible for heterogeneous airflow seen among these airways (Fuller and Freed 1995). While *ex-vivo* animal and human lungs have the ability to present airway closure following treatment with acetylcholine (Gunst and Stropp 1988), histamine and methacholine (Murtagh, Proctor et al. 1971; Gunst, Warner et al. 1988), it is unknown how much this can occur in normal excised equine lungs. The degree to which normal equine lungs present airway collapse (if any) following excision is also unknown, and would make a fine follow up study to the present one.

### 1.1.2.2. Retrograde catheter and alveolar capsule techniques for the location of greatest resistance in the airway tree.

The standard lung mechanics technique allows measuring lung properties of the whole respiratory system, including the upper airways. To measure the resistance to airflow from only a section of the lung, it is necessary to measure airflow as well as (and simultaneously) the drop in pressure between two points in the airway tree. In addition, to understand better the contribution of peripheral lung tissues to the whole lung mechanics, it is also necessary to measure pressures in the very periphery of the lung, next to its surface. Techniques like the retrograde catheter and
alveolar capsule allow performing these measurements and were used widely in lung physiology studies (Bates and Lutchen 2005).

*Ex-vivo-retrograde catheter:*

The first direct investigations of the lung periphery were provided by the retrograde catheter, introduced by Macklem and Mead (Macklem and Mead 1967). These investigators pulled a thin flexible catheter along the airways by means of a piano wire, and out through a hole in the parenchyma until its bell-shaped end became wedged in a small airway (Figure 5). By measuring the pressure at the other end of the catheter during ventilation of the lung, they were able to determine that most of the resistance to airflow in the normal airway tree was located proximally, thus establishing the notion of the silent zone (Bates and Lutchen 2005). Subsequent calculations based on the dimensions of the various branches of the tree also indicated that most of its flow resistance occurred in the first 10 or so airway generations (Pedley, Schroter et al. 1970). The retrograde catheter technique, however, was restricted to use in isolated lungs as opposed to *in-vivo* use, and it also suffered from the frequency-response problems, which is out of phase recordings inherent to measuring pressure through a long thin conduit. Both these limitations were overcome with the development of the alveolar capsule method described shortly. Furthermore, many studies of the day relied on paper tracings and oscilloscopes to record their pressure readings and to calculate resistance. With more sensitive differential pressure transducers and powerful computing power, these problems have largely been prevented.
Figure 5: Illustration of the retrograde catheter technique used to measure pressures in peripheral airways. Image adapted from Macklem and Mead. (1967) J Appl Physiol. 22(3): 395-401.

Whereas most studies addressing the contribution of peripheral airways to total lung resistance (R_L) using the retrograde catheter technique (Macklem and Mead 1967; Hogg, Macklem et al. 1968; Hogg, Agarawal et al. 1972) were performed in dogs, some also have replicated this technique in excised human lungs (Macklem and Mead 1967; Hogg, Macklem et al. 1968; Hogg, Agarawal et al. 1972; Silvers, Maisel et al. 1974). Hogg et al. reported that small airways with an internal diameter (ID) 2-3mm or less account for 25 % of R_L at transpulmonary pressure (P_{lp}) of 5.1 cm H_2O (Hogg, Macklem et al. 1968), which corresponds to physiological normal breathing effort, whereas Silvers et al. found the small airways to contribute as much as 54 % at vital capacity (V_C), which corresponds to the maximum amount of air expelled from the lungs following maximal inhalation. The apparent discrepancy is proposed to be the result of differences in experimental technique, such as Forced oscillation technique (FOT) in Hogg et
al.’s work vs. passive expiration performed by Silvers et al., and from different breathing frequencies and tidal volumes. Interestingly, Van Brabandt et al. compared small airway resistances between excised human and dog lungs of 2.4mm ID or less using the FOT (Van Brabandt, Cauberghs et al. 1983; Brusasco, Warner et al. 1989). They reported markedly larger contributions of peripheral airway resistance (R_p) to R_L in humans (44 to 96% ) than in dogs (41 to 59%) (Van Brabandt, Cauberghs et al. 1983).

Ex-vivo-alveolar capsule:

The alveolar capsule technique was originally developed by Sasaki et al.(Sasaki, Takishima et al. 1977) and subsequently perfected by Fredberg et al. (Fredberg, Keefe et al. 1984). It consists of a small chamber with a flanged end glued to the pleural surface of the lung with cyanoacrylate, so a small section of pleura, usually a few millimeters across may be sampled. For this purpose, a modified 200 µL pipette tip works well. Careful puncturing of the pleura within the capsule then brings the sub-pleural alveoli into direct communication with the capsule chamber. A miniature piezoresistive pressure transducer lodged into the capsule provides a continuous recording of alveolar pressure (Figure 6), however placing the transducer at the end of 10 cm of communicating tube is an alternative method.
Figure 6. The alveolar capsule technique with the capsule glued to the surface of the lung and plural punctures allowing for the sampling of alveolar pressures. Image adapted from Bates et al. 2005 Respir Physiol Neurobiol 148(1-2): 153-164.

Most studies with the alveolar capsule have been applied to the lungs after the chest is opened and widely retracted, for obvious practical reasons. Consequently, the measurements of alveolar pressure obtained are not influenced by the mechanical properties of the chest wall. However some studies have successfully applied the technique in closed-chest dogs (Bates, Abe et al. 1989). The alveolar capsule technique has increased the understanding of pulmonary mechanics, primarily through its extensive use in many species including dogs (Bates, Ludwig et al. 1988; Ludwig, Romero et al. 1990), cats (Hantos, Adamicza et al. 1992), rabbits (Tepper, Sato et al. 1992) and mice (Tomioka, Bates et al. 2002). Furthermore, the technique has shown that sub-pleural alveolar pressures at different locations throughout the normal lung are almost identical when ventilation occurs within the frequency range of normal breathing (Bates and Lutchen 2005). The normal lung behaves in a remarkably homogeneous fashion despite its structural
complexity, with a substantial fraction of the lung resistance determined at physiological respiratory rate consisting of a component due to viscous dissipation of energy in the lung tissues (Ludwig, Dreshaj et al. 1987; Bates, Ludwig et al. 1988; Bates and Lutchen 2005). The preceding studies found that lung resistance decreases with breathing frequency. However multiple alveolar capsules installed simultaneously on the same lung have shown the development of bronchoconstriction and regional heterogeneities in alveolar pressure as frequency increases (Fredberg, Keefe et al. 1984; Fredberg, Ingram et al. 1985; Ludwig, Romero et al. 1990).

The alveolar capsule technique has its limitations, particularly in terms of its ability to resolve the nature of a regional lung function. Having more capsules for example would be better able to detect heterogeneity of alveolar pressures throughout the lung. This is particularly true following the administration of bronchoconstrictive agents (Ludwig, Robatto et al. 1991). However this is not possible in smaller animals such as mice where only a single capsule may be used (Tomioka, Bates et al. 2002), or where only a small area of open chest is available (Hantos, Adamicza et al. 1992). Using multiple capsules would still represents a gross under-sampling of the tens of thousands of acini within the entire lung (Bates and Lutchen 2005).

Furthermore, this technique does not allow one to decide if differences in alveolar pressure are due to changes in local airway resistance or local tissue stiffness. Moreover, the contribution of tissue resistance tends to be overestimated by the capsule when the airway tree constricts heterogeneously (Lutchen, Greenstein et al. 1996). Tissue resistance varies with lung volume, and refers to the resistance offered by lung tissue when it expands. The alveoli at high lung volumes are more distended, and the elastic recoil tension in their walls is greater.
To overcome this, one needs to know how the flow entering the airway tree is partitioned between the various regions whose pressures are being monitored by direct measurement using the retrograde catheter, or by applying known oscillatory flows into the lung via the alveolar capsule while simultaneously measuring capsule pressure thereby measuring an alveolar impedance ($Z_A$) (Davey and Bates 1993). Recent investigations have recorded pressure and flow signals measured from the airway opening while sending high frequency oscillations to probe the lung periphery. The subtracted differences, which should represent the influence of all lung regions is a major challenge, and a subject of current research (Bates and Lutchen 2005).

1.1.2.3. Location of greatest resistance in the airway trees of various species.

More recently in dogs, the addition of the alveolar capsule technique (Fredberg, Keefe et al. 1984; McNamara, Castile et al. 1987; Warner 1990) allowed sampling of small airways located directly under the parenchyma. Using this technique, Brusasco et al. reported sub-pleural resistance found in the tissue ($R_t$) to comprise 70% of $R_L$ in excised dog lungs when upper airway resistance was excluded (Brusasco, Warner et al. 1989), and concluded resistance found in the airways ($R_{aw}$) to be negligible. Upper respiratory tract structures contribute most to airflow resistance in a healthy respiratory system, and are therefore normally excluded when studying lung resistance.

Numerous values have been reported on the location(s) of greatest resistance in various mammalian species; however, these values are based on measurements of lung resistance and compliance using transpulmonary pressure ($P_{tp}$). Only limited conclusions can be made when instruments or techniques used to probe the lung periphery are not used. Using such techniques would reveal how the smaller interior airways contribute to the entire lung, and to lung resistance.
and compliance. By additionally using the retrograde catheters and alveoli capsule, many have used this model to quantify areas of greatest resistance whereby partitioning various components of the lung reveals the “bottleneck”, or location of greatest resistance, in various species and conditions.

Lung resistance to airflow ($R_L$) can be calculated when $P_{tp}$ is in phase with airflow and changes in lung compliance can be determined based on changes in volume for healthy lungs. It has long been thought that large changes in lung resistance and lung compliance result from non-uniform constriction of airways causing heterogeneous ventilation (Ludwig, Dreshaj et al. 1987), however more information from additional measurements using alveolar capsule technique have shown that tissue resistance is also a contributing factor. The resistance found across the lung parenchyma, tissue resistance ($R_{tissue}$), possesses a flow resistance component due to its viscoelastic properties, known as dynamic hysteresis of lung recoil, which is discussed in later chapters (Loring, Drazen et al. 1981). Unlike airways, lung tissues respond in proportion to the volume of air applied (Hildebrandt 1969). The contribution of tissue resistance to total lung resistance can be magnified by the following conditions and serve to minimize the contribution of the airway component: large lung volume changes and low flow rates (Ludwig, Dreshaj et al. 1987). Indeed, the effect of large lung volumes can increase the viscoelastic effects originating from the tissue whereas low flow rates would favor small airways collapse, thereby increasing the relative contribution of $R_{tissue}$ to $R_L$. Depending on which experimental frequencies and/or volumes are employed when ventilating lungs, exploring the contribution of tissue resistance could help explain conflicting results reported in multiple species both in vivo and ex-vivo.

1.2. Non-septic respiratory pathologies in horses.
Alberta is the province which currently has the largest horse population in Canada (Alberta 2005) and where horses are important to both economy and culture. Respiratory diseases account for the second most common reason horse owners seek veterinary intervention (Traub-Dargatz, Salman et al. 1991). Several non-septic respiratory diseases exist in horses, of which Recurrent Airway Obstruction (RAO) (also known as Heaves) and Inflammatory Airway Disease (IAD) are the most frequent.

1.2.1. **Heaves and IAD.**

*Heaves*: The environmental inflammatory disease known as Recurrent Airway Obstruction (RAO) is similar to human asthma. It is characterized by recurrent episodes of labored breathing due to bronchoconstriction, mucus accumulation and inflammation of the lower airways (Robinson 2001; Leguillette 2003). Clinical signs can include exercise intolerance, respiratory distress at rest, coughing, nasal discharge, increased respiratory effort and weight loss (Leguillette 2003). The diagnosis of RAO is based on clinical signs and increased neutrophil percentages in the bronchoalveolar lavage (BAL) fluid (Robinson 2001; Leguillette 2003), Similarly to asthma, RAO horses exhibit a 3-fold increase in airway smooth muscle (ASM) mass as a result of remodelling and thickening of small airways (Herszberg, Ramos-Barbon et al. 2006).

*IAD*: Inflammatory Airway Disease is also an environmental inflammatory disease that presents more subtle clinical signs than RAO. A recent consensus statement described IAD as an inflammatory lung disease associated with respiratory clinical signs during exercise, but no labored breathing at rest, even after an allergic challenge with moldy hay for example (Couetil, Hoffman et al. 2007). Clinical signs can be latent or can include abnormal lung sounds, nasal discharge, and weight loss (Moore 1996; Viel 1997). Since poor performance is often the only
sign, IAD can go unnoticed for many years (Hoffman, Mazan et al. 1998). Horses with IAD have more severe exercise induced hypoxemia than health controls (Couetil and Denicola 1999) resulting from airway restriction.

1.2.1.1 Treatments for Heaves and IAD.

*Heaves:* Corticosteroids and bronchodilators are the most common veterinary treatments for RAO. They can however be problematic and expensive to horse owners. Steroids in particular have been perceived to cause laminitis (although this is controversial), a potentially fatal condition causing severe lameness in the hoof. Furthermore, some horses have been known to develop resistance to steroids when used for long periods of time (Stamper 2002). Bronchodilators are an efficient means to relieving acute clinical signs but do not decrease the lung inflammation which is the root of the pathophysiology of RAO (Leguillette 2003). In addition, bronchodilators do not have long term activity in horses (Leguillette 2003). Another concern is the use of steroids and bronchodilators for competitions where drugs with performance enhancing capabilities are prohibited. For these reasons, the cornerstone for managing horses with RAO is the limitation of the exposure to organic dust. To date, the best management practices for this disease are maintaining hygienic environments with reduced aero-allergen content. It also should be noted that horses have been proposed as a potential model for the study of human respiratory disease (Snapper 1986) and asthma due in part to their physiological and anatomical similarities (Tyler, Gillespie et al. 1971). Furthermore RAO is a naturally occurring disease that does not require pre-sensitisation. The lack of safe or practical therapeutic options available combined with the difficulties of preventing exposure of horses to organic dust justify the quest for a non-pharmaceutical treatment for RAO that would be of great benefit to the equine industry.
IAD: Much of the knowledge for treating IAD is borrowed from therapies used in RAO, as a result of both being non-septic lung inflammatory diseases. Inhaled or systemic corticosteroids and bronchodilators have been prescribed for IAD, however bronchodilator efficacy in maintaining airway patency has not been studied (Couetil, Hoffman et al. 2007). The effect of corticosteroids has been shown to decrease airway hyperreactivity and hypersensitivity in IAD (Tohver T. 2009)

1.2.1.2 Thermoplasty in horses?

Human asthma is a chronic inflammatory condition of the airways characterized by episodic symptoms of breathlessness, cough, and wheezing, which can increase or decrease in severity over time (Gildea, Khatri et al. 2011). Bronchial thermoplasty in humans involves delivery of radiofrequency energy to the airway wall, which ablates the airway smooth muscle (ASM) layer, lessening bronchoconstriction and improving symptoms (Gildea, Khatri et al. 2011). In cows, airway smooth muscle actin and myosin interactions are directly disrupted by high temperatures through the denaturation of motor proteins (Dyrda, Tazzeo et al. 2011). Using radiofrequency energy in dogs’ airways reduced airway resistance and hyperresponsiveness during challenge with methacholine (Danek, Lombard et al. 2004). Although long term clinical trials have shown that thermoplasty improved asthma symptoms, quality of life, and reduced health care utilization, these effects could not however be directly attributed to airway smooth muscle ablation itself (Cox, Miller et al. 2004; Castro, Rubin et al. 2010; Castro, Rubin et al. 2011; Thomson, Rubin et al. 2011). Treatments in people are performed during three separate procedures, with the right lower lobe being treated in the first procedure, the left lower lobe in
the second, and the two upper lobes in the third (Figure 7). The right middle lobe is not treated (Gildea, Khatri et al. 2011).

![Diagram of lung lobes](image)


Recently in 2010, Alair Bronchial Thermoplasty System and protocols were approved by the Food and Drug Administration for the treatment of severe persistent asthma in patients 18 years and older whose asthma is not well controlled with inhaled corticosteroids and long acting beta agonists (Asthmatx 2010).

1.3. **Measurement of respiratory physiological parameters.**

There are several lung function testing methods of measuring lung function, including airway hyper-responsiveness, in horses. The older standard lung mechanics methods are less appropriate for clinical practice, as they require use of an esophageal balloon catheter,
pneumotachograph, and tight environmental conditions. This setup can be readily employed in human or veterinary laboratory settings, but not in the field or in client horses (Hoffman 2009). This technique is described in details below in this section. Two entirely different systems for lung function testing in horses are emerging: (1) Oscillometry measures the effects of introducing tiny oscillatory waves of pressure into the airways, and ignores the mechanics of natural breathing. A comparison between the pressure input to the system and the flow output (P/F) gives a measure of resistance at varying input frequencies. Elevated resistance at lower frequencies is indicative of small airway disease (Hoffman 2009). (2) Flow-metrics utilizes natural breathing and compares thoraco-abdominal volume displacement with volume displacement at the airway opening (nares). Volume displacements, measured in terms of a rate (flow) should be equal and virtually synchronous. As airway obstruction develops, asynchrony develops, which is readily observed as divergence of the two flow curves that represent each volume displacement for each breath (Hoffman 2009). Lung mechanics, oscillometry or flow-metrics provide a baseline for testing airway reactivity with a series of increasing nebulised histamine agonist doses. Lung function testing is performed after each nebulised dose of histamine, and a dose-response curve is produced. The dose of histamine that evokes a given response, or that increases respiratory system resistance by 100% (PC100RRS) is interpolated on the dose-response curve, where the slope is the index of airway reactivity (Hoffman 2009). Standard lung mechanics and oscillometry can also be used in ex-vivo conditions.

Standard lung mechanics:

Physiological respiratory parameters are measured in-vivo using standard lung mechanics equipment. It usually includes a pneumotachograph for measuring airflow and a method of measuring changes in thoracic pressure. To this end piezoelectric pressure transducers are used
to measure pressure changes which are recorded using a computer, but other forms of recording
device have been used in the past. DuBois et al. (DuBois, Botelho et al. 1956) first reported a
method of measuring changes in volume within an organ (plethysmography) using a sealed
chamber, in which conscious human subjects are seated in an airtight chamber. The subject
breathes ambient air for five-minutes, and the pressure of air in the plethysmograph relative to
atmospheric pressure is measured by a manometer. Similar equipment has been developed and is
still in use today. The principle is based on Boyle's law where volume of a gas varies in inverse
proportion to the pressure to which it is subjected.

Other more invasive methods used to perform lung mechanics in-vivo are by directly
measuring pleural pressure using a blunt needle inserted into the pleural cavity (Sasse 1971), or
indirectly using an esophageal balloon attached to a tube (Milic-Emili, Mead et al. 1964;
Derksen and Robinson 1980). Both techniques communicate directly with (a) pressure
transducer(s) and are coupled with a pneumotachograph measuring flow, allowing for pulmonary
resistance to be calculated.

The simplest and most widely employed model of pulmonary mechanics consists of a
single compartment served by a single conduit (Bates and Lutchen 2005), and offers good
physiological insight into experimental data. An elastic component \(E\) is assigned to the lung
and a resistance \(R\) to flow \(\frac{dV}{dt}\) can be calculated once transpulmonary pressure \(P_{tp}\) is
known using the following equation:

\[
P_{tp}(t) = RV(t) + EV(t) + P_0
\]

In animal models, airway resistance and lung elastance (the mathematical inverse of compliance)
have long been the traditional variables for assessing pulmonary function. Using the equation
above, airway resistance can be linked to the conducting structure in an idealized lung model
with laminar flow and cylindrical rigid tubes for airways (Mitzner 2007). Lung elastance is a simple variable to both define and measure the stiffness of the non-conducting airways in the same idealized lung model. A stiffer lung is one that takes a greater pressure change to reach a given volume change, but very many factors can alter elastance in both normal and pathologic lungs, including lung volume, smooth muscle contraction, surface tension, and the rate at which the measurement is made (Mitzner 2007).

In addition to the above description of resistance and compliance, measurements of impedance can also be measured using a forced oscillation technique (FOT) described in detail by Landsér et al. (Landser, Nagles et al. 1976). Respiratory impedance is defined simply as the ratio of time-varying pressure and flow, and is often displayed in graphs with real and imaginary phase components plotted as a function of frequency (Mitzner 2007). A noise signal which consists of a complex oscillation with frequencies of 2, 4, 6, 8 etc… up to 32 Hz is produced by means of a loudspeaker at the inlet of the main bronchus. A Fourier analysis of pressure and flow signals yields a mean resistance for each harmonic (Van Brabandt, Cauberghs et al. 1983). Flow is measured with a pneumotachograph and pressure is measured with a pressure transducer connected to a side tap close to the main bronchus opening, and to the two catheters wedged in respective larger and smaller airways. To estimate the amount of noise, a coherence function is calculated (Landser, Nagles et al. 1976).

*Ex-vivo* studies, which employ whole excised lungs or dissected lobes only, can be used to measure lung mechanics also using forced oscillation or negative pressure vacuum box depending on pulmonary impedance or pulmonary resistance being of interest respectively. Impedance measurements have been more popular in small animal lungs due in part to their prohibitively small size making either retrograde catheter or alveolar capsule application
difficult. Impedance studies can be performed in much the same manner as in-vivo experiments described above. Other ex-vivo studies employ a leak-proof negative pressure vacuum box by which the thoracic cavity is essentially replicated. The lung (or lobe) of interest is cannulated at the main bronchus, the lumen of which opens to atmosphere and is either suspended vertically or laid prone prior to negative pressure application to the box. Lung mechanics are performed using a pneumotachograph to measure airflow at the main bronchus, and pressure transducers measuring pressure either inside the box or inside lung airways of interest. Resistance and elastance are calculated using the above formula similarly to in-vivo studies. This technique has been described to measure lung resistance ex-vivo in cats (Macklem and Mead 1967; Hildebrandt 1969) dogs (Macklem and Mead 1967; Sasaki, Takishima et al. 1977; Van Brabandt, Cauberghs et al. 1983; Fredberg, Keefe et al. 1984; Allen, Frantz et al. 1987; Gunst and Stropp 1988; Brusasco, Warner et al. 1989; Vettermann, Warner et al. 1989), rabbits, cattle (Gustin, Lomba et al. 1987), horses (Van Erck, Votion et al. 2004) (Tepper, Sato et al. 1992), non-human primates (Macklem and Mead 1967) and humans (Macklem and Mead 1967; Van Brabandt, Cauberghs et al. 1983).

1.3.1 Measuring lung / airway resistance in ex-vivo horse lungs.

Airway resistance and airway impedance can be calculated from measurements obtained using similar methods to those described above. To the author’s knowledge, only 2 studies have investigated airway resistances using excised equine lungs. Robinson investigated the species differences in response, including horses, to airway obstruction. They hypothesized microscopic lung anatomy, particularly variation in interlobular septa anatomy between species, was responsible for differences in observed airway obstruction (Robinson and Sorenson 1978). They employed a double lumen catheter to obstruct a peripheral airway, where delivered air from the
outer catheter escaped via collateral channels. Segmental pressure was recorded from the inner catheter during a static lung maneuver, and collateral resistance was calculated, however no pressure–volume loops from horses were recorded due to leaks. They found significantly higher collateral resistance in horses than in dogs, and suspected the presence of incomplete lobulated fibrous septa, similar to that seen in humans, was responsible (dogs have none).

Only one study has addressed the question of lung impedance using FOT, whereby impedance was calculated using measurements obtained from a whole equine lung. As shown in figure 6, the lung was placed in a rigid plexiglas box, which was used to simulate the thoracic cavity, and a flexible tube was tightly connected to the main circular opening on the lateral side of the box. The lumen of the flexible tube was open to the atmosphere and a vacuum pump was used to create negative pressure oscillations in the box as to inflate the lung. A calibrated pressure transducer was used to determine the pressure in the box, where $P_{tp}$ was determined by subtracting the pressure inside the box from atmospheric pressure. The impulse oscillometry system (IOS) used to perform FOT was tightly connected to the main opening and impedance was calculated later (Van Erck, Votion et al. 2004).
Figure 8. A system used to perform FOT lung mechanics on *ex-vivo* equine lungs. Modified from Van Erck *et al.* (2004); *The Veterinary Journal* **168** (2004) 259–269.
2. **AIMS AND OBJECTIVES.**

The aim of this study was to partition pulmonary resistance in healthy horses’ lungs using an *ex-vivo* technique where the lungs are artificially breathing in a sealed box with oscillating negative pressure, thus allowing measurement of peripheral lung resistances when several respiratory parameters are changed. A similar technique was previously described in calves (Gustin, Lomba et al. 1987) and dogs (Brusasco, Warner et al. 1989) that allowed observing how partitioned lung resistances varied at various respiratory rates (RR) and transpulmonary pressures ($\Delta P_{tp}$). Achieving this would provide a significant contribution to our understanding of lung mechanics in horses.

The specific objectives were to:

1) Partition total lung resistance into contributing peripheral resistances: $R_{\text{large}}$, $R_{\text{middle}}$, $R_{\text{small}}$, and $R_{\text{tissue}}$.

2) Measure the effect of changing RR and $\Delta P_{tp}$ on these various peripheral resistances.

To address these objectives we performed an *ex-vivo* study using healthy horses’ lungs put in various physiological conditions. We compared our data to *ex-vivo* measurements reported in humans, dogs, calves and horses (Robinson and Sorenson 1978; Van Erck, Votion et al. 2004) No study to our knowledge has used both the retrograde catheter and alveolar capsule technique in horses to directly measure peripheral lung resistances.
3. MATERIALS AND METHODS.

3.1. Ex-vivo horse lungs and cranial lobes.

The following is a description of tissue procurement, preparation and necropsy protocols, as well as a detailed description of all instrumentation used during the experiments.


A total of fifty-six entire pairs of lungs were obtained on 8 separate occasions from Bouvry abattoir in Ft McLeod, Alberta, during the development and data analysis of the project. The lungs were kept in a cooler with cold saline and ice packs for transportation (Gustin, Lomba et al. 1987). Care was taken to avoid damage caused during removal from the animal, and to avoid saline from entering the trachea. The lungs were dissected within 3 hours and the lung mechanics measurements were completed within 12 hours of the death of the horses. The cranial lobes, which in horses are independent of other lobes, were dissected off the main lobes and used to do the measurements. This was achieved by cutting an overestimated portion of the lung, including a significant part of the caudal lobe, then the lobe of interest was carefully separated. Only through practicing on 15 practice lungs was it possible to develop adequate technique necessary to remove cranial lobes while preventing leaks from peripheral airways. Small leaks were observed on several occasions as a result of dissection at the level of the heart and major arteries. We exsanguinated the lungs by briefly inverting the lobes, and manipulating any clots along the pulmonary arteries/veins adjacent to the central bronchus. Others have found excised lungs become edematous during prolonged perfusion, and that they can gain as much as 30g in fluid weight (Zardini and West 1966). Using the cranial lobes only allowed performing the experiments using a smaller box volume (see details below), which provided better and quicker control on the internal box pressure generated by an external vacuum pump.
After the dissection, the cranial lobes were prepared for the lung mechanics by cannulating the airways, placing retrograde catheters and an alveolar capsule. The main bronchus was first cannulated using stiff polypropylene tubing with internal diameter (ID) of 1cm and external diameter (ED) of 1.4 cm that was inserted to a length of approximately 8cm. A plastic lock and grip collar was used to seal the bronchial tube to the bronchus.

3.1.2. **Retrograde catheterization of lung.**

Please refer to section 1.1.2.3 for listings of studies that have employed the retrograde catheter technique, as well as Figure 5. Two retrograde catheters were inserted in a larger first generation bronchus, as well as in a smaller third generation bronchus respectively, following the technique described by Macklem and Mead (Macklem and Mead 1967). Briefly, a 10 cm piece of Poly Ethylene tubing (1 mm ID, 2 mm OD) was guided through the bronchial tube and into a respective airway of the cranial lobe using a piano cord. The distal end of this catheter was then directed through the visceral surface of the uninflated lobes and carefully withdrawn until its proximal bell shaped end, wedged in respective small airways an average of 10cm and 4cm from the outer pleural surface as seen following dissection of the un-inflated lobes (at a $P_{tp} = 0$ mmH$_2$O) after concluding the experiment. The distal end was attached to a differential pressure transducer located outside the lung box for measurement of peripheral airway pressure. Air leaks on the visceral surface of the lung at the retrograde catheters exit points were prevented by the application of a small drop of cyanoacrylate. Aspiration from the retrograde catheter always yielded free air and in no case yielded blood. During experimentation, the catheter only rarely became obstructed with moisture or mucus, which was easily cleared by gentle flushing with a 5 mL air bolus. It is well accepted and it was assumed that the pressure measured by the retrograde catheter represented simultaneous pressure of all airways having the same dimension as the
retrograde catheter (Macklem and Mead 1967). For the purposes of this project the two catheters measured pressure in a middle and in a small sized airway respectively, denoted by the naming conventions of \( P_{\text{middle}} \) and \( P_{\text{small}} \).

Because catheters were inserted blindly, it was difficult to verify the final airway generation until after lung mechanics were completed and the lungs could be dissected. Since the equine cranial lobe exhibits monopodal branching (described above) care was taken to consistently aim for the same airways among all lungs. Using the cranial lobe binomial numbering system adapted from Smith et al. seen in Figure 2, the large retrograde catheter would usually reside in airway number 1.1.1 (or farther into 1.1.1.5) in the right lobe (and equivalent airway number 2.1.1 (or farther into 2.1.1.5) in the left lobe). The small retrograde catheter would usually reside in airway number 1.1.13 (or farther into 1.1.14/15) in the right lobe (and equivalent airway number 2.1.13 (or farther into 2.1.14/15) in the left lobe). The exact locations and airway sizes were determined and measured using a caliper at the conclusion of the experiment, and histological samples of the airways were also taken for microscopic analysis.

3.1.3. Alveolar capsule technique.

The alveolar capsule technique was used to measure regional alveolar pressures (\( P_a \)). The capsule was fabricated from the end of a 200 \( \mu \text{L} \) pipette tip, with the narrow end removed to match the diameter of the polyethylene (PE) tubing used for attachment to the pressure transducer. One alveolar capsule was glued using superglue to the surface of each lobe as previously described (Sasaki, Takishima et al. 1977; Fredberg, Keefe et al. 1984). The capsule had a conical shape (3cm long, 5mm ID brim in contact with lobe). Having a capsule of greater size and internal volume may have resulted in additional frequency response artifacts also seen when using large diameter tubing. Gas compression (and consequent frequency delay) is more
likely in larger diameter conduits under pressure, particularly when the communicating pressure arises from small apertures. The possibility exists that this was the case between the pleural punctures and the alveolar capsule, however we endeavored to reduce the size of all communicating conduits where possible as to best replicate the characteristics of a cable. Cable properties, like gas moving through small diameter tubes and apertures, allow almost instantaneous communication from a start point to a terminal end. Capsules were placed on a distant portion of the lobe from where the retrograde catheter exited the pleural surface to ensure that airways that potentially led directly to the capsule were not distorted or obstructed by the retrograde catheters. Care was also taken to place the alveolar capsule on a location of the lung that would be fully recruited throughout all box pressures and respiratory rates used during the experimental protocol. The lobe was inflated to no more than 15cm H$_2$O for 30 seconds as to allow placement on inflated parenchyma. An inflated lung was needed to maximize the surface area occupied by the capsule, and to facilitate adequate adhesion using the glue. Four to six 2-mm-deep punctures located inside the lumen of the capsule were made with a 20-gauge needle in the pleural surface (Lauzon, Dechman et al. 1995). Finally, the capsules were attached to a short piece of flexible tubing to form a closed volume of gas in direct communication with alveolar gas and the pressure transducer. Capsules occasionally became occluded, usually by moisture in the PE tubing and rarely by blood/surfactant on the pleural surface where the punctures were made. Unlike the retrograde catheters, it was difficult to flush with a bolus of air due to the small punctures acting somewhat like a one-way valve, however increasing vacuum pressure and ventilation frequency could sometimes dislodge obstructions. Other in-vivo studies with similar problems would simply remove the capsule to another location on the lung (Fuller and Freed 1995), a technique not afforded by our investigations because we aspired to insure our data was
congruent. Simply removing and re-gluing the capsule on a different location would have introduced error not afforded to our study.

Prior to our investigations, it became clear that pressure data obtained from the capsule needed to be validated. First, the pressure within the capsule may not equal the pressure of alveolar gas underneath the capsule. To assess this, variations of pressure in an airtight box were recorded directly by a pressure transducer and simultaneously by the capsule system. Secondly, resistance from pleural punctures was simulated by covering the entry port of the capsule with tape in which four punctures were made. These values were verified with lung data obtained during troubleshooting.

Following placement of both retrograde catheters and alveolar capsule, the lobes were inflated to check for any final leaks and to recruit the maximal number of collapsed airways caused during cold transportation. To do so, lungs were forcibly immersed in saline and inflated through the main airway cannula to a pressure of no more than 25 cmH₂O for 5 minutes, which corresponds to the maximal pressure reached in our protocol. Furthermore, we assumed that the inflation would restore tissue compliance back to physiological levels (Robinson and Sorenson 1978) Since both the retrograde catheter and alveolar capsule technique required technical expertise, at least 20 lungs obtained from the abattoir were sacrificed as practice in order to perfect the techniques.

3.1.4. **Lung fixation and sectioning of lung lobes for histopathology.**

Following the assessment of lung mechanics and upon dissection, the diameters of the catheterized airways were measured using a caliper. It was then confirmed that the retrograde catheters were wedged in bronchi of two different sizes: a larger and a smaller diameter airway. Data from lungs not fulfilling these criteria was not used. Approximately 3cm³ sections of lung
were removed for histopathology before and after lung mechanics were performed. Pre-experiment samples were taken off the main lungs adjacent to the location where cranial lobes were excised, while post-experiment samples were taken directly from the cranial lobes at a location adjacent to retrograde catheter placement. Care was taken to include excised sections of airway to rule out airway smooth muscle pathology that would introduce some error from changes in the mechanics of the lungs. The 26 before and after samples were placed in 50mL Falcon tubes with 10% buffered formalin, and delivered to The University of Calgary, Faculty of Veterinary Medicine’s Department of Pathology. Samples were embedded in paraffin and processed with a microtome, then mounted, stained and examined by Dr. Jan Bystrom, Director of Anatomic Pathology Service. All samples were free of gross and microscopic pathology. A copy of the pathology report can be found in Appendix A.

3.1.5. Lung box construction.

To simulate the chest cavity we built a leak proof Plexiglas box of our own design which we submitted to oscillating vacuum using an external pump (Figure 8). The pump, manufactured by Emmerson™, allowed for changes in negative box pressure and respiratory rate. The 5/16” plexiglass was cut and glued together using 3M acrylic welding solvent to form a 16” x 16” x 24” box, save for the opening of a door along one of the 16” x 24” sides on which a 21” x 30” frame was adhered to. Rubber gasket was subsequently cut to the shape of the frame. The gasket was glued to the frame with non-adhesive centrifuge silicon. 20 x 5/16” holes were drilled and tapped into both the frame and new corresponding gasket, in which 3/8” x 1 ½” galvanized machine screws were carefully inserted and sealed with silicone to prevent air leaks. A removable 3/8” thick door corresponding to the 21” x 30” frame was fitted and drilled with a
larger ½” bit to each of the 20 corresponding holes so that it could be sealed in place by 20 silicon coated washers-wing nuts tightened to finger-tension. An airtight seal with the frame was achieved using Corning Vacuum Grease, and the door was sealed and removed before and after each experiment while re-applying sealant as needed.

5 x 5/8” holes were drilled and tapped, and natural gas fittings (3/4” outside fine thread and inside ½” fine thread) obtained from Fairview Fittings were inserted. ¾”threaded washers were added and silicone to ensure airtight communication between the interior and exterior of the box. A multitude of male hose fittings could therefore be changed in the ½” inside threads as need be, the smallest being 1/16” to accommodate the PE240 tubing communicating with the retrograde catheter / alveolar capsule and the largest ½” communicating with hoses attached to the vacuum pump. One of the five ¾” outside fine thread and inside ½” fine thread fitting was applied to the roof of the box, where the cannulated bronchi were attached and ventilated.

Since a leak proof box with minimum -30 cmH₂O pressure was specified to follow the experimental protocol, the door sealed and all natural gas fittings were plugged (except those in communication with the vacuum pump). The 5/16” plexiglass was found inadequate during -30 mmH₂O pressure due to warping and , as a result 6 x 3/8” thick x 1” wide plexiglass joists were welded inside the box to prevent distension and to support structural integrity. As an additional safety check for leaks, the box was filled to capacity with 26.6 Gallons of water (16” x 16”x 24” = 6155 inches³) containing red food dye additive. Any additional leaks were marked and welded, then filled with silicon. A final check for leaks was performed as described below before each lung experiment by making sure there was no pressure loss in the box for several minutes after it was put under vacuum.
3.1.5.1. Piezoresistive differential pressure transducers.

A pressure sensor is a generic term for an instrument that measures pressure and will activate at a predetermined value, versus a transducer which takes a non-electrical measurement and converts it to an electrical value. Piezoresistive materials, usually silicon, change resistance to the flow of current when they are compressed or strained, where twice as much pressure results in twice as large a change in resistance. When the Wheatstone bridge, a circuit running small amounts of current through the silicon surfaces, becomes unbalanced, less current passes through the pressure sensor and is reported as a change in pressure (Maxim 2002).

For our purposes, we used six piezoresistive differential pressure transducers obtained from SCIREQ Inc. Montreal (Figure 9). Since out protocol required precise measurement of negative pressures ranging from very high to the very low, several types of transducers were required. The following is an inventory of differential pressure transducers which are denoted by their maximal pressure sensing capability: A 75 KPa transducer measured the difference in pressure between the lung box’s interior and the inside of the cannulated main bronchus. 2 x 25 KPa transducers measured the pressure found between the cannulated main bronchus and the retrograde catheters, and between the retrograde catheters and alveolar capsule respectively. An additional 25 KPa transducer measured the pressure between the cannulized main bronchus and the alveolar capsule, a measurement that would later be used to help validate data from the other 25 KPa transducers. The more sensitive 7.5 KPa transducer measured the pressure between the alveolar capsule and the lung box’s interior while the most sensitive 0.2 KPa transducer was attached to the ports of the pneumotachograph used to measure the velocity of air moving in and out of the main bronchus. Airflow is estimated in the pneumotachograph by measuring the pressure drop induced by a grid inducing a small resistance to airflow.
3.1.5.2. Pneumotachograph.

A Fleisch #1 pneumotachograph was attached to the outside of the lung box in direct communication with the main bronchus, and had the capability to measure a maximum airflow of 1.2 L/s (Bird 2006). Calibration was achieved by pushing air using a calibrated 1 L syringe (from SCIREQ Inc.) through the pneumotachograph and recording the airflow with the flexiware software. The software was calculating the air volume that passed through the pneumotachograph (by integrating air flow over time) and it had to be exactly 1L to be considered valid. A calibration flow performed too rapidly or slowly resulted in a prompt for the user re-calibrate. Calibration of the pneumotachograph, in addition to all other pressure transducers was performed prior to each experiment.

3.2 Lung box setup

The lungs were suspended from the gas fitting located on the top of the box by the main bronchus cannula (Figure 9 A and B).
Figure 9A: Cranial lobe suspended in the lungbox by the main bronchus open to atmosphere.
Figure 9B. Experimental setup of the lungbox; Six Differential pressure transducers were used to calculate the following: (1) Flow from pneumotachograph (2)\(R_L\). (3)\(R_{large}\). (4)\(R_{small}\). (5)\(R_{alway}\). (6) \(R_{tissue}\)

The opening of the tube was connected to a pneumotachograph, which measured airflow, and opened to atmosphere. The box had a port connecting to the vacuum pump that could control the maximal peak to peak vacuum pressure (equivalent to trans-pulmonary pressure, \(\Delta P_{tp}\)), the inspiratory and expiratory time, the respiratory rate and relative vacuum pressure exerted inside the box. The pump was essentially a portable “Iron Lung” and was found to be ideal for our purposes (Pfister and Bullas 1985). Prior to each day of experimentation, the box was sealed and subjected to a -25 cmH\(_2\)O leak test for 1 minute ensuring no outside air could enter. Both retrograde catheters and the alveolar capsule, by means of additional PE240 tubing as needed,
were connected to aforementioned male gas fittings located on the sides of the box. The door was sealed and the lungs were ventilated slowly to a maximum of -25 cmH₂O for 1 minute to ensure they were indeed leak free. Upon finding any leaks during this step, a lung would be removed and the leak would be super-glued by adding a small drop onto the suspicious site. While in the box, the lungs were subjected to fully-sinusoidal pressure changes. Temperature and humidity were observed using a sensor where temperature was maintained at 20°C while humidity was maintained at 80% using a tray of warm water located in the box below the suspended lung.

### 3.2.1 Data acquisition:

**Measured outputs:**

The pneumotachograph was used to measure lung inspiratory and expiratory airflow (dV/dt). Furthermore, five differential pressure transducers located outside the box were used to measure trans-pulmonary pressure (∆P_{tp}), central airway (P_c), peripheral airway (P_p), and alveolar pressures (P_a). Each pressure transducer was calibrated using a water column manometer before every experiment.

Tubing used for the retrograde catheters and alveolar capsule located inside the box was connected to the pressure transducers located outside the box through the leak-proof fittings, which was tested for integrity before each experiment. All tubing located outside the box connecting the pressure transducers was stiff (made of polyvinyl carbonate) and of very small diameter (1mm ID and 3mm OD).

Both retrograde catheters were attached to a 3 way valve which allowed for pressure measurement of P_p_large and P_p_small at different times, but still allowed consistent measurement throughout the various experimental conditions (Figure 8). Connected to the switch was a “Y”
connection, which allowed communication to two separate differential pressure transducers (Transducers 3 and 4 in Figure 9B).

One alveolar capsule, measuring alveolar pressure ($P_{a}$), was glued to the parenchyma and a 20 cm length of PC 240 tubing was connected to its distal end. This same tube was connected outside the box to three separate pressure transducers (transducers 4, 5 and 6 in Figure 9B).

*Calculated outputs:*

Connecting different transducers together allowed calculating the partitioned peripheral lung resistances by combining their signal to the airflow measurements.

The whole lung resistance ($R_{L}$) was calculated using pressure drop between the the main bronchus and the box (transducer #2) combined with airflow (transducer #1).

Similarly, the whole lung elastance ($E_{L}$) was calculated using pressure drop between the the main bronchus and the box (transducer #2) combined with calculated inspiratory/ expiratory and tidal volume. Lung inspiratory, expiratory and tidal volumes were obtained by integrating the flow signal from the pneumotachograph over time (transducer #1) (Figure 9).

$R_{large}$ was calculated using pressure drop from the central bronchus to the retrograde catheter via the 3 way switch mentioned above (transducer #3) combined with airflow (transducer #1) (Figure 9B and 10).

$R_{small}$ was calculated using the pressure drop from the retrograde catheter to the alveolar capsule (transducer #4) and airflow (transducer #1) (Figures 9B and 10).

As a control, a redundant resistance measure was added to the design with direct measurement of the airways only ($P_{airway}$) used to calculate airway resistance ($R_{airway}$). $R_{airway}$ was
calculated using the pressure drop from central airway to alveolar capsule (transducer #5) combined with airflow (transducer #1) (Figure 9B and 10).

\[ R_{\text{tissue}} \] was calculated using the pressure drop from the alveolar capsule to the box (transducer #6) divided by airflow (transducer #1) (Figure 9B and 10).

### 3.2.2. Data analysis

One problem with the retrograde catheter data still existed; \( P_{\text{small}} \) and \( P_{\text{large}} \) were not measured simultaneously in 2 separate timeframes due to the Y switch mentioned above. This was due to a limitation in the number of pressure transducers available for the study. To rectify this, airway resistances were partitioned (Figure 10) as follows: Large airway resistance (\( R_{\text{large}} \)), middle sized airway resistance (\( R_{\text{middle}} \)) and small airway resistances (\( R_{\text{small}} \)). \( R_{\text{tissue}} \) and \( R_{L} \) remained the same. By performing breath by breath analysis using this method, a better partitioning of resistances in the lung could be achieved.
Figure 10. Simplified schematic of resistances calculated while ventilating a cranial lung lobe in the lung box.

\( R_{\text{large}} \) became the resistance found between the central main bronchus and the large catheter (Figure 10) while \( R_{\text{small}} \) was now the resistance found between the alveolar capsule and the small catheter. \( R_{\text{middle}} \) was calculated using the average between the following: the difference between resistance data for \( R_p \text{ large} \) and \( R_p \text{ small} \) and the difference between \( R_c \text{ small} \) and \( R_c \text{ large} \). To qualify as a lung to be used in the final data set, these resistance values were compared to the independent redundant \( R_{\text{aw}} \) data as before.
3.2.3. **Calculations performed by Flexiware™ software.**

Prior to computerized data acquisition and analysis, resistance and elastance were estimated based on waveform recordings on oscilloscopes or strip-chart recorders. However, techniques used at the time, such as the Mead and Whittenberger technique (Mead, Whittenberger et al. 1957), relied on a few singular points in the breathing cycle, making the results susceptible to measurement noise and not necessarily representative of the entire breath. Unless using the forced oscillation technique, resistance and elastance are most commonly estimated using mathematical model-fitting techniques such as Multiple Linear Regression that take all data points into account as seen in previous sections. Data was sampled at a rate of 100 Hz using the SCIREQ’s flexiware data acquisition software using the formula below.

\[ P_{ip}(t) = R\dot{V}(t) + EV(t) + P_0 \]
3.2.4. Pressure-Volume curves.

When plotting the pressure needed to inflate a lung vs. the volume present during the same time period, a characteristic curve called a Pressure-Volume curve develops (Figure 11).

![Pressure-Volume curve diagram](image)

Figure 11. Comparison of pressure-volume curves of air-filled and saline filled lungs (cat). Open circles, inflation; closed circles, deflation. Note that the saline-filled lung has a higher compliance than the air-filled lung and exhibits no hysteresis. Adapted from EP Radford: *Tissue Elasticity*. Washington, D.C., American Physiological Society, 1957, p177.

The tracings of the Pressure-Volume curve can be explained by differences in the forces involved during inhaling and exhaling. During inspiration, surface tension creating a collapsing force must first be overcome. Lungs surfactant, dipalmitoyl phosphatidylcholine (DPPC) produced by alveolar type II pneumocytes greatly reduces, though not entirely, the surface tension. As a result, relatively little change in volume occurs despite a significant pressure increase until surface tension is overcome. In other words some initial pressure is required to "pop" open the alveoli and respiratory bronchi. Volume then follows pressure in somewhat of a
linear manner until the lung reaches maximum capacity and air compression begins to occur. During expiration there is little holding the lung open other than the air present at that particular time, so volume decreases linearly as pressure decreases. Figure 11 shows that inflation is easier in a saline-filled lung. This is because surface tension (or reduction of H-bonding between water molecules and surroundings) is greatly reduced, and both filling and emptying curves resemble the expiration curve of the air-filled lung. The difference between the inspiratory and expiratory curve is due to hysteresis in the system. Hysteresis is therefore dissipating energy applied to the system during inspiration that is not recovered in expiration (West 2012), and is quantified as it applies to the area between the ascending and descending portions of the pressure-volume curve.

Pressure-volume loops are useful for determining elastance, compliance and work performed by the system (described in proceeding sections), however they are also useful in critical care medicine when mechanical ventilators are concerned (George, Light et al. 2005). The most common methods to estimate pressure pressure-volume curves in a clinical setting are as follows: 1) The super syringe method where recoil pressures are recorded during static stepwise inflations from a syringe (or ventilator). However they are time consuming while difficult to perform, and requiring paralysis of the patient while exposing them to oxygen desaturation (Roupie, Dambrosio et al. 1995; Lu and Rouby 2000). 2) The inspiratory occlusion technique which involves measuring plateau pressures at different tidal volumes during successive end inspiratory occlusions. The pressure–volume curve is constructed from different plateau pressures particular to peak administered volumes. It has the advantage of the patient not being disconnected from the ventilator, and the loss of oxygen is negligible because only 3 seconds is needed for each manoeuvre (Lu and Rouby 2000). 3) The quasistatic method using a continuous inflation at a constant flow is the simplest technique to obtain the pressure–volume
curve using mechanical ventilation and most closely approximates the methods used in our study. Using a similar linear regression formula as described in previous sections, P-V loops could be dynamically computed using similar methods seen in our study using a pneumotachograph attached to the ventilator. Flow and time could then be used to calculate volume, which would then be plotted against the resistive pressure in the airway (Lu and Rouby 2000). Advantages are that the patient could be kept attached to the ventilator and little loss of lung volume due to lung oxygen uptake occurs, with the entire procedure taking 10s and analysis taking 2 min(Lu and Rouby 2000).

By taking the slope of the inspiratory and expiratory pressure-volume loop, one can then obtain lung compliance as well as its reciprocal elastance (E=1/C).

3.2.5. Elastance and Compliance.

Elastance (or stiffness) is the ability of the lung or the tissue to resist deformation and to return to original shape following deformation, whereas compliance is the volume change per unit pressure change. In other words, compliance is a measure of how easy it is to stretch, or distend the lungs (West 2012). In equation form:

\[ \uparrow \text{elastic tissue} \rightarrow \uparrow \text{stiffness} \rightarrow \downarrow \text{compliance}. \]

As a convention, we chose to describe elastance over compliance as it was easier to conceptualize the effect of tissue viscoelasticity, summarized as a box and spring cartoon seen in Figure 12. In addition, compliance of the lung depends on its size, where a human (or horse) lung would be more compliant than say that of a mouse. For this reason specific compliance, or per unit volume of lung, is often measured when drawing conclusions about elastic properties. Viscoelasticity then is the ability of the lung`s parenchyma to resist deformation and return back to its original shape, which includes a dampening effect of the cylinder seen in the below figure.
In short, and similar to a ball of clay, the lung can be deformed more easily when done more slowly, whereas a rapid maneuver using the same force results in little deformation as predicted with the addition of a dampening piston to the model.

Figure 12. Box and spring model of viscoelasticity showing the effect of elasticity coupled with the dampening effect of the piston.

Elastin and collagen fibers, and the proteoglycans dermatan and heparin sulfate to some degree (McGowan, Liu et al. 1993), are responsible for the lung’s elastic behavior and tendency to return back to its resting volume following distension (West 2012). Furthermore, others have measured the effect in normal rats and have found rises in elastance with $P_{tp}$, and speculate remodeling (or breakdown) of collagen and elastin to be partly responsible for pathologies such as emphysema (Brewer, Sakai et al. 2003).
Additionally, the effect of elastic tissue may have an effect on airway resistance through a process known as airway tethering, simply because airways are tethered to the parenchyma in which they reside. Because lung parenchyma is viscoelastic, the rate at which it is distorted during breathing would have an influence on its apparent stiffness (Bates and Lauzon 2007), and possibly on airway diameter, which could in turn influence ventilation heterogeneities during challenge. Shen *et al.* found in rabbits that when Ptp varied between 0 and 20 cmH2O, airway diameter changed by between 15 and 45% of its value at 20 cmH2O depending on airway generation number (Shen, Ramchandani et al. 2000). Because we studied lung mechanics in horses *ex-vivo*, chest wall compliance components has not been factored in our study. At the level of the alveoli, support offered to lung units surrounding them is termed ”interdependence”, and may play an important role in the prevention of atelectasis. So important is this that some believe interdependence might be as important as pulmonary surfactant in maintaining small airspaces (West 2012).
3.3. **Protocol for experimental conditions and outputs.**

We based our experimental conditions on two factors:

1) Adherence to respiratory parameters of physiological significance.

2) The maximum, mean and minimum rates capable by the Emerson pump controlling the box.

We therefore chose to test the lungs at respiratory rates (RR) of 10, 20 and 25 bpm, and box maximal negative pressures (ΔPtp) of -30, -20 and -15 cmH2O. Combining changes in each of these parameters thus provided 9 experimental conditions to perform the measurements (RR 10bpm/ ΔPtp -30cmH2O or ΔPtp -20cmH2O or ΔPtp -15cmH2O; RR 20bpm/ ΔPtp -30cmH2O or ΔPtp-20cmH2O or ΔPtp-15cmH2O; RR 25bpm/ ΔPtp -30cmH2O or ΔPtp -20cmH2O or ΔPtp -15cmH2O).

Since lungs are viscoelastic (see above) and were transported in cold saline after slaughter, we judged that re-recruitment could best be achieved first at high volume. To this end, we first performed a 5 minute leak test to confirm that both the lung and box were viable, and then began the experiment by ventilating at a RR of 10 bpm and pressure of -30 cmH2O. The pressure was then decreased to -20 cmH2O following 15 breaths or 5 minutes of recording, with at least 2 minutes allowed in between for the lung to “adapt” to the new condition. Pressure was finally dropped to -15 cmH2O while using the same respiratory rate. The ventilation rate was then changed to 20 bpm while returning the pressure back to -30 cmH2O, followed by -20 and -15 cmH2O until data for a total of nine conditions was collected, ending at a final condition of 25 bpm and -15 cmH2O. Maintaining the quality of each condition was done by following actual readouts on the computer presented in Flexiware. Furthermore, the pump produced fully sinusoidal tracings where expiratory time remained congruent with inspiratory time.
Pressures from the differential pressure transducers and airflow from the pneumotachograph (Figure 9) were integrated in real-time by the SCIREQ Flexiware software which presented the following recorded outputs live and in graphical format: Time, airflow, pressure from transducers #2 – #6, lung and partitioned resistances, elastances and compliances. Minute ventilations and tidal volumes were also calculated, as was a coefficient of determination (COD) for each pressure transducer indicating the goodness of fit to the model used for a particular airway resistance and elastance calculation (see formula above).

3.4. Statistical analysis.

The generalized linear mixed model (GLMM) is a statistical test that is both powerful but challenging. Many basic statistics use the assumption of normally distributed data, which is simply not the case in our study. In addition, the variation among units, or within lungs is such in our data that basic statistics, such as Student’s t-test and the ANOVA (Analysis Of Variance) were not recommended. Researchers faced with non-normally distributed data often attempt transforming data to achieve normality or homogeneity of variance using non parametric tests, or even still rely solely on the robustness of classical ANOVA (Bolker, Brooks et al. 2009). However, in doing so, random effects may be inadvertently ignored and /or violate statistical assumptions such as homogeneity of variance across a particular group (Bolker, Brooks et al. 2009).

The GLMM is considered a conditional parametric test. This is because it uses parametric regression (differently than an ANOVA) in the form of logistic and Poisson regression analysis instead of repeated measures variance. The GLMM accounts for variance by adding random
effects in the form of conditional means into the regression model resulting in a model with both fixed and random effects (Barchia, Herron et al. 2003).

Instead of forcing data into classical tests mentioned above, researchers should use appropriate statistical approaches matching their data. GLMMs combine the properties of two statistical frameworks; such as the incorporation of random effects using linear models and the handling of non-normally disturbed data using generalized linear models (Bolker, Brooks et al. 2009). GLMMs are the best tool for analyzing non-normal data that involve random effects, yet it is a surprisingly difficult tool to use among researchers and even statisticians mainly because of debate surrounding null hypothesis testing (Johnson and Omland 2004) and the use of Bayesian statistics (discussed shortly). Furthermore, the GLMM is known to have been used inappropriately 58% of the time when performing a search in Google Scholar (Bolker, Brooks et al. 2009). A thorough understanding of the statistical test pivotal to our results is required to validate our results.

To illustrate the difficulty face when using GLMMs, various software packages estimate parameters using several techniques: 1) Penalized quasi-likelihood has the advantage of being flexible and widely implemented, but it is also biased by small means and large variance. 2) Laplace approximation has more power than the Penalized quasi-likelihood but tends to lack flexibility when analyzing multiple treatment variables. 3) Gauss-Hermite quadrature is advantageous because it is more accurate and has more statistical power than Laplace, but it is limited to 2-3 random effects. 4) Markov chain Monte Carlo is highly flexible, accurate and allows for an arbitrary number of random effects, still it relies on Bayesian framework (or best fit model) (Bolker, Brooks et al. 2009). The latter of these is employed by the software package STATA, the software package we used for our data analysis.
Markov chain Monte Carlo algorithms sample both fixed and random effect parameters in a way that assumes normal distribution around the mean (Bolker, Brooks et al. 2009). It also allows for the inclusion of missing values. Because we found high variation, or heterogeneity, in the data between lungs, and to improve statistical accuracy from only 13 lungs, the GLMM allowed for the consideration of significant effects. Furthermore, it allowed us to dichotomize the effect of pressure or respiratory rate on various calculated resistance or elastance despite our small sample size. The GLMM included “a goodness of fit” in the form of AIC (Akaike information criterion) and BIC (Bayesian information criterion). AIC is said to describe the tradeoff between bias and variance in model construction, or loosely speaking between accuracy and complexity of the model (Burnham 2004), whereas BIC is based, in part, on the likelihood of function and it is closely related to AIC (Burnham 2004). For the purposes of our study, it was determined a lower AIC and BIC was advantageous and more statistically relevant where significance was found. In short, independent variables (pressure and respiratory rate) each improved the GLMM on their own, but the greatest improvement was seen when both independent variables were included. Additionally, using the above described Bayesian approach allowed confidence intervals to be computed, which was useful as we were better able to find significance during post-hock testing.

The GLMM was used in preference to the ANOVA (analysis of variance). Simply put, ANOVA predicts whether or not the means of several groups are equal. However complicating factors such as small sample sizes, high degrees of variation between samples and missing values often result in an insignificant estimate of variance. A similar and analogous situation would be using a t-test where an ANOVA was preferred, where performing multiple t-tests would result in an increased chance of observing a significant difference due to a Type I error. Type I error is the
incorrect rejection of the null (or expected) hypothesis ($H_0$) in favour of the alternative hypothesis, akin to “crying wolf” when there is in fact no wolf.

The repeated measure ANOVA was considered as an appropriate test for our purposes since it compares the means of the same subjects under several different experimental factors (Field 2011). An essential criterion for this test is that there be at least one element of comparison between “within-subject” treatments, or an effect estimated by observing before and after treatment in the same individual. This is different to the “between subject” effect where effects of say a particular drug differ between subject test group A and subject test group B (Field 2011). In addition, the repeated measures ANOVA is appropriate when all subjects get the same treatments, receive all levels of the independent variable and where treatments are carried out in serial (one after the other). This test also requires fewer subjects than tests examining independent groups to maintain power, since these tests need an equal minimum number of different participants for each situation (Howitt 2011). The disadvantage of the repeated measures ANOVA as it pertains to our study is that it assumes normal distribution (Field 2011); an observation clearly not seen in a sample size of 13 lungs with missing values. Using this test would have resulted in a lack of statistical power to detect significant differences between the effects of pressure or respiratory rate on the dependent variable of interest, particularly following post-hock analysis. In spite of this, there are additional methods to test for abnormal distributions.

Non-parametric tests, such as the Kruskal-Wallis or the Friedman test (an alternative to the repeated measures ANOVA) are useful statistical analyses for non-normally distributed data. Despite this, non-parametric tests lack power (or statistical robustness) when missing data and/or randomization is not present (Field 2011). This would lead to a lack of sensitivity especially as it
pertains to finding differences between 2 or more independent variables such as the effect of pressure and respiratory rate on the variable of interest.

Because of the problems mentioned above, mixed modeling was the most appropriate and sensitive method for our purpose due to the following: 1) Missing data is appropriately included in the test rather the individual being completely dropped as is the case with multivariate approach (Barchia, Herron et al. 2003). 2) Post hoc testing allows identifying statistical differences between two (or more) variables as is the case with our study seen between pressure and respiratory rate.

Correlation between airway size and airway resistance were tested using both a spearman rank test, and Pearson correlation.

4. RESULTS.

4.1. Lung data exclusion:

Twenty lungs were dissected and ventilated to troubleshoot our experimental technique. Fifty-six lungs in total were used in our experiments, however only 13 lungs did fit our inclusion criteria. This was due primarily to the effects of mucous plugs in the alveolar capsule and due to leaks developing on the surface or at the interface of the lung and the retrograde catheter. The Flexiware™ software calculated a coefficients of determination (COD) with the regression model described above, and any value demonstrating less than an 80% COD was subsequently removed from the data set. Data from lungs exhibiting alveolar capsule obstructions was not used, and this procedure proved to be most the most challenging element of the study. Pressure data from the
capsule often lacked consistency despite all efforts, and for this reason upwards of 40 lungs were dismissed from the study.

4.2. **Validation of data collection software and inputs.**

![Graph](image)

Figure 13. ΔP<sub>tp</sub> and volume vs. time. Validating the absence of time lag between recorded pressure and Flexiware calculated volume for Lung #1.

Recorded ΔP<sub>tp</sub> and volume calculated by Flexiware at 100Hz for 3 breaths were plotted against time to verify reliability when reporting and then comparing values to other literature. As illustrated in Figure 13, both curves are synchronous for Lung # 1 at a Ptp of -30 and RR of 10 in addition to there being no time lag between both outputs.
Figure 14. Comparison of calculated $E_L$ and $E_{tissue}$ vs. time. Higher time resolution scale shows similar results.

As shown in Figure 14, $E_L$ and $E_{tissue}$ were calculated by Flexiware at 100Hz and 3 breaths were plotted for Lung # 1 at a Ptp of -30 and RR of 10 to demonstrate there was no time lag between the two outputs.
4.3. Validation of data analysis: Pressure-Volume loops.

Figure 15. Pressure volume loops from lung #1 seen for all conditions. Lung elastance in hatched blue and tissue elastance in solid red are shown with their respective slope equations.

To validate data obtained from SCIREQ’s acquisition software, pressure-volume loops were manually calculated and elastances were computed. With generous engineering help from Chris Boumeester and Dr. Tyberg, areas under a time vs. airflow plot were manually calculated by taking the integral to obtain volume for 1 breath. Pressures from two individual differential transducers (#2: main bronchus-box, #6: Alveolar capsule-box) were plotted against calculated volume to obtain total lung elastance (E_L) and tissue elastance (E_t) respectively by taking the inverse slope of the curves. An example taken from lung #1 (Figure 15) illustrates not only an
increase in slope (or compliance) with a decrease in pressure, but also the opposite for when respiratory rate was increased. Slopes in figure 15 were calculated in Microsoft excel™ and they used data calculated at 100Hz. Most data points in the above pressure-volume loops are located at the top of the loop during the apex of inspiration, and the slopes of the lines were drawn accordingly with a bias towards that fact.

Using lung #1 as an example, manually calculated $E_L$ and $E_t$ were compared to $E_L$ and $E_t$ values generated by SCIREQ flexiware (Table 1) using methods described above, and no significant differences ($p=0.23$) were found using a two sample T-test.

### Table 1. Comparison of calculated total lung and lung tissue elastance to computer generated values during 1 breath for Lung #1 sampled at 100 Hz. at various respiratory rates (RR) and negative pressures ($P_{tp}$) seen in both the large and small catheter.

<table>
<thead>
<tr>
<th></th>
<th>1 / slope of Lung P-V Loop</th>
<th>Lung elastance [(cm H2O/sec.) / (L/sec)] from Flexiware</th>
<th>1 / slope of Tissue P-V Loop</th>
<th>Tissue elastance [(cm H2O/sec.) / (L/sec)] from Flexiware</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large cath RR 10 $P_{tp}$ - 30</td>
<td>10.5</td>
<td>9.8</td>
<td>9.9</td>
<td>9.5</td>
</tr>
<tr>
<td>Small cath RR 10 $P_{tp}$ - 30</td>
<td>10.7</td>
<td>9.9</td>
<td>10.1</td>
<td>9.5</td>
</tr>
<tr>
<td>Large cath RR 10 $P_{tp}$ - 20</td>
<td>8.9</td>
<td>8.2</td>
<td>8.3</td>
<td>7.9</td>
</tr>
<tr>
<td>Small cath RR 10 $P_{tp}$ - 20</td>
<td>9.2</td>
<td>8.5</td>
<td>8.7</td>
<td>8.2</td>
</tr>
<tr>
<td>Large cath RR 10 $P_{tp}$ - 15</td>
<td>7.2</td>
<td>6.6</td>
<td>6.7</td>
<td>6.4</td>
</tr>
<tr>
<td>Small cath RR 10 $P_{tp}$ - 15</td>
<td>7.0</td>
<td>6.5</td>
<td>6.5</td>
<td>6.2</td>
</tr>
<tr>
<td>Large cath RR 20 $P_{tp}$ - 30</td>
<td>13.0</td>
<td>11.4</td>
<td>12.3</td>
<td>11.0</td>
</tr>
<tr>
<td>Small cath RR 20 $P_{tp}$ - 30</td>
<td>14.0</td>
<td>12.2</td>
<td>13.3</td>
<td>11.8</td>
</tr>
<tr>
<td>Large cath RR 20 $P_{tp}$ - 20</td>
<td>9.9</td>
<td>8.8</td>
<td>9.3</td>
<td>8.5</td>
</tr>
<tr>
<td>Small cath RR 20 $P_{tp}$ - 20</td>
<td>9.9</td>
<td>8.8</td>
<td>9.3</td>
<td>8.5</td>
</tr>
<tr>
<td>Large cath RR 20 $P_{tp}$ - 15</td>
<td>6.7</td>
<td>5.9</td>
<td>6.2</td>
<td>5.7</td>
</tr>
<tr>
<td>Small cath RR 20 $P_{tp}$ - 15</td>
<td>6.9</td>
<td>6.1</td>
<td>6.4</td>
<td>5.9</td>
</tr>
<tr>
<td>Large cath RR 25 $P_{tp}$ - 30</td>
<td>15.5</td>
<td>13.3</td>
<td>14.7</td>
<td>12.8</td>
</tr>
<tr>
<td>Small cath RR 25 $P_{tp}$ - 30</td>
<td>15.2</td>
<td>13.2</td>
<td>14.4</td>
<td>12.7</td>
</tr>
<tr>
<td>Large cath RR 25 $P_{tp}$ - 20</td>
<td>9.6</td>
<td>8.5</td>
<td>9.0</td>
<td>8.2</td>
</tr>
<tr>
<td>Small cath RR 25 $P_{tp}$ - 20</td>
<td>10.2</td>
<td>8.9</td>
<td>9.5</td>
<td>8.6</td>
</tr>
<tr>
<td>Large cath RR 25 $P_{tp}$ - 15</td>
<td>7.0</td>
<td>6.2</td>
<td>6.5</td>
<td>6.0</td>
</tr>
<tr>
<td>Small cath RR 25 $P_{tp}$ - 15</td>
<td>7.0</td>
<td>6.2</td>
<td>6.5</td>
<td>5.9</td>
</tr>
</tbody>
</table>
Table 2. Comparison of calculated total lung and lung tissue elastance to computer generated values during 1 breath for all lungs sampled at 100 Hz.

<table>
<thead>
<tr>
<th>RR 10 Ptp 30</th>
<th>1 / slope of LUNG P-V Loop</th>
<th>Lung Elastance [(cm H2O/sec.) / (L/sec)] from Flexiware</th>
<th>SE</th>
<th>1 / slope of TISSUE P-V Loop</th>
<th>Tissue elastance [(cm H2O/sec.) / (L/sec)] from Flexiware</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>RR 10 Ptp 20</td>
<td>11.3</td>
<td>10.4</td>
<td>±0.6</td>
<td>11.5</td>
<td>10.8</td>
<td>±0.6</td>
</tr>
<tr>
<td>RR 10 Ptp 15</td>
<td>8.7</td>
<td>8.2</td>
<td>±0.4</td>
<td>8.7</td>
<td>8.3</td>
<td>±0.6</td>
</tr>
<tr>
<td>RR 20 Ptp 30</td>
<td>7.3</td>
<td>6.8</td>
<td>±0.5</td>
<td>7.2</td>
<td>6.9</td>
<td>±0.6</td>
</tr>
<tr>
<td>RR 20 Ptp 20</td>
<td>12.6</td>
<td>11.4</td>
<td>±0.2</td>
<td>12.5</td>
<td>11.5</td>
<td>±0.3</td>
</tr>
<tr>
<td>RR 20 Ptp 15</td>
<td>9.2</td>
<td>8.5</td>
<td>±0.5</td>
<td>9.0</td>
<td>8.6</td>
<td>±0.6</td>
</tr>
<tr>
<td>RR 25 Ptp 30</td>
<td>7.4</td>
<td>6.9</td>
<td>±0.6</td>
<td>7.2</td>
<td>6.9</td>
<td>±0.6</td>
</tr>
<tr>
<td>RR 25 Ptp 20</td>
<td>13.5</td>
<td>12.1</td>
<td>±0.6</td>
<td>13.3</td>
<td>12.2</td>
<td>±0.6</td>
</tr>
<tr>
<td>RR 25 Ptp 15</td>
<td>9.0</td>
<td>8.2</td>
<td>±0.5</td>
<td>8.8</td>
<td>8.2</td>
<td>±0.5</td>
</tr>
<tr>
<td>RR 25 Ptp 15</td>
<td>7.4</td>
<td>6.8</td>
<td>±0.5</td>
<td>7.2</td>
<td>6.8</td>
<td>±0.6</td>
</tr>
</tbody>
</table>

Using data calculated from all lungs, manually calculated $E_L$ and $E_t$ using the same method above were compared to $E_L$ and $E_t$ values generated by SCIREQ flexiware (table 2), and no significant differences (p= 0.45) and (p= 0.59) respectively were found using a Two-Sample T-test.
Table 3A. Calculated areas inside the pressure-volume loops for LUNG and TISSUE seen in Lung #1.

<table>
<thead>
<tr>
<th>RR</th>
<th>Ptp</th>
<th>Area under the P-V loop (in L * cmH20) LUNG</th>
<th>Area under the curve (in L * cmH20) TISSUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>30</td>
<td>11.2</td>
<td>9.3</td>
</tr>
<tr>
<td>10</td>
<td>20</td>
<td>9.1</td>
<td>7.2</td>
</tr>
<tr>
<td>10</td>
<td>15</td>
<td>7.5</td>
<td>5.7</td>
</tr>
<tr>
<td>20</td>
<td>30</td>
<td>11.7</td>
<td>9.8</td>
</tr>
<tr>
<td>20</td>
<td>20</td>
<td>7.1</td>
<td>5.6</td>
</tr>
<tr>
<td>20</td>
<td>15</td>
<td>5.8</td>
<td>4.1</td>
</tr>
<tr>
<td>25</td>
<td>30</td>
<td>9.2</td>
<td>8.0</td>
</tr>
<tr>
<td>25</td>
<td>20</td>
<td>7.1</td>
<td>5.6</td>
</tr>
<tr>
<td>25</td>
<td>15</td>
<td>6.1</td>
<td>4.9</td>
</tr>
</tbody>
</table>

Table 3B. Calculated area inside the pressure-volume loop for LUNG and TISSUE seen in 7 lungs (Average and standard error are indicated).

<table>
<thead>
<tr>
<th>RR</th>
<th>Ptp</th>
<th>Area under the curve (in L* cmH20) LUNG</th>
<th>SE</th>
<th>Area under the curve (in L* cmH20) TISSUE</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>Ptp-30</td>
<td>16.4</td>
<td>±3.9</td>
<td>12.9</td>
<td>±3.4</td>
</tr>
<tr>
<td>10</td>
<td>Ptp-20</td>
<td>9.1</td>
<td>±0.7</td>
<td>6.3</td>
<td>±0.9</td>
</tr>
<tr>
<td>10</td>
<td>Ptp-15</td>
<td>9.0</td>
<td>±1.7</td>
<td>6.1</td>
<td>±1.4</td>
</tr>
<tr>
<td>20</td>
<td>Ptp-30</td>
<td>9.8</td>
<td>±1.4</td>
<td>6.7</td>
<td>±1.3</td>
</tr>
<tr>
<td>20</td>
<td>Ptp-20</td>
<td>6.4</td>
<td>±0.7</td>
<td>3.9</td>
<td>±0.6</td>
</tr>
<tr>
<td>20</td>
<td>Ptp-15</td>
<td>5.4</td>
<td>±0.6</td>
<td>3.0</td>
<td>±0.4</td>
</tr>
<tr>
<td>25</td>
<td>Ptp-30</td>
<td>8.4</td>
<td>±1.1</td>
<td>5.8</td>
<td>±1.0</td>
</tr>
<tr>
<td>25</td>
<td>Ptp-20</td>
<td>6.0</td>
<td>±0.6</td>
<td>3.4</td>
<td>±0.6</td>
</tr>
<tr>
<td>25</td>
<td>Ptp-15</td>
<td>4.8</td>
<td>±0.4</td>
<td>2.7</td>
<td>±0.5</td>
</tr>
</tbody>
</table>

Areas inside the pressure-volume loops were calculated by taking the integral of the ascending inspiratory arm and then subtracting it from the integral of the descending expiratory arm. The units of L*cmH2O represent the work done, or energy lost to the system, during inspiration. Lung and tissue hysteresis then is illustrated in the above tables. At high lung volumes, maximum lung and tissue hysteresis was observed. Of note, one lung in particular
(Lung # 3) contributed an unusually high area inside the curve, particularly at a RR of 10 and $P_{tp}$ of -30 (data not shown), and was the greatest source of variation seen during data analysis.

4.4. Sizes of airway measured by the retrograde catheter.

Table 4. Sizes of catheterized airways determined by caliper measurement following lung mechanics, with the middle airway size calculated from the average of the large and small airways (see methods and discussion).

<table>
<thead>
<tr>
<th>Lungs</th>
<th>L. Airway (in cm):</th>
<th>Middle (in cm) = avg(L,S)</th>
<th>S. Airway (in cm):</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung #1</td>
<td>0.53</td>
<td>0.34</td>
<td>0.14</td>
</tr>
<tr>
<td>Lung #2</td>
<td>0.72</td>
<td>0.55</td>
<td>0.37</td>
</tr>
<tr>
<td>Lung #3</td>
<td>0.56</td>
<td>0.44</td>
<td>0.32</td>
</tr>
<tr>
<td>Lung #4</td>
<td>0.56</td>
<td>0.44</td>
<td>0.32</td>
</tr>
<tr>
<td>Lung #5</td>
<td>0.44</td>
<td>0.35</td>
<td>0.25</td>
</tr>
<tr>
<td>Lung #6</td>
<td>0.52</td>
<td>0.4</td>
<td>0.28</td>
</tr>
<tr>
<td>Lung #7</td>
<td>0.49</td>
<td>0.38</td>
<td>0.26</td>
</tr>
<tr>
<td>Lung #8</td>
<td>0.61</td>
<td>0.44</td>
<td>0.27</td>
</tr>
<tr>
<td>Lung #9</td>
<td>0.53</td>
<td>0.33</td>
<td>0.12</td>
</tr>
<tr>
<td>Lung #10</td>
<td>0.69</td>
<td>0.46</td>
<td>0.22</td>
</tr>
<tr>
<td>Lung #11</td>
<td>0.57</td>
<td>0.42</td>
<td>0.26</td>
</tr>
<tr>
<td>Lung #12</td>
<td>0.49</td>
<td>0.26</td>
<td>0.23</td>
</tr>
<tr>
<td>Lung #13</td>
<td>0.51</td>
<td>0.19</td>
<td>0.32</td>
</tr>
<tr>
<td>Mean</td>
<td>0.56</td>
<td>0.39</td>
<td>0.26</td>
</tr>
<tr>
<td>SD</td>
<td>±0.079</td>
<td>±0.092</td>
<td>±0.071</td>
</tr>
</tbody>
</table>

No significant correlation between airway size and airway resistance were found using both a spearman rank test, and Pearson correlation. It should be noted that middle airway size was obtained from averaging the large and small airway sizes rather than by direct measurement.
4.5. **Results of histological report.**

The histology report performed by Dr. Jan Bystrom at the University of Calgary’s Faculty of Veterinary is presented in appendix A.

Histological observations revealed that the lung sections were judged to be from healthy lungs and that the ventilation experiment did not induce significant damage. The presence of abnormal edema following post experiment was also verified making it easy to identify the pre- from the post-ventilation samples by the pathologist.

There were very subtle differences in individual slides as follows:

1) There were a few lung sections showing peribronchiolar inflammatory cell aggregates. At the number and size of these, they are considered within normal range in adult horses and not likely to be of clinical relevance. There were very small localized areas of chronic inflammation in 5 samples that were likely responses to inhaled pathogens or irritants at some time in the horse's past life.

2) Three lung sections showed peribronchiolar fibrous tissue slightly more prominently compared to others. Again, this is not likely to be of clinical relevance when it is this mild and in the absence of other findings.

3) All sections showed a mild degree of autolysis with sloughing of epithelial cells seen and with it being difficult to resolve cilial detail.
4.6. Tidal volume and minute ventilation.

Table 5. Tidal volume ($V_t$) in litres and minute ventilation ($V_E$) in litres / minute.

<table>
<thead>
<tr>
<th>RR 10</th>
<th>RR 10</th>
<th>RR 10</th>
<th>RR 20</th>
<th>RR 20</th>
<th>RR 25</th>
<th>RR 25</th>
<th>RR 25</th>
</tr>
</thead>
<tbody>
<tr>
<td>$P_{tp}$ -30</td>
<td>$P_{tp}$ -20</td>
<td>$P_{tp}$ -15</td>
<td>$P_{tp}$ -30</td>
<td>$P_{tp}$ -20</td>
<td>$P_{tp}$ -15</td>
<td>$P_{tp}$ -30</td>
<td>$P_{tp}$ -20</td>
</tr>
<tr>
<td>$V_t$</td>
<td>2.8 †</td>
<td>2.5</td>
<td>2.1</td>
<td>2.4 †*</td>
<td>2.1 *</td>
<td>2.0 *</td>
<td>2.3 †x</td>
</tr>
<tr>
<td></td>
<td>(2.4 -</td>
<td>(2.1 -</td>
<td>(1.8 -</td>
<td>(1.9 -</td>
<td>(1.7 -</td>
<td>(1.5 -</td>
<td>(1.7 -</td>
</tr>
<tr>
<td></td>
<td>3.2)</td>
<td>2.8)</td>
<td>2.6)</td>
<td>2.7)</td>
<td>2.5)</td>
<td>2.3)</td>
<td>2.5)</td>
</tr>
<tr>
<td>$V_E$</td>
<td>28.7 †</td>
<td>24.3</td>
<td>19.5</td>
<td>50.7 †</td>
<td>43.7</td>
<td>40.0</td>
<td>55.2 †x</td>
</tr>
<tr>
<td></td>
<td>(24.7 -</td>
<td>(21.1 -</td>
<td>(17.4 -</td>
<td>(39.0 -</td>
<td>(33.6 -</td>
<td>(30.0 -</td>
<td>(41.8 -</td>
</tr>
<tr>
<td></td>
<td>31.6)</td>
<td>25.5)</td>
<td>23.6)</td>
<td>57.5)</td>
<td>49.1)</td>
<td>45.3)</td>
<td>60.5)</td>
</tr>
</tbody>
</table>

† ≠ $\Delta P_{tp}$ of -20 and -15 cmH$_2$O  
x ≠ RR of 20 and 10 bpm  
* ≠ RR of 10 bpm

Tidal volume and minute ventilation.

At a respiratory rate (RR) of 10 bpm and $\Delta P_{tp}$ of -30 cm H$_2$O, $V_t$ was at its maximum value of 2.0L (± SD 0.79L). Moreover, a minimum average $V_t$. (± SD 0.38L) was identified at 25bpm and $\Delta P_{tp}$ of -15 cm H$_2$O. Tidal volume ($V_t$) tended to decrease as $\Delta P_{tp}$ was decreased and respiratory rate was increased.

Using the generalized linear mixed model, $V_t$ was significantly greater at $\Delta P_{tp}$ of -30 compared to -20 and to -15 cmH$_2$O (p< 0.00 for both) (Table 1). $V_t$ was significantly different between RR of 25 or 20 to 10bpm (p< 0.00 and 0.00 respectively).

A maximum average minute ventilation ($V_E$) value of 55.2 L/min (± SD 17.8 L/min.) at a RR of 25 bpm and $P_{tp}$ of -30 cm H$_2$O was observed, while a minimum of 21.1 L/min. (± SD 3.7 L/min.) was identified at 10 bpm and $\Delta P_{tp}$ of -15 cm H$_2$O. $V_E$ tended to decrease as pressure in the box decreased, conversely $V_E$ tended to increased with respiratory rate. $V_E$ was significantly greater at $\Delta P_{tp}$ of -30 compared to -20 or to -15 cmH$_2$O (p< 0.00 for both) (Table 5). $V_E$ was significantly different between all RR’s (p< 0.00 for all).
4.7. Effect of pressure and respiratory rate on total lung resistance.

A. Effect of Pressure on Lung Resistance

B. Effect of Respiratory Rate on Lung Resistance

Figure 16 A & B. Variation in total lung resistance ($R_L$) with respiratory rate (RR) and transpulmonary pressure ($\Delta P_{tp}$). † indicates significantly different from $ptp$ -15 and $ptp$ -20, x indicates significantly different from $rr$ 10 and $rr$ 20.
To investigate the effect of $\Delta P_{tp}$ and RR and on $R_L$, we ventilated the lungs at increasing $\Delta P_{tp}$ s and RR’s. At an RR of 10 bpm and $\Delta P_{tp}$ of -30 cm H$_2$O, the average lung resistance ($R_L$) was at its maximum value of 0.85 cm H$_2$O/L/sec (± SD 0.32 cm H$_2$O/L/sec.). Moreover, a minimum average $R_L$ value of 0.54 cm H$_2$O/L/sec. (± SD 0.22 cm H$_2$O/L/sec.) was observed at 25bpm and $\Delta P_{tp}$ of -15 cm H$_2$O.

$R_L$ tended to decrease as $\Delta P_{tp}$ was decreased for all 3 respiratory rates (Figure. 16A). Conversely, $R_L$ tended to increase when RR was decreased (fig 1b) for all 3 $\Delta P_{tp}$.

Using the generalized linear mixed model, $R_L$ values were significantly greater at $\Delta P_{tp}$ of -30 compared to -20 or -15 cmH$_2$O (p< 0.00 for both) (Figure16B). Similarly, $R_L$ values were significantly different between RR of 25 and 20 or 10bpm (p< 0.00 and 0.00 respectively) (Figure. 16B).
4.8. Comparing measured with calculated values for airway resistances.

![Box plot of airway resistance values](image)

**Figure 17.** Values obtained for objective measurements between $R_{\text{airway}}$ and the sum of $R_{\text{large}}$, $R_{\text{middle}}$, $R_{\text{small}}$. Hatched bars represent $R_{\text{airway}}$; Dark bars represent the sum of $R_{\text{large}}$, $R_{\text{middle}}$, $R_{\text{small}}$.

$R_{\text{airway}}$ was measured independently and compared to the sum of $R_{\text{large}}$, $R_{\text{middle}}$, $R_{\text{small}}$ to validate the measures of $R_{\text{large}}$ and $R_{\text{small}}$ as well as the calculated values for $R_{\text{middle}}$. $R_{\text{airway}}$ is denoted in hatched bars while the sum of $R_{\text{large}}$, $R_{\text{middle}}$, $R_{\text{small}}$ is represented by dark bars. There were no significant differences between $R_{\text{airway}}$ and the sum of $R_{\text{large}}$, $R_{\text{middle}}$, $R_{\text{small}}$ in any experimental condition (Figure 17). Moreover, neither pressure of respiratory rate had significant effects on the change in $R_{\text{airway}}$. 
4.9. Lung and tissue elastance

Figure 18. Values obtained for objective measurements between total lung elastance $E_L$ and average tissue elastance $E_t$.

We wanted to assess the contribution of tissue stiffness to total lung elastance $E_L$. There was no significant difference between $E_L$ and $E_t$ for all lungs over all conditions. Total lung elastance $E_L$ is seen in hatched bars while average tissue elastance $E_t$ is seen in dark bars. The greatest values of $E_L$ and $E_t$ were $11.3 \text{ cm H}_2\text{O/sec./L/sec.}$ (± SD $3.0 \text{ cm H}_2\text{O/sec./L/sec.}$) and $10.7 \text{ cm H}_2\text{O/sec./L/sec.}$ (± SD $2.8 \text{ cm H}_2\text{O/sec./L/sec.}$) respectively at a $\Delta P_{tp}$ of $-30 \text{ cm H}_2\text{O}$ and a RR of $25 \text{ bpm}$ in both cases (Figure 18).
4.10. Effect of Pressure and respiratory rate on $R_{\text{large}}$, $R_{\text{small}}$ and $R_t$.

Figure 19 A & B. Partitioning of pulmonary resistance. The variation of $R_{\text{large}}$, $R_{\text{middle}}$, $R_{\text{small}}$ and $R_{\text{tissue}}$ with respiratory rate (RR) and transpulmonary pressure ($\Delta P_{tp}$).
For all 3 respiratory rates (Figure 19A), the trend was a decrease in $R_{\text{large}}$, $R_{\text{middle}}$ and $R_{\text{tissue}}$ as $\Delta P_{tp}$ in the box was decreased. Conversely, $R_{\text{large}}$, $R_{\text{middle}}$ and $R_{\text{tissue}}$ demonstrated an opposite trend when RR was decreased (Figure 19B) for all 3 pressures.

Using the generalized linear mixed model, $R_{\text{tissue}}$ values (Figure 19A) decreased significantly between $\Delta P_{tp}$ of -30 compared to -20 or -15 cmH$_2$O ($p< 0.00$ for both). Furthermore, $R_{\text{tissue}}$ decreased significantly between -20 compared to -15 cmH$_2$O ($p= 0.00$).

$R_{\text{large}}$ significantly decreased only following $\Delta P_{tp}$ changes, but not during changes in RR. $R_{\text{large}}$ declined significantly between RR of 25 compared to 20 or 10bpm ($p<0.00$ and 0.00 respectively), as well as between 20 and 10 bpm ($p< 0.00$) (Figure 19B). A significant decrease between 10 and 30 bpm for $R_{\text{tissue}}$ was also found ($p=0.00$).

For both $\Delta P_{tp}$ and RR, $R_{\text{tissue}}$ contributed the most resistance to $R_L$, followed by $R_{\text{small}}$, and $R_{\text{large}}$. 
4.11. Relative contribution of $R_{\text{large}}$, $R_{\text{middle}}$, $R_{\text{small}}$ and $R_{t}$ to $R_{L}$.

Figure 20 A&B. Percent contributions of $R_{\text{large}}$, $R_{\text{middle}}$, $R_{\text{small}}$ and $R_{t}$ to total lobar resistance $R_{L}$. 
As pressure in the lung box was decreased (Figure 20A), $R_{large}$ and $R_{small}$ tended to contribute more to $R_L$, however the contribution of $R_t$ tended to decline under the same conditions.

The contributions of $R_{large}$ and $R_{small}$ increased when we reduced box $\Delta P_{tp}$, between -30 and -20 or -15 cmH$_2$O ($R_{large}$; $p<0.00$ and $p<0.02$ / $R_{small}$; $p<0.00$ for both), as well as between -20 or -15 cmH$_2$O ($R_{large}$; $p<0.00$ / $R_{small}$; $p=0.00$),

The contribution however of $R_{tissue}$ to $R_L$ decreased under the same conditions ($p<0.00$ and 0.00). $R_{middle}$ did not participate significantly with changes in pressure.

Adjusting respiratory rate (Figure 20B) had no significant effect on the contributions of $R_{middle}$ and $R_{tissue}$ to $R_L$. In spite of this, the contribution of $R_{large}$ decreased ($p=0.01$) while the contribution of $R_{small}$ increased ($p=0.01$) between 10 and 25bpm.
5. **DISCUSSION.**

The aim of this study was to partition pulmonary resistance in healthy horses’ lungs using a similar technique as one previously described in calves (Gustin, Lomba et al. 1987) and in dogs (Brusasco, Warner et al. 1989). Furthermore, we wanted to dissect the lung resistance to airflow so that the “bottleneck” between lower airways of various sizes could be identified. This method may further aid the evaluation of regional differences in various airways ex vivo, and may help in identifying mechanisms responsible for possible obstructive locations without requiring direct in vivo measurements of small airways. Additionally, veterinarians could use this data when treating lower airway lung inflammation in horses, and ultimately researchers may develop treatments that are targeted to the airways contributing the most to the lung resistance to airflow. A better understanding of the effect of RR and Pressure on lung resistances at different levels would also provide a significant contribution to our understanding of lung mechanics in horses.

Our specific goals were to partition total lobar resistance (from the cranial lobes of horses’ lungs) using ex-vivo lungs ventilated under physiological conditions, and to assess the effect of increasing respiratory rate and negative chest pressure on lung mechanics at the level of different airway sizes. To address these objectives we performed an ex-vivo study using horses’ lungs and compared our data to ex-vivo experiments reported in humans, dogs, calves and horses (Robinson and Sorenson 1978), (Van Erck, Votion et al. 2004). No study to our knowledge has used both the retrograde catheter and alveolar capsule technique in horses to directly measure lung resistances. For comparison purposes and since many of the previous ex-vivo studies have additionally investigated the effects of changes in tidal volume on lung resistance, we have assumed that changes in transpulmonary pressure (ΔP<sub>tp</sub>) in our study were a comparable measure to changes in tidal volume. This assumption allowed comparing our data to other
studies in the literature reporting changes in tidal volume but not in transpulmonary pressure. It should be however stressed that the relationship between transpulmonary pressure and tidal volume is not linear and shifts to a plateau shape at low and high pressures. In addition, the respiratory rate also affects this relationship, making the comparison between data difficult under certain conditions. A similar relationship would be seen throughout all the lobes of the lungs as long as they are healthy with limited ventilation heterogeneity.

We found that $R_L$ and $R_{\text{tissue}}$ decreased significantly with decreasing $\Delta P_{\text{tp}}$ and with increasing RR, while $R_{\text{large}}$ did the same with RR only. Furthermore, $R_{\text{tissue}}$ contributed most to $R_L$, while $R_{\text{small}}$ was highest among airways.

5.1. Validation of data acquisition software and inputs.

Conventional data acquisition and signal conditioning systems are often designed by and for engineers. Researchers in the life sciences are often challenged by complicated procedures to adjust and calibrate data acquisition hardware and software settings. Furthermore, a significant amount of time must be spent to configure a system for a specific application. The SCIREQ Flexiware system proved to be a relatively turn-key system complete with personal engineering assistance from Dr. Thomas Schuessler, president and CEO of SCIREQ. A personalized lung mechanics software template was additionally developed for our purposes, however good practice necessitates a degree of skepticism as it pertains to data acquisition and simultaneous recordings. In short, we wanted to confirm all recorded and calculated data were represented in the same timeframe with no evidence of time-lag.

Earlier studies circumvented the problem of time-frame dependency simply because all calculations of resistance, compliance and elastance were performed by hand. Macklem and Mead were first to employ the retrograde catheter technique (Macklem and Mead 1967), and like
all similar studies of the era, oscilloscopes and paper tracings were the preferred collection method for pressure and flow data. In particular, the Sanborn Poly-Viso electrocardiograph machines could produce accurate paper tracing of pressure readings, and pressure-flow curves could be monitored and stored using such instruments as the Tektronix oscilloscope series. No time discrepancies between pressure and flow readings were observed simply due to redundancy of measurement techniques, and careful breath by breath analysis by investigators. Resistances could be obtained either by storing and tracing pressure-flow curves on the oscilloscope, or by dividing the amplitude of pressure obtained from Sanborn paper tracings by the amplitude of flow. A common method to obtain $R_L$ meant oscillating lungs at a preferred frequency and manually relating flow to the pressure of interest (Dubois, Botelho et al. 1956; Ferris, Mead et al. 1964). With the introduction of the retrograde catheter came the ability to partition airways of the lung into various component resistances, however early investigation found large phase differences between pressure and flow particularly when measuring peripheral lung pressures. Two techniques were used to compensate; measuring pressures and flows at peak points where no net acceleration was present, or manually subtracting phase lags by matching pressure and flow signals or tracings (the latter could later be done electronically using more advanced oscilloscopes equipment) (Macklem and Mead 1967; Macklem, Woolcock et al. 1969; Mead 1969). Another method was to simply photograph the screen of the oscilloscope using a Polaroid, and compare it to the trace outputs from the Sanborn (Hildebrandt 1969). It should be recognized that none of these compensation techniques are perfect and they can be the source of some error, particularly at extreme flow or respiratory rate values.

Needless to say, the methods described above were both time consuming and taxing, however they paved the groundwork for software seen in today’s mechanical ventilators. To
verify that our data contained no phase lags, we plotted pressures from various transducers with volume calculated by Flexiware against time (Figure 13). No apparent time delay was observed among 5 lungs at several test conditions which helped serve to validate our findings. Similar validation calculations have been performed for the above reasons in other studies (Romero, Rodriguez et al. 1998). The significance of this became apparent during manual calculations of volume used later to generate pressure-volume loops discussed in the following chapter, since only breath by breath alignments would yield meaningful numbers. Furthermore manually calculated elastance values were essential to substantiating data collected from Flexiware. To this end we plotted lung and tissue elastance against time (Figure 14) to verify they were in phase, and to therefore confirm we could compare manually calculated elastance from P-V loops to those calculated by software. Lung and tissue elastances taken during 5 breaths for 5 lungs at several conditions were compared and no lag time was observed, permitting us to compare them to manually calculated elastance values. Changes in elastance with pressure and respiratory rate are discussed in later chapters.

5.2. Validation of data analysis: Pressure-Volume Loops.

To affix an additional degree of certainty to our calculated software data, pressure-volume loops for all lungs and conditions were plotted by calculating the area under airflow-time curve during 1 breath sampled at 100Hz, then calculating the volume using the integration of the signal over time. Volume was then plotted against pressure from 2 separate differential transducers (see Figure 9): Main bronchus-to-box (transducer #2) and alveolar capsule-to-box (transducer #6) for transpulmonary and tissue pressures respectively to generate elliptical curves (Figure 15). Taking the inverse slopes of the curves yielded $E_L$ and $E_t$, which could be compared
to software-created $E_L$ and $E_t$ data. Table 1 illustrates the inverse slopes of the P-V loops for Lung #1 in all conditions. No significant differences using a 2 sample t-test were found between calculated elastance from the P-V loops and Flexiware generated elastance produced by the linear model described in previous chapters ($p=0.23$: $1/\text{slope}$, $p=0.25$: $E_L$ from Flexiware). A two sample t-test was employed over a paired t-test because 2 independent samples were compared. Of note and as also seen in Table 1, there were no significant differences between data generated during the large and small catheter timeframes using a 2 sample t-test ($p=0.47$: $1/\text{slope}$, $p=0.46$: $E_L$ from flexiware) for lung #1.

### 5.2.1. Elastance results from Pressure-Volume Loops.

Similar to the P-V loop analysis performed by Hildebrandt et al. (Hildebrandt 1969) in cats, we found increases in elastance with frequency (Table 2). The reasons for this are discussed in the chapter on viscoelasticity but in short, increases in tensile spring force are met by an equal resistive force, and the lung is more likely to “stretch” when tensile forces are applied more slowly. Hildebrandt et al. did not report on changes in volume, but we also observed increases in elastance with pressure. Interestingly we found elastance tended to increase at high pressures (or tidal volumes), and we rationalize that viscoelastic effects were most pronounced under these conditions. Positive pressure ventilation over 30cmH$_2$O are deemed high for the lung with risk of barotrauma increasing from 30cmH$_2$O to 45cmH$_2$O (Lapinsky, Aubin et al. 1999). Like the present study and analogous to an increase in elastance with frequency, Brusasco et al. found decreased lung compliances with increasing frequency (Brusasco, Warner et al. 1989). Using excised dog lungs and the alveolar capsule technique, they found significantly different mean lung compliance values ranging from 0.0610 l*cmH$_2$O$^{-1}$ at 7 bpm to 0.0525 l*cmH$_2$O$^{-1}$ at 45
bpm. By taking the inverse of these compliance values respectively to evaluate elastance, a range of 16.6 and 19.05 (cm H2O/sec.) / (L/sec) can be contrasted against our range of 11.3 to 13.5 (cm H2O/sec.) / (L/sec) from our manual inverse slope calculations, or a range of 10.4 to 12.1 (cm H2O/sec.) / (L/sec) from the Flexiware generated data at a frequency range of 10 to 25 bpm. The authors concluded that changes in compliance were caused by the viscoelastic properties of the lung tissue rather than heterogeneity or regional lung ventilation.

The inspiratory and expiratory curves of the pressure-volume loop are differently shaped and furthermore, the loop generally widens with tidal volume. The reason for this is because the pressure during expiration is less than during inspiration, and the elastic recoil on expiration is always less than the pressure gradient required to inflate the lung. If we were to think of the lung as a spring, Hooke’s Law states the change in length of a spring is proportional to the tension exerted on the spring, and a release of tension results in a loss of length (West 2012). In addition, the lung is an elastic structure resisting deformation with some exceptions (see chapter on viscoelasticity), and the area encompassed inside the loop is termed lung hysteresis.

5.2.2. Hysteresis: Calculated area inside the P-V loops.

Hysteresistivity is defined most rigorously as the ratio energy dissipated per cycle to the stored potential energy at maximum volume (Fredberg and Stamenovic 1989). Borrowing from engineering and even geological principles, hysteretic stress-strain relationships have been used to describe the behaviour of soil, rock, metals, electric displacement fields of a ferroelectric material and any structure capable of dissipating energy at granular or crystalline interfaces (Fredberg and Stamenovic 1989). The fact that a single relationship describes data from lungs of different sizes from three (or even four) animal species is remarkable. Agostini et al. (Agostoni, Thimm et al. 1959) found that specific compliances and hysteresis were quite similar between
cats, dogs, humans and even monkeys (Bachofen and Hildebrandt 1971), with added credibility to such findings seen in summary papers (Fredberg and Stamenovic 1989; Bates and Lutchen 2005). As mentioned in introductory chapters, hysteresis is an empirically determined variable that quantifies the dependence of dissipative energy on elastic process. Furthermore, interaction and hysteretic matching of the lung with the chest wall tissues in-vivo is thought to be a necessary to guarantee synchronous expansion of the organ (Barnas, Heglund et al. 1989).

In the present study, we calculated the area between the curves by subtracting the integral of the expiratory curve from the integral of the inspiratory curve for 1 breath in all conditions in 7 lungs (Table 3A) and in 1 example lung (Table 3B). It has been demonstrated in the lungs of humans and dogs that the width of a quasi-static pressure-volume hysteresis loop increases with increasing tidal volume (Mead, Whittenberger et al. 1957), Hildebrandt et al. (Hildebrandt 1969) also found that by doubling tidal volume, hysteresis is roughly quadrupled but is independent of frequency. Our results support the notion of width increasing with tidal volume (or ΔP_tp) and furthermore, we found areas of hysteresis were greatest at high tidal volumes (or ΔP_tp) and lowest at decreased tidal volumes (or ΔP_tp). Others have observed similar results in open chested dogs (Loring, Drazen et al. 1981), where Loring et al. found increases in hysteresis with pressure during open airway dynamic maneuvers with values of 3.09 and 3.47 ΔP/ΔV at pressures of 5 and 10 cmH}_2O respectively. Sugihara et al. (Sugihara, Hildebrandt et al. 1972) also found pressure-volume hysteresis increased in any state where increases in lung elastance were seen, a result echoed by our findings. In table 3B, a distinctive outlier at a RR of 10 and ΔP_tp 30 was evident and resulted from lung #3, which had come from a large draft horse. We suspect this lung was capable of containing high volumes at increased pressures, which resulted in an abnormally high value for hysteresis.
In regards to frequency and using lung data from table 3B at a low pressure of 15 cmH₂O, we found hysteretic area tended to decrease with increasing frequencies ranging from 9.0 L* cmH₂O at 10 bpm to 4.8 L* cmH₂O at 25 bpm. Others have found little evidence of dependence of hysteresis on cycling frequency below 2 Hz (Hildebrandt 1969). Hysteresis increased exponentially beyond 2 Hz, however we were unable to confirm this due to a maximum frequency reached of 25 bpm (= 25*1/60= 0.42 Hz) which is physiological.

In inorganic elastic systems mentioned above, dissipative energy often manifests in the form of heat. The possibility of significant temperature changes in the lung arising from repeated cycling has been considered by Hildebrandt et al. (Hildebrandt 1969). The work done during a compression of 40 ml is roughly 1 cal., and heat dissipated in 10g of parenchyma causes a temperature rise of 0.1°C. However most heat is dissipated and the heating effect on the lung as a result of compression-expansion cycles appears negligible, where the maximal energy loss due to hysteresis itself is about 0.001 cal/cycle.

5.3 Airway sizes.

We measured airway sizes of 0.56 ± 0.079mm, 0.39 ± 0.092mm and 0.26 ± 0.071mm which was comparable to other measured airway sizes using similar technique (Macklem and Mead 1967; Hogg, Agarawal et al. 1972; Van Brabandt, Cauberghs et al. 1983; Gustin, Lomba et al. 1987). The lungs were dissected using scissors until the bell-shaped end of the retrograde catheter was found, which was then removed and the airway of interest was measured using a caliper. Since the airways of interest contained cartilaginous rings which held the airway open and prevented collapse, we are confident our measurements were representative of in-vivo diameters. We were however unable to find any significant relationships between catheterized
airway size (and calculated R\textsubscript{middle}) and airway resistance due in part to the high variability of resistance data seen between lungs (Table 4). Additional to our lungs being harvested from multiple horse breeds of various size, sex and age, we included both the right and left cranial lobes in our data set as a result of having a limited sample size. Although 56 separate cranial lobes were ventilated in our experiments between technique development and data collection, 13 lungs satisfied all the inclusion criteria for valid data analysis.

Other studies have however found significant associations between airway size and resistance. Using forced oscillation technique, retrograde catheters and tantalum bronchograms, Hahn \textit{et al.} measured airway diameters in excised dog lungs at various transpulmonary pressures, and found diameters increased with pressure up to a \(\Delta P\textsubscript{tp}\) of 30 cmH\textsubscript{2}O (Hahn, Graf et al. 1976). Moreover, they found airway resistance decreased between 0 and 10 cmH\textsubscript{2}O but increased again between 10 and 30 cmH\textsubscript{2}O, and concluded excised lungs have bronchomotor tone that narrows airways at high \(\Delta P\textsubscript{tp}\) values. We found highest airway and lung resistance values at high pressure but were unable to measure bronchomotor tone. Furthermore, it is possible that we saw a high degree of variation in resistance as a result of changes in bronchomotor tone observed between our lungs.

Hoppin \textit{et al.} using retrograde catheters in open chested dogs also found difficult reconciling relationships between lung resistance and lung volume with airway diameters when they were measured by bronchographic and anatomical means (Hoppin, Green et al. 1978). They noted increases in resistance at high volume using the retrograde catheter technique (similar to us, and that narrowing of the airways was not generally noted. However others (Kilburn 1960; Colgan 1964; Hahn, Graf et al. 1976) have found changes in airway caliber when lungs are ventilated either with positive or negative pressure, confirmed also by Tisi \textit{et al.} (Tisi, Minh et al.
1975) who showed bronchographically in dogs that 1 mm airways changed size proportionately more than did larger airways. Hughes et al. (Hughes, Hoppin et al. 1972) found in general that all airways down to 2 mm changed size proportionately using ex-vivo dog lungs. Since small airways appear to change in diameter under ventilation, one can presume knowing static airway diameter would be of little use for making hypotheses concerning airway resistance. Airway size, and hence resistance, appear to be more the result of pressure and volume changes. It is unknown if this is also the case in ex-vivo lungs.
5.4  **Tidal volume (V_t) & minute ventilation (V_E).**

We observed a significant decreased in V_t with ΔP_{tp} and with RR, and V_E tended to decrease as pressure in the box decreased. Conversely V_E tended to increase with respiratory rate, and was significantly greater at ΔP_{tp} of -30 compared to -20 or to -15 cmH_2O (p< 0.00 for both) (Table 1). V_E was significantly different between all RR’s (p< 0.00 for all).

We found maximum average V_t values of 2.8 L (2.4 – 3.2 L) at P_{tp} -30 / RR 10 and V_E 55.2 L min^{-1} (41.8 – 60.5 L min^{-1}) at P_{tp} -30 / RR 25. It is difficult to compare our values to live horses and intact lungs for two reasons: 1) Normal adult horses at rest have breathing rates of 10 – 24 bpm and up to 120 bpm during heavy exercise. While we simulated breathing rates at rest and during low level exercise, we could not ventilate at respiration rates seen during heavy exercise. 2) No one, to our knowledge, has measured breathing patterns *ex-vivo* using equine cranial lobes. Nonetheless, tidal volume and minute ventilation patterns for live horses at rest have been measured at 6.2 ± 3.8 L and 76.8 ± 21.2 L min^{-1} respectively (Koterba, Kosch et al. 1988). Other studies have found tidal volumes at rest and at canter of 8.45 ± 1.41 L and 16.3 ± 2.65 L respectively and minute ventilations at rest and at canter of 89.1 ± 30.2 L min^{-1} and flow rates of 860.7 ± 250.3 L/ min respectively (LaFortuna and Saibene 1990). Others however have found flow rates as high as 1500 L/min during high-speed gallop. Comparing our maximal tidal volumes to literature values seen at rest and during intense exercise in normal adult horses with two caudal, two cranial and one accessory lobe, we find single equine cranial lobes comprise 45.1 % and 17.2 % of V_t respectively. Doing the same for minute ventilation yields values of 62.0% and 6.4% respectively. Although these results are actually comparing in-vivo with ex-vivo lungs in very different conditions, they suggest that equine cranial lobes may have the ability to hold comparatively large volumes when compared to other lobes. We can hypothesize
from these results that cranial lobes of horses are preferentially recruited during periods of intense physical exertion. Furthermore and as seen in the introduction with equine airway tree structure, the cranial lobes are located lateral to left and right main bronchi at angles of 90 degrees. This anatomical arrangement suggests that it would be difficult to have direct laminar airflow to the lobes, and that the additional contribution of cranial lobes to lung ventilation may occur only at higher airflows and pressures than seen at rest. To our knowledge, this effect has not been studied in horses.

5.5. **Total lung resistance (R<sub>L</sub>).**

We performed lung mechanics (Figure 16) on 13 lungs using the methods mentioned above, and found similar values for R<sub>L</sub> as seen in other ex-vivo studies (Hogg, Macklem et al. 1968; Fredberg, Keefe et al. 1984). In normal ex-vivo human lungs for example, Hogg *et al.* (Hogg, Macklem et al. 1968) found similar values (0.8 – 0.6 cmH<sub>2</sub>O/L/sec. of R<sub>L</sub>) at lower pressures ranging from 5 – 15 cm H<sub>2</sub>O. It is important however to recognize that while examining the variability in R<sub>L</sub> with changes in pressure and respiratory rate, understanding changes in airway and tissue components is essential. Changes in R<sub>L</sub> have been widely interpreted as reflecting changes in airway resistances, but R<sub>tissue</sub> was the predominant component of R<sub>L</sub> over most ranges of pressure and respiratory rates we studied. Furthermore, numerous methods other than our own have been used to partition lung resistance.

Despite the wide range of R<sub>L</sub> reported in the literature, our results often matched others’ in the manner by which R<sub>L</sub> changed with respiratory rate and/or pressure (or volume). Surprisingly, we found R<sub>L</sub> decreased significantly with decreasing ΔP<sub>IP</sub>. In contrast, Macklem *et al.* (Macklem, Woolcock et al. 1969) found in dogs R<sub>L</sub> decreased with increasing lung volume,
however $R_L$ became independent of lung volume following vagotomy. (Living vagotomised lungs and excised lungs are similar). In addition, Macklem et al. (Macklem, Woolcock et al. 1969) used positive pressure ventilation. We additionally found our values for $R_L$ agreed with the literature observed by a decrease with RR (decreased with frequency / when RR was decreased). Gustin et al. (Gustin,omba et al. 1967) found $R_L$ increased with higher pressures in Friesian calves, and Brusasco et al. (Brusasco, Warner et al. 1989) found $R_L$ significantly decreased with frequency. However, they reported a decrease in $R_L$ with increasing $V_t$ using ex-vivo and in-vivo dog lungs. Van Erck et al. (Van Erck, Votion et al. 2004) also found a decreasing $R_L$ with decreasing negative pressure by means of the Impulse Oscillometry System (IOC), a system similar to Forced Oscillation Technique (FOT) / High Frequency Oscillation (HFO).

Our data differed to that of Macklem and Mead (Macklem and Mead 1967), who using ex-vivo dog lungs found greatest values of $R_L$ (3.9 cm H$_2$O/L/sec ) at low volumes, or 10 % $V_C$, and lowest values of $R_L$ (1.2 cm H$_2$O/L/sec ) at a $V_C$ of 70%. However after 70% $V_C$, their values of $R_L$ suddenly increased to 1.5 cm H$_2$O/L/sec. ). Our result increased likewise at higher pressures; however we did not observe the same profile for $R_L$ at low pressures. In addition, our experimental setup differed to that of Macklem and Mead in several ways. 1) Our lungs were ventilated under negative pressure, thereby better approximating the conditions found in the thoracic cavity. 2) The lungs were supported by the main bronchus rather than from below. Van Brabandt et al. (Van Brabandt, Cauberghs et al. 1983) verified this does not noticeably change the resistance values. 3) We measured $R_L$ not only under changes in pressure, but also during changes in respiratory rate. We suggest that the wide range of variation between reported values and trends for $R_L$ is the result of a combination of the above factors. Moreover the contribution
of $R_{\text{tissue}}$ to $R_L$ has been high in many experiments, and is thought to be due to an overestimation of the tissue component when using the alveolar capsule (Lauzon, Dechman et al. 1995).

5.6. **Resistance of the airways ($R_{\text{airway}}$).**

As a control and to validate our partitioned resistances, we calculated the resistance between the alveolar capsule and the central bronchus ($R_{\text{airway}}$) using transducer #5, and compared it to the sum of $R_{\text{large}}$, $R_{\text{middle}}$ and $R_{\text{small}}$, and found no significant differences (Figure 17). Brusasco et al. (Brusasco, Warner et al. 1989) found a likewise $R_{\text{airway}}$ decrease with rising frequency, and a small increase with pressure (though not significant) was also observed. We found a slight increase in $R_{\text{airway}}$ with RR, but no significance was confirmed following post-hoc analysis.

5.7. **Lung elastance ($E_L$) and tissue elastance ($E_{\text{tissue}}$).**

Additionally we found no significant variation between total lung elastance and tissue elastance, lending us to conclude the tissue parenchyma was responsible for most of the elastance found in the lung. See sections 4.2. and 4.2.1 for more information pertaining to elastance and comparison between species.

5.8. **Partitioning of pulmonary resistances.**

5.8.1. **Tissue resistance ($R_{\text{tissue}}$).**

$R_{\text{tissue}}$ was measured between the alveolar capsule and the box, and was the resistance in our study contributing the most to $R_L$. Occasionally $R_{\text{tissue}}$ is called tissue hysteresis or viscance (Hildebrandt 1969; Loring, Drazen et al. 1981), and has been estimated by different methods.

Because of differences in technique, a comparison between our work and data obtained by others is difficult. Our $R_{\text{tissue}}$ values (Figure 19) ranged from 0.22 – 0.50 cmH$_2$O/ L/sec, or 46.2% - 60.0% of $R_L$ respectively. Other studies have reported relatively high values for $R_{\text{tissue}}$ in calves (Gustin, Lomba et al. 1987) and in dogs using HFO (Loring, Drazen et al. 1981; Ludwig, Dreshaj et al. 1987; Ludwig, Romero et al. 1989). In dogs using a similar technique to us but different conditions, Brusasco et al. (Brusasco, Warner et al. 1989) found $R_{\text{tissue}}$ contributed 93% to $R_L$ at low frequency and high tidal volume, and 41% at high frequency and small tidal volumes. We also observed a similar significant decrease in $R_{\text{tissue}}$ with volume (or pressure), and a decreased trend for $R_{\text{tissue}}$ with increasing frequency as the above. Like our $R_{\text{tissue}}$ data, it has been shown that $R_{\text{tissue}}$ increases with tidal volume (Mount 1956; Bachofen 1968; Hildebrandt 1969; Loring, Drazen et al. 1981), and decreases with frequency (Brusasco, Warner et al. 1989).

In contrast with the above, in Bachofen’s study (Bachofen 1968) lung tissue resistance accounted for only 17 % of $R_L$ during rapid shallow breathing at low lung volume, but accounted for 3% of $R_L$ during slow deep breathing at high volumes. Brusasco et al. (Brusasco, Warner et al. 1989) found for any $V_t$, the relative contribution of $R_{\text{tissue}}$ to $R_L$ decreased with increasing frequency. This finding is in agreement with those from previous studies.

Several possibilities may contribute to the high contribution of $R_{\text{tissue}}$ to $R_L$ we observed in this study. First, horses have more extensive interlobular septa than dogs, an observation confirmed by Robinson et al. (Robinson and Sorenson 1978) who using ex-vivo lungs found higher collateral resistances in horses than in dogs. Furthermore, Bachoven et al. found that a given tidal volume in a small individual is equivalent to a much larger breath in large individuals.
(Bachofen and Duc 1968). Therefore, the tissue resistance seen in children, small adults or ex-vivo equine cranial lobes may appear misleadingly high.

Second, we did not use isovolume technique, and high frequencies of ventilation. It is also possible \( R_{\text{tissue}} \) was high as a result of viscoelastic inerance of our system, especially at high respiratory rates. This however is unlikely since we found that changing respiratory rate did not significantly change the contribution of \( R_{\text{tissue}} \) to \( R_L \). The apparent source of disagreement among reported values for \( R_{\text{tissue}} \) was proposed by Bachofen and Hildebrandt (Bachofen 1968; Hildebrandt 1969; Bachofen and Hildebrandt 1971), who showed that lung recoil hysteresis cannot be characterized as viscoelastic resistance because it depends primarily on the magnitude of change in tidal volume and is independent of frequencies below 120 bpm (Loring, Drazen et al. 1981; Brusasco, Warner et al. 1989). In dogs, Brusasco et al. (Brusasco, Warner et al. 1989) found the greatest changes in \( R_{\text{tissue}} \) occurred at frequencies less than 25 bpm, which could explain some of the variation we observed. Even so, we waited no less than 3 minutes following any change in experimental condition prior to subsequent data collection at the new condition. This allowed tissue resistance, and presumably tissue viscoelastance, to adapt to the new pressure and respiratory rate. More recently in normal rats and while studying tissue mechanics, Brewer et al. (Brewer, Sakai et al. 2003) reported a decrease in hysteresivity with pressure.

Third, air trapping at low frequencies and low lung volumes may have contributed to high \( R_{\text{tissue}} \) values seen in other studies (Hoppin, Green et al. 1978; Fredberg, Keefe et al. 1984). However, the alveolar capsule is adequate for determining regional differences, but does not allow one to decide if this is due to changes in local airway resistances or local tissue stiffness (Bates and Lutchen 2005). In humans (Hajari, Yablonskiy et al. 2012), others have found alveolar duct radii increase only slightly during inspiration, suggesting that total lung volume
increases are largely by alveolar recruitment. It is difficult to cleanly partition airway vs. tissue components where tissue is overestimated, and furthermore the capsule only samples half a dozen acini compared to the tens of thousands (Bates and Lutchen 2005). Imparting more weight to this argument is Romero and Ludwig (Romero and Ludwig 1991) who used alveolar capsules when measuring tissue resistance in rabbits. Peripheral airway resistance was thought to participate in viscance \( (R_p + R_t) \), where they found its contribution to \( R_L \) was 65% ± 15%.

We found the contribution of \( R_{\text{tissue}} \) to \( R_L \) significantly decreased (figure 5) when pressure was decreased. Brusasco et al. (Brusasco, Warner et al. 1989) found for any \( V_t \), the relative contribution of \( R_{\text{tissue}} \) to \( R_L \) decreases with increasing frequency. This finding is in agreement with those from previous studies in humans (Bachofen 1968), and in dogs (Loring, Drazen et al. 1981; Ludwig, Dreshaj et al. 1987). The dependence of \( R_{\text{tissue}} \) on frequency may explain the wide variability in the reported relative contributions of \( R_{\text{tissue}} \) to \( R_L \). Our data suggests a similar trend; however we found no significance for the percent contribution of \( R_{\text{tissue}} \) to \( R_L \) with frequency. Of note is \( R_{\text{tissue}} \) is variable and often a large component of \( R_L \), where partitioning of \( R_L \) between tissue and airways has been controversial. This in large is the result if \( R_{\text{tissue}} \) being sensitive to host of factors such as frequency and lung volume history (Fredberg and Stamenovic 1989). In short, lung viscoelastic properties are and have been difficult to both predict and to model.

5.8.2. Large airway resistance \( (R_{\text{large}}) \).

Like Gustin’s study in calves, we found \( R_{\text{large}} \) (also called \( R_{\text{central}} \) or \( R_c \) in other studies) to be the airway size contributing the second most to \( R_L \) after \( R_{\text{small}} \). \( R_{\text{large}} \) decreased (though not significantly) following \( \Delta P_{lp} \) changes and is consistent with the findings of Hogg (Hogg, Williams et al. 1970) and Wood (Wood, Engel et al. 1976), but not with Gustin (Gustin, Lomba...
et al. 1987). Macklem and Mead (Macklem, Woolcock et al. 1969) found at high volumes, $R_c$ contributed most to $R_L$ at high volumes, however their results were obtained *in-vivo* and $R_c$ was estimated by subtracting $R_p$ from $R_L$ rather than by direct measurement. We found the percent contribution of $R_{\text{large}}$ (Figure 20) to $R_L$ significantly increased with decreasing pressure, however it did the opposite with respiratory rate. This finding is unique, but what we called $R_{\text{large}}$ is by convention the grouping of $R_c$ and $R_p$ found in other literature. Hence our results were obtained from partitioning a portion of the lung rather than just a single central bronchus. Upper airway resistance is another important factor that can influence the measured values of $R_L$ *in vivo*, notably in horses, where the sedation or head position can affect calculated values of $R_L$ (Lavoie, Pascoe et al. 1992). Using *ex vivo* lungs removes the effect of upper airways as a confounding factor, but caution should be taken when comparing $R_L$ data obtained using excised lungs to *in-vivo* experimental models.

**5.8.3. Middle airway resistance ($R_{\text{middle}}$).**

$R_{\text{middle}}$ was an attempt to refine the partitioning of lung resistance by adding an intermediate airway diameter. It was done by use of pressure data averaged from the retrograde catheters recorded in distinct time frames using the same differential pressure transducer and a three-way stopper. No studies to our knowledge have addressed the partitioning of pulmonary resistance similarly, and this method was not without question. To review, $R_{\text{middle}}$ was calculated by subtracting small catheter from large catheter flexiware resistance data obtained from pressure transducer #4 (Figure 9) over 15 breaths. Similar resistance data obtained over 15 breaths but gained from transducer # 5 by subtracting large catheter from small catheter time frame values was averaged with that from transducer #4 since little difference between the two safety
measures was observed. Furthermore, middle airway diameter was determined by averaging the measured airway sizes catheterized by both the small and large retrograde catheters (Figure 9). No significant conclusions however were found when attempting to reconcile airway size with respective resistances. Several challenges were immediately evident when examining calculated data for $R_{\text{middle}}$: 1) $R_{\text{middle}}$ contributed the least resistance to partitioned resistances. This most likely resulted from it being a calculated value; the difference between the large and small catheter seen in Figure 8, the spring and box model. This meant there was proportionately less difference in pressure measured between both retrograde catheters than between the alveolar capsule and the catheters, or from between the central airway and catheters. As mentioned, most studies have partitioned pulmonary lung resistance into central airway and peripheral airway resistances (and tissue resistance in some), however our design differed with the use of two retrograde catheters.

5.8.4. Small airway resistance ($R_{\text{small}}$).

Similar to Gustin et al. (Gustin, Lomba et al. 1987) and among the airway sizes investigated, $R_{\text{small}}$ was the resistance that contributed the most to $R_L$ as measured by the retrograde catheters and alveolar capsule. Despite finding no significant differences resulting from changes in pressure, $R_{\text{small}}$ tended to decrease with pressure followed by an unexpected increase. $R_{\text{small}}$ increased likewise with RR, though not significantly.

Averaging $R_{\text{small}}$ over all conditions, we found it contributed $38.7\% \pm 6.0$ to $R_L$. Similar results have been found in dogs (Hogg, Macklem et al. 1968) and in humans (Hogg, Williams et al. 1970) with peripheral airway resistance accounting for 25% of $R_L$. Using the HFO technique,
Hoppin et al. (Hoppin, Green et al. 1978) found $R_p$ to be 34% of $R_L$ at 1.1 Hz. While Kappos et al. (Kappos, Rodarte et al. 1981) observed a 41% $R_p$ to $R_L$ at 5 Hz and $\Delta P_{tp}$ of 5 cm H2O.

While the contribution of $R_{tissue}$ to $R_L$ decreased with $\Delta P_{tp}$ and tended to decrease with increased RR, the contribution of $R_{small}$ to $R_L$ under the same conditions did the opposite. We found the significantly contribution of $R_{small}$ went from 35.1% and 42.7% as $\Delta P_{tp}$ was decreased, and from 32.4% to 42.0% as RR was increased. In dogs, Macklem and Mead (Macklem, Woolcock et al. 1969) found $R_p$ became increasingly a larger fraction of $R_L$ as volume diminished. While investigating dog and human lungs under HFO, Van Braband et al. (Van Brabandt, Cauberghs et al. 1983) found $R_p$ (equivalent to $R_{small}$) in dogs represented a smaller percentage of pulmonary resistance than in human lungs, and that $R_p$ increased at low and high volumes. Our $R_{small}$ results are aligned more closely with those found in the more anatomically similar human lung, and we propose this resulted in a higher $R_{small}$ or ($R_p$) than those found in dogs. While examining human subjects using insoluble gas, Crawford et al. (Crawford, Makowska et al. 1985) found a tidal volume-dependent decrease in heterogeneities of the lung periphery. This perhaps indicates tidal volume alters small airway diameter, and hence small airway resistance.

6. CONCLUSIONS AND FUTURE DIRECTIONS.

6.1. Conclusions

In summary, the data collection and calculations were legitimate as a result of rigorously verifying that there were no possible time delays between signals, and that elastance data from manually generated pressure-volume curves matched the one generated by the Flexiware software. Furthermore, we confirmed that data collected from the alveolar capsule and retrograde...
catheters were valid and in phase with other recordings, which were compared to our redundant measure of $R_{\text{airway}}$.

We found that $R_{\text{tissue}}$ contributed the most to $R_L$ and that $R_L$ decreased with pressure and with increasing respiratory rate. Furthermore, $R_{\text{small}}$ was the airway contributing the most to airflow resistance among airways, and the relative contributions of $R_{\text{large}}$ and $R_{\text{small}}$ to $R_L$ increased when box pressure decreased, whereas the relative contribution of $R_{\text{tissue}}$ to $R_L$ did the opposite. In addition, the effect of $\Delta P_{tp}$ on $R_L$ tended to predominate over the change in respiratory rate.

Consistent with these findings, we maintain that thermoplasty treatment used to address airway smooth muscle remodeling in horses would not produce the desired results, for the reason that most resistance to airflow in the equine cranial lung lobe resides both in the tissue and in the small airways. Clearly additional study is required and we believe there is now sufficient support for further investigations, regardless of conformation for the effectiveness of thermoplasty.

### 7.2. Future directions

An additional investigation using ex-vivo horse lungs would be the appropriate next step of challenging newly excised horse lungs with histamine or methacholine to determine patterns of homogeneous ventilation. Similar *in-vivo* studies have been performed in dogs (Fredberg, Ingram et al. 1985; Ludwig, Shore et al. 1988; Ludwig, Shore et al. 1988; Ludwig, Romero et al. 1990; Ludwig, Robatto et al. 1991) and in swine (Olson 1991) and they have found heterogeneous lung ventilations develops during challenge. With the limitations in the number of channels available for pressure transducers, we however would require to select carefully which
differential pressure transducers we would remove from the study so that we could measure pressures in 2 or possibly 3 alveolar capsules.

We currently have animal care approval to attempt the partitioning of pulmonary lung resistance in-vivo with the following questions in mind: 1) What are the effects of frequency of ventilation using alveolar capsule technique on total lung resistance (R_L), total tissue resistance (R_tissue) and on the homogeneity of ventilatory distribution throughout the equine lung? 2) What are the effects of changing volume during ventilation using alveolar capsule technique on R_L, R_tissue and on the homogeneity of ventilatory distribution throughout the equine lung? 3) What are the effects of histamine / methacholine challenge using alveolar capsule technique R_L, R_tissue and on the homogeneity of ventilatory distribution throughout the equine lung?

![Diagram](image)

Figure 21. Proposed experimental setup to partition pulmonary lung resistance \textit{in vivo} in horses.

We hypothesize R_L and R_tissue would increase as a result of increasing frequency and volume variables. Furthermore, heterogeneity of ventilatory distribution would increase as a
result of increasing frequency and volume variables based on results found in dogs. Six alveolar capsules would potentially be required. This project would challenge both researcher and surgical clinicians as not only the data recording would be challenging, but the opened chest surgery exposing enough lung for the experiments on an anesthetized horse would also be difficult. Nevertheless, such data would shed valuable insight into the mechanisms of the lung periphery in live horses.
REFERENCES.


APPENDICES.

Appendix A: Necropsy report.

Report
Accession No. 11-72
Pathologist: Dr. Jan Bystrom

Date Received: 04/08/11
Date Reported: 08/18/11

Clinic: UOVM - VCDS
Animal NameID:
Species: Equine
Breed:
Age:
Sex: U
Owner: Nicol, James

Billing: RT757145

Specimens Received
Lung

Case History
Research study. Lung tissue from 8 horses submitted, pretube and post tube, plus 1 tube labelled "lung 4 left Sept 10."

Final Diagnosis:
LUNG SECTIONS X 17: WITHIN NORMAL RANGE

Comments
Final Interpretation: August 18, 2011

These lung sections are all judged within normal range. There are very subtle differences in individual slides as follows:
- generally it is easy to distinguish preventilation lung with one exception, lung 1 right lobe done August 13 and 14/08.
- there are few lung sections showing peribronchial inflammatory cell aggregates. At the number and size of these, they are considered within normal range in adult horses and not likely to be of clinical relevance. They are very small localized areas of chronic inflammation that are likely responses to inhaled pathogens or irritants at some time in the horse’s past life. Lungs so affected are: Lung 1 right Sept 11, Lung 2 right Aug 14, Lung 3 left Aug 13, Lung 4 left Aug 13 and Lung 4 left Sept 10.
- a few lung sections show peribronchial fibrous tissue; slightly more prominently compared to others. These are Lung 3 left Aug 13, Lung 3 left Aug 20 and Lung 7 right Aug 21. Again, this is not likely to be of clinical relevance when it is this mild and in the absence of other findings.
- all sections are showing a mild degree of autolysis with sloughing of epithelial cells seen and with it being difficult to resolve cilia detail.

Descriptions have been arranged in numerical order starting with lung 1, then in chronological order by date. If there are questions or further clarification that is needed as research papers are written, please do not hesitate to contact the DSU for assistance.
HISTOLOGICAL FINDINGS:

Slide 8: LUNG 1, RIGHT LOBE PRE Aug 13/08: Slide 7 and 8 show no appreciable difference in inflation with both showing patchy areas of good inflation with intervening areas of moderate atelectasis.

Slide 7: LUNG 1, RIGHT LOBE POST Aug 14/08: See slide 8 immediately above.

Slide 14: LUNG 1, RIGHT LOBE PRE Sept 10/08: Quite atelectatic with little open alveolar space showing in section examined.

Slide 13: LUNG 1, RIGHT LOBE POST Sept 11/08: Much more inflated vs. slide 14 immediately above. One very small peribronchial/perivascular mononuclear, mostly lymphocytic, aggregate seen.

Slide 15: LUNG 2, RIGHT LOBE PRE Aug 13/08: See slide 16 immediately below.

Slide 16: LUNG 2, RIGHT LOBE POST Aug 14/08: No appreciable difference in inflation vs. slide 15 above. This one shows two small peribronchial mononuclear, mostly lymphocytic, aggregates.

Slide 9: LUNG 3, LEFT LOBE PRE Aug 13/08: Section quite atelectatic. There are two small peribronchial lymphoid aggregates, one associated with a small irregular focus of crystalline material that is birefringent. Peribronchial fibrous tissue is slightly more prominent than expected.

Slide 10: LUNG 3, LEFT LOBE POST Aug 14/08: No appreciable difference vs. slide 9 above. Again, a few small peribronchial mononuclear, mostly lymphocytic, aggregates.

Slide 5: LUNG 3, LEFT LOBE PRE Aug 20/08: Section shows patchy atelectasis and mildly prominent peribronchial fibrous tissue, often not circumferential.

Slide 6: LUNG 3, LEFT LOBE POST Aug 21/08: Section a little more inflated vs. slide 5 above. Peribronchial fibrosis is not quite as prominent with greater inflation.

Slide 1: LUNG 4, LEFT LOBE PRE Aug 13/08: Section examined is quite atelectatic with small areas of moderate inflation only. There is a small aggregate of mononuclear cells, mostly lymphocytes, between a cartilage plaque and a small tertiary bronchus. A few mitotic figures are seen within the lymphoid population.

Slide 2: LUNG 4, LEFT LOBE POST Aug 14/08: This section is more inflated, not completely but with large areas where alveolar walls are distinctly monolayered. There are very small mononuclear aggregates beside a single small bronchus. Cell population as above, mostly lymphocytes, with plasma cells occasionally seen.

Slide 17: LUNG 4, LEFT LOBE PRE Sept 10/08: Section is quite atelectatic. There is one small peribronchial mononuclear, mostly lymphocytic, aggregate.

Slide 11: LUNG 7, RIGHT LOBE PRE Aug 20/08: See slide 12 below.

Slide 12: LUNG 7, RIGHT LOBE POST Aug 21/08: More inflated vs. slide 11. Both show very mildly prominent peribronchial fibrous tissue.

Slide 3: LUNG 8, LEFT LOBE PRE Aug 20/08: Section is quite atelectatic with small areas of moderate inflation only. Bronchi often contain ghost-like material, interpreted as sloughed epithelial cells, mixed with erythrocytes and small bits of amorphous eosinophilic debris interpreted as proteinaceous fluid.

Slide 4: LUNG 8, LEFT LOBE POST Aug 21/08: Section is moderately more inflated compared to slide 3 above with areas of atelectasis remaining. Similar material is seen within bronchi, less ghost-like in appearance, with cell detail consistent with
sloughed epithelial cells.

08/18/11
Dr. Jan Bystrom, DVM, MSc, Veterinary Pathologist

End of Report
Samples submitted for testing become the property of the University of Calgary Faculty of Veterinary Medicine and may be used for teaching purposes.
It is the responsibility of the referring veterinarian to release results to the owner.