The Comparison of Airway Responses of Normal Horses Fed Round Bale versus Square Bale Hay

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ABSTRACT

Background – Feeding horses round bale hay (RBH) has been associated with airway inflammation. The purpose of this study was to determine if horses fed RBH for a 6-week period demonstrated more evidence of airway inflammation than horses fed square bale hay (SBH) of comparable quality.

Hypothesis - The respiratory health of horses fed RBH will not differ from horses fed SBH of comparable quality.

Animals – Two feeding groups of 15 healthy horses (mixed ages, breeds) from the University riding program.

Methods – This was a prospective study performed during fall of 2009. At the beginning and end of a 6-week feeding trial, horses were examined (physical, upper airway endoscopic) and samples (tracheal aspirate (TA), bronchoalveolar lavage (BAL)) collected for cytology and/or bacterial/fungal culture. Hay was analyzed for nutritional value and bacterial/fungal content.

Results – Horses fed RBH demonstrated an increase in pharyngeal lymphoid hyperplasia ($p=0.0143$) and percentage neutrophils ($p=0.0078$) in the TA samples post-feeding as compared to pre-feeding values. Nutritional analysis of hay and measurements of bacterial/fungal load did not differ over time and/or between hay types.

Conclusions and clinical importance – The identification of airway inflammation in the horses fed RBH indicates that factors associated with the manner in which the hay is fed and consumed contribute to the development of subclinical airway inflammation. RBH affords horses continuous daily exposure to hay and as horses bury their muzzles in the bale, exposure to particulate matter is likely increased. These factors may partially explain the response in horses fed RBH. Further studies are required to confirm these predictions

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ABBREVIATIONS

RBH Round bale hay

SBH Square bale hay

BAL Bronchoalveolar lavage

BALF Bronchoalveolar lavage fluid

TA Tracheal aspirate

TM Tracheal mucus

PLH Pharyngeal lymphoid hyperplasia

PDA Potato dextrose agar

ASP *Aspergillus* selective media

FSM *Fusarium* selective media

RAO Recurrent airway obstruction

IAD Inflammatory airway disease

FLD Farmer's lung disease

BW Body weight

UV Ultraviolet

BSA Bovine serum albumin

Chapter 1

Literature Review

1.1 Economics of Horse Ownership

Costs associated with owning a horse include the initial expenses of the animal and associated supplies, and equipment and the on-going costs of veterinary care (including vaccinations, dental exams and deworming), farrier care, feed (grain and hay), bedding and/or boarding fees, among others. Over the past 10 years (2000 – 2010), the price of small square bale horse (SBH) hay has nearly doubled, while the cost of round bale hay (RBH) has remained relatively unchanged[1]. Packaging hay in large rounds bales appreciably reduces harvesting expenditures. In Virginia in March of 2012, the price of large "good quality" round bales (>1000 pounds (lbs.)) of grass hay was approximately $0.03/lb. (based on a weight of 1000 lbs.), while equivalent quality small square bales (35-45 lbs.) averaged $0.13/lb.[1] (based off an estimate of 40 lbs.). A horse that weighs 1,000 lbs. will consume approximately 20 lbs. of hay (2% of body weight) per day[2]. Using the above prices, this equates to approximately $0.60 per horse per day ($18.00/month) if feeding round bales and $2.54 per horse per day ($76.20/month) if feeding square bales. Round bales save in direct cost, and also offer ease of labor as compared to square bales if the equipment is available. Moving round bales from the field takes one-half the machinery time and one-tenth the labor of moving square bales from the ground to the barn[3].

1.2 The Unwanted Horse

The unwanted horse has become an ever increasing problem in the United States, especially after closure of the horse slaughter plants in 2007. The Unwanted Horse Coalition (UHC) is an organization that developed out of a 2005 American Association of Equine Practitioners (AAEP) initiative and is financially supported by participating organizations in the American Horse Council (AHC), which includes national, regional and local associations of veterinarians, breed registries, horse associations, breeders, performance groups, owners and equine publications[4]. The mission statement of the UHC is: "To effectively reduce the number of unwanted horses in the United States, and to improve their welfare through education and the efforts of organizations committed to the health, safety and responsible care of the horse[4]. The "unwanted horse" was defined by AAEP in 2005 and has since been adopted by the UHC as "horses which are no longer wanted by their current owner because they are old, injured, sick, unmanageable, fail to meet their owner's expectations, or their owner can no longer afford them[4].

In 2007, it was estimated that each year approximately 170,000 additional horses become "unwanted". Few studies or surveys have been performed to document the facts of unwanted horses in the United States, but horse industry experts and horse owners have hypothesized that the number of

unwanted horses is increasing and that the problem is becoming more severe due to the declining economy, rising costs of hay, the drought that has affected several parts of the United States, the costs of euthanasia and carcass disposal, and closing of the nation's horse processing facilities[4]. In 2008, the UHC conducted a survey (published as the "2009 Unwanted Horses Survey") that consisted of two questionnaires (one for horse owners, one for industry stakeholders) that were focused specifically on the problem of unwanted horses with three objectives in mind:

- Develop a comprehensive assessment and magnitude of the unwanted horse problem
- Provide factual evidence for decisions relating to solution-based programs and policies.
- Establish a baseline for measuring progress in generating awareness, education and action.

This survey indicated that the number of unwanted horses, including the number of those neglected and abused, is growing and has become a bigger problem within the last year (2007) compared to what it was three years prior (2005)[4]. Most of the survey participants (87%) indicated that in the past year the number of unwanted horses has become a "big problem" compared with only 22% who said it was an issue three years prior. Sixty-three percent of rescue facilities reported that they were at full or near full capacity and on average turn away 38% of horses that are presented to them. The reasons why horses became unwanted varied in this report, but the number one most commonly cited reason a horse became unwanted was economics (affordability)[4].

It was estimated that the annual budget to care for one horse at Rescue/Retirement/Adoption Facilities was $2,300[4]. Survey participants were asked to choose the "most appealing solution to the problem of unwanted horses", and in the top 10 of most appealing choices was "increase options to find free or low-cost feed for unwanted horses". Virginia is an important player in the national horse industry, ranking 12th in the number of horses, according to estimates made for the American Horse Council. In March of 2011, a study was published that looked at the economic impact of the horse industry in Virginia[5]. The survey population included Virginia horse owners, horse farms, breeding facilities, boarding facilities and other horse-related private and commercial operations that had horses. The survey used expenditure data for horse operations and equine population figures that were reported in the *2006 Virginia Equine Survey Report*, and each category of expenditure was inflated to 2010 price levels. This adjustment corrected for price increases in items such as feed and bedding, which have fluctuated widely since the survey. Feed and bedding was the highest expenditure category. In adjusted 2010 prices, the total expenditure reported for feed and bedding in Virginia was $116,019,108, which when broken down averaged $2,830 per operation or $540 per horse (for one year)[5]. With the average price per pound of round bale hay being approximately a quarter of that of square bale hay, feeding round bale hay appears to be a viable option (for those equipped to handle round bales) for reducing the annual budget to care for a horse[4,5].

1.3 Forage Sources

Horses, being herbivores, should be fed a diet based on forages. There are a variety of forms of forages that can be safely fed to horses, and local availability often influences how popular a specific variety of hay is[6]. Hay can be harvested and processed several different forms that have specific benefits and/or disadvantages, including[7]:

- Square bales: Relatively small bales varying significantly in weight (40-80 lbs.) that are easy to handle and store.
- Large round bales: Large bales weighing between 800-1200 lbs. that save on labor, but may be difficult to move, and can feed a large number of horses at one time.
- Hay cubes: Made from a variety of types of hay and can be bagged, with the composition guaranteed on the bag. Usually these have little waste, but are expensive.
- Chopped hay: Hay that has been chopped to 1-inch in length and then packaged. This type may be more palatable and easier to masticate for horses that cannot chew well, but is also expensive.
- Hay pellets: Easy form of hay to feed with less wastage by horses, takes up less storage space, and is cheaper and easier to transport. Increased wood chewing has been associated with pellet feeding (when compared to long-stem forage feeding)[8,9].
- Hay Silage: Can be made from forages harvested at moisture levels from 50-70% and stored, but the risk of spoilage and toxic substances is higher. Silage should only be fed if it was managed correctly and if it will be consumed quickly after being exposed to air.

Several factors should be considered when deciding the type of hay to feed to horses, including cleanliness, nutrient value and the intended use of the of horse being fed [6]. Factors that influence the nutrient value of hay include type of hay and the stage of maturity at which it was harvested[6]. Alfalfa and red clover, both legumes, are usually higher in protein and calcium and can be higher in total energy and digestible nutrients than grass hays, such as timothy, orchardgrass and bermudagrass. Hay harvested during early maturity is usually more energy dense and palatable. Hay harvested during late maturity usually has lower nutrient values and is not as palatable[6]. To determine the nutrient content of hay, a chemical analysis should be performed on samples collected with a forage core sampler. A typical nutritional analysis will determine moisture, crude protein, neutral and acid detergent fiber levels (determines energy content), calcium and phosphorus levels[6].

1.4 Round Bale Hay

The round hay baler was invented by a Nebraskan farmer named Ummo F. Luebben (1867 – 1953) in the early 1900s and patented in 1910[10]. The manufacturing rights were then sold to the company, Allis-Chalmers, in 1940, which further developed the machine and sold it as the Roto-Baler in 1947[10].

The Roto-Baler produced bales 36 inches in length with a diameter of 14-22 inches. These bales were small, weighing anywhere from 40 to 100 pounds. Twine was wrapped but not tied around the bales, making them prone to breaking open if not handled carefully[11]. The next innovation with round bales came in the 1971 when Gary Vermeer (one of the founders of Vermeer Manufacturing Company) and engineers developed the "One-Man Hay System"[12]. This design used belts to further compacted hay into the large familiar round bales of today[13]. The round baler has since become a popular method for harvesting forage that can later be fed to horses, cows and other livestock species[14]. Round bale hay is easier and cheaper to harvest compared to square bale hay because the packaging systems allow for one person to harvest, store and feed large quantities of hay; however, the round bale's size and weight may make it difficult to feed, depending on horse-housing situation (pasture vs. stall) or if the necessary equipment to move round bales is unavailable[15]. Disadvantages of harvesting hay as round bales includes decreased salability (compared to square bales) in the open hay market, difficulty in transporting round bales for long distances, and potential losses from baling, transporting, storage and feeding[15]. During the harvesting process, baling losses of 1-5% can be expected for square bale alfalfa hay, whereas losses from 3-30% can be expected for large round bales. The moisture content of the hay at harvesting, baler pick-up and baler chamber mechanisms all contribute to the amount of hay lost[15]. Hay baled at greater than 15% moisture has less leaf loss than hay baled below 15% moisture (dryer hay leads to increased leaf breaking and crumbling). The pickup mechanism of the round baler also contributes to hay loss (typically 1-3%, but up to 12%)[15]. Pick up losses can be reduced if the baler's forward speed matches its rotational speed so that the windrow (row of cut hay) moves into the baler smoothly, with little disruption. The windrow's width should also match that of the baler to reduce the amount of hay lost at either end as its picked up[15]. In regards to bale chamber losses, there are several contributing factors, including hay moisture levels (same mechanism as mentioned previously), windrow size, field speed, bale rotating speed and wrapping of twine. The feed rate should be as high as possible (utilizing large windrows with high forward speeds) to minimize the number of turns within the bale chamber, which also reduces chamber losses. It has been shown that bale chamber losses are minimized when the amount of time to create a bale is also minimized[15]. The amount of hay lost in the bale chamber is typically greater than the amount of hay lost during pickup, meaning it is better to accept some pick up losses by driving faster in order to reduce the amount of time needed to form a bale. When wrapping round bales in twine, the minimum number of rotations to secure the bale should be made. This reduces the amount of loose hay pieces and leaves that fall out of the bale[15].

1.5 Factors Affecting Hay Quality

Moisture

The primary factor that affects the development of mold and dust in hay is the moisture content at the time when it is baled. It is also the largest single factor that contributes to leaf loss[15]. The moisture level of the hay as it enters the baler will also determine the amount of field loss and quality of hay after storage. The moisture content at baling will affect the quality of both round and rectangular bales, but tends to be more critical with round bales because they are larger and tend to be more densely packed[15]. Unless a preservative is used, the moisture content in the hay at the time of baling should be no greater than 18-20%[16]. When hays are too wet at baling, microbial respiration causes hay to heat spontaneously[17]. Consequences of this heating include oxidation of nonstructural carbohydrates[18], mold growth and production of associated toxins[19], and increased concentrations of fiber components and heat-damaged nitrogen[20].

Harvesting hay can also be affected by poor drying conditions (high humidity, heavy dew), poor harvesting techniques and rainfall, and in an attempt to avoid rainfall, hay is often baled before it has dried adequately to recommended moisture levels. This results in the development of mold and poorer quality forage[18,19,21-25]. The recommended moisture at the time of baling is dependent on several factors, including bales size[25]. A threshold moisture of 20% is recommended for small rectangular bales[26], but the threshold moisture recommendations for satisfactory storage may be less than this for larger bales, such as large round bales. Hays baled at a moisture level of less than 15% are assumed to be relatively stable and show hardly any evidence of microbial respiration[27], but this hasn't been studied well in larger bale types or for cool-season grass hays[17,25]. A study published by Muhonen, et. al, in 2009, investigated wrapping individual round bales of grass hay (timothy and meadow fescue) baled at 35% moisture in plastic[28]. This study found that bales wrapped at this moisture level exhibited minimal fermentation, and horses tolerated being fed it with no adverse health effects[28]. Another study, published in 2011 by K. Martinson, et. al, studied the effects of initial bale moisture and plastic wrapping on internal bale temperatures, forage quality and mold formation in large round bales of orchard-grass hay[25]. This study included 40 round bales (1.2 x 1.5 m^2) that were baled and tied with three revolutions of net wrap (Deere and Co., Moline, IL). The bales were made over a two year period (16 in 2008, 24 in 2009). The forage was harvested at three different targeted moisture ranges: 1) Low moisture (LM) at < 15% moisture; intermediate moisture (IM) at 18-25% moisture and high moisture (HM) at 30-25% moisture. The bales were then stored outside on a sod-surface (well drained); in one continuous row running east and west. Bales within each treatment group were set tightly against one another. A 'nontreatment' bale (bale not included in the study) was placed between each treatment group and on each ends of the row. In 2008, there were four treatment groups: LM, IM, HM and HM wrapped with plastic (HMW). In 2009, there were six treatment

groups: LM, LMW, IM, IMW, HM, and HMW. Each bale was cored six times after baling and analyzed for forage quality. After sampling, three temperature data loggers were placed in each bale at different depths from the top of the bale, and the hays in the HMW (2008) and LMW, IMW and HMW (2009) groups were wrapped six times with one mil plastic wrap with a bale wrapper. Temperatures were recorded every hour for seven (2008) or 10 (2009) weeks and heating degree-days (HDD) computed as the summations of the daily increment by which the average internal temperature was greater than 30°C[29]. Upon removal of the temperature sensors, the bales were sampled again for forage quality and mold counts and mold identification. Orchard-grass hay baled at LM (12.4%) had low mold counts and lower HDD accumulations compared to unwrapped hays baled at higher moisture levels. Orchard grass hay baled at a moisture level of 16.6% had HDD accumulations, mold counts and negative changes in forage quality similar to hay baled at 23.2% or 29.8% moisture. Martinson's 2011 study concluded that large round bales of orchard grass are susceptible to molding and forage quality loss at relatively LM concentrations. Forage quality and a reduced mold growth can be maintained if hay is baled dry (12.4% moisture) or if wrapped (regardless of initial moisture levels), but the longevity of forage quality of higher moisture bales after unwrapping (especially during the summer months or with intermittent feeding) needs further investigation[25]. Martinson's 2011 results are also in agreement with previous research that determined alfalfa and mixed grass-legume hays baled at moisture concentrations less than 15% are relatively stable in regards to mold growth and forage quality[23,25,30].

Packaging

In 1999, G. Ranalli, et. al., published a study[31] that investigated the influence of hay-packing techniques on the presence of *Saccharopolyspora rectivirgula*, a thermophilic actinomycete found in moldy hay that has been known to cause extrinsic allergic alveolitis (farmer's lung disease) in farm workers. In this paper, small prismatic bales (average weight of 55-66 lbs., baled at 17-20% moisture) were compared to large cylindrical bales (average weight of 1100 lbs., baled at 20-25% moisture). The bales were harvested from the same field on the same day. After baling, small prismatic (rectangular) bales were picked up and stored under a shed at 24 hours, whereas the large cylindrical bales were picked up and stored in vertical piles under haylofts (with ventilation) after one week. The bales were stored for 3-6 months before being fed to cows and were sampled before and after feeding. When combining the viable microbial counts of micromycetes, thermophilic actinomycetes and aerobic heterotrophic bacteria (total bacteria) in samples taken from small prismatic and large cylindrical bales from the two farms, the large cylindical bales had significantly higher counts (of all three types of organisms) compared to the small prismatic bales. Overall, *S. rectivirgula* was present in 66.6% of all samples from the small prsismatic bales and in 100% of all samples from large cylindrical bales. Although this paper indicates large cylindrical bales are more "moldy" compared to small prismatic bales, this conclusion should be

taken lightly because the two hay groups were not equal. The large cylindrical bales were baled at higher moisture content and were exposed to the outdoor elements (rain, dew) for six days longer than the small prismatic bales were, before being stored. It is possible these two elements also influenced the microflora content of each hay type.

Storage

Large round bale hay may be stored in a variety of ways. Storage outside on the ground represents the cheapest method of hay storage, but is accompanied by the potential of having the greatest losses in dry matter due to weathering. Most of the loss occurs on the bottom of the bale (high moisture levels, little air movement). To reduce storage losses of round bale hay stored outside, hay should be placed off the ground. Several substrates can be used as a barrier between the hay and ground, such as poles, pallets, tires, and crushed rock. Placing bales on one of these types of materials can reduce storage losses by as much as 38%[32]. If stored outside, round bales should ideally be placed in a well-drained, sloping location with a barrier between the hay and ground and end-to-end in long rows as tightly as possible (facing south or southeast to maximize drying from the sun) so that the bales retain their shape. If more than one row is required, rows should be spaced at least three feet apart so that the hay receives sufficient air flow and sunlight reaches the back row of to help dry it out[15,32].

Circumferentially wrapping large round bales (plastic or net wraps) is another way to reduce dry matter losses. Some research has shown that wrapping round bales in ultraviolet light-stabilized plastic reduces dry matter losses to 7% as compared to 35% from unwrapped bales stored outside on the ground[32]. Net wraps are porous and are designed to shed water and allow greater airflow to the bale. Hay loss with net wraps varies between losses of bales wrapped in either plastic or twine (and stored outside)[32]. Other options for protecting round bales stored outside includes plastic tarps, plastic bale sleeves, and covered storage facilities. When tarps are used, they should be durable and treated to tolerate UV light. Bale sleeves can be added to twine-tied bales, and storage losses are similar to those bales wrapped in plastic wraps stored inside. The disadvantage of plastic sleeves includes higher material cost (in addition to requiring twine) and additional labor needed to move the bales[32]. Table 1 shows the comparison of several storage systems in regards to storage life, approximate cost per 1,000 pound bale, and dry matter losses.

Table 1: System life, approximate cost and dry matter loss with various hay storage systems[32]

Storage System	System Life (Years)	Approximate Cost ($/bale/year)	Dry Matter Loss (%)
Conventional shed	20	5.00	4-7
Pole structure with plastic roof on pad	4	3.00	4-7
Resuable tarp on pad (stacked bales)	5	3.00	4-7
Bale sleeve on ground	1	3.00	4-7
Plastic wrap on ground	1	1.50	15-25
Elevated stack on pad (rock plus filter fabric)	20	2.62	13-17
Net wrap on ground	1	1.50	15-25
Stacked on ground (cost is twine)	1	0.75	25-35

Much of the hay fed to livestock is packaged as round bale hay for a variety of reasons, as mentioned previously. The size of these bales and rapid baling rates minimizes the labor requirements for packaging and local transport[32]. Unfortunately, storage losses with large round bales are greater than those of small square bales, which in part is due to their shape. A seemingly insignificant layer of damaged material on the outside of the bale actually represents a large loss of yield and quality based on the total volume of hay[32]. For example, a two-inch layer of damaged material on the outside of a round bale (four feet by four feet) represents 16% of the bale volume, even though only less than 7% of the diameter is affected (this calculation excludes the end of the bale, but little loss occurs there)[32]. Most of the in storage loss appears to result from storage outside and no protection from the weather[32]. Twine-tied round bales stored outside suffer losses due to weathering and from moisture that wicks into the bales from the ground. In addition to actual hay losses, weathered hay also deteriorates in regards to forage quality[32]. When considering the economics of feeding round bale hay, aside from the costs associated with wrapping hay and dry matter losses, other factors should be considered, such as the cost and estimated life of the storage structure/method and its value in terms of the amount of hay saved[32].

A study published by R. L. Belyea, et. al, in 1985 compared the storage and feeding losses of alfalfa hay packaged as both round bale and square bale hay[33]. The round bales were stored in a variety of fashions; 1) in a barn, 2) outside in single rows and uncovered, 3) outside in two-high stacks and covered and 4) outside in three-high stacks and covered. Square bales were stored in a storage barn. The hay was harvested in July and August. Bales remained on the field for several days before being placed in their respective storage locations and were fed to dairy heifers during the winter. Hay was placed in feed bunks under cover so heifers had constant access (square bales were broken up, round bales were cut

manually into circles). Enough hay was fed so that each group had 5-10% feed refusal. Refused hay was gathered, weighed and sampled for moisture daily and the body weight of each heifer was measured on two consecutive days at three intervals during the study (beginning, middle and end). The results of this study showed that storage and feeding losses for round bale hay were the highest for the round bales stored outside, uncovered (15.0 and 24.7%, respectively). Storage losses were the least for the round bales stored inside the barn (2.5%). There was no difference in the feeding losses of round bale hay stored inside or outside and covered (12.4% (inside), 14.5% (two-high, covered), 13.4% (three-high, covered)). Feed intake and weight gains were the greatest for the small square bales (2.35 kg/100kg body weight and 0.77 kg/day, respectively) and least for the large round bales stored outside uncovered (2.11 kg/100 kg body weight and 0.54 kg/day). For the round bales stored outside, rain penetrated 10-25 cm into the bales which resulted in the weathering and deterioration of approximately 40% of the hay (in dry weight).

1.6 Round Bale Feeding Practices

For good digestive function, horses require a minimum of one percent of their body weight daily as long-stem forage[7]. Limited acreage, poorly productive pastures, seasonal variation in rainfall and the need to house horses separately or indoors may necessitate the feeding of hay. Round bale hay can allow horses constant access to hay. Horses with ab libitum access to forage may spend up to 64% of their time eating[34,35]. If round bale hay is fed to horses, it should be fed in a manner so that the number of horses present will consume the hay within a few days[6]. If left to sit on a pasture for too long, round bale hay will mold, or horses may consume too much and over eat. As mentioned previously, waste from round bale hay occurs both during storage and feeding. The storage losses of round bales can range from 2-40% of dry matter, depending on the type of forage, storage method, environment and storage length[33,34,36,37]. Hay feeders can reduce hay waste and improve economics during horse feeding. Feeding hay in a hay feeder (as opposed to loose on the ground) reduces the amount of hay horses waste by 20% or more[6]. A study published in 2012 by K. Martinson, et.al, compared nine round-bale feeders and a no-feeder control and found that feeder design did not affect hay intake[34]. All the hay feeders resulted in an estimated hay intake of 2.0-2.4% body weight (BW). The no-feeder control actually resulted in a reduced intake of 1.3% BW and resulted in greater pen (group) BW loss than six of the nine feeders tested[34]. Feeder design affected hay waste, which ranged from means of 5% - 33%, while mean hay waste from the no feeder control was at 57%[34]. This study indicates that a round-bale feeder, regardless of design, is necessary to avoid the 57% mean hay waste, reduced hay intake and body weight lost observed when not using a hay feeder. Although economics were affected both by the purchase price of the hay feeder and hay waste reduction, all round-bale feeders repaid their cost within 20 months[34].

In summary, round bale feeding can be a more economical method of feeding horses. To get the greatest financial and quality benefits and minimize waste from this food source, round bale hay should be baled at ≤ 15% moisture, wrapped or stored under cover and not directly on the ground and fed from a feeder. The size (weight) of the round bale should be chosen based on the number of horses to be fed such that the entire bale is consumed by all the horses within a few days. In situations where there are only few horses eating hay, it may be necessary to feed square bale hay. One potential option for owners of only a few horses (who would still like to feed round bale hay) is to feed round bale hay from a covered feeder off the ground, which may prolong its "pasture life".

1.7 Health Concerns Associated With Feeding Round Bale Hay

While round bales are more economical for feeding horses, owners are reluctant to feed them because of a perceived increased risk to the horse's health. Specifically, feeding round bales has been associated with outbreaks of botulism in horse herds. In addition, feeding round bales is thought to increase the horse's exposure to respirable debris because the animals tend to bury their muzzle in the hay as they fed. The quality of round bale hay may also differ significantly from square bales depending on how the hay is packaged and stored. In addition to round bale hay causing an increased risk associated with botulism, people may have an increased risk of developing farmer's lung disease (FLD). Both conditions are described below.

Botulism

Botulism is a term used to describe the disease that results from toxins produced by *Clostridium botulinum*, an anaerobic, gram positive rod bacterium that is present in the soil, vegetable matter and rotting carcasses[38,39]. Botulism has been reported in most mammals as well as birds and fish in the United States and worldwide[40]. The strains of botulism are classified based on the neurotoxin they produce, including A, B, C2, D, E, F and G, and their distribution varies geographically[38,39]. Types A, B, E, and F cause human botulism, and types A, B, C and D cause most cases of botulism in animals[41]. Reports of botulism in horses have been documented with the strains A-D, but most cases (80%) in North America involve strain B[41]. The spores of *C. botulinum* type B can be found in the soil in most regions of the United States, but are more frequently found in the northeastern and Appalachian regions. *C. botulinum* type C occurs mostly in Florida, whereas *C. botulinum* type A occurs more frequently in the western United States[41]. The botulism toxin is considered to be the most potent biologic toxin identified, and horses are exquisitely sensitive to it[38]. All toxins, whether ingested through feed sources, or produced in the intestines or in a wound, interfere with the release of acetylcholine at the neuromuscular junction by irreversibly binding to the presynaptic membrane, thus resulting in paresis and flaccid paralysis[38,42]. The botulism neurotoxins do not affect the central nervous system or sensory nerves[38]. Once inside a cell,

botulinum toxin is resistant to inactivation by antitoxin, and recovery occurs as new nerve terminals with functional neuromuscular junctions are formed[42-44]. The higher the dose of *C. botulinum* toxin at the neuromuscular junction, the more quickly the disease progresses and the poorer the prognosis[42]. Death usually occurs secondary to respiratory failure from diaphragmatic paralysis[38].

There are three routes of infection with botulism recognized in horses[42]; 1) toxicoinfectious botulism (also known as "shaker foal syndrome"), 2) forage poisoning, and 3) wound botulism. Toxicoinfectious botulism is seen primarily in foals from 2-8 weeks of age, when they begin nibbling hay and grass and consume *C. botulinum* spores from the soil[41]. Ingested spores proliferate and produce their toxin (Type B), which is then absorbed from the immature gastrointestinal tract and transported to the neuromuscular junction[42]. It is believed that the normal intestinal flora of healthy adults horses (and humans) inhibits the growth of botulism spores, which limits the toxicoinfectious form to young animals[42]. Wound botulism occurs when *C. botulinum* infects a wound, sporulates and produces toxin under anaerobic conditions[42]. Wound botulism has been associated with castration, injection abscesses, trauma and surgery in adult horses, and in foals with omphalophlebitis, umbilical hernias (treated with clamps) and in infected leg wounds[39,45,46]. Forage poisoning is the most common route of infection and occurs when horses ingest preformed toxin in feed (typically forages versus grains) or water[42].

Clinical signs of botulism include flaccid paralysis, weakness, muscle tremors, and decreased eyelid, tail and tongue tone, and dysphagia[39-41]. Bilateral, weak palpebral reflexes may be an early sign of botulism, as is decreased tongue tone[47,48]. Colic may develop due to decreased intestinal motility from the effects of the botulinium toxin on intestinal smooth muscle[38]. Dysphagia may lead to anorexia, dehydration, hypoglycemia and/or aspiration pneumonia. Paresis may lead to paralysis and respiratory failure[38]. Clinical signs of botulism from preformed toxin ingestion may develop within hours or days of ingestion of the toxin, and are similar to those mentioned previously with toxicoinfectious botulism[38]. Varying degrees of weakness, muscle tremors, exercise intolerance, reluctance to stand, excessive salivation, dysphagia and reduced eyelid, tongue and tail tone are common[38,42]. Pupillary light response may also be delayed. In severe cases of botulism, horses are unable to stand and may die from respiratory failure, and occasionally sudden death is the only clinical sign[38]. Horses with botulism often appear alert and bright until problems associated with dehydration and aspiration pneumonia develop[38].

The diagnosis of botulism is usually made based on clinical signs and history[38,49]. Confirmation of botulism can be achieved by detecting the toxin in feed, serum, gastrointestinal contents, feces or wound debris, recovering spores in gastrointestinal contents or feed, or detecting antibodies in a recovered patient[49]. The tests available for detecting botulism toxin include the mouse neutralization assay or enzyme-linked immunosorbent assay (ELISA)[49]. The mouse neutralization test is sensitive and takes several days to complete[49]. A negative result with this test does not rule out botulism. Horses are

especially sensitive to the botulinum toxin, so it is possible for the toxin to not reach measurable levels before affected horses begin showing clinical signs (and are tested)[49]. The ELISA test has the advantage of a quick turn-around time (24 hours), but is less specific due to cross reactivity with other clostridial toxins[49].

The primary goals when treating botulism is to neutralize circulating toxin (both multi- and mono-valent antitoxins are available), and provide supportive care (which may include supportive respiratory therapy (oxygen insufflation and or mechanical ventilation)) and antibiotics to reduce the risk of secondary infection from aspiration and/or recumbency[38].

The prognosis of horses with botulism depends on the type of botulism, how severely the horse is affected and how soon treatment is initiated. Survival may be as high a 96% in foals with the toxicoinfectious form of botulism, but approximately 30% of foals will require mechanical ventilation[38]. Cost of supportive care is the primary factor that affects prognosis in foals, and without aggressive supportive care, mortality rates are high[38]. The prognosis of horses that ingest preformed toxin (forage poisoning) depends in the severity of the disease. Horses that are found or become recumbent have a grave prognosis due to the problems associated with prolonged recumbency and the difficulty of providing mechanical ventilation to adult horses[38]. Prompt antitoxin administration is likely one of the most important factors in regards to treating affected horses, but its cost makes it impossible in some cases[38].

The risk of a horse developing botulism can be minimized by following certain management practices. A toxoid vaccine is currently available for type B botulism, which is an effective way of reducing the risk of toxicoinfectious botulism in endemic areas because foals can be protected by ingesting an adequate amount of antibodies in colostrum from appropriately vaccinated mares[38]. Many horse owners vaccinate their horses if round bale hay is to be fed. Vaccination against type B botulism does not offer immunity against exposure to other strains[38]. Proper production and handling of feed is another method horse owners should follow when feeding horses to prevent botulism. Silage and haylage should be considered "high-risk" feed because these types of feeds, if not fermented properly (pH maintained below 4.5) allow for the growth of *C. botulinum* and production of its toxin[38,40].

Fear of forage poisoning is one reason that some horse owners are reluctant to feed round bale hay. This type of botulism mainly affects adult horses and results from the ingestion of preformed toxin. Spoiled hay, haylage and silage are the most common types of feeds implicated, but other forms of feed may also be involved[38], such as small (square bale) alfalfa baled under wet conditions, processed alfalfa hay cubes, plastic-packaged hay that was rained on before baling, feed trough dirt, grass clippings, breweres' grains, vegetable waste and decomposing animal parts[45,50-53]. Occasionally forage poisoning occurs when a wild animal (that has died from botulism) becomes incorporated into the feed material.

Outbreaks of botulism occasionally occur because the affected feed source is often fed to multiple animals[38]. In early July of 1999, 8 out of 15 yearlings and 1 of 2 stallions from one farm developed clinical signs of botulism[54]. The yearlings all died, and the stallion, the most mildly affected, survived. These horses had all been fed large round bale hay. The bales fed to these horses had been stored outdoors, directly on the ground, and the interior portion of the bales showed evidence of mold and moist decomposition and were warm to the touch. No evidence of decomposing animal parts was found. The farm owner reported that the hay was harvested in late May and early June of 1999. In April of 1999, lime and fertilizer had been applied to the hay fields. The hay that had been stored outdoors had been subjected to heavy rain fall for several weeks prior to the outbreak of the botulism. *C. botulinum* was isolated from hay samples taken from the two large round bales that had been fed to the yearlings before the outbreak. This, in addition to the clinical signs of the other horses and favorable response of one stallion to botulism antitoxin administration, provided strong evidence for the diagnosis of equine botulism at this farm[54]. It was postulated that application of lime to the hayfield before harvesting provided an alkaline environment that improved the conditions needed for multiplication of the vegetative form of *C. botulinum* after the spores had germinated, and the heavy rain fall resulted in moist decomposition of the interior of the hay bales. The conditions in the hay bales at this farm (decomposing vegetation, anaerobic environment, high temperature and pH) provided the perfect medium for germination of *C. botulinum* spores, multiplication of the organism and subsequent toxin production[54].

To minimize the risk of a horse developing botulism from round bale hay, the harvesting and storage practices mentioned previously (to produce high quality hay with low mold counts and reduced storage and feeding wastes) should be followed. If horse owners are purchasing hay from an outside source and do not have access to the harvesting data, hay should be inspected for evidence of weathering and spoilage (moist, "moldy" smelling/looking hay) and not purchased if found. The hay should be closely inspected for animal carcasses as it is being unloaded or stored and as the bale is being fed. Hay should be fed so that it is consumed quickly and does not have time spoil. Hay that has been soiled with urine and feces and excessively wet hay from rain should be removed. Additionally, horses can be vaccinated with the botulism type B toxoid annually to help prevent disease from that specific type of botulism.

Hay Exposure and Airway Health: Farmer's Lung Disease

Increased exposure to inhaled debris and the development of airway inflammation is a second concern of horse owners who are reluctant to feed round bale hay. The relationship between an increase in exposure to hay dust and particulate matter and development of airway disease is not well documented in normal horses, but has been recognized problem in human farmers. Farmer's Lung Disease (FLD) is a well-documented respiratory disease of people that is associated with workers in agricultural practice who

deal with hay. Recurrent airway obstruction (RAO) and inflammatory airway disease (IAD) are two respiratory diseases of horses where the clinical signs can be exacerbated with increased exposure to inhaled debris.

Farmer's lung disease is a granulomatous pneumonitis often referred to as allergic alveolitis, and is caused by repeated exposure to high concentrations or prolonged exposure to low concentrations of inhaled antigens (bacterial and/or fungal products) in moldy hay or straw[55-57]. It was the first hypersensitivity pneumonia (HSP) or extrinsic allergic alveolitis described[58]. Unrecognized or untreated, FLD can cause severe disability and death[59]. Farmer's lung disease is often diagnosed based on history alone, and avoiding moldy hay often effectively treats the condition[58]. Classic symptoms of FLD include fever, cough and dyspnea within 4-6 hours after exposure to moldy hay[58]. These symptoms occur due to alveolar and interstitial inflammation in the lung. Farmer's lung disease is more common in wetter climates, especially after a wet harvest season[60]. It may present acutely (classic form) or as a chronic disease with insidious progression[58]. If FLD is not recognized and antigenic exposure continues, 50% of people affected will become disabled within five years and 10% will eventually die due to chronic lung disease (granulomas and inflammation lead to localized or diffuse interstitial fibrosis and pulmonary architectural disruption)[59,61].

The microbial agent classically said to induce FLD is *Saccharopolyspora rectivirgula* (previously known as *Micropolyspora faeni*), a thermophilic actinomycete[57,62]; however, in some regions, such as Doubs, France, *S. rectivirgula* has rarely been isolated, despite a high prevalence of FLD (ranging from 2-4% of farmers) and a previous serological study suggests most farmers in this region had never been exposed to this organism[57,63,64]. Other agents, often in combination, that have been associated with FLD include *Thermoactinomyces vulgaris*, *Thermoactinomyces candidus*, *Thermoactinomyces sacchari*, *Aspergillus spp*., and *Saccharamonospora viridis*[58,65]. In an attempt to identify the putative cause of FLD in eastern France, one study showed that sera collected in FLD patients specifically reacted with some molds, including *Absidia corymbifera*, *Eurotium amstelodami* and *Wallemia sebi,* but *S. rectivirgula* gave negative results[55,57]. The results of this study are also in agreement with another study conducted in Finland, where the authors found the level of IgG against *A. corymbifera* to be three times higher in farmers with FLD than in exposed control farmers[57,66].

Farmer's lung disease is a hypersensitivity reaction to inhaled organic antigens found in moldy hay[61]. The acute form of the disease is best explained by a type III cell reaction[61,67]: moldy hay antigens bind with antibodies in the lung, which in turn bind complement and attract neutrophils. The neutrophils then cause inflammation by releasing enzymes and free radicals[61,67]. The chronic form of FLD is more consistent with a type IV cell-mediated immune reaction[61]. Chronic disease manifests as mononuclear cell inflammation and granulomas. It has also been suggested that sensitized pulmonary alveolar macrophages

become activated by antigen, attract neutrophils and modulate T-cell activity, which leads to the appearance of mononuclear cells and granulomas[61,68].

The only way to prevent FLD is to avoid the causative antigens. Acute FLD is usually self-limiting and is treated symptomatically (supplemental oxygen, antipyretics) and with rest. Each case must be managed individually. Farmers may have to used feed other than hay, apply mold inhibitors, purchase higher quality hay and store it properly with good ventilation. Portable respirators and masks are also effective, but cumbersome to work in. If management changes do not alleviate clinical signs, it is recommended affected individuals leave the farming environment and lifestyle[58].

1.8 Respiratory Immune Function and Response to Airway Contamination

Immune Function

The immune system of the equine respiratory tract is extremely important in protecting the horse from airway and pulmonary disease. It is estimated that a horse inhales approximately 100,000 liters of air daily into its respiratory tract, which has an area of approximately 2,000 square meters[69]. This means that each day and with each breath, the horses' respiratory tract is exposed to thousands of micro-organisms and micro particles[70]. The respiratory defense mechanisms of the horse are well adapted and efficient at removing inhaled particular matter, so under normal circumstances inhalation of this debris does not evoke significant airway inflammation. The equine respiratory system has several defense mechanisms that can be categorized into three broad categories: 1) Mechanical, 2) Innate Immunity and 3) Adaptive Immunity[71] (Table 2). The respiratory immune system is not congruent between the upper and lower airways and alveolar space; each has its own defense mechanisms[70].

Table 2: Defense mechanisms of the respiratory tract[71]

Mechanical	Innate Immunity	Adaptive Immunity
Upper respiratory tract (nasopharynx and larynx)		
• Ciliated epithelium • Sneezing • Arytenoid cartilages and vocal folds • Mucus	• Complement • Proteases • Lactoferrin	• Secretory IgA and IgM • IgG subclasses (IgGa and IgGb) in mucus layer and lamina propria • Full complement of lymphocyte subsets (including inflammatory CD4 cells and cytotoxic lymphocytes in organized (tonsils) and dispersed lymphoid tissue in mucosa
Lower respiratory tract (tracheobronchial tree)		
• Mucociliary clearance • Coughing	• Recruited neutrophils • Alveolar macrophages	• Secretory IgA and IgM (as in upper respiratory tract), plus increasing amounts of IgG • Lymphocyte subsets (as in upper respiratory tract)
Lung parenchyma (alveoli and lung interstitium)		
• None	• Surfactant products • Phagocytic cells (alveolar macrophages, neutrophils) & their products	• Parenchymal lymphocytes and recruited lymphocytes (inflammatory response)

Taken from Lunn, et al, 2007, which was adapted from Pilette et al, 2001

The initial mechanical defense mechanism of the respiratory tract is found within the nasal and oropharyngeal passages. Here, particulate material is deposited on the surface of these structures and is eliminated through coughing, sneezing and mucociliary clearance[70]. Particles inhaled further into the respiratory system area also removed in this fashion and if highly soluble, may move into the bronchial circulation and lymphatics[72]. Highly soluble noxious gases are primarily absorbed in the upper respiratory tract, and less soluble noxious gases are inhaled deeper into the lungs. Mucociliary clearance is an extremely important function of the horse's respiratory immune system. Ciliated epithelium is present in much of the horses' nasopharynx, upper and lower airways, extending down to the level of the terminal bronchioles[73]. Mucociliary clearance rate is determined by ciliary amplitude and beat frequency, and the physical properties off mucus[74,75]. Mucociliary transport is affected by gravity and is faster when a horse's head is down compared to when it is up. The average velocity of tracheal mucus in a normal healthy horse is 2 cm/minute, but this rate may vary greatly between individual horses[76]. A loss of 50% of the cilia is required for there to be a reduction in the clearance rate[75]. The deeper lung parenchyma does not have any mechanical defense mechanisms, and therefore relies on the phagocytic system to remove particles and micro-organisms[77].

In addition to physical defense mechanisms, adaptive immunity and innate immune responses are also important in protecting the respiratory system from pathogens and harmful micro particles. The innate immune system may become activated from a variety of sources, including respiratory epithelial cells. Cytokines are released, which recruits phagocytic myeloid cells and lymphocytes[71]. These cells then serve as antigen presenting cells to begin the adaptive immune responses by lymphoid cells[71].

Local mucosal immunity is extremely important in dealing with respired debris at its site of deposition and is composed of mucosal lymphoid tissue, mucosal mast cells, mucosal plasma cells (which produce immunoglobulins), local free immunoglobulins, lymphocytes and macrophages[78]. Immune responses that are initiated at one location are transmitted throughout the mucosal immune system by lymphocytes[71]. Secretory immunoglobulin A is the primary immunoglobulin produced by the mucosal immune system. This immunoglobulin protects the respiratory system from bacteria and viruses by immune exclusion (the molecule itself physically prevents the microbes from attaching to the mucosal surface)[71].

Particle Deposition

As horses respire, they are constantly exposing their respiratory tract to airborne particles, gases and microorganisms. Where the particles distribute depends on the particle size and density. Larger particles (5-10 microns in size) typically become trapped in the nose, mouth, pharynx and/or larynx. Smaller particles (< 5 microns in size) can move into the deeper airways[79]. Particles and aerosols are deposited into the respiratory tract by three main mechanisms: 1) inertial impaction, 2) gravitational sedimentation and 3) brownian movement[79]. Inertial impaction occurs when larger particles (> 1 micron in diameter) experience fast flow rates (≥ 0.5 L/sec) in the section of the airway where total cross sectional area is low (trachea and large bronchi)[80]. Sharp bifurcations influence the deposition of large particles, as they are unable to stay suspended as airflow changes directions within the airways. These particles tend to be deposited in areas such as the carina or other bronchiole bifurcations[81].

Particles less than 5 microns traveling at lower velocities penetrate into the smaller airways and are deposited by gravitational sedimentation. At these lower air velocities, the gravitational force is no longer opposed by air movement, which causes particles to settle onto the airway walls[80,81]. Particle sedimentation increases when respiratory rate increases (example: with exercise) or during periods of time when the breath is held or when breathing is slow and steady[80].

Finally, very small particles (< 0.5 μm) that are inhaled into the smaller airways are deposited by Brownian diffusion as they are displaced by gas molecules (moving randomly) and connect with the airway walls[81]. Brownian diffusion deposition occurs when airflow is extremely slow and particles are too small/not dense enough to settle into the bronchiole wall by gravity. Most of these particles (90%), however, remain suspended in the air and are exhaled[80,81].

Dust particles may deposit into the upper airways, tracheobronchial tree, and respiratory or alveolar region by the three methods described above. The sizes of typical particles found in dust are listed in Table 3, and factors influencing deposition are listed in Table 4.

Table 3: Typical sizes for common aerosol particles[81]

- Tobacco smoke: < 1μm
- Pollens: Approximately 10 – 30 μm
- Mold spores: Approximately 2-50 μm
- Actinomycete spores: Approximately 1 – 2 μm
- Foundry dusts: Approximately 50 mm

Table 4: Major factors determining particle deposition[81]

- Respiratory tract anatomy
- Patterns of airflow
- Mode of inhalation
- Particle characteristics (size, density, charge)

Airborne dust found in equine stables varies and is composed of a mixture of bacteria, viruses, molds, mite debris, feces, plant material, bacterial endotoxins, β-glucans and inorganic dusts[82]. The probability that a certain respiratory challenge induces respiratory disease in a horse depends on the susceptibility of the horse, pathogenicity of the organism, and the number of active pathogenic organisms retained within the respiratory tract at any one time[83]. These organisms and particles induce airway inflammation by a variety of mechanisms, including initiating infection, inducing allergy or as a direct toxicant, or indirectly by overwhelming the pulmonary defense mechanisms[84]. There are three main components that must be studied when assessing the risk associated with particle inhalations: 1) the quantity of particles, 2) the proportion of respirable (particles that will enter the intrathoracic respiratory system) and non-respirable fractions and 3) the composition of the dust, especially the number of pro-inflammatory agents, such as microbes, endotoxins, aeroallergens and proteinases[84]. There are several sources of dust in a stable, but the main ones include feed and bedding with the highest exposure of respirable particles coming from forage and/or bedding contaminated with mold[84].

In humans breathing through their nose, 90% of particles 2 – 20 μm in diameter are retained in the upper nasal airways[81]. Approximately 50% of particles 1-5 μm in diameter remain in the trachea and bronchi, and only a few particles 10-20 μm in size reach the tracheal and bronchial areas. Approximately

50% of particles 1-5 μm in size remain in the alveolar space. Rarely do particles greater than 10 μm reach the alveoli[81]. As mentioned above, the primary sources of dust in a horse stable are feed and bedding. While a stall is being cleaned/mucked out, dust levels may be as high as 10-15 mg/m^3, with 20-60% of that being respirable particles. It has been reported that grain elevator operators exposed continuously to dust levels of 5 mg/m^3 show a severe decline in pulmonary function, as demonstrated by a decrease in forced expiratory volume in one second (FEV_1) associated with bronchial hyperreactivity[85]. Unfortunately the threshold level of dust known to contribute to respiratory disease is not known. Dust levels greater than 2 mg/m^3 are considered high and it is recommended to keep levels below 0.5 – 1.0 mg/m^3 until more defined limits can be established[86].

Ventilation (the process of "changing" or replacing air in a space) is the primary mechanism for effective clearance of mold spores[83], however it would be best to eliminate them at their source[87]. A ventilation rate between 4 and 8 air changes per hour is recommended to minimize exposure to airborne molds and ammonia, which occurs in most normal stables when the barn doors and top half of each stall door is left open at all times[86,88]. An ideal stable has an opening (0.3 m^2) in each stall and an opening of 0.15 m^2 in the ceiling[84,89]. When all stable doors are closed (which is common in winter months), ventilation rates are below 4 air changes per hour[86]. When hay and straw are used (as in a classic equine management system), the airborne dust levels may be 6-7 times greater in the horse's breathing zone than the stall itself. Dust levels in the horse's immediate breathing zone are not affected by the ventilation rate, which is why changing the quality of forage and bedding is essential to decrease dust levels[84,90].

1.9 Pathogenesis of Airway Disease

There are several factors involved in the pathogenesis of lower airway disease in horses, including exposure to respiratory viruses, bacterial infections, specific hypersensitivity (antigen-antibody reactions), inhalation of dusts, endotoxins or irritating gases, or as part of the exercise induced pulmonary hemorrhage (EIPH) complex[91]. Equine tonsilar tissue comes into contact with a great number of foreign material and antigens on a daily basis. Immunological tolerance, the property of the immune system that allows for discrimination of self and non-self-antigens, is important because it is the process through which the immune system protects the host from external pathogens without provoking autoimmune disease[92].

Inflammation is the primary cause of almost all the changes that occur in chronic airway diseases[93]. Once the inflammatory cascade is initiated and cytokines and inflammatory mediators released, and each one has its own effect on the airways. RAO, a chronic lower respiratory disease in horses characterized by an increased breathing effort, coughing, airway hyperreactivity and mucus and neutrophil accumulation in the airways[93], is the disease in horses in which these events have most

extensively been studied[94]. The exact mechanism in which the environment leads to chronic airway inflammation and clinical signs of RAO is not completely known, but it has been suggested that the disease results from a non-specific inflammatory response (airway neutrophilia) to inhaled pro-inflammatory agents, such as molds, endotoxins, particles and noxious gases[95]. Moldy hay exacerbates RAO, so it has also been described as a hypersensitivy reaction to molds, with the spores of thermophilic actinomycetes such as *Aspergillus fumigatus*, *Thermoactinomyces vulgaris,* and *Faeni rectivirgula* being most important[91]. After exposure to stable dusts, a RAO susceptible horse experiences an influx of neutrophils into the lung within 7 hours[96], which is detected by cytological examination of bronchoalveolar lavage fluid (BALF). Along with development of lower airway neutrophilia, proinflammatory mediators are released, which induce bronchospasm[91]. Proinflammatory mediators involved with bronchospasm may include histamine and leukotrienes[91,97,98].

Airway hyperresponsiveness is a term that describes when the airways over react to a nonallergenic or non-sensitizing stimulus[91,99,100]. As an example, a normal horse inhaling a solution containing 0.1 mg/mL of histamine does not develop bronchospasm, but a RAO affected horse does, and quite severely[91]. The RAO affected horse is then said to have hyperreactive airways, which is associated with spontaneous bronchiolitis and bronchoconstriction after exposure to antigens, such as hay molds. It is not known how much stable dust is required to induce hyperresponsiveness, but a RAO affected horse stabled for seven hours will have hyper responsive airways that persist for at least 72 hours[91,101].

Subclinical airway inflammation is thought to be common in horses, but little information is available on its prevalence and none on risk factors[102]. It is well known that a relationship between stabling, hay feeding and respiratory disease exists, and stabling exposes horses to high levels of debris which induce airway inflammation[90,103,104]. In addition airway obstruction can be induced in RAO-susceptible horses by stabling them, as well as exposure to hay dust; the obstruction can be reversed when dust levels are reduced[103,105-109]. In 2006, N.E. Robinson, et. al., attempted to determine the prevalence of airway inflammation and elucidate risk factors in pleasure horses in Michigan[102]. This was the first investigation of the risk factors for airway inflammation in pleasure horses in North America and showed that the type of roughage being consumed was a major determinant of the number of neutrophils in the trachea[102]. Horses eating hay had more neutrophils in their airway secretions than horses on pasture, and horses eating round bale hay had more neutrophils in their secretions than horses eating square bales. Round bale hay increased the risk of horses having > 20% neutrophils in their transtracheal wash samples by almost five-fold, and pasture halved the risk compared to square bales[102]. Unfortunately, this study did not look at any of the hay characteristics that might have influenced the association of increased tracheal neutrophils with round bale hay feeding, such as hay quality (nutritional, moisture levels and mold and dust levels), and the storage and feeding practices of the round bale hay. K. Kobinger, et. al, published a

study in 2011 that was aimed at determining if upper airway endoscopic scores were associated with the severity of airway inflammation measured using BAL cytology. Although not an objective, this study also showed that feeding conditions (round bale hay vs. square bale hay) were significantly different between horses with moderate and severe inflammatory BAL categories[110]. Again, no attempt was made to characterize the factors associated with the type of hay fed that may have been responsible for the airway neutrophilia.

1.10. Non-Septic Airway Disease

While the evidence that links exposure to hay and airway disease in normal horses is scant, it is well established that increased exposure to respirable debris can exacerbate pre-existing non-septic airway diseases that occur in horses. Recurrent airway obstruction (RAO, also known as "heaves" and formally called chronic obstructive pulmonary disease (COPD)[111]) and inflammatory airway disease (IAD) are two examples of commonly recognized non-septic inflammatory respiratory diseases of horses. While the actual cause of these diseases is not completely understood it is likely that an increased exposure to aerosolized debris exacerbates or provokes the clinical manifestation of both RAO and IAD. RAO is an obstructive airway disease that is typically found in middle-aged to older horses that often results in overt increased respiratory effort at rest[100]. IAD can affect horses of any age, and clinical signs at rest are usually subtle[100]. In both conditions, exercise intensifies clinical signs[112,113].

Recurrent Airway Obstruction

RAO tends to be more prevalent in horses in the northern hemisphere, where they are stabled and fed hay for a large portion of their lives. Summer pasture-associated obstructive pulmonary disease (SPAOD) occurs in the southeastern United States, Britain and California, and is thought to be the same disease with different initiating factors[111]. Clinical signs, initiated by the inhalation of organic dusts, (most commonly from hay and bedding[90,104,114]) include signs of respiratory distress; nasal flaring, elevated respiratory rate, abdominal effort while breathing and anxiousness[111]. Other clinical signs include nasal discharge, coughing (especially during feeding or exercise), sensitivity and coughing upon tracheal palpation, reduced exercise tolerance and delayed recovery from exercise[111]. Auscultation of the lungs often reveal abnormal lung sounds, which vary depending on the severity of disease and may include increased breath sounds, crackles or wheezes[95,111]. Chronic elevated respiratory effort often leads to development of a "heave line," which is hypertrophy of the external abdominal oblique muscle[111]. Components found in the dust that are thought to initiate pulmonary inflammation include specific allergens, endotoxin, components of molds such as beta-glucan, and small particulates[111]. Within 6-8 hours of being moved from a pasture to a stall and fed hay, neutrophils accumulate in the lung and airway lumen. Bronchospasm, mucus accumulation and inflammatory changes within the walls of the airway

lead to airway obstruction[111]. A characteristic feature of horses with RAO is increased non-specific airway hyperesponsiveness[105,115], which means the airways of these horses constrict in an exaggerated response to a variety of stimuli. Airway hyperresposiveness is most severe during acute bouts of RAO, and is much less severe when horses are out on pasture and airway inflammation is at a minimum[111].

The diagnosis of RAO is made often made based on signalment, clinical signs, and after infectious pulmonary diseases have been ruled out. A complete blood count and routine blood chemistry screen in affected horses is usually within normal limits. Evaluation of blood gases can be done to determine the magnitude of gas exchange compromise, and response to treatment[111]. On endoscopy, horses with RAO and SPAOPD have variable amounts of mucopus within the trachea, and the carina may be thickened and tracheal and bronchial mucosa hyperemic[95,116]. Radiographs of the thorax are often unremarkable, but may reveal an increased bronchointerstitial pattern or flattening or concavity of the diaphragm, indicating alveolar hyperinflation[95]. The severity of pulmonary inflammation can be evaluated by cytological evaluation of bronchoalveolar lavage fluid (BALF). In normal horses, BALF is composed primarily of lymphocytes and macrophages. In RAO affected horses, the percentage of neutrophils increases (can be over 50% in severely affected animals) and are non-degenerate[111]. In horses with RAO, elevated levels if interleukin (IL)-4, IL-5 and IL—13 messenger ribonucleic acid (mRNA) has been demonstrated from cells in BALF and peripheral blood mononuclear cells, which is indicative of a T-helper 2 (Th2) response[117-119]. Increased levels of IL—8 and IL-1β proteins in BALF and IL-8 and IL-1β mRNA concentrations in BAL cells of RAO-affected horses have been found, which is likely due to increased neutrophils in airways, as equine neutrophils are a source of these cytokines[117,120-123].

Reduction of exposure to environmental dust (or specific allergens, if known) is necessary for the successful long-term management of horses with RAO and SPAOPD. If possible, horses with RAO should be kept on pasture year long and horses with SPAOPD should either be placed on a different pasture or stabled[95]. If this is not possible, feedstuff and bedding should be changed to those that are less dusty and management practices altered to reduce dust exposure, such as cleaning the stalls and aisle ways when the horses are not stalled and increasing ventilation to remove airborne dust[90,95,124]. Alternatives to hay include pelleted or cubed hay, hay silage and hydroponic hay. Soaking hay for 2-4 hours before feeding may control RAO in some horses but not others. Wood shavings, sand, shredded paper, peanut kernels and peat moss are alternative bedding options to straw[95]. If heaves cannot be managed by changing hay types and/or soaking it and/or changing bedding, horses may have to be stalled on rubber mats with no bedding (when necessary) and fed a complete pelleted feed.

Horses with RAO may require medications if rapid resolution of clinical signs is necessary and/or if environmental dust control cannot be fully achieved[95]. Corticosteroids are the most potent anti-inflammatory drugs currently available to treat RAO. They reduce mucus production, potentiate the

bronchodilatory effects of catecholamines and improve airway function by decreasing smooth muscle contraction by inhibiting the effects of inflammatory cells and their mediators[95]. Corticosteroids may be administered enterally, parenterally or by inhalation. Bronchodilators can be used to relieve airway obstruction caused by smooth muscle contraction and to aid the clearance of airway secretions. Agents used for bronchodilation in horses include β_2-adrenergic agonists, antimuscarinic agents, and methylxanthine derivatives[95].

RAO and SPAOPD are considered reversible conditions, as improvements in airway function are seen when horses are properly managed. Unfortunately there are no prognostic indicators for long-term outcome[95]. When following up on horses diagnosed with RAO and treated with environmental changes and oral steroid therapy, 78% of horses occasionally experienced an episode of RAO, and 41% of horse owners believed their horses had compromised athletic ability[125]. Horses rarely die from RAO directly, but owners of severely affected horses may have them euthanized if they are unable or unwilling to perform environmental modifications, if they are unable to deal with the anxiety of a horse in respiratory distress, or if the horse fails to respond to therapy[95].

Inflammatory Airway Disease

Inflammatory airway disease, also a non-septic inflammatory airway disease, affects horses of all ages. In the United Kingdom, studies suggest that IAD is most prevalent in horses being introduced to the training environment, regardless of age[113]. Inflammatory airway disease has been diagnosed in all types of horses, including foals and older adults[126]. The pathogenesis of it is not well defined and is characterized by airway inflammation with no obvious signs of systemic disease or respiratory distress in horses at rest[126]. The term IAD has been used to describe a condition of respiratory tract inflammation in racehorses, which occurs in up to 11-50% of horses in training[127]. Clinical signs and findings include coughing, accumulation of tracheal mucus, cytological evidence of airway inflammation, nasal discharge, poor performance, and/or delayed recovery following exercise[100,126]. Affected horses usually do not show overt signs of increased respiratory effort at rest, and have no evidence of illness based on a complete blood count and serum biochemical profile. Horses with IAD usually have a normal attitude and appetite[113], unless airway inflammation is severe[113]. Clinical signs in horses with IAD are very similar to those of horses with RAO. Horses with IAD tend to not have increased respiratory effort at rest or severe exercise intolerance as horses with (clinical) RAO do, and tend to be younger[126]. Although horses with RAO and IAD each show airway neutrophilia in BALF, the neutrophilic inflammation is usually less in horses with IAD (<20%). Horses with IAD (but typically not RAO) may also have increased percentages of mast cells and/or eosinophils in their BALF as well[127]. Horses with IAD have been shown to have increased expression of mRNA for IL-1β, IL-23, and TNF-α, but not IL-2, IFN-gamma, IL-4 or IL-13, in their BALF compared to control horses. This suggests that the airway neutrophilia is associated with

active cell recruitment and distal airway inflammation and is likely not due to a polarized T-cell response[128].

Risk factors for IAD in racehorses include animal, environmental and management factors. The risk of IAD (or signs related to IAD) decreases with age in racehorses[129-131]. Poor ventilation and bedding type also influence the risk of IAD in young racehorses[124,127,129]. Noninfectious agents are likely the most important factor in the development of IAD[100]. Horses housed in stables are exposed to high levels of aerosolized particles and gases, which accumulate with time. Several studies have shown that introducing horses to a stable is a risk factor for them developing IAD[103,132] and high dust concentrations are common in normal horse stables[90,104]. The contribution of environmental and stable factors to the development of IAD is unknown, but the presence of an increase in eosinophils and mast cells in BALF fluid of some horses with IAD suggests aeroallergens are a causative agent[100,133,134]. It also is not known if environmental pollutants, cold and dry environments or infectious agents also lead to the development of this condition [100].

It is not known if a relationship between IAD and RAO exists. Some researchers believe IAD represents a milder or earlier phase of RAO, while others believe they are different[127]. The etiology of IAD likely varies between horses and populations. Some potential initiating causes of IAD include infection with bacteria and/or mycoplasma, as a consequence to exercise induce pulmonary hemorrhage (EIPH), inhalation of airborne pro-inflammatory agents (such as endotoxin) in stables with poor air hygiene, exposure to noxious gases (such as ammonia from urine), deep inhalation of particulate matter associated with training and racing, recurrent or persistent viral infection, or a type-I hypersensitivity reaction to environmental molds[127].

A diagnosis of IAD should be made after a full physical exam (with or without a CBC or serum biochemical profile to rule out systemic illness) and examination of the respiratory tract (thoracic auscultation, upper airway endoscopy, and cytology and bacteriology of the airways)[127]. On endoscopy, an increase in tracheal mucus is common, but the amount that is considered abnormal is unclear[127]. On airway cytology, an increase in neutrophils, mast cells and/or eosinophils is one of the main diagnostic criteria for IAD[127]. Both a tracheal aspirate and bronchoalveolar lavage should be collected when trying to diagnose IAD, as collection of one sample may provide an incomplete clinical database[127]. Bacterial pneumonia should be ruled out as well, which can be done based on clinical signs, physical exam findings and diagnostic imaging (thoracic radiographs and/or thoracic ultrasonography).

Although the different subcategories of IAD are not well defined, it is recommended to group horses into two categories to help direct therapy – bacterial IAD and non-bacterial IAD[127]. Bacterial – IAD should be considered when pathogenic bacteria (or *Mycoplasma spp*.) in numbers greater than 10^3 colony-forming units/ml of tracheal aspirate fluid are identified in conjunction with neutrophilic

inflammation (and absence of evidence of upper airway contamination)[127]. Horses with bacterial IAD should be treated with antimicrobials, preferably based on culture and sensitivity, for a period of 7-10 days[113]. If bacteria are not considered to be an etiology of IAD (non-bacterial IAD), cytological evaluation of BALF or TA fluid should be used to categorize the horse into one of three categories: 1) mixed inflammation with a high total nucleated cell count, neutrophilia (>5% of BAL cells, > 20% TA cells), 2) increased metachromatic cells (>2% mast cells), and 3) eosinophilic inflammation (>5%)[127].

Treatment of IAD depends on the inciting cause and is often based off of a regimen that has been successful in the past. Horses are typically affected by IAD for an average of 7 weeks[126]. Therapy should be aimed at providing an environment with low levels of airborne dust, removal of inciting causes (if known) and treatment of pathological sequelae, such as airway inflammation and bronchoconstriction[127]. Environmental management should be similar to that described for treating horses with RAO. Medications used to treat IAD include antimicrobials (if indicated), systemic corticosteroids, +/- bronchodilators, sodium cromoglycate, interferon-α, acetylcysteine and/or a topical throat spray[126]. Bronchoconstriction is not typically present in horses with IAD, but bronchodilators may be indicated to reduce minor degrees of small airway obstruction and reduce exercise-induced cough[127]. Horses with IAD and elevated numbers of mast cells may improve when treated with the aerosol sodium cromoglycate, a mast cell stabilizer[127]. Airway inflammation in racehorses with IAD has been shown to be reduced after treatment with interferon-α with a concurrent reduction in BALF immunoglobulins and inflammatory mediator concentrations[135,136,137]. Horses with eosinophilic IAD do not respond to interferon-α treatment[127]. Inflammatory airway disease is a cause of poor exercise performance in horses. The impact of IAD on exercise performance is dependent on type, length and severity of airway inflammation, as well as the type of exercise the horse is performing[127]. The withdrawal time of medications should be observed in treated horses returning to the racing circuit[126].

While the airways of RAO and IAD affected horses are poised to react to inhaled debris, only the few studies (described previously) have assessed the relationship between exposure to round bales and the resultant airway inflammation in normal horses, and these studies failed to differentiate between the effects of hay quality from the way in which hay is baled. It is possible that management of round bales and the effect on hay quality is the cause of the increased airway neutrophilia rather than a potential increase in exposure to inhaled debris due to the manner in which the horses eat the hay. This distinction is important since hay quality can be affected by baling and management practices, while feeding behavior is a factor over which the horse owner has far less control. To better define the aspects of feeding round bale hay that provoke airway neutrophilia in normal horses, a systematic approach must be taken to assess the airway and rule out other potential causes of inflammation.

1.11 Diagnostic Methods and Detection of Airway Disease

When evaluating a horse that displays evidence of airway disease, the approach requires a through clinical examination, careful auscultation of the thorax, and visual examination and sampling of the airway. The decision to collect respiratory tract samples for analysis in a horse demonstrating signs of airway disease will be determined based on the history, clinical signs, likely differential diagnoses and owner finances. The ease of sample retrieval (and patient behavior) may also influence the selection of which tests/procedure is chosen[138]. In the horse, the nasal cavity, nasopharynx, guttural pouches, upper and lower airways and thorax can all be imaged and sampled. Clinical signs indicating imaging and /or sampling of the nasal cavity, nasopharynx and guttural pouches include nasal discharge, epistaxis, dyspnea, inspiratory stridor, malodorous breath, exercise intolerance and dysphagia. Clinical signs indicating imaging and/or sampling of the lower airways and pleural cavity include coughing, poor performance, mucopus in trachea, epistaxis post-exercise, fever of unknown origin, dyspnea and pleural effusion. Table 5 (page 27) lists the possible sites of origin and approach to diagnosing different respiratory conditions.

Table 5: Possible sites and origin and their approach to making a diagnosis for differing clinical manifestations of disease relating to the respiratory tract[138].

Clinical Sign	Site					Examination Techniques
	Sinonasal Cavity	Naso-Pharynx	Guttural Pouch	Lungs	Pleural Cavity	
Nasal Discharge (unilateral)	+++	++	+++	+	-	PE, endoscopy, radiography (most likely rewarding). Swab, wash or FNA/biopsy the lesion(s) in nasopharynx. Wash from GP.
Nasal Discharge (bilateral)	++	++	+	++	+	PE, endoscopy, radiography (most diagnostically rewarding). If LRT, include TA, BAL. Culture samples from nasal cavity, nasopharynx and GP washings for diagnosing strangles
Epistaxis (unilateral)	+++	++	+++	+	-	PE, endoscopy (esp. GPs), radioigraphy most likely rewarding. If LRT involved, include TA, BAL
Epistaxis (bilateral)	+	++	+++	+++	+	As for unilateral epistaxis
Dysphagia	-	+++	+++	-	-	Endoscopy and radiography of nasopharynx, GPs and neck
Swelling of sinonasal or parotid	+	++	++	-	-	PE, US, endoscopy, radiography most likely rewarding. Aspirate or biopsy lesion in nose, culture swabs from nose +/- GP washes for strangles
Horner Syndrome	-	-	+++	-	+	PE, neurological exam, endoscopy and radiography. Look in GPs.
Inspiratory Stridor	+++	+++	++	-	-	PE, endoscopy, radiography. FNA or biopsy if observed in the nares, nasopharynx or parotid.
Dyspnea	+	+	+	+++	+++	PE, endoscopy and radiography. Possible thoracic ultrasound. If LRT, +/- TA, BAL +/- thoracocentesis
Cough	-	-	-	+++	+	As for dyspnea
Poor exercise performance	+	+++	+	+++	+++	As for dyspnea
FUO	+	+	++	+++	+++	As for dyspnea
Pleurodynia	-	-	-	+	+++	As for dyspnea, ultrasound thorax +/- thoracocentesis.

A horse evaluated for a respiratory complaint should undergo a thorough physical exam which includes the examination and palpation of structures of the head and neck and auscultation of the trachea and lungs. External examination should include inspection for the presence of ocular or facial swelling, ocular or nasal discharge, nostril patency, percussion of the paranasal sinuses, palpation of the submandibular/throat latch region for lymphadenopathy and palpation of the laryngeal and proximal tracheal regions for defects, surgical scars and sensitivity. A thorough respiratory examination also includes careful auscultation of both lung fields (including the distal cervical trachea), before and after application of a rebreathing bag. Each lung field should be examined for a minimum of one complete breath, and attention should be paid to the costal arch to determine where in the breath cycle the audible sounds are heard[139].

Common diagnostic procedures for respiratory disease include: skull radiographs, sinus trephination and/or sinusoscopy, upper airway endoscopy, transtracheal wash/tracheal aspiration (for culture and/or cytology), and bronchoalveolar lavage (culture and/or cytology), thoracic ultrasound, thoracocentesis (for culture and cytology), and /or pulmonary biopsy. In humans, it is known that exposure to airway debris from hay and straw can be detrimental, and elements in hay (dust, mold spores, endotoxin, etc.) also have a negative impact on horses with pre-existing disease, such as RAO or IAD. The impact of round bales on the respiratory health of normal horses is not well understood. Because it is known that hay and dust exacerbates airway inflammation in susceptible horses, the diagnostic procedures chosen for this study included physical examination, auscultation of the lungs (with and without application of a rebreathing bag), sinus percussion, clinical score assessment, upper airway endoscopy and assignment of a PLH and TM score, and the collection of tracheal and bronchial fluid for diagnostic testing (bacterial and fungal culture (TA), and cytology (BALF and TA)).

1.12 Study Purpose and Hypotheses

Round bale hay is cheaper and easier to feed for owners who are equipped to handle and move them. Despite the economic advantages of feeding round bale hay, horsemen have been reluctant to feed it in part due to the potential risk of botulism and respiratory related problems. Studies have shown a correlation between hay exposure and airway disease in both humans and animals. However, it is unclear from the available reports whether hay quality or eating behavior associated with round bale feeding is the primary reason for the development of airway inflammation reported in horses fed round bales. To better define the factors that contributed to the development of airway inflammation, we designed a study in which horses were fed either round or square bale hay from the same pasture and harvested at same time. Storage of round and square bales was also designed to minimize exposure to moisture and mold which was confirmed by measuring and comparing the nutritional composition as well as bacterial and mold

content of both hays at the beginning and end of the study period. Two groups of 15 normal horses were fed either the RBH or the SBH for six weeks. Airway health of these horses was assessed before and at the end of the feeding period to determine if horses fed RBH had greater evidence of airway inflammation at the end of the study as compared to horse fed SBH. Specifically stated, the goal of this study was to test the HYPOTHESIS that *the respiratory health of horses fed RBH will not differ from horses fed SBH of comparable quality*. The results of this investigation indicate that horses experience a minimal difference in airway health when fed RBH of comparable quality to SBH for a period of six weeks. These findings suggest that hay quality likely plays predominant role in the increase in airway inflammation associated with feeding RBH.

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Chapter 2

Publication

COMPARISON OF AIRWAY RESPONSES IN HORSES FED ROUND BALE VERSUS SQUARE BALE HAY

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Running Head: round bale hay, square bale hay, airway inflammation

Key Words: airway inflammation, hay, RAO, respiratory

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Abbreviations

RBH round bale hay

SBH square bale hay

BAL bronchoalveolar lavage

BALF bronchoalveolar lavage fluid

TA tracheal aspirate

TM tracheal mucus

PLH pharyngeal lymphoid hyperplasia

PDA Potato dextrose agar

ASP *Aspergillus* selective media

FSM *Fusarium* selective media

RAO Recurrent airway obstruction

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2.1 ABSTRACT

Background – Feeding horses round bale hay (RBH) has been associated with airway inflammation. The purpose of this study was to determine if horses fed RBH for 6-weeks demonstrated more evidence of airway inflammation than horses fed square bale hay (SBH) of comparable quality.

Hypothesis - The respiratory health of horses fed RBH will not differ from horses fed SBH of comparable quality.

Animals – Two feeding groups of 15 healthy adult horses (mixed ages, breeds).

Methods – Horses were fed RBH or SBH for a period of 6-weeks. At the beginning and end of the feeding period, horses were examined (physical, upper airway endoscopy) and samples (tracheal brushing, tracheal aspirate (TA), bronchoalveolar lavage (BAL)) were collected for cytology and/or bacterial/fungal culture. Hay was analyzed for nutritional value and bacterial/fungal content.

Results – Horses fed RBH demonstrated an increase in pharyngeal lymphoid hyperplasia ($p=0.0143$) and percentage neutrophils ($p=0.0078$) in the TA samples post-feeding compared to pre-feeding values. Nutritional analysis of hay and measurements of bacterial/fungal load did not differ over time and/or between hay types.

Conclusions/clinical importance – The identification of upper airway inflammation in the horses fed RBH indicates that factors associated with the manner in which the hay was consumed contributed to the development of subclinical airway inflammation. RBH fed in bulk feeders affords horses continuous daily exposure to hay and as horses often bury their muzzles in the bale, upper airway exposure to particulate matter could be increased.

2.2 INTRODUCTION

In recent years, the price of small square bale horse (SBH) hay has nearly doubled[1]. Packaging hay in large rounds bales appreciably reduces harvesting expenditures. In Virginia in 2010, the price of large average quality round bales (>1000 lbs.) was approximately $0.032/lb., while equivalent quality small square bales (35-45 lbs.) averaged $0.065/lb[1]. A horse that weighs 1,000 lbs. will consume approximately 20 lbs. of hay (2% of body weight) per day[2]. Using the above prices, this equates to about $0.64 per horse per day if feeding round bales and $1.30 per horse per day if feeding square bales. Round bales save in direct cost, and also offer ease of labor as compared to square bales since machinery is used to move the bales and hay doesn't have to be fed daily. Moving round bales from the field takes one-half the machinery time and one-tenth the labor of moving square bales from the ground to the barn[3].

Despite the apparent economic advantages, horse owners have historically been reluctant to feed round bale hay (RBH) in part due to the perceived risk of round bales contributing to respiratory health-related problems. The lack of storage space and equipment to move round bales also deters some horse owners from purchasing and feeding round bale hay. An association with feeding round bale hay and airway inflammation was recently demonstrated in a large cross sectional study of horse herds in Michigan[4]. In this study researchers discovered that horses on pasture had less neutrophils in their tracheas than horses fed hay, and horses fed round bale hay had more neutrophils than horses fed square bale hay[4]. Unfortunately the results of this study did not take into account pre-existing disease in horses, hay quality or factors that affect hay quality such as packaging and storage.

Several studies have shown a correlation between hay exposure and airway disease. In humans, Farmer's Lung Disease is a common ailment among farm workers caused by chronic inhalation of microorganisms from moldy hay[5-8]. In cattle, acute interstitial pneumonia is associated with exposure to allergens in moldy hay[9]. Bovine allergic pneumonitis, a chronic, fatal pulmonary disease of adult cattle also shares similarities with chronic farmer's lung disease[10]. An association between overt respiratory disease and moldy feed and bedding has also been demonstrated in horses[11]. In addition, acute exacerbation of recurrent airway obstruction (heaves) in horses can be provoked by exposure to dust and mold in hay[11-14]. Square bale hay is typically fed to horses once to twice daily whereas round bale hay is typically placed in the field and replenished when most or the entire bale has been consumed. As a result, large round bales offer more prolonged exposure to the irritant particles simply because the hay is constantly available. Horses eating from round bales also likely increase their exposure to dust particles and fungal spores because they often immerse their muzzles into the bales as they eat. Pasture generally provides little dust exposure because of the high water content of grass[4,15].

Additional problems are correlated with improper management of round bales. When uncovered bales are stored outside, rain can deteriorate approximately 40% of the original bale (in dry weight)[3,16].

Aside from dry matter losses, hay quality also decreases, as evidenced by the oxidation of nonstructural carbohydrates, presence of mold growth and production of associated toxins, and increased concentrations of fiber components and heat damaged nitrogen[17]. These changes inevitably affect the feed value of the forage and may lead to decreased animal performance (cattle). As a result, horse owners and cattle producers often decide against the use of RBH[16,18,19]. However, the relationship between risk of disease and hay quality has not been explored.

The purpose of this study was to determine if horses with continuous access to RBH for a 6 week period demonstrated more evidence of airway inflammation than horses fed similar quality SBH. To minimize the effects of hay quality, the hay used in this study was harvested from the same pasture, at the same time, and baled as either RB or SB. It was then stored undercover on substrate (gravel) and evaluated both at the beginning and end of the feeding period for nutritional composition, and fungal and bacterial content by culturing on selective and non-selective media. *We hypothesized that there would be no difference in the airway responses of normal, healthy horses fed comparable RBH or SBH harvested and stored in this manner.*

2.3 MATERIALS AND METHODS

The study was performed from October 26, 2009 through December 13, 2009. Thirty healthy University owned adult horses of mixed ages (range 3- 22 years, median 12 years old) and breeds (warmbloods, Thoroughbreds and Quarter Horses) were used in this study (Table 1). Horses were divided into their respective study groups (11 geldings, 4 mares per group) prior to the initiation of the study and housed on pastures. Two of the pastures were 2-3 acres in size and contained four mares each. The other two pastures were 8-10 acres in size and contained 11 geldings each. All horses received routine preventative care, were healthy, and had no clinical signs or history of respiratory disease. The University Animal Care and Use Committee approved the experimental protocol.

Horses included in this study were used in the University riding program, and select horses spent between four and nine hours in a stall on days when in work. Groups were balanced based on horse use and stall time (Table 1). Horses in the stall for 4-5 hours received 1 -2 flakes of hay (alfalfa/grass mix square bale, different from the study hay, from a single source) and were typically ridden once daily. Horses in the stall for 8-9 hours per day were fed 2-3 flakes of this hay (same hay fed to the horses in a stall for 4-5 hours) and typically ridden twice daily. The horses not brought into a stall did not receive any of this alfalfa/grass mix hay. Some horses were also fed a commercial grain (sweet feed) product depending on the body condition score of the horse and work load. Horses were mainly ridden in an indoor facility by undergraduate university students (once or twice per day, 1 hour per session, flat work or jumping) and were occasionally taken to a show on the weekend. Water was provided free choice both

in the stalls and on pasture. Records were kept for the daily use of each horse, including the amount of time ridden per day and what type of riding activity was performed.

Experimental Design

For the study, half of the horses (one pasture of 11 geldings, one pasture of 4 mares) received square bale hay, and half of the horses (one pasture of 11 geldings, one pasture of 4 mares) received round bale hay as their predominate feed source. Both hay types were from a second cutting of University mixed grass hay harvested from the same field at the same time in August of 2009. At the time of harvest, round bales were covered with nylon-mesh and stored stacked in an open-faced shed on top of gravel substrate. Square bales were stored stacked in a covered hay loft. When fed, round bales were placed in a metal round bale feeder on the ground and replenished as needed (one new bale every 4-5 days). Square bales were supplied twice daily as individual flakes on the ground. Approximately one-half of a square bale was fed per horse per day.

Hay Sampling

Hay was sampled prior to the start and at the completion of the study. Ten percent of the bales from each group were randomly sampled with a Stihl Model BT 45 gas powered drill[a] with a Penn State Forage Sampler probe[b]. This probe cut a core sample 0.75 inches in diameter and 18-inches long. For each bale, one 9-inch "shallow" core sample and one 18-inch (9-27 inches from the surface) "deep" core sample was collected for analysis. For square bales, samples were taken from the center of the short end. For round bales, samples were taken from 9 locations (see Figure 1) on the short, rounded side. Core samples were evaluated for mold and bacterial content and analyzed for nutritional content[c].

Three media were used for bacterial and fungal cultures of hay samples; a non-selective potato-dextrose agar (PDA), and *Aspergillus* (ASP) and *Fusarium* (FSM) selective media. Media was prepared in 1 L aliquots in a 2 L Erlenmeyer flask containing a stir bar. The flask was maintained on a warming plate during mixing and the solution was not allowed to boil. The media was autoclaved on a wet (liquid) cycle at 121^{o}Celsius for 40 minutes on slow exhaust to achieve a pressure of 15-20 psi. Media was allowed to cool and solidify at room temperature for 24 hours before being placed in cold storage at 4^{o}C until used. For the ASP and FSM media, all ingredients except antibiotics were combined and autoclaved. After autoclaving, media was cooled to the point where the flask could safely be handled and the antibiotic powder(s), dissolved in 10 ml of sterile deionized water, was(were) added. The antibiotic(s) and media were blended using a stir plate on the medium setting. The PDA was composed of 1000 ml distilled water, 6 g potato dextrose broth and 15 g BD Bacto™ Agar[d]. The ASP was composed of 1000 ml distilled water, 15 g Bacto™ Agar[d], 20 g yeast extract, 10 g peptone, 0.5 g ferric ammonium citrate, 0.1 g chloramphenicol, and 1 ml of a 0.2% stock solution of dicloran (0.2 g dicloran with 100 mls 99% ethanol). The FSM was composed of 1000 ml distilled water, 15 g BD Bacto™ Agar[d], 15 g Peptone, 1 g

potassium phosphate, 0.5 g magnesium sulfate, 1 g terrachlor, 1 g streptomycin sulfate and 0.35 g neomycin sulfate.

All cultures were repeated in duplicate. Both wet and dry culture preparations were performed to provide the optimum opportunity to identify fungi and molds. For the dry preparations, two quantities (0.05 g and 0.10 g) of sample were sprinkled across the media. For the wet preparations, 7.5 grams of hay were added to 375 ml of distilled water to create a 2% solution (1X). Serial dilutions were performed to yield 1:10 and 1:100 concentrations. One hundred µl of each concentration were applied to the media. The amounts and concentrations of hay were selected based on previous preliminary results (unpublished results, Buechner-Maxwell) of a study which allowed for ideal colony growth without compromising the ability to accurately count colonies.

Bacterial and fungal growth was counted as colony forming units (CFUs) daily for a total of 5 days. If a plate reached greater than 75 CFUs, the number of colonies were deemed too numerous to count and the plate was discarded. The number of colonies was then determined by going to the next higher dilution (0.05 g for the dry preparation and 1:10X for the wet preparation) and the number of CFUs were multiplied by 2 and 10 respectively to allow statistical analysis of the number of colonies based on 0.10 grams dry preparation and 100 µl wet preparation (CFU/0.1 grams hay and CFU/100 µl of a 2% solution).

Evaluation of Horses

Prior to the beginning of the study, and at the completion of the study, each horse received a physical examination and an upper airway endoscopic examination. For the physical examination, basic vital parameters (temperature, per rectum, heart rate, respiratory rate) were recorded as well as mucous membrane color and capillary refill time. The eyes and nares were examined for the presence of ocular or nasal discharge, the paranasal sinuses were percussed, and a rebreathing examination was performed. For the rebreathing examination, a small plastic garbage bag was placed over each horse's muzzle and held in place for approximately 30 – 60 second or until the horse began to take deep breathes, and then it was removed. The lungs were ausculted using a stethoscope during the application of and after removal of the garbage bag. Each horses' respiratory effort was scored with a nasal flare score, abdominal component score, and then each was added together to get a total clinical score[20] (Table 2). Weight was approximated with a weight tape[e] and a body condition score[21] (on a scale of 1-9) assigned. All evaluators (6 total, all veterinarians) were blinded as to the group of each horse. The same evaluators examined the horses at each time point. Following physical examination, horses were sedated with xylazine hydrochloride[f] (0.5 mg/kg) and butorphanol tartrate[g] (0.01 mg/kg) intravenously for upper airway endoscopic evaluation and sample collection. The following structures were assessed during endoscopy: nasal passages, pharynx,

larynx, right and left guttural pouches (medial and lateral compartments) and trachea. Any abnormalities were recorded.

Scoring Upper Airway Inflammation and Function

The airways of each horse were examined by two different observers (V.B.M and J.L.L.) for evidence of inflammation using a 1-meter endoscope. The examiners were blinded as to the group of each horse. Horses received a score of 1-4 based on the degree of pharyngeal lymphoid hyperplasia (PLH) that was noted[22]. The presence of debris was noted in each nasal passage, as well as any other abnormalities (example: melanomas in the guttural pouch, left laryngeal hemiplegia). The amount of mucus visible in the trachea was graded on a scale of 0 (no visible mucus), 1 (singular, small blobs), 2 (multiple blobs only partly confluent), 3 (mucus ventrally confluent), 4 (large ventral pool), and 5 (profuse amounts of mucus occupying more than 25% of tracheal lumen), as described by Gerber V, *et al.,* 2004[23].

Tracheal Mucus Collection, Sample Processing and Cytology

A 1-meter endoscope was passed into the proximal 1/3 of the trachea, and a tracheal brushing sample was collected for culture (aerobic and anaerobic bacterial culture, fungal culture) by passing a guarded sterile gastroscopy cytology brush[h] through the biopsy port. Once removed from the endoscope, the cytology brush was swabbed with a sterile microbiology culturette[i] for aerobic and anaerobic bacterial and fungal culture. Cultures were performed by the clinical microbiology laboratory at the Virginia-Maryland Regional College of Veterinary Medicine using standard techniques. For statistical analyses, cultures were recorded as either positive or negative if bacteria were isolated or not. A tracheal aspirate (TA) was then performed by passing a triple lumen catheter[j] through the biopsy port of the endoscope and infusing and retrieving by gentle suction 30-60 mL's of sterile saline. The amount infused and retrieved was recorded, placed in a sterile 50-mL conical tube and set on ice until processed (within 2-4 hours). For processing, samples were centrifuged (300 x g x 15 minutes). The resulting supernatant was discarded and the cell pellet was washed twice in 30 mL of sterile DPBS (500 x g x 8 minutes). Following removal of the final supernatant, the pellet was resuspended in 500 µl of sterile DPBS. The total nucleated cell counts per ml were determined by using an automated cell counter[k] and the total number of cells was calculated based on the volume of aspirated tracheal wash fluid. Slides for differential cell counts were prepared by cytospin[l] using 300 µL of sample and stained[m] with a modified Wright's stain. A total of 300 cells per slide were counted by use of light microscopy (40x). The following cells were identified and counted: macrophages, neutrophils, mast cells, eosinophils and lymphocytes.

Bronchoalveolar Lavage Collection, Sample Processing and Cytology

Following the endoscopic exam, a broncoalveolar lavage (BAL) was performed using a bronchoalveolar lavage fluid (BALF) collection tube[n] with an inflatable cuff passed blindly via the nose

and wedged in a peripheral bronchus. At the beginning of the procedure, the cough reflex was suppressed by infusing 40 mL of 0.5% lidocaine[o] as the BAL tube was advanced into the proximal trachea[24]. Once wedged, the cuff was inflated with 5-10 mL of room air and 240 mL of sterile saline was gently infused and aspirated in 120 mL aliquots. The volume retrieved was recorded and the aliquots were combined into a sterile specimen cup and placed on ice until processing (within 2-4 hours). The BAL samples were processed and slides prepared in a similar fashion to the TA samples.

Statistical Analysis

For parametric data measured on a continuous scale, a mixed model analysis of variance (ANOVA) was used to test for differences within and across groups. Data was natural log transformed as necessary to achieve normal distribution. Nonparametric continuous data was compared using the Wilcoxon 2 sample and Friedman's Chi-square tests. Categorical data was compared using the Fisher's exact test. All statistical analyses were compiled utilizing SAS® software[p]. Differences were considered significant when the p-value was < 0.05.

2.4 RESULTS

Physical Exam Findings (Table 3)

Mean body temperature increased significantly in the SBH group over time (mean rectal temperature in October was 37.1°C and 37.5°C in December ($p = 0.0053$) while mean respiratory rate decreased in each group (16 bpm and 11 bpm, in October and December, respectively for each group ($p < 0.0001$ for SBH, $p = 0.0001$ for RBH)). There were no differences over time or between groups in ocular or nasal discharge, findings associated with auscultation of the thorax or lungs, or the mean value of physical parameters other than as noted above.

Clinical Scores (Table 3)

There was no difference in nasal flare score for either group. The RBH group had a higher median abdominal component score in December compared to October (1.5 vs. 1.0, $p = 0.033$). Similarly, the total clinical score was increased in the RBH group over time (2.0 in October vs. 2.5 in December, $p = 0.035$), while it decreased in the SBH over time (3.0 in October vs. 2.5 in December, $p = 0.034$).

Upper Airway Endoscopic Exam

The SBH group had more abnormal findings in the nasal passages (abnormal = the presence of foam, food or mucus) over time ($p = 0.0092$). In October, 12/15 SBH horses had normal nasal passages and 3/15 had abnormal nasal passages; whereas in December, 4/15 horses had normal nasal passages, whereas 11/15 had abnormal nasal passages. Table 4 includes the median (± SD) PLH scores for each group at each examination time point. Median scores are displayed due to the nonparametric nature of the data. The RBH group had a higher PLH score in December compared to October. The SBH group had a

lower mean (but same median) PLH score in December compared to October (mean PLH score of 1.0 in December and 1.2 in October), and a lower PLH score compared to the RBH group in December. There was no difference between groups or over time in any other parameter evaluated by endoscope examination, including TM scores (median ± SD scores are listed in Table 4).

Tracheal Aspirate Culture and Cytology, Broncoalveolar Lavage Cytology

Both groups of horses had lower total cells per milliliter in their TA samples in December compared to October. The RBH group had a greater percentage of neutrophils in their TA samples in December compared to October (Table 5A and Figure 2A). There was no difference in any of the other cell types between either groups or over time. Both groups of horses had fewer positive cultures for aerobic bacteria in their TA samples in December compared to October. For the SBH group, 9/15 samples grew bacteria in October compared to 2/15 samples in December ($p = 0.021$). For the RBH group, 12/15 samples grew bacteria in October compared to 2/15 samples in December ($p = 0.00068$). Although there was no statistical differences in the number of TA cultures that grew fungi between groups or over time, horses in the RBH group had more positive cultures in December vs. October whereas horses in the SBH had less positive cultures. For horses in the SBH group, 5/15 TA cultures grew fungi in October (3/5 were *Aspergillus* spp., 1/5 *Fusarium*), and 3/15 cultures grew fungi in December (1/3 was *Aspergillus* spp.). For the RBH group, 5/15 TA cultures were positive for fungi in October (one sample was heavily contaminated; neither *Aspergillus* nor *Fusarium* spp. were isolated from any culture) and 8/15 were positive for fungi in December (7/8 grew *Aspergillus* spp.). Anaerobic bacteria were not isolated from any sample at any time.

For the BAL samples, there was less volume recovered in December for both groups compared to October. There was no difference in the percentages of all cell types for either group or over time (Table 5B), including neutrophils (Figure 2B).

Hay Data

There was no difference in the nutrient analysis of either hay type over time. There were no differences in the number of CFUs of bacteria or fungi grown from either hay type in either preparation (wet vs. dry) on any media (PDA, FSM and ASP) over time. Figures 3A – 3D show the median daily CFU of fungi grown on each culture day for each hay preparation (wet vs. dry) on each selected media type.

2.5 DISCUSSION

Several differences in the physical exam parameters were noted within each group and/or over time, including rectal temperature, respiratory rate, pharyngeal lymphoid hyperplasia scores and abdominal component and total clinical scores. The changes in respiratory rate and rectal temperature

were not considered clinically relevant, as each mean value fell within the normal range for adult horses. Based on the increase in PLH score, abdominal component and total clinical score for horses in the RBH group, these horses displayed mild evidence of increased upper airway inflammation and respiratory effort compared to horses in the SBH; however, the authors do not consider these changes clinically relevant. Pharyngeal lymphoid hyperplasia is typically scored on a scale of 1 – 4[22]. If the scores had been rounded to whole numbers, the PLH scores for the RBH group (1.0 in October, 1.3 in December) would not have changed over time. When using the scoring system published by Robinson, *et. al*[20], to grade the severity of clinical signs in horses with RAO, a total clinical score of 2 is considered normal while a score of 3-4 is considered to be mildly affected[20]. Using this system, horses in the RBH group went from "normal" to between "normal" and "mildly affected" over the course of the study. Robinson and coworkers in 2000 showed that considerable changes in pulmonary function had occurred before evidence of this compromise could be detected clinically, and it was not until horses reached a total clinical score of 5 before the evaluator could confidently detect signs of airway obstruction[20]. Based on this work, the authors of this study conclude that the changes in abdominal component and total clinical scores were clinically inconsequential for the horses in this study.

Horses in the RBH group showed a mild increase in airway inflammation in December compared to October, as shown by having a significantly higher percentage of neutrophils in their tracheal wash samples in December compared to October (geometric means were 25.3% and 18%, respectively). One possible explanation for this is that horses eating round bale hay tend to bury their noses in the bale as they eat, increasing their exposure to irritant respirable particles that are deposited in the trachea. The respiratory tract acts like a filter, keeping most inhaled dust from reaching the alveoli[25]. The location where particles deposit in the equine respiratory tract is determined by their size, hydrophilic or hydrophobic nature, shape and density, all which can be described by the term "aerodynamic particle diameter"[25]. Larger particles (> 5 μ) tend to deposit in the upper airway (nasal turbinates, pharynx and bifurcation of the large airways) whereas smaller particles (0.5 – 5 μ) are deposited deeper in the lung (including terminal bronchioles) by gravitational settlement where they can induce pulmonary inflammation[25]. Other factors influence the location where particles deposit including the respiratory pattern, tidal volume, lung volume, respiratory rate and flow rate[25]. Typical sizes of common aerosol particles include: a) most bacterial cells/spores - approximately 0.3 – 10 μm, b) fungal spores – approximately 2.0 – 5.0 μm and c) viruses – approximately 0.02 – 0.30 μm[26]. Thus, mold spores inhaled during hay consumption could be deposited in both the upper and lower airways, including the terminal bronchioles.

The most important factor that determines the microbial concentration in hay is the moisture content when it is baled[25]. When hay is baled at a moisture content of 35-50%, the hay may heat and

become contaminated with thermotolerant microbes[25]. A horse eating heated hay may inhale 10^{10} dust particles per breath[27], but even a horse exposed to good quality hay with lower levels of dust and microbes with its head constantly buried in hay will likely have higher exposure than a horse that eats with its head above the hay. A study by Mahieu and colleagues (2000) found no correlation between the air contamination rates (of *Aspergillus*) in the neonatal intensive care unit (NICU) of a hospital and the nasopharyngeal colonization rate (of *Aspergillus*) in neonatal infants[28]. The current study, and the lack of increase in upper (or lower) airway inflammation of the horses in the SBH group, suggests that the method of eating RBH may contribute to the increase in airway inflammation. Additionally, although the differences were not significant, based on the greater number of positive fungal TA cultures, increased frequency of *Aspergillus* spp. isolation, and decrease in number of positive bacterial TA cultures over time, perhaps the cause of this inflammation is due to larger particles, such as mold spores.

Interestingly, horses in both groups had significantly less total cells per milliliter in their TA samples in December compared to October, where the cause for this is unknown. It is possible that there were other environmental factors affecting the airways in October vs. December. The horses were sampled in two groups in October and all at once in December. During the sampling process, horses were kept in stalls with no feed. One possible explanation for the decreased concentration of cells in the TA samples in December is that the horses had increased time (compared to October) in the stalls where they were not being exposed to respirable particles, and more time for any debris in the trachea to be cleared by mucocillary clearance. The order in which horses were sampled was not recorded, so the authors were unable to test this hypothesis.

Feeding RBH or SBH hay did not appear to cause an increase in bronchiole or alveolar airway inflammation over period of 6 weeks, as there was no difference in the percentage of any cell type, including neutrophils, over this time period in the BAL fluid samples. However, it should be noted there are differences in the literature as to normal BAL differential cell counts[29]. If BAL fluid cytology is considered normal when it contains less than 5% neutrophils[30,31], then the mean percentage of neutrophils in BAL fluid from both groups exceeded this number at each time point. One study[29] found using 5% neutrophils in BAL fluid as a cut-off value did not show any differences in lung inflammation between horses with normal versus those with mild to moderate lower airway inflammation. When the threshold for normal neutrophils in a BAL was increased from 5% to 10%, the significance of the results did not change, but the number of horses with a "normal" BAL increased and the number of horses with a "mild-moderate inflammatory" BAL decreased[29]. If a normal BAL cut-off value of 10% neutrophils is applied to results in Table 5B, horses in the SBH in October went from having a "normal-to-increased" percentage of BAL fluid neutrophils in October to being "mildly increased" in December, whereas horses in the RBH group would have been "mildly increased" at both time points. The "greater than 5%

neutrophils" in each group's BALF may also have been due to other unknown environmental factors and/or individual horse(s). It is possible that individual horse(s) affected the BALF neutrophil percentages. Looking at individual horse data (not published), in the SBH group, 8 horses had 5% or less neutrophils in their BALF in October, whereas only 3 horses had 5% or less neutrophils in their BALF in December. Similarly, 9 horses in the RBH group had 5% or less neutrophils in their BALF in October, and only 3 horses had 5% or less neutrophils in their BALF in December. Also in the RBH group, 3 horses had greater than 25% neutrophils in their BALF at each time point, and two horses in the SBH group had greater than 19% neutrophils at each time point. Although not compared statistically, the neutrophil percentages in the 3 horses in the RBH group with greater than 25% neutrophils in their BALF were considerably higher than the neutrophil percentages in the 2 horses in the SBH group with greater than 19% neutrophils, even at the beginning of the study.

Significantly less BAL fluid was retrieved for both groups of horses in December compared to October (Table 5B). This procedure was performed by the same evaluators at each time point using the same type of tubing and same type and volume of isotonic crystalloid fluid. The typical amount of fluid retrieved from a BAL procedure is 50-80% of the instilled volume[32]. All mean volumes retrieved were within this percentage range, thus can be considered normal. Airway bronchospasm and edema, which can be induced with suctioning during the BAL procedure, leads to airway lumen obstruction and can be a reason for decreased BAL fluid retrieval[32]. However, since the BAL procedures were performed by the same evaluators at each time point, it is unlikely that the procedures performed in December were done using a consistently different method than in October.

Based on nutritional analysis and culture results, the storage method for both types of hay appeared to be appropriate for the time length that the hays were stored. There was no change in the nutritional value or increase in the bacterial and fungal load of either hay type during the storage period. *Aspergillus* and *Fusarium* were the two types of species of fungi chosen to be isolated and counted in this study. Both of these fungi are commonly found in insufficiently dried hay, and can lead to the production of mycotoxins[33,34]. Growth of *Fusarium* spp. is favored when hay is harvested during cool, wet weather. As the hay is stored, this species tends to die while other fungi (deemed "storage fungi") proliferate rapidly. The main species associated with stored hay include *Aspergillus* and *Penicillium*[33], where exposure to *Aspergillus* can induce clinical signs of RAO in susceptible horses[35]. Numerous other studies have been performed comparing storage methods to hay quality and storage losses and how they affect production for food animal species. When hay is too wet at baling, microbial respiration causes the hay to heat spontaneously[36]. This heating process oxidizes nonstructural carbohydrates[37], increases mold growth (and associated production of toxins)[33], and increases the concentration of fiber components and unavailable nitrogen[18]. Losses of dry matter and quality during the harvest of hay (alfalfa) can be

extremely large and can range from 15-25% (under good conditions) to 35-100% (hay damaged by rain)[38]. Losses are typically categorized based on where they occur during the hay making process and the cause of the loss[38]. One study[39] compared alfalfa-orchard grass hay baled and stored by several methods (conventional rectangular bales stored inside, large round bales stored inside and large round bales stored outside) to the utilization by lactating dairy cows and the milk production response to each feeding method[39]. This study concluded that cows fed round bales stored inside produced milk as well as cows fed conventional rectangular bales stored inside when no heat damage was present, but cows fed round bales stored outside and uncovered produced significantly less milk than cows fed round bales protected from the weather (plastic wraps)[39]. Another study[40] assessed the influence of hay-packing techniques (25-30 kg prismatic and 500 kg cylindrical bales) on the presence of *Saccharopolyspora rectivirgula*, a thermophilic actinomycete, which has been implicated as one of the causative agents for "farmer's lung disease" in people[40]. All bales were stored under cover; prismatic bales were stored 24 hours after baling and the cylindrical bales were stored 7 days after baling. Results from this study indicated that hay packing technique influenced the microbiological quality of hay, with large cylindrical bales containing higher levels of *S. rectivirgula* than the prismatic bales, however, it did not state if the round bales were stored off the ground and what the moisture content was at the time they were fed[40]. Ranalli's study differs from the current study where in this study there were no differences in the fungal load of each hay type, and factors such as storage and moisture content were accounted. It is possible that the increased number of days that the round bales were on pasture in Ranalli's study affected their fungal load.

There were several limitations of the current study, including horse housing variability (which included the addition of square bale hay from a different source to those horses brought into the barn for a half or full day), the length of time the hay was fed (6 weeks), or factors that did not get measured, such as the change in RBH quality during the period over which it was being consumed and exposed to the elements, and the types of bacteria isolated from each of the hay types. Although bacteria cultured from the hay samples and all fungal species were not identified, there were no differences in the number colony forming units cultured from either hay type (RBH vs. SBH) or hay preparation (dry vs.wet) over time, thus indicating both hay types were similar in the bacterial and fungal (*Aspergillus* and *Fusarium*) load at the time of feeding (approximately 2-3 months after the hay was harvested), and this level did not change after 6 weeks of storage. One thing that was not assessed was the change in bacterial and fungal content of the round bale hay over the amount of time (approximately 4-5 days) it was out on pasture being consumed. There were no differences in the number of CFUs of bacteria, *Aspergillus* and *Fusarium* cultured on each of the 5 days for either hay type or hay preparation (wet vs. dry) at the beginning and end of the study. Based on the bacterial cultures (data not shown) of the wet hay preparations, maximum median levels were reached on day 4 of culture for the October samples and on day 2 of culture for the

December samples. These maximum levels were maintained through the entire culture time period (5 days). Bacterial levels increased more slowly in in the dry preparations. *Fusarium* and *Aspergillus* levels did not appear to increase until the third culture day for the dry RBH preparations, and fourth culture day for the wet RBH preparations. There were no differences in the number of positive fungal TA cultures obtained from the RBH group in December vs. October, and there were significantly less positive bacterial TA cultures in December compared to October (12/15 of samples grew bacteria in October, 2/12 grew bacteria in December, $p = 6.789 \times 10^{-4}$). Considering this information and the way the cultures were performed (daily monitoring for 5 days of the changes in bacterial and fungal content of an initial hay samples), it can be postulated that the quality of the RBH, as it sits uncovered on pasture for a period of 4-5 days, does not change significantly enough to affect the airway health of horses, but further studies investigating this and comparing it to the respiratory health of horses eating round bales under cover are warranted.

There was also no attempt to measure the presence of anerobic bacteria, like *Clostridium botulinum*, in the hay samples collected. Feeding round bales is thought to increase the risk of horses (and other farm animals) for botulism, which is a disease caused by a neurotoxin produced by the bacterium *Clostridium botulinum*[41]. *C. botulinum* is a spore-forming, gram-positive anaerobic rod bacterium that produces eight different exotoxins, including A, B, C1, C2, D, E, F and G[42,43]. Type B botulism, also known as forage poisoning, is the most common form of botulism that affects horses and is usually caused by proliferation and production of the toxin in decaying vegetable matter[43]. Spoiled hay, haylage and silage are the most commonly implicated feed sources[44], but other feed sources have also been involved, such as alfalfa baled under wet conditions, processed alfalfa hay cubes, big bale silage and plastic-packaged hay that had been rained on prior to baling[42,45-48]. The goal of this study was not to test the hays for the presence of *C. botulinum* bacteria or toxins, so the risk of botulism (and thus the need for vaccination) should be considered when feeding this forage.

It is well established that exposure to hay and stable dusts exacerbates airway inflammation in horses with the respiratory disease known as Recurrent Airway Obstruction (RAO). This inflammation is characterized by airway neutrophilia[14]. Horses without RAO could also demonstrate more severe clinical changes and airway neutrophilia when fed RBH for prolonged periods and/or if hay quality is inferior. Exposure of healthy horses to moldy hay invokes lower airway neutrophilia characterized by increased percentages of neutrophils in BAL fluid[14]. Recently, two studies have suggested that feeding round bale hay is linked with an increase number of neutrophils in tracheal wash[4] and BAL samples[49]; however factors affecting hay quality were not accounted for in these reports. Although the RBH fed in this study did not clinically affect the respiratory health of normal horses, its potential negative effects should be

considered with long term feeding and before feeding it to horses with a propensity to develop respiratory disease, such as RAO.

In summary, when hay quality is held constant with regard to nutritional value and microbial contamination, feeding round bale hay for a period of 6 weeks induces significant changes associated with mild airway inflammation in horses as compared to feeding square bales of the same quality. These changes include an increase in PLH score, abdominal component, total clinical score, and percentage of neutrophils in the TA samples over time. In contrast, evidence of airway inflammation in bronchioles or alveoli was not detected in horses fed either RB or SB hay. As confirmation that the hay quality was held constant throughout the study, the quality (nutritional and bacterial/fungal content) of each hay type remained unchanged over a period of several months. These findings suggest that factors other than difference in hay quality may contribute to the airway inflammation associated with feeding RBH. These factors may include more continuous exposure to hay, and/or propensity for some horses to bury their muzzles in the hay, however, no attempt was made to determine the effects of these behaviors. This study also did not examine the relationship between hay quality and horse's response, the effect of a more prolonged exposure (i.e., greater than 6 weeks), changes that occur in hay quality once RBs were placed in a feeder (and exposed to the environment), or the potential for other risks associated with round bale hay (such as botulism). Further studies investigating the daily change in hay microflora while it is on pasture may help determine the optimal time frame for horse exposure to a single round bale in regards to respiratory health. Prior to feeding RBH to horses, factors should be considered such as preexisting respiratory disease, vaccination against botulism, RBH storage facilities and the ability to move them, the number of horses to be fed (and thus amount of days a RB may sit out on the pasture), and the quality of the RBH and how it was stored prior to purchasing. Additional studies are also needed to assess the consequences of a similar but longer feeding trial on equine respiratory health, which may indicate that particle exposure is the key factor in round bale hay that leads to airway inflammation.

2.6 TABLES AND FIGURES

Table 1: Distribution of horse characteristics (age, sex, amount of time in a stall daily (which corresponds to the amount of time ridden daily) for each group

ROUND BALE HAY GROUP	GROUP	SQUARE BALE HAY
	SEX	
11	Geldings	11
4	Mares	4
	AGE (YEARS)	
2	< 5	1
5	5-10	7
5	11-15	4
2	16-20	3
1	21-25	0
	AMOUNT OF TIME IN STALL (HOURS)	
4	0	4
6	4-5	6
5	8-9	5

The numbers in the first and last columns indicate the number of horses in that particular group

Figure 1**:** Round bale sampling sites (Picture taken by Dr. Virginia Buechner-Maxwell, October 2009)

Table 2: Scoring system used to rate degree of RAO in horses[20]

Characteristic	Score
Abdominal respiratory effort	
No abdominal component to breathing	1
Slight abdominal component	2
Moderate abdominal component	3
Severe, marked abdominal component	4
Nostril flaring	
No flaring	1
Slight, occasional flaring of nostrils	2
Moderate nostril flaring	3
Severe, continuous flaring during each respiration	4
RAO clinical score (combination of above scores)	2
No signs	3-4
Mild signs	5-6
Moderate signs	7-8
Severe signs	

Figure 2**:** Box-and-whisker plot showing the median percentage of neutrophils in the A) Tracheal aspirate samples and B) Bronchoalveolar lavage samples of horses in both groups over time

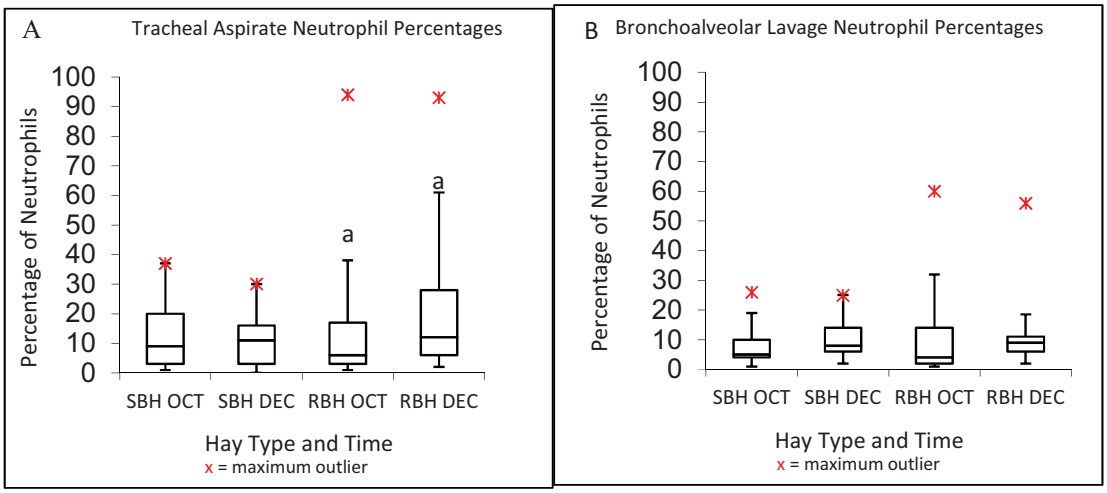

Figure A: a = Difference in the percentage of neutrophils in tracheal aspirate samples from RBH horses between October and December (p = 0.01)

Figure 3: Histograms showing the median (± SD) daily fungal colony forming units cultured from: A) dry hay on ASP media, B) dry hay on FSM media, C) wet hay on ASP media, and D) wet hay on FSM media, from RBH in October (white bar with black dots), RBH in December (grey solid bar), SBH in October (white bar with black squares) and SBH in December (solid black bar). Each day shows the median number of fungal colony forming units that were counted for each hay type and preparation on that day.

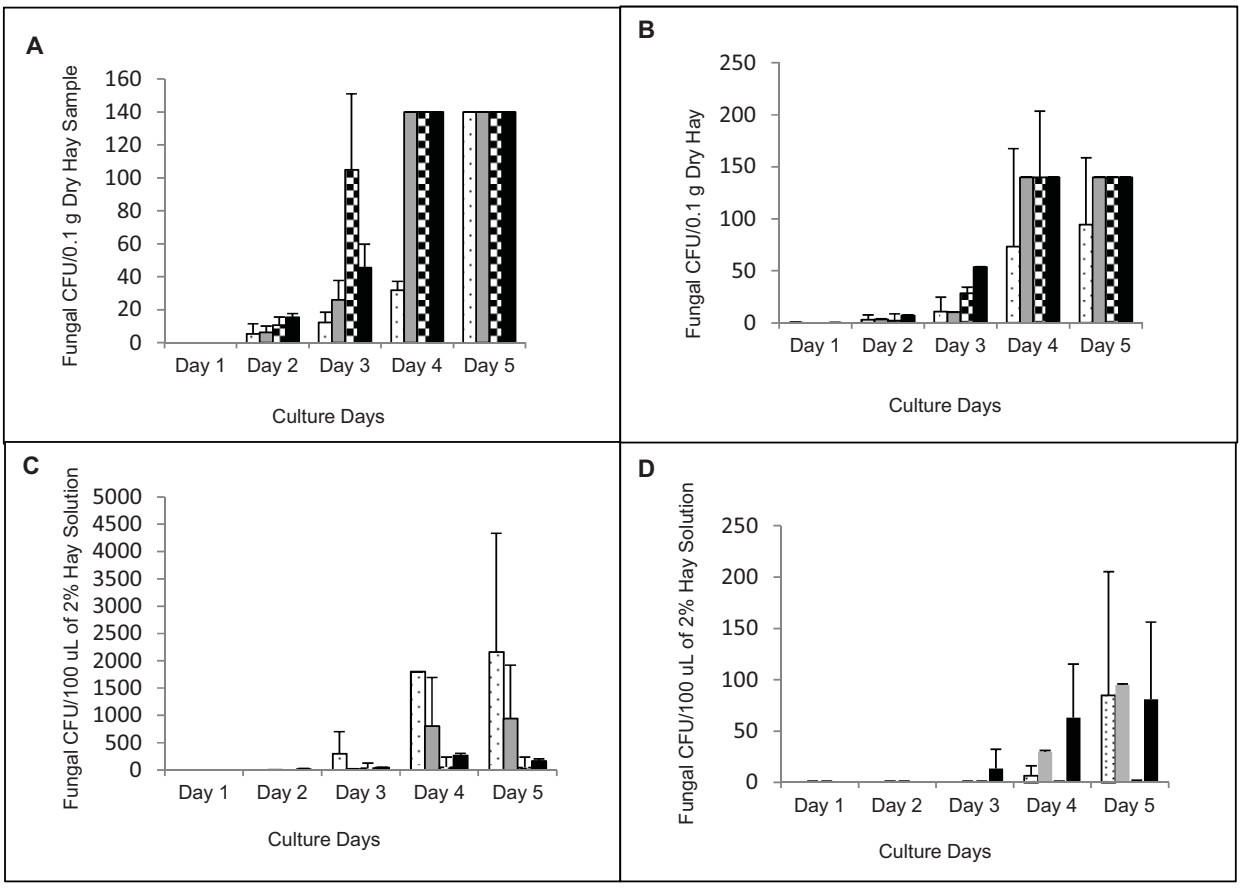

Table 3: Mean (± SEM) values for temperature, heart rate and respiratory rate, and median (± SD) scores for nasal flare, abdominal component and total clinical scores

Hay Type	Sample Time	Temperature (^{o}C)	Heart Rate (bpm)	Respiratory Rate (bpm)	Nasal Flare Score	Abdominal Component Score	Total Clinical Score
SBH	OCT	37.1 ± 0.09^{a}	36 ± 1.48	16 ± 1.08^{b}	1.0 ± 0.46	1.0 ± 0.49	3.0 ± 0.63^{e}
SBH	DEC	37.5 ± 0.11^{a}	36 ± 1.52	11 ± 0.65^{b}	1.0 ± 0.13	1.5 ± 0.25	2.5 ± 0.31^{e}
RBH	OCT	37.2 ± 0.07	37 ± 0.85	16 ± 1.07^{c}	1.0 ± 0.41	1.0 ± 0.35^{d}	2.0 ± 0.72^{f}
RBH	DEC	37.5 ± 0.10	35 ± 1.01	11 ± 0.37^{c}	1.0 ± 0.37	1.5 ± 0.28^{d}	2.5 ± 0.48^{f}

a = Difference in rectal temperature between Oct and Dec for SBH ($p = 0.01$), b,c = Difference in respiratory rate between Oct and Dec for ^{b}SBH ($p < 0.0001$) and ^{c}RBH ($p = 0.0001$), d = Difference in abdominal component score between Oct and Dec for RBH ($p = 0.03$), e,f = Difference in total clinical score between Oct and Dec for ^{e}SBH ($p = 0.03$) and ^{f}RBH ($p = 0.03$)

Table 4: Median (± SD) Tracheal Mucus and Pharyngeal Lymphoid Hyperplasia Scores

Hay Type	Sample Time	TM Score	PLH Score
SBH	OCT	0.0 ± 0.6	1.0 ± 0.26^{a}
SBH	DEC	0.0 ± 0.8	1.0 ± 0.13^{ab}
RBH	OCT	0.0 ± 0.6	1.0 ± 0.21^{c}
RBH	DEC	0.5 ± 0.3	1.3 ± 0.36^{cb}

a = Difference in PLH score for SBH Group for October vs. December ($p = 0.03$), b = Difference in PLH score in December for RBH vs. SBH ($p = 0.01$), c = Difference in PLH score for RBH Group for October vs. December ($p = 0.01$)

Table 5: Mean volume recovered, concentration of cells and percentages of each cell type for the A) Tracheal Aspirate samples and B) Bronchoalveolar Lavage samples.

A	Volume Recovered (mL)	Total Cells (x 10^{5}/mL)*	Neutrophils (%)*	Mast Cells (%)	Macrophages (%)	Lymphocytes (%)	Eosinophils (%)
SBH OCT	17.8 ± 2.4	$26.7^{a} \pm 7.1$	13.1 ± 3.0	6.6 ± 2.2	60.1 ± 3.9	17.3 ± 2.2	2.9 ± 1.1
SBH DEC	17.4 ± 2.2	$6.48^{a} \pm 2.0$	10.3 ± 2.2	7.2 ± 1.6	62.3 ± 2.5	14.7 ± 1.7	5.3 ± 2.3
RBH OCT	19.6 ± 3.2	$39.2^{b} \pm 21.0$	$18.0^{c} \pm 7.6$	4.1 ± 0.7	61.4 ± 6.2	14.7 ± 2.2	1.9 ± 0.6
RBH DEC	19.0 ± 1.6	1.9 ± 0.6^{b}	$25.3^{c} \pm 7.5$	4.3 ± 0.8	56.8 ± 6.5	12.3 ± 3.4	1.4 ± 0.5

Values reported are mean ± SEM, or *geometric mean ± SEM (data was natural log transformed to achieve normal distribution). a,b - Difference in total cells/mL between OCT and DEC for ^{a}SBH ($p = 0.01$) and ^{b}RBH ($p = 0.01$) c - Difference in % neutrophils between OCT and DEC for RBH ($p = 0.01$)

B	Volume Recovered (mL)	Total Cells (x 10^5/mL)*	Neutrophils (%)*	Mast Cells (%)	Macrophages (%)	Lymphocytes (%)	Eosinophils (%)
SBH OCT	155.3[a] ± 5.8	20.1 ± 2.8	7.9 ± 1.8	5.3 ± 0.8	49.5 ± 3.0	36.5 ± 3.5	0.8 ± 0.4
SBH DEC	137.9[a] ± 6.7	13.8 ± 1.7	10.3 ± 1.7	6.3 ± 0.9	42.9 ± 3.6	40 ± 3.3	0.3 ± 0.2
RBH OCT	151.3[b] ± 6.5	22.3 ± 3.8	12.7 ± 4.6	5.4 ± 0.9	46.2 ± 4.3	35.3 ± 3.5	0.3 ± 0.1
RBH DEC	132.5[b] ± 8.0	15 ± 1.9	13.3 ± 3.6	6.1 ± 1.4	47.8 ± 3.9	32.3 ± 3.8	0.5 ± 0.2

Values reported are mean ± SEM or *geometric mean ± SEM (for data that was natural log transformed to achieve normal distribution). a,b - Differences between BAL volume recovered between OCT and DEC for [a]SBH ($p = 0.03$) and [b]RBH ($p = 0.01$)

2.7 FOOT NOTES

a) STIHL Inc., Virginia Beach, VA

b) Nasco, Fort Atkinson, WI

c) Equi-analytical Laboratories, Ithaca, NY

d) BD Bacto™ Agar, BD Diagnostics Systems, Sparks, Maryland

e) Nasco, Fort Atkinson, WI

f) AnaSed, Lloyd, Inc., Shenandoah, IA

g) Torbugesic®, Fort Dodge, Fort Dodge, IA

h) Endoscopy Support Services, Inc., Brewster, NY

i) BBL™Culture Swab™ Plus, Copan for Becton, Dickinson & Company, Sparks, MD

j) Tracheal Wash/Aspiration triple stage catheter, MILA International, Inc., Erlanger, KY

k) Auto T4 Cellometer®, Nexcelom Bioscience LLC, Lawrence, MA

l) Cytospin 3 by Shandon, Thermo Electron Corporation, Waltham, MA

m) Hema-Tek 2000, Bayer Health Car, LLC, Mishawaka, IN

n) Bivona, Gary, IN

o) VEDCO, Inc., St. Joseph, MO

p) The SAS system version 9.2 ,Cary, NC

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Chapter 3

Standard Operating Procedures

3.1 Clinical Evaluation of Horses

A complete physical examination of each horse included the measurement of rectal temperature and calculation of heart rate (beats per minute) and respiratory rate (breathes per minute). A digital thermometer was used to measure rectal temperature. The heart rate was calculated by auscultating the heart with a stethoscope and counting the number of beats in 15 seconds and multiplying it by four. The respiratory rate was calculated by counting the number of thoracic excursions in 15 seconds and multiplying that number by four. Cardiovascular and hydration status was assessed by observing the mucous membranes for color, moisture level and capillary refill time. The gastrointestinal tract, respiratory system and heart were all evaluated by auscultation with a stethoscope. Also as part of the complete physical exam, the paranasal sinuses were percussed and the eyes and nares monitored for the presence of discharge.

For clinical scoring, nasal flare and abdominal push were subjectively evaluated according to previously described methods[1]. Nasal flare was evaluated by assessing the extent of deviation of the alar cartilages from normal resting position. Abdominal push is the degree of involvement of the abdominal musculature in expiration. A scale of 1 to 4 is used with a grade of 1 being normal and grade 4 being most severely affected. The clinical score is the cumulative score of the nasal flare and abdominal push scores (Table 2, Chapter 2, page 55)

A manufactured weight tape for horses[a] was used to calculate body weight. The tape was placed circumferentially around the horse's heart girth (over the highest point of the withers and directly behind the elbows), pulled taught and then relaxed. The weight was recorded as the number where the end of the weight tape rested as it was relaxed. Each horse's weight was measured by two evaluators and averaged.

A body condition score (Figure 1)[2] was assigned to each horse by two evaluators and averaged. A 1-9 scoring system was used with 1 = emaciated, 5 = moderate, and 9 = extremely fat. The body condition score was made after palpating and assessing the amount of adipose tissue in the following places: nuchal ligament, withers, shoulder region, ribs, spine, and tail head.

3.2 Upper Airway Endoscopy

Horses were sedated with xylazine hydrochloride[b] (0.5 mg/kg) and butorphanol tartrate[c](0.01 mg/kg) intravenously for upper airway endoscopy. A 1-meter portable endoscope[d] was inserted into the ventral meatus of the right nostril and advanced into the pharynx. Any abnormalities (mucous, foam, feed material) within the right nostril were recorded. Once in the pharynx, laryngeal function (movement and

symmetry of the arytenoids) was assessed and abnormalities recorded. True laryngeal function could not be completely assessed because the horses were sedated. The pharyngeal region was assessed for the presence of mucous, and a pharyngeal lymphoid hyperplasia (PLH) score (Table 1)[3] was assigned. The scores were based off of a four point grading scale such that Grade 1 = normal pharynx with small in active lymphoid follicles, Grade 2 = numerous small inactive follicles with some exhibiting hyperemia, Grade 3 = many numerous hyperemic follicles covering the dorsum and walls of the pharynx and Grade 4 = large edematous follicles coalescing into polypoid structures. Two evaluators assigned each horse a PLH score, and scores were averaged. The endoscope was then passed into the right guttural pouch with the aid of a stylet[e], and abnormalities (mucous, lymphoid hyperplasia, masses (melanomas)) were recorded. The endoscope was then withdrawn from the right nostril and reinserted into the ventral meatus of the left nostril. Abnormalities within the left nostril were recorded. The left guttural pouch was then entered in a similar fashion as the right, and abnormalities recorded. Once the left guttural pouch was assessed, the endoscope was advanced into the trachea. The amount of mucous within the tracheal lumen was graded. The amount of mucus visible in the trachea was graded on a scale of 0 (no visible mucus), 1 (singular, small blobs), 2 (multiple blobs only partly confluent), 3 (mucus ventrally confluent), 4 (large ventral pool), and 5 (profuse amounts of mucus occupying more than 25% of tracheal lumen), as described by Gerber V, *et al.,* 2004 (Figure 2)[4]. As with the PLH and body condition scores, two evaluators each assigned a tracheal mucous score, which were then averaged.

3.3 Tracheal Brushing for Bacterial/Fungal Culture

With the scope advanced into the proximal 1/3 of the trachea, and a tracheal brushing sample was collected for culture (aerobic and anaerobic bacterial culture, fungal culture) by passing a sterile gastroscopy cytology brush[f] through the biopsy port of the endoscope. Once the sterile plastic sleeve (containing the cytology brush) was advanced beyond the end of the endoscope, the handle was depressed, advancing the brush beyond the end of the plastic sleeve. The cytology brush was then passed back and forth over the mucosa of the trachea, withdrawn into the protective sleeve, and the entire brush was removed from the endoscope. The end of the plastic sleeve of the gastroscopy cytology brush was wiped with alcohol; the brush was advanced out of the plastic sleeve and swabbed with a sterile microbiology culturette[g] for aerobic and anaerobic bacterial and fungal culture. Cultures were performed by the clinical microbiology laboratory at the Virginia-Maryland Regional College of Veterinary Medicine. For statistical analyses, cultures were recorded as either positive or negative.

3.4 Tracheal Aspirate Fluid Collection and Cytology

After the gastroscopy cytology brush was removed from the endoscope, a tracheal aspirate (TA) was then performed by passing a triple lumen catheter[h] through the biopsy port of the endoscope and infusing and retrieving by gentle suction 30-60 mL's of sterile saline. The amount infused and retrieved was recorded, placed in a sterile 50-mL conical tube and set on ice until processed. For processing, samples were centrifuged (300 x g x 15 minutes). The resulting supernatant was discarded and the cell pellet was washed twice in 30 mL of sterile DPBS (500 x g x 8 minutes). Following removal of the final supernatant, the pellet was resuspended in 500 µl of sterile DPBS. The total nucleated cell counts per milliliter were determined by use of an automated cell counter[i] and the total number of cells was calculated based on the volume of aspirated tracheal wash fluid. Slides for differential cell counts were prepared by cytospin[j]. A glass microscope slide and plastic cytofunnel sample chamber with a filter was loaded into a stainless steel clip. Bovine serum albumin[k] (BSA; 50 µL of 30% BSA) was added to each sample chamber funnel by circling the micropipette tip around the bottom of the funnel. After applying the BSA, a volume of fluid that contained about 100,000 cells (usually 100 µl of 1,000,000 cells per ml) was added to the sample chamber funnel. Samples were then spun for 5 minutes at 700 rpm on the "high acceleration" setting. After spinning, the clip was released, chamber funnel removed, and the slide set on the counter to dry. After drying, the slides were stained[l] with a modified Wright's stain. A total of 300 cells (3 x 100) per slide were counted and averaged. Epithelial cells were not included in the total counts.

3.5 Bronchoalveolar Lavage Fluid (BALF) Collection and Cytology

Following the endoscopic exam, a broncoalveolar lavage (BAL) was performed using a bronchoalveolar lavage fluid (BALF) collection tube[m] with an inflatable cuff passed blindly via the nose (in the ventral meatus) and wedged in a peripheral bronchus. At the beginning of the procedure, the cough reflex was suppressed by infusing 40 mL of 0.5% lidocaine[n] as the BAL tube was advanced into the proximal trachea[5]. Once wedged, the cuff was inflated with 5-10 mL of room air and 240 mL of sterile saline was gently infused and aspirated in 120 mL aliquots. The volume retrieved was recorded and the aliquots were combined into a sterile specimen cup and placed on ice until processing. BAL samples were processed and slides prepared in a similar fashion to the TA samples.

3.6 Collection of Hay Samples for Nutritional Analysis and Bacterial and Fungal Cultures

Hay was sampled prior to the start and at the completion of the study. Approximately ten percent of the bales from each group were randomly sampled with a Stihl Model BT 45 gas powered drill[o] with a Penn State Forage Sampler probe[p] at each time point. This probe cuts a core sample 0.75 inches in diameter and 18-inches long. For each bale, one 9-inch (0-9 inches from the surface) "shallow" core

sample and one 18-inch (9-27 inches from the surface) "deep" core sample was collected for analysis. For square bales, samples were taken from the center of the short end. For round bales, samples were taken from 9 locations (Figure 1, Chapter 2, page 54) on the short, rounded side (18 samples per round bale) of ten percent of approximately 30 bales (3 bales sampled). Each of the 18 samples was divided in half; one half of each sample was frozen at -50 °F for later use, and the remaining halves of each sample were combined into a single common bag (one common bag per round bale). Each common bag was then further divided in half, and one half from each common bag was used for nutritional analysis, and while the other half was used for fungal and bacterial culture. Based on this design, there were three common round bale samples sent for nutritional analysis and three set up for bacterial and fungal culture from the first sample date (pre-feeding) and the second sample date (post feeding). The sampling process for the square bales was similar to the round bales. Ten percent of the 300 square bales were sampled (30 bales sampled, total). Sampled bales were selected randomly during the hay pick-up and storage. From the short (square) end of each bale, a "shallow" and "deep" core sample was obtained. Each sample was marked by hay bale number and "deep" or "shallow" sample. All samples were weighed and separated into two bags. One half of each sample was labeled and stored at -50 °F for possible future analysis. The second half of each sample from 10 bales was added to a common bag. Since 30 bales were sampled, this resulted in the formation of 3 common square bale bags. The content of these bags was thoroughly mixed and divided into two bags. One bag was used submitted for nutritional analysis while the second was used to set up cultures. Based on this design, there were three samples from the common square bale sample bags sent for nutritional analysis and three that were set up for fungal and bacterial cultures from the first sample date (pre-feeding) and the second sample date (post feeding). Nutritional analysis was performed by Equi-analytical in Ithaca, New York.

3.7 Media Preparation for Bacterial and Fungal Cultures of Hay Samples

Three media were used for bacterial and fungal cultures - a non-selective potato-dextrose agar (PDA), an *Aspergillus*-selective media and a *Fusarium*-selective media. The potato-dextrose agar was composed of 1000 milliliters distilled water, 6 g potato dextrose broth and 15 g BD Bacto Agar[q]. The media was made in 1 liter quantities in a 2 liter Erlenmeyer flask and a stir bar was used on a medium setting during the mixing process. The components were mixed and the solutions were gradually warmed until they formed a solution. The flask was maintained on a warming plate during mixing and the solution was not allowed to boil. The media was autoclaved on a wet (liquid) cycle at 121 degrees Celsius for 40 minutes on slow exhaust to achieve a pressure of 15-20 psi. The media was then poured into 4 inch plates and allowed to cool for 24 hours before being placed in cold storage at 4 degrees Celsius. The *Aspergillus*-selective media was composed of 1000 mls distilled water, 15 g BD Bacto

Agar[l], 20 g yeast extract, 10 g peptone, 0.5 g ferric ammonium citrate, and 1 ml of a 0.2% stock solution of dicloran (0.2 g dicloran with 100 mls 99% ethanol). After the solution was mixed and autoclaved as previously described, 0.1 g chloramphenical dissolved in 10 mls of sterile distilled water was added to the media prior to pouring into plates. *Fusarium*-selective media was composed of 1000 mls distilled water, 15 g BD Bacto Agar[l], 15 g peptone, 1 g potassium phosphate, 0.5 g magnesium sulfate, and 1 g Terrachlor[m]. The components were mixed and autoclaved as previously described and 1 g streptomycin sulfate and 0.35 g neomycin sulfate dissolved in 10 mls of sterile distilled water were added prior to pouring into plates.

3.8 Bacterial and Fungal Cultures of Hay Samples

Specific methods for quantifying fungal and bacterial growth in hay samples for the purpose of this study were adapted from previous published information[6-8]. All cultures were performed in duplicate using a wet and dry methodology. For the dry preparations, two quantities (0.05 g and 0.10 g) of hay were applied directly to the media. For the wet preparations, 7.5 grams of hay were added to 375 ml of distilled water to create a 2% (weight to volume) solution which was considered the 1X concentration. Serial dilutions were performed to yield 1:10X and 1:100X concentrations. One hundred microliters of each concentration were applied to and spread across the media. These amounts and concentrations of hay were selected based on a the results of a series of pilot studies aimed at determining the optimal concentration that allowed for colony growth over time without compromising the ability to accurately count the colonies (significant overgrowth). Once the samples were applied to the plates, the plates were cultured at room temperature.

Bacterial and fungal growth was counted as colony forming units (CFUs) daily for a total of 5 days. Bacterial CFUs were determined based on growth on PDA only since *Aspergillus* and *Fusarium* selective media contained antibiotics. Fungal CFUs were counted on all medias. If a plate reached greater than 75 CFUs, the number of colonies were deemed too numerous to count and the plate was discarded. The number of colonies were then counted from the next lower concentration (0.05 g for the dry preparation and 1:10X for the wet preparation) and the number of CFUs were multiplied by 2 and 10 respectively to allow statistical analysis of the number of colonies based on 0.10 grams dry preparation and 100 ul wet preparation (CFU/0.1 grams hay and CFU/100 ul of a 2% solution).

Figure 1: Body Condition Scoring Chart[2]

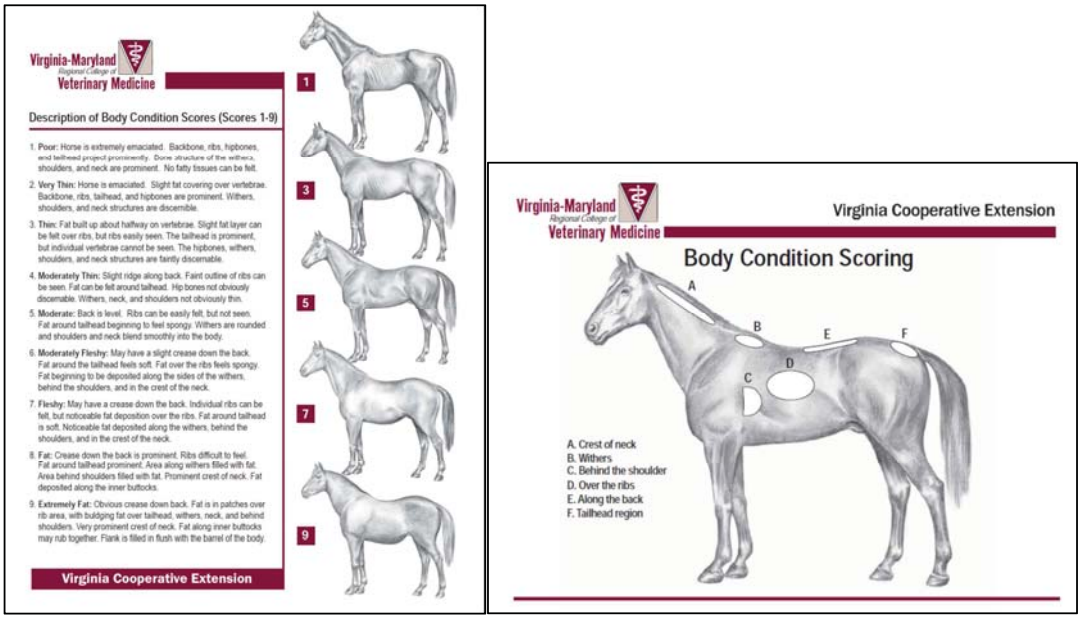
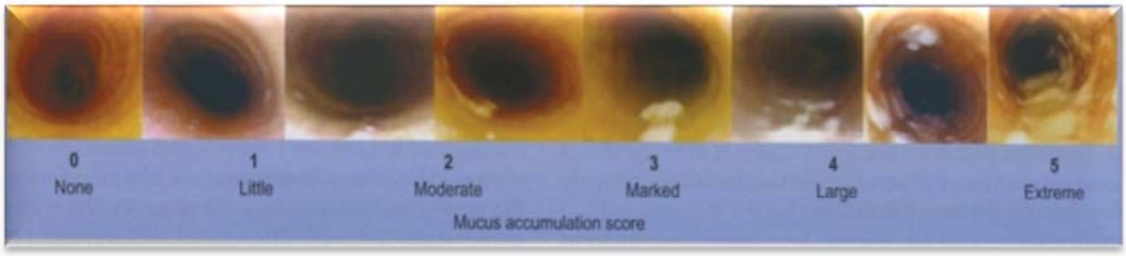


Table 1: Pharyngeal lymphoid hyperplasia scoring system[3]

1	Small # of inactive, small white follicles over dorsal pharyngeal wall. Normal finding in horses of all ages.
2	Many small, white inactive follicles over the dorsal & lateral walls of the pharynx to the level of the guttural pouch. Follicles are larger, pink & edematous.
3	Many large pink follicles, some shrunken white follicles. Distributed over dorsal & lateral walls of the pharynx, in some cases extending onto the dorsal surface of the soft palate & into the pharyngeal diverticula.
4	More numerous pink & edematous follicles packed close together covering the entire pharynx & dorsal surface of the soft palate and epiglottis & the lining of the guttural pouches. Large accumulations appear as polyps.

Figure 2: Tracheal Mucus Scoring Chart[4]



3.9 Footnotes

a) Nasco, Fort Atkinson, WI

b) AnaSed, Lloyd, Inc., Shenandoah, IA

c) Torbugesic®, Fort Dodge, Fort Dodge, IA

d) Olympus, Olympus America Inc., Melville, NY

e) Disposable Biopsy Forceps, Olympus America Inc., Melville, NY

f) Endoscopy Support Services, Inc., Brewster, NY

g) BBL™Culture Swab™ Plus, Copan for Becton, Dickinson & Company, Sparks, MD

h) Tracheal Wash/Aspiration triple stage catheter, MILA International, Inc., Erlanger, KY

i) Auto T4 Cellometer®, Nexcelom Bioscience LLC, Lawrence, MA

j) Cytospin 3 by Shandon, Thermo Electron Corporation, Waltham, MA

k) Sigma-Aldrich, St. Louis, MO

l) Hema-Tek 2000, Bayer Health Car, LLC, Mishawaka, IN

m) Bivona, Gary, IN

n) VEDCO, Inc., St. Joseph, MO

o) STIHL Inc., Virginia Beach, VA

p) Nasco, Fort Atkinson, WI

q) BD Bacto™ Agar, BD Diagnostics Systems, Sparks, Maryland

3.10 References

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Chapter 4

Additional Data and Discussion

The following chapter contains data and some discussion of results that were not presented earlier in Chapter 2. Specifically, the species of fungi and bacteria that were isolated from the tracheal aspirates, the bacterial CFUs cultured from each hay group, and a table containing the mean body weights and median body condition scores for the horses over time.

4.1 Accuracy of tracheal aspirate and bronchoalveolar lavage cell counts of a novice cytologist

Purpose: To check the accuracy of the cell counts performed by the author, a novice cytologist, an experienced respiratory cytologist (J. Hodgson) also performed cell counts on 10% of each of the TA and BAL samples. The results were then compared using a paired t-test or Wilcoxon signed rank test using SAS® software[a], with a value less than 0.05 considered significant.

Table 1: Mean ± SEM percentages of cell types in tracheal aspirate and bronchoalveolar lavage samples of a novice cytologist (J. Larson (JL), author) and experienced cytologist (J.Hodgson (JH), committee member).

	Macrophages (%)	Mast Cells (%)	Neutrophils (%)	Lymphocytes (%)	Eosinophils (%)
JH	41.7 ± 5.3[a]	4.8 ± 1.3	18.2 ± 5.2	33.4 ± 3.8[b]	2.0 ± 1.0
JL	52.1 ± 3.8[a]	6.0 ± 1.4	14.5 ± 4.0	24.4 ± 3.4[b]	3.0 ± 1.4

a = difference in the percentage of macrophages counted between JH and JL ($p = 0.0291$), b = difference in the percentage of lymphocytes counted between JH and JL ($p = 0.0261$).

Discussion/Conclusion: The percentage of each cell type in the tracheal aspirate and bronchoalveolar lavage samples were averaged together and compared as single values. The only differences in cell counts between the experienced cytologist and inexperienced cytologist were within the macrophage and lymphocyte subsets. When all mononuclear cells were combined and compared as a group, there was no difference in the counts made the either the experienced or novice cytologist.

When performing differential cell counts on equine tracheal aspirates and bronchoalveolar lavage samples, it may be difficult to discriminate between certain cell types. BALF macrophages are easy to recognize when large and vacuolated, but small macrophages are similar in appearance to large lymphocytes[1]. BALF mast cells are more readily recognized with certain stains such as a Leishman stain as compared to the Giemsa stain. BALF neutrophils are easily recognized based on their multilobed nuclei, as are eosinophils with their large, refractile eosinophilic intracytoplasmic granules[1].

The ability to accurately identify cells can be improved using different staining techniques. Neef and Hodgson compared two staining methods and techniques on equine TA samples using a modified Wright-Giemsa stain (Diff Quick®) versus immunocytochemistry (Dako EnVision™ with an epithelial cell marker (mouse anti-human pan-cytokeratin AE1/AE3 (Serotec®)) as the primary antibody[2])[3]. In this study, significant differences between certain cell types were found using each staining method[3]. With the immunocytochemistry stain, significantly more epithelial cells and macrophages were counted compared to the Diff Quick® stain, and fewer "unknown" cells were observed. No differences were observed in the percentage of neutrophils, lymphocytes and eosinophils when the two stains were compared, which suggests a Diff Quick® stain is adequate for them[3]. Inter-observer variability was also assessed by correlating cell counts between the experienced and inexperienced cytologist for each stain type. Correlation between cytologists was better with the immunocytochemistry stain, especially with macrophages. Correlation between cytologists was excellent for neutrophils and poor for lymphocytes and unknown cells, regardless of which stain was used[3].

Although intra-observer variability was not assessed between the experienced cytologist (J.H.) and inexperienced cytologist (J.L.) in this study, a similar trend was noted as in Neef and Hodgson's study; the main differences in cell counts between cytologists were within the macrophage and lymphocyte subsets, whereas no differences in cell counts were noted within the other cell types (neutrophils, mast cells and eosinophils).

4.2 Speciation of Fungi and Bacteria Isolated from Tracheal Aspirates

Purpose: The purpose of this procedure was to document the bacteria and fungi present in each horse's trachea prior to and at the completion of the study.

Table 2A-D: Species of Fungi and Bacteria isolated from Tracheal Aspirates

2A: Fungi isolated from the tracheal aspirates of horses in the square bale hay group

Square Bale Hay		
Horse Number	OCTOBER	DECEMBER
1	None	None
4	None	*Aspergillus gaucus*
8	*Aspergillus terreus*	None
13	*Fusarium* spp.	None
15	None	None
16	None	None
17	None	None
18	None	None
19	*Aspergillus fumigatus*	*Chaetomium* spp.
20	None	*Chrysosporium* spp.
21	None	None
24	None	None
25	*Cladosporium* spp.	None
27	*Aspergillus fumigatus*	None
28	None	None

2B: Bacteria isolated from the tracheal aspirates of horses in the square bale hay group

Square Bale Hay		
Horse Number	OCTOBER	DECEMBER
1	None	None
4	*Actinetobacter lwoffi, Staphylococcus xylosus*	None
8	*Pasteurella trehalosi*	None
13	Grossly contaminated	None
15	Grossly contaminated	None
16	Grossly contaminated	None
17	*Klebsiella pneumonia* ss. *pneumonia, Acinetobacter* spp.	None
18	None	*Streptomyces*
19	*Pasteurella alcaligenes*	None
20	None	None
21	None	*Streptococcus suis*
24	*Actinetobacter lwoffi, Staphylococcus xylosus*	None
25	Grossly contaminated	None
27	None	None
28	None	None

2C: Fungi isolated from the tracheal aspirates from horses in the round bale hay group

Round Bale Hay		
Horse Number	OCTOBER	DECEMBER
2	*Cladosporium* spp.	*Aspergillus glaucus*
3	*Cladosporium* spp.	*Aspergillus glaucus*
5	Grossly contaminated	None
6	None	None
7	None	*Aspergillus fumigatus*
9	None	None
10	None	*Aspergillus fumigatus*
11	None	Mucor spp.
12	None	*Aspergillus fumigatus*
14	None	None
22	None	*Aspergillus fumigatus*
23	None	None
26	None	None
29	*Mucor* spp.	None
30	*Verticillium* spp.	*Aspergillus fumigatus*

2D: Bacteria isolated from the tracheal aspirates of horses in the round bale hay group

ROUND BALE HAY		
Horse Number	OCTOBER	DECEMBER
2	None	None
3	Grossly contaminated, *Actinetobacter lwoffi*	None
5	Coagulase negative *Staphylococcus* spp.	None
6	*Actinetobacter lwoffi* *Acinetobacter baumannii* *Klebsiella pneumonia ss pneumoniae*	None
7	*Actinetobacter lwoffi* *Staphylococcus xylosus*	None
9	Grossly contaminated	None
10	Grossly contaminated	None
11	*Actinetobacter lwoffi* Gram positive coccobacilli CDC Enteric GR 76	None
12	Coagulase negative *Staphylococcus* spp. Gram positive coccobacilli	None
14	*Actinetobacter lwoffi* *Staphylococcus xylosus* *Pasteurella alcaligenes*	None
22	*Pantoea agglomerans*	None
23	*Pantoea agglomerans*	None
26	None	Grossly contaminated
29	None	*Staphylococcus xylosus* *Achromobacter xylo ss denitrificans*
30	*Actinetobacter lwoffi* Coagulase negative *Staphylococcus* spp.	None

Discussion/Conclusion: The most commonly identified fungi in TA samples collected from horses have been *Aspergillus* spp., *Penicillium* spp., and *Mucor* spp.[4]. The fungal pathogens that primarily cause respiratory disease in the horse include the systemic yeasts, such as *Coccidioides immitis*, *Blastomyces dermatitidis*, *Cryptococcus neoformans* and *Histoplasma capsulatum* and opportunists such as *Aspergillus* spp., *Pneumocystis carinni* and *Emmonsia crescens*. These organisms cause fungal pneumonia in immunocompromised or neutropenic horses or those with enteritis, colitis, bacterial pneumonia or neoplasms[5-9]. *Klebsiella pneumonia* is a bacterial pathogen that has been considered a pulmonary pathogen in horses with clinical respiratory disease[10] . For the two horses in this study in which *K. pneumonia* was isolated, it was likely a secondary opportunistic invader because these horses never developed clinical signs of respiratory disease. The other bacteria that were isolated can be found in the environment, are commensals on the skin of humans and animals (*Staphylococcus xylosus)* and/or cause

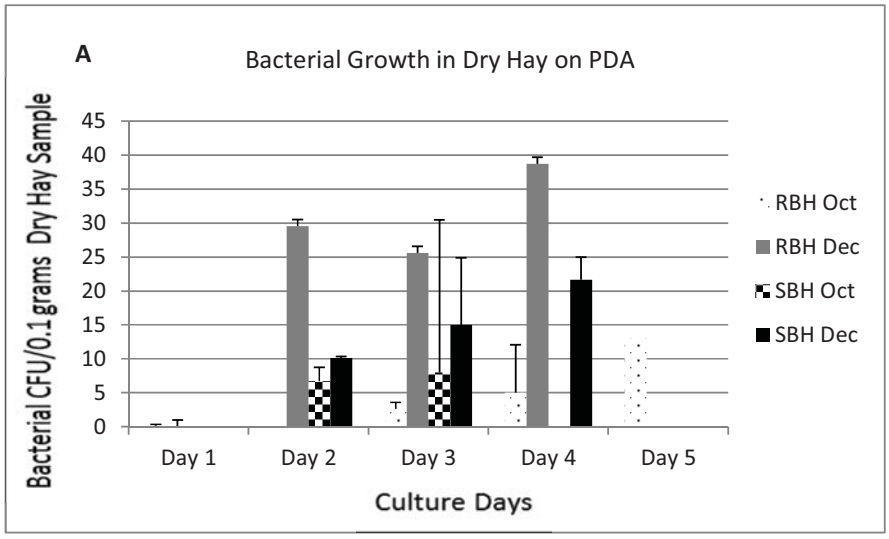
disease in other species (*Streptococcus suis*) and were not considered to be significant respiratory pathogens in this study.

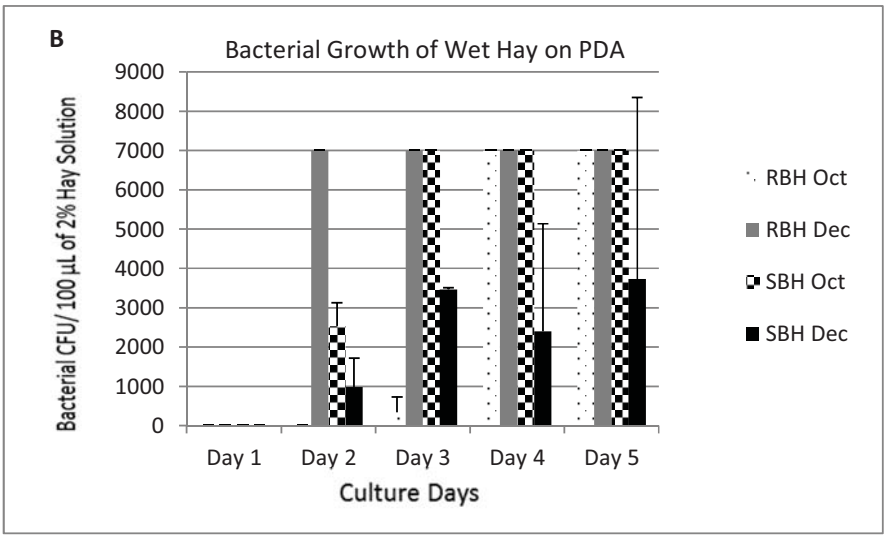
Although not compared statistically, it can be noted that horses in the RBH group appeared to have an increased prevalence of *Aspergillus* spp. in their TA samples after eating RBH for a period of 6 weeks, and the horses in the SBH group did not. *Aspergillus fumigatus* is known to exacerbate clinical signs in RAO-affected horses[11]. The data presented in the tables above indicates horses eating RBH (potentially due the method at which horses consume the hay (head buried)) have increased exposure to *Aspergillus* spp., which could predispose susceptible horses to the airway disease, such as RAO, and may exacerbate clinical signs in horses that already suffer from it.

4.3 Daily Bacterial Growth on PDA media

Purpose: The purpose of this data was to compare the quality of each hay type (in regards to bacterial levels) over a 6 week period and to assess if hay type or storage method had an effect on the bacterial levels.

Figure 1: Histograms showing the median (+ SD) daily bacterial CFU cultured from: A) dry hay on PDA media, B) dry hay on PDA media. Each day shows the median number of bacterial colony forming units that were counted for each hay type and preparation on that day.





Discussion/Conclusion: There was no difference in the number of bacterial colonies cultured from either the wet or dry hay preparations for either hay type at each time. This indicates that hay quality, in regards to bacterial levels, was not affected by the bale type and storage method. Unfortunately some results weren't recorded for all the dry hay prep samples, so this data could not be fully evaluated.

4.4 Horse Weight and Body Condition Scores

Purpose: The purpose of measuring each horse's body weight and body condition score was to see if free choice hay feeding resulted in excessive weight gain.

Table 3: Mean (± SEM) body weight (kilograms) and median (± SD) body condition scores

Hay Type	Sample Time	Body Weight (Kilograms)	Body Condition Score
SBH	OCT	559.4 ± 12^{a}	6.0 ± 0.78
SBH	DEC	531.7 ± 8.5^{a}	5.5 ± 0.67
RBH	OCT	597.6 ± 18.1^{b}	7.0 ± 0.31
RBH	DEC	570.2 ± 14.1^{b}	6.0 ± 0.19

a,b – significant difference in mean body weight over time for ^{a}SBH ($p = 0.0007$) and ^{b}RBH ($p = 0.0008$)

Discussion/Conclusion: Despite being fed hay in a manner designed to be free choice, horses in both groups of hay lost a significant amount of weight over time. The difference in mean body weight (kilograms) in horses fed SBH over time was 27.7 kg ($p = 0.0007$), and the difference in mean body weight (kilograms) in horses fed RBH was 27 kg ($p = 0.0008$). One explanation for the weight loss despite being fed free choice hay could be due to the nutritional quality and palatability of the hay. Relative feed value (RFV) is a number assigned to nutritionally analyzed hay. This value reflects the

overall quality of the hay, and a higher number indicates a higher quality, greater intake, and higher digestibility[12]. The nutritional analyses of each hay type at each time point are listed below in Table 4. Hay with a RFV greater than 151 is considered to be prime quality, whereas hay with a RFV of 86-75 is considered to be poor quality. Hay with a RFV less than 74 should be rejected[12]. Although there were no differences in the nutritional quality of the RBH and SBH at any time point, the RFV of both hays at each time point was between 81 and 85, putting it between the "poor" and "reject" categories, and was likely not very palatable or digestible.

Although not significant, the median body condition score of each group of horses also decreased over time. The median BCS in horses fed SBH dropped by 0.5, and the median BCS in horses fed RBH dropped by 1.0. It is interesting that the drop in BCS for the horses fed RBH was greater than that of horses fed SBH considering the mean weight lost was similar. This is likely due to differences in body condition scoring by the evaluators.

Table 4: Nutrient analysis of hay samples from equi-analytical laboratories for Round Bale Hay and Square Bale Hay in October and December

	RBH OCT	RBH DEC	SBH OCT	SBH DEC
Moisture (%)	7.3	9.2	7.3	8.8
Dry Matter (%)	92.7	90.8	92.7	91.2
Digestible Energy (Mcal/kg)	1.98	1.92	2.05	1.96
Crude Protein (%)	12.3	12.7	11.6	12.5
Estimated Lysine (%)	0.43	0.49	0.4	0.49
Acid Detergent Fiber (ADF) (%)	37.7	39.2	39	36.6
Neutral Detergent Fiber (NDF) (%)	67.2	67.2	64.4	66
Water Soluble Carbohydrates (WSC) (%)	6.1	6.5	9.3	8.6
Simple Sugars (ESC) (%)	3.1	5.8	5.1	5
Starch (%)	4	0.2	3.7	0.3
Non Fiber Carbohydrates (NFC) (%)	11.3	10.1	14.8	11.5
Calcium (%)	0.49	0.47	0.49	0.46
Phosphorus (%)	0.41	0.39	0.44	0.43
Magnesium (%)	0.34	0.36	0.34	0.34

	RBH OCT	RBH DEC	SBH OCT	SBH DEC
Potassium (%)	2.26	2.11	2.15	2.15
Sodium (%)	0.01	0.02	0.005	0.007
Iron (ppm)	152	158	111	95
Zinc (ppm)	23	26	20	18
Copper (ppm)	8	9	7	7
Maganese (ppm)	130	70	103	81
Molybdenum (ppm)	1.2	1.8	2.2	1.9
Relative Feed Value	82	81	85	85

4.5 Study Conclusions

In summary, this study showed that normal horses eating round bale hay for a period of six weeks showed evidence of an increase in airway inflammation compared to horses eating small square bale hay of similar quality based on the increase in abdomoinal component and total clinical scores, pharyngeal lymphoid hyperplasia score and percentage of TA neutrophils over time. There was no difference in the tracheal mucus score or distribution of cell types in the BAL samples. The inflammatory changes mostly occured in the large airways where air velocity was increased, suggesting the inflammation was potentially caused by a greater exposure to larger particles, which deposit by impaction. The method of which horses consume round bale hay (with their muzzles buried in the hay) could explain this phenomenom.

Several questions not tested in this study, whose answers may help elucidate why horses eating round bale hay have increased airway inflammation compared to horses eating square bale hay, include:

- Would poorer hay quality result in a more profound inflammation?
- Is the cause of the inflammation due to changes in RBH quality once its placed on the pasture?
- Does airway inflammation increase with more prolonged exposure to RBH hay?
- Are there other environmental factors (temperature, dust) that could amplify the effect of RBH exposure?
- Would horses with preexisting airway disease (RAO, IAD) have a more profound response?

Limitations that were beyond the scope of this study included a short feeding period, lack of identification of other factors in hay that known to cause airway inflammation (dust, β-glucans,

endotoxin), not identifying the changes that occur in round bale hay quality as it sits on pasture, and not identifying and or quantifying "what" the horse is exposed to as it consumes hay. Future studies that could be developed from the results of this one include: monitoring the airway responses of horses fed 1) RBH and SBH for a longer feeding period, 2) hays of varying qualities, and 3) round bale hay fed in a different manner (large bale vs. rolled on the ground), monitoring the changes in round bale hay (nutrition, bacterial/fungal/dust levels) as it sits on pasture being consumed, quantifying and characterizing "what" horses are exposed to as they consume RBH or SBH, and comparing the airway responses of horses with pre-existing airway diseases (RAO, IAD) fed RBH and SBH.

4.6 Footnotes

a) The SAS system version 9.2 ,Cary, NC

4.7 References

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