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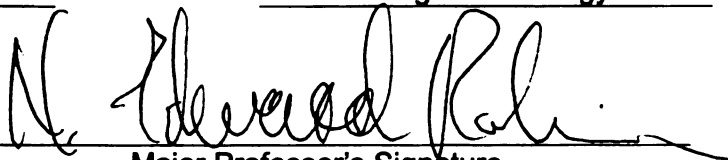
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THE AIR IN THERE – SHOULD WE CARE?
AN INVESTIGATION INTO THE RELATIONSHIP BETWEEN INDOOR
AIR QUALITY AND TRACHEAL MUCUS IN THOROUGHBRED
RACEHORSES

By

Melissa Millerick-May

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ABSTRACT

THE AIR IN THERE – SHOULD WE CARE? AN INVESTIGATION INTO THE RELATIONSHIP BETWEEN INDOOR AIR QUALITY AND TRACHEAL MUCUS IN THOROUGHBRED RACEHORSES

By

Melissa Millerick-May

Tracheal mucus accumulation is a significant problem in racehorses, as its presence in sufficient quantities negatively affects racing performance. The cause is likely multifactorial in nature, but the stable environment has been implicated as one risk factor, as it has been shown that some healthy horses brought into the stable from pasture experience airway inflammation. The purpose of my work was to describe the range of concentrations of airborne particulate matter within the stable, and factors that influence its presence. I hypothesized that tracheal mucus accumulations result, at least in part, from exposure to particulate matter (PM) in size ranges (less than 10 and less than 2.5 microns in diameter) determined to be important in human respiratory disease. I hypothesized that area particle concentration and personal particulate exposure varies with season, stable, stall location within a stable, and activity level, and correlates in an exposure-dependent manner with established measures of inflammatory airway disease (IAD). Using direct-reading instruments that measure particulate concentrations, I demonstrated that dusts present in the breathing zone of horses during routine stable activities (cleaning, feeding, and riding) contained concentrations of PM₁₀ and PM_{2.5} that would be considered unacceptable in industrial environments, but that consistent use of easy to implement dust-suppression techniques more than adequately reduced exposures. Investigations in stables at

a Midwestern Thoroughbred racetrack, again using direct-reading instruments, revealed significant variations in area PM₁₀ and PM_{2.5} concentrations by season (month), time of day, stable, and certain stalls within stables. PM was lowest during months following periods of wet weather and highest after periods of hot dry weather. Early morning PM concentrations, when feeding, cleaning, and training activities occur, were highest in comparison to mid-day and afternoon concentrations when activity was minimal. Enclosed stables with few sources of natural dilution ventilation experienced the highest concentrations of PM as compared to the open-aired stables designed for maximum ventilation. Stalls located in either high-traffic areas or areas devoid of natural dilution ventilation contained PM concentrations significantly higher than adjacent stalls. The presence of tracheal mucus was significantly associated with area PM concentrations, and overall prevalence was highest during months and in stables and stalls where area PM concentrations were the greatest. Personal particulate exposure monitoring (PM₁₀) in horses both with and without tracheal mucus suggests that personal idiosyncratic activities did not influence personal exposures to the same degree as area PM; furthermore, overall prevalence of tracheal mucus shows a striking association to personal PM exposures that likely reflects ambient concentrations (e.g. monthly variations in PM). If this is true, then implementation of dust suppression techniques within stables should significantly reduce the overall prevalence of significant quantities of tracheal mucus. Percent neutrophils present in tracheal wash samples did not correlate with the presence of tracheal mucus, and I therefore suggest that its use in diagnosing IAD be reconsidered, as endoscopic examination scoring of tracheal mucus is easy to perform, and likely the most accurate predictor of airway disease sufficient to affect performance.

For Star – The inspiration behind these investigations

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LIST OF TERMS

ACGIH	American Conference of Governmental Industrial Hygienists
ANOVA	Analysis of variation
BAL	Bronchoalveolar lavage
CAPs	Concentrated ambient particles
COPD	Chronic Obstructive Pulmonary Disease
GEE	General estimating equation
IAD	Inflammatory airway disease
MS	Mucus score
MS=0	Mucus score equal to zero
MS $\geq$ 1	Mucus score greater than or equal to 1
MS $\geq$ 2	Mucus score greater than or equal to 2
mg/kg	Milligram per kilogram
mg/m ³	Milligram per cubic meter of air
NAAQS	National Ambient Air Quality Standard
OELs	Occupational exposure limits
OSHA	Occupational Safety and Health Administration
PM	Particulate matter
PNOC	Particles not otherwise classified
RAO	Recurrent airway obstruction
TWA	Time-weighted average

INTRODUCTION

Airway inflammation and associated tracheobronchial mucus accumulation in racehorses has been shown to reduce racing performance (Holcombe *et al.* 2006; MacNamara *et al.* 1990b), a significant problem for trainers and owners, as the overall prevalence has been reported to be 13% (Wood *et al.* 2005a). Mucus accumulations are generally a consequence of airway inflammation, the cause of which is most likely multifactorial in nature. Risk factors of interest in racehorses are infection and exposure to particulate matter (dust). Many of the horses in racing stables found to have mild to moderate airway disease do not culture positive for bacteria (Christley *et al.* 2001a; Wood *et al.* 1993; Wood *et al.* 2005a, c), therefore our focus has turned to the evaluation of exposures to particulate matter.

It is likely that multiple factors acting alone or synergistically cause the severity of inflammation that is responsible for mucus production and accumulation. Of particular relevance to the development of airway inflammation in racehorses are the observations in humans that increases in ambient 'coarse' (<10 micron in diameter) and 'fine' (<2.5 micron in diameter) particulates are associated with worsening of disease (mucus production, cough, wheeze) in patients with asthma and COPD, and increased upper respiratory infection visits to hospitals in individuals without pre-existing disease (Pope 1991a; Pope III 2000 ; Pope III *et al.* 2002; Schwartz 2004).

Sources of particulate matter in the racehorse environment include feed and bedding, flooring materials, track footing, road dust, vehicle exhaust, and emissions from adjacent industrial operations. Particle sizes of interest in this study are classified as those less than 10 (PM10) and less than 2.5 (PM2.5) microns in diameter. A small percentage of PM10 have the potential to reach the lower airways but the majority of these particles are deposited in the central airways such as the trachea (ACGIH 2005a). The smaller particles represented by PM2.5 penetrate deep within the airways.

Our long-range goal is to understand the causes of inflammatory airway disease (IAD) in racehorses. My investigations focused on the role of airborne particulates as a cause of airway inflammation, and resultant mucus production. In order to test the hypothesis that area particle concentration and personal particulate exposure varies with stable, stall location within a stable, and activity level, and correlates in an exposure-dependent manner with established measures of airway inflammation (mucus score, and inflammatory cell counts) in racehorses with and without IAD, I did the following:

- Evaluated the range of concentrations of PM10 and PM2.5 in the breathing zone of horses during routine stable activities using real-time particulate monitors (chapter 1)
- Investigated factors that influence area (background) concentrations of PM10 and PM2.5 in 3 stables of differing design and management at a midwestern Thoroughbred racetrack (chapter 2)

- Determined the relationship between area concentrations of PM10 and PM2.5 by stable, stall, month, and time of day, and the overall prevalence of tracheal mucus in a population of Thoroughbred racehorses (chapter 3)
- Used a case-control study design to identify significant differences in PM10 exposures between cases and their matched controls (chapter 4)

The individual studies (chapters) have been written such that they can be read independently, with references following each chapter. The studies are written in first person plural form (e.g. 'we investigated'), as a team of individuals was necessary to perform these investigations.

Literature Review

Definition of IAD

Inflammatory airway disease (IAD) of racehorses is a syndrome characterized by cough, exercise intolerance, excess tracheobronchial mucus that is visible upon endoscopic exam, and inflammatory cells in tracheal aspirates and bronchoalveolar lavage (BAL) (Anon 2003). Up to 45% of racehorses in a stable may be affected at any one time (Newton *et al.* 2003a; Wood *et al.* 2005b), leading to a significant financial impact as relatively small amounts of mucus in the airways result in horses finishing further back in a race (Holcombe *et al.* 2006; MacNamara *et al.* 1990b). Due to the potential impact of IAD on the racing industry, investigators have focused on elucidating its cause, which is most likely multifactorial in nature.

Risk Factors for IAD

IAD is common in young racehorses, with incidence declining as the horse ages and length of time in training increases (Christley *et al.* 2001a; Newton *et al.* 2003b; Wood *et al.* 2005a, c), suggesting an infectious component, as increasing time spent in a stable and exposure to bacterial and viral pathogens would confer immunity, similar in nature to that of a child entering daycare. As a result, several investigations have focused on the role of infectious agents in the etiology of IAD. Bacterial, and not viral infections are associated with neutrophilic inflammation and mucus accumulation in racehorse populations (Chapman *et al.* 2000; Christley *et al.* 2001b; Wood *et al.* 1993; Wood *et al.* 2005b, c), and in one study as age of the population increased, the prevalence and incidence of *Streptococcus zooepidemicus* and *Streptococcus pneumoniae* infection and

IAD decreased at the same rate (Wood *et al.* 2005a). Bacterial infections, however, are not the only cause of IAD in racehorses, as many racehorses (over 20%) with airway inflammation culture negative (Chapman *et al.* 2000; Christley *et al.* 2001a; Wood *et al.* 2005a). The stable environment has been identified as a second risk factor for airway inflammation (Malikides and Hodgson 2005), as young racehorses are kept primarily outdoors until, when at around the age of two, they are brought into a training facility or racetrack to begin their careers.

Mucus, Airway Inflammation, and Particulates

Mucus secretion is a response to local irritation or inflammation through either neural reflexes or secretion/recruitment of inflammatory cells (Wanner *et al.* 1996). Particulates comprised of organic compounds, bacterial components (endotoxin), and transition metals have been demonstrated to catalyze reactive oxygen species production (Gonzalez-Flecha 2004; Shukla *et al.* 2000) potentially stimulating the release of stored mucosubstance via signaling or cytotoxic events (Gonzalez-Flecha 2004). Inhalation of particulate matter initiates an inflammatory response in both humans and animal models, with a common component of this response being neutrophil recruitment (Ghio and Devlin 2001; Ghio *et al.* 2006; Li *et al.* 1997). Exposure to organic dusts commonly found in stables, dispersed from feed and bedding, have been shown to be associated with airway neutrophilia and mucus accumulation in *healthy* horses (Gerber V 2004; Holcombe *et al.* 2001; Tremblay *et al.* 1993).

Particle Composition

Particle composition in a stable is a complex mixture. Airborne exposure to organic dusts (mold and fungi) and endotoxin resulting from agitation of feed and bedding materials result in airway neutrophilia and an increase in mucus production in both healthy horses (Gerber V 2004; Holcombe *et al.* 2001; Tremblay *et al.* 1993), and those diagnosed with recurrent airway obstruction (RAO) (Bartner *et al.* 2005; Berndt *et al.* 2006, 2007; Derksen *et al.* 1985; Gerber *et al.* 2004a; McGorum *et al.* 1993a, b; McGorum BC 1998 Sep; Robinson *et al.* 2006). Inorganic components of particulate matter including metals (iron) and crustal materials (crystalline silica) are present in the dusts to which horses are exposed (unpublished data), and have been associated with the development and worsening of airway disease in humans and other animal models (ACGIH 2005a). As yet, these materials have not been evaluated in terms of their impact on equine airway inflammation/disease (Ghio *et al.* 2006).

Co-exposures

As particles in a dust-cloud are not of uniform size or composition, exposures over a period of time are varied. For example, as a racehorse eats its hay in the morning, it may be exposed to a myriad of particles of differing composition. Airborne mold spores and endotoxin may be elevated during morning cleanout and feeding activities, and diesel exhaust persists throughout the day as a result of vehicle and tractor movement. Evaluations of co-exposures in horses are limited, however, one such study involving challenge with a hay dust suspension both with and without endotoxin (endotoxin depleted hay dust suspension), demonstrated a 'synergistic proinflammatory

response' (increased airway neutrophilia and dysfunction) in horses with RAO (Pirie *et al.* 2003b). Low dose co-exposure in rats to particles harvested from rural and urban locations revealed that regardless of sampling location and metal composition, the coarse fraction (PM₁₀) induced neutrophilic inflammation in the lung, presumably due to their endotoxin content (Schins *et al.* 2004). Human studies reveal that relatively small increases in fine particles (PM_{2.5}) are associated with increased hospital admissions due to increased upper airway infection (Peel *et al.* 2005), and exposure studies in rats have demonstrated a compromised ability of the lung to handle streptococcal infections (Zelikoff *et al.* 2003; Zelikoff *et al.* 2002).

Particle Size

Airborne PM (dust) in racehorse stables originates from feed and bedding, flooring materials, racetrack footing materials, and emissions from neighboring roadways and industrial operations. As there are many sources of PM in the environment of the racehorse, size and composition of the particulate matter is not uniform. In regards to human disease, particulate matter is generally classified in size ranges that include inhalable (< 125 micron in diameter), thoracic (< 25 micron in diameter), and respirable (< 10 micron in diameter) (ACGIH 2005a). Approximately 50% of particles 10 micron in diameter have the ability to be deposited in the thoracic region (anywhere in the lung airways), while that number goes up to approximately 90% for particles 2.5 micron in diameter (ACGIH 2005a). On the same note, approximately 1% of particles 10 micron in diameter have the ability to be deposited in the respirable region (gas exchange region), increasing to 95% of particles 2.5 micron in diameter (ACGIH 2005a). The

Environmental Protection Agency (EPA) has classified these particles as ‘coarse’ or PM₁₀ (2.5-10 micron in diameter) and ‘fine’ or PM_{2.5} (<2.5 micron in diameter)(USEPA).

Consequence of Particulate Exposures

Human epidemiology studies consistently show that small increases in ambient (background) concentrations of PM₁₀ and PM_{2.5} of mixed composition results in a worsening of symptoms in patients with pre-existing airway disease (Brunekreef and Forsberg 2005; Oberdorster 1996; Pope 1991b; Pope *et al.* 2002), an increase in hospital admissions (Peel *et al.* 2005; Pope 1991a), days away from school (Morgenstern *et al.* 2007; Pierse *et al.* 2006; Schwartz 2004), and lost work days (Pope 1991a; Pope III 2000 ; Pope III *et al.* 2002; Schwartz 2004; Sunyer 2001). Overloading of the lungs by particulate matter that is considered to be relatively inert in nature has the potential to impair macrophage function resulting in a prolonged inflammatory response (Morrow 1992). Mechanisms that have been suggested in the ‘overload’ model include neutrophil and alveolar macrophage activation and release of inflammatory cytokines (Morrow 1992; Oberdorster 1995). In-vitro exposure studies in mice with fine (PM_{2.5}) concentrated air particles (CAPs) resulted in impaired macrophage interactions (binding, internalization, and killing) with *Streptococcus pneumoniae* (Zhou and Kobzik 2007). A similar mechanism could be responsible for the high prevalence of streptococcal infections when racehorses first enter training.

Particulate Monitoring in Stables

With regard to particulate effects on the airways in horses, organic dust challenges have been used to induce airway inflammation (Derksen *et al.* 1985; Pirie *et al.* 2003a), area particulate concentrations have been measured in a riding stable (Crichlow *et al.* 1980), the dust-generating potential of various feeds and bedding have been reported (Vandenput *et al.* 1997), and particulate load has been measured with personal samplers under various management systems (McGorum *et al.* 1998; Webster *et al.* 1987; Woods *et al.* 1993). Previous studies conducted in controlled settings as well as private boarding stables utilizing traditional filter/pump methods (Clarke and Madelin 1987; Clarke *et al.* 1987; Crichlow *et al.* 1980; Kirschvink *et al.* 2002; McGorum *et al.* 1998; Woods *et al.* 1993) and direct reading instruments (Clements and Pirie 2007a, b) have shown that dusts present in the breathing zone of horses are composed of particles that include those in size ranges (PM₁₀ and PM_{2.5}) consistent with those described in the human literature.

Justification of Our Studies in Relation to the Current Literature

Our studies are the first to investigate the association between airborne exposure to particulates and the presence of tracheal mucus at an American Thoroughbred Racetrack. The first step in characterizing the environment of the racetrack is to survey the stables (particulate mapping) to better understand the impact of stable design, ventilation status, and management practices on area concentrations and numbers of particles in size ranges (PM₁₀ and PM_{2.5}) known to influence disease in humans and

animal models. Because overexposure to particulate matter is recognized as a contributing factor in airway inflammation (Morrow 1992; Oberdorster 1995), and is the basis for setting exposure limits for ‘particulates not otherwise classified’ (PNOC) in occupational settings (ACGIH 2005a), we chose to utilize real-time particulate monitors which determine average and peak PM₁₀ and PM_{2.5} particulate concentrations.

‘Mapping’ particulates, the act of determining PM concentrations at points equally distributed throughout a pre-determined space, is also an important step in determining the contribution of area particulates dispersed by sources not actively influenced by the horse (e.g. stall cleaning, the act of feeding, ventilation status, exhaust and dust from roadways). Careful tracking of activities and ventilation status (doors/windows open or closed, air moving fans, etc.) gives insight into the relative contribution of these external sources on area particulate concentrations, and is crucial for developing the rationale for cost-effective and comprehensive sampling protocols focusing on composition.

Area particulate concentrations are important to understand in the racehorse environment as the majority of the epidemiological investigations of the effects of air quality on respiratory health, as measured by excess morbidity and mortality, focus on concentrations of PM₁₀ and PM_{2.5}, and these values would likely be the minimum concentrations to which a horse is exposed. However, depending on the activity in which an individual is involved, area concentrations may or may not reflect personal exposure. In the case of horses, individual behavior may determine daily exposure to particulates. Some horses bury their faces in hay when they eat, others sniff and dig at the bedding, and some lie down more than others. All of these idiosyncrasies have the potential to

affect both average and peak exposures. For this reason, we used small-scale personal monitors, with the sampling point located at the nostril of the horse. Our sampling protocol, for the first time, allowed us to characterize average, minimum, and maximum particulate concentrations, and the length of time these exposures occur. Previous particulate sampling technology used in IAD and RAO studies, consisting of pump and filter apparatus, measured only average concentration values and did not allow for the evaluation of 'peak' exposures. Use of real-time monitors allowed us to determine whether or not there were significant differences (both average and peak values) in exposures of horses both with and without tracheal mucus, and the factors that lead to those exposures.

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CHAPTER 1

Indoor Air Quality in a Boarding Stable

Summary

Reasons for Performing Study

Recurrent airway obstruction (RAO) and inflammatory airway disease (IAD) are equine respiratory syndromes that are characteristically associated with inhalation of organic dust. Feedstuffs, bedding, and arena footing are all sources of particulate matter that, upon agitation, have the potential for dispersion into the horse's breathing zone.

Objectives

Our study had four objectives: 1) to measure particulate concentrations in the breathing zone of horses eating a variety of feeds and during a variety of routine stable activities, including riding in an arena; 2) to determine the amount of some crustaceous metals and crystalline silica in arena footing and dust on ledges; 3) to investigate the efficacy of adding water to reduce particulate generation concentrations arising from feeds and activities; and 4) to determine stall ventilation and ammonia concentrations in stalls during winter and summer.

Methods

Two aerosol photometers measured coarse and fine particulate concentrations in the breathing zone of the horses eating hay, alfalfa cubes, complete pelleted feed, sweet feed, and a "senior" diet. Particle concentrations also were measured during stall

cleanout, raking of aisles, removal of cobwebs, and during 1-hour riding lessons in the indoor arena with footing of varying water content. Particle size distribution and the content of several metals and crystalline silica were measured in arena footing and dust on ledges. Stall ventilation was evaluated by measurement of ammonia concentrations in the air.

Results

In all situations, concentrations of coarse particles always were greater than those of fine. Eating dry hay and alfalfa cubes generated the highest concentrations of particulates, with lower concentrations originating from complete feed and sweet feeds. Stall cleaning generated fewer particles than aisle sweeping or removal of cobwebs. Adding moisture reduced particle concentrations dramatically. Riding in the arena generated large excessive concentrations of dust unless moisture content was greater than 10 percent. Stall ammonia concentrations were greater in summer than in winter.

Conclusions

Horses may be routinely exposed to levels of particulate matter and ammonia that exceed occupational exposure limits for people. Inorganic components of dust sampled included iron and crystalline silica, both known to induce respiratory disease in occupational settings. Wetting was very effective in controlling dusts in the breathing zone of horses. Location of stall, season, and proximity to source of natural ventilation affects ammonia concentrations.

Potential Relevance

Understanding sources of dust and the importance of breathing zone concentrations will help in managing horses with respiratory disease. Horse owners need to know that dust can be suppressed by use of water, and both dust and ammonia can be removed by improved ventilation. Real-time monitors are an effective tool in the education of stable owners about inhalation hazards present in their stables. Particulate exposures in horses may be greatly underestimated.

Introduction

Recurrent airway obstruction (RAO) and inflammatory airway disease (IAD) are two equine respiratory syndromes that are characteristically associated with poor air quality in stables (Anon 2001, 2003). Feedstuffs and bedding materials are common sources of particulate matter in the equine environment. If sufficiently agitated, as occurs during eating and movement of the horse within the stall, these materials have the potential to disperse particulate into the horse's breathing zone in significant concentrations (Vandenput *et al.* 1997; Woods *et al.* 1993).

Human epidemiology studies suggest a strong association between exposure to coarse and fine airborne particulate matter and worsening of disease in patients with asthma and COPD (Oberdorster 1996; Pope 1991b; Pope *et al.* 2002). Coarse particulates have an aerodynamic diameter between 2.5 and 10 μ (PM₁₀), and fine particulates are those with a mean aerodynamic diameter of less than 2.5 μ (PM_{2.5}). In the US, the Environmental Protection Agency (EPA) has established National Ambient Air Quality Standards (NAAQS) for particles in these two size ranges. In addition, the

American Conference of Governmental Industrial Hygiene (ACGIH) has established chemical and size-specific occupational exposure limits (OELs) for coarse and fine particulates, but their size ranges differ from those referred to in the NAAQS (ACGIH 2005b; Anon 2006). They are inhalable ($<100\ \mu$ diameter), thoracic ($1\text{-}25\ \mu$ in diameter), and respirable ($<10\ \mu$ in diameter) particulates.

With regard to particulate effects on the airways in horses, organic dust challenges have been used to induce airway inflammation (Derksen *et al.* 1985; Pirie *et al.* 2003a), area particulate concentrations have been measured in a riding stable (Crichlow *et al.* 1980), the dust-generating potential of various feeds and bedding have been reported (Vandenput *et al.* 1997), and particulate load has been measured with personal samplers under various management systems (McGorum *et al.* 1998; Webster *et al.* 1987; Woods *et al.* 1993). These investigations have been conducted in relatively controlled academic environments, and none have measured particulate load in the breathing zone of horses in stables during a variety of routine activities. This paper begins to correct this deficit by measuring $PM_{2.5}$ and PM_{10} in a large boarding stable that a) uses various forms of feedstuffs as specified by each horse owner, b) is managed as a cooperative, allowing for routine tasks to be performed by multiple individuals, and c) has an active riding/lesson schedule allowing for sampling to occur in an indoor riding arena. Through the use of real-time particle samplers, not only the average values but peak exposures were measured. The bulk dust samples were analyzed for inorganic components known to cause respiratory disease in humans. Measurements of concentrations of ammonia allowed estimation of regional ventilation in the stable.

Materials and Methods

The Boarding Stable

The boarding stable consists of two buildings connected by an aisle (Figure 1). The larger building measures 66 x 27 m, and consists of a 40 x 20 m indoor arena with 13 stalls along the east wall, each measuring approximately 3 x 3.5 m. The stalls are separated from the arena by an aisle, and a wall measuring 1.2 m in height. Flooring in the stalls consists of packed clay on top of which sawdust is partially replaced daily. Flooring in the aisle consists of packed dirt/clay with rubber matting down the center. Arena footing consists of a solid clay base underneath a mixture of sand (approximately 20 cm in depth). The footing has not been completely changed for over 10 years, but has been added to approximately every other year. There is a large sliding door (4.5 x 6 m) at one end of the arena, and a sliding door (2.4 x 3 m) at each end of the aisle. The older and smaller of the two buildings is wooden with a hayloft that spans the entire length and width of the structure. It contains five stalls (3 x 3.5 m) and measures 10 x 14 m overall.

Particulate Measurements

All particulate air sampling was conducted by use of two aerosol photometers (DustTrak, TSI, Shoreview, Minnesota). Each photometer was fitted with either a 10 μ or a 2.5 μ particle size conditioner, and was calibrated before and after measurements according to manufacturer instructions (Anon 2005).

Feed

Feedstuffs utilized in this study were baled alfalfa/timothy hay, commercially prepared sweet feed, a complete feed, and a senior feed. All feed, with the exception of the alfalfa/timothy hay, was consumed from a feed tub suspended in the front corner of the stall. The hay was fed from the floor. To determine the effect of wetting on particulate concentrations, feedstuffs were watered down immediately prior to feeding via a spray nozzle attached to a garden hose. Five sets of measurements were taken for each type of feed under both wet and dry conditions. The flexible tube (0.64 mm IAD) was attached to the particle size conditioners on each of the two aerosol photometers and its end was held adjacent to the nostril of the horse for the duration of feeding in order to make simultaneous measurements of PM_{10} and $PM_{2.5}$ in the breathing zone of the horse. For each measurement, minimum, maximum, and average values were recorded in mg/m^3 .

Stable Activities

Five sets of measurements were taken during aisle sweeping, stall cleaning and removal of cobwebs, and grading the arena. Arena grading was performed by pulling a metal harrow (drag) over the arena for 13 minutes with a four-wheeled utility vehicle. To determine the effect of wetting, a garden hose with a spray nozzle was utilized to dampen the surface of the arena footing, aisle footing, and stall sawdust immediately before each activity began. It was not possible to study removal of cobwebs in the wet conditions because the aerosol photometer views water aerosol as particulate.

A horse was placed in crossties in the center of the aisle during sweeping and arena grading, and measurements were taken adjacent to the nostril. The horse was placed back in its stall during stall cleaning and removal of cobwebs, with all measurements taken adjacent to the nostril. Measurements were taken for the duration of the activity, and the minimum, maximum, and average values in mg/m^3 were recorded.

Riding

Five students rode at a walk, trot, and canter for 60 minutes in each of six trials. Five trials were conducted with varying moisture content in the footing. The sixth was performed after application of a commercial dust suppressant containing calcium chloride. Moisture content was measured by weighing a sample of footing, and drying it to constant weight.

Both aerosol photometers were placed side-by-side in the center of the arena approximately 1 m above the floor in order to approximate the breathing zone of the horse. The monitors ran continuously throughout each 60-minute trial. Data were recorded every second, and minimum, maximum, and average values were computed for each trial.

Bulk Samples

Three bulk dust samples were taken from the arena footing, and four samples from ledges on the wall of the stable at least 3 m above the ground. The height of sampling was selected so that particulates represented those that would have passed through the breathing zone and settled on a flat surface. For both footing and wall

samples, the individual samples were combined and mixed and a representative sample was taken from each. Each sample was split and sent to the laboratories for measurement of particle size distribution and analysis of composition. Particle size analysis was performed using an LS13 320 particle size analyzer with the Tornado dry powder sample module, and a specified size range of 0.4 μ to 2000 μ in diameter (Beckman-Coulter (2006) http://www.beckmancoulter.com/products/instrument/partChar/pc_ls13320.asp). Composition analysis of the arena footing included crystalline silica (quartz, cristobalite, tridymite) using the NIOSH 7500 method (Galson 2006) and x-ray diffraction, and ten metals (silver, cadmium, cobalt, chromium, copper, iron, manganese, nickel, lead, and zinc) using a "modified" NIOSH 7300 method and ICAP/ICP-MS (Galson 2006). Dust samples taken from the ledges were analyzed for crystalline silica and iron, as well as particle size distribution via methods described above. Metal content was reported in mg/kg and crystalline silica was reported as a percentage of total dust.

Gas Sampling

Gas sampling for ammonia was performed utilizing pumps and sampling tubes (Dragar, Accuro hand-held pump, Luebeck, Germany). Seven stalls were identified for this portion of the sampling scheme (Figure 1) and each stall was sampled once in winter when all stable doors were closed, and once in summer while all stable doors were open. Samples were taken before cleaning out, in the center of each stall, 1 m above the ground, approximating the breathing zone on a horse.

Statistical Analysis

Effects of feeding and stable activities on particle loads in the PM₁₀ and PM_{2.5} ranges were compared by ANOVA. When significant defects were identified, differences between means were compared by Student Neuman Keuls procedure. Ammonia concentrations in summer and winter were compared by a paired t-test. In all analyses, significance was set at $p < 0.05$.

Results

Feeds

In all feeds, the average and maximum load of PM₁₀ particles was significantly greater than those of PM_{2.5}. Dry hay provided the greatest average and maximum PM₁₀ and PM_{2.5} particulate load in the horse's breathing zone (Figure 2). The maximal PM₁₀ value was underestimated, because in two samples, the particle load was greater than the readable range of the aerosol photometer, i.e. greater than 150 mg/m³. These high values originated from hay that was taken from the bottom of the stack. The average but not the maximum particulate load from dry alfalfa cubes was significantly less than that from dry hay. Pouring the cubes into the feed tub resulted in a visible cloud of dust that remained suspended while the horse ate. Complete pelleted feed generated a significantly smaller particle load than hay or alfalfa cubes, but the load was greater than that from sweet feed or the commercially available feed for older horses.

Stable Activities

In the case of each stable activity, PM₁₀ particle average and maximum load was significantly greater than PM_{2.5}. Cobweb removal resulted in the greatest particle load in the horse's breathing zone for both size ranges (Figure 3). Aisle sweeping produced significantly less PM₁₀ load than cobweb removal but not significantly less PM_{2.5}. Stall cleaning and bedding with sawdust generated significantly less PM₁₀ and PM_{2.5} than either of the former procedures. Wetting the aisles and the stalls decreased the average and maximum load of both PM₁₀ and PM_{2.5} in the horse's breathing zone to less than 2 mg/m³.

Riding

In the absence of riding, the area particulate load averaged 0.045 mg/m³ for PM₁₀ and 0.021 mg/m³ for PM_{2.5}. During a one-hour riding lesson with five horses under dry footing conditions, average and maximum load were 9.1 and 55.3 mg/m³ and 2.3 and 60.6 mg/m³ for PM₁₀ and PM_{2.5}, respectively. Figure 4 shows how the particle load varied during the hour, increasing during warm-up and decreasing during cooling down. As moisture content of footing increased, particulate load decreased. When moisture content was ten percent or greater, particulate load was almost one hundredth that of dry footing (Figure 5). PM₁₀ particulates were maximally reduced when water content was 6.1 percent or greater. In the case of PM_{2.5}, maximal reduction occurred at 9.33 percent water content. After application of the commercial dust suppressant, average and maximum load were 0.03 and 2.2 mg/m³ and 0.02 and 0.17 mg/m³ for PM₁₀ and PM_{2.5},

respectively, similar values to those resulting from a water content of 9.33 percent or greater.

Bulk Samples

The distribution of particle sizes in footing and wall samples is shown in Figure 6. In the footing samples, particles greater than 10 μ in diameter constituted virtually the entire sample. While larger particles also constituted most of the wall sample, there was a considerable percentage below 10 μ and a secondary peak centered around 1 μ .

Crystalline silica (quartz) comprised the bulk of both footing and wall samples. Iron was present in both samples, with the concentration being greater in wall sample. Manganese and copper were also present in the footing samples. Other metals were below the laboratory limit of detection (Table 1-1).

Gases

In both summer and winter, concentrations of ammonia were below the limits of detection in the stable aisle. The mean concentration of ammonia in stalls was significantly higher in summer (31 ± 19 ppm, mean $\pm$ SD) than in winter. In summer, the concentration of ammonia was highest in the center of the stable and decreased toward the doors at each end with the lowest value being in stall number 7 (Figure 1) where the prevailing wind entered the stable.

Discussion

In this first investigation of particulate concentrations in the breathing zone of horses in a large boarding stable, average particulate concentrations only occasionally exceeded recommended occupational exposure limits for humans (ACGIH 2005b), but peak exposures regularly exceeded the excursion limits for both PM_{10} and $PM_{2.5}$, i.e., inhalable and respirable sizes. Eating certain feeds generated the highest exposures followed by cleaning and riding. Results from gas sampling indicated that ventilation within the stable was not uniform, so that suspended particulates and irritant gases such as ammonia were not removed.

The process of eating generates dust concentrations in the breathing zone of the horse that are generally not accurately perceived by the observer who is breathing the air several meters away from the horse's nose (Woods *et al.* 1993). When the horse consumes hay, the act of biting and pulling the captured wisp of hay from the flake or bale results in the immediate dispersion of high concentrations of dusts at the point of the nostril. The results of our study indicate that the peak concentrations in the breathing zone regularly exceeded 87.5 mg/m^3 PM_{10} and 20 mg/m^3 $PM_{2.5}$ while eating hay. These values are more than two times greater than the excursion limits of 30 mg/m^3 and 9 mg/m^3 PM_{10} and $PM_{2.5}$ allowed in an occupational setting (ACGIH 2005b). The hay utilized in our study was all grown in the same field, and was baled and hauled the same day. Despite this common source, dust levels varied considerably between the bales of hay over the course of the study. Hay that was fed close to the time of bailing resulted in less dust generation during feeding than the hay that was stored and fed several months

later. Interestingly, none of the hay that was fed during the study was considered to be dusty or moldy by observers, even the hay that resulted in breathing zone concentrations beyond our sampler's maximal range, which was 150 mg/m³.

Frequently, horses that are diagnosed with heaves are fed cubed hay or complete pelleted feed to reduce particulate exposure. Our results suggest that this should be done with great caution, especially in the case of cubed hay, because peak dust levels in the breathing zone are similar to those found during consumption of baled hay. In this stable, cubes are consumed from feed tubs suspended in the corner of the stall. When cubes were poured into the feed tubs, particle clouds formed immediately adjacent to the horse's nostrils, and did not disperse for several minutes. This phenomenon also occurred when complete pelleted feed was taken from the bottom of the bag and poured into the feed tubs.

Sweet feeds and senior feeds resulted in very low dust exposures. Similar levels were measured during feeding of complete pelleted feeds especially when the pellets were taken from the top of the bag. Molasses used in sweet feeds and senior feeds are a flavor enhancer but also act as a binding agent, thus dramatically reducing particle liberation. For this reason, these types of feeds should be optimal in the management of horses with heaves.

As might be expected, routine stable-cleaning activities also resulted in high exposures to particulates. Removal of cobwebs resulted in very high exposures to small particles, while raking and sweeping of the aisles dispersed clouds of particles

predominantly in the PM₁₀ range. Stall cleaning resulted in moderate increases in dust levels of predominantly larger particles (PM₁₀). Exposure concentrations during stall cleaning may have been underestimated as the wood shavings available during this study appeared to be particularly clean in relation to other bulk loads received. Straw was not available at this boarding stable, but breathing zone particulate concentrations during cleanout at another stable were similar to those found in the breathing zone of horses eating hay (unpublished data).

Under dry conditions, riding in the arena or grading its surface generated high concentrations of both small and large size particles. This is a common observation by stable owners and riders who see the dust, and experience upper respiratory responses such as itchy and watery eyes, sneezing, and cough. For this reason, dust management practices are often focused primarily on the arena. Dust concentrations in both size ranges increased progressively during the one-hour riding lesson, and then decreased rapidly to background concentrations when riding ceased. In this particular stable, riding in the arena does not continue throughout the day, thus allowing for dust levels to return to background concentrations. This may underestimate exposure potentials in other stables, where, in many cases, riding occurs continually throughout the day, so the dust does not have time to settle out. Unfortunately, we did not have sufficient samplers to concurrently measure particulate exposure in the stalled horses while the arena was in use. However, in one quick measurement made during arena use under dry conditions, particle load in the breathing zone of a stalled horse was only slightly less than that recorded in the arena.

Crystalline silica and iron were the two components of the dust identified in bulk samples from the stable that are known to result in occupational respiratory morbidity in overexposure situations. Particulates in general and silica in particular can induce generation of reactive oxygen species and production of inflammatory cytokines (e.g. IL-6, IL-8, TNF-alpha) and metals such as iron contribute to the toxic effects of such particulates (Dye *et al.* 1999; Fubini and Hubbard 2003; Ghio *et al.* 1992). Their role in equine pulmonary disease is presently unknown. The actual exposures to silica and iron could not be determined in the present study because our sampling equipment did not distinguish between the various components of dust. Occupational exposure limits for iron and crystalline silica are significantly lower than the established guidelines for nuisance dust, which is what was measured in this study and is our reference point. Future studies should include personal sampling specifically for components of inorganic dusts such as those mentioned above in order to begin to estimate exposures over a represent period of time as dictated by established sampling methods.

Ammonia is a well-known respiratory irritant (Wang *et al.* 1996). Before stall cleaning, concentrations of ammonia in the stall hovered near occupational exposure levels with some sample concentrations exceeding the 15-minute short-term exposure limits for humans (ACGIH 2005c). Ammonia concentrations in summer were significantly higher than those observed in the winter, even though ventilation in summer was superior to that in winter when all stable doors were closed. This can be explained by the increased volatilization of ammonia during the warmer summer months. During

summer when stable doors were opened, ammonia concentrations increased in stalls towards the center of the stable.

Fortunately, implementing wet methods to reduce particulate exposures is easy and inexpensive. In all cases, exposures during feeding, cleaning, and riding were dramatically reduced after wetting. In arena footing, water content of 10 percent or higher provides maximal dust suppression. The commercial dust suppressant was as effective as water in reducing particulate concentrations. Wetting (not soaking) of feedstuffs immediately prior to initiation of feeding reduces exposures to that of background levels.

In conclusion, our investigation has reinforced the fact that particulate concentrations can be very high in the breathing zone of horses eating hay and other dry feeds (Vandenput *et al.* 1997; Woods *et al.* 1993). To decrease exposures to concentrations of particles and gases in the microenvironment of the stall, it is important to improve ventilation within the stable as much as possible and to use clean feeds and dust suppression techniques in order to reduce the potential for particle generation and dispersion. A little water goes a long way in suppressing dust. Unfortunately, stable and horse owners tend to rely on their own senses to evaluate whether or not a stall is well ventilated and dust free. Decisions made from the aisle do not reflect what is going on in the breathing zone of the horse. Use of real-time particulate monitors is useful to educate stable owners about the inhaled hazards present in a stable.

Table 1-1. Metal (mg/kg) and crystalline silica (% of total) concentration in bulk samples taken from the arena footing and wall ledges.

Metals	Footing concentration (mg/kg)	Wall ledge concentration (mg/kg)
Silver	<99.4	
Cadmium	<5	
Cobalt	<15	
Chromium	<50	
Copper	11	
Iron	3800	11000
Manganese	160	
Nickel	<9.9	
Lead	<25	
Zinc	<248	
Crystalline Silica	Footing concentration (% of total)	Wall ledge concentration (% of total)
Quartz	22	22
Cristobalite	<1	<1
Tridymite	<1	<1

Table 1-2. Summer and winter ammonia concentrations (ppm) in each of seven stalls.

Stall number	Ammonia	
	Winter	Summer
1	13	41
2	20	47
3	4	59
4	10	30
5	2	30
6	9	10
7	11	20
Mean $\pm$ SD	9.1 $\pm$ 5.3	31 $\pm$ 19

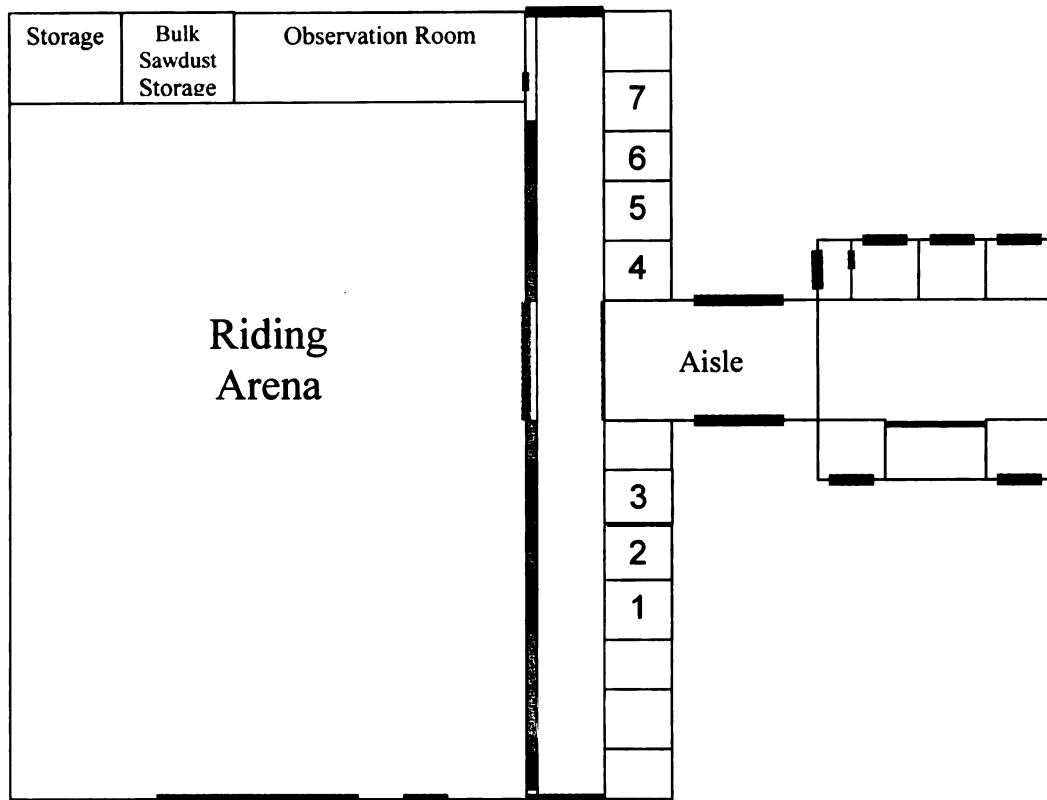


Figure 1-1. Diagram of the boarding stable showing the location of the stalls in which ammonia concentrations were measured.

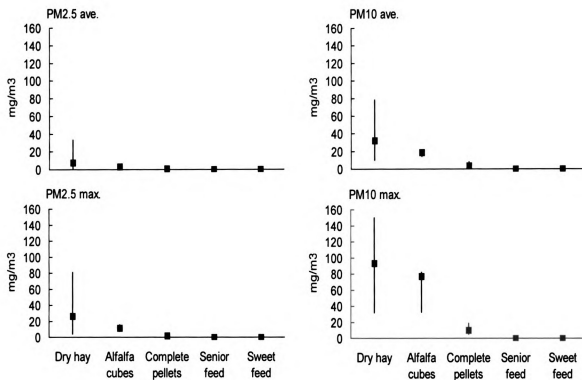


Figure 1-2. Breathing zone concentration (mg/m^3) of PM₁₀ and PM_{2.5} during consumption of various dry feedstuffs. Median values and range are shown for both average and peak particle concentration. Values with similar superscripts do not differ significantly.

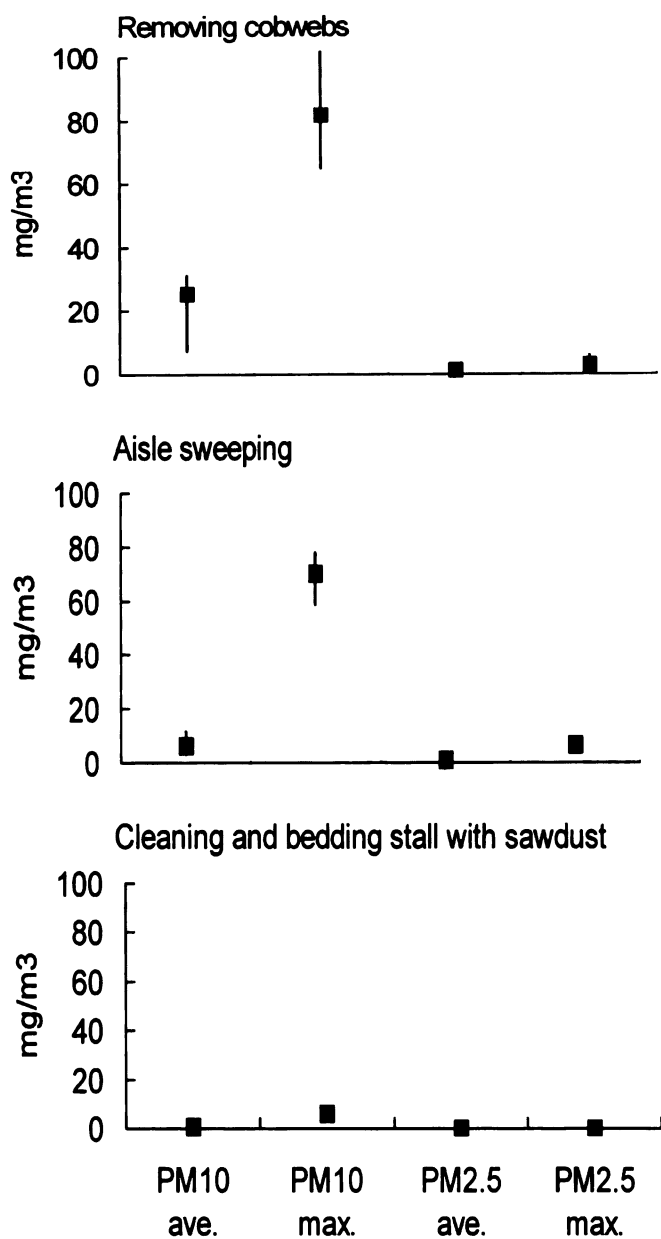


Figure 1-3. Breathing zone concentration (mg/m^3) of PM_{10} and $\text{PM}_{2.5}$ during routine stable activities. Median values and range are shown for both average and peak particle concentration.

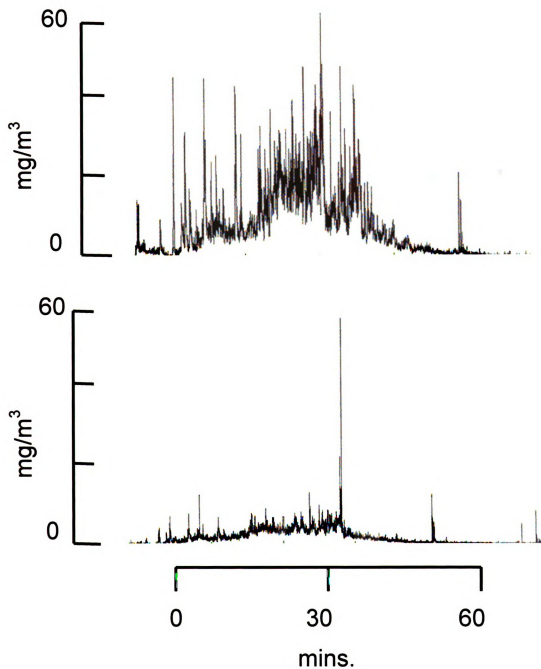


Figure 1-4. PM₁₀ (top) and PM_{2.5} (bottom) concentrations (mg/m³) in the riding arena during a single riding event. Aerosol photometer samples were recorded every second for the duration of the activity.

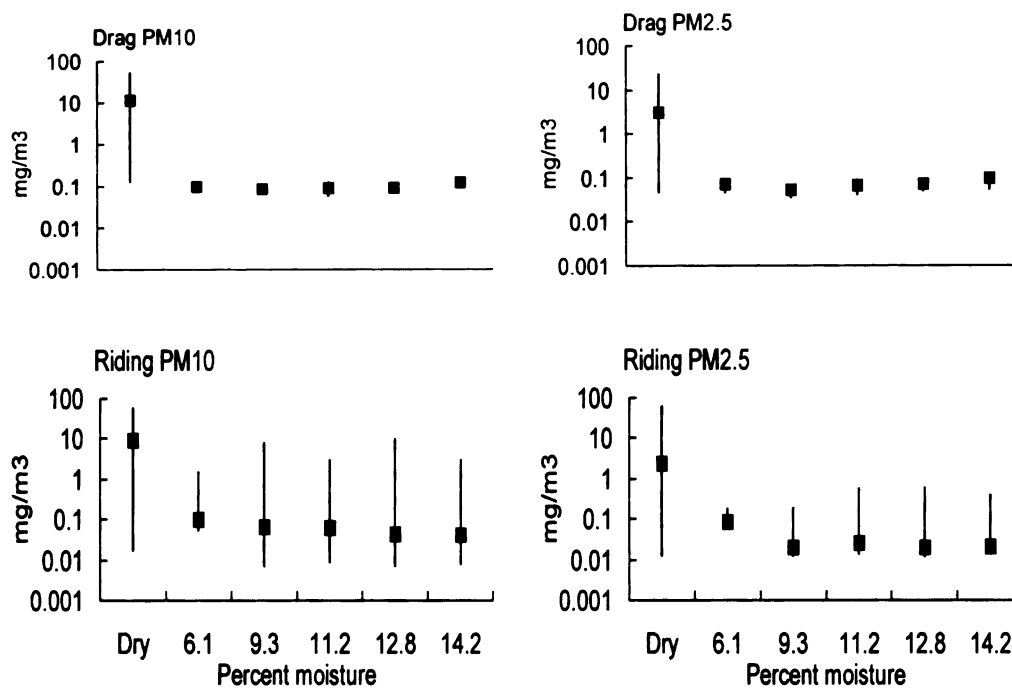


Figure 1-5. Effect of moisture content on breathing zone concentration (mg/m³) of PM₁₀ (left) and PM_{2.5} (right) during arena grading (upper) and riding (lower). Values are maximum, minimum and mean.

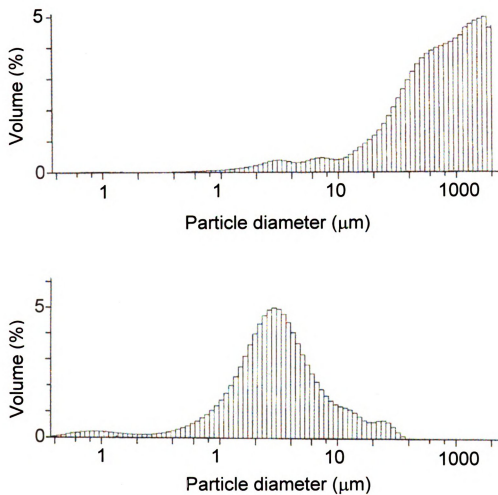


Figure 1-6. Distribution of particle diameters (micron) in bulk samples taken from the arena footing (top) and wall ledges (bottom).

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CHAPTER 2

Evaluation of Factors Affecting the Concentration of Particulate Matter (PM₁₀ and PM_{2.5}) in Stables at an American Thoroughbred Racetrack

Summary

Reasons for Performing Study

Inflammatory Airway Disease (IAD) is common in Thoroughbred Racehorses with an estimated overall prevalence of 13%, and is most likely multifactorial in nature. Particles present in the environment of the racehorse are in size ranges known to result in worsening of pre-existing respiratory disease in humans, and compromised ability to handle streptococcal infections in laboratory animals.

Objectives

The purpose of this study was to determine the size ranges and concentrations of airborne small particles in the stable environment of the racehorse, and the factors that influence their presence.

Methods

Direct reading instruments were utilized to determine the concentration and factors affecting the concentration of particulate matter (PM₁₀ and PM_{2.5}) in three stables at an American Thoroughbred Racetrack. Particulate mapping took place three times daily (early morning, midday, late afternoon) during each of three months (early,

mid, and late racing season) corresponding with times of varying activity within the stable, and changes in racing activity and season.

Results

There was a significant effect of month, time of day, and stable on concentrations of both PM₁₀ and PM_{2.5}. Average PM₁₀ and PM_{2.5} concentrations were highest in September following a period of dry weather, and lowest in July following a period of rainy weather. November concentrations of PM₁₀ and PM_{2.5} were slightly lower than those of September even though monitoring took place during a period of snowy/wet weather. Early morning concentrations of PM were significantly higher ($P < 0.0001$) than those measured throughout the rest of the day. The stable that was completely enclosed and relatively devoid of natural sources of ventilation, consistently had significantly higher average concentrations of both PM₁₀ and PM_{2.5} than the open-sided stable. There was a significant difference in number of stalls within a stable that exceeded the 75th percentile for both PM₁₀ and PM_{2.5}.

Conclusions

We report significant effects of month, time of day, stable, and stalls within a stable on particle concentrations. PM₁₀ and PM_{2.5} concentrations were lowest overall in the month following a period of rain, and highest in the morning hours during peak activity. The stable which was most enclosed, fed from hay nets, and had the most activity had significantly higher average PM₁₀ and PM_{2.5} concentrations than the stable

with open sides and less activity. Specific stalls within stables have consistently elevated concentrations of PM compared to adjacent stalls.

Potential relevance

Understanding sources and factors influencing concentrations of particulate matter in the stables of racehorses will allow for the development of strategies aimed at reduction of exposures and thus potentially a reduction in the prevalence of IAD.

Introduction

Inflammatory airway disease (IAD) of racehorses is a syndrome characterized by cough, exercise intolerance, excess tracheobronchial mucus that is visible upon endoscopic examination, and inflammatory cells in tracheal aspirates and bronchoalveolar lavage (BAL) (Anon 2003). There is an overall prevalence of IAD of approximately 13% of Thoroughbreds in training (Wood *et al.* 2005c), with up to 45% of racehorses in a stable affected at any one time (unpublished data) leading to a significant financial impact as relatively small amounts of mucus in the airways result in horses finishing further back in a race (Holcombe *et al.* 2006; MacNamara *et al.* 1990b). Due to the potential impact of IAD on the racing industry, investigators have focused on elucidating its cause, which is most likely multifactorial in nature.

IAD is common in young racehorses, with incidence declining as the horse ages and length of time in training increases, suggesting an infectious component, as increasing time spent in a stable and exposure to bacterial and viral pathogens would be

expected to confer immunity (Wood *et al.* 2005c). As a result, several investigations have focused on the role of infectious agents in the etiology of IAD. Bacterial, but not viral infections have been associated with neutrophilic inflammation and mucus accumulation in asymptomatic racehorse populations (Chapman *et al.* 2000; Christley *et al.* 2001b; Wood *et al.* 1993; Wood *et al.* 2005a, c). Infections, however, most likely are not the only cause of IAD in racehorses, as many racehorses (over 20%) with airway inflammation culture negative (Chapman *et al.* 2000; Christley *et al.* 2001a; Wood *et al.* 2005a). The stable environment has been identified as a second risk factor for airway inflammation in thoroughbred racehorses (Malikides and Hodgson 2005), as they are kept primarily outdoors until, when at around the age of two, they are brought into a training facility or racetrack to begin their careers. Exposure to organic dusts commonly found in stables, dispersed from feed and bedding, have been shown to be associated with airway neutrophilia and mucus accumulation in healthy horses (Gerber V 2004; Holcombe *et al.* 2001; Tremblay *et al.* 1993). For these reasons, our attention has turned to the evaluation of exposures to particulate matter (dust) in the etiology of IAD in Thoroughbred racehorses.

Airborne particulate matter (dust) in racehorse stables can originate from feed and bedding, flooring materials, and racetrack footing materials. As there are many sources of particulate matter in the environment of the racehorse, size and composition of the particulate matter would not be expected to be uniform. Particle sizes of interest in this study are classified as those less than 10 (PM10) and less than 2.5 (PM2.5) microns in diameter. In humans, a small percentage of PM10 have the potential to reach the lower

airways but the majority of these particles are deposited in the central airways such as the trachea. The smaller particles represented by PM_{2.5} penetrate deep within the airways.

Previous studies conducted in controlled settings as well as private boarding stables utilizing traditional filter/pump methods (Clarke and Madelin 1987; Clarke *et al.* 1987; Crichlow *et al.* 1980; Kirschvink *et al.* 2002; McGorum *et al.* 1998; Woods *et al.* 1993) and direct reading instruments (Clements and Pirie 2007a, b) have shown that dusts present in the breathing zone of horses are composed of particles that include those in size ranges (PM₁₀ and PM_{2.5}) consistent with those described in the human literature. When airborne concentrations of particles in these size ranges increase, there is worsening of symptoms in people with pre-existing airway disease, increased lost school/work days, increased hospital admissions, and increased mortality (Brunekreef and Forsberg 2005; Oberdorster G 1996; Peel *et al.* 2005; Pope 1991a; Pope III *et al.* 2002; Schwartz 2004).

The current study is part of a larger study that aims to describe the relationship between exposure to particulate matter and the presence of IAD in Thoroughbred racehorses at an American Thoroughbred Racetrack. The purpose of this first study is to characterize area particulate concentrations and the factors that influence their presence.

Materials and Methods

Stables

This study was conducted at a Thoroughbred racetrack in the Midwestern United States, where there are stables of various ages and styles. The three stables used in this study were selected because of their different designs and management styles, as well as the cooperation of the trainers that use them.

Stable 1 was of newer construction and design. Orientation of the stable was north-south with roll-up shutters along the entire length (east and west sides) of the stable in combination with high vaulted ceilings, large sliding doors on both ends (front and back), and open fronted stalls (bars and gates) that face the outdoors. This stable is located immediately adjacent to the main roadway and a common parking area west of the stable.

Stable 2 is of brick construction and the long axis of the stable is oriented in the north-south direction. This stable has 3 meter ceilings, closed-front stalls, and small, high windows (only in stalls that are on outer walls) that are kept closed. A single row of stalls is located along each of the outer walls of the stable, with a double row (back-to-back) of stalls down the center. The main source of ventilation is from two sets of large doorways both at the north and south ends of the stable, and one set of smaller doorways in the center of the west side of the stable. A large manure handling facility is located 6 meters from the north end of the stable, with another stable of similar design located a similar distance from the south end. Stable management attempts to improve ventilation in the summer months by placing large air-moving fans in each end doorway. This is the

only stable in the study that feeds from hay nets. This stable is also located immediately adjacent to a busy racetrack road along the length of the western wall, and a busy city road along the east side of the stable.

Stable 3 is of identical construction to Stable 2, however some differences do exist. The stable is oriented with its long axis east to west, and has open windows during the warm months. This stable is located in a secluded area (away from roads), but is adjacent to a common parking area along its western end.

Measurement of particle concentration and numbers

Concentration and number of area airborne particles were evaluated using two TSI DustTrak¹ monitors and one MetOne HHPC-6 Airborne Particle Counter², respectively. These machines are small and portable and use optical methods to quantify particle concentrations and numbers. One DustTrak was set to measure the concentration (mg/m³ of air) of particles less than 10 microns and the other to measure particles less than 2.5 microns. The particle counter provided the number of particles within a given size range: 0.5, 0.7, 1.0, 2.0, 5.0 and 10.0 microns. Equipment calibration and cleaning was performed prior to the start of each day of sampling per manufacturers instructions, and periodic flow evaluations were made throughout each sampling day.

All measurements were one minute in duration, taken at the front-center of each stall 1 meter above the ground, approximating the height of the nostril of the horse. In an attempt

¹ TSI Incorporated, 500 Cardigan Road, Shoreview, MN 55126-3996

² Hach Ultra, 481 California Ave., Grants Pass, OR 97526

to minimize our influence in the stable, horses remained in the stalls during monitoring, and all activities (cleaning, grooming, feeding, etc.) took place as scheduled.

Experimental Design

Three visits were made to the racetrack over the 2005 racing season (June, September, and November), staying 3 days at each visit. The visits were selected to be a) early in the racing season, b) at the height of the season when conditions were usually driest, and c) when the weather was cooler and damp and the new stable (Stable 1) had closed its outer shutters. The weather (rain/snow), temperature, and humidity were recorded during our visit. Information on total rainfall for one week prior to each visit was purchased from Weather Source, LLC.

We measured particulate concentrations in one stable each day during the visit. Concentrations were measured for one minute in every stall three times of day - a) early morning during clean out, feeding and grooming, b) mid-day when there was less activity, and c) late afternoon which coincided with feeding and racing.

Data Analysis

Particle concentrations measured in each stall within each stable were analyzed using Wilcoxon rank sums and the Kruskal-Wallis test of significance to determine if there were regional pockets of high particulate concentrations. Maps were prepared to show the stalls within each stable that exceeded the 50th and 75th percentile of particulate concentrations overall. PROC CROSSTABS was used to determine

significant differences in the number of stalls in each stable with PM concentrations greater than the 75th percentile overall. Mixed models were employed in SAS v.9.1. (PROC MIXED). This procedure is designed to handle hierarchical data that is collected repeatedly on stalls over time. In addition, it is able to handle missing data and has the power to analyze a number of variance covariance structures while accounting for within-stall correlations. Then, the possible covariance structure including unstructured (UN), spatial Gaussian (SP(GAU)), first order autoregressive (AR(1)), TOEPLITZ (TOEP), and compound symmetry (CS) were tested. The best covariance structure was chosen based on the Akaike Information Criterion, which was TOEPLITZ for all models. Using PROC MIXED, the effects of month, stable, and time of day on particle concentrations (PM10 and PM2.5) within each stable, and between stables were determined. Level of significance was set at $P \leq 0.05$.

Results

A total of 717 measurements were taken throughout the sampling season (Table 2-1), with each consisting of average, minimum, and maximum values recorded for PM10 and PM2.5 during the one minute measurement. The average PM10 and PM2.5 concentrations ranged between 0.078 and 4.090 mg/m³ and 0.030 and 1.170 mg/m³, respectively. Peak PM10 and PM2.5 concentrations ranged from 0.124 to 12.800 and 0.052 to 4.16 mg/m³, respectively. The median PM10 and PM2.5 average, minimum, and maximum concentrations for all samples are given in Table 2-1. While the larger particles ($PM_{5.0} \leq PM_{10}$) comprised the majority of the mass of the measurements that

were taken, those particles smaller than 5 microns in diameter (PM 5.0) made up the largest percentage of particles (84-99%) (data not shown).

Effect of Month and Time of Day

Average particle concentrations (PM10 and PM 2.5) were significantly ($P < 0.0001$) affected by month (Figure 2-1) with highest concentrations occurring in September and the lowest in July. PM2.5 minimum concentrations were significantly lower in November than in July.

Average concentrations of PM10 and PM2.5 changed significantly ($P < 0.0001$) throughout the day (Figure 2-2). Concentrations were highest in the morning during cleanout and decreased by the midday and late afternoon sampling periods. Average concentrations of PM10 and PM 2.5 were significantly different between the morning and late afternoon (reference measurement) ($P < 0.0001$), but were not significantly different between the midday and late afternoon concentrations. This is in contrast to the minimum PM2.5 concentrations in which both the morning and midday measurements were significantly different than those taken during the late afternoon ($P < 0.0001$).

Effect of Stable

There was a highly significant ($P < 0.0001$) effect of stable on PM10 and PM2.5 concentrations (Figure 2-3) with Stable 2 having the highest concentrations of both PM10 and PM 2.5. However, stable 1 had significantly higher concentrations of average and minimum PM2.5 than Stable 3 (referent stable). Figure 2-4 shows the interaction

between stable and month. While PM10 and PM2.5 average concentrations are always higher in Stable 2 than Stable 1, overall trends are identical with average concentrations increasing from July to September, and decreasing slightly in November. Stable 3 did not follow a similar trend. By contrast, trends through the time of day were similar in all stables (data not shown).

Effect of Stall Location

There was a significant ($P < 0.001$) effect of location of a stall within a stable on PM10 average concentrations. Significant effects of stall location also existed for PM10 ($P < 0.0005$) and PM2.5 maxima ($P < 0.0008$) and PM2.5 minimum ($P < 0.005$) concentrations. With regard to PM10 and PM2.5 average measurements, Stable 2 contained significantly more stalls in the 75th percentile than Stables 1 and 3 (Table 2-2, Figures 2-5 and 2-6). In general, Stable 2 consistently had the highest number of stalls which exceeded both the 50th and 75th percentiles for both PM10 and PM2.5; however, in the case of PM 2.5 min, all the stalls of Stable 1 exceeded the 50th percentile (Table 2-2). Stalls exceeding the 50th percentile for both PM10 and PM2.5 average concentrations were consistently located along the west aisle of Stable 1 (Figures 2-5 and 2-6).

Discussion

These are the first reported measurements of indoor air quality (real-time particle concentration) in American racing stables. Our measurements indicate that there are significant effects of time of day, month, stable, and location of stalls within a stable on particulate matter in sizes and concentrations of interest in terms of airway inflammation

in both humans and laboratory animals (Becker *et al.* 1996; Ghio *et al.* 2006; Gilmour *et al.* 1996; Kappos *et al.* 2004; Li *et al.* 1997; Peel *et al.* 2005; Zelikoff *et al.* 2003).

Particle concentrations and numbers increased from July to September, and then decreased slightly during November. These observations are consistent with weather patterns, ventilation status, and numbers of horses in the stables. While Stable 2 consistently had higher monthly PM₁₀ and PM_{2.5} average concentrations than Stable 1, particulate concentrations both followed the same increasing pattern from July to September, and then experienced a slight decrease in November, suggesting a component aside from stable construction/orientation and management style that affects particle concentrations (e.g. rainfall, humidity, etc.). While it rained just prior to our visit in July, and snowed during our visit in November, it was very dry just prior to and during our visit in September. Wet/humid weather attenuated particle concentrations in July, but not to the same degree in November, as the stable doors and windows were closed tight due to the cold weather, thus eliminating all sources of natural dilution ventilation. Particle concentrations in Stable 3 did not follow a similar pattern, presumably because the trainer regularly shipped horses in and out of the stable, altering which stalls were occupied within. Furthermore, this stable was the only one of the three oriented east-west and away from major roadways, so external influences may have played a smaller role than in the other two stables.

As might be expected, the new construction stable (Stable 1) with roll-up (open-air) sides and vaulted ceilings had significantly lower concentrations of large particles

(PM10) throughout the day than the older brick style building with low ceilings, closed windows, and hay fed from hay nets rather than the floor (Stable 2). Not surprisingly, all particles were at their highest concentration during the morning measurement period when cleaning, feeding, and preparation for exercise were occurring. The average concentration of PM10 and PM 2.5 fell significantly by mid-day, but only PM 2.5 minimum values continued to decrease significantly throughout the remainder of the day. The rapid decrease of PM10 can be explained by the higher rate of settling of the larger particles. In contrast, the smaller and lighter PM 2.5 settles out more slowly.

In addition to particles (PM10 and PM2.5) generated and dispersed within the stable due to activity (feeding, cleaning, etc.), factors external to the stable appear to influence particle concentrations within the stable environment. Stable 1 can illustrate this point with its open sides and high vaulted ceilings that allow for complete mixing with outside air. In this stable all 30 stalls had median PM2.5 minimum values meeting or exceeding the 50th percentile (Table 2-2). If all measured PM2.5 were generated indoors, Stable 2 rather than Stable 1 would have had the greatest number of such stalls as it does for all other PM values. This is not the case, and therefore, other factors must be affecting the small particles. The consistently elevated levels of minimum PM 2.5 in Stable 1 are consistent with its location in proximity to a busy roadway and the main parking area, as small particles can originate from road dust (Gillies *et al.* 2001; Singh *et al.* 2006).

We observed a significant variation in management style between stables that probably explained particle concentrations throughout the day with Stable 2 consistently

having high particle concentrations. Stables 1 and 3 were busy in the morning hours during feeding/cleanout activities and preparations for training, and then quiet throughout the rest of the day. In these two stables, activity began daily between 5 and 5:30 a.m., and was usually at a minimal level by around 9 a.m. Both stables fed grain from feeders suspended in the corner of the stall, and hay was fed from the floor. In each of these stables, no more than 3 individuals took part in the morning activities (cleaning, grooming). In contrast, Stable 2 assigned one groom per 4-5 horses. This allowed for simultaneous cleaning/grooming in a minimum of six stalls at any one time – at least double that of the other two stables, resulting in greater dispersion of particles. Stable 2 also fed grain from a corner feeder, but fed hay from hay nets that were filled each morning by the grooms. Hay for each net was taken from the bale and shaken in the aisle to loosen it before it was packed into hay nets and hung near the doorway of each stall. In order to eat from the hay net, horses pulled the hay-filled net into the doorway, which both released particles and obstructed ventilation of the stall. Aisle raking took place continuously throughout the morning until the training session ended and all horses were returned to their stalls. Activity in Stable 3 decreased by 11 a.m. daily, approximately 2 hours later than in Stables 1 & 2.

Our data indicate a significant effect of stall location within the stable on PM concentrations. Stable 2 consistently had stalls that exceeded the 75th percentile in terms of PM₁₀ and PM_{2.5} average and maximum concentrations. These stalls were located along the center partition, an area furthest from doorways and windows, and likely devoid of natural mixing/dilution.

In humans and laboratory animals, inhalation of PM10 typically results in a neutrophil influx into the lung (Li *et al.* 1997) and upregulation of pro-inflammatory cytokines (Becker *et al.* 1996). The pathogenesis of PM10 damage has been related to their metal content, which leads to oxidative stress (Gilmour *et al.* 1996). PM2.5, because of their large surface area per unit mass, provide a large capacity to generate free radicals and are inherently more damaging than coarse particles (Oberdorster *et al.* 2000). Exposing rats to PM 2.5, in concentrations observed at this racetrack, has been shown to compromise the ability of the lung to handle streptococcal infections (Zelikoff *et al.* 2003). Compounds such as endotoxin attached to the particle surface also may be responsible for particle-induced injury or may prime the airway for particle-induced inflammation (O'Grady *et al.* 2001; Oberdorster *et al.* 2000). Increases in ambient PM2.5 are consistently associated with increased morbidity and mortality from respiratory disease (Kappos *et al.* 2004), while increases in ambient PM10 tend to be linked to short-term transient worsening of disease (Brunekreef and Forsberg 2005).

A required first step in any thorough Indoor Air Quality (IAQ) assessment of facilities not previously evaluated is a survey of the building environment. This 'survey' typically consists of identification of building construction, type(s) of ventilation present (forced or dilution), location(s) and characteristics of ventilation sources, identification of structural components (walls, beams, etc.) that influence airflow within the building, and identification of potential indoor air contaminants and factors influencing their presence. This information is then used to develop process controls to reduce exposures, exposure

risk assessments, and targeted air sampling plans for future contaminant specific personal exposure monitoring.

We have conducted a thorough survey of particle concentrations (PM10 and PM2.5) and factors that affect them in 3 stables at an American Thoroughbred racetrack which follows the rationale mentioned above for conducting IAQ assessments. We have identified multiple factors that influence particulate concentrations within these stables, and have shown that particle concentrations consistently vary between stables, by season, time of day, and location of the stable with regard to neighboring roads. Additionally, we have identified regions (stalls) within stables that consistently experience higher concentrations of PM, thus allowing for the identification of horses potentially at increased risk for airway inflammation.

Table 2-1. Summary of number of stalls and total number of observations within each stable, including the median, 5th, and 95th percentile values for PM10 and PM2.5 (average, minimum, and maximum).

	Stable 1 (30 stalls) (Total Obs. 270)			Stable 2 (31 stalls) (Total Obs. 276)			Stable 3 (20 stalls) (Total Obs. 171)			All Stables (81 stalls) (Total Obs. 717)		
	Med	5th pctl	95th pctl	Med	5th pctl	95th pctl	Med	5th pctl	95th pctl	Med	5th pctl	95th pctl
PM Avg Value												
PM 10 Avg (mg/m ³)	0.273	0.112	0.829	0.432	0.156	1.390	0.308	0.150	1.130	0.331	0.129	1.09
PM 2.5 Avg (mg/m ³)	0.131	0.052	0.300	0.169	0.052	0.452	0.097	0.042	0.359	0.122	0.049	0.386
PM 10 Max (mg/m ³)	0.510	0.185	1.570	0.734	0.272	2.780	0.582	0.215	1.950	0.582	0.218	2.080
PM 2.5 Max (mg/m ³)	0.273	0.075	0.863	0.497	0.081	1.750	0.362	0.092	1.480	0.351	0.081	1.420
PM 10 Min (mg/m ³)	0.178	0.066	0.501	0.252	0.088	0.771	0.169	0.082	0.596	0.196	0.076	0.616
PM 2.5 Min (mg/m ³)	0.089	0.043	0.228	0.062	0.017	0.311	0.056	0.025	0.136	0.068	0.024	0.253

Med = Median

Table 2-2. Number of stalls within each stable that exceed the 50th and 75th percentile for PM10 and PM2.5 (Average, Minimum, and Maximum).

	Stable 1 (30 stalls)		Stable 2 (31 stalls)		Stable 3 (20 stalls)		All Stables (81 stalls)	
<u>PM Values</u> <u>(50th/75th</u> <u>percentiles</u> <u>(mg/m³)</u>	<u>>50th</u> <u>pctl</u>	<u>>75th</u> <u>pctl</u>	<u>>50th</u> <u>pctl</u>	<u>>75th</u> <u>pctl</u>	<u>>50th</u> <u>pctl</u>	<u>>75th</u> <u>pctl</u>	<u>>50th</u> <u>pctl</u>	<u>>75th</u> <u>pctl</u>
PM 10 Avg (0.331/0.556)	10	0	26	7	11	2	47	9
PM 2.5 Avg (0.122/0.229)	13	0	23	3	9	0	45	3
PM 10 Max (0.582/0.977)	8	1	23	6	14	2	45	9
PM 2.5 Max (0.351/0.636)	6	0	26	8	12	2	44	10
PM 10 Min (0.196/0.298)	6	1	27	6	8	1	41	8
PM 2.5 Min (0.068/0.158)	30	1	5	0	5	0	40	1

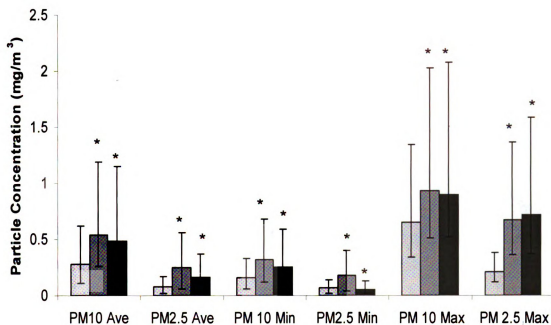


Figure 2-1. Effect of month on mean particle concentrations (PM10 and PM2.5) in mg/m^3 . Light gray bars denote July, dark gray bars denote September, and black-striped bars indicate November measurement values. Error bars indicate the 25th and 75th percentile. An * denotes significant difference from referent month (July).

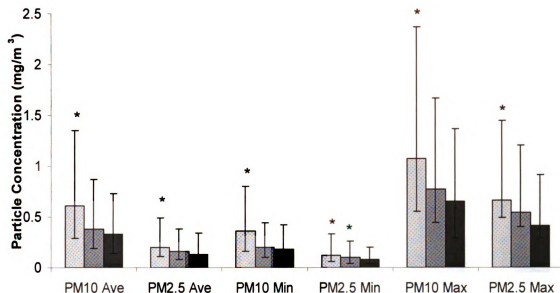


Figure 2-2. Effect of time of day on mean particle concentrations (PM10 and PM2.5) in mg/m^3 . Light gray bars denote early morning, dark gray bars denote mid-day, and black-striped bars indicate late afternoon measurement values. Late afternoon measurements were set as the referent group. Error bars indicate the 25th and 75th percentile. An * denotes significant difference from referent time of day (late afternoon).

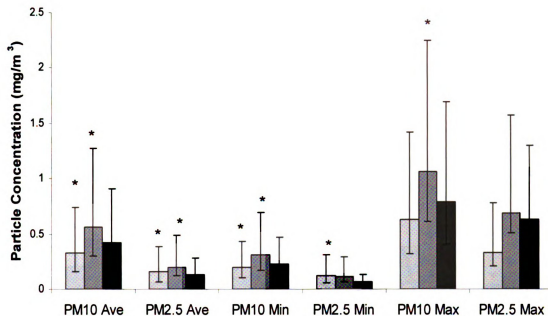


Figure 2-3. Effect of stable on mean particle concentrations (PM10 and PM2.5) in mg/m^3 . Light gray bars denote Stable 1, dark gray bars denote Stable 2, and black-striped bars indicate Stable 3. Error bars indicate the 25th and 75th percentile. An * denotes significant difference from referent stable (stable 3).

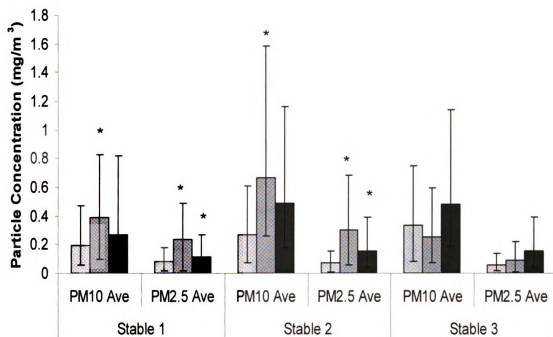


Figure 2-4. Effect of stable/month interaction on mean particle concentrations (PM10 and PM2.5) in mg/m^3 . Light gray bars denote July, dark gray bars denote September, and black-striped bars indicate November measurement periods. Stable 3 and the month of July were used as the referent group. Error bars indicate the 25th and 75th percentile. An * denotes significant difference from referent groups.

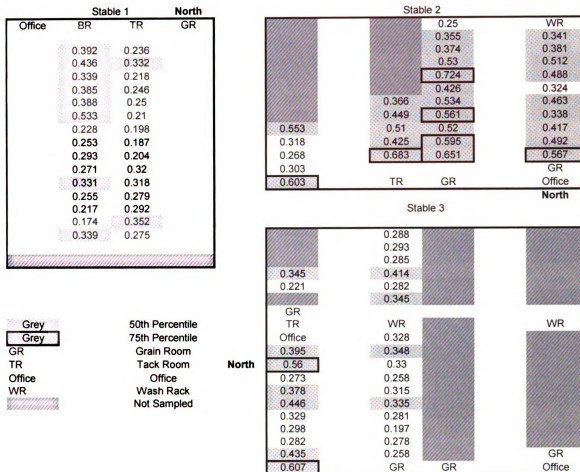


Figure 2-5. Map showing the locations of stalls within each stable that exceed the 50th and 75th percentiles for PM10 Average values. Note that almost all of the stalls in Stable 2 exceed the 50th percentile, while less than half of all stalls in both Stables 1&3 exceed the 50th percentile. Also, Stable 2 contains the majority of the stalls that exceed the 75th percentile while Stable 1 does not contain any. The 'North' notation located adjacent to each diagram denotes stable orientation.

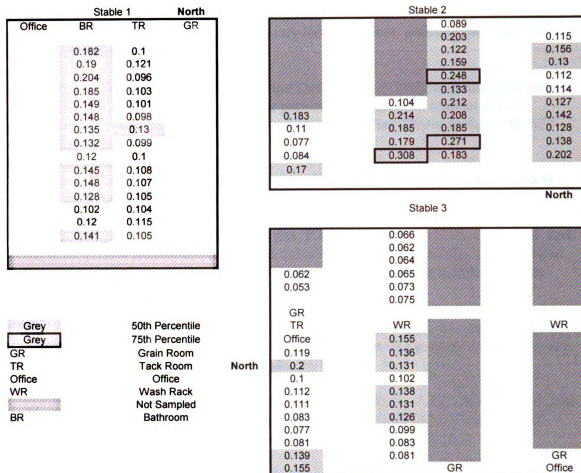


Figure 2-6. Map showing the locations of stalls within each stable that exceed the 50th and 75th percentiles for PM_{2.5} Average values. Note that almost all of the stalls in Stable 2 exceed the 50th percentile, while less than half of all stalls in both Stables 1&3 exceed the 50th percentile. Also, Stable 2 contains the majority of the stalls that exceed the 75th percentile while Stable 1 does not contain any. The 'North' notation located adjacent to each diagram denotes stable orientation.

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CHAPTER 3

Horse-Environment Interaction: The Relationship between Environmental Particulate Matter and Airway Inflammation in Thoroughbred Racehorses

Summary

Reason for Performing Study

Inflammatory airway disease (IAD) is characterized by excess tracheobronchial mucus, which negatively affects performance. The stable environment has been implicated as a risk factor for IAD in Thoroughbred racehorses.

Objectives

The purpose of the present study was to determine the relationship between area concentrations of PM₁₀ and PM_{2.5} as measured in three racing stables and indices of airway inflammation (i.e. tracheal mucus and inflammatory cells in tracheal wash) in the horses that inhabit them.

Methods

We studied 107 racehorses from 3 stables, in 3 different months. Horses were endoscopically examined from the nares to the trachea, assigned a mucus score, and tracheal lavage was performed. Bivariate procedures, general estimating equations (GEE), and mixed models were applied to estimate the association between repeated measurements of PM each stall and stable, mucus presence, and inflammatory cells.

Conclusions

Month, stable, and PM were significantly associated with the presence of tracheal mucus, which had an overall prevalence of 67%. GEE models show statistically significant association between PM10 [OR 5.8, 95% CI (1.64-20.56), $p < 0.0064$] and PM2.5 [OR 4.5, CI (1.35-14.90), $p < 0.0151$]. The prevalence of tracheal mucus was highest in the stables and months with highest PM and least in the open-sided stable with low PM and after a period of wet weather when the PM concentration was low. Of all tracheal wash samples, 68% contained $> 20\%$ neutrophils, and there was a significant association between PM2.5 and inflammatory cells.

Potential Relevance

Airborne particulates affect the presence of tracheal mucus. Further studies are warranted to investigate associations of PM and severity of mucus, allowing for recommendations that would reduce overall prevalence of tracheal mucus. More than 20% neutrophils in 80% of horses without visible mucus underscores the need to reevaluate the use of inflammatory cells in the definition of IAD.

Introduction

Inflammatory airway disease (IAD) is a syndrome with an overall prevalence of 13% (Wood *et al.* 2005a), and affects up to 45% of racehorses in a training stable at any one time. Racehorses with IAD may not show clinical signs of respiratory illness (i.e. cough, nasal discharge, malaise), however, the presence of excess tracheobroncheal mucus visible upon endoscopic examination, and inflammatory cells in tracheal aspirates and/or bronchoalveolar lavage fluid are used to define this syndrome (Anon 2003). Previous studies have shown that the presence of visible mucus in the trachea of racehorses may result in a significant negative financial impact to owners and trainers, as these horses tend to finish further back in a race (Holcombe *et al.* 2006; MacNamara *et al.* 1990a).

Bacterial infection (Newton *et al.* 2003b; Wood *et al.* 1993; Wood *et al.* 2005a, c) and stable environment (Malikides and Hodgson 2005) are two factors that have been implicated as causative agents of IAD in racehorses. As more than 20% of racehorses have been diagnosed with IAD culture negative from the trachea (Chapman *et al.* 2000; Christley *et al.* 2001a; Wood *et al.* 2005a), the stable environment may be responsible for airway inflammation in healthy horses (Gerber *et al.* 2004a; Holcombe *et al.* 2001; Tremblay *et al.* 1993). Because particulate exposure is associated with airway disease in people and experimental animals, we focused on the exposure of racehorses to dusts (particulate matter) in the stable environment, and its relationship to measures of airway inflammation (i.e. tracheal mucus and inflammatory cells in tracheal aspirates).

Sources of particulate matter in the stable environment are many, and may include feed and bedding, flooring materials, and particulates that while originating outside of the stable environment (e.g. racetrack footing, road dust), have the potential to disperse and be carried indoors. These sources of dust all contribute to area particulate concentrations in the stalls. Multiple studies investigating particulate matter both in controlled settings as well as in private stables have demonstrated that the aerodynamic diameter of particulates found in the stable environment (Clarke and Madelin 1987; Clarke *et al.* 1987; Crichlow *et al.* 1980; Kirschvink *et al.* 2002; McGorum *et al.* 1998; Woods *et al.* 1993) are consistent with those known to result in worsening of pre-existing respiratory disease in humans, increased lost work/school days, and increased mortality (Brunekreef and Forsberg 2005; Pope 1991a; Pope III 2000 ; Pope III *et al.* 2002; Schwartz 2004).

We have previously demonstrated that particles less than 10 and 2.5 μ in diameter (PM10 and PM2.5, respectively) consistent with worsening of disease in humans are present in racing stables, and that their concentrations are influenced by month, activity level (time of day), stable, and stalls within a stable (Millerick-May *et al.* 2008a). Concentrations of PM10 and PM2.5 are high in the morning hours during peak activity (e.g. feeding, cleaning), and in sampling months following a period of dry weather. Large particles (PM10) settle out quickly, while the smaller/lighter particles (PM2.5) remain suspended. Stable design may affect the rate of decline, with the more enclosed stable experiencing higher PM10 and PM2.5 peak and average concentrations throughout the day as compared to the stable with a more open design. Low-level concentrations of

small particles (PM 2.5) potentially representing chronic low-level exposures are affected by stable design and orientation to track roadways, with the open-air stable experiencing the highest concentrations. The location of stalls within each stable also is significantly associated with PM concentration, with stalls located in high-traffic areas and/or away from doors and windows experiencing significantly higher concentrations than adjacent stalls.

The purpose of the present study was to determine the relationship between area concentrations of PM₁₀ and PM_{2.5} as measured in three racing stables (Millerick-May *et al.* 2008a) and indices of airway inflammation (i.e. tracheal mucus and inflammatory cells in tracheal wash) in the horses that inhabit them. We hypothesized that indices of airway inflammation would have a high prevalence during the months in which particulate concentrations are increased, in stables with the highest overall PM concentrations, and in horses whose stalls had the highest concentrations of PM.

Materials and Methods

This study was conducted as repeated measurement in July, September, and November 2005 at a Thoroughbred racetrack in the Midwestern United States, where, Holcombe *et al.*, (2006) demonstrated that the presence of mucus in the airways negatively affected racing performance (Holcombe *et al.* 2006), and where we investigated factors affecting area particulate concentrations in stables (Millerick-May *et al.* 2008a). Three trainers with differing management styles and inhabiting stables of differing construction were chosen for inclusion into this study.

Experimental Design

Three visits were made to the racetrack during the 2005 racing season (June, September, and November), staying 3 days at each visit. The visits were selected to be a) early in the racing season, b) at the height of the season when conditions were usually driest, and c) when the weather was cooler and damp and the new stable (Stable 1) had closed its outer shutters.

The upper airway and trachea of each horse was endoscopically evaluated, and a tracheal aspirate obtained one day during each of the three visits. The name, age, sex, stable, and stall location of each horse was recorded along with mucus score. As reported in Millerick-May et al. 2008, particulate concentrations were measured in one stable each day during the visit. Concentrations of PM₁₀ and PM_{2.5} were measured for one minute in every stall three times of day - a) early morning during clean out, feeding and grooming, b) mid-day when there was less activity, and c) late afternoon, which coincided with feeding and racing.

Endoscopic Examination

Endoscopic examination was performed with the horse in its own stall. A member of the trainer's staff restrained the horse; no chemical restraint was used. The nasopharynx, larynx, and trachea were examined. The amount of mucus visible in the trachea was graded on a scale of 0-5: 0 = no visible mucus; 1 = singular small drops; 2 = multiple partially confluent drops; 3 = a ventral stream of mucus; 4 = a ventral pool; and

5 = profuse amounts of mucus occupying more than 25% of tracheal lumen (Gerber *et al.* 2004b). None of the horses in this study scored grade 5.

Tracheal Lavage

After the mucus accumulation was scored, 10 mL of saline was infused into the upper trachea through a sterile polyethylene catheter placed in the biopsy channel of the endoscope. The saline flowed down the trachea and formed a pool at the thoracic inlet. The endoscope was then advanced caudally until the saline pool was visible and a sample was aspirated through the catheter. After collection, samples were placed in storage tubes on ice until they were analyzed (within 20 minutes). The outside of the endoscope was thoroughly cleaned with betadine solution and distilled water between horses.

Measurements of total cell count and preparation of slides for differential cell counts were conducted at a temporary laboratory that was set up in one of the stables. The tracheal lavage samples were diluted 1:4 in sterile buffer. In the case of extremely viscous samples, further dilutions were made. For total cell count, 10 microliters of diluted sample was loaded into one side of a hemacytometer. Leukocytes were counted in 4 chambers and total cell count was calculated by using the formula: cell concentration/mL = total cell count in 4 squares x 2500 x dilution factor. In addition, 2 slides were prepared by use of a cytocentrifuge. Each slide was loaded with 175 microliters of sample and the positively charged slides spun at 600 rpm for 8 minutes, removed from the cassette, air dried and sprayed with histological fixative for transport back to Michigan State University. Differential cell counts were conducted at the

University on the cytopsin slides stained with Wright Geimsa and examined under a microscope by use of a 40X objective. A total of 200 leukocytes were counted in order to determine the percentage of neutrophils, macrophages, lymphocytes, eosinophils, and mast cells. Absolute cell counts were calculated from the total and differential cell counts.

Stables

Stable 1 was of newer construction and design. Orientation of the stable was north-south with roll-up shutters along the entire length (east and west sides) of the stable in combination with high vaulted ceilings, large sliding doors on both ends (front and back), and open fronted stalls (bars and gates) that face the outdoors. This stable, while consistently experiencing the lowest PM₁₀ and PM_{2.5} average and maximum concentrations, has the highest PM_{2.5} minimum concentrations, presumably from road dust dispersed from adjacent roadways and parking lots.

Stable 2 was of brick construction and the long axis of the stable was oriented in the north-south direction with busy track and city roadways flanking the lengths of the stable. This stable had 3-meter ceilings, closed-front stalls, and small, high windows (only in stalls that are on outer walls) that were kept closed. A single row of stalls was located along each of the outer walls of the stable, with a double row (back-to-back) of stalls down the center. The main source of ventilation was from two sets of large doorways both at the north and south ends of the stable, and one set of smaller doorways in the center of the west side of the stable. Large buildings located close to the main

doorways served to impede natural airflow. This was the only stable in the study to feed from hay nets, and had the greatest number of individuals simultaneously working within the stable. This stable consistently experienced the highest PM10 and PM2.5 average and peak concentrations.

Stable 3 was of identical construction to Stable 2, however some differences existed. The stable was oriented with its long axis east to west, and had open windows during the warm months. This stable was located in a secluded area (away from roads), but was adjacent to a common parking area along its western end.

Measurement of Particle Concentration

The stables and protocol for determining particle concentrations has been described previously (Millerick-May *et al.* 2008a), and is therefore only briefly reported here. Briefly, concentration of particles were assessed with two TSI DustTrak³ monitors. Using particle size conditioning inlets, one was set to measure concentrations of PM10, and the other PM2.5. Equipment calibration and cleaning was performed prior to the start of each day of sampling per manufacturers instructions, and periodic flow evaluations were made throughout each sampling day.

All measurements were one minute in duration, taken at the front-center of each stall 1 meter above the ground, approximating the height of the nostril of the horse. In an attempt

³ TSI Incorporated, 500 Cardigan Road, Shoreview, MN 55126-3996

to minimize our influence in the stable, horses remained in the stalls during monitoring, and all activities (cleaning, grooming, feeding, etc.) took place as scheduled.

Data Analysis

Bivariate Analysis

Pearson's chi-square tests were performed to determine which factors (e.g. age, month, stable, and particulate concentrations (PM10 and PM2.5)) were associated with tracheal mucus scores. For purposes of this analysis, we grouped tracheal mucus scores into two categories. A tracheal mucus score of (MS) ≥ 1 includes the presence of any amount of mucus in the trachea observed during endoscopic examination. MS ≥ 2 corresponds with a mucus score of 2 or above, that which has previously been determined to negatively impact racing performance (Holcombe *et al.* 2006). Average, minimum, and maximum PM10 and PM2.5 concentrations were also grouped into categories which 1) represented concentrations across all day times (morning, noon, afternoon) and sampling months (e.g. PM10Ave), 2) by day time across all months (e.g. PM10Ave morning). To facilitate analysis, PM concentrations in each measurement category were grouped into quartiles.

In order to determine the association between the above exposures and continuous variables (i.e. cell count data from tracheal lavage), Spearman correlation coefficients were estimated. Inflammatory cell counts by cell type (e.g. neutrophils), as well as percent of each type of inflammatory cell were analyzed to determine whether or not

there was a significant effect of particulate exposure. PM₁₀ and 2.5 concentrations were grouped as stated above.

Multivariate Analysis

Generalized estimating equation (GEE) models were conducted (GENMOD procedure) to determine the association between the categorical variables of MS $\geq$ 1 and MS $\geq$ 2 and factors found to be significant in bivariate analyses (e.g. age, month, PM concentrations). The outcome variables were measured three times (July, September, and November) with horses being potentially evaluated endoscopically up to three times. GEE analysis allowed for the determination of the effect of PM exposures on the aforementioned dichotomous outcome variables (MS $\geq$ 1 and MS $\geq$ 2), while controlling for within-horse variability (Zeger and Liang 1986). The potential effect of stable on the presence of tracheal mucus was considered, however, not included in the models as horses were not randomly assigned to stable. Because concentrations of particulates were strongly associated with stable, the latter was a possible surrogate for exposure level, and therefore could not be evaluated independently.

Linear mixed models (MIXED procedure) were conducted to determine the effect of the above exposures on continuous outcome variables (inflammatory cell counts). This procedure is designed to handle hierarchical data that is collected repeatedly on subjects (horses) over time. In addition, it is able to handle missing data and has the power to analyze a number of variance covariance structures while accounting for within-horse variability (Littel *et al.* 1996). The regular maximum likelihood (REML) method

was applied, assuming the random effects and error vector were normally distributed. This was achieved by log-transforming the cell count data, which were then transformed in geometric means. As possible covariance structures, unstructured (UN), spatial Gaussian (SP [GAU]), first order autoregressive (AR[1]), TOEPLITZ (TOEP), and compound symmetry (CS) were tested. The best covariance structure was chosen based on the Akaike Information Criterion, which was TOEPLITZ for all models.

All data analysis was conducted using SAS v.9.1. Level of significance was set at $P \leq 0.05$.

Results

Study Population

The population consisted of a total of 107 horses from 3 stables. Each horse that was present during each of our 3 visits was examined (July, September, and November of 2005). Thirty-eight percent of horses were examined once, with thirty-one percent of horses each examined a second and third time, resulting in a total of 206 individual examinations. Of the 206 attempted examinations, endoscopy was not possible 18 times, resulting in a total of 188 mucus score/tracheal lavage combinations (Table 1). Fourteen tracheal lavage samples were not of sufficient quality to perform analysis, leaving 174 samples of sufficient quality for total and differential cell count analysis.

Tracheal Mucus

Of the 188 endoscopic evaluations performed, 67% (n=126) had a mucus score of greater than or equal to 1 ($MS \geq 1$), and 19% (n=35) had a mucus score of greater than or equal to 2 ($MS \geq 2$) (Table 1).

Bivariate analysis (Table 2) showed that the following had a significant effect on $MS \geq 1$: age, month, stable, PM10 and PM2.5 average and maximum values, as well as PM10 and PM2.5 average and maximum values during the early morning sampling periods. PM10 minimum concentrations, as well early morning PM10 minimum concentrations were also significantly associated with $MS \geq 1$ (Table 2). Age and stable were the only two factors significantly associated with $MS \geq 2$ (Table 2).

Multivariate Analysis

$MS \geq 1$

There was a statistically significant positive association between PM10 average concentrations (4th quartile - OR 5.8, 95% CI (1.64-20.56), $p < 0.0064$) and a mucus score of ≥ 1 . There was also a statistically significant association between PM2.5 average concentrations (1st quartile – OR 4.5, CI (1.35-14.90), $p < 0.0151$) and the presence of tracheal mucus. The overall trend between PM10 and PM2.5 average concentrations by stable, and the association with $MS \geq 1$ can be seen in Figure 1. In a model that included age, gender, sample month, and PM2.5 average concentrations (quartiles), there was a significant effect of month and of PM2.5 average on $MS \geq 1$ (Figure 2), with the July measurement period being the lowest in terms of $MS \geq 1$ prevalence (OR 0.10, CI (0.03-

0.38), $p < 0.0007$) and of PM_{2.5} concentration. There were no significant effects of age or gender on MUC ≥ 1 .

MS ≥ 2

Bivariate analysis shows a significant relationship between age and MS ≥ 2 (Table 2). This association remained significant in the models (GEE), which were developed to evaluate the association between average, minimum, and maximum particle concentrations, sampling month, and age, and the presence of tracheal mucus in quantities that affect racing performance (MS ≥ 2). Two-year olds had a significantly greater risk (OR 3.36-3.96, 95% CI (1.08-13.46), $p < 0.0275 - 0.0371$) of MS ≥ 2 as compared to horses 4 years old and older (reference group).

While the GEE models used to determine the effect of particle concentrations on MUC₂ or greater did not show a significant association, the overall trend remained consistent with that of MS ≥ 1 , with a greater prevalence of MS ≥ 2 in Stable 2 as compared to Stables 1&3 (Figure 3), and an increasing trend between the months of July and September, with a slight decline in November (data not shown).

Inflammatory Cells

In this study, 68% of tracheal wash samples contained 20% or more neutrophils. Chi-square analysis revealed no significant association between inflammatory cell counts (grouped in tertiles) and MS ≥ 1 . However, there was a significant association of MS ≥ 2 with numbers of neutrophils ($p < 0.02$), macrophages ($p < 0.02$), and lymphocytes ($p < 0.01$).

The construction of GEE models using $MS \geq 1$ and $MS \geq 2$ as outcome variables, and inflammatory cell count data as predictor variables confirmed that there was no association between inflammatory cell counts and $MS \geq 1$, but a significant negative association between number of lymphocytes and $MS \geq 2$ remained (<0.0009).

Chi-square analysis showed a significant association between percent neutrophils and lymphocytes, and PM 2.5 average concentrations, PM 2.5 average concentrations during morning and evening sampling periods, and PM2.5 minimum concentrations (all) and sampling time points (morning, midday, and evening) (Table 2). Significant associations between percent neutrophils are similar to those of numbers of neutrophils and particle concentrations, in that associations remain with PM 2.5 average concentrations and the morning time point, as well as a PM2.5 maximum evening time point (Table 2).

Mixed model analysis did not reveal any significant relationship between PM10 and PM2.5, age, gender, or month, and numbers or percentage of inflammatory cells in tracheal wash samples. However, the trend in numbers and percentage of neutrophils is similar to that of PM10 and PM2.5 average concentrations by month (Figures 4 & 5), with increasing values from July to September, and then a decline in November.

Discussion

These are the first reported measurements of the association between indices of airway inflammation in Thoroughbred racehorses and area concentrations of particulate

matter (PM10 and PM2.5) in American racing stables. Previously, a study conducted by our laboratory (Holcombe *et al.* 2006) revealed that horses with $MS \geq 2$ finished further back in a race. This finding prompted racetrack management and trainers to inquire as to the etiology of mucus in the airways, and supported our efforts to determine whether or not there was an association between area (background) particle concentrations (PM10 and PM2.5), and measures of airway inflammation (tracheal mucus, inflammatory cells).

Briefly, we found a significant effect of month, time of day, stable, and stall on area concentrations of PM10 and PM2.5 (Millerick-May *et al.* 2008a). Measurements of PM in the month following a period of hot/dry weather revealed the highest concentrations, with those following a period of wet/rainy weather the lowest. As to be expected, times of day had a significant effect on particle concentrations, with the early morning hours during activities such as feeding and stall cleaning giving rise to the highest concentrations of PM, and evening measurements the lowest. We found a significant effect of stable, with the busy, completely enclosed brick-style stable with low ceilings (Stable 2) having the highest concentrations, and the open-sided stable with high vaulted ceilings, the lowest.

Based on the aforementioned information, we hypothesized that if exposures to area particle concentrations were to affect measures of airway inflammation, we would expect the prevalence of airway inflammation to be greatest during the month of highest PM concentrations (September), lowest during the month of lowest concentrations (July),

and highest in the stable which consistently had the highest concentrations of PM (Stable 2).

In support of this hypothesis, bivariate analysis revealed significant effects of month, stable, and concentrations of PM10 & PM2.5, on the presence of tracheal mucus ($MS \geq 1$). PM10 and PM2.5 average and maximum concentrations as measured during the early morning period, when peak activity was taking place, was also determined to have a significant effect on $MS \geq 1$. Furthermore, multivariate analysis also revealed a significant effect of PM10 & PM2.5 average concentrations on the presence of tracheal mucus ($MS \geq 1$), and also a significant effect of month (PM2.5 average) on $MS \geq 1$, with the month of July (following a period of wet weather) having the lowest concentrations of PM2.5 average measurements, and the lowest prevalence of $MS \geq 1$ (Figure 2). While there was not a significant effect of PM on the prevalence of $MS \geq 2$ as there was with $MS \geq 1$, there was a similar trend between the prevalence of $MS \geq 1$ or $MS \geq 2$ and stable (Figure 3).

We were not able to include 'stable' as a variable in the model as it is a surrogate for PM concentrations, however, further investigation into which stable contributed the highest percentage of PM measurements to quartiles deemed to be significant, indeed revealed that Stable 2 had the greatest influence (Figure 1), as it was the most enclosed and busiest of the three, contributed the greatest percent of PM10 and PM2.5 average measurements to each of the 4th quartiles (highest concentrations), and was found previously to contain the greatest number of stalls whose PM concentrations were in the

4th quartile (Millerick-May *et al.* 2008a), with the 4th quartile of PM10 (average) being significantly associated to $MS \geq 1$.

There was a significant effect of age on $MS \geq 2$, as was determined by both bivariate and multivariate analysis. 2-year olds, in comparison to those aged 4 and older, had a higher prevalence of $MS \geq 2$. Others investigating factors affecting the presence of IAD in racehorses have also found an association between age and the prevalence of airway inflammation (Christley *et al.* 2001a; Wood *et al.* 1993; Wood *et al.* 2005c), however, more recent reports suggest an association between length of time in training, rather than age, and indices of airway inflammation (Wood *et al.* 2005c), with those horses that have been in training longer, having a lower prevalence. While we were not able to collect data regarding length of time in training, we did include length of time in stable (data not shown) in each of our multivariate models. Length of time in stable was estimated by number of months included in our study and reports of ‘shipped in’ dates by our trainers. We found no significant association between length of time in stable and either $MS \geq 1$ or $MS \geq 2$. Others have hypothesized that young horses recently brought into a stable environment would be more susceptible to respiratory infection, and over time would become more resistant (Wood *et al.* 2005a). If this were the case, one would expect the highest prevalence of $MS \geq 1$ or $MS \geq 2$ in our population to occur during our initial sampling period (July), and decline throughout the rest of the year. This was not the case in our population, as our highest prevalence of both $MUC \geq 1$ and $MUC \geq 2$ occurred in September.

Our data suggest that small amounts of mucus ($MUC=1$) are a result of a local irritant response to particulate exposure. We reach this conclusion for the following reasons: 1) the prevalence of $MS \geq 1$ tracks particulate concentrations by month and by stable, and is strongly associated with PM_{10} and $PM_{2.5}$, 2) the prevalence of $MS \geq 1$ is not related to either the percent or number of inflammatory cells, and 3) the prevalence of $MS \geq 2$ is associated with changes in inflammatory cell populations. The fact that two-thirds of the observations of $MS > 1$ are comprised of $MUC=1$ (Table 1), suggests therefore that $MUC=1$ is not associated with the presence of inflammatory cells, but rather is a local response to particulates perhaps mediated through the epithelium.

Our overall study was designed to determine the effects of month, time of day, and stable (design features and management) on area particle concentrations, and resulting measures of airway inflammation (tracheal mucus and inflammatory cell counts). As such, our study design required repeated measures in the same stables over time. This study was not designed or sufficiently powered to distinguish between concentrations of particulate matter, and degrees tracheal mucus (i.e. $MS \geq 2$) previously found to be associated with racing performance (Holcombe *et al.* 2006)). Based on the trends observed in the present study, a more extensive investigation of this association is warranted. As we have shown that the prevalence of $MS \geq 1$ is significantly associated with the 4th quartile PM_{10} Ave concentrations, and that prevalence of $MS \geq 2$ follows a similar pattern, if data from a larger study provided more clarity as to the area concentrations of PM that are associated with degrees of tracheal mucus, it should be possible to set area $PM_{10}/PM_{2.5}$ exposure limits to reduce the occurrence of tracheal

mucus in quantities shown to be associated with racing performance (Holcombe *et al.* 2004).

The usefulness of inflammatory cell counts is an unresolved issue with regard to diagnosis of IAD (Anon 2003) as there are other newer and better references. The phenotype for IAD has been defined as the presence of tracheal mucus observed during endoscopic examination, and >20% neutrophils in tracheal wash/BAL samples. However, in the present study, 68% of tracheal wash samples contained greater than 20% neutrophils. Furthermore, 82% of horses with no visible tracheal mucus (MUC=0), had greater than 20% neutrophils. Almost exactly the same prevalence was observed by (Robinson *et al.* 2006), in a study of pleasure horses. These observations indicate that the definition of IAD may need to be revisited, with tracheal mucus score rather than inflammatory cells used as the gold standard for diagnosis.

Table 3-1. Summary of number of examinations performed per stable, age, gender, and assigned mucus score, as well as the prevalence of MS_≥1 and MS_≥2.

		Age				Gender		Mucus Score*				Mucus Present (MS _≥ 1)		Mucus Affecting Racing (MS _≥ 2)	
Stable	# Examinations	2	3	≥4	Unk.	M	F	0	1	2	≥3	#	%	#	%
1	67	13	7	46	1	54	13	29	25	1	1	27	48	2	4
2	84	44	25	12	3	43	41	20	41	14	7	62	76	21	26
3	55	16	18	17	4	38	17	13	25	12	0	37	74	12	24
Total	206	73	50	75	8	135	71	62	91	27	8	126	67	35	19

Table 3-2. Result of bivariate analysis of factors affecting mucus scores and inflammatory cells. P-values <0.05 have been listed.

	$MS_{\Delta 1}$	$MS_{\Delta 2}$	% Neutrophils	# Neutrophils	% Lymphocytes	# Lymphocytes	% Eosinophils	# Eosinophils	% Macrophages	# Macrophages	% Mast Cells	# Mast Cells
Age	0.0471	0.0106								0.0389	0.0012	
Month	0.0144											0.0024
Stable	0.0017	0.0025										
PM10Avg	0.0013											
PM2.5Avg	0.0277		0.0144	0.0204	0.0035						0.0147	0.0247
PM10Max	0.0013											
PM2.5Max	0.0092										0.0258	
PM10Min	0.0031										0.042	0.0521
PM2.5Min			0.0048		0.0002							
PM10Avg1	0.0291											
PM10Avg2											0.0363	
PM10Avg3												
PM2.5Avg1	0.0305		0.002	0.0168	0.0004						0.0236	0.0424

Table 3-2 (cont'd).

	MS ₁	MS ₂	% Neutrophils	# Neutrophils	% Lymphocytes	# Lymphocytes	% Eosinophils	# Eosinophils	% Macrophages	# Macrophages	% Mast Cells	# Mast Cells
PM2.5Avg2											0.0481	
PM2.5Avg3			0.0177		0.0033						0.0079	0.0278
PM10Max1	0.0414											
PM10Max2									0.0257			
PM10Max3												
PM2.5Max1	0.0416										0.0052	0.0076
PM2.5Max2						0.0311					0.0454	
PM2.5Max3				0.0401							0.0078	0.0282
PM10Min1	0.0098											
PM10Min2											0.0203	0.0459
PM10Min3											0.07	
PM2.5Min1			0.0009		0.0001							
PM2.5Min2			0.0075		0.0039							
PM2.5Min3			0.0057		0.0005		0.0474	0.0266				

Table 3-3. Concentrations of particulate matter (PM) within each quartile utilized for GEE and mixed model analysis.

Average (avg), maximum (max), and minimum (min) values are presented, with numeric notations indicating time of day sampling occurred (1 - morning, 2 - midday, 3 - evening).

Quartile	PM10 ave	PM2.5 ave	PM10 max	PM2.5 max	PM2.5 min	PM10 ave1	PM2.5 ave1	PM2.5 ave3	PM10 max1	PM2.5 max1	PM2.5 max3	PM10 min1	PM2.5 min1	PM2.5 min2	PM2.5 min3
1	0.120 - 0.272	0.054 - 0.083 0.496	0.183 - 0.496	0.086 - 0.147 0.496	0.016 - 0.055 0.496	0.093 - 0.324	0.030 - 0.096 0.496	0.042 - 0.062 0.496	0.168 - 0.518	0.052 - 0.162 0.496	0.054 - 0.109 0.496	0.038 - 0.189	0.005 - 0.060 0.496	0.015 - 0.056 0.496	0.013 - 0.042 0.496
2	0.273 - 0.369	0.083 - 0.129 0.688	0.499 - 0.688	0.148 - 0.418 0.688	0.056 - 0.073 0.688	0.331 - 0.517	0.097 - 0.156 0.517	0.063 - 0.094 0.517	0.526 - 0.855	0.164 - 0.441 0.855	0.111 - 0.294 0.855	0.192 - 0.290	0.061 - 0.089 0.290	0.057 - 0.065 0.290	0.043 - 0.053 0.290
3	0.377 - 0.558	0.131 - 0.236 0.992	0.692 - 0.992	0.419 - 0.701 0.992	0.074 - 0.186 0.992	0.520 - 0.759	0.162 - 0.300 0.759	0.097 - 0.203 0.759	0.871 - 1.34	0.442 - 0.779 1.34	0.303 - 0.500 1.34	0.291 - 0.450	0.091 - 0.203 0.450	0.066 - 0.129 0.450	0.054 - 0.116 0.450
4	0.569 - 2.02	0.239 - 0.643 4.70	1.01 - 4.70 4.70	0.706 - 7.78 4.70	0.190 - 0.323 4.70	0.763 - 4.09	0.303 - 1.17 4.09	0.204 - 0.673 4.09	1.37 - 6.63 4.09	0.795 - 22.9 4.09	0.502 - 4.16 4.09	0.472 - 1.570	0.204 - 0.389 1.570	0.158 - 0.830 1.570	0.117 - 0.309 1.570

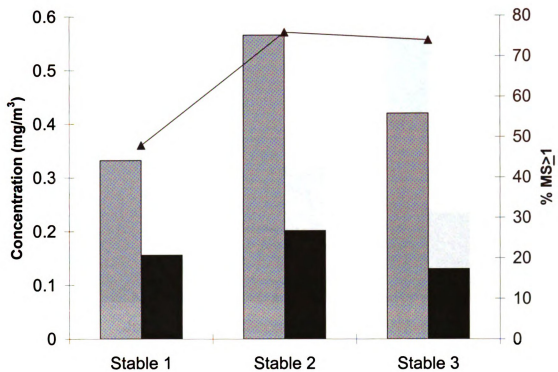


Figure 3-1. Effect of particle concentrations on overall prevalence of $MS_{\geq 1}$ (black line) by stable. Gray bars indicate average PM10 concentrations (mg/m^3), and black bars indicate average PM2.5 concentrations (mg/m^3).

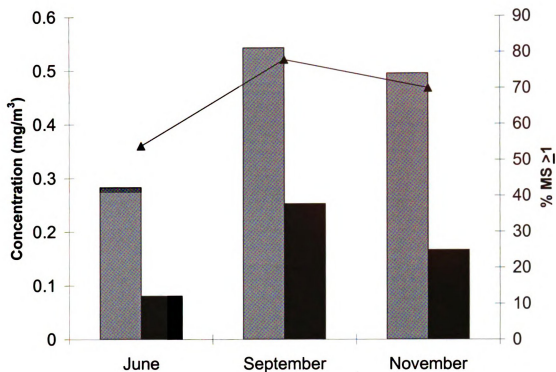


Figure 3-2. Effect of particle concentrations on overall prevalence of MS>1 (black line) by sampling month. Gray bars indicate average PM10 concentrations (mg/m³), and black bars indicate average PM2.5 concentrations (mg/m³).

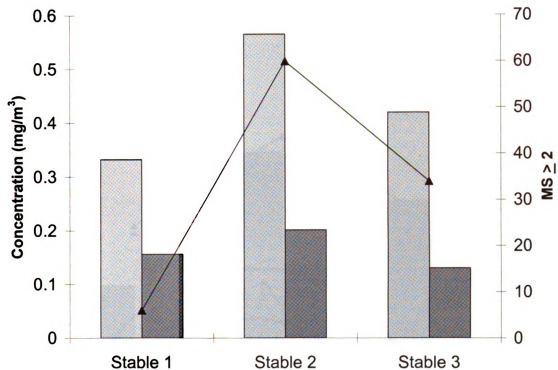


Figure 3-3. Effect of particle concentrations on overall prevalence of MS>2 (black line) by stable. Light gray bars indicate average PM10 concentrations (mg/m³), and dark gray bars indicate average PM2.5 concentrations (mg/m³).

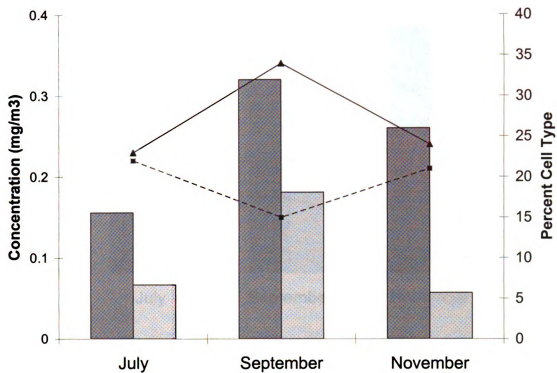


Figure 3-4. Effect of particle concentrations on percent neutrophils (solid line) and lymphocytes (dashed line) by month. Dark gray bars indicate average PM10 concentrations (mg/m3), and light gray bars indicate average PM2.5 concentrations (mg/m3).

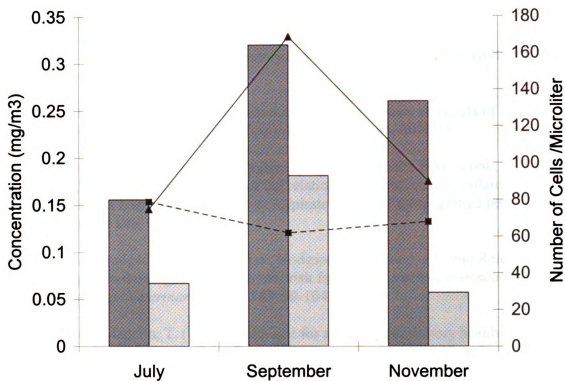


Figure 3-5. Effect of particle concentrations on numbers of neutrophils (solid line) and lymphocytes (dashed line) by month. Dark gray bars indicate average PM10 concentrations (mg/m³), and light gray bars indicate average PM2.5 concentrations (mg/m³).

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CHAPTER 4

Airborne Particulates (PM₁₀) and Tracheal Mucus: A Case-Control Study at an American Thoroughbred Racetrack

Summary

Reasons for Performing Study

The presence of sufficient quantities of tracheal mucus ($MS \geq 2$) negatively affects racing performance. We have previously demonstrated a significant association between concentrations of particulate matter (PM₁₀ and PM_{2.5}) and the presence of tracheal mucus in a population of Thoroughbred racehorses.

Objectives

Previous measurements of particulates (PM₁₀ and PM_{2.5}) by our laboratory were of background concentrations rather than at the horse's nostril, which would account for not only area concentrations of PM, but also that which is generated as a result of personal idiosyncrasies of the horse (manner of eating, in-stall activity, rolling, etc.). The objective of this study was to determine the differences in personal particulate exposures between those with ($MS \geq 2$) and without ($MS = 0$) tracheal mucus visible upon endoscopic exam in quantities previously associated with poor racing performance.

Methods

We studied 653 racehorses from 6 stables over seven months (April – October 2006). Horses were endoscopically examined, and assigned a mucus score. Nine cases

and controls were selected and age matched each month based on the presence ($MS \geq 2$) or absence ($MS = 0$) of tracheal mucus. Tracheal wash was performed on all cases and controls. Each month, the selected case-control pairs wore direct reading personal particulate monitors for on average 17.5 hours. The personal monitors were attached to vaulting surcingles and a sufficient length of Tygon tubing to span the crest of the horse, with the end of the sampling tube secured approximately 2 inches above the nostril. The monitors were set to take samples every second during 3 time periods (mid-day, evening, and overnight). Particulate concentrations (PM_{10}) were recorded after each time period.

Results

Overall prevalence of $MS \geq 2$ was 23% during the 7-month duration of the study. Bivariate analysis revealed a significant effect of trainer and month on prevalence of $MS \geq 2$, and a significant effect of month on personal PM concentrations and percent neutrophils. There was a significant difference between exposures to PM_{10} concentrations in cases and controls during the evening (maximum) and overnight (minimum) time periods, as well as significant differences in absolute, but not percent neutrophils between cases and controls, with both groups having greater than 20% neutrophils in their tracheal wash fluid.

Conclusions

The fact that overall prevalence of $MS \geq 2$ tracked closely with monthly personal PM exposure data, that personal particulate concentrations between cases and controls were not significantly different in most instances, and were similar to area concentrations

measured in a prior study, suggests that area PM is what predominantly influences personal exposures. We report a significant difference in neutrophil counts between cases and controls, but there was no difference between percent neutrophils, a value which is frequently used in the diagnosis of IAD.

Potential Relevance

If indeed it is the area concentrations of PM that predominantly influence personal exposures as our data suggests, and it is these concentrations that are associated with tracheal mucus ($MS \geq 2$), following low-dust stable management practices may greatly reduce the prevalence of tracheal mucus.

Introduction

The presence of tracheal mucus in sufficient quantities negatively affects racing performance, potentially resulting in a significant financial impact to the racing industry (Holcombe *et al.* 2006; MacNamara *et al.* 1990b). Tracheal mucus is one feature of a syndrome known as Inflammatory Airway Disease (IAD), which has an overall prevalence in racing Thoroughbreds of 13% (Wood *et al.* 2005c), with up to 45% of horses in a single racing stable affected at any one time (unpublished data).

The cause of IAD is likely multifactorial in nature, however, bacterial infection (Newton *et al.* 2003a; Wood *et al.* 2005a, c) and the environment (Malikides and Hodgson 2005) are two factors that have been implicated in otherwise young and healthy racehorses. Airway inflammation, as determined by the presence of tracheal mucus and increased inflammatory cells in tracheal wash or bronchoalveolar lavage fluid (BALF), occurs in healthy horses upon exposure to the stable environment (Gerber V 2004; Holcombe *et al.* 2001; Tremblay *et al.* 1993).

We have previously demonstrated a significant association between concentrations of particles less than 10 and 2.5 μ in diameter (PM₁₀ and PM_{2.5}), and the presence of tracheal mucus in Thoroughbred racehorses (Millerick-May *et al.* 2008b), as well as significant associations between month, time of day, stable, and stall on area concentrations of these particles (Millerick-May *et al.* 2008a). Area concentrations of particulate matter, however, do not reflect personal exposures as they may underestimate

actual exposures. Area measurements in stables reflect particles in the outside air that permeate throughout the building, as well as those dispersed as a result of stable activity (i.e. cleaning, feeding, and raking), and are useful in determining ventilation patterns and ‘hot spots’/dead zones within the stable. These measurements are invaluable in formulating/recommending control strategies to reduce overall concentrations.

Personal exposure concentrations, as would be measured at the level of the nostril (breathing zone), reflect not only the area concentrations mentioned above, but the additional particulates generated as a result of ‘personal’ activities of the horse (e.g. eating, rolling, and stall walking). These concentrations would be expected to be different for each individual as personal behavior differs. Examples of this would be the vigor with which an animal pulls hay from the flake/hay net, the number of times a horse rolls or lies down in a day, and movement (walking) behaviors in the stall, etc.

Dusts (particulates) measured in the breathing zone of horses have been recorded in concentrations known to induce airway inflammation in humans and laboratory animals (Millerick-May *et al.* 2007; Woods PS 1993), and originate from bedding, feedstuffs, and footing materials. Components of these dusts are both organic (e.g. molds and endotoxin) (Vandenput *et al.* 1997; Woods PS 1993) and inorganic (e.g. iron and crystalline silica) (Millerick-May *et al.* 2007) in nature, many of which have occupational exposure limits (ACGIH 2005a) aimed at preventing occupationally induced lung disease in people.

The purpose of the present study, which used a case-control approach, was to determine the association between breathing zone concentrations of particulate matter in a population of Thoroughbred racehorses, and the presence of tracheal mucus in quantities previously determined to negatively affect racing performance (Holcombe *et al.* 2006). We hypothesized that horses with $MS \geq 2$ would experience greater personal PM exposures as compared to horses with $MS=0$.

Materials and Methods

This study was conducted at the same Thoroughbred racetrack in the Midwestern United States, where, Holcombe *et al.*, (2006) demonstrated that the presence of mucus in the airways negatively affected racing performance (Holcombe *et al.* 2006), where we investigated factors affecting area particulate concentrations in stables (Millerick-May *et al.* 2008a), and where we demonstrated an association between particulate matter in racing stables and the presence of tracheal mucus in resident horses (Millerick-May *et al.* 2008b).

Experimental Design

Seven visits were made to the racetrack during the 2006 racing season (April through October), staying 4 days at each visit. Horses stabled in 6 racing stables were chosen for inclusion into this study. Cases and controls were selected from these racing stables, and their personal PM₁₀ exposures recorded over a period of 24 hours.

In each of the 7 months, the upper airway and trachea of approximately 100 horses was endoscopically evaluated. The name, age, gender, stable, and stall location of each horse was recorded along with mucus score. From these horses, 9 cases and 9 controls were identified based on the presence or absence of tracheal mucus. Each horse was fitted with a personal particulate monitor, and particulate concentrations (PM10) were measured over most of a 24-hour period of time.

Endoscopic Examination

Endoscopic examination was performed with the horse in its own stall. A member of the trainer's staff restrained the horse; no chemical restraint was used. The nasopharynx, larynx, and trachea were examined. The amount of mucus visible in the trachea was graded on a scale of 0-5: 0 = no visible mucus; 1 = singular small drops; 2 = multiple partially confluent drops; 3 = a ventral stream of mucus; 4 = a ventral pool; and 5 = profuse amounts of mucus occupying more than 25% of tracheal lumen (Gerber *et al.* 2004b). None of the horses in this study scored grade 5.

Tracheal Lavage

Tracheal lavage was performed only in horses identified as either a case or control. Ten milliliters of saline was infused into the upper trachea through a sterile polyethylene catheter placed in the biopsy channel of the endoscope. The saline flowed down the trachea and formed a pool at the thoracic inlet. The endoscope was then advanced caudally until the saline pool was visible and a sample was aspirated through the catheter. After collection, samples were placed in storage tubes on ice until they were

analyzed (within 20 minutes). The outside of the endoscope was thoroughly cleaned with betadine solution and distilled water between horses.

Measurements of total cell count and preparation of slides for differential cell counts were conducted at a temporary laboratory that was set up in one of the stables. The tracheal lavage samples were diluted 1:4 in sterile buffer. In the case of extremely viscous samples, further dilutions were made as necessary. For total cell count, 10 microliters of diluted sample was loaded into one side of a hemacytometer. Leukocytes were counted in 4 chambers and total cell count was calculated by using the formula: cell concentration/ml = total cell count in 4 squares x 2500 x dilution factor. In addition, 2 slides were prepared by use of a cytocentrifuge. Each slide was loaded with 175 microliters of sample and the positively charged slides spun at 600 rpm for 8 minutes, removed from the cassette, air dried and sprayed with histological fixative for transport back to Michigan State University. Differential cell counts were conducted at the University on the cytospin slides stained with Wright Geimsa and examined under a microscope by use of a 40X objective. A total of 200 leukocytes were counted in order to determine the percentage of neutrophils, macrophages, lymphocytes, eosinophils, and mast cells. Absolute cell counts were calculated from the total and differential cell counts.

Selection of Cases and Controls

Nine cases and nine controls were selected each month from the approximately 100 horses that were endoscopically examined. Pools of potential cases were identified

as horses with a tracheal mucus score of 2 or greater ($MS \geq 2$), and controls as those without tracheal mucus ($MS=0$). The 9 cases and 9 controls were randomly chosen from the pool and were matched by age. Individuals that had previously been identified as a case were no longer eligible to be a case in subsequent months, and the same selection rule applied to controls. That said - an individual that had been previously selected as a case was eligible to be a control during a subsequent month, and vice versa. Once horses had been used both as a case and as a control, they were excluded from further study.

Measurement of Breathing Zone Concentrations

Concentrations of breathing zone particulates were evaluated using TSI SidePak AM510⁴ personal monitors. These monitors are small, lightweight, and utilize light-scattering technology to determine real-time particle concentrations. Using particle size conditioning inlets, each monitor was set to measure concentrations of PM₁₀. Equipment calibration (flow and zero filter) was performed prior to the start of each sampling segment, with cleaning occurring once daily per manufacturers' instructions.

Samplers were affixed to the horse by the use of vaulting surcingles (Figure 1) secured just behind the shoulder. Each sampler was secured within (encircled by) a handle located on the surcingle, the intent of which was to offer additional protection to the monitor during lying down/rolling. Six feet of TygonTM tubing (1/4" ID) was attached at one end of the sampler to the inlet, run along the crest of the horses' neck (secured to the mane),

⁴ TSI Incorporated, 500 Cardigan Road, Shoreview, MN 55126-3996

down the right side of the halter, with the other end of the tubing located approximately two inches above the nostril (Figure 2).

The use of four feet of tubing or less was recommended by the manufacturer (personal communication) in order to minimize sampling losses due to impaction/adhesion onto the inner walls of the tubing. However, to allow the horse to have full range of motion of its head and neck and be assured that the sampling tube remain connected to the sampler, it was necessary to extend the length of the tube to six feet. In order to determine potential losses due to impaction/sedimentation in the sampling tube, we took 6 matched one minute samples (six different horse/tubing set-ups) at the nostril of the horse using a TSI DustTrak¹ monitor and compared 1-minute average concentrations to those measured by the personal monitor/tubing configuration.

Personal monitors were programmed to record PM₁₀ concentrations every second (Figure 3) for the duration of each of three sampling periods, which were midday (11 a.m. to 4 p.m.), evening (5 p.m. to 11 p.m.), and overnight (11:30 p.m. until 6 a.m.). It was necessary to remove the monitors between sampling periods to download data, clean/calibrate/check flow rates, and recharge/replace battery packs. We were unable to take measurements between 6 a.m. and 11 a.m. due to stable and training activities.

Data Analysis

For each horse and each sampling time segment (mid-day, evening, and overnight), average, maximum, and minimum PM10 concentrations were recorded. In addition, the number of 1 second concentrations exceeding 0.569 mg/m^3 (a concentration equal to or above which is associated with the presence of tracheal mucus) (Millerick-May *et al.* 2008b), 1 mg/m^3 , and 10 mg/m^3 was calculated. Time weighted average (TWA) concentrations for each horse were calculated from average concentrations/length of time sampled (in .5 hour segments) recorded during each of the three sampling segments. In the event of equipment failure, any concentrations measured during that sampling period were not used.

Pearson's chi-square analysis was used to determine which factors (age, stable, month) were associated with $MS \geq 2$ (Holcombe *et al.* 2006). As personal monitoring data reflects not only concentrations of particulates liberated in close proximity to the nostril, but also area concentrations of dust previously determined to be influenced by other factors (i.e. month, stable, and time of day) (Millerick-May *et al.* 2008a), we utilized the personal monitoring data collected during the case/control portion of our study to determine whether or not these samples were influenced by such factors (month). For these analyses, it was necessary to use Fisher's exact test in lieu of Pearson's chi-square, as the expected frequency in certain cells was determined to be less than 5. Therefore, the particulate data were grouped into 'high' and 'low' categories for each of the sampling time points (mid-day, evening, and overnight) and were analyzed by month.

Effect of month on inflammatory cell counts and percentages were also determined using Fisher's exact test. In order to determine the association between the particle concentrations and cell count data from tracheal lavage, Spearman correlation coefficients were employed. Inflammatory cell counts by cell type (e.g. neutrophils), as well as percent of each type of inflammatory cell were analyzed to determine whether or not there was a significant effect of particulate exposure.

Conditional logistic regression procedures (PROC PHREG) were employed to determine if there were significant differences in personal exposures to particulates during mid-day, evening, and overnight sampling periods within matched cases and controls. Time-weighted average exposures, as well as numbers of 1 second samples above 0.569, 1, and 10 mg/m³ were also considered. These procedures were also utilized to determine whether there was a difference in total neutrophils between cases and their matched controls. Odds ratios and 95% confidence limits were calculated.

All data analysis was conducted using SAS v.9.1. Level of significance was set at $P \leq 0.05$.

Results

The population consisted of a total of 653 horses from 6 racing stables. All but 4 horses (n=649) were endoscopically examined and a mucus score of 0-5 assigned (Table 1). Characteristics (age, gender, and stable) of horses selected for inclusion into the study are listed in Tables 2&3. At the conclusion of the 7-month sampling period, we had obtained

personal monitoring data on a total of 113 cases and controls, with a total of 104 matched pairs. We obtained an average of 17.5 hours of personal particulate monitoring data per horse, giving rise to a total of 6,557,000 1-second data points for inclusion in our analysis. Median PM concentrations, and numbers of 1-second samples above 3 thresholds (0.569, 1, and 10 mg/m³) for cases and controls are presented in Table 4.

Validation of Particulate Measurements

Comparison of simultaneous 1-minute measurements of particle concentrations at the end of the tubing by the nostril with those recorded by the monitor located on the surcingle, revealed losses due to the tubing attachment ranged from 9-11% (data not shown).

Mucus Scores and Particle Concentrations

In the 649 endoscopic examinations, the overall prevalence of MS_≥2 was 23.1 percent (n=150) (Table 1). Bivariate analysis revealed a significant effect of trainer (p<0.0153) and month (p<0.0430) on MS_≥2.

There was a significant effect of month on the following measurements of particle concentrations: midday average (p<0.0077) and minimum (p<0.0280) concentrations, evening average (p<0.001) (Figure 5) and minimum (p<0.0395) concentrations, overnight average (p<0.0005) (Figure 4), minimum (p<0.0028), and maximum (p<0.0008) concentrations, time-weighted average concentrations (0.0001) and number of data points (seconds) (p<0.0187) exceeding 1 mg/m³.

Differences in mean concentrations of PM₁₀ throughout the three sampling periods were negligible, however, significant differences did exist between evening and overnight minimum concentrations ($p<0.0066$), as well as between midday and evening minimum concentrations ($p<0.02$) with evening concentrations being the greatest in both instances.

Inflammatory Cell Counts

There was a significant effect of month on percent of neutrophils ($p<0.0049$), macrophages ($p<0.0013$), and eosinophils ($p<0.0237$) in tracheal wash and also on number of eosinophils ($p<0.0201$).

Evening average ($p<0.0002$), mid-day average ($p<0.0280$), overnight average ($p<0.0234$), and time-weighted average ($p<0.0005$) PM₁₀ concentrations were significantly associated with absolute neutrophil counts, and evening average concentrations were significantly associated with numbers of macrophages ($p<0.0066$) and lymphocytes ($p<0.0324$). Number of eosinophils was associated with mid-day average ($p<0.0164$) and overnight maximum ($p<0.0017$) PM concentrations. There was a significant difference between cases and their matched controls in numbers of neutrophils ($p<0.0486$, OR 1.863, 95%CI (1.004 – 3.456), with cases having greater numbers in relation to controls.

Variation in PM Exposures between Matched Cases ($MS\geq 2$) and Controls ($MS=0$)

Analysis of differences between matched case/control exposures to PM during three sampling periods (evening, overnight, and mid-day) revealed significant differences only in exposure to evening maximum PM concentrations ($p<0.0265$, OR 2.083, 95% CI (1.090-

3.092), and overnight minimum PM concentrations ($p < 0.0309$, OR 2.560, 95% CI (1.090-6.013)). Significant differences in exposure between cases and controls were not identified with the remaining measures of PM₁₀.

Discussion

This is the first reported large-scale case-control study of the association between personal exposures to airborne particulates (PM₁₀) in horses with (MS $\geq$ 2) and without (MS=0) visible accumulations of tracheal mucus. In a previous study, we reported significant associations of time of day, month, stable, and stall on concentrations of PM, and the presence (MS $\geq$ 1) of tracheal mucus, but no significant association between area concentrations and MS $\geq$ 2 even though the data showed similar trends to that of MS $\geq$ 1. As a result, we hypothesized that greater personal rather than area PM exposure was responsible for MS $\geq$ 2.

Particulate concentrations in the breathing zone of horses have previously been measured by both the traditional pump and filter methods (Woods PS 1993), allowing for gravimetric analysis and speciation of dusts over a set period of time, as well as the use of real-time monitors (Clements and Pirie 2007a, b; Millerick-May *et al.* 2007), which provide data in terms of average, minimum, and maximum particle concentrations recorded during the sampling period. There are benefits to using both methods, however, the real-time monitoring used in the present study allowed for measurement of the previously uncharacterized range of PM concentrations (peak and minimum) experienced over a long sampling period. The anatomy of the horse necessitated use of longer

sampling tubes than recommended by the manufacturer and indeed, about 10 percent of particulates were lost. However, because all personal sampler/tubing configurations were standardized, matched cases and controls were monitored during the same sampling period, and our goal was to determine *relative* differences in exposures rather than *absolute* values, these losses were deemed acceptable for the purposes of this study.

Despite any losses due to impaction/sedimentation in the tubing, the PM concentrations measured with the personal samplers were similar to those previously measured with the area monitors during the midday and evening time periods (we could not compare morning measurements because personal samplers had to be removed during that time). The similarities between personal and area concentrations suggest that PM exposures are primarily dependent on area concentrations rather than additional contributions by personal idiosyncratic activities of the horse, such as nostril placement while eating (leaving nose inside hay pile), rolling, or sleeping while lying down rather than standing.

The overall prevalence of $MS \geq 2$ was 19% in our previous study as compared to 23% in our present study. The latter may be a more accurate reflection of true prevalence because horses were examined for 7 consecutive months during this study as compared to only 3 months (July, September, and November) in the previous study. Overall trends between the two studies were consistent; the lowest prevalence occurred during the cooler early summer months, and the highest during hot dry weather.

While there was a significant difference between cases and controls and numbers of neutrophils in tracheal wash fluids, percent neutrophils were not found to be significantly different. Median values for percent neutrophils in both cases and controls exceeded 27%, with 20% being recognized as the value above which a diagnosis of airway inflammation is generally made. In fact, 59% of horses with mucus scores of 0 (MS=0) had neutrophil percentages that exceeded the 20% threshold for IAD.

In support of our hypothesis that cases had higher PM exposures than controls, we found significant differences in their exposures to evening maximum, and overnight minimum concentrations of PM₁₀. In our previous investigation of the relationship between area PM and the presence of visible mucus, $MUC \geq 1$ also was significantly associated with both maximum and minimum PM₁₀ concentrations. Our failure to find more case/control differences in PM concentrations may be a function of study design. Although the number of cases and controls were similar in each stable, they were not evenly distributed among the stables (Table 3). This was because it was sometimes challenging to find an adequate numbers of controls (MS=0) that fulfilled our matching criteria. As a consequence, more than half of the horses recruited (both cases and controls) came from 2 stables (Table 3). Because stable design and management determine area PM concentrations (Chapter 3), and the latter rather than individual factors is the most important determinant of personal exposure, having large numbers of horses from the same two stables likely reduced our chances in finding a difference in PM exposures.

We have previously reported that both stable and month influence area concentrations of PM and the presence of tracheal mucus and inflammatory cells (Millerick-May *et al.* 2008b). If, as we suspect that personal exposures are largely the result of area concentrations, then the data from personal samplers should reflect monthly changes in area PM. For this reason, we examined temporal trends in personal PM concentrations and prevalence of MS $\geq$ 2 (Figure 4 and 5). Indeed, as personal PM concentrations fluctuated month-by-month, they were tracked by MS $\geq$ 2 prevalence. There was a similar tracking between personal PM₁₀ and percent neutrophils (Figure 6). These observations strongly suggest that area particulate exposure is an important determinant of the presence of mucus in the airways.

Elevations in area concentrations of particulate matter (PM₁₀ & PM_{2.5}) are known to result in worsening of pre-existing respiratory disease in humans, increased lost work/school days, and increased mortality (Brunekreef and Forsberg 2005; Pope 1991a; Pope III 2000 ; Pope III *et al.* 2002; Schwartz 2004). Control of area particulate concentrations within the stable environment is much easier than attempting to control individual behaviors (e.g. eating, rolling, pacing, etc.) that influence personal dust exposures. If indeed it is the area concentrations of PM that predominantly influence personal exposures as our data suggests, and it is these concentrations that, in turn, are associated with tracheal mucus (MS $\geq$ 2), efforts to improve ventilation within stables, and to follow dust management practices aimed at minimizing dispersion of dust within the stable environment will reduce the prevalence of tracheal mucus in horses within those

stables, and during months where factors external to the stable environment (e.g. weather conditions) also influence PM concentrations.

Table 4-1. Summary of number of examinations performed per stable, age, gender, and assigned mucus score, as well as the prevalence of MS $\geq$ 2. *4 horses unable to be endoscopically examined

Stable	Age				Gender		Mucus Score*				Mucus Affecting Racing (MS $\geq$ 2)	
	2	3	$\geq$ 4	Unk.	M	F	0	1	2	$\geq$ 3	#	%
1	5	27	53		61	24	22	49	12	1	13	15.5
2	116	96	26		112	126	75	95	51	16	67	28.3
3	22	31	34		44	44	23	51	10	3	13	14.9
4	22	57	82		88	73	66	59	25	10	35	21.9
5	4	17	21	1	23	19	12	15	11	4	15	35.7
6	0	21	18		21	18	5	27	6	1	7	18
Total	169	249	234	1	349	304	203	296	115	35	150	23.1

Table 4-2. Summary of age and gender of cases and controls included in the study

	Age			Gender	
	2	3	≥ 4	M	F
Case ($MS \geq 2$)	15	24	14	26	27
Control ($MS=0$)	16	25	19	29	31
Total	31	49	33	55	58

Table 4-3. Summary of numbers of cases and controls selected from each stable

<u>Stable</u>	<u># of Cases</u>	<u># of Controls</u>	<u>Total</u>
1	7	7	14
2	24	24	48
3	3	5	8
4	11	17	29
5	5	6	11
6	3	1	4
Total	53	60	113

Table 4-4. Description of the average, minimum, maximum, and time-weighted average concentrations of PM10 (mg/m³) during each of three sampling periods, as well as the number of 1-second samples exceeding 0.569, 1, and 10 mg/m³ as experienced by cases and controls.

	<u>Case</u> Median (5th-95th pctl)	<u>Control</u> Median (5th-95th pctl)
Evening Average (mg/m ³)	0.075 (0.023 - 0.254)	0.059 (0.022 - 0.235)
Evening Maximum (mg/m ³)	12.346 (1.940 - 19.140)	9.402 (2.758 - 19.392)
Evening Minimum (mg/m ³)	0.003 (0 - 0.056)	0.002 (0 - 0.056)
Midday Average (mg/m ³)	0.073 (0.024 - 0.197)	0.072 (0.017 - 0.149)
Midday Maximum (mg/m ³)	10.933 (3.868 - 19.071)	11.311 (3.189 - 19.496)
Midday Minimum (mg/m ³)	0 (0 - 0.046)	0 (0 - 0.031)
Overnight Average (mg/m ³)	0.059 (0.017 - 0.178)	0.054 (0.018 - 0.179)
Overnight Maximum (mg/m ³)	9.733 (1.659 - 19.100)	11.463 (3.001 - 19.406)
Overnight Minimum (mg/m ³)	0 (0 - 0.055)	0 (0 - 0.037)
Time-Weighted Average (mg/m ³)	0.070 (0.030 - 0.178)	0.075 (0.022 - 0.167)
# of points >0.569 mg/m ³	353 (46 - 892)	231 (32 - 1020)
# of points > 1 mg/m ³	3 (0 - 18)	4 (0-14)
# of points > 10mg/m ³	726 (122 - 1977)	522 (100 - 1778)

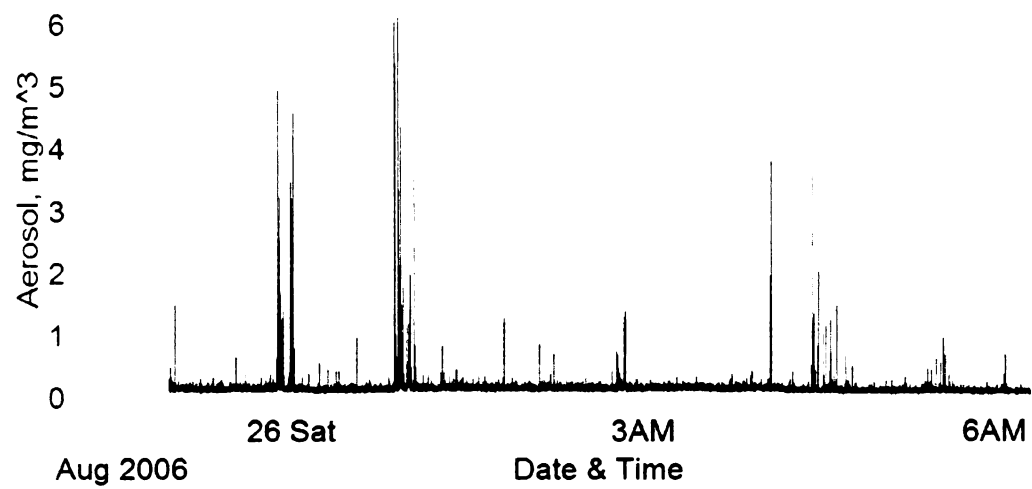


Figure 4-1. Example of 1 second tracings of PM10 concentrations of a selected case.

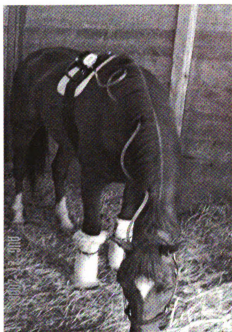


Figure 4-2. Picture of personal monitor attached to a surcingles, with sample tubing along the length of the horse's neck, and ending near the nostril.

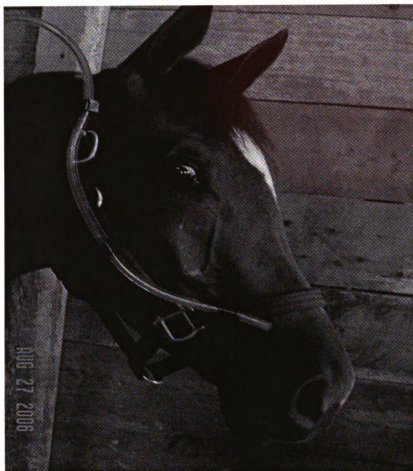


Figure 4-3. Location of sampling tube close to the nostril

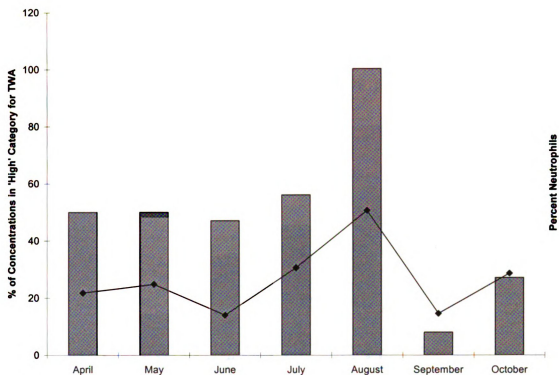


Figure 4-4. Percent of PM10 concentrations (grey bars) that fall into the 'high' calculated time-weighted average (TWA) category by sampling month, and percent neutrophils (black line) by month plotted along the same axis.

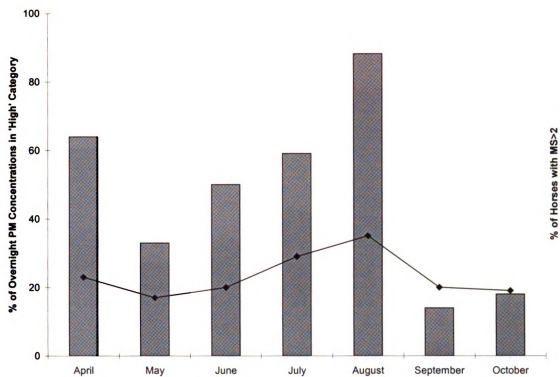


Figure 4-5. Percent of overnight PM₁₀ concentrations (grey bars) that fall into the 'high' category by sampling month, and percent of horses with $MS \geq 2$ (black line) by month plotted along the same axis.

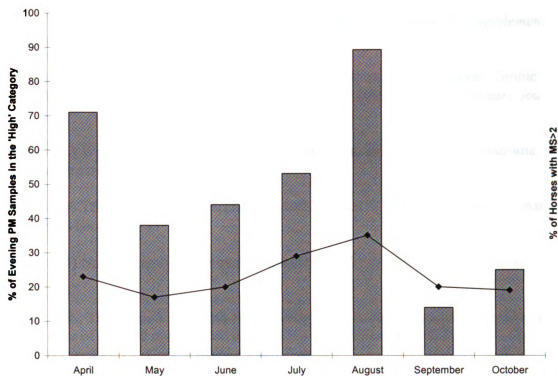


Figure 4-6. Percent of evening PM10 concentrations (grey bars) that fall into the 'high' category by sampling month, and percent of horses with $MS \geq 2$ (black line) by month plotted along the same axis.

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CONCLUSIONS

This investigation focused on the exposure of horses to airborne particulate matter (PM) within the stable and its effects on measures of airway inflammation (tracheal mucus and inflammatory cells) in Thoroughbred racehorses. The purpose of my work was to quantify these exposures, better understand the factors that influence them, and determine whether or not there was a significant effect on airway inflammation. My overall hypothesis was that ambient particle load and personal particulate exposure varies with stable, stall location within a stable, and activity level, and correlates in an exposure-dependent manner with established measures of airway inflammation (mucus score, and inflammatory cell counts) in racehorses.

Determining concentrations of PM that accurately reflect area concentrations or personal exposures is difficult in indoor environments when sources of PM generation, dispersion, and ventilation are relatively constant, as these measurements are simply a 'snapshot in time'. Add to this the complexity of monitoring an environment such as a stable in which there is a potential for substantial variation in PM generation and dispersion due to factors that include: weather patterns, fixed (doors/windows) and non-fixed (fans) sources of ventilation, inconsistencies in human work habits, variability in feed/bedding quality, and adaptation of equipment originally developed for human use to be worn by horses, and one has a very inconsistent environment in which to attempt to characterize PM concentrations. Attempts were made to overcome these variations in the present indoor air quality/airway inflammation investigation by utilizing a repeated measures study design in a limited number of stables, allowing me to determine whether or not there were relatively 'constant' differences in PM concentrations over time and between stables. While this study

design allowed me to investigate variations in PM concentrations and measures of airway inflammation by month (PM and inflamm.), time of day (PM and inflamm.), stable (PM only), and stall (PM and inflamm.), I was unable to perform an analysis that would incorporate the human element (effect of management style/stable) on airway inflammation as it was likely a surrogate for PM concentration. As horses were endoscopically examined only once per month and PM monitoring took place during the same time frame, I was only able to make statements regarding PM concentrations and their association to airway inflammation (mucus score and inflammatory cells) at that particular point in time. This study design did not allow me to track PM concentrations and airway inflammation throughout each month and over several months, which would allow for further clarity in terms of duration of time horses experienced particular levels of airway inflammation (e.g. moving through a range of mucus scores) and their association to PM concentrations measured over prolonged periods of time (days/months).

For the case-control study, it would have been ideal to be able to monitor each horse over several days. However, in order to achieve the power required to detect a difference in exposure between cases and controls, it was necessary to study each horse for no more than 24 hours. Therefore, it is possible that I was not able to truly capture the variability in personal exposures due to intra-animal variation in behavior over time (e.g. reduced movement due to exhaustion after a race). I was unable to monitor the horses during the early morning period, a time at which I determined there to be significant elevations in area PM concentrations, due to the training schedule of the horses, which also resulted in an underestimation of personal PM exposures. While I made every attempt to select cases/controls from a 'pool' of horses that were evenly distributed through multiple stables,

I was unable to do so, ultimately resulting in a skewed distribution of cases/controls coming from each stable. This was unavoidable as there were several months in which it was difficult to find horses that would meet the criteria to become a 'control' and I had to take them where I could find them. In addition, I quickly found that there were horses from certain stables that would not tolerate the sampling equipment for one reason or another (nervousness, etc.), thus reducing the total 'pool' of horses from which to choose. I also had to inevitably drop one stable from our study as the type of bedding used was routinely plugging the personal monitors resulting in lost/missing data and the inability to utilize those monitors for the rest of the monthly sampling period.

Despite these inherent limitations to this study, I was able to determine with certainty that there was an association between airborne PM concentrations as measured by personal and area monitors, and airway inflammation in racehorses. Based on what I have learned from conducting this study, and the results that are summarized in the following sections, future studies will be designed to take these limitations into account.

Air Quality in a Boarding Stable

Prior to initiating activities at the racetrack, I wanted to better understand the range of PM concentrations in the breathing zone of the horse as a result of routine activities and management practices which commonly occur in stables. As a result, I conducted a small-scale field study in a boarding stable where I was confident that I would have complete cooperation by horses and people alike.

I chose to use direct-reading instruments in my investigations as they have the ability to provide not only average concentrations of PM, but also peak and minimum concentrations, which we hypothesized may be important in the etiology of IAD, as short-term/peak exposures, or chronic low-level exposures had not been previously quantified in this particular environment. These monitors are ideal for 'mapping' particle concentrations, allowing the investigator to determine regional 'hot spots' of PM, which indicate areas of poor ventilation, and I had utilized them in the past in industrial settings.

I found that even when horses ate what would be considered by horse owners to be good quality hay, particle concentrations at the nostril of the horse exceeded short-term excursion limits set for industrial workers, and that eating 'slightly dusty hay' exceeded the capacity of the monitor (150 mg/m^3). Veterinarians often recommend feeding horses with recurrent airway obstruction (RAO) a diet that includes pellets or hay cubes in order to minimize dust exposure, but surprisingly, breathing zone concentrations of PM while consuming these types of feeds were high as well, as they are fed out of deep bins that essentially trap the particles in the immediate vicinity of the horses nostril. Activities such as de-cobwebbing, sweeping/raking of the aisles, dragging and riding in the indoor arenas, also dispersed very high concentrations of PM, which remained suspended in the horses breathing zone. Fortunately, however, a small trial using simple dust suppression techniques commonly utilized in industry (application of water) virtually eliminated the dust when water was applied just prior to initiating the activity (feeding, cleaning, etc.).

I used portable pump/colorimetric tube methods to determine ammonia concentrations within stalls, and found seasonal variations (high in summer, low in winter), consistent with what I'd expect due to the volatile nature of gasses in the heat/cold. What was interesting was that ammonia concentrations increased in stalls located furthest from the doorways (natural ventilation). I also discovered that even though the entire front of the stalls were fitted with bars to allow for movement of air, the air exchange within these stalls was minimal, as any air movement/exchange predominantly occurred in the aiseways, leading me to believe that the majority of decision making in terms of ventilation status (e.g. doors open vs. doors closed), temperature regulation, and sense of 'air quality' (e.g. dustiness) occurs 'from the aisleway' where the stable management, owners, and riders spend the majority of their time (rather than in the stall with the horse).

Bulk samples of dust were collected from ledges greater than six feet off the ground to estimate the composition of dust that would pass up through the breathing zone of the horse and then settle-out. These dusts were analyzed for both particle composition and size, and I was interested to find that two components known to result in airway inflammation/chronic disease in occupational lung disease were present in the stable environment, namely crystalline silica (sand footing/flooring materials) and iron (crustal materials).

These initial findings, which verified that 1) particulates common in the breathing zone of horses were of the same size (PM10 and PM2.5) and in concentrations that were of interest in the current human and animal literature, 2) there were regional variations in

ventilation within the stable resulting in ‘hot spots’ of PM, 3) there were certain activities that generated significant concentrations of dust in the breathing zone of horses, and 4) that components of these dusts were of biological importance, armed me with the knowledge necessary to go forward and attempt a similar study on a much larger scale.

Indoor Air Quality and Airway Inflammation in Thoroughbreds

I was very fortunate to be able to continue a relationship with racetrack veterinarians and trainers that had been built previously during a study conducted by our laboratory which ultimately determined that there was a significant effect of tracheal mucus ($MS \geq 2$) on racing performance. As a result of this study, these stakeholders were very interested in the cause(s) of tracheal mucus, and allowed us to perform our study in the hopes that we would be able to provide recommendations back to them in order to reduce the prevalence of airway mucus.

I was able to demonstrate that indeed there were significant effects of season (month), time of day, stable, and location within a stable on area concentrations of PM₁₀ and PM_{2.5}. The sampling month that followed a period of hot dry weather resulted in the highest concentrations of PM, with the lowest occurring after a period of rainy weather.

Morning PM measurements were always the highest because this is when all of the activity at a racetrack occurs (feeding, cleaning, grooming, training, etc.). Large particles (PM₁₀) tended to settle out rather quickly, and our mid-day measurements were not

significantly different from the late afternoon. The smaller particles (PM_{2.5}) settled out more slowly, particularly in the stable that was the most enclosed.

There was a significant effect of stable on area PM concentrations; however, it was difficult to separate out the effect of stable design vs. management style. The newer open-air design stable consistently had the lowest concentrations of PM, but also had the least amount of human activity throughout the day. The two older-style enclosed brick buildings of the same design differed from one another in terms of PM concentrations most likely due to orientation of buildings along roadways as well as management/activity level. The stable that consistently experienced the highest concentrations of PM was flanked on both sides by busy roadways, but also was the only stable to feed from hay nets, and had the greatest number of employees, which performed all chores (cleaning, grooming, raking) simultaneously. Interestingly, during the month of cold weather when the doors and windows/shutters were closed tight on all three stables, the magnitude of variation in PM between the stables was reduced.

There was a significant effect of stall location on PM concentrations. Stalls that were located either in high-traffic areas or the furthest from natural sources of dilution ventilation had the highest concentrations of PM as compared to adjacent stalls.

Taking all of this into consideration, I expected to see the highest prevalence of tracheal mucus during the months with highest PM concentrations, and in the stables and stalls with the highest PM concentrations. Indeed, the highest prevalence of the presence of

tracheal mucus occurred during our September sampling month when PM concentrations were the greatest, in the stable that consistently had the highest concentrations of PM, and in those stalls with the highest PM concentrations. I was unable to take stable and stall into consideration in the hierarchical statistical models, as they were most likely a surrogate for PM exposure, but bivariate analysis did determine significance.

As the study was designed to determine the differences in PM concentrations between months, stables, and stalls, repeated measures design was necessary. As a result, I did not have sufficient power to distinguish the relationship between ambient concentrations of PM and differing degrees of mucus accumulation.

Interpretation of total and percent inflammatory cells in regards to airway inflammation was not straight-forward, as numbers and percent of inflammatory cells tracked nicely with mucus prevalence by month, however, they were not significantly associated with the presence ($MS \geq 1$) of tracheal mucus.

Case-Control Study

Based on the particle concentrations that were measured at the level of the nostril in the boarding stable, and my hypothesis that horses with significant ($MS \geq 2$) quantities of tracheal mucus were exposed to higher concentrations of PM than their counterparts without visible mucus ($MS = 0$), I decided to use *personal* real-time monitors to track particle concentrations over as much of a 24 hour period as possible.

Incredibly, we were able, on average, to monitor the horses over a 17.5 hour period of time, with the monitors taking samples every second so as to 'catch' peak exposures that would result from the horse's personal activities (eating, rolling, pacing, etc.). We were unable to monitor the horses during the early morning hours, the time period in our last study in which PM concentrations were the greatest.

PM concentrations measured with personal samplers in cases and their matched controls did not differ in most instances; however, the data also suggested that they did not differ greatly from ambient PM concentrations. Overall prevalence of $MS \geq 2$ in all horses endoscopically examined tracked convincingly with personal monitoring results. As in the previous study, there was a significant effect of month, with the lowest PM concentrations occurring in the early summer months during periods of wet weather, and peaking late summer during hot/dry periods.

While there was a significant difference between cases and controls in numbers of neutrophils in tracheal wash fluids, there was no difference in percent neutrophils. As with the previous study, percent neutrophils tracked nicely with PM concentrations stratified by month. Based on our data, I question the usefulness of utilizing percent neutrophils in tracheal wash samples as a diagnostic criterion for IAD. Our data consistently show no significant differences in percent neutrophils between horses with and without tracheal mucus, and in fact, in our second study, 82% of horses with no visible tracheal mucus had greater than 20% neutrophils, similar to what has been found in other studies. As

endoscopic examination is readily available, perhaps the criteria for diagnosis of IAD should be limited to tracheal mucus score.

Practical Implications from the Investigation

The fact that my data suggest that personal monitoring reflects primarily area PM exposures, and that it is area PM that is associated with tracheal mucus, is very exciting. This means that individuals armed with the right equipment can enter stables and measure the area PM concentrations during times of peak activity, determine where 'pockets' of high PM/low ventilation exist, and make appropriate recommendations as to how to maximize ventilation and implement dust management practices within that facility in order to reduce the prevalence of IAD. Furthermore, because I have demonstrated that simple changes in management can dramatically reduce particulate load, it should be easy to implement control measures.

Future Investigations

As I have determined an effect of season and time of day on PM concentrations, it is no longer necessary to perform repeated monitoring in stables and repeated examinations of horses. To be able to obtain significant power to determine the association between area particulate concentrations and degree of tracheal mucus, any future study should encompass additional stables and horses, thus providing additional clarity as to an area concentration/threshold above which one would expect to find horses with significant quantities of tracheal mucus. A similar study in non-racing stables would be beneficial in order to understand the variation in areat PM concentrations as a result of stable

construction/design and management practices, as currently insufficient stables have studied to define a 'normal' and a 'dusty' environment. Limited monitoring of specific PM components 'of concern' (endotoxin, mold, iron, crystalline silica, etc.) would be useful to begin to understand what is commonly found in stables, and in what concentrations/ratios. Finally, an 'intervention' study should be performed at a racing stable to determine the effect of a dust control strategy on the prevalence of mucus within that stable.

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